

Analytica Chimica Acta

Supplementary Information

**Sensitive monitoring of monoterpene metabolites in human urine
using two-step derivatisation and positive chemical ionisation–
tandem mass spectrometry**

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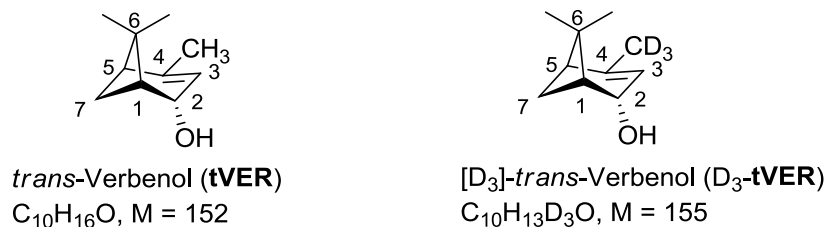
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Analytical Instrumentation for Standard Characterization and Synthesis

¹H-NMR spectra were recorded on a Varian MERCURY-Vx300 spectrometer (300 MHz), Agilent 400MR spectrometer (400 MHz) and Varian INOVA 600 MHz system. Chemical shifts (δ) are given in ppm relative to TMS, using the signal of residual CHCl₃ in CDCl₃ (δ =7.24 ppm). Multiplicities of signals are described as singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m). GC-MS was performed on a Thermo Scientific gas chromatograph Focus GC with the Thermo Scientific DSQ II GC-MS EI/CI mainframe (70 eV ionization energy in electron impact (EI) mode and using methane gas in chemical ionization (CI) mode). For gas chromatographic separation, a capillary ZB-5ms column (20 m \times 0.18 mm \times 0.18 μ m) was used at a constant flow of helium (1.4 mL/min) as a carrier gas. The initial column temperature of 50 °C was held for 2 min, then raised at a rate of 20 °C/min to 270 °C, and held at this level for 5 min. Injector temperature was 220 °C. Mass spectra of the synthesized standards were obtained with electrospray ionization (ESI) using LCQ and ESI-TOF mass spectrometers (MICROTOF (focus), Bruker, Bremen, Germany). A Knauer smartline gradient HPLC system (Knauer, Berlin, Germany) including two pumps (1050), UV detector (2520), column thermostat (25 °C) was used for separation of the commercial (–)-carveol mixture (Sigma-Aldrich, mixture of *cis*- and *trans*-isomers). A Eurospher-100 C₁₈ column (5 μ m, 250 \times 20 mm, Knauer, Berlin, Germany) was used as a preparative column at a flow rate of 25 mL/min. HPLC grade water containing 0.1 % (v/v) trifluoroacetic acid (TFA) was used as solvent A and acetonitrile containing 0.1 % TFA as solvent B. The detection of *cis*- and *trans*-(–)-carveols was carried out at 210 nm. Purity of the standards was controlled by thin-layer chromatography on MERCK ready-to-use plates with silica gel 60 (F254), using 10 % molybdenum-phosphoric acid in ethanol (Sigma-Aldrich, Seelze, Germany) as a developer. Column chromatography was carried out on MERCK silica gel (grade 60, 0.063 mm).

Reference compounds and internal standards

Synthesis of *trans*-verbenol and *D*₃-*trans*-verbenol



(1*R*,2*S*,5*R*)-*trans*-verbenol (tVER) and *rel*-(1*R*,2*S*,5*R*)-4-(²H₃-Methyl)-6,6-dimethylbicyclo[3.1.1]hept-3-en-2-ol, (D₃-tVER) were prepared by oxidation of (1*R*)-(+)- α -pinene and [*D*₃]- α -pinene with lead(IV) acetate in benzene followed by saponification of the corresponding acetates with KOH in methanol; exactly as described [1].

tVER (C₁₀H₁₆O, M =152). GC-MS: t_R = 5.31 min (100%); EI-MS: m/z (rel. int. %) = 151 (2) [M-H]⁺, 135 (10) [M-OH]⁺, 109 (12) [M-C₃H₇]⁺, 93 (100) [M-C₃H₇O]. ¹H-NMR (400 MHz, CDCl₃): δ = 1.08 (s, 3 H, C⁶-CH₃), 1.31 (d, 1 H, J = 9.2 Hz, H⁷), 1.35 (s, 3 H, C⁶-CH₃), 1.59 (br. s, 1 H, OH), 1.74 (t, J = 1.7 Hz, 3 H, C⁴-CH₃), 1.97 (dt, J = 5.5 and 1.4 Hz, 1 H, H⁵), 2.29 (ddd, J = 10.5, 6.5 and 4.0 Hz, 1 H, H¹), 2.45 (ddd, J = 9.0, 6.2 and 5.2 Hz, 1 H, H⁷), 4.46 (br. s, 1 H, H⁴), 5.37 (dq, J = 3.3 and 1.7 Hz, 1 H, CH⁴=) ppm. ¹H-NMR Spectrum (400 MHz, CDCl₃) is given in Figure S-3 [for comparison with reported 500 MHz ¹H-NMR, see: Lajunen et al. (2000)[2]].

D₃-tVER (C₁₀H₁₃D₃O, M =155). GC-MS: t_R = 5.20 min (*cis*-isomer, 8%) 5.34 min (*trans*-isomer, 92%); EI-MS of both peaks are identical: m/z (rel. int. %) = 154 (1) [M-H]⁺, 138 (8) [M-OH]⁺, 112 (9) [M-C₃H₇]⁺, 96 (100) [M-C₃H₇O]. ESI-MS (positive mode): m/z (rel. int. %) = 333 (100) [2M+Na]⁺, 178 (89) [M+Na]⁺; isotopic purity >98%.

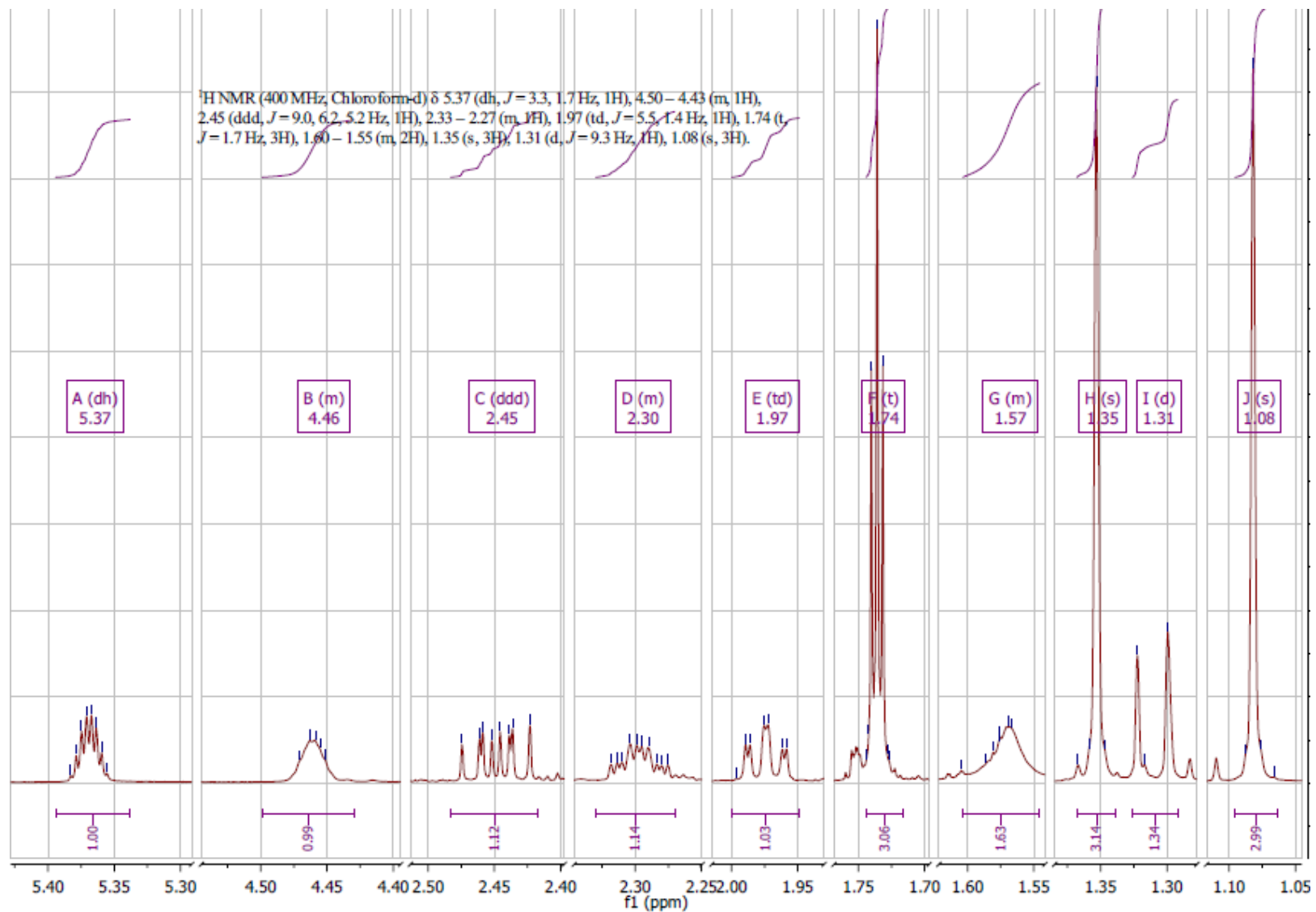
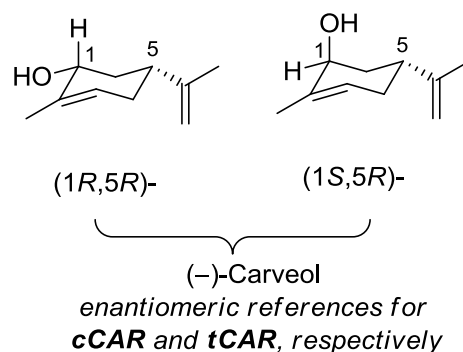


Figure S-1 400 MHz ¹H-NMR spectrum of (1*R*,2*S*,5*R*)-*trans*-verbenol (tVER) in CDCl₃

Separation and identification of *cis*- and *trans*-carveols

Separation of the diastereomers cCAR and tCAR from the commercial (–)-carveol mixture was carried out using a Eurospher 100 C18 HPLC column (20 × 250 mm; Knauer, Berlin, Germany) and UV detection at 210 nm [3]. The mobile phase was MeCN/H₂O with 0.1% TFA at a flow rate of 25 mL/min and a gradient of 20/80 to 50/50 in 25 min. Two peaks with retention times (t_R) of 26.3 and 27.0 min were resolved (ratio of peak areas 2:3). Both peak eluates were collected separately, cooled and neutralised with saturated aqueous NaHCO₃. Then, solid NaCl was added to the fractions, they were diluted with water and extracted several times with pentane, until cCAR and tCAR diastereomers could not be detected on TLC plates. Pentane solutions were washed with brine, dried (Na₂SO₄), and evaporated. The residues (ca. 2–5 mg of oils) were dissolved in CDCl₃, and their ¹H NMR spectra (600 MHz) were recorded. The minor component of the commercial (–)-carveol mixture (with lower t_R in HPLC and higher GC- t_R) turned out to be *trans*-(1*S*,5*R*)-diastereomer and the major component – *cis*-(1*R*,5*R*)-isomer (with higher t_R in HPLC and lower GC- t_R).

cis-(1*R*,5*R*)-(–)-Carveol: ¹H-NMR (600 MHz, CDCl₃): δ = 1.49 (dt, J = 12.2 and 9.5 Hz, 1 H, H^{6a}), 1.52 (br. s, 1 H, OH), 1.72 (t, J = 1.1 Hz, 3 H, CH₃-C²), 1.74 (m, 3 H, CH₃C=), 1.93 (m, 1 H, H^{4a}), 2.05 (m, 1 H, H^{4c}), 2.14 (ddt, J = 12.3, 5.9 and 2.3 Hz, 1 H, H^{6c}), 2.25 (dddd, J = 12.5, 10.7, 5.1 and 2.6 Hz, 1 H, H^{5a}), 4.17 (m, $\Delta\nu_{1/2}$ = 20 Hz, 1 H, H^{1a}), 4.71 (s, 2 H, CH₂=), 5.48 (m, 1 H, H³) ppm.

trans-(1*S*,5*R*)-(-)-Carveol: $^1\text{H-NMR}$ (600 MHz, CDCl_3): $\delta = 1.52$ (br. s, 1 H, OH), 1.58 (dt, $J = 13.0$ and 4.1 Hz, 1 H, H^{6a}), 1.73 (s, 3 H, $\text{CH}_3\text{-C}^2$), 1.78 (br. s, 3 H, $\text{CH}_3\text{C=}$), 1.84 (m, 1 H, H^{4a}), 1.92 (dq, $J = 13.6$ and 2.0 Hz, 1 H, H^{6e}), 2.12 (dt, $J = 17.5$ and 5.2 Hz, 1 H, H^{4e}), 2.31 (br. t, $J = 12.8$ Hz, 1 H, H^{5a}), 4.00 (br. s, $\Delta\nu_{1/2} = 9$ Hz, 1 H, H^{1e}), 4.71 (s, 1 H, $\text{CH}_2\text{=}$), 4.73 (s, 1 H, $\text{CH}_2\text{=}$), 5.57 (dt, $J = 5.4$ and 1.8 Hz, 1 H, H^3) ppm.

