Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Materials

for

Glycyrrhetinic Acid as Hepatocyte Targeting Unit for Anticancer Drug Delivery System with Enhanced Cell Type Selectivity

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General Information

All the reagents and solvents were used as received from chemical suppliers like Sigma Aldrich, Alfa Aesar, TCI Korea and Merck. Column chromatography purifications were performed by using silica gel 60 (70 ~ 230 mesh) as a stationary phase. Analytical thin-layer chromatography (TLC) was conducted using TLC silica gel 60, visualized under ultraviolet light. The ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker 500 NMR instruments. Chemical shifts are reported as δ values (ppm) with (residual) solvent as internal standard (DMSO-d_6; ^{1}H: δ = 2.50, ^{13}C: δ = 39.52 and $CDCl_3$; ^{1}H: δ = 7.26, ^{13}C: δ = 77.16). The mass spectra were recorded on an IonSpecHiRes ESI Shimadzu LC/MS-2020 mass spectrometer. All UV-Vis. and the fluorescence spectral studies were carried out on S-3100 and RF-5301PC spectrophotometer, respectively. Stock solutions of **Epi-GA** and **Cou-GA** (each, 5 mM) were prepared in pure DMSO. For solution studies, each stock solution was diluted with phosphate saline buffer (PBS, pH 7.4, 37 ^{o}C) containing 10% DMSO to make the final working solution with concentration of 5 μM. The total volume of solution was kept fixed at 3.0 ml. The excitation wavelengths at 494 nm (for **Epi-GA**) and 414 nm (for **Cou-GA**) with all excitation and emission slit widths at 5 nm were used. To monitor the drug release, the working solution of **Epi-GA** (5 μM) was first incubated with esterase (10 units) at 37 ^{o}C for different time intervals and corresponding fluorescence spectra were recorded.

Cell culture and confocal microscopy imaging

The cell lines of human colon adenocarcinoma HCT-116, Chang liver cells, and human cervical carcinoma HeLa were cultured with Dulbecco's Modified Eagle's medium (DMEM, Gibco), and the cells of human hepatocellular carcinoma HepG2, human breast carcinoma MCF7, and human lung carcinoma A549 were cultured with RPMI 1640 medium (Gibco, CA, USA). Both media contain 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). Cells were cultured in a humidified air and 5% CO_2 atmosphere at 37℃. For maintenance, the cells were cultured every 3 or 4 days. For confocal microscopic study. The cells were seeded on a cover glass bottomed dish (SPL Lifesciences Co., Ltd.), and were incubated for 24 hours. After the cells were replaced with culture medium containing the probes for 12 hours, the confocal images were obtained with annexin V (5 μL) and propidium iodide (1 μM) for 15 min using a

confocal laser scanning microscope (Zeiss LSM 510, Zeiss, Oberko, Germany). The excitation wavelength and the emission filters were 633 nm with a long pass (> 650 nm) and 543 nm with a band pass 585 nm ~ 615 nm filter for Annexin V (Alexa Fluor ™ 647 conjugate) and propidium iodide, respectively. Images were analyzed using Zen imaging software (ZEISS, Oberko, Germany).

Fluorescence microscopy

Various cell lines were plated in a 35 mm glass bottom dish, and the probe was added to the cell culture to a final concentration of 5 μM. Cells were incubated at 37 °C, 5% CO_2. Before the images were taken, the cells were washed with PBS. The fluorescence microscope (NeoScience Co., Ltd., Suwon, Korea) was excited at 400-410 nm detected with long pass emission 455 nm filter. The fluorescence images were analyzed by using Zen imaging software.

MTT analysis

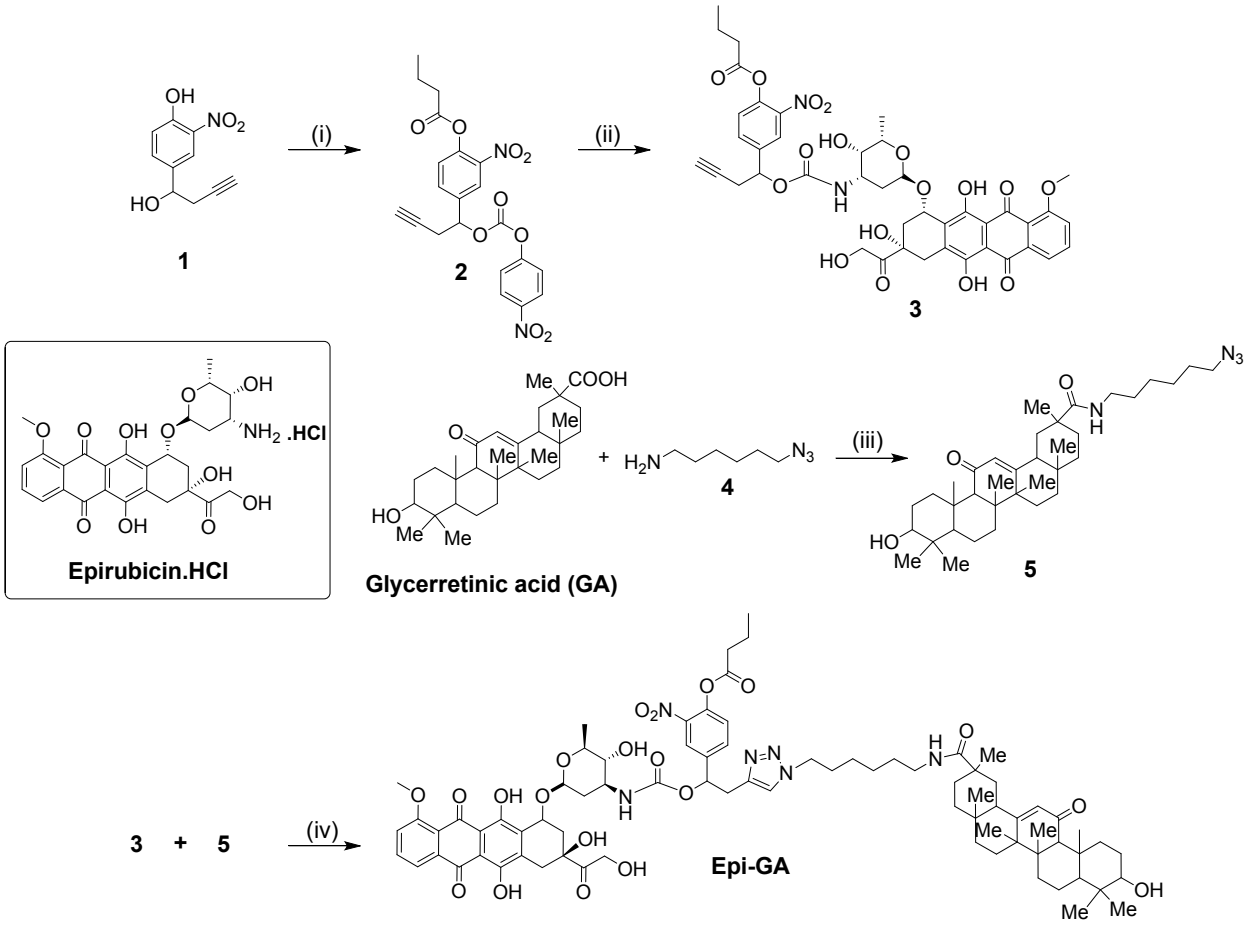
The cells' reducing ability of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used for the effect of **Epi-GA** and epirubicin on cell viability. HepG2 cells, Chang liver cells, A549 cells, MCF7 cells, HeLa cells, and HCT116 cells were seeded in a 96 well plate for 0.5 × 10^4cells. Cells were treated with **Epi-GA** and epirubicin at various concentrations for 48 hours. The cells were replaced with 100 μL medium containing 0.5 mg/mL MTT for 50 minutes. The absorbance at 570 nm was measured using VICTOR ™ X3 ELISA Multilable Plate Reader (Perkin Elmer Inc, Waltham, USA).

Animal Studies

All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Ulsan College of Medicine, South Korea, and conformed to the ARRIVE guidelines.[1] For a tumour xenograft model, the mice were anesthetized by inhalation of isoflurane gas (N_2O:O_2/70%:30%) and then subcutaneously injected with HepG2 cells (1 × 10^6 cells) suspended in a 50% solution of Matrigel (BD Bioscience) in PBS. Tumors were allowed to establish, at which time the mice were randomized into experimental groups. Intraperitoneal administration of PBS (vehicle) Epirubicin (**Epi**) and GA-conjugated Epirubicin (**Epi-GA**)

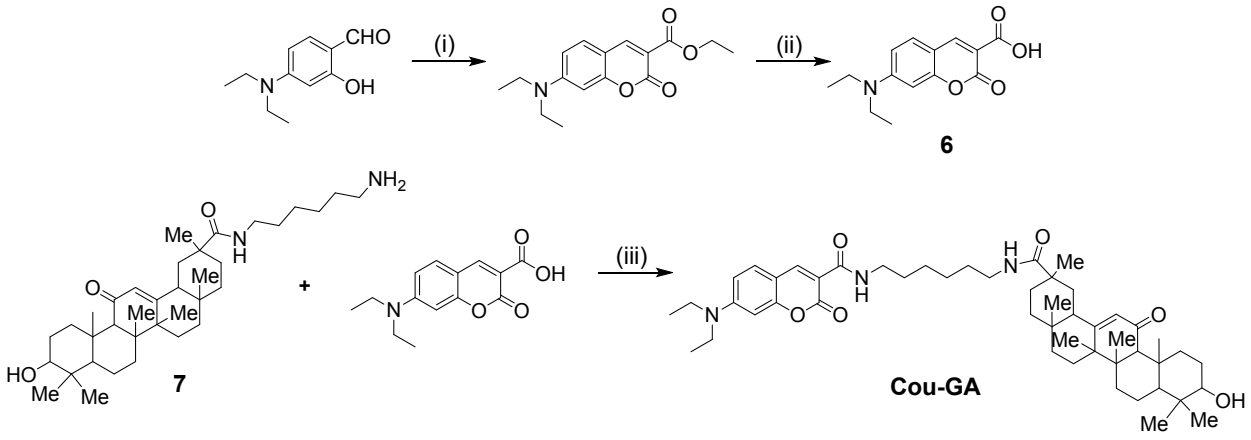
compound was begun 6 days after cell injection and repeated every 3 days. The diameters of the growing tumours was measured using a caliper and then the volume was calculated according to the $V=axb^2/2$, where a and b denote the longer and shorter superficial diameters, respectively.

