For *Chemical Communications*

Supplementary Information

Sterically Bulky Caging for Light-Inducible Protein Activation

Satoshi Takamori[1], Satoshi Yamaguchi[1*], Noriyuki Ohashi[1], and Teruyuki Nagamune[1,2*]

[1]*Department of Chemistry and Biotechnology, Graduate School of Engineering,* [2]*Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

E-mail: yamaguchi@bio.t.u-tokyo.ac.jp

E-mail: nagamune@bio.t.u-tokyo.ac.jp

Biotinylation of HEWL and conjugation with streptavidin

For biotinylation of HEWL, 36 μM hen egg white lysozyme (HEWL, from Seikagaku Kogyo, Tokyo, Japan) in 100 mM sodium borate buffer (500 μl, pH 8.3) was mixed with 5.8 or 11.6 mM *N*-hydroxysuccinimidyl-biotin (biotin-NHS) in dried DMSO (25 μl). The final molar ratio of HEWL to biotin-NHS was 1 to 8 or 16. The reaction mixture was incubated for 3 hours at room temperature and the reaction was then quenched by addition of 1 M Tris acetate buffer (50 μl, pH 8.1). To remove free biotin derivatives, biotinylated HEWL (BHEWL) was purified by gel filtration using Sephadex^{TM} NAP-5 columns (from GE Healthcare, Buckingham, UK) equilibrated with 100 mM Tris-acetate buffer (pH 8.1). The BHEWLs, biotinylated at molar ratios 1 to 8 or 16, are abbreviated as BHEWL1 and BHEWL2, respectively.

The modification number of biotin per BHEWL molecule was evaluated by measuring the mass spectra with a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS, Voyager-DE^{TM} STR, from Applied Biosystems, Carlsbad, Germany): in the BHEWL1 sample, HEWL with two biotins (calc. MS: 14759.6) obs. MS: 14797.83, with three biotins (calc. MS: 14985.9) obs. MS: 15025.68, with four biotins (calc. MS: 15212.2) obs. MS: 15254.00, with five biotins (calc. MS:15438.5) obs. MS: 15481.23 were identified; in the BHEWL2 sample, HEWL with four biotins obs. MS 15255.02, with five biotins obs. MS: 15484.53, with six biotins (calc. MS: 15664.8) obs. MS: 15712.91, were identified.

For conjugation with streptavidin (SA, from Wako Chemicals, Osaka, Japan), 27 μM BHEWL in Tris acetate buffer (60 μl, pH 8.1) was mixed with 4 mM SA in water (7.5 μl). The final molar ratio of BHEWL to SA was 1 to 20. The mixture was incubated for 15 min at room temperature. This mixture was directly used for bacteriolytic activity assay and sodium dodecylsulfonate-poly (acrylamide) gel electrophoresis (SDS-PAGE) without further purification.

To confirm the conjugation of BHEWL and SA, the mixture was analyzed by SDS-PAGE. The mixture solution was diluted with Laemmle's buffer and not boiled before loading into a gel, so as not to dissociate the SA tetramer. As a result, the bands of BHEWL disappeared with excess SA and were replaced by ladder bands appearing above the SA band (Fig. S1, lanes 5 and 6). On the other hand, the band for intact HEWL did not change after addition of excess SA (Fig. S1, lane 4). These results indicate that SA was conjugated with the biotin moieties on BHEWLs.

