18α-glycyrrhetinic Acid Monoglucuronide as Anti-inflammatory Agent through Suppression of NF-κB and MAPK Signaling Pathway

Bo Li,[a] Yongan Yang,[b] Liuzeng Chen,[a] Shichao Chen,[a] Jing Zhang,[*c] and Wenjian Tang[*a]

[a] *School of Pharmacy, Anhui Medical University, Hefei 230032, China. E-mail: ahmupharm@126.com; Fax & Tel: (+86) 551-65161115.*

[b] *Elion Nature Biological Technology Co., Ltd, Nanjing 210038, China.*

[c] *Anhui Prevention and Treatment Center for Occupational Disease, Anhui No. 2 Province People's Hospital, Hefei 230022, China.*

Content

1. Synthesis Procedures of Glycyrrhizin Analogs

18β-GA was provided by Jiangsu Nature Biological Engineering Technology Co., Ltd., Jiangsu, P. R. China. *Aspergillus* sp Ts-1, a kind of β-glucuronidase, selectively hydrolyzed the terminal–glucuronyl linkage of **18β-GA** to produce **18β-GAMG** in 54% yield. **18α-GA** and **18α-GAMG** were respectively synthesized in yields of 23% and 21% after recrystallization from **18β-GA** and **18β-GAMG** by alkaline isomerization. **18α-GCCS** and **18β-GCCS** were respectively prepared in yields of 75% and 72% from **18α-GA** and **18β-GA** by acid hydrolysis (Scheme 1). The structures of Glycyrrhizin analogs were elucidated by mass spectra, ^{1}H-NMR and ^{13}C-NMR spectra.

18β-GA: White powder; m.p.: 234−236 °C; $[\alpha]_{20}^{D}$ = +52 (*c* = 1.0, MeOH); ^{13}C-NMR (75 MHz, DMSO-d_6): Table S1. TOF-HRMS: *m/z* $[M + Na]^+$ calcd for $C_{42}H_{62}NaO_{16}$: 845.3930; found: 845.3935.

18β-GAMG: This compound was prepared from **18β-GA** *via* biotransformation reaction. *Aspergillus* sp. Ts-1 on glucose yeast agar slant was inoculated into a 250 mL Erlenmeyer flask containing 100 mL of seed medium consisting of 1.0 g glucose, 0.2 g yeast, 1.0 g agar, 0.1 g KH_2PO_4 and 0.025g $MgSO_4$ in distilled water (pH 7.0). The culture media were sterilized at 121 °C for 20 min and the fermentation was carried out at 30 °C on a rotary shaker at 200 rpm. After 24 h of inoculation, 30 mL sterilized medium was inoculated into a 1,000 mL Erlenmeyer flask containing 300 mL pre-culture sample consisting of 15 g GA, 0.30 g KH_2PO_4, 3.0 g urea and 0.24 g $MgSO_4$ in distilled water and the pH value was adjusted to 6.0. The culture media were sterilized at 121 °C for 20 min and the fermentation was carried out at 30 °C on a rotary shaker at 250 rpm.

After 72 h of inoculation, the culture solution was filtered and the filtrate was extracted with ethyl acetate. The extract was concentrated under the reduced pressure. The residue (14.5 g) was applied to a silica gel column (800 g, 5.0 × 100 cm) and eluted with $CHCl_3$–MeOH in a gradient manner from 100:1 to 1:1. By TLC analysis, fractions I–IX was obtained. Fractions VI–VIII was concentrated *in vacuo* and recrystallization from aqueous MeOH to give **18β-GAMG** (6.35 g, 54% yield) as a white crystalline powder. M.p.: 237−239 °C; $[\alpha]_{20}^{D}$ = +91 (*c* = 1.0, MeOH); ^{1}H-NMR (500 MHz,

DMSO-d_6) δ (ppm): 0.76 (s, 3H, 24-CH_3), 0.77 (s, 3H, 28-CH_3), 0.99 (s, 3H, 23-CH_3), 1.06 (s, 2 × 3H, 25-CH_3, 26-CH_3), 1.10 (s, 3H, 29-CH_3), 1.34 (s, 3H, 27-CH_3), 2.34 (s, 1H, 9-H), 3.01 (m, 1H, 4'-H), 3.08 (dd, 1H, J_1 = 4.8 Hz, J_2 = 11.2 Hz, 3-H), 3.15 (t, 1H, J = 9.0 Hz, 3'-H), 3.30 (m, 1H, overlapped, 2'-H), 3.58 (d, 1H, J = 9.7 Hz, 5'-H), 4.25 (d, 1H, J = 7.8 Hz, 1'-H), 5.40 (s, 1H, 12-H); ^{13}C-NMR (125 MHz, DMSO-d_6): Table S1. TOF-HRMS: *m/z* $[M + Na]^+$ calcd for $C_{36}H_{54}NaO_{10}$: 669.3609; found: 669.3608.