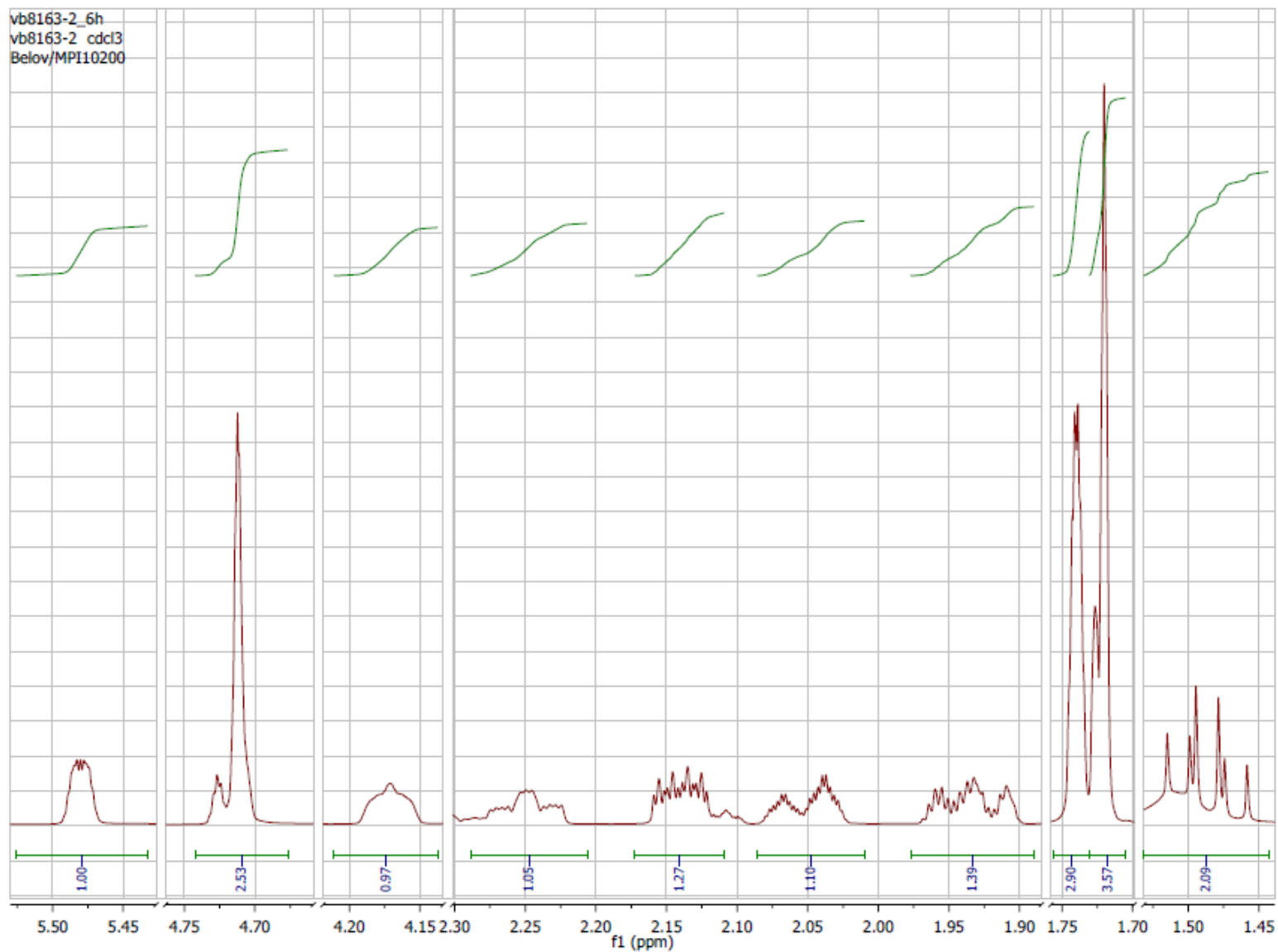


Figure S-2 600 MHz $^1\text{H-NMR}$ spectrum of *cis*-(1*R*,5*R*)-(-)-carveol in CDCl_3

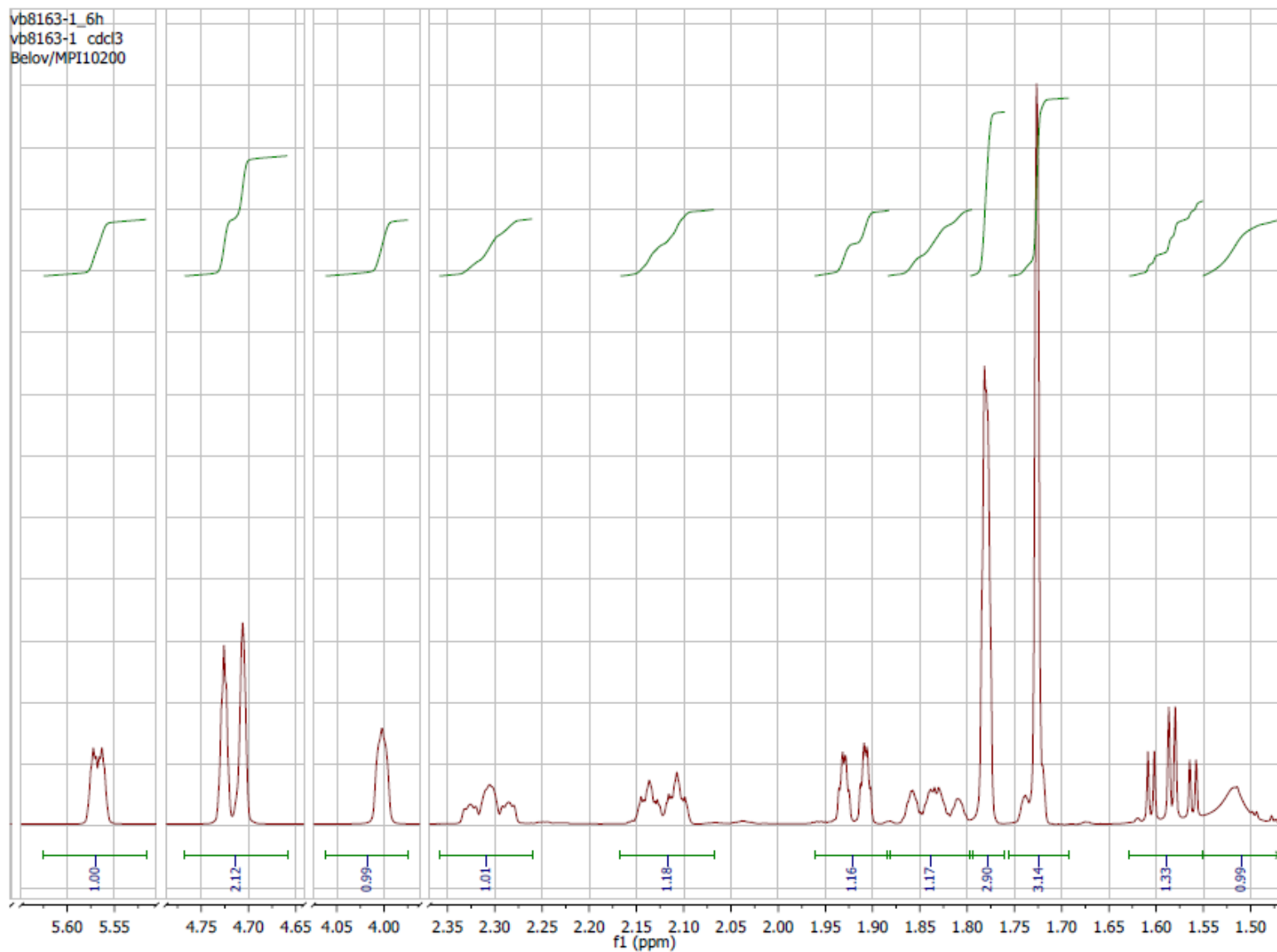
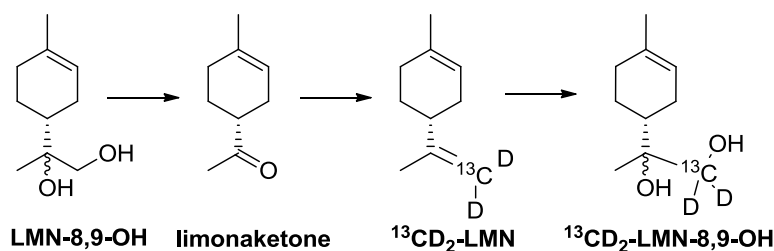


Figure S-3 600 MHz ^1H -NMR spectrum of *trans*-(1*S*,5*R*)-(-)-carveol in CDCl_3

Synthesis of limonene-8,9-diol and $^{13}\text{CD}_2$ -limonene-8,9-diol

Limonene-8,9-diol (2-[(1*R*)-4-methyl-3-cyclohexen-1-yl]-1,2-propanediol; 1:1 mixture of 2 diastereomers, uroterpenol, (4*R*)-*p*-menth-1-ene-8,9-diol; LMN-8,9-OH) and (4*R*)-acetyl-1-methylcyclohexene (limonaketone) were prepared from (*R*)-(+)-limonene essentially as described by Dean et al. (1967) [4]. LMN-8,9-OH was purified by chromatography (SiO_2 , ether) and isolated as an oil. ESI-MS ($\text{C}_{10}\text{H}_{18}\text{O}_2$ [$M=170$], positive mode): m/z (rel. int. %) = 363 (100) [$2M+\text{Na}$] $^+$, 193 (78) [$M+\text{Na}$] $^+$; HRMS: 193.1201 (found for $M+\text{Na}$), 193.1199 (calc.). $^1\text{H-NMR}$ (300 MHz, CDCl_3 , 1:1 mixture of 2 diastereomers): δ = 1.07/1.11 (two singlets, total 3 H, CH_3), 1.16–1.39 (m, 1 H, H^4), 1.62 (br. s, 3 H, $\text{CH}_3\text{-C=}$), 1.66–1.80 (m, 1 H), 1.80–2.13 (m, 7 H), 3.39/3.43 (two A-parts of AB-system, $J_{\text{AB}} = 12.6$ Hz, total 1 H, $\text{CH}^{\text{A}}\text{H}^{\text{B}}\text{OH}$), 3.54/3.58 (two B-parts of AB-system, $J_{\text{AB}} = 12.5$ Hz, total 1 H, $\text{CH}^{\text{A}}\text{H}^{\text{B}}\text{OH}$), 5.29–5.44 (m, 1 H, CH=) ppm.

Limonaketone was synthesized from limonene-8,9-diol (10 mmol) by oxidation with NaIO_4 (11 mmol) [4]. Saturated aq. solution of NaIO_4 was added to the stirred warm solution of limonene-8,9-diol (1.7 g) in THF–water mixture (1:1, 100 mL). After stirring for 20 min at room temperature, the reaction mixture was extracted with hexane (2 x 200 mL). Organic solutions were combined, washed with brine, dried (MgSO_4) and evaporated in vacuo. Limonaketone was isolated from the residue by chromatography on SiO_2 (100 g; elution with hexane – ether mixture) and distilled in Kugelrohr: b.p. $\sim 80^\circ\text{C}$ at 10 Torr; yield 0.8 g (58%).

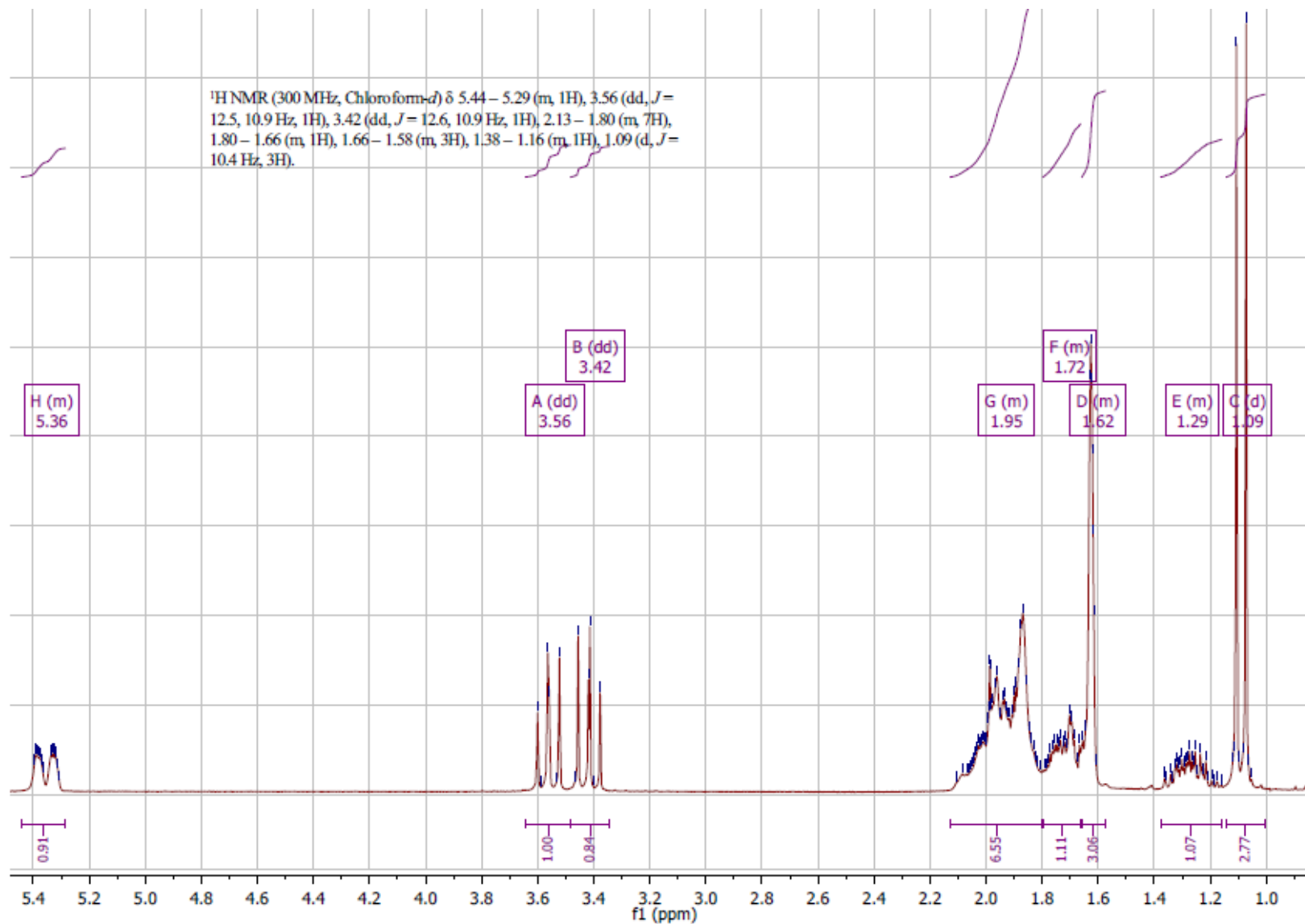


Figure S-4 300 MHz ¹H-NMR spectrum of limonene-8,9-diol (2-[(1*R*)-4-methyl-3-cyclohexen-1-yl]-1,2-propanediol; 1:1 mixture of 2 diastereomers) in CDCl₃

[9-¹³C,²H₂]-(*R*)-(+)-limonene was synthesized from [¹³C,²H₃]-methyl triphenylphosphonium iodide (2.0 g, 4.9 mmol) and limonaketone (550 mg, 4.0 mmol) in THF (120 mL) using 1.6 M *n*BuLi in hexane (3.1 mL, 5.0 mmol) [5]. THF was distilled-off from the reaction mixture using Vigreux-column at 400 mbar (bath tem. – 60 °C), the residue was taken-up with pentane, filtered, and the filtrate was passed through a short pad of silica gel (to remove Ph₃PO). The filter-cake was washed with pentane; fractions containing [9-¹³C,²H₂]-(*R*)-(+)-limonene (TLC in pentane) were combined and evaporated in vacuo (200 mbar, room temp. – 45°C) to yield 390 mg of the residue (with ca. 10% of hexane according to ¹H NMR; yield 65%). [9-¹³C,²H₂]-(*R*)-(+)-Limonene was used for the preparation of ¹³CD₂-LMN-8,9-OH according to the method of Dean et al. (1967) [4].

¹³CD₂-LMN-8,9-OH was purified by chromatography (SiO₂, ether) and isolated as an oil. GC-MS: *t*_R = 7.73 min (100%); EI-MS: *m/z* (rel. int. %) = 155 (28) [M-H₂O]⁺, 139 (44) [M-2xOH]⁺, 121 (100) [M-H₂O-¹³CD₂OH]⁺. ESI-MS (C₉¹³CH₁₆D₂O₂ [M=173], positive mode): *m/z* (rel. int. %) = 196 (100) [M+Na]⁺, isotopic purity 81% . ¹H-NMR (300 MHz, CDCl₃, 1:1 mixture of 2 diastereomers): δ = 1.09/1.13 (two doublets, ³J(¹³C-H) = 4.6 Hz, total 3 H, CH₃), 1.22–1.37 (m, 1 H, H⁴), 1.65 (br. s, 3 H, CH₃-C=), 1.66–1.80 (m, 1 H), 1.82 (s, 2 H, OH), 1.87–2.13 (m, 5 H), 5.29–5.44 (m, 1 H, CH=) ppm. ¹H-NMR Spectrum (400 MHz, CDCl₃) is given in Figure S-7.

