Electronic Supplementary Information

Establishment of Evaluation Criteria for the Development of high quality ERα-targeted fluorescent probes

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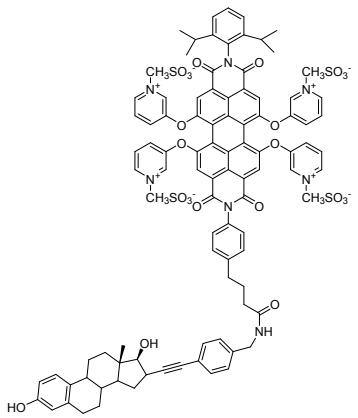
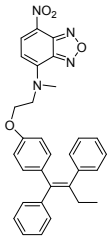
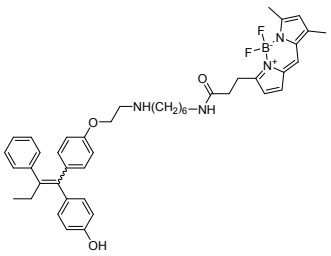
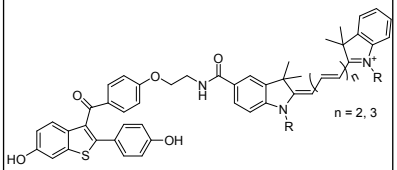
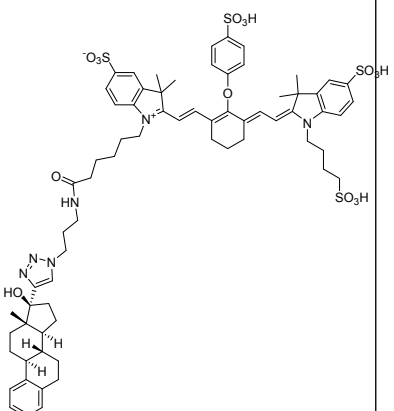
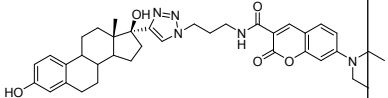
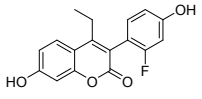
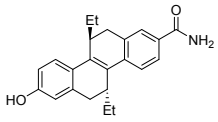
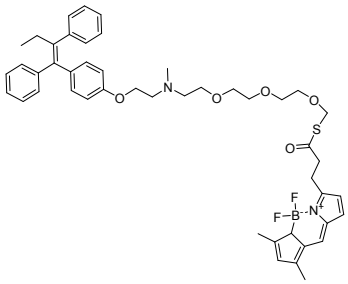
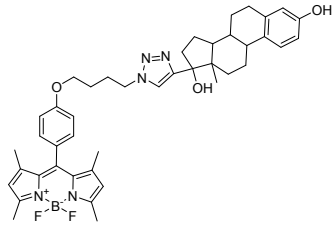
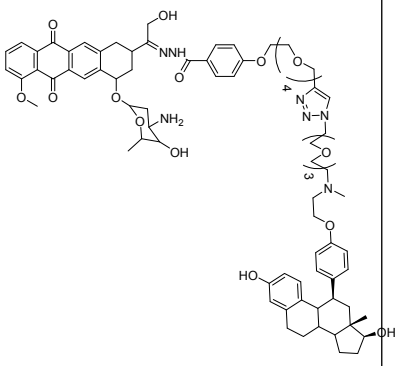
1. Evaluation aspects of ERα-targeted fluorescent probes used in cellular ERα imaging

Table S1 Evaluation aspects of ERα-targeted fluorescent probes used in cellular ERα imaging[a,b,c]

	structure	ERα binding affinity	Fluorescent quantum yield	Cytotoxicity	ERα tracking capacity	ERα selectivity	ERα labeling ability
1[1]		—	√	—	√	√	√
2[2]		√	—	√	√	—	√
3[3]		√	—	√	√	—	√
4[4]		√	√	—	√	√	√
5[5]		√	—	—	√	—	√ 1

6[6]		√	√	—	√	—	—
7[7]		√	√	√	√	√	—
8[8]		√	√	—	√	√	—
9[9]		—	—	—	√	√	—
10[10]		—	√	√	√	√	—
11[11]		—	—	√	√	—	—

[a] Most of the ERα-targeted fluorescent probes that were used in cellular ERα imaging are listed in **Table S1**. ERα-targeted fluorescent probes not being applied in living cell imaging can be found in ref 12, Science Direct Copyright 2019.

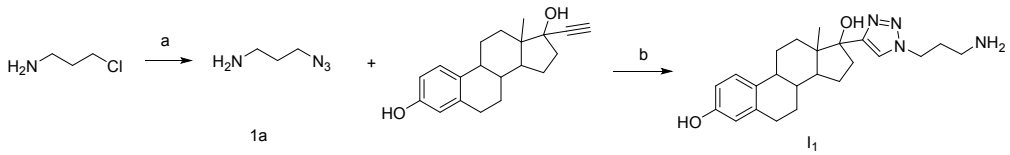
[b] " √ " means the experiment was conducted, "—" means the experiment was not carried out.

[c] None of the probes were used to study the motion characteristics of ERα.

2. Materials and instruments.

All the starting materials were purchased commercially and used directly without further purification. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker Biospin AV400 (400 MHz) instrument. Chemical shifts were reported in ppm (parts per million) and were referenced to tetramethylsilane. Melting points were measured on the X-4 Beijing Tech melting point apparatus, the data were not corrected. UV spectra and fluorescence spectra were recorded with SHIMADZU UV-2600 and HITACHI F-4600, respectively. Cell imaging was observed with Leica-LCS-SP8 confocal laser scanning microscope.

3. Synthesis and characterization of probes 1-3.

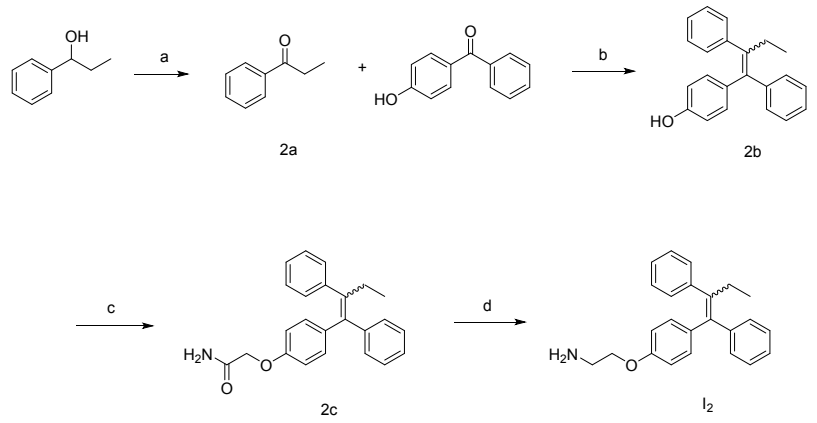


Scheme S1. Synthesis of intermediate I_1. Reagents and conditions: (a) NaN_3, H_2O, 80 °C, 12 h; (b) ascorbic acid, $CuSO_4 \cdot 5H_2O$, *t*BuOH/H_2O, 25 °C, 12 h.

