Supporting Information

Fluorogenic Photoaffinity Labeling of Proteins in Living Cells

Tewoderos M. Ayele, Steve D. Knutson, Satheesh Ellipilli, Hyun Hwang, and Jennifer M. Heemstra*

Department of Chemistry, Emory University, Atlanta, GA 30302, United States

Table of Contents

_p_-amino-leucomalachite green (1)

This molecule was synthesized using the same protocol described by Deng and coworkers[1]. First *p*-nitro-malachite green was synthesized by reacting *p*-nitrobenzaldehyde (250 mg, 1.6543 mmol) and *N,N*-dimethylaniline (0.503 ml, 3.9704 mmol) in the presence of zinc chloride (451 mg, 3.3086 mmol) at 100 ºC for 5 h. The reaction was then cooled down to room temperature and purified using the same procedure as reported by Den and coworkers to obtain 70% yield. *p*-nitro-leucomalachite green (100 mg, 0.2665 mmol) was then reduced using Pd/C (5% w/w) and 9 ml of methanol and THF solution (1/2 v/v) under hydrogen for 3h. The solution was then filtered through celite and concentrated under reduced pressure to yield a light blue solid. Yield = 91.0 mg, 98.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.90 (s, 12H), 3.53 (s, 2H), 5.27 (s, 1H), 6.58-6.66 (m, 6H), 6.89-6.98 (m, 6H)

(9*H*-fluoren-9-yl)methyl (1-((4-(bis(4-(dimethylamino)phenyl)methyl)phenyl)amino)-5-N,N-diboc-guanidino-1-oxopentan-2-yl)carbamate (2)

To a solution of compound **1** (50.00mg, 0.1483 mmol) in pyridine (500.0 uL), *N,N'*-Diisopropylcarbodiimide (70.0 uL, 0.4449 mmol) and Fmoc-Arg(Boc)$_2$-OH (106.0 mg, 0.1780 mmol) were added at room temperature and the reaction mixture stirred for 12 h. After completion of the reaction, the solvent was removed *in vacuo* and the product was extracted with dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and purified using column chromatography (50% EtOAc/hexane) to obtain compound **2**. Yield = 30.0 mg, 23%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.36 (s, 9H), 1.49 (s, 9H), 1.65-1.72 (m, 2H), 1.78-1.91 (m, 2H), 2.89 (s, 12H), 3.41-3.55 (m, 2H), 4.20 (t, 1H, *J* = *8.0 Hz*), 4.36-4.47 (m, 2H), 5.33 (s, 1H), 6.30 (d, 1H, *J* = *8.0 Hz*), 6.63 (d, 4H, *J* = *8.0 Hz*), 6.94 (d, 4H, *J* = *8.0 Hz*), 7.06 (d, 2H, *J* = *8.0 Hz*), 7.26-7.39 (m, 5H), 7.57-7.62 (m, 2H), 7.74 (d, 2H, *J* = *8.0* Hz); 8.41 (s, 1H), 8.48 (t, 1H, *J* = *8.0 Hz*), 11.44 (s, 1H); LRMS (ESI-TOF) *m/z* Calcd for $C_{56}H_{67}N_5O_7$ $[M + H]^+$ 924.5024; Found 924.5016.

(*Z*)-*N*-(4-((4-(5-(2,3-bis(*tert*-butoxycarbonyl)guanidino)-2-(3-(3-methyl-3*H*-diazirin-3-yl)propanamido)pentanamido)phenyl)(4-(dimethylamino)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)-*N*-methylmethanaminium (3)

To a solution of compound **2** (30.00mg, 0.0325 mmol) in DMF (500.0 uL), 0.5 mL of 20% piperidine in DMF was added and stirred room temperature for 1h. The free amine was purified by preparative TLC using 2% MeOH/DCM, then coupled with NHS-diazirine (22.0 mg, 0.0975 mmol) in DMF. The solvent was evaporated in vacuo and the coupled product was subjected to oxidation using chloranil (16.0 mg, 0.0650 mmol) and acetic acid (30.0 uL) in chloroform. The reaction mixture was refluxed for 4h. After completion of the reaction, the solvent was removed *in vacuo*, and the compound **3** was purified by preparative TLC using 10% MeOH/DCM. Yield = 20.0 mg, 76%. ^{1}H NMR (400 MHz, CD_3OD) δ 1.00 (d, 3H, *J* = *4.0 Hz*), 1.42-1.51 (m, 18H), 1.66-1.70 (m, 4H), 2.14-2.22 (m, 2H), 2.89 (s, 6H), 3.31 (s, 6H), 3.34-3.44 (m, 2H), 4.44-4.61 (m, 3H), 6.68 (d, 2H, *J* = 8.0 Hz), 7.03 (d, 2H, *J* = 8.0 Hz), 7.17 (d, 2H, *J* = 8.0 Hz), 7.33 (m, 2H), 7.42(d, 2H, *J* = 8.0 Hz), 7.86 (d, 2H, *J* = 8.0 Hz); LRMS (ESI-TOF) *m/z* Calcd for $C_{44}H_{60}N_9O_6^+$ $[M]^+$ 810.4661; Found 810.4664.

