Chemo-enzymatic cascade for the generation of fragrance aldehydes

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Experimental Details General: ATP was obtained from Roche Diagnostics. NADPH and MES were purchased from Roth, IPTG from Serva, and $MgCl_2$ from Merck. HPLC–MS grade acetonitrile was purchased from J.T.Baker/Avantor Performance Materials, Deventer, The Netherlands. All other chemicals were obtained from Sigma–Aldrich or Roth and used without further purification. *E. coli* cells were cultivated in an RS 306 shaker (Infors, Bottmingen, Switzerland) and Multitron shakers (Infors AG), and the cells were harvested with an Avanti J-20 centrifuge (Beckman Coulter). Cell pellets were disrupted by a 102C converter with a Sonifier 250 (Branson, Danbury, CT), and the cell-free extract was obtained by centrifugation in an Ultracentrifuge OptimaLE80K (Beckman). Enzymes were purified using a gravity flow protocol according to instructions by the supplier. The protein-containing fractions were pooled, the buffer exchanged for 50 mM MES buffer, pH 7.5, containing 10mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT, and aliquots of the protein solution were shock frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined with a Nanodrop 2000c spectrophotometer (Thermo Scientific). Reactions were performed on a Thermomixer comfort (Eppendorf). HPLC/MS analysis was carried out on an Agilent Technologies 1200 Series equipped with G1379B degasser, G1312B binary pump SL, G1367C HiP-ALS SL autosampler, aG1314C VWD SL UV detector, G1316B TCC SL column oven, and aG1956B MSD mass selective detector. For HPLC/UV analysis an Agilent Technologies 1100 Series equipped with aG1379A degasser, 1200 Series Quaternary pump G1379A, G1367A autosampler, G1330B autosampler thermostat, G1316A thermostated column compartment, and aG1315B Diode Array Detector was used. The following gradient was used for 1: 0-5.00 min/25%-55% ACN; 5.00-7.20 min/55%-70% ACN; 7.20-7.50 min/70-90% ACN; 7.50-9.00 min/90% ACN; 9.01-15.00 min/25% ACN. Compounds were detected at 254 nm using a Diode Array Detector (DAD).

Protein Expression: A codon-optimized synthetic gene coding for N-terminally HIS-tagged sequence from XP_955820.1 was ordered from GenScript custom cloned into the pETDUET1 vector with phosphopantetheinyltransferase from *E. coli* (CAQ31055.1) in the upstream multiple cloning site. After sequencing, *E. coli* K12 MG1655 RARE was transfected with the plasmid pETDUET1:*Ec*PPTaseHT*Nc*CAR and colonies selected on LB/Amp. For protein expression, the autoinduction protocol was used. After 24 h at 20°C, the cells were harvested by centrifugation and stored at -20°C for protein purification or used for biotransformations.

Protein Purification: Thawed cells were disrupted by sonication and the protein purified by nickel affinity chromatography. After purification the resulting proteins, *Nc*CAR and *Nc*CAR:OYE1, were desalted via a PD 10 column and stored at -80°C in 50 mM MES buffer, pH 7.5, containing 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT.

Spectrophotometric Assay: An NADPH depletion assay was used to determine the activity on selected substrates of *Nc*CAR. Therefore, several carboxylic acids were dissolved in equal amounts of KOH. The

assay composition was as follows: the substrate (10 µL of 100 mM stock solution) was added to 160 µL of Tris-HCl buffer (100 mM, pH 7.5, containing 10 mM $MgCl_2$). Subsequently, 10 µL NADPH (10 mM in water), 10 µL of ATP (20 mM in water), and 10 µL of CAR preparation from Ni-affinity chromatography (0.7-1.5 mg mL^{-1}) were added. The depletion of NADPH was followed on a Synergy Mx Platereader (BioTek) at 340 nm and 28°C for 10 min. Appropriate blank reactions were carried out in parallel, and each reaction was carried out at least in triplicate.

ABAO Assay to screen resting cell biocatalysts in high-throughput format. Ninety-six-deep-well plates (DWP) filled with initial growth medium (LB 0.8G) containing 100 µg/mL ampicillin were inoculated with colonies of *Nc*CAR in *E. coli*. Specifically, 8 wells were used for negative control (e.g., pETDuet:*Ec*PPTase*Ni*CARΔA, an A-domain deletion variant of *Nocardia iowensis* CAR, 8 wells sterile control, and 4 wells *E. coli* RARE expressing each CAR. These master plates were incubated for 5 h at 37 °C and 1000 rpm on a Heidolph Titramax 1000 until cells were in the exponential growth phase. Freshly prepared 96-well DWPs filled with 800 µL LB-5052 containing 50 µg/mL ampicillin were inoculated from the master plates. Expression plates were incubated for 4 h at 37 °C and moved into a 20 °C incubator for expression ON. After 18 h total time for growth and expression, cells were harvested by centrifugation at 3220 x g for 20 min, and the supernatant was removed. The remaining cell pellet was resuspended in 400 µl of conversion buffer (50 mM HEPES pH7.5, 10 mM $MgSO_4$, 50 mM glucose, and 5 mM of substrate in KOH). The plates were incubated at 20 °C and 1000 rpm for 1 h. Next, 400 µl of the ABAO solution (sodium acetate buffer 100 mM, pH 4.5 containing 5% DMSO and 10 mM of ABAO were added and the plates were centrifuged at 3220 x g for 30 min. One hundred fifty microliters of supernatants were transferred to a fresh plate via multichannel pipette and analyzed at 380 nm.

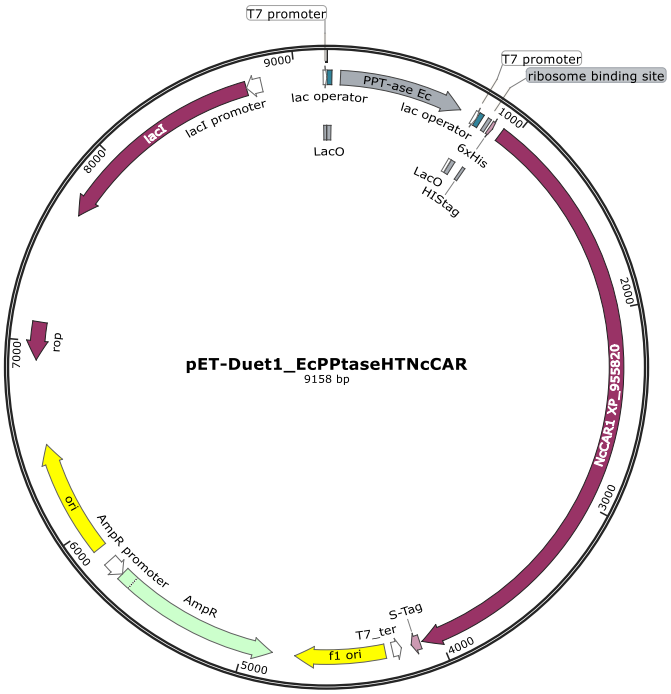


Figure S 1: Expression vector pETDuet1_EcPPTaseHTNcCAR [1]

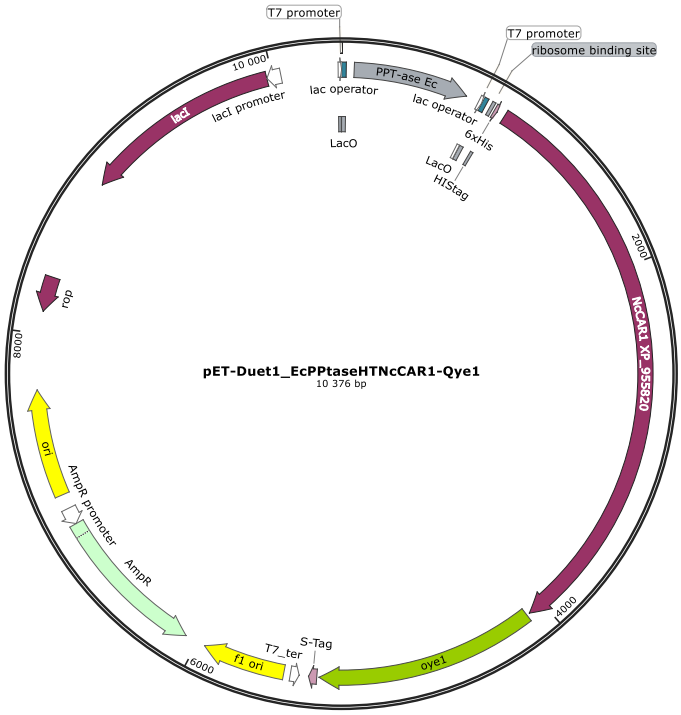


Figure S 2: Illustration of the expression vector pETDuet1_EcPPTaseHTNcCAR_Oye1. The plasmid encodes one T7 promoter in front of both expression cassettes, the T7 terminator behind the second expression cassette, an ampicillin resistance gene, the lacI gene for the two lac operators in front of every gene, his tag at the 5' end of the NcCAR and a pBR322-derived high copy origin of replication. The copy number is downregulated by the product of the rop gene and reaches a medium copy number. The two genes encoding EcPPTase and NcCAR were cloned in the two-consecutive multiple cloning sites with NcoI and HindIII for EcPPTase and NdeI and XhoI for NcCAR.

A 166.3 kDa fusion protein consisting of NcCAR and OYE1 is produced.

