Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

A Preliminary Study for the Development of Cleavable Linkers Using Activatable Fluorescent Probes Targeting Leucine Aminopeptidase

Julie Kang, Seul-Ki Mun, Eu-Jin Choi, Jong-Jin Kim, Sung-Tae Yee, and Dong-Jo Chang

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A. Synthetic procedures

Materials

All reagents and solvents were obtained from commercial sources Sigma-Aldrich Chemical Co., Combi-Block, Tokyo Chemical Industries, Daejung Chemicals, and Alfa Aesar and used without purification. Anhydrous solvents were purchased from Sigma-Aldrich and used under dry nitrogen atmosphere. Ultrapure water was obtained from a water ultra-purification system. Completion of reaction was confirmed by thin layer chromatography (TLC) on Kiesegel $60F_{254}$ from Merck and all synthesized compounds were purified by flash column chromatography using ZEOprep 60 (40-63) μM silica gel from ZEOCHEM. ^{1}H and ^{13}C NMR spectra were measured on JEOL JNM-ECZ400s/L1 (400 MHz) and $CDCl_3$ or DMSO-d_6 was used as the NMR solvents. The chemical shifts were quoted in parts per million (ppm) and the coupling constant (*J)* were reported in hertz unit (Hz). Chemical shifts (in ppm) were referenced to tetramethylsilane (δ = 0 ppm) in $CDCl_3$ as an internal standard. ^{13}C NMR spectra were reported in ppm referenced to the center line of the triplet at 77.0 ppm for $CDCl_3$ or 39.5 ppm for DMSO-d_6. All *in vitro* enzyme assays were performed by taking the absorbance and emission on Synergy™ H1 microplate reader from BioTek. Leucine aminopeptidase from porcine kidney and other enzymes were purchased from Sigma-Aldrich.

A. Synthesis

General procedure A for amide coupling

To a solution of fluorophore (1.2 eq.) in CH_2Cl_2 was added EEDQ (1.2 eq.) and Boc-Leu-OH (1.0 eq.) The reaction mixture was stirred at RT for 4 h under N_2 atmosphere. After completion of reaction, the solvent was removed under reduced pressure and the residue was purified by flash column chromatography on silica gel to give the desired probe with a amide linkage.

General procedure B for the carbamate linkage

To a solution of fluorophore with a terminal -NH_2 group (1.0 eq) in CH_2Cl_2 was added a stock solution of phenyl chloroformate (1 M in CH_2Cl_2) and DIPEA (1 M in CH_2Cl_2) at 0 ^oC. The reaction mixture was stirred at RT for 2 h. The reaction was quenched with water and extracted with CH_2Cl_2. The organic layer was washed with water and dried over Na_2SO_4. The organic layer was filtered and concentrated under reduced pressure. The crude residue was purified by flash column chromatography on silica gel to afford the desired phenyl carbamate. The obtained phenyl carbamate (1.00 eq) and DBU (1.00 eq.) was added to a solution of the benzyl alcohol **41** (1.00 eq.) in acetone. The mixture was stirred at RT for 1h and then the solvent was removed under reduced pressure. The crude residue was purified by flash column chromatography on silica gel to produce the desired probe with a carbamate linkage.

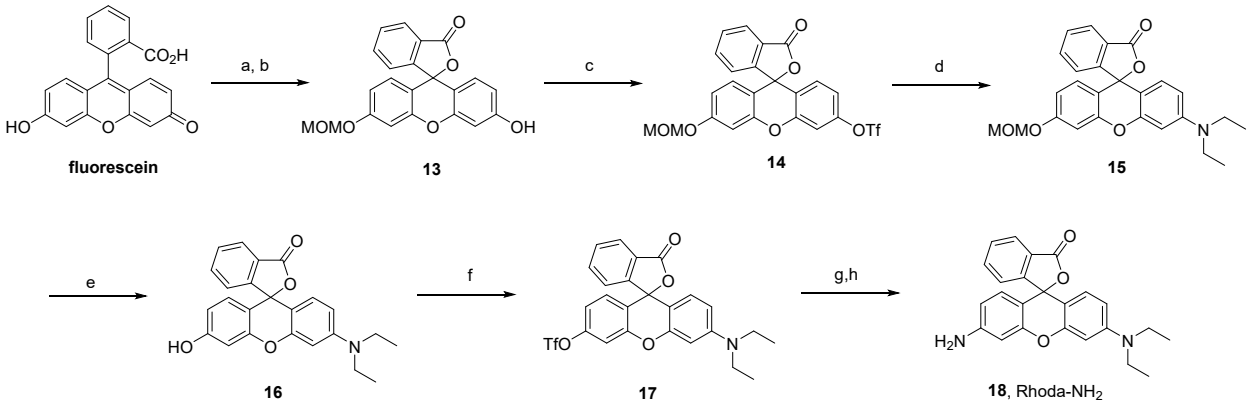
General procedure C for the ether linkage

To a solution of fluorophore with a terminal -OH group (1.0 eq.) in toluene was added the benzyl bromide **42** (1.2 eq.) and Ag_2O (1.5 eq.). The reaction mixture was heated at 120 ^oC for 2 to 12 h under N_2 atmosphere. After completion of reaction, the solvent was removed under reduced

pressure and the crude residue was purified by flash column chromatography on silica gel to give the desired probe with a ether linkage.

General procedure D for the *N*-Boc-deprotection

To a solution of *N*-Boc-protected probe (10 mg) in anhydrous CH_2Cl_2 (1 mL) was added dropwise a 30% solution of TFA in CH_2Cl_2 (0.6 mL) at -20 °C and was stirred overnight at -20 °C. The saturated aqueous $NaHCO_3$ solution was added to the reaction until pH 8 and the mixture was extracted with CH_2Cl_2. The combined organic layers were washed with water and dried over Na_2SO_4. The crude residue was purified by flash column chromatography on silica gel to produce the desired product.



Scheme S1. Synthesis of rhodamine fluorophore **18**. Reagents and conditions: (a) MOMCl, K_2CO_3. DMF, rt; (b) NaOH, THF/WATER, reflux, 13 % for 2 steps; (c) Tf_2O, pyridine, CH_2Cl_2, 0 °C to rt, 82 %; (d) Diethylamine, $Pd(OAc)_2$, Cs_2CO_3, BINAP, toluene, reflux, 76 %; (e) TFA, CH_2Cl_2, rt, 90 %; (f) N-Phenyl-bis-(trifluoromethanesulfonimide), K_2CO_3, CH_3CN, 0 °C to rt, 49 %; (g) Benzophenone Imine, $Pd(OAc)_2$, Cs_2CO_3, BINAP, toluene, reflux; (h) aq. 1N-HCl, THF, rt, 89 % for 2 steps.

3'-Hydroxy-6'-(methoxymethoxy)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-one (13)

To a solution of fluorescein (10 g, 30 mmol) and K_2CO_3 (10.4 g, 75 mmol) in DMF (150 mL) was added chloromethyl methyl ether (7.7 g, 90 mmol) at RT for 12 h under nitrogen atmosphere. The mixture was extracted with EtOAc and washed with 1N HCl solution, water and brine. The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure to provide a solid. The obtained solid was dissolved in a mixture of THF (150 mL) and aqueous NaOH solution (3 M, 0 mL). The mixture was heated to reflux for 2 h and cooled to RT. The residue was acidified with aqueous 1N HCl to pH 2 and then extracted with EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by column chromatography on silica gel (EtOAc: Hexane = 1: 2) to afford the compound **13** (1.47 g, 13 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.05 (d, J = 6.9 Hz, 1H), 7.75-7.83 (m, 2H), 7.71 (d, J = 2.3 Hz, 1H), 7.39 (d, J = 7.8 Hz, 1H), 7.25 (dd, J = 8.9, 2.5 Hz, 1H), 7.06 (d, J = 1.8 Hz, 2H), 6.84 (dd, J = 8.9, 2.5 Hz, 1H), 6.74-6.77 (m, 1H), 6.62 (s, 1H), 5.27 (d, J = 1.8 Hz, 2H), 3.38 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.36, 158.66, 152.05, 151.24, 150.99, 149.67, 136.05, 130.59, 129.18, 125.41, 125.05, 124.96, 124.15, 119.76, 117.42, 113.97, 111.42, 110.75, 103.30, 102.29, 93.94, 80.81, 55.83; HRMS (ESI$^+$): m/z Calcd for $C_{22}H_{16}O_6$ $[M]^+$: 376.0947, Found: 376.0949.

3'-(Methoxymethoxy)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl trifluoromethanesulfonate (14)

To a solution of compound **13** (100 mg, 0.266 mmol) and pyridine (85 mg, 1.064 mmol) in anhydrous CH_2Cl_2 (5 mL) was added dropwise trifluoromethanesulfonic anhydride (153 mg, 0.532 mmol) at 0 °C under nitrogen atmosphere. The mixture was stirred at RT for 3 h under nitrogen

atmosphere and then was quenched with water. The residue was extracted with CH_2Cl_2 and washed with 1N HCl, water and brine. The organic layer was dried with Na_2SO_4 and concentrated. The product was isolated by flash column chromatography on silica gel (EtOAc: Hexane = 1: 2) to yield the compound **14** (110 mg, 82 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (d, *J* = 7.8 Hz, 1H), 7.68 (dtd, *J* = 19.4, 7.4, 1.1 Hz, 2H), 7.25 (d, *J* = 2.3 Hz, 1H), 7.18 (d, *J* = 7.3 Hz, 1H), 7.00 (d, *J* = 2.3 Hz, 1H), 6.96 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.90 (d, *J* = 8.7 Hz, 1H), 6.78 (dd, *J* = 9.1, 2.3 Hz, 1H), 6.73 (d, *J* = 8.7 Hz, 1H), 5.21 (s, 2H), 3.48 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.8,159.1,152.2,151.9,149.8,135.2,130.0,129.7,128.8,126.1,125.1,123.8,119.7,118.5,116.4,113.8 ,111.6,110.1,103.5,94.2,81.4,55.8; HRMS (ESI^+): m/z Calcd for $C_{23}H_{15}F_3O_6S$ $[M]^+$: 508.0440, Found: 508.0438.

3'-(Diethylamino)-6'-(methoxymethoxy)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-one (15)

An oven-dried round bottom flask was charged with $Pd(OAc)_2$ (59 mg, 0.256 mmol), Cs_2CO_3 (1.18 g, 3.584 mmol) and BINAP (244 mg, 0.384 mmol) and was flushed with nitrogen gas for 10 min. A solution of compound **14** (1.3 g, 2.56 mmol) and diethylamine (1.91 g, 25.6 mmol) in anhydrous toluene (83 mL) was added, stirred under nitrogen atmosphere at RT for 20 min and heated at 100 ℃ for 20 h. After completion of the reation, the reaction mixture was cool down to RT, filtered through a pad of Celite and washed with CH_2Cl_2. The filtrate was concentrated and purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 2) to afford the compound **15** (1.1 g, 76 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (d, *J* = 7.8 Hz, 1H), 7.78 (dd, *J* = 7.3, 6.4 Hz, 1H), 7.71 (t, *J* = 7.3 Hz, 1H), 7.27 (d, *J* = 7.8 Hz, 1H), 6.96 (d, *J* = 2.3 Hz, 1H), 6.75 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.64 (d, *J* = 8.7 Hz, 1H), 6.45 (s, 3H), 5.24 (s, 2H), 3.41 (m, 4H), 3.37 (s, 3H), 1.08 (t, *J* = 6.9 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.78, 158.30, 152.38,

152.22, 149.26, 135.57, 130.10, 129.05, 128.68, 124.60, 124.10, 112.77, 112.52, 108.70, 103.26, 96.88, 93.92, 83.43, 55.80, 54.94, 43.81, 12.33; HRMS (ESI^+): m/z Calcd for $C_{26}H_{25}NO_5$ $[M]^+$: 431.1733, Found: 431.1738.

3'-(Diethylamino)-6'-hydroxy-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-one (16)

To a solution of compound **15** (100 mg, 0.23 mmol) in anhydrous CH_2Cl_2 (1 mL) was added dropwise the solution of trifluoroacetid acid (1 mL) at 0 ℃. The reaction mixture was stirred at 0 ℃ for 30 min and then warmed to RT followed by stirring of 1h. After completion of reaction, the mixture was quenched with aqueous $NaHCO_3$ to pH 7 and extracted with EtOAc, washed with water. The organic layer was dried with Na_2SO_4. The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 5: 1) to produce **16** (80 mg, 90 %). ^1H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, *J* = 8.7 Hz, 1H), 7.60-7.53 (m, 2H), 7.11 (d, *J* = 8.7 Hz, 1H), 6.93-6.83 (m, 2H), 6.74 (d, *J* = 2.3 Hz, 1H), 6.62-6.53 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.3, 166.5, 161.9, 159.0, 157.5, 156.7, 133.7, 132.5, 132.1, 131.2, 131.1, 130.9, 130.2, 130.1, 117.0, 116.5, 115.6, 115.2, 102.0, 96.0, 46.2, 11.6; HRMS (ESI^+): m/z Calcd for $C_{24}H_{21}NO_4$ $[M]^+$: 387.1471, Found: 387.1472.