***N*-(4-((4-(dimethylamino)phenyl)(4-(5-guanidino-2-(3-(3-methyl-3*H*-diazirin-3-yl)propanamido)pentanamido)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)-*N*-methylmethanaminium (5)**

Compound **3** (20.00mg, 0.0246 mmol) was subjected to Boc-deprotection using 2.0 mL of 50% TFA/DCM for 1 h to obtain compound **4**. The solvent was removed *in vacuo* and the product was treated with 10% *aq.* HCl in order to remove excess TFA to obtain pure compound **5**. Yield = 14.8 mg, 98%. ^{1}H NMR (400 MHz, CD_3OD) δ 1.01 (s, 3H), 1.67-1.72 (m, 2H), 1.77-1.98 (m, 4H), 2.20 (t, 2H, *J* = 8.0 Hz), 3.22-3.26 (m, 2H), 3.32 (s, 12H), 4.52-4.57 (m, 1H), 7.04 (d, 2H, *J* = 8.0 Hz), 7.34 (d, 2H, *J* = 8.0 Hz), 7.42 (d, 2H, *J* = 8.0 Hz), 7.87 (d, 2H, *J* = 8.0 Hz); LRMS (ESI-TOF) *m/z* Calcd for $C_{34}H_{44}N_9O_2^+$ $[M]^+$ 610.3612; Found 610.3589.