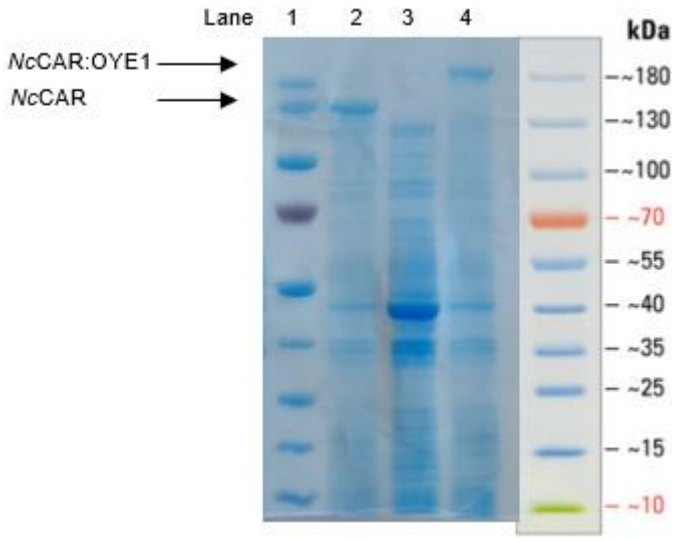


Figure S 3: Expression of *Nc*CAR and *Nc*CAR:OYE1 fusion protein in *E. coli* RARE 30 µg of cell-free extract per lane, lane 1: PageRuler Prestained Protein Ladder Mix, lane 2: *E. coli* RARE [pETDuet1_*Ec*PPTase_HT*Nc*CAR], lane 3: *E. coli* RARE [pJ401_OYE1], lane 4: *E. coli* RARE pETDuet1_*Ec*PPTase_*Nc*CAR:Oye1]

As visible in Figure S 3 *Nc*CAR and the *Nc*CAR:OYE1 fusion protein are expressed in soluble form in *E. coli* RARE. *Nc*CAR corresponds to a size of 120.9 kDa, OYE1 has 45 kDa, and *Nc*CAR:OYE1 has 166.3 kDa. OYE1 was expressed from a second plasmid with an additional antibiotic resistance, but the fusion protein proved to be more stable in the cell.

Cloning of *Nc*CAR was described previously [1].

Cloning of *Nc*CAR:OYE1: The vector containing the sequence for the fusion protein *Nc*CAR:OYE1 was constructed with pETDuet1_EcPPTase_HTNcCAR and pJExpress401_OYE1 as templates. The fragment containing OYE1 was amplified with the primers Oye1_fwd_LinkerNcCAR and Qye1_rev_Linker_pETDuet from the plasmid pJExpress401_OYE1. The PCR was performed using the following thermal cycle parameters: initial denaturation at 98 °C for 30 s, followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 2 min with the Q5™ High-Fidelity DNA Polymerase. The PCR products were purified via agarose gel electrophoresis, and the desired fragments were extracted using the GeneJET Gel Extraction Kit (Thermo Scientific). The vector backbone with the *Nc*CAR and the *Ec*PPTase was amplified with the primers pETDuet1:*Ec*PPTase fwd and *Nc*CAR_rev_Linker_OYE1 with pETDuet1_*Ec*PPTase_HT*Nc*CAR as template. The PCR was performed using the following thermal cycle parameters: initial denaturation at 98 °C for 30 s, followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 30 s, extension at 72 °C for 3 min 30 s, and a final extension at 72 ◦C for 7 min with the Q5™ High-Fidelity DNA Polymerase. The PCR products were purified via agarose gel electrophoresis.

Both products were assembled *via* Gibson assembly according protocol [2]. After transformation and plasmid preparation, the sequence was validated by Sanger sequencing.

Table S 1: Primers used in this study

Oye1_fwd_LinkerNcCAR (52-mer):
GGAACTTTtccggtagcgcagcgggcatgtcatttgtaaaagattttaagcc
Qye1_rev_Linker_pETDuet (55-mer):
CGCAGCAGCGGTTTCTTTACCAGACTCGAGttacttttttgtcccagcctaatttg
pETDuet1:EcPPTase fwd (25-mer):
CTCGAGTCTGGTAAAGAAACCGCTG
NcCAR_rev_Linker_OYE1 (48-mer):
caaatgacatgcccgctgcgctaccggaAAAGTTCCATTGGCGACACC

Reaction control vial HPLC:

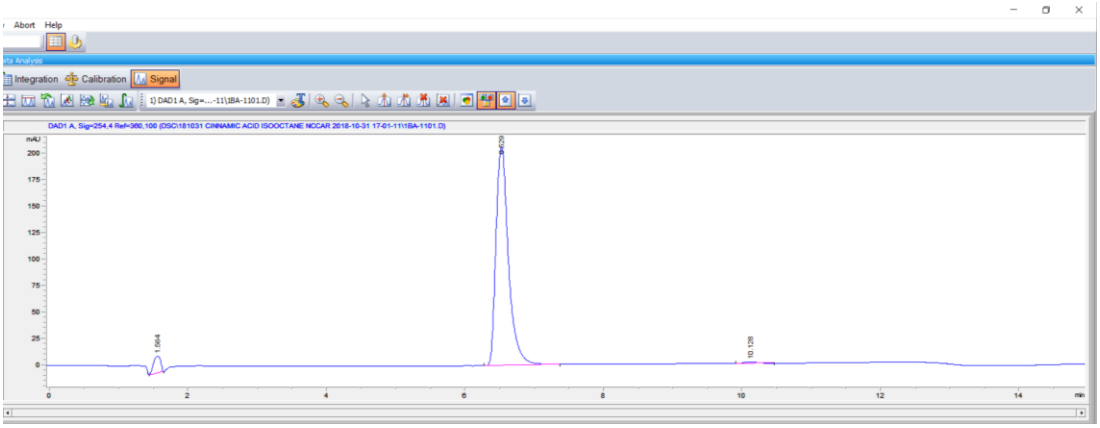


Figure S 4: Conversion 25 mM isolated CA with Isoocatane, 0 h, 6.6 min ***1b***

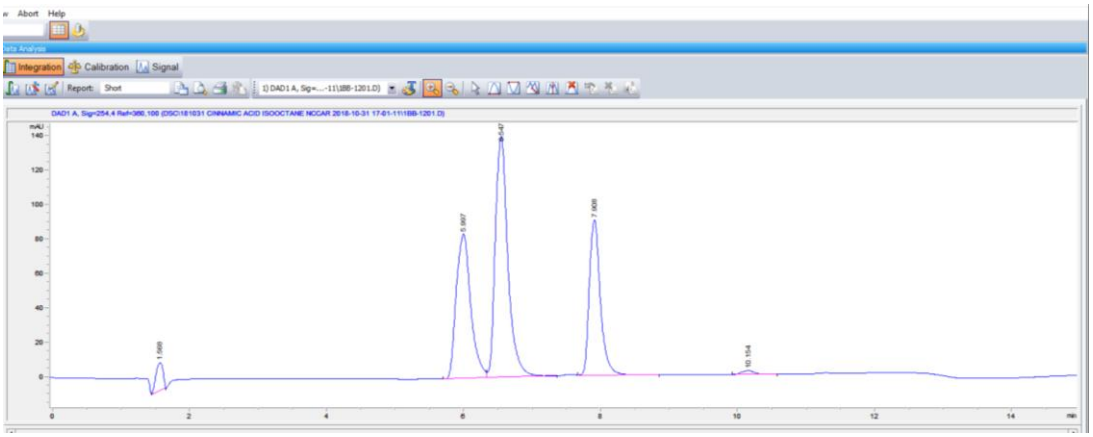
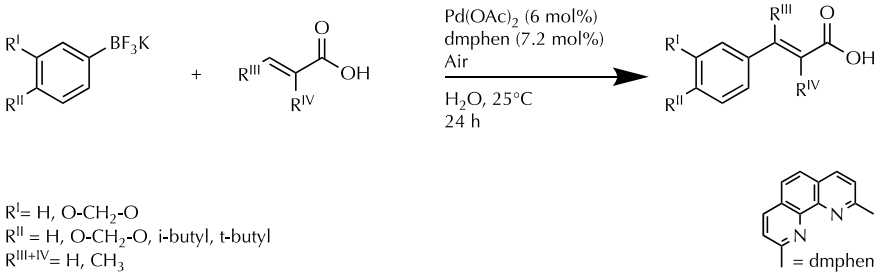


Figure S 5: Conversion 25 mM with Isooctane, 1 h, 5.9 min ***1e,*** *6.6 min* ***1b,*** *7.9 min* ***1c***

Heck coupling protocol of cinnamic acids with potassium aryltrifluoroborates



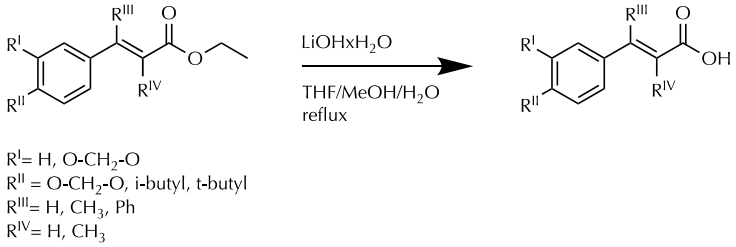
A 6.7 mM catalyst stock solution was prepared by adding $Pd(OAc)_2$ (5.99 mg, 26.7 µmol, 6.7 mM), 2,9-dimethyl-1,10-phenanthroline (6.66 mg, 8.0 mM), and deionized water (4.00 mL) to an 8 mL glass vial. The mixture was stirred at room temperature for approximately 2 h until a homogeneous, orange suspension was formed. The suspension was sonicated 5 min at room temperature to further homogenize it. The catalyst stock suspension showed no loss in activity after storage at room temperature over a period of 5 days.

The reactions were performed in triplicates in a 10 mM scale (concentration based on limiting substrate) in 2 mL of deionized water.

A 30 mL glass vial with magnetic stirrer and septum was charged with potassium aryltrifluoroborate (0.02 mmol, 1 equ.) and olefin (0.04 mmol, 2 equ.), water and catalyst stock suspension (180 µL, 6 mol%). An air balloon was applied, and the reaction was stirred at 25°C for 24 h. The reaction process was followed by GC analysis.

The reaction mixture was acidified with 2 M HCl (200 µL) and extracted into internal standard (5.00 mL, 1 mM methyl benzoate in ethyl acetate). One thousand microliters of the organic layer was dried over anhydrous Na_2SO_4, centrifuged 2 min at 13 krpm, and flashed through a pad of celite and cotton wool in a Pasteur pipette. Yields were determined by GC-FID based on standard calibration of reference compounds.

