Studying Mass Balance and the Stability of (*Z*)-Ligustilide from *Angelica sinensis* Helps to Bridge a Botanical Instability-bioactivity Chasm

■ SUPPORTING INFORMATION

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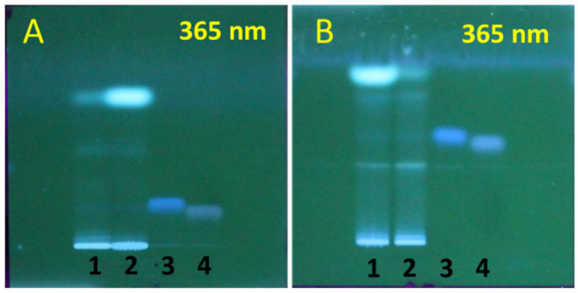
S1. Identification and Verification of the Plant Material

HPTLC identification

Plant material (BC440) and a genuine plant voucher (acquired from National Institute for Food and Drug Control, China) were extracted with MeOH:$CHCl_3$ (7:3) in a sonication for 40 min. The extracts were passed thru 0.20 µm PFET syringe filter before using CAMAG Automatic TLC Sampler 4 loading samples on a glass based HPTLC Nano-SIL HD plates which is manufactured Macherey-Nagel (Düren, Germany) with 0.2 mm Silica gel 60 with fluorescent indicator UV_{254}. The TLC plates were developed with two different solvent systems.



1. Genuine *A. sinensis* Plant Ext
2. *A. sinensis* (BC440) Ext
3. Ferulic acid
4. Isoferulic acid

- Mobile phase

A: hexances:EtOAc (4:5)

B: $CHCl_3$/MeOH/formic acid (85:15:1)

Verification via DNA Barcoding

All DNA preparations were checked using a Thermo Scientific NanoDrop 1000 spectrophotometer (Waltham, MA, USA). Polymerase chain reactions (PCRs) were performed with Big Dye Terminator v3.1 chemistry, and the amplicons were electrophoresed on an ABI 3730xl DNA analyzer (Life Technologies, Carlsbad, CA, USA). DNA sequence chromatograms were edited and analyzed with Sequencher v5.2 (Gene Codes, Ann Arbor, MI, USA).

Qiagen HotStar Taq PCR 1X Mix

3 µL HotStar 10X Buffer with 15mM $MgCl_2$
0.25 µL HotStar Taq Polymerase (5U/µL)
0.75 µL dNTP's (10 mM each)
0.75 µL BSA (20 mg/mL)
2.5 µL For Primer (5 µM)
2.5 µL Rev Primer (5 µM)
1uL total DNA (~10-25 ng/µL)
PCR Grade H20 up to 30µL

Eppendorf or Techne PCR Program

1. 94°C for 15 minutes (1 cycle)
2. 94°C for 20 seconds
3. 55°C for 30 seconds
4. 72°C for 1 minute

Go to 2 for 44 more cycles

5. 72°C for 8 minutes (1 cycle)
6. 4°C Hold

PCR Primers

ITS:
ITS5F1 GGAAGTAAAAGTCGTAACAAGG
ITS4R1 TCCTCCGCTTATTGATATGC
(White et al. 1990; made to amplify Fungi)

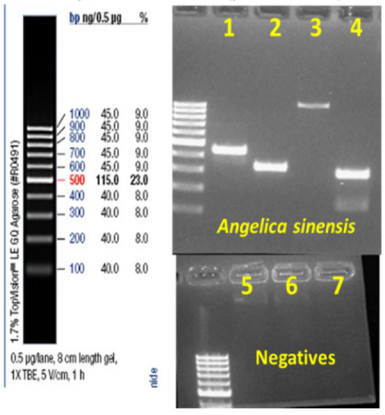
matK:
3F_KIM CGTACAGTACTTTTGTGTTTACGAG
1R_KIM ACCCAGTCCATCTGGAAATCTTGGTTC
(Sun et al 2012 - CBOL http://barcoding.si.edu)

rbcL:
rbcL m3F TATCTTAGCGCCATTCCGAGTA
rbcL m4R CGCGGATAATTTCATTACCTTC
(Gao et al 2008)
1Fm (rbcL) ATGTCACCACAAACAGAGAC
(Sun et al 2012; Fay et al. 1997)

psbA-trnH:
psbAF GTTATGCATGAACGTAATGCTC
trnH2 CGCGCATGGTGGATTCACAATCC
(Tate & Simpson 2003)

