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Electronic Supplementary Information

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

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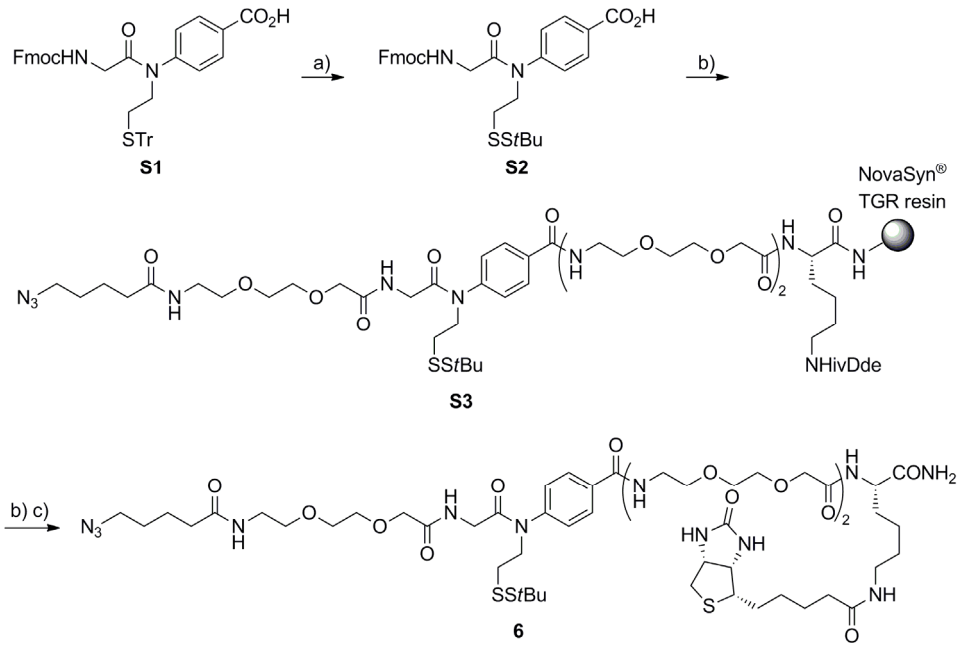
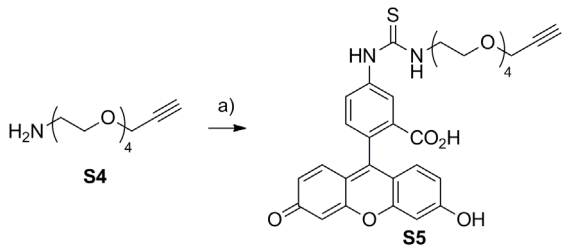
Abbreviations

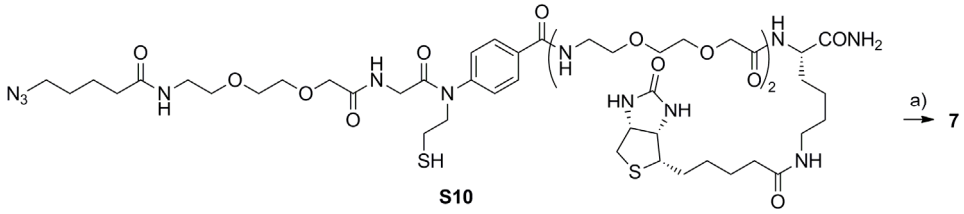
MPAA	4-mercaptophenylacetic acid
TCEP	tris(2-carboxyethyl)-phosphine hydrochloride



Scheme S1 Reagents and conditions: a) *t*BuSH, I_2, CH_2Cl_2, 79%; b) Fmoc SPPS; c) TFA/triethylsilane/H_2O = 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%.

