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Electronic Supplementary Information

Synthesis and biological activity evaluation of novel peroxo-bridged derivatives as potential anti-hepatitis B virus agents

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Table of Contents

Bio-Evaluation Methods

Cell culture

The cell lines HepG2.2.15 (clonal cells derived from human hepatoma cell line G2,) was provided by the 302 Military Hospital of China. Cultures were maintained as monolayers in DMEM (GIBCO) with nonessential amino acids, sodium pyruvate (90%) and 10% (v/v) heat inactivated fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2. The sterol derivatives under study were dissolved in ethanol and added at required concentrations to the cell culture. Cells incubated without the preparations served as the control.

Cytotoxicity Assay

The cytotoxicity of the compounds was evaluated on HepG2.2.15 cells by the standard MTS assay. In short, exponentially growing cells were seeded into 96-well plates at a density 1.0×10^5 cell/mL. The plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. After the various concentrations of the test drugs being added into cultures treated 4d, the media was removed. MTS solution was added to all the wells, and incubation continued at 37 °C for 3 h. Untreated cells were used as control. The cell pathological changes were observed by microscope and then the absorbance was quantified at a wavelength of 490 nm with a BIORAD 550 spectrophotometer (CA, USA), Inhibition ratio (%) was calculated based on the equation (1):

$$\% \text{ inhibition} = (1 - \text{Sample group OD/Control group OD}) \times 100\% \quad (1)$$

Assays for HBsAg and HBeAg in the Cell Culture

HepG2.2.15 cells at a density 1.0×10^5 cells/mL were incubated in 96-well plates for the measurement of HBV antigens. After incubation with various concentrations of **1d-1f** , **2d-2f** or 3TC for 4 or 8 days, the culture medium was collected, cell debris was removed, and the supernatant was collected and performed at −20 °C. The levels of Hepatitis B surface antigen (HBsAg) and Hepatitis B e antigen (HBeAg) in culture supernatants of HepG2.2.15 cells were determined with an ELISA kit (DaAn Gene Corp. of Sun Yat-sen University, Guangzhou, China). The optical density (OD) was measured at a wavelength of 450 nm using a BIORAD 550 spectrophotometer. Inhibition ratio (%) was calculated based on the equation (1).

Acute Toxicity

Compound **1f** was further investigated for its approximate LD_{50} and 95% confidence interval in mice. Kunming mice (Beijing Vital River Laboratory Animal Technology Company Limited, Beijing, China) of both sexes, weighing 18-22 g, were divided into six groups per six individuals matched in weight and size. The six groups were given intraperitoneal injection of compound **1f** in dose of 56, 178, 350, 422, 750 and 1000 mg/kg respectively. The general behavior of the mice was observed continuously for 1, 4 and 24 h after the treatment. The mice were further observed up to 14 days. Behavior, toxicity effects and mortality response were recorded.

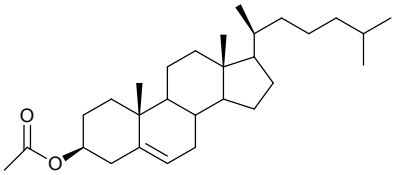
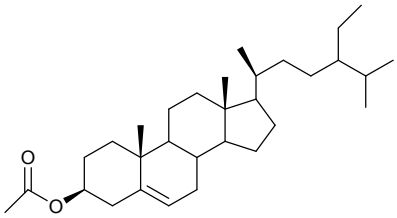
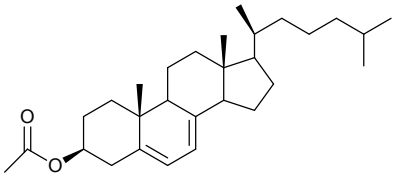
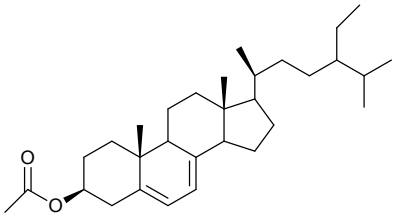
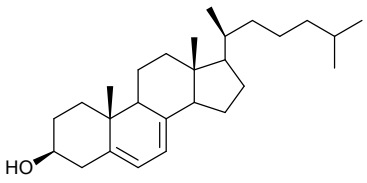
Table 1 Inhibitory effect of **1d-1f** and **2d-2f** on HBsAg and HBeAg secretion in HepG2.2.15 cells at 3.13 μg/mL.

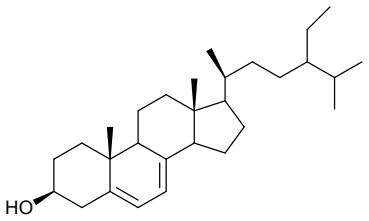
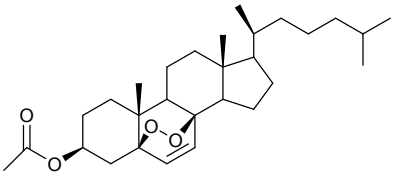
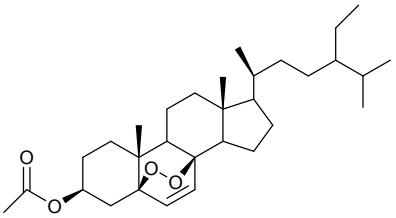
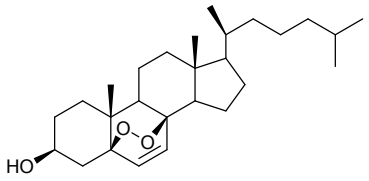
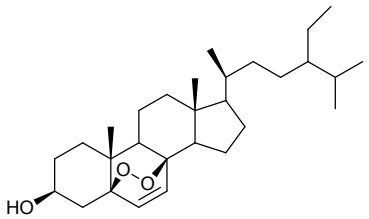
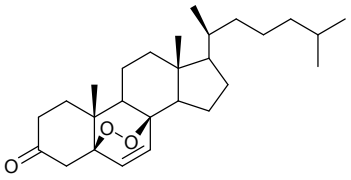
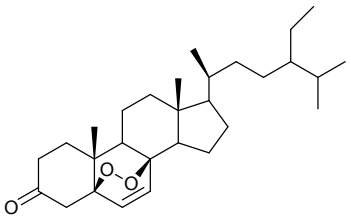
Compound	4day		8day	
	HBsAg (%)	HBeAg (%)	HBsAg (%)	HBeAg (%)
1d	12.96±3.45	3.51±2.65	13.77±4.23	7.56±4.78
1e	1.20±4.67	1.35±4.87	1.56±3.12	2.55±5.01
1f	72.28±6.00	54.10±5.06	77.45±6.01	58.73±8.64
2d	26.67±2.98	25.64±3.23	28.79±4.34	25.78±3.54
2e	19.01±2.65	2.88±5.45	20.04±3.12	10.35±3.56
2f	1.91±4.65	3.28±4.98	5.70±4.13	2.36±5.37
3TC	5.68±3.78	4.94±4.99	-0.44±3.85	-9.22±6.32

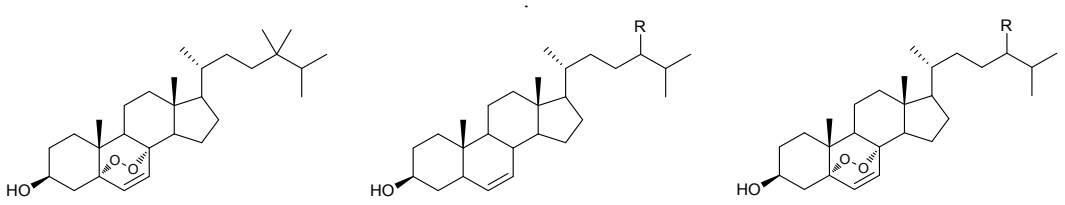
Table 2 Acute toxicity test of compound **1f**

Dose(mg/kg)	Mice Number Start/End	Death rate (%)	LD_{50} (mg/kg)	95%CIs
1000	6/6	100	362.46	233.83-561.85
750	6/6	100		
422	6/4	67		
350	6/3	50		
178	6/0	0		
56	6/0	0		

Table 3 The structures of cholesterol and sitosterol derivatives **1a-1f** and **2a-2f**

Compound	Structure	inventory	output	Yield
1a		Cholesterol 11.598g (30.00 mmol)	1a 11.616g (27.24mmol)	90.4%
2a		β-sitosterol 12.441g (30.00 mmol)	2a 12.921g (28.28mmol)	94.3%
1b		1a 4.284g (10.00 mmol)	1b 3.646g (8.55mmol)	85.5%
2b		2a 4.564g (10.00 mmol)	2b 3.394g (7.47mmol)	74.7%
1c		1b 2.132g (5mmol)	1c 1.64g (4.27mmol)	85.4%

2c		2b 2.272g (5mmol)	2c 1.87g (4.53mmol)	90.7%
1d		1b 1.279g (3mmol)	1d 0.48g (1.05mmol)	35.0%
2d		2b 1.363g (3mmol)	2d 0.543g (1.12mmol)	37.2%
1e		1c 1.153g (3mmol)	1e 0.94g (2.26mmol)	75.3%
2e		2c 1.237g (3mmol)	2e 1.182g (2.66mmol)	88.7%
1f		1e 0.624g (1.5mmol)	1f 0.535g (1.29mmol)	86.1%
2f		2e 0.667g (1.5mmol)	2f 0.547g (1.24mmol)	82.5%



SC($C_{29}H_{48}O_3$) Cholesterol:R=H ; Sitosterol:R=C_2H_5 Novel peroxide-bridged compounds

Figure 1. the structures of SC, cholesterol, β-sitosterol and the novel peroxo-bridged derivatives.

