Supporting Information

Fluorescent Light-Up Probe with Aggregation-Induced Emission Characteristics for In Vivo Imaging of Cell Apoptosis

Haibin Shi, [†a] *Na Zhao,* [†b] *Dan Ding,*[a] *Jing Liang,*[a] *Ben Zhong Tang**[b,c] *and Bin Liu**[a,d]

[a] Department of Chemical & Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, 117576
[b] Department of Chemistry, Division of Biomedical Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China.
[c] SCUT–HKUST Joint Research Laboratory, Guangdong Innovative Research Team, South China University of Technology, Guangzhou 510640, China.
[d] Institute of Materials Research Engineering, 3 Research Link, 117602, Singapore
[*]Corresponding author (Email: cheliub@nus.edu.sg; tangbenz@ust.hk)
[†] Dr. H. Shi and Dr. N. Zhao contributed equally to this work.

Experimental Section

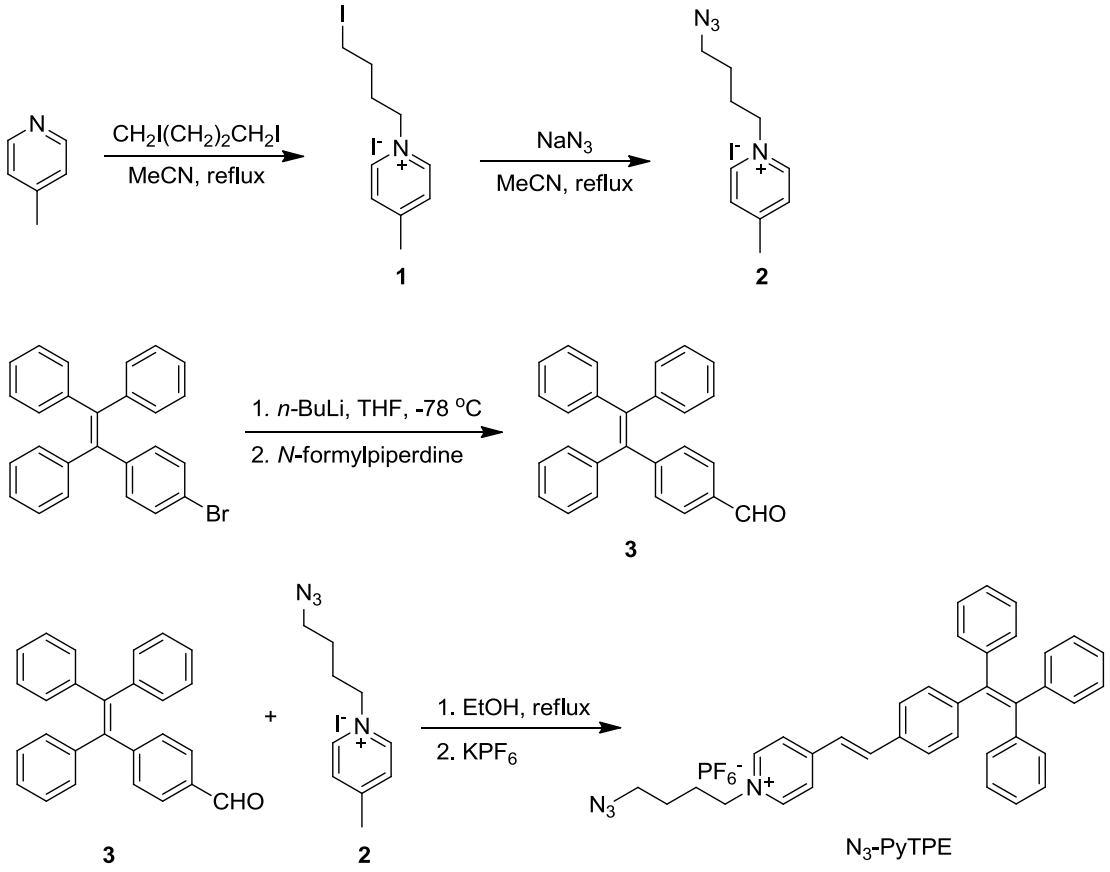
Materials.

Lysozyme, cathepsin B, pepsin, pepain and trypsin were purchased from Sigma. Recombinant human caspase-3, caspase-7 and caspase-1 were purchased from R&D Systems. Inhibitor 5-[(S)-(+)-2-(methoxymethyl)pyrrolidino]sulfonylisatin was purchased from Calbiochem. Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb (#9664) was purchased from Cell Signaling. Mouse anti-rabbit IgG-FITC was purchased from Santa Cruz. Annexin V-Alexa Fluor was purchased from Invitrogen. Fetal bovine serum (FBS) and trypsin-EDTA solution were purchased from Gibco (Lige Technologies, AG, Switzerland). Staurosporine was purchased from Biovision. Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation, Breford, USA). MCF-7 breast cancer cell line was provided by American Type Culture Collection. Tetrahydrofuran (THF) was distilled from sodium benzophenoneketyl immediately prior to use. *n*-butyllithium, copper(II) sulfate, sodium ascorbate, dimethyl sulfoxide (DMSO), piperazine-*N,N′*-bis(2-ethanesulfonic acid (PIPES),

ethylenediaminetetraacetic acid (EDTA), 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonic acid (CHAPS) and solvents were all purchased from Sigma-Aldrich and used as received without further purification. Other chemicals were purchased from Aldrich and used as received without further purification.

Characterization.

^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. Chemical shifts were reported in parts per million (ppm) referenced with respect to residual solvent ($CDCl_3$ = 7.26 ppm, $(CD_3)_2SO$ = 2.50 ppm or tetramethylsilane $Si(CH_3)_4$ = 0 ppm). High-resolution mass spectra (HRMS) were recorded on a Finnigan MAT TSQ 7000 Mass Spectrometer System operating in a MALDI-TOF mode. The HPLC profiles and ESI mass spectra were acquired using a Shimadzu IT-TOF. A 0.1% TFA/H_2O and 0.1% TFA/acetonitrile were used as eluents for all HPLC experiments. The flow rate was 0.6 mL/min for analytical HPLC and 3 mL/min for preparative HPLC. UV-vis absorption spectra were taken on a Milton Ray Spectronic 3000 array spectrophotometer. Photoluminescence (PL) spectra were measured on a Perkin-Elmer LS 55 spectrofluorometer. All PL spectra were measured with an excitation wavelength of 405 nm. Average particle size and size distribution of N_3-PyTPE was determined by laser light scattering (LLS) with particle size analyzer (90 Plus, Brookhaven Instruments Co. USA) at a fixed angle of 90^{o} at room temperature, excited using semiconductor Laser diode at laser wavelength of 659 nm and power of ~35 mW. The cells were imaged by confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena, Germany) with imaging software (Fluoview FV500). The images were analyzed by Image J 1.43× program (developed by NIH, http://rsbweb.nih.gov/ij/).



Scheme S1. The synthetic route of N_3-PyTPE.

1-(4-Iodopropyl)-4-methylpyridinium iodide (1): 4-Picoline (460.00 mg, 5 mmol) and 1,4-diiodopropane (5.92 g, 20 mmol) were refluxed in acetonitrile (10 mL) for 4 h. After cooling to room temperature the solvent was removed under vacuum. Ethyl acetate was added to precipitate the crude product, which was recrystallized from ethanol to afford **1** as white solid (1.75 g, 87%). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.91 (d, J = 6.4 Hz, 2H), 8.00 (d, J = 6.4 Hz, 2H), 4.56 (t, J = 6.8 Hz, 2H), 3.28 (t, J = 6.8 Hz, 2H), 2.60 (s, 3H), 2.00–1.93 (m, 2H), 1.78–1.71 (m, 2H). ^{13}C NMR (100 MHz, MeOH-d_4), δ (ppm): 159.3, 142.8, 128.0, 58.9, 31.1, 29.0, 20.1, 3.0. HRMS (MALDI-TOF): m/z 275.9458 [$(M–I)^+$, calcd 276.0249].

