Electronic Supplementary Material (ESI) for Green Chemistry.

Supporting Information

Design of Recombinant Whole-Cell Catalysts for Double Reduction of C=C and C=O Bonds in Enals and Application in the Synthesis of Guerbet Alcohols as Industrial Bulk Chemicals for Lubricants

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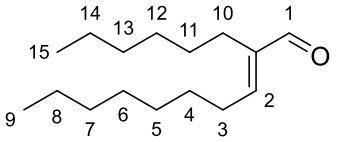
1 Synthesis of substrates and reference compounds

The synthesis of (*E*)-2-butyl-2-octenal (**1**) and *rac*-2-butyloctanal (**2**) was described previously.[1] The same procedures were applied for the synthesis of the following substrates and reference compounds.

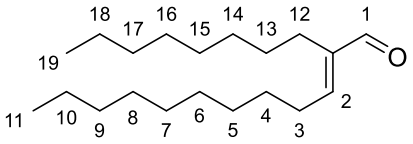
1.1 Synthesis of (*E*)-2-hexyl-2-decenal (6)



6
$C_{16}H_{30}O$
238.42 g/mol

(*E*)-2-hexyl-2-decenal (**6**, 5.68 g, 23.8 mmol, 60%) was obtained as a colorless liquid.
^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 0.86 - 0.90 (6H, m, H-**C9**/**C15**), 1.26 - 1.34 (16H, m, H-**C5**-**8**/**C11**-**14**), 1.46 - 1.52 (2H, m, H-**C4**), 2.22 (2H, t, H-**C10**), 2.32 - 2.36 (2H, m, H-**C3**), 6.43 (1H, t, H-**C2**), 9.35 (1H, s, H-**C1**).
MS (ESI): *m/z* = 261.2 $[M+Na]^+$, 499.4 $[2M+Na]^+$.
Bp (at 0.019 mbar) = 95…100°C.

1.2 Synthesis of (*E*)-2-octyl-2-dodecenal (7)

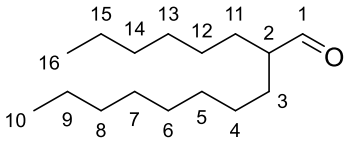


7
$C_{20}H_{38}O$
294.92 g/mol

(*E*)-2-octyl-2-dodecenal (**7**, 5.70 g, 19.3 mmol, 32%) was obtained as a colorless liquid.
^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 0.86 - 0.89 (6H, m, H-**C11**/**C19**), 1.25 - 1.32 (24H, m, H-**C5**-**10**/**C13**-**18**), 1.46 - 1.51 (2H, m, H-**C4**), 2.22 (2H, t, H-**C12**), 2.32 - 2.38 (2H, m, H-**C3**), 6.43 (1H, t, H-**C2**), 9.36 (1H, s, H-**C1**).
MS (ESI): *m/z* = 317.3 $[M+Na]^+$, 611.5 $[2M+Na]^+$.
Bp (at 0.0075 mbar) = 140…146°C.

1 [1] M. Biermann, H. Gruß, W. Hummel and H. Gröger, *ChemCatChem*, 2016, **8**, 895-899.

1.3 Synthesis of *rac*-2-hexyldecanal (10)



10
$C_{16}H_{32}O$
240.43 g/mol

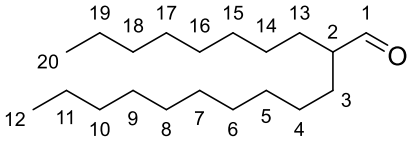
rac-2-hexyldecanal (**10**, 4.10 g, 17.1 mmol, 28%) was obtained as a colorless liquid.

^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 0.86 - 0.89 (6H, m, H-**C10**/**C16**), 1.25 - 1.29 (20H, m, H-**C4-9**/**C12-15**), 1.39 - 1.46 (2H, m, H-**C11**), 1.57 - 1.65 (2H, m, H-**C3**), 2.19 - 2.25 (1H, m, H-**C2**), 9.55 (1H, d, H-**C1**).

MS (ESI): *m/z* = 263.3 $[M+Na]^+$, 503.3 $[2M+Na]^+$.

Bp (at 0.018 mbar) = 95…100°C.

