

# Supporting Information

# **Evolving the Promiscuity of** *Elizabethkingia meningoseptica* Oleate Hydratase for the Regio- and Stereoselective Hydration of Oleic Acid Derivatives

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anie\_201901462\_sm\_miscellaneous\_information.pdf

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# **Experimental Procedures**

# General

Unless stated otherwise, standard laboratory reagents were obtained from Sigma-Aldrich® (Steinheim, Germany) or Carl Roth GmbH & Co. KG (Karlsruhe, Germany) with the highest purity available. Oleic acid (OA) and esters thereof (methyl, ethyl, *i*-propyl), oleyl alcohol and oleyl amine were purchased from Sigma-Aldrich® (Steinheim, Germany). The OA methyl and ethyl ester and oleyl alcohol were distilled prior to use to a purity >90% according to GC-FID analysis. (*R*)-10-hydroxy stearic acid was obtained from DSM Innovative Synthesis B.V. (Geleen, Netherlands). Other starting materials used for the investigations were synthesized from suitable precursors as described below or used as received from Sigma-Aldrich®. Flash column chromatography was performed on Acros Organics silica gel 0.035-0.070 mm, 60 Å. Analytical thin layer chromatography (TLC) was performed using TLC-plates from Merck (TLC aluminium foil, silica gel 60 F254) and subsequent visualization with cerium ammonium molybdate stain. The specific optical rotations were determined on a Perkin Elmer Polarimeter 341 with an integrated sodium vapor lamp. All samples were measured at the D-line of the sodium light ( $\lambda$  = 589 nm).

# Plasmid and expression strain construction

Restriction enzymes were acquired from Thermo Scientific (St. Leon-Rot, Germany). Sterile water was purchased from Fresenius Kabi (Graz, Austria). Molecular cloning of the expression vector was performed according to standard procedures<sup>[1]</sup> and correct integration of the insert was confirmed by sequencing (LGC Genomics, Berlin, Germany). For gene amplification, Phusion® High Fidelity DNA polymerase (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany) was utilized in accordance with the recommended PCR protocol. A codon-optimized gene variant of OhyA (*Elizabethkingia meningoseptica* XP\_001209325 oleate hydratase) was purchased from DNA2.0 (Menlo Park, CA). For expression of recombinant OhyA, a modified pMS470 expression vector, pMS470-HISTEV-OhyA was constructed as described previously.<sup>[2]</sup> For all cloning steps and plasmid replication, *E. coli* Top10 F' (F'[*Iacl*<sup>q</sup> Tn*10*(tet<sup>R</sup>)] *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) φ80*IacZ*ΔM15 Δ*IacX*74 *deoR nupG recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL*(Str<sup>R</sup>) *endA1* λ<sup>·</sup>) from Life technologies (Vienna, Austria) was used. Recombinant OhyA was expressed in *E. coli* BL21Star<sup>TM</sup> (DE3) (F- *ompT hsdS*<sub>B</sub> (r<sub>B</sub><sup>-m</sup><sub>B</sub><sup>-</sup>) *gal dcm rne131* (DE3)) (Life technologies, Vienna, Austria).

# Amino acid sequence alignments

The protein sequence of OhyA was compared to the amino acid sequences in the Hydratase Engineering Database (HyED), in which a total of 2046 sequences are collected.<sup>[3]</sup> Since OhyA is categorized in homologous family 11 (HFam11) of the HyED, all amino acid sequences from HFam11 were selected for the multiple sequence alignment. Sequences were extracted from the database for a multiple sequence alignment with the Clustal Omega sequence alignment tool using default settings,<sup>[4]</sup> and were visualized with the Unipro UGene software.

#### Site-directed mutagenesis

Amino acid exchange variants of OhyA were generated by site-directed mutagenesis using a modified Stratagene QuikChange<sup>™</sup> site-directed mutagenesis protocol. Twenty-five µL of two separate PCR reactions containing forward and reverse primers, respectively, were prepared (**Fehler! Verweisquelle konnte nicht gefunden werden.**Table S1). After five cycling steps, PCR reactions were combined and the PCR was continued for 20 additional cycles. Mutated plasmids were verified by sequencing of the coding regions of the constructs.

Primer name	Primer sequence (from 5' to 3')
Fw(OhyA_Gln265Ala)	GTTTCCGAAGTACAAT <u>GCA</u> TATGACACGTTTGTC
Rv(OhyA_GIn265Ala)	GACAAACGTGTCATA <u>TGC</u> ATTGTACTTCGGAAAC
Fw(OhyA_GIn265Glu)	GTTTCCGAAGTACAAT <u>GAA</u> TATGACACGTTTGTC
Rv(OhyA_GIn265Glu)	GACAAACGTGTCATATTGTACTTCGGAAAC
Fw(OhyA_GIn265Lys)	GTTTCCGAAGTACAATAAATATGACACGTTTGTC

Table S1. Primers used for introduction of point mutations into the OhyA nucleotide sequence. The underlined bases mark the mutated codons.