3'-(Diethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl trifluoromethanesulfonate (17)

To a solution of compound **16** (200 mg, 0.516 mmol) in MeCN (20 mL) was added phenyl triflimide (372 mg, 1.032 mmol) and K_2CO_3 (287 mg, 2.064 mmol). The reaction mixture was stirred at RT for 12 h. After completion of the reaction, the solvent was removed under reduced pressure and the residue was extracted with CH_2Cl_2 and water. The organic layer was washed with brine and dried over Na_2SO_4. The crude residue was purified by flash column chromatography on

silica gel (EtOAc: Hexane = 1: 8) to produce **17** (130 mg, 49 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 7.8 Hz, 1H), 7.73-7.61 (m, 2H), 7.23-7.17 (m, 2H), 6.92 (dd, *J* = 8.7, 2.3 Hz, 1H), 6.87 (d, *J* = 8.7 Hz, 1H), 6.58 (d, *J* = 48.5 Hz, 3H), 3.38 (d, *J* = 6.9 Hz, 4H), 1.19 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.16, 152.60, 149.89,

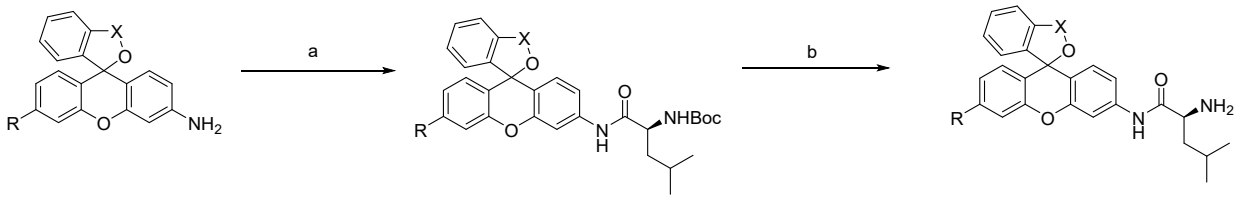
135.06, 130.97, 130.01, 128.80, 126.92, 125.12, 124.00, 120.28, 119.78, 117.65, 116.11, 110.31, 109.07, 104.42, 97.59, 82.79, 44.53, 12.45; HRMS (ESI^+): m/z Calcd for $C_{25}H_{20}FNO_6S$ $[M+H]^+$: 519.0963, Found 520.1035.

3'-Amino-6'-(diethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-one (18, Rhoda-NH_2)

An oven-dried round bottom flask was charged with $Pd(OAc)_2$ (16 mg, 0.023 mmol), Cs_2CO_3 (227 mg, 0.69 mmol) and BINAP (24 mg, 0.037 mmol) and flushed with nitrogen gas for 10 min. A solution of compound **17** (120 mg, 0.23 mmol) and benzophenone imine (53 mg, 0.276 mmol) in anhydrous toluene (6 mL) was added and stirred under nitrogen atmosphere at RT for 20 min and then heated at 100 ℃ for 4 h. The mixture was filtered through a pad of Celite and washed with CH_2Cl_2. The filtrate was concentrated and re-dissolved in THF (2.4 mL). The mixture was added dropwise 1N HCl (0.8 mL). The reaction mixture was stirred to RT for 1 h. The reaction was neutralized with saturated aqueous $NaHCO_3$ to pH 7-8. The residue was extracted with EtOAc and washed with water. The organic layer was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by column chromatography on silica gel (CH_2Cl_2: MeOH = 10: 1) to afford **18** (**Rhoda-NH_2**, 78 mg, 89 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.96 (d, *J* = 7.8 Hz, 1H), 7.76 (t, *J* = 7.3 Hz, 1H), 7.68 (t, *J* = 7.5 Hz, 1H), 7.24 (d, *J* = 7.3 Hz, 1H), 6.54-6.39 (m, 4H), 6.34 (q, *J* = 8.8 Hz, 2H), 5.78 (s, 1H), 3.33 (d, *J* = 6.9 Hz, 5H), 1.13-1.01 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.78, 168.09, 152.86, 152.74,

149.52, 135.09, 130.34, 129.83, 128.74, 127.17, 124.84, 124.51, 111.27, 108.63, 99.01, 96.92, 48.62, 43.86, 12.38; MS (ESI$^+$): m/z Calcd for $C_{24}H_{22}N_2O_3$ $[M+H]^+$: 387.17, Found 387.15.

Amide linkage without spacer



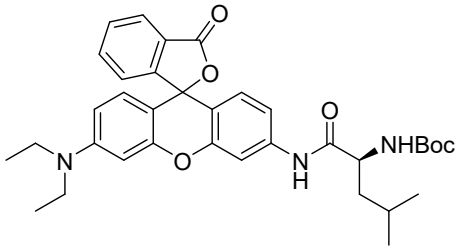
18, R=NEt_2, X=CO
19, R=OCH_3, X=CO
20, R=NEt_2, X=CH_2
21, R=OCH_3, X=CH_2

22, R=NEt_2, X=CO
23, R=OCH_3, X=CO
24, R=NEt_2, X=CH_2
25, R=OCH_3, X=CH_2

1, R=NEt_2, X=CO (**Rhoda-P1**)
2, R=OCH_3, X=CO (**Rhodo-P1**)
7, R=NEt_2, X=CH_2 (**Red Rhoda-P1**)
8, R=OCH_3, X=CH_2 (**Red Rhodo-P1**)

Scheme S2. Synthesis of LAP-responsive activatable fluorescent probes via direct amide linkage. Reagents and conditions: (a) Boc-Leu-OH, EEDQ, CH_2Cl_2, rt, **22** = 92 %, **23** = 67 %, **24** = 56 %, **25** = 81 %; (b) 30 % TFA in CH_2Cl_2, -20 ^oC, **1** = 30 %, **2** = 80 %, **7** = 52 %, **8** = 29 %.

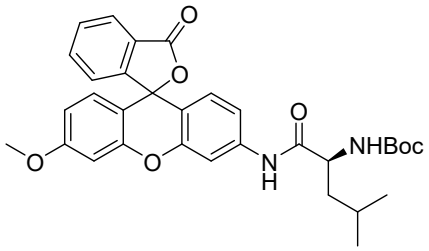
***tert*-Butyl ((2*S*)-1-((3'-(diethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (22)**



Compound **22** was synthesized by the amide coupling of **18** (**Rhoda-NH_2**, 30 mg, 0.078 mmol) with Boc-Leu-OH (15 mg, 0.065 mmol) and EEDQ (19 mg, 0.078 mmol) according to the general procedure A. The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 30: 1) to produce **22** in 92 % of yield (37 mg). ^{1}H-NMR (400 MHz, DMSO-d_6)

δ 10.24 (s, 1H), 7.98 (d, *J* = 7.8 Hz, 1H), 7.85 (s, 1H), 7.78 (t, *J* = 7.3 Hz, 1H), 7.70 (t, *J* = 7.5 Hz, 1H), 7.26 (d, *J* = 7.8 Hz, 1H), 7.11 (dd, *J* = 13.7, 8.2 Hz, 2H), 6.68 (d, *J* = 8.7 Hz, 1H), 6.47 (d, *J* = 14.2 Hz, 3H), 4.11 (d, *J* = 5.0 Hz, 1H), 3.36-3.32 (m, 4H), 1.63-1.51 (m, 2H), 1.43-1.30 (m, 1H), 1.37 (s, 9H), 1.08 (t, *J* = 6.9 Hz, 6H), 0.88 (t, *J* = 4.8 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 172.5, 168.8, 155.5, 152.5, 152.2, 151.3, 149.3, 141.0, 135.5, 130.0, 128.6, 128.4, 126.3, 124.6, 124.0, 114.9, 113.6, 108.6, 106.3, 104.5, 97.0, 83.3, 78.1, 53.6, 43.8, 28.2, 24.3, 22.9, 21.5, 12.3; HRMS (ESI$^+$): m/z Calcd for $C_{35}H_{41}N_3O_6$ $[M+H]^+$: 600.3029, Found: 600.3069.

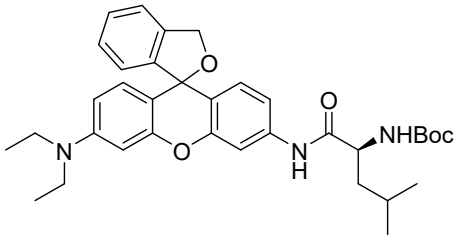
tert-Butyl ((2*S*)-1-((3'-methoxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (23)



Compound **23** was synthesized by the amide coupling of **19** (**Rhodo-NH$_2$**, 90 mg, 0.259 mmol) with Boc-Leu-OH (51 mg, 0.216 mmol) and EEDQ (65 mg, 0.259 mmol) according to the general procedure A. The crude residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 4) to produce **23** in 67 % of yield (80 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.28 (s, 1H), 8.01 (d, *J* = 7.8 Hz, 1H), 7.89 (d, *J* = 3.7 Hz, 1H), 7.79 (t, *J* = 7.1 Hz, 1H), 7.72 (t, *J* = 7.5 Hz, 1H), 7.26 (d, *J* = 7.1 Hz, 1H), 7.18 (d, *J* = 8.2 Hz, 1H), 7.10 (d, *J* = 8.2 Hz, 1H), 6.97 (d, *J* = 2.3 Hz, 1H), 6.78-6.63 (m, 3H), 4.14-4.01 (m, 1H), 3.81 (s, 3H), 1.62-1.65 (m, 1H), 1.58-1.46 (m, 1H), 1.37 (s, 9H), 1.3 (s, 1H), 0.92-0.82 (m, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 172.54, 168.68, 161.10, 155.51, 152.57, 151.79, 150.82, 141.16, 135.76, 130.24, 128.95, 128.48,

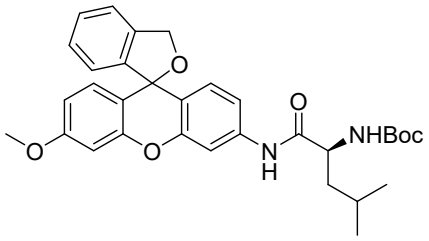
125.76, 124.76, 123.97, 115.40, 113.15, 112.13, 110.72, 106.24, 100.85, 82.13, 78.08, 55.69, 53.65, 28.19, 24.34, 22.93, 21.52; HRMS (ESI^+): m/z Calcd for $C_{32}H_{34}N_2O_7$ $[M+H]^+$: 559.2400, Found: 559.2447.

tert-Butyl ((2*S*)-1-((3'-(diethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (24)



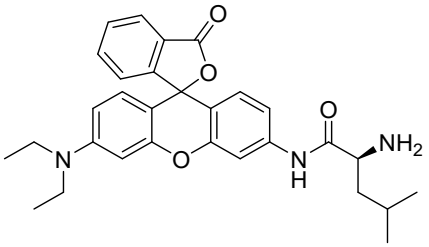
Compound **24** was synthesized by the amide coupling of **20** (**Red Rhoda-NH_2**, 50 mg, 0.134 mmol) with Boc-Leu-OH (26 mg, 0.112 mmol) and EEDQ (34 mg, 0.134 mmol) according to the general procedure A. The crude residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 4) to produce **24** in 56 % of yield (34 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.73 (q, J = 1.8 Hz, 1H), 7.43 (d, J = 7.3 Hz, 1H), 7.35 (t, J = 7.3 Hz, 1H), 7.23 (t, J = 7.3 Hz, 1H), 7.13 (dq, J = 8.6, 1.9 Hz, 1H), 6.81 (d, J = 8.2 Hz, 1H), 6.76 (d, J = 7.8 Hz, 1H), 6.66 (d, J = 8.7 Hz, 1H), 6.40 (td, J = 8.1, 2.4 Hz, 2H), 5.21 (s, 2H), 3.34 (d, J = 7.3 Hz, 5H), 2.07 (s, 2H), 1.79-1.69 (m, 1H), 1.50-1.43 (m, 1H), 1.36-1.29 (m, 1H), 1.08 (t, J = 6.9 Hz, 6H), 0.88 (dd, J = 10.1, 6.9 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 175.2, 151.0, 150.0, 148.2, 145.3, 139.4, 138.7, 129.4, 128.9, 128.1, 127.8, 123.1, 121.0, 120.0, 114.4, 111.4, 108.1, 105.8, 96.8, 82.7, 71.4, 54.0, 44.0, 43.6, 30.6, 24.2, 23.1, 21.8, 12.4; HRMS (ESI^+): m/z Calcd for $C_{35}H_{43}N_3O_5$ $[M+H]^+$: 586.3236, Found: 586.3278.

***tert*-Butyl ((2*S*)-1-((3'-methoxy-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (25)**



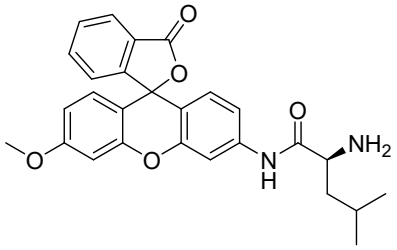
Compound **25** was synthesized by the amide coupling of **21** (**Red Rhodo-NH$_2$**, 50 mg, 0.151 mmol) with Boc-Leu-OH (30 mg, 0.126 mmol) and EEDQ (38 mg, 0.151 mmol) according to the general procedure A. The crude residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 3) to produce **25** in 81 % of yield (56 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.09 (d, J = 11.0 Hz, 1H), 7.74-7.72 (m, 1H), 7.45 (d, J = 7.3 Hz, 1H), 7.36 (t, J = 3.9 Hz, 1H), 7.23 (t, J = 4.1 Hz, 1H), 7.12 (d, J = 11.4 Hz, 1H), 6.90-6.81 (m, 3H), 6.74 (d, J = 7.8 Hz, 1H), 6.66 (dd, J = 8.7, 2.7 Hz, 1H), 5.28 (s, 2H), 4.18-4.06 (m, 1H), 3.77 (s, 3H), 1.68-1.59 (m, 1H), 1.55-1.47 (m, 1H), 1.44-1.39 (m, 1H), 1.36 (s, 9H), 0.91-0.86 (m, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 172.3, 154.6, 150.8, 148.8, 144.5, 139.5, 138.1, 131.5, 128.9, 128.1, 126.7, 124.6, 121.2, 120.2, 113.7, 111.6, 98.7, 95.6, 94.5, 79.1, 66.9, 62.2, 48.6, 41.4, 32.8, 30.5, 28.2, 22.8, 21.6; HRMS (ESI$^+$): m/z Calcd for $C_{32}H_{36}N_2O_6$ $[M+H]^+$: 545.2607, Found: 545.2659.