1-(4-Azidepropyl)-4-methylpyridinium iodide (2): 1-(4-Iodopropyl)-4-methyl pyridinium iodide (403.00 mg, 1.00 mmol) and NaN_3 (162.50 mg, 2.50 mmol) were refluxed in acetonitrile (20 mL) for 24 h. After cooling to room temperature the solution was filtered and

solvent was evaporated under vacuum. Methylene chloride (20 mL) was added, and the resulting brown solution was filtered again. Evaporation of the solvent yielded product **2** as brown viscous oil (286.20 mg, 90%). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.94 (d, J = 6.4 Hz, 2H), 8.00 (d, J = 6.0 Hz, 2H), 4.56 (t, J = 6.2 Hz, 2H), 3.38 (t, J = 6.8 Hz, 2H), 2.60 (s, 3H), 1.98–1.90 (m, 2H), 1.54–1.47 (m, 2H). ^{13}C NMR (100 MHz, MeOH-d_4), δ (ppm): 159.2, 142.9, 127.9, 59.4, 49.8, 27.5, 24.3, 20.3. HRMS (MALDI-TOF): m/z 190.9841 [$(M–I)^+$, calcd 191.1297].

4-(1,2,2-Triphenylvinyl)benzaldehyde (3): This compound was prepared according to the previous publications.[1] ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 9.90 (s, 1H), 7.61 (d, J = 8.2 Hz, 2H), 7.19 (d, J = 8.2 Hz, 2H), 7.11 (m, 9 H), 7.02 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 191.2, 149.9, 142.4, 142.3, 142.2, 139.1, 133.6, 131.3, 130.7, 130.6, 128.5, 127.3, 127.1, 126.4, 126.2, 126.1. HRMS (MALDI-TOF): m/z 360.1520 [M^+, calcd 360.1514].

1-(4-Azidepropyl)-4-methylpyridinium hexafluorophosphate (N_3-PyTPE): A solution of **3** (200 mg, 0.55 mmol) and **2** (169 mg, 0.55 mmol) in dry EtOH (15 mL) was refluxed under nitrogen for 48 h. After cooling to ambient temperature, the solvent was evaporated under reduced pressure. The solid was dissolved in acetone (5 mL) and a saturated aqueous solution of KPF_6 (5 mL) was then added. After stirring for 30 min, the solution was evaporated to dryness. The residue was purified by silica gel column chromatography using dichloromethane and acetone mixture (v:v = 5:1) as eluent to give N_3-PyTPE as a yellow product (119 mg, 32%). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.92 (t, J = 6.8 Hz, 2H), 8.18 (t, J = 6.8 Hz, 2H), 7.90 (t, J = 16.4 Hz, 1H), 7.51 (t, J = 8.4 Hz, 2H), 7.42 (d, J = 16.0 Hz, 1H), 7.11–7.18 (m, 9H), 7.06 (d, J = 8.4 Hz, 2H), 6.96–7.02 (m, 6H), 4.50 (t, J = 7.2 Hz, 2H), 3.39 (t, J = 6.8 Hz, 2H), 1.92–1.99 (m, 2H), 1.50–1.57 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6), δ (ppm): 152.70, 145.39, 144.06, 142.79, 142.71, 142.55, 141.43, 140.23, 139.73, 133.09, 131.26, 131.26, 130.53, 130.51, 130.43, 127.78, 127.66, 127.50, 126.68, 126.60,

126.56, 123.63, 122.98, 59.00, 49.82, 27.68, 24.75. HRMS (MALDI-TOF): *m*/*z* 533.2691 [$(M–PF_6)^+$, calcd 533.2728].

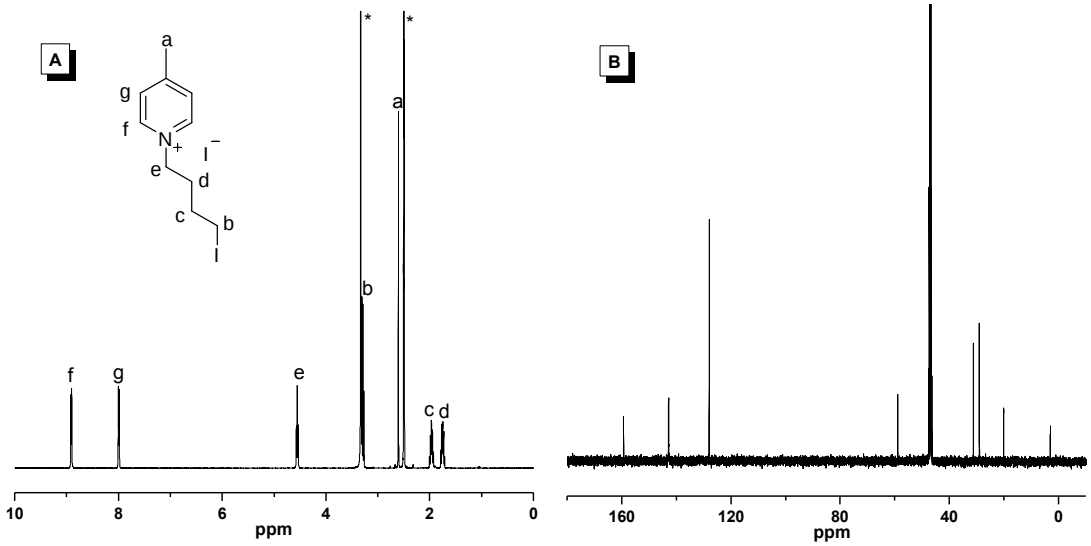


Fig. S1 (A) ^{1}H (DMSO-d_6) and (B) ^{13}C NMR (MeOH-d_4) spectra of compound **1.**

