Supporting Information for

Synthesis, Biological Evaluation of Fluorescent 23-Hydroxybetulinic Acid Probes and Their Utility in Cellular Localization Studies

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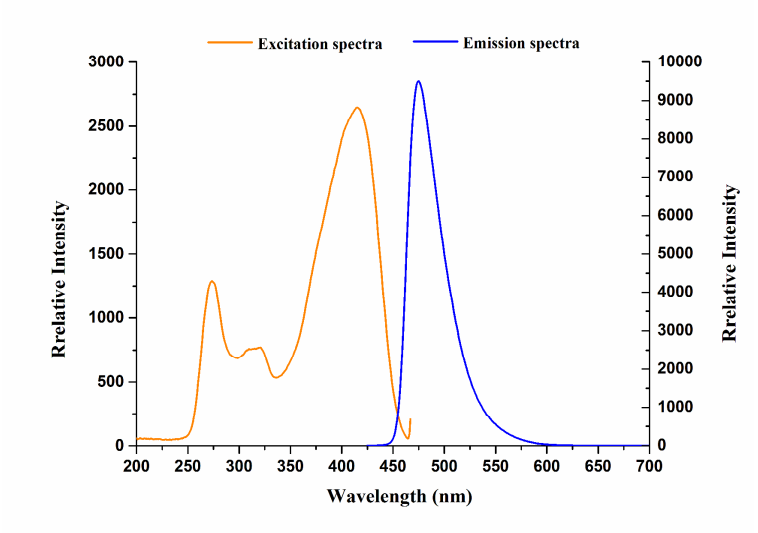


Figure S1. Excitation and emission spectra of probe **26c**. Compound **26c** had large stokes shift (60 nm) with λ_{exc} at ~415 nm and λ_{em} at ~475 nm.

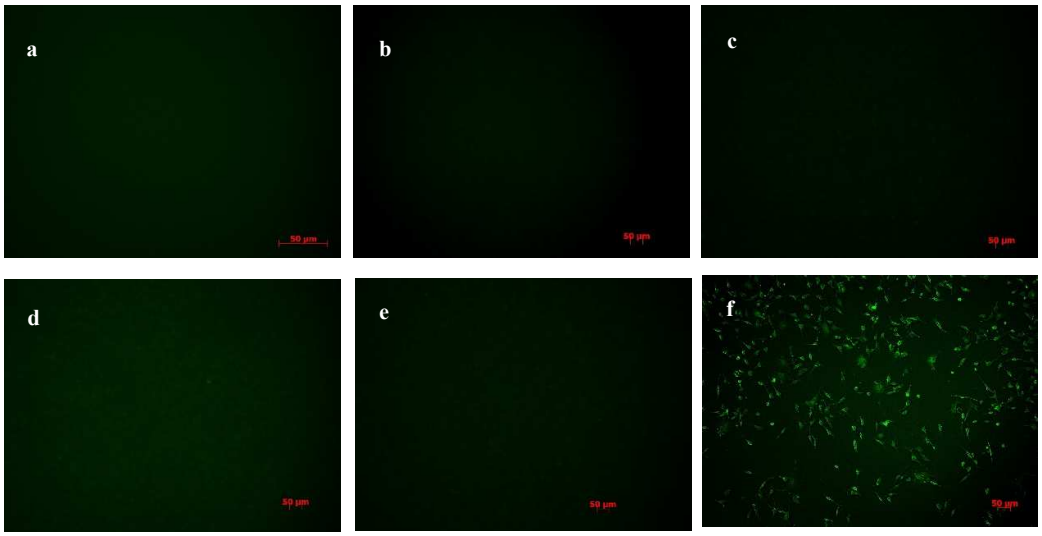


Figure S2. Time-course uptake of 2 μM probe **26c** in B16F10 cells. (a) 15 min; (b) 30 min; (c) 1 h; (d) 2 h; (e) 4 h; (d) 24 h.

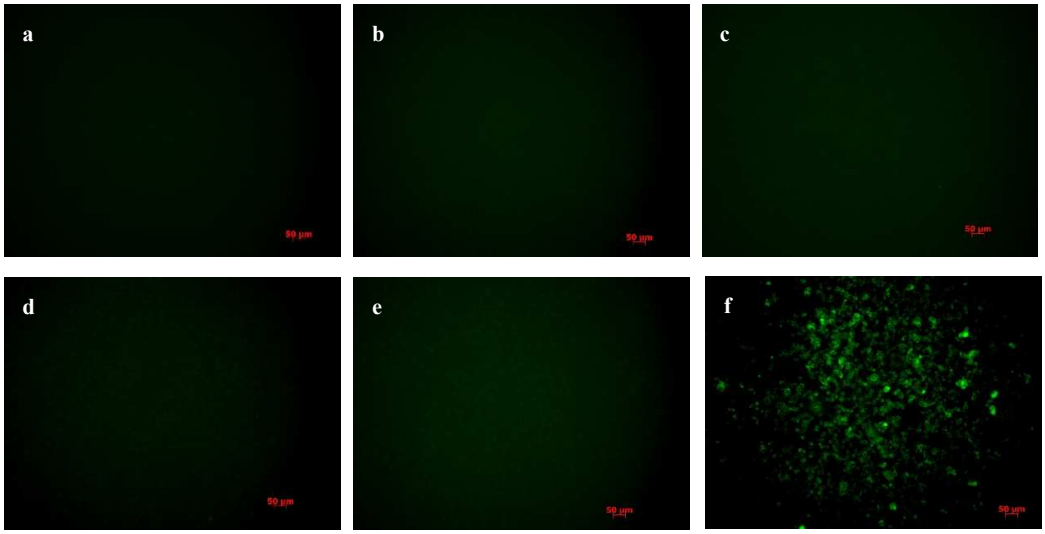


Figure S3. Time-course uptake of 4 μM probe **26c** in B16F10 cells. (a) 15 min; (b) 30 min; (c) 1 h; (d) 2 h; (e) 4 h; (d) 24 h.

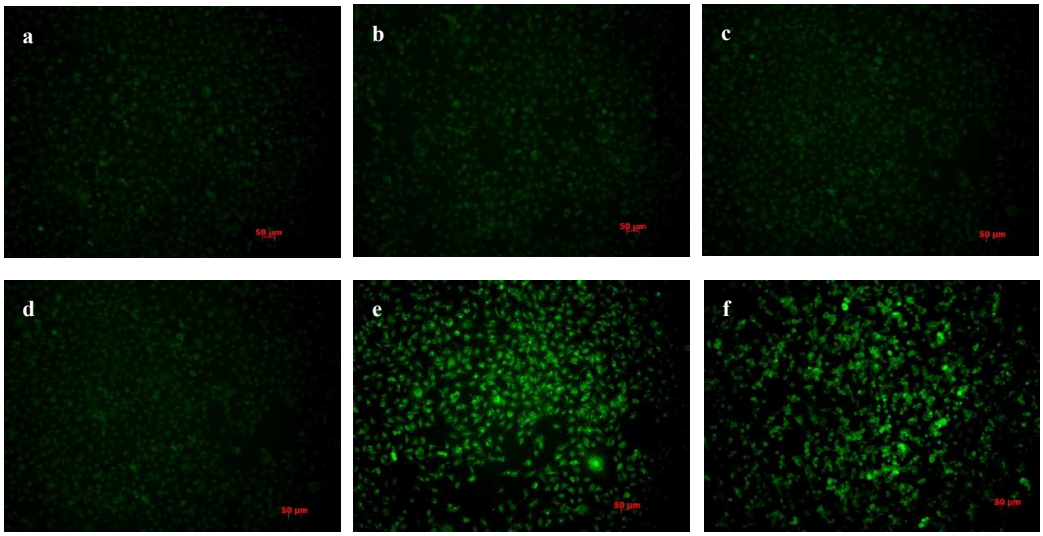


Figure S4. Time-course uptake of 10 μM probe **26c** in B16F10 cells. (a) 15 min; (b) 30 min; (c) 1 h; (d) 2 h; (e) 4 h; (d) 24 h.

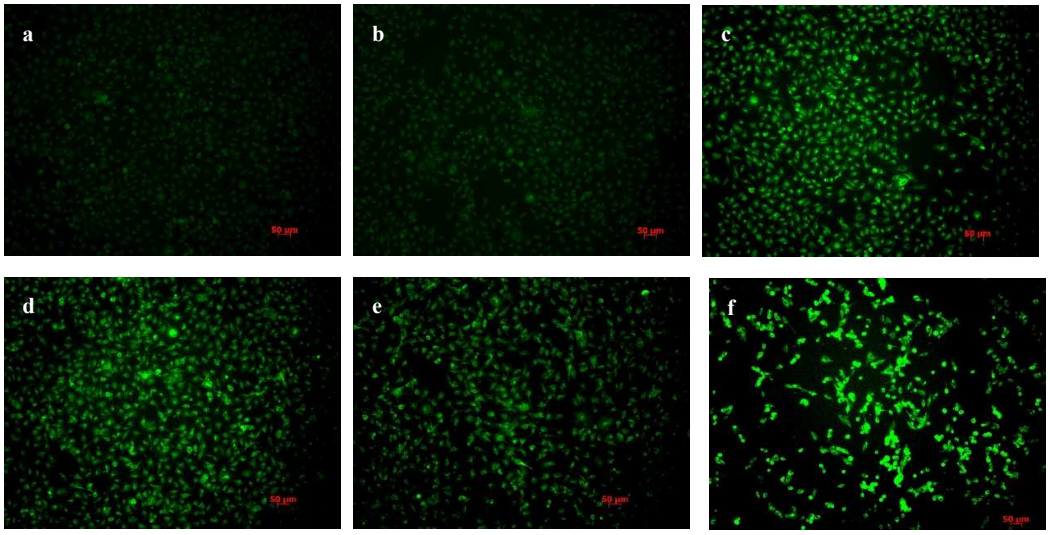


Figure S5. Time-course uptake of 20 μM probe **26c** in B16F10 cells. (a) 15 min; (b) 30 min; (c) 1 h; (d) 2 h; (e) 4 h; (d) 24 h.

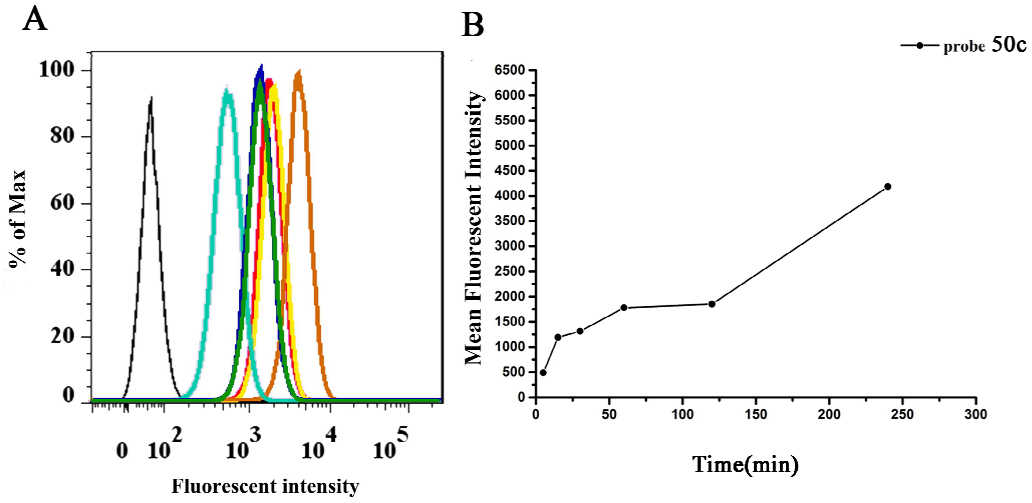


Figure S6. (A) Flow cytometry histograms of B16F10 cells exposed to 10 μM **26c** for 0 min (black), 5 min (aquamarine), 15 min (blue), 30 min (green), 60 min (red), 120 min (yellow) and 240 min (orange). (B) Plot of **26c** signal versus time in B16F10 cells.

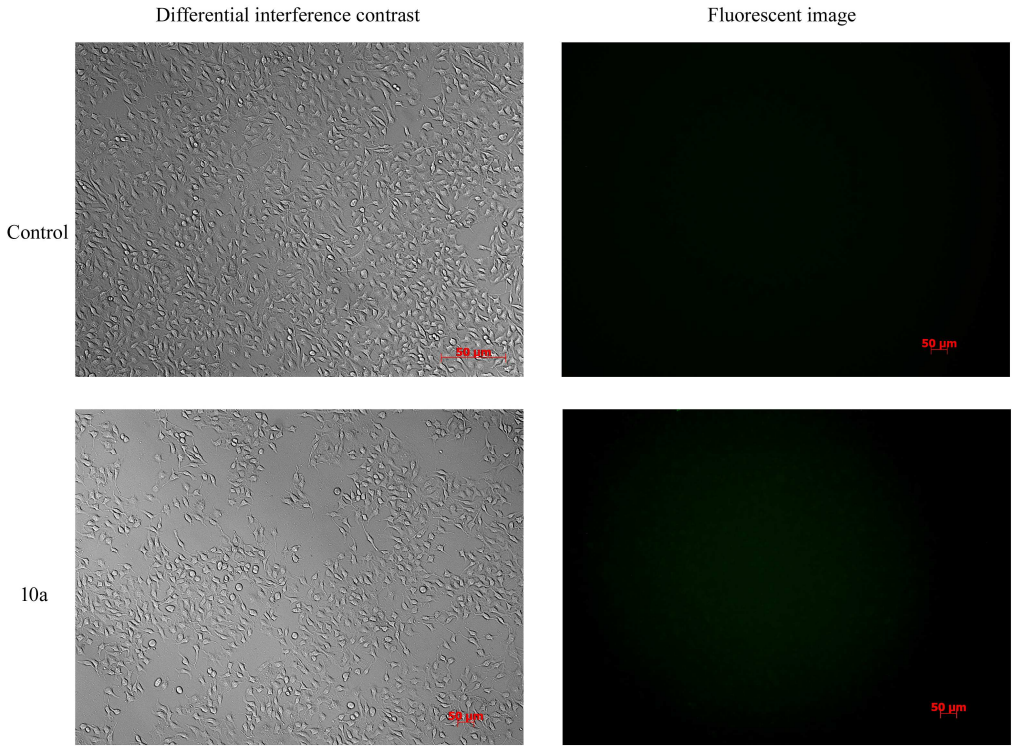


Figure S7. Live-cell imaging of blank control and coumarin dye **10a** (50 μM for 1 h).

