Supplementary Information

Cultivated Sea Lettuce is a Multiorgan Protector from Oxidative and Inflammatory Stress by Enhancing the Endogenous Antioxidant Defense System

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Supplementary Materials and Methods

Chemicals, Reagents and Cells. Cell culture media, Dulbecco's Modified Eagle Medium (DMEM), and Eagle's Minimum Essential Medium (EMEM) were purchased from Invitrogen (Carlsbad, CA), RPMI 1640 from Fisher Scientific (Pittsburgh, PA) and fetal bovine serum (FBS) purchased from HyClone (Logan, UT). *Tert*-butylhydroquinone (tBHQ) was purchased from Acros Organics (St. Louis, MO) and sulforaphane from Sigma (St. Louis, MO). FUGENE HD transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN), Opti-MEM reduced serum medium from Invitrogen and siLentFect from Bio-Rad laboratories (Hercules, CA). BriteLite reagent for ARE reporter assay was purchased from PerkinElmer, Life and Analytical Sciences, Inc. (Waltham, MA). IMR-32 neuroblastoma cells, LNCaP human prostate carcinoma cells and the mouse macrophagic cell line RAW264.7 were purchased from American Type Culture Collection (Manassas, VA). Mouse embryonic fibroblasts (wild type, $Nrf2^{-/-}$, $Keap1^{-/-}$) were kindly provided by Dr. Albena Dinkova-Kostova. DMSO and Cremophor®EL used for animal work and purchased from Sigma-Aldrich (St Louis, MO) were either USP grade or filtered through 5 µ filter before use as a vehicle in feeding experiments. All other chemicals and solvents used for extraction, fractionation and isolation of compounds were purchased from Fisher Scientific unless specified.

Cell culture. All cells were maintained at 37 °C in humidified CO_2 atmosphere in respective media (IMR-32: EMEM; LNCaP: RPMI 1640; RAW264.7: DMEM; MEFs: Iscove's MDM) supplemented with 10% heat-inactivated FBS from HyClone (Logan, UT).

RNA Extraction and Quantitative RT-PCR (Fractions). IMR-32 (6×10^5 cells/well), LNCaP (4×10^5 cells/well) and RAW264.7 (2.5×10^5 cells/well) were seeded in 6-well plates 24 h prior to treatment. Following incubation, cells were treated with *Ulva* fractions and respective positive controls and vehicle control (0.5% DMSO or EtOH) and allowed to incubate for further 12 h. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Animal tissues (50–100 mg) were sonicated in Trizol (Invitrogen) and total RNA was extracted by phase separation following manufacturer's protocol. RNA was reverse-transcribed from 2 µg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen), 1 mM dNTP and Oligo $(dT)_{12\text{-}18}$ primer (Invitrogen) 0.5µg/µL. Real-time PCR was performed by using 12.5 µL of TaqMan 2× universal master mix (Applied Biosystems), 1.25 µL of 20× TaqMan gene expression assay mix, 2 µL of cDNA and 9.25 µL of sterile water, in a total volume of 25 µL per well reaction in 96-well plate (Applied Biosystems) by using the ABI 7300 sequence detection systems (Applied Biosystems). The thermocycler program consisted of 2 min at 50 ºC, 10 min at 95 ºC, and 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Each assay was carried out in triplicate. Real-time PCR analyses were carried out using probes for mouse *Nqo1* and human *NQO1*, *NRF2*, mouse *iNOS* and *Cox2*. Human *GAPDH* or mouse *β-actin* (Applied Biosystems) expression was used as internal control for normalization.

Immunoblot Analysis. IMR-32 cells (6×10^5 cells/well) were plated in 6-well plates 24 h before treatment. Cells were transfected with compounds or vehicle, and incubated for 24 h.

The whole-cell lysates were prepared by using PhosphoSafe lysis buffer (Novagen). Alternatively, nuclear and cytoplasmic proteins were separated by using the NE-PER reagent kit (Pierce). The protein concentrations of the samples were determined using the BCA method (Pierce), and cell lysates containing equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membranes, probed with antibodies and detected with the Supersignal Femto Western blotting kit (Pierce). NQO1and Nrf2 antibodies were obtained from Abcam (Cambridge, MA), Akt (C67E7), Phospho-Akt (Ser473) (D9E) XP®, Phospho-GSK-3α/β (Ser21/9), GSK-3α/β (D75D3) XP®, β-actin, β-tubulin, secondary anti-rabbit and anti-mouse antibodies from Cell Signaling (Beverly, MA), and Oct1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

RT-qPCR for *NRF2* and *NQO1* Expression in IMR-32 Cells (Purified Compounds). IMR-32 cells (1×10^6 cells/well) were seeded in 6-well plates one day before treatment. Compounds were treated with variable concentrations for 12 h. Total RNA was extracted with the RNeasy Mini Kit (Qiagen). cDNA was synthesized from 2 µg of total RNA by using SuperScript II Reverse Transcriptase (Invitrogen) and Oligo$(dT)_{12\text{-}18}$ primer (Invitrogen). Real-time PCR was performed in triplicate using *GAPDH* expression as internal control for normalization.

Glutathione Assays. IMR-32 cells were seeded in 6-well dishes (1.5×10^6 cells/well). 24 h later, the cells were treated with fractions or vehicle for 2, 8, 16, or 24 h. The treated cells were washed twice with PBS and centrifuged at 600*g* for 6 min to obtain the cell pellet. The volume of the cell pellet was measured and resuspended in 3 volumes of 5% sulfosalicylic acid solution. The cell suspensions were frozen and thawed twice (using liquid nitrogen to freeze and a 37 ºC water bath to thaw) and incubated for 5 min at 4 °C, and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was used as glutathione stock. The concentrations of total (reduced and oxidized) glutathione (GSH+GSSG) were assessed using Glutathione Assay Kit (Sigma), following the manufacturer's instructions.

