Development of N–S-acyl-transfer-based chemical tool for target identification of drug candidates

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Abbreviations

ABP: activity-based probe
Alloc: Allyloxycarbonyl
aq.: aqueous solution
BSA: bovine serum albumin
^{t}Bu: *tert*-butyl
CBB: Coomassie Brilliant Blue
concn.: concentration
DIC: *N*,*N'*-diisopropylcarbodiimide
DIPEA: *N,N*-diisopropylethylamine
DMF: *N*,*N*-dimethylformamide
DMSO: dimethyl sulfoxide
ESI: electrospray ionization
FCG: forward chemical genetics
Fmoc: 9-fluorenylmethoxycarbonyl
FTC: fluorescein thiocarbamoyl
HATU: *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate
HOBt: 1-hydroxybenzotriazole
HPLC: high performance liquid chromatography
HRMS: high resolution mass spectrometry
ivDde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl
LRMS: low resolution mass spectrometry
Lys: lysine
Me: methyl
mini-PEG: 8-amino-3,6-dioxaoctanoic acid
MPAA: 4-mercaptophenylacetic acid
MS: mass spectrometry
NCL: native chemical ligation
NP40: Nonidet P-40
Oxyma pure®: ethyl cyanoglyoxylate-2-oxime
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PVDF: polyvinylidene fluoride
SAv-HRP: streptavidin-horseradish peroxidase conjugate
SDS: sodium dodecyl sulfate

SEAlide: *N*-sulfanylethylanilide
SECmide: *N*-Sulfanylethylcoumarinyl amide
SPPS: solid-phase peptide synthesis
TBS: tris buffered saline
TBTA: tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
TCEP: tris(2-carboxyethyl)phosphine
TES: triethylsilane
TFA: trifluoroacetic acid
THF: tetrahydrofuran
Tris: tris(hydroxymethyl)aminomethane
Trt: triphenylmethyl
UV: ultraviolet

Contents

Preface

In the fields of chemical biology and drug discovery, identification of the target proteins of biologically active ligands is essential. Enrichment of the target proteins is crucial step for the identification and recently traceable linkers which enable enrichment and selective labeling of the target proteins have been developed for facilitation of target identification. Protocol for enrichment and selective labeling of target utilizing the traceable linkers is as follow (Figure 1.): (1) introduction of a biotin- and azide-modified traceable linker to an alkynylated target protein by click reaction between the azide and alkyne units; (2) adsorption the biotinylated protein on streptavidin beads; (3) selective linker cleavage-induced elution of the target from the beads followed by chemoselective labeling of the target protein with a tag such as fluorescein.

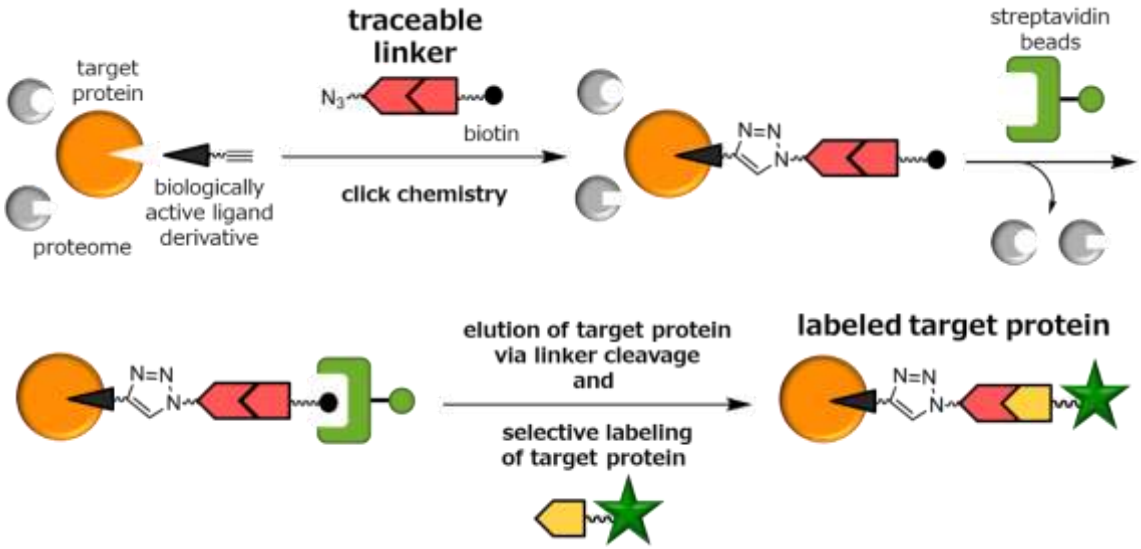


Figure 1. Purification and selective labeling of target proteins using traceable linker.

In this study, I attempted to develop a novel traceable linker which allows for the selective cleavage and labeling by an N–S acyl transfer reaction in the presence of phosphate salt.

In Chapter 1, development of SEAlide-based traceable linker for purification and selective labeling of target proteins is described. Development of turn-on fluorescent traceable linker using SECmide is presented in Chapter 2.

Chapter 1

Development of SEAlide-based traceable linker for purification and selective labeling of target proteins

1.1 Introduction

Many bioactive compounds, represented by natural products, peptides/proteins, and synthetic small molecules, exhibit their biological activities through their specific interactions with target proteins such as receptors, ion channels and enzymes. Therefore, identification of unknown target proteins interacting with bioactive compounds is indispensable for understanding their biological significance, thereby sometimes leading to development of a new therapeutic agent. Pull-down experiments of a target protein using a biologically active ligand constitute an indispensable step. Therefore, extensive efforts for improving efficacy of the pull-down experiments have been devoted, in which efficiency improvement of a conventional affinity purification protocol is involved. Conventional affinity purification process is conducted as follows: (1) attachment of a tag moiety to a target protein using a suitably functionalized biologically active ligand; (2) tag-based enrichment of the target; and (3) sequence analysis of the target protein using proteomic protocol including determination of amino acid sequence followed by Edman degradation and/or mass spectrometry (MS) measurement[1]. As shown in Figure 1.1., attachment of a tag moiety (e.g., an alkyne) on the target is achieved by photo-affinity labeling[1a,b,2] or an activity-based probe approach[1c-e]. Next, the resulting tagged target protein is connected to a biotin moiety by a tag-specific reaction through a linker and purifying it on streptavidin beads based by strong streptavidin-biotin interaction[1,3]. Then, elution of the target protein from the streptavidin beads is required for sequence analysis.

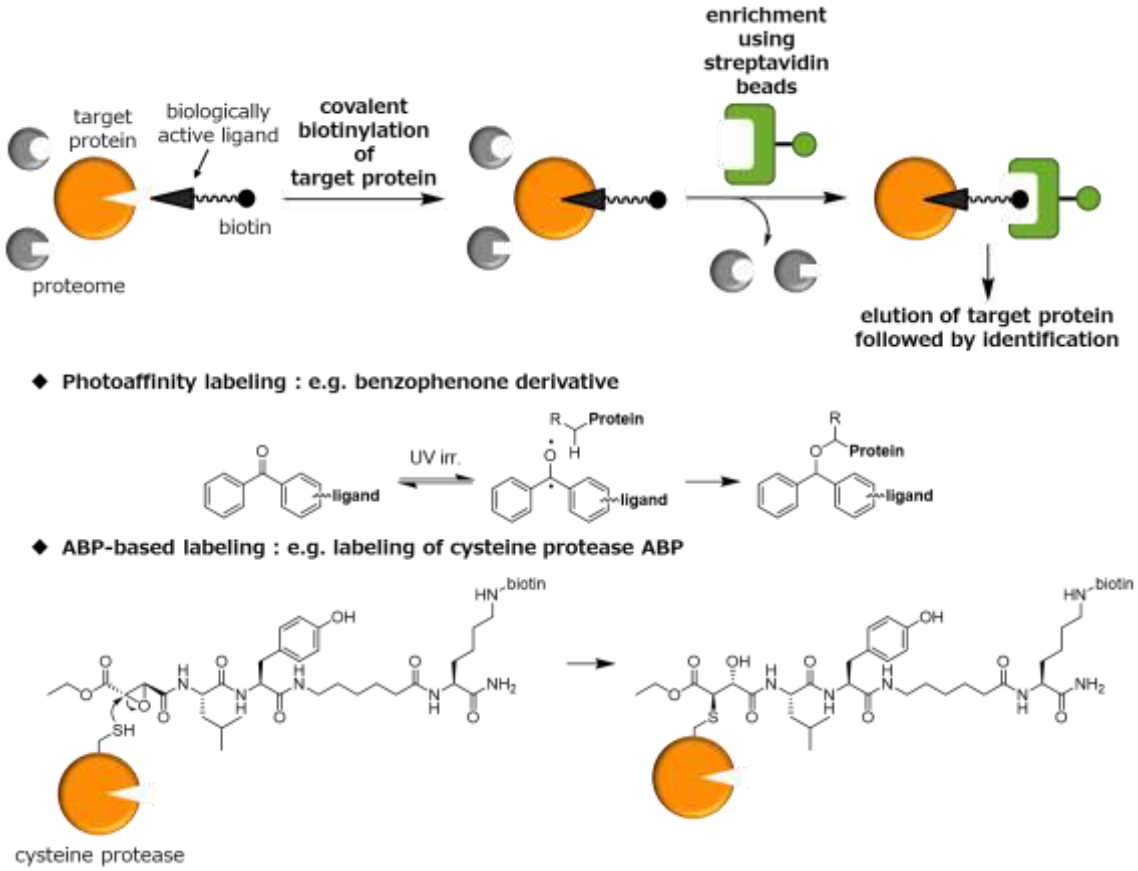


Figure 1.1. Affinity purification utilizing streptavidin-biotin interaction.

However, the elution efficiency of target proteins under mild conditions is typically low due to the high affinity of the streptavidin-biotin interaction ($K_d = 10^{-15}$ M)[4]. The efficiency of the elution could be improved by the use of harsh conditions; however, elution of nonspecifically adsorbed proteins is generally accompanied.

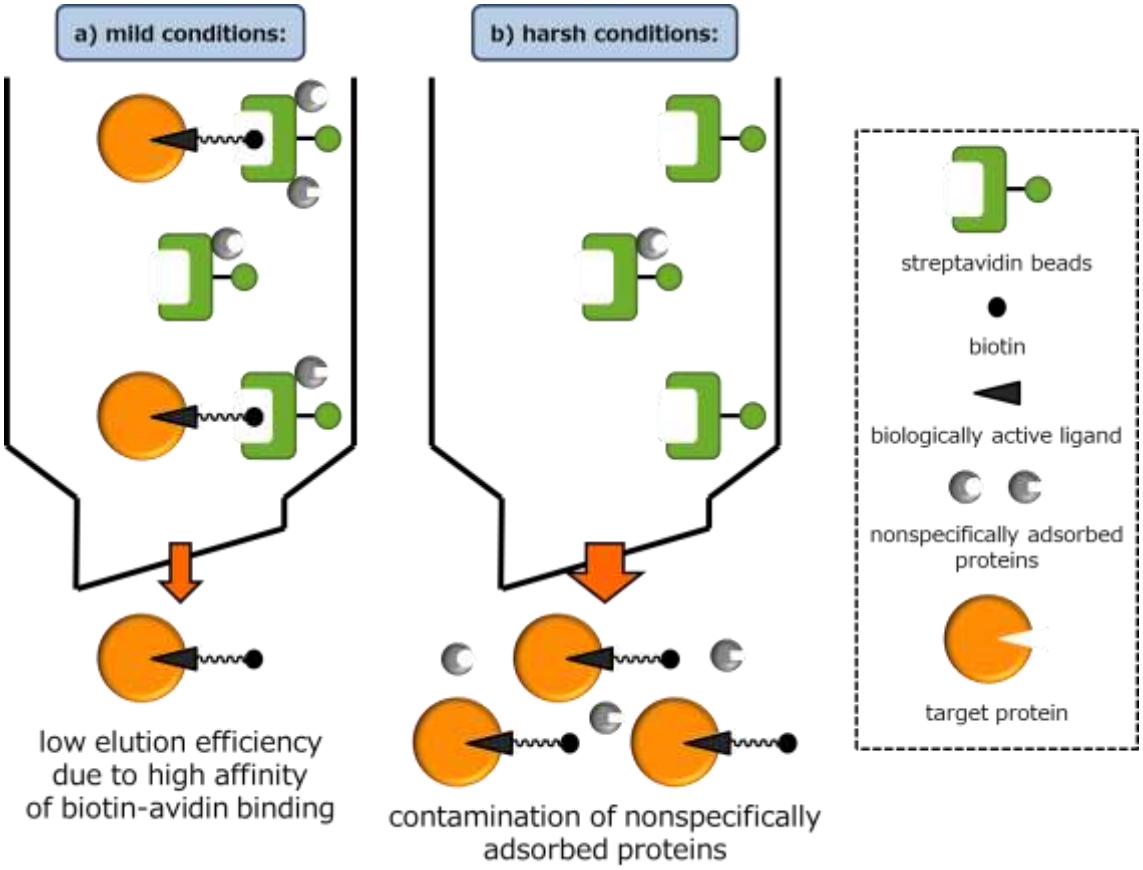


Figure 1.2. Problems encountered during elution of target protein from streptavidin beads.

To overcome these problems, the first-generation cleavable linkers which can be split under mild reaction conditions have been introduced between the biologically active ligands and biotin[5] as shown Figure 1.3. The use of such a linker allows for the efficient elution of the target protein from the streptavidin beads upon cleavage of the linker, but free from any contamination of nonspecifically adsorbed proteins remains to be achieved by the conventional linker[6].

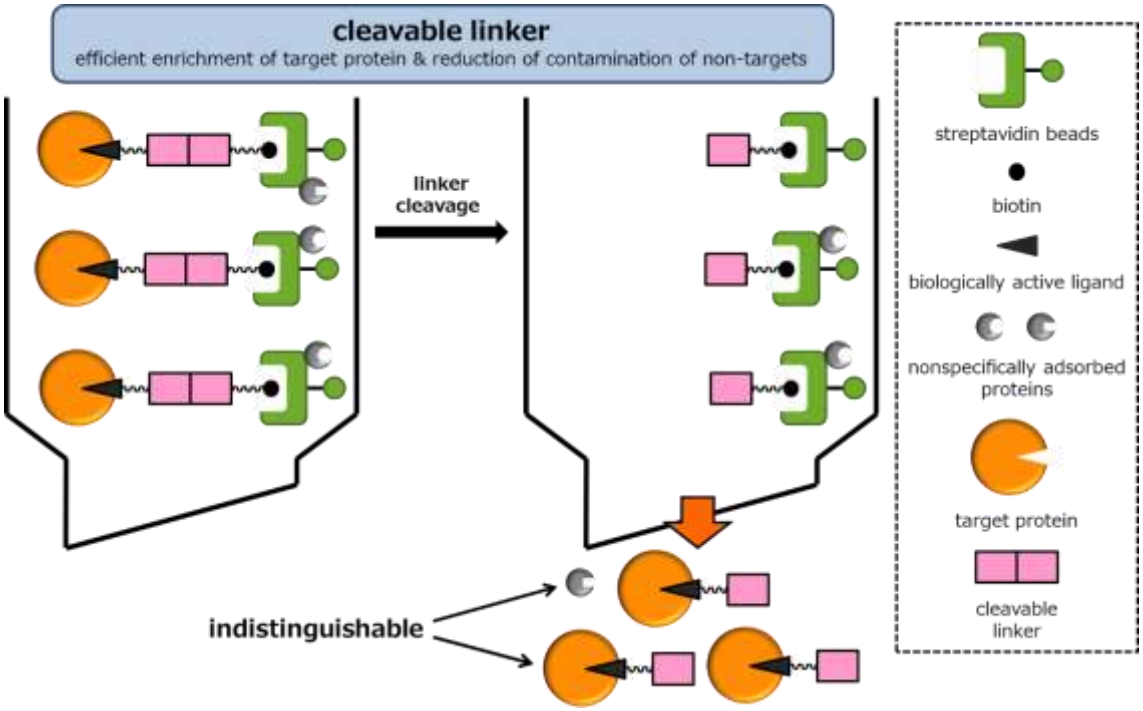


Figure 1.3. Strategy of enrichment of target protein utilizing cleavable linker.

Such contamination of off-target proteins observed in the elution process hampers the subsequent identification of target, prompting several research groups including ours to engage in the development of the second-generation traceable linkers as an advanced cleavable system which enables the selective labeling of target proteins from the mixture consisting of targets and off-targets[7]. Cleavage of the traceable linker conducted during the elution step generates the mixture of both the off-target proteins themselves and split-linker-containing target proteins. Here, of significance is that the split-linker-containing target has an orthogonal functional group generally which is not seen in proteins including off-targets. And chemoselective modification of the orthogonal group with labeling reagents including a fluorescent dye or an isotopically-enriched unit facilitates the easy discrimination of the target from protein mixture by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or MS analysis, respectively.

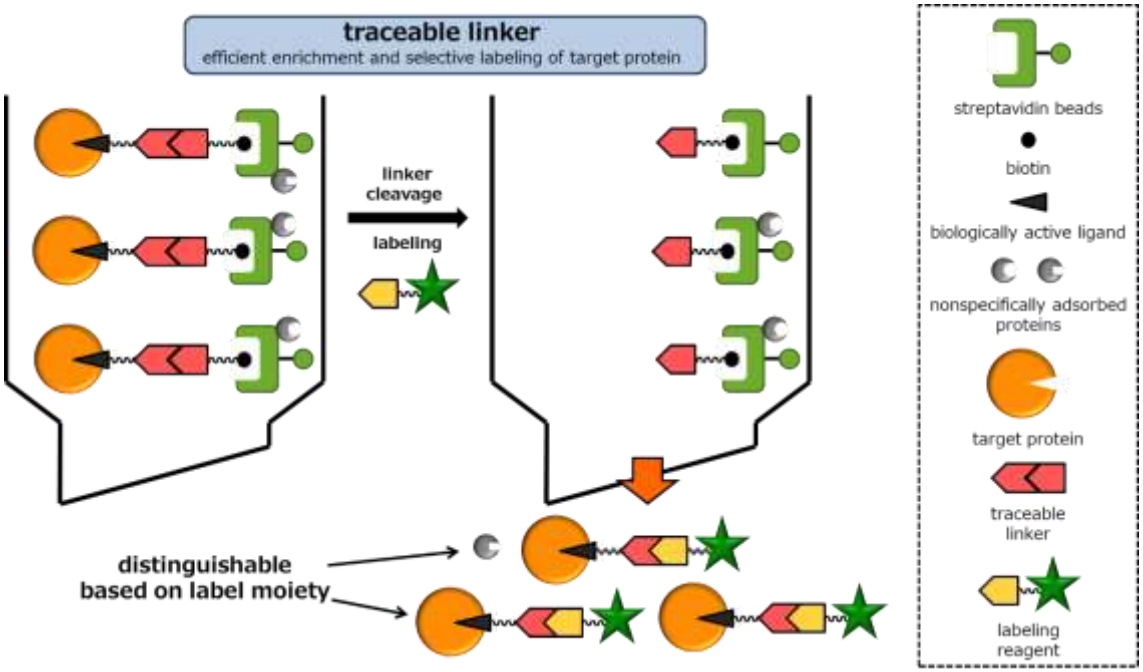


Figure 1.4. Strategy of enrichment and selective labeling of target protein utilizing traceable linker.

Reversible reactions including hydrazone/hydrazine[7e] or hydrazine/oxime[7d] exchange reaction for linker cleavage and labeling were utilized for the design of Kohn's or Dawson's linker, respectively. Aa an alternative, we developed the methodology based on the cleavage of an acyl-aminooxy linkage by using a stimulus-responsive processing amino acid followed by oxime formation of the resulting aminooxy group with an aldehyde-based reporter[7b,c]. Although the oxime linkage is stable enough for labeling the targets, the use of hydrazone linkage requires the carefully controlled experimental conditions[7d]. Thus, robust linkage highly applicable to various working conditions has been still demanded. From this point of view, I have developed a traceable linker in which both selective cleavage and introduction of a reporter can be performed using an irreversible mild reaction.

1.2 *Synthesis of SEAlide-based traceable linker*

We previously reported that the *N*-sulfanylethylanilide (SEAlide) unit works as a thioester precursor applicable to native chemical ligation (NCL) protocol which represents chemoselective amide-forming reaction of thioesters with N-terminal cysteine residue[8]. The amide-type SEAlide peptide remains almost intact in the absence of phosphate salts, whereas addition of phosphate salts induces the N–S-acyl-transfer-mediated transformation of the amide unit to give the corresponding thioester[9]. Such generated thioester subsequently reacts with N-terminal cysteinyl peptides as shown in Figure 1.5. to give ligated peptides via NCL[10]. On the basis of the observed conversion of the amide-type SEAlide linkage to the thioester-type followed by reaction with a cysteine unit, I envisioned that the SEAlide unit should be used for development of a new traceable linker which operates in an irreversible manner.

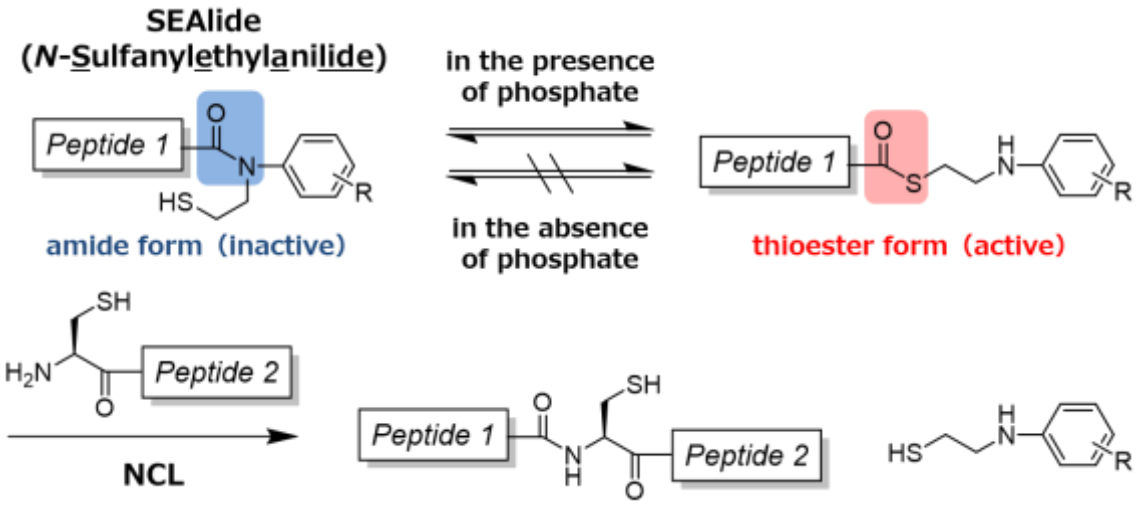


Figure 1.5. Phosphate-induced NCL of SEAlide unit with cysteinyl peptide.

With this in mind, I designed a SEAlide-based traceable linker in which two indispensable units, azide and biotin, are connected through the SEAlide (Figure 1.6.). In the designed linker, the thiol moiety on the SEAlide was protected by tert-butylsulfanyl group (S-^{t}Bu) to avoid any complications resulted from disulfide formation or accidental cleavage of the linker due to the presence of free thiol group of the amide-type SEAlide unit. The introduced S-^{t}Bu group can be removed under NCL conditions in the presence of reductants such as 4-mercaptophenylacetic acid (MPAA) and tris(2-carboxyethyl)phosphine (TCEP). Enrichment followed by the selective labeling of target proteins with a cysteine unit using the new linker was performed as follows (Figure. 1.6.). (i) Initially, the new traceable linker was introduced to an alkynylated target protein in proteome by click chemistry[11]; (ii) Then, the resulting conjugate was enriched over

streptavidin beads; (iii) Finally, exposure of the streptavidin beads to NCL with an N-terminal cysteinyl labeling reagent in the presence of phosphate salts allowed for cleavage of the SEAlide-incorporated linker to give the covalent conjugate of the target and labeling reagent. In this chapter, I describe the examination of enrichment of an alkynylated model protein using the SEAlide-based traceable linker.

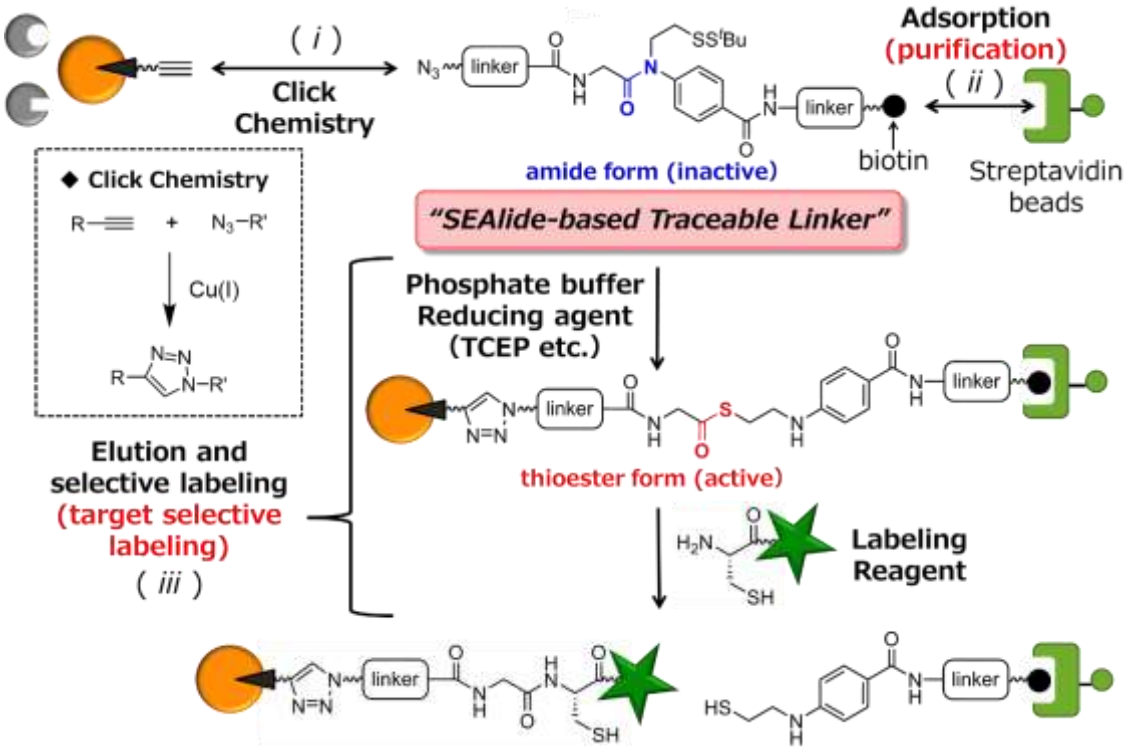
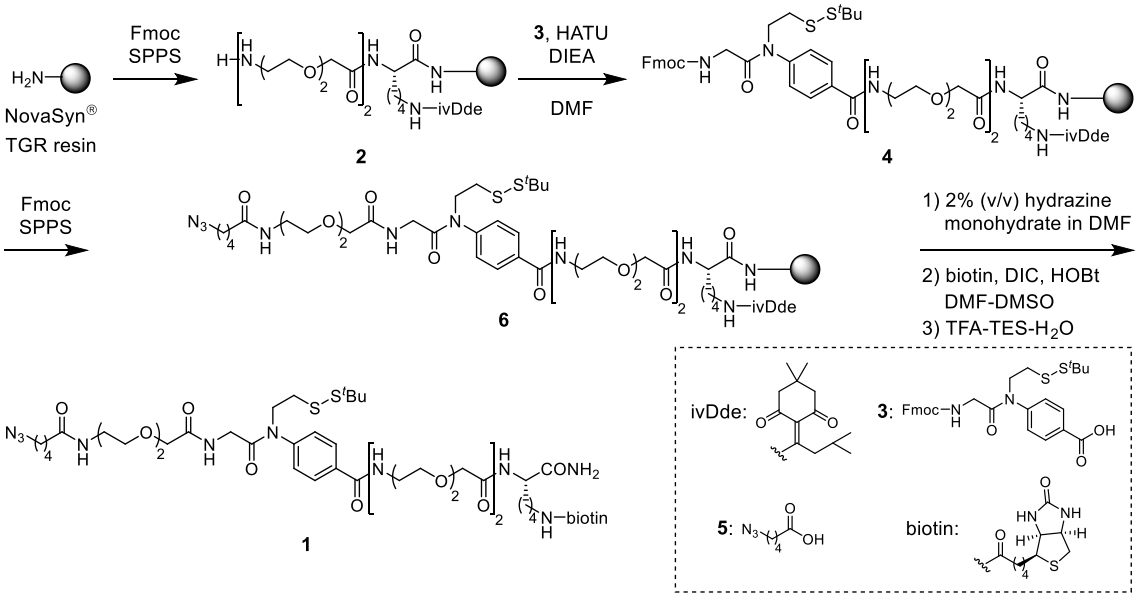


Figure 1.6. Strategy of the enrichment and selective labeling of target protein using SEAlide-based traceable linker.