Botanical DNA Authentication PCR

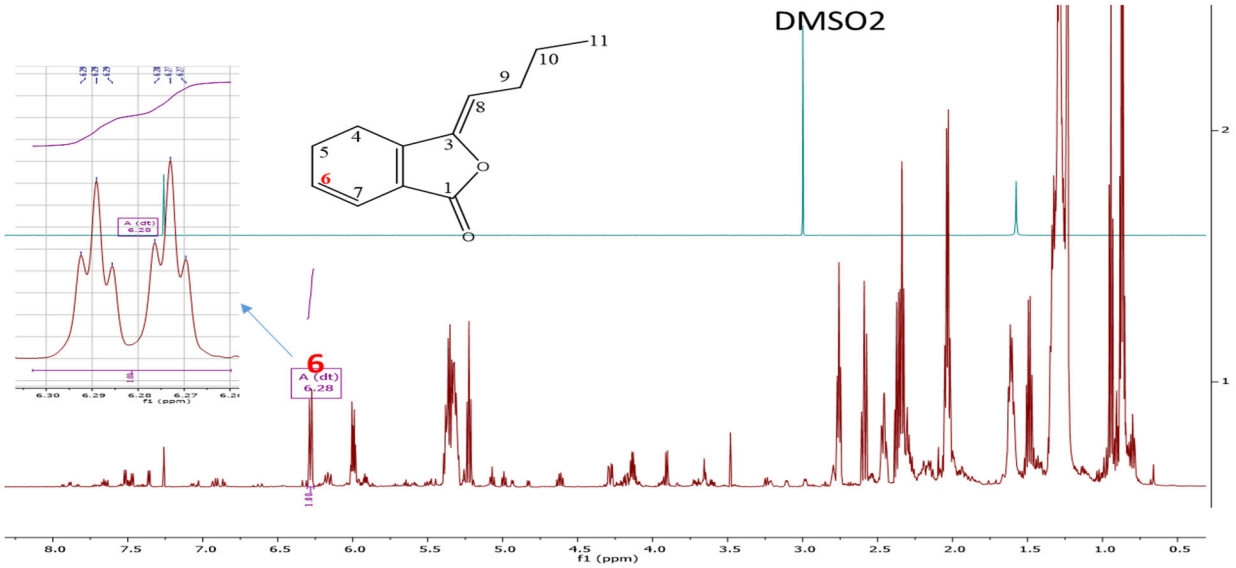
Primer Reactions:
1,5 = ITS5.8 JA F1 + ITS4 (*ITS2*)
2,6= ITS 5A + ITS5.8 JA R6 (*ITS1*)
3,7= 3F_KIM + 1R_KIM (*matK*)
4 = psbAF + trnH2 (*psbA-trnH*)



Botanical Sample Authentication Results

Sample	Description	Barcode Loci	Mixture Detected	Correct Species
Angelica sinensis **(BC440)**	**Whole Root**	**ITS1,2; trnH-psbA; matK**	**No**	**Yes**

S2. (*Z*-)Ligustilide qHNMR Quantification Using $DMSO_2$ as External Calibrant (EC)



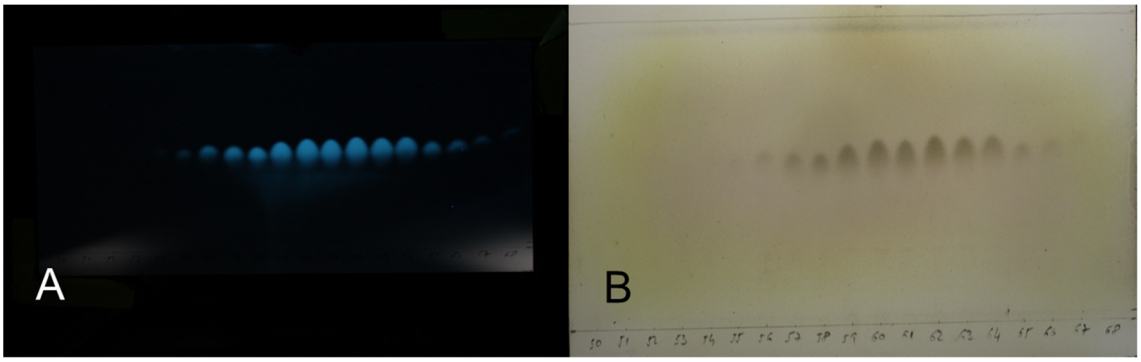
Representative quantitative ^{1}H NMR spectrum ($CDCl_3$, 360 MHz) of the supercritical fluid extract (SFE) of *A. sinensis* essential oil (EO). The table below provides the quantification of (*Z*-)ligustilide (**1**) using $DMSO_2$ as external calibrant (EC).