1. Chemistry

1.1 General

Most chemicals and solvents were purchased from commercial sources. Further purification and drying by standard methods were employed when necessary. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AV-300 or ACF 500 spectrometer in the indicated solvents (TMS as internal standard): the values of the chemical shifts are expressed in δ values (ppm) and the coupling constants (J) in Hz. EI-MS spectra were recorded on an Agilent1100- LC-MSD-Trap/SL spectrometer and High-resolution mass spectra were recorded using an Agilent QTOF 6520.

1.2. Ethyl 7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxylate (**4**)

To a solution of **2** (5 g, 25.9 mmol) and **3** (4.9 g, 31.1 mmol) in dry MeOH (30 mL) was added piperidine (1.5 mL). The reaction mixture was heated under reflux for 14h, then filtrated and concentrated in vacuo. The crude product was transferred to the next stage without further purification.

1.3. 7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (**5**)

To a solution of **4** (7 g, 24.2 mmol) in MeOH was added NaOH (2.9 g, 72.6 mmol). The reaction mixture was heated under reflux for 4h, and then, the solvent was removed under reduced pressure. The residue was dissolved in dichloromethane (50 mL) and acidified by 10% HCl, washed with water and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by chromatography on silica gel (CH_2Cl_2/CH_3OH 60 : 1) to give compound **5** as a saffron yellow solid (5 g, 79%). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 1.27 (6H, t, J = 7.1 Hz), 3.50 (4H, q, J = 7.1 Hz), 6.53 (1H, d, J = 2.2 Hz, Ar-H), 6.71 (1H, dd, J = 9.0 Hz, J = 2.4 Hz, Ar-H), 7.45 (1H, d, J = 9.0 Hz, Ar-H), 8.65 (1H, s, Ar-H) , 12.37 (1H, s). MS(ESI)*m/z*: 262.3 $[M+H]^+$.

1.4. 2, 5-dioxopyrrolidi-*N*-1-yl 7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxylate (**6**)

To a solution of **5** (2 g, 7.7 mmol) in dichloromethane (15 mL) were added successively *N*-hydroxysuccinimide (1.3 g, 11.5 mmol) and EDCI (1.8 g, 9.2 mmol).

The mixture was stirred for 11 h at room temperature. Then, the reaction mixture was poured into water and extracted with dichloromethane (30 mL × 3). The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel (CH_2Cl_2/CH_3OH 100 : 1) to give **6** (2 g, 72.9%) as a yellow solid. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 1.26 (6H, t, J = 7.1 Hz), 2.90 (4H, s), 3.48 (4H, q, J = 7.1 Hz), 6.45 (1H, d, J = 1.9 Hz, Ar-H), 6.65 (1H, dd, J = 9.0 Hz, J = 2.2 Hz, Ar-H), 7.38 (1H, d, J = 9.0 Hz, Ar-H), 8.59 (1H, s, Ar-H). HRMS (*m/z*) (ESI): calcd for $C_{18}H_{19}N_2O_6$ $[M + H]^+$: 359.1238; found: 359.1226.

1.5. General procedure for the preparation of **7a-b**

To a solution of **6** (700 mg, 1.95 mmol) in DMF/MeOH (9 mL/4.5 mL) were added corresponding amino acids (1.5 equiv mol) and DIPEA (1.4 mL). The mixture was stirred at room temperature for 3 h and acidified by 10% HCl. Then, the mixture was extracted with dichloromethane (30 mL). The organic layer was washed with water and brine, dried with anhydrous Na_2SO_4, and concentrated in vacuo. The residue was chromatographed to afford corresponding products.

1.6. 4-(7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxamido)butanoic acid (**7a**)

Yield: 88.6%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 1.25 (6H, t, J = 7.1Hz), 1.98 (2H, m), 2.45 (2H, t, J = 7.1 Hz), 3.47 (4H, m), 3.53 (2H, m), 6.50 (1H, d, J = 2.1 Hz, Ar-H), 6.66 (1H, q, J = 9.0 Hz, J = 2.3 Hz, Ar-H), 7.44 (1H, d, J = 9.0 Hz, Ar-H), 8.71

(1H, s, Ar-H), 9.04 (1H, t, *J* = 5.8 Hz). HRMS (*m/z*) (ESI): calcd for $C_{18}H_{23}N_2O_5$ [M + H]$^+$: 347.1601; found: 347.1603.

1.7. 6-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido)hexanoic acid (**7b**)

Yield: 83.2%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 1.24 (6H, t, *J* = 7.1Hz), 1.36 (2H, m), 1.75 (4H, m), 2.42 (2H, t, *J* = 7.1 Hz), 3.47 (4H, m), 3.55 (2H, m), 6.48 (1H, d, *J* = 2.4 Hz, Ar-H), 6.63 (1H, q, *J* = 9.0 Hz, *J* = 2.4 Hz, Ar-H), 7.46 (1H, d, *J* = 9.0 Hz, Ar-H), 8.72 (1H, s, Ar-H), 9.21 (1H, t, *J* = 5.7 Hz).HRMS (*m/z*) (ESI): calcd for $C_{20}H_{27}N_2O_5$ [M + H]$^+$: 375.1914; found: 375.1910.

1.8. General procedure for the preparation of **8a-b**

To a solution of **6** (700 mg, 1.95 mmol) in dichloromethane (15 mL) were added corresponding *N*-Boc-diamines (1.5 equiv mol) and DIPEA (1.4 mL). The mixture was stirred at room temperature for 3 h and extracted with dichloromethane (30 mL). The organic layer was washed with water and brine, dried with anhydrous Na_2SO_4, and concentrated in vacuo. The residue was chromatographed to afford corresponding products and transferred to the next stage.

1.9. General procedure for the preparation of **9a-b**

To a solution of **8a** or **8b** in dichloromethane (15 mL) was added trifluoroacetic acid (3 mL). The mixture was stirred for 2 h at room temperature. Excess TFA was removed in vacuo. Then, the free amine of **9a** or **9b** basified by 10% NaOH was

regenerated from TFA salt. The crude product was transferred to the next stage without further purification.

1.10. 4-((2-(7-(Diethylamino)-2-oxo-2H-chromene-3-carboxamido)ethyl)amino)-4-oxobutanoic acid (**10a**)

To a solution of **9a** (500 mg, 1.65 mmol) in dichloromethane (15 mL) were successively added succinic anhydride (247 mg, 2.47 mmol) and DMAP (101 mg, 0.82 mmol). The mixture was stirred overnight at room temperature and washed with 10% HCl, water and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by chromatography on silica gel (CH_2Cl_2/CH_3OH 20 : 1) to afford compound **10a** (600 mg, 90%) as a yellow solid. HRMS (*m/z*) (ESI): calcd for $C_{20}H_{26}N_3O_6$ $[M + H]^+$: 404.1816; found: 404.1819.

1.11. 4-((4-(7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxamido)butyl)amino)-4-oxobutanoic acid (**10b**)

Compound **10b** was synthesized by the same steps as depicted in preparation of compound **10a**. ^{1}H-NMR (DMSO, 300 MHz) δ: ppm 1.14 (6H, t, J = 6.7 Hz), 1.46 (4H, m), 2.29 (2H, m), 2.38 (2H, m), 3.04 (2H, m), 3.29 (2H, m), 3.58 (2H, m), 6.62 (1H, s, Ar-H), 6.81 (1H, d, J = 8.0 Hz, Ar-H), 7.69 (1H, d, J = 8.9 Hz, Ar-H), 7.96 (1H, s), 8.66 (2H, s), 14.56 (1H, s). HRMS (*m/z*) (ESI): calcd for $C_{22}H_{30}N_3O_6$ $[M + H]^+$: 432.2129; found: 432.2124.

1.12. 2-hydroxyethyl 7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxylate (**13a**)

To a solution of **5** (500 mg, 1.91 mmol) in dichloromethane (15 mL) were added ethylene glycol (1783 mg, 28.72 mmol), EDCI (367 mg, 1.91 mmol) and DMAP (70 mg, 0.57 mmol). The mixture was stirred for 6 h at room temperature, and then, diluted with dichloromethane (20 mL). The organic layer was washed with water and brine, dried with anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/CH_3OH 100 : 1) to give **13a** (500 mg, 85.7%). HRMS (*m/z*) (ESI): calcd for $C_{16}H_{20}NO_5$ $[M + H]^+$: 306.1336; found: 306.1332.

1.13. 2-(2-hydroxyethoxy)ethyl7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxylate (**13b**)

Compound **13b** was synthesized by the same steps as depicted in preparation of compound **13a.** ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 1.24 (6H, t, J = 7.1 Hz), 3.45 (4H, q, J = 7.1 Hz), 3.69 (2H, m), 3.77 (2H, s), 3.86 (2H, t, J = 4.7 Hz), 4.78 (2H, t, 4.6 Hz), 6.43 (1H, d, J = 2.1 Hz, Ar-H), 6.61 (1H, dd, J = 9.0 Hz, J = 2.3 Hz, Ar-H), 7.37 (1H, d, J = 9.0 Hz, Ar-H), 8.46 (1H, s, Ar-H). HRMS (*m/z*) (ESI): calcd for $C_{18}H_{24}NO_6$ $[M + H]^+$: 350.1598; found: 350.1584.

1.14. 2-(2-(2-hydroxyethoxy)ethoxy)ethyl7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxylate (**13c**)

Compound **13c** was synthesized by the same steps as depicted in preparation of

compound **13a**. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 1.24 (6H, t, J = 7.1 Hz), 3.45 (4H, m), 3.63 (2H, m), 3.73 (6H, m), 3.85 (2H, m), 4.48 (2H, m), 6.44 (1H, s, Ar-H), 6.61 (1H, m, Ar-H), 7.38 (1H, d, J = 8.7 Hz, Ar-H), 8.49 (1H, s, Ar-H). HRMS (*m/z*) (ESI): calcd for $C_{20}H_{28}NO_7$ $[M + H]^+$: 394.1860; found: 394.1849.

1.15. General procedure for the preparation of **14a-c**

Compound **14a-c** were synthesized by the same steps as depicted in preparation of compound **10a**.

1.16. 4-(2-(2-((7-(diethylamino)-2-oxo-2*H*-chromene-3-carbonyl)oxy)ethoxy) ethoxy)-4-oxobutanoic acid (**14b**)

Yield: 85%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 1.24 (6H, t, J = 7.1Hz), 2.68 (4H, s), 3.45 (4H, q, J = 7.1 Hz), 3.77 (2H, t, J = 4.6 Hz), 3.85 (2H, m), 4.28 (2H, t, J = 4.4 Hz), 4.47 (2H, t, J = 4.6 Hz), 6.46 (1H, d, J = 2.1 Hz, Ar-H), 6.62 (2H, dd, J = 9.0 Hz, J = 2.3 Hz, Ar-H), 7.38 (1H, d, J = 9.0 Hz, Ar-H), 8.48 (1H, s, Ar-H). HRMS (*m/z*) (ESI): calcd for $C_{22}H_{28}NO_9$ $[M + H]^+$: 450.1759; found: 450.1760.

1.17. Benzyl 3,23-dihydroxy-lup-20(29)-e*N*-28-oate (**24**)

To a solution of 23-HBA (1.00 g, 2.12 mmol) in DMF (20 mL) was successively added K_2CO_3 (1.00 g, 7.24 mmol) and benzyl bromide (0.3 mL, 2.52 mmol). The mixture was stirred for 12 h at room temperature and then poured into water (15 mL) and extracted with ethyl acetate (30 mL × 3). The organic layer was washed with

water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether-ethyl acetate 2:1) to afford compound **24** as a white solid (1.07 g, 89.9%). HRMS (*m/z*) (ESI): calcd for $C_{37}H_{55}O_4$ $[M + H]^+$: 563.4095; found: 563.4089.

1.18. General procedure for the preparation of **25a-j**

To a solution of corresponding coumarin acid (1 equiv mol) in dry dichloromethane (15 mL) was added oxalyl chloride (1.2 equiv mol) and DMF as catalyst. The mixture was stirred for 10 min at room temperature. The solvent was removed under reduced pressure. Then, the residue was dissolved in dry dichloromethane (15 mL) followed by adding **24** (300 mg, 0.53 mmol) and triethylamine (0.15 mL, 1.07 mmol). The mixture was stirred for 30 min at room temperature, and then, diluted with dichloromethane (20 mL). The organic layer was washed with water and brine, dried with anhydrous Na_2SO_4, and concentrated in vacuo. The residue was chromatographed to afford corresponding ester compounds.

1.19. Compound **25a**

^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.74 (3H, s), 0.83 (3H, s), 0.87 (3H, s), 0.93 (3H, s), 1.65 (3H, s), 2.13-2.25 (2H, m), 2.34-2.39 (2H, m), 3.01 (1H, m, H-19), 3.39-3.46 (7H, m), 3.82 (1H, d, J = 11.4 Hz, H-23a), 4.15 (1H, d, J = 11.5Hz, H-23b), 4.56, 4.71 (each 1H, s, H-29), 5.01-5.12 (2H, m), 6.49 (1H, d, J = 1.9 Hz, Ar-H), 6.65 (1H, dd, J = 8.9 Hz, J = 2.2 Hz, Ar-H), 7.32-7.44 (6H, m), 8.69 (1H, s, Ar-H), 8.84

(1H, s). HRMS (*m/z*) (ESI): calcd for $C_{55}H_{75}N_2O_8$ $[M + H]^+$: 891.5518; found: 891.5512.

1.20. Compound **25b**

^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.73 (3H, s), 0.85 (3H, s), 0.87 (3H, s), 0.89 (3H, s), 1.62 (3H, s), 2.12-2.29 (2H, m), 2.41-2.44 (2H, m), 2.99 (1H, m, H-19), 3.41-3.49 (6H, m), 3.52-3.69 (1H, m, H-3), 3.82 (1H, d, *J* = 11.4 Hz, H-23a), 4.12 (1H, d, *J* = 11.4Hz, H-23b), 4.56, 4.69 (each 1H, s, H-29), 5.09-5.12 (2H, m), 6.50 (1H, s, Ar-H), 6.62-6.69 (1H, m, Ar-H), 7.33-7.35 (5H, m), 7.43 (1H, d, *J* = 9.0 Hz, Ar-H), 8.70 (1H, s, Ar-H), 8.83-8.92 (1H, m). HRMS (*m/z*) (ESI): calcd for $C_{57}H_{79}N_2O_8$ $[M + H]^+$: 919.5831; found: 919.5835.