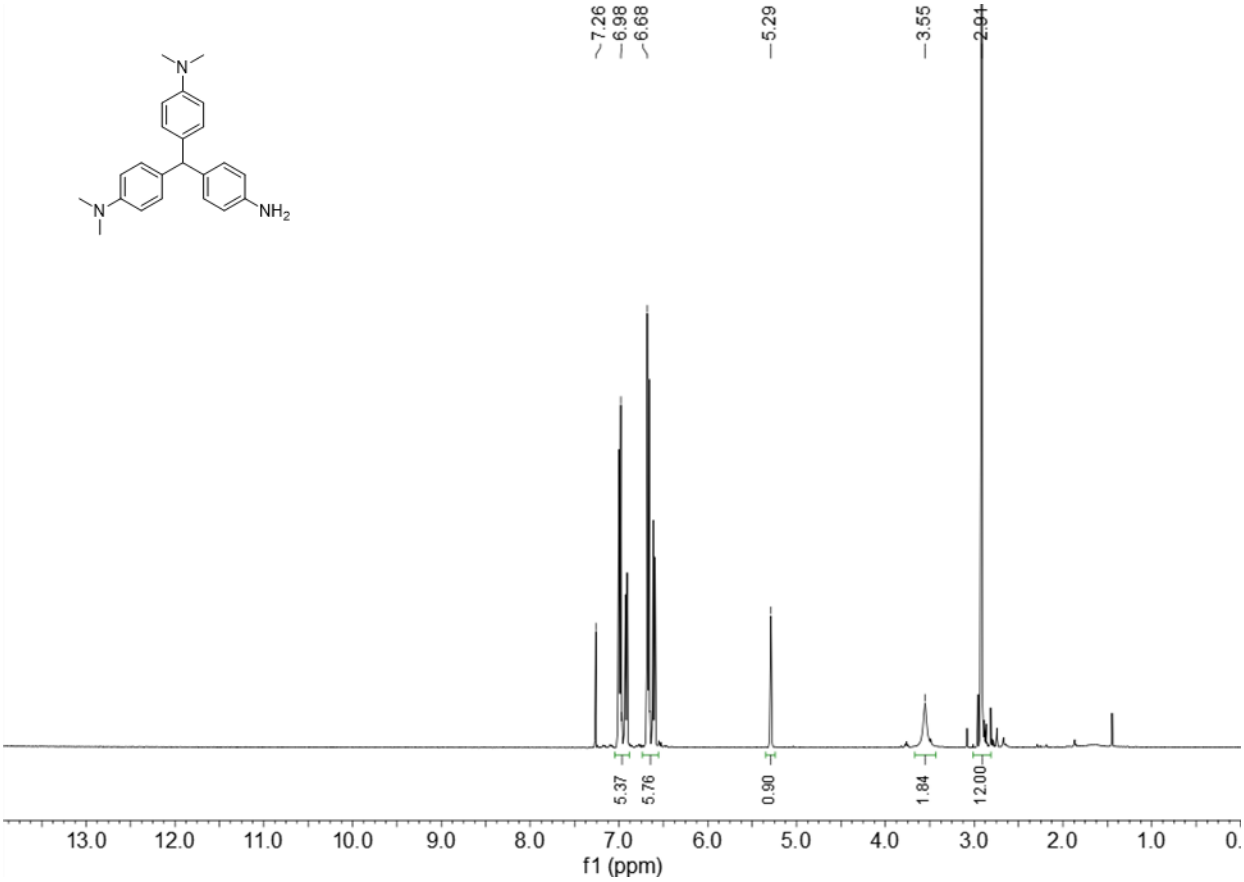


Figure S1. ^{1}H NMR of compound 1

