Supporting Information

A Bioreductive Prodrug of Cucurbitacin B Significantly Inhibits Tumor Growth in the 4T1 Xenograft Mice Model

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HPLC method. HPLC was performed at room temperature using an XBridge^{TM} C18 3.5 μm (2.1 mm × 150 mm) and a mobile phase gradient from 45% ACN for 10 min, 45% ACN to 70% ACN for another 40 min, a flow rate of 0.2 μL/min, and plotted at 254 nm. This method was used to determine the purity for the tested compounds and also used in cellular uptake experiments.

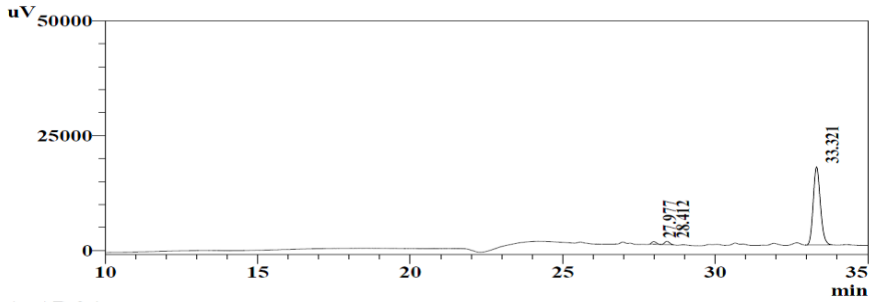
Cell culture. MCF-7 and Vero cells were cultured with DMEM supplemented with 10% (v/v) fetal bovine serum. Cells were incubated at 37 °C with 5% CO_2 in a humidified incubator. 4T1 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were cultured at 37 °C in 5% CO_2.

MTT assay. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 100 μL of medium with FBS then cultured in a CO_2 incubator at 37 °C for 24 h. After which, the cells were incubated for another 48 h with 100 μL of control (DMSO) and sample compounds. After incubation, 20 μL MTT stock solution (5 mg/mL in phosphate-buffered saline, PBS) was added to each well, incubated for 4 h at 37 °C and the solution was then removed. Formazan crystals were solubilized with 150 μL of DMSO and the solution was then measured at 570 nm.

Cellular uptake. MCF-7 cells were plated in small dish (2×10^6 cells/dish) and then treated with prodrug (25 μM) for 24 h. After 24 h incubation, the culture medium was collected and centrifuged at 13000 rpm for 10 min, and the supernatant was analyzed by HPLC. The cells were lysed with 0.5 mL of the lysis solution (Beyotime, Nanjing, China). The cell lysate was also centrifuged at 13000 rpm for 10 min and the supernatant was analyzed by HPLC.

Animal studies. Tumor allografts were generated via subcutaneous implantation of 4T1 cells (1×10^6 cells/0.1 mL) into the left and right flanks of each mouse on day 0. When the tumor reached about 50–100 mm^3, a total of 36 female BALB/c mice were randomly assigned into 6 groups (n = 6). On day 3, mice were treated with control (0.9% NaCl/H_2O), positive control (tamoxifen, 5 mg/kg/d), cucurbitacin B (3 mg/kg/d) and prodrug **1** (3, 5 and 10 mg/kg/d). Tumor size and mouse body weight were monitored by digital calipers every day. Tumor volume was calculated using the following equation: tumor volume (mm^3) = 0.5 × length × $width^2$. Mice were given injections every day intraperitoneally for 2 weeks.

A



1 AD 2 /
2 PDA Multi 1 / 254nm 4nm

PeakTable

Ch1 254nm 4nm

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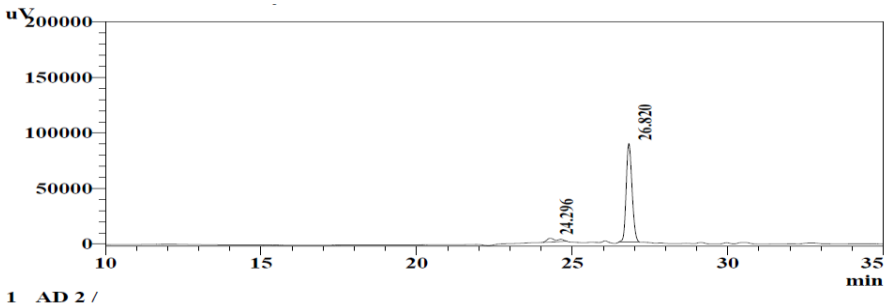
2	28.412	7945	721	0.956	2.943
3	33.321	276580	17017	98.306	95.060
Total		290202	18286	100.000	100.000

B



1 AD 2 /
2 PDA Multi 1 / 254nm 4nm

PeakTable

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1	24.296	78472	3390	3.019	3.684
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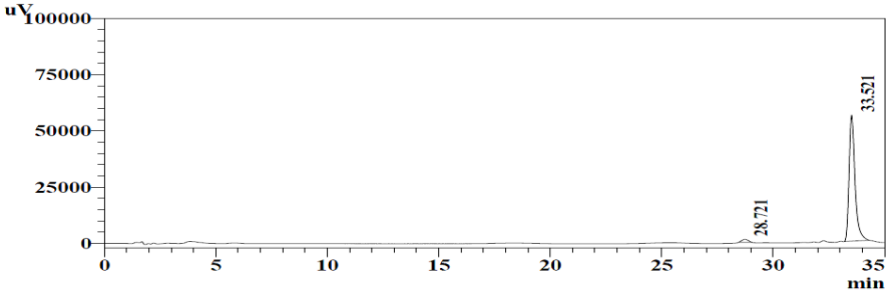
C



1 AD 2 /
2 PDA Multi 1 / 254nm 4nm

PeakTable

Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	28.721	25547	1262	2.416	2.205
2	33.521	1032078	55973	97.584	97.795
Total		1057625	57235	100.000	100.000

Figure S1. HPLC purity chromatograms of prodrugs **1** (A), **2** (B) and **3** (C).

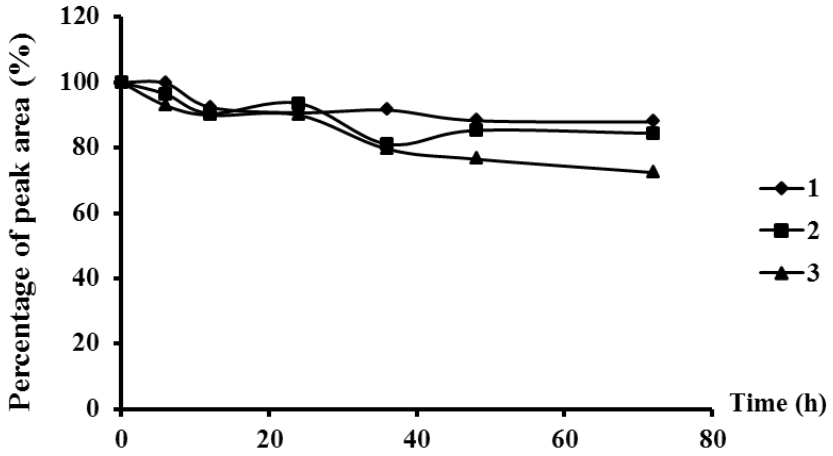


Figure S2. Stability of prodrugs **1**, **2** and **3** in DMEM with 10% FBS.

Table S1. *In Vitro* Cytotoxicity Activity of Lactone 5, Cyclic ureas X and Y

Compound	IC_{50}[a] (μM)	
	MCF-7	Vero[b]
Lactone **5**	>200	>200
Cyclic urea **X**	>200	>200
Cyclic urea **Y**	>200	>200

[a]Each value was reproduced in three experiments. [b]African green monkey kidney.

HPLC assay for drug release studies

Prodrugs **1**, **2** and **3** were dissolved in DMSO as solutions (50 mM). Phosphate buffer solution (PBS) was used to dilute the stock solutions. The incubation was initiated by the addition of compound (50 mM) to PBS (10 mM, pH 7.4) buffer to obtain a final concentration of 50 μM and then added cofactor NADPH (500 μM) and NQO1 (10 U/mL). The solution was incubated at 37°C and conducted in triplicate. Prodrugs **1**, **2** and **3** were taken at appropriate time intervals and directly analyzed by HPLC analysis. Agilent 1200 HPLC and DAD detector with conditions: Agilent C18 column (4.6 × 150 mm, 4.6 mm); Mobile phase: a gradient of 45% ACN for 10 min, 45% ACN to 70% ACN for another 40 min; Flow rate: 0.3 mL/min.

The release of CuB from the prodrugs **1**, **2** and **3** in response to NQO1 and cofactor NADPH was studied using the HPLC assay (Figure S3). As for prodrugs **1**, **2** and **3**, the time dependence of release profiles of the prodrugs were observed. With increasing incubation time, the percentage of CuB and lactone **5** increased steadily and reached a

plateau in 120 min, coming up to ~25%, ~15% and ~48% (for CuB) and ~48%, ~31% and ~72% (for the lactone **5**), respectively (Figure S3C and S3D), while the percentage of the prodrugs **1, 2** and **3** decreased steadily and reached a plateau in 120 min (Figure S3A).

The rate of NQO1 catalysis (slope) and half life were calculated during the first 30 min to determine the susceptibility of the prodrugs **1**, **2** and **3** on target enzyme NQO1. The shorter rate of catalytic activation indicating that the prodrug **3** was highly susceptible to the NQO1 than the prodrugs **2** and **1**, respectively.

All prodrugs underwent the bioreductive activation by NQO1 due to the structural constraint from the trimethyl lock. However, the presence of the linkers in the prodrugs **1** and **2** may slow down the rate of activation due to one more step of elimination of the linkers than that required in the prodrug **3**. The enzyme catalyzed bioreductive activation is the first step of reaction which is rate determining step followed by spontaneous cyclization reaction. The N-methyl groups in the prodrug **1** might lead to steric hindrance for enzyme catalyzed bioreductive activation as well as elimination of the linker by cyclization reaction. This might be the reasons why the prodrug **1** exhibited slower rate of activation than the prodrug **2**.

However, this NQO1 susceptibility was tested in the *in vitro*. The stability and the cellular uptake ability of prodrugs also play pivotal role on the anticancer effect of the prodrugs. After cellular uptake, the prodrugs might encounter metabolic instability and degradation in the cells. The remaining prodrugs will undergo bioreductively activated by NQO1 and ultimately lead to the anticancer action.

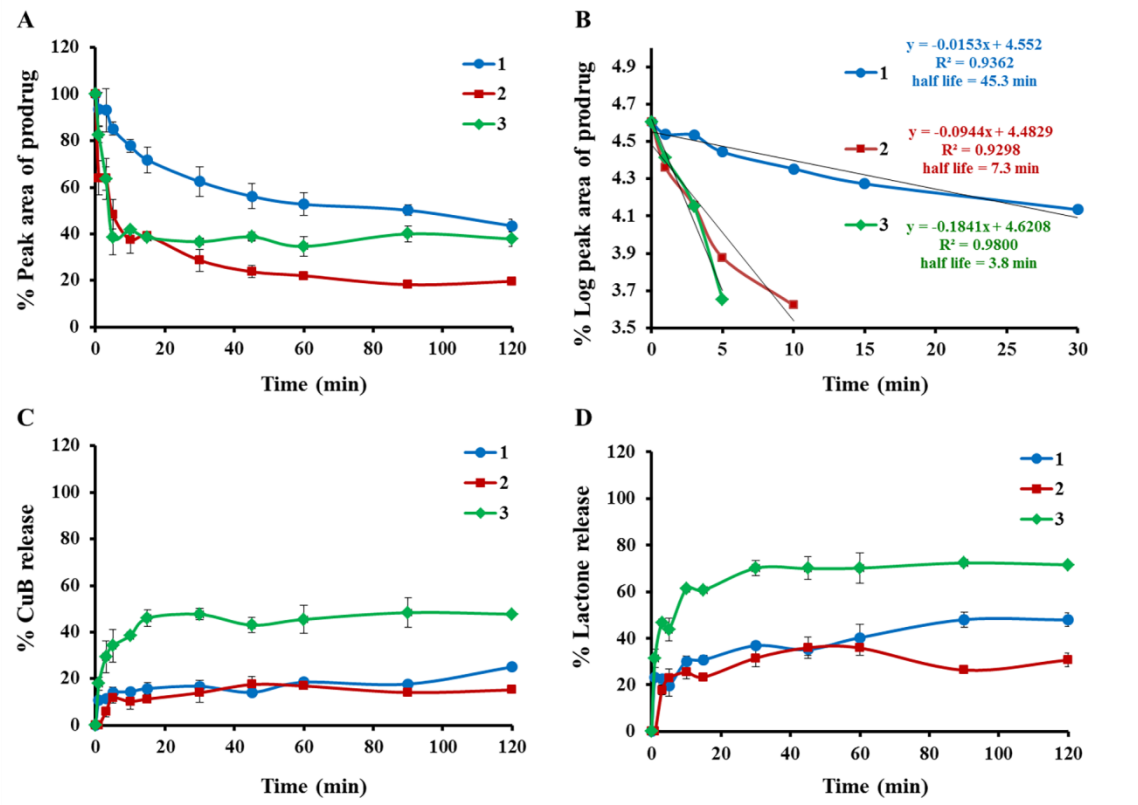
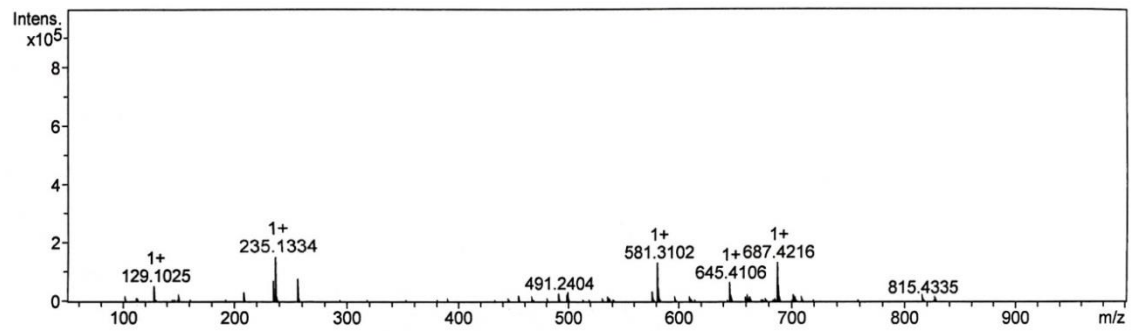


Figure S3. Drug release studies, as a function of time in the presence of NQO1 (10 U/mL) and NADPH (500 μM) in PBS, (n = 3). (A) Percentage of prodrugs **1**, **2** and **3** remaining (as determined by HPLC) at various time intervals. (B) Rate (slope) and half-life values of prodrugs **1**, **2** and **3** in response to NQO1 and cofactor NADPH. (C) Percentage of CuB and (D) percentage of lactone **5** (as determined by HPLC) released from **1**, **2** and **3** by NQO1 at various time intervals.

