Supporting Information

Phorbasones A and B, Sesterterpenoids Isolated from the Marine Sponge *Phorbas* sp. and Induction of Osteoblast Differentiation

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Contents

General Experimental Method

General Instruments

Optical rotations were measured on a JASCO P-1010 polarimeter with a 5 cm cell. UV spectra were obtained in MeOH using a Varian Cary 50 and IR spectra were measured on a JASCO FT/IR 4100 spectrometer. NMR spectra of compounds **1** and **2** were recorded on a Varian VNMRS 500 spectrometer in CD_3OD solutions, and those of compound **3** on a Bruker AVANCE 400 spectrometer in CD_3OD solutions. The measured concentrations of phorbasone A and B were 3 mg / 300 μl and 2 mg / 300 μl, respectively. Chemical shifts of the proton and carbon spectra measured in CD_3OD solution were reported in reference to residual solvent peaks at 3.30 ppm and 49.0 ppm, respectively. HPLC was carried out on a Varian ProStar 230 solvent delivery system with ProStar 355 Refractive Index detector. High resolution mass spectra were obtained on a JEOL JMS-700 spectrometer at Korea Basic Science Research Institute, Daegu, Korea.

Animal Material

The specimen of *Phorbas* sp. (Sample No. 08G-11) were collected by hand using SCUBA at Gageo Island at 25m depth in July 2008, Southwest Sea, Korea. The sponge is massive, measure 30 × 20 ㎜ and 15 ㎜ thick. The surface has many oscular bearing papillae. Oscules are rare. The texture is soft. The colour in life is red and gradually changing to dark brown in alcohol. In skeleton, megascleres are tornotes (315-440 × 4-8 ㎛), acanthostyles (227-460 × 8-15 ㎛), and microscleres are isochelaes (37.5-50 ㎛). A voucher specimen (registry No. Spo. 50) is deposited at the Natural History Museum, Hannam University, Korea.

Extraction and Isolation

The collected specimen was frozen on site and delivered to the laboratory under dry ice, and then kept in a refrigerator at -25℃ until study. Freshly thawed sponge was cut into small pieces and extracted twice with MeOH at room temperature. The methanolic extract was partitioned between

CH_2Cl_2 and H_2O solvents. The organic layer was evaporated under reduced pressure and repartitioned between *n*-hexane and 15% aqueous MeOH for defatting. Then the aqueous MeOH fraction (*ca* 5 g) was subjected to reversed phase silica gel flash column chromatography eluting with solvents of decreasing polarity (MeOH / H_2O = 50 / 50; 60 / 40; 70 / 30; 80 / 20; 90 / 10; 100% MeOH; 100% acetone) to give seven fractions. The fraction eluted with MeOH / H_2O (90 / 10) solvent consisted in large part of phorbaketal A compound reported by our group and showed conspicuous effect on calcium deposition in mesenthymal C3H10T1/2 cells. This fraction (~1.4 g) was separated by reversed phase preparative HPLC [C18, YMC ODS-H80; column, 150 mm × 20 mm ID) eluting with AcCN / H_2O (65 / 35) with a flow rate of 5.5 ml / min] to give the major compound, phorbaketal A (1.0 g) and a minor compound, phorbasone A (**1**, 22 mg) at a retention time of 43 min. Additionally, phorbasone B (**2**, 5.0 mg) was isolated from the MeOH / H_2O (80 / 20) solvent fraction by reversed phase semi-preparative HPLC [C18, YMC ODS-A; column, 250 mm × 10 mm ID) eluting with MeOH / H_2O (70 / 30) with a flow rate of 2.0 ml / min].

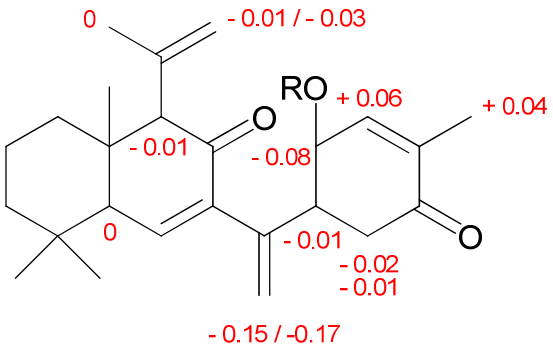
Phorbasone A (**1**): yellowish oil, $[\alpha]^{25}_{D}$ -107.2 (*c* 0.15 MeOH); IR (film) ν_{max} 3433, 2926, 1672, 1448 cm^{-1}; UV (MeOH) λ_{max} 225 nm (log ε 4.05), 261 (log e 3.69), 273 (log e 3.61), 285 (log e 3.10);; HRFABMS *m/z* 405.2410 $[M + Na]^+$ (calcd for $C_{25}H_{34}O_3Na$, 405.2406); ^1H and ^{13}C NMR data is given in Table 1.

Phorbasone B (**2**): colorless oil, $[\alpha]^{25}_{D}$ -47.1 (*c* 0.10 MeOH); IR (film) ν_{max} 3409, 2928, 1664, 1447 cm^{-1}; UV (MeOH) λ_{max} 230 nm (log ε 3.90), 257 (log e 3.76), 268 (log e 3.75), 280 (log e 3.62); HRFABMS *m/z* 439.2460 $[M + Na]^+$ (calcd for $C_{25}H_{36}O_5Na$, 439.2460); ^1H and ^{13}C NMR data is given in Table S1.

MTPA reaction of Phorbasone A(1)

To a stirred solution of phorbasone A (**1**, 3 mg) and dried pyridine (20 μl) in dry CH_2Cl_2 (0.5 ml) at room temperature, *R*-MTPA-Cl (150 μl) was added. The reaction progress was monitored by TLC chromatography on silica gel (Hex : EtOAc = 4 : 1). After ~ 10 hrs, the reaction mixture was quenched by the addition of H_2O and dimethyl ether. The organic layer was concentrated in *vacuo*.

The mixture was subjected on silica-phased HPLC using hexane / ethyl acetate (5 : 1) to give the *S*-MTPA-ester **1*S***. ^{1}H NMR (500MHz, $CDCl_3$) δ 6.84 (dd, *J* = 5.9, 1.5 Hz, H-14), 6.76 (d, *J* = 2.0 Hz, H-6), 5.39 (dd, *J* =5.9, 2.5 Hz, H-13), 5.09 (s, H-24), 5.02 (s, H-18), 4.81 (s, H-18), 4.79 (s, H-24), 3.60 (brd, *J* = 12.0 Hz, H-12), 2.71 (dd, *J* = 16.6, 12.0 Hz, H-17), 2.65 (dd, *J* = 16.6, 4.9 Hz, H-17), 2.58 (s, H-9), 2.56 (d, *J* = 2.0 Hz, H-5), 1.87 (s, H_3-22), 1.83 (d, *J* = 1.5 Hz, H_3-25). In an entirely analogous way, the *R*-MTPA-ester **1*R*** was obtained using *S*-MTPA-Cl.: ^{1}H NMR (500MHz, $CDCl_3$) δ 6.78 (dd, *J* = 5.9, 1.5 Hz, H-14), 6.79 (d, *J* = 2.0 Hz, H-6), 5.47 (dd, *J* =5.9, 2.7 Hz, H-13), 5.17 (s, H-18), 5.10 (s, H-24), 4.98 (s, H-18), 4.82 (s, H-24), 3.61 (brd, *J* = 12.5 Hz, H-12), 2.73 (dd, *J* = 16.6, 12.5 Hz, H-17), 2.66 (dd, *J* = 16.6, 4.4 Hz, H-17), 2.59 (s, H-9), 2.56 (d, *J* = 2.0 Hz, H-5), 1.87 (s, H_3-22), 1.79 (d, *J* = 1.5 Hz, H_3-25).



R = *S*-MTPA (**1*S***)
R-MTPA (**1*R***)

^{1}H NMR chemical shifts differences ($\Delta\delta^{S-R}$) in ppm for S/R-MTPA esters of **1**.

Synthesis of 3: Acetal formation of Phorbasone A(1)

A solution of HCl in anhydrous MeOH (6.5 µl, 0.1 M) was added to a stirred solution of phorbasone A (5 mg) in anhydrous MeOH (1 ml) at room temperature. The resulting reaction mixture was stirred for 24 hours at room temperature. The reaction was quenched with saturated aqueous solution of $NaHCO_3$ (3 ml). The crude product was extracted with ethyl acetate (5 ml x 3). The combined organic layer was dried over sodium sulfate, filtered, and concentrated in *vacuo*. The crude product mixture was purified by silica gel column chromatography with hexane / ethyl acetate (10 / 1) to afford the cyclized phorbasone A (**3**, 2.5mg, 48%).

Cell culture and osteoblast differentiation

C3H10T1/2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (100 units/ml penicillin, 100ug/ml streptomycin). To induce osteoblast differentiation, C3H10T1/2 cells were seeded on 24 well culture plates at a density of 2×10^4 cells/cm² and 48hr later the culture media were changed with DMEM containing 50 μM ascorbic acid, 10 mM β-glycerophosphate and 10% FBS (Differentiation media) for 6 days. The media (Differentiation media) was replaced every 2 days.

Calcium deposition activity

Calcium deposition activity was analyzed by Alizarin Red S staining. Briefly, cell layers were washed twice with cold PBS, fixed with 70 % ethanol at –20 °C for 1 h and then rinsed once with cold PBS before staining with a 40 mM Alizarin Red S (Sigma) solution in water for 20 min. Staining was followed by three washes with distilled water and one with 70% ethanol.

Table S1. The spectral data for Phorbasone B (2).

No	δ_C	δ_H, multi (*J* Hz)
1a	37.6, CH_2	1.37, br d (13.2)
1b		1.41, ddd (13.2, 13.2, 3.4)
2a	19.4, CH_2	1.53, br d (13.2)
2b		1.78, m
3a	42.3, CH_2	1.27, m
3b		1.54, m
4	33.7, C	
5	49.3, CH	2.59, d (2.0)
6	152.2, CH	7.31, d (2.0)
7	139.8, C	
8	202.0, C	
9	71.2, CH	2.52, s
10	43.0, C	
11	79.6, C	
12	42.2, CH	2.85, dd (13.7, 2.7)
13	65.3, CH	4.66, dd (5.9, 1.7)
14	144.2, CH	6.76, dq (5.9, 1.2)
15	137.5, C	
16	202.5, C	
17a	35.3, CH_2	2.06, dd (13.7, 2.7)
17b		2.81, dd (13.7, 13.7)
18a	67.2, CH_2	3.96, d (10.7)
18b		4.00, d (10.7)
19	33.3, CH_3	1.09, s
20	23.5, CH_3	1.02, s
21	23.4, CH_3	1.12, s
22	27.6, CH_3	1.83, s
23	141.7, C	
24a	115.3, CH_2	4.94, s
24b		5.01, br s
25	15.5, CH_3	1.75, d (1.2)

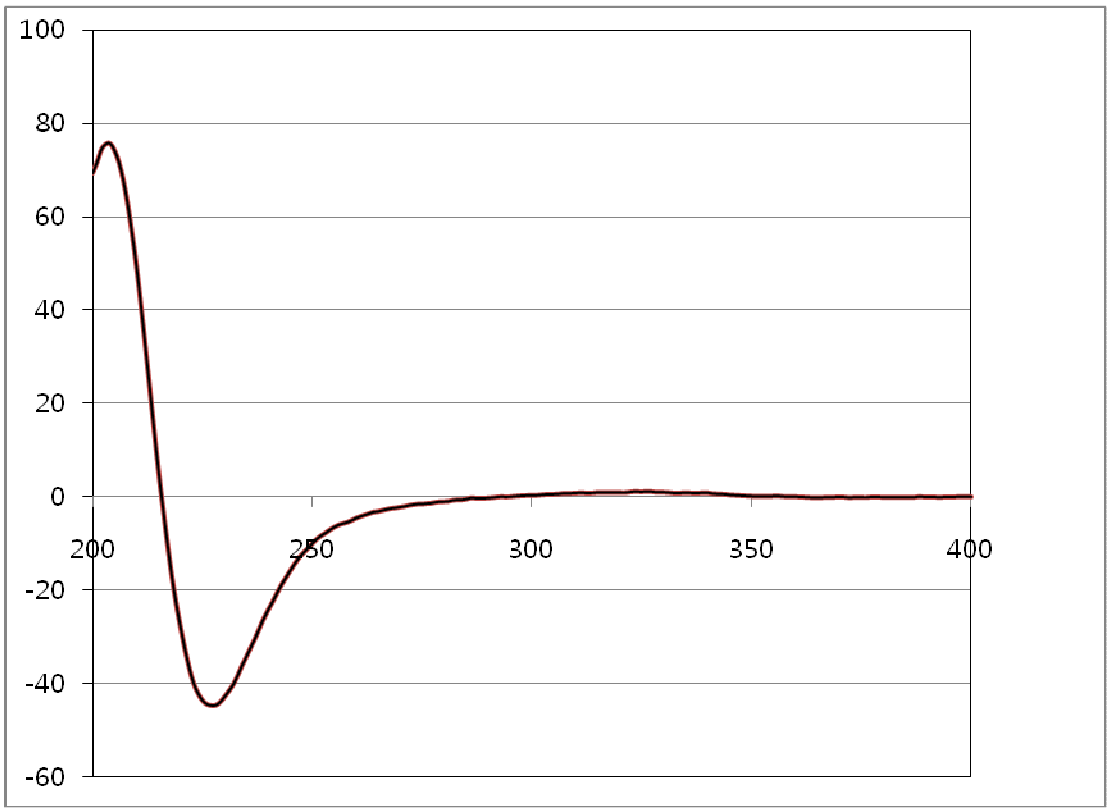
All assignments were performed using ^1H, ^{13}C, COSY, HSQC, HMBC and NOESY on a Varian VNMRS 500 spectrometer (500MHz for ^1H) in CD_3OD solutions

Table S2. The spectral data for cyclized phorbasone A (3).

