Advanced Synthesis & Catalysis

Supporting Information

Alteration of Chain Length Selectivity of *Candida antarctica* Lipase A by Semi-Rational Design for the Enrichment of Erucic and Gondoic Fatty Acids

Katja Zorn[a1], Isabel Oroz-Guinea[a1], Henrike Brundiek[b], Mark Dörr[a] and Uwe T. Bornscheuer[a]*

[a]University of Greifswald, Institute of Biochemistry, Dept. of Biotechnology & Enzyme Catalysis, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany

Fax: (+49) 3834 420 744367; phone: (+49) 3834 420 4367; email: uwe.bornscheuer@uni-greifswald.de

[b]Enzymicals AG, Walther-Rathenau-Str. 49a, 17489 Greifswald, Germany.

[1]Equal contribution.

Supplementary information

Table S1. Comparison of different methods to generate site-directed saturation mutagenesis libraries, including the number of primers used, the number of codons contained, the number of amino acids and stop codons encoded, and the screening effort required for the simultaneous saturation of 1, 2 or 3 positions to achieve a 95 % library coverage.

Degenerate primers	Number of primers	Number of codons	Number of aa (Stop codon)	1 Pos.	2 Pos.	3 Pos.
NNN	1	64	20 (3)	192	12 288	786 432
NNK	1	32	20 (1)	96	3 072	98 304
22c-trick[19a]	3	22	20 (0)	66	1 450	31 899
20c-trick[19b]	4	20	20 (0)	60	1 200	24 000
NDT	1	9	9 (0)	36	432	5 184

Table S2. Amino acid changes present in the CAL-A variants chosen from the high-throughput screening for further analysis.

Variant	V238	V286	V290	Additional mutation
V1	L	-	L	
V2	Y	-	N	
V3	I	-	M	
V4	M	H	-	
V5	L	H	-	
V6	L	H	M	
V7	I	-	-	
V8	S	-	T	
V9	-	Q	W	
V10	L	Q	-	
V11	L	-	T	P399Q
V12	L	-	N	
V13	-	R	E	E335K
V14	L	-	H	
V15	I	-	L	N285D

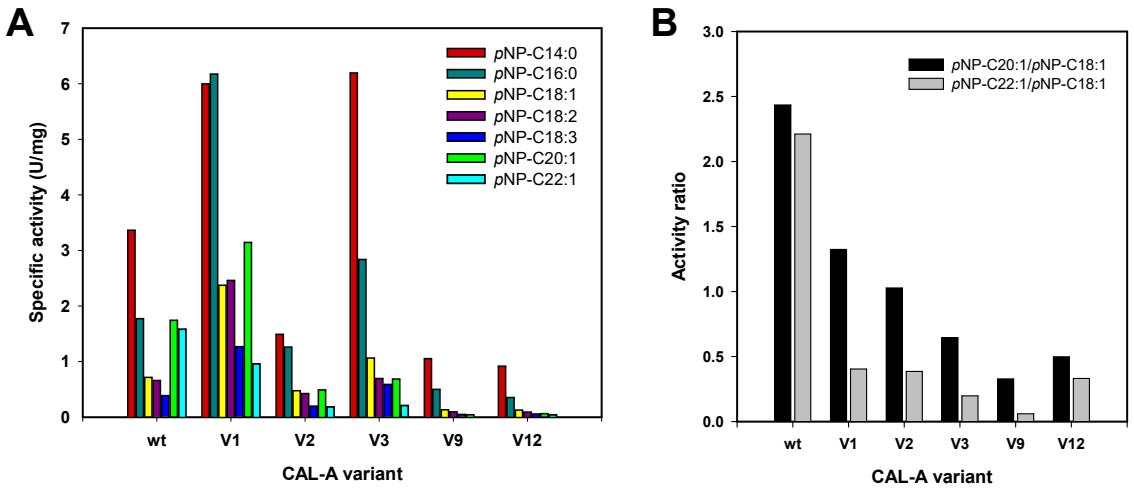


Figure S1. (**A**) Chain length profile displayed by the selected CAL-A variants (wt, V1, V2, V3, V9 and V12) overexpressed in *P. pastoris* towards *p*NP-C14:0 (■), -C16:0 (■), -C18:1 (■), -C18:2 (■), -C18:3 (■), -C20:1 (■) and –C22:1 (■). (**B**) Activity ratios calculated for the aforementioned CAL-A variants: *p*NP-C20:1/*p*NP-C18:1 (■), *p*NP-C22:1/*p*NP-C18:1 (■).

Table S3. Protein concentration present in the supernatant obtained during the small scale *P. pastoris* overexpression of the different CAL-A variants. All the samples were previously desalted before quantification. 1Mut: single mutant. 2Mut: double mutant. 3Mut: triple mutant.

