

SUPPLEMENTARY MATERIAL

Trypethelone and Phenalenone derivatives isolated from the mycobiont culture of *Trypethelium eluteriae* Spreng. and their anti-mycobacterial properties

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Abstract

The metabolites of the mycobiont culture of the lichen *Trypethelium eluteriae* were isolated by column chromatography and preparative TLC. Nine compounds (**1–9**) including two new trypethelones, 8-methoxytrypethelone (**6**) and 5'-hydroxy-8-ethoxytrypethelone (**9**), together with four known trypethelones (**3–4**, **7–8**), and two known phenalenones (**1–2**) were characterized. It is the first report of 8-methoxytrypethelone methyl ether (**5**) purification as a racemic mixture in *T. eluteriae*. Earlier, 7-hydroxyl-8-methoxyltrypethelone (**10**) was reported as new compound with erroneous spectroscopic data. This compound was identified later as 8-hydroxytrypethelone methyl ether (**4**). X-ray crystallographic structures of compounds **5–7** were elucidated for the first time. Phenalenones (**1–2**) and trypethelones (**5–6** and **9**) were the additional compounds discovered in the cultured mycobiont of *T. eluteriae*. Six compounds (**1–2**, **5–8**) were screened against *Mycobacterium tuberculosis* H37Rv and two compounds (**7–8**) against non-tuberculosis mycobacteria and other human pathogenic bacteria. Compound (**7**) inhibited *M. tuberculosis* H37Rv strain with an MIC of 12.5 µg/mL.

Keywords: *Trypethelium eluteriae*; Trypetheliaceae; Mycobiont culture; Trypethelone; Phenalenone; *Mycobacterium tuberculosis* H37Rv

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Experimental

1.1. General Experimental Procedures

All melting points were determined by the open capillary method on a Dolphin micro melting point apparatus and were uncorrected. Optical rotations were obtained in a Rudolph Polarimeter (A22109) Autopol I automatic connected with the 3.03 software version using CH₃OH. UV spectra were recorded on a Shimadzu UV-1800 spectrophotometer installed with a 2.03 UV probe, and ECD spectra were obtained on a JASCO J-815 spectropolarimeter using CH₃OH. FT-IR was performed on a Spectrum One FT-IR spectrometer, Perkin-Elmer using KBr disc. ¹H (500 MHz) and ¹³C (125 MHz) NMR spectra were recorded in CDCl₃ or CD₃OD using a Bruker AVANCE III with tetramethylsilane as an internal reference standard. HRESIMS was performed on a Thermo Scientific, Orbitrap Elite Mass spectrometer. X-ray diffraction was performed using a Bruker AXS Kappa Apex II CCD Diffractometer. Analytical HPLC was conducted using the Shimadzu HPLC system with an LC8A pump, equipped with Phenomenex Luna 5 μm C18 (2) column (250 × 4.60 mm) and UV detector. Column chromatography was performed on open glass columns at atmospheric pressure using silica gel (100–200 mesh). HPTLC was carried out on pre-coated silica gel aluminium plates Kieselgel 60 F₂₅₄ (20 × 20 cm) Merck (Darmstadt, Germany), using a Camag instrument connected with Linomat 5 and TLC Scanner 4 installed with winCATS software version 1.4.8 (Muttentz, Switzerland). Spots were visualized under a UV cabinet (254 and 366 nm) followed by spraying with 10% aqueous H₂SO₄ then derivatized at 110 °C for 20 min. Preparative TLC was performed for fractions containing two closely-associated major spots. The sample solution was prepared using a suitable solvent and applied onto the TLC plate (20 × 20 cm) at 15 mm from the bottom of the plate using a Linomat 5 applicator with a 500 μL syringe as a streak ranging from 10 to 190 mm in length. The spotted TLCs were developed in the respective solvent system and allowed to air-dry thoroughly at room temperature. TLCs were redeveloped again in the same mobile phase to achieve the complete and compact separation of spots as distinct bands. The silica gel at the spot of interest was scraped separately from the TLC plate and loaded immediately on the ideal glass column. The compound was desorbed from the silica gel using a suitable solvent, concentrated and analyzed in TLC prior to spectroscopic analysis.

1.2. Lichen specimen, Mycobiont culture, and Molecular identity

Trypethelium eluteriae Spreng. (Trypetheliaceae); Index Fungorum
(<http://www.indexfungorum.org/names/NamesRecord.asp?RecordID=122402>). Specimen

examined: India, Tamil Nadu, Kanchipuram district, Vadanemmeli, (N 12°44' 08.9", E 80°14' 04.4", alt. 6m MSL), bark of *Anacardium occidentale* collected by S. Muthukumar, S. Karthik and G.N. Hariharan on 21.01.2014, Voucher No. MSSRF/H/TRYe/102/2014 deposited at the Lichen Ecology and Bioprospecting Laboratory, M.S. Swaminathan Research Foundation, Chennai, India. The specimens were authenticated by a lichenologist (Dr. G.N. Hariharan). The hymenial layer (containing the mature ascospores, young and undehisced asci along with interascal filaments) excised from the ascomata was used as the inoculum to initiate mycobiont cultures. After pre-culture in malt-yeast extract (MYE) medium (malt extract 10 g, yeast extract 4 g, agar 18 g, H₂O 1 l, pH 6.6) lacking any sucrose (MYE 0%), the mycobionts were sub-cultured in MYE supplemented with 6% sucrose (MYE 6%), and incubated at 25 °C and 70% relative humidity with a 12 h light-dark cycle (light intensity: 50–100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PPFD). The growth and biomass production were observed at 30-day intervals until the 240th day, when red crystals were observed on the mycelium. In order to obtain an appreciable quantity of metabolites for isolation and characterization, the mycelia were sub-cultured in 55 macro disposable Petri dishes (120 × 45 mm). Genomic DNA was isolated from the harvested mycelia using the CTAB method (Gargas and Taylor 1992). The conserved region of the mitochondrial small subunit (mtSSU) rDNA was amplified using forward and reverse primers (Zoller et al. 1999). The PCR amplification conditions included an initial denaturation at 95 °C (5 min), 30 cycles each of denaturation at 95 °C (1 min), annealing at 56 °C (1 min) and extension at 72 °C (1 min), and a final extension step at 72 °C (7 min). The PCR product was cloned in a T/A vector (MBI Fermentas), and sequenced using M13 forward and reverse universal primers in an automated ABI PRISM 3130 sequencer (Applied Biosystems). The molecular identity of the cultured mycobiont was confirmed by comparing the sequenced PCR product of mtSSU region (873 bp) with the existing sequence available at NCBI through a blast search. The results of this blast search revealed 99% similarity between the sequence data of the mycobiont culture of *T. eluteriae* (KC592291) collected from Florida, USA. Our sequence data were submitted to NCBI with accession no. KY418158.

1.3. Extraction and isolation of compounds

The mycobiont cultures were harvested from the medium using a scalpel and 148.2 g of the harvested material was dried in a hot air oven at 45 °C for two days, and powdered using a mixer grinder, yielding dry weight of 64.4 g. The mycelial powder was subjected to cold extraction at room temperature (28 ±1 °C) using acetone, and repeated four times (250

mL \times 4) every 48 h for complete extraction of the compounds. The crude extract was filtered with Whatman No.1 filter paper, and the filtrate was concentrated using a rotary evaporator under reduced pressure yielding 5.62 g of solid residue. In addition, the metabolites that diffused from the mycelium and were deposited on the upper surface of the medium were removed carefully, and dried at 50 °C for four days to eliminate moisture and prevent fungal growth, resulting in a dry weight of 119.8 g. The dried metabolites were powdered and extracted with acetone (500 mL \times 4) as described above, producing a gummy substance (4.89 g). The solid residue and gummy substance were combined for subsequent purification.

The crude extract was subjected to silica gel column chromatography, and the column was eluted using a gradient of n-hexane and n-hexane-chloroform mixture, with an increasing amount of chloroform, and finally with chloroform alone. The eluates were separated by thin layer chromatography and the fractions were combined based on their TLC profiles. Eight major fractions were obtained (**F1–F8**), of which **F1** was eluted with 100% n-hexane as a mixture of waxy residue and was not studied further. **F2** was eluted with n-hexane–chloroform (75:25), and resulted in three sub-fractions (**F2S_{1–3}**) of which **F2S₁** did not contain any characteristic compound. **F2S₂** was chromatographed again with n-hexane–chloroform (70:30) and yielded compound **1** (0.37 g), and **F2S₃** yielded compound **2** (50 mg) with n-hexane–chloroform (65:35) elution. **F3** was fractionated with n-hexane–chloroform (60:40) and two sub-fractions (**F3S_{1–2}**) were obtained; **F3S₁** was purified using the preparative TLC toluene-ethyl acetate (3:1) to remove traces of impurities from compound **3** (35 mg) (24 plates), after which **F3S₂** was chromatographed again with n-hexane–chloroform (50:50), yielding compound **4** (40 mg) and the major compound **5** (0.98 g). **F5** was eluted with n-hexane–chloroform (40:60) yielding a mixture of two compounds; further separation was achieved by preparative TLC using toluene-ethyl acetate (1:1) separating compound **6** (160 mg) (62 plates) and subsequently compound **7** (0.15 g) (55 plates). **F7** was fractionated with n-hexane–chloroform (10:90) as a dark red gummy solid, which after washing with diethyl ether yielded compound **8** (0.35 g). **F8** was eluted with chloroform (100%) yielding a compound with traces of impurities, and was purified by preparative TLC using toluene-ethyl acetate (1:4.5) (28 plates) finally yielding compound **9** (30 mg).

1.3.1. 8-methoxytryptethelone methyl ether (**5**)

Red plates (crystallized from chloroform: ether); mp 144–146 °C; $[\alpha]_D^{25} -5^\circ$ (*c* 0.01, CH₃OH); UV (CH₃OH) λ_{\max} 277, 369, 476 nm; IR (KBr) ν_{\max} 1691, 1636, 1567, 928, 900,

874, 782 cm^{-1} ; ^1H and ^{13}C NMR data in Table S1; HRESIMS m/z 317.1378 $[\text{M}+\text{H}]^+$ (calcd. for $\text{C}_{18}\text{H}_{21}\text{O}_5$, 317.1389).

1.3.2. 8-methoxytryptethelone (**6**)

Dark purple-red crystals (crystallized from tetrahydrofuran); mp 170–172 $^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{25} +502^{\circ}$ (c 0.0050, CH_3OH); UV (CH_3OH) λ_{max} 276, 307, 367, 487 nm; ECD (CH_3OH) λ_{max} 303 & 495 (+), 279 & 342 (–) nm; IR (KBr) ν_{max} 3247, 1690, 1620, 1547, 936, 873, 787, 708 cm^{-1} ; ^1H and ^{13}C NMR data in Table S1; HRESIMS m/z : 303.1228 $[\text{M}+\text{H}]^+$ (calcd. for $\text{C}_{17}\text{H}_{19}\text{O}_5$, 303.1227).

1.3.3. 5'-hydroxy-8-ethoxytryptethelone (**9**)

Red amorphous powder; $[\alpha]_{\text{D}}^{25} +533^{\circ}$ (c 0.001, CH_3OH); UV (CH_3OH) λ_{max} 277, 310, 372, 490 nm; ECD (CH_3OH) λ_{max} 306 & 495 (+), 281 & 345 (–) nm; IR (KBr) ν_{max} 3359, 1687, 1622, 1596, 938, 881, 789 cm^{-1} ; ^1H and ^{13}C NMR data in Table S1; HRESIMS m/z : 333.1315 $[\text{M}+\text{H}]^+$ (calcd. for $\text{C}_{18}\text{H}_{21}\text{O}_6$, 333.1333).

1.4. Crystallization

The compounds were dissolved in a single or a combination of two solvents ($v/v = 10:1$), and heated in a water bath at 65 $^{\circ}\text{C}$. The solutions were cooled and left undisturbed at room temperature (28 ± 1 $^{\circ}\text{C}$) to allow slow evaporation of the solvent and the development of crystals. This process was repeated continuously until good diffraction-quality crystals were obtained. Compounds **5**, **6** and **7** yielded diffraction-quality crystals and the following solvents were suitable for the crystallization of these compounds: Chloroform-ether mix – red plates (compound **5**); tetrahydrofuran – dark purple-red crystals (compound **6**); chloroform-ether mix – dark violet crystals (compound **7**).

1.4.1. X-ray crystallography studies (Compounds **5**–**7**)

X-ray crystallography was performed with Bruker AXS Kappa Apex II CCD Diffractometer equipped with graphite monochromated Mo ($\text{K}\alpha$) ($\lambda = 0.7107$ Å) radiation. The automatic cell determination routine with 36 frames at three different orientations of the detector was employed to collect reflections for unit cell determination. Furthermore, intensity data for structure determination were collected through an optimized strategy which gave an average 4-fold redundancy. The APEX2-SAINT program (Bruker 2004) was used for integrating the frames. Four-fold redundancy per reflection was utilized for achieving good multi-scan absorption correction using the SADABS program (Bruker 2004). In addition to absorption, Lorentz, polarization and decay corrections were applied to intensity during data reduction. The structures were solved by direct methods using SIR92 (Altomare

et al. 1993), and refined by full-matrix least squares technique using SHELXL–2014/7 (Sheldrick 2015) program. Molecular graphics were drawn using ORTEP3 (Farrugia 1997). The crystallographic data have been deposited in the Cambridge Crystallographic Data Centre under the reference numbers 1411890 (compound **5**), 1411971 (compound **6**) and 1520256 (compound **7**). The CCDC data are freely available in the database.

1.5. Bioassays (Compounds 1, 2, 5–8)

1.5.1. Antimycobacterial assay

Minimum inhibitory concentration (MIC) was determined against mycobacterial strains using the 7H11 Middlebrook agar dilution method (Hacek 1992). Standardized inocula of mycobacterial strains were grown on drug-free media and media containing graded concentrations of the drugs to be tested, and incubated at 37 °C under 5–10% CO₂. Colony-forming units (CFU) were determined after eight weeks of incubation. The concentration of the compound that inhibited growth of mycobacteria was referred to as the MIC of that drug. Rifampicin was included in the assay as a positive control and its MIC was determined to be 8 µg/mL, which is satisfactory according to WHO standards for testing rifampicin.

1.5.2. Antibacterial assay

The biological activity of the isolated compounds against pathogenic Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Vibrio cholera*, *Salmonella typhi*, *S. typhi-murium*, *Shigella dysenteriae*, *Escherichia coli*, *Klebsiella pneumoniae*) was determined by the minimum inhibitory concentration (MIC) method (Wiegand et al. 2008) using Muller Hinton agar. Standardized inocula of the different bacteria were seeded on drug-free media and media containing graded concentrations of the drugs to be tested, and incubated at 37 °C under 5–10% CO₂. The presence or absence of bacterial growth was measured after 18–24 h of incubation.