Synthetic Scheme for prodrug Epi-GA



Scheme S1. Reagents and conditions (i) Butyryl chloride, Et_3N, THF, RT, (ii) (a) p-Nitro chloroformate, Et_3N, THF, r.t.; (b) Epirubicin.HCl, Et_3N, DMF, RT, (iii) EDC, HOBt, DMAP, DMF, RT, (iv) Sodium ascorbate, $CuSO_4.5H_2O$, DMF, RT.

Synthetic Scheme for fluorescent probe Cou-GA



Scheme S2. Reagents and conditions (i) Diethyl malonate, piperidine, EtOH, reflux, (ii) NaOH, EtOH, RT, (iii) EDC, HOBt, DMAP, DMF, RT.

Detailed Synthetic Procedures

Compounds **1**[2], **4**[3], **6**[4] and **7**[5] were synthesized by following literature procedures. The detailed synthesis of other derivatives and final product is described below:

Synthesis of compound 2

To a cold mixture of compound **1** (1.0 g, 4.83 mmol) and Et_3N (0.61 g, 6.03 mmol) in dry DCM (10 ml) was added butyryl chloride (0.64 g, 6.03 mmol) drop-wise with continuous stirring. After 1 hours, the mixture was quenched with cold water. The DCM layer was separated, washed with brine, dried over Na_2SO_4 and finally concentrated to get the crude. The product was purified by column chromatography to yield **3** in 70% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.14 (s, 1H), 7.69 (d, J = 8.0 Hz, 1H), 7.22 (d, J = 8.0 Hz, 1H), 4.95 (t, J = 8.0 Hz, 1H), 2.68-2.61 (m, 4H), 2.12 (s, 1H), 1.85-1.76 (m, 2H), 1.05 (t, J = 8.0 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.54, 143.74, 141.67, 132.14, 125.50, 123.43, 79.53, 72.34, 70.97, 36.06, 29.67, 18.22, 13.84.

Synthesis of compound 5

A mixture of glycyrrhetinic acid (GA) (0.5 g, 1.06 mmol), EDCI (0.25 g, 1.32 mmol), HOBt (0.18 g, 1.32 mmol) and DMAP (0.16 g, 1.32 mmol) in 15 ml DMF was stirred under nitrogen at room

temperature (RT) for 1 hour. Amine derivative **5** (0.19 g, 1.32 mmol) was then added and stirring was continue overnight. Upon completion of reaction, the DMF was evaporated to get the crude which was purified by column chromatography using 60% EtOAc/hexane to yield **5** in 65% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.66 (s, 1H), 5.59 (t, J = 10.0 Hz, 1H), 3.32-3.23 (m, 5H), 2.83-2.79 (m, 1H), 2.35 (s, 1H), 2.19-2.15 (m, 1H), 2.09-2.03 (m, 1H), 1.95 (d, J = 10.0 Hz, 1H), 1.88-1.82 (m, 1H), 1.75 (d, J = 10.0 Hz, 2H), 1.67-1.64 (m, 3H), 1.61-1.59 (m, 5H), 1.57-1.51 (m, 2H), 1.44-1.35 (m, 10H), 1.30-1.26 (m, 2H), 1.23-1.20 (m, 1H), 1.15-1.14 (m, 9H), 1.02 (s, 5H), 0.82 (d, J = 5.0 Hz, 6H), 0.71 (d, J = 15 Hz, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 200.13, 175.67, 169.40, 128.36, 78.64, 61.81, 54.94, 51.31, 48.21, 45.36, 43.53, 43.22, 41.78, 39.29, 39.18, 39.14, 37.48, 37.05, 32.74, 31.88, 31.45, 29.68, 29.38, 28.72, 28.51, 28.12, 27.22, 26.47, 26.45, 26.37, 26.35, 23.33, 18.66, 17.46, 16.33, 15.61. MS (ESI): m/z calcd. for $C_{36}H_{58}N_4O_3$: 594 Found: 593 $[M-1]^+$.

Synthesis of Cou-GA

A mixture of **6** (0.5 g, 1.91 mmol), EDCI (0.45 g, 2.39 mmol), HOBt (0.32 g, 2.39 mmol) and DMAP (0.29 g, 2.39 mmol) in 15 ml DMF was stirred under nitrogen at RT for 1 hour. Amine derivative **7** (1.35 g, 2.39 mmol) was then added and stirring was continue overnight. Upon completion of reaction, the DMF was evaporated to get the crude which was purified by column chromatography using 5% MeOH/DCM to yield **Cou-GA** in 68% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.81 (t, J = 5.0 Hz, 1H), 8.70 (s, 1H), 7.44 (d, J = 10 Hz, 1H), 6.65 (dd, J = 5.0 Hz, 1H), 6.51 (d, J = 5.0 Hz, 1H), 5.74 (t, J = 5.0 Hz, 1H), 5.68 (s, 1H), 3.51-3.43 (m, 7H), 3.29-3.25 (m, 3H), 2.82-2.78 (m, 1H), 2.34 (s, 1H), 2.21-2.18 (m, 1H), 2.09-2.04 (m, 1H), 1.94 (d, J = 10.0 Hz, 1H), 1.88-1.78 (m, 2H), 1.72 (d, J = 10.0 Hz, 1H), 1.68-1.59 (m, 8H), 1.56-1.51 (m, 2H), 1.44-1.38 (m, 11H), 1.27-1.21 (m, 9H), 1.15-1.14 (m, 8H), 1.02 (s, 4H), 0.82 (d, J = 10.0 Hz, 6H), 0.71 (d, J = 10.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 200.09, 175.60, 169.25, 163.11, 162.81, 157.58, 152.46, 148.01, 131.10, 128.51, 110.37, 109.92, 108.38, 96.53, 78.75, 61.80, 54.93, 48.06, 45.33, 45.07, 43.54, 43.20, 41.82, 39.32, 39.27, 39.14, 37.51, 37.07, 32.76, 31.90, 31.55, 29.71, 29.64, 29.50, 28.54, 28.11, 27.29, 26.46, 26.45, 26.42, 23.38, 18.68, 17.48, 16.37, 15.60, 12.43; MS (ESI): m/z calcd. for $C_{50}H_{73}N_3O_6$: 812 Found: 812 $[M]^+$.

Synthesis of 3

To an ice-cold mixture of compound **2** (0.2 g, 0.72 mmol) and Et_3N (0.086 g, 0.86 mmol) in 10 ml dry DCM was added solution of 4-nitrophenyl chloroformate (0.17 g, 0.86 mmol) in 5 ml dry DCM drop-wise and reaction mix was stirring at RT under nitrogen. After reaction was completed, the mixture was washed with 0.5 N $NaHCO_3$ and then brine. The DCM layer was separated, dried over Na_2SO_4 and finally concentrated to get the crude which was used as such for next step. The crude was dissolved in 5 ml dry DMF and drug epirubicin hydrochloride (0.41 mg, 0.72 mmol) was added followed by addition of excess Et_3N (0.5 ml). The mixture was stirred at room temperature until the completion of reaction. The solvent was evaporated under reduced pressure to get the crude which was then purified by column chromatography using 5% MeOH/DCM to yield **3** in 58% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.11-8.07 (m, 1H), 8.03-8.00 (m, 1H), 7.81-7.77 (m, 1H), 7.63-7.54 (m, 1H), 7.42-7.39 (m, 1H), 7.21-7.08 (m, 1H), 5.78-5.67 (m, 1H), 5.48 (br s, 1H), 5.27 (br s, 1H), 5.03-4.98 (m, 1H), 4.77 (br s, 2H), 4.58-4.54 (m, 1H), 4.10 (s, 1H), 4.08 (s, 1H), 3.84-3.80 (m, 1H), 3.67 (br s, 1H), 3.37 (br s, 1H), 3.26-3.12 (m, 2H), 3.07 (br s, 1H), 2.97-2.92 (m, 1H), 1.27-2.70 (m, 2H), 2.64-2.57 (m, 2H), 2.37 (d, J = 15 Hz, 1H), 2.19-2.16 (m, 2H), 2.10-2.06 (m, 1H), 2.00-1.97 (m, 1H), 1.82-1.74 (m, 3H), 1.72-1.66 (m, 1H), 1.37-1.34 (m, 3H), 1.27 (br s, 1H), 1.07-1.01 (m, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 213.78, 186.63, 128.25, 171.125, 160.88, 156.08, 155.33, 154.70, 143.75, 141.46, 138.06, 135.77, 135.13, 133.69, 133.58, 133.17, 132.89, 127.78, 125.18, 123.74, 122.95, 120.45, 120.04, 119.71, 118.49, 111.27, 111.12, 100.04, 78.38, 72.71, 72.10, 71.85, 70.04, 69.60, 65.44, 56.60, 56.59, 50.52, 35.75, 33.65, 26.27, 17.93, 17.74, 13.59; MS (ESI): m/z calcd. for $C_{42}H_{42}N_2O_{17}$: 846 Found: 869 $[M + 23]^+$.