***In Vivo* Studies.** ARE-human placental alkaline phosphatase transgenic mice were bred and genotyped using 51 basepair segment of the rat *Nqo1* promoter upstream of a heat-stable human placental alkaline phosphatase (hPAP) reporter gene construct as described.[1] The presence of the transgene was confirmed by PCR amplification of a portion of the inserted gene. ARE-hPAP negative littermates were used as background controls for endogenous alkaline phosphatase activity. The animals were housed at the University of Florida animal facility and treated in accordance with all IACUC regulations and maintained under standard laboratory conditions.

General Procedures for Extraction, Isolation and Characterization of Compounds. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. ^{1}H and 2D NMR spectra for compounds were recorded in $CDCl_3$, DMSO-d_6 or CD_3OD on a Bruker Avance II 600 MHz spectrometer equipped with a 5 mm TXI cryogenic probe or Bruker Avance 500 MHz spectrometer equipped with a 2.5 mm TXI probe, using residual solvent signals as internal reference. HSQC and HMBC experiments were optimized for $^1J_{CH}$ = 145 Hz, $^nJ_{CH}$ = 7 Hz respectively. Other ^{1}H NMR spectra were recorded on a Varian 400 MHz spectrometer. ^{13}C

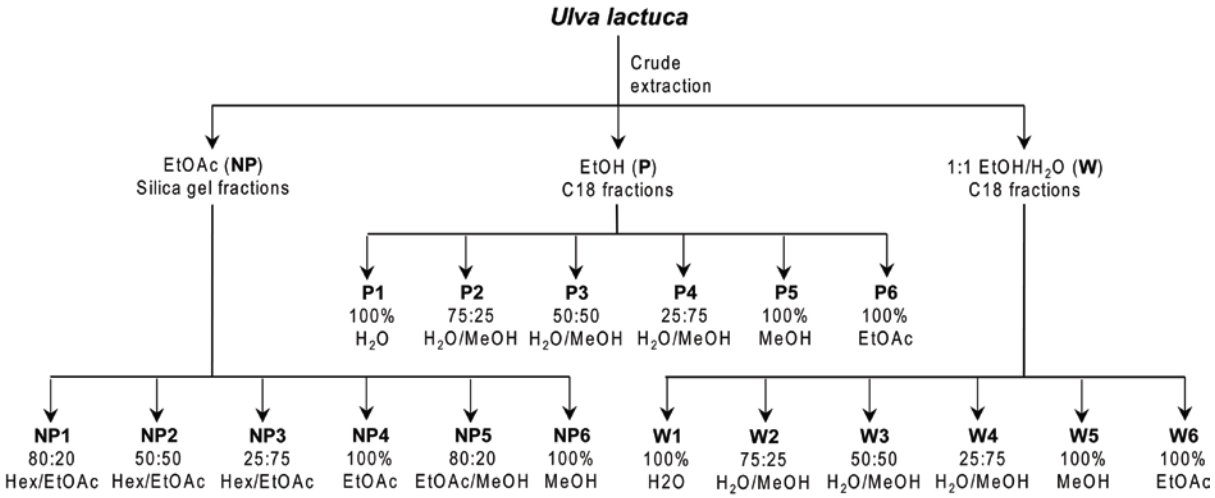
NMR experiments were recorded on a Bruker 500 MHz or Varian 400 MHz spectrometer (5 mm probe), operating at 125 MHz and 100 MHz, respectively. HRMS data were obtained using an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source, and low-resolution mass spectra were obtained on a A3200 Q TRAP LC/MS/MS (hybrid triple quadrupole linear ion trap mass spectrometer, Applied Biosystems, USA) with an electrospray ionization (ESI) interface operated in positive mode. HPLC-based compound isolation was performed on a Shimadzu LC-20AT Prominence LC system with peak detection by a Shimadzu SPD-M20A Prominence diode array detector.

Extraction of Cultivated *Ulva* sp. and Bioassay-guided Isolation. Material from cultured *Ulva* was freeze-dried (2.23 kg) and extracted with nonpolar (EtOAc, NP), and polar organic (EtOH, P) and polar aqueous (1:1 EtOH/H_2O, W) solvents, as shown (Supplementary Scheme S1). The nonpolar (NP) extract was concentrated to dryness (14.07 g) and chromatographed on Si gel eluting in the order of 80:20 hexanes/EtOAc, 50:50 hexanes/EtOAc, 25:75 hexanes/EtOAc, followed by 100% EtOAc, 80:20 EtOAc/MeOH and 100% MeOH to yield six fractions (NP1–NP6). Both polar (P and W) extracts were similarly concentrated and chromatographed on C_{18} eluting first with 100% H_2O followed by increasing gradients of MeOH, to 100% MeOH and finally with 100% EtOAc to elute any nonpolar residual constituents. The resulting Si gel fractions (NP1–NP6) from the nonpolar EtOAc extract and the C_{18} fractions from both EtOH (P1–P6) and 1:1 EtOH/H_2O (W1–W6) extracts were investigated for ARE activity to establish previously identified activity profiles. NP3 (424 mg), NP4 (129 mg), NP5 (354 mg) from the nonpolar extract and P4 (420 mg) from the polar EtOH extract were designated as prioritized fractions based on preliminary biological evaluation for ARE activity.