Table S1. ^1H and ^{13}C NMR spectroscopic data of Compounds **4–6** and **9** (^1H 500 MHz, ^{13}C 125 MHz)

Position	4^a		5^a		6^a		9^b	
	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}
1		186.9		182.6		182.4		182.4
2		174.4		175.8		174.8		175.5
3		122.8		121.7		121.6		125.1
4		170.6		170.9		171.5		174.7
5		132.7		135.4		136.3		136.7
6	6.71, s	119.8	6.81, s	119.6	6.99, s	123.4	6.95, s	123.9
7		152.5		156.4		152.9		155.7
8		156.0		151.5		149.1		149.1
9		114.0		125.4		123.7		116.8
10		115.6		118.1		118.3		116.7
11							4.03, q (7.0)	69.5
12							1.44, t (7.0)	14.2
1'	1.44, d (6.5)	14.7	1.44, d (6.5)	14.7	1.46, d (6.5)	14.7	1.64, d (7.0)	13.1
2'	4.61, q (6.5)	92.4	4.59, q (6.5)	92.1	4.63, q (6.5)	92.3	4.77, q (6.5)	92.3
3'		43.0		43.0		42.9		48.1
4'	1.24, s	20.2	1.24, s	20.3	1.27, s	20.3	1.36, s	19.4
5'	1.43, s	25.6	1.42, s	25.7	1.45, s	25.7	3.80, d (11.5) 3.71, d (11.5)	62.7
5-CH ₃	2.54, s	22.2	2.59, s	22.8	2.57, s	22.6	2.54, s	21.3
7-OCH ₃	3.94, s	56.2	3.91, s	56.1				
8-OCH ₃			3.88, s	61.3	3.92, s	62.1		
8-OH	13.15, s							

^a Data collected in CDCl₃^b Data collected in CD₃OD

Figure S1. Key HMBC (red arrows), NOESY (double-headed black arrows) and COSY (blue bold) correlations of Compounds **5**, **6** and **9**

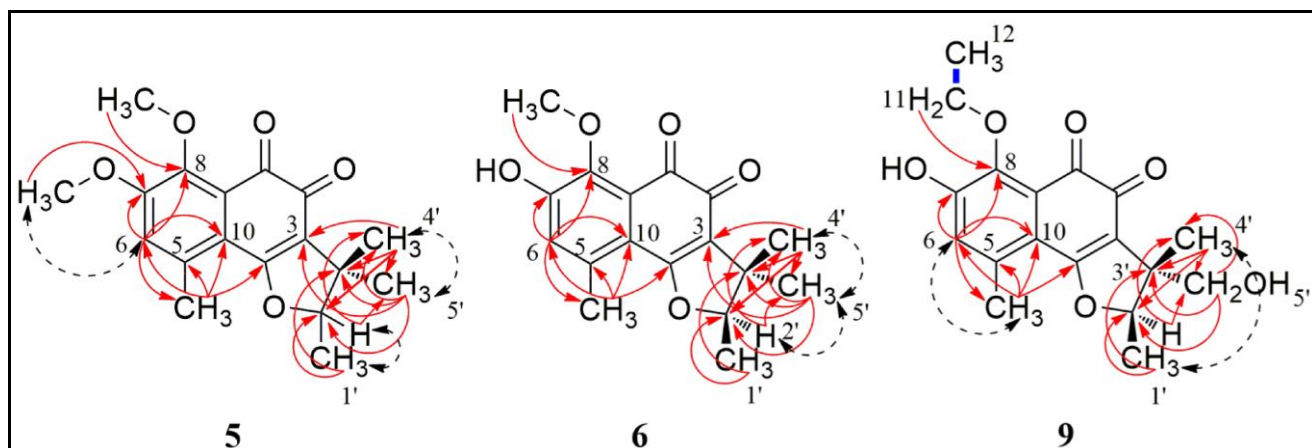


Figure S2. X-ray ORTEP of Compound **5** showing racemic mixture

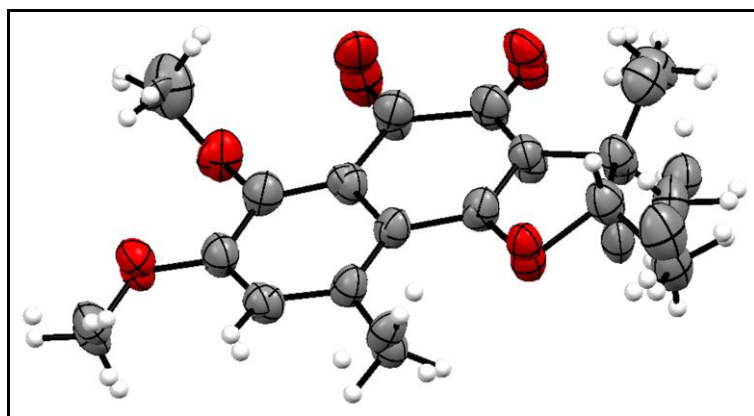


Figure S3. ECD spectra of Compounds **6** and **9**

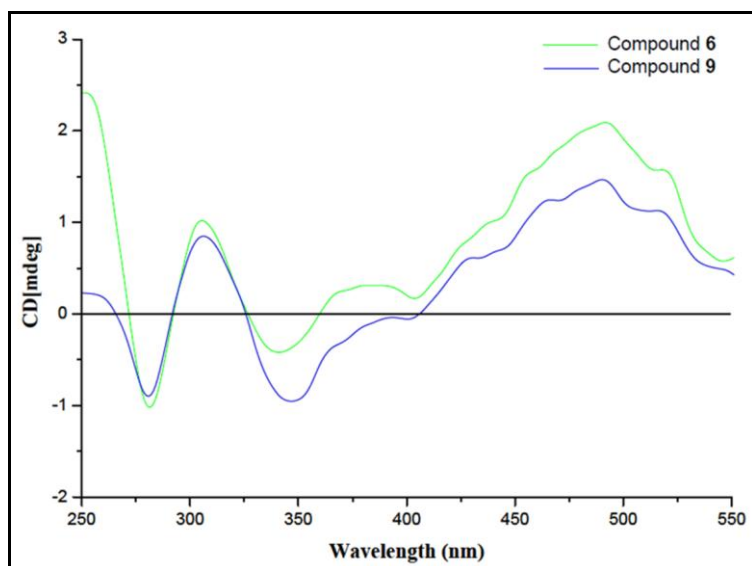


Table S2. Antimycobacterial activity of Compounds **7** and **8** against selected non-tuberculosis mycobacterial strains.

S.No.	Non-tuberculosis mycobacterial strain	MIC (µg/mL)	
		7	8
1	<i>M. phlei</i>	25	50
2	<i>M. chitae</i>	25	50
3	<i>M. fortuitum</i>	>100	>100
4	<i>M. thermoresistibile</i>	>100	>100
5	<i>M. smegmatis</i>	>100	>100
6	<i>M. parafortuitum</i>	25	50
7	<i>M. flavescens</i>	25	50
8	<i>M. simiae</i>	>100	>100
9	<i>M. asiaticum</i>	50	>100
10	<i>M. szulgai</i>	25	50
11	<i>M. intracellulare</i>	50	50
12	<i>M. avium</i>	50	50
13	<i>M. kansasii</i>	25	50
14	<i>M. gordonae</i>	>100	>100

Compounds: **7** - tryptethelone; **8** - 4'-hydroxy-8-methoxytryptethelone methyl ether.

Table S3. Antibacterial activity of Compounds **7** and **8** against Gram positive and Gram negative bacterial species.

S.No.	Strain	MIC (µg/mL)	
		7	8
1	<i>Staphylococcus aureus</i>	25	25
2	<i>Vibrio cholera</i>	>100	>100
3	<i>Salmonella typhi</i>	>100	>100
4	<i>S. typhi-murium</i>	>100	>100
5	<i>Shigella dysenteriae</i>	>100	>100
6	<i>Escherichia coli</i>	>100	>100
7	<i>Klebsiella pneumoniae</i>	>100	>100

Compounds: **7** - tryptethelone; **8** - 4'-hydroxy-8-methoxytryptethelone methyl ether.

Figure S4. ^1H NMR (500 MHz, CDCl_3) spectrum of Compound **5**

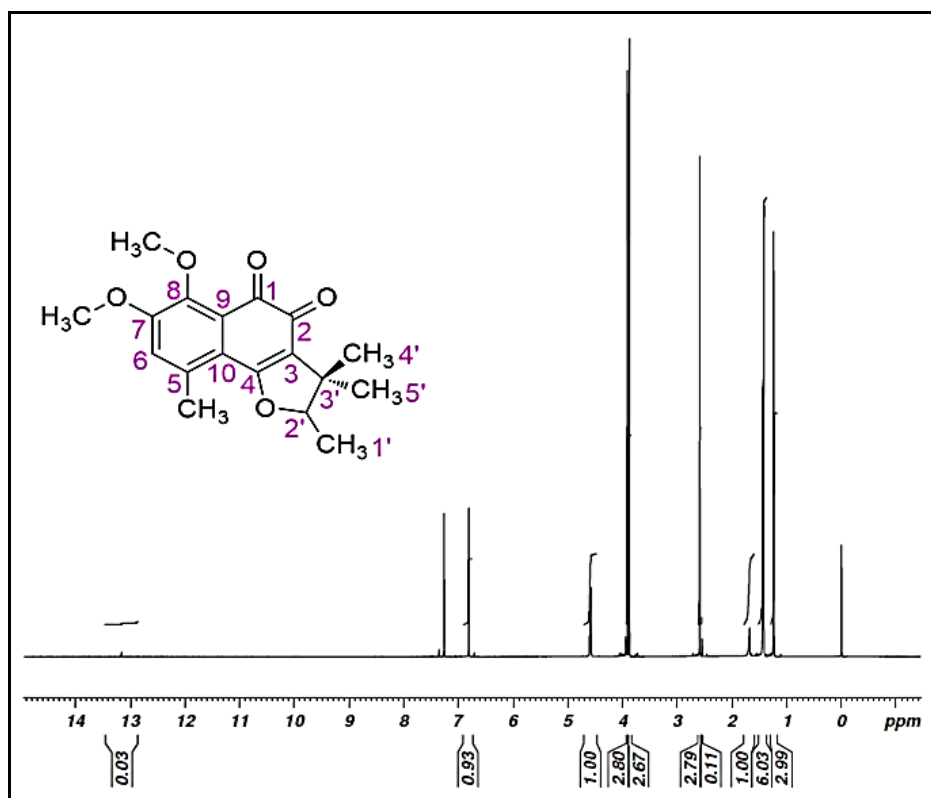


Figure S5. ^1H NMR (500 MHz, CDCl_3) spectrum of Compound **5** (Expansion 1)

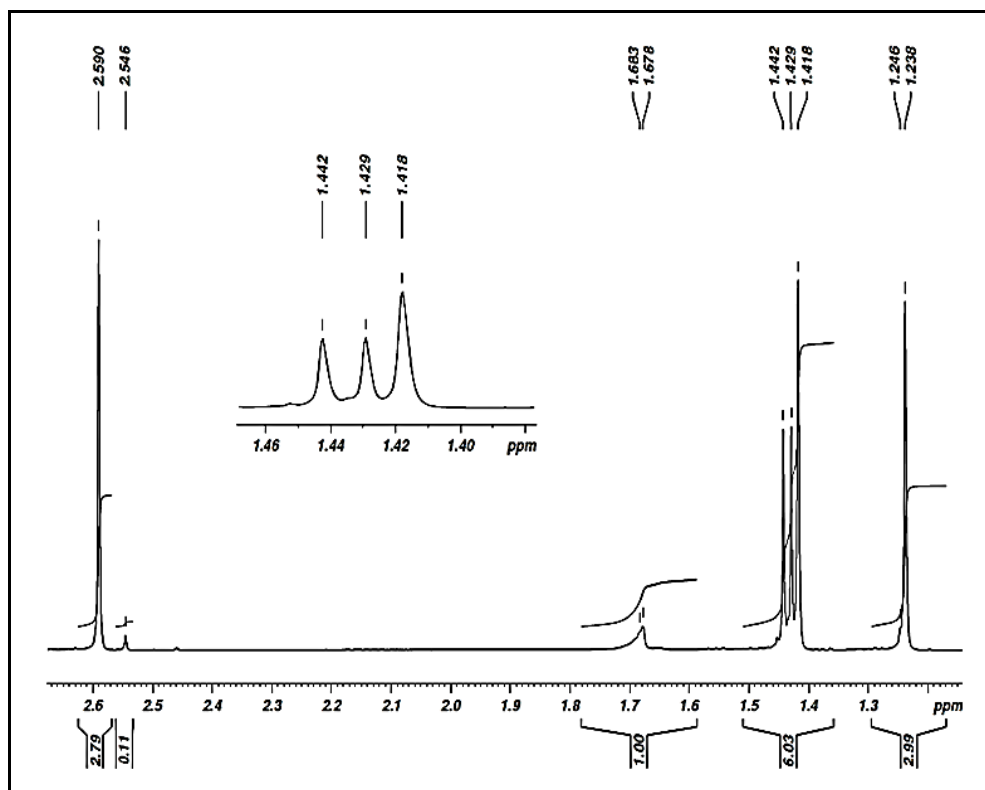


Figure S6. ^1H NMR (500 MHz, CDCl_3) spectrum of Compound **5** (Expansion 2)