(2*S*)-2-Amino-*N*-(3'-(diethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)-4-methylpentanamide (1, Rhoda-P1)



Probe **1** (**Rhoda-P1**) was synthesized from **22** (35 mg, 0.078 mmol) according to the general procedure D. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 10: 1) to give probe **1** (**Rhoda-P1**) in 30 % of yield (9 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.98 (d, J = 7.3 Hz, 1H), 7.88 (s, 1H), 7.78 (t, J = 7.5 Hz, 1H), 7.70 (t, J = 7.3 Hz, 1H), 7.26 (d, J = 7.8 Hz, 1H), 7.18 (dd, J = 8.5, 1.6 Hz, 1H), 6.66 (d, J = 8.7 Hz, 1H), 6.47 (d, J = 12.8 Hz, 3H), 3.37-3.32 (m, 5H), 1.79-1.69 (m, 1H), 1.49-1.42 (m, 1H), 1.36-1.29 (m, 1H), 1.08 (t, J = 6.9 Hz, 6H), 0.88 (dd, J = 10.3, 6.6 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 176.0, 169.2, 152.9, 152.7, 151.7, 149.7, 141.4, 136.0, 130.4, 129.0, 128.7, 126.7, 125.0, 124.5, 115.4, 114.0, 109.0, 106.6, 104.9, 97.4, 83.8, 54.6, 44.4, 44.2, 24.6, 23.6, 22.3, 12.8; HRMS (ESI$^+$): m/z Calcd for $C_{30}H_{33}N_3O_4$ $[M+H]^+$: 500.2505, Found: 500.2542.

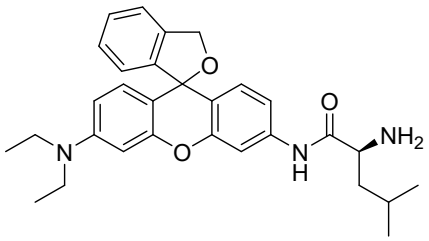
(2*S*)-2-Amino-*N*-(3'-methoxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)-4-methylpentanamide (2, Rhodo-P1)



Probe **2** (**Rhodo-P1**) was synthesized from **23** (30 mg, 0.54 mmol) according to the general procedure D, using 30 % trifluoroacetic acid in CH_2Cl_2 (1.8 mL). The residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 10: 1) to give 20 mg of probe **2** (**Rhodo-P1**) in 80 % of yield. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.01 (d, J = 7.3 Hz, 1H), 7.94 (d, J = 8.2 Hz, 1H), 7.79 (t, J = 7.5 Hz, 1H), 7.72 (t, J = 7.1 Hz, 1H), 7.31-7.18 (m, 2H), 6.97 (d, J = 2.3 Hz, 1H), 6.77-6.64 (m, 3H), 3.81 (s, 3H), 3.38 (s, 1H)3.35 (s, 2H), 1.80-1.67 (m, 1H), 1.52-1.42 (m,

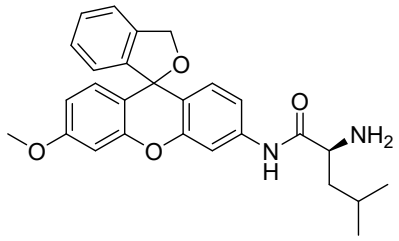
1H), 1.40-1.29 (m, 1H), 0.88 (dd, J = 9.6, 6.9 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 175.31, 168.66, 161.10, 152.53, 151.80, 150.83, 141.09, 135.74, 130.23, 128.95, 128.38, 125.78, 124.76, 123.96, 115.44, 113.10, 112.10, 110.73, 106.18, 100.83, 82.16, 55.68, 54.04, 43.78, 24.18, 23.14, 21.84; HRMS (ESI$^+$): m/z Calcd for $C_{27}H_{26}N_2O_5$ [M+H]$^+$: 459.1875, Found: 459.1917.

(2*S*)-2-Amino-*N*-(3'-(diethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)-4-methylpentanamide (7, Red Rhoda-P1)

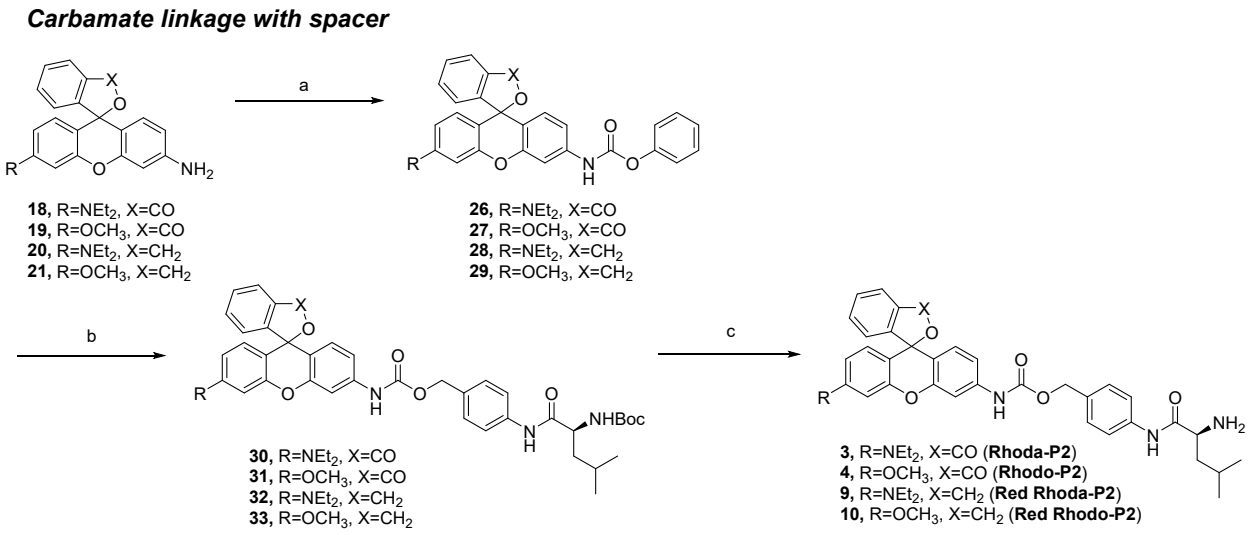


Probe **7** (**Red Rhoda-P1**) was synthesized of compound **24** (30 mg, 0.055 mmol) according to the general procedure D. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2: EA = 5: 1) to give probe **7** (**Red Rhoda-P1**) in 52 % of yield (14 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.74 (q, J = 2.1 Hz, 1H), 7.44 (d, J = 7.3 Hz, 1H), 7.35 (t, J = 7.3 Hz, 1H), 7.23 (t, J = 7.3 Hz, 1H), 7.13 (qd, J = 4.2, 2.2 Hz, 1H), 6.81 (d, J = 8.7 Hz, 1H), 6.76 (d, J = 7.3 Hz, 1H), 6.66 (d, J = 8.7 Hz, 1H), 6.40 (td, J = 8.7, 2.3 Hz, 2H), 5.21 (s, 2H), 3.35 (m, 5H), 2.53 (s, 2H), 1.79-1.69 (m, 1H), 1.49-1.42 (m, 1H), 1.35-1.28 (m, 1H), 1.08 (t, J = 6.9 Hz, 6H), 0.88 (dd, J = 10.1, 6.9 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 175.3, 151.0, 150.0, 148.2, 145.4, 139.5, 138.7, 129.5, 129.0, 128.1, 127.8, 123.2, 121.1, 120.0, 114.4, 111.3, 108.1, 105.8, 96.8, 82.7, 71.4, 54.1, 44.0, 43.7, 40.4, 24.2, 23.2, 21.9, 12.4; HRMS (ESI$^+$): m/z Calcd for $C_{30}H_{35}N_3O_3$ [M+H]$^+$: 486.2712, Found: 486.2750.

(2*S*)-2-Amino-*N*-(3'-methoxy-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)-4-methylpentanamide (8, Red Rhodo-P1)

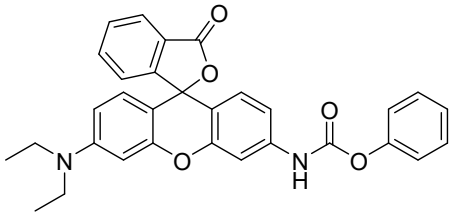


Probe **8** (**Red Rhodo-P1**) was synthesized from compound **25** (20 mg, 0.37 mmol) according to the general procedure D, using 30 % trifluoroacetic acid in CH_2Cl_2 (1.2 mL). The residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 10: 1) to give 5 mg of probe **8** (**Red Rhodo-P1**) in 29 % of yield. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.75 (q, J = 1.8 Hz, 1H), 7.45 (d, J = 7.3 Hz, 1H), 7.35 (t, J = 7.1 Hz, 1H), 7.23 (t, J = 7.1 Hz, 1H), 7.16 (dq, J = 8.5, 1.8 Hz, 1H), 6.89-6.80 (m, 3H), 6.73 (d, J = 7.3 Hz, 1H), 6.66 (dd, J = 8.9, 2.5 Hz, 1H), 5.28 (s, 2H), 3.77 (s, 3H), 2.07 (s, 2H), 1.81-1.69 (m, 1H), 1.51-1.43 (m, 1H), 1.36-1.29 (m, 1H), 0.93-0.85 (m, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 175.3, 159.9, 150.5, 149.6, 145.3, 139.6, 138.3, 129.6, 128.9, 128.2, 128.0, 123.0, 121.2, 119.6, 117.2, 114.9, 111.2, 105.8, 100.2, 82.4, 71.9, 55.4, 55.3, 54.1, 44.0, 30.6, 24.2, 23.1, 21.8; HRMS (ESI$^+$): m/z Calcd for $C_{27}H_{28}N_2O_4$ $[M+H]^+$: 445.2083, Found: 445.2128.



Scheme S3. Synthesis of LAP-responsive fluorescent probes via carbamate linkage with a spacer. Reagents and conditions: (a) Phenyl chloroformate, DIPEA, CH_2Cl_2, rt, **26** = 57 %, **27** = 69 %, **28** = 64 %, **29** = 85 %; (b) **41**, DBU, acetone, rt, **30** = 79 %, **31** = 73 %, **32** = 47 %, **33** = 60 %; (c) 30 % TFA in CH_2Cl_2, -20 ^{o}C, **3** = 47 %, **4** = 78 %, **9** = 47 %, **10** = 48 %.

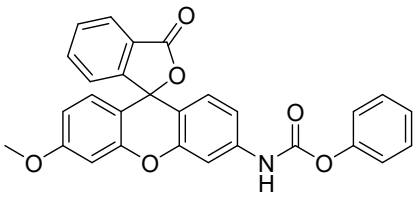
Phenyl (3'-(diethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (26)



Compound **26** was synthesized by carbamate of **18** (**Rhoda-NH_2**, 45 mg, 0.12 mmol) with 1 M Phenyl chloroformate (0.24 mL, 0.24 mmol) and 1 M DIPEA (0.24 mL, 0.24 mmol) in CH_2Cl_2 according to the general procedure B. The crude residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 4) to produce **26** in 57 % of yield (34 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.53 (s, 1H), 7.99 (d, J = 7.3 Hz, 1H), 7.80-7.76 (m, 1H), 7.71 (t,

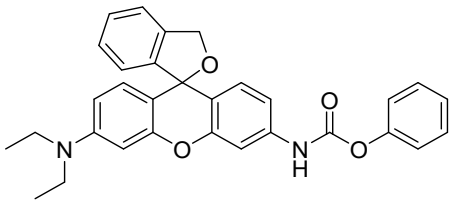
J = 7.5 Hz, 1H), 7.54 (d, *J* = 1.8 Hz, 1H), 7.43 (t, *J* = 7.8 Hz, 2H), 7.28-7.26 (m, 2H), 7.24-7.22 (m, 2H), 7.19 (dd, *J* = 8.7, 1.8 Hz, 1H), 6.70 (d, *J* = 8.7 Hz, 1H), 6.48 (d, *J* = 16.0 Hz, 3H), 3.37-3.32 (m, 4H), 1.08 (t, *J* = 7.1 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 174.9, 169.0, 153.5, 152.6, 152.4, 151.6, 149.4, 141.5, 139.1, 135.7, 131.0, 130.2, 129.2, 128.8, 128.7, 126.5, 124.8, 124.2, 119.2, 113.2, 105.2, 104.7, 97.2, 83.6, 54.1, 44.2, 44.0, 24.4, 23.4, 22.0, 12.5.

Phenyl (3'-methoxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (27)



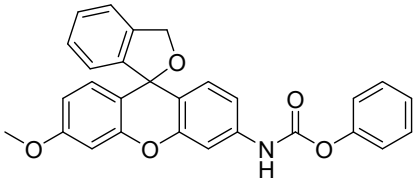
Compound **27** was synthesized from **19** (**Rhodo-NH$_2$**, 100 mg, 0.215 mmol) according to the general procedure B, using 1 M phenyl chloroformate (0.43 mL, 0.43 mmol) and 1 M DIPEA (0.43 mL, 0.43 mmol) in CH_2Cl_2. The residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 2) to give 68 mg of **27** in 69 % of yield. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.57 (s, 1H), 8.01 (d, *J* = 7.3 Hz, 1H), 7.80 (t, *J*= 7. 3, 1H), 7.72 (t, *J* = 7.1 Hz, 1H), 7.60 (s, 1H), 7.47-7.39 (m, 2H), 7.28 (d, *J* = 7.3 Hz, 2H), 7.24 (d, *J* = 7.8 Hz, 3H), 6.99 (d, *J* = 2.3 Hz, 1H), 6.76 (d, *J* = 8.7 Hz, 1H), 6.71 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.68 (s, 1H), 3.80 (s, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 168.66, 161.11, 152.52, 151.73, 151.63, 150.99, 150.30, 140.99, 135.75, 130.24, 129.49, 129.36, 128.94, 128.74, 125.76, 125.65, 124.78, 123.96, 121.93, 118.78, 115.20, 113.03, 112.15, 110.75, 105.39, 100.86, 82.08, 55.69; HRMS (ESI$^+$): m/z Calcd for $C_{28}H_{19}NO_6$ $[M+H]^+$: 466.1246, Found: 466.1284.

Phenyl (3'-(diethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (28)



Compound **28** was synthesized from **20** (**Red Rhoda-NH_2**, 40 mg, 0.107 mmol) with 1 M phenyl chloroformate (0.21 mmol, 0.21 mL) and 1 M DIPEA (0.21 mmol, 0.21 mL) in CH_2Cl_2 according to the general procedure B. The crude residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 2) to produce **28** in 64 % of yield (34 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.39 (s, 1H), 7.44-7.41 (m, 4H), 7.35 (t, *J* = 7.3 Hz, 1H), 7.25 (dd, J = 16.7, 7.5 Hz, 4H), 7.15 (dd, *J* = 8.7, 1.8 Hz, 1H), 6.85 (d, *J* = 8.7 Hz, 1H), 6.77 (d, *J* = 7.3 Hz, 1H), 6.67 (d, *J* = 8.7 Hz, 1H), 6.42-6.39 (m, 2H), 5.21 (s, 2H), 3.31 (t, *J* = 7.1 Hz, 3H), 1.07 (t, *J* = 7.1 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 152.3, 151.6, 151.0, 150.8, 148.8, 146.0, 139.9, 139.3, 130.1, 130.0, 128.8, 128.5, 126.2, 123.8, 122.6, 121.7, 120.6, 114.3, 112.0, 108.8, 105.7, 97.4, 83.2, 72.1, 55.5, 44.3, 13.0; HRMS (ESI$^+$): m/z Calcd for $C_{31}H_{28}N_2O_4$ $[M+H]^+$: 493.2083, Found: 493.2129.

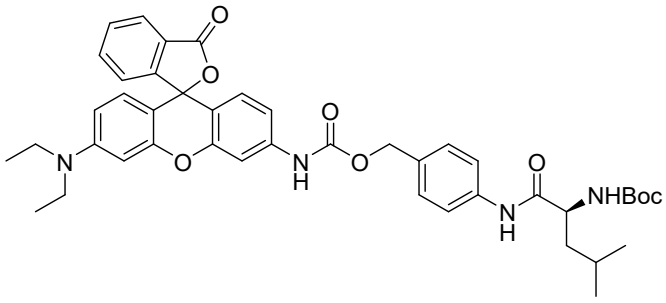
Phenyl (3'-methoxy-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (29)



Compound **29** was synthesized from **21** (**Red Rhodo-NH_2**, 20 mg, 0.06 mmol) according to the general procedure B, using 1 M phenyl chloroformate (0.12 mmol, 0.12 mL) and 1 M DIPEA (0.12 mmol, 0.12 mL) in CH_2Cl_2. The residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 2) to give 23 mg of compound **29** in 85 % of yield. ^{1}H-NMR (400 MHz,

DMSO-d_6) δ 10.43 (s, 1H), 7.44 (q, J = 8.1 Hz, 4H), 7.36 (t, J = 7.3 Hz, 1H), 7.28-7.22 (m, 4H), 7.18 (dd, J = 8.7, 1.8 Hz, 1H), 6.92 (d, J = 8.7 Hz, 1H), 6.87-6.84 (m, 2H), 6.76 (d, J = 7.3 Hz, 1H), 6.66 (dd, J = 8.7, 2.7 Hz, 1H), 5.29 (s, 2H), 3.77 (s, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 159.9, 151.6, 150.4, 150.4, 149.8, 145.4, 139.5, 138.3, 129.7, 129.5, 129.3, 128.3, 128.0, 125.6, 123.0, 121.9, 121.2, 119.6, 117.2, 114.2, 111.3, 105.0, 100.2, 82.4, 72.0, 55.5; HRMS (ESI$^+$): m/z Calcd for $C_{28}H_{21}NO_5$ $[M+H]^+$: 452.1453, Found: 452.1496.

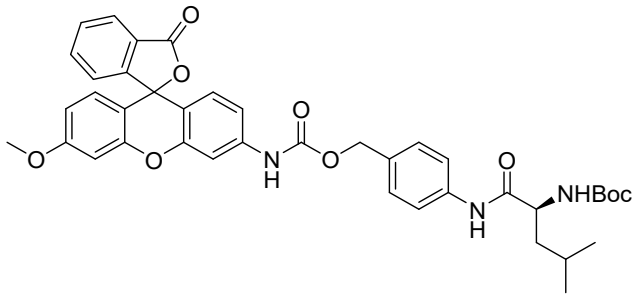
4-((*S*)-2-((*tert*-Butoxycarbonyl)amino)-4-methylpentanamido)benzyl (3'-(diethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (30)



Compound **30** was synthesized from **26** (28 mg, 0.055 mmol) with **41** (19 mg, 0.055 mmol) and DBU (9 mg,0.055 mmol) according to the general procedure B. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 20: 1) to give compound **30** in 79 % of yield (31 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.05 (s, 1H), 10.01 (s, 1H), 7.97 (d, J = 7.3 Hz, 1H), 7.77 (td, J = 7.5, 1.1 Hz, 1H), 7.72-7.68 (m, 1H), 7.61 (d, J = 8.2 Hz, 2H), 7.52 (d, J = 1.8 Hz, 1H), 7.36 (d, J = 8.2 Hz, 2H), 7.26 (d, J = 7.8 Hz, 1H), 7.13 (dd, J = 8.7, 2.3 Hz, 1H), 7.03 (d, J = 8.2 Hz, 1H), 6.64 (d, J = 8.7 Hz, 1H), 6.47 (d, J = 17.8 Hz, 3H), 5.10 (s, 2H), 4.11 (dd, J = 13.7, 9.1 Hz, 1H), 3.35 (t, J = 7.1 Hz, 4H), 1.62 (td, J = 13.4, 6.7 Hz, 1H), 1.55-1.48 (m, 1H), 1.41 (dd, J = 8.5, 5.3 Hz, 1H), 1.36 (s, 9H), 1.08 (t, J = 7.1 Hz, 6H), 0.88 (q, J = 3.2 Hz, 6H); ^{13}C-NMR

(100 MHz, DMSO- d_6) δ 171.9, 168.8, 158.2, 155.5, 153.3, 152.4, 152.2, 151.4, 149.2, 141.2, 139.0, 135.5, 130.9, 130.0, 129.0, 128.6, 128.5, 126.3, 124.5, 124.0, 119.1, 114.0, 112.9, 108.6, 105.0, 104.5, 97.0, 83.4, 78.0, 65.8, 53.5, 43.7, 40.6, 28.2, 24.3, 23.0, 21.5, 12.3; HRMS (ESI^+): m/z Calcd for $C_{43}H_{48}N_4O_8$ $[M+H]^+$: 749.3506, Found: 749.3558.

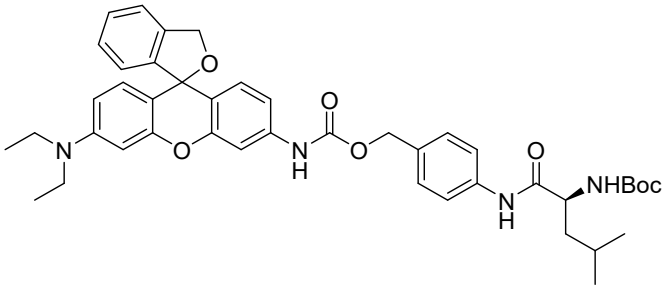
4-((*S*)-2-((*tert*-Butoxycarbonyl)amino)-4-methylpentanamido)benzyl (3'-methoxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (31)



Compound **31** was synthesized by the reaction of intermediate **27** (50 mg, 0.11 mmol) with benzyl alcohol **41** (37 mg, 0.11 mmol) and DBU (17 mg, 0.11 mmol) according to the general procedure B. The crude residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 2: 3) to produce **31** in 73 % of yield (57 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.09 (s, 1H), 10.01 (s, 1H), 8.00 (d, J = 7.8 Hz, 1H), 7.78 (t, J = 7.5 Hz, 1H), 7.71 (t, J = 7.3 Hz, 1H), 7.62 (d, J = 8.2 Hz, 2H), 7.57 (d, J = 1.4 Hz, 1H), 7.36 (d, J = 8.2 Hz, 2H), 7.26 (d, J = 7.8 Hz, 1H), 7.16 (dd, J = 8.7, 1.8 Hz, 1H), 7.03 (d, J = 7.8 Hz, 1H), 6.97 (d, J = 2.3 Hz, 1H), 6.70 (q, J = 4.1 Hz, 2H), 6.65 (d, J = 8.7 Hz, 1H), 5.10 (s, 2H), 4.11 (m, 1H), 3.80 (s, 3H), 1.62 (q, J = 6.4 Hz, 1H), 1.57-1.45 (m, 1H), 1.45-1.25 (m, 10H), 0.88 (q, J = 3.2 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 171.89, 168.66, 161.09, 155.46, 153.29 , 152.50, 151.78, 150.98, 141.47, 139.04, 135.73, 130.87, 130.22, 129.03, 128.57, 125.80, 124.76, 123.97, 119.09, 114.45, 112.44, 112.07, 110.79,

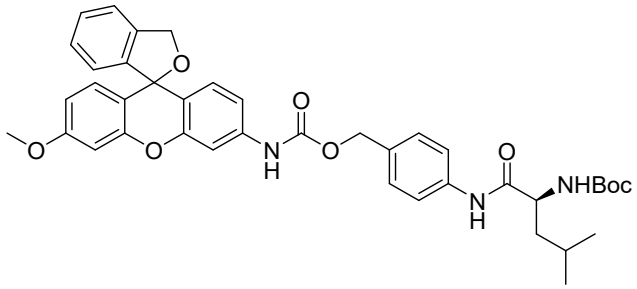
105.00, 100.85, 82.18, 78.00, 65.86, 55.68, 53.50 , 40.60, 28.19, 24.34, 22.97, 21.53; HRMS (ESI$^+$): m/z Calcd for $C_{40}H_{41}N_3O_9$ $[M+H]^+$: 708.2876, Found: 708.2926.

4-((*S*)-2-((tert-Butoxycarbonyl)amino)-4-methylpentanamido)benzyl (3'-(diethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (32)



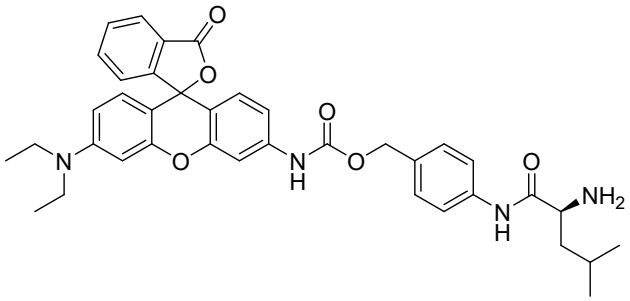
Compound **32** was synthesized from intermediate **28** (30 mg, 0.061 mmol) with benzyl alcohol **41** (21 mg, 0.061 mmol) and DBU (10 mg,0.061 mmol) according to the general procedure B. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 30: 1) to give **32** in 47 % of yield (21 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.08 (s, 1H), 7.71 (d, J = 7.3 Hz, 2H), 7.56-7.48 (m, 1H), 7.40 (d, J = 7.8 Hz, 2H), 7.37-7.30 (m, 1H), 7.09 (dd, J = 9.6, 2.7 Hz, 1H), 6.81-6.72 (m, 2H), 6.58 (d, J = 8.7 Hz, 1H), 6.48 (d, J = 8.7 Hz, 1H), 6.30 (q, J = 2.4 Hz, 2H), 5.29 (s, 2H), 5.11 (s, 2H), 3.36 (d, J = 14.6 Hz, 4H), 3.16 (d, J = 5.5 Hz, 1H), 1.60-1.39 (m, 1H), 1.39-1.27 (m, 1H), 1.25 (m, 1H), 1.22 (s, 9H), 1.07 (t, J = 6.9 Hz, 6H), 0.91-0.78 (m, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 172.9, 168.8, 155.5, 153.3, 152.4, 152.2, 151.4, 147.2, 140.5, 139.2, 132.5, 130.9, 130.0, 129.0, 128.6, 128.5, 125.3, 124.5, 122.3, 119.8, 116.3, 112.4, 108.6, 105.1, 104.8, 97.1, 83.4, 81.1, 66.0, 54.0, 44.2, 42.6, 28.5, 24.5, 23.4, 21.9, 12.5; HRMS (ESI$^+$): m/z Calcd for $C_{43}H_{50}N_4O_7$ $[M+H]^+$: 735.3713, Found: 735.3771.

4-((*S*)-2-((*tert*-Butoxycarbonyl)amino)-4-methylpentanamido)benzyl (3'-methoxy-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (33)



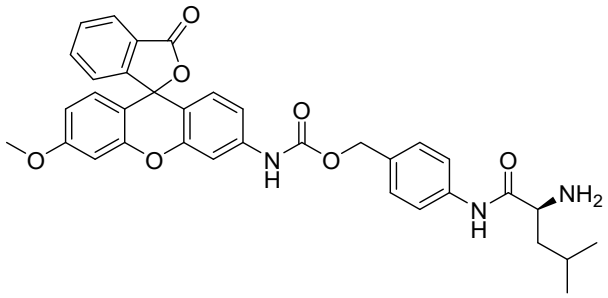
Compound **33** was synthesized by the reaction of intermediate **29** (35 mg, 0.078 mmol) with benzyl alcohol **41** (26 mg, 0.078 mmol) and DBU (12 mg, 0.078 mmol) according to the general procedure B. The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: EA = 20: 1) to produce compound **33** in 60 % of yield (32 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.03 (s, 1H), 9.94 (s, 1H), 7.62 (d, J = 8.7 Hz, 2H), 7.45 (d, J = 6.9 Hz, 2H), 7.35 (t, J = 7.1 Hz, 3H), 7.22 (t, J = 7.3 Hz, 1H), 7.12 (dd, J = 8.7, 1.8 Hz, 1H), 7.04 (d, J = 7.8 Hz, 1H), 6.76-6.73 (m, 3H), 6.60 (dd, J = 8.7, 2.3 Hz, 1H), 6.54 (d, J = 8.2 Hz, 1H), 5.27 (s, 2H), 5.09 (s, 2H), 4.14-4.08 (m, 1H), 3.76 (s, 3H), 1.62 (q, J = 6.6 Hz, 1H), 1.55-1.48 (m, 1H), 1.42 (dd, J = 8.5, 5.3 Hz, 1H), 1.36 (s, 9H), 0.88 (q, J = 3.1 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 173.3, 159.9, 155.5, 153.3, 150.7, 149.8, 145.5, 140.0, 139.0, 138.3, 129.7, 129.3, 129.0, 128.3, 128.1, 123.0, 121.0, 119.1, 117.6, 110.7, 100.1, 82.8, 78.0, 71.2, 65.7, 56.8, 55.4, 53.5, 44.4, 40.6, 28.2, 24.3, 23.0, 21.5; HRMS (ESI$^+$): m/z Calcd for $C_{40}H_{43}N_3O_8$ $[M+H]^+$: 694.3084, Found: 694.3133.