General Procedure of Alkaline Isomerization of the 18β-isomer to the 18α-isomer: A solution of **18β-GA** (5.00 g, 6.1 mmol) in 5.0 M NaOH solution (100 mL) was heated and stirred for 12 h at 90 °C. After the reaction mixture was cooled to < 5 °C, the pH was adjusted to 2.5 with concentrated HCl. After 12 h, the mixture was filtrated, washed with water, dried. The product **18α-GA** (1.17 g, 23% yield) was obtained as a white crystalline powder by crystallization from ethanol/EtOAc. M.p.: 211−216 °C; $[\alpha]_{20}^{D}$ = +20 (c = 1.0, MeOH); ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): 0.65 (s, 3H, 28-CH_3), 0.73 (s, 3H, 24-CH_3), 0.95 (s, 3H, 23-CH_3), 1.04 (s, 3H, 26-CH_3), 1.10 (s, 3H, 25-CH_3), 1.16 (s, 3H, 29-CH_3), 1.33 (s, 3H, 27-CH_3), 4.31 (d, 1H, J = 7.3 Hz, 1'-H), 4.49 (d, 1H, J = 7.6 Hz, 1''-H), 5.33 (s, 1H, 12-H); ^{13}C-NMR (75 MHz, DMSO-d_6): Table S1. TOF-HRMS: *m/z* $[M + Na]^+$ calcd for $C_{42}H_{62}NaO_{16}$: 845.3930; found: 845.3938.

18α-GAMG: According to the above procedure, **18α-GAMG** (0.83 g, 21% yield) was obtained from **18β-GAMG** (4.00 g, 4.9 mmol) as a white crystalline powder. M.p.: 229−231 °C; $[\alpha]_{20}^{D}$ = +24 (c = 1.0, MeOH); ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): 0.65 (s, 3H, 28-CH_3), 0.77 (s, 3H, 24-CH_3), 0.92 (s, 3H, 23-CH_3), 0.98 (s, 3H, 25-CH_3), 1.04 (s, 3H, 26-CH_3), 1.16 (s, 3H, 29-CH_3), 1.33 (s, 3H, 27-CH_3), 2.27 (overlapped, 9-H), 3.01 (t, 1H, J = 8.4 Hz, 4'-H), 3.07 (dd, 1H, J_1 = 6.5 Hz, J_2 = 9.7 Hz, 3-H), 3.15 (t, 1H, J = 9.0 Hz, 3'-H), 3.30 (t, 1H, J = 9.8 Hz, 2'-H), 3.58 (d, 1H, J = 9.7 Hz, 5'-H), 4.24 (d, 1H, J = 7.8 Hz, 1'-H), 5.33 (s, 1H, 12-H); ^{13}C-NMR (75 MHz, DMSO-d_6): Table S1. TOF-HRMS: *m/z* $[M + Na]^+$ calcd for $C_{36}H_{54}NaO_{10}$: 669.3609; found: 669.3600.

General Procedure of Acid Hydrolysis of Glycyrrhizin: 18β-GA (2.00 g, 2.43 mmol) was added to a solution of AcOH (100 mL) and 5.0 M HCl (20 mL), the reaction mixture was heated and stirred for 1 h at 100 °C. After the reaction mixture was cooled to < 10 °C, the mixture was filtrated, washed with water, ethanol. The filter cake was dried to obtain the product **18β-GCCS** (0.82 g, 72% yield) as a white

crystalline powder. M.p.: > 250 ºC, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm): δ = 0.65 (3 H, s, 28-CH_3), 0.69 (3 H, s, 24-CH_3), 0.90 (3 H, s, 23-CH_3), 1.04 (3 H, s, 25-CH_3), 1.09 (3 H, s, 26-CH_3), 1.16 (3 H, s, 29-CH_3), 1.33 (3 H, s, 27-CH_3), 2.27 (1 H, m, 9-H), 3.01 (1 H, m, 3-H), 4.31 (1 H, d, J = 1.9, 3-OH), 5.33 (1 H, s, 12-H), 12. 17 (1 H, s, COOH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm): Table S1. HRMS (ESI-TOF) calcd for $C_{30}H_{46}NaO_4$ $[M+Na]^+$ 493.3288, found 493.3285.

18α-GCCS: According to the above procedure, **18α-GCCS** (0.86 g, 75% yield) was obtained from **18α-GA** (2.00 g, 2.43 mmol) as a white crystalline powder. M.p.: > 250 ºC, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm): δ = 0.65 (3 H, s, 28-CH_3), 0.69 (3 H, s, 24-CH_3), 0.90 (3 H, s, 23-CH_3), 1.04 (3 H, s, 25-CH_3), 1.09 (3 H, s, 26-CH_3), 1.16 (3 H, s, 29-CH_3), 1.33 (3 H, s, 27-CH_3), 2.27 (1 H, m, 9-H), 3.01 (1 H, m, 3-H), 4.31 (1 H, d, J = 1.9, 3-OH), 5.33 (1 H, s, 12-H), 12.17 (1 H, s, COOH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm): Table S1. HRMS (ESI-TOF) calcd for $C_{30}H_{46}NaO_4$ $[M+Na]^+$ 493.3288, found 493.3280.