1.4 Synthesis of *rac*-2-octyldodecanal (11)



11
$C_{20}H_{40}O$
296.54 g/mol

rac-2-octyldodecanal (**11**, 1.35 g, 4.6 mmol, 15%) was obtained as a colorless liquid.

^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 0.86 - 0.89 (6H, m, H-**C12**/**C20**), 1.23 - 1.31 (12H, m, H-**C4**-**11**/**C14**-**19**), 1.41 - 1.45 (2H, m, H-**C13**), 1.56 - 1.64 (2H, m, H-**C3**), 2.19 - 2.24 (1H, m, H-**C2**), 9.55 (1H, d, H-**C1**).

MS (ESI): *m/z* = 319.3 $[M+Na]^+$, 615.4 $[2M+Na]^+$.

Bp (at 0.0081 mbar) = 118…124°C.

2 Construction of expression vectors

Escherichia coli strains BL21(DE3) and DH5α, as well as the vectors (pACYCDuet-1 and pETDuet-1) were purchased from Novagen (Madison, USA). The strain DH5α was used as a host organism for cloning, whereas BL21(DE3) was implemented for expression. The amplification of the genes was obtained *via* standard PCR-techniques from the cloning vectors pGDH, pET28a_PpADH and pET28a_Gox8. The *gdh* gene was cloned into the low copy expression vector pACYCDuet1 with the restriction sites *Nco*I and *Xho*I. The genes of the alcohol dehydrogenase *PpADH* and the enoate reductase gene *Gox8* were subcloned into each "multi-cloning-site" (MCS) of the medium copy expression vector pETDuet1. For cloning the genes in the MCS1 without a 6xHis-tag, the restriction enzymes *Nco*I and *Not*I were used, for cloning the *PpADH* gene with N-terminal 6xHis-tag restriction enzymes *EcoR*I and *Not*I were used. For cloning the genes in the MCS2, the restriction enzymes *Nde*I and *Xho*I were used. For cloning the PpADH with N-terminal 6xHis-tag in MCS2, the His-tag were included in the primer. All used primer with appropriate restriction site were listed in table 1. All the constructed vectors are under the control of T7-promoter. The correct in-frame DNA sequence and the absence of any mutations were confirmed by sequencing (Eurofins genomics).

Table 1: Oligonucleotides used for PCR amplification in this study.

Primer	Sequence (5' -> 3')	restriction site
PpADH_for	AGCGCACCATGGGA**ATG**GTTTCTAAGGTTTTATTGAC	*Nco*I
PpADH_for2	AGCGCACAT**ATG**GTTTCTAAGGTTTTATTGAC	*Nde*I
PpADH_for3	AGCGCAGAATTC**ATG**GTTTCTAAGGTTTTATTGAC	*EcoR*I
PpADH_for4	AGTGCACAT**ATG**GGCAGCAGC*CATCACCATCATCACCAC*AG CCAGGTTTCTAAGGTTTTA	*Nde*I
PpADH_rev	AGCCGAGCGGCCGC**TTA**TTTATTAGCACGCAATATC	*Not*I
PpADH_rev2	AGCCGACTCGAG**TTA**TTTATTAGCACGCAATATC	*Xho*I
Gox8_for	AACCAACCATGGGA**ATG**CCAACCCTGTTCGATCCC	*Nco*I
Gox8_for2	ACCCAACAT**ATG**CCAACCCTGTTCGATCCC	*Nde*I
Gox8_rev	AATTAAGCGGCCGC**TTA**GTTGGGGCCGGAGGTGGCGGA	*Not*I
Gox8_rev2	AATTAACTCGAG**TTA**GTTGGGGCCGGAGGTGGCGGA	*Xho*I
GDH_for	ACCAACCACC**ATG**GGCATGTATCCGGATTTAAA	*Nco*I
GDH_rev	ATAATAACTCGAG**TTA**ACCGCGGCCTGC	*Xho*I

The restriction enzyme sites were underlined, the start- or stop codon were in bold and the 6xHis-Tag coding region were italic typed.

3 Cultivation of the whole cell catalysts

Starting cultures of *E. coli* BL21(DE3) cells carrying the recombinant plasmids were cultivated overnight at 37 °C in 5 mL LB medium, containing 80 µg mL^{-1} carbenicillin and 28 mL^{-1} chloramphenicol. These cultures were used to inoculate the main cultures in TB-medium (24 g L^{-1} yeast extract, 12 g L^{-1} casein hydrolysate, 5 g L^{-1} glycerol and 50 g L^{-1} glucose in 100 mM potassium phosphate buffer (KP_i) pH 7.0) containing 80 µg mL^{-1} carbenicillin and 28 µg mL^{-1} chloramphenicol for expression in shaking flasks at a final concentration of 0.05 optical density at 600 nm (OD_{600}). When the OD_{600} reached a value between 0.6 and 0.8, the production of the recombinant proteins was induced by the addition of isopropyl-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM. The cultures were shaken for 72 h at 15 °C and harvested by centrifugation.

4 Preparation of crude extract

A cell suspension (25% w/v) was prepared in 100 mM phosphate buffer (KP_i), pH 7. Cells were disrupted by three sonication cycles of 3 min (10-20% power output) with cooling periods in-between cycles. The lysed cells were centrifuged at 17,000 x g for 30 min at 4 °C and the supernatant was used for determine the activity.

5 Protein analysis

Protein overexpression was monitored by SDS-PAGE according to Bradford using BSA as a standard. For the expected mass of 39.2 kDa for the enoate reductase and alcohol dehydrogenase and 28.1 kDa for the glucose dehydrogenase, a tris-glycine gel containing 15% acrylamide was used. Samples were incubated for 10 min at 95 °C in loading buffer and 10 µg were loaded on the gel. The gels were stained with coomassie brilliant blue R-250 and molecular mass under denaturing conditions was determined by comparison with standard markers (Thermo Scientific, Dreieich, Germany).

6 Determination of enzyme activity and protein content

The enzyme activity of whole cell catalyst (WCC) crude extracts and purified enzymes was determined according to procedures in sections 7.1 and 7.2. Bradford assay with BSA as standard was used for determination of the protein content. The results are summarized in table 2.

Table 2: Enzyme activities and protein content of whole cell catalyst and isolated enzymes.

catalyst	glucose (ox.)[a]	2-butyl-2-octenal (1) (red.) [a]	2-butyloctanal (2) (red.) [a]	protein content [b]
WCC1	7.93	0.20	0.10	56.8
WCC2	9.52	0.26	0.16	79.7
WCC3	6.91	0.17	0.04	58.4
WCC4	8.33	0.19	0.14	80.3
Gox-ER	n.d.	3.76	n.d.	n.d.
PpADH	n.d.	0.77	2.11	n.d.

[a] activities in U mg^{-1}, [b] protein content in mg protein g^{-1} WCC, n.d. = not determined

6.1 Activity screening in reductive mode

The initial activities were measured spectrophotometrically at 340 nm determining the consumption of NADPH reducing their respective substrate. For a total volume of 1 mL, 970 µL of 20 mM substrate solution in KP_i buffer (pH 7, 50 mM), 10 µL of 20 mM NADPH solution (final concentration: 0.2 mM) and 20 µL enzyme solution of appropriate dilution ($\Delta E\ t^{-1} \approx 0.1\ min^{-1}$) were used. Activities were measured as triplets and calculated according to the following equation:

$$\text{activity [U mL}^{-1}] = \frac{\Delta E}{t} \cdot \frac{v_t \cdot f}{\varepsilon \cdot v_s \cdot d}$$

v_t = volume of cuvette (here: 1 mL)

f = dilution factor

ε = extinction coefficient (here: 6.3 $mL \cdot \mu mol^{-1} \cdot cm^{-1}$)

v_s = volume of enzyme solution

d = thickness of cuvette (here: 1 cm)

6.2 Activity screening in oxidative mode

The initial activities were measured spectrophotometrically at 340 nm determining the production of NADPH by oxidation of D-glucose. For a total volume of 1 mL, 970 µL of 100 mM solution of D-glucose in KP_i buffer (pH 7, 50 mM), 10 µL of 200 mM $NADP^+$ solution (final concentration: 2 mM) and 20 µL enzyme solution of appropriate dilution ($\Delta E/t \approx 0.1\ min^{-1}$) were used. Activities were measured as triplets and calculated according to the equation in section 6.1.

7 Gene- and amino acid sequences

Gene sequence of the enoate reductase Gox8 (genbank entry: WP_011252080.1) (1086 bp)

ATGCCAACCCTGTTCGATCCCATTGATTTCGGTCCCATTCACGCGAAAAACCGGATCGT
GATGTCCCCGCTGACGCGCGGACGTGCTGACAAGGAGGCCGTTCCGACCCCCATCAT
GGCGGAATACTACGCCCAGCGCGCCAGTGCCGGGCTGATCATCACGGAAGCCACGGG
TATCTCCCGCGAAGGTCTGGGCTGGCCGTTCGCACCGGGAATCTGGTCCGATGCGCAG
GTCGAAGCCTGGAAGCCGATCGTGGCCGGTGTGCATGCAAAGGGCGGAAAGATCGTC
TGCCAGCTCTGGCACATGGGCCGCATGGTCCACTCGTCCGTGACCGGAACGCAGCCC
GTCTCGTCCTCCGCCACCACGGCCCCCGGCGAGGTCCATACCTATGAAGGCAAGAAGC
CGTTCGAGCAGGCCCGCGCAATCGATGCCGCGGATATCAGCCGTATTCTGAACGACTA
TGAGAACGCCGCCCGCAATGCGATCCGCGCCGGCTTCGACGGGGTGCAGATCCACGC
CGCCAATGGCTACCTCATCGACGAGTTCCTGCGAAACGGTACGAATCACCGCACAGAT
GAATACGGCGGCGTCCCCGAAAACCGCATCCGTTTCCTGAAGGAAGTCACCGAGCGCG
TGATCGCTGCCATCGGTGCCGACCGCACAGGCGTGCGCCTGTCCCCCAACGGCGATA
CGCAGGGCTGCATCGACAGCGCACCTGAGACGGTCTTTGTCCCGGCGGCAAAGCTGC
TTCAGGATCTGGGCGTGGCCTGGCTCGAACTGCGCGAACCCGGCCCGAACGGCACCT
TCGGCAAGACGGACCAGCCCAAACTGTCCCCGCAGATCCGCAAGGTGTTCCTGCGCCC
GCTGGTGCTCAATCAGGACTATACGTTCGAGGCAGCACAGACCGCGCTGGCAGAAGGG
AAGGCTGATGCGATCGCCTTCGGTCGCAAGTTCATCTCGAACCCCGACCTGCCGGAGC
GCTTCGCCCGCGGCATCGCCCTGCAGCCGGATGATATGAAAACCTGGTACAGTCAGGG
CCCCGAAGGATACACGGACTACCCGTCCGCCACCTCCGGCCCCAACTAA

Amino acid sequence of the enoate reductase Gox8 (361 aa)

MPTLFDPIDFGPIHAKNRIVMSPLTRGRADKEAVPTPIMAEYYAQRASAGLIITEATGISREGL
GWPFAPGIWSDAQVEAWKPIVAGVHAKGGKIVCQLWHMGRMVHSSVTGTQPVSSSATTA
PGEVHTYEGKKPFEQARAIDAADISRILNDYENAARNAIRAGFDGVQIHAANGYLIDEFLRNG
TNHRTDEYGGVPENRIRFLKEVTERVIAAIGADRTGVRLSPNGDTQGCIDSAPETVFVPAAK
LLQDLGVAWLELREPGPNGTFGKTDQPKLSPQIRKVFLRPLVLNQDYTFEAAQTALAEGKA
DAIAFGRKFISNPDLPERFARGIALQPDDMKTWYSQGPEGYTDYPSATSGPN

Gen sequence of the alcohol dehydrogenase PpADH (Genbank entry: XM_002492630.1) (1065 bp)