Each of the four prioritized fractions was chromatographed further employing size exclusion chromatography (Sephadex LH20), and subsequently by several sequences of reversed-phase HPLC as described below. The resulting LH20 chromatography (eluent: 1:1 DCM/MeOH) fractions (1–8) of NP3 (100 mg) were analyzed by ^{1}H NMR and LCMS. Supplementary LH20 chromatography (eluent: 2:5:1 Hexanes/DCM/MeOH) and subsequent purification by semipreparative reversed-phase HPLC (Phenomenex Phenyl-Hexyl, 250 × 10 mm, 4 μm; flow rate, 2.0 mL/min) using a linear gradient of MeOH / H_2O (40%–100% MeOH in 20 min and then 100% MeOH for 10 min) of fraction 7 afforded the three known compounds loliolide (**1**) (t_R 16.3 min, 0.7 mg), isololiolide (**2**) (t_R 14.4 min, 0.7 mg) and 3,5,6-trihydroxy-7-megastigmen-9-one (**3**) (t_R 15.5 min, 1.2 mg). Purification of fraction 8 utilizing similar chromatographic conditions resulted a new oxylipin analog, 8-chloro-6,7-dihydroxy-deca-2,4-dienal (**4**) (t_R 17.9 min, 0.24 mg). The three remaining prioritized fractions were chromatographed under similar LH20 conditions (eluent: 1:1 DCM/MeOH) and fractions assessed for the respective chemical profiles based on ^{1}H NMR and HPLC-DAD analyses.

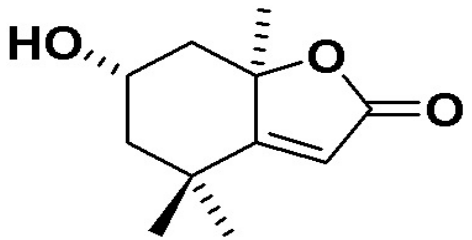
Supplementary Reference

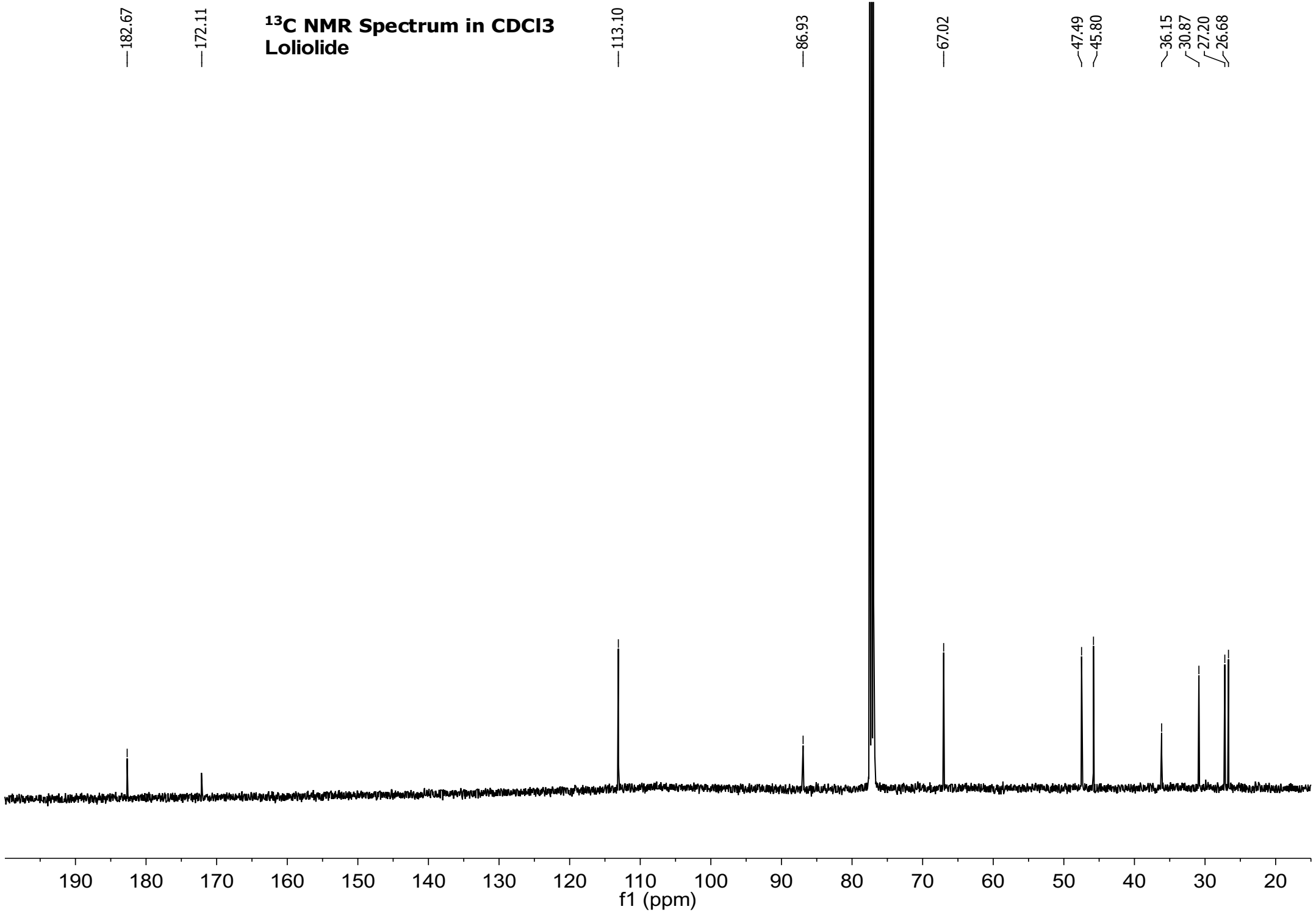
1. Johnson DA, Andrews GK, Xu W, Johnson JA. Activation of the antioxidant response element in primary cortical neuronal cultures derived from transgenic reporter mice. J Neurochem 2002;81:1233–1241.



Supplementary Scheme S1. Extraction and initial fractionation.

