

Development of novel protein labeling reagents utilizing
N-sulfanylethylanilide unit

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Abbreviations

Boc	<i>tert</i> -butoxycarbonyl
DBU	1,8-diazabicyclo[5,4,0]undec-7-ene
DIC	diisopropylcarbodiimide
DIPEA	diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethylsulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EZA	ethoxyzolamide
FITC	fluorescein-5-isothiocyanate
Fmoc	9-fluorenylmethoxycarbonyl
FTC	fluorescein-5-aminothiocarbonyl
HATU	<i>O</i> -(7-azabenzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HBS	HEPES-buffered Saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOBt	1-hydroxybenzotriazole
HRP	horseradish peroxidase
ivDde	1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl
Me	methyl
mini-PEG	8-amino-3,6-dioxaoctanoic acid
PAGE	polyacrylamide gel electrophoresis
PBS-T	phosphate buffered saline with Tween 20
PEG	polyethylene glycol
PVDF	polyvinylidene fluoride
rt	room temperature
SDS	sodium dodecylsulfate
SPPS	solid phase peptide synthesis
Su	succinimide
TES	triethylsilane

TFA trifluoroacetic acid
Tris tris(hydroxymethyl)aminomethane

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Preface

Proteins play important roles in living organisms. Therefore, elucidation of these protein functions is an important task for understanding the biological profiles of these organisms. Notably, the elucidation of functions of a wide variety of proteins in protein mixtures (proteomes) is of great importance for understanding vital phenomena, because proteins work within proteomes. Tracing the dynamic behavior of proteins in cells leads to a powerful tool for elucidating biological functions of proteins, in which attachment of reporter molecules such as fluorescent dyes on proteins of interest is needed.¹ The ligand-dependent incorporation of a reporter onto the target protein using affinity-based labeling systems has thus emerged as an important strategy (Figure 1).^{2,3} In this strategy, generally used reagents are composed of three functional units, namely (1) a ligand unit for the selective interaction with a target protein; (2) a reporter unit for the visualization of a target protein; (3) a reactive unit for the covalent attachment of the reporter unit to a target protein. Because the conventional labeling reagents have a slightly activated reactive unit, there is a potential risk of a non-target protein being labeled by the activated reagent in a ligand-independent manner. Therefore, it was envisaged that the activation of labeling reagents through interaction between a target protein and its ligand could potentially overcome the problems associated with conventional labeling reagents. With this in mind, I attempted to adapt *N*-sulfanylethylamide (SEALide) unit developed by our group as reactive unit of a labeling reagent.

In Chapter 1, design and synthesis of SEAL-tag is described. The application of SEAL-tag for selective labeling of human carbonic anhydrase (hCA) and cyclooxygenase 1 (COX-1) is reported in Chapter 2.

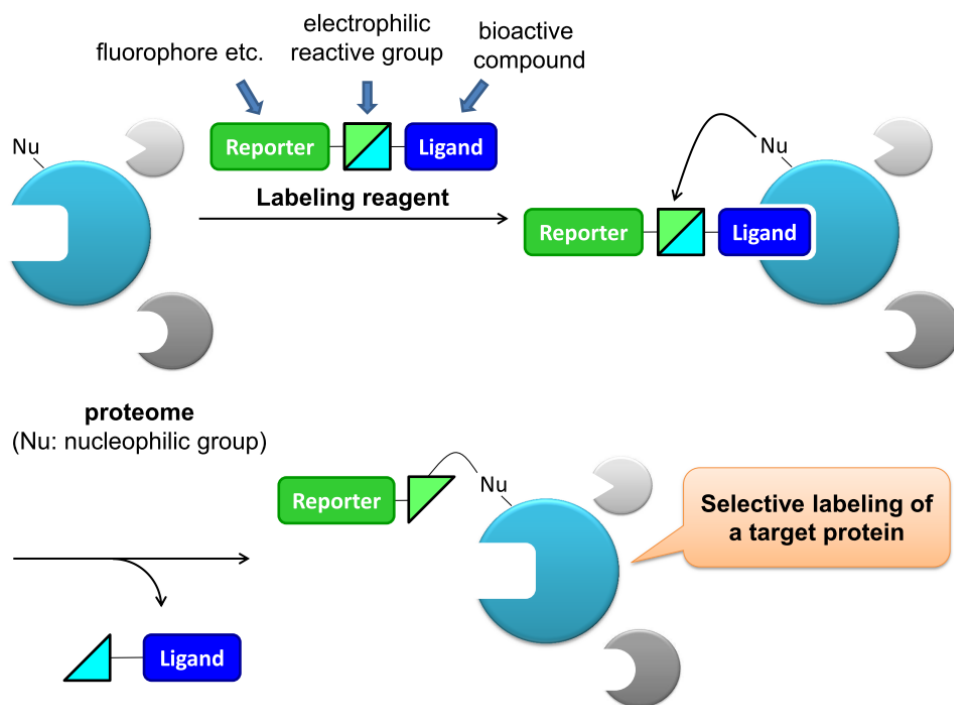


Figure 1. Selective labeling of a target protein using an affinity-based labeling method.

Chapter 1 Development of SEALide-based labeling tag (SEAL-tag)

1.1 Background

Affinity-based protein labeling has recently attracted considerable attention as an efficient strategy for introduction of a reporter unit (e.g., fluorescent dyes or biotin) onto target proteins of bioactive compounds. In this method, employed affinity-based labeling reagents are generally composed of three functional units, including (1) a ligand unit for the selective recognition of a target protein; (2) a reporter unit for the visualization of the labeled protein; and (3) a reactive unit for a covalent installation of the reporter units onto the target protein.

Recently, Hamachi and co-workers developed several labeling reagents based on ligand-directed tosyl (LDT) chemistry (Figure 2a)⁴ and ligand-directed acyll imidazole (LDAI) chemistry (Figure 2b).⁵ An LDT reagent bearing phenylsulfonate as a reactive unit was applied to label several target proteins, such as carbonic anhydrase (CA) and FK506-binding protein (FKBP12), and an LDAI reagent bearing acyl imidazole as a reactive unit was applied to modify target proteins such as dihydrofolate reductase (DHFR) and a membrane-bound folate receptor (FR). Additionally, Sodeoka's group developed a labeling reagent bearing *O*-nitrobenzoxadiazole (*O*-NBD) unit as reactive unit, with the reagent employed for selective labeling of a partner protein of translocator protein (TSPO), voltage-dependent anion channel (Figure 2c).⁶

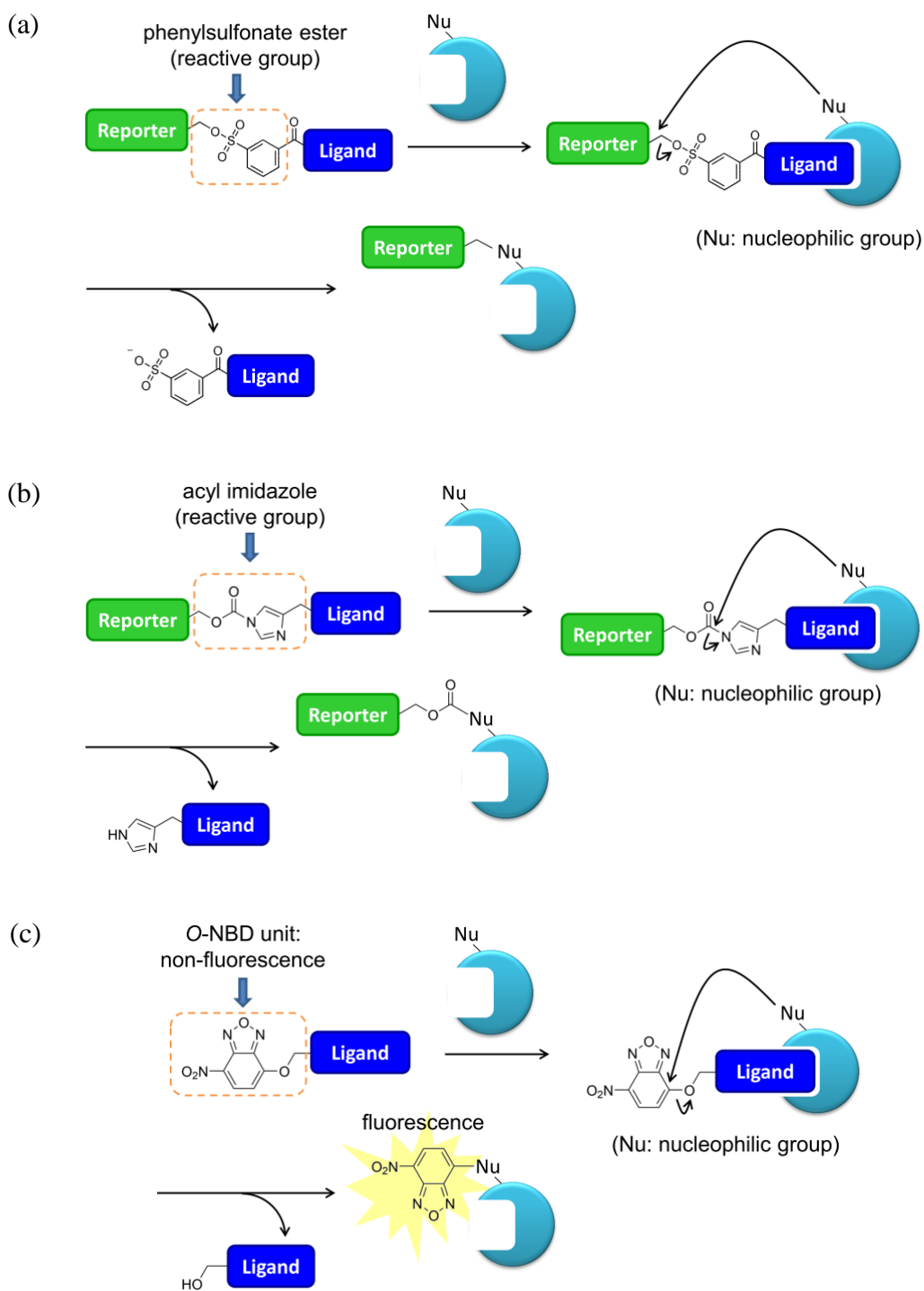


Figure 2. Schematic illustrations of protein labeling methods. (a) Ligand-directed tosylate (LDT) chemistry developed by Hamachi's group. (b) Ligand-directed acyl imidazole (LDAI) chemistry developed by Hamachi's group. (c) *O*-NBD method developed by Sodeoka's group.

However, the reactive units of such labeling reagents were slightly and constitutively activated, which sometimes resulted in the labeling of non-target proteins in a ligand-independent manner. Some groups carefully examined reactivity against non-targets and demonstrated stability of their labeling reagents under physiological conditions.^{4(c),7} Although such labeling of non-targets with the activated reagent has been documented to be mostly not significant, it has been considered that an unprecedented labeling reagent that remains inert in the absence of targets, but is activated upon its binding to targets, would be ideal for the protein labeling (Figure 3). In this context, the chemical basis of the SEALide involved in native chemical ligation (NCL) was considered to enable the development of this unprecedented reagent.