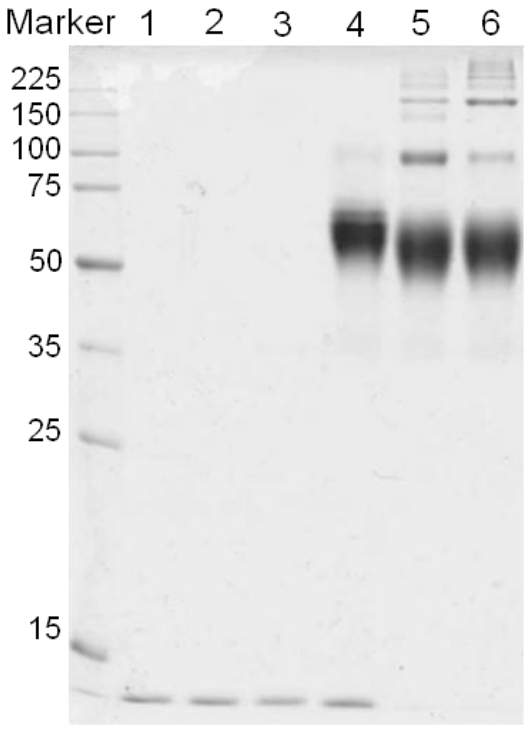

Figure S1. SDS-PAGE gel image of biotinylated lysozyme with and without streptavidin. Native lysozyme and the biotinylated lysozyme samples (biotinylation at molar ratios of HEWL to *N*-hydroxysuccinimidyl-biotin of 1 to 8 or 16) were loaded into lanes 1, 2 and 3, respectively. Each sample was mixed with excess streptavidin, and loaded into lanes 4, 5 and 6, respectively. All samples were loaded without prior boiling.

Activity assay

Micrococcus lysodeiktcus cells (from Sigma-Aldrich, St Louis, MO) were suspended (0.15 mg dried cell/ml) in 66 mM sodium phosphate buffer (pH 6.2, 2 ml), and placed into a cuvette with a stirrer chip. 20 μl of a sample solution including intact HEWL, BHEWL or caged HEWL with/without excess SA (0.18 mg/ml HEWL in 0.1 M Tris acetate buffer (pH 8.1)) was added to the cuvette and stirred. The initial rate of enzymatic lysis of the *M. lysodeikticus* cells at 20 °C was obtained by measuring the decrease in absorbance at 450 nm using a UV spectrophotometer V550 (from Jasco, Tokyo, Japan). The specific activity was expressed as the ratio relative to the initial lysis rate of a native HEWL sample.

Synthesis of biotinylated caging reagent 1

Biotinylated caging reagent **1** was synthesized in four steps from a commercially available starting compound **2** (4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy] -butyric acid, from Sigma-Aldrich) (Scheme **1**).

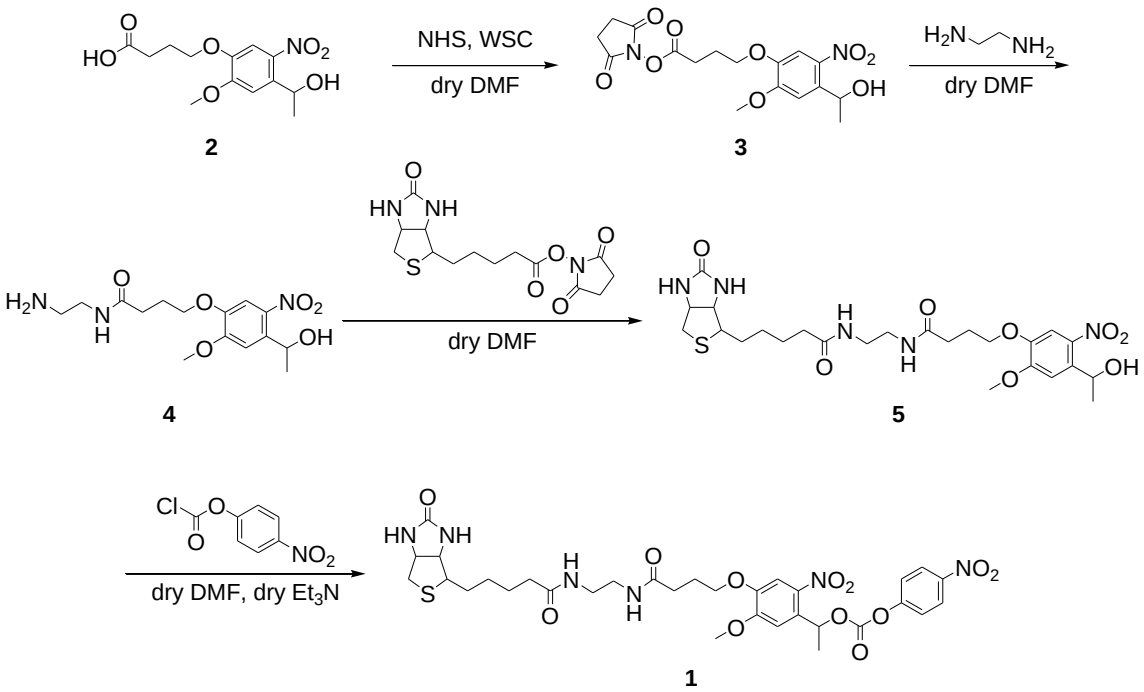

Figure S2. Synthesis of biotinylated caging reagent **1**

Synthesis of 3

To a solution of **2** (320.0 mg, 1.07 mmol) and NHS (197.8 mg, 1.72 mmol) in DMF (4 ml) was added WSC (307.2 mg, 1.60 mmol). After stirring for 20 h, NHS (105 mg, 0.94 mmol) and WSC (153 mg, 0.80 mmol) were added to the reaction mixture and stirred for 18 h. After evaporation, the resulting residue was suspended in water and the precipitate was collected by filtration and dried to give compound **4** as a yellow powder (369.3 mg, 87%).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.59 (s, 1H), 7.31 (s, 1H), 5.58-5.57 (q, 1H), 4.18-4.16 (t, 2H), 3.99 (s, 3H), 2.90-2.89 (t, 2H), 2.88-2.82 (t, 4H), 2.32-2.27 (m, 2H), 1.57-1.56 (d, 3H).

Synthesis of 4

To a solution of anhydrous ehylene diamine (5 ml, 75 mmol) in DMF (5 ml) was slowly added a solution of **3** (205.4 mg, 0.52 mmol) in DMF (10 ml). After stirring for 1 h at room temperature, the solvent and excess ethylene diamine was removed under vacuum and the resulting residue was re-dissolved in water (6 ml). The crude solution was purified by reversed-phase HPLC equipped with a ODS column, to give compound **4** as an orange powder (129.8 mg, 55%).

^{1}H-NMR (CD_3OD, 500 MHz): δ 7.58 (s, 1H), 7.39 (s, 1H), 5.45-5.44 (q, 1H), 4.10-4.09 (t, 2H), 3.95 (s, 3H), 3.45 (t, 2H), 3.04 (t, 2H), 2.46-2.45 (t, 2H), 2.12-2.11 (t, 2H), 1.47-1.46 (d, 3H).

Synthesis of 5

To a solution of **4** (129.8 mg, 0.29 mmol) in DMF (3 ml) were added anhydrous triethylamine (Et_3N, 200 μl, 1.4 mmol) and biotin *N*-succinimidyl ester (102.5 mg, 0.30 mmol). After stirring for 1 h at room temperature, the solvent was removed under vacuum and the resulting residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH = 5/1). For further purification, the resulting residue was suspended in water and the precipitate was collected by filtration and dried to give compound **5** as a yellow powder (118.6 mg, 73%).

^{1}H-NMR (CD_3OD, 500 MHz): δ 7.58 (s, 1H), 7.39 (s, 1H), 5.45-5.44 (q, 1H), 4.48-4.46 (q, 1H), 4.30-4.28 (q, 1H), 4.09-4.07 (t, 2H), 3.95 (s, 3H), 3.19-3.18 (m, 1H), 2.92-2.67(m, 2H), 2.42-2.39 (t, 2H), 2.20-2.17 (t, 2H), 2.12-2.09 (t, 2H), 1.64-1.58 (br, 4H), 1.47-1.46 (d, 3H), 1.43-1.40 (br, 2H).