Synthesis of prodrug Epi-GA

A mixture of **3** (0.10 g, 0.12 mmol), **5** (0.08 g, 0.13 mmol), and sodium ascorbate (10 mol %) in 5 ml DMF was stirred for 15 minutes at room temperature. Then, 5 mol % of $CuSO_4$ in 2 ml DMF was added and reaction mixture was purged with nitrogen for 5 minutes. The mixture was then allowed to stir at RT overnight. After removal of the solvent under reduced pressure, the crude mixture was purified over silica gel using 5% MeOH/DCM to yield final product **Epi-GA** as a dark red solid in 52% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 10.42 (s, 1H), 8.06-8.04 (m, 1H), 7.89 (s, 1H),

7.82-7.78 (m, 1H), 7.48-7.40 (m, 2H), 7.22 (s, 1H), 7.02 (d, J = 10.0 Hz, 1H), 5.85-5.72 (m, 2H), 5.62-5.61 (m, 1H), 5.41 (d, J = 5.0 Hz, 1H), 5.27 (br s, 1H), 5.05 (br s, 1H), 4.79 (br s, 2H), 4.69 (br s, 1H), 4.30 (t, J = 5.0 Hz, 2H), 4.11 (s, 2H), 4.09 (s, 1H), 3.84-3.81 (m, 1H), 3.49 (q, J = 10.0 Hz, 1H), 3.32-3.13 (m, 6H), 3.05-3.01 (m, 3H), 2.77-2.70 (m, 2H), 2.40-2.33 (m, 2H), 2.31 (s, 1H), 2.19 (s, 1H), 2.17-1.95 (m, 4H), 1.86-1.84 (m, 3H), 1.75 (br s, 2H), 1.64-1.60 (m, 8H), 1.53-1.44 (m, 5H), 1.41-1.37 (m, 10H), 1.27 (s, 3H), 1.23 (t, J = 5.0 Hz, 2H), 1.14 (d, J = 10.0 Hz, 8H), 1.07 (d, J = 10.0 Hz, 2H), 1.02 (br s, 4H), 0.90 (t, J = 5.0 Hz, 1H), 0.82 (br s, 6H), 0.70 (t, J = 10.0 Hz, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 214.17, 214.00, 200.44, 186.94, 186.54, 176.00, 175.75, 169.91, 161.00, 156.27, 155.59, 154.56, 154.53, 135.87, 135.79, 135.75, 135.37, 133.83, 128.23, 128.20, 122.62, 120.71, 120.15, 119.77, 118.50, 111.39, 111.21, 100.31, 78.65, 64.48, 65.46, 61.84, 56.64, 54.94, 54.91, 48.31, 45.42, 45.41, 43.55, 43.28, 43.26, 41.88, 41.87, 39.18, 39.13, 37.46, 37.09, 35.79, 32.73, 31.90, 31.37, 30.32, 29.68, 29.57, 29.50, 29.48, 29.33, 29.21, 28.54, 28.11, 27.20, 26.47, 26.37, 26.01, 25.96, 25.64, 23.34, 23.30, 18.67, 17.96, 17.46, 17.45, 16.39, 15.61, 13.59; MS (ESI): m/z calcd. for $C_{78}H_{100}N_6O_{20}$: 1441 Found: 1464 $[M+23]^+$

Photo-physical Data

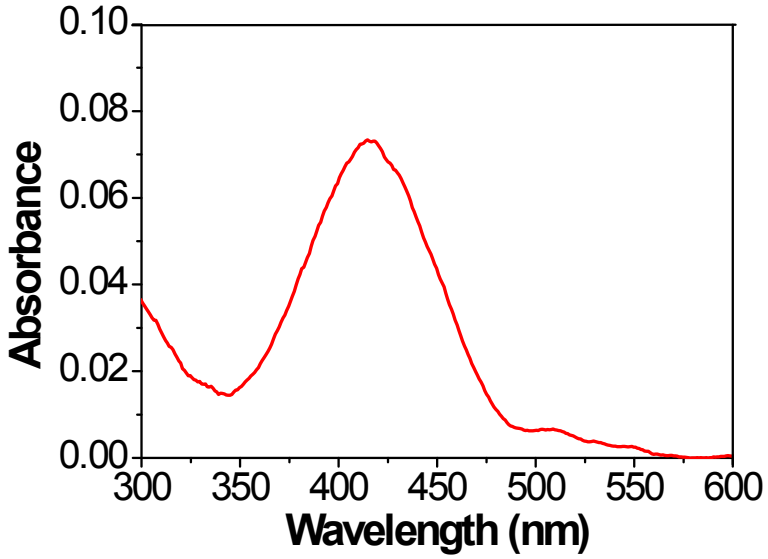


Figure S1. The absorption spectrum of **Cou-GA** (5 μM) in phosphate buffered saline (PBS, pH 7.4, 37 ^{o}C).

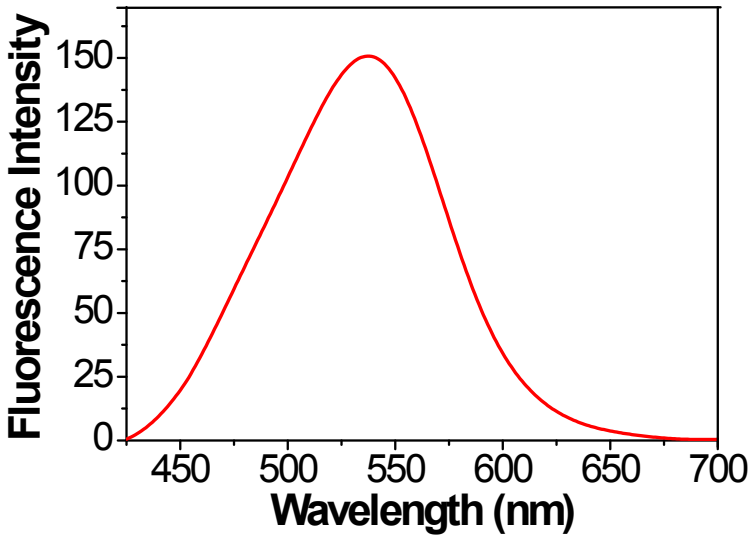


Figure S2. The emission spectrum of **Cou-GA** (5 μM) in phosphate buffered saline (PBS, pH 7.4, 37 ^{o}C). Excitation wavelength ($\lambda_{ex.}$) was 415 nm.

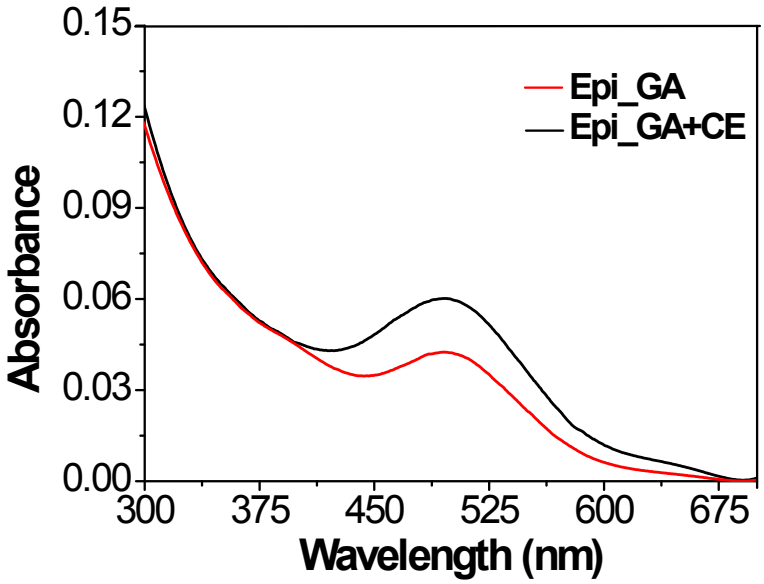


Figure S3. The change in absorption spectra of **Epi-GA** (5 μM) in the presence of carboxyesterase (5 U/3 mL). The spectra were recorded in phosphate buffered saline (PBS, pH 7.4, 37 ^{o}C).

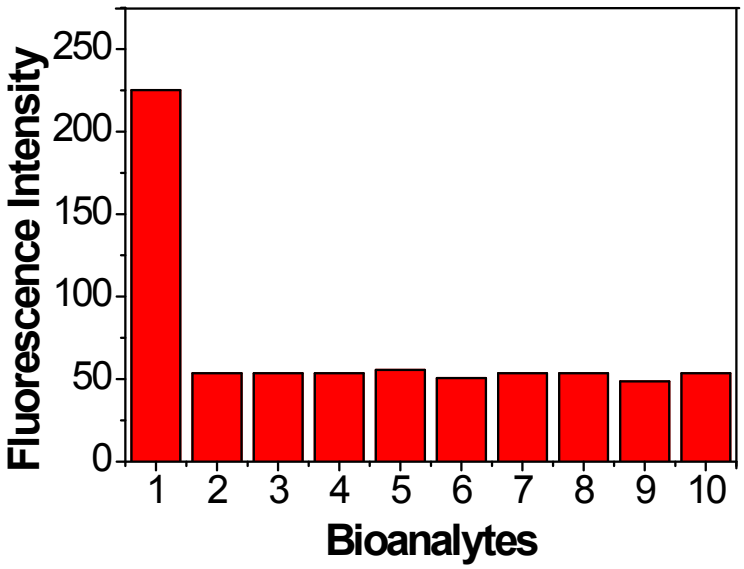


Figure S4. Bar diagram representing the selective response of **Epi-GA** to carboxylesterase over other bioanalytes: (1) carboxylesterase, (2) L-ascorbic acid, (3) L-Glutamic acid, (4) NADH, (5) Pepsin, (6) cysteine (7) Homo-cysteine (8) L-Glutathione, (9) H_2O_2, (10) hypochlorite ion (OCl^-). All spectra were recorded in phosphate buffered saline (PBS, pH 7.4, 37 ^{O}C). Excitation wavelength ($\lambda_{ex.}$) was 495 nm.