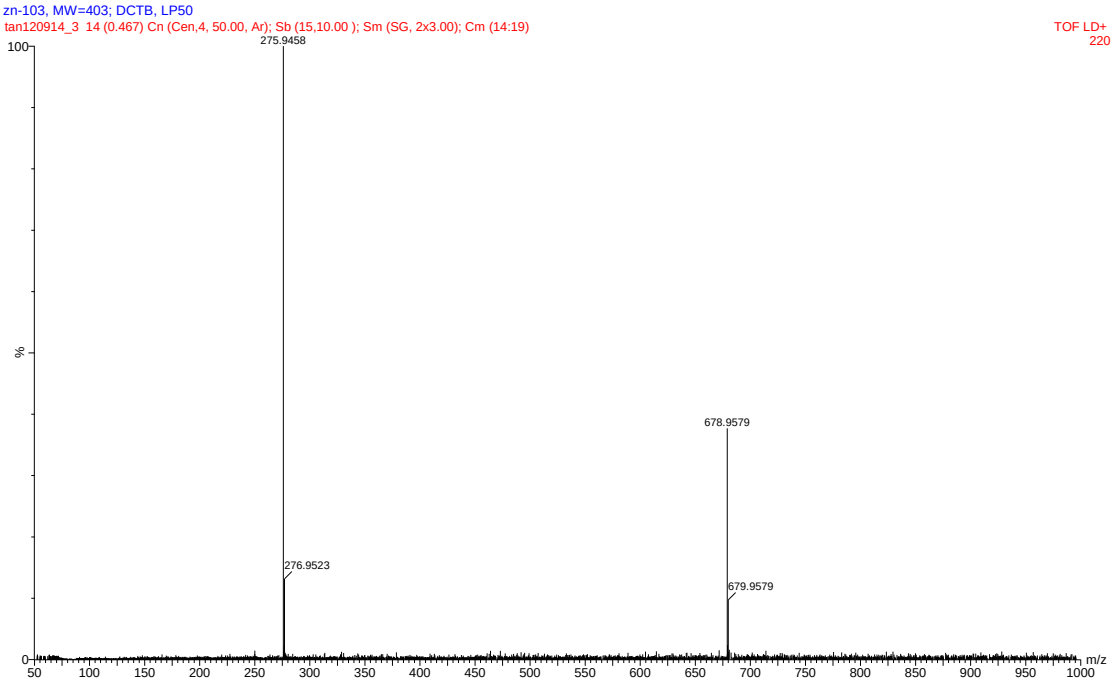


Fig. S2 High resolution mass spectrum (MALDI-TOF) of compound **1**.

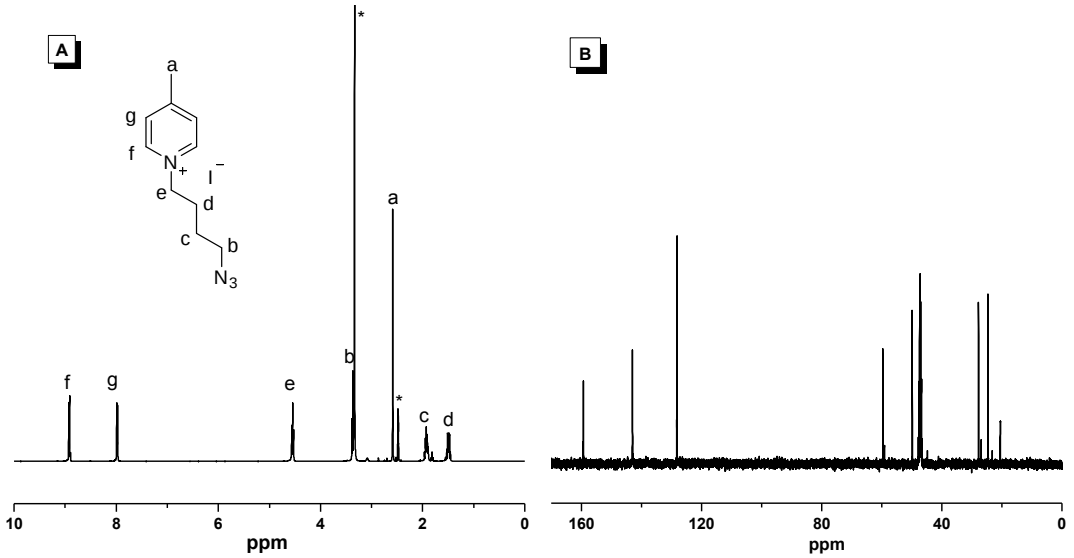


Fig. S3 (A) ^{1}H (DMSO-d_6) and (B) ^{13}C NMR (MeOH-d_4) spectra of compound **2.**

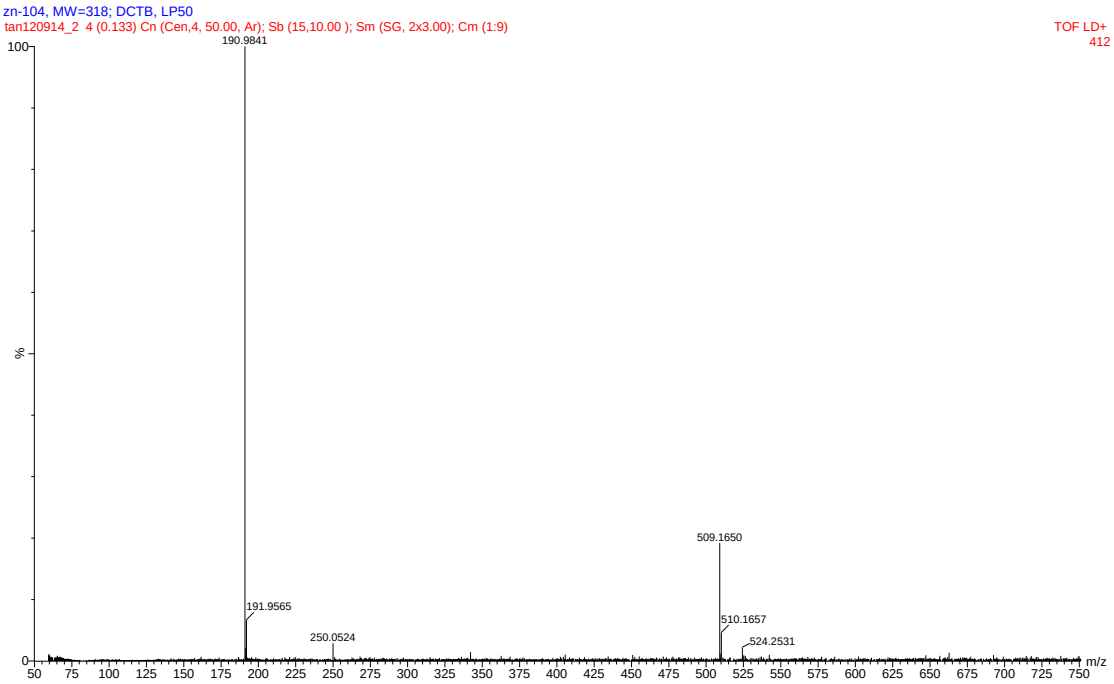


Fig. S4 High resolution mass spectrum (MALDI-TOF) of compound **2**.

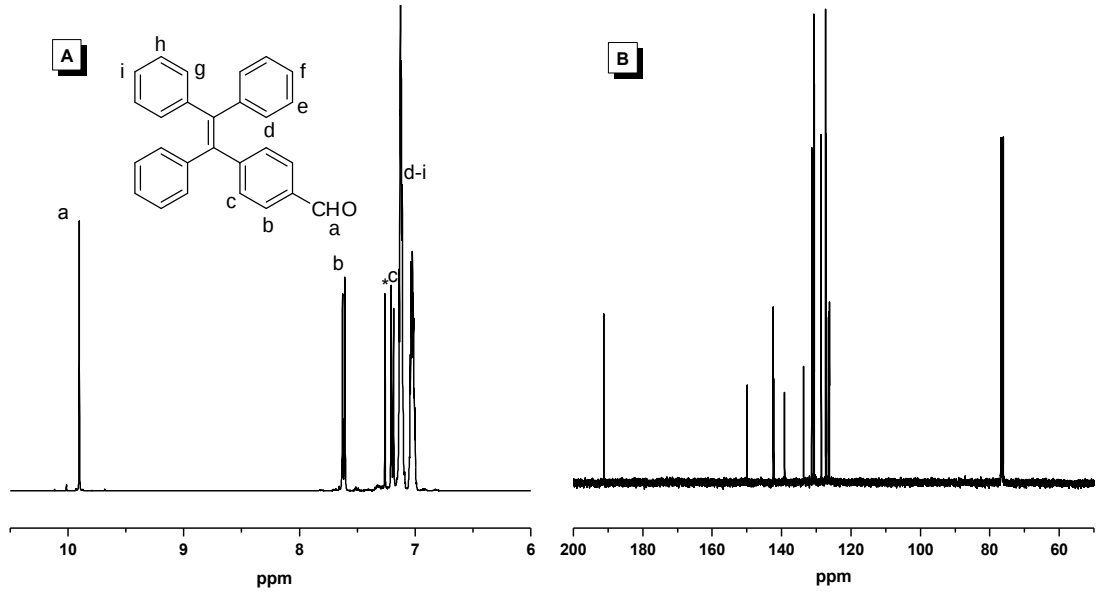


Fig. S5 (A) ^{1}H ($CDCl_3$) and (B) ^{13}C NMR ($CDCl_3$) spectra of compound **3.**