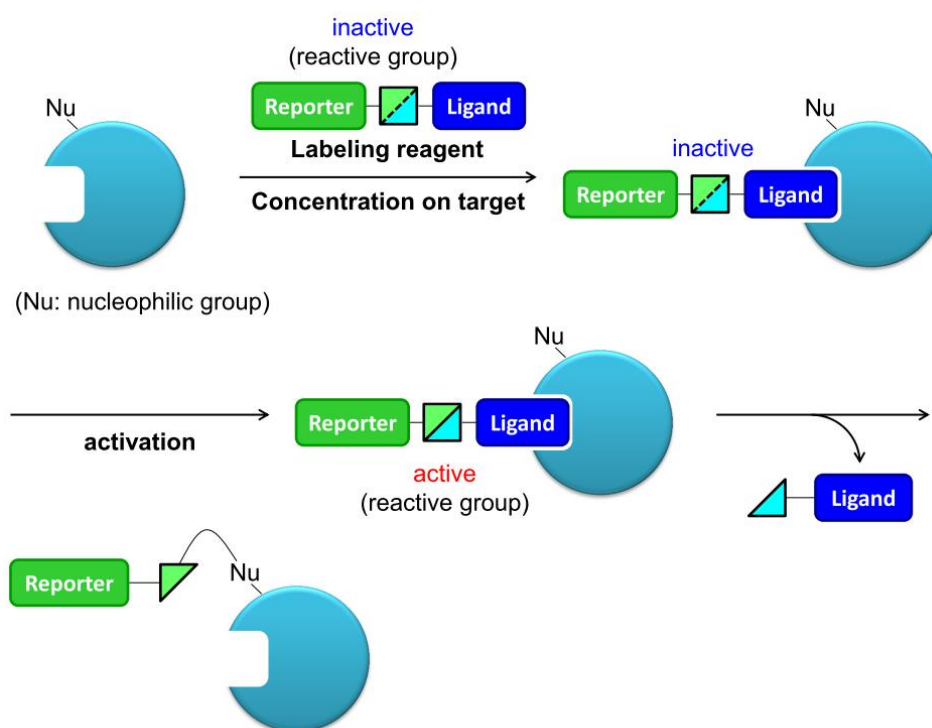
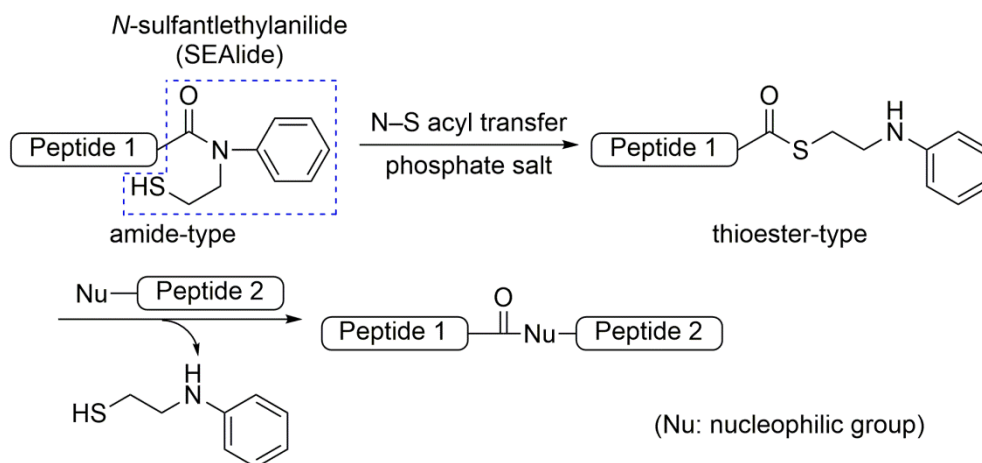


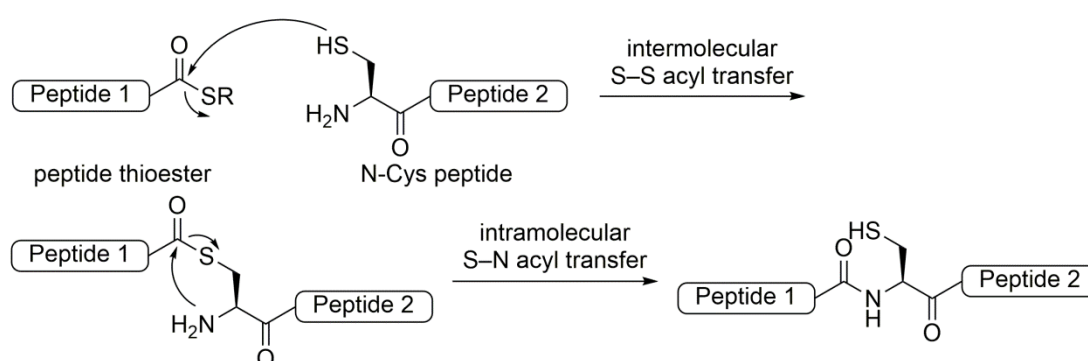
Figure 3. Selective labeling of a target protein by an affinity-based labeling reagent activated by binding to targets.

The SEALide was developed as a crypto-thioester by our group and has been successfully applied to the chemical synthesis of proteins, being used in a one-pot/N-to-C-directed sequential NCL reaction (Scheme 1).^{8,9}



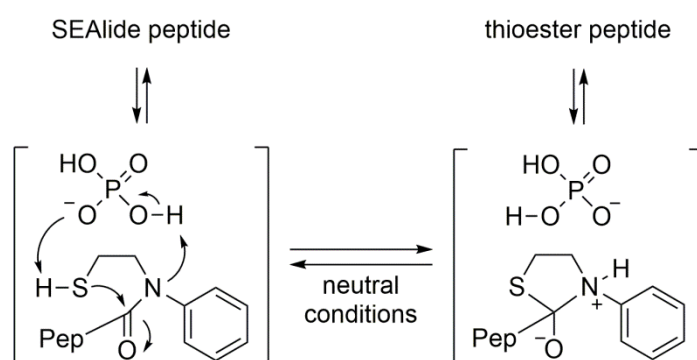
Scheme 1. SEALide peptide as a potential peptide thioester equivalent.

The NCL, which features the chemoselective coupling of peptide thioesters with N-terminal cysteinyl peptide, is among the most reliable fragment-coupling reactions (Scheme 2).¹⁰



Scheme 2. Reaction mechanism of native chemical ligation.

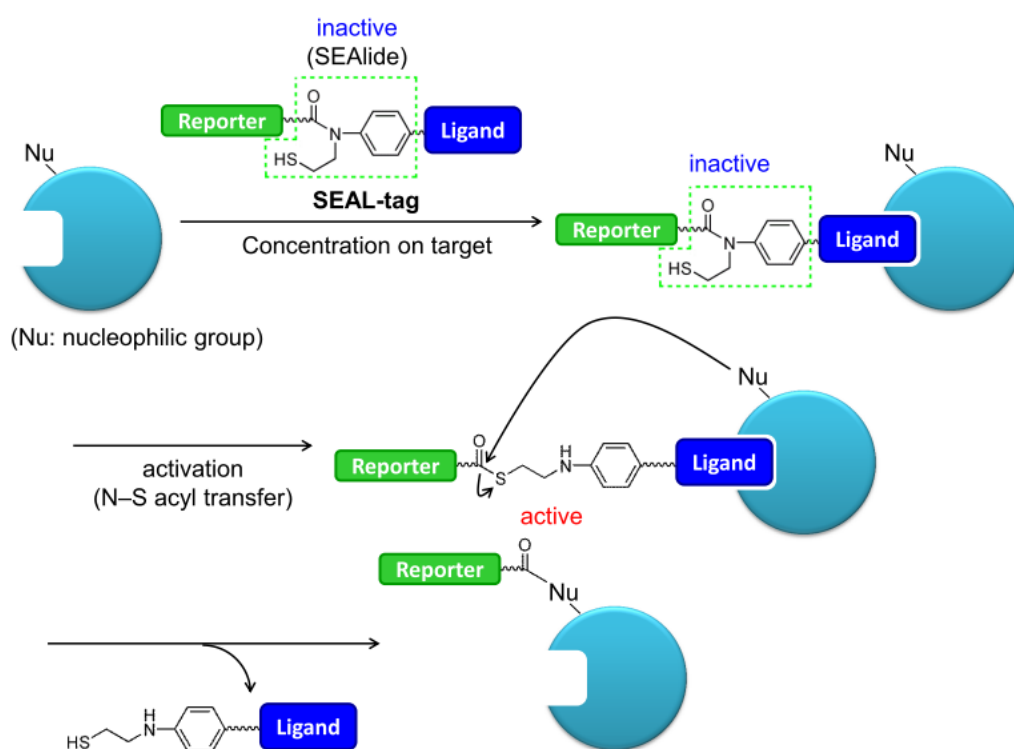
The SEALide unit generally remains electrophilically inert as the amide-form under neutral conditions; however, participation of the SEALide unit in the NCL reaction requires the conversion of the amide-form to the corresponding thioester form as an electrophilically active species. Our research on the SEALide, involving its use in chemical synthesis of proteins, has disclosed that the unreactive amide-type SEALide could be easily converted to the reactive thioester-type SEALide and participate in NCL reaction only in the presence of phosphate salts under neutral conditions. Our unexpected finding suggested that phosphate salts could function as a general acid-base catalyst for the intramolecular N–S acyl transfer, promoting the reaction of the SEALide with nucleophiles such as the N-terminal cysteinyl residue (Scheme 3).^{9(f)} I thus envisioned that the unprecedented labeling reagent could be developed on the basis of the unique nature of the SEALide.



Scheme 3. Potential involvement of phosphate salts in N–S acyl transfer in SEALide unit.

Incidentally, protein surfaces are composed of various polar functional groups, including hydroxyl, amino, carboxyl, imidazole, and guanidine groups. The cluster of functional groups could cooperatively work as general acid-base catalysts, potentially promoting the N–S acyl transfer of the SEALide unit. With this in mind, I hypothesized that the immobilization of the stable amide-type SEALide on protein surfaces could induce the activation of the SEALide to the corresponding thioester through N–S acyl transfer. The generated electrophilically active thioester part could potentially behave as the reactive unit of affinity-based labeling reagents. Based on my hypothesis, a SEALide-based labeling tag (SEAL-tag) bearing the SEALide unit as the reactive unit of the labeling reagent was designed. Without interacting with target proteins, the

SEAL-tag would remain inert as its amide form. This would therefore prevent the SEAL-tag from reacting with non-targets, because it would not be efficiently concentrated on the protein surface, which would be required as an acid-base catalyst. On the other hand, the specific binding of the SEAL-tag with the target protein via interaction between the ligand and the target would allow the labeling reagent to be concentrated on the target protein for a certain period, with the cluster of functional groups on the target protein working as general acid-base catalysts. This would therefore result in the N–S-acyl-transfer of SEALide followed by acylation of the nucleophilic residues on the target protein in a ligand-dependent manner (Scheme 4). Based on my hypothesis, I prepared unreactive amide-type SEAL-tags and applied SEAL-tags to label two model proteins, human carbonic anhydrase (hCA) and cyclooxygenase 1 (COX-1).



Scheme 4. Strategy for the selective labeling of a target protein using SEAL-tag.

1. 2 Molecular design and synthesis of the SEAL-tag

The incorporation of an S-protected SEALide unit as a stable synthetic intermediate enabled the desired SEAL-tags to be readily prepared using standard Fmoc-based solid-phase protocols, representing a considerable advantage over other techniques generally used for the synthesis of labeling reagents.

General structures of SEAL-tags are shown in Figure 4. In this study, I chose hCA and COX-1 as model target proteins of bioactive compounds. The cytosolic protein hCA participates in several biological processes, including regulation of pH, transportation of CO₂ and HCO₃⁻, and balance of water and electrolyte.¹¹ Because (1) Co-crystal structures of hCA–benzenesulfonamide (SA) derivatives have been previously reported;¹² (2) ethoxzolamide (EZA), which is a strong binder against the SA-binding pocket of hCA,¹³ can be readily obtained from commercial sources, I selected hCA as one of the model target proteins and SA, as a specific ligand of hCA, was chosen and used as the ligand unit of SEAL-tag.