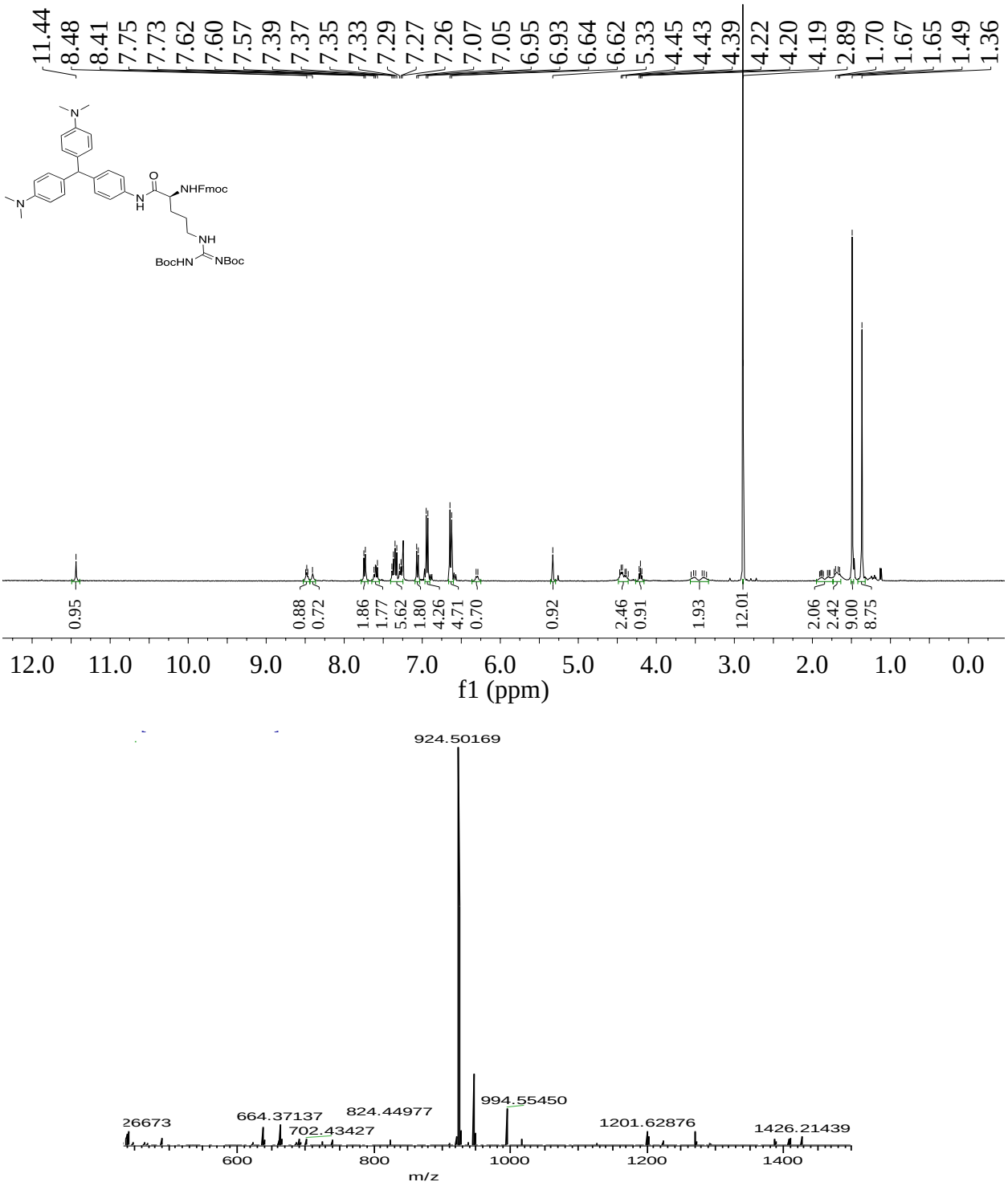
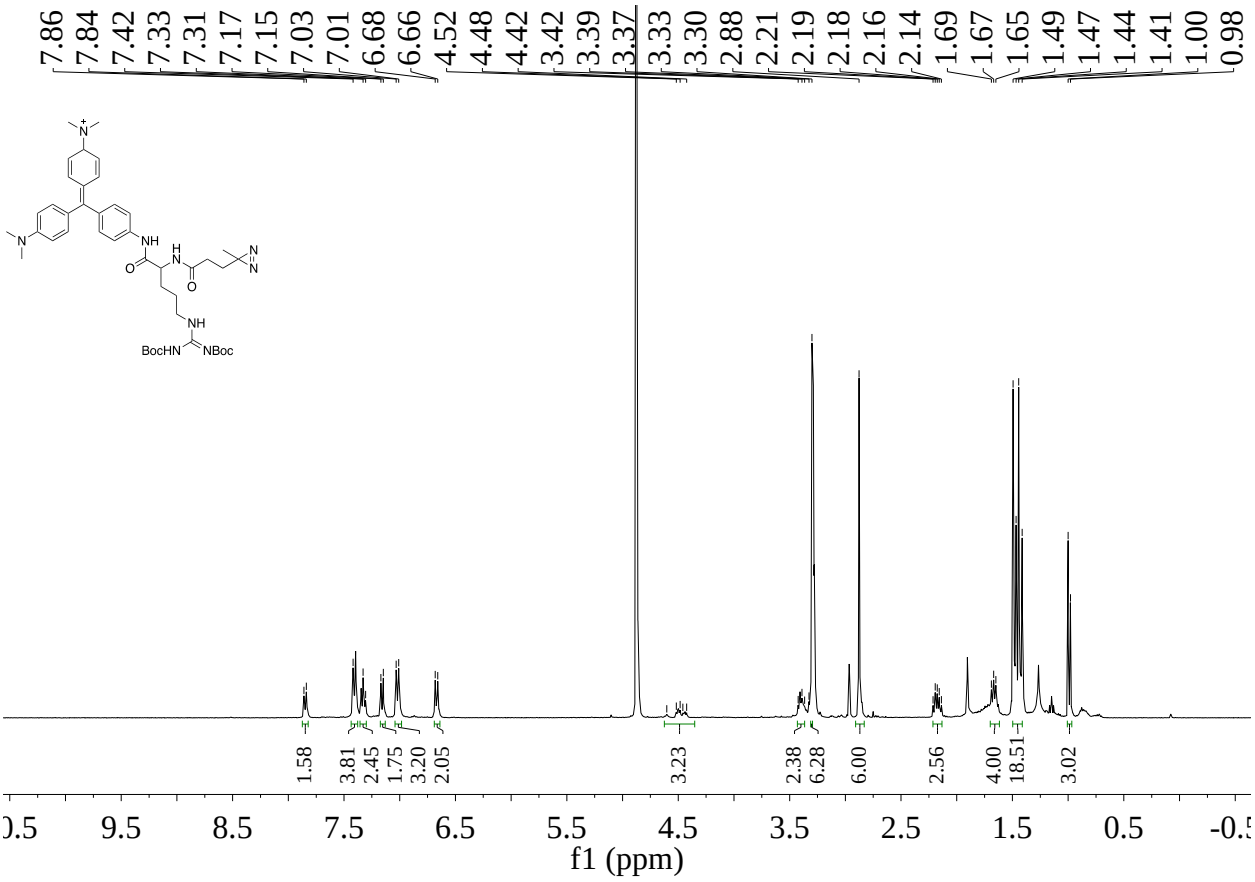


