Electronic Supplementary Information

for

Photoaffinity casting of coumarin flag for rapid identification of ligand-binding sites within proteins

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General
Melting points were measured on a Yanaco MP-S3 micro melting point apparatus, and uncorrected. Fmoc amino acids and Alko PEG resin were purchased from Watanabe Chemical Industries, Streptavidin-HRP conjugate from New England BioLabs, and non-fluorescence water was purchased from Dojindo Laboratories. Pierce Streptavidin UltraLink Resin was purchased from Thermo Scientific. Kieselgel 60 (70-230 mesh, Merck) was used for column chromatography. All chemicals were of analytical grade and were used without further purification. $^1$H, $^{13}$C, and $^{19}$F NMR spectra were recorded on a JEOL ECX400P spectrometer (400 MHz) and a Varian UNITYplus500 spectrometer (500 MHz), with chemical shifts (δ) reported in ppm relative to internal standards (Me$_4$Si for $^1$H and $^{13}$C, CFCl$_3$ for $^{19}$F) and coupling constants (J) reported in Hz. Mass spectra and high-resolution mass spectra (HRMS) were recorded by electron impact ionization (EI) on a JEOL JMS-GCmate II or electrospray ionization (ESI) on a Thermo LTQ Orbitrap XL ETD. UV/Vis spectra were obtained by a Shimazdu UV-1800 spectrometer, and fluorescent spectra were measured on a JASCO FP-6500 spectrometer. Peptides were prepared by Shimadzu PSSM-8 peptide synthesizer. Photoreactions performed by HP-30M (ATTO), Transilluminator FTI-15L (Funakoshi), and REX-250 high-pressure mercury lump (Asahi Spectra). Chemiluminescence detection was performed by Chemi-Print CX-EpiUV system (Relyon).

Synthesis of probe 1

(E)-Ethyl 3-[2-hydroxy-4-(3-trifluoromethyl-3H-diazirin-3-yl)phenyl-2-methylacrylate (2). To a benzene solution (40 mL) of 2-hydroxy-4-(3-trifluoromethyl-3H-diazirin-3-yl)benzaldehyde (2.0 g, 8.8 mmol)$^1$ was added [1-(ethoxycarbonyl)ethylidene] triphenylphosphorane (3.5 g, 9.7 mmol), and the reaction mixture was stirred at room temperature overnight under argon. After removal of the solvent, the product was purified by a column chromatography on silica gel eluted with n-hexane/ethyl acetate (5:1) to give a pale yellow solid (2.2 g) in 79%. Mp 69-70 °C; $^\text{1}$$^1$H (500 MHz; CDCl$_3$) 7.72 (1 H, s), 7.24 (1 H, d, J 8.1), 6.75 (s, 1 H), 6.70 (1 H, d, J 8.1), 4.29 (2 H, q, J 7.3), 2.00 (3H, s), 1.35 (3 H, t, J 7.3); $^\text{13}$$^1$C (126 MHz; CDCl$_3$) 168.8 (s), 154.2 (s), 133.1 (d), 131.3 (s), 130.8 (d), 130.4 (s), 124.4 (s), 122.6 (q, $^1$J$_{C,F}$ 273), 118.0 (d), 113.8 (d), 61.4 (t), 28.3 (q, $^2$J$_{C,F}$ 41), 14.1 (q), 14.2 (q); $^\text{19}$$^1$F (376 MHz; CDCl$_3$) -65.6 (3F, s); $\lambda_{max}$/nm (ε) (MeOH) 352 (sh, 1,270); HRMS (EI) m/z 314.0872 ([M$^+$], C$_{14}$H$_{13}$F$_3$N$_2$O$_3$ requires 314.0878).
(E)-2-Hydroxy-4-(3-Trifluoromethyl)-3H-diazirin-3-yl[phenyl-2-methylacrylic acid (3). To a methanol solution (1 mL) of compound 2 (2.0 g, 6.4 mmol) was slowly added a 3 M NaOH (50 mL) at 0 °C and the mixture was stirred at room temperature for 2 hours. The mixture was poured into cold 3 M HCl, and the products were extracted with ethyl acetate. The organic phase was washed with brine and dried over MgSO4. After removal of the solvent, the product was recrystallized from CHCl3 to give pale yellow leaflets (1.52 g) in 83%. Mp 66-67 °C; 1H (500MHz; CD3OD) 7.78 (1 H, s), 7.36 (1 H, d, J 8.1), 6.72 (1 H, s), 6.65 (1 H, d, J 8.1), 1.99 (3 H, s); 13C (126 MHz; CD3OD) 171.8 (s), 157.5 (s), 135.0 (d), 131.9 (d), 131.4 (s), 130.5 (s), 126.3 (s), 123.6 (q, 1JCF 273), 117.8 (d), 114.1 (d), 29.4 (q, 2JCF 41), 14.4 (q); 19F (376 MHz; CD3OD) -64.9 (3F, s); λmax/nm (ε) (MeOH) 347 (sh, 1,100); HRMS (EI) m/z 286.0565 (M+ +) C12H9F3N2O3 requires 286.0565).