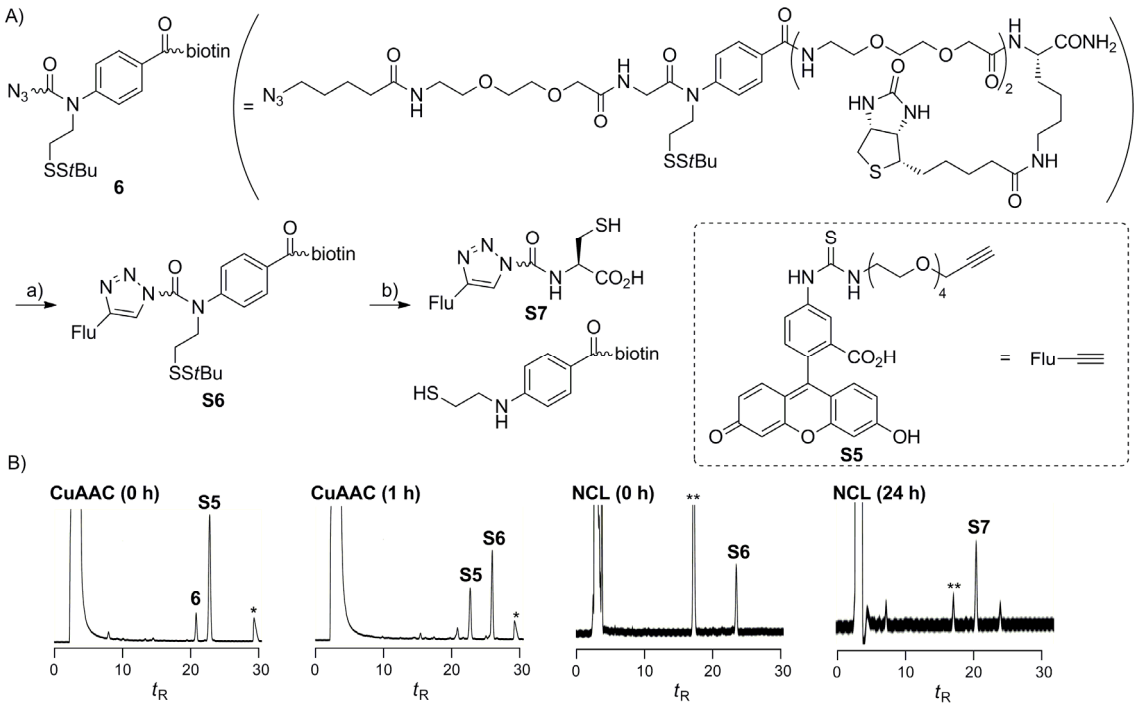


Fig. S1 CuAAC of the traceable linker **6** followed by NCL. A) Reagents and conditions: **S5**, $CuSO_4$, Na ascorbate, TBTA, PBS, DMF (*Non-peptidic peak derived from reagents); b) MPAA, TCEP, Na phosphate buffer, NP40, 37 °C (**MPAA). B) Reaction profiles of the CuAAC and NCL. HPLC conditions for 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 $CuSO_4$, 1.0 mM Na ascorbate, 1% (w/v) SDS, 100 µM TBTA, PBS, 1 h.

