Supporting Information

A Self-immobilizing Near-infrared Fluorogenic Probe for Sensitive Imaging of Extracellular Enzyme Activity in vivo

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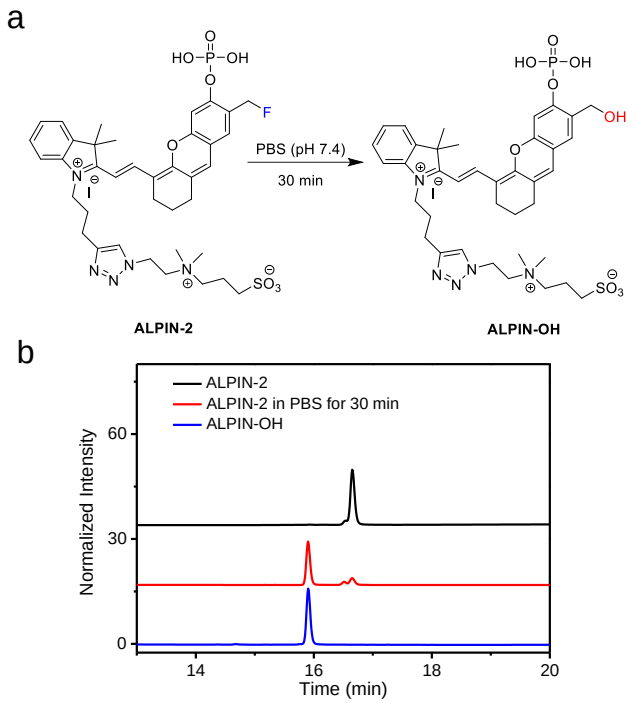


Figure S1. (a) Hydrolysis of ALPIN-2 in PBS (pH 7.4). (b) HPLC traces of ALPIN-2 (10 μM) before and after incubation in PBS at rt for 30 min.

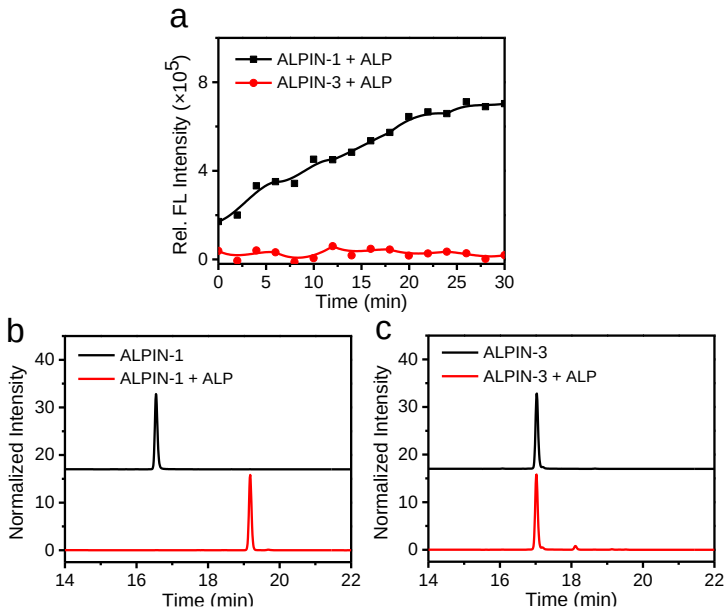


Figure S2. (a) Time course fluorescence intensity of ALPIN-1 or ALPIN-3 (10 μM) upon incubation with ALP (2 U/mL) in PBS (pH 7.4). (b) HPLC traces of ALPIN-1 and ALPIN-3 (10 μM) (c) before and after incubated with ALP (2 U/mL) at 37 ^{o}C for 30 min.

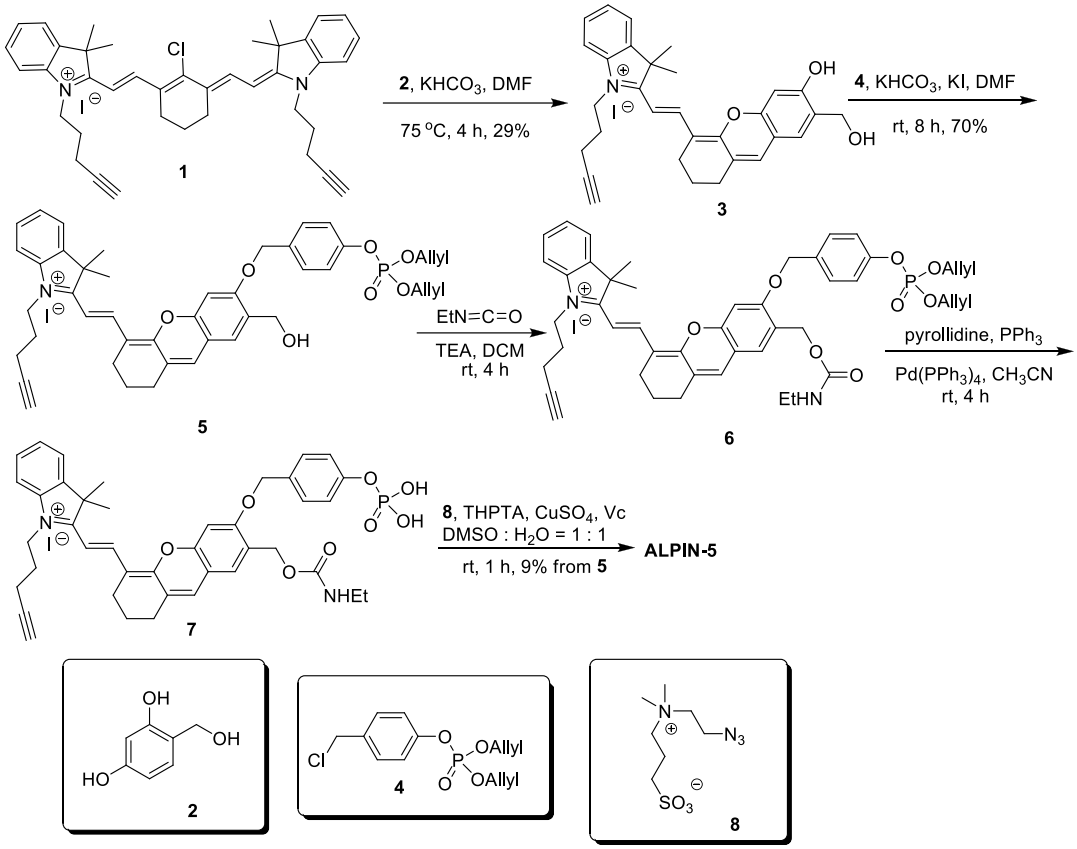


Figure S3. Synthesis of self-immobilizing probe ALPIN-5.

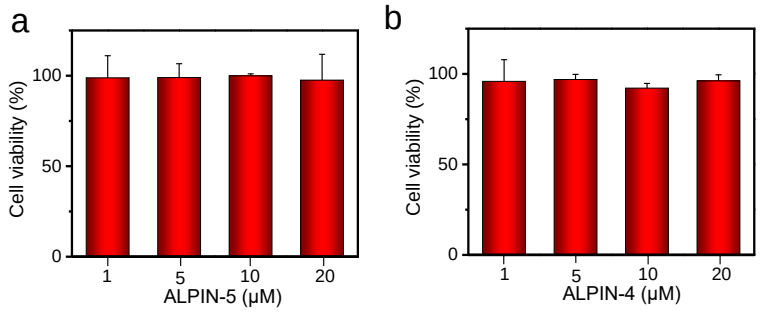


Figure S4. Test of cell viability. HeLa cells were incubated with ALPIN-5 (a) and ALPIN-4 (b) at 0, 1, 5, 10, 20 μM for 24 h, and the cell viability was determined by MTT assay. Error bars mean ± s.d. (n = 3 technical replicates).

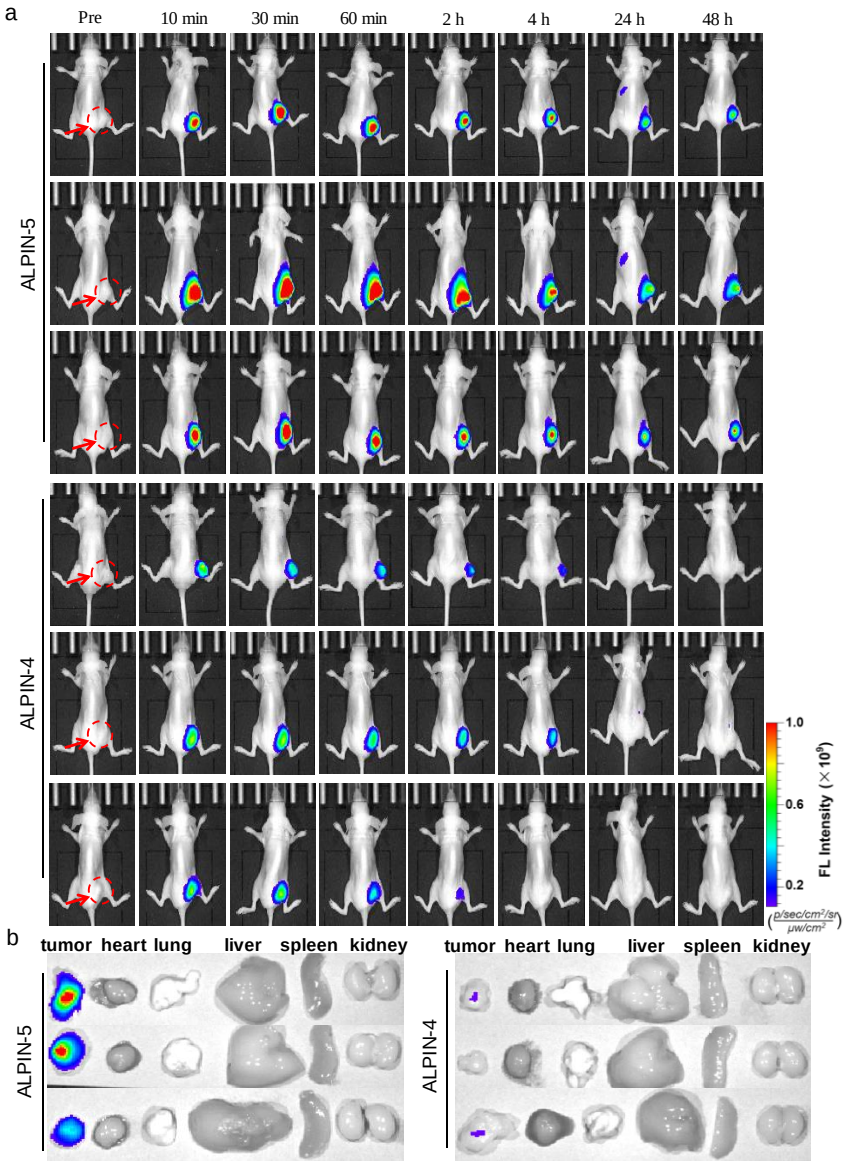


Figure S5. (a) Fluorescence images of ALP activity in HeLa tumor-bearing mice after i.t. injection of ALPIN-5 and ALPIN-4. (b) Fluorescence images of tumor and main organs at 48 h post injection. $\lambda_{ex}/\lambda_{em}$ = 660/710 nm..