4-((*S*)-2-Amino-4-methylpentanamido)benzyl (3'-(diethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (3, Rhoda-P2)



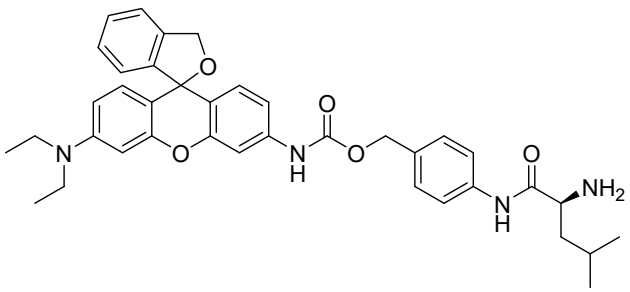
Probe **3** (**Rhoda-P2**) was synthesized from **30** (30 mg, 0.043 mmol) according to the general procedure D, using 30 % trifluoroacetic acid in CH_2Cl_2 (1.8 mL). The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 5: 1) to give 12 mg of probe **3** (**Rhoda-P2**) in 47 % of yield. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.01 (s, 1H), 7.94 (d, J = 7.3 Hz, 1H), 7.73 (t, J = 7.3 Hz, 1H), 7.67 (d, J = 7.3 Hz, 1H), 7.61 (d, J = 8.7 Hz, 2H), 7.48 (s, 1H), 7.32 (d, J = 8.7 Hz, 2H), 7.21 (d, J = 7.8 Hz, 1H), 7.09 (dd, J = 8.7, 1.8 Hz, 1H), 6.60 (d, J = 8.7 Hz, 1H), 6.43 (d, J = 16.9 Hz, 3H), 5.06 (s, 2H), 3.31 (m, 4H), 2.45 (t, J = 1.6 Hz, 1H), 1.73-1.66 (m, 1H), 1.46-1.39 (m, 1H), 1.31-1.24 (m, 1H), 1.04 (t, J = 7.1 Hz, 6H), 0.84 (dd, J = 10.1, 6.4 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 172.54, 168.68, 161.10, 155.51, 152.57, 151.79, 150.82, 141.16, 135.76, 130.24, 128.95, 128.48, 125.76, 124.76, 123.97, 115.40, 113.15, 112.13, 110.72, 106.24, 100.85, 82.13, 78.08, 55.69, 53.65, 28.19, 24.34, 22.93, 21.52; HRMS (ESI$^+$): m/z Calcd for $C_{38}H_{40}N_4O_6$ $[M+H]^+$: 649.2981, Found: 649.3028.

4-((*S*)-2-Amino-4-methylpentanamido)benzyl (3'-methoxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (4, Rhodo-P2)



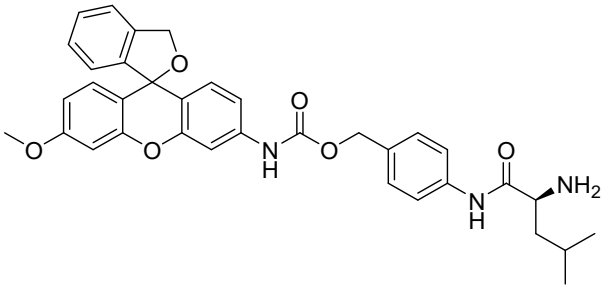
Probe **4** (**Rhodo-P2**) was synthesized of compound **31** (20 mg, 0.028 mmol) according to the general procedure D. The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: EA = 10: 1) to give probe **4** (**Rhodo-P2**) in 78 % of yield (22 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.08 (s, 1H), 8.00 (d, J = 7.3 Hz, 1H), 7.78 (t, J = 7.1 Hz, 1H), 7.71 (t, J = 7.3 Hz, 1H), 7.65 (d, J = 8.7 Hz, 2H), 7.58 (s, 1H), 7.36 (d, J = 8.7 Hz, 2H), 7.26 (d, J = 7.8 Hz, 1H), 7.16 (d, J = 8.7 Hz, 1H), 6.97 (d, J = 2.3 Hz, 1H), 6.75-6.62 (m, 3H), 5.10 (s, 2H), 3.80 (s, 3H), 1.80-1.67 (m, 1H), 1.51-1.41 (m, 1H), 1.36-1.26 (m, 1H), 0.88 (dd, J = 10.1, 6.4 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 174.90, 168.65, 161.08, 153.29, 152.49, 151.77, 150.97, 141.46, 138.94, 135.71, 130.78, 130.21, 129.02, 128.93, 128.56, 125.80, 124.75, 123.96, 119.01, 114.43, 112.44. 112.05, 110.79, 104.98, 100.85, 82.17, 65.88, 55.68, 53.93, 44.07, 24.21, 23.20, 21.82; HRMS (ESI$^+$): m/z Calcd for $C_{35}H_{33}N_3O_7$ $[M+H]^+$: 608.2352, Found: 608.2404.

4-((*S*)-2-Amino-4-methylpentanamido)benzyl (3'-(diethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (9, Red Rhoda-P2)



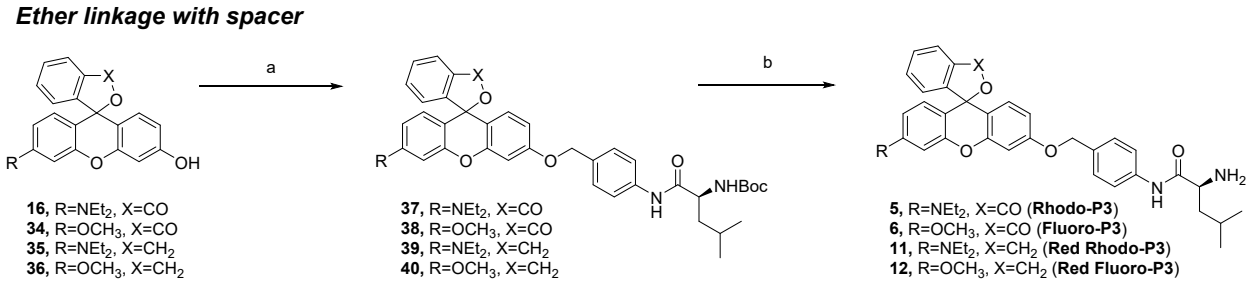
Probe **9** (**Red Rhoda-P2**) was synthesized from compound **32** (15 mg, 0.02 mmol) according to the general procedure D, using 30 % trifluoroacetic acid in CH_2Cl_2 (0.9 mL). The residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 5: 1) to give 6 mg of probe **9** (**Red Rhoda-P2**) in 47 % of yield. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.88 (s, 1H), 7.64 (dd, *J* = 6.9, 1.8 Hz, 2H), 7.43 (d, *J* = 7.3 Hz, 1H), 7.38-7.32 (m, 4H), 7.23 (t, *J* = 7.1 Hz, 1H), 7.09 (dd, *J* = 8.5, 2.1 Hz, 1H), 6.77 (dd, *J* = 12.8, 8.2 Hz, 2H), 6.65 (d, *J* = 8.7 Hz, 1H), 6.42-6.37 (m, 2H), 5.21 (d, *J* = 13.3 Hz, 2H), 5.10 (d, *J* = 12.3 Hz, 2H), 3.34 (d, *J* = 6.9 Hz, 4H), 3.16 (s, 1H), 1.80-1.69 (m, 1H), 1.49-1.42 (m, 1H), 1.34-1.27 (m, 1H), 1.07 (t, *J* = 6.9 Hz, 6H), 0.88 (dd, *J* = 10.5, 6.4 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 175.5, 151.5, 150.7, 148.7, 145.9, 139.5, 139.3, 130.0, 129.7, 129.6, 128.7, 128.3, 123.7, 121.6, 119.9, 119.5, 111.9, 108.6, 105.2, 97.3, 83.2, 66.2, 54.5, 44.6, 44.2, 24.7, 23.8, 22.4, 12.9; HRMS (ESI$^+$): m/z Calcd for $C_{38}H_{42}N_4O_5$ $[M+H]^+$: 635.3189, Found: 635.3226.

4-((*S*)-2-Amino-4-methylpentanamido)benzyl (3'-methoxy-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)carbamate (10, Red Rhodo-P2)



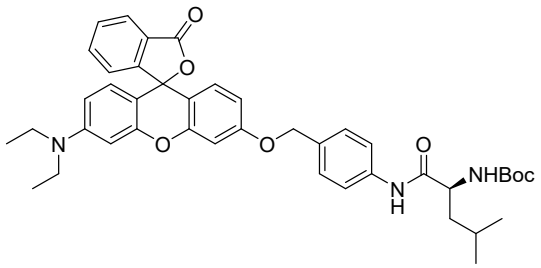
Probe **10** (**Red Rhodo-P2**) was synthesized of compound **33** (25 mg, 0.042 mmol) according to the general procedure D. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2: EA = 10: 1) to give probe **10** (**Red Rhodo-P2**) in 48 % of yield (10 mg). ^{1}H-NMR (400

MHz, DMSO-d_6) δ 9.94 (s, 1H), 7.65 (d, J = 8.7 Hz, 2H), 7.45 (d, J = 7.3 Hz, 2H), 7.37-7.33 (m, 3H), 7.22 (t, J = 7.1 Hz, 1H), 7.12 (dd, J = 8.5, 2.1 Hz, 1H), 6.87-6.82 (m, 3H), 6.74 (d, J = 7.8 Hz, 1H), 6.66 (dd, J = 8.7, 2.7 Hz, 1H), 5.27 (s, 2H), 5.09 (s, 2H), 4.10 (d, J = 7.3 Hz, 1H), 3.77 (s, 3H), 1.79-1.69 (m, 1H), 1.48-1.42 (m, 1H), 1.34-1.27 (m, 1H), 0.88 (dd, J = 10.5, 6.4 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO- d_6) δ 175.0, 159.9, 153.3, 150.5, 149.7, 145.4, 140.0, 138.9, 138.3, 130.9, 129.7, 129.2, 129.0, 128.3, 128.0, 123.0, 121.2, 119.0, 117.2, 111.2, 100.2, 82.4, 71.9, 65.8, 55.5, 54.9, 54.0, 48.6, 44.1, 24.2, 23.2, 21.8; HRMS (ESI$^+$): m/z Calcd for $C_{35}H_{35}N_3O_6$ $[M+H]^+$: 594.2559, Found: 594.2597.



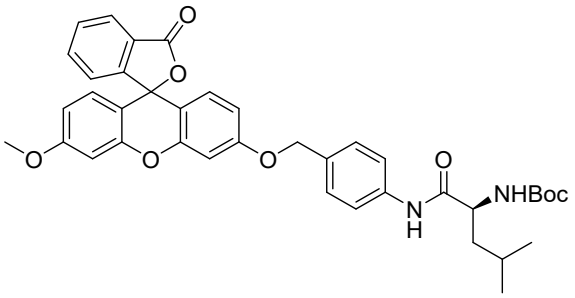
Scheme S4. Synthesis of LAP-responsive fluorescent probes via ether linkage with a spacer. Reagents and conditions: (a) **42**, Ag_2O, toluene, 120 °C, **37** = 52 %, **38** = 49 %, **39** = 24 %, **40** = 22 %; (b) 30 % TFA in CH_2Cl_2, -20 °C, **5** = 58 %, **6** = 42 %, **11** = 59 %, **12** = 48 %.

***tert*-Butyl ((2*S*)-1-((4-(((3'-(diethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)methyl)phenyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (37)**



Compound **37** was synthesized by the alkylation of **16** (**Rhodo-OH**, 50 mg, 0.129 mmol) with benzyl bromide **42** (62 mg, 0.155 mmol) and Ag_2O (45 mg, 0.194 mmol) according to the general procedure C. The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 20: 1) to produce **37** in 52 % of yield (47 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.00 (s, 1H), 7.98 (d, *J* = 7.3 Hz, 1H), 7.77 (t, *J* = 7.5 Hz, 1H), 7.70 (t, *J* = 7.5 Hz, 1H), 7.62 (d, *J* = 8.7 Hz, 2H), 7.38 (d, *J* = 8.2 Hz, 2H), 7.26 (d, *J* = 7.8 Hz, 1H), 7.03 (d, *J* = 7.8 Hz, 1H), 6.93 (d, *J* = 2.3 Hz, 1H), 6.73 (dd, *J* = 8.7, 2.3 Hz, 1H), 6.61 (d, *J* = 8.7 Hz, 1H), 6.45 (d, *J* = 1.8 Hz, 3H), 5.09 (s, 2H), 4.11 (dd, *J* = 14.4, 8.0 Hz, 1H), 3.40-3.35 (m, 4H), 1.64-1.58 (m, 1H), 1.55-1.48 (m, 1H), 1.42 (dd, *J* = 8.7, 5.5 Hz, 1H), 1.36 (s, 9H), 1.08 (t, *J* = 7.1 Hz, 6H), 0.88 (q, *J* = 3.4 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 168.7, 159.9, 155.4, 152.4, 152.2, 152.1, 149.2, 138.9, 135.5, 131.1, 130.0, 128.9, 128.6, 128.4, 126.3, 124.5, 124.0, 119.2, 112.2, 111.5, 104.5, 101.6, 96.9, 83.5, 78.0, 69.4, 53.5, 43.8, 40.6, 28.2, 24.3, 22.9, 21.6, 12.3; HRMS (ESI$^+$): m/z Calcd for $C_{42}H_{47}N_3O_7$ $[M+H]^+$: 706.3448, Found: 706.3492.

