Supporting Information

Photo-click construction of targetable and activatable two-photon probe imaging protease in apoptosis

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Materials and General methods

All chemicals were available commercially and the solvents were purified by conventional methods before use. The ^{1}H and ^{13}C NMR spectra were acquired on a 400 MHz Bruker AVANCE III–400 spectrometer with tetramethylsilane (TMS) as internal standard. Data for 1H NMR spectra are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broadened); coupling constants are reported as a J value in Hertz (Hz); the number of protons (n) for a given resonance is indicated nH, and based on the spectral integration values. High-resolution mass spectra were collected using a SHIMADZU-2020 LC/MS system with Electrospray Ionization (ESI). The fluorescence spectra were measured with a Shimadzu RF-5301 PC spectrofluorimeter with a 10 mm quartz cuvette. Fluorescence spectrophotometer with slit widths were set at 3 and 5 nm for excitation and emission, respectively. Fluorescent microscopy images were acquired on confocal laser scanning microscopy. HPLC was carried out on Agilent 1200 LC (analytic) or Waters 2535 LC (preparative) with CH_3CN/H_2O (1‰ CF_3COOH) as eluents.

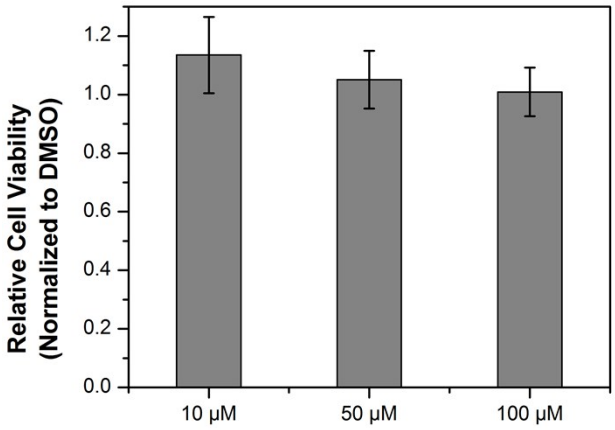


Fig S1 MTT results of NP-1. 10^6 cell mL^{-1} HeLa cells were dispersed within 96-well microtiter plates to a total volume of 200 μL $well^{-1}$. Plates were maintained at 37 °C in a 5% CO_2/95% air incubator for 24 h. Then HeLa cells were incubated for 12 h upon different probe concentrations of 10, 50, and 100 μM. MTT solution (5 mg mL^{-1}, HEPES) was then added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a Triturus microplate reader.

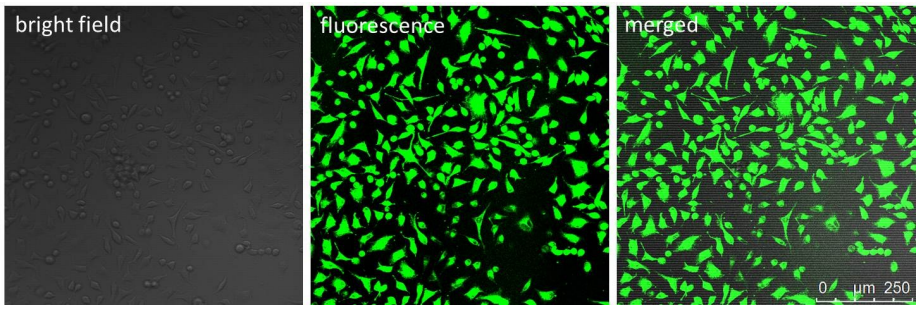


Fig S2 Two-photon imaging of NP-1. 10^6 cell mL^{-1} HeLa cells were incubated with 5 μM **NP-1** for 30 minutes at 37 °C in a 5% CO_2/95% air in confocal dish, and after 30 minutes the cells were washed with cold PBS for three times in order to remove the redundant **NP-1** adequately. Then Leica two-photon laser confocal scanning microscopy are used to imaging the HeLa cells.

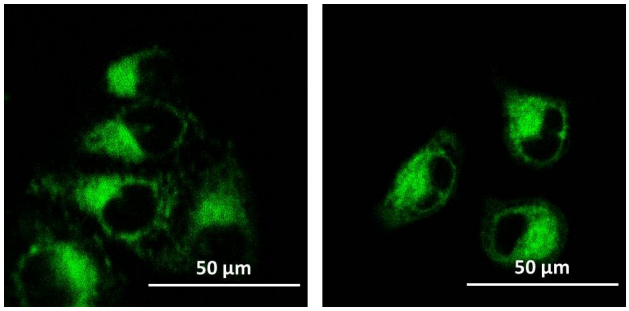
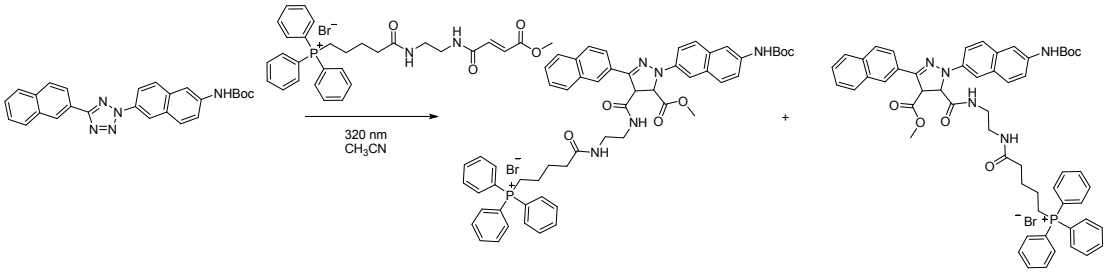
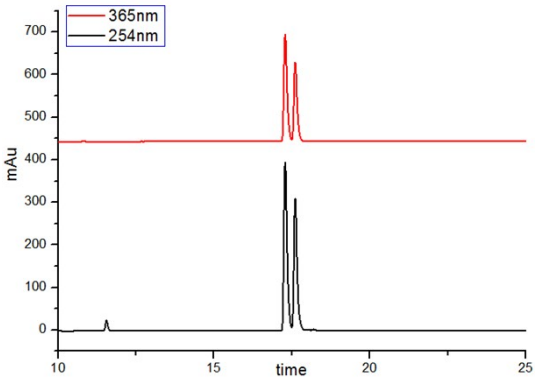


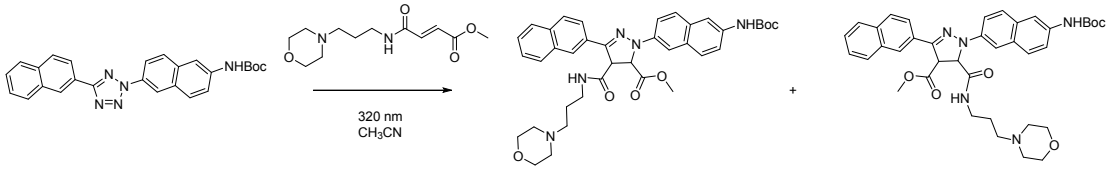
Fig S3 Two-photon imaging comparison of the two regioisomers of NP-1a. 10^6 cell mL^{-1} MCF-7 cells were incubated with 5 μM two regioisomers for 30 minutes at 37 °C in a 5% CO_2/95% air in confocal dish, and after 30 minutes the cells were washed with cold PBS for three times in order to remove the redundant compounds adequately. Then Leica two-photon laser confocal scanning microscopy is used to imaging the two MCF-7 cells. It is obviously that there is almost no difference in two-photon imaging of these two regioisomers products obtained from photo-click reaction.

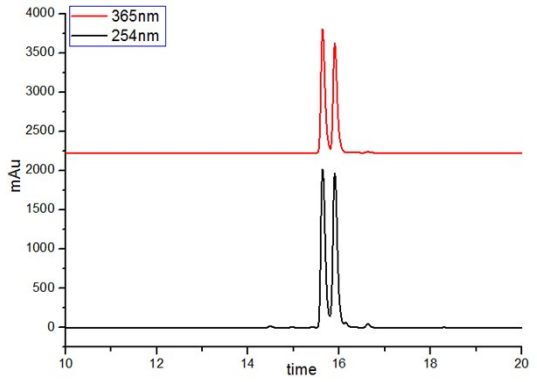
(a) The photo-click process to obtain mitochondria targetable NP-1a.