CAL-A 1Mut variants	**V238L**	**V238I**	**V238Y**	**V286Q**	**V290L**	**V290M**	**V290N**	**V290W**
Protein conc. (mg/ml)	1.7	1.6	1.6	2.1	1.6	1.7	1.6	1.7

CAL-A 2Mut variants	**V1**	**V2**	**V3**	**V9**	**V12**
Protein conc. (mg/ml)	1.7	1.6	2.0	1.7	2.1

CAL-A 3Mut variants	**V16**	**V17**	**V18**	**V19**	**V20**
Protein conc. (mg/ml)	1.7	1.7	2.0	1.7	2.1

CAL A	**wt**
Protein conc. (mg/ml)	1.8

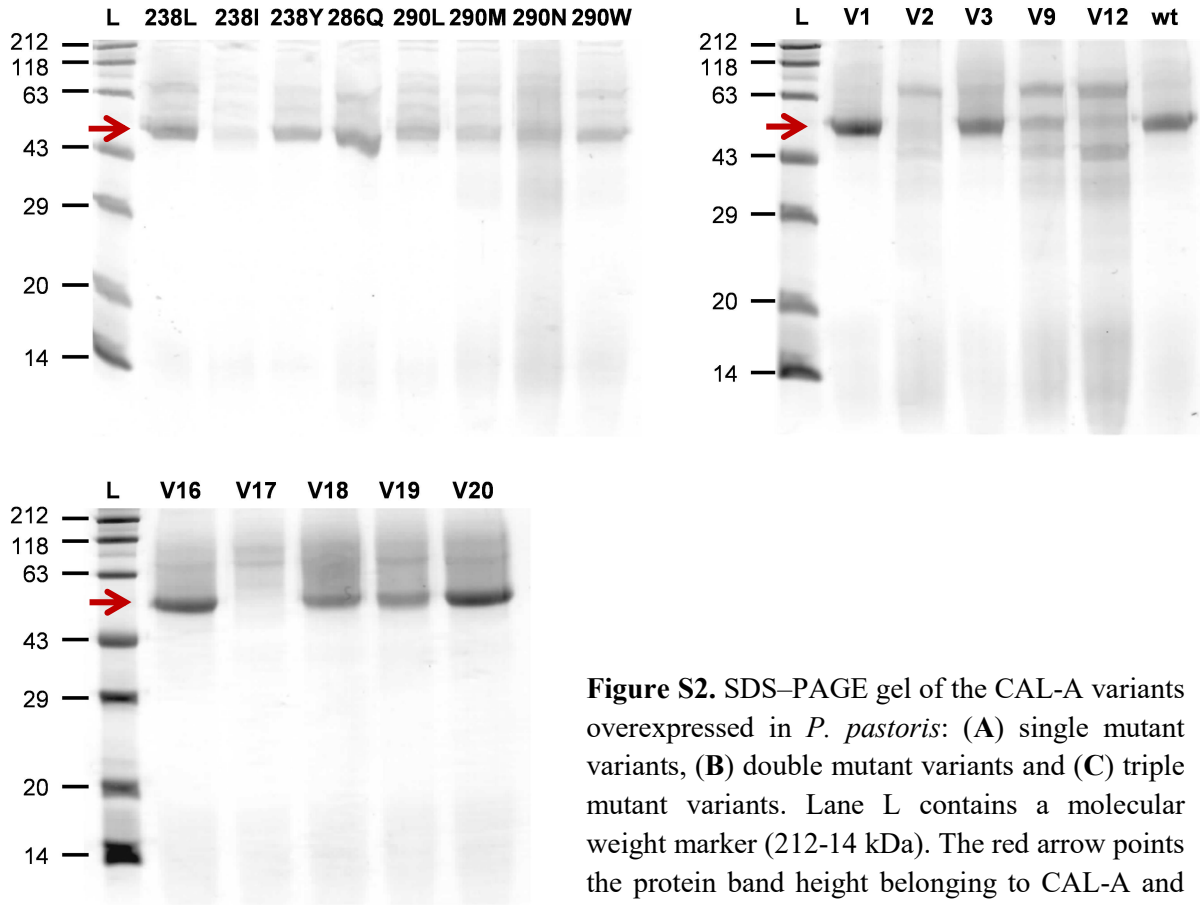
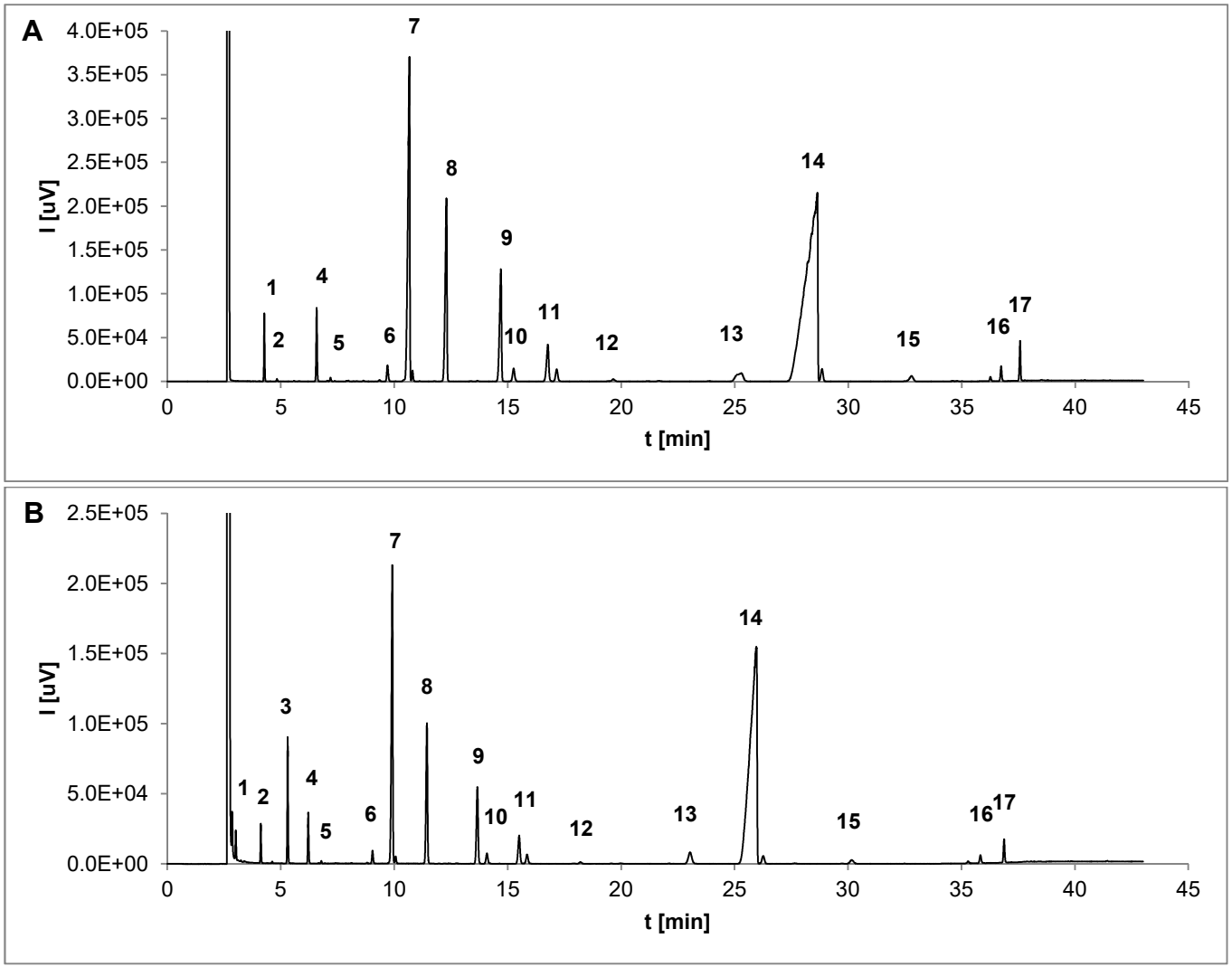