1.21. Compound **25c**

Yield: 40%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.73 (3H, s), 0.74 (3H,s), 0.82 (3H, s), 0.95 (3H, s), 1.67 (3H, s), 2.14-2.29 (2H, m), 2.67 (4H, s), 3.01 (1H, m, H-19), 3.38-3.48 (5H, m), 3.69-3.72 (6H, m), 3.78-3.85 (3H, m), 4.18 (1H, d, *J* = 11.4 Hz, H-23), 4.25 (2H, m), 4.46 (2H, t, *J* = 4.6 Hz), 4.60, 4.73 (each 1H, s, H-29), 5.12 (2H, m), 6.46 (1H, s, Ar-H), 6.61 (1H, d, *J* = 9.0 Hz, Ar-H), 7.36 (6H, m, Ar-H), 8.45 (1H, s, Ar-H). HRMS (*m/z*) (ESI): calcd for $C_{57}H_{78}N_3O_9$ $[M + H]^+$: 948.5733; found: 948.5733.

1.22. General procedure for the preparation of **26a-j**

To a solution of correspongding benzyl ester in THF/MeOH (10 mL/0.5 mL) was added 10% Pd on carbon. The mixture was subjected to 1 atm of H_2 and was stirred for 1 h at 50 ^oC. The mixture was filtered and concentrated. The residue was chromatographed to give **26a-j** as a yellow solid.

1.23. Fluorescent probe **26a**

Yield: 30%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.74 (3H, s), 0.85 (3H, s), 0.89 (3H, s), 0.91 (3H, s), 1.67 (3H, s), 2.12-2.24 (2H, m), 2.42-2.45 (2H, m), 2.99 (1H, m, H-19), 3.33-3.49 (6H, m), 3.57-3.64 (1H, m, H-3), 3.82 (1H, d, J = 11.4 Hz, H-23a), 4.14 (1H, d, J = 11.4Hz, H-23b), 4.58, 4.70 (each 1H, s, H-29), 6.50 (1H, s, Ar-H), 6.65 (1H, m, Ar-H), 7.45 (1H, d, J = 8.9 Hz, Ar-H), 8.72 (1H, s, Ar-H), 8.92 (1H, m); ^{13}C NMR (CDCl3, 75 MHz) δ: 11.9, 12.4, 14.7, 16.0, 16.7, 18.0, 19.3, 20.8, 25.2, 25.4, 26.3, 29.3, 30.6, 31.7, 32.2, 34.0, 37.0, 38.3, 38.6, 40.6, 42.4, 45.1, 46.9, 48.0, 49.2, 50.6, 56.3, 66.5, 71.9, 96.5, 108.4, 109.6, 109.9, 110.0, 131.3, 148.3, 150.5, 152.6, 157.6, 162.8, 163.6, 173.4, 181.5; ESI-MS *m/z*: 801.4 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{48}H_{68}N_2O_8$ $[M + H]^+$: 801.5048; found: 801.5052.

1.24. Fluorescent probe **26b**

Yield: 35%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.74 (3H, s), 0.84 (3H, s), 0.87 (3H, s), 0.94 (3H, s), 1.64 (3H, s), 2.13-2.25 (2H, m), 2.34 (2H, m), 2.98 (1H, m, H-19), 3.39-3.46 (7H, m), 3.80 (1H, d, J = 11.4 Hz, H-23a), 4.13 (1H, d, J = 11.5Hz,

H-23b), 4.46, 4.70 (each 1H, s, H-29), 6.46 (1H, d, *J* = 1.9 Hz, Ar-H), 6.62 (1H, dd, *J* = 8.9 Hz, *J* = 2.2 Hz, Ar-H), 7.41 (1H, d, *J* = 9.0 Hz, Ar-H), 8.70 (1H, s, Ar-H), 8.85 (1H, t, *J* = 5.4 Hz); 13C NMR (CDCl3, 75 MHz) *δ*: 11.9, 12.4, 14.7, 16.0, 16.6, 18.1, 19.3, 20.8, 22.6, 24.9, 25.4, 26.3, 26.6, 29.3, 29.6, 30.6, 32.1, 34.0, 34.4, 37.0, 38.3, 38.5, 39.4, 40.6, 42.1, 42.4, 45.1, 46.9, 48.1, 49.2, 50.7, 56.3, 66.5, 72.2, 96.5, 108.4, 109.6, 110.0, 110.1, 131.2, 148.2, 150.5, 152.5, 157.6, 162.8, 163.3, 173.9, 181.5; ESI-MS *m/z*: 829.4 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{50}H_{72}N_2O_8$ $[M + H]^+$: 829.5361; found: 829.5366.

1.25. Fluorescent probe **26c**

Yield: 25%; ^{1}H-NMR ($CDCl_3$, 300 MHz) *δ*: ppm 0.63 (3H, s), 0.83 (3H, s), 0.89 (3H, s), 0.97 (3H, s), 1.68 (3H, s), 2.15-2.28 (2H, m), 2.51-2.56 (2H, m), 2.63-2.76 (2H, m), 3.01 (1H, m, H-19), 3.45-3.50 (7H, m), 3.58 (2H, m), 3.77 (1H, d, *J* = 11.3 Hz, H-23a), 4.17 (1H, d, *J* = 11.5 Hz, H-23b), 4.60, 4.73 (each 1H, s, H-29), 6.49 (1H, d, *J* = 1.9 Hz, Ar-H), 6.66 (1H, dd, *J* =8.9 Hz, *J* = 2.1 Hz, Ar-H), 6.95 (1H, s), 7.44 (1H, d, *J* = 9.0 Hz, Ar-H), 8.67 (1H, s, Ar-H), 9.09 (1H, m); ^{13}C NMR (CDCl3, 75 MHz) *δ*: 11.9, 12.4, 12.7, 14.7, 16.0, 16.6, 18.1, 19.3, 20.9, 25.4, 26.3, 29.7, 29.8, 30.6, 31.1, 32.2, 34.0, 37.0, 38.3, 38.5, 39.2, 40.6, 40.8, 42.1, 42.4, 45.1, 46.9, 48.2, 49.2, 50.5, 56.3, 67.2, 72.1, 96.5, 108.3, 109.4, 109.6, 110.1, 131.3, 148.4, 150.5, 152.8, 157.7, 162.7, 164.8, 171.9, 173.4, 181.0; ESI-MS *m/z*: 858.4 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{50}H_{71}N_3O_9$ $[M + H]^+$: 858.5263; found: 858.5251.

1.26. Fluorescent probe **26d**

Yield: 20%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.73 (3H, s), 0.84 (3H, s), 0.90 (3H, s), 0.97 (3H, s), 1.68 (3H, s), 2.18-2.24 (2H, m), 2.55 (2H, m), 2.70 (2H, m), 3.01 (1H, m, H-19), 3.30 (2H, m), 3.46 (7H, m), 3.80 (1H, d, J = 11.4 Hz, H-23a), 4.18 (1H, d, J = 11.4 Hz, H-23b), 4.60, 4.73 (each 1H, s, H-29), 6.40 (1H, s), 6.49 (1H, d, J = 2.0 Hz, Ar-H), 6.65 (1H, dd, J = 9.0 Hz, J = 2.2 Hz, Ar-H), 7.44 (1H, d, J = 9.0 Hz, Ar-H), 8.70 (1H, s, Ar-H), 8.90 (1H, m); ^{13}C NMR (CDCl3, 75 MHz) δ: 11.8, 12.4, 14.7, 16.0, 16.6, 18.1, 20.9, 25.4, 26.3, 26.4, 27.3, 29.7, 29.8, 30.6, 31.1, 32.2, 34.0, 37.1, 38.3, 38.5, 39.1, 39.3, 40.6, 42.1, 42.4, 45.1, 46.9, 48.2, 49.2, 50.5, 56.3, 67.3, 72.2, 96.5, 108.4, 109.6, 109.9, 110.0, 131.2, 148.2, 150.5, 152.6, 157.6, 162.8, 163.6, 171.6, 173.5, 181.0; ESI-MS *m/z*: 886.4 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{52}H_{75}N_3O_9$ $[M + H]^+$: 886.5576; found: 886.5575.

1.27. Fluorescent probe **26e**

Yield: 45%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.73 (3H, s), 0.87 (3H, s), 0.95 (3H, s), 0.98 (3H, s), 1.68 (3H, s), 2.14-2.28 (2H, m), 2.70 (4H, s), 3.01 (1H, m), 3.43 (5H, m), 3.82 (1H, d, J = 11.4 Hz, H-23a), 4.18 (1H, d, J = 11.5Hz, H-23b), 4.45 (2H, m), 4.51 (2H, m), 4.60, 4.73 (each 1H, s, H-29), 6.46 (1H, d, J = 2.1 Hz, Ar-H), 6.62 (1H, dd, J = 9.0 Hz, J = 2.3 Hz, Ar-H), 7.40 (1H, d, J = 9.0 Hz, Ar-H), 8.45 (1H, s, Ar-H); ^{13}C NMR (CDCl3, 75 MHz) δ: 11.7, 12.4, 14.6, 15.9, 16.5, 18.1, 19.3, 20.8, 25.4, 26.2, 29.0, 29.1, 29.6, 30.5, 32.0, 33.9, 37.0, 38.3, 38.4, 40.5, 42.0, 42.3, 45.1, 46.8, 48.1, 49.1, 50.5, 56.3, 62.5, 67.1, 72.1, 96.6, 107.6, 109.6, 131.2, 149.6, 150.3,

153.0, 158.5, 163.8, 172.0, 172.6, 181.9; ESI-MS *m/z*: 860.3 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{50}H_{69}NO_{11}$ $[M + H]^+$: 860.4943; found: 860.4936.

1.28. Fluorescent probe **26f**

Yield: 50%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.73 (3H, s), 0.85 (3H,s), 0.92 (3H, s), 0.98 (3H, s), 1.69 (3H, s), 2.18-2.28 (2H, m), 2.68 (4H,s), 2.92-3.01 (1H, m, H-19), 3.32-3.49 (5H, m), 3.38-3.84 (5H, m), 4.18 (2H, d, J = 11.5 Hz, H-23), 4.27 (2H, m), 4.46 (2H, m), 4.60, 4.73 (each 1H, s, H-29), 6.45 (1H, s, Ar-H), 6.61 (1H, d, J = 8.9 Hz, Ar-H), 7.34 (1H, d, J = 9.0 Hz, Ar-H), 8.46 (1H, s, Ar-H); ^{13}C NMR (CDCl3, 75 MHz) δ: 11.7, 12.3, 12.7, 14.6, 15.9, 16.5, 16.6, 18.0, 19.3, 29.9, 25.4, 26.2, 29.1, 29.3, 29.6, 30.4, 32.0, 33.8, 37.0, 38.3, 40.5, 42.0, 42.3, 45.0, 46.8, 49.1, 56.2, 63.8, 63.9, 69.0, 96.6, 109.5, 110.1, 131.1, 149.4, 150.4, 152.9, 158.4, 164.0, 172.2, 172.6, 173.2, 181.7; ESI-MS *m/z*: 904.4 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{52}H_{73}NO_{12}$ $[M + H]^+$: 904.5206; found: 904.5192.

1.29. Fluorescent probe **26g**

Yield: 45%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.73 (3H, s), 0.85 (3H,s), 0.92 (3H, s), 0.98 (3H, s), 1.69 (3H, s), 2.15-2.29 (2H, m), 2.67 (4H, s), 3.01 (1H, m, H-19), 3.38-3.49 (5H, m), 3.68-3.74 (6H, m), 3.81-3.86 (3H, m), 4.18 (1H, d, J = 11.5 Hz, H-23), 4.26 (2H, t, J = 4.4 Hz), 4.46 (2H, t, J = 4.6 Hz), 4.60, 4.73 (each 1H, s, H-29), 6.45 (1H, d, J = 1.9 Hz, Ar-H), 6.62 (1H, dd, J = 8.9 Hz, J = 2.2 Hz, Ar-H), 7.37 (1H, d, J = 8.9 Hz, Ar-H), 8.46 (1H, s, Ar-H); ^{13}C NMR (CDCl3, 75 MHz) δ: 11.8, 12.4,

12.7, 14.7, 16.0, 16.6, 16.7, 18.1, 19.3, 20.9, 25.4, 26.3, 29.1, 29.2, 29.4, 29.6, 30.6, 32.1, 33.9, 36.8, 37.0, 38.3, 40.6, 42.1, 42.4, 45.1, 46.9, 48.1, 49.2, 50.3, 50.5, 56.3, 63.9, 64.1, 67.0, 69.0, 69.2, 70.6, 72.1, 75.2, 96.6, 107.6, 109.6, 131.2, 149.5, 150.5, 153.0, 158.5, 164.0, 172.2, 172.6, 173.3, 181.8; ESI-MS *m/z*: 948.4 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{54}H_{77}NO_{13}$ $[M + H]^+$: 948.5468; found: 948.5452.

1.30. Fluorescent probe **26h**

Yield: 55%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.73 (3H, s), 0.84 (3H, s), 0.90 (3H, s), 0.98 (3H, s), 1.68 (3H, s), 2.15-2.28 (2H, m), 2.70 (4H, s), 3.01 (1H, m), 3.46 (5H, m), 3.72 (2H, m), 3.83 (1H, d, J = 11.3 Hz, H-23a), 4.18 (1H, d, J = 11.5 Hz, H-23b), 4.29 (2H, t, J = 5.3 Hz), 4.59, 4.73 (each 1H, s, H-29), 6.49 (1H, d, J = 2.0 Hz, Ar-H), 6.66 (1H, dd, J = 9.0 Hz, J = 2.2 Hz, Ar-H), 7.44 (1H, d, J = 9.0 Hz, Ar-H), 8.71 (1H, s, Ar-H), 9.06 (1H, t, J = 5.6 Hz); ^{13}C NMR (CDCl3, 75 MHz) δ: 11.78, 12.4, 14.6, 15.9, 16.5, 18.0, 19.3, 20.8, 25.4, 26.3, 29.1, 29.2, 29.6, 30.5, 32.1, 33.9, 37.0, 38.3, 38.4, 40.6, 42.0, 42.3, 45.0, 46.9, 48.0, 49.1, 50.5, 56.2, 63.4, 67.0, 72.0, 96.5, 108.3, 109.6, 110.0, 131.2, 148.4, 150.4, 152.6, 157.6, 162.7, 163.6, 172.0, 172.5, 181.4; ESI-MS *m/z*: 859.4 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{50}H_{70}N_2O_{10}$ $[M + H]^+$: 859.5103; found: 859.5092.