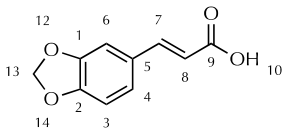
Synthesis of cinnamic acid derivatives – reference compounds



A reaction flask with magnetic stirrer and reflux condenser was supplemented with ethyl ester (1 eq., 50 mM), $LiOHxH_2O$ (1.5 eq.) and a solvent mixture (THF:MeOH:H_2O = 4:1:1, 20 mL/mmol). The reaction was stirred at reflux until complete consumption of ester (verified by TLC).

The reaction was acidified with 2 M HCl. After addition of brine, the aqueous layer was extracted 3 times with diethyl ether. The organic layers were combined, washed with brine, dried over Na_2SO_4, filtrated and concentrated under reduced pressure to yield the carboxylic acids.

Synthesis of (2*E*)-3-(1,3-benzodioxol-5-yl) acrylic acid (3b)



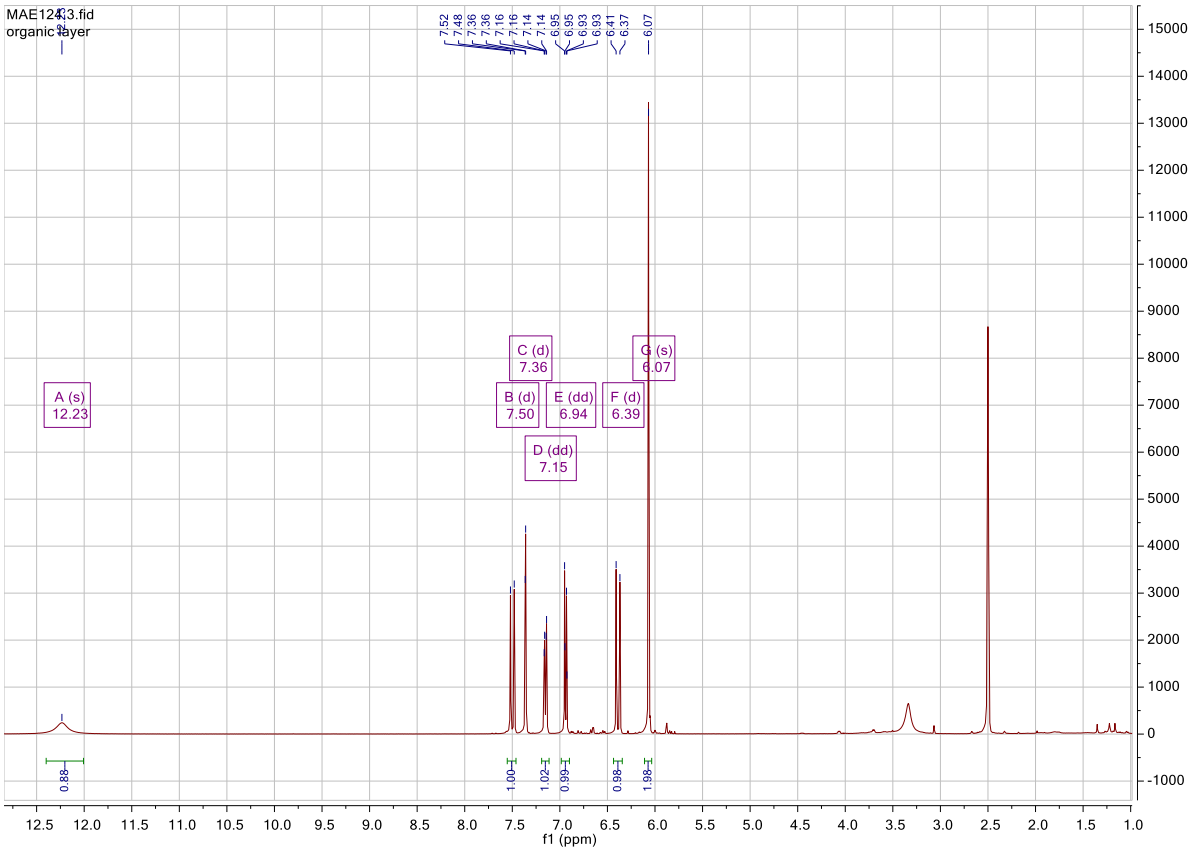
D1

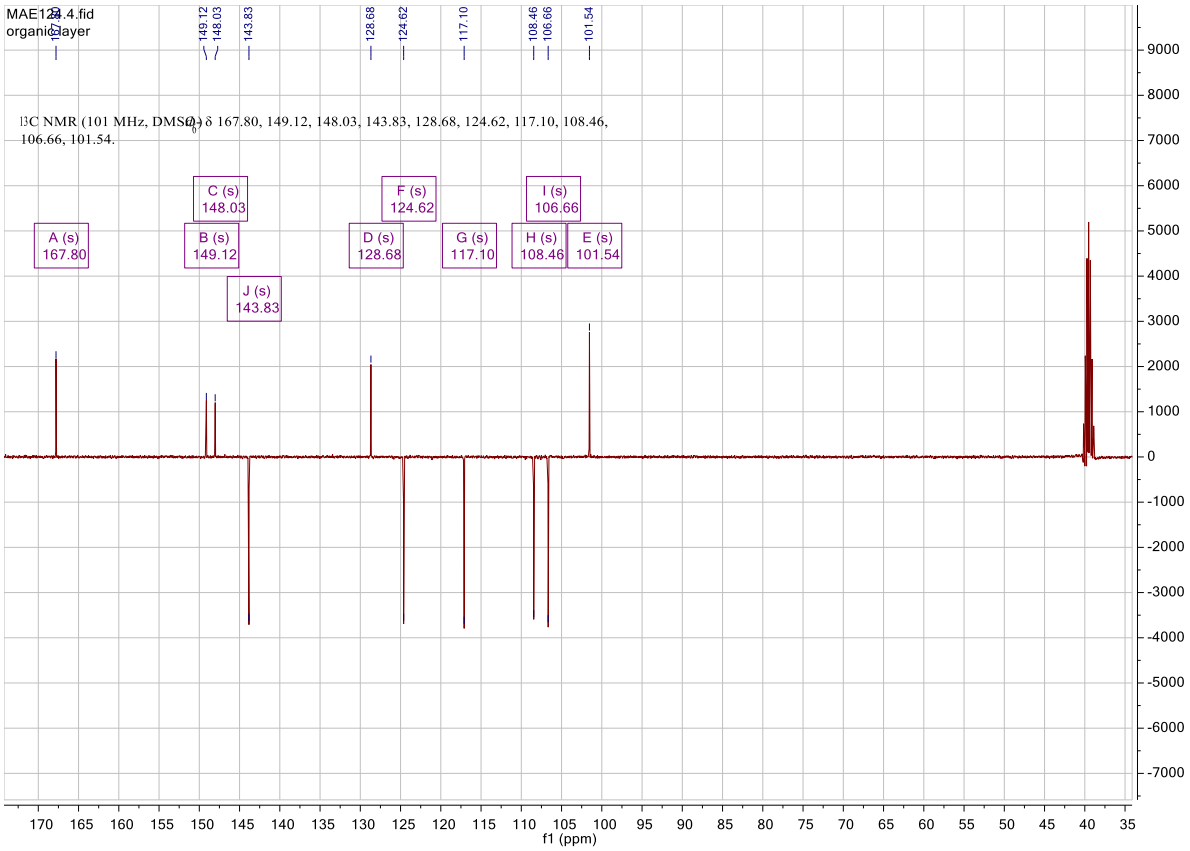
The synthesis was performed according to general procedure B. A1 (0.3490 g, 1.58 mmol, 1 eq.), $LiOHxH_2O$ (0.2331 g, 3.5 eq.), and THF/MeOH/H_2O solvent mixture (31.7 mL) were used. D1 was obtained as off white solid (0.2605 g, 1.36 mmol, 86% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.23 (s, 1H), 7.50 (d, *J* = 15.9 Hz, 1H), 7.36 (d, *J* = 1.8 Hz, 1H), 7.15 (dd, *J* = 8.1, 1.7 Hz, 1H), 6.94 (dd, *J* = 8.2, 1.5 Hz, 1H), 6.39 (d, *J* = 15.9 Hz, 1H), 6.07 (s, 2H).

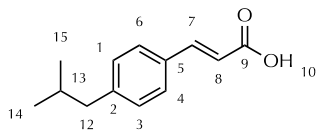
^{13}C NMR (101 MHz, DMSO-d_6) δ 167.80 (s, 1C), 149.12 (s, 1C), 148.03 (s, 1C), 143.83 (s, 1C), 128.68 (s, 1C), 124.62 (s, 1C), 117.10 (s, 1C), 108.46 (s, 1C), 106.66 (s, 1C), 101.54 (s, 1C).

GC-MS: 192.07 (100, M^+), 191.08 (48), 175.08 (19), 146.07 (19), 145.05 (25), 89.05 (44), 63.04 (33), 62.04 (18).




MAE124.4.fid
organic layer
167.80
149.12
148.03
143.83
128.68
124.62
117.10
108.46
106.66
101.54
13C NMR (101 MHz, DMSO-d6) δ 167.80, 149.12, 148.03, 143.83, 128.68, 124.62, 117.10, 108.46, 106.66, 101.54.
A (s) 167.80
B (s) 149.12
C (s) 148.03
D (s) 128.68
E (s) 101.54
F (s) 124.62
G (s) 117.10
H (s) 108.46
I (s) 106.66
J (s) 143.83
f1 (ppm)

Synthesis of (2*E*)-3-(4-isobutylphenyl) acrylic acid (5b)



D2

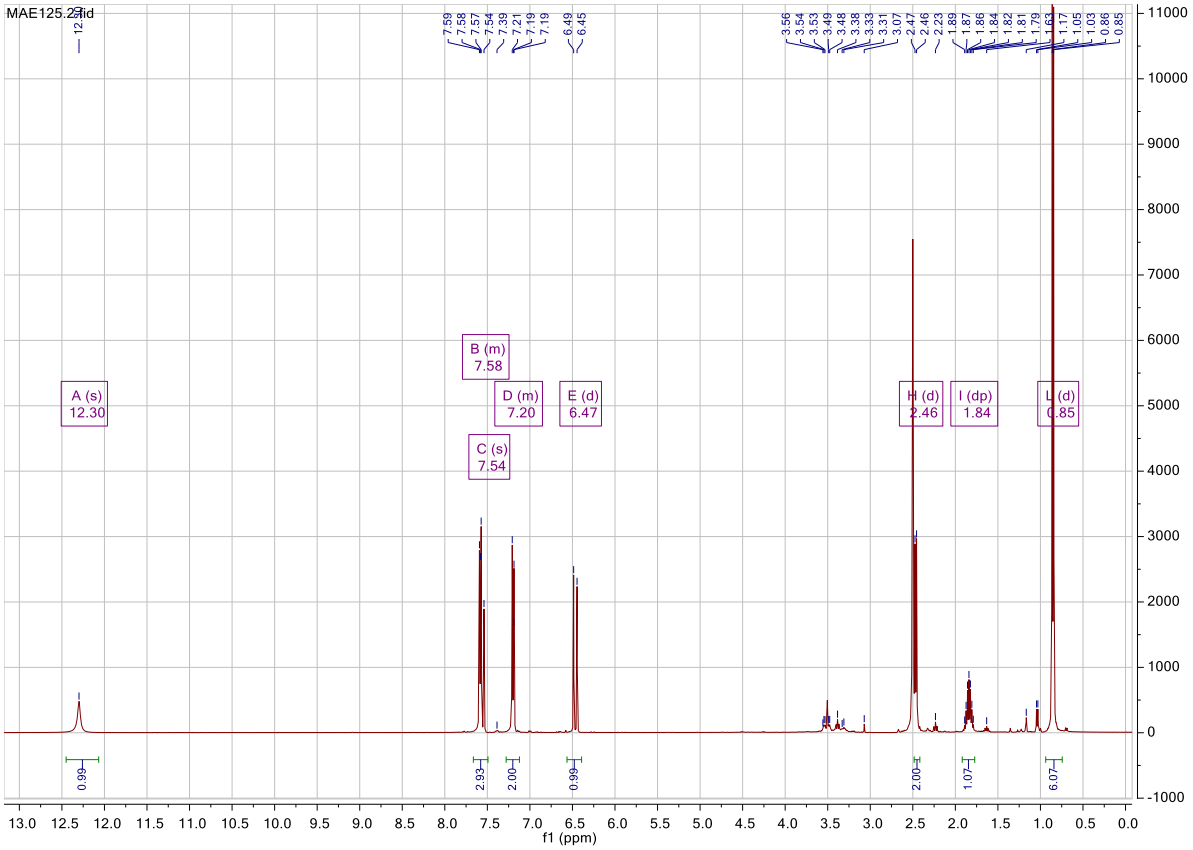
The synthesis was performed according to general procedure B. A2 (0.3045 g, 1.31 mmol, 1 eq.), LiOHxH_2O (0.1928 g, 3.5 eq.), and THF/MeOH/H_2O solvent mixture (26.2 mL) were used. D1 was obtained as off white solid (0.2655 g, 1.32 mmol, 99% yield).

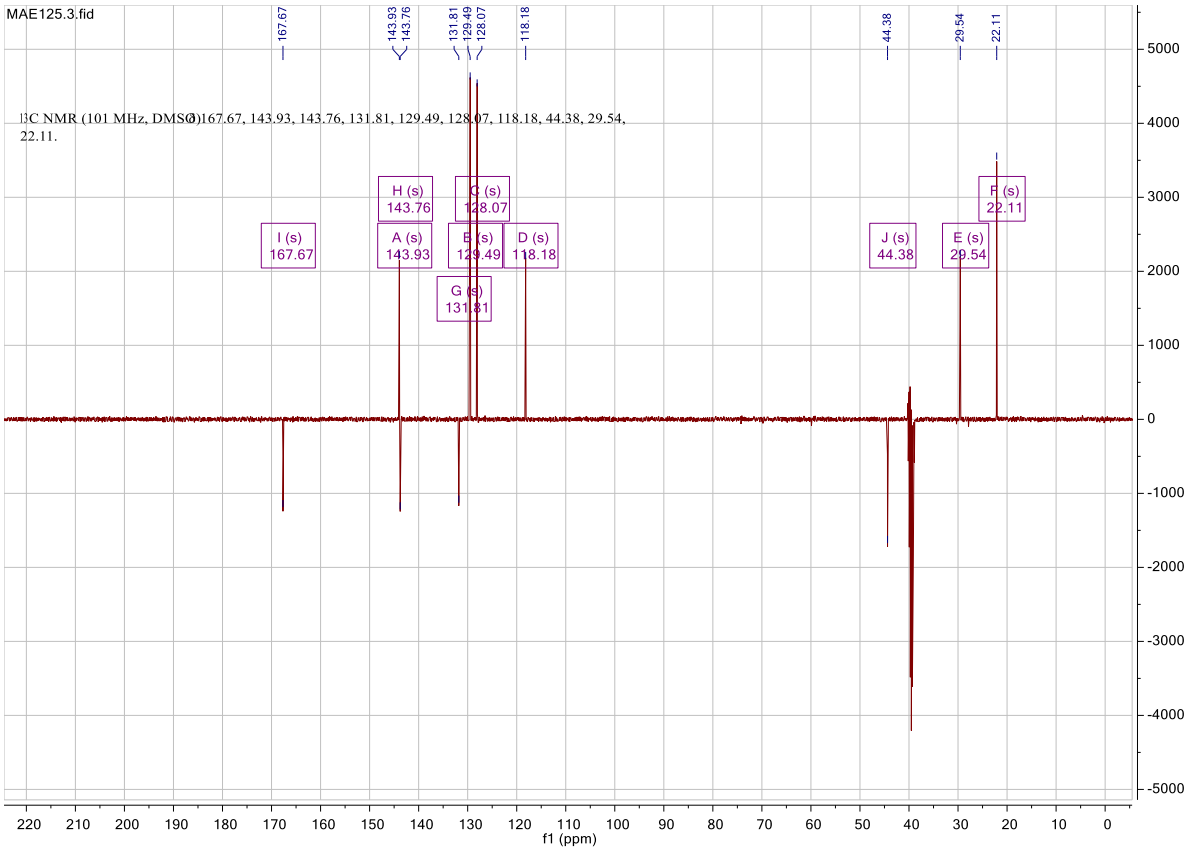
M_p: 159 -163°C (Apollo Scientific 168 – 169°C)

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.30 (s, 1H), 7.58 (d, *J* = 8.0 Hz, 2H), 7.56 (d, *J* = 15.9 Hz, 1H), 7.20 (d, *J* = 7.9 Hz, 2H), 6.47 (d, *J* = 16.0 Hz, 1H), 2.47 (d, *J* = 7.2 Hz, 2H),1.83 (m, 1H), 0.85 (d, *J* = 6.6 Hz, 6H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 167.67 (s, 1C), 143.93 (s, 1C), 143.76 (s, 1C), 131.81 (s, 1C), 129.49 (s, 2C), 128.07 (s, 2C), 118.18 (s, 1C), 44.38 (s, 1C), 29.54 (s, 1C), 22.11 (s, 2C).

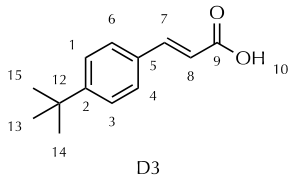
GC-MS: 204.13 (35, M+), 162.11 (40), 161.09 (100), 144.08 (18), 116.09 (16), 115.06 (62), 91.06 (13), 77.04 (9)





^{13}C NMR (101 MHz, DMSO) 167.67, 143.93, 143.76, 131.81, 129.49, 128.07, 118.18, 44.38, 29.54, 22.11.

Synthesis of (2*E*)-3-(4-tertbutylphenyl) acrylic acid (7b)



D3

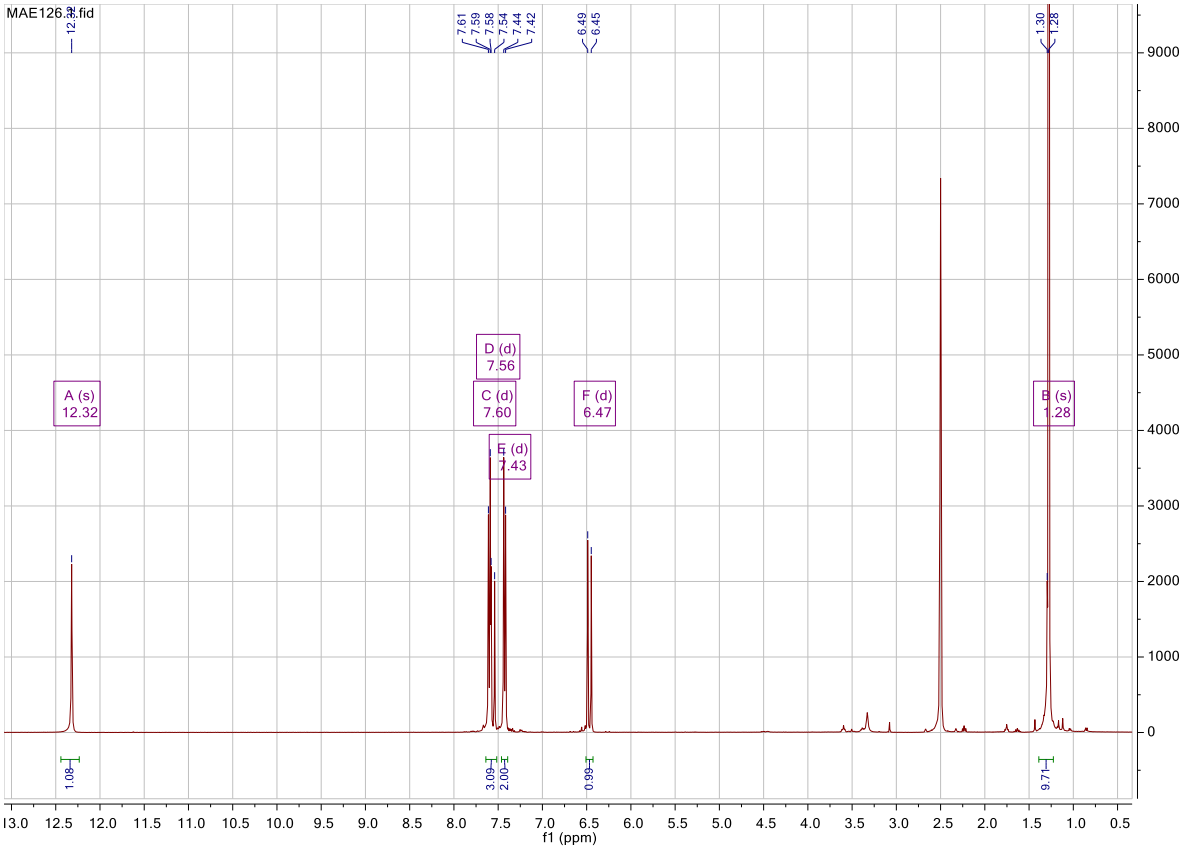
The synthesis was performed according to general procedure B. A3 (0.2727 g, 1.17 mmol, 1 eq.), $LiOHxH_2O$ (0.1717 g, 3.5 eq.), and THF/MeOH/H_2O solvent mixture (26.2 mL) were used. D1 was obtained as off white solid (0.2258 g, 1.11 mmol, 94% yield).

