**Supplementary Information**

# *Bis*-Arylidene oxindole-betulinic acid conjugate: A fluorescent cancer cell detector with potent anticancer activity

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**Experimental Section:**

**General Information**

Chemicals and solvents were purchased from commercial suppliers and used as received. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker Avance III HD (300 MHz) or an Avance III 500 (500 MHz) spectrometer. Chemical shifts were reported in parts per million (ppm), and the residual solvent peak was used as an internal reference: proton (chloroform δ 7.26), carbon (chloroform δ 77.16) or tetramethylsilane (TMS δ 0.00) was used as a reference. Multiplicity was indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), br s (broad singlet), app t (apparently triplet). Coupling constants were reported in Hertz (Hz). High resolution mass spectra were obtained on a Micromass/Q-Toff. micro™ spectrometer. Melting points were determined on a Thomas Hoover capillary melting point apparatus. IR spectra were measured on Thermo Scientific Nicolet 380 instrument. For thin layer chromatography (TLC), Merck precoated TLC plates (Merck 60 F254) were used, and compounds were visualized with a UV light at 254 nm. Further visualization was achieved by staining with iodine. Flash chromatography separations were performed on SRL 230-400 mesh silica gel.

**1-(4-methoxybenzyl)indolin-2,3-dione (2):**

To a suspension of NaH (180 mg, 7.5 mmol) in anhydrous THF (15mL) at 0°C was added dropwise a solution of isatin (**1**) (1.0 g, 6.8 mmol) in DMF (6 mL). After the addition was complete, the cooling bath was removed and the mixture stirred at rt for 1 h. A solution of p-methoxybenzyl bromide (PMB-Br) (1.51g, 7.5 mmol) in DMF (10 mL) and a catalytic amount of tetrabutylammonium iodide (TBAI) were added. After stirring at rt under argon for 5 h, the reaction mixture was poured into water (100 mL) and extracted with EtOAc (3×50 mL). The organic phase was washed with water (50 mL), brine (50 mL), dried and concentrated in vaccuo. The residue was purified by flash chromatography on (230-400 mesh) silica gel (3/1 pet ether/EtOAc) to give (**2**) ($R_f$ ≈0.45, 1.42 g, 78%) as a yellow solid. **$^{1}$H NMR** (300 MHz, $CDCl_3$): *δ* 7.77 (d, *J*=7.4 Hz, 1H), *δ* 7.65 (dd, *J*= 7.8 Hz, *J*= 1.0 Hz, 1H), *δ* 7.45 (app t, *J*= 4.3 Hz, 2H), *δ* 7.26 (t, *J*=7.5Hz, 1H), *δ* 7.05 (d, *J*= 8.6Hz, 2H), *δ* 6.99 (d, *J*= 8.0Hz, 1H), *δ* 5.04 (s, 2H), 3.96 (s, 3H). **$^{13}$C NMR** (75 MHz, $CDCl_3$): 183.5, 159.5, 158.3, 150.9, 138.4, 129.0, 126.6, 125.5, 123.9, 117.8, 114.5, 111.1, 55.42, 55.36, 43.6. **HRMS** calcd for $C_{16}H_{13}NO_3Na$ $(M+Na)^+$ : 290.0793, found 290.0793.

**3-(bis(4-hydroxyphenyl)methylene)-1-(4-methoxybenzyl)indolin-2-one [Is-PMB (3)]:**

To a suspension of Zn dust (314 mg, 4.8 mmol) in THF (10 mL), $TiCl_4$ (0.26 mL, 2.4 mmol) was added at -10°C. The cooling bath was removed, and the mixture was refluxed for 1 h. After cooling to rt, anhydrous pyridine (0.38 mL, 4.8 mmol) was added and the mixture was stirred for 5 min. A

solution of **2** (215 mg, 0.8 mmol) and 4,4'-dihydroxybenzophenone (171 mg, 0.8 mmol) in THF (4 mL) was added and the mixture was refluxed for 2 h (monitered by TLC). After cooling to rt, the mixture was hydrolyzed with 10 mL of 8% aqueous $K_2CO_3$. The reaction mixture was extracted with several 10 mL portions of EtOAc. The organic phase was washed with water (2x20 mL) and brine (20 mL), dried over $Na_2SO_4$, and evaporated to dryness. The crude product was flash chromatographed on silica gel (230-400 mesh) with 3/2 pet ether/EtOAc as an eluent to obtain first unreacted oxindole moiety [$R_f \approx 0.7$; 15 %] followed by the product **(3)**[$R_f \approx 0.45$; 235 mg, 65%] and finally the homo coupled product derived from the corresponding benzophenone moiety [$R_f \approx 0.35$; 20%]. **$^1$H NMR** (300 MHz, Acetone-$d_6$): $\delta$ 8.78 (s, 1H), $\delta$ 8.64 (s, 1H), $\delta$ 7.16 (d, *J*=8.3Hz, 2H), $\delta$ 7.09 (d, *J*= 8.3Hz, 2H), $\delta$ 6.99 (d, *J*= 8.3 Hz, 2H), $\delta$ 6.87 (t, *J*= 7.6Hz, 1H), $\delta$ 6.79 (d, *J*= 8.3Hz, 2H), $\delta$ 6.73-6.64 (m, 5H), $\delta$ 6.48 (t, *J*= 7.5Hz, 1H), $\delta$ 6.34 (d, *J*= 7.7Hz, 1H), $\delta$ 4.73 (s, 2H), $\delta$ 3.59 (s, 3H). **$^{13}$C NMR** (75MHz, Acetone-$d_6$): 166.5, 159.23, 159.17, 159.03, 155.8, 141.9, 133.4, 133.0, 132.0, 131.5, 129.3, 128.8, 127.6, 124.6, 122.4, 121.5, 120.9, 115.7, 114.4, 114.0, 108.6, 54.7, 42.2. **HRMS** calcd for $C_{29}H_{24}NO_4$ $(M+H)^+$ : 450.1705, found 450.1703. M.P: 254-256°C.

**3-((4-(3-bromopropoxy)phenyl)(4-hydroxyphenyl)methylene)-1-(4-methoxybenzyl) indolin-2-one [Is-Bromide (4)]:**

Compound **3** (200 mg, 0.45 mmol), dissolved in acetone (10 mL), was treated with anhydrous potassium carbonate (185 mg, 1.35 mmol). After the mixture had been refluxed for 1 h at 60°C, a solution of 1,3-dibromo propane (50 µL, 0.49 mmol) in acetone was added and the mixture was heated under reflux for 3 h. After extraction in EtOAc and solvent removal, the crude product was isolated by flash chromatography on (230-400 mesh) silica gel with 3/2 pet ether/EtOAc as an eluent to obtain first di-substituted derivative [$R_f \approx 0.7$; 15 %], followed by the product (**4**) [$R_f \approx 0.5$; 152 mg, 60%] and finally the *bis*-phenol starting material [$R_f \approx 0.35$; 25%]. **$^1$H NMR** (500 MHz, $CDCl_3$): $\delta$ 7.31-7.24 (m, 4H), $\delta$ 7.18 (d, *J*=8.5Hz, 1H), $\delta$ 7.12 (d, *J*=8.5Hz, 1H), $\delta$ 7.05-6.99 (m, 1H), $\delta$ 6.93-6.90 (m, 1H), $\delta$ 6.87-6.81 (m, 3H), $\delta$ 6.75 (t, *J*=7.5Hz, *J*=7.0Hz 1H), $\delta$ 6.70-6.63 (m, 3H), $\delta$ 6.60-6.55 (m, 1H), $\delta$ 4.90 (d, *J*=23.0 Hz 2H), $\delta$ 4.16-4.06 (m, 2H), $\delta$ 3.74 (s, 3H), $\delta$ 3.63-3.52 (m,2H), $\delta$ 2.37-2.23 (m, 2H). **$^{13}$C NMR** (125 MHz, $CDCl_3$): 168.0, 167.5, 160.3, 159.3, 159.1, 159.0, 157.8, 141.7, 141.2, 134.0, 133.3, 133.2, 133.1, 133.0, 132.7, 132.54, 132.48, 132.3, 131.3, 128.9, 128.7, 128.5, 127.8, 127.7, 124.7, 124.5, 122.7, 122.5, 121.9, 121.7, 121.45, 121.36, 115.9, 115.6, 114.9, 114.7, 114.3, 114.2, 113.8, 108.9, 108.7, 65.6, 65.4, 55.4, 43.3, 43.1, 32.4, 32.0, 30.1, 30.0. **HRMS** (ESI-TOF): Calcd for $C_{32}H_{29}NO_4Br$ $(M+H)^+$: 570.1280, found 570.1343 and 572.1262.

**3-((4-(3-azidopropoxy)phenyl)(4-hydroxyphenyl)methylene)-1-(4-methoxybenzyl) indolin-2-one [Is-Azide (5)]:**

Compound **4** (150 mg, 0.26 mmol) was treated with sodium azide (25 mg, 0.39 mmol) in DMSO and stirred overnight at 40°C. After extraction in EtOAc and solvent removal, the crude product was passed through a filtering column to obtain the pure product (**5**) (137 mg, 98%). **$^1$H NMR** (500 MHz, $CDCl_3$): *δ* 7.32-7.25 (m, 4H), *δ* 7.19 (d, *J*=8.5Hz, 1H), *δ* 7.13 (d, *J*=8.5Hz, 1H), *δ* 7.06-7.00 (m, 1H), *δ* 6.94-6.91 (m, 1H), *δ* 6.88-6.82 (m, 3H), *δ* 6.76 (t, *J*=7.5Hz, *J*=7.0Hz 1H), *δ* 6.71-6.64 (m, 3H), *δ* 6.61-6.57 (m, 1H), *δ* 4.91 (d, *J*=23.5Hz 2H), *δ* 4.14-4.02 (m, 2H), *δ* 3.75 (s, 3H), *δ* 3.57-3.45 (m, 2H), *δ* 2.10-1.99 (m, 2H). **$^{13}$C NMR** (125 MHz, $CDCl_3$): 168.0, 167.5, 160.3, 160.2, 159.3, 159.1, 159.0, 158.1, 157.8, 141.7, 141.2, 134.0, 133.3, 133.2, 133.1, 132.7, 132.54, 132.47, 132.3, 131.3, 128.9, 128.7, 128.5, 127.8, 127.7, 124.7, 124.5, 122.7, 122.5, 121.7, 121.44, 121.36, 115.9, 115.6, 114.9, 114.6, 114.3, 114.2, 113.7, 108.9, 108.7, 64.7, 64.6, 55.4, 48.3, 43.3, 43.1, 28.9, 28.8. **HRMS** (ESI-TOF): Calcd for $C_{32}H_{29}N_4O_4$ $(M+H)^+$ : 533,2189, found 533.2181.