No	δ_C	δ_H, mult (*J* Hz)	HMBC correlations	NOE's
1	39.7, CH_2	1.32, m	C-3, 5, 10, 21	H-5, 9, 21, 22
2a	19.7, CH_2	1.41, m	C-1, 5	
2b		1.58, m		H-20, 21
3a	45.4, CH_2	1.19, m	C-1, 4	
3b		1.39, m		H-19
4	34.5, C			
5	41.8, CH	1.93, d (10.2)	C-4, 6, 9, 10, 20, 21	H-1, 3a, 24a, OMe
6	75.8, CH	4.64, d (10.2)	C-4, 5, 7, 8, 11, OMe	H-18b, 20, 21
7	109.5, C			
8	156.9, C			
9	61.9, CH	2.34, s	C-4,7, 8, 10, 21, 22, 23, 24	H-1, 21, 22, 24a
10	39.1, C			
11	138.0, C			
12	40.3, CH	3.19, m	C-14	H-13, 17a, 18a
13	73.5, CH	4.98, m	C-8, -11, 14, -15	H-12, 17a
14	145.8, CH	6.52, m	C-16	H-13, 24a
15	139.5, C			
16	199.4, C			
17a	41.0, CH_2	2.63, dd (16.6, 3.8)	C-12, 13, 15, 16	H-12, 13, 17b
17b		2.96, dd (16.6, 5.2)		H-17a, 18a
18a	108.8, CH_2	4.86, d (2.0)	C-7, 11, 12	H-12, 17b, 18b
18b		5.53, d (2.0)		H-6, 18a
19	35.3, CH_3	1.11, s	C-3, 4, 5, 20	H-3b, 5, OMe
20	23.1, CH_3	1.07, s	C-3, 4, 5, 19	H-2b, 6
21	23.8, CH_3	1.01, s	C-1, 5, 9, 10	H-1, 2b, 6, 9
22	27.0, CH_3	1.88, s	C-9, 23, 24	H-1, 9, 24b
23	146.3, C			
24a	115.7, CH_2	4.91, s	C-9, 22	H-5, 9, 14, OMe
24b		5.13, br s		H-22, 24a
25	15.7, CH_3	1.67, d (1.7)	C-14, 15, 16	H-OMe
OMe	48.6, CH_3	2.84, s	C-6	H-5, 19, 24a, 25

All assignments were performed using ^1H, ^{13}C, COSY, HSQC, HMBC and NOESY on a Bruker AVANCE 400 spectrometer (400MHz for ^1H) in CD_3OD solutions

Figure S1. CD spectrum of phorbasone A (1) recorded in MeOH.



SPECTROMETER/DATA SYSTEM	
ORIGIN	JASCO-815
LOCALE	1042
RESOLUTION	
DELTAX	-1
XUNITS	NANOMETERS
YUNITS	ARBITRARY UNITS
FIRSTX	400
LASTX	200
NPOINTS	201
FIRSTY	0.07732
MAXY	75.59769
MINY	-44.531

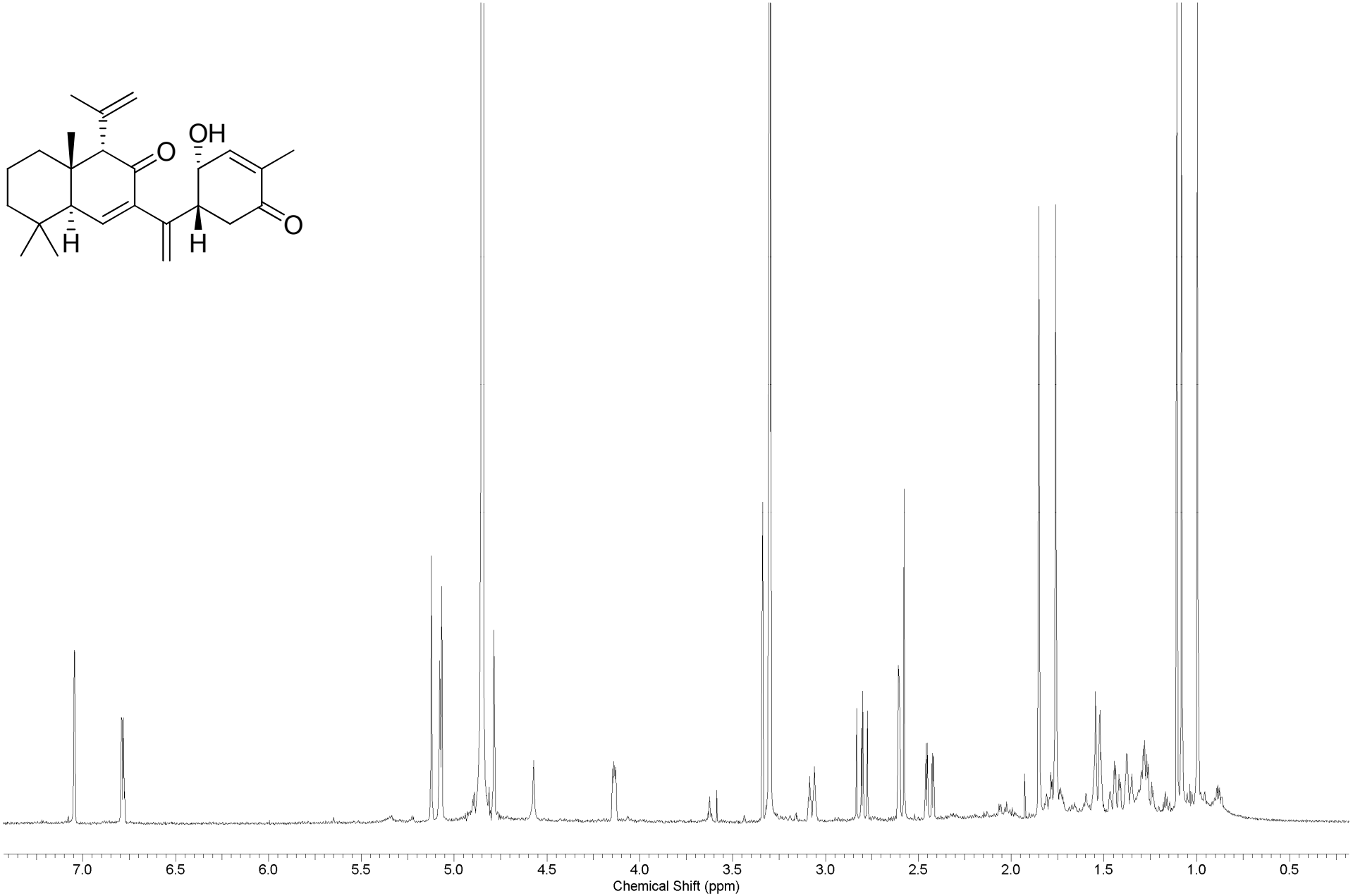


Figure S2. ^{1}H NMR spectrum of phorbasone A (1) in CD_3OD.

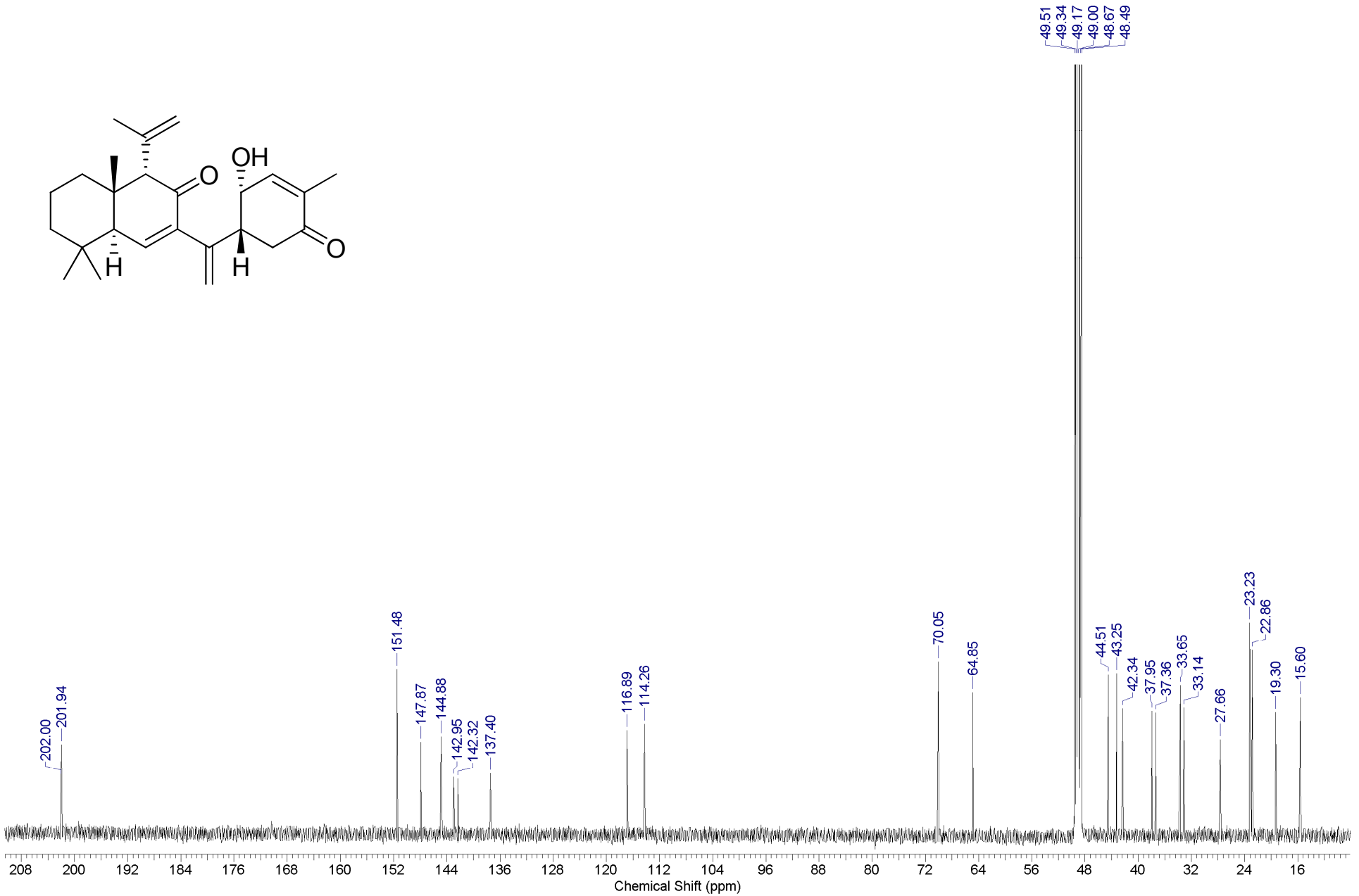


Figure S3. ^{13}C NMR spectrum of phorbasone A (1) in CD_3OD.

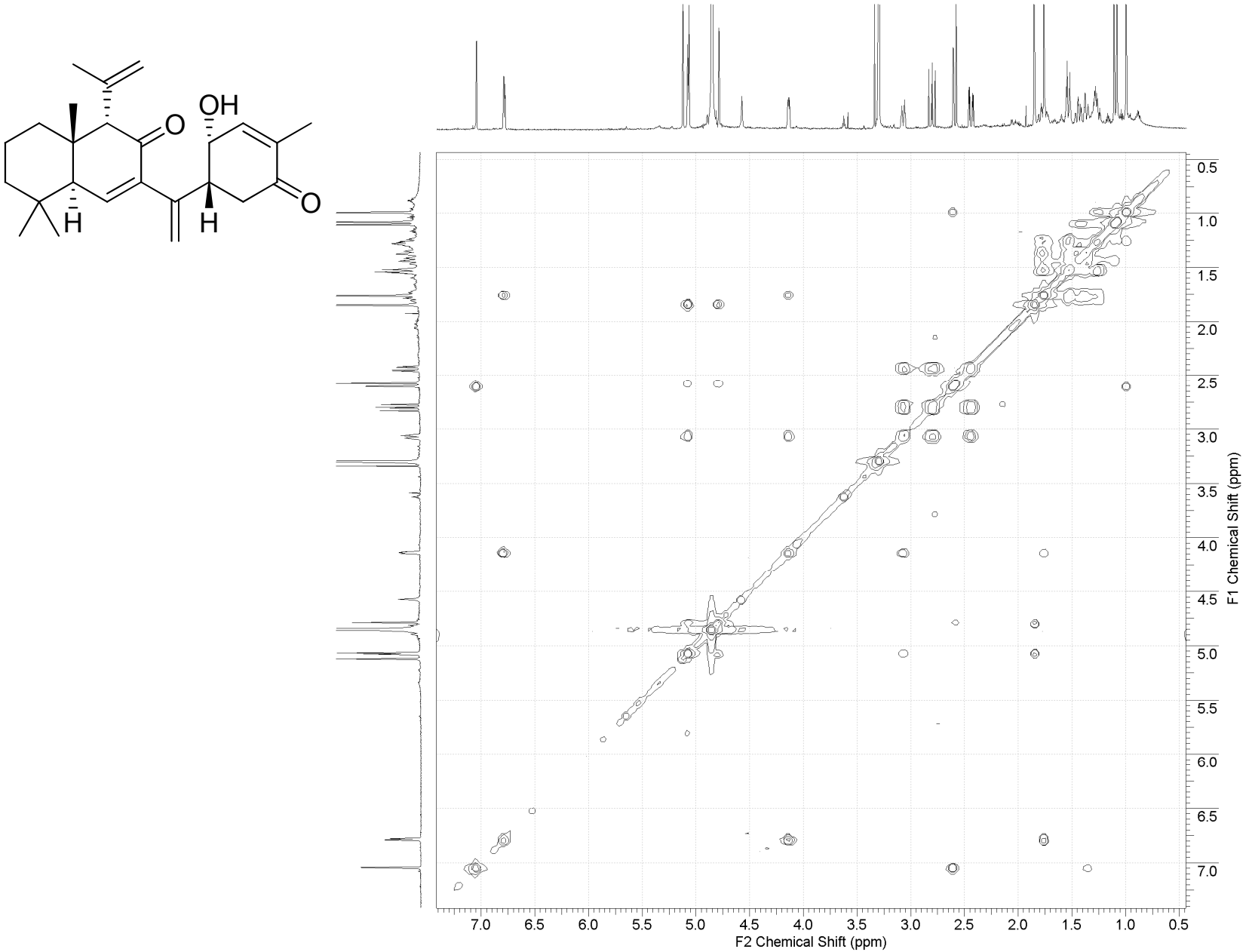


Figure S4. COSY NMR spectrum of phorbasone A (1) in CD_3OD.

