Development of Stimulus-responsive Amino Acids and Their Use in Chemical Biology Field

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Development of Stimulus-responsive Amino Acids and Their Use in Chemical Biology Field

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Abbreviations

Ac	acetyl
Bn	benzyl
Boc	*tert*-butoxycarbonyl
BSA	bovine serum albumin
Bu	butyl
CuAAC	Cu-catalyzed azide-alkyne cycloaddition
DEAD	diethyl azodicarboxylate
DIC	*N*,*N'*-diisopropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DMNB	4,5-dimethoxy-2-nitrobenzyl
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
ESI-MS	electrospray ionization mass spectrometry
Et	ethyl
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
ivDde	1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl
Me	methyl
MESNa	2-mercaptoethanesulfonic acid sodium salt
MOPS	3-morpholinopropanesulfonic acid
NIR	near-infrared
NP40	Nonidet P-40
Oxyma pure	ethyl cyanoglyoxylate-2-oxime
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCC	pyridinium chlorochromate
PDC	pyridinium dichromate
PEG	polyethylene glycol
PG	protective group
Ph	phenyl

*p*Ns	*p*-nitrobenzenesulfonyl
PVDF	polyvinylidene fluoride
rt	room temperature
SAv-HRP	streptavidin-horseradish peroxidase conjugate
SDS	sodium dodecylsulfate
SPPS	solid phase peptide synthesis
Su	succinimide
TBAF	tetrabutylammonium fluoride
TBDPS	*tert*-butyldiphenylsilyl
TBS	*tert*-butyldimethylsilyl
TBTA	tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
THF	tetrahydrofuran
TMAD	*N*,*N*,*N*',*N*'-tetramethylazodicarboxamide
Trt	triphenylmethyl
UV	ultraviolet

Contents

Preface

Development of a methodology to control the function of peptides and proteins in a spatiotemporal manner is indispensable in various fields such as chemical biology and drug delivery. Photo-induced bond cleavage or conformational change in the peptide/protein backbone has been successfully applied to convert inactive (or active) peptides and proteins into their active (or inactive) forms at the desired time and location.[1,2] Recently, we have developed a stimulus-responsive amino acid that induces amide bond cleavage after stimulus-induced removal of a phenolic protective group (PG) followed by lactonization of a trimethyl lock moiety (Figure 1).[3,4]

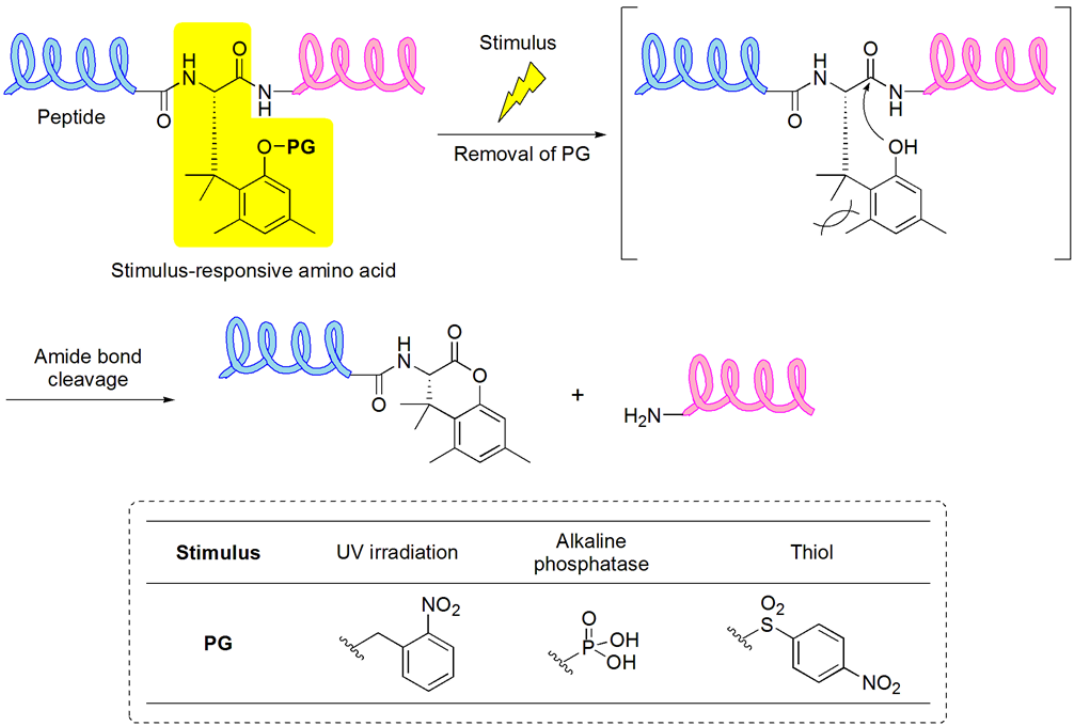

Figure 1. Stimulus-responsive amide bond cleavage induced by a stimulus-responsive amino acid (**PG**: a protective group removable by an appropriate stimulus).

The stimulus-responsive amino acid can be used to respond to various stimuli by replacing the phenolic protective group. We have already developed ultraviolet (UV) irradiation (PG = *o*-nitrobenzyl)-, phosphatase (PG = phosphate)-, and thiol (PG = *p*-nitrobenzenesulfonyl)-responsive amino acids with their practical applications.[3a,c]

In this study, I examined the development of novel stimulus-responsive amino acids and their applications to chemical biology including purification of target proteins.

In Chapter 1, development of a near-infrared two-photon excitation-responsive peptide and its photo-responsive amide bond cleavage reaction are described. Photophysical properties of the peptide with the near-infrared two-photon excitation-responsive amino acid are also presented.

Application of a stimulus-responsive amino acid attached to a traceable linker for enrichment and selective labeling of target proteins is presented in Chapters 2 and 3.

In Chapter 2, I describe the development of a prototype traceable linker possessing a fluoride-responsive amino acid and examine a pull-down experiment for a model protein.

In Chapter 3, development of a thiol-responsive traceable linker is described. The resulting traceable linker was successfully applied to pull-down enrichment and selective labeling of a target protein in a protein mixture.

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Chapter 1. Development of a Near-infrared Two-photon Excitation-responsive Peptide Capable of Photo-responsive Amide Bond Cleavage

Summary

A near-infrared two-photon excitation-responsive amino acid possessing a 4,5-dimethoxy-2-nitrobenzyl group as a phenolic protection was developed. By introducing this amino acid into a model peptide, amide bond cleavage could be induced by near-infrared two-photon excitation. Furthermore, the photophysical properties of the photo-responsive model peptide were also clarified.

Methodologies to spatially and temporally control peptide/protein functions by a stimulus have received increased attention owing to those potential usefulness in various fields such as chemical biology and drug delivery. UV irradiation-induced bond cleavage or conformational change of a peptide/protein backbone has been successfully applied to control peptide/protein function.[1,2] A typical example is a UV-responsive inactivation system developed by Kron et al., where an active yeast peptide pheromone analog containing a UV-responsive 2-nitro-β-phenylalanine was converted to an inactive form by UV-induced main chain cleavage (Figure 2).[1g]

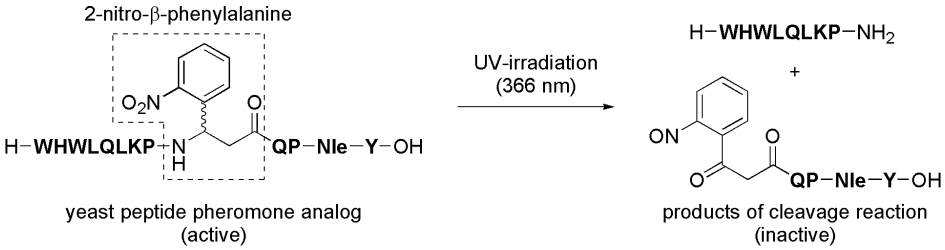


Figure 2. UV-responsive functional control of the peptide pheromone analog (**Nle**: L-norleucine; **H**: histidine; **K**: lysine; **L**: leucine; **P**: proline; **Q**: glutamine; **W**: tryptophan; **Y**: tyrosine).

UV irradiation, however, sometimes causes serious damage to living organisms. In addition, UV one-photon photolysis occurs along the optical path in an imprecise three-dimensional manner. These problems could be overcome by near-infrared (NIR) two-photon photolysis. Compared with UV, NIR is less damaging to cells because its wavelength is longer. Furthermore, NIR two-photon photolysis occurs with three-dimensional precision owing to simultaneous absorption of two photons at the

point of focus of a femtosecond pulsed laser (Figure 3).[3] Therefore, if NIR two-photon photolysis could induce amide bond cleavage, the problems arising from the UV irradiation would be overcome. To my knowledge, amide bond cleavage triggered by NIR two-photon photolysis has yet to be reported.

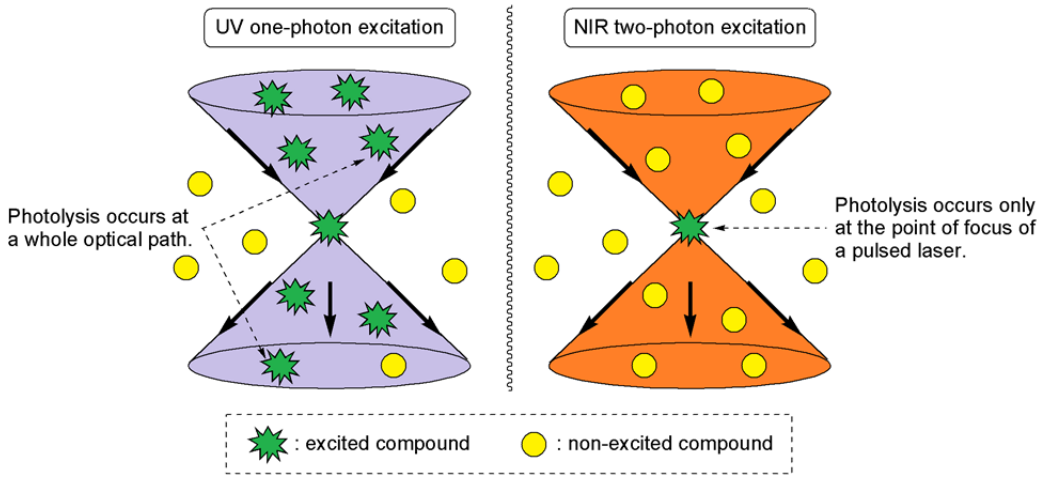
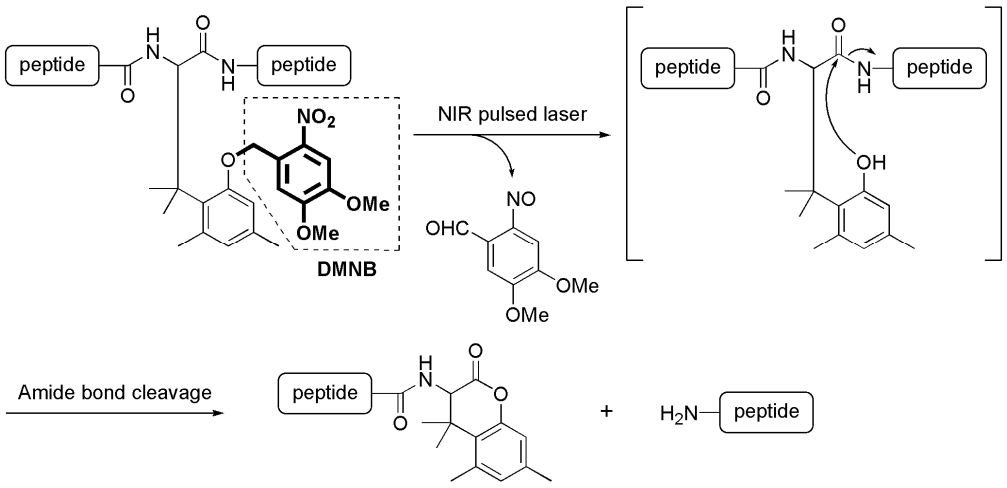


Figure 3. Comparison of UV one-photon excitation and NIR two-photon excitation. The double cones symbolize beams of UV and NIR.

In this chapter, I describe the development of an NIR two-photon excitation (2PE)-responsive peptide and its photo-reactivity when exposed to a focused NIR pulsed laser.

Previously, we developed a stimulus-responsive processing (amide bond cleavage) device and used this device to control peptidyl function in living cells.[4] In the UV one-photon excitation (1PE)-responsive system developed by our group,[4b,4c] UV-induced removal of PG on phenolic groups triggers amide bond cleavage (Figure 1, PG = *o*-nitrobenzyl). I envisioned that NIR 2PE-induced amide bond cleavage could be achieved by introduction of phenolic protective groups that are susceptible to removal by NIR 2PE.

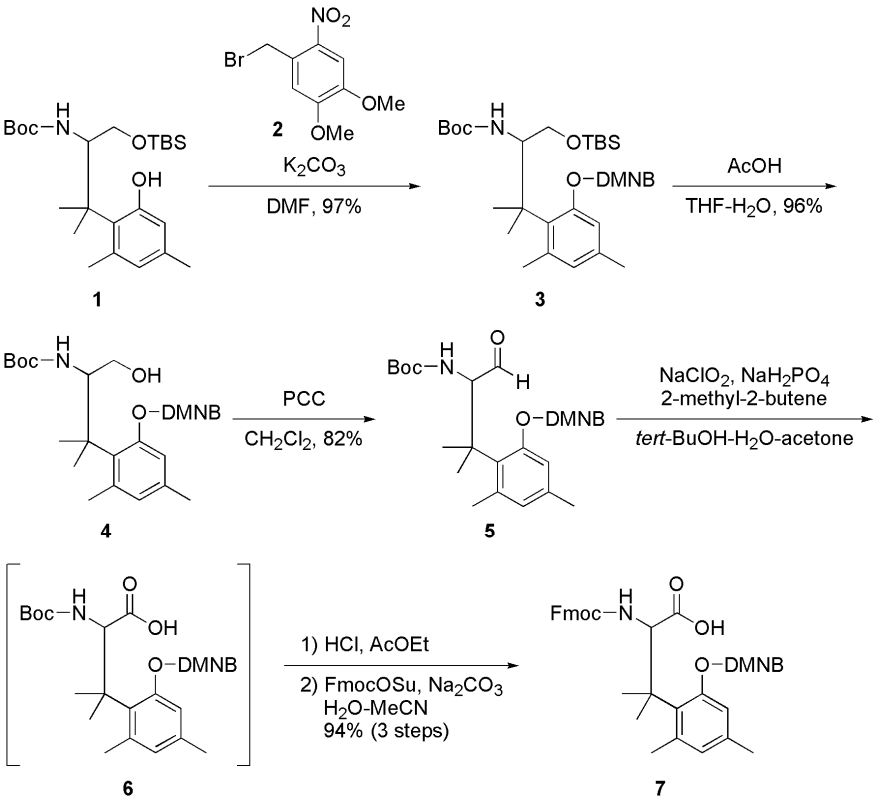
A 4,5-dimethoxy-2-nitrobenzyl (DMNB) group was originally developed by Patchornik et al. as a UV 1PE-responsive protective group,[5] and it has recently been applied to NIR 2PE-responsive caged compounds to control function in living cells.[6] I designed a system of NIR 2PE-responsive amide bond cleavage in which the DMNB group serves as the phenolic protective group of the stimulus-responsive amino acid (Scheme 1).



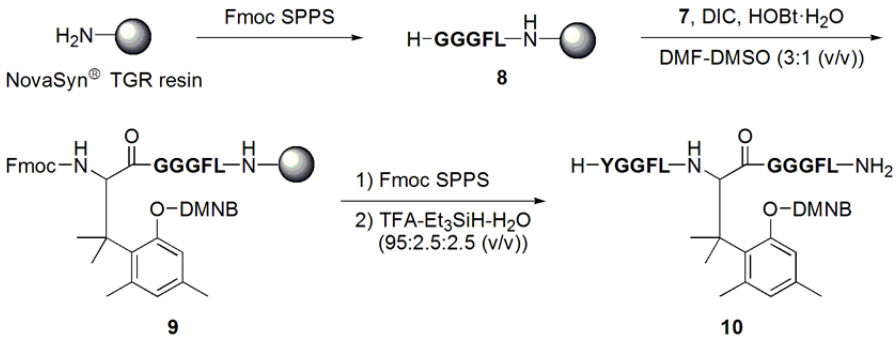
Scheme 1. Design of the NIR 2PE-responsive amide bond cleavage system.

At first, racemic Fmoc-protected NIR 2PE-responsive amino acid **7** was synthesized (Scheme 2). Phenol **1**[4c] was alkylated with DMNB bromide **2**[7] in the presence of K_2CO_3 to yield DMNB ether **3**. After removal of the TBS group of **3** under acidic conditions, the generated hydroxyl group of **4** was oxidized with PCC to give aldehyde **5**. After transformation of aldehyde **5** into carboxylic acid **6** by Pinnick oxidation, the Boc group of **6** was replaced with an Fmoc group by treatment with an acid followed by reaction with FmocOSu to generate the desired Fmoc-protected NIR 2PE-responsive amino acid **7**. The total yield of the conversion from phenol **1** to the amino acid derivative **7** was 72% over six steps.

Next, the resulting racemic NIR 2PE-responsive amino acid derivative **7** was incorporated into model peptide **10** using Fmoc-based solid phase peptide synthesis (Fmoc SPPS) (Scheme 3). Peptide resin **8** was prepared using standard Fmoc SPPS starting from NovaSyn® TGR resin. The Fmoc amino acid derivative **7** was attached to the peptide resin **8** by *N*,*N'*-diisopropylcarbodiimide (DIC) and 1-hydroxylbenzotriazole (HOBt) in DMF-DMSO (3:1 [v/v])[8] to produce peptide resin **9**. After elongation of the peptide chain of **9** using Fmoc SPPS, the protected peptide resin was treated with TFA-Et_3SiH-H_2O (95:2.5:2.5 [v/v]) to generate NIR 2PE-responsive model peptide **10**. The diastereomeric peptides derived from racemic NIR 2PE-responsive amino acid derivative **7** were separated by preparative HPLC, and the diastereomerically pure peptide **10** (eluted earlier) was used for subsequent photolysis experiments.

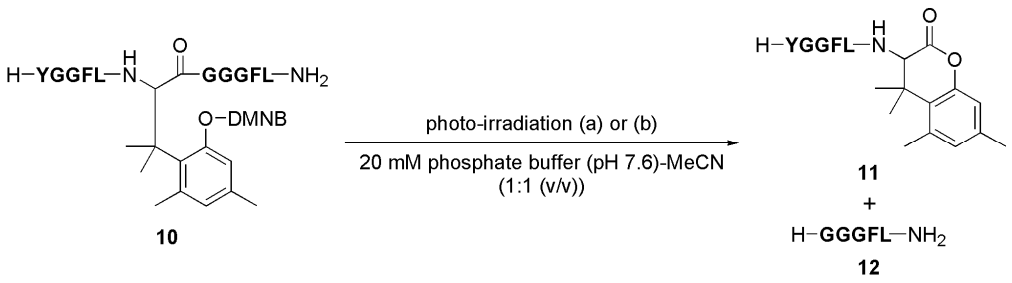


Scheme 2. Synthesis of NIR 2PE-responsive amino acid **7**.



Scheme 3. Synthesis of NIR 2PE-responsive model peptide **10** (**F**: phenylalanine; **G**: glycine).

The photo-responsive amide bond cleavage reactions of the model peptide **10** were next examined (Scheme 4). Prior to the NIR two-photon photolysis experiment, I performed a UV one-photon photolysis experiment to obtain standard processing products (Scheme 4a). Model peptide **10** in 50% (v/v) acetonitrile in 20mM phosphate buffer (pH 7.6) was irradiated by UV (>365 nm) for 3 min and incubated at 37 ˚C.



Scheme 4. Photo-responsive amide bond cleavage reaction of the model peptide **10** by (a) UV (>365 nm) or (b) NIR pulsed laser (740 nm) irradiation.