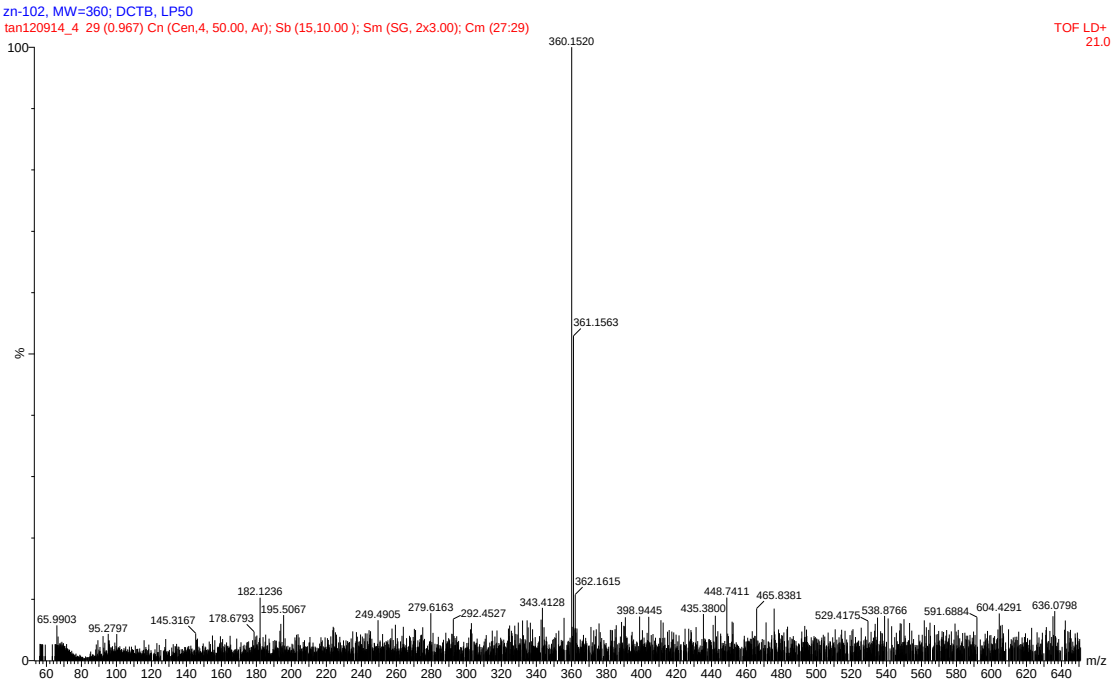


Fig. S6 High resolution mass spectrum (MALDI-TOF) of compound **3**.

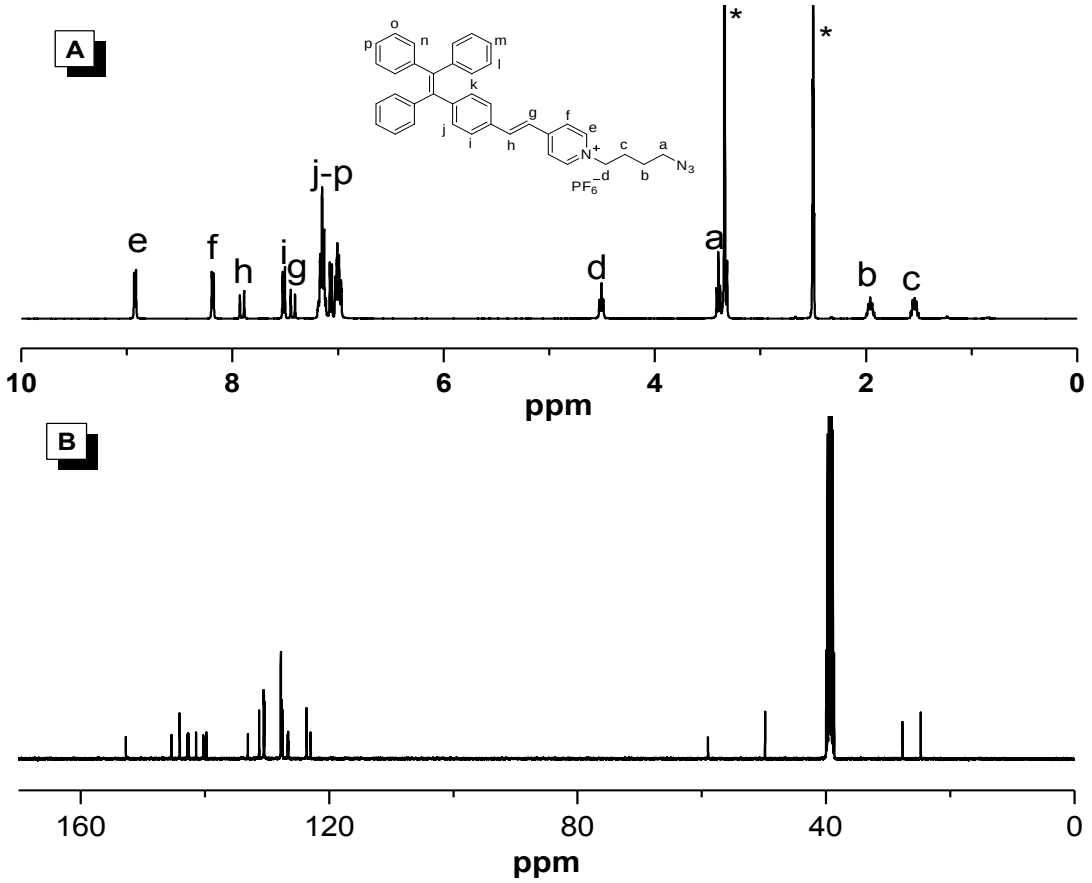


Fig. S7 (A) ^{1}H (DMSO-d_6) and (B) ^{13}C NMR (DMSO-d_6) spectra of compound N_3-PyTPE.