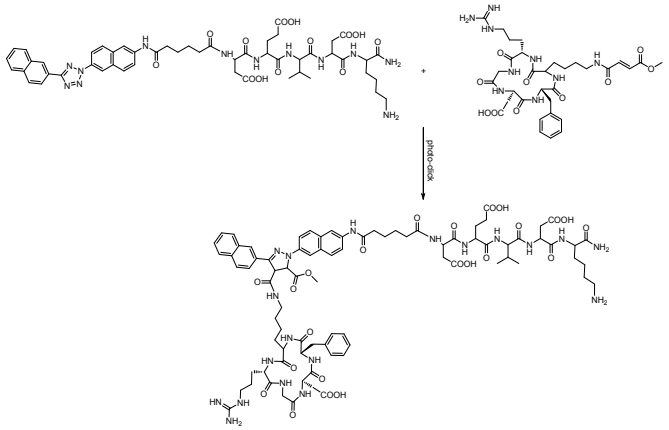


(b) The photo-click process to obtain lysosome targetable NP-1b.

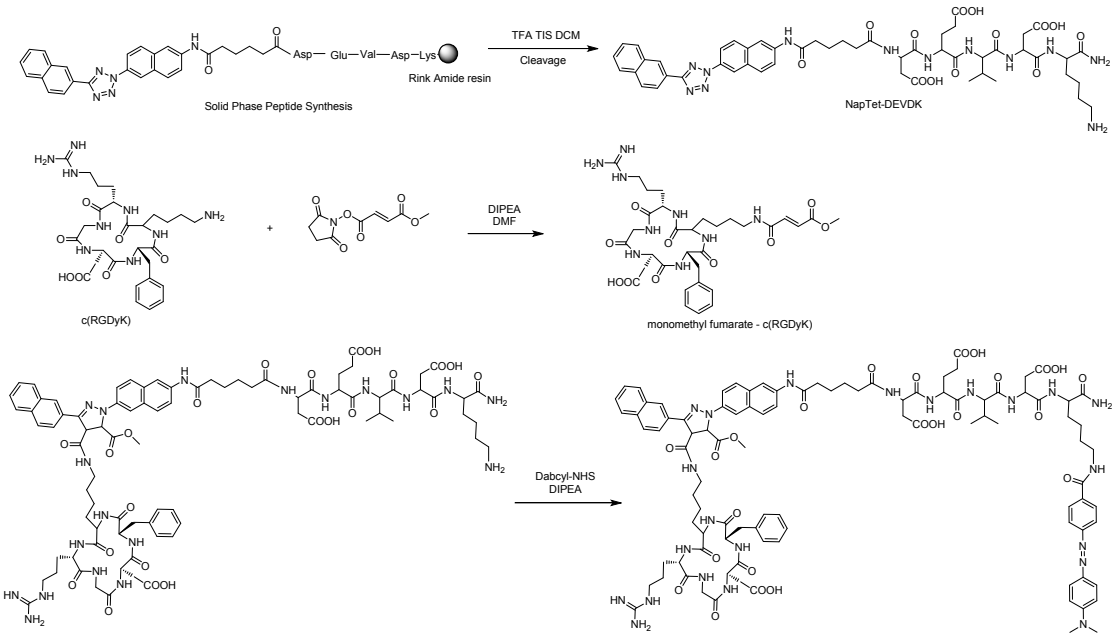




Scheme S1 The photo-click reactions of NT with monomethyl fumarate linked to dimethyl fumarate derivated triphenylphosphine(a) or morpholine(b) and each corresponding HPLC monitored results. The photo-click reactions were conducted under a 302nm hand-held UV lamp in CH_3CN.



Scheme S2 Photo-click reaction of NT-2 with cyclic RGD peptide (cRGDyK) linked with monomethyl fumarate. The photo-click reactions were conducted under a 302nm hand-held UV lamp in CH_3CN.



Scheme S3 Synthesis route of probe **NP-2**

Naptet-DEVDK was obtained from the solid phase peptide synthesis methods by using Rink-Amide resin and cleavage by TFA in DCM. Without further purified, the photo click reaction was conducted with the monomethyl fumarate modified cycle(RGDyK) and obtained the RGD targeted two-photon fluorophore linked caspase 3 substrates DEVDK. Then the commercial 4-[4-(Dimethylamino)phenylazo] benzoic acid N-succinimidyl ester (dabcyl-NHS) was coupled to the preceding step products to acquired target probe **NP-2**. The **NP-2** was then purified by Preparative-High Performance Liquid Chromatography (Flowing phrase from 0 min 5% acetonitrile/95% H_2O with 0.1% TFA to 30 min 95% acetonitrile/5% H_2O with 0.1% TFA) and freeze-drying.

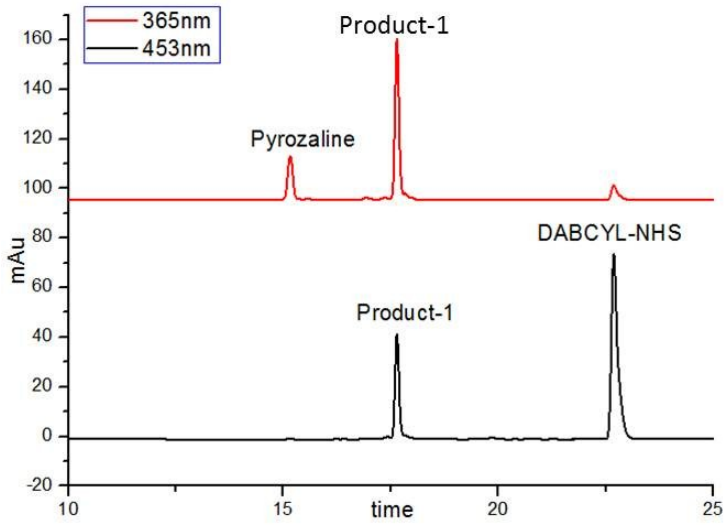


Figure S4 The coupling progress with Dabycl-NHS was monitored by HPLC.

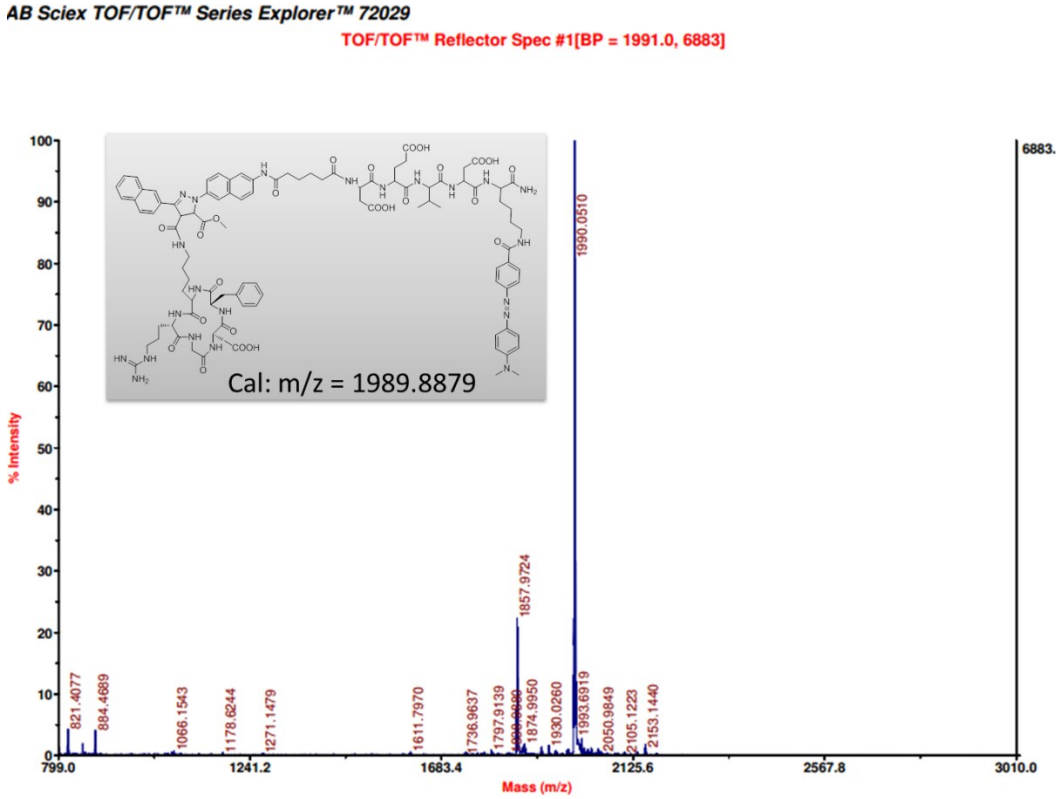


Fig S5 The MALDI-TOF-MS result of NP-2.