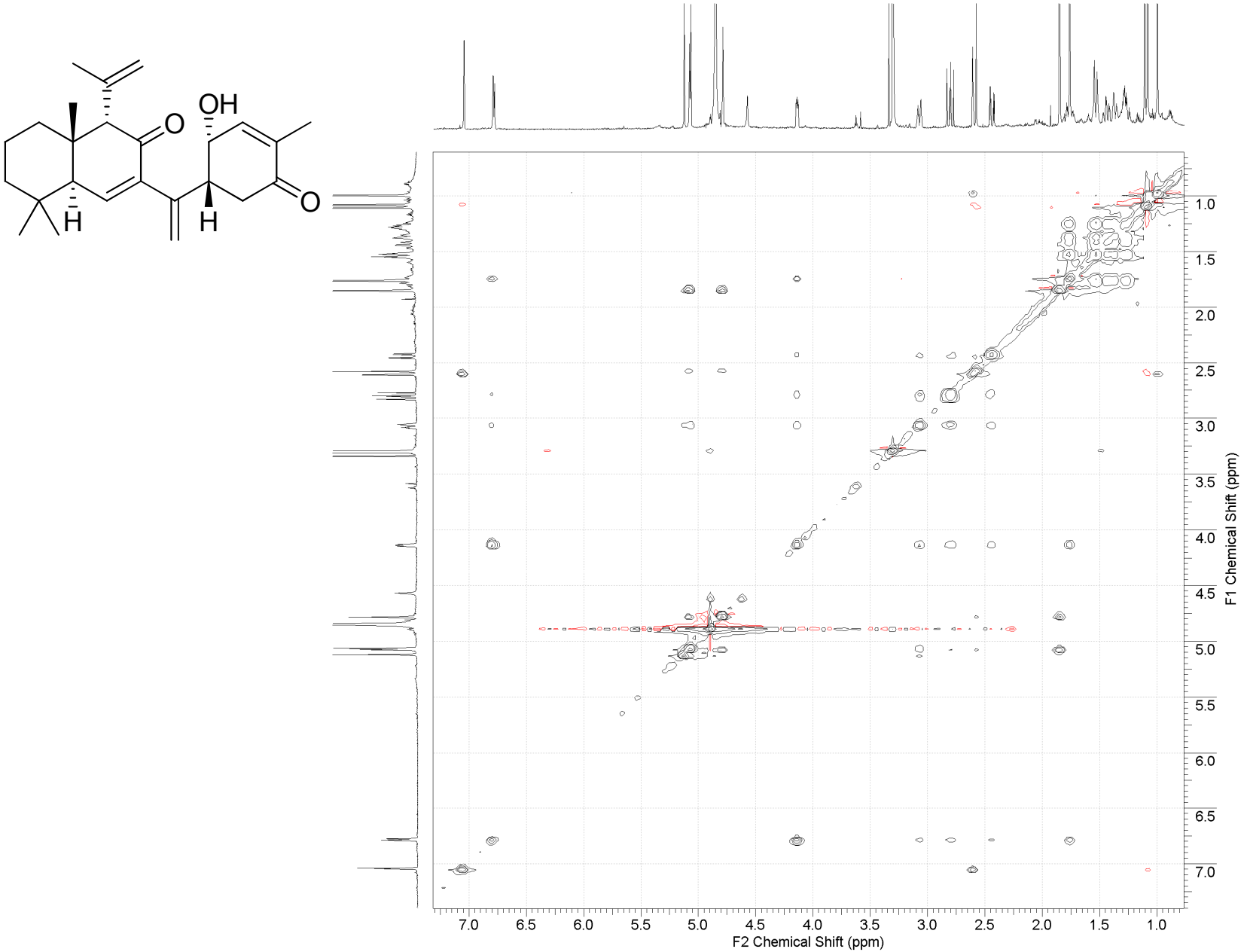


Figure S5. TOCSY NMR spectrum of phorbasone A (1) in CD_3OD.

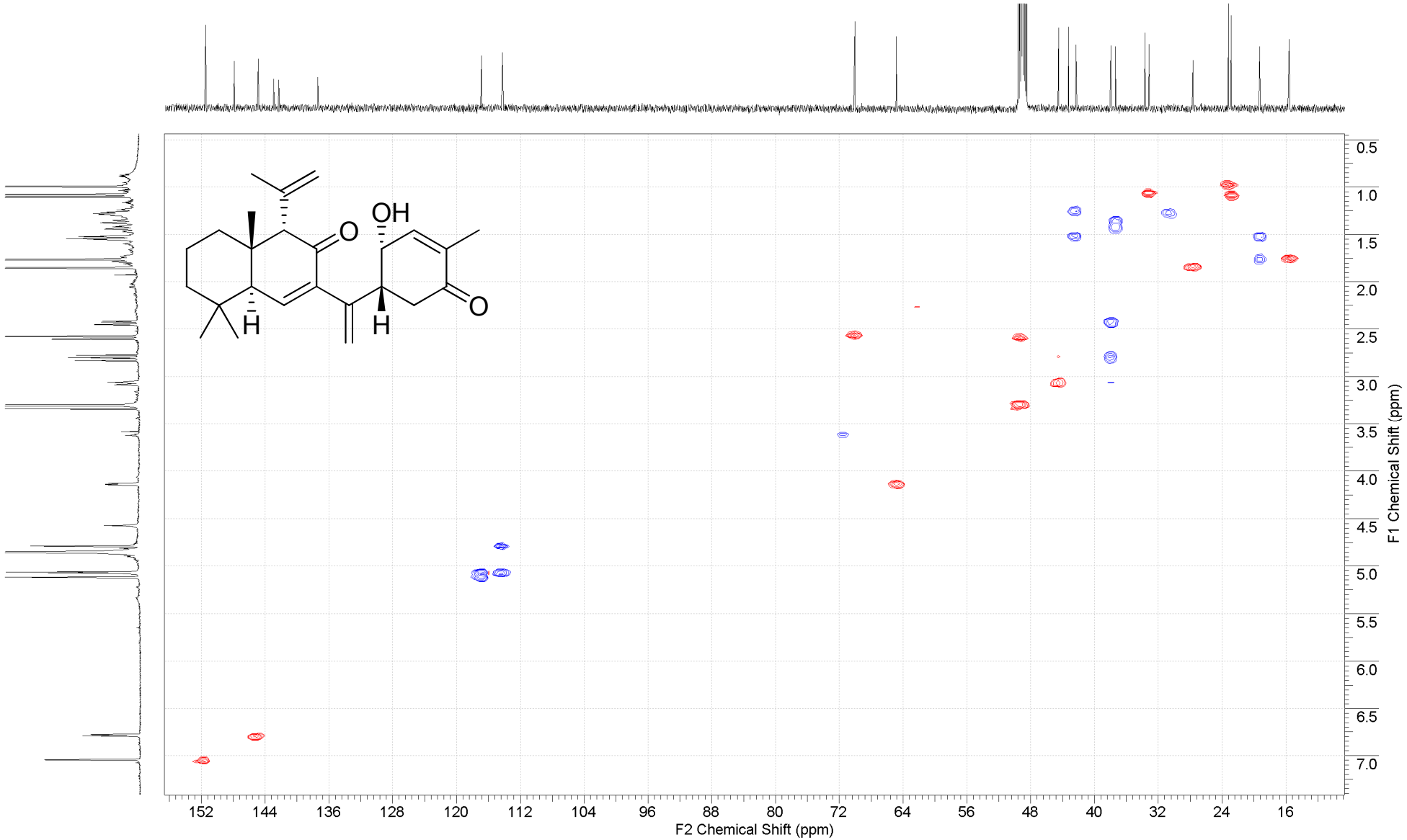


Figure S6. HSQC NMR spectrum of phorbasone A (1) in CD_3OD (Red: CH + CH_3, Blue: CH_2).

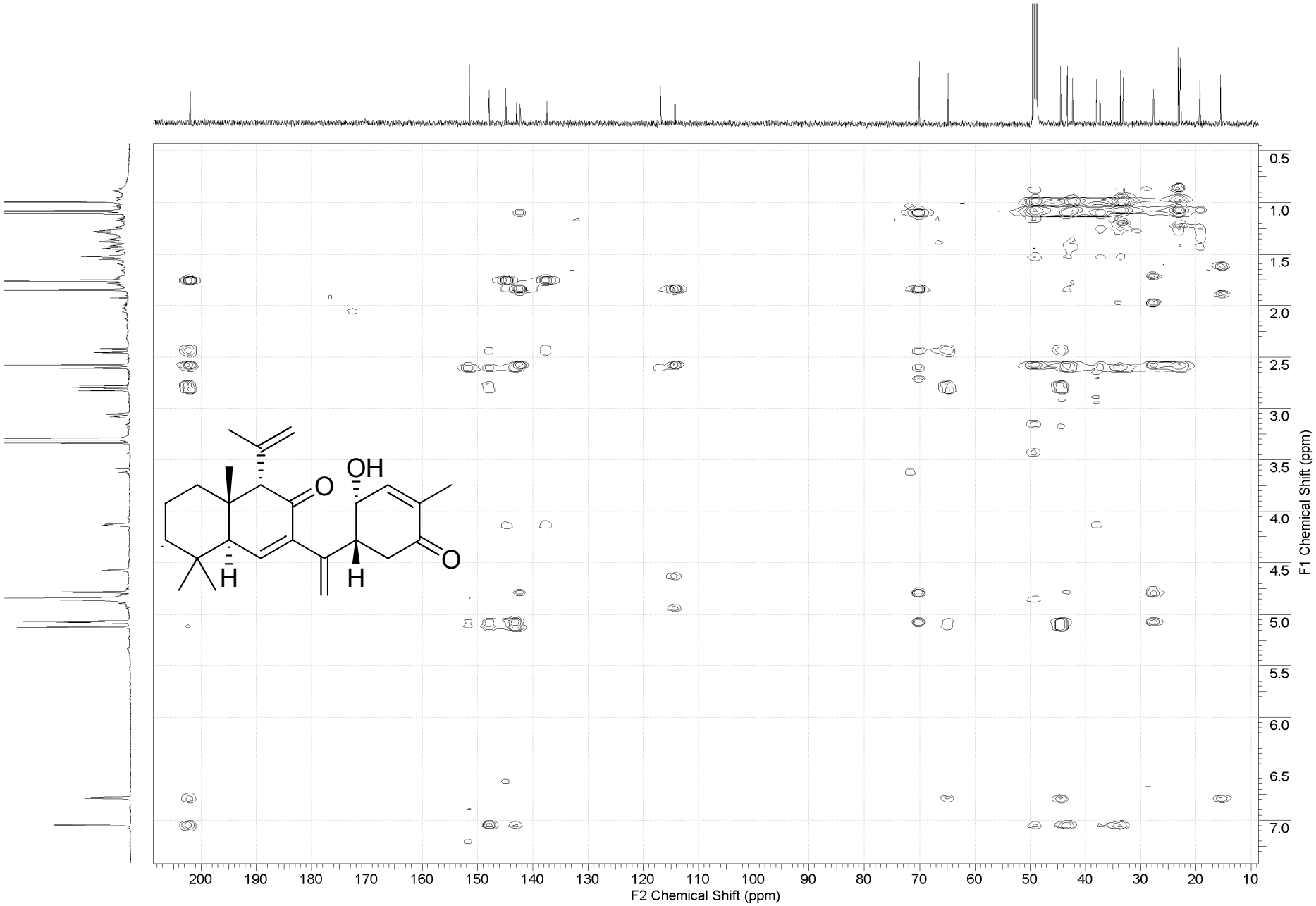


Figure S7. HMBC NMR spectrum of phorbasone A (1) in CD_3OD.

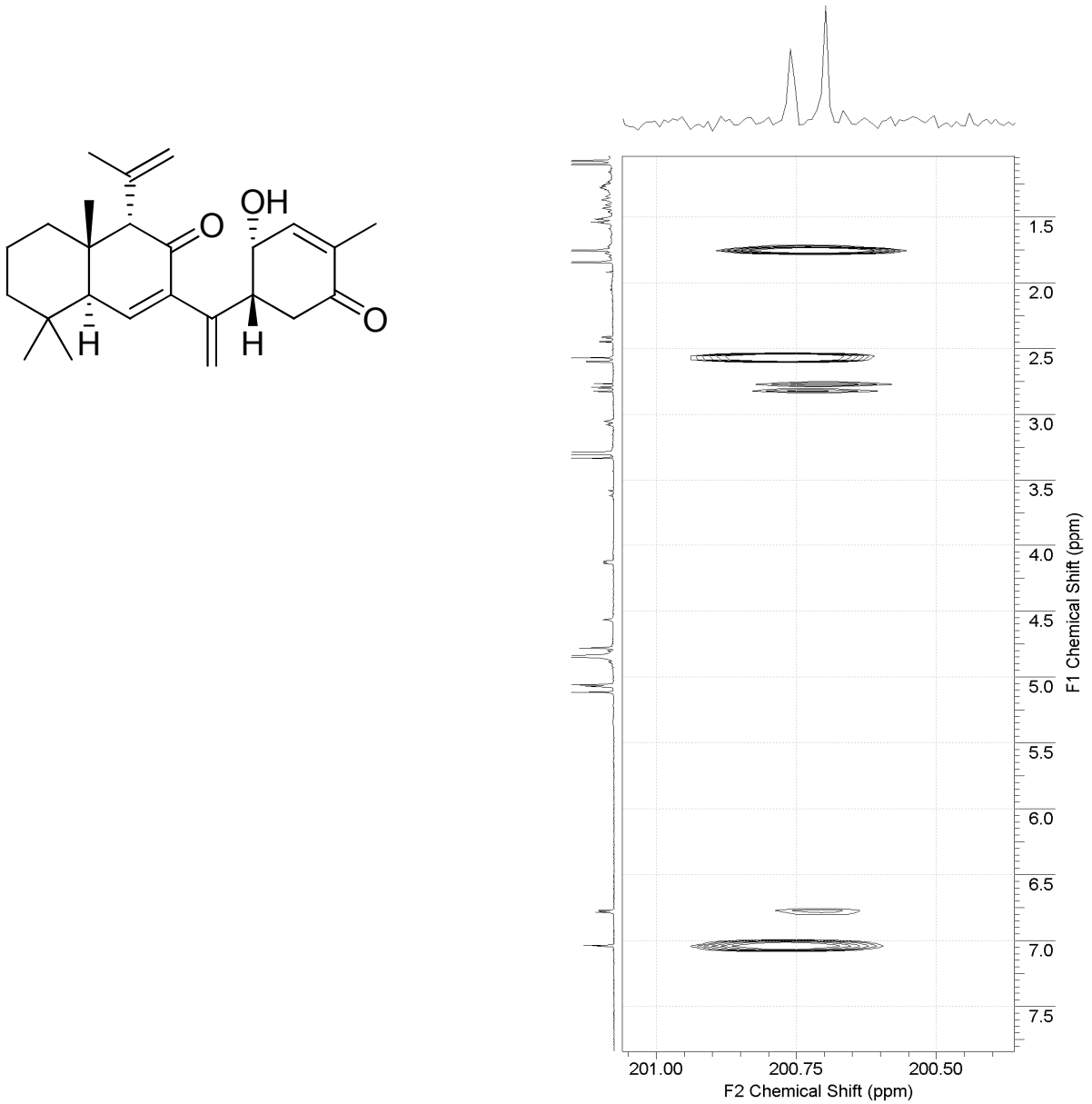


Figure S8. Selective HMBC NMR spectrum (doenfield area) of phorbasone A (1) in CD_3OD.