Table S1. ^{13}C-NMR data for **18α-GA**, **18α-GAMG**, **18α-GCCS**, **18β-GA**, **18β-GAMG** and **18β-GCCS** in DMSO-d_6 (δ, ppm, J in Hz)

Position	18α-GA	18α-GAMG	18α-GCCS	18β-GA	18β-GAMG	18β-GCCS
1	38.6	38.3	38.4	38.7	38.4	38.5
2	25.5	25.7	26.2	25.8	25.6	26.9
3	88.3	87.9	76.6	88.3	87.7	76.5
4	39.0	39.1	38.7	39.1 [a]	39.1 [a]	38.7
5	54.4	54.3	54.1	54.4	54.1	54.1
6	17.1	17.1	17.2	17.0	16.9	17.1
7	33.2	33.2	33.2	32.2	32.1	32.1
8	43.4	43.4	43.3	44.9	44.9	44.8
9	59.9	59.9	59.9	61.1	61.0	61.1
10	36.2	36.2	36.4	36.4	36.3	36.6
11	198.8	198.7	198.7	199.1	198.9	198.9
12 *	123.1	123.1	123.0	127.4	127.3	127.3
13 *	166.2	166.1	166.0	169.8	169.7	169.5
14	44.7	44.7	44.6	43.0	42.9	42.9
15	26.3	26.3	26.9	26.1	26.1	26.0
16 *	36.8	36.7	36.6	25.9	25.8	25.8

17 *	35.4	35.3	35.1	31.6	31.5	31.5
18 *	39.6 [a]	39.8 [a]	40.1	48.1	48.0	48.0
19 *	31.5	31.4	31.4	40.7	40.6	39.1
20 *	41.7	41.6	41.6	43.2	43.1	43.0
21 *	28.5	28.4	28.3	30.4	30.4	30.3
22	35.2	35.1	35.2	37.6	37.5	37.5
23	27.3	27.5	28.2	27.2	27.4	27.8
24	16.2	16.45	16.1	16.0	16.2	15.9
25	16.4	16.49	16.4	16.3	16.4	16.1
26	18.2	18.2	18.2	18.4	18.3	18.3
27 *	20.4	20.4	20.4	23.0	23.0	23.0
28 *	15.7	15.7	15.6	28.5	28.4	28.3
29 *	20.7	20.6	20.6	27.9	27.8	28.1
30	179.7	179.5	179.4	177.8	177.6	177.6
1'	103.5	105.6		104.8	105.5	
2'	81.0	75.6		82.7	76.1	
3'	74.2	73.7		76.3	75.6	
4'	72.0/71.9	71.6		71.6/71.3	71.5	
5'	76.6	76.1		74.9	73.7	
6'	172.4	170.6		172.4	170.5	
1"	103.7			103.5		
2"	75.0			75.7		
3"	75.2			75.9		
4"	72.0/71.9			71.6/71.3		
5"	76.0			75.2		
6"	172.0			172.0		

* Carbon atoms of chemical shifts with significant difference between the 18β-isomer and the 18α-isomer. [a] Overlapped with solvent.

2. Biological Assays and Experimental Procedures

2.1. Cell Culture

RAW264.7 murine macrophages were cultured in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 ºC in a 5% CO2 humidified atmosphere.

2.2. Assay for NO Production

RAW264.7 cells were inoculated at 1×10^5 cells per well in 24-well plate and cultured for 20 h. The cells were then pre-treated with 50 μM compounds which were prepared in serum-free medium for 2 h before stimulation with LPS (1 μg/mL). After stimulated for 24 h by LPS. The NO production was determined by detecting the nitrite level using Griess reagent (Beyotime, China) according to the manufacturer's instructions. then measured absorbance of the samples at 540 nm (OD_{540}) in a microplate reader (MQX200, Bio-Tek, USA). NO inhibition rate = [control (OD_{540}) - compound (OD_{540})] / [control (OD_{540}) - blank (OD_{540})] × 100%.

Control: treated with LPS only.

Compound: treated with LPS and compounds.

Blank: cultured with fresh medium only.

2.3. Cell Cytotoxicity

Cell cytotoxicity was evaluated by methyl thiazolyl tetrazolium (MTT) assay. RAW264.7 cells were inoculated at 6×10^3 cells per well in 96-well plate. After cultured for 24 h, the cells were treated with different compounds which were diluted in DMEM for 24 h. Then 20 μL of 0.5 mg/mL MTT reagent was added into the cells and incubated for 4 h. After 4 h, cell culture was removed and then 150 μL DMSO was added to dissolve the formazan. The optical density was measured at 570 nm (OD_{570}). Cell viability was calculated from three independent experiments. The density of formazan formed in blank group was set as 100% of viability. Cell viability (%) = compound (OD_{570} / blank (OD_{570}) × 100%

Blank: cultured with fresh medium only.

Compound: treated with compounds or LPS.

Table S1. Effect of Glycyrrhizin analogs on the viability of RAW264.7 cells[a]

Compound	Concentrations	Cell viability (%)[a]
Blank		100
18β-GA	40 μM	103.6 ± 2.8
18α-GA	40 μM	101.7 ± 3.4
18β-GAMG	40 μM	97.5 ± 2.6
18α-GAMG	40 μM	98.1 ± 2.0
18β-GCCS	40 μM	96.6 ± 2.7
18α-GCCS	40 μM	101.3 ± 3.0
LPS	1 μg/mL	99.5 ± 4.5

[a] The results were showed as means ± SD of at least three independent experiments.

2.4. Measurement of IL-6

RAW264.7 cells (7×10^4 cells/well) were cultured in 24-well plate. After cultured for 24 h, and pretreated with 10 and 40 μM of compounds for 2 h, and then LPS was added. The production of IL-6 was stimulated by the addition of 1 μg/mL LPS and incubated for 20 h. The levels of IL-6 in the supernatant were determined using the mouse ELISA kit (IL-6: R&D SYSTEMS, DY406-05) which is operated according to the manufacturer's instructions.