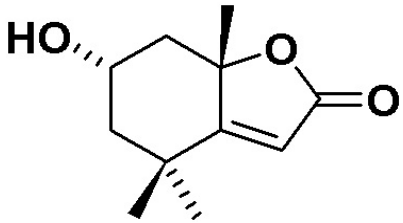
^{1}H NMR Spectrum in CDCl3
Loliolide

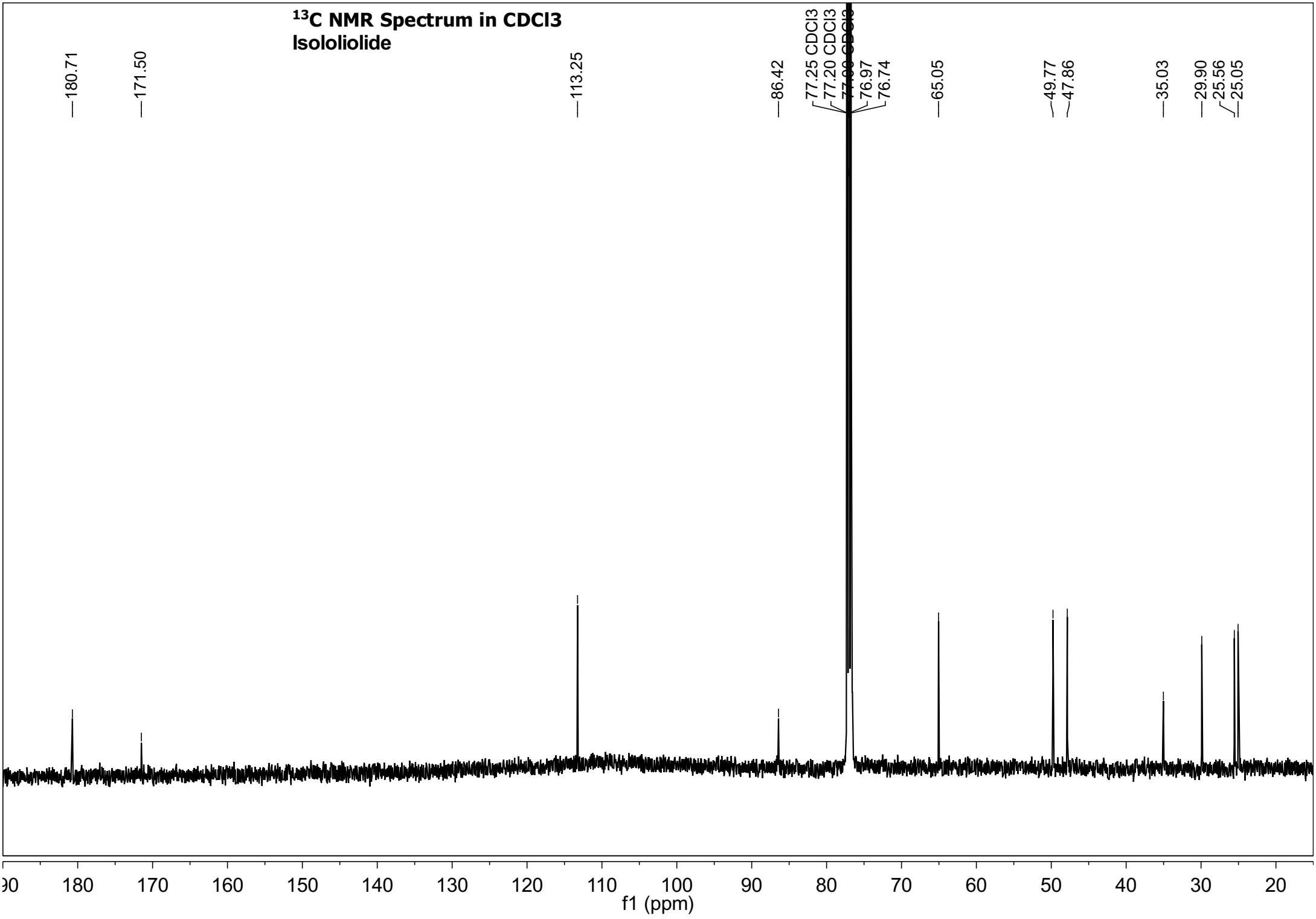




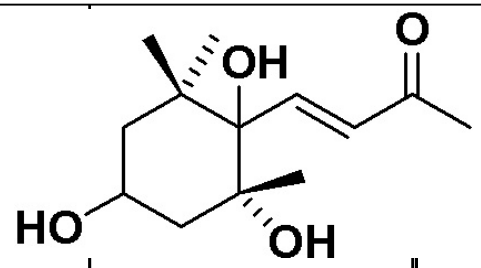
^{13}C NMR Spectrum in CDCl3
Loliolide

^{1}H NMR Spectrum in CDCl3
Isololiolide




13C NMR Spectrum in CDCl3
Isololiolide
180.71
171.50
113.25
86.42
77.25 CDCl3
77.20 CDCl3
76.97
76.74
65.05
49.77
47.86
35.03
29.90
25.56
25.05
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)

