SUPPLEMENTARY INFORMATION

FOR

Convenient Modular Method for Affinity Labeling (MoAL Method) Based on a Catalytic Amidation

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Experimental details

General methods. ^{1}H NMR spectra were recorded on a Bruker DPX 400 spectrometer. Chemical shifts are reported as δ values relative to tetramethylsilane as internal standard. Infrared spectra were recorded on a Nicolet FT-IR AVATER 360 spectrometer. Mass spectra were measured on a Micromass Zq2000 spectrometer (ESI-MS) and Bruker Daltonics micrOTOFQ II (ESI-MS). UV-visible spectra were recorded on a SHIMADZU vis recording spectrophotometer UV-2400 PC. Fluorescence analysis was performed on a JASCO Spectrofluorometer FP-6500.

Cascade Blue ethylenediamine, trisodium salt (CBA)[1], 6-(biotinylamino)hexanoic acid[2], and 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT)[3] were prepared according to the literature. Other chemicals were obtained from commercial sources and used as received unless otherwise noted. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % horse serum and 5 % fetal bovine serum.

1. Preparation of the biotinylated ligand catalyst **1** (Fig. S-1)

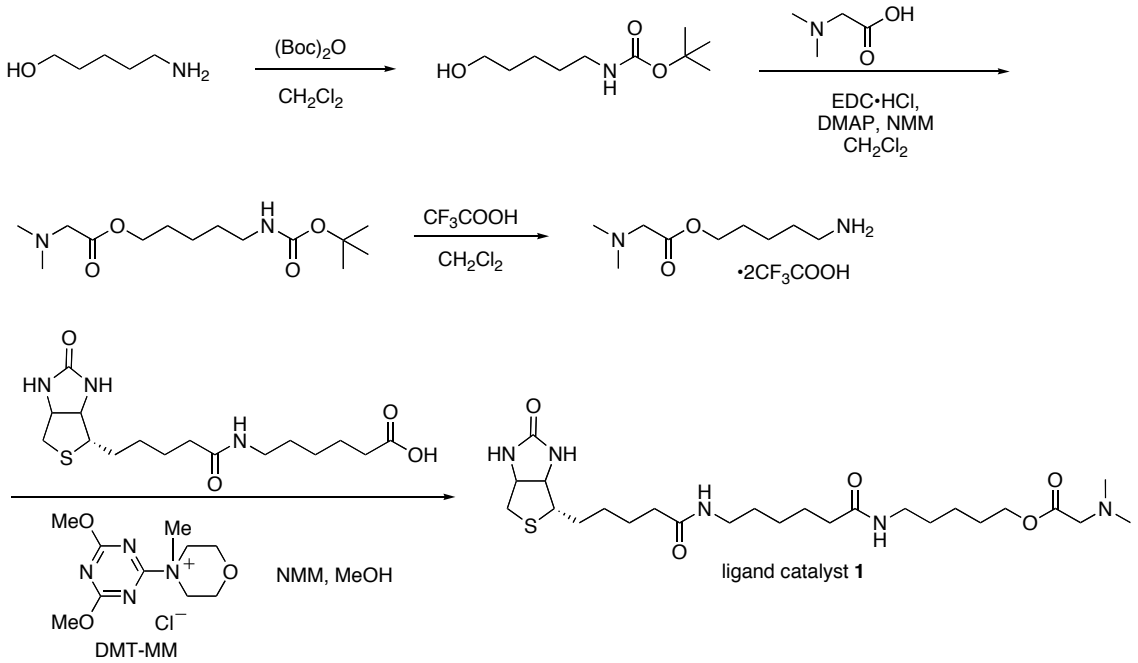

Figure S-1. Reaction scheme for preparation of **1**.

t-Butyl 5-hydroxypentylcarbamate

Di-*t*-butyl dicarbonate (7.6 g, 34.9 mmol) was added to a solution of 5-amino-1-pentanol (3.0 g, 29.1 mmol) in CH_2Cl_2 (29 mL) at 0 °C. After being stirred

for 1 h at RT, the reaction mixture was loaded on a silica gel column and eluted with hexane/AcOEt (1:1). Further purification by distillation (2 mmHg, 220 °C) gave the desired compound as a colorless oil (5.9 g, 60% yield).