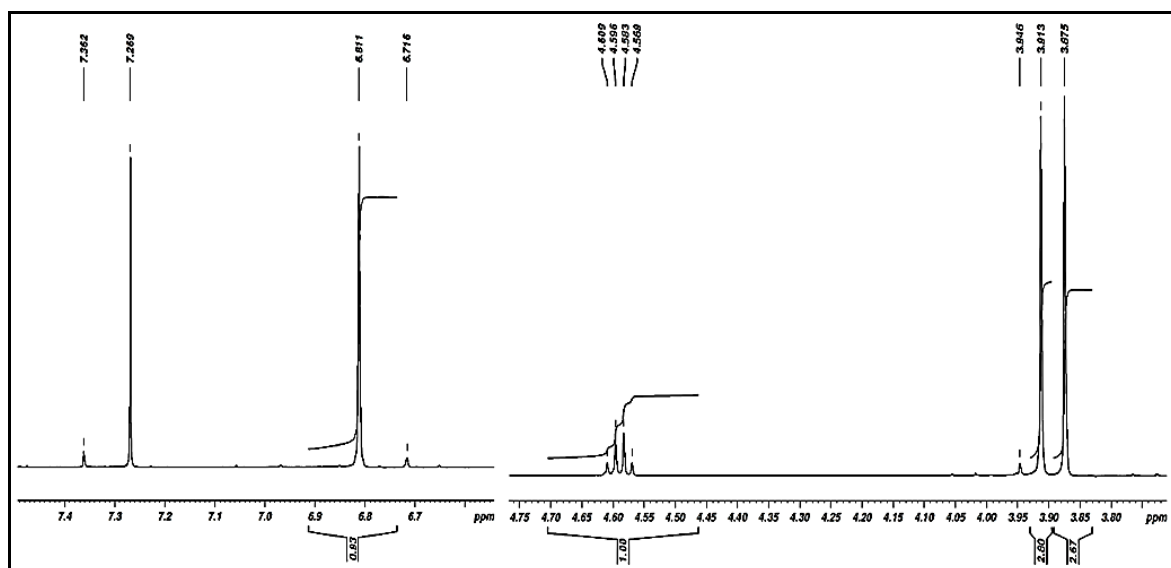


Figure S7. ^{13}C NMR (125 MHz, CDCl_3) spectrum of Compound **5**

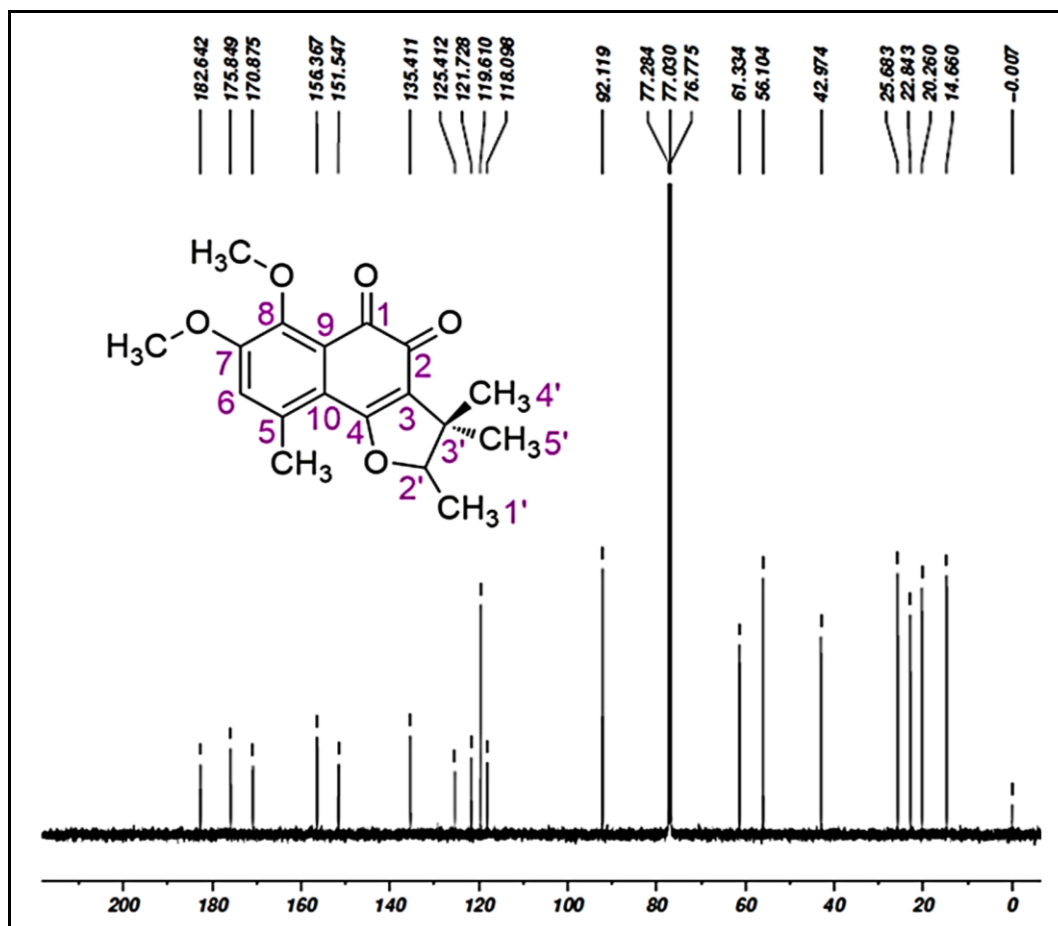


Figure S8. ^{13}C NMR DEPT 135 (125 MHz, CDCl_3) spectrum of Compound **5**

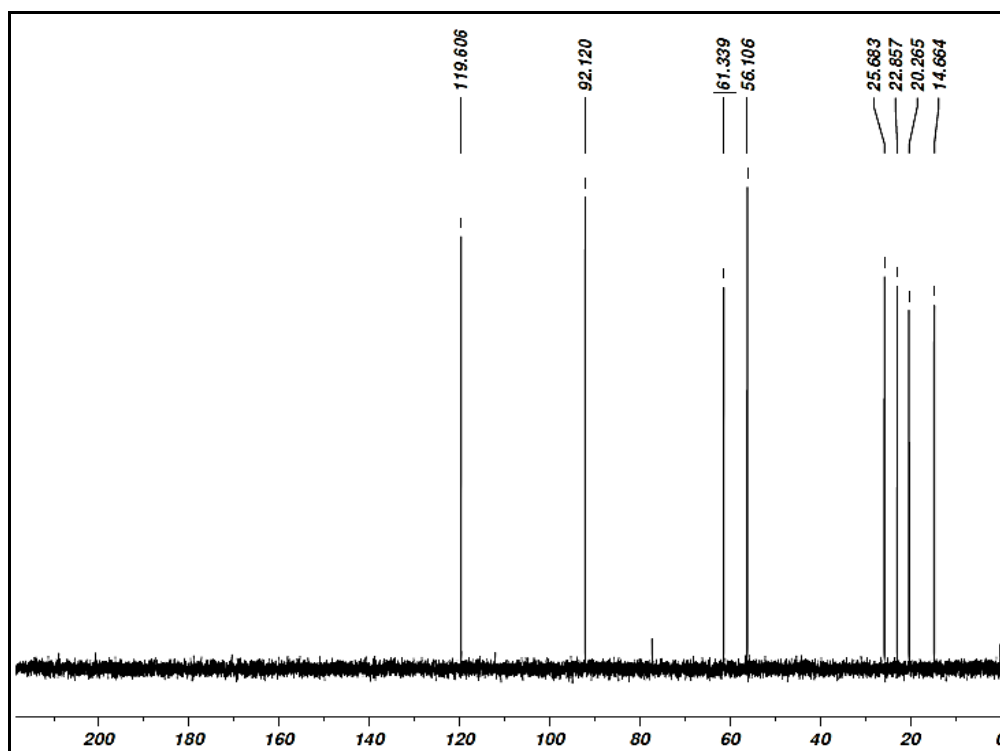


Figure S9. ^1H - ^{13}C HSQC (^1H 500 MHz, ^{13}C 125 MHz, CDCl_3) spectrum of Compound **5**

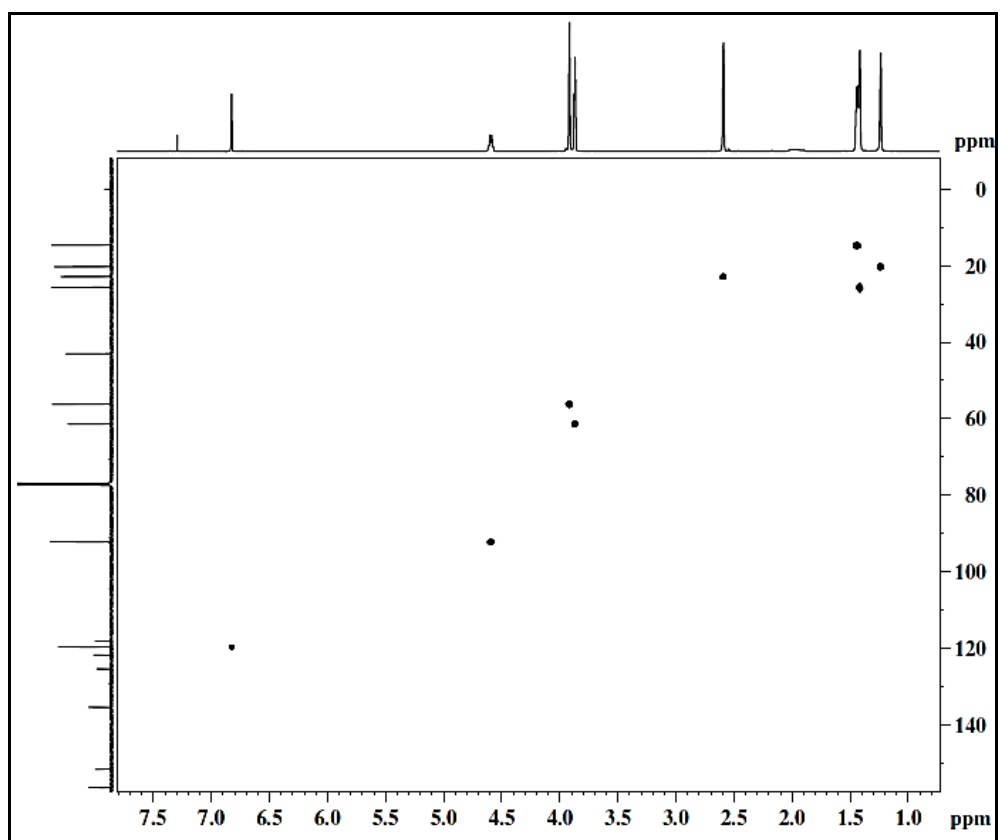


Figure S10. ^1H - ^{13}C HMBC (^1H 500 MHz, ^{13}C 125 MHz, CDCl_3) spectrum of Compound **5**