ATGGTTTCTAAGGTTTTATTGACAGGTGCTTCTGGTTACATCGCCCAACACATCACTAAT
GAATTACTTTCCCACGGCTTTAAGGTCATTGGAACTGTAAGAAGACAGGAGCAGGCAGA
CCAATTGCATAAGCAATTTTCAGAGGAAAGTTCTGTTTTACAAAAAGATCCCTCCCTTCT
GACCTATGTCCTTGTCCCAGATATTGGTGCTTCGGATGCTTTTGATGAGGTATTGAAGTC
AACCCCCGACATCACTTATGTCCTTCACACAGCTTCCCCATTTATTTTCAATGACGACAG
AGCTCTTGAGGACGTTTATTTGAAGCCTGCTGTTGAGGGTACCAGAAACATCTTAAGTG
CTATCAAGAAGTTTGCCAATGACAGTGTCAAGAACGTCGTGGTAACTTCCTCCTTTGCA
GCAATTTTGAACGCTGACAAGTTTGAAGACAAGAGTTTCATTCACACTGAGAAGGTGTG
GAATAACAACACCTGGGATCAAACCAAGAGTGGAGACAGAGGTGTTGCTTACATCGTTT
CCAAGAAGGAGGCTGAGAAAGCTGCCTGGGACTTCGTTGAGAAGGAGAAACCAAACTT
TAAATTGACAACAGTAAACCCTCCTTATGTTTTTGGTCCTCAGAAATTCGATGCTTCGGC
TAAGAAGGAATCTTTGAACACCTCAGCTGAGATCGTTGGATCTCTGTTGCACACAAAGTA
TCCATCTGATGACAAGTTGTTTGATGATCCCCTCAATTTATCCGTTGATGTTAGAGACGT
AGCTCTCTACCATGTTCTGCCACTTTTGAATGCTGATTTGGCTTCCAGGAGATTGCTTGT
TGTTCAATCCAAGTTCAGCGCTCAGAGAATCCTGAACATTATCAACGAGAATTTTCCTGA
ATTAAAGGGCAAGATCGCAGTTGGAAAGCCCGAGGAAACGGCAAGAGTGGAAGCTATT
AAGGGCCCGGAGTATAACAACAGTGTTACTGTAGGCCTAACTGGAGTTGACCCCATCCC
ACTGGAGAAAACGGTTGTGGACTCTGTCAAACAGATATTGCGTGCTAATAAATAA

Amino sequence of the alcohol dehydrogenase PpADH (354 aa)

MVSKVLLTGASGYIAQHITNELLSHGFKVIGTVRRQEQADQLHKQFSEESSVLQKDPSLLTY
VLVPDIGASDAFDEVLKSTPDITYVLHTASPFIFNDDRALEDVYLKPAVEGTRNILSAIKKFAN
DSVKNVVVTSSFAAILNADKFEDKSFIHTEKVWNNNTWDQTKSGDRGVAYIVSKKEAEKAA
WDFVEKEKPNFKLTTVNPPYVFGPQKFDASAKKESLNTSAEIVGSLLHTKYPSDDKLFDDPL
NLSVDVRDVALYHVLPLLNADLASRRLLVVQSKFSAQRILNIINENFPELKGKIAVGKPEETAR
VEAIKGPEYNNSVTVGLTGVDPIPLEKTVVDSVKQILRANK

8 Biotransformations

8.1 General procedure for small scale biotransformations

In a Schlenk flask, $NADP^+$ (5 mol%) and D-glucose (4 eq) were dissolved in 3 mL degassed KPi buffer. The whole cell catalyst (100 mg thawed wet cell mass) was thoroughly resuspended in 900 µL KPi buffer and added to the reaction mixture. The flask was degassed and flooded with argon. 1 mL KPi buffer (pH 7, 200 mM) or methanol to a final reaction volume of 5 mL and the substrate were added. The flask was tightly sealed and the mixture was stirred at room temperature. The reaction mixture was acidified with 1 mL 0.5 M hydrochloric acid and extracted with methyl *tert*-butyl ether (3 x 5 mL). Centrifugation was used to promote phase separation.

Table 3: Initial screening of whole-cell catalysts.

WCC	conversion [%]	2-butyl-octanal (2) [%]	2-butyl-1-octanol (3) [%]	2-butyloct-2-en-1-ol (4) [%]
1	93.7	4.3	94.7	1.8
2	>99.9	<0.1	99.7	0.3
3	>99.9	39.1	60.8	0.1
4	93.5	1.3	97.9	0.8

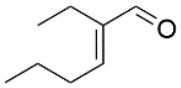
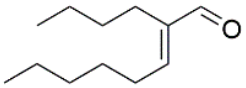
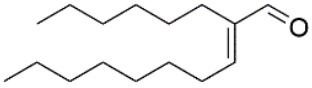
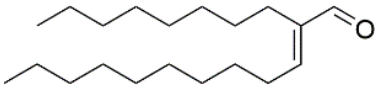
conditions: 5 mL scale, 20 mM substrate in KPi buffer (200 mM, pH 7), 20 mg mL^{-1} whole-cell catalyst (wet cell mass), 5 mol% $NADP^+$, 80 mM D-glucose, 48 h, room temp., under argon