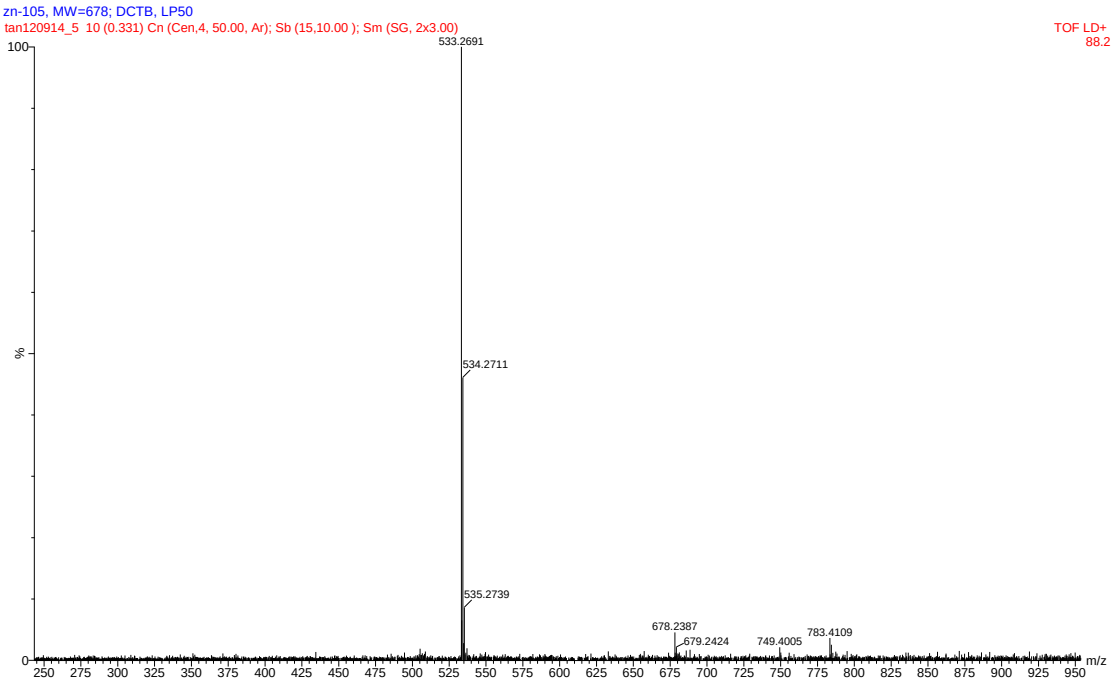
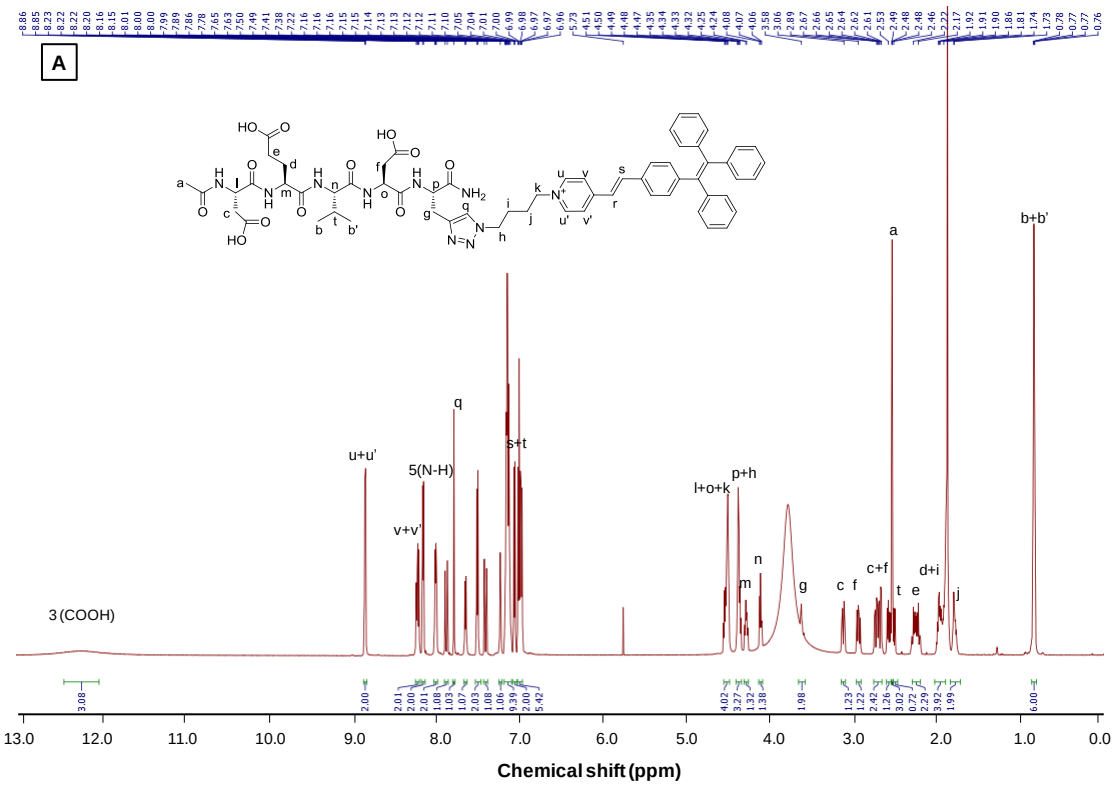


Fig. S8 High resolution mass spectrum (MALDI-TOF) of compound N_3-PyTPE.

"Click" Synthesis of Probe Ac-DEVD-PyTPE. Alkyne-functionalized Ac-DEVD (9.3 mg, 15 μmol) and N_3-PyTPE (12.3 mg, 18 μmol) were dissolved in 50 μL of DMSO. A mixture of DMSO/H_2O solution (v/v = 1/1; 1.0 mL) was subsequently added and the reaction was shaken for a few minutes to obtain a clear solution. The "click" reaction was initiated by sequential addition of catalytic amounts of sodium ascorbate (1.2 mg, 6.0 μmol) and $CuSO_4$ (4.8 mg, 3.0 μmol). The reaction was continued with shaking at room temperature for another 24 h. The final product was purified by prep-HPLC and further characterized by NMR and HRMS. ^{1}H NMR (600 MHz, DMSO-d_6), δ (TMS, ppm): 12.24 (s, 3H), 8.85 (d, J = 6.7 Hz, 2H), 8.22 (dd, J = 11.5 Hz, 7.4, 2H), 8.15 (d, J = 6.7 Hz, 2H), 8.00 (dd, J = 7.8, 2.8 Hz, 2H), 7.87 (d, J = 16.3 Hz, 1H), 7.78 (s, 1H), 7.64 (d, J = 8.1 Hz, 1H), 7.50 (d, J = 8.3 Hz, 2H), 7.40 (d, J = 16.3 Hz, 1H), 7.22 (s, 1H), 7.17-7.08 (m, 9H), 7.05 (d, J = 8.3 Hz, 2H), 7.01-6.95 (m, 5H), 4.52-4.45 (m, 4H), 4.33 (dt, J = 14.0, 7.2 Hz, 3H), 4.25 (dd, J = 13.4, 8.3 Hz, 1H), 4.09-4.05 (m, 1H), 3.61-3.53 (m, 2H), 3.07 (dd, J = 14.9, 4.7 Hz, 1H), 2.89 (dd, J = 14.9, 8.9 Hz, 1H), 2.65 (m, 2H), 2.52 (dd, J = 16.8, 7.9 Hz, 1H), 2.49-2.47 (m, 3H), 2.45 (d, J = 8.4 Hz, 1H), 2.19 (m, 2H), 1.90 (m, 4H), 1.78-1.66 (m, 2H), 0.77 (dd, J = 6.4, 4.9 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6), δ (TMS, ppm): 174.5, 172.7, 172.4, 172.2, 171.6, 170.0, 158.6, 158.4, 153.3, 146.0, 144.6, 143.5, 143.3, 143.2, 142.1, 140.8, 140.4, 133.7, 131.9, 131.2, 131.1, 131.9, 128.4, 128.3, 128.1, 127.3, 127.2, 124.3, 123.6, 123.3, 67.5, 59.5, 58.3, 52.9, 52.5, 50.2, 50.1, 48.9, 36.4, 36.1, 30.8, 30.5, 28.4, 28.1, 27.3, 26.8, 25.6, 22.9, 19.5, 18.4. HRMS (MALDI-TOF): m/z 1145.5104 ($[M]^+$, calcd 1145.5091). The HPLC condition is: 20-100% B for 10 min, then 100% B for 2 min, 20% B for 5 min (Solvent A: 100% H_2O with 0.1% TFA; Solvent B: 100% CH_3CN with 0.1% TFA).