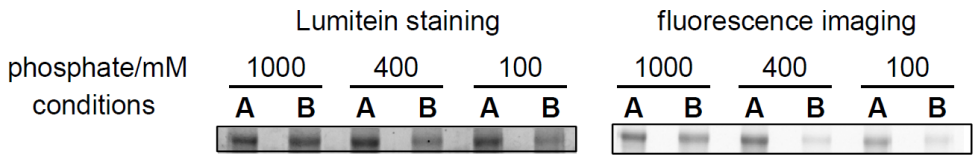
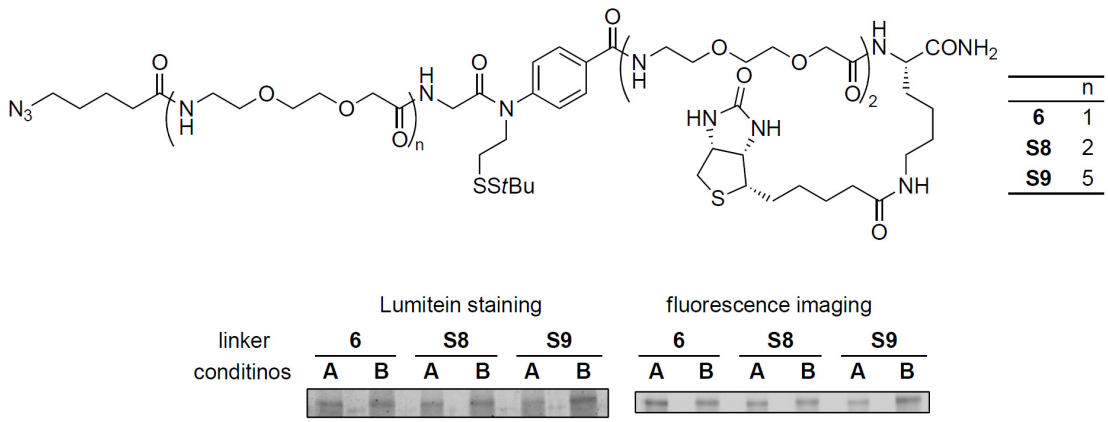


Fig. S3 SDS-PAGE of the BSA-traceable linker **6** conjugate eluted from the streptavidin beads. Elution conditions **A**: 100 µM **8**, 50 mM MPAA, 40 mM TCEP, 0.1% SDS, Na phosphate buffer (concentration of the phosphate: 1000, 400 or 100 mM), pH 7.4, 37 °C, 24 h; elution conditions **B** for the elution 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_{ex} = 460 nm, λ_{em} > 515 nm.



	n
6	1
S8	2
S9	5

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, λ_{ex} = 460 nm, λ_{em} > 515 nm.

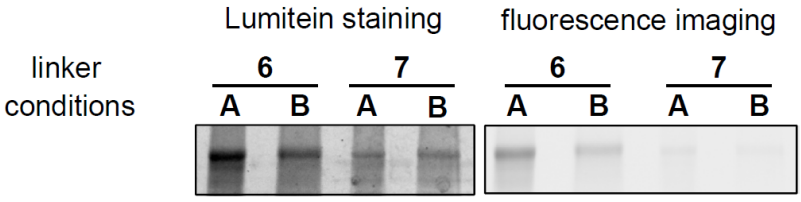


Fig. S5 SDS-PAGE of the BSA-linker conjugate eluted from the streptavidin beads. Elution conditions **A** and **B** are shown in Figure S4 Fluorescence imaging, λ_{ex} = 460 nm, λ_{em} > 515 nm.

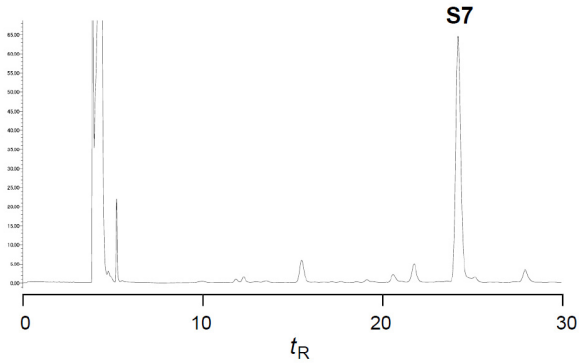


Fig. S6 An HPLC profile of the eluent from streptavidin beads. Following to adsorption of **S6** on streptavidin beads, the beads were treated with 100 μM cysteine, 50 mM MPAA, 40 mM TCEP and 0.1% SDS in 400 mM Na phosphate buffer (pH 7.4) at 37 °C for 24 h. The obtained eluent was analyzed by HPLC and 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 (λ_{ex} = 495 nm, λ_{em} = 520 nm)