Figure S2. ^{1}H NMR and mass spec of compound 2



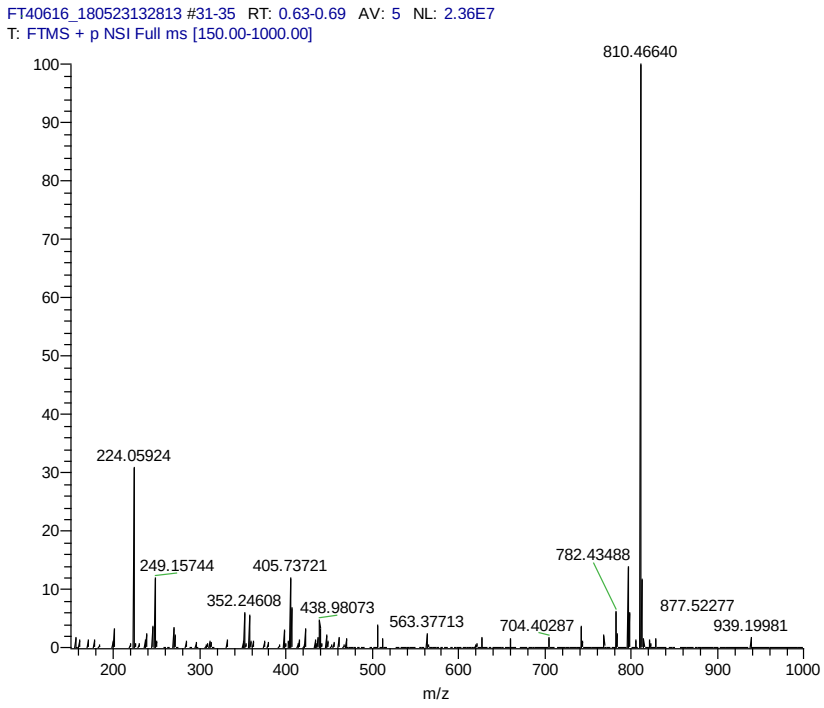
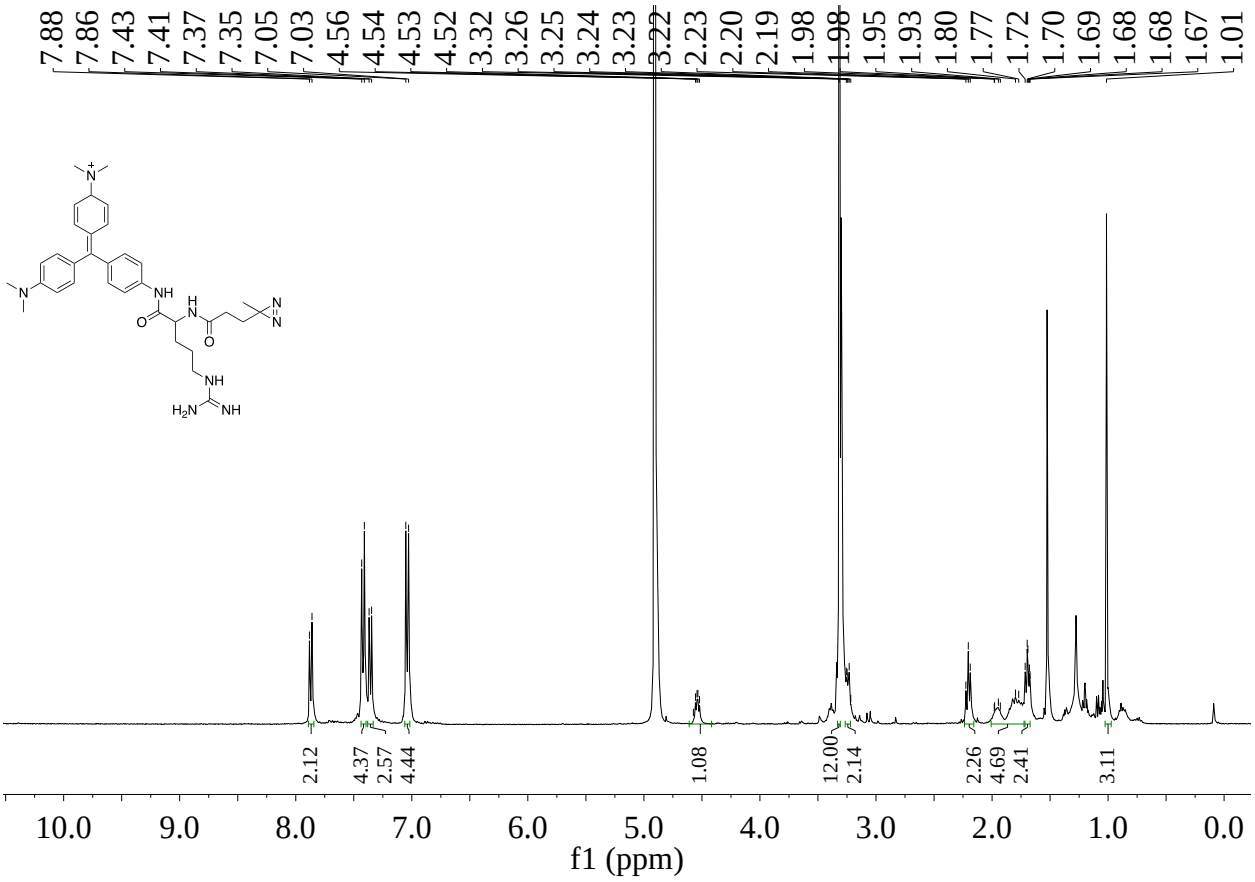


Figure S3. ^{1}H NMR and mass spec of compound 3



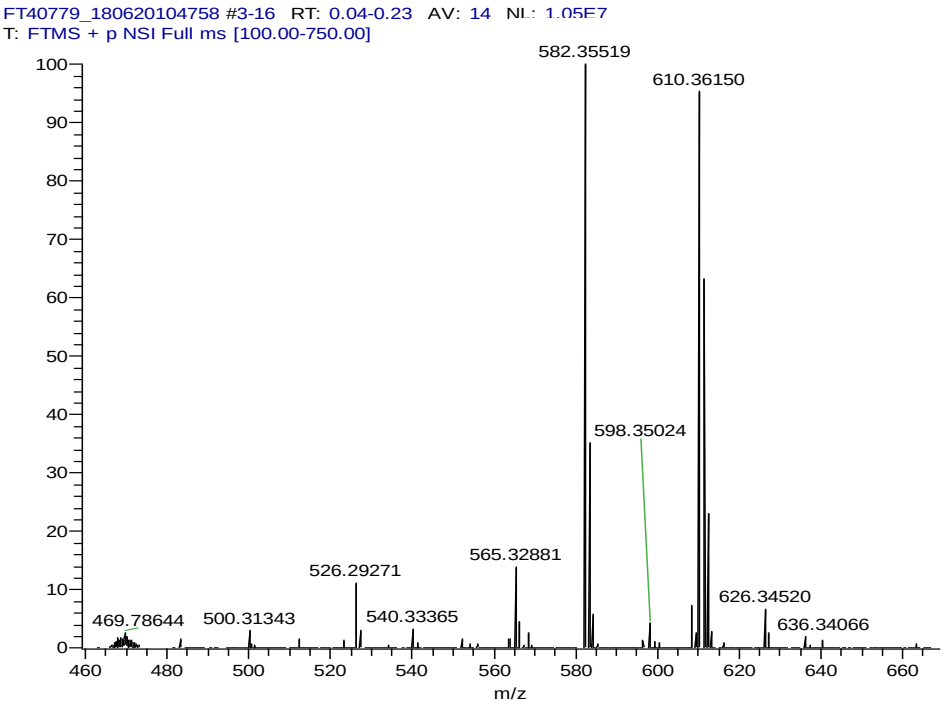


Figure S4. ^{1}H NMR and mass spec of compound 5. The other major fragment at 582.35519 is likely due to the loss of the diazirine group during ionization.

Kinetic experiment for protein concentration

50 µM MG-diazarine was incubated at 37 ºC in a solution containing different FAP concentrations for 30 min. After the incubation time, the solutions were then mixed 1:1 (v/v) with SDS denaturing loading dye and were heated to 90 ºC for 10 min and allowed to cool down to room temperature. These samples were loaded on SDS-PAGE and the fluorescence of the bands were quantified using the same technique described in the manuscript.

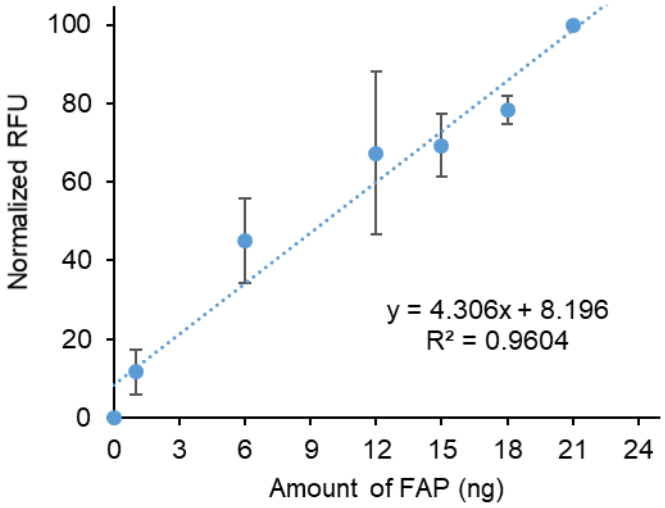


Figure S5. Protein concentration dependent kinetic experiment.