Mechanism of drug activation

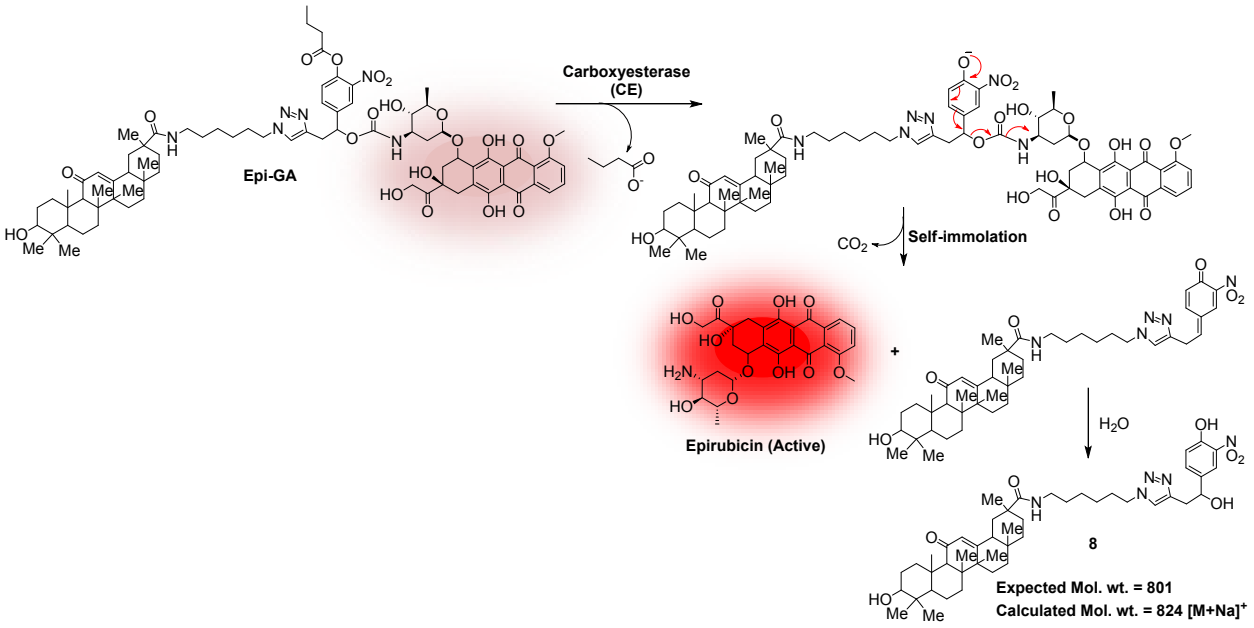


Figure S5. Mechanism of drug activation triggered by carboxylesterase (CE).

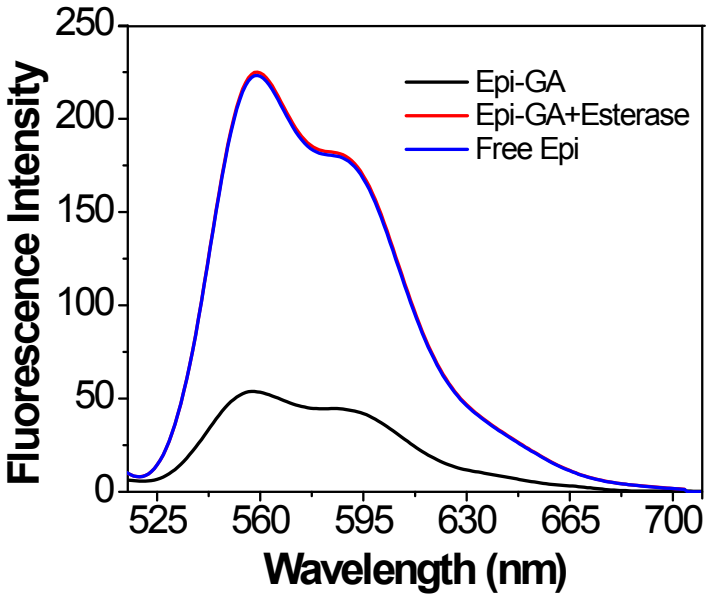


Figure S6. Fluorescence emission spectra of **Epi-GA** (5 μM) in the presence and absence of carboxylesterase and free epirubicin (**Epi**, 5 μM) in phosphate buffered saline (PBS, pH 7.4, 37 ^{o}C). The excitation wavelength was 495 nm.

Confocal Imaging

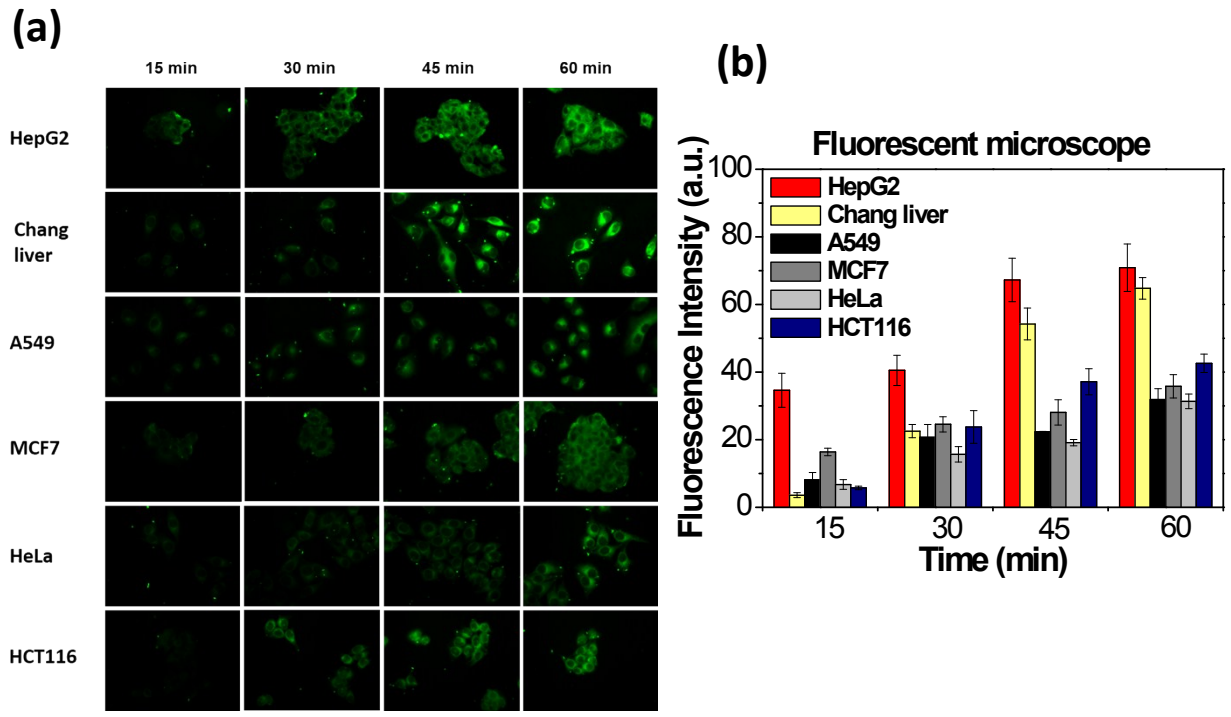


Figure S7. Cell selective uptake of **Cou-GA (a)** Confocal microscopy images of various cell lines incubated with 5 µM of **Cou-GA** for different time intervals at 37 °C. (b) The corresponding mean fluorescence intensity measured in each cell lines. Excitation wavelength was 410 nm and emission was collected at 455 nm. The images were obtained using zen imaging software. The data are presented as mean ± S.D. (n = 3).

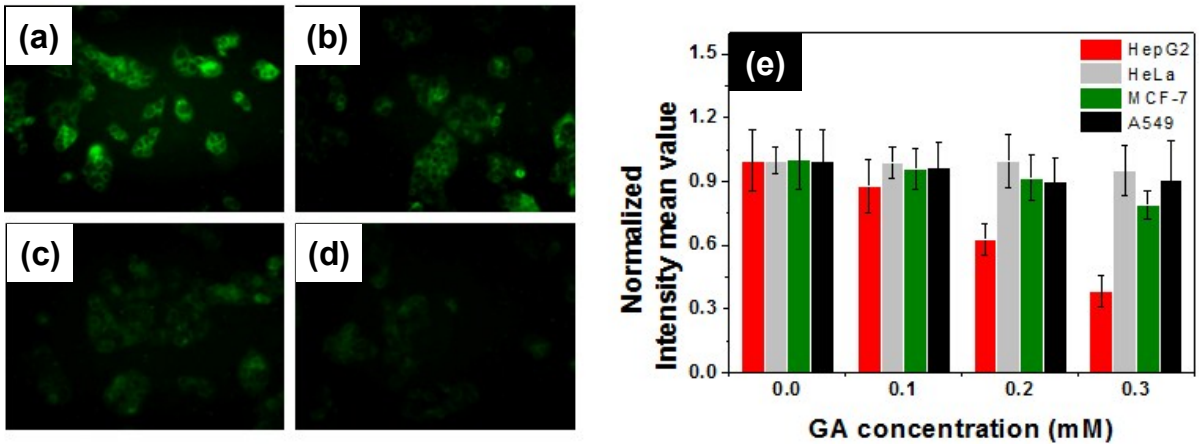


Figure S8. Effect of free GA on cellular uptake behavior of **Cou-GA**. (a-d) Fluorescence microscopic images of HepG2 cells pre-treated with different concentrations of free GA (from a to d; 0, 0.1, 0.2 and 0.3 mM) upon incubation with 5 µM Cou-GA, (e) Corresponding mean fluorescence intensity of **Cou-GA** measured in different cells lines (HepG2, HeLa, MCF7, A549). Excitation wavelength was 410 nm and emission was collected at 455 nm. The images were obtained using image J software. The data are presented as mean ± S.D. (n = 5).

