

**Development of Novel Chemical Approaches
for Detection and Engineering of Endogenous Proteins**

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Preface and Acknowledgements

The studies presented in this dissertation have been carried out under the direction of Professor Itaru Hamachi at Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, from April 2010 to March 2014, which are focused on the development of novel chemical approaches for the detection and engineering of endogenous proteins.

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General Introduction

Introduction

Proteins are biological macromolecules involved in almost all biological and physiological phenomena such as recognition, catalysis, storage, transport and signaling. Many intra- and extracellular events depend on specific changes of both protein structures and functions. If the intrinsic properties of proteins are disordered, various diseases are induced. Therefore, analyzing their complicated structures, functions and dynamics are essential for understanding living systems in molecular level. For instance, proteomics based analyses have provided useful information how proteins interact with each other, which would offer the keys to curing diseases or providing the targets for drug development. However, they are not applicable to real-time analyses for protein behaviors. Therefore, protein detection or engineering methods for real-time analysis is still challenging. Especially, *in cell* or *in vivo* detection, imaging and engineering of natural proteins, which are not genetically encoded proteins but endogenously expressed proteins bearing twenty kinds of natural amino acids, is highly demanding. In this chapter, I focus current chemical and biological methods for protein detection/imaging and protein labeling/engineering under crude conditions.

Protein detection/imaging methods (mainly non-genetic methods)

Protein detection/imaging techniques are one of the most fundamental techniques in chemical biology. Various modalities to visualize the proteins of interest (POI) under live cell condition have been recently developed and established.

1. Fluorescence techniques

Fluorescent imaging is the most powerful method for the observation of dynamic events in living cells. Recent progress in the genetically encoded protein tags such as green fluorescent protein¹ (GFP) readily allows to image the behaviors of POI in real-time manner as described below (see next section : **protein labeling/engineering methods**). In this subsection, I focus on the fluorescent imaging without genetic methods.

An antibody, also known as an immunoglobulin, selectively recognizes a part of an antigen. Immunofluorescence technology with antibodies conjugated with fluorophores is generally used for antigens staining.² The imaging using antibodies with various fluorophores is only available for endogenous proteins on cell surface. As an excellent work³, Urano and Kobayashi *et al.* developed the activatable fluorescent antibody (trastuzumab) against human epidermal growth factor receptor type 2 (HER2) modified with an acidic pH-activatable BODIPY probe based on photoinduced electron transfer for imaging viable cancer cells. This agent is fluorescently activated after endocytic internalization by sensing the pH change in lysosome even *in vivo*. The concept of antibody-fluorophore conjugates can be widely adapted to endogenous protein imaging (Figure 1).

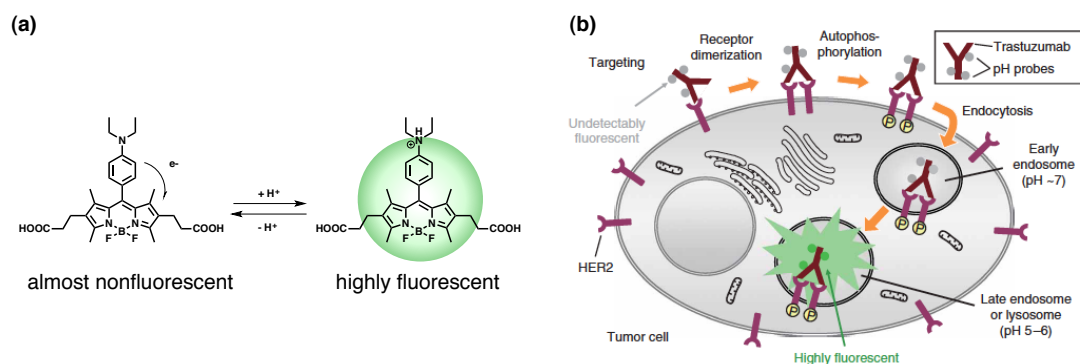


Figure 1. (a) Scheme for the reversible and acidic pH-induced fluorescence activation of a probe. (b) Schematic illustration of tumor imaging with an activatable fluorescence probe-antibody conjugate.³

Compared to the antibody-based imaging, the small molecule-based imaging is absolutely useful for intracellular imaging because of the relatively high membrane-permeability. Ligand-tethered fluorophores are valuable for protein imaging. For instance, Marnett *et al.* reported fluorescent imaging agents for cyclooxygenase 2 (COX2) *in vivo*, in which indomethacin as an inhibitor for COX2 was attached to rhodamine derivatives (Figure 2a, b).⁴ The always-on fluorescent probes have the disadvantage of high background signal, which need the washout process of the excess probes. On this contrary, activatable probes which can be activated upon recognizing the target analytes provide lower background signals. Development of activatable fluorescent probes for ligand-recognition proteins is highly challenging because of the insufficient advances in the signal-switching mechanism. One of the impressive examples is the turn-on fluorescent probe using aggregation-induced emission (AIE). Tang and Liu *et al.* developed the AIE-based probe by integrating tetraphenylsilole with Arg-Gly-Asp (cRGD) tripeptides, which is a ligand to integrin $\alpha_v\beta_3$ receptor on cell surface.⁵ Recently, Hamachi *et al.* developed the self-assembling turn-on nanoprobe for ligand-binding proteins.⁶ The nanoprobe was designed based on recognition-driven disassembly of a ligand-tethered fluorophore with the aggregation properties. They succeeded in specific visualization of overexpressed folate receptor (FR) and hypoxia-inducible carbonic anhydrase (CA) on cancer cells without washout process (Figure 2c, d).

Fluorescent imaging of enzymes, not ligand-binding proteins, which specifically convert a substrate into a product, have been conducted. Enzyme reaction is one of the most important functions of proteins. Because a substrate undergoes in a substantial structural change through enzyme reaction, the development of turn-on fluorescent probes is relatively easy. Many activatable probes have been developed, which are based on some fluorescence-switching mechanisms for selective detection/imaging of enzyme activity. Fluorescence resonance energy transfer (FRET) is a mechanism describing energy transfer between two chromophores, which occurs between a fluorescence donor and a fluorescence acceptor that are in molecular proximity of each other if the emission spectrum of the donor overlaps the excitation spectrum of the acceptor. As a pioneered example of FRET-based imaging, Tsien *et al.* succeeded in ratiometric imaging of β -lactamase activity within live cells, which allows to reveal quantitative heterogeneity in real-time gene expression of β -lactamase.⁷ Photoinduced electron transfer (PeT) is the widely accepted phenomena for fluorescence quenching in which electron transfer diminishes the fluorescence. Urano and

Nagano *et al.* have established the rational design strategy using PeT mechanism for novel fluorescein-based probe, which yielded the turn-on probes for β -galactosidase activity.⁸ Some fluorophores form aggregates such as homodimer, resulting in the quenching of the fluorescence signal, which allows Weissleder *et al.* to develop polymer-based turn-on probes by the self-quenching of a near-infrared fluorophore for *in vivo* imaging of protease activities of cathepsin and matrix metalloproteinase.⁹

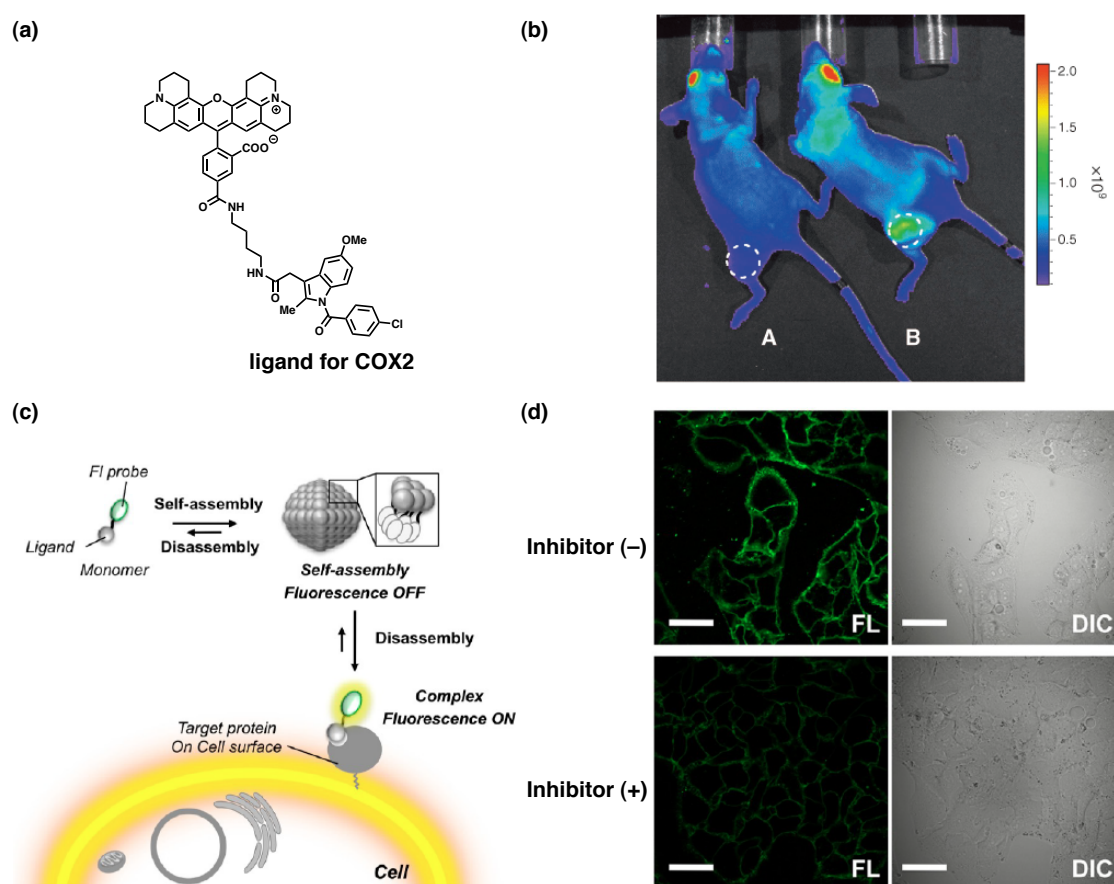


Figure 2. (a) Chemical structure of COX2 imaging agent (b) *In vivo* imaging of COX2-expressing xenografts. Nude mice with HCT116 xenograft (A; COX2 negative) or 1483 xenograft (B; COX2 positive), where white dotted line indicates tumor perimeter.⁴ (c) Schematic illustration of a self-assembling turn-on fluorescent probe for cell surface protein imaging. (d) Transmembrane-type CA imaging of A549 cells (top) without or (bottom) with ethoxazolamide (a strong inhibitor for CA) cultured under hypoxic conditions. Scale bars, 40 μm .⁶

2. Magnetic resonance techniques

Magnetic resonance imaging (MRI) is well known to be useful for the *in vivo* imaging technique to investigate the anatomy of our body.¹⁰ However, signal-switching strategies for MRI are as yet quite limited. As a pioneering example, ^1H magnetic resonance (MR)-type switching probes have been reported as powerful tools for *in vivo* enzyme imaging by Meade's group.¹¹ The switching mechanisms are mainly based on paramagnetic relaxation enhancement (PRE) using paramagnetic metal complexes and their coordinated water molecules.¹²

For target-specific imaging, heteronuclear MRI techniques have also attracted considerable attention. In particular, fluorine (^{19}F)-MRI is a promising method for protein imaging¹³. Kikuchi *et al.* have developed the novel signal-switching mechanism for detecting protease activity in ^{19}F -MRI. The ^{19}F -MRI signal is quenched by the intramolecular paramagnetic effect from Gd^{3+} .¹⁴ The intramolecular Gd^{3+} dramatically attenuated the ^{19}F probe signal by the paramagnetic effect, which was cancelled by the probe hydrolyzation by caspase-3 (Figure 3a). Hamachi *et al.* have also developed the self-assembling turn-on ^{19}F probe for the specific detection of ligand-binding proteins.¹⁵ The ligand-tethered probes clearly exhibit a disassembly-driven turn-on signal change upon specific binding to target proteins (Figure 3b).

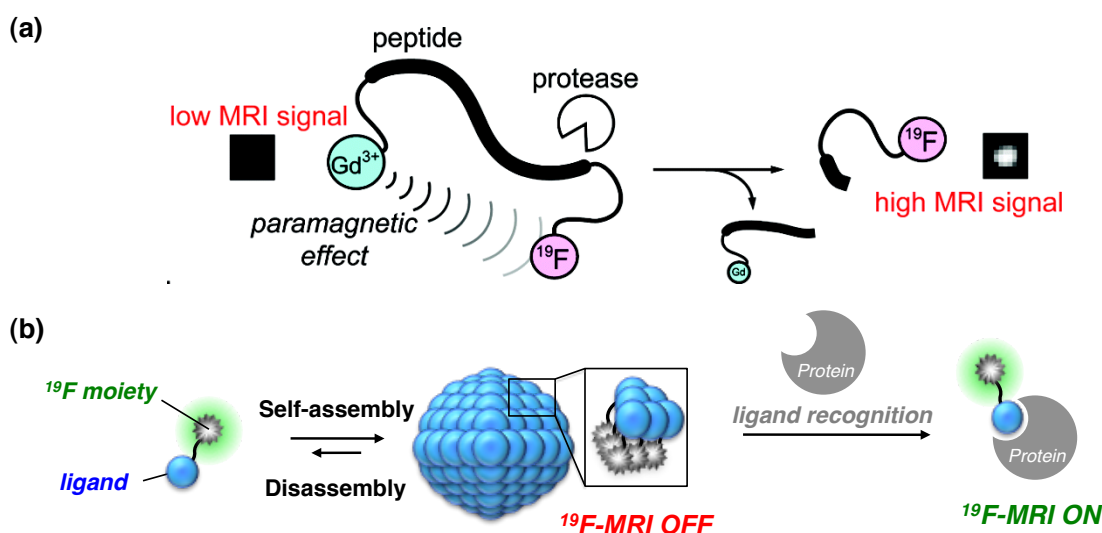


Figure 3. Schematic illustration of the novel signal-switching mechanisms in ^{19}F -NMR/MRI based on (a) paramagnetic effect¹⁴ and (b) self-assembly¹⁵. Adapted with permission from reference ¹⁴. Copyright 2008 American Chemical Society.

Furthermore, the recent development of nuclear magnetic resonance (NMR) techniques is achieved. For instance, not only the analyses of protein activity or protein recognition but also protein structural analyses are available even in mammalian cells. The in-cell NMR techniques¹⁶ are expected to clarify the stability and dynamics of a target protein in live cell. The in-cell NMR spectra shows the formation of specific complexes of FKBP12 with FK506 or rapamycin and also demonstrate that ubiquitin exhibits much higher hydrogen exchange rates in the intracellular environment than that *in vitro*.

The detection/imaging in magnetic resonance techniques are really useful for bio-imaging tools, but often suffers from the low sensitivity, especially in heteronuclear magnetic resonance techniques. However, hyperpolarization, which is the nuclear spin polarization far beyond thermal equilibrium conditions, is a highly promising technique for improving the sensitivity of magnetic resonance chemical probes. The enhanced sensitivity by a hyperpolarization technique can reduce detection limits. Sando *et al.* succeeded the direct and efficient detection of mouse lactate dehydrogenase in crude biological mixtures by monitoring the ¹³C-NMR signal of hyperpolarized product of enzyme reaction.¹⁷

3. Other techniques

Enormous strides have developed many technologies for protein detection/imaging. Positron emission tomography (PET) as a nuclear medicine imaging technique is widely used for medical diagnosis.¹⁸ PET imaging of tumor metabolism with 2-[¹⁸F]fluoro-2-deoxy-D-glucose is widely used for detecting *in vivo* and the reagent is also in clinical use for detection of normal and pathologic function in brain and heart. Mazitschek *et al.* recently reported that the ¹⁸F-suberoylanilide hydroxamic acid (¹⁸F-SAHA), which is an inhibitor for histone deacetylases, was used for PET based protein imaging *in vivo* (Figure 4).¹⁹ The single-photon-emission computed tomography (SPECT) and photoacoustic tomography (PAT) are also used for protein imaging techniques. The multi-modal imaging, which is the combination of plural modalities, is expected to the next-generation of medical and molecular imaging techniques.

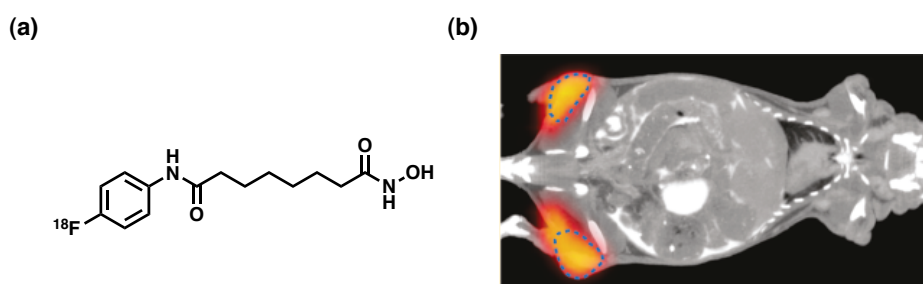


Figure 4. (a) Chemical structure of PET imaging probe for histone deacetylases. (b) PET-CT scan of tumor xenograft after injection of ¹⁸F-SAHA, where blue dotted line indicates tumor perimeter.¹⁹ Adapted with permission from reference ¹⁹. Copyright 2011 American Chemical Society.

Protein labeling/engineering methods

The specific protein labeling methods under crude conditions enable target proteins not only to be visualized, but also to be arbitrarily manipulated for the creation of novel bioanalytical tools *in vitro* and *in vivo*. In this section, I briefly summarized the recent progress in the protein labeling/engineering techniques.

Protein labeling methods based on genetic technology

1. Fluorescent proteins and protein/peptide tag technology

The most powerful methods for selective protein visualization, manipulation and engineering under biochemically complex conditions are undoubtedly the protein labeling with genetically encoded fluorescent proteins and peptide tags.

The fluorescent protein such as GFP composed of 238 amino acid residues (ca. 27 kDa) that exhibits green fluorescence, is genetically and covalently fused with POI, which can precisely and directly analyze protein expression and localization under live cell conditions. Furthermore, the fluorescent proteins are manipulated to produce the attractive bio-tools for analyzing the life phenomena. As an excellent work of fluorescent protein-based imaging of enzyme activity, Yoshida *et al.* have developed a genetically encoded FRET-based probe for histone deacetylase activity, termed Histac, consisting of an acetylation-binding domain, a flexible linker, a substrate histone H4 and the two different-colored mutants of fluorescent proteins (CFP and Venus), which serve as FRET pair.²⁰ The acetylation-binding domain dynamically induces a conformational change when the H4 peptide was acetylated. Histac allows the monitor the dynamic fluctuation of histone H4 acetylation levels (Figure 5). However, fluorescent proteins and the other types of protein tags such as SNAP and Halo tags have some concern to interfere with the function of POI due to their large size and the fusion site (mainly limited to the N or C terminus of POI).

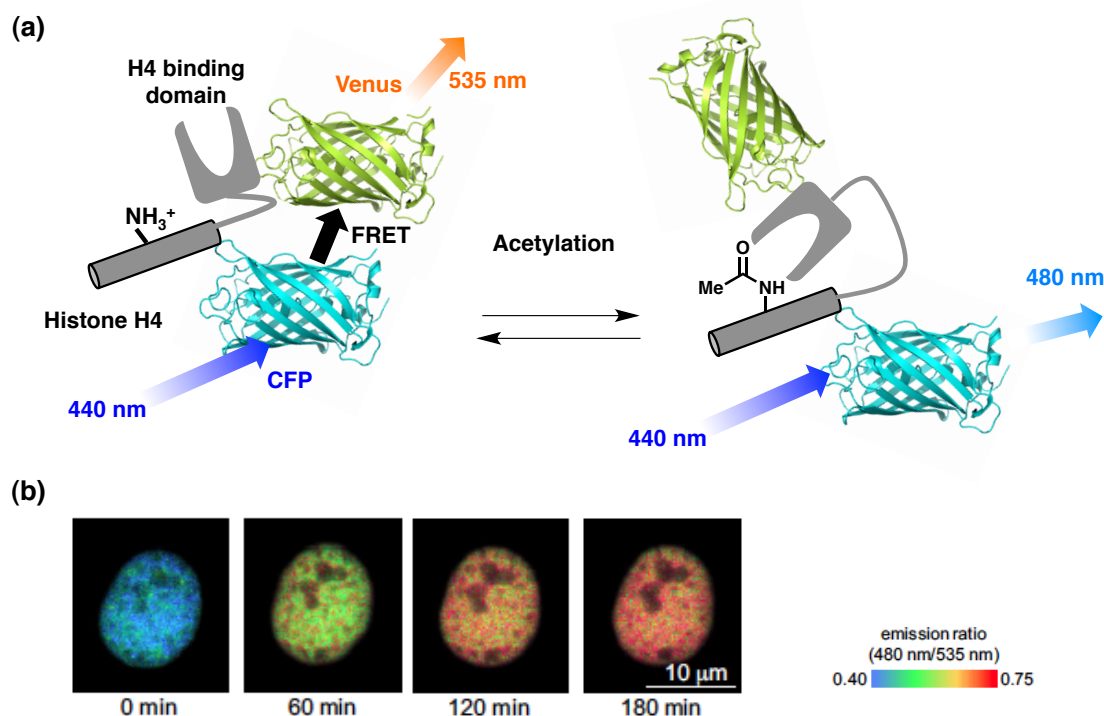


Figure 5. (a) Schematic illustration of Histac. (b) Pseudocolored images and a time course of the emission ratio in the nucleus of a COS7 cell expressing Histac.²⁰

To overcome these drawbacks, another labeling strategy has been proposed using short peptide tags, which can minimize the functional disorder of target proteins due to their small size. The peptide tags are applicable to selective labeling in cellular contexts. As a representative work, Tsien *et al.* reported the pioneering work that peptide tags with the consensus sequence CCXXCC are labeled by the biarsenical probes such as the green fluorophore FAsH²¹ (Figure 6a). Taking advantage of FAsH, Kodadek *et al.* have developed label transfer chemistry for analyzing protein-protein interaction. In their chemistry, POI is tagged with a tetracysteine sequence (FAsH receptor) that specifically and non-covalently interacts with DOPA (3,4-dihydroxyphenylalanine)-biotin-FAsH (Figure 6b). When oxidized with sodium periodate, DOPA moiety is cross-linked to nucleophiles on the surface of the partner protein.²² They also succeeded to detect the protein-protein interaction not only *in vitro* but also in *E. coli* lysate (Figure 6c).

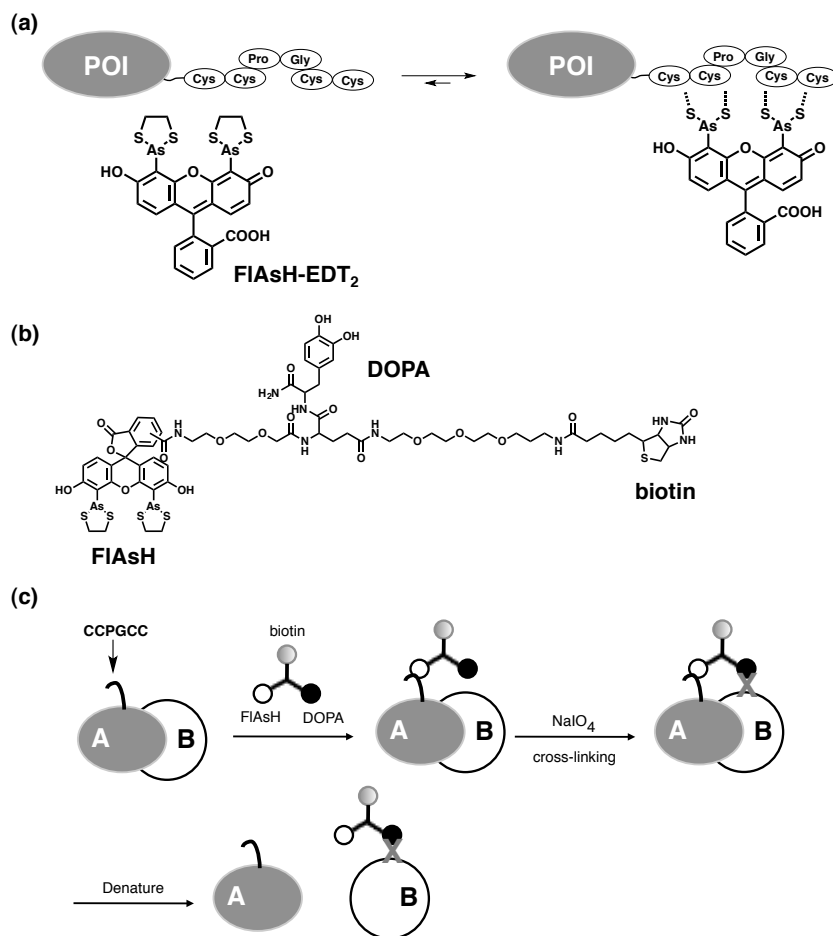


Figure 6. (a) FIAsh-based protein labeling. (b) Chemical structure of DOPA-biotin-FIAsh. (c) Label transfer system by using DOPA-biotin-FIAsh.

2. Site-directed mutagenesis

Site-directed mutagenesis, which can be used to change particular base pairs in a piece of DNA, is the powerful tool to study protein function, identify enzyme active sites and engineer proteins. The most classical method is to use the cysteine mutant of corresponding proteins, which can be specifically labeled by thiol-reactive molecules.

As the pioneering work for the activity-controllable proteins, Trauner and Kramer *et al.* synthesized with a thiol-reactive azobenzene derivative bearing protein ligands which are covalently attached to the cysteine of the mutated proteins by classical cysteine-maleimide conjugation reaction.^{23a} Their approach allows to photoregulate channel proteins activity with high spatiotemporal precision in a reversible manner caused by *cis-trans* isomerization of azobenzene

derivatives (Figure 7). Their method could be applied to a variety of different channel proteins, including the ionotropic glutamate receptor (GluR)^{23b} and metabotropic GluR (one of G protein coupled receptors)^{23c}. Furthermore, it could be used to manipulate the neuronal activity with light even in living zebrafish^{23e}, as well as in cultured cells.

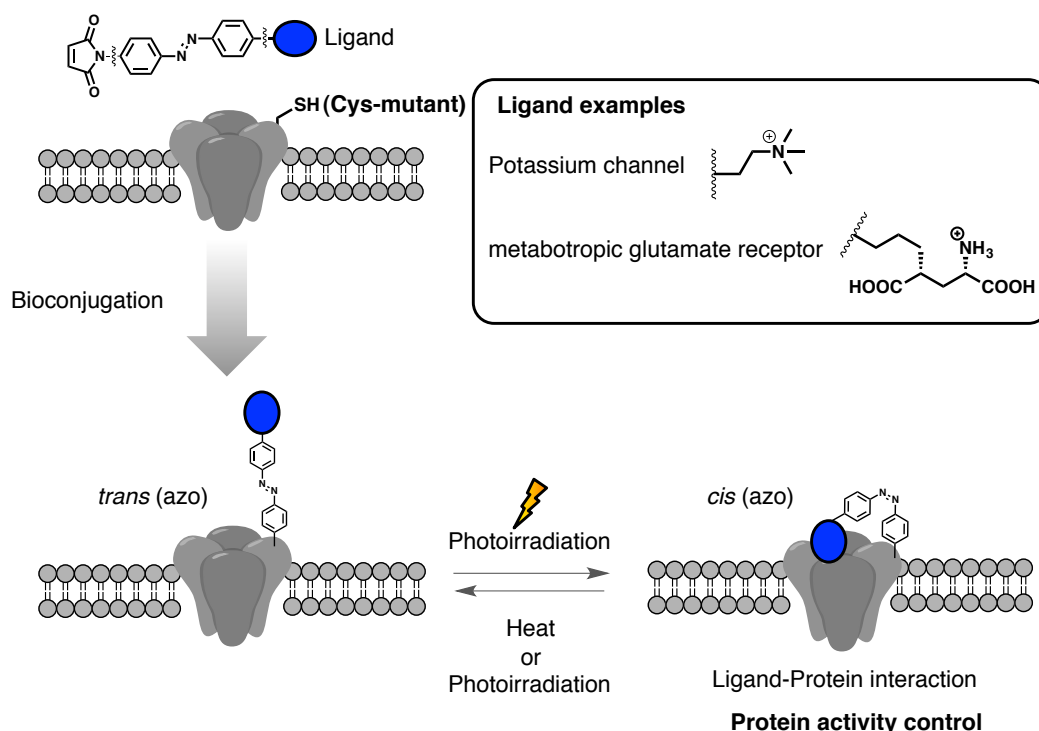


Figure 7. Azobenzene-based reversible photocontrol of channel proteins.

Bayley *et al.* also developed a pore protein-sensor using α -hemolysin (α -HL), whose seven subunits made the nanopore on cell membrane²⁴. Their approaches using the engineered α -HL-based nanopore were applied to the measurement of current changes induced by various kinds of analytes such as the guest recognition, reaction process, and nucleic acids detection. They attached β -cyclodextrin (β -CD) covalently within the α -HL pore in stable orientations via disulfide bond formation with the cysteine residues of α -HL. Also, oligo-DNA and DNA aptamers were conjugated to α -HL for bioanalysis at a single molecule level. The current changes associated with chemical reaction processes were observed using engineered α -HL by photo-caged molecules or azobenzene derivatives. This β -barrel type sensors were flexibly utilized to observe their analytes according to

the characteristics of the conjugated molecules to α -HL (Figure 8).

As described above, the site-directed mutagenesis is undoubtedly useful to construct the engineered proteins, but it is only applicable to purified proteins.

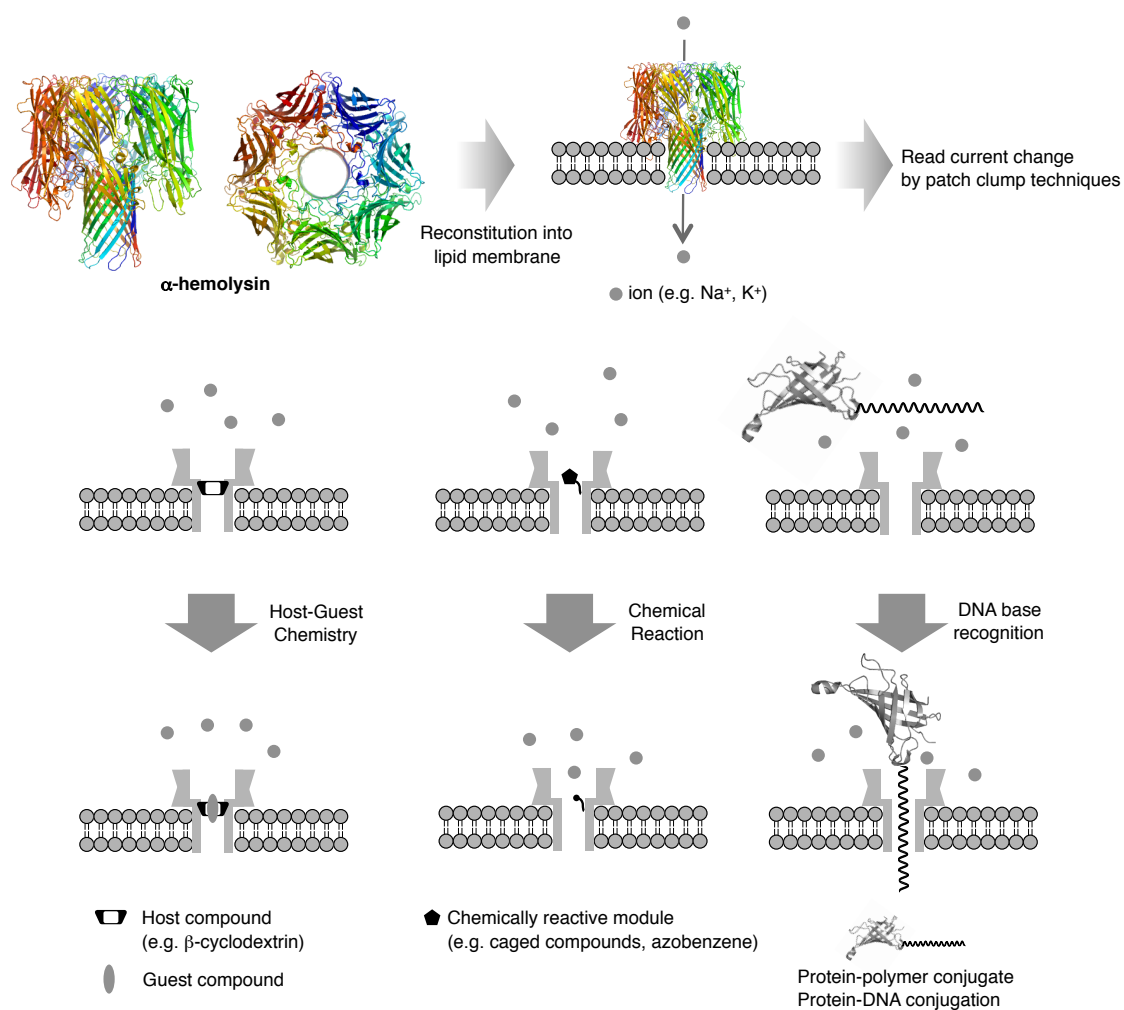


Figure 8. The representative works of Bayley's group. The various types of α -HL based sensors.

3. Unnatural amino acids incorporation and bioorthogonal reactions

An alternative to tag mediated protein labeling has been developed, which is to produce the chemically and specifically reactive proteins using expanded genetic codes even under live cell condition. As the pioneer, Schultz *et al.* have developed a powerful tool for direct incorporation of non-natural amino acids into proteins by their original genetic modification system exploiting the expanded genetic codes²⁵. Nowadays, many kinds of unnatural amino acids, for example amino acids with keto, azido and alkynyl groups, and photoreactive and redox-active amino acids, have been genetically encoded into target proteins using the unique triplet and quadruplet codons, which would require the unique tRNA/aminoacyl tRNA-synthetase pairs.

Chin *et al.* developed the synthesis and site-specific genetic incorporation of the photo-caged amino acids such as caged lysine and caged tyrosine into kinases such as MEK1 in mammalian cells (Figure 9).²⁶ This elegant method enables to measure the kinetics of nuclear import via the photochemical control of protein localization in cells owing to the high spatiotemporal control of the activity of the caged kinase by photoirradiation.

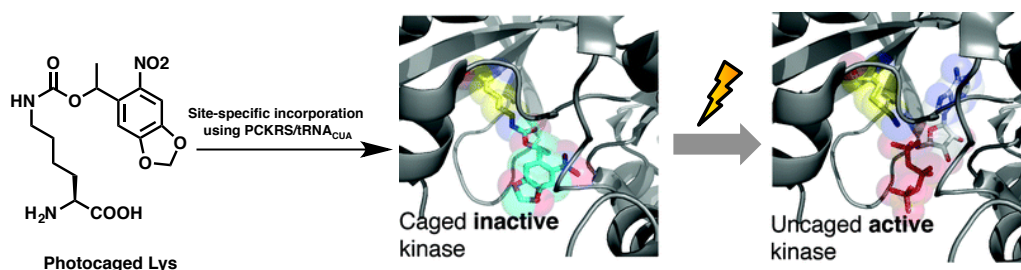


Figure 9. Photocaged enzyme using unnatural amino acid.²⁶ Adapted with permission from reference ²⁶. Copyright 2011 American Chemical Society.

Recently, some researchers have performed site-specific labeling of protein in live cells using the combination of incorporation of reactive unnatural amino acids with bioorthogonal reactions, which are conducted inside of living systems without interfering with various substances²⁷. Among bioorthogonal reactions, the most useful and ideal reaction handle is azide-alkyne-based coupling reaction, termed click reaction²⁸. Chemically reactive unnatural amino acids with azide and alkyne groups are introduced into target proteins by genetic methods, followed by the copper (I) catalyzed click reaction (1,3-dipolar cycloaddition) for selective labeling under live cell conditions (Figure 10a).

For instance, Tirelle *et al.* reported the metabolic incorporation of azidohomoalanine into outer membrane protein, OmpC, one of the abundant outer membrane porins of *E. coli*.²⁹ The azide-functionalized OmpC was successfully labeled by copper (I) catalyzed click chemistry and analyzed. In addition, Bertozzi *et al.* reported a copper-free Huisgen cycloaddition reaction using strained cyclooctyne reagents³⁰, to avoid the use of toxic Cu(I) (Figure 10b). This improvement significantly expanded the utility of this ligation reaction in complicated biological systems, including zebrafish embryo, as well as living mouse.

Other reactive handles of bioorthogonal chemical ligation have been developed (Figure 10c to 10f). For instance, Davis *et al.* have developed the protein labeling method based on Pd-mediated Suzuki-Miyaura coupling reaction with 4-iodo-phenylalanine displayed on protein surface under aqueous condition (Figure 10d).^{31a} Recently, they have also discovered the enhanced Pd ligand for aqueous Suzuki-Miyaura reaction, that is 1,1-dimethylguanidine, which enabled site-specific protein ¹⁸F-labeling using the [¹⁸F]4-fluorophenylboronic acid for PET detection.^{31b} Lin *et al.* also reported the Pd-mediated protein labeling based on copper-free Sonogashira cross coupling, which enabled selective modification of a homo-propargylglycine-encoded ubiquitin protein in *E. coli* (Figure 10e).³² They also developed another bioorthogonal reaction, that is the tetrazole-based photoclick chemistry (Figure 10f). The photoinducible 1,3-dipolar cycloaddition reactions of alkenes with tetrazole derivatives can be employed to selectively label the genetically encoded protein bearing alkene group inside *E. coli*.³³

However, these methods are limited only to applying genetically encoded proteins, which are not native proteins. It is keenly desirable to develop native protein labeling approaches for the study of proteins in native tissue, in their physiological context and at physiological protein concentrations.

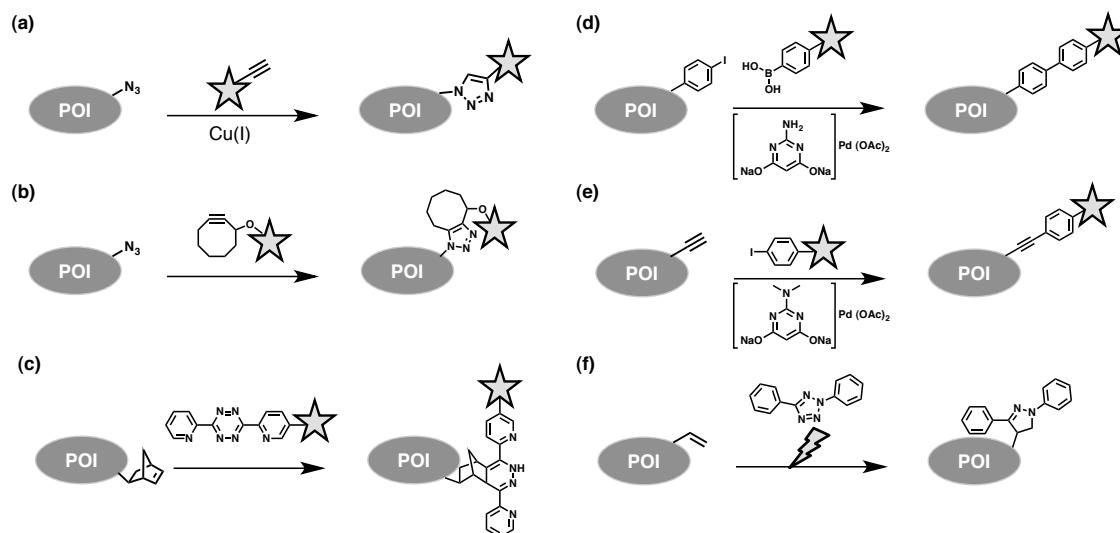


Figure 10. Bioorthogonal reactions. (a) copper (I)-catalyzed click chemistry. (b) copper-free click chemistry. (c) Diels–Alder cycloaddition. (d) Suzuki–Miyaura coupling reaction. (e) Sonogashira coupling reaction. (f) photoclick reaction.

Protein labeling method based on non-genetic technology

All natural proteins expose chemically reactive functional groups on their surfaces, such as thiol (Cys), amine (Lys), carboxyl (Asp, Glu), hydroxyl (Ser, Thr, Tyr), guanidine (Arg), imidazole (His) and indole (Trp) which can be modified by traditional chemical reactions. For instance, sulfhydryl coupling reactions such as Cys-maleimide and amine (Lys) coupling reactions with active esters or isocyanates are widely utilized. One of the fatal drawbacks of these chemical bioconjugation methods was their low selectivity in targeting a protein among many others, and/or modifying a specific site of the target protein. Therefore, these have not been regarded as suitable for specific protein labeling under crude conditions such as living cells, tissues and animals.

1. Affinity labeling

Affinity protein labeling³⁴ has been widely utilized to specifically label natural proteins under crude conditions. A photochemically or chemically reactive handle is appended to a ligand such as drugs or natural products capable of binding a target protein selectively. It is expected that the ligand-protein interactions facilitate protein labeling in crude contexts with the greater selectivity. The most conventional photo-affinity labeling employs diazirine, phenylazide and benzophenone as suitable precursors to generate carbene, nitrene and carbonyl radical species, respectively. This technique, while useful for identifying and characterizing target proteins of the specific ligands, often suffers from low yields of cross-linking products. Recent progresses have been made in affinity labeling using proximity-driven nucleophilic reactions with moderate reactivity, providing the rather high yields. Sames *et al.*, for instance, demonstrated by their systematic studies that tuning the reactivity of reactive handles is essential for specific and efficient labeling of a model protein, purified CA.³⁵ Although such affinity labeling approach may allow for selective modification of a target protein, the covalently modified ligands remained even after labeling so as to inhibit the protein activity by masking the protein active pockets. Therefore, one can neither analyze nor image the labeled proteins in their active states.

Activity-based protein profiling (ABPP) also allows the application to endogenous proteins for the study of specific classes of enzymes based on the catalytic mechanism (a mechanism-based inhibitor). Cravatt *et al.* reported the functional proteomic application of chemical reaction to ABPP and used phenyl sulfonate derivatives bearing rhodamine or biotin molecules that targeted serine

hydrolases, which allows to accelerate the discovery of enzyme activities with discrete physiological and/or pathological states.³⁶

2. Ligand-directed chemistry

In order to overcome the further perturbation, Hamachi *et al.* have improved the conventional affinity labeling method and developed ligand-directed chemistry for one-step traceless labeling of endogenous protein. To date, they have achieved two kinds of ligand-directed chemistry, that is ligand-directed tosyl (LDT)³⁷ and acyl imidazole (LDAI)³⁸ chemistry (Figure 11a,11b). The S_N2-type reaction (for LDT) or acyl transfer reaction (for LDAI) induced by proximity effect between the reactive group and a nucleophilic amino acid residue result in the release of the ligand moiety upon the labeling reaction.