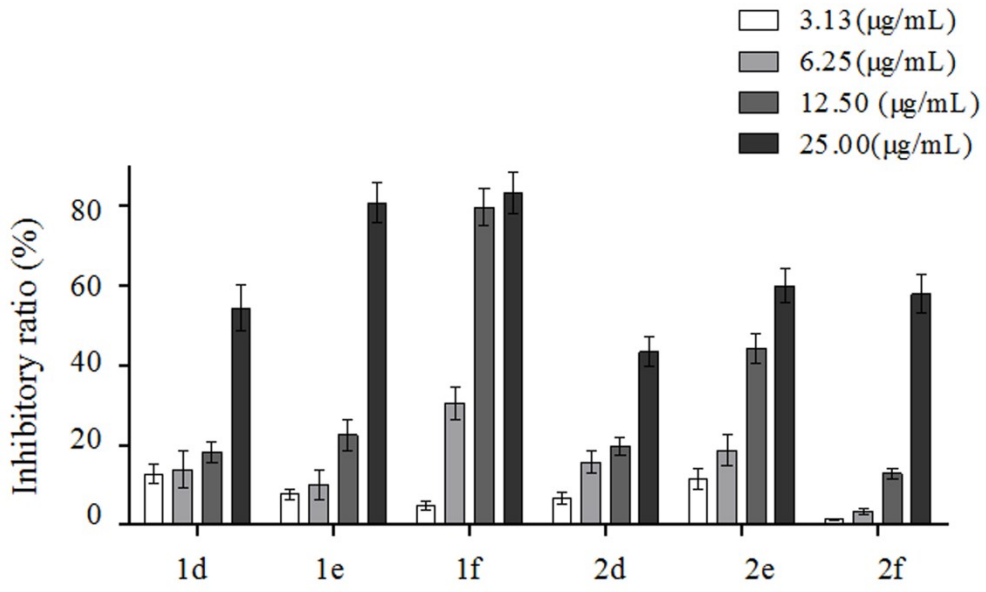


Figure 2. Cytotoxicity effect of **1d-1f** and **2d-2f** on HepG2.2.15 cells line

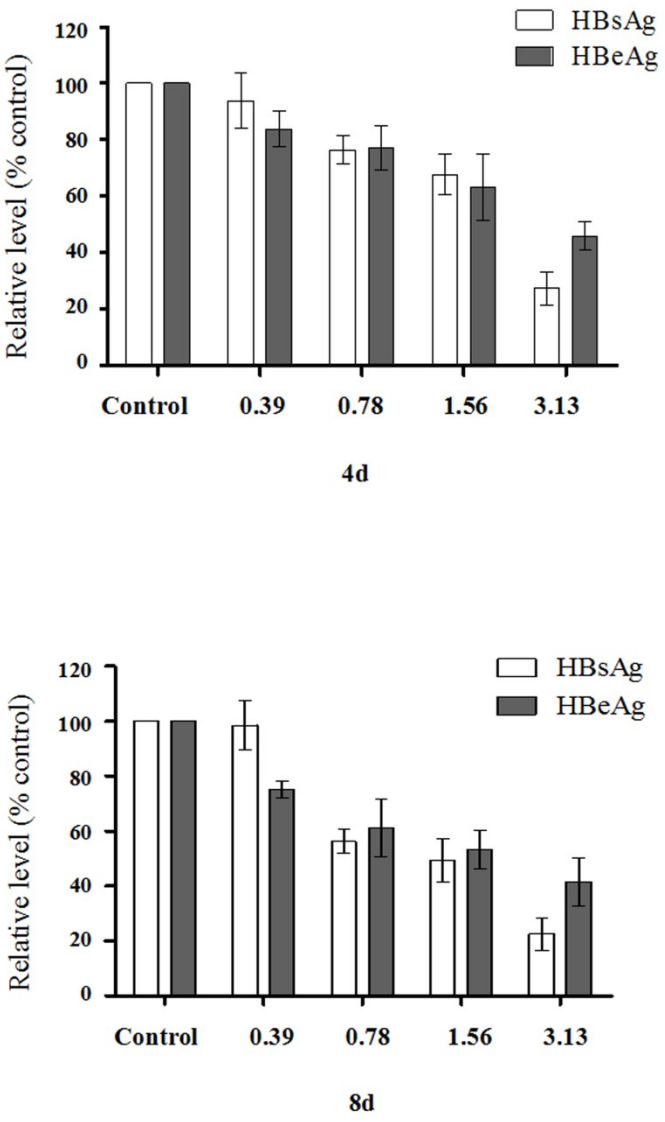


Figure 3. The inhibition ratio of compound **1f** on HBsAg and HBeAg in HepG2.2.15 cells.

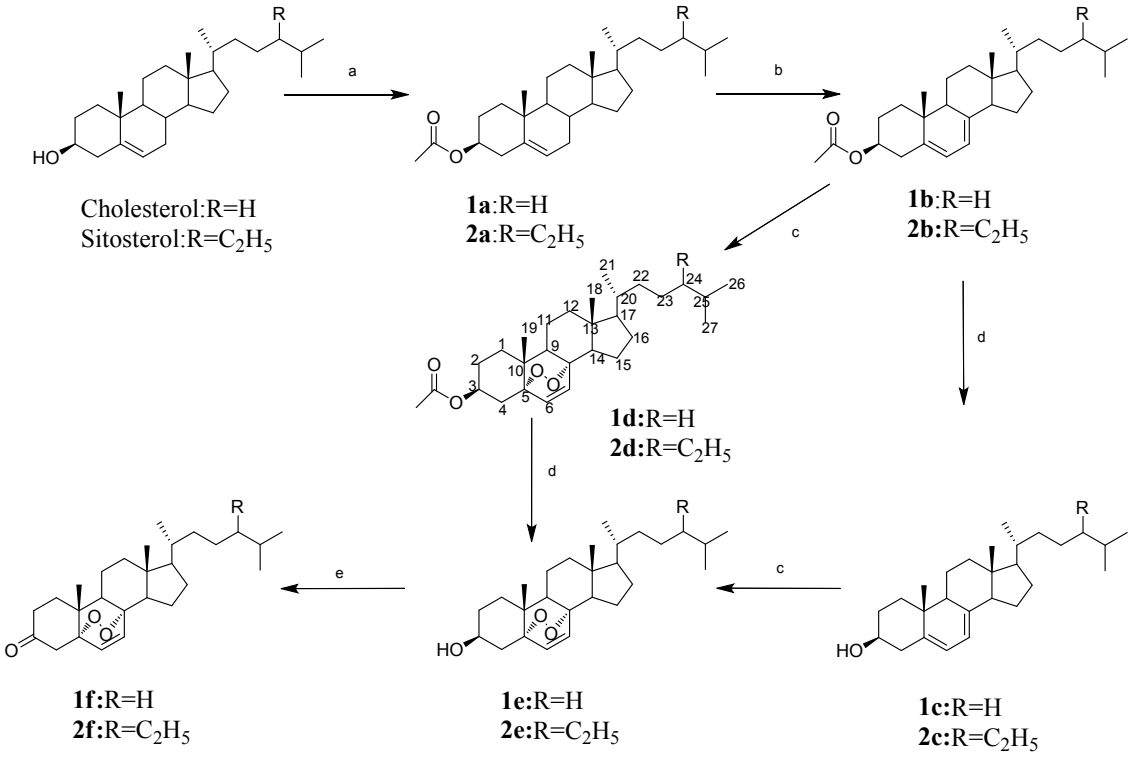
HepG2.2.15 cells were treated with four concentrations (0.39, 0.78, 1.56, and 3.13 μg/ml) of compound **1f** for 4d or 8d. The cultural media of each treatment were collected for viral HBsAg and HBeAg via ELISA analysis. The data are expressed as the mean and the standard deviation of the mean. ($n = 3$, $p<0.05$).