Cyclooxygenase, involved in the biosynthesis of prostaglandins from arachidonic acid (AA), was selected as the second model target protein.¹⁴ Because non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin or ibuprofen inhibit the COX-1 mediated synthesis of prostaglandins,¹⁵ competitively binding to the active site of COXs, the indomethacin was chosen as the ligand and introduced into the SEAL-tag. A fluorescein-thiocarbamoyl (FTC) group or biotin was introduced as the reporter into SEAL-tag, because FTC or biotin-modified protein is easily detected by fluorescence gel imaging (FI) or Western blotting using streptavidin-HRP, respectively. In addition, flexible ethylene glycol (mini-PEG) linkers of various lengths were also embedded into the reagents in consideration of reactivity property of the SEAL-tags with targets and their solubility in aqueous environments.^{4(d),16}

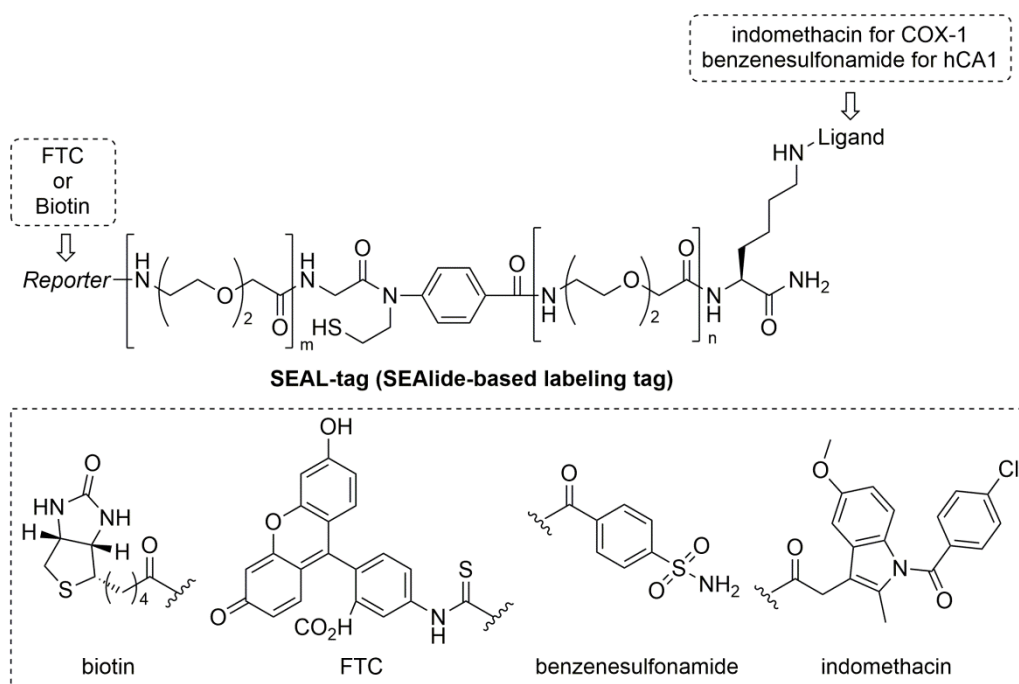
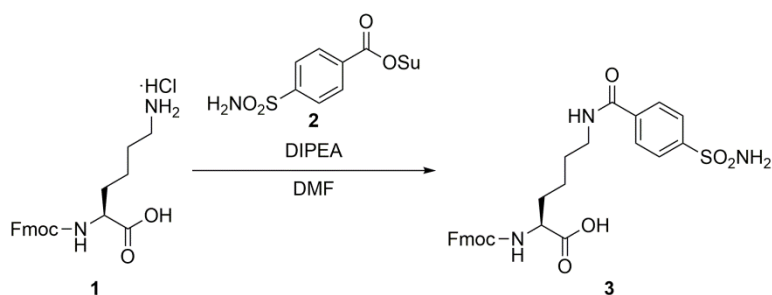


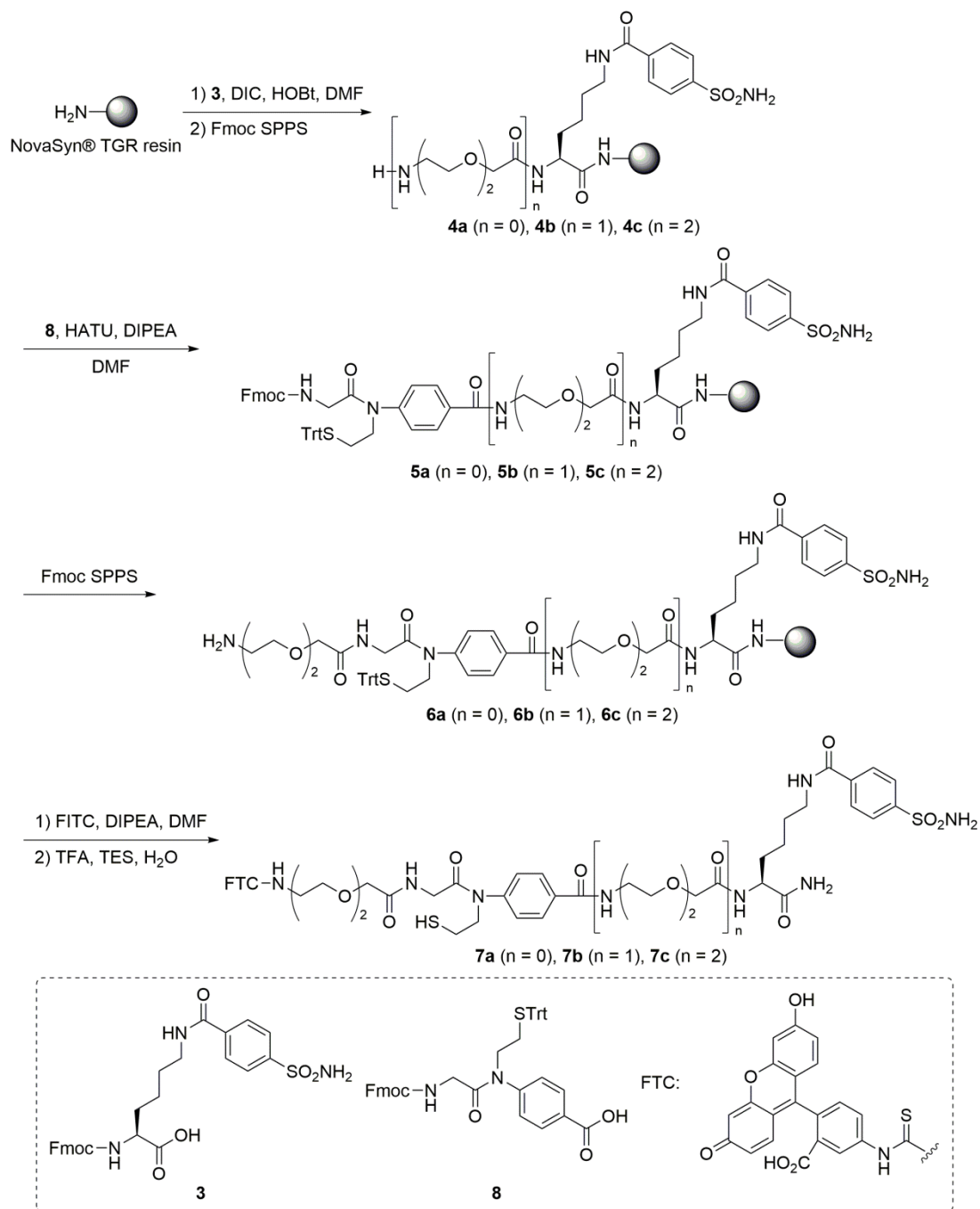
Figure 4. Structure of SEAL-tags.

Our synthetic strategy of SEAL-tag for labeling of hCA is shown in Scheme 6. First, the synthetic intermediate **3** was prepared. Na⁺ Fmoc-protected Lys derivative **1** was reacted with an activated SA N-hydroxysuccinimide ester **2**^{4(h)} to afford Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH **3** in 80% yield (Scheme 5).



Scheme 5. Synthesis of Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH **3**.

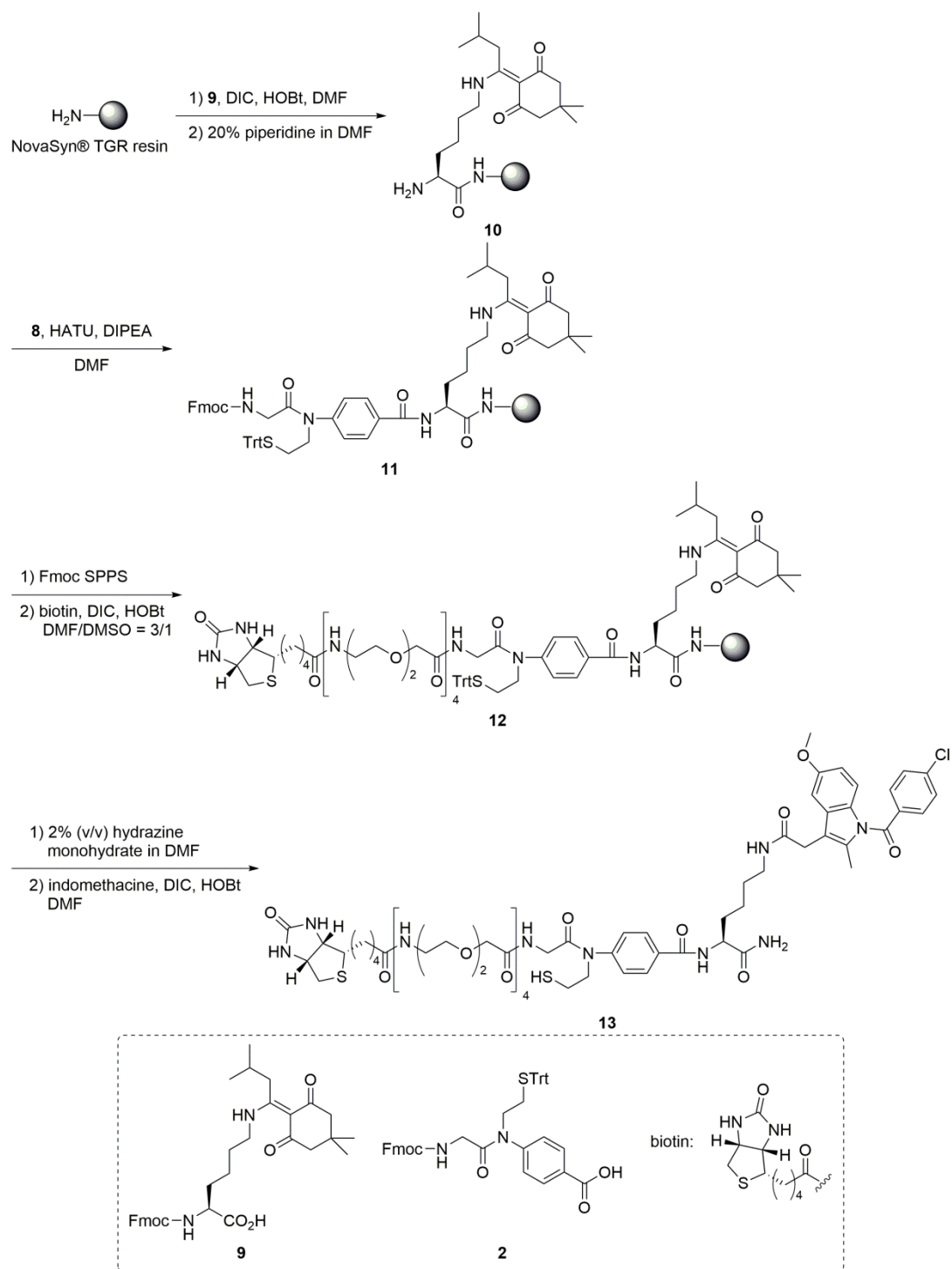
The resulting Lys derivative **3** and Fmoc-miniPEGTM were successively incorporated by standard Fmoc SPPS protocol using the DIC/HOBt system on a NovaSyn[®] TGR resin. After coupling of the Fmoc-Gly-SEAlide-OH **8**^{9(h)} using *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) and *N,N*-diisopropylethylamine (DIPEA), the DIC/HOBt system was used to incorporate Fmoc-miniPEGTM. The resulting resins **6a-c** were reacted with FTC, and the resin-bound SEAL-tags were finally treated with TFA-Et₃SiH-H₂O (95:2.5:2.5 [v/v]) to generate the desired SEAL-tag **7a-c**.



Scheme 6. Synthesis of SEAL-tag **7a-c**.

In Scheme 7, I described a synthetic strategy of SEAL-tag **13** for labeling of COX-1. SEAL-tag **13** was synthesized in a manner similar to that employed for the synthesis of SEAL-tag **7a-c**. In this strategy, N ϵ ivDde-protected Lys derivative **9**¹⁷ was employed as synthetic material, because ivDde group was stable under standard Fmoc SPPS conditions, whereas ivDde group was selectively removed by treatment with 2% (v/v) hydrazine hydrate in DMF.¹⁸ I expected that the use of ivDde-protected Lys derivative would enable ready introduction of various bioactive ligands into the SEAL-tag in late step of the synthesis.

ivDde-protected Lys derivative **9** was successively incorporated by standard Fmoc SPPS protocol using the DIC/HOBt system on a NovaSyn[®] TGR resin. After coupling of the Fmoc-Gly-SEAlide-OH **8** using HATU and DIPEA, the DIC/HOBt system was used to incorporate Fmoc-miniPEG[™] and biotin. The resulting resin was treated with 2% (v/v) hydrazine hydrate in DMF, and indomethacin was condensed on the resulting ivDde-deprotected resin using the DIC/HOBt protocol. The resin-bound SEAL-tag was finally treated with TFA-Et₃SiH-H₂O (95:2.5:2.5 [v/v]) to generate the desired SEAL-tag **13**.



Scheme 7. Synthesis of SEAL-tag **13**.

1. 3 Conclusion

I developed a novel affinity-based labeling reagent, SEALide-based labeling tag (SEAL-tag). The SEAL-tag is readily prepared using standard Fmoc-based solid-phase protocol. For the labeling of hCA and COX-1, facile synthesis of the SEAL-tag bearing benzenesulfonamide (for hCA) or indomethacin (for COX-1) as a specific ligand was achieved.

Chapter 2 Labeling of target proteins using SEAL-tag

2.1 Optimization of the structure of the SEAL-tag

Initially, I determined a suitable length of the mini-PEG linker for the most efficient labeling of hCA1. FTC-modified SEAL-tags **7a-c** bearing various repeats of the mini-PEG unit were prepared and incubated with commercially available hCA1 (10 μ M) at 37 °C in 50 mM HEPES buffer (pH 7.2).^{4(h)} Aliquots of the different reaction mixtures containing FTC-labeled hCA1 were sampled after 72 h incubation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorescence gel imaging (FI). So far examined here, incubation using **7a** (n=0 for the chain length) afforded maximal fluorescence intensity (Figure 5b, lane 1). However, this is not always the case for other protein materials, because suitable fitting of SEAL-tags with ligand-binding cavities is crucial for efficient labeling.

To monitor the progress of hCA1 labeling, SEAL-tag **7a** was incubated with hCA1 at 37 °C in 50 mM HEPES buffer (pH 7.2) and aliquots of different reaction mixture containing FTC-labeled hCA1 were collected every 24 h. The collected samples were analyzed by SDS-PAGE followed by FI. The results revealed that the labeling of hCA1 with **7a** reached a plateau within 48 h (Figure 5c).

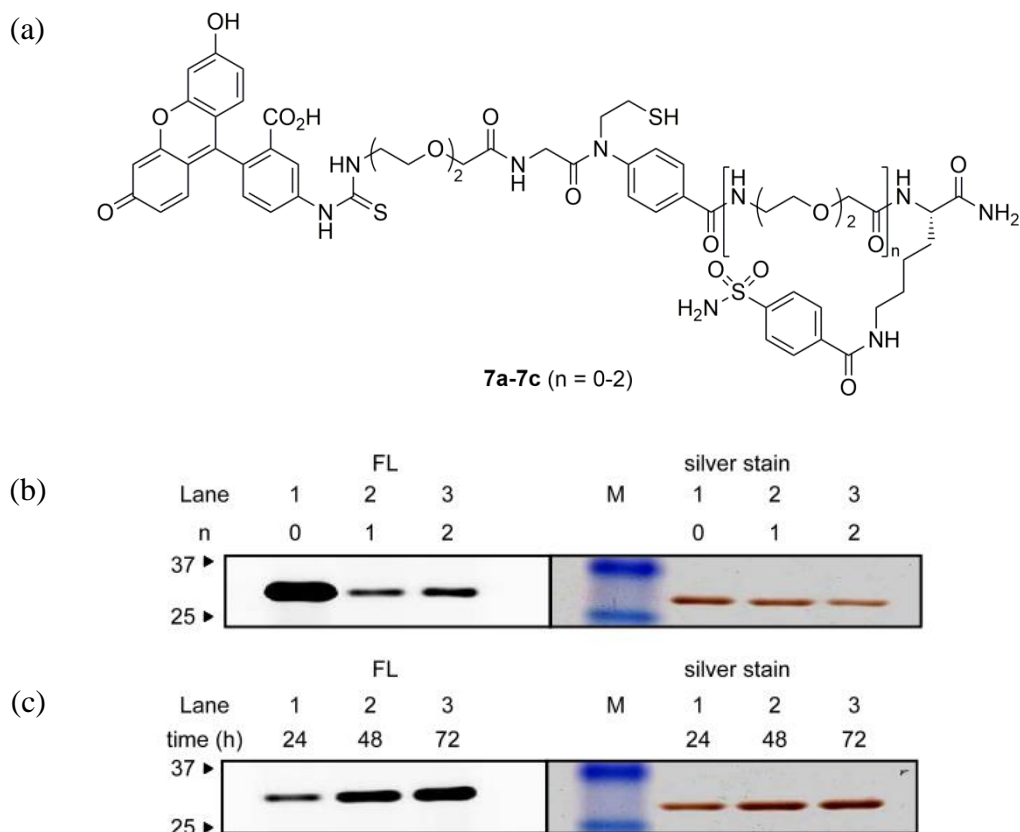


Figure 5. Optimization of the structure of the SEAL-tag. (a) Chemical structures of SEAL-tag **7a-7c**. (b) SDS-PAGE analysis of the hCA1-labeling with SEAL-tag **7a-7c**. The gel was analyzed by in-gel fluorescence imaging (left, FL) and stained using silver staining protocol (right, silver stain). Reaction conditions: purified hCA1 (10 μ M) was incubated with SEAL-tag **7a**, **7b**, or **7c** (100 μ M) in 50 mM HEPES buffer (pH 7.2) at 37 $^{\circ}$ C for 72 h. (c) Time-course analysis of the hCA1 labeling with SEAL-tag **7a** using SDS-PAGE. The gel was analyzed by in-gel fluorescence imaging (left, FL) and stained using silver staining protocol (right, silver stain). Reaction conditions: purified hCA1 (10 μ M) was incubated with SEAL-tag **7a** in 50 mM HEPES buffer (pH 7.2) at 37 $^{\circ}$ C for the specified time. (M: molecular-weight marker)

I also established whether SEAL-tags site-specifically labeled nucleophilic amino acids. In this experiment, a dual-modified SEAL-tag **14** containing biotin and FTC group was used for labeling of hCA1, because the labeled and unlabeled hCA1 proteins could be individually detected by SDS-PAGE (Figure 6). Purified hCA1 (10 μ M) was incubated with SEAL-tag **14** (100 μ M) at 37 $^{\circ}$ C in 50 mM HEPES buffer (pH 7.2) for 48 h. The labeled hCA1 was isolated from protein mixture containing unlabeled hCA1 by SDS-PAGE, and digested by trypsin in the gel.¹⁹ The resulting digested peptide fragments were extracted from the gel and analyzed by nanoLC-MS/MS. The results revealed that Lys137 (Figure 7c), which is located in close proximity to the ligand-binding site, was selectively labeled with the reporter (Figure 7b).^{12(a)}

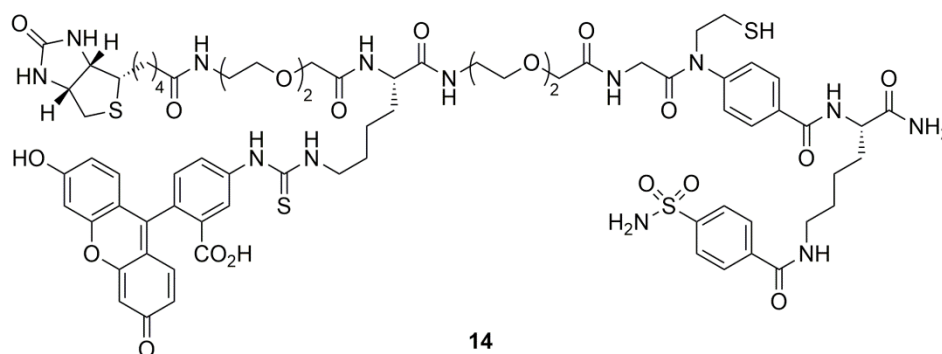


Figure 6. Chemical structure of SEAL-tag **14**, bearing FTC and biotin as a reporter.

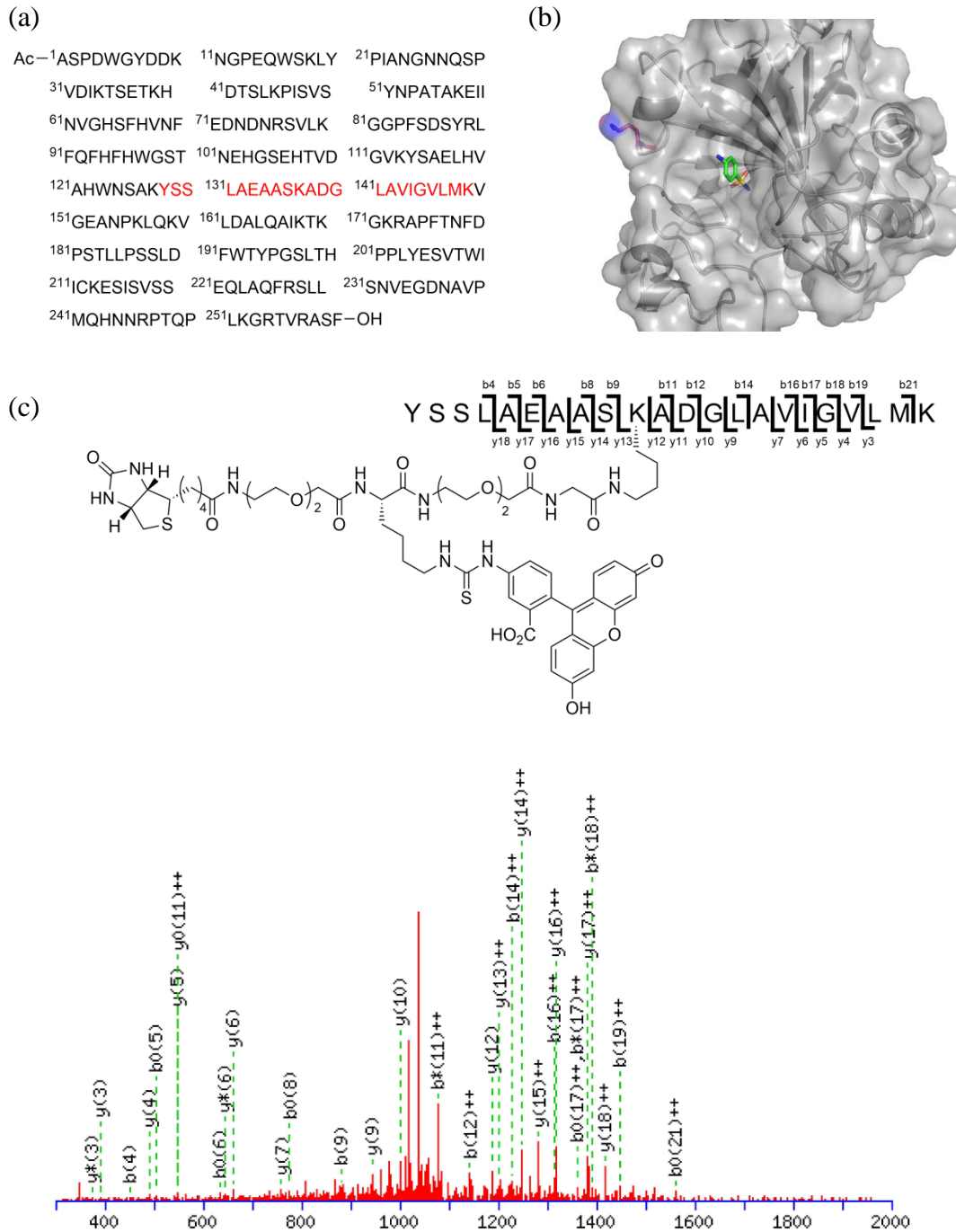


Figure 7. Mass spectral analysis of the labeling site of hCA1. (a) The primary sequence of hCA1. (b) Crystal structure of hCA1 complexed with 3-actoxymercuri-4-aminobenzenesulfonamide (PDB ID: 1CZM). The amino acid (Lys 137) modified by SEAL-tag **14** is highlighted by a colored stick model. (c) nanoLC-MS/MS analysis of the fragment labeled at Lys137.

2.2 Selective labeling of target proteins

Subsequently, I evaluated the target labeling using SEAL-tags. A protein mixture containing hCA1, enolase,²⁰ ovalbumin (OVA)²¹ and glutathione S-transferase (GST)²² (10 μ M each) was incubated with SEAL-tag **7a** (100 μ M) at 37 °C in 50 mM HEPES buffer (pH 7.2) for 48 h. Excess amount of the labeling reagent in the samples was removed by ultrafiltration,²³ and the resulting samples were analyzed by SDS-PAGE followed by FI. The analysis showed that a strong fluorescent band corresponding to the FTC-labeled hCA1 was presented (Figure 8b). These results therefore demonstrated that I could achieve the selective labeling of a target protein in a protein mixture using the SEAL-tag system, while leaving the non-target protein unchanged.