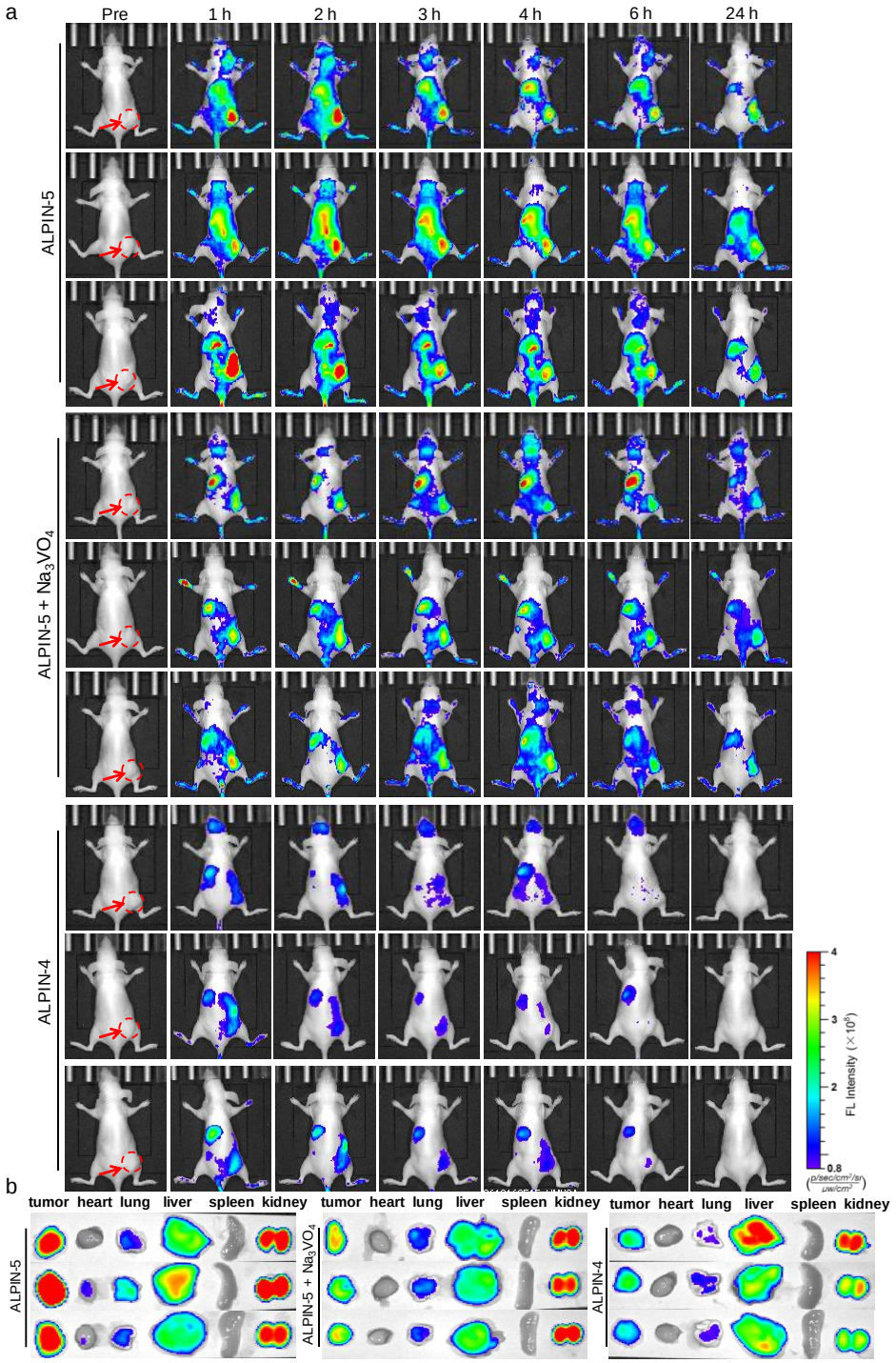


Figure S6. (a) Fluorescence images of endogenous ALP activity in HeLa tumor-bearing mice after i.v. injection of probes or together with i.t. injection of Na_3VO_4 (10 mM, 50 μL) before i.v. injection of ALPIN-5. (b) Fluorescence images of tumor and main organs at 2 h after i.v. injection of ALPIN-5, ALPIN-4, or ALPIN-5 together with i.t. injection of Na_3VO_4.

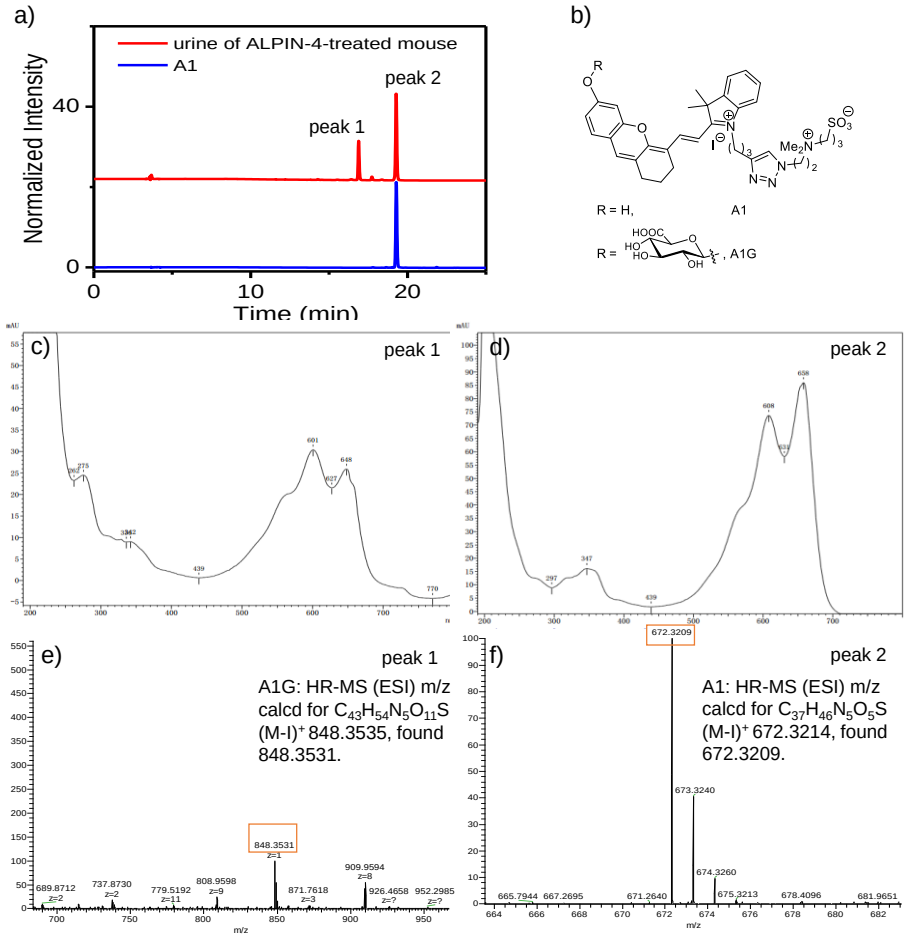


Figure S7. Analysis of urine from ALPIN-4-treated mouse at 0.5 h post injection. (a) HPLC trace of urine at 600 nm. (b) Chemical structures of A1 and A1G (c) UV-Vis spectrum of peak 1 (c) or peak 2 (d) at (a). (e) HR-MS (ESI) analysis of peak 1 (e) or peak 2 (f).

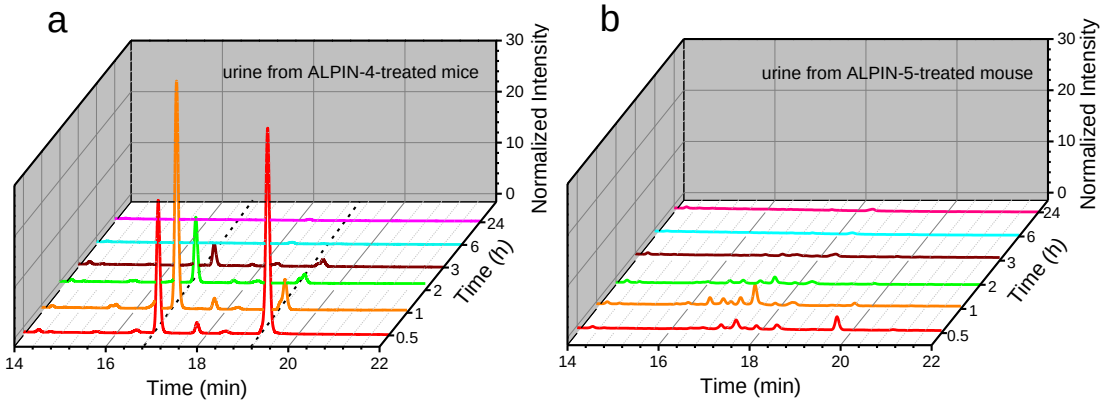


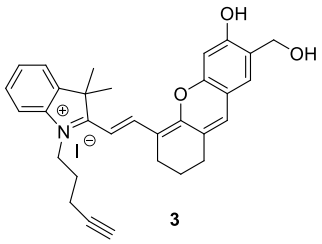
Figure S8. Representative HPLC traces of urine from ALPIN-4-treated mouse (a) or ALPIN-5-treated mouse (b) collected at 0.5 h, 1 h, 2 h, 3 h, 6 h, 24 h post-injection, respectively.

General information

Unless otherwise noted, all reagents were obtained commercially and used without further purification. The alkaline phosphatase (ALP from bovine intestinal mucosa) was purchased from Sigma-Aldrich and BSA (bovine serum albumin) from Aladdin. HeLa cells and HEK293 cells were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Matrigel™ Basement Membrane Matirx for living tumor inoculation in mice were purchased from BD (UAS).

The ^{1}H and ^{13}C NMR spectra were taken on Bruker nuclear magnetic resonance spectrometer (400 MHz for ^{1}H; 100 MHz for ^{13}C NMR; 150 MHz for ^{13}C NMR). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane (δ = 0.00 ppm). Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). High-resolution mass spectra (HRMS) were recorded on a Bruker micro-TOF-QII time of flight mass spectrometer with electrospray ionization. HPLC was performed on a Shimadzu HPLC System equipped with a LC-20AT gradient pump and an inline diode array UV-Vis detector. A reversed-phase C18 (Inertsil ODS-SP, 5 μm, 4.6 × 250 mm or phenomenex, 5 μm, 21.2 x 250 mm) column was used with a MeCN/H_2O gradient mobile phase containing 0.1% trifluoroacetic acid at a flow of 1 or 12 mL/min for the analysis or purification. A number of synthesized chemicals were purified with a SepaBean machine equipped with Sepaflash columns produced by Santai Technologies Inc. in China. Absorbance spectrum determined by UV1800 Series UV-Vis spectrophotometer (Shimadzu, Japan). Fluorescence spectrum determined by a wavelength- calibrated FluoroMax-3 fluorometer (Horiba Jobin Yvon, France). In-gel fluorescence scanning was taken using Gel Documentation and Typhoon TRIO Variable Mode Imager System (GE Healthcare, USA). Absorbance for MTT assay was determined in a microplate reader (Molecular Devices, SpectraMax *i*3). Cell images were taken using fluorescence microscope (Leica, Germany). Image processing was made on image J software (National Institutes of Health, USA). In vivo images were obtained by IVIS Lumina XRMS Series III in Vivo Imaging System (PerkinElmer, Inc. USA).

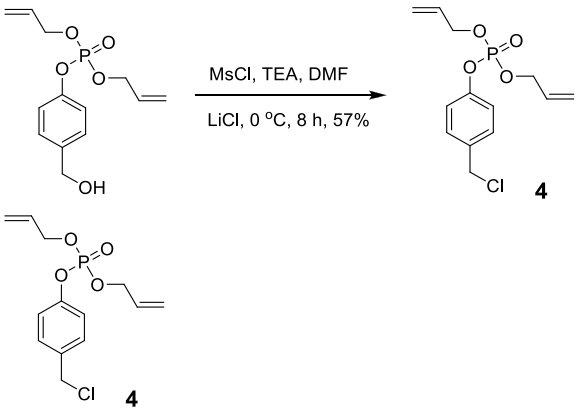
Chemical synthesis and characterization of probes



3

(E)-2-(2-(6-hydroxy-7-(hydroxymethyl)-2, 3-dihydro-1H-xanthen-4-yl)vinyl)-3, 3 -dimethyl-1-(pent-4-ynyl)-3H-indolium iodide (3)

Under N_2 atmosphere, to a solution of **1**[1] (951.6 mg, 1.332 mmol), **2**[2] (1.29 g, 9.214 mmol) , $KHCO_3$ (411.5 mg, 4.115 mmol) in DMF (4 mL) were stirred at 75 ºC for 4 h. After dilution with ethyl acetate (10 mL), the solution was washed with water (10 mL x 3). The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by chromatography on silica gel column to afford title compound as blue solid (370.1 mg, 47%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.76 (d, *J* = 14.8 Hz, 1H), 7.63 (d, *J* = 7.2 Hz, 1H), 7.60 – 7.47 (m, 4H), 7.46 – 7.37 (m, 1H), 6.89 (s, 1H), 6.52 (d, *J* = 14.8 Hz, 1H), 4.69 (s, 2H), 4.42 (t, *J* = 7.4 Hz, 2H), 2.84 – 2.77 (m, 2H), 2.74 (t, *J* = 6.0 Hz, 2H), 2.55 (t, *J* = 2.4 Hz, 1H), 2.44 – 2.36 (m, 2H), 2.11 – 2.04 (m, 2H), 2.00 – 1.89 (m, 2H), 1.82 (s, 6H). ^{13}C NMR (101MHz, CD_3OD) δ 176.68, 162.69, 159.72, 153.98, 145.02, 141.64, 141.63, 135.86, 128.76, 128.34, 126.69, 126.48, 126.26, 122.33, 114.87, 114.48, 111.86, 101.99, 100.77, 82.36, 70.31, 58.54, 50.19, 43.19, 28.53, 27.15, 25.98, 23.86, 20.23, 15.13. HRMS (ESI) m/z calcd for $C_{31}H_{32}NO_3$ $(M\text{-}I)^+$ 466.2382, found 466.2383.