2.5. Western Blot Analysis

RAW264.7 cells were seeded into a 6-well culture plate at a density of 2×10^6 cells per well, and then cultured for 24 h. Then, the culture medium was replaced by fresh medium containing 10、20 and 30 μM compounds, and 1 μg/mL LPS was added. After cultured for another 30 min or 20 h, the cells were harvested and lysed with IP buffer (Beyotime, P0013) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF: Beyotime, ST506) and incubated on ice for 30 min. The cell lysates were centrifuged at 14,000 × g for 10 min at 4 ℃ to remove insoluble materials and the supernatant was collected. Total protein concentration was determined using a BCA protein assay kit (Thermo Scientific, 23227). Each protein sample was mixed with a quarter volume of 5X SDS-PAGE sample loading buffer (100 mmol/L Tris-HCl pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue) and

boiled for 10 min. Equal amounts of total cellular protein were loaded per well in 12.5% precast SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Bio-Rad) for over 60 min at 300 mA. The membranes were blocked with 5% non-fat dry milk in TBS plus 0.1% Tween 20 (TBST) for 2 h at room temperature, washed 3 times in TBST for 5 min each, incubated with the primary antibody (anti-phosphorylation of SAPK/JNK, anti-SAPK/JNK, anti-phosphorylation of ERK1/2, anti-ERK1/2, anti-phosphorylation of p38, anti-p38, anti-phosphorylation of IkBa , anti-IkBa, antiphosphorylation of NF-kB p65, and anti-NF-κB p65) at 4 ºC overnight (all the primary antibodies were purchased from Cell Signaling Technology and diluted at the ratio of 1:1000),washed 3 times in TBST for 5 min each, incubated with anti-rabbit or anti-mouse secondary antibody (1:5000 in TBST, Cell Signaling Technology) for 90 min, washed in TBST and exposed to ECL reagents.

2.6. Immunofluorescence Assay

RAW264.7 cells (7×10^4 cells/well) were cultured in 24-well plate. After cultured for 24 h, and pretreated with 20 and 30 μM of 18α-GAMG for 2 h, and then treated with LPS (1 μg/mL) for 3 h. The cells were washed twice with cold PBS, fixed with 4% formaldehyde for 15 min, and then permeabilized with 0.3% Triton X-100 in PBS for 10 min. After that, the cells were blocked for 0.5 h with 5% BSA. Cells were later incubated with primary antibody anti-NF-κB p65 antibody for overnight, followed by Alexa Fluor 488-labeled goat anti-rabbit IgG secondary antibody. After a wash step, stained with DAPI for 5 min and the images were acquired.

2.7. 18α-GAMG Alleviated CCl_4-induced Hepatic Fibrosis

Healthy male C57BL6 mice were randomly divided into 4 groups: control group, model group, high-dose 18α-GAMG group and low-dose 18α-GAMG group, and each with 10. All mice except normal group were given 20% CCl_4 olive oil (2 mL/kg) by hypodermic injection，two times a week for 4 weeks. Control group was given 0.9% sodium chloride by hypodermic injection, two times a week for 4 weeks. 18α-GAMG (200 mg/kg and 100 mg/kg) was given by intra-gastric administration to high-dose 18α-GAMG group and low-dose 18α-GAMG, two times a week for 4 weeks at beginning of third week. All mice were killed at 4th week. Pathological changes in hepatic tissue were observed by HE and Masson staining.

