Electronic Supplementary Material (ESI) for Chemical Communications
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Electronic Supplementary Information

for

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

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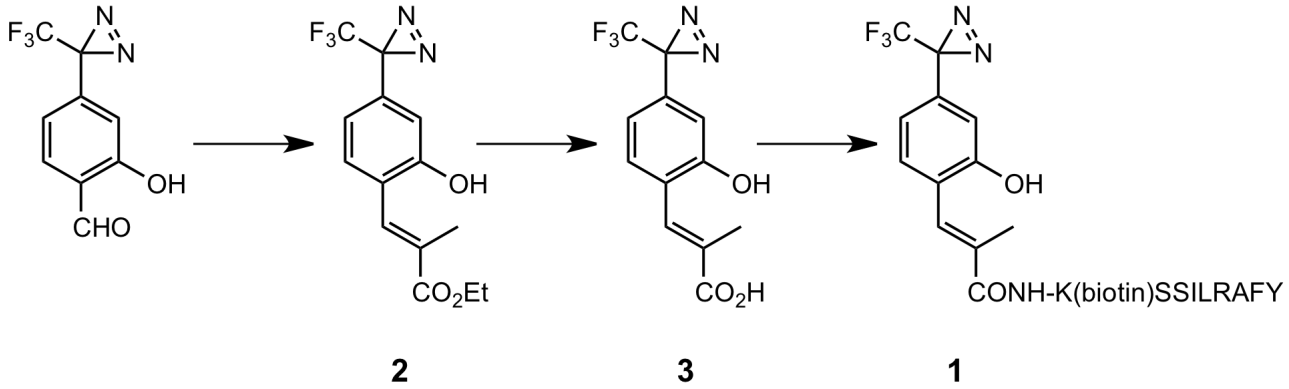
General

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_4Si 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-3*H*-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; ™$_H$ (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); ™$_C$ (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, $^{1}J_{C\text{-}F}$ 273), 118.0 (d), 113.8 (d), 61.4 (t), 28.3 (q, $^{2}J_{C\text{-}F}$ 41), 14.1 (q), 14.2 (q); ™$_F$ (376 MHz; $CDCl_3$) -65.6 (3F, s); λ_{max}/nm (ε) (MeOH) 352 (sh, 1,270); HRMS (EI) *m/z* 314.0872 ((M^+), $C_{14}H_{13}F_3N_2O_3$ 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 $MgSO_4$. After removal of the solvent, the product was recrystallized from $CHCl_3$ to give pale yellow leaflets (1.52 g) in 83%. Mp 66-67 °C; ™$_H$ (500MHz; CD_3OD) 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); ™$_C$ (126 MHz; CD_3OD) 171.8 (s), 157.5 (s), 135.0 (d), 131.9 (d), 131.4 (s), 130.5 (s), 126.3 (s), 123.6 (q, $^1J_{C\text{-}F}$ 273), 117.8 (d), 114.1 (d), 29.4 (q, $^2J_{C\text{-}F}$ 41), 14.4 (q); ™$_F$ (376 MHz; CD_3OD) -64.9 (3F, s); λ_{max}/nm (ε) (MeOH) 347 (sh, 1,100); HRMS (EI) *m/z* 286.0565 (M^+) $C_{12}H_9F_3N_2O_3$ 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 a 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), thioanisol (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^+) $C_{73}H_{103}F_3N_{17}O_{17}S$ 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 $CaCl_2$, 0.4% chaps, 0.02% $NaN_3$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

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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 photoproduct 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.[2] 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.

