Algal pheromone biosynthesis: stereochemical analysis and mechanistic implications in gametes of *Ectocarpus siliculosus*

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- Supporting Information -

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General Experimental Methods

Solvents were distilled prior to use. All reactions were conducted under argon in flame dried glassware. NMR: 500 MHz spectrometer, $CDCl_3$ as solvent. Chemical shifts of ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) are given in ppm relative to the solvent peak respectively at 7.26 and 77.0 ppm. ^1H NMR for (16*R*)-[$^2H_3$]-**9** was acquired in benzene-d_6, solvent peak at 7.15 ppm and 120.8 ppm. GLC-MS used for synthetic purposes: GLC column DB-5 30 m x 0.25 mm, ion trap, EI, 70 eV. GLC-MS used for analytical purposes and mass spectrometry characterisation of all compounds: quadrupole, EI, 70 eV, He as carrier gas. The following columns were used: Zebron ZB-5 (15 m x 0.25 mm); Hydrodex-β-6TBDM (heptakis-(2, 3-di-*O*-methyl-6-*O*-t-butyldimethyl-silyl)-β-cyclodextrin) 25 m x 0.25mm; Hydrodex-β-3P (heptakis-(2, 6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin) 25 m x 0.25 mm. For the analysis of oxylipins as PFB-oximes in negative ion mode (CI, CH_4) a GLC-MS system equipped with a RTX-200 column (0.25mm x 30m) was used. HRMS for all compounds and additional GLC-MS of methyl arachidonate **25** were obtained with a sector field analyser. For FTIR Spectra all samples were thin film between NaCl disks. The HPLC system was equipped with the following columns: LiChrocart column RP-C18 250 x 10 mm, 5µm; Nucleosil column 50-7, 250 x 10 mm and Nucleosil column 50-5, 250 x 4 mm. Column chromatography was done with silica gel Si 60 (0.200-0.063 mm) and florisil® (0.400 mm). Thin layer chromatography was performed on aluminium sheets coated with silica gel 60 F254. Sonication of cell suspensions was carried out with a sonotrode set to 50 % intensity. SPME was done with 100 µm polydimethylsiloxane, bonded phase.

Synthetic procedures

(2*S*)-Glycidyl benzyl ether [(2*S*)-14]. Hydrolytic kinetic resolution[1] of (±)-glycidyl benzyl ether (5 g, 30 mmol) yielded the epoxide (2*S*)-**14** as a colourless liquid (2.27 g, 46 %

yield). The enantiomeric ratio was determined by GLC-MS on a chiral column Hydrodex-β-3-P; er = 97.6 ± 0.1 % (n = 3). Other data (^{1}H NMR, ^{13}C NMR, MS) were in agreement with literature.[2]

(2*S*)-1-(Benzyloxy)hex-5-en-2-ol (15). A cold (-40 °C) suspension of CuI (200 mg, 1 mmol, 0.07 eq.) in 25 ml of dry THF, was gradually treated with a 1 M solution of allyl magnesium bromide in THF (24 ml, 24 mmol) and stirred for 15 min. Benzyl-(S)-glycidyl ether (2.25 g, 13.6 mmol) dissolved in THF (2 ml) was added with a syringe and the mixture was stirred for 3 h. After GLC control, the excess allyl magnesium bromide was hydrolysed with 2N HCl. The aqueous phase was extracted with ether; the collected organic phases were washed with a saturated solution of ammonium chloride and with brine. The organic phase was dried with sodium sulphate and the residue was filtered through a pad of silica (PE/E 1/1). After removal of the solvent a colourless liquid was obtained (2.55 g; 91 % yield). Determination of ee: an aliquot of 1µl of the diol **15** was transferred in a GLC microvial and treated with 4 µl of R-(+)-1-phenylethyl isocyanate. A second aliquot was treated with 2 µl of the same reagent and 2 µl of its opposite enantiomer. After heating both samples at 60 °C for two hours, 30 µl of methanol were added and further heating followed for 15 min. After solvent evaporation and dilution with dichloromethane, GLC-MS was performed on the ZB-5 column using temperature programmed elution: 40 °C to 185 °C at 40 °C/min, then 185 °C for 35 min, then to 280 at 10 °C/min; er = 96.4 ± 0.1 % (n = 4). Spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR, MS) were in agreement with the literature for the (2*R*)-enantiomer.[3]

(2*R*)-[2,5,6-$^{2}H_3$]-Hexan-1-ol (18). A 50 mL flask equipped with a cock and a septum was loaded with 135 mg of palladium on charcoal (Pd-C 5%). A hydrogen balloon connected with a Pasteur pipette was applied through a septum and the flask was flushed with hydrogen for 10 min, then 30 ml of dichloromethane and the benzyl ether **17** (1.35 g, 6.9 mmol) were added. After 3 hours the precursor was consumed (GLC-MS), and the suspension was filtered

on celite. Solvent and toluene were removed at 60 °C and 55 mbar in a rotary evaporator equipped with a Vigreux column and a colourless liquid was obtained (611 mg, 84%).

^{1}H NMR ($CDCl_3$, for peak assignment see correlation spectra HSQC and HMBC): δ 3.63 (d, *J*=6.7 Hz, 2H, C(1)); 1.58-1.50 (m, 2H, C(2), (-OH)); 1.38-1.30 (m, 2H, C(3)); 1.30-1.20 (m, 3H, C(4), C(5)); 0.9-0.8 (m, 2H, C(6)); ^{13}C NMR: δ 63.0 (C(1)); 32.3 (t, ^{CD}J=19 Hz; C(2)); 31.5 (C(4)); 25.3 (C(3)); 22.1 (t, ^{CD}J=19 Hz C(5)); 13.6 (t, ^{CD}J=19 Hz C(6)). MS of trimethylsilyl derivative *m/z* (%): 162 ($M^{+\bullet}$-15, 100); 116 (8); 103 (15); 89 (19), 75 (26); 73 ($^{+\bullet}SiC_3H_9$, 35). IR (cm^{-1}): 3500-3000, 2921, 2854, 2166, 1456, 1043; HRMS (EI) cacld for $C_9H_{19}D_3OSi$ 177.162824, found 177.162314.

Methyl 5-oxopentanoate (21). DOWEX EX50-WX8 (200 mg) was added to a solution of technical grade δ-valerolactone (**20**) (4 g, 40 mmol) in 50 ml of methanol and stirred under reflux for 3 hours in a 100 ml flask. After addition of the same volume of ethyl acetate and evaporation of methanol, IBX[4] (14.0 g, 50 mmol) was added and the mixture was refluxed for 3 more hours (GLC-MS control). After cooling on ice, filtration and evaporation of the solvent, the residue was distilled in a glass oven (130 °C; 20 mbar) and a colourless liquid was obtained (3.14 g; 60 %). IR (cm^{-1}): 2955, 2836, 2730, 1736, 1438, 1370, 1167; MS *m/z* (%):102 ($[M-18]^{+\cdot}$, 19); 99 (47), 98 (45), 74 (100), 59 (40). The remaining spectroscopic data (^{1}H NMR, ^{13}C NMR) were in agreement with the literature.[5]

Methyl 7-(1,3,-dioxan-2-yl)-hept-(5*Z*)-en-oate (23). Carefully dried 2-(1,3-dioxan-2-yl)-ethyltriphenylphosphonium bromide (**22**) (2.51 g, 5.5 mmol) was suspended in 40 ml of dry THF at -78 °C and a solution of potassium bis(trimethylsilyl)amide (0.91 M, 6.1 ml, 5.5 mmol) was gradually added. After heating to room temperature and cooling (-78 °C), the orange solution was treated with a solution of the oxoester **21** (650 mg, 5 mmol) in THF was added in 10 min. After 2 hours the solution was diluted with petrol ether and the base was neutralised with 2 N HCl; the upper phase was separated and dried over Na_2SO_4. The solvent was evaporated at reduced pressure, the residue was dissolved in THF/PE 1/10 and crystals of

triphenylphosphine oxide were removed by filtration. The residue was purified through a silica pad (PE/E 1/1) to give a colourless liquid (595 mg, yield 52 %).

^{1}H NMR ($CDCl_3$): δ 5.51-5.46 (m, 2H, C(5), C(6)), 4.52 (t, *J*= 5 Hz, 1H, C(8)), 4.12-4.08 (m, 2H, C(1’’)), 3.78-3.73 (m, 2H, C(3’’)), 3.66 (s, 3H, C(1´)), 2.37-2.33 (m, 2H, C(7)), 2.31 (t, *J*=7.5 Hz, 2H, C(2)), 2.13-2.02 (m, 3H, C(4), C(2’’)), 1.69 (quint. *J*= 7.8 Hz, 2H, C(3)), 1.34-1.31 (m, 1H, C(2’’)). ^{13}C NMR ($CDCl_3$): δ 174.0 (C(1)), 131.2 (C(5)), 124.5 (C(6)), 101.8 (C(8)), 67.0 (C(1’’) and C(2’’)), 51.5 (C(1´)), 33.5 (C(7)), 33.4 (C(2)), 26.8 (C(4), 25.7(C(2’’)), 24.7 (C(3)); MS *m/z* (%): m/z 228 ($M^{+\cdot}$, 7), 197 (4), 152 (6), 87 (100), 59 (18). IR (cm^{-1}): 3018, 2955, 2851, 1738, 1657, 1435, 1378. HRMS (EI): cacld for $C_{12}H_{20}O_4$ 228.136159, found 228.135072.

Methyl 8,8-dimethoxyoct-(5*Z*)-enoate (24). Methyl 7-(1,3-dioxan-2-yl)-hept-5-en-oate (**23**) (588 mg, 2.6 mmol) in 100 ml of methanol in a 250 ml flask was stirred with DOWEX EX50-WX8 (400 mg) and refluxed for 5 hours. After removal of the resin, 2 ml of saturated solution of NaCl and 50 mL of ethyl acetate were added and two thirds of the solvent were evaporated. The upper phase was dried and concentrated to give **24** as a colourless liquid (495 mg, 88 %).

^{1}H NMR ($CDCl_3$): δ 5.50-5.38 (m, 2H, C(5), C(6)), 4.36 (t, *J*= 5.5 Hz, 1H, C(8)), 3.66 (s, 3H, C(1´)), 3.32 (s, 6H, C(1’’/1’’’)), 2.38-2.34 (m, 2H, C(7)), 2.32 (t, J= 7.5 Hz, 2H, C(2)), 2.12-2.06 (m, 2H, C(4)), 1.70 (quint. J= 7.4 Hz, 2H, C(3)); APT ^{13}C NMR ($CDCl_3$): δ 174.0 (C(1)), 131.1 (C(5), 124.6 (C(6), 104.2 (C(8), 53.0 (C(1’’), C(1’’’), 51.4 (C(1´), 33.4 (C(7), 31.0 (C(2), 26.7 (C(4), 24.7 (C(3). MS *m/z* (%): 185 ($[M-31]^{+\cdot}$, 9); 153 (15), 75 (100). IR (cm^{-1}): 2952, 1739, 1657, 1438, 1364. HRMS cacld for $C_{11}H_{20}O_4$ 216.136159, found 216.135547.

Methyl 8-oxooct-(5*Z*)-enoate (19). Dimethyl acetal **24** (490 mg, 2.3 mmol) was dissolved in 70 mL of pentane, then 2 ml of formic acid were added and the mixture was stirred vigorously at 20 °C. After 1,5 hours the upper phase was separated and the lower phase was carefully extracted with pentane. The collected organic phases were washed with brine

until the washing solution had pH 6, then they were dried and evaporated at 35 °C. The light yellow oil obtained contained the product **19**, as well as the α,β-unsaturated isomer and impurities of the acetal **23** in the ratio 84:8:8 mol % as determined by ^{1}H NMR (9.66 ppm, 0.81H, CHO; 9.49 ppm, 0.08H, CHO; 4.51 ppm, 0.08H, CH). Since the side products do not interfere with the next reaction and the product is prone to isomerisation already under mild acidic conditions, it was used directly for the olefination. (360 mg, 77 % w/w purity, yield 71 %). Isomeric purity was determined by GLC-MS as *Z:E* = 95:5. The remaining spectroscopic data (^{1}H NMR, ^{13}C NMR, IR) are in agreement with the literature.[6]

(16*R*)-[16,19,20-$^{2}H_3$]-Arachidonic acid (16*R*)-[$^{2}H_3$]-9. The hydrolysis of the methyl ester was carried out in small batches prior to use. An aqueous solution of LiOH (1 M; 1 ml) was added with a syringe pump in 30 min to a 3 ml degassed THF solution of the ester **25** (13 mg; 0.04 mmol) at 0 °C. After cooling to 20 °C the mixture was stirred for 11 hours (TLC control, Pentane/E/HAc = 85/14/1, Rf = 0.15). After that, it was poured into a 5 mL flask containing 1 ml of 2N HCl and 1 ml of diethyl ether stirred in an ice bath. Extraction with diethyl ether (5 x 1 ml), drying of the organic layers and evaporation at 18 °C afforded the raw product. The residue was purified by preparative reversed phase HPLC with the following conditions. Eluent A: H_2O/CH_3CN 95/5 + 0.05 % HCOOH; eluent B: CH_3CN + 0.05 % HCOOH; flow: 3.6 ml/min; UV detection 210 nm, column RP-C18 250 x 10 mm, 5µm. Instrument method: 85 % B for 3 min; then from 85 % to 100 % B in 15 min; hold 3 min, to 85 % B in 2 min. The collected fractions were pooled and concentrated at 100 mbar and 25 °C, the residue was extracted with pentane (4x1 ml) and dried on a small pad of sodium sulphate. Evaporation under a stream of argon gave a colourless oil (6 mg, 48 %).

^{1}H NMR (benzene-d_6, for peak assignment see correlation spectra HSQC and HMBC): δ 5.50-5.35 olefinic (m, 7H, C (6/8/9/11/12/14/15)); 5.30-5.25 olefinic (m, 1H, C(5)), 4.25 (s, DMSO), 2.90-2.84 (m, 4H, C(7), C(13)), 2.80-2.75 (m, 2H, C(10)), 2.03 (t, *J*=7.5 Hz, 3H, C(2) superimposed to C(16)), 1.95-1.89 (m, 2H, C(4)), 1.52 (quint., *J*=7.5 Hz, 2H, C(3)),

1.35-1.30 and 1.25-1.21 (m, 5H, C(17/18/19)), 0.88-0.83 (m, 2H, C(20)); ^{13}C NMR ($CDCl_3$): δ 176.9 (C(1)), 130.5 (C(15)), 129.1 (C(5)), 128.7 (C(6)), 128.6 (C(12)), 128.3 (C(8)), 128.1 (C(14)), 127.9 (C(11), 127.6 (C(9)), 32.8 (C(2)), 31.4 (C(18)), 29.2 (C(17)), 26.9 (t, ^{CD}J=19 Hz, C(16)), 26.5 (C(4)), 25.63 (C(7/10/13), 24.5 (C(3)), 22.1 (t, ^{CD}J=19 Hz, C(19)), 13.7 (t, ^{CD}J=19 Hz, C(20)); MS of trimethylsilyl derivative *m/z* (%): 379 ($M^{+\cdot}$, 2); 364 ($M^{+\cdot}$-15, 4); 153 (51); 117 (100).