17-(1-(3-Aminopropyl)-1*H*-1,2,3-triazol-4-yl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[α]phenanthrene-3,17-diol (I_1)[5].

NaN_3 (0.9 g, 13.8 mmol) was mixed into a solution of 3-chloropropylamine hydrochloride (0.6 g, 4.6 mmol) in H_2O (15 mL). This solution was heated to 80 °C for 12 h, cooled down, basified by KOH (1 mol/L) to pH = 9. The residue was dissolved in saturated ammonium chloride aqueous solution (20 mL) and extracted with ethyl acetate (30 mL × 3). The organic layers were combined and dried over Na_2SO_4, concentrated *in vacuo* to obtain **1a** as white oil. **1a** was used without further purification. A mixture of **1a** (100 mg, 1 mmol) and ethinyl estradiol (300 mg, 1 mmol) in *t*-BuOH (5 mL) was supplemented in H_2O (5 mL), ascorbic acid (20 mg, 0.11 mmol) and $CuSO_4 \cdot 5H_2O$ (30 mg, 0.12 mmol) were added, the mixture was stirred at 25 °C for 12 h. The crude mixture was diluted using ethyl acetate (40 mL) and 4:1 saturated NH_4Cl/NH_4OH (40 mL). The organic layer was separated and washed with 4:1 saturated NH_4Cl/NH_4OH (3 × 40 mL), dried, and concentrated *in vacuo* to yield **I_1** (240 mg, 61.7%) as a white solid.

^{1}H NMR (400 MHz, MeOD) δ 7.84 (s, 1H), 7.10 (d, J = 5.4 Hz, 1H), 6.99 (d, J = 8.1 Hz, 1H), 6.53-6.46 (m, 2H), 4.47 (d, J = 15.8 Hz, 2H), 2.92 (s, 1H), 2.76 (s, 3H), 2.47 (t, J = 8.7 Hz, 1H), 2.12 (m, 4H), 1.93 (m, 4H), 1.75 (d, J = 5.4 Hz, 1H), 1.64 (d, J = 8.2 Hz, 2H), 1.48 -1.36 (m, 3H), 1.32 (d, J = 7.5 Hz, 2H), 1.05 (s, 3H).



Scheme S2. Synthesis of intermediate I_2. Reagents and conditions: (a) TCICA, pyridine, EtOAc, 95%; (b) Zn, $TiCl_4$, THF, 60%; (c) ICH_2CONH_2, acetone, K_2CO_3, 50%; (d) LAH, $AlCl_3$, THF, 75%.

Propiophenone (2a).

TCICA (1 g, 4.30 mmol) in ethyl acetate (10 mL) was added to the mixture of 1-phenylpropan-1-ol (1.36 g, 10 mmol), pyridine (0.95 g, 12 mmol) in ethyl acetate (10 mL) carefully and stirred for 5 min at rt. The mixture was filtered, and the filtrate was collected and washed with 1 M HCl (10 mL) and 5% $NaHCO_3$ respectively, extracted with ethyl acetate (30 mL × 3). The organic layers were combined and dried over Na_2SO_4, concentrated *in vacuo* to obtained **2a** as transparent oil (1.27 g, 95%).

4-(1,2-Diphenylbut-1-en-1-yl)phenol (2b)[13].

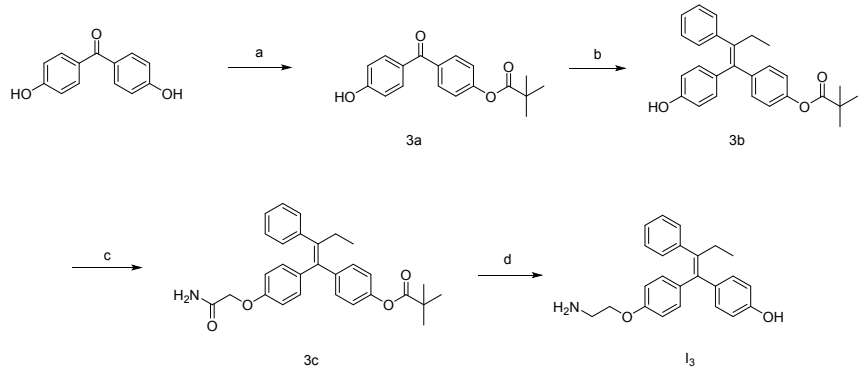
Zinc powder (1.30 g, 20 mmol) was suspended in dry THF (20 mL), and the mixture was cooled to 0 °C. $TiCl_4$ (1.2 mL, 10 mmol) was added dropwise under argon. When the addition was complete, the mixture was warmed to room temperature and heated to reflux for 2 h. After cooling down, a solution of 4-hydroxybenzophenone (0.50 g, 2.53 mmol) and **2a** (1.10 g, 8.18 mmol) in dry THF (10 mL) was added at 0 °C and the mixture was heated at reflux in the dark for 2.5 h. After being cooled to room temperature, the zinc dust was filtered off and THF was evaporated. The residue was dissolved with saturated ammonium chloride aqueous solution (50 mL) and extracted with ethyl acetate (50 mL × 6). The organic layers were combined and dried over Na_2SO_4, concentrated *in vacuo*, and further purified by silica gel column chromatography (hexane/ethyl acetate = 30:1) to provide **2b** as a faint yellow solid (0.45 g, 60%).

(*E*,*Z*)-2-(4-(1,2-Diphenylbut-1-en-1yl)phenoxy)acetamide (2c).

A suspension of **2b** (0.84 g, 2.8 mmol) and K_2CO_3 (2.18 g, 15.75 mmol) in acetone (20 mL) was heated to reflux for 10 min. A solution of 2-iodoacetamide (2.08 g, 11.25 mmol) in acetone (20 mL) was added, and the mixture was stirred for 3 h. After cooling down, acetone was evaporated and the residue was dissolved in saturated ammonium chloride aqueous solution (50 mL) and extracted with ethyl acetate (50 mL × 5). The organic layers were combined, dried over Na_2SO_4, concentrated *in vacuo*, and further purified by silica gel column chromatography (hexane/ethyl acetate = 4:1) to provide the **2c** as a faint yellow solid (0.50 g, 50%) of a 1:1 mixture of *E* and *Z* isomers.

(*E*,*Z*)-2-(4-(1,2-Phenylbut-1-en-1-yl)phenoxy)ethan-1-amine (I_2).