Synthesis

Materials and Methods

Reactions were monitored by TLC using silica gel coated aluminum sheets (Qingdao, China) and visualized in UV light (254 nm). ^{1}H-NMR and ^{13}C-NMR assays were recorded on a Bruker AVANCE 500 NMR spectrometer and chemical shifts are reported in form of δ (ppm). 2, 6-dimethyl-pyridine, CCl_4, N-bromobutanimide (NBS) was served as the solvent (Beijing, China). HRMS spectra were performed on High-resolution ESI mass spectrum (Solarix 9.4T, Bruker, Germany). Melting points were measured at a rate of 5 °C/min using an X-5 micro melting point apparatus (Beijing, China). Cellular morphologies were observed using an inverted fluorescence microscope

(Tokyo, Japan). Silica-gel column chromatography was performed using 200–300 mesh silica-gel. The yields were calculated based on the last step reaction. All solvents and chemicals used were analytical or high-performance liquid chromatography grade.



Scheme 1. Synthesis of the cholesterol and β-sitosterol derivatives. Reagents and Conditions: (a) Toluene, acetic anhydride, pyridine, reflux, 4h. (b) CCl_4, N-bromobutanimide (NBS), lighted, reflux, 4 h. (c) Eosin Y, anhydrous ethanol, lighted, r.t., 10 h. (d) Ethanol, 10% sodium hydroxide, 80 °C, 30 min. (e) Acetone, chromic acid, ice-bath, 3h.

The molecular formula of **1f** was assigned as $C_{27}H_{42}O_3$ through the basis of the positive high-resolution mass spectrometer (HR-MS) m/z 437.30087 $[M+Na]^+$. Especially, product **1f** was further identified by HMQC, HMBC and ^1H-^1H COSY 2D-NMR spectra. Combined with the significant HMQC, HMBC and ^1H-^1H COSY correlations, CH-6 [δH 6.53 (d, 1H, J = 8.5 Hz, =CH-)] was connected with δC 83.4 (C-5), and CH-7 [δH 6.27 (d, 1H, J = 8.5 Hz, =CH-)] was connected with δC 80.0 (C-8), which were similar with **1b**. Besides, there were δC 83.4 (C-5) and δC 80.0 (C-8) in the ^{13}C-NMR spectrum of compound **1f**, which were very different from while δC 141.6 (C-5) and δC 138.6 (C-8) in the ^{13}C-NMR spectrum of **1b**. It suggested that the peroxide bridge of compound **1f** was synthesized according to all of the above analysis. And This inference can also be applied to compounds **1d** and **1e**.

General procedure for the derivatives (1a-1f, 2a-2f method as shown in Scheme 1)

Preparation of compound 1a and 2a:

Cholesterol (30.00 mmol) or β- sitosterol (30.00 mmol) was added to a solution of toluene (60.00 mmol) and acetic anhydride (60.00 mL) in pyridine (12.41 mmol).The mixture stirred with heating at reflux until the crude material was absence. After cooling the reaction mixture, the combined organic layer were washed with 0.1% hydrochlochric for two times, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel to give desired products.

3β-O-Acetyl-cholesterol (1a). White solid, m.p.: 110.5-111.3 °C. ^{1}H-NMR (500MHz, $CDCl_3$, δ ppm): 0.69 (s, 3H, -C<u>H</u>$_3$), 0.88 (m, 3H, -C<u>H</u>$_3$), 0.91 (d, 3H, J = 6.5 Hz, -C<u>H</u>$_3$), 1.01 (s, 3H, -C<u>H</u>$_3$), 2.05 (s, 3H, -C<u>H</u>$_3$), 2.32 (d, J= 7.0 Hz, 2H), 4.63 (m, 1H), 5.37 (s, 1H, =C<u>H</u>-), 1.06–2.53 (29H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 170.6 (<u>C</u>=O), 140.0, 122.7, 74.0, 56.7, 56.1, 50.0, 42.3, 39.7, 39.5, 38.1, 37.0, 36.6, 36.2, 35.8, 31.9, 31.8, 28.2, 28.0, 27.8, 24.3, 23.8, 22.8, 22.5, 21.4, 21.0, 19.3, 18.7, 11.9. HRMS (ESI) *m/z*: 451.35465 $[M+Na]^+$. calcd. for $C_{29}H_{48}O_2$ 428.36543.

3β-O-Acetyl-β-sitosterol (2a). White solid, m.p.: 186.1-186.8 °C. ^{1}H-NMR (500MHz, $CDCl_3$, δ ppm): 0.67 (s, 3H, -C<u>H</u>$_3$), 0.80 (d, 3H, J = 7.0 Hz, -C<u>H</u>$_3$), 0.84 (d, 3H, J = 7.0 Hz, -C<u>H</u>$_3$), 0.91 (d, 3H, J = 6.0 Hz, -C<u>H</u>$_3$), 1.01 (s, 3H, -C<u>H</u>$_3$), 2.02 (s, 3H, -C<u>H</u>$_3$), 2.32 (d, J = 7.0 Hz, 2H), 4.62 (m, 1H), 5.37 (s, 1H, =C<u>H</u>-), 1.04–2.43 (30H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 170.5 (<u>C</u>=O), 139.7, 122.7, 74.0, 56.7, 56.7, 50.1, 42.4, 39.8, 38.2, 37.0, 36.6, 36.2, 36.2, 31.9, 31.9, 28.3, 28.2, 27.8, 24.3, 23.1, 21.4, 21.0, 19.3, 18.9, 16.2, 11.8. HRMS (ESI) *m/z*: 479.38595 $[M+Na]^+$. calcd. for $C_{31}H_{52}O_2$ 456.39673.

Preparation of compound 1b and 2b:

Compound **1a** or **2a** (10.00 mmol) and NBS (10.00 mmol) was dissolved in dry tetrachloromethane (30.00 mmol). The mixture was refluxed at 85 °C for 4h with magnetic stirring, and illuminated by the meantime until the material **1a** or **2a** was absence. The crude product was redissolved with hot ethylacetate then was filtered, and then the filtrate was cooled overnight to obtained crude crystals, which was purified by recrystallization from ethylacetate to produce a light yellow solid.

$^{\Delta}$5,7-diene-3β-O-Acetyl-cholesterol (1b) White solid, m.p.: 105.4-105.9 °C. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 0.61 (s, 3H, -C<u>H</u>$_3$), 0.87 (m, 3H, -C<u>H</u>$_3$), 0.95 (drs, 6H, -C<u>H</u>$_3$), 2.04 (s, 3H, -C<u>H</u>$_3$), 2.36 (d, J = 7.0 Hz, 2H), 4.70 (m, 1H), 5.37 (m, 1H, =C<u>H</u>-), 5.56 (m, 1H, =C<u>H</u>-), 1.06–2.53 (26H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 170.5 (<u>C</u>=O), 141.6, 138.5, 120.2, 116.2, 72.8, 55.8, 55.4, 46.0, 42.9, 39.5, 39.1, 37.9, 37.1, 36.6, 36.2, 36.1, 28.1, 28.1, 28.1, 23.9, 23.0, 22.8, 22.6, 21.5, 21.0, 18.9, 16.2, 11.8. HRMS (ESI) *m/z*: 449.33900 $[M+Na]^+$. calcd. for $C_{29}H_{46}O_2$ 426.34978.

3β-O-Acetyl-$^{\Delta}$5,7-diene-β-Sitosterol (2b) White solid, m.p.: 117.6-118.4 °C ^{1}H-NMR (500 MHz, CDCl3, δ ppm): 0.72 (s, 3H, -C<u>H</u>$_3$), 0.78 (m, 3H, -C<u>H</u>$_3$), 0.84 (d, 3H, J = 7.0 Hz, -C<u>H</u>$_3$), 0.96 (s, 3H, -C<u>H</u>$_3$), 0.98 (d, 3H, J = 6.5 Hz, -C<u>H</u>$_3$), 2.02 (s, 3<u>H</u>, -C<u>H</u>$_3$), 2.36 (m, 2H), 4.50 (m, 1H), 5.37 (m, 1H, =C<u>H</u>-), 5.60 (m, 1H, =C<u>H</u>-), 0.70–2.52 (27H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz $CDCl_3$, δ ppm): 170.5 (<u>C</u>=O), 141.6, 138.5, 120.2, 116.3, 72.8, 55.6, 54.5, 45.8, 42.9, 41.6, 41.0, 40.8, 39.1, 37.9, 37.1, 36.6, 36.5, 33.9, 28.1, 29.1, 26.1, 23.0, 21.0, 18.9, 19.7, 19.1, 17.1, 16.2, 12.0, 11.9. HRMS (ESI) *m/z*:477.37030 $[M+Na]^+$. calcd. for $C_{31}H_{50}O_2$ 454.38108.

Preparation of compound 1c and 2c:

Compound **1b** or **2b** was added (5.00 mmol) into ethanol (60.00 mL) and sodium hydroxide (12.50 mmol), then the mixture was heated to 80 °C with magnetic stirring until the end of the experiment. After extracting the solution with ethyl acetate, the organic layer was washed with distilled water to neutral. Then the organic layer was dried over anhydrous sodium sulfate and evaporating the solvent in vacuum pressure, the crude compound was obtained. Then the crude product was recrystallized from ethanol.