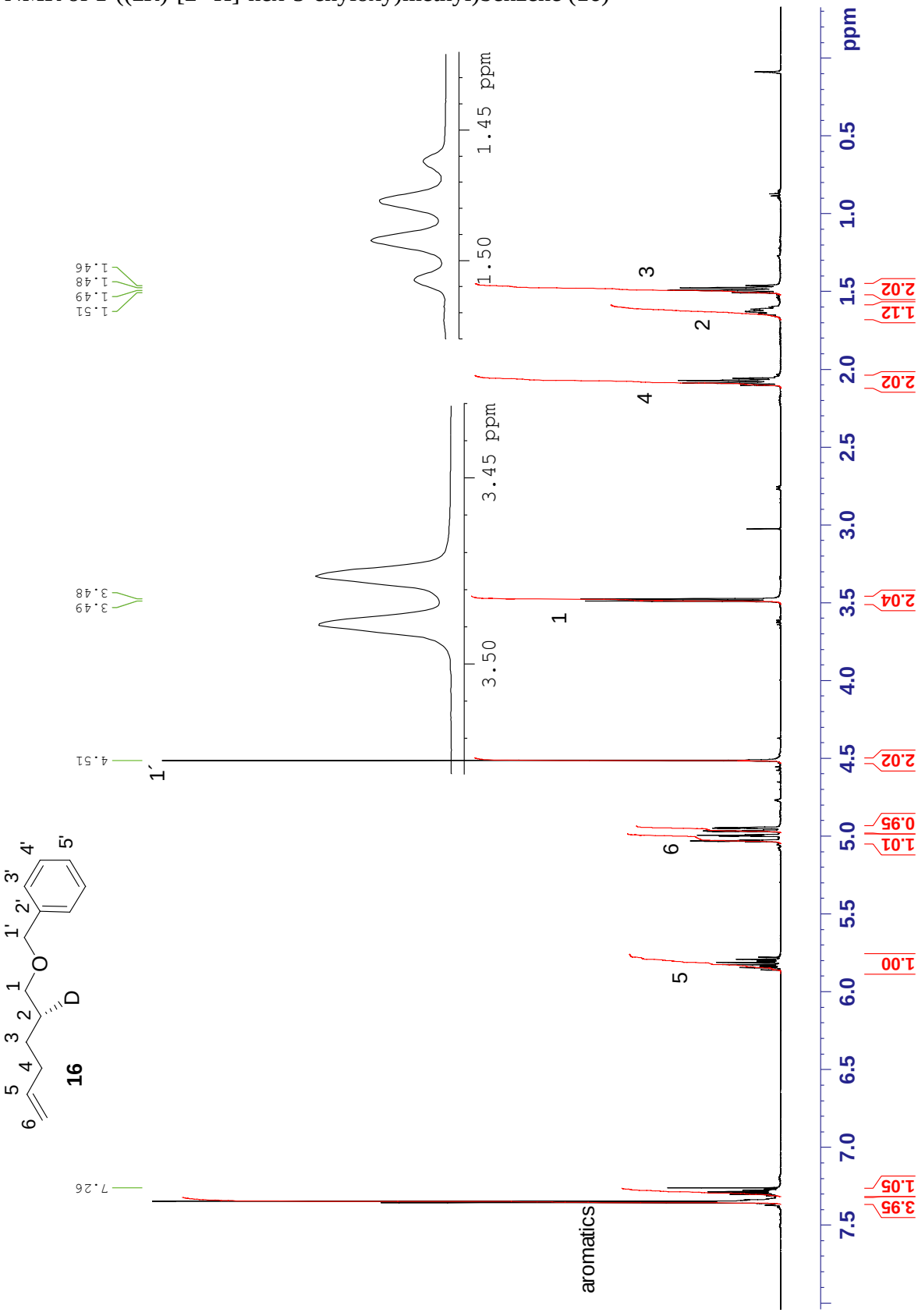
Cultivation of *E. siliculosus* and gamete release. Axenic cultures of female gametophytes of *E. siliculosus* (New Zealand strain) on agarised medium were kindly provided by Prof. D.G. Müller. Fragments of thalli were transferred in artificial sea water medium[7] and kept in a climate chamber at 13°C, with a period of Light/Darkness 14:10 hours and light intensity around 10-15 μE/m^2•s. The growing filaments were transferred into fresh medium every 5 days using sterile Pasteur pipettes under a sterile bench. During the first 30 days of cultivation, Petri dishes of 6 cm diameter sealed with parafilm and containing around 20 ml of medium were used, later glass vessels with plastic cap with around 100 ml of medium. Formation of gametangia was monitored with a microscope and was complete around 90 days from the inoculation in liquid medium. Prior to gamete release the cultures were kept in darkness at 4 °C overnight. After transferring filaments in empty Petri dishes under illumination, and removing the residual cold water with Pasteur pipette, to every culture was added a small amount (around 0.5 ml) of medium at 16 °C. Released gametes were collected with a Pasteur pipette and pooled into a graduated cylinder. Part of the suspension was used for cell counting with a hemocytometer (typical value 1 x 10^7 cells/ml), the rest was divided in aliquots for incubation with labelled fatty acids or collection of the released volatiles.

Determination of the enantiomeric excess of dictyotene. Female gametes of *E. siliculosus* were collected and three aliquots of 2 mL were transferred in 4 mL vials (8.3 x 10^6 cell/ml). After 6 h volatiles were collected by SPME for 60 minutes and analysed with GLC-

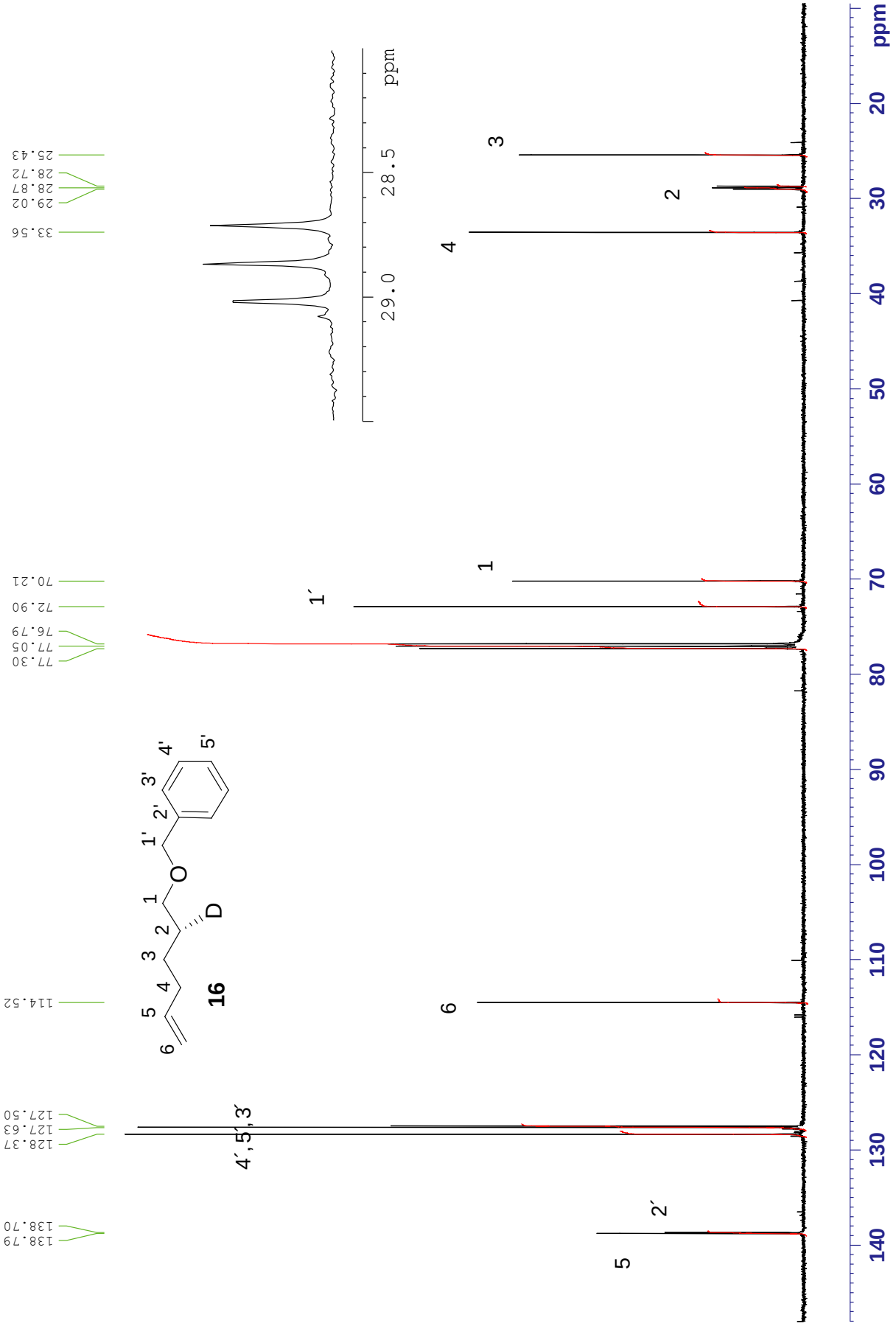
MS on the Hydrodex-β-6-TBDMS column, 0.25 mm x 25 m. Program: 50 min at 60°C then to 200 °C at 20°C/min. Inlet temperature 220 °C. The enantiomeric ratio is er = 97.9 ± 0.3 % (n = 3).

NMR spectra

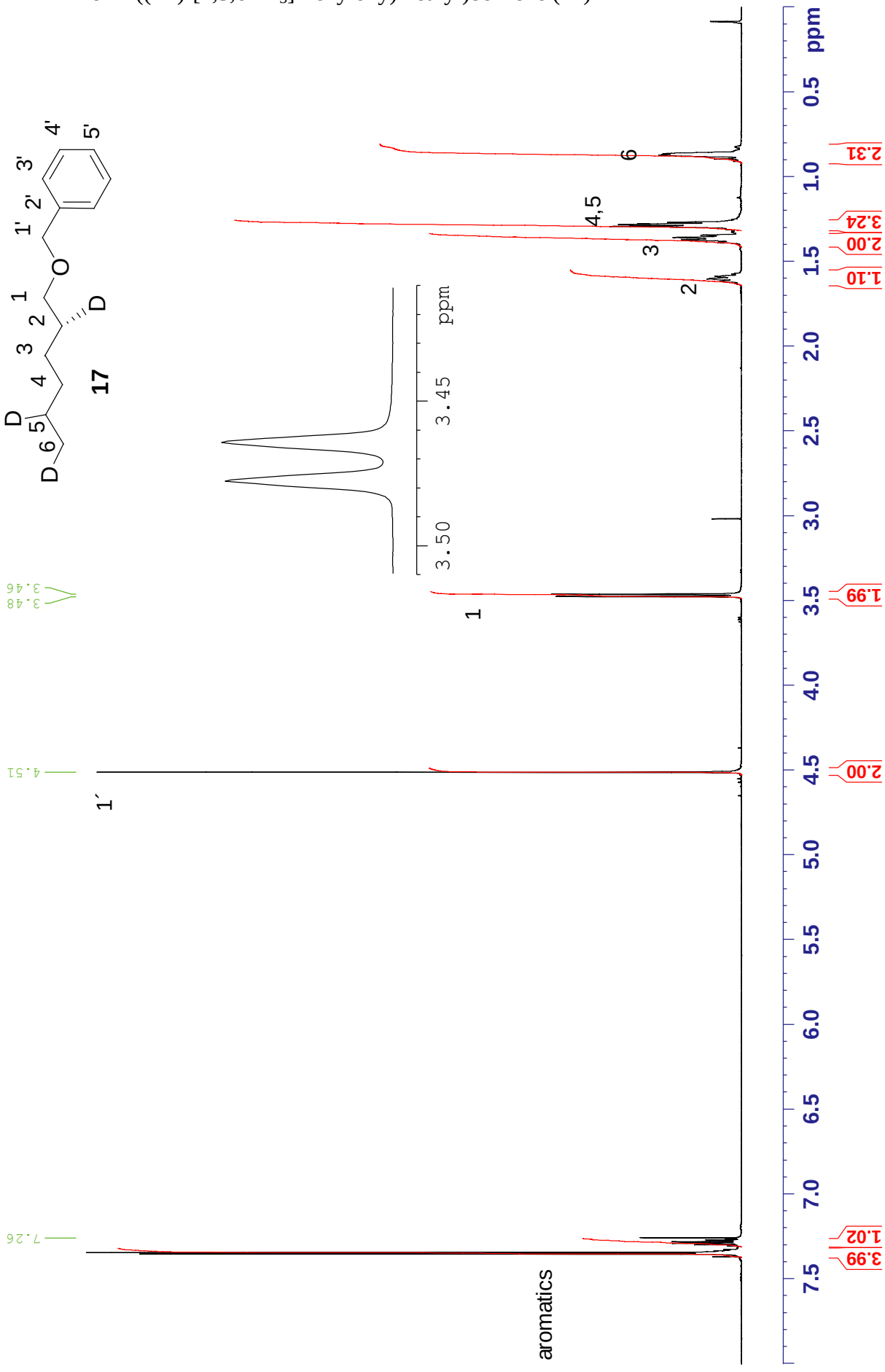
^{1}H NMR of 1-((2*R*)-[2-^{2}H]-hex-5-enyloxy)methyl)benzene (**16**)



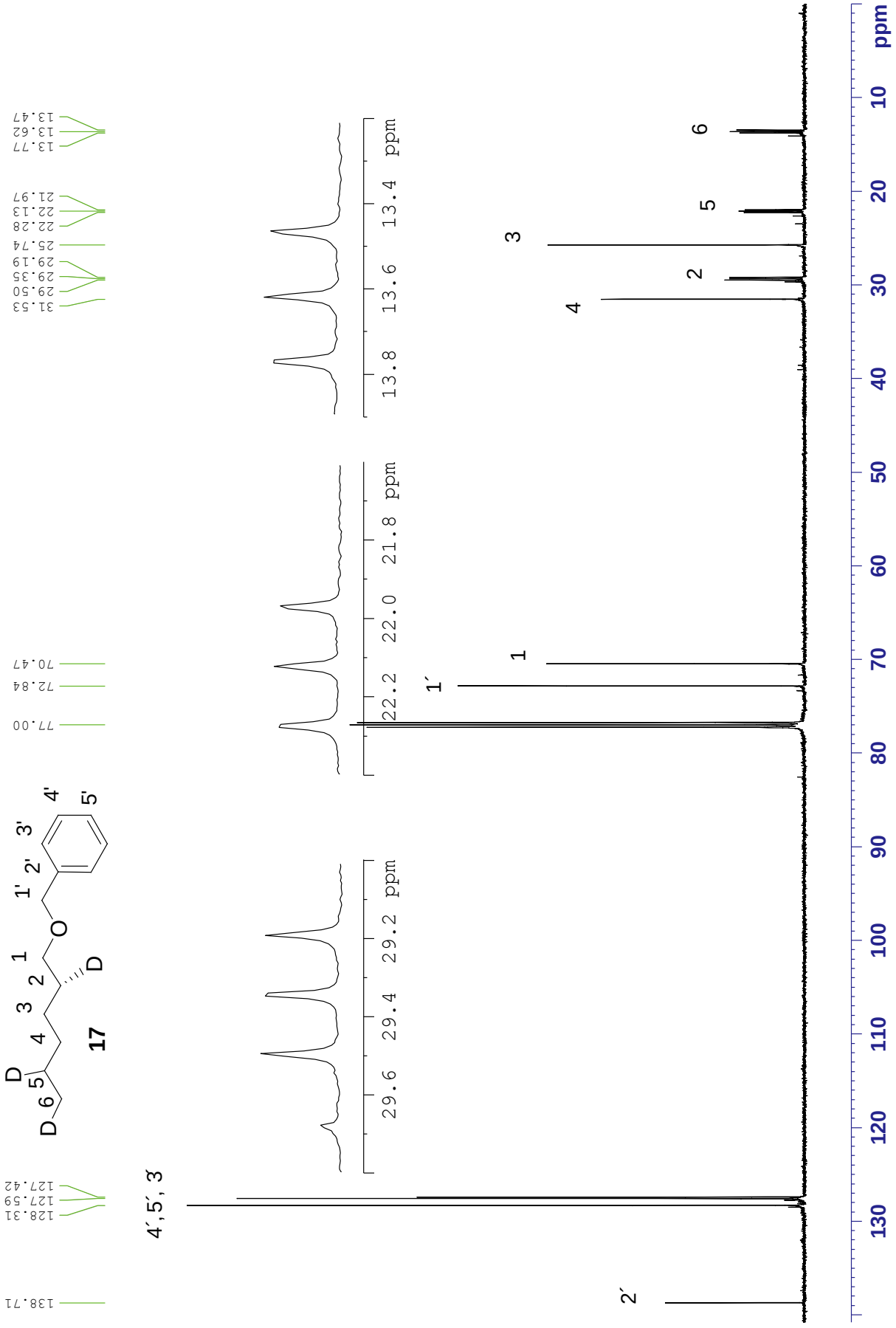
^{13}C NMR of 1-((2*R*)-[2-^{2}H]-hex-5-enyloxy)methyl)benzene (**16**)



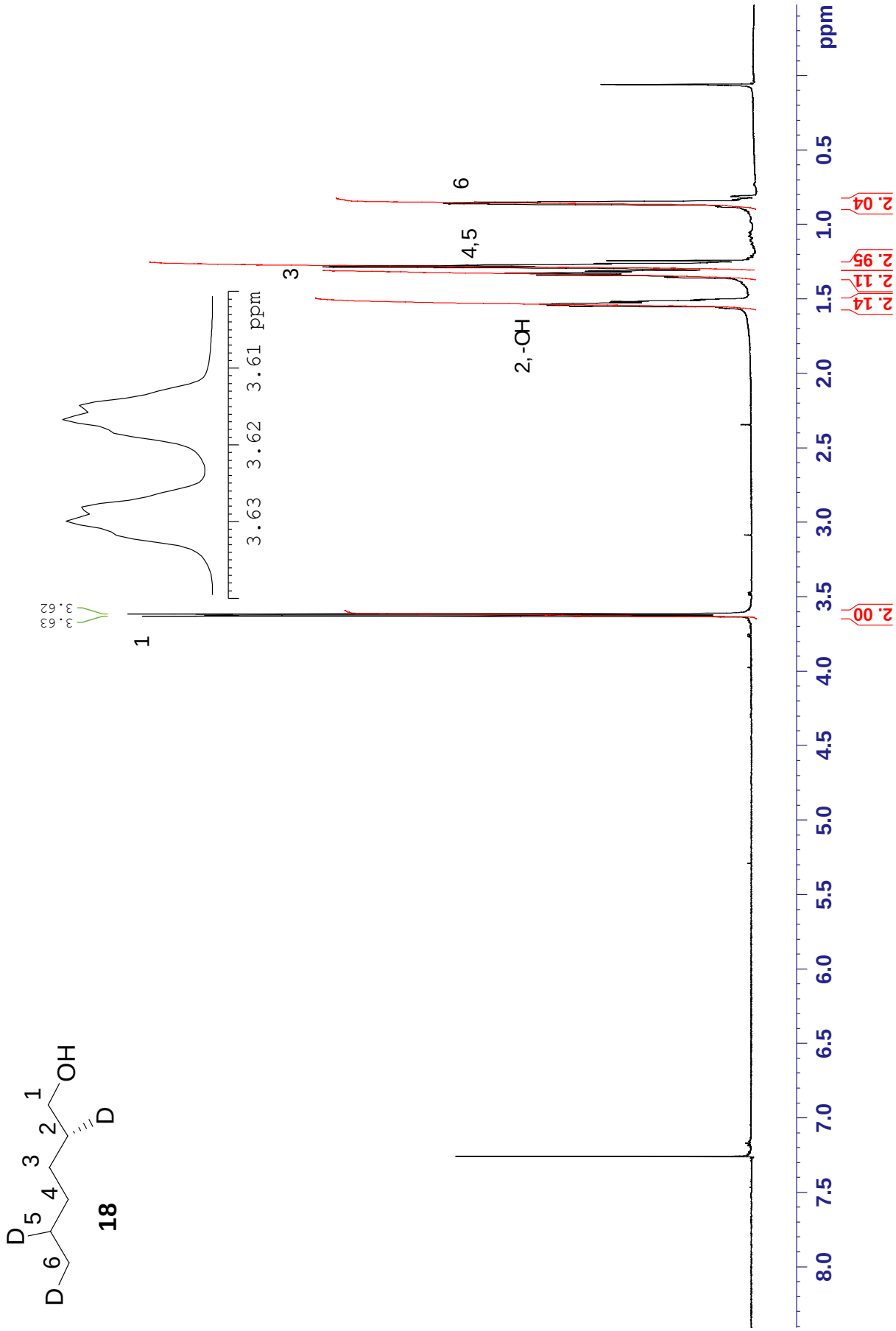
^{1}H NMR of 1-((2*R*)-[2,5,6-$^{2}H_3$]-hexyloxy)methyl)benzene (**17**)



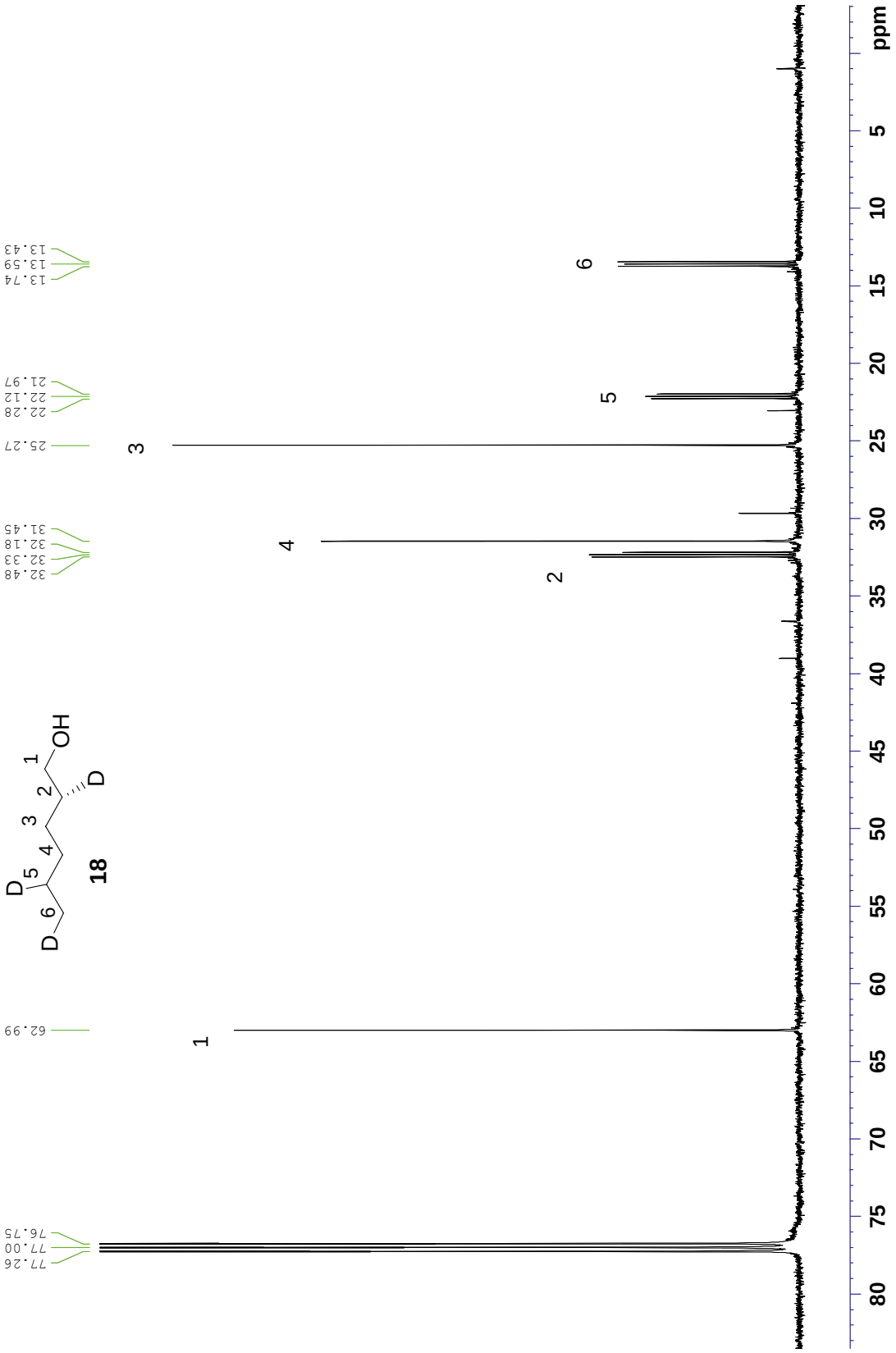
^{13}C NMR of 1-((2*R*)-[2,5,6-$^{2}H_3$]-hexyloxy)methyl)benzene (**17**)



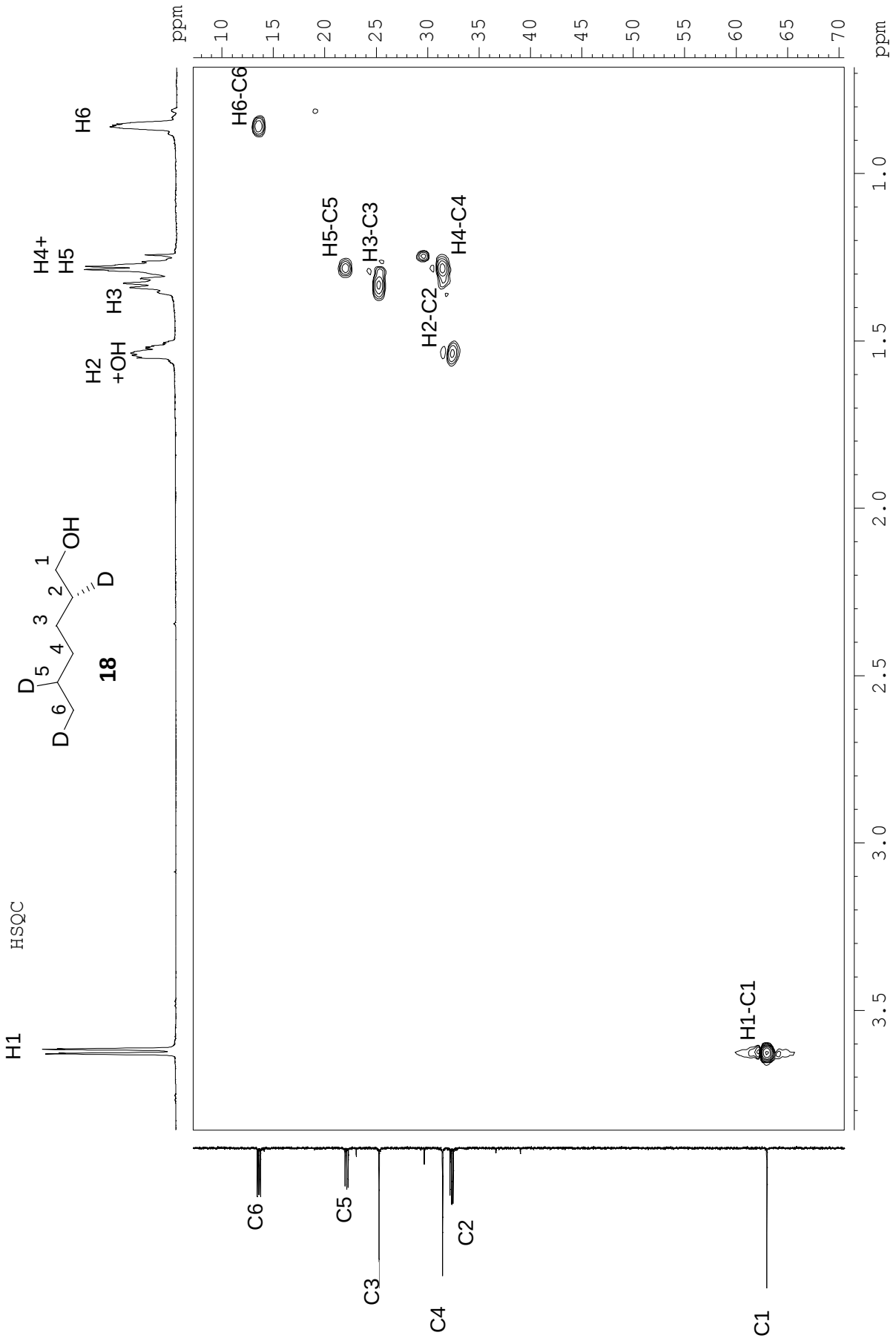
^{1}H NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexan-1-ol (**18**)



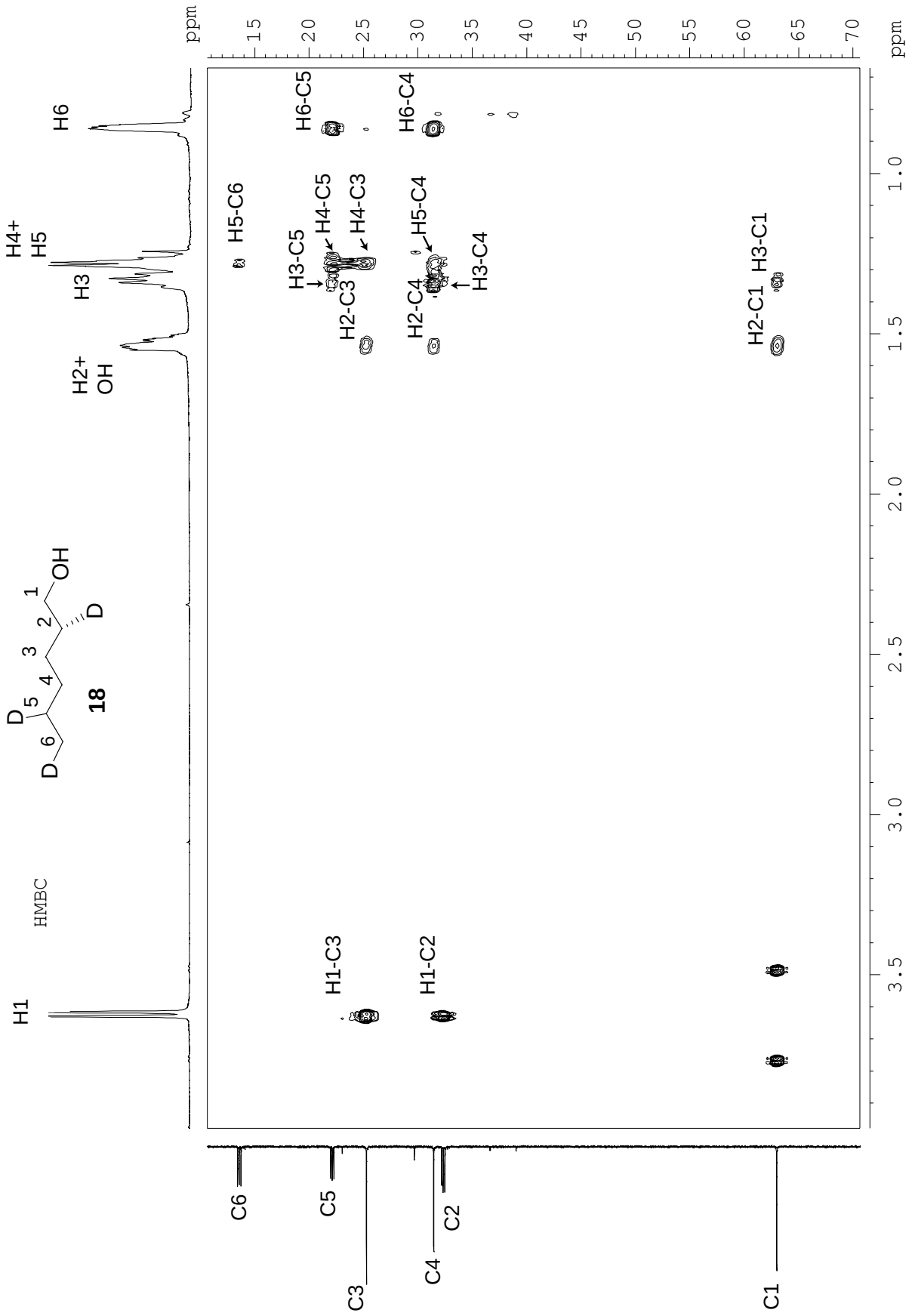
^{13}C NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexan-1-ol (**18**)



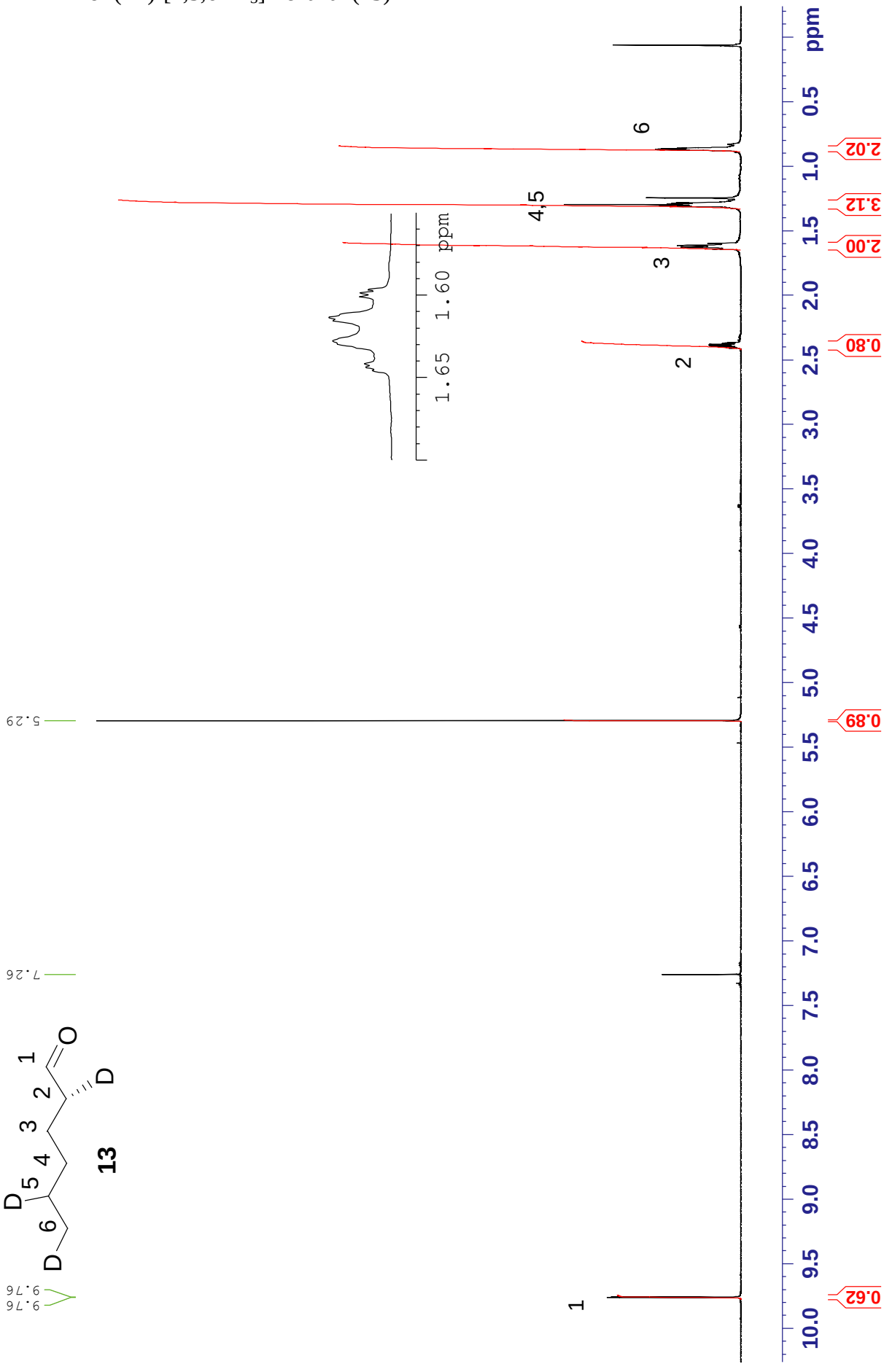
HSQC of (2*R*)-[2,5,6-$^{2}H_{3}$]-hexan-1-ol (**18**)



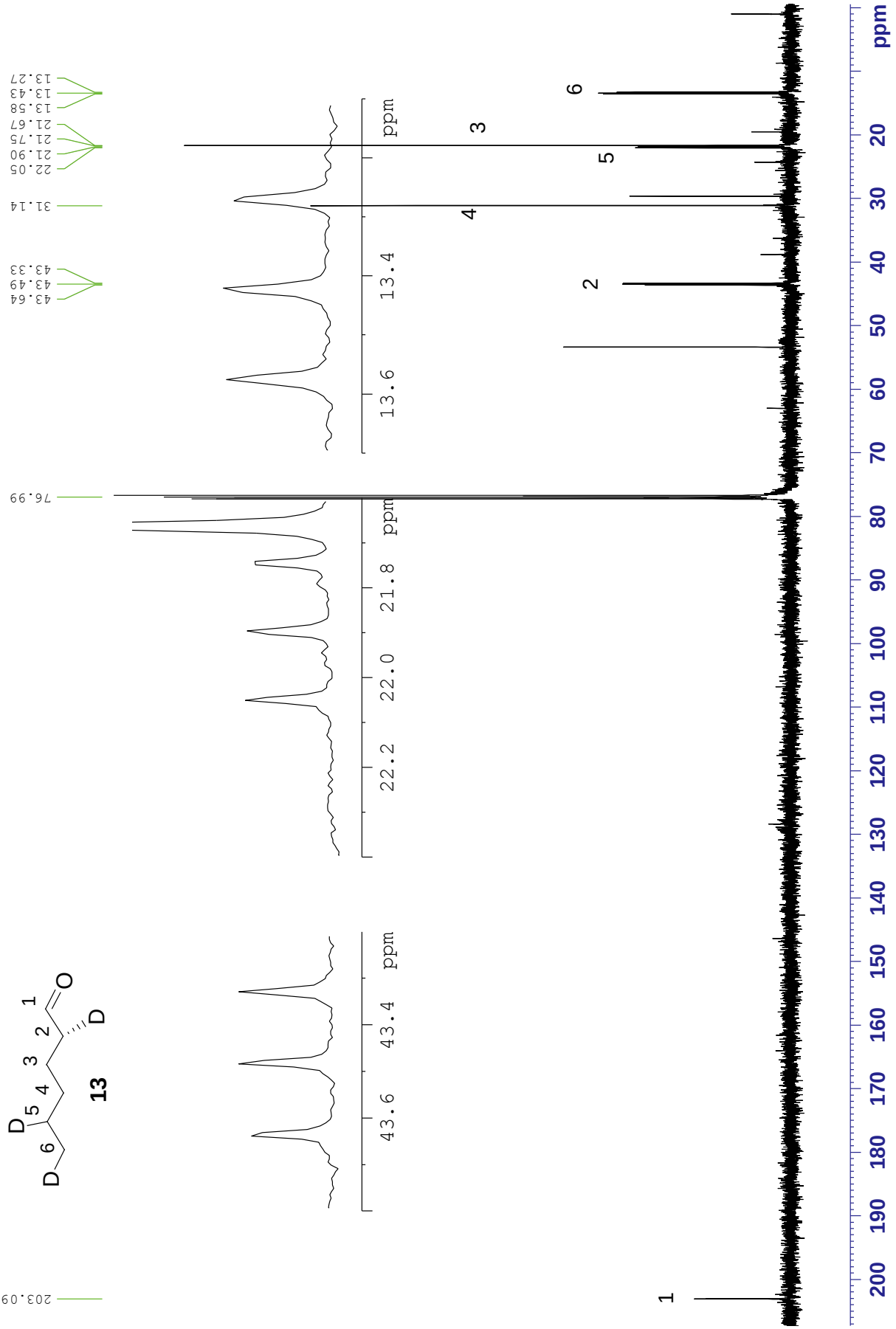
HMBC of (2*R*)-[2,5,6-$^{2}H_{3}$]-hexan-1-ol (**18**)



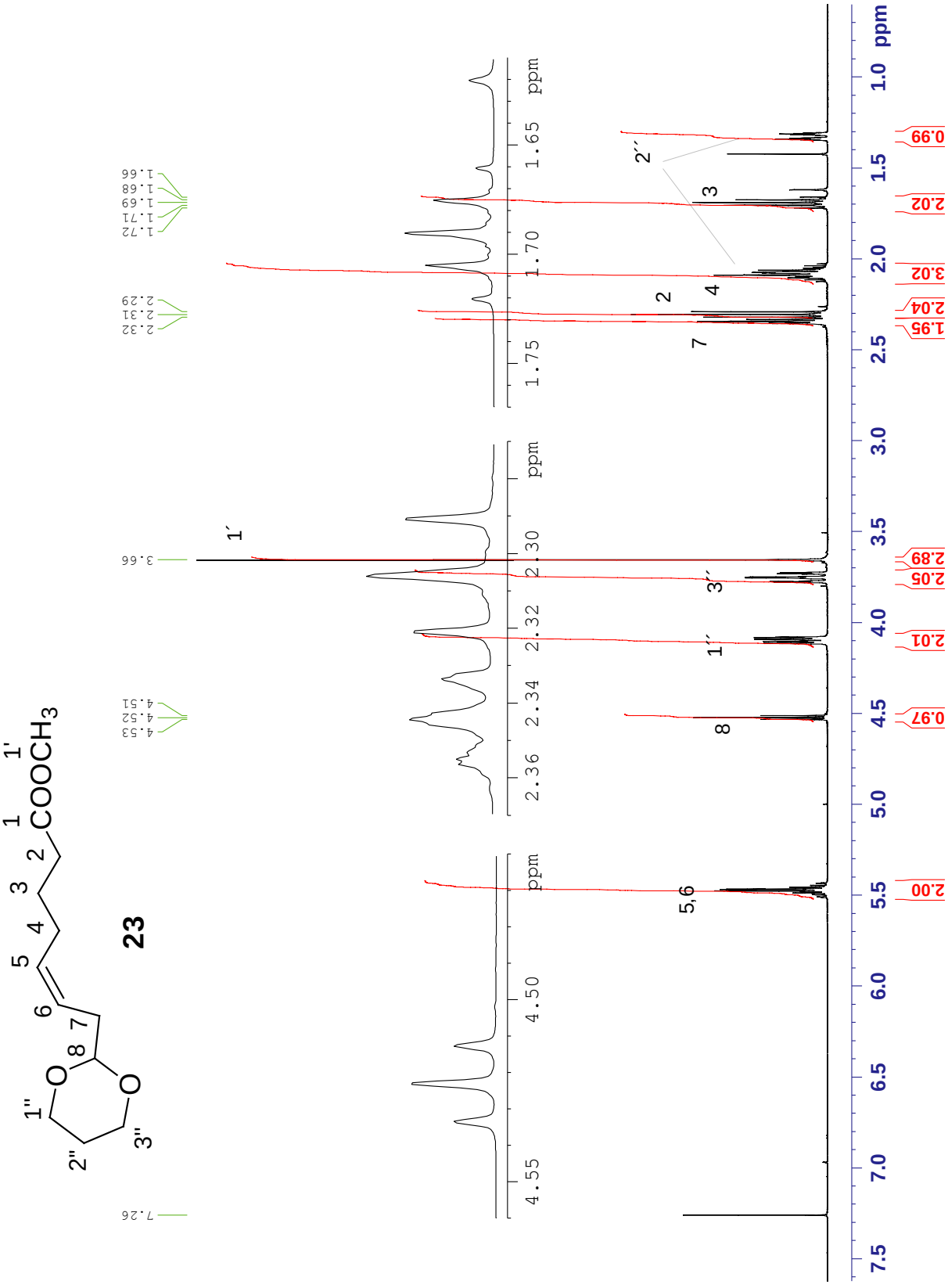
^{1}H NMR of (2*R*)-[2,5,6-$^{2}H_3$]-hexanal (**13**)