Synthesis of 1

To a solution of **5** (38.9 mg, 0.069 mmol) in DMF (1.5 ml) were added Et_3N (50 μl, 0.36 mmol) and 4-nitrophenyl chloroformate (21.8 mg, 0.11 mmol). After stirring for 3 h at room temperature, Et_3N (100 μl, 0.72 mmol) and 4-nitrophenyl chloroformate (24.1 mg, 0.120 mmol) were further added to the reaction mixture and stirred for 17 h at room temperature. After evaporation, the crude residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH = 4/1) to give compound **1** as a white powder (20.5 mg, 41%).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.26-8.24 (d, 2H), 7.61 (s, 1H), 7.36-7.34 (d, 2H), 7.11 (s, 1H), 6.50-6.51 (q, 1H), 4.52 (br, 1H), 4.33 (br, 1H), 4.12 (t, 2H), 4.00 (s, 3H), 3.39 (br, 4H), 3.13 (br, 1H), 2.89-2.70 (m, 2H), 2.44 (t, 2H), 2.23 (t, 2H), 2.18-2.17 (t, 2H), 1.78-1.67 (br, 7H), 1.46 (br, 2H).

^{13}C-NMR (DMSO-d_6, 125.6 MHz): δ 21.2, 24.6, 25.2, 28.0, 28.2, 30.8, 31.6, 35.2, 38.3, 38.5, 55.4, 56.4, 59.2, 61.0, 68.3, 73.0, 108.4, 115.8, 122.6, 125.4, 130.2, 139.8, 145.2, 147.4, 151.2, 153.6, 155.1, 162.8, 171.6, 172.2.
MALDI-TOF MS (CHCA) *m*/*z*: 755.6042 $[M + Na]^+$, 771.5813 $[M + K]^+$ (calc. MS: 732.76).

Caging of HEWL with 1 and conjugation with streptavidin

For caging of HEWL, 72 μM HEWL in 100 mM sodium borate buffer with 30% DMSO (500 μl, pH 7.7) was mixed with 46 mM of **1** in dried DMSO (25 μl). The final molar ratio of HEWL to **1** was 1 to 32. The reaction mixture was incubated for 24 hours at room temperature under orbital stirring. The reaction was quenched by addition of 1 M Tris acetate buffer (50 μl, pH 8.1). To remove byproducts, caged HEWL (CHEWL) was purified by gel filtration using Sephadex^{TM} NAP-5 column equilibrated with 100 mM Tris acetate buffer (pH 8.1) with 30% DMSO.

The modification number of caging groups per CHEWL molecule was evaluated by MALDI-TOF MS as described above: HEWL with no caging group (calc. MS: 14307.0) obs. MS: 14350.01, with one caging group (calc. MS: 14900.7) obs. MS: 14943.98, with two caging groups (calc. MS: 15494.3) obs. MS: 15541.26, with three caging groups (calc. MS: 16088.0) obs. MS: 16119.7 were identified.

For conjugation with SA, 27 μM CHEWL in Tris acetate buffer (264 μl, pH 8.3, 30 % DMSO) was mixed with 0.3 mM SA in water (96 μl). The final molar ratio of CHEWL to SA was 1 to 8. The mixture was incubated for 15 min at room temperature. This mixture was directly used for the bacteriolytic activity assay and SDS-PAGE before and after light irradiation.

Light irradiation

Sample solutions including intact HEWL or caged HEWL with/without excess SA were irradiated with UV light at 365 nm, 1~10 J/cm^2. An LAX102 illuminator (Asahi Spectra, Tokyo, Japan) equipped with a Xenon arc lamp was used as the UV source. After irradiation, the irradiated samples were evaluated by the bacteriolytic activity assay and SDS-PAGE, as described above.