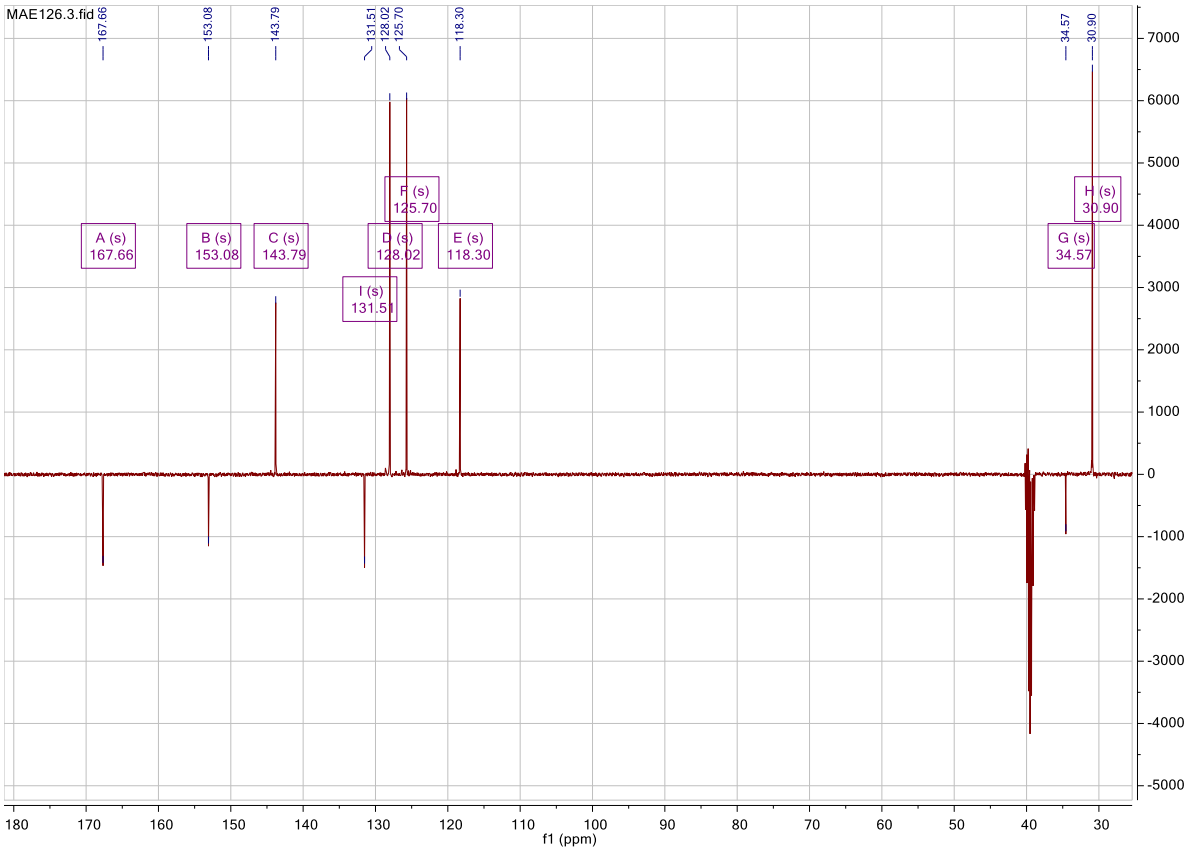
M_p: 203 – 206°C

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.32 (s, 1H), 7.60 (d, *J* = 8.4 Hz, 2H), 7.56 (d, *J* = 16.1 Hz, 1H), 7.43 (d, *J* = 8.4 Hz, 2H), 6.47 (d, *J* = 16.0 Hz, 1H), 1.28 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 167.66 (s, 1C), 153.08 (s, 1C), 143.79 (s, 1C), 131.51 (s, 1C), 128.02 (s, 1C), 125.70 (s, 1C), 118.30 (s, C1), 34.57 (s, 1C), 30.90 (s, 3C).

GC-MS: 204.13 (22, M+), 190.15 (13), 189.12 (100), 161.10 (21), 131.07 (11), 129.11 (10), 128.09 (18), 115.08 (19)




MAE126.3.fid
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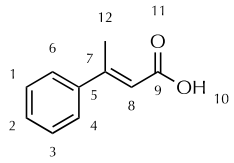
153.08
143.79
131.51
128.02
125.70
118.30
34.57
30.90
A (s) 167.66
B (s) 153.08
C (s) 143.79
I (s) 131.51
D (s) 128.02
F (s) 125.70
E (s) 118.30
G (s) 34.57
H (s) 30.90
f1 (ppm)

Synthesis of (2*E*)-β-methyl cinnamic acid (2b)



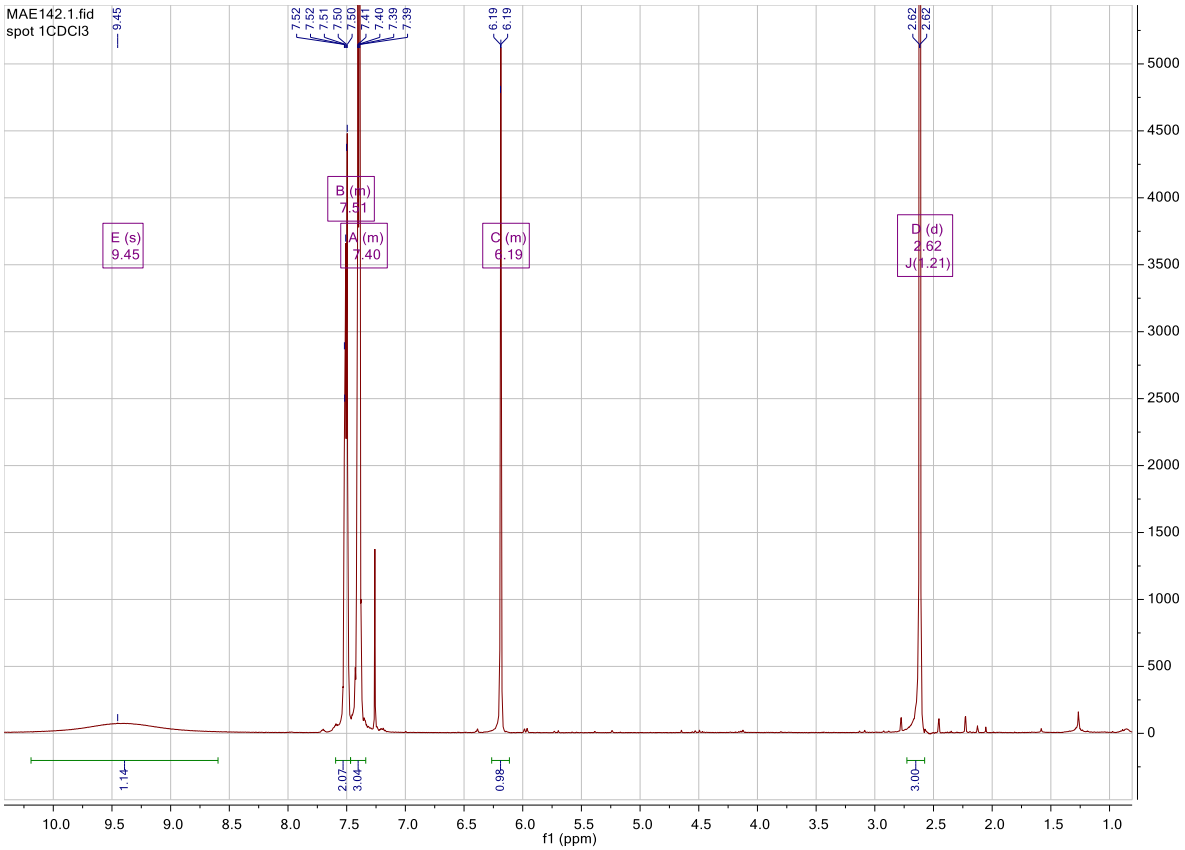
The synthesis was performed according to general procedure B. β-methyl ethyl cinnamate (191.9 mg, 1.01 mmol, 1 eq.), $LiOHxH_2O$ (167.6 mg, 3.50 mmol, 3.5 eq.), and THF/MeOH/H_2O solvent mixture (20 mL) were used. The crude product contained a mixture of isomers. The *E* isomer was isolated as white solid (69.8 mg, 43% yield) after purification by column chromatography (33.33% of ethyl acetate in petrol ether).

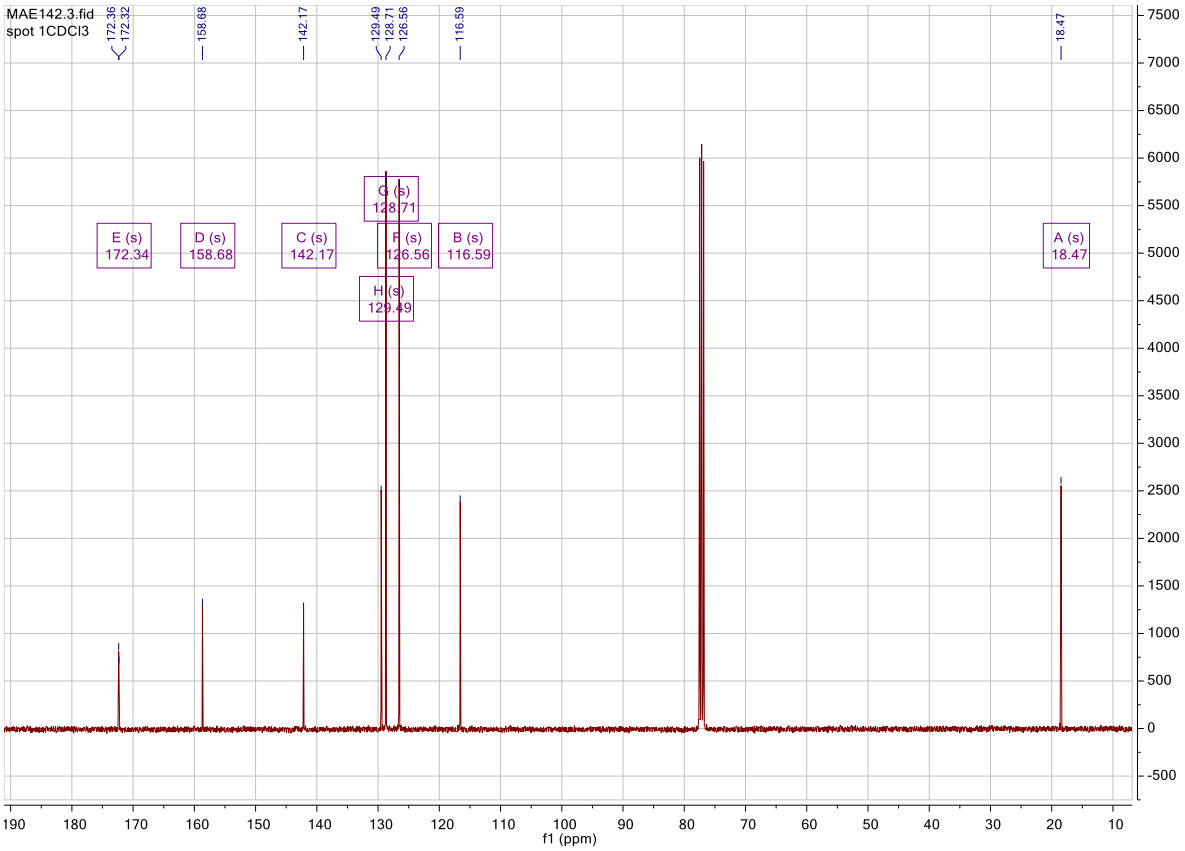
M_P: 95 – 96°C

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.44 (s, 1H), 7.56 – 7.46 (m, 2H), 7.40 (m, 3H), 6.19 (m, 1H, C8), 2.62 (d, *J* = 1.3 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.36 (1C, C9), 158.68 (1C, C7), 142.17 (1C, C5), 129.49 (2C, C2), 128.71 (2C, C1+3), 126.56 (2C, C4+6), 116.59 (1C, C8), 18.47 (1C, C12).

GC-MS: 162.09 (73, M^+), 161.08 (73), 144.06 (53), 117.09 (26), 116.09 (53), 115.06 (100), 91.06 (30), 77.05 (20).

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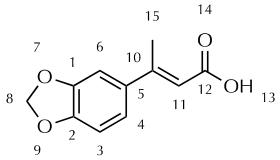
172.32
158.68
142.17
129.49
128.71
126.56
116.59
18.47
E (s)
172.34
D (s)
158.68
C (s)
142.17
G (s)
128.71
F (s)
126.56
B (s)
116.59
H (s)
129.49
A (s)
18.47
f1 (ppm)

Synthesis of (*2E*)-3-(1,3-benzodioxol-5-yl) crotonic acid (4b)



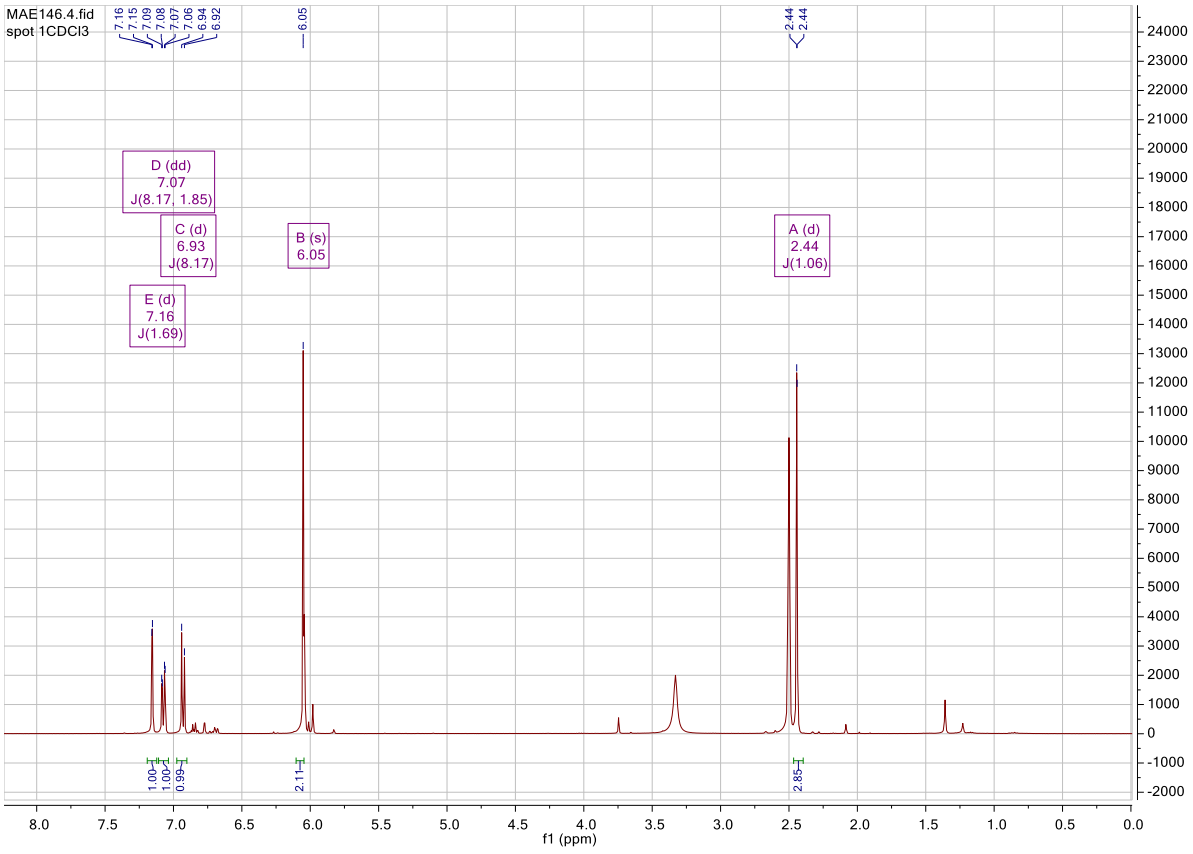
The synthesis was performed according to general procedure B. C1-E (235.0 mg, 1.00 mmol, 1 eq.), $LiOHxH_2O$ (146.9 mg, 3.50 mmol, 3.5 eq.), and THF/MeOH/H_2O solvent mixture (20 mL) were used. The crude product contained a mixture of isomers. The *E* isomer was isolated as white solid (27.9 mg, 14% yield) after purification by column chromatography (33.33% of ethyl acetate in petrol ether).