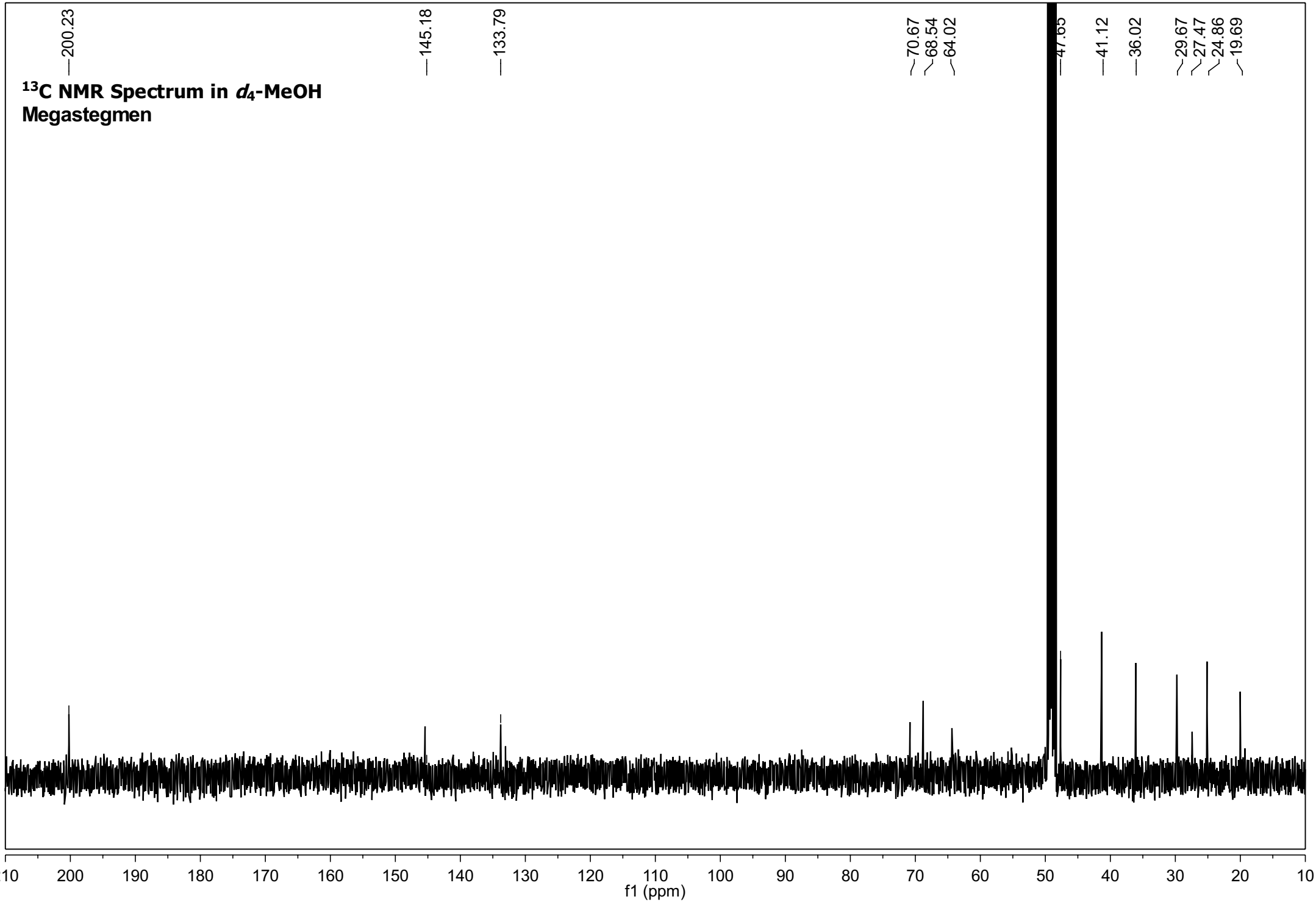
^{1}H NMR Spectrum in d_4-MeOH
Megastegmen



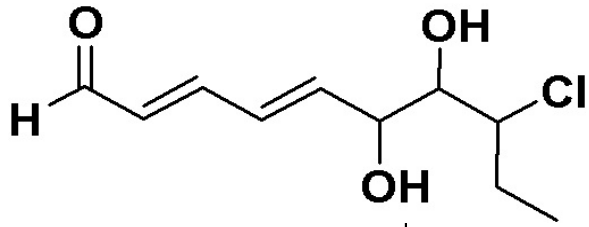
4.88

7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0

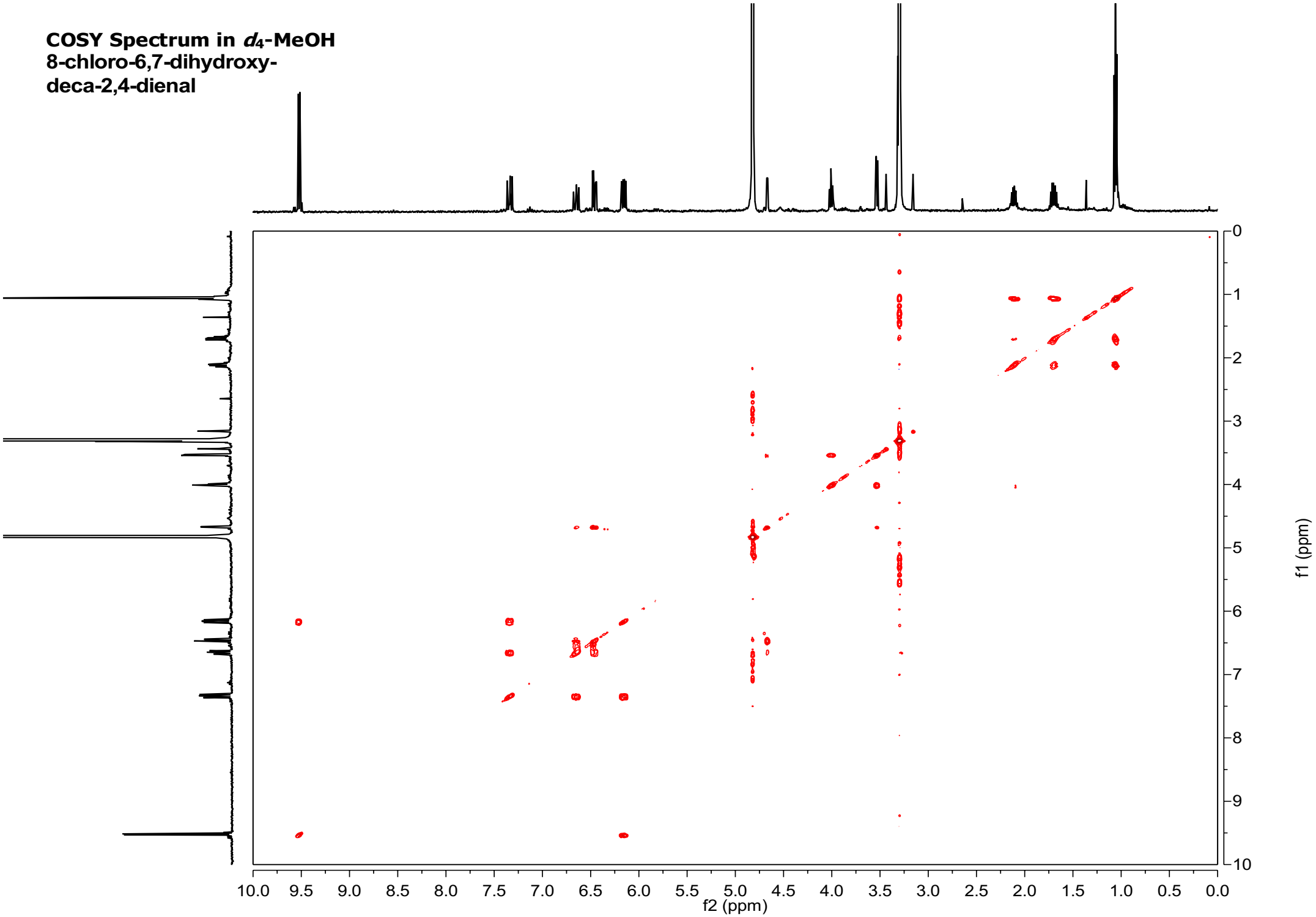
f1 (ppm)