Figure S2. SDS–PAGE gel of the CAL-A variants overexpressed in *P. pastoris*: (**A**) single mutant variants, (**B**) double mutant variants and (**C**) triple mutant variants. Lane L contains a molecular weight marker (212-14 kDa). The red arrow points the protein band height belonging to CAL-A and its variants.

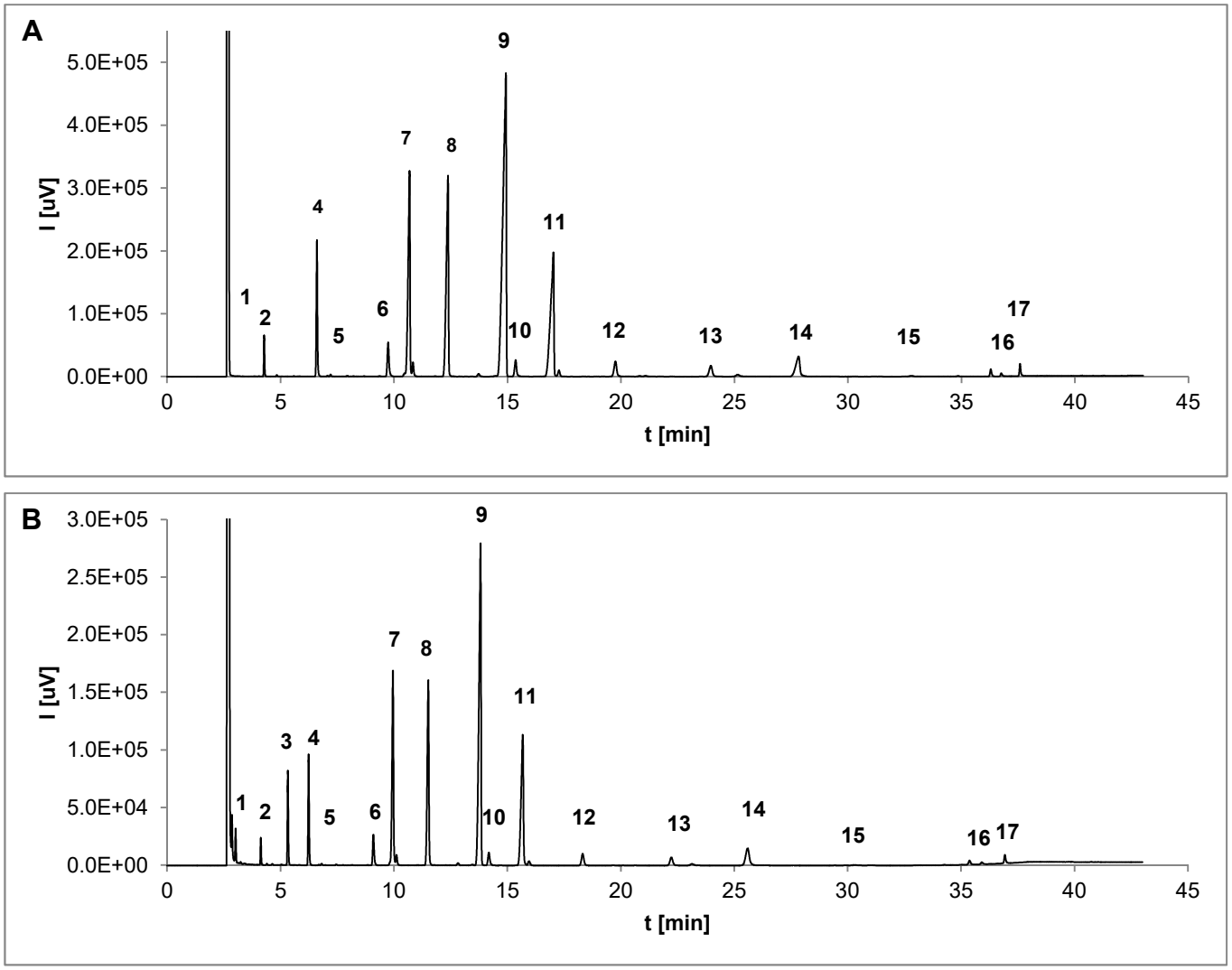
Table S4. Summary of the protein concentration and the hydrolytic activity towards *p*NP-C14:0 quantified for the fermented CAL-A variants. Hydrolytic activity was determined twice, firstly for the supernatant collected at the end of the fermentation (t=162 h; U/ml_{SN}), and subsequently for the same sample after a desalting step (U/ml_{dSN}). Protein concentration was determined for the latter aliquot.