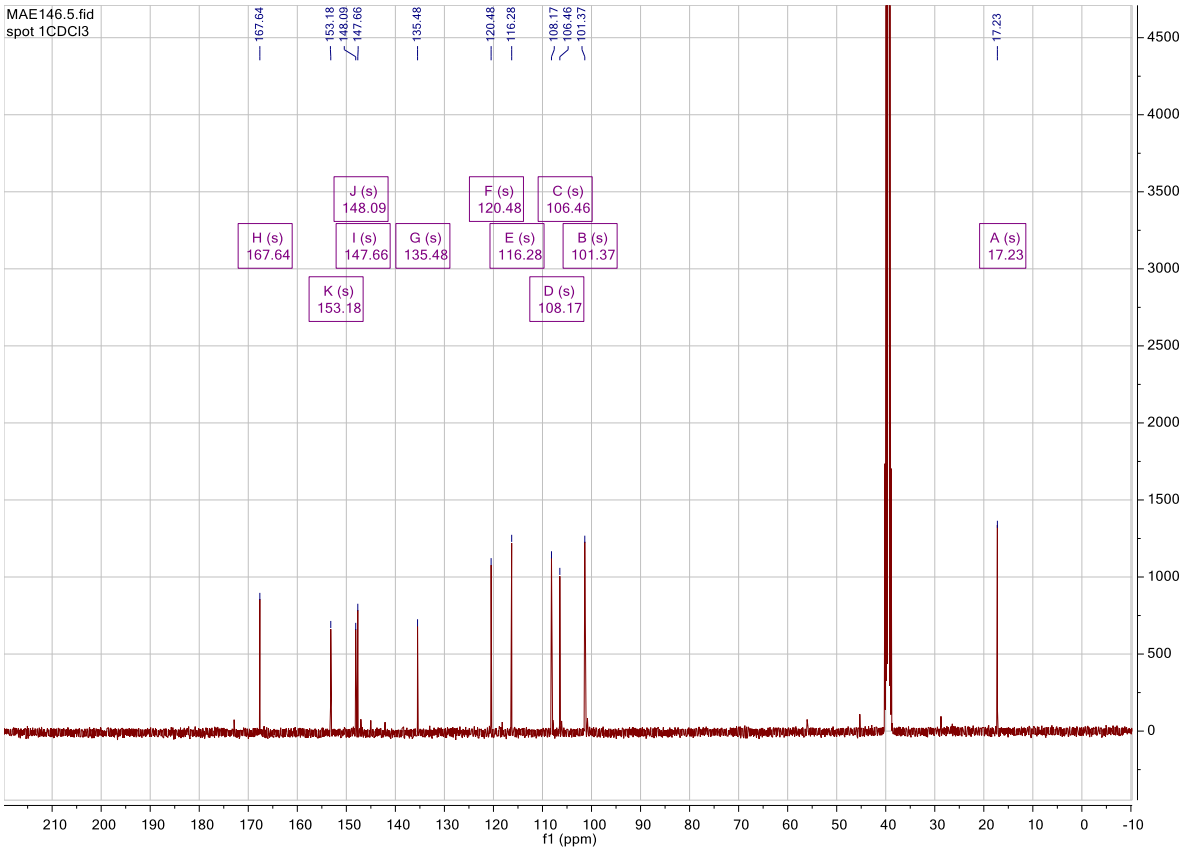
M_P: 160 – 165°C

^{1}H NMR (400 MHz, DMSO) δ 7.16 (d, *J* = 1.7 Hz, 1H), 7.07 (dd, *J* = 8.2, 1.9 Hz, 1H), 6.93 (d, *J* = 8.2 Hz, 1H), 6.05 (s, 2H), 6.04 (m, 1H), 2.44 (d, *J* = 1.1 Hz, 3H).

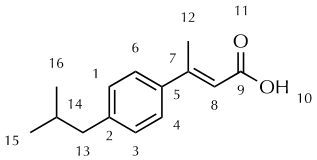
^{13}C NMR (101 MHz, DMSO) δ 167.64 (1C), 153.18 (1C), 148.09 (1C), 147.66 (1C), 135.48 (1C), 120.48 (1C), 116.28 (1C), 108.17 (1C), 106.46 (1C), 101.37 (1C), 17.23 (1C).

GC-MS: 206.07 (100, M^+), 188.06 (26), 160.06 (29), 122.04 (34), 121.04 (20), 103.05 (36), 102.06 (20), 77.04 (27).




MAE146.5.fid
spot 1CDCl3
167.64
153.18
148.09
147.66
135.48
120.48
116.28
108.17
106.46
101.37
17.23
J (s)
148.09
F (s)
120.48
C (s)
106.46
H (s)
167.64
I (s)
147.66
G (s)
135.48
E (s)
116.28
B (s)
101.37
A (s)
17.23
K (s)
153.18
D (s)
108.17
4500
4000
3500
3000
2500
2000
1500
1000
500
0
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

Synthesis of (2*E*)-3-(4-isobutylphenyl) crotonic acid (6b)



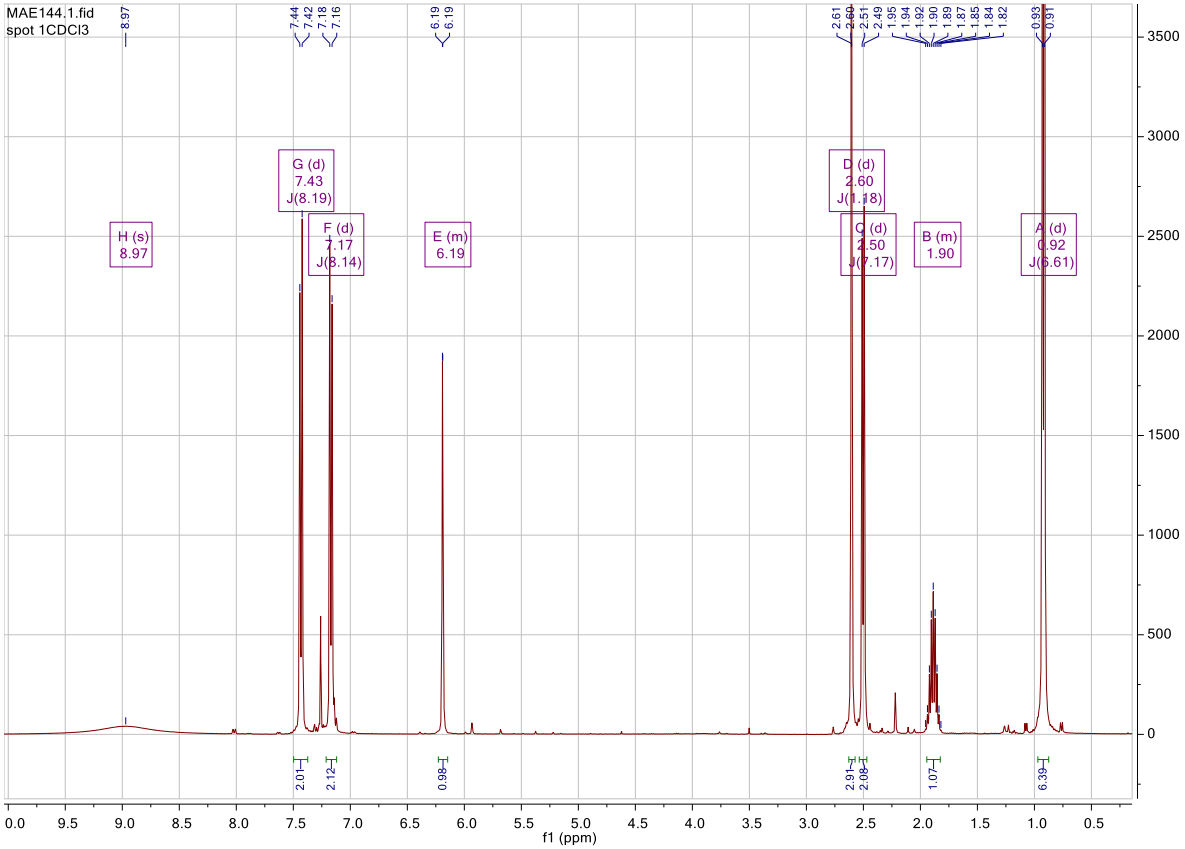
The synthesis was performed according to general procedure B. C2-E (246.6 mg, 1.00 mmol, 1 eq.), $LiOHxH_2O$ (145.9 mg, 3.48 mmol, 3.5 eq.), and THF/MeOH/H_2O solvent mixture (20 mL) were used. The crude product contained a mixture of isomers. The *E* isomer was isolated as white solid (119.7 mg, 55% yield) after purification by column chromatography (33.33% of ethyl acetate in petrol ether).

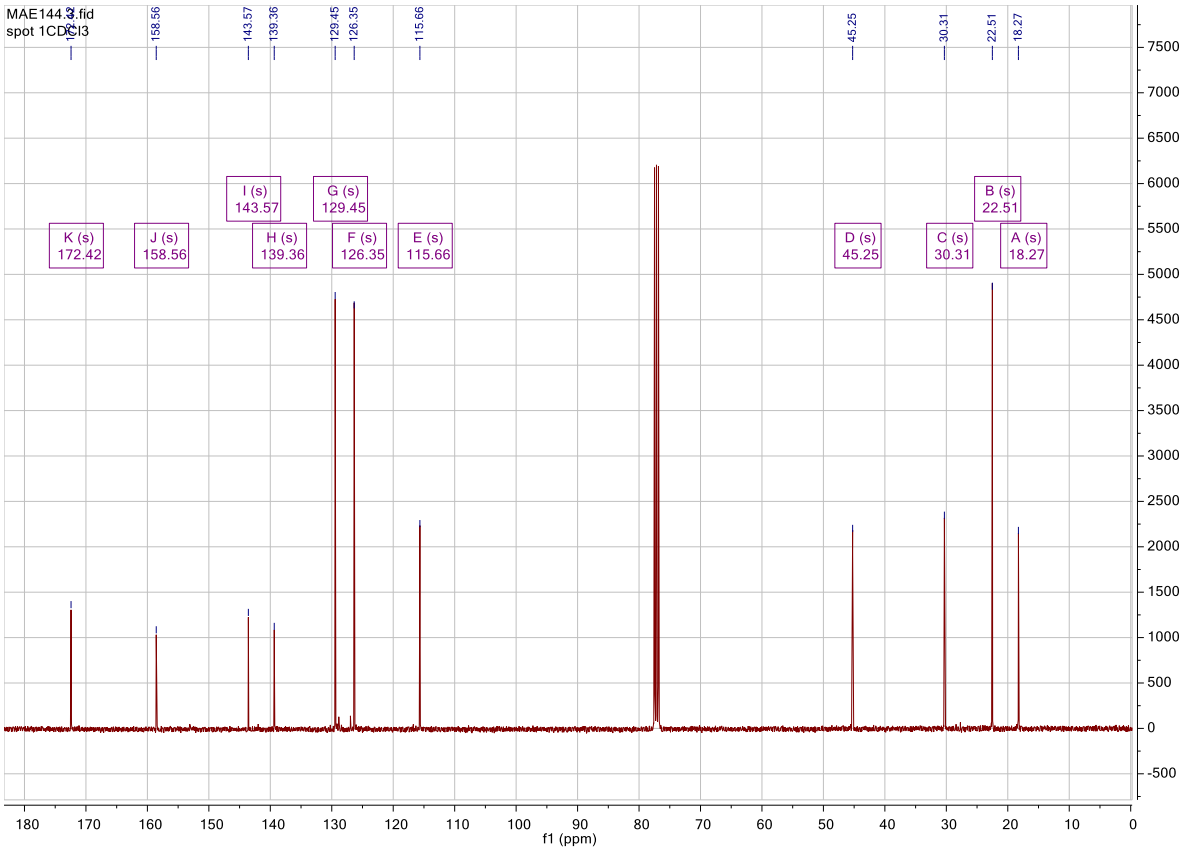
M_P: 105 – 110°C

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.97 (s, 1H), 7.43 (d, *J* = 8.2 Hz, 2H), 7.17 (d, *J* = 8.2 Hz, 2H), 6.19 (m,1H), 2.60 (d, *J* = 1.3 Hz, 3H), 2.50 (d, *J* = 7.2 Hz, 2H), 1.89 (m, 1H), 0.92 (d, *J* = 6.6 Hz, 6H).