^{1}H NMR ($CDCl_3$): δ 1.33-1.63 (m, 6H), 1.44 (s, 9H), 3.12 (td, 2H, *J*=6.2, 6.4Hz), 3.64 (t, 2H, *J*=6.4Hz), 4.64 (s, 1H); IR (neat): 3469, 2932, 1678, 1644, 1536, 1169 cm^{-1}; MS (ESI): 204 $(M+H)^+$.

5-(*t*-Butoxycarbonylamino)pentyl 2-(dimethylamino)acetate

To a solution of *t*-butyl 5-hydroxypentylcarbamate (2.2 g, 10.8 mmol), *N,N*-dimethylglycine hydrochloride (1.5 g, 10.8 mmol), *N*-methylmorpholine (NMM, 1.1 g , 10.8 mmol), and 4-dimethylaminopyridine (0.7 g, 5.37 mmol) in dry CH_2Cl_2 (32 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (3.1 g, 16.2 mmol) under nitrogen atmosphere at 0 °C. After being stirred overnight at RT, the reaction mixture was poured into water, and extracted with CH_2Cl_2. The organic layer was washed with Na_2CO_3 and brine, and then dried over $MgSO_4$. The crude mixture was purified by silica gel column chromatography (AcOEt) to give 2.4 g of the desired compound as a colorless oil (2.4 g, 78% yield).

^{1}H NMR ($CDCl_3$): δ 1.33-1.56 (m, 4H), 1.44 (s, 9H), 1.62-1.72 (m, 2H), 2.35 (s, 6H), 3.12 (q, 2H, *J*=6.4Hz), 3.16 (s, 2H), 4.13 (t, 2H, *J*=6.8Hz); IR (neat): 3441, 2938, 1692, 1641, 1166 cm^{-1}; MS (ESI): 289 $(M+H)^+$.

5-Aminopentyl *N,N*-dimethylglycine bis(trifluoroacetate)

To a solution of 5-(*t*-butoxycarbonylamino)pentyl 2-(dimethylamino)acetate (1.0 g, 3.47 mmol) in CH_2Cl_2 (6.3 mL) was added trifluoroacetic acid (3.9 g , 34.7 mmol) at RT under nitrogen. After being stirred for 3 h at RT, the solvent was removed under vacuum. The residue was dissolved in CH_2Cl_2 (ca. 6 mL) followed by removing the solvent, and this procedure repeated three times. The crude compound was purified by decantation with AcOEt–Et_2O at -78 °C to give the desired compound as a white solid (1.4 g, 100% yield).

mp: 51-55 °C. ^{1}H NMR (methanol-d_4): δ 1.42-1.54 (m, 2H), 1.63-1.82 (m, 4H), 2.93 (t, 2H, *J*=7.7Hz), 2.97 (s, 6H), 4.15 (s, 2H), 4.28 (t, 2H, *J*=6.6Hz); IR(KBr): 3452, 2955,1742, 1676, 1468, 1425, 1202, 1136 cm^{-1}; MS(ESI): 189 $(M+H)^+$.

5-[6-(Biotinylamino)hexanoylamino]pentyl 2-(dimethylamino)acetate (1)

To a solution of 5-aminopentyl *N,N*-dimethylglycine bis(trifluoroacetate) (346 mg, 0.83 mmol), 6-(biotinylamino)hexanoic acid (300 mg, 0.83 mmol), and *N*-methylmorpholine (252 mg, 2.49 mmol) in MeOH (21 mL) was added 4-(4,6-dimetoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM, 574 mg, 2.07 mmol) at RT. After being stirred overnight, the MeOH was partially removed under vacuum followed by addition of Et_2O to form a precipitate. The resulting solid was collected and loaded on silica gel column and eluted with MeOH containing 1% Et_3N to give **1** as a trifluoroacetate. The salt was neutralized with 0.05 M KOH in MeOH, and then recrystallized from MeOH–Et_2O to give **1** as colorless crystals (262.8 mg, 60% yield).