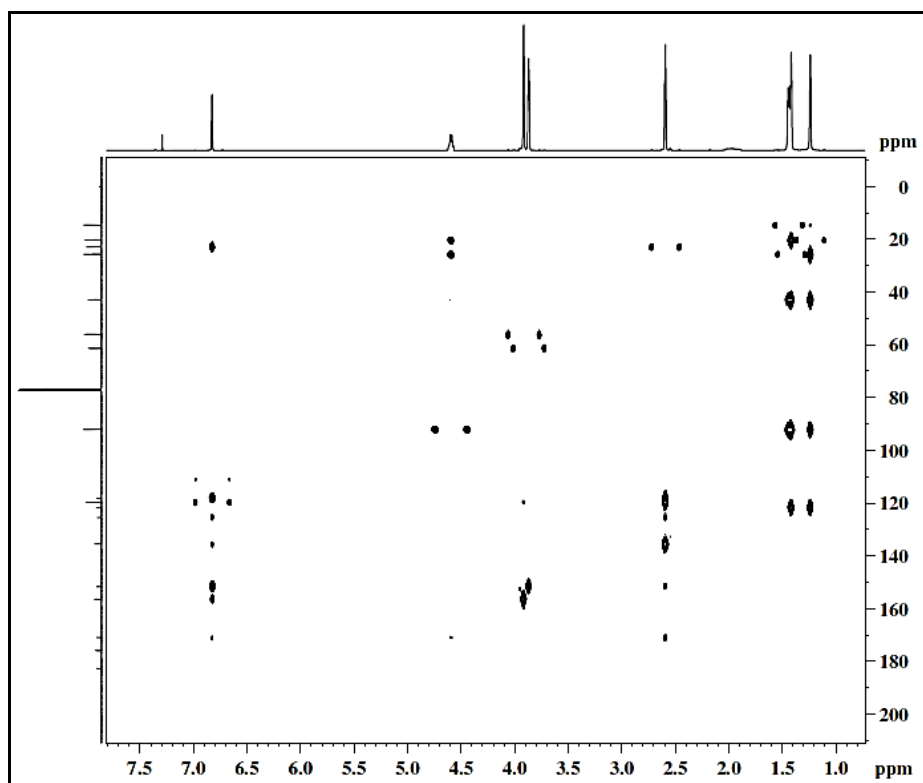


Figure S11. ^1H - ^1H NOESY (500 MHz, CDCl_3) spectrum of Compound **5**

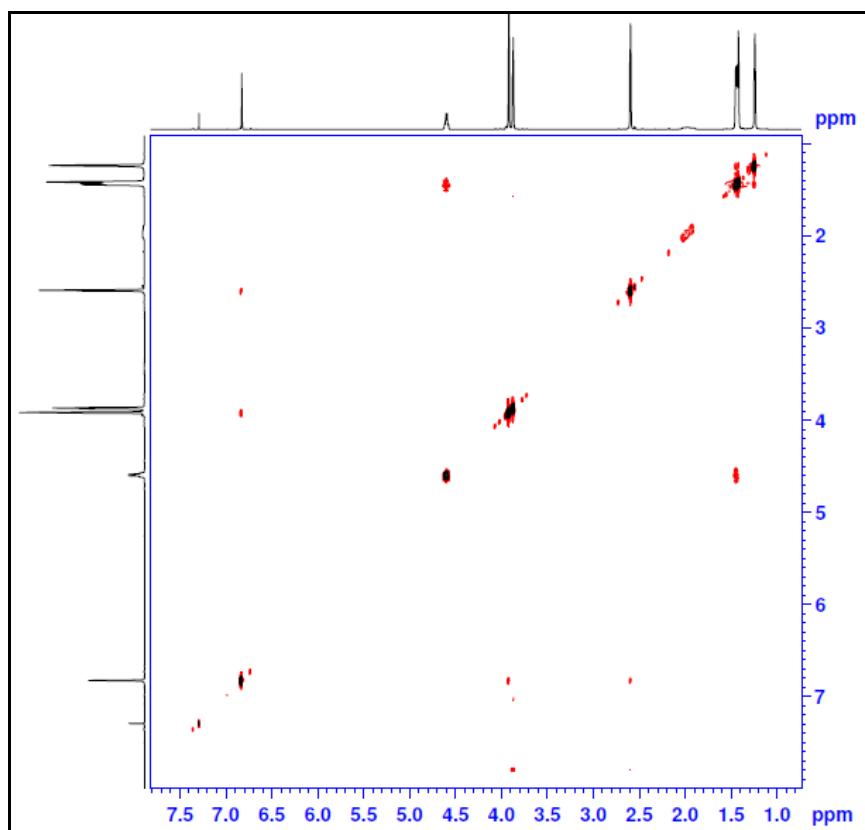


Figure S12. ^1H - ^1H COSY (500 MHz, CDCl_3) spectrum of Compound **5**

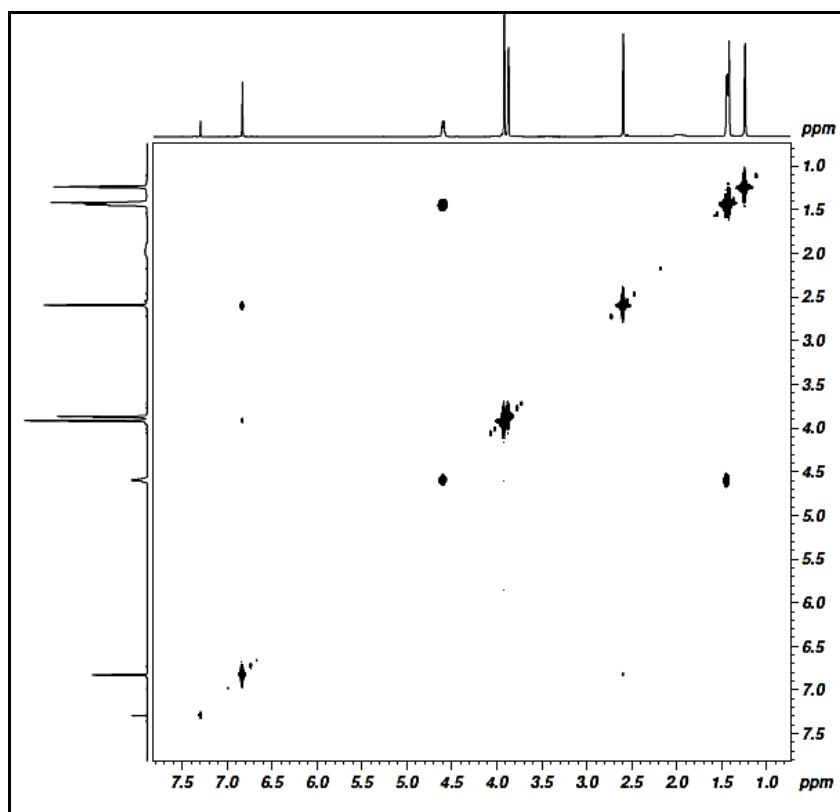


Figure S13. HRESIMS (positive mode) spectrum of Compound **5**

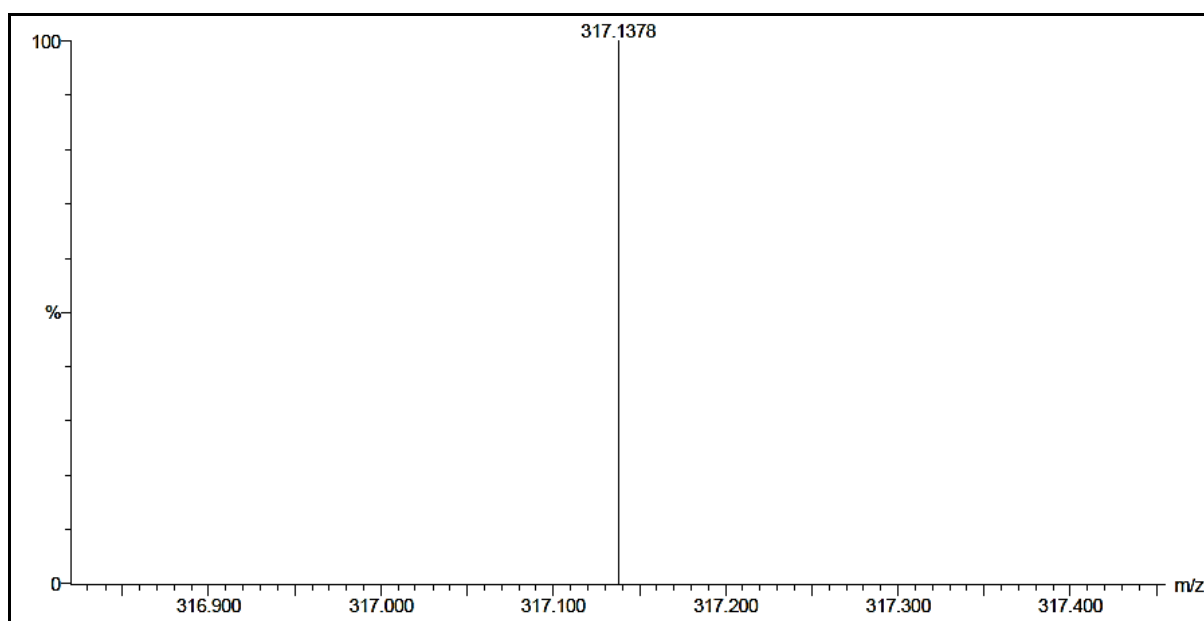


Figure S14. ^1H NMR (500 MHz, CDCl_3) spectrum of Compound **6**

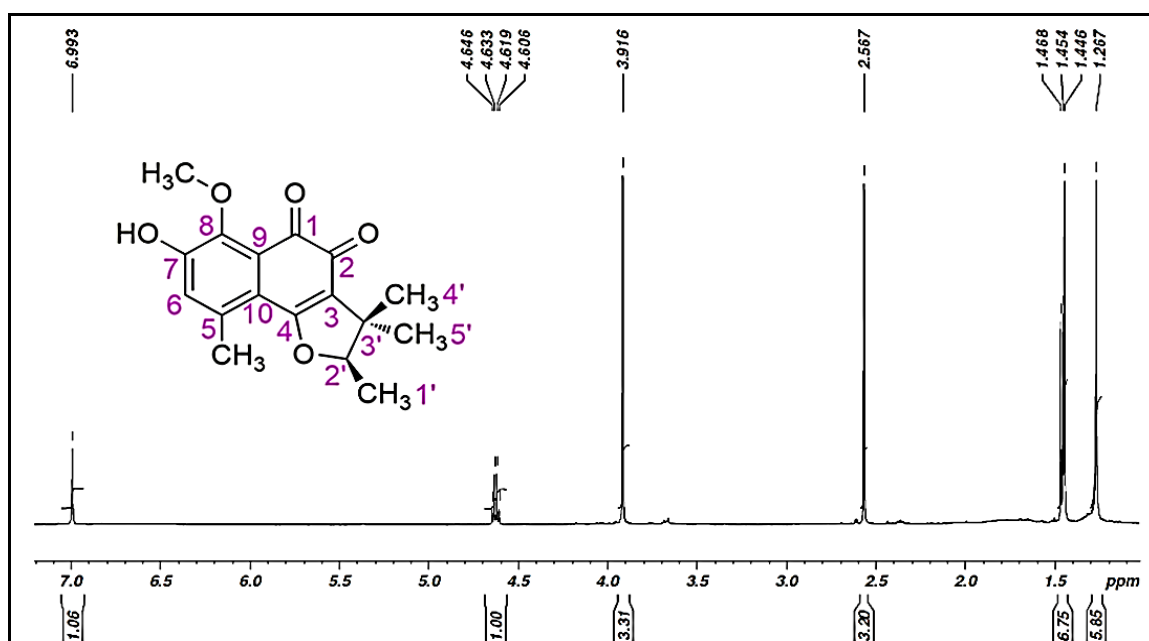


Figure S15. ^{13}C NMR (125 MHz, CDCl_3) spectrum of Compound **6**

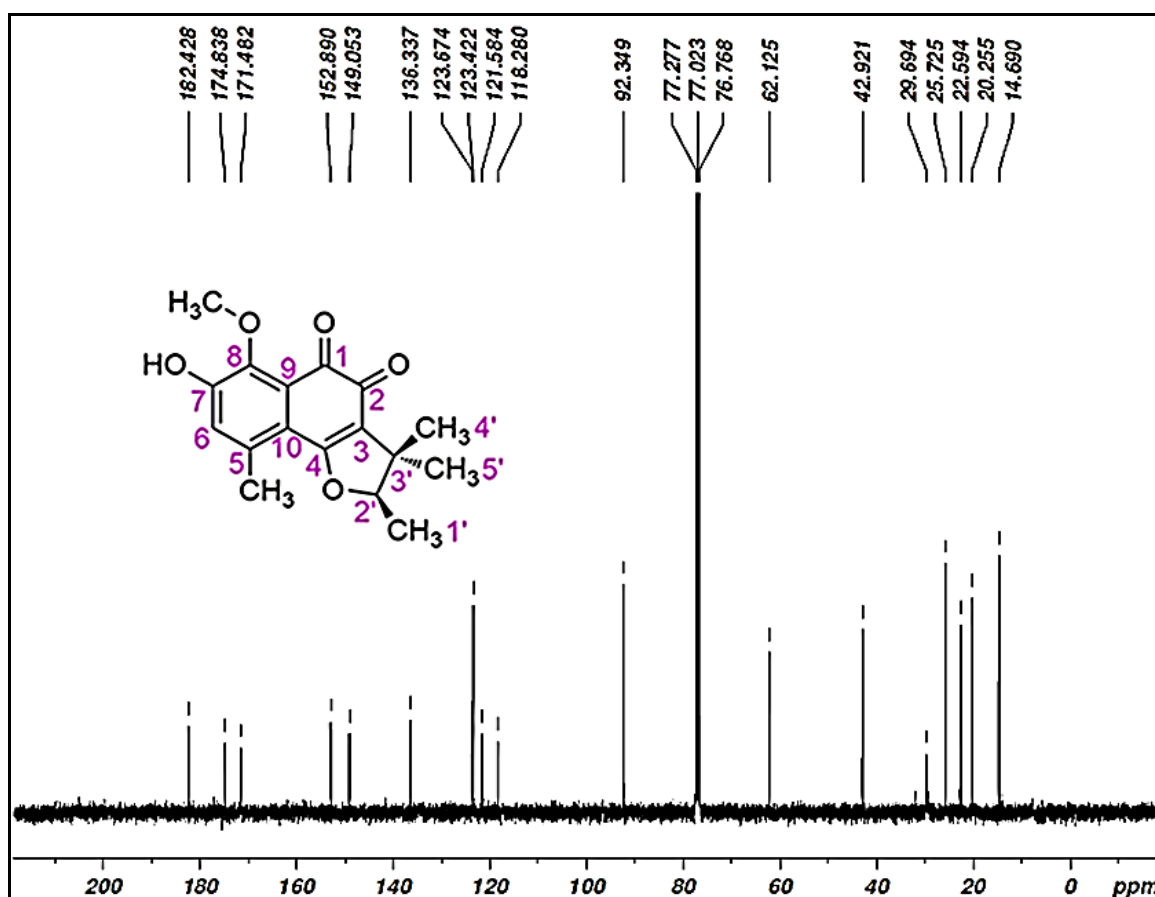


Figure S16. ^{13}C NMR DEPT 135 (125 MHz, CDCl_3) spectrum of Compound **6**

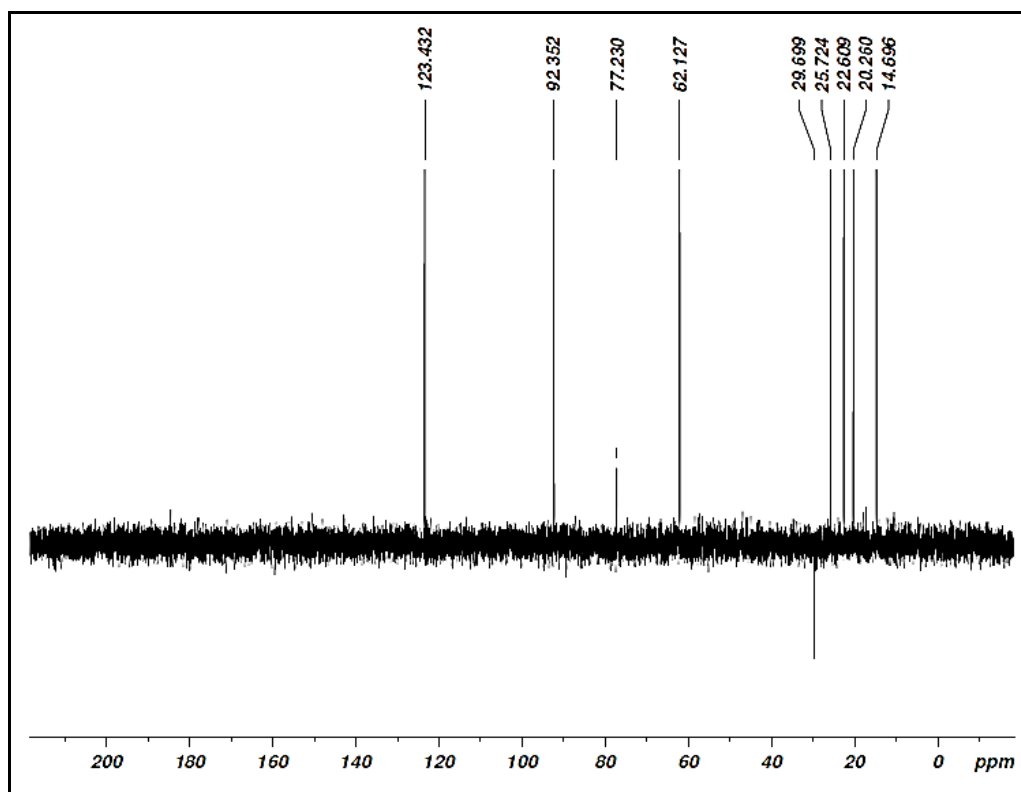


Figure S17. ^1H - ^{13}C HSQC (^1H 500 MHz, ^{13}C 125 MHz, CDCl_3) spectrum of Compound **6**

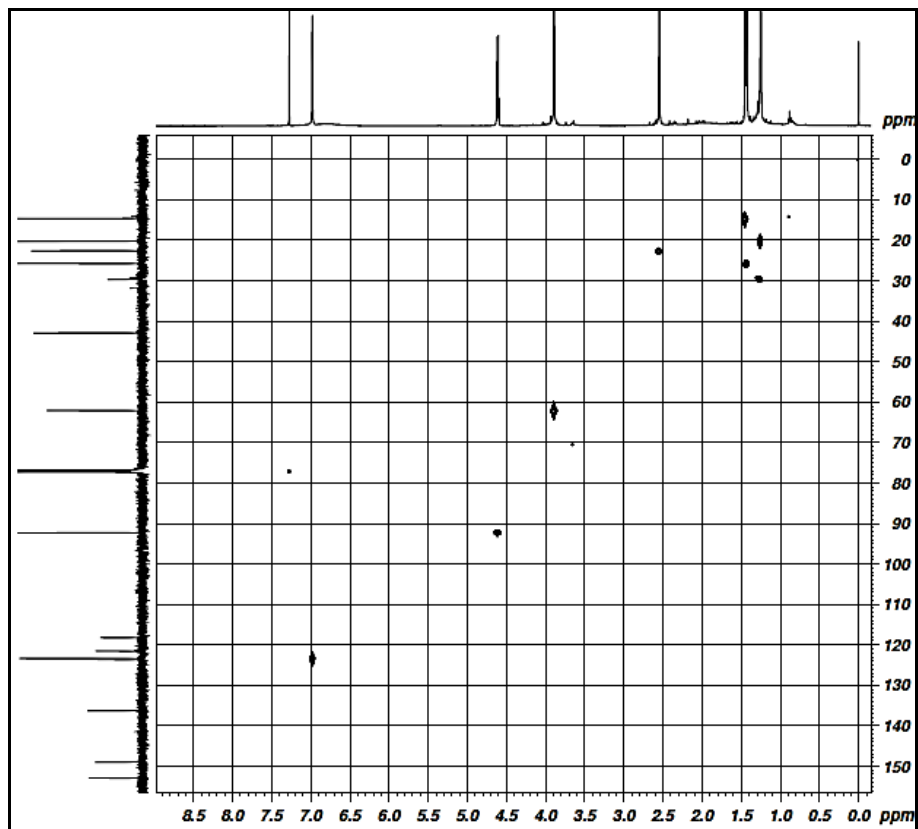


Figure S18. ^1H - ^{13}C HMBC (^1H 500 MHz, ^{13}C 125 MHz, CDCl_3) spectrum of Compound **6**