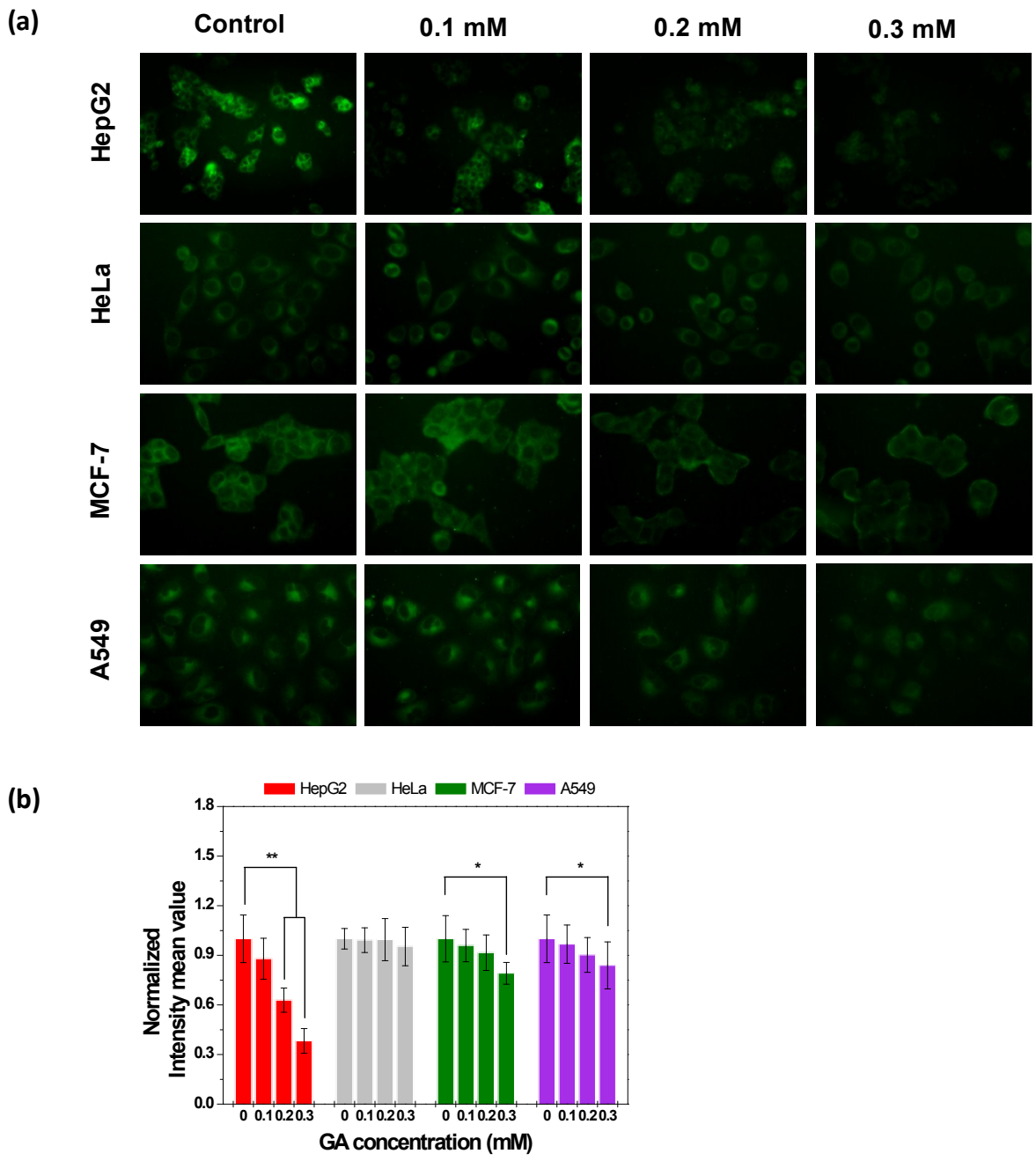


Figure S9. Effect of free GA on cellular uptake behavior of **Cou-GA** (a) Fluorescence microscopic images of cell lines (HepG2, HeLa, MCF-7, A549), pre-treated with different concentrations of free GA (0, 0.1, 0.2, and 0.3 mM) upon incubation with 5 μM **Cou-GA**. (b) Corresponding mean normalized fluorescence intensity of **Cou-GA** measured in other cell lines, including HepG2 cells. Excitation wavelength was 410 nm and emission was collected at 455 nm. The images were obtained using image J software. The data are presented as mean ± S.D. (n = 9). *P < 0.05 and **P < 0.01, compared to control in respectively cell lines. Unmarked, no significant.

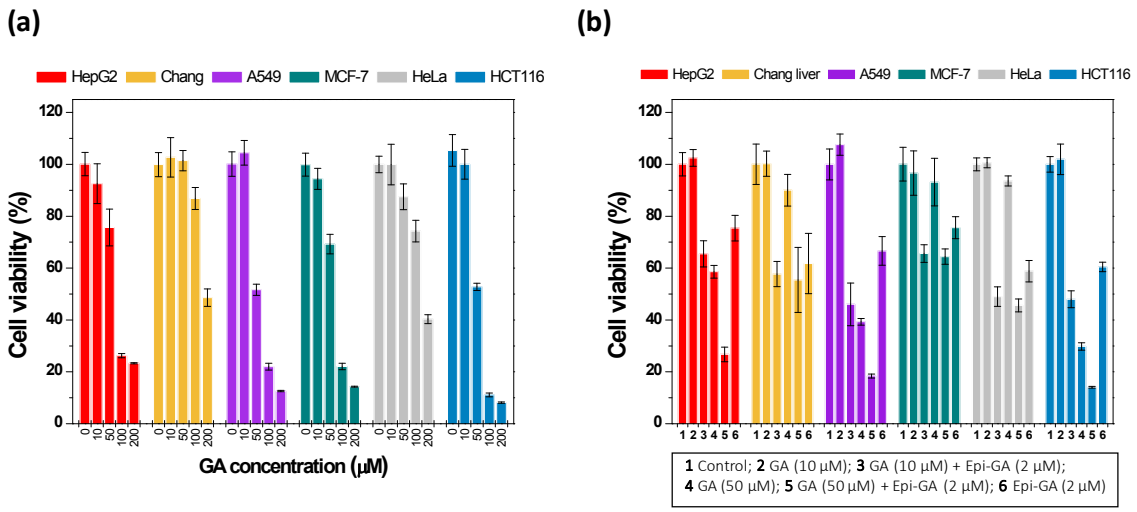


Fig S10. (a) MTT assay showing the comparison of cell viability of different cancer cell lines (HepG2, Chang liver, A549, MCF7, HeLa and HCT116 cell) treated with GA (0, 10, 50, 100, 200 μM) for 48 hours at 37 ^{o}C. The data are presented as mean ± S.D. (n = 6). (b) MTT assay showing the comparison of cell viability of different cancer cell lines (HepG2, Chang liver, A549, MCF7, HeLa and HCT116 cell). Cells were treated with free GA (various concentrations) and **Epi-GA** (2 μM) for 48 hours at 37 ^{o}C. The data are presented as mean ± S.D. (n = 6).

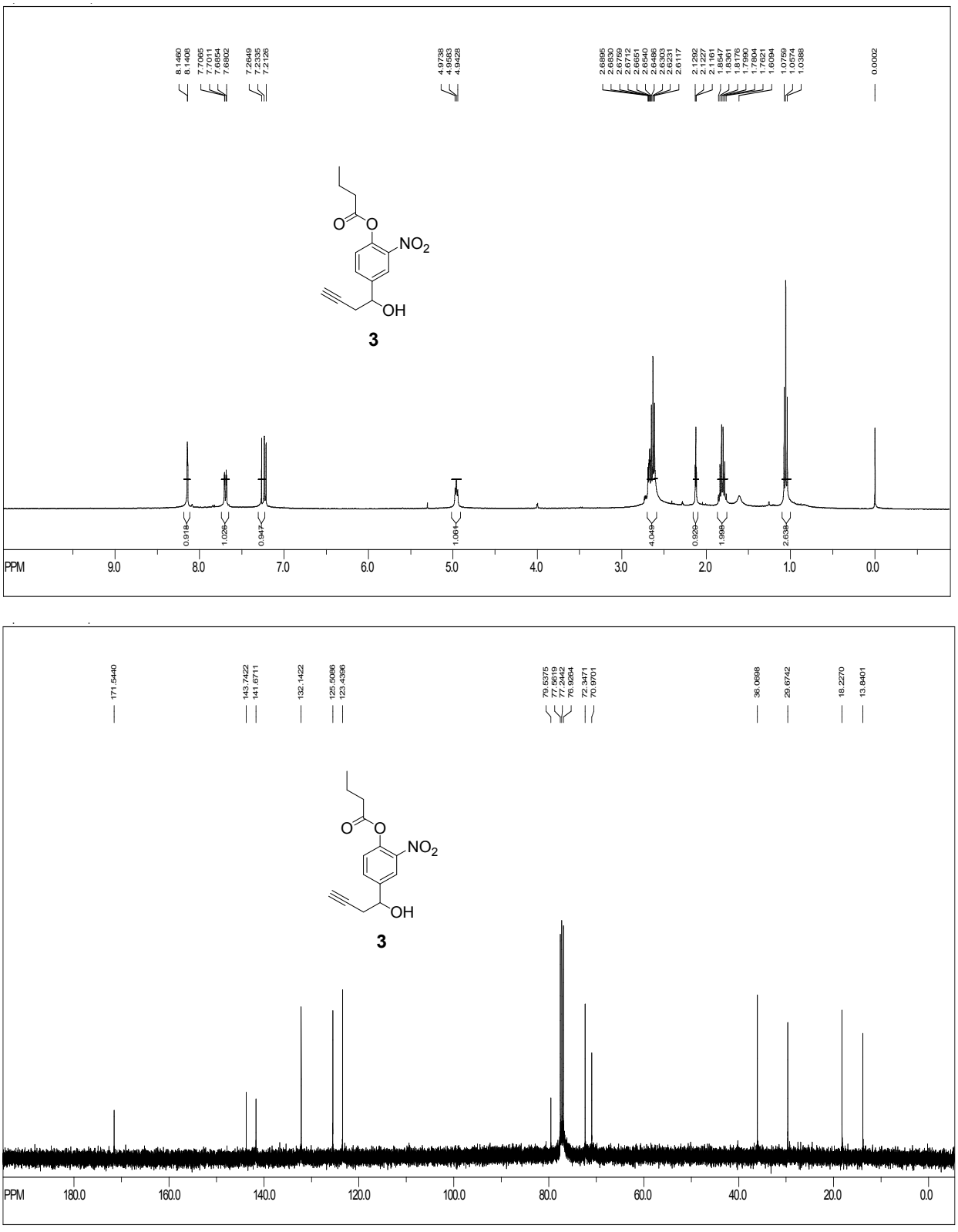


Fig. S11. ^{1}H and ^{13}C NMR spectrum of **3** in $CDCl_3$.