Preparation and photoreaction of diazirine-based photoprobe (1)

Peptide, H-K(biotin)SSILRAFY-OH, was prepared by Fmoc solid-phase peptide synthesis on an Alko PEG resin (0.78 mmol/g, 50 mg). N-Hydroxysuccinimide ester of compound 3 was freshly prepared. To a DMF solution (1 mL) of compound 3 (40 mg, 0.14 mmol) and N-hydroxysuccinimide (17 mg, 0.15 mmol) was added a DMF solution (0.5 mL) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (28 mg, 0.15 mL). After disappearance of compound 3 on TLC (thin layer chromatography), the reaction mixture (760 µL) and triethylamine (40 µL) were added to the resin and reacted overnight at room temperature in a shaking apparatus. After washing with DMF and MeOH, the resin was treated with TFA (340 µL), m-cresol (20 µL), thiaoanisol (20 µL), and triisopropyl silane (20 µL) together for 1 hour. Ether (10 mL) was added to the supernatant and the white solid was precipitated by centrifugation. This treatment was repeated six times. The photoprobe was purified by reverse-phase HPLC on ODS (SHISEIDO CAPCELL PAK C18, 4.6 mm x 150 mm) with a liner gradient of 2–60% acetonitrile-water containing 0.1% TFA over 50 min at a flow rate of 1 mL/min. The 37.6-min peak was collected and freeze-dried. HRMS (ESI+) m/z 1578.7294 (MH+) C73H103F3N17O17S requires 1578.7391.

Photocross-linking of photoprobe 1 to GmVSR and photocleavage of cross-linked product

Photoaffinity labeling of GmVSR with the photoprobe 1 was generally performed as follows. A buffered solution (9 µL) of GmVSR (2 pmol) containing 20 mM Hepes (pH 7.0), 150 mM NaCl, 1 mM CaCl2, 0.4% chaps, 0.02% NaN3 was incubated with a 1% DMF aqueous solution (1 µL) of the photoprobe (2 pmol) for 1 hour at room temperature. The mixture was irradiated at 0 °C for 10 min with 360-nm light. After photolysis, an appropriate volume of SDS sample buffer (0.1 M Tris-HCl (pH 6.8), 0.4% SDS, 1.7 M 2-mercaptoethanol, 0.2% v/v glycerol, 0.005% w/v bromophenol blue) was added to the sample solution. The samples were stand at room temperature for 1 hour and separated by 10% SDS-PAGE. After electroblotting onto a PVDF membrane, the photocross-linked products were detected by chemiluminescence method using avidin-HRP conjugate. The product of the first photoreaction was continuously irradiated with 315-nm light for 60 min at
25 °C. The samples were then subjected to 10% SDS-PAGE and detected by fluorescent method for a coumarin tag.

**Purification of labeled GmVSR using avidin-immobilized gel and the digested products**

A Hepes buffered solution (100 µL) of GmVSR (0.2 nmol) and the photoprobe (0.2 nmol) was incubated for 1 hour at room temperature, and was then irradiated at 0 °C for 10 min with 360-nm light. The photoproduce was treated with SDS sample buffer for 1 hour at room temperature, and was then dialyzed to remove non-cross-linked probe. After incubation with an avidin-immobilized agarose gel for 2 hours at room temperature, the gel was washed with 0.2% SDS-containing PBS solution (100 µL) for 5 times by centrifugation, and was then exposed to 315-nm light in PBS solution (20 µL) for one hour at 25 °C. The supernatant was subjected to 10% SDS-PAGE. The band corresponding to GmVSR was cut in small pieces and afforded in-gel digestion by lysyl endopeptidase (100 ng/µL in 50 mM of Tris buffer, pH 8.5) after treatment of iodoacetamide as an alkylating agent according to the previous report. The products were extracted 50% acetonitrile solution containing 5% TFA (50 µL x 1, 25 µL x 1) and stored in a freezer. The digested sample was purified by reverse-phase HPLC on ODS (4.6 mm x 150 mm) with a liner gradient of 10–90% acetonitrile-water containing 0.1% TFA over 150 min, and with an isocratic of 90% for 30 min at a flow rate of 0.75 mL/min. The 98.7-min (peak 1) and 123.8-min (peak 2) peaks were analyzed by ESI-MS/MS.
**Fig. S1** Competition assay of cross-linking of GmVSR with photoprobe 1 in the presence of VSD analogs. Photolysis of the solution including GmVSR and the photoprobe 1 was carried out with 360-nm light for 10 min at 0 °C in the absence or presence of VSD analogs at 1, 5, 25 equivalent mol against the photoprobe 1. Percent of competition was calculated from the emission intensity using chemiluminescence method by comparison with the intensity of the sample in the absence of competitor.
**Fig. S2** Photoreaction of photoprobe 1 under irradiation with 315-nm light.

Photoprobe 1 was dissolved in 50% CH$_3$OH-H$_2$O solution (1 µM) and irradiated with 315-nm light at 25 °C in a sealed quartz cell. Emission spectra ($\lambda_{ex} = 320$ nm) was measured at 20 °C.
Fig. S3 ESI (+) -MS of photoprobe 1 (panel A), HPLC peaks 1 (panel B) and 2 (panel C) of digested products.

(A) HRMS of photoprobe 1
1550.6 for [MH-N$_2$]$^+$: carbene species produced by heat decomposition.

(B) Peak 1 (861.4 for [FVVEK-coumarin + H]$^+$)
(C) Peak 2 (985.4 for [VWNAQK-coumarin + H]^+)

![Mass spectrum image]

VWNAQK+H+tag (985.44)

985.2 [MH+tag]^+

967.1 [b6+tag]^+ or [MH-H2O+tag]^+

969.9 [MH-NH+tag]^+

304.2 [b2-H2O]^+

286.9 [b2]^+

417.7 [c3]^+

698.8 [x4+tag-CO]^+

571.3 [as]^+

823.5 [b5-NH2+tag]^+

699.3 [y5-OH+tag]^+

868.1 [y4+tag]^+

701.0 [y4+tag]^+

1010.0 [y3+tag]^+

1250.5 [y2+tag]^+

1550.7 [y1+tag]^+

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**Fig. S4** NMR data of compounds

(A) Compound 2
References