General Procedure for Enzymatic Assay. DMSO stock solutions of Ac-DEVD-PyTPE were diluted with caspase-3/-7 assay buffer (50 mM PIPES, 100 mM NaCl, 1 mM EDTA, 0.1% w/v CHAPS, 25% w/v sucrose, pH = 7.2) to make 10 μM working solutions. 5 μL of the recombinant caspase-3 and -7 (~0.05 μg/μL stock solution in assay buffer) was added into the

above working solution. The reaction mixture was incubated at room temperature for 60 min and was then diluted to a total of 600 μL with deionized water for photoluminescence measurement. The solution was excited at 405 nm and the emission was collected from 430 to 800 nm.



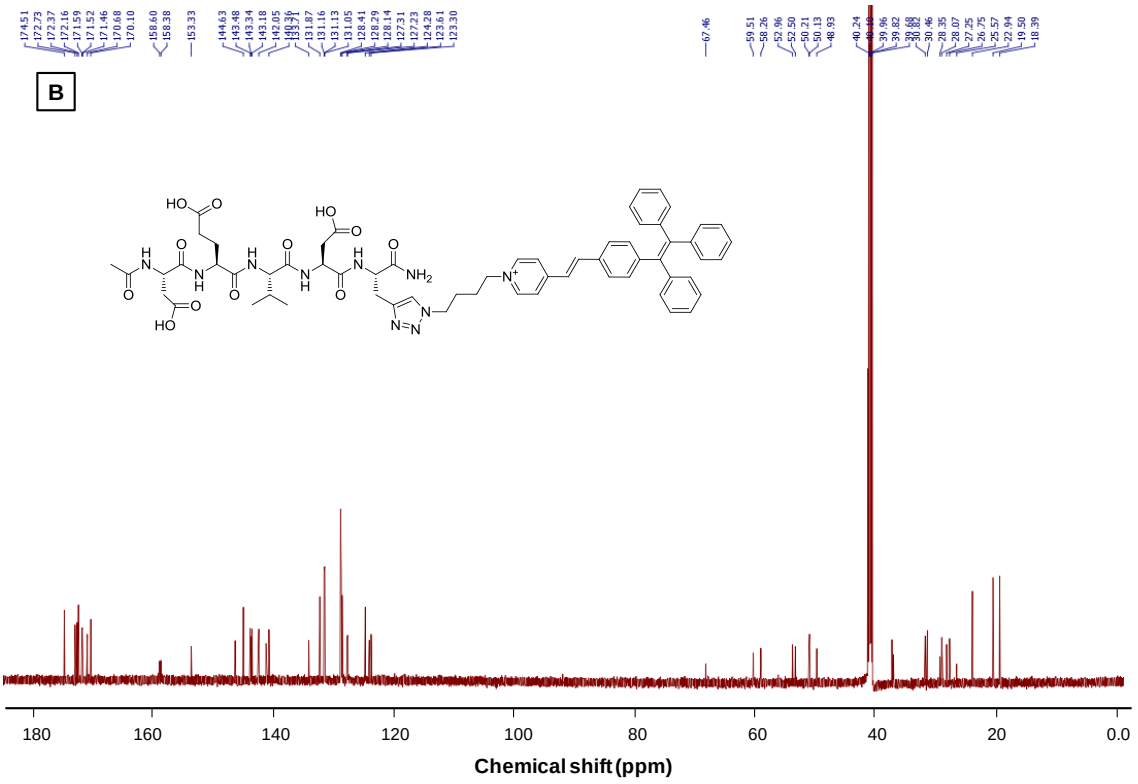


Fig. S9 (A) ^{1}H and (B) ^{13}C NMR spectra of Ac-DEVD-PyTPE in DMSO-d_6.

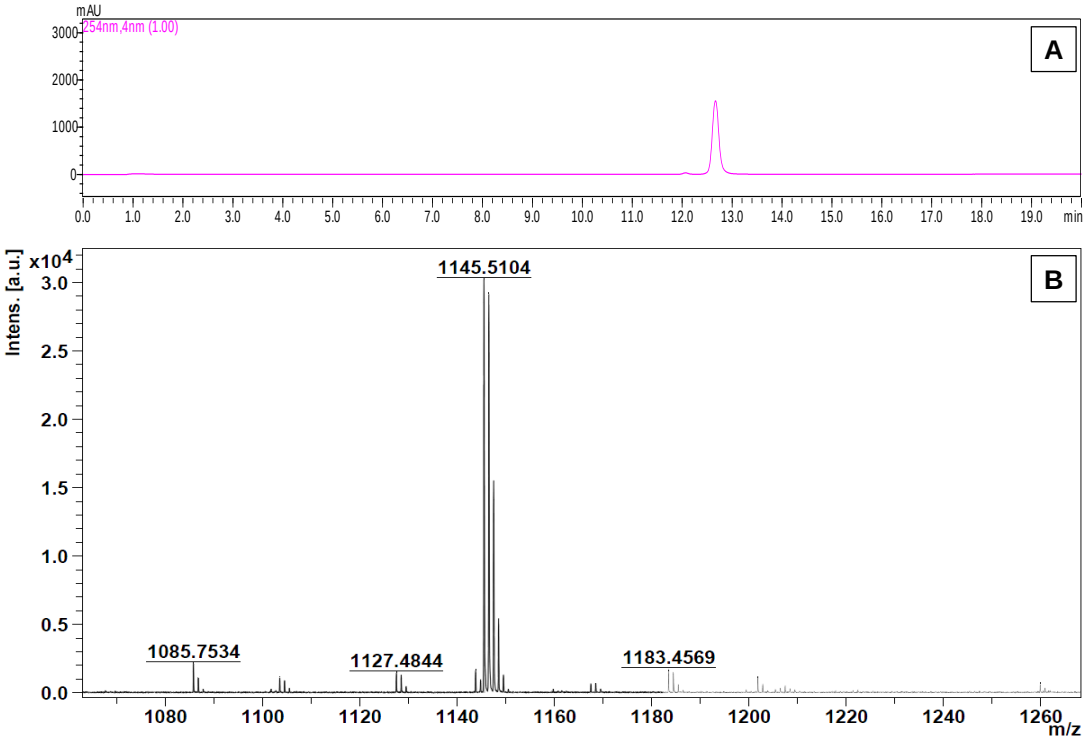


Fig. S10 (A) HPLC and (B) High Resolution Mass (HRMS) Characterization of Ac-DEVD-PyTPE.