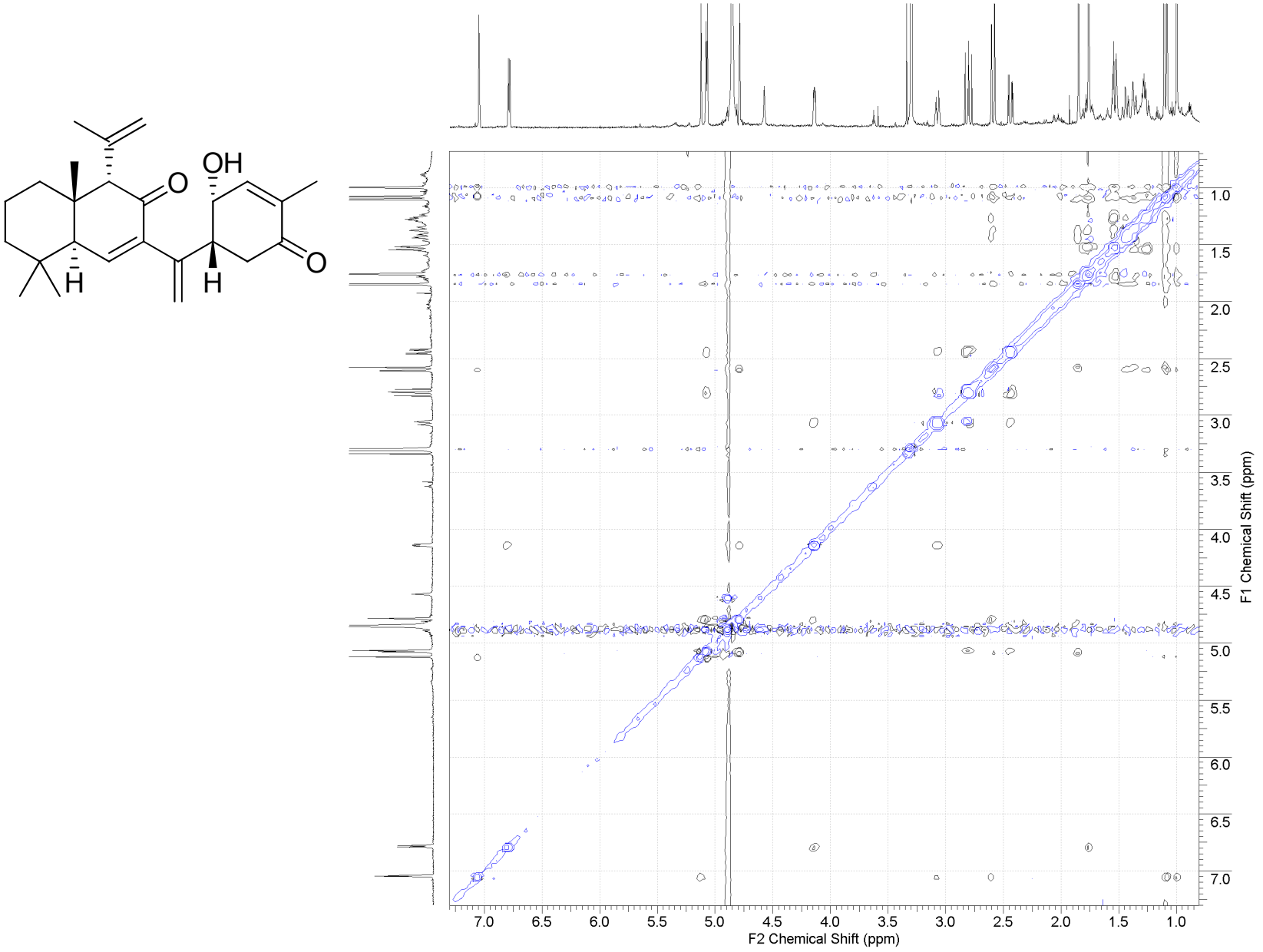


Figure S9. NOESY NMR spectrum of phorbasone A (1) in CD_3OD.

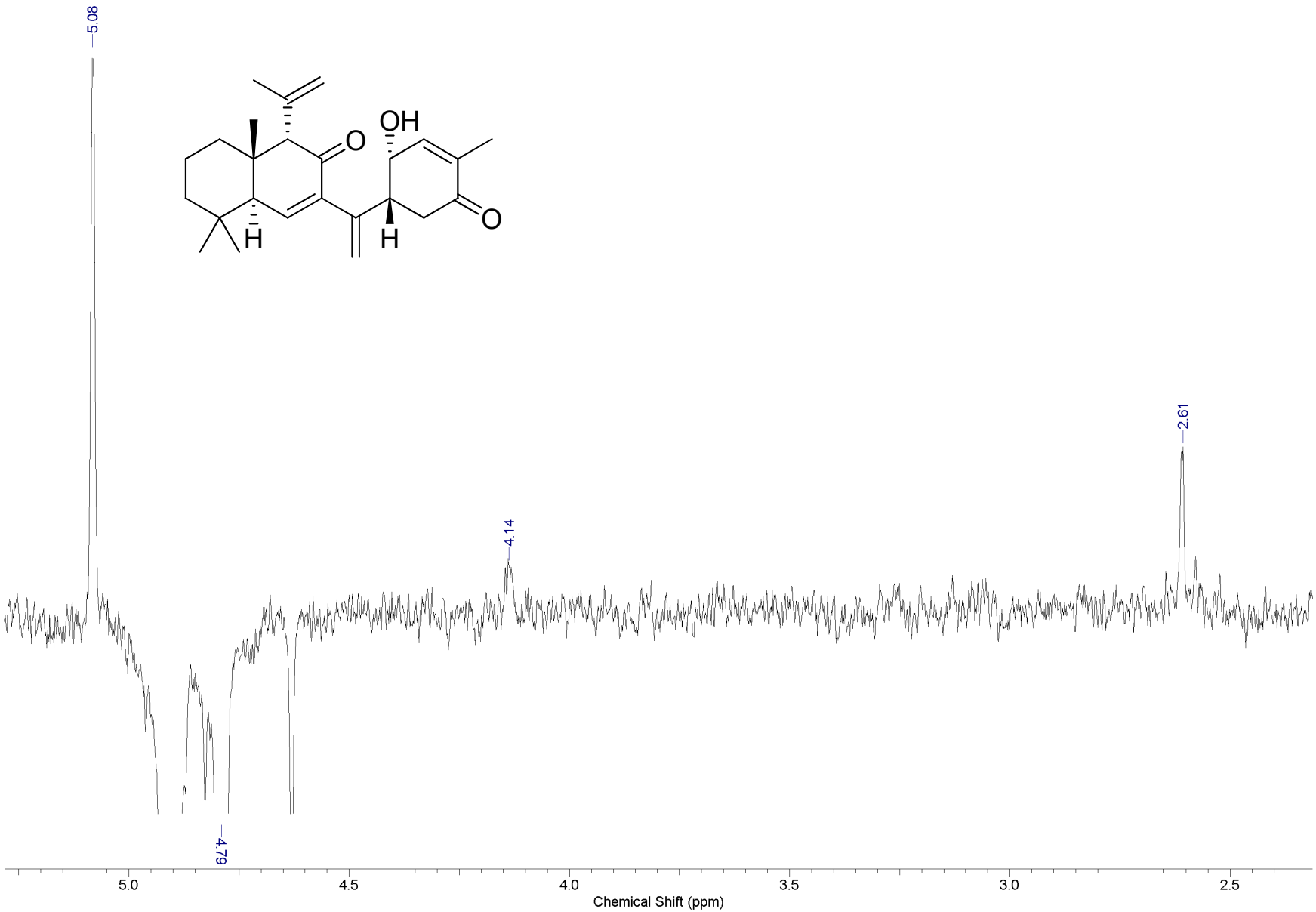


Figure S10. NOESY1D NMR spectrum of phorbasone A (1) irradiated at δ 4.79 in CD_3OD.

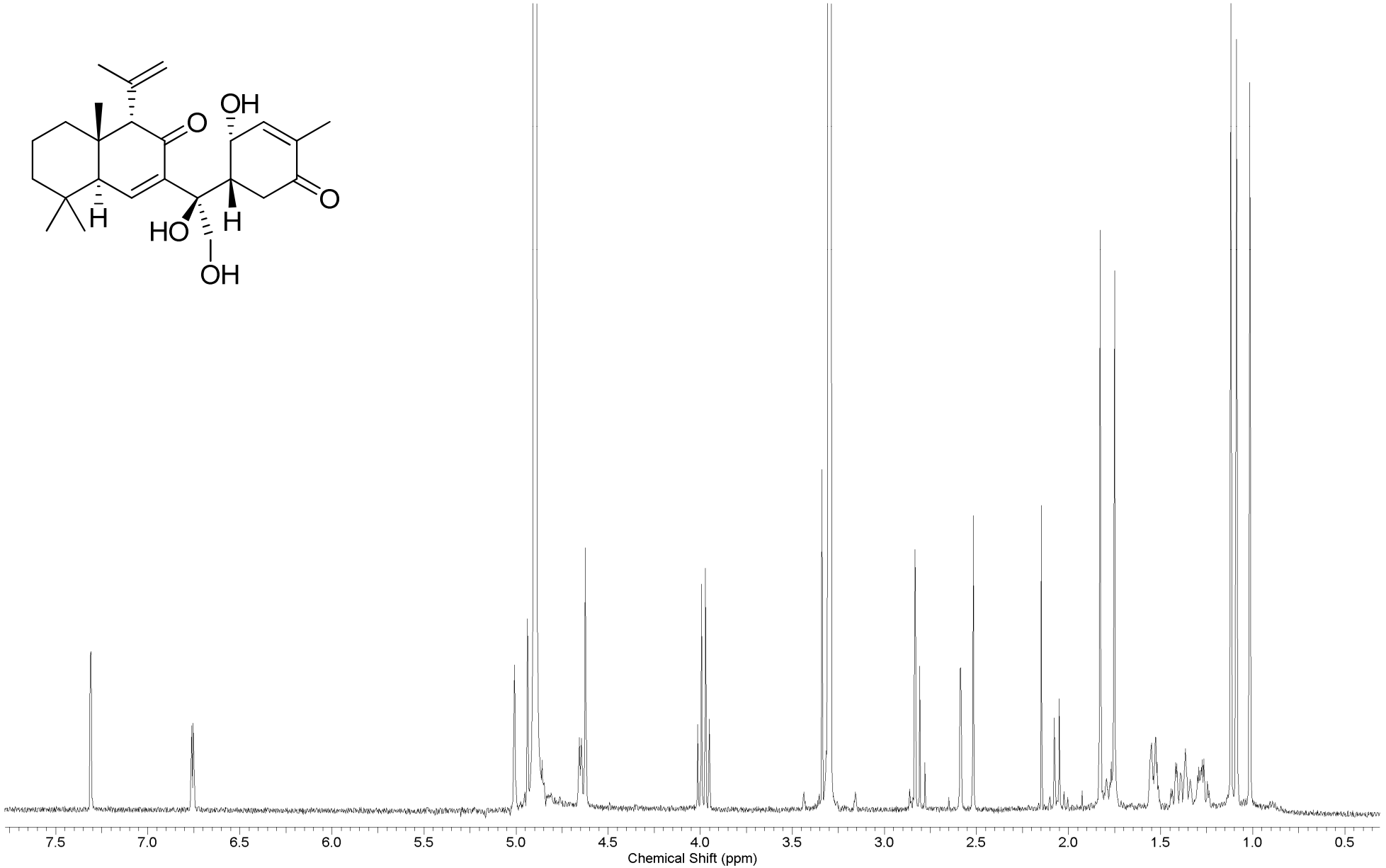


Figure S11. ^{1}H NMR spectrum of phorbasone B (2) in CD_3OD.