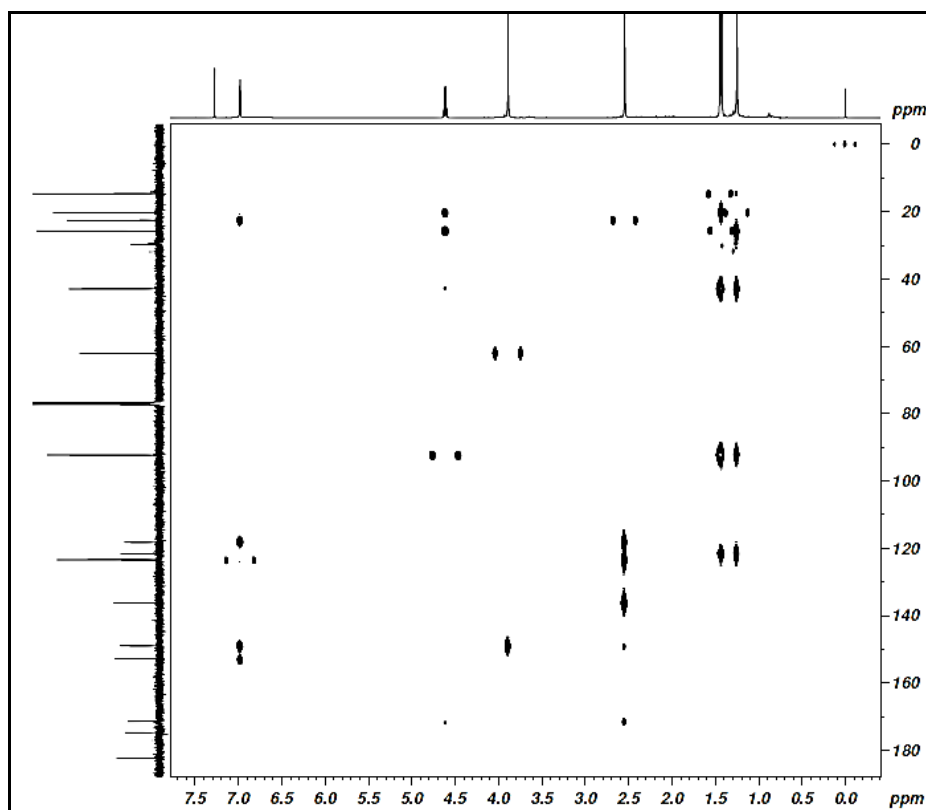


Figure S19. ^1H - ^1H NOESY (500 MHz, CDCl_3) spectrum of Compound **6**

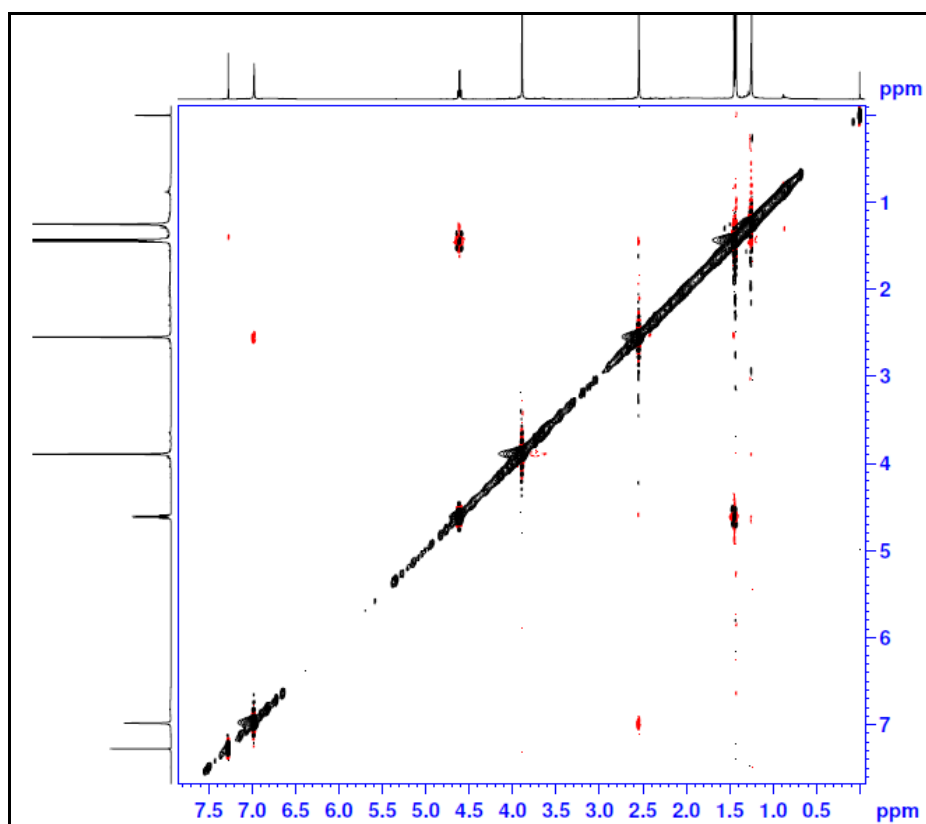


Figure S20. ^1H - ^1H COSY (500 MHz, CDCl_3) spectrum of Compound **6**

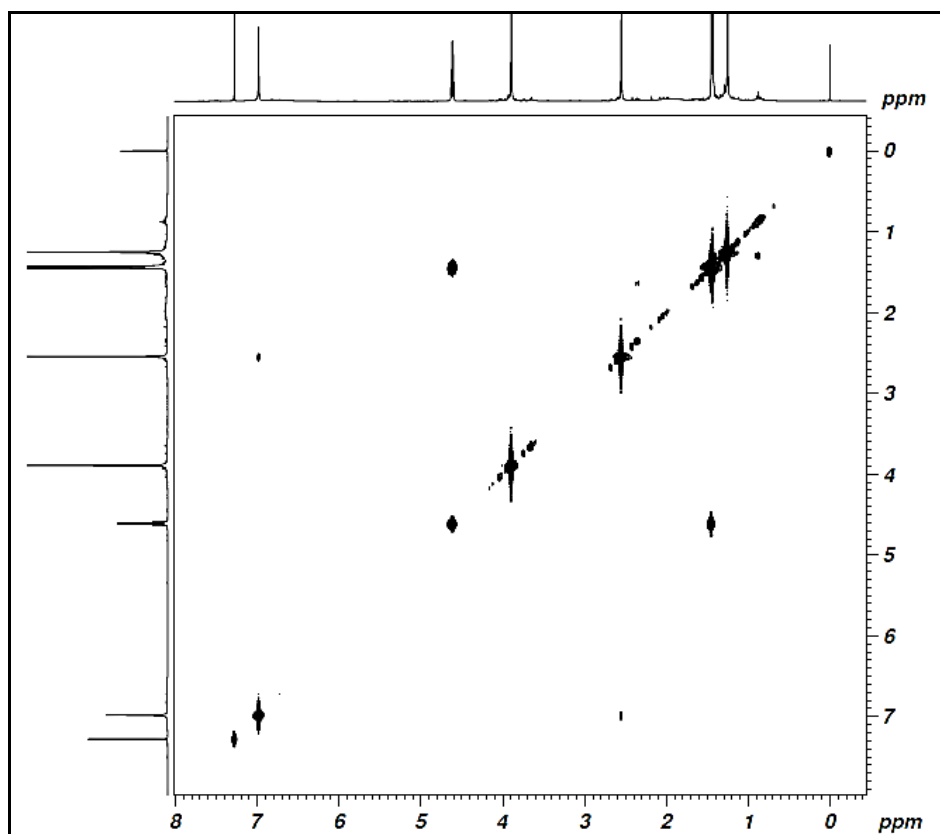


Figure S21. HRESIMS (positive mode) spectrum of Compound **6**

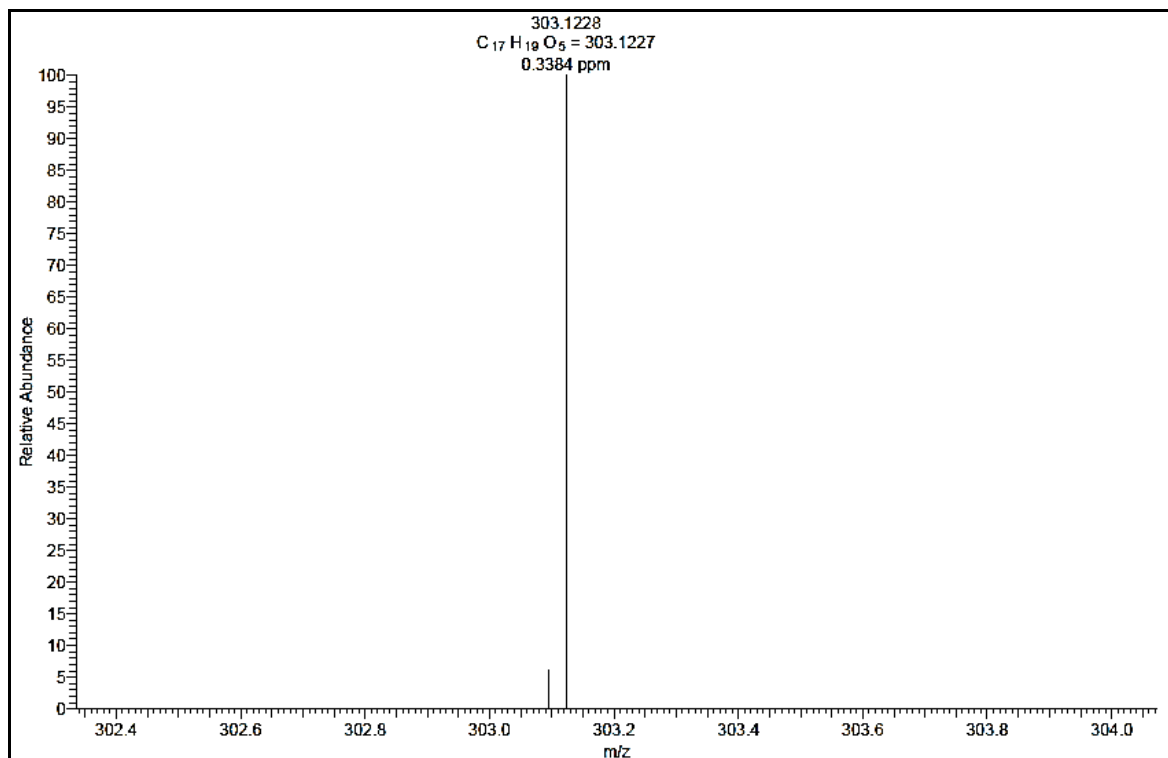


Figure S22. ^1H NMR (500 MHz, CD_3OD) spectrum of Compound **9**

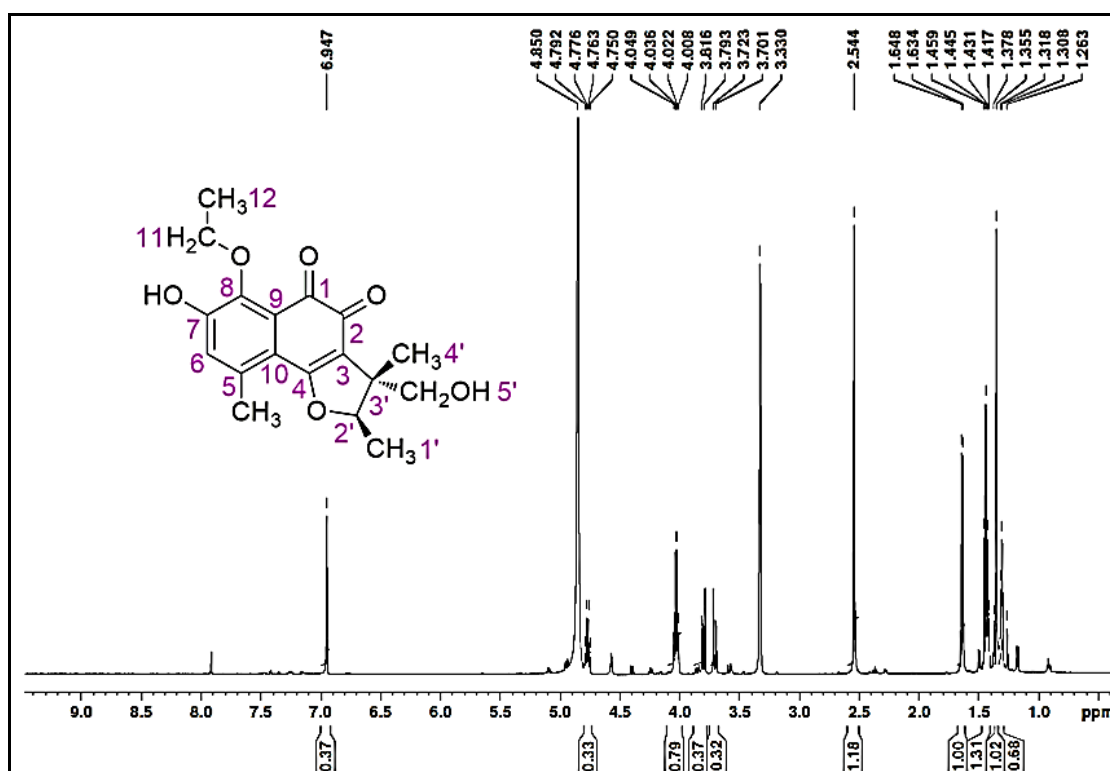


Figure S23. ^{13}C NMR (125 MHz, CD_3OD) spectrum of Compound **9**

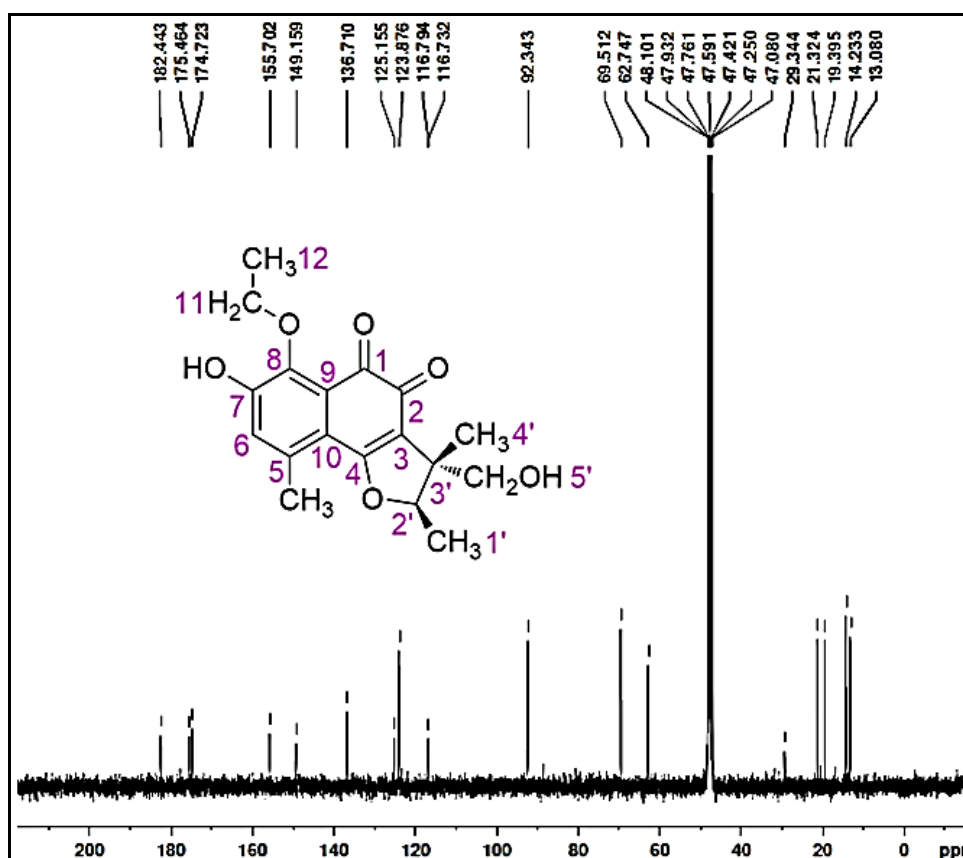


Figure S24. ^{13}C NMR DEPT 135 (125 MHz, CD_3OD) spectrum of Compound **9**

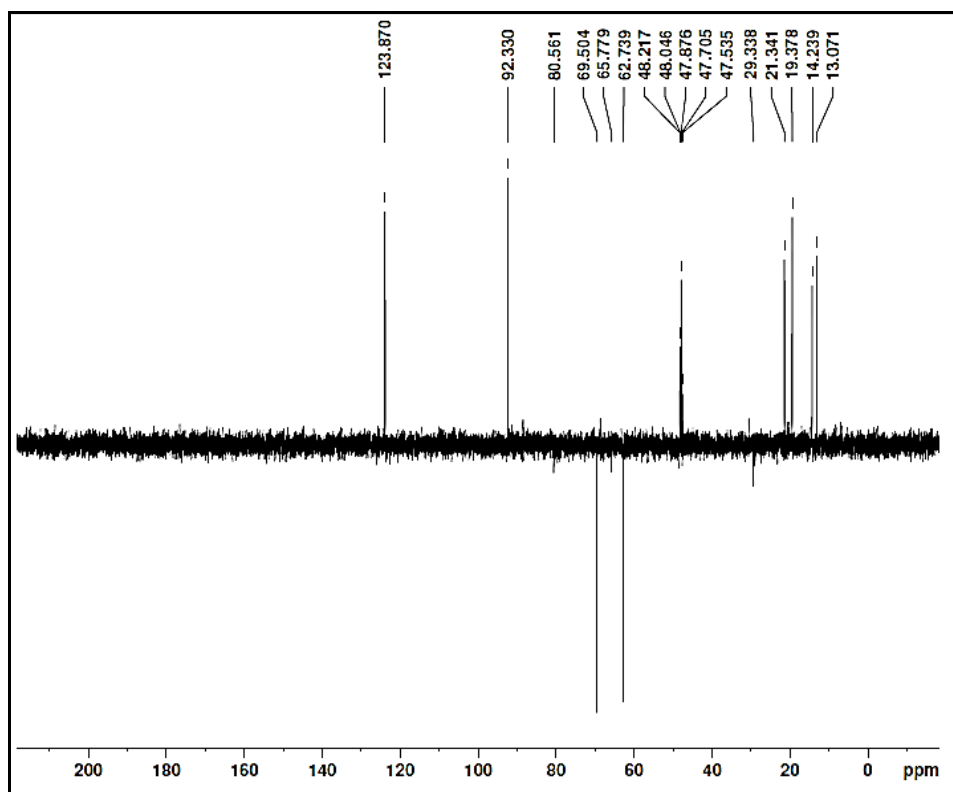


Figure S25. ^1H - ^{13}C HSQC (^1H 500 MHz, ^{13}C 125 MHz, CD_3OD) spectrum of Compound **9**