	$P_{analyte}$	$I_{analyte}$	I_{EC}	$N_{analyte}$	N_{EC}	$M_{analyte}$	M_{EC}	$W_{analyte}$	W_{EC}	P_{EC}
SFE EO1	**15.55%**	5577.01	1618.70	1	6	190.238	94.13	26.8	0.1	0.9973
SFE EO2	**14.26%**	5325.30	1618.70	1	6	190.238	94.13	27.9	0.1	0.9973
SFE EO3	**14.57%**	5362.70	1618.70	1	6	190.238	94.13	27.5	0.1	0.9973

$$P_{analyte} = \frac{I_{analyte} \times N_{EC} \times M_{analyte} \times W_{EC} \times P_{EC}}{I_{EC} \times N_{analyte} \times M_{EC} \times W_{sample}}$$

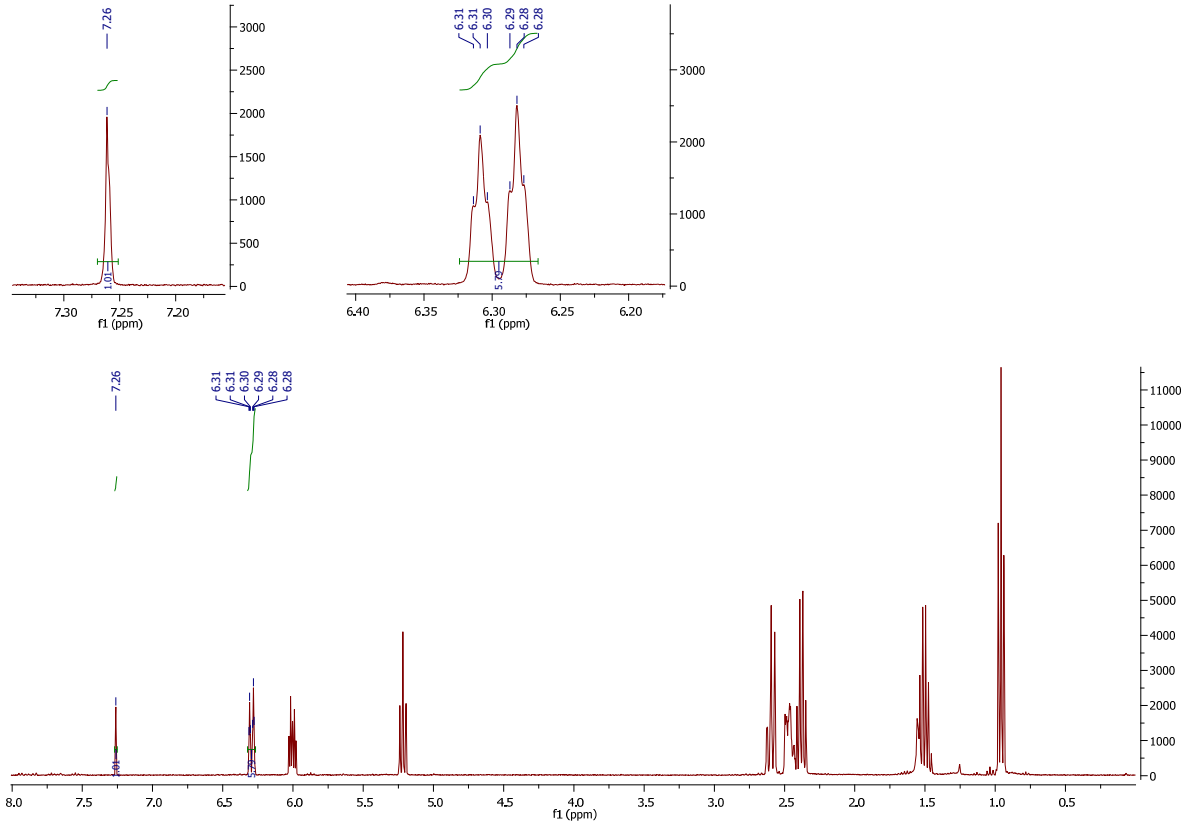
P - % purity of the analyte; I - absolute integral value; N - Number of protons in the integrated signal; M- Molar mass; W - Gravimetric weight (in mg); EC – External calibrant; SFE EO1, SFE EO2 and SFE EO3 – SFE essential oil samples.

S3. TLC Identification of the (*Z*)-Ligustilide (1) Fractions



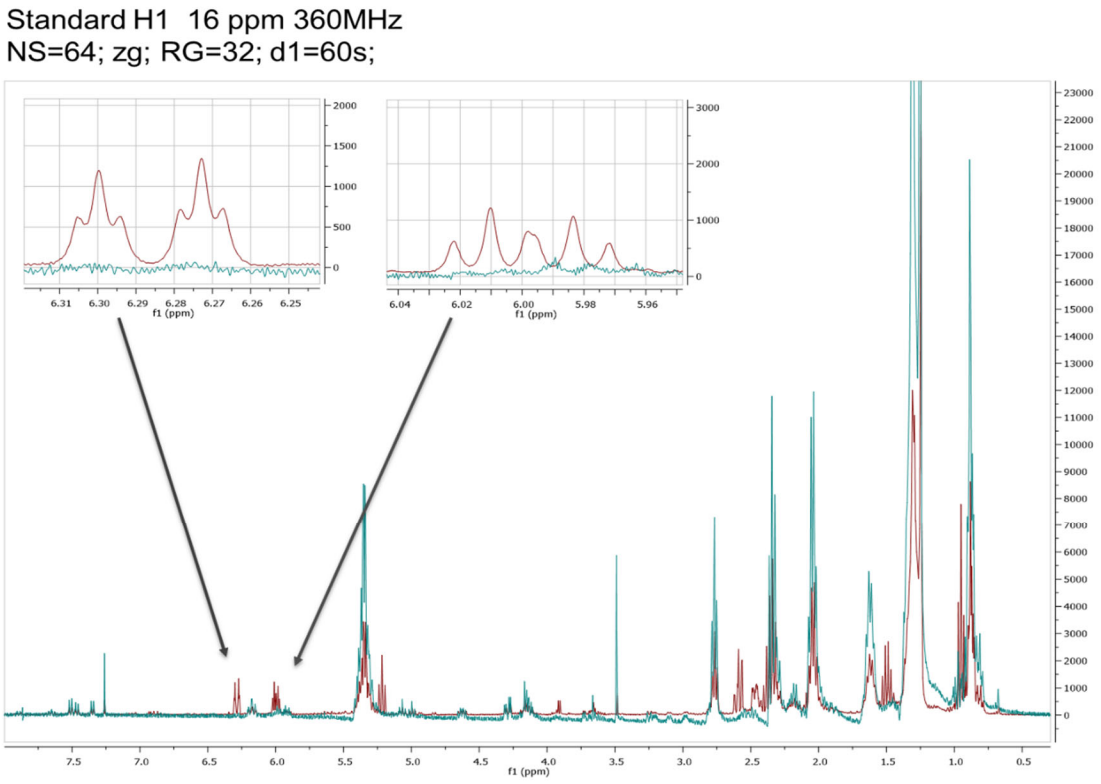
TLC identification of (*Z*-)ligustilide fractions obtained by high speed countercurrent chromatography (HSCCC). Using normal phase TLC, with the upper phase of *n*-hexane-EtOAc-MeOH-water (9:1:9:1, v/v) as mobile phase, a single spot at R*f* value of 0.45 exhibited a blue-gray color under UV at 356 nm (A) and it was clearly visible, after spraying with vanillin-H_2SO_4 reagent (B).

S4. The ^{1}H NMR Spectrum of (*Z*-)Ligustilide (1) Purified from the SFE Essential Oil of *A. sinensis* by HSCCC in a Single Step (93.4% purity)



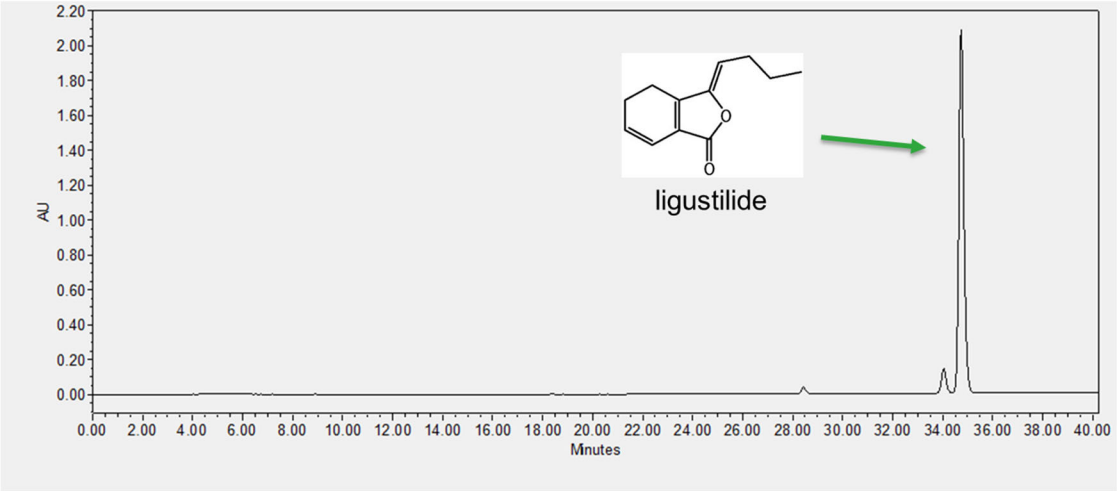
^{1}H ($CDCl_3$, 360 MHz) NMR spectra of (*Z*-)ligustilide separated from SFE essential oil by HSCCC in a single step (93.4% purity determined by qHNMR with $DMSO_2$ as an external calibrant).

S5. NMR Spectra of the (*Z*-)Ligustilide (1) Knockout Extract (KOE) of *A. sinensis* Essential Oil Compared with that of the Purified (*Z*-)Ligustilide Fraction.



^{1}H NMR ($CDCl_3$, 360 MHz) spectra of (*Z*-)ligustilide knockout extract (KOE) of *Angelica sinensis* essential oil obtained by HSCCC (blue) overlaid with the (*Z*-)ligustilide fraction (red).

S6. Preparative HPLC Purification of (*Z*-)Ligustilide (1) after HSCCC Pre-purification



Preparative HPLC was conducted in order to obtain highly purified (Z-)ligustilide (98.2% w/w determined by qHNMR, with $DMSO_2$ as an external calibrant) suitable for the investigation of the degradation products by mass balance-qHNMR methodology.

Flow rate: 1.6 mL/ min

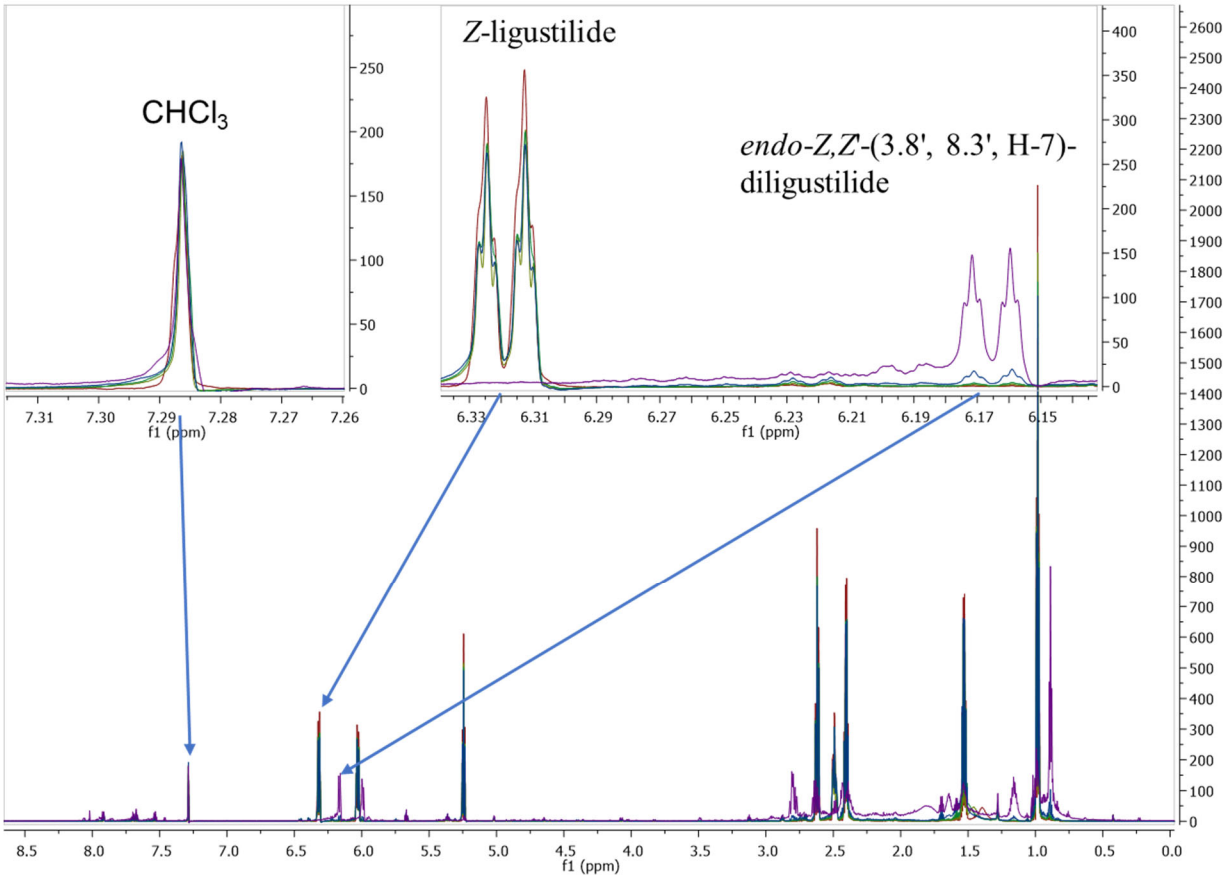
Solvent system: 0-2 min: 87% to 13% acetonitrile in H_2O;

2-10 min: 50% to 50% acetonitrile in H_2O;

10-35 min: 5% to 95% acetonitrile in H_2O;

36-40 min: 87% to 13% acetonitrile in H_2O.

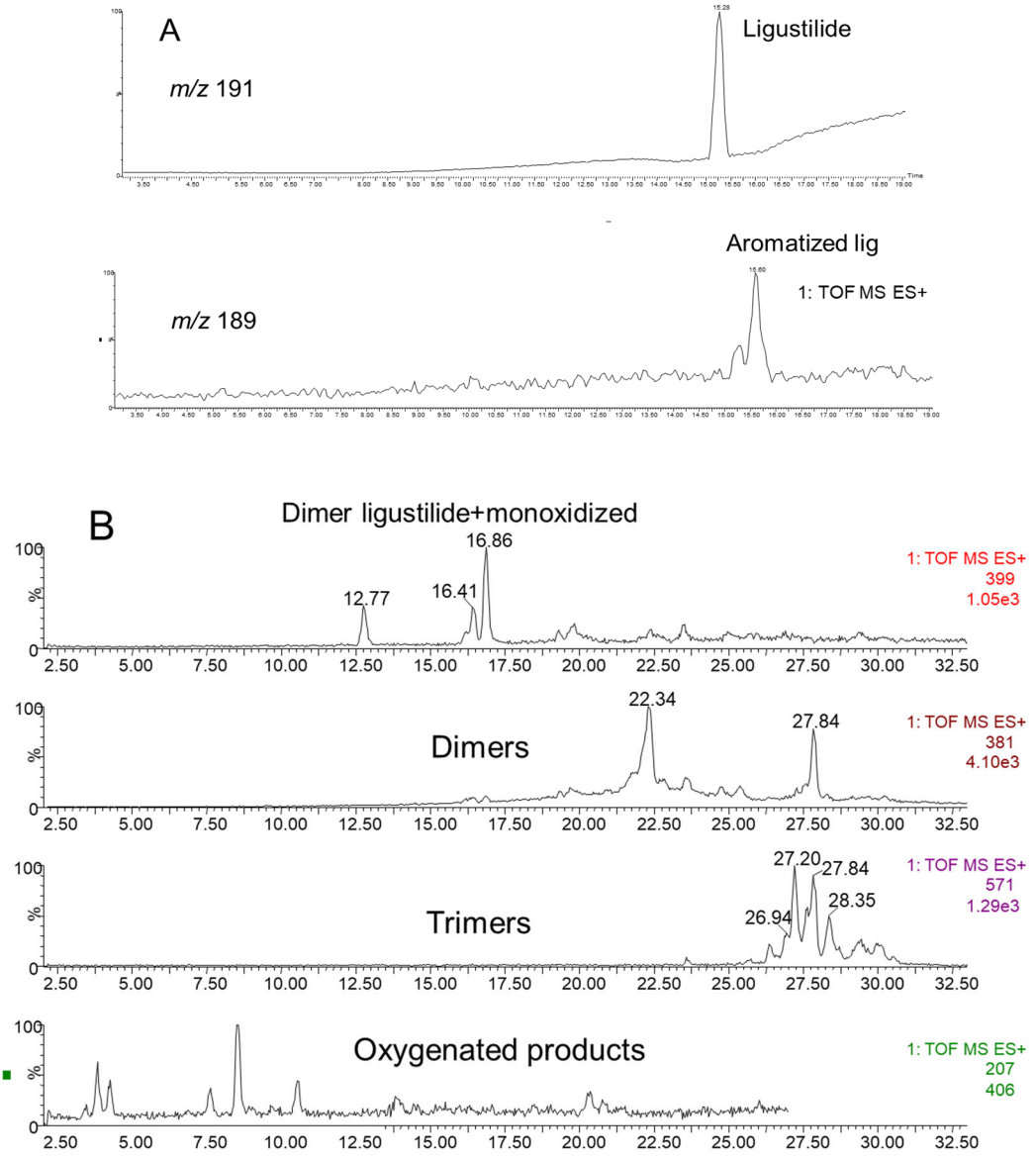
S7. Time-Course of the Degradation of (Z-)Ligustilide (1) Forming Its Main Degradation Product, *endo-Z,Z'*-(3.8',8.3', H-7)-diligustilide (*m/z* 381) in $CHCl_3$



^{1}H NMR spectra ($CDCl_3$, 800 MHz) of the chemical transformation of *(Z-)*ligustilide into *endo-Z,Z'*-(3.8',8.3', H-7)-diligustilide.

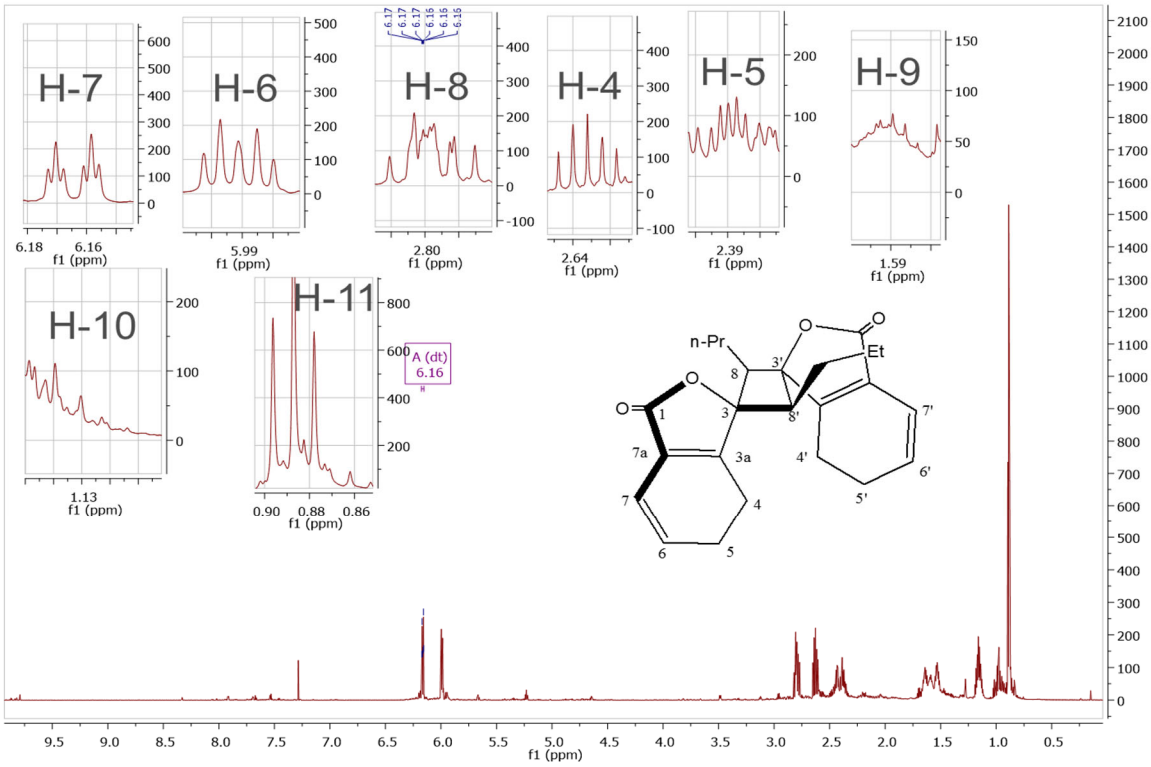
The time-course of the degradation of C1 is reflected by the colors: day 1 (red), day 3 (light green), day 14 (dark green); day 30 (blue), and day 41 (violet).