mp: 124-133 °C. ^{1}H NMR (methanol-d_4): δ 1.25-1.80 (m, 18H), 2.18 (t, 2H, *J*=7.4Hz), 2.19 (t, 2H, *J*=7.3Hz), 2.33 (s, 6H), 2.70 (d, 1H, *J*=12.7Hz), 2.93 (dd, 1H, *J*=4.9, 12.7Hz), 3.11-3.24 (m, 7H), 4.13 (t, 2H, *J*=6.6Hz), 4.30 (dd, 1H, *J*=4.5, 7.8Hz), 4.49 (dd, 1H, *J*=4.9, 7.8Hz); ^{13}C NMR (methanol-d_4): δ 23.3, 25.7, 25.9, 26.6, 28.3, 28.5, 28.8, 29.0, 29.1, 35.8, 36.0, 39.1, 39.2, 40.0(CH_2-S), 44.2($2CH_3$), 56.0(CH-S), 59.3(CH_2-N), 60.6(CH-N), 62.4(CH-N), 64.9(CH_2-O), 165.1(N-CO-N), 170.1(CO-O), 174.9(CO-NH), 175.0(CO-NH); IR(KBr): 3302, 2924, 2853, 1738, 1701, 1638, 1560 cm^{-1}; MS (ESI): 528 $(M+H)^+$; HRMS (ESI-TOF): calcd for $C_{25}H_{46}N_5O_5S$ $(M+H)^+$ 528.3214; found 528.3211.

2. Specific labeling experiment of avidin by modular affinity labeling (MoAL) method.

2-1. General procedure for labeling of avidin

Avidin (10 μL of 0.15 mM solution in 50 mM phosphate buffer, pH 8.0) and the ligand catalyst **1** (5 μL of 1.2 mM solution in 50 mM phosphate buffer, pH 8.0) were mixed and allowed to stand for 30 min at RT. To the solution was added CBA (20 μL of 6.0 mM solution in 50 mM phosphate buffer, pH 8.0), CDMT (10 μL of 12 mM solution in 50 mM phosphate buffer containing 5% MeOH, pH 8.0), and 50 mM phosphate buffer (30 μL, pH 8.0), and the resulting solution was shaken by Vortex and allowed to stand at RT for 8 h. The concentration of the solutes in the resulting solution (75 μL) was as follows: avidin: 20 μM; ligand catalyst **1**: 80 μM; CBA: 1.6 mM; CDMT: 1.6 mM. The

solution was loaded on a gel filtration column (Sephadex G-50 medium, 0.7 × 35 cm), and eluted with 50 mM phosphate buffer (pH 8.0) containing 0.1 M NaCl at RT. The labeling yield of avidin was determined by UV-visible spectral analysis (Table S-1, entry 1). For control experiments, γ-globulins or dimethylglycine ethyl ester **2** was used instead of avidin or **1**, respectively (entries 2, 3).

2-2. Competitive inhibition of the labeling by addition of (+)-biotin

The experiment was performed in the manner described above for avidin labeling (2-1) except for addition of 2.0 mM of (+)-biotin (Table S-1, entry 4).

2-3. Quantification of labeling yield by UV-visible spectroscopy[4]

Table S-1. Labeling yields of proteins by CAL.

entry	protein	catalyst	average[a)]
1	Avidin	ligand catalyst **1**	169%
2	Avidin	ethyl ester **2**	10%
3	g-Globulins	ligand catalyst **1**	11%
4	Avidin [b)]	ligand catalyst **1**	9%

a) Average value of three times of experiments. b) The labeling reaction was conducted in the presence of 25 equiv of biotin

The labeling yield of avidin was determined by measuring the absorbance of labeled avidin at 280 nm (A_{280}) and 399 nm (A_{399}). (Fig. S-2)

The molar extinction coefficients ($M^{-1}cm^{-1}$) of avidin and CBA at 280 nm and 399 nm are:

CBA: $\varepsilon_{280} = 18792$, $\varepsilon_{399} = 26400$

Avidin: $\varepsilon_{280} = 93960$, $\varepsilon_{399} = 0$

The labeling yield of avidin (L_{avd}) was calculated by

L_{avd} (%) = 100 × C_{cba} / C_{avd}

where C_{avd} and C_{cba} are concentration (M) of avidin and CBA connecting to avidin, respectively. They are determined by the following equations.

$C_{cba} = A_{399}$ / (26400 × 1 cm)

$$C_{avd} = (A_{280} - 18792 \times C_{cba}) / (93960 \times 1\ cm)$$
$$= (A_{280} - 18792 \times A_{399} / 26400) / 93960$$

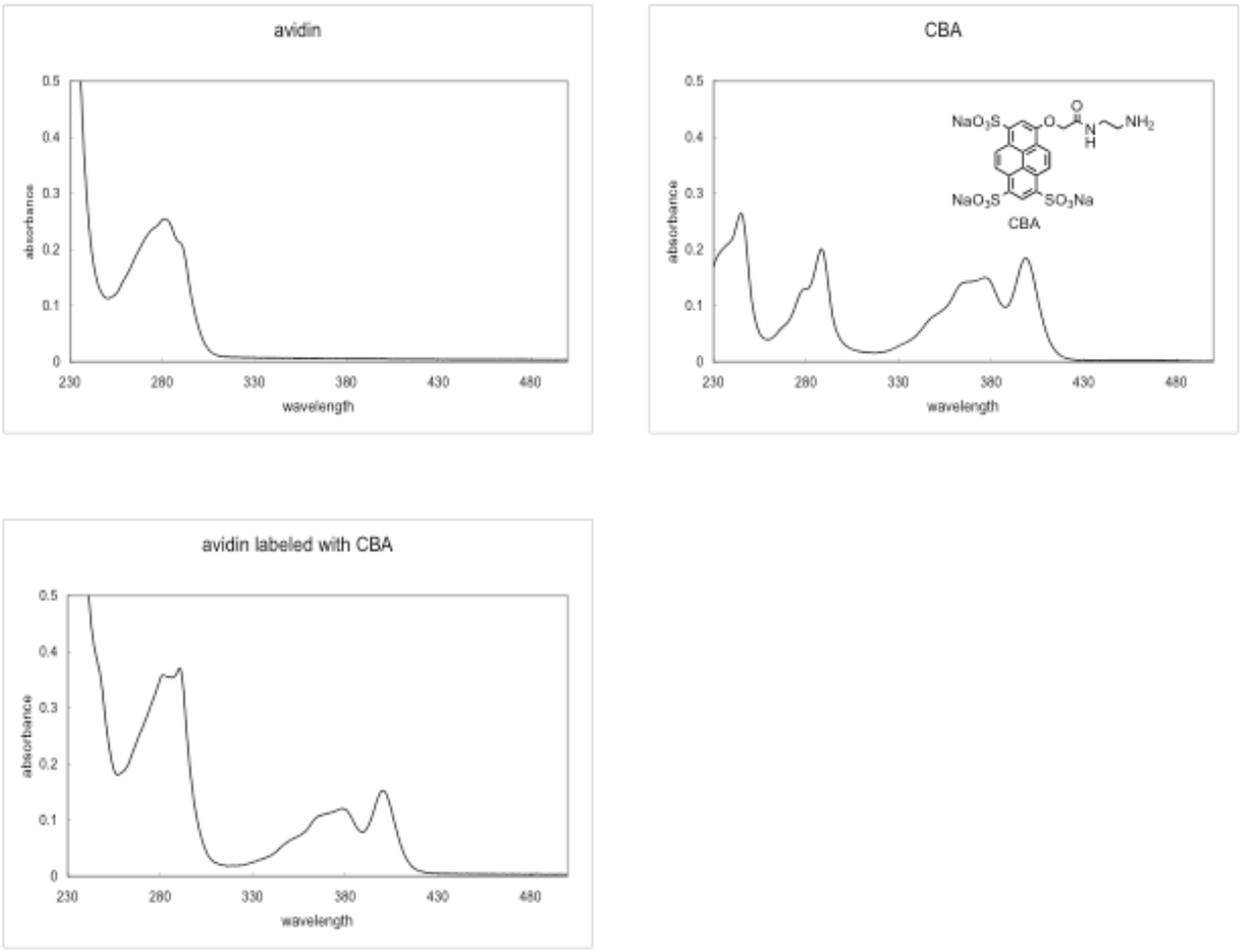

Figure S-2. UV-visible spectra of avidin, CBA, and CBA-labeled avidin.