Table S1. Tabular data for protein concentration dependent kinetic experiment

FAP concentration (ng)	Average Normalized RFU	Standard deviation
0	0.000	0.000
1	11.621	5.588
6	45.025	10.903
12	67.384	20.615
15	69.342	8.058
18	78.356	3.528
21	100.000	0.000

MG-diazarine concentration dependent kinetic experiment

20 µg of FAP was dissolved in 1XPBS. MG-diazarine was added to this solution to give different final concentration of MG-diazarine. The solution was then incubated at 37 ºC for 30 min. After the initial incubation time the protein was denature by missing 1:1 (v/v) with SDS denaturing loading dye and were incubated at 90 ºC for 10 min and allowed to cool down to room temperature. These samples were loaded on SDS-PAGE and the fluorescence of the bands were quantified using the same technique describe in the manuscript.

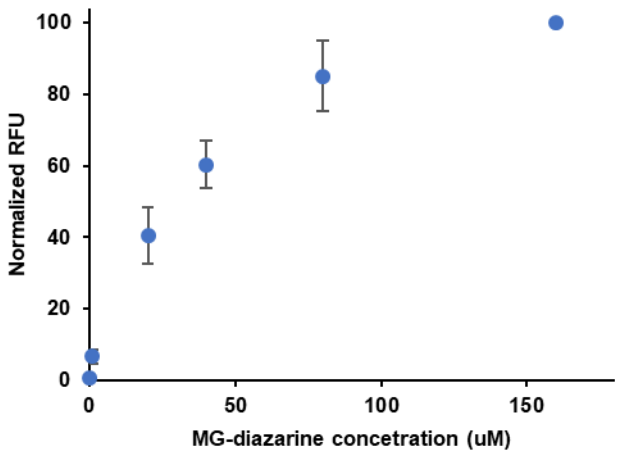


Figure S6. Kinetic experiment for MG-diazarine concentration.

Table S2. Tabular data for protein concentration dependent kinetic experiment

MG-diazarine concertation (µM)	Average RFU	Standard deviation
0	0.609	0.542
1	6.653	2.057
20	40.474	7.762
40	60.360	6.617
80	85.145	9.752
160	100.000	0.000

Cell viability experiment

The effect of UV-irradiation on cell viability was tested using HeLa cells. The cells were grown on 96-well plates containing 100 µl media until they reached 90% confluency and were exposed to UV-irradiation for different amount to time. A well without cells containing 100 µl media was used as a negative control. After the UV-irradiation, the 96-well plate was placed back in 37 ºC cell incubator for 12 h. Then 10 µl of WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) (Thermo Fisher) was added to each well and allowed to incubate at 37 ºC cell incubator for 4 h. After the 4h incubation, the plate was shaken thoroughly for 1 min on a shaker and 440 nm absorbance of each of the wells was measured.

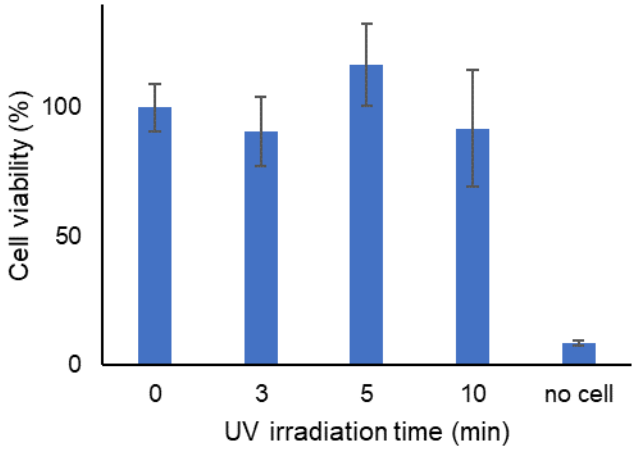


Figure S7. Cell viability

Table S3. Tabular data for cell viability experiment

UV-irradiation Time (min)	0	3	5	10	no cell
Normalized cell viability	100.000	90.570	116.700	91.886	8.755
Standard deviation	9.111	13.490	15.992	22.554	0.966