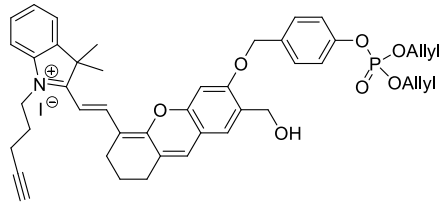


4

Diallyl-4-(chloromethyl)phenyl phosphate (4)

Under N_2 atmosphere, to a solution of diallyl-4-(hydroxymethyl)phenyl phosphate[3] (136.7 mg, 0.481 mmol) in DMF (1 mL) at 0 ºC were added triethylamine (334 µL, 2.402 mmol), methanesulfonyl chloride (185 µL, 2.390 mmol), subsequently. The resulting mixture were stirred for 3 h before LiCl (102.8 mg, 2.425 mmol) was added

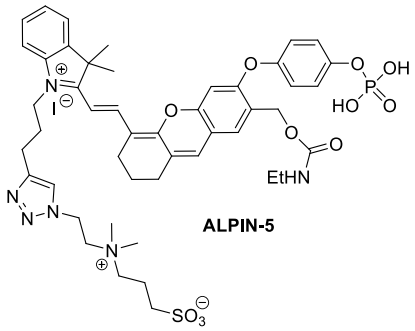
and stirred at 0 °C for 5 h. After dilution with ethyl acetate (10 mL), the solution was washed with water (10 mL x 3). The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by chromatography on silica gel column to afford title compound as yellow oil (82.6 mg, 57 %).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, *J* = 8.4 Hz, 2H), 7.21 (t, *J* = 8.0 Hz, 2H), 5.99 – 5.89 (m, 2H), 5.37 (dd, *J* = 16.8, 1.2 Hz, 2H), 5.27 (dd, *J* = 10.4, 1.2 Hz, 2H), 4.72 – 4.58 (m, 4H), 4.56 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 150.52 (d, J = 6.7 Hz), 134.41, 131.98 (d, J = 6.9 Hz), 130.11, 120.33 (d, J = 5.0 Hz), 118.81, 68.95 (d, J = 5.5 Hz), 45.47. HRMS (ESI) m/z calcd for $C_{13}H_{16}ClNaO_4P$ $(M+Na)^+$ 325.0372, found 325.0371.



5

(E)-2-(2-(6-(4-(bis(allyloxy)phosphoryloxy)benzyloxy)-7-(hydroxymethyl)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-1-(pent-4-ynyl)-3H-indolium iodide (5)

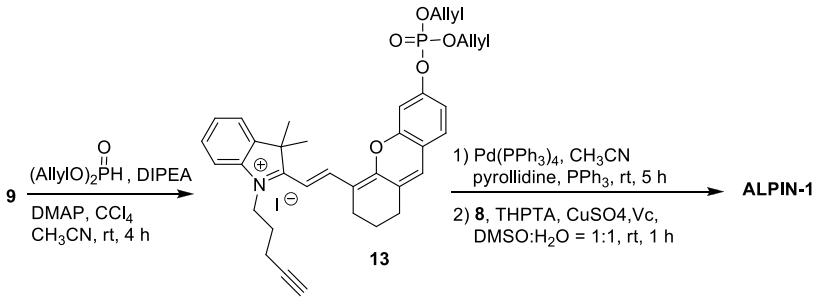
Under N_2 atmosphere, to a solution of **3** (50.1 mg, 0.084 mmol), **4** (49.4 mg, 0.163 mmol), KI (142.3 mg, 0.857 mmol), $KHCO_3$ (42.7 mg, 0.427 mmol), 18-crown-6 (30.4 mg, 0.115 mmol) in DMF (1 mL) were stirred at rt for 8 h. After dilution with DCM (10 mL), the solution was washed with water (10 mL x 3). The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by chromatography on silica gel column to afford title compound as blue solid (48.1 mg, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (d, *J* = 14.4 Hz, 1H), 7.71 (s, 1H), 7.54 (d, *J* = 7.2 Hz, 1H), 7.45 – 7.35 (m, 4H), 7.29 (d, *J* = 10.4 Hz, 2H), 7.21 (d, *J* = 8.4 Hz, 2H), 7.11 (s, 1H), 6.33 (d, *J* = 14.4 Hz, 1H), 5.99 – 5.89 (m, 2H), 5.38 (dd, *J* = 17.2, 1.2 Hz, 2H), 5.27 (dd, *J* = 10.4, 1.2 Hz, 2H), 5.06 (s, 2H), 4.77 (s, 2H), 4.64 (t, *J* = 7.4 Hz, 4H), 4.33 (t, *J* = 7.0 Hz, 2H), 2.69 – 2.62 (m, 4H), 2.44 – 2.38 (m, 2H), 2.13 – 2.06 (m, 1H), 2.12 – 2.06 (m, 2H), 1.88 – 1.78 (m, 8H). ^{13}C NMR (151 MHz, CD_3OD) δ 177.32, 162.31, 159.46, 153.89, 153.05 (d, *J* = 6.7 Hz), 145.40, 141.93, 141.56, 134.90, 134.33 (d, *J* = 7.8 Hz), 130.95, 129.65 (d, *J* = 2.5 Hz), 128.76, 128.43, 127.29, 126.74, 126.00, 124.85, 122.50, 120.19 (d, *J* = 5.1 Hz), 115.44, 115.06, 114.53, 112.06, 102.64, 99.28, 82.38, 70.35, 70.28, 69.59, 66.38 (d, *J* = 5.5 Hz), 58.27, 50.47, 48.46, 43.36, 28.62, 27.20, 26.06, 23.92, 20.18, 15.15. HRMS (ESI) m/z calcd for $C_{44}H_{47}NO_7P$ $(M-I)^+$ 732.3090, found 732.3089.

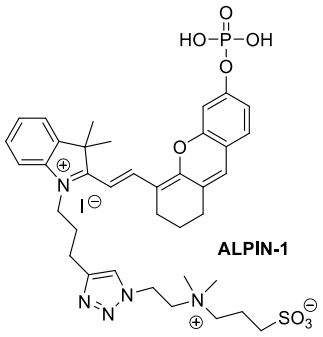


(E)-3-((2-(4-(3-(2-(2-(7-((ethylcarbamoyloxy)methyl)-6-(4-(phosphonooxy) benzyloxy)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-3H-indolium-1-yl) propyl)-1H-1,2,3-triazol-1-yl)ethyl)dimethylammonio)propane-1-sulfonate iodide (ALPIN-5)

To a solution of **5** (42.9 mg, 0.050 mmol) in DCM (1 mL) at rt were added isocyanatoethane (40 μL, 0.505 mmol), triethylamine (35 μL, 0.252 mmol) and the resulting mixture were stirred for 4 h. Volatile solvent and excess reagents were then removed under vacuum to afford **6** as crude product, which was used in the next step without further purification.

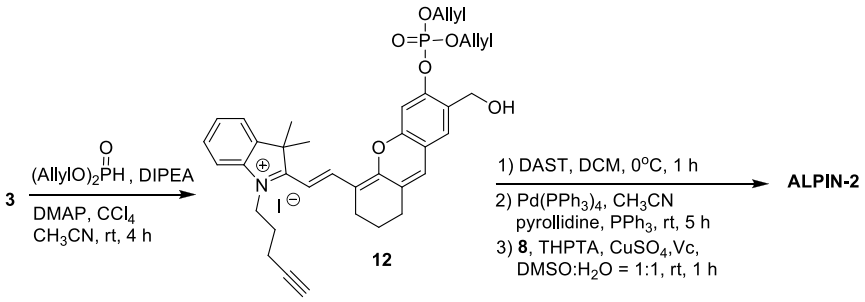
Under N_2 atmosphere, a mixture of **6** prepared above, PPh_3 (5.3 mg, 0.020 mmol), $Pd(PPh_3)_4$ (11.4 mg, 0.010 mmol), and pyrrolidine (25 μL, 0.304 mmol) in MeCN (1 mL) were stirred at rt for 4 h. Volatile solvent and excess reagents were then removed under vacuum to afford **7** as crude product, which was used in the next step without further purification. A mixture of **7** prepared above, **8**[4] (17.8 mg, 0.075 mmol), tris(3-hydroxypropyl triazolylmethyl)amine (3.5 mg, 0.008 mmol), $CuSO_4$ (5.1 mg, 0.032 mmol), ascorbic acid (33.9 mg, 0.192 mmol), DMSO (0.5 mL), and H_2O (0.5 mL) were added and the resulting mixture were stirred at rt for 1 h. The title compound was obtained as blue solid (4.6 mg, 9%) after purification by preparative RP-HPLC on a C18 column. ^{1}H NMR (600 MHz, *d6*-DMSO) δ 8.58 (d, *J* = 10.0 Hz, 1H), 8.09 (s, 1H), 7.81 (d, *J* = 5.6 Hz, 1H), 7.71 (d, *J* = 6.0 Hz, 1H), 7.57 – 7.51 (m, 4H), 7.47 (t, *J* = 5.0 Hz, 1H), 7.28 (t, *J* = 3.6 Hz, 1H), 7.23 (d, *J* = 5.6 Hz, 2H), 7.21 (s, 1H), 6.61 (d, *J* = 10.0 Hz, 1H), 5.34 (s, 2H), 5.06 (s, 2H), 4.91 (t, *J* = 4.2 Hz, 2H), 4.50 (t, *J* = 5.2 Hz, 2H), 3.83 (t, *J* = 4.8 Hz, 2H), 3.54 – 3.51 (m, 2H), 3.07 (s, 6H), 3.05 – 3.00 (m, 2H), 2.86 (t, *J* =4.8 Hz, 2H), 2.74 (t, *J* = 3.8 Hz, 2H), 2.66 (t, *J* = 3.8 Hz, 2H), 2.45 (t, *J* = 4.6 Hz, 2H), 2.20 – 2.15 (m, 2H), 2.01 – 1.96 (m, 2H), 1.88 – 1.82 (m, 2H), 1.80 (s, 6H), 1.05 – 1.01 (m, 3H). HRMS (ESI) m/z calcd for $C_{48}H_{60}N_6O_{11}PS$ $(M\text{-}I)^+$ 959.3778, found 959.3785.