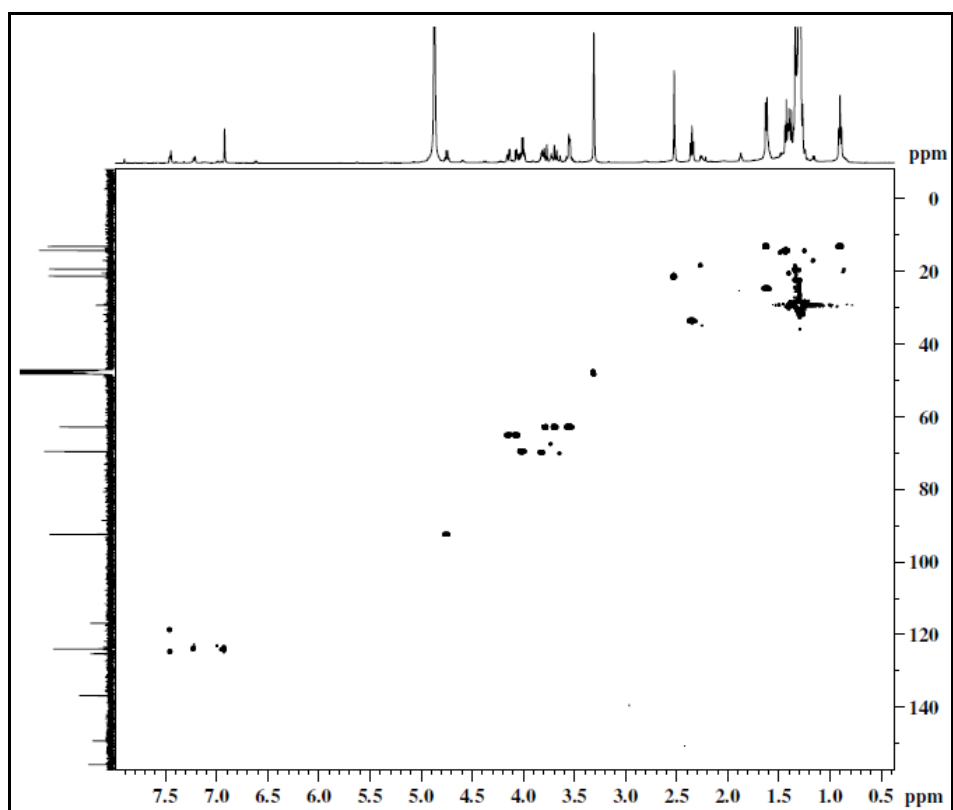


Figure S26. ^1H - ^{13}C HMBC (^1H 500 MHz, ^{13}C 125 MHz, CD_3OD) spectrum of Compound **9**