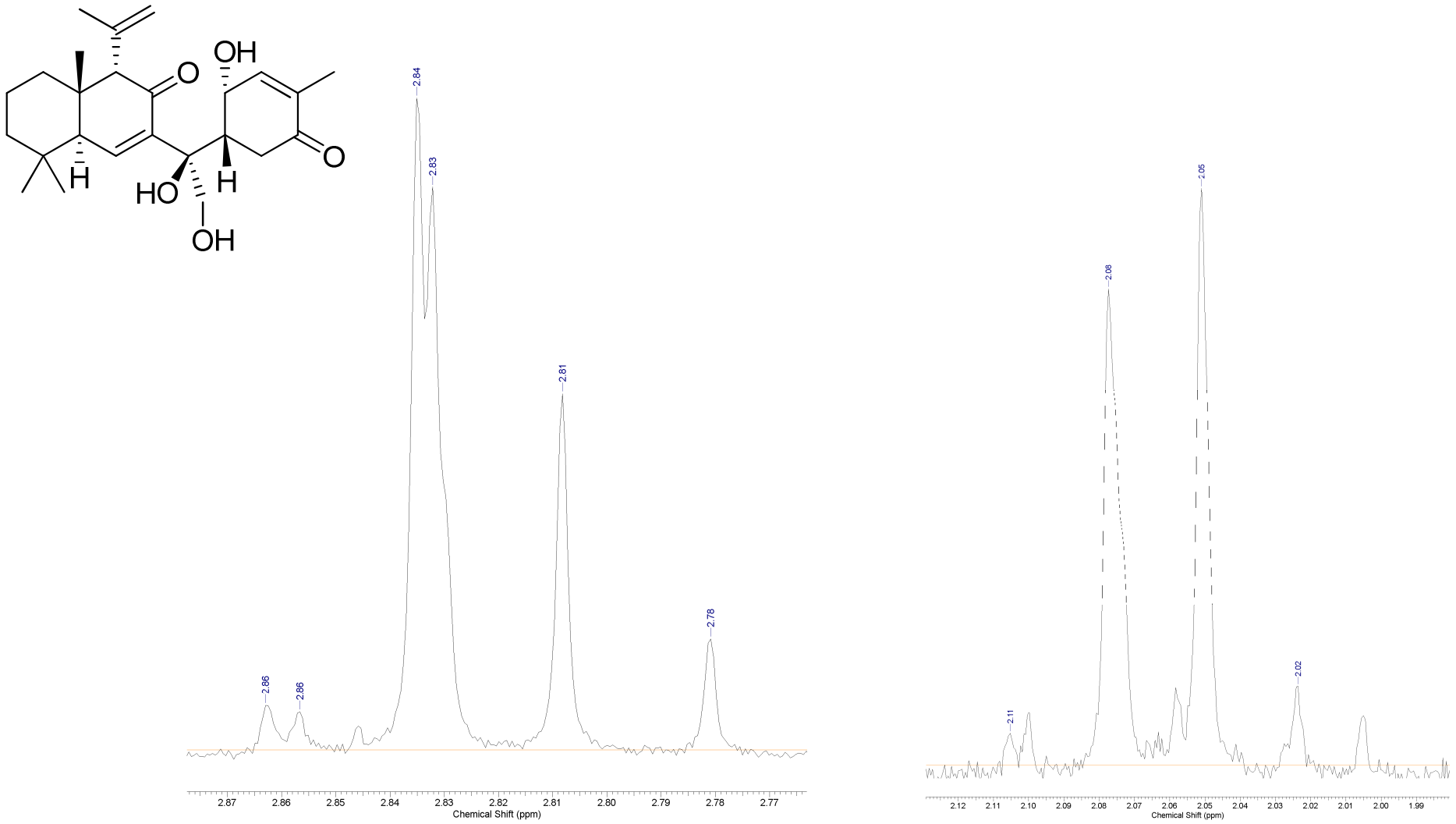


Figure S12. Expanded ^{1}H NMR spectrum of phorbasone B (2) in CD_3OD.

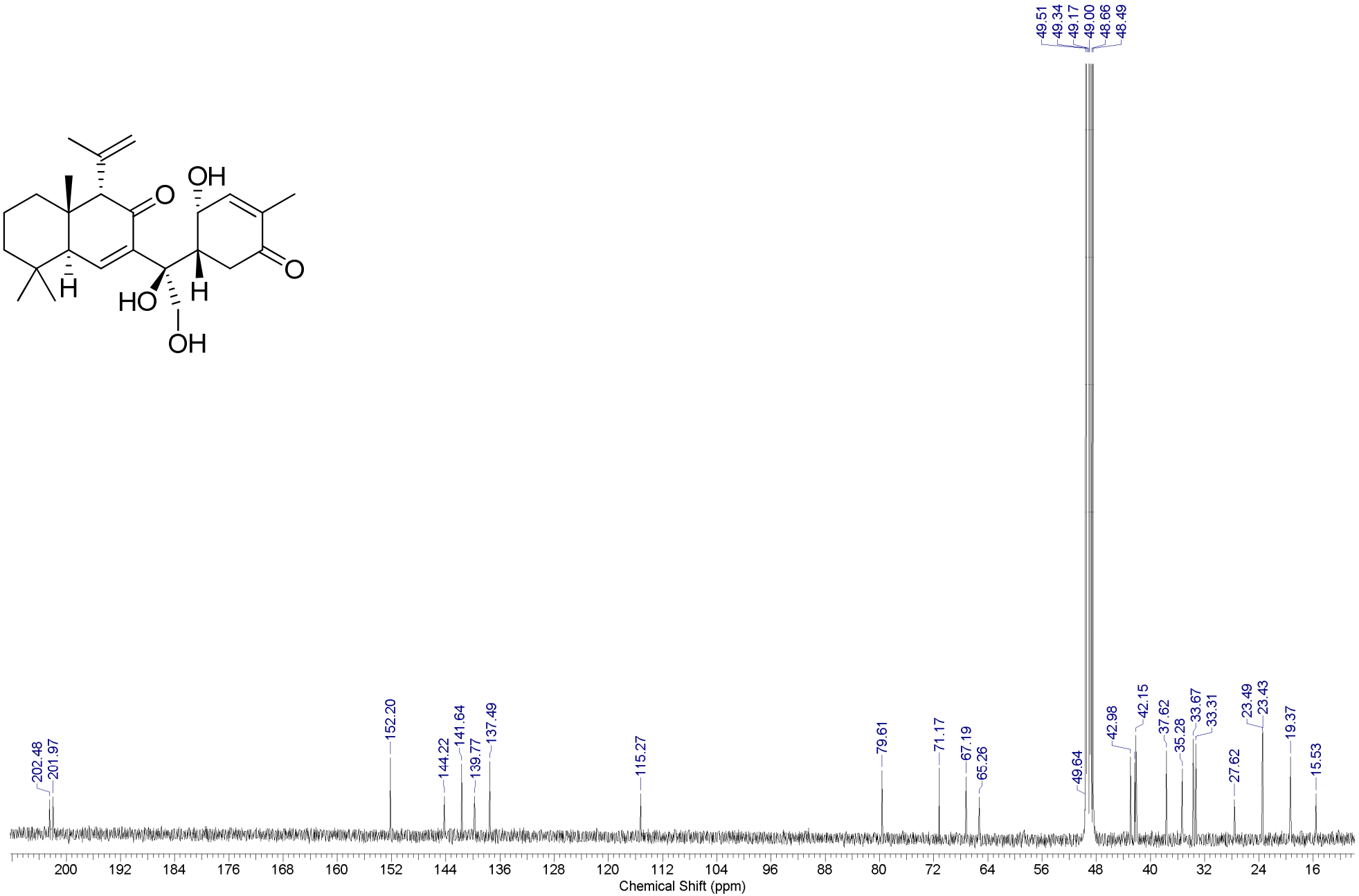


Figure S13. ^{13}C NMR spectrum of phorbasone B (2) in CD_3OD.

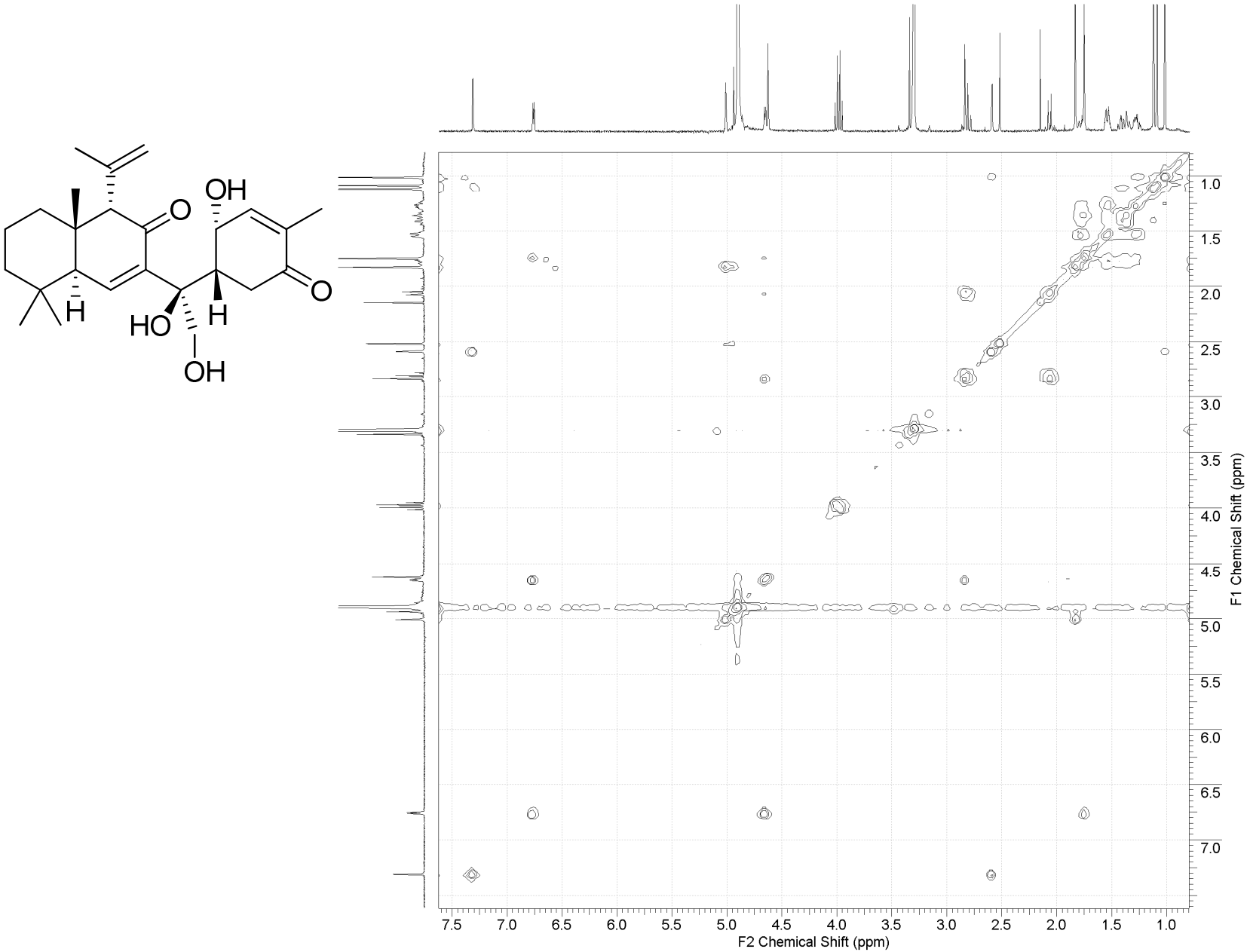


Figure S14. COSY NMR spectrum of phorbasone B (2) in CD_3OD.

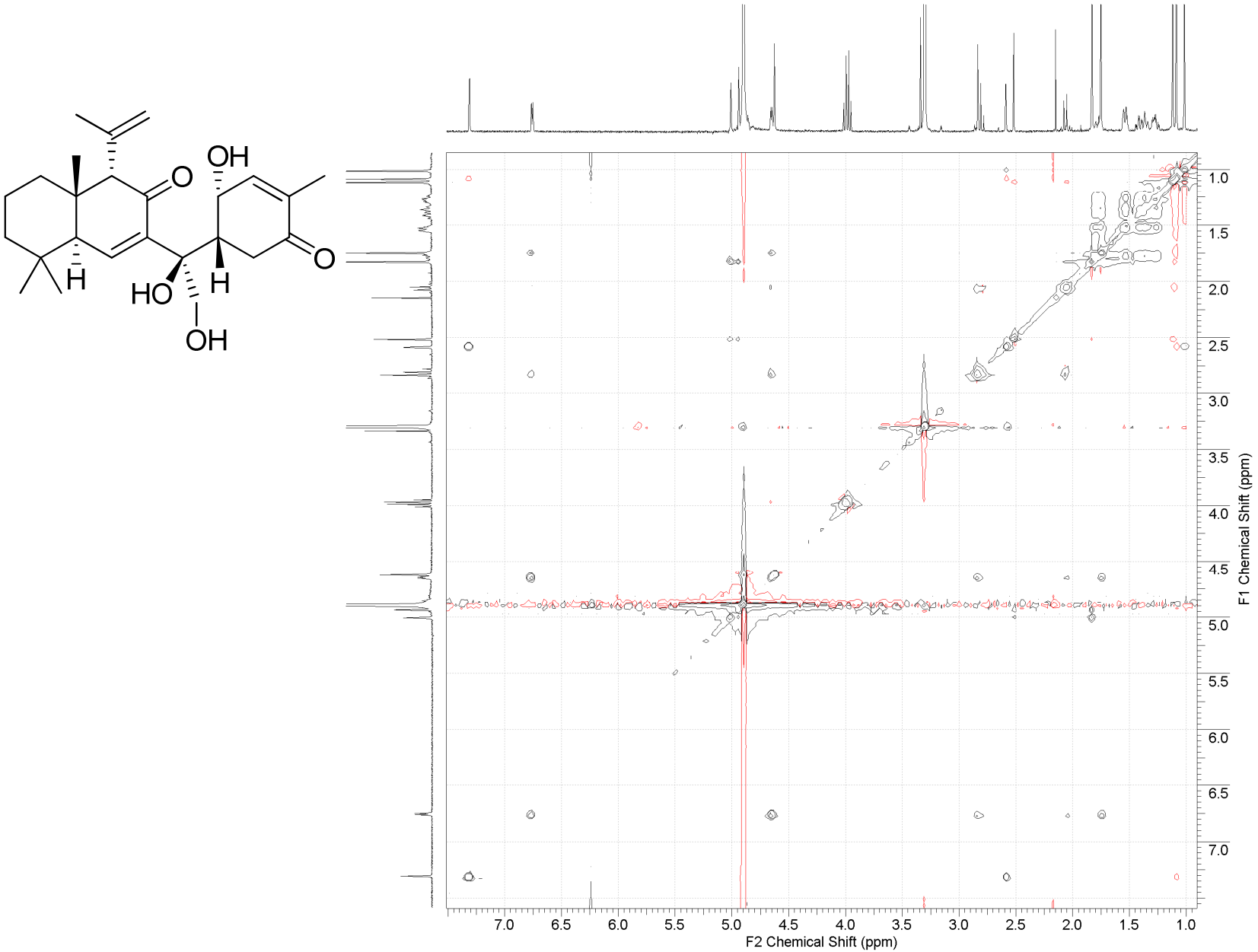


Figure S15. TOCSY NMR spectrum of phorbasone B (2) in CD_3OD.