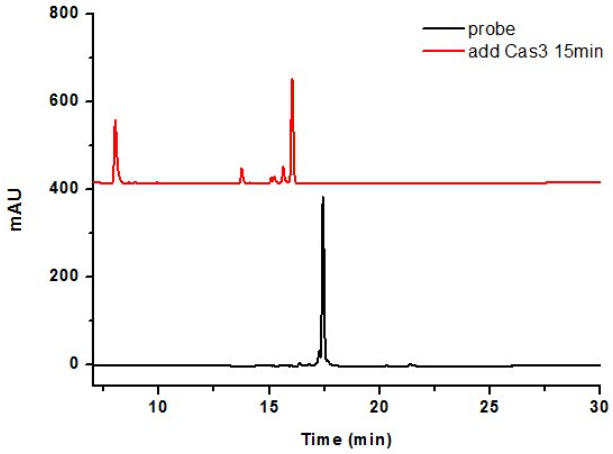


Fig S6 The HPLC monitored result of probe NP-2 response to Caspase-3. The enzyme assay was conducted in caspase-3 assay buffer by 25 μM probe with 1.8 μg/mL Caspase-3 incubated at 37°C for 15 min.

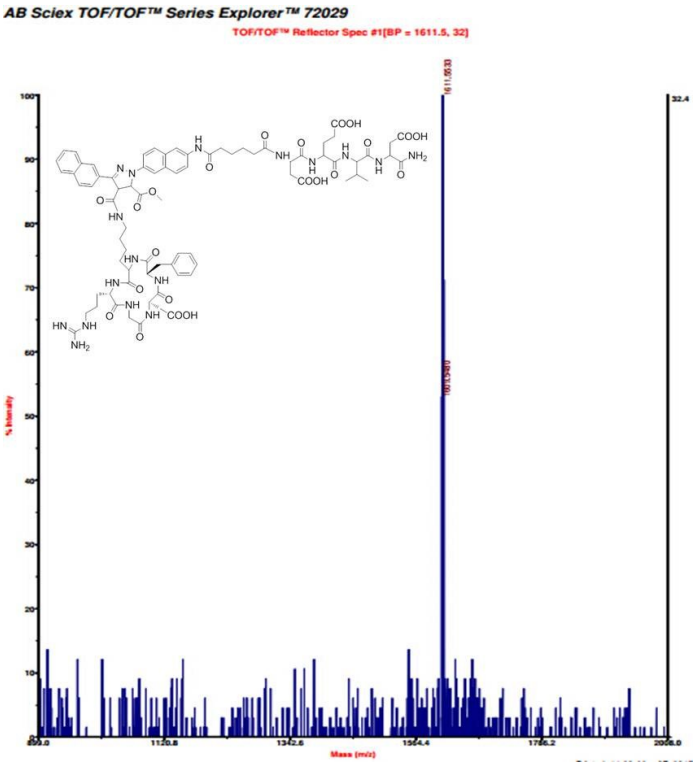


Fig S7 MALDI-TOF-MASS result of Caspase-3 cleaved residue. After Caspase-3 incubating for 15min, the reaction products were confirmed by MALDI-TOF-MASS.

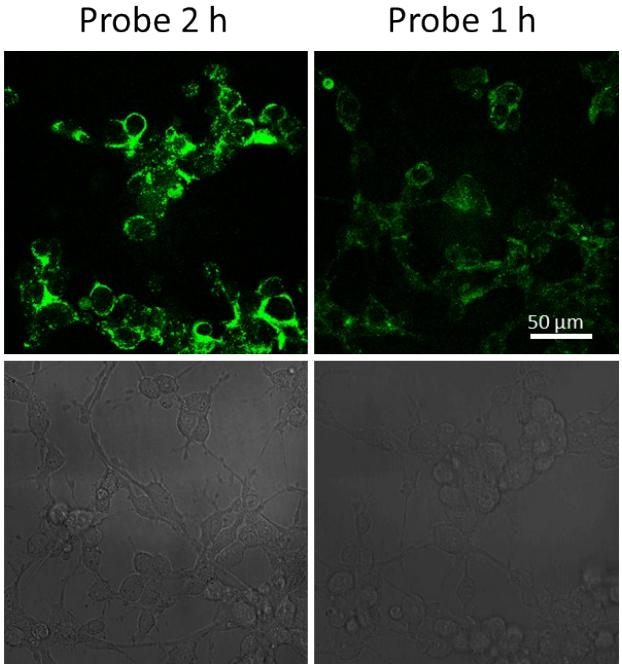


Fig S8 Incubating the probe (2 µM) for 1or 2h at 37°C in U87MG cell first, then the cell were treatd with STS (2 µM) for 1h and imaging.

Tissue 3D Imaging

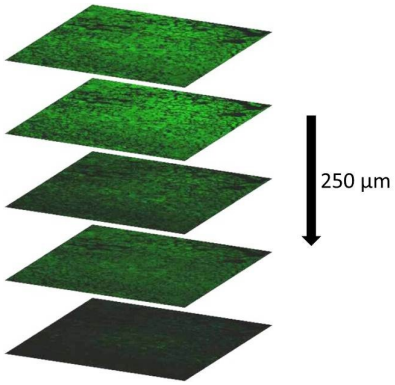
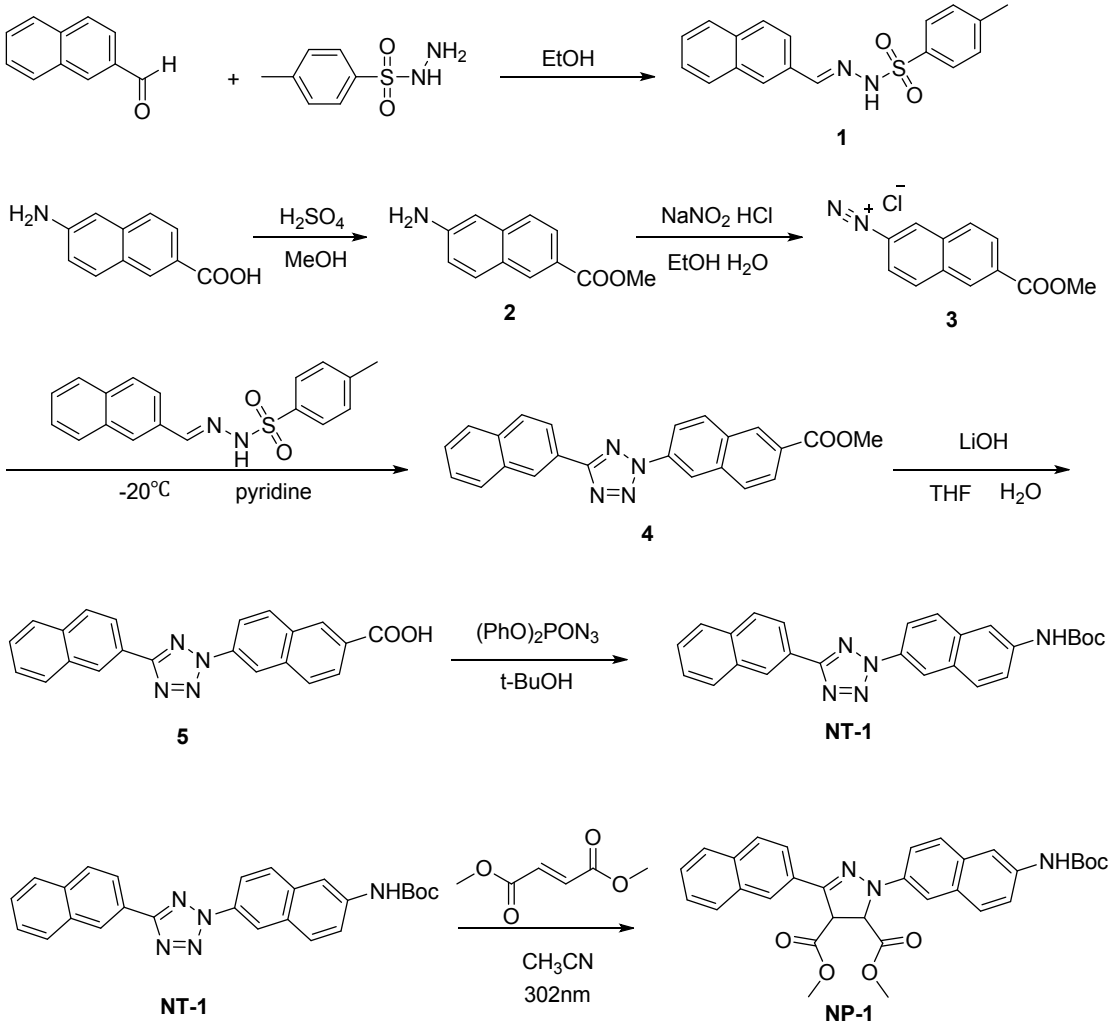