CAL-A variant	Concentration (mg_{prot}/ml_{dSN})	Activity) (U/ml_{SN})	Activity) (U/ml_{dSN})	Activity (U/mg_{prot})
wt	1.7	1028.1	634.35	368.9
V290W	2.2	1667.8	1234.58	558.1
V1	1.1	2377.5	1857.84	1,672.4
V2	2.3	544.6	422.03	183.7
V3	1.8	1552.9	1016.85	576.6
V9	1.6	536.6	435.90	271.9
V16	2.3	496.0	358.90	153.3
V18	1.1	793.8	438.47	389.9
V19	1.6	1673.4	1248.25	762.3
V20	1.9	318.4	231.65	120.1



Peak	FAEE	FAEE x_i [%]	FAME x_i [%]
1	*C13:0*	*2 mM*	*2 mM*
2	C14:0	0.05	0.05
	C15:0		*6 mM*
4	C16:0	1.77	1.81
5	C16:1	0.10	0.09
6	C18:0	0.62	0.64
7	C18:1	16.85	16.13
8	C18:2	8.65	7.66
9	C18:3	5.69	4.69
10	C20:0	0.72	0.73
11	C20:1	2.95	2.81
12	C20:2	0.11	0.13
13	C22:0	1.53	1.49
14	C22:1	58.68	57.66
15	C22:2	0.45	0.41
16	C24:0	0.52	0.49
17	C24:1	1.14	1.08

Figure S3. Chromatograms of the FAEE derived from *Crambe* oil: (**A**) unmodified FAEE *Crambe* derivatives and (**B**) the total FA as FAME. Both samples contained ethyl tridecanoate (1) and tripentadecanoin (3) as external standards. In the table the mole fraction (x_i) of the FA in the *Crambe* FAEE is displayed.



Peak	FA	FAEE x_i [%]	FAME x_i [%]
1	*C13:0*	*2 mM*	*2 mM*
2	C14:0	0.05	0.08
	C15:0	-	*6 mM*
4	C16:0	5.46	5.68
5	C16:1	0.07	0.09
6	C18:0	2.18	2.33
7	C18:1	15.31	15.98
8	C18:2	15.81	15.56
9	C18:3	37.95	35.37
10	C20:0	1.20	1.31
11	C20:1	15.38	16.26
12	C20:2	1.45	1.47
13	C22:0	1.24	1.16
14	C22:1	3.06	3.15
15	C22:2	0.09	0.10
16	C24:0	0.18	0.17
17	C24:1	0.60	0.64

Figure S4. Chromatograms of the FAEE derived from *Camelina* oil: (**A**) unmodified FAEE *Camelina* derivatives and (**B**) the total FA as FAME. Both samples contained ethyl tridecanoate (1) and tripentadecanoin (3) as external standards. In the table the mole fraction (x_i) of the FA in the *Camelina* FAEE is displayed.

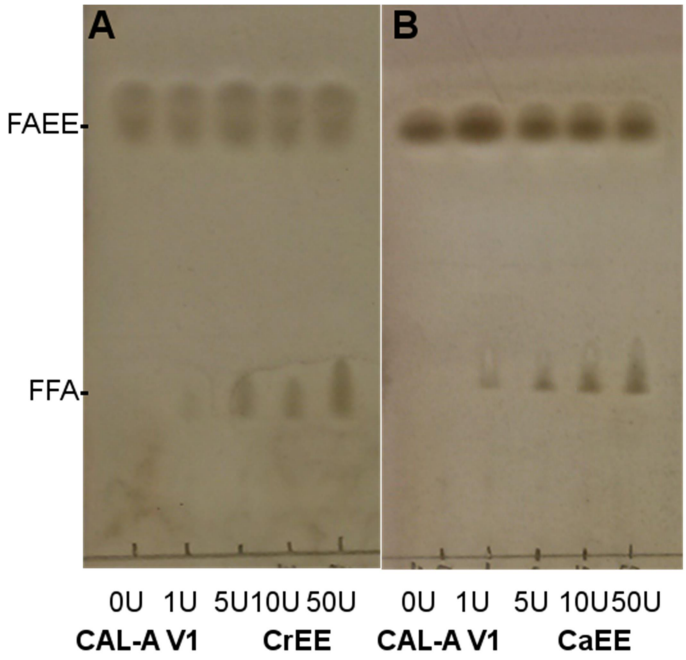


Figure S5. Exemplary thin-layer chromatography showing the reactions of 0, 1, 5, 10, and 50 U CAL-A V1 with *Crambe* and *Camelina* FAEE on aluminum silica foils dyed with potassium permanganate solution.