200.23
145.18
133.79
70.67
68.54
64.02
47.65
41.12
36.02
29.67
27.47
24.86
19.69
13C NMR Spectrum in d4-MeOH
Megastegmen
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

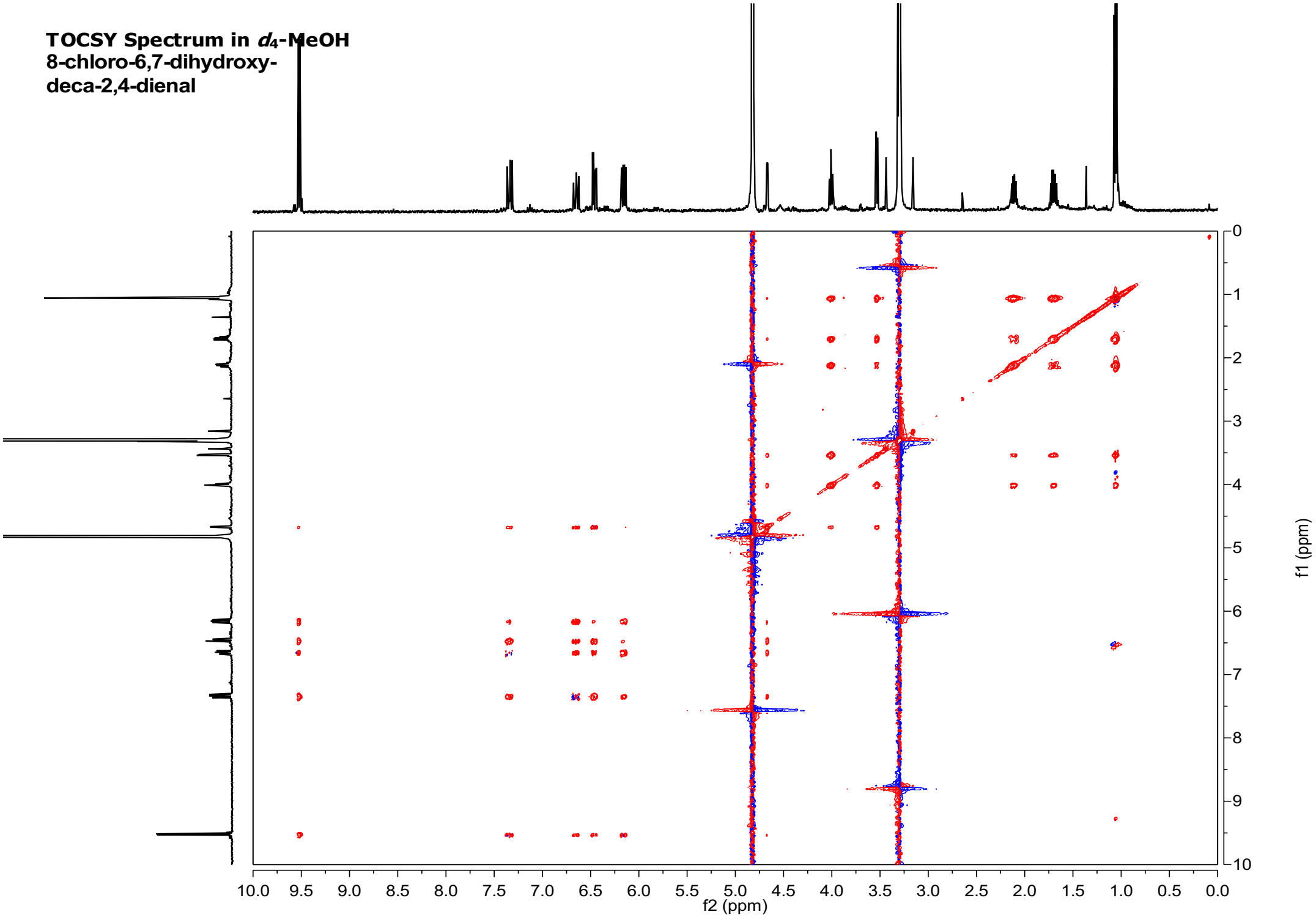
^{1}H NMR Spectrum in d_4-MeOH
8-chloro-6,7-dihydroxy-
deca-2,4-dienal