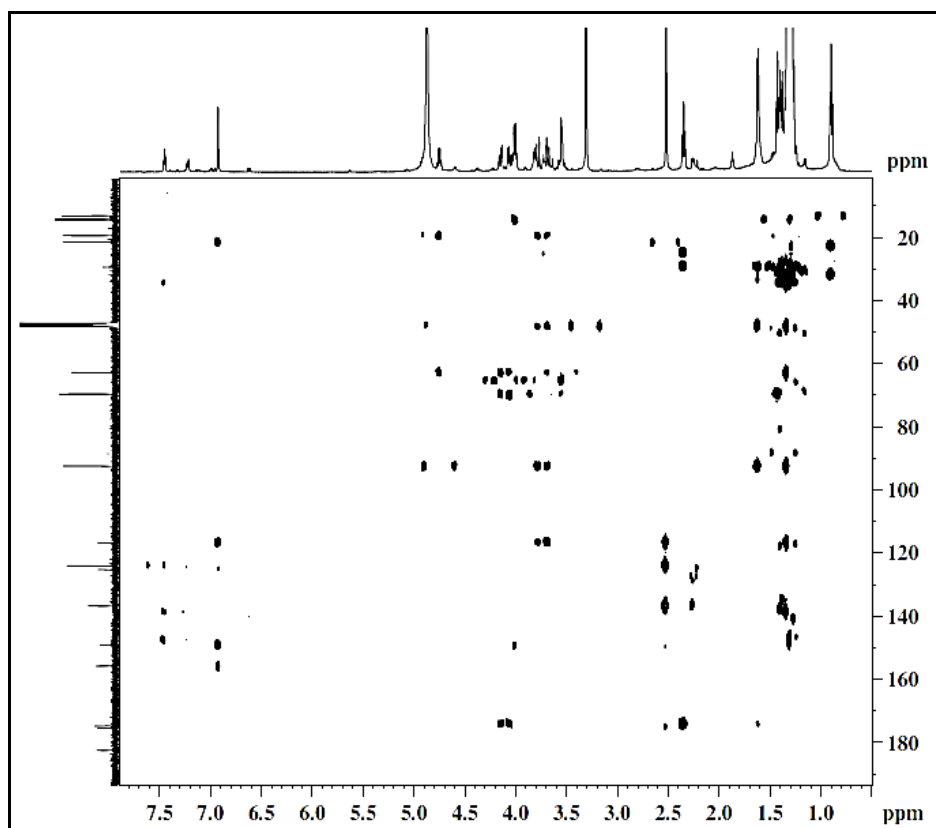


Figure S27. ^1H - ^1H NOESY (500 MHz, CD_3OD) spectrum of Compound **9**

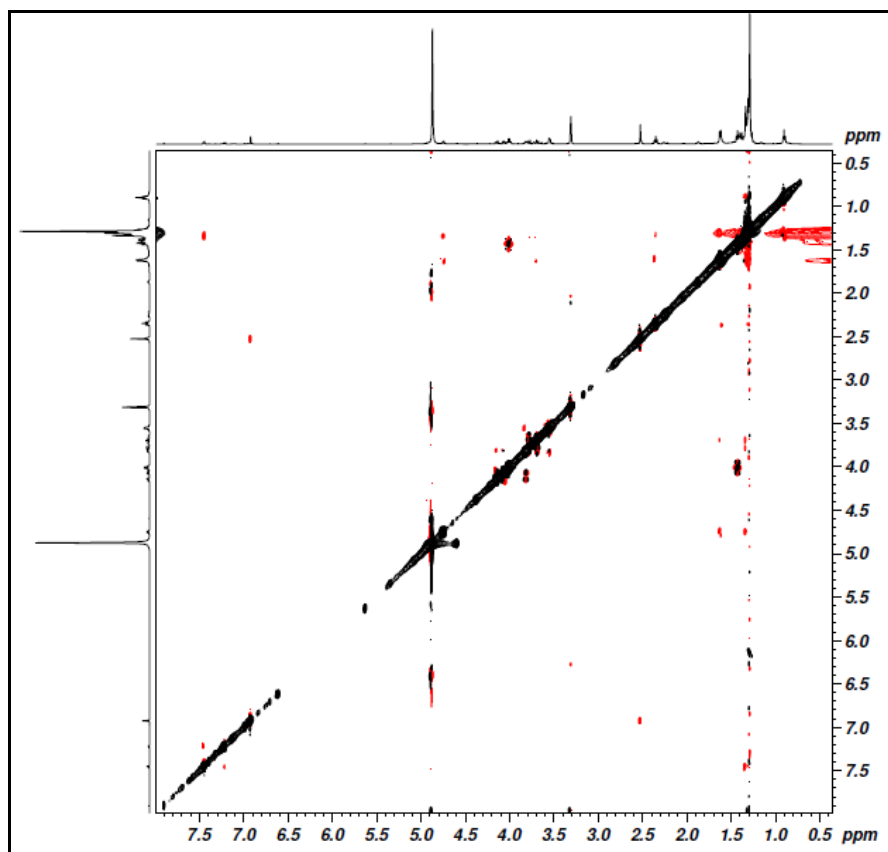


Figure S28. ^1H - ^1H COSY (500 MHz, CD_3OD) spectrum of Compound **9**

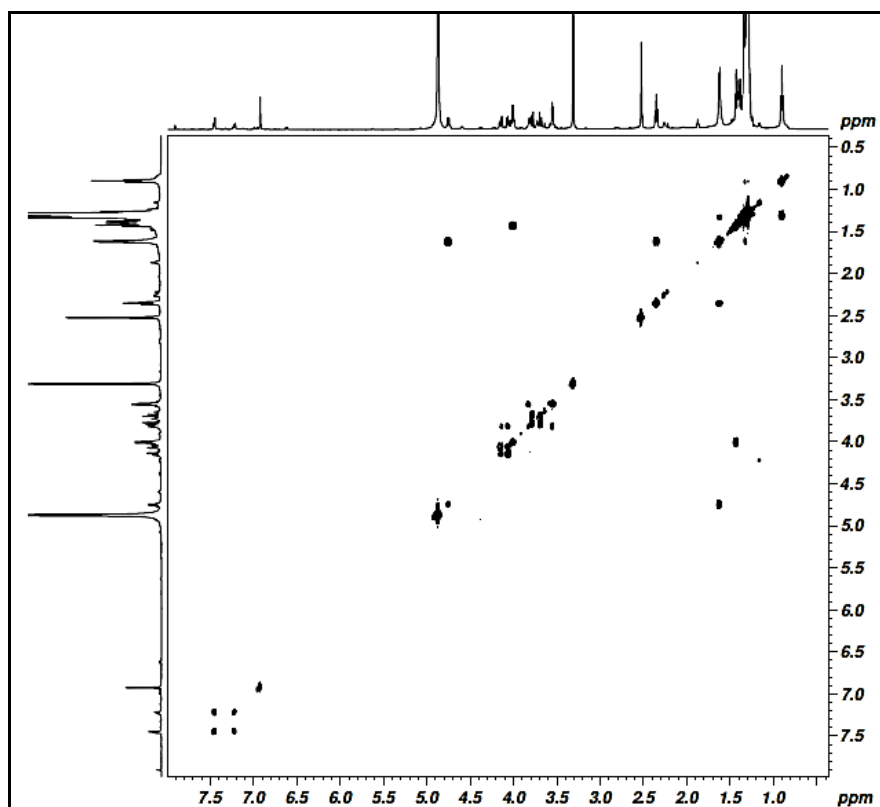


Figure S29. HRESIMS (positive mode) spectrum of Compound **9**

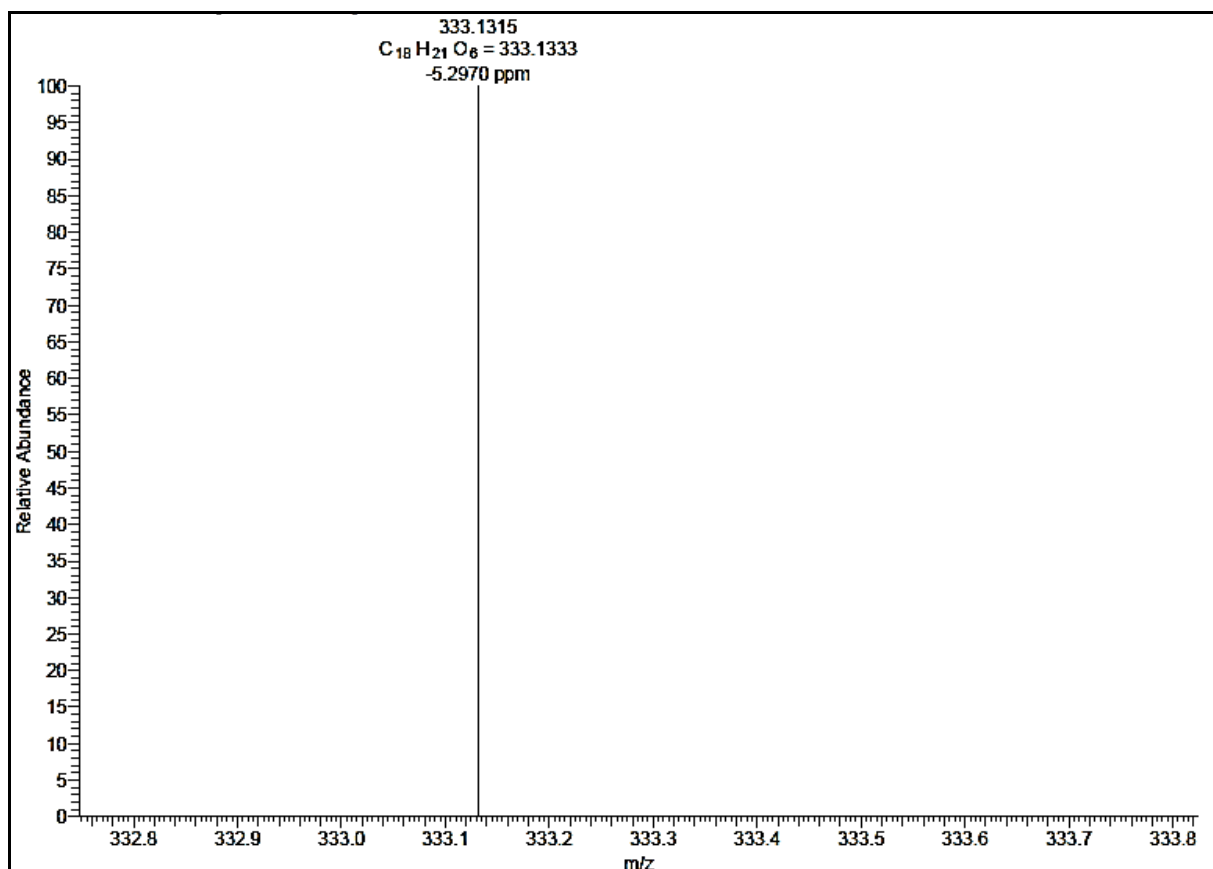


Figure S30. ^1H NMR (500 MHz, CDCl_3) spectrum of Compound **4**

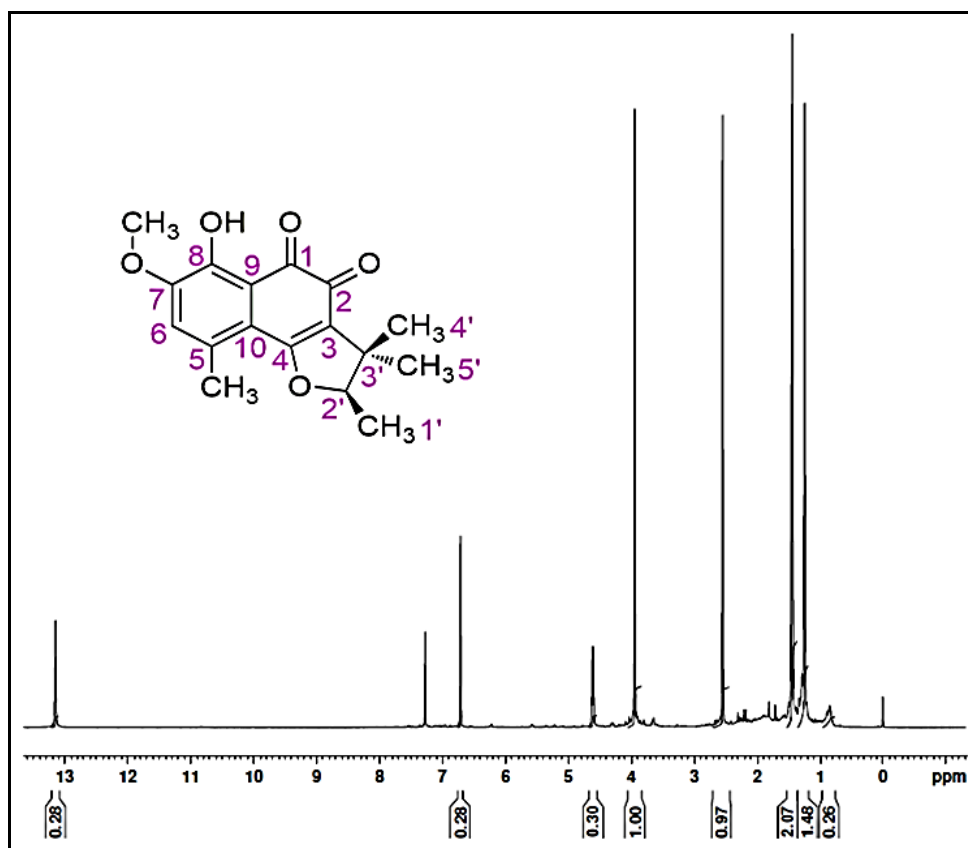


Figure S31. ^1H NMR (500 MHz, CDCl_3) spectrum of Compound **4** (Expansion 1)

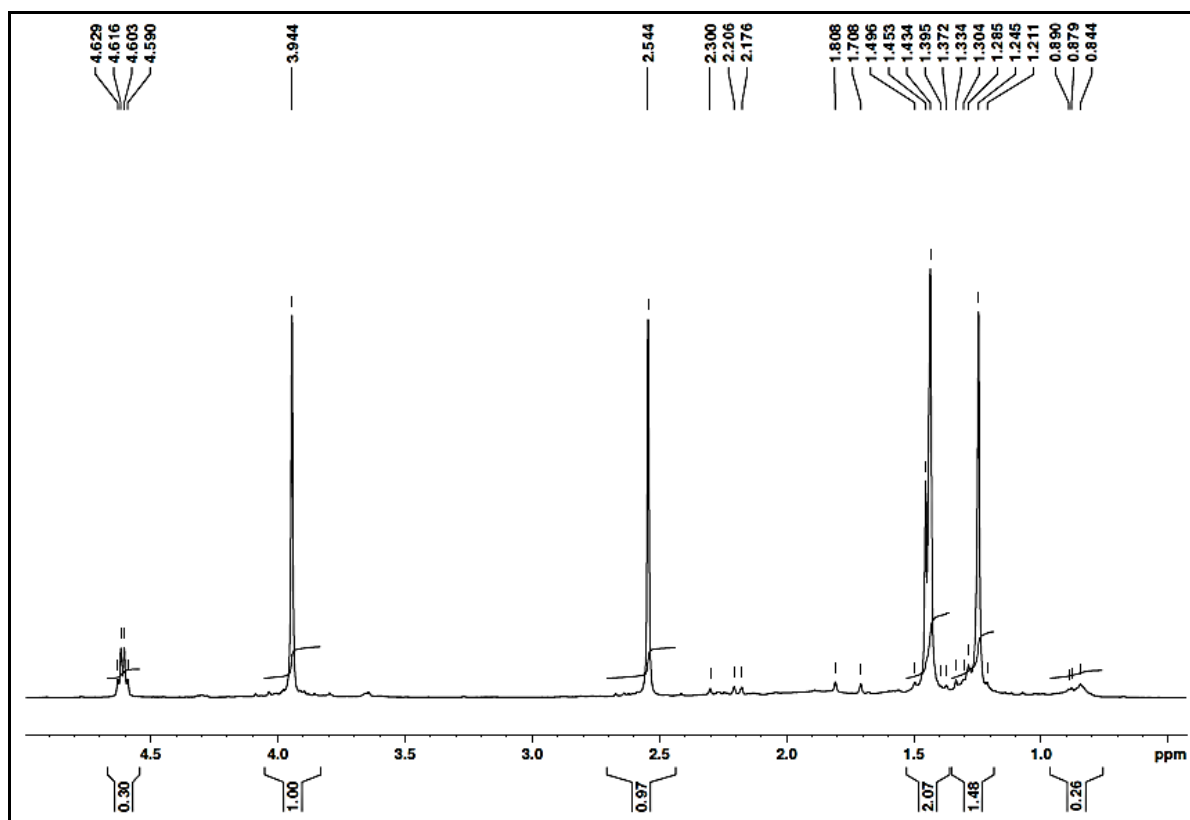


Figure S32. ^1H NMR (500 MHz, CDCl_3) spectrum of Compound **4** (Expansion 2)