**3-((4-(3-aminopropoxy)phenyl)(4-hydroxyphenyl)methylene)-1-(4-methoxybenzyl) indolin-2-one [Is-Amine (6)]:**

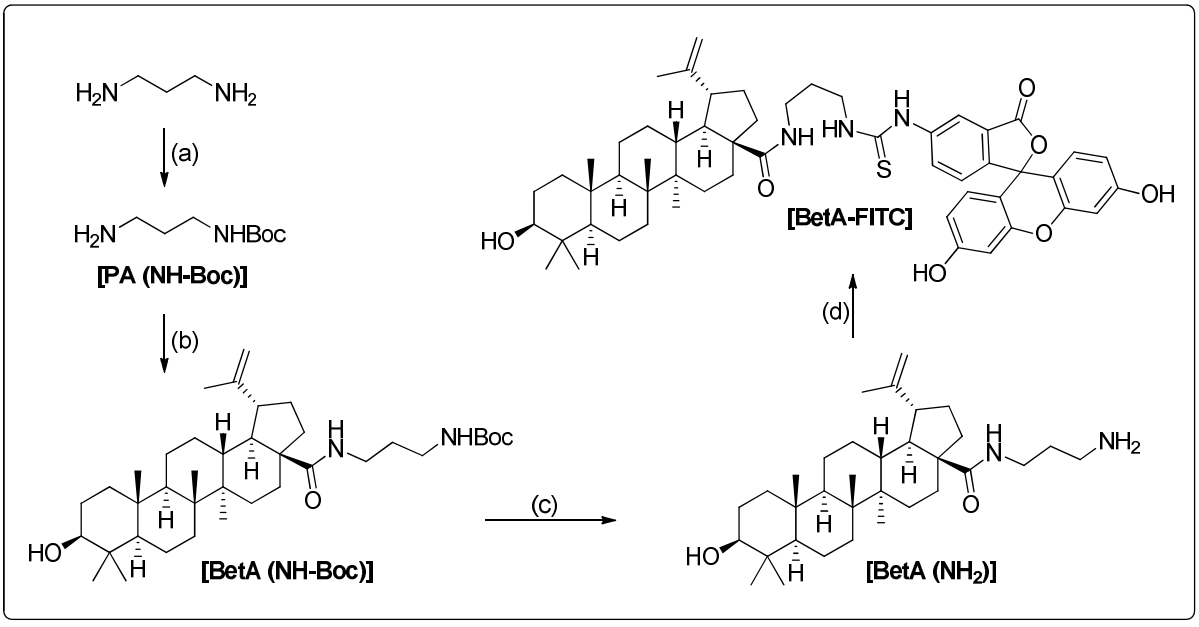
Compound **5** (100 mg, 0.19 mmol), dissolved in dry THF (5 mL), was treated with $PPh_3$ (74 mg, 0.28 mmol) at 0°C. After stirring the reaction mixture for 2 h at 0°C, few drops of water were added the reaction was kept stirring for overnight at rt. The crude reaction mixture was flash chromatographed on silica gel (230-400 mesh) with (9:1) DCM/MeOH as an eluent to obtain the pure product (**6**) [$R_f \approx 0.4$, 72 mg, 75%]. **$^1$H NMR** (300 MHz, $CDCl_3$): *δ* 7.26-7.17 (m, 4H), *δ* 7.1 (d, *J*=5.1Hz, 1H), *δ* 6.99 (t, *J*=4.5Hz, 1H), *δ* 6.87-6.63 (m, 9H), *δ* 6.52 (d, *J*=4.8Hz, 1H), *δ* 4.86 (d, *J*=7.5Hz, 2H), *δ* 4.05 (d, *J*=12.9Hz, 2H), *δ* 3.73 (s, 3H), *δ* 3.65 (br s, 2H), *δ* 2.95 (d, *J*=13.2Hz, 2H), *δ* 1.97 (d, *J*=15.6Hz, 2H). **$^{13}$C NMR** (125 MHz, $CDCl_3$): 167.6, 167.5, 160.30, 160.28, 159.6, 159.1, 159.0, 156.9, 156.4, 141.7, 141.5, 134.0, 133.5, 133.3, 132.7, 132.6, 132.4, 131.1, 128.9, 128.8, 128.7, 127.8, 127.7, 124.7, 124.6, 122.7, 122.6, 121.8, 121.5, 121.4, 116.1, 115.4, 114.6, 114.24, 114.20, 113.7, 108.8, 108.7, 66.1, 66.0, 55.4, 43.2, 43.1, 39.2, 32.1, 31.7. **HRMS** (ESI-TOF): Calcd for $C_{32}H_{31}N_2O_4$ $(M+H)^+$ : 507.2284, found 507.2276.

**(1R,3aS,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-9-hydroxy-N-(3-(4-((4-hydroxyphenyl) (1-(4-methoxybenzyl)-2-oxoindolin-3-ylidene)methyl)phenoxy)propyl)-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)icosahydro-1H-cyclopenta[a]chrysene-3a-carboxamide [Is-BetA (7)]:**

To a solution of betulinic acid (20 mg, 0.0438 mmol) and HOBt (6.5 mg, 0.0482 mmol) in dry DMF, compound **6** (22 mg, 0.0438 mmol), DMAP (12 mg, 0.096 mmol) and DCC (11 mg, 0.0525 mmol) were sequentially added. The reaction mixture were kept stirring overnight at rt. After

extraction in EtOAc and solvent removal, the crude product was isolated by flash column chromatography on (230-400 mesh) silica gel with (24:1) DCM/MeOH as an eluent to obtain the product (**7**, orange solid) [$R_f \approx 0.5$, 35 mg, 85%]. **$^1$H NMR** (500 MHz, $CDCl_3$): $\delta$ 7.31 (d, $J$=8.5Hz, 1H), $\delta$ 7.28-7.25 (m, 3H), $\delta$ 7.21 (d, $J$=8.5Hz, 1H), $\delta$ 7.16 (d, $J$=8.0Hz, 1H), $\delta$ 7.03-7.02 (m, 1H), $\delta$ 6.91-6.89 (m, 2H), $\delta$ 6.86-6.81 (m, 3H), $\delta$ 6.76-6.63 (m, 3H), $\delta$ 6.53 (d, $J$=7.5Hz, 1H), $\delta$ 6.18-6.03 (m, 1H), $\delta$ 4.88 (d, $J$=15.5Hz, 2H), $\delta$ 4.72 (s, 1H), $\delta$ 4.58 (s, 1H), $\delta$ 4.22 (d, $J$=7.0Hz, 2H), $\delta$ 4.12-4.06 (m, 2H), $\delta$ 3.76 (s, 3H), $\delta$ 3.55-3.37 (m, 3H), $\delta$ 3.18-3.13 (m, 2H), $\delta$ 2.45 (t, $J$=10.0Hz, $J$=10.5Hz, 1H), $\delta$ 2.05-1.92 (m, 8H), $\delta$ 1.77-1.73 (m, 2H), $\delta$ 1.70-1.67 (m, 9H), $\delta$ 1.64-1.45 (m, 9H), $\delta$ 1.43-1.35 (m, 10H), $\delta$ 1.24 (t, $J$=7.0Hz, 3H), $\delta$ 1.18-1.06 (m, 7H), $\delta$ 0.96-0.88 (m, 10H), $\delta$ 0.78 (d, $J$=4.5Hz, 3H), $\delta$ 0.73 (d, $J$=6.5Hz, 3H), $\delta$ 0.66 (d, $J$=9.0Hz, 1H). **$^{13}$C NMR** (125 MHz, $CDCl_3$): 176.6, 167.5, 167.4, 160.2, 160.1, 159.1, 159.0, 158.7, 157.1, 156.7, 155.9, 151.1, 151.0, 141.8, 141.6, 134.3, 133.3, 133.2, 132.9, 132.5, 132.3, 131.4, 129.0, 128.8, 128.7, 127.9, 127.8, 124.6, 124.5, 122.8, 122.5, 122.1, 121.7, 121.5, 121.4, 116.0, 115.3, 114.6, 114.22, 114.19, 113.6, 109.6, 109.5, 108.8, 108.6, 79.20, 79.16, 67.1, 66.9, 58.6, 55.84, 55.81, 55.6, 55.4, 50.80, 50.77, 50.33, 50.31, 49.3, 47.0, 43.2, 43.1, 42.6, 40.9, 39.0, 38.9, 38.6, 37.98, 37.96, 37.6, 37.5, 37.4, 34.6, 34.1, 34.0, 33.9, 31.1, 29.6, 29.4, 29.3, 28.1, 27.6, 25.8, 25.7, 25.0, 21.1, 19.7, 19.6, 18.5, 18.4, 16.4, 16.3, 16.2, 15.5, 14.8. **IR** (KBr, $cm^{-1}$): 3326.8, 2929.5, 2850.9, 1670.0, 1663.5, 1627.0, 1605.0, 1576.4, 1535.1, 1511.4, 1466.9, 1346.3, 1245.3, 1171.4, 1088.4, 1044.5, 1032.2. **HRMS** (ESI-TOF): Calcd for $C_{62}H_{76}N_2O_6Na$ $(M+Na)^+$: 967.5601, found 967.5595. **M.P**: 170-172°C.

**Synthesis of BetA-FITC:**




**Reactions and conditions:** (a) $(BoC)_2O$, $CHCl_3$, rt, 24h; (b) BetA, EDC, $NEt_3$, DMAP (cat), HOBt, DMF, rt, overnight, 92%; (c) 20% (v/v) TFA in DCM, rt, 2h; (d) FITC, $NEt_3$, DCM, rt, overnight, 53%.

**Experimental Procedures for the preparation of [BetA-FITC]:**

**Synthesis of tert-butyl (3-aminopropyl) carbamate [PA (NH-Boc)]:**

To a stirred solution of 1,3-diaminopropane (1.0 g, 13.5 mmol) in 15 mL anhydrous $CHCl_3$, $(BoC)_2O$ (0.31 mL, 1.35 mmol) in 5 mL $CHCl_3$ was added dropwise at 0°C under argon atmosphere. The reaction mixture was warmed to room temperature and stirred for 24 h. Then the solvent was removed under reduced pressure and the thick oil so obtained was diluted with $CH_2Cl_2$. The organic layer was washed with brine (3 × 20 mL), dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure to give product as gummy oil. The crude oily substance was then purified through a filtering column, which was used for the next step. **$^1$H NMR** (300 MHz, $CDCl_3$): *δ* 4.94 (br s, 1H), *δ* 3.18 (d, *J*=6.0Hz, 2H), *δ* 2.76-2.71 (m, 2H), *δ* 1.63-1.54 (m, 2H), *δ* 1.41 (s, 9H), *δ* 1.36 (br s, 2H). [Ref. *J. Med. Chem.* **2014**, *57*, 4263-4272.]

**Synthesis of tert-butyl (3-((1R,3aS,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-9-hydroxy-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)icosahydro-1H-cyclopenta[a]chrysene-3a-carboxamido)propyl)carbamate [BetA (NH-Boc)]:**

To a solution of betulinic acid (30 mg, 0.0657 mmol) and HOBt (10.0 mg, 0.0723 mmol) in dry DMF, compound (11.5 mg, 0.0657 mmol), $NEt_3$ (20µL, 0.1445 mmol), DMAP (10 mol %), EDC (14 mg, 0.0723 mmol) were sequentially added. The reaction mixture were kept stirring overnight at rt. After extraction in EtOAc and solvent removal, the crude product was isolated by flash column chromatography on (230-400 mesh) silica gel with (24:1) DCM/MeOH as an eluent to obtain the pure product [BetA (NH-Boc)] [$R_f \approx 0.5$, 37 mg, 92%]. **$^1$H NMR** (300 MHz, $CDCl_3$): *δ* 6.33 (br s, 1H), *δ* 4.90 (br s, 1H), *δ* 4.74 (s, 1H), *δ* 4.58 (s, 1H), *δ* 3.39-3.11 (m, 5H), *δ* 2.50-2.40 (m, 1H), *δ* 2.04-1.87 (m, 2H), *δ* 1.80-1.71 (m, 2H), *δ* 1.68 (s, 3H), *δ* 1.63-1.47 (m, 9H), *δ* 1.44 (s, 9H), *δ* 1.41-1.30 (m, 4H), *δ* 1.25 (s, 6H), *δ* 1.17-1.14 (m, 1H), *δ* 0.96 (s, 3H), *δ* 0.95 (s, 3H), *δ* 0.92 (s, 3H), *δ* 0.89-0.85 (m, 2H), *δ* 0.81 (s, 3H), *δ* 0.74 (s, 3H), *δ* 0.67 (d, *J*=9.0Hz, 1H). **HRMS** (ESI-TOF): Calcd for $C_{38}H_{65}N_2O_4$ $(M+H)^+$: 613.4944, found 613.4937.