1.31. Fluorescent probe **26i**

Yield: 60%; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: ppm 0.73 (3H, s), 0.84 (3H, s), 0.90 (3H, s), 0.98 (3H, s), 1.68 (3H, s), 2.15-2.28 (2H, m), 2.70 (4H, s), 3.01 (1H, m), 3.46

(5H, m), 3.72 (2H, m), 3.83 (1H, d, *J* = 11.3 Hz, H-23a), 4.18 (1H, d, *J* = 11.5 Hz, H-23b), 4.29 (2H, t, *J* = 5.3 Hz), 4.59, 4.73 (each 1H, s, H-29), 6.49 (1H, d, *J* = 2.0 Hz, Ar-H), 6.66 (1H, dd, *J* = 9.0 Hz, *J* = 2.2 Hz, Ar-H), 7.44 (1H, d, *J* = 9.0 Hz, Ar-H), 8.71 (1H, s, Ar-H), 9.06 (1H, t, *J* = 5.6 Hz); ^{13}C NMR (CDCl3, 75 MHz) *δ*: 11.78, 12.4, 14.6, 15.9, 16.5, 18.0, 19.3, 20.8, 25.4, 26.3, 29.1, 29.2, 29.6, 30.5, 32.1, 33.9, 37.0, 38.4, 40.6, 42.1, 42.3, 45.0, 46.9, 48.0, 49.2, 50.5, 56.3, 63.4, 67.0, 72.0, 96.5, 108.3, 109.6, 110.0, 131.2, 148.4, 150.4, 152.6, 157.7, 162.7, 163.6, 172.0, 172.5, 181.5; ESI-MS *m/z*: 859.9 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{50}H_{70}N_2O_{10}$ $[M + H]^+$: 859.5103; found: 859.5097.

1.32. Fluorescent probe **26j**

Yield: 50%; ^{1}H-NMR ($CDCl_3$, 300 MHz) *δ*: ppm 0.76 (3H, s), 0.85 (3H, s), 0.90 (3H, s), 0.95 (3H, s), 1.68 (3H, s), 2.16-2.28 (2H, m), 3.01 (1H, m), 3.45 (5H, m), 3.67 (4H, m), 3.70-3.72 (2H, m), 3.77-3.78 (2H, m), 3.92 (1H, d, *J* = 11.3 Hz, H-23a), 4.18-4.23 (3H, m), 4.60, 4.73 (each 1H, s, H-29), 6.71 (1H, d, *J* = 2.0 Hz, Ar-H), 6.65 (1H, dd, *J* = 8.9 Hz, *J* =2.2 Hz, Ar-H), 7.43 (1H, d, *J* = 8.9 Hz, Ar-H), 8.70 (1H, s, Ar-H), 9.07 (1H, s); ^{13}C NMR (CDCl3, 75 MHz) *δ*: 11.8, 12.4, 12.7, 14.6, 16.0, 16.5, 17.0, 18.2, 19.3, 20.9, 25.4, 26.4, 29.7, 30.6, 32.1, 34.0, 36.7, 37.0, 38.3, 39.5, 40.6, 42.0, 42.3, 42.4, 45.1, 46.8, 46.9, 48.4, 49.2, 50.3, 56.3, 64.3, 67.8, 68.9, 69.9, 70.5, 70.6, 70.9, 71.1, 72.4, 75.5, 96.5, 108.4, 109.6, 109.9, 131.2, 148.2, 150.5, 152.6, 157.6, 162.6, 163.5, 170.1, 171.7, 181.3; ESI-MS *m/z*: 861.4 $[M+H]^+$; HRMS (*m/z*) (ESI): calcd for $C_{50}H_{72}N_2O_{10}$ $[M + H]^+$: 861.5260; found: 861.5263.

2. Biology

2.1. Cell lines and culture conditions

B16F10 (mice skin melanoma), MCF-7 (human breast cancer) and Helf (Human Embryonic Lung Fibroblast) were obtained from Chinese Academy of Sciences Committee Type Culture Collection. All cell lines were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicillin streptomycin (Gibco) at 37 °C in the presence of 5% CO_2.

2.2. Antiproliferative screening using MTT assay

The antiproliferative activity of the compounds was determined using MTT assay. Cells were seeded in 96-well micro culture plates, then incubated for 24 h at 37 °C in CO_2 incubator. All of the tested betulinic acid derivatives were dissolved in DMSO while the positive control doxorubicin was dissolved in PBS. These tested compounds of different concentrations were added into wells and cells were treated at 37 °C for 72 h. Then MTT (5 mg/mL, in PBS) was added into each well and cultured for another 4 h. The optical density was detected in a microplate reader at 570 nm. IC_{50} values were calculated according to the dose-dependent curves.

2.3. Determination of fluorescence properties of compound **26c**

Componud **26c** (1 mg) was dissolved in DMSO (10 mL) followed with dilution to ten times to afford 10 μM solution. With 100 μL in hand, the different wavelengths

of incident light excited compound **26c** using F-7000 fluorescence spectrophotometer Then, generated fluorescent light irradiating to the detector throngh an emission monochromator with a fixed wavelength was detected its fluorescent strength. Subcequently, light intensity-versus-excitation wavelengths curve which was so called excitation spectra was obtained by a recorder. With λ_{ex}= 415 nm in hand, emission spectra of compound **26c** was got. Based on the data, the best emission wavelength was λ_{em}= 475 nm.

2.4. Time-coure and dose-dependent imaging studies

To observe the cellular uptake and localization of **26c** in time-course and dose-dependent process, cells were detected using Fluorescence Microscopy. Briefly, 5×10^4 B16F10 cells were seeded in 24-wells plate for 24h, and the medium was replaced with 1.5mL of Serum-free 1640 medium without phenol red containing **26c**. The concentration of **26c** was 2 μM, 4 μM, 10 μM and 20 μM. Then , at each concentration, the cells were incubated for 15 min, 30 min, 1 h, 2 h, or 4h at 37□ in 5% CO_2, respectively. Subsequently, the cells were washed three times with PBS and observed using Fluorescence Microscopy at 415 nm laser excitation.

2.5. Cellular uptake of **26c** detected by flow cytometry measurements

For flow cytometric analysis, B16F10 cells were seeded into 6-well plates at 5×10^5 cells per well with 1.5mL of complete RIM-1640 culture medium for 24h. The medium was replaced with 1.5mL of Serum-free 1640 medium without phenol red

containing **26c**. The concentration of **26c** was 10 μM. The cells were further incubated for specific periods of time, and then washed three times with cold PBS, trypsinized and harvested in PBS. The samples were then assessed with BD Calibur flow cytometry (BD Co., USA) to determine the fluorescence intensity of **26c** at 375 nm laser excitation. The date were processed by FlowJo software and expressed as the mean fluorescent intensities.

2.6. Subcellular Localization of **26c**

The subcellular localization of **26c** was determined by the colocalization of organelle specific dyes including MitoTracker® Red CMXRos (Mitochondria specific dye, Shanghai Yi St. Biotechnology Co., Ltd.). The subcellular localization was assessed using confocal laser scanning microscopy (CLSM). Briefly, Place sterilized coverslips into the wells of a 24-well plate. 5×10^4 B16F10 cells were seeded in 24-well plate with sterilized coverslips for 24h, and the medium was replaced with Serum-free 1640 medium without phenol red, which included **26c**. The concentration of **26c** was 4 μM and the cells were then incubated for 1 h at 37□. Subsequently, the cells were washed thress times with PBS followed by staining with 100 nM MitoTracker® Red CMXRos for 30min at 37□. The cells were washed three times with PBS and fixed with fresh 4% paraformaldehyde for 10 min at room temperature. Then, the cells were washed three times with PBS and ruptured with 0.1% Triton® X-100 for 5min at room temperature. Afterwards, the cells were washed three times with PBS and blocked with fresh 1% bovine serum albumin (BSA) for 30 min at

room temperature. Green fluorescence of **26c**, red fluorescence of MitoTracker® Red CMXRos were observed using a Zeiss LSM780 CLSM (Zeiss Co.,Germany).

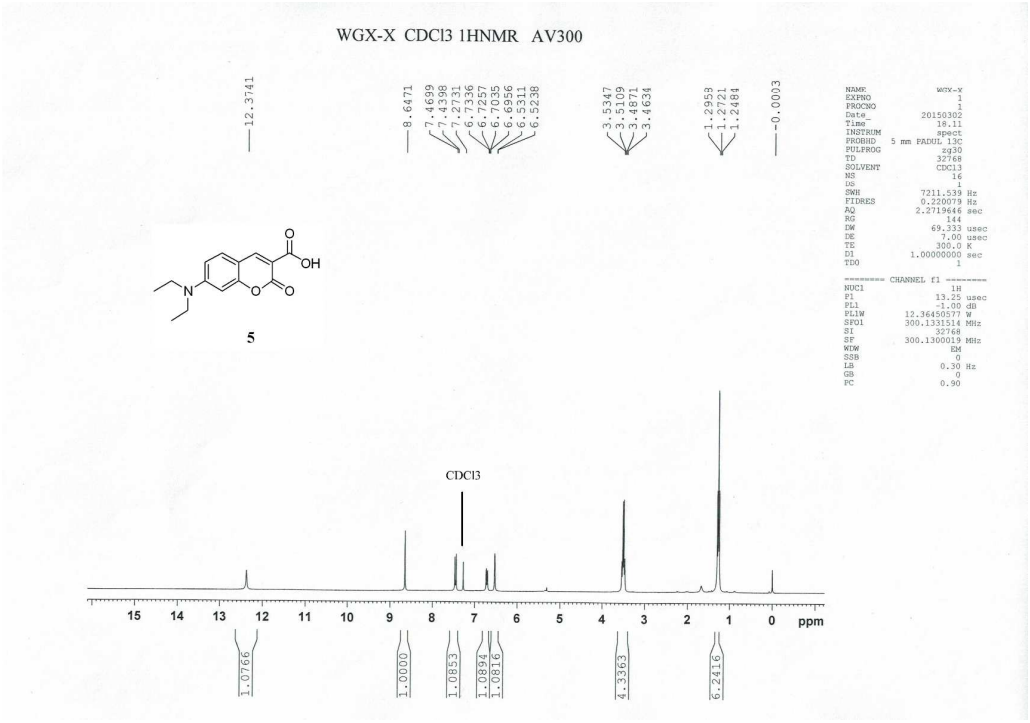
2.7. Cell mitochondrial membrane potential assay

B16F10 cells were cultured overnight and incubated in triplicate with the test compounds (0, 7.5, 15, 30 μM of 23-HBA and 0, 1.5, 3.0, 6.0 μM of **26c**) or vehicle for 48 h. The cells were stained with the lipophilic cationic dye JC-1, according to the manufacturer's instruction (Keygen, KGA601). The percentage of cells with healthy or collapsed mitochondrial membrane potentials was monitored by flow cytometry analysis.

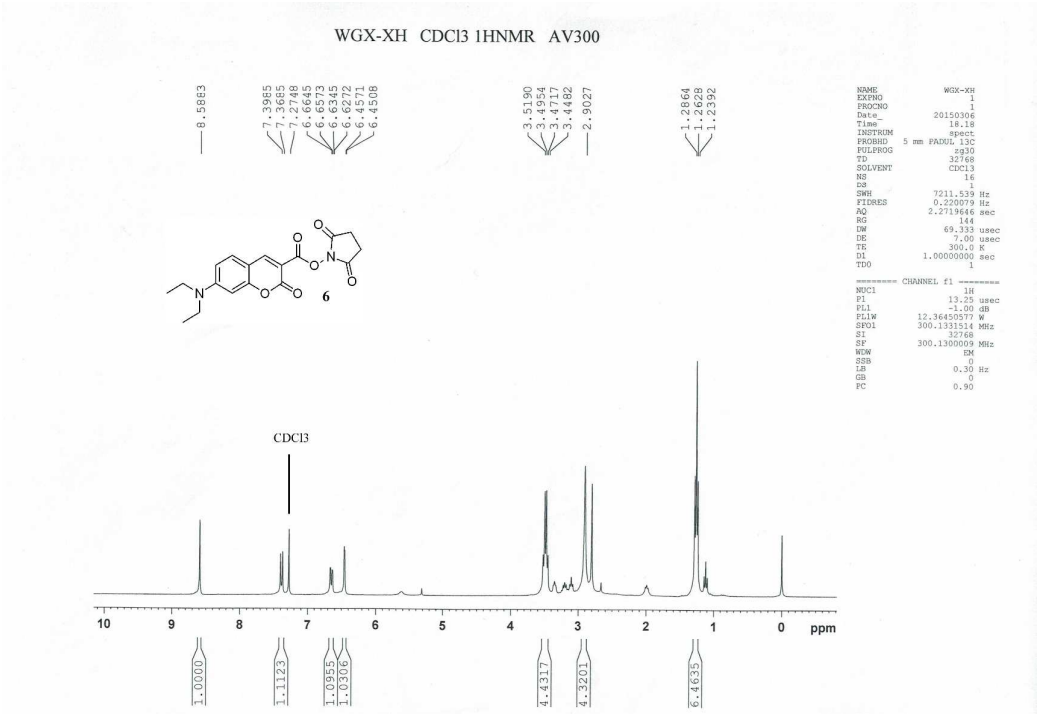
2.8. Western blot analysis

B16F10 cells were incubated in triplicate with different dose of test compounds (0, 7.5, 15, 30 μM of 23-HBA and 0, 1.5, 3.0, 6.0 μM of **26c**) for 48 h. After the protein concentrations were determined, individual cell lysates (50 μg per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel, SDS-PAGE) and transferred onto nitrocellulose membranes. After being blocked with 5% fat-free milk, the target proteins in the membranes were probed with monoclonal anti-Bax, anti-Bcl2, anti-cyto C, and anti-GAPDH antibodies (KGA731, KeyGEN Biotech, Nanjing, China), respectively. The relative levels of each signaling event to control GADPH were determined by densimetric scanning.