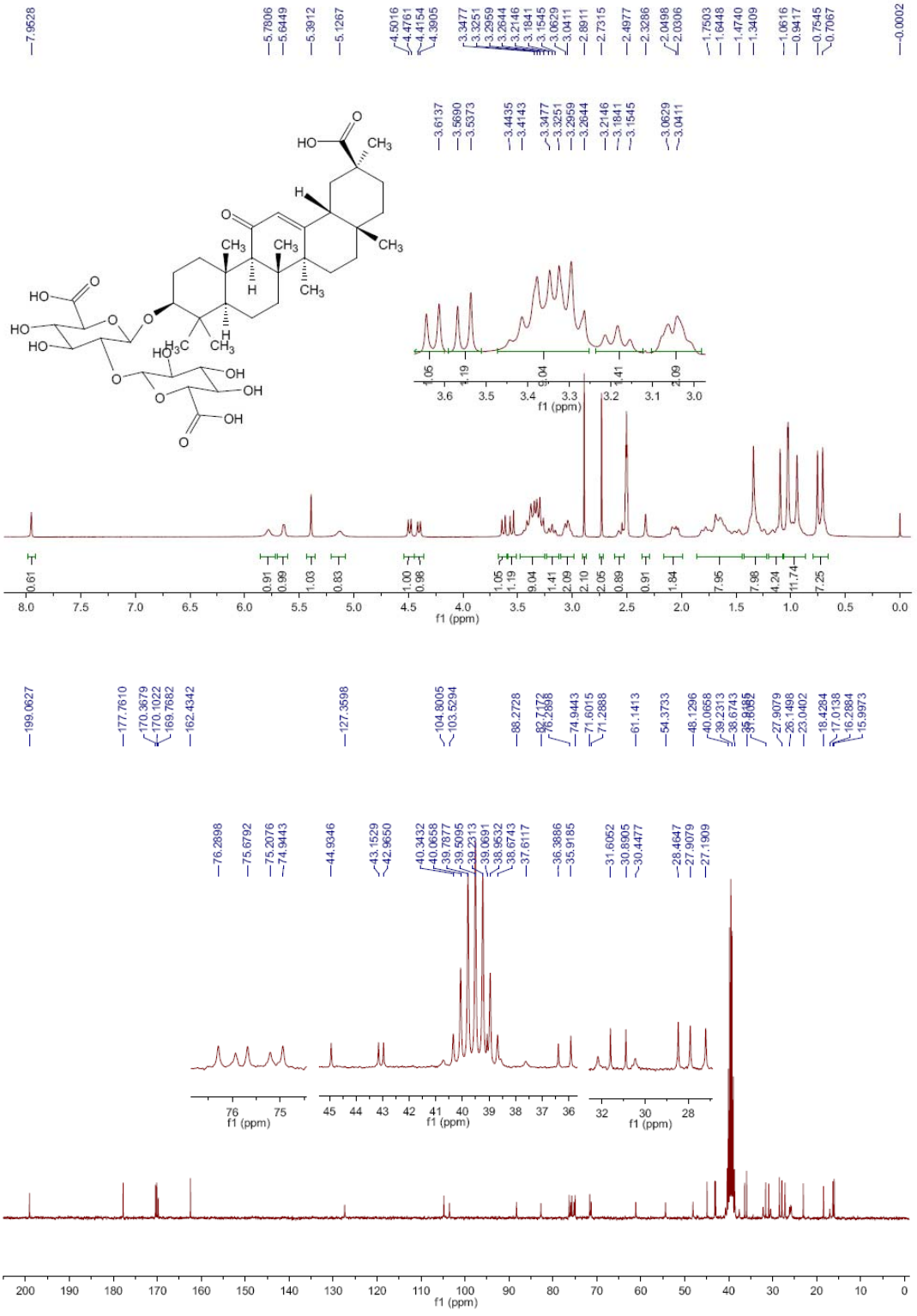

Figure S1. ^{1}H-NMR and ^{13}C-NMR spectra of **18β-GA**.

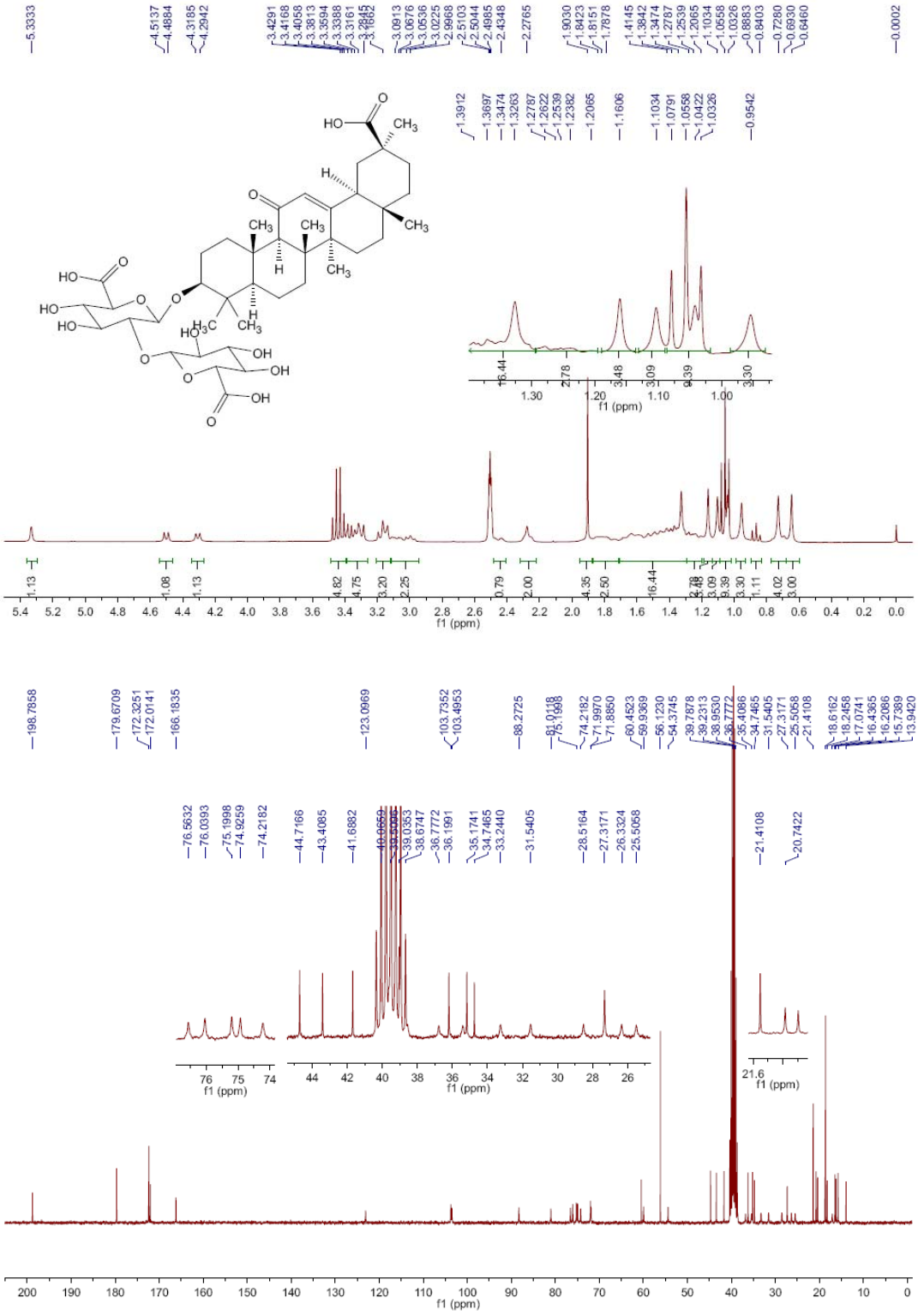

Figure S2. ^{1}H-NMR and ^{13}C-NMR spectra of **18α-GA**.