2-4. Avidin specific labeling in the presence of γ-globulins

This experiment was performed in the manner described above for avidin labeling (2-1) except for addition of 20 μM of γ-globulins. The resulting solution was loaded on a gel filtration column (Sephadex G-100 superfine, 1.5 × 40 cm), and eluted with 50 mM phosphate buffer (pH 8.0) containing 0.1 M NaCl at RT.

The elution profiles of the proteins are shown in Fig. 2a in the text and Supporting Fig. S-3.

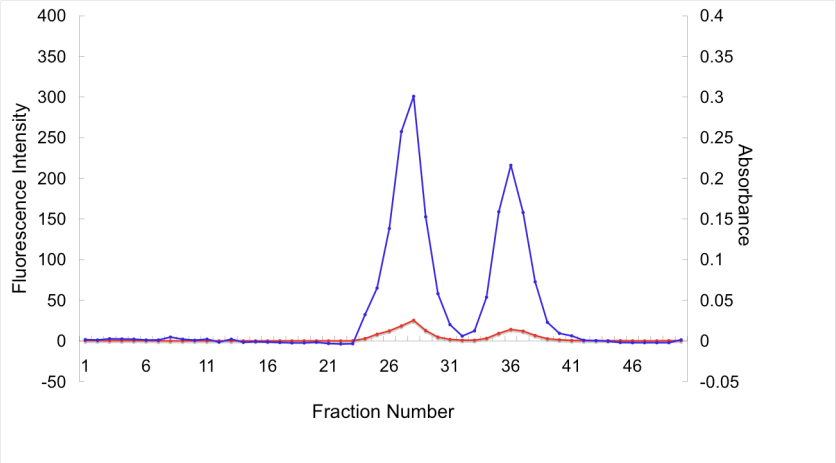

Figure S-3. Elution profile of proteins obtained in the control experiment using dimethylglycine ethyl ester **2**. UV absorption at 280 nm (blue line) and fluorescence intensity (λ_{ex} 399 nm, λ_{em} 423 nm: red line). No significant fluorescence emission was observed in the fractions containing proteins.

2-5. SDS-PAGE experiment

SDS-PAGE was carried out according to the standard procedure of Laemmli.[5]

Reducing sample buffer: 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.02 % bromophenol blue

Gel: 4% stacking gel, 12% resolving gel

Running buffer: 0.025 M Tris-HCI, pH 8.3, 0.192 M glycerol, 0.1% SDS

The reducing sample buffer (10 μL) was added to the protein solution (10 μL of 1 g/L solution) resulting from labeling experiments. The resulting solution was heated at 100 °C for 30 min, loaded on the gel and resolved by SDS-PAGE.

2-6. ESI-MS analysis of labeled avidin

Avidin was labeled with BBA according to the method described above, followed by SDS-PAGE separation. The band containing the monomeric subunit of avidin was subjected to in-gel tryptic digestion, and then analyzed on HPLC–ESI-MS.

LC: Performed on HP1100 nanoLC

column: MonoCap for fast flow (0.05 × 150mm, GL sciences) and Zorbax 300SB C18

(0.3 × 5cm, Agilent)

solvent A: 0.1% formic acid/water; solvent B : 0.1% formic acid/MeCN

flow rate: 0.3 μL/min (analytical), 10 μL/min(loading)

gradient: 0 min/(A : B = 95 : 5) – 5 min/(A : B = 95 : 5) – 35 min/(A : B = 40 : 60) – 40 min/(A : B = 10 : 90) – 45min/(A : B = 10 : 90)