Rv(OhyA\_GIn265Lys) Fw(OhyA\_GIn265Ser) Rv(OhyA\_GIn265Ser) Fw(OhyA\_Thr436Ala) Rv(OhyA\_Thr436Ala) Fw(OhyA\_Thr436Asn) Rv(OhyA\_Thr436Asn) Fw(OhyA\_Thr436Asp) Rv(OhyA\_Thr436Asp) Fw(OhyA\_Thr436Lys) Rv(OhyA\_Thr436Lys) Fw(OhyA\_Asn438Ala) Rv(OhyA\_Asn438Ala) Fw(OhyA\_Asn438Arg) Rv(OhyA\_Asn438Arg) Fw(OhyA\_Asn438Asp) Rv(OhyA\_Asn438Asp) Fw(OhyA\_Asn438Lys) Rv(OhyA\_Asn438Lys) Fw(OhyA\_Asn438Ser) Rv(OhyA\_Asn438Ser) Fw(OhyA\_His442Ala) Rv(OhyA\_His442Ala) Fw(OhyA\_His442Asn) Rv(OhyA\_His442Asn) Fw(OhyA\_His442Asp) Rv(OhyA\_His442Asp) Fw(OhyA\_His442GIn) Rv(OhyA\_His442GIn) Fw(OhyA\_His442Glu) Rv(OhyA\_His442Glu) Fw(OhyA\_His442Tyr) Rv(OhyA\_His442Tyr) Fw(OhyA\_ Asn438Ala/His442Ala) Rv(OhyA\_Asn438Ala/His442Ala) Fw(OhyA\_Thr436Asp/Asn438Asp) Rv(OhyA\_Thr436Asp/Asn438Asp) Fw(OhyA\_Thr436Asp/Asn438Ser) Rv(OhyA\_Thr436Asp/Asn438Ser)

GACAAACGTGTCATA<u>TTT</u>ATTGTACTTCGGAAAC GTTTCCGAAGTACAAT<u>TCT</u>TATGACACGTTTGTC GACAAACGTGTCATA<u>AGA</u>ATTGTACTTCGGAAAC TGGTTGATGAGCTTT<u>GCG</u>TGCAATCGCCAGCCG CGGCTGGCGATTGCACGCAAAGCTCATCAACCA TGGTTGATGAGCTTT<u>AAC</u>TGCAATCGCCAGCCG CGGCTGGCGATTGCA<u>GTT</u>AAAGCTCATCAACCA TGGTTGATGAGCTTTGATTGCAATCGCCAGCCG CGGCTGGCGATTGCAATCAAAGCTCATCAACCA TGGTTGATGAGCTTT<u>AAA</u>TGCAATCGCCAGCCG CGGCTGGCGATTGCATTTAAAGCTCATCAACCA GATGAGCTTTACCTGC<u>GCA</u>CGCCAGCCGCATTTCC GGAAATGCGGCTGGCG<u>TGC</u>GCAGGTAAAGCTCATC GATGAGCTTTACCTGC<u>CGC</u>CGCCAGCCGCATTTCC GGAAATGCGGCTGGCG<u>GCG</u>GCAGGTAAAGCTCATC GATGAGCTTTACCTGC<u>GCA</u>CGCCAGCCGCATTTCC GGAAATGCGGCTGGCGTGCGCAGGTAAAGCTCATC GATGAGCTTTACCTGC<u>AAA</u>CGCCAGCCGCATTTCC GGAAATGCGGCTGGCG<u>TTT</u>GCAGGTAAAGCTCATC GATGAGCTTTACCTGC<u>AGC</u>CGCCAGCCGCATTTCC GGAAATGCGGCTGGCG<u>GCT</u>GCAGGTAAAGCTCATC CTGCAATCGCCAGCCG<u>GCC</u>TTCCCGGAGCAGCCGG CCGGCTGCTCCGGGAAGGCCGGCTGGCGATTGCAG CTGCAATCGCCAGCCGAATTTCCCGGAGCAGCCGG CCGGCTGCTCCGGGAAAATTCGGCTGGCGATTGCAG CTGCAATCGCCAGCCG<u>GAT</u>TTCCCGGAGCAGCCGG CCGGCTGCTCCGGGAAAