Supplementary Information:

Labelling Studies on the Biosynthesis of Terpenes in *Fusarium fujikuroi*

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I. General Information

Chemicals were obtained from Acros Organics (Geel, Belgium) or Sigma Aldrich Chemie GmbH (Steinheim, Germany) and used without purification. All reactions were performed under an inert atmosphere (N_2) in flame-dried flasks. Solvents were purified by distillation and dried according to standard methods. Thin-layer chromatography was performed with 0.2 mm precoated plastic sheets Polygram® Sil G/UV254 (Machery-Nagel). Column chromatography was carried out using Merck silica gel 60 (70-200 mesh). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AV II-300 (300 MHz), DRX-400 (400 MHz), AV III-400 (400 MHz) or AV II-600 (600 MHz) spectrometers, and were referenced against TMS (δ = 0.00 ppm) for ^{1}H-NMR and $CDCl_3$ (δ = 77.01 ppm) for ^{13}C NMR. For measurements in [$^2H_6$]DMSO it was referenced against TMS (δ = 0.00 ppm) for ^{1}H NMR and [$^2H_6$]DMSO (δ = 39.51 ppm) for ^{13}C NMR. IR spectra were recorded with a Bruker Tensor 27 ATR (attenuated total reflectance). GC-MS analyses were carried out with an HP6890 gas chromatograph connected to an HP5973 mass selective detector fitted with a BPX-5 fused silica capillary column (25 m, 0.25 mm i. d., 0.25 µm film). Instrumental parameters were (1) inlet pressure, 77.1 kPa, He 23.3 mL min^{-1}, (2) injection volume, 2 µL, (3) transfer line, 300 °C, and (4) electron energy 70 eV. The GC was programmed as follows: 5 min at 50 °C increasing at 10 °C min^{-1} to 320 °C, and operated in split mode (20:1, 60 s valve time). The carrier gas was He at 1 mL min^{-1}. Retention indices (*I*) were determined from a homologous series of *n*-alkanes (C_8-C_{38}).

II. Culture Conditions

For the analyses of volatile terpenes cultures of *Fusarium fujikuroi* IMI58289 were grown on CM-agar amended with a 2 mM concentration of [2-^{13}C]mevalonolactone for 5 d at 28 °C and then analysed by CLSA.[1] In order to gain larger amounts of material for NMR analysis overexpression strains of *Fusarium* were used that have been reported in a previous work.[2] In particular, for the collection of α-acorenol the mutant strain SC6 was used, while for collection of koraiol SC4 was cultivated. For the extraction of cyclonerodiol and gibberellins 5 pieces of a CM agar plate were used to inoculate a Darken preculture that was grown for 3 d at 28 °C. For analysis of cyclonerodiol, 500 µL of the preculture were transferred into 10% ICI medium (50 mL) containing 2 mM [2-^{13}C]mevalonolactone, and cultivation was extended for 6 d prior to filtration and extraction with hexane. For gibberellin analysis, 300 µL of the preculture were transferred into 10% ICI medium (30 mL) and grown for 6 d at 28 °C on a rotary shaker prior to filtration and extraction with ethyl acetate. After one and two days of cultivation, 2 mM [2-^{13}C]mevalonolactone were added to the culture. The organic layers were dried and the solvent was removed under reduced pressure followed by NMR analysis.

CM medium: salt solution (50 mL), trace element solution (1 mL), vitamin solution (1 mL), yeast extract (1.0 g), peptone (2.0 g), casamino acids (1.0 g), glucose (10 g), agar (15 g), H_2O (950 mL).

Salt solution for CM: KCl (10.4 g), $MgSO_4$ x 7 H_2O (10.4 g), KH_2PO_4 (30.4 g), H_2O (1000 mL).

Trace element solution for CM: $FeSO_4$ x 7 H_2O (10 g), $MgSO_4$ x 7 H_2O (50 g), H_2O (1000 mL).

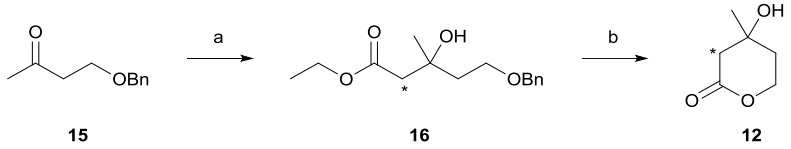
Vitamin solution for CM: biotin (500 mg), nicotinic acid (50 g), *p*-aminobenzoic acid (16 g), pyridoxal hydrochloride (20 g), H_2O (1000 mL).

Darken preculture: saccharose (30 g), corn steep solids (15 g), $CaCO_3$ (7.0 g), $(NH_4)_2SO_4$ (0.5 g), H_2O (1000 mL).

10% ICI medium: glucose (80 g), $MgSO_4$ x 7 H_2O (1.0 g), KH_2PO_4 (0.5 g), NH_4NO_3 (0.5 g), trace element solution (2 mL), H_2O (998 mL).

Trace element solution for ICI: H_3BO_3 (2.9 g), $MnCl_2$ x 4 H_2O (1.8 g), $ZnSO_4$ x 7 H_2O (222 mg), $Na_2Mo_2O_7$ x 2 H_2O (79 mg), $Co(NO_3)_2$ x 6 H_2O (49 mg), H_2O (100 mL).

III. Synthesis of [2-^{13}C]mevalonolactone



15 **16** **12**

Scheme 1 Synthesis of [2-^{13}C]mevalonolactone (**12**): a) LDA, [2-^{13}C]ethyl acetate, -78 °C, THF, 89%; b) H_2, Pd/C, 40 bar, 40 °C, methanol, 71%.

The synthesis of [2-^{13}C]mevalonolactone (**12**) was carried out as outlined in Scheme 1 in a procedure that is very similar to previously reported approaches by Tanabe.[3,4] Starting from ketone **15**[5] an aldol addition of [2-^{13}C]ethyl acetate gave **16** in high yield. Subsequent deprotection under H_2 atmosphere gave **12** after filtration from the catalyst.

Synthesis of [2-^{13}C]ethyl 5-(benzyloxy)-3-hydroxy-3-methylpentanoate (16): A solution of diisopropylamine (574 mg, 5.7 mmol, 2.1 eq.) in 25 mL of dry THF was cooled to 0 °C and treated with a 1.6 M solution of *n*-BuLi (3.6 mL, 5.7 mmol, 2.1 eq.) in hexane. After stirring for 1 h at 0 °C it was cooled to -78 °C and [2-^{13}C]ethyl acetate (500 mg, 5.7 mmol, 2.1 eq.) was added in 10 mL of dry THF dropwise. Stirring was continued for 30 min, then ketone **15**[5] (482 mg, 2.7 mmol, 1.0 eq.) was added in 6 mL dry THF. The reaction mixture was stirred for further 45 min at -78 °C and 45 min at room temperature. It was quenched by the addition of distilled water and extracted three times with ethyl acetate. The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. Column chromatography with hexane/ethyl acetate (5:1) yielded **16** (641 mg, 2.4 mmol, 89%) as colourless oil.[6]

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.36 (m, 5H), 4.50 (s, 2H), 4.17 – 4.09 (m, 2H), 3.68 (ddd, *J* = 6.3, 1.1 Hz, 2H), 2.70 (dd, *J* = 33.0, 15.3 Hz, 1H), 2.37 (dd, *J* = 33.0, 15.0 Hz, 1H), 1.93 – 1.89 (m, 2H), 1.29 (d, 4.2 Hz, 3H), 1.25 (t, *J* = 7.2 Hz, 3H) ppm.
^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.4 (d, *J* = 56.6 Hz, CO), 137.9 (C_q), 128.3 (2x CH), 127.6 (CH), 127.5 (2x CH), 73.2 (CH_2), 70.7 (d, *J* = 37.4, C_q), 66.9 (d, *J* = 2.2 Hz, CH_2), 60.4 (CH_2), 45.5 ($^{13}CH_2$), 45.0 (d, *J* = 17.8 Hz, CH_2), 27.2 (d, *J* = 17.8 Hz, CH_3), 14.1 (CH_3) ppm.
MS (EI, 70 eV): *m*/*z* (%) = 267 (<1) $[M]^+$, 234 (1), 179 (1), 160 (15), 143 (8), 132 (7), 107 (7), 91 (100), 77 (17), 65 (20), 43 (38).
GC (BPX-5): *I* = 1909.
IR (ATR): $\tilde{\nu}$ = 3499 (br), 3064 (w), 2977 (w), 1715 (s), 1454 (m), 1369 (m), 1325 (m), 1192 (s), 1096 (s), 1027 (s), 698 (s) cm^{-1}.

Synthesis of [2-^{13}C]mevalonolactone (12): Compound **16** (481 mg, 1.8 mmol, 1.0 eq.) was dissolved in 20 mL methanol and treated with 5% Pd/C (200 mg, 0.1 mmol, 0.05 eq.). The reaction mixture was stirred for 2 h under an atmosphere of H_2 (40 bar) at 40 °C. After cooling to room temperature it was filtered over a pad of silica and concentrated under reduced pressure. The residue was taken up into 20 mL of CH_2Cl_2. A catalytic amount of *p*-TsOH was added and it was stirred over night at room temperature. Another silica gel filtration and removal of the solvent gave **12** (170 mg, 1.3 mmol, 71%) as colourless oil.

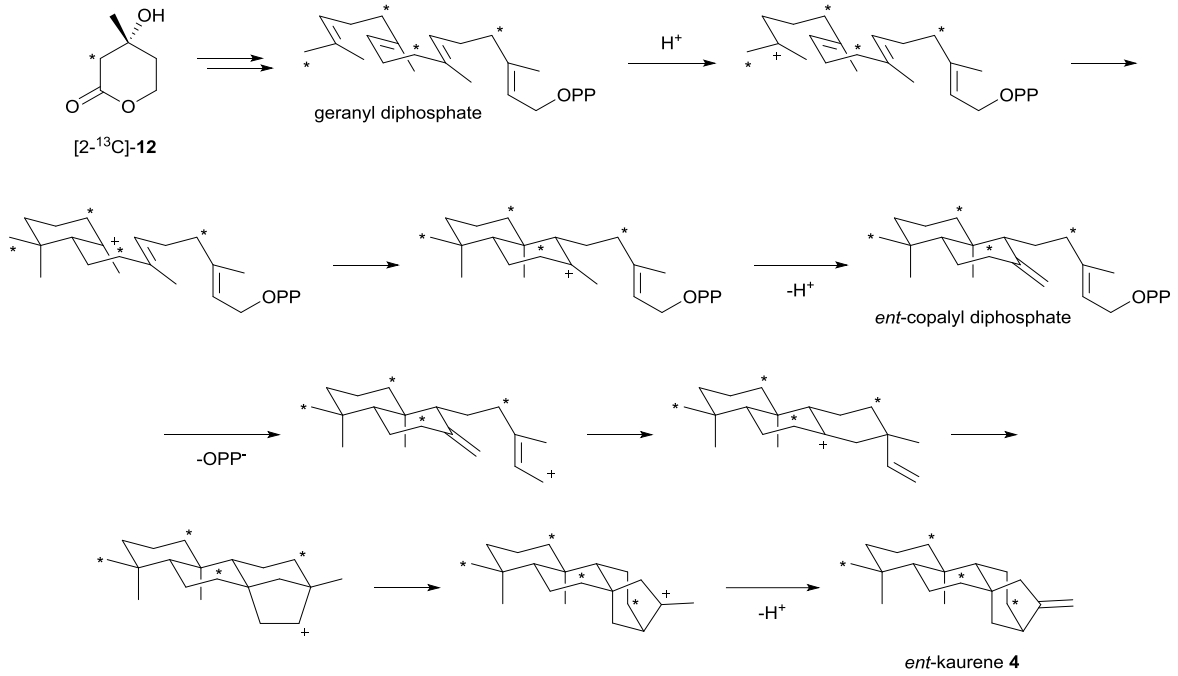
^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.61 (ddd, *J* = 11.3, 8.6, 5.9 Hz, 1H), 4.36 (ddd, *J* = 11.3, 9.1, 4.6 Hz, 1H), 2.86 – 2.65 (m, 1H), 2.52 – 2.33 (m, 1H), 1.94 – 1.89 (m, 2H), 1.40 (d, *J* = 4.4 Hz, 3H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.9 (d, J = 50.2 Hz, CO), 68.0 (d, J = 39.1 Hz, C_q), 66.1 (d, J = 1.7 Hz, CH_2), 44.6 ($^{13}CH_2$), 36.0 (d, J = 19.3 Hz, CH_2), 27.2 (t, J = 2.0 Hz, CH_3) ppm.
MS (EI, 70 eV, MSTFA): m/z (%) = 202 (<1) $[M]^+$, 188 (16), 145 (100), 116 (48), 101 (15), 83 (18), 75 (70), 45 (32).
GC (BPX-5, MSTFA): I = 1385.
IR (ATR): $\tilde{\nu}$ = 3418 (br), 2972 (w), 2929 (w), 1702 (s), 1457 (w), 1397 (m), 1262 (s), 1228 (s), 1126 (s), 1068 (s), 1023 (m) cm^{-1}.

Synthesis of α-cedrene (14): A CLSA extract obtained from *Fusarium fujikuroi* SC4 containing ^{13}C-labelled α-acorenol after feeding of [2-^{13}C]mevalonolactone was concentrated with a gentle stream of nitrogen. The residue was stirred in 88% aqueous formic acid for 10 min. The resulting product was extracted with 1 mL of $CDCl_3$, dried over $MgSO_4$ and directly subjected to NMR analysis.[7]

^{13}C NMR (150 MHz, $CDCl_3$): δ = 119.2 (CH), 36.1 (CH_2), 27.6 (CH_3, major peak, ca. 90%), 25.6 (CH_3, minor peak, ca. 10%) ppm.

IV. Biosynthesis of *ent*-kaurene



Scheme 2 Biosynthesis of labelled *ent*-kaurene **4** from [2-^{13}C]-**12**.

V. Literature

1 K. Grob and F. Zürcher, *J. Chromatogr.*, 1976, **117**, 285.

2 N. L. Brock, K. Huss, B. Tudzynski and J. S. Dickschat, *ChemBioChem*, 2013, **14**, 311.

3 J. A. Lawson, W. T. Colwell, J. I. De Graw, R. H. Peters, R. L. Dehn and M. Tanabe, *Synthesis*, 1975, 729.

4 M. Tanabe and R. H. Peters, *Org. Synth.*, 1981, **60**, 92.

5 J. S. Dickschat, C. A. Citron, N. L. Brock, R. Rîclea and H. Kuhz, *Eur. J. Org. Chem.*, 2011, 3339.

6 L. O. Zamir and C.-D. Nguyen, *J. Label. Compd. Radiopharm.*, 1988, **25**, 1189.

7 T. G. Randall and R. G. Lawton, *J. Am. Chem. Soc.*, 1969, **91**, 2127.

VI. NMR-Spectra

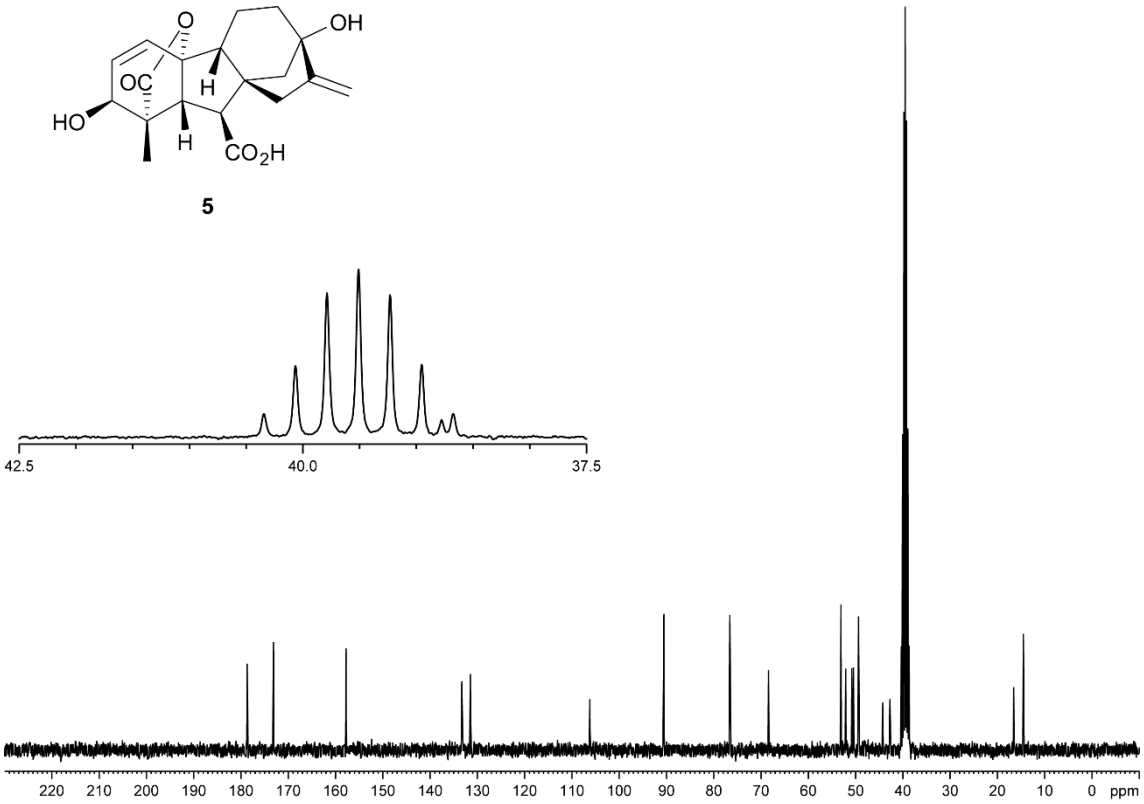


Figure 1 ^{13}C NMR spectrum of commercially available gibberellic acid (**5**) in [$^{2}H_6$]DMSO.

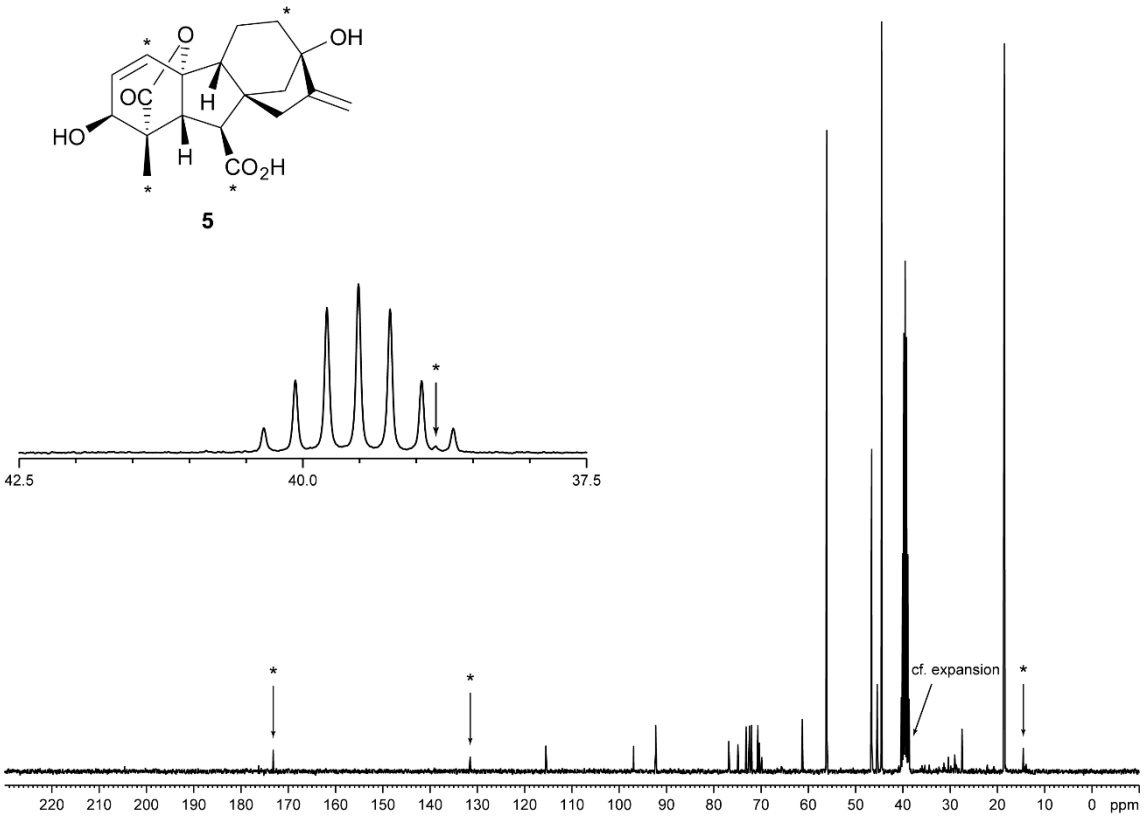


Figure 2 ^{13}C NMR spectrum of a crude ethyl acetate extract from *Fusarium fujikuroi* after feeding of [2-^{13}C]-**12**. Asterisks indicate ^{13}C-labelled carbons of GA_3 and arrows point to the respective enhanced ^{13}C signals due to incorporation of labelling.

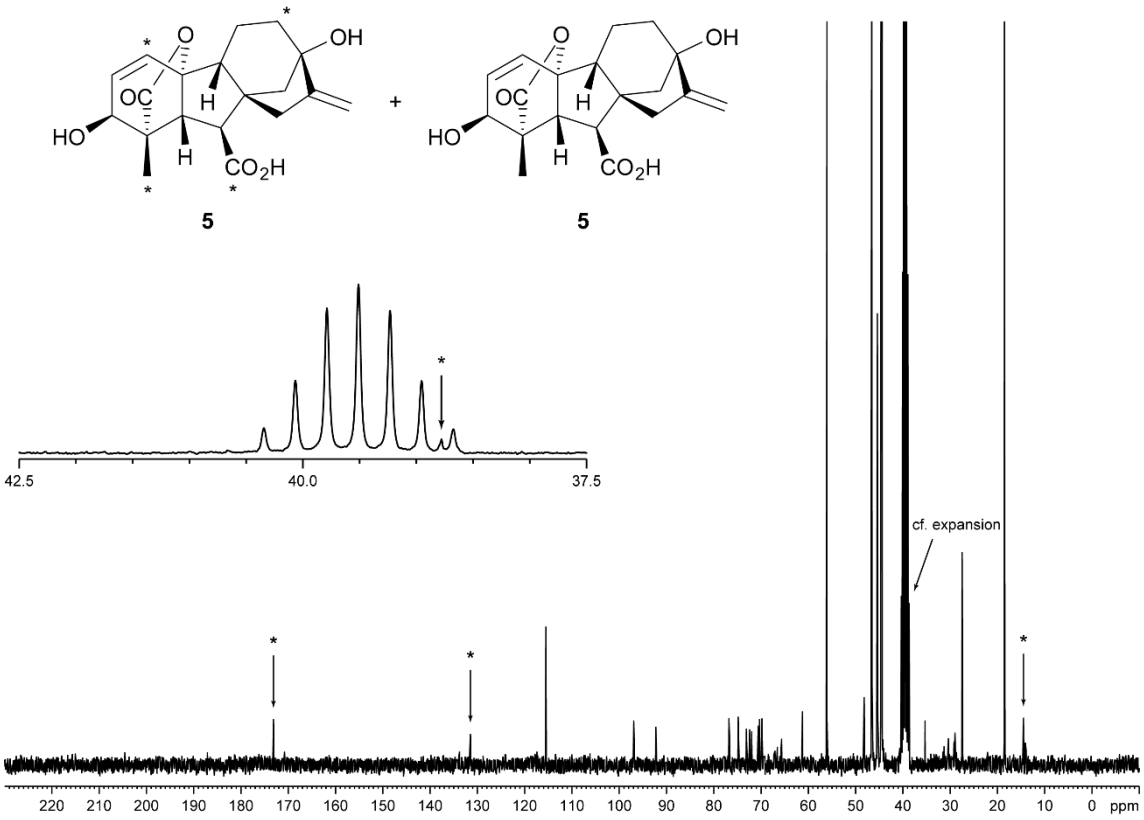


Figure 3 ^{13}C NMR spectrum of the crude ethyl acetate extract from *Fusarium fujikuroi* after feeding of [2-^{13}C]-**12** mixed with unlabelled gibberellic acid (**5**). Asterisks indicate ^{13}C-labelled carbons of GA_3 and arrows point to the respective enhanced ^{13}C signals due to incorporation of labelling.

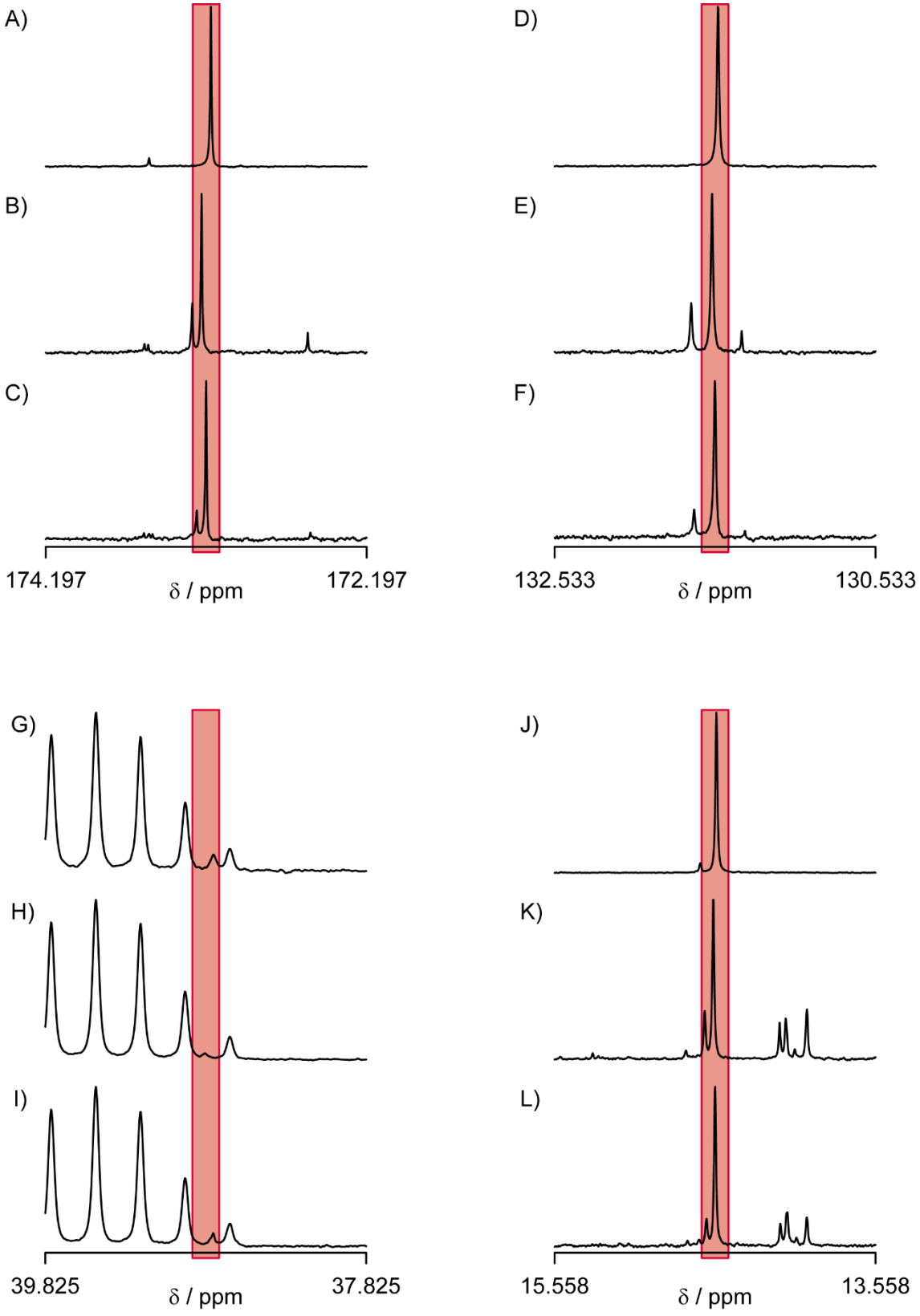


Figure 4 (A, D, G, J) Expansions of ^{13}C NMR spectrum of commercially available gibberellic acid (**5**); (B, E, H, K) Expansions of ^{13}C NMR spectrum of the crude ethyl acetate extract from *Fusarium fujikuroi* after feeding of [2-^{13}C]-**12**; (C, F, I, L) Expansions of ^{13}C NMR spectrum of the crude ethyl acetate extract from *Fusarium fujikuroi* after feeding of [2-^{13}C]-**12** mixed with unlabelled gibberellic acid (**5**). Relevant ^{13}C NMR peaks of carbons into which labelling from [2-^{13}C]-**12** was incorporated are shown in red boxes.

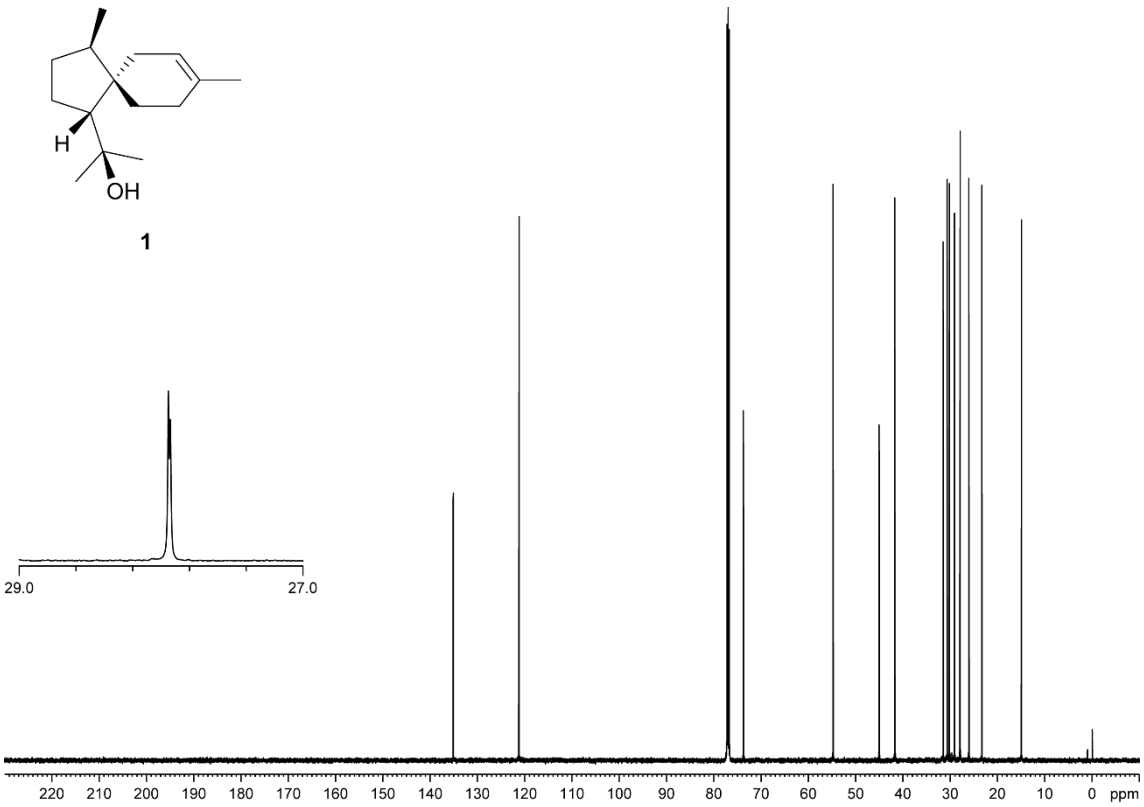


Figure 5 ^{13}C NMR spectrum of α-acorenol (**1**).

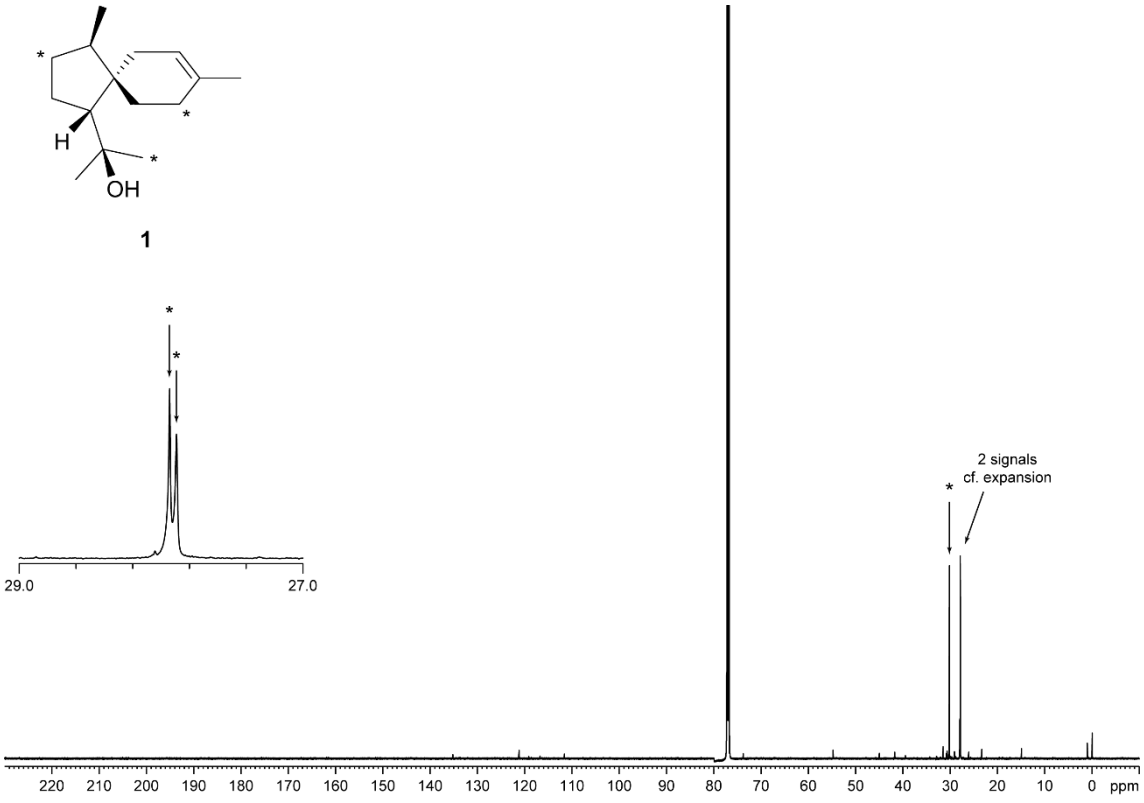


Figure 6 ^{13}C NMR spectrum of a crude CLSA headspace extract from *F. fujikuroi* SC6 after feeding of [2-^{13}C]-**12**. Asterisks indicate ^{13}C-labelled carbons of α-acorenol (**1**) and arrows point to the respective enhanced ^{13}C signals due to incorporation of labelling. GC-MS analysis of the headspace extract showed that labelled **1** was the main compound in the headspace extract that coeluted with an authentic standard.

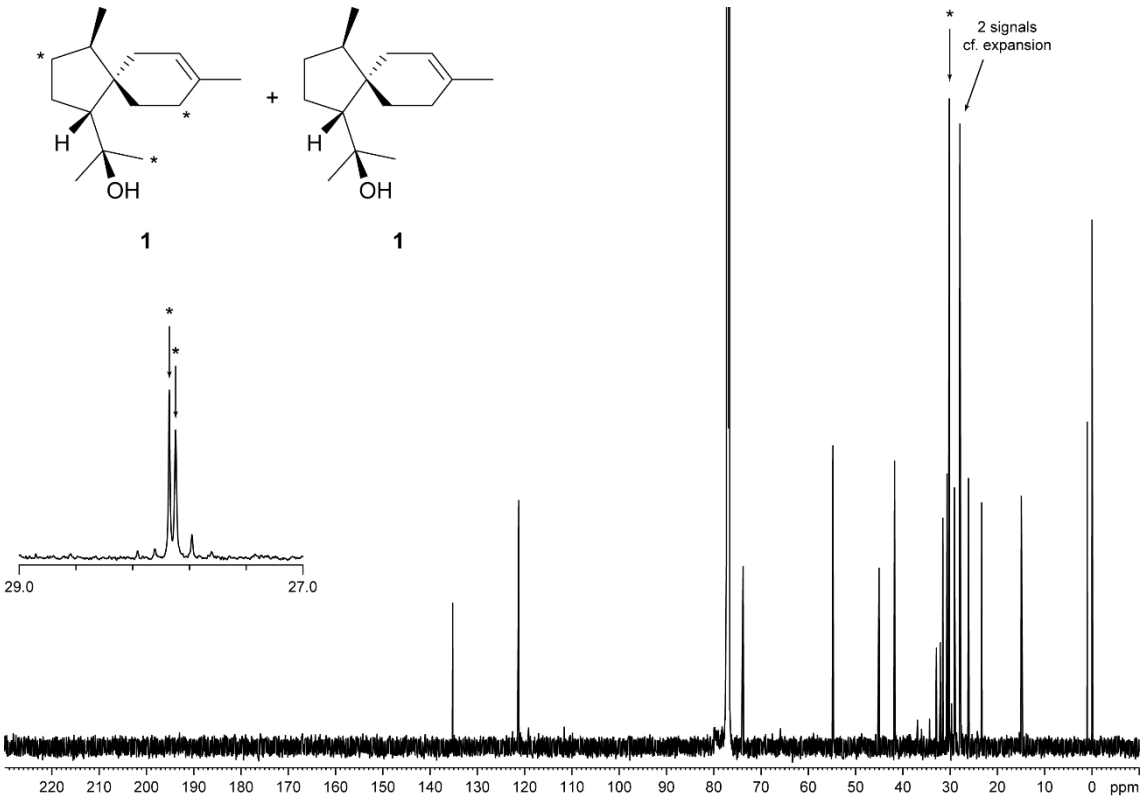


Figure 7 ^{13}C NMR spectrum of the crude CLSA headspace extract from *F. fujikuroi* SC6 after feeding of [2-^{13}C]-**12** mixed with unlabelled α-acorenol (**1**). Asterisks indicate ^{13}C-labelled carbons of **1** and arrows point to the respective enhanced ^{13}C signals due to incorporation of labelling.

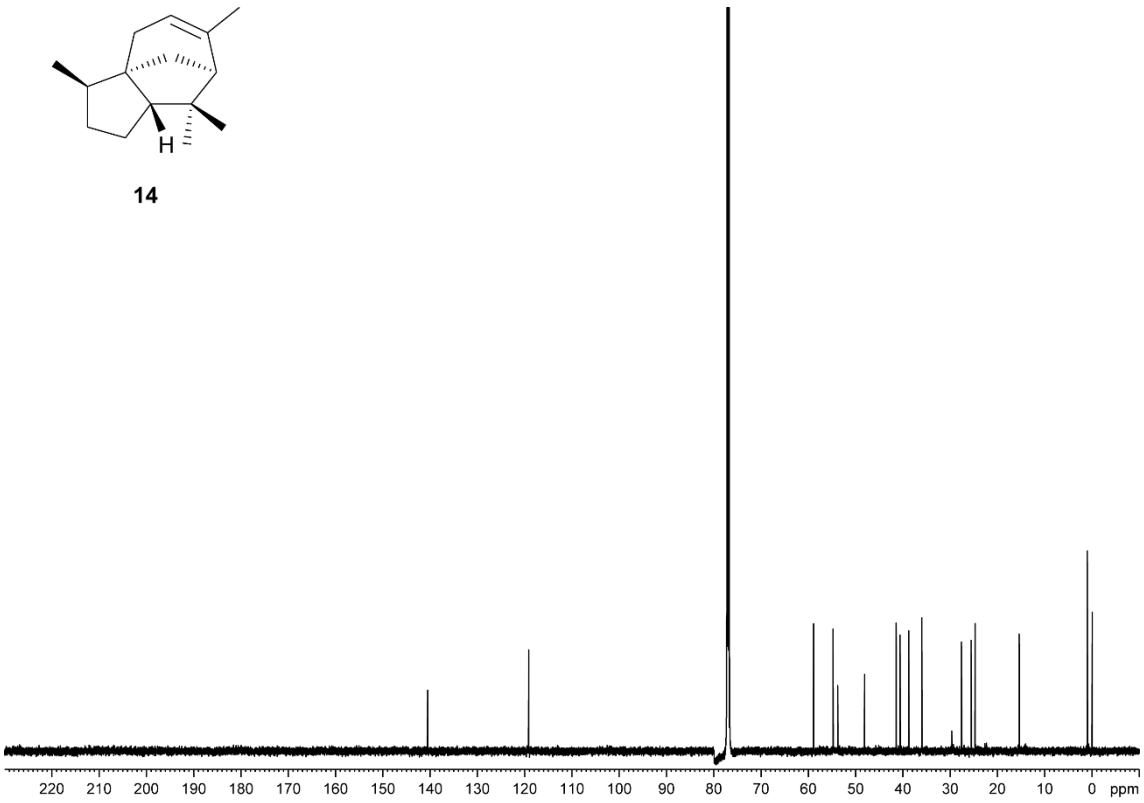


Figure 8 ^{13}C NMR spectrum of α-cedrene (**14**).

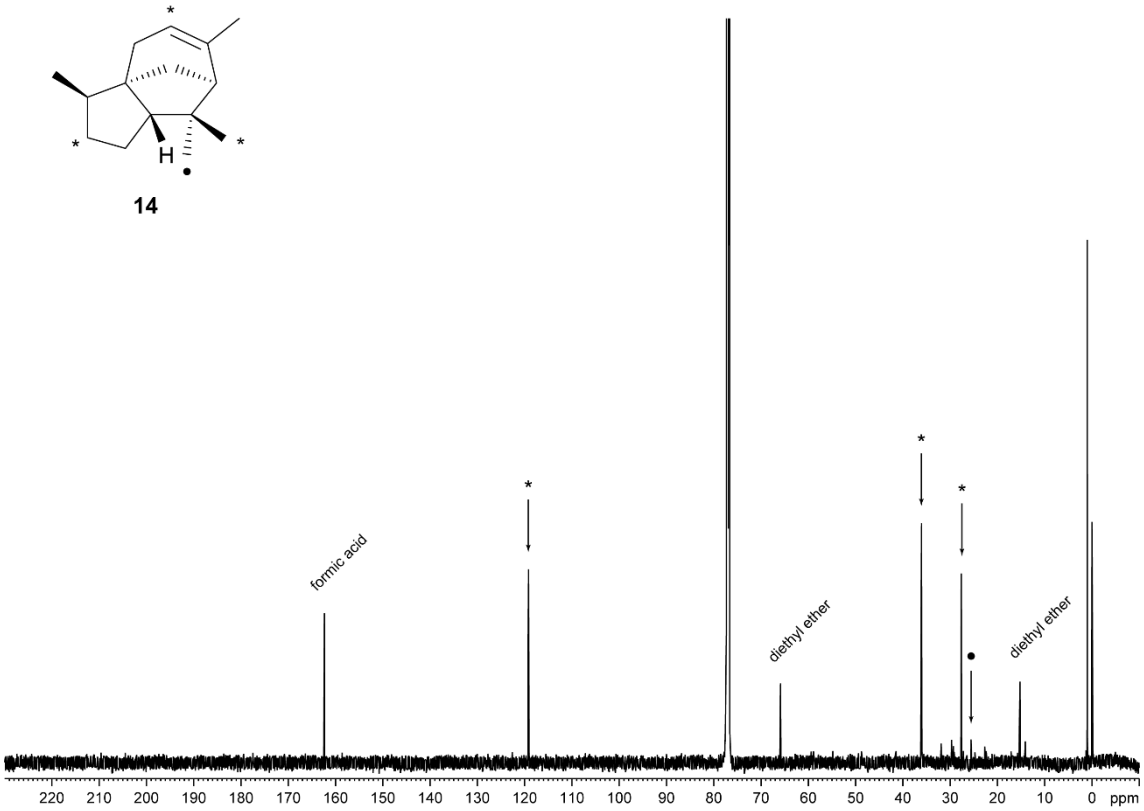


Figure 9 ^{13}C NMR of the CLSA extract obtained from *F. fujikuroi* SC6 after feeding of [2-^{13}C]-**12** and conversion of α-acorenol to α-cedrene (**14**) with formic acid (NMR of corresponding α-acorenol sample: Figure 6). Asterisks indicate ^{13}C-labelled carbons of **14** and arrows point to the respective enhanced ^{13}C signals due to incorporation of labelling. The ^{13}C signal and methyl group labelled by a dot indicate minor incorporation (ca. 10%) into the second geminal methyl group.

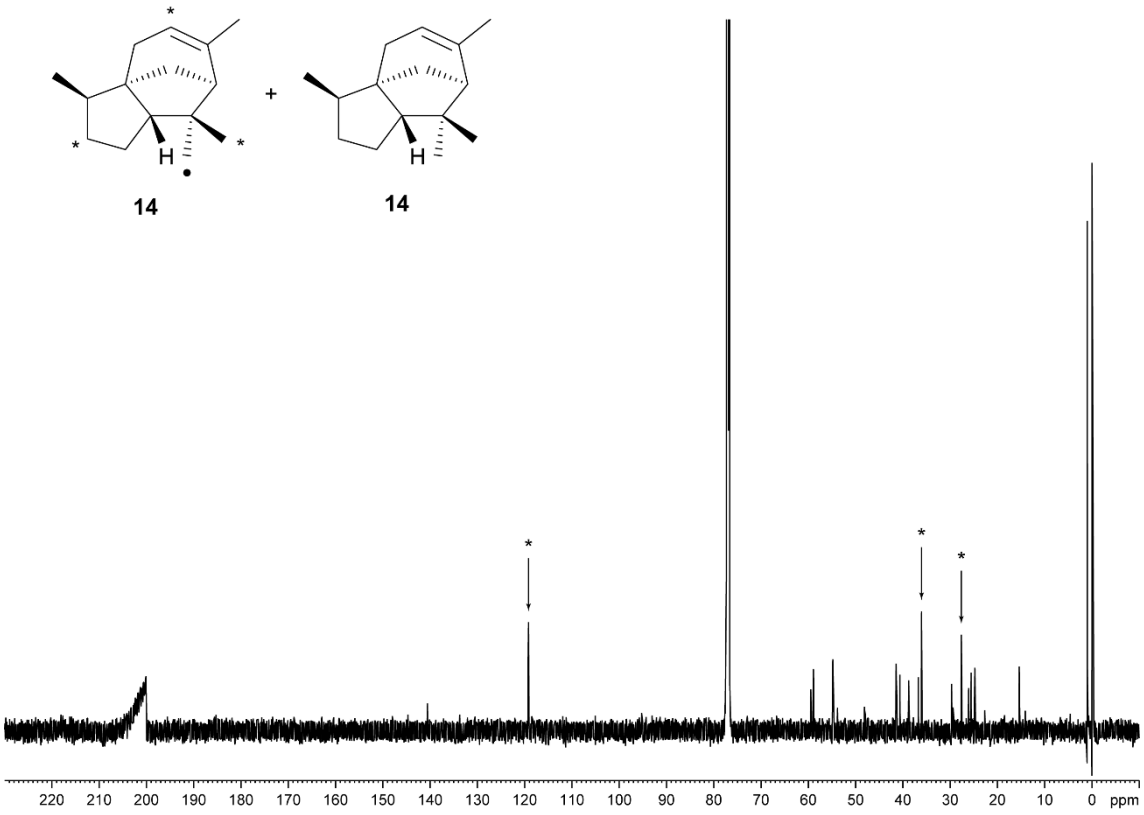


Figure 10 ^{13}C NMR spectrum of the crude CLSA headspace extract from *F. fujikuroi* SC6 after feeding of [2-^{13}C]-**12** and conversion of α-acorenol to α-cedrene with formic acid mixed with unlabelled α-cedrene (**14**). Asterisks indicate ^{13}C-labelled carbons of **14** and arrows point to the respective enhanced ^{13}C signals due to incorporation of labelling.

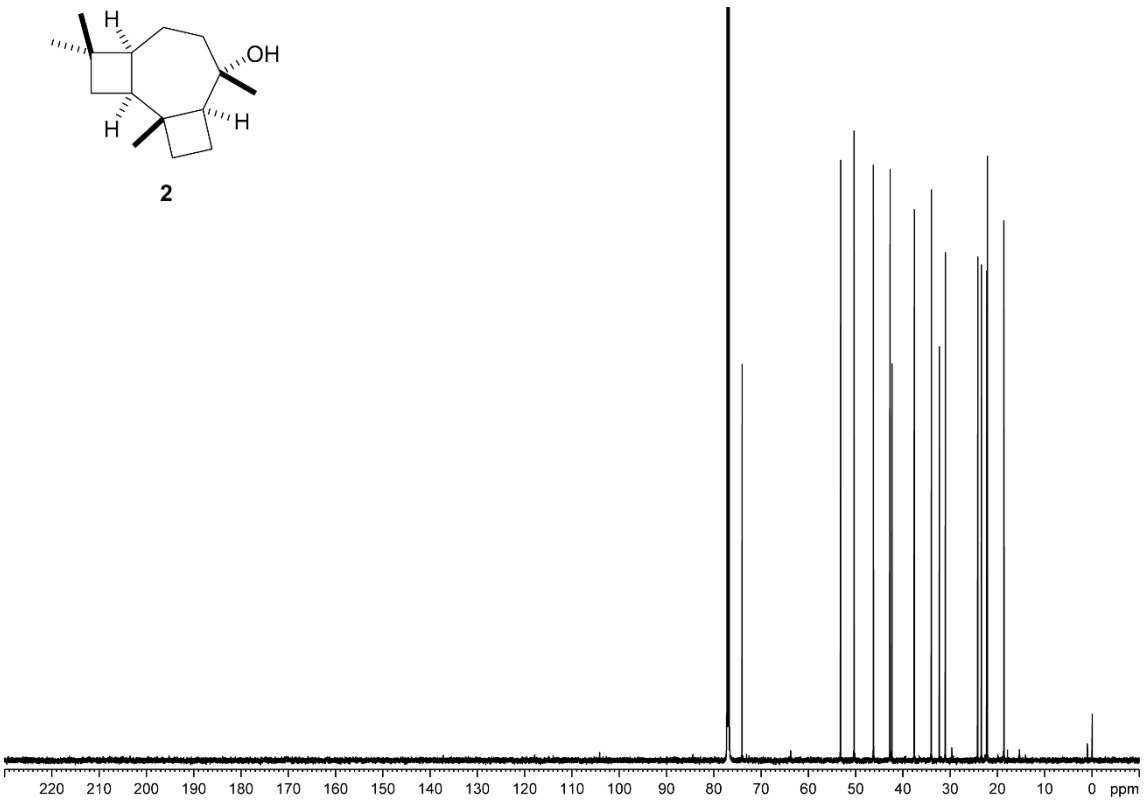


Figure 11 ^{13}C NMR spectrum of koraiol (**2**).

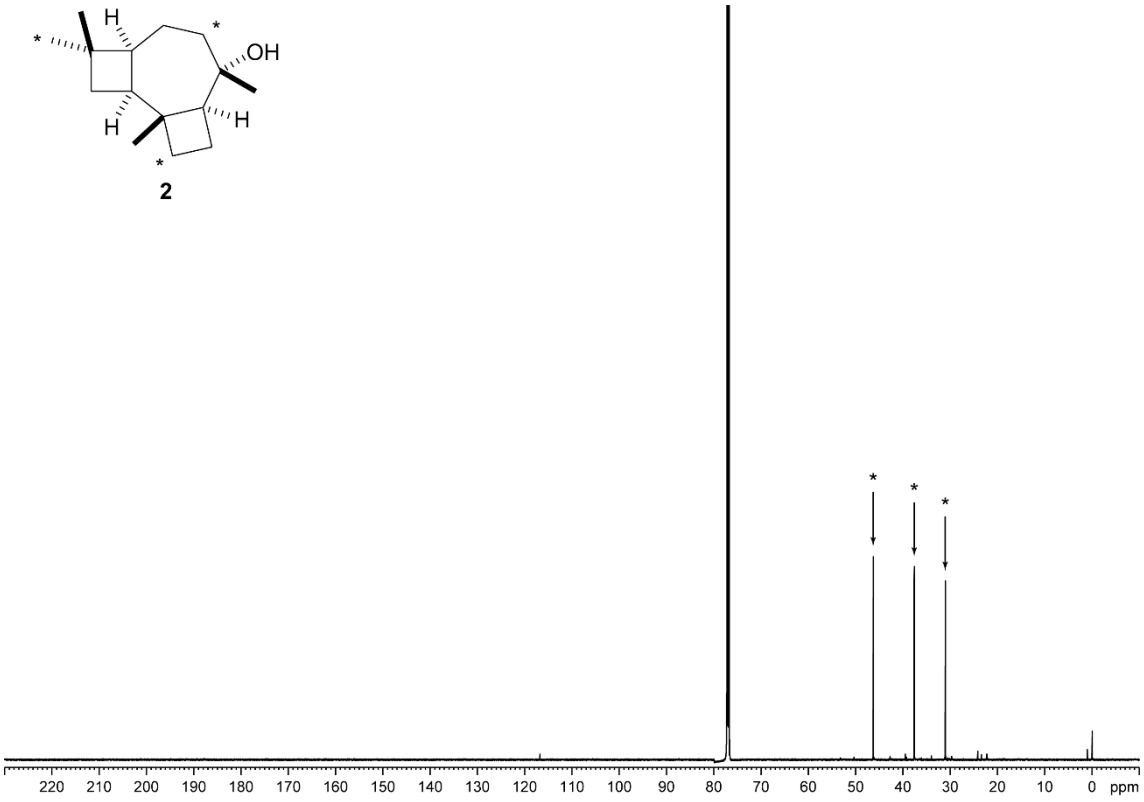


Figure 12 ^{13}C NMR spectrum of a crude CLSA headspace extract from *F. fujikuroi* SC4 after feeding of [2-^{13}C]-**12**. Asterisks indicate ^{13}C-labelled carbons of koraiol (**2**) and arrows point to the respective enhanced ^{13}C signals due to incorporation of labelling. GC-MS analysis of the headspace extract showed that labelled **2** was the main compound in the headspace extract that coeluted with an authentic standard.

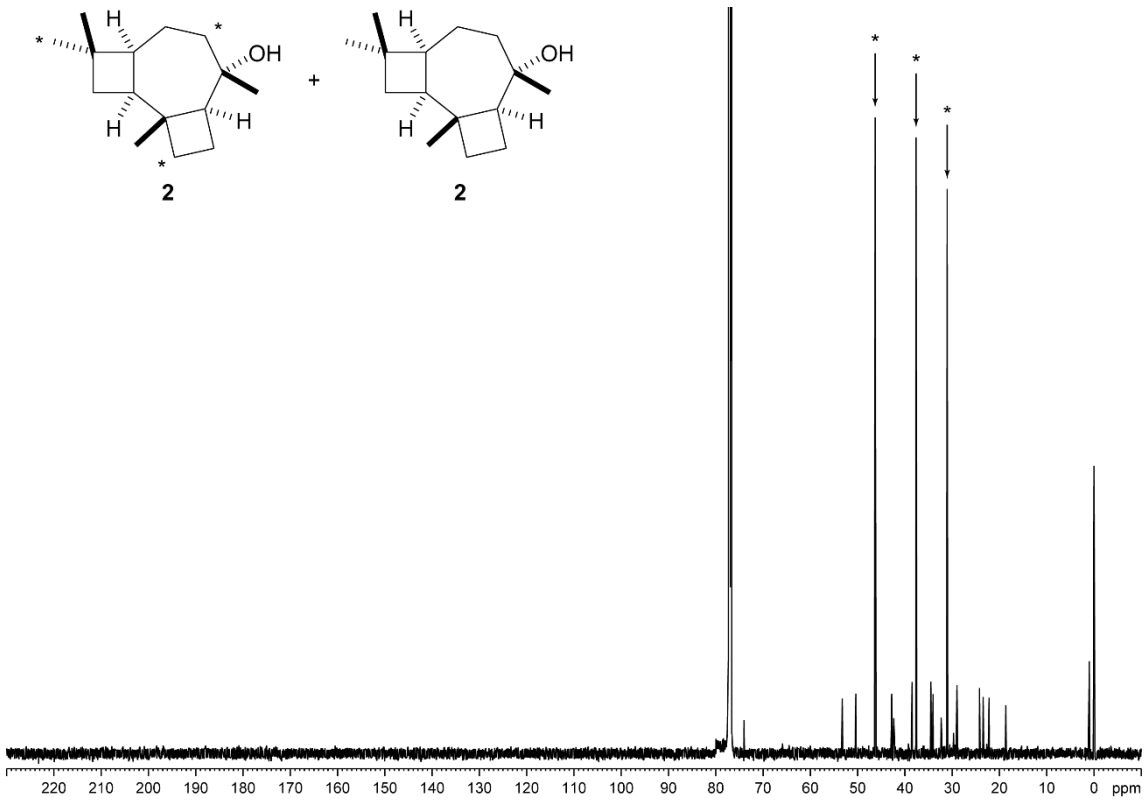


Figure 13 ^{13}C NMR spectrum of the crude CLSA headspace extract from *F. fujikuroi* SC4 after feeding of [2-^{13}C]-**12** mixed with unlabelled koraiol (**2**). Asterisks indicate ^{13}C-labelled carbons of **2** and arrows point to the respective enhanced ^{13}C signals due to incorporation of labelling.

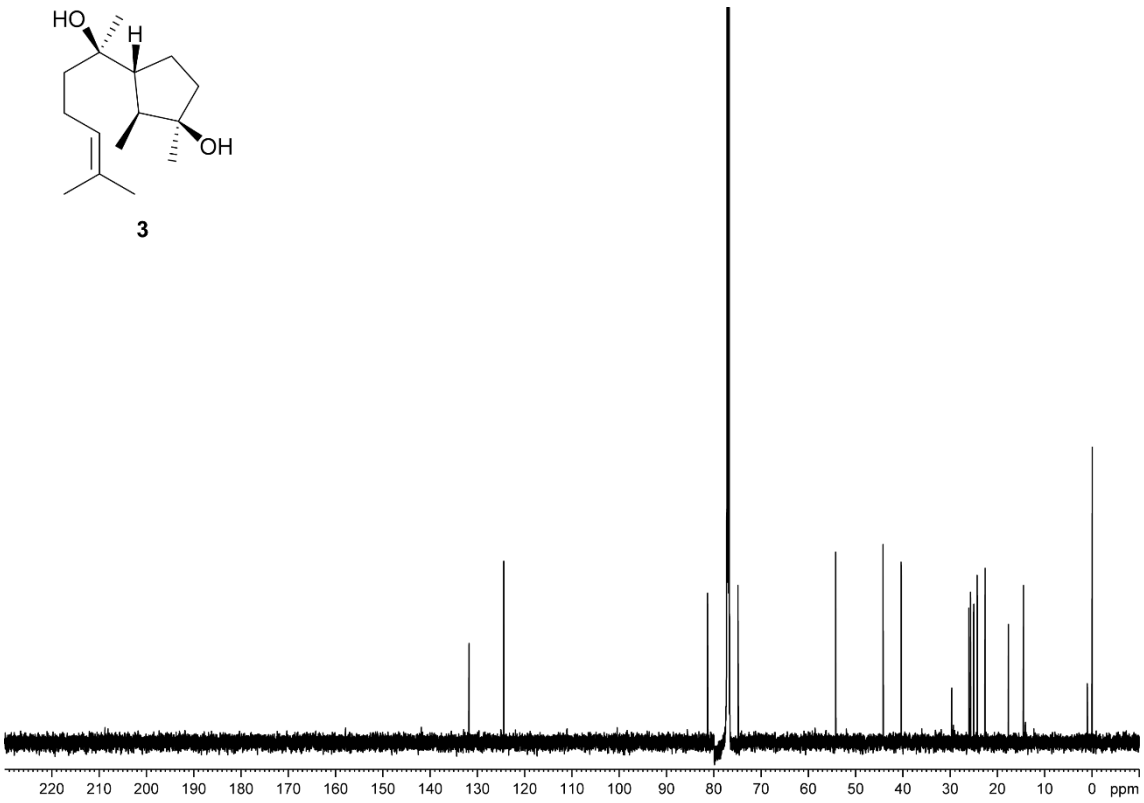


Figure 14 ^{13}C NMR spectrum of cyclonerodiol (**3**).

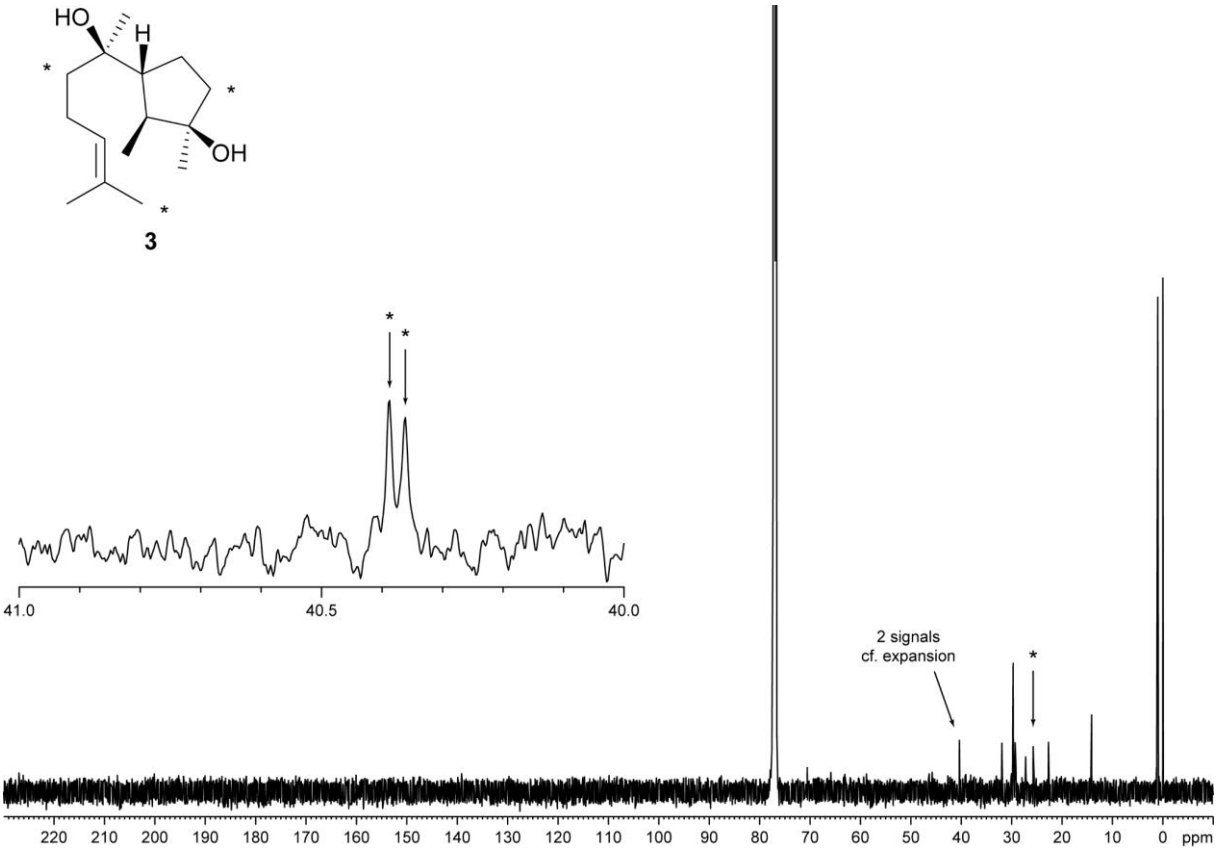


Figure 15 ^{13}C NMR spectrum of a crude ethyl acetate extract from *Fusarium fujikuroi* cultivated in 10% ICI after feeding of [2-^{13}C]-**12**. Asterisks indicate ^{13}C-labelled carbons of cyclonerodiol (**3**) and arrows point to the respective enhanced ^{13}C signals due to incorporation of labelling. GC-MS analysis of the liquid culture extract showed that labelled **3** was one of the major compounds that coeluted with an authentic standard.