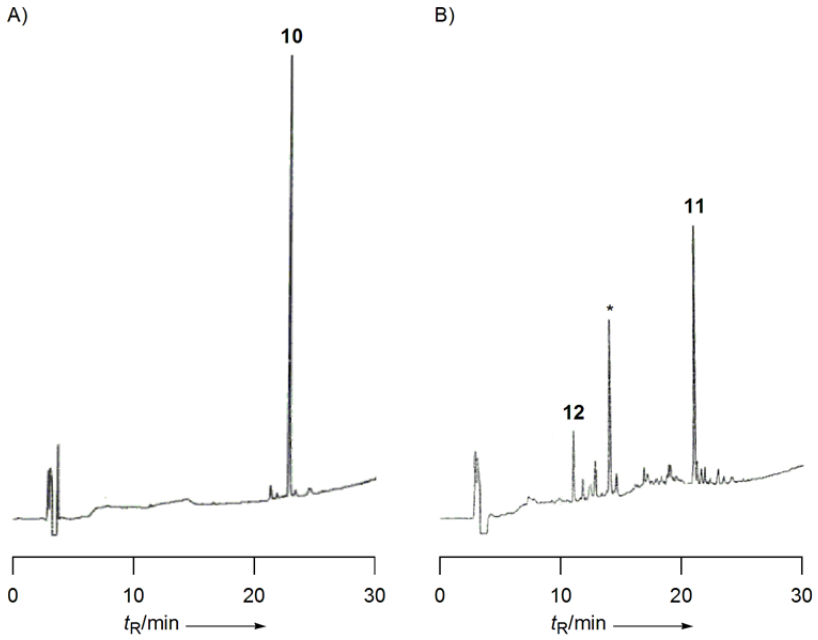


Figure 4. HPLC monitoring of the reaction as shown in Scheme 4a. (A) Before UV irradiation. (B) After 3 min of UV irradiation followed by 4 h of incubation at 37 ˚C. Analytical HPLC conditions: linear gradient of 0.1% (v/v) TFA-MeCN in 0.1% (v/v) TFA aq., 10 to 80% over 30 min. *4,5-dimethoxy-2-nitrosobenzaldehyde generated by photolysis of DMNB groups.

Reaction progress was monitored by HPLC and the resulting peptides were characterized by electrospray ionization mass spectrometry (ESI-MS). After 4 h of incubation, peptide **10** was completely converted to the corresponding processing products **11** and **12** (Figure 4). The time course of photolysis by irradiation with 365 nm UV light (1.41×10^{16} photon·s^{-1}) indicated that the uncaging (photo-induced removal of

DMNB group) reaction of peptide **10** showed first-order dependence on the concentration of peptide **10** (Figure 5). Details of the photophysical properties of peptide **10** will be discussed later.

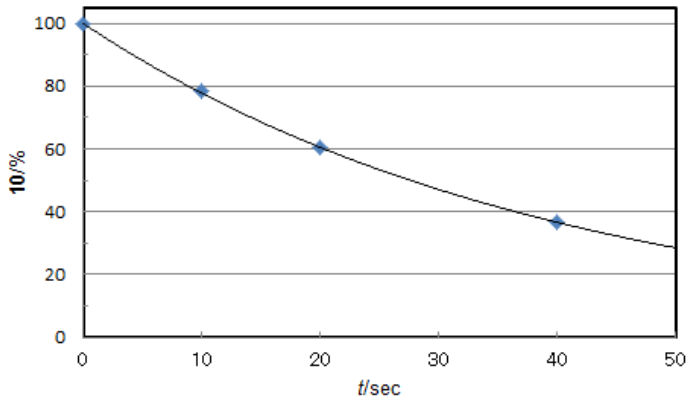


Figure 5. Time course study for photolysis of peptide **10** carried out under UV irradiation at 365 nm (light intensity: 1.41×10^{16} photon·s^{-1}).

Next, the two-photon excitation reaction with peptide **10** using a focused NIR pulsed laser was examined (Scheme 4b). The solution of peptide **10** in 50% (v/v) acetonitrile in 20 mM phosphate buffer (pH 7.6) was irradiated with a focused NIR pulsed laser (740 nm, 3.48×10^{12} photon·s^{-1}) and the time course of photolysis was monitored by HPLC. It was found that the uncaging reaction of peptide **10** induced by focused NIR pulsed laser irradiation also showed first-order dependence on the concentration of peptide **10** (Figure 6).

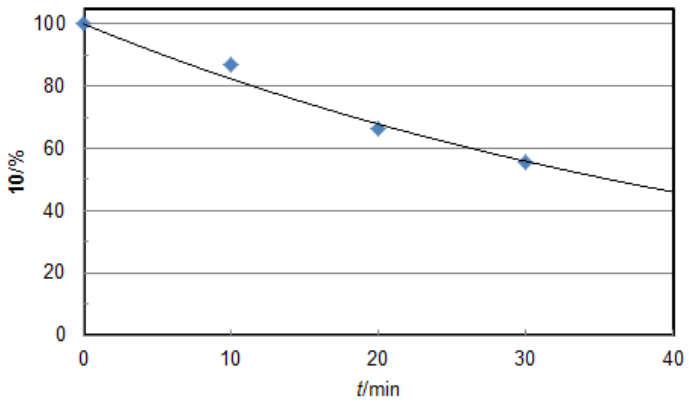


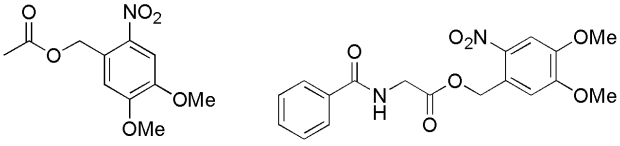
Figure 6. Time course study for photolysis of peptide **10** carried out under NIR pulsed laser irradiation at 740 nm (light intensity: 3.48×10^{12} photon·s^{-1}).

Based on the results of Figures 5 and 6, the photophysical properties of peptide **10** were examined. The quantum yield of peptide **10** for UV 1PE was estimated based on the decay curve (Figure 5) and molar extinction coefficient. The results are summarized in Table 1.

Table 1. Photophysical properties of photo-responsive compounds.

	10[a]	**13**[b]	**14**[c]
ε_{365}[d]	5,890	5,200	3,180[e]
Φ_{365}[f]	0.080	0.005	0.080
$\Phi\varepsilon_{365}$[g]	471	26	254[h]
δ_{u740}[i]	0.23	0.03	-

[a] Solvent: 50% (v/v) MeCN in 20 mM phosphate buffer (pH 7.6).
[b] Solvent: K-MOPS buffer (pH 7.2, 10.0 mM MOPS, 100 mM KCl).
[c] Solvent: 60% (v/v) MeCN in H_2O.
[d] Molar absorptivity at 365 nm ($M^{-1}\cdot cm^{-1}$).
[e] Molar absorptivity at 380 nm ($M^{-1}\cdot cm^{-1}$).
[f] Quantum yield of disappearance of starting materials upon 365 nm irradiation.
[g] Product of photolysis quantum yield and molar absorptivity at 365 nm ($M^{-1}\cdot cm^{-1}$).
[h] Product of photolysis quantum yield and molar absorptivity at 380 nm ($M^{-1}\cdot cm^{-1}$).
[i] Two-photon uncaging action cross-section at 740 nm (GM).



13 **14**

The molar extinction coefficient of peptide **10** at 365 nm (ε_{365}) is similar to that of reference compound **13**,[9] which also possesses a DMNB group, whereas the quantum yield of peptide **10** disappearance at 365 nm (Φ_{365}) is higher than that of compound **13** disappearance. Although the reason for this high quantum yield is unclear so far, I speculate that it is not due to solvent effects[10] but rather to the influence of an amino acid moiety. Actually, the photophysical parameters (ε_{365} and Φ_{365}) of **13** in 50% (v/v) acetonitrile in 20 mM phosphate buffer (pH 7.6) were estimated as 5,063 $M^{-1}\cdot cm^{-1}$ and 0.003, respectively. And these values are almost identical to those in 3-morpholinopropanesulfonic acid (MOPS) buffer. Furthermore, my hypothesis is also supported by Singh's report in which DMNB derivative **14** possessing an amino acid moiety showed high quantum yield (0.080).[11]

Based on the decay curve depicted in Figure 6, the two-photon uncaging action cross-section at 740 nm (δ_{u740}) of photolyzable caged peptide **10** was estimated as 0.23 GM (Goeppert- Meyer, 10^{-50} $cm^4\cdot s\cdot photon^{-1}$) (Table 1). This δ_{u740} value is higher than

that reported for DMNB derivatives including reference compound **13**,[9,12] presumably due to high quantum yield of peptide **10** as mentioned above. According to the literature,[9] a δ_u value exceeding 0.1 GM is preferable for biological application of NIR 2PE-responsive caged compounds. The NIR 2PE-responsive amide bond cleavage system, therefore, can be potentially applicable for biological studies.

In conclusion, development of an NIR 2PE-responsive peptide with potential applicability to biological studies was achieved. The amide bond at the C-terminal position of the photo-responsive amino acid was successfully cleaved by irradiation of a focused NIR pulsed laser at 740 nm to yield processing products, and the δ_{u740} value was sufficient for application in biological studies. To my knowledge, this is the first example of an amide bond cleavage reaction triggered by NIR 2PE.

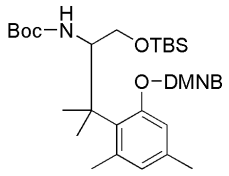
Experimental Section

General Methods

All reactions of small molecules were carried out under a positive pressure of argon. For column chromatography, silica gel (KANTO KAGAKU N-60, Kanto Chemicals) was used. Thin layer chromatography was performed on precoated plates (0.25 nm, silica gel Merck Kiesegel $60F_{245}$). Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™ or a Bruker Esquire2000T. NMR spectra were recorded using a JEOL GSX400 spectrometer at 400 MHz frequency for ^{1}H and a JEOL JNM-AL300 at 75 MHz frequency for ^{13}C in $CDCl_3$. Chemical shifts were calibrated to the solvent signal. For HPLC separations, a Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1 mL/min) or a $5C_{18}$-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) was used, and eluting products were detected by UV at 220 nm. For HPLC elution, linear gradient of 0.1% (v/v) TFA in MeCN (solvent B) in 0.1% (v/v) TFA aqueous solution (solvent A) over 30 min was employed. Photolysis by UV irradiation was performed using a Moritex MUV-202U with the filtered output (>365 nm) of a 3000 mW/cm^2 Hg-Xe lamp. Femtosecond near-infrared pulses from a mode-locked Ti-sapphire laser (Tsunami pumped by Millenium V; Spectra-Phisics) were used for an NIR two-photon excitation experiment.

Preparation of NIR Two-photon Excitation-responsive Amino Acid

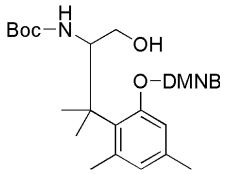
{1-(*tert*-Butyldimethylsilanyloxymethyl)-2-[2-(4,5-dimethoxy-2-nitrobenzyloxy)-4,6-dimethylphenyl]-2-methylpropyl}carbamic acid *tert*-butyl ester (3)



To a stirred solution of phenol **1**[4c] (841 mg, 1.92 mmol) in DMF (17 mL) were added K_2CO_3 (637 mg, 4.61 mmol) and 4,5-dimethoxy-2-nitrobenzyl bromide **2**[7] (636 mg, 2.31 mmol), and the resulting suspension was stirred at room temperature overnight. After addition of saturated aqueous solution of NH_4Cl (sat. NH_4Cl aq.), the reaction mixture was stirred for 30 min. To the reaction mixture was added H_2O, and the obtained mixture was extracted with diethyl ether. The organic phase was washed with H_2O, sat. NH_4Cl aq. and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by column chromatography (SiO_2, hexane/EtOAc = 20/1) and 1.17 g of ether **3** (1.86 mmol, 97%) was obtained as a yellow amorphousness: ^{1}H NMR

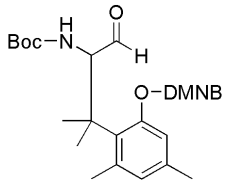
($CDCl_3$, 400 MHz) δ = –0.04 (3H, s), –0.03 (3H, s), 0.85 (9H, s), 1.36 (9H, s), 1.57 (3H, s), 1.58 (3H, s), 2.17 (3H, s), 2.55 (3H, s), 3.53 (1H, dd, *J* = 10.4 and 4.2 Hz), 3.58 (1H, dd, *J* = 10.4 and 4.2 Hz), 3.94 (3H, s), 3.97 (3H, s), 4.70 (1H, dt, *J* = 10.1 and 4.2 Hz), 4.84 (1H, d, *J* = 10.1 Hz), 5.51 (1H, d, *J* = 16.0 Hz), 5.57 (1H, d, *J* = 16.0 Hz), 6.48 (1H, s), 6.58 (1H, s), 7.40 (1H, s), 7.78 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = –5.6, –5.5, 18.1, 20.6, 25.8, 25.9, 27.6, 28.3, 29.0, 45.0, 56.3, 56.3, 56.6, 63.7, 69.5, 78.4, 108.0, 110.4, 114.1, 128.4, 130.4, 131.6, 136.4, 138.4, 138.8, 147.8, 154.2, 155.9, 158.5; HRMS (ESI-TOF) *m*/*z* calcd for $C_{33}H_{52}N_2NaO_8Si$ ($[M + Na]^+$): 655.3391, found: 655.3362.

{2-[2-(4,5-Dimethoxy-2-nitrobenzyloxy)-4,6-dimethylphenyl]-1-hydroxymethyl-2-methylpropyl}carbamic acid *tert*-butyl ester (4)



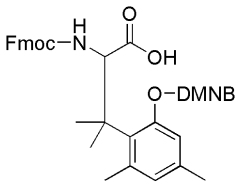
Glacial acetic acid (15 mL) and H_2O (5.0 mL) were added to a solution of silyl ether **3** (1.17 g, 1.86 mmol) in THF (5.0 mL). The reaction mixture was stirred at room temperature overnight. After extraction with EtOAc, the obtained organic phase was washed with H_2O (× 3) and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by column chromatography (SiO_2, hexane/EtOAc = 2/1) and 926 mg of alcohol **4** (1.79 mmol, 96%) was obtained as a yellow amorphousness: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.35 (9H, s), 1.54 (3H, s), 1.55 (3H, s), 2.17 (3H, s), 2.53 (3H, s), 3.53 (1H, dd, *J* = 10.5 and 7.8 Hz), 3.66 (1H, d, *J* = 10.5 Hz), 3.94 (3H, s), 3.97 (3H, s), 4.70 (1H, dd, *J* = 9.3 and 7.8 Hz), 4.80 (1H, d, *J* = 9.3 Hz), 5.52 (1H, d, *J* = 15.6 Hz), 5.56 (1H, d, *J* = 15.6 Hz), 6.50 (1H, s), 6.59 (1H, s), 7.33 (1H, s), 7.79 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 20.7, 26.0, 27.6, 28.3, 28.7, 44.2, 56.4, 56.7, 58.9, 64.0, 69.5, 79.3, 108.2, 110.4, 114.1, 128.7, 130.0, 130.8, 136.8, 138.1, 139.0, 148.0, 154.3, 157.0, 158.5; HRMS (ESI-TOF) *m*/*z* calcd for $C_{27}H_{39}N_2O_8$ ($[M + H]^+$): 519.2706, found: 519.2697.

{2-[2-(4,5-Dimethoxy-2-nitrobenzyloxy)-4,6-dimethylphenyl]-1-formyl-2-methyl-propyl}carbamic acid *tert*-butyl ester (5)



To a stirred solution of alcohol **4** (926 mg, 1.79 mmol) in CH_2Cl_2 (13 mL) was added PCC (1.54 g, 7.14 mmol), and the resulting suspension was stirred at room temperature for 6 h. After addition of the Cerite 535, the reaction mixture was filtered through the Cerite 535. The obtained organic phase was washed with sat. NH_4Cl aq., dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography (SiO_2, hexane/EtOAc = 8/1 then 4/1) and 756 mg of aldehyde **5** (1.46 mmol, 82%) was obtained as a yellow amorphousness: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.38 (9H, s), 1.54 (3H, s), 1.63 (3H, s), 2.20 (3H, s), 2.54 (3H, s), 3.93 (3H, s), 3.98 (3H, s), 5.13 (1H, d, J = 8.8 Hz), 5.35 (1H, d, J = 8.8 Hz), 5.54 (1H, d, J = 15.6 Hz), 5.60 (1H, d, J = 15.6 Hz), 6.54 (1H, s), 6.64 (1H, s), 7.27 (1H, s), 7.79 (1H, s), 9.51 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 20.9, 26.0, 27.9, 28.4, 28.6, 44.3, 56.5, 56.8, 66.0, 69.5, 79.7, 108.3, 110.7, 114.1, 128.7, 128.9, 129.5, 137.7, 138.4, 139.3, 148.2, 154.3, 156.0, 158.3, 201.3; HRMS (ESI-TOF) *m*/*z* calcd for $C_{27}H_{37}N_2O_8$ ($[M + H]^+$): 517.2550, found: 517.2545.

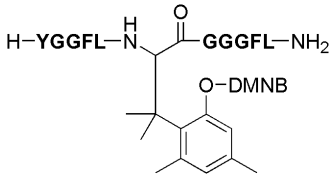
3-[2-(4,5-Dimethoxy-2-nitrobenzyloxy)-4,6-dimethylphenyl]-2-(9*H*-fluoren-9-yl-methoxycarbonylamino)-3-methylbutyric acid (7)



2-Methyl-2-butene (262 μL, 2.47 mmol), NaH_2PO_4 (66.0 mg, 0.549 mmol) and $NaClO_2$ (217 mg, 1.92 mmol) were added to a solution of aldehyde **5** (189 mg, 0.366 mmol) in acetone-*tert*-BuOH-H_2O (17:12:3 [v/v], 13 mL), and the resulting mixture was stirred at room temperature for 4 h. To the reaction mixture was added sat. NH_4Cl aq., and the obtained mixture was extracted with EtOAc. The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. Hydrogen chloride in EtOAc (4 M, 2.8 mL) was added to the crude product, and the resulting mixture was stirred at room temperature for 1.5 h.

After concentration *in vacuo*, the obtained residue was dissolved in MeCN-10% (w/v) aqueous solution of Na_2CO_3 (3:1 [v/v], 8.0 mL). To the resulting solution was added FmocOSu (136 mg, 0.403 mmol), and the reaction mixture was stirred at room temperature for 6 h. After being acidified by addition of 5% (w/v) aqueous solution of $KHSO_4$, the reaction mixture was extracted with diethyl ether. The organic phase was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The obtained crude product was purified by column chromatography (SiO_2, chloroform then chloroform/MeOH = 100/1) and 226 mg of Fmoc derivative **7** (0.345 mmol, 94%) was obtained as a yellow amorphousness: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.63 (3H, s), 1.66 (3H, s), 2.14 (3H, s), 2.50 (3H, s), 3.83 (3H, s), 3.93 (3H, s), 4.06–4.14 (1H, m), 4.18 (1H, dd, *J* = 10.5 and 6.8 Hz), 4.33 (1H, dd, *J* = 10.5 and 6.8 Hz), 5.49 (1H, d, *J* = 9.5 Hz), 5.51–5.65 (1H, m), 5.54 (1H, d, *J* = 15.6 Hz), 5.61 (1H, d, *J* = 15.6 Hz), 6.48 (1H, s), 6.56 (1H, s), 7.21–7.40 (5H, m), 7.43 (1H, d, *J* = 7.3 Hz), 7.48 (1H, d, *J* = 7.3 Hz), 7.73 (2H, d, *J* = 7.3 Hz), 7.76 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 20.9, 25.9, 27.9, 28.6, 44.6, 47.2, 56.4, 56.7, 59.9, 67.1, 69.7, 108.2, 110.2, 114.0, 120.1, 125.1, 125.2, 127.1, 127.1, 127.8, 127.9, 128.7, 128.9, 130.1, 137.4, 138.0, 139.0, 141.3, 143.9, 148.0, 154.3, 156.1, 158.7, 176.5; HRMS (ESI-TOF) *m*/*z* calcd for $C_{37}H_{38}N_2NaO_9$ ([M + Na]$^+$): 677.2475, found: 677.2498.