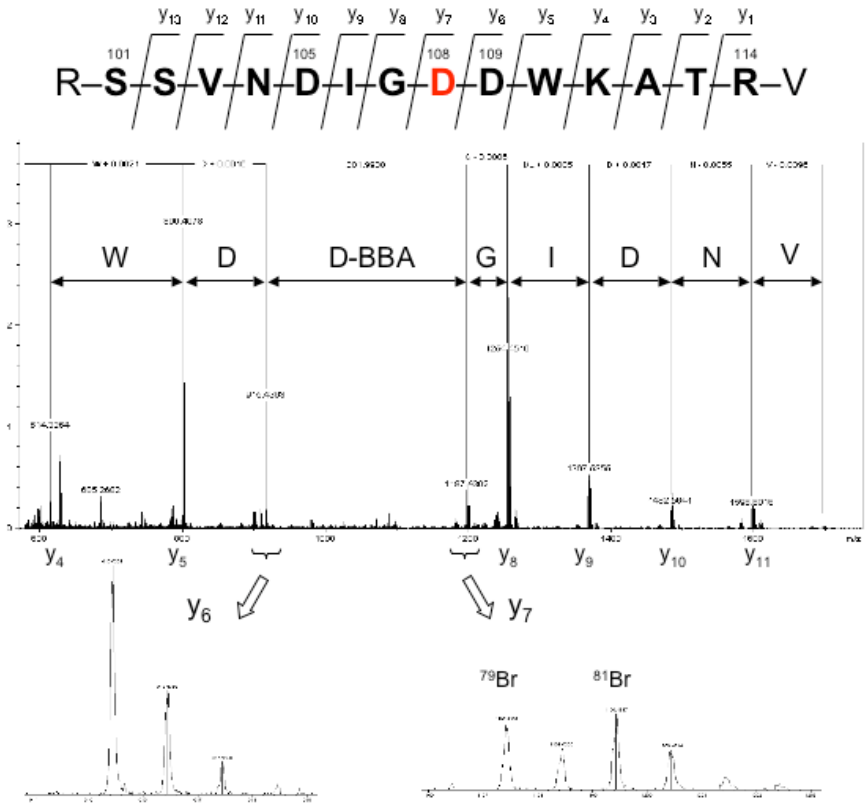

Figure S-4. Tandem mass spectra of the fragments of labeled avidin obtained by tryptic digestion. A HPLC fraction corresponding to the fragment compromised of residues Ser-101 through Arg-114 was found to contain bromine atom, which was analyzed by ESI-MS/MS. Location of BBA to Asp-108 is unambiguously determined by y-series fragmentations (fragmentation of an amide bond with charge retention on the carboxyl terminus) on a collision-induced dissociation (CID) spectrum. The amino acid residue with 282 m/z detached from the y_7 ion corresponds to Asp residue coupled with BBA. Two equivalent isotopic peaks resulting from the existence of bromine were observed with y_7 and lager ions (y_8–y_{11}) whereas such isotopic peaks disappeared with the y_6 ion resulting from cleavage of Asp-108.

2-7. Preparation of the complex structure

The structure of the complex of monomeric avidin and **1** binding to the triazinyl group (Fig. S-5) was prepared based on the X-ray crystal structure of the complex of avidin with 6-(biotinylamino)hexanoic acid (PDB ID: 1LEL).[6]