A requisite SEAlide-based traceable linker **1** possessing azide and biotin units was synthesized by Fmoc-based solid-phase peptide synthesis (SPPS). Azide and biotin units were introduced on N- and C-terminal ends of linker, respectively. For the introduction of biotin unit at the C-terminal end, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) group as an ε-amino protection of lysine (Lys) was used (Scheme 1.1.). Starting from NovaSyn® TGR resin, Fmoc-Lys(ivDde)-OH as amino acid for biotin incorporation and Fmoc-miniPEG-OH were successively incorporated by standard Fmoc SPPS protocols using *N*,*N*'-diisopropylcarbodiimide (DIC)/Oxyma pure® system. After coupling of disulfide-form-protected SEAlide derivative **3** using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIPEA), Fmoc-miniPEG-OH and the azide derivative **5**[12] were also incorporated using DIC/Oxyma pure® system. To remove the ivDde group from

the lysine side chain, the completed resin **6** resulted from chain elongation was treated with 2% (v/v) hydrazine monohydrate in *N*,*N*-dimethylformamide (DMF). After incorporation of biotin on the regenerating ε-amino group using DIC/1-hydroxybenzotriazole (HOBt) system, the resulting resin was then treated with trifluoroacetic acid (TFA)-triethylsilane (TES)-H_2O (95:2.5:2.5 [v/v]) to generate the desired SEAlide-based traceable linker **1**.

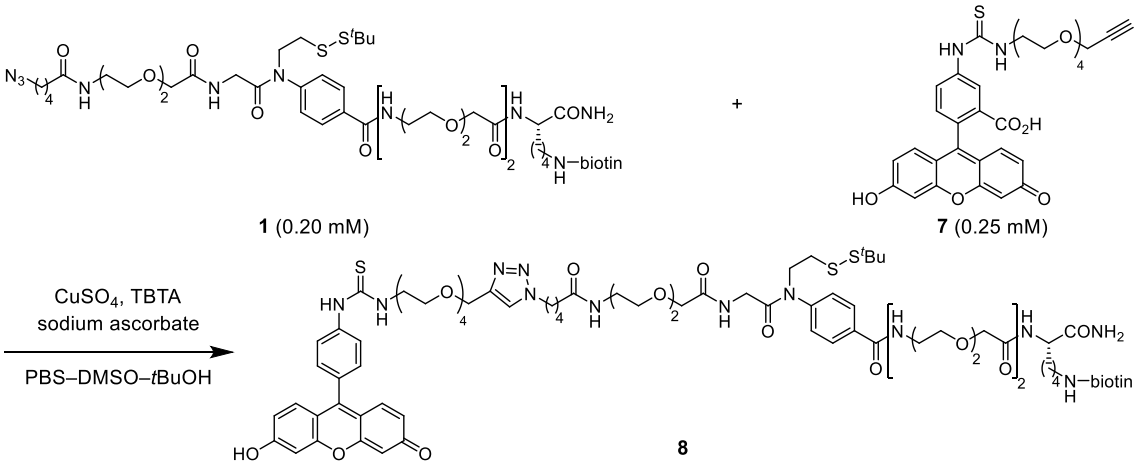
Scheme 1.1. Synthesis of SEAlide-based traceable linker **1**.



1.3 *Evaluation of Click Chemistry and NCL using a model FTC derivative*

Prior to the examination of enrichment and successive selective labeling of an alkynylated protein, model reactions were conducted using an alkynylated fluorescein thiocarbamoyl (FTC) derivative **7** as a model substrate. Linking of traceable linker **1** with FTC derivative **7** by click chemistry was performed in a mixed solvent consisting of phosphate buffered saline (PBS), DMF, and ^{t}BuOH in the presence of $CuSO_4$, sodium ascorbate, and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA) (Scheme 1.2.). After 1 h of the reaction at ambient temperature, the desired traceable linker-fluorescein conjugate **8** was generated in high purity (Figure 1.7.).

Scheme 1.2. Click chemistry between traceable linker **1** and FTC derivative **7**.



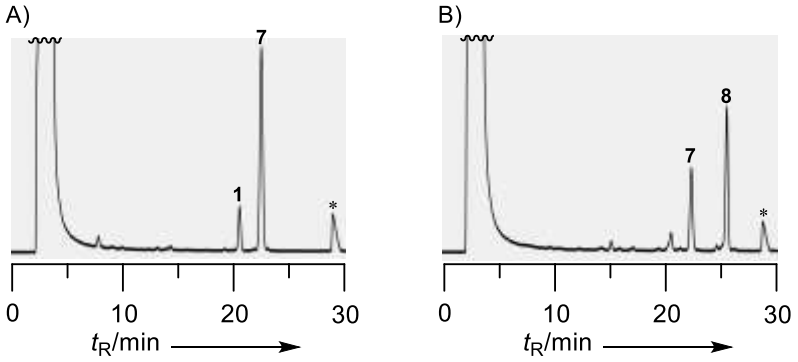
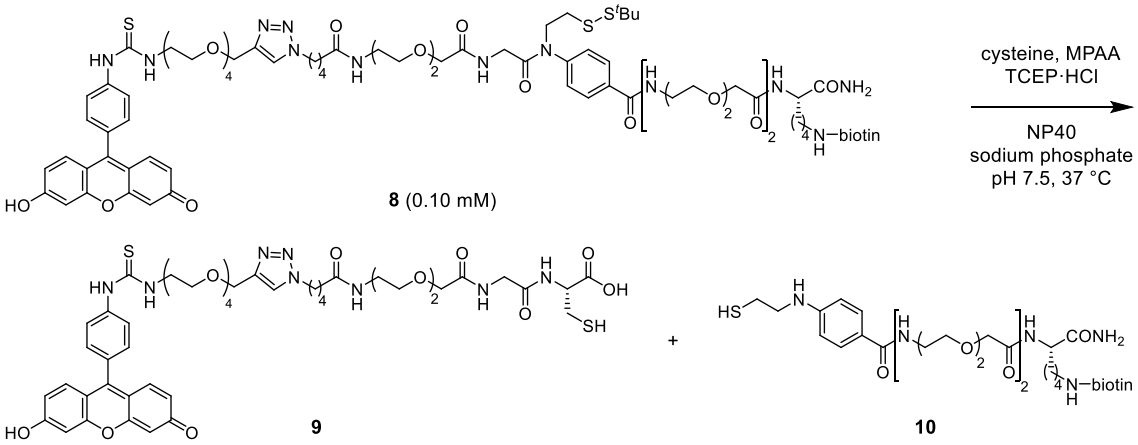


Figure 1.7. high performance liquid chromatography (HPLC) monitoring of the reaction as shown in scheme 1.2. (A) before reaction. (B) after 1 h of the reaction. Analytical HPLC conditions: linear gradient of 0.1% (v/v) TFA–MeCN in 0.1% (v/v) TFA aq., 30 to 40% over 30 min. Detection at 220 nm. *Non-peptidic small molecule.

Next, I attempted the NCL-mediated cleavage of the obtained model conjugate **8** by reaction with cysteine under reductive conditions (MPAA and TCEP) in the presence of phosphate salts (Scheme 1.3.). Progress of the reaction was monitored by HPLC analysis and the resulting products were characterized by electrospray ionization mass spectrometry (ESI-MS) (Figure 1.8.). The HPLC-purified conjugate **8** was treated with cysteine, MPAA, TCEP at 37 °C in sodium phosphate buffer (pH 7.5) containing Nonidet P-40 (NP40). Attempted NCL-mediated cleavage of the conjugate **8** proceeded to almost completion within 24 h to yield cysteine-incorporated NCL product **9**.

Scheme 1.3. NCL reaction of linker-FTC conjugate **8** with cysteine.



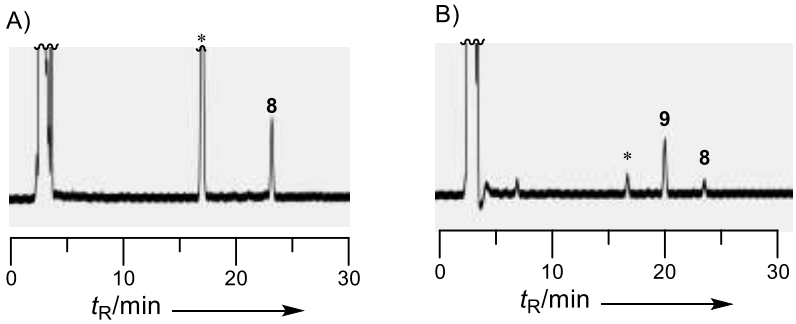
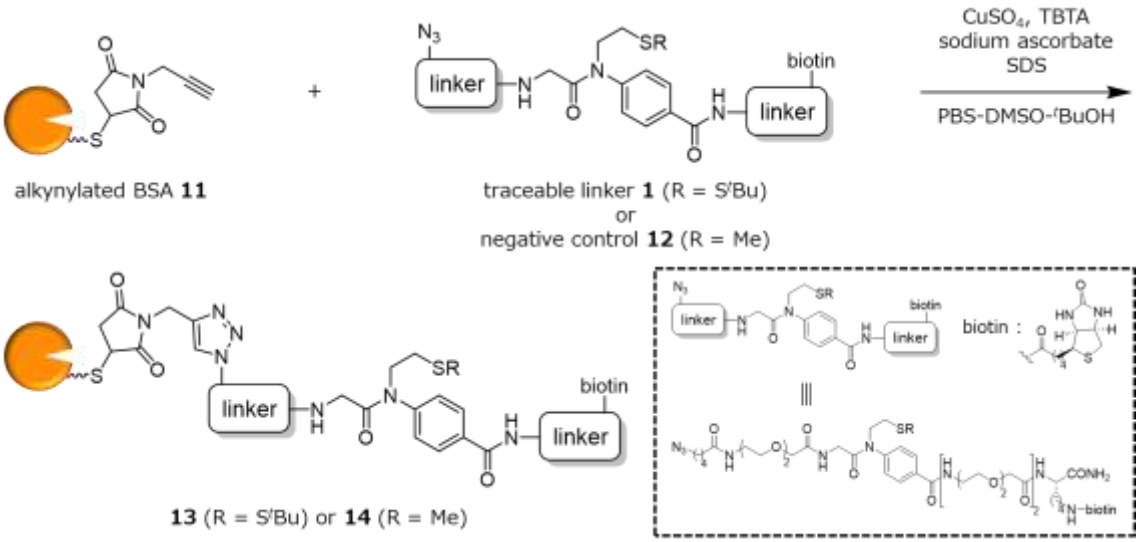


Figure 1.8. HPLC monitoring of the reaction as shown in Scheme 1.3. (A) before reaction. (B) after 24 h of the reaction. Analytical HPLC conditions: linear gradient of 0.1% (v/v) TFA-MeCN in 0.1% (v/v) TFA aq., 25 to 35% over 30 min. Detection at 220 nm. *MPAA.

1.4 Purification and Selective Labeling of an alkynylated model protein

Since the model reaction of conjugate **8** indicated that the cleavage followed by the incorporation of cysteine as a label was efficiently achieved in homogeneous solution system, I next evaluated the enrichment of an alkynylated model protein using traceable linker **1**, followed by NCL-mediated elution of selectively labeled protein from the streptavidin beads. In this experiment, alkynylated bovine serum albumin (BSA) was used as a target model protein[7a]. An S-alkylated negative control linker **12**, which cannot be cleaved via the N–S acyl transfer reaction involving in the NCL reaction, was also prepared. Initially, the click reaction of traceable linker **1** or negative control **12** with alkynylated BSA was examined. Alkynylated BSA was treated with the linker **1** or **12** in phosphate buffered saline (PBS) in the presence of $CuSO_4$, sodium ascorbate, TBTA and sodium dodecyl sulfate (SDS) to afford the corresponding biotin-incorporated protein **13** or **14**, respectively. After 1 h reaction, progress of the click reaction was examined by SDS-PAGE separation of the reaction, followed by western blot analysis using streptavidin-horseradish peroxidase conjugate (SAv-HRP) and silver staining. It was found that both linkers **1** and **12** were successfully attached to the alkynylated BSA (Figure 1.9.)