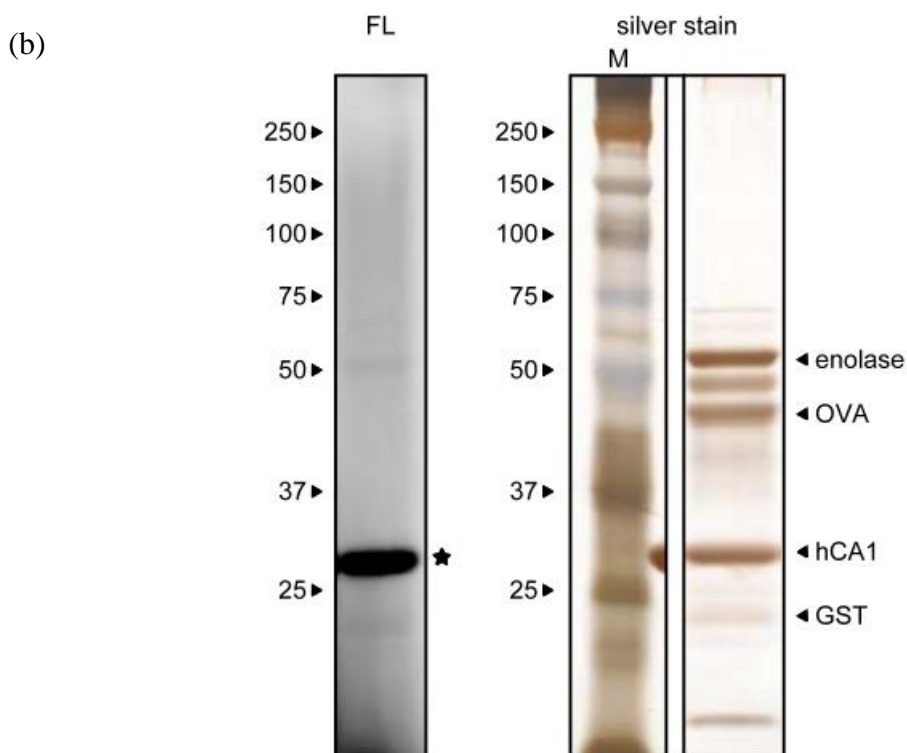
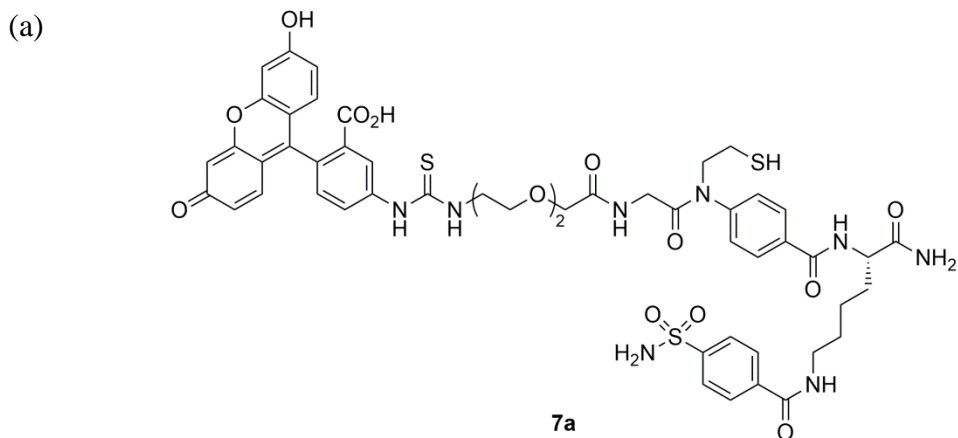


Figure 8. Selective labeling of hCA1 in a protein mixture. (a) Chemical structure of SEAL-tag **7a**. (b) SDS-PAGE analysis of the selective labeling of hCA1 with SEAL-tag **7a** in a protein mixture consisting of four different proteins. The gel was analyzed by in-gel fluorescence imaging (left, FL) and stained using silver staining protocol (right, silver stain). Reaction conditions: SEAL-tag **7a** (100 μ M) was incubated in 50 mM HEPES buffer (pH 7.2) containing four different proteins (10 μ M each; enolase, GST, hCA1, and OVA) at 37 $^{\circ}$ C for 48 h.

To verify that the versatility of the selective labeling of target proteins using the SEAL-tag resulted, I attempted to label COX-1 in a protein mixture using SEAL-tag containing indomethacin (IM) as a specific ligand for COX-1. Three proteins consisting of bovine serum albumin (BSA),²⁴ COX-1, and enolase (1.4 μ M each) with SEAL-tag **13** (14 μ M) were incubated in 80 mM Tris·HCl buffer (pH 8.0) at 4 °C for 48 h. Notably, this experiment was conducted at 4 °C because of instability of COX-1 at 37 °C in aqueous buffer without a stabilizer.²⁵ The incubation mixture was subsequently analyzed by SDS-PAGE, which revealed the presence of a single biotin-labeled protein using a streptavidin-horseradish peroxidase conjugate (SAv-HRP). Figure 9b shows a single band corresponding biotinylated-COX-1 that was detected at approximately 70 kDa. These results showed that the SEAL-tags could be used for the various target proteins of bioactive compound labeling in protein mixtures.

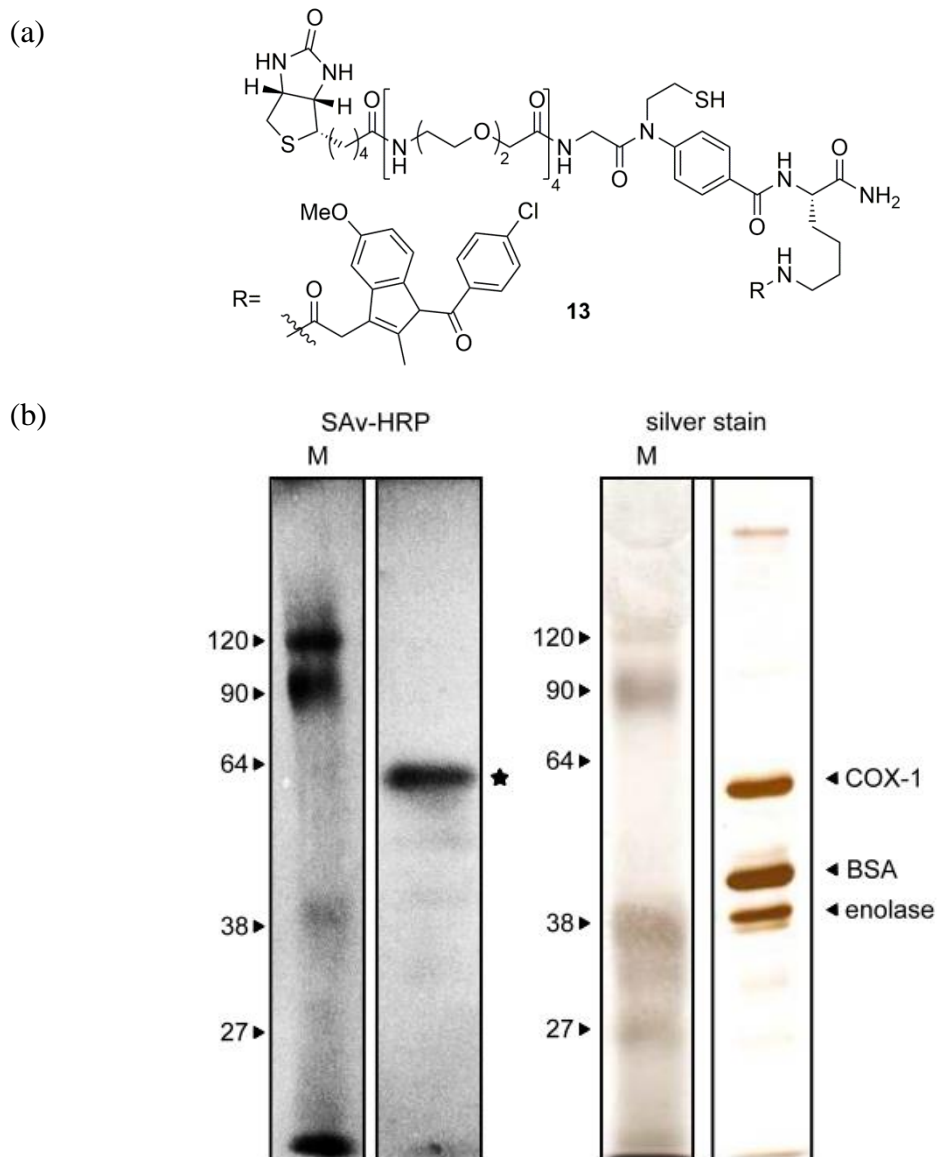


Figure 9. Selective labeling of COX-1 in a protein mixture. (a) Chemical structure of SEAL-tag **13**, bearing indomethacin (IM) as a specific ligand for COX-1 labeling. (b) Western blotting analysis of biotin-labeled protein using the biotin–streptavidin-HRP method (left, SAv-HRP), and all proteins in the gel were visualized by silver staining (right, silver stain). Reaction conditions: SEAL-tag **13** (14 μM) was incubated in 80 mM Tris·HCl buffer (pH 8.0) containing three different proteins (1.4 μM each; BSA, COX-1, and enolase) at 4 $^{\circ}\text{C}$ for 48 h.

2.3 Selective labeling of endogenous hCA

We also investigated the application of this strategy for the labeling of endogenous hCA in human red blood cells (RBCs) or the cell lysate using the SEAL-tag.^{11(b)}

First, we performed the selective labeling of hCA in the lysate of RBCs. The lysate of RBC was incubated with SEAL-tag **14** at 37 °C for 48 h. The incubation mixture was analyzed by SDS-PAGE followed by detection with SA_v-HRP, which revealed the presence of a single band corresponding to biotin-modified protein. Figure 10b shows the single band corresponding biotin-modified hCA at approximately 29 kDa. These results showed that the endogenous target protein that was present in minute amounts in cells could be labeled and visualized using the SEAL-tag.

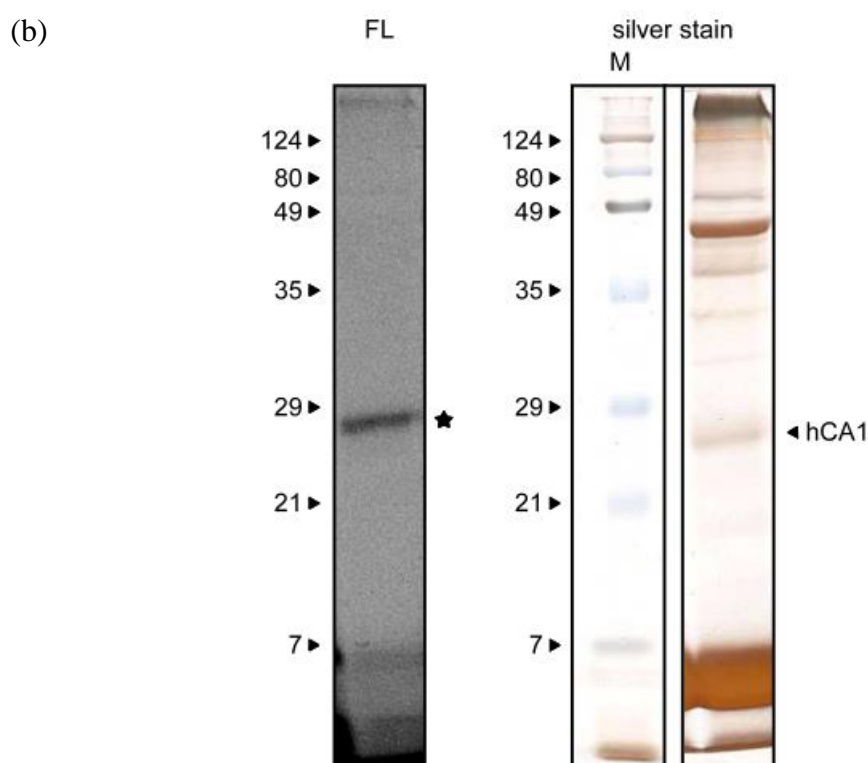
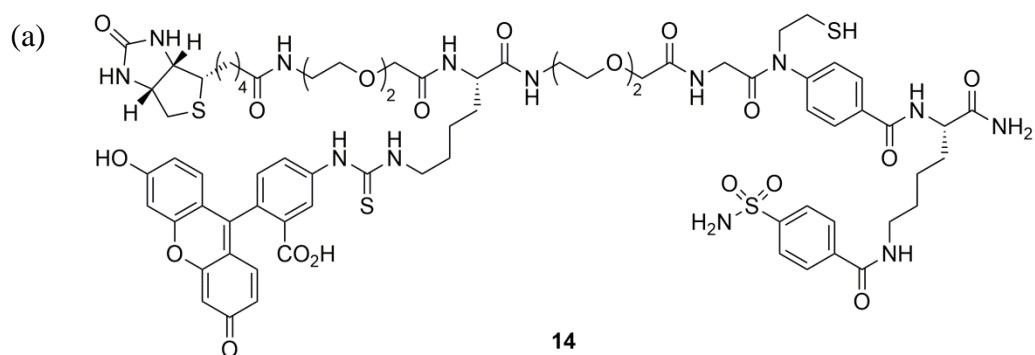


Figure 10. Selective labeling of hCA in lysate of RBC. (a) Chemical structure of SEAL-tag **14**. (b) SDS-PAGE analysis of the FTC-labeled protein in RBC lysate with SEAL-tag **14**. The gel was analyzed by in-gel fluorescence imaging (left, FL) and stained using silver staining protocol (right, silver stain). Reaction conditions: SEAL-tag **14** (100 μ M) was incubated in RBC lysate diluted with 50 mM HEPES buffer (pH 7.2) at 37 $^{\circ}$ C for 48 h.