FAP expression and purification

Recombinant FAP was expressed in Rosetta-gami 2(DE3) *E. coli* cells. Plasmid (pET21) harboring a 10XHis-GST-HRV-FAP fusion was transformed into competent cells and plated on LB agar plates containing 50 µg/mL ampicillin. Individual colonies were picked and grown in 5 mL LB overnight cultures. The following day, these were transferred into 1 L of LB supplemented with 100 mM phosphate, 20 mM succinic acid, 0.4% glycerol, 12.5 µg/mL tetracycline, 34 µg/mL chloramphenicol, and 50 µg/mL ampicillin. Cultures were grown at 37 °C to an approximate OD of 0.8, at which point the temperature was reduced to 22 °C for 1 hour. Cultures were then supplemented with 500 µM IPTG and 0.4% glucose and allowed to grow for an additional 18 h at 22 °C. Cells were then pelleted and frozen at −20 °C, followed by lysis in B-PER™ Bacterial Protein Extraction Reagent (Thermo Fisher) and purification using Ni-NTA agarose beads (Thermo Fisher) according to manufacturer guidelines. FAP was released using GST-HRV-3C protease (Thermo Fisher), and 10XHis-GST was removed using immobilized glutathione agarose (Thermo Fisher). The supernatant containing released FAP was concentrated and buffer-exchanged into PBS using 10kDa MWCO ultracentrifugal filters (Amicon). SDS-PAGE was used to evaluate purity and concentration was determined using the Micro BCA Protein Assay (Thermo Fisher).

MG-diazarine labeling specificity

A-172 whole-cell lysate (Rockland Antibodies & Assays) was used for this experiment. To test the specificity of FAP labeling, 74 µg of whole-cell lysate was spiked with FAP and incubated in 50 µM MG-diazarine for 30 min. A negative control group was also prepared by mixing 74 µg of whole-cell lysate in 50 µM of MG-diazarine and incubated for 30 min. After the incubation time, both samples were UV irradiated for 5 min and analyzed by SDS-PAGE.

Cell culture

HeLa cells were cultured on tissue culture dishes (Cell Star) in DMEM (Sigma Aldrich, MO) media supplemented with 10% FBS at 37 ºC, 5% CO_2 and 95% humidity.

Fluorescent imaging

Fluorescent images were obtained using Leica DMi8 confocal fluorescence microscope with 10x objective with the following settings: Texas Red excitation laser (7.0 laser intensity, 259.3% gain), 640-700 nm emission (band-pass). Image processing was done using ImageJ software.

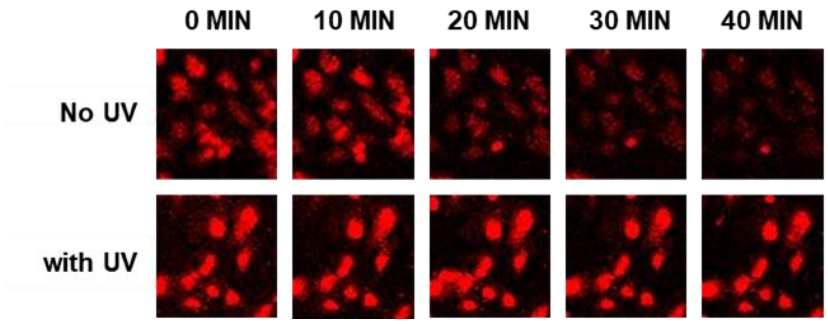


Figure S8. Wash-out of MG-diazarine from HeLa cells.

Image processing

The fluorescent images were opened using ImageJ software and individual cells were selected using the magic wand tool plugin. Using this plugin, the mean brightness of the cells was measured. The background is then selected and subtracted from the mean brightness measurement to quantitate the correct brightness of the cells. Similar measurement was done to the same cells over the course of 40 min wash-out time.

Table S1. Tabular Data for Reported *in vitro* Labeling Fluorescence Data

UV Irradiation Time (min)	Normalized RFU
0	0.000
2	0.258
4	0.416
6	0.537
8	0.768
10	0.651
14	0.822
18	0.917
22	1.000

Table S2. Tabular Data for Reported Cell-Washout experiment

Wash-out time (min)	no UV normalized RFU	with UV normalized RFU
0	1	1.048
10	0.940	1.053
20	0.603	0.988
30	0.328	0.956
40	0.258	0.936

References

(1) Xing, W.; He, L.; Yang, H.; Sun, C.; Li, D.; Yang, X.; Li, Y.; Deng, A. (2009) Development of a Sensitive and Group-Specific Polyclonal Antibody-Based Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Malachite Green and Leucomalachite Green in Water and Fish Samples. *J. Sci. Food Agric. 89* (13), 2165–2173.

(2) Wang, Y.; Telmer, C. A.; Schmidt, B. F.; Franke, J. D.; Ort, S.; Arndt-Jovin, D. J.; Bruchez, M. P. (2014) Fluorogen Activating Protein−Affibody Probes: Modular, No-Wash Measurement of Epidermal Growth Factor Receptors. *Bioconjugate Chem. 26* (1), 137–144.