$^{\Delta}$5,7-diene-3β-O-cholesterol (1c). White solid, m.p.: 103.7-104.4 °C. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 0.62 (s, 3H, -C<u>H</u>$_3$), 0.86 (m, 3H, -C<u>H</u>$_3$), 0.87 (m, 3H, -C<u>H</u>$_3$), 0.94 (s, 3H, -C<u>H</u>$_3$), 1.90 (m, 3H, -C<u>H</u>$_3$), 2.27 (m, 2H), 3.63 (m, 1H), 5.39 (m, 1H, =C<u>H</u>-), 5.57 (m, 1H, =C<u>H</u>-), 0.62–2.47 (24H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 141.5, 139.8, 119.9, 116.3, 70.5, 55.9, 54.5, 46.3, 40.8, 39.5, 39.2, 38.4, 37.1, 36.2, 36.2, 36.2, 32.1, 28.1, 28.1, 23.9, 23.0, 22.8, 22.6, 21.2, 18.9, 16.3, 11.9. HRMS (ESI) *m/z*: 407.32844 $[M+Na]^+$. calcd. for $C_{27}H_{44}O$ 384.33922.

$^{\Delta}$5,7-diene-3β-β-Sitosterol (2c). White solid, m.p.: 117.0-118.0 °C. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 0.67 (s, 3H, -C<u>H</u>$_3$), 0.80 (brs, 3H, -C<u>H</u>$_3$), 0.84 (m, 6H, -C<u>H</u>$_3$), 0.94 (s, 3H, -C<u>H</u>$_3$), 1.00 (s, 3H, -C<u>H</u>$_3$), 2.27 (m, 2H), 3.60 (m, 1H), 5.37 (m, 1H, =C<u>H</u>-), 5.57 (m, 1H, =C<u>H</u>-), 0.62–2.50 (25H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 141.5, 139.7, 119.6, 116.2, 70.5, 55.9, 54.5, 46.2, 42.9, 39.1, 38.4, 37.2, 37.0, 36.5, 33.9, 29.1, 28.7, 28.7, 26.5, 23.1, 23.0, 21.0, 21.1, 19.8, 19.0, 18.9, 16.3, 12.0, 11.8. HRMS (ESI) *m/z*: 435.35974 $[M+Na]^+$. calcd. for $C_{29}H_{48}O$ 412.37052.

Preparation of compound 1d and 2d:

Product **1b** or **2b** (3.00 mmol) and Eosin Y (0.31 mmol) was added to anhydrous ethanol (100.00 mL). The air was bubbled into the liquid for approximately 2h by a micro air pump until the compound **1b** or **2b** was not

detected. Then the solvent was evaporated under vacuum, chromatographed on the silica gel column and eluted with ethyl acetate/petroleum ether (10/1) to give compound **1d** or **2d**.

3β-O-Acetyl-5α,8α-cyclicobioxygen-6-vinyl-cholesterol (1d). White solid, m.p.: 137.5-138.2 °C. ^{1}H-NMR (500MHz, $CDCl_3$, δ ppm): 0.79 (s, 3H, -C$\underline{H}_3$), 0.85 (d, J = 2.5 Hz, 3H, -C$\underline{H}_3$), 0.86 (d, J = 2.5 Hz, 3H, -C$\underline{H}_3$), 0.88 (s, 3H, -C$\underline{H}_3$), 2.01 (s, 3H, -C$\underline{H}_3$), 2.11 (m, 2H), 4.97 (m, 1H), 6.23 (d, J = 8.5 Hz, 1H, =C$\underline{H}$-), 6.49 (d, J = 8.5 Hz, 1H, =C$\underline{H}$-), 0.57–2.38 (26H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 170.1 (C=O), 135.4, 130.9, 81.7 (-C-O-O-), 79.3 (-C-O-O-), 69.5, 56.4, 51.5, 51.0, 44.7, 39.4, 39.4, 37.0, 35.9, 35.2, 34.3, 33.2, 28.2, 28.0, 26.2, 23.8, 23.4, 22.8, 22.5, 21.3, 20.6, 18.6, 18.1, 12.6. HRMS (ESI) *m/z*: 481.32718 $[M+Na]^+$. calcd. for $C_{29}H_{46}O_4$ 458.33961.

3β-O-Acetyl-5α,8α-cyclicobioxygen-6-vinyl-β-Sitosterol (2d). White solid, m.p.: 186.1-186.8 °C. ^{1}HNMR (500 MHz, $CDCl_3$, δ ppm): 0.79 (s, 3H, -C$\underline{H}_3$), 0.84 (brs, 6H, -C$\underline{H}_3$), 0.89 (brs, 6H, -C$\underline{H}_3$), 2.01 (s, 3H, -C$\underline{H}_3$), 2.12 (m, 2H), 4.98 (m, 1H), 6.28 (d, 1H, J = 7.0 Hz, =C$\underline{H}$-), 6.50 (d, 1H, J = 7.0 Hz, =C$\underline{H}$-), 0.75–2.15 (27H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 170.1 (C=O), 135.0, 130.9, 81.7 (-C-O-O-), 79.4 (-C-O-O-), 69.5, 56.3, 51.5, 51.0, 45.8, 44.7, 39.4, 37.0, 35.9, 34.3, 33.7, 33.2, 29.1, 28.2, 28.2, 26.2, 23.4, 23.0, 21.3, 20.6, 19.8, 19.0, 18.6, 18.1, 12.6, 12.0. HRMS (ESI) *m/z*: 509.35904 $[M+Na]^+$. calcd. for $C_{31}H_{50}O_4$ 486.37091.

Preparation of compound 1e and 2e:

There are two routes to get compound **1e** or **2e**.

Route (1): Compound **1c** or **2c** (3.00 mmol) was dissolved in anhydrous ethyl alcohol (100.00 mL), and Eosin Y (0.31 mmol) was added. In this way, the yield of was 75.3%.

Route (2): Compound **1d** or **2d** (0.50 mmol), ethanol (30.00 mL) and 10% sodium hydroxide solution (2.50 mL) were added to a flask. The residue was chromatographed on silica gel column to give compound **1e** or **2e**, and the yield was 90.1%.

5α,8α-cyclicobioxygen-6-vinyl-3β-cholesterol (1e). White solid, m.p.: 154.8-155.4 °C. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 0.85 (s, 3H, -C$\underline{H}_3$), 0.88 (d, 3H, J = 2.0 Hz, -C$\underline{H}_3$), 0.89 (d, 3H, J = 2.0 Hz, -C$\underline{H}_3$), 0.92 (d, 3H, J = 6.5 Hz, -C$\underline{H}_3$), 1.07 (s, 3H, -C$\underline{H}_3$), 1.90 (m, 2H), 3.97 (m, 1H), 6.29 (d, 1H, J = 8.5 Hz, =C$\underline{H}$-), 6.59 (d, 1H, J = 8.5 Hz, =C$\underline{H}$-), 0.75–2.16 (24H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 135.4, 130.8, 82.2 (-C-O-O-), 79.5 (-C-O-O-), 66.5, 56.4, 51.6, 51.1, 44.7, 39.4, 39.4, 37.0, 36.9, 36.0, 35.2, 34.7, 30.1, 28.2, 28.0, 23.8, 23.4, 22.8, 22.6, 20.6, 18.6, 18.2, 12.7. HRMS (ESI) *m/z*: 439.31665$[M+Na]^+$. calcd. for $C_{27}H_{44}O_3$ 416.32905.

5α,8α-cyclicobioxygen-6-vinyl-3β-β-Sitosterol (2e). White solid, m.p.: 138.9-140.5 °C. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 0.79 (s, 3H, -C$\underline{H}_3$), 0.82 (d, 3H, J = 4.0 Hz, -C$\underline{H}_3$), 0.83 (s, 3H, -C$\underline{H}_3$), 0.87 (s, 3H, -C$\underline{H}_3$), 0.90 (d, 3H, J = 6.5 Hz, -C$\underline{H}_3$), 1.20 (m, 3H, -C$\underline{H}_3$), 1.90 (m, 2H), 3.96 (m, 1H), 6.25 (d, 1H, J = 8.5 Hz, =C$\underline{H}$-), 6.49 (d, 1H, J = 8.5 Hz, =C$\underline{H}$-), 0.75–2.16 (25H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 135.4, 130.8, 81.2 (-C-O-O-), 79.5 (-C-O-O-), 66.5, 56.3, 51.6, 51.0, 45.8, 44.7, 39.4, 37.0, 36.9，35.6, 34.7, 33,7, 30.1, 29.1, 28.3, 26.0, 23.4, 23.0, 20.6, 19.8, 19.0, 18.6, 18.1, 12.6, 12.0. HRMS (ESI) *m/z*: 467.34860 $[M+Na]^+$: calcd. for $C_{29}H_{48}O_3$ 444.36035.