Preparation of NIR Two-photon Excitation-responsive Peptide (**10**)



Peptide **10** was synthesized on NovaSyn® TGR resin (7.5 μmol) using Fmoc chemistry reported in the previous report (2.2 mg, 20%).[4c] Analytical HPLC conditions: 20 to 80%. Retention times = 18.5 and 19.9 min, respectively for each diastereomer. Preparative HPLC conditions: 42 to 52%. LRMS (ESI-Ion Trap) *m*/*z* calcd for [M + H]$^+$: 1400.7, found: 1401.0 and 1401.0. Diastereomerically pure peptide **10** eluted earlier was used for subsequent photolysis experiments.

UV One-photon Excitation Experiment

Diastereomerically pure peptide **10** (0.10 mg, 70 nmol) in MeCN (320 μL) was added to 20 mM phosphate buffer (pH 7.6, 320 μL), and the resulting mixture was irradiated by UV (>365 nm) for 3 min. The reaction mixture was then incubated at 37 ˚C and the

reaction was monitored by analytical HPLC. Analytical HPLC conditions: 10 to 80%. **10**: Retention time = 23.4 min. **11**: Retention time = 21.8 min, LRMS (ESI-Ion Trap) *m*/*z* calcd for $[M + H]^+$: 757.4, found: 757.5. **12**: Retention time = 11.5 min, LRMS (ESI-Ion Trap) *m*/*z* calcd for $[M + H]^+$: 449.3, found: 449.3.

Estimation of Quantum Efficiency for One-photon Excitation

Typical procedure: Light intensity was determined as 1.41×10^{16} photon·s^{-1} using a potassium ferrioxalate actinometer. As the reaction solvent, 20 mM phosphate buffer (pH 7.6)-MeCN (1:1 [v/v]) was used. Quantum yield of disappearance of peptide **10** was estimated according to a previous report.[13] To calculate a remaining percentage of **10**, *m*-cresol was used as an internal standard. Product of the photolysis quantum yield and the molar absorptivity at 365 nm was calculated as 470 $M^{-1}\cdot cm^{-1}$ based on the time course for photolysis of peptide **10** (Figure 5). Because molar absorptivity of peptide **10** was determined as 5,889 $M^{-1}\cdot cm^{-1}$ (Figure 7), quantum yield for disappearance of peptide **10** was estimated as 0.080.

NIR Two-photon Excitation Experiment

NIR two-photon excitation experiment and calculation of δ_u value were performed as similar to that reported in previous report.[9] Irradiation intensity was estimated as 3.48×10^{12} photon·s^{-1} when referenced to a fluorescein, for which fluorescence quantum yield Φ_F (0.9) and two-photon absorption cross-section δ_F (30 GM at 740 nm) have been characterized.[14] A 10 μM solution of diastereomerically pure peptide **10** in 20 mM phosphate buffer (pH 7.6)-MeCN (1:1 [v/v]) was subjected to photolysis.

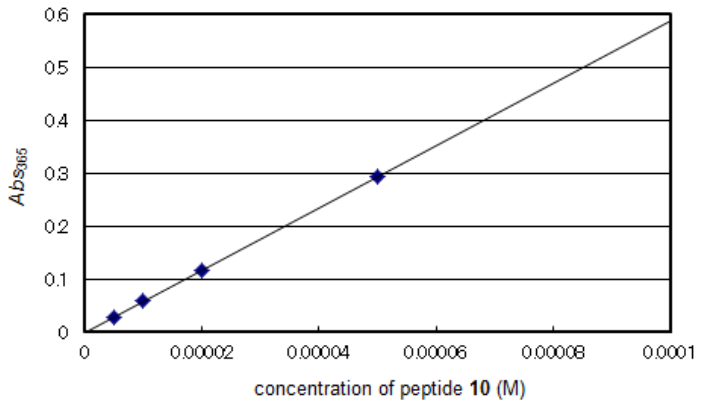


Figure 7. Plot of concentration of peptide **10** versus absorbance at 365 nm.

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Chapter 2. Development of a Fluoride Ion-responsive Traceable Linker That Possesses a Fluoride Ion-responsive Amino Acid

Summary

An efficient method of synthesizing an enantiomerically pure fluoride ion-responsive amino acid possessing a 4-(tert-butyldiphenylsiloxy)benzyl group as a phenolic protection was developed. This amino acid was attached to a fluoride ion-responsive traceable linker. The introduction of this traceable linker into alkynylated protein by click chemistry, adsorption of the obtained protein on streptavidin beads, and elution of the adsorbed protein from the beads by treatment with fluoride ion were achieved. It was, however, suggested that the elution efficiency was not high enough for enrichment of a target protein in a protein mixture.

A wide variety of bioactive compounds, such as natural products, peptides, and synthetic small molecules, exhibit their biological activities through specific interactions with a target biological macromolecule. Among the macromolecules present in living systems, proteins including enzymes, receptors, and ion channels represent one of the most important target classes. In the fields of chemical biology and drug discovery, the identification of unknown target proteins capable of interaction with biologically active compounds is indispensable to understanding complex biological signaling pathways and to developing novel therapeutic agents, but such efforts are time-consuming and laborious. In forward chemical genetic protocols, the pull-down assay using biologically active ligands for the identification of a target protein constitutes an indispensable experimental step.[1] Pull-down methods used for identification of a target protein involve (1) immobilizing a target protein on solid-phase support possessing a lead compound used as bait; (2) elution of the immobilized target protein; and (3) analysis of the target protein sequence (e.g., using Edman degradation or mass spectrometry).[2] The biotin-streptavidin pull-down assay is among the most widely used methods for the enrichment of target proteins (Figure 8). In this case, a biotinylated target protein is selectively adsorbed on streptavidin beads.[2,3] Then, the adsorbed target protein can be eluted from the streptavidin beads by disrupting the biotin-streptavidin interaction. The elution efficiency of the target protein under mild conditions is, however, typically low owing to the high affinity of the biotin-streptavidin interaction (K_d = 10^{-15} M) (Figure 9a).[4] On the other hand, elution efficiency is improved under harsh conditions but contamination of the eluted target protein with

nonspecifically adsorbed proteins becomes formidable (Figure 9b).

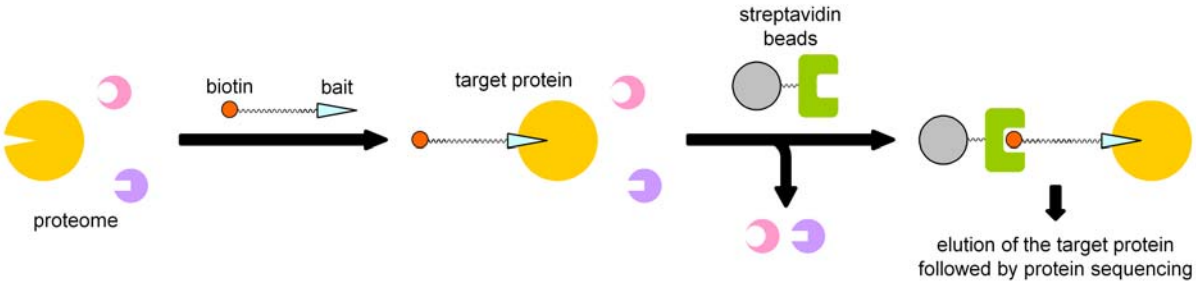


Figure 8. Pull-down assay using biotin-streptavidin interaction.

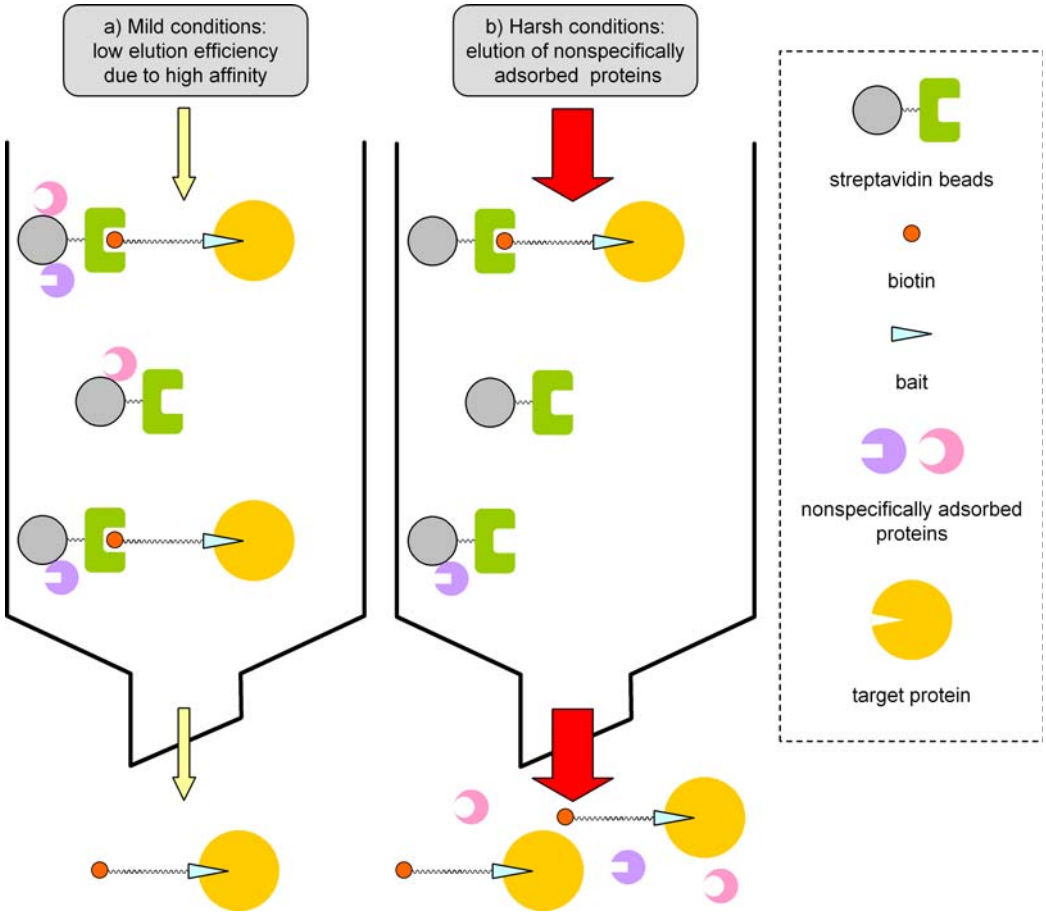


Figure 9. Problems encountered during elution of the target protein from streptavidin beads.

To overcome these problems, cleavable linkers have been introduced between biotin and the bait moiety.[5] The use of such linkers allows the efficient elution of the target protein from the streptavidin beads via cleavage of the linker, but contamination

of the eluted target protein with nonspecifically adsorbed proteins is still problematic for the identification of the target protein in some cases.[6] The development of a linker which is stable under physiological conditions, and can be cleaved under mild conditions with generation of an orthogonal functional group lacking in natural proteins is, therefore, strongly desired. The use of the orthogonal functional group enables chemoselective introduction of an isotopic or fluorescent label that facilitates identification of the eluted target protein by mass spectrometry (isotopic label) or SDS-PAGE (fluorescent label). Kohn's group and Dawson's group independently developed equilibrium-based hydrazone type linkers that enable simultaneous cleavage of the linker moiety and labeling of the target protein (Figure 10).[7] Selective labeling of the target proteins was achieved by cleavage of these hydrazone type linkers followed by labeling of the resulting aldehyde moiety with a hydrazine or hydroxylamine derivative.

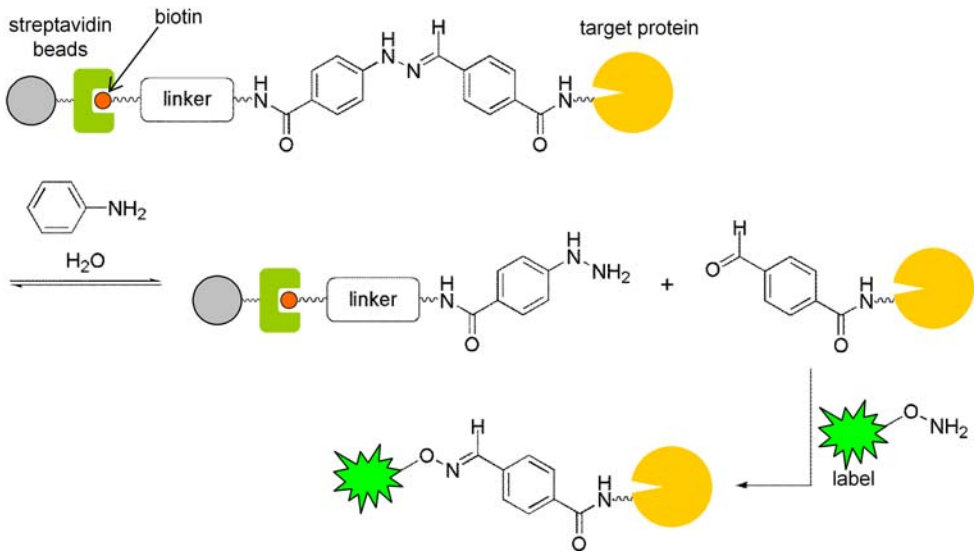


Figure 10. Cleavage followed by labeling of the target protein using Dawson's linker.

I envisioned that incorporation of the stimulus-responsive amino acid (Figure 1)[8] into a cleavable linker would allow the enrichment and selective labeling of target proteins, although two factors need to be considered: (1) the cleavage of the linker should be mediated by a reagent that does not react irreversibly with the target protein and (2) only the cleaved products should react with labeling reagents to allow the target protein to be discriminated from the contaminating non-target proteins. With these requirements in mind, I designed an advanced cleavable linker termed a "traceable linker" (Figure 11). The traceable linker would be more stable and easier to cleave on

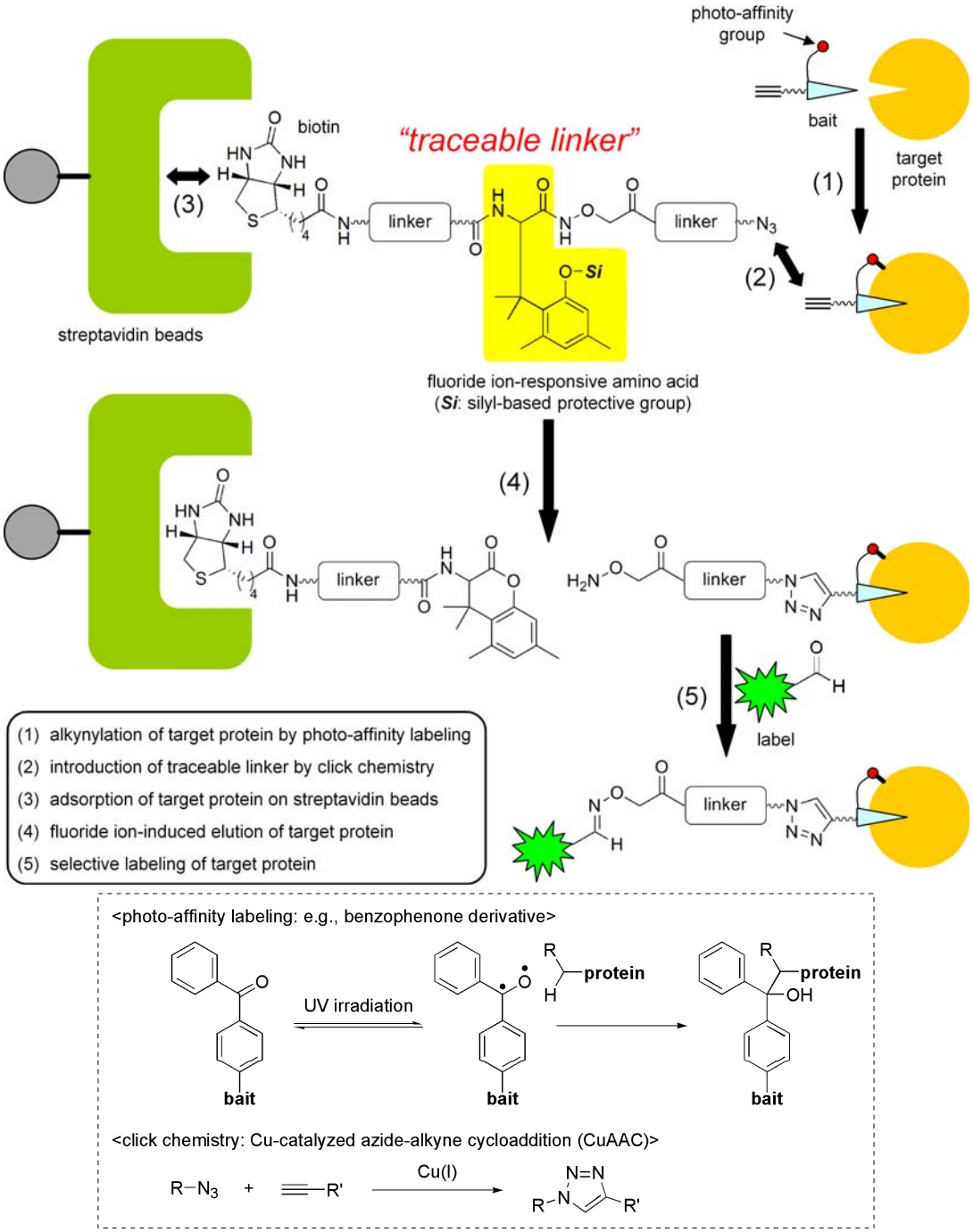
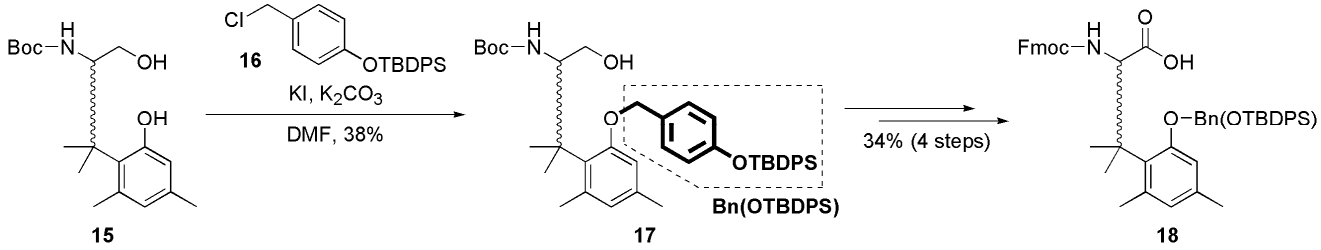


Figure 11. Strategy of the enrichment and selective labeling of target proteins using the fluoride ion-responsive traceable linker.

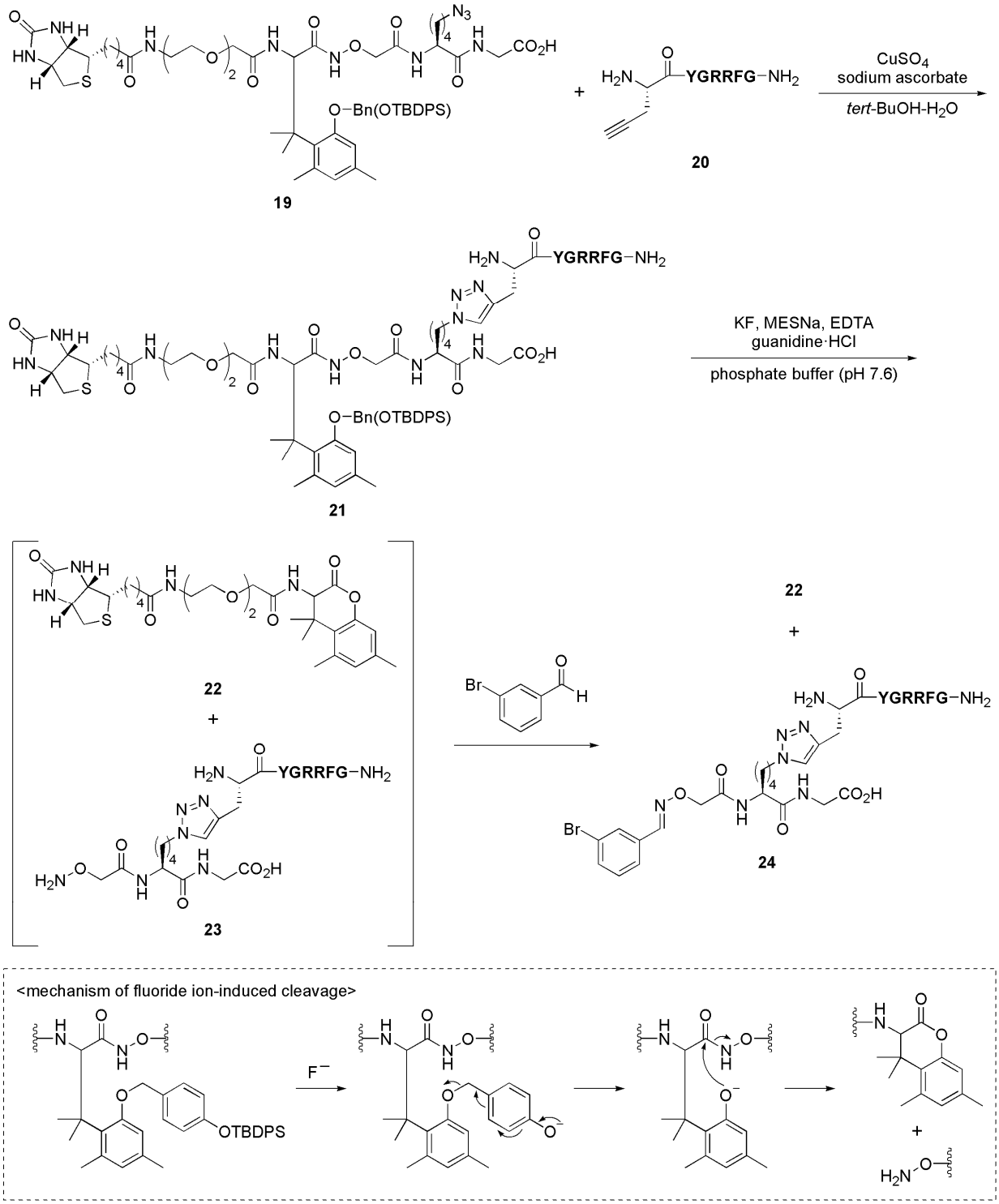
demand than equilibrium-based hydrazone type linkers described previously. I speculated that a fluoride ion was a suitable trigger of the linker cleavage reaction owing to its quite low concentration in serum, which is insufficient to accidentally

cleave the linker.[9] An aminooxy group was used for the chemoselective labeling of the eluted protein, because this functional group would react selectively with the aldehyde group on the labeling reagent.[10] An azide group was placed at one end of the linker and a biotin was installed at the other end to enable the introduction of the linker to the alkynylated target protein and the enrichment of the target protein-linker conjugate using streptavidin beads, respectively. The enrichment and selective labeling of target proteins using the fluoride ion-responsive traceable linker comprised a sequence of processes as follows (Figure 11): (1) alkynylation of the target protein by photo-affinity labeling[2a,b,11] using a bait compound containing an alkyne and photo-affinity group; (2) introduction of the traceable linker to the alkynylated target protein using click chemistry;[12] (3) adsorption of the biotinylated target protein on streptavidin beads; (4) elution of the adsorbed target protein by fluoride ion-induced linker cleavage; and (5) chemoselective labeling of the eluted target protein with a label containing an aldehyde group.

In my laboratory, the development of a fluoride ion-responsive amino acid derivative **18** was previously reported (Scheme 5).[13] The 4-(*tert*-butyldiphenylsiloxy)-benzyl (Bn[OTBDPS]) group on the phenol moiety can be removed via fluoride ion-induced cleavage of the *tert*-butyldiphenylsilyl (TBDPS) group followed by elimination of quinone methide. Furthermore, development of a fluoride ion-responsive traceable linker **19** was achieved, where were included conjugation of linker **19** with alkyne-containing model peptide **20** by click chemistry, linker cleavage of conjugate **21** by treatment with KF and 2-mercaptoethanesulfonic acid sodium salt (MESNa) as a scavenger of quinone methide, and selective labeling of cleavage product **23** using 3-bromobenzaldehyde (Scheme 6).



Scheme 5. A previous synthetic method of the fluoride ion-responsive amino acid derivative.

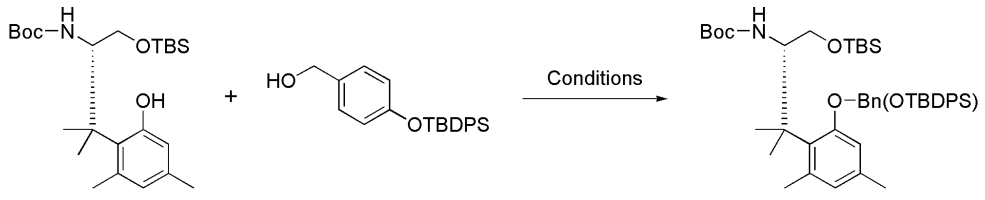


Scheme 6. Selective labeling of model peptide **20** using fluoride ion-responsive traceable linker **19** (**R**: arginine).

In this chapter, I describe the development of an efficient method of synthesizing the enantiomerically pure fluoride ion-responsive amino acid derivative and examination of the enrichment of an alkynylated model protein using the fluoride ion-responsive traceable linker.

At first, I screened the reaction conditions of homochiral fluoride ion-responsive amino acid derivative synthesis because the Fmoc-protected fluoride ion-responsive amino acid **18** in the previous report was racemic and the yield of the attachment of a Bn(OTBDPS) group to a phenol group was not sufficient. I speculated that the synthesis of ether **17** did not proceed efficiently owing to the instability of Bn(OTBDPS) chloride **16**. The synthesis of ether **27** by the Mitsunobu reaction of an enantiomerically pure phenol **25**[14] with Bn(OTBDPS) alcohol **26**[15] was, therefore, attempted (Table 2). The Mitsunobu reaction under the standard conditions using diethyl azodicarboxylate (DEAD) and triphenylphosphine did not proceed efficiently (entry 1), whereas modified conditions using *N*,*N*,*N'*,*N'*-tetramethylazodicarboxamide (TMAD)[16] and tri-*n*-butylphosphine produced compound **27** in high yields (entries 2–4). Furthermore, the amounts of TMAD and tri-*n*-butylphosphine could be reduced to three equivalents (entry 5).

Table 2. Synthetic attempts to prepare compound **27** by the Mitsunobu reaction.

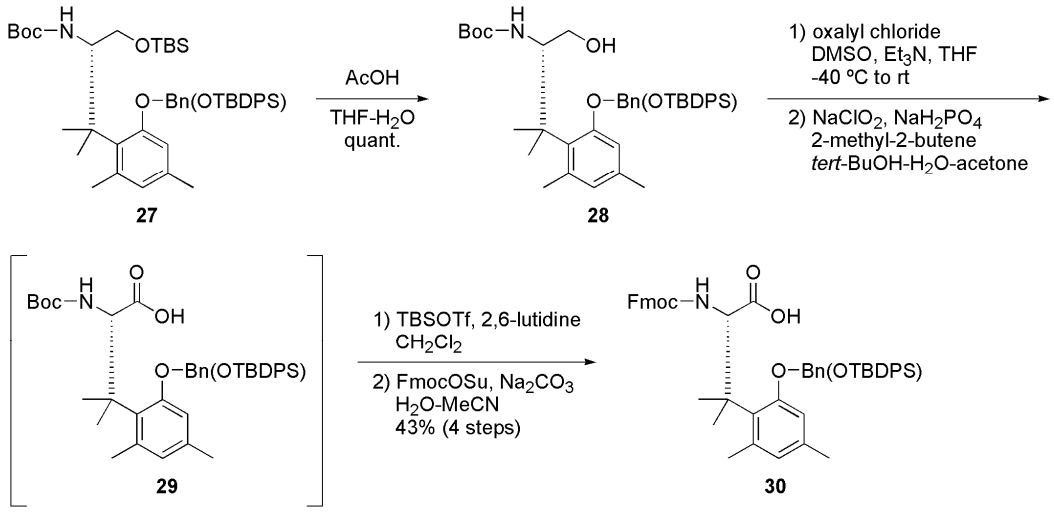


25 **26** **27**

Entry	Conditions	Yield (%)
1	**26** (3 eq.), DEAD (5 eq.), Ph_3P (5 eq.), THF	41
2	**26** (1.5 eq.), TMAD (5 eq.), *n*-Bu_3P (5 eq.), THF, 0 °C to rt	87
3	**26** (1.5 eq.), TMAD (5 eq.), *n*-Bu_3P (5 eq.), CH_2Cl_2, 0 °C to rt	91
4	**26** (1.5 eq.), TMAD (5 eq.), *n*-Bu_3P (5 eq.), toluene, 0 °C to rt	95
5	**26** (1.5 eq.), TMAD (3 eq.), *n*-Bu_3P (3 eq.), toluene, 0 °C to rt	98

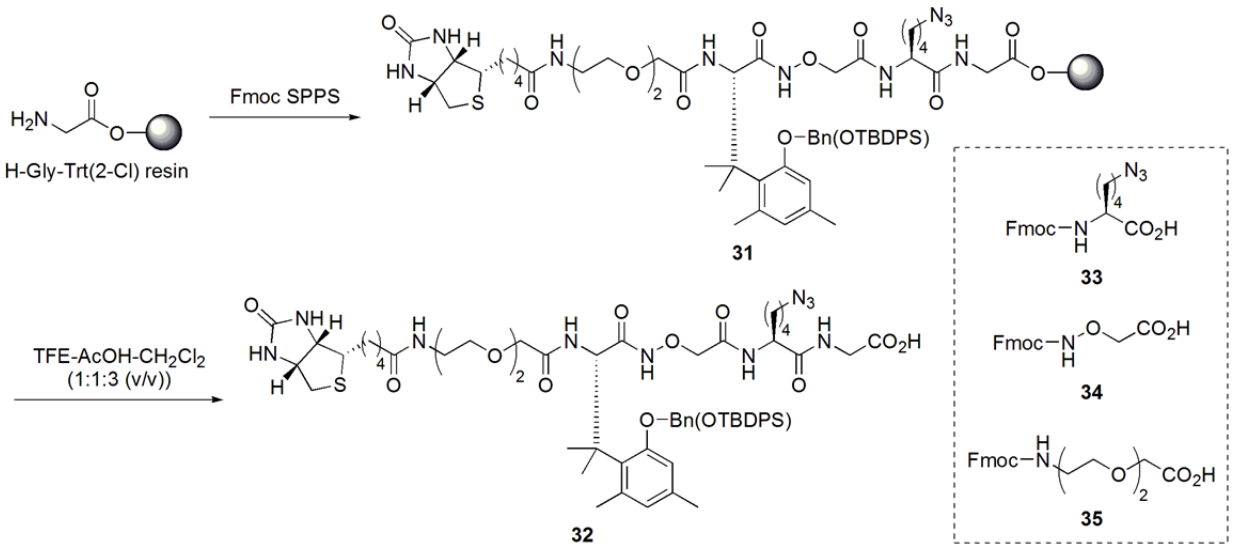
Next, the obtained compound **27** was converted to enantiomerically pure fluoride ion-responsive amino acid derivative **30** (Scheme 7). The TBS group of ether **27** was removed under acidic conditions to yield alcohol **28**. After transformation of alcohol **28** into carboxylic acid **29** by Swern oxidation followed by Pinnick oxidation, the Boc group of carboxylic acid **29** was removed. In this case, TFA was not suitable as

a reagent because of undesired cleavage of the Bn(OTBDPS) group. After screening several sets of conditions, I found that Ohfune's protocol gave the best result without any problem.[17] The obtained amine was finally protected with an Fmoc group to yield the desired Fmoc-protected fluoride ion-responsive amino acid **30**. The total yield of the conversion from phenol **25** to the amino acid derivative **30** was 43% over six steps.



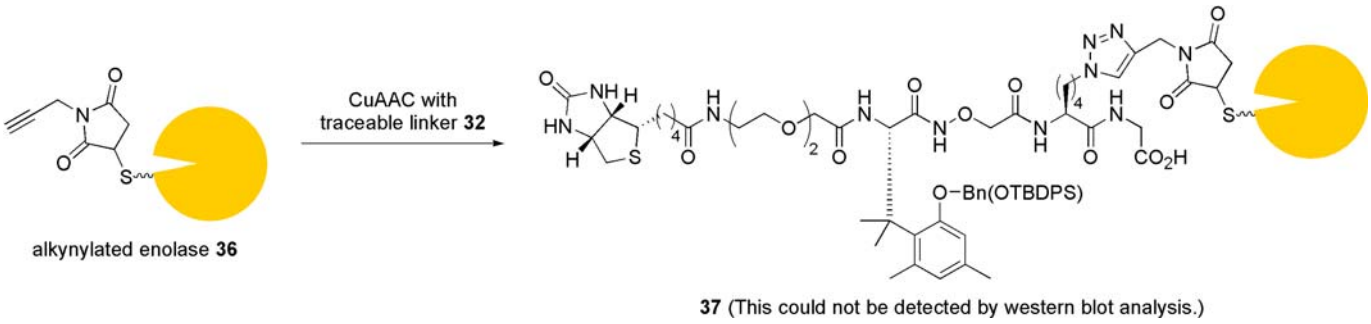
Scheme 7. Enantioselective synthesis of fluoride ion-responsive amino acid derivative.

Fluoride ion-responsive traceable linker **32** was next prepared using Fmoc SPPS (Scheme 8). Because of the presence of the acid-labile Bn(OTBDPS) group, release of the fluoride ion-responsive traceable linker from resins using standard TFA conditions was not compatible. Commercially available glycine-preloaded 2-chlorotrityl resin (H-Gly-Trt[2-Cl] resin) was therefore employed because the product can be released from 2-chlorotrityl resin under weaker acidic conditions. Starting from an H-Gly-Trt(2-Cl) resin, azide derivative **33**[18] and aminooxy derivative **34**[19] were incorporated by standard Fmoc SPPS conditions using the DIC/HOBt system. After coupling of the fluoride ion-responsive amino acid **30** 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 miniPEG unit **35** and biotin. The resin-bound traceable linker **31** was finally treated with a 2,2,2-trifluoroethanol (TFE)-AcOH-CH_2Cl_2 cocktail to generate the desired fluoride ion-responsive traceable linker **32** without concomitant removal of the Bn(OTBDPS) group.



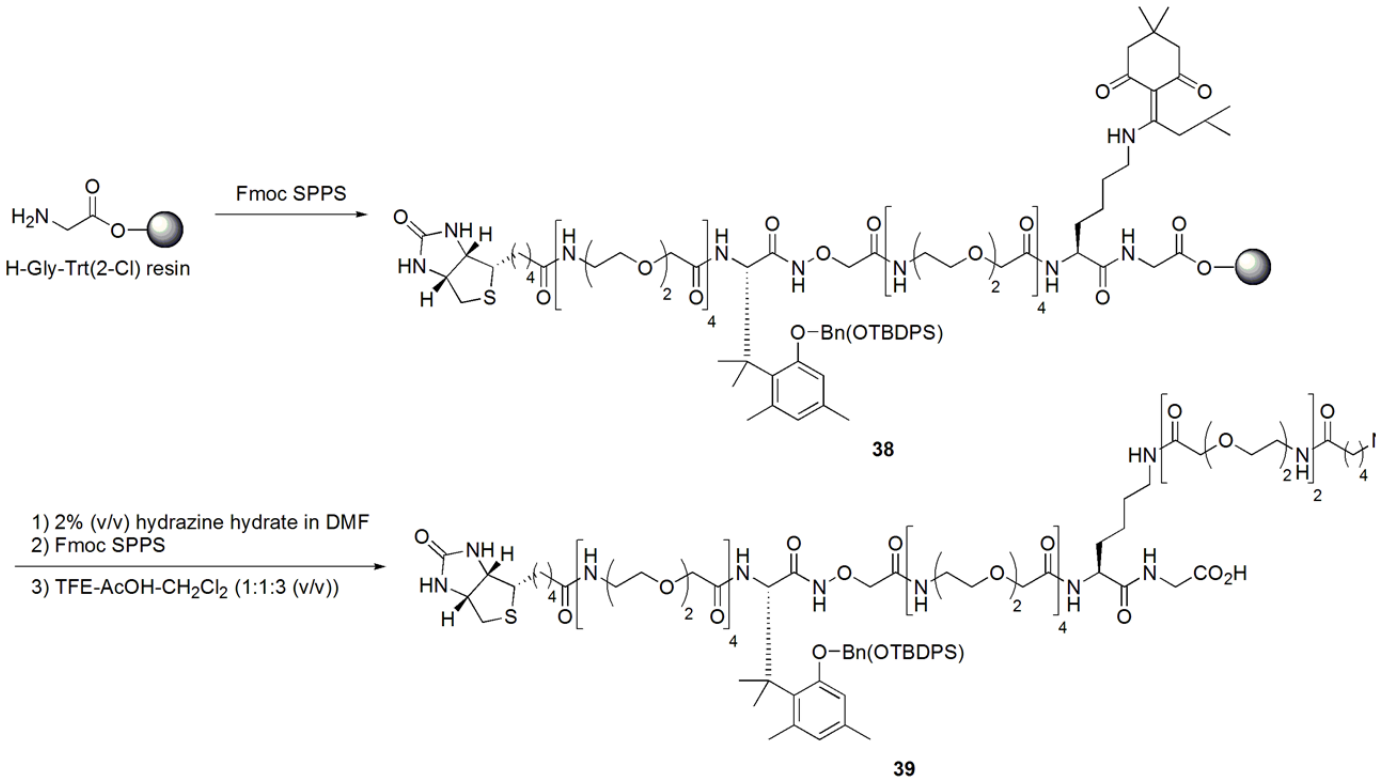
Scheme 8. Synthesis of fluoride ion-responsive traceable linker **32**.

Since traceable linker **32** has already been shown to be responsive to fluoride ion-induced cleavage and selective labeling processes with alkynylated model peptide in a previous report,[13] the applicability of the traceable linker strategy to the enrichment of an alkynylated protein was evaluated. In this study, an alkynylated enolase **36** was used as a target protein.[7b] The Cu-catalyzed azide-alkyne cycloaddition (CuAAC) of the traceable linker **32** with the alkynylated enolase **36** was attempted (Scheme 9), but no biotinylated enolase was detected by western blot analysis using streptavidin-horseradish peroxidase conjugate (SAv-HRP) (data not shown). I speculated that the CuAAC did not proceed owing to steric hindrance around the azide group on the traceable linker **32**.



Scheme 9. CuAAC between traceable linker **32** and alkynylated enolase **36**.

Therefore, the elongated fluoride ion-responsive traceable linker **39** possessing a longer miniPEG moiety than that of linker **32**, which can place its biotin moiety in a favorable position to interact with SAv-HRP, was synthesized by Fmoc SPPS using a 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) group as an ε-amino protective group (Scheme 10). Starting from an H-Gly-Trt(2-Cl) resin, resin **38** was prepared using synthetic protocols similar to those employed for the preparation of **31**. For removal of an ivDde group on the lysine residue, resin **38** was treated with 2% (v/v) hydrazine hydrate in DMF. After elongation of the chain using standard Fmoc SPPS conditions, the desired elongated fluoride ion-responsive traceable linker **39** was obtained by treatment with a TFE-AcOH-CH_2Cl_2 cocktail.



Scheme 10. Synthesis of the elongated fluoride ion-responsive traceable linker **39**.

The CuAAC of the elongated traceable linker **39** with the alkynylated enolase **36** followed by the enrichment of the enolase was then examined (Figure 12). Fluoride ion-intact linker **40**[20] was used as a negative control for the fluoride ion-induced cleavage required for elution of the target protein from streptavidin beads. The CuAAC of linkers **39** or **40** was initially examined. The alkynylated enolase **36** was treated with the linker **39** or **40** in phosphate buffered saline (PBS) in the presence of $CuSO_4$, sodium

ascorbate, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA),[21] and sodium dodecyl sulfate (SDS). Following a reaction for 1 h and subsequent separation by SDS-PAGE, the biotinylated proteins and all proteins were visualized by western blot analysis using SAv-HRP and silver stain, respectively. The linkers were successfully introduced to the enolase in both cases (Figure 13A). Although I examined several conditions including reductant (sodium ascorbate or tris[2-carboxyethyl] phosphine) and SDS (presence or absence during the reaction), the above click reaction conditions gave a better result (data not shown).

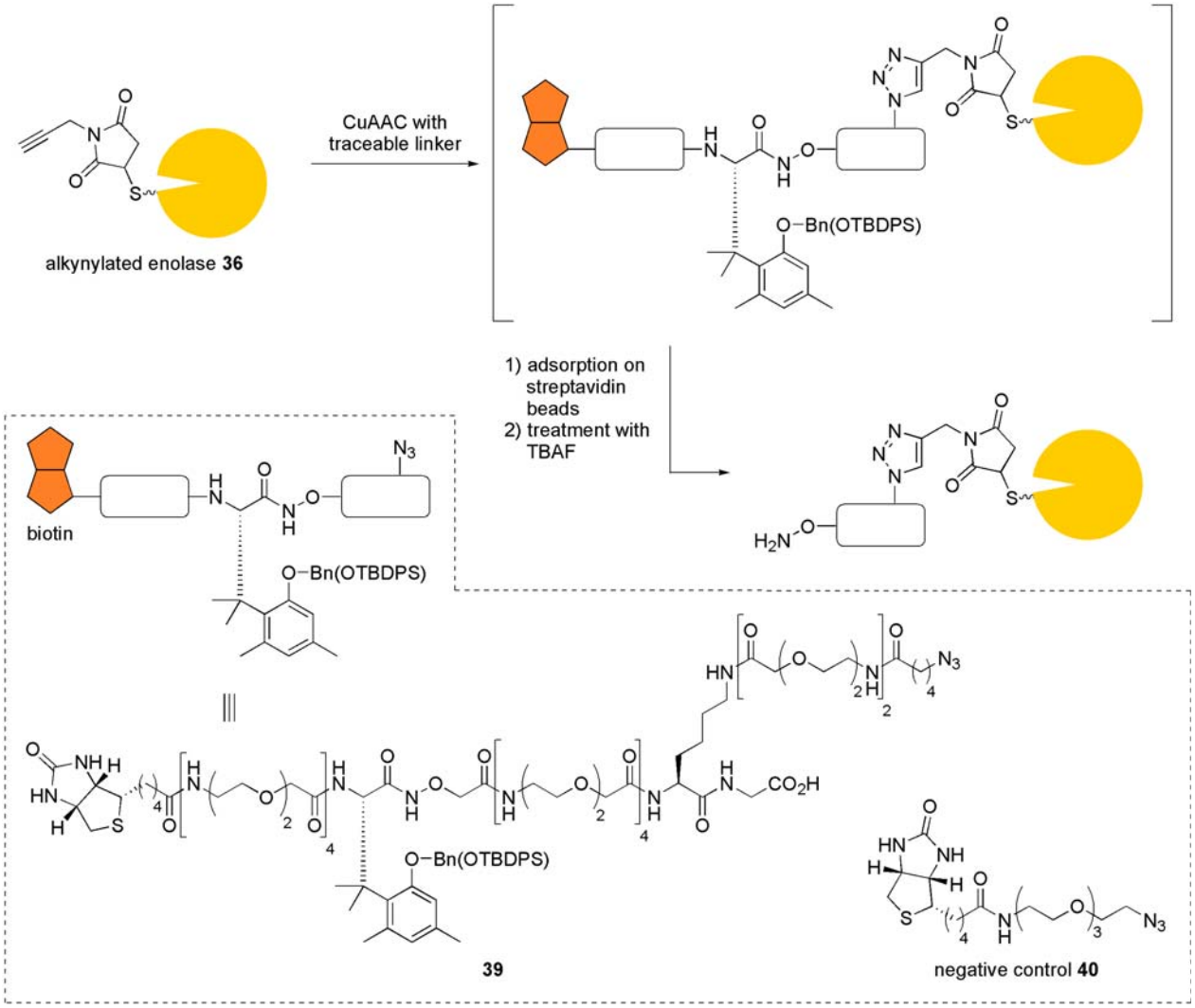


Figure 12. Schematic representation of CuAAC and enrichment of alkynylated enolase using fluoride ion-responsive traceable linker.

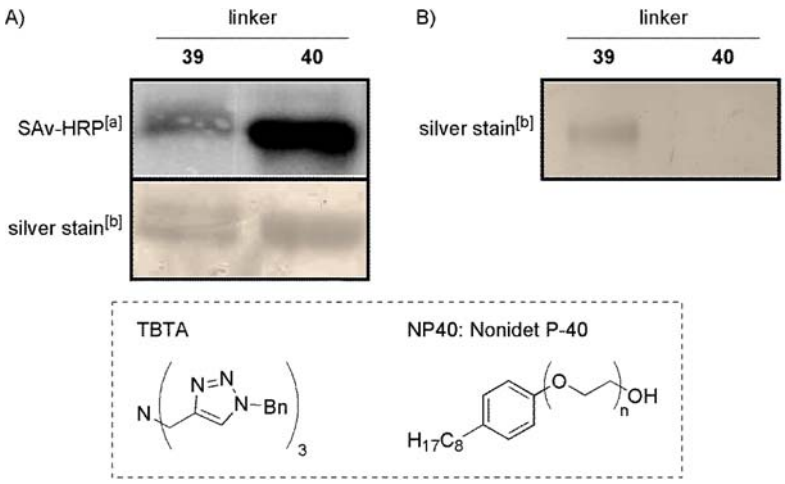


Figure 13. Monitoring of the reactions shown in Figure 12 using SDS-PAGE. (A) After CuAAC. Traceable linker **39** or negative control **40** (0.10 mM) was introduced to the alkynylated enolase (0.50 g/L) using a mixture of $CuSO_4$ (1.0 mM), sodium ascorbate (0.50 mM), TBTA (0.10 mM), SDS (1% [w/v]), PBS, and co-solvents for 1 h. (B) Adsorption on streptavidin beads followed by fluoride ion-induced elution. Proteins were treated with streptavidin beads for 1 h following the CuAAC. After washing, the beads were reacted with TBAF (100 mM), MESNa (100 mM) and NP40 (1.3% [v/v]) in 200 mM sodium phosphate buffer (pH 7.6) at 37 ˚C for 48 h. [a] Biotinylated proteins were detected by western blot analysis using SAv-HRP. [b] All proteins were visualized by silver stain.

After the click reaction, the obtained products were treated with streptavidin beads for 1 h. After washing with PBS, the beads were reacted with tetrabutylammonium fluoride (TBAF) and MESNa in 200 mM sodium phosphate buffer (pH 7.6) containing Nonidet P-40 (NP40) at 37 ˚C for 48 h. When the traceable linker **39** was employed, the fluoride ion-induced elution of the adsorbed enolase proceeded, whereas when the fluoride ion-intact negative control **40** was employed, the adsorbed protein was not released (Figure 13B). Because the enolase-linker conjugate derived from the CuAAC with linker **40** was released by heating the resulting beads in SDS-PAGE sample loading buffer at 100 ˚C for 5 min, its adsorption to streptavidin beads was regarded as successful (data not shown). These observations suggested that the traceable linker enabled the fluoride ion-responsive release of the target protein in a manner similar to that of conventional cleavable linkers. I examined the conditions of the elution of the adsorbed protein including fluoride ion source (TBAF, KF, HF, or H_2SiF_6), time (24, 48, or 72 h) and NP40 (presence or absence during the reaction), but

the elution efficiency could not be improved (data not shown).

In conclusion, development of the efficient method of homochiral fluoride ion-responsive amino acid synthesis and its application to the fluoride ion-responsive traceable linker were achieved. The traceable linker was successfully coupled to an alkynylated protein using click chemistry, and fluoride ion-induced elution of the adsorbed protein from streptavidin beads was also achieved. It was, however, suggested that the elution efficiency was not enough for the enrichment of target proteins in a protein mixture. The improvement of the traceable linker will be described in the next chapter.

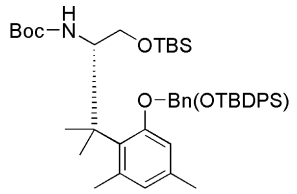
Experimental Section

General Methods

^{1}H NMR spectra were recorded using a Bruker AV400N at 400 MHz frequency in $CDCl_3$. Optical rotations were measured using a JASCO P-2200 polarimeter (concentration in g/100 mL). ECL signals from the western blot analysis were detected using LAS-4000mini (Fujifilm). For Mitsunobu reaction, distilled tri-*n*-butylphosphine stored in a sealed in thin glass ampoules was used. All of other reagents purchased from the suppliers were used without further purifications. Other methods were similar to those described in the Experimental Section in Chapter 1.

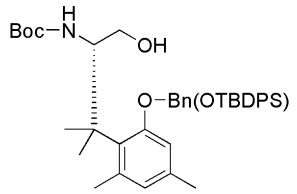
Preparation of Fluoride Ion-responsive Amino Acid

(*S*)-(1-(*tert*-Butyldimethylsilanyloxymethyl)-2-{2-[4-(*tert*-butyldiphenylsilanyloxy) benzyloxy]-4,6-dimethylphenyl}-2-methylpropyl)carbamic acid *tert*-butyl ester (27)



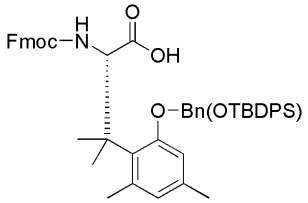
To a stirred solution of phenol **25**[14] (200 mg, 0.457 mmol), Bn(OTBDPS) alcohol **26**[15] (250 mg, 0.690 mmol) and TMAD (236 mg, 1.37 mmol) in toluene (4.6 mL) was added tri-*n*-butylphosphine (342 μL, 1.37 mmol) at 0 ˚C. After being stirred at 0 ˚C for 30 min and at room temperature for 3 h, the resulting mixture was quenched by the addition of H_2O and extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography (SiO_2, hexane/EtOAc = 20/1) and 349 mg of ether **27** (0.446 mmol, 98%) was obtained as a colorless oil: $[\alpha]^{20}_D$ –22.3 (*c* 1.29, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = –0.06 (3H, s), –0.04 (3H, s), 0.86 (9H, s), 1.13 (9H, s), 1.40 (9H, s), 1.49 (3H, s), 1.50 (3H, s), 2.20 (3H, s), 2.55 (3H, s), 3.47 (1H, dd, *J* = 10.6 and 5.0 Hz), 3.56 (1H, dd, *J* = 10.6 and 4.0 Hz), 4.54 (1H, ddd, *J* = 10.0, 5.0 and 4.0 Hz), 4.85 (1H, d, *J* = 10.0 Hz), 4.94 (1H, d, *J* = 11.6 Hz), 4.99 (1H, d, *J* = 11.6 Hz), 6.55 (1H, s), 6.57 (1H, s), 6.78 (2H, d, *J* = 8.5 Hz), 7.21 (2H, d, *J* = 8.5 Hz), 7.35–7.47 (6H, m), 7.71–7.77 (4H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = –5.6, –5.5, 18.1, 19.5, 20.7, 25.8, 25.8, 26.5, 27.7, 28.4, 29.3, 44.5, 56.7, 63.6, 70.7, 78.3, 112.7, 119.7, 127.4, 127.7, 128.6, 129.8, 130.0, 131.0, 132.9, 135.5, 136.0, 138.5, 155.1, 156.0, 158.7; HRMS (ESI-TOF) *m*/*z* calcd for $C_{47}H_{68}NO_5Si_2$ ($[M + H]^+$): 782.4636, found: 782.4610.

(*S*)-(2-{2-[4-(*tert*-Butyldiphenylsilanyloxy)benzyloxy]-4,6-dimethylphenyl}-1-hydroxymethyl-2-methylpropyl)carbamic acid *tert*-butyl ester (28)



Glacial acetic acid (6.0 mL) and H_2O (2.1 mL) were added to a solution of silyl ether **27** (298 mg, 0.381 mmol) in THF (2.1 mL). The reaction mixture was stirred at room temperature for 9 h. After the addition of H_2O, the resulting mixture was extracted with EtOAc. The organic phase was washed with H_2O and brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography (SiO_2, hexane/EtOAc = 4/1 then 2/1) and 258 mg of alcohol **28** (0.386 mmol, quant.) was obtained as a colorless oil: $[\alpha]^{21}_D$ –6.10 (*c* 1.04, $CHCl_3$); ^{1}H NMR, ^{13}C NMR and HRMS spectra were identical with those of the racemic one.[13]

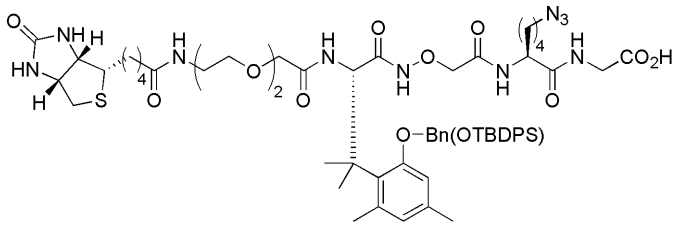
(*S*)-3-{2-[4-(*tert*-Butyldiphenylsilanyloxy)benzyloxy]-4,6-dimethylphenyl}-2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methylbutyric acid (30)



To a cooled (–78 ˚C) solution of oxalyl chloride (38.0 μL, 0.444 mmol) in CH_2Cl_2 (3.6 mL) were added DMSO (63.1 μL, 0.888 mmol) and a solution of alcohol **28** (198 mg, 0.296 mmol) in CH_2Cl_2 (660 μL) slowly, and the resulting solution was stirred at –40 ˚C for 30 min. Then, triethylamine (206 μL, 1.48 mmol) was added dropwise. After being stirred at the same temperature for 30 min, the resulting mixture was allowed to warm to room temperature with stirring for 30 min. The resulting mixture was quenched by the addition of H_2O and extracted with CH_2Cl_2. The organic phase was washed with sat. NH_4Cl aq., dried over Na_2SO_4 and concentrated *in vacuo*. To a solution of the crude product in acetone-*tert*-BuOH-H_2O (6:4:1 [v/v], 10 mL) were added 2-methyl-2-butene (212 μL, 2.00 mmol), NaH_2PO_4 (53.3 mg, 0.444 mmol) and $NaClO_2$ (176 mg, 1.55 mmol). The resulting mixture was stirred at room temperature for 2.5 h. To the reaction mixture was added sat. NH_4Cl aq., and the obtained mixture was extracted with EtOAc. The resulting organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. To the

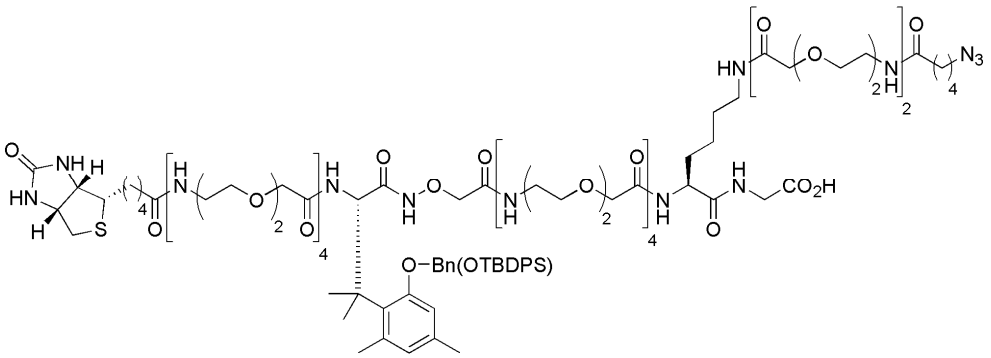
crude product in CH_2Cl_2 (6.3 mL) were added 2,6-lutidine (207 μL, 1.78 mmol) and TBSOTf (272 μL, 1.18 mmol), and the reaction mixture was stirred at room temperature for 2 h. The resulting mixture was concentrated *in vacuo*, and the obtained residue was dissolved in MeCN-10% (w/v) Na_2CO_3 aqueous solution (3:1 [v/v], 6.3 mL). To the resulting solution was added FmocOSu (120 mg, 0.355 mmol), and the reaction mixture was stirred at room temperature overnight. The reaction mixture was acidified by 5% (w/v) $KHSO_4$ aqueous solution, and the obtained mixture was extracted with diethyl ether. The organic phase was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography (SiO_2, chloroform) and 103 mg of Fmoc derivative **30** (0.128 mmol, 43%) was obtained as a beige amorphousness: $[\alpha]^{22}_D$ –5.58 (*c* 0.64, $CHCl_3$); ^{1}H NMR, ^{13}C NMR and HRMS spectra were identical with those of the racemic one.[13]

Preparation of Fluoride Ion-responsive Traceable Linker (**32**)



Preparation of resin-bound traceable linker **31** was performed on H-Gly-Trt(2-Cl) resin (loading: 0.86 mmol/g, 23 mg, 20 μmol) by using Fmoc SPPS reported in the previous report.[13] The resin **31** was treated with TFE-AcOH-CH_2Cl_2 (1:1:3 [v/v], 7.0 mL) for 2 h. After filtration of the resin, the filtrate was concentrated to provide the crude traceable linker. To the obtained residue was added 0.1% (v/v) TFA in MeCN-0.1% (v/v) TFA aq. (1:1 [v/v]) to afford clear solution, which was then subjected to preparative HPLC to yield linker **32** (4.4 mg, 18%). Analytical HPLC conditions: 70%. Retention time = 12.8 min. Preparative HPLC conditions: 55 to 65%. LRMS (ESI-Ion Trap) *m*/*z* calcd for $[M + H]^+$: 1237.6, found: 1237.5.

Preparation of Fluoride Ion-responsive Traceable Linker (**39**)



Preparation of resin **38** was performed by using Fmoc SPPS on H-Gly-Trt(2-Cl) resin (loading: 0.86 mmol/g, 51 mg, 45 μmol). Treatment with 20% (v/v) piperidine in DMF was used for the removal of Fmoc protection, and DIC (22 μL, 3.2 equiv.)/HOBt·H_2O (18 mg, 3.0 equiv.) in DMF was employed for coupling of Fmoc-Lys(ivDde)-OH (78 mg, 135 μmol), Fmoc-aminooxy acetic acid **34**[19] (42 mg, 135 μmol) or Fmoc-mini-PEG™ **35** (51 mg, 135 μmol). Fmoc derivative **30** (72 mg, 90 μmol) was coupled by using HATU (33 mg, 86 μmol) and DIPEA (15 μL, 86 μmol) in DMF overnight. (+)-Biotin (54 mg, 225 μmol) was coupled with the aid of DIC (35 μL, 5.0 equiv.)/HOBt·H_2O (30 mg, 5.0 equiv.) in DMF-DMSO (1:1 [v/v]). The ivDde group on Lys residue was removed by treatment with 2% (v/v) hydrazine hydrate in DMF for 2 days followed by successive coupling of Fmoc-mini-PEG™ **35** under the conditions mentioned above and 6-azidohexanoic acid[22] (33 mg, 225 μmol) using DIC (35 μL, 5.0 equiv.)/HOBt·H_2O (30 mg, 5.0 equiv.) in DMF. The resulting completed resin was treated with TFE-AcOH-CH_2Cl_2 (1:1:3 [v/v], 5.5 mL) for 2 h. After filtration of the resin, the filtrate was concentrated to provide the crude traceable linker. To the obtained residue was added AcOH-H_2O-MeCN (2:4:1 [v/v]) to afford clear solution, which was then subjected to preparative HPLC to yield linker **39** (20 mg, 17%). Analytical HPLC conditions: 50 to 90%. Retention time = 9.0 min. Preparative HPLC conditions: 48 to 58%. LRMS (ESI-Ion Trap) *m*/*z* calcd for $[M + 2H]^{2+}$: 1321.7, found: 1322.4.