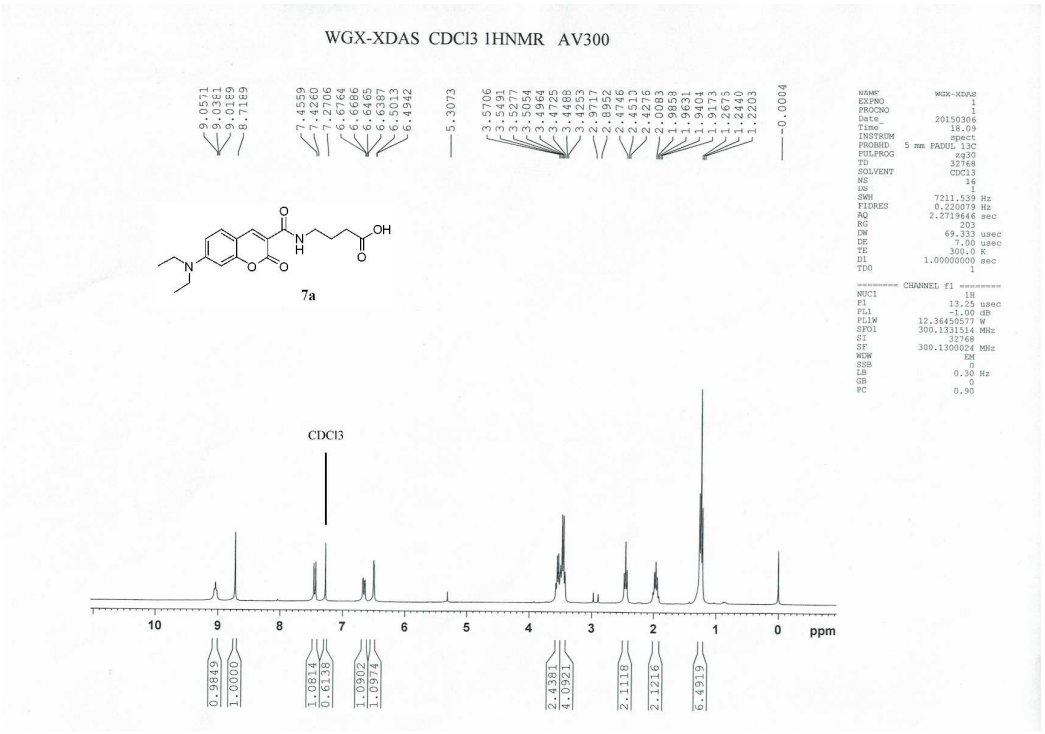
Copies of ^{1}H NMR of **5**



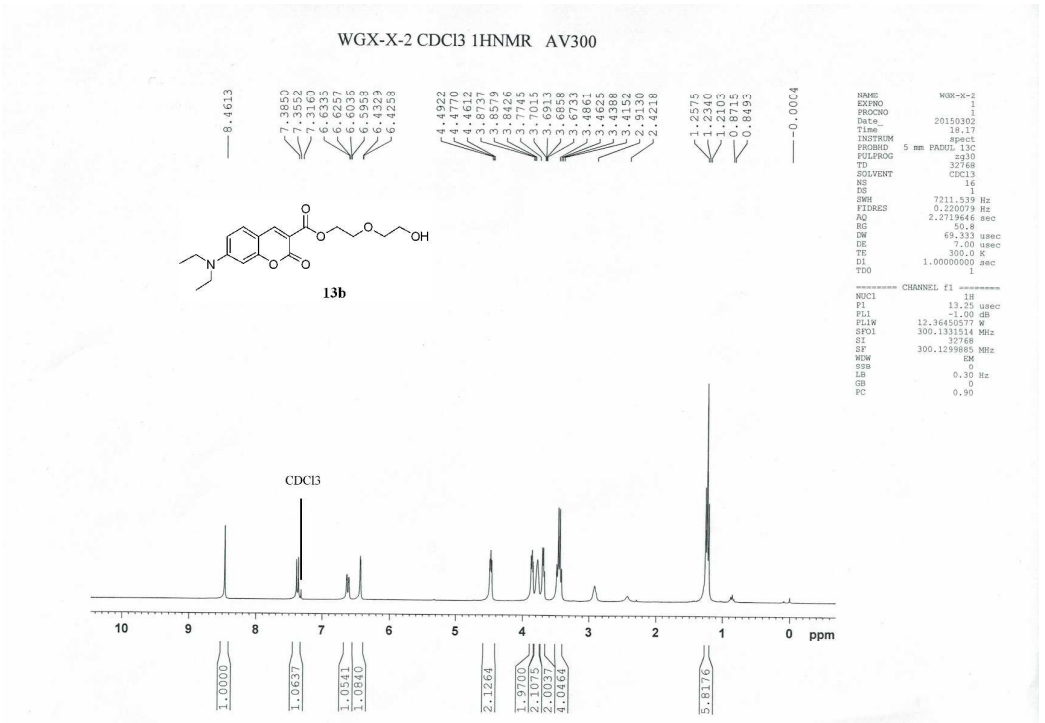
Copies of ^{1}H NMR of **6**



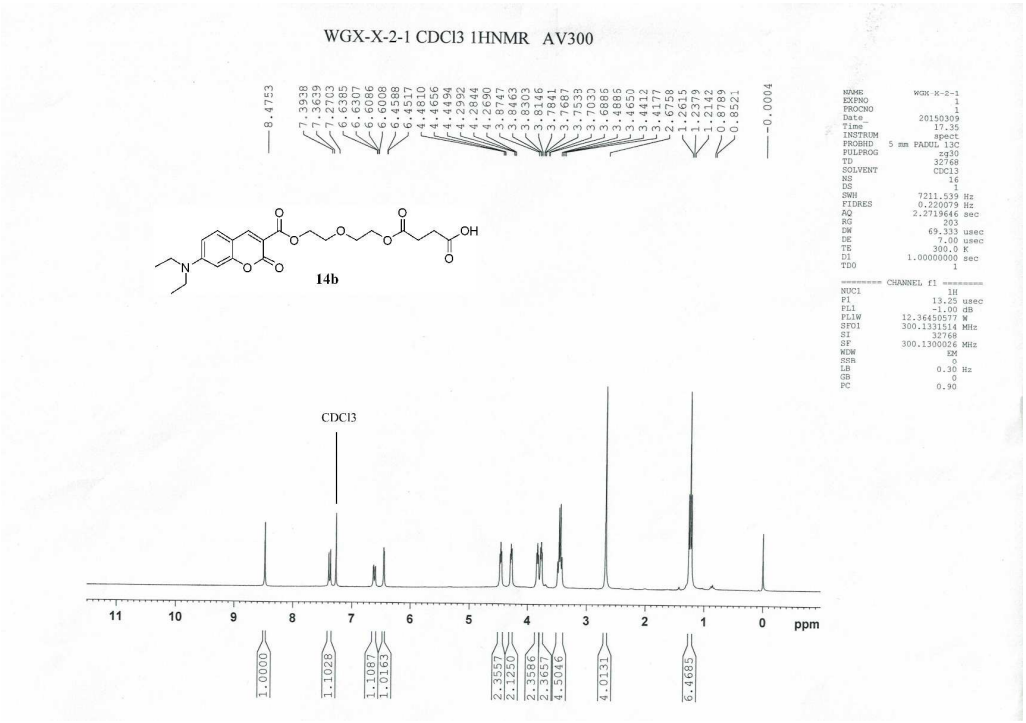
Copies of ^{1}H NMR of **7a**



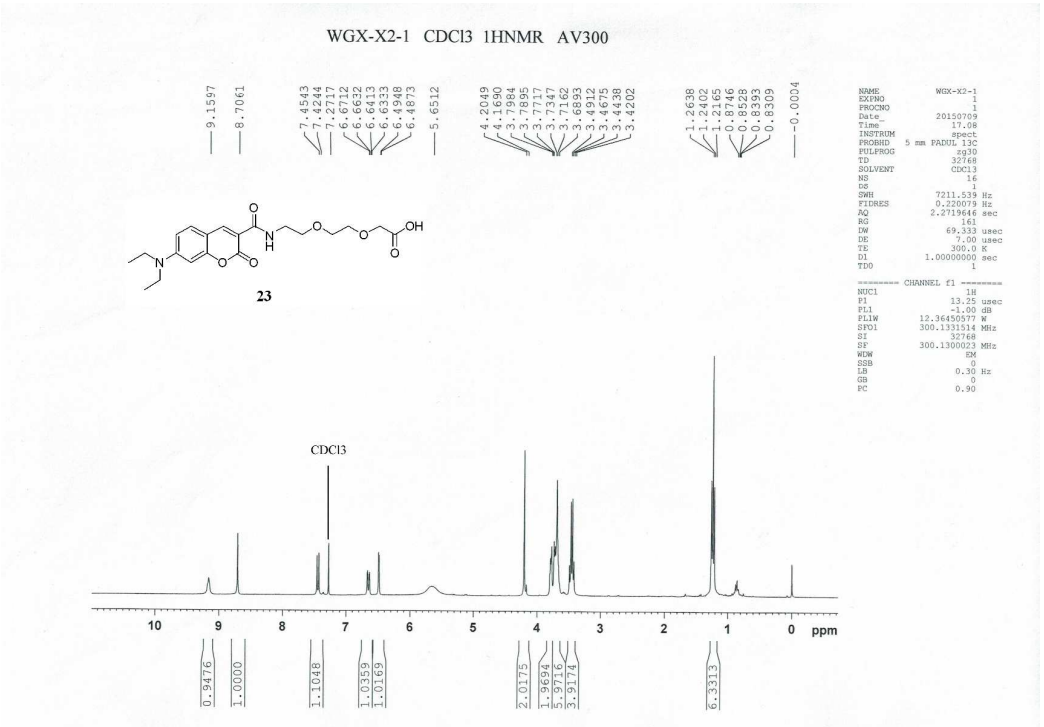
Copies of ^{1}H NMR of **13b**



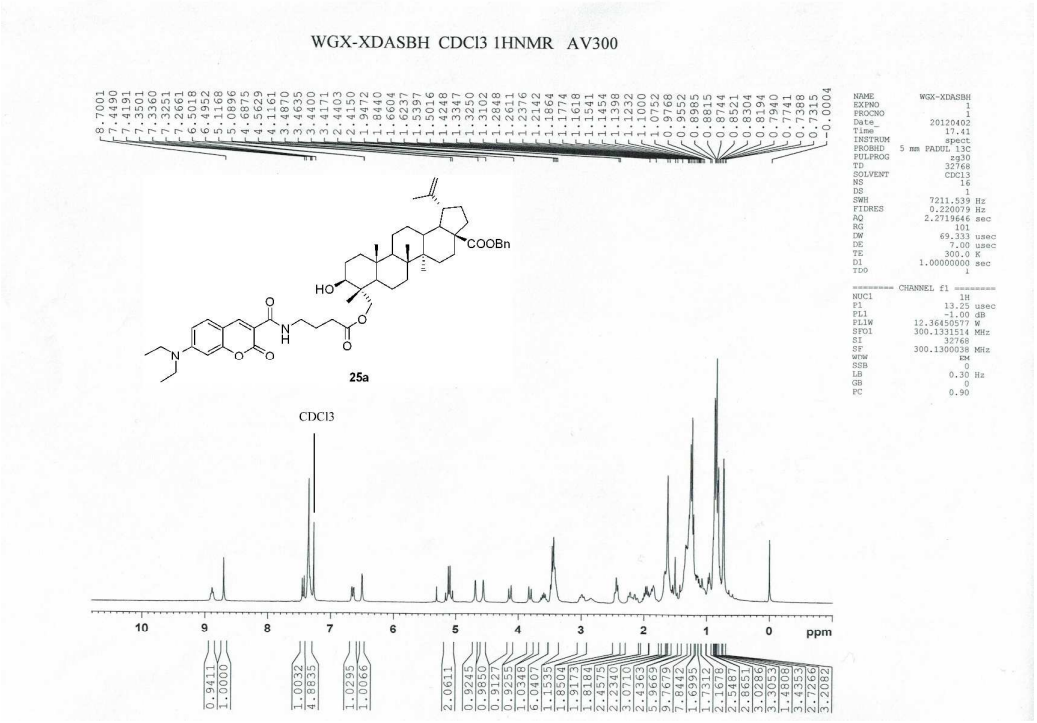
Copies of ^{1}H NMR of **14b**



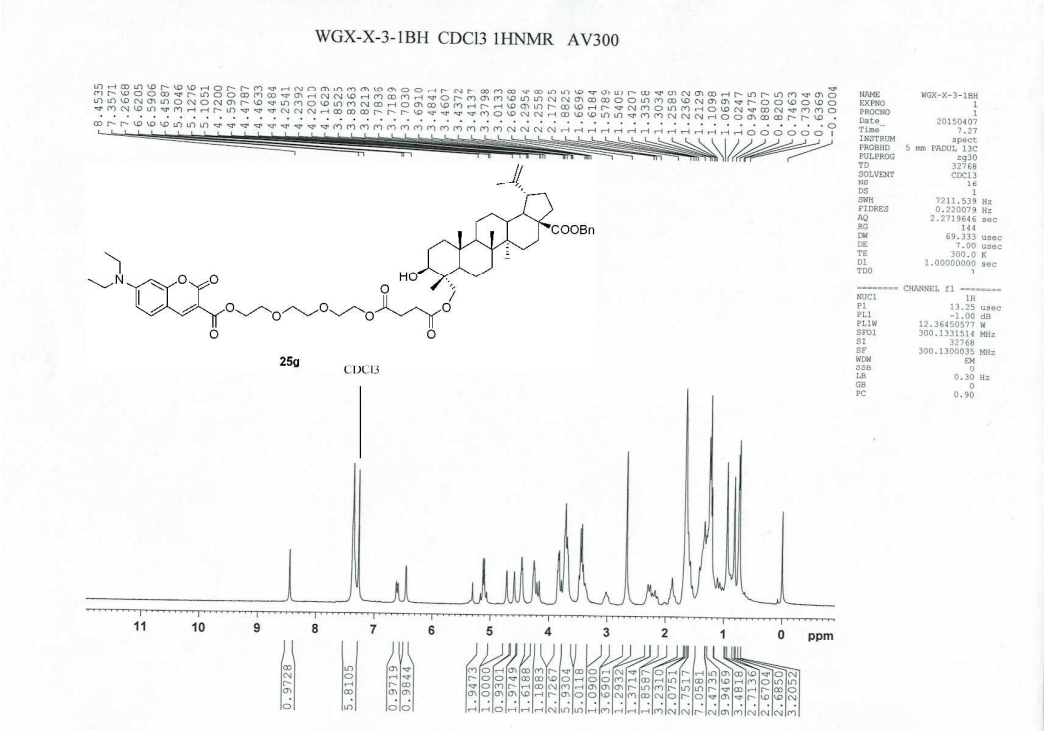
Copies of ^{1}H NMR of **23**