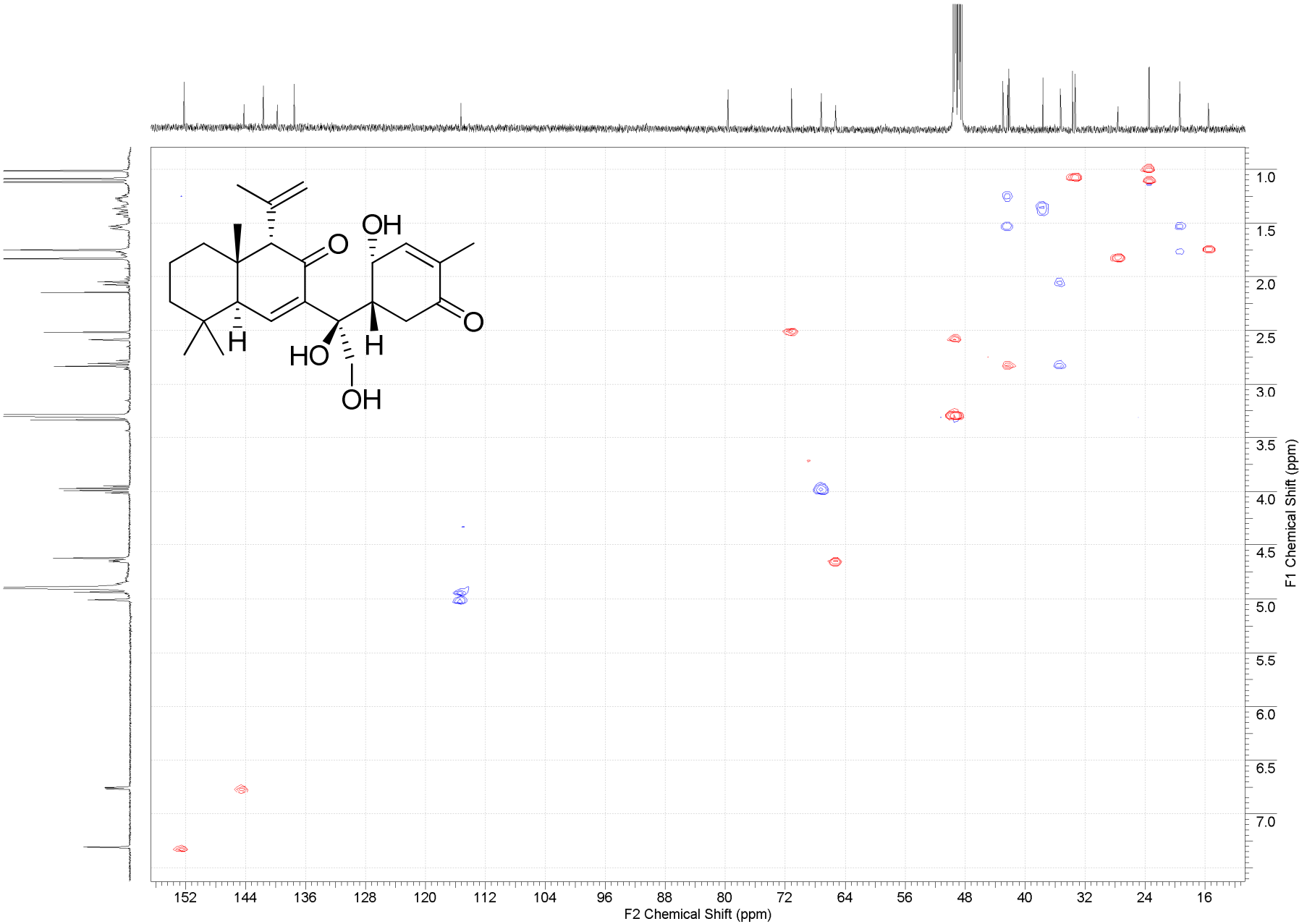


Figure S16. HSQC NMR spectrum of phorbasone B (2) in CD_3OD (Red: CH + CH_3, Blue: CH_2).

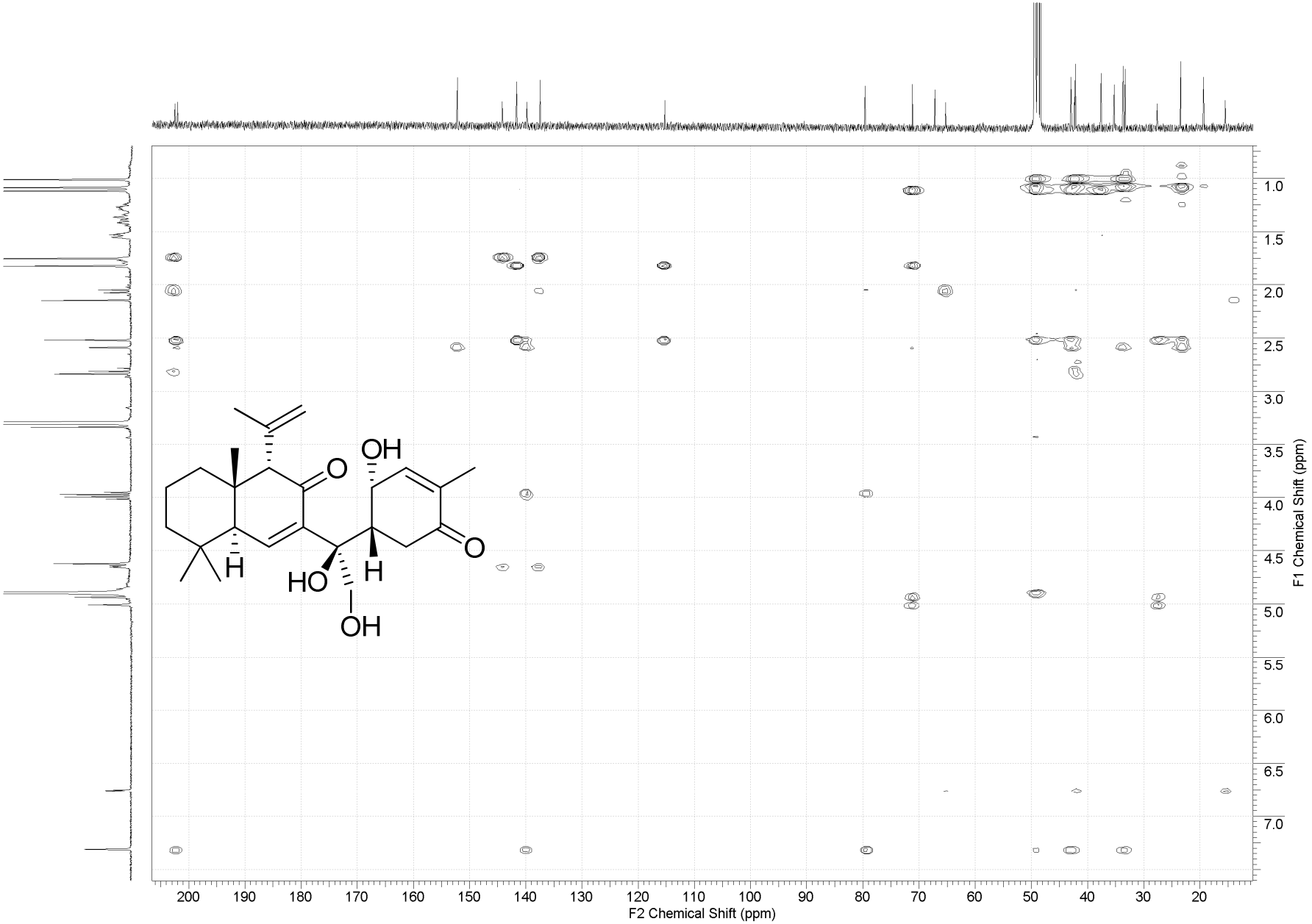


Figure S17. HMBC NMR spectrum of phorbasone B (2) in CD_3OD.

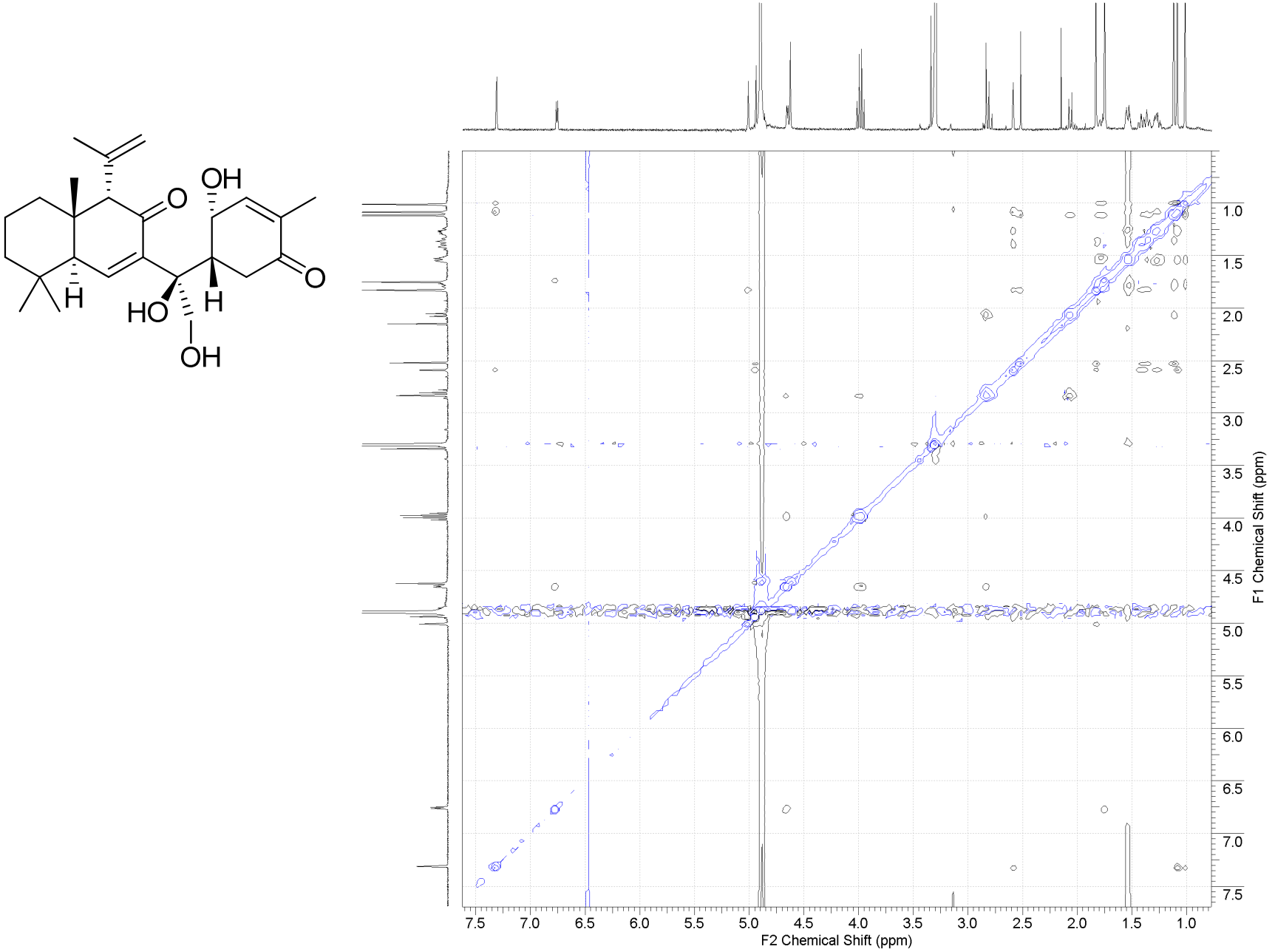


Figure S18. NOESY NMR spectrum of phorbasone B (2) in CD_3OD.

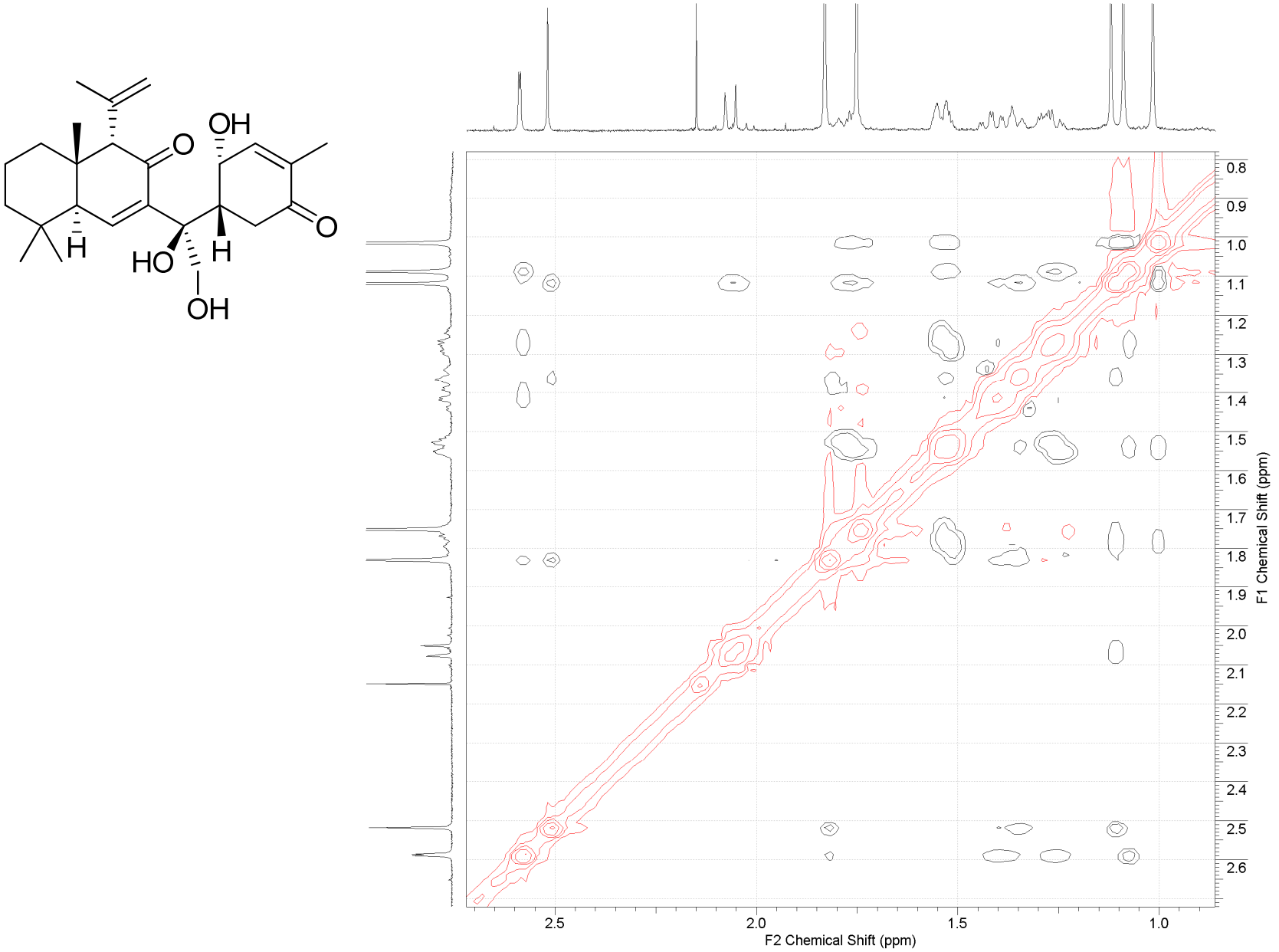


Figure S19. Expanded NOESY NMR spectrum of phorbasone B (2) in CD_3OD.