Preparation of compound 1f and 2f:

Compound **1e** or **2e** (1.50 mmol) was dissolved by acetone (50.00 mL), equipped with dropping funnel, surrounded by an ice-bath. The chromic acid solution (1.60 mmol) that had already been prepared was added into the mixture, and continued stirring 1h. Then the contents were poured into a breaker with chipped ice, the solid was separated by filtration. The residue was purified by silica gel column chromatograph and recrystallized from alcohol.

5α,8α-cyclicobioxygen-6-vinyl-3-cholesterone (1f). White solid, m.p.: 127.1-127.7 °C. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 0.82 (s, 3H, -C$\underline{H}_3$), 0.85 (m, 3H, -C$\underline{H}_3$), 0.90 (d, 3H, J = 6.5 Hz, -C$\underline{H}_3$), 1.05 (s, 3H, -C$\underline{H}_3$), 1.56 (m, 3H, -C$\underline{H}_3$), 1.92 (m, 2H), 2.86 (m, 1H), 6.27 (d, 1H, J = 8.5 Hz, =C$\underline{H}$-), 6.53 (d, 1H, J = 8.5 Hz, =C$\underline{H}$-), 0.74–2.90 (22H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 207.0 (C=O), 134.3, 131.6, 83.4 (-C-O-O-), 80.0 (-C-O-O-), 56.4, 51.4, 51.1,44.9, 43.6, 39.4, 39.4, 39.3, 37.3, 36.7, 35.9, 35.2, 28.3, 28.0, 23.8, 23.5, 22.8, 22.5, 20.5, 18.7, 17.5, 12.8. HRMS (ESI) *m/z*: 437.30087 $[M+Na]^+$. calcd. for $C_{27}H_{42}O_3$ 414.31340.

5α,8α-cyclicobioxygen-6-vinyl-3β-β-Sitosterol (2f). White solid, m.p.: 127.1-127.7 °C. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 0.82 (m, 9H, -C$\underline{H}_3$), 0.92 (d, 3H, J = 6.5 Hz, -C$\underline{H}_3$), 1.05 (s, 3H, -C$\underline{H}_3$), 1.57 (m, 3H, -C$\underline{H}_3$), 1.94 (m, 2H), 2.86 (m, 1H), 6.27 (d, 1H, J = 8.5 Hz, =C$\underline{H}$-), 6.58 (d, 1H, J = 8.5 Hz, =C$\underline{H}$-), 0.80–2.90 (23H, methyl- and methylene- of steroid structure). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 207.0 (C=O), 134.3, 131.6, 83.4 (-C-O-O-), 79.9 (-C-O-O-), 56.3, 51.5, 51.2, 45.8, 44.9, 43.7, 39.4, 37.4, 36.8, 35.6, 35.3, 33.7, 29.1, 28.3, 26.0, 23.5, 23.1, 20.6, 19.9 191, 18.7, 17.5, 12.8, 12.0. HRMS (ESI) *m/z*: 443.35104$[M+H]^+$.calcd. for $C_{29}H_{46}O_3$ 442.34470.

The optical rotation data of all six compounds were showed in Table 3. The optical rotation were measured by

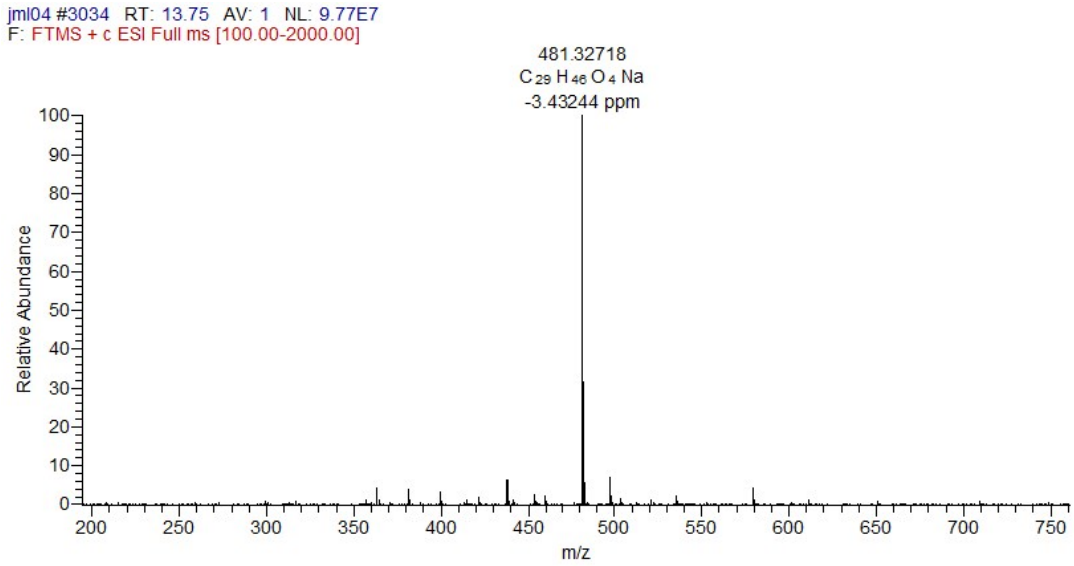
AUTOPOLⅢ AUTOMATIC POLARIMETER (RUDOLPH RESEARCH ANALYTICAL).

Table 3 The optical rotation of the compounds

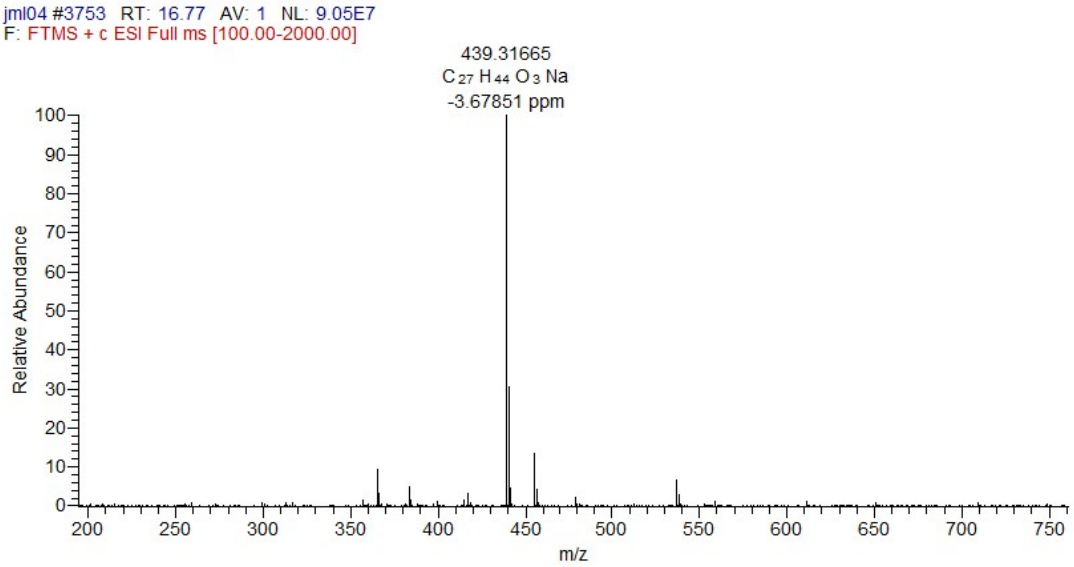
Compound	The optical rotation
1d	**+5.6**
1e	**+6.9**
1f	**+77.5**
2d	**+124.8**
2e	**+67.9**
2f	**+83.7**

Here we list the HRMS spectrogram of these six compounds with peroxide bridge.

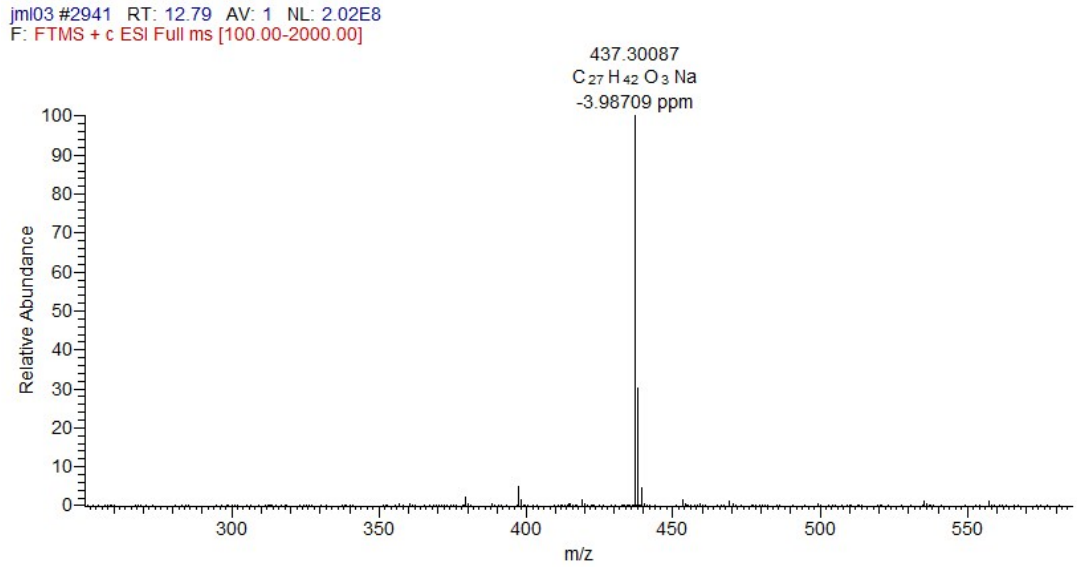
1d: $C_{29}H_{46}O_4$:458.33906____[M+Na]:481.32718