Image analysis of a SDS-PAGE gel

Coomassie Blue-stained bands on SDS-PAGE gels of light-irradiated samples (shown in Fig. 3b) were quantified by image analysis with Image J. The density of the bands of free HEWL in each lane were quantified and expressed as the ratio to the density in the

lane for native HEWL (Fig. 3b, lane 1). As shown in Fig. S3, the band density of free HEWL increased in proportion to the dose of light. Finally, 70% of the HEWL was liberated from the CHEWL-SA conjugates at 10 J/cm^2.

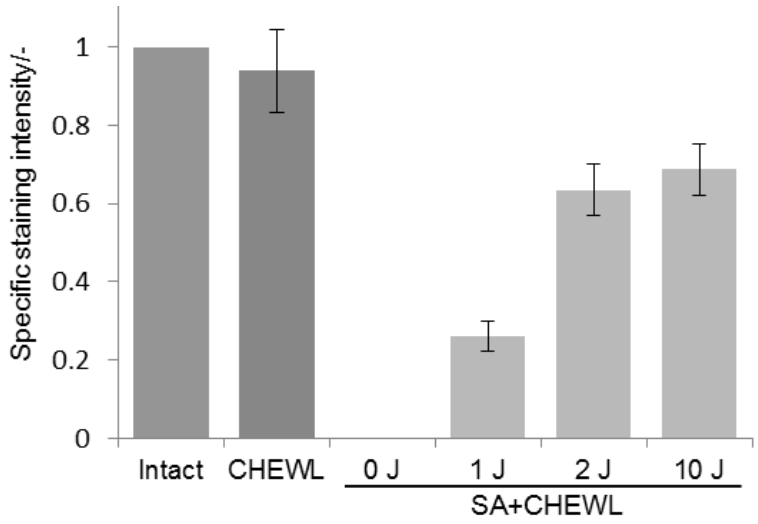

Figure S3. Staining intensity of the bands on the SDS-PAGE gel of light-irradiated CHEWL-SA conjugate samples (shown in Fig. 3b). The density of the bands derived from free HEWL (14 kDa) in each lane was quantified by Image J. Each bar represents the mean ± S.E. (n = 3).

Kinetic analysis for the caging process

To evaluate the kinetics of the coupling reaction, the ratios of absorbance at 365 nm to at 280 nm were obtained after coupling for various times (Fig. S4a). As described above, CHEWL was caged and purified with gel filtration, and the absorbances of CHEWL solutions at 365 and 280 nm were measured. The former absorbance peak is derived from the caging group, and the latter one is from HEWL. Moreover, the activities of CHEWL-SA complexes after binding to SA are measured and plotted as a function of the coupling time (Fig. S4b). The conditions of complexation to SA and activity measurement were the same as described above.

As shown in Fig. S4a, under our optimal experimental condition (pH 7.7), the coupling reaction proceeded and finished almost at 24 h. And, after coupling for 24 h, the activity of CHEWL was almost perfectly inhibited by binding to SA.

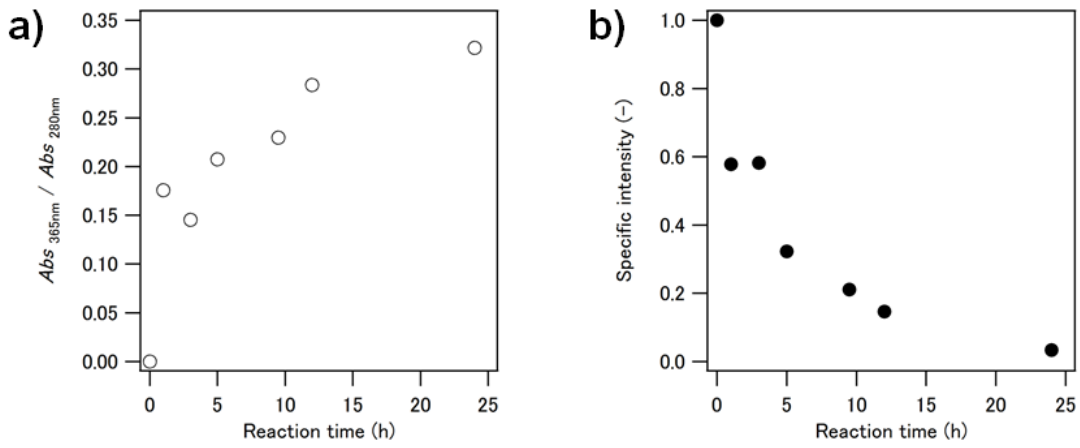

Figure S4. The kinetic analysis for the coupling reaction between HEWL and the caging reagent **1** and for overall inactivation of HEWL activity. (a) The ratios of absorbance of CHEWL at 365 nm to at 280 nm were plotted as a function of the coupling reaction time. (b) The activities of CHEWL-SA complexes were as a function of the coupling reaction time.

Gel filtration chromatography of CHEWL-SA conjugates

Gel filtration chromatography was performed using an AKTA-FPLC system (GE Healthcare). 500 µl of the CHEWL-SA conjugate sample, native SA sample (50 µM), and native HEWL sample (12 µM) were injected and fractionated by gel filtration with a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 100 mM Tris acetate buffer with 10% DMSO (pH 7.7) at a flow rate of 1.0 ml/min. The UV absorbance at 280 nm was recorded. As shown in Fig. S5a, three peaks were detected in the elution curve of the conjugate sample. The eluted fractions were analyzed by SDS-PAGE (Fig. S5b). Fractions containing CHEWL-SA conjugates were then separated into three in the chromatographic elution curve (5~6th, 7~8th, and 11~12th fractions were collected as shoulder **1**, peak **2**, and peak **3**, respectively). These three separated CHEWL-SA conjugates solutions were evaluated by SDS-PAGE and enzymatic activity assay before and after light irradiation (Fig. S5c, d).

Considering the molecular sizes estimated from the bands on SDS-PAGE (Fig. S5b), peak **3** mainly included free SA (58 kDa) and CHEWL conjugated with one SA (approx. 72 kDa, abbreviated as CHEWL-1SA), and also included a little CHEWL with two SAs (approx. 130 kDa, CHEWL-2SA). Peak **2** mainly consisted of CHEWL-2SA, and shoulder **1** sample was CHEWL-2SA, CHEWL with three SAs (approx. 190 kDa, CHEWL-3SA) and oligomers. In the lanes of shoulder **1** and peak **2** samples, bands

corresponding to free HEWL and free SA appeared after light irradiation, simultaneously with disappearance of CHEWL-SA conjugate bands (Fig. S5c). This result clearly shows that the conjugates were degraded by light. With regard to the sample of peak **3**, the CHEWL band with one SA (approx. 72 kDa) overlapped with that of free SA, and therefore the band for this conjugate was not clearly confirmed as disappearing. However, the appearance of free HEWL indicates light-induced degradation of the conjugates.

Figure S5d shows the residual activities, normalized with respect to those of light-irradiated CHEWL. Activities in all samples before irradiation were completely inhibited, compared with their activities after light irradiation. This result strongly indicates that conjugates of CHEWL with only one SA also completely lost activity prior to irradiation.