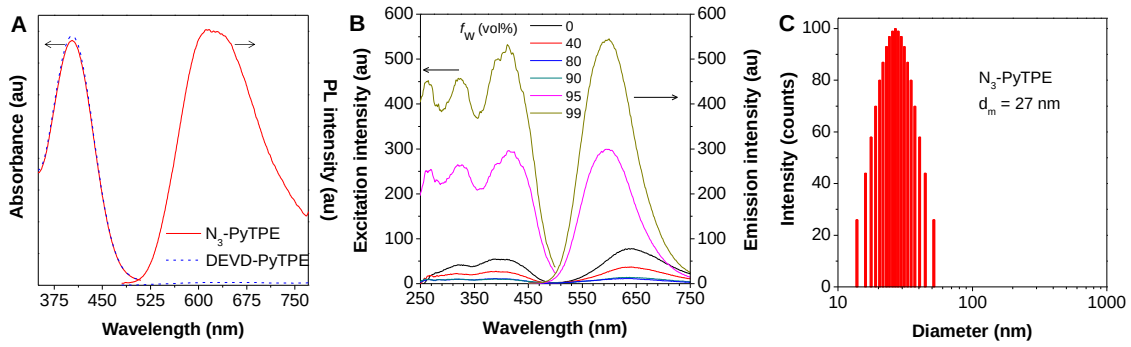


Fig. S11 (A) UV-vis absorption and PL spectra of N_3-PyTPE (red, solid line) and Ac-DEVD-PyTPE (blue, dashed line) in DMSO/water (v/v = 1/99). [N_3-PyTPE] = [Ac-DEVD-PyTPE] = 10 μM. λ_{ex} = 405 nm. (B) Excitation and PL spectra of N_3-PyTPE in DMSO/water mixtures with different water fractions (f_w). (C) Hydrodynamic diameters of N_3-PyTPE in DMSO/water (v/v = 1/99).

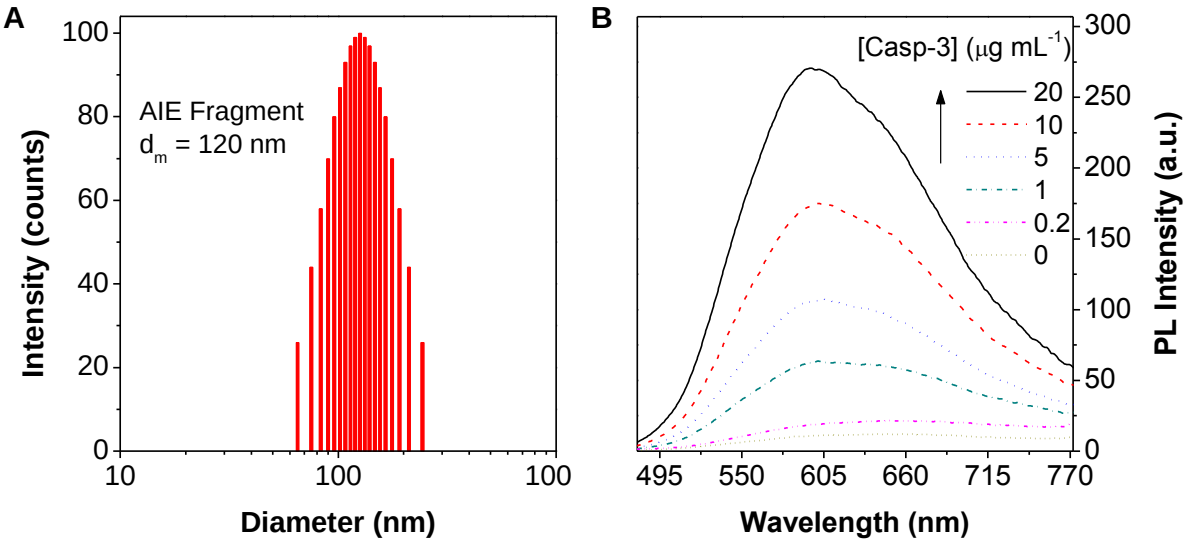
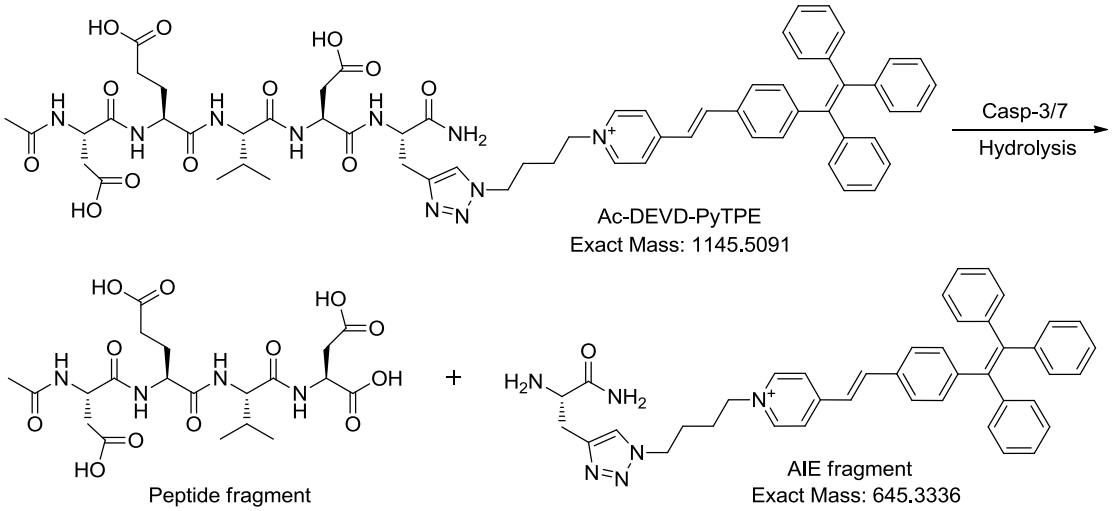


Fig. S12 (a) Hydrodynamic diameters of the AIE fragment of Ac-DEVD-PyTPE after caspase-3 cleavage in PIPES buffer obtained from LLS. (b) PL spectra of Ac-DEVD-PyTPE in the presence of different amounts of caspase-3 (0, 0.2, 1, 5, 10 and 20 μg mL^{-1}), [Ac-DEVD-PyTPE] = 10 μM, λ_{ex} = 405 nm.



Scheme S2. Caspase-3/7 catalyzed hydrolysis of Ac-DEVD-PyTPE.

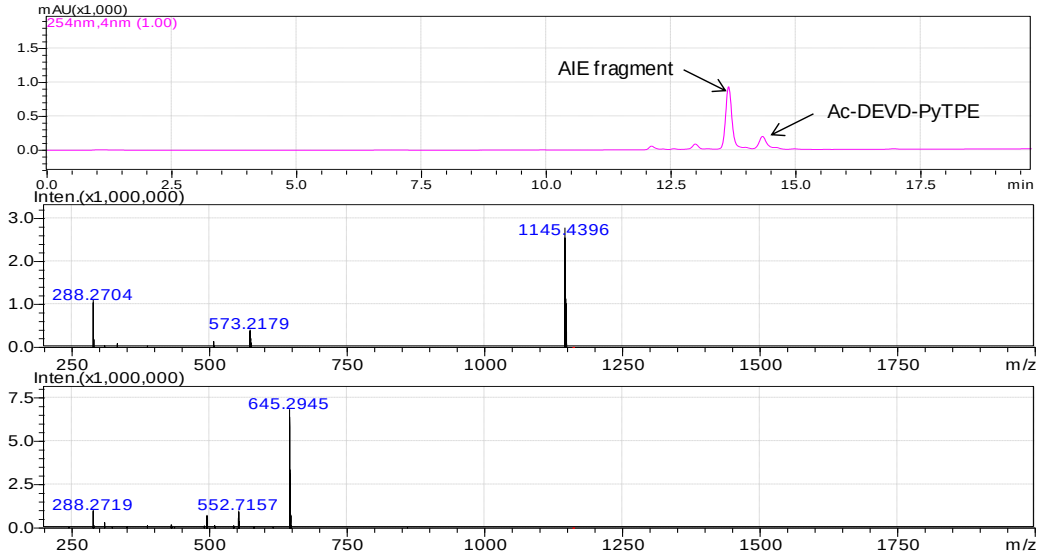


Fig. S13 The caspase-catalyzed hydrolysis of Ac-DEVD-PyTPE monitored by LC-MS.