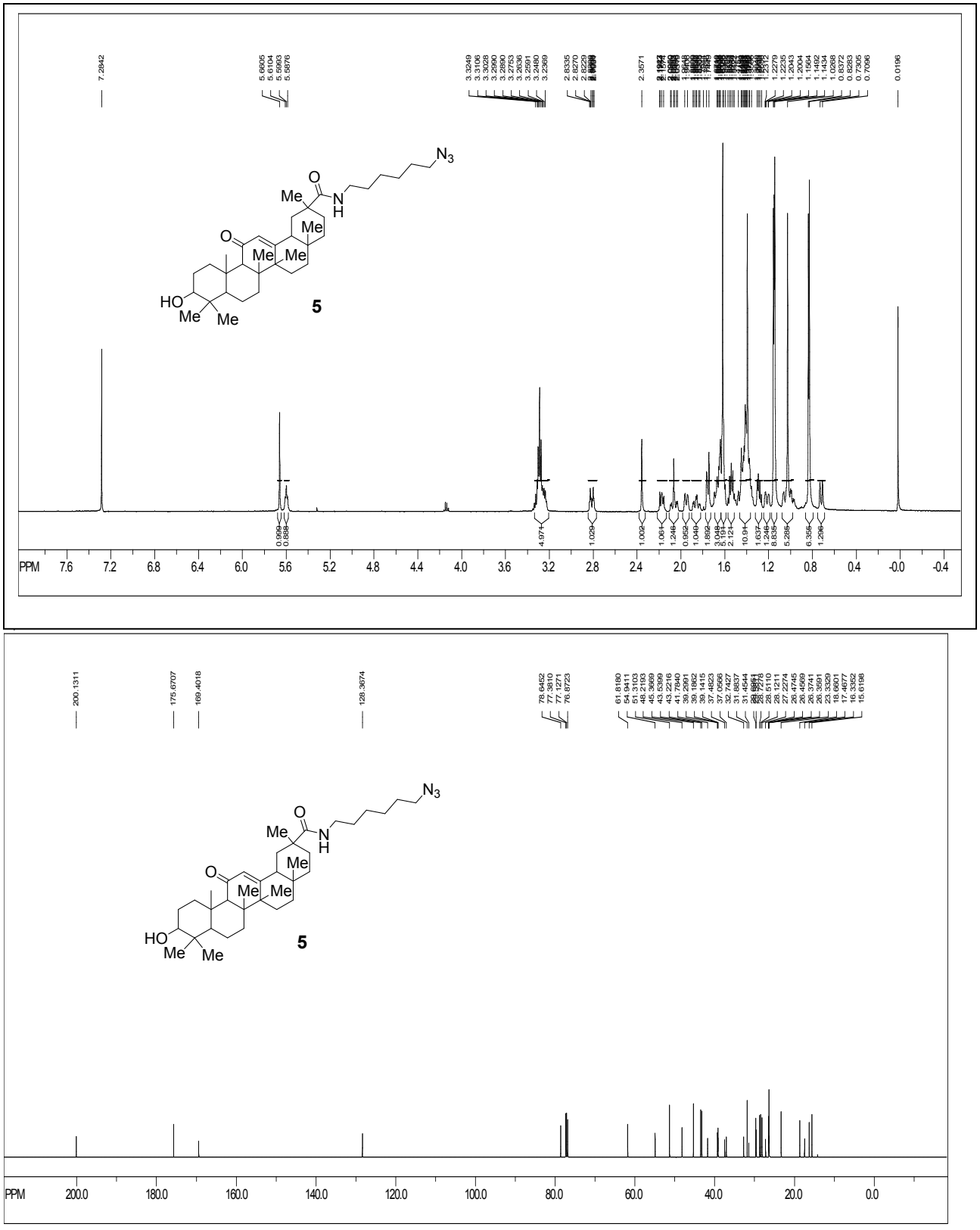


Fig. S12. ^{1}H and ^{13}C NMR spectrum of **5** in $CDCl_3$.

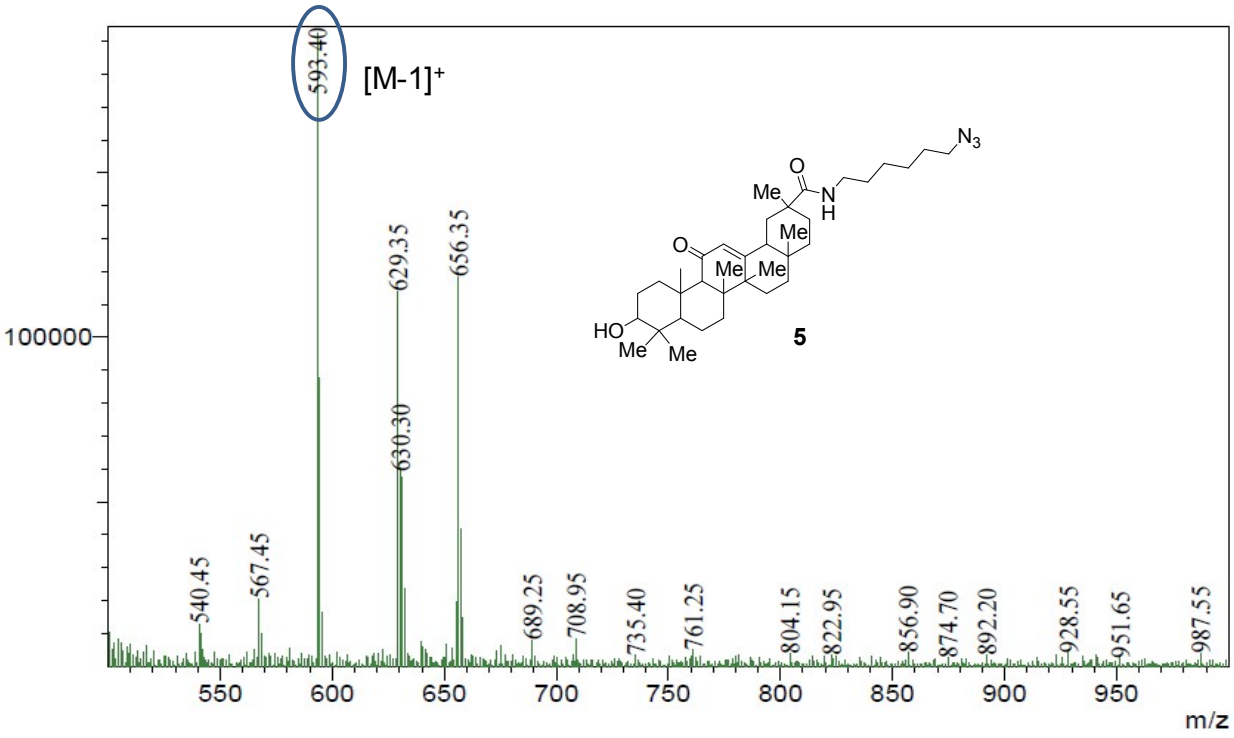


Fig. S13. Mass spectrum of **5** in $CDCl_3$.

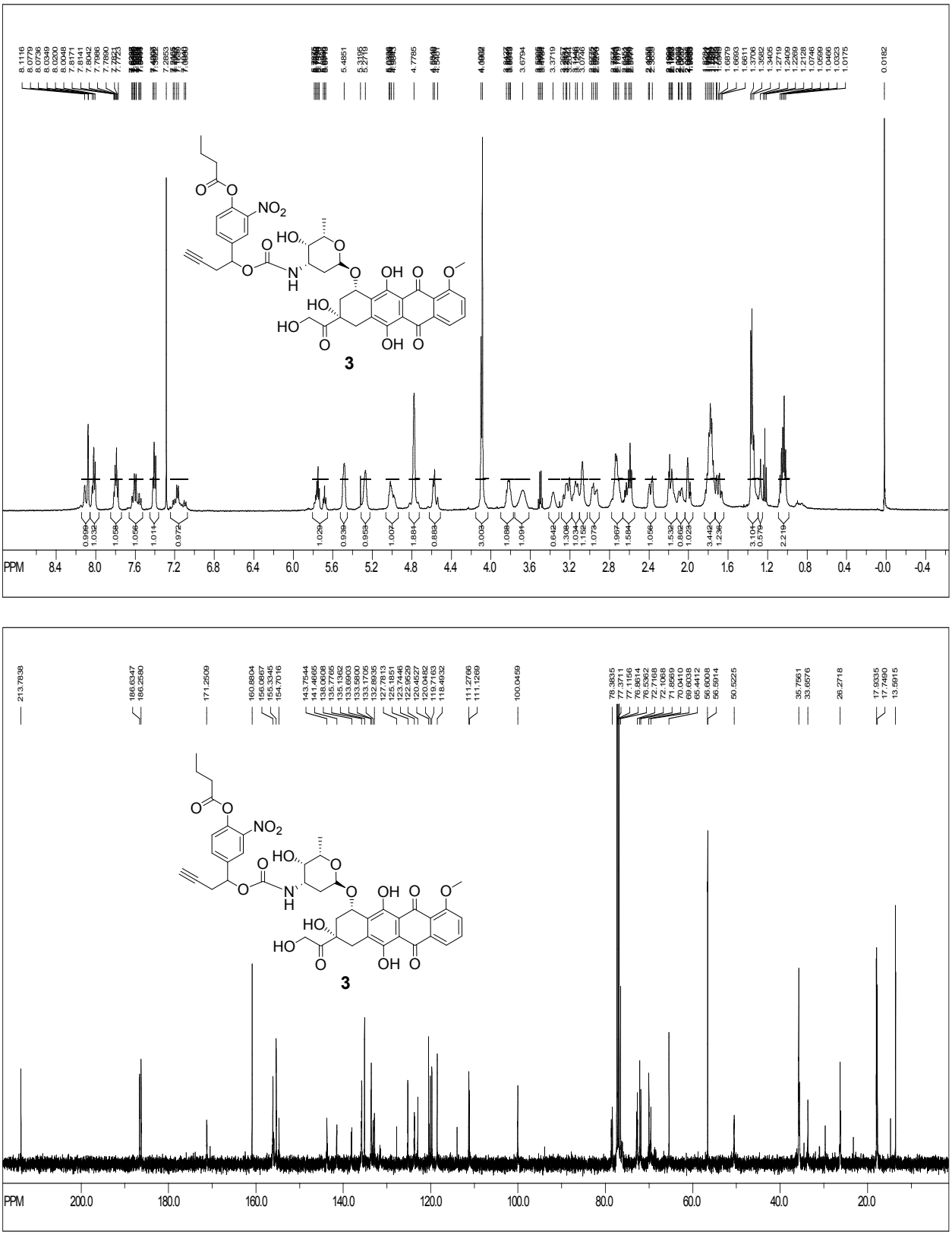


Fig. S14. ^{1}H and ^{13}C NMR spectrum of **3** in $CDCl_3$.