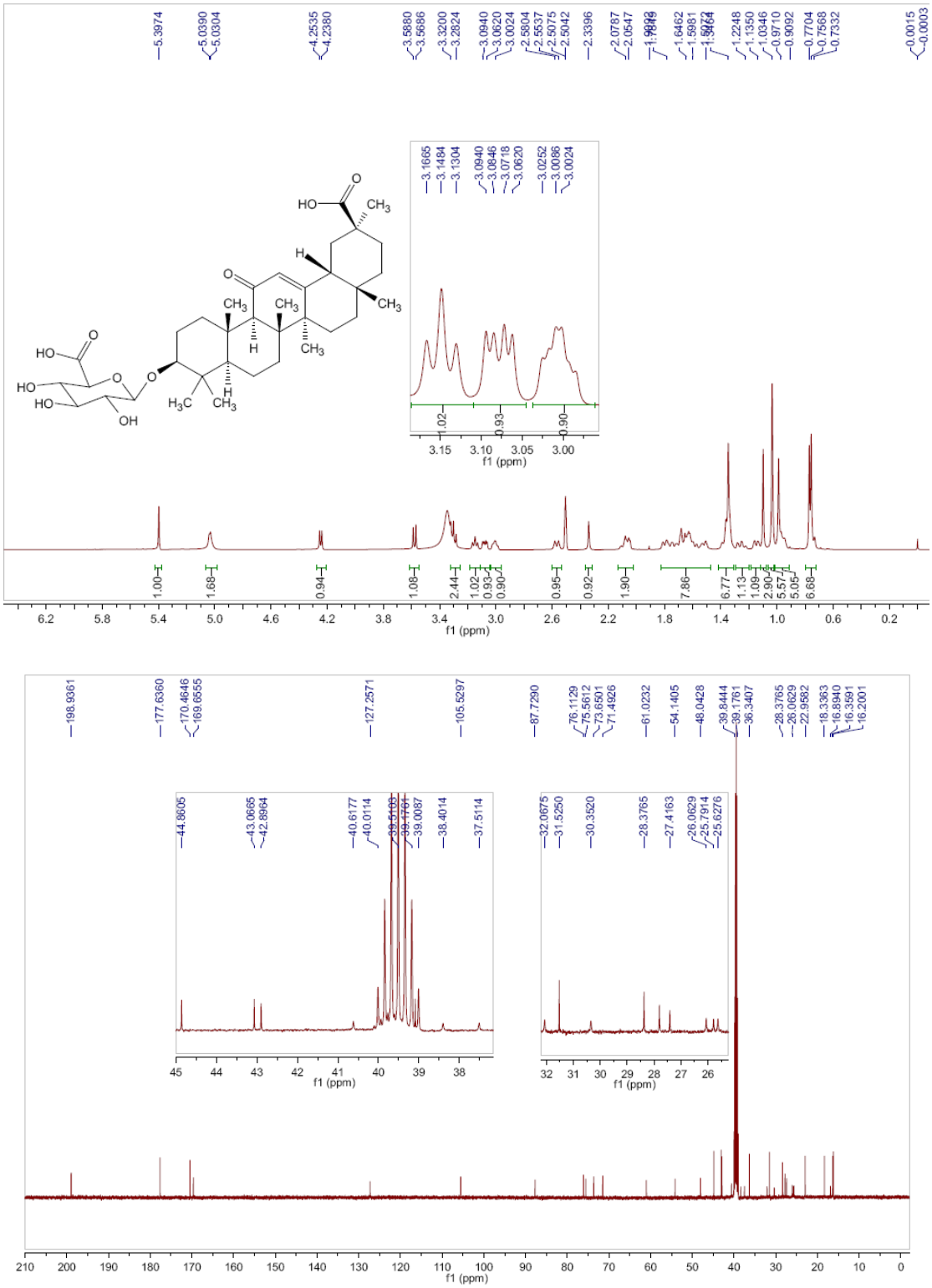

Figure S3. ^{1}H-NMR and ^{13}C-NMR spectra of **18β-GAMG**.