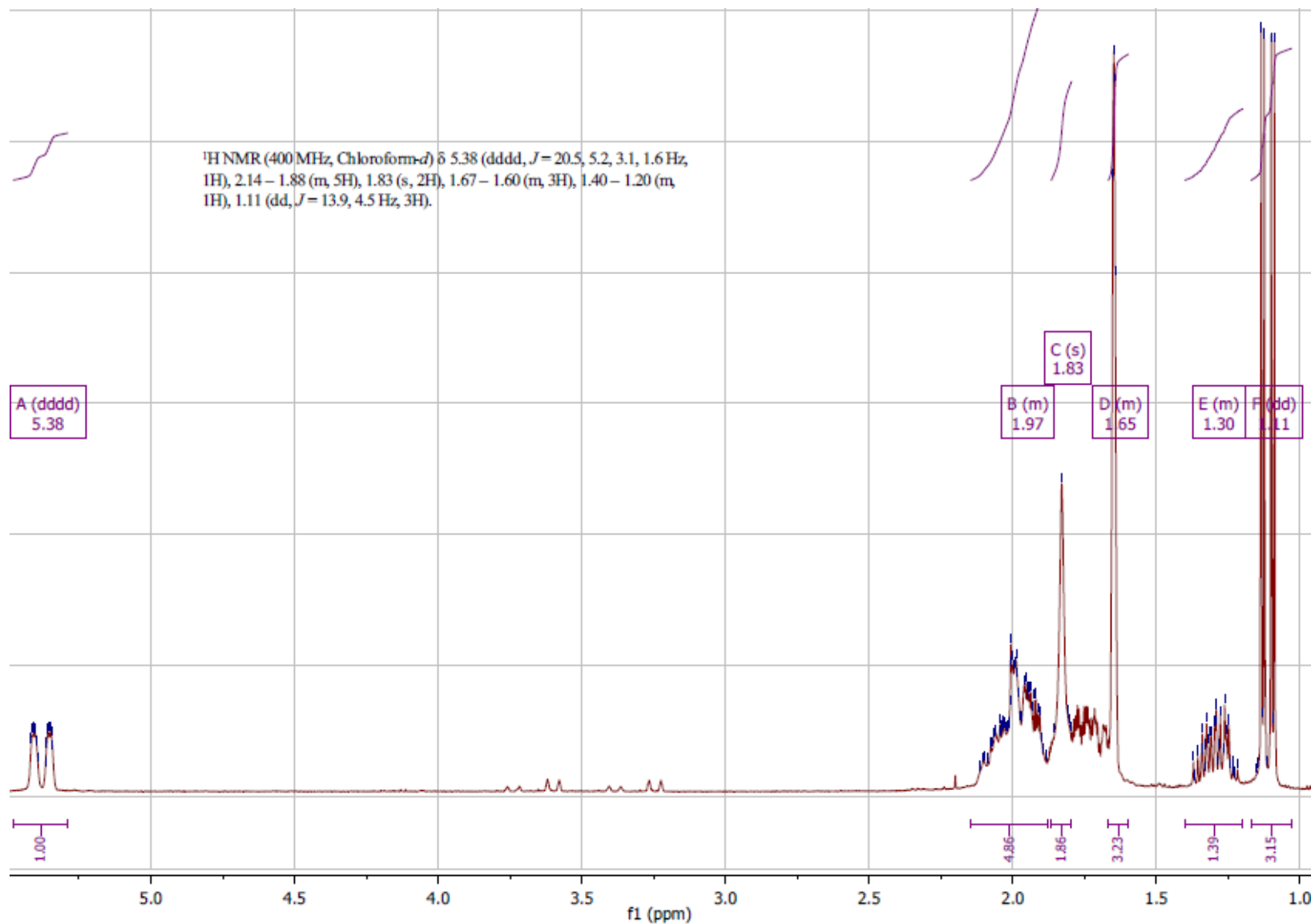
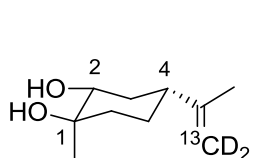
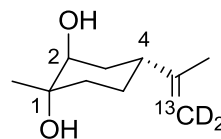


Figure S-4 400 MHz ^1H -NMR spectrum of $^{13}\text{C}_2$ -limonene-8,9-diol ($^{13}\text{C}_2$ -LMN-8,9-OH) in CDCl_3

Synthesis of $^{13}\text{CD}_2$ -(1*R*,2*R*,4*R*)- and $^{13}\text{CD}_2$ -(1*S*,2*S*,4*R*)-limonene-1,2-diol



$[^{13}\text{CD}_2]$ -(1*R*,2*R*,4*R*)-limonene-1,2-diol



$[^{13}\text{CD}_2]$ -(1*S*,2*S*,4*R*)-limonene-1,2-diol

$[9\text{-}^{13}\text{C},^2\text{H}_2]$ -(*R*)-(+)-Limonene was also converted into the diastereomeric mixture of *cis*- and *trans*-1,2-epoxides [6] which was not separated, but used for the synthesis of the individual $^{13}\text{CD}_2$ -(1*R*,2*R*,4*R*)- and $^{13}\text{CD}_2$ -(1*S*,2*S*,4*R*)-limonene-1,2-diols [$^{13}\text{CD}_2$ -(1*R*,2*R*)- and $^{13}\text{CD}_2$ -(1*S*,2*S*)-LMN-1,2-OH]. Under neutral conditions and in the presence of HgCl_2 , (1*R*,2*R*,4*R*)-limonene-1,2-diol is selectively formed in aqueous solutions from a diastereomeric mixture of 1,2-epoxides (structures not shown); reduction with NaBH_4 removes Hg(II) from the intermediate complex and completes this reaction [7, 8]. Another diastereomer of 1,2-epoxy-(4*R*)-limonene was isolated and used for the preparation of (1*S*,2*S*,4*R*)-limonene-1,2-diol which turned out to be identical (in respect of retention time) to commercially available (+)-(1*S*,2*S*,4*R*)-limonene-1,2-diol (Sigma-Aldrich). For that, acid-catalysed ring-opening reaction was used (diluted H_2SO_4 in aq. THF) [9].

$^{13}\text{CD}_2$ -(1*R*,2*R*,4*R*)-limonene-1,2-diol: white solid with m. p. 63°C , $[\alpha]_{\text{D}}^{20} = -4.1^\circ$ ($c = 0.1$), CHCl_3). ESI-MS ($\text{C}_9^{13}\text{CH}_{16}\text{D}_2\text{O}_2$ [$M = 173$], positive mode): m/z (rel. int. %) = 196 (100) [$M + \text{Na}$] $^+$, isotopic purity 93%. $^1\text{H-NMR}$ (600 MHz, CDCl_3): $\delta = 1.00$ (s, 3 H, $\text{CH}_3\text{-C}^1$), 1.09–1.16 (m, 2 H, $\text{H}^{3a} + \text{H}^{6a}$), 1.30 (td, $J = 13.4$ and 4.0 Hz, H^{6e} , 1 H), 1.48–1.53 (m, 1 H, H^5), 1.55 (d, 3 H, $^3J(^{13}\text{C-H}) = 5.8$ Hz, $\text{CH}_3\text{-C=}$), 1.59 (td, $J = 13.6$ and 3.6 Hz, H^5 , 1 H), 1.71 (ddd, $J = 12.8$, 4.6 , and 3.5 Hz, H^{3e} , 1 H), 1.88 (tt, $J = 12.3$ and 4.1 Hz, H^{4e} , 1 H), 3.36 (dd, $J = 12.0$ and 4.3 Hz, H^{2a} , 1 H), 3.97 (s, 2 H, OH) ppm. $^1\text{H-NMR}$ Spectrum (600 MHz, CDCl_3) is given in Figure S-8.

$^{13}\text{CD}_2$ -(1*S*,2*S*,4*R*)- limonene-1,2-diol: white solid with m. p. 70°C, $[\alpha]_{\text{D}}^{20} = +18^\circ$ ($c=0.1$), CHCl_3). ESI-MS ($\text{C}_9^{13}\text{CH}_{16}\text{D}_2\text{O}_2$ [$M=173$], positive mode): m/z (rel. int. %) = 196 (100) $[\text{M}+\text{Na}]^+$, isotopic purity 93%. $^1\text{H-NMR}$ (600 MHz, CDCl_3): $\delta = 1.12$ (s, 3 H, $\text{CH}_3\text{-C}^1$), 1.38–1.45 (m, 2 H, H^5+H^6), 1.45–1.51 (m, 1 H, H^5/H^6), 1.55 (td, $J = 13.7$ and 3.9 Hz, H^{3e} , 1 H), 1.58–1.64 (m, 1 H, H^6/H^5), 1.62 (d, 3 H, $^3J(^{13}\text{C-H}) = 5.8$ Hz, $\text{CH}_3\text{-C}=\text{C}$), 1.80 (ddd, $J = 14.3$, 11.7 and 2.8 Hz, H^{3a} , 1 H), 2.16 (tq, $J = 11.8$ and 4.1 Hz, H^{4e} , 1 H), 3.36 (m, $J = 4.2$ and 2.9 Hz, H^{23} , 1 H), 3.46 (m, 2 H, OH) ppm. $^1\text{H-NMR}$ Spectrum (600 MHz, CDCl_3) is given in Figure S-9.