Stability of prodrugs under the GSH activation

Each of the prodrugs **1**, **2** and **3** in DMSO (0.002 mmol) was treated with glutathione (GSH) (0.2 mmol) in PBS solution (pH 7.4, 10 mM) at 37 ^{o}C for 1 h and possible progress of the reaction was analyzed by thin layer chromatography (TLC) eluting with *n*-hexane:ethyl acetate solvent system (1:4 v/v for prodrugs **1** and **2** and 1:1 v/v for prodrug **3**). HPLC monitoring using the same conditions employed in the drug release studies has also been conducted. Only the starting prodrugs were detected in both TLC and HPLC monitoring (data not shown). The results indicated that the prodrugs **1**, **2** and **3** are stable under GSH reducing conditions.

A



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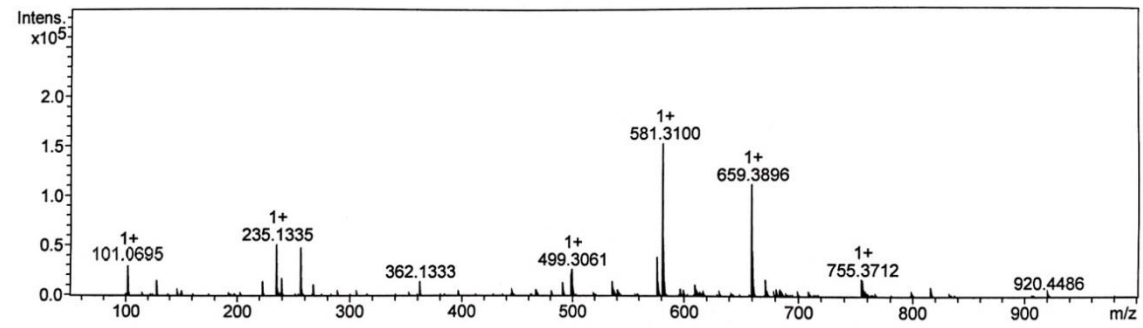

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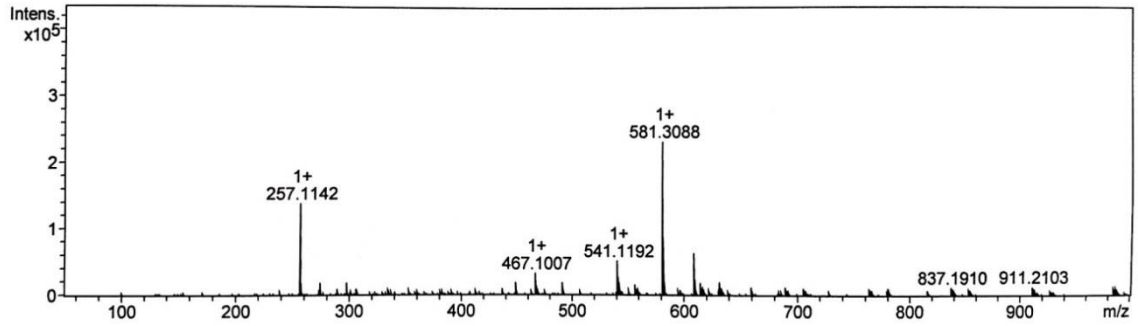


Figure S4. The original HRMS analysis (positive ion mode, extended HRMS *m/z* range to 1000) of prodrugs **1** (A), **2** (B) and **3** (C) in cell lysate after 24 h incubation.

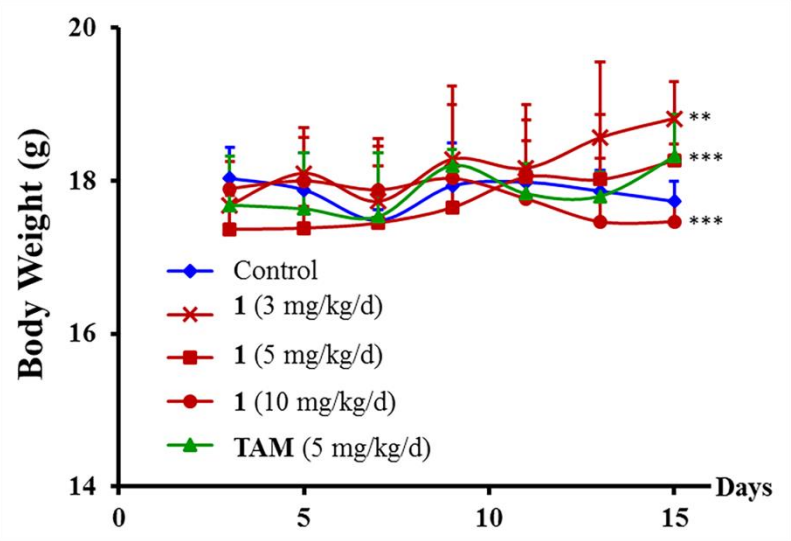


Figure S5. *In vivo* antitumor activity of prodrug **1** on mice body weight. Mice body weights were plotted against days post-inoculation.

Table S2. *In Vivo* Effects of Prodrug 1 and CuB

Compound	Tumor growth inhibition[a] (TGI, %)	Relative change of body weight[b] (%)
Control	–	–1.48 ± 0.58
CuB	–	–
1 (3 mg/kg/d)	48.66 ± 6.43	6.46 ± 0.85
1 (5 mg/kg/d)	53.79 ± 4.64	5.27 ± 0.74
1 (10 mg/kg/d)	70.79 ± 4.28	–2.49 ± 0.88
TAM (5 mg/kg/d)	54.96 ± 4.75	4.99 ± 1.15

[a]The difference between the tumor volume of a test group and control group.

[b]Body weight change compared with that of the day treatment was started.

General. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 FT-NMR spectrometer, operating at 400 (^{1}H) and 100 (^{13}C) MHz. For the spectra taken in $CDCl_3$, the residual nondeuterated solvent signals at δ 7.24 and the solvent signals at δ 77.0 were used as references for ^{1}H and ^{13}C NMR spectra, respectively. Low resolution ESI mass spectra were recorded on a Shimadzu LCMS-2020 instrument. High resolution mass spectra (ESI-TOFMS) were recorded on a Bruker micrOTOF-II mass spectrometer or an Agilent 6540 UHD Accurate-mass Q-TOF LC/MS. Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. Column chromatography was carried out using Merck silica gel 60 (<0.063 mm). For TLC, Merck precoated silica

gel 60 F254 plates were used. Spots on TLC were detected under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating. Human NQO1 (D1315; Sigma-Aldrich), NADPH (Sigma-Aldrich), glutathione (GSH; Sigma-Aldrich) were used. Tamoxifen (TAM) was purchased from Energy Chemical (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, fetal bovine serum (FBS), and antibiotic/antimycotic were purchased from HyClone (Carlsbad, CA). Human breast cancer cell lines MCF-7 was generously provided by the Department of Biological Sciences of Nanjing University. Murine breast cancer cell line 4T1 was purchased from the cell bank of type culture collection of the Chinese Academy of Sciences (Shanghai, China). Female BALB/c mice (6–8 weeks, 17−18 g) were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). The animal protocol used was carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) and the related ethical regulations of Nanjing University. The license number is SCXK 2015-0001.

Isolation of cucurbitacin B

The dried fruits of *T. cucumerina* used in this study were collected from Takli district, Nakhonsawan province, Thailand in 2009 and the plant species was identified by Nopporn Damrongsiri, Department of Biology, Faculty of Science, Ramkhamhaeng University. The voucher specimen (Apichart Suksamrarn No. 058) is deposited at the Faculty of Science, Ramkhamhaeng University. The peels and seeds were removed and the fruit fibers were chilled in liquid N_2, milled to small pieces and extracted successively with *n*-hexane, EtOAc and MeOH. The extracts were evaporated to dryness under reduced pressure at temperature 40–45 °C. The hexane extract was fractionated and isolated by column chromatography to afford CuB.

Cucurbitacin B (CuB). White solid, mp 177–179 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.04 (d, J = 15.6 Hz, 1H), 6.45 (d, J = 15.6 Hz, 1H), 5.77 (m, 1H), 4.39 (dd, J = 13.0, 5.9 Hz, 1H), 4.35 (t, J = 8.2 Hz, 1H), 4.23 (s, 1H), 3.58 (s, 1H), 3.21 (d, J = 14.5 Hz, 1H), 2.71 (d, J = 13.5 Hz, 1H), 2.66 (d, J = 14.5 Hz, 1H), 2.47 (d, J = 6.9 Hz, 1H), 2.39 (dd, J = 19.7, 7.6 Hz, 1H), 2.29 (ddd, J = 13.5, 5.9, 3.5 Hz, 1H), 1.98 (s, 3H), 1.97 (m, 1H), 1.95 (m, 1H), 1.85 (dd, J = 12.8, 9.2 Hz, 1H), 1.52 (s, 3H), 1.54 (s, 3H), 1.41 (s, 3H), 1.40 (m, 1H), 1.32 (s, 3H), 1.33 (s, 3H), 1.25 (s, 3H), 1.21 (d, J = 13.5 Hz, 1H), 1.06 (s, 3H), δ 0.96 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 213.0, 212.0, 202.4, 170.2, 151.9, 140.4, 120.4, 120.3, 79.3,

78.2, 71.6, 71.2, 58.2, 50.7, 50.2, 48.6, 48.4, 48.1, 45.3, 42.4, 35.9, 33.7, 29.3, 26.4, 25.9, 23.9, 23.8, 21.9, 21.2, 20.0, 19.8, 18.8; HRMS (ESI, TOF): *m/z* 581.3101 $[M + Na]^+$, calcd for $C_{32}H_{46}O_8Na$: 581.3085.

Synthesis of compounds 5, 6, 8a, 8b, 9, 10a, 10b, 11a and 11b

*Compound **5***. Trimethylhydroquinone (20 mg, 0.13 mmol) was added into dry methanesulfonic acid (2 mL, excess) followed by the addition of 3,3-dimethylacrylic acid (13 mg, 0.13 mmol). The reaction mixture was stirred at 70 °C for 30 min and was then cooled to room temperature. Water was added and the crude mixture was extracted with DCM (3 × 50 mL). The combined organic phase was washed with H_2O, dried over anhydrous Na_2SO_4 and the solvent was removed under vacuum. The residue was purified by column chromatography under isocratic condition using DCM to give compound **5** as a white solid (24.5 mg, 80%), mp 183–184 ^oC. ^1H NMR (400 MHz, $CDCl_3$) δ 2.52 (s, 2H), 2.34 (s, 3H), 2.19 (s, 3H), 2.16 (s, 3H), 1.43 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.8, 148.8, 143.5, 128.2, 123.4, 121.8, 118.9, 46.1, 35.5, 27.7, 14.4, 12.5, 12.3; HRMS (ESI, TOF): *m/z* 257.1119 $[M + Na]^+$, calcd for $C_{14}H_{18}ONa$: 257.1148.

*Compound **6***. To a solution of compound **5** (20 mg, 0.09 mmol) in acetone–H_2O (5:1, 2 mL) was added *N*-bromosuccinimide (NBS) (20 mg, 0.11 mmol). The reaction mixture was stirred at ambient temperature for 45 min; 10% aq. $NaHCO_3$ was added and the mixture was extracted with EtOAc (3 × 50 mL). The combined organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and the solvent was evaporated in vacuo. The residue was purified by column chromatography under isocratic condition using DCM–MeOH (5 : 0.1) to yield compound **6** as a yellow solid (20.2 mg, 90%), mp 99–100 ^oC. ^1H NMR (400 MHz, $CDCl_3$) δ 9.06 (s, 1H), 2.97 (s, 2H) 2.10 (s, 3H), 1.91 (s, 3H), 1.89 (s, 3H), 1.39 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.8, 187.4, 177.7, 152.1, 143.0, 138.9, 138.3, 47.2, 37.9, 28.7, 14.2, 12.4, 12.0; HRMS (ESI, TOF): *m/z* 273.1081 $[M + Na]^+$, calcd for $C_{14}H_{18}O_4Na$: 273.1097.

*Compound **8a***. To the stirred solution of compound **7a** (20 mg, 0.2 mmol) in DCM (3 mL) was slowly added di-*tert*-butyldicarbonate (Boc_2O) (43 mg, 0.2 mmol) in DCM (2 mL) and stirring was continued at 0 °C for 30 min. Water was added and the reaction mixture was extracted with DCM (2 × 50 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography using DCM–MeOH (95 : 5) as eluting solvent to

afford compound **8a** (27.8 mg, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.24 (m, 2H), 2.83 (t, J = 6.6 Hz, 2H), 2.77 (s, 3H), 2.59 (s, 3H), 1.93 (m, 2H), 1.36 (s, 9H); HRMS (ESI, TOF): *m/z* 203.1710 $[M + H]^+$, calcd for $C_{10}H_{23}N_2O_2$: 203.1754.

*Compound **8b***. Compound **7b** (20 mg, 0.27 mmol) was subjected to the reaction in similar manner to that of compound **7a** to give compound **8b** (22.3 mg, 64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (s, 1H), 2.91 (m, 2H), 2.47 (t, J = 6.3 Hz, 2H), 1.35 (m, 2H), 1.16 (s, 9H); HRMS (ESI, TOF): *m/z* 175.1420 $[M + H]^+$, calcd for $C_8H_{19}N_2O_2$: 175.1441.

*Compound **9***. To a solution of CuB (20 mg, 0.04 mmol) in dry DCM (2 mL) was added 4-nitrophenyl chloroformate (8.1 mg, 0.04 mmol) and triethylamine (TEA) (3 drops) at 0 °C. The reaction mixture was stirred at 0 °C for 45 min. The reaction was worked up by H_2O and extracted with EtOAc (3 × 40 mL). The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4 and the solvent was evaporated to dryness. The crude product was purified by column chromatography using hexane–EtOAc (1 : 1) to yield compound **9** as a white solid (22.6 mg, 78%), mp 132–134 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (d, J = 9.0 Hz, 2H), 7.40 (d, J = 9.0 Hz, 2H), 7.04 (d, J = 15.6 Hz, 1H), 6.45 (d, J = 15.6 Hz, 1H), 5.83 (m, 1H), 5.39 (dd, J = 13.5, 5.4 Hz, 1H), 4.34 (t, J = 7.8 Hz, 1H), 3.22 (d, J = 14.6 Hz, 1H), 2.82 (d, J = 12.3 Hz, 1H), 2.69 (d, J = 14.6 Hz, 1H), 2.48 (d, J = 7.0 Hz, 1H), 2.41 (dd, J = 19.3, 7.5 Hz, 1H), 2.32 (m, 1H), 1.99 (s, 3H), 1.94 (m, 2H), 1.87 (dd, J = 12.9, 9.4 Hz, 1H), 1.56 (s, 3H), 1.52 (s, 3H), 1.45 (m, 2H), 1.42 (s, 3H), 1.34 (s, 3H), 1.32 (s, 3H), 1.31 (s, 3H), 1.09 (s, 3H), 0.97 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.2, 204.6, 202.3, 170.2, 155.5, 152.0, 151.7, 145.5, 139.0, 125.2, 121.8, 121.1, 120.3, 79.3, 78.2, 77.7, 71.2, 58.2, 51.2, 50.6, 48.7, 48.3, 48.0, 45.2, 42.3, 34.1, 31.8, 28.6, 26.4, 25.9, 23.9, 23.8, 21.9, 21.1, 20.0, 19.9, 18.8; HRMS (ESI, TOF): *m/z* 746.3167 $[M + Na]^+$, calcd for $C_{39}H_{49}NO_{12}Na$: 746.3147.