**Synthesis of (1R,3aS,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-N-(3-aminopropyl)-9-hydroxy-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)icosahydro-1H-cyclopenta[a] chrysene-3a-carboxamide [BetA ($NH_2$)]:**

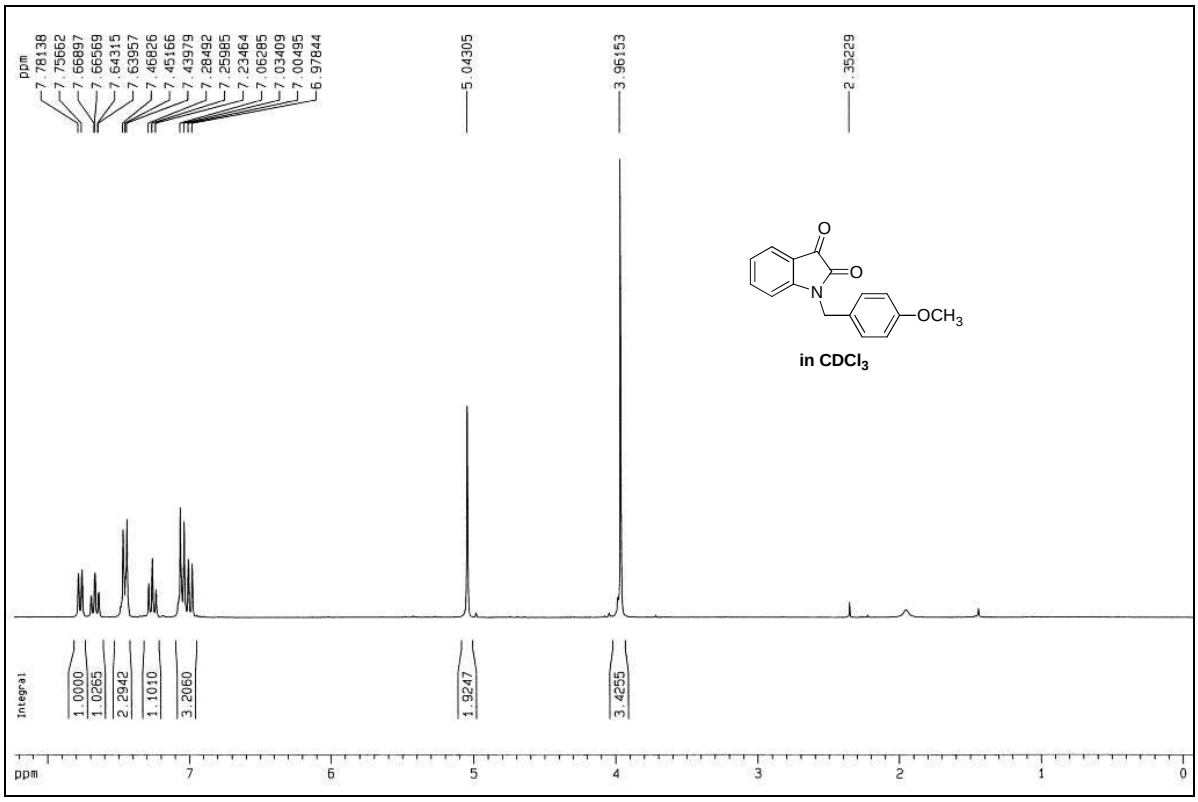
Compound **[BetA (NH-Boc)]** (10 mg, 0.0163 mmol) was treated with 20 % TFA in DCM at 0°C. After the addition was complete, the cooling bath was removed and the mixture stirred at rt for 2h. Then the solvent was removed under reduced pressure and the crude mixture thus obtained was used for the next step without further purification. **HRMS** (ESI-TOF): Calcd for $C_{33}H_{57}N_2O_2$ $(M+H)^+$: 513.4420, found 513.4417.

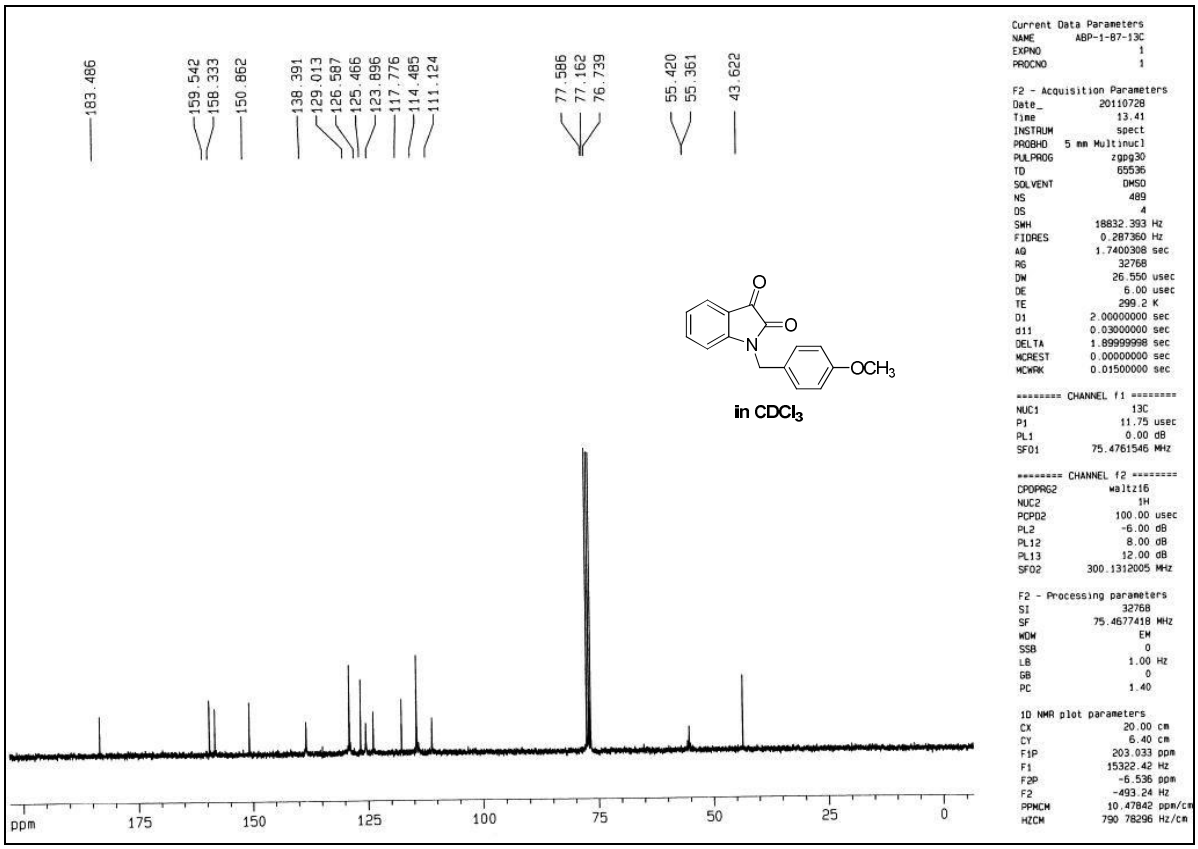
**Synthesis of (1R,3aS,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-N-(3-(3-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)propyl)-9-hydroxy-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)icosahydro-1H-cyclopenta[a]chrysene-3a-carboxamide [BetA-FITC]:**

To a solution of **[BetA ($NH_2$)]** (5.3 mg, 0.0103 mmol) in dry DCM, FITC (4.0 mg, 0.0103 mmol) and $NEt_3$ (4.6 µL, 0.033 mmol) were sequentially added. The reaction mixture was kept stirring overnight at rt under dark condition. After extraction in EtOAc and solvent removal, the crude product was purified by flash column chromatography on (230-400 mesh) silica gel with (19:1) DCM/MeOH as an eluent [$R_f \approx 0.4$, 5.0 mg, 53%]. **$^1$H NMR** (500 MHz, DMSO-$d_6$): $\delta$ 10.15 (s, 2H), $\delta$ 8.35 (br s, 1H), $\delta$ 7.80 (br s, 1H), $\delta$ 7.16 (d, $J$=8.0Hz, 1H), $\delta$ 6.69 (s, 2H), $\delta$ 6.57 (dd, $J$= 15.0Hz, $J$=7.5Hz, 5H), $\delta$ 4.70 (m, 1H), $\delta$ 4.30-4.26 (m, 1H), $\delta$ 4.11-4.05 (m, 1H), $\delta$ 3.62-3.50 (m, 3H), $\delta$ 3.16 (d, $J$=3.0Hz, 2H), $\delta$ 3.14-3.11 (m, 1H), $\delta$ 2.99-2.96 (m, 1H), $\delta$ 1.82-1.56 (m, 12H), $\delta$ 1.45-1.14 (m, 15H), $\delta$ 1.08-0.65 (m, 20H). **HRMS** (ESI-TOF): Calcd for $C_{54}H_{68}N_3O_7S$ $(M+H)^+$ : 902.4778, found 902.4774.

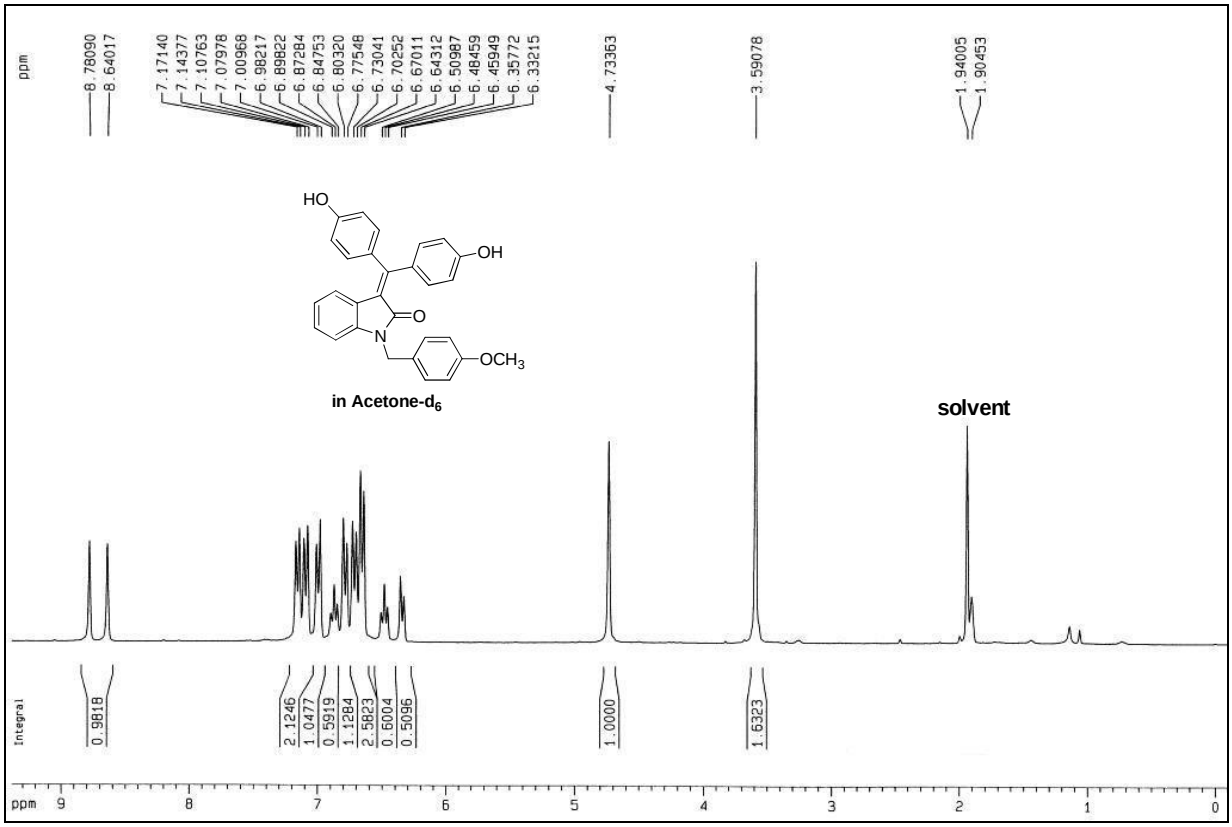
## Relevant $^{1}H$ and $^{13}C$ spectrums:

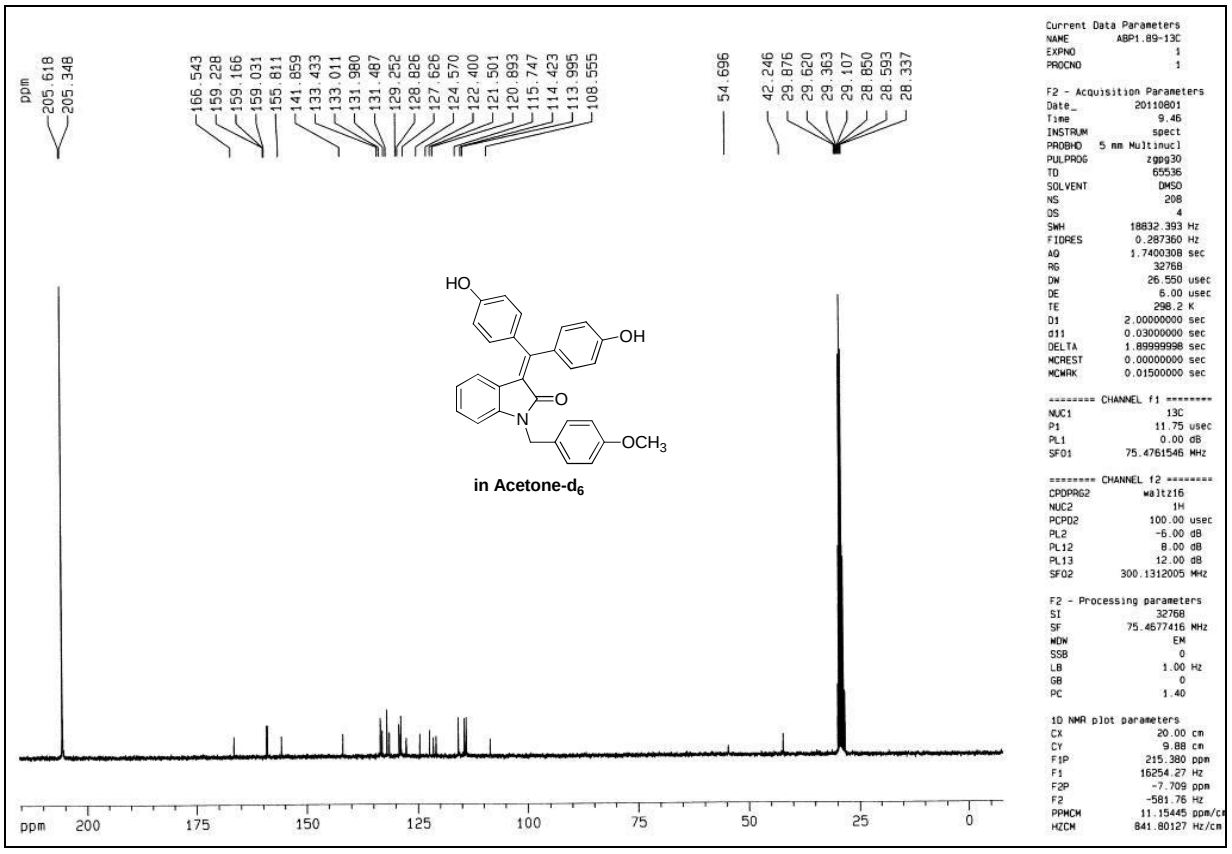
### Compound 2:



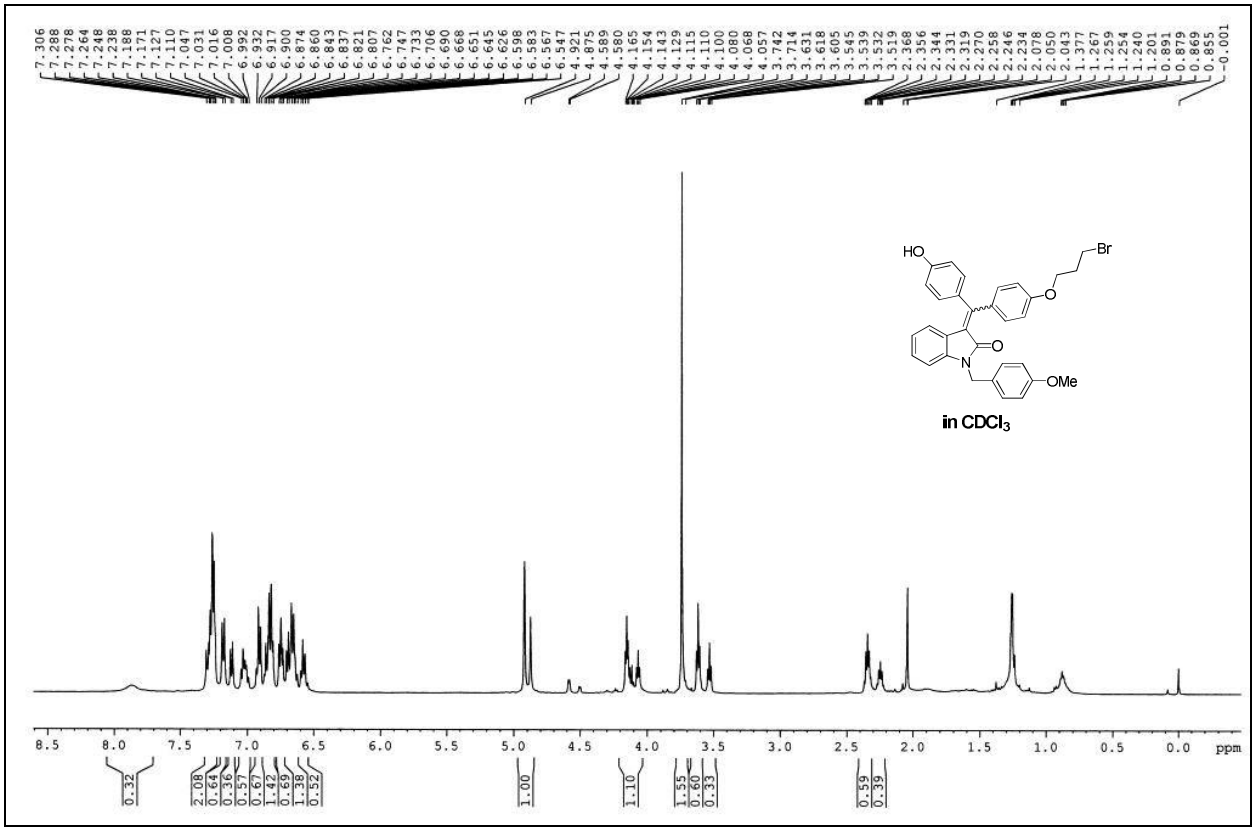



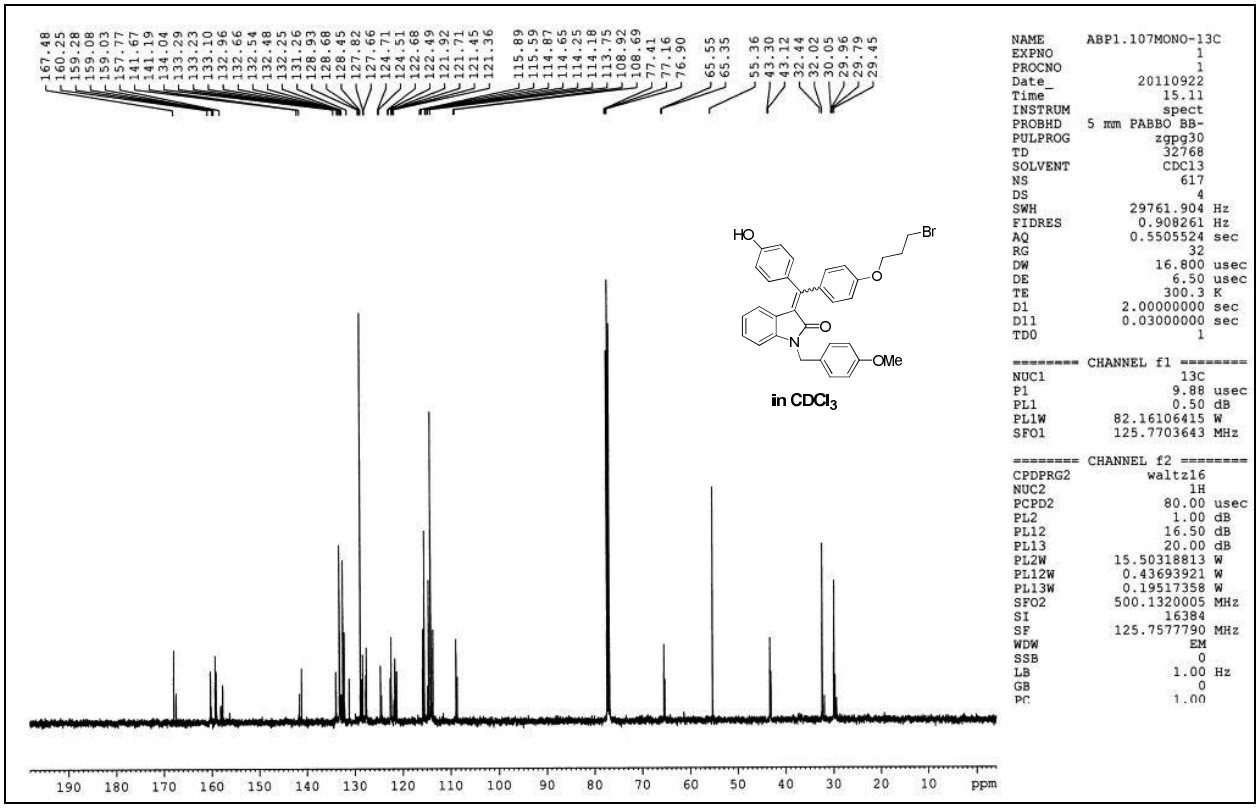
**Compound 3 (Is-PMB):**



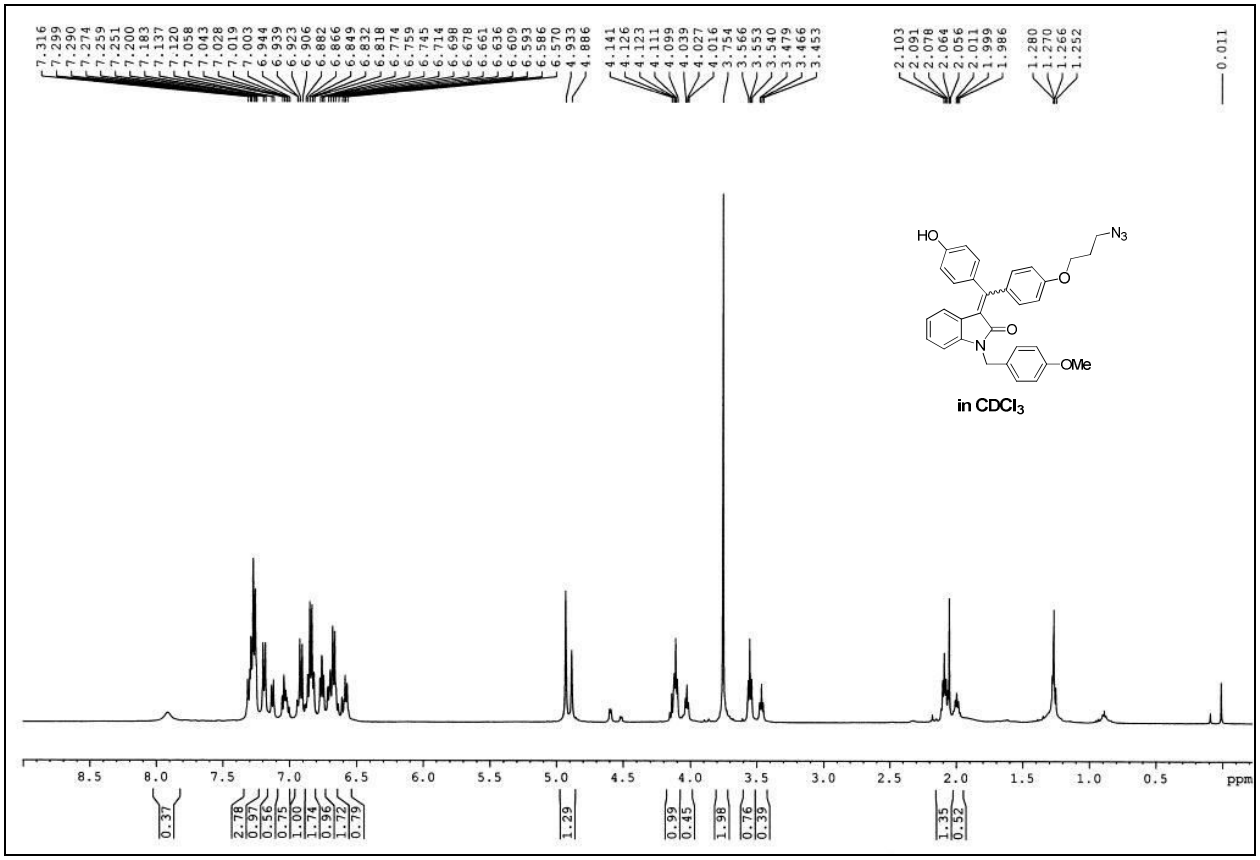



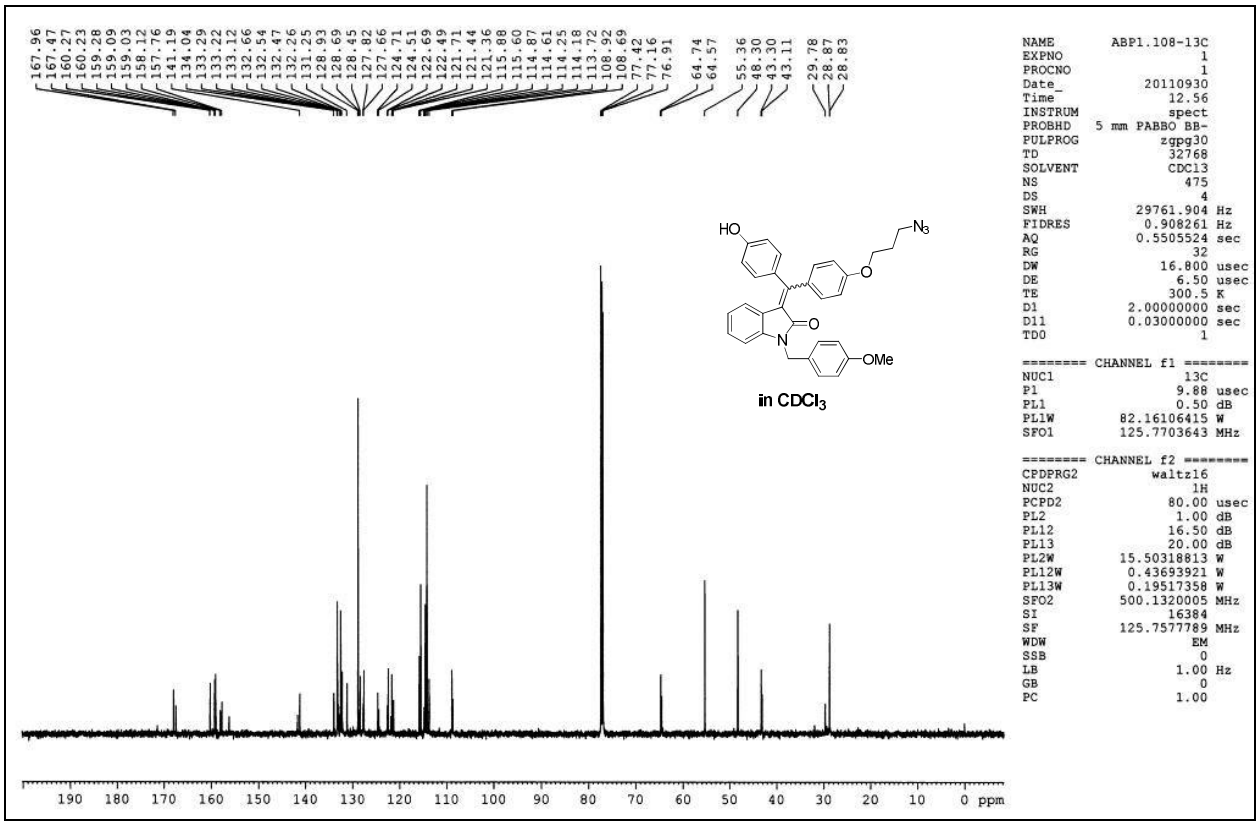
**Compound 4 (Is-Bromide):**



**Compound 5 (Is-Azide):**




| | |
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| Date_ | 20110930 |
| Time | 12.56 |
| INSTRUM | spect |
| PROBHD | 5 mm PABBO BB- |
| PULPROG | zgpg30 |
| TD | 32768 |
| SOLVENT | CDCl3 |
| NS | 475 |
| DS | 4 |
| SWH | 29761.904 Hz |
| FIDRES | 0.908261 Hz |
| AQ | 0.5505524 sec |
| RG | 32 |
| DW | 16.800 usec |
| DE | 6.50 usec |
| TE | 300.5 K |
| D1 | 2.00000000 sec |
| D11 | 0.03000000 sec |
| TD0 | 1 |
| ======== CHANNEL f1 ======== | |