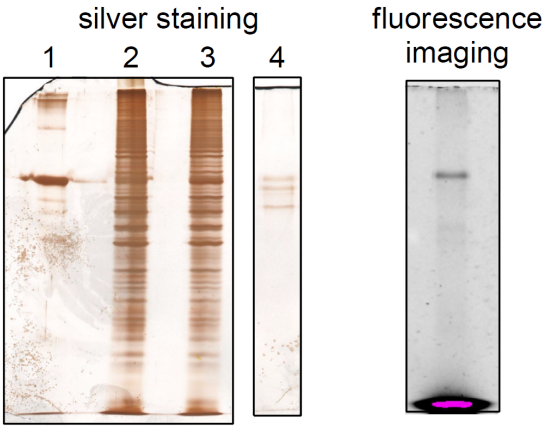


Fig. S7 Enrichment and selective labelling of the alkynylated BSA in HCT116 cell lysate using traceable linker **6**. Details of the conditions are shown in the experimental section. Silver staining: lane 1: alkynylated BSA; Lane 2: lysate of HCT116 cells; Lane 3: a mixture of the alkynylated BSA and 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 (λ_{ex} = 460 nm, λ_{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 (λ_{ex} = 495 nm, λ_{em} = 520 nm). A solvent system consisting of 0.1% (v/v) TFA in H_2O and 0.1% TFA (v/v) in MeCN using linear gradient over 30 min was employed for HPLC elution. ECL signals from the western blot analysis and fluorescence were detected using a LAS-4000mini (Fujifilm). Composition of an SDS-PAGE sample loading buffer is as follow: 50 mM Tris-HCl, 2.0% (v/v) SDS, 6.0% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.050% (w/v) bromophenol blue in H_2O.

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

4-{2-([{(9*H*-fluoren-9-yl)methoxy}carbonyl]amino)-*N*-(2-[*tert*-butylsulfinothioyl]ethyl)acetamido}benzoic acid (S2)

To a stirred solution of trityl derivative **S1**[S1] (200 mg, 278 μmol) in CH_2Cl_2 (9.27 mL) were added *t*BuSH (156 μL, 1.39 mmol) and I_2 (529 mg, 4.17 mmol) at room temperature, and the mixture was stirred at same temperature for 1 h. Then 0.5 M $Na_2S_2O_3$ aq. was added to the resulting mixture until its dark red color disappeared. The obtained mixture was extracted with EtOAc, and the combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The product was purified by column chromatography ($CHCl_3$/MeOH = 100/0, 99/1, then 0/100 (v/v)) and 124 mg of **S2** (220 μmol, 79%) was obtained as white amorphousness: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.29 (9H, s), 2.85 (2H, t, J = 7.5 Hz), 3.81 (2H, s), 4.05 (2H, t, J = 6.8 Hz), 4.20 (1H, t, J = 6.8 Hz), 4.35 (2H, d, J = 7.3 Hz), 5.89 (1H, s), 7.30 (2H, t, J = 7.6 Hz), 7.35 (2H, d, J = 8.4 Hz), 7.39 (2H, t, J = 7.5 Hz), 7.59 (2H, d, J = 7.5 Hz), 7.75 (2H, d, J = 7.4 Hz), 8.12 (2H, d, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 30.0, 37.1, 43.8, 47.2, 48.2, 49.1, 67.6, 120.1, 125.3, 127.2, 127.9, 128.4, 132.2, 141.4, 143.9, 144.6, 156.7 ; HRMS (ESI-TOF) m/z calcd for $C_{30}H_{32}N_2NaO_5S_2$ ($[M + Na]^+$) 587.1650, found 587.1631.

