Electronic Supplementary Information

N-Sulfanylethylanilide-based traceable linker for enrichment and selective labelling of target proteins

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## Abbreviations

- MPAA: 4-mercaptophenylacetic acid
- TCEP: tris(2-carboxyethyl)-phosphine hydrochloride
Scheme S1 Reagents and conditions: a) tBuSH, I₂, CH₂Cl₂, 79%; b) Fmoc SPPS; c) TFA/triethylsilane/H₂O = 95/2.5/2.5 (v/v).

Scheme S2 Reagents and conditions: a) fluorescein 5-isothiocyanate, triethylamine, THF, EtOH, 93%.

Scheme S3 Reagents and conditions: a) methyl iodide, DIPEA, DMF, 19%.
Fig. S1 CuAAC of the traceable linker 6 followed by NCL. A) Reagents and conditions: S5, CuSO4, Na ascorbate, TBTA, PBS, DMF (*Non-peptidic peak derived from reagents); b) MPAA, TCEP, Na phosphate buffer, NP40, 37 °C (**MPAA). B) Reaction profiles of the CuAAC and NCL. HPLC conditions for CuAAC: 0.1% (v/v) TFA in acetonitrile/0.1% (v/v) TFA aq., 30 to 40% over 30 min, detection by UV absorption (220 nm); HPLC conditions for NCL: 0.1% (v/v) TFA in acetonitrile/0.1% (v/v) TFA aq., 25 to 35% over 30 min, detection by UV absorption (220 nm).

Fig. S2 CuAAC of traceable linker 6 and alkynylated BSA. A: before CuAAC; B: after CuAAC with traceable linker 6; C: after CuAAC with negative control 7. Reagents and conditions: 3.2 μM alkynylated BSA, 100 μM traceable linker 6, 1.0 mM CuSO4, 1.0 mM Na ascorbate, 1% (w/v) SDS, 100 μM TBTA, PBS, 1 h.

Fig. S3 SDS-PAGE of the BSA-traceable linker 6 conjugate eluted from the streptavidin beads. Elution conditions A: 100 μM 8, 50 mM MPAA, 40 mM TCEP, 0.1% SDS, Na phosphate buffer (concentration of the phosphate: 1000, 400 or 100 mM), pH 7.4, 37 °C, 24 h; elution conditions B for the elution of the proteins remaining on the beads after the application of conditions A: sample buffer, 100 °C, 5 min. Lumitein staining allowed for the visualization of all proteins as similar to silver staining. Fluorescence imaging, λ<sub>ex</sub> = 460 nm, λ<sub>em</sub> > 515 nm.
Fig. S4 SDS-PAGE of the BSA-linker 6, S8 or S9 conjugate eluted from the streptavidin beads. Elution conditions A: 100 μM 8, 50 mM MPAA, 40 mM TCEP, 0.1% SDS, 400 mM Na phosphate buffer, pH 7.4, 37 °C, 24 h; elution conditions B for the elution of the proteins remaining on the beads after the application of conditions A: sample buffer, 100 °C, 5 min. Fluorescence imaging, $\lambda_{\text{ex}} = 460$ nm, $\lambda_{\text{em}} > 515$ nm.

Fig. S5 SDS-PAGE of the BSA-linker conjugate eluted from the streptavidin beads. Elution conditions A and B are shown in Figure S4. Fluorescence imaging, $\lambda_{\text{ex}} = 460$ nm, $\lambda_{\text{em}} > 515$ nm.

Fig. S6 An HPLC profile of the eluent from streptavidin beads. Following to adsorption of S6 on streptavidin beads, the beads were treated with 100 μM cysteine, 50 mM MPAA, 40 mM TCEP and 0.1% SDS in 400 mM Na phosphate buffer (pH 7.4) at 37 °C for 24 h. The obtained eluent was analyzed by HPLC and MS. HPLC conditions: 0.1% (v/v) TFA in acetonitrile/0.1% (v/v) TFA aq., 25 to 35% over 30 min, detection by fluorescence ($\lambda_{\text{ex}} = 495$ nm, $\lambda_{\text{em}} = 520$ nm).
Fig. S7 Enrichment and selective labelling of the alkynylated BSA in HCT116 cell lysate using traceable linker 6. Details of the conditions are shown in the experimental section. Silver staining: lane 1: alkynylated BSA; Lane 2: lysate of HCT116 cells; Lane 3: a mixture of the alkynylated BSA and HCT116 cell lysate; Lane 4: eluted proteins after the CuAAC reaction of the mixture with 6, adsorption of the biotinylated material onto streptavidin beads and the treatment of the beads with cysteine-fluorescein conjugate 8 in the presence of phosphate (elution by linker cleavage using elution conditions A shown in Figure S4); Fluorescence imaging: the sample applied to lane 4 was analyzed ($\lambda_{\text{ex}} = 460$ nm, $\lambda_{\text{em}} > 515$ nm).
General Methods

All reactions of small molecules were carried out under a positive pressure of argon. Column chromatography of the small molecules was performed using Silica Gel 60 N (spherical, neutral, Kanto Chemical Co., Inc.). Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™ (ESI-TOF) or a Bruker Esquire2000T (ESI-Ion Trap). NMR spectra were recorded using a Bruker AV400N at 400 MHz frequency for $^1$H, and JEOL JNM-AL300 at 75 MHz frequency for $^{13}$C. Optical rotations were measured using a JASCO P-2200 polarimeter (concentration in g/100 mL). For HPLC separations, a Cosmosil 5C$_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil 5C$_{18}$-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min) or a Cosmosil 5C$_{18}$-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10.0 mL/min) was employed, and eluting products were detected by UV absorption (220 nm) or fluorescence ($\lambda_{ex} = 495$ nm, $\lambda_{em} = 520$ nm). A solvent system consisting of 0.1% (v/v) TFA in H$_2$O and 0.1% TFA (v/v) in MeCN using linear gradient over 30 min was employed for HPLC elution. ECL signals from the western blot analysis and fluorescence were detected using a LAS-4000mini (Fujifilm). Composition of an SDS-PAGE sample loading buffer is as follow: 50 mM Tris-HCl, 2.0% (v/v) SDS, 6.0% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.050% (w/v) bromophenol blue in H$_2$O.

Synthesis of SEAlide-based Traceable Linker 6 as Shown in Scheme S1


To a stirred solution of trityl derivative S1 (200 mg, 278 µmol) in CH$_2$Cl$_2$ (9.27 mL) were added tBuSH (156 µL, 1.39 mmol) and I$_2$ (529 mg, 4.17 mmol) at room temperature, and the mixture was stirred at same temperature for 1 h. Then 0.5 M Na$_2$S$_2$O$_3$ aq. was added to the resulting mixture until its dark red color disappeared. The obtained mixture was extracted with EtOAc, and the combined organic layer was dried over MgSO$_4$ and concentrated in vacuo. The product was purified by column chromatography (CHCl$_3$/MeOH = 100/0, 99/1, then 0/100 (v/v)) and 124 mg of S2 (220 µmol, 79%) was obtained as white amorphousness: $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ = 1.29 (9H, s), 2.85 (2H, t, $J$ = 7.5 Hz), 3.81 (2H, s), 4.05 (2H, t, $J$ = 6.8 Hz), 4.20 (1H, t, $J$ = 6.8 Hz), 4.35 (2H, d, $J$ = 7.3 Hz), 5.89 (1H, s), 7.30 (2H, t, $J$ = 7.6 Hz), 7.35 (2H, d, $J$ = 8.4 Hz), 7.39 (2H, t, $J$ = 7.5 Hz), 7.59 (2H, d, $J$ = 7.5 Hz), 7.75 (2H, d, $J$ = 7.4 Hz), 8.12 (2H, d, $J$ = 8.4 Hz); $^{13}$C NMR (CDCl$_3$, 75 MHz) $\delta$ = 30.0, 37.1, 43.8, 47.2, 48.2, 49.1, 67.6, 120.1, 125.3, 127.2, 127.9, 128.4, 132.2, 141.4, 143.9, 144.6, 156.7; HRMS (ESI-TOF) m/z calcd for C$_{30}$H$_{32}$N$_2$NaO$_5$S$_2$ ([M + Na]$^+$) 587.1650, found 587.1631.
**Preparation of Traceable Linker 6 via Peptide Resin S3**

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

<table>
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<th>building block</th>
<th>reagents</th>
<th>solvent</th>
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<tr>
<td><strong>S2 (2 eq.)</strong></td>
<td>HATU (1.9 eq.), DIPEA (1.9 eq.)</td>
<td>DMF</td>
</tr>
<tr>
<td>(+)-biotin (5 eq.)</td>
<td>DIC (5 eq.), HOBt-H2O (5 eq.)</td>
<td>DMSO/DMF = 1/1 (v/v)</td>
</tr>
<tr>
<td>N3(CH2)4CO2HS2 (5 eq.)</td>
<td>DIC (5.3 eq.), Oxyma Pure3 (5 eq.)</td>
<td>DMF</td>
</tr>
<tr>
<td>Others (3 eq.)</td>
<td>DIC (3.2 eq.), Oxima Pure (3 eq.)</td>
<td>DMF</td>
</tr>
</tbody>
</table>

Abbreviations. DIC: N,N'-diisopropylcarbodiimide; DIPEA: N,N'-diisopropylethylamine; HATU: 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; HOBt: 1-hydroxybenzotriazole; Oxyma pure: ethyl cyanoglyoxylate-2-oxime.

For removal of an ivDde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl) group, the peptide resin was treated with 2% (v/v) hydrazine hydrate in DMF (twice for 2 h followed by once overnight). Following to completion of the peptide elongation, the resin was subjected to global deprotection using TFA/triethylsilane/H2O (95:2.5:2.5 (v/v)) for 2 h at room temperature. After filtration of the resin and subsequent removal of TFA by N2 flow, the obtained residue was neutralized by the addition of sat. NaHCO3 aq. followed by solid NaHCO3. The obtained mixture was dissolved in 33% (v/v) AcOH aq., and then purified by a preparative HPLC.

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

**Preparation of Traceable Linkers S8 and S9 and Negative Control Linker 7**

Linkers **S8, S9** (Fig. S4) and **S10** (Scheme S3) were prepared as similar to traceable linker 6. For preparation of negative control 7, SEAlide S1 was employed instead of S2. Then, methyl iodide (15 µL, 0.24 mmol) and DIPEA (15 µL, 86 µmol) were added to a solution of **S10** (3.5 mg, 3.0 µmol) in DMF (3.4 mL). The resulting mixture was stirred at room temperature for 1 h, and the obtained solution was purified by preparative HPLC to yield linker 7 (0.67 mg, 19% yield).

**S8:** A white lyophilized powder; 25% yield; Analytical HPLC conditions: 10 to 90%. Retention time = 16.3 min; Preparative HPLC conditions: 32 to 42%; LRMS (ESI-Ion Trap) m/z calcd for [M + H]+ 1401.7, found 1401.8.
S8

S9: A white lyophilized powder; 36% yield; Analytical HPLC conditions: 10 to 90%. Retention time = 16.0 min; Preparative HPLC conditions: 31 to 41%; LRMS (ESI-Ion Trap) m/z calcd for [M + H]^+ 1836.9, found 1836.4.

S10: A white lyophilized powder; 24% yield; Analytical HPLC conditions: 10 to 90%. Retention time = 14.2 min; Preparative HPLC conditions: 25 to 35%; LRMS (ESI-Ion Trap) m/z calcd for [M + H]^+ 1168.5, found 1168.0.

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

**Click Chemistry of Traceable Linker 6 with Alkynylated Small Molecule S5 Followed by Linker Cleavage with Cysteine**

**Preparation of alkyne derivative S5:** To fluoresceinisothiocyanate isomer-I (FITC) (70.1 mg, 180 µmol) were added S4 (50.0 mg, 216 µmol) in THF/EtOH (2/3 (v/v), 10.5 mL) and triethylamine (30.2 µL, 216 µmol) at 0 °C, and the resulting mixture was stirred at room temperature for 1 h. After evaporation, the resulting residue was purified by column chromatography (CHCl_3/MeOH = 98/2, 97/3, 90/10, then 0/100 (v/v)) and 104 mg of S5 (168 µmol, 93%) was obtained as a yellow powder: 1H NMR (CD3OD, 400 MHz) δ = 2.81 (1H, t, J = 2.5 Hz), 3.63-3.81 (16H, m), 4.14 (2H, d, J = 2.5 Hz), 6.58 (2H, dd, J = 2.5 Hz and 8.8Hz), 6.71-6.73 (4H, m), 7.81 (2H, dd, J = 1.7 Hz and 8.3 Hz), 8.19 (2H, d, J = 1.8 Hz); 13C NMR (CD3OD, 75 MHz) δ = 45.5, 59.0, 70.0, 70.1, 71.3, 71.43, 71.49, 71.54, 75.9, 80.6, 103.5, 112.1, 114.2, 126.1, 129.2, 130.6, 142.6, 154.7, 162.3, 170.8, 182.9; HRMS (ESI-TOF) m/z calcd for C32H32N2NaO9S ([M + Na]^+) 643.1726, found 643.1706.