Taking advantage of LDT chemistry, endogenous proteins can be easily converted into biosensors for sensing their ligand binding not only *in vitro* but also *in cell* without genetic engineering. Hamachi's group reported the adequate characterization of the ligand-bound and unbound structures of ¹⁹F-labeled carbonic anhydrase I by X-ray crystallography, and an unprecedented quantitative comparison of the protein's dynamics was conducted in RBCs and *in vitro* using ¹⁹F exchange spectroscopy NMR. They also reported that LDT chemistry was applicable to the construction of the photo-reactive FK506-binding protein 12 (FKBP12) by tethering a photo-cross linker in mammalian cells. The UV-induced covalent cross-linking of FKBP12 with its interacting proteins was achieved in living cells. As well as LDT chemistry, LDAI chemistry was utilized to manipulate and analyze an endogenous protein. The fluorescein-labeled endogenous FR by LDAI chemistry works as a fluorescent biosensor on the live KB cell surface, which allowed to carry out *in situ* ligand binding kinetic analyses.

In addition to LDT and LDAI chemistries, they have developed a new protein labeling strategy called Affinity-Guided DMAP (AGD)³⁹ chemistry (Figure 11c), which conducted a catalytic reaction on a protein surface for native proteins labeling using a series of ligand-tethered DMAP catalysts and thiophenylester acyl donors, which showed rapid and selective protein modifications on live cell expressing the target protein. Recently, the AGD chemistry-based semisynthetic reactive lectins (sugar-binding proteins) were successfully constructed, which are able to selectively label glycoproteins. The reactive lectins coupled with immunoprecipitation and mass spectrometry

(MS)-fingerprinting techniques allowed to develop a new tool for labeling and profiling glycoproteins on living cells.

These results show that ligand-directed chemistry, which can target endogenous proteins, allows the specific and traceless detection/imaging and the one-step construction of functionalized proteins. Other groups also followed the design concept of ligand-directed chemistry, reporting other types of ligand-directed chemistry, using acyl phenol group for acylation⁴⁰ and 5-sulfonyl tetrazole group and *O*-nitrobenzoxadiazole group for S_NAr reaction^{41,42} as the reactive handles (Details are shown in chapter 3).

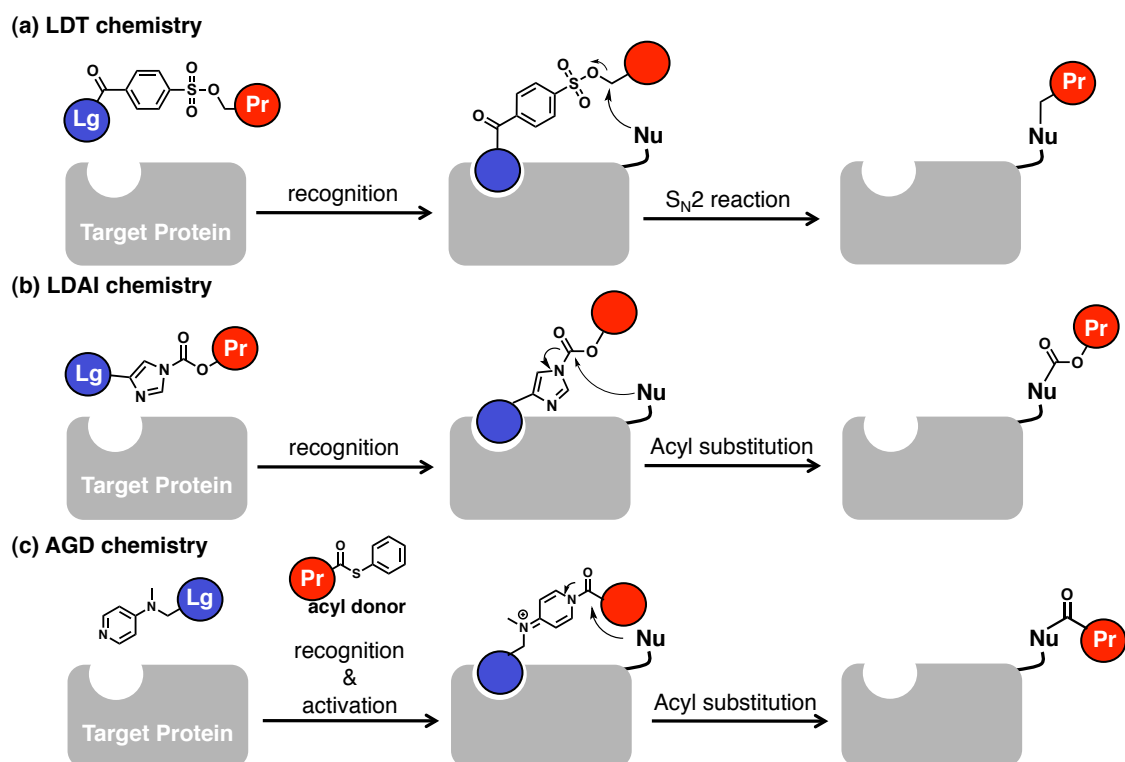


Figure 11. Schematic illustration of (a) LDT, (b) LDAI and (c) AGD chemistry. Lg, protein ligand; Pr, chemical probe; Nu, nucleophilic amino acid.

Summary of this thesis

As described above, many scientists have developed the various methodologies for detection/imaging or labeling/engineering of proteins. However, it still remains extremely challenging to develop the tools and techniques for monitoring or imaging of protein activity and to construct proteins with potential for improved performance and new catalytic activities without genetic engineering. In this thesis, I developed the novel chemical approaches for detection and engineering of endogenous proteins, which allows to visualize protein activity and to construct a caged enzyme. This thesis mainly consists of three chapters, the contents of which are briefly summarized as follows:

In chapter 1, I describe the development of signal-amplifiable self-assembling ^{19}F -MRI probes for the specific detection and imaging of enzyme activity. The supramolecular based turn-on probe (that is, self-assembly and enzymatic cleavage-triggered disassembly of the probe) was applicable for two different enzymes, nitroreductase and matrix metalloproteinase (MMP). Using the ^{19}F probe for MMP, the activity of endogenously secreted MMP in the media of tumor cells was detected by ^{19}F -MRI phantom techniques.

In chapter 2, I describe a rational one-step construct of caged enzyme by LDAI chemistry based labeling method, which is based on the transient tethering of a moderate inhibitor to the active site on an enzyme surface. I successfully demonstrated that the activity of the caged carbonic anhydrase was almost completely suppressed and fully recovered by photoirradiation in the crude conditions (such as cell lysate) as well as in test tube settings.

In chapter 3, I describe the development of the novel traceless protein sulfonylation method, called LDSP (ligand-directed *N*-sulfonyl Pyridone) chemistry. I demonstrated the detailed properties of LDSP chemistry, which enables the rapid and specific modification of endogenous proteins not only *in vitro* but also under living cell conditions.

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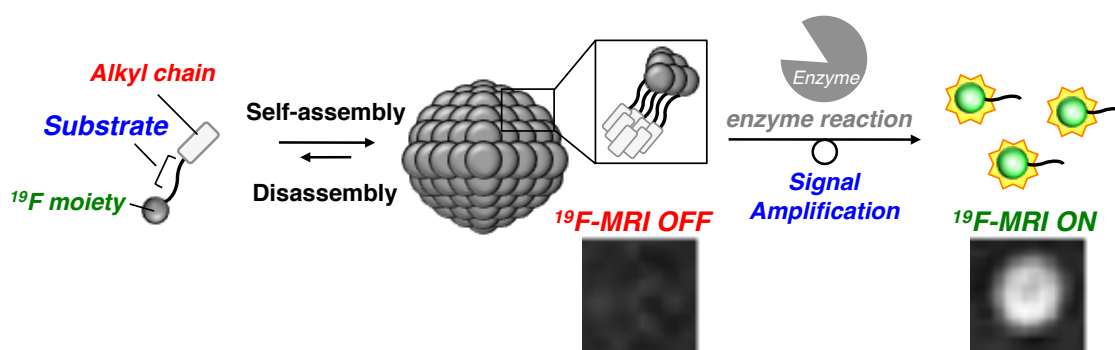
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***Specific Detection and Imaging of Enzyme Activity by Signal-Amplifiable
Self-Assembling ^{19}F -MRI Probes***

Abstract

Specific turn-on detection of enzyme activities is of fundamental importance in drug discovery research, as well as medical diagnostics. Although magnetic resonance imaging (MRI) is one of the most powerful techniques for noninvasive visualization of enzyme activity, both *in vivo* and *ex vivo*, promising strategies for imaging specific enzymes with high contrast have been very limited to date. I describe herein a novel signal-amplifiable self-assembling ^{19}F -NMR/MRI probe for turn-on detection and imaging of specific enzymatic activity. In NMR spectroscopy, these designed probes are “silent” when aggregated, but exhibit a disassembly-driven turn-on signal change upon cleavage of the substrate part by the catalytic enzyme. Using these ^{19}F probes, nanomolar levels of two different target enzymes, nitroreductase (NTR) and matrix metalloproteinase (MMP), could be detected and visualized by ^{19}F -NMR spectroscopy and MRI. Furthermore, I have succeeded in imaging the activity of endogenously secreted MMP in cultured media of tumor cells by ^{19}F -MRI, depending on the cell lines and the cellular conditions. These results clearly demonstrate that the turn-on ^{19}F probes may serve as a screening platform for the activity of MMPs.



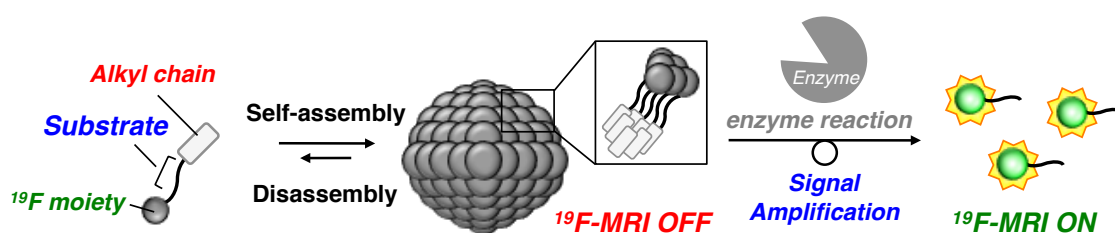
1-1. Introduction

There are many uses for the specific detection and imaging of enzyme activity *in vivo* in both drug discovery research and medical diagnostics. Chemical “switchable” probes, which induce detectable signal changes in response to specific enzyme reactions, are particularly useful for imaging enzyme activities because they allow selective and sensitive detection with high signal-to-noise ratios.^{1,2} Many fluorescent switching probes for detecting enzyme activities have now been developed.³ However, fluorescence techniques are, in general, not suitable for *in vivo* imaging due to the limited light transmission and its scattering in animal bodies. Although magnetic resonance imaging (MRI) is known to be an adequate technique for *in vivo* studies,⁴ switching strategies for MRI are as yet quite limited. As a pioneering example, ¹H magnetic resonance (MR)-type switching probes have been reported as powerful tools for *in vivo* enzyme imaging by Meade’s group.^{4a} The switching mechanisms are mainly based on paramagnetic relaxation enhancement (PRE) using paramagnetic metal complexes and their coordinated water molecules.⁵ For target-specific imaging, heteronuclear MRI techniques have also attracted considerable attention. In particular, ¹⁹F-MRI is anticipated to be a promising method because ¹⁹F has a high NMR sensitivity approaching that of ¹H (83 % relative to ¹H) and there are no background signals even *in vivo*.⁶ Despite the potential importance of ¹⁹F-MRI for medical diagnostics, promising strategies for imaging specific enzymes with high contrast are as yet very limited.⁷ There are also some problems associated with ¹⁹F-MRI that have yet to be overcome, namely low sensitivity and poor efficiency of probe delivery. Therefore, ¹⁹F-MRI is an immature technique and both more fundamental studies on ¹⁹F probes and improvement of the instrumentation are needed to establish it as an *in vivo* imaging tool.

Hamachi *et al.* have developed a unique self-assembling turn-on ¹⁹F probe for the specific detection of target proteins composed of a hydrophilic protein ligand and a hydrophobic ¹⁹F detection modality (Figure 1a).⁸ These ligand-tethered probes clearly exhibit a disassembly-driven turn-on signal change upon specific binding to the target proteins.^{8,9} Hamachi *et al.* have recently established that moderate stability of the aggregates is critical for obtaining an ideal off/on response, and the stability can be modulated by the change of hydrophobicity/hydrophilicity balance of the probe (Figure 1b).¹⁰ However, since this turn-on sensing requires 1:1 complex formation between a

reaction. Using these new self-assembling ^{19}F probes, detection and imaging of two different enzymes, nitroreductase (NTR) and matrix metalloproteinase (MMP), at the nanomolar level has been accomplished by ^{19}F -NMR spectroscopy and MRI. Furthermore, I have succeeded in the turn-on imaging of the activity of MMP endogenously secreted in the cultured media of tumor cells by the ^{19}F -MRI phantom technique, which clearly demonstrated that the substrate-based turn-on probes are capable of serving as a platform for screening the MMP activities of several mammalian cell lines.

Scheme 1. Schematic illustration of a substrate-based self-assembling ^{19}F probe for turn-on detection of enzymatic activity.



1-2. Results and discussion

1-2-1. Substrate-based ^{19}F -MRI probes for turn-on detection of nitroreductase activity

As proof-of-principle experiments, I initially employed nitroreductase (NTR) as a target enzyme.¹¹ According to guidelines established by ligand-tethered self-assembling turn-on probes, a new ^{19}F probe **1** was designed with the ability to self-assemble, composed of a hydrophobic alkyl chain appended *para*-nitrobenzene as a substrate moiety for NTR, and a hydrophilic ^{19}F -containing detection moiety. This ^{19}F moiety (3,5-bis(trifluoromethyl)benzene (FB)-appended aspartate; compound **2**) was linked to *para*-nitrobenzene through a carbonate ester bond.¹² This “substrate-based probe” alone is “NMR-silent” because of its self-assembly, but should give a distinct ^{19}F signal from the monomeric FB unit produced upon its cleavage triggered by nitro group reduction with NTR (Figure 2 a).

To test the turn-on property of probe **1**, I initially monitored the ^{19}F -NMR spectra

with or without NTR. When **1** (50 μ M) was dissolved in a buffer solution, no ^{19}F -NMR signal was observed. In contrast, a sharp signal appeared at $\delta = -62.9$ ppm upon addition of NTR (0.83 μ M) and incubation for 60 min at 37 $^{\circ}\text{C}$ (Figure 2b). This new signal could be assigned to compound **2** by ^{19}F -NMR and HPLC analyses, which was produced by an NTR-catalyzed tandem reaction. These results clearly showed that nitro group reduction followed by β -1,6-elimination indeed occurred in the presence of NTR, resulting in the turn-on signal change. Probe **1** alone showed relatively high light scattering at 600 nm (O.D.₆₀₀, Figure 2c) in a buffer solution. Dynamic light-scattering (DLS) measurements of **1** alone also showed aggregates with a mean diameter of 100 nm (Figure 2d), whereas negligible scattering intensity was observed after 60 min incubation with NTR. These results indicated that the enzymatic reaction induced destruction of the aggregates. Thus, it is clear that **1** can serve as an off/on-type probe driven by enzymatic reaction-triggered disassembly. Furthermore, probe **1** allowed to visualize the enzymatic activity in a ^{19}F MRI phantom technique owing to its ideal off/on response, as shown in Figure 2e.

1-2-2. Development of an MMP2 substrate-based ^{19}F probe and detection of MMP2 activity in a test tube

Benefiting from the modular design of my self-assembling probes, a new substrate-based probe was readily extended to detection of the activities of other enzymes. Matrix metalloproteinase 2 (MMP2), a target enzyme of next substrate-based self-assembling probe, is a protease secreted from cell membranes and is involved in cell migration and remodeling processes through its degradation action on the extracellular matrix.¹³ MMP2 and MMP9 are also related to tumor invasion, metastasis, and angiogenesis, making them valuable targets for cancer diagnosis and treatment. Similar to the NTR probe, an off/on-type ^{19}F probe **3a**, containing a hydrophilic MMP2 substrate peptide (GPLG-VRG) was designed and synthesized.¹⁴ In this probe, a hydrophobic dodecyl chain (C12) is attached to the *N*-terminus of the substrate through a tetraethyleneglycol linker (O4 linker), and FB is tethered to the substrate through an additionally incorporated lysine at the C-terminus (Figure 3). Upon cleavage by MMP2, a hydrophilic peptide containing FB (VRGK(FB), compound **5**) is expected to be produced, which should give a clear ^{19}F NMR signal.

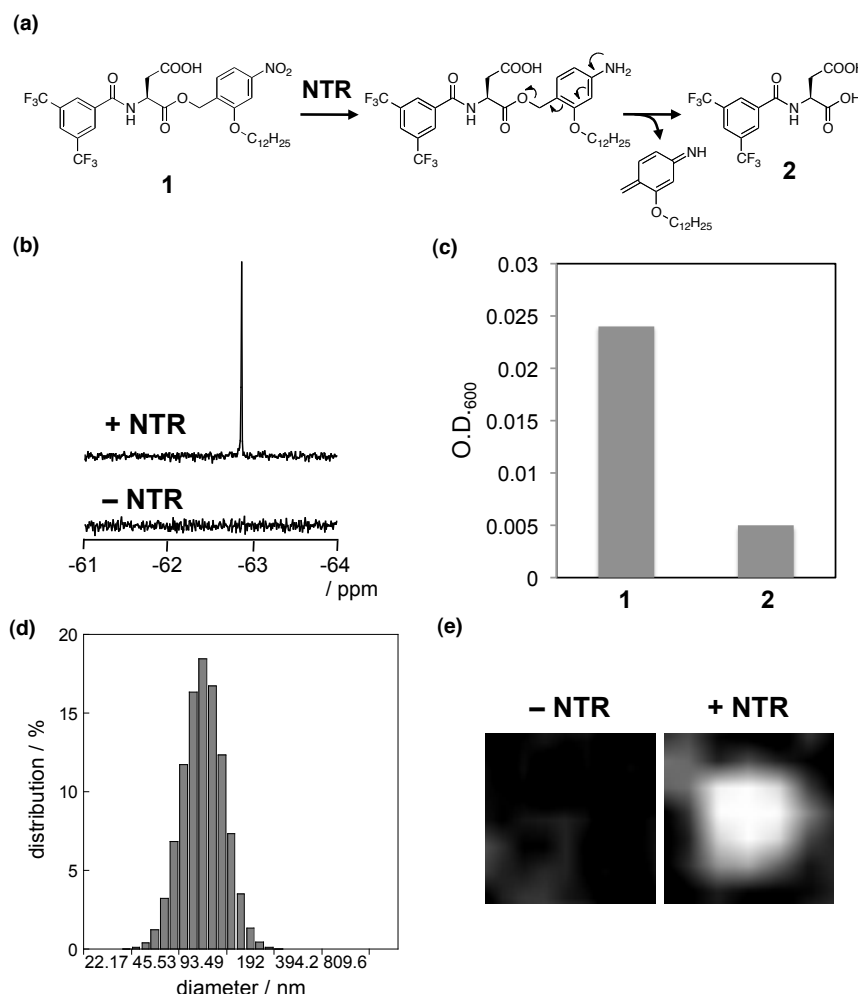


Figure 2. Substrate-based turn-on ¹⁹F probe for detection of nitroreductase activity. (a) Chemical structure of probe **1**, and proposed reduction and ensuing elimination mechanism with NTR. (b) ¹⁹F NMR spectra of probe **1** in the absence of NTR (bottom) and after incubation with NTR (0.83 μM) at 37°C for 60 min (top). (c) Optical densities of probe **1** (50 μM, left) and compound **2** (50 μM, right). (d) DLS analysis of the particle-size distribution of probe **1** (50 μM). (e) ¹⁹F MR images of probe **1** in the absence of NTR (left) and after incubation with NTR (0.83 μM) at 37°C for 60 min (right). All experiments illustrated in this figure were performed in 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (pH 7.2, containing nicotinamide adenine dinucleotide phosphate (NADPH; 1.0 mM), D₂O (10%), and trifluoroacetic acid (TFA; 0.1 mM) as an internal standard (δ = -75.6 ppm)).

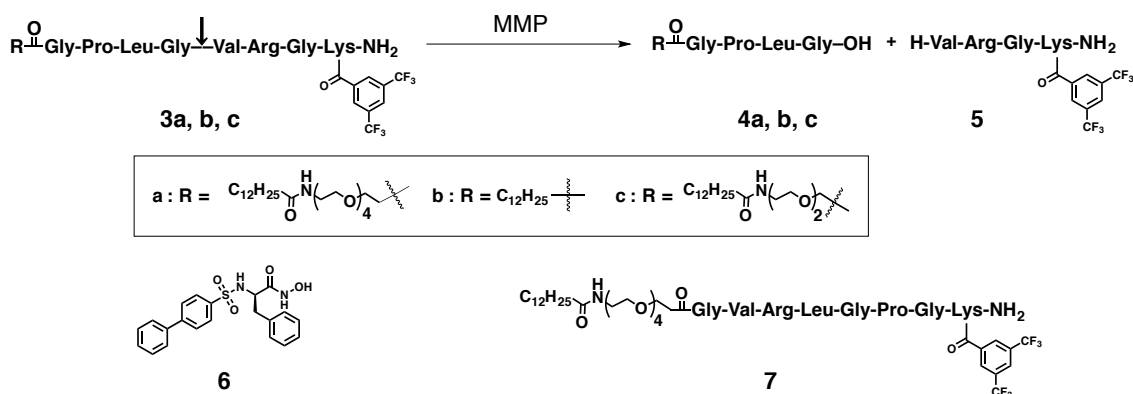


Figure 3. Chemical structures of probes **3a–c**, **7**, cleaved compounds **4a–c**, **5** and MMP2 inhibitor **6**. The down-pointing arrow represents the cleavage site by MMP2.

Firstly, it was confirmed that probe **3a** alone (50 μ M) showed no ^{19}F -NMR signal in a buffer solution. I then found that a sharp signal appeared at $\delta = -62.9$ ppm upon addition of MMP2 (15 nM) and incubation for 24 h at 37 $^{\circ}\text{C}$, which was assigned compound **5** by HPLC analyses (Figure 4a,b). In contrast, no ^{19}F signal recovery was observed after incubation of probe **3a** with MMP2 in the presence of an MMP2 inhibitor (compound **6**, 100 μ M, Figure 4c).¹⁵ Probe **7**, in which I replaced the substrate part with a nonsubstrate peptide (GVRLGPG), also gave no response with MMP2 (Figure 4d).^{14a} Given these results, I demonstrated that an ideal turn-on ^{19}F probe for the specific detection of MMP2 activity could be successfully prepared in a test tube. More importantly, the detection limit of MMP2 with probe **3a** was found to be less than 0.5 nM (Figure 4e), which is at least 10,000-fold lower than those of ligand-tethered probes Hamachi *et al.* previously reported (i.e., 5 μ M in the case of a benzenesulfonamide-appended ^{19}F probe for the detection of human carbonic anhydrase I).^{8a}

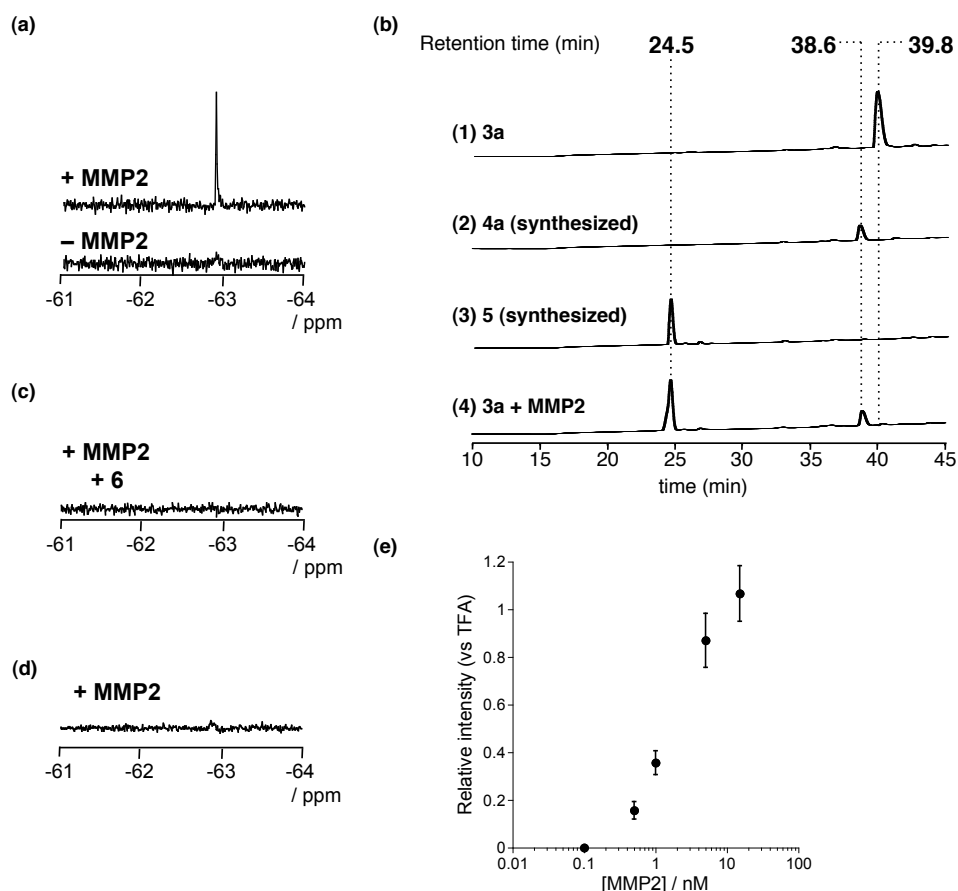


Figure 4. Substrate-based turn-on ^{19}F probes for the detection of matrix metalloproteinase-2 (MMP2) activity. (a) ^{19}F -NMR spectra of probe **3a** (100 μM) in the absence of MMP2 (bottom) and after incubation with MMP2 (15 nM) at 37°C for 24 h (top). (b) (1-3) Reversed-phase HPLC diagrams of purified compounds **3a** (1), **4a** (2) or **5** (3). (4) Reversed-phase HPLC diagram of the reaction mixture of **3a** (50 μM) with MMP2 (15 nM) after 24 h incubation at 37°C. Eluent: A (acetonitrile containing 0.1% TFA) : B (0.1% TFAaq) = 20 : 80 (0 min) $\rightarrow$ 20 : 80 (10 min) $\rightarrow$ 90 : 10 (45 min). (c) ^{19}F -NMR spectrum of probe **3a** (100 μM) in the presence of MMP2 (15 nM) and inhibitor **6** (100 μM). (d) ^{19}F -NMR spectra of scrambled-type probe **7** incubated with MMP2 (15 nM) at 37°C for 18 h. (e) Semi-log plot of relative ^{19}F -NMR signal intensity versus MMP2 concentration. Error bars represent standard deviations of three experiments. All experiments illustrated in this figure were performed in 50 mM HEPES buffer (pH 7.5 containing NaCl (100 mM), CaCl_2 (10 mM), D_2O (10%), and TFA (0.1 mM) as an internal standard, $\delta = -75.6$ ppm).

1-2-3. Examination of the self-assembling properties and MMP2 response of the MMP2-substrate-based ^{19}F probes

Next, I investigated the self-assembling properties of the MMP2-type ^{19}F probes, in order to explore the response mechanism in detail. Fluorescent microscopy of aggregates of **3a** loaded with Nile Red showed spherical shapes with diameters ranging from 0.5 to 1 μm (Figure 5a).¹⁶ From DLS measurements, a buffer solution containing **3a** alone showed aggregates with a mean diameter of 600 nm (Figure 5b), whereas negligible scattering intensity was detected upon cleavage by MMP2. Aggregation of **3a** resulted in a dramatic increase in the apparent molecular mass (M_r) from 1.5×10^3 (monomer) to 7×10^7 Da. It is reasonable that such an increase in M_r caused a significant increase in the ^{19}F relaxation rate, affording no NMR signals, as in the case of the ligand-tethered ^{19}F probes.⁸

Hamachi *et al.* previously clarified that the stability of the aggregates of the ligand-tethered probes was critical for their off/on response.¹⁰ In order to examine the relationship between the aggregation property and reactivity of the substrate-based probes, I additionally prepared two further ^{19}F probes **3b** and **3c**. Compound **3a** has a tetraethyleneglycol (O4) linker between the hydrophobic alkyl chain and the *N*-terminus of the substrate, whereas **3b** has no linker and **3c** has a diethyleneglycol (O2) linker (Figure 3). These two probes alone showed no ^{19}F -NMR signals in buffer solutions, indicating that they both formed self-assembled aggregates like probe **3a**. The critical aggregation concentration (CAC) values of all three probes (**3a**, **3b** and **3c**) were about 5 μM (Figure 5c).¹⁶ The CAC values of compounds **4a** and **5** (the two products from probe **3a** hydrolyzed by MMP2, as shown in Figure 3), on the other hand, were higher than 100 μM , which clearly indicated that the self-assembled aggregates of **3a** collapsed due to the MMP2 enzymatic reaction under the present conditions. Interestingly, the initial rates of ^{19}F -NMR signal recovery were different among probes **3a**, **3b** and **3c**, specifically 20, 7.5, and 15 $\mu\text{M h}^{-1}$, respectively (Figure 5d). These findings suggested that the flexibility of the linker moiety is critical for the efficient access of MMP2 so as to result in a rapid response of these substrate probes. The k_{cat} value for MMP2 determined by using the ideal off/on-type probe **3a** was in good agreement with that reported by using a monodisperse MMP2 substrate (Table 1 and Figure 5e).¹⁷

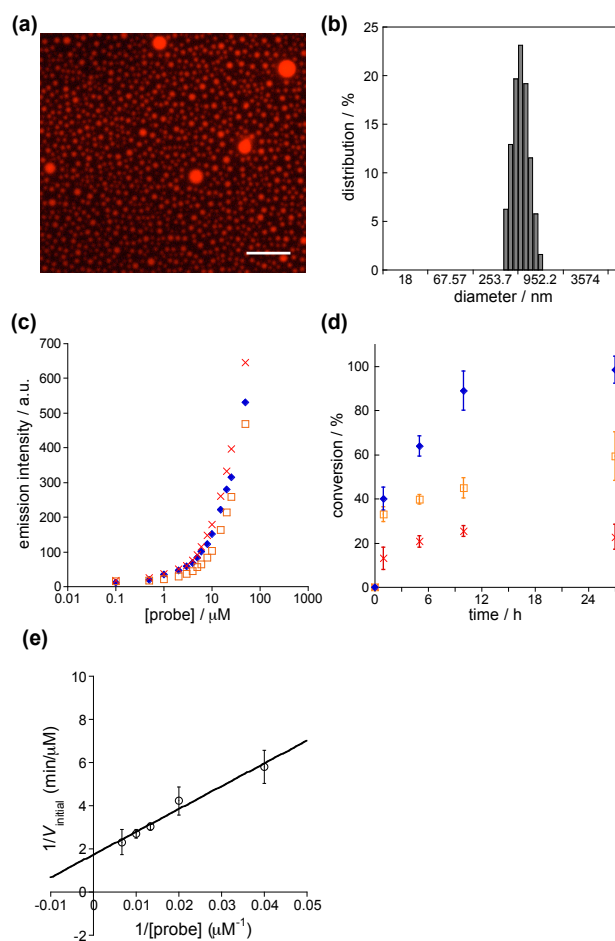


Figure 5. (a, b) Self-assembling properties of MMP2-type probe **3a**. (a) Fluorescent microscopy image of probe **3a** (50 μM) loaded with Nile Red (5 μM). Scale bar 5 μm . (b) DLS analysis of the particle-size distribution of probe **3a** (50 μM). (c) Fluorescent intensity changes of Nile Red (5 μM) with the addition of probe **3a** (◆), **3b** (×), or **3c** (□) ($\lambda_{\text{em}} = 575 \text{ nm}$). (d) Time profiles of hydrolysis reactions of probes **3a** (◆), **3b** (×), and **3c** (□) with MMP2. All experiments illustrated in this figure were performed in 50 mM HEPES buffer (pH 7.5 containing NaCl (100 mM), CaCl_2 (10 mM), D_2O (10%), and TFA (0.1 mM)). (e) Lineweaver-Burk plot of probe **3a** with MMP2 (3 nM). Varying concentrations of **3a** (25, 50, 75, 100 and 150 μM) were incubated with 3 nM MMP2 at 37°C in 50 mM HEPES buffer (pH 7.5, 100 mM NaCl, 10 mM CaCl_2). At intervals, aliquots were diluted in equal volume of 200 mM HEPES (pH 6.8 containing 20 mM EDTA). A solution of 0.1 mg/mL fluorescamine in acetone was added with continuous mixing to an equal volume of the HEPES/EDTA mixture and 15 min incubated at room temperature. Fluorescence was read in a plate reader (TECAN Infinite® 200 PRO). Excitation was at 390 nm and emission was measured at 475 nm.

Table 1. Kinetic parameters of purified MMP2 for the hydrolysis of self-assembling ^{19}F -probe and monodispersed-type peptide.

Substrate derivatives	kinetic parameters		
	k_{cat} (s^{-1})	$1/K_{\text{m}}$ (M^{-1})	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1}\text{M}^{-1}$)
Probe 3a ^[a]	3.9	1.6×10^4	6.3×10^4
Ac-PLGVRG-OC ₂ H ₅	4.1	3.4×10^3	1.4×10^4

1-2-4. Detection and imaging of MMP activity in MMP-secreted cultured media from tumor cells

With the substrate-based turn-on ^{19}F probe **3a** for MMP2 in hand, I finally applied it for the detection of MMP activity in a medium of cultured tumor cells. HT-1080 cells, a model cell line secreting MMP2 or MMP9,¹⁸ were cultured without serum for 48 h and then the supernatant was collected. The supernatant was mixed with 50 μM probe **3a**, and then incubated for 24 h in the absence or presence of MMP2 inhibitor (compound **6**, 100 μM). A sharp ^{19}F -NMR signal appeared in the absence of the inhibitor **6**, whereas no signal was detected in its presence (Figure 6a). ^{19}F -MRI phantom experiments by using this turn-on probe (**3a**) allowed to visualize the MMP activity with a high signal-to-noise ratio. As shown in Figure 6b, a clear ^{19}F -MR image was observed for the supernatant of HT-1080 cells, whereas no clear ^{19}F -MR image was obtained in the presence of inhibitor **6**.¹⁹ Figure 6c shows the dependence of contrast on the number of scans accumulated, and the contrast was distinctly higher with increasing number of accumulations. An image with sufficient contrast was nevertheless successfully obtained after just 900 accumulations (about 15 min).

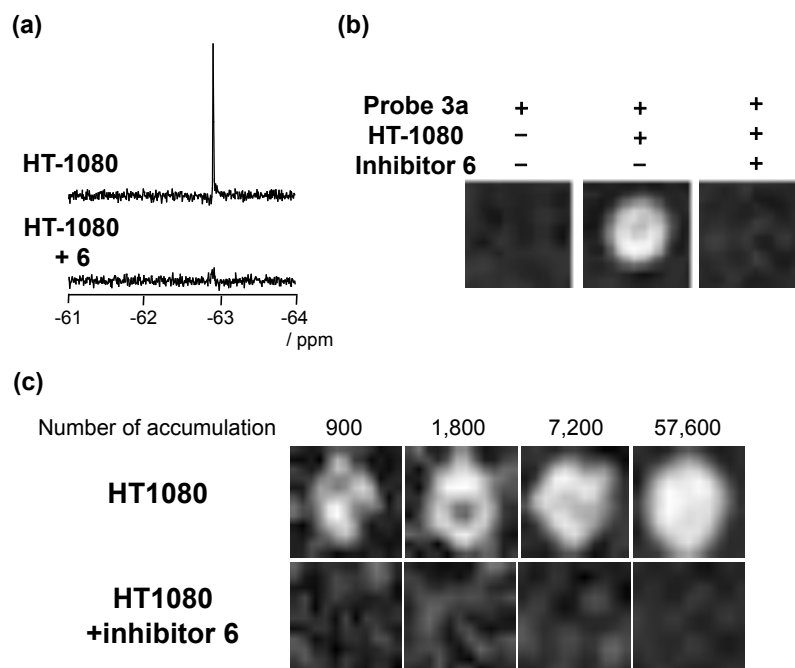


Figure 6. (a) ^{19}F -NMR spectra of probe **3a** (50 μM) in the supernatant of HT-1080 cells in the absence or presence of inhibitor **6** (100 μM). The samples were reacted at 37°C for 48 h in the cultured media (collected from 48 h pre-incubated cells without serum, including D_2O (10%) and TFA (0.1 mM) as an internal standard). (c) ^{19}F -MR images of probe **3a** (50 μM) in the supernatant of HT-1080 cells in the absence or presence of inhibitor **6** (100 μM). The samples were incubated at 37°C for 48 h in each cultured medium (collected from 48 h pre-incubated cells without serum, including D_2O (10%) and TFA (0.1 mm) as an internal standard).

Similarly, well-defined ^{19}F -NMR/MRI signals were observed for HeLa cells, which have previously been reported to be an MMP2-secreting cell line (Figure 7a).²⁰ In contrast, no signals were detected for Jurkat²¹ or COS7 cells,²² two non-MMP2-secreting cell lines. Therefore, it is clear that our self-assembling ^{19}F probe for imaging of enzymatic activity may serve as a screening platform for the activity of MMPs. More interestingly, in the case of human breast cancer MCF7 cells, distinct ^{19}F -NMR and MR signals were only observed when the cells were cultured under conditions of hypoxia, and not under normoxia (Figure 7b). Similarly, by treatment of MCF7 cells with antimycin A or rotenone, a sharp ^{19}F -NMR signal appeared (Figure

7c). It has recently been reported that conditions of hypoxia or addition of these compounds can induce or promote MMP2 secretion in live MCF7 cells by producing reactive oxygen species (ROS).²³ The present results demonstrate that the turn-on ^{19}F probes may potentially be useful for visualizing drug-triggered enzyme induction in live tumor cells.

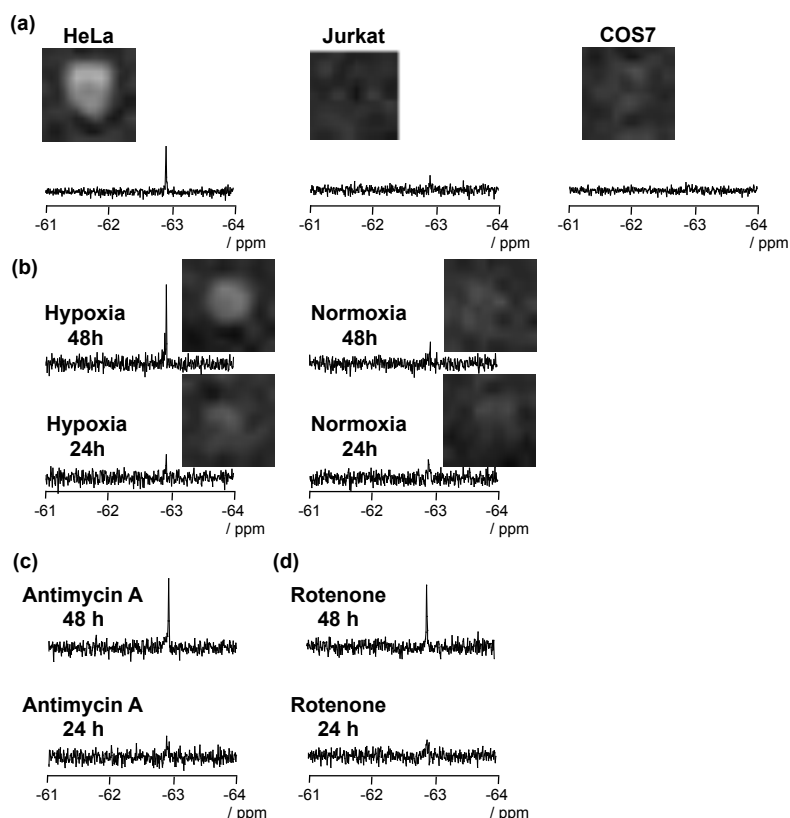


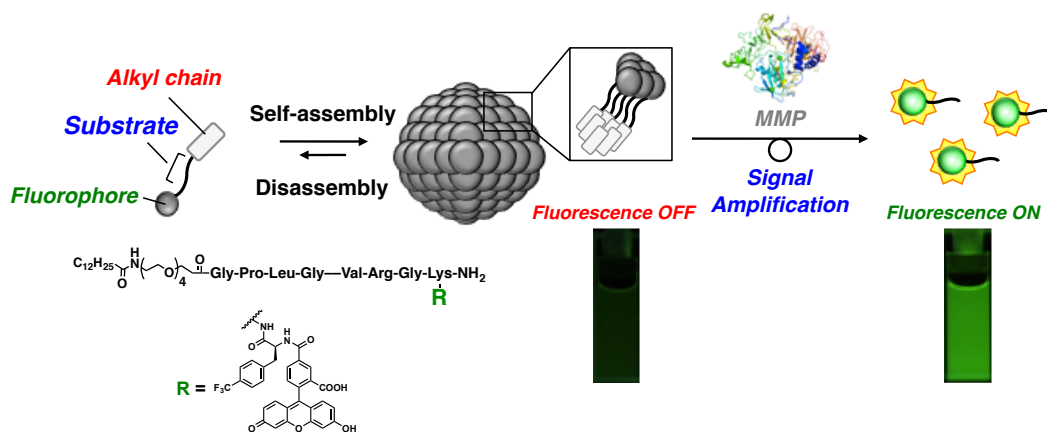
Figure 7. (a) ^{19}F -NMR spectra and MR images of probe **3a** in the supernatants of various cell lines (HeLa, Jurkat, and COS7 cells). The samples were incubated at 37°C for 48 h in each cultured medium (collected from 48 h pre-incubated cells without serum, including D_2O (10%) and TFA (0.1 mM) as an internal standard). (b) ^{19}F -NMR spectra and MR images of probe **3a** in the supernatants of MCF7 cells cultured under the indicated conditions. The samples were incubated at 37°C for 48 h in the cultured media (collected from pre-incubated cells under hypoxia or normoxia conditions (24 and 48 h pre-incubation), including D_2O (10%) and TFA (0.1 mM) as an internal standard). (c, d) ^{19}F -NMR spectra of probe **3a** (50 μM) in the supernatant of MCF7 cells treatment of antimycin A (5 μM , c) or rotenone (5 μM , d). Each cultured medium was collected in the pre-indicated time as shown in this figure. The samples were reacted at 37°C for 48 h in these cultured media including 10% D_2O and 0.1 mM TFA as an internal standard.