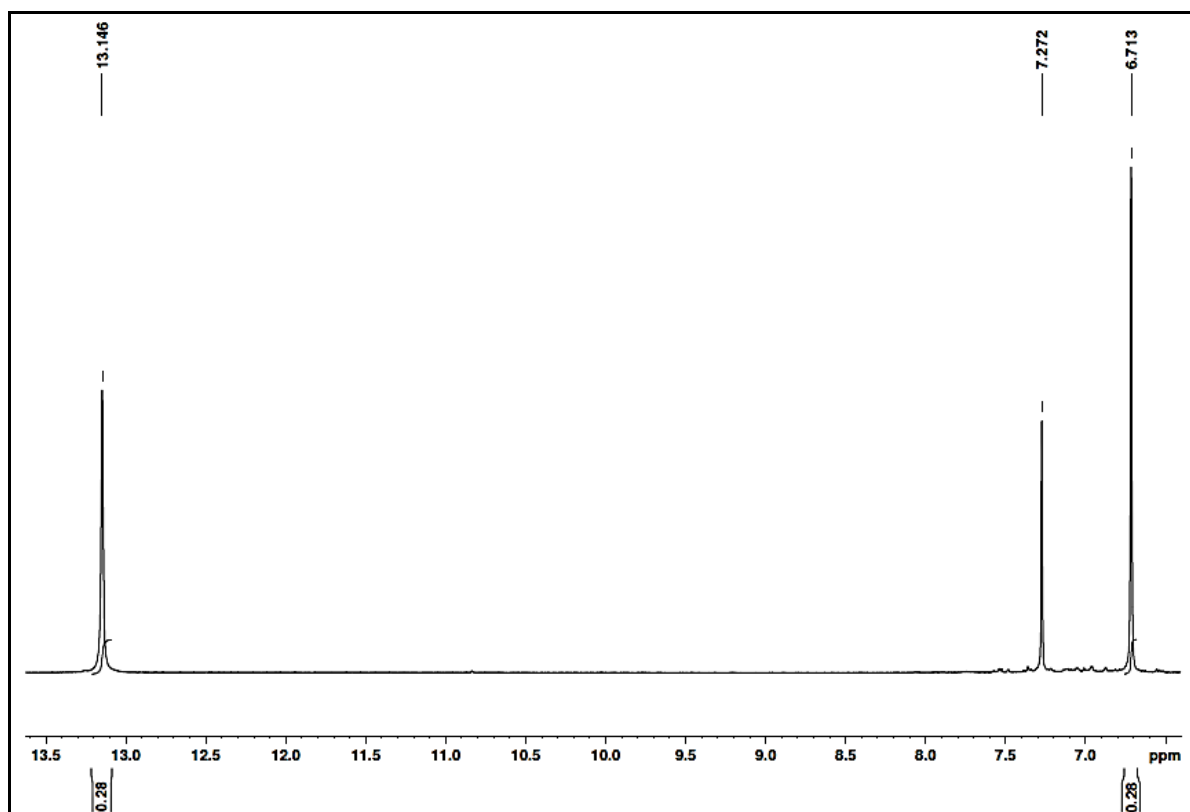


Figure S33. ^{13}C NMR (125 MHz, CDCl_3) spectrum of Compound **4**

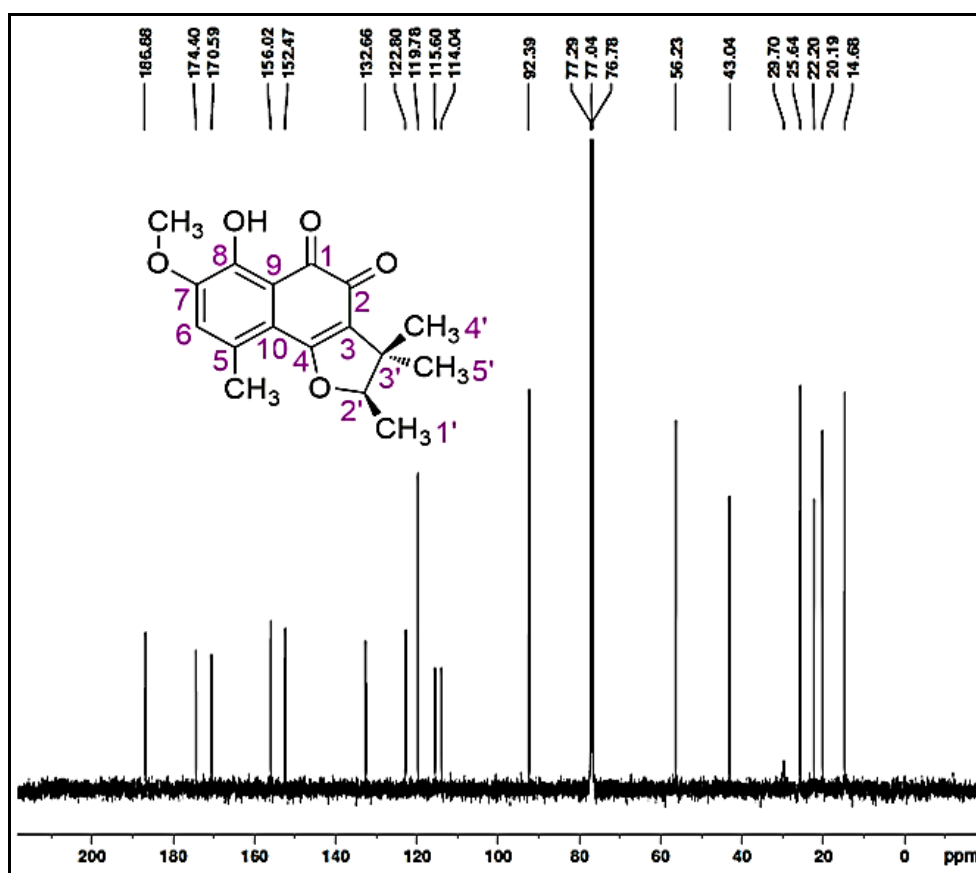


Figure S34. ^{13}C NMR DEPT 135 (125 MHz, CDCl_3) spectrum of Compound **4**

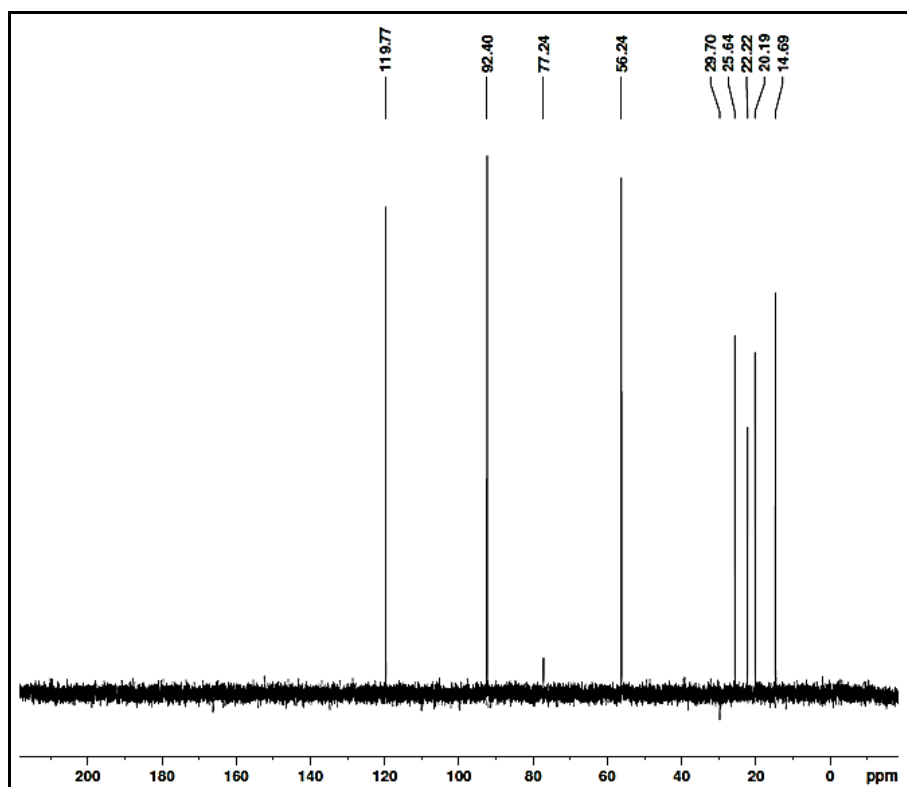


Figure S35. ^1H NMR (500 MHz, CD_3OD) spectrum of Compound **7**

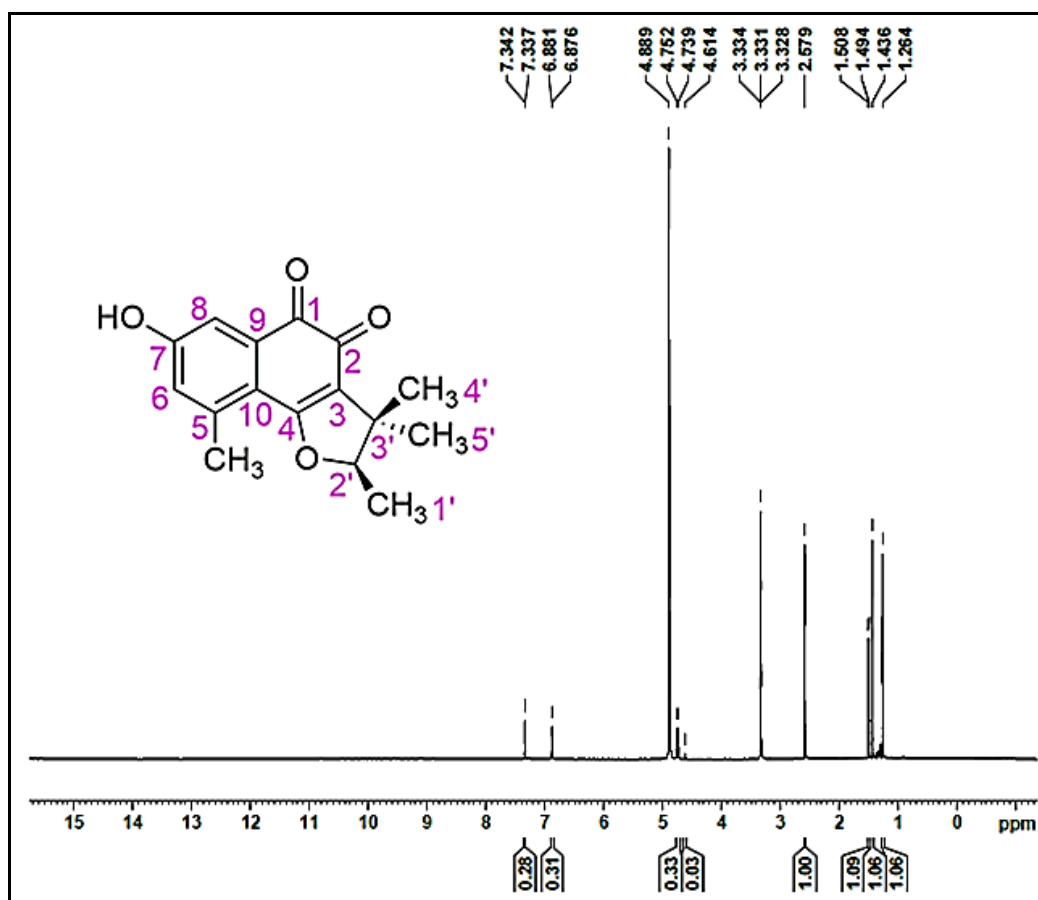


Figure S36. ^1H NMR (500 MHz, CD_3OD) spectrum of Compound **7** (Expansion 1)

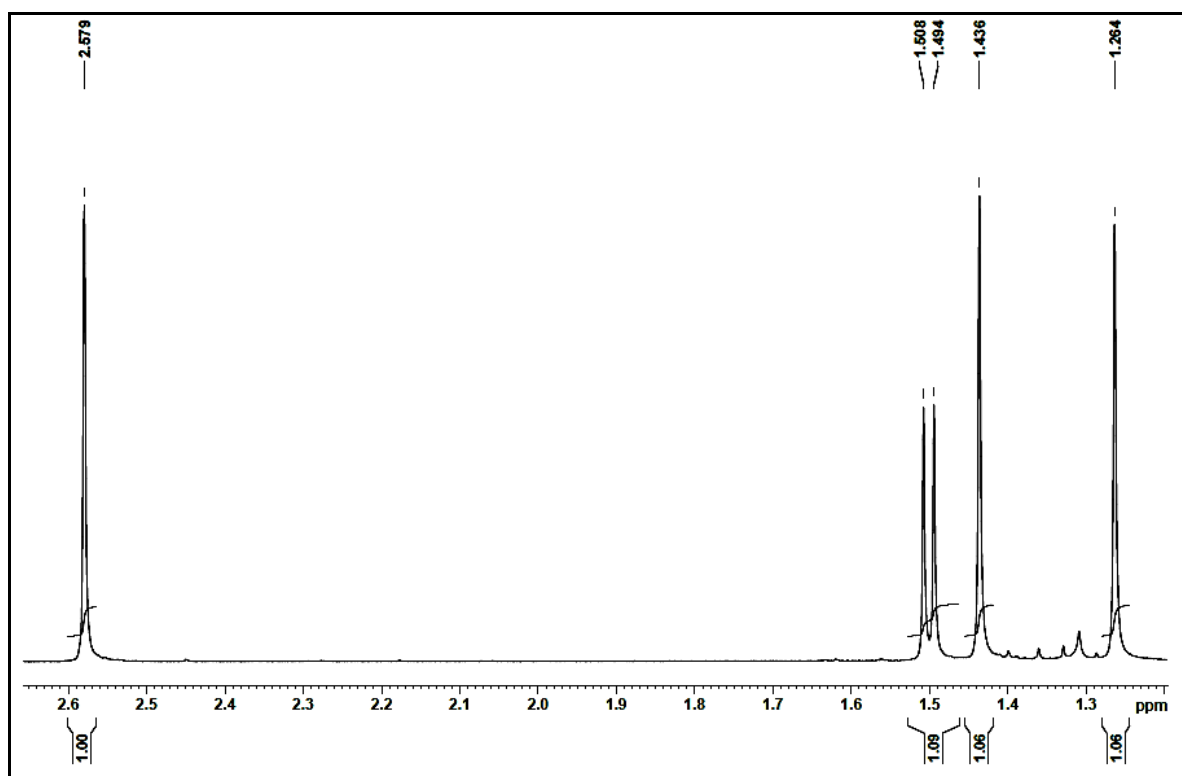


Figure S37. ^1H NMR (500 MHz, CD_3OD) spectrum of Compound **7** (Expansion 2)

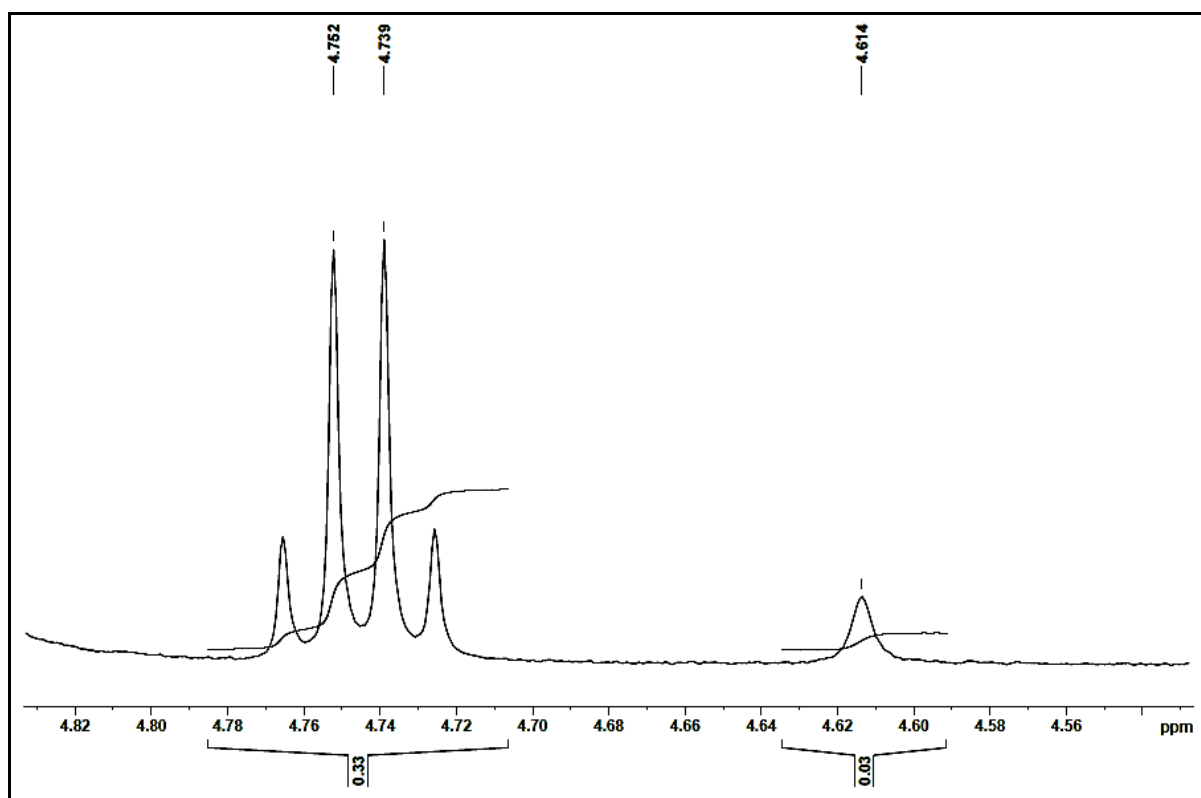


Figure S38. ^1H NMR (500 MHz, CD_3OD) spectrum of Compound **7** (Expansion 3)

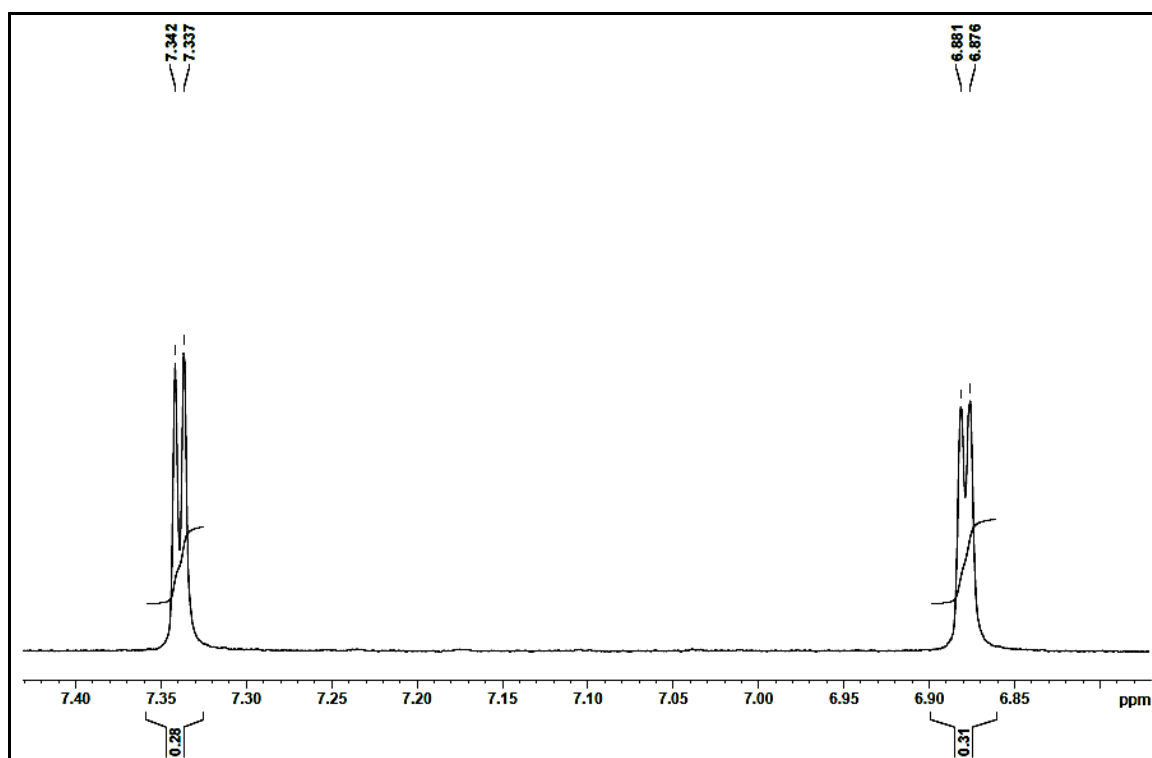


Figure S39. ^{13}C NMR (125 MHz, CD_3OD) spectrum of Compound **7**

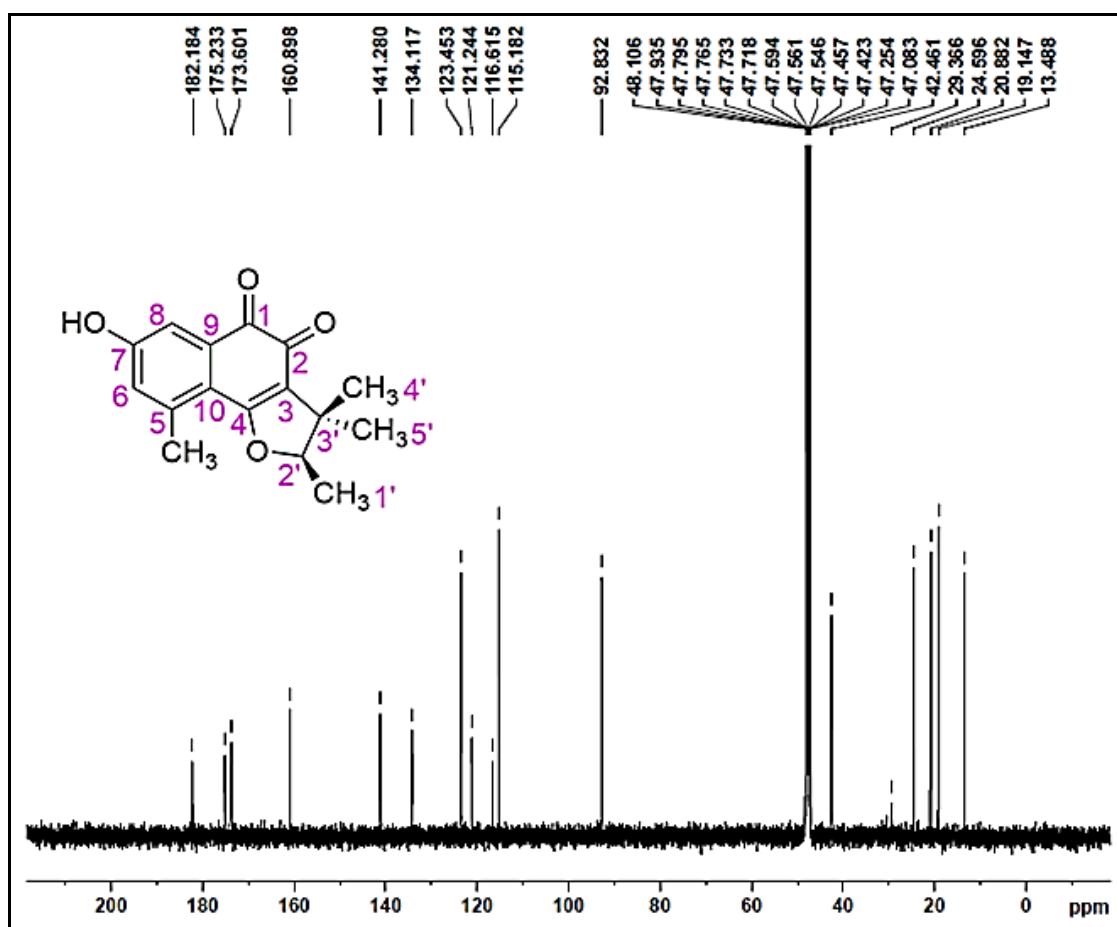


Figure S40. ^{13}C NMR DEPT 135 (125 MHz, CD_3OD) spectrum of Compound **7**