Preparation of Alkynylated Enolase (**36**)

Starting from a commercially available enolase (7.5 mg, Sigma), the alkynylated enolase was prepared according to the literature.[7b] After the reaction, unreacted small molecules were removed by dialysis (Slide-A-Lizer® G2 Dialysis Cassette, Thermo SCIENTIFIC) with PBS instead of gel filtration. Volume of the resulting solution was adjusted to 1.8 to 2.0 mL, and it was used as 4.2 g/L to 3.8 g/L solution of the

alkynylated enolase in PBS.

Introduction of Traceable Linker onto Alkynylated Enolase

CuAAC: To a mixture of PBS (1.10 mL) and H_2O (855 μL) were added the alkynylated enolase in PBS (3.8 g/L, 400 μL, final concn. 0.50 g/L), traceable linker **39** or negative control **40**[20] in DMSO (6.0 mM, 50.0 μL, final concn. 0.10 mM), TBTA[21] in 20% (v/v) DMSO-*tert*-BuOH (1.7 mM, 176 μL, final concn. 0.10 mM), $CuSO_4$ in H_2O (50 mM, 60.0 μL, final concn. 1.0 mM), sodium ascorbate in H_2O (25 mM, 60 μL, final concn. 0.50 mM), and SDS in H_2O (10% [w/v], 300 μL, final concn. 1% [w/v]). After the reaction at room temperature for 1 h, small molecules were removed by dialysis (Slide-A-Lizer® G2 Dialysis Cassette, Thermo SCIENTIFIC) with PBS.

SDS-PAGE: After addition of 2 × 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). For silver staining of all proteins in gel, silver stain KANTO III (Kanto Chemicals) was employed.

Adsorption on Streptavidin Beads Followed by Linker Cleavage

Adsorption on Streptavidin Beads: After CuAAC, NeutrAvidin™ Agarose Resins (70 μL, Thermo SCIENTIFIC) was added to the reaction mixture containing ca. 100 μg enolase and its derivatives. After the adsorption at room temperature for 1 h, the resulting resin was washed with PBS five times and it was subjected to subsequent reaction.

Linker Cleavage: To the resulting streptavidin beads was added a cleavage cocktail (152 μL) consisting of TBAF (100 mM), MESNa (100 mM), NP40 (1.3% [v/v]) in 200 mM sodium phosphate buffer (pH 7.6). After the reaction at 37 ˚C for 48 h, to the reaction mixture was added sodium phosphate buffer (48 μL, 200 mM, pH 7.6). After centrifugation of the resulting mixture (2,000 rpm, 2 min), supernatant was desalted using Pierce® SDS-PAGE Sample Prep Kit (Thermo SCIENTIFIC). Then the obtained mixture was subjected to the SDS-PAGE as similar to those described in section "Introduction of Traceable Linker onto Alkynylated Enolase".

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Chapter 3. Development of a Traceable Linker Containing a Thiol-responsive Amino Acid for the Enrichment and Selective Labeling of Target Proteins

Summary

An improved traceable linker that possesses a thiol-responsive amino acid was developed. Its application to the enrichment and selective labeling of a target protein (alkynylated enolase) in a protein mixture consisting of the alkynylated enolase, bovine serum albumin, and ovalbumin was achieved.

The identification of unknown target proteins with which biologically active compounds can interact is indispensable in the fields of chemical biology and drug discovery. Methodologies for the identification of target proteins using cleavable linkers have been developed by many groups, but these approaches are sometimes not applicable due to the contamination of the target protein with non-target proteins.[1,2]

In Chapter 2, I described a fluoride ion-responsive traceable linker which enables the elution of adsorbed protein on streptavidin beads via cleavage of the linker by treatment with fluoride ion (Figure 11). The elution efficiency was, however, insufficient for the enrichment of the target protein in a protein mixture for two reasons: (1) the length of the linker was probably too long for efficient adsorption of the biotinylated protein to streptavidin beads; (2) the fluoride ion in aqueous buffer probably did not have enough nucleophilicity for the removal of the silyl group before linker cleavage. Therefore, I surmised that shorter linker length and replacement of the fluoride ion-responsive amino acid with another appropriate stimulus-responsive amino acid (Figure 1)[3] would improve elution efficiency.

In this chapter, I describe the development of a thiol-responsive traceable linker and its application to the enrichment and selective labeling of a target protein in a protein mixture.

We previously reported that thiols, which are compatible with biomolecules, can be used as a suitable stimulus for amide bond cleavage by using the thiol-responsive amino acid bearing a *p*-nitrobenzenesulfonyl (*p*Ns) group as a phenolic protective group.[3a] In order to apply this amino acid to a traceable linker strategy, I designed thiol-responsive traceable linkers possessing flexible miniPEG moiety **41** and **42**, as well as rigid proline rod **43** (Figure 14).[4]

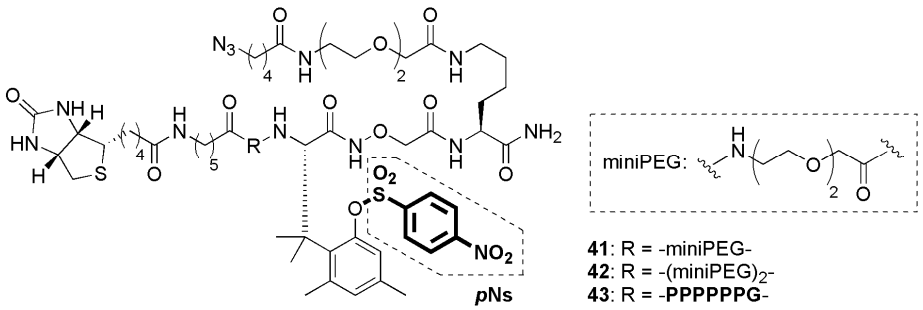
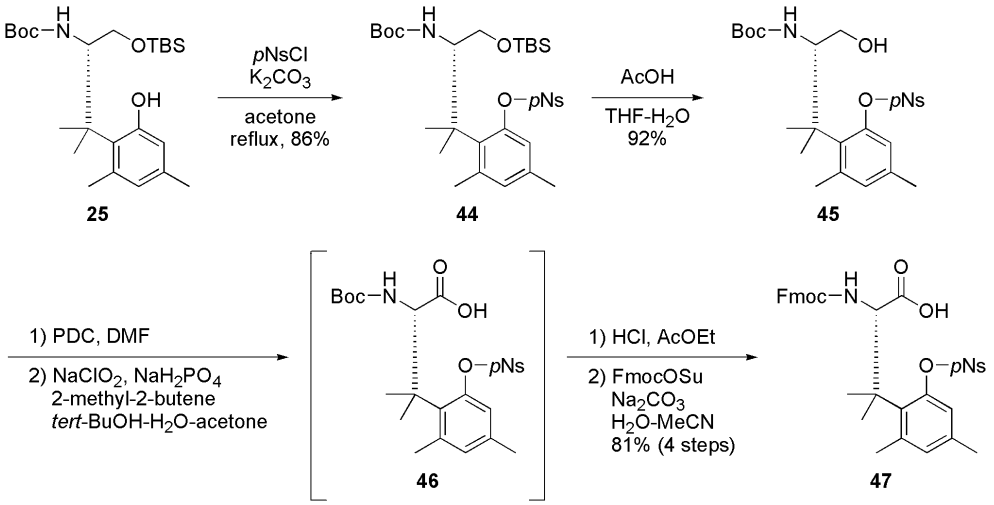


Figure 14. Structure of thiol-responsive traceable linkers.

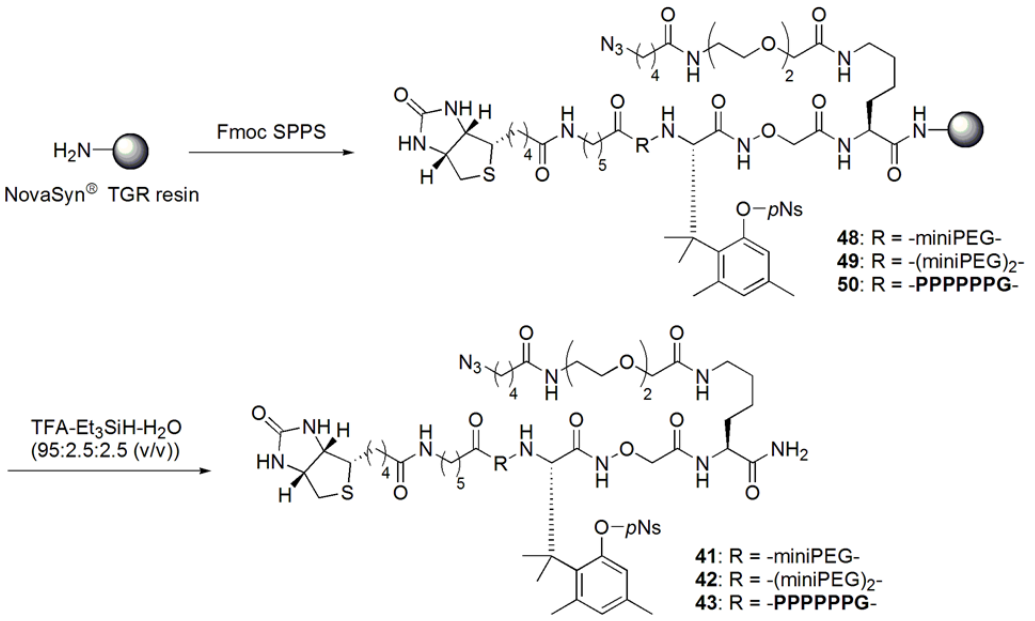
At first, a homochiral thiol-responsive amino acid derivative **47** was synthesized (Scheme 11). The enantiomerically pure phenol **25**[5] was sulfonylated with a *p*Ns chloride in the presence of K_2CO_3 to generate *p*Ns derivative **44**. The TBS group of **44** was removed under acidic conditions to produce alcohol **45**. After transformation of alcohol **45** into carboxylic acid **46** by oxidation with pyridinium dichromate (PDC) followed by Pinnick oxidation, the Boc group was replaced with an Fmoc group by treatment with an acid followed by reaction with FmocOSu to yield the desired Fmoc-protected thiol-responsive amino acid **47**. The total yield of the conversion from phenol **25** to the amino acid derivative **47** was 64% over six steps.



Scheme 11. Enantioselective synthesis of thiol-responsive amino acid derivative.

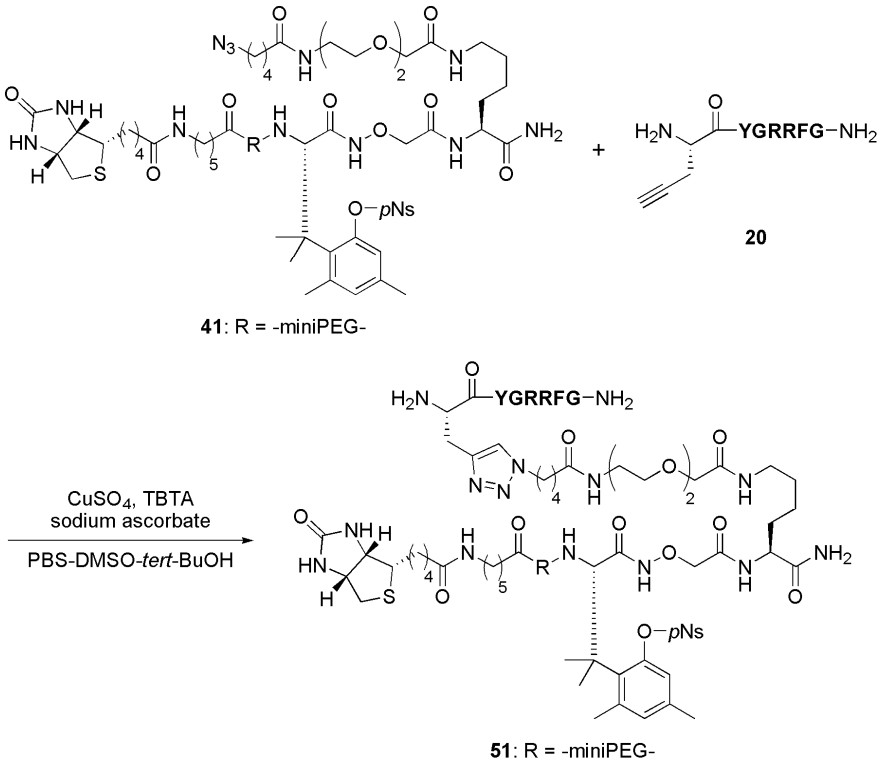
The thiol-responsive amino acid derivative **47** was next incorporated into the traceable linkers by Fmoc SPPS using an ivDde group (Scheme 12). Starting from

NovaSyn® TGR resin, the resin-bound traceable linkers **48**, **49**, and **50** were prepared using protocols similar to those employed for the preparation of fluoride ion-responsive traceable linker **39**. The resins **48**, **49**, and **50** were then treated with TFA-Et_3SiH-H_2O (95:2.5:2.5 [v/v]) to generate the desired thiol-responsive traceable linkers **41**, **42**, and **43**.



Scheme 12. Synthesis of thiol-responsive traceable linkers **41**, **42**, and **43**.

Prior to the examination of the enrichment and selective labeling of an alkynylated protein, a model reaction was conducted using an alkynylated model peptide **20**. CuAAC of traceable linker **41** with peptide **20** was performed in PBS with organic co-solvents in the presence of $CuSO_4$, sodium ascorbate, and TBTA[6] (Scheme 13). After 1 h of the reaction at ambient temperature, the traceable linker-peptide conjugate **51** was generated in high purity (Figure 15).



Scheme 13. CuAAC between traceable linker **41** and alkynylated model peptide **20**.

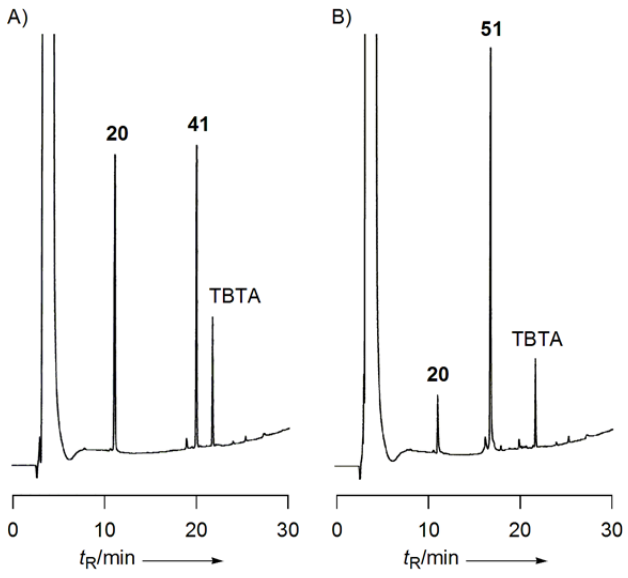
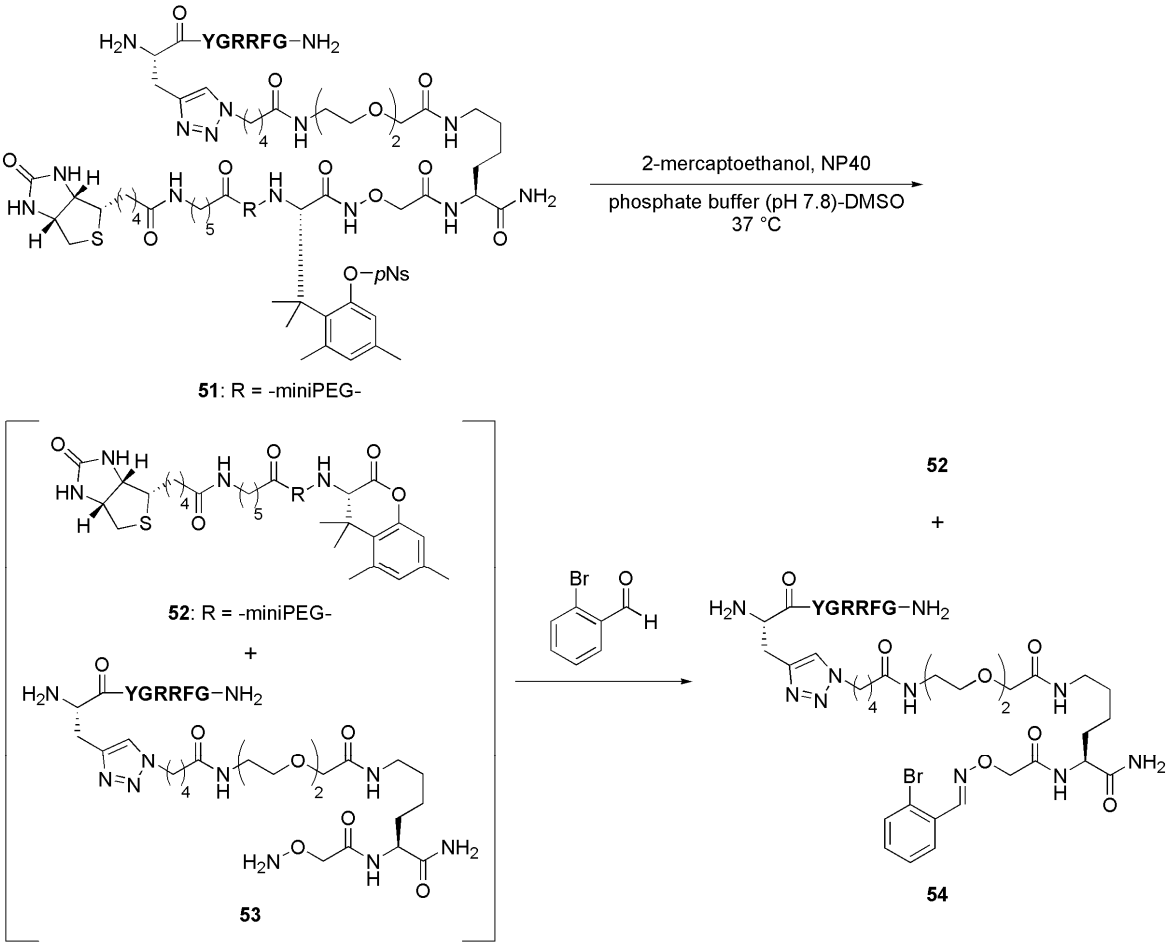


Figure 15. HPLC monitoring of the reaction as shown in Scheme 13. (A) Before the CuAAC (without $CuSO_4$ and sodium ascorbate). (B) After the CuAAC (reaction time = 1 h). Analytical HPLC conditions: linear gradient of 0.1% (v/v) TFA-MeCN in 0.1% (v/v) TFA aq., 5 to 90% over 30 min.

Next, I investigated the thiol-induced cleavage and labeling of the model peptide conjugate in a one-pot manner (Scheme 14). Reaction progress was monitored by HPLC and the resulting products were characterized by ESI-MS (Figure 16). The HPLC-purified conjugate **51** was treated with 2-mercaptoethanol at 37 ˚C in 10 mM sodium phosphate buffer (pH 7.8) containing DMSO and NP40. The cleavage of the linker went to completion within 24 h to yield biotin derivative **52** and model peptide **53** bearing an aminooxy group. 2-Bromobenzaldehyde was then added to the reaction mixture as a bromine-based labeling reagent[7] followed by the addition of aniline, which was used to accelerate the formation of the oxime.[8] Following a reaction for 1 h, the selective labeling of the model peptide was completed to yield the labeled peptide **54** and intact biotin derivative **52**.



Scheme 14. Thiol-induced cleavage of conjugate **51** and selective labeling of model peptide **53**.