1-3. Conclusion

I have developed new signal-amplifiable self-assembling ^{19}F -NMR/MRI probes for the sensitive detection and imaging of specific enzymatic activity. The simple principle for the off/on response (that is, self-assembly and enzymatic cleavage-triggered disassembly of the probe) should be applicable for various target enzymes by appropriately replacing the substrate module, as shown in this chapter for two different enzymes (NTR and MMP2). The detection limit of these substrate-based probes was found to be less than 0.5 nM, which is at least 10,000-fold lower than that of the ligand-tethered ^{19}F probes. Moreover, by using novel substrate-based self-assembling probe, I succeeded in imaging the activity of endogenously secreted matrix metalloproteinase (MMP, a valuable target for cancer diagnosis and treatment) in the media of tumor cells by ^{19}F -MRI phantom techniques, depending on the cell lines and the cellular conditions. From a practical viewpoint, it may be fair to say that the sensitivity of ^{19}F is considerably lower than that of conventionally used Gd-based ^1H -MRI probes. However, further advances in both instrumentation and chemistry (e.g., increasing the number of equivalents of fluorine in the probe for higher sensitivity) may overcome this limitation, and the ^{19}F -NMR/MRI technique may become a more powerful modality for target-specific *in vivo* imaging.

Also, this supramolecular chemistry-based approach could be applied to the specific fluorescent detection of MMP activity, not only the ^{19}F detection, in the conditioned media of tumor cells²⁴, which implied that the signal-amplifiable self-assembling probes were flexibly designed for various detection modalities (Scheme 2). I believe that the signal-switching mechanism based on self-assembling turn-on probes should be in use for monitoring enzyme activities in cells and *in vivo*.

Scheme 2. Schematic illustration and results of a substrate-based self-assembling fluorescent nanoprobe for turn-on detection of enzymatic activity in cultured media from tumor cells.



1-4. Experimental Section

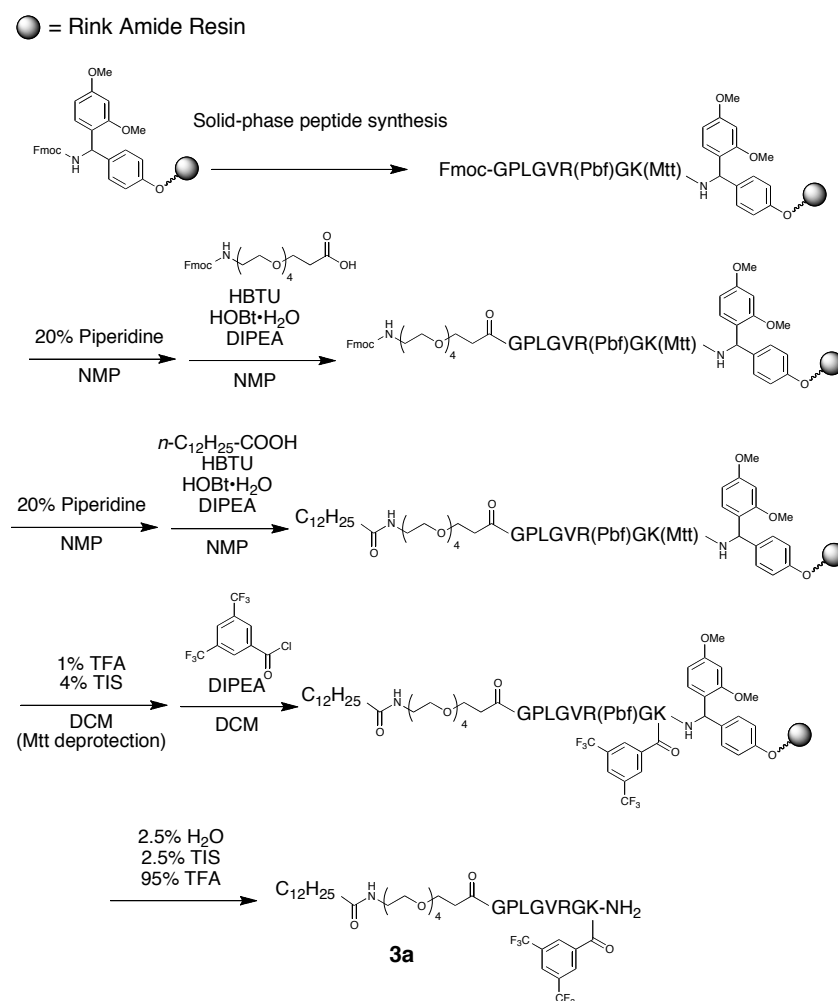
General materials and methods

All proteins and chemicals were obtained from commercial suppliers (Aldrich, Tokyo Chemical Industry (TCI), Wako Pure Chemical Industries, Sasaki Chemical, Watanabe Chemical Industries, Calbiochem) and were used without further purification. Thin-layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ precoated aluminum sheets (Merck) and spots were visualized by fluorescence quenching or ninhydrin staining. Chromatographic purifications were conducted by flash column chromatography on silica gel 60 N (neutral, 40–50 μ m, Kanto Chemical). ¹H-NMR spectra were recorded from solutions in deuterated solvents on a Varian Mercury 400 (400 MHz) spectrometer and calibrated to the residual solvent peak or tetramethylsilane (δ = 0 ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, dd = doublet of doublets. ¹⁹F-NMR spectra were recorded on a JEOL ECX-400P (376.5 MHz) spectrometer and calibrated with TFA (δ = –75.6 ppm). Standard parameters were used: spectral width 36 kHz, pulse length 8 μ s, acquisition time 0.46 s, and relaxation delay 0.50 s. A 0.1 Hz line broadening was applied. The number of accumulations was 1,024. ¹⁹F-MR images were obtained on a 7 T Bruker-Biospec 70/20 USR system (282 MHz for ¹⁹F) with 72 mm i.d.

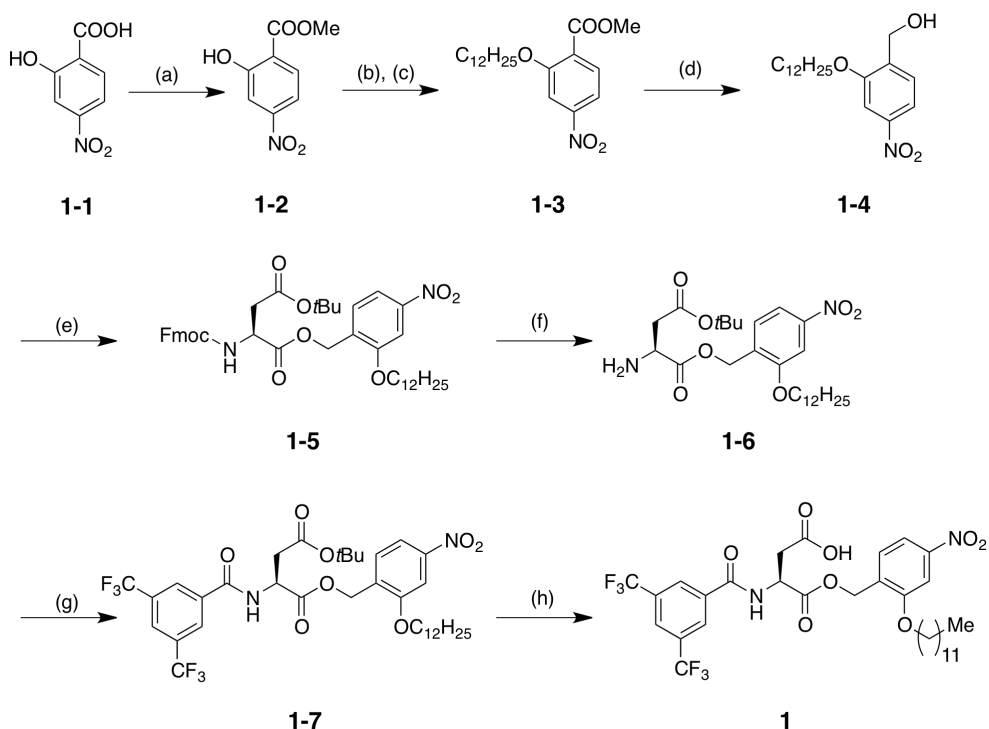
$^1\text{H}/^{19}\text{F}$ radiofrequency (RF) volume coil (Bruker Biospin, Germany). UV/Vis spectra were recorded on a Shimadzu UV-2550 spectrometer. DLS measurements were performed with a NICOMP 380zls apparatus (wavelength of the laser 520 nm). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on an Autoflex II instrument (Bruker Daltonics) by using α -cyano-4-hydroxycinnamic acid (CHCA) as the matrix. High-resolution electrospray ionization quadrupole Fourier-transform mass spectroscopy (HR-ESI Qq-LTMS) was carried out on a Bruker Apex-Ultra (7 T) mass spectrometer. HPLC purification was conducted with a Lachrom chromatograph (Hitachi, Japan).

Synthesis

Scheme 3. Synthetic scheme of probe **3a**.



Probe **3 a** was synthesized manually on Rink amide resin (Novabiochem) by a standard Fmoc-based solid-phase peptide synthesis protocol (Scheme 3). Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Lys(Mtt)-OH were used as building blocks (Pbf: 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl; Mtt: 4-methyltrityl; Fmoc: fluorenylmethyloxycarbonyl). Fmoc deprotection was performed with 20 % piperidine in *N*-methylpyrrolidone (NMP); Mtt deprotection was performed in dichloromethane containing 1 % TFA and 4 % triisopropylsilane (TIS). Coupling reactions were performed with a mixture of Fmoc-amino acid (3 equiv), *O*-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU; 3 equiv), 1-hydroxybenzotriazole hydrate (HOBt·H₂O; 3 equiv), and diisopropylethylamine (DIPEA; 6 equiv) in NMP at room temperature. All coupling and Fmoc/Mtt deprotection steps were monitored by the Kaiser test. Following chain assembly, global deprotection and cleavage from the resin was performed with TFA containing 2.5 % TIS and 2.5 % H₂O. The crude peptide products were precipitated by the addition of diethyl ether and purified by reversed-phase HPLC (eluent: A (acetonitrile containing 0.1 % TFA)/B (0.1 %aq. TFA)=20:80 (0 min)→20:80 (10 min) → 90:10 (45 min)). The obtained compound was lyophilized, and the residue was dissolved in 5 % hydrogen chloride/methanol reagent (TCI). The solution was concentrated and the residue was dried *in vacuo* to yield **3 a** as the hydrogen chloride salt. Probe **3 a** was characterized by reversed-phase HPLC and MALDI-TOF MS (calcd for $[M+H]^+$: 1465.83; found: 1465.55).

Scheme 4. Synthetic scheme of **1**.

Reaction conditions: (a) SOCl_2 in MeOH, (b). 1-dodecanol, MsCl , TEA in DCM, (c) K_2CO_3 in DMF, (d) DIBAL in THF, (e) Fmoc-Asp(OTBu)-OH, EDCI, DMAP in DMF, (f) piperidine in DMF, (g) 3,5-bis(trifluoromethyl)benzoyl chloride, DIPEA in DCM (h) TFA in DCM

Compound 1-2 To 10 mL of MeOH cooled to 0°C was added dropwise 1.5 mL of SOCl_2 (20 mmol). To the resulting clear solution was added 1831 mg (10 mmol) of **1-1**, and the mixture was refluxed for 6 h, at which time TLC indicated the reaction to be complete. The resulting solution was cooled to room temperature, and the solvent was evaporated as much as possible. The residue was partitioned between CHCl_3 ($100\text{ mL} \times 3$) and water (100 mL). The collected organic layer was washed with sat. NaHCO_3 aq (100 mL) and brine (150 mL), collected, dried over MgSO_4 . Filtration, and concentration *in vacuo* gave 1904 mg (97 %) of **1-2** as a yellow solid.

$^1\text{H-NMR}$ (400 MHz, CDCl_3 , TMS): δ/ppm , 11.0 (s, 1H), 8.02 (d, $J_H = 8.4$ Hz, 1H), 7.82 (s, 1H), 7.71 (dd, $J_H = 2.3, 8.8$ Hz, 1H), 4.03 (s, 1H).

Compound **1-3****[First step]**

A solution of 1-dodecanol (1863 mg, 10 mmol) in dry DCM (5 mL) is treated at 0°C with TEA (1.5 mL, 11 mmol) and methanesulfonyl chloride (774 μ L, 11 mmol). The reaction mixture is stirred at 0°C for 15 min. The mixture was partitioned between CHCl_3 (50 mL $\times$ 3) and water (50 mL). The collected organic layer was washed with brine (100 mL), collected, dried over MgSO_4 . Filtration, and concentration *in vacuo* gave 2801 mg (quantitative) of **C₁₂H₂₅OMs** as a yellow solid. $^1\text{H-NMR}$ (400 MHz, CDCl_3 , TMS): δ /ppm, 4.22 (t, J_H = 6.4 Hz, 2H), 3.00 (s, 3H), 1.75 (quin, J_H = 6.8 Hz, 2H), 1.43–1.26 (m, 18H), 0.88 (t, J_H = 6.8 Hz, 3H).

[Second step]

To a solution of **1-1** (700 mg, 3.55 mmol) in DMF (70 mL) was added **C₁₂H₂₅OMs** (1005 mg, 3.8 mmol) and K_2CO_3 (525 mg, 3.8 mmol). The mixture was stirred for 8h at 60°C. The reaction mixture was poured into water (150 mL) and the whole was extracted with AcOEt (150 mL $\times$ 3). The AcOEt layer was separated, washed with brine (150 mL), and was dried over MgSO_4 . Filtration, concentration *in vacuo*, purification by silica gel flash column chromatography (*n*-hexane/AcOEt = 1/0 to 5/1) gave 981 mg (76%) of **1-3** as a light yellow solid.

$^1\text{H-NMR}$ (400 MHz, CDCl_3 , TMS): δ /ppm, 7.87 (d, J_H = 8.4 Hz, 1H), 7.82 (d, J_H = 2.0 Hz, 1H), 7.78 (m, 1H), 4.13 (m, 2H), 3.93 (s, 3H), 1.85 (quin, J_H = 6.4 Hz, 2H), 1.54–1.24 (m, 18H), 0.88 (t, J_H = 7.2 Hz, 3H).

Compound **1-4**

To a solution of **1-3** (981 mg, 2.69 mmol) in dry THF (15 mL) at 0°C was added DIBAL (10 mL of a 1.0 M solution in hexane, 10 mmol) slowly. The reaction was stirred for 30 min, then quenched with saturated solution of Rochelle's salt *aq.* (100 mL) and the biphasic mixture stirred for 60 min. The mixture was extracted with AcOEt (100 mL $\times$ 3). The AcOEt layer was separated, washed with brine (100 mL), and was dried over MgSO_4 . Filtration, concentration *in vacuo*, purification by silica gel flash column chromatography (*n*-hexane/AcOEt = 1/0 to 5/1) gave 648 mg (71%) of **1-4** as light yellow solid.

$^1\text{H-NMR}$ (400 MHz, CDCl_3 , TMS): δ /ppm, 7.85 (dd, J_H = 2.0, 8.4 Hz, 1H), 7.70 (d, J_H = 2.0 Hz, 1H), 7.51 (d, J_H = 8.0 Hz, 1H), 4.78 (d, J_H = 6.0 Hz, 2H), 4.10 (t, J_H = 6.4 Hz,

2H), 2.16 (t, $J_H = 6.4$ Hz, 1H), 1.87 (quin, $J_H = 7.6$ Hz, 2H), 1.47 (m, 2H), 1.37–1.19 (m, 18H), 0.89 (t, $J_H = 6.8$ Hz, 3H).

Compound 1-5

To a stirred solution of Fmoc-Asp (OtBu)-OH (658 mg, 1.6 mmol), DMAP (12 mg, 0.1 mmol) and EDCI (307 mg, 1.6 mmol) in dry DMF (20 mL) was added **1-4** (536 mg, 1.59 mmol). The reaction mixture was stirred at room temperature for 3 hours. The mixture was diluted with water (100 mL), and then extracted with AcOEt (100 mL×3). The combined organic layer was washed with brine (100 mL) and dried over MgSO₄. Filtration, concentration *in vacuo* and purification by silica gel flash column chromatography (*n*-hexane/AcOEt = 1/0 to 4/1) gave **1-5** (454 mg, 39%) as a light yellow solid.

¹H-NMR (400 MHz, CDCl₃, TMS): δ /ppm, 7.77–7.74 (m, 3H), 7.66 (d, $J_H = 2.0$ Hz, 1H), 7.58 (m, 2H), 7.54–7.38 (m, 3H), 7.29 (m, 2H), 5.86 (d, $J_H = 9.2$ Hz, 1H), 5.31 (s, 2H), 4.71 (m, 1H), 4.45–4.33 (m, 2H), 4.22 (m, 1H), 4.05 (t, $J_H = 6.4$ Hz, 2H), 3.00–2.79 (m, 2H), 1.82 (quin, $J_H = 7.2$ Hz, 2H), 1.57–1.27 (m, 27H), 0.88 (t, $J_H = 6.4$ Hz, 3H).

Compound 1-6

To a solution of **1-5** (180 mg, 0.35 mmol) in dry DMF (5 mL) was added piperidine (85 mg, 0.39 mmol) at room temperature for 5 h. The solution was poured into water (100 mL) and the whole was extracted with AcOEt (100 mL×2). The AcOEt layer was separated, washed with brine (100 mL), and was dried over MgSO₄. Filtration, concentration *in vacuo* and purification by silica gel flash column chromatography (*n*-hexane /AcOEt = 2/1) gave 103 mg (58%) of **1-6** as yellow oil.

¹H-NMR (400 MHz, CD₃OD): δ /ppm, 7.82 (dd, $J_H = 2.0, 8.4$ Hz, 1H), 7.70 (d, $J_H = 2.4$ Hz, 1H), 7.46 (d, $J_H = 8.0$ Hz, 1H), 5.28 (s, 2H), 4.08 (t, $J_H = 6.8$ Hz, 2H), 3.84 (t, $J_H = 5.2$ Hz, 1H), 1.84 (overlap, 2H), 1.48 (overlap, 2H), 1.43 (s, 9H), 1.36–1.27 (m, 16H), 0.88 (t, $J_H = 6.8$ Hz, 3H).

Compound 1-7

To a solution of **1-6** (103 mg, 0.20 mmol) in dry DCM (5 mL) was added 3,5-bis(trifluoromethyl)benzoyl chloride (54 μ L, 0.28 mmol) and DIPEA (39 mg, 0.30

mmol) and the mixture was stirred for 45 min at room temperature. After removal of the solvent by evaporation, the residue was purified by silica gel flash column chromatography (*n*-hexane/AcOEt = 4/1) to give 140 mg (100%) of **1-7** as a light yellow solid.

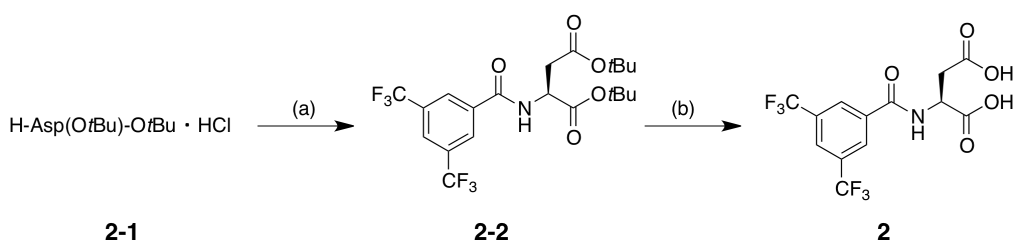
¹H-NMR (400 MHz, CDCl₃, TMS): δ /ppm, 8.23 (s, 2H), 8.04 (s, 1H), 7.81 (dd, J_H = 2.0, 8.4 Hz, 1H), 7.71 (d, J_H = 2.0 Hz, 1H), 7.45 (d, J_H = 8.4 Hz, 1H), 7.38 (d, J_H = 8.0 Hz, 1H), 5.35 (s, 2H), 5.11 (m, 1H), 4.08 (t, J_H = 6.4 Hz, 2H), 3.15–2.90 (m, 2H), 1.85 (quin, J_H = 7.2 Hz, 2H), 1.55–1.26 (m, 23H), 1.43 (s, 9H), 0.88 (t, J_H = 6.8 Hz, 3H).

Compound 1

To a stirred solution of **1-7** (140 mg, 0.20 mmol) in dry DCM (5 mL) was added TFA (1 mL). The reaction mixture was stirred at room temperature for 8 hours. After removal of the solvent by evaporation, the residue was purified by silica gel flash column chromatography (*n*-hexane/AcOEt = 1/3) to give 94 mg (74%) of **1** as a light yellow solid.

¹H-NMR (400 MHz, CDCl₃, TMS): δ /ppm, 8.23 (s, 2H), 8.04 (s, 1H), 7.80 (dd, J_H = 2.0, 8.4 Hz, 1H), 7.69 (d, J_H = 2.0 Hz, 1H), 7.44 (d, J_H = 8.4 Hz, 1H), 7.28 (d, J_H = 7.2 Hz, 1H), 5.36 (s, 2H), 5.15 (m, 1H), 4.07 (t, J_H = 6.4 Hz, 2H), 3.24–3.13 (m, 2H), 1.83 (quin, J_H = 7.6 Hz, 2H), 1.46 (m, 2H), 1.31–1.26 (m, 16H), 0.88 (t, J_H = 6.8 Hz, 3H).

HR-ESI MS : calcd for C₃₂H₃₈O₈N₂F₆Na [M+Na]⁺ = 715.2425; found 715.2424.

Scheme 5. Synthetic scheme of **2**.

Reaction conditions: (a) 3,5-bis(trifluoromethyl)benzoyl chloride, DIPEA in DCM, (b) TFA in DCM

Compound **2-2**

To a stirred solution of **2-1** (282 mg, 1.0 mmol) and DIPEA (284 mg, 2.2 mmol) in dry DCM (10 mL) was added 3,5-bis(trifluoromethyl)benzoyl chloride (277 mg, 1.0 mmol). The reaction mixture was stirred at room temperature for 30 min. After removal of the solvent by evaporation, the residue was purified by silica gel flash column chromatography (hexane/CHCl₃ = 1/1) to give **2-2** (486 mg, 100%) as colorless oil.

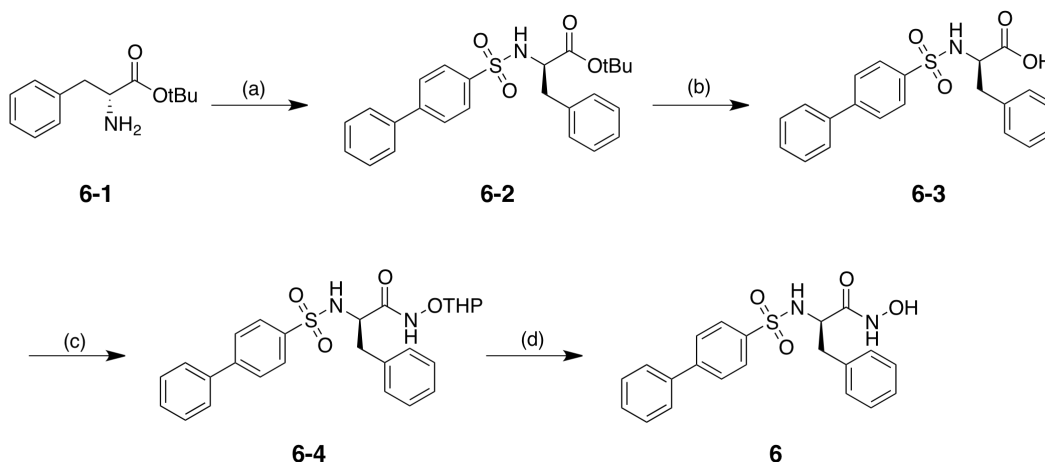
¹H-NMR (400 MHz, CDCl₃, TMS): δ /ppm, 8.25 (s, 2H), 8.02 (s, 1H), 7.33 (d, J_H = 8.4 Hz, 1H), 4.89–4.85 (m, 1H), 3.04–2.85 (m, 2H), 1.50 (s, 9H), 1.46 (s, 9H).

Compound **2**

To a stirred solution of **2-2** (486 mg, 1.0 mmol) in dry DCM (10 mL) was added TFA (3 mL). The reaction mixture was stirred at room temperature for 2.5 hours. After removal of the solvent by evaporation, the residue was washed by hexane to give **2** (350 mg, 94%) as colorless oil.

¹H-NMR (400 MHz, DMSO-*d*₆): δ /ppm, 9.31 (d, J_H = 7.6 Hz, 1H), 8.49 (s, 2H), 8.35 (s, 1H), 4.79–4.74 (m, 1H), 2.89–2.68 (m, 2H).

HR-ESI MS : calcd for C₁₃H₉O₅NF₆Na [M+Na]⁺ = 396.0283; found 396.0278.

Scheme 6. Synthetic scheme of **6**.

Reaction conditions: (a) 4-biphenylsulfonyl chloride, DMAP, DIPEA in DCM, (b) TFA in DCM, (c) NH_2OTHP , EDCI, $\text{HOBT}\cdot\text{H}_2\text{O}$, DIPEA in DMF, $\text{TsOH}\cdot\text{H}_2\text{O}$ in MeOH.

Compound **6-2**

To a solution of **6-1** (516 mg, 2.0 mmol) in DCM (3 mL) and DIPEA (870 μL , 5.0 mmol) was added 4-biphenylsulfonyl chloride (505 mg, 2.0 mmol) and DMAP (1 mg). The mixture was stirred for 3 h at room temperature. After removal of the solvent by evaporation, the residue was purified by silica gel flash column chromatography ($n\text{-hexane}/\text{CHCl}_3 = 1/2$ to $0/1$) to give 800 mg (91%) of **6-2** as a white solid.

^1H -NMR (400 MHz, CDCl_3 , TMS): δ/ppm , 7.83 (d, $J_H = 8.0$ Hz, 2H), 7.64 (d, $J_H = 8.4$ Hz, 2H), 7.55 (m, 2H), 7.47 (m, 2H), 7.42 (m, 1H), 7.16 (m, 2H), 5.11 (d, $J_H = 9.2$ Hz, 1H), 4.13 (m, 1H), 3.04 (d, $J_H = 6.4$ Hz, 2H), 1.18 (s, 9H).

Compound **6-3**

To a stirred solution of **6-2** (800 mg, 1.83 mmol) in DCM (10 mL) was added TFA (2 mL). The reaction mixture was stirred at room temperature for 3 hours. The solvent was removed by evaporation to give 697 mg (quantitative) of **6-3** as a white solid. ^1H -NMR (400 MHz, $\text{CD}_3\text{OD} + \text{CDCl}_3$ (1/1), TMS): δ/ppm , 7.75 (d, $J_H = 8.0$ Hz, 2H), 7.60 (m, 2H), 7.43 (m, 2H), 7.32 (m, 2H), 7.21 (m, 1H), 7.13 (m, 2H), 4.13 (m, 2H), 4.15 (m, 1H), 3.01 (m, 2H).

Compound **6-4**

A solution of **6-3** (191 mg, 0.50 mmol), NH₂OTHP (1.0 mmol), EDCI (1.0 mmol), HOBt·H₂O (1.0 mmol), and DIPEA (871 μ L, 5.0 mmol) in 5 mL of DMF was stirred at room temperature for 8 hours. The reaction mixture was poured into brine (100 mL) and extracted with AcOEt (100 mL). The organic layer was separated and dried over MgSO₄. Filtration, evaporation of the solvent *in vacuo*, and purification by silica gel flash column chromatography (hexane/AcOEt = 1/2) gave 204.4 mg (86 %) of **6-4** as a light yellow solid.

¹H-NMR (400 MHz, CDCl₃, TMS): δ /ppm, 9.25 (s, 1H), 9.11 (s, 1H), 7.70 (m, 4H), 7.59 (m, 5H), 7.18 (m, 5H), 5.50 (t, J_H = 7.2 Hz, 2H), 4.79 (m, 1H), 4.70 (m, 1H), 3.88 (m, 2H), 3.54 (m, 2H), 2.96 (m, 2H), 1.66 (m, 2H), 1.52 (m, 2H).

Compound **6**

To a solution of **6-4** (204 mg, 0.43 mmol) in MeOH (10 mL) was added TsOH·H₂O (8.1 mg, 0.04 mmol). The reaction mixture was stirred for 4 h at room temperature. After removal of the solvent by evaporation, the residue was purified by silica gel flash column chromatography (*n*-hexane/AcOEt = 1/2 to 0/1) to give 102 mg (60%) of **6** as a white solid.

¹H-NMR (400 MHz, CD₃OD): δ /ppm, 7.71 (d, J_H = 8.0 Hz, 2H), 7.63 (m, 4H), 7.40 (m, 2H), 7.36 (m, 1H), 7.09 (m, 5H), 3.88 (t, J_H = 7.6 Hz, 1H), 2.83 (m, 2H).

HR-ESI MS : calcd for C₂₁H₂₁O₄N₂S [M+H]⁺ = 397.1217; found 397.1218.

Measurement of dynamic light scattering (DLS) of probes.

DLS measurements were performed on a NICOMP 380zls at 25°C in 50 mM HEPES buffer (pH 7.2, 100 mM NaCl, 10mM CaCl₂) using a plastic cuvette (3 mL volume). All measurements were carried out in triplicate.

Evaluation of Critical Aggregation Concentrations (CACs).

The fluorescence spectra of Nile Red with different concentrations of probes were measured using a PerkinElmer LS55 spectrometer. Plotting the fluorescence intensity (at 575 nm) of Nile Red (ex. 535 nm) affords an inflection point, which represented CAC.

Fluorescent microscopy image of probe 3a.

Probe **3a** (50 µM) was added in 50 mM HEPES (pH 7.5, 100mM NaCl, 10mM CaCl₂, 100 µL) loaded with Nile Red (final concentration 5 µM). Fluorescent microscopy image was obtained using an inverted fluorescence microscope (IX-71, Olympus) with 530–550 nm band pass filter.

¹⁹F-MRI experiments *in vitro*.

Probe **1** (50 µM) was added to 50 mM HEPES buffer (pH 7.2, containing 1.0 mM NADPH) and incubated with NTR (0.83 µM) at 37°C for 60 min. Probe **3a** (100 µM) was added to 50 mM HEPES buffer (pH 7.5 containing 100 mM NaCl, 10 mM CaCl₂) and incubated with MMP2 (15 nM) in the absence or presence of inhibitor **6** (100 µM) in a test tube. In the ¹⁹F-MR imaging experiments, samples of 2 mL were loaded into sample tubes (depth of sample tube 20 mm). ¹⁹F-MR images of samples were obtained by fast spin echo with repetition time/echo time of 1000/5.5 ms, echo train length of 32, field of view of 32×8 cm² without slice selection, matrix size of 128×32, and the number of accumulations of 1,200. The excitation pulse width was 1,370 Hz. All the images were acquired at 20°C.

Cell culture

HT-1080, HeLa, COS7, and MCF7 cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose L⁻¹) supplemented with 10 % fetal

bovine serum (FBS), penicillin ($100 \text{ units mL}^{-1}$), and streptomycin ($100 \mu\text{g mL}^{-1}$) under a humidified atmosphere of 5 % CO_2 in air. Jurkat cells were cultured in RPMI-1640 medium supplemented with 10 % FBS, penicillin ($100 \text{ units mL}^{-1}$), and streptomycin ($100 \mu\text{g mL}^{-1}$). For all experiments, cells were harvested from subconfluent (<80 %) cultures by using a trypsin/ethylenediaminetetraacetic acid (EDTA) solution and then resuspended in fresh medium. A subculture was performed every 2–3 days.

^{19}F -NMR/MR images of probe 3a in the supernatants of various cells

All cells were plated at a density of 5.0×10^6 cells per 60 mm dish and cultured for 24 h at 37°C in air with 5 % CO_2 . The cells (except for MCF7 cells) were washed three times, and cultured in serum-free DMEM or RPMI-1640 for 24 h. The MCF7 cells were washed three times, and cultured in serum-free DMEM for 12, 24, or 48 h at 37°C under normoxic conditions (air with 5 % CO_2) or hypoxic conditions (<0.1 % O_2) generated with an AnaeroPack (Mitsubishi Gas Chemical Company, Inc.) and a rectangular jar. The conditioned media were then collected and centrifuged, and probes were added to give a final concentration of $50 \mu\text{M}$. After incubation for 48 h at 37°C , magnetic resonance measurements were conducted as follows. In ^{19}F -NMR experiments, samples (conditioned medium) of $450 \mu\text{L}$ were added to $50 \mu\text{L}$ of D_2O containing TFA (final concentration $100 \mu\text{M}$). ^{19}F -NMR spectra were then measured at 20°C . In ^{19}F -MR imaging experiments, aliquots of conditioned medium (2 mL) were loaded into sample tubes (depth of each sample tube 20 mm). ^{19}F -MR images of samples were obtained by fast spin echo with a repetition time/echo time of 1000/5.5 ms, an echo train length of 32, a field of view of $32 \times 8 \text{ cm}^2$ without slice selection, a matrix size of 128×32 , and 57,600 accumulations. The excitation pulse width was 1370 Hz. Zero-filling (256×64) was applied to the ^{19}F -MR images. All images were acquired at 20°C .

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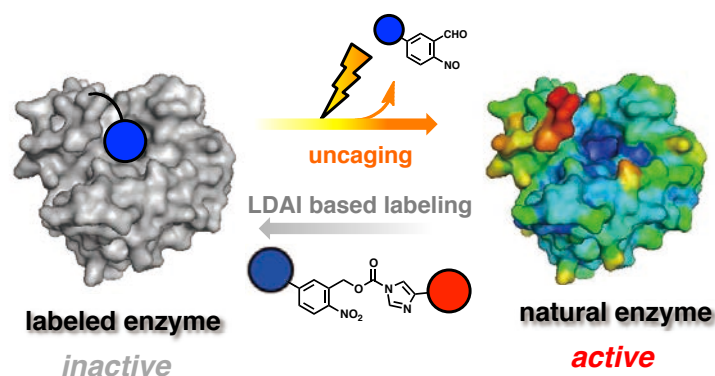
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***One-step Construction of Caged Carbonic Anhydrase I
Using a Ligand-directed Acyl Imidazole-based Protein Labeling Method***

Abstract

Caged enzymes whose activities can be controlled by light represent a powerful tool for various biological analyses. However, limited methods are available for the construction of caged proteins and enzymes. Hamachi et al. developed a novel protein labeling method termed ligand-directed acyl imidazole (LDAI) chemistry, which allows to selectively modify natural dihydrofolate reductase and folate receptor in the test tube and under live cell contexts. In this chapter, the reaction characteristics of the LDAI chemistry was examined in detail using carbonic anhydrase 1 (CA1) as a model enzyme. In addition to modifying Lys residues with a carbamate bond, the LDAI method modified Ser and Tyr residues with a carbonate bond. Owing to the relatively labile carbonate bond formed, the LDAI chemistry was demonstrated to be applicable for a rational one-step construction of caged enzymes. This method is simple and based on the transient tethering of an inhibitor with moderate activity that is directed to the active site on an enzyme surface. I successfully showed that the activity of the caged CA1 was almost completely suppressed by LDAI-based labeling and fully recovered by photoirradiation in the crude conditions (such as cell lysate) as well as in test tube settings.



2-1. Introduction

Light-induced manipulation of bioactive molecules is a valuable strategy for the precise elucidation of complicated biological phenomena. As one of the most representatives, various types of caged compounds have been developed.¹ Caged glutamate, for example, was prepared by partial masking of a moiety of glutamate essential for its activity (a hot spot), which was unmasked by the photo-triggered cleavage of the masking group *o*-nitrobenzyl carbamate (Figure 1a).² The light-stimuli responsive unmasking of small molecules leads to drastic changes in their concentration with spatiotemporal control, and thereby subsequent events are substantially affected.³ The similar design strategy was successfully applied to other small molecules,⁴ however, it is not readily suitable for use with biopolymers such as DNA, RNA⁵ and proteins/enzymes. This is because the identification of the hot spots is rather difficult in these biopolymers, and the hot spots, if identified, are generally too large to be masked, compared to small molecules. Moreover, current methodologies for the precise modification of the hot spots on biopolymers are limited. Therefore, regardless of its potential use in photo-control of enzyme activity,⁶ insufficient examples have been reported for caged proteins and enzymes.^{7,8} While recent advances by Chin's group have demonstrated that the expanded genetic code method is a promising approach for the synthesis of caged proteins (Figure 1b),⁹ chemistry-based methods should be also desirable for caging/uncaging natural proteins and enzymes.¹⁰

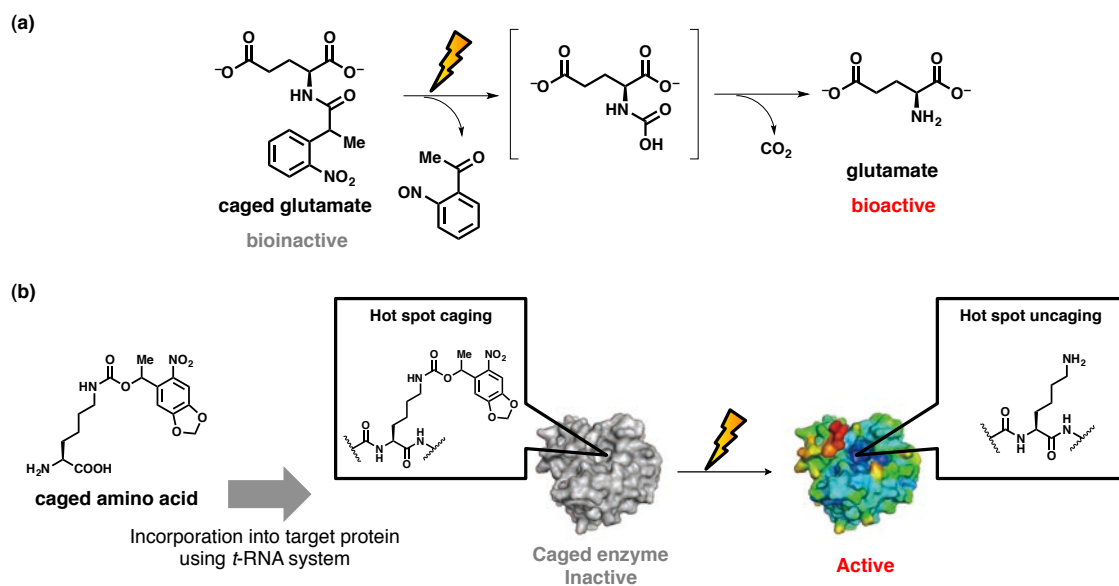


Figure 1. Light-induced manipulation of bioactive molecules. (a) Caged glutamate as a light-induced neurotransmitter. (b) Genetic construction of caged enzymes reported by Chin's group.

Hamachi *et al.* reported a new protein labeling method using ligand-directed acyl imidazole, so-called LDAI chemistry, which enables the selective modification of natural dihydrofolate reductase (DHFR) and folate receptor (FR) in the test tube and under live cell contexts.¹¹ LDAI is based on a typical nucleophilic acyl substitution reaction using a modified acyl imidazole (AI) functionality that is stable in aqueous solution. Like another ligand-directed chemistry, LD-Tosyl (LDT, Figure 2a),¹² LDAI is driven by the proximity effect and capable of releasing the ligand moiety upon covalent labeling of a target protein (Figure 2b). In this chapter, I and my predecessors in Hamachi group examined in detail the reactivity of LDAI chemistry using carbonic anhydrase 1 (CA1) as a model protein, and characterized the amino acid preference of the LDAI method. The results showed that in addition to Lys being modified with a carbamate bond, Ser and Tyr can be modified with a carbonate bond, when they are located around the active site. It was also revealed that both the labeling efficiency and the site selectivity were controlled by the spacer length and the probe structure. The careful design of an LDAI reagent enabled the rapid and highly site-selective labeling of CA1. Taking advantage of these fundamental features of LDAI chemistry, I

successfully developed a novel strategy for the one-step construction of a caged CA1 by covalent tethering of an inhibitor moiety to the CA1 surface through the photo-cleavable carbonate bond (Figure 2c).