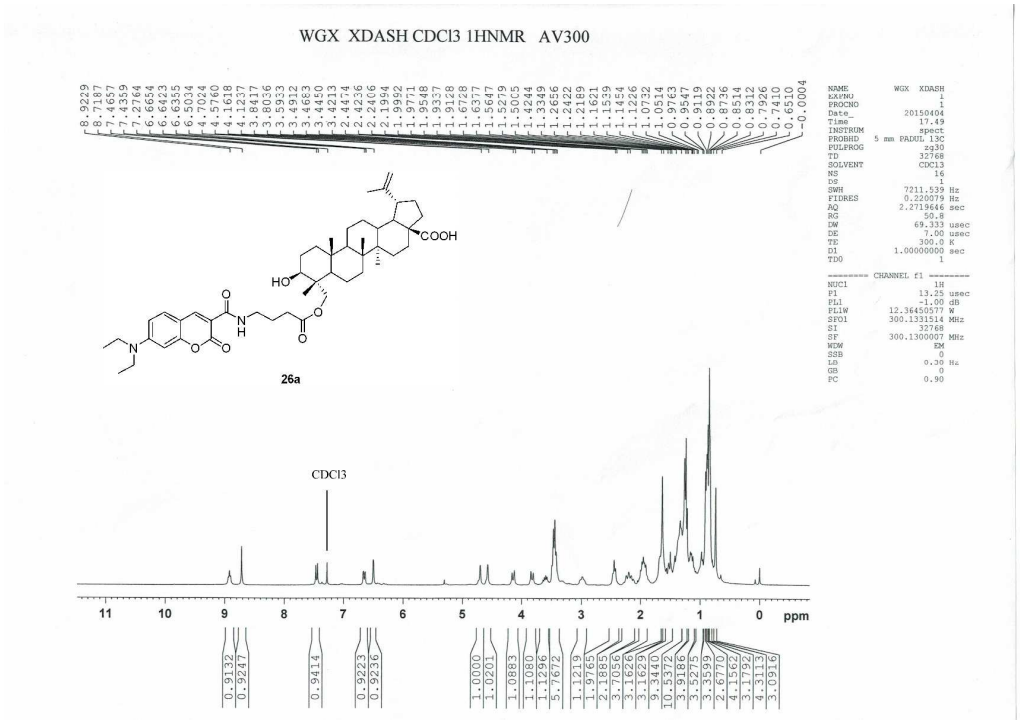
Copies of ^{1}H NMR of **25a**

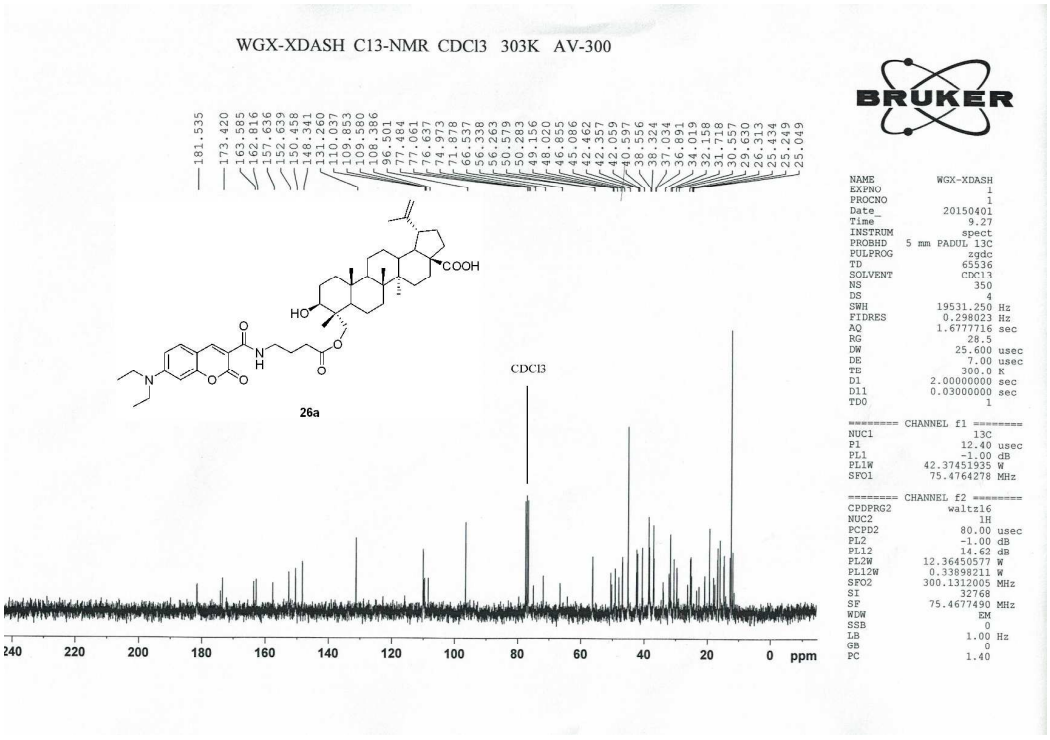


Copies of ^{1}H NMR of **25g**

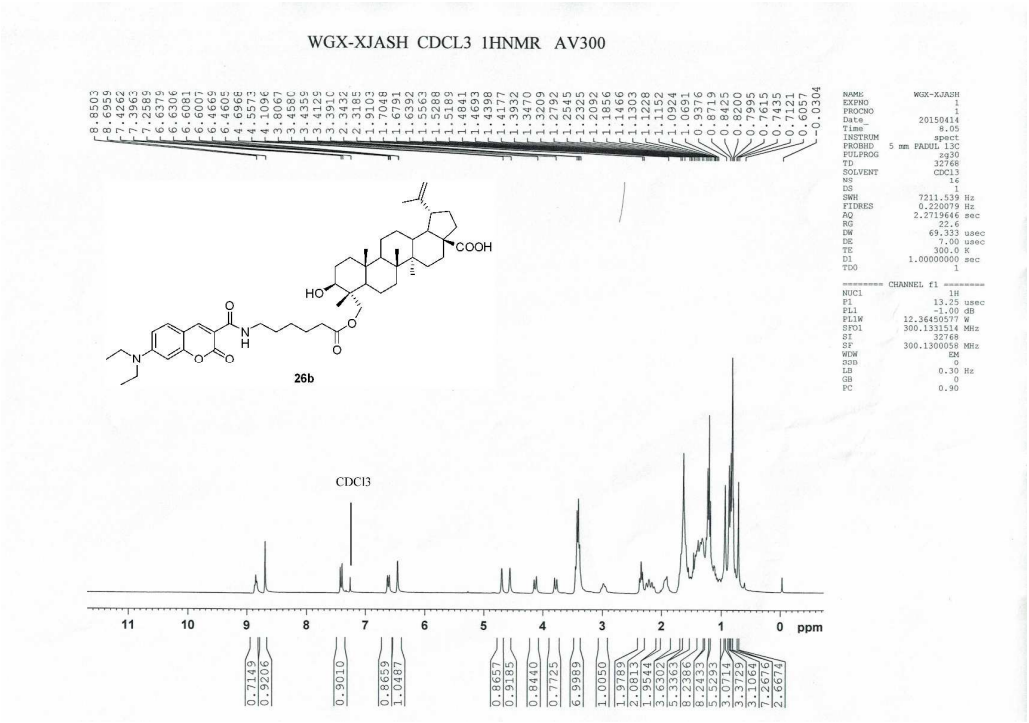


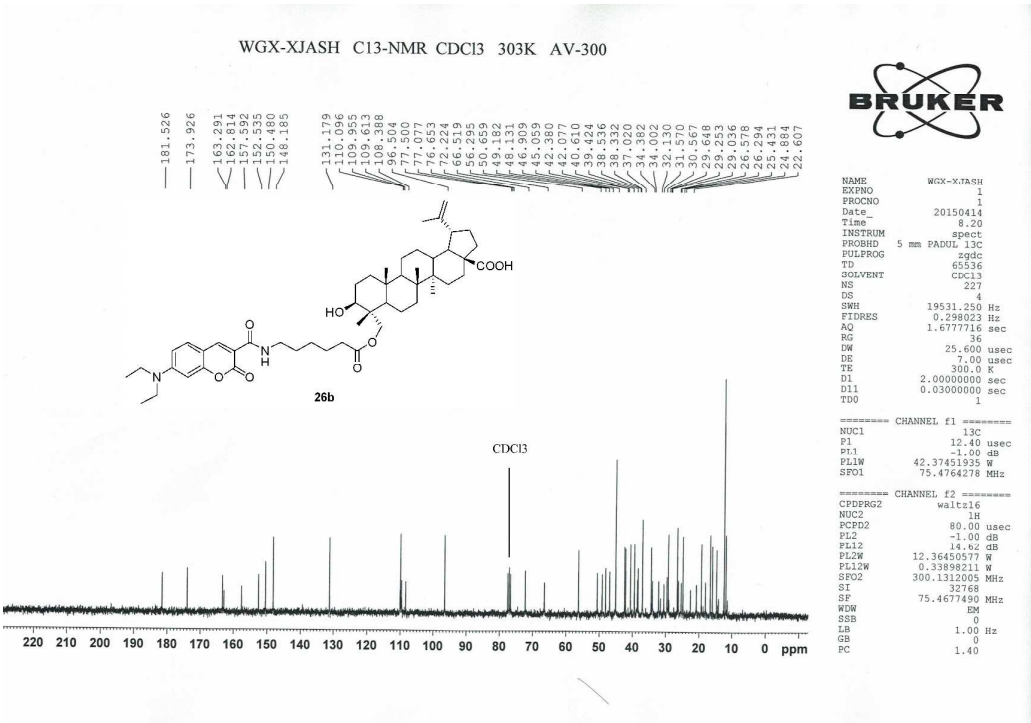
Copies of ^{1}H NMR and ^{13}C NMR of **26a**



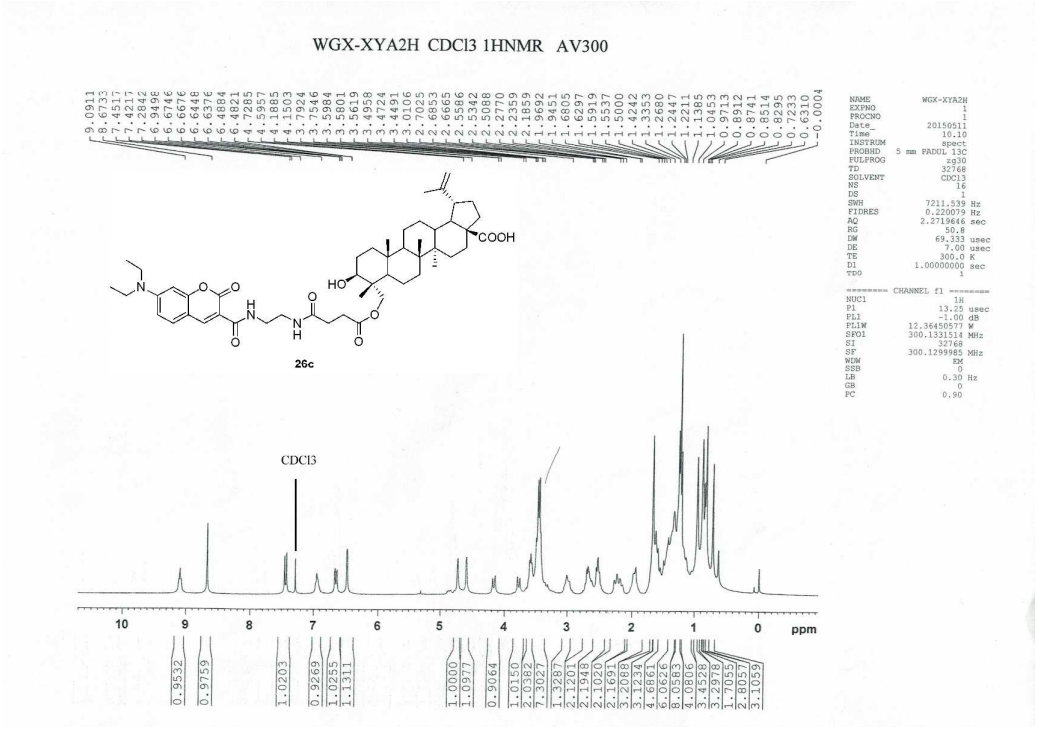


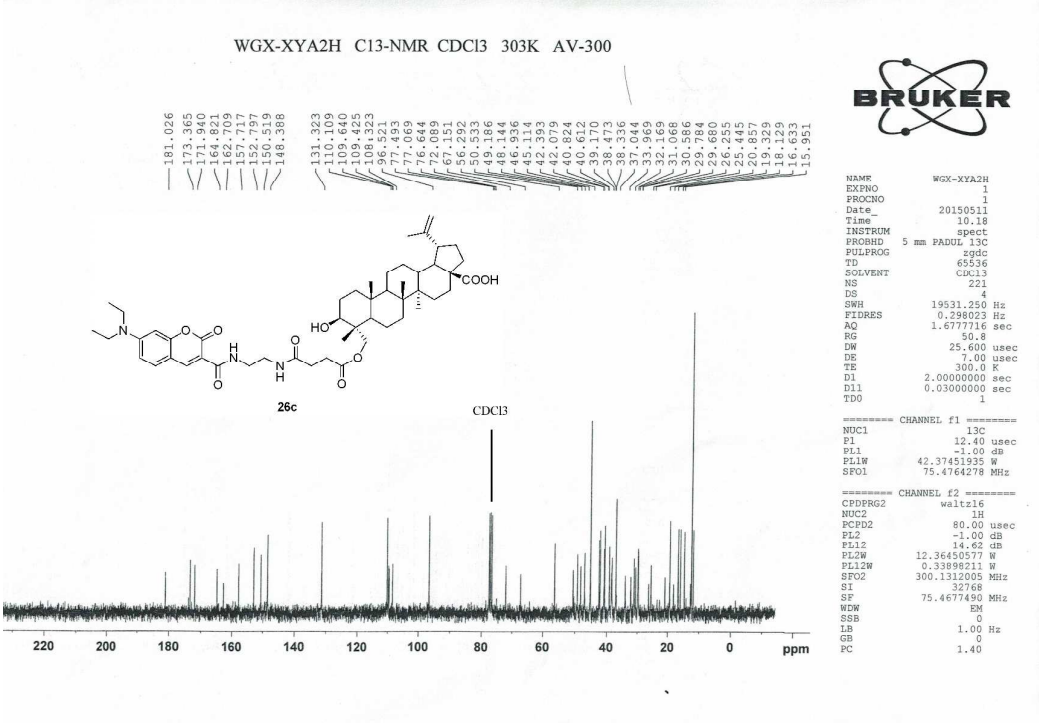
Copies of ^{1}H NMR and ^{13}C NMR of **26b**