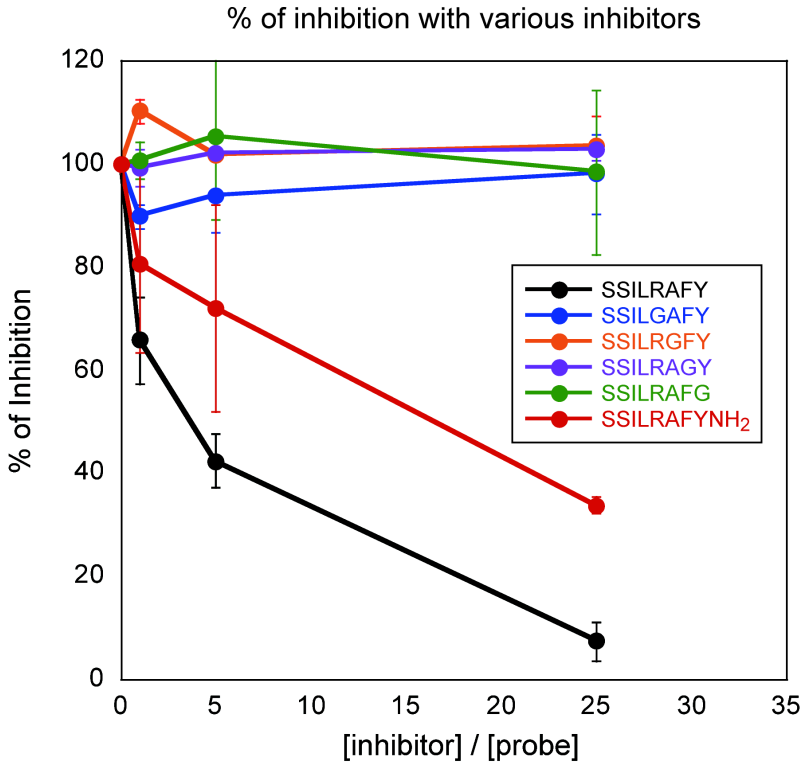


Fig. S1 Competition assay of cross-linking of GmVSR with photoprobe **1** in the presence of VSD analogs. Photolysis of the solution including GmVSR and the photoprobe **1** was carried out with 360-nm light for 10 min at 0 °C in the absence or presence of VSD analogs at 1, 5, 25 equivalent mol against 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.

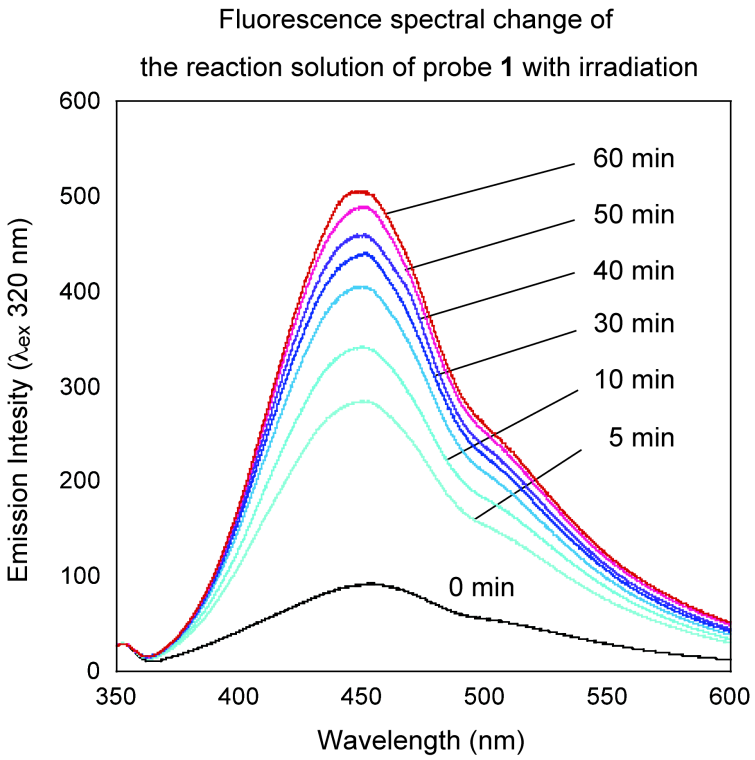


Fig. S2 Photoreaction of photoprobe **1** under irradiation with 315-nm light.