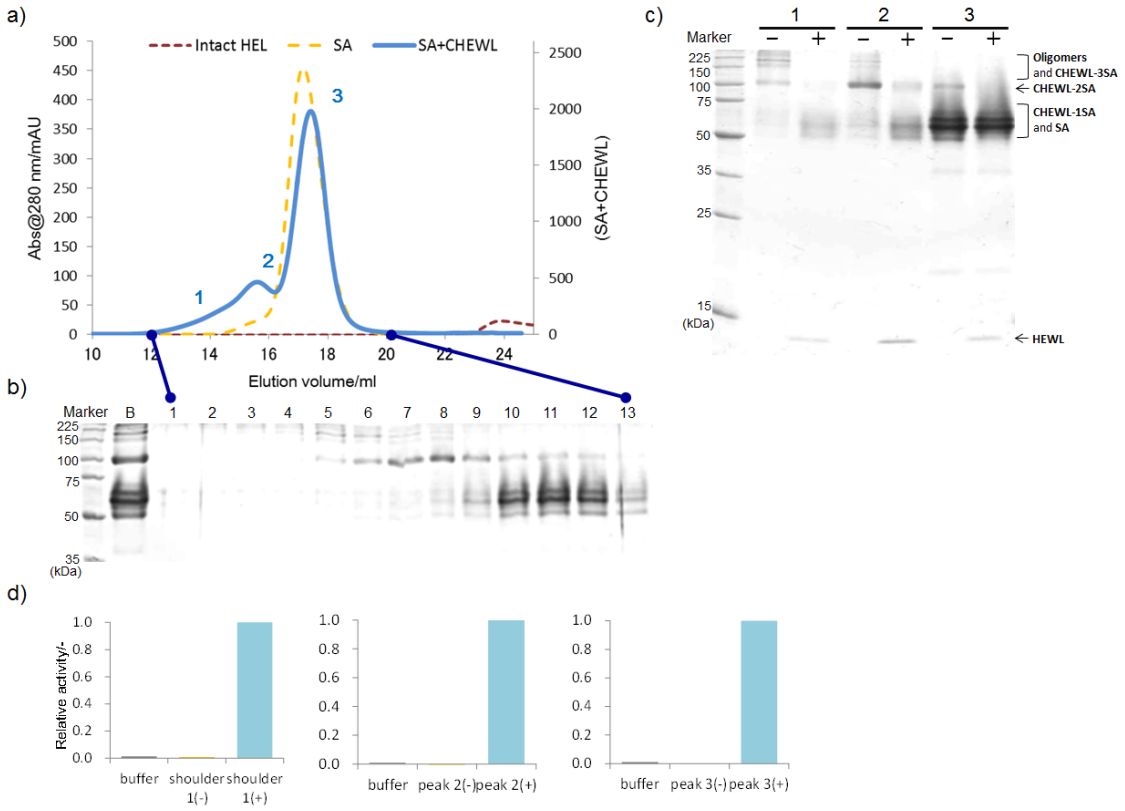

Figure S5. Gel filtration chromatographic separation of caged lysozyme-streptavidin conjugates and their enzymatic activities before and after light irradiation. (a) Gel filtration chromatographic elution curve of caged lysozyme (CHEWL) in the presence of excess streptavidin (SA) (blue solid line). One shoulder and two peaks were detected,

abbreviated as shoulder **1**, peak **2**, and peak **3**, respectively. The curves of intact HEWL (red dotted line) and SA (yellow broken line) are also shown as a control. (b) The image of bands of eluted CHEWL with excess SA on an SDS-PAGE gel. The mixture before gel filtration was also loaded (shown as lane "B"). For the subsequent experiment, the 5~6th, 7~8th, and 11~12th fractions were collected as shoulder **1**, peak **2**, and peak **3**, respectively. (c) Bands of each CHEWL-SA conjugate separated in the three peak areas before (-) and after (+) light irradiation on SDS-PAGE gel. (d) Enzymatic activities of fractionated CHEWL with SA before and after light irradiation (365 nm, 10 J/cm^2). The specific activities were normalized with respect to the activity of light-irradiated CHEWL.

Caging of HEWL with 6 and evaluation of CHEWL-SA conjugates

The synthesis of 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc)-based caging reagent **6** was previously reported[1]. All experiments were performed under the same condition where biotinylated caging reagent **1** was employed.

As a result, similar to the results obtained by using nitrobenzyl group (Fig. 3), HEWL was modified with 6 (Fig. S6b), and CHEWL was confirmed to bind to SA (Fig. S6c). Moreover, complexation with streptavidin (SA) dramatically inactivated CHEWL, and after light irradiation, most of the activity was recovered (Fig. S6d).