*Compound **10a***. To a solution of compound **9** (20 mg, 0.03 mmol) in dry DCM (2 mL) was added compound **8a** (16 mg, 0.08 mmol) and TEA (3 drops). The reaction mixture was stirred at ambient temperature for 45 min. H_2O was added and the mixture was extracted with EtOAc (3 × 30 mL). The combined organic phase was washed with H_2O and dried over anhydrous Na_2SO_4, the solvent was evaporated and the residue was chromatographed using EtOAc–hexane (2 : 1) to give compound **10a** as a white solid (22.6 mg, 96%), mp 88–90 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.04 (d, J = 15.6 Hz, 1H), 6.45 (d, J = 15.6 Hz, 1H), 5.76 (m, 1H), 5.39 (m, 1H), 4.33 (m, 1H), 4.27 (s, 1H), 3.21 (d, J = 14.5 Hz, 1H), 3.20-3.29 (m, 4H), 2.90 (d, J = 10.6 Hz,1H), 2.90 (m, 2H), 2.82 (s, 6H), 2.65 (d,

J = 14.5 Hz, 1H), 2.48 (d, *J* = 6.9 Hz, 1H), 2.40 (dd, *J* = 12.2, 7.0 Hz, 1H), 2.10 (d, *J* = 11.8 Hz, 1H), 1.99 (s, 3H), 1.96 (m, 1H), 1.94 (m, 1H), 1.84 (m, 1H), 1.55 (s, 3H), 1.52 (s, 3H), 1.42 (m, 2H), 1.42 (s, 12H), 1.33 (s, 3H), 1.30 (s, 3H), 1.26 (s, 3H), 1.06 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.4, 207.0, 202.4, 170.2, 155.7, 155.4, 152.0, 140.0, 120.3, 79.3, 78.2, 74.1, 71.3, 58.1, 51.2, 50.7, 48.7, 48.6, 48.1, 47.0, 46.7, 45.3, 42.4, 34.4, 34.1, 32.3, 28.9, 28.4, 26.4, 26.0, 23.9, 23.8, 21.9, 21.3, 19.9, 19.8, 18.8; HRMS (ESI, TOF): *m/z* 809.4564 $[M + Na]^+$, calcd for $C_{43}H_{66}N_2O_{11}Na$: 809.4564.

*Compound **10b***. Compound **9** was prepared in similar manner described for the preparation of **10a** from **9**, but using compound **8b** instead of **8a**, to yield compound **10b** as a white solid (16.9 mg, 74%), mp 115–117 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.03 (d, *J* = 15.6 Hz, 1H), 6.45 (d, *J* = 15.6 Hz, 1H), 5.76 (m, 1H), 5.39 (dd, *J* = 13.4, 5.2 Hz, 1H), 4.89 (s, 1H), 4.33 (t, *J* = 7.6 Hz, 1H), 3.19 (d, *J* = 14.4 Hz, 1H), 3.15-3.23 (m, 6H), 2.81 (d, *J* = 11.7 Hz, 1H), 2.65 (d, *J* = 14.4 Hz, 1H), 2.48 (d, *J* = 6.9 Hz, 1H), 2.36 (dd, *J* = 12.1, 7.0 Hz, 1H), 2.09 (d, *J* = 11.8 Hz, 1H), 1.99 (s, 3H), 1.94 (m, 2H), 1.87 (dd, *J* = 12.4, 9.9 Hz, 1H), 1.54 (s, 3H), 1.52 (s, 3H), 1.42 (s, 9H), 1.41 (m, 2H), 1.41 (s, 3H), 1.33 (s, 3H), 1.29 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.3, 207.1, 202.4, 170.2, 156.3, 155.8, 151.9, 139.9, 120.4, 120.3, 79.3, 78.2, 73.6, 71.2, 58.1, 51.2, 50.6, 48.6, 48.5, 48.0, 45.3, 42.4, 37.8, 37.1, 34.3, 32.2, 28.9, 28.3, 26.4, 26.0, 23.9, 23.8, 21.9, 21.3, 19.8, 18.8; HRMS (ESI, TOF): *m/z* 781.4258 $[M + Na]^+$, calcd for $C_{41}H_{62}N_2O_{11}Na$: 781.4256.

*Compound **11a** and **11b***. A portion of 10% TFA (5 drops) was added to a solution of individual compounds **10a** and **10b** in DCM (2 mL), in parallel, and the reaction mixture was stirred at ambient temperature for 2 h. The reaction mixture was evaporated under reduced pressure to give compounds **11a** and **11b** (**11a**, 19.1 mg, 93%; **11b**, 18.9 mg, 96%).

*Compound **11a***. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (s, 1H), 7.03 (d, *J* = 15.6 Hz, 1H), 6.45 (d, *J* = 15.6 Hz, 1H), 5.78 (m, 1H), 5.33 (dd, *J* = 13.8, 5.8 Hz, 1H), 4.33 (t, *J* = 7.8 Hz, 1H), 3.24 (d, *J* = 14.6 Hz, 1H), 3.08 (m, 6H), 2.93 (m, 1H), 2.93 (s, 3H), 2.62 (d, *J* = 14.6 Hz, 1H), 2.60 (s, 3H), 2.49 (d, *J* = 7.0 Hz, 1H), 2.39 (m, 1H), 2.37 (m, 1H), 2.15 (m, 1H), 1.98 (s, 3H), 1.95 (m, 2H), 1.84 (dd, *J* = 13.8, 9.2 Hz, 1H), 1.54 (s, 3H), 1.51 (s, 3H), 1.48 (m, 2H), 1.41 (s, 3H), 1.33 (s, 3H), 1.27 (s, 3H), 1.24 (s, 3H), 1.07 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.5, 207.8, 202.5, 170.3, 157.5, 152.0, 139.3, 121.0, 120.3, 79.3, 78.2, 75.6, 71.2, 58.1, 51.2, 50.6, 48.6, 48.5, 48.0, 45.5, 45.3, 44.5, 42.3, 34.2, 33.8, 33.1, 31.7, 28.9, 26.4, 26.0, 23.9, 23.7, 21.8, 21.3, 19.9, 18.7; HRMS (ESI, TOF): *m/z* 687.4217 $[M + H]^+$, calcd for $C_{38}H_{59}N_2O_9$: 687.4215.

*Compound **11b***. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (s, 2H), 7.03 (d, J = 15.7 Hz, 1H), 6.48 (d, J = 15.7 Hz, 1H), 6.11 (s, 1H), 5.76 (m, 1H), 5.41 (m, 1H), 4.33 (m, 1H), 3.28 (d, J = 14.8 Hz, 1H), 3.08 (m, 6H), 2.88 (d, J = 9.9 Hz, 1H), 2.63 (d, J = 14.8 Hz, 1H), 2.50 (d, J = 6.3 Hz, 1H), 2.38 (m, 1H), 2.08 (m, 1H), 1.98 (s, 3H), 1.94 (m, 2H), 1.85 (m, 1H), 1.54 (s, 3H), 1.51 (s, 3H), 1.41 (m, 2H), 1.41 (s, 3H), 1.33 (s, 3H), 1.29 (s, 3H), 1.23 (s, 3H), 1.03 (s, 3H), 0.94 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 213.0, 208.3, 202.7, 170.4, 156.7, 152.0, 139.7, 120.6, 120.3, 79.4, 78.3, 74.3, 71.2, 58.2, 51.3, 50.7, 48.7, 48.5, 48.0, 45.3, 42.4, 37.3, 37.1, 34.0, 32.3, 28.8, 26.4, 25.9, 23.9, 24.0, 21.9, 21.2, 19.9, 18.8; HRMS (ESI, TOF): *m/z* 659.3900 $[M + H]^+$, calcd for $C_{36}H_{55}N_2O_9$: 659.3902.

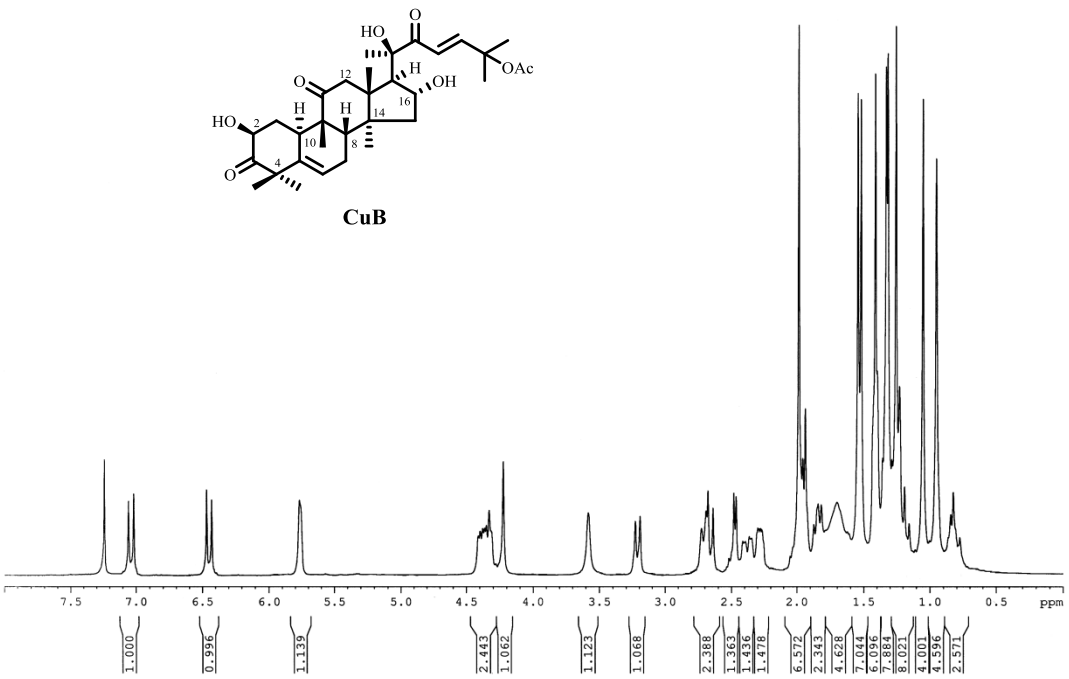
Synthesis of prodrugs 1, 2 and 3

*Compound **1***. To a solution of compound **11a** (20 mg, 0.03 mmol), *N,N*-dimethyl-4-aminopyridine (DMAP) (1.8 mg, 0.02 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (6 mg, 0.039 mmol) in DCM (2 mL) was added a solution of compound **6** (7.5 mg, 0.03 mmol) in DCM (2 mL). After stirring at ambient temperature for 3 h, the reaction was worked up by H_2O and extracted with EtOAc (3 × 40 mL). The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4 and the solvent was evaporated to dryness. The crude product was purified by column chromatography using EtOAc–hexane (3 : 1) to yield compound **1** as a yellow solid (19.8 mg, 72%), mp 100–102 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.03 (d, J = 15.6 Hz, 1H), 6.44 (d, J = 15.6 Hz, 1H), 5.76 (m, 1H), 5.40 (m, 1H), 4.32 (t, J = 7.8 Hz, 1H), 3.20–3.30 (m, 4H), 3.21 (d, J = 14.8 Hz, 1H), 2.95 (m, 2H), 2.95 (s, 5H), 2.87 (d, J = 7.4 Hz, 1H), 2.79 (s, 3H), 2.65 (d, J = 14.5 Hz, 1H), 2.47 (d, J = 7.0 Hz, 1H), 2.38 (dd, J = 19.2, 7.8 Hz, 1H), 2.09 (m, 1H), 2.09 (s, 3H), 1.98 (s, 3H), 1.94 (m, 2H), 1.90 (s, 3H), 1.87 (s, 3H), 1.81 (m, 1H), 1.54 (s, 3H), 1.52 (s, 3H), 1.42 (s, 3H), 1.39 (m, 2H), 1.39 (s, 6H), 1.33 (s, 3H), 1.29 (s, 3H), 1.25 (s, 3H), 1.06 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.4, 206.9, 202.4, 191.2, 187.7, 171.8, 170.2, 155.2, 154.8, 152.0, 143.3, 139.9, 138.0, 135.9, 120.4, 120.2, 79.3, 78.1, 74.1, 71.2, 58.1, 51.2, 50.6, 48.6, 48.5, 48.0, 47.7, 47.4, 46.6, 45.3, 42.3, 37.4, 34.8, 34.3, 33.2, 32.3, 28.8, 28.6, 26.4, 25.9, 23.9, 23.8, 21.9, 21.3, 19.9, 19.8, 18.8, 14.1, 12.6, 12.0; HRMS (ESI, TOF): *m/z* 941.5127 $[M + Na]^+$, calcd for $C_{52}H_{74}N_2O_{12}Na$: 941.5134.

*Compound **2***. Compound **11b** was reacted with compound **6** in similar manner to that of compound **1** to give compound **2** as a yellow solid (19.8 mg, 74%), mp 120–122 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.04 (d, J = 15.6 Hz, 1H), 6.45 (d, J = 15.6 Hz, 1H), 6.11 (s, 1H), 5.77 (m, 1H), 5.35 (dd, J = 13.4, 5.2 Hz, 1H), 5.20 (s, 1H), 4.33 (t, J = 7.8 Hz, 1H),

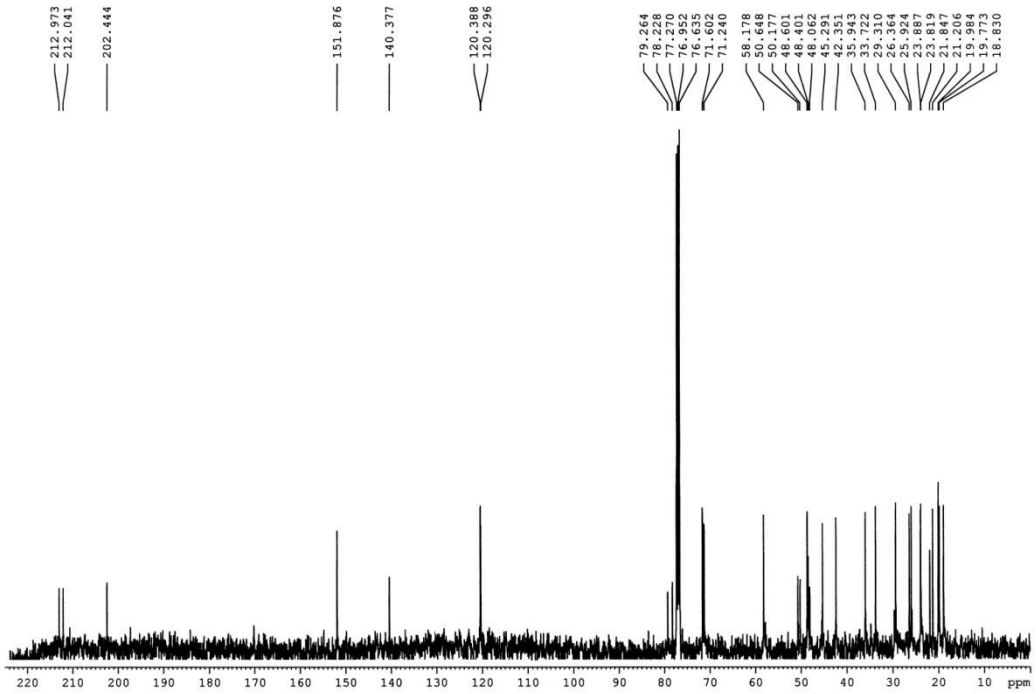
3.22 (d, *J* = 14.5 Hz, 1H), 3.09–3.21 (m, 4H), 2.80 (m, 1H), 2.80 (s, 2H), 2.74 (m, 2H), 2.66 (d, *J* = 14.5 Hz, 1H), 2.48 (d, *J* = 7.0 Hz, 1H), 2.39 (dd, *J* = 18.7, 6.8 Hz, 1H), 2.09 (s, 3H), 2.09 (m, 1H), 1.99 (s, 3H), 1.99 (m, 2H), 1.95 (s, 3H), 1.92 (s, 3H), 1.85 (dd, *J* = 13.2, 9.6 Hz, 1H), 1.55 (s, 3H), 1.52 (s, 3H), 1.42 (m, 2H), 1.42 (s, 3H), 1.39 (s, 6H), 1.34 (s, 3H), 1.31 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H), 0.96 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.4, 207.3, 202.4, 191.2, 187.6, 172.2, 170.2, 156.0, 153.7, 152.0, 143.7, 139.7, 137.8, 137.3, 120.5, 120.3, 79.3, 78.2, 73.7, 71.3, 58.1, 53.4, 51.2, 50.7, 49.1, 48.7, 48.5, 48.1, 45.3, 42.3, 38.1, 37.8, 35.8, 34.3, 32.2, 29.9, 28.9, 28.7, 26.4, 25.9, 23.9, 23.8, 21.9, 21.3, 19.9, 19.8, 18.8, 14.1, 12.7, 12.1; HRMS (ESI, TOF): *m/z* 913.4822 $[M+Na]^+$, calcd for $C_{50}H_{70}N_2O_{12}Na$: 913.4821.

*Compound **3***. CuB was reacted with compound **6** in similar manner to that of compound **1** to give compound **3** as a yellow solid (22.1 mg, 70%), mp 110–112 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.03 (d, *J* = 15.6 Hz, 1H), 6.43 (d, *J* = 15.6 Hz, 1H), 5.74 (d, *J* = 4.0 Hz, 1H), 5.32 (dd, *J* = 13.5, 5.2 Hz, 1H), 4.33 (t, *J* = 7.8 Hz, 1H), 3.18 (d, *J* = 14.4 Hz, 1H), 3.09 (d, *J* = 16.3, 1H), 3.01 (d, *J* = 16.3, 1H), 2.74 (d, *J* = 12.7, 1H), 2.64 (d, *J* = 14.4 Hz, 1H), 2.46 (d, *J* = 6.9 Hz, 1H), 2.37 (dd, *J* = 19.2, 7.6 Hz, 1H), 2.13 (s, 3H), 2.02 (m, 1H), 1.98 (s, 3H), 1.96 (s, 3H), 1.94 (s, 3H), 1.94 (s, 2H), 1.84 (dd, *J* = 13.0, 9.0 Hz , 1H), 1.54 (s, 3H), 1.52 (s, 3H), 1.46 (s, 6H), 1.41 (s, 3H), 1.39 (m, 2H), 1.30 (s, 3H), 1.24 (s, 3H), 1.23 (s, 3H), 1.03 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.3, 205.1, 202.4, 190.6, 187.6, 171.5, 170.2, 152.3, 152.0, 143.1, 139.6, 138.9, 138.3, 120.5, 120.3, 79.3, 78.2, 73.2, 71.3, 58.1, 51.2, 50.6, 48.7, 48.4, 48.0, 47.4, 45.3, 42.3, 38.2, 34.2, 31.9, 28.9, 28.7, 26.4, 25.9, 23.9, 23.8, 21.9, 21.3, 20.0, 18.8, 14.2, 12.6, 12.1; HRMS (ESI, TOF): *m/z* 813.4204 $[M+Na]^+$, calcd for $C_{46}H_{62}O_{11}Na$: 813.4184.

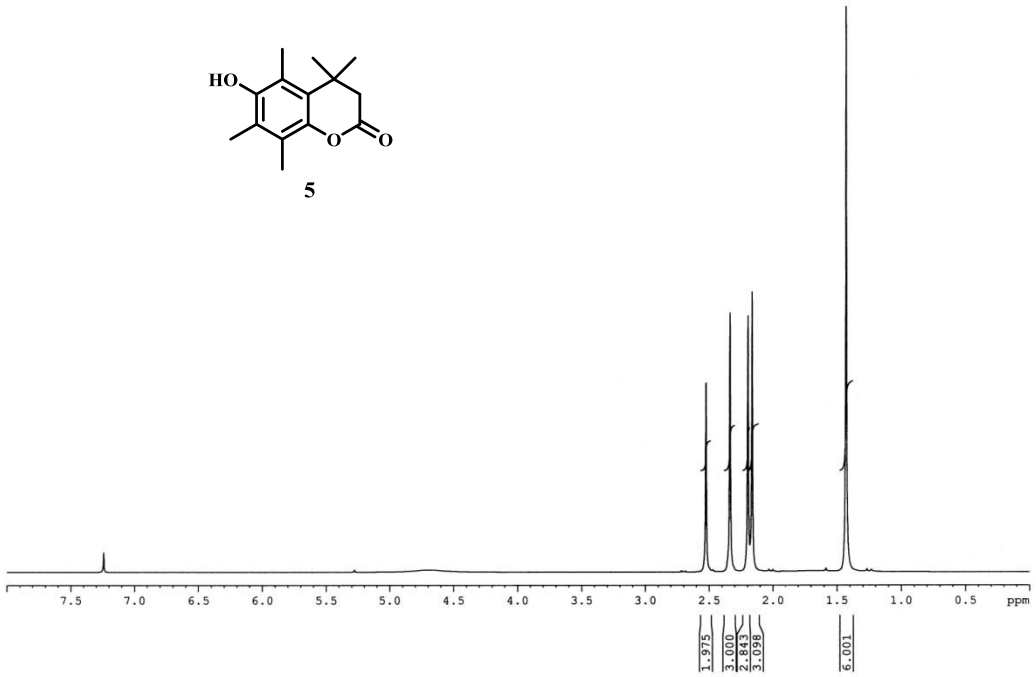


CuB

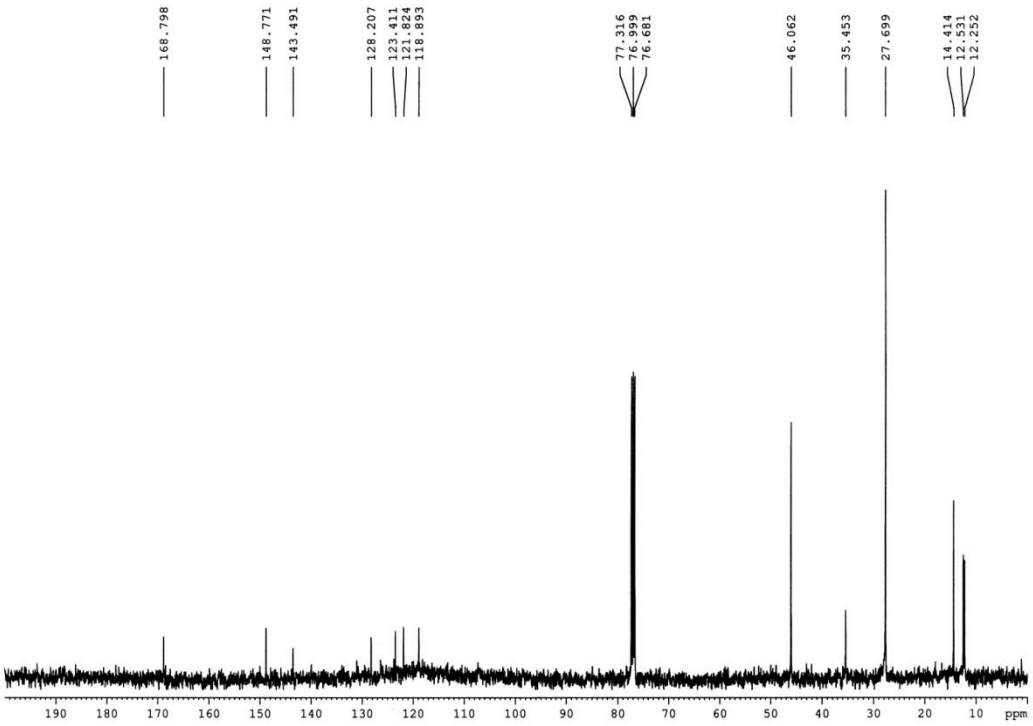
^{1}H NMR (400 MHz) of cucurbitacin B (CuB) in $CDCl_3$



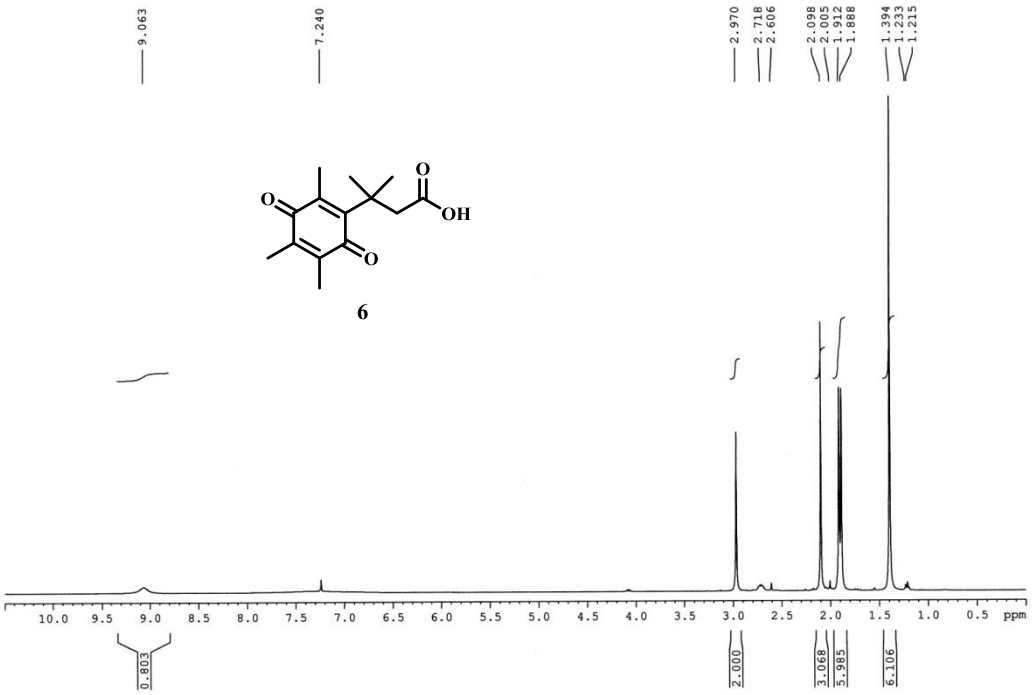
^{13}C NMR (100 MHz) of cucurbitacin B (CuB) in $CDCl_3$



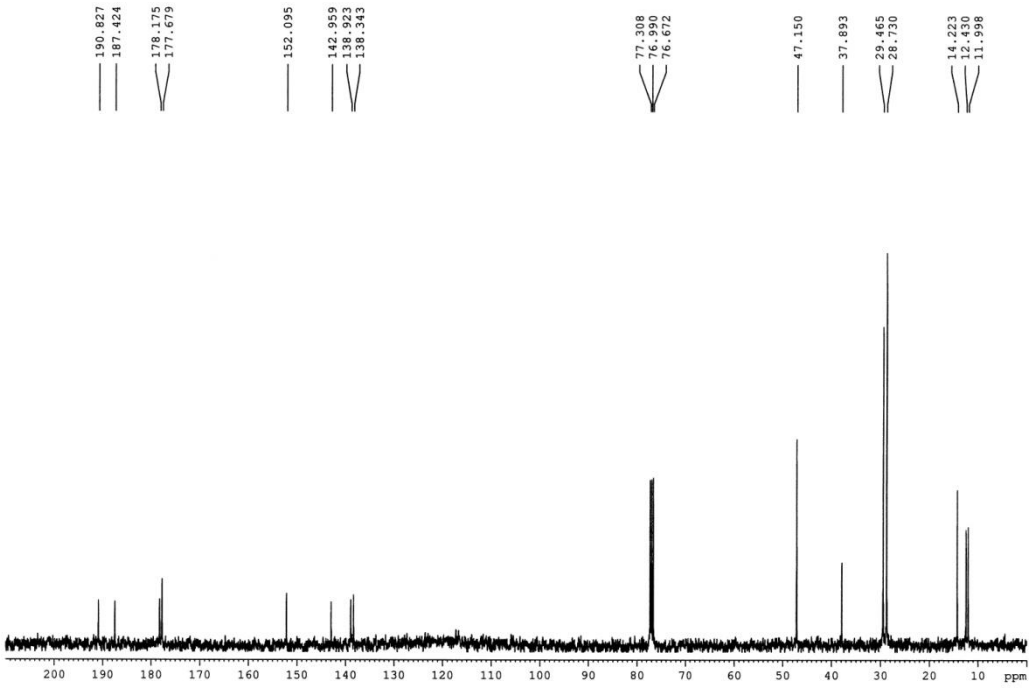
^{1}H NMR (400 MHz) of compound **5** in $CDCl_3$



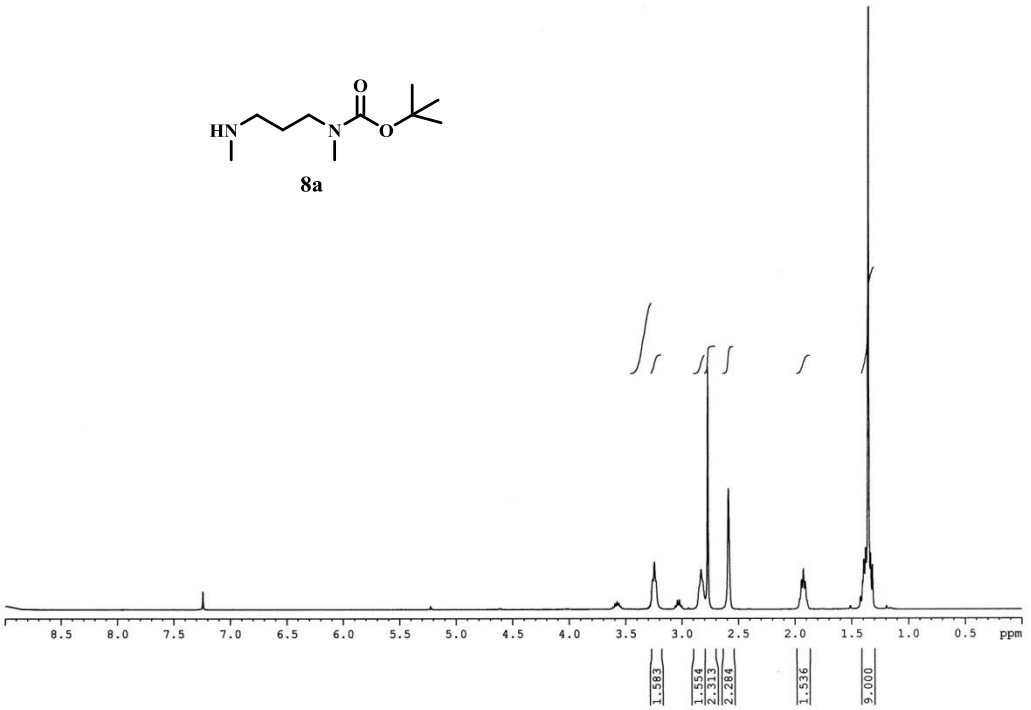
^{13}C NMR (100 MHz) of compound **5** in $CDCl_3$



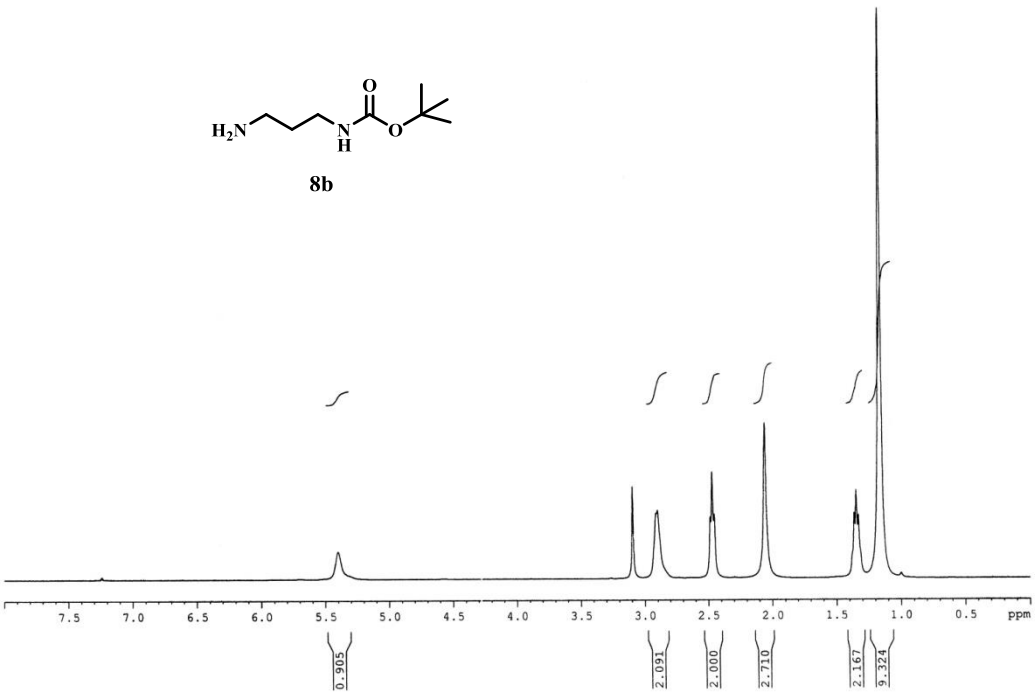
^{1}H NMR (400 MHz) of compound **6** in $CDCl_3$



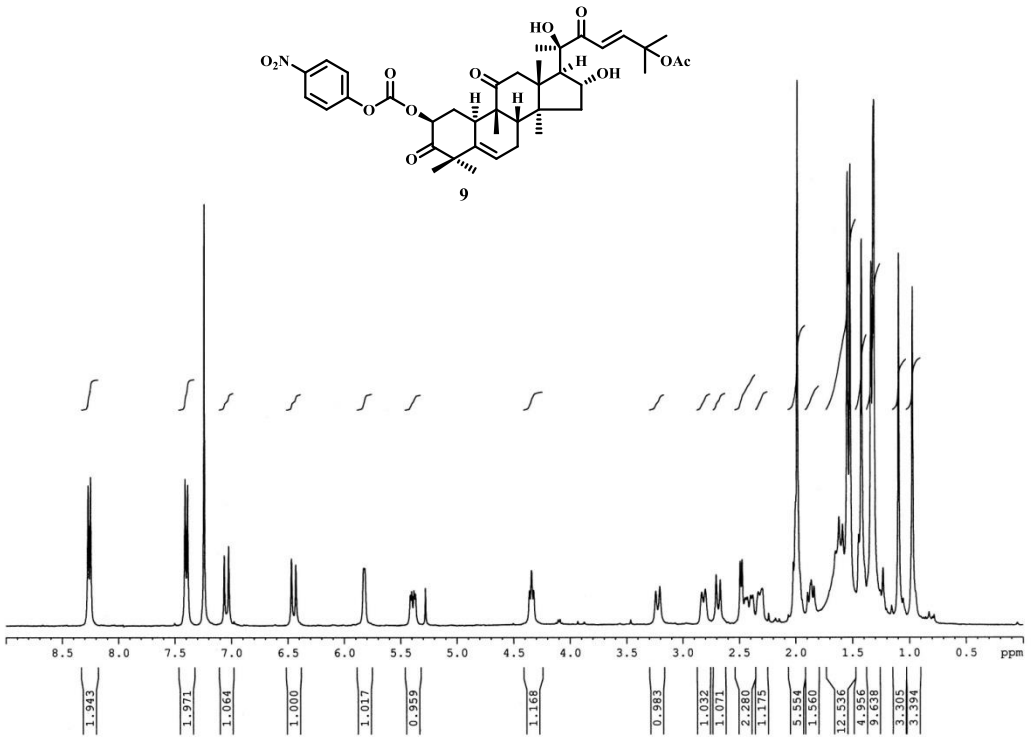
^{13}C NMR (100 MHz) of compound **6** in $CDCl_3$



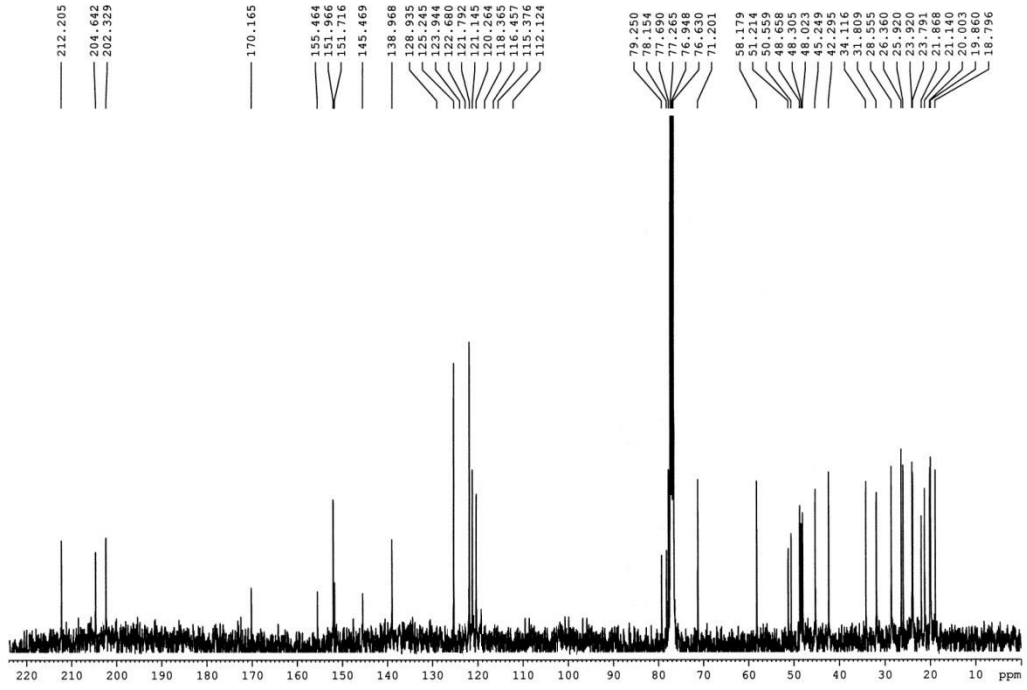
^{1}H NMR (400 MHz) of compound **8a** in $CDCl_3$



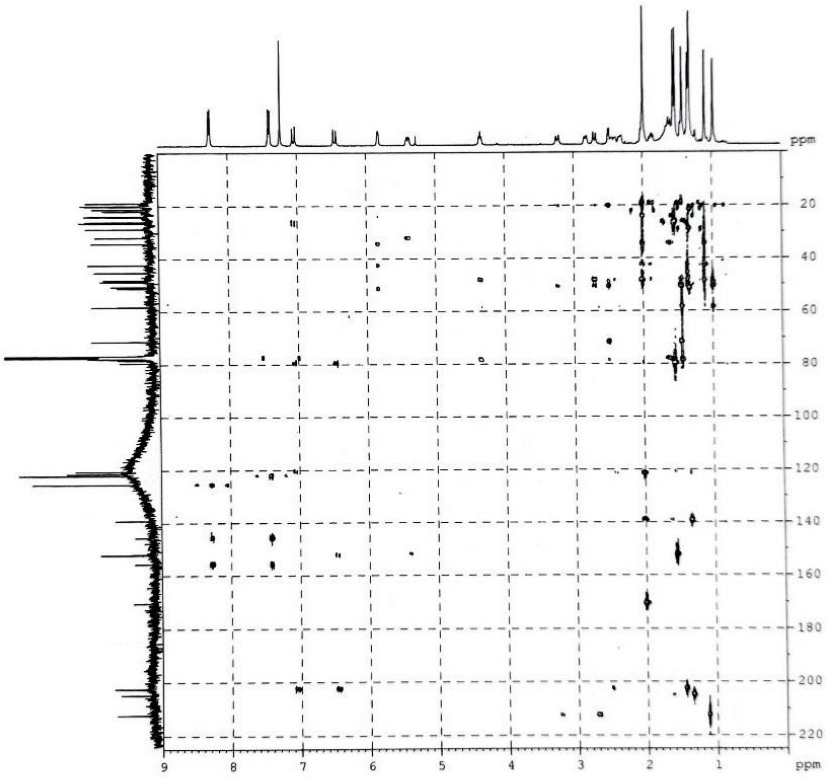
^{1}H NMR (400 MHz) of compound **8b** in $CDCl_3$



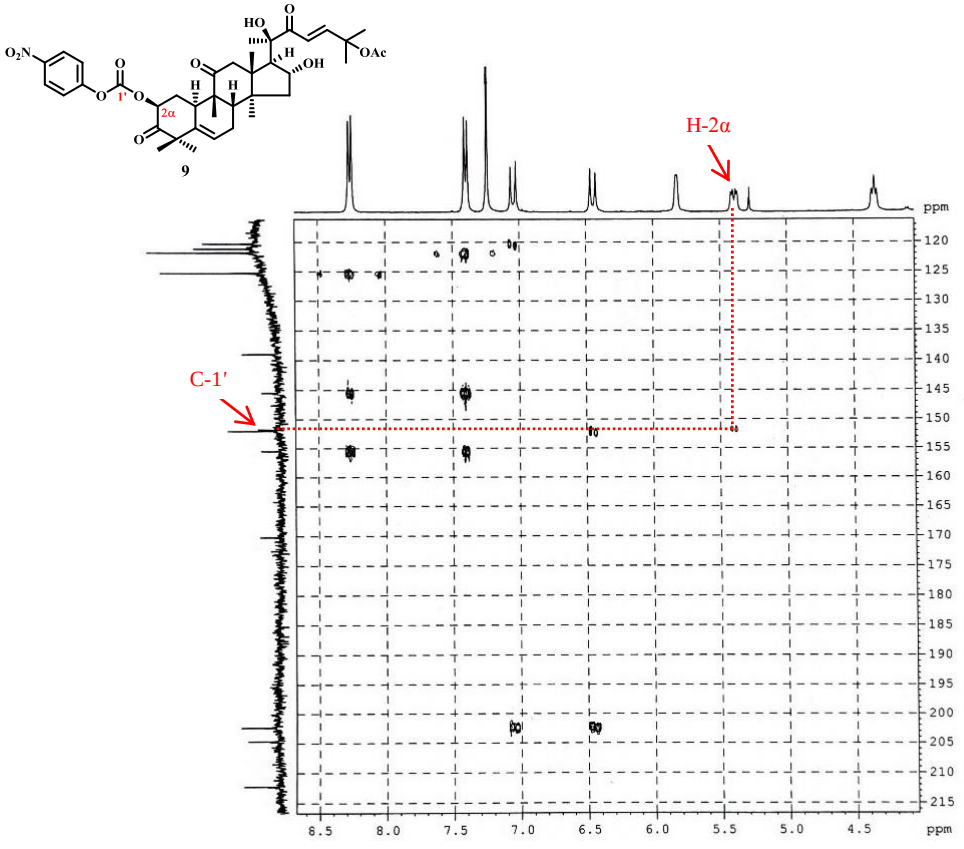
^{1}H NMR (400 MHz) of compound **9** in $CDCl_3$



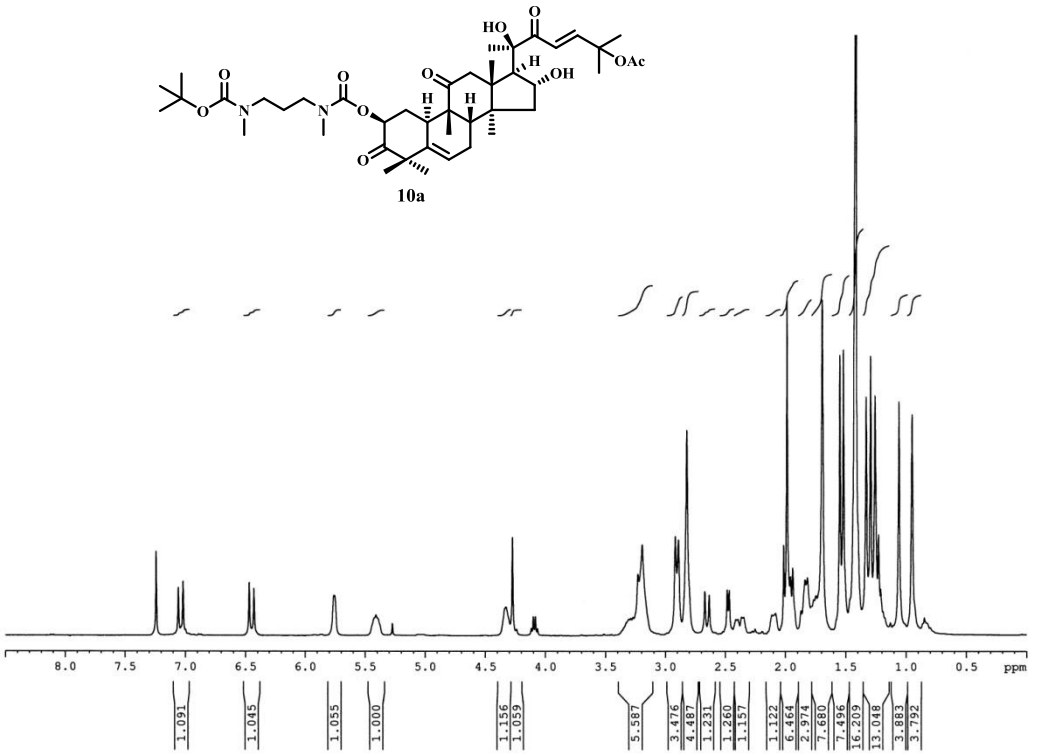
^{13}C NMR (100 MHz) of compound **9** in $CDCl_3$



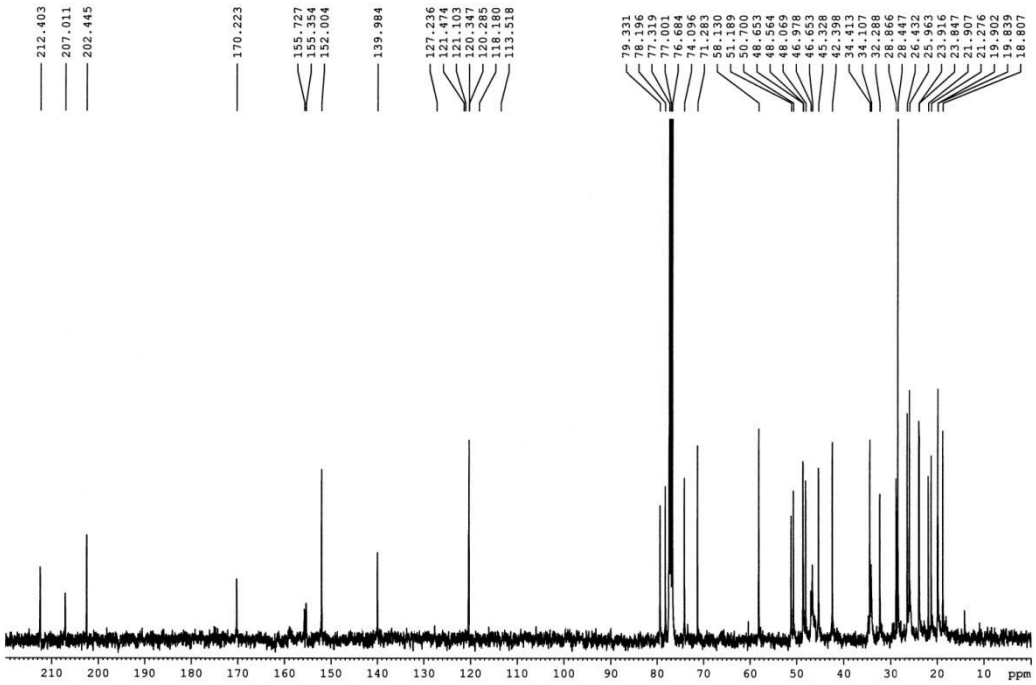
HMBC of compound **9** in $CDCl_3$



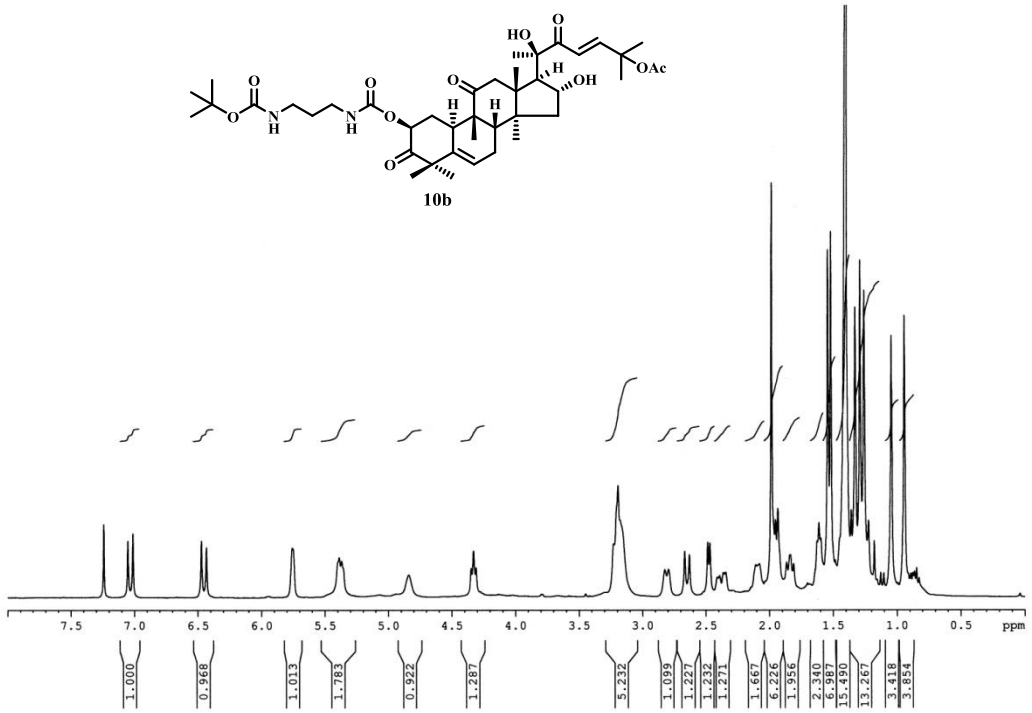
Expansion of HMBC of compound **9** in $CDCl_3$



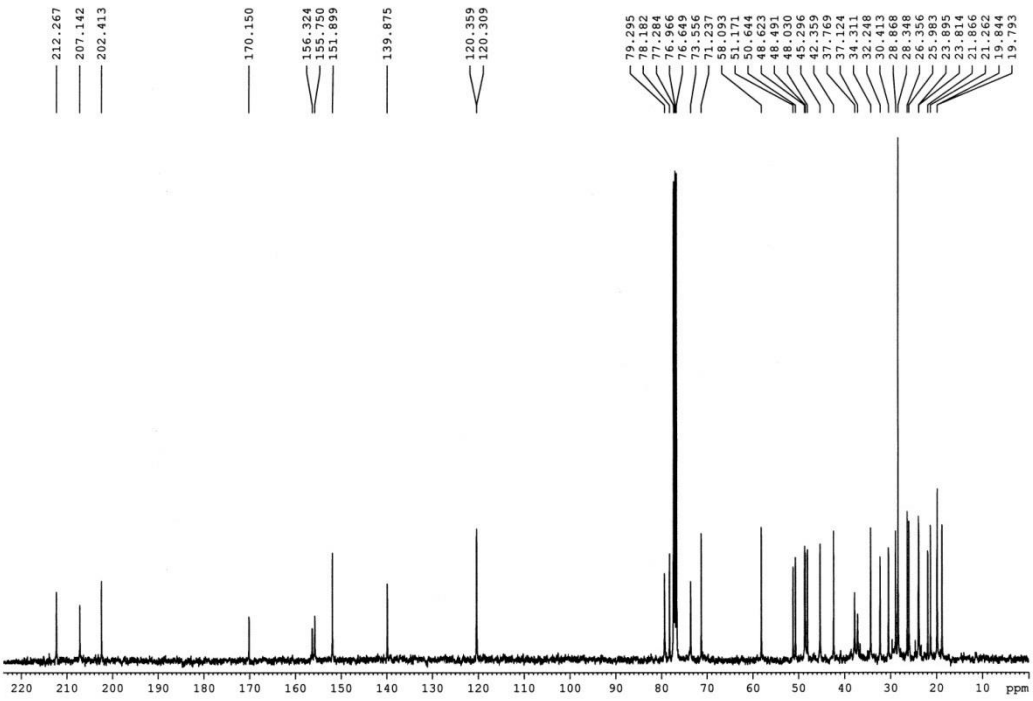
^{1}H NMR (400 MHz) of compound **10a** in $CDCl_3$



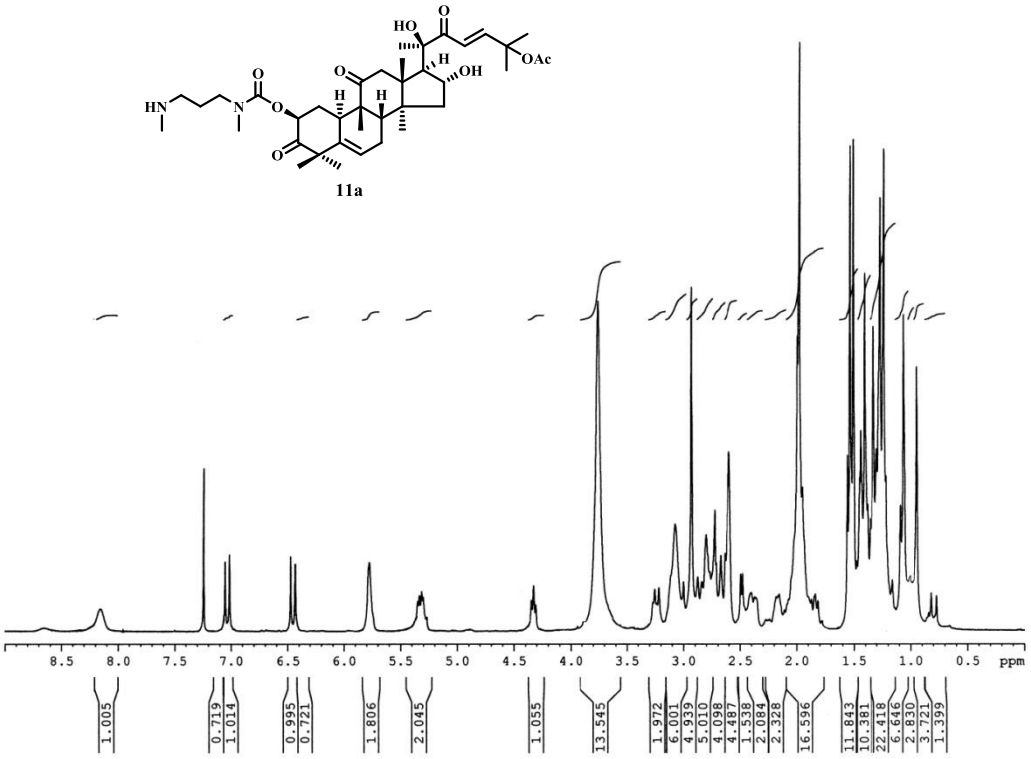
^{13}C NMR (100 MHz) of compound **10a** in $CDCl_3$



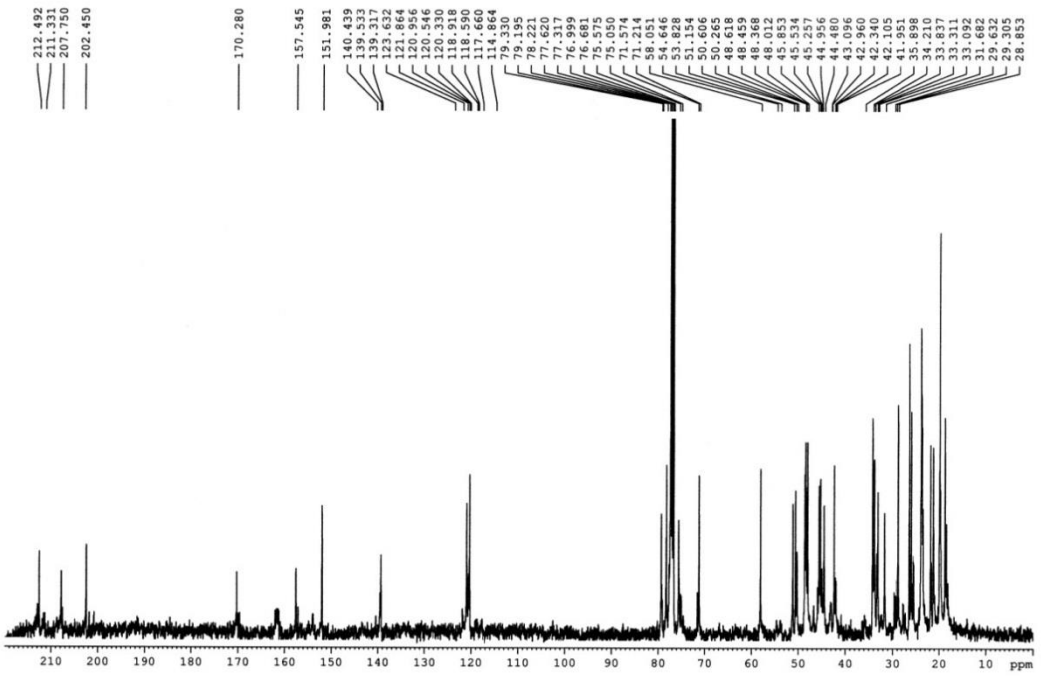
^{1}H NMR (400 MHz) of compound **10b** in $CDCl_3$



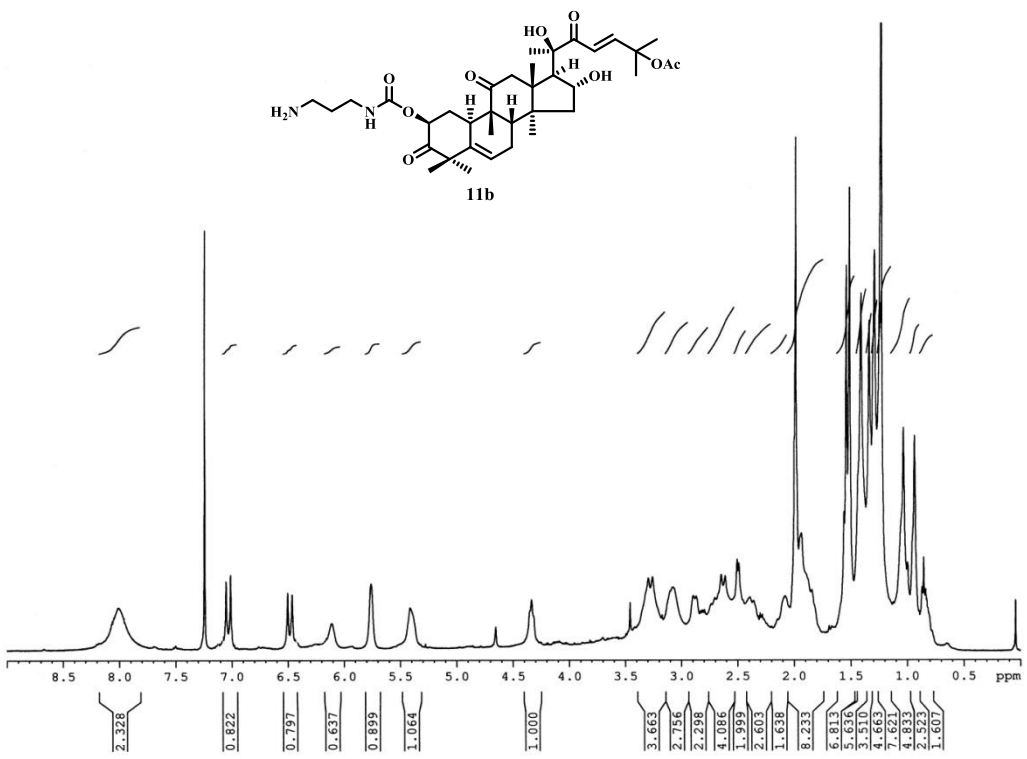
^{13}C NMR (100 MHz) of compound **10b** in $CDCl_3$



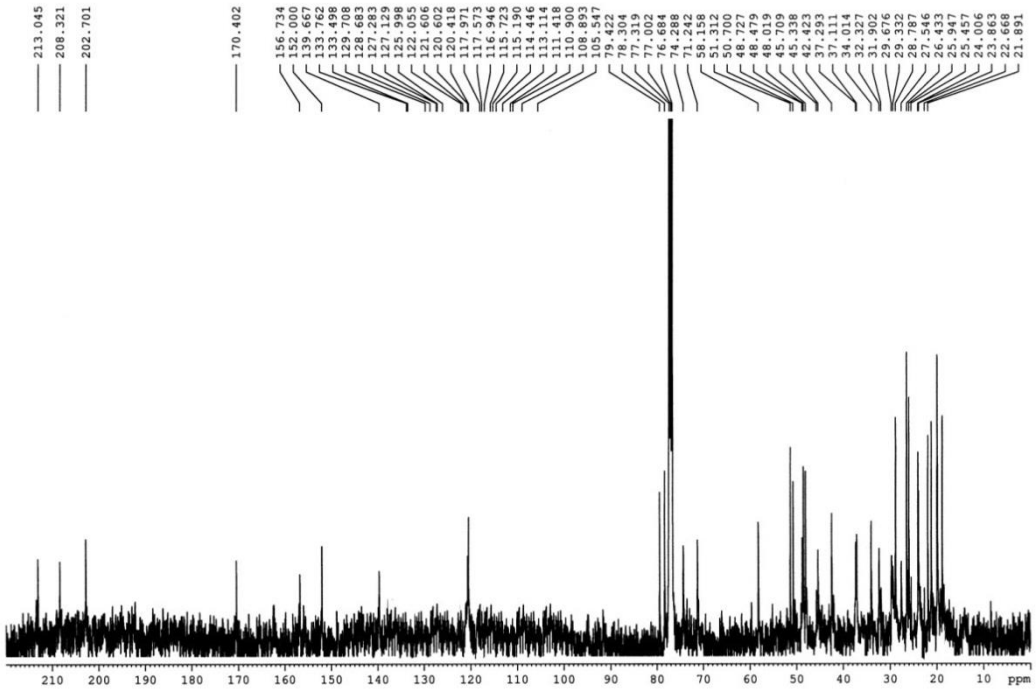
^{1}H NMR (400 MHz) of compound **11a** in $CDCl_3$



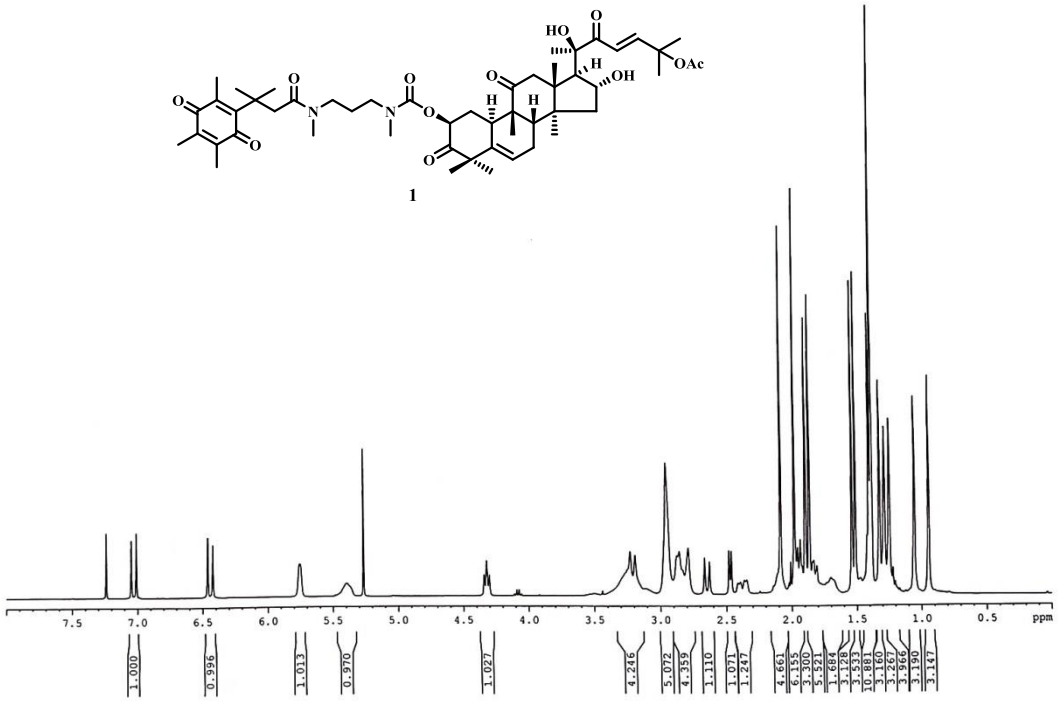
^{13}C NMR (100 MHz) of compound **11a** in $CDCl_3$



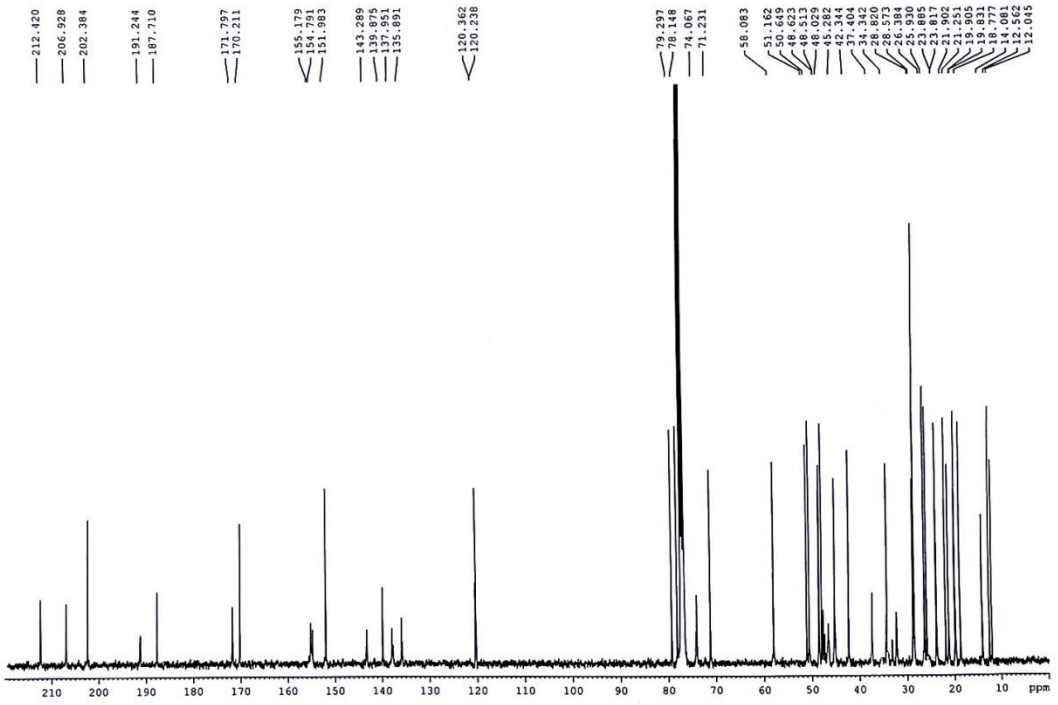
^{1}H NMR (400 MHz) of compound **11b** in $CDCl_3$



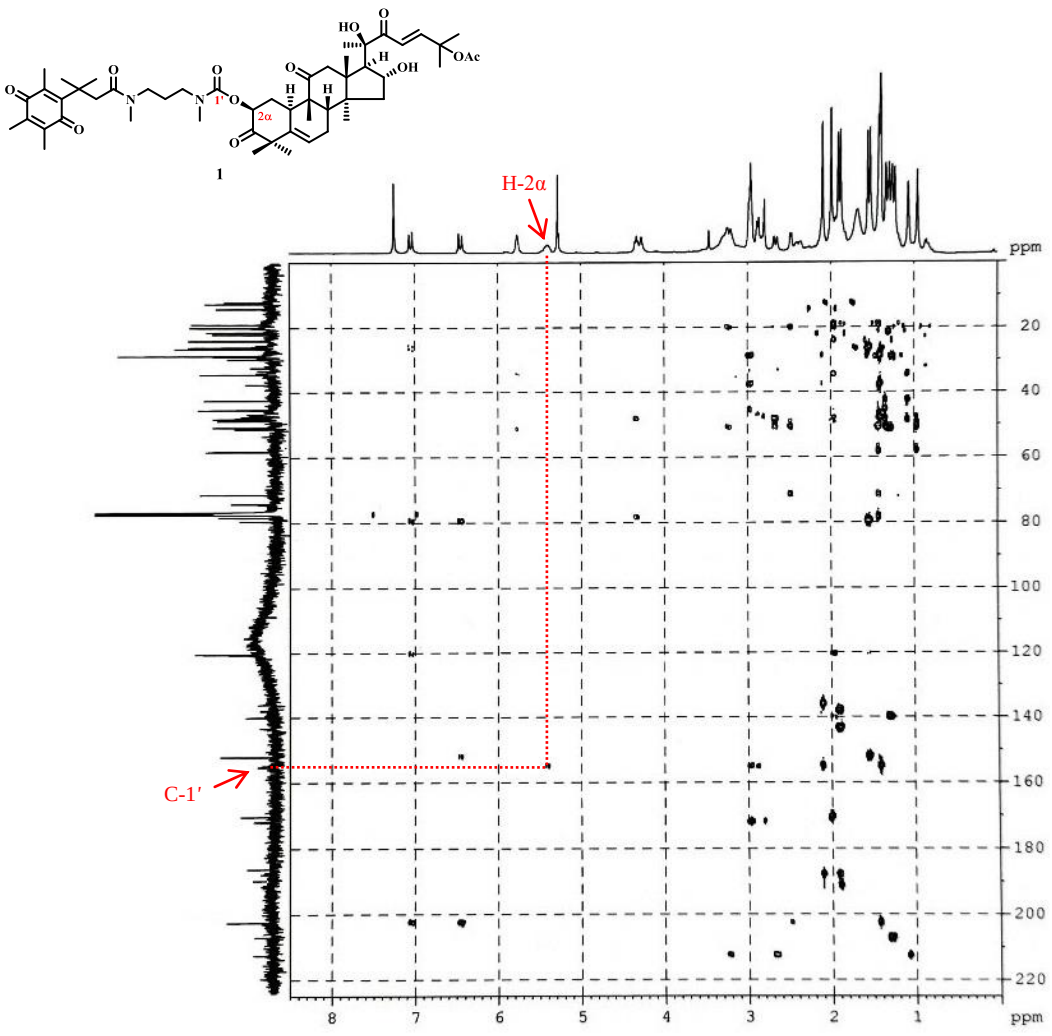
^{13}C NMR (100 MHz) of compound **11b** in $CDCl_3$



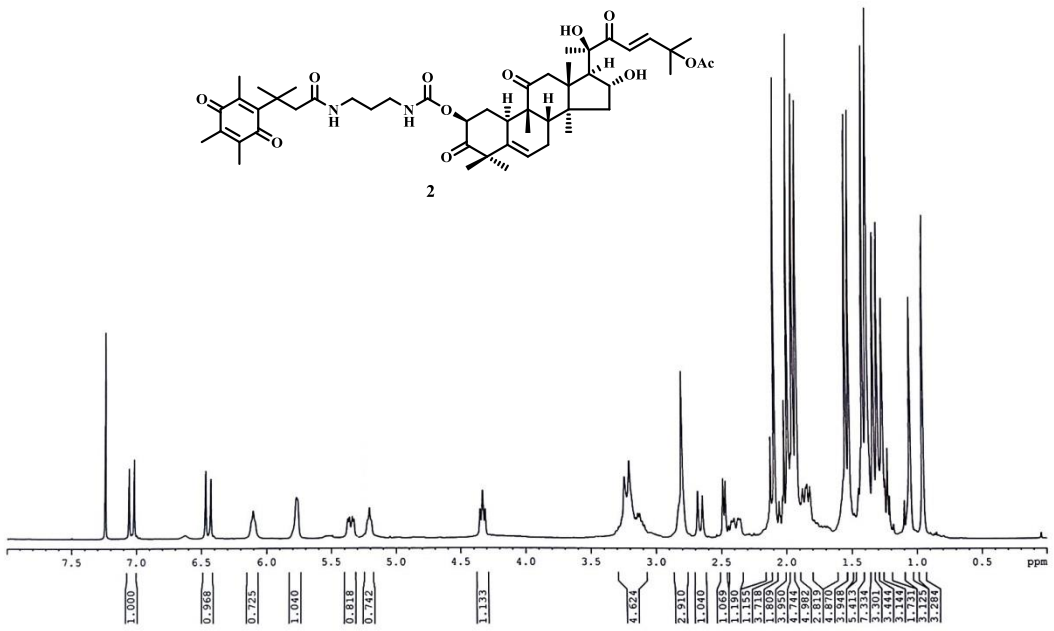
^{1}H NMR (400 MHz) of compound **1** in $CDCl_3$