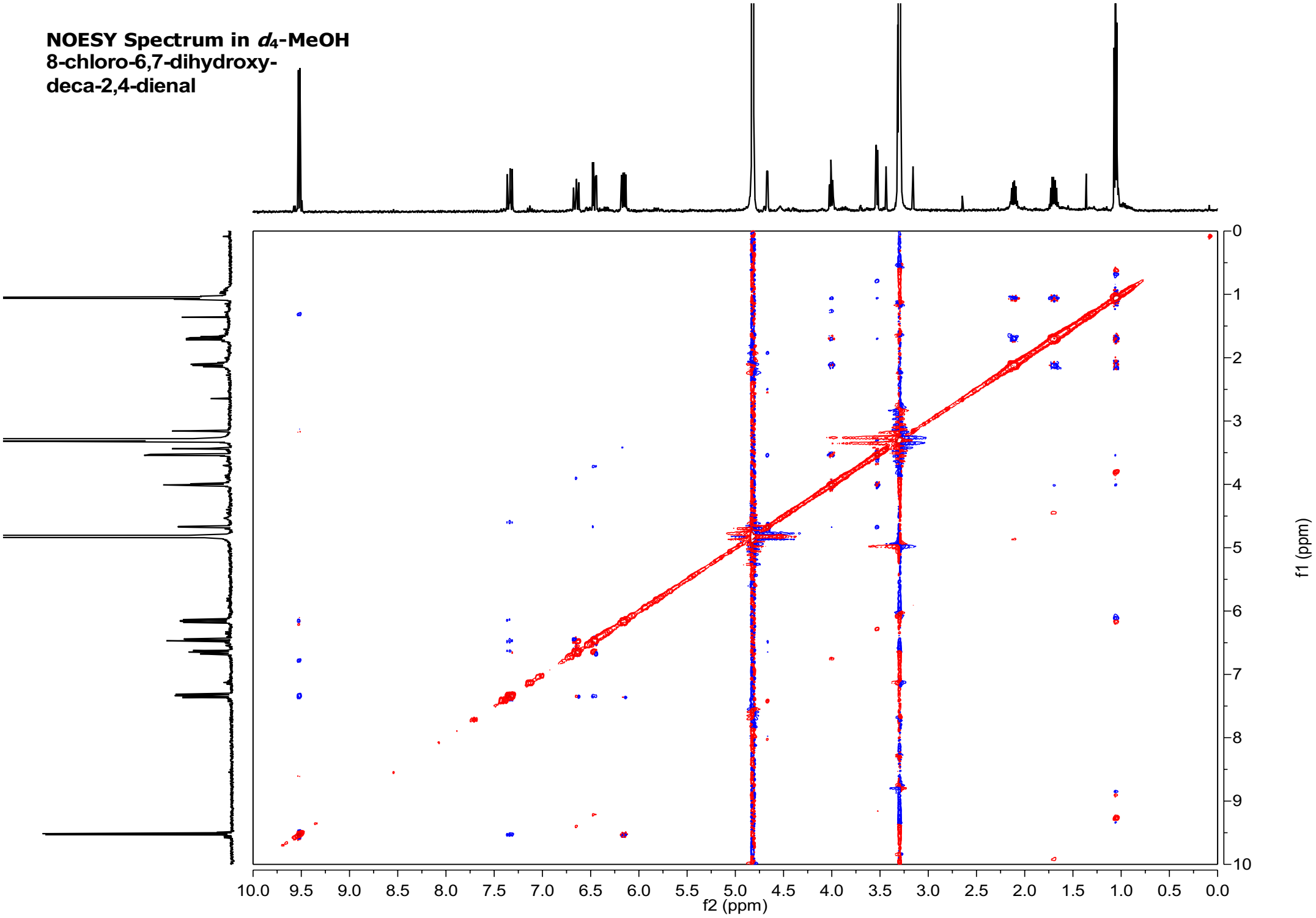
COSY Spectrum in d_4-MeOH
8-chloro-6,7-dihydroxy-
deca-2,4-dienal



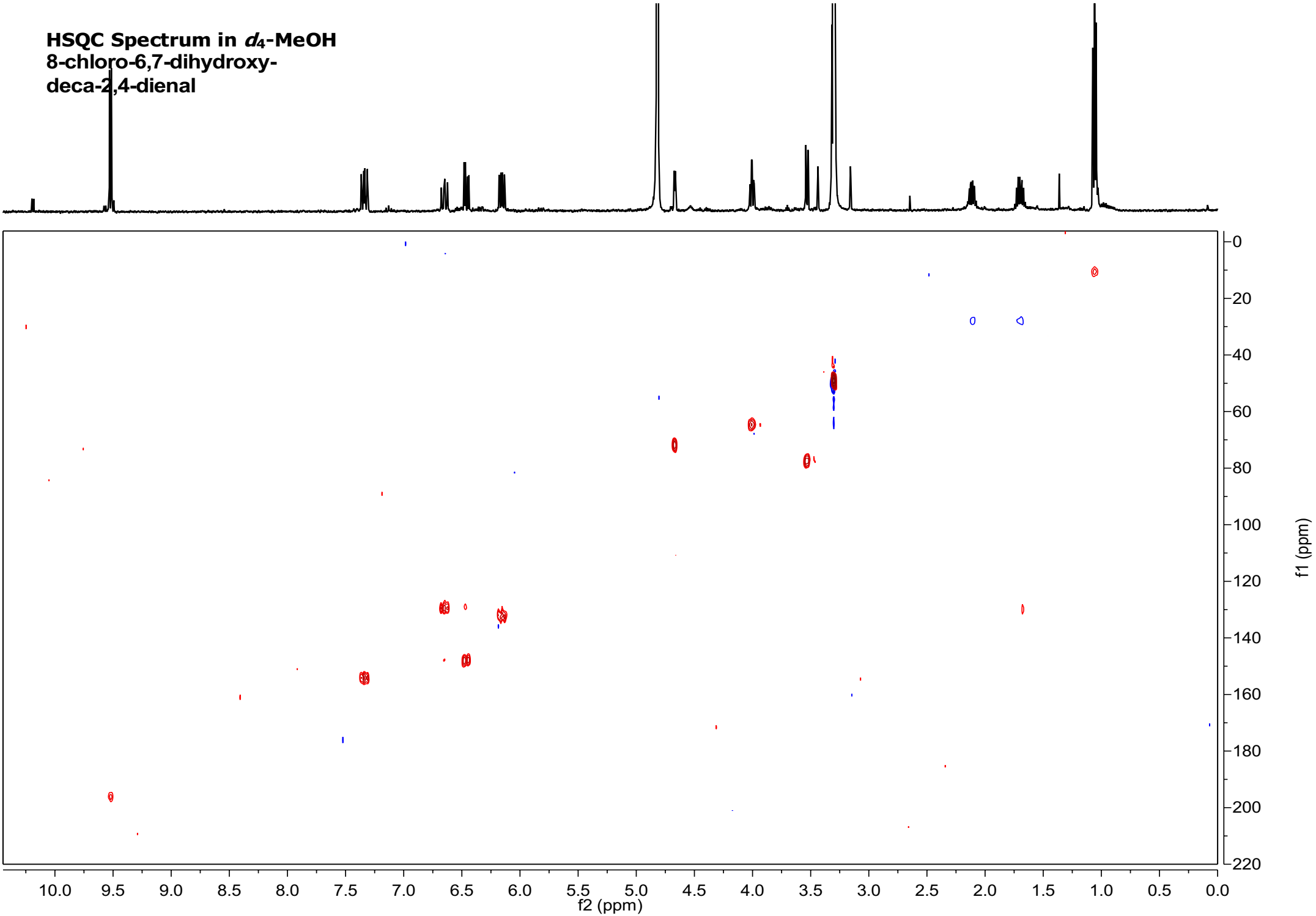
TOCSY Spectrum in d_4-MeOH
8-chloro-6,7-dihydroxy-deca-2,4-dienal