A suspension of $AlCl_3$ (0.84 g, 6.32 mmol) and $LiAlH_4$ (1.22 g, 32.17 mmol) in dry THF (20 mL) was stirred under argon and cooled to 0 °C. A solution of **2c** (0.47 g, 1.30 mmol) in dry THF (10 mL) was added. The mixture was warmed to room temperature and stirred under argon for 3h. The reaction was quenched with H_2O (8 mL), and THF was evaporated. The residue was dissolved in saturated ammonium chloride aqueous solution (20 mL) and extracted with ethyl acetate (25 mL × 4). The organic layers were combined, dried over Na_2SO_4, concentrated *in vacuo*, and further purified by silica gel column chromatography (methanol/dichloromethane = 1:15) to provide the **I_2** as a white solid (0.33 g, 75%) consisting of a 1:1 mixture of E and Z isomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44-7.35 (m, 1H), 7.30 (t, *J* = 7.2 Hz, 1H), 7.26-7.11 (m, 6H), 7.03 (t, *J* = 6.8 Hz, 2H), 6.93 (d, *J* = 7.7 Hz, 2H), 6.83 (d, *J* = 8.4 Hz, 1H), 6.59 (d, *J* = 8.4 Hz, 1H), 4.05 (t, *J* = 4.7 Hz, 1H), 3.88 (t, *J* = 4.7 Hz, 1H), 3.13 (s, 1H), 3.03 (s, 1H), 2.54 (q, *J* = 12.3 Hz, 2H), 1.08-0.90 (t, *J* = 7.2 Hz, 3H).



Scheme S3. Synthesis of intermediate I_3. Reagents and conditions: (a) NaH, *t*-BuCOCl, THF, 51%; (b) propiophenone, Zn, $TiCl_4$, THF, 59%; (c) ICH_2CONH_2, acetone, K_2CO_3, 57%; (d) LAH, $AlCl_3$, THF, 70%.

4-Hydroxy-4'-(trimethylacetoxy)benzophenone (3a).

Sodium hydride in 60% dispersion in mineral oil (0.44 g, 18.52 mmol) was added to a solution of 4,4'-dihydroxybenzophenone (2.14 g, 10 mmol) in dry THF (20 mL) under argon. The solution was stirred at rt for 30 min, cooled to 0 ℃, treated with trimethylacetyl chloride (1.32 g, 10.95 mmol) and stirred for 1h after removing the ice-water bath. The reaction mixture was quenched with distilled water (10 mL) and extracted with ethyl acetate (50 mL × 3). The combined organic phase was dried and concentrated and further purified by silica gel column chromatography (dichloromethane/ethyl acetate = 70:1) to produce **3a** as a white solid. (1.52 g, 51%)

(*E*)-4-(1-(4-Hydroxyphenyl)-2-phenylbut-1-enyl)phenyl Pivalate (3b)[14].

3b was synthesized similar to the procedure of **2b**. The crude **3b** was provided by silica gel column chromatography (hexanes/ethyl acetate = 25:1) as a faint yellow solid (425 mg, 74%). Further trituration with methanol (2 mL) provided **3b** as the pure *E* isomer (*E*:*Z* > 100:1), a white solid (339 mg, 59%).

(*E*)-4-(1-(4-(2-Amino-2-oxoethoxy)phenyl)-2-phenylbut-1-enyl)-phenyl Pivalate (3c).

3c was synthesized similar to the procedure of **2c**. The preliminary **3c** was obtained by silica gel column

chromatography (hexanes/ethyl acetate = 4:1) as a faint yellow solid (203 mg, 78.5%). Trituration with methanol (3 mL) provided **3c** as the pure E isomer (*E*:*Z* > 25:1), a white solid (147 mg, 57%).

(*Z*)-4-(1-(4-(2-Aminoethoxy)phenyl)-2-phenylbut-1-enyl)phenol (I_3).

I_3 was synthesized similar to the procedure of I_2. Purification by silica gel column chromatography (methanol/dichloromethane = 1:70) to provide **I_3** as a white solid (100 mg, 70%). The NMR spectrum indicated an *E*:*Z* ratio of 1:4.4. ^{1}H NMR (400 MHz, MeOD) δ 7.13-7.05 (m, 7H), 7.03 (m, 1.58H, *E* isomer), 6.95 (d, *J* = 8.5 Hz, 0.47H, *E* isomer), 6.78 (dd, *J* = 8.4, 5.0 Hz, 4H), 6.66 (d, *J* = 8.5 Hz, 0.47H, *E* isomer), 6.58 (d, *J* = 8.6 Hz, 2H), 6.42 (d, *J* = 8.5 Hz, 0.45H, *E* isomer), 4.05 (t, *J* = 5.2 Hz, 0.47H, *E* isomer), 3.88 (t, *J* = 5.2 Hz, 2H), 3.04 (t, *J* = 4.7 Hz, 0.45H, *E* isomer), 2.94 (t, *J* = 4.5 Hz, 2H), 2.50 (q, *J* = 7.5 Hz, 2H), 0.92 (t, *J* = 7.4 Hz, 3.5H).

General synthetic procedure of probes 1-3.

Equivalent **FITC** (1 mmol) and **I_1**, **I_2**, **I_3** were added to a flask under argon atmosphere, respectively. Et_3N (5 mL) was then added to the mixture and stirred in the dark at 25 °C for 12 h and monitored by TLC. When the reaction was completed, the purification was carried out by column chromatography (methanol / dichloromethane = 1 : 20) to obtain probes **1**-**3** respectively as yellow solids.

Probe 1. Yield: 56 mg (52%), yellow solid (mp > 320 °C). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.22 (s, 2H), 8.04 (d, J = 7.7 Hz, 1H), 8.00-7.87 (m, 2H), 7.82 (d, J = 8.2 Hz, 1H), 7.71 (s, 1H), 7.69 (s, 1H), 7.49 (s, 1H), 7.36 (d, J = 8.1 Hz, 1H), 6.96 (d, J = 8.3 Hz, 1H), 6.70 (s, 2H), 6.59 (m, 4H), 6.47 (d, J = 8.3 Hz, 1H), 6.42 (s, 1H), 4.46 (t, J = 6.7 Hz, 1H), 4.42-4.29 (m, 1H), 3.17 (s, 2H), 2.86-2.76 (m, 1H), 2.69 (s, 2H), 2.34 (d, J = 4.6 Hz, 1H), 2.11 (m, 2H), 2.01-1.90 (m, 2H), 1.81 (m, 3H), 1.68-1.57 (m, 1H), 1.53-1.40 (m, 2H), 1.24 (t, J = 8.4 Hz, 3H), 0.93 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 181.13, 174.86, 162.81, 155.96, 155.38, 155.36, 154.53, 154.49, 137.58, 130.91, 130.87, 130.84, 130.25, 130.11, 126.47, 123.26, 115.36, 113.15, 111.11, 111.09, 102.96, 81.55, 70.24, 48.00, 43.58, 37.68, 35.59, 33.14 , 31.75, 29.75, 29.49 , 29.04, 27.66, 27.02, 25.58, 24.02, 22.56, 14.41. HRMS (ESI) calcd for $C_{44}H_{43}N_5O_7S$ $[M+H]^+$, 786.2956; found 786.2936.