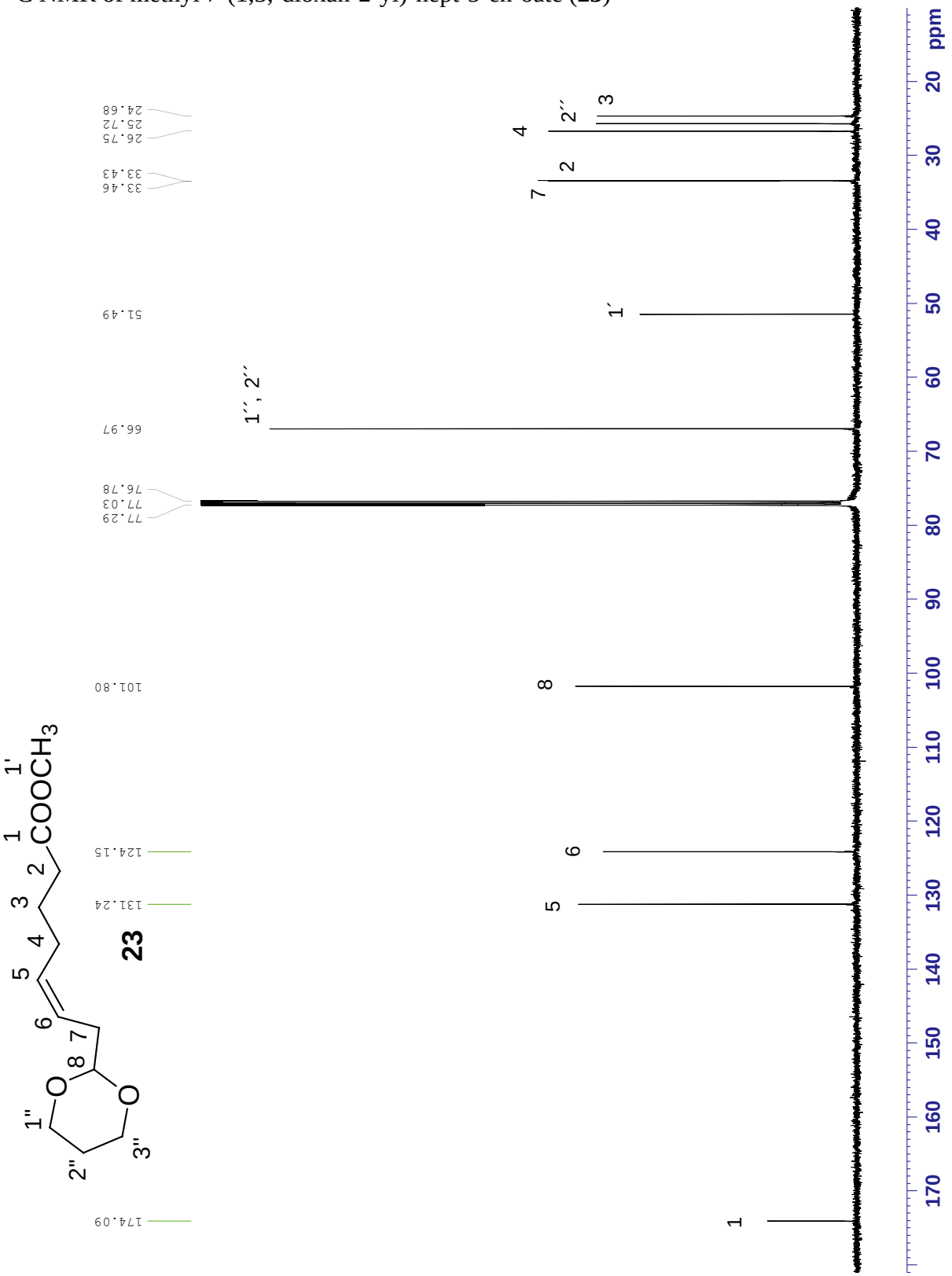
^{13}C NMR of 2(*R*)-[2,5,6-$^{2}H_3$]-hexanal (**13**)



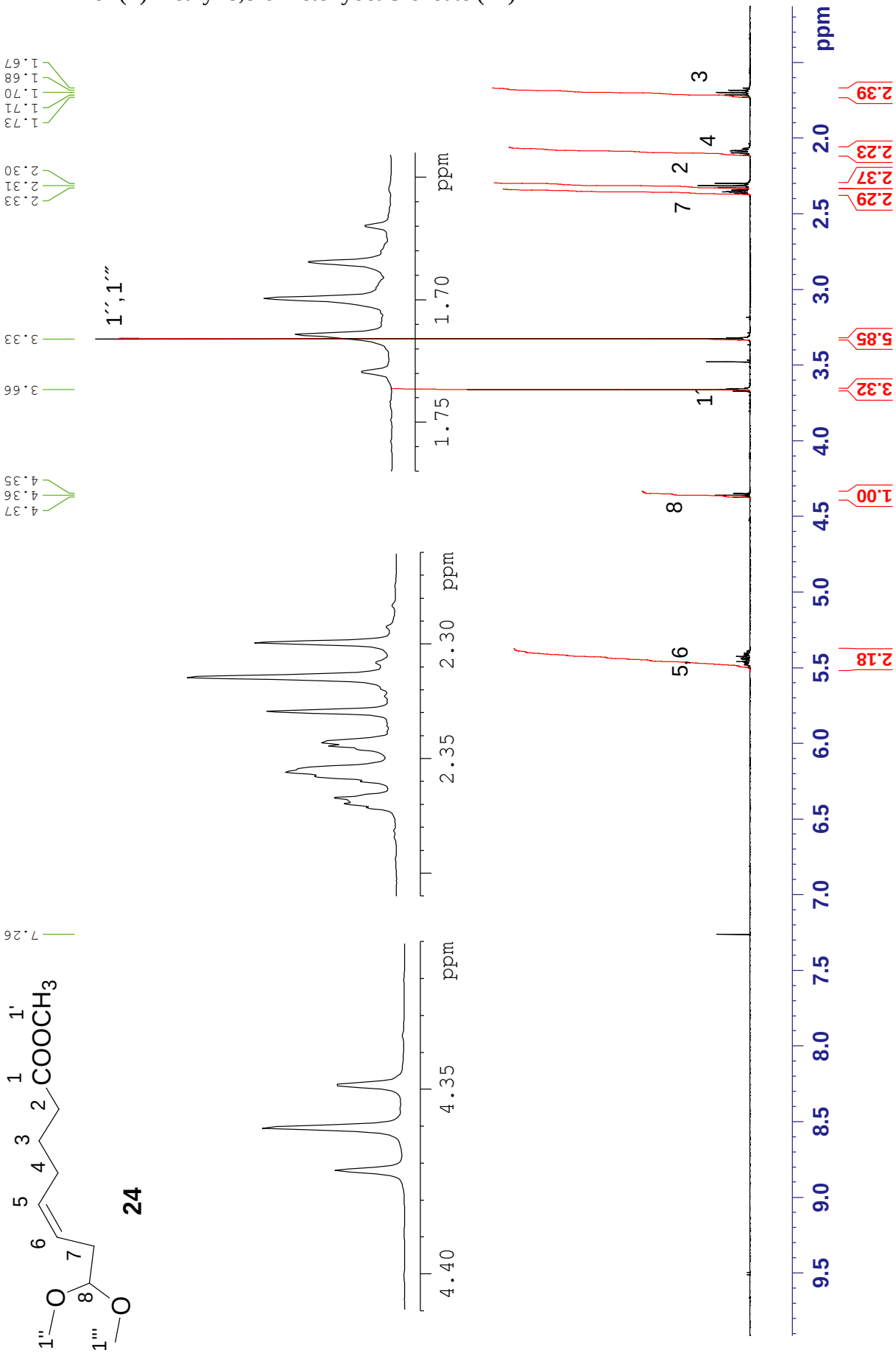
^{1}H NMR of methyl 7-(1,3,-dioxan-2-yl)-hept-5-en-oate (**23**)



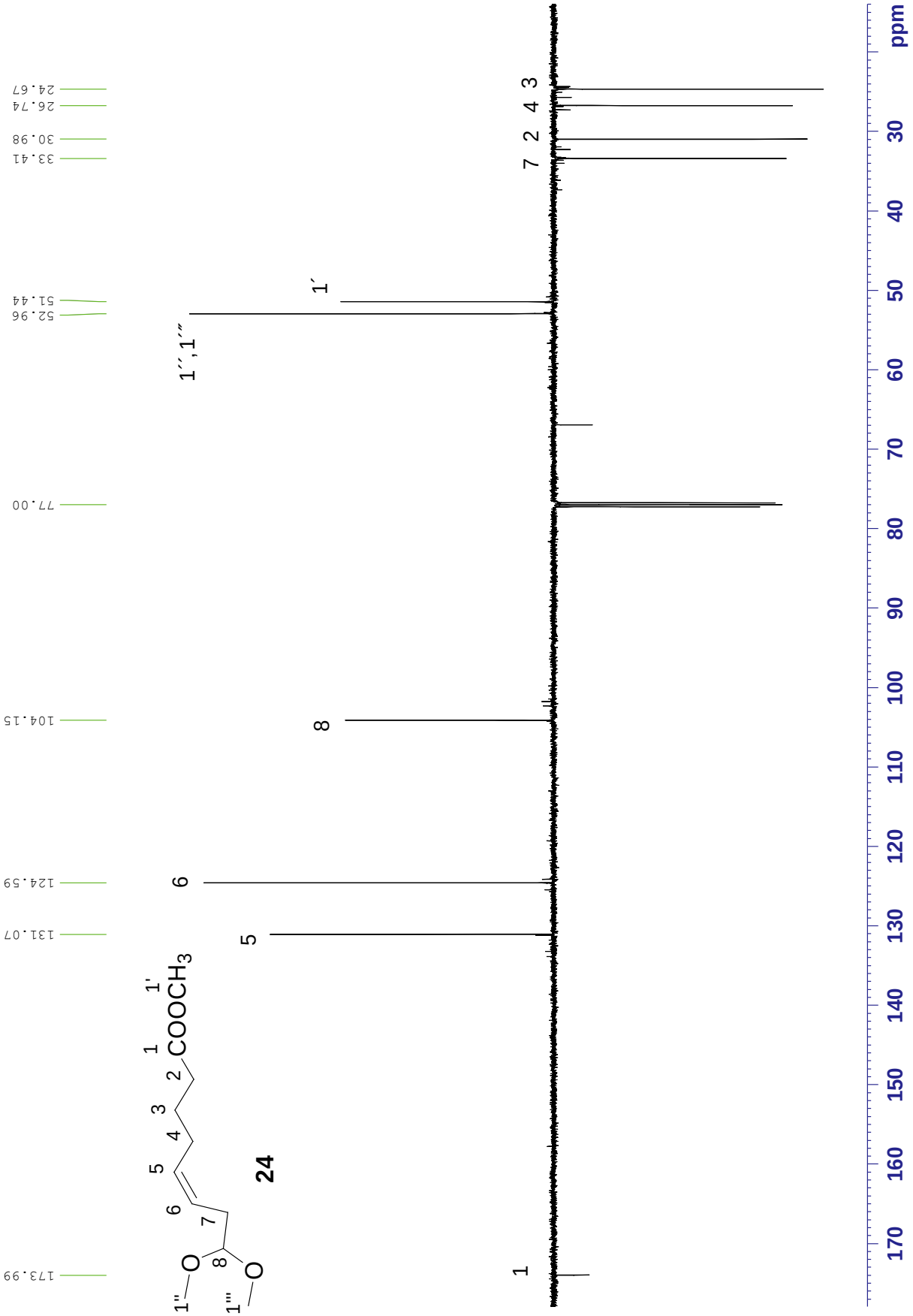
^{13}C NMR of methyl 7-(1,3,-dioxan-2-yl)-hept-5-en-oate (**23**)



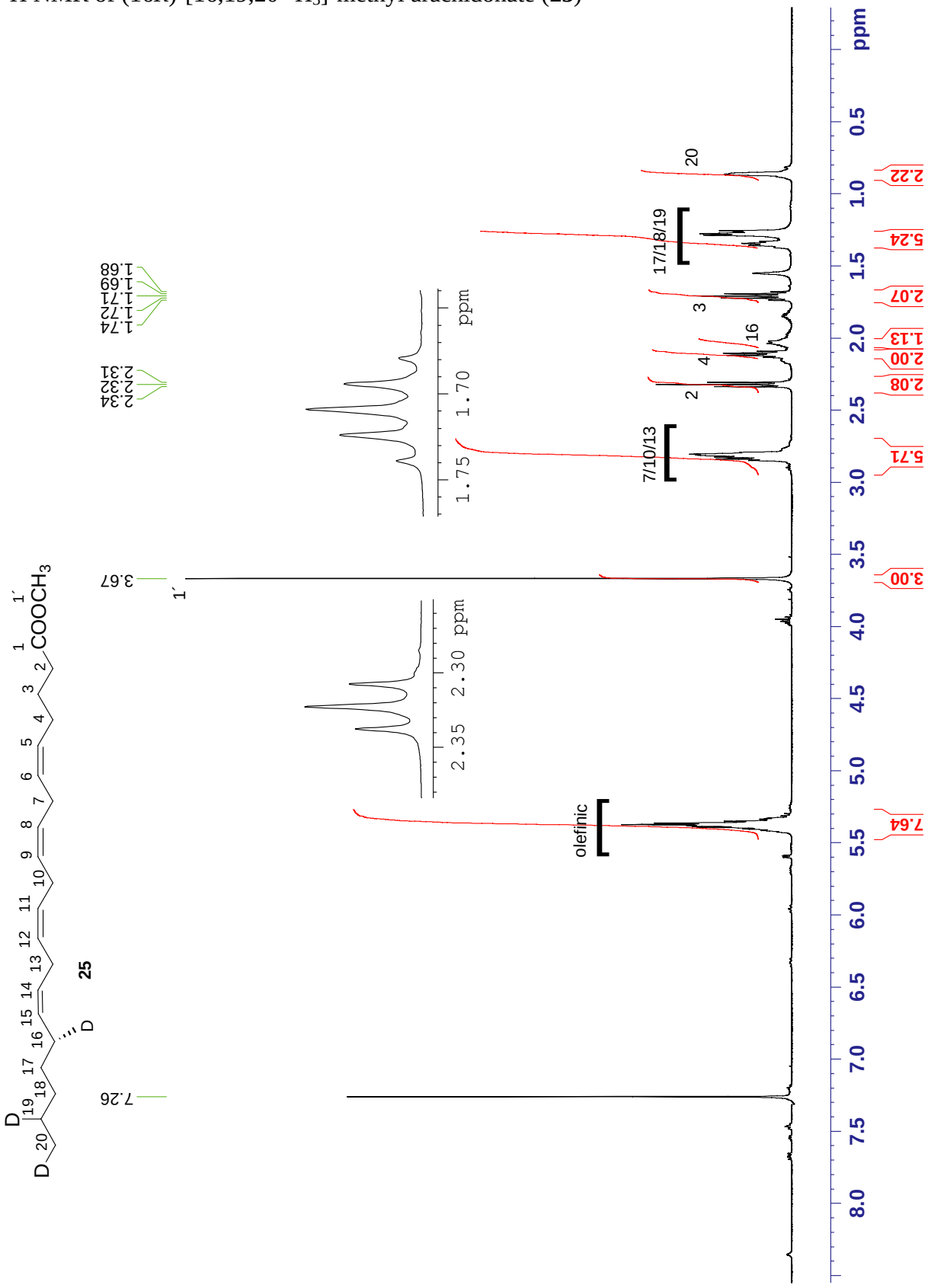
^{1}H NMR of (*Z*)-methyl 8,8-dimetoxyoct-5-enoate (**24**)