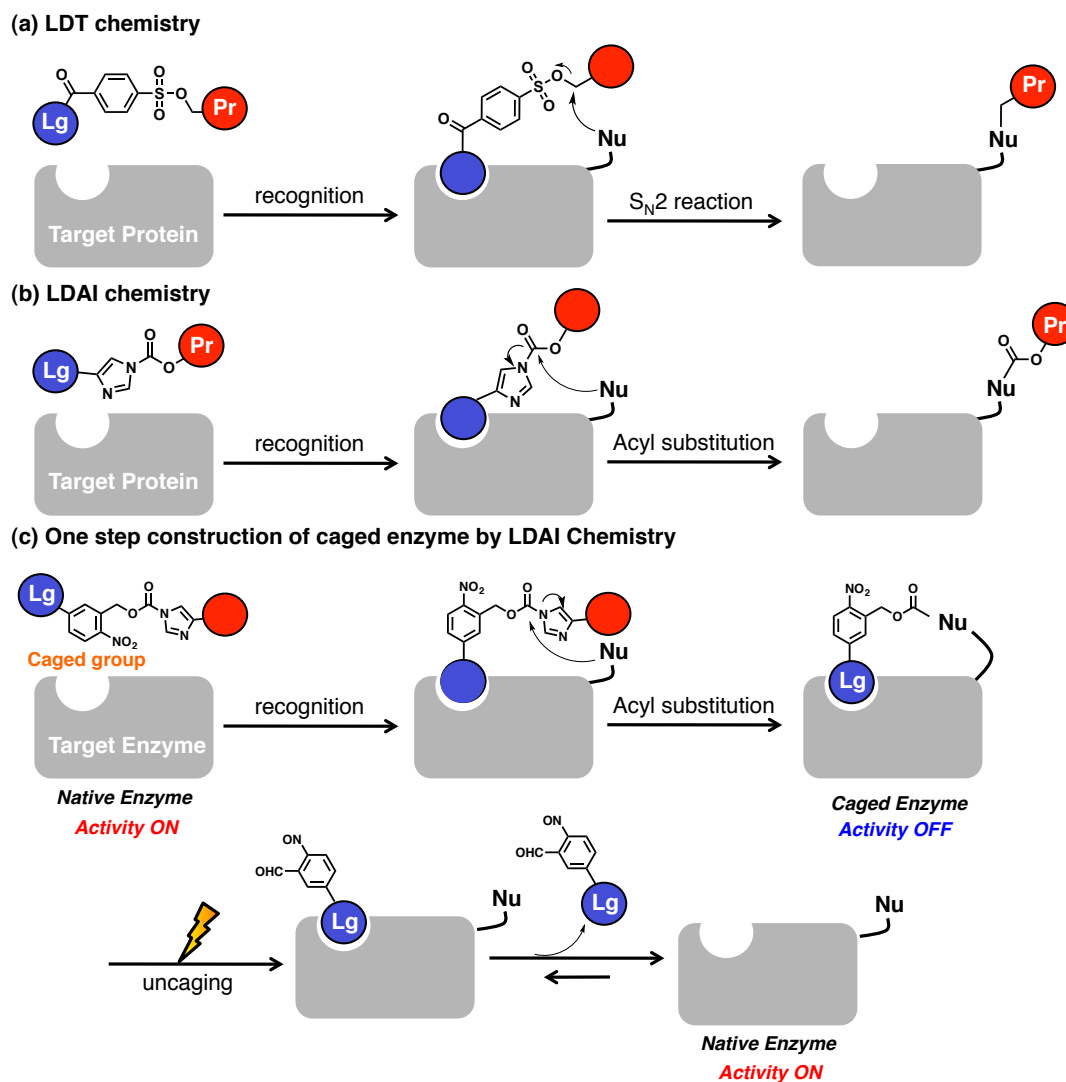


Figure 2. Schematic illustrations of (a) ligand-directed tosyl (LDT) chemistry, (b) ligand-directed acyl imidazole (LDAI) chemistry and (c) one-step construction of caged enzyme by LDAI chemistry and photo-uncaging: (1) chemical labeling by LDAI chemistry, (2) uncaging by photoirradiation and (3) ligand elimination by equilibrium recovers to native enzyme. Lg, protein ligand; Pr, chemical probe; Nu, nucleophilic amino acid.

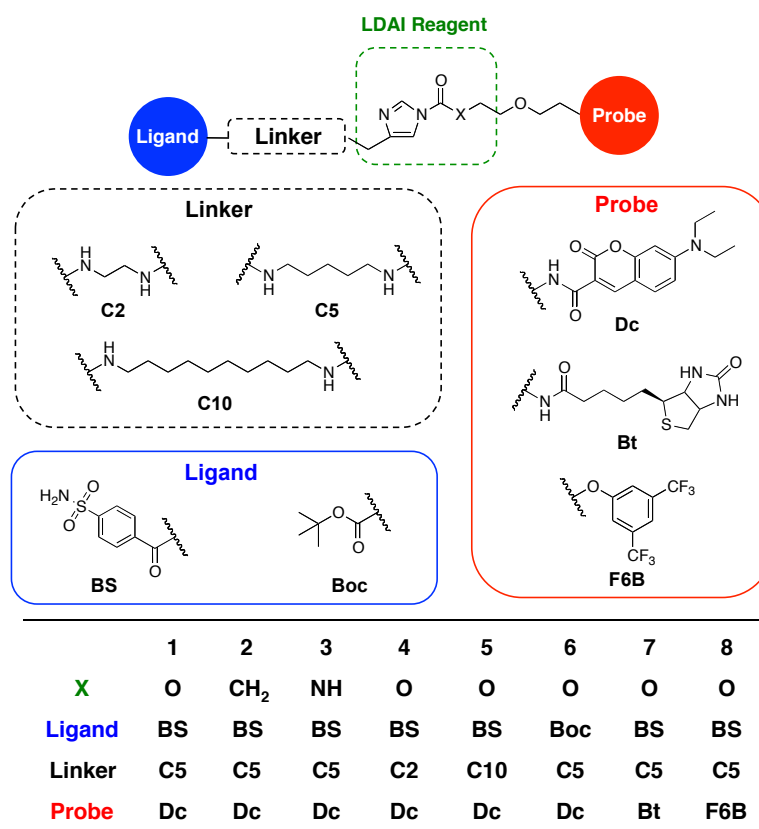


Figure 3. LDAI reagents used in the present study: **1–5** with 7-diethylaminocoumarin (Dc), **7** with biotin (Bt) and **8** with 3,5-bis(trifluoromethyl)benzene (F6B) for CA. **6** with Dc for control experiments.

2-2. Results & Discussion

2-2-1. Labeling of CA1 by various LDAI reagents

Prior to developing a design strategy for caged enzymes by LDAI chemistry, the characteristics of the LDAI-based protein labeling reaction was initially unveiled. In order to reveal the validity of the LDAI method, human CA1 was employed herein as a target protein, while DHFR and FR were used in previous studies.¹¹ According to the scheme of LDAI chemistry, several types of new LDAI labeling reagents were designed (Figure 3). Owing to its modular feature, an appropriate group for each module can be flexibly chosen for the following experiments. Benzenesulfonamide (BS) was used as the ligand module for CA1,¹³ and several functional molecules such as

7-diethylaminocoumarin (Dc), biotin (Bt) or a ^{19}F -NMR probe (3,5-bis(trifluoromethyl)benzene: F6B)^{12a,c} were tethered as the probe modules. Also, the reactivity of AI derivatives such as alkoxyacylimidazole (A(O)I), alkylacylimidazole (A(C)I) and carbamoylimidazole (A(N)I) was carefully examined for optimizing the reactivity of the electrophilic CA1 that underwent a nucleophilic acyl substitution reaction on the CA1 surface. Thus, LDAI reagents **1-3** containing a fluorescent Dc probe were prepared. Among them, the alkylcarbonyl-I (A(C)I) derivative **2** was too unstable to be isolated (data not shown). After LDAI reagent **1** or **3** was incubated with purified CA1 in an aqueous buffer solution at 37 °C, the labeling efficiency was determined by SDS-PAGE (Figure 4). The alkoxyacyl type of the LDAI reagent (**1**) was more appropriate to CA1 labeling relative to the carbamoyl type (**3**). The predecessors varied the distance between the ligand moiety and the A(O)I site (using LDAI reagents **1**, **4** and **5**) and evaluated the distance dependency of the labeling reaction. The labeling efficiency by **1** was about 2- or 4-fold larger than that by **4** or **5** (Figure 4, lanes 4, 6 and 7). This result indicates that there is an optimal distance between the ligand and the reaction site for efficient labeling by LDAI. A similar distance dependency was observed for LDT chemistry.^{12d} In addition, the labeling occurred neither in the presence of ethoxyzolamide¹⁴ (EZA, lane 8), a strong inhibitor for CA1, nor using LDAI reagent **6** lacking the BS moiety (lane 9). Given these results, it was demonstrated that CA1 labeling by LDAI was effectively driven by the ligand-protein recognition.

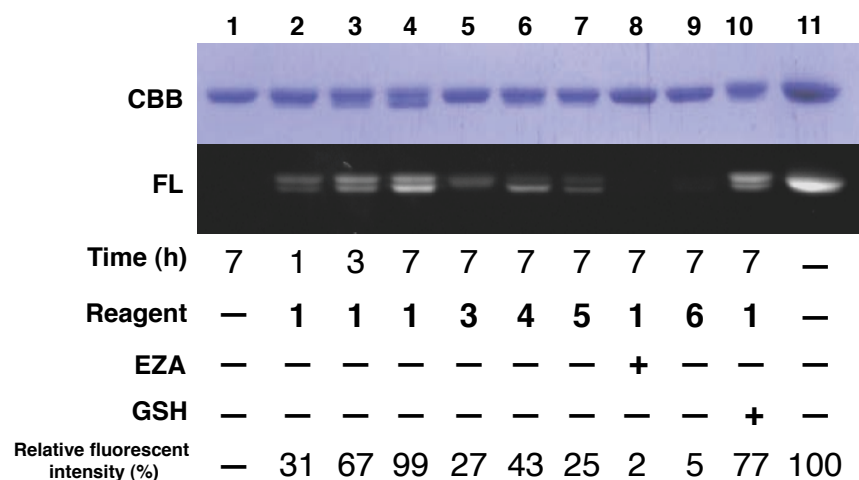


Figure 4. CA1 labeling by LDAI chemistry. In-gel fluorescence analysis of the labeling reaction. CA1 (5 μ M) was mixed with **1**, **3**, **4**, **5** or **6** (10 μ M) in HEPES buffer (50 mM, pH 7.2) at 37 °C. The band intensity in the lane 11 (loaded with the same amount of carbonic anhydrase 2 (CA2) that is quantitatively labeled with a Dc dye) was used as a standard reference.^{12a}

Besides, the labeling reaction by **1** was not substantially affected by the presence of a high concentration (10 mM) of the nucleophilic glutathione (GSH, Figure 4a, lane 10). In contrast to the behaviors of LDAI reagents with a Dc probe (**1**, **3**, **4**, **5**) or biotin (**7**), the rapid and single labeling took place by **8** with F6B. To my surprise, the labeling reaction by **8** completed within 2 h at neutral pH in almost quantitative labeling yield (100%) (Figure 5), which was about 3-fold faster than that by **1**. The MALDI-TOF mass chart showed that a new peak appeared and a native (unlabeled) CA1 peak concurrently disappeared. The observed molecular weight (M.W. 29,214) of the new peak well corresponded to the sum of native CA1 and the LDAI fragment (CA1 + 344), indicating that the purely single labeling of CA1 occurred. The sharp difference of **8** against **1** and **7** suggested that LDAI labeling was controlled not only by the spacer length, but also the probe structure.

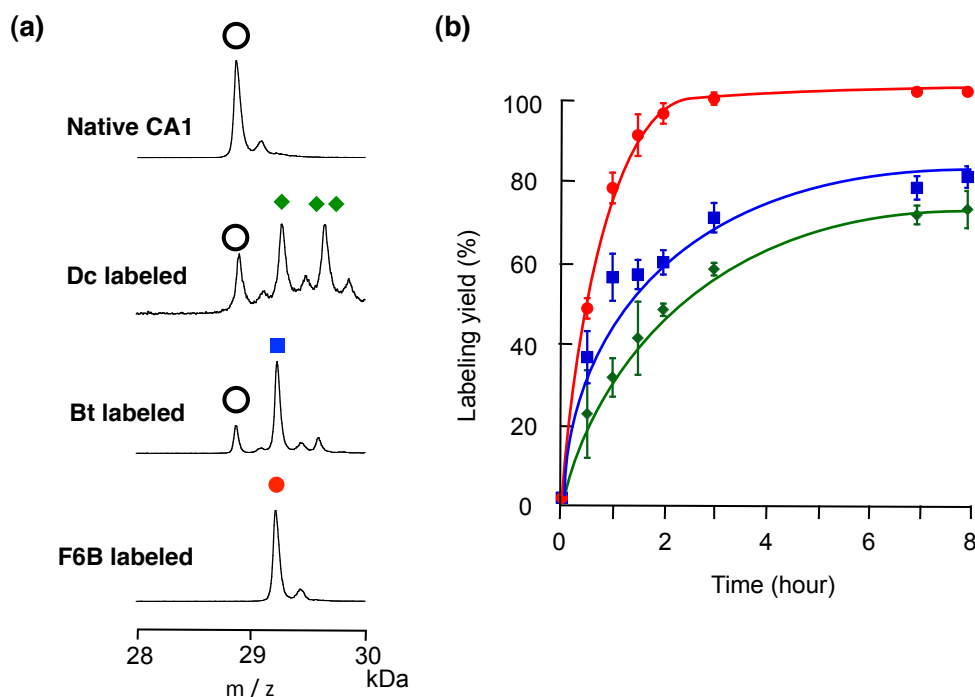


Figure 5. Labeling of CA1 using labeling reagents with Dc probe **1**, Bt probe **7** and F6B probe **8**. (a) MALDI-TOF mass analysis of CA1 (10 μ M) labeling by **1**, **7** or **8** (20 μ M) in HEPES buffer (50 mM, pH 7.2) at 37 $^{\circ}$ C for 7 h. $\circ$, native CA1 (M.W. 28,870); $\blacklozenge$, Dc-labeled CA1 (M.W. 29,214); $\blacksquare$, Bt-labeled CA1 (M.W. 29,227); $\bullet$, F6B-labeled CA1 (M.W. 29,214). (b) The time course of the reaction yields of CA1 labeling by several LDAI reagents **1** ($\blacklozenge$), **7** ($\blacksquare$) or **8** ($\bullet$). Error bars represent the S.D. of triplicate measurements.

2-2-2. Determination of the labeling sites of CA1

The labeling sites of CA1 by **1** and **8** were determined using conventional protease digestion followed by tandem mass analysis. In the case of **1** with the fluorescent Dc probe, it was found that several amino acids were labeled with Dc; Ser3 (70%), Tyr21 (11%), Lys58 (4%), Ser137 (8%) and Lys171 (7%) (Figures 4a, b). Examination of the crystal structure of human CA1 shows that all of these residues are located around the entrance of the CA1 active site (Figure 6e). This selectivity is one of the most important features in ligand-directed chemistry, which explicitly indicates that LDAI labeling is indeed driven by the ligand-protein binding, as observed for the LDT method. On the

basis of the labeled amino acids, it was reasonable to consider that a carbonate bond (for Ser, Tyr) or a carbamate bond (for Lys) should form by LDAI chemistry, which are distinct from those bonds form by LDT.^{12a,d} In addition to Lys, which was previously identified in DHFR labeling, Ser and Tyr were newly identified as a reactive amino acids by LDAI in this CA1 labeling reaction. In sharp contrast to the results of **1**, only Ser3 was labeled when the LDAI reagent **8** was used, indicating that **8** exhibits high site selectivity, as well as a rapid labeling rate (Figures 7). Additionally, it is rather remarkable that such a less activated OH group located on the protein surface, unlike in an enzyme active site,¹⁵ can undergo the efficient reaction under aqueous conditions where competitive water molecules are abundant. To the best of my knowledge, the selective carbonate bond formed on the protein surface is unprecedented. The distinct labeling properties between **1** and **8** clearly showed that the probe moiety represents an additional controlling factor for the labeling reaction both in its reaction rate and the site-selectivity. From the crystallographic analysis of ¹⁹F-labeled CA1 reported by Hamachi group, it was suggested that F6B moiety weakly interacted with a rather hydrophobic pocket (consisting L132, L199 and Y205) proximal to the active site cavity.^{16,17} This may allow me to propose that such hydrophobic subsite partially contribute to regulating the orientation of the bound LDAI reagent **8** via the two point fixation using both BS and F6B moieties, so that the labeling rate and selectivity toward Ser2 was enhanced.

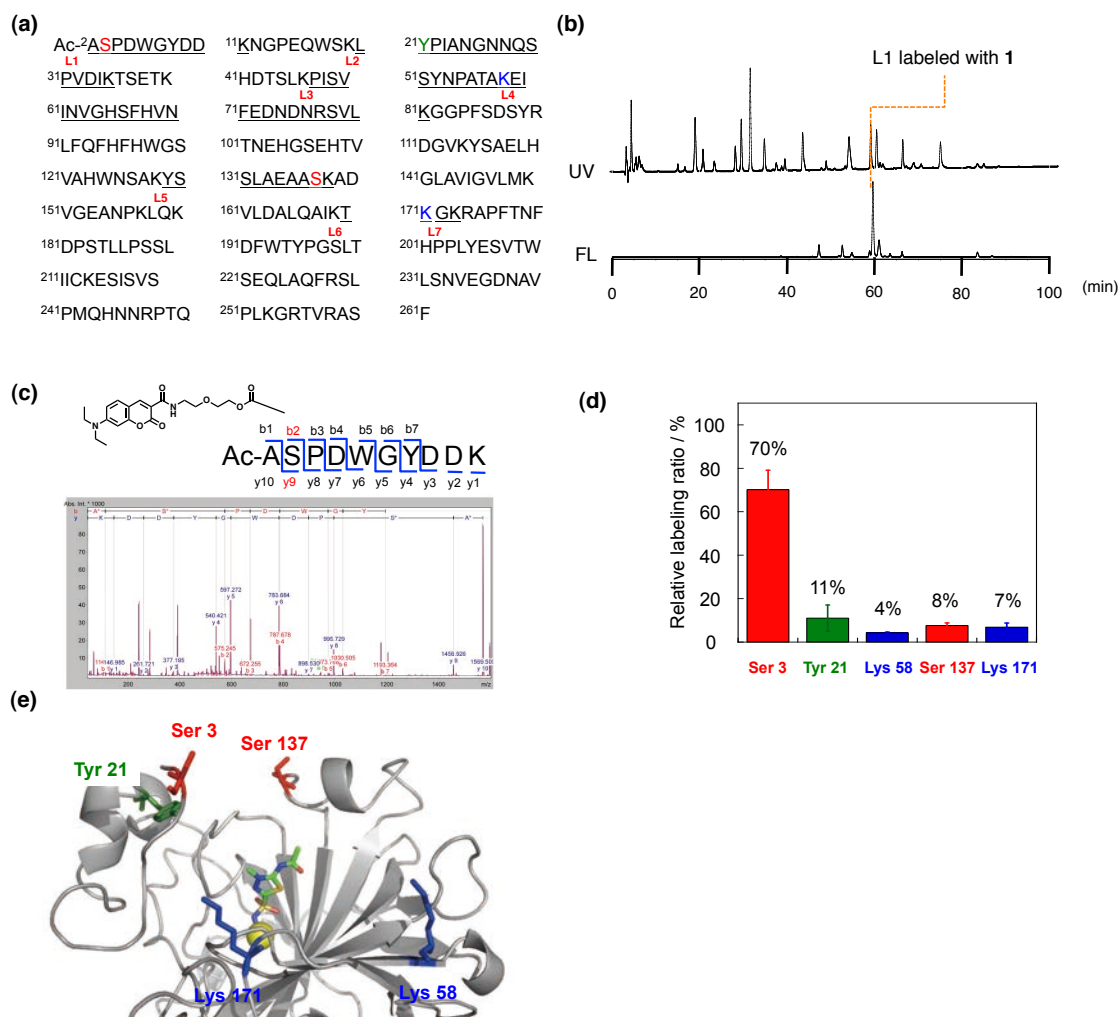


Figure 6. Evaluation of labeling sites and site-selectivity in CA1 labeling by reagent **1**. (a) The primary sequence of CA1 and the assignment of each fragment generated by lysyl endopeptidase (LEP) digestion. (b) RP-HPLC traces of LEP-digested Dc-labeled CA1. The peaks were monitored by absorbance detection ($\lambda = 220$ nm) and fluorescence detection ($\lambda_{em} = 473$ nm, $\lambda_{ex} = 427$ nm). (c) MALDI-TOF MS/MS analysis of the Dc labeled L1 as one example. (d) Relative amino acid (reaction site) selectivity. (e) The crystal structure of the CA1 complex (PDB:1BZM). The amino acids (Ser3, Tyr 21, Lys 58, Ser137, Lys171) modified by **1** are highlighted by a colored stick model. The zinc ion in the active site is shown as a yellow ball.

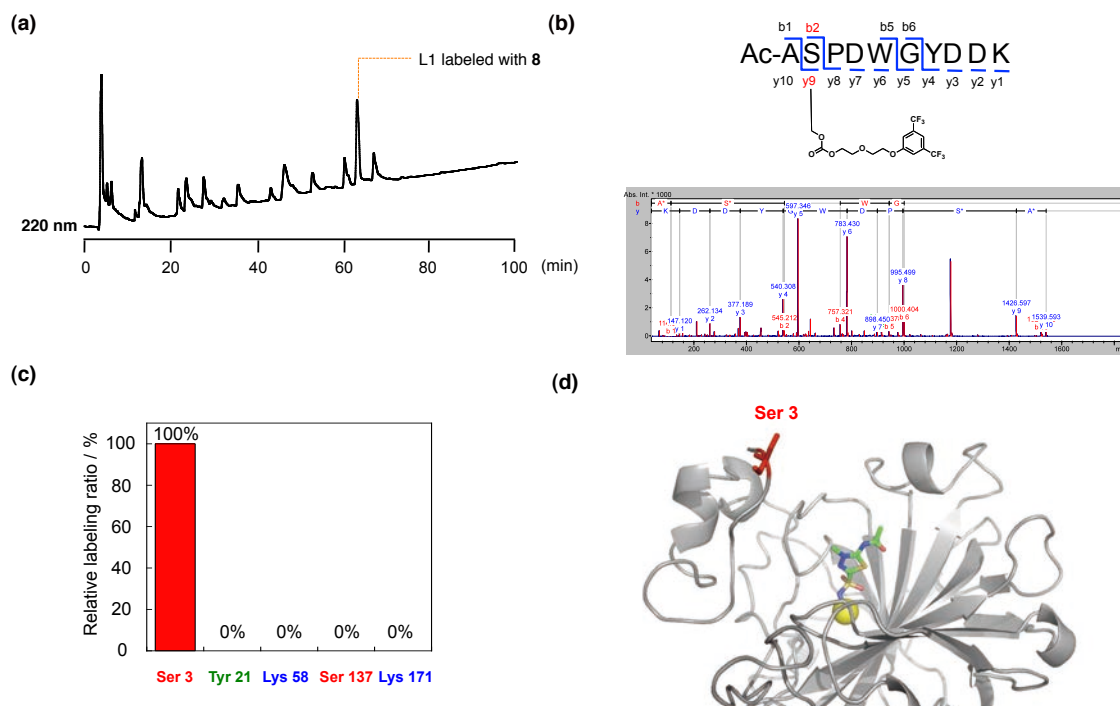


Figure 7. Evaluation of labeling sites and site-selectivity in CA1 labeling by reagent 8. (a) RP-HPLC traces of LEP-digested F6B-labeled CA1. The peaks were monitored by absorbance detection ($\lambda = 220$ nm). (b) MALDI-TOF MS/MS analysis of the F6B-labeled L1. (c) Relative amino acid (reaction site) selectivity. (d) The crystal structure of the CA1 complex (PDB:1BZM). The amino acids (Ser3) modified by 1 are highlighted by a colored stick model. The zinc ion in the active site is shown as a yellow ball.

2-2-3. Semisynthetic strategy of caged CA1 by LDAI and its function

The rapid, quantitative and active-site directed “carbonate bond” formation by the LDAI chemistry may represent promising features for one-step semisynthesis of caged CA1. In order to construct the caged enzyme whose activity is tentatively masked, however, a strategy that can covalently restore an affinity ligand instead of a probe in the labeled protein, is required. Moreover, for light-triggered uncaging of the activity, an appropriate photo-cleavable module should be inserted between the ligand and the reactive AI sites. I thus newly designed an LDAI reagent **9** for the construction of the caged CA1, where the ligand part and the F6B (probe) part were linked with the AI module in the position opposite to the original LDAI labeling reagent **8**.¹⁶ An *o*-nitrobenzyl group was placed as a photo-cleavage site between the BS and the reactive AI moieties. After CA1 labeling by **9**, the photo-triggered cleavage reactions may be expected to proceed on the CA1 surface. That is, upon UV light irradiation, the attack of the excited *o*-nitrophenyl group to the benzyl moiety of the carbonate bond terminal takes place, followed by the decarboxylation to reproduce an original nucleophilic amino acid residue. It is also rational that the noncovalent inhibitor (BS, $K_d = 2 \mu\text{M}$)¹⁸ is released from the CA1 site after reaching the equilibrium, because of its moderate affinity.

Preparation of the caged CA1 is simple, and was achieved by mixing the LDAI reagent **9**¹⁹ with CA1 in aqueous buffer (pH 7.2), followed by incubation at 25 °C. The CA1 labeling proceeded almost quantitatively (95% by MALDI-TOF mass) for 10 h, although the labeling reaction was relatively slower than that by the original F6B-reagent **8** (Figures 5 and 8). The MALDI-TOF mass data indicated that selective single labeling occurred, similar to the case of **8**. The observed mass value agreed well with the M.W. that the ligand (BS) part was labeled to CA1 instead of the probe (F6B) part. The caged CA1 was then purified by gel filtration chromatography. Using the purified caged CA1, the labeling site using compound **9** was confirmed to be only Ser3 (Figure 8d, e), which strongly suggested that a carbonate bond formed to link the inhibitor BS with Ser3. An efficient photo-cleavage reaction was anticipated, because the carbonate bond formed is more labile than C–N and C–O bonds.

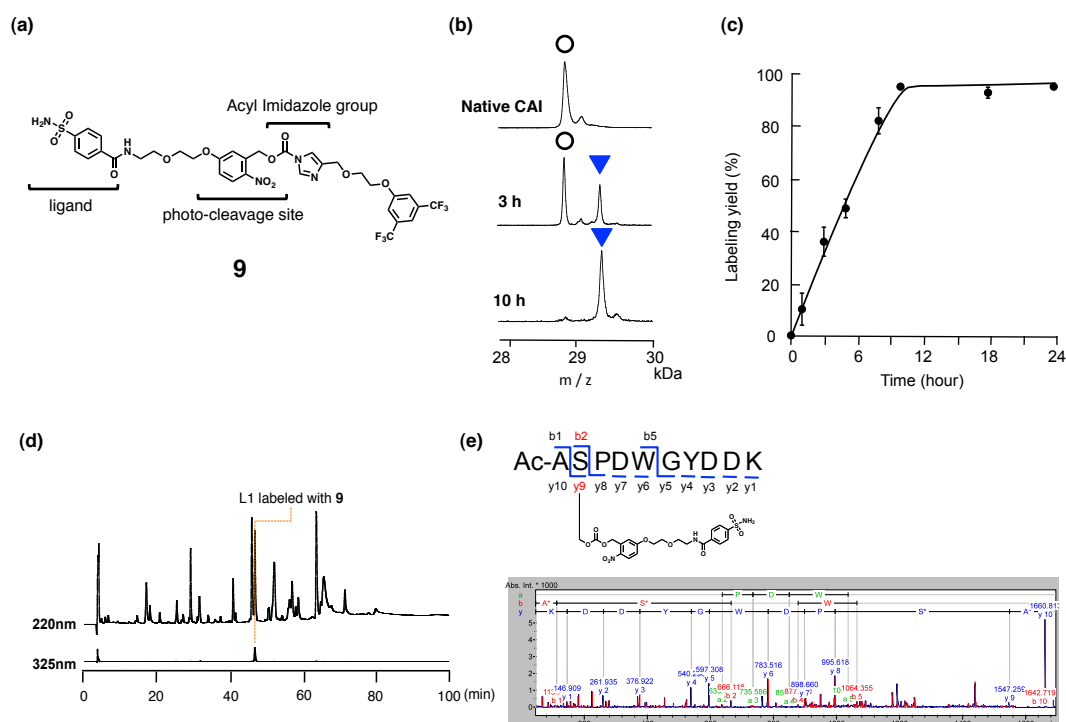


Figure 8. Labeling of CA1 using the caged labeling reagent **9**. (a) Chemical structure. (b) MALDI-TOF mass analysis of the CA1 labeling by **9**. Reaction conditions: 10 μ M CA1 and 20 μ M **9** in HEPES buffer (50 mM, pH 7.2) at 25 °C for 0 h (top), 3 h (middle) or 10 h (bottom). $\bigcirc$, native CA1 (M.W. 28,870); $\blacktriangledown$, caged-CA1 (M.W. 29,335). (c) The time course of the reaction yields of CA1 labeling by **9**. Error bars represent the S.D. of triplicate measurements. (d) RP-HPLC traces of LEP-digested **9**-labeled CA1. The peaks were monitored by absorbance detection ($\lambda = 220, 325$ nm). (e) MALDI-TOF MS/MS analysis of the **9**-labeled L1.

With the produced caged CA1 in hand, the photo-responsive CA1 activity was examined by the standard enzyme activity assay using *p*-nitrophenyl acetate (*p*-NPA) as the substrate.²⁰ Successful caging of the CA1 activity was initially demonstrated by the kinetic analysis of the caged CA1, as shown in Figure 9a. From the initial rate, the activity of caged CA1 was suppressed by 10-fold when compared with the rate of native CA1. The background activity may be due to residual unlabeled CA1 (< 5%). After UV light irradiation, the initial rate significantly recovered to a value almost identical the rate of native CA1, clearly indicating that both caging of CA1 by the LDAI reagent **9**

and the subsequent photo-uncaging were successfully carried out. Recovery of the initial rate was dependent on the photo-irradiation time (Figure 9a), thereby supporting the concept that the light-triggered removal of the ligand part was critical for restoring the CA1 activity.²¹

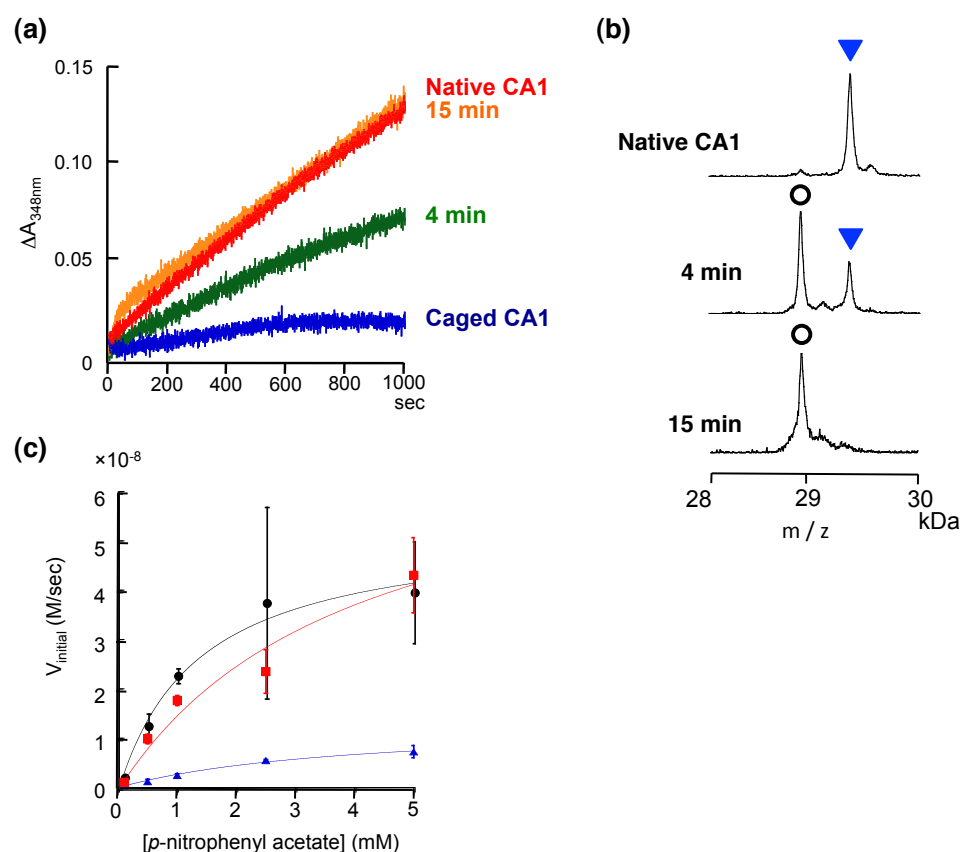


Figure 9. (a) Photoirradiation time dependency of caged CA1 activity with *p*-nitrophenyl acetate. Photoirradiation time: 4 or 15 min. All measurements were performed under the following conditions: [enzyme] = 0.10 μ M, [substrate] = 1.0 mM, in HEPES buffer (50 mM, pH 7.2) at 25 °C. (b) MALDI-TOF mass spectra of photoirradiation experiments of caged CA1 (10 μ M) using 500 W mercury lamp with a 330–380 nm band-pass filter in HEPES buffer (50 mM, pH 7.2) at 25 °C for 4 min or 15 min. ○, native CA1 (M.W. 28,870); ▼, caged-CA1 (M.W. 29,335). (c) Michaelis-Menten plots of caged CA1 (▲), uncaged CA1 (■) and native CA1 (●). All enzymes concentrations were 0.10 μ M. Error bars represent the S.D. of triplicate measurements.

I also monitored the photo-uncaging process by MALDI-TOF mass analysis. Here, the peak representing native CA1 reappeared during UV irradiation, whereas the peak representing the caged CA1 concurrently disappeared (Figures 9b). This interconversion occurred almost quantitatively, clearly showing that the carbonate bond was photo-cleaved so as to regenerate the OH group of native Ser3 in a traceless manner. In the detailed analysis, I found that the photo-recovery was also dependent on the caged CA1 concentration. At 1 μM of the caged CA1 employed, the initial rate was not fully recovered (only 30% recovery), whereas almost 100% recovery in the initial rate was observed at 0.1 μM of CA1 (Figure 9a). This was ascribed to the potency of the cleaved ligand module (BS). Since BS is a moderate and reversible inhibitor ($K_d = 2 \mu\text{M}$), it was not effective at the rather low concentration, but effective at the higher concentration. The substrate dependent kinetics for the caged CA1 and uncaged CA1 showed typical saturation kinetics against the *p*-NPA concentration in both cases, giving the kinetic parameters of $k_{\text{cat}} = 0.12 \text{ sec}^{-1}$ and $K_m = 3.3 \times 10^{-3} \text{ M}$ for the caged CA1, and $k_{\text{cat}} = 0.75 \text{ sec}^{-1}$ and $K_m = 4.0 \times 10^{-3} \text{ M}$ for uncaged CA1 (Figure 9c and Table 1). The k_{cat}/K_m value for the uncaged CA1 was 5-fold greater than that for the caged CA1 and also roughly comparable to that of native CA1.

Table 1. Kinetic parameters of native CA1, caged CA1 and uncaged CA1 for the hydrolysis of *p*-nitrophenyl acetate.

	native	caged	uncaged
$k_{\text{cat}} (\text{sec}^{-1})$	0.53 ± 0.05	0.12 ± 0.01	0.75 ± 0.02
$K_m (\text{M})$	$(1.4 \pm 0.4) \times 10^{-3}$	$(3.3 \pm 0.9) \times 10^{-3}$	$(4.0 \pm 2.1) \times 10^{-3}$
$k_{\text{cat}}/K_m (\text{M}^{-1}\text{sec}^{-1})$	3.9×10^2	36	1.9×10^2

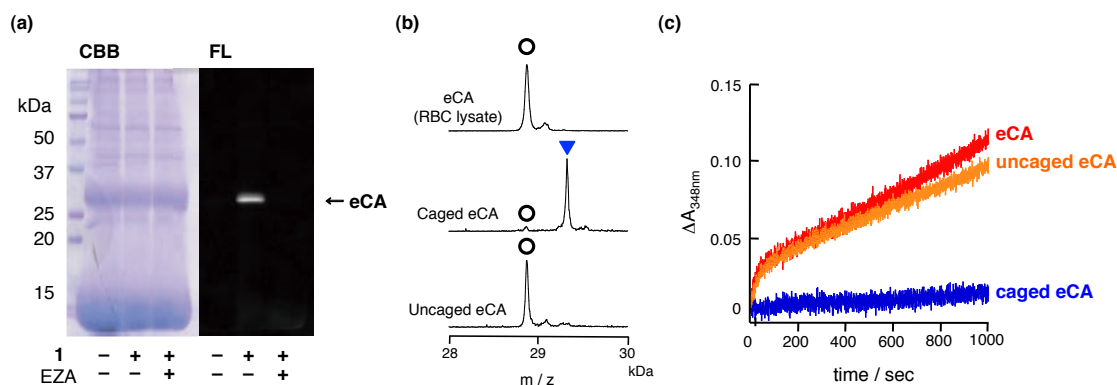


Figure 10. SDS-PAGE analyses (a) and MALDI-TOF mass analyses (b) of eCA labeling by **9** and uncaging in RBC lysate (20-fold diluted with HEPES-buffered saline). Labeling conditions: 100 μ M of **9** was incubated in RBC lysate (20-fold diluted) at 25 °C for 20 h. Uncaging conditions: Caged eCA in RBC lysate (ca. 30 μ g/mL calculated by BCA assay) was photoirradiated using 100 W mercury lamp with a 330–380 nm band-pass filter in HEPES buffer (50 mM, pH 7.2) at 25 °C for 120 min. ○, eCA; ▼, caged eCA. (c) Enzyme activities of eCA, caged eCA and uncaged eCA. All measurements were performed under the following conditions: [RBC lysate] = ca. 3.0 μ g/mL, [substrate] = 1.0 mM, in HEPES buffer (50 mM, pH 7.2) at 25 °C.

LDAI-based caging strategy was finally applied to endogenous CA (eCA) in human red blood cell (RBC) lysate. The specific labeling for eCA in RBC lysate was confirmed by SDS-PAGE gel fluorescence analysis using Dc-type labeling reagent **1** (Figure 10a). In the case of caged-type reagent **9**, MALDI-TOF mass analyses confirmed that the labeling reaction of eCA occurred quantitatively even in the RBC lysate (Figure 10b). The caged eCA thus prepared showed almost no activity from the initial rate of the hydrolysis of *p*-NPA in the roughly purified RBC lysate, as shown in Figure 10c.²² Moreover, after photolysis of caged eCA, the peak corresponding to native eCA appeared in MALDI-TOF mass analysis, and concurrently the initial rate of the hydrolysis reaction was almost fully recovered, as shown in Figure 10c. These results clearly revealed that both the sufficient masking of enzyme activity by the one-step LDAI method, and the photo-triggered reactivation of the caged CA1 were achieved, even in the crude conditions as well as in test tube settings.

2-3. Conclusions

Similar to the LDT chemistry, it was clear that LDAI was highly selective towards amino acids surrounding the protein active pocket. More interestingly, the amino acid preference is different to that of LDT, which is reactive with His, Tyr and Glu. Furthermore, the labeling rate of LDAI is faster than the LDT chemistry under optimal conditions. I concluded that LDAI nicely complements LDT in the available chemistry-based natural protein labeling methods. Owing to the relatively labile bond formed, I also demonstrated that LDAI chemistry is applicable for a rational one-step construction of caged enzymes. This method is simple and based on transient tethering of an inhibitor with moderate activity with a carbonate bond on an enzyme surface directed to the active site.

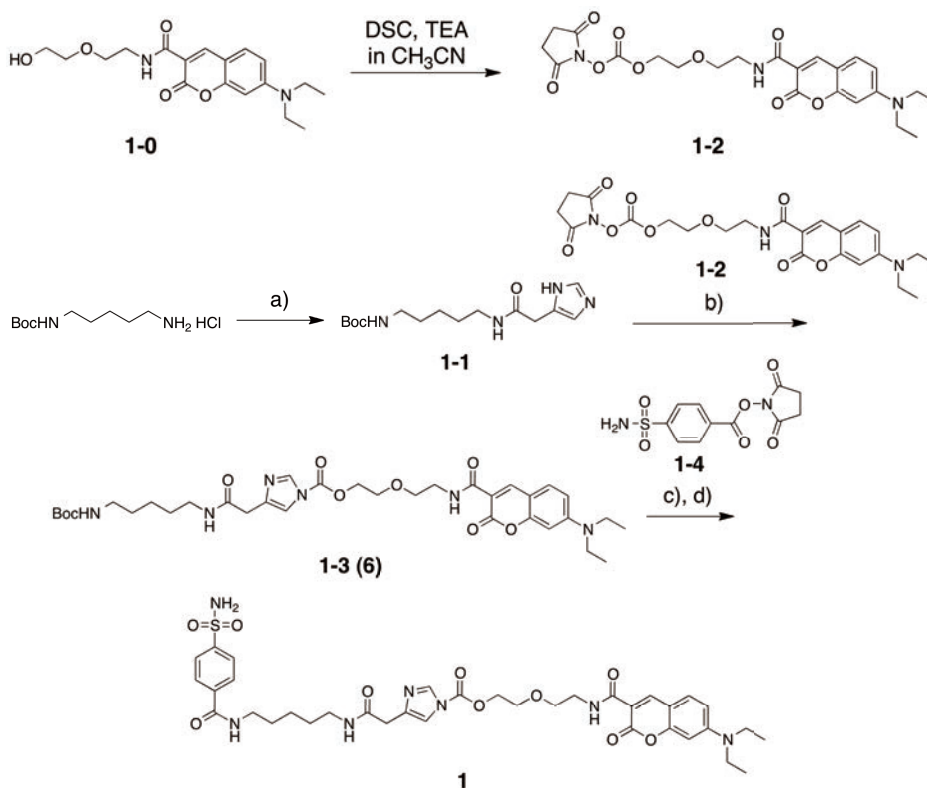
2-4. Experimental procedures

General materials and methods for organic synthesis

All chemical reagents and solvents were obtained from commercial suppliers (Aldrich, Tokyo Chemical Industry (TCI), Wako Pure Chemical Industries, Acros Organics, Sasaki Chemical, or Watanabe Chemical Industries) and used without further purification.

Thin layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ precoated aluminium sheets (Merck) and visualized by fluorescence quenching or ninhydrin staining. Chromatographic purification was conducted by flash column chromatography on silica gel 60 N (neutral, 40–50 μ m, Kanto Chemical). ¹H NMR spectra were recorded in deuterated solvents on a Varian Mercury 400 (400 MHz) spectrometer and calibrated to the residual solvent peak or tetramethylsilane (= 0 ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, dd = double doublet, br s = broad singlet. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) was measured by Autoflex II (Bruker Daltonics, Billerica, MA) and Ultraflex III (Bruker Daltonics, Billerica, MA) spectrometers using α -cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SA) as the matrix. High-resolution electrospray ionization quadrupole fourier transform mass spectroscopy (HR-ESI Qq-LTMS) were measured by a Bruker apex-ultra (7T) mass spectrometer, respectively.

Synthesis

Scheme 1. Synthesis of **1**

Reaction conditions: (a) *1H*-imidazole-4-acetic acid hydrochloride, EDCI•HCl, HOBT•H₂O, DIPEA in DMF, (b) **1-2**, pyridine in DCM, (c) TFA in DCM and (d) **1-4**, DIPEA in DMF.