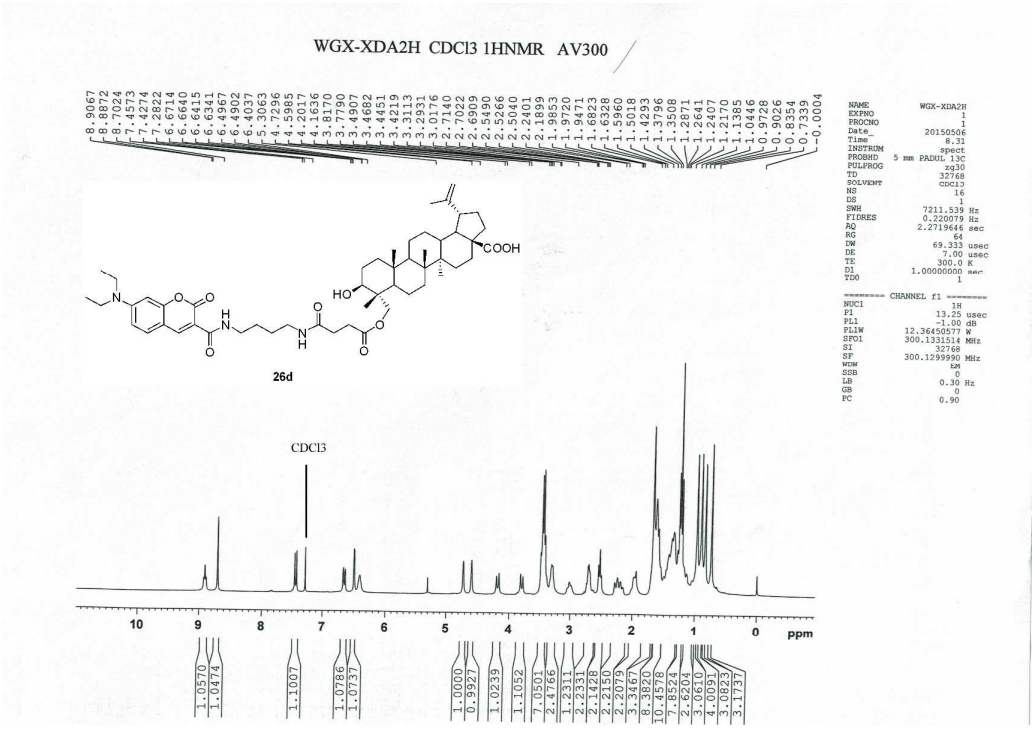


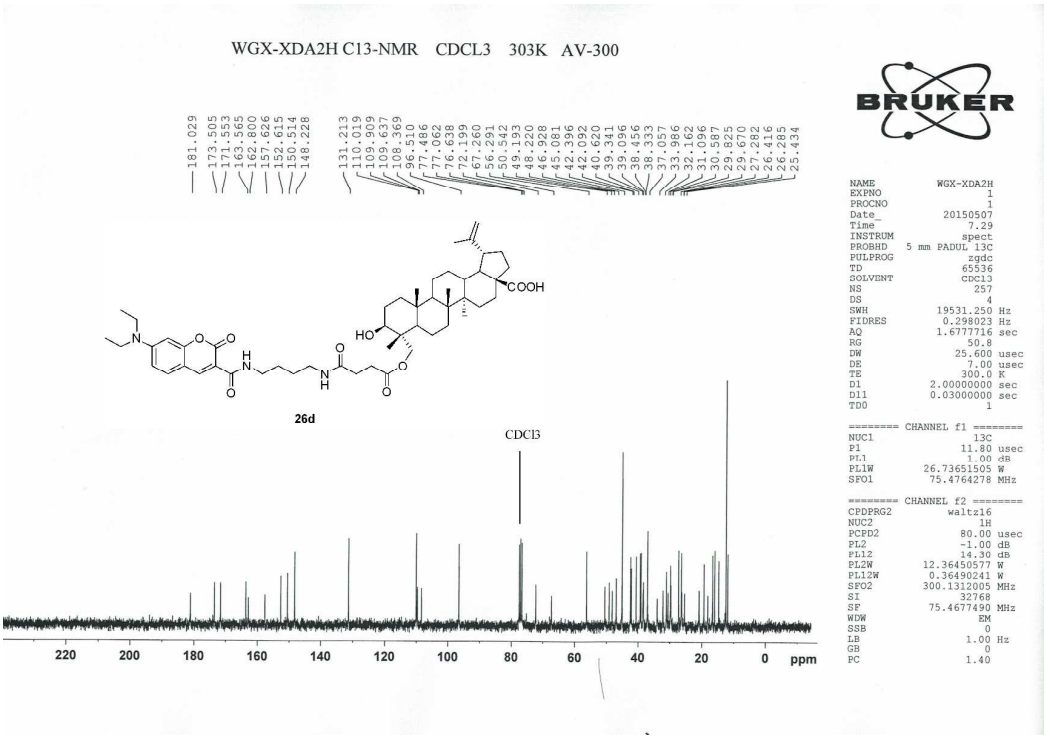
Copies of ^{1}H NMR and ^{13}C NMR of **26c**



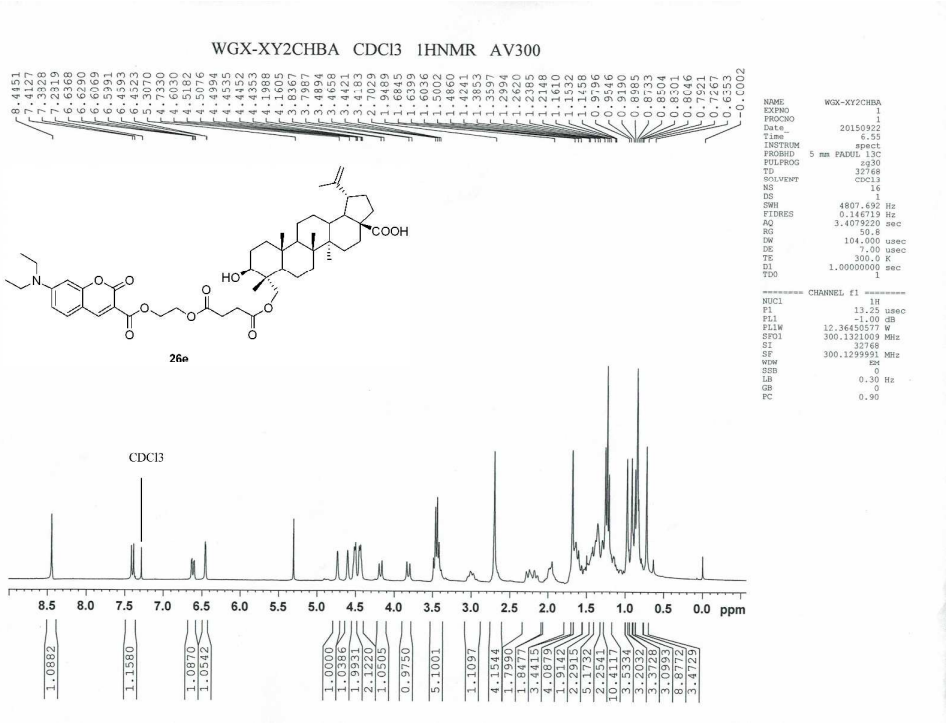


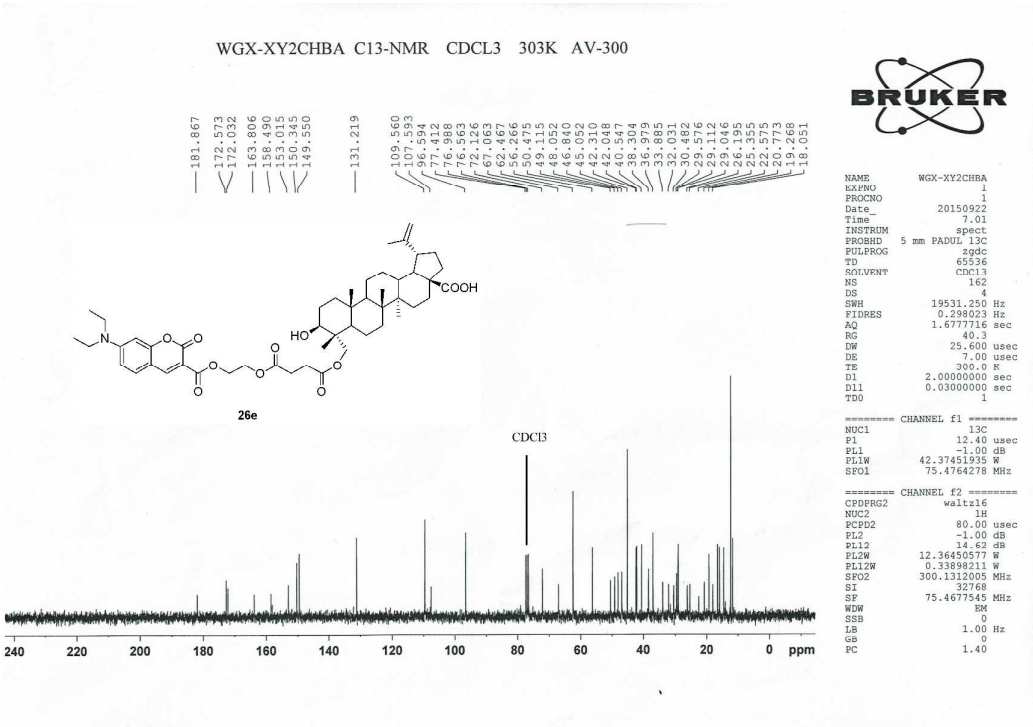
Copies of ^{1}H NMR and ^{13}C NMR of **26d**



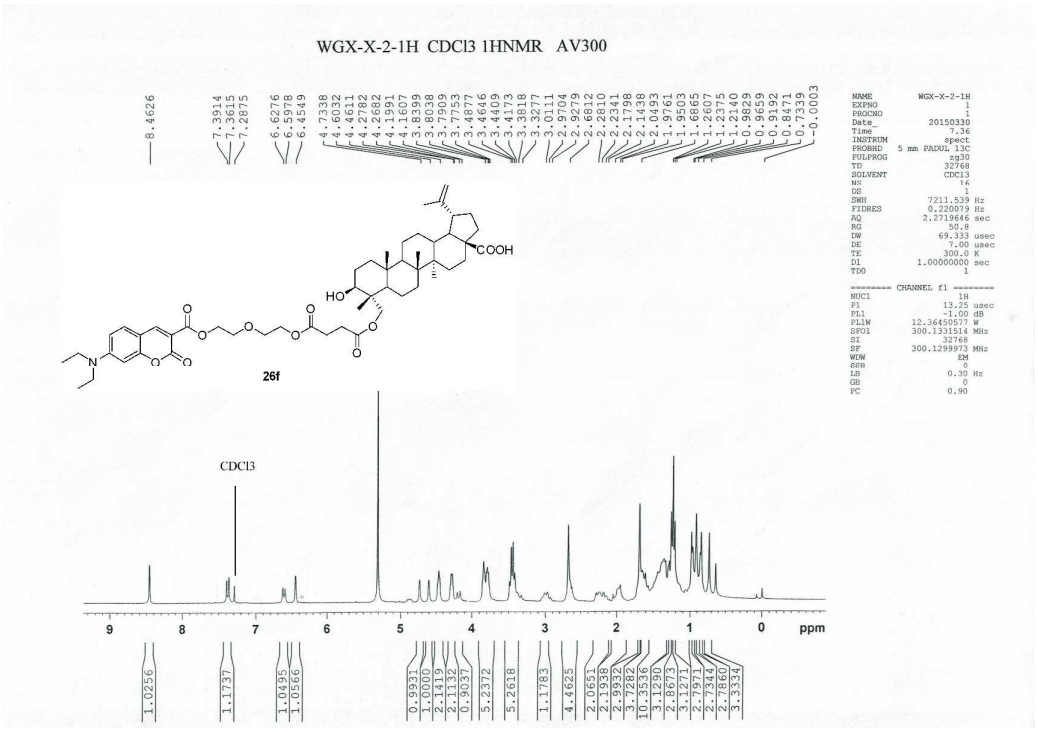


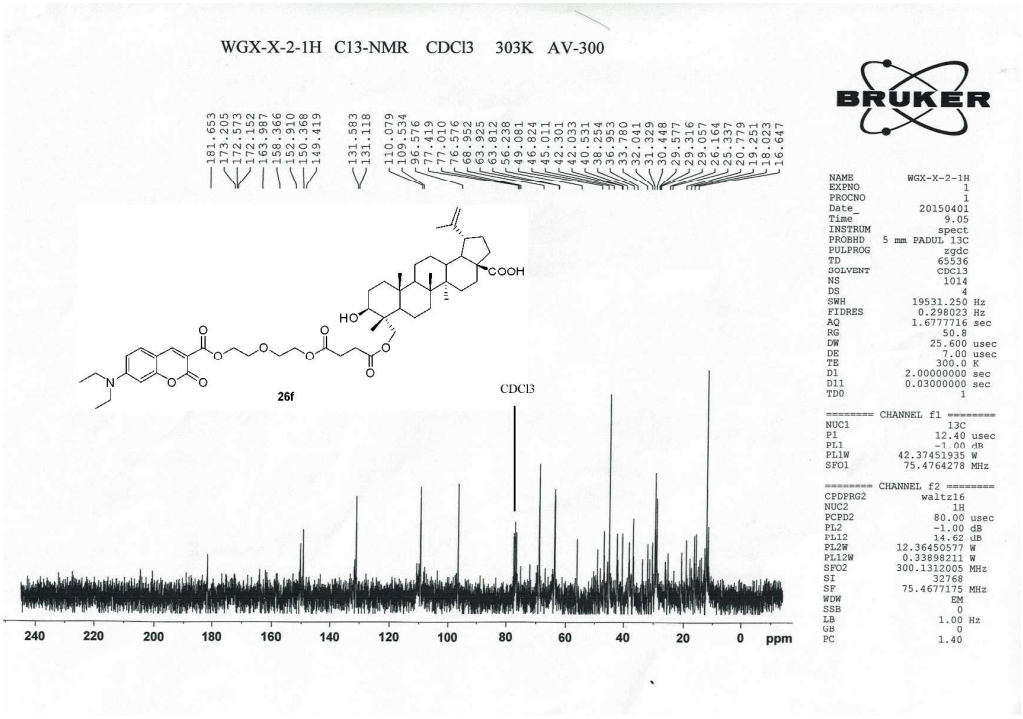
Copies of ^{1}H NMR and ^{13}C NMR of **26e**