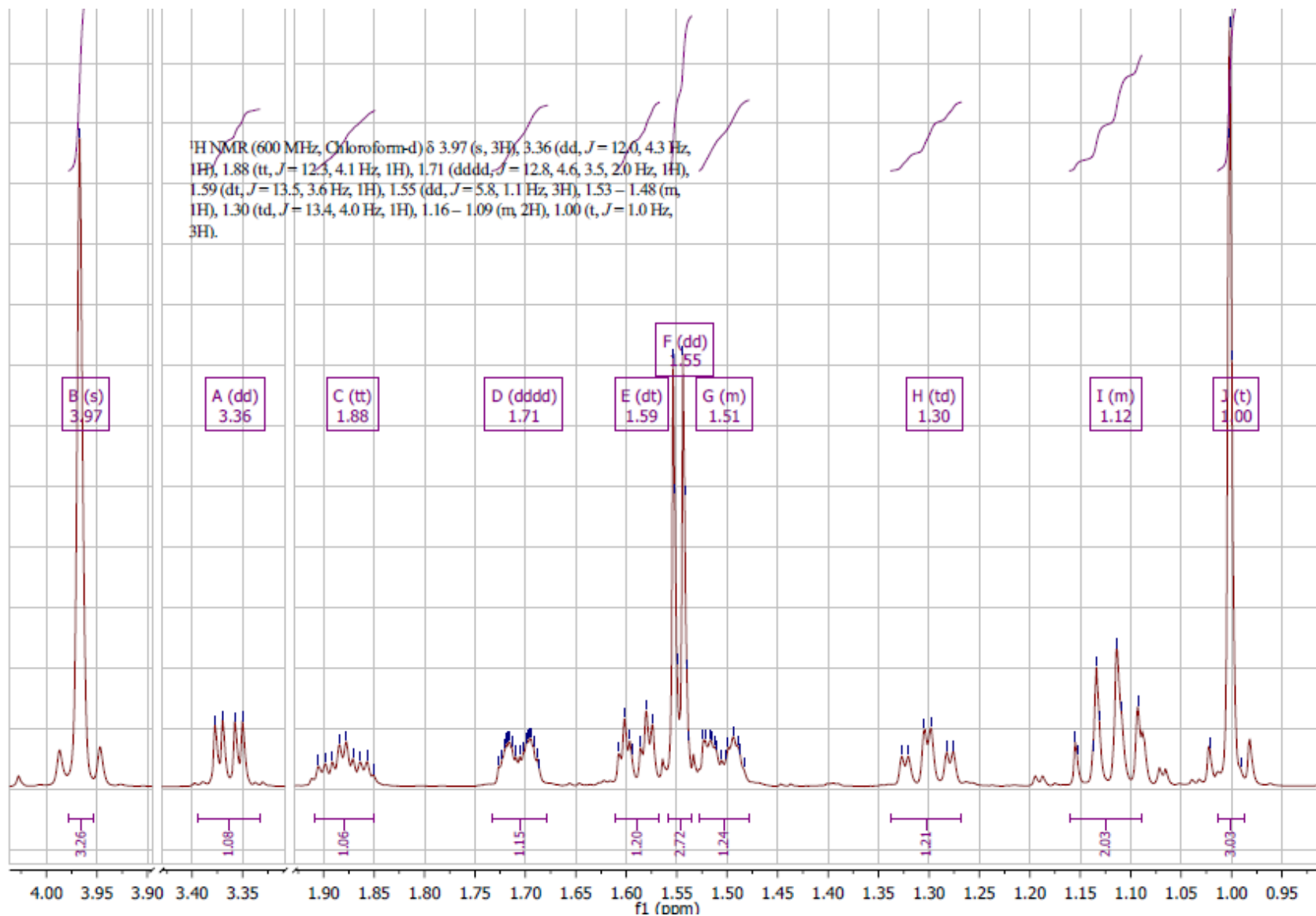


Figure S-6 ¹H NMR Spectrum (600 MHz, CDCl₃) of ¹³CD₂-(1R,2R,4R)-limonene-1,2-diol in CDCl₃