Compound **1-2**

A solution of **1-0** (120 mg, 0.34 mmol), *N,N'*-disuccinimidyl carbonate (DSC) (264 mg, 1.02 mmol) and triethylamine (101 mg, 1.02 mmol) in anhydrous CH₃CN (2 mL) was stirred for 6 h at room temperature (rt). After dilution with water, the resulting mixture was extracted with AcOEt. The organic layer was washed with sat. NaHCO₃aq and dried over Na₂SO₄ followed by concentration *in vacuo*. The residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH = 40 : 1) to give **1-2** (115 mg, 70%) as a yellow amorphous powder. ¹H-NMR (400 MHz, CDCl₃) δ 8.99 (s, 1H), 8.68 (s, 1H), 7.42 (d, *J* = 9.2 Hz, 1H), 6.65 (d, *J* = 6.4 Hz, 1H), 6.49 (s, 1H), 4.48 (t, *J* = 4.8 Hz, 2H), 3.79 (t, *J* = 4.4 Hz, 2H), 3.67-3.69 (m, 4H), 3.45 (q, *J* = 7.2 Hz, 4H), 1.24 (t, *J* = 7.2 Hz, 6H). HR-ESI MS *m/z* calcd for [M+H]⁺ 490.1820, found 490.1817.

Compound **1-1**

A solution of *N*-(*tert*-Butoxycarbonyl)-1,5-diaminopentane hydrochloride (1.00 g, 4.94 mmol), imidazole-4-acetic acid hydrochloride (0.96 g, 5.93 mmol), HOBT•H₂O (1.14 g, 7.41 mmol), EDCI•HCl (1.42 g, 7.41 mmol) and DIPEA (2.58 mL, 14.8 mmol) in dry DMF (100 mL) was stirred for 4 h at rt. After removal of the solvent by evaporation, the residue was diluted with AcOEt. The organic layer was extracted with 5% citric acid *aq*. Then, the aqueous layer was alkalized with aqueous NH₃ solution (pH = 9) and extracted with AcOEt. – The organic layer was dried over Na₂SO₄ and concentrated by evaporation. – The residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH : NH₃ = 100 : 10 : 1) to give **1-1** (705 mg, 50 %) as a colorless amorphous powder. ¹H-NMR (400 MHz, CD₃OD) δ 7.61 (s, 1H), 6.95 (s, 1H), 3.48 (s, 2H), 3.18 (t, *J* = 6.8 Hz, 2H), 3.01 (t, *J* = 6.8 Hz, 2H), 1.43-1.60 (m, 13H), 1.32 (quin, *J* = 7.2 Hz, 2H). HR-ESI MS *m/z* calcd for [M+H]⁺ 311.2078, found 311.2076.

Compound **1-3 (6)**

A solution of **1-1** (55 mg, 180 μ mol), **1-2** (83 mg, 180 μ mol), and pyridine (14 mg, 180 μ mol) in CH₂Cl₂ (7 mL) was stirred overnight at rt. After removal of the solvent by evaporation, the residue was dissolved in AcOEt. The organic layer was washed with sat. NaHCO₃ *aq* and 1 N HCl. After drying over Na₂SO₄, the solvent was removed *in vacuo* to give **1-3 (6)** (92 mg, 75%) as a yellow amorphous powder. ¹H-NMR (400 MHz, CDCl₃) δ 9.09 (br s, 1H), 8.67 (s, 1H), 8.19 (s, 1H), 7.44 (d, *J* = 3.6 Hz, 1H), 7.41 (s, 1H), 6.65 (d, *J* = 9.2 Hz, 1H), 6.55 (s, 1H), 4.57 (t, *J* = 4.4 Hz, 2H), 3.83 (t, *J* = 4.4 Hz, 2H), 3.66-3.71 (m, 4H), 3.53 (s, 2H), 3.47 (q, *J* = 7.2 Hz, 4H), 3.22 (q, *J* = 6.0 Hz, 2H), 3.07 (q, *J* = 6.0 Hz, 2H), 1.43-1.50 (m, 13H), 1.23-1.33 (m, 8H). HR-ESI MS *m/z* calcd for [M+H]⁺ 685.3556, found 685.3560.

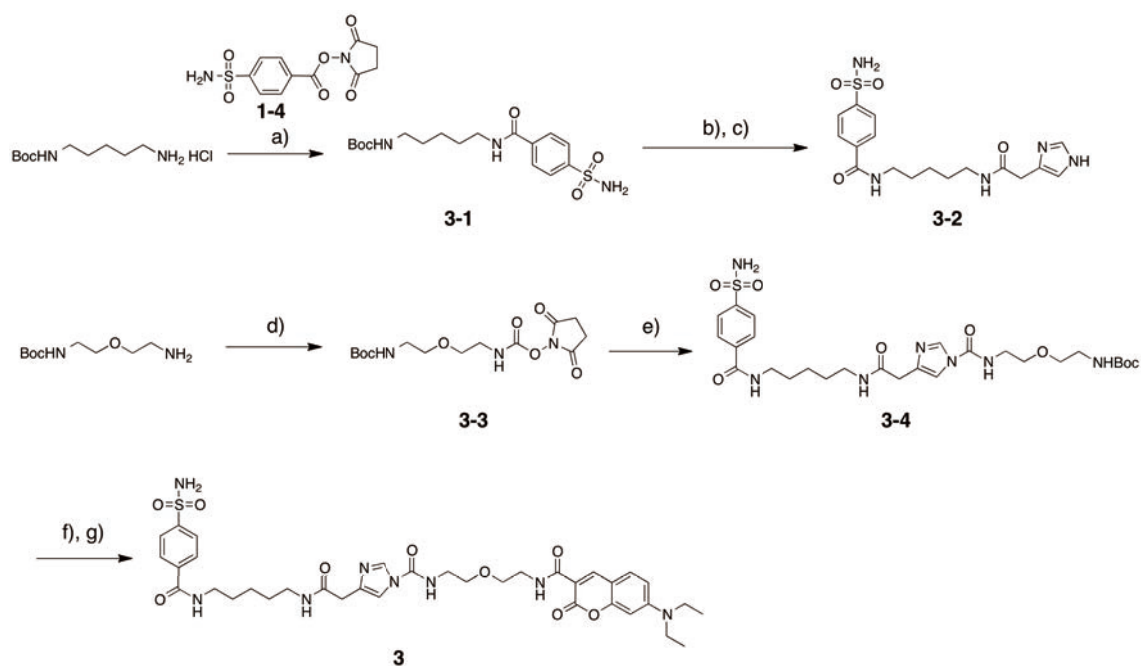
Compound **1**

A solution of **1-3** (50 mg, 80 μ mol) in CH₂Cl₂ (2 mL) / TFA (1.5 mL) was stirred for 2 h at rt. After removal of the solvent *in vacuo*, the residual TFA was removed with toluene (twice) to give deprotected **1-3**.

A mixture of deprotected **1-3**, **1-4**^{S1} (24 mg, 80 μ mol), DIPEA (55 μ L, 320 μ mol) in dry DMF (5 mL) was stirred for 10 h at rt. After removal of the solvent *in vacuo*, the

residue was dissolved in CHCl_3 . The organic layer was washed with sat. NaHCO_3 *aq* and dried over Na_2SO_4 followed by concentration *in vacuo*. The residue was purified by flash column chromatography on SiO_2 (CHCl_3 : MeOH = 10 : 1 to 5 : 1) to give **1** (45 mg, 59%) as a yellow amorphous powder. ^1H -NMR (400 MHz, CDCl_3 : CD_3OD = 1 : 1) δ 9.19 (br s, 1H), 8.62 (s, 1H), 8.19 (s, 1H), 7.97 (d, J = 8.4 Hz, 2H), 7.94 (d, J = 8.4 Hz, 2H), 7.50 (d, J = 9.2 Hz, 1H), 7.49 (s, 1H), 6.75 (d, J = 9.2 Hz, 1H), 6.55 (s, 1H), 4.61 (m, 2H), 3.88 (t, J = 4.8 Hz, 2H), 3.73 (t, J = 4.8 Hz, 2H), 3.64 (q, J = 4.4 Hz, 2H), 3.52 (q, J = 7.2 Hz, 4H), 3.48 (s, 2H), 3.40 (t, J = 6.4 Hz, 2H), 3.22 (q, J = 6.0 Hz, 2H), 1.64 (quin, J = 7.2 Hz, 2H), 1.56 (quin, J = 7.6 Hz, 2H), 1.40 (quin, J = 7.2 Hz, 2H), 1.27 (t, J = 7.2 Hz, 6H). HR-ESI MS m/z calcd for $[\text{M}+\text{H}]^+$ 768.3021, found 768.3029.

Scheme 2. Synthesis of **3**



Reaction conditions: (a) **1-4**, DIPEA in DMF, (b) TFA in DCM, (c) *1H*-imidazole-4-acetic acid hydrochloride, EDCI•HCl, HOBT•H₂O, DIPEA in DMF, (d) DSC, TEA in DMF at 40 °C, (e) **3-2**, pyridine in DMF, (f) TFA in DCM and (g) 7-diethylaminocoumarin-3-carboxylic acid, EDCI•HCl, HOBT•H₂O, DIPEA in DMF.

Compound **3-2**

A solution of **1-4** (200 mg, 0.67 mmol), *N*-(*tert*-butoxycarbonyl)-1,5-diaminopentane (136 mg, 0.67 mmol), and DIPEA (0.446 mL, 2.68 mmol) in dry DMF (20 mL) was stirred for 2 h at rt. After removal of the solvent *in vacuo*, the residue was dissolved in CHCl₃. The organic layer was washed with water and dried over Na₂SO₄ followed by concentration *in vacuo* to give **3-1** (247 mg, 95%) as a white solid.

A solution of **3-1** (150 mg, 390 μmol) in TFA (4 mL) was stirred for 1.5 h at rt. After removal of the solvent *in vacuo*, the residual TFA was further removed with toluene (twice). The residue was dissolved in dry DMF (17 mL), and the solution was mixed with DIPEA (389 μL, 1.16 mmol), imidazole-4-acetic acid hydrochloride (59 mg, 85 μmol), HOBT•H₂O (88 mg, 580 μmol) and EDCI•HCl (111 mg, 580 μmol). The reaction mixture was stirred overnight at rt. After removal of the solvent *in vacuo*, the residue was purified by flash column chromatography on SiO₂ (CHCl₃ / MeOH = 3 / 1) to give **3-2** (103 mg, 67%) as a colorless oil. ¹H-NMR (400 MHz, CD₃OD) δ 7.96 (m, 4H), 7.79 (s, 1H), 7.00 (s, 1H), 3.52 (s, 2H), 3.38 (t, *J* = 7.2 Hz, 2H), 3.19-3.23 (m, 2H), 1.64 (m, 2H), 1.56 (m, 2H), 1.40 (m, 2H). HR-ESI MS *m/z* calcd for [M+H]⁺ 394.1544, found 394.1539.

Compound **3-3**

To a solution of *N,N'*-disuccinimidyl carbonate (279 mg, 1.09 mmol) in dry DMF (2 mL) was added dropwise the solution of **3-1** (44 mg, 0.215 mmol) and TEA (0.151 mL, 1.09 mmol) in dry DMF (10 mL) at 40 °C. The reaction mixture was stirred for 2 h at rt. After removal of the solvent *in vacuo*, the residue was dissolved in AcOEt. The organic layer was washed with 1N HCl and dried over Na₂SO₄. After removal of the solvent *in vacuo*, **3-3** (102 mg, containing a small amount of impurities) was obtained as a white solid. HR-ESI MS *m/z* calcd for [M+Na]⁺ 368.1428, found 368.1427.

A solution of **3-3** (27 mg, 79 μmol), **3-2** (31 mg, 79 μmol), and pyridine (6 μL, 79 μmol) in dry DMF (2 mL) was stirred for 3 days at rt. After removal of the solvent *in vacuo*, the residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH = 10 : 1 to 8 : 1) to give **3-4** (15 mg, 42% in 2 steps) as a white powder. ¹H-NMR (400 MHz, CDCl₃ : CD₃OD = 1 : 1) δ 8.12 (s, 1H), 7.91-7.97 (m, 5H), 7.49 (br s, 1H), 7.42 (s, 1H), 5.66 (br s, 1H), 3.61-3.68 (m, 6H), 3.52 (q, *J* = 5.2 Hz, 4H), 3.28 (q, *J* = 5.2 Hz, 2H), 3.24 (q, *J* = 5.2 Hz, 2H), 1.65 (quin, *J* = 7.2 Hz, 2H), 1.56

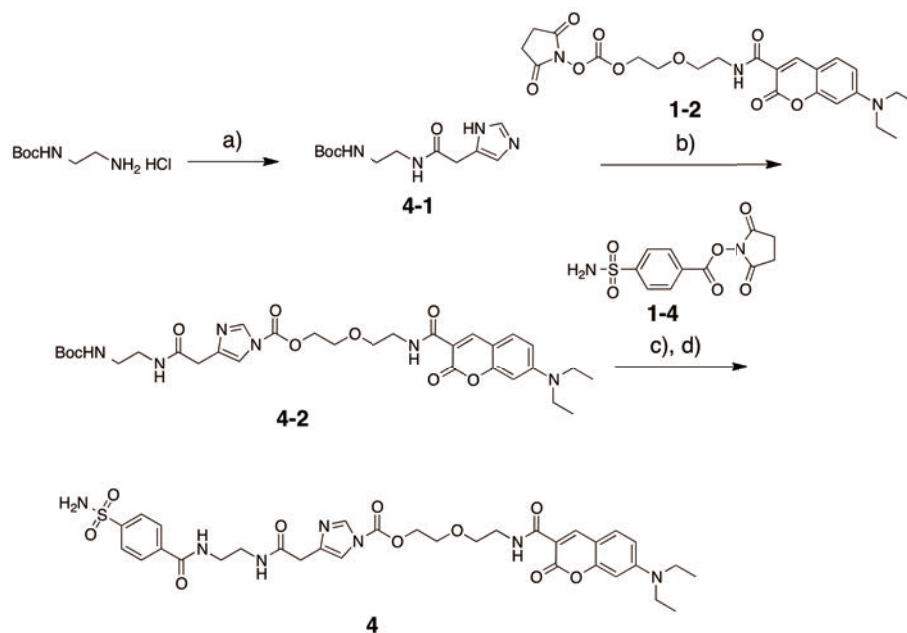
(quin, $J = 7.2$ Hz, 2H), 1.39-1.43 (m, 11H). HR-ESI MS m/z calcd for $[M+H]^+$ 624.2810, found 624.2811.

Compound 3

A solution of **3-4** (5 mg, 8.0 μ mol) in TFA (2 mL) was stirred for 1 h at rt. After removal of the solvent *in vacuo*, the residual TFA was further removed with toluene (twice) to give the deprotected **3-4**.

A solution of the deprotected **3-4**, 7-diethylaminocoumarin-3-carboxylic acid (3 mg, 10 μ mol), EDCI $\cdot$ HCl (2 mg, 12 μ mol), HOBt $\cdot$ H₂O (2 mg, 12 μ mol), and DIPEA (20 μ L) in dry DMF (2 mL) was stirred for 10 h at rt. After removal of the solvent *in vacuo*, the residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH = 8 : 1) to give **3** (2 mg, 33 % in 2 steps) as a yellow powder. ¹H-NMR (400 MHz, CDCl₃ : CD₃OD = 4 : 1) δ 9.21 (br s, 1H), 8.64 (s, 1H), 8.19 (s, 1H), 7.93-7.98 (m, 4H), 7.55 (s, 1H), 7.48 (d, $J = 8.8$ Hz, 1H), 6.70 (d, $J = 8.8$ Hz, 1H), 6.55 (s, 1H), 3.59 (t, $J = 4.4$ Hz, 2H), 3.37-3.52 (m, 14H), 3.23 (q, $J = 6.4$ Hz, 2H), 1.63 (quin, $J = 7.6$ Hz, 2H), 1.54 (quin, $J = 7.6$ Hz, 2H), 1.37 (quin, $J = 7.6$ Hz, 2H), 1.26 (t, $J = 6.0$ Hz, 6H). HR-ESI MS m/z calcd for $[M+H]^+$ 767.3181, found 767.3188.

Scheme 3. Synthesis of 4



Reaction conditions: (a) 1*H*-imidazole-4-acetic acid hydrochloride, EDCI•HCl, HOBT•H₂O, DIPEA in DMF, (b) **1-2**, pyridine in DCM, (c) TFA in DCM and (d) **1-4**, DIPEA in DMF.

Compound 4-1

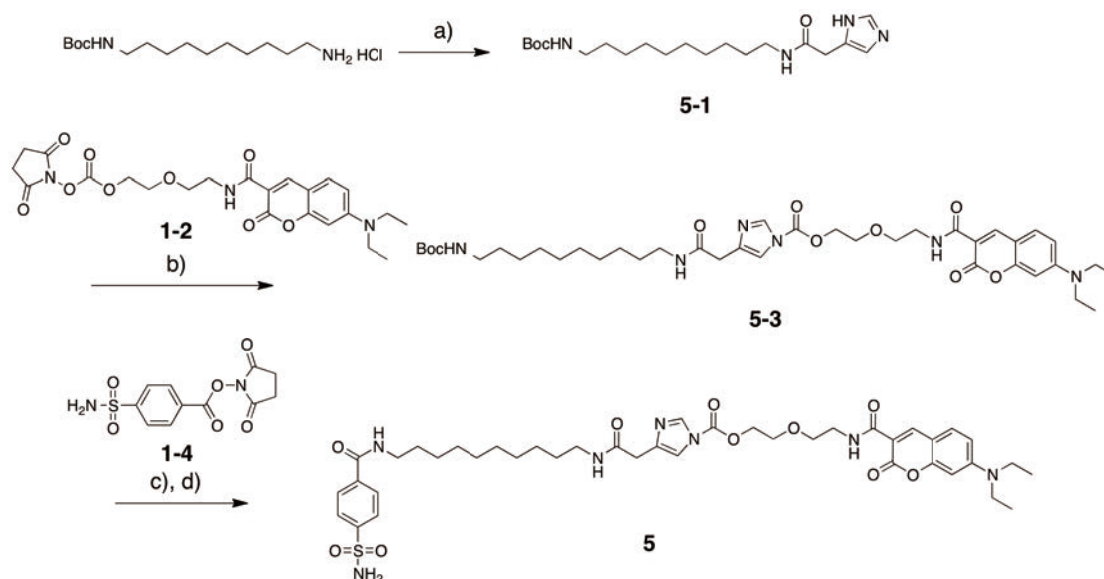
N-(*tert*-Butoxycarbonyl)-1,2-diaminoethane hydrochloride (100 mg, 0.51 mmol) was used as a starting material. By the same procedure described for the synthesis of **1-1**, **4-1** (75 mg, 55%) was obtained as a colorless amorphous powder. ¹H-NMR (400 MHz, CD₃OD) δ 7.61 (s, 1H), 6.95 (s, 1H), 3.50 (s, 2H), 3.27 (t, *J* = 6.0 Hz, 2H), 3.14 (t, *J* = 6.0 Hz, 2H), 1.43 (s, 9H). HR-ESI MS *m/z* calcd for [M+H]⁺ 269.1608, found 269.1608.

Compound 4-2

4-1 (30 mg, 0.11 mmol) was used as a starting material. By the same procedure described for the synthesis of **1-3**, **4-2** (72 mg, quant.) was obtained as a yellow amorphous powder. ¹H-NMR (400 MHz, CDCl₃) δ 9.09 (br s, 1H), 8.67 (s, 1H), 8.17 (s, 1H), 7.45 (d, *J* = 5.2 Hz, 1H), 7.42 (s, 1H), 6.65 (d, *J* = 9.2 Hz, 1H), 6.56 (s, 1H), 4.57 (t, *J* = 4.4 Hz, 2H), 3.83 (t, *J* = 4.4 Hz, 2H), 3.65-3.71 (m, 4H), 3.55 (s, 2H), 3.49 (q, *J* = 7.2 Hz, 4H), 3.36 (q, *J* = 6.0 Hz, 2H), 3.24 (t, *J* = 5.6 Hz, 2H), 1.40 (s, 9H), 1.25 (t, *J* = 7.2 Hz, 6H). HR-ESI MS *m/z* calcd for [M+H]⁺ 643.3086, found 643.3080.

Compound 4

4-2 (72 mg, 110 μmol) was used as a starting material. By the same procedure described for the synthesis of **1**, compound **4** (22 mg, 27%) was obtained as a yellow amorphous powder. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 9.03 (br s, 1H), 8.59 (s, 1H), 8.02 (s, 1H), 7.85 (d, $J = 8.0$ Hz, 2H), 7.77 (d, $J = 8.4$ Hz, 2H), 7.58 (br s, 1H), 7.40-7.43 (m, 2H), 6.65 (d, $J = 8.8$ Hz, 1H), 6.52 (s, 1H), 5.68 (br s, 1H), 4.56 (br s, 2H), 3.83 (br s, 2H), 3.70 (t, $J = 4.8$ Hz, 2H), 3.64 (t, $J = 5.2$ Hz, 2H), 3.53-3.55 (m, 6H), 3.47 (q, $J = 7.2$ Hz, 4H), 1.25 (t, $J = 7.2$ Hz, 6H). HR-ESI MS m/z calcd for $[\text{M}+\text{H}]^+$ 726.2552, found 726.2558.

Scheme 4. Synthesis of **5**

Reaction conditions: (a) 1H-imidazole-4-acetic acid hydrochloride, EDCI·HCl, HOBT·H₂O, DIPEA in DMF, (b) **1-2**, pyridine in DCM, (c) TFA in DCM and (d) **1-4**, DIPEA in DMF.

Compound **5-1**

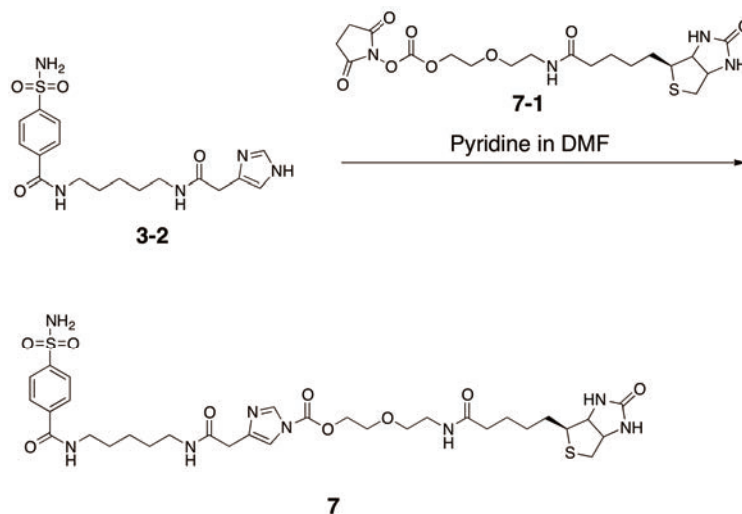
N-(*tert*-Butoxycarbonyl)-1,10-diaminodecane hydrochloride (1.00 g, 3.23 mmol) was used as a starting material. By the same procedure described for the synthesis of **1-1**, **5-1** (700 mg, 57%) was obtained as a colorless amorphous powder. ¹H-NMR (400 MHz, CD₃OD) δ 7.61 (s, 1H), 6.94 (s, 1H), 3.48 (s, 2H), 3.17 (t, *J* = 6.8 Hz, 2H), 3.01 (t, *J* = 7.2 Hz, 2H), 1.43-1.52 (m, 13H), 1.30 (br s, 12H). HR-ESI MS *m/z* calcd for [M+H]⁺ 381.2860, found 381.2859.

Compound **5-2**

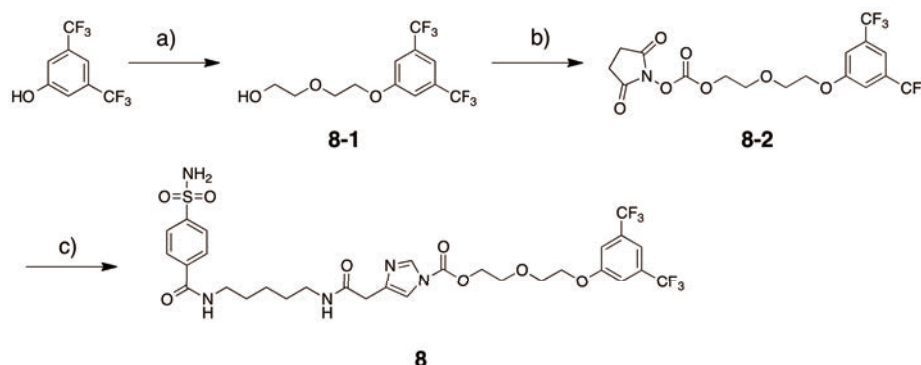
5-1 (60 mg, 0.15 mmol) was used as a starting material. By the same procedure described for the synthesis of **1-3**, **5-2** (98 mg, 86%) was obtained as a yellow amorphous powder. ¹H-NMR (400 MHz, CDCl₃) δ 9.10 (t, *J* = 4.8 Hz, 1H), 8.67 (s, 1H), 8.18 (s, 1H), 7.45 (d, *J* = 5.6 Hz, 1H), 7.42 (s, 1H), 6.65 (d, *J* = 8.8 Hz, 1H), 6.56 (s, 1H), 4.57 (t, *J* = 4.4 Hz, 2H), 3.83 (t, *J* = 4.4 Hz, 2H), 3.70 (t, *J* = 4.8 Hz, 2H), 3.67 (t, *J* = 4.8 Hz, 2H), 3.53 (s, 2H), 3.47 (q, *J* = 7.2 Hz, 4H), 3.21 (q, *J* = 6.8 Hz, 2H), 3.09 (q, *J* = 6.0 Hz, 2H), 1.44 (m, 13H), 1.23-1.28 (m, 18H). HR-ESI MS *m/z* calcd for [M+H]⁺ 755.4338, found 755.4340.

Compound **5**

5-2 (50 mg, 66 μ mol) was used as a starting material. By the same procedure described for the synthesis of **1**, compound **5** (42 mg, 76%) was obtained as a yellow amorphous powder. ¹H-NMR (400 MHz, CDCl₃ : CD₃OD = 1 : 1) δ 9.19 (br s, 1H), 8.63 (s, 1H), 8.20 (s, 1H), 7.93 (d, *J* = 8.4 Hz, 2H), 7.90 (d, *J* = 8.4 Hz, 2H), 7.48 (s, 1H), 7.47 (d, *J* = 9.2 Hz, 1H), 6.69 (d, *J* = 9.2 Hz, 1H), 6.53 (s, 1H), 4.59 (m, 2H), 3.71 (t, *J* = 5.2 Hz, 2H), 3.65 (t, *J* = 6.0 Hz, 2H), 3.46-3.51 (m, 4H), 3.36-3.42 (m, 6H), 3.18 (q, *J* = 6.0 Hz, 2H), 1.62 (m, 2H), 1.46 (m, 2H), 1.21-1.34 (m, 20H). HR-ESI MS *m/z* calcd for [M+H]⁺ 838.3804, found 838.3806.

Scheme 5. Synthesis of **7**Compound **7**

To a stirred solution of **3-2** (22 mg, 56 μmol) in dry DMF (2 mL) was added **7-1**^{S2} (32 mg, 67 μmol), pyridine (5.1 μL) at room temperature. The reaction mixture was allowed to stir for 12 h at rt. The reaction mixture was purified by column chromatography (CHCl_3 : MeOH = 10 : 1 to 5 : 1) to yield **7** (239 mg). The reaction mixture was purified by HPLC [(0.1 % TFA MeCN : 0.1 % TFA H_2O , linear gradient) = 5 : 95 (0 min), 30 : 70 (50 min), 100 : 0 (60 min) to 5 : 95 (70 min)] to yield **7** (13 mg, 30 %) as a white powder. ^1H -NMR (400 MHz, CD_3OD) δ 8.20 (s, 1H), 7.95 (m, 4H), 7.45 (s, 1H), 4.59 – 4.55 (m, 2H), 4.47 (dd, J = 4.8, 7.6 Hz, 1H), 4.28 (dd, J = 4.8, 7.6 Hz, 1H), 3.81 (t, J = 5.6 Hz, 2H), 3.57 (t, J = 5.6 Hz, 2H), 3.48 (s, 2H), 3.40 – 3.33 (m, 3H), 3.18 – 3.15 (m, 4H), 2.90 (dd, J = 4.8, 12.8 Hz, 1H), 2.69 (d, J = 12.8 Hz, 1H), 2.17 (t, J = 7.2 Hz, 2H), 1.73 – 1.54 (m, 8H), 1.45 – 1.38 (m, 4H). HR-ESI MS m/z calcd for $[\text{M}+\text{H}]^+$ 751.2902, found 751.2902.

Scheme 6. Synthesis of **8**

Reaction conditions: (a) ethylene glycol mono-2-chloroethyl ether, K_2CO_3 , KI in DMF at 60 °C (b) DSC, TEA in DMF and (c) **3-2**, pyridine in DMF.

Compound **8-1**

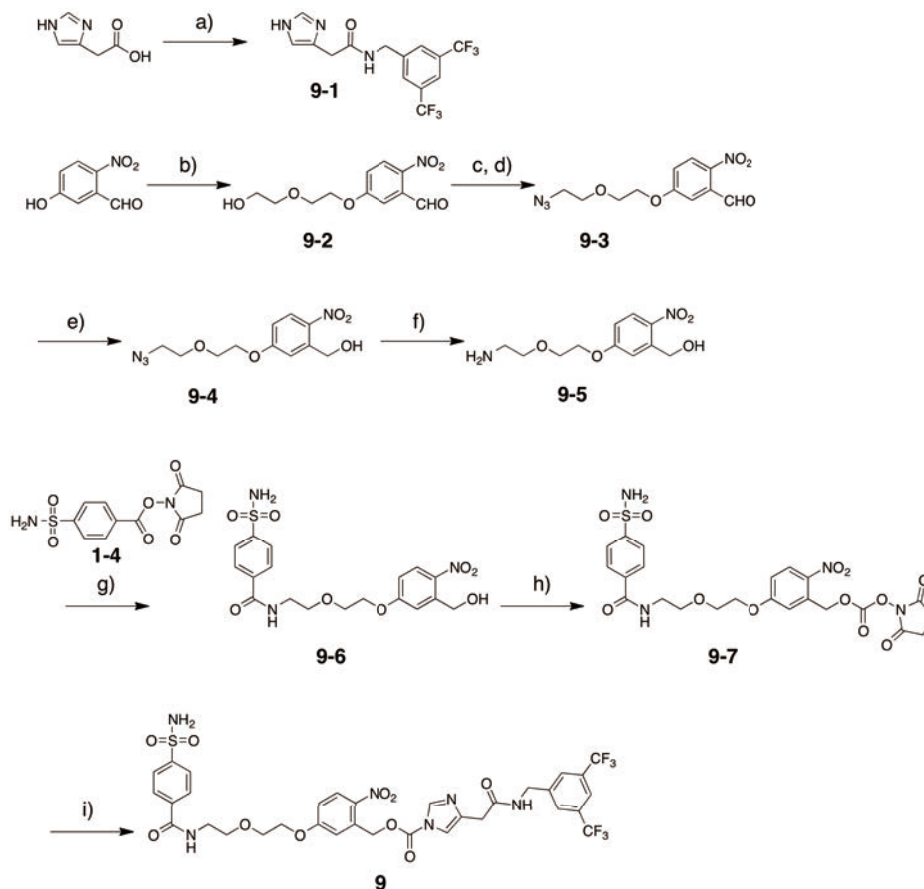
To a stirred solution of 3,5-bis(trifluoromethyl)phenol (800 mg, 3.48 mmol), K_2CO_3 (1.92 g, 13.9 mmol), KI (1.16 g, 6.96 mmol) in dry *N,N*-dimethylformamide (3 mL) was added ethylene glycol mono-2-chloroethyl ether (1.30 g, 10.4 mmol). The reaction mixture was allowed to stir for 40 h at 60 °C. To the reaction mixture was added sat. $NaHCO_3$ *aq* to quench redundant ethylene glycol mono-2-chloroethyl ether. The reaction mixture was allowed to stir for 7 h at 60 °C. The reaction mixture was diluted with AcOEt. The solution was washed with sat. $NaHCO_3$, 5 % citric acid *aq*, brine. The organic layer was dried over $MgSO_4$, filtered, evaporated to yield **8-1** (1.26 g, quantitative yield) as pale yellow oil. 1H -NMR (400 MHz, $CDCl_3$) δ 7.47 (s, 1H), 7.35 (s, 1H), 4.23 (t, J = 4.4 Hz, 2H), 3.91 (t, J = 4.4 Hz, 2H), 3.78 (t, J = 4.4 Hz, 2H), 3.69 (t, J = 4.4 Hz, 2H), 1.95 (br, 1H).

Compound **8-2**

To a stirred solution of **8-1** (60 mg, 0.189 mmol) in dry DMF (3 mL) was added *N,N'*-disuccinimidyl carbonate (145 mg, 0.567 mmol), triethylamine (78.6 μ L, 0.567 mmol) at room temperature. The reaction mixture was allowed to stir for 6 h at room temperature. The reaction mixture was purified by column chromatography ($CHCl_3$) to yield **8-2** (70 mg, 80%) as pale yellow oil. 1H -NMR (400 MHz, $CDCl_3$) δ 7.47 (s, 1H), 7.37 (s, 1H), 4.50 (t, J = 4.4 Hz, 2H), 4.24 (t, J = 4.4 Hz, 2H), 3.92 (t, J = 4.4 Hz, 2H), 3.87 (t, J = 4.4 Hz, 2H), 2.84 (s, 4H).

Compound **8**

To a stirred solution of **3-2** (16 mg, 41 μ mol) in dry DMF (3 mL) was added **8-2** (23 mg, 49 μ mol, 1.2 eq), pyridine (3.7 μ L, 49 mmol) at rt. The reaction mixture was allowed to stir for 6 h at rt. The reaction mixture was purified by column chromatography (CHCl_3 : MeOH = 20 : 1 to 10 : 1) to yield **8** (12 mg, 41%). $^1\text{H-NMR}$ (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ = 7/3) δ 8.12 (s, 1H), 7.94 (d, J = 4.4 Hz, 4H), 7.56 (s, 1H), 7.45 (s, 2H), 7.40 (s, 1H), 4.60 (t, J = 4.4 Hz, 2H), 3.94 (t, J = 4.4 Hz, 2H), 3.45 (t, J = 4.4 Hz, 2H), 3.42 (t, J = 4.4 Hz, 2H), 3.36 (t, J = 1.6 Hz, 1H), 3.27 – 3.22 (m, 2H), 1.65 – 1.63 (m, 2H), 1.57 – 1.54 (m, 2H), 1.47 – 1.40 (m, 2H). HR-ESI MS m/z calcd for $[\text{M}+\text{H}]^+$ 738.2027, found 738.2025.

Scheme 7. Synthesis of **9**

Reaction conditions: (a) 3,5-bis(trifluoromethyl)benzyl amine, EDCI•HCl, HOBT•H₂O, DIPEA in DMF, (b) 2-(2-chloroethoxy)ethanol, K₂CO₃ in DMF at 50 °C, (c) MsCl, TEA in DCM at 0 °C, (d) NaN₃ in DMF at 50 °C, (e) NaBH₄ in THF, (f) PPh₃ in THF/H₂O, (g) **1-4**, DIPEA in DMF, (h) DSC, DIPEA in DMF and (i) **9-1**, DIPEA in DMF

Compound **9-1**

To a stirred solution of 1H-imidazole-4-acetic acid hydrochloride (446 mg, 3.0 mmol), EDCI•HCl (578 mg, 3.0 mmol), HOBT•H₂O (105 mg, 3.0 mmol) and DIPEA (1433 μL) in DMF (20 mL) was added 3,5-bis(trifluoromethyl)benzyl amine (734 mg, 2.74 mmol). The reaction mixture was stirred at rt for 1 hour. The mixture was diluted with sat. NaHCO₃ aq and then extracted with CHCl₃. The combined organic layer was washed with brine and dried over MgSO₄. Filtration and concentration *in vacuo* gave **9-1** (597 mg, 62%) as a light yellow solid. ¹H-NMR (400 MHz, CDCl₃ : CD₃OD = 1 : 1) δ 7.75 (s, 1H), 7.72 (s, 2H), 7.60 (s, 1H), 6.96 (s, 1H), 4.51 (s, 1H), 3.57 (s, 1H).

Compound **9-2**

To a stirred solution of 5-hydroxy-2-nitrobenzaldehyde (1000 mg, 6.0 mmol), KI (166 mg, 1.0 mmol) and K_2CO_3 (1244 mg, 9.0 mmol) in DMF (20 mL) was added 2-(2-chloroethoxy)ethanol (1246 mg, 10 mmol). The reaction mixture was stirred at 50 °C for 24h. The mixture was extracted with AcOEt. The combined organic layer was washed with brine and dried over $MgSO_4$. Filtration, concentration *in vacuo* and purification by silica gel flash column chromatography ($CHCl_3$) gave 1409 mg (92 %) of **9-2** as a white solid. 1H -NMR (400 MHz, $CDCl_3$) δ 10.48 (s, 1H), 8.17 (dd, J = 1.6, 9.2 Hz, 1H), 7.38 (d, J = 2.8 Hz, 1H), 7.18 (dd, J = 2.8, 9.2 Hz, 1H), 4.30 (t, J = 4.4 Hz, 1H), 3.91 (t, J = 4.8 Hz, 1H), 3.78 (t, J = 4.4 Hz, 1H), 3.67 (t, J = 4.0 Hz, 2H).

Compound **9-3**

A solution of **9-2** (1400 mg, 5.5 mmol) in DCM (20 mL) is treated at 0 °C with TEA (1.5 mL) and methanesulfonyl chloride (387 μ L, 5.5 mmol). The reaction mixture is stirred at 0 °C for 1h. The mixture was partitioned between $CHCl_3$ and water. The collected organic layer was washed with brine, collected, dried over $MgSO_4$. Filtration, and concentration *in vacuo* gave mesylated **9-2** as a yellow solid. Without further purification, mesylated **9-2** was used in next step.

To a solution of mesylated **9-2** in DMF (20 mL) was added NaN_3 (650 mg, 10 mmol) and K_2CO_3 (1382 mg, 10 mmol). The mixture was stirred for 8h at 50 °C. The reaction mixture was poured into water and the whole was extracted with AcOEt. The AcOEt layer was separated, washed with brine, and was dried over $MgSO_4$. Filtration, concentration *in vacuo*, purification by silica gel flash column chromatography ($CHCl_3$: AcOEt = 10 : 1) gave 1314 mg (85%) of **9-3** as a white solid. 1H -NMR (400 MHz, $CDCl_3$) δ 10.49 (s, 1H), 8.15 (d, J = 9.2 Hz, 1H), 7.36 (d, J = 2.8 Hz, 1H), 7.19 (dd, J = 3.2, 9.3 Hz, 1H), 4.29 (t, J = 4.4 Hz, 1H), 3.91 (t, J = 4.4 Hz, 1H), 3.74 (t, J = 4.8 Hz, 1H), 3.67 (t, J = 5.2 Hz, 2H).

Compound **9-4**

To a stirred suspension of **9-3** (1314 mg, 4.69 mmol) in THF (15 mL) was added $NaBH_4$ (190 mg, 5.0 mmol). The reaction mixture was stirred at rt for 30 min. The solvent was removed by evaporation. The residue was diluted with water, and then

extracted with AcOEt. The combined organic layer was washed with sat. NaHCO_3 *aq* and brine and dried over MgSO_4 . Filtration, and concentration *in vacuo* gave **9-4** (1324 mg, 100%) as a white solid. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.18 (d, $J = 8.8$ Hz, 1H), 7.26 (overlapped with CHCl_3 , 1H), 6.92 (dd, $J = 2.8, 9.2$ Hz, 1H), 5.00 (s, 2H), 4.27 (t, $J = 4.4$ Hz, 1H), 3.91 (t, $J = 4.4$ Hz, 1H), 3.75 (t, $J = 4.4$ Hz, 1H), 3.42 (t, $J = 4.8$ Hz, 2H).

Compound **9-5**

To a stirred suspension of **9-4** (779 mg, 2.76 mmol) in THF/ H_2O (10 mL / 1 mL) was added PPh_3 (796 mg, 3.04 mmol). The reaction mixture was stirred at rt for 24h. The solvent was removed by evaporation. The residue was purified by silica gel flash column chromatography (CHCl_3 saturated by NH_3 *aq* : MeOH = 4 : 1) gave 587 mg (83 %) of **9-5** as a white solid. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.16 (d, $J = 9.2$ Hz, 1H), 7.39 (d, $J = 2.8$ Hz, 1H), 6.90 (dd, $J = 3.2, 9.2$ Hz, 1H), 5.01 (s, 2H), 4.30 (t, $J = 4.8$ Hz, 1H), 3.86 (t, $J = 4.8$ Hz, 1H), 3.86 (t, $J = 4.8$ Hz, 1H), 2.91 (t, $J = 4.4$ Hz, 2H).

Compound **9-6**

To a stirred solution of **9-5** (254 mg, 1.0 mmol) and DIPEA (348 μL) in DMF (2 mL) and DCM (10mL) was added **1-4** (298 mg, 1.0 mmol). The reaction mixture was stirred at rt for 2 hours. The mixture was diluted with sat. NaHCO_3 *aq* and then extracted with AcOEt. The combined organic layer was washed with brine and dried over MgSO_4 . Filtration, concentration *in vacuo* and purification by silica gel flash column chromatography (CHCl_3 : MeOH = 8 : 1) gave 369 mg (84 %) of **9-6** as a white solid. $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ 8.12 (dd, $J = 3.6, 9.2$ Hz, 1H), 7.94 – 7.87 (m, 4H), 7.48 (m, 1H), 6.88 (d, $J = 8.8$ Hz, 1H), 4.99 (s, 2H), 4.29 (m, 2H), 3.91 (m, 1H), 3.75 (m, 1H), 3.63 (m, 2H).