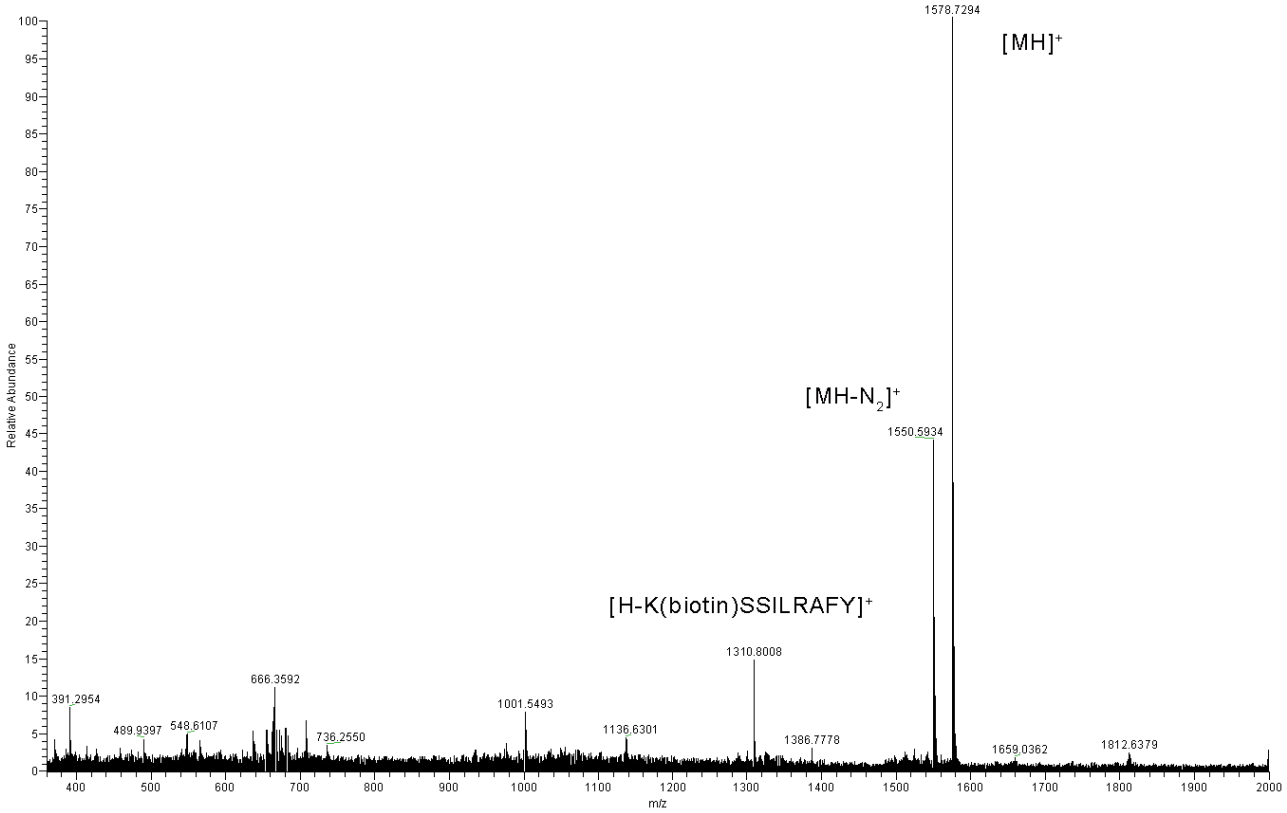
Photoprobe **1** was dissolved in 50% CH_3OH-H_2O solution (1 μM) and irradiated with 315-nm light at 25 °C in a sealed quartz cell. Emission spectra (λ_{ex} = 320 nm) was measured at 20 °C.

Fig. S3 ESI (+) -MS of photoprobe **1** (panel A), HPLC peaks **1** (panel B) and **2** (panel C) of digested products.