Fig S9 Two-photon tissue 3D images accumulated along the Z-direction at depth of 250 μm.

Solid-phase peptide synthesis (SPPS). The tetrazole linked peptides were synthesized using standard solid phase peptide synthesis (SPPS) protocol. All the peptide substrate for protease were prepared by SPPS using 2-chlorotrityl chloride resin (100~200 mesh and ~1.0 mmol/g) and *N*-Fmoc-protected amino acids. The resin swelled in dry dichloromethane (DCM) for 5 minutes, then the first amino acid was loaded onto resin at its C-terminal with Fmoc-protected amino acid (1.1 equiv.) and *N,N*-diisopropylethylamine (DIPEA) in DCM for 1 hour. After washed with DMF (3 × 3 mL), the resin was agitated with the blocking solution (16:3:1 of DCM/MeOH/DIPEA) for 20 minutes to deactivate the unreacted sites. Then the resins were treated with 20% piperidine (in DMF) for 20 minutes to remove the protecting group, followed by coupling Fmoc-protected amino acid (3 equiv.) to the free amino group on the resin using HBTU as the coupling reagent. These two steps were repeated to elongate the peptide chain, which were carried out by the standard Fmoc SPPS protocol. The resin was washed with DMF for 3~5 times after each step. As the final step, the peptide was cleaved with TFA in DCM with TIS for 2 hours and the resulted crude products were purified by reverse phase HPLC.

Chemical synthesis of NT-1



Scheme S4. Synthesis of **NT-1**.

Compound **1**, **2** *and* **3** *were synthesized according to the literature procedure.*

Synthesis of compound **4**

6-(methoxycarbonyl)naphthalene-2-diazonium chloride **3** was prepared by adding a cooled solution of sodium nitrite (5 mmol) in 2mL of water to a solution of methyl 6-amino-2-naphthoate (5 mmol) and 1.3 mL of concentrated hydrochloric acid in 8 mL of 50% ethanol below 4 °C. The resulted solution of **3** was directly added dropwise over a period of 30 minutes into a solution of 4-methyl-N'-(naphthalen-2-ylmethylene)benzenesulfonohydrazide (5 mmol) in 30 mL pyridine at -15 ~ -10 °C. The reaction mixture was extracted with dichloromethane and water. The chloroform layer was washed with dilute hydrochloric acid and dried with Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (dichloromethane / methanol = 20:1) to give a white powder (932mg, 49.21%).

Synthesis of compound **5**

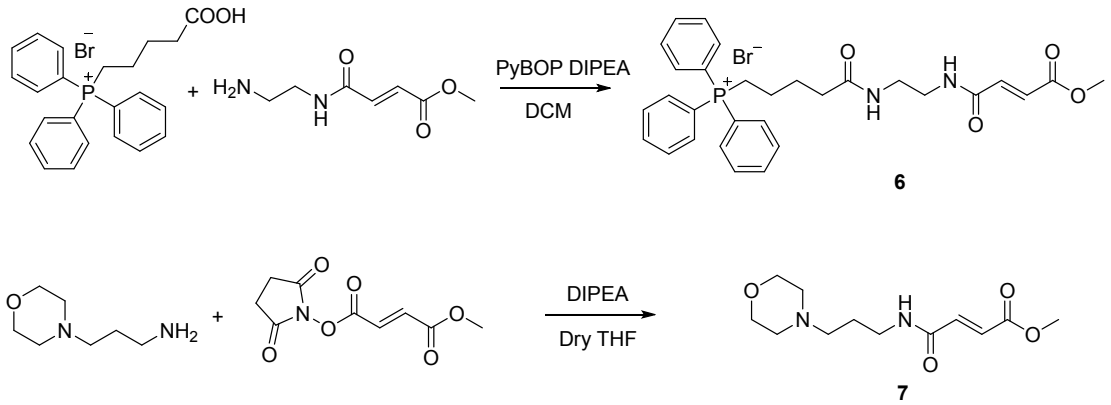
900mg (2.4mmol) compound **4** dissolved in tetrahydrofuran and H_2O (V/V=1) reacted at 70℃ for 4h. TLC (dichloromethane / petroleum ether = 2:1) monitored the reaction was complete. 2M HCl was used to adjust the pH to acidic. Then the mixture was extracted with ethyl acetate and dried with Na_2SO_4. The solvent was removed under reduced pressure and got the grey white product. (823mg, 94.9%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.31 (s, 1 H), 8.92 (s, 1 H), 8.86 (s, 1 H), 8.76 (s, 1 H), 8.46 (m, 2 H), 8.32 (m, 2 H), 8.15 (m, 3 H), 8.04 (m, 1 H), 7.65 (m, 2 H). HRMS (ESI): *m/z* calcd. for $C_{22}H_{15}N_4O_2^+$ [$(M+H)^+$]: 367.1190; found: 367.1523.

Synthesis of compound **NT-1**

366mg (1mmol) compound **5** was dissolved in 20mL anhydrous toluene with 100mg 4A molecular sieve, then 30mL tertiary butanol, 2mmol triethylamine, 2mmol diphenyl azidophosphate were added, and reflux at 80℃ overnight. Then the molecular sieve were removed by filtration, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (petroleum ether/ ethyl acetate = 5:1) to give a white powder (87.5mg, 20.1%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.79 (s, 1 H), 8.83 (s, 1 H), 8.68 (s, 1 H), 8.27 (m, 3 H), 8.15 (m, 2 H), 8.10 (m, 2 H), 8.04 (m, 1 H), 7.64 (m, 3 H), 1.52 (s, 9 H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.03, 153.28, 139.37, 134.39, 133.28, 132.62, 129.82, 129.79, 129.60, 129.17, 128.98, 128.33, 128.06, 127.56, 126.93, 124.37, 124.03, 121.57, 118.67, 118.60, 113.66, 80.05, 28.59. HRMS (ESI): *m/z* calcd. for $C_{26}H_{25}N_5O_2^+$ [$(M+H)^+$]: 438.1925; found: 438.1893.

Synthesis of compound **NP-1**

NT-1 (43.7 mg, 0.1 mmol) in 50 mL CH_3CN/PBS = 1:1 was irradiated with a hand-held 302 nm UV lamp for 30 minutes. CH_3CN was removed and the aqueous layer was extracted with ethyl acetate and further was purified by flash chromatography on silica gel to yield the desired product as a yellow solid (51mg, 92.1%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.44 (s, 1 H), 8.15 (s, 1 H), 8.11 (dd, J =1.61, 8.68 Hz, 1 H), 7.97 (m, 4 H), 7.75 (d, J =8.96 Hz, 1 H), 7.68 (d, J =8.96 Hz, 1 H), 7.57 (m, 3 H), 7.45 (dd, J =2.13, 8.96 Hz, 1 H), 7.30 (d, J =2.13 Hz, 1 H), 5.65 (d, J =3.66 Hz, 1H), 5.21 (d, J =3.66 Hz, 1 H), 3.71 (s, 3 H), 3.68 (s, 3 H), 1.51 (s, 9 H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.08, 163.50, 136.92, 129.46, 66.98, 75.72, 53.62, 52.18, 39.73, 24.43. HRMS (ESI): *m/z* calcd. for $C_{32}H_{31}N_3O_6Na^+$ [$(M+Na)^+$]: 577.2178; found:577.2095.



Scheme S5. Synthesis of triphenylphosphine (**6**) and morpholine (**7**) linked monomethyl fumarate.

Synthesis of compound **6**

To a solution of 4-(Carboxybutyl)triphenylphosphonium Bromide (221 mg, 0.5 mmol) in 10 ml DCM, PyBOP (286 mg, 0.55 mmol) and DIEA (259.5 µL, 1.5 mmol) was added. The mixture was stirred at room temperature for 10 min and then ethylenediamine derivate monomethyl fumarate (86 mg, 0.5 mmol) was added. After stirring for 3 h, the solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel to yield the desired product as a white solid (185 mg, 62%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1 H), 8.75 (s, 1 H), 7.80 (m, 3 H), 7.68 (m, 13 H), 7.04 (d, *J*=16.0 Hz, 1 H), 6.71 (d, *J*=16.0 Hz, 1 H), 3.71 (s, 3 H), 3.34 (m, 6 H), 2.41 (t, *J*=6.0 Hz, 2 H), 1.89 (m, 2 H), 1.66 (m, 2 H). HRMS (ESI): *m/z* calcd. for $C_{30}H_{35}N_2O_4P^+$ [$(M+H)^+$]: 517.2251; found: .517.2203

Synthesis of compound **7**

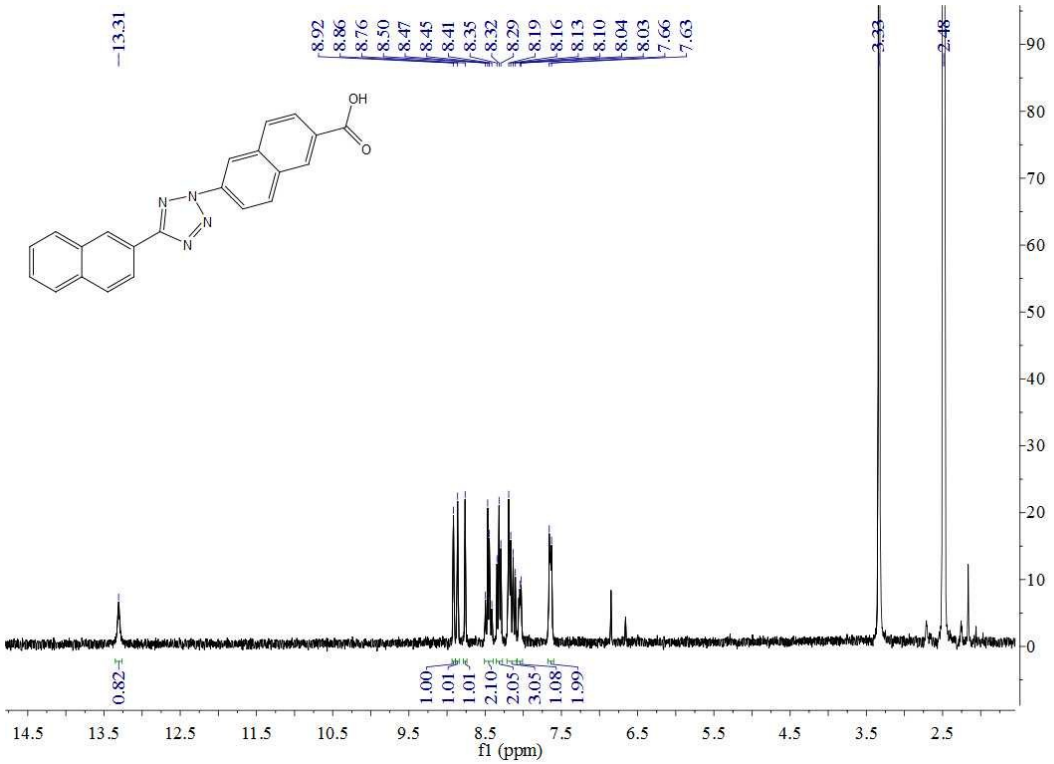
To a solution of N-(3-aminopropyl)morpholine (14.4 mg, 0.1 mmol) in 15 ml dry THF, DIEA (52 µL, 0.3 mmol) was added. Then, MMF-NHS (22.7 mg, 0.1 mmol) was added slowly at room temperature. The reaction mixture was stirred for 2 h and then removed the solvent under reduced pressure and the residue was purified by flash chromatography on silica gel to yield the desired product as a white solid (21 mg, 82%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (s, 1 H), 6.89 (d, *J*=16.0 Hz, 1 H), 6.80 (d, *J*=16.0 Hz, 1 H), 3.80 (s, 3 H), 3.74 (m, 4 H), 3.46 (m, 2 H), 2.51 (m, 6 H), 1.75 (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.08, 163.50, 136.92, 129.46, 66.98, 75.72, 53.62, 52.18, 39.73, 24.43. HRMS (ESI): *m/z* calcd. for $C_{12}H_{21}N_2O_4^+$ [$(M+H)^+$]: 257.1496; found: 257.1493.

Synthesis of compound **NP-1a**

The compound **NP-1a** was prepared according to general photo-click procedure like **NP-1**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.44 (s, 1 H), 8.88 (s, 1 H), 8.13 (s, 1 H), 8.10 (d, *J*=1.72 Hz, 1 H), 8.08 (d, *J*=1.72 Hz, 1 H), 7.96 (m, 3 H), 7.91 (m, 2 H), 7.82 (m, 4 H), 7.75 (m, 14 H), 7.66 (d, *J*=9.32 Hz, 1 H), 7.57 (dd, J=2.27, 9.32 Hz, 1 H), 7.52 (m, 2 H), 7.45 (m, 1 H), 7.22 (d, *J*=2.27 Hz, 1 H), 5.23 (d, *J*=4.96 Hz, 1 H), 4.75 (d, *J*=4.96 Hz, 1 H), 3.74 (s, 3 H), 3.09 (m, 6 H), 2.00 (m, 4 H), 1.62 (m, 2 H), 1.51 (s, 9 H). HRMS (ESI): *m/z* calcd. for $C_{56}H_{57}N_5O_6P^+$ [$(M)^+$]:926.4041; found: 926.4041.