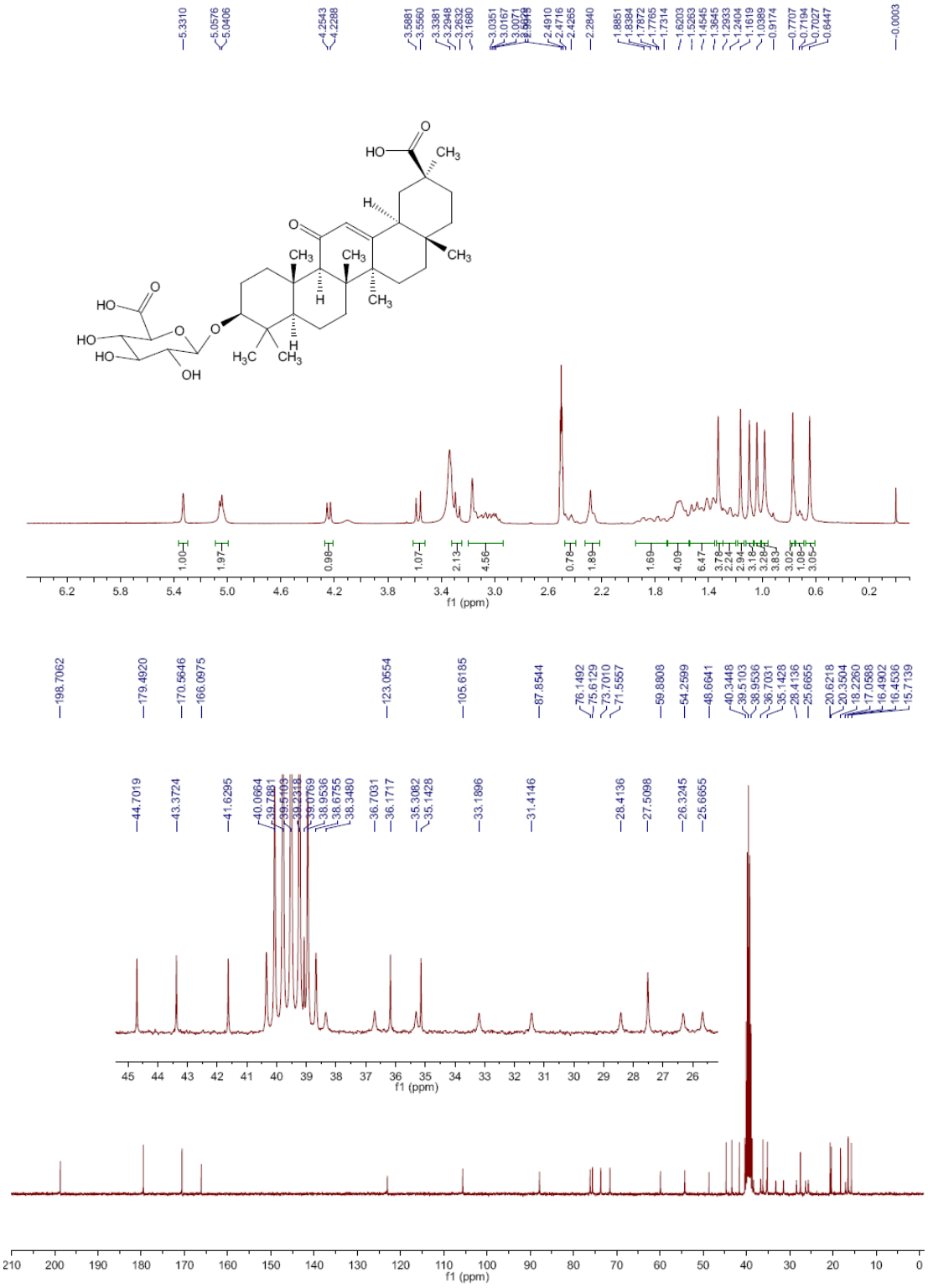

Figure S4. ^{1}H-NMR and ^{13}C-NMR spectra of **18α-GAMG**.