Scheme 1.4. Click chemistry between alkynylated BSA **11** and linker **1** or **12**.



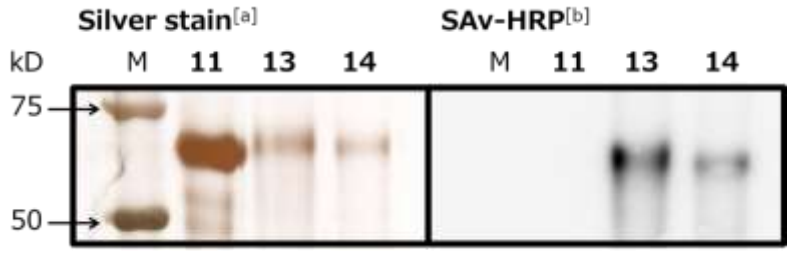
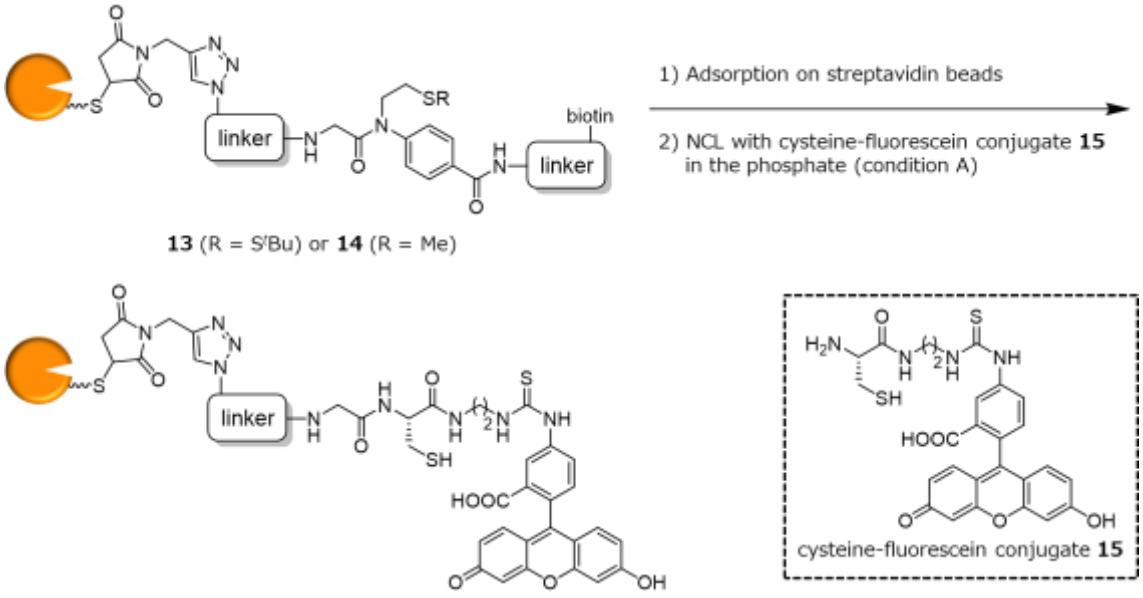


Figure 1.9. Monitoring of the click chemistry of traceable linker **1** or negative control **12** with alkynylated BSA **11** using SDS-PAGE. The linker **1** or **12** (0.10 mM) was introduced to alkynylated BSA **11** (0.50 g/L) using a mixture of $CuSO_4$ (1.0 mM), sodium ascorbate (1.0 mM), TBTA (0.10 mM) and SDS (1.0% [v/v]) in PBS, and dimethyl sulfoxide (DMSO)/^{t}BuOH (1:4) for 1 h. [a] All proteins were visualized by silver staining. [b] Biotinylated proteins were detected by western blot analysis using SAv-HRP. M = marker.

Next, I examined the NCL-mediated elution of a selectively labeled protein from streptavidin beads by the action of a cysteinyl labeling reagent in phosphate buffer. For facile visualization of SDS-PAGE gel, cysteine-fluorescein conjugate **15**[13] was used as a labeling reagent possessing ability of cleavage of the linker. The resulting click product **13** or **14** was immobilized on streptavidin beads by incubation of protein samples in PBS containing SDS for 1 h. After washing with PBS, each model protein-loaded beads was treated with 100 μM cysteine-fluorescein conjugate **15**, 50 mM MPAA, 40 mM TCEP in 0.4 M sodium phosphate buffer (pH 7.4) containing SDS at 37 °C for 24 h (condition A).

Scheme 1.5. Enrichment and selective labeling of linker-BSA conjugate **13** or **14**.

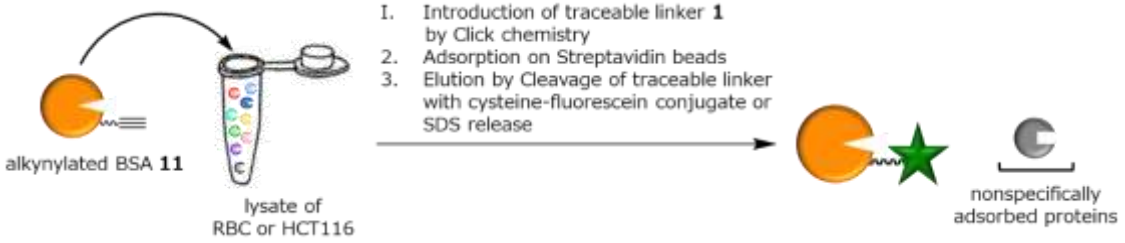


After separation by SDS-PAGE, the fluorescein-labeled proteins and all proteins were visualized by fluorimetry without staining (λ_{ex} = 460 nm, λ_{em} > 515 nm) and by lumitein staining, respectively. When traceable linker **1** was employed, the NCL-mediated elution and labeling of the BSA proceeded successfully. In the case of S-alkylated negative control **12**, a small amount of the protein was eluted from the beads, presumably because of the SDS-mediated denaturation of streptavidin. However, the elution efficiency of the negative control was lower than that of the corresponding traceable linker **1**. These results therefore demonstrate that linker **1** may be used to achieve an efficient elution profile by the cleavage of the linker. Furthermore, the eluted protein was detected by fluorescence imaging (FL) when **1** but not **12** was used as the linker.

Figure 1.10. Monitoring of the elution from streptavidin beads and labeling of BSA conjugate **13** or **14** using SDS-PAGE. Proteins were treated with streptavidin beads at rt for 1 h following the click chemistry described in Scheme 1.4. and Figure 1.9. After washing with PBS, the beads were allowed to react with MPAA (50 mM), TCEP (40 mM), cysteine-fluorescein conjugate **15** (0.10 mM) and SDS (0.10% [v/v]) in 0.40 M sodium phosphate buffer (pH 7.4) at 37 °C for 24 h. [a] All proteins were visualized by lumitein staining. [b] fluorescein-labeled proteins were detected at λ_{ex} = 460 nm and λ_{em} > 515 nm without staining. [c] Proteins after eluent of the reaction. [d] Proteins remaining on streptavidin beads after the reaction. After centrifugation and removal of the supernatant, the beads were suspended in SDS-PAGE sample loading buffer, and the suspension was heated at 100 °C for 5 min. After centrifugation, the supernatant was analyzed.

Finally, we examined the enrichment and selective labeling of the alkynylated protein using a mixture of the alkynylated BSA and lysate of red blood cell (RBC) or HCT116 (Scheme 1.6.). This mixture was treated with traceable linker **1**, to perform click chemistry. The resulting mixture was then incubated with streptavidin beads, and the adsorbed proteins were treated under denaturation conditions or the cysteine-fluorescein conjugate **15** presence conditions to carry out elution.

Scheme 1.6. Purification and selective labeling of alkynylated BSA in protein mixture.



The eluted mixture was analyzed by SDS-PAGE and the proteins were visualized by silver staining or fluorescence imaging. When the elution was performed by the denaturation of streptavidin in a manner similar to that used with conventional linkers, the enrichment of the BSA was observed (Figure 1.11., silver stain, lane 4). The elution of the proteins by the cleavage of the linker in the presence of cysteine–fluorescein conjugate **15** gave slightly better enrichment results compared with those obtained by the denaturation of streptavidin (Figure 1.11., silver stain, lane 5). Here, of note is the fact that the traceable linker **1** allowed for the visualization of the target, therefore showing potential utility in specific identification of target proteins from proteome samples (Figure 1.11., FL, lane 5). However, one imperative limitation with the use of the linker **1** is that strong fluorescence derived from the labeling reagent is observed in the low molecular weight region, which provably obstructs the identification of low molecular weight target (Figure 1.11., FL, lane 5).

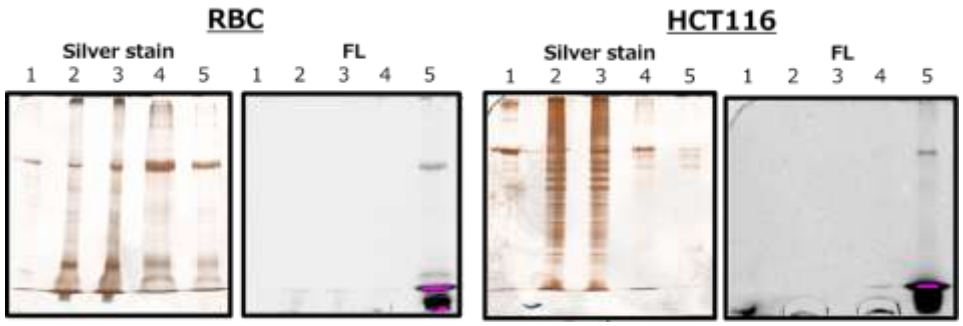


Figure 1.11. Enrichment and selective labeling of the alkynylated BSA in lysate of RBC or HCT116 using traceable linker **1**. Detailed conditions are provided in Experimental Section. Lane 1: alkynylated BSA; lane 2: lysate; lane 3: a mixture of the alkynylated BSA and lysate; lane 4: eluted proteins after the click chemistry of the mixture with **1**, adsorption of the biotinylated material onto streptavidin beads and the treatment of the beads with sample buffer at 100 °C for 5 min (elution by denaturation of streptavidin); lane 5: eluted proteins after the click chemistry of the mixture with **1**, adsorption of the biotinylated material onto streptavidin beads and the treatment of the beads with cysteine-fluorescein conjugate **15** in the presence of phosphate (elution conditions: condition A); fluorescence imaging (FL), λ_{ex} = 460 nm, λ_{em} > 515 nm.

1.5 Conclusion

The SEAlide-based traceable linker was developed as an advanced cleavable linker. This new system not only enables phosphate-induced cleavage of linkers for enrichment of target proteins in a manner similar to that of conventional cleavable linkers, but also allows the selective labeling of the target proteins so that it can be distinguished from contaminant non-target proteins. This-traceable-linker-based technique therefore represents a novel and powerful methodology for the facile identification of target proteins of the biologically active compounds, including drug candidates. Work toward the application of this traceable linker to identify the targets of target-unknown bioactive compounds is currently in progress.

Chapter 2

Development of turn-on fluorescent traceable linker using SECmide

2.1 Introduction

Identification of unknown target proteins with which biologically active compounds can interact is indispensable in drug discovery and chemical biology. Methodologies for identification of target proteins using traceable linkers with an advanced cleavable system which enables selective labeling of a target protein from proteome consisting of targets and off-targets have been developed by several research groups, but previously reported traceable linkers use reversible reactions for the cleavage of the linker and/or selective labeling. Because a traceable linker based on a reversible reaction has risk of accidental cleavage of the linker and/or removal of reporter moieties, further improvement has been demanded[7].

In Chapter 1, I described the irreversible reaction-based traceable linker using SEAlide unit, which enables elution of the adsorbed protein on streptavidin beads and selective labeling by cleavage of the linker with phosphate-induced NCL. However, the SEAlide-based linker **1** is not applicable to detection of low molecular weight proteins because strong fluorescence derived from residual labeling reagent such as the cysteine-fluorescein conjugate **15** interferes with the detection of labeled low molecular weight target proteins. In this context, I envisioned that introduction of a fluorescent precursor into a traceable linker, resulting in yielding a fluorescence after elution from the linker, allows for visualization of only a target protein without addition of the fluorescent labeling reagent. This type of the traceable linker is termed here a “turn-on fluorescent traceable linker”.

In this chapter, I describe the development of turn-on fluorescent traceable linker and its application to the enrichment and selective visualization of a target protein in a protein mixture.

2.2 *Synthesis of SECmide-based Traceable Linker*

We previously reported that *N*-sulfanylethylcoumarinyl amide (SECmide) unit functions as a precursor for C-terminal thioester to be used in chemical protein synthesis[14]. The amide-type SECmide peptide remains almost intact and non-fluorescent in the absence of phosphate salts, whereas addition of phosphate salts induces the N–S-acyl-transfer-mediated transformation of the amide unit to the corresponding thioester and the resulting non-N-acylated SECmide unit produces fluorescence (Figure 2.1.). In situ generated thioester was subsequently reacted with a variety of nucleophiles including cysteine unit followed by NCL-mediated release of a coumarin fluorophore as fluorescent molecule. On the basis of such conversion of the non-fluorescent amide-type SECmide linkage to the fluorescent thioester-type followed by reaction with a nucleophile, I envisioned that the SECmide unit should be used for development of a turn-on fluorescent traceable linker.