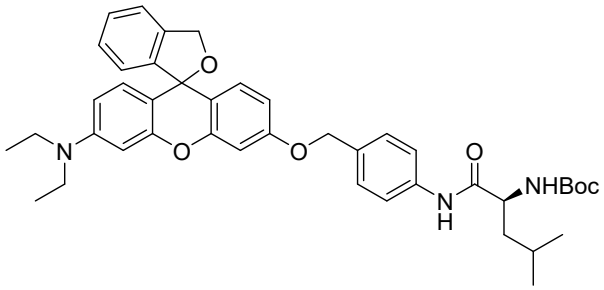
tert-Butyl ((2*S*)-1-((4-(((3'-methoxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)methyl)phenyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (38)



Compound **38** was synthesized by the reaction of **34** (**Fluor-OH**, 50 mg, 0.144 mmol) with benzyl bromide **42** (69 mg, 0.173 mmol) and Ag_2O (51 mg, 0.216 mmol) according to the general

procedure C. The crude residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 2) to produce **38** in 49 % of yield (47 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.98 (s, 1H), 8.00 (d, *J* = 7.3 Hz, 1H), 7.78 (t, *J* = 7.3 Hz, 1H), 7.71 (t, *J* = 7.5 Hz, 1H), 7.62 (d, *J* = 8.2 Hz, 2H), 7.38 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 7.3 Hz, 1H), 7.05-6.95 (m, 2H), 6.92 (d, *J* = 2.3 Hz, 1H), 6.78 (dd, *J* = 8.7, 2.3 Hz, 1H), 6.71 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.66 (dd, *J* = 8.9, 2.1 Hz, 2H), 5.11 (s, 2H), 4.10 (t, *J* = 7.1 Hz, 1H), 3.81 (s, 3H), 2.07 (s, 9H), 1.63 (t, *J* = 6.2 Hz, 1H), 1.59-1.46 (m, 1H), 0.88 (q, *J* = 3.2 Hz, 9H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 206.44, 171.80, 168.57, 161.05, 160.08, 152.43, 151.74, 151.66, 138.82, 135.67, 131.10, 130.19, 128.95, 128.40, 125.86, 124.69, 123.94, 119.22, 112.67, 111.98, 111.03, 110.83, 101.65, 100.77, 82.30, 77.99, 69.47, 55.63, 53.52, 37.91, 30.64, 28.16, 24.32, 22.90, 21.54; HRMS (ESI$^+$): m/z Calcd for $C_{39}H_{40}N_2O_8$ $[M+H]^+$: 665.2818, Found: 665.2861.

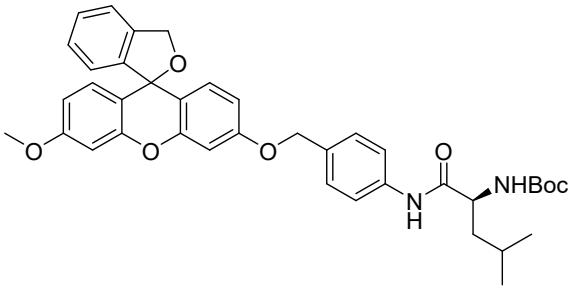
***tert*-Butyl ((2*S*)-1-((4-(((3'-(diethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)methyl)phenyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (39)**



Compound **39** was synthesized by the alkylation of **35** (**Red Rhodo-OH**, 40 mg, 0.107 mmol) with benzyl bromide **42** (51 mg, 0.128 mmol) and Ag_2O (37 mg, 0.161 mmol) according to the general procedure C. The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 30: 1) to produce **39** in 24 % of yield (18 mg). ^{1}H-NMR (400 MHz, DMSO-

d_6) δ 9.99 (s, 1H), 7.61 (d, J = 8.7 Hz, 2H), 7.43 (d, J = 7.8 Hz, 1H), 7.37 (d, J = 8.2 Hz, 2H), 7.33 (d, J = 7.3 Hz, 1H), 7.23 (t, J = 7.3 Hz, 1H), 7.02 (d, J = 7.8 Hz, 1H), 6.77 (td, J = 8.1, 2.9 Hz, 3H), 6.69-6.63 (m, 2H), 6.40 (dd, J = 8.7, 2.3 Hz, 1H), 6.34 (d, J = 2.3 Hz, 1H), 5.18 (s, 2H), 5.05 (s, 2H), 4.11 (q, J = 7.2 Hz, 1H), 3.42-3.37 (m, 4H), 3.29 (m, 1H), 1.66-1.59 (m, 1H), 1.55-1.48 (m, 1H), 1.44-1.40 (m, 1H), 1.36 (s, 9H), 1.07 (t, J = 7.1 Hz, 6H), 0.88 (q, J = 3.4 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 171.9, 158.7, 155.5, 151.0, 148.1, 145.3, 138.8, 131.4, 129.8, 129.6, 128.4, 128.2, 127.8, 119.2, 117.7, 111.4, 96.7, 82.7, 78.0, 69.2, 67.2, 63.1, 53.5, 49.5, 43.7, 40.6, 29.0, 28.2, 24.4, 23.0, 21.6, 12.4; HRMS (ESI$^+$): m/z Calcd for $C_{42}H_{49}N_3O_6$ $[M+H]^+$: 692.3655, Found: 692.3702.

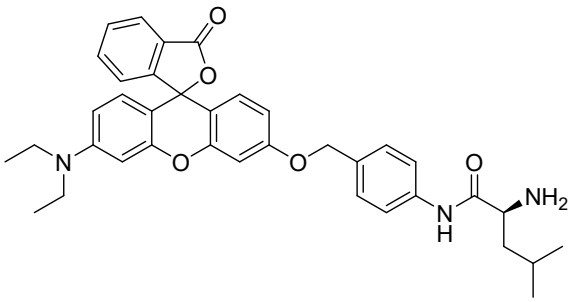
tert-Butyl ((2*S*)-1-((4-(((3'-methoxy-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)methyl)phenyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (40)



Compound **40** was synthesized by the reaction of **36** (**Red Fluor-OH**, 100 mg, 0.30 mmol) with benzyl bromide **42** (144 mg, 0.36 mmol) and Ag_2O (104 mg, 0.45 mmol) according to the general procedure C. The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 20: 1) to produce **40** in 22 % of yield (43 mg). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.00 (s, 1H), 7.61 (d, J = 8.7 Hz, 1H), 7.61 (D, J = 8.7 Hz, 2H), 7.36 (q, J = 7.3 Hz, 3H), 7.23 (t, J = 7.3 Hz, 1H), 7.03 (d, J = 8.2 Hz, 1H), 6.83 (q, J = 4.3 Hz, 3H), 6.65 (dd, J = 8.7, 2.7 Hz, 1H), 6.56 (d, J = 2 Hz, 1H), 6.50 (dd, J = 8.5, 2.5 Hz, 1H), 5.25 (s, 2H), 5.07 (s, 2H), 4.10 (d, J =

15.1 Hz, 1H), 3.77 (s, 3H), 1.60 (d, *J* = 7.3 Hz, 1H), 1.58-1.46 (m, 1H), 1.41 (d, *J* = 8.2 Hz, 1H), 1.37 (s, 9H), 0.88 (q, *J* = 3.2 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 159..56, 157.90, 150.34, 150.26, 145.13, 138.28, 129.58, 129.50, 128.00, 127.70, 122.88, 120.90, 117.09, 115.59, 111.74, 110.81, 101.36, 99.90, 82.33, 71.37, 56.21, 40.39, 27.98, 24.82, 24.14, 21.34; HRMS (ESI$^+$): m/z Calcd for $C_{39}H_{42}N_2O_7$ $[M+H]^+$: 651.3026, Found: 651.3067.

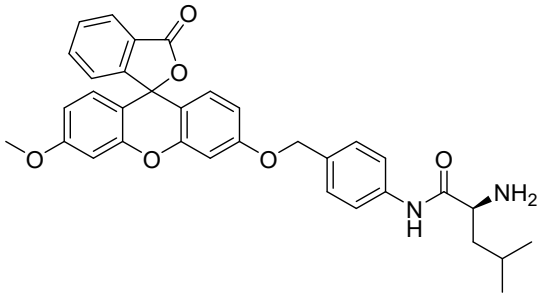
(2*S*)-2-Amino-N-(4-(((3'-(diethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)methyl)phenyl)-4-methylpentanamide (5, Rhodo-P3)



Probe **5** (**Rhodo-P3**) was synthesized from **37** (40 mg, 0.057 mmol) according to the general procedure D, using 30 % trifluoroacetic acid in CH_2Cl_2 (2.4 mL). The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 5: 1) to give 20 mg of probe **5** (**Rhodo-P3**) in 58 % of yield. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.98 (d, *J* = 7.8 Hz, 1H), 7.77 (td, *J* = 7.4, 1.1 Hz, 1H), 7.70 (td, *J* = 7.5, 0.9 Hz, 1H), 7.65 (d, *J* = 8.2 Hz, 2H), 7.38 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 7.8 Hz, 1H), 6.93 (d, *J* = 2.3 Hz, 1H), 6.73 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.61 (d, *J* = 8.7 Hz, 1H), 6.45 (d, *J* = 2.7 Hz, 3H), 5.09 (s, 2H), 3.36-3.29 (m, 5H), 1.79-1.69 (m, 1H), 1.49-1.42 (m, 1H), 1.37-1.27 (m, 1H), 1.08 (t, *J* = 7.1 Hz, 6H), 0.88 (dd, *J* = 10.5, 6.4 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 174.9, 168.7, 159.9, 152.4, 152.2, 152.1, 149.2, 138.8, 135.5, 131.1, 130.0, 128.9, 128.6, 128.4, 126.4, 124.5, 124.0, 119.1, 112.2, 111.5, 108.6, 104.5, 101.6, 96.9,

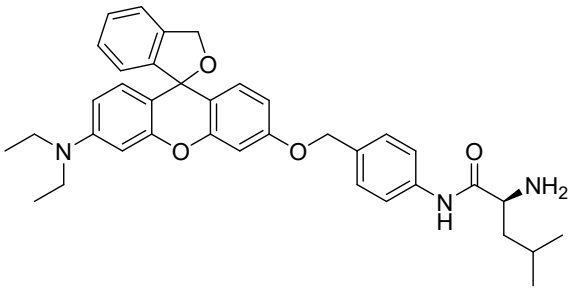
83.5, 69.4, 54.0, 44.1, 43.8, 24.2, 23.2, 21.9, 12.3; HRMS (ESI^+): m/z Calcd for $C_{37}H_{39}N_3O_5$ $[M+H]^+$: 606.2923, Found: 606.2973.

(2*S*)-2-Amino-N-(4-(((3'-methoxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)methyl)phenyl)-4-methylpentanamide (6, Fluor-P3)



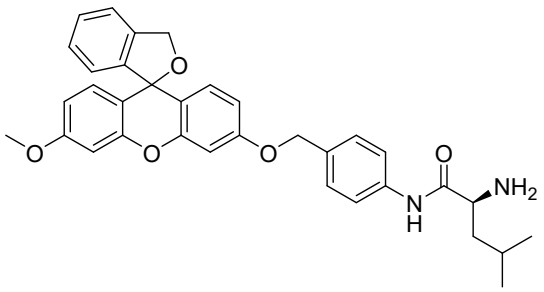
Probe **6** (**Fluor-P3**) was synthesized from **38** (20 mg, 0.03 mmol) according to the general procedure D, using 30 % trifluoroacetic acid in CH_2Cl_2 (1.2 mL). The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 15: 1) to give 7 mg of probe **6** (**Fluor-P3**) in 42 % of yield. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.97 (d, J = 7.3 Hz, 1H), 7.74 (t, J = 7.1 Hz, 1H), 7.68 (t, J = 7.3 Hz, 1H), 7.61 (d, J = 8.2 Hz, 2H), 7.35 (d, J = 8.7 Hz, 2H), 7.23 (d, J = 7.3 Hz, 1H), 6.91 (dd, J = 19.4, 2.5 Hz, 2H), 6.74 (dd, J = 8.7, 2.7 Hz, 1H), 6.69-6.62 (m, 3H), 5.07 (s, 2H), 3.77 (s, 3H), 3.34-3.31 (m, 1H), 1.68 (td, J = 13.7, 6.5 Hz, 1H), 1.48-1.40 (m, 1H), 1.33-1.26 (m, 1H), 0.84 (dd, J = 9.8, 6.6 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 168.6, 161.1, 160.1, 152.5, 151.7, 151.7, 138.7, 135.7, 131.1, 130.2, 129.0, 128.5, 125.9, 124.7, 124.0, 119.1, 112.7, 112.0, 111.0, 110.8, 101.6, 100.8, 82.3, 69.5, 55.7, 53.8, 43.8, 42.1, 40.4, 29.0, 24.2, 23.1, 21.9; HRMS (ESI^+): m/z Calcd for $C_{34}H_{32}N_2O_6$ $[M+H]^+$: 565.2294, Found: 565.2334.