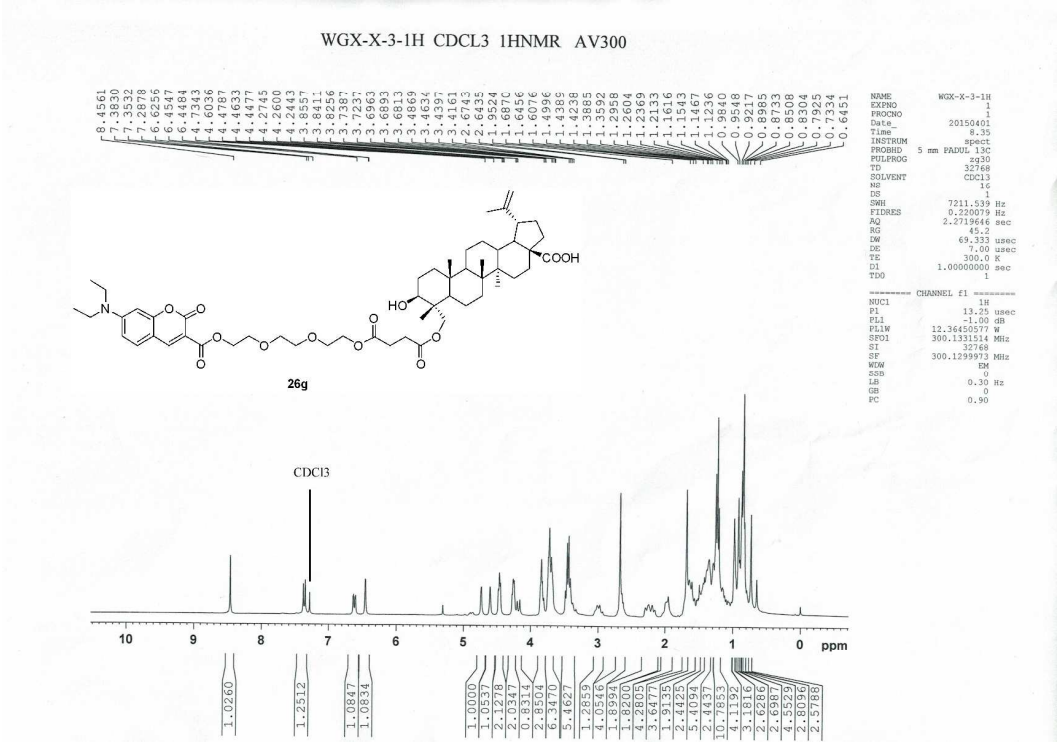


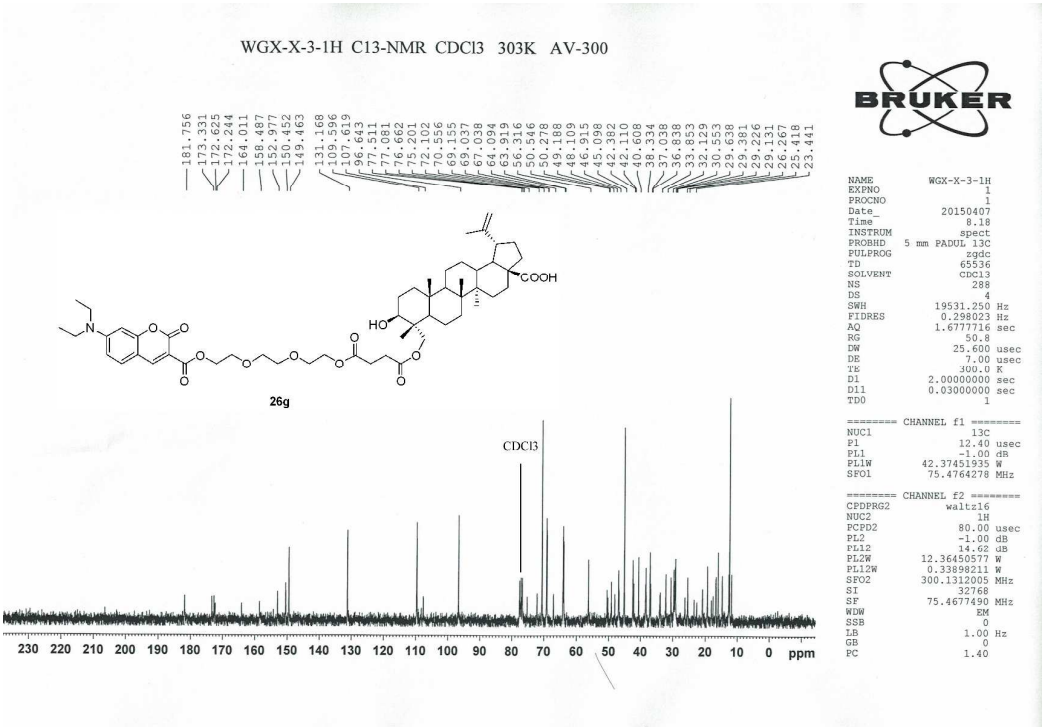
Copies of ^{1}H NMR and ^{13}C NMR of **26f**



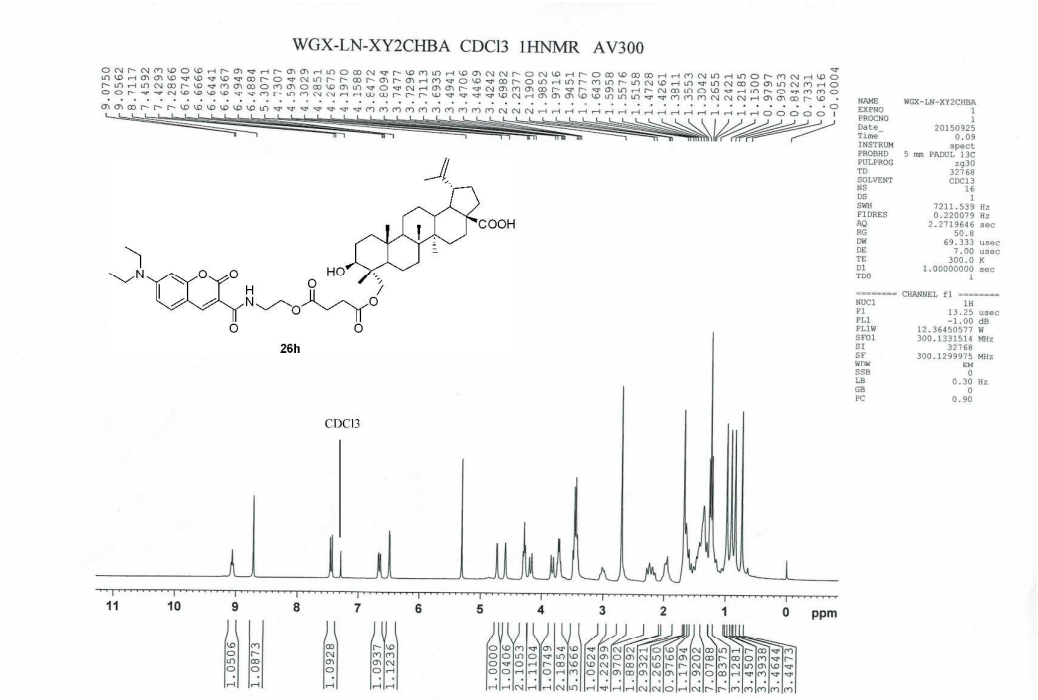


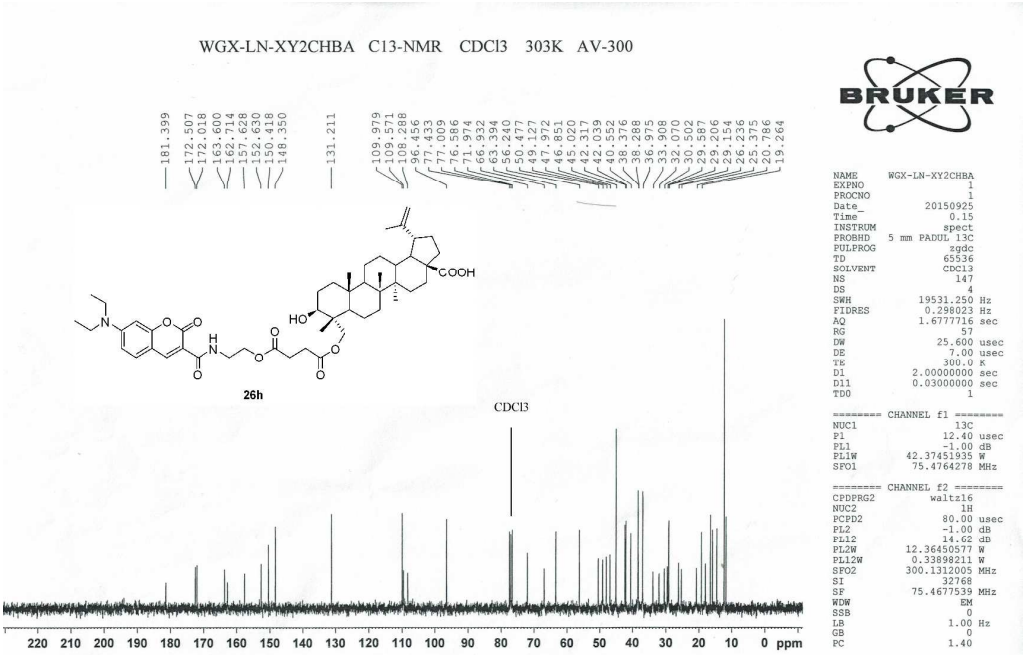
Copies of ^{1}H NMR and ^{13}C NMR of **26g**



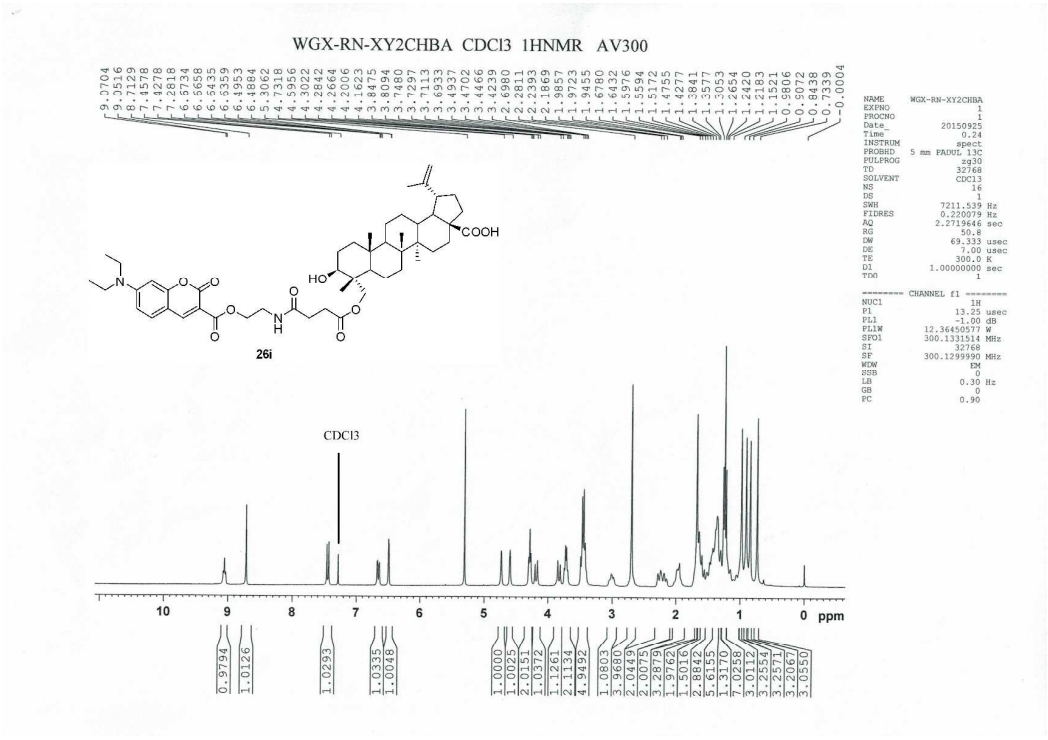


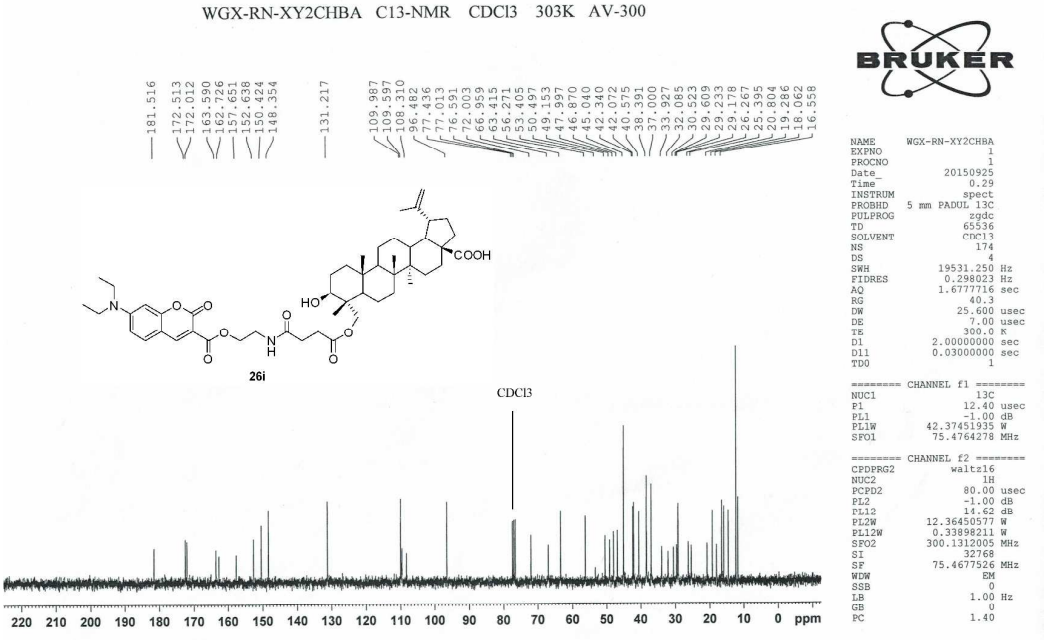
Copies of ^{1}H NMR and ^{13}C NMR of **26h**





Copies of ^{1}H NMR and ^{13}C NMR of **26i**





Copies of ^{1}H NMR and ^{13}C NMR of **26j**