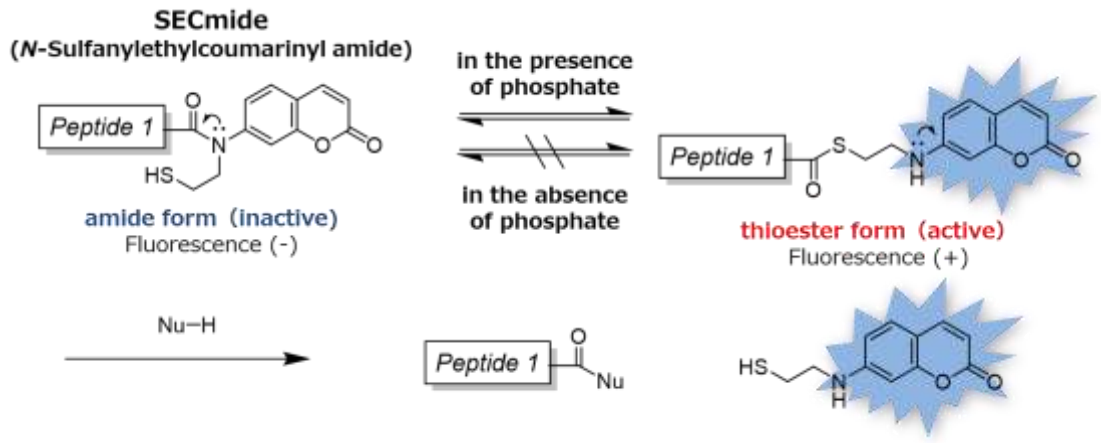


Figure 2.1. Phosphate-triggered activation of SECmide unit.

With this in mind, I designed a turn-on fluorescent traceable linker in which two indispensable units, azide and biotin, are connected through the SECmide (Figure 2.2.). In the designed linker, the thiol moiety on the SECmide was protected by tert-butylsulfanyl group (S-^{t}Bu) as described in Chapter 1. Enrichment followed by selective visualization of target proteins with a nucleophile using the new linker was performed as follows (Figure 2.2.). (i) Initially, the SECmide-based traceable linker was introduced to an alkynylated target protein in proteome by click chemistry[11]; (ii) Then, the resulting conjugate was enriched over streptavidin beads; (iii) Finally, exposure of the streptavidin beads to reaction with a nucleophile in the presence of phosphate salts allowed for cleavage of the SECmide-incorporated linker leading to visualization of a target by means of the fluorescent signal of the non-acylated SECmide unit. In this chapter, I describe the

examination of enrichment of an alkynylated model protein using the SECmide-based traceable linker.

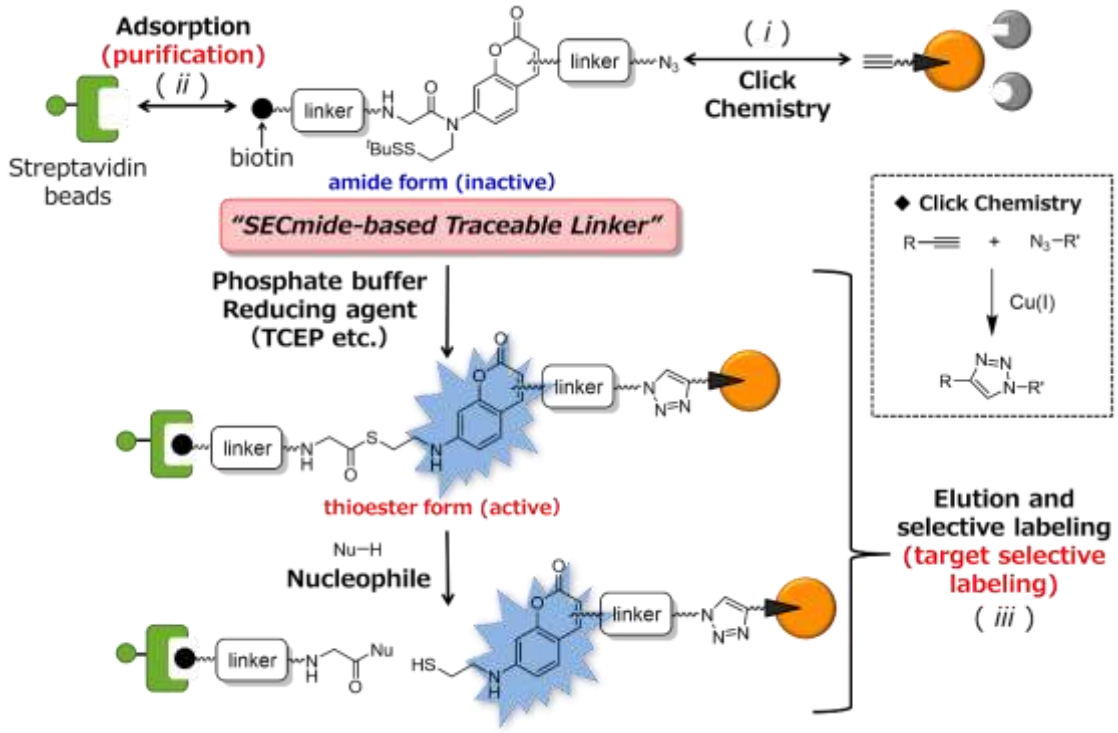
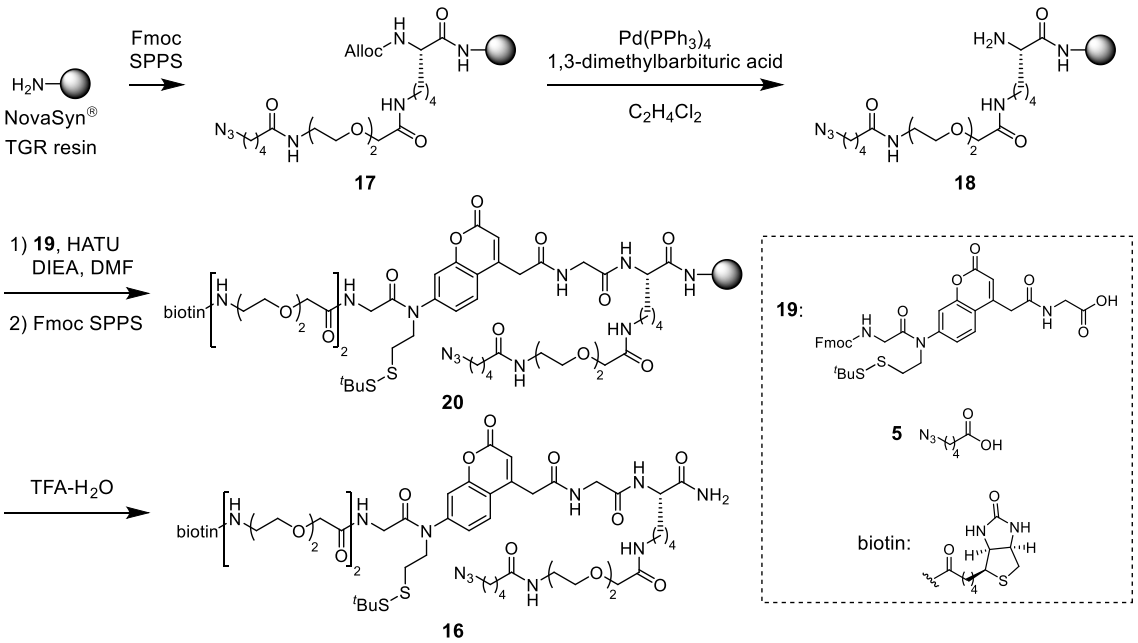


Figure 2.2. Strategy of the enrichment and selective visualization of target proteins using SECmide-based traceable linker.

A requisite SECmide-based traceable linker **16** possessing azide and biotin units was synthesized by Fmoc SPPS (Scheme 2.1.). Biotin and azide units were introduced on N- and C-terminal ends of linker, respectively. Starting from NovaSyn® TGR resin, Alloc-Lys(Fmoc)-OH as amino acid for azide incorporation, Fmoc-miniPEG-OH, and the azide derivative **5**[12] were successively incorporated by standard Fmoc SPPS protocols using DIC/HOBt system on the ε-amino group of lysine side chain to give the intermediate protected resin **17**. Chain elongation along main chain backbone was initiated by removal of the Alloc group of the resulting resin **17** by treatment with 1,3-dimethylbarbituric acid and $Pd(PPh_3)_4$ in $C_2H_4Cl_2$. After coupling of disulfide-form-protected SECmide derivative **19** using HATU and DIPEA on the Alloc-removed resin, Fmoc-miniPEG-OH and biotin were successively incorporated using DIC/HOBt system. Finally, the completed resin **20** was treated with TFA-H_2O (97.5:2.5 [v/v]) to generate the desired SECmide-based traceable linker **16**.

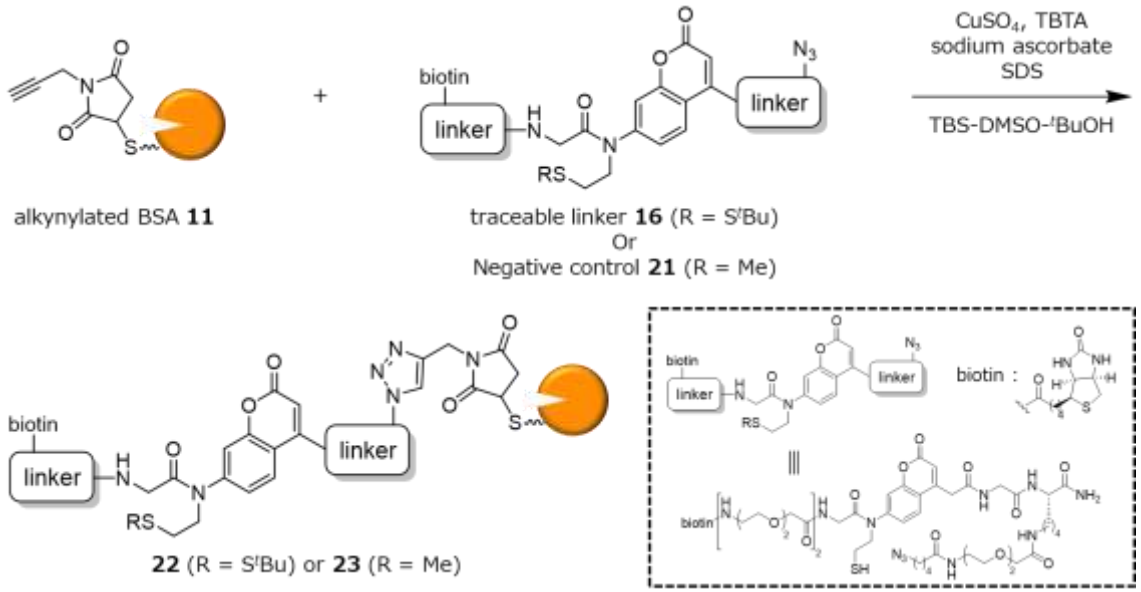
Scheme 2.1. Synthesis of SECmide-based traceable linker **16**.



2.3 *Evaluation of Click Chemistry and elution of an alkynylated model protein*

Next, I evaluated enrichment of an alkynylated model protein using traceable linker **16** and subsequent linker-cleavage-mediated elution of the fluorescent-visualized model protein from streptavidin beads. In this experiment, which is shown in Scheme 2.2., alkynylated BSA was used as a model target protein as described in Chapter 1[7a]. An S-alkylated negative control linker **21**, which cannot be cleaved via the phosphate-induced N–S acyl transfer reaction, was also prepared. Initially, the click reaction of traceable linker **16** or negative control **21** with alkynylated BSA was examined. Alkynylated BSA was treated with the linker **16** or **21** in tris buffered saline (TBS) in the presence of $CuSO_4$, sodium ascorbate, TBTA and SDS to afford the corresponding biotin-incorporated protein **22** or **23**, respectively. After 1 h reaction, progress of the click reaction was examined by SDS-PAGE separation of the reaction, followed by western blot analysis using SAv-HRP and Coomassie Brilliant Blue (CBB). It was found that both linkers **16** and **21** were successfully attached to the alkynylated BSA (Figure 2.3.)

Scheme 2.2. Click chemistry between alkynylated BSA **11** and linker **16** or **21**.



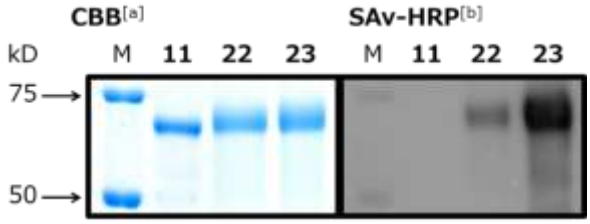
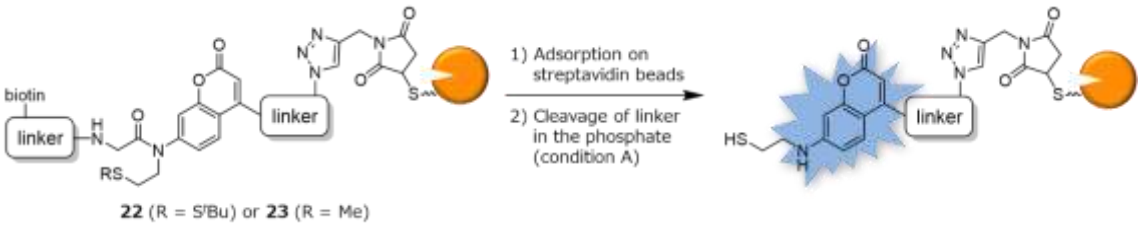


Figure 2.3. Monitoring of the click chemistry of traceable linker **16** or negative control **21** with alkynylated BSA **11** using SDS-PAGE. The linker **16** or **21** (0.10 mM) was introduced to alkynylated BSA **11** (0.50 g/L) using a mixture of $CuSO_4$ (1.0 mM), sodium ascorbate (1.0 mM), TBTA (0.10 mM) and SDS (1.0% [v/v]) in TBS, and DMSO/^{t}BuOH (1:4) for 1 h. [a] All proteins were visualized by CBB. [b] Biotinylated proteins were detected by western blot analysis using SAv-HRP. M = marker.