| NUC1 | 13C |
| P1 | 9.88 usec |
| PL1 | 0.50 dB |
| PL1W | 82.16106415 W |
| SFO1 | 125.7703643 MHz |
| ======== CHANNEL f2 ======== | |
| CPDPRG2 | waltz16 |
| NUC2 | 1H |
| PCPD2 | 80.00 usec |
| PL2 | 1.00 dB |
| PL12 | 16.50 dB |
| PL13 | 20.00 dB |
| PL2W | 15.50318813 W |
| PL12W | 0.43693921 W |
| PL13W | 0.19517358 W |
| SFO2 | 500.1320005 MHz |
| SI | 16384 |
| SF | 125.7577789 MHz |
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| LB | 1.00 Hz |
| GB | 0 |
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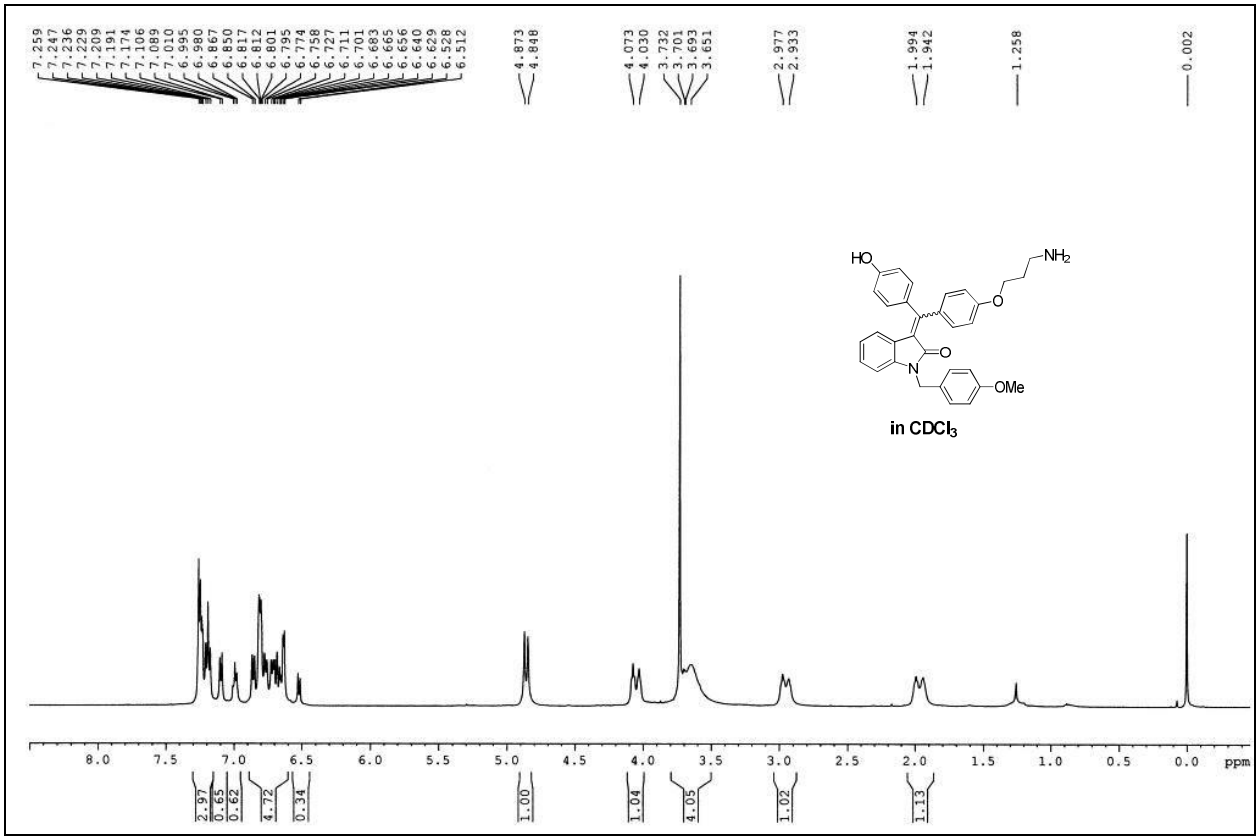
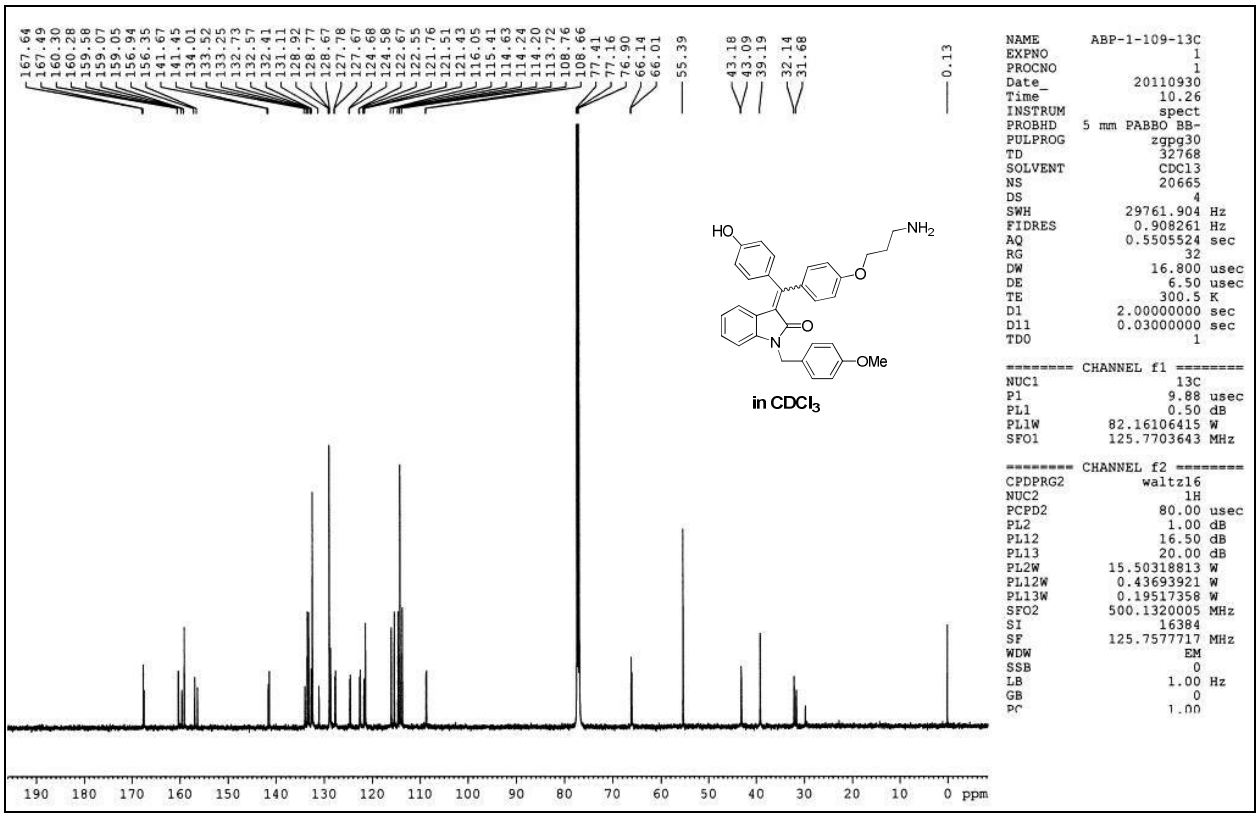
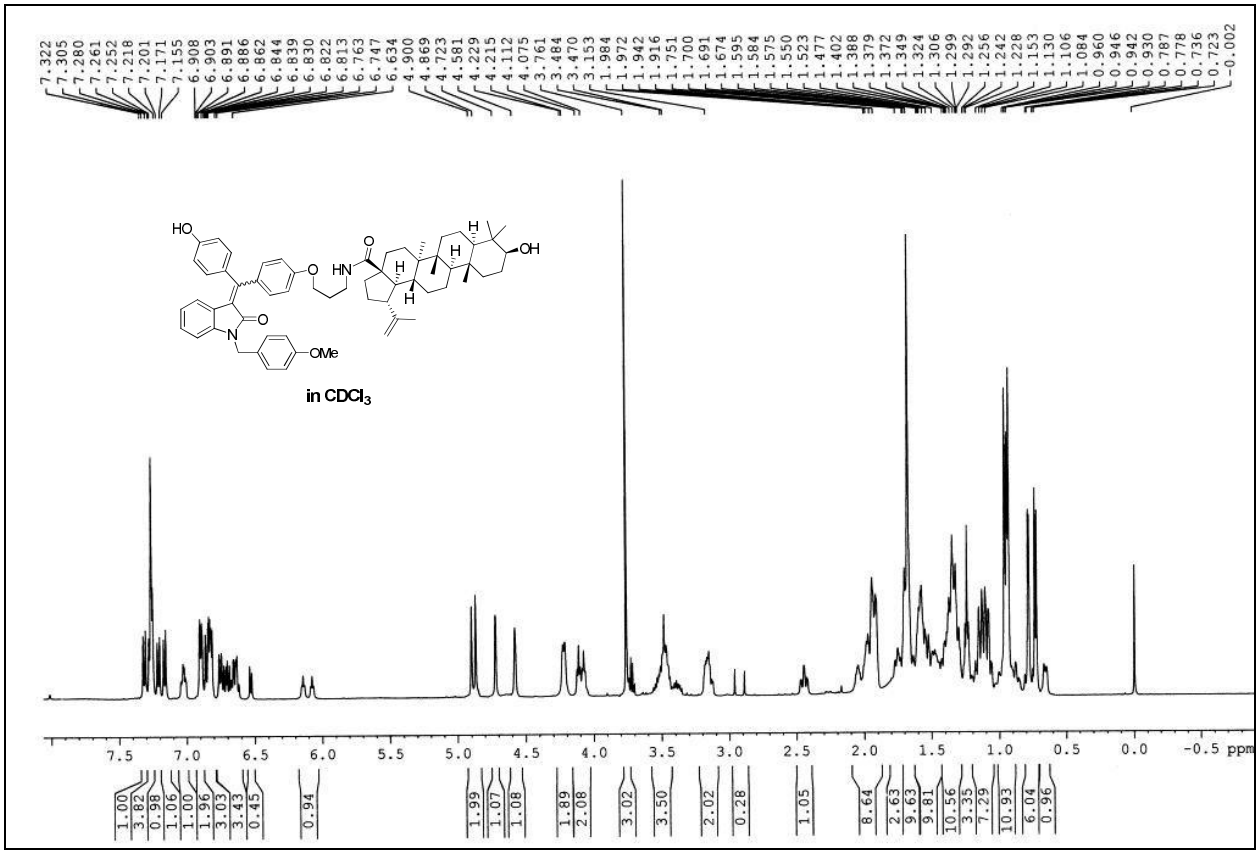
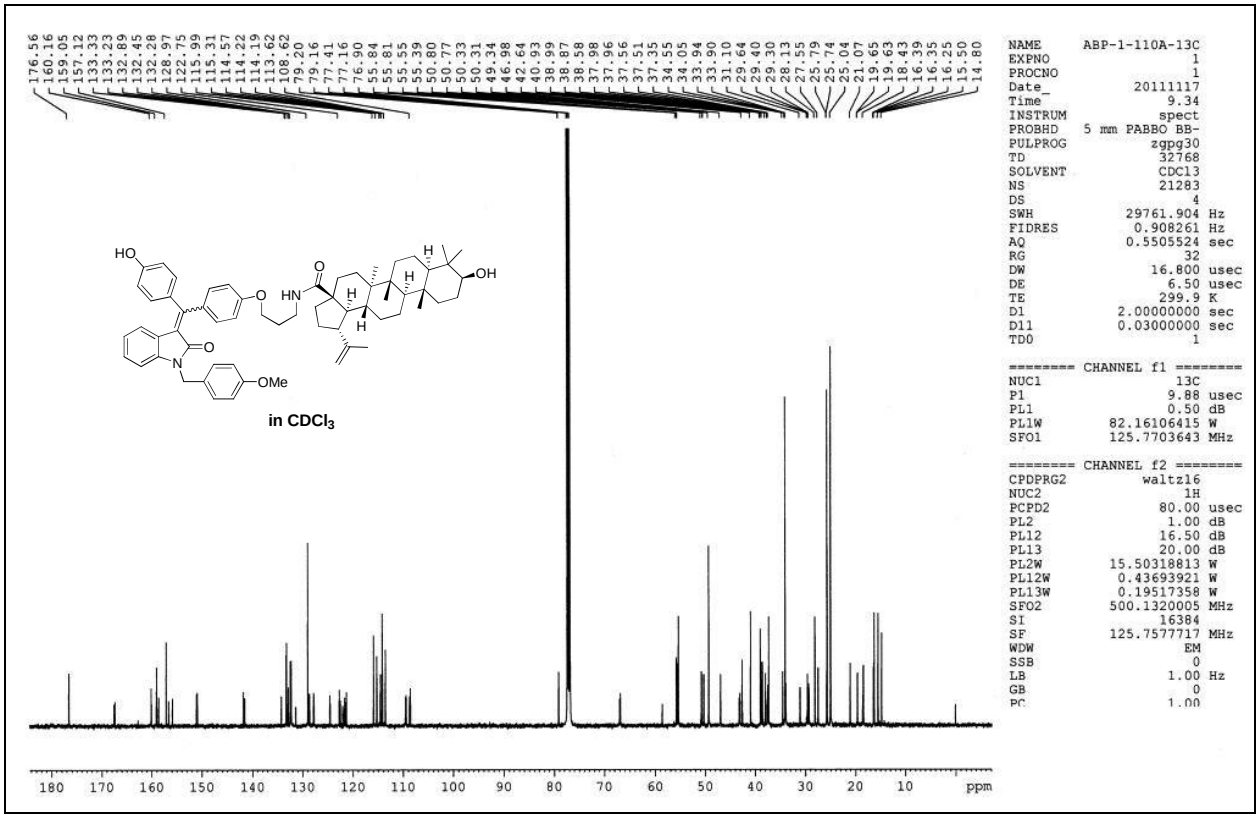