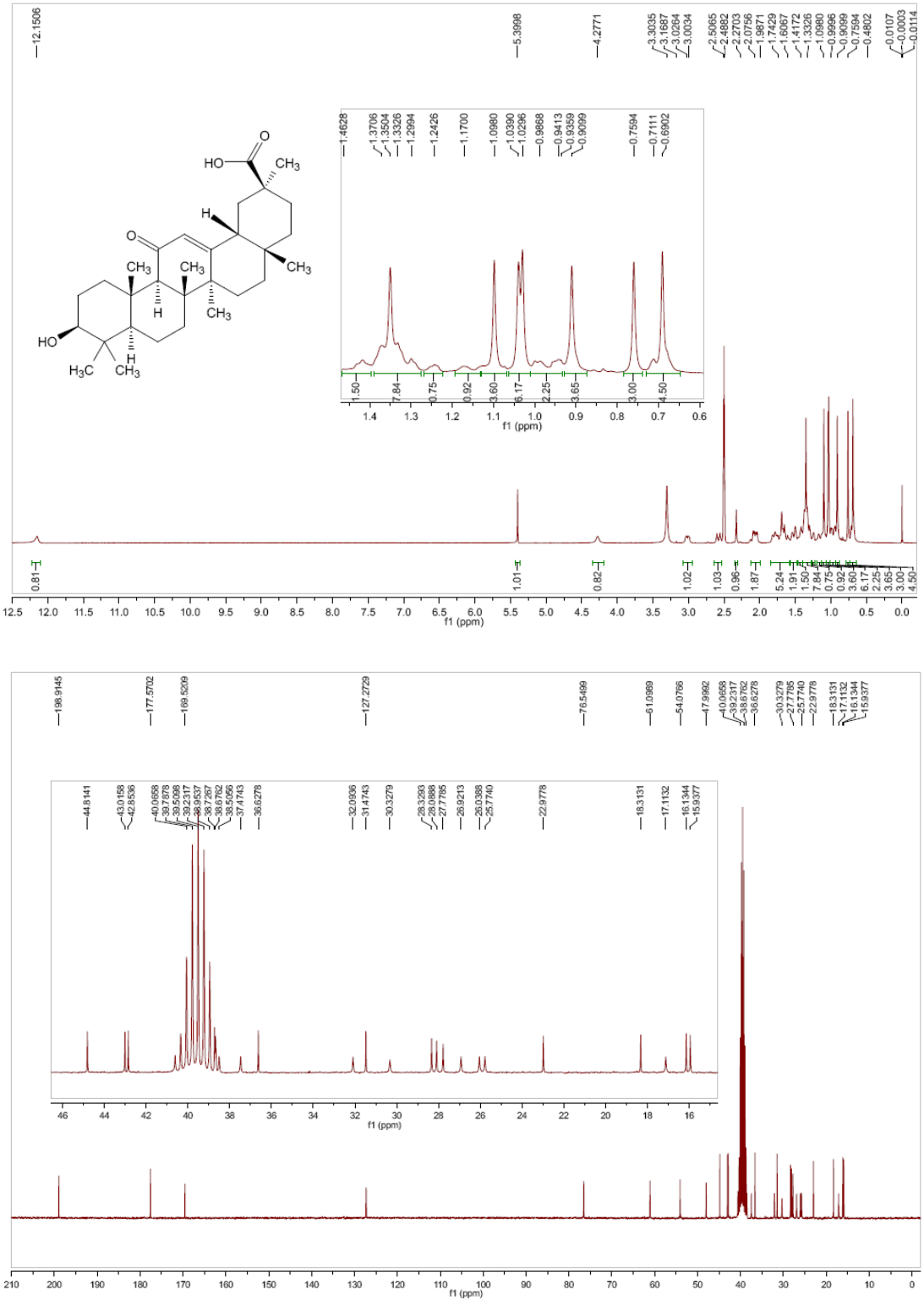

Figure S5. ^{1}H-NMR and ^{13}C-NMR spectra of **18β-GCCS**.

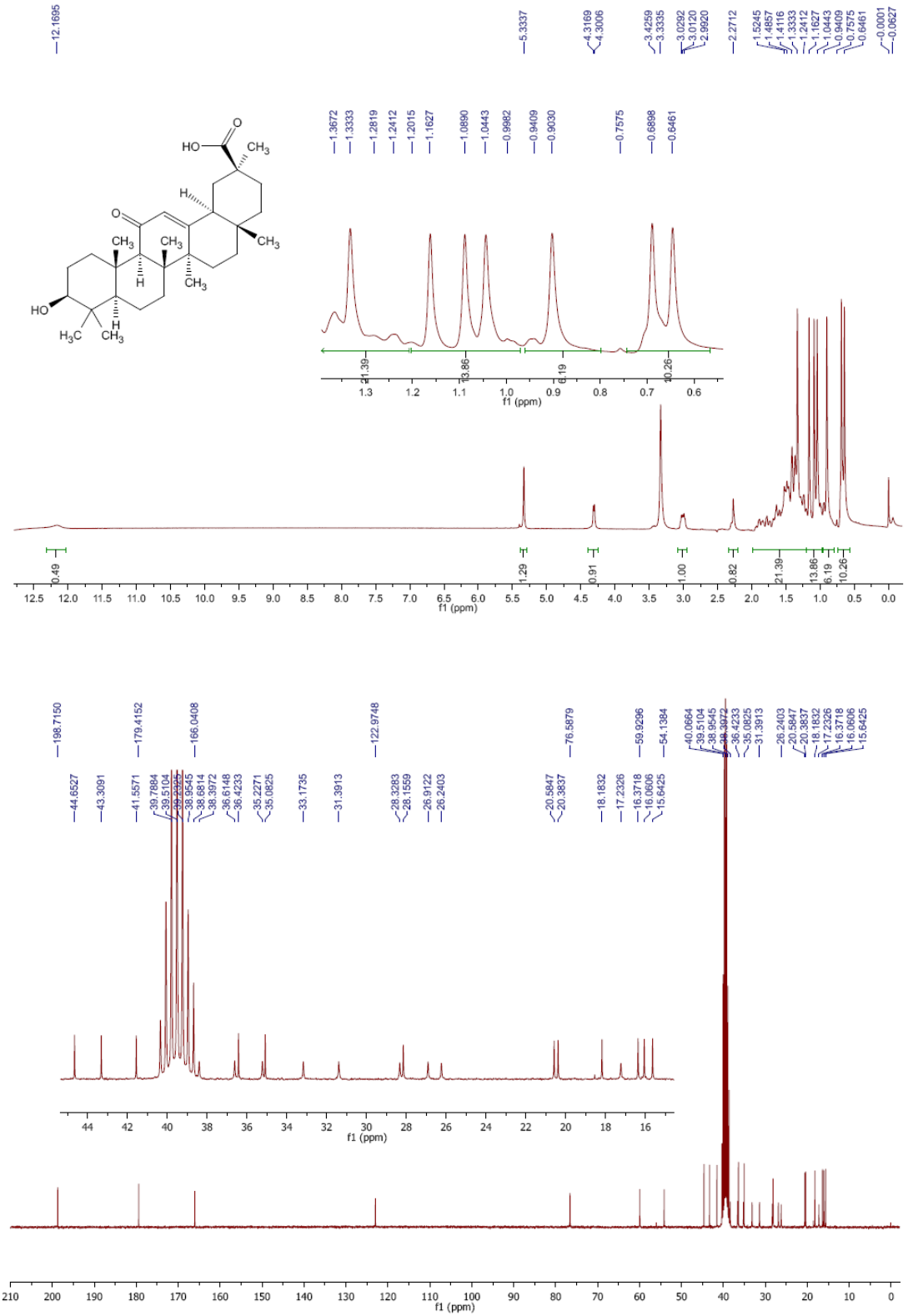

Figure S6. ^{1}H-NMR and ^{13}C-NMR spectra of **18α-GCCS**.