Synthesis of compound **NP-1b**

The compound **NP-1b** was prepared according to general photo-click procedure like **NP-1**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.45 (s, 1 H), 8.87 (s, 1 H), 8.21 (s, 1 H), 8.11 (d, *J*=8.6 Hz, 1 H), 7.96 (m, 4 H), 7.76 (d, *J*=9.09 Hz, 1 H), 7.68 (d, *J*=9.09 Hz, 1 H), 7.57 (m, 3 H), 7.47 (d, *J*=8.6 Hz, 1 H), 7.23 (s, 1 H), 5.22 (d, *J*=5.29 Hz, 1 H), 4.82 (d, *J*=5.29 Hz, 1 H), 3.77 (s, 3 H), 3.46 (m, 4 H), 3.26 (m, 1 H), 3.06 (m, 1 H) 2.16 (m, 6 H), 2.00 (m, 2 H), 1.52 (s, 9 H). HRMS (ESI): *m/z* calcd. for $C_{38}H_{44}N_5O_6^+$ [$(M+H)^+$]:666.3286; found: 666.3222.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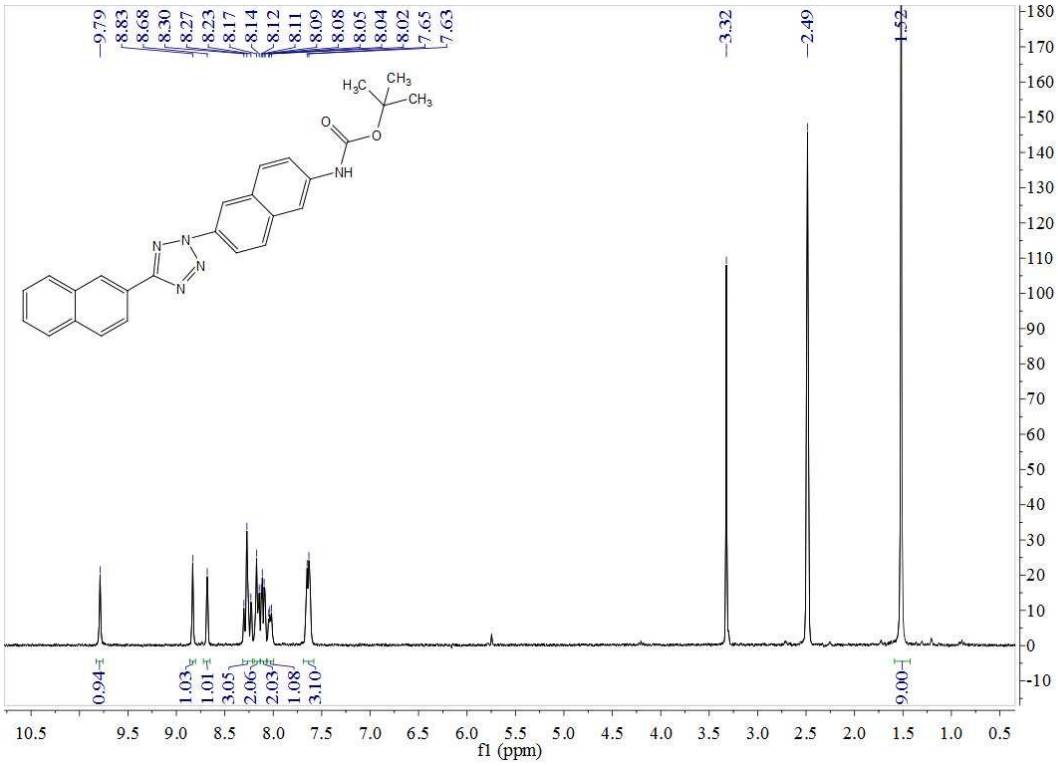
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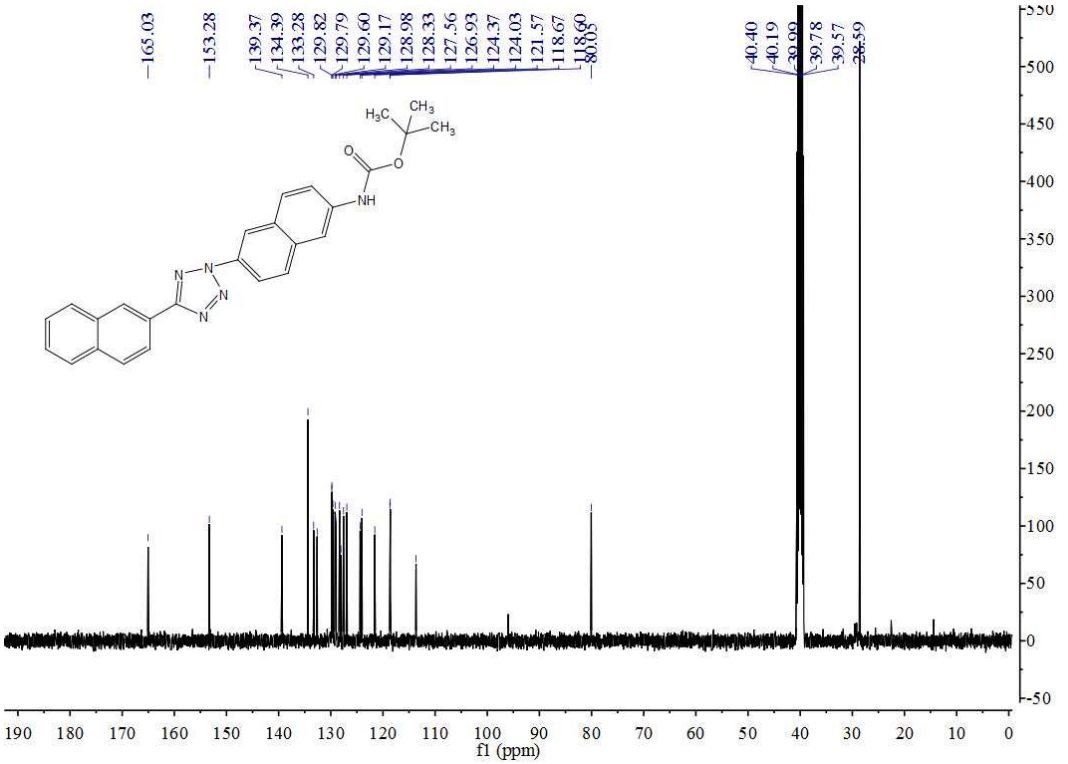
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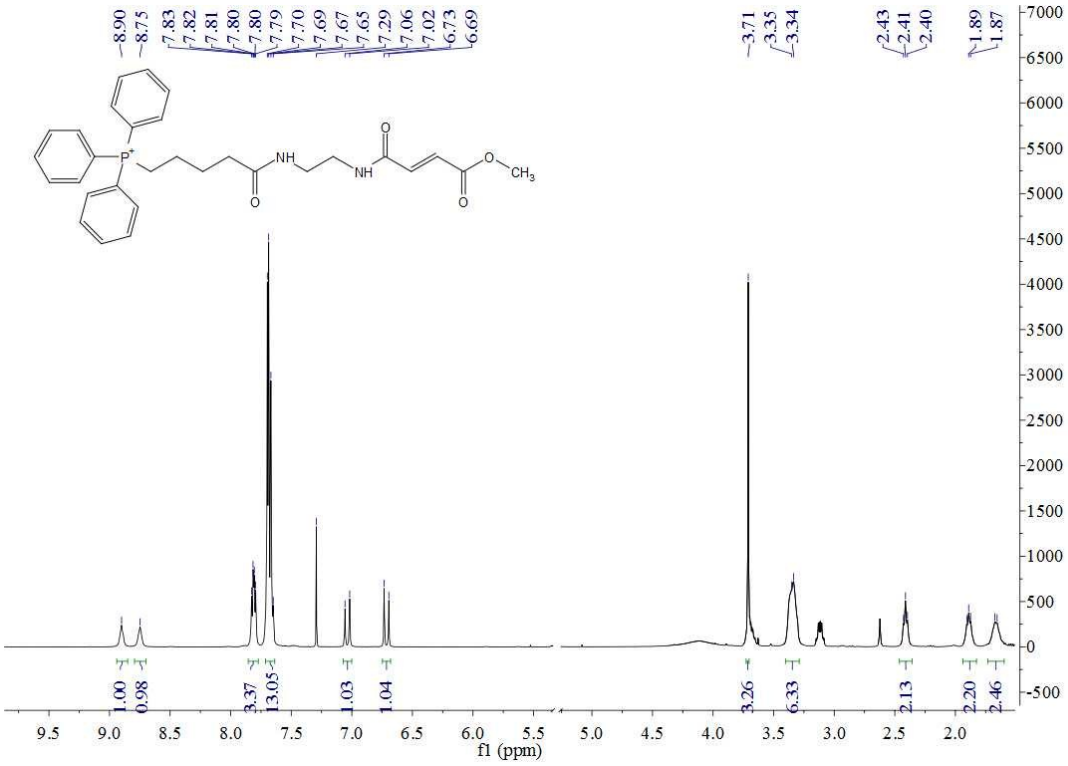
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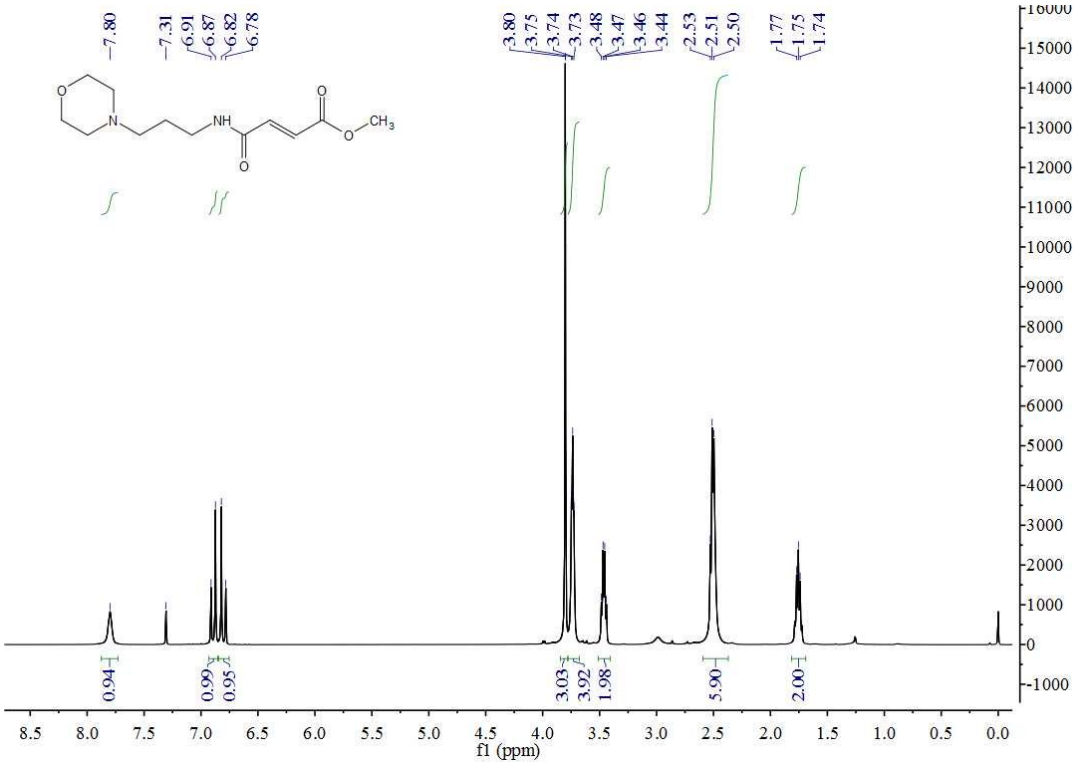
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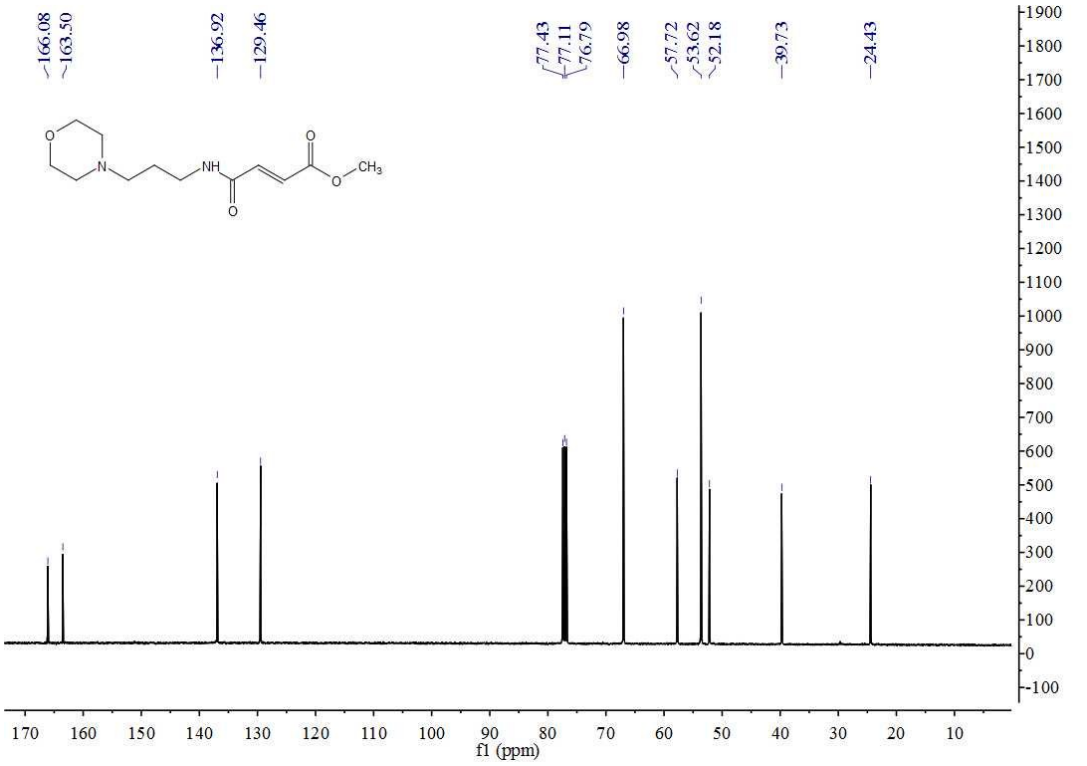
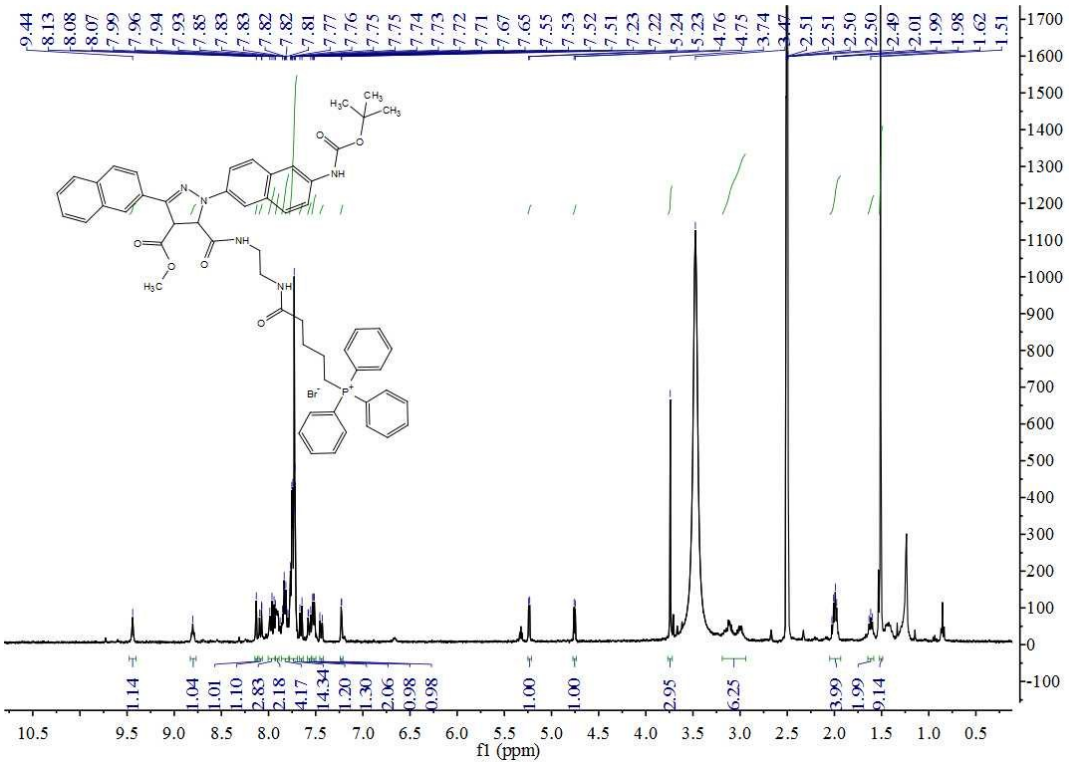
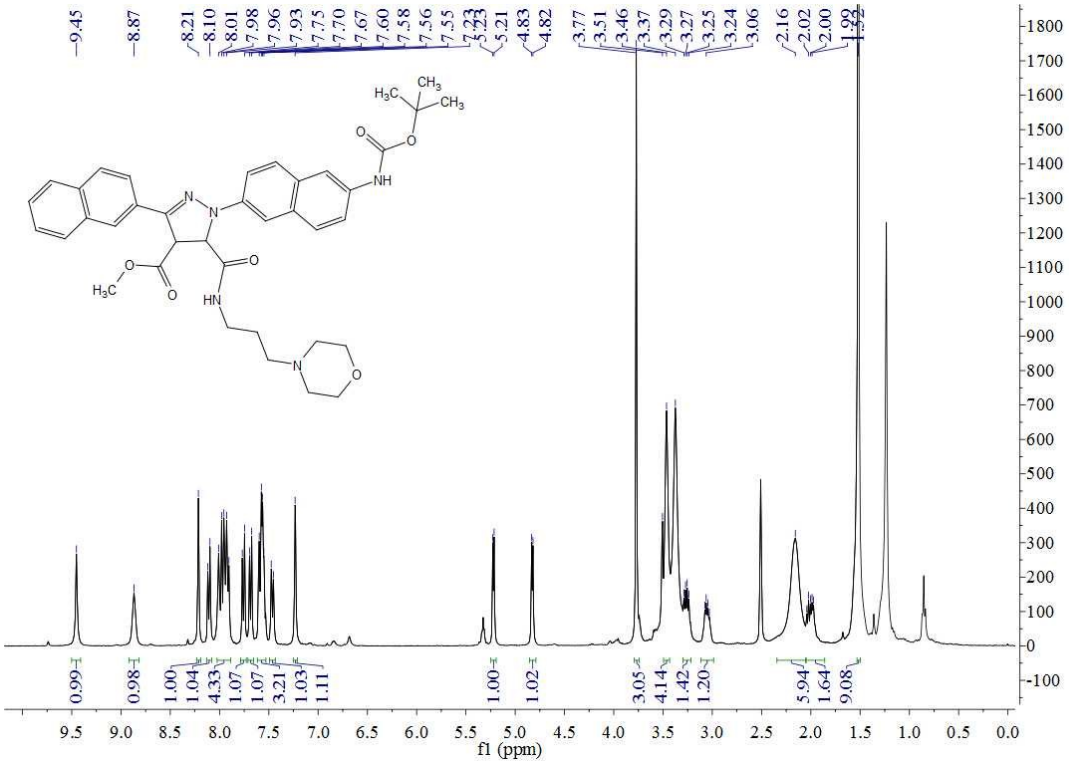