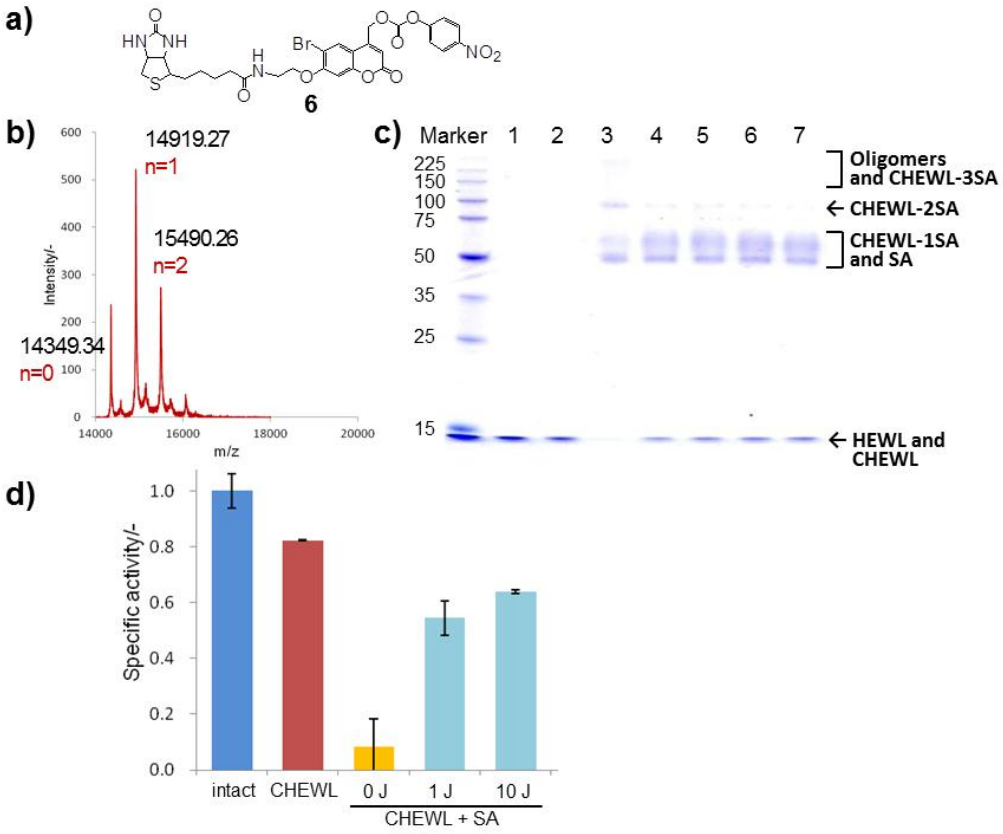

Figure S6. Chemical structure of **6**, mass spectra, electrophoretic mobilities and enzymatic activities of caged lysozyme with **6**. (a) Chemical structure of a caging reagent **6**. (b) MALDI-TOF MS spectra of caged lysozyme. n is the number of biotinylated photo-cleavable groups modified on each lysozyme. (c) Bands of caged lysozyme (CHEWL) with and without streptavidin (SA) on an SDS-PAGE gel. Intact and caged lysozyme were loaded into lanes 1 and 2, respectively. The conjugate of caged lysozyme and streptavidin was loaded after irradiation of ultraviolet light (365 nm) at 0, 1, 2, 5 and 10 J/cm^2 into lanes 3, 4, 5, 6 and 7, respectively. Conjugates of CHEWL with one, two and three SAs are abbreviated as CHEWL-1SA, CHEWL-2SA and CHEWL-3SA, respectively. All samples were loaded without boiling. (c) Relative activities of caged lysozyme to that of native lysozyme were measured with and without streptavidin (SA) after irradiation by ultraviolet light (365 nm, 0 – 10 J/cm^2). Each bar represents the mean ± S.E. (n = 3)

References

1. S. Yamaguchi, Y. Chen, S. Nakajima, T. Furuta, and T. Nagamune, *Chem. Commun.*, 2010, **46**, 2244-2246.