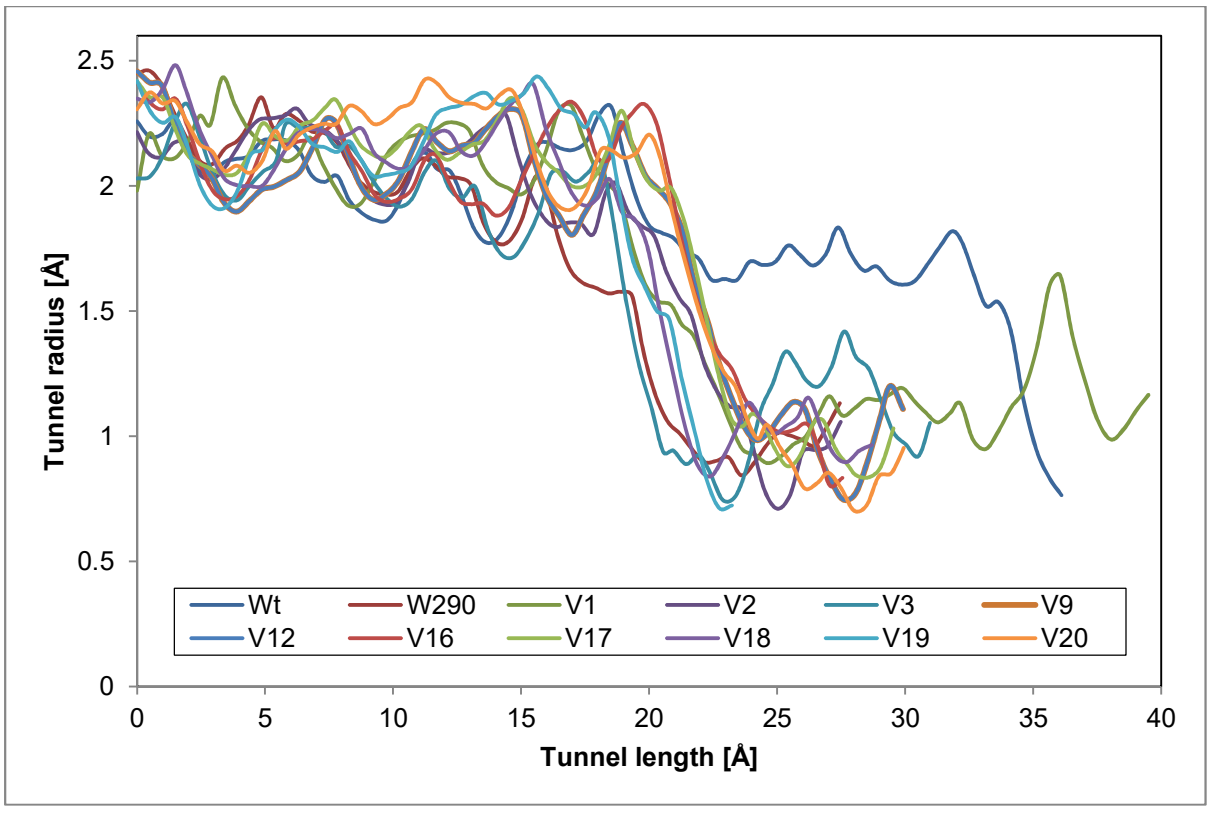


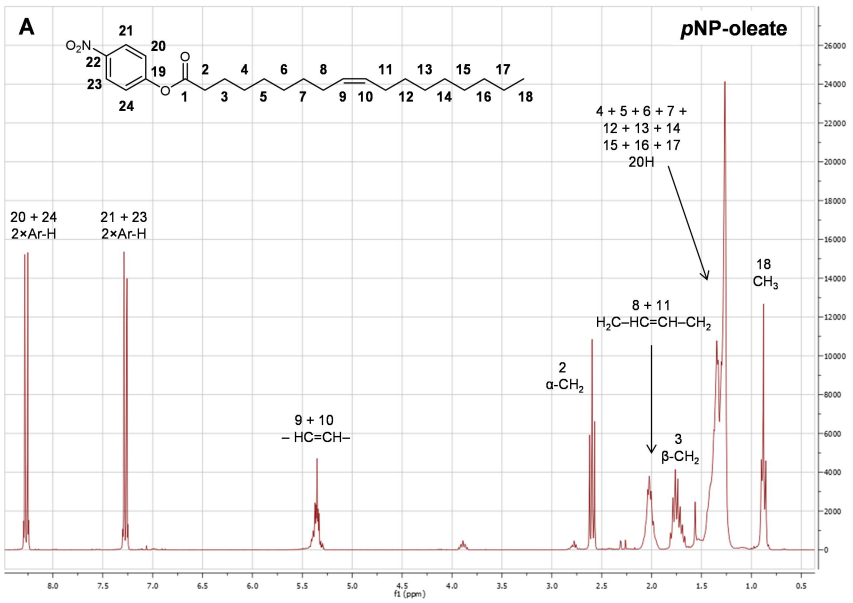
Figure S6. Tunnel calculations with Caver Analyst 1.0 for CAL-A Wt, the single mutant V290W, the double mutants V1, 2, 3, 9, and 12, and the triple mutants V16-20. The tunnel graphs are displayed for the fatty acid binding tunnel calculated from the catalytic Ser185 with a min. probe radius of 0.6-0.7.