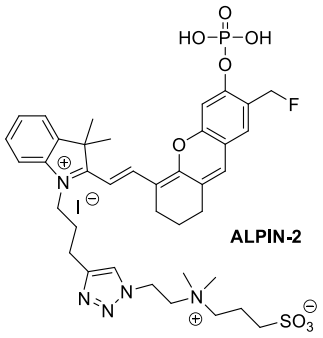




(E)-3-((2-(4-(3-(3,3-dimethyl-2-(2-(6-(phosphonooxy)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3H-indolium-1-yl)propyl)-1H-1,2,3-triazol-1-yl)ethyl)dimethylammonio) propane-1-sulfonate iodide (ALPIN-1)

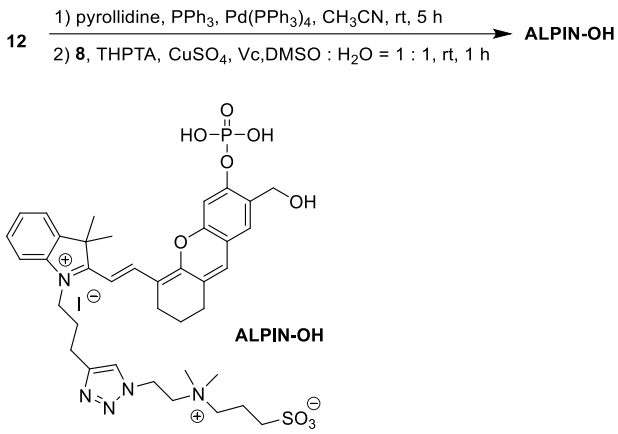
Following a synthetic method similar to **ALPIN-5**, the title compound was obtained after RP-HPLC purification on a C18 column. The purity of this compound was confirmed by HPLC analysis. HRMS (ESI) m/z calcd for $C_{37}H_{47}N_5O_8PS$ $(M\text{-}I)^+$ 752.2877, found 752.2864.





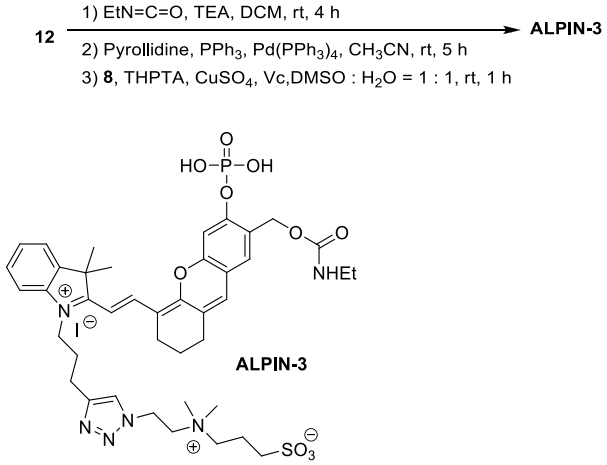
(E)-3-((2-(4-(3-(2-(2-(7-(fluoromethyl)-6-(phosphonooxy)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-3H-indolium-1-yl)propyl)-1H-1,2,3-triazol-1-yl)ethyl)dimethylammonio)propane-1-sulfonate iodide (ALPIN-2)

Following a synthetic method similar to **ALPIN-5**, the title compound was obtained after RP-HPLC purification on a C18 column. The purity of this compound was confirmed by HPLC analysis. HRMS (ESI) m/z calcd for $C_{38}H_{48}FN_5O_8PS$ $(M\text{-}I)^+$ 784.2945, found 784.2944.



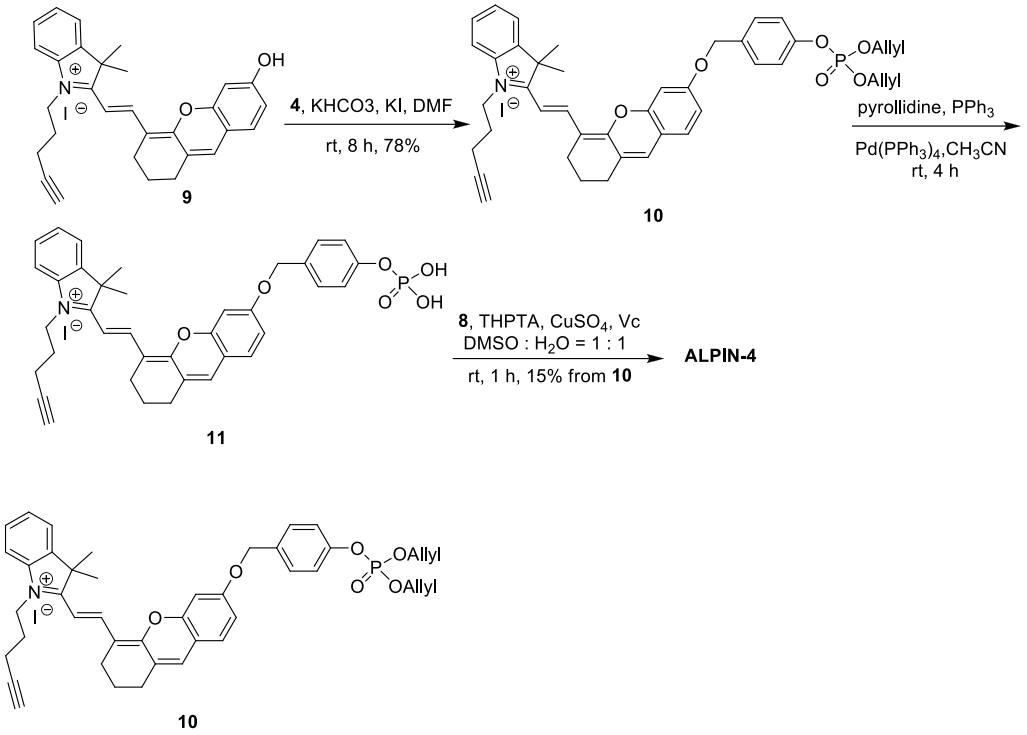
(E)-3-((2-(4-(3-(2-(2-(7-(hydroxymethyl)-6-(phosphonooxy)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-3H-indolium-1-yl)propyl)-1H-1,2,3-triazol-1-yl)ethyl)dimethylammonio)propane-1-sulfonate iodide (ALPIN-OH)

Following a synthetic method similar to **ALPIN-5**, the title compound was obtained after RP-HPLC purification on a C18 column. The purity of this compound was confirmed by HPLC analysis. HRMS (ESI) m/z calcd for $C_{38}H_{49}N_5O_9PS$ $(M\text{-}I)^+$ 782.2989, found 782.2988.



(E)-3-((2-(4-(3-(2-(2-(7-((ethylcarbamoyloxy)methyl)-6-(phosphonooxy)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-3H-indolium-1-yl)propyl)-1H-1,2,3-triazol-1-yl)ethyl)dimethylammonio)propane-1-sulfonate iodide (ALPIN-3)

Following a synthetic method similar to **ALPIN-5**, the title compound was obtained after RP-HPLC purification on a C18 column. The purity of this compound was confirmed by HPLC analysis. HRMS (ESI) m/z calcd for $C_{41}H_{54}N_6O_{10}PS$ $(M\text{-}I)^+$ 853.3360, found 853.3361.

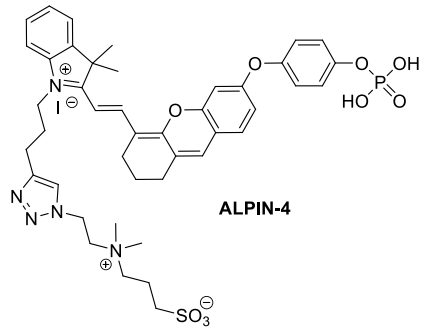


9 **10** **11**

10

(E)-2-(2-(6-(4-(bis(allyloxy)phosphoryloxy)benzyloxy)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-1-(pent-4-ynyl)-3H-indolium iodide (10)

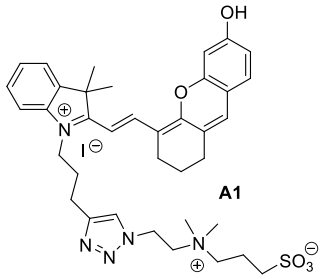
Under N_2 atmosphere, to a solution of **9**[5] (27.1 mg, 0.048 mmol), **4** (28.6 mg, 0.094 mmol), KI (82.6 mg, 0.497 mmol), $KHCO_3$ (27.0 mg, 0.270 mmol), 18-crown-6 (13.6 mg, 0.051 mmol) in DMF (460 μL) were stirred at rt for 8 h. After dilution with DCM (10 mL), the solution was washed with water (10 mL x 3). The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by chromatography on silica gel column to afford title compound as blue solid (32.3 mg, 78%).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (d, *J* = 14.8 Hz, 1H), 7.54 – 7.43 (m, 4H), 7.42 7.37 (m, 3H), 7.28 (d, *J* = 8.8 Hz, 2H), 7.25 (s, 1H), 7.01 – 6.89 (m, 2H), 6.51 (d, *J* = 14.8 Hz, 1H), 6.00-5.90 (m, 2H), 5.38 (dd, *J* = 17.2, 1.2 Hz, 2H), 5.28 (dd, *J* = 9.6 Hz, 0.4 Hz, 2H), 5.18 (s, 2H), 4.66 (t, *J* = 6.8 Hz, 4H), 4.44 (t, *J* = 7.0 Hz, 2H), 2.75 (t, *J* = 5.2 Hz, 2H), 2.69 (t, *J* = 5.4 Hz, 2H), 2.41 (t, *J* = 4.6 Hz, 2H), 2.18 – 2.04 (m, 3H), 1.96 – 1.90 (m, 2H), 1.80 (s, 6H). ^{13}C NMR (151 MHz, CD_3OD) δ 177.89, 162.23, 161.97, 154.45, 150.32 (d, *J* = 6.9 Hz), 145.89, 142.06, 141.50, 134.06, 133.84, 132.02 (d, *J* = 6.1 Hz), 129.05, 128.81 (d, *J* = 6.6 Hz), 127.37, 126.98, 122.43, 120.03 (d, *J* = 4.2 Hz), 117.74, 116.09, 114.58, 114.02, 112.30, 103.23, 101.33, 82.30, 70.35, 69.64, 68.98 (d, *J* = 5.5 Hz), 50.62, 43.50, 28.65, 27.02, 26.10, 23.87, 20.17, 15.12. HRMS (ESI) m/z calcd for $C_{43}H_{45}NO_6P$ $(M\text{-}I)^+$ 702.2984, found 702.2983.



(E)-3-((2-(4-(3-(3,3-dimethyl-2-(2-(6-(4-(phosphonooxy)benzyloxy)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3H-indolium-1-yl)propyl)-1H-1,2,3-triazol-1-yl)ethyl)dimethylammonio)propane-1-sulfonate iodide (ALPIN-4)

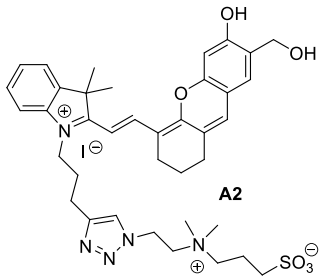
Under N_2 atmosphere, to a solution of **10** (24.9 mg, 0.030 mmol), PPh_3 (3.9 mg, 0.014 mmol), $Pd(PPh_3)_4$ (16.2 mg, 0.014 mmol) in MeCN (0.46 mL) were added pyrrolidine (23 μL, 0.280 mmol) at rt and the mixture were stirred at the same temperature for 3 h. Volatile solvent and excess reagents were then removed under vacuum to afford **11** as crude product, which was used in the next step without further purification.

To **11** prepared above were added **8** (22.9 mg, 0.097 mmol), tris(3-hydroxypropyl triazolylmethyl)amine (2.7 mg, 0.006 mmol), $CuSO_4$ (1.8 mg, 0.011 mmol), ascorbic acid (40.8 mg, 0.232 mmol), DMSO (0.2 mL), and H_2O (0.2 mL) and the resulting mixture were stirred at rt for 1 h. The reaction was purified by preparative RP-HPLC on a C18 column to afford title compound as blue solid (4.5 mg, 15%). HRMS (ESI) m/z calcd for $C_{44}H_{53}N_5O_9PS$ $(M\text{-}I)^+$ 858.3302, found 858.3301.



(E)-3-((2-(4-(3-(2-(2-(6-hydroxy-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-3H-indolium-1-yl)propyl)-1H-1,2,3-triazol-1-yl)ethyl)dimethylammonio) propane-1-sulfonate iodide (A1)

Following a synthetic method similar to **ALPIN-4**, the title compound was obtained after RP-HPLC purification on a C18 column. The purity of this compound was confirmed by HPLC analysis. HRMS (ESI) m/z calcd for $C_{37}H_{46}N_5O_5S$ $(M\text{-}I)^+$ 672.3214, found 672.3221.