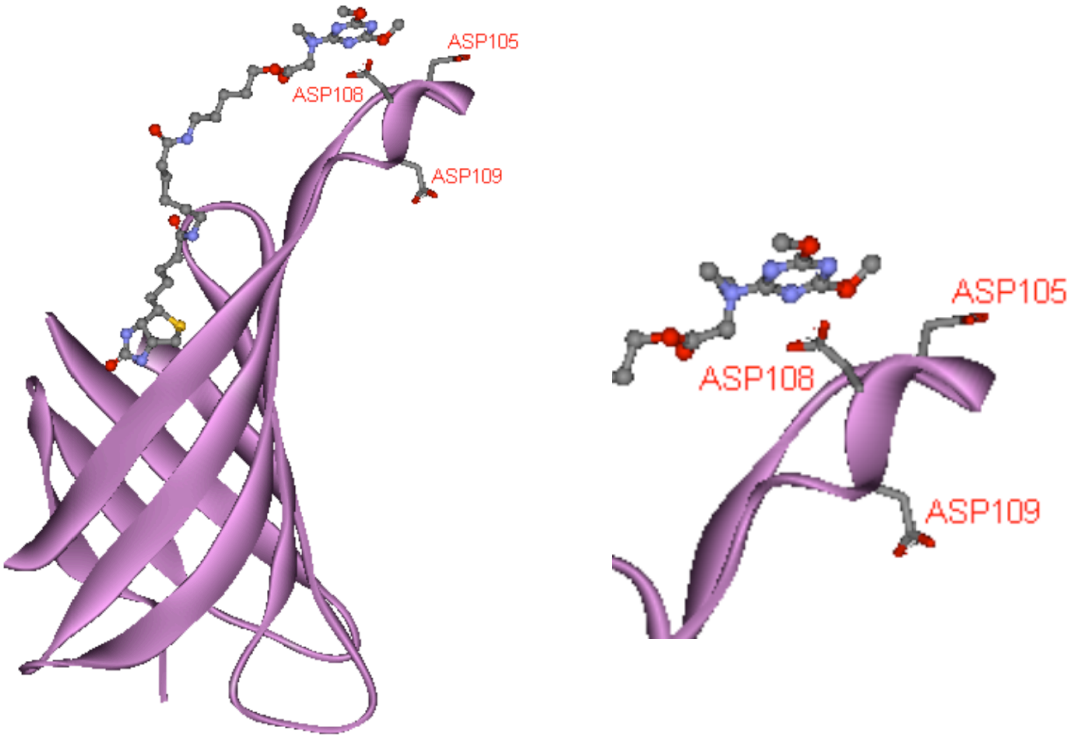

Figure S-5. A structure of the complex of monomeric subunit of avidin and **1** connecting to the triazinyl group (the activated ligand catalyst **1**) during the labeling reaction. The structure was prepared based on the X-ray crystal structure of avidin (PDB ID: 1LEL) and was not energy minimized. The activated ligand catalyst **1** is shown as a stick figure, and three Asp residues (105, 108, and 109) are shown with labels. When the triazinyl group is positioned in close proximity to the domain of the labeled peptide fragment, the carboxyl group of Asp-108 faces to the triazinyl group.

2-8. Specific labeling experiment of avidin in cell lysates by the MoAL method

Preparation of PC12 cell lystes: PC12 cells were sonicated at 0 °C in a phosphate buffer (20 mM, pH 8.0) containing 2 mM EDTA, 1 mM EGTA, and 1% Halt^{TM} Protease Inhibitor Cocktail 100X (Thermo). The resulting crude lysate was centrifuged at 100,000 × *g* at 4 °C for 60 min, and the supernatant was collected and stored at -80 °C for the following steps. The protein concentration of the solution was determined to be

3.75 mg/mL by Pierce BCA Protein Assay Kit.

Specific labeling experiment of avidin in the presence of cell lysates: The buffer solution of the PC12 cell lysates (62.7 μL) was replaced with 50 mM phosphate buffer (pH 8.0) by using Microcon filter units (10-kDa; Millipore) to remove EDTA and EGTA dissolved, and the volume of the resulting solution was adjusted to be 8 μL. To the solution was added avidin (4 μL of 18 μM solution in 50 mM phosphate buffer, pH 8.0) and the ligand catalyst **1** (5 μL of 400 μM solution in 50 mM phosphate buffer, pH 8.0), and the mixture was allowed to stand for 30 min at 4 °C. To the solution was added CBA (4 μL of 1.6 mM solution in 50 mM phosphate buffer, pH 8.0) and CDMT (4 μL of 1.6 mM solution in 50 mM phosphate buffer containing 5% MeOH, pH 8.0) to initiate the labeling reaction, and the resulting solution was allowed to stand at 4 °C for 20 h. After removal of the excess of reactants (**1**, CBA, and CDMT) and other small molecules generated during the reaction by the Microcon filter units, the resulting mixture was analyzed by SDS-PAGE.

References

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