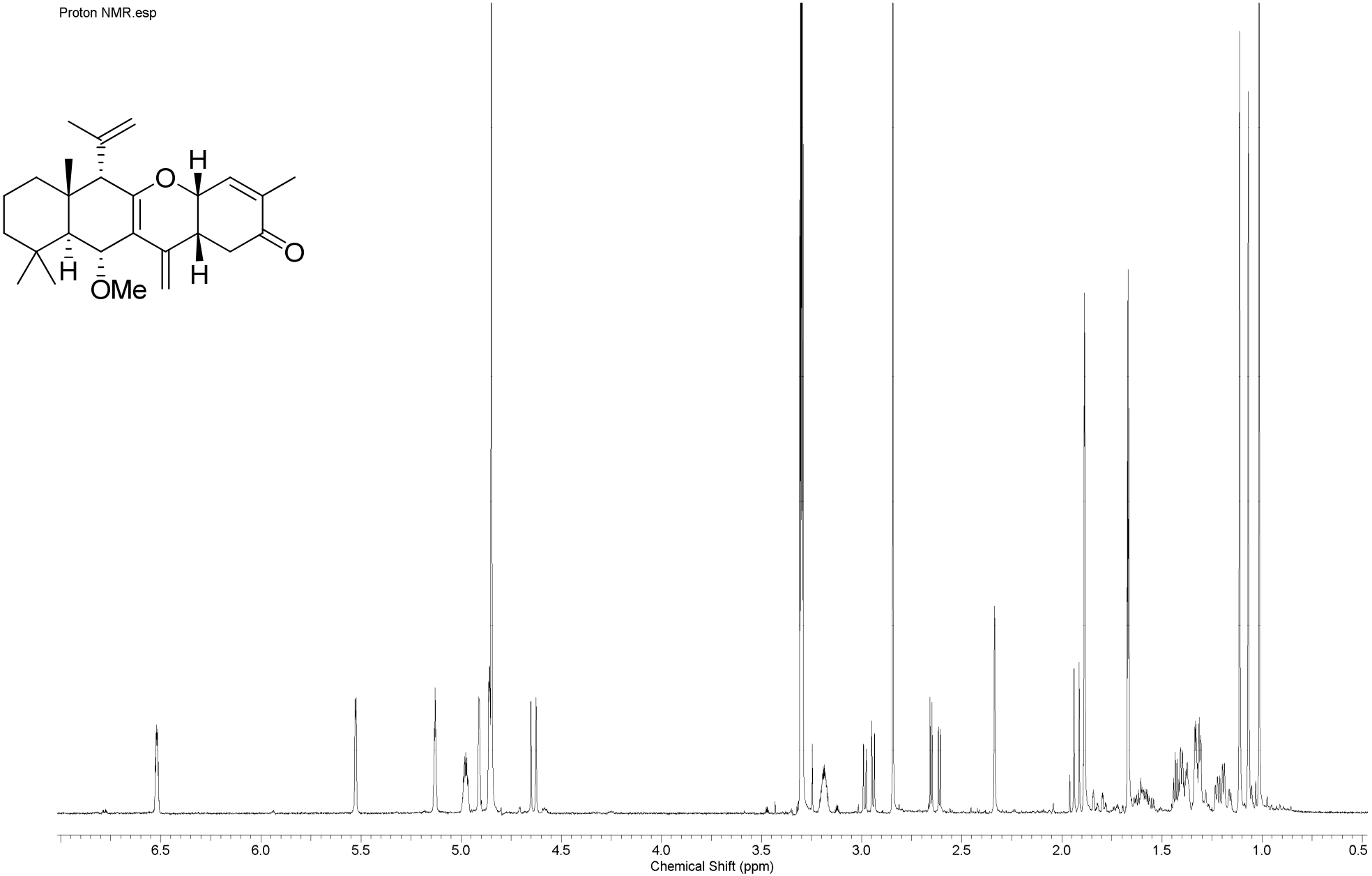


Figure S20. ^{1}H NMR spectrum of cyclized phobasone A (3) in CD_3OD.

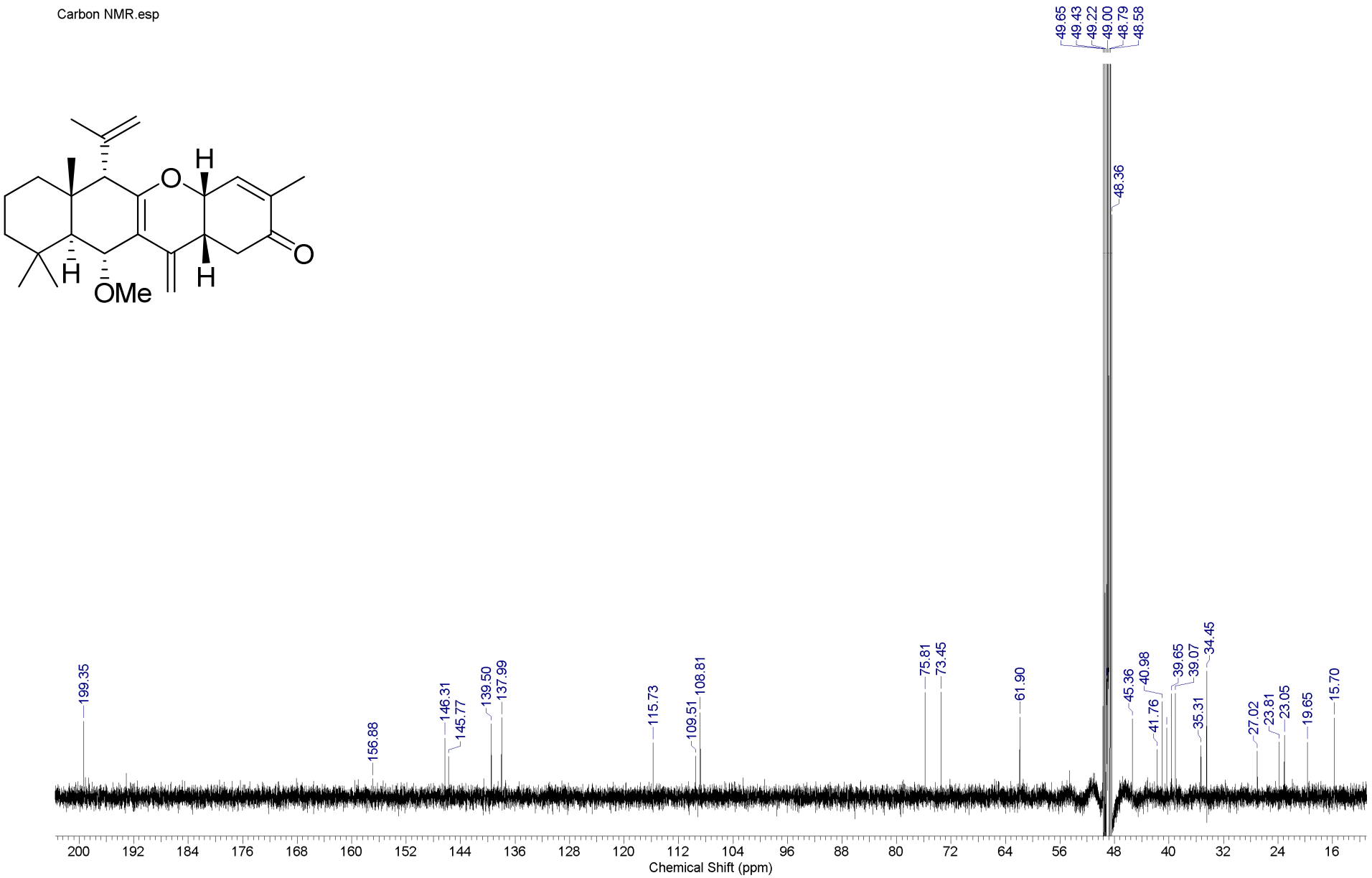


Figure S21. ^{13}C NMR spectrum of cyclized phobasone A (3) in CD_3OD.

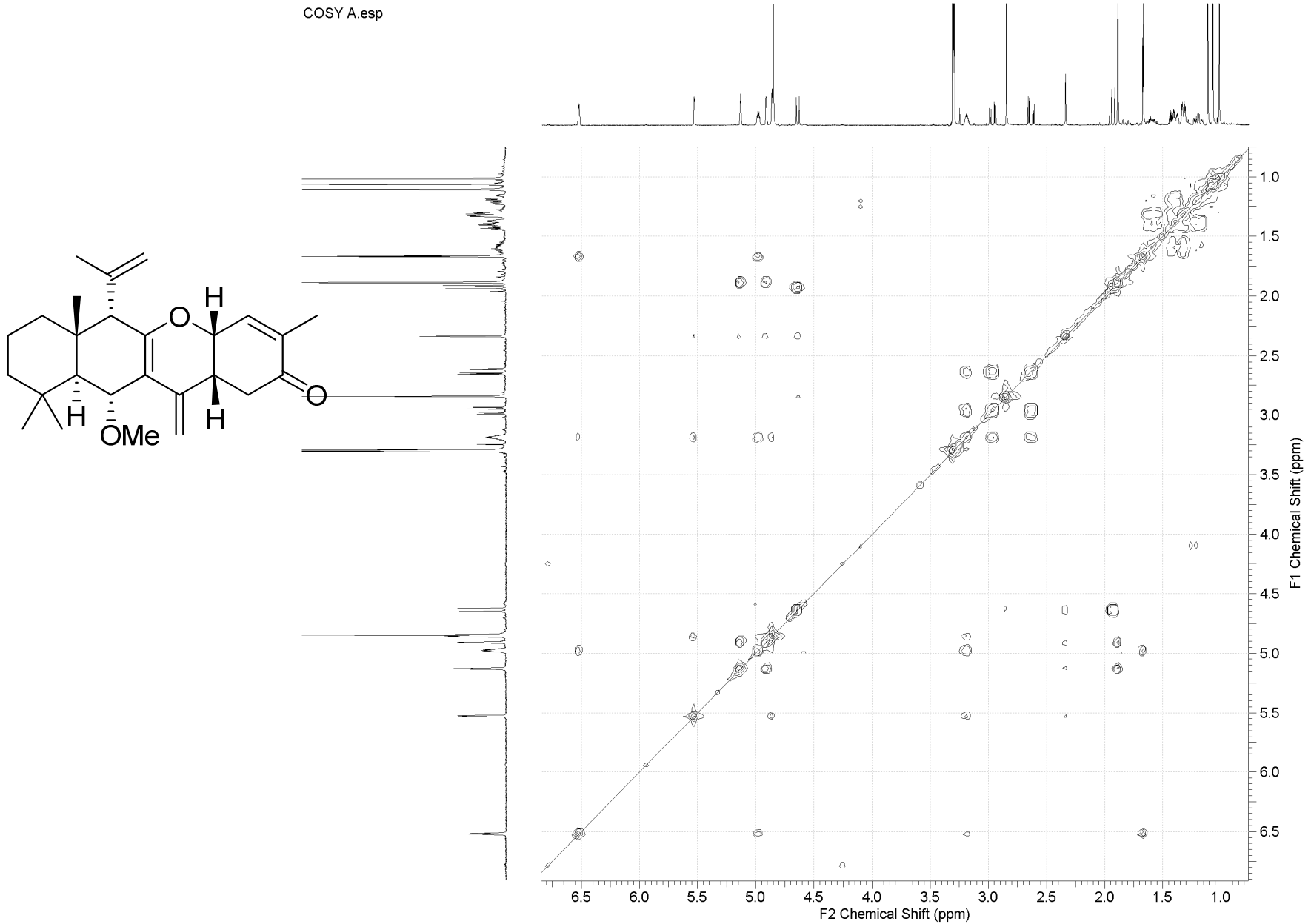


Figure S22. COSY NMR spectrum of cyclized phobasone A (3) in CD_3OD.