Compound **9-7**

To a stirred solution of **9-6** (50 mg, 114 μmol) and DIPEA (75 μL) in DMF (3 mL) was added *N,N'*-disuccinimidyl carbonate (143 mg, 563 μmol). The reaction mixture was stirred at rt for 5 hours. The mixture was diluted with sat. NaHCO_3 *aq* and then extracted with AcOEt. The combined organic layer was washed with brine and dried over MgSO_4 . Filtration, concentration *in vacuo* gave 35.1 mg (53 %) of **9-7** as a white

solid. ^1H -NMR (400 MHz, CD_3OD) δ 8.20 (d, $J = 9.6$ Hz, 1H), 7.97 – 7.85 (m, 4H), 7.45 (m, 1H), 7.16 (m, 1H), 5.72 (s, 2H), 4.28 (m, 1H), 3.80 (m, 1H), 3.62 (m, 1H), 3.46 (m, 1H), 2.80 (s, 4H).

Compound 9

To a stirred solution of **9-7** (30.2 mg, 52 μmol) and DIPEA (32 μL) in DMF (2 mL) was added **9-1** (18.6 mg, 53 μmol). The reaction mixture was stirred at rt for 2 hours. The mixture was diluted with sat. NaHCO_3 aq, and then extracted with AcOEt. The combined organic layer was washed with brine and dried over MgSO_4 . Filtration, concentration *in vacuo* and purification by silica gel flash column chromatography (CHCl_3 : MeOH = 1 : 0 to 10 : 1) gave 21.4 mg (50 %) of **9** as a white solid. ^1H -NMR (400 MHz, CDCl_3 : CD_3OD = 1 : 1) δ 8.23 (m, 1H), 8.21 (s, 1H), 7.95 – 7.88 (m, 4H), 7.79 (s, 1H), 7.77 (s, 1H), 7.58 (d, $J = 2.8$ Hz, 1H), 7.49 (s, 1H), 7.05 (m, 1H), 5.83 (s, 2H), 4.53 (s, 2H), 4.29 (m, 1H), 3.91 (m, 1H), 3.63 (m, 1H). HR-ESI MS m/z calcd for $[\text{M}+\text{H}]^+$ 817.1721, found 817.1710.

Peptide mapping of the Dc labeled CA1

A solution of CA1 (30 μ M) and **1** (60 μ M) in HEPES buffer (50 mM, pH 7.2) was incubated for 12 h at 37 °C. The labeled CA1 was purified by size-exclusion chromatography (TOYOPEARL HW-40F) and dialyzed against Tris buffer (100 mM, pH 8.0) using a Spectra/Por[®] dialysis membrane (MWCO: 10,000). After concentration by ultrafiltration (Centricon Ultracel YM-10, Millipore), the labeling yield of CA1 (63%) was determined based on the relative absorbance at 280 nm and 430 nm. For protein digestion, the solution was incubated with lysyl endopeptidase (LEP) (LEP/substrate ratio = 1/3 (w/w)) at 37 °C for 4 h in the presence of urea (2 M). Native (unlabeled) CAI was also subjected to LEP digestion under the same conditions. The digested sample was subjected to RP-HPLC (column; YMC-Pack Triat C18, 250 $\times$ 4.6 mm, mobile phase; CH₃CN (containing 0.1% TFA) : H₂O (containing 0.1% TFA) = 5 : 95 to 55 : 45 (linear gradient over 100 min), flow rate; 1.0 mL/min, detection; UV (220 nm) and fluorescence (excitation at 427 nm, emission at 473 nm)). For the peptide mapping, the labeled fragments were further analyzed by MALDI-TOF MS/MS.

Peptide mapping of the F6B labeled CA1

A solution of CA1 (10 μ M) and **8** (20 μ M) in HEPES buffer (50 mM, pH 7.2) was incubated for 8 h at 37 °C. The labeled CA1 was purified by size-exclusion chromatography (TOYOPEARL HW-40F). After concentration by ultrafiltration (Centricon Ultracel YM-10, Millipore), the labeling yield of CA1 (100%, 17 μ M) was determined based on the relative absorbance at 280 nm and ¹⁹F-NMR. For protein digestion, the solution was incubated with lysyl endopeptidase (LEP) (LEP/substrate ratio = 1/5 (w/w)) at 37 °C for 4.5 h in the presence of urea (2 M). Native (unlabeled) CA1 was also subjected to LEP digestion under the same conditions. The digested sample was subjected to RP-HPLC (column; YMC-Pack Triat C18, 250 $\times$ 4.6 mm, mobile phase; CH₃CN (containing 0.1% TFA) : H₂O (containing 0.1% TFA) = 5 : 95 to 55 : 45 (linear gradient over 100 min), flow rate; 1.0 mL/min, detection; UV (220 nm). All of the purified digested fragments were analyzed by MALDI-TOF mass. For the peptide mapping, the labeled fragments were further analyzed by MALDI-TOF MS/MS.

CA1 Labeling by **9** and Peptide Mapping of Caged CA1.

A solution of human CA1 (10 μ M, SIGMA, C4396) and **9** (20 μ M) in HEPES buffer (50 mM, pH 7.2) was incubated for 35 h at 25 °C. Aliquots were taken at different time points, and the labeling yields were determined by MALDI-TOF mass. The labeled CA1 was purified by size-exclusion chromatography (TOYO-PEARL HW-40F). After concentration by ultrafiltration (Centricon Ultracel YM-10, Millipore), the labeling yield of CA1 (95%, 20 μ M) was determined based on the relative absorbance at 280 and 325 nm. For protein digestion, the solution was incubated with lysyl endopeptidase (LEP) (LEP/substrate ratio = 1/3 (w/w)) at 25 °C for 4 h in the presence of urea (2 M). Native (unlabeled) CA1 was also subjected to LEP digestion under the same conditions. The digested sample was subjected to RP-HPLC (column; YMC-Pack Triat C18, 250 $\times$ 4.6 mm, mobile phase; CH₃CN (containing 0.1% TFA) : H₂O (containing 0.1% TFA) = 5 : 95 to 55 : 45 (linear gradient over 100 min), flow rate; 1.0 mL/min, detection; UV-Vis (220–400 nm). All of the purified digested fragments were analyzed by MALDI-TOF mass. For the peptide mapping, the labeled fragments were further analyzed by MALDI-TOF MS/MS.

Photo-Irradiation Conditions.

Photoirradiation experiments in quartz cuvettes (containing 200 μ L of 10 μ M caged CA1 solution in 50 mM HEPES buffer, pH 7.2) were carried out by using the light source (100 W mercury lamp (Ushio Inc., Japan) of a fluorescence microscope (Olympus IX71) or 500 W mercury lamp (Ushio Inc., Japan) with a 330–380 nm band-pass filter.

Enzyme Assay Protocols.

The hydrolytic (esterase) activities of the unlabeled, caged, and uncaged CA1s were assayed in HEPES buffer (50 mM, pH 7.2, 25 °C) using *p*-nitrophenyl acetate as the substrate. Reaction conditions were as follows: the protein concentration was 0.10 or 1.0 μ M, the samples were native CA1, caged CA1, uncaged CA1 and native CA1 with the cleaved ligand module (BS) (1 eq), and the substrate concentrations were 0.1–5 mM *p*-nitrophenyl acetate. Initial rates of *p*-nitrophenyl acetate hydrolysis were determined by measuring the increase in absorbance at 348 nm ($\Delta\epsilon_{348} = 5,150 \text{ M}^{-1}\text{cm}^{-1}$)²³ for

200–800 sec (for 0.10 μM enzyme) or 10–100 sec (for 1.0 μM enzyme). The background hydrolysis of *p*-NPA was observed, and the rate was subtracted in advance. Kinetic parameters were obtained by fitting a plot of the initial rates as a function of the substrate concentrations to the Michaelis-Menten equation.

Preparation of RBC lysate and caging/uncaging of eCA.

Human RBC lysate was prepared by a slight modification of the chloroform-ethanol extraction procedure.²² Briefly, 1 mL of RBC was lysed with 1 mL of HEPES buffered saline (20 mM HEPES, 107 mM NaCl, 6 mM KCl, 1.2 mM MgSO_4 , 2 mM CaCl_2 , 11.5 mM glucose, pH 7.4) using ultrasonic homogenizer. The homogenized RBC was centrifuged at 13500 rpm for 10 min at 4 °C and the supernatant was obtained as RBC lysate, to which **1** or **9** was added for eCA labeling performed. For enzyme assay, hemoglobin was removed as follows. 200 μL of 40% ethanol and 100 μL of chloroform were added to 200 μL of the RBC lysate on ice. The mixture was vigorously shaken for 5 minutes and centrifuged at 1500 rpm for 10 min at 4 °C. eCA was included in aqueous ethanol layer. This was repeated three times. The enzyme assay was performed using the combined solution.

2-5. References

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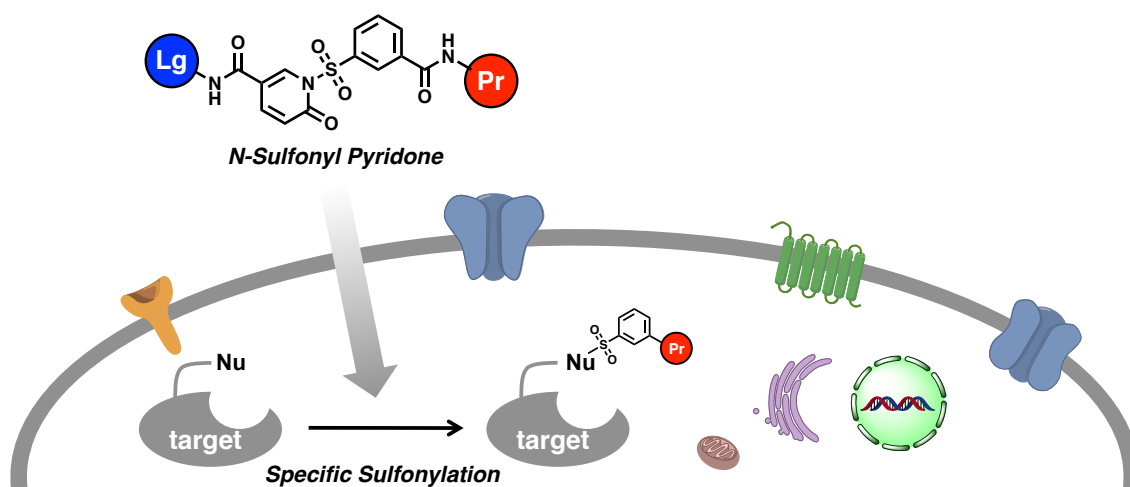
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***Specific Natural Proteins Sulfonation under live cell condition by
Ligand-directed N-Sulfonyl Pyridone Chemistry***

Abstract

It is challenging for chemists to conduct chemical reactions under strict control in living cells including a variety of reactive substances. One of such challenges represents specific modification of endogenously expressed proteins (natural proteins) under crude condition, which should be achieved only by chemistry-based approaches. Ligand-directed chemistry allows for traceless and specific protein labeling under live cell condition. Here, I describe the development of the novel protein sulfonation method using *N*-sulfonyl pyridone functionality, that is ligand-directed *N*-sulfonyl pyridone (LDSP) chemistry. I demonstrate the detailed properties of LDSP chemistry, which enables the rapid and specific modification of the target natural proteins not only *in vitro* but also under living cell conditions.



3-1. Introduction

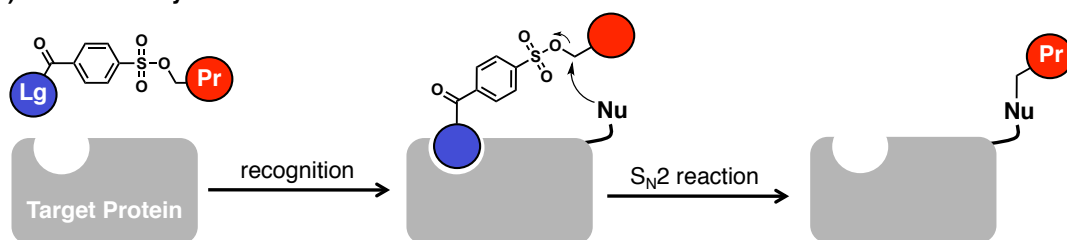
Chemical modification and functional regulation of proteins is one of the primary goals in chemical biology since proteins play a major role in most cellular events such as recognition, catalysis, storage, transport and signaling. The selective modification of proteins under live cell condition allows the analyses of enzyme activities and the alteration of their functions, which can contribute to not only fundamental science but also pharmaceutical and medical applications such as drug discovery. In order to accomplish such goals, the genetic encoding methods with unnatural amino acids bearing alkyne or azide groups are well known¹. In the approaches, genetically expressed proteins display bioorthogonal functionalities on their surfaces, followed by conducting bioorthogonal reactions² such as copper(I)-catalyzed azide-alkyne cycloaddition for highly selective modification. However, the methodology requires the pre-identification of target protein to utilize genetic manipulation, limiting the scope of the method. Meanwhile, the chemistry-based labeling approaches is applicable not only to genetically encoded proteins but also endogenously expressed proteins, natural proteins bearing twenty kinds of natural amino acids. However, the traditional bioconjugation reactions³ such as maleimide-Cys reaction under aqueous and crude conditions unfortunately lack the selectivity for target proteins and their uses are limited to purified proteins. Therefore, the development of specific and biocompatible reaction for natural protein labeling under cellular environment is extremely challenging, where there are infinite of substances such as ions, lipids, carbohydrates, nucleic acids and proteins, closely and complicatedly interacted with each other.

As a result of their continuing efforts to develop chemistries for specific and traceless protein labeling under live cell condition, Hamachi *et al.* have reported two excellent approaches, ligand-directed tosyl⁴ / acyl imidazole⁵ (LDT/LDAI) chemistries. The labeling is driven by coupling of protein recognition with specific ligands and chemical reaction with amino acids around ligand binding sites. In LDT chemistry, the phenylsulfonate (tosyl) group as the reactive handle undergoes the proximity-accelerated S_N2 reaction on the surfaces of target proteins. While LDT chemistry-based labeling reaction is with high selectivity even *in vivo*, it often suffers from slow rate and low labeling efficiency. For instance, membrane bound proteins were not effectively labeled by LDT chemistry to date. In contrast, LDAI chemistry, where a reactive acyl imidazole allows specific oxycarbonylation of natural proteins,

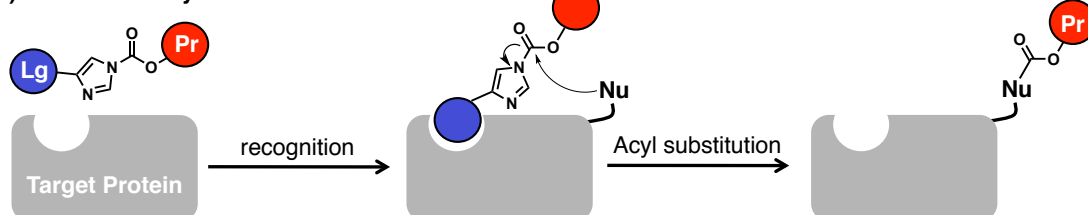
allowed the efficient and rapid labeling of cell surface proteins, but the subcellular proteins labeling by LDAI chemistry has not been observed so far. In addition to these Hamachi's works, other groups also have recently developed the different types of ligand-directed chemistry using acylation W. Fenical *et al.* reported⁶ and S_NAr reaction H. Kakeya *et al.* and M. Sodeoka *et al.* reported⁷. However, in all reported ligand-directed chemistries, the scope of applicable proteins was not clearly demonstrated, which might demand for the selection of labeling chemistry suitable for target proteins through trial and errors.

In this chapter, I describe the novel ligand-directed chemistry, ligand-directed *N*-sulfonyl pyridone chemistry (LDSP), for specific labeling of natural proteins on/in live cell conditions. *N*-Sulfonyl pyridone as an appropriate reactive handle is accelerated only by the proximity effect, result in specific sulfonylation of target protein. I have also demonstrated that LDSP chemistry offers more rapid and specific labeling than LDT and LDAI chemistries not only *in vitro* but also in/on cells and it is applicable to various proteins.

(a) LDT chemistry



(b) LDAI chemistry



(c) Acyl transfer reaction reported by W. Fenical

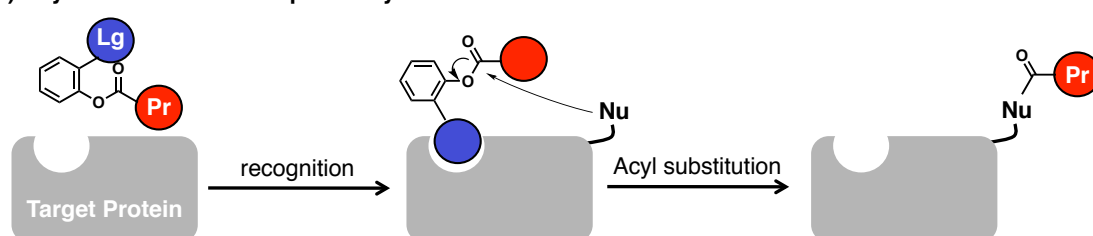
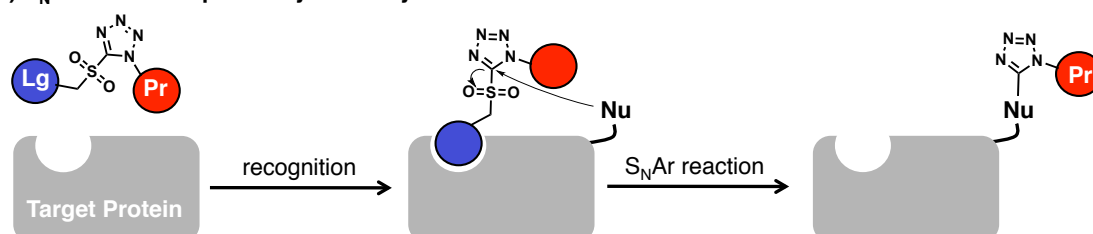
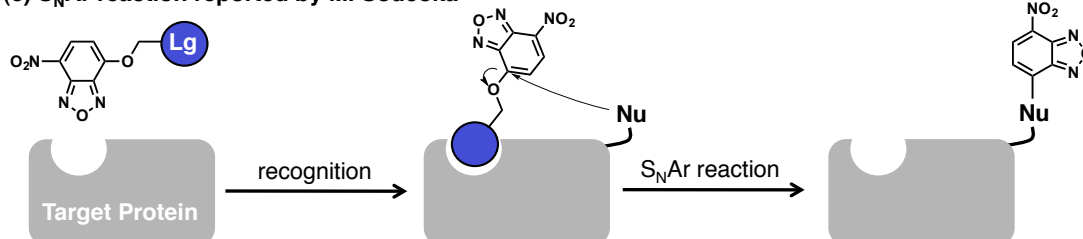
(d) S_NAr reaction reported by H. Kakeya(e) S_NAr reaction reported by M. Sodeoka

Figure 1. Ligand-directed chemistries. (a) LDT chemistry. (b) LDAI chemistry. (c) Acyl transfer reaction reported by W. Fenical *et al.* (d) and (e) S_NAr reaction reported by H. Kakeya *et al.* and by M. Sodeoka *et al.* Lg, protein ligand; Pr, chemical probe; Nu, nucleophilic amino acid.

3-2. Results & Discussion

3-2-1. Protein sulfonylation based on ligand-directed chemistry

Sulfonylation (sulfonyl transfer reaction) of proteins is one of the representatives in bioconjugation chemistry. It has almost exclusively relied on sulfonyl chloride derivatives such as Texas Red, which are difficult to handle and not amenable to long-term storage. Except for sulfonyl chloride species, to the best of my knowledge, some groups of activated sulfonyl functionalities such as *N*-sulfonyl compounds (*N*-sulfonyl methylimidazolium⁸ and *N*-sulfonylbenzotriazole⁹) and *O*-sulfonyl compounds (trichlorophenyl sulfonate¹⁰ and pentafluorophenyl sulfonate¹¹) were reported. None of them, however, have been applied to bioconjugation reactions. While these are applicable to random (non-selective) modification, it must be impossible to label the target proteins specifically under crude condition because of high reactivity and lack of selectivity. I focused here on the characteristics of 2-pyridone, which is known as a tautomer of 2-pyridinol¹² as a leaving group in order to tune the reactivity for selective protein sulfonylation.

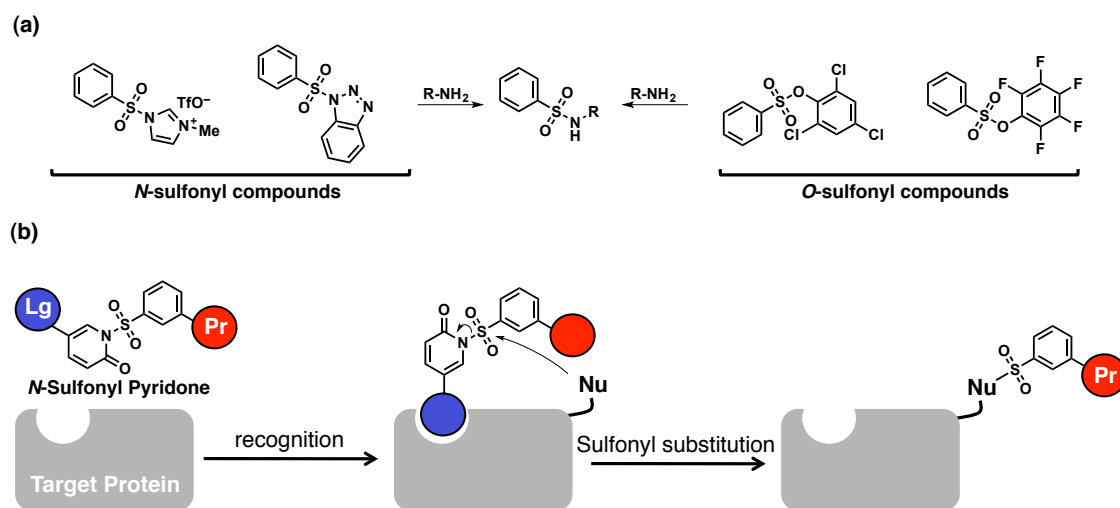
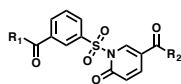


Figure 2. (a) Activated sulfonyl derivatives. *N*-Sulfonyl compounds are *N*-sulfonyl methylimidazolium and *N*-sulfonylbenzotriazole. *O*-Sulfonyl compounds are trichlorophenyl sulfonate and pentafluorophenyl sulfonate. (b) LDSP chemistry.

Lg, protein ligand; Pr, chemical probe; Nu, nucleophilic amino acid.



No.	1	2	3	4	5	6	7
R ₁	Dc	Al	Alexa	Al	Al	Al	Al
R ₂	BS	BS	BS	FA	Erl	IMC	Cbz

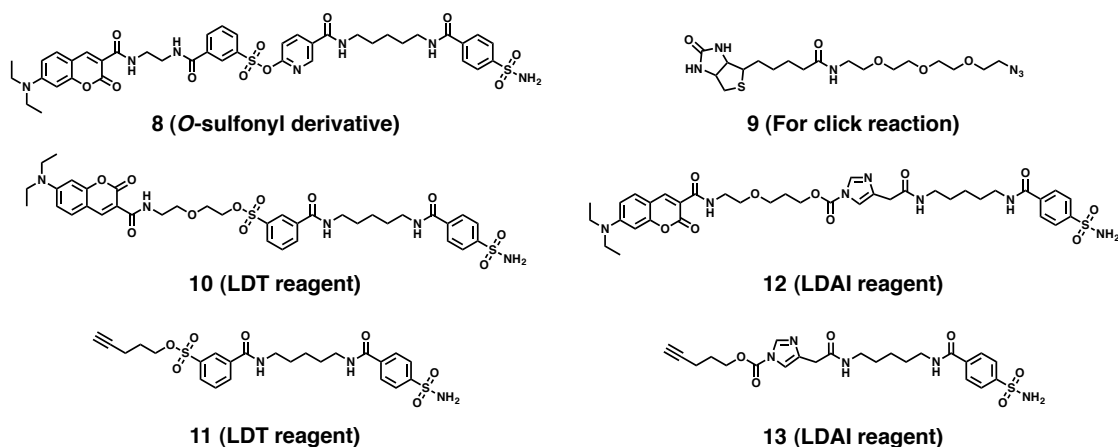
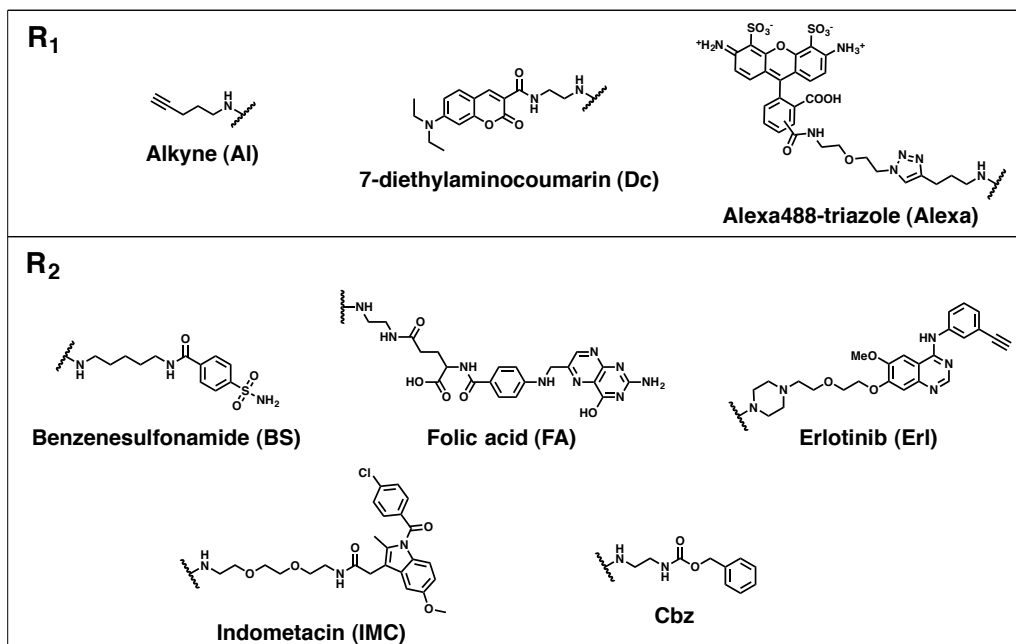


Figure 3. LDSP, LDT and LDAI reagents used in this study: LDSP chemistry based labeling reagents **1**, **2** and **3** for CA family, **4** for FR, **5** for EGFR, **6** for COX-2, and **7** and **8** for control experiments. **9** for click reaction. LDT chemistry based labeling reagents **10** and **11** for CA family. LDAI chemistry based labeling reagents **12** and **13** for CA family.

In the first proof-of-principle experiments of specific protein sulfonylation, I sought to label purified carbonic anhydrases 1 and 2 (CA1 and CA2). The labeling reagent **1** was designed, containing a fluorescent coumarin dye (Dc) as a probe and benzenesulfonamide (BS) as the common ligand¹³ for CA family such as CA 1, 2 and 12 (Figure 3). In the synthesis of the sulfonyl pyridone derivative **1**, the reaction of the corresponding 2-pyridone derivatives and sulfonyl chloride derivatives in the presence of triethylamine expectedly gave *N*-sulfonylation product **1** and *O*-sulfonylation product **8**, which could be separated by HPLC purification. CA2 labeling by *N*- or *O*-sulfonyl derivatives (**1** or **8**) revealed the much more efficient modification of CA occurred with *N*-sulfonyl pyridone **1** than *O*-sulfonyl pyridinol **8** (Figure 4a). Hence, I used the *N*-sulfonyl pyridone derivatives to label proteins in the following studies and this ligand-directed chemistry is called LDSP chemistry.

In LDSP chemistry, by connecting an affinity ligand and a probe through an electrophilic *N*-sulfonyl pyridone group, the surface of the target protein would be specifically labeled by an S_N2-type reaction on the sulfur atom with the concomitant release of the ligand molecule bearing 2-pyridone group. The calculation by MMFF94s method revealed that in *N*-sulfonyl pyridone derivative, the pyridone and the phenyl sulfonyl moieties were almost orthogonal to each other. It was expected that the carbonyl group of *N*-sulfonyl pyridone, which could work as a hydrogen bond acceptor, might facilitate the labeling reaction with nucleophilic amino acids located at the target protein surfaces. I should also point out that the electrophilic *N*-sulfonyl pyridone groups undergo nonproductive hydrolysis under the water-rich labeling conditions. However, the labeling reagent **1** was hardly hydrolyzed by incubation in the buffer solution (>90% remaining after 24h in 50mM HEPES buffer, pH 7.4, 37°C), which was appropriate for protein labeling (Figure 4c).

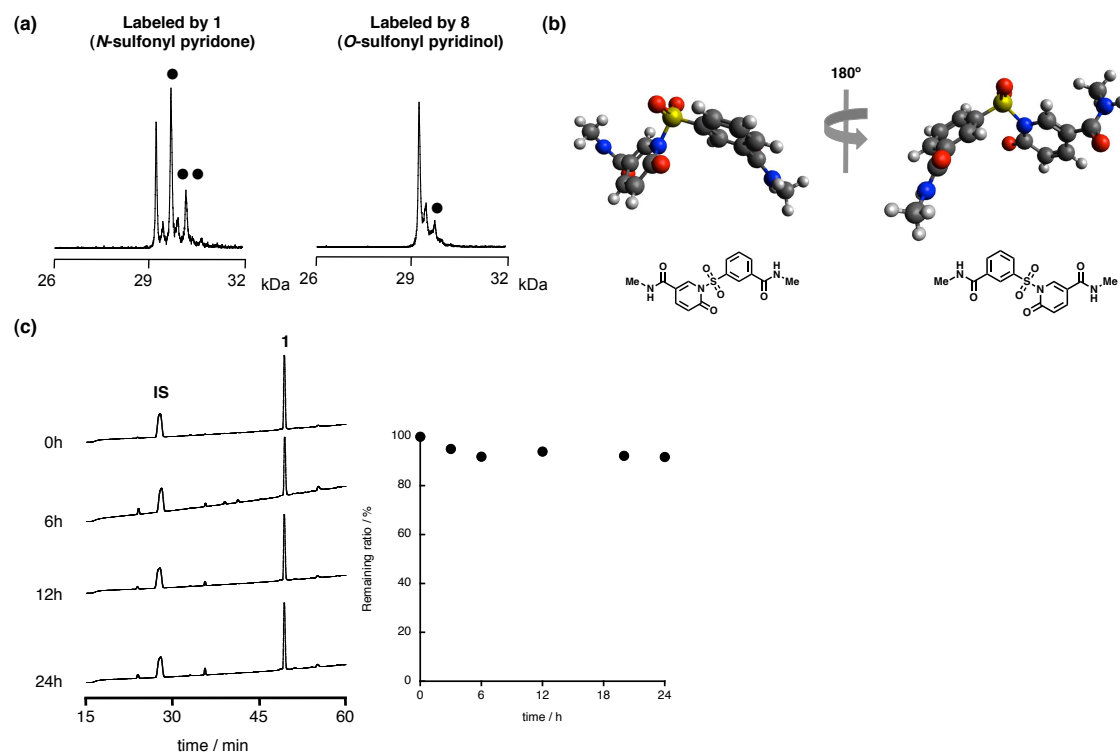


Figure 4. (a) MALDI-TOF MS analyses of CA2 labeling *in vitro* using compounds **1** and **8** at 37°C in 50 mM HEPES buffer (pH 8.0) for 6h. ●, singly labeled CA2; ●●, doubly labeled CA2. (b) The structure of *N*-sulfonyl pyridone calculated by MMFF94s. (c) Hydrolysis analysis of labeling reagent **1** at 37°C in 50 mM HEPES buffer (pH 7.4) with 100 mM NaCl. RP-HPLC analyses of hydrolysis of **1** (5 μM) at 0, 6, 12, 24 h incubation were monitored by absorbance detection ($\lambda = 220$ nm). Internal standard (IS) is *p*-nitrobenzenesulfonamide (40 μM).

3-2-2. *In vitro* protein labeling by LDSP chemistry

The reactivity of LDSP reagents **1** (10 μ M) with purified CA1 and CA2 (each 5 μ M) in 50mM HEPES buffer solution (pH 7.4) at 37°C was examined and analyzed by in-gel fluorescence analyses. The fluorescent band appeared within 1h, showing that target proteins were covalently and effectively labeled with fluorescent Dc (CA1 in Figure 5a, CA2 in Figure 5b). The Dc modification yields after 24h was about 140% for CA1 and 100% for CA2. In contrast, the labeling did not occur at all in the presence of 100 μ M ethoxzolamide¹⁴ (EZA, lane 6 in Figure 5), a strong inhibitor for CA family. Based on these results, it is conceivable that the labeling by LDSP was driven by ligand-protein recognition. Moreover, the labeling reaction was not significantly affected by the presence of a high concentration (1 mM) of nucleophilic reduced glutathione (GSH, lane 7 in Figure 5). Next, I compared the labeling kinetics of LDSP chemistry with that of LDT and LDAI chemistries (Figure 6). By SDS-PAGE analyses, the labeling velocity (3.7 μ M/hour) of LDSP chemistry was about 20-fold greater than that (0.17 μ M/hour) of LDT chemistry with **10**. It was 2-fold greater than that (2.0 μ M/hour) of LDAI chemistry with **12** in CA1 labeling. It was almost same as in the case of CA2 (LDSP 2.4 μ M/hour, LDT 0.24 μ M/hour, LDAI 1.3 μ M/hour).

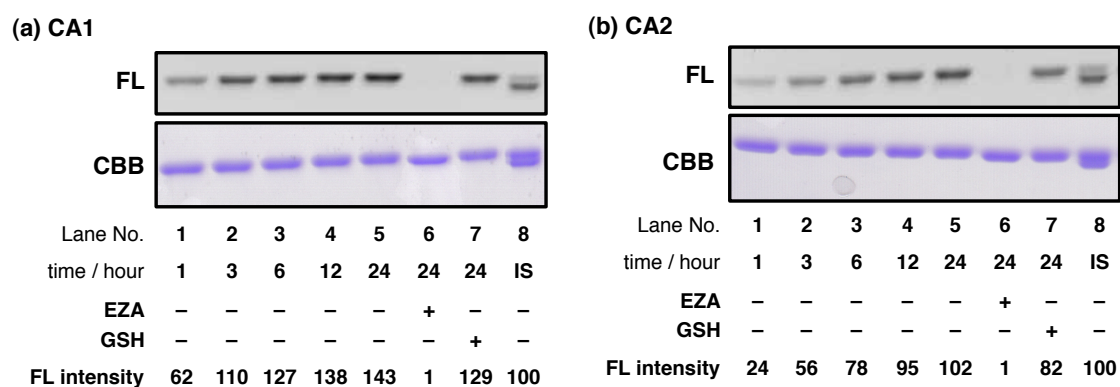


Figure 5. CA protein labeling by LDSP chemistry *in vitro*. (a) In-gel fluorescence analysis of the CA1 labeling reaction. CA1 protein (5 μ M) was mixed with **1** (10 μ M) in 50 mM HEPES buffer (100 mM NaCl, pH 7.4) at 37 °C. (b) In-gel fluorescence analysis of the CA2 labeling reaction. CA2 protein (5 μ M) was mixed with **1** (10 μ M) in 50 mM HEPES buffer (100 mM NaCl, pH 7.4) at 37 °C.

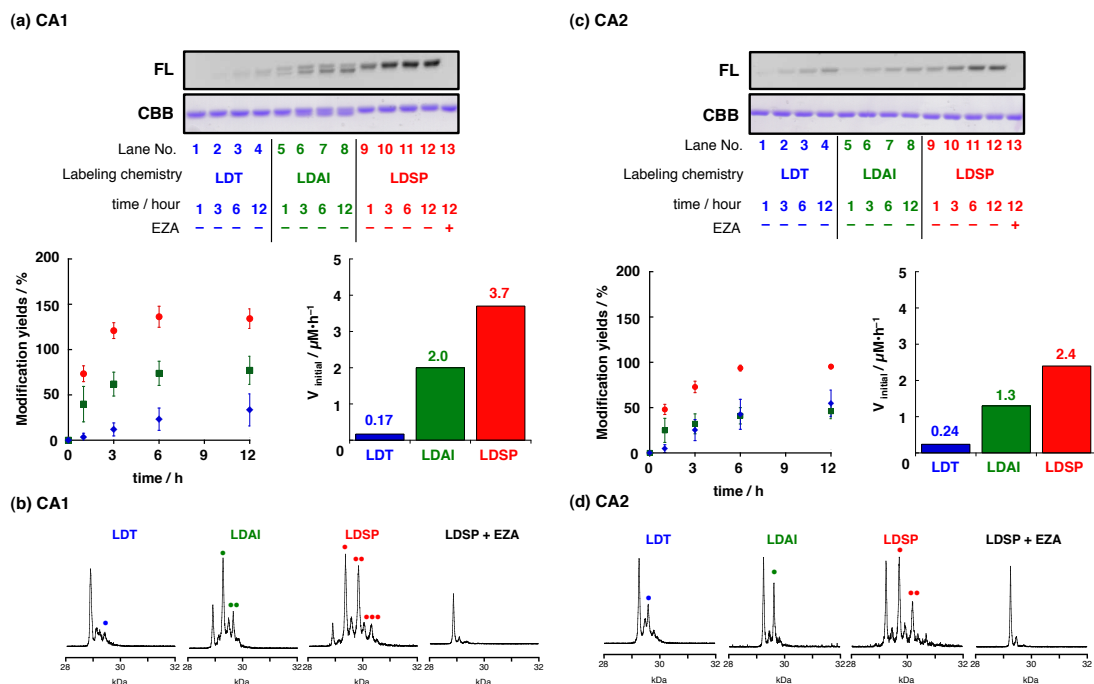


Figure 6. The comparison of CA protein labeling by ligand-directed chemistries, LDT, LDAI and LDSP chemistries *in vitro*. (a) In-gel fluorescence analysis and time plots of the CA1 labeling reaction. CA1 protein (5 μM) was mixed with **1**, **10**, **12** (10 μM) in 50 mM HEPES buffer (100 mM NaCl, pH 7.4) at 37 $^{\circ}\text{C}$. The initial rates of CA1 labeling by **1**, **10**, **12** were described in the bar graph. (b) MALDI-TOF mass analysis of the labeling reaction of CA1 protein with **1**, **10**, **12** (10 μM) in 50 mM HEPES buffer (100 mM NaCl, pH 7.4) at 37 $^{\circ}\text{C}$ for 12h. : ●, singly labeled CA1; ●●, doubly labeled CA1; ●●● triply labeled CA1. (c) In-gel fluorescence analysis and time plots of the CA2 labeling reaction. CA2 protein (5 μM) was mixed with **1**, **10**, **12** (10 μM) in 50 mM HEPES buffer (100 mM NaCl, pH 7.4) at 37 $^{\circ}\text{C}$. The initial rates of CA1 labeling by **1**, **10**, **12** were described in the bar graph. (d) MALDI-TOF mass analysis of the labeling reaction of CA2 protein with **1**, **10**, **12** (10 μM) in 50 mM HEPES buffer (100 mM NaCl, pH 7.4) at 37 $^{\circ}\text{C}$ for 12h. : ●, singly labeled CA2; ●●, doubly labeled CA2.

The sulfonylation sites of CA1 and CA2 by **1** were determined using standard proteolytic digestion and tandem mass/mass measurements. The peptide mapping analysis identified that the main labeling sites, that is, Tyr21 (60%) and Lys171 (40%) for CA1 and Tyr7 (50%) and Lys169 (25%) for CA2. Like other ligand-directed methods, all these amino acids were localized around the active pockets (Figure 7, 8). Since Tyr or Lys possesses nucleophilic phenol-OH or alkyl-NH₂ groups respectively, arylsulfonate ester or sulfonamide should be formed. Compared with other ligand-directed chemistries, Tyr (LDT and LDAI) and Lys (LDAI) are also observed, but the preference of labeled amino acids is entirely different, which implies that LDSP chemistry could expand the repertoire of labeling methods for endogenous proteins. I also confirmed that the labeled CA1 and CA2 did not decompose at all by incubation for 48 h in 50mM HEPES buffer (pH 7.4) at 37 °C (>90% remaining after 48h), indicating the sufficient stability of arylsulfonate ester or sulfonamide bonds (Figure 9). All these *in vitro* results of LDSP chemistry are appropriate in protein labeling properties, which encourage me to apply LDSP chemistry to in/on live cell labeling.

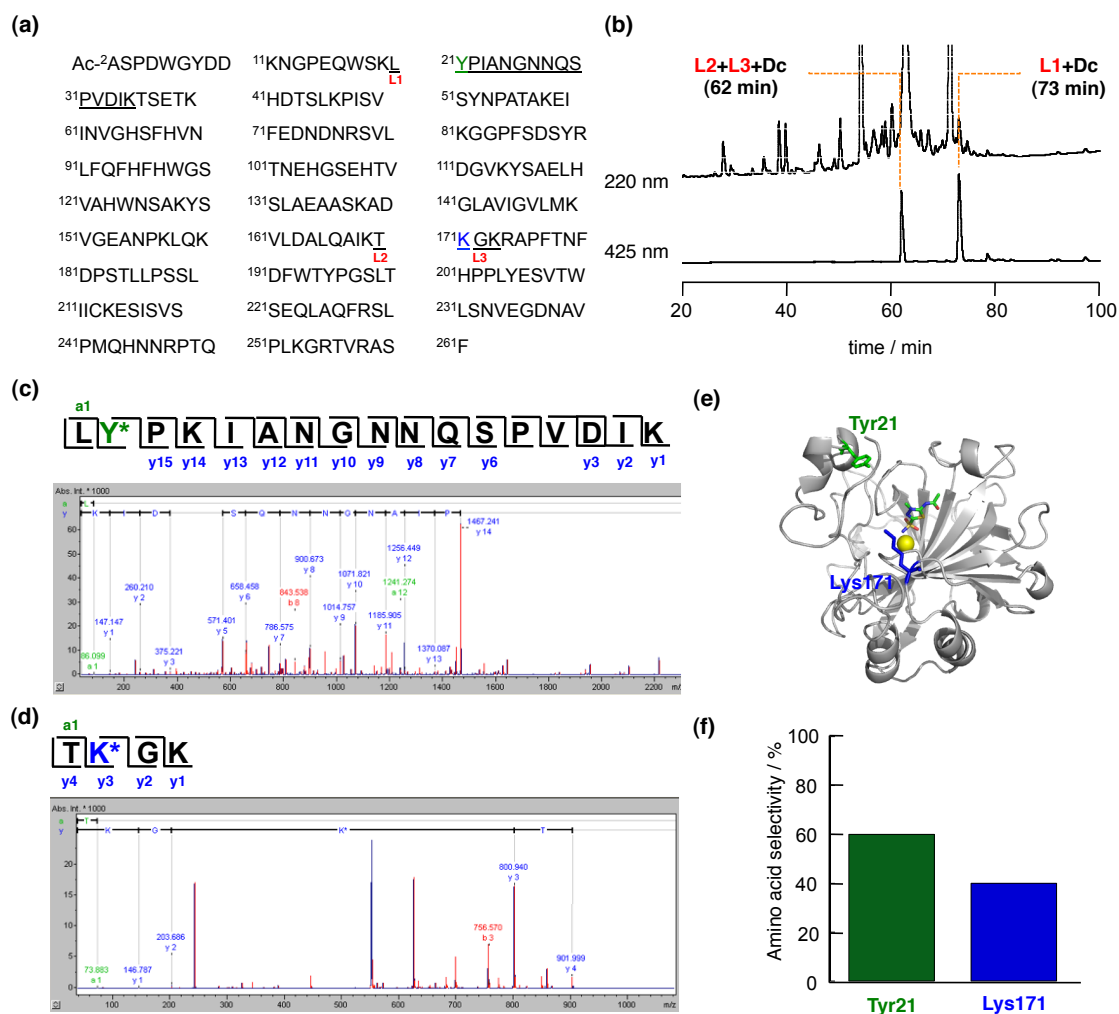


Figure 7. Evaluation of labeling sites and site-selectivity in CA1 labeling. (a) The primary sequence of CA1 and the assignment of each fragment generated by lysyl endopeptidase (LEP) digestion. (b) RP-HPLC traces of LEP-digested Dc-labeled CA1. The peaks were monitored by absorbance detection ($\lambda = 220$ and 425 nm). (c) MALDI-TOF MS/MS analysis of the Dc labeled L1 (HPLC retention time; 73min). (d) MALDI-TOF MS/MS analysis of the Dc labeled L2+L3 (HPLC retention time; 62min). (e) The crystal structure of the CA1 complex (PDB:1BZM). The amino acids (Tyr 21, Lys 171) modified by **1** are highlighted by a colored stick model. The zinc ion in the active site is shown as a yellow ball. (f) Relative amino acid (reaction site) selectivity.