S8. LC-MS Chromatograms of Days 1 and 41 During the Characterization of the (Z-)Ligustilide (1) Degradation Products



On the first day, LC-MS chromatogram showed two main components (**A**): the top spectrum with the protonated molecule at *m/z* 191 and the bottom spectrum with the protonated molecule at *m/z* 189. Comparing the HNMR data with the LC-MS data showed the presence of **1** and **3**. After 41 days, the LC-MS chromatograms (**B**) indicated presence of oxygenated products (*m/z* 207) and dimers at *m/z* 381. Interestingly, multiple peaks eluting between 26 and 30 min with the protonated molecules at *m/z* 571 were also observed. The elemental composition of these ions $C_{36}H_{42}O_6$ suggests that they might be trimers of (*Z*-)ligustilide. **1**: (*Z*)-ligustilide (*m/z* 190), **2**: *endo-Z,Z'*-(3.8',8.3', H-7)-diligustilide (*m/z* 381), **3**: (*Z*)-butylidenephthalide (*m/z* 189), **4**: phthalic acid anhydride (*m/z* 148), **5**: butyraldehyde.

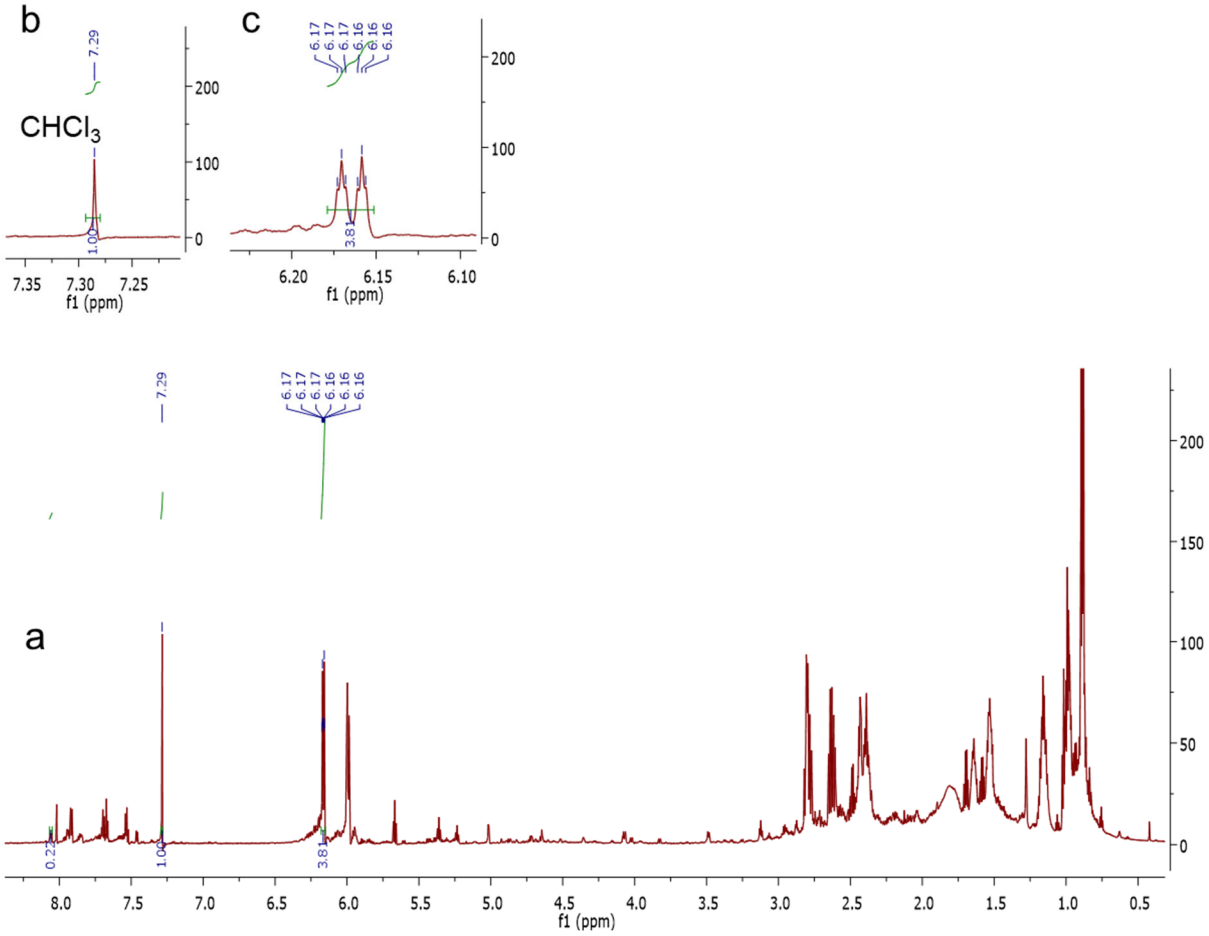
S9. The ^{1}H NMR Spectrum ($CDCl_3$, 800 MHz) of *endo-Z,Z'*-(3.8', 8.3', H-7)-Diligustilide