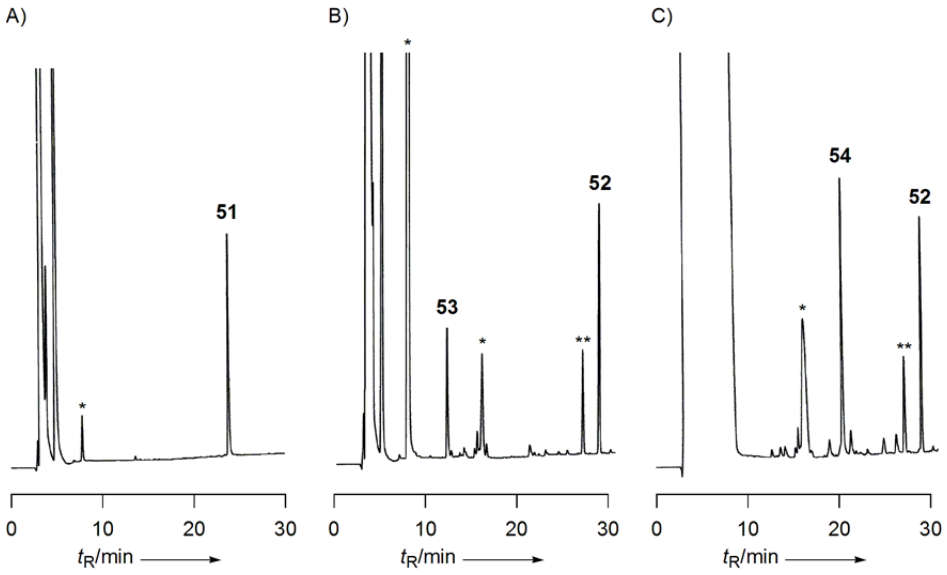


Figure 16. HPLC monitoring of the reaction as shown in Scheme 14. (A) After the linker cleavage (reaction time < 5 min). (B) After the linker cleavage (reaction time = 24 h). (C) After treatment with 2-bromobenzaldehyde and aniline. Analytical HPLC conditions: linear gradient of 0.1% (v/v) TFA-MeCN in 0.1% (v/v) TFA aq., 10 to 50% over 30 min. *Peaks observed when 2-mercaptoethanol was incubated in sodium phosphate buffer with DMSO. **A derivative of the *p*Ns group.

Since traceable linker **41** has been shown to be responsive to thiol-induced cleavage and selective labeling processes with alkynylated model peptide, I evaluated the enrichment and selective labeling of an alkynylated protein (Figure 17). In this study, alkynylated enolase **36**[9] was used as a target protein as described in Chapter 2. The thiol-intact linker **55** was also prepared as a negative control for thiol-induced cleavage used to elute the target protein from streptavidin beads. The CuAAC of traceable linkers **41**–**43** or negative control **55** with the alkynylated enolase **36** was initially examined. The alkynylated enolase **36** was treated with the linkers in PBS under optimized conditions as described in Chapter 2. Following a reaction for 1 h and subsequent separation by SDS-PAGE, the biotinylated proteins and all proteins were visualized by western blot analysis using SAv-HRP and lumitein staining, respectively. The linkers were successfully attached to the enolase in all cases (Figure 18).

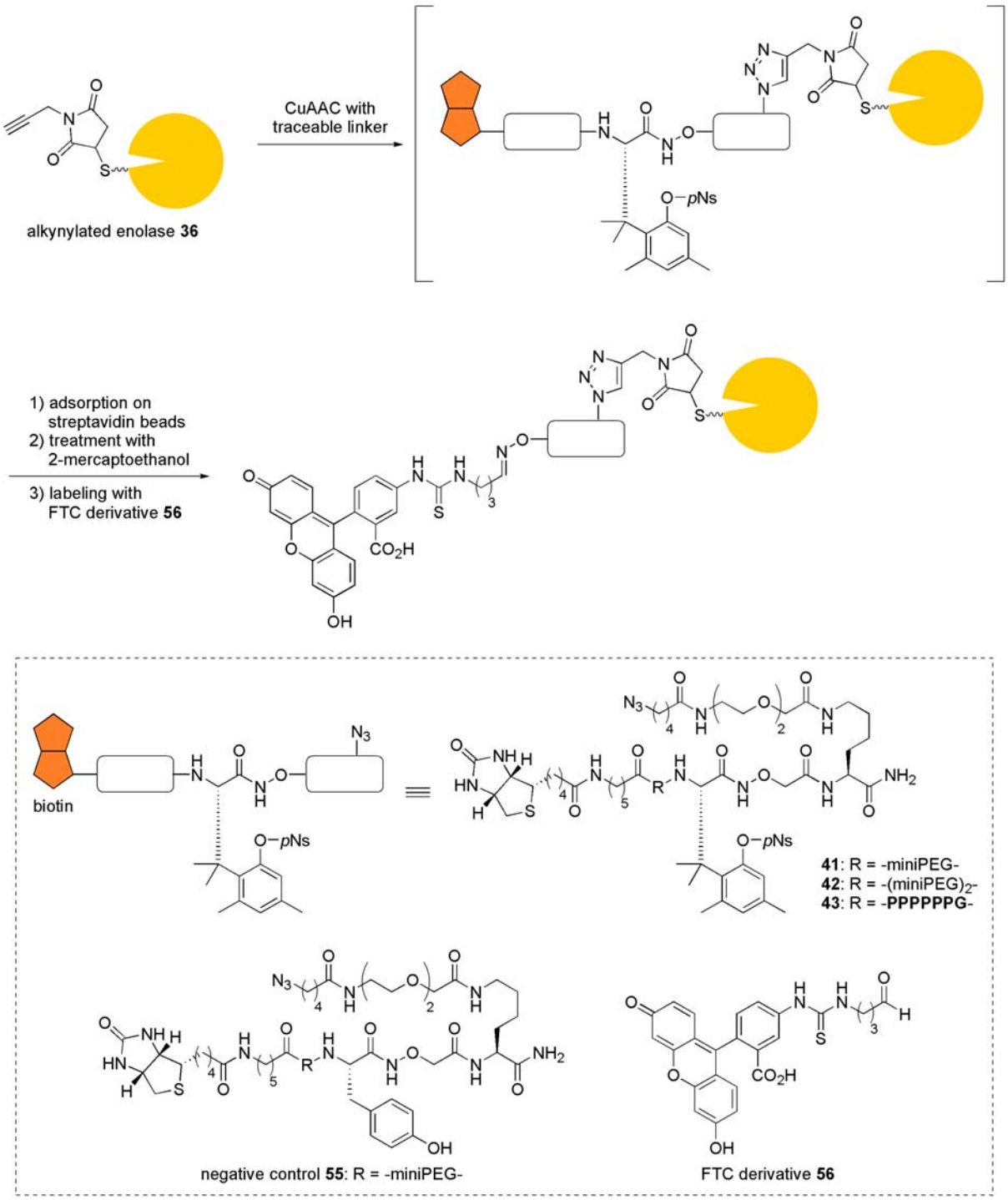


Figure 17. Schematic representation of CuAAC, enrichment, and labeling of alkynylated enolase **36** using the thiol-responsive traceable linkers.

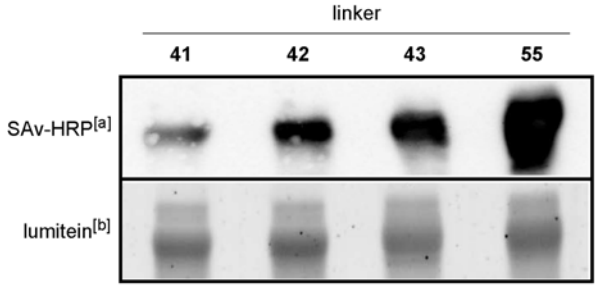


Figure 18. Monitoring of the CuAAC of traceable linkers **41**–**43** or negative control **55** with alkynylated enolase **36** using SDS-PAGE. Linker (0.10 mM) was introduced to alkynylated enolase **36** (0.50 g/L) using a mixture of $CuSO_4$ (1.0 mM), sodium ascorbate (0.50 mM), TBTA (0.10 mM), SDS (1% [w/v]), PBS, and co-solvents for 1 h. [a] Biotinylated proteins were detected by western blot analysis using SAv-HRP. [b] All proteins were visualized by lumitein staining.

Before the examination of thiol-induced elution of the adsorbed protein from streptavidin beads, I attempted to optimize the reaction time for the adsorption of the biotinylated protein to streptavidin beads. After CuAAC of traceable linker **43** with the alkynylated enolase **36**, the obtained products were treated with streptavidin beads for 3, 6, 12, 24, or 48 h. After washing the beads with PBS, the adsorbed proteins were released by heating the beads in SDS-PAGE sample loading buffer at 100 ˚C for 5 min. After separation by SDS-PAGE, the eluted proteins were visualized using silver stain. The amounts of the eluted protein increased time-dependently from 3 to 24 h, but remained unchanged between 24 and 48 h (data not shown). Therefore, the optimal reaction time for the adsorption of the biotinylated protein to streptavidin beads was 24 h.

Next, I examined the thiol-induced elution and selective labeling of the target protein. After the click reaction, the obtained products were treated with streptavidin beads for 24 h. After washing with PBS, the beads were reacted with 100 mM 2-mercaptoethanol in 10 mM sodium phosphate buffer (pH 7.8) containing NP40 at 37 ˚C for 24 h. The resulting mixture was centrifuged and the supernatant was treated with fluorescein thiocarbamoyl (FTC) derivative **56**,[10] bearing an aldehyde group in the presence of aniline for 24 h (the optimization of labeling conditions is shown in Figure 22 in the Experimental Section). After separation by SDS-PAGE, the FTC-labeled proteins and all proteins were visualized by fluorimetry without staining (λ_{ex} = 488 nm, λ_{em} = 530 nm) and by lumitein staining, respectively. When traceable linker **41**, **42**, or **43** was employed, the thiol-induced elution and labeling of the enolase proceeded

successfully, whereas small amounts of the proteins remained on the streptavidin beads after treatment with a thiol (Figure 19). In the case of thiol-intact negative control **55**, although the biotinylated enolase was successfully adsorbed onto the streptavidin beads, it was not released by treatment with a thiol. These observations suggested that the traceable linkers enabled the thiol-responsive release of the target protein and then the labeling of the aminooxy group on the cleavage products. Although I examined the influence of several conditions including thiol (2-mercaptoethanol, dithiothreitol, MESNa, 2-mercaptoethyl-*N*,*N*,*N*-trimethylammonium chloride,[11] 2-(dimethylamino)-ethanethiol, PhSH, 4-mercaptophenylacetic acid, or NaSH), pH (6.0, 7.8, or 9.0), temperature (37 or 50 ˚C), the kind of streptavidin beads (Pierce® Streptavidin UltraLink® Resin [Thermo SCIENTIFIC], NeutrAvidin™ Agarose Resins [Thermo SCIENTIFIC] or FG beads Streptavidin beads [TAMAGAWA SEIKI]) and thiol concentration (10, 100, 1000, or 10000 mM), the above conditions for the elution of the adsorbed enolase (100 mM 2-mercaptoethanol in 10 mM sodium phosphate buffer (pH 7.8) containing NP40 at 37 ˚C for 24 h using Pierce® Streptavidin UltraLink® Resin) gave a better result (data not shown). Furthermore, the adsorbed enolase was not eluted by 10 mM thiol treatment from streptavidin beads. This result indicated that the thiol-responsive traceable linker would be stable enough to be introduced into target proteins in a proteome because the concentration of glutathione in cytoplasm is 0.2–10 mM.[12] Although the elution efficiencies were 63–68% in all cases using thiol-responsive traceable linker **41**, **42**, or **43**, linker **41** gave the darkest band of all of the linkers to FTC-labeled enolase. Therefore, traceable linker **41** was used in all of the subsequent experiments.

Next, the orthogonal nature of the aminooxy group generated by the cleavage of the linker was investigated. The enolase-linker conjugate derived from CuAAC with traceable linker **41** or negative control **55** was treated with 2-mercaptoethanol followed by FTC derivative **56** in a manner similar to that shown in the footnote of Figure 19. After separation by SDS-PAGE, the FTC-labeled enolase was detected only when traceable linker **41** was used (Figure 20). From this result, the cleaved traceable linker proved to be suitable for the selective labeling of the target protein in the presence of other proteins.

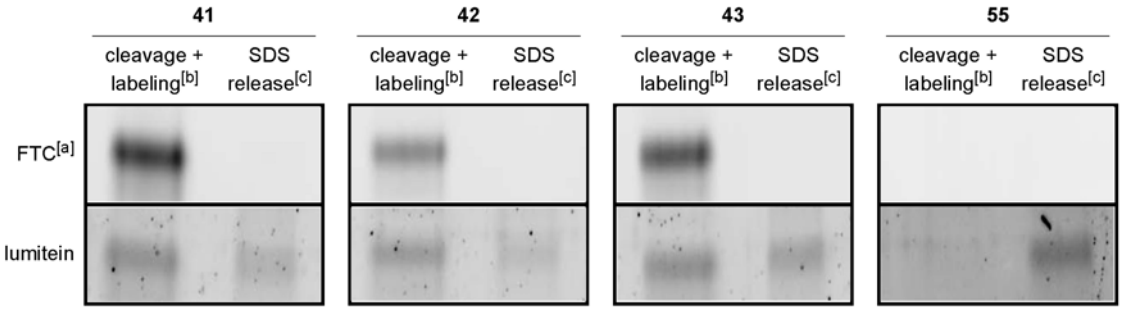


Figure 19. Monitoring of the enrichment and labeling of enolase using SDS-PAGE. Proteins were treated with streptavidin beads for 24 h following CuAAC. After washing, the beads were reacted with 2-mercaptoethanol (100 mM) and NP40 (1% [v/v]) in 10 mM sodium phosphate buffer (pH 7.8) at 37 ˚C for 24 h. The product was centrifuged and the supernatant was treated with FTC derivative **56** (0.10 mM) and aniline (100 mM) for labeling purposes. The reaction mixture was stirred at room temperature for 24 h. [a] FTC-labeled proteins were detected at λ_{ex} = 488 nm and λ_{em} = 530 nm without staining. [b] Proteins after the thiol treatment followed by the labeling reaction. [c] Proteins remaining on streptavidin beads after the thiol treatment. 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.

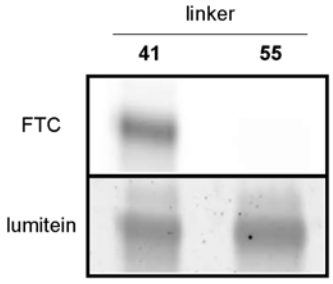


Figure 20. Examination of the orthogonal nature of the aminooxy group attached to the enolase following cleavage of the linker. After the CuAAC of traceable linker **41** or negative control **55** with the alkynylated enolase **36**, the resulting product was reacted with 2-mercaptoethanol followed by labeling reagent **56**. The reaction conditions used were similar to those described in the footnote of Figure 19.

Finally, pull-down experiments were conducted for the enrichment and selective labeling of the alkynylated enolase **36** in a protein mixture (Figure 21). A mixture consisting of alkynylated enolase **36**, bovine serum albumin (BSA), and

ovalbumin (1:1:1 [w/w]) was subjected to enrichment (by CuAAC with traceable linker **41**, adsorption on streptavidin beads, and treatment with a thiol) and to selective labeling with FTC derivative **56**. A procedure similar to that described for the alkynylated enolase **36** shown in Figure 19 was used in this particular case. The experimental details have been provided in the Experimental Section. After enrichment using streptavidin beads, ovalbumin was excluded from the protein mixture (Figure 21; lumitein staining: proteins adsorbed to the beads before treatment with a thiol [SDS release] and proteins eluted from the beads by treatment with a thiol [cleavage + labeling]). In this experiment, the eluent was found to be contaminated with BSA, in the same way that conventional cleavable linker systems can be contaminated with non-target proteins.[2] The eluent containing the enolase and the BSA contaminant was then treated with FTC derivative **56** in the presence of aniline for 24 h, which resulted in the selective labeling of the enolase and allowed the target enolase to be distinguished from the non-target contaminants (Figure 21; FTC detection: cleavage + labeling). These results indicated that the thiol-responsive traceable linker can be used to enrich and selectively label the target proteins for facile identification, even when the target protein is contaminated with non-target proteins in an eluent from the streptavidin beads.

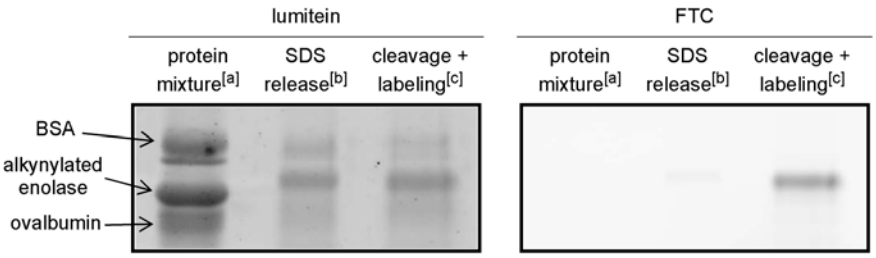


Figure 21. Pull-down experiments for alkynylated enolase **36** in a protein mixture consisting of alkynylated enolase **36**, BSA, and ovalbumin. Enrichment (CuAAC with traceable linker **41**, adsorption on streptavidin beads, and treatment with a thiol) and selective labeling with FTC derivative **56** were performed in a manner similar to that described in the footnote of Figure 19. [a] Mixture of alkynylated enolase **36**, BSA, and ovalbumin (1:1:1 [w/w]). [b] The beads were suspended in SDS-PAGE sample loading buffer prior to the thiol treatment. The mixture was heated at 100 ˚C for 5 min and then centrifuged, and the supernatant was analyzed. [c] Samples after enrichment and selective labeling of alkynylated enolase **36**.

In conclusion, the thiol-responsive traceable linker was developed as an advanced cleavable linker. This new linker not only enabled thiol-induced cleavage of linkers for target protein enrichment in a manner similar to that of conventional cleavable linkers, but also allowed the selective labeling of the target protein so that it could 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.

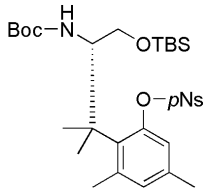
Experimental Section

General Methods

A Molecular Imager FX Pro and a Quantity One 1-D Analysis Software (Bio-Rad Laboratories) were employed for fluorescence gel images and its analyses, respectively. Other methods were similar to those in the Experimental Section in Chapters 1 and 2.