Next, I examined the phosphate-induced elution of the target model protein with its visualization achieved by fluorescence of the SECmide moiety. The resulting click product **22** or **23** was immobilized on streptavidin beads by incubation of protein samples in TBS containing SDS for 1 h. After washing with TBS, each model protein-loaded beads was treated with 100 μM cysteine as nucleophile under optimized conditions as described in Chapter 1 (condition A).

Scheme 2.3. Enrichment and selective visualization of linker-BSA conjugate **22** or **23**.



After separation by SDS-PAGE, the fluorescent (coumarin)-labeled proteins and all proteins were visualized by fluorimetry without staining (λ_{ex} = 302 nm, λ_{em} = 530 ± 14 nm) and by CBB staining, respectively. When traceable linker **16** was employed, the phosphate-induced elution of the model target BSA protein which is visualized by the coumarin fluorophore proceeded successfully. In the case of S-alkylated negative control **21**, a small amount of the protein was eluted from the beads, presumably because of SDS-mediated denaturation of streptavidin. However, the elution efficiency observed by the use of the negative control linker **21** was lower than that of the SECmide-based traceable linker **16**. Furthermore, the eluted protein only with the use of **16** was detected by fluorescence imaging (FL). Therefore, observed results demonstrated that the SECmide-based traceable linker **16** showed an efficient and unprecedented elution profile in which the visualization of the target was achieved in a “turn-on” manner.

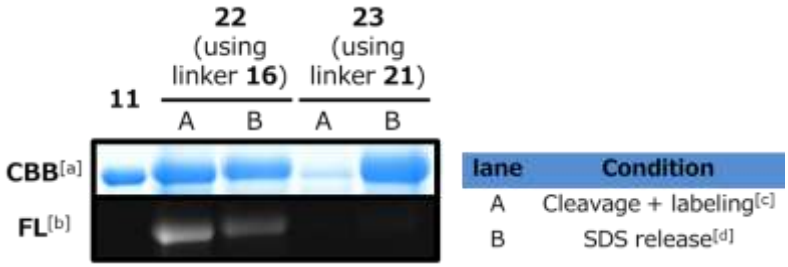
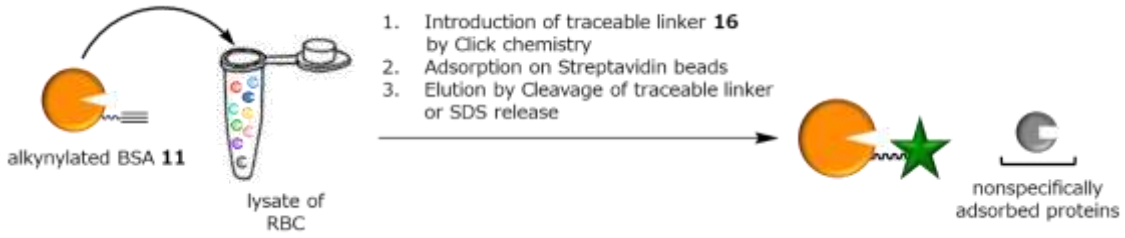


Figure 2.4. Monitoring of the elution from streptavidin beads and labeling of BSA conjugate **22** or **23** using SDS-PAGE. Proteins were treated with streptavidin beads at rt for 1 h following to click chemistry described in Scheme 2.2. and Figure 2.3. After washing with TBS, the beads were reacted with MPAA (50 mM), TCEP (40 mM), cysteine (0.10 mM) and SDS (0.10% [v/v]) in 0.40 M sodium phosphate buffer (pH 7.0) at 37 °C for 24 h. [a] All proteins were visualized by lumitein staining. [b] coumarin-labeled proteins were detected at λ_{ex} = 302 nm and λ_{em} 530 ± 14 nm without staining. [c] Proteins after eluent of the reaction. [d] Proteins remaining on streptavidin beads after the reaction. The beads after centrifugation followed by removal of the supernatant were suspended in SDS-PAGE sample loading buffer, and the suspension was heated at 100 °C for 5 min. After centrifugation, the supernatant was analyzed.

2.4 *Purification and Selective visualization of an alkynylated model protein*

Finally, we examined the enrichment and selective visualization of the alkynylated protein using a mixture of the alkynylated BSA and red blood cell lysate. This mixture was treated with traceable linker **16**, to perform click chemistry. The resulting mixture was then treated with streptavidin beads, and the adsorbed proteins were eluted. The eluted mixture was analyzed by SDS-PAGE and the proteins were visualized by CBB or fluorescence imaging.

Scheme 2.4. Purification and selective labeling of alkynylated BSA **11** in protein mixture.



When elution was performed by denaturation of streptavidin in a manner similar to that used with conventional linkers, enrichment of the BSA was observed (Figure. 2.5., CBB, lane 4). Elution of the proteins by cleavage of the linker with cysteine in phosphate buffer gave slightly better enrichment results compared with those obtained by denaturation of streptavidin (Figure. 2.5., CBB, lane 5). Here of note is that the SECmide-based traceable linker **16** allowed for visualization of the target by fluorescence without adding a fluorescence labeling reagent for cleavage of the linker. This is not the case for the use of the SEAlide-based linker **1** as described in Chapter 1. (Figure. 2.5., FL, lane 5). Furthermore, no strong fluorescence was observed in the low molecular weight region. These results indicated that the SECmide-based linker **16**, which can add the fluorescent unit on the target in "turn-on" manner, allows for facile detection of not only high molecular weight target proteins but also low molecular weight target from complex proteome mixture.

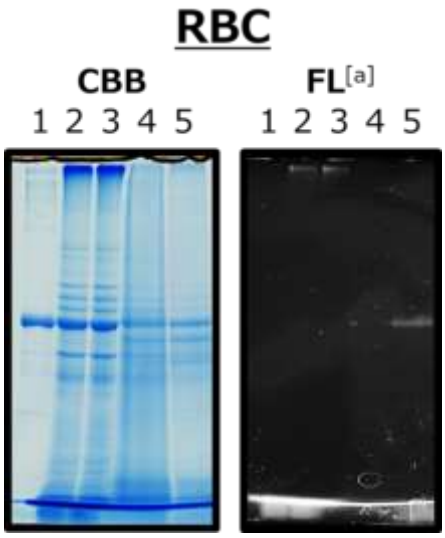


Figure 2.5. Enrichment and selective labeling of the alkynylated BSA in RBC lysate using traceable linker **16**. Detailed conditions are provided in Experimental Section. Lane 1: alkynylated BSA; lane 2: RBC lysate; lane 3: a mixture of the alkynylated BSA and RBC lysate; lane 4: eluted proteins after the click chemistry of the mixture with **16**, adsorption of the biotinylated material onto streptavidin beads and the treatment of the beads with sample buffer at 100 °C for 5 min (elution by denaturation of streptavidin); lane 5: eluted proteins after the click chemistry of the mixture with **16**, adsorption of the biotinylated material onto streptavidin beads and the treatment of the beads with cysteine in the presence of phosphate (elution conditions: 0.10 mM cysteine, 50 mM MPAA, 40 mM TCEP, 0.1% SDS, 0.40 M Na phosphate buffer, pH 7.0, 37 °C, 24 h); [a] FL, λ_{ex} = 302 nm, λ_{em} = 530 ± 14 nm.

2.5 Conclusion

Preventing the contamination of fluorescence labeling reagent in low molecular weight region observed with the use of the SEAlide-based traceable linker, the turn-on fluorescent traceable linker using SECmide unit was developed. This new linker allowed for visualization of the eluted target protein by fluorescence without addition of a fluorescence labeling reagent. Such an unprecedented character enabled fluorescence-based detection of low molecular weight targets, which cannot be achieved by the SEAlide-based linker.

Chapter 3

Conclusions

1. SEAlide-based traceable linker has been developed. Enrichment (click chemistry, adsorption on streptavidin beads, and elution by the phosphate-induced NCL) and selective labeling of the BSA in a protein mixture (lysate of red blood cell or HCT116) were achieved. Work toward the application of this traceable linker to identification of the targets of target-unknown bioactive compounds is currently in progress.

2. Turn-on fluorescent traceable linker using SECmide has been developed. Enrichment (click chemistry, adsorption on streptavidin beads, and elution by the phosphate-induced reaction) and selective visualization of the BSA in a protein mixture (lysate of red blood cell) were achieved. In terms of the identification of low molecular weight targets, the second-generation turn-on type SECmide based linker compensates for the shortcoming of the SEAlide-based linker.

Experimental Section

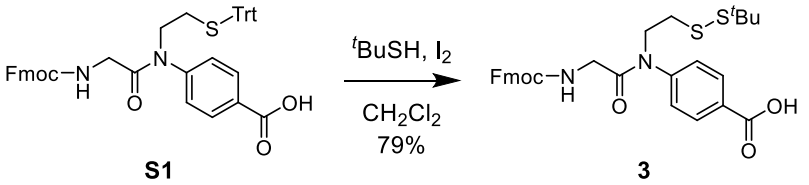
Experimental Section

All reactions of small molecules were carried out under a positive pressure of argon. Column chromatography of the small molecules was performed using Silica Gel 60 N (spherical, neutral, Kanto Chemical Co., Inc.). Mass spectra were recorded on a Waters MICROMASS® LCT PREMIERTM (ESI-TOF) or a Bruker Esquire2000T (ESI-Ion Trap). NMR spectra were recorded using a Bruker AV400N at 400 MHz frequency for ^{1}H, and JEOL JNM-AL300 at 75 MHz frequency for ^{13}C. Optical rotations were measured using a JASCO P-2200 polarimeter (concentration in g/100 mL). For HPLC separations, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil 5C_{18}-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min) or a Cosmosil 5C_{18}-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10.0 mL/min) was employed, and eluting products were detected by UV absorption (220 nm) or fluorescence (λ_{ex} = 495 nm, λ_{em} = 520 nm). A solvent system consisting of 0.1% (v/v) TFA in H_2O and 0.1% TFA (v/v) in MeCN using linear gradient over 30 min was employed for HPLC elution. ECL signals from the western blot analysis and fluorescence were detected using a LAS-4000mini (Fujifilm) and ChemiDoc™ XRS+ (Bio-rad). Composition of an SDS-PAGE sample loading buffer is as follow: 50 mM Tris-HCl, 2.0% (v/v) SDS, 6.0% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.050% (w/v) bromophenol blue in H_2O.

Chapter 1

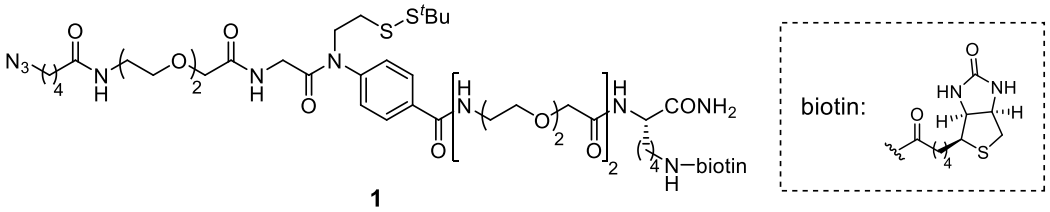
Preparation of disulfide **3**

4-{2-([{(9H-fluoren-9-yl)methoxy}carbonyl]amino)-N-(2-[tert-butylsulfinothioyl]ethyl)acetamide}benzoic acid (3)



To a stirred solution of trityl derivative **S1**[9d] (200 mg, 278 μmol) in CH_2Cl_2 (9.27 mL) were added ^{t}BuSH (156 μL, 1.39 mmol) and I_2 (529 mg, 4.17 mmol) at room temperature, and the mixture was stirred at same temperature for 1 h. Then 0.5 M $Na_2S_2O_3$ aq. was added to the resulting mixture until its dark red color disappeared. The obtained mixture was extracted with EtOAc, and the combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The product was purified by column chromatography ($CHCl_3$/MeOH = 100/0, 99/1, then 0/100 (v/v)) and 124 mg of **3** (220 μmol, 79%) was obtained as white amorphousness: 1H NMR ($CDCl_3$, 400 MHz) δ = 1.29 (9H, s), 2.85 (2H, t, J = 7.5 Hz), 3.81 (2H, s), 4.05 (2H, t, J = 6.8 Hz), 4.20 (1H, t, J = 6.8 Hz), 4.35 (2H, d, J = 7.3 Hz), 5.89 (1H, s), 7.30 (2H, t, J = 7.6 Hz), 7.35 (2H, d, J = 8.4 Hz), 7.39 (2H, t, J = 7.5 Hz), 7.59 (2H, d, J = 7.5 Hz), 7.75 (2H, d, J = 7.4 Hz), 8.12 (2H, d, J = 8.4 Hz); 13C NMR ($CDCl_3$, 75 MHz) δ = 30.0, 37.1, 43.8, 47.2, 48.2, 49.1, 67.6, 120.1, 125.3, 127.2, 127.9, 128.4, 132.2, 141.4, 143.9, 144.6, 156.7 ; HRMS (ESI-TOF) m/z calcd for $C_{30}H_{32}N_2NaO_5S_2$ ($[M + Na]^+$) 587.1650, found 587.1631.