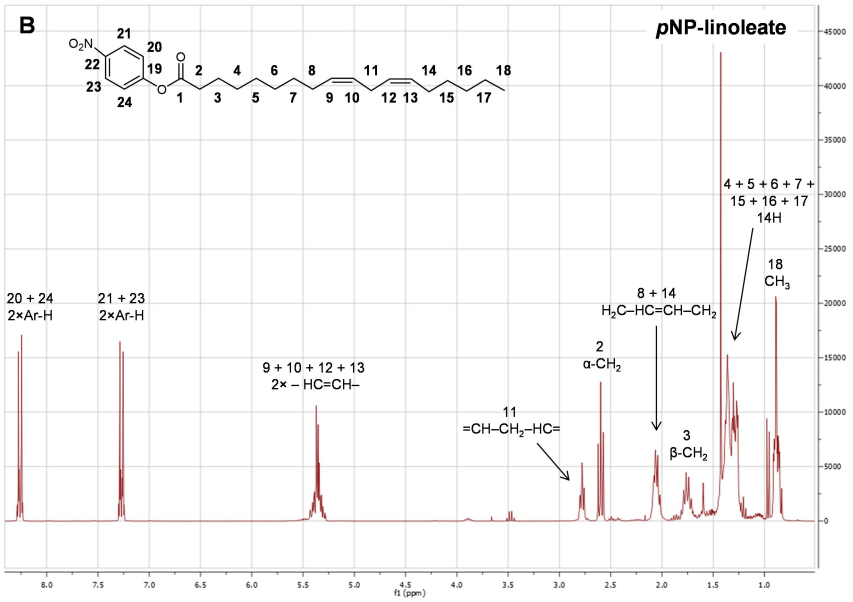
Table S5. Estimated dimensions of the fatty acid molecules.

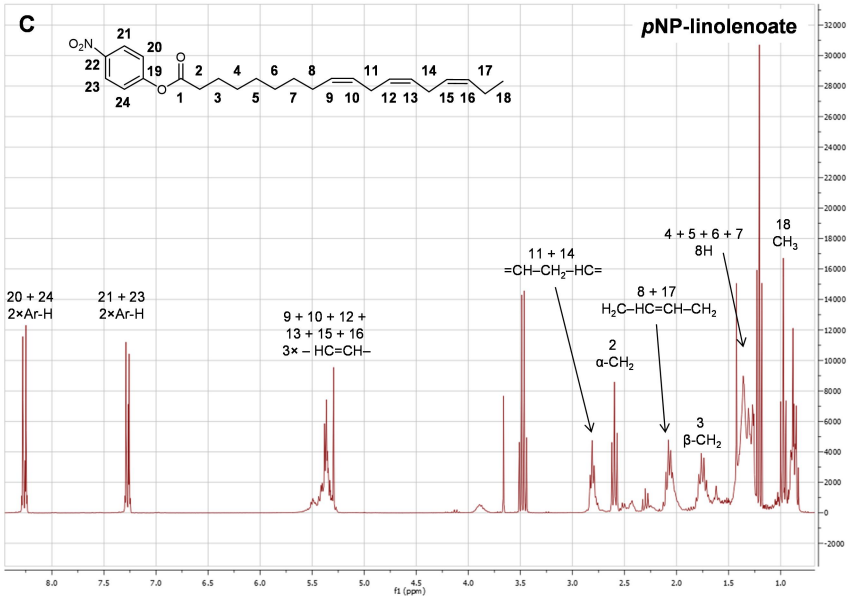
Fatty acid	Length [Å]	Distance double bond [Å]
Oleic acid	19.0 - 21.3	9.5 - 11.2
Gondoic acid	20.4 - 23.8	10.4 - 13.6
Erucic acid	21.8 - 26.3	13.2 - 16.1

Table S6. Primers used to generate CAL-A libraries I to III

Primer name		Primer Sequence
15.15	pET-RP rv	CTAGTTATTGCTCAGCGG
16.19	QC/MW1 CAL-A V286X rv	CAGGCTAAA**CAC**GTTCAGAAACGGATAG
		CAGGCTAAA**NDT**GTTCAGAAACGGATAG
		CAGGCTAAA**VHG**GTTCAGAAACGGATAG
		CAGGCTAAA**TGG**GTTCAGAAACGGATAG
16.20	QC V290X fw	TTTAGCCTG**GTG**AACGATACCAACCTG
		TTTAGCCTG**NDT**AACGATACCAACCTG
		TTTAGCCTG**VHG**AACGATACCAACCTG
		TTTAGCCTG**TGG**AACGATACCAACCTG
16.21	MW1/MW2 V238X fw	CCGGT**GTG**AGCGGTCTGAGC
		CCGGT**NDT**AGCGGTCTGAGC
		CCGGT**VHG**AGCGGTCTGAGC
		CCGGT**TGG**AGCGGTCTGAGC
16.22	MW2 V290X rv	GTATCGTT**CAC**CAGGCTAAACACGTTC
		GTATCGTT**NDT**CAGGCTAAACACGTTC
		GTATCGTT**VHG**CAGGCTAAACACGTTC
		GTATCGTT**TGG**CAGGCTAAACACGTTC


A
pNP-oleate
20 + 24
2×Ar-H
21 + 23
2×Ar-H
9 + 10
– HC=CH–
2
α-CH2
8 + 11
H2C–HC=CH–CH2
3
β-CH2
4 + 5 + 6 + 7 +
12 + 13 + 14
15 + 16 + 17
20H
18
CH3
f1 (ppm)