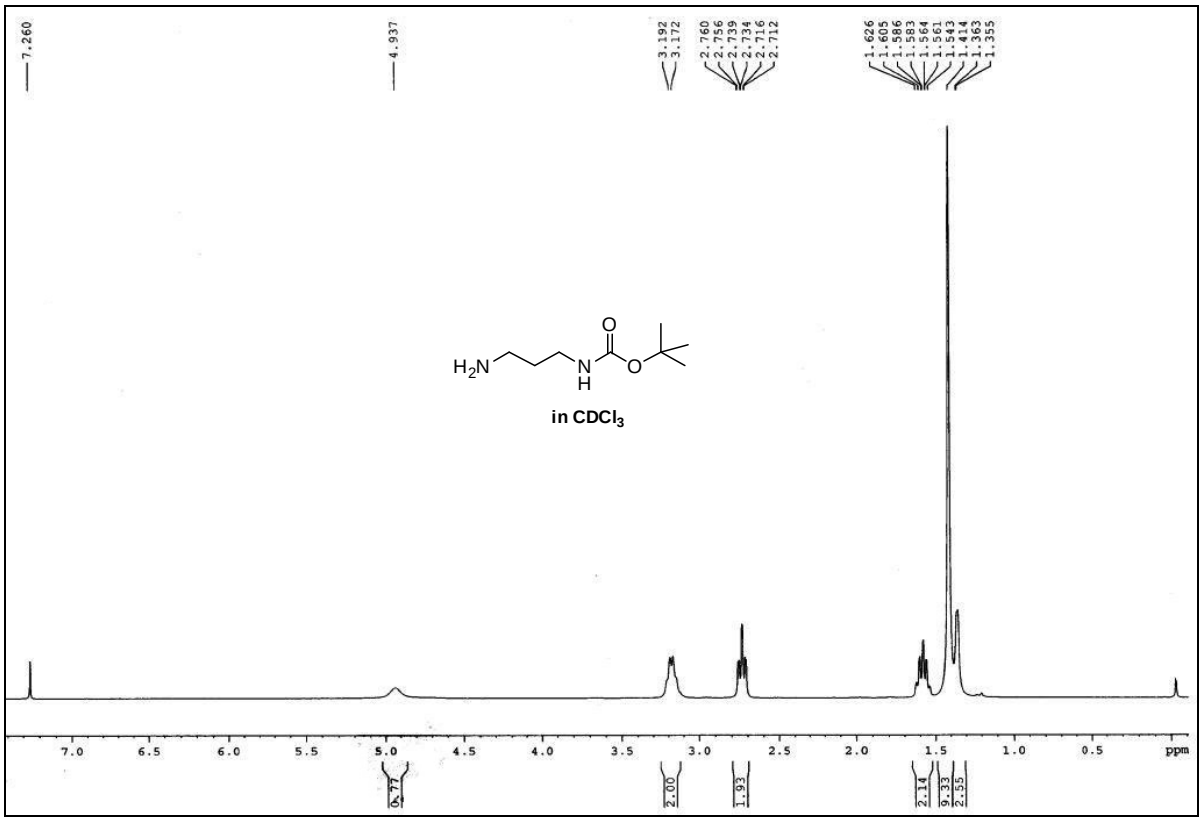



**Compound 7 (Is-BetA):**

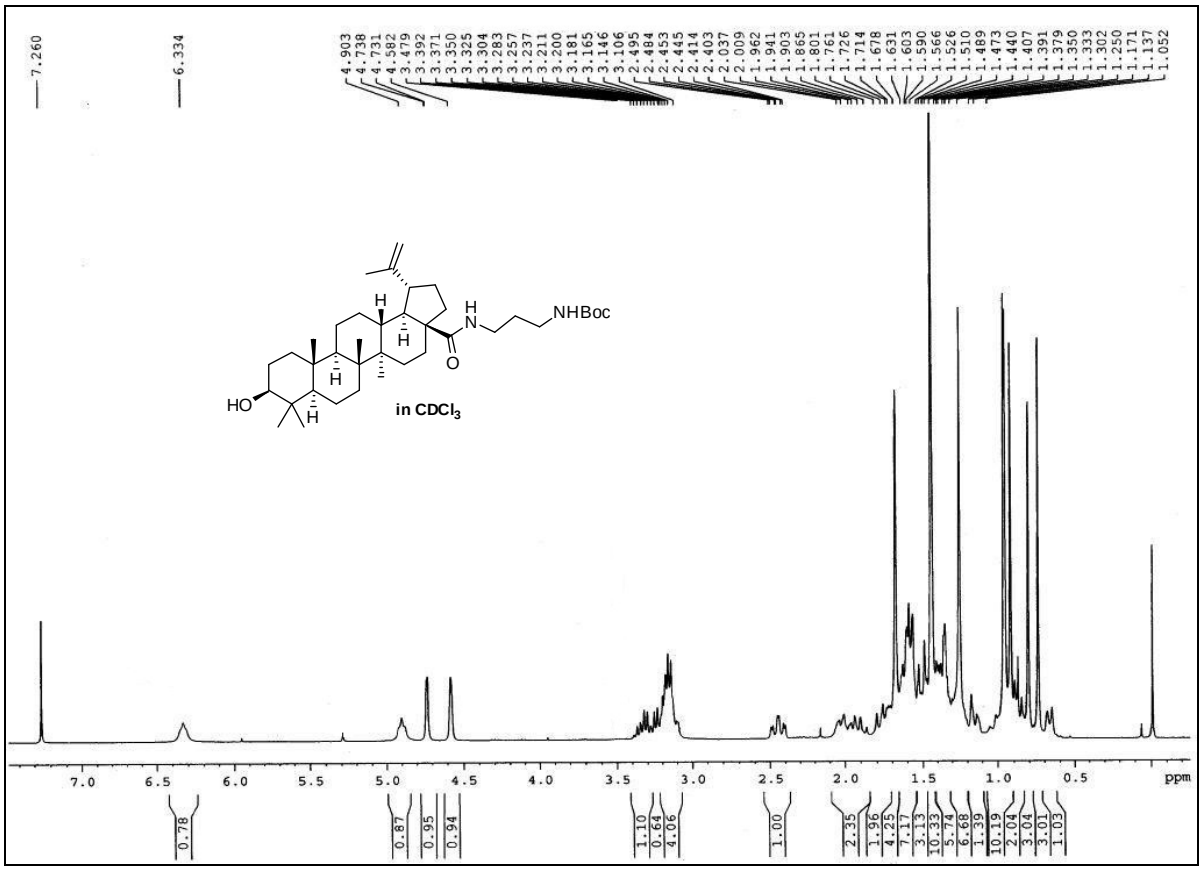


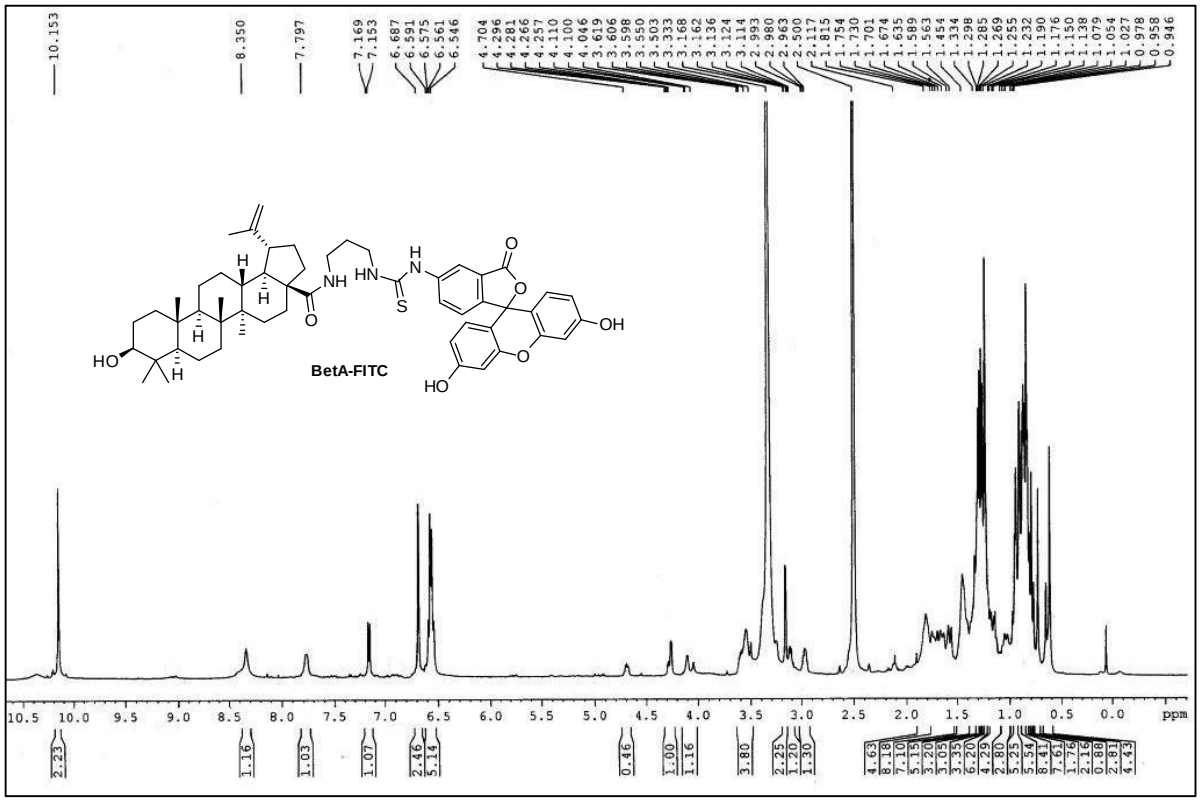
**$^1$H NMR of [PA (NH-Boc)]:**




**$^1$H NMR of [BetA (NH-Boc)]:**



**$^{1}$H NMR of [BetA-FITC]:**



## Experimental Section

## Biological data:

### Cell Culture:

Cells like MCF-7, MDA-MB-231, NIH3T3, CHO, HEK-293T, B16F10, A549, OVCAR3, PANC1 were bought from the ATCC (USA). DMEM medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (Lonza, US), 50µg/ml penicillin, 50µg/ml streptomycin and 100µg/ml kanamycin (Sigma Aldrich, US) as preventive antibiotics and gentamycin (10 µg/ml) (Abbott Laboratories, India) and amphotericin B (2 µg/ml) (Bharat Serums and Vaccines Ltd., India) as additional, preventive, antimycotic and antifungal agents were chosen as the cell culture medium. Cells were cultured at 37°C in a humidified atmosphere of 5% $CO_2$ in air. All the cells were mycoplasma free. Cultures of 85–90% confluency were maintained for all of the experiments. The cells were trypsinized, counted and seeded in 96-well plates for viability studies or in 6-well plates for apoptosis and cellular uptake studies. The cells were allowed to adhere overnight before they were used for experiments.

### Cytotoxicity Studies:

Cytotoxicities of the compounds were evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay as described earlier. Briefly, cells were seeded at a density of 5,000 cells/well in a 96-well plate usually 18–24h before experiment. Compounds were dissolved in DMSO to make their stock solutions. Serum containing media containing respective concentrations of compounds were added to triplicate wells. The final volume of DMSO was never more than 0.5% of final volume of solution treated to cells. In this study all the molecules at different concentrations were treated continuously to the cells for 72h. Following the termination of experiment, cells were washed and promptly assayed for viability using MTT. Results were expressed as percent viability = [$A_{550}$(treated cells) – background/$A_{550}$(untreated cells) – background] × 100. Cytotoxicity studies were repeated at least 6 times in MCF-7, B16F10, PANC1 and NIH3T3 cell lines and at least 3 times in other cell lines. $IC_{50}$ values were obtained as a mean of 3 results obtained from individual cytotoxicity experiments.

### Cellular Uptake studies under Fluorescence Microscope:

Cells were seeded at a density of 200,000 cells/well in a 6-well plate usually. After 18-24 hrs, the cells were either kept untreated (UT) or treated with the bis-arylidene oxindole derivatives (Is-BetA, Is-PMB, Is-Amine) and the other control molecules like Doxorubicin (Dox) and Betulinic Acid (BetA) at concentrations of 2µM for 4 hrs for all the molecules, before they were

washed with 1x PBS and placed under the Fluorescence Microscope, (Nikon) for observing the cellular uptake through elucidation of their respective fluorescence emission. All the images were taken at 20x objective.