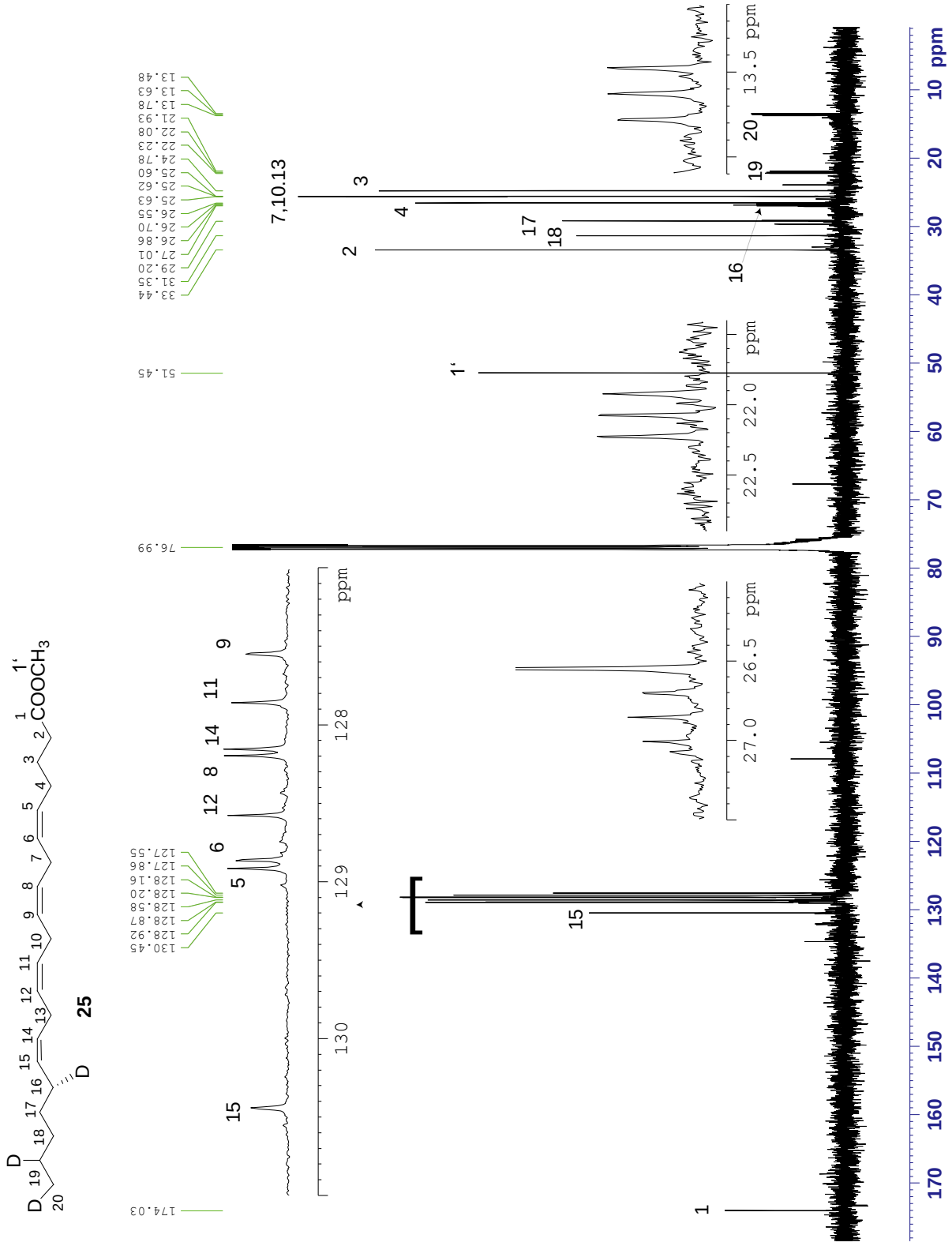
^{13}C NMR of (*Z*)-methyl 8,8-dimetoxyoct-5-enoate (**24**)



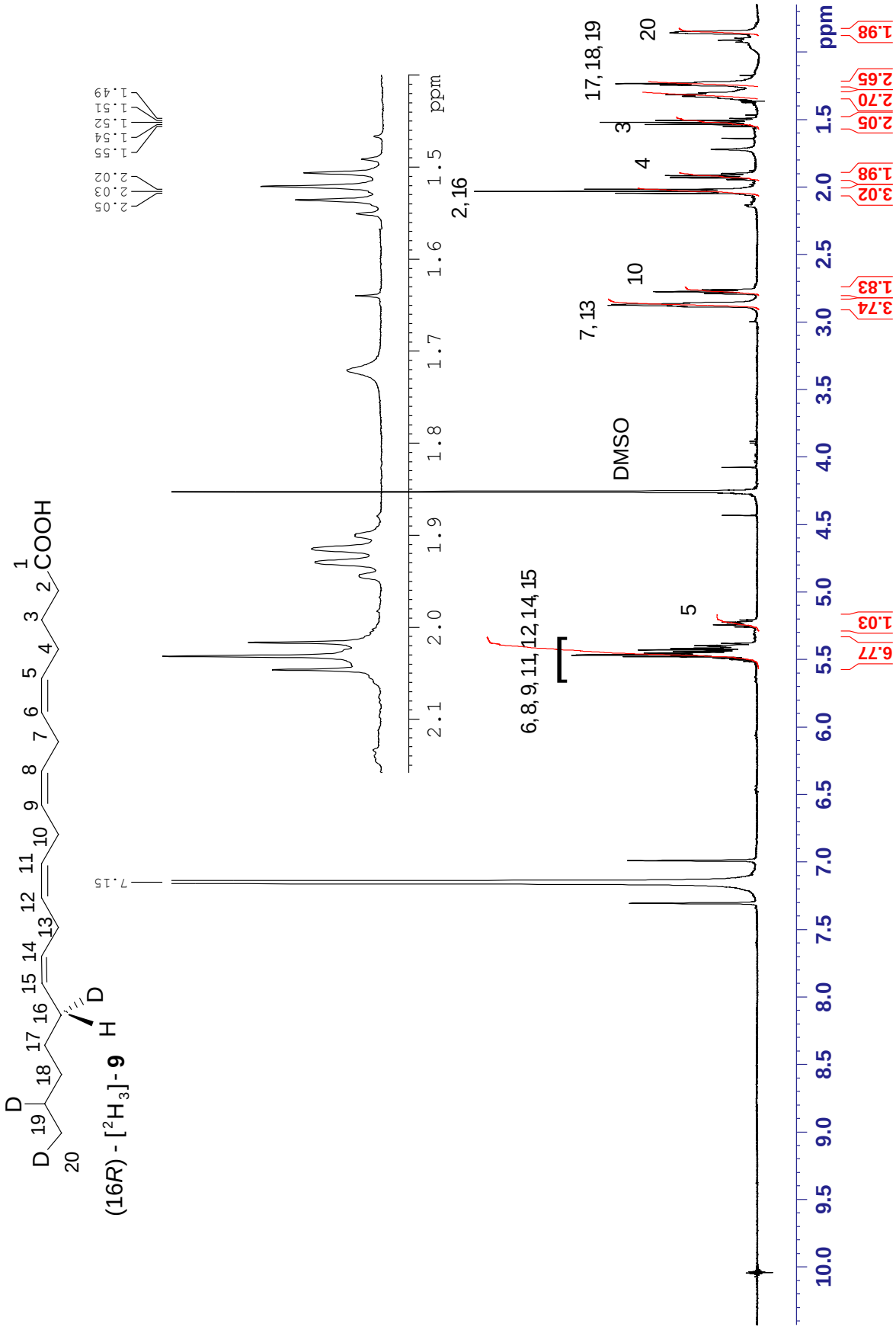
^{1}H NMR of (16*R*)-[16,19,20-$^{2}H_3$]-methyl arachidonate (**25**)



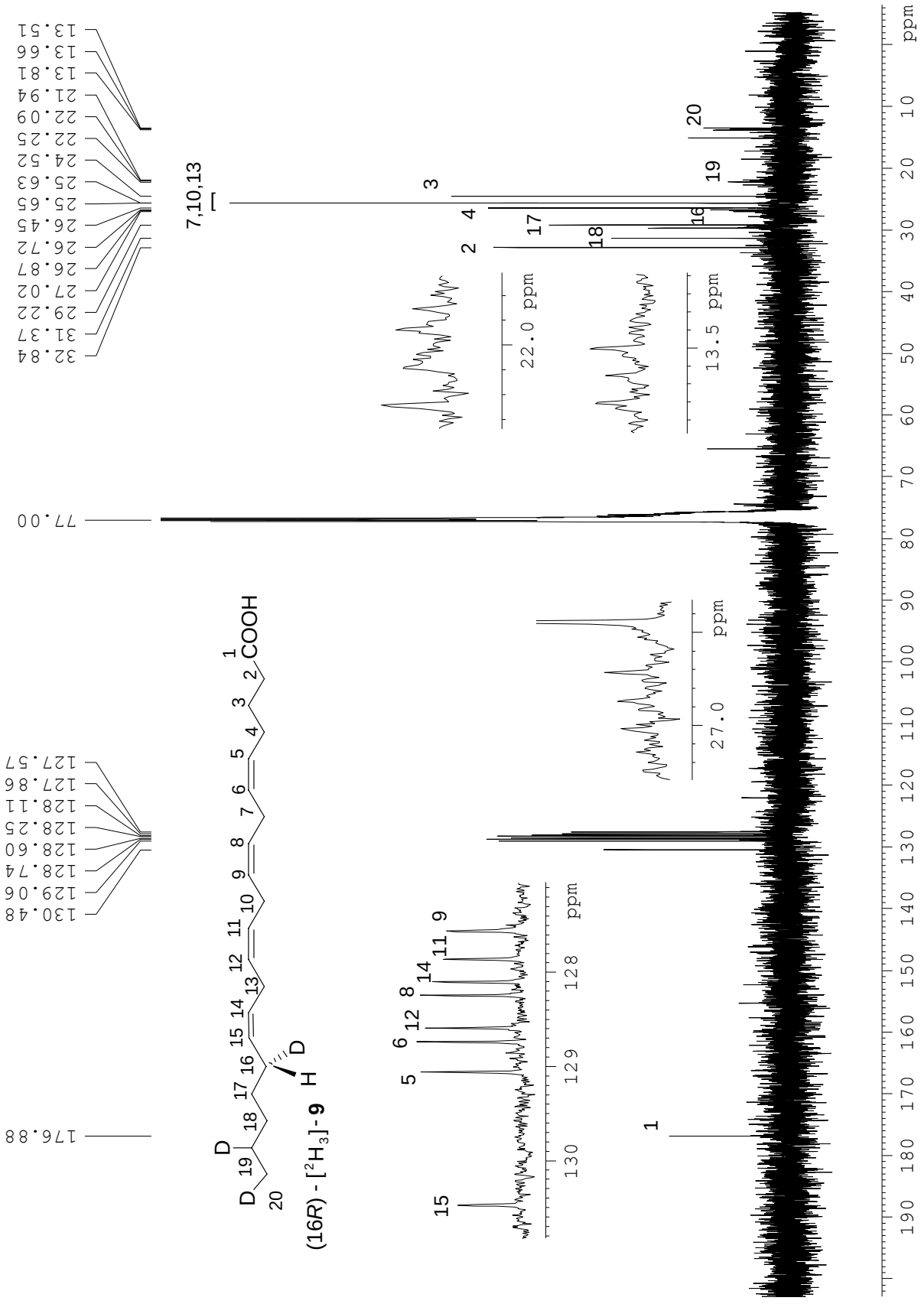
^{13}C NMR of (16*R*)-[16,19,20-$^{2}H_3$]-methyl arachidonate (**25**)