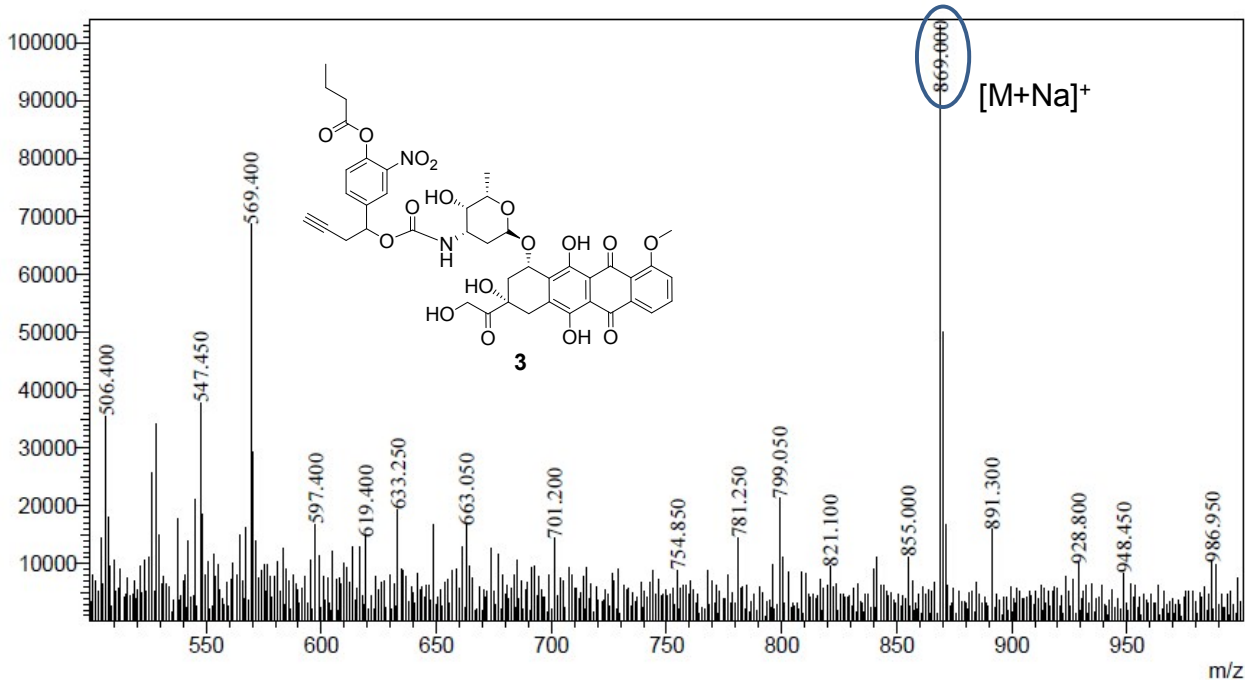


Fig. S15. Mass spectrum of **3** in $CDCl_3$.

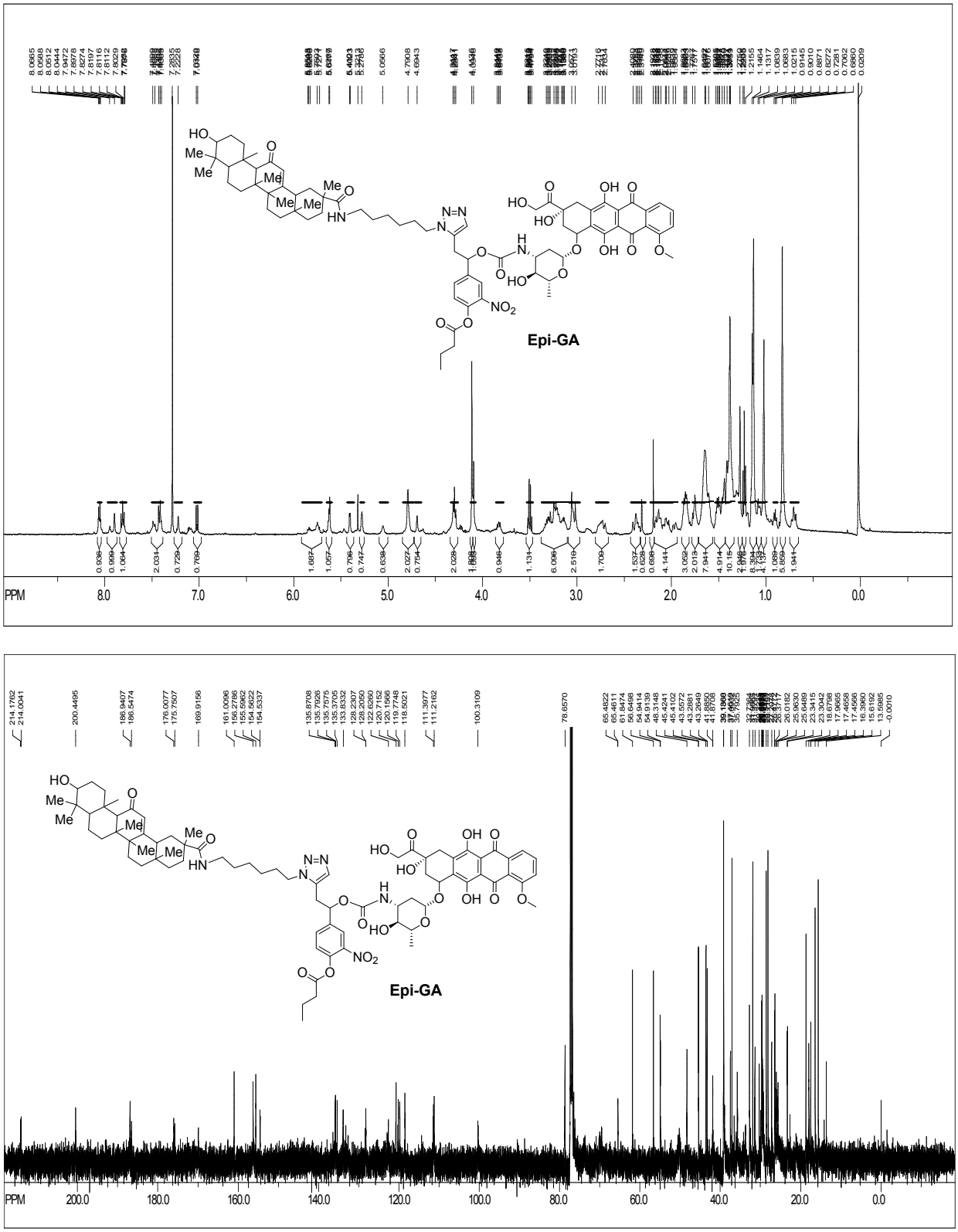


Fig. S16. ^{1}H and ^{13}C NMR spectrum of **Epi-GA** in $CDCl_3$.

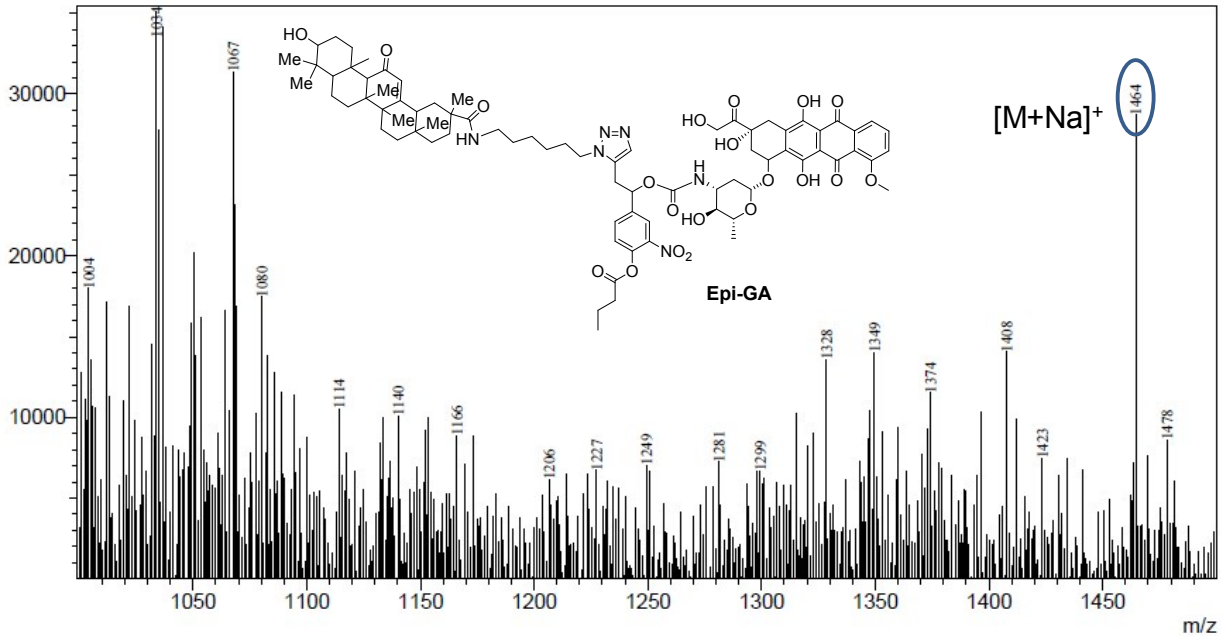


Fig. S17. Mass spectrum of **Epi-GA** in $CDCl_3$.