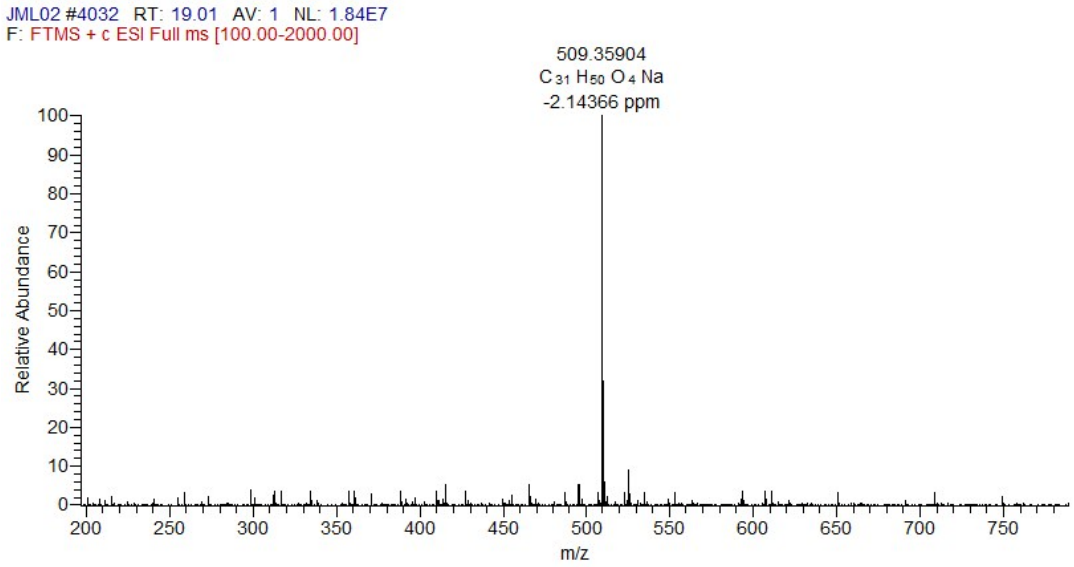
1e:$C_{27}H_{44}O_3$:416.32850____[M+Na]:439.31665



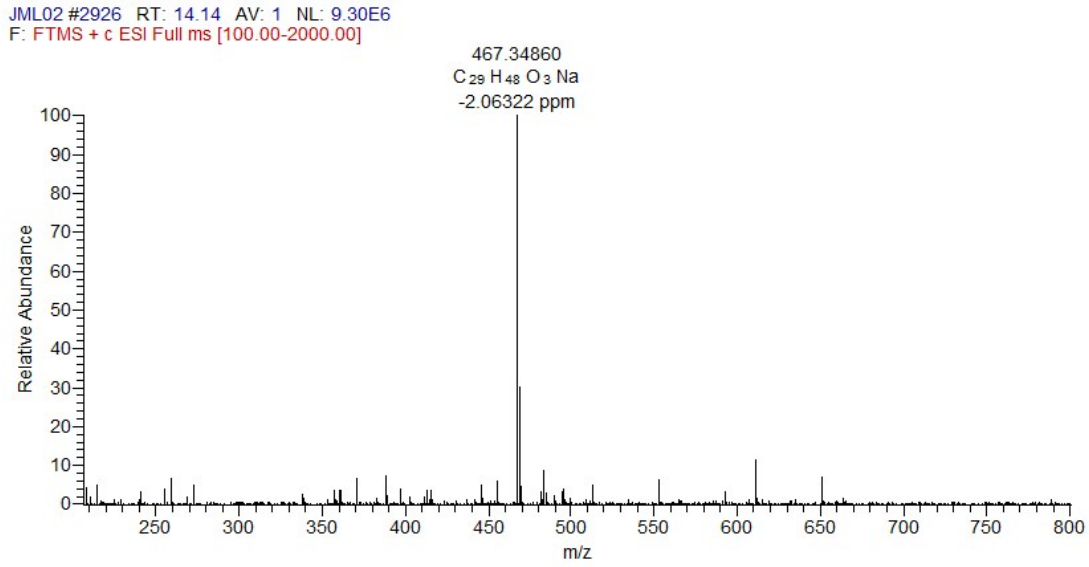
1f:$C_{27}H_{42}O_3$:414.31285____[M+Na]437.30087



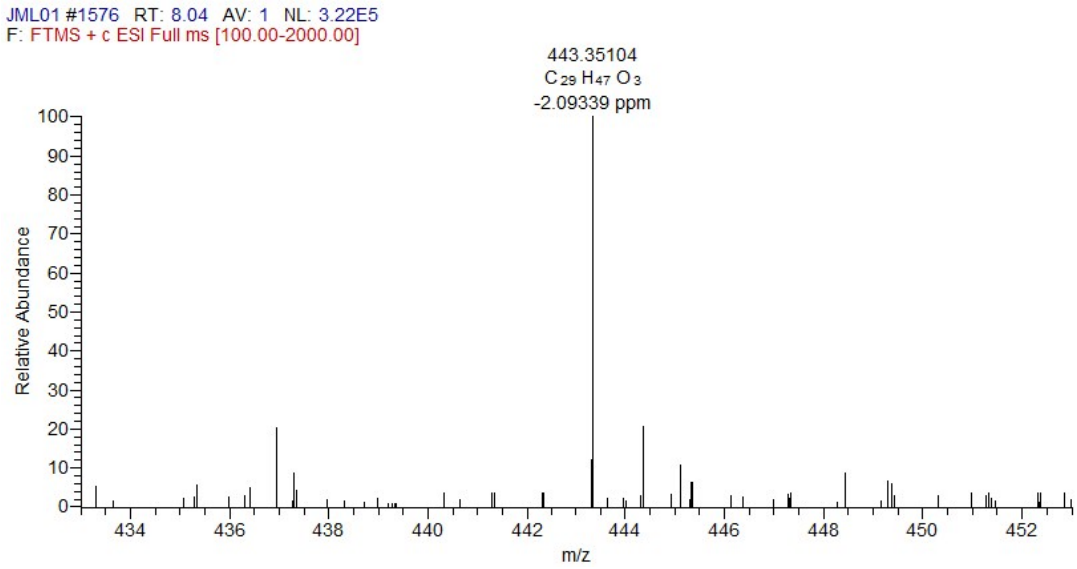
2d: $C_{31}H_{50}O_4$:486.37036____[M+Na]:509.35904