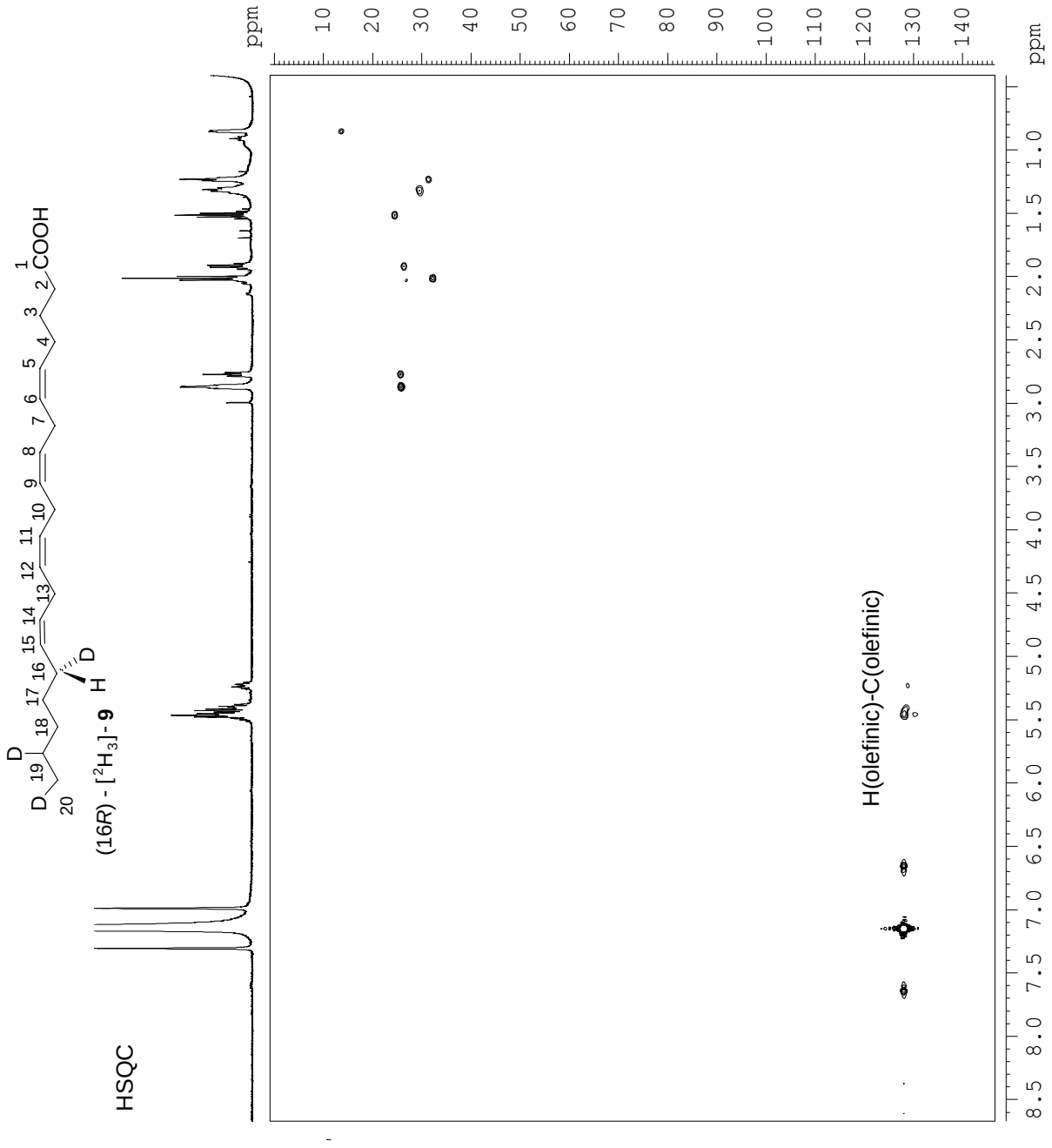
^{1}H NMR of (16*R*)-[16,19,20-$^{2}H_3$]-arachidonic acid (16*R*)-[$^{2}H_3$]-**9**



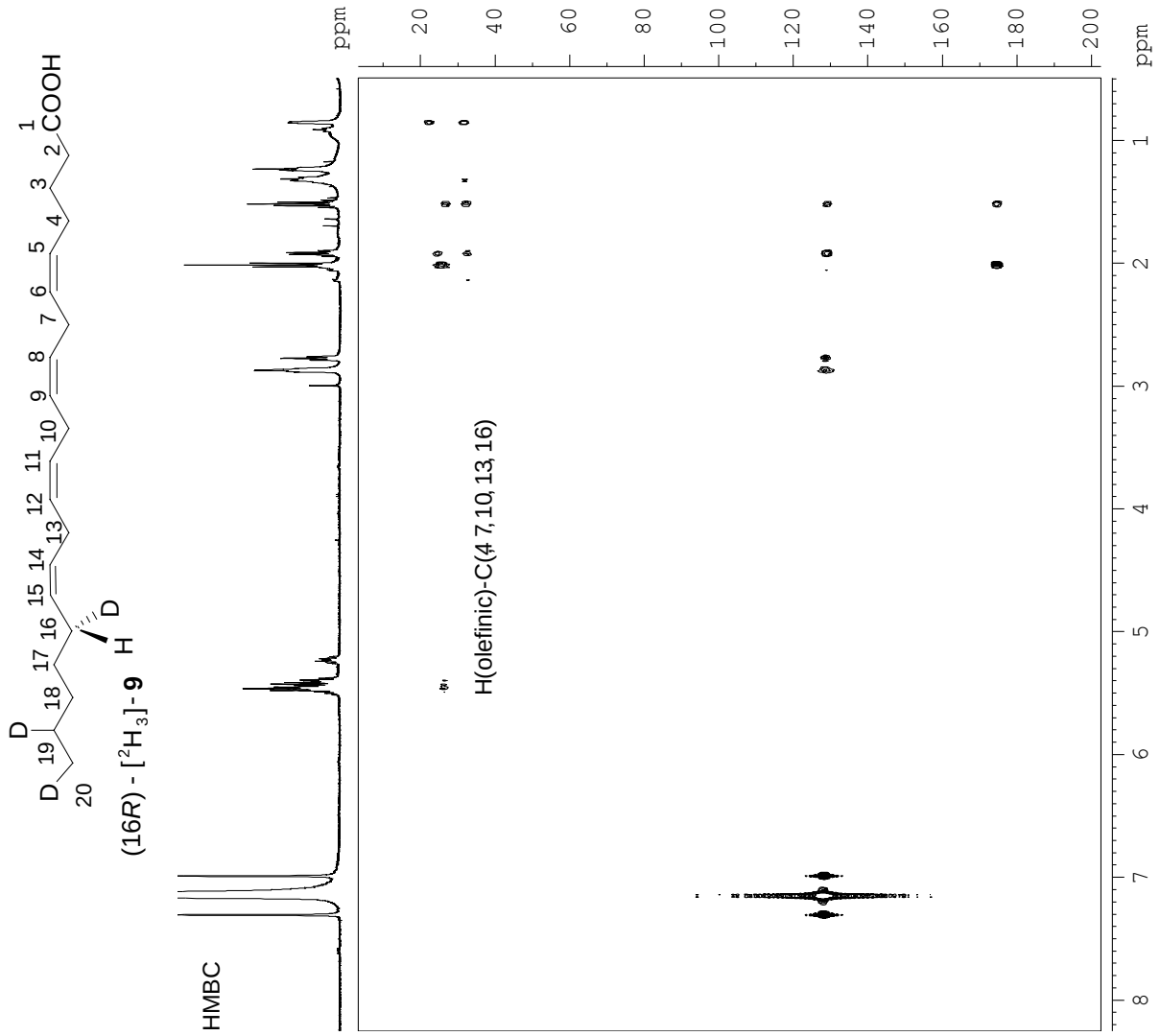
^{13}C NMR of (16*R*)-[16,19,20-$^{2}H_3$]-arachidonic acid (16*R*)-[$^{2}H_3$]-**9**



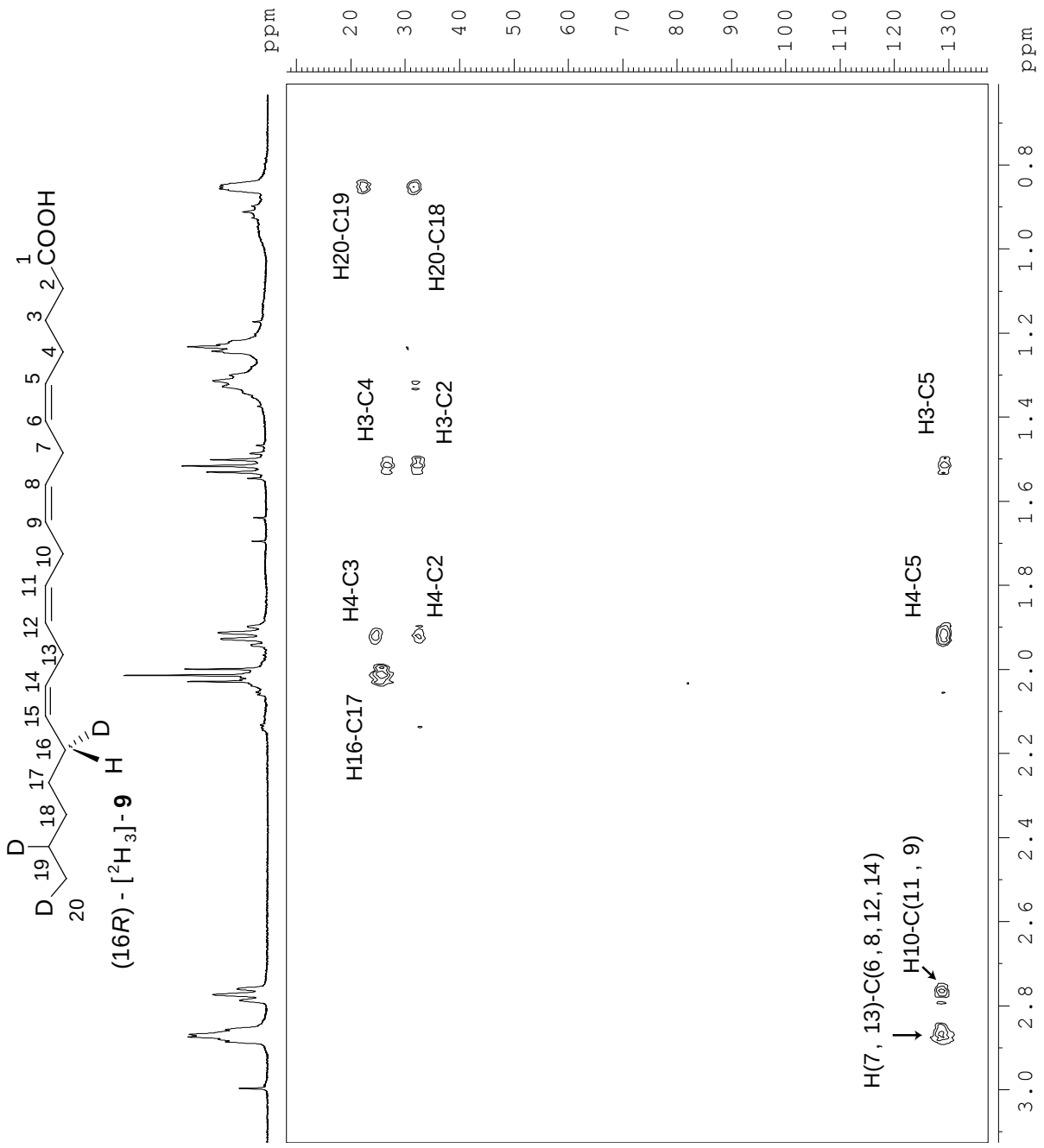
HSQC NMR of (16*R*)-[16,19,20-$^{2}H_3$]-arachidonic acid (16*R*)-[$^{2}H_3$]-**9**



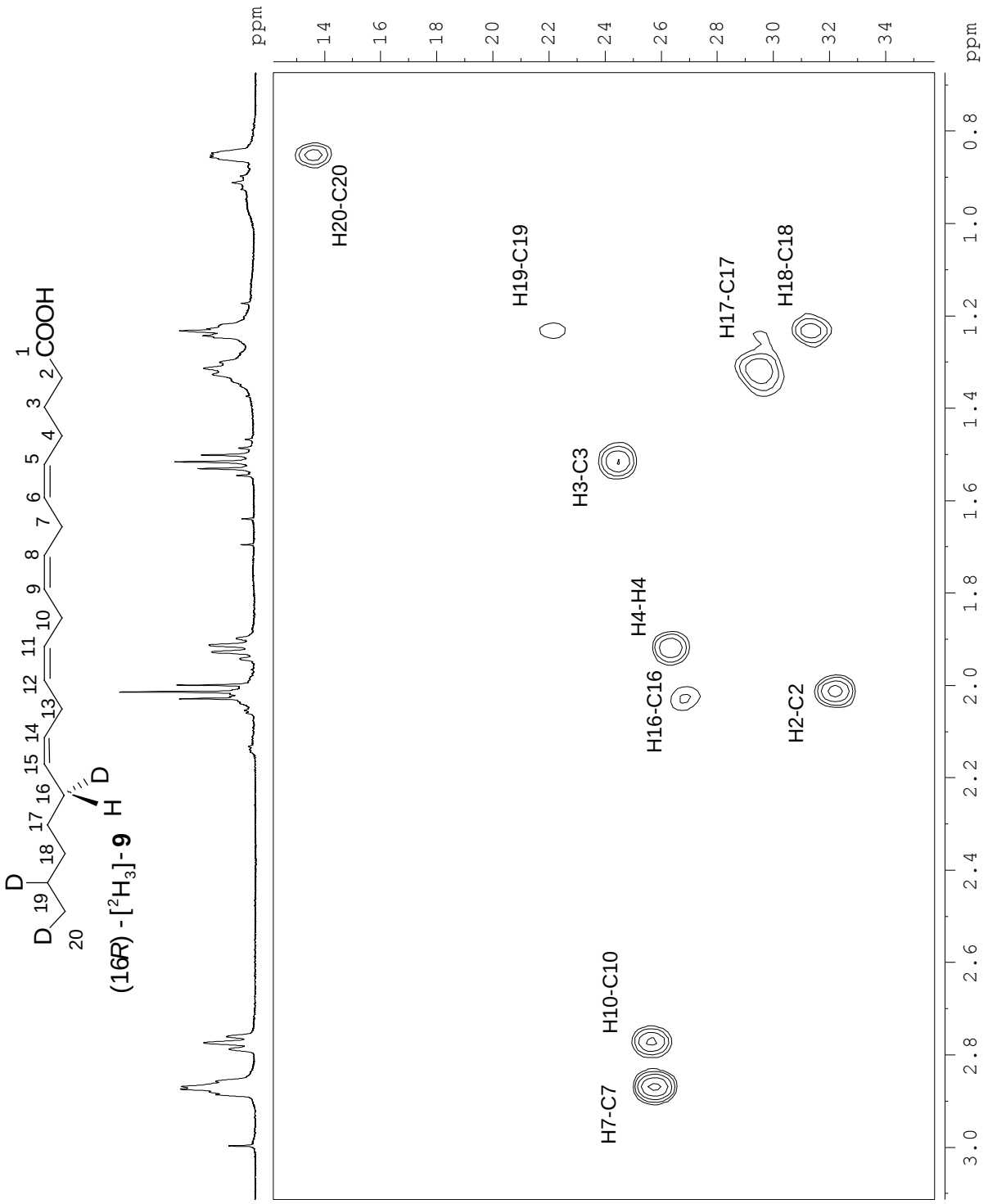
HMBC NMR of (16*R*)-[16,19,20-$^2H_3$]-arachidonic acid (16*R*)-[$^2H_3$]-**9**



Enlarged view of HSQC NMR of (16*R*)-[16,19,20-$^2H_3$]-arachidonic acid (16*R*)-[$^2H_3$]-**9**