(E)-3-((2-(4-(3-(2-(2-(6-hydroxy-7-(hydroxymethyl)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-3H-indolium-1-yl)propyl)-1H-1,2,3-triazol-1-yl)ethyl)dimethylammonio)propane-1-sulfonate iodide (A2)

Following a synthetic method similar to **ALPIN-5**, the title compound was obtained after RP-HPLC purification on a C18 column. The purity of this compound was confirmed by HPLC analysis. HRMS (ESI) m/z calcd for $C_{38}H_{48}N_5O_6S$ $(M\text{-}I)^+$ 702.3325, found 702.3326.

Cell experiments

Cell culture conditions

HeLa cells or HEK293 cells were cultured in high-glucose DMEM (Gibco) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) in 5% CO_2 humidified atmosphere (95%) at 37 °C.

Cell viability assays

The cytotoxicity of ALPIN-5 or ALPIN-4 to HeLa cells was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation and Cytotoxicity assay following manufacture's protocol. In brief, cells (4×10^3 cells/well) in DMEM (supplemented with 10% FBS, 100 μL) were seeded in 96-well plate and incubated at 37 ºC overnight. Medium was then changed and cells were treated with ALPIN-5 or ALPIN-4 at a serial of concentrations (0, 1, 5, 10 and 20 μM) in DMEM (supplemented with 10% FBS, 100 μL) at 37 °C with 5% CO_2 and 95% humidity for 24 h. After the removal of medium, DMEM (90 μL) supplemented with MTT (10 μL, 5 mg/mL) was added to each well and incubated at 37 ºC with 5% CO_2 and 95% humidity for 4 h. Upon formation of visible purple crystals (formazan), medium was aspirated and dimethyl sulfoxide (DMSO, 100 μL) was added to dissolve the forming formazan for 10 min. Absorbance at 490 nm was measured with microplate reader and cell viability was calculated based on untreated wells. Each experiment was triplicated.

Fluorescence imaging of cells

Cells were dispersed onto a glass-bottom dish (8×10^4 cells) (Cellvis, D35-20-1-N, 35 mm Dish with 20 mm bottom Well) and allowed to grow overnight. ALPIN-5 or ALPIN-4 (10 μM) in FBS free DMEM was added into dishes and incubated in 5% CO_2 humidified atmosphere at 37 ºC for 1 h.

For the inhibition experiments, cells were pretreated with ALP inhibitor Na_3VO_4 (10 mM) for 20 min before incubation with ALPIN-5 (10 μM) for 1 h. Culture medium was removed and the cells were washed three times with PBS. After adding phenol red-free DMEM, Cell images were taken using fluorescence microscope (Leica, Germany), with excitation/emission at Cy5.5 channel.

For wash-free imaging of ALP activity in HeLa cells, HeLa cells were incubated with ALPIN-5 (1 μM) in FBS-free DMEM and the fluorescence images were captured with fluorescence microscope (Leica, Germany) at 10-60 min without washing. The cell images were processed using the ImageJ software.

Flow cytometry

HeLa cells (5×10^5 cells per sample) were incubated in microcentrigue tube containing ALPIN-5 or ALPIN-4 (10 μM) at 37 °C with 5% CO_2 and 95% humidity for 1 h. After being washed with PBS, cells were then resuspended in PBS (200 μL). The fluorescence of cell samples was analyzed with a flow cytometer (CytoFLEX S,

Beckman, USA).

Animals and Tumor Models

Ethics statement

All animal studies were performed in agreement with the guidelines set by the Institutional Animal Care Use Committee of Shanghai Jiao Tong University (Shanghai, China).

Tumor models

BALB/c female mice at 4 – 6 weeks old were obtained from Shanghai Laboratory Animal Research Center. Mice were housed at 25 ºC with free access to food and water. Xenograft tumor model of mouse was established by subcutaneously injection of 4×10^6 HeLa cells suspended in 200 μL of 1:4 (v/v) mixture of Matrigel and DMEM. The tumors were allowed to grow to reach the size of around 0.1 cm^3(around 2 – 3 weeks).

Fluorescence imaging of ALP activity in mice

To compare the retention of ALPIN-4 and ALPIN-5 in tumor, living mice bearing subcutaneous HeLa tumors were intratumorally injected with ALPIN-5 or ALPIN-4 (0.143 μmol kg^{-1}) in 100 μL PBS. Whole body fluorescence images were acquired before injection, as well as 10 min, 30 min, 1 h, 2 h, 4 h, 24 h, and 48 h post injection. The whole-body fluorescence images were acquired on an IVIS Lumina XRMS Series III imaging system at indicated time point with excitation wavelength at 660 nm and emission wavelength at 710 nm. The fluorescence intensities were quantified by the ROIs measurement using Living Image Software (PerkinElmer, U.S.A). Each experiment was conducted in three mice.

To investigate the accumulation of these probes to ALP-overexpressing-tumor, mice were injected through tail vein with ALPIN-5 or ALPIN-4 (0.381 μmol kg^{-1}) in 200 μL PBS. For the inhibition study, Na_3VO_4 (10 mM) in 50 uL PBS was injected into tumors 30 min before tail vein injection of ALPIN-5. Whole body fluorescence images were acquired before injection, as well as 1 h, 2 h, 3 h, 4 h, 6 h and 24 h post injection. The whole-body fluorescence images were acquired on an IVIS Lumina XRMS Series III imaging system at indicated time point with excitation wavelength at 660 nm and emission wavelength at 710 nm. The fluorescence intensities were quantified by the ROIs measurement using Living Image Software (PerkinElmer, U.S.A). Each experiment was conducted in three mice.

Bio-distribution studies

At 2 h or 48 h post-injection, tumor-bearing mice were sacrificed and dissected. Fluorescence images of tumor tissues and main organs were obtained with IVIS Lumina XRMS Series III in Vivo Imaging System (PerkinElmer, Inc. USA) with excitation at 660 nm and emission at 710 nm.

References

[1] J. Y. Hyun, N. R. Kang, I. Shin, *Org. Lett.* **2018**, *20*, 1240-1243.

[2] S. A. Lermontov, A. N. Malkova, N. A. Sipyagina, E. A. Straumal, A. E. Baranchikov, K. E. Yorov, V. K. Ivanov, *J. Fluorine .Chem.* **2017**, *193*, 1-7.

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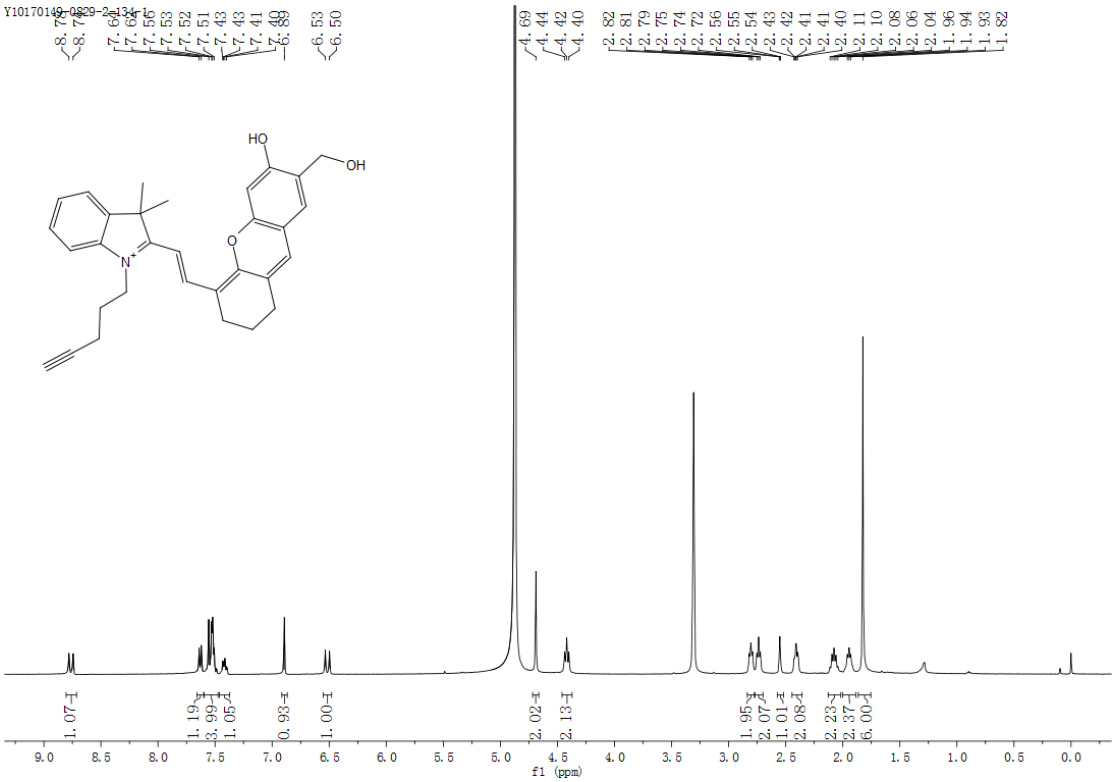
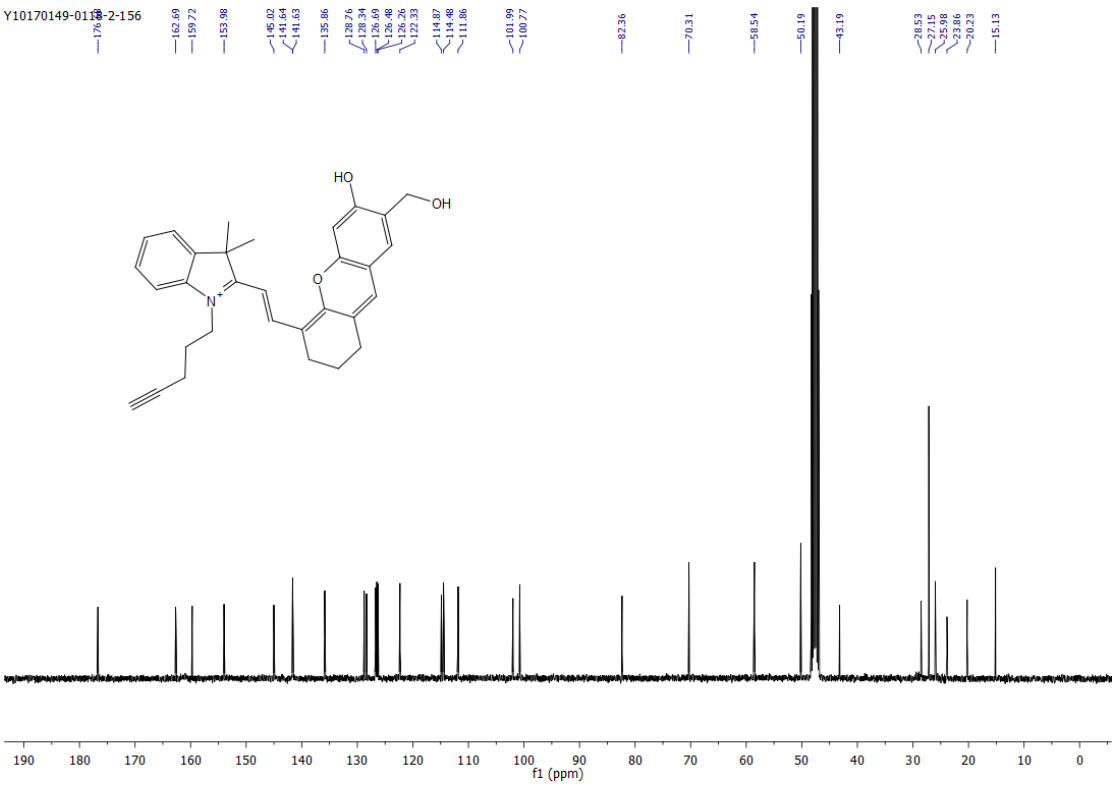
[4] J. Huang, W. Xu, *Journal of J. Appl. Polym. Sci.* **2011**, *122*, 1251-1257.