B
pNP-linoleate
20 + 24
2×Ar-H
21 + 23
2×Ar-H
9 + 10 + 12 + 13
2× – HC=CH–
11
=CH–CH2–HC=
2
α-CH2
8 + 14
H2C–HC=CH–CH2
3
β-CH2
4 + 5 + 6 + 7 +
15 + 16 + 17
14H
18
CH3
f1 (ppm)


C
pNP-linolenoate
20 + 24
2×Ar-H
21 + 23
2×Ar-H
9 + 10 + 12 +
13 + 15 + 16
3× – HC=CH–
11 + 14
=CH–CH2–HC=
2
α-CH2
8 + 17
H2C–HC=CH–CH2
3
β-CH2
4 + 5 + 6 + 7
8H
18
CH3
f1 (ppm)

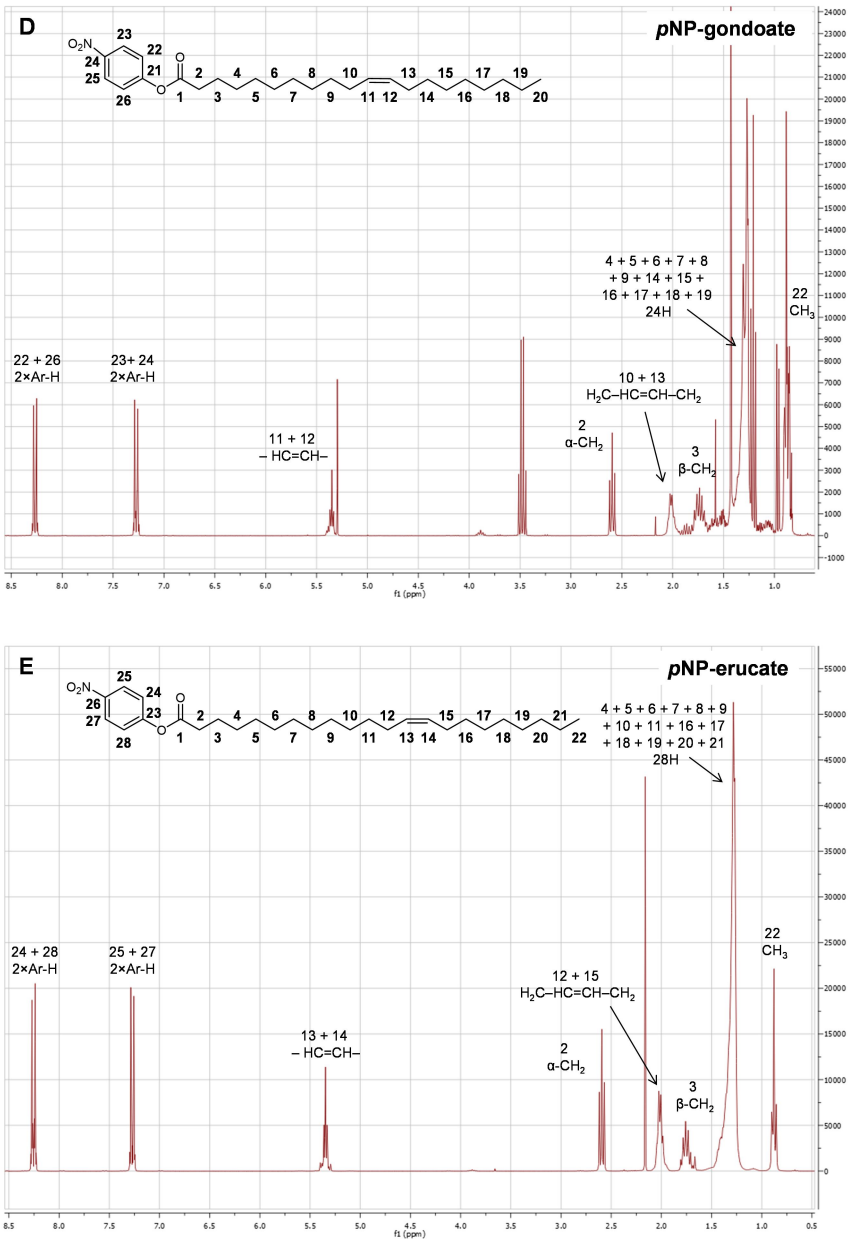


Figure S7. Observed ^{1}H NMR spectra of (**A**) *p*NP-oleate, (**B**) *p*NP-linoleate, (**C**) *p*NP-linolenoate, (**D**) *p*NP-gondoate, (**E**) *p*NP-erucate.

Table S7. Primers and plasmids used to create the single, double and triple mutant vectors for *P. pastoris* overexpression.