Next, I designed and synthesized a new SEAL-tag for the labeling of a target protein in cells. In general, low-molecular-weight hydrophobic compounds could more efficiently permeate through cell membranes than high-molecule-weight hydrophilic compounds.²⁶ With this in mind, we designed and synthesized SEAL-tag **15** to provide improved membrane permeability and enhanced labeling efficiency towards intracellular proteins compared with SEAL-tag **7a**. Notably, the characteristics of SEAL-tag **15** are (1) a lower molecular weight than SEAL-tag **7a**; and (2) the absence of hydrophilic mini-PEG linker (Figure 11a). Using SEAL-tag **15**, I also attempted to label hCA in RBCs. RBCs with SEAL-tag **15** were incubated in HEPES-buffered saline (HBS) for 48 h at 37 °C, before being washed three times with fresh HBS. The resulting RBCs were lysed and the resulting lysate was centrifuged for separating soluble fraction. The soluble fraction was collected and analyzed by SDS-PAGE followed by detection with SA_v-HRP. A single band corresponding to biotinylated-hCA at approximately 37 kDa was detected (Figure 11b lane 1).

In the presence of ethoxzolamide (EZA), which is a strong binder against the SA-binding pocket of hCA, I did not notably observe the SA-dependent labeling of hCA (Figure 11b lane 2). These results therefore demonstrated that the use of hydrophobic and low molecular weight SEAL-tag bearing a SA ligand could be used as an efficient strategy for the intracellular labeling of hCA in a ligand-dependent fashion.

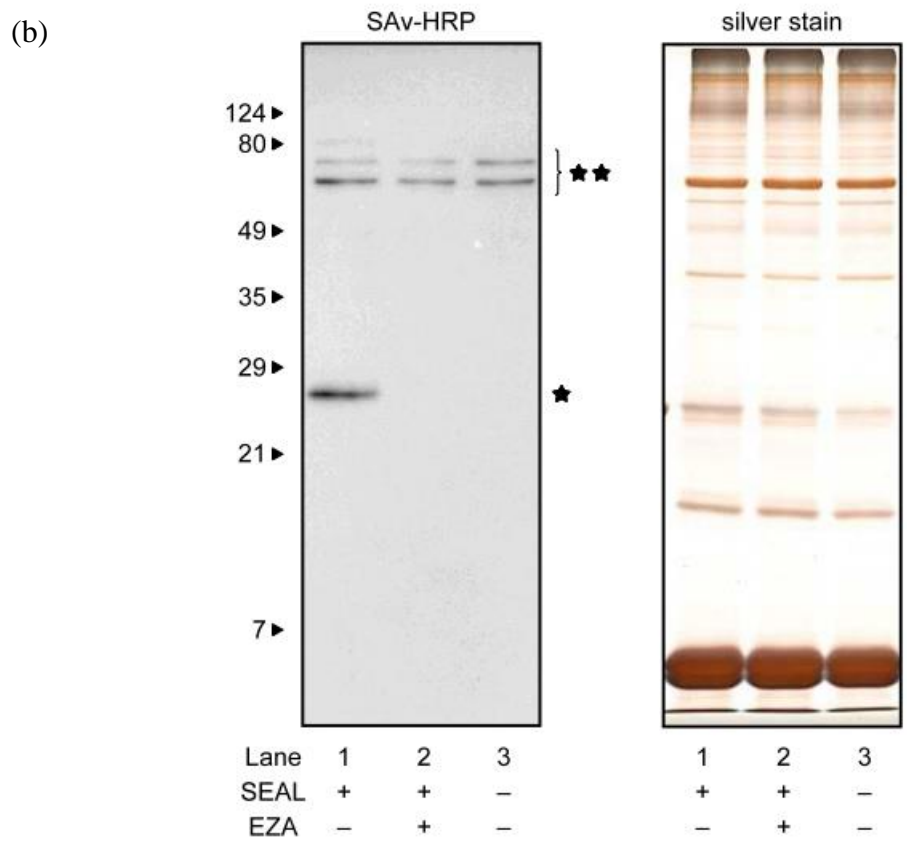
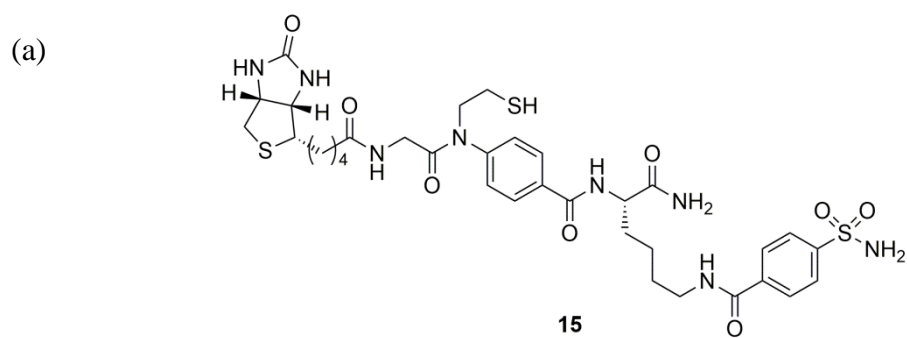


Figure 11. Selective labeling of hCA in RBCs. (a) Chemical structure of SEAL-tag **15**. (b) Western blotting analysis of the biotin-labeled protein in RBCs using the biotin–streptavidin-HRP method (left, SAV-HRP), and all proteins in the gel were visualized by silver staining (right, silver stain). The band with the single asterisk (*) correspond to hCA, whereas those with the double asterisk (**) correspond to non-specific detected with streptavidin-HRP band. Reaction conditions: RBCs and SEAL-tag **15** (100 μ M) were incubated in HEPES-buffered saline (HBS, pH 7.2) at 37 $^{\circ}$ C for 48 h.

2.4 Elucidation of the mechanism of this SEAL-mediated labeling process

I examined whether N–S acyl transfer of SEALide unit is necessary for protein labeling process using the SEAL-tag **16** bearing S-protected SEALide unit. Mixture of COX-1 and SEAL-tag **13** or **16** was subjected to labeling reaction in 80 mM Tris·HCl buffer (pH 8.0) for 48 h at 4 °C and the reaction liquids were collected and analyzed by SDS-PAGE. Using SEAL-tag **13** for the labeling reaction, I detected a single band corresponding to biotinylated-COX-1 at approximately 70 kDa. On the other hand, no band was detected using SEAL-tag **16**, and thereby these results indicated that the N–S acyl transfer reaction was a critical step in the labeling process of proteins.

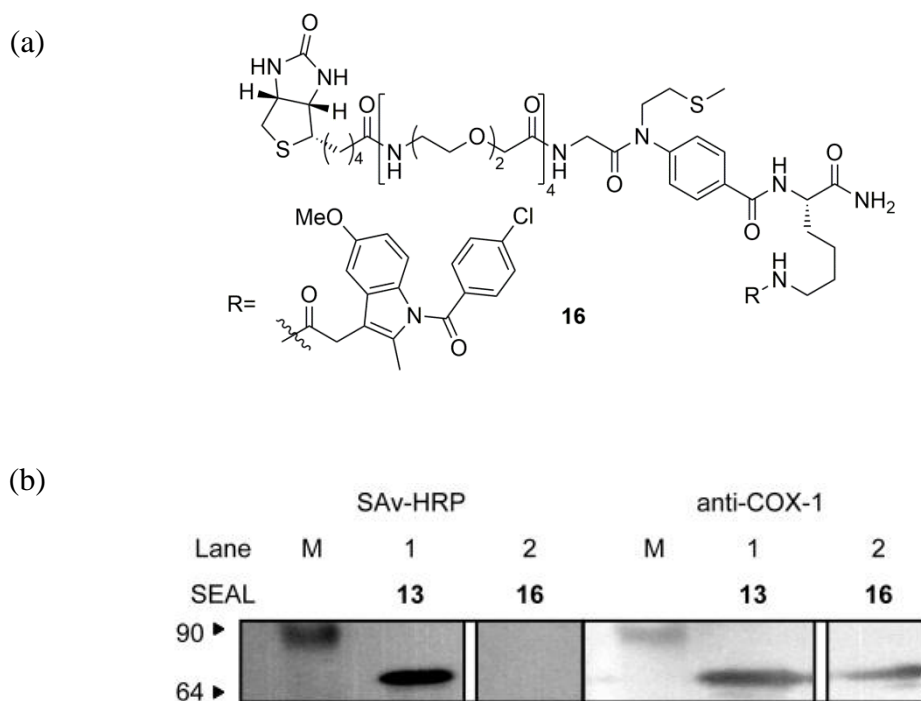


Figure 12. Elucidation of the mechanism for the SEAL-tag-mediated labeling of the protein. (a) Chemical structure of SEAL-tag **16**. (b) Western blotting analysis of biotin-labeled COX-1, detected with streptavidin-HRP (left, SAv-HRP), anti-COX-1 antibody (right, anti-COX1). Reaction conditions: SEAL-tag **13** or **16** (14 μ M) was incubated in 80 mM Tris·HCl buffer (pH 8.0) containing purified-COX-1 (1.4 μ M) at 4 °C for 48 h.

Finally, I attempted to address the issue of whether the surface of the protein acts as an acid-base cluster catalyst to enhance the N–S acyl transfer reaction. In the presence of a large amount of nucleophile, I confirmed that SEALide remained intact in the absence of an acid-base catalyst in an acylation reaction. The SEAL-tag (10 μM) was incubated with butylamine (1 M) in 50 mM HEPES buffer (pH 7.2) at 37 $^{\circ}\text{C}$ and aliquots of incubated solution were collected every 24 h and analyzed by high performance liquid chromatography (HPLC). The analysis showed that the SEAL-tag did not react with amine. Although I have not yet collected any direct evidence to suggest that the N–S acyl transfer reaction is accelerated by acid-base residues on protein surfaces, I have collected the following anecdotal evidence: (1) SEAL-tag **15** remained intact in the presence of a large amount of amine in HEPES buffer. (2) SEAL-tags immobilized on protein surfaces in a ligand-dependent manner could modify Lys- ϵ -amino groups, which would present themselves in a stoichiometric ratio relative to the SEAL-tag under the labeling conditions.

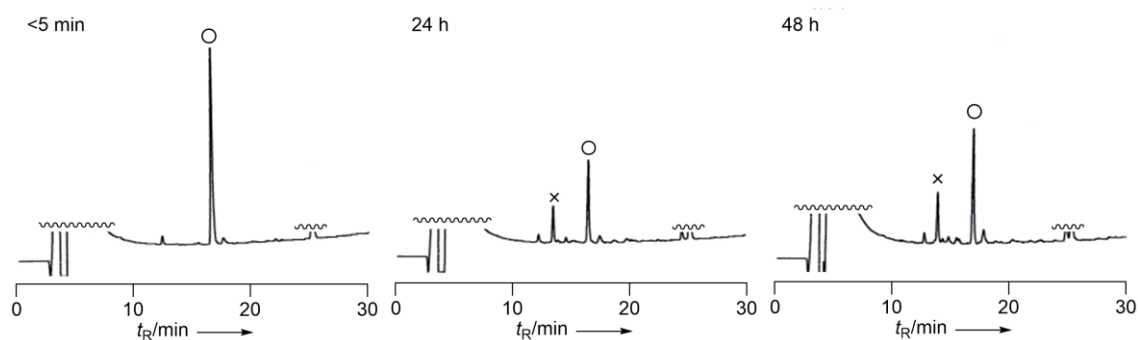


Figure 13. HPLC analysis of the SEAL-tag **15** (○, 10 μM) with butylamine (1 M) in 50 mM HEPES buffer (pH 7.2) at 37 $^{\circ}\text{C}$ for 5 min > (left), 24 h (middle), or 48 h (right) of incubation. × denotes oxidated SEAL-tag **15**; however, the aminolysis product was not detected.