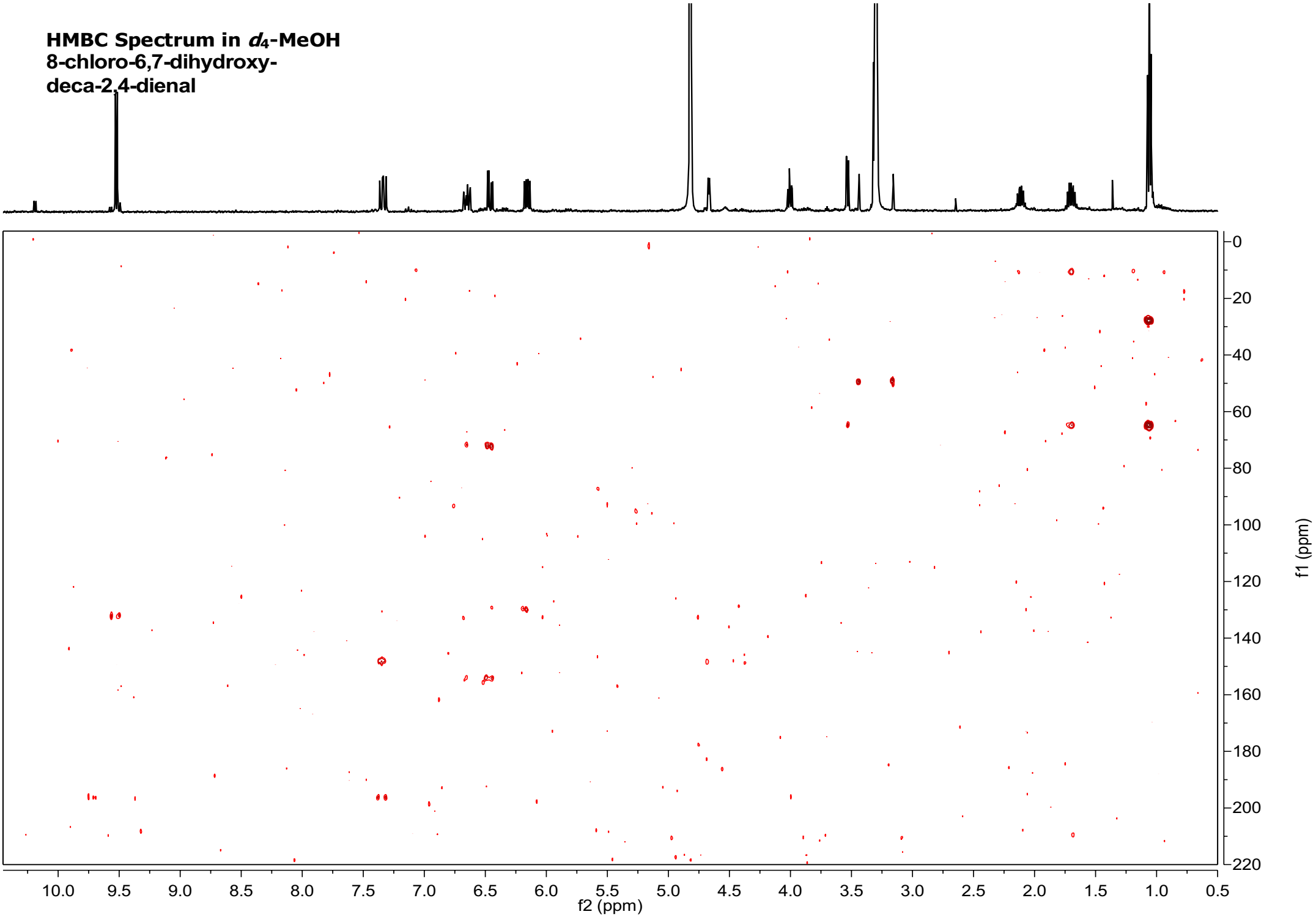
NOESY Spectrum in d_4-MeOH
8-chloro-6,7-dihydroxy-deca-2,4-dienal



HSQC Spectrum in d_4-MeOH
8-chloro-6,7-dihydroxy-
deca-2,4-dienal



HMBC Spectrum in d_4-MeOH
8-chloro-6,7-dihydroxy-deca-2,4-dienal



^{1}H NMR Spectrum in d_6-DMSO
8-chloro-6,7-dihydroxy-
deca-2,4-dienal