CAL-A variant	Resulting plasmid	Plasmid template	Primer fw name	Primer rv name
V238L	pGAPZα-CALA-His V238L	pGAPZα-CALA-His	CALA V238L QC fw	CALA V238L QC rv
	pPICZα-CALA-His V238L	pPICZα-CALA-His		
V238I	pGAPZα-CALA-His V238I	pGAPZα-CALA-His	CALA V238I QC fw	CALA V238I QC rv
	pPICZα-CALA-His V238I	pPICZα-CALA-His		
V238Y	pGAPZα-CALA-His V238Y	pGAPZα-CALA-His	CALA V238Y QC fw	CALA V238Y QC rv
	pPICZα-CALA-His V238Y	pPICZα-CALA-His		
V286Q	pGAPZα-CALA-His V286Q	pGAPZα-CALA-His	CALA V286Q QC fw	CALA V286Q QC rv
	pPICZα-CALA-His V286Q	pPICZα-CALA-His		
V290L	pGAPZα-CALA-His V290L	pGAPZα-CALA-His	CALA V290L QC fw	CALA V290L QC rv
V290M	pGAPZα-CALA-His V290M	pGAPZα-CALA-His	CALA V290M QC fw	CALA V290M QC rv
V290N	pGAPZα-CALA-His V290N	pGAPZα-CALA-His	CALA V290N QC fw	CALA V290N QC rv
V290W	pGAPZα-CALA-His V290W	pGAPZα-CALA-His	CALA V290W QC fw	CALA V290W QC rv
	pPICZα-CALA-His V290W	pPICZα-CALA-His		
V1	pPICZα-CALA-His V238L V290L	pPICZαB-CALA-His V238L	CALA V290L QC fw	CALA V290L QC rv
V2	pPICZα-CALA-His V238Y V290N	pPICZαB-CALA-His V238Y	CALA V290N QC fw	CALA V290N QC rv
V3	pPICZα-CALA-His V238I V290M	pPICZαB-CALA-His V238I	CALA V290M QC fw	CALA V290M QC rv
V9	pPICZα-CALA-His V286Q V290W	pPICZαB-CALA-His V286Q	CALA (V286Q) V290W QC fw	CALA (V286Q) V290W QC rv
V12	pPICZα-CALA-His V238L V290N	pPICZαB-CALA-His V238L	CALA V290N QC fw	CALA V290N QC rv
V16	pPICZα-CALA-His V238L V286Q V290L	pPICZα-CALA-His V238L, V290L	CALA V286Q (V290L) QC fw	CALA V286Q (V290L) QC rv
V17	pPICZα-CALA-His V238Y V286Q V290N	pPICZα-CALA-His V238Y, V290N	CALA V286Q (V290N) QC fw	CALA V286Q (V290N) QC rv
V18	pPICZα-CALA-His V238I V286Q V290M	pPICZα-CALA-His V238I, V290M	CALA V286Q (V290M) QC fw	CALA V286Q (V290M) QC rv
V19	pPICZα-CALA-His V238L V286Q V290W	pPICZα-CALA-His V286Q, V290W	CALA V238L QC fw	CALA V238L QC rv
V20	pPICZα-CALA-His V238I V286Q V290W	pPICZα-CALA-His V286Q, V290W	CALA V238I QC fw	CALA V238I QC rv

Table S8. Sequences of the primers used to create the single, double and triple mutant vectors for *P. pastoris* overexpression.

Primer name	Sequence fw	Sequence rv
CALA V238L QC	5'-GCCCTGGCGGGTCTTTCGGGTCTCT-3'	5'-AGAGACCCGAAAGACCCGCCAGGGC-3'
CALA V238I QC	5'-TGCCCTGGCGGGTATTTCGGGTCTCTC-3'	5'-GAGAGACCCGAAATACCCGCCAGGGCA-3'
CALA V238Y QC	5'-TTGCCCTGGCGGGTTATTCGGGTCTCTCGC-3'	5'-GCGAGAGACCCGAATAACCCGCCAGGGCAA-3'
CALA V286Q QC	5'-CCTACCCCTTCCTCAACCAGTTCTCGCTGGTCAACGA-3'	5'-TCGTTGACCAGCGAGAACTGGTTGAGGAAGGGGTAGG-3'
CALA V290L QC	5'-AACGTCTTCTCGCTGCTCAACGACACGAACC-3'	5'-GGTTCGTGTCGTTGAGCAGCGAGAAGACGTT-3'
CALA V290M QC	5'-CAACGTCTTCTCGCTGATGAACGACACGAACCTGC-3'	5'-GCAGGTTCGTGTCGTTCATCAGCGAGAAGACGTTG-3'
CALA V290N QC	5'-TCAACGTCTTCTCGCTGAACAACGACACGAACCTGC-3'	5'-GCAGGTTCGTGTCGTTGTTCAGCGAGAAGACGTTGA-3'
CALA V290W QC	5'-CTCAACGTCTTCTCGCTGTGGAACGACACGAACCTGCTC-3'	5'-GAGCAGGTTCGTGTCGTTCCACAGCGAGAAGACGTTGAG-3'
CALA (V286Q) V290W QC	5'-CTCAACCAGTTCTCGCTGTGGAACGACACGAACCTGCTC-3'	5'-GAGCAGGTTCGTGTCGTTCCACAGCGAGAACTGGTTGAG-3'
CALA (V290L) V286Q QC	5'-CCTACCCCTTCCTCAACCAGTTCTCGCTGCTCAACGA-3'	5'-TCGTTGAGCAGCGAGAACTGGTTGAGGAAGGGGTAGG-3'
CALA (V290N) V286Q QC	5'-CCTACCCCTTCCTCAACCAGTTCTCGCTGAACAACGA-3'	5'-TCGTTGTTCAGCGAGAACTGGTTGAGGAAGGGGTAGG-3'
CALA (V290M) V286Q QC	5'-CCTACCCCTTCCTCAACCAGTTCTCGCTGATGAACGA-3'	5'-TCGTTCATCAGCGAGAACTGGTTGAGGAAGGGGTAGG-3'