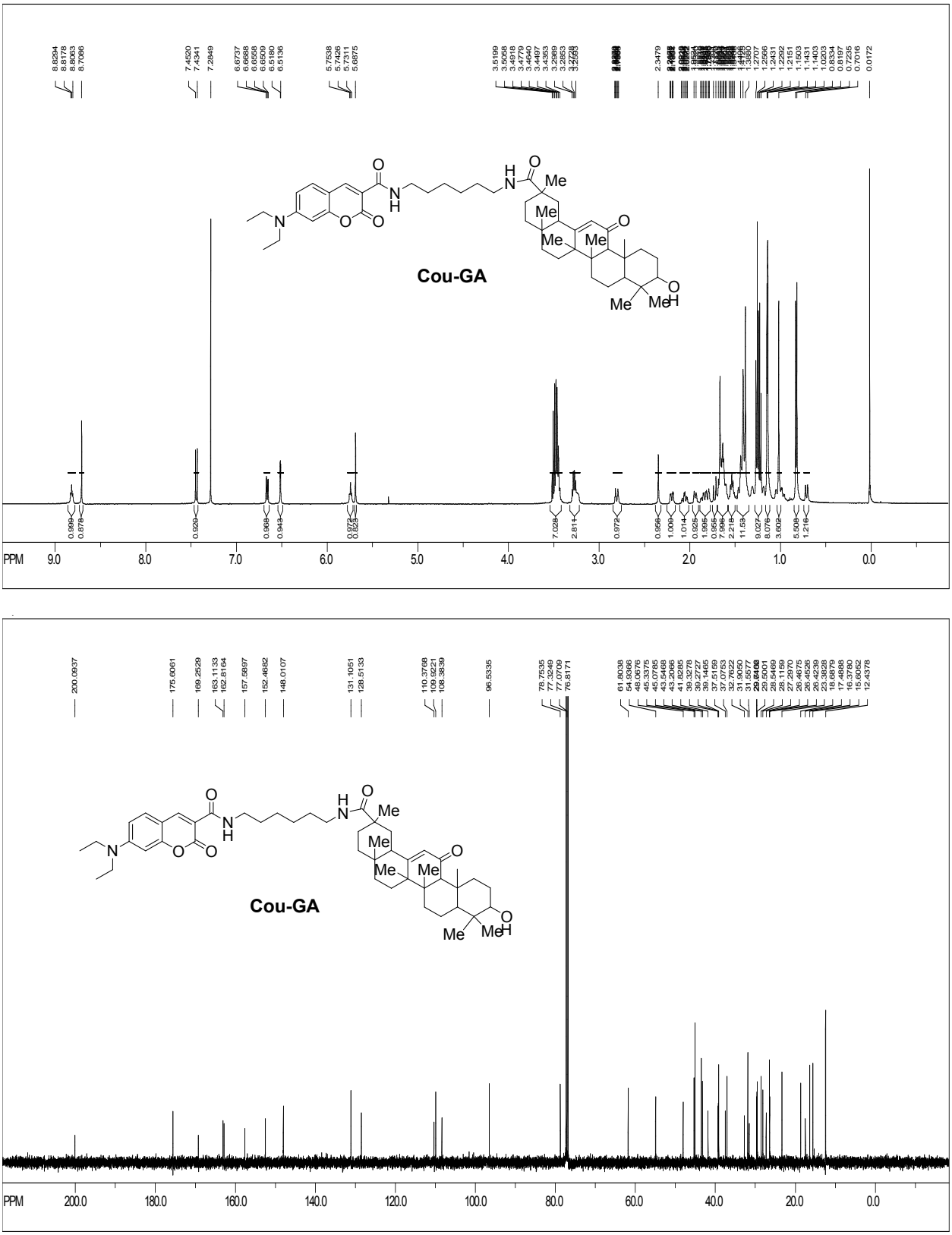


Fig. S18. ^{1}H and ^{13}C NMR spectrum of **Cou-GA** in $CDCl_3$.

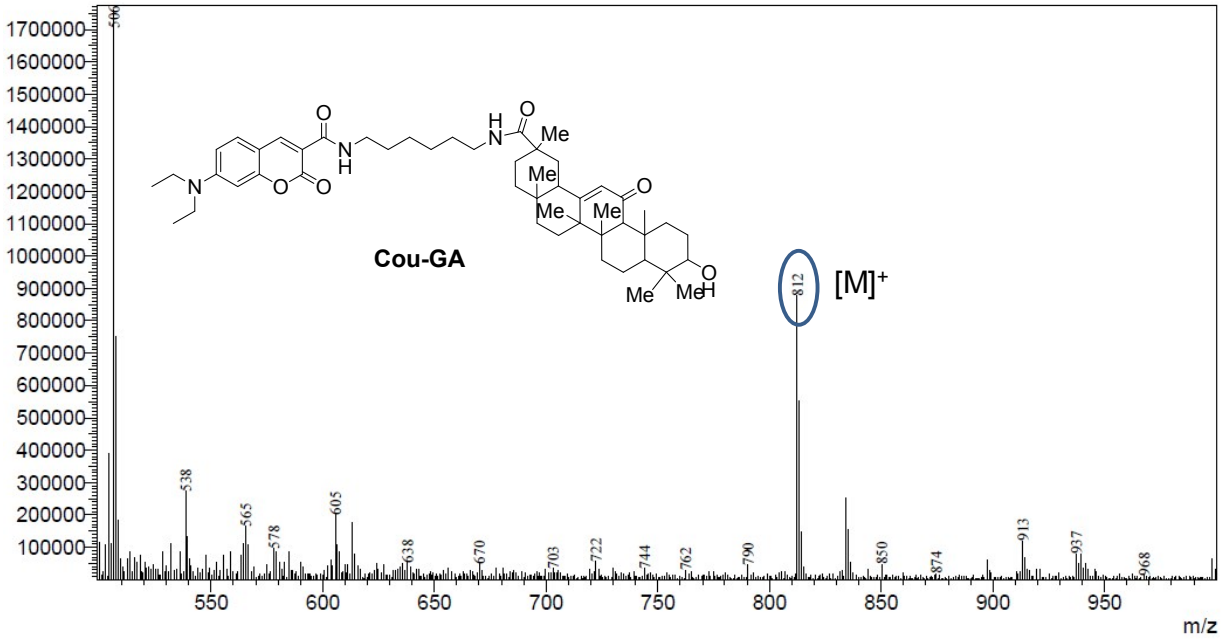


Fig. S19. Mass spectrum of **Cou-GA** in $CDCl_3$.

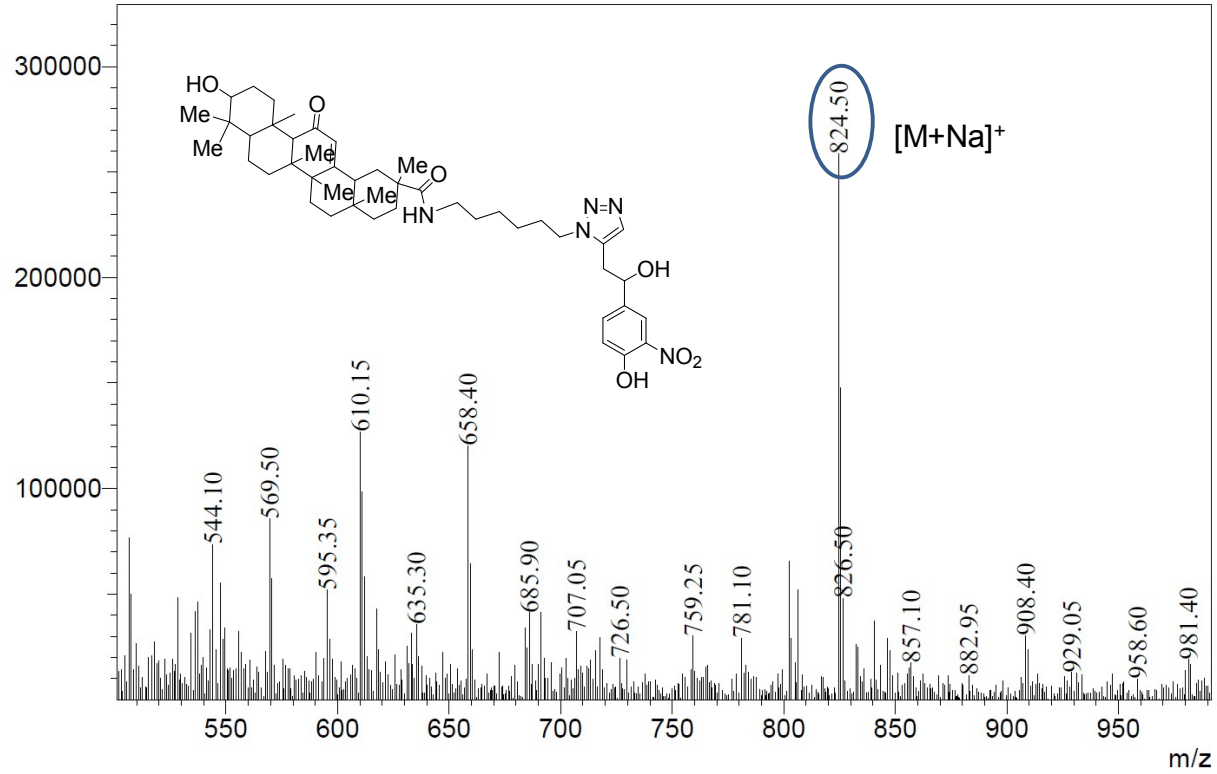


Fig. S20. MS spectrum of **Epi-GA** after incubation with carboxylesterase (5 U/3 mL) for 2 h at 37°C in PBS.

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