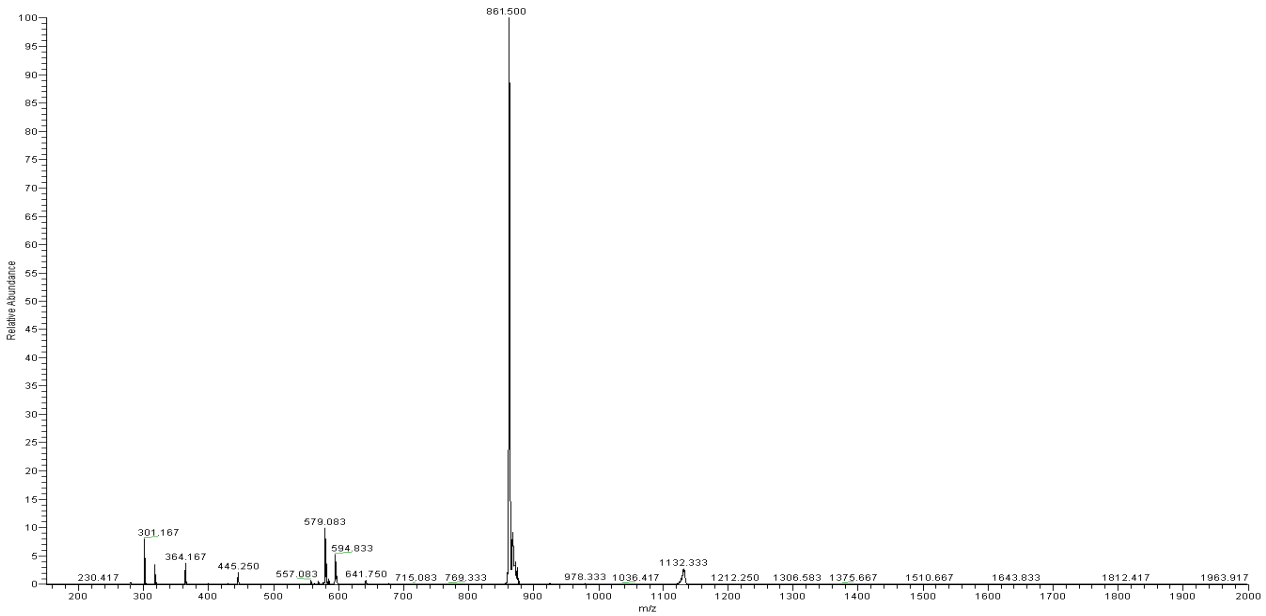
(A) HRMS of photoprobe **1**

1550.6 for $[MH\text{-}N_2]^+$: carbene species produced by heat decomposition.

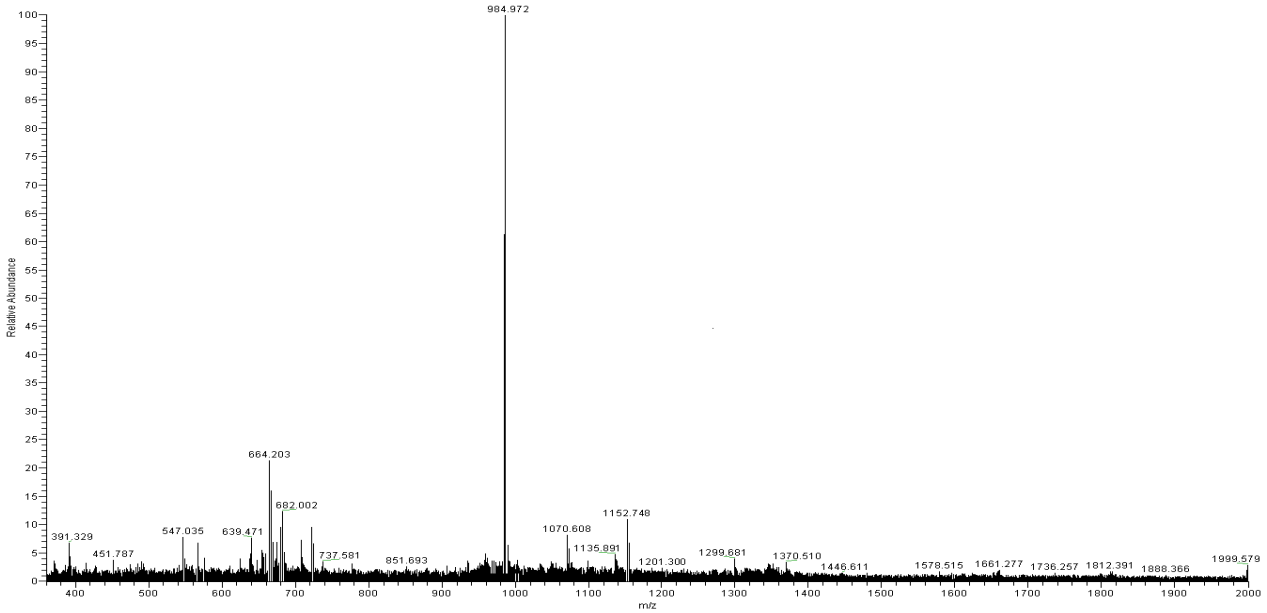
1310.8 for [MH - cross-linker unit]$^+$: peptide [H-K(biotin)SSILRAFY] produced by intramolecular cyclization reaction.



(B) Peak **1** (861.4 for [FVVEK-coumarin + H]$^+$)



(C) Peak **2** (985.4 for [VWNAQK-coumarin + H]$^+$)



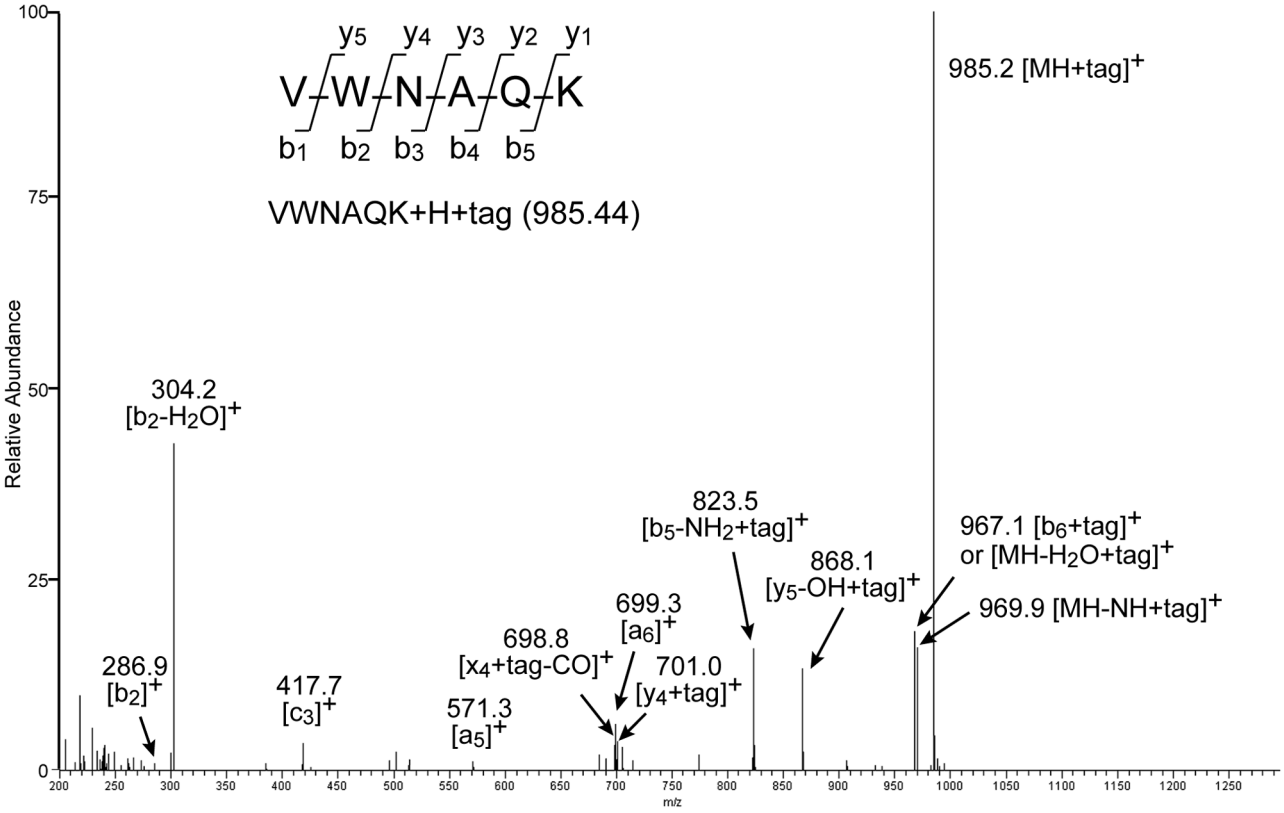
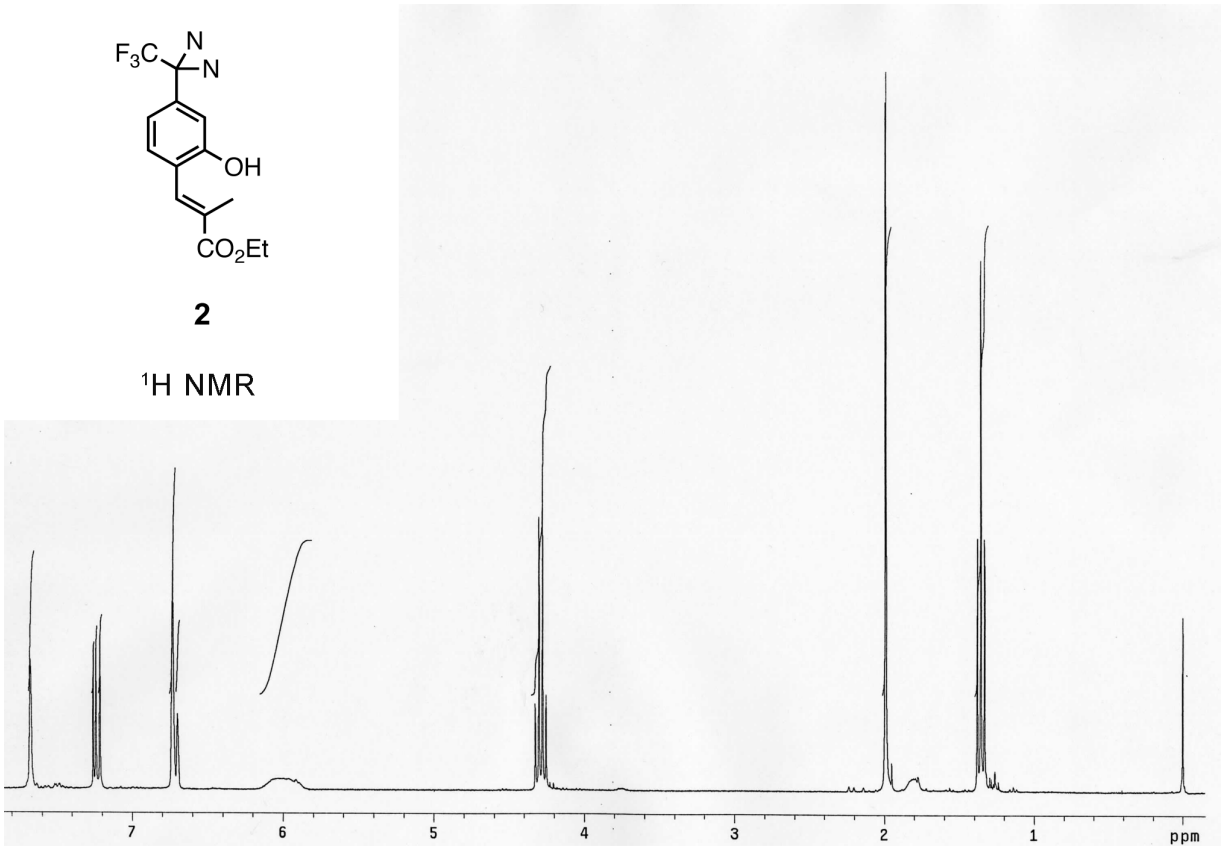
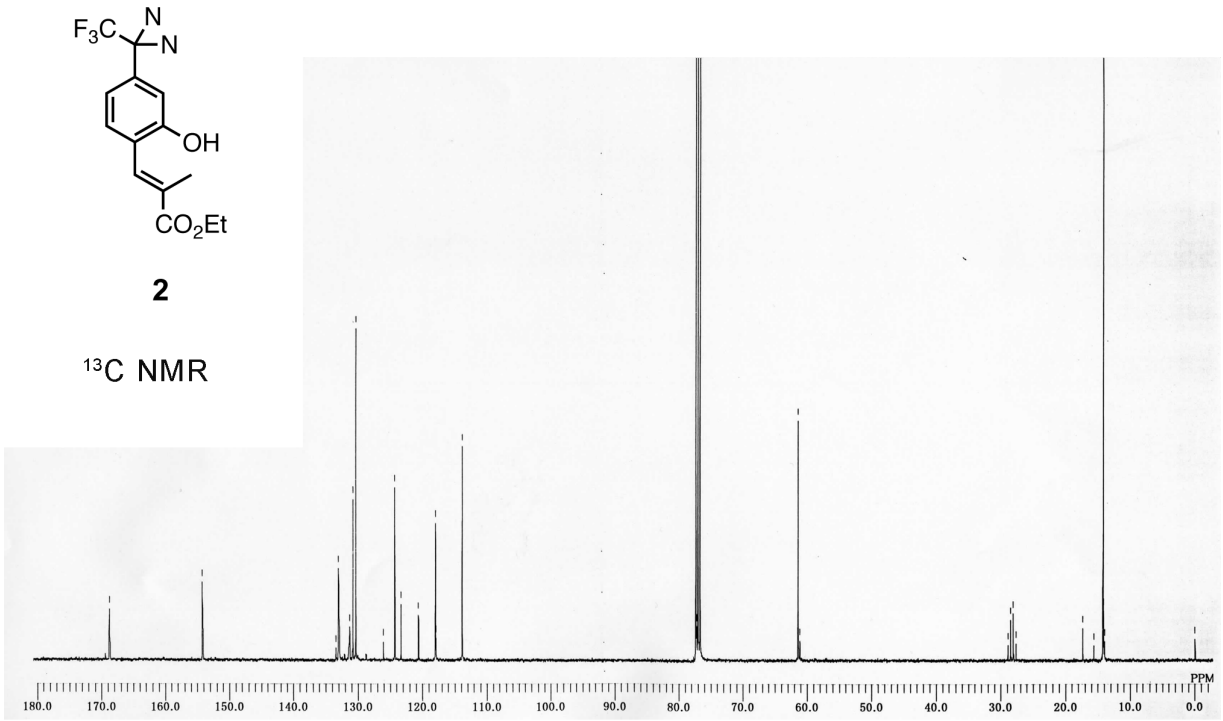