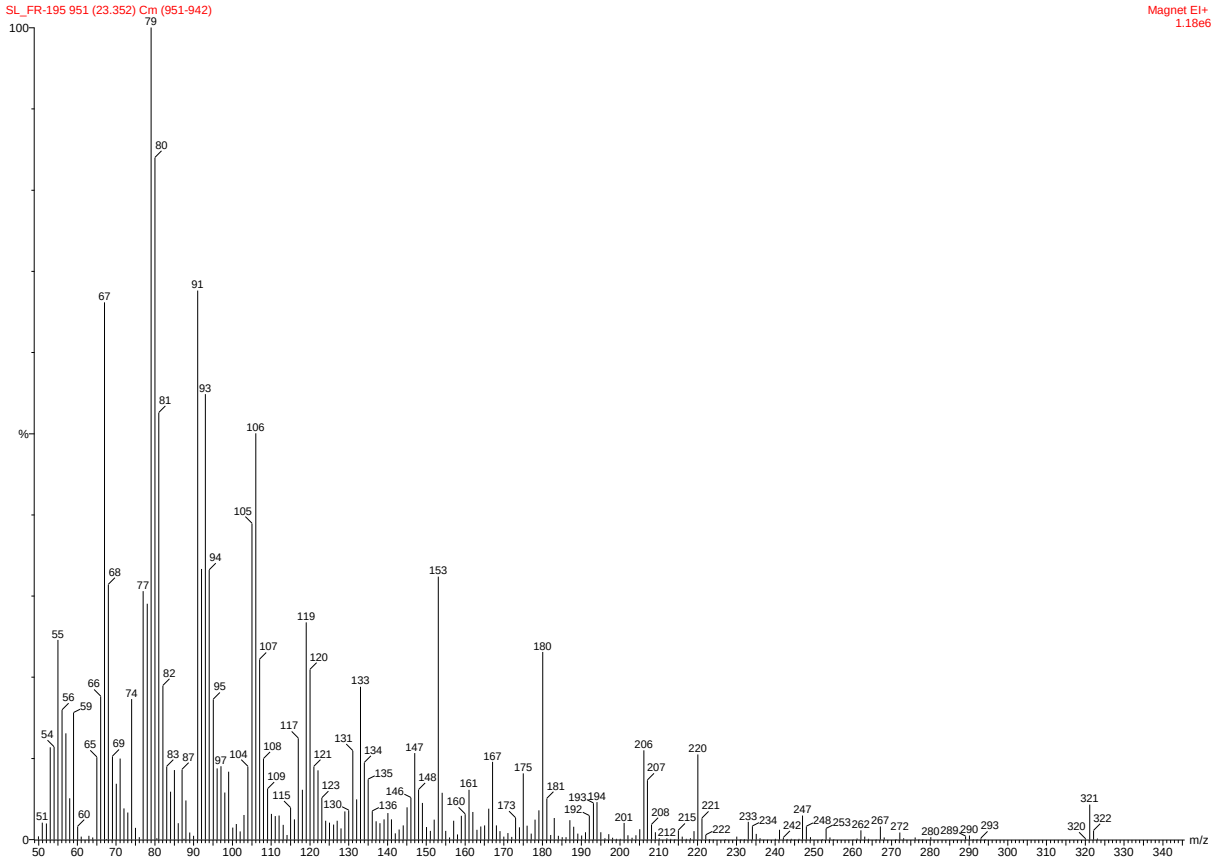


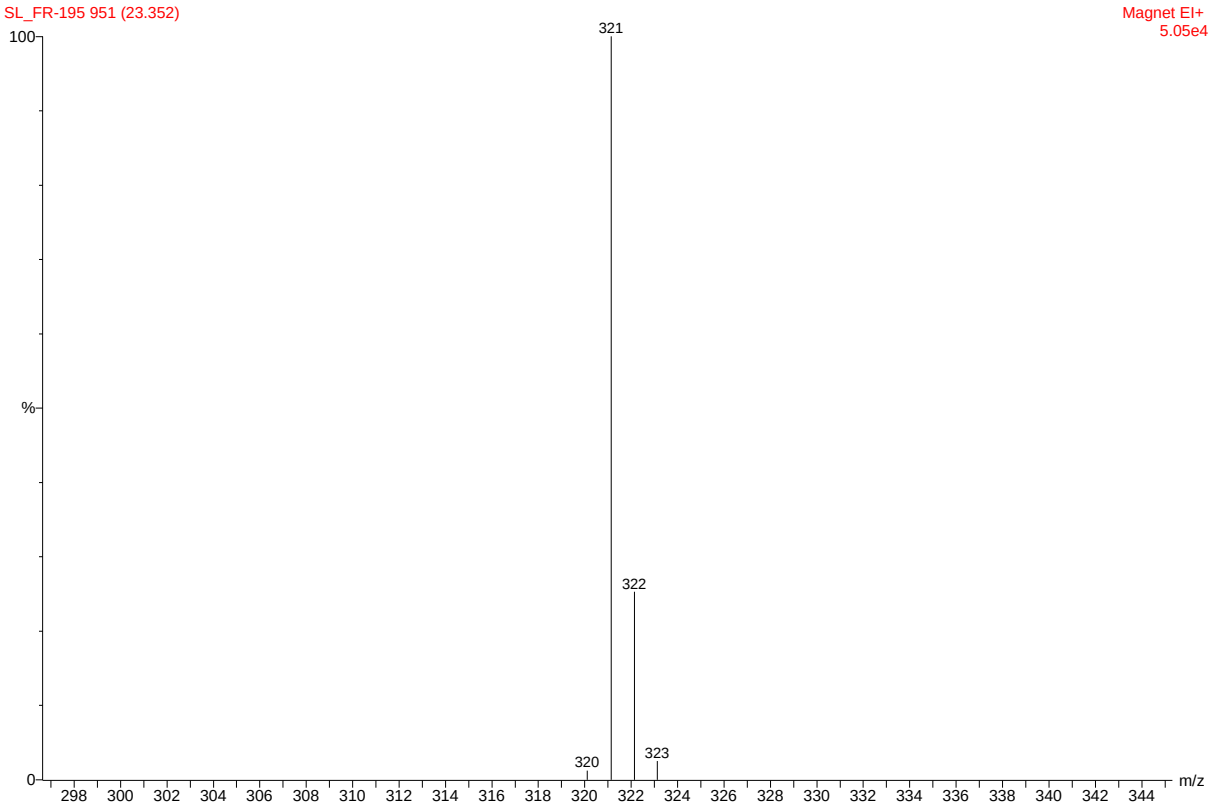
Enlarged view of HMBC NMR of (16*R*)-[16,19,20-$^{2}H_3$]-arachidonic acid (16*R*)-[$^{2}H_3$]-**9**



GLC-MS Spectra

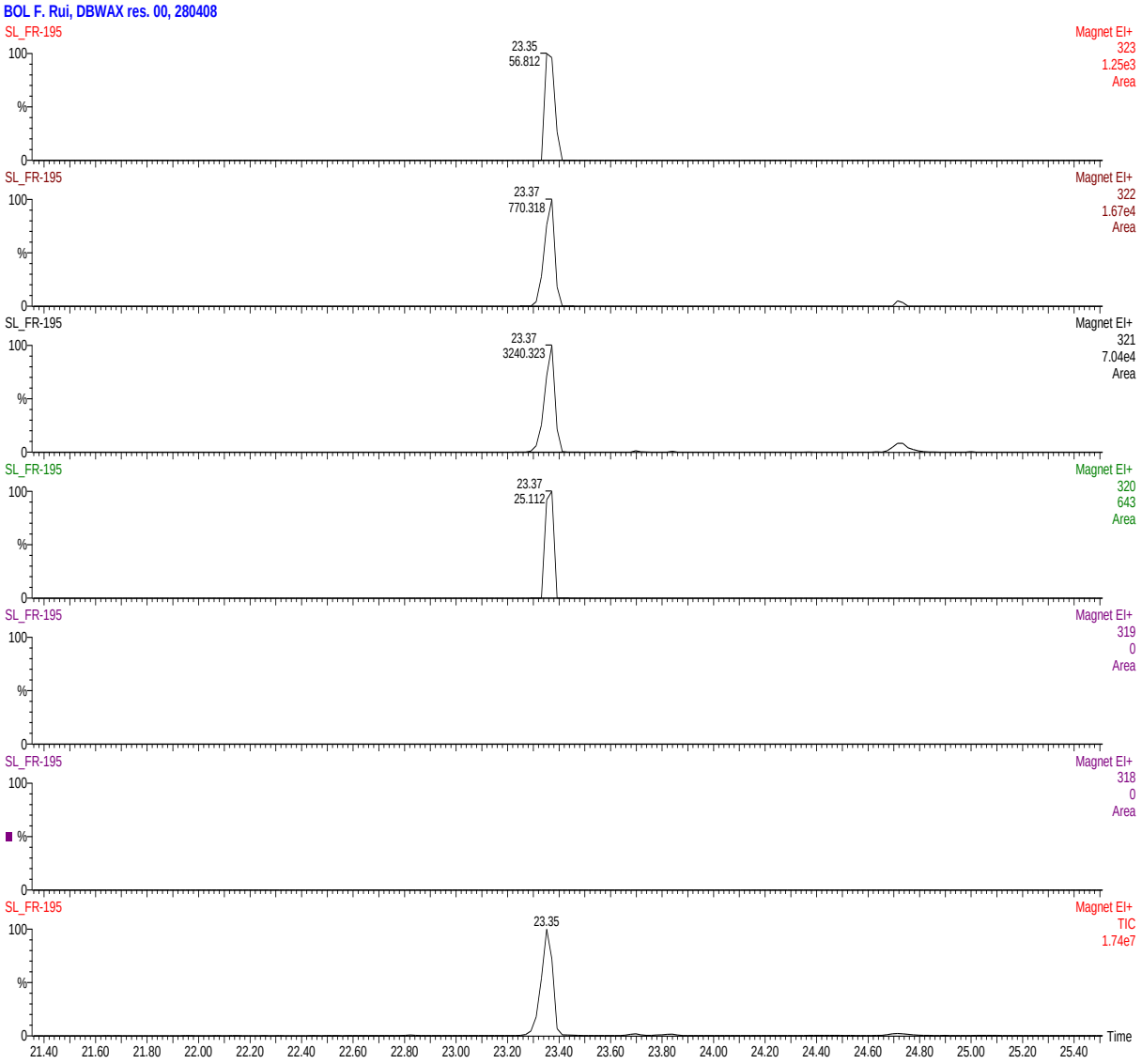
GLC-MS analysis of (16*R*)-[16,19,20-$^{2}H_3$]-methyl arachidonate (**25**)




SL_FR-195 951 (23.352)
Magnet EI+
5.05e4
100
%
0
321
322
320
323
298 300 302 304 306 308 310 312 314 316 318 320 322 324 326 328 330 332 334 336 338 340 342 344
m/z

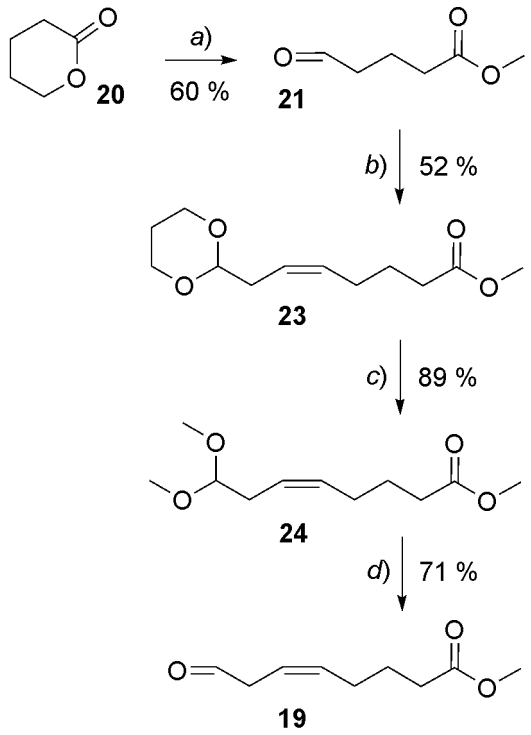
GLC-MS analysis of (16*R*)-[16,19,20-$^{2}H_3$]-methyl arachidonate (**25**), extracted ion chromatograms

Ion trace (m/z)	Deuterium content	RT	Area	Area corrected for the contribution of ^{13}C	%
323	d_5	23.372	57	<5	<0.2
322	d_4	23.372	770	22	0.7
321	d_3	23.372	3240	3233	98.5
320	d_2	23.372	25	25	0.8
319	d_1	-	not detected	-	<0.2
318	d_0	-	not detected	-	<0.2



Additional Graphics

SCHEME 4. Synthesis of oxoester **19** [a]



[a] Reagents and conditions: *a*) 1. cation exchange resin/MeOH, reflux, 2. IBX/AcOMe, reflux; *b*) 2-(1,3-dioxan-2-yl)-ethyltriphenylphosphonium bromide (**22**)/KN[Si(CH_3)$_3$]$_2$/THF, - 78 °C; *c*) 1. cation exchange resin/MeOH, reflux; *d*) HCOOH/pentane.

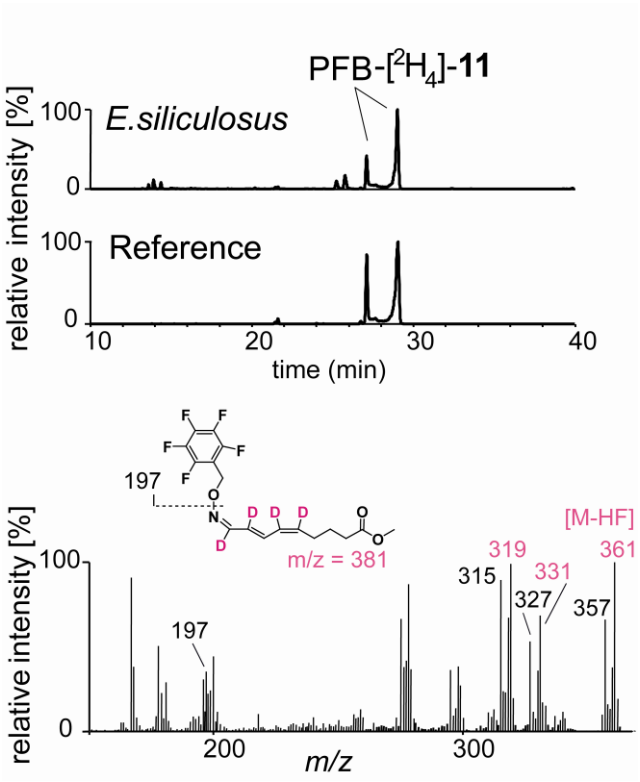


FIGURE 5. GLC-MS of derivatised polar metabolites after incubation with (16*R*)-[$^2H_3$]-**9** – CI-NEG.

References

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