Preparation of traceable linker 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).

building block	reagents	solvent
S2 (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)
$N_3(CH_2)_4CO_2H$[S2] (5 eq.)	DIC (5.3 eq.), Oxyma Pure[S3] (5 eq.)	DMF
Others (3 eq.)	DIC (3.2 eq.), Oxima Pure (3 eq.)	DMF

Abbreviations. DIC: *N*,*N'*-diisopropylcarbodiimide; DIPEA: *N*,*N*-diisopropylethylamine; HATU: 1-[bis(dimethylamino)methylene]-1*H*-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/H_2O (95:2.5:2.5 (v/v)) for 2 h at room temperature. After filtration of the resin and subsequent removal of TFA by N_2 flow, the obtained residue was neutralized by the addition of sat. $NaHCO_3$ aq. followed by solid $NaHCO_3$. The obtained mixture was dissolved in 33% (v/v) AcOH aq., and then purified by a preparative HPLC.

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.

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**[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: ^{1}H NMR (CD_3OD, 400 MHz) δ = 2.81 (1H, t, J = 2.5 Hz), 3.63-3.81 (16H, m), 4.14 (2H, d, J = 2.5 Hz), 6.58 (2H, dd, J = 2.5 Hz and 8.8Hz), 6.71-6.73 (4H, m), 7.81 (2H, dd, J = 1.7 Hz and 8.3 Hz), 8.19 (2H, d, J = 1.8 Hz); ^{13}C NMR (CD_3OD, 75 MHz) δ = 45.5, 59.0, 70.0, 70.1, 71.3, 71.43, 71.49, 71.54, 75.9, 80.6, 103.5, 112.1, 114.2, 126.1, 129.2, 130.6, 142.6, 154.7, 162.3, 170.8, 182.9; HRMS (ESI-TOF) *m/z* calcd for $C_{32}H_{32}N_2NaO_9S$ ($[M + Na]^+$) 643.1726, found 643.1706.

Click Chemistry: Traceable linker **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)[S5] in 20% (v/v) DMSO/*t*BuOH (1.7 mM, 118 μL), $CuSO_4$ in water (50 mM, 40.0 μL), sodium ascorbate in water (25 mM, 40.0 μL), and PBS (416 μL) were added to 1.00 mL of water (final concn.: 0.20 mM **6**, 0.25 mM **9**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 0.50 mM sodium ascorbate). After 1 h of the reaction at room temperature, reaction mixture was injected into a preparative HPLC to yield conjugate **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.

S7: Analytical HPLC conditions: 25 to 35%. Retention time = 19.4 min; LRMS (ESI-Ion Trap) *m/z* calcd for $[M + H]^+$ 1069.4, found 1069.3.

Preparation of Alkynylated BSA

Starting from a commercially available BSA (6.6 mg), the alkynylated BSA was prepared according to the literature.[S6] 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/*t*BuOH (1.7 mM, 88.0 μL), $CuSO_4$ aq. (50 mM, 30.0 μL), sodium ascorbate aq. (50 mM, 30.0 μL), and SDS aq. (10% (w/v), 150 μL) (final concn.: 0.50 g/L alkynylated BSA, 0.10 mM **6**, **S8**, **S9** or **7**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 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%), Gn·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 vender's website.[S7] 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% CO_2-95% air. The cells were washed twice with cold PBS, harvested, and then sonicated in HEPES buffered saline (HBS). The cell extracts were centrifuged at 20,000 × g for 10 min at 4 °C. Protein concentration of resultant supernatants was

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

Click Chemistry in the Red Blood Cell Lysate: To the alkynylated BSA 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/*t*BuOH (1.7 mM, 88 μL), $CuSO_4$ aq. (50 mM, 30 μL), sodium ascorbate aq. (50 mM, 30 μL), SDS aq. (10% (w/v), 150 μL) and PBS (57 μL) (final concon.: 0.10 mM **6**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 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/*t*BuOH (1.7 mM, 11.6 μL), $CuSO_4$ aq. (50 mM, 4.0 μL), sodium ascorbate aq. (50 mM, 4.0 μL), SDS aq. (10% (w/v), 19.8 μL) and PBS (52.7 μL) (final concon.: 0.10 mM **6**, 0.10 mM TBTA, 1.0 mM $CuSO_4$, 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”.

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