[5] Z. Fang, Y. Li, H. Xie, *Tetrahedron Lett.* **2019**, *60*, 1658-1662.

^{1}H and ^{13}C NMR Spectra

^{1}H and ^{13}C NMR Spectra of 3





^{1}H and ^{13}C NMR Spectra of 4

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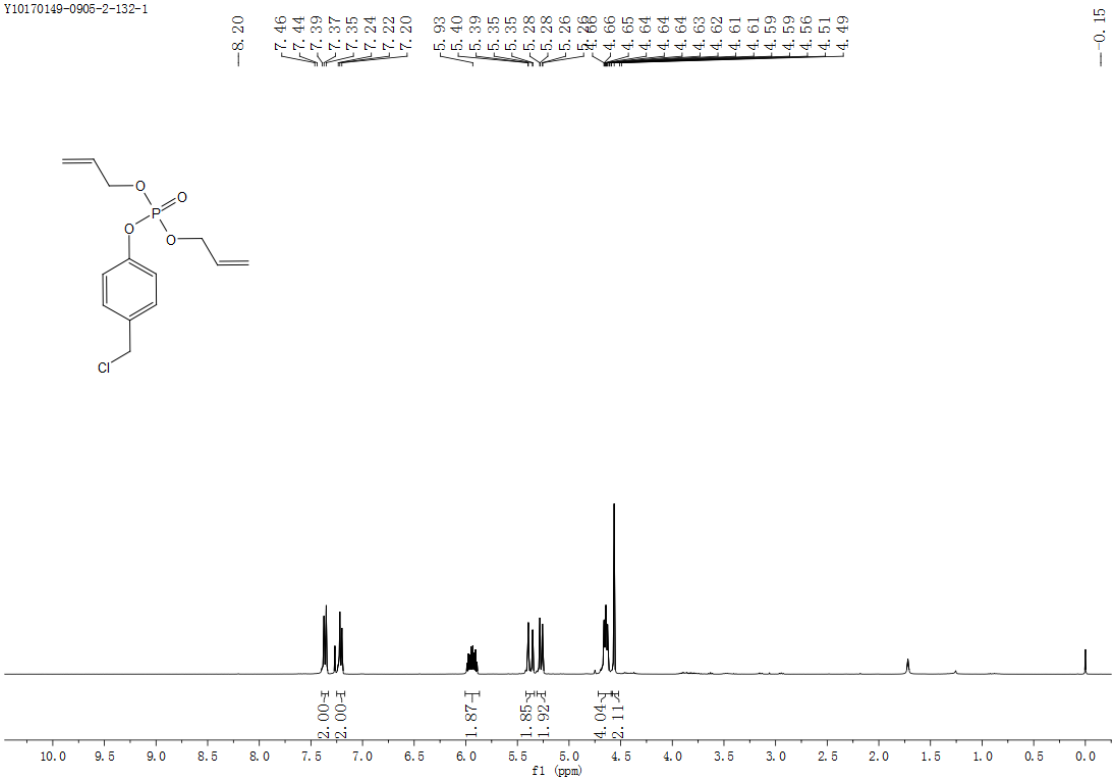

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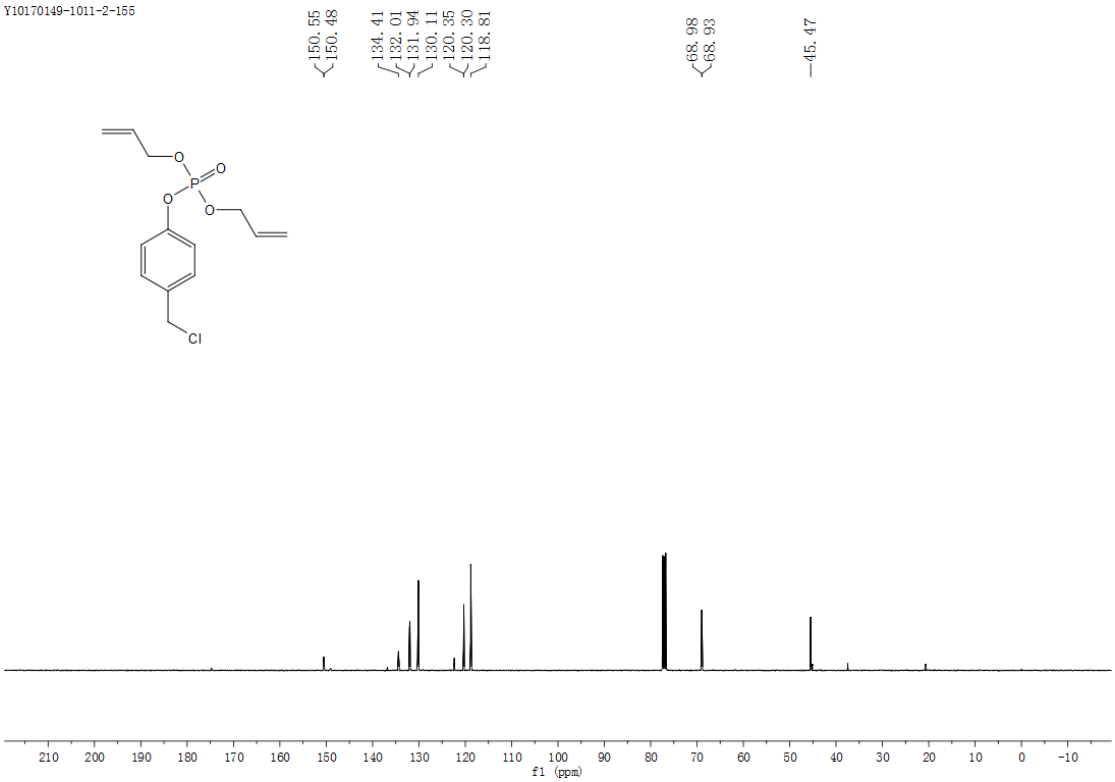
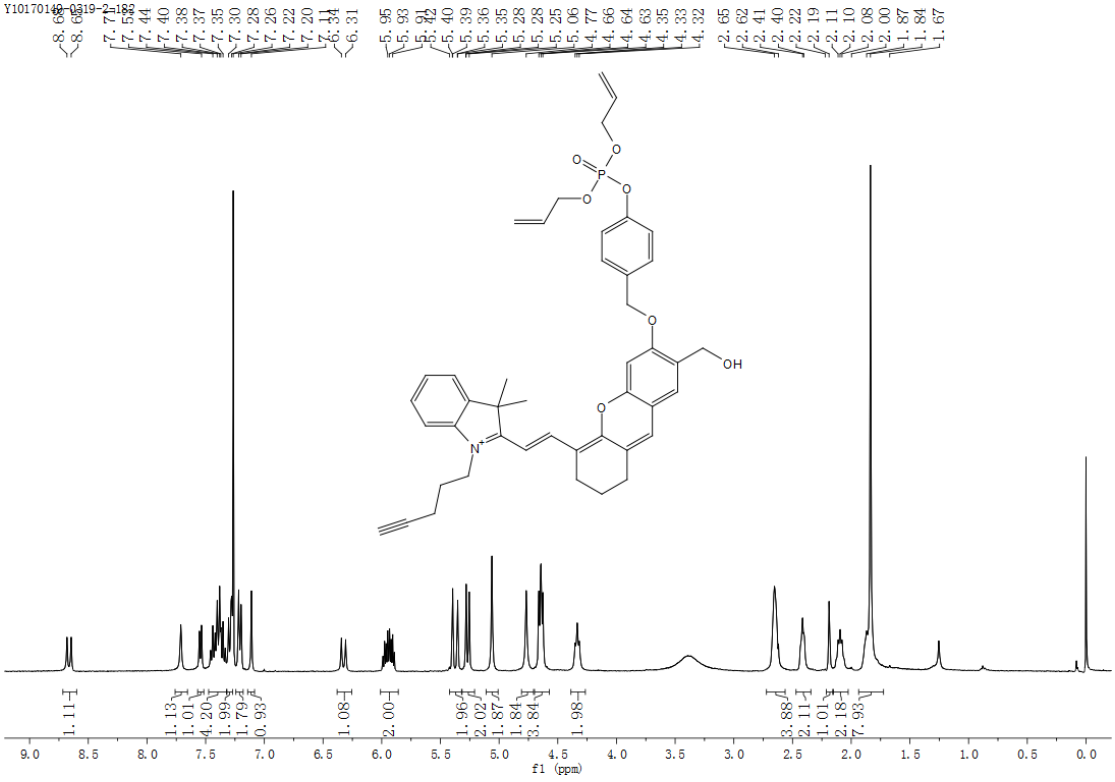
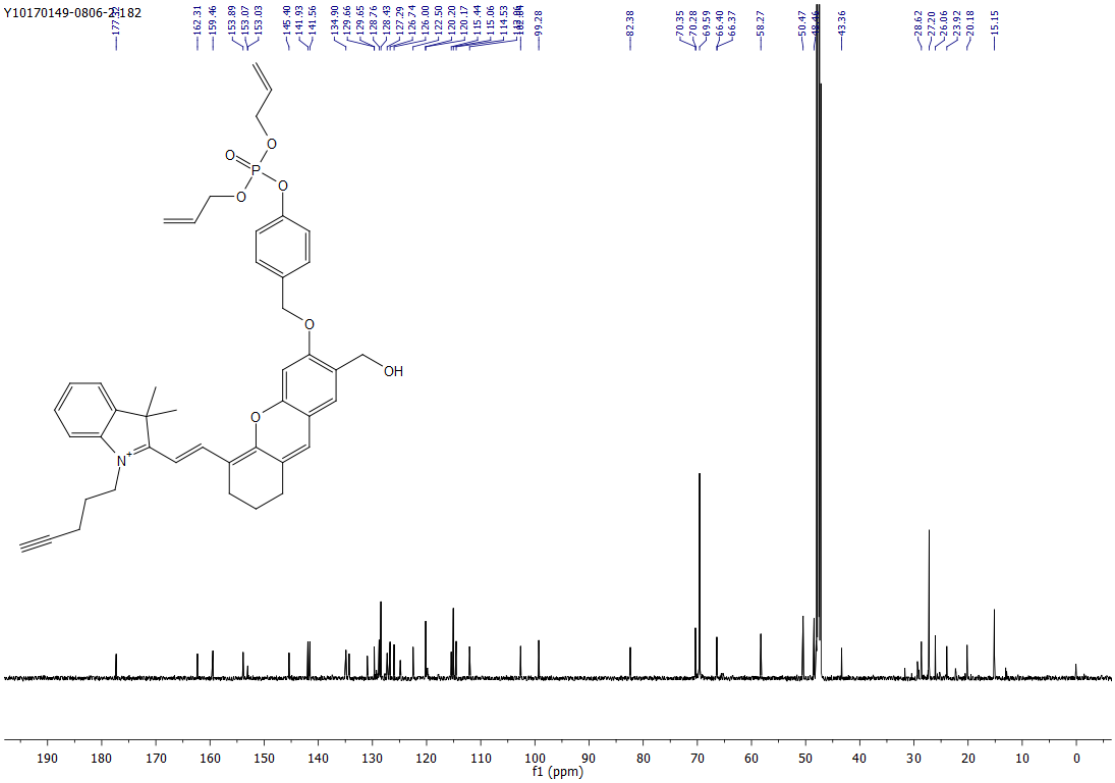
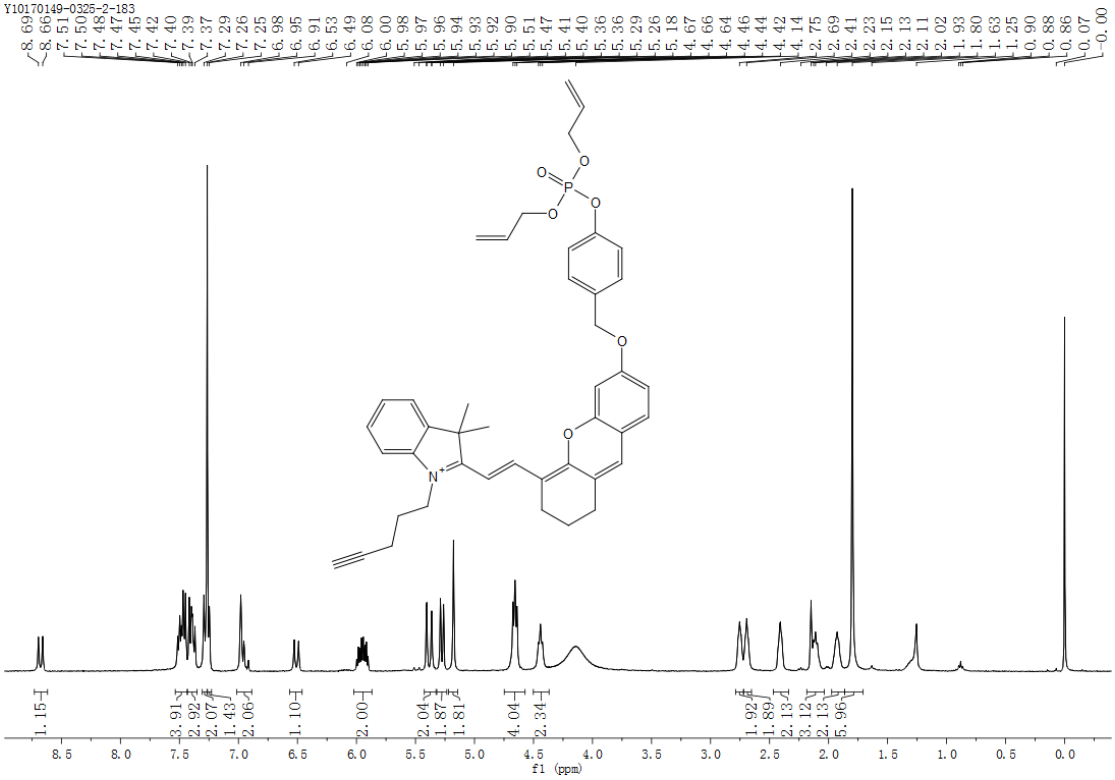