**Cellular Uptake Quantification:**

Cells were seeded at a density of 10,000 cells/well in a 96-well plate. After 18-24 hrs, the cells were either kept untreated or treated with bis-arylidene oxindole derivative molecules and also other control molecules, at concentration of 2μM for 4 hrs. The cells were then washed twice with PBS after complete removal of the media and 50μl of cell lysis buffer (RIPA Buffer, Sigma Aldrich) was added in each well and kept in the shaker for around 20-30 minutes, for proper lysis. After the lysis, 10μl of the lysates of each sample were taken out for protein estimation which was done with BCA protein estimation kit (Thermo Scientific) as described in the manufacturer protocol. 10μl of the remaining lysate of each sample along with the free molecules(i.e. without treating in the cell) were taken in a 96-well black plate and the respective fluorescence was measured in the multimode fluorescence reader (Biotek Instruments). Results were expressed in the form of fluorescence percentage uptake ({fluorescence of the cell treated samples-fluorescence of the untreated samples/fluorescence of the free molecules} x 100) per μg protein.

**ROS studies:**

Cells were seeded at a density of 200,000 cells/well in a 6-well plate. After 18-24 hrs, cells were either kept untreated or treated with bis-arylidene oxindole derivatives and other control molecules, at a concentration of 2μM for 36 hrs for all the molecules. The samples were then treated with DCFDA {2,4-dichloro fluorescin diacetate}(Sigma Aldrich), 30 minutes before ROS analysis. The cells were then washed twice with PBS and observed under Fluorescence Microscope (Nikon) using green filter.

**Cellular Apoptosis studies:**

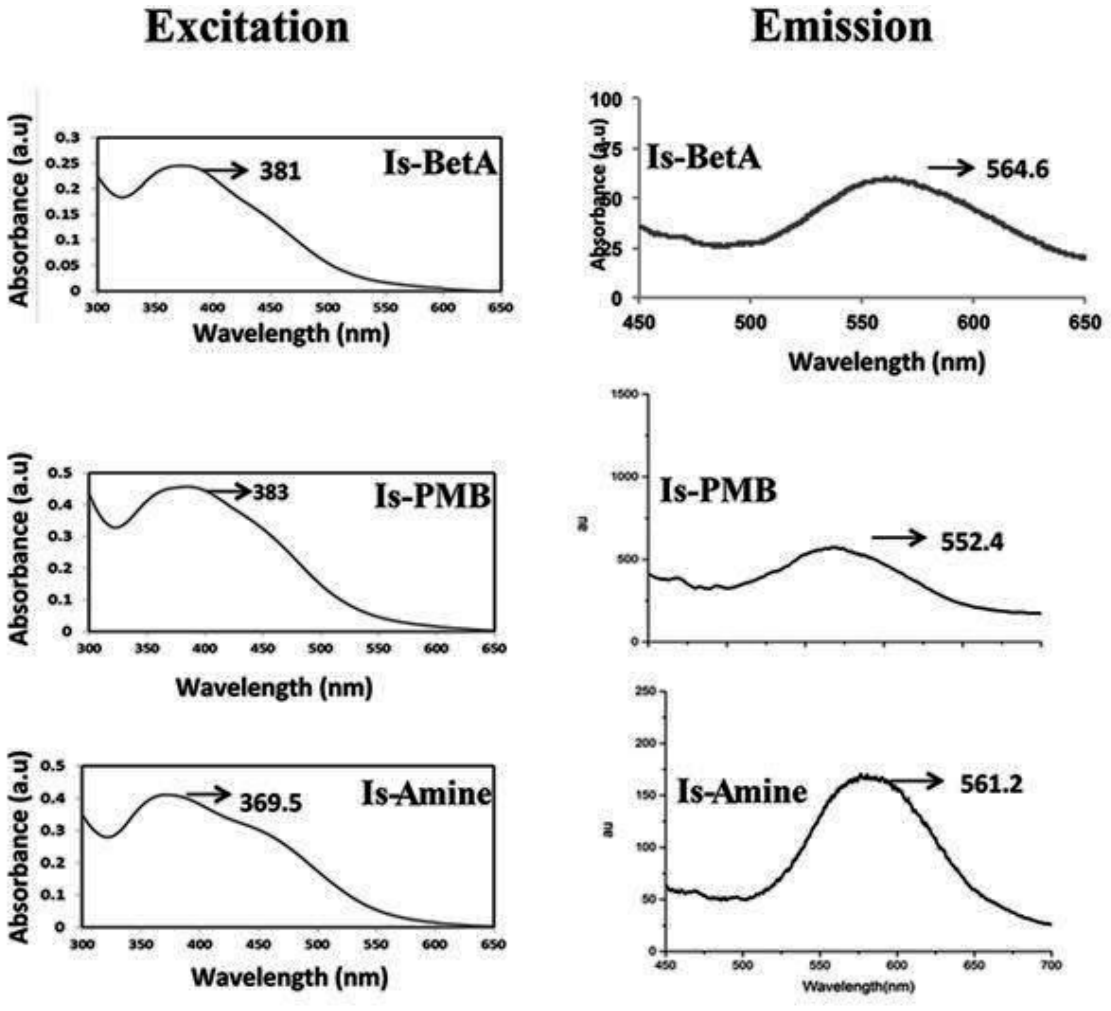
Cells were seeded at a density of 200,000 cells/well. After 18-24 hrs, the cells were either kept untreated or treated with bis-arylidene oxindole derivatives and the other control molecules at concentrations of 2μM for 45 hrs for all the molecules. The cells were then trypsinised and subjected to apoptosis studies by using the Annexin V FITC Apoptosis kit (BioLegend), as described in the manufacturer protocol.

**Determination of absorbance and emission ranges for bis-arylidene oxindole derivative molecules:**

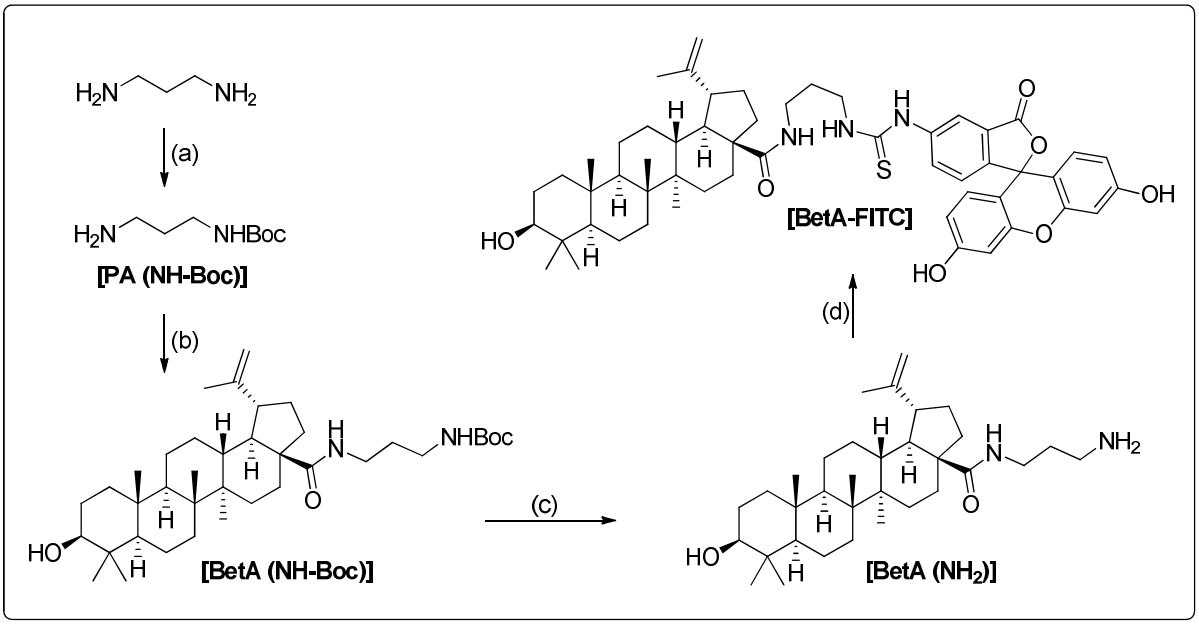
The respective absorbances i.e. excitation ranges of the bis-arylidene oxindole derivatives were determined by using Hitachi 4700 Spectrometer. The stock solution of the samples were prepared in DMSO. The samples were diluted 1000 times in water and DMSO in water was taken as blank. The samples were then measured and their respective absorbance were determined. The emission ranges of the samples were determined by using the same Hitachi 4700 Spectrometer after keeping the blank and the dilution factor same as in case of absorbance.