**Click Chemistry:** Traceable linker 6 in DMSO (6.0 mM, 66.6 µL), alkyne 9 in PBS (1.25 mM, 400 µL), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) in 20% (v/v) DMSO/tBuOH (1.7 mM, 118 µL), CuSO4 in water (50 mM, 40.0 µL), sodium ascorbate in water (25 mM, 40.0 µL), and PBS (416 µL) were added to 1.00 mL of water (final concn.: 0.20 mM 6, 0.25 mM 9, 0.10 mM TBTA, 1.0 mM CuSO4, 0.50 mM sodium ascorbate). After 1 h of the reaction at room temperature, reaction mixture was injected into a preparative HPLC to yield conjugate S6.

S6: A yellow lyophilized powder; 0.61 mg, 65% yield; Analytical HPLC conditions: 30 to 40%. Retention time = 25.5 min; Preparative HPLC conditions: 35 to 45%; LRMS (ESI-Ion Trap) m/z calcd for [M + 2H]^2+ 938.9, found 938.7.
Linker Cleavage via NCL: To sodium phosphate buffer (1.0 M, pH 7.5, 147.5 μL) containing NP40 (1.0% (v/v)), TCEP-HCl (40 mM), MPAA (100 mM) and cysteine (5.0 mM) was added conjugate 5 in DMF (6.0 mM, 2.5 μL, final concn. 0.10 mM). After incubation at 37 °C for 24 h under argon, completion of cleavage of the linker was confirmed using HPLC and the products were characterized by MS analyses.

Preparation of Alkynylated BSA

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

SDS-PAGE Conditions

After addition of SDS-PAGE sample loading buffer followed by heating at 100 °C for 5 min, the reaction mixture was analyzed using SDS-PAGE in 10% polyacrylamide gels. For the chemiluminescence imaging of the biotinylated proteins, the proteins were transferred to Amersham Hybond-P PVDF Membrane (GE Healthcare) and detected with a SAv-HRP (GE Healthcare) and ECL plus Western Blotting Detection System (GE Healthcare). For visualization of all proteins, silver stain KANTO III (KANTO CHEMICAL CO., INC.) or Lumitein™ Protein Gel Stain (Nacalai Tesque) was employed.

Introduction of Traceable Linker 6, S8, S9 or negative control 7 onto Alkynylated BSA

Click Chemistry: To a mixture of PBS (540 μL) and water (437 μL) were added the alkynylated BSA in PBS with 0.1% SDS (5.0 g/L, 200 μL), traceable linker 6, S8, S9 or negative control linker 7 in DMSO (6.0 mM, 25.0 μL), TBTA in 20% (v/v) DMSO/tBuOH (1.7 mM, 88.0 μL), CuSO₄ aq. (50 mM, 30.0 μL), sodium ascorbate aq. (50 mM, 30.0 μL), and SDS aq. (10% (w/v), 150 μL) (final concn.: 0.50 g/L alkynylated BSA, 0.10 mM 6, S8, S9 or 7, 0.10 mM TBTA, 1.0 mM CuSO₄, 1.0 mM sodium ascorbate, 1% (w/v) SDS). After the reaction at room temperature for 1 h, small molecules were removed by acetone precipitation.

Adsorption on Streptavidin Beads Followed by Elution of BSA Conjugate

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

**Elution of BSA Conjugate by Linker Cleavage:** To the resulting streptavidin beads was added FTC-derivative 8 (10 mM, 2.0 μL, final concn. 0.10 mM) in sodium phosphate buffer (0.40 M, pH 7.4, 198 μL) containing SDS (0.1%), Gdn-HCl (6.0 M), TCEP-HCl (40 mM) and MPAA (50 mM). The reaction was conducted at 37 °C for 24 h under argon. After centrifugation of the resulting mixture (2000 rpm, 2 min), supernatant was collected and the precipitate was suspended in 100 μL PBS with 0.1% SDS. The suspension was subjected to centrifugation (2000 rpm, 2 min) again and the obtained supernatant was combined with the first one.