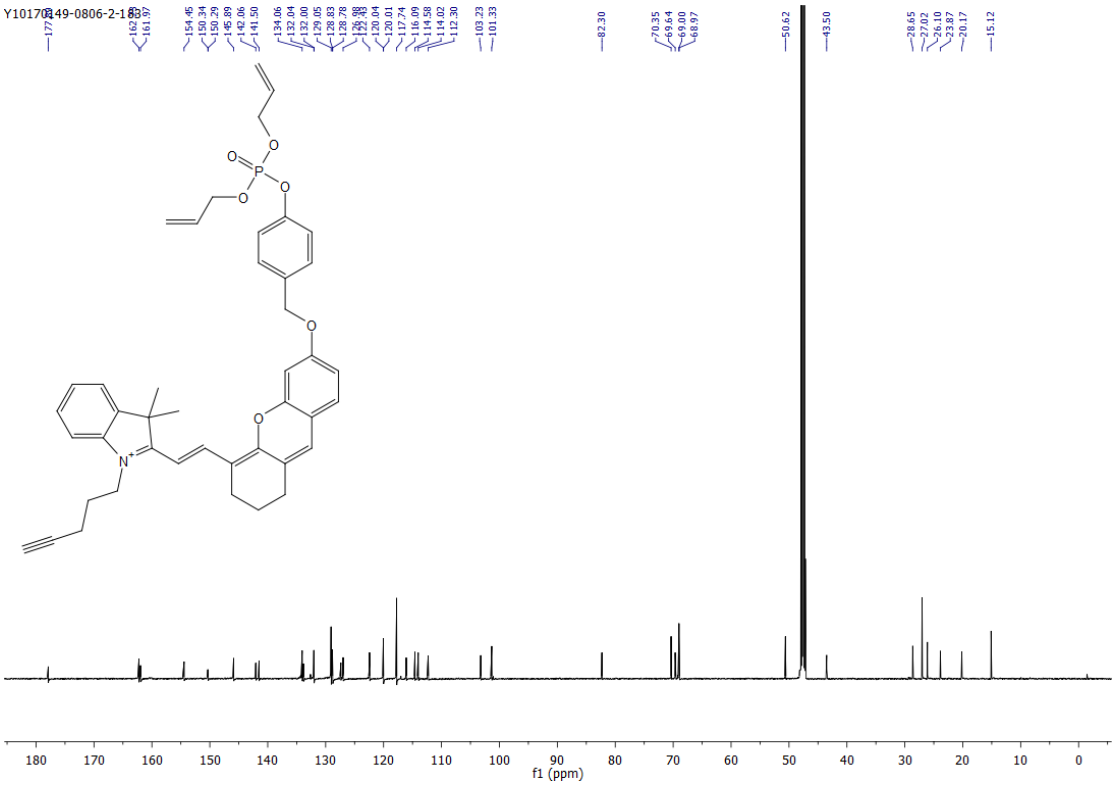
^{1}H and ^{13}C NMR Spectra of 5



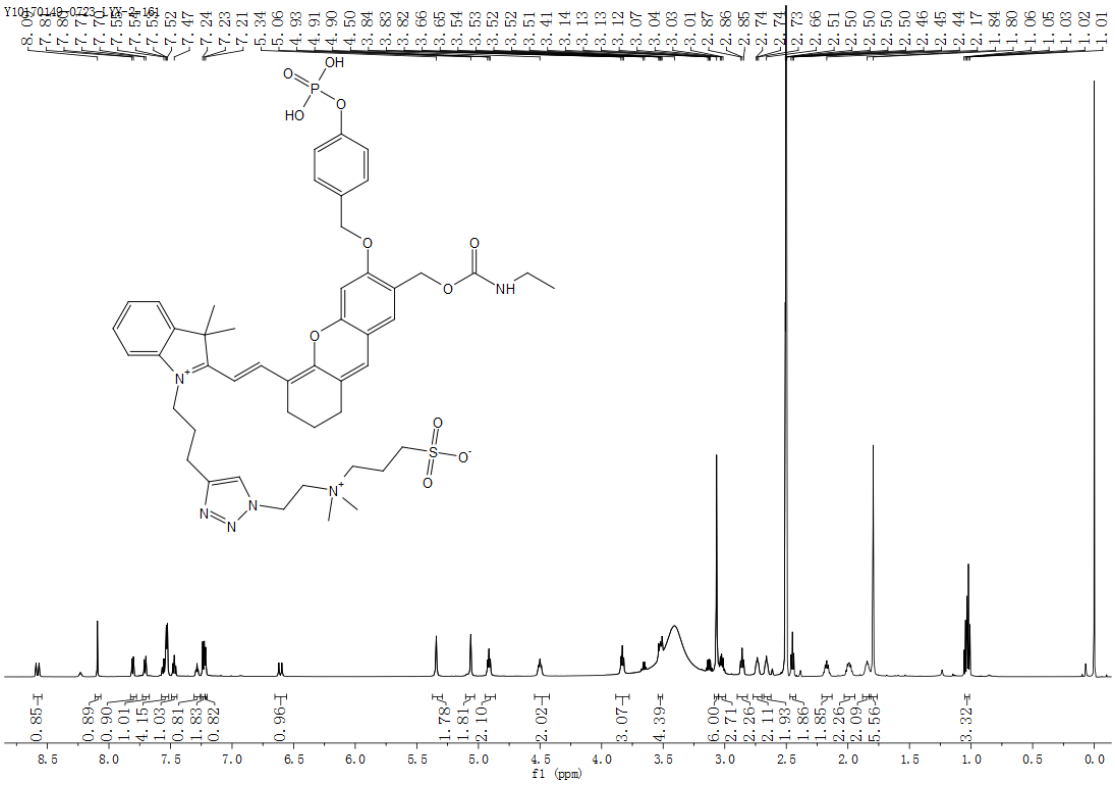


^{1}H and ^{13}C NMR Spectra of 10



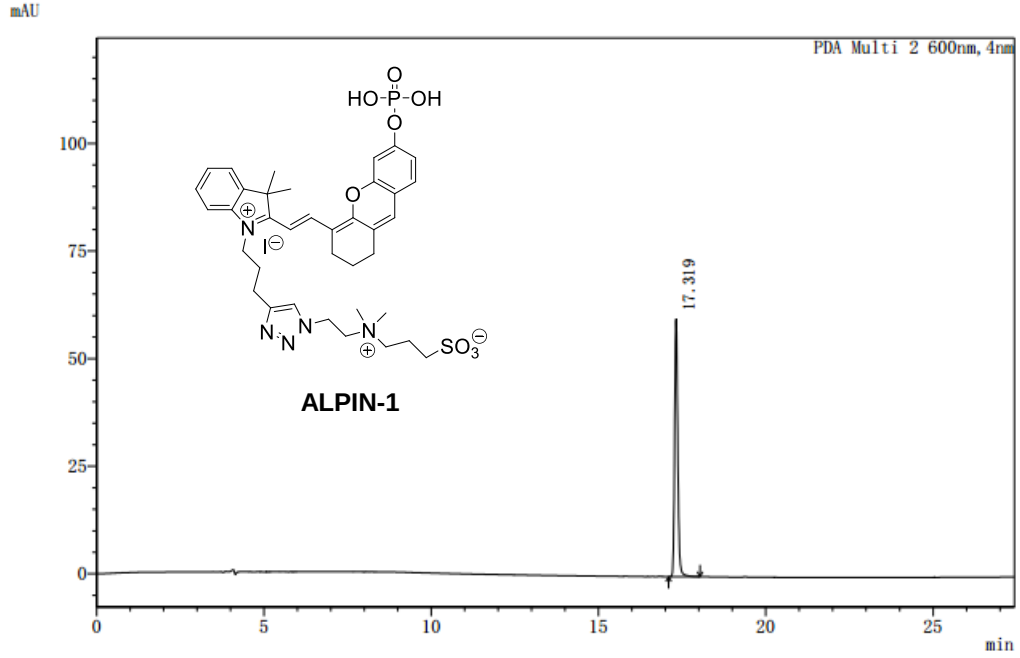


^{1}H Spectra of ALPIN-5

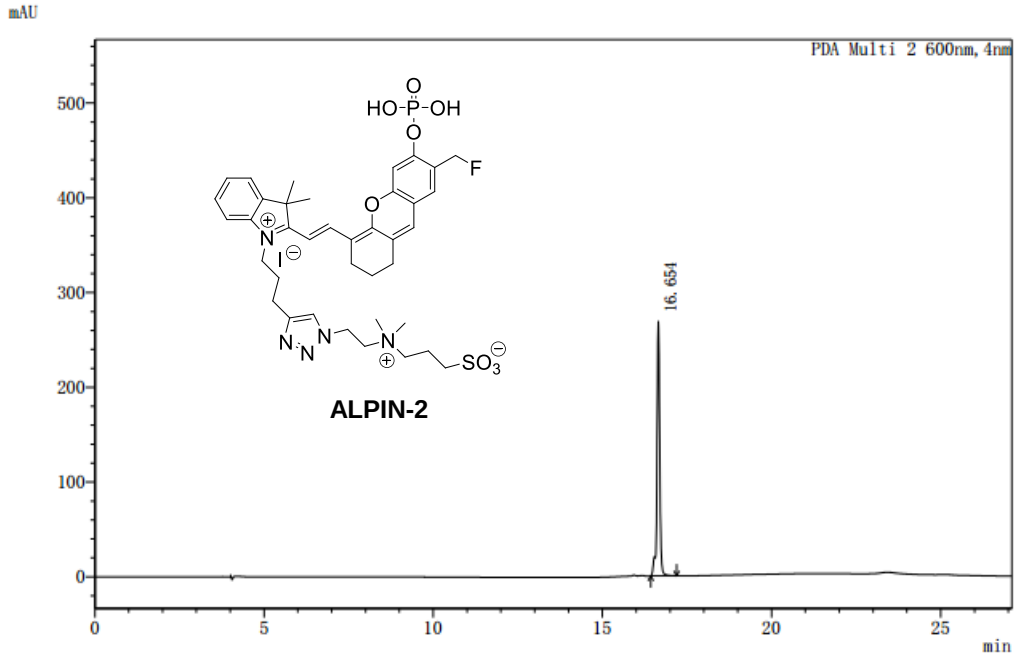


HPLC trace

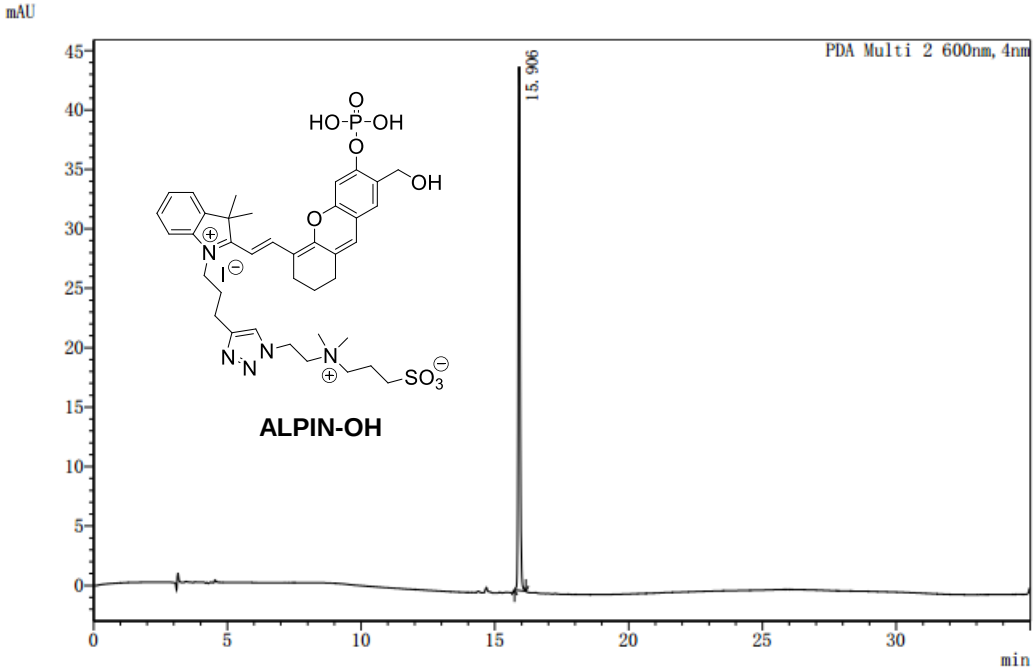