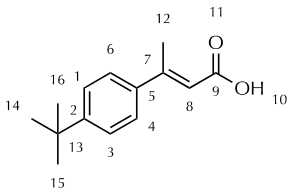
^{13}C NMR (101 MHz, $CDCl_3$) δ 172.42 (1C), 158.56 (1C), 143.57 (1C), 139.36 (1C), 129.45 (2C), 126.35 (2C), 115.66 (1C), 45.25 (1C), 30.31 (1C), 22.51 (2C), 18.27 (1C).

GC-MS: 218.13 (39, M^+), 176.10 (27), 175.07 (100), 158.08 (27), 129.08 (15), 128.07 (14), 115.04 (21), 91.05 (13).




MAE144.3.fid
spot 1CDCl3
172.42
158.56
143.57
139.36
129.45
126.35
115.66
45.25
30.31
22.51
18.27
K (s) 172.42
J (s) 158.56
I (s) 143.57
H (s) 139.36
G (s) 129.45
F (s) 126.35
E (s) 115.66
D (s) 45.25
C (s) 30.31
B (s) 22.51
A (s) 18.27
f1 (ppm)

Synthesis of Synthesis of (2*E*)-3-(4-*tert*-butylphenyl) crotonic acid (8b)



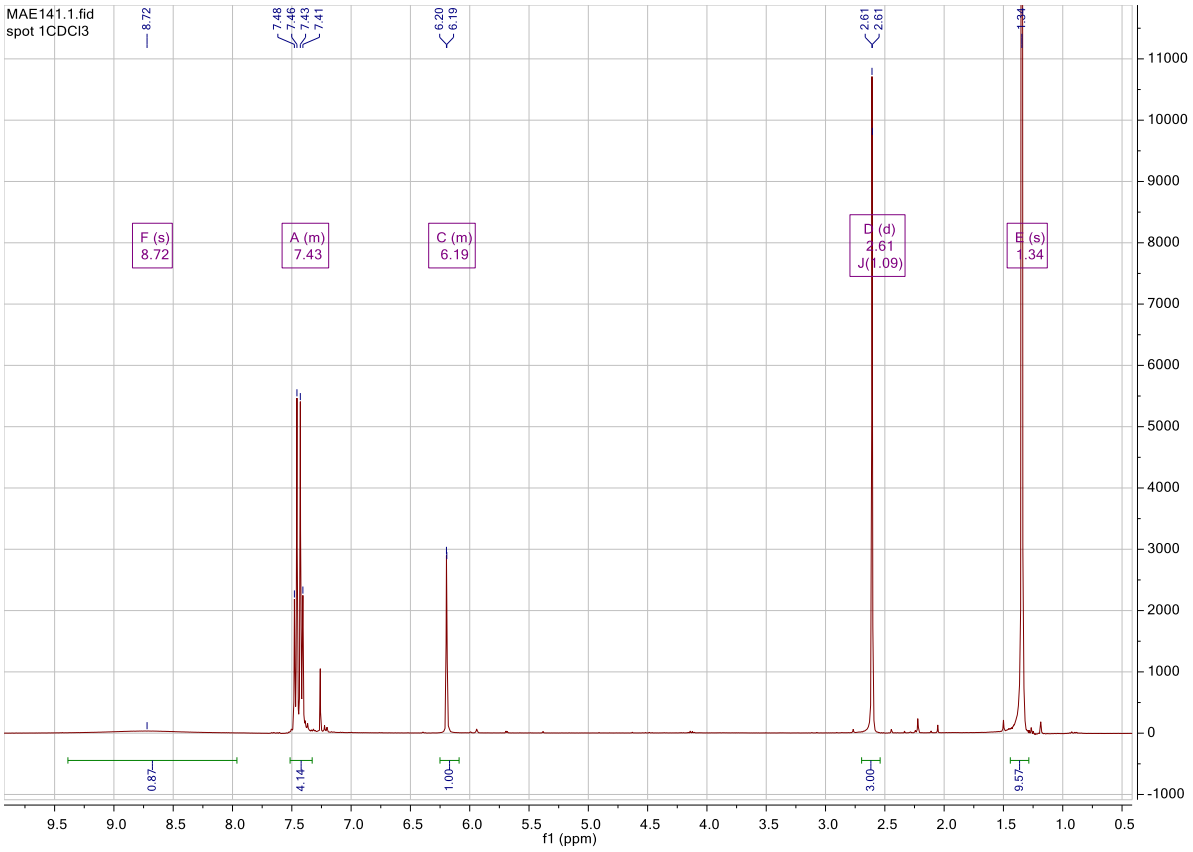
The synthesis was performed according to general procedure B. C3-E (247.4 mg, 1.00 mmol, 1 eq.), $LiOHxH_2O$ (168.0 mg, 4.00 mmol, 4.0 eq.), and THF/MeOH/H_2O solvent mixture (20 mL) were used. The crude product contained a mixture of isomers. The *E* isomer was isolated as white solid (60.3 mg, 28% yield) after purification by column chromatography (33.33% of ethyl acetate in petrol ether).

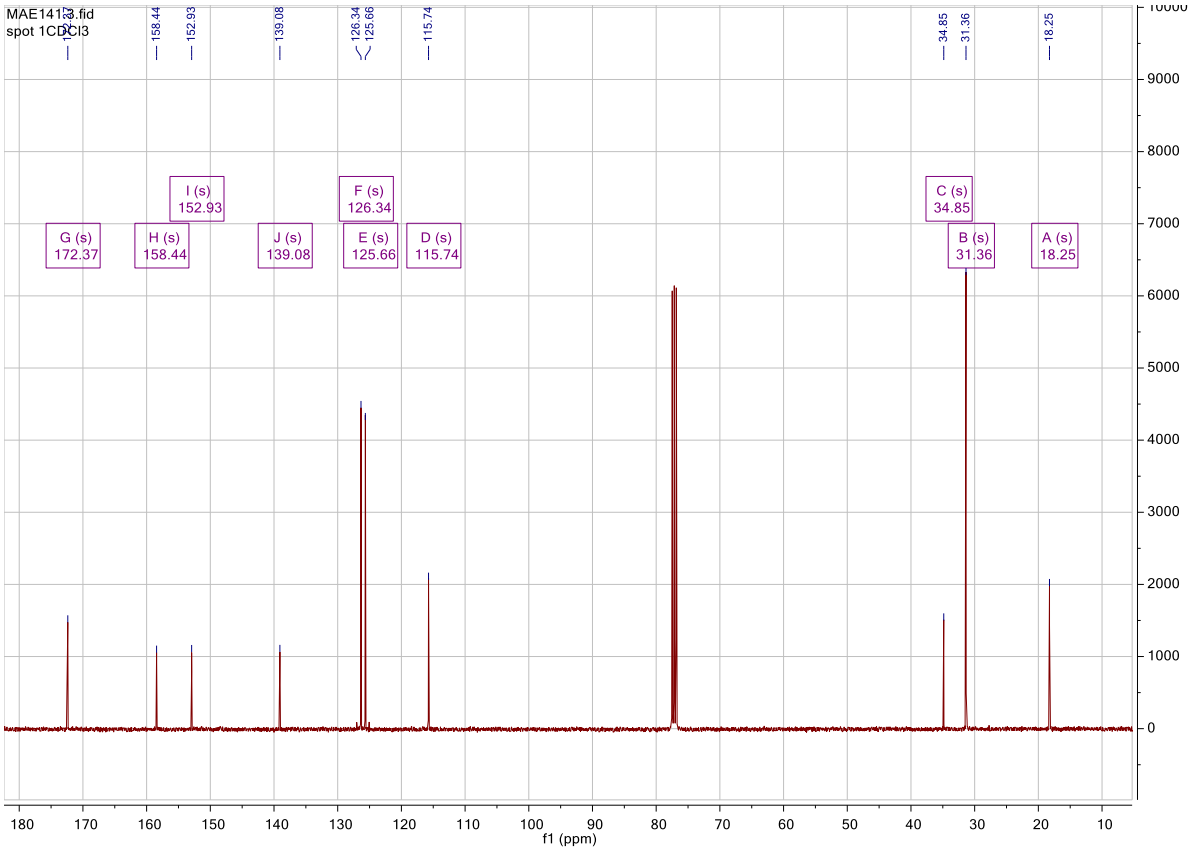
M_P: 133 – 136°C

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (bs, 1H), 7.50 – 7.37 (m, 4H), 6.19 (m, 1H), 2.61 (d, *J* = 1.3 Hz, 3H), 1.34 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.38 (1C), 158.44 8(1C), 152.93 (1C), 139.08 (1C), 126.34 (2C), 125.66 (2C), 115.74 (1C) 34.85 (1C), 31.36 (3C), 18.25 (1C).

GC-MS: 218.11 (28, M^+), 204.13 (14), 203.11 (100), 175.07 (12), 157.08 (7), 129.08 (8), 128.06 (8), 115.04 (13).





1. Schwendenwein, D., Fiume, G., Weber, H., Rudroff, F. & Winkler, M. Selective Enzymatic Transformation to Aldehydes in vivo by Fungal Carboxylate Reductase from Neurospora crassa. *Adv. Synth. Catal.* **358**, 3414–3421 (2016).

2. Gibson, D. G. et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **6**, 343–345 (2009).