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

**Structural Analysis of Eluted Target:** In this experiment, S6 was employed instead of the BSA-traceable linker conjugate. Adsorption of S6 on streptavidin beads was performed as mentioned above (5.34 μL S6 (3 mM in DMSO), 20 μL beads and 200 μL PBS). Then the beads were treated with 100 μM cysteine, 50 mM MPAA, 40 mM TCEP and 0.1% SDS in 400 mM Na phosphate buffer (pH 7.4) at 37 °C for 24 h. Then the eluent was analysed by HPLC. Analytical HPLC conditions: 25 to 35%. Retention time of S7 = 22.4 min; LRMS (ESI-Ion Trap) m/z calcd for [M + 2H]⁺ 535.2, found 535.2.

**Enrichment and Selective Labelling of BSA in Protein Mixture**

**Preparation of the protein mixture:** A lysate of red blood cells (BIZCOM JAPAN) in PBS was prepared according to a protocol on vendor’s website. To the lysate (1.1 mL) was added the alkynylated BSA in PBS with 0.1% SDS (20 μL) and the obtained mixture was used for the following experiments. For preparation of a lysate of HCT116 cells, HCT116 cells were cultured in DMEM (Dulbecco’s modified Eagle medium) containing 10% fetal bovine serum and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin) at 37 °C in a humidified incubator continuously flushed with a mixture of 5% CO₂-95% air. The cells were washed twice with cold PBS, harvested, and then sonicated in HEPES buffered saline (HBS). The cell extracts were centrifuged at 20,000 × g for 10 min at 4 °C. Protein concentration of resultant supernatants was

S10
determined with a DC protein assay kit (Bio-Rad) with BSA as the standard. To the obtained protein mixture (4.26 mg/mL, 100 μL) was added the alkynylated BSA in PBS with 0.1% SDS (2.6 μL) and the obtained mixture was used for following experiments.

Click Chemistry in the Red Blood Cell Lysate: To the alkynylated BSA in the red blood cell lysate (1.12 mL) were added traceable linker 6 in DMSO (6.0 mM, 25 μL), TBTA in 20% (v/v) DMSO/tBuOH (1.7 mM, 88 μL), CuSO₄ aq. (50 mM, 30 μL), sodium ascorbate aq. (50 mM, 30 μL), SDS aq. (10% (w/v), 150 μL) and PBS (57 μL) (final concn.: 0.10 mM 6, 0.10 mM TBTA, 1.0 mM CuSO₄, 1.0 mM sodium ascorbate, 1% (w/v) SDS). Following to reaction at room temperature for 1 h, the reagents were removed by acetone precipitation. Then, the obtained precipitate was dissolved in 0.1% SDS in PBS (1.2 mL) for the following experiments.

Click Chemistry in the Lysate of HCT116 Cells: To the alkynylated BSA in the lysate of HCT116 cells (102.6 μL) were added traceable linker 6 in DMSO (6.0 mM, 3.3 μL), TBTA in 20% (v/v) DMSO/tBuOH (1.7 mM, 11.6 μL), CuSO₄ aq. (50 mM, 4.0 μL), sodium ascorbate aq. (50 mM, 4.0 μL), SDS aq. (10% (w/v), 19.8 μL) and PBS (52.7 μL) (final concn.: 0.10 mM 6, 0.10 mM TBTA, 1.0 mM CuSO₄, 1.0 mM sodium ascorbate, 1% (w/v) SDS). Following to reaction at room temperature for 1 h, the reagents were removed by acetone precipitation. Then, the obtained precipitate was dissolved in 0.1% SDS in PBS (600 μL) for the following experiments.

Adsorption on Streptavidin Beads Followed by Cleavage: It was performed as similar to that described in a section “Adsorption on Streptavidin Beads Followed by Elution of BSA Conjugate”.

Enrichment without Linker Cleavage as Similar to Conventional Linkers: After adsorption of the protein mixture on streptavidin beads, the proteins on the beads were eluted and analyzed as mentioned in a section “Elution of Proteins Remaining on Streptavidin Beads”.

References


S7 A lysate of red blood cells was prepared using sonication according to a vendor’s protocol. See, “http://www.gelifesciences.co.jp/technologies/protein_preparation/lysis.html” (accessed December 6, 2014).