(2*S*)-2-Amino-N-(4-(((3'-(diethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)methyl)phenyl)-4-methylpentanamide (11, Red Rhodo-P3)

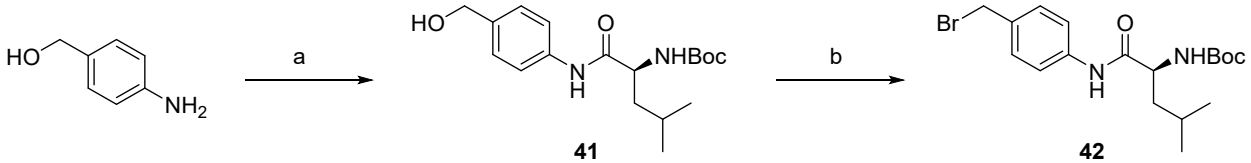


Probe **11** (**Red Rhodo-P3**) was synthesized from **39** (20 mg, 0.029 mmol) according to the general procedure D, using 30 % trifluoroacetic acid in CH_2Cl_2 (1.2 mL). The crude residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 5: 1) to give 10 mg of probe **11** (**Red Rhodo-P3**) in 59 % of yield. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.65 (d, J = 8.2 Hz, 2H), 7.43 (d, J = 7.3 Hz, 1H), 7.37 (d, J = 8.2 Hz, 2H), 7.34 (d, J = 7.3 Hz, 1H), 7.23 (t, J = 7.3 Hz, 1H), 6.80-6.73 (m, 3H), 6.69-6.64 (m, 2H), 6.40 (dd, J = 8.9, 2.5 Hz, 1H), 6.34 (d, J = 2.7 Hz, 1H), 5.18 (s, 2H), 5.06 (s, 2H), 3.31 (s, 5H), 3.18-3.29 (2H), 1.79-1.69 (m, 1H), 1.50-1.43 (m, 1H), 1.36-1.29 (m, 1H), 1.07 (t, J = 6.9 Hz, 6H), 0.88 (dd, J = 10.1, 6.9 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 174.6, 158.7, 151.0, 150.8, 148.1, 145.3, 138.8, 138.7, 131.4, 129.8, 129.5, 128.4, 128.1, 127.8, 123.2, 121.0, 119.1, 117.7, 111.3, 108.1, 101.0, 96.7, 82.7, 71.2, 69.2, 53.9, 43.9, 43.7, 24.2, 23.2, 21.9, 12.4; HRMS (ESI$^+$): m/z Calcd for $C_{37}H_{41}N_3O_4$ $[M+H]^+$: 592.3131, Found: 592.3177.

(2*S*)-2-Amino-N-(4-(((3'-methoxy-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)oxy)methyl)phenyl)-4-methylpentanamide (12, Red Fluor-P3)



Probe **12** (**Red Fluor-P3**) was synthesized from **40** (25 mg, 0.038 mmol) according to the general procedure D, using 30 % trifluoroacetic acid in CH_2Cl_2 (1.5 mL). The residue was purified by flash column chromatography on silica gel (CH_2Cl_2: MeOH = 10: 1) to give 10 mg of probe **12** (**Red Fluor-P3**) in 48 % of yield. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.65 (d, J = 8.7 Hz, 2H), 7.45 (d, J = 7.8 Hz, 1H), 7.36 (t, J = 8.7 Hz, 3H), 7.23 (t, J = 7.5 Hz, 1H), 6.83 (q, J = 2.9 Hz, 3H), 6.78-6.71 (m, 3H), 6.66 (dd, J = 8.7, 2.3 Hz, 1H), 5.25 (s, 2H), 5.07 (s, 2H), 3.77 (s, 3H), 3.40-3.37 (m, 1H), 1.73 (q, J = 7.0 Hz, 1H), 1.49-1.42 (m, 1H), 1.34-1.27 (m, 1H), 0.88 (dd, J = 10.4, 6.5 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 159.8, 158.9, 150.5, 150.4, 145.3, 138.7, 138.4, 129.8, 128.4, 128.3, 128.0, 123.1, 121.2, 119.1, 117.4, 117.2, 111.9, 111.2, 101.0, 100.1, 82.4, 71.8, 69.3, 55.4, 53.9, 44.1, 28.7, 24.2, 23.2, 21.9; HRMS (ESI$^+$): m/z Calcd for $C_{34}H_{34}N_2O_5$ $[M+H]^+$: 551.2501, Found: 551.2546.



Scheme S5. Synthesis of Boc-Br-PABA-Leu (**42**). Reagents and conditions: (a) Boc-Leu-OH, EEDQ, CH_2Cl_2, rt, 90 %; (b) PPh_3, NBS, rt, 64 %

***tert*-Butyl *(S)*-(1-((4-(hydroxymethyl)phenyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (41)**

To a solution of Boc-Leu-OH (1 g, 4.3 mmol) in CH_2Cl_2 (40 mL) was added (4-aminophenyl)methanol (650 mg, 5.2 mmol) and EEDQ (1.3 g, 5.2 mmol) under nitrogen atmosphere and stirred at RT for 12 h. After completion of the reaction, the solvent was evaporated and the crude residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 3: 2) to produce benzyl alcohol **41** (1.3 g, 90 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.88 (s, 1H), 7.53 (d, J = 8.2 Hz, 2H), 7.22 (d, J = 8.2 Hz, 2H), 7.00 (d, J = 8.2 Hz, 1H), 5.09 (t, J = 5.7 Hz, 1H), 4.42 (d, J = 5.9 Hz, 2H), 4.15-4.04 (m, 1H), 1.72-1.57 (m, 1H), 1.56-1.45 (m, 1H), 1.44-1.40 (m, 1H), 1.37 (s, 9H), 0.88 (q, J = 3.4 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.61, 155.42, 137.64, 137.33, 126.87, 118.95, 77.97, 62.58, 53.47, 40.68, 28.20, 24.34, 22.96, 21.56; HRMS (ESI$^+$): m/z Calcd for $C_{18}H_{28}N_2O_4$ $[M+Na]^+$: 359.2083, Found 359.1938.

***tert*-Butyl (*S*)-(1-((4-(bromomethyl)phenyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (42)**

To a solution of benzyl alcohol **41** (100 mg, 0.3 mmol) in anhydrous CH_2Cl_2 (10 mL) was added triphenylphosphine (94 mg, 0.36 mmol) and NBS (65 mg,0.36 mmol) and stirred at RT for 1.5 h. After completion of the reaction, the mixture was extracted with CH_2Cl_2 and washed with water. The organic layer was washed with brine and dried over Na_2SO_4. The crude residue was purified by flash column chromatography on silica gel (EtOAc: Hexane = 1: 4) to produce benzyl bromide **42** (77 mg, 64 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.95 (s, 1H), 7.57 (d, J = 8.2 Hz, 2H), 7.26 (d, J = 8.2 Hz, 2H), 4.42 (s, 2H), 4.13-4.07 (m, 1H), 1.68-1.58 (m, 1H), 1.57-1.45 (m, 1H), 1.45-1.27 (m, 11H), 0.88 (dd, J = 6.4, 4.1 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.76, 155.45, 138.38 , 133.08, 128.22, 119.05 , 77.98, 70.86, 53.51, 40.63, 28.20, 24.35, 22.96, 21.57.

B. General procedure for fluorescence analysis

All spectroscopic readings were measured with BioTek Synergy™ H1 spectrophotometer, using 96 well microplate. A parent stock solution of probes was dissolved in DMSO to obtain 10 mM. Leucine aminopeptidase was dissolved in deionized water to obtain 1mg/mL. All spectra measurements were carried out in PBS buffer solution (PBS buffer, 10 mM, pH 7.4, 37 ℃). The final volume in 96 well microplate was adjusted to 200 μL. RPMI 1640 was obtained from Thermo SCIENTIFIC (Waltham, MA, USA). Bestatin hydrochloride (LAP inhibitor), DMSO and 2-mercaptoethanol were purchased from Sigma Aldrich (St. Louis, Mo, USA). All experiments were performed three times independently under identical or similar conditions and the data were expressed as the mean ± standard deviation.

C. pH-Dependent fluorescence of fluorophores

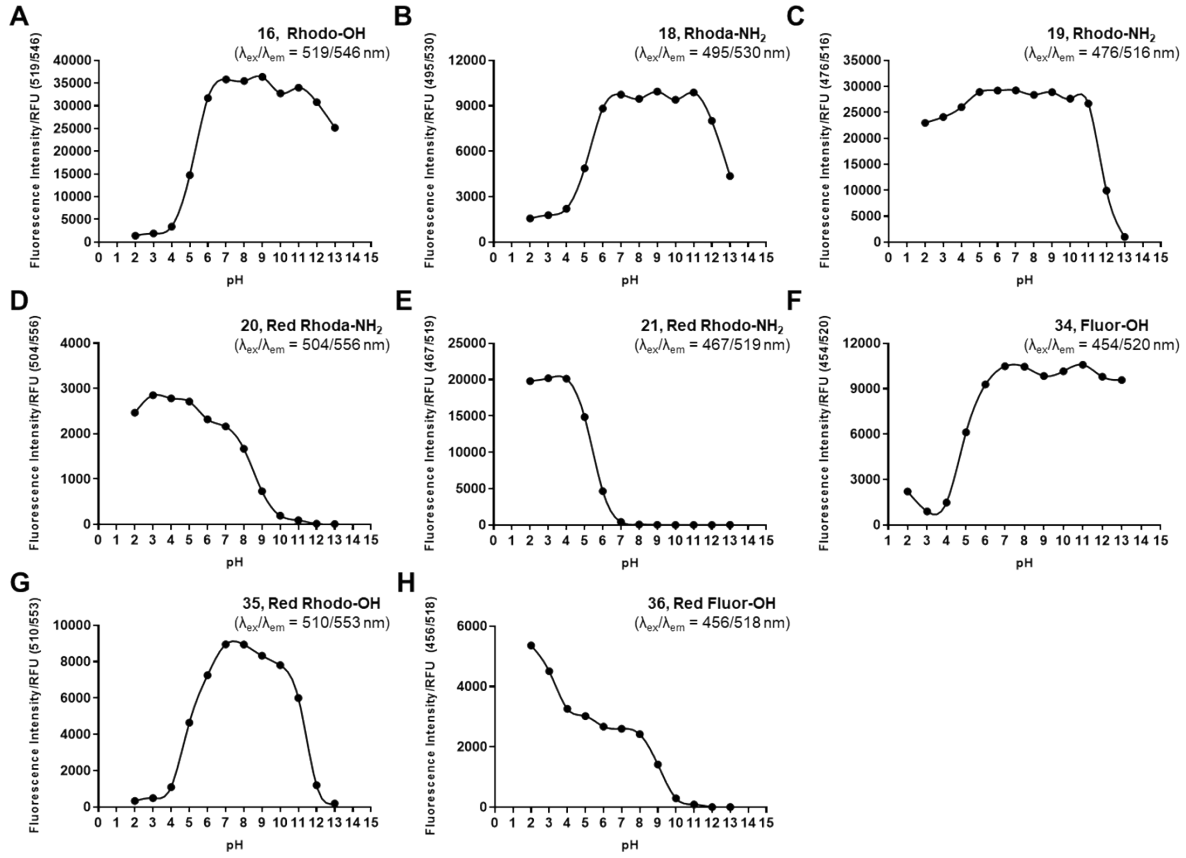


Figure S1. pH-Dependent fluorescence spectra of fluorophores (Normalized fluorescence intensity plotted against pH at 1.0 μM) in buffers with pH ranging from 2 to 13.

D. Thermal and pH-dependent stability of activatable fluorescent probes

Thermal stability was evaluated by the incubation of 1 μM of the probe in PBS buffer (pH 7.4) at different temperatures ranging from 25 to 45 ^{o}C for 20 min and reading fluorescence at each temperature. The pH stability test was performed by the incubation with 1 μM probe in different

pH buffers (2 to 13) at 25 °C and recording fluorescence at each pH. All experiments were performed in triplicate and the data were expressed as the mean ± standard deviation.

E. Concentration optimization of LAP reaction

The concentration optimization of LAP kinetic study was performed by incubation of various concentrations (1, 5, 10, 20, 50 and 100 μg/mL) of LAP with 1 μM probe in PBS buffer (10 mM, pH 7.4) at 37 °C for 2 h and recording the fluorescence spectra at each concentration of LAP in Synergy H1 microplate reader (Figure S4A). LAP (10 μg/mL) was incubated with different concentrations of probe (0.05, 0.1, 0.2, 0.5, 1 μM) in PBS buffer (10 mM, pH 7.4) at 37 ℃ for 2 h and recording the fluorescence spectra at each concentration of probe in Synergy H1 microplate reader (Figure S4B and S4C). All experiments were performed in triplicate and data are expressed as the mean ± standard deviation.

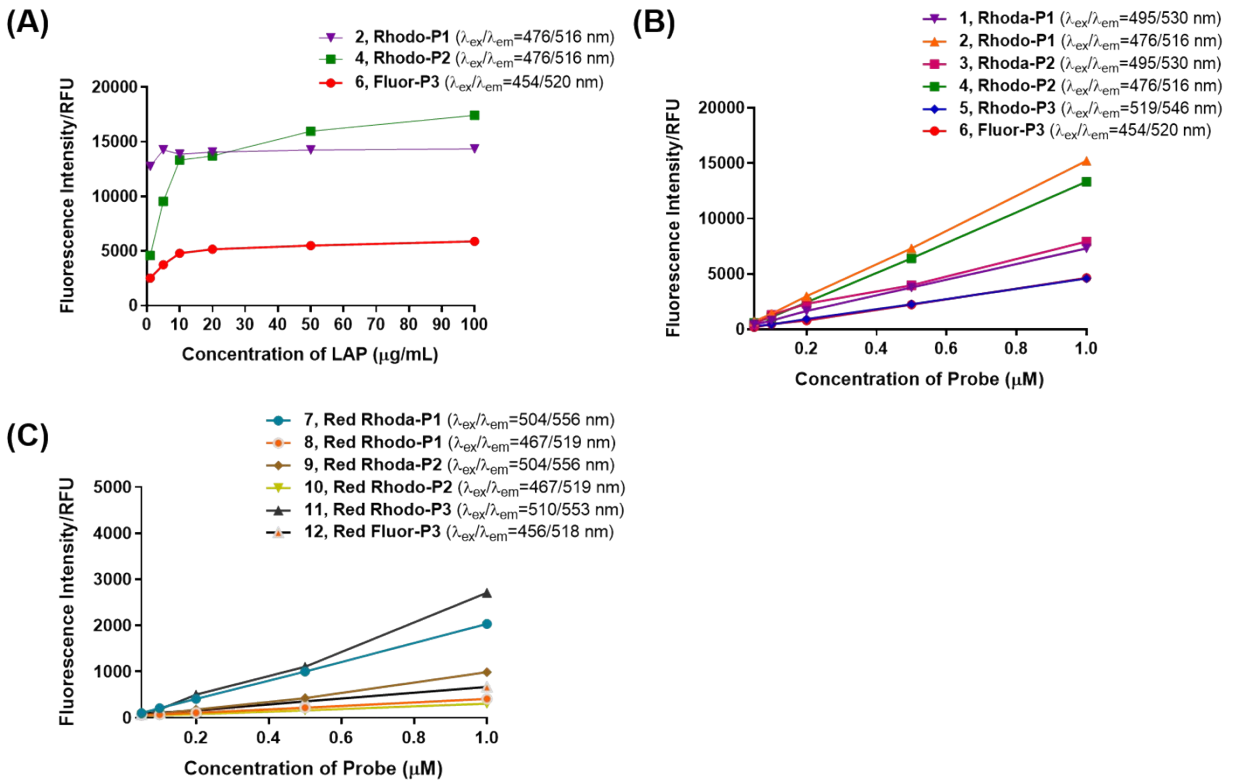


Figure S2. Concentration optimization of LAP reaction. **A.** LAP reaction of the fluorescent probes (1.0 μM) on various concentrations of LAP (1, 5, 10, 20, 50, 100 μg/ mL), **B.** LAP reaction of the typical xanthene-based fluorescent probes (0.05, 0.1, 0.2, 0.5, 1 μM) on the LAP (10 μg/ mL), **C.** LAP reaction of the reduced xanthene-based fluorescent probes (0.05, 0.1, 0.2, 0.5, 1 μM) on the LAP (10 μg/ mL) in PBS buffer (10 mM, pH 7.4) at 37 ℃ (n=3).