2. 5 Conclusion

Ligand-dependent selective labeling of hCA in a protein mixture or human red blood cells was achieved. NanoLC-MS/MS analysis of the labeled hCA revealed that modification site-specifically occurred at Lys137 located in close proximity to the ligand-binding site. Furthermore, the SEAL-tag bearing indomethacin as specific ligand for COX-1 also specifically modified COX-1 in a protein mixture. These results clearly indicated that the newly developed SEAL-tag served as ligand-mediated labeling reagent.

Chapter 3 Conclusions

On the basis of the unique chemical nature of the SEALide unit, in that the electrophilically inert amide-type SEALide is converted to the corresponding active thioester-type SEALide through N-S acyl transfer catalyzed by phosphate salts as a general acid-base catalyst, the SEALide-based labeling tag (SEAL-tag) as a novel ligand-mediated-labeling reagent was developed. Synthesis of the inert amide-type SEAL-tag was efficiently effected by incorporation of the SEALide moiety into the SEAL-tag using Fmoc-SPPS protocols. Ligand-mediated concentration of the SEAL-tag on target proteins with the inert amide part activated through N-S acyl transfer possibly catalyzed by the acid-base cluster on the surface of the target proteins enabled the specific labeling of the target protein. As model cases, specific labeling of hCA and COX-1 using the SEAL-tags-bearing ligands specific for the target proteins was achieved.

There remains an unsolved issue as to whether the acid-base cluster on the protein surface is actually involved in the activation of the inert amide-type SEALide unit, whereas indirect evidence for such the involvement was obtained. At this stage, the direct evidence rationalizing the activation has yet to be acquired.

In addition to research to obtain the direct evidence, identification of a target protein using a SEAL-tag bearing a target-unknown ligand should be conducted to expand the utility of the SEAL-tag in chemical biology fields.

Experimental Section

General Methods

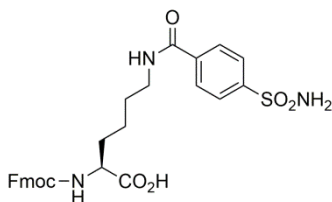
All reactions of small molecules were carried out under a positive pressure of argon. For column chromatography, silica Gel 60 N (spherical, neutral, Kanto Chemical Co. Inc.) was employed. Thin layer chromatography was performed on precoated plates (0.25 mm, silica gel Merck Kiesegel 60F245). Mass spectra were recorded on a Waters MICROMASS[®] LCT PRIME[™] (ESI-TOF) or a Bruker Esquire2000T (ESI-Ion trap). NMR spectra were recorded using a Bruker AV400N at 400 MHz frequency for ¹H and a JEOL JNM-AL300 at 75 MHz frequency for ¹³C in methanol-d₄. Chemical shifts are calibrated to the solvent signal. For HPLC separations, a Cosmosil 5C₁₈-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1 mL/min) or a 5C₁₈-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% (v/v) TFA aqueous solution (solvent A) and 0.1% (v/v) TFA in MeCN (solvent B) was used for HPLC elution. Fluorescence and chemiluminescent signals were detected with LAS-4000mini (Fujifilm).

Chapter 1

Synthetic of SEAL-tags

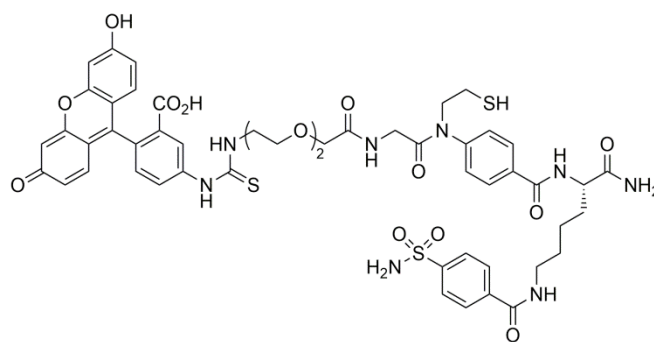
SEAL-tags were prepared by Fmoc-solid phase protocols using Fmoc-miniPEG™, Fmoc-Gly-SEAlide-OH,^{9(h)} Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH (for hCA1 labelling) and Fmoc-L-Lys(ivDde)-OH (for COX-1 labelling).

Synthesis of Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH (3)



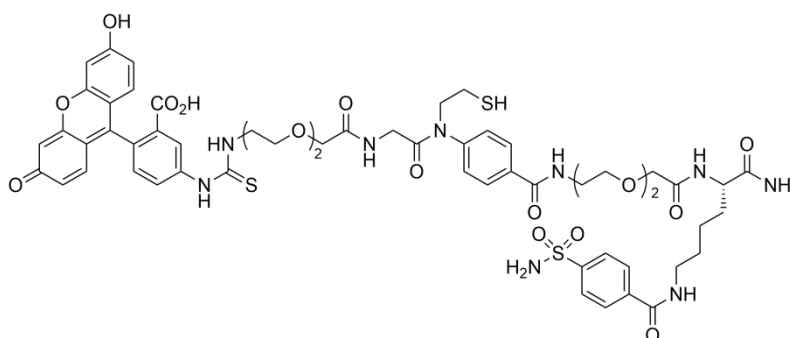
To a stirred solution of Fmoc-L-Lys-OH (346 mg, 0.940 mmol) in DMF (2.5 mL) were added 4-sulfamoylbenzoic acid N-hydroxysuccinimide ester (365 mg, 1.22 mmol) and DIPEA (486 μ L, 2.82 mmol), and the reaction mixture was stirred for 3.5 h. The mixture was concentrated in vacuo, and the obtained residue was dissolved in EtOAc. The organic layer was washed with 5% (w/v) aqueous citric acid, H₂O and brine, dried over MgSO₄ and concentrated in vacuo. The crude product was purified by column chromatography (CHCl₃/MeOH = 9:1) and 427 mg of Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH (**3**) (0.754 mmol, 80%) was obtained as a white powder: $[\alpha]_D^{19}$ 2.79 (c 0.64, CH₃OH); ¹H NMR (400 MHz, CD₃OD) δ = 1.45-1.58 (m, 2H), 1.58-1.81 (m, 3H), 1.91 (m, 1H), 3.42 (t, J = 7.0 Hz, 2H), 4.18 (dd, J = 4.4, 9.2 Hz, 1H), 4.24 (t, J = 6.8 Hz, 1H), 4.34 (dd, J = 6.8, 10.7 Hz, 1H), 4.39 (dd, J = 6.8, 10.7 Hz, 1H), 7.31 (t, J = 7.6 Hz, 1H), 7.40 (t, J = 7.0 Hz, 2H), 7.68 (d, J = 7.6 Hz, 2H), 7.70 (d, J = 7.6 Hz, 1H), 7.81 (d, J = 7.6 Hz, 1H), 7.95 (d, J = 8.8 Hz, 2H), 7.98 (d, J = 8.8 Hz, 2H), ¹³C NMR (75 MHz, CD₃OD): δ = 24.4, 29.9, 32.3, 40.9, 55.2, 67.9, 120.9, 126.2, 127.3, 128.1, 128.8, 129.0, 142.6, 145.2, 145.3, 147.6, 158.7, 168.8, 175.9; HRMS (ESI-TOF) m/z calcd for C₂₈H₂₉N₃O₇NaS ([M+Na]⁺) 574.1624, found 574.1625)

Synthesis of SEAlide-based Labeling Tag (SEAL-tag) (7a)



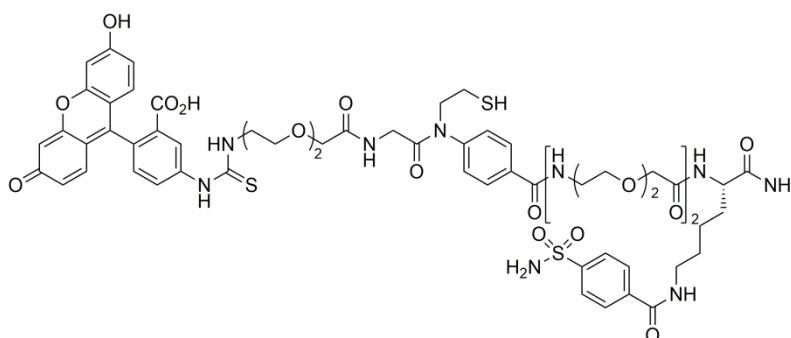
Synthesis of SEAL-tag **7a** was performed by using Fmoc-based solid-phase protocols on NovaSyn[®] TGR resin (loading: 0.22 mmol/g, 45 mg, 0.010 mmol). Coupling of Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH (17 mg, 0.030 mmol) with the aid of DIC (4.6 μ L, 0.030 mmol)/HOBt-H₂O (4.6 mg, 0.030 mmol) in DMF was performed, and treatment with 20% (v/v) piperidine in DMF was used for the removal of Fmoc protection to give a resin. Fmoc-Gly-SEAlide-OH (1.4 mg, 0.020 mmol) was coupled on the resulting resin by HATU (7.2 mg, 0.019 mmol) and DIPEA (3.3 μ L, 0.019 mmol) in DMF at room temperature in DMF for 2 h. After deprotection of Fmoc group with 20% (v/v) piperidine in DMF, coupling of Fmoc-mini-PEGTM (12 mg, 0.030 mmol) in DMF were conducted by Fmoc-based solid-phase protocols. To obtained resin were added fluorescein-5-isothiocyanate (FITC, 9.7 mg, 0.025 mmol) and DIPEA (43 μ L, 0.025 mmol) in DMF, and the reaction was performed for 2 h. The resulting completed resin was treated with TFA-TES-H₂O (95:2.5:2.5, 3.3 mL) for 2h. After filtration of the resin, the filtrate was concentrated to provide the crude product. The obtained crude product was purified by preparative HPLC to yield SEAL 1 (1.51 mg, 14%, an yellow lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 20.9 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 30 to 40% over 30 min. MS (ESI-TOF) m/z calcd for C₅₁H₅₄N₈O₁₄S₃ ([M+H]⁺): 1099.3, found 1099.6.

Synthesis of SEAL-tag (7b)



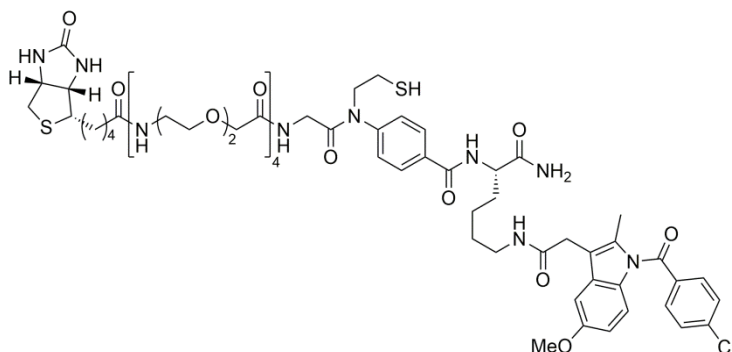
SEAL-tag **7b** was synthesized in a manner similar to that employed in the synthesis of SEAL-tag **7a**. NovaSyn® TGR resin (loading: 0.22 mmol/g, 45.0 mg, 0.01 mmol) was used to yield SEAL-tag **7b** (2.85 mg, 23%, an yellow lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 20.7 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 30 to 40% over 30 min. MS (ESI-TOF) m/z calcd for $C_{57}H_{65}N_9O_{17}S_3$ ($[M+H]^+$): 1244.4, found 1244.8.

Synthesis of SEAL-tag (7c)



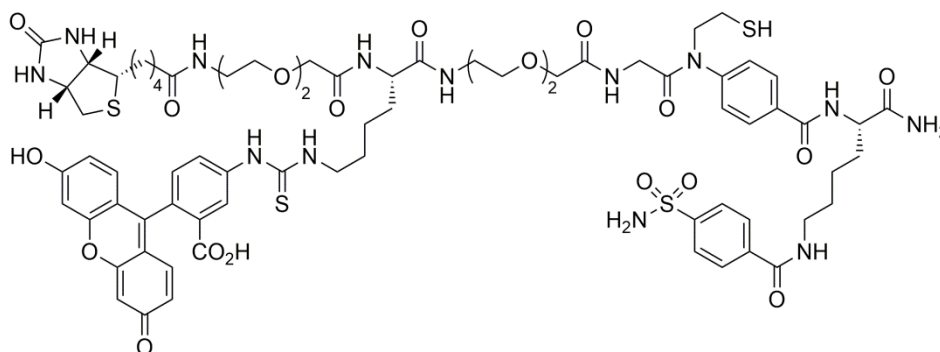
SEAL-tag **7c** was synthesized in a manner similar to that employed in the synthesis of SEAL-tag **7a**. NovaSyn® TGR resin (loading: 0.22 mmol/g, 45.0 mg, 0.01 mmol) was used to yield SEAL **7c** (2.90 mg, 21%, an yellow lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 20.4 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 29 to 39% over 30 min. MS (ESI-TOF) m/z calcd for $C_{63}H_{76}N_{10}O_{20}S_3$ ($[M+H]^+$): 1389.5, found 1389.9.