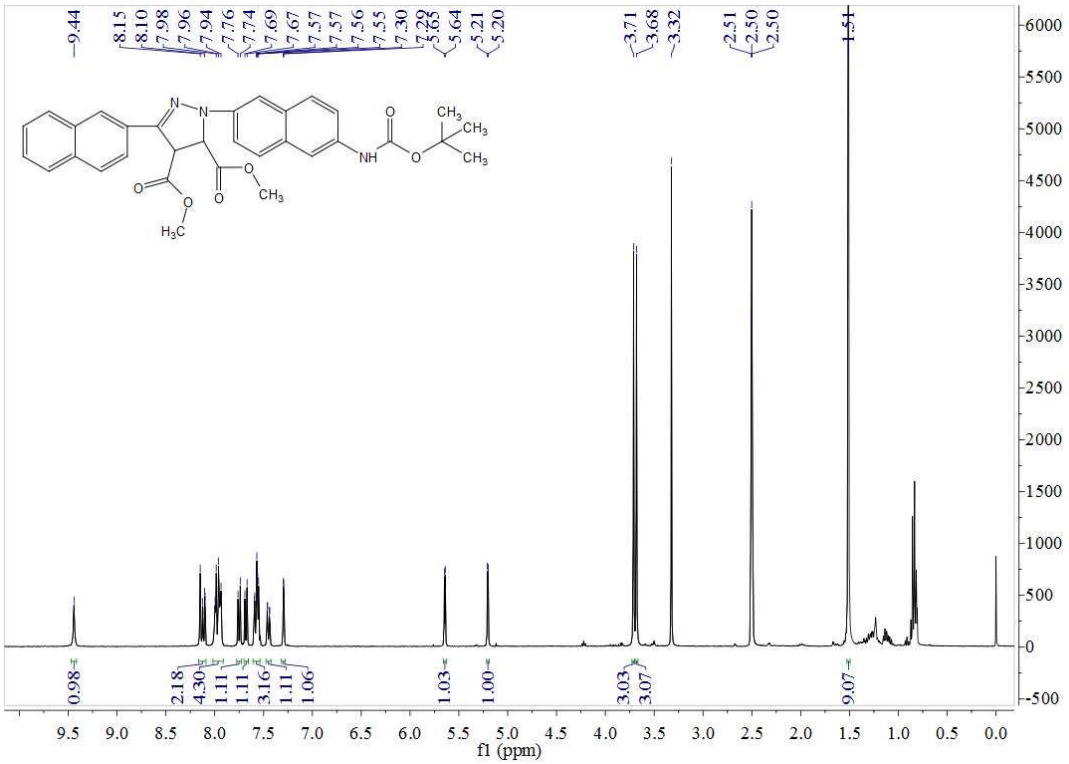
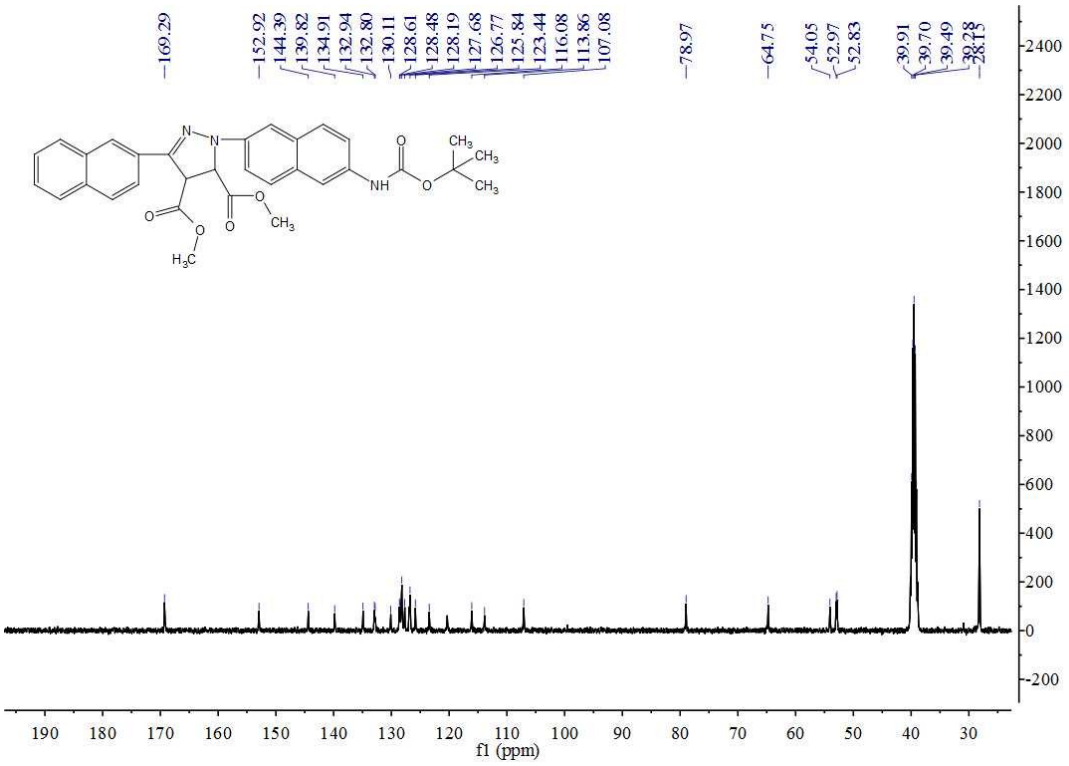
2.00
f1 (ppm)


166.08
163.50
136.92
129.46
77.43
77.11
76.79
66.98
57.72
53.62
52.18
39.73
24.43
f1 (ppm)


f1 (ppm)


f1 (ppm)


9.44
8.15
8.10
7.98
7.96
7.94
7.76
7.74
7.69
7.67
7.57
7.57
7.56
7.55
7.30
5.65
5.64
5.21
5.20
3.71
3.68
3.32
2.51
2.50
2.50
1.51
0.98
2.18
4.30
1.11
1.11
3.16
1.11
1.06
1.03
1.00
3.03
3.07
9.07
f1 (ppm)


169.29
152.92
144.39
139.82
134.91
132.94
132.80
130.11
128.61
128.48
128.19
127.68
126.77
125.84
123.44
116.08
113.86
107.08
78.97
64.75
54.05
52.97
52.83
39.91
39.70
39.49
39.28
28.15
f1 (ppm)