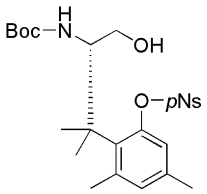
Preparation of Thiol-responsive Amino Acid

(*S*)-2-*tert*-Butoxycarbonylamino-3,3-dimethyl-3-[2,4-dimethyl-6-(nitrobenzene-4-sulfonyloxy)-phenyl] propanol *tert*-butyldimethylsilyl ether (44)



To a stirred solution of phenol **25**[5] (200 mg, 0.457 mmol) in acetone (16 mL) were added K_2CO_3 (632 mg, 4.57 mmol) and *p*Ns chloride (270 mg, 1.10 mmol), and the resulting suspension was refluxed for 4 h. The resulting mixture was quenched by the addition of H_2O and extracted with EtOAc. The combined organic phase was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by column chromatography (SiO_2, hexane/EtOAc = 20/1 then 10/1) and 244 mg of ether **44** (0.392 mmol, 86%) was obtained as a pale yellow oil: $[\alpha]^{20}_{D}$ –35.6 (*c* 1.22, $CHCl_3$); ^{1}H NMR, ^{13}C NMR and HRMS spectra were identical with those of the racemic one.[3a]

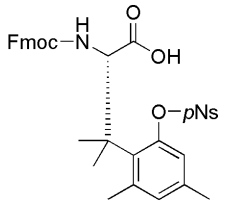
(*S*)-2-*tert*-Butoxycarbonylamino-3,3-dimethyl-3-[2,4-dimethyl-6-(nitrobenzene-4-sulfonyloxy)-phenyl]propanol (45)



Glacial acetic acid (2.8 mL) and H_2O (0.96 mL) were added to a solution of silyl ether **44** (231 mg, 0.371 mmol) in THF (0.96 mL). The reaction mixture was stirred at room temperature overnight. After the addition of H_2O, the resulting mixture was extracted with EtOAc. The organic phase was washed with H_2O and brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography

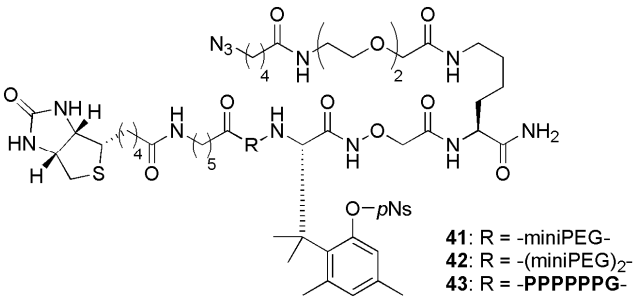
(SiO_2, hexane/EtOAc = 4/1 then 1/1) and 173 mg of alcohol **45** (0.339 mmol, 92%) was obtained as a white amorphousness: $[\alpha]^{20}_D$ –38.3 (*c* 1.13, $CHCl_3$); ^{1}H NMR, ^{13}C NMR and HRMS spectra were identical with those of the racemic one.[3a]

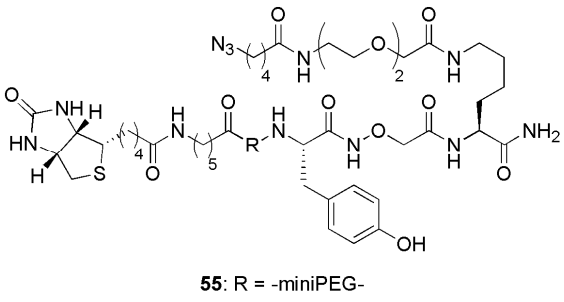
(*S*)-3,3-Dimethyl-3-[2,4-dimethyl-6-(nitrobenzene-4-sulfonyloxy)phenyl]-2-(9-fluorenylmethyl-carbonylamino)propionic acid (47)



To a stirred solution of alcohol **45** (160 mg, 0.315 mmol) in DMF (1.5 mL) was added PDC (592 mg, 1.57 mmol), and the resulting suspension was stirred at room temperature overnight. After addition of the Cerite 535, the reaction mixture was filtered through the Cerite 535. The obtained organic phase was washed with 5% (w/v) $KHSO_4$ aqueous solution and brine, dried over Na_2SO_4 and concentrated *in vacuo*. To a solution of the crude product in acetone-*tert*-BuOH-H_2O (6:4:1 [v/v], 6.8 mL) were added 2-methyl-2-butene (234 μL, 2.21 mmol), NaH_2PO_4 (60.5 mg, 0.504 mmol) and $NaClO_2$ (214 mg, 1.89 mmol). The resulting mixture was stirred at room temperature for 3.5 h. To the reaction mixture was added sat. NH_4Cl aq., and the obtained mixture was extracted with EtOAc. The resulting organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. Hydrogen chloride in EtOAc (4 M, 2.5 mL) was added to the crude product, and the resulting mixture was stirred at room temperature for 1 h. After concentration *in vacuo*, the obtained residue was dissolved in MeCN-10% (w/v) aqueous solution of Na_2CO_3 (1:1 [v/v], 2.8 mL). To the resulting solution was added FmocOSu (112 mg, 0.331 mmol), and the reaction mixture was stirred at room temperature overnight. After being acidified by addition of 5% (w/v) $KHSO_4$ aqueous solution, the reaction mixture was extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The obtained crude product was purified by column chromatography (SiO_2, chloroform then chloroform/MeOH = 99/1) and 165 mg of Fmoc derivative **47** (0.256 mmol, 81%) was obtained as a pale yellow amorphousness: $[\alpha]^{20}_D$ –19.6 (*c* 1.02, $CHCl_3$); ^{1}H NMR, ^{13}C NMR and HRMS spectra were identical with those of the racemic one.[3a]

Preparation of Thiol-responsive Traceable Linkers **41**, **42**, and **43**, and negative control **55**





General Procedure: The linkers 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.

building block	reagents	solvent
47 (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])
$N_3(CH_2)_4CO_2H$[13] (5 eq.)	DIC (5.3 eq.), Oxyma pure[14] (5 eq.)	DMF
Others[15] (3 eq.)	DIC (3.2 eq.), Oxyma pure (3 eq.)	DMF

Oxyma pure: ethyl cyanoglyoxylate-2-oxime.

For removal of an ivDde group, the resin was treated with 2% (v/v) hydrazine hydrate in DMF (twice for 2 h followed by once overnight). Following to completion of the chain elongation, the resin was subjected to global deprotection using TFA-Et_3SiH-H_2O (95:2.5:2.5 [v/v]) at room temperature for 2 h. After the resin was filtered off, cooled diethyl ether was added to the filtrate. The resulting precipitate was collected by centrifugation, washed with diethyl ether, and then purified by a preparative HPLC. When an amount of the precipitate was not sufficient, the filtrate after the general deprotection was concentrated using N_2 flow and neutralized by addition of $NaHCO_3$. Then it was purified by a preparative HPLC without the precipitation step.

41: A white lyophilized powder. 13% yield. Analytical HPLC conditions: 10 to 90%. Retention time = 18.8 min. Preparative HPLC conditions: 37 to 47%. LRMS (ESI-Ion Trap) *m/z* calcd for $[M + H]^+$: 1377.6, found: 1378.0.

42: A white lyophilized powder. 13% yield. Analytical HPLC conditions: 10 to 90%. Retention time = 18.6 min. Preparative HPLC conditions: 35 to 45%. LRMS (ESI-TOF) *m/z* calcd for $[M + 2H]^{2+}$: 761.9, found: 761.7.

43: A white lyophilized powder. 14% yield. Analytical HPLC conditions: 10 to 90%. Retention time = 18.0 min. Preparative HPLC conditions: 35 to 45%. LRMS (ESI-TOF) *m/z* calcd for $[M + 2H]^{2+}$: 936.5, found: 936.2.

55: A white lyophilized powder. 33% yield. Analytical HPLC conditions: 10 to 90%. Retention time = 13.2 min. Preparative HPLC conditions: 22 to 32%. LRMS (ESI-Ion Trap) *m/z* calcd for $[M + H]^{+}$: 1136.6, found: 1137.1.

Model Reactions Using Alkynylated Peptide **20**

Preparation of Model Peptide 20: The peptide was synthesized on NovaSyn® TGR resin (0.22 mmol amine/g) using Fmoc SPPS. Fmoc protected amino acids (3 eq.) were coupled at room temperature for 2 h by using DIC (3.2 eq.) and Oxyma Pure (3 eq.) in DMF. After treatment with TFA-Et_3SiH-H_2O (95:2.5:2.5 [v/v]) at room temperature for 1.5 h, the resin was filtered off and cooled diethyl ether was added to the filtrate. The resulting precipitate was collected by centrifugation, washed with diethyl ether, and then purified by a preparative HPLC.

20: A white lyophilized powder. 67% yield. Analytical HPLC conditions: 5 to 30%. Retention time = 16.7 min. Preparative HPLC conditions: 15 to 25%. LRMS (ESI-TOF) *m/z* calcd for $[M + H]^{+}$: 849.5, found: 849.2.

CuAAC: Traceable linker **41** in DMSO (6.0 mM, 66.6 μL, final concn. 0.20 mM), peptide **20** in PBS (1.25 mM, 400 μL, final concn. 0.25 mM), TBTA[6] in 20% (v/v) DMSO-*tert*-BuOH (1.7 mM, 118 μL, final concn. 0.10 mM), $CuSO_4$ in H_2O (50 mM, 40.0 μL, final concn. 1.0 mM), sodium ascorbate in H_2O (25 mM, 40.0 μL, final concn. 0.50 mM), and PBS (416 μL) were added to 1.00 mL of H_2O. After 1 h of the reaction at room temperature, the reaction mixture was purified by a preparative HPLC to yield conjugate **51**.

51: A white lyophilized powder. 56% yield. Analytical HPLC conditions: 5 to 90%. Retention time = 16.7 min. Preparative HPLC conditions: 30 to 39%. LRMS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$: 742.7, found: 742.6.

Thiol-induced cleavage: To 10 mM sodium phosphate buffer (pH 7.8, 137 μL) were added conjugate **51** in DMSO (6.0 mM, 2.37 μL, final concn. 0.10 mM), 2-mercaptoethanol (0.99 μL, final concn. 100 mM), and NP40 (1.42 μL, final concn. 1% [v/v]). 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 ESI-MS.

52: Analytical HPLC conditions: 10 to 50%. Retention time = 28.2 min. LRMS (ESI-TOF) *m*/*z* calcd for $[M + H]^+$: 704.4, found: 704.2.

53: Analytical HPLC conditions: 10 to 50%. Retention time = 11.9 min. LRMS (ESI-TOF) *m*/*z* calcd for $[M + 2H]^{2+}$: 669.4, found: 669.3.

Labeling with 2-bromobenzaldehyde: To the reaction mixture after the linker cleavage (72.0 μL) were added 10 mM sodium phosphate buffer (pH 7.8, 634 μL), 2-bromobenzaldehyde in DMSO (10 mM, 7.20 μL, final concn. 0.10 mM), and aniline (6.56 μL, final concn. 100 mM). The reaction was performed at room temperature for 1 h, and labeled product **54** was characterized using ESI-MS.

54: Analytical HPLC conditions: 10 to 50%. Retention time = 19.8 min. LRMS (ESI-TOF) *m*/*z* calcd for $[M + 2H]^{2+}$: 752.3 (^{79}Br deriv.) and 753.3 (^{81}Br deriv.), found: 752.2 and 753.2.

Introduction of Traceable Linker onto Alkynylated Enolase

CuAAC: To a mixture of PBS (550 μL) and H_2O (447 μL) were added the alkynylated enolase **36** in PBS (4.2 g/L, 180 μL, final concn. 0.50 g/L), traceable linker **41**, **42**, **43**, or negative control **55** in DMSO (6.0 mM, 25.0 μL, final concn. 0.10 mM), TBTA in 20% (v/v) DMSO-*tert*-BuOH (1.7 mM, 88.0 μL, final concn. 0.10 mM), $CuSO_4$ in H_2O (50 mM, 30.0 μL, final concn. 1.0 mM), sodium ascorbate in H_2O (25 mM, 30.0 μL, final concn. 0.50 mM), and SDS in H_2O (10% [w/v], 150 μL, final concn. 1% [w/v]). After the reaction at room temperature for 1 h, small molecules were removed by dialysis (Slide-A-Lyzer® G2 Dialysis Cassette, Thermo SCIENTIFIC) with PBS.

SDS-PAGE: After addition of 5 × non-reducing SDS-PAGE sample loading buffer (5 × SDS-PAGE sample loading buffer without 2-mercaptoethanol) followed by heating at 100 ˚C for 5 min, the reaction mixture was analyzed using SDS-PAGE in 12% 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 SAv-HRP (GE Healthcare) and ECL plus Western Blotting Detection System (GE Healthcare). For fluorescence imaging of all proteins in gel, Lumitein™ Protein Gel Stain (Nacalai Tesque) was employed.

Optimization of Reaction Conditions for Selective Labeling of Protein Containing Aminooxy Group

After the CuAAC of the traceable linker **43** or negative control **40**[16] with the alkynylated enolase **36**, 2-mercaptoethanol (0.63 μL, final concn. 100 mM), NP40 (0.90 μL, final concn. 1% [v/v]) and 200 mM sodium phosphate buffer (pH 7.6, 76.5 μL) were added to the reaction mixture containing ca. 4.5 μg enolase and its derivatives. After incubation at 37 ˚C for 24 h, the resulting mixture was treated with FTC derivative **56**[10] (final concn. 1, 10, 100 or 1000 μM) with or without aniline (final concn. 100 mM). After standing at room temperature for 1 or 24 h, the resulting mixture was desalted using Pierce® SDS-PAGE Sample Prep Kit (Thermo SCIENTIFIC). Then the obtained mixture was subjected to the SDS-PAGE as similar to those described in section “Introduction of Traceable Linker onto Alkynylated Enolase”. In this case, 2 × SDS-PAGE sample loading buffer was used instead of 5 × non-reducing SDS-PAGE sample loading buffer.

Figure 22. Optimization of the reaction conditions for selective labeling of the protein containing an aminooxy group. After the CuAAC of traceable linker **43** or negative control **40** with the alkynylated enolase **36**, the resulting product was reacted with 2-mercaptoethanol followed by labeling reagent **56**. After separation by SDS-PAGE, the FTC-labeled proteins and all proteins were visualized by fluorimetry without staining (FTC: λ_{ex} = 488 nm, λ_{em} = 530 nm) and by silver stain, respectively. Proteins were reacted with 2-mercaptoethanol (100 mM) and NP40 (1% [v/v]) in 200 mM sodium phosphate buffer (pH 7.6) at 37 ˚C for 24 h. The resulting mixture was treated with FTC derivative **56** and stood at room temperature. (A) Examination of the concentration of FTC derivative **56** (1, 10, 100, or 1000 μM) for 24 h without aniline. (B) Examination of the time (1 or 24 h) and 100 mM aniline (presence or absence during the reaction) with 100 μM FTC derivative **56**.

Adsorption on Streptavidin Beads, Linker Cleavage and Labeling of Enolase Conjugate

Adsorption on Streptavidin Beads: After the CuAAC of the linkers with the alkynylated enolase **36**, Pierce® Streptavidin UltraLink® Resin (35 μL, Thermo SCIENTIFIC) was added to the reaction mixture containing ca. 100 μg enolase and its derivatives. After the adsorption at room temperature for 24 h, the resulting resin was

washed with PBS five times and then subjected to subsequent reactions.

Linker Cleavage: To the resulting streptavidin beads was added a cleavage cocktail consisting of 2-mercaptoethanol (1.40 μL, final concn. 100 mM), NP40 (2.00 μL, final concn. 1% [v/v]) in 10 mM sodium phosphate buffer (pH 7.8, 197 μL). The reaction was conducted at 37 ˚C for 24 h under N_2 atmosphere. After centrifugation of the resulting mixture (2,000 rpm, 2 min), supernatant was collected and the precipitate was suspended in 100 μL PBS. The suspension was subjected to centrifugation (2,000 rpm, 2 min) again and the obtained supernatant was combined with the first one.

Labeling: To the obtained supernatant were added FTC derivative **56** (final concn. 0.10 mM) and aniline (final concn. 100 mM), and the mixture was stirred at room temperature for 24 h. After concentration using Amicon® Ultra-0.5, Ultracel-10 Membrane, 10 kDa (Merk Millipore) (14,000 × g, 15 min), addition of PBS and the concentration was repeated four times to remove the excess of the non-reacted fluorophore. Then the obtained mixture was subjected to the SDS-PAGE as similar to those described in section "Introduction of Traceable Linker onto Alkynylated Enolase". In this case, SDS-PAGE sample loading buffer was used instead of the non-reducing SDS-PAGE sample loading buffer. The FTC-labeled enolase was detected by fluorescence imaging (λ_{ex} = 488 nm, λ_{em} = 530 nm) without staining or the use of western blot analysis.

Elution of Proteins Remaining on Streptavidin Beads After Thiol-induced Cleavage: The resin obtained after the linker cleavage as mentioned above was suspended in 2 × SDS-PAGE sample loading buffer (25 μL) and H_2O (25 μL), and the mixture was heated at 100 ˚C for 5 min. After centrifugation as mentioned in the section "Linker Cleavage", the combined supernatant was concentrated and analyzed using SDS-PAGE as similar to those described in section "Labeling".

Examination of Orthogonality of Aminooxy Group on Enolase

After CuAAC of traceable linker **41** or negative control **55** with the alkynylated enolase **36** followed by treatment with 2-mercaptoethanol and NP40, the product was treated with FTC derivative **56** and aniline. The obtained mixture was analyzed using SDS-PAGE followed by fluorimetric detection. Reaction conditions as similar to those described in section "Introduction of Traceable Linker onto Alkynylated Enolase" and "Adsorption on Streptavidin Beads, Linker Cleavage and Labeling of Enolase

Conjugate" were employed.

Enrichment and Selective Labeling of Enolase in Protein Mixture

As a protein mixture, solution containing the alkynylated enolase **36**, BSA, and ovalbumin (1/1/1 [w/v]) was used.

CuAAC: The alkynylated enolase **36** in PBS (3.9 g/L, 127 μL, final concn. 0.50 g/L), BSA in PBS (3.3 g/L, 150 μL, final concn. 0.50 g/L), ovalbumin in PBS (3.3 g/L, 150 μL, final concn. 0.50 g/L), the traceable linker in DMSO (6.0 mM, 17 μL, final concn. 0.10 mM), TBTA in 20% (v/v) DMSO-*tert*-BuOH (1.7 mM, 59 μL, final concn. 0.10 mM), $CuSO_4$ in H_2O (50 mM, 20 μL, final concn. 1.0 mM), sodium ascorbate in H_2O (25 mM, 20 μL, final concn. 0.50 mM), SDS in H_2O (10% [w/v], 100 μL, final concn. 1% [w/v]), PBS (100 μL), and H_2O (257 μL) were mixed. Following to reaction at room temperature for 1 h, the resulting solution was dialyzed using Slide-A-Lyzer® G2 Dialysis Cassette (Thermo SCIENTIFIC) with PBS.

Adsorption on Streptavidin Beads, Cleavage of Linker and Labeling: It was performed as similar to that described in a section "Adsorption on Streptavidin Beads, Linker Cleavage and Labeling of Enolase Conjugate".

Enrichment without Use of Thiol-induced Linker Cleavage: After the adsorption of the protein mixture on streptavidin beads following CuAAC, the proteins on the beads were eluted and analyzed as mentioned in a section "Elution of Proteins Remaining on Streptavidin Beads After Thiol-induced Cleavage".

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Chapter 4. Conclusions

1. A near-infrared (NIR) two-photon excitation (2PE)-responsive peptide has been developed. The amide bond at the C-terminal position of the NIR 2PE-responsive amino acid possessing a 4,5-dimethoxy-2-nitrobenzyl group as a phenolic protection was successfully cleaved by a focused NIR pulsed laser irradiation at 740 nm. The two-photon uncaging action cross-section at 740 nm (δ_{u740}) of the model peptide was sufficient for application in biological studies.

2. A fluoride ion-responsive traceable linker that possesses a fluoride ion-responsive amino acid has been developed. The introduction of the traceable linker onto an alkynylated enolase by click chemistry, adsorption of the resulting biotinylated enolase on streptavidin beads, and elution of the adsorbed enolase from the beads by treatment with TBAF were achieved.

3. A thiol-responsive traceable linker that possesses a thiol-responsive amino acid has been developed. The enrichment (click chemistry, adsorption on streptavidin beads, and elution by treatment with 2-mercaptoethanol) and selective labeling of the enolase in a protein mixture (enolase, BSA, and ovalbumin) were achieved.

Taken together, my findings demonstrate that novel stimulus-responsive amino acids have been developed and that the developed stimulus-responsive amino acids can be applied to chemical biology including purification of target proteins. These findings should provide a new methodology for functionally investigating peptides/proteins and easily identifying target proteins of bioactive compounds, such as drug candidates, using stimulus-responsive amino acids. The search for additional applications of stimulus-responsive amino acids is currently underway in my laboratory.

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List of Publications

This study was or will be published in following papers.

1. Development and Photo-responsive Peptide Bond Cleavage Reaction of Two-photon Near-infrared Excitation-responsive Peptide
   Akira Shigenaga, Jun Yamamoto, Yoshitake Sumikawa, Toshiaki Furuta and Akira Otaka
   Tetrahedron Lett. **2010**, *51*, 2868–2871.

2. Enantioselective Synthesis of Stimulus-responsive Amino Acid via Asymmetric α-Amination of Aldehyde
   Akira Shigenaga, Jun Yamamoto, Naomi Nishioka and Akira Otaka
   Tetrahedron **2010**, *66*, 7367–7372.

3. Development of a Fluoride-responsive Amide Bond Cleavage Device and Its Application to a Fluoride-responsive Traceable Linker
   Jun Yamamoto, Nami Maeda, Tomohiro Tanaka, Masaya Denda, Koji Ebisuno, Wataru Nomura, Hirokazu Tamamura, Youichi Sato, Aiko Yamauchi, Akira Shigenaga and Akira Otaka
   to be submitted.

4. Development of a Traceable Linker Containing a Thiol-responsive Amino Acid for the Enrichment and Selective Labeling of Target Proteins
   Jun Yamamoto, Nami Maeda, Miku Kita, Chiaki Komiya, Tomohiro Tanaka, Wataru Nomura, Hirokazu Tamamura, Youichi Sato, Aiko Yamauchi, Akira Shigenaga and Akira Otaka
   to be submitted.