Preparation of traceable linker **1** via peptide resin **6**



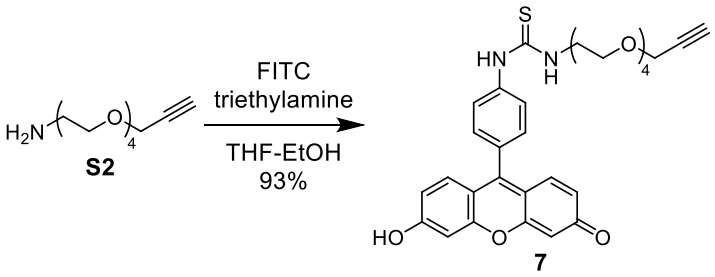
General procedure: The peptides were synthesized using Fmoc SPPS. Building blocks were coupled on NovaSyn® TGR resin (0.22 mmol amine/g). Reagents and solvents are listed below. All coupling reactions were performed for 2 h. Fmoc-removal was achieved using 20% (v/v) piperidine in DMF (10 min).

building block	reagents	solvent
3 (2 eq.)	HATU (1.9 eq.), DIPEA (1.9 eq.)	DMF
(+)-biotin (5 eq.)	DIC (5 eq.), HOBt·H_2O (5 eq.)	DMSO/DMF = 1/1 (v/v)
$N_3(CH_2)_4CO_2H$[12] (5 eq.)	DIC (5.3 eq.), Oxyma Pure®[15] (5 eq.)	DMF
Others (3 eq.)	DIC (3.2 eq.), Oxyma Pure® (3 eq.)	DMF

For removal of an ivDde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl) group, the peptide resin was treated with 2% (v/v) hydrazine hydrate in DMF (twice for 2 h followed by once overnight). After completion of the peptide elongation, the resin was subjected to global deprotection using TFA/triethylsilane/H_2O (95:2.5:2.5 (v/v)) for 2 h at room temperature. After filtration of the resin and subsequent removal of TFA by N_2 flow, the obtained residue was neutralized by the addition of sat. $NaHCO_3$ aq. followed by solid $NaHCO_3$. The obtained mixture was dissolved in 33% (v/v) AcOH aq., and then purified by a preparative HPLC.

1: A white lyophilized powder; 41% yield; Analytical HPLC conditions: 10 to 90%. Retention time = 18.2 min; Preparative HPLC conditions: 36 to 46%; LRMS (ESI-Ion Trap) m/z calcd for $[M + H]^+$ 1256.5, found 1256.7.

Click chemistry of traceable linker **1** with alkynylated small molecule **7** followed by linker cleavage with cysteine



Preparation of alkyne derivative 7: To fluorescein isothiocyanate isomer-I (FITC) (70.1 mg, 180 μmol) were added **S2**[16] (50.0 mg, 216 μmol) in THF/EtOH (2/3 (v/v), 10.5 mL) and triethylamine (30.2 μL, 216 μmol) at 0 °C, and the resulting mixture was stirred at room temperature for 1 h. After evaporation, the resulting residue was purified by column chromatography ($CHCl_3$/MeOH = 98/2, 97/3, 90/10, then 0/100 (v/v)) and 104 mg of **7** (168 μmol, 93%) was obtained as a yellow powder: 1H NMR (CD_3OD, 400 MHz) δ = 2.81 (1H, t, J = 2.5 Hz), 3.63-3.81 (16H, m), 4.14 (2H, d, J = 2.5 Hz), 6.58 (2H, dd, J = 2.5 Hz and 8.8Hz), 6.71-6.73 (4H, m), 7.81 (2H, dd, J = 1.7 Hz and 8.3 Hz), 8.19 (2H, d, J = 1.8 Hz); 13C NMR (CD_3OD, 75 MHz) δ = 45.5, 59.0, 70.0, 70.1, 71.3, 71.43, 71.49,

71.54, 75.9, 80.6, 103.5, 112.1, 114.2, 126.1, 129.2, 130.6, 142.6, 154.7, 162.3, 170.8, 182.9; HRMS (ESI-TOF) m/z calcd for $C_{32}H_{32}N_2NaO_9S$ ($[M + Na]^+$) 643.1726, found 643.1706.

Click chemistry: Traceable linker **1** in DMSO (6.0 mM, 66.6 μL), alkyne **7** in PBS (1.25 mM, 400 μL), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA)[17] in 20% (v/v) DMSO/^{t}BuOH (1.7 mM, 118 μL), $CuSO_4$ in water (50 mM, 40.0 μL), sodium ascorbate in water (25 mM, 40.0 μL), and PBS (416 μL) were added to 1.00 mL of water (final concn.: 0.20 mM **1**, 0.25 mM **7**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 0.50 mM sodium ascorbate). After 1 h of the reaction at room temperature, reaction mixture was injected into a preparative HPLC to yield conjugate **8**.
8: A yellow lyophilized powder; 0.61 mg, 65% yield; Analytical HPLC conditions: 30 to 40%. Retention time = 25.5 min; Preparative HPLC conditions: 35 to 45%; LRMS (ESI-Ion Trap) m/z calcd for $[M + 2H]^{2+}$ 938.9, found 938.7.

Linker cleavage via NCL: To sodium phosphate buffer (1.0 M, pH 7.5, 147.5 μL) containing NP40 (1.0% (v/v)), TCEP·HCl (40 mM), MPAA (100 mM) and cysteine (5.0 mM) was added conjugate **8** in DMF (6.0 mM, 2.5 μL, final concn. 0.10 mM). After incubation at 37 °C for 24 h under argon, completion of cleavage of the linker was confirmed using HPLC and the products were characterized by MS analyses.
9: Analytical HPLC conditions: 25 to 35%. Retention time = 19.4 min; LRMS (ESI-Ion Trap) m/z calcd for $[M + H]^+$ 1069.4, found 1069.3.

Preparation of alkynylated BSA **11**

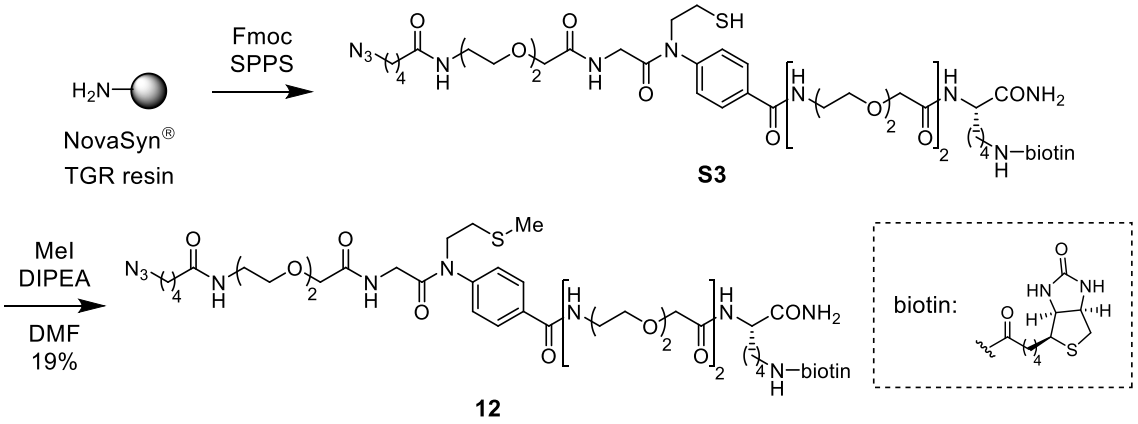
Starting from a commercially available BSA (6.6 mg), the alkynylated BSA **11** was prepared according to the literature[7a]. Briefly, *N*-(1-propynyl)maleimide was added to BSA in PBS (final concentration: 20 μM BSA; 2 mM maleimide). After stirring for 12 h, the protein was purified by acetone precipitation and was dissolved in PBS with 0.1% SDS (1.32 mL) for the following experiments.

SDS-PAGE conditions

After addition of SDS-PAGE sample loading buffer followed by heating at 100 °C for 5 min, the reaction mixture was analyzed using SDS-PAGE in 10% 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 SAv-HRP (GE Healthcare) and ECL plus Western Blotting Detection System (GE

Healthcare). For visualization of all proteins, silver stain KANTO III (KANTO CHEMICAL CO., INC.) or Lumitein™ Protein Gel Stain (Nacalai Tesque) was employed.

Preparation of negative control linker **12**



Negative control **12** were prepared in a manner similar to that of traceable linker **1**. For preparation of negative control **12**, SEAlide **S1** was employed instead of **2**. Then, methyl iodide (15 μL, 0.24 mmol) and DIPEA (15 μL, 86 μmol) were added to a solution of **S3** (3.5 mg, 3.0 μmol) in DMF (3.4 mL). The resulting mixture was stirred at room temperature for 1 h, and the obtained solution was purified by preparative HPLC to yield linker **12** (0.67 mg, 19% yield).

S3: A white lyophilized powder; 24% yield; Analytical HPLC conditions: 10 to 90%. Retention time = 14.2 min; Preparative HPLC conditions: 25 to 35%; LRMS (ESI-Ion Trap) m/z calcd for $[M + H]^+$ 1168.5, found 1168.0.

12: A white lyophilized powder; Analytical HPLC conditions: 10 to 60%. Retention time = 18.9 min; Preparative HPLC conditions: 10 to 60%; LRMS (ESI-Ion Trap) m/z calcd for $[M + H]^+$ 1182.6, found 1182.6.

Introduction of traceable linker **1** and negative control **12** onto alkynylated BSA **11**

Click chemistry: To a mixture of PBS (540 μL) and water (437 μL) were added the alkynylated BSA **11** in PBS with 0.1% SDS (5.0 g/L, 200 μL), traceable linker **1**, negative control linker **12** in DMSO (6.0 mM, 25.0 μL), TBTA in 20% (v/v) DMSO/^{t}BuOH (1.7 mM, 88.0 μL), $CuSO_4$ aq. (50 mM, 30.0 μL), sodium ascorbate aq. (50 mM, 30.0 μL), and SDS aq. (10% (w/v), 150 μL) (final concn.: 0.67 g/L alkynylated BSA, 0.10 mM **1**, or **12**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 1.0 mM sodium ascorbate, 1% (v/v) SDS). After the reaction at room temperature for 1 h, small molecules were removed by acetone precipitation.

Adsorption on streptavidin beads followed by elution of BSA conjugate

Adsorption on streptavidin beads: After the click chemistry, Pierce® Streptavidin UltraLink® Resin (50 μL, Thermo SCIENTIFIC) was added to the reaction mixture containing ca. 100 μg BSA and its derivatives in PBS with 0.1% SDS (200 μL). After the adsorption at room temperature for 1 h, the resulting resin was washed with 0.1% SDS in PBS five times and it was subjected to subsequent reactions.

Elution of BSA conjugate by linker cleavage: To the resulting streptavidin beads was added cysteine-fluorescein conjugate **15** (10 mM, 2.0 μL, final concn. 0.10 mM) in sodium phosphate buffer (0.40 M, pH 7.4, 198 μL) containing SDS (0.1%), TCEP·HCl (40 mM) and MPAA (50 mM). The reaction was conducted at 37 °C for 24 h under argon. After centrifugation of the resulting mixture (2000 rpm, 2 min), supernatant was collected and the precipitate was suspended in 100 μL PBS with 0.1% SDS. The suspension was subjected to centrifugation (2000 rpm, 2 min) again and the obtained supernatant was combined with the first one.

Elution of proteins remaining on streptavidin beads by denaturation: The resin obtained after the linker cleavage as mentioned above was suspended in 2 × SDS-PAGE sample loading buffer (25 μL) and water (25 μL), and the mixture was heated at 100 °C for 5 min. After centrifugation as mentioned in the section "Elution of BSA Conjugate by Linker Cleavage", the combined supernatant was concentrated by ultrafiltration (Amicon® Ultra-0.5, Ultracel-10 Membrane, 10 kDa, Merk Millipore, 14000 × g, 15 min) and analyzed using SDS-PAGE.

Enrichment and Selective Labeling of BSA in Protein Mixture

Preparation of the protein mixture: A lysate of red blood cells (BIZCOM JAPAN) in PBS was prepared according to a protocol on Vender's protocol[18]. To the lysate (1.1 mL) was added the alkynylated BSA **11** in PBS with 0.1% SDS (20 μL) and the obtained mixture was used for the following experiments. For preparation of a lysate of HCT116 cells, HCT116 cells were cultured in DMEM (Dulbecco's modified Eagle medium) containing 10% fetal bovine serum and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin) at 37 °C in a humidified incubator continuously flushed with a mixture of 5% CO_2-95% air. The cells were washed twice with cold PBS, harvested, and then sonicated in HEPES buffered saline (HBS). The cell extracts were centrifuged at 20,000 × g for 10 min at 4 °C. Protein concentration of resultant supernatants was determined with a DC protein assay kit (Bio-Rad) with BSA as the standard. To the obtained protein mixture (4.26 mg/mL, 100 μL) was added the alkynylated BSA in PBS with 0.1% SDS (2.6 μL) and the obtained mixture was used for following experiments.

Click chemistry in the Red Blood Cell lysate: To the alkynylated BSA **11** in the red blood cell lysate (1.12 mL) were added traceable linker **1** in DMSO (6.0 mM, 25 μL), TBTA in 20% (v/v) DMSO/^{t}BuOH (1.7 mM, 88 μL), $CuSO_4$ aq. (50 mM, 30 μL), sodium ascorbate aq. (50 mM, 30 μL), SDS aq. (10% (w/v), 150 μL) and PBS (57 μL) (final concon.: 0.10 mM **1**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 1.0 mM sodium ascorbate, 1% (v/v) SDS). After reaction at room temperature for 1 h, the reagents were removed by acetone precipitation. Then, the obtained precipitate was dissolved in 0.1% SDS in PBS (1.2 mL) for the following experiments.