^{13}C NMR (100 MHz) of compound **1** in $CDCl_3$

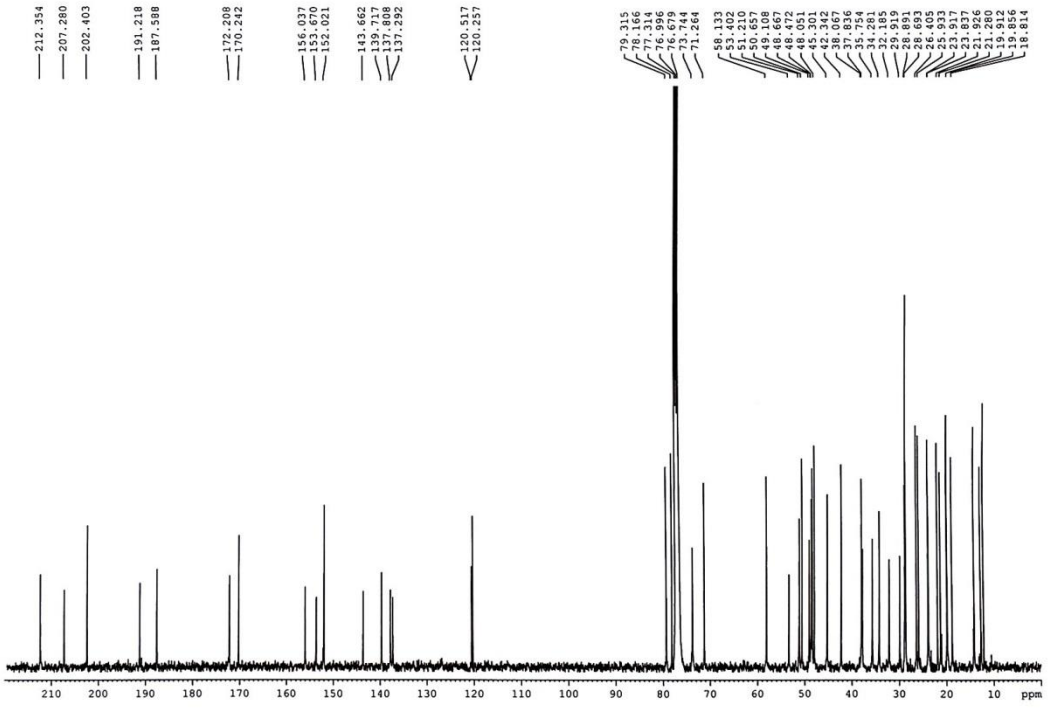


HMBC of compound **1** in $CDCl_3$

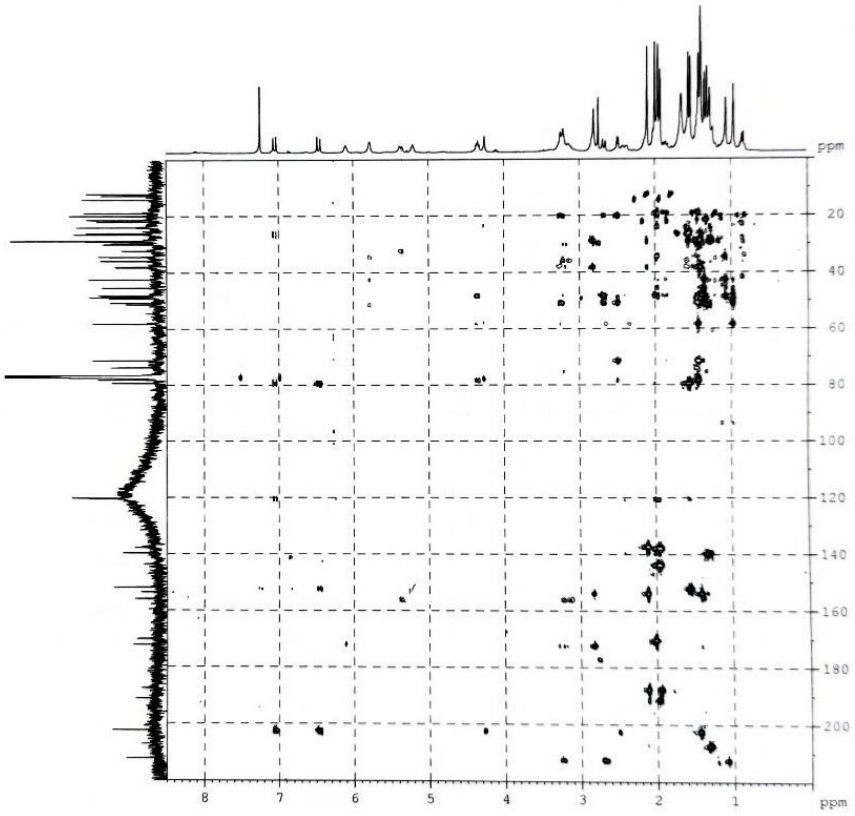


2

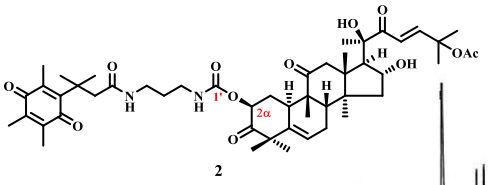
^{1}H NMR (400 MHz) of compound **2** in $CDCl_3$



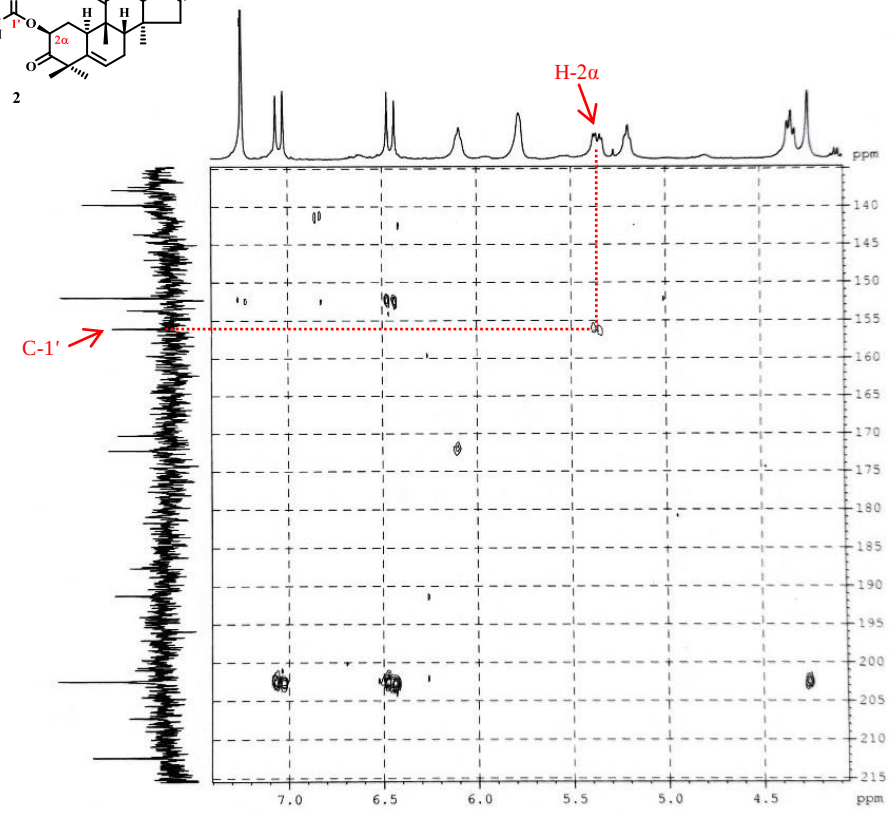
^{13}C NMR (100 MHz) of compound **2** in $CDCl_3$



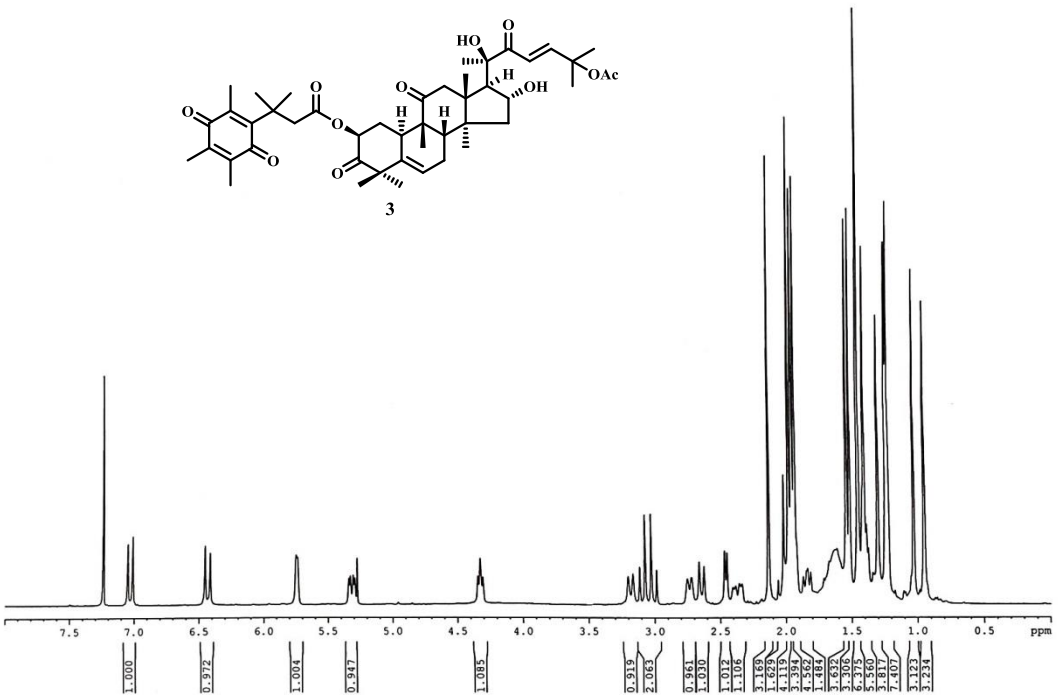
HMBC of compound **2** in $CDCl_3$



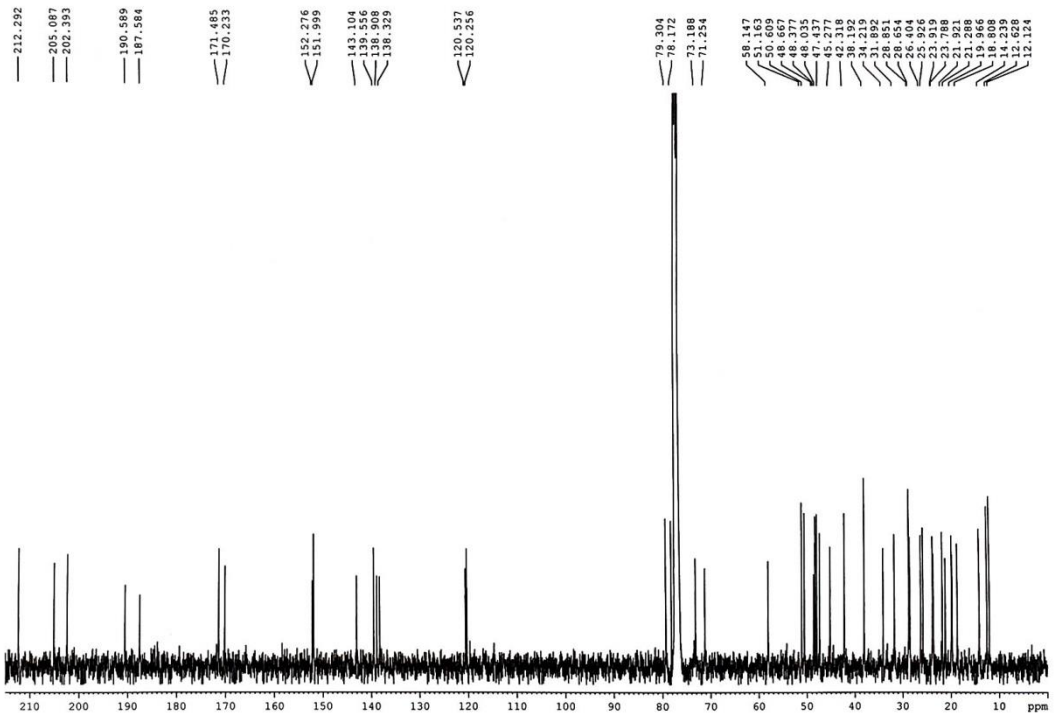
2



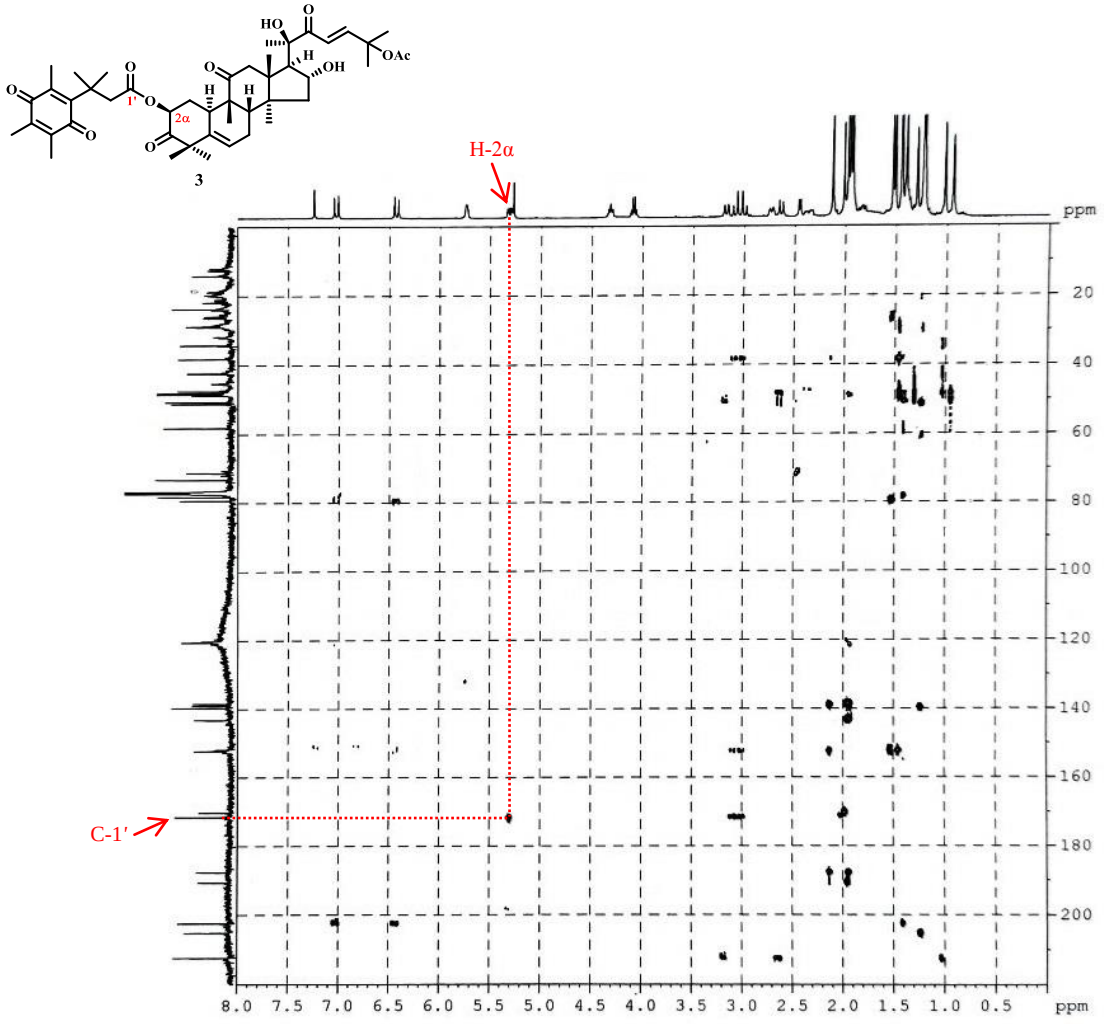
Expansion of HMBC of compound **2** in $CDCl_3$



^{1}H NMR (400 MHz) of compound **3** in $CDCl_3$



^{13}C NMR (100 MHz) of compound **3** in $CDCl_3$



HMBC of compound **3** in $CDCl_3$