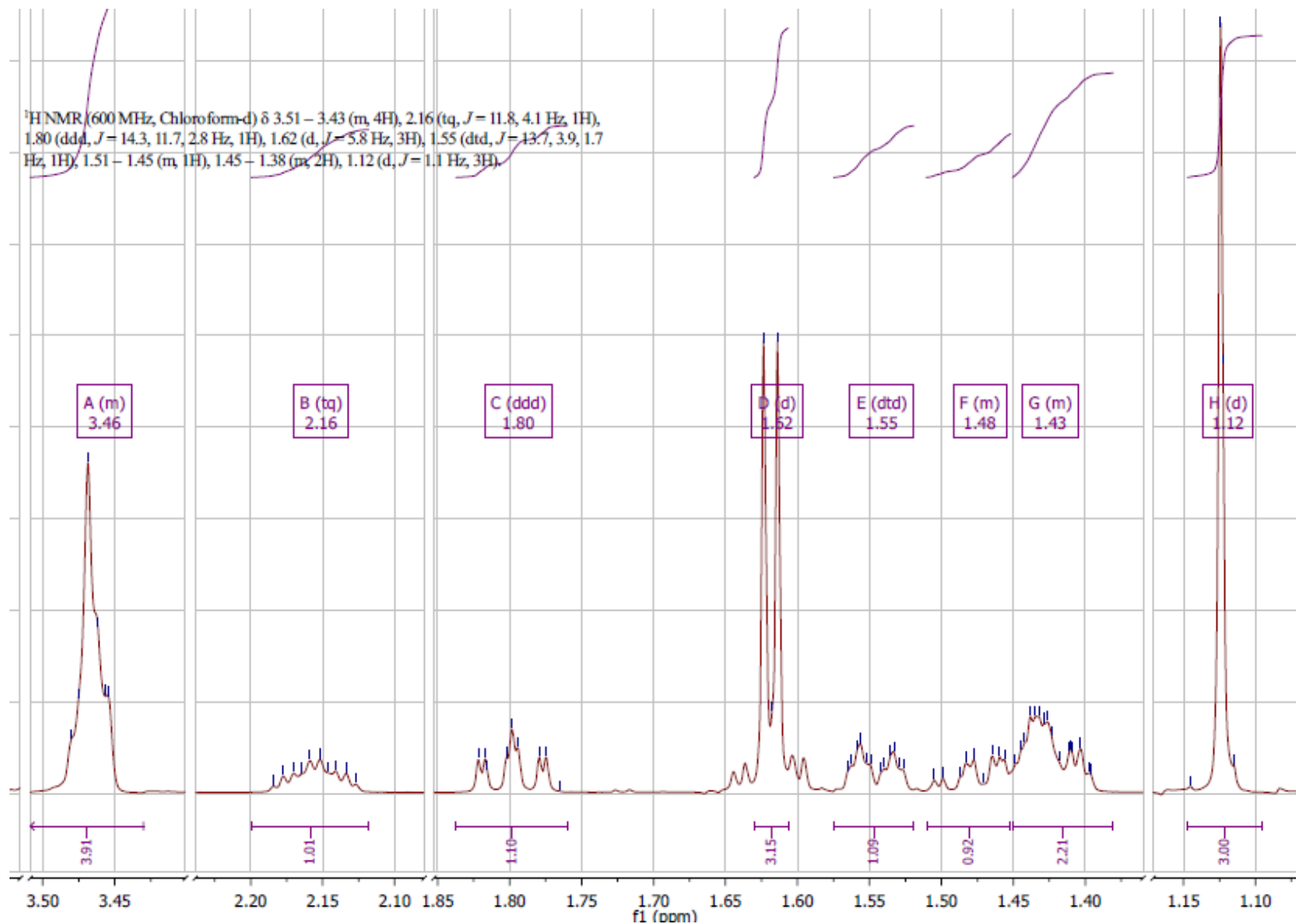
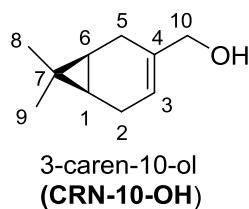


Figure S-7 ¹H NMR Spectrum (600 MHz, CDCl₃) of ¹³CD₂-(1*S*,2*S*,4*R*)-limonene-1,2-diol in CDCl₃

Synthesis of 3-carene-10-ol

3-carene-10-ol (CRN-10-OH) was prepared from (+)- Δ^3 -carene according to the known methods [10, 11]. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ = 0.61–0.73 (m, 1 H, H^1/H^6), 0.76 (s, 3 H, H^8/H^9), 0.75–0.79 (m, 1 H, H^6/H^1), 1.04 (s, 3 H, H^9/H^8), 1.38 (br. s, 1 H, OH), 1.85–2.08 (m, 2 H, H^2/H^5), 2.27–2.48 (m, 2 H, H^5/H^2), 3.97 (br. s, 2 H, H^{10}), 5.56 (m, 1 H, H^3) ppm. $^1\text{H-NMR}$ spectrum (400 MHz, CDCl_3) is given in Figure S-10.

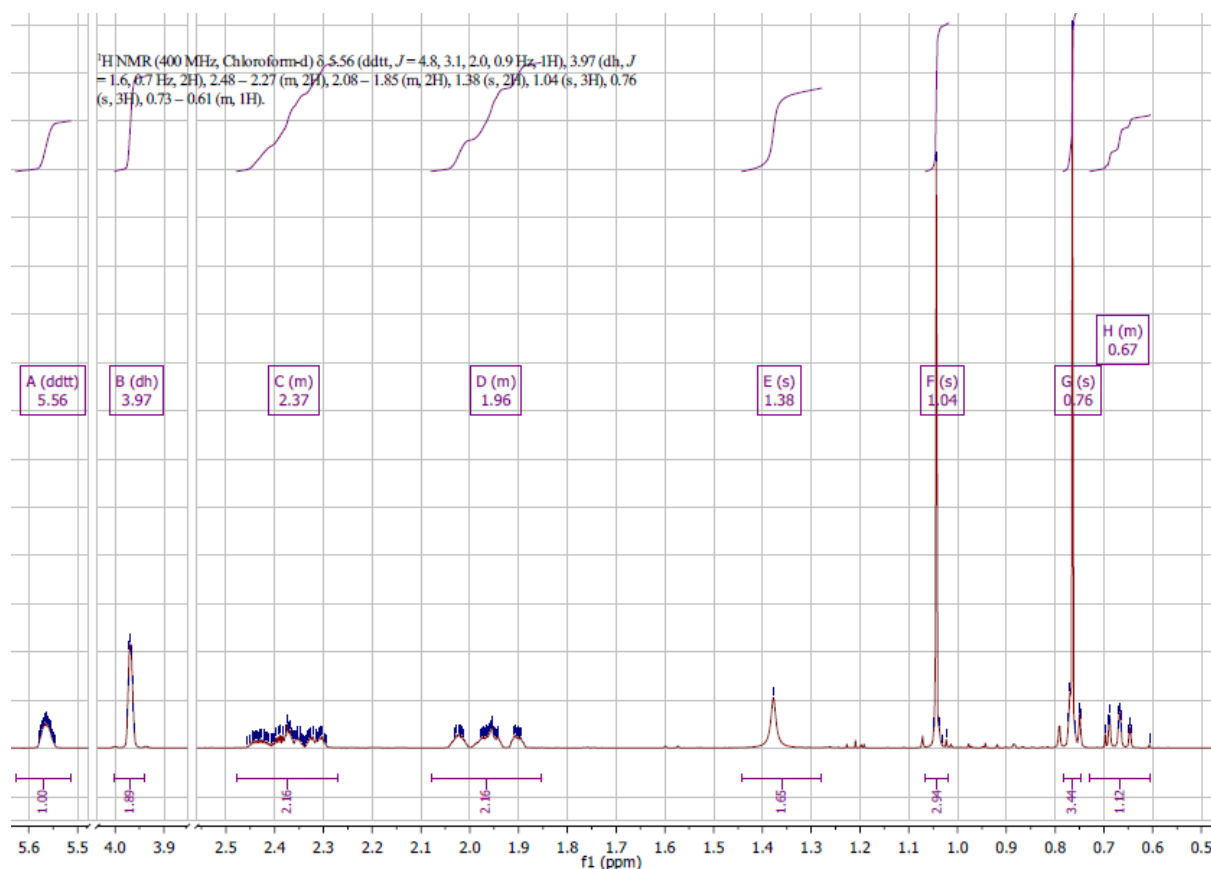


Figure S-8 ^1H NMR Spectrum (400 MHz, CDCl_3) of 3-carene-10-ol (CRN-10-OH) in CDCl_3

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