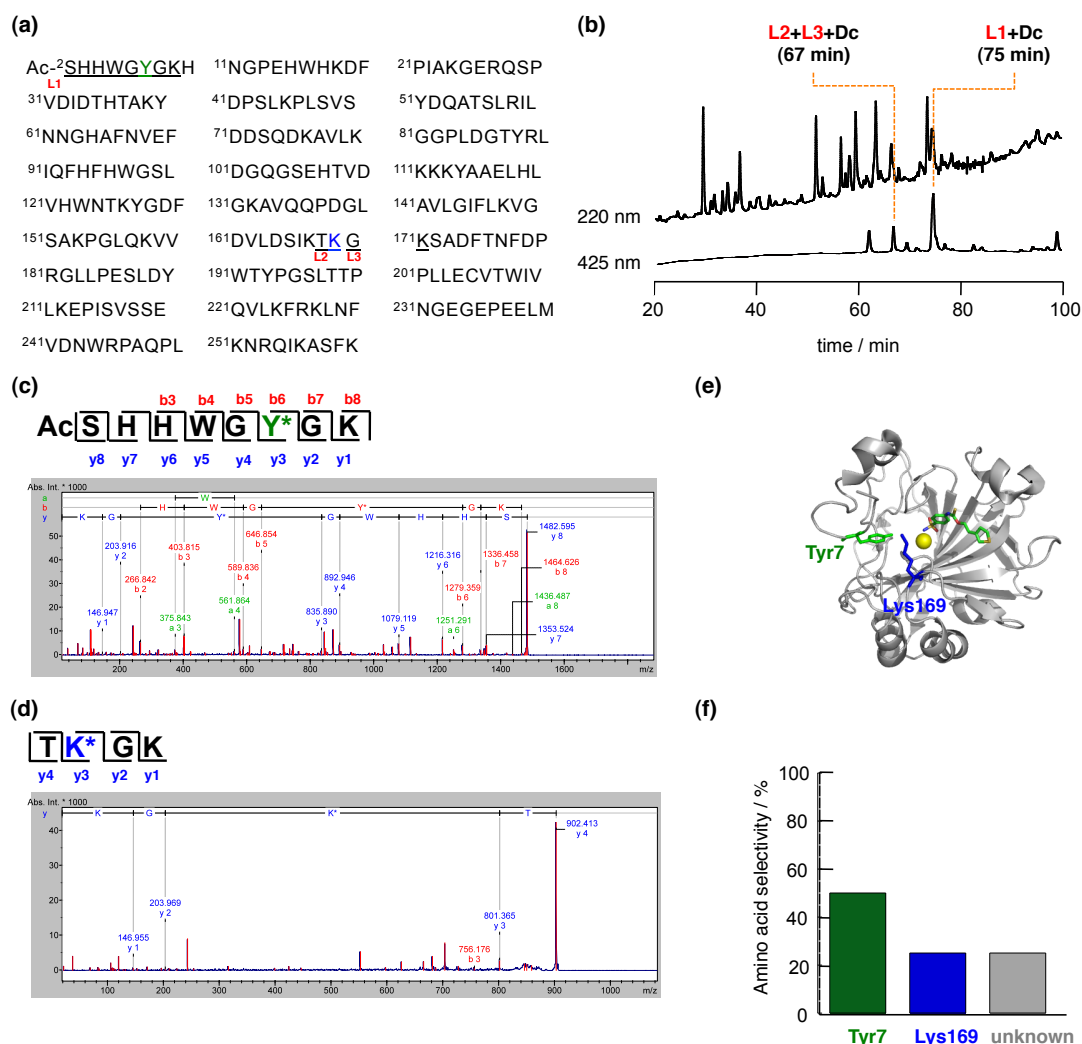


Figure 8. Evaluation of labeling sites and site-selectivity in CA1 labeling. (a) The primary sequence of CA2 and the assignment of each fragment generated by LEP digestion. (b) RP-HPLC traces of LEP-digested Dc-labeled CA2. The peaks were monitored by absorbance detection ($\lambda = 220$ and 425 nm). (c) MALDI-TOF MS/MS analysis of the Dc labeled L1 (HPLC retention time; 75min). (d) MALDI-TOF MS/MS analysis of the Dc labeled L2+L3 (HPLC retention time; 67min). (e) The crystal structure of the CA2 (PDB:3K34). The amino acids (Tyr 7, Lys 169) modified by **1** are highlighted by a colored stick model. The zinc ion in the active site is shown as a yellow ball. (f) Relative amino acid (reaction site) selectivity.

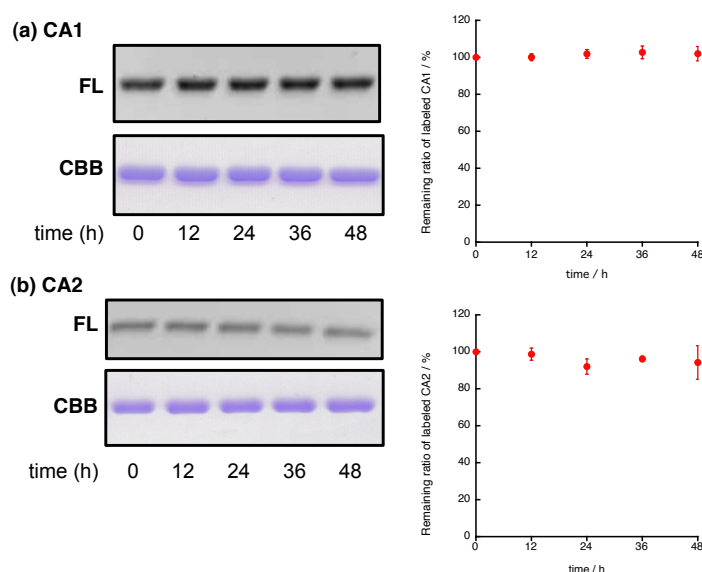


Figure 9. Evaluation of the hydrolysis stability of the labeled protein with LDSP reagent 1. (a) SDS-PAGE analysis of labeled CA1 (5 μ M, modification yields 115%) Time-trace plot of the remaining rate of the labeled CA1 (5 μ M) in 50mM HEPES buffer (pH 7.4, 100mM NaCl) at 37 $^{\circ}$ C. (b) SDS-PAGE analysis of labeled CA2 (5 μ M, modification yields 60%) Time-trace plot of the remaining rate of the labeled CA2 (5 μ M) in 50mM HEPES buffer (pH 7.4, 100mM NaCl) at 37 $^{\circ}$ C.

3-2-3. Specific proteins labeling by LDSP chemistry in/on cells

Next, I sought to modify cytosolic CA2 in living cells. Endogenous CA2-specific labeling in MCF7 cells (human breast cancer cell line) is highly challenging. To confirm in cell labeling ability of LDSP chemistry, endogenous CA2 in MCF7 cells was labeled by reagent **2** bearing alkyne group as a chemical reporter molecule, of which the subsequent click chemistry with biotin-azide compound **9** allowed western blotting analysis using streptavidin–horseradish peroxidase conjugate (SAv-HRP) detection. I observed the specific single band at ca 30 kDa, which was well correspondent with the results of anti-human CA2 antibody (Figure 10a). The further evidence was confirmed by immunoprecipitation (IP) method (Figure 10b). The addition of EZA completely suppressed the labeling, which implies that this labeling was driven by the ligand-CA interaction even inside of mammalian cells. I subsequently compared the in cell labeling of CA2 by LDAI and LDT methods with by LDSP chemistry (Figure 11). No labeling bands by LDAI-type reagent **13** were observed, which might not be due to the less membrane-permeability of **13**. The corresponding band to CA2 was slightly observed by LDT-type reagent **11**, but the non-specific labeling based bands at the higher molecular weight position were also detected. LDSP-type reagent **2** showed the most rapid and specific labeling even in cells as well as *in vitro*, which indicates that LDSP chemistry is the most appropriate for in cell protein labeling.

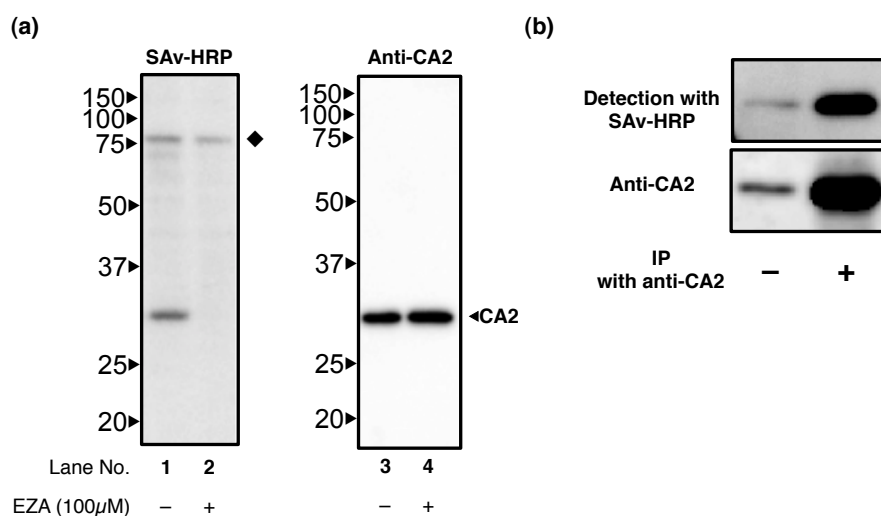


Figure 10. Chemical labeling of endogenous CA2 in MCF7 cells. Reaction conditions: MCF7 cells (2×10^5 cells), 5 μ M reagent **2**, serum-free DMEM, 37 °C, 20 h. After labeling, the cells were washed and lysed. Click reaction with **9** was conducted with the cell lysates and they were analyzed by western blotting. (a) Detection by SAV-HRP and anti-CA2 antibody. $\blacklozenge$, endogenously biotinylated protein (b) Immunoprecipitant with anti-CA2 antibody analyses.

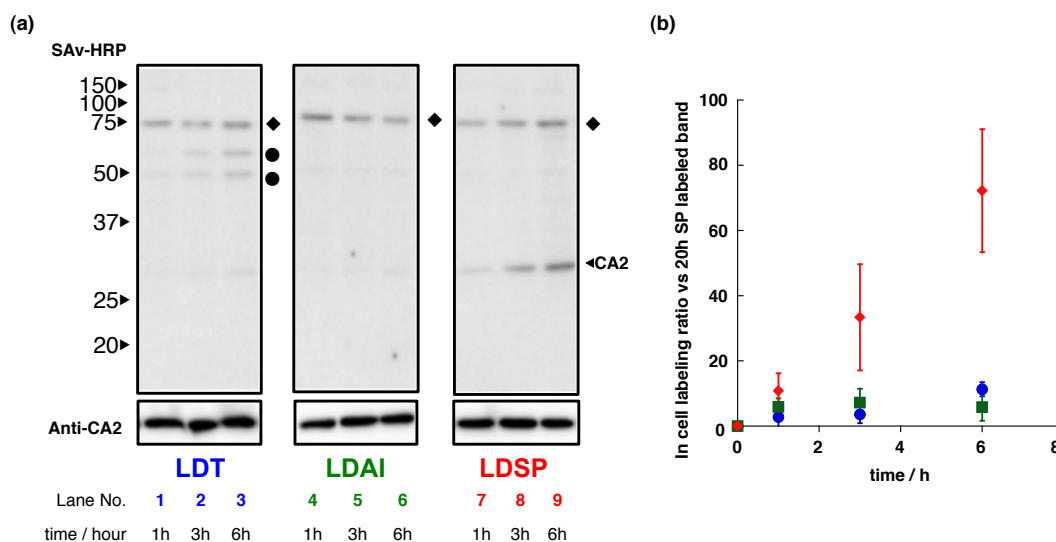


Figure 11. Chemical labeling of endogenous CA2 in MCF7 cells. Reaction conditions: MCF7 cells (2×10^5 cells), 5 μ M reagent **2**, **11** and **13**, serum-free DMEM, 37 °C, 1, 3, 6 h. After labeling, the cells were washed and lysed. Click reaction with **9** was conducted with the cell lysates and they were analyzed by western blotting. (a) Detection by SAV-HRP and anti-CA2 antibody. $\blacklozenge$, endogenously biotinylated protein. $\bullet$, non-specific bands. (b) Plots of in cell labeling efficiency.

A membrane-bound protein labeling was carried out under the live cell conditions using LDSP chemistry. CA12 is a transmembrane enzyme and is used as a tumor marker endogenously expressed on MCF7 cell membrane (Figure 12). I preliminarily noticed that reagent **2** bearing an alkyne probe underwent the insufficient labeling of CA12. This may be because it was membrane-permeable so that the efficient cytosolic CA2 labeling took place. Therefore, I designed compound **3** with membrane-impermeable probe, Alexa 488. This showed the sufficient labeling of CA12 as shown in Figure 12. It is clear that LDSP chemistry is applicable to not only in cell but also on-cell protein labeling, which implies the exquisite bioorthogonality and biocompatibility.

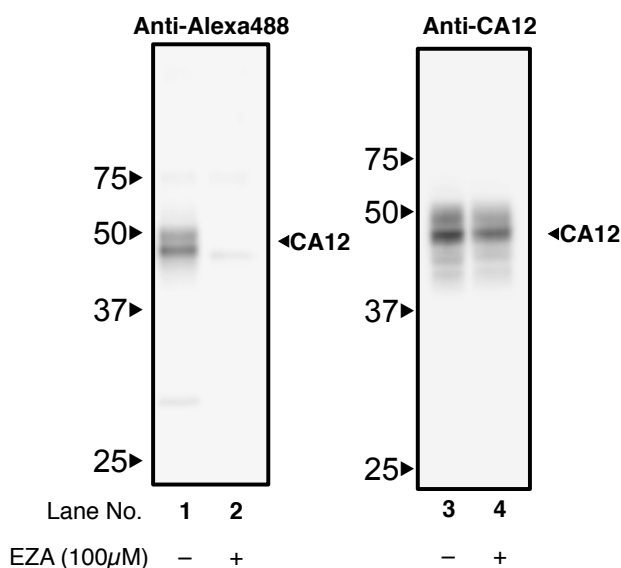


Figure 12. Chemical labeling of endogenous CA12 on MCF7 cells. Reaction conditions: MCF7 cells (2×10^5 cells), 1 μ M reagent **3**, serum-free DMEM, 37 $^{\circ}$ C, 20 h. After labeling, the cells were washed and lysed. They were analyzed by western blotting. (a) Detection by anti-Alexa488 and anti-CA12 antibodies.

To further demonstrate the broad generality of LDSP chemistry, membrane-bound proteins (extracellular domain: folate receptor, FR¹⁵; intracellular domain: epidermal growth factor receptor, EGFR¹⁶) and the inducible protein (cyclooxygenase 2, COX2¹⁷) are selected as target proteins other than CA family. Due to a lot of flexibility in designing LDSP labeling reagents, target proteins are easily expanded by the replacement of the ligand module with the corresponding one. The specific ligands in this study are as follows. Folic acid (FA) is a strong agonist for FR, erlotinib¹⁸ (Erl) is a strong inhibitor for kinase domain of EGFR and indomethacin¹⁹ (IMC) is one of NSAIDs (nonsteroidal anti-inflammatory drugs) for COX2. As a result, labeling reagent **4**, **5** and **6** were designed and synthesized. Using these reagents, all target proteins were effectively labeled by LDSP chemistry. Outer-membrane region of FR on KB cells (human mouth epidermal carcinoma cell line) were labeled by reagent **4**, whereas the labeling did not occur in the presence of FA as a competitive inhibitor (Figure 13a). Not only extracellular region but also intracellular region of transmembrane protein, the kinase domain of EGFR in A431 cells (human epithelial carcinoma cell line) was successfully labeled with reagent **5**, and the labeled band was not observed in the presence of excess erlotinib (Figure 13b). In contrast, COX2 is localized at endoplasmic reticulum, nuclear membrane and somewhere, which is involved in prostaglandin biosynthesis, is induced by lipopolysaccharide (LPS) stimuli in RAW264.7 cells (mouse macrophage-like cell line). I designed the labeling reagent **6** having alkyne for COX2 labeling by LDSP chemistry. I tried to detect the labeled COX2 by SAv after click reaction with biotinyne-azide **9**, but the band of COX-2 (70kDa) is overlapped with bovine serum albumin or endogenous biotinylated proteins. Therefore, I confirmed the labeling of endogenous COX2 by IP methods as was done in CA2, which indicated that the labeled COX2 from LPS stimulated cells was only detected by labeling reagent **6**. On the other hand, reagent **7** lacking IMC never modified COX2 (Figure 14). Given these results, it is demonstrated that LDSP chemistry based labeling is generally applicable to a variety of endogenous proteins.

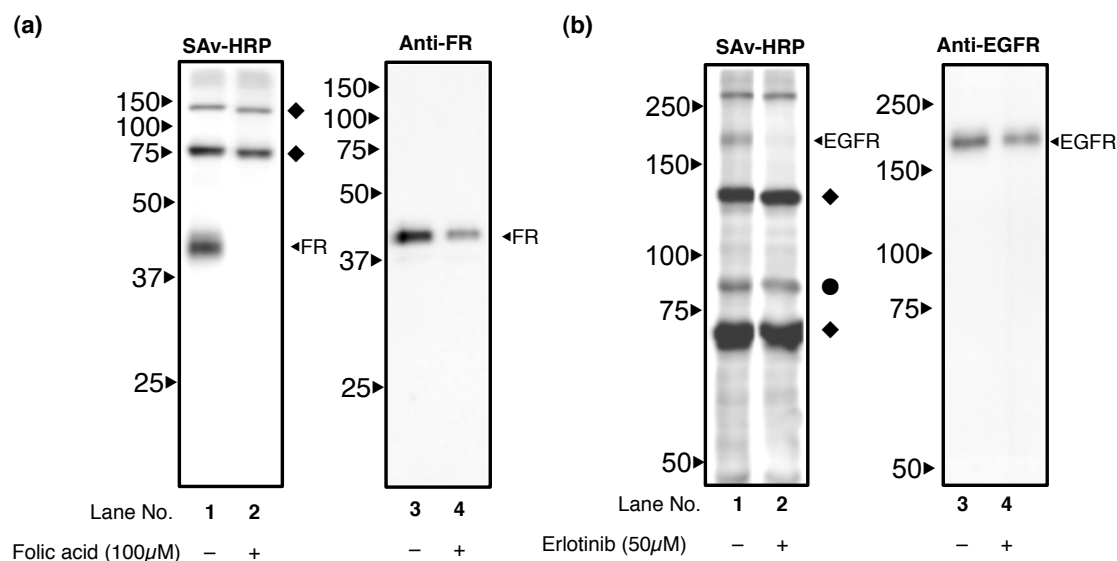


Figure 13. Chemical labeling of endogenous FR on KB cells and EGFR in A431 cells. (a) Reaction conditions: KB (2×10^5 cells), 1 μ M reagent 4, serum-free RPMI, 37 $^{\circ}$ C, 18 h. After labeling, the cells were washed and lysed. Click reaction with 9 was conducted with the cell lysates and they were analyzed by western blotting. Detection by SAy-HRP and anti-FR antibody. $\blacklozenge$, endogenously biotinylated protein. (b) Reaction conditions: A431 (2×10^5 cells, pre-incubation for 48 h), 1 μ M reagent 5, serum-free DMEM, 37 $^{\circ}$ C, 8 h. After labeling, the cells were washed and lysed. Click reaction with 9 was conducted with the cell lysates and they were analyzed by western blotting. Detection by SAy-HRP and anti-EGFR antibody. $\blacklozenge$, endogenously biotinylated protein. $\bullet$, non-specific bands.

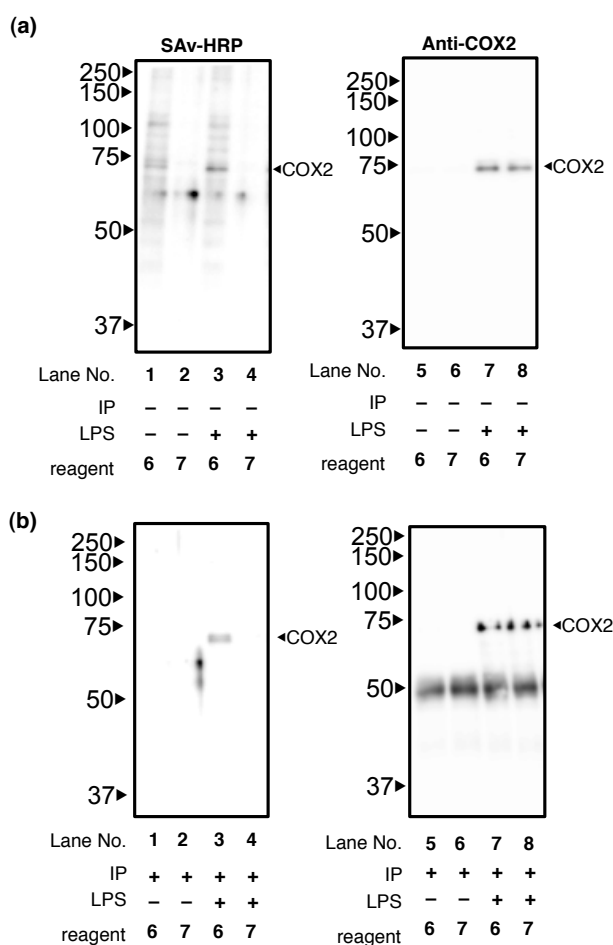


Figure 14. Chemical labeling of LPS-induced COX2 in RAW264.7 cells. Reaction conditions: RAW264.7 (2×10^5 cells), 1 μ M reagent **6**, serum-free DMEM in the absence or presence of LPS (1 ng/mL), 37 °C, 18 h. After labeling, the cells were washed and lysed. Preclear was conducted with Av beads. Click reaction with **9** was conducted with the precleared cell lysates and they were analyzed by western blotting. (a) Detection by SAv-HRP and anti-COX2 antibody. (b) Immunoprecipitant with anti-COX2 antibody analyses.

3-3. Conclusion

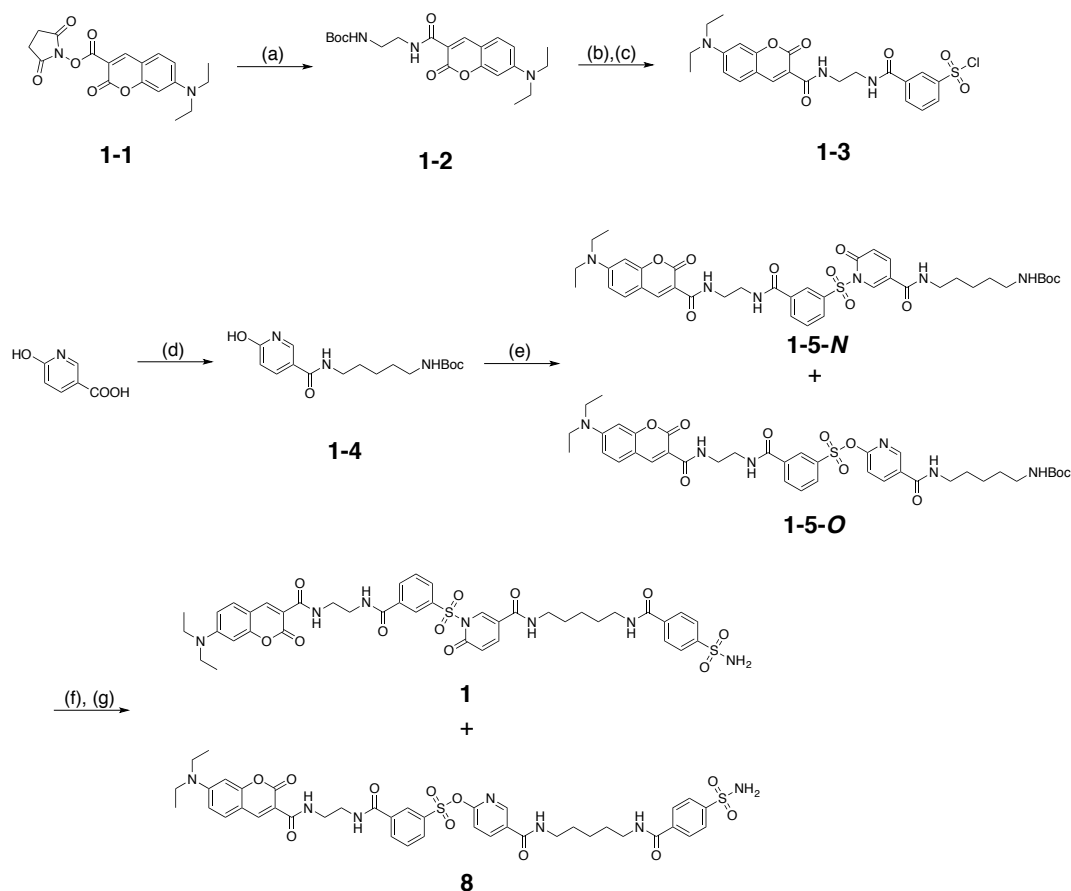
I have successfully developed *N*-sulfonyl pyridone group as the novel reactive site for selective protein labeling under live cell condition. This is the first example of specific protein sulfonylation to the best of my knowledge. I demonstrated in this chapter that the LDSP chemistry was generally applicable to various endogenous proteins in/on cells including CA2, CA12, FR, EGFR and COX2. Furthermore, LDSP chemistry-based labeling exhibited the most rapid and selective labeling not only in test tube, but in live cell among three ligand-directed chemistries. I believe that LDSP chemistry is the valuable chemical approach for traceless labeling methods for endogenous proteins.

3-4. Experimental procedures

Materials and Methods. All chemical and biochemical reagents were purchased from commercial sources (Wako Pure Chemical, TCI Chemical, Sigma-Aldrich, and Watanabe Chemical) and were used without further purification. Thin-layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ precoated aluminum sheets (Merck) and visualized by fluorescence quenching or ninhydrin staining. Chromatographic purification was performed using flash column chromatography on silica gel 60 N (neutral, 40–50 μ m, Kanto Chemical).

Physical Measurements: ¹H-NMR spectra were recorded on 400 MHz Varian Mercury spectrometer. Chemical shifts were referenced to residual solvent peaks or tetramethylsilane (δ = 0 ppm). UV-vis absorption spectra were acquired on a Shimadzu UV-2550 spectrophotometer. Matrix-assisted laser ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra were recorded on an Autoflex III (Bruker Daltonics, Bremen, Germany) using α -cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SA) as a matrix. High resolution mass spectra were measured on an Exactive (Thermo Scientific, CA, USA) equipped with electron spray ionization (ESI). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi Chromaster system equipped with a diode array and fluorescence detectors, and an YMC-Pack Triat C18 or ODS-A column. All runs used linear gradients of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B).

Synthesis

Scheme 1. Syntheses of **1** and **8**.

Reaction conditions: (a) *N*-(*tert*-Butoxycarbonyl)-1,2-diaminoethane, triethylamine (TEA) in DMF, (b) TFA in DCM, (c) 3-(chlorosulfonyl)benzoyl chloride, TEA in DCM (dichloromethane), (d) Boc-NH-(CH₂)₅-NH₂•HCl, EDCI•HCl (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide), HOBt•H₂O (1-hydroxybenzotriazole monohydrate), TEA in DMF (*N,N*-dimethylformamide), (e) **1-3**, TEA in DCM/DMF, (f) TFA (trifluoroacetic acid) in DCM, (g) SA-OSu, TEA in DMF.

Compound **1-2**

A solution of **1-1** (385 mg, 1.0 mmol), *N*-(*tert*-Butoxycarbonyl)-1,2-diaminoethane (320 mg, 2.0 mmol), and TEA (420 μ L, 3.1 mmol) in DMF (8 mL) was stirred for 4 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH = 10 : 1) to give **1-2** (350 mg, 87 %).

as a yellow solid. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.96 (br, 1H), 8.70 (s, 1H), 7.43 (d, $J = 8.8$ Hz, 1H), 6.65 (dd, $J = 2.4, 8.8$ Hz, 1H), 5.06 (br, 1H), 3.56 (m, 2H), 3.45 (q, $J = 6.8$ Hz, 4H), 3.36 (m, 2H), 1.24 (t, $J = 7.2$ Hz, 6H).

Compound **1-3**

A solution of **1-2** (100 mg, 0.25 mmol) and TFA (1 mL) in DCM (2 mL) was stirred for 4 h at rt. After removal of the solvent, the residue in DCM/DMF (2mL/2mL) was dropwise added to the solution of 3-(chlorosulfonyl)benzoyl chloride (100 mg, 0.40 mmol) and TEA (108 μL , 0.80 mmol) in DCM (10 mL) and the reaction mixture was stirred for 1 h at 0°C . After removal of the solvent by evaporation, the residue was roughly-purified by flash column chromatography on SiO_2 (CHCl_3 : AcOEt : MeOH = 10 : 30 : 1) to give crude **1-3** (62 mg, 49 %) as a yellow solid, which was intermediately utilized in the next reaction.

Compound **1-4**

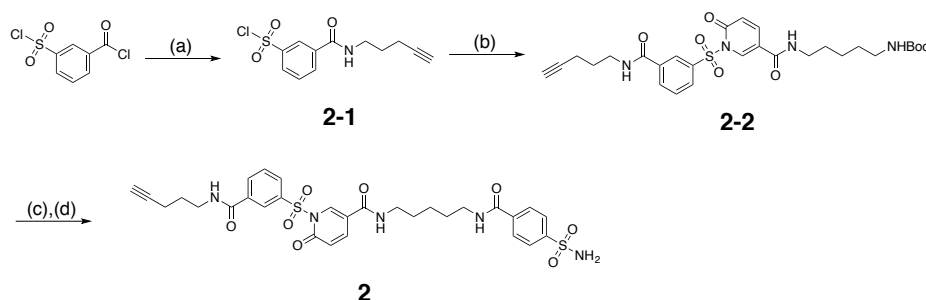
A solution of Boc-NH-(CH_2)₅-NH₂•HCl (1432 mg, 6.0 mmol), 6-hydroxynicotinic acid (696 mg, 5.0 mmol), HOBT•H₂O (920 mg, 6.0 mmol), EDCI•HCl (1150 mg, 6.0 mmol) and TEA (4 mL, 30 mmol) in DMF (10 mL) was stirred for 12 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (CHCl_3 : MeOH : NH_3 = 100 : 10 : 1) to give **1-4** (1440 mg, 89 %) as colorless solid. $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ 8.02 (m, 1H), 7.96 (dd, $J = 2.8, 9.6$ Hz, 1H), 6.02 (d, $J = 9.6$ Hz, 1H), 3.32 (m, 2H), 3.03 (t, $J = 7.2$ Hz, 2H), 1.60 (quin., $J = 6.8$ Hz, 2H), 1.52 (m, 2H), 1.42 (s, 9H), 1.38 (m, 2H).

Compound **1-5** (mixture of **1-5-N** and **1-5-O**)

A solution of **1-3** (62 mg, 144 μmol) and TEA (2.58 mL, 14.8 mmol) in DMF (0.5 mL) and DCM (2 mL) was added to the solution of **1-4** (5.0 mg, 15 μmol) in DMF (0.5 mL) and DCM (1mL). The reaction mixture was stirred for 4 h at rt. After removal of the solvent by evaporation, the residue was roughly purified by flash column chromatography on SiO_2 (CHCl_3 : MeOH : NH_3 = 10 : 1) to give the mixture of **1-5-N** and **1-5-O** (total 5 mg, 10 %) as a yellow solid. The mixture was used for next reaction without further purification.

Compound **1** and **8**

The solution of the mixture of **1-5-N** and **1-5-O** (10 mg, 12.6 μmol), TFA (0.5 mL) in DCM (2 mL) was stirred for 3 h at rt. After removal of the solvent by evaporation, the residue was added to the solution of SA-OSu (7.5 mg, 25 μmol) and TEA (13 μL , 100 μmol) in DMF (100 mL). The reaction mixture was stirred for 1 h at rt. After removal of the solvent by evaporation, the residue was purified by HPLC to give **1** (1.2 mg, 11 %) and **8** (1.6 mg, 14%) as yellow solids, which were confirmed only by HR-ESI MS m/z calcd for $[\text{M}+\text{H}]^+$ 876.2691, found 876.2814 (**1**) and 876.2747 (**8**). **1** and **8** were assigned, based on the case of **7** and its *O*-sulfonyl derivative.

Scheme 2. Synthesis of **2**

Reaction conditions: (a) 4-Pentyn-1-amine, TEA in DCM, (b) **1-4**, TEA in DCM, (c) TFA in DCM, (d) SA-OSu, TEA in DMF.

Compound **2-1**

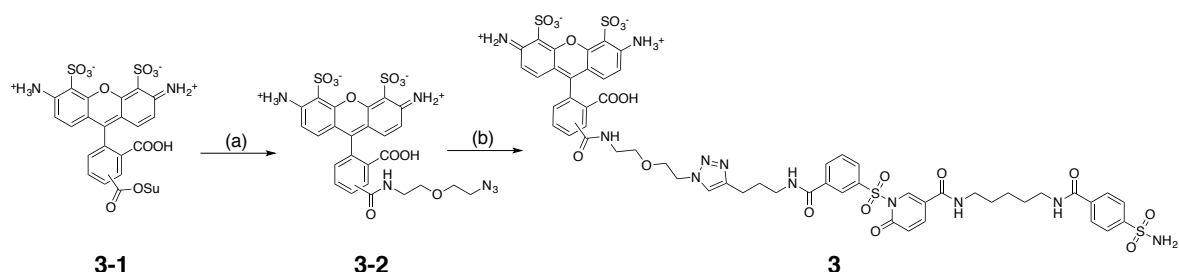
To the solution of 3-(chlorosulfonyl)benzoyl chloride (287 mg, 1.2 mmol) and TEA (280 μL , 2.0 mmol) in DCM (5 mL) was added dropwise the solution of 4-pentyn-1-amine (83 mg, 1.0 mmol) in DCM (10 mL). The reaction mixture was stirred for 1 h at 0°C. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (CHCl_3 : AcOEt = 8 : 1) to give **1-3** (274 mg, 96 %) as a colorless oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.40 (s, 1H), 8.23 (m, 2H), 8.19 (m, 2H), 7.75 (t, J = 8.0 Hz, 1H), 6.87 (br, 1H), 3.66 (m, 2H), 2.39 (m, 1H), 2.14 (m, 1H), 1.93 (quin, J = 6.4 Hz, 1H).

Compound **2-2**

To the solution of **1-4** (32 mg, 0.10 mmol) and TEA (70 μ L, 0.50 mmol) in DCM (10 mL) was added the solution of **2-1** (100 mg, 0.35 mmol) in DCM (2 mL). The reaction mixture was stirred for 5 h at rt. After removal of the solvent by evaporation, the residue was roughly purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH = 10 : 1) to give the mixture (total 25 mg, 44%) of **1-3** (*N*-sulfonyl derivative) and *O*-sulfonyl derivative as a colorless oil. The mixture was used for next reaction without further purification.

Compound **2**

The solution of the mixture of crude **1-3** (25 mg, 0.43 mmol), TFA (1 mL) in DCM (2 mL) was stirred for 3 h at rt. After removal of the solvent by evaporation, the residue was added to the solution of SA-OSu (149 mg, 0.50 mmol) and TEA (136 μ L, 1.0 mmol) in DMF (3 mL). The reaction mixture was stirred for 1 h at rt. After removal of the solvent by evaporation, the residue was purified by HPLC to give **2** (99 mg, 35 %) as colorless oil. ¹H-NMR (400 MHz, CD₃OD) δ 8.79 (s, 1H), 8.53 (s, 1H), 8.27 (d, *J* = 8.0 Hz, 1H), 8.20 (d, *J* = 7.2 Hz, 1H), 7.85 (dd, *J* = 2.0, 9.6 Hz, 1H), 7.74 (t, *J* = 8.0 Hz, 1H), 7.30 (m, 4H), 6.42 (d, *J* = 9.6 Hz, 1H), 3.49 (m, 2H), 3.40 (m, 4H), 2.27 (m, 3H), 1.83 (m, 2H), 1.69 (m, 4H), 1.48 (m, 2H). HR-ESI MS *m/z* calcd for [M+H]⁺ 656.1843, found 656.1890.

Scheme 3. Synthesis of **3**

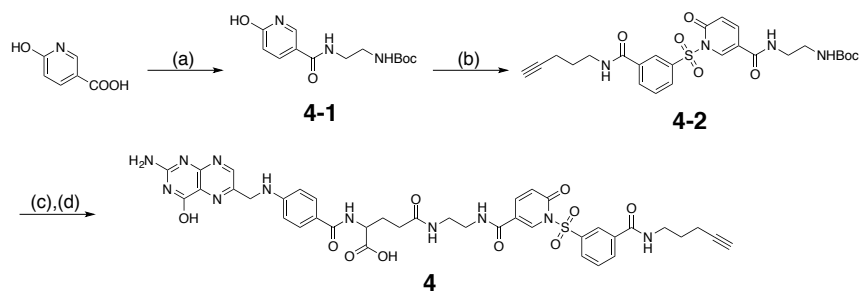
Reaction conditions: (a) 2-(2-azidoethoxy)ethan-1-amine, TEA in DMF, (b) **2**, tetrakis(acetonitrile)copper(I) hexafluorophosphate, tris(2-carboxyethyl)phosphine in DMF/50mM HEPES buffer (pH 7.2).

Compound **3-2**

The solution of the mixture of **3-1** (isomer mixture : 5 mg, 7.8 μmol), TEA (31.4 mg, 312 μmol) and 2-(2-azidoethoxy)ethan-1-amine (4.9 mg, 21 μmol) in DMF (2 mL) was stirred for 16 h at rt. After removal of the solvent by evaporation, the residue was purified by HPLC to give **3-2** (single isomer : 1.9 mg, 38 %) as a yellow solid, which was only confirmed by MALDI-TOF MS m/z calcd for $[\text{M}+\text{H}]^+$ 645, found 646.9.

Compound **3**

The solution of the mixture of **2** (0.54 mg, 1.5 μmol), **3-2** (1.9 g, 3.0 μmol), tetrakis(acetonitrile)copper(I) hexafluorophosphate (5.5 mg, 15 μmol) and tris(2-carboxyethyl)phosphine (8.5 mg, 30 μmol) in DMF (3.5 mL) and 50mM HEPES buffer (pH 7.2) (3 mL) was stirred for 8h at rt. After removal of the solvent by evaporation, the residue was purified by HPLC to give **3-2** (242 μg , 6 %), which was only confirmed by MALDI-TOD MS m/z calcd for $[\text{M}+\text{Na}]^+$ 1324.2, found 1324.2.

Scheme 4. Synthesis of **4**

Reaction conditions: (a) *N*-Boc-ethylenediamine, EDCI, HOBt•H₂O, TEA in DMF, (b) **2-1**, TEA in DCM/DMF, (c) TFA in DCM, (d) Folic acid-OSu, TEA in DMF.

Compound **4-1**

A solution of *N*-Boc-ethylenediamine (3.2 g, 20 mmol), 6-hydroxynicotinic acid (1391 mg, 10 mmol), HOBt•H₂O (2.3 g, 15 mmol), EDCI•HCl (2.9 g, 15 mmol) and TEA 2.8 mL, 20 mmol) in DMF (10 mL) was stirred for 18 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography

on SiO₂ (CHCl₃ : MeOH : NH₃ = 100 : 10 : 1) to give **4-1** (2.3 g, 82%) as colorless solid. ¹H-NMR (400 MHz, CD₃OD) δ 8.03 (d, J = 2.0 Hz, 1H), 7.96 (m, 1H), 6.51 (d, J = 9.6 Hz, 1H), 3.40 (t, J = 6.0 Hz, 2H), 3.24 (t, J = 6.0 Hz, 2H), 1.40 (s, 9H).