Bruker 800 MHz Avance NMR spectrometer with 5mm RT BBO probe; T=298K, NS 64, RG 128, D1 60sec, P1 9.22usec.

The main chemical transformation product of C2 was the dimer **2** (*m/z* 381), characterized by a doublet of triplets at δ 6.165 (1H, dt, *J* = 1.9, 1.9, 9.5, H-7).

S10. The ^{1}H NMR Monitoring ($CDCl_3$, 800 MHz) of the Degradation of (*Z*-)Ligustilide (1) Forming *endo-Z,Z'*-(3.8',8.3', H-7)-diligustilide as Main Degradation Product during 41 Days and Calculation of the Target Signal to Residual Solvent (TSRS) Ratio



a: ^{1}H NMR spectra ($CDCl_3$, 800 MHz) of the degradation of (*Z*-)ligustilide (**1**) into the main degradation product *endo-Z,Z'*-(3.8',8.3', H-7)-diligustilide with a TSRS of 3.81.

b: $CHCl_3$ Integral, absolute: 1857.47; f1 from: 7.293 to 7.280 ppm

c: *endo-Z,Z'*-(3.8',8.3', H-7)-diligustilide, identified by its doublet of a triplet at δ 6.165 (1H, dt, *J* = 9.5 and 1.9 Hz; H-7), Integral, absolute: 7079.83; f1 from 6.179 to 6.151 ppm

The ratio of the integral value of target signal: residual solvent (TSRS) = 7079.83:1857.47 = 3.81

S11. Time Course Analysis of (Z-)Ligustilide (1) and Its Identified Degradation Products in Sealed NMR Tubes in the Presence of Ar

Table S11-1. Course time of (*Z*)-ligustilide (**1**) and its identified degradation products in sealed NMR tubes in presence of air (C1), quantified by calculating the ratio of the integral value of its target signal to that of the residual solvent ($CHCl_3$) in the sample.

Substance	**1**	**2**	**3**	**4**	**5**
Time course					
day 1	8.43	0.02	0.16	0	0.06
day 3	7.91	0.09	0.21	0.01	0.14
day 14	7.54	0.13	0.26	0.01	0.19
day 30	6.62	0.49	0.34	0.03	0.25
day 41	0	3.81	0.48	0.22	0.54

1: (*Z*)-ligustilide, **2**: *endo-Z,Z'*-(3.8',8.3', H-7)-diligustilide, **3**: (*Z*)-butylidenephthalide, **4**: phthalic acid anhydride, and **5**: butyraldehyde.

Table S11-2. Course time of (*Z*)-ligustilide (**1**) and its identified degradation products in sealed NMR tubes in presence of Ar (C2), quantified by calculating the ratio of the integral value of its target signal to that of the residual solvent ($CDCl_3$) in the sample.

Substance	**1**	**2**	**3**	**4**	**5**
Time course					
day 1	13.25	0.18	0.16	0	0.04
day 3	13.16	0.30	0.15	0	0.05
day 14	13.39	0,51	0.19	0	0.08
day 30	13.21	2.25	0.24	0	0.13
day 41	0.20	13.05	0.33	0.04	0.30

1: (*Z*)-ligustilide, **2**: *endo-Z,Z'*-(3.8',8.3', H-7)-diligustilide, **3**: (*Z*)-butylidenephthalide, **4**: phthalic acid anhydride, and **5**: butyraldehyde.