HPLC trace of ALPIN-1



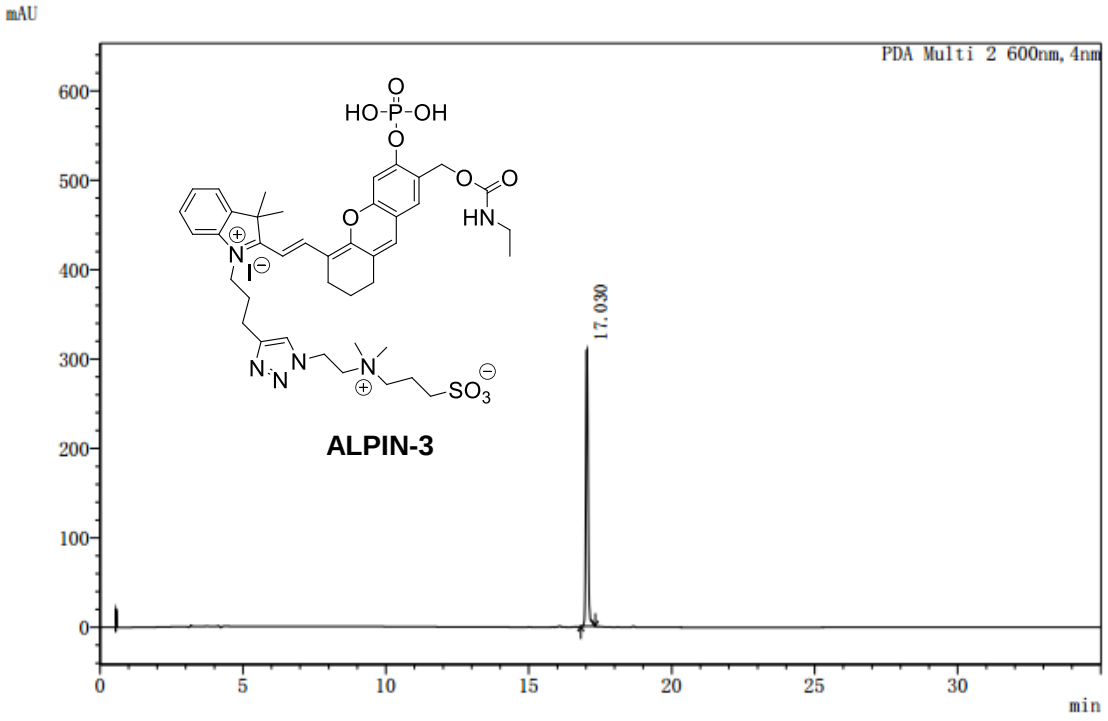
HPLC trace of ALPIN-2



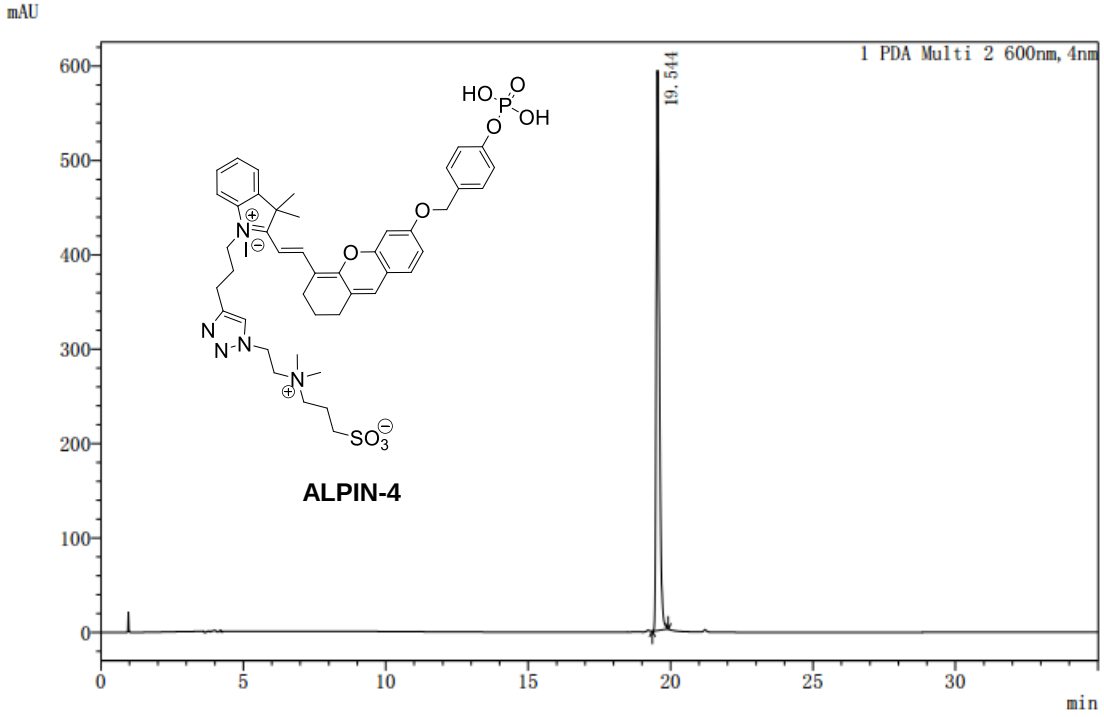
HPLC trace of ALPIN-OH



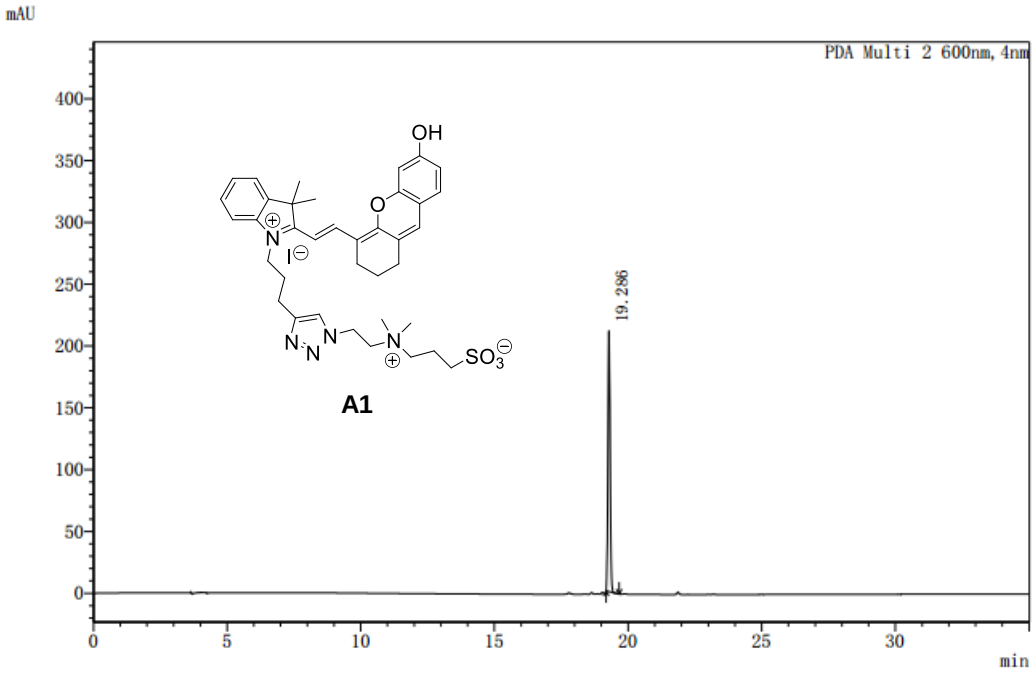
HPLC trace of ALPIN-3



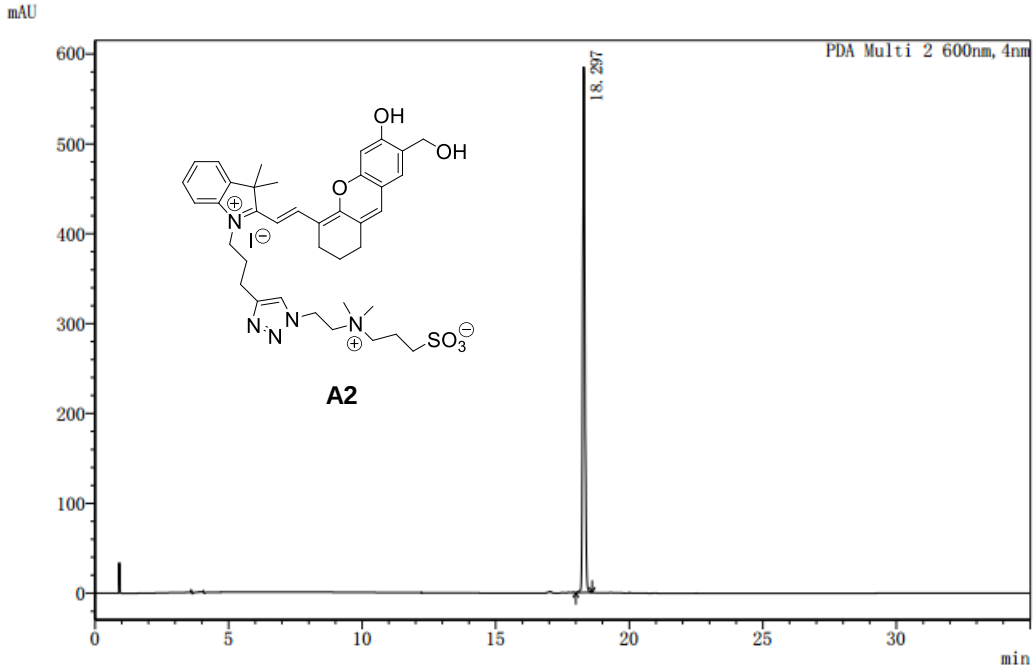
HPLC trace of ALPIN-4



HPLC trace of A1



HPLC trace of A2



A2