Table 4: Determination of optimal pH for whole-cell catalyst WCC 2.

pH	conversion [%]	2-butyl-octanal (2) [%]	2-butyl-1-octanol (3) [%]	2-butyloct-2-en-1-ol (4) [%]
6.0	75.2	3.5	95.5	0.6
6.5	95.5	1.2	98.0	0.8
7.0	96.5	1.0	97.2	1.8
7.5	87.7	5.6	93.1	1.3
8.0	27.2	4.8	94.6	0.6

conditions: 5 mL scale, 20 mM substrate in KPi buffer (200 mM, pH 7), 20 mg mL^{-1} whole-cell catalyst 2 (wet cell mass), 5 mol% $NADP^+$, 80 mM D-glucose, 24 h, room temp., under argon.

Table 5: Substrate screening.

substrate	conversion [%]	intermediate[a] [%]	product[b] [%]	by-product[c] [%]
 **5**	>99.9	<0.1	98.3	1.7
 **1**	>99.9	<0.1	99.7	0.3
 **6**	87.3	18.1	72.7	9.2
 **7**	11.9	47.9	42.9	9.2

conditions: 5 mL scale, 20 mM substrate in KPi buffer (200 mM, pH 7), 20 mg mL^{-1} whole-cell catalyst 2 (wet cell mass), 5 mol% $NADP^+$, 80 mM D-glucose, 48 h, room temp., under argon. [a] 2-alkylalkanal, [b] 2-alkyl-1-alkanol (Guerbet alcohol), [c] 2-alkylalk-2-en-1-ol.

Table 6: Increase of substrate concentration with (+) and without (-) 20% v/v methanol as a co-solvent.

entry	substrate [mM]	cosolvent	conversion [%]	2-butyloctanal (2) [%]	2-butyl-1-octanol (3) [%]	2-butyloct-2-en-1-ol (4) [%]
1[a]	20	-	>99.9	0.0	98.5	1.5
2	20	-	84.2	16.4	82.5	1.1
3	50	-	53.5	11.2	88.0	0.8
4	100	-	33.5	3.3	95.2	1.5
5	50	+	92.7	3.3	95.5	1.2
6	100	+	76.9	1.8	96.9	1.3

conditions: 5 mL scale, KPi buffer (500 mM, pH 7), 20 mg mL^{-1} whole-cell catalyst 2 (wet cell mass), 2 mol% $NADP^+$, 4 eq D-glucose, 48 h, room temp., under argon. [a] KPi buffer (200 mM, pH 7) used.

8.2 General procedure for large scale biotransformations

In three-necked round bottom flask, $NADP^+$ (2 mol%) and D-glucose (4 eq) were dissolved in degassed KPi buffer (50 mM, pH 7, 60% of final reaction volume). A 10% w/v suspension of the whole cell catalyst (20% of final reaction volume) in KPi buffer was added (20 g/L final cell concentration). 2-butyl-2-octenal (**1**, 100-500 mM), dissolved in methanol (20% of the final reaction volume), was added. The flask was equipped with the titration head and pH electrode of a pH stat apparatus as well as an argon supply. It was tightly sealed and the mixture was stirred at room temperature. The pH was kept constant by the addition aqueous sodium hydroxide solution. The reaction mixture was extracted with methyl *tert*-butyl ether (3 x 100% of reaction volume). The combined organic phases were dried over magnesium sulfate and the solvent was evaporated *in vacuo*. The purity of crude products was determined using dimethyl terephathalate as internal standard in ^{1}H-NMR.

9 Calculation of logP values of substrates

The ACDLabs prediction tool was used for calculation of logP values of substrates (table 7).

Table 7: LogP values of substrates.

compound	**logP** (±0.32)
2-ethyl-2-hexenal (**5**)	2.66
2-butyl-2-octenal (**1**)	4.78
2-hexyl-2-decenal (**6**)	6.91
2-octyl-2-dodecenal (**7**)	9.04