# Supporting Information for:

# Biosynthetic pathway of sesquiterpene lactones in *Smallanthus sonchifolius* and their localization in leaf tissues by MALDI imaging

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## Experimental

### Chemicals

The labeled precursors 1-$^{13}$C-D-glucose, 1,2-$^{13}C_2$-mevalonolactone and 2-$^{13}$C-sodium acetate (99% isotopic abundance) were purchased from Sigma-Aldrich®. L-[U-$^{13}C_6$]-isoleucine (>98%) was obtained from Cambridge Isotope Laboratories, Inc.

### General procedure for *S. sonchifolius in vitro* solid cultures

A voucher specimen from *S. sonchifolius* was deposited in the herbarium of the University of Ribeirão Preto (voucher 1413), Ribeirão Preto, SP, Brazil. Nodal segments excised from *S. sonchifolius* were quickly washed in tap water, soaked in 1 % (w/v) cercobin solution and 1 % (w/v) captan solution for 2 h under constant agitation and then washed with sterile water. The samples were then submerged in 0.5% (w/v) sodium hypochlorite solution for 30 min. Disinfested explants were inoculated into glass tubes (8.5 cm × 2.5 cm) containing 5 mL of Murashige & Skoog culture medium,[1] supplemented with 3% (w/v) 1-$^{13}$C-D-glucose, and jelled with 0.2% Phytagel®, in which the was pH adjusted to 6.0. After inoculation, the culture flasks were covered with polypropylene lids (Bellco™), sealed with plastic film, stored at 25 ± 2°C (55-60% relative humidity with a 16-h photoperiod of 40 µmol $m^{-2}$ $s^{-1}$ intensity, provided by 85W cool-white GE fluorescent lamps) and incubated for 56 days (8 weeks). After this period, the fresh shoots (*n*=33) from *S. sonchifolius* were extracted with acetone for 12 h to obtain the crude extract.

**General procedure for *S. sonchifolius in vitro* liquid cultures** The feeding experiments with 1-$^{13}$C-D-glucose (3% m/v), 1,2-$^{13}C_2$-mevalonolactone (6.8 mM)[2], 2-$^{13}$C-sodium acetate (0.25% m/v)[3] and L-[U-$^{13}C_6$]-isoleucine (2.5 mM)[4] were performed in liquid culture medium. Segments of *S. sonchifolius* plantlets were inoculated in liquid MS medium supplemented with 1-$^{13}$C-D-glucose, 1,2-$^{13}C_2$-mevalonolactone, 2-$^{13}$C-sodium acetate and L-[U-$^{13}C_6$]-isoleucine, transferred into glass tubes (8.5 cm × 2.5 cm) containing 2.5 mL of Murashige & Skoog liquid culture medium. After inoculation, the culture flasks were covered with polypropylene lids (Bellco™), sealed with plastic film, stored at 25 ± 2°C and incubated for 63 days (9 weeks). After this period, the fresh shoots (*n*=30) from *S. sonchifolius* were extracted with acetone for 12 h to obtain the crude extract. Unsuccessful

biosynthetic experiments: the first one was carried out using field-grown plant. Ten leaves, freshly excised from 5-month-old-plats, were incubated separately in 2 mL eppendorf tubes and each incubated with 10% solution of 1-$^{13}$C-D-glucose in water at 25 °C for 72 hours. After this period, the leaves from *S. sonchifolius* were extracted with acetone for 12 h to obtain the crude extract. Compounds **1** and **2** were isolated as described in the general procedure for isolation. $^{13}$C NMR analyses showed no significant $^{13}$C-enrichment for compound **1** and **2**. The second experiment was carried out using full-grown plantlets. Plantlets (*n*=30) from *S. sonchifolius in vitro* solid cultures (lifecycle lasting 6-8 weeks) were transferred into glass tubes (8.5 cm × 2.5 cm) containing 2 mL of Murashige & Skoog liquid culture medium supplemented aseptically with 2-$^{13}$C-sodium acetate (0.25% m/v) and incubated for 15 days as well as for 30 days. After these periods, the fresh shoots from *S. sonchifolius* were extracted with acetone for 12 h to obtain the crude extract. The incorporation of $^{13}$C labels from 2-$^{13}$C-sodium acetate into **1** and **2** was subsequently monitored by GC-MS. No incorporation of $^{13}$C labels from 2-$^{13}$C-sodium acetate into **1** and **2** were detected. However, the mass spectra showed an increase in the relative abundances of $[M+1]^+$, $[M+2]^+$ and $[M+3]^+$ species of other compounds present in the crude extract. These results suggested that **1** and **2** are biosynthesized and accumulated during the early stages of leaves' development.

**General procedure for isolation of STL 1 and 2**

After eight weeks, fresh shoots (*n*=33) from *S. sonchifolius in vitro* solid cultures were extracted with acetone for 12 h. The crude extract (212 mg) was fractionated via column chromatography over silica gel (70-230 mesh; Merck, column size 8.0 × 1.0 cm) to yield eight fractions, using *n*-hexane and increasing amounts of EtOAc (up to 30%) as the eluent. Fraction 6 (16.6 mg) was further purified via column chromatography over silica gel (70-230 mesh; Merck, column size 5.0 × 1.2 cm) using *n*-hexane-EtOAc (7:3) to yield enriched uvedalin (**1**, 5.0 mg). Fraction 7 (12 mg) was purified by column chromatography over silica gel (70-230 mesh; Merck, column size 4.5 × 1.0 cm) using *n*-hexane-EtOAc (3:2) from which two fractions were collected. Fraction 7.2 (3 mg) was purified in a HPLC Shimadzu apparatus using analytical CLC-CN column (250 × 4.6 mm; 5µ particle size) with an elution gradient of MeCN/$H_2O$ (5:95) increasing up to MeCN (100%) at a flow of 1

mL $min^{-1}$, λ= 225 nm, to yield enriched enhydrin (**2**, 1.0 mg). After nine weeks, fresh shoots (*n*=30) from *S. sonchifolius in vitro* liquid cultures were extracted with acetone for 12 h to obtain the crude extract. The same chromatography procedure described above was carried out to yield enriched uvedalin (**1**, 3.0 mg) and enhydrin (**1**, 2.0 mg) from *S. sonchifolius in vitro* liquid cultures.

**NMR analysis**

NMR spectra were recorded on a Varian INOVA 500 MHz (enhydrin and uvedalin from *S. sonchifolius in vitro* solid cultures) and Bruker 400 MHz spectrometers (enhydrin and uvedalin from *S. sonchifolius in vitro* liquid cultures) using $CDCl_3$ as solvent and internal standard. The relative $^{13}C$ enrichments were obtained by comparing the relative intensity of the labeled signal with that of the natural abundance of STL peaks. HMBC and HMQC spectra were acquired for **1** and **2** and allowed the assignment of all the carbon shifts except for C-12 for compound **1** and C-12, C-1', C-2' e C-3' for compound **2**.[5]

**LC-HRESIMS analysis**

LC-HRESIMS analyses were performed on a Shimadzu LC-20A liquid chromatograph with a diode array detector (CBM20A, Shimadzu), using a Shimadzu CLC-ODS (C-18) column (5 μm, 4.6 × 250 mm) protected with a 4-Pack end-capped guard column from Agilent Technologies (12.5 × 4.6 mm), and coupled to an UltrOTOFq (Bruker Daltonics) ESI-qTOF mass spectrometer. The elution gradient started with MeCN/$H_2O$ (5:95 v/v) and was increased to MeCN (100%) over 30 minutes, at a flow rate of 1 mL.$min^{-1}$ (split at a ratio of 3:1), injection of 20 μL and λ= 225 nm. MS TIC chromatograms were recorded between *m/z* 50 and 900 in positive mode with the mass spectrometer in full scan mode (1000 scans per second; spectrum interval: 2 s; drying gas flow: 5.0 $min^{-1}$; drying gas temperature: 180 °C; nebulising gas pressure: 4 bar). Collision-induction was performed using $N_2$ as collision gas with collision energies that were between 10 and 20 eV.

**General procedure for MALDI Imaging experiments**

The equipment used was a MALDI-TOF/TOF UltrafleXtreme (Bruker Daltonics, Bremen, Germany). MALDI-MS parameters: 100 ns PIE, 1000 Hz frequency of Laser, reflector mode and 300 shots and for MALDI-MS/MS: 100 ns PIE, 19 kv voltage of LIFT1, 1000 Hz frequency of Laser, reflector mode and 300 shots. Both analyses were done in positive mode. Transversal sections of *S. sonchifolius* (cultivated *in vitro*) were cut handily at a thickness 100 μm. These sections were pasted with double-sided tape (3M Co., USA) in ITO–coated conductive slides for MALDI (Bruker Daltonics). The analyses were done duplicates in positive mode, including the MALDI-MS/MS images of **1** and **2**, in the intact surface and transversal sections from leaves. Matrix solution used was 10 mg/mL CHCA (α-cyan 4-hydroxycinnamic acid) and 0.15 $mg.mL^{-1}$ NaCl in 50% of MeCN. It was applied by an ImagePrep station (Bruker Daltonics, Germany) using nitrogen flux in the entire spraying process.

**General procedure for standards and extract analyses by MALDI**

The equipment used was a MALDI-TOF/TOF UltrafleXtreme (Bruker Daltonics, Bremen, Germany). A mixture of flavonoids and ions from the matrix were used in the external and internal calibrations. The ions were accelerated at 20 kV. For MS analyses, the experimental conditions used were: pulsed ion extraction of 150 ns, laser frequency of 1000 Hz, reflectron mode, positive ion mode and 1000 shots were averaged to record a mass spectrum. The ions selected were accelerated to 19 kV in the LIFT cell for MS/MS analyses. About 81.6 mg of dried leaves were washed with 4 mL of chloroform during 30 seconds. The leaf extract was dried with nitrogen, it was solubilized in 50% MeCN and a mix (1:1) with the same matrix used in MALDI imaging experiments. The standards of uvedalin (**1**) and enhydrin (**2**) were prepared at 2 $mg.mL^{-1}$ and mix with matrix as described for extract analysis.

**Photomicroscopy of leaves from *S. sonchifolius***

The images were obtained using a Leica DM 4500B photomicroscope coupled to a Leica DFC 320 digital camera.

**Table S1.** $^{13}C$ NMR data of sesquiterpene lactone uvedalin (**1**) isolated from *S. sonchifolius* after incorporation of 1-$^{13}C$-D-glucose (**a**), 2-$^{13}C$-sodium acetate (**b**) and L-[U-$^{13}C_6$]-isoleucine (**c**) into cultures ($CDCl_3$, 25°C).

| **C** | δ | 1-$^{13}C$-D-glucose (**a**) | | | 2-$^{13}C$-sodium acetate (**b**) | | | L-[U-$^{13}C_6$]-isoleucine (**c**) | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Relative intensity of signal** | | ΔC | **Relative intensity of signal** | | ΔC | **Relative intensity of signal** | |
| | | **L** | **U** | | **L** | **U** | | **L** | **U** |
| **1**[a] | *148.6* | 1.1 | 1.1 | 1.1 | 0.3 | 0.3 | 1.1 | 0.3 | 0.3 |
| **2** | *26.3* | 1.8 | 1.1 | **1.8** | 0.2 | 0.2 | 1.1 | 0.2 | 0.2 |
| **3** | *37.1* | 1.0 | 1.2 | 1.1 | 0.2 | 0.2 | 1.1 | 0.3 | 0.3 |
| **4** | *130.8* | 0.5 | 0.5 | 1.1 | 0.6 | 0.5 | 1.3 | 0.5 | 0.5 |
| **5** | *126.3* | 1.1 | 1.2 | 1.1 | 0.3 | 0.3 | 1.1 | 0.4 | 0.4 |
| **6** | *75.4* | 1.8 | 1.3 | **1.5** | 0.4 | 0.4 | 1.1 | 0.4 | 0.4 |
| **7** | *51.1* | 1.0 | 1.3 | 1.1 | 0.4 | 0.5 | 1.1 | 0.5 | 0.5 |
| **8** | **71.3* | 2.1 | 1.3 | **1.8** | 0.4 | 0.4 | 1.1 | 0.4 | 0.4 |
| **9** | **71.3* | - | - | - | - | - | - | - | - |
| **10** | *134.6* | 0.5 | 0.5 | 1.1 | 0.5 | 0.5 | 1.1 | 0.5 | 0.5 |
| **11** | *138.8* | 0.5 | 0.5 | 1.1 | 0.4 | 0.4 | 1.1 | 0.4 | 0.4 |
| **12** | *169.3* | 0.2 | 0.2 | 1.1 | 0.3 | 0.3 | 1.1 | 0.3 | 0.3 |
| **13** | *121.7* | 2.4 | 1.2 | **2.2** | 0.2 | 0.2 | 1.1 | 0.3 | 0.3 |
| **14** | *166.1* | 0.7 | 0.4 | **2.0** | 0.5 | 0.4 | 1.3 | 0.4 | 0.4 |
| **15** | *17.1* | 1.8 | 0.9 | **2.2** | 0.7 | 0.6 | 1.3 | 0.7 | 0.7 |
| **1'** | *168.7* | 0.6 | 0.3 | **2.2** | 1.5 | 0.4 | **4.1** | **m** | 0.4 |
| **2'** | *59.6* | 0.4 | 0.5 | 1.1 | 0.7 | 0.6 | 1.3 | **m** | 0.6 |
| **3'** | *60.1* | 2.9 | 0.4 | **2.3** | 1.4 | 0.4 | **3.8** | **m** | 0.4 |
| **4'** | *13.8* | 2.0 | 1.1 | **2.0** | 1.3 | 0.3 | **4.8** | **m** | 0.3 |
| **5'** | *19.3* | 2.8 | 1.3 | **2.4** | 0.6 | 0.5 | 1.3 | **m** | 0.6 |
| **1''** | *170.4* | 0.5 | 0.4 | 1.3 | 0.5 | 0.5 | 1.1 | 0.5 | 0.5 |
| **2''** | *21.1* | 2.5 | 0.9 | **3.0** | 1.2 | 0.6 | **2.2** | 0.6 | 0.6 |
| **1'''** | *52.6* | 2.5 | 1.0 | **2.8** | 0.8 | 0.7 | 1.2 | 0.7 | 0.7 |

U: control experiments with unlabeled precursor; L: labeling experiment with $^{13}C$ precursor; ΔC= 1.1% x L/U: increase in the relative intensity (significant increases in bold for enriched carbons).

[a] Used as reference.

* Overlapped signals

**Table S2.** $^{13}C$ NMR data of sesquiterpene lactone enhydrin (**2**) isolated from *S. sonchifolius* after incorporation of 1-$^{13}C$-D-glucose (**a**), 2-$^{13}C$-sodium acetate (**b**) and L-[U-$^{13}C_6$]-isoleucine (**c**) into cultures ($CDCl_3$, 25°C). S7

| **C** | δ | 1-$^{13}C$-D-glucose (**a**) | | | 2-$^{13}C$-sodium acetate (**b**) | | | L-[U-$^{13}C_6$]-isoleucine (**c**) | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Relative intensity of signal** | | ΔC | **Relative intensity of signal** | | ΔC | **Relative intensity of signal** | |
| | | **L** | **U** | | **L** | **U** | | **L** | **U** |
| **1**[a] | *149.4* | 1.1 | 1.1 | 1.1 | 0.2 | 0.3 | 1.1 | 0.3 | 0.4 |
| **2** | *24.7* | 2.2 | 1.0 | **2.4** | 0.2 | 0.2 | 1.1 | 0.2 | 0.2 |
| **3** | *35.4* | 1.2 | 1.1 | 1.2 | 0.2 | 0.2 | 1.1 | 0.2 | 0.2 |
| **4** | *59.3* | 0.5 | 0.5 | 1.1 | 0.4 | 0.4 | 1.1 | 0.4 | 0.4 |
| **5** | *62.7* | 1.3 | 1.1 | 1.3 | 0.3 | 0.3 | 1.1 | 0.3 | 0.3 |
| **6** | *75.9* | 2.4 | 1.1 | **2.4** | 0.3 | 0.4 | 1.1 | 0.4 | 0.4 |
| **7** | *45.5* | 1.0 | 1.2 | 1.1 | 0.2 | 0.3 | 1.1 | 0.2 | 0.2 |
| **8** | *71.2* | 2.1 | 1.2 | **2.0** | 0.3 | 0.4 | 1.1 | 0.4 | 0.4 |
| **9** | *70.4* | 1.1 | 1.2 | 1.1 | 0.3 | 0.3 | 1.1 | 0.3 | 0.3 |
| **10** | *130.0* | 0.5 | 0.5 | 1.1 | 0.4 | 0.4 | 1.1 | 0.4 | 0.4 |
| **11** | *133.3* | 0.5 | 0.5 | 1.1 | 0.4 | 0.4 | 1.1 | 0.4 | 0.4 |
| **12** | *167.9* | 0.2 | 0.2 | 1.1 | 0.2 | 0.2 | 1.1 | 0.3 | 0.3 |
| **13** | *122.8* | 2.5 | 1.0 | **2.8** | 0.2 | 0.2 | 1.1 | 0.2 | 0.2 |
| **14** | *165.5* | 0.7 | 0.3 | **2.6** | 0.3 | 0.3 | 1.1 | 0.3 | 0.3 |
| **15** | *17.5* | 2.6 | 1.0 | **2.9** | 0.6 | 0.5 | 1.3 | 0.5 | 0.5 |
| **1'** | *168.3* | 0.6 | 0.3 | **2.2** | 1.0 | 0.3 | **3.7** | **m** | 0.4 |
| **2'** | *59.3* | 0.7 | 0.7 | 1.1 | 0.4 | 0.4 | 1.1 | **m** | 0.5 |
| **3'** | *59.8* | 3.1 | 1.1 | **3.1** | 1.0 | 0.4 | **2.8** | **m** | 0.4 |
| **4'** | *13.6* | 2.1 | 1.0 | **2.3** | 1.0 | 0.5 | **2.2** | **m** | 0.5 |
| **5'** | *19.0* | 3.0 | 1.0 | **3.3** | 0.4 | 0.5 | 1.1 | **m** | 0.5 |
| **1''** | *170.3* | 0.5 | 0.4 | 1.3 | 0.4 | 0.4 | 1.1 | 0.4 | 0.4 |
| **2''** | *20.8* | 2.7 | 0.9 | **3.3** | 1.0 | 0.5 | **2.2** | 0.5 | 0.5 |
| **1'''** | *52.5* | 2.6 | 0.9 | **3.2** | 0.6 | 0.6 | 1.1 | 0.5 | 0.5 |

U: control experiments with unlabeled precursor; L: labeling experiment with $^{13}C$ precursor; ΔC= 1.1% x L/U: increase in the relative intensity (significant increases in bold for enriched carbons).

[a] Used as reference.

* Overlapped signals

**Fig. S1** $^{13}C$ NMR (125 MHz, $CDCl_3$) spectra of **1** with natural $^{13}C$-isotopic abundance *(a)* and **1** after *S. sonchifolius* plantlets grew on solid culture with 1-$^{13}C$-D-glucose *(b)*.

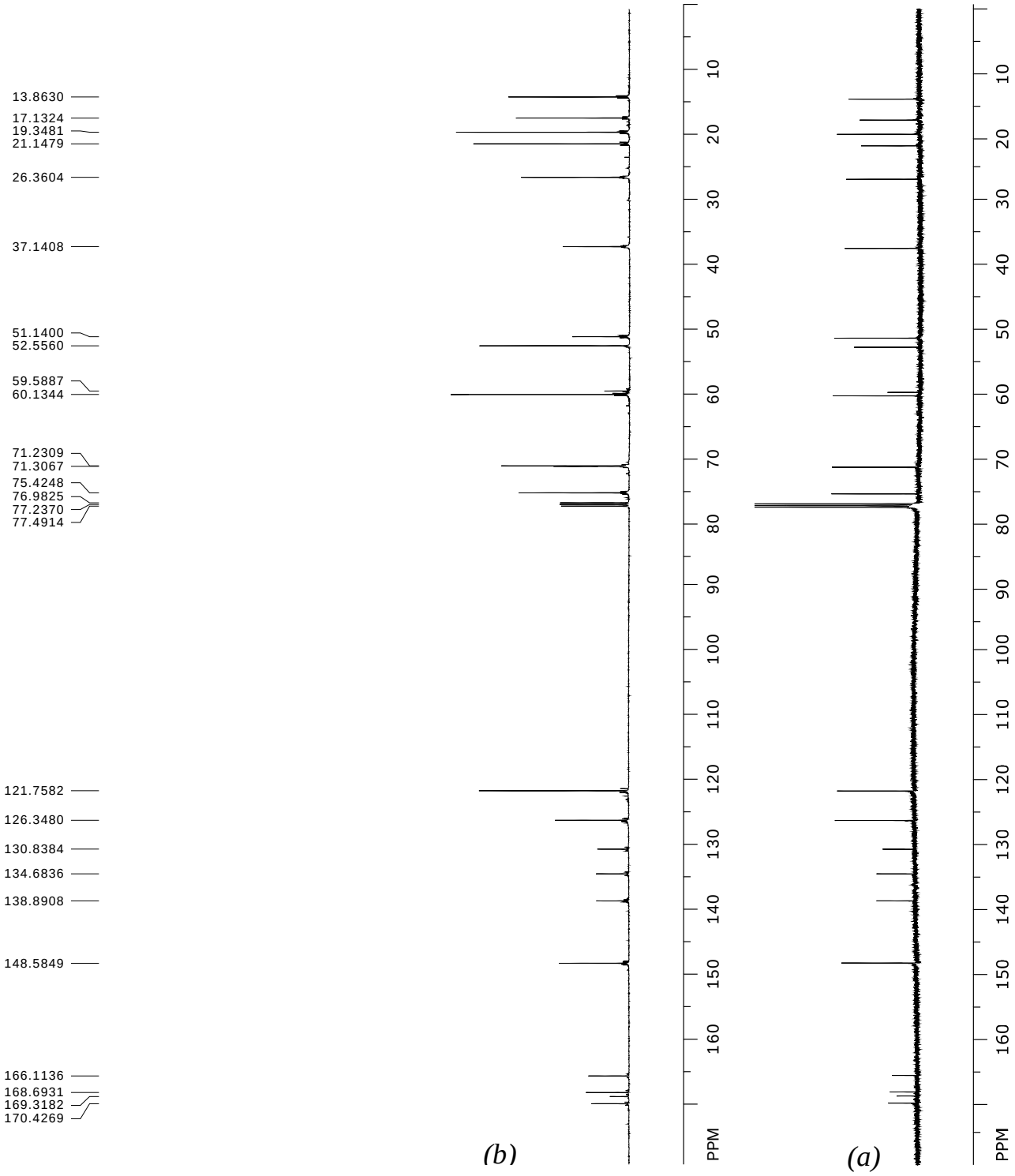


**Fig. S2** $^{13}C$ NMR (125 MHz, $CDCl_3$) spectra of **2** with natural $^{13}C$-isotopic abundance *(a)* and **2** after *S. sonchifolius* plantlets grew on solid culture with 1-$^{13}C$-D-glucose *(b)*.

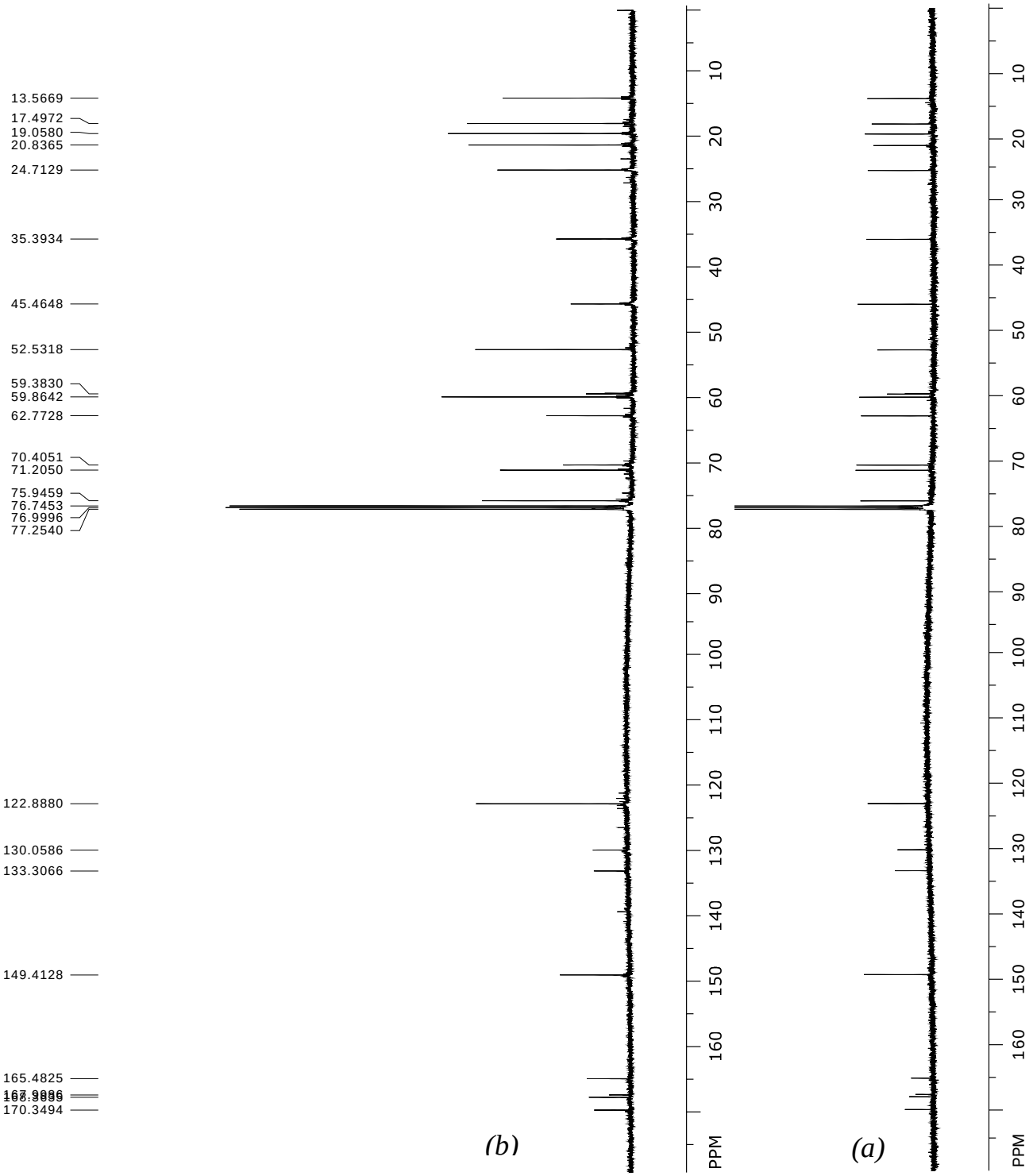


**Fig. S3** $^{13}C$ NMR (125 MHz, $CDCl_3$) spectra of **1** with natural $^{13}C$-isotopic abundance *(a)* and **1** after *S. sonchifolius* plantlets grew on liquid culture with 1-$^{13}C$-D-glucose *(b)*.

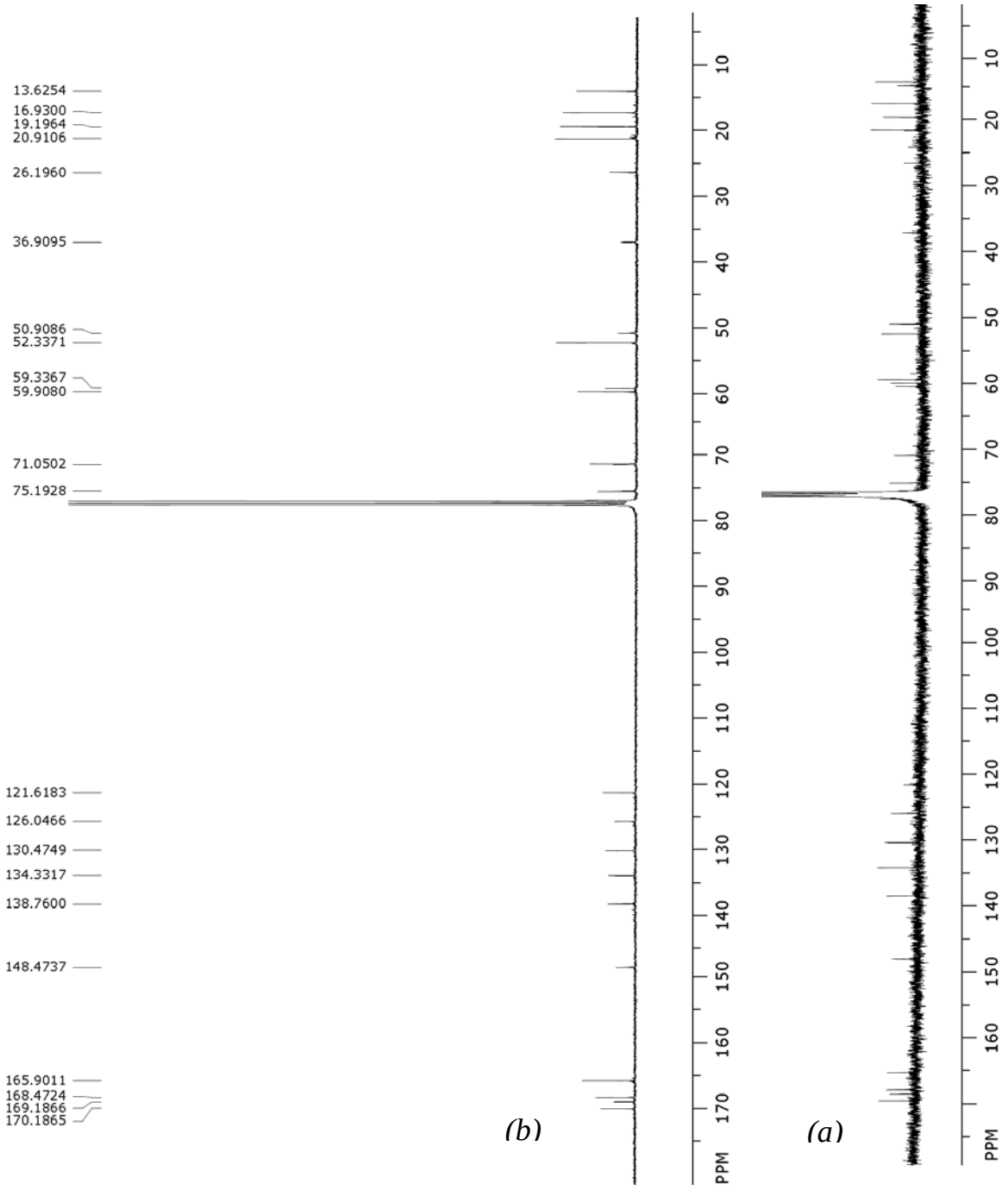


**Fig. S4** $^{13}C$ NMR (125 MHz, $CDCl_3$) spectra of **1** with natural $^{13}C$-isotopic abundance *(a)* and **1** after *S. sonchifolius* plantlets grew on liquid culture with 2-$^{13}C$-sodium acetate *(b)*.

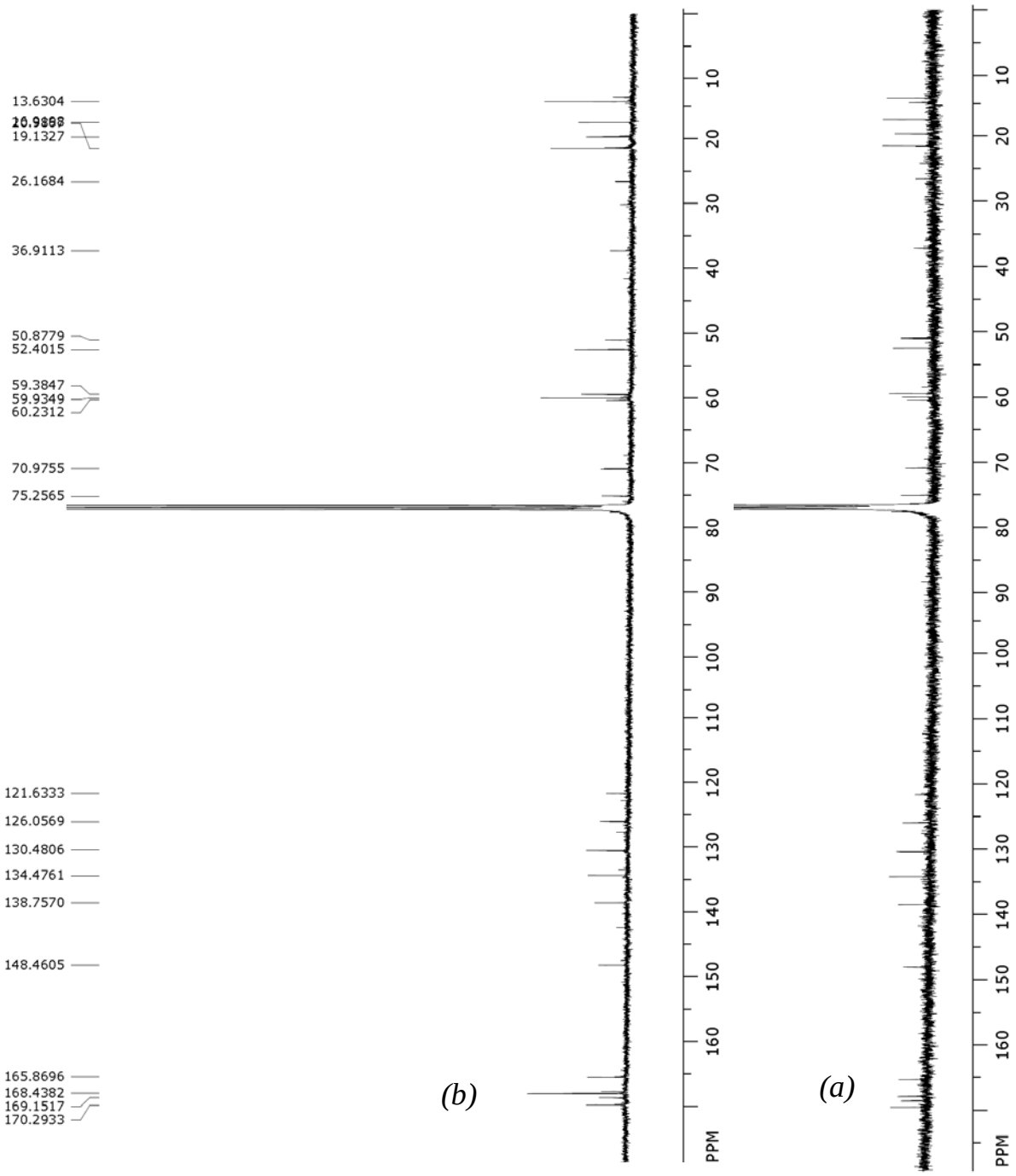


**Fig. S5** $^{13}C$ NMR (125 MHz, $CDCl_3$) spectra of **1** with natural $^{13}C$-isotopic abundance *(a)* and **1** after *S. sonchifolius* plantlets grew on liquid culture with L-[U-$^{13}C_6$]-isoleucine *(b)*.

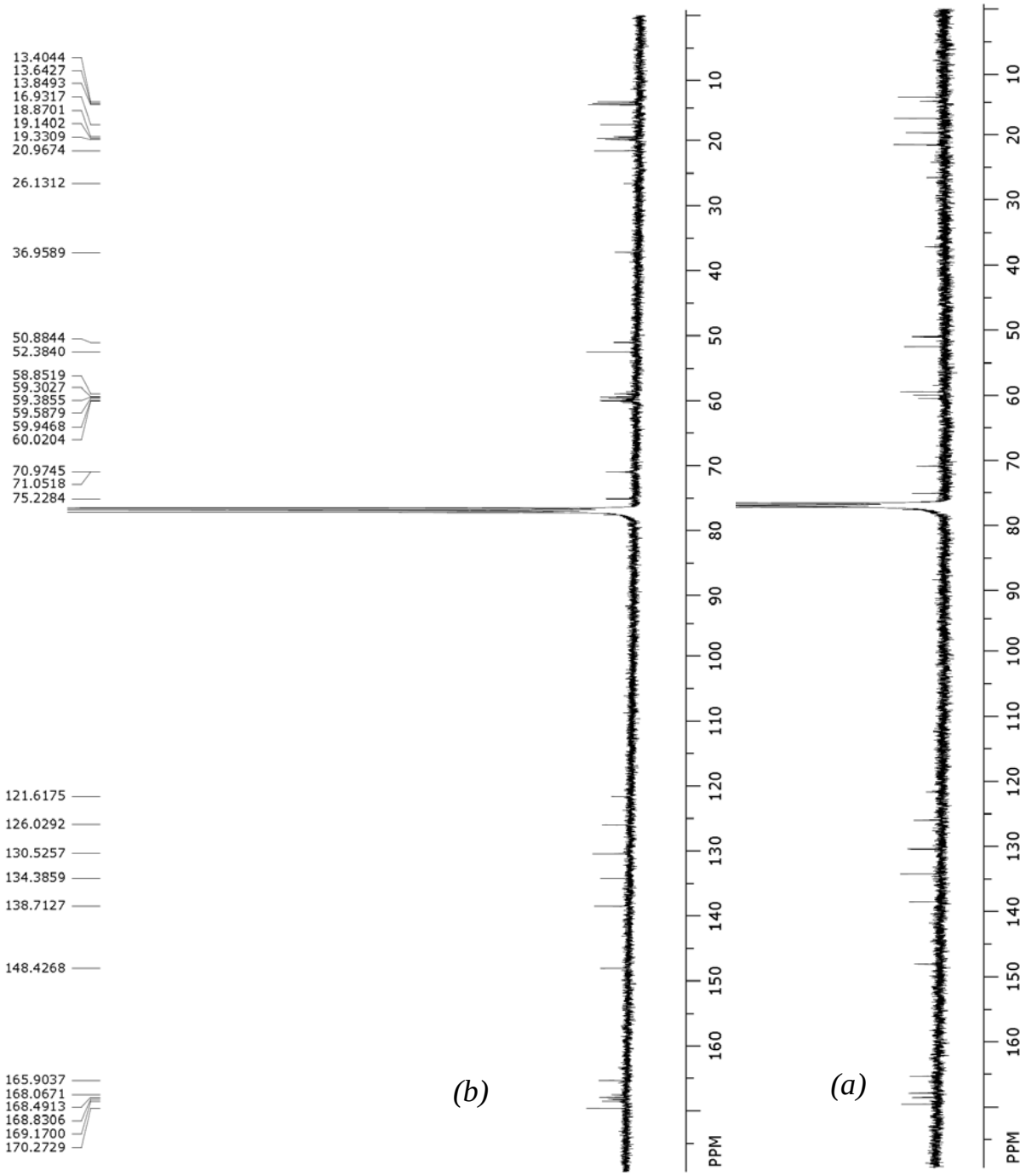


**Fig. S6** Expansion of $^{13}C$ NMR (125 MHz, $CDCl_3$) spectrum of **1** after *S. sonchifolius* plantlets grew on liquid culture with L-[U-$^{13}C_6$]-isoleucine.

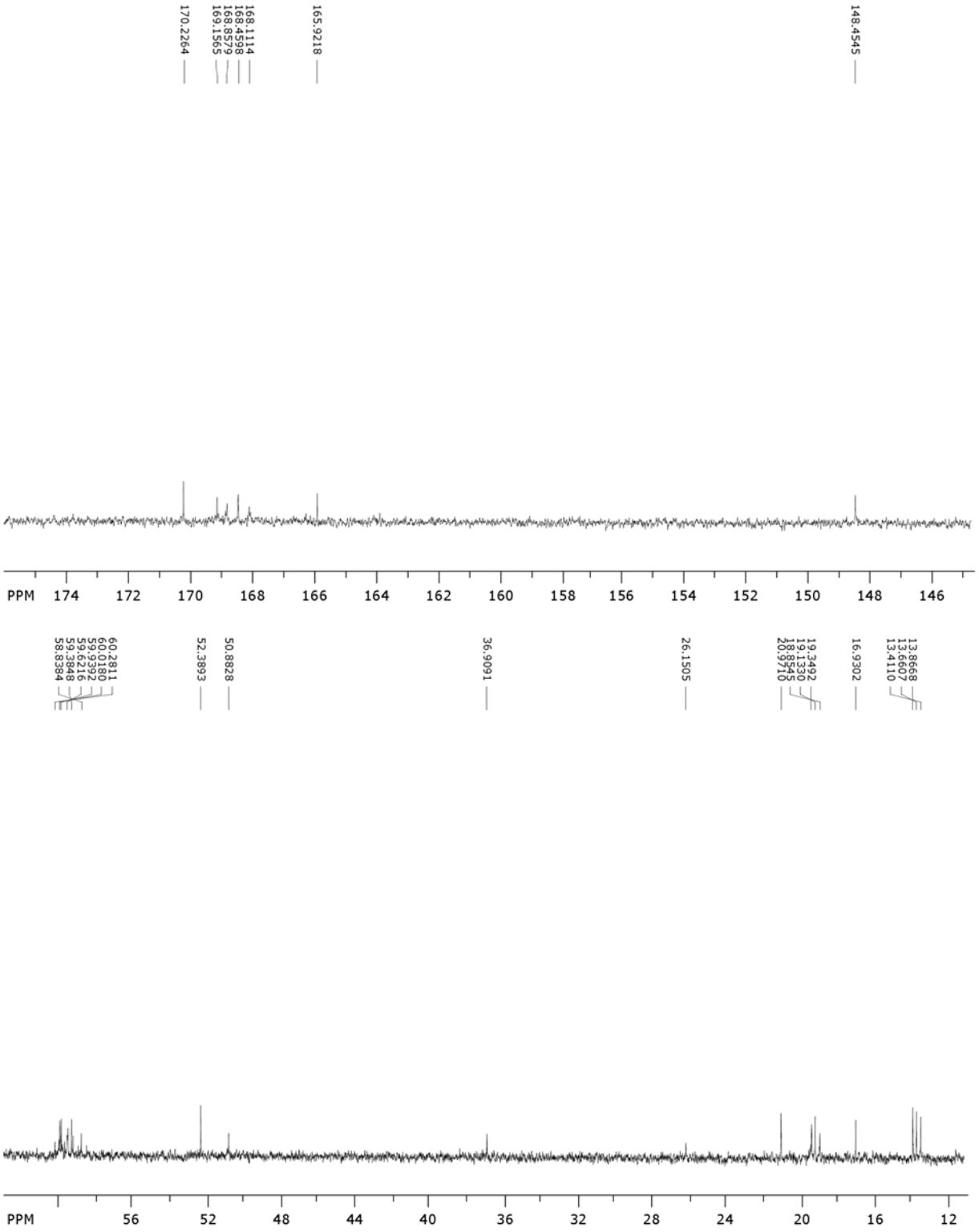


**Fig. S7** $^{13}C$ NMR (125 MHz, $CDCl_3$) spectra of **2** with natural $^{13}C$-isotopic abundance *(a)* and **2** after *S. sonchifolius* plantlets grew on liquid culture with 2-$^{13}C$-sodium acetate *(b)*.

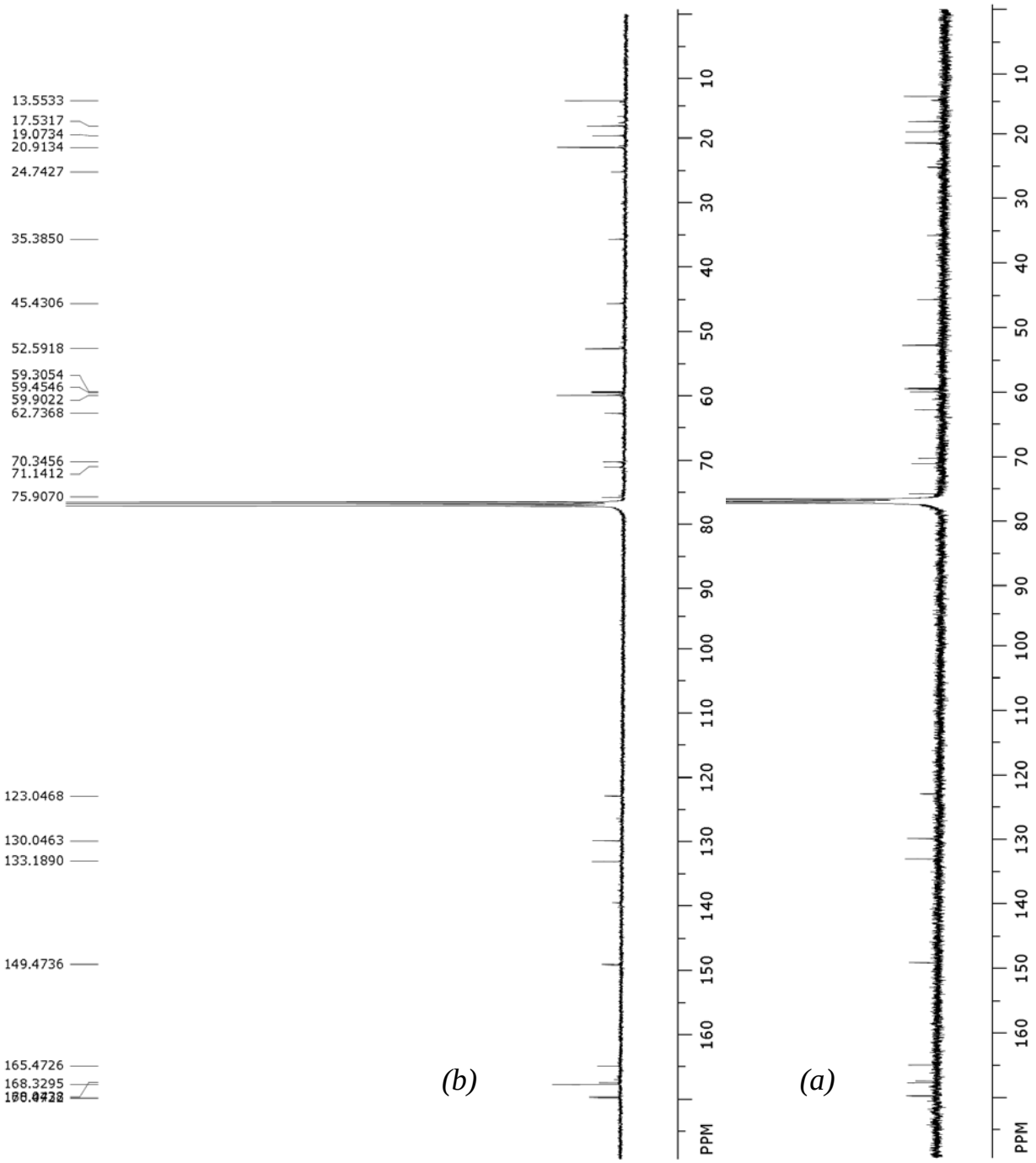



**Fig. S8** $^{13}C$ NMR (125 MHz, $CDCl_3$) spectra of **2** with natural $^{13}C$-isotopic abundance *(a)* and **2** after *S. sonchifolius* plantlets grew on liquid culture with L-[U-$^{13}C_6$]-isoleucine *(b)*.

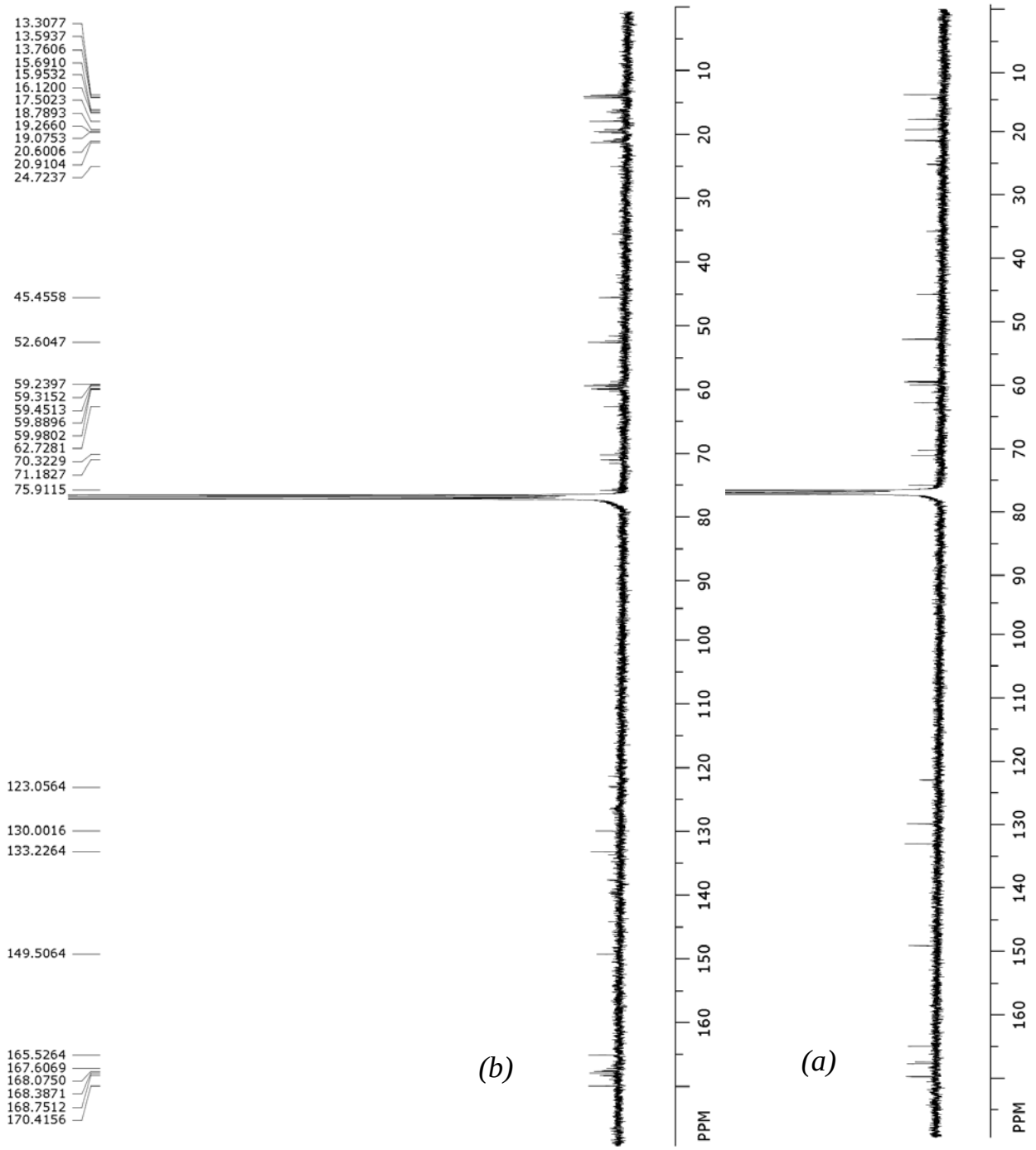
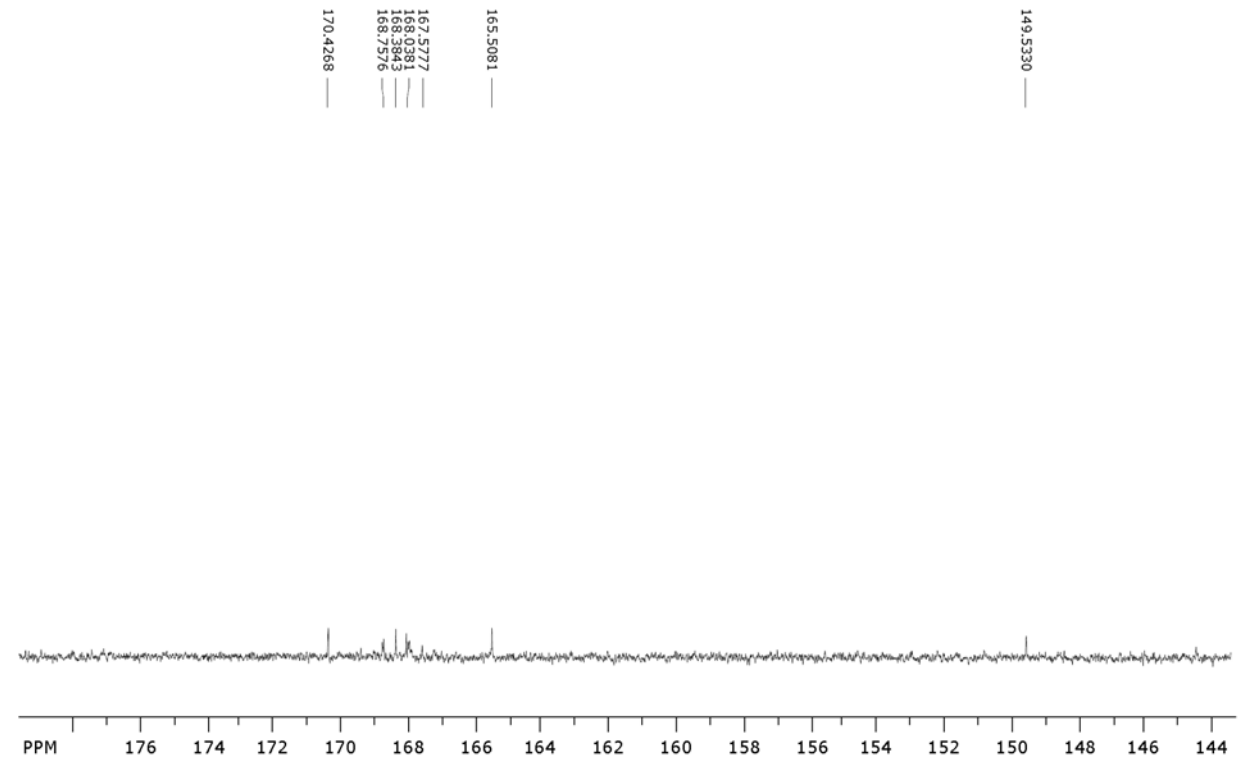



**Fig. S9** Expansion of $^{13}C$ NMR (125 MHz, $CDCl_3$) spectrum of **2** after *S. sonchifolius* plantlets grew on liquid culture with L-[U-$^{13}C_6$]-isoleucine.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168.3843
168.0381
167.5777
165.5081
149.5330
PPM
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162
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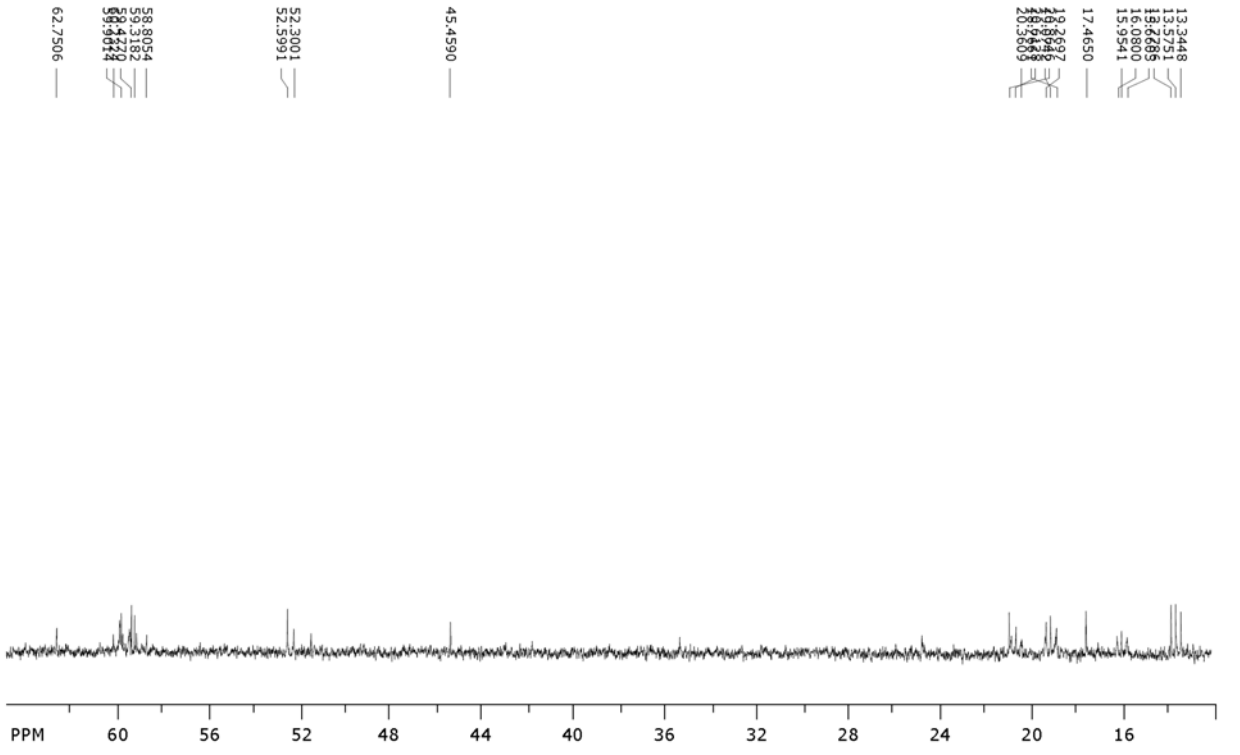
154
152
150
148
146
144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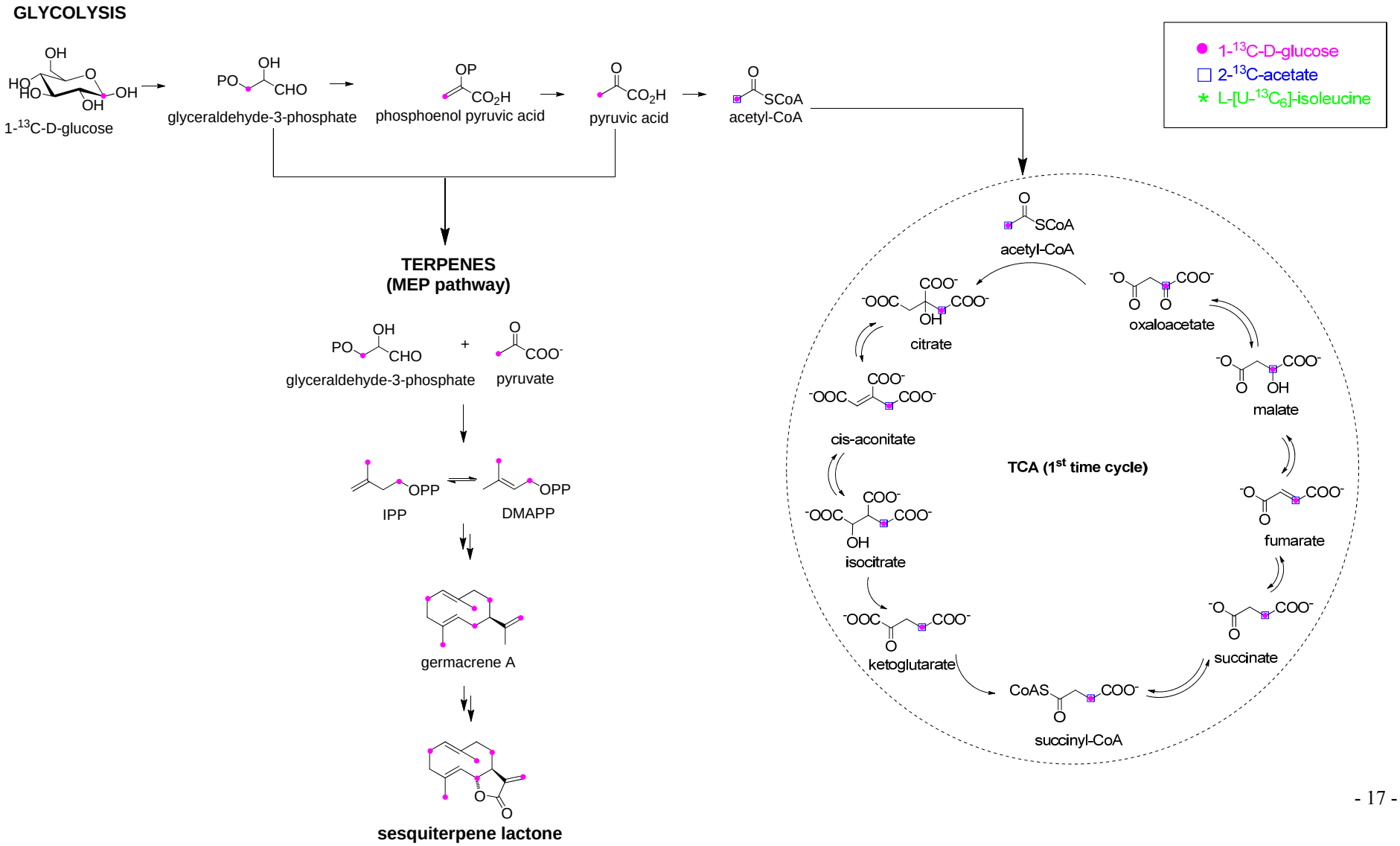
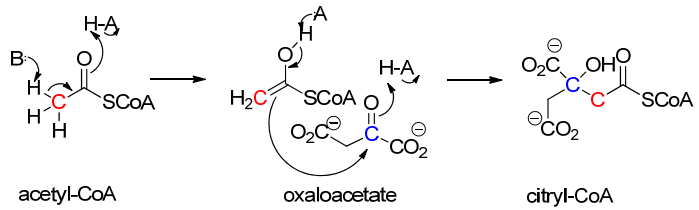
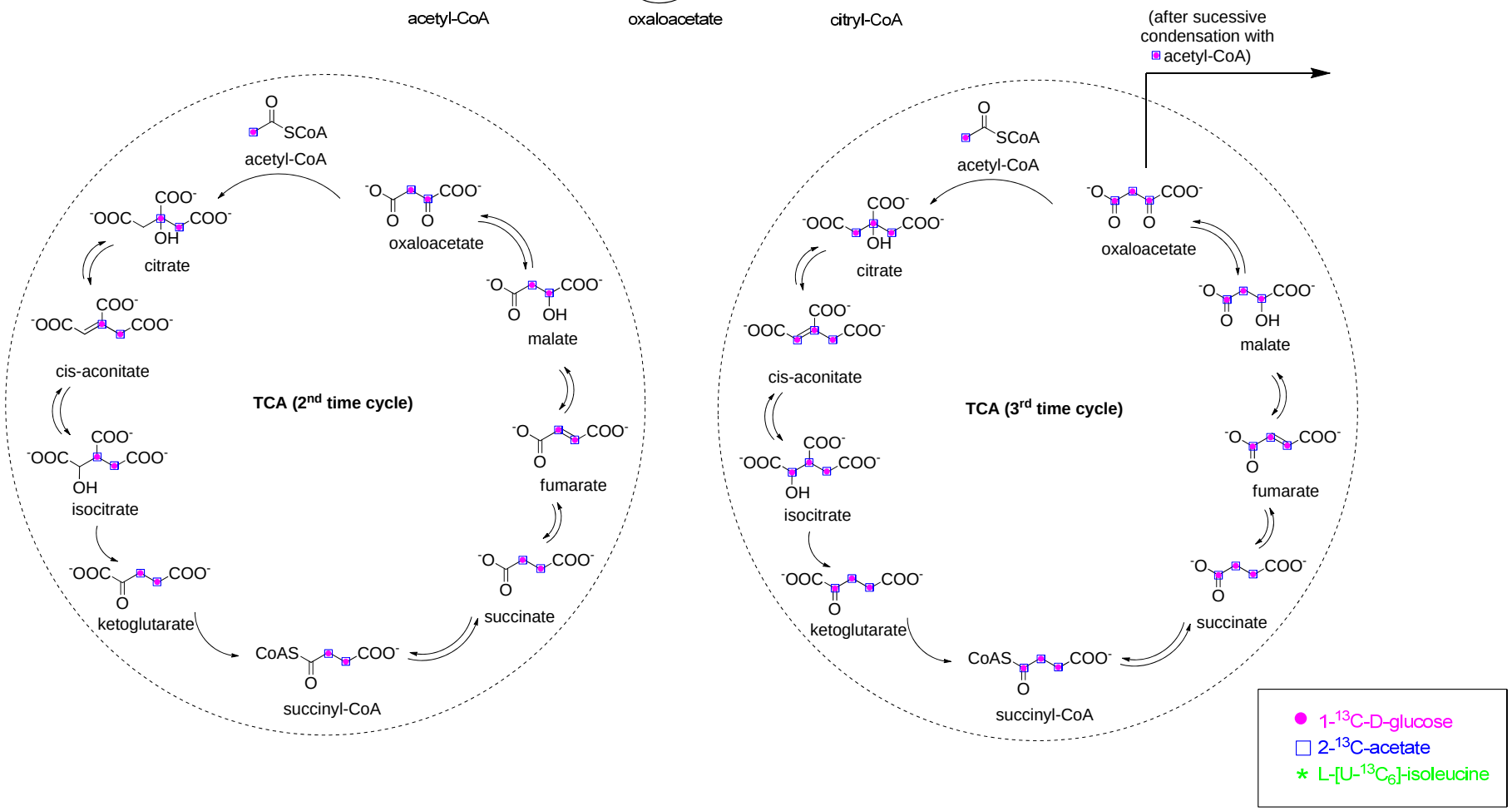
52.5991
52.3001
45.4590
20.3609
19.2697
17.4650
16.0800
15.9541
13.3448
PPM
60
56
52
48
44
40
36
32
28
24
20
16

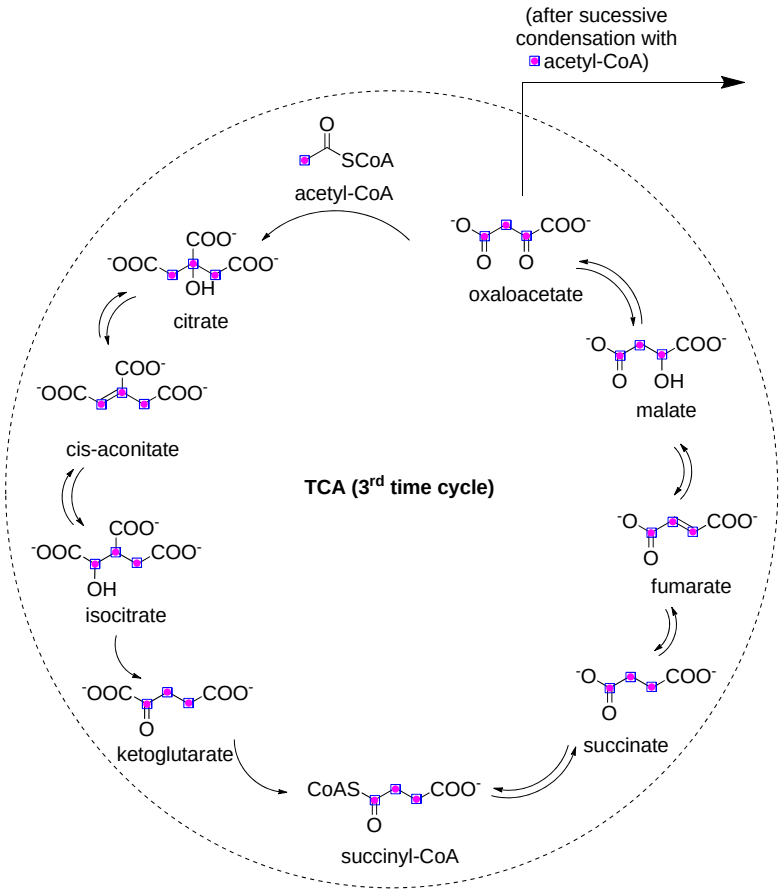
**Fig. S10** Biosynthetic pathway to acyl side chain esters and terpenoid scaffolds (derived MEP pathway) from 1-$^{13}$C-D-glucose, 2-$^{13}$C-sodium acetate and L-[U-$^{13}C_6$]-isoleucine.

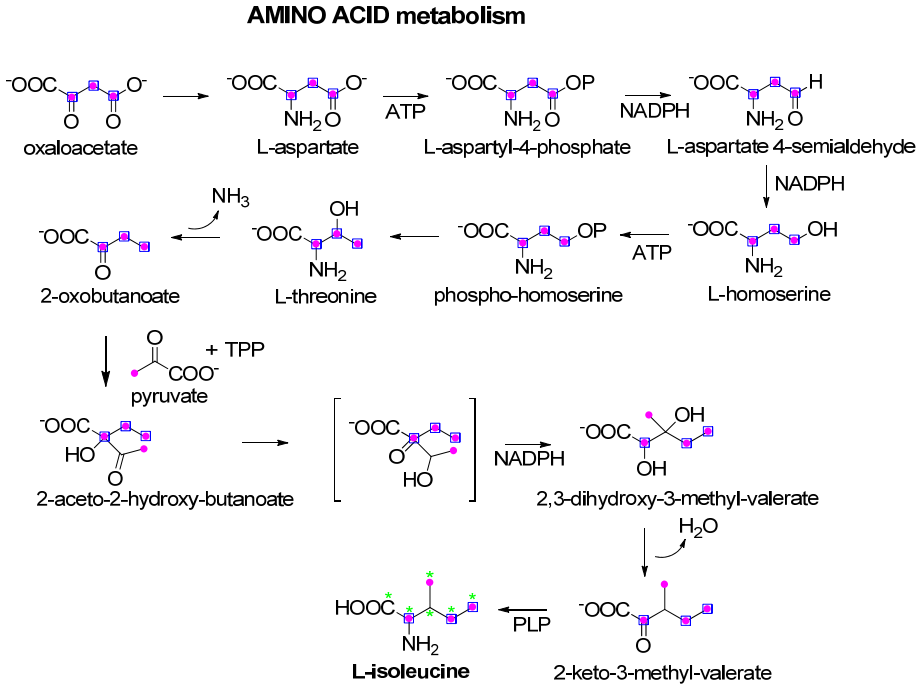


Condensation of acetyl CoA with oxaloacetate (mechanism):






● 1-$^{13}$C-D-glucose
□ 2-$^{13}$C-acetate
* L-[U-$^{13}C_6$]-isoleucine

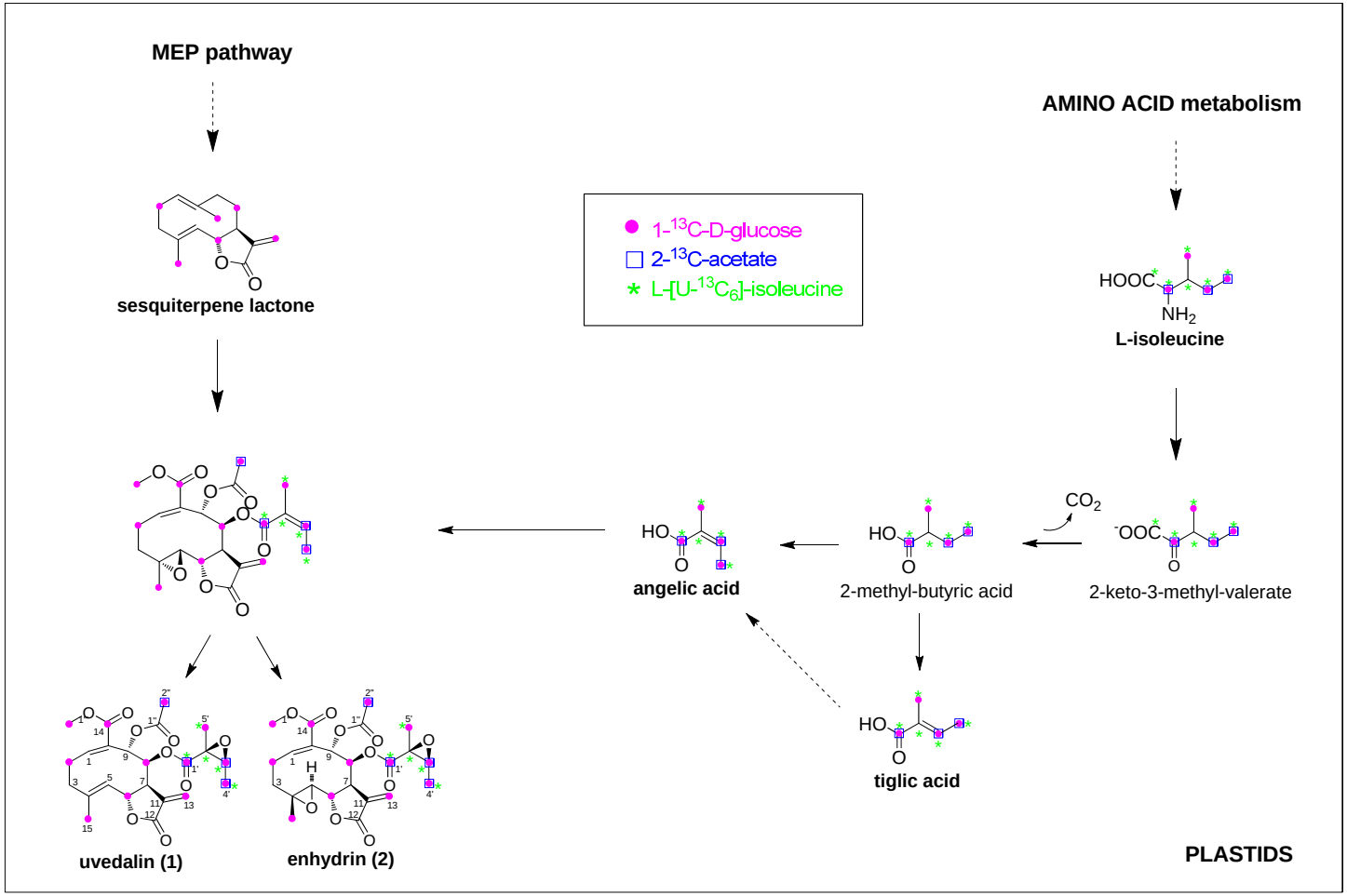
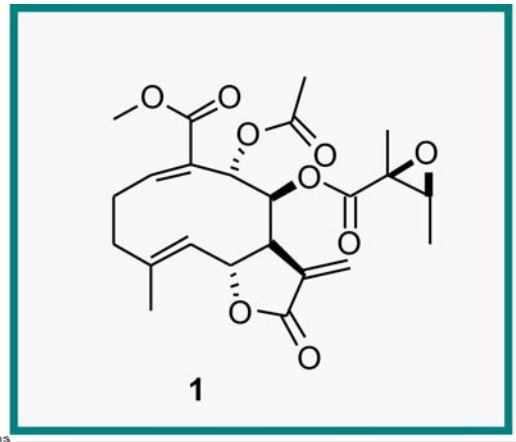
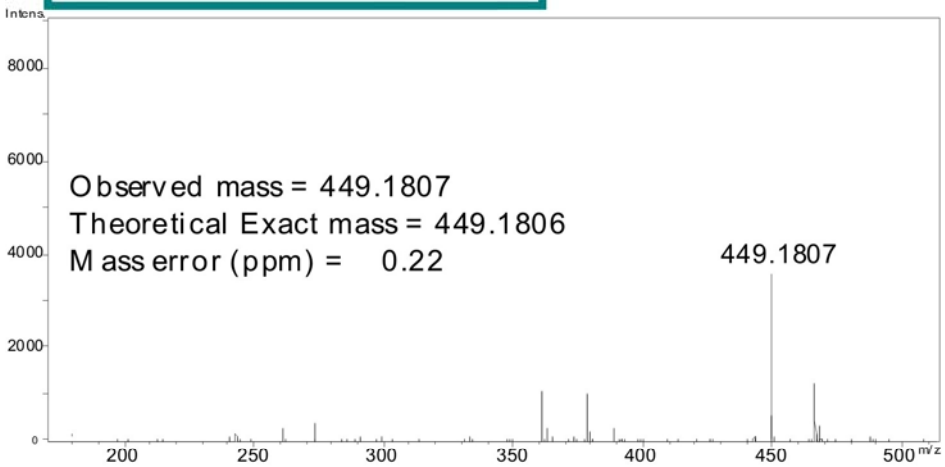
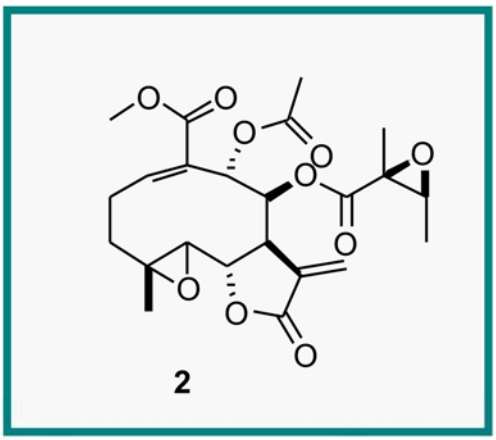


**Fig. S11** High resolution mass spectra of **1** and **2**.



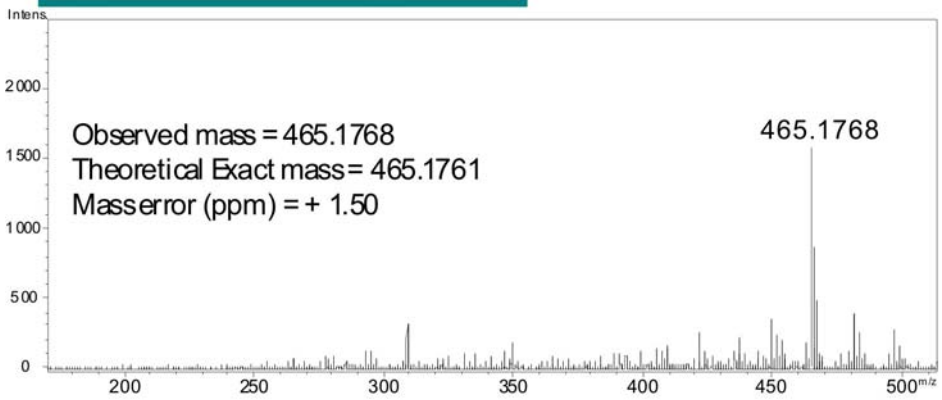
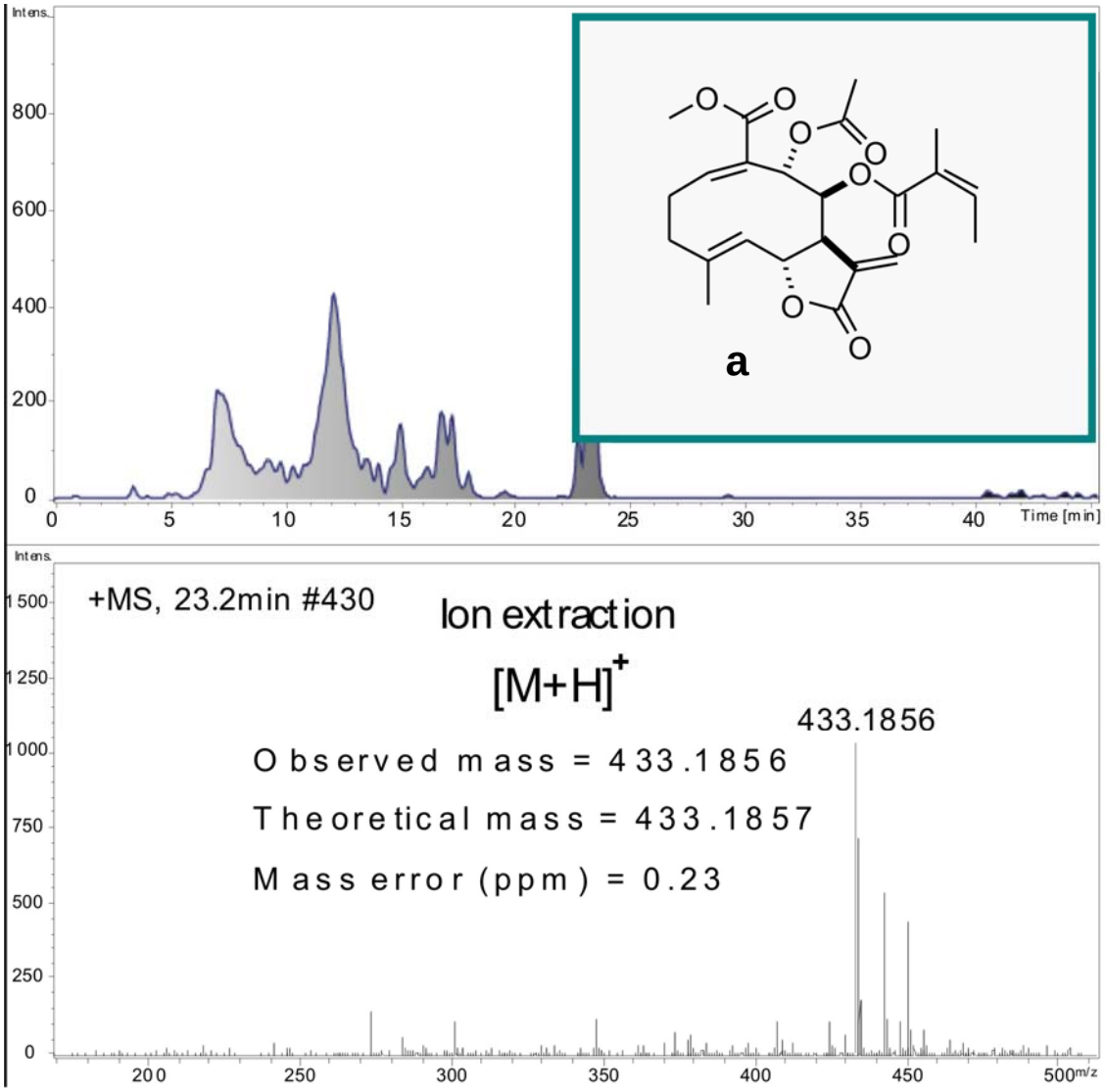



**Fig. S12** Analysis of the STL intermediates (**a** and **b**) by LC-HRESIMS operating in ion extraction mode. All data were obtained in high resolution, and accuracy is reported as mass error.



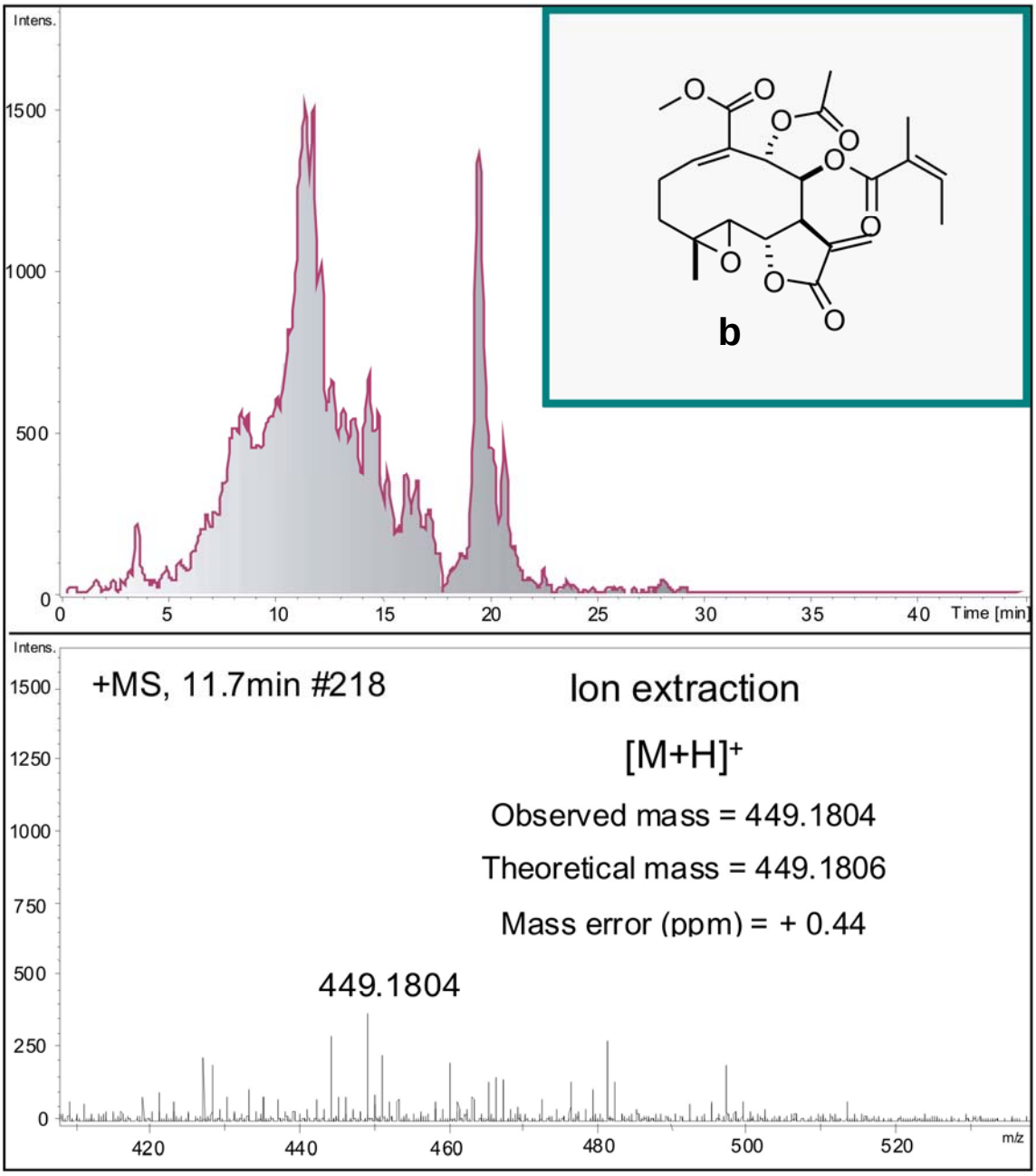

Intens.
1500
1000
500
0
0
5
10
15
20
25
30
35
40
Time [min]
b
Intens.
+MS, 11.7min #218
Ion extraction
[M+H]+
Observed mass = 449.1804
Theoretical mass = 449.1806
Mass error (ppm) = + 0.44
449.1804
1500
1250
1000
750
500
250
0
420
440
460
480
500
520
m/z

**Fig. S13** Chemical structures and accurate mass measurements of the STL intermediates (**a** and **b**) involved in the branched-chain ester biosynthesis obtained by LC-HRESIMS analysis.

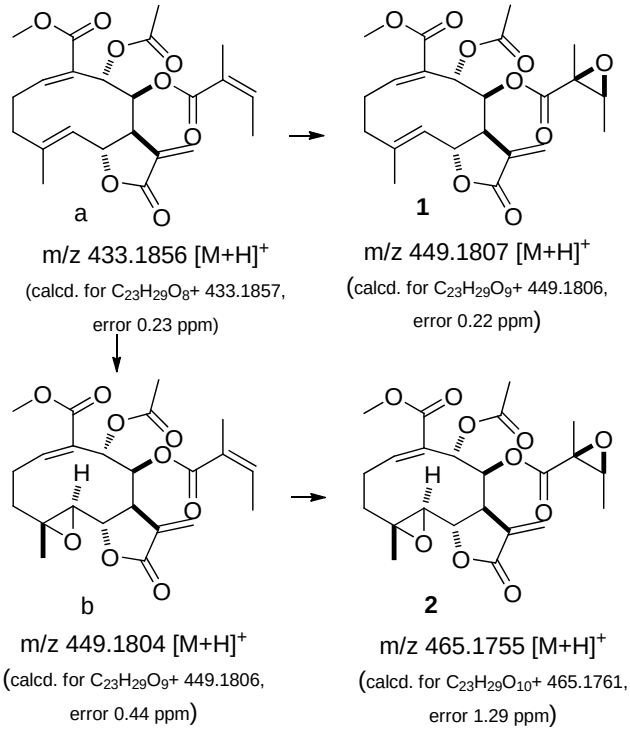



**Fig. S14** Photomicroscopy of leaves from *S. sonchifolius* (adaxial side). Glandular trichomes of cultivated plant (a) and *in vitro* plant (b).

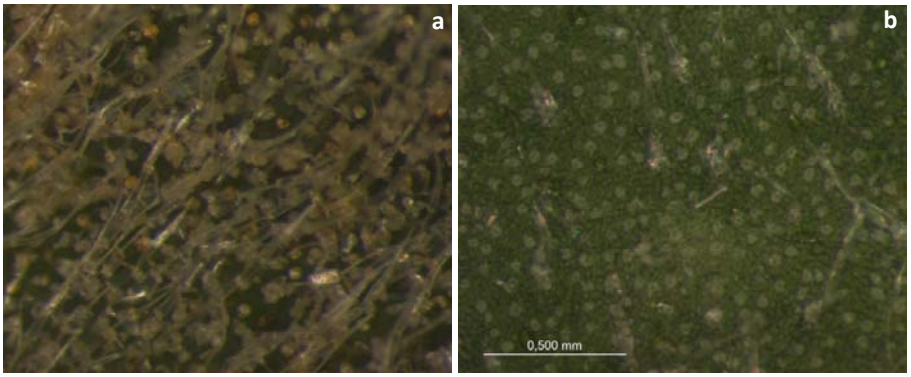


**Fig. S15** MS/MS spectrum of enhydrin

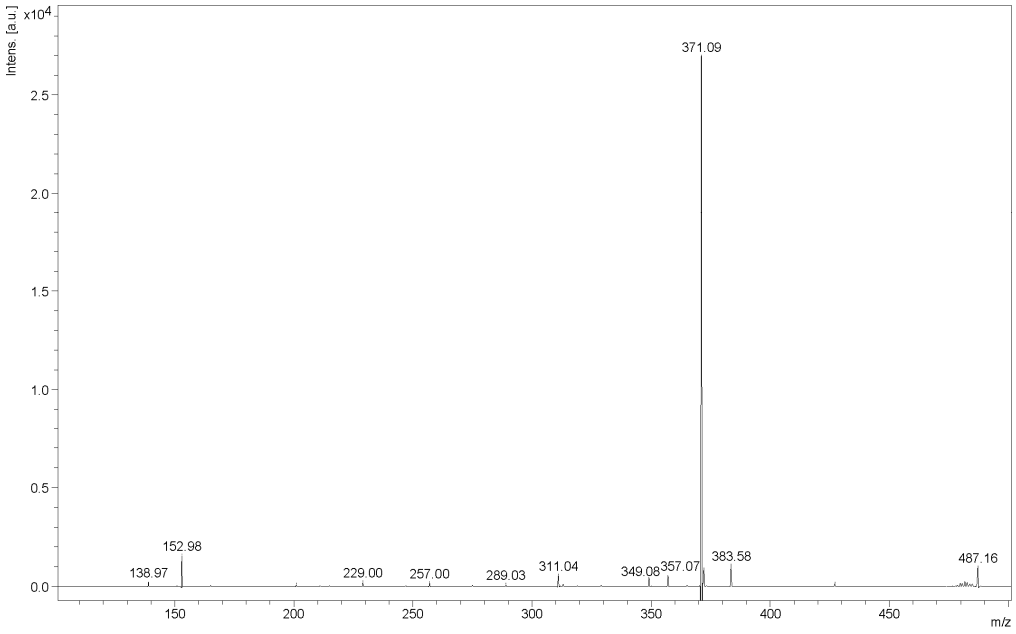



**Fig. S16** MS/MS spectrum of uvedalin

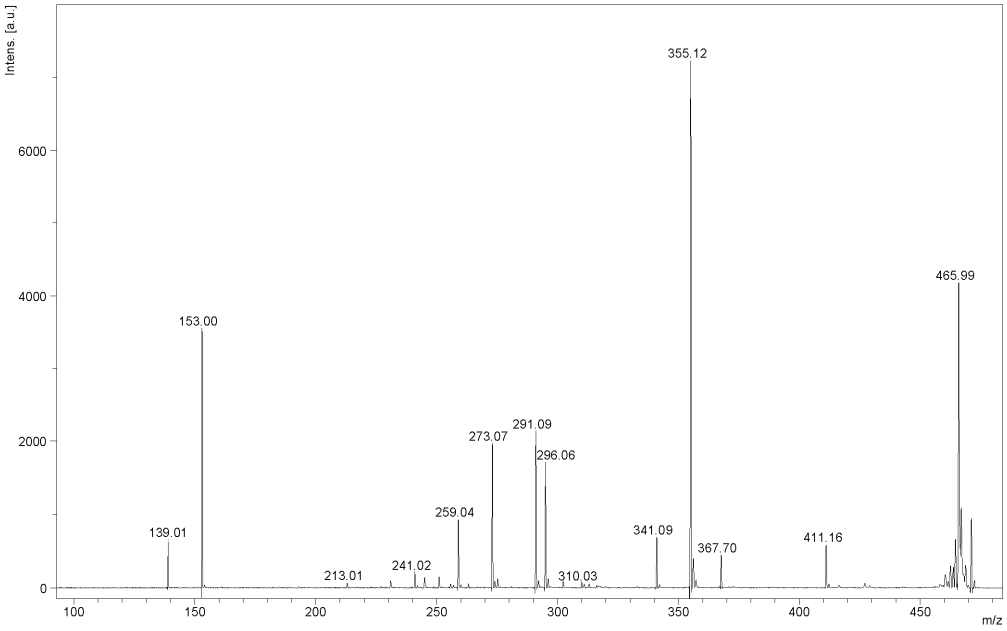
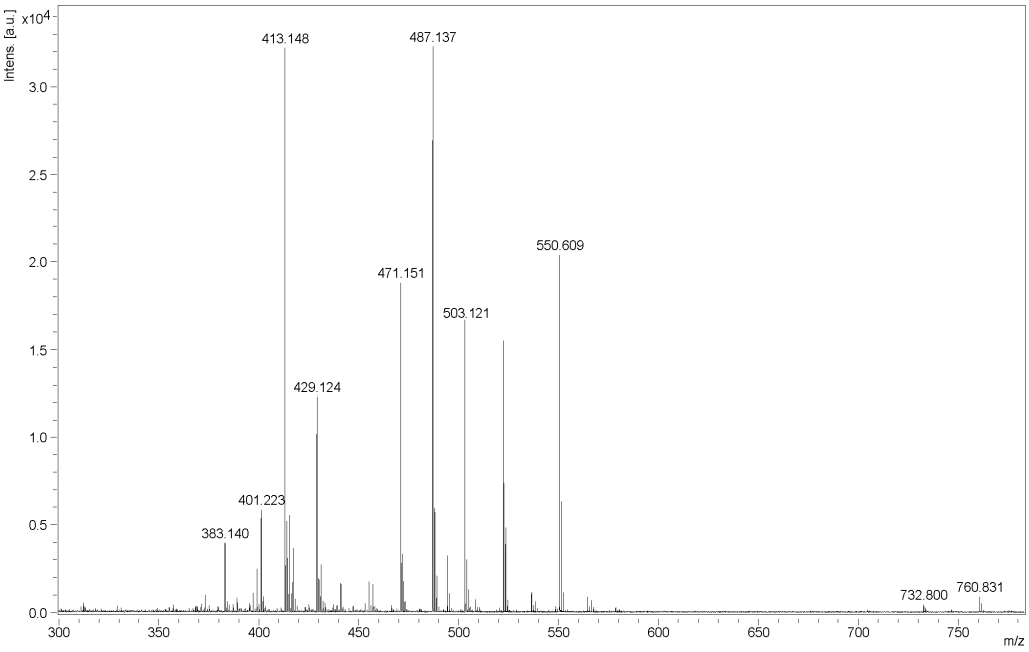


**Fig. S17** MS spectrum of leaf extract

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