F. Calibration curves of fluorophores

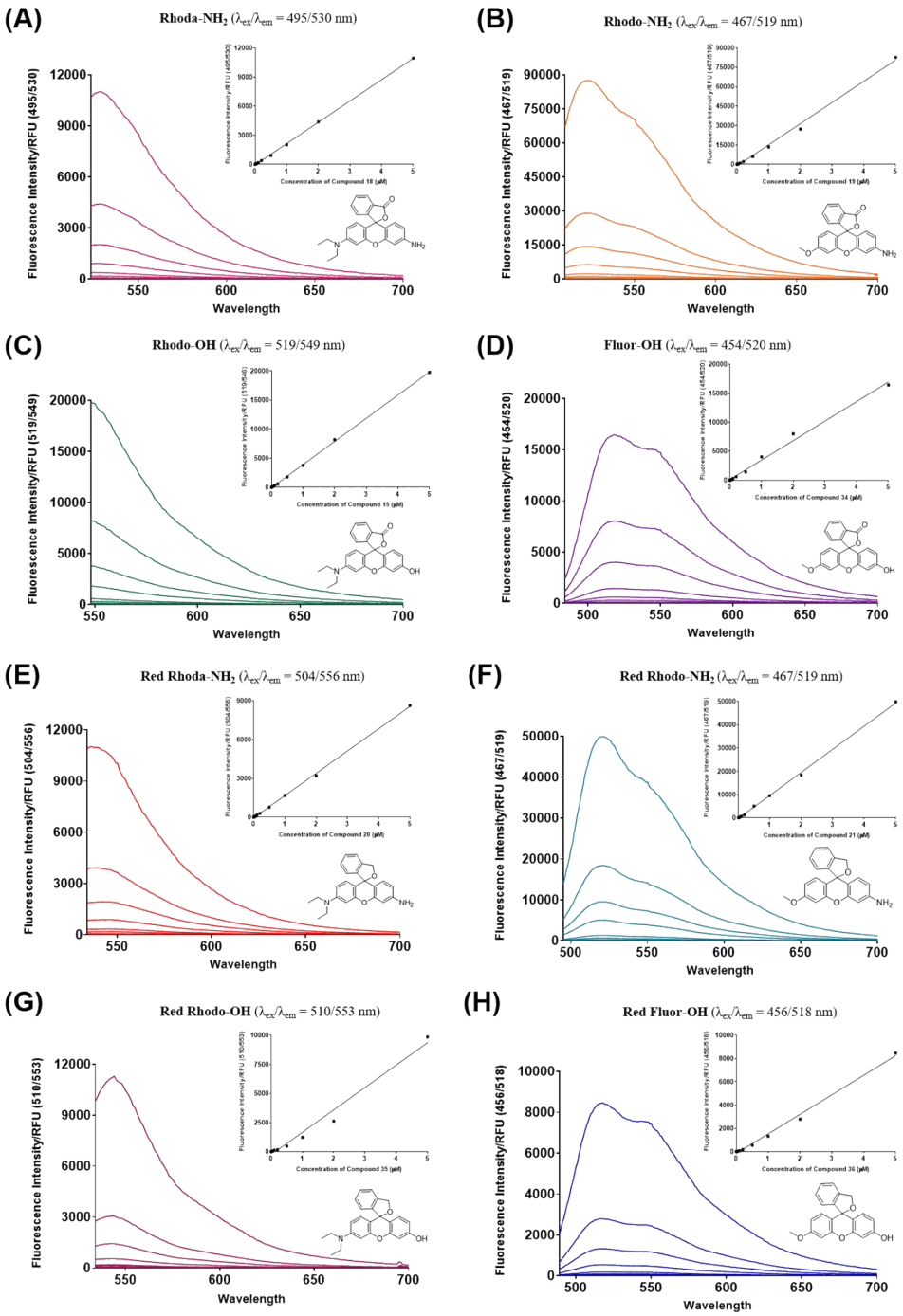


Figure S3. Calibration curves of fluorophores for determining the concentration of released fluorophores from activatable fluorescent probes (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 μM, pH 7.4 PBS buffer). Inset: Linear regression graph of fluorescence enhancement change of concentration of fluorophore.

G. Enzymatic kinetic study of LAP-responsive fluorescent probes

All spectroscopic readings were recorded on Synergy™ H1 using a 96-well microplate. The kinetic study for LAP reaction was performed by incubation of 2 μg of LAP (20 μg / 100 μL) with 1 μM probe in PBS buffer (10 mM, pH 7.4). The plate was incubated at 37 ℃ for 80 min with continuous shaking, and the emission spectra were recorded at the corresponding wavelength over time. Experiments were performed in triplicate and repeated three times with similar results.

H. Competitive assay of LAP-responsive fluorescent probes with LAP inhibitor

A solution of LAP (2 μg/200 μL) was pretreated with bestatin (0, 1, 10, 50 and 100 μM) in PBS (10 mM, pH 7.4) at 37 ℃ for 30 min, and then 1 μM of probe was added. The mixture was incubated at 37 ℃, and the fluorescence was recorded on Synergy™ H1 using a 96-well microplate. All experiments were performed in triplicate and the data were expressed as the mean ± standard deviation.

I. Cell culture

Hep G2 cells (Human liver adenocarcinoma cell line, KCLB No. 88065) were purchased from the Korean Cell Line Bank (Seoul, South Korea). They were all propagated in cell culture flask (75T, SPL Life Sciences, South Korea) cultured at 37 °C under a humidified 5 % CO_2 atmosphere in RPMI 1640 medium, which were supplemented with 10 % fetal bovine serum (Hyclone, Logan,

UT, USA) and 1 % penicillin-streptomycin (10000 U/mL penicillin and 10 mg/mL streptomycin) (Invitrogen, Carlsbad, CA, USA) and 50 μM of 2-mercaptoethanol.

J. Kinetic and competitive study in HepG2 cells

HepG2 cells (3×10^4 cells/well) were seeded in black-walled, transparent bottomed 96-well plates and cultured overnight. Cells were pretreated with LAP inhibitor (100 μM) for another 1 h, then cells were treated with 1 μM of probe. Kinetic study was performed in Synergy™ H1 microplate reader from Biotek. Experiments were performed in triplicate and repeated three times with similar results. All experiments were performed in triplicate and data are expressed as the mean ± standard deviation.

K. Cytotoxicity of LAP-responsive fluorescent probes in HepG2 cells

The cell viability was determined by CCk-8 assay. Briefly, HepG2 ($3x10^4$) cells were seeded in 96 well plate and incubated for over-night. The cells were then treated with various concentrations of probes and incubated for 24h at 37°C with 5% CO_2. After, 10 μL of CCK-8 solution was added to the wells and incubated for 2h at 37°C with 5% CO_2. The absorbance was detected at 450nm with a Micro plate Reader (VersaMax, Molecular Devices, Sunyvale, CA). All experiments were performed in triplicate and the data were expressed as the mean ± standard deviation.

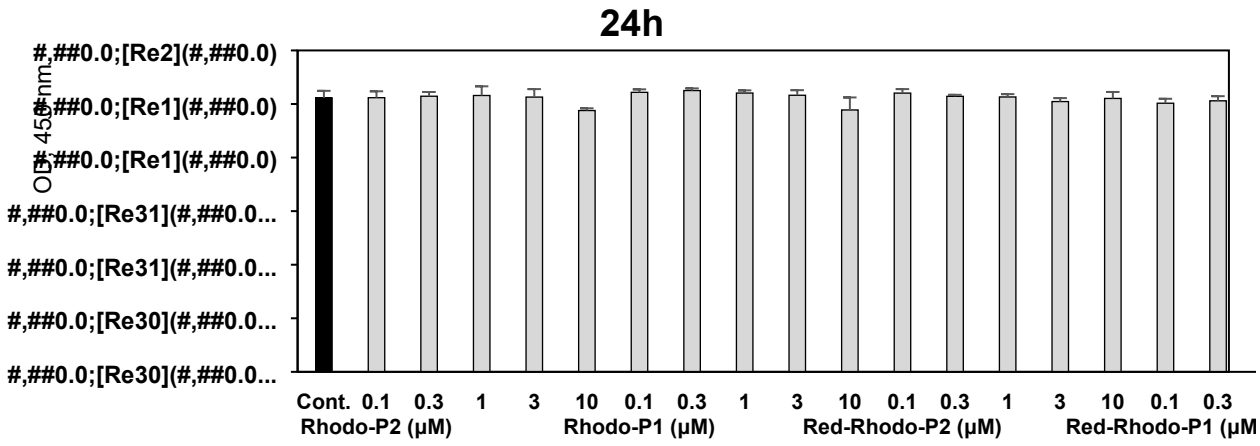


Figure S4. HepG2 cells were cultured with each compound for 24 hours. Cell viability was measured by CCK-8 assay in the 450 nm wavelength (n=3).

L. Confocal live cell imaging of LAP-responsive fluorescent probes in HepG2 cells

HepG2 cells were maintained in DMEM medium containing 10% FBS and 1% antibiotics in the 15 μ-plate 96 well black (ibidi, Germany). HepG2 cells were incubated with or without 500 μM of bestatin, LAP inhibitor, for 1 h and subsequently treated with 1 μM of probe and Lyso-Tracker™ Red (Thermo Scientific, USA) for 1 h. The treated cells were washed three times with PBS and treated with 10 μM of DAPI (Thermo Scientific, USA) for 2 h. Imaged were collected by Leica confocal microscopy (TCS SP5 AOBS/Tandem, Leica, Germany). Lyso-Tracker™ Red was excited by 514 nm and detected by 570-620nm, compounds were excited by 476 nm and detected by 505-545nm, DAPI was excited by 405 nm and detected by 420-470 nm. All experiments were performed three times.

M. Selectivity of LAP-responsive fluorescent probes

1 μM of probe was incubated with various bioanalytes containing 1 mM of NaOCl, H_2O_2, $CaCl_2$, $ZnCl_2$, KCl, NaCl, GSH, vitamin C, *L*-cystein, *L*-arginine, *L*-alanine and 1 U/mL of α-amylase, lysozyme, cellulase under 0.05 U/mL of LAP in PBS (10 mM, pH 7.4) at 37 ℃ for 1 h. The fluorescence was recorded on Synergy™ H1 microplate reader. All experiments were performed in doublet and calculated as means ± S.D.

N. *Ex vivo* study for plasma stability of LAP-responsive fluorescent probe in mice

Animal was treated strictly according to the guidelines for laboratory animal care and use issued by the Sunchon National University Institutional Animal Care and Use Committee (SCNU IACUC). Female C57/BL6 mice (7 weeks old), weighing about 17–20 g, were purchased from Orientbio (Orientbio Inc., Seongnam, Korea). Blood was collected from the posterior orbital plexus in normal mice and centrifuged (5000 rpm, 4 °C, 5 minutes) to obtain serum. The stability study was performed by incubation of the fluorescent probe (10 μM) or rhodol fluorophore (0.1, 0.3, 1, 3 and 10 μM) with serum at 37 ℃ during 48 h. The fluorescence was detected on Synergy™ H1 microplate reader from Biotek. All experiments were performed in triplicate and the data were expressed as the mean ± standard deviation.

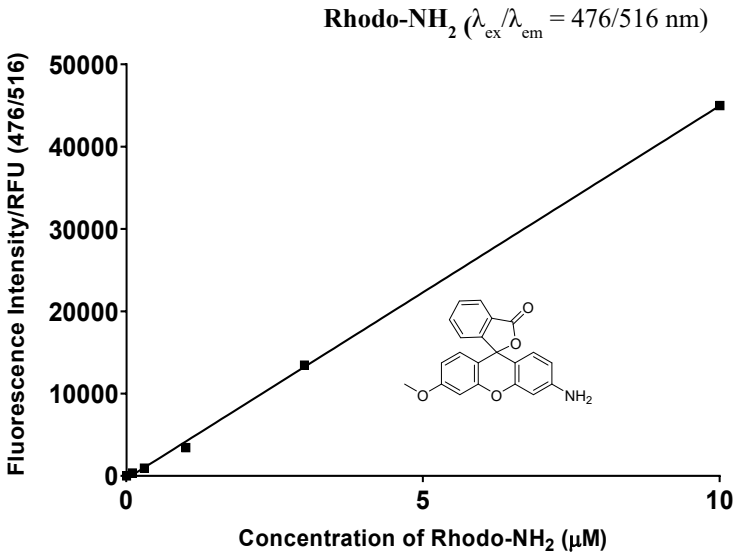


Figure S5. Fluorescence calibration curve of Rhodo-NH_2 fluorophore for determining the concentration of fluorophore released from LAP probes. Inset: Linear regression graph of fluorescence enhancement change of concentration of fluorophore.

References

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