2e:$C_{29}H_{48}O_3$:444.35980____[M+Na]:467.34860



2f: $C_{29}H_{46}O_3$:442.34415____[M+H]:443.35104



All the spectra of the compound 1f

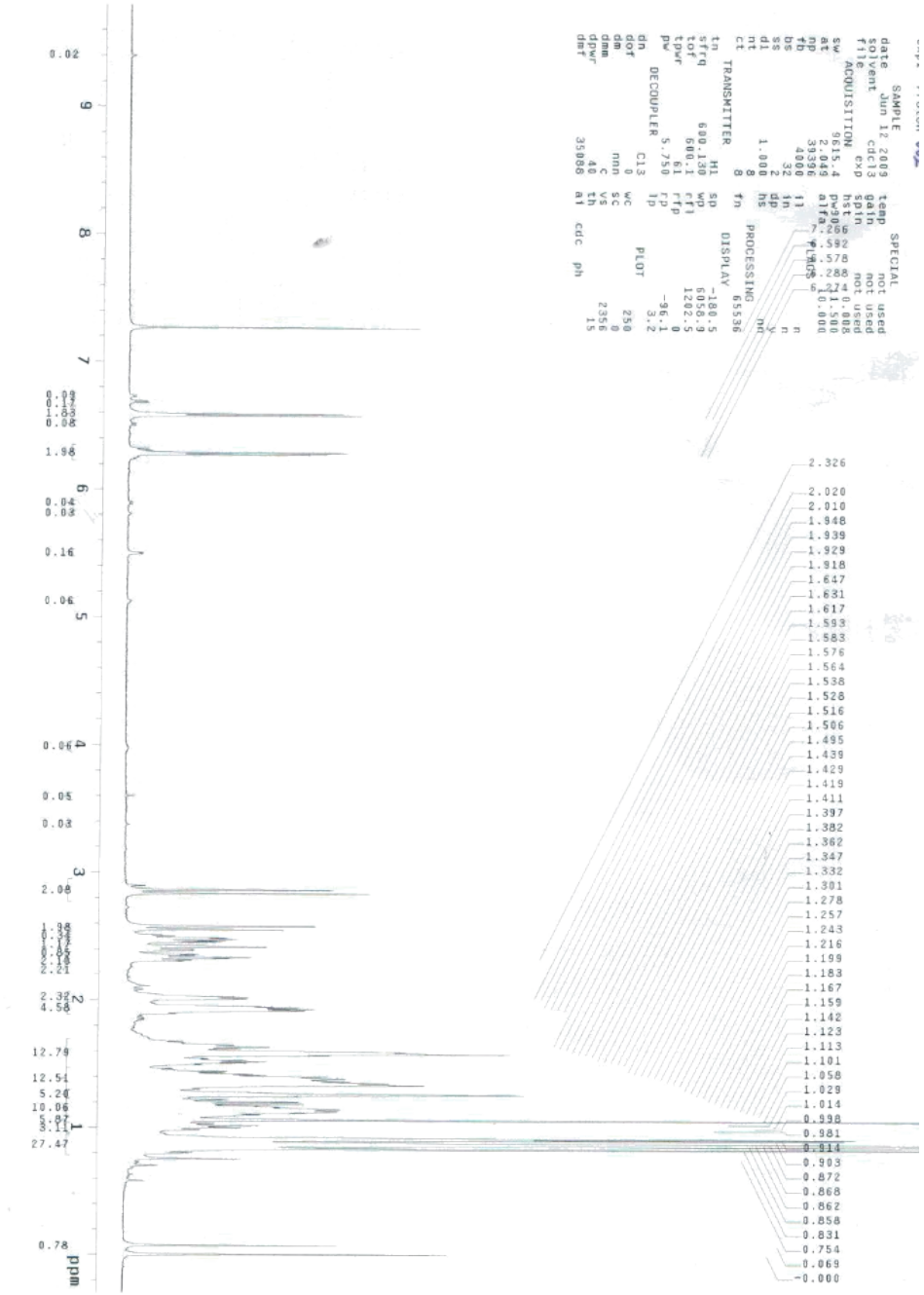


Figure 4 ^{1}H-NMR of the compound **1f**

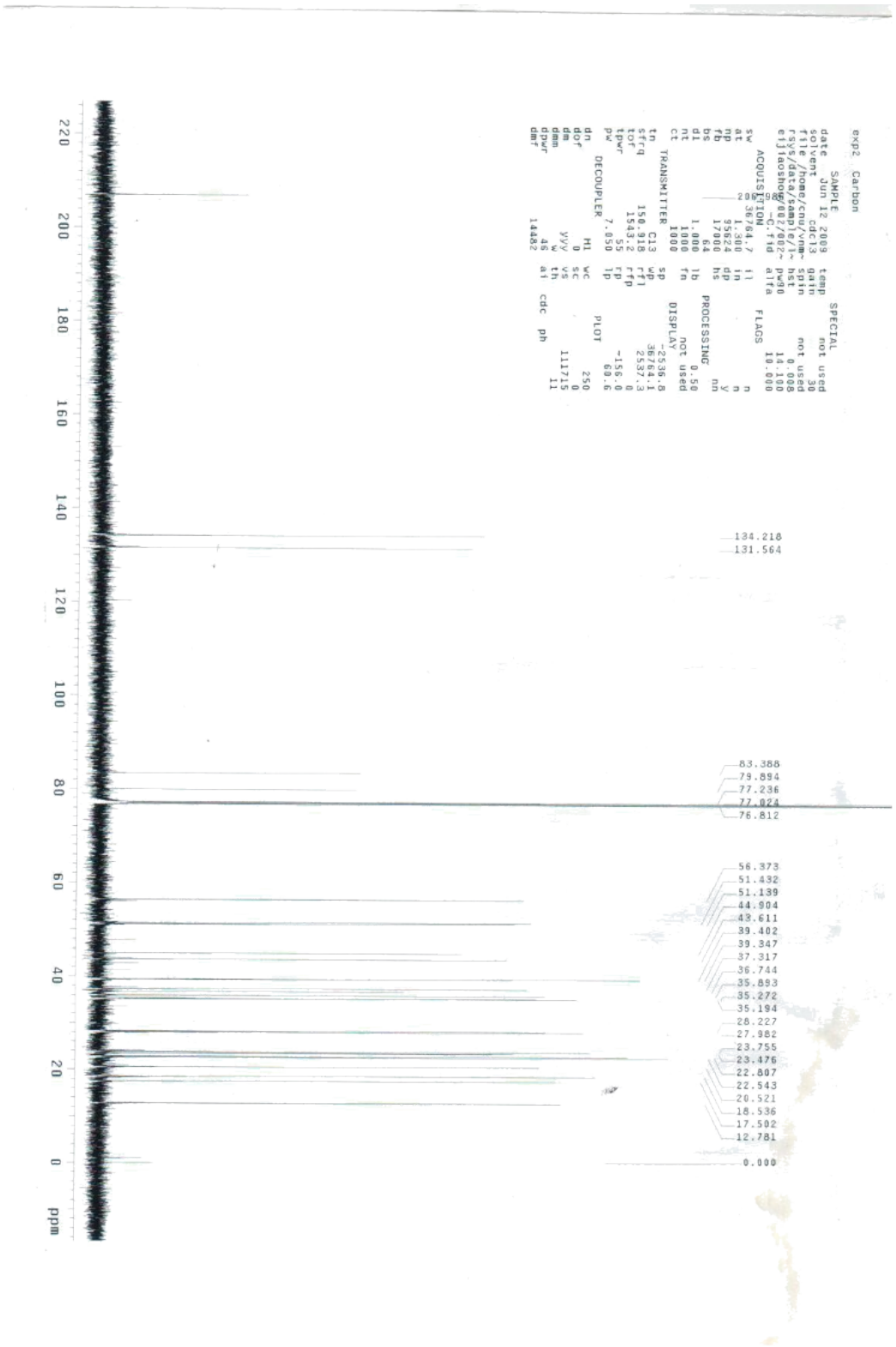