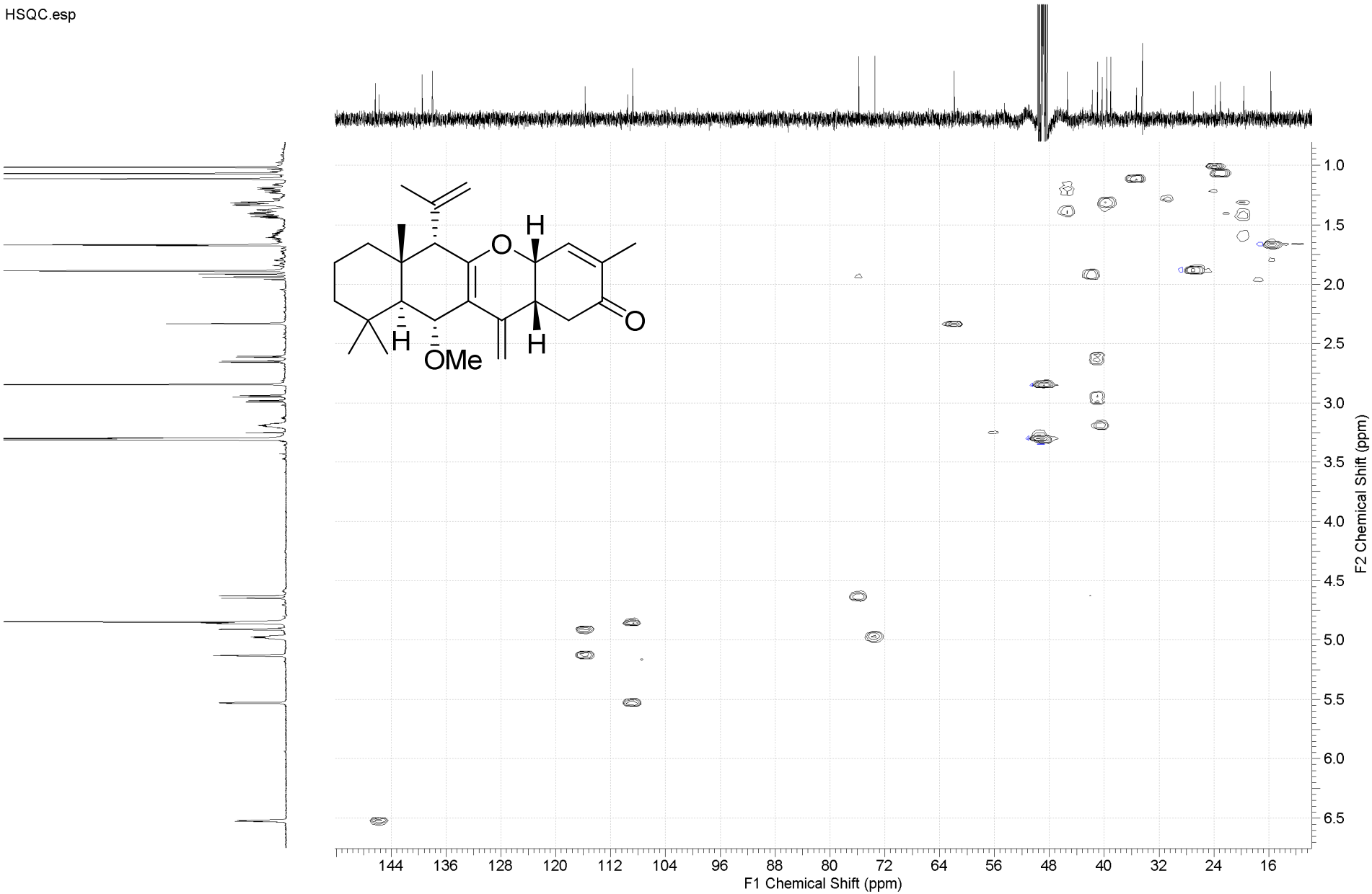


Figure S23. HSQC NMR spectrum of cyclized phobasone A (3) in CD_3OD.

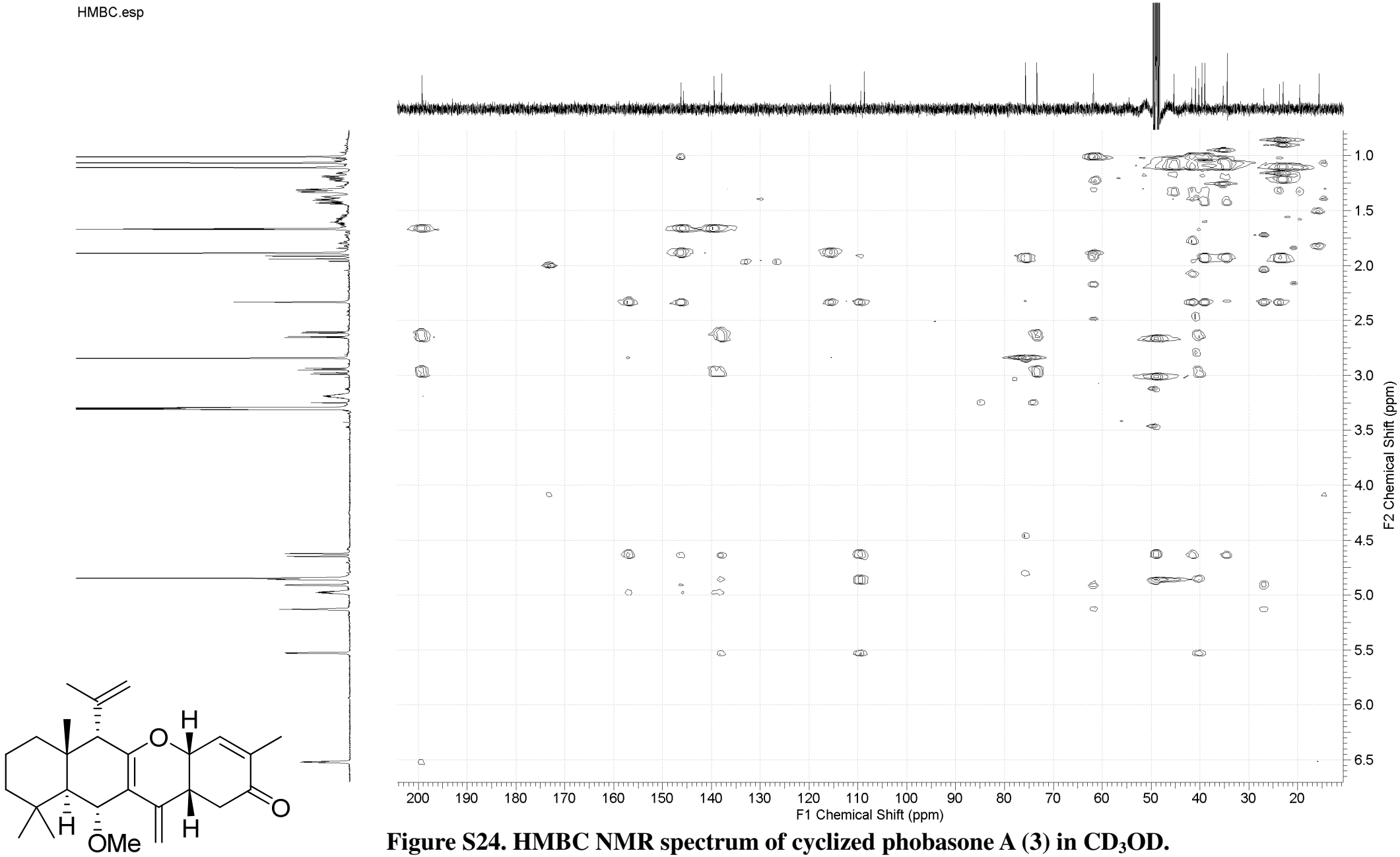


Figure S24. HMBC NMR spectrum of cyclized phobasone A (3) in CD_3OD.

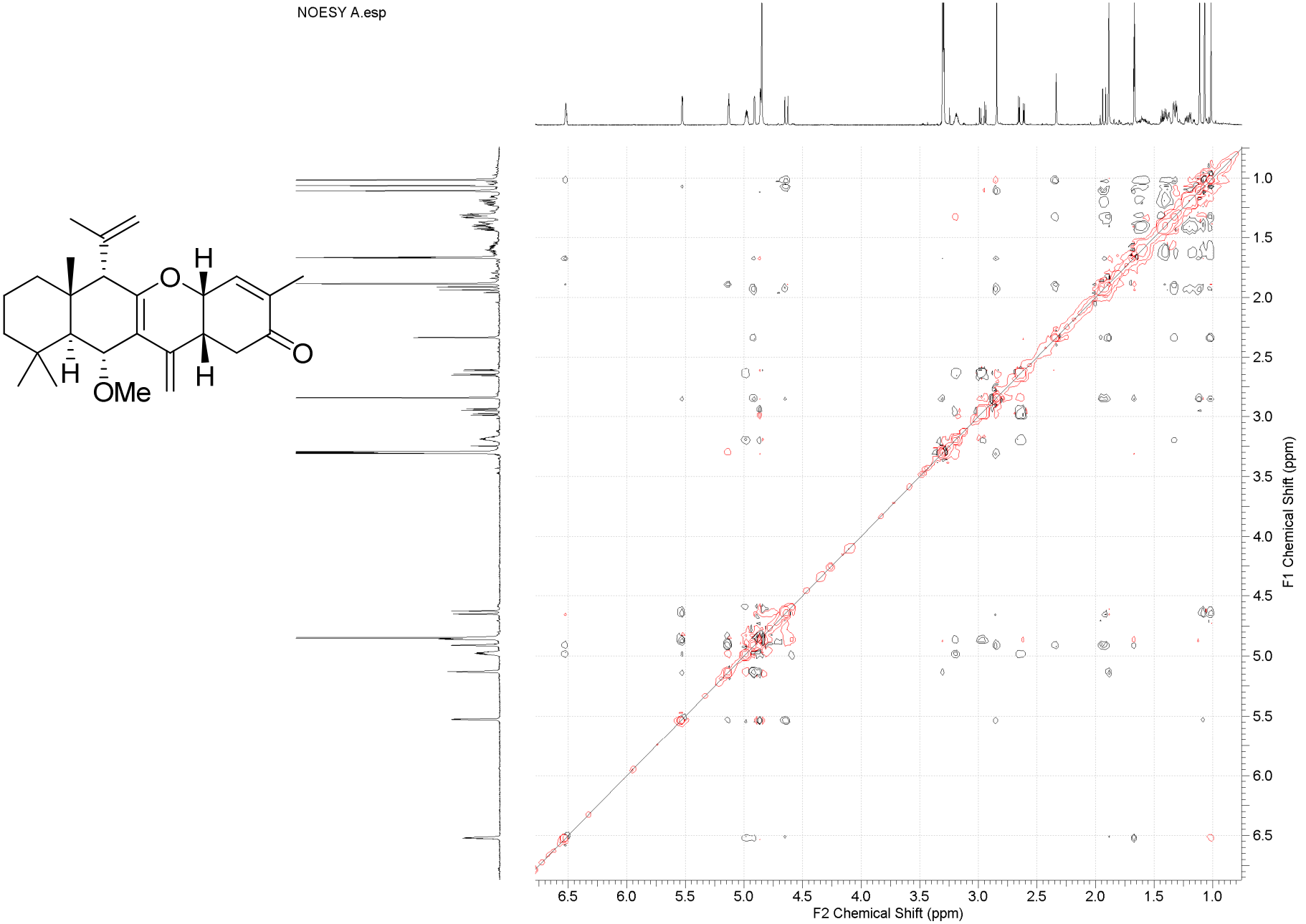


Figure S25. NOESY NMR spectrum of cyclized phobasone A (3) in CD_3OD.

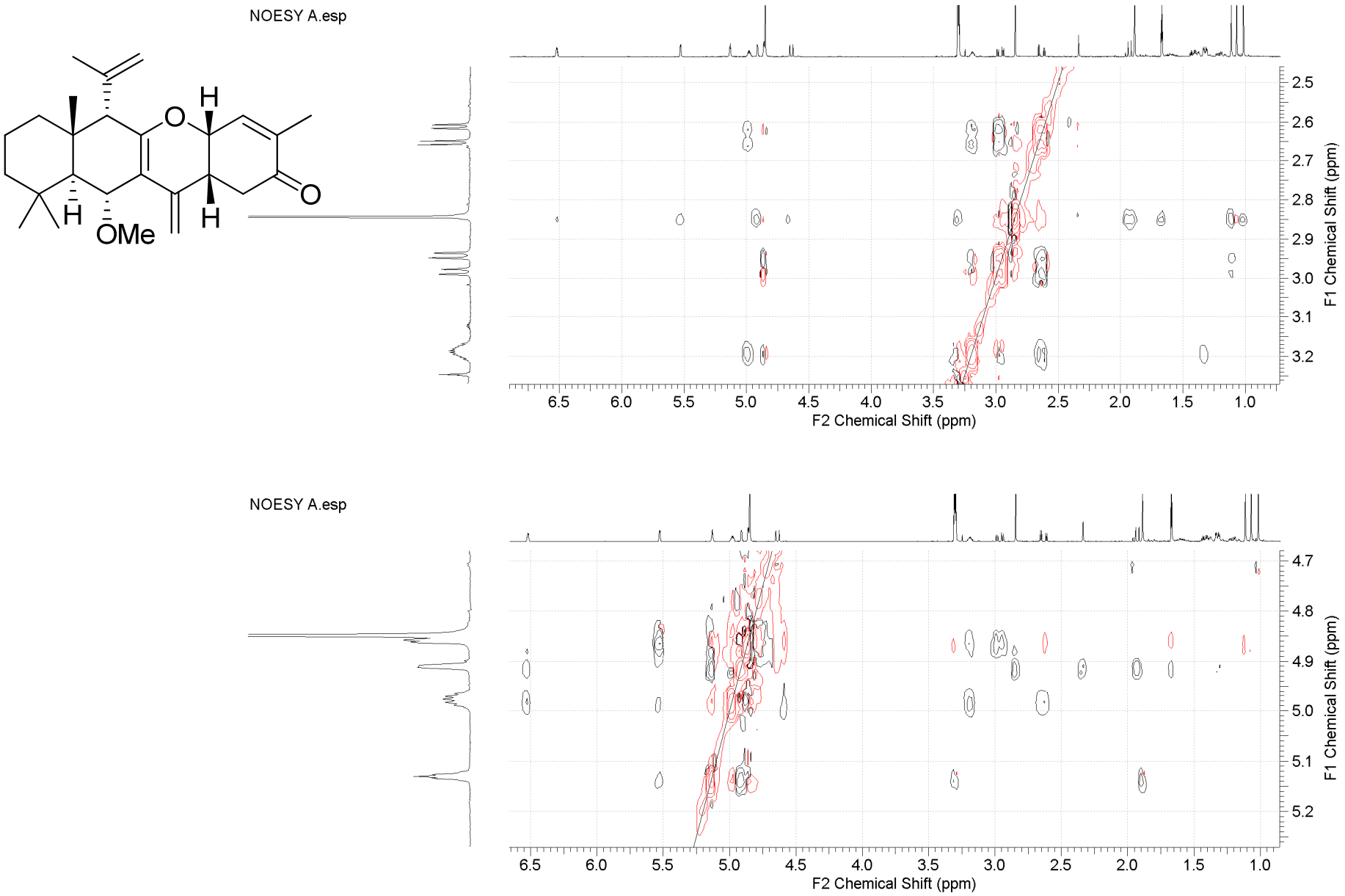


Figure S26. Expanded NOESY NMR spectrum of cyclized phobasone A (3) in CD_3OD.