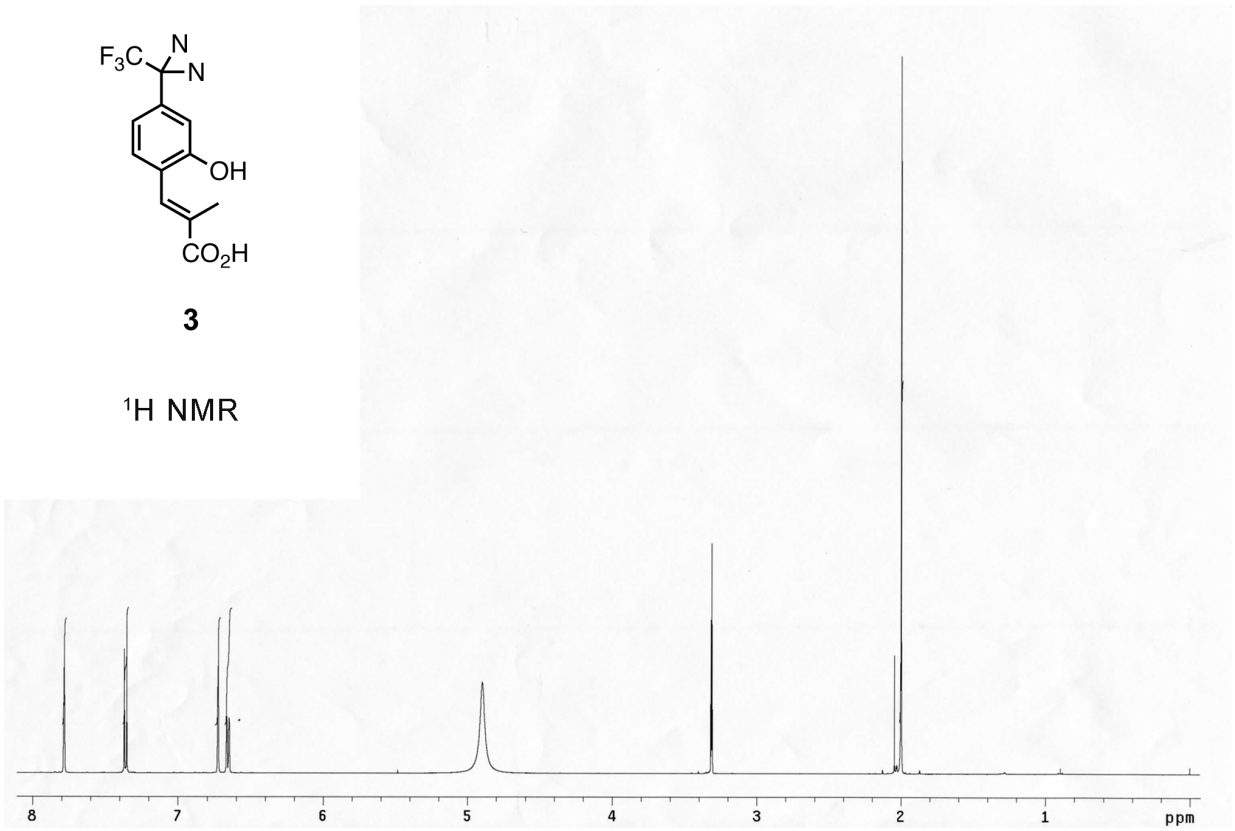
Fig. S4 NMR data of compounds

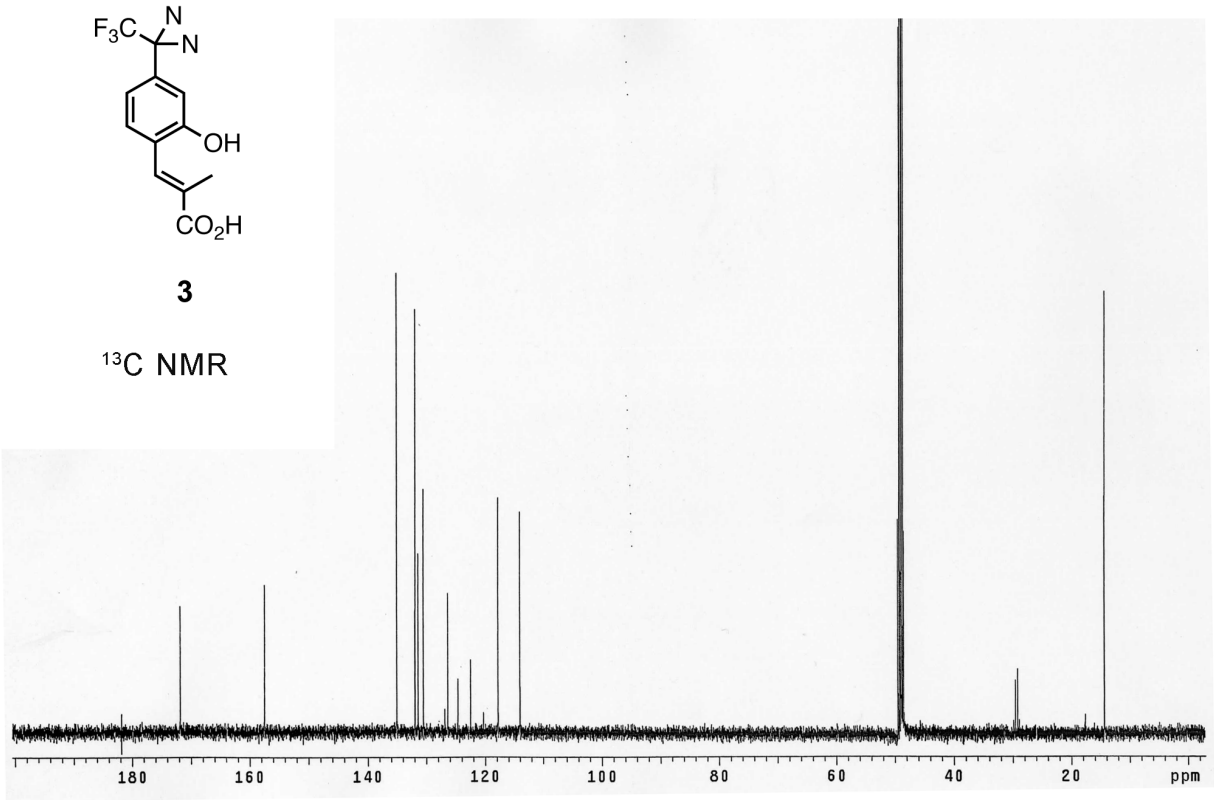
(A) Compound **2**





(B) Compound **3**





References

1) M. Hashimoto, Y. Kanaoka, and Y. Hatanaka, *Heterocycles*, 1997, **46**, 119.

2) A. E. Speers and B. F. Cravatt, *J. Am. Chem. Soc.*, 2005, 127, 10018.