Probe 2. Yield: 48 mg (66%), yellow solid (mp > 320°C). ^{1}H NMR (400 MHz, MeOD) δ 8.22 (s, 1H), 8.20 (s, 1H), 7.85 (s, 1H), 7.65 (d, J = 8.6 Hz, 2H), 7.60 (s, 1H), 7.18 (s, 1H), 7.17-7.13 (m, 2H), 7.12-7.06 (m, 4H), 7.02 (t, J = 8.4 Hz, 2H), 6.80 (m, 2H), 6.71-6.59 (m, 6H), 6.55 (m, 2H), 6.42 (d, J = 8.5 Hz, 1H), 4.26 (t, J = 9.3 Hz, 1H), 4.09 (t, J = 9.6 Hz, 1H), 3.40 (t, J = 7.5 Hz, 1H), 3.29 (t, J = 7.2 Hz, 1H), 2.48 (q, J = 14.9, 2H), 0.91 (t, J = 8.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 175.32, 168.62, 162.99, 158.74, 156.87, 156.09, 152.45, 149.73, 143.38, 142.21, 141.80, 138.35, 135.83, 131.68, 130.64, 130.47, 129.79, 129.38, 128.75, 128.30, 127.90, 127.15, 126.21, 124.26, 119.61, 114.29, 113.51, 112.84, 110.67, 106.12, 102.55, 79.49, 63.81, 49.06, 28.9, 13.77. HRMS (ESI) calcd for $C_{45}H_{36}N_2O_6S$ $[M+H]^+$, 733.2367; found 733.2333.

Probe 3. Yield: 63 mg (61%), yellow solid (mp > 320°C). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.23 (s, 1H), 10.16 (s, 1H), 8.42 (s, 1H), 8.29 (t, J = 12.2 Hz, 1H), 7.74 (t, J = 9.7 Hz, 1H), 7.38 (t, J = 7.5 Hz, 1H), 7.29 (d, J = 7.3 Hz, 1H), 7.23-7.18 (m, 2H), 7.17-7.13 (m, 3H), 7.10 (d, J = 5.6 Hz, 3H), 7.02 (m, 3H), 6.80 (m, 2H), 6.68 (m, 3H), 6.62 (t, J = 6.8 Hz, 2H), 6.57 (d, J = 8.5 Hz, 2H), 4.21 (d, J = 4.7 Hz, 1H), 4.11-4.00 (m, 1H), 3.93 (d, J = 3.2 Hz, 1H), 3.83 (d, J = 4.9 Hz, 1H), 2.40 (m, 2H), 0.87 (t, J = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 181.26, 169.03, 162.80, 159.97, 157.59, 156.74 152.36, 147.67, 143.72, 143.47, 142.22, 141.78, 141.26, 138.47 135.99, 131.88, 130.68, 130.05, 129.82, 129.49, 129.42, 128.74, 128.42, 128.30, 127.91, 127.13, 126.62, 126.19, 124.50, 114.77, 113.98, 113.06, 110.16, 102.74, 83.53, 66.12, 49.08, 29.50, 29.00, 13.81. HRMS (ESI) calcd for $C_{45}H_{36}N_2O_7S$ $[M+H]^+$, 749.2316;

found 749.2299.

4. Real-time imaging ability of probes 1-3

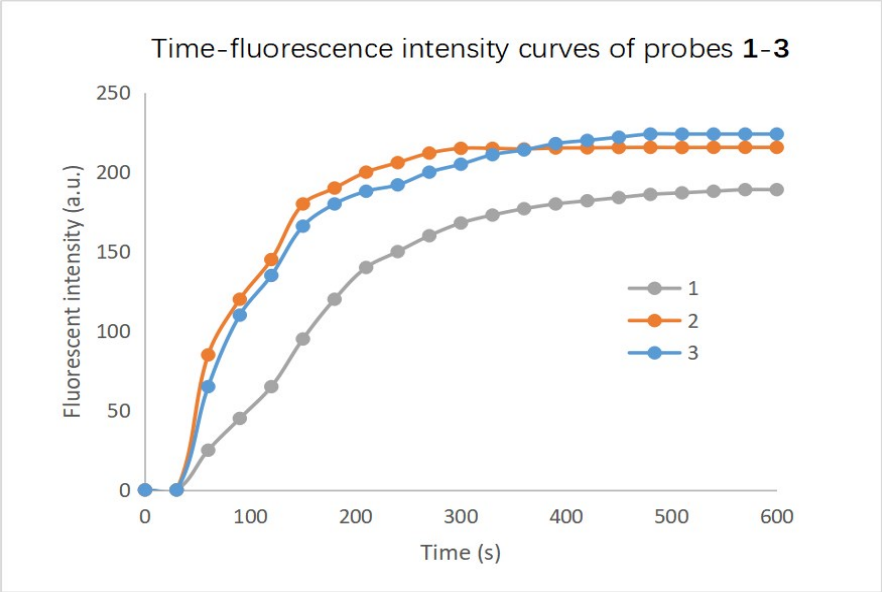
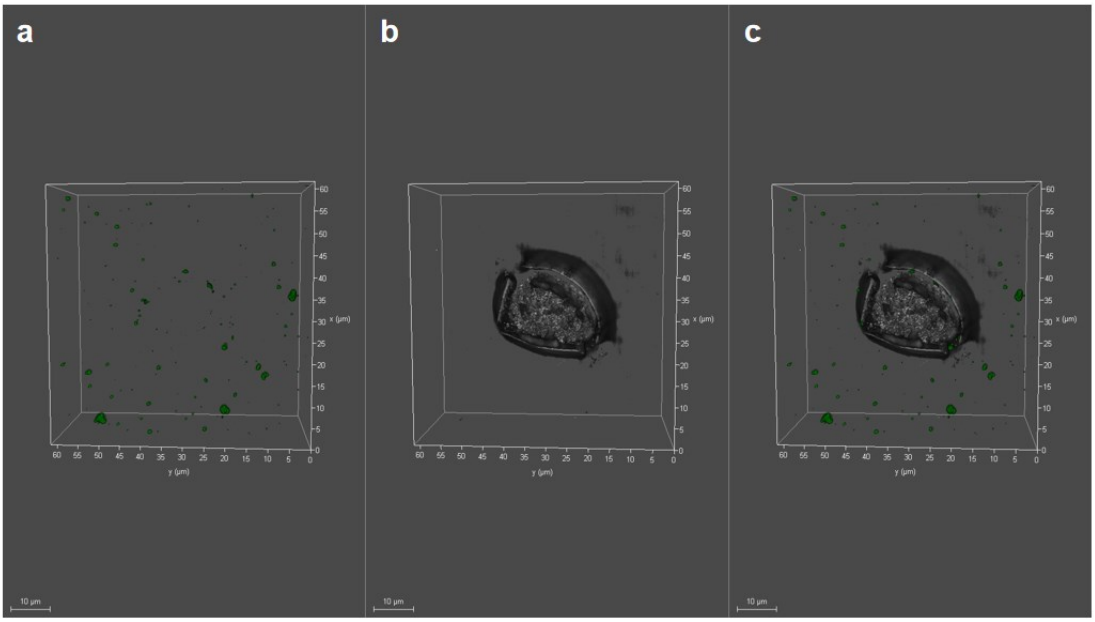


Figure S1 Time-fluorescence intensity curves of probes **1**-**3**. MCF-7 cells were incubated with probes **1**-**3** (10 μM) respectively and the fluorescence intensity was monitored the minute the probe was added till the fluorescence intensity reached saturation.