Click chemistry in the lysate of HCT116 cells: To the alkynylated BSA **11** in the lysate of HCT116 cells (102.6 μL) were added traceable linker **1** in DMSO (6.0 mM, 3.3 μL), TBTA in 20% (v/v) DMSO/^{t}BuOH (1.7 mM, 11.6 μL), $CuSO_4$ aq. (50 mM, 4.0 μL), sodium ascorbate aq. (50 mM, 4.0 μL), SDS aq. (10% (w/v), 19.8 μL) and PBS (52.7 μL) (final concon.: 0.10 mM **1**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 1.0 mM sodium ascorbate, 1% (v/v) SDS). After reaction at room temperature for 1 h, the reagents were removed by acetone precipitation. Then, the obtained precipitate was dissolved in 0.1% SDS in PBS (600 μL) for the following experiments.

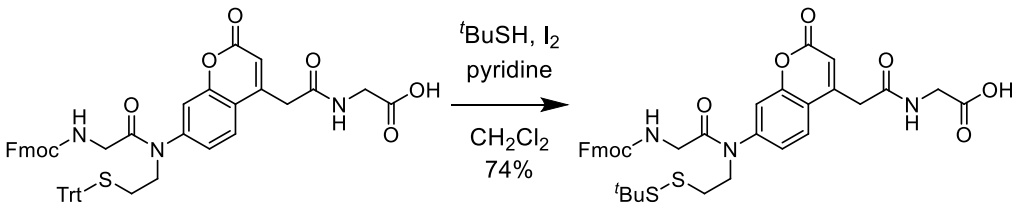
Adsorption on streptavidin beads followed by cleavage: It was performed in a manner similar to that described in a section “Adsorption on Streptavidin Beads Followed by Elution of BSA Conjugate”.

Enrichment without linker cleavage as similar to conventional linkers: After adsorption of the protein mixture on streptavidin beads, the proteins on the beads were eluted and analyzed as mentioned in a section “Elution of Proteins Remaining on Streptavidin Beads”.

Chapter 2

Preparation of disulfide **19**

(2-{7-[N-(Fmoc-Gly)-N-(2-tert-butylsulfinothioylethyl)amino]coumarin-4-acetylamino})-acetic acid (19)



S4 **19**

To a stirred solution of trityl derivative **S4**[14] (429 mg, 0.50 mmol) in CH_2Cl_2 (16.7 mL) were added ^{t}BuSH (281 μL, 2.5 mmol), I_2 (952 mg, 7.5 mmol) and pyridine (816 μL, 10.5 mmol) at room temperature, and the mixture was stirred at the same temperature for 2 h. Then 0.5 M $Na_2S_2O_3$ aq. was added to the resulting mixture until its dark red color disappeared. The obtained mixture was extracted with EtOAc, and the combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The product was purified by column chromatography ($CHCl_3$/MeOH = 20/1 (v/v)) and 259 mg of **19** (0.37 mmol, 75%) was obtained as white amorphousness: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.30 (9H, s), 2.87 (2H, t, J = 6.8 Hz), 3.69 (2H, br s), 3.79 (2H, s,), 3.94 (2H, s,), 4.05 (2H, t, J = 6.5 Hz), 4.16 (1H, t, J = 6.5), 4.31 (2H, d, J = 7.0 Hz), 5.94 (1H, br s), 6.51 (1H, s), 7.15 (1H, d, J = 8.8 Hz), 7.25-7.30 (3H, m), 7.38 (2H, t, J = 7.4 Hz), 7.55 (2H, d, 7.5 Hz), 7.73-7.75 (3H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 29.8, 37.0, 39.9, 41.2, 43.6, 46.9, 48.1, 48.9, 67.4, 116.6, 117.6, 119.2, 120.0, 124.4, 125.0, 127.1, 127.8, 141.2, 143.3, 143.6, 148.8, 154.2, 156.5, 160.1, 168.0, 168.5, 171.5 ; HRMS (ESI-TOF) m/z calcd for $C_{36}H_{37}N_3NaO_8S_2$ ($[M + Na]^+$) 726.1920, found 726.1910.

Preparation of traceable linker **16** via peptide resin **20**

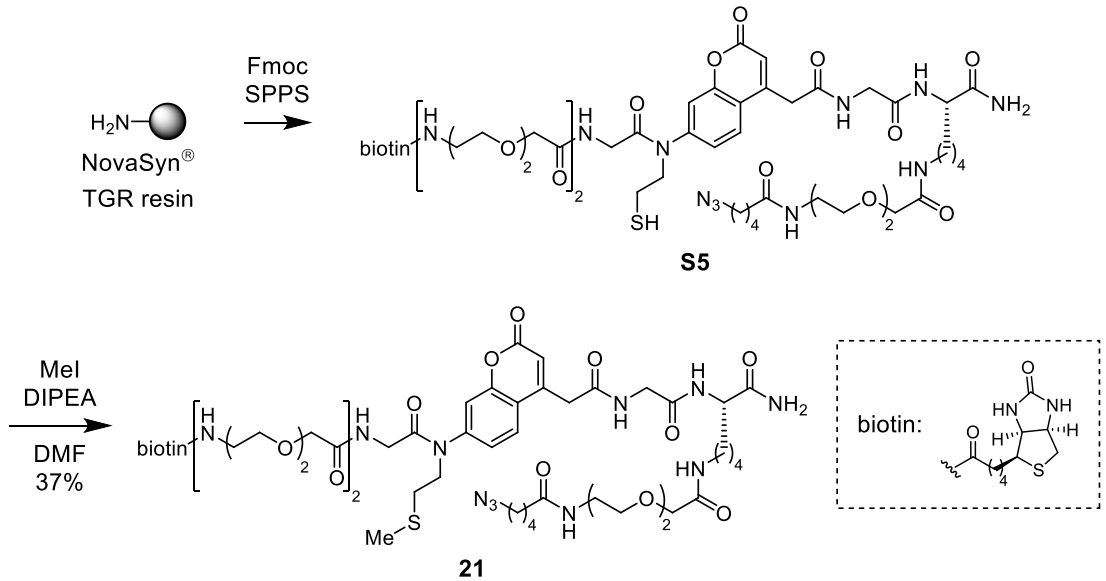
General procedure: The peptides were synthesized using Fmoc SPPS. Building blocks were coupled on NovaSyn® TGR resin (0.22 mmol amine/g). Reagents and solvents are listed below. All coupling reactions were performed for 2 h. Fmoc-removal was achieved using 20% (v/v) piperidine in DMF (10 min).

building block	reagents	solvent
19 (2 eq.)	HATU (1.9 eq.), DIPEA (1.9 eq.)	DMF
(+)-biotin (5 eq.)	DIC (5 eq.), HOBt·H_2O (5 eq.)	DMSO/DMF = 1/1 (v/v)
$N_3(CH_2)_4CO_2H$[12] (3 eq.)	DIC (3.3 eq.), HOBt·H_2O (3 eq.)	DMF
Others (3 eq.)	DIC (3.3 eq.), HOBt·H_2O (3 eq.)	DMF

For removal of an Alloc group, the peptide resin was treated with 1,3-dimethylbarbituric acid and $Pd(PPh_3)_4$ in $C_2H_4Cl_2$. After completion of the peptide elongation, the resin was subjected to global deprotection using TFA/H_2O (97.5:2.5 (v/v)) for 2 h at room temperature. After filtration of the resin and subsequent removal of TFA by N_2 flow, the obtained residue was neutralized by the addition of sat. $NaHCO_3$ aq. followed by solid $NaHCO_3$. The obtained mixture was dissolved in 20% (v/v) CH_3CN/H_2O containing 0.1% TFA, and then purified by a preparative HPLC.

16: A white lyophilized powder; 23% yield; Analytical HPLC conditions: 10 to 60%. Retention time = 22.8 min; Preparative HPLC conditions: 27 to 37%; LRMS (ESI-Ion Trap) m/z calcd for $[M + H]^+$ 1395.7, found 1395.7.

Preparation of negative control linker **21**



Negative Linker **21** were prepared in a manner similar to that of traceable linker **16**. For preparation of negative control **21**, SECmide **S4** was employed instead of **19**. Then, methyl iodide (8.46 μL, 0.136 mmol) and DIPEA (8.37 μL, 48.8 μmol) were added to a solution of **S5** (2.22 mg, 1.7 μmol) in DMF (1.7 mL). The resulting mixture was stirred at room temperature for 1 h, and the obtained solution was purified by preparative HPLC

to yield linker **21** (0.84 mg, 37% yield).

S5: A white lyophilized powder; 20% yield; Analytical HPLC conditions: 10 to 60%. Retention time = 17.8 min; Preparative HPLC conditions: 18 to 28%; LRMS (ESI-Ion Trap) m/z calcd for $[M + H]^+$ 1307.5, found 1307.7.

21: A white lyophilized powder; Analytical HPLC conditions: 10 to 60%. Retention time = 18.18 min; Preparative HPLC conditions: 1% for 10 min, then 10 to 50% over 30 min; LRMS (ESI-Ion Trap) m/z calcd for $[M + H]^+$ 1321.5, found 1321.8.

SDS-PAGE conditions

After addition of SDS-PAGE sample loading buffer followed by heating at 100 °C for 5 min, the reaction mixture was analyzed using SDS-PAGE in 10% 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 SAv-HRP (GE Healthcare) and ECL plus Western Blotting Detection System (GE Healthcare). For visualization of all proteins, CBB (Nacalai Tesque) was employed.

Introduction of traceable linker **16** and negative control **21** onto alkynylated BSA **11**

Click chemistry: To a mixture of TBS (135 μL) and water (109 μL) were added the alkynylated BSA **11** in TBS (5.0 g/L, 50 μL), traceable linker **16**, negative control linker **21** in DMSO (6.0 mM, 6.25 μL), TBTA in 20% (v/v) DMSO/^{t}BuOH (1.7 mM, 22.0 μL), $CuSO_4$ aq. (50 mM, 7.5 μL), sodium ascorbate aq. (50 mM, 7.5 μL), and SDS aq. (10% (w/v), 37.5 μL) (final concn.: 0.67 g/L alkynylated BSA, 0.10 mM **16**, or **21**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 1.0 mM sodium ascorbate, 1% (v/v) SDS). After the reaction at room temperature for 1 h, small molecules were removed by acetone precipitation.

Adsorption on streptavidin beads followed by elution of BSA conjugate

Adsorption on streptavidin beads: After the click chemistry, Pierce™ Streptavidin Agarose Resin (50 μL, Thermo SCIENTIFIC) was added to the reaction mixture containing ca. 20 μg BSA and its derivatives in TBS with 0.1% SDS (200 μL). After the adsorption at room temperature for 1 h, the resulting resin was washed with 0.1% SDS in TBS five times and it was subjected to subsequent reactions.

Elution of BSA conjugate by linker cleavage: To the resulting streptavidin beads was added cysteine (10 mM, 2.0 μL, final concn. 0.10 mM) in sodium phosphate buffer (0.40 M, pH 7.0, 198 μL) containing SDS (0.1%), TCEP·HCl (40 mM) and MPAA (50 mM). The reaction was conducted at 37 °C for 24 h under argon. After centrifugation of the

resulting mixture (2000 rpm, 2 min), supernatant was collected and the precipitate was suspended in 100 μL TBS with 0.1% SDS. The suspension was subjected to centrifugation (2000 rpm, 2 min) again and the obtained supernatant was combined with the first one.

Elution of proteins remaining on streptavidin beads by denaturation: The resin obtained after the linker cleavage as mentioned above was suspended in 2 × SDS-PAGE sample loading buffer (25 μL) and water (25 μL), and the mixture was heated at 100 °C for 5 min. After centrifugation as mentioned in the section "Elution of BSA Conjugate by Linker Cleavage", the combined supernatant was concentrated by ultrafiltration (Amicon® Ultra-0.5, Ultracel-10 Membrane, 10 kDa, Merk Millipore, 14000 × g, 15 min) and analyzed using SDS-PAGE.

Enrichment and selective labeling of BSA in protein mixture

Preparation of the protein mixture: A lysate of red blood cells (BIZCOM JAPAN) in TBS was prepared according to a protocol as described in Chapter 1. To the lysate (8.5 mL) was added the alkynylated BSA in TBS with 0.1% SDS (7.65 μL) and the obtained mixture was used for the following experiments.

Click chemistry in the red blood cell lysate: To the alkynylated BSA in the red blood cell lysate (1.18 mL) were added traceable linker **16** in DMSO (6.0 mM, 25 μL), TBTA in 20% (v/v) DMSO/*t*BuOH (1.7 mM, 88 μL), $CuSO_4$ aq. (50 mM, 30 μL), sodium ascorbate aq. (50 mM, 30 μL), SDS aq. (10% (w/v), 150 μL) (final concon.: 0.10 mM **16**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 1.0 mM sodium ascorbate, 1% (v/v) SDS). After reaction at room temperature for 1 h, the reagents were removed by acetone precipitation. Then, the obtained precipitate was dissolved in 0.1% SDS in TBS (1.0 mL) for the following experiments.

Adsorption on streptavidin beads followed by cleavage: It was performed in a manner similar to that described in a section "Adsorption on Streptavidin Beads Followed by Elution of BSA Conjugate".

Enrichment without linker cleavage as similar to conventional linkers: After adsorption of the protein mixture on streptavidin beads, the proteins on the beads were eluted and analyzed as mentioned in a section "Elution of Proteins Remaining on Streptavidin Beads".

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List of publications

This study was published in the following papers.

Chapter 1

An *N*-sulfanylethylanilide-based traceable linker for enrichment and selective labelling of target proteins

Takuya Morisaki, Masaya Denda, Jun Yamamoto, Daisuke Tsuji, Tsubasa Inokuma, Kohji Itoh, Akira Shigenaga and Akira Otaka

Chem.Commun. **2016**, *52*, 6911–6913.

Chapter 2

A turn-on fluorescent traceable linker using SECmide for purification and selective visualization of target proteins

Takuya Morisaki, Akira Shigenaga and Akira Otaka

manuscript in preparation