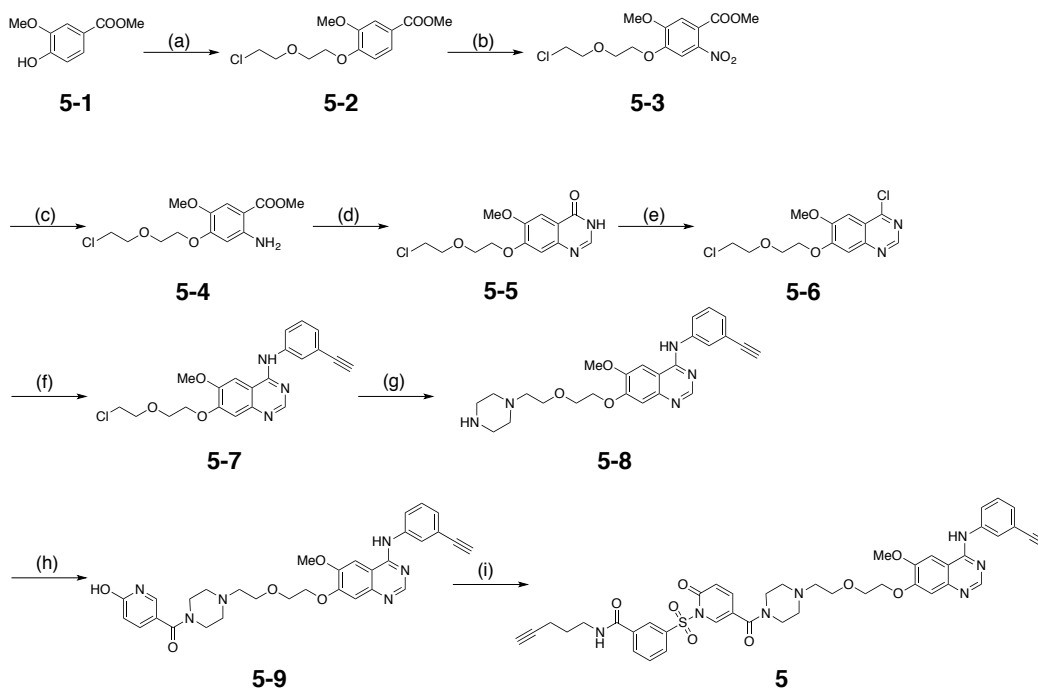
Compound **4-2**

To the solution of **4-1** (20 mg, 71 μ mol) and TEA (28 μ L, 0.2 mmol) in DCM (5 mL) was added the solution of **2-1** (86 mg, 0.30 mmol) in DCM (3 mL). The reaction mixture was stirred for 2 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH = 8 : 1) to give the mixture (total 20 mg, 49%) of **4-2** (*N*-sulfonyl derivative) and *O*-sulfonyl derivative as colorless oil. The mixture was used for next reaction without further purification.

Compound **4**

The solution of the mixture of crude **4-2** (20 mg, 35 μ mol), TFA (1 mL) in DCM (2 mL) was stirred for 3h at rt. After removal of the solvent by evaporation, the residue was added to the solution of folic acid-OSu (the mixture of alpha and gamma substitutes, 50 mg, 93 μ mol) and TEA (14 μ L, 0.1 mmol) in DMF (2 mL). The reaction mixture was stirred for 1 h at rt. After removal of the solvent by evaporation, the residue was purified by HPLC to give **4** (8 mg, 27 %) as colorless solid. ¹H-NMR (400 MHz, CD₃OD) δ 8.71 (m, 1H), 8.62 (m, 1H), 8.49 (m, 1H), 8.23 (m, 2H), 7.88 (m, 1H), 7.66 (t, J = 8.0 Hz, 1H), 7.63 (m, 2H), 6.61 (t, J = 8.8 Hz, 1H), 6.43 (m, 1H), 4.46 (s, 2H), 4.30 (m, 1H), 3.30 (overlap, 6H), 2.20 (m, 7H), 1.70 (m, 2H). HR-ESI MS m/z calcd for [M+H]⁺ 854.2675, found 854.2814.

Scheme 5. Synthesis of 5



Reaction conditions: (a) 2-(2-chloroethoxy)ethyl methanesulfonate, K_2CO_3 in DMF, (b) HNO_3 (fuming) in acetic acid, (c) Fe, HCl_{aq} in EtOH, (d) formamidine acetate in ethanol, (e) $SOCl_2$, DMF (cat.), (f) 3-ethynylaniline in *i*-propanol (g) piperazine in DMF, (h) 6-hydroxynicotinic acid, EDCI, $HOBT \cdot H_2O$, TEA in DMF, (i) **2-1**, TEA in DCM/DMF.

Compound **5-2**

A mixture of **5-1** (8.5 g, 47 mmol), 2-(2-chloroethoxy)ethyl methanesulfonate (13 g, 65 mmol), and potassium carbonate (13.8 g, 100 mmol) in DMF (50 mL) was heated at 70 °C for 7 h. The reaction mixture was cooled to room temperature, then poured slowly into ice water while stirring constantly. The solid formed was filtered off and washed with cold water. The off-white product was recrystallized from ethyl acetate (40 mL) to give 11.3 g of **5-2** in 83% yield. 1H -NMR (400 MHz, $DMSO-d_6$) δ 7.55 (dd, $J = 2.0, 8.4$ Hz, 1H), 7.43 (d, $J = 2.0$ Hz, 1H), 7.08 (d, $J = 8.4$ Hz, 1H), 4.16 (t, $J = 4.8$ Hz, 2H), 3.80 (m, 3H+3H+2H), 3.73 (m, 2H+2H).

Compound **5-3**

Fuming nitric acid (830 μ L, 20 mmol) was added dropwise to a stirred solution of **5-2** (1.2 g, 4.2 mmol) in acetic acid (10 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then poured into ice-water and extracted with dichloromethane. The organic phase was washed with water, aqueous NaHCO₃ and brine; dried over sodium sulfate; filtered; and concentrated to give **5-3** as a yellow oil (1.3 g, 93%). ¹H-NMR (400 MHz, CDCl₃): δ 7.55 (s, 1H), 7.08 (s, 1H), 4.28 (dt, J = 1.6, 4.4 Hz, 2H), 3.96 (m, 3H+2H), 3.91 (s, 3H), 3.84 (t, J = 6.0 Hz, 2H), 3.65 (t, J = 6.4 Hz, 2H) .

Compound **5-4**

Powder iron (1.0 g, 18 mmol) was added portionwise to a solution of **5-3** (600 mg, 1.8 mmol) in ethanol (30 mL), and 2M hydrochloric acid aq (10 mL). The mixture was stirred at reflux for 30 min. The mixture was then cooled to room temperature and adjusted to pH 8 with 10% sodium hydroxide. The formed solid was removed by filtration and the filtrate was concentrated in vacuo to leave a residue. The residue was extracted with dichloromethane, washed with brine, dried over sodium sulfate, filtered, and concentrated to give **5-4** as a yellow solid (437 mg, 80%). ¹H-NMR (400 MHz, CDCl₃): δ 7.31 (s, 1H), 6.20 (s, 1H), 5.55 (br, 2H), 4.18 (t, J = 5.2 Hz, 2H), 3.92 (t, J = 4.8 Hz, 2H), 3.86 (m, 3H+2H), 3.80 (s, 3H), 3.66 (t, J = 2.0 Hz, 2H).

Compound **5-5**

A mixture of **5-4** (437 g, 1.4 mmol), formamidine acetate (750 g, 7.2 mmol) in ethanol (30 mL) was refluxed for 5 h. The mixture was cooled to room temperature and was evaporated *in vacuo*. The residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH = 8 : 1) to give **5-5** as a brown solid (314 mg, 73%). ¹H-NMR (400 MHz, DMSO-*d*₆): δ 7.96 (s, 1H), 7.43 (s, 1H), 7.14 (s, 1H), 4.23 (t, J = 4.8 Hz, 2H), 3.83 (m, 2H+3H), 3.74 (m, 2H+2H).

Compound **5-6**

5-5 (150 mg, 0.50 mmol) was added to thionyl chloride (20 mL) with magnetic stirring. DMF (100 μ L) was then slowly added dropwise and the mixture was heated to reflux for 15 h. Most of the excess of thionyl chloride was then removed under reduced pressure and the yellow residue was dissolved in chloroform, washed with a saturated solution of sodium carbonate and brine, and dried over Na_2SO_4 . The chloroform was then removed under reduced pressure to give the product **5-6** (131 mg, 82%) as off-white solid. ^1H -NMR (400 MHz, CDCl_3): δ 8.87 (s, 1H), 7.40 (s, 1H), 7.35 (s, 1H), 4.37 (t, J = 4.8 Hz, 2H), 4.06 (s, 3H), 4.02 (t, J = 6.4 Hz, 2H), 3.88 (t, J = 6.0 Hz, 2H), 3.70 (t, J = 3.68 Hz, 2H).

Compound **5-7**

A mixture of **5-6** (100 mg, 0.32 mmol) and 3-ethynylaniline (74 mg, 0.63 mmol) in isopropanol (10 mL) was stirred at reflux for 8 h. The mixture was evaporated and the residue was purified by flash column chromatography on SiO_2 (CHCl_3 : MeOH = 8 : 1) to give **5-7** (115 mg, 90%) as a yellow solid. ^1H -NMR (400 MHz, $\text{CDCl}_3+\text{CD}_3\text{OD}$): δ 8.58 (s, 1H), 8.05 (s, 1H), 7.85 (s, 1H), 7.71 (m, 1H), 7.46 (m, 3H), 4.44 (t, J = 4.8 Hz, 2H), 4.06 (s, 3H), 4.05 (t, J = 4.4 Hz, 2H), 3.91 (t, J = 5.6 Hz, 2H), 3.71 (t, J = 5.2 Hz, 2H), 3.27 (s, 1H).

Compound **5-8**

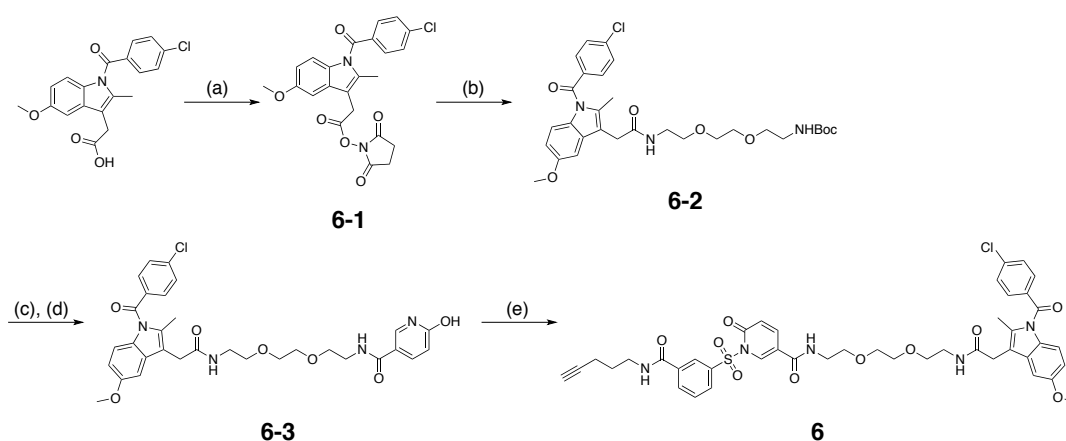
5-7 (100 mg, 0.25 mmol) and potassium iodide (50 mg) were added to the solution of piperadine (433 mg, 5 mmol) in DMF (15 mL). The solution was stirred at 70 $^\circ\text{C}$ for 14h. The reaction mixture was removed by evaporation and the residue dissolved in chloroform, washed with brine, and then dried over Na_2SO_4 . The solvent was removed under vacuum. The crude product was purified by flash column chromatography on SiO_2 (CHCl_3 : MeOH = 7 : 1) to afford white powder (76 mg, 68%). ^1H -NMR (400 MHz, CDCl_3) δ 8.64 (s, 1H), 8.16 (s, 1H), 7.85 (s, 1H), 7.82 (m, 1H), 7.82 (m, 3H), 4.31 (t, J = 4.8 Hz, 2H), 3.96 (s, 3H), 3.92 (t, J = 4.8 Hz), 3.71 (t, J = 5.6 Hz, 2H), 3.42 (m, 4H), 3.09 (s, 1H), 2.62 (t, J = 5.2 Hz, 2H), 2.44 (m, 4H), 1.45 (s, 9H).

Compound **5-9**

A solution of **5-8** (70 mg, 0.16 mmol), 6-hydroxynicotinic acid (28 mg, 0.20 mmol), HOBt•H₂O (31 mg, 0.20 mmol), EDCI•HCl (38 mg, 0.20 mmol) and TEA (174 μ L, 1.0 mmol) in DMF (5 mL) was stirred for 8 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH : NH₃ = 80 : 10 : 1) to give **4-1** (52 mg, 57%) as colorless solid. ¹H-NMR (400 MHz, CD₃OD) δ 8.45 (s, 1H), 7.91 (t, J = 2.0 Hz, 1H), 7.77 (m, 1H+1H), 7.68 (m, 1H), 7.58 (dd, J = 2.4, 9.6 Hz, 1H), 7.37 (t, J = 8.0 Hz, 1H), 7.27 (m, 1H), 7.16 (s, 1H), 6.50 (d, J = 9.6 Hz, 1H) 4.32 (m, 2H), 4.01 (s, 3H), 3.95 (m, 2H), 3.87 (m, 2H), 3.72 (m, 4H), 3.71 (s, 1H), 3.03 (m, 2H), 2.99 (m, 4H).

Compound **5**

To the solution of **5-9** (15 mg, 26 μ mol) and TEA (28 μ L, 200 μ mol) in DCM (5 mL) was added the solution of **2-1** (29 mg, 100 μ mol) in DCM (2 mL). The reaction mixture was stirred for 5 h at rt. After removal of the solvent by evaporation, the residue was purified by HPLC to give **4-2** (1.1 mg, 5 %) as a colorless solid, which was only confirmed by HR-ESI MS m/z calcd for [M+H]⁺ 818.2967, found 818.2982.

Scheme 6. Synthesis of **6**

Reaction conditions: (a) NHS, EDCI in DMF, (b) *N*-Boc-2,2'-(ethylenedioxy)diethylamine, TEA in DMF, (c) TFA in DCM, (d) 6-hydroxynicotinic acid, EDCI, HOBt•H₂O in DMF, (e) **2-1**, TEA in DCM

Compound **6-1**

A solution of indomethacin (3.58 g, 10 mmol), NHS (2.3 g, 20 mmol) and EDCI•HCl (3.8 g, 20 mmol) in DMF (20 mL) was stirred for 3 h at rt. After removal of the solvent by evaporation, the residue was dissolved in ethyl acetate, washed with a saturated NaHCO_3 aq and brine, and dried over Na_2SO_4 . The solvent was removed under vacuum to give **6-1** (4.5 g, 99 %) as colorless solid. ^1H -NMR (400 MHz, $\text{DMSO}-d_6$) δ 7.65 (m, 4H), 7.11 (d, $J = 2.4$ Hz, 1H), 6.93 (d, $J = 8.8$ Hz, 1H), 6.72 (dd, $J = 2.4$, 9.2 Hz, 1H), 4.23 (s, 2H), 3.79 (s, 3H), 2.79 (s, 4H), 2.42 (s, 3H).

Compound **6-2**

A solution of **6-1** (341 mg, 0.75 mmol), *N*-Boc-2,2'-(ethylenedioxy)diethylamine (124 mg, 0.50 mmol) and TEA (140 μL , 1.0 mmol) in DMF (8 mL) was stirred for 3 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (CHCl_3 : MeOH = 20 : 1) to give **6-2** (270 mg, 92 %) as colorless amorphous. ^1H -NMR (400 MHz, CDCl_3) δ 7.67 (m, 2H), 7.48 (d, $J = 8.4$ Hz, 2H), 6.85 (d, $J = 8.8$ Hz, 1H), 6.68 (dd, $J = 2.4$, 9.2 Hz, 1H), 3.82 (s, 3H), 3.64 (s, 2H), 3.45 (m, 10H), 3.24 (q, $J = 5.2$ Hz, 2H), 2.39 (s, 3H), 1.43 (s, 9H).

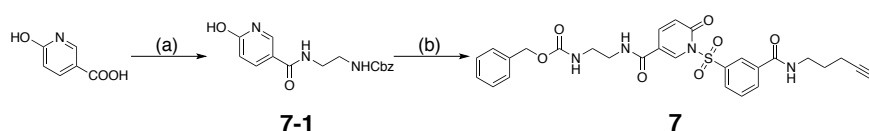
Compound **6-3**

A solution of **6-2** (100 mg, 0.17 mmol), TFA (1 mL) in DCM (3 mL) was stirred for 3 h at rt. After removal of the solvent by evaporation, the residue was added to the mixture of 6-hydroxynicotinic acid (28 mg, 0.20 mmol), HOBt• H_2O (31 mg, 0.20 mmol), EDCI•HCl (35 mg, 0.18 mmol) and TEA (70 μL , 0.50 mmol) in DMF (3 mL). The reaction mixture was stirred for 16 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (CHCl_3 : MeOH : NH_3 = 150 : 10 : 1) to give **6-3** (65 mg, 63 %) as light yellow solid. ^1H -NMR (400 MHz, CD_3OD) δ 7.97 (d, $J = 2.4$ Hz, 1H), 7.91 (dd, $J = 2.8$, 9.6 Hz, 1H), 7.66 (d, $J = 8.4$ Hz, 2H), 7.53 (d, $J = 8.4$ Hz, 2H), 6.98 (d, $J = 2.4$ Hz, 1H), 6.90 (d, $J = 9.2$ Hz, 1H), 6.64 (dd, $J = 2.8$, 9.2 Hz, 1H), 6.45 (d, $J = 9.6$ Hz, 1H), 3.78 (s, 3H), 3.60 (s, 2H), 3.55 (m, 8H), 3.45 (m, 2H), 3.63 (m, 2H), 2.28 (s, 3H).

Compound 6

To the solution of **6-3** (10 mg, 16 μ mol) and TEA (14 μ L, 100 μ mol) in DCM (2.5 mL) was added the solution of **2-1** (17 mg, 60 μ mol) in DCM (2.5 mL). The reaction mixture was stirred for 2 h at rt. After removal of the solvent by evaporation, the residue was purified by HPLC to give **4-2** (4.4 mg, 32 %) as light yellow solid, which was only confirmed by HR-ESI MS m/z calcd for $[M+H]^+$ 858.2570, found 858.2598.

Scheme 7. Synthesis of 7



Reaction conditions: (a) *N*-Cbz-ethylenediamine, EDCI, HOBT•H₂O, TEA in DMF, (b) **2-1**, TEA in DCM/DMF.

Compound 7-1

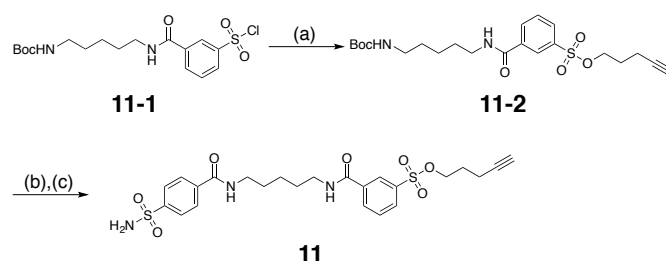
6-Hydroxynicotinic acid (278 mg, 2.0 mmol), *N*-Cbz-ethylenediamine (583 mg, 3.0 mmol), HOBT•H₂O (382 mg, 2.5 mmol), EDCI•HCl (479 mg, 2.5 mmol) and TEA (700 μ L, 5.0 mmol) in DMF (10 mL) was stirred for 16 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH : NH₃ = 100 : 10 : 1) to give **7-1** (499 mg, 79 %) as colorless solid. ¹H-NMR (400 MHz, DMSO-*d*₆) δ 8.25 (t, br, J = 5.2 Hz, 1H), 7.94 (d, J = 2.4 Hz, 1H), 7.80 (dd, J = 2.8, 6.0 Hz, 1H), 7.31 (m, 5H), 6.32 (d, J = 9.6 Hz, 1H), 4.99 (s, 2H), 3.23 (q, J = 5.6 Hz, 2H), 3.11 (q, J = 5.6 Hz, 2H).

Compound 7

To the solution of **7-1** (15 mg, 48 μ mol) and TEA (140 μ L, 1.0 mmol) in DCM (2 mL) and DMF (0.5 mL) was added the solution of **2-1** (57 mg, 200 μ mol) in DCM (2 mL). The reaction mixture was stirred for 2 h at rt. After removal of the solvent by evaporation, the residue was purified by HPLC to give **7-2** (10 mg, 38 %) as colorless

solid. $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ 8.69 (m, 1H), 8.44 (m, 1H), 8.18 (d, $J = 8.8$ Hz, 1H), 8.12 (dd, $J = 2.0, 8.4$ Hz, 1H), 7.72 (dd, $J = 2.4, 9.6$ Hz, 1H), 7.65 (t, $J = 7.6$ Hz, 1H), 7.18 (m, 5H), 6.31 (d, 9.6 Hz, 1H), 4.99 (s, 2H), 3.38 (m, 2H+2H), 3.24 (m, 2H), 2.20 (m, 1H+2H), 1.73 (m, 2H). HR-ESI MS m/z calcd for $[\text{M}+\text{H}]^+$ 565.1751, found 565.1790. O-Sulfonyl derivatives was also acquired as colorless solid. $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ 8.83 (br, 1H), 8.69 (br, 1H), 8.57 (d, $J = 2.4$ Hz, 1H), 8.47 (t, $J = 1.6$ Hz, 1H), 8.23 (dd, $J = 2.4, 8.4$ Hz, 1H), 8.17 (m, 2H), 7.73 (t, $J = 8.0$ Hz, 1H), 7.29 (m, 6H), 5.04 (s, 2H), 3.49 (m, 2H+2H), 3.34 (m, 2H), 2.28 (m, 1H+2H), 1.83 (quin., $J = 7.2$ Hz, 2H). HR-ESI MS m/z calcd for $[\text{M}+\text{H}]^+$ 565.1751, found 565.1620.

Scheme 8. Synthesis of **11**



Reaction conditions: (a) 3-butyn-1-ol, DMAP (cat.), TEA in DMF, (b) TFA in DCM, (c) SA-OSu, TEA in DMF

Compound **11-2**

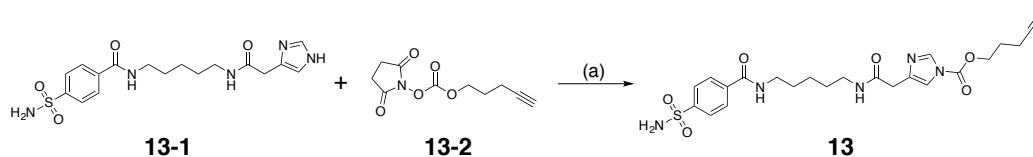
To the solution of **11-1**^{4a} (273 mg, 0.67 mmol), DMAP (2 mg) and TEA (300 μL , 2.1 mmol) in DMF (5 mL) was added the solution of 3-butyn-1-ol (67 mg, 0.80 mmol) in DCM (2 mL). The reaction mixture was stirred for 4 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 ($\text{CHCl}_3 : \text{MeOH} = 25 : 1$) to give **11-2** (240 mg, 79 %) as colorless amorphous.

Compound **11**

The solution of **11-2** (100 mg, 0.22 mmol) and TFA (1 mL) in DCM (3 mL) was stirred for 3h at rt. After removal of the solvent by evaporation, the residue was added to the solution of SA-OSu (75 mg, 0.25 mmol) and TEA (140 μL , 1.0 mmol) in DMF (3 mL). The reaction mixture was stirred for 1 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2

(CHCl₃ : MeOH = 10 : 1) to give **11** (81 mg, 69 %) as colorless solid. ¹H-NMR (400 MHz, CD₃OD) δ 8.19 (d, J = 1.2 Hz, 1H), 7.96 (m, 1H+4H), 7.44 (d, J = 1.2 Hz, 1H), 4.53 (t, J = 6.4 Hz, 2H), 3.40 (m, 2H), 3.22 (m, 2H), 2.38 (m, 2H), 2.28 (m, 1H), 2.01 (m, 2H), 1.66 (m, 2H), 1.53 (m, 2H), 1.44 (m, 2H).

Scheme 9. Synthesis of **13**



Reaction conditions: (a) TEA in DMF

Compound **13**

The solution of **13-1** (25 mg, 64 μ mol), **13-2** (22 mg, 96 μ mol) and TEA (140 μ L, 1.0 mmol) in DMF (5 mL) was stirred for 3 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH = 10 : 1) to give **13** (23 mg, 72 %) as colorless solid. ¹H-NMR (400 MHz, CD₃OD) δ 8.35 (m, 1H), 8.13 (dd, J = 2.0, 6.4 Hz, 1H), 8.05 (dd, J = 2.0, 8.0 Hz, 1H), 7.96 (m, 4H), 7.72 (t, J = 7.6 Hz, 1H), 4.82 (t, J = 6.0 Hz, 1H), 3.44 (m, 2H+2H), 2.22 (m, 2H), 3.02 (m, 1H), 1.80 (m, 2H), 1.68 (m, 2H+2H), 1.48 (m, 2H).

Biochemical experiments: SDS-PAGE and western blotting were performed using a Bio-Rad Mini-Protean III electrophoresis apparatus. Fluorescence and chemical luminescent signals were detected with a ChemiDoc XRS system equipped with a 520DF30 filter (ChemiDoc, Bio-Rad laboratory) and Imagequant LAS 4000 (GE Healthcare), respectively.

In vitro labeling of CA1&CA2

CA1&CA2 (5 μ M) was incubated with LDSP reagent (10 μ M) in the absence or presence of EZA (100 μ M) in HEPES buffer (50 mM, pH 7.4, 100mM NaCl) at 37 °C for 24 h. The aliquots were taken at different time points and the labeling yields were

determined by MALDI-TOF MS and SDS-PAGE.

Peptide mapping of the Dc labeled CA1&2

A solution of CA1 or CA2 (10 μ M) and **1** (20 μ M) in HEPES buffer (50 mM, pH 7.2) was incubated for 12 h at 37 °C. The labeled CA1 or CA2 was purified by size-exclusion chromatography (TOYOPEARL HW-40F) and dialyzed against HEPES buffer (100 mM, pH 8.0) using a Spectra/Por[®] dialysis membrane (MWCO: 10,000). After concentration by ultrafiltration (Centricon Ultracel YM-10, Millipore), the solution was incubated with lysyl endopeptidase (LEP) (LEP/substrate ratio = 1/3 (w/w)) at 37 °C for 8 h in the presence of urea (2 M). The digested sample was subjected to RP-HPLC (column; YMC-Pack Triat C18, 250 $\times$ 4.6 mm, mobile phase; CH₃CN (containing 0.1% TFA) : H₂O (containing 0.1% TFA) = 5 : 95 to 55 : 45 (linear gradient over 100 min), flow rate; 1.0 mL/min, detection; UV (220–600 nm). For the peptide mapping, the labeled fragments were further analyzed by MALDI-TOF MS/MS.

Cell Culture: MCF7, A431 and RAW264.7 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose/L) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μ g/mL) under a humidified atmosphere of 5% CO₂ in air. KB cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μ g/mL) without folic acid. For all experiments, cells were harvested from subconfluent (< 80%) cultures using a trypsin-EDTA solution or cell scraper methods and then resuspended in fresh medium. A subculture was performed every 2–3 days.

Endogenous protein labeling in mammalian cells.

For CA2 labeling

MCF7 cells (2×10^5 cells) were incubated at 37 °C in DMEM without FBS containing **2** (10 μ M) in the absence or presence of EZA (100 μ M). After 20 h, the cells were washed three times with phosphate-buffered saline (PBS), lysed using RIPA (radio immunoprecipitation assay) buffer containing 1% protease inhibitor cocktail set III (Calbiochem[®]) on ice. After centrifugation (13,500rpm, 10min), the supernatant was

reacted with **9** under click condition (BPAA, TCEP, Cu^+) for 2 hours. The resulting solution was mixed with 2 × SDS-PAGE loading buffer (pH 6.8, 125 mM Tris•HCl, 20% Glycerol, 4% SDS and 0.01% Bromophenol blue, 100mM DTT) and it was vortexed for 30 min at room temperature. The samples were resolved by 12.5% SDS-PAGE and electrotransferred onto an Immun-Blot PVDF membrane (Bio-Rad). The labeled products were detected with SAV-HRP (Invitrogen, ×5000). The immunodetection of CA2 was carried out with an anti-CA2 antibody (Abcam) and anti-rabbit IgG antibody-HRP conjugate (GE Healthcare, ×5000). The HRP signal was detected with LAS 4000 imaging system (FujiFilm) using ECL plus western blotting detection reagents (GE Healthcare).

Identification of CA2 by immunoprecipitant method

Immunoprecipitation was carried out by a standard procedure provided by GE healthcare with slight modifications. In brief, after labeling and click reaction the resulting solution was dissolved in RIPA buffer. Each sample was pre-treated with nProtein A sepharose 4 Fast flow (GE healthcare) for 30 min. The sample was then incubated with anti-CA2 at 4 °C for 1 hour, followed by addition of nProtein A sepharose 4 Fast flow and further incubation at 4 °C for 8 hours. The sepharose was washed 3 times with RIPA and once with PBS(-). Protein was eluted by addition of 5× SDS-PAGE sample buffer containing 200 mM DTT and boiling in heatblock for 5min, and resolved by SDS-PAGE, which was analyzed with SAV-HRP (Invitrogen, ×5000).

For CA12 labeling

MCF7 cells (2×10^5 cells) were incubated at 37 °C in DMEM without FBS containing **3** (1 μM) in the absence or presence of EZA (100 μM). After 20 h, the cells were washed three times with PBS, lysed using RIPA buffer containing 1% protease inhibitor cocktail set III on ice. After centrifugation (13,500rpm, 10min), the supernatant was mixed with 2 × SDS-PAGE loading buffer and it was vortexed for 30 min at room temperature. The samples were resolved by 12.5% SDS-PAGE and electrotransferred onto an Immun-Blot PVDF membrane. The labeled products were detected with

Anti-Alexa488 antibody (Abcam, $\times 1000$) and anti-rabbit IgG antibody-HRP conjugate (GE Healthcare). The immunodetection of CA12 was carried out with an anti-CA12 antibody (Cellsignaling, $\times 1000$) and anti-rabbit IgG antibody-HRP conjugate (GE Healthcare, $\times 5000$). The HRP signal was detected with LAS 4000 imaging system.

For FR labeling

KB cells (2×10^5 cells) were incubated at 37 °C in RPMI-1640 without folic acids and FBS containing **4** (1 μ M) in the absence or presence of folic acid (100 μ M). After 20 h, the cells were washed three times with PBS, lysed using RIPA buffer containing 1% protease inhibitor cocktail set III on ice. After click reaction with **9**, the resulting solution mixed with 2 $\times$ SDS-PAGE loading buffer and it was vortexed for 30 min at room temperature. The samples were resolved by 12.5% SDS-PAGE and electrotransferred onto an Immun-Blot PVDF membrane. The labeled products were detected with SAV-HRP (Invitrogen, $\times 5000$). The immunodetection of FR was carried out with an anti-FR antibody (Abcam, $\times 500$) and anti-rabbit IgG antibody-HRP conjugate (GE Healthcare, $\times 5000$). The HRP signal was detected with LAS 4000 imaging system.

For EGFR labeling

A431 cells (2×10^5 cells), which were pre-incubated at 37 °C in DMEM for 48h, were incubated at 37 °C in DMEM without FBS containing **5** (5 μ M) in the absence or presence of erlotinib (20 μ M). After 8 h, the cells were washed three times with PBS, lysed using RIPA buffer containing 1% protease inhibitor cocktail set III on ice. After click reaction with **9**, the resulting solution was mixed with 2 $\times$ SDS-PAGE loading buffer and it was vortexed for 30 min at room temperature. The samples were resolved by 7.5% SDS-PAGE and electrotransferred onto an Immun-Blot PVDF membrane. The labeled products were detected with SAV-HRP (Invitrogen, $\times 5000$). The immunodetection of EGFR was carried out with an anti-EGFR antibody (Cellsignaling, $\times 1000$) and anti-rabbit IgG antibody-HRP conjugate (GE Healthcare, $\times 5000$). The HRP signal was detected with LAS 4000 imaging system.

For COX2 labeling

RAW264.7 cells (2×10^5 cells), which were pre-incubated at 37 °C in DMEM with/without LPS (1 ng/mL) for 12h, were incubated at 37 °C in DMEM containing **6** or **7** (each 5 μ M). After 12 h, the cells were washed three times with PBS, lysed using RIPA buffer containing 1% protease inhibitor cocktail set III on ice. After centrifugation (13,500rpm, 10min), the endogenous biotinylated proteins were precleared by incubating with immobilized NeutrAvidin protein beads (Thermo) at 4 °C for 1 h. After removal of the beads, click reaction reacted with **9** was conducted for 2 hours. The resulting solution was immunoprecipitated with COX2 antibody (Abcam) by the same methods of CA2. The samples were resolved by 10% SDS-PAGE and electrotransferred onto an Immun-Blot PVDF membrane. The labeled products were detected with SAv-HRP (Invitrogen, $\times 5000$). The immunodetection of FR was carried out with an anti-COX2 antibody (Abcam, $\times 1000$) and anti-rabbit IgG antibody-HRP conjugate (GE Healthcare, $\times 5000$). The HRP signal was detected with LAS 4000 imaging system.

3-5. References

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List of Publications

Chapter 1 Specific Detection and Imaging of Enzyme Activity by Signal Amplifiable Self-Assembling ^{19}F -MRI Probes

Kazuya Matsuo, Rui Kamada, Keigo Mizusawa, Hirohiko Imai, Yuki Takayama, Michiko Narazaki, Tetsuya Matsuda, Yousuke Takaoka, Itaru Hamachi
Chem. Eur. J. **2013**, *19*, 12875-12883.

Chapter 2 One-step construction of caged carbonic anhydrase I using a ligand-directed acyl imidazole-based protein labeling method

Kazuya Matsuo, Yoshiyuki Kioi, Ryosuke Yasui, Yousuke Takaoka, Takayuki Miki, Shohei Fujishima, Itaru Hamachi
Chem. Sci. **2013**, *4*, 2573-2580.

Chapter 3 Specific Natural Proteins Sulfonylation under Live Cell Condition by Ligand-directed *N*-Sulfonyl Pyridone Chemistry

Kazuya Matsuo, Marie Masuda, Itaru Hamachi
Manuscript in preparation.

Other Publications

1) Photoactivatable HNO-releasing compounds using the retro-Diels-Alder reaction

Yusuke Adachi, Hidehiko Nakagawa, Kazuya Matsuo, Takayoshi Suzuki, Naoki Miyata
Chem. Commun. **2008**, 5149-5151.

2) Alternative photoinduced release of HNO or NO from an acyl nitroso compound, depending on environmental polarity

Kazuya Matsuo, Hidehiko Nakagawa, Yusuke Adachi, Eri Kameda, Hiroki Tsumoto, Takayoshi Suzuki, Naoki Miyata.
Chem. Commun. **2010**, 46, 3788-3790.

3) Systematic Study of Protein Detection Mechanism of Self-Assembling ¹⁹F NMR/MRI Nanoprobes toward Rational Design and Improved Sensitivity

Yousuke Takaoka, Keishi Kiminami, Keigo Mizusawa, Kazuya Matsuo, Michiko Narazaki, Tetsuya Matsuda, Itaru Hamachi
J. Am. Chem. Soc. **2011**, 133, 11725-11731.

4) Photoinduced Upregulation of Calcitonin Gene-Related Peptide in A549 Cells through HNO Release from a Hydrophilic Photocontrollable HNO Donor

Kazuya Matsuo, Hidehiko Nakagawa, Yusuke Adachi, Eri Kameda, Kazuyuki Aizawa, Hiroki Tsumoto, Takayoshi Suzuki, Naoki Miyata
Chem. Pharm. Bull. **2012**, 60, 1055-1062.

5) Piloty' s acid derivative with improved nitroxyl-releasing characteristics

Kazuyuki Aizawa, Hidehiko Nakagawa, Kazuya Matsuo, Kodai Kawai, Naoya Ieda,
Takayoshi Suzuki, Naoki Miyata

Bioorg. Med. Chem. Lett. **2013**, 23, 2340-2343.

6) Disassembly-driven Turn-on Sensing of Enzyme Activity by using Substrate-based
Fluorescent Nanoprobe

Yousuke Takaoka, Yoshiaki Fukuyama, Kazuya Matsuo, Itaru Hamachi

Chem. Lett. **2013**, 42, 1426-1428.

Reviews and Books

1) 生細胞でのタンパク質選択的なケミカルラベリングの新手法

松尾 和哉、浜地 格

生化学, 第 83 巻, 第 10 号, 920-929 (2011)

2) Ligand-directed tosyl/acyl imidazole chemistry

Kazuya Matsuo, Itaru Hamachi

Chemoselective and Bioorthogonal Ligation Reactions: Concepts and Applications

John Wiley & Sons: New York, *in press*.

List of Presentations

International Symposium

1) NOVEL PHOTOINDUCED HNO DONORS

Kazuya Matsuo, Hidehiko Nakagawa, Yusuke Adachi, Eri Kameda, Hiroki Tsumoto,
Takayoshi Suzuki, Naoki Miyata

The 5th Joint Meeting of The Societies For Free Radical Research Australasia and Japan,
Sydney, Australia, **2009**.

Domestic Symposium

1) Design and synthesis of novel photoinducible HNO donors

Kazuya Matsuo, Hidehiko Nakagawa, Yusuke Adachi, Takayoshi Suzuki, Naoki Miyata
The 27th Medicinal Chemistry Symposium. Osaka, November **2008**.

2) Photoreactivity in RNS formation from hydrophilic hetero Diels Alder cycloadducts

Kazuya Matsuo, Hidehiko Nakagawa, Yusuke Adachi, Takayoshi Suzuki, Naoki Miyata
The 129th Annual Meeting of the Pharmaceutical Society of Japan. Kyoto, March **2009**.

3) Development of photocontrollable hydrophilic HNO donors and their reactivity

Kazuya Matsuo, Hidehiko Nakagawa, Yusuke Adachi, Hiroki Tsumoto, Takayoshi Suzuki,
Naoki Miyata
The 62nd Annual Meeting of the Japanese Oxidative Stress Society. Fukuoka, June **2009**.

4) The photo-reactivity of photo-induced HNO donors

Kazuya Matsuo, Hidehiko Nakagawa, Yusuke Adachi, Hiroki Tsumoto, Takayoshi Suzuki,
Naoki Miyata
The 28th Medicinal Chemistry Symposium. Tokyo, November **2009**.

5) Development of novel photocontrollable HNO releasers

Kazuya Matsuo, Hidehiko Nakagawa, Yusuke Adachi, Hiroki Tsumoto, Takayoshi Suzuki,
Naoki Miyata

Medicinal Chemistry Forum 2009. Gifu, December **2009**.

6) Novel photoinduced HNO releasers

Kazuya Matsuo, Hidehiko Nakagawa, Yusuke Adachi, Hiroki Tsumoto, Takayoshi Suzuki,
Naoki Miyata

The 130th Annual Meeting of the Pharmaceutical Society of Japan. Okayama, March **2010**.

7) Development of self-assembling ^{19}F -NMR/MRI OFF/ON probes for the selective detection of proteins

Kazuya Matsuo, Yousuke Takaoka, Shinya Tsukiji, Itaru Hamachi

The 22nd Symposium on Biofunctional Chemistry Summer School, Kameyama, July **2010**.

8) Self-assembled probes (2): Application of ^{19}F NMR/MRI OFF/ON probes to enzymes

Kazuya Matsuo, Yousuke Takaoka, Daishiro Minato, Shinya Tsukiji, Itaru Hamachi

91st The Chemical Society of Japan Annual Meeting, Yokohama, March **2011**.

9) Imaging of enzyme activity using self-assembling ^{19}F -NMR/MRI OFF/ON probes

Kazuya Matsuo, Yousuke Takaoka, Keigo Mizusawa, Michiko Narazaki, Tetsuya Matsuda,
Itaru Hamachi

The 5th Symposium on Biorelevant Chemistry CSJ. Tsukuba, September **2011**.

10) The stability of ^{19}F probe aggregates controls the OFF/ON response in ^{19}F -MRI detection

Kazuya Matsuo, Yousuke Takaoka, Keishi Kiminami, Keigo Mizusawa, Michiko Narazaki, Tetsuya Matsuda, Itaru Hamachi

The 14th Forum on Biomolecular Chemistry. Wakayama, December **2011**.

11) Self-assembling probe (1) : Systematic study of protein response mechanism in ^{19}F MRI probes and sensitivity improvements

Kazuya Matsuo, Yousuke Takaoka, Keishi Kiminami, Keigo Mizusawa, Michiko Narazaki, Tetsuya Matsuda, Itaru Hamachi

92nd The Chemical Society of Japan Annual Meeting, Yokohama, March **2012**.

12) Development of Caged Enzyme Using Protein Labeling by LDAI Chemistry

Kazuya Matsuo, Shohei Fujishima, Yoshiyuki Kioi, Yousuke Takaoka, Itaru Hamachi

The 7th Annual Meeting of Japanese Society for Chemical Biology. Kyoto, June **2012**.

13) Development of the novel approach of caging enzyme activity using the efficient protein labeling by LDAI chemistry

Kazuya Matsuo, Shohei Fujishima, Yoshiyuki Kioi, Yousuke Takaoka, Itaru Hamachi

The 23rd Symposium on Biofunctional Chemistry Summer School. Fukuoka, July **2012**.

14) One-step Construction of Caged Enzyme Using LDAI Chemistry

Kazuya Matsuo, Shohei Fujishima, Yoshiyuki Kioi, Yousuke Takaoka, Itaru Hamachi

The 6th Symposium on Biorelevant Chemistry CSJ. Sapporo, September **2012**.

15) Biosensing using Innovative Chemical Probes (1) Turn-On Imaging of Enzyme Activity using Self-Assembling Nano-Probes

Kazuya Matsuo, Yoshiaki Fukuyama, Rui Kamada, Kaigo Mizusawa, Hirohiko Imai, Yuki Takayama, Michiko Narazaki, Tetsuya Matsuda, Yousuke Takaoka, Itaru Hamachi
93rd The Chemical Society of Japan Annual Meeting, Kusatsu, March **2013**.

List of Patents

1) Anthracene Derivative Cycloaddition with Acylnitroso Derivative, and Light-Activated HNO Donor

Naoki Miyata, Hidehiko Nakagawa, Kazuya Matsuo, Takayoshi Suzuki
WO2010052993 A1, May 14th, 2010.

List of Honors

- 1) Japan Society for the Promotion of Science (JSPS) Predoctoral Fellowship (DC2, 2011-2013)
- 2) Student presentation award at the 93rd annual meeting of Chemical Society of Japan