5. 3D and 2D stack images of competitive experiment



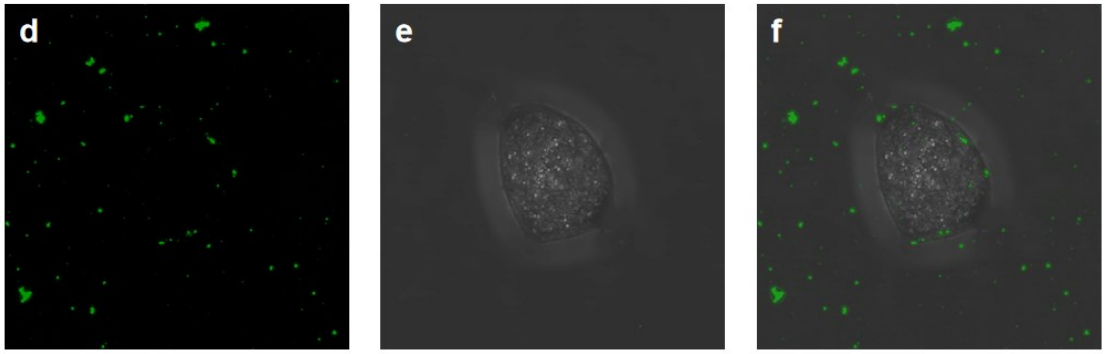


Figure S2 3D and 2D stack images of MCF-7 cells incubated with both the probe 3 (10 μM) and ES (100 μM), a high affinity ligand of ERα, to block the interaction between the probe and ERα. (a-c) 3D images and (d-f) corresponding 2D stack images. (a) (d) Fluorescence channel. (b) (e) bright field. (c) (f) Merged images of (a) and (b), (d) and (e) respectively.

6. Fluorescence recovery after photobleaching of probe 2

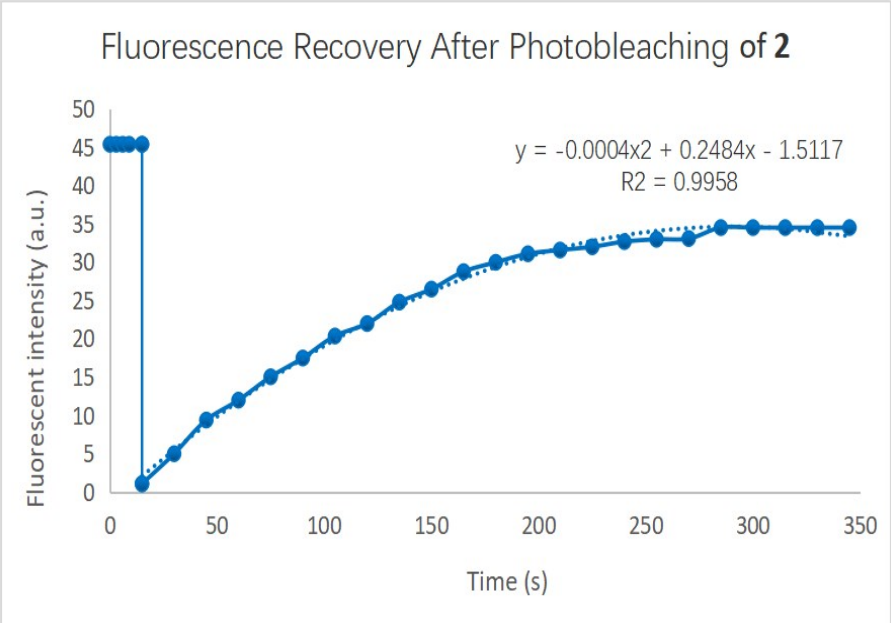


Figure S3 Fluorescence recovery after photobleaching of probe **2**. MCF-7 cells were incubated with probes **2** (10 μM) for 30 min, followed by a wash procedure to remove the unbound probe, then the region of interest (ROI) was photobleached, fluorescence recovery was observed for 10 min.

7. Confocal images of fluorescence recovery after photobleaching of probe 3

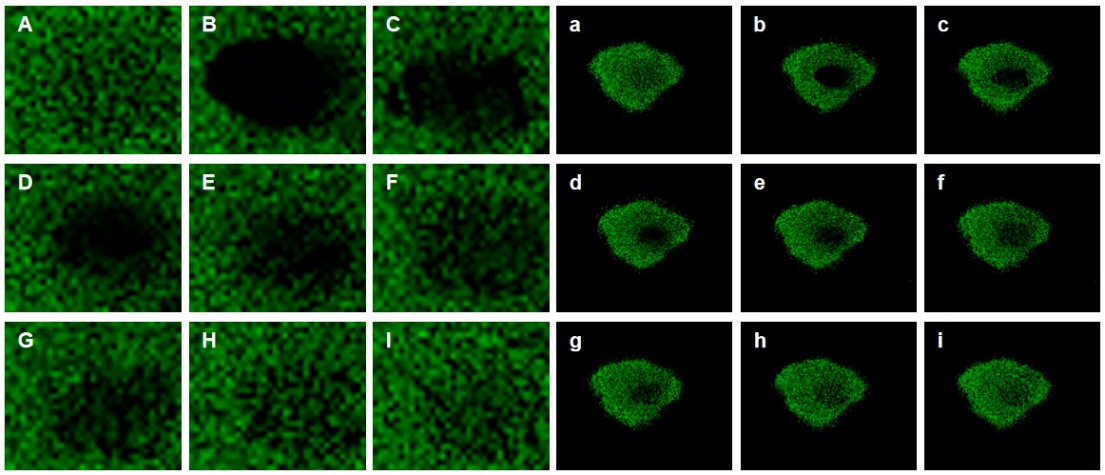
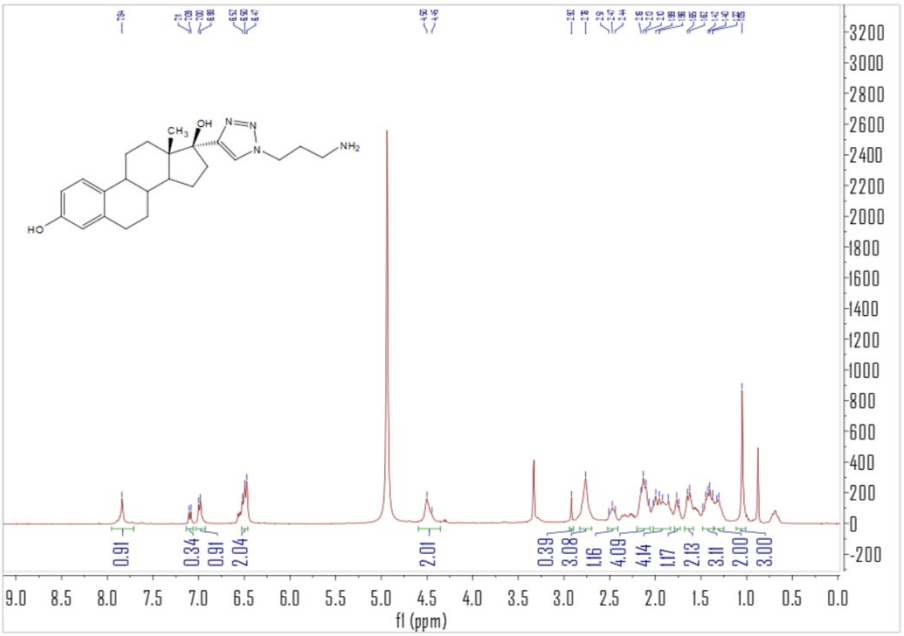


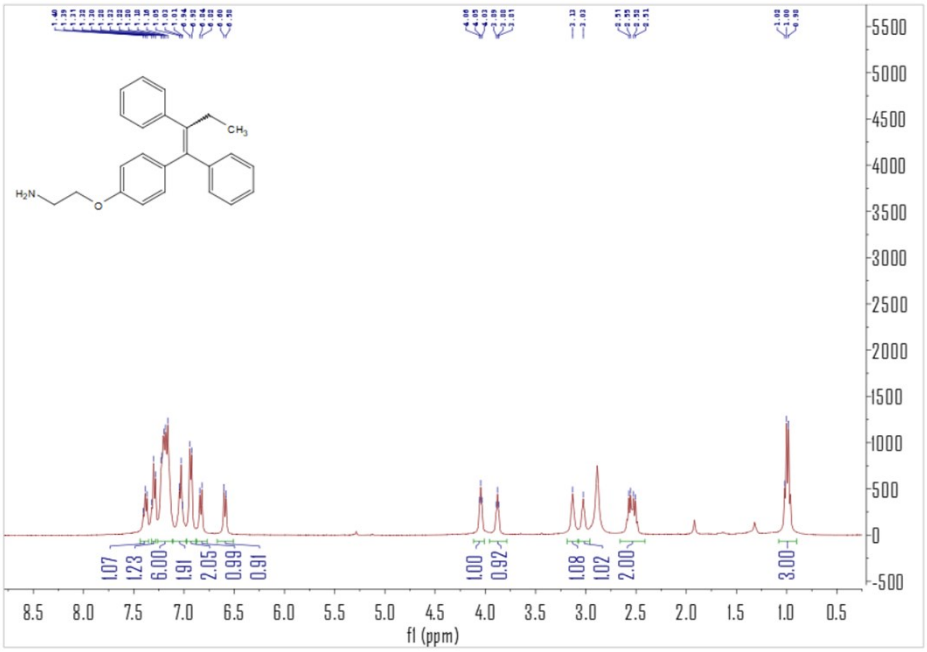
Figure S4 Confocal images of fluorescence recovery after photobleaching of probe **3**. MCF-7 cells were incubated with **3** (10 µM) for 30 min, after a wash procedure the ROI was photobleached, and the fluorescence recovery was observed for 10 min. The region of interest (ROI) was magnified for (A-I). (A) (a) Images taken before photobleaching. (B-I) (b-i) Images taken during fluorescence recovery after photobleaching. (I) (i) Images taken when recovered fluorescent intensity reached plateau.

8. ^{1}H NMR and ^{13}C Spectra.

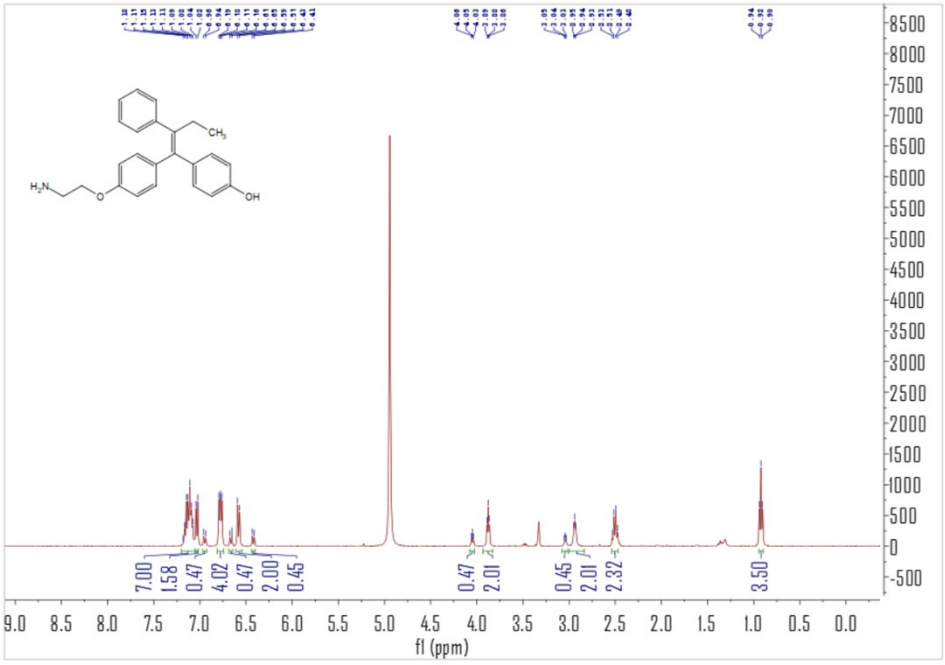
^{1}H NMR spectrum of (13*S*,17*S*)-17-(1-(3-aminopropyl)-1H-1,2,3-triazol-4-yl)-13-methyl-7,8,9,11,12,13,14,15,16, 17-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diol (I_1)



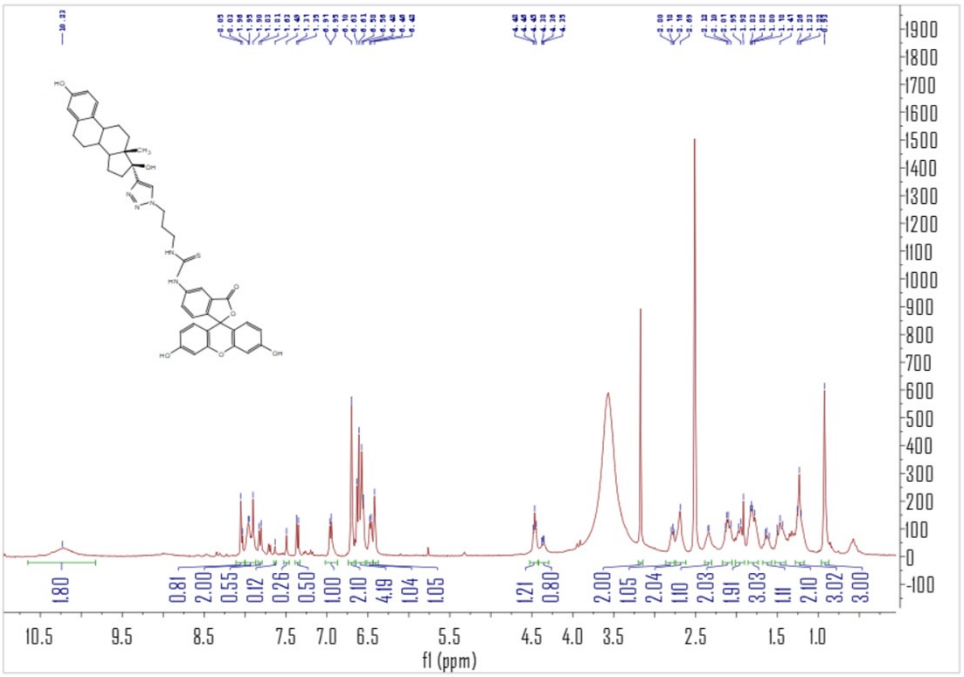
^{1}H NMR spectrum of (*E*,*Z*)-2-(4-(1,2-phenylbut-1-en-1-yl)phenoxy)ethan-1-amine (I_2).



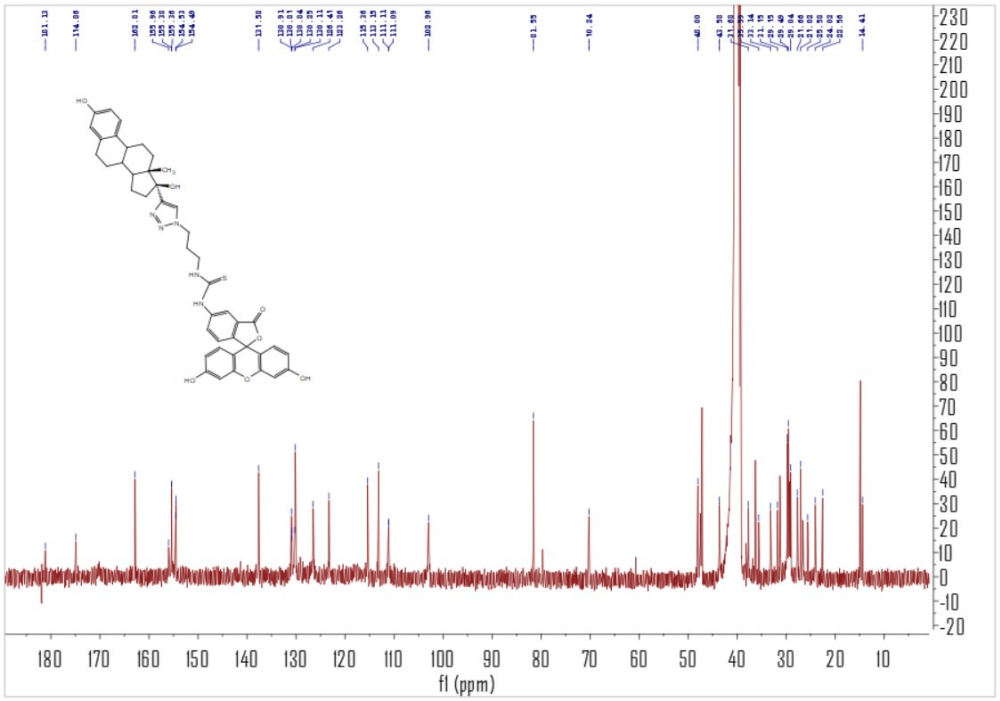
^{1}H NMR spectrum of (*Z*)-4-(1-(4-(2-Aminoethoxy)phenyl)-2-phenylbut-1-enyl) phenol (I_3).



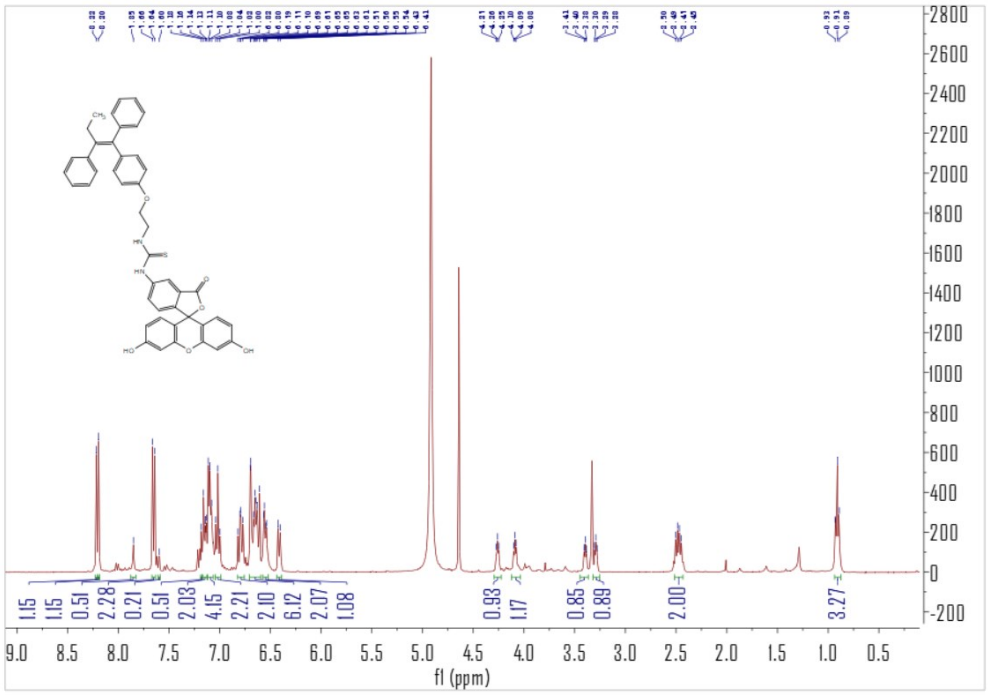
^{1}H NMR spectrum of 1-(3-(4-((13*S*,17*S*)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[a]phenanthren-17-yl)-1*H*-1,2,3-triazol-1-yl)propyl)-3-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thiourea (1)



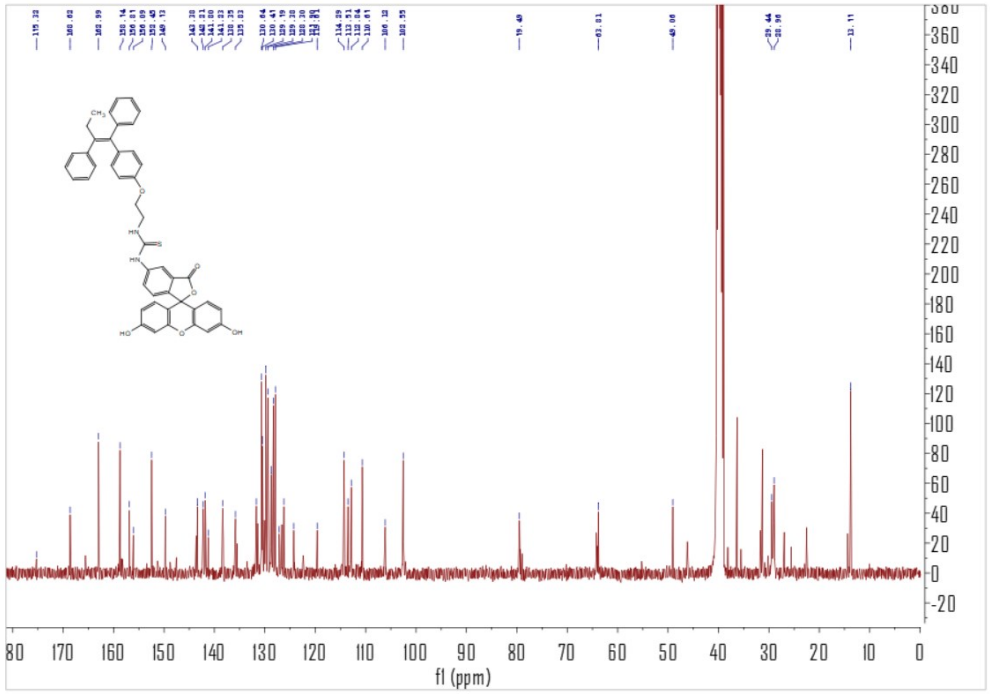
^{13}C NMR spectrum of 1-(3-(4-((13*S*,17*S*)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1*H*-1,2,3-triazol-1-yl)propyl)-3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thiourea (1)



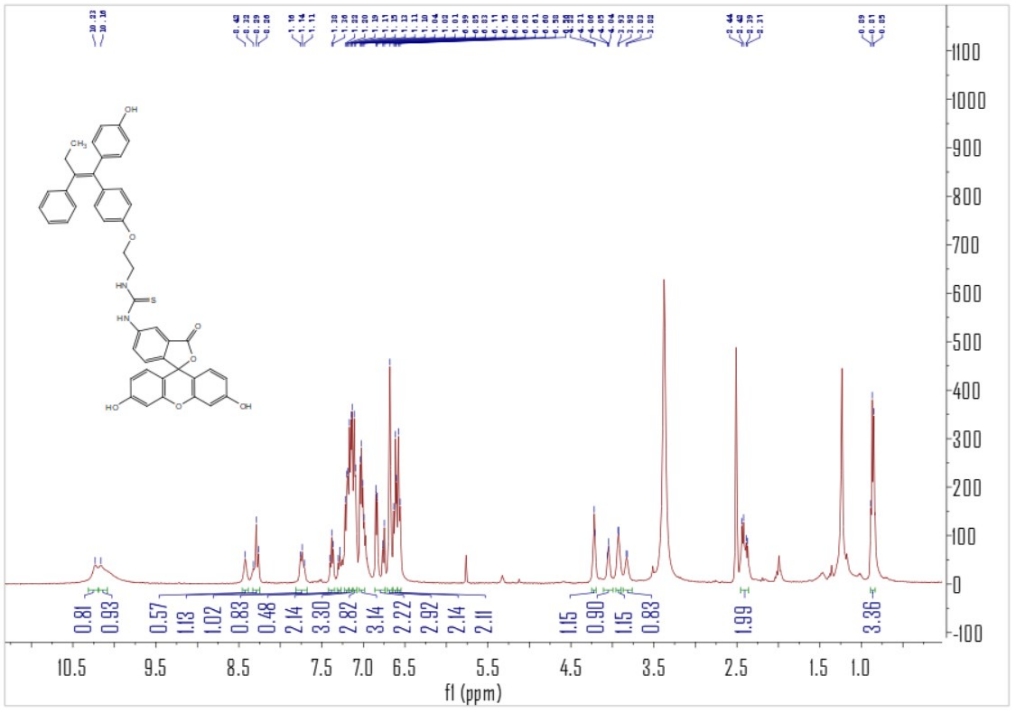
^{1}H NMR spectrum of 1-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)-3-(2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)thiourea (2)



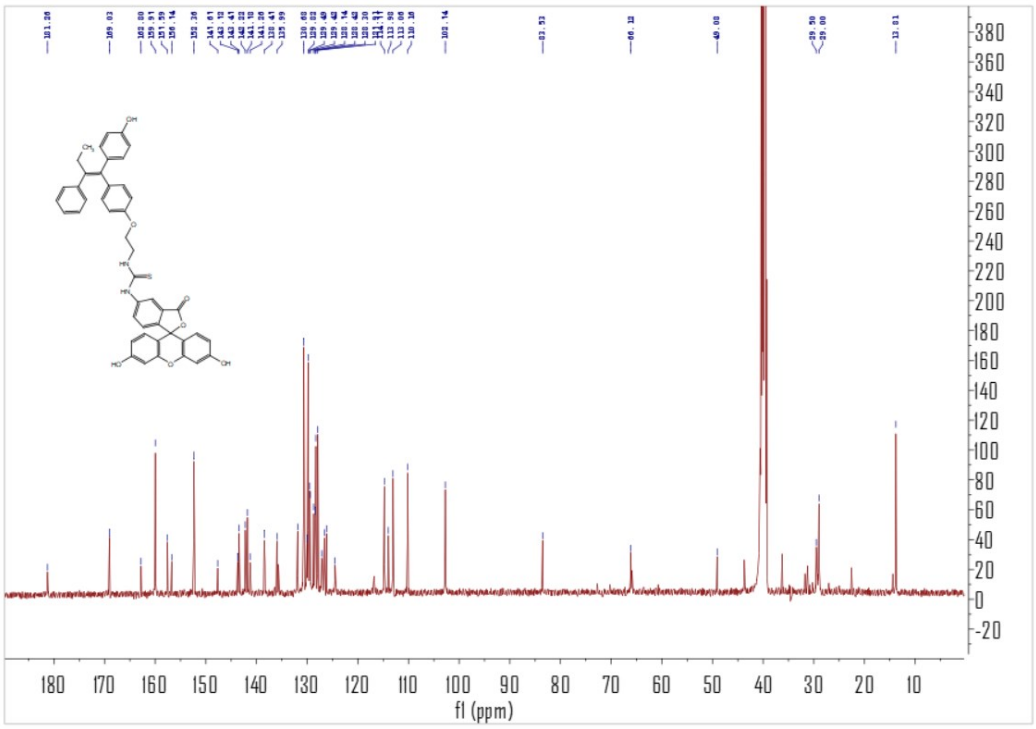
^{13}C NMR spectrum of 1-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)-3-(2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)thiourea (2)



^{1}H NMR spectrum of 1-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)-3-(2-(4-(1-(4-hydroxyphenyl)-2-phenylbut-1-en-1-yl)phenoxy)ethyl)thiourea (3)



^{13}C NMR spectrum of 1-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)-3-(2-(4-(1-(4-hydroxyphenyl)-2-phenylbut-1-en-1-yl)phenoxy)ethyl)thiourea (3)



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