Synthesis of SEAL-tag (13)



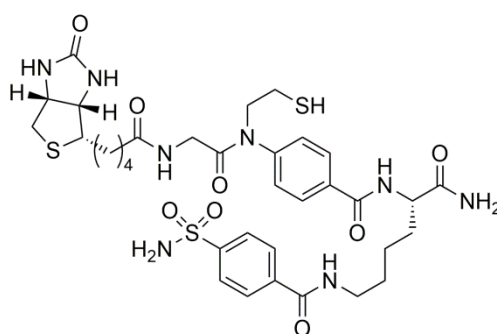
SEAL-tag **13** was synthesized in a manner similar to that employed in the synthesis of SEAL-tag **7a** on NovaSyn® TGR resin (loading: 0.25 mmol/g, 200 mg, 0.050 mmol). Coupling of Fmoc-L-Lys(ivDde)-OH (86 mg, 0.15 mmol) with the aid of DIC (23 μ L, 0.15 mmol)/HOBt·H₂O (20 mg, 0.15 mmol) in DMF was performed, and treatment with 20% (v/v) piperidine in DMF was used for the removal of Fmoc protection to give a resin. Fmoc-Gly-SEAlide-OH (72 mg, 0.10 mmol) was coupled on the resulting resin by using HATU (36 mg, 0.095 mmol) and DIPEA (17 μ L, 0.095 mmol) in DMF at room temperature for 2 h. After deprotection of Fmoc group of the resulting resin with 20% (v/v) piperidine in DMF, stepwise coupling of Fmoc-miniPEG™ (58 mg, 0.15 mmol) in DMF and biotin (61 mg, 0.25 mmol) in DMF-DMSO (3:1 (v/v)) were conducted by Fmoc-based solid-phase protocols. The ivDde group on the Lys residue was removed by treatment with 2% (v/v) hydrazine monohydrate in DMF for 17 h, and the generated amine and indomethacin (54 mg, 0.15 mmol) in DMF were conducted by Fmoc-based solid-phase protocols. The resulting completed resin was treated with TFA-TES-H₂O (95:2.5:2.5, 16 mL) for 2h. After filtration of the resin, the filtrate was concentrated to provide a crude product. The obtained crude product was purified by preparative HPLC to yield SEAL-tag **13** (0.38 mg, 0.6%, a white lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 25.7 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 38 to 48% over 30 min. MS (ESI-TOF) m/z calcd for C₇₀H₉₉ClN₁₂O₂₀S₂ ([M+H]⁺): 1527.6, found: 1527.9.

Synthesis of SEAL-tag (14)



SEAL-tag **14** was synthesized in a manner similar to that employed in the synthesis of SEAL-tag **13**. NovaSyn® TGR resin (loading: 0.22 mmol/g, 227 mg, 0.050 mmol) was used to yield SEAL **14** (22.0 mg, 28%, an yellow lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 19.5 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 28 to 38% over 30 min. MS (ESI-TOF) *m/z* calcd for C₇₉H₁₀₂N₁₄O₂₃S₃ ([M+2H]²⁺): 799.6, found 799.5.

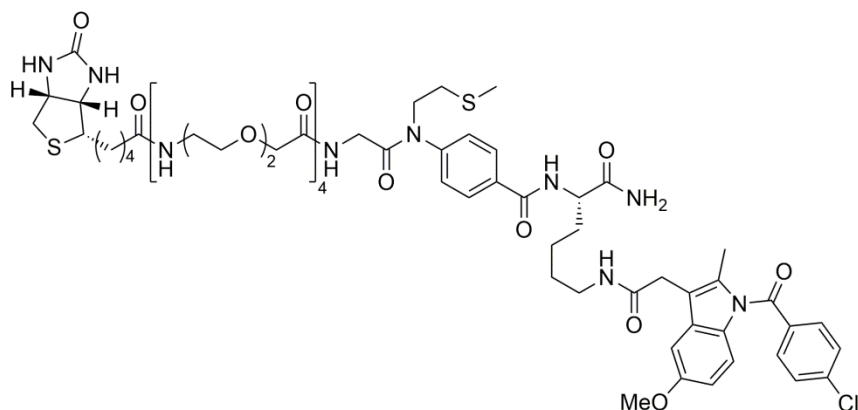
Synthesis of SEAL-tag (15)



SEAL-tag **15** was synthesized in a manner similar to that employed in the synthesis of SEAL-tag **7a**. NovaSyn® TGR resin (loading: 0.24 mmol/g, 208 mg, 0.050 mmol) was used to yield SEAL **6** (13 mg, 33%, a white lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 16.8 min. Preparative HPLC conditions: linear

gradient of solvent B in solvent A, 23 to 33% over 30 min. MS (ESI-TOF) m/z calcd for $C_{34}H_{46}N_8O_8S_3$ ($[M+H]^+$): 791.3, found: 791.1.

Synthesis of SEAL-tag (16)



To a solution of SEAL-tag **13** (10.6 mg, 6.87 μmol) in DMF was added MeI (69.0 μL , 1.11 mmol) and DIPEA (69.0 μL , 6.87 μmol) with stirring at room temperature for 1 h. The reaction mixture was purified by preparative HPLC to yield SEAL-tag **16** (4.43 mg, 42%, a white lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 25.9 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 35 to 49% over 30 min. MS (ESI) m/z calcd for $C_{71}H_{101}ClN_{12}O_{20}S_2$ ($[M+H]^+$): 1541.7, found 1541.7.

Chapter 2

Optimization of the structure of SEAL-tags

Purified human carbonic anhydrase 1 (hCA1) was purchased from SIGMA-Aldrich and used without further purification. The hCA1 (10 μ M) with **7a-c** (100 μ M) was incubated at 37 °C in 50 mM HEPES buffer (pH 7.2). At every 24 h, the reaction aliquots were subjected to ultrafiltration (14,000 g, 15 min, himac CT 15RE (Hitachi Koki Co., Ltd.)) using Amicon[®] Ultra-0.5 Centrifugal Filter Devices (Merck Millipore) and the concentrate was mixed with the same volume of 2 \times SDS sample loading buffer followed by heating at 100 °C for 5 min. The heated reaction mixtures were analyzed using SDS-PAGE in 14% in polyacrylamide gels. For the chemiluminescence imaging of the biotinylated proteins, the proteins were transferred to Amersham Hybond-P PVDF Membrane (GE Healthcare) and detected with a streptavidin-horseradish peroxidase conjugate (SAv-HRP, GE Healthcare) and ECL plus Western Blotting Detection System (GE Healthcare). For silver staining of all proteins, proteins in a gel were stained with Silver Stain KANTOIII (KANTO CHEMICAL CO., INC.).

To determine that the labelling of hCA1 was site-specifically occurred, purified hCA1 (10 μ M) was labelled using **14** (100 μ M) according to the method described above. The labelled hCA1 was subjected to SDS-PAGE in 14% in polyacrylamide gel and stained with CBB Stain One (Nacalai tesque). The gel containing of the labelled hCA1 with trypsin was incubated at 37 °C in Tris·HCl buffer (pH 8.0) for 20 h. The digested labelled-hCA1 was analyzed on nanoLC-MS/MS.

LC: UltiMate 3000 RSLCnano system (Thermo Fisher Scientific Inc.)

MS: Orbital Elite (Thermo Fisher Scientific Inc.)

Column: Acclaim PepMap RSLC Nano Column (75 μ m \times 15 cm, Thermo Fisher Scientific Inc.)

Solvent A: 0.1% formic acid aq.; solvent B 0.08% formic acid/80% MeCN aq.

Selective labeling of hCA1 in a protein mixture

A protein mixture containing of enolase, GST, hCA1 and OVA (10 μ M each, purchased from SIGMA) with **7a** (100 μ M) was incubated in 50 mM HEPES buffer (pH 7.2) at 37 °C for 48 h. The reaction mixture was subjected to ultrafiltration and the concentrated mixture was blended with the same volume of 2 \times SDS sample loading

buffer followed by heating at 100 °C for 5 min. The heated samples were applied to 14% SDS-PAGE, and analyzed by *in-gel* fluorescence imaging and silver-staining.

Selective labeling of COX-1 in a protein mixture

As a protein mixture, BSA purchased from SIGMA, cyclooxygenase 1 (COX-1) purchased from Cayman Chemical and enolase (1.4 μM each) was used. The mixture of proteins with **13** was incubated in 80 mM Tris-HCl buffer (pH 8.0) at 4 °C for 48 h. The reaction mixture was analyzed using SDS-PAGE in 10% polyacrylamide gels followed by chemiluminescence imaging according to the methods described above. For silver staining of all proteins, proteins in a gel were stained with Silver Stain KANTO III.

Selective labeling of endogenous hCA in RBC lysate

Human red blood cells (hRBCs) were purchased from BizCom Japan, Inc., and purified by the use of a centrifugation (3,000 g, 15 min). Purified hRBCs were resuspended in 50 mM HEPES buffer (pH 7.2) and lysed by sonication for 15 min at 0 °C. The resulting lysate and **14** was incubated with at 37 °C for 48 h. The reaction mixture was subjected to ultrafiltration and the concentrated mixture was blended with the same volume of 2 × SDS sample loading buffer followed by heating at 100 °C for 5 min. The heated samples were applied to 14% SDS-PAGE and analyzed by *in-gel* fluorescence imaging and silver-staining.

Selective labeling of endogenous hCA in RBCs

hRBCs purified by the use of a centrifugation (3,000 g, 15 min). Purified hRBCs were resuspended in HEPES-buffered saline (HBS) and incubated with **15** (200 μM) in the absence or presence of ethoxzolamide (EZA, 2 mM) at 37 °C for 48 h. The incubated hRBCs were washed three times with HBS and lysed by sonication. The resulting lysate was centrifuged (12,000 g, 30 min) and the resulting supernatant was collected and separated with 5 by the use of ultrafiltration. The resulting mixture was analyzed using SDS-PAGE in 10% polyacrylamide gels followed by chemiluminescence imaging

according to the methods described above. For silver staining of all proteins, proteins in a gel were stained with Silver Stain KANTOIII.

Elucidation of a mechanism of the labeling using a SEAL-tag

Purified COX-1 (1.4 μM) was incubated with **13** or **16** (14 μM) in 80 mM Tris·HCl buffer at 4 °C for 48 h. The reaction mixtures were subjected to ultrafiltration followed by SDS-PAGE analysis. The biotinylated COX-1 was detected with SAv-HRP using ECL plus Western Blotting Detection System.

The immunodetection of COX-1 was accomplished with COX-1 monoclonal antibody and anti-mouse IgG-HRP conjugate (both Santa Cruz Biotechnology).

To demonstrate of the stability of **15**, butylamine (1 M) and **15** (10 μM) were incubated in 50 mM HEPES buffer (pH 7.2) at 37 °C. At every 24 h, reaction aliquots containing **15** were collected and analyzed by HPLC. Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min.

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I express my deepest gratitude and sincere, wholehearted appreciation to Prof. Akira Otaka (Department of Bioorganic Synthetic Chemistry, Institute of Biomedical Sciences and Graduate School of Pharmaceutical Sciences, Tokushima University) for his kind guidance, constructive support and hearty encouragement provided throughout this study. In addition, I feel honored to have been given the opportunity of being the one to study organic and peptide chemistry from the beginning.

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List of publication

This study was published in the following paper

1. Labelling of endogenous target protein via N–S acyl transfer-mediated activation of *N*-Sulfanylethylanilide

Masaya Denda, Takuya Morisaki, Taiki Kohiki, Jun Yamamoto, Kohei Sato, Ikuko Sagawa, Tsubasa Inokuma, Youichi Sato, Aiko Yamauchi, Akira Shigenaga and Akira Otaka

Org. Biomol. Chem. **2016**, *14*, 6244-6251.

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