Cytotoxicity of Ac-DEVD-PyTPE. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were used to assess the metabolic activity of MCF-7 cancer cells or study the cytotoxicity of Ac-DEVD-PyTPE. MCF-7 cells were seeded in 96-well plates (Costar, IL, USA) at an intensity of 4×10^4 cells mL^{-1}. After 24 h incubation, the medium was replaced by the Ac-DEVD-PyTPE suspension at concentrations of 5, 10 and 20 μM, and the cells were then incubated for 12, 24 and 48 h. After the designated time intervals, the wells were washed twice with 1 × PBS buffer, and 100 μL of freshly prepared MTT (0.5 mg mL^{-1})

solution in culture medium was added into each well. The MTT medium solution was carefully removed after 3 h incubation in the incubator at 37 °C. DMSO (100 μL) was then added into each well and the plate was gently shaken to dissolve all the precipitates formed. The absorbance of MTT at 570 nm was monitored by microplate reader (GeniosTecan). Cell viability was expressed by the ratio of absorbance of the cells incubated with Ac-DEVD-PyTPE suspension to that of the cells incubated with culture medium only.

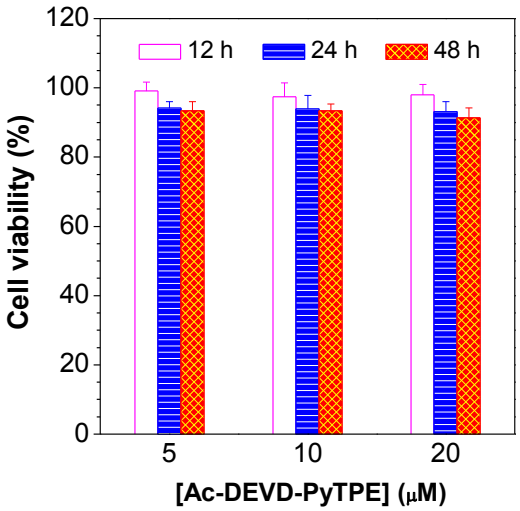


Fig. S14 Metabolic viability of MCF-7 cancer cells after incubation with Ac-DEVD-PyTPE at concentration of 5, 10 and 20 μM for 12, 24 and 48 h.

Cell Culture. MCF-7 cell lines were provided by American Type Culture Collection. MCF-7 breast cancer cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Scientific) and maintained in a humidified incubator at 37 ℃ with 5% CO_2. Before experiment, the cells were pre-cultured until confluence was reached.

Microscopy Imaging. MCF-7 cells were cultured in the chambers (LAB-TEK, Chambered Coverglass System) at 37 °C. After 80% confluence, the adherent cells were washed twice with 1 × PBS buffer. The Ac-DEVD-PyTPE solution (3 μM) was then added to the chamber. After incubation for 2 h at 37 °C, the cells were washed once with 1 × PBS buffer, and treated with 3 μM apoptosis inducers (staurosporine, sodium ascorbate and cisplatin) for another 2 h.

The cells were washed one time with 1× PBS buffer. For co-localization with Annexin V-Alexa Fluor, the cells were further incubated with a mixture of Annexin V-Alexa Fluor/FBS-free DMEM (1:799 by vol) for 15 min at room temperature, washed with 1× PBS buffer once. The cells were then kept in fresh FBS-free DMEM for cell imaging. For co-localization with active caspase-3 antibody, the cells were first fixed for 15 min with 3.7% formaldehyde in 1× PBS at room temperature, washed twice with cold 1× PBS again, and permeabilized with 0.1% Triton X-100 in 1× PBS for 10 min. The cells were then blocked with 2% BSA in 1× PBS for 30 min, washed twice with 1× PBS. The cells were subsequently incubated with a mixture of anti-caspase-3 antibody/1× PBS (1:99 by vol) for 1 h at room temperature (or overnight at 4 ℃), washed with 1× PBS buffer for once and then incubated with mouse anti-rabbit IgG-FITC (0.8 μg mL^{-1}) in 1× PBS for 1 h, following by washing with 1× PBS again. The imaging was done with confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena, Germany) with imaging software (Fluoview FV500). The images were analyzed by Image J 1.43× program.

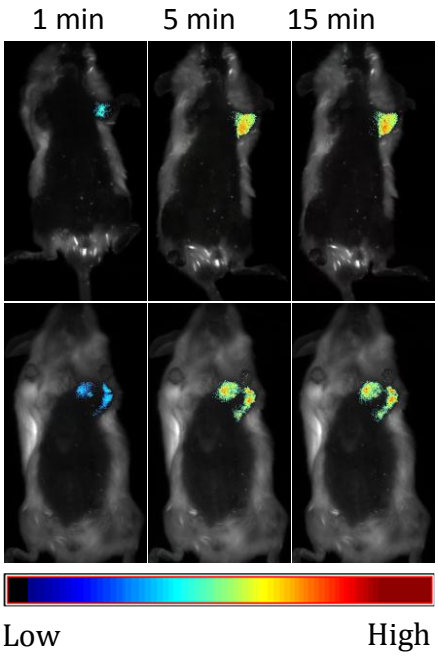


Fig. S15 In vivo fluorescence images of subcutaneous C6 tumor-bearing mice after intratumoral injection of Ac-DEVD-PyTPE with or without pretreatment of staurosporine (STS) for 12 h before the probe injection using normal mice as control.

Real-time Imaging of Cell Apoptosis. MCF-7 cells were cultured in the 8-well chambers (LAB-TEK, Chambered Coverglass System) at 37 ^{o}C. After 80% confluence, the adherent cells were washed twice with 1× PBS buffer. The Ac-DEVD-PyTPE solution (3 μM) was then added to the chamber. After 2 h incubation at 37 ^{o}C, the cells were washed twice with 1× PBS buffer, and staurosporine (3 μM) was added to the chambers. The chambers were then placed on the confocal microscopy platform immediately before image collection. The fluorescence images were acquired every 15 min.

Detection of Apoptosis in Mice. All animal experiments were performed in compliance with guidelines set by the Institutional Animal Care and Use Committee (IACUC), Singapore General Hospital. In brief, C6 glioma cells (1×10^6 cells in 0.1 mL of culture medium) were subcutaneously injected into the flank of mice. Three mice were used for each group. STS (100 μL, 3 μM) was then intravenously injected into the subcutaneous C6 tumor. After 12 h incubation, Ac-DEVD-PyTPE (100 μL, 3 μM) was directly injected into tumor. After designated time intervals post injection, the mice were imaged using an IVIS Spectrum imaging system (Caliper Life Sciences) while under anesthesia. The fluorescence images were recorded with 1 second exposure using a filter 610/20 nm upon excitation at 405 nm. Scans were carried out at 1, 5 and 15 min. The autofluorescence was removed using the software of IVIS Spectrum imaging system.

Reference:

1. R. Hu, J. L. Maldonado, M. Rodriguez, C. Deng, C. K. W. Jim, J. W. Y. Lam, M. M. F. Yuen, G. Ramos-Ortiz and B. Z. Tang, *J. Mater. Chem.*, 2011, **22**, 232.