Figure 5 ^{13}C -NMR of the compound **1f**

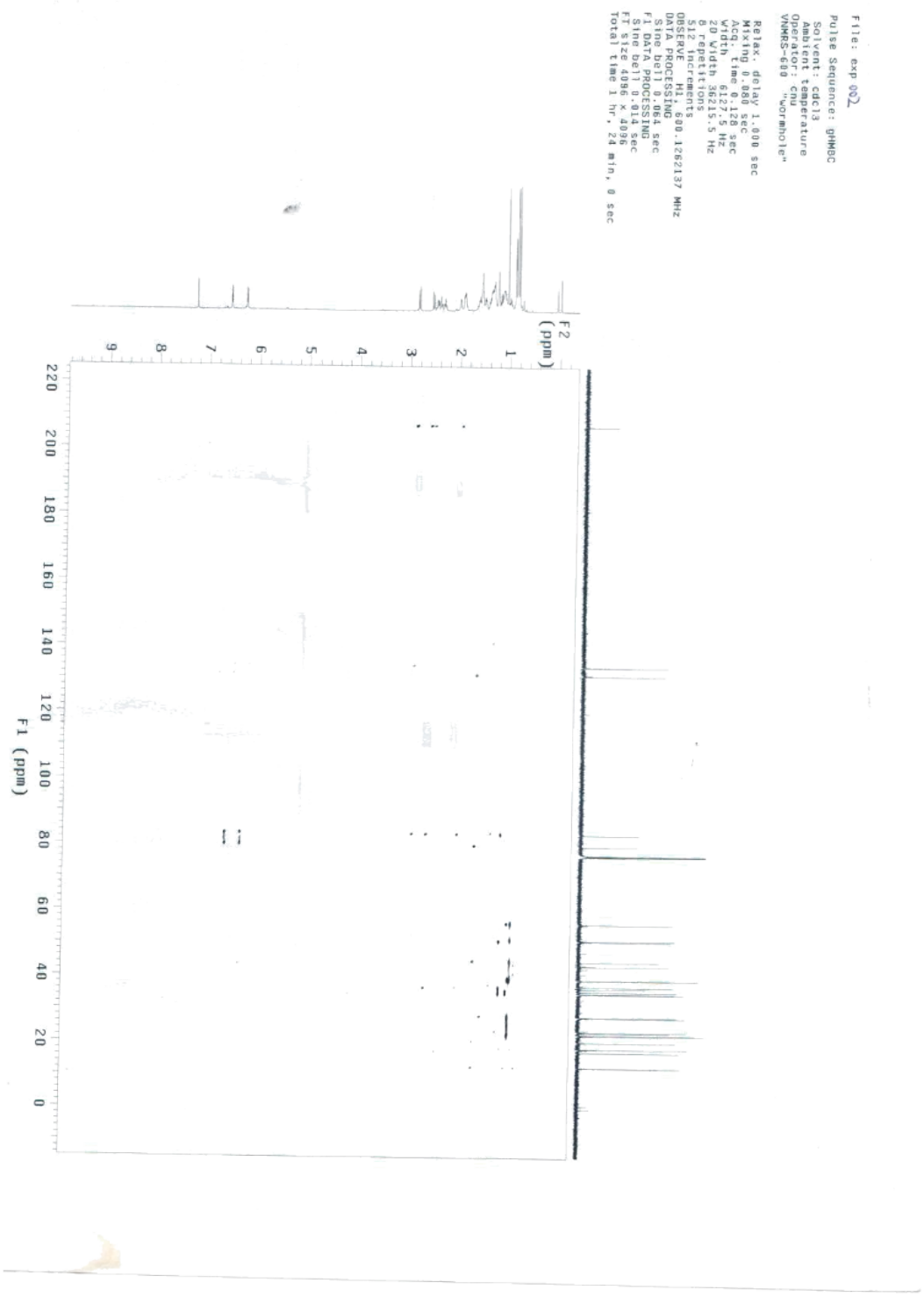


Figure 6HMBC of the compound **1f**

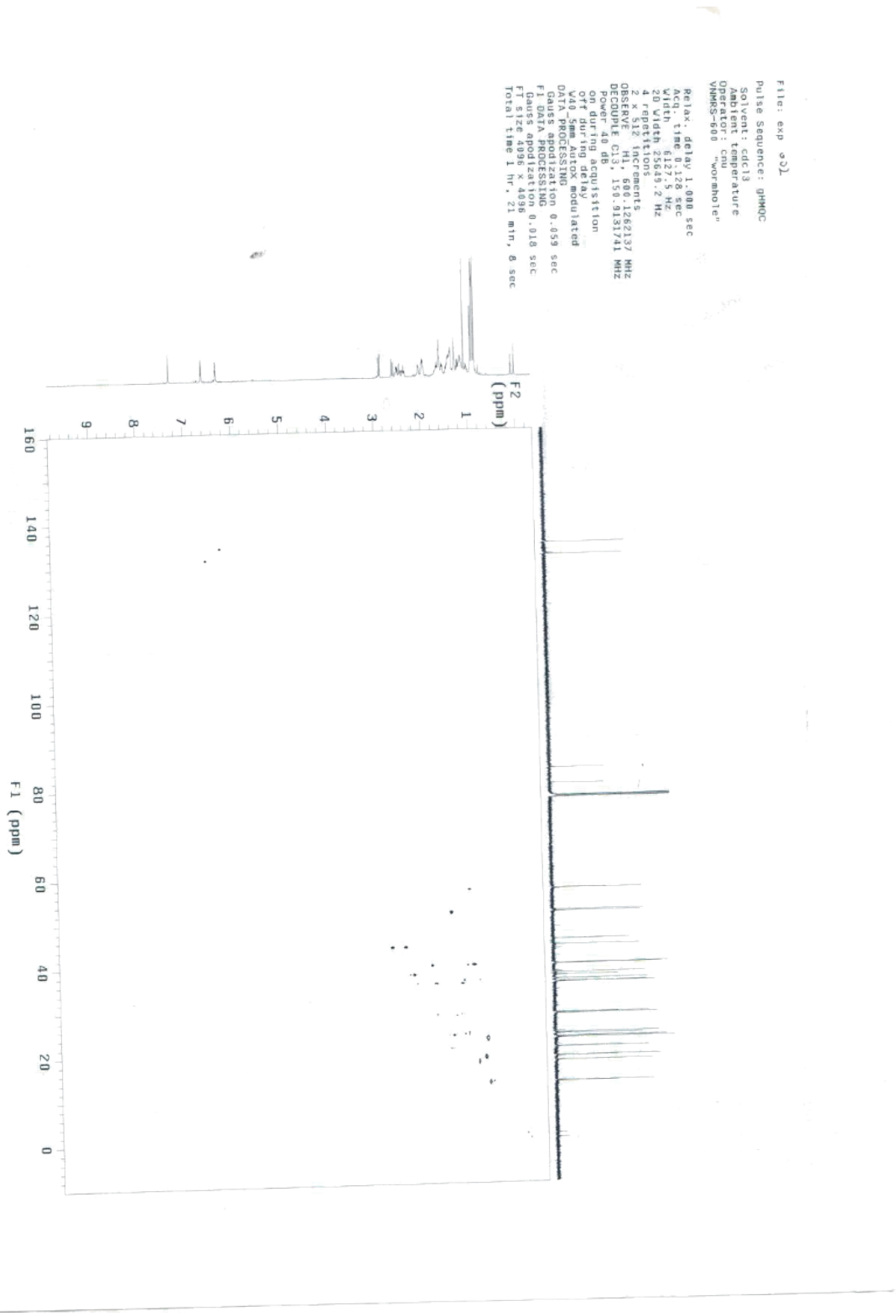


Figure 7 HMQC of the compound **1f**

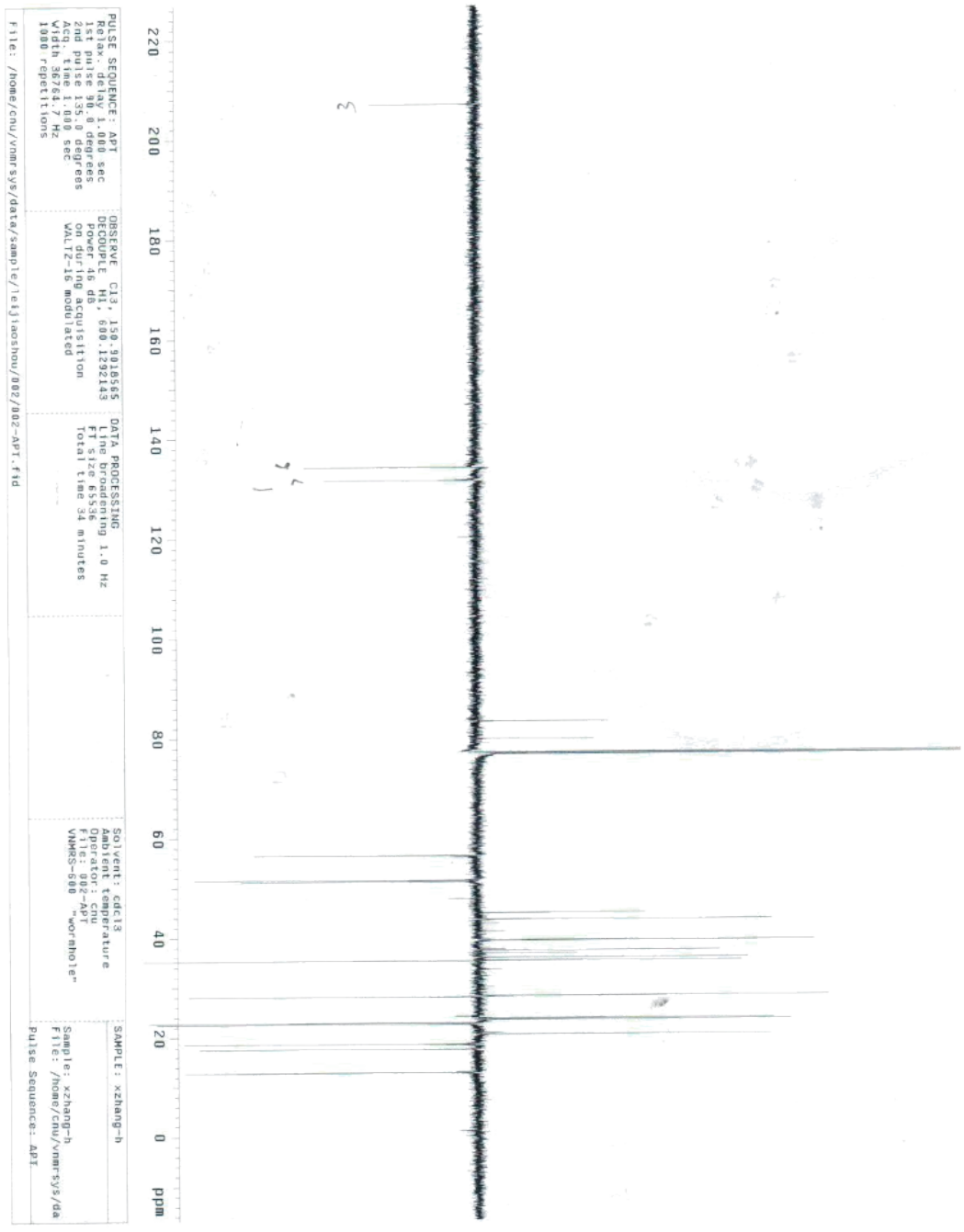


Figure 8 ^{1}H-^{1}H COSY of the compound **1f**