**Supplementary Figures and Figure Legends**

**Supplementary figure S1:** The excitation and emission curves of the compounds Is-BetA, Is-PMB and Is-Amine. The excitation ranges of the compounds vary between 369-385nm and their emission ranges vary between 550-565nm. Excitation wavelengths of Is-BetA, Is-PMB and Is-Amine are 381nm, 383nm and 369.5nm respectively. Emission wavelengths of Is-BetA, Is-PMB and Is-Amine are 564.6nm, 552.4nm and 561.2nm respectively.

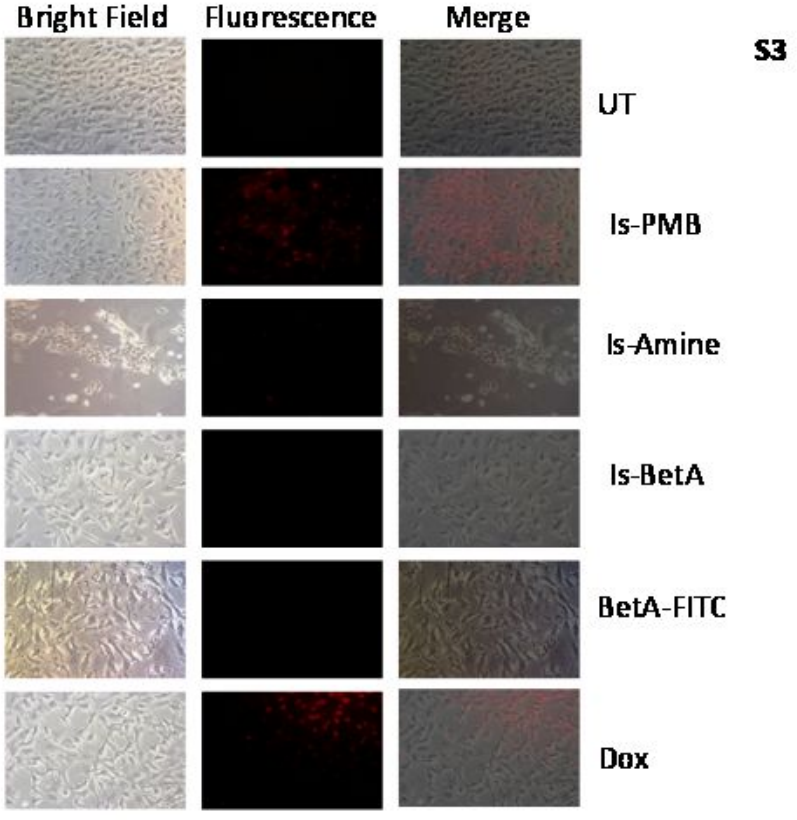


**Supplementary Figure S2: Synthesis of BetA-FITC**

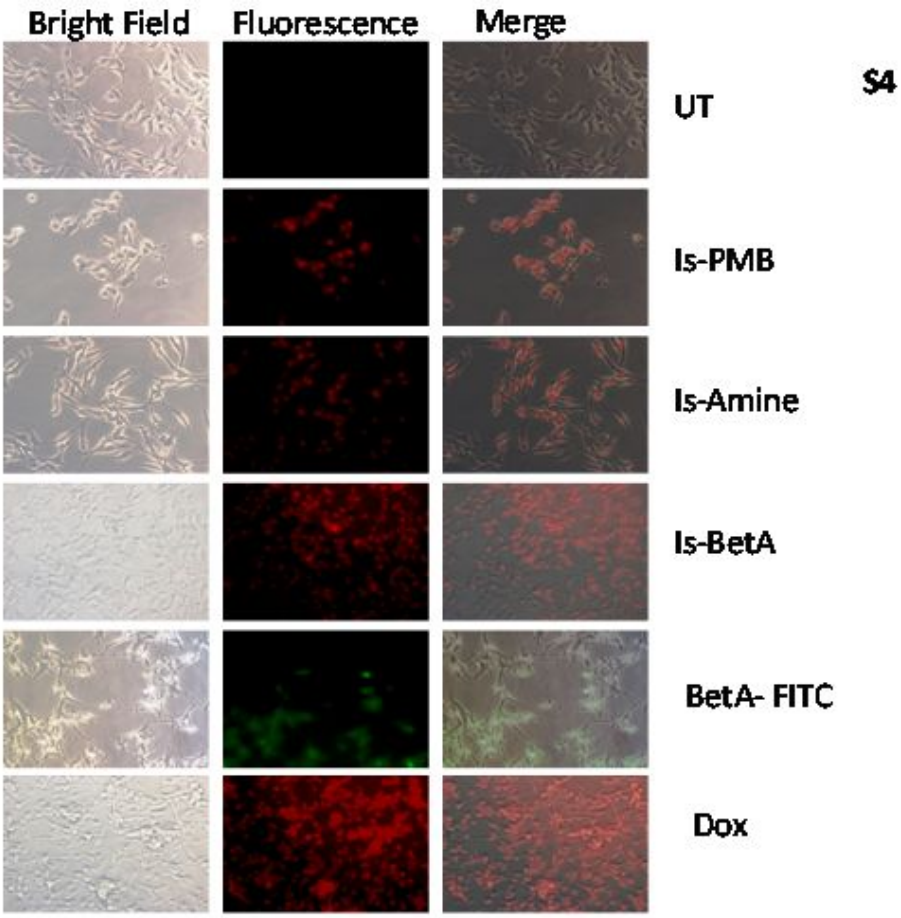



**Reactions and conditions:** (a) $(BoC)_2O$, $CHCl_3$, rt, 24h; (b) BetA, EDC, $NEt_3$, DMAP (cat), HOBt, DMF, rt, overnight (yield-92%); (c) 20% (v/v) TFA in DCM, rt, 2h; (d) FITC, $NEt_3$, DCM, rt, overnight (yield-53%).

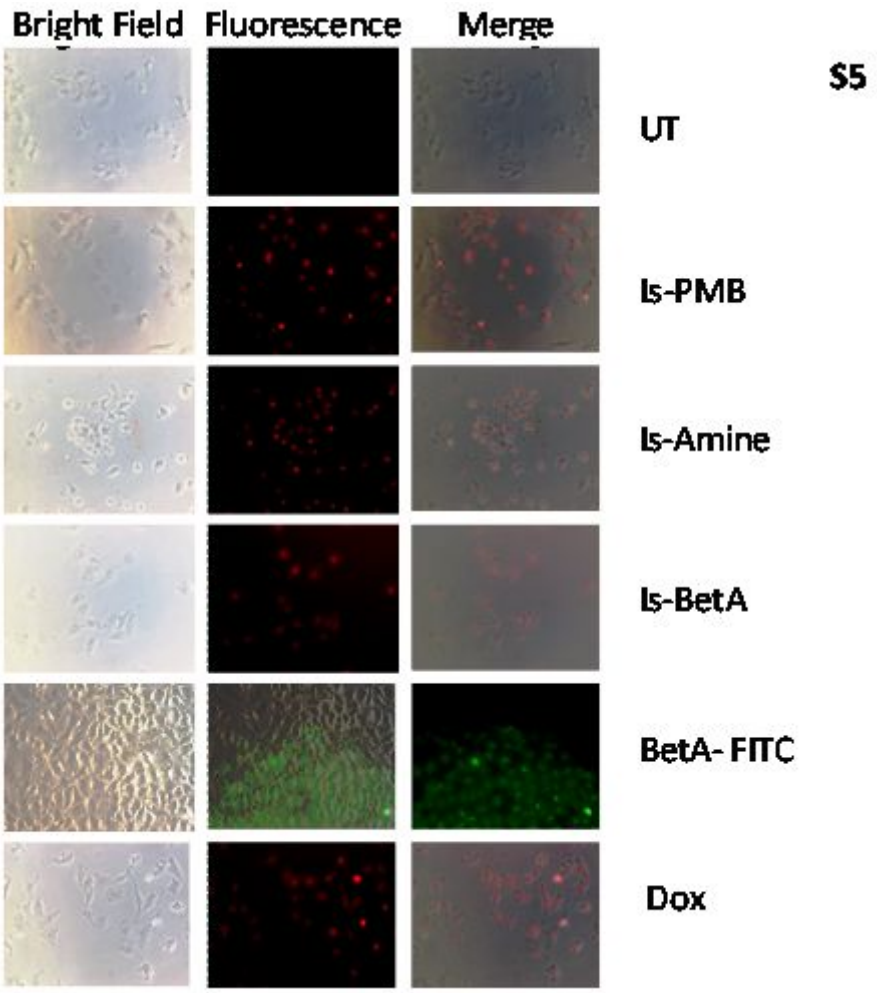
**Supplementary figure S3:** Bright field, fluorescence and their corresponding merged images of the bis-arylidene oxindole derivatives and the control molecules in NIH3T3 Cell line. The images were taken in 20x objective. The compounds Is-PMB, Is-Amine, Is-Beta and Doxorubicin (Dox) give red fluorescence upon emission, while BetA-FITC gives green fluorescence.



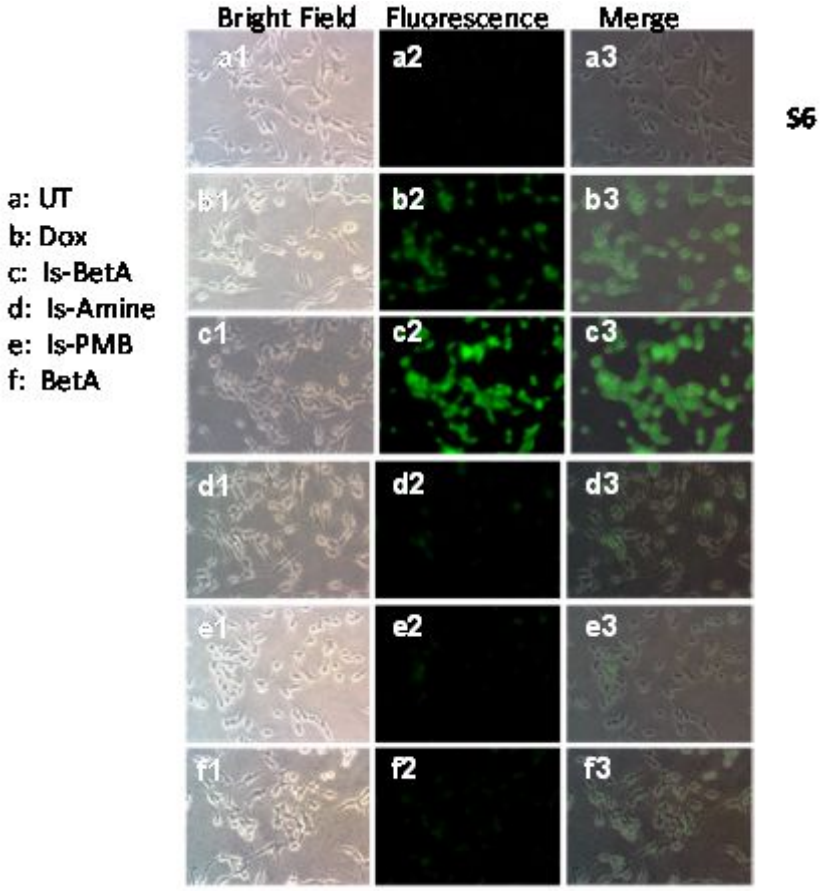
**Supplementary figure S4:** Bright field, fluorescence and their corresponding merged images of the bis-arylidene oxindole derivatives and the control molecules in B16F10 Cell line. The images were taken in 20x objective. The compounds Is-PMB, Is-Amine, Is-BetA and Doxorubicin (Dox) give red fluorescence upon emission, while BetA-FITC gives green fluorescence.



**Supplementary figure S5:** Bright field, fluorescence and their corresponding merged images of the bis-arylidene oxindole derivatives and the control molecules in PANC1 Cell line. The images were taken in 20x objective. The compounds Is-PMB, Is-Amine, Is-Beta and Doxorubicin (Dox) give red fluorescence upon emission, while BetA-FITC gives green fluorescence.



**Supplementary figure S6:** Bright field, fluorescence and their corresponding merged images showing ROS activity (a1-f3) upon treatment with bis-arylidene oxindole derivatives and the other control molecules (a-f) in B16F10 Cell line. For ROS detection DCFDA (2,4-dichloro fluorescin diacetate) was treated in each sample 30 minutes before observing under the microscope. The DCFDA dye gives green fluorescence upon emission.



**Supplementary figure S7:** Bright field, fluorescence and their corresponding merged images showing ROS activity (a1-f3) upon treatment with bis-arylidene oxindole derivatives and the other control molecules (a-f) in NIH3T3 Cell line. For ROS detection DCFDA (2,4-dichloro fluorescin diacetate) was treated in each sample 30 minutes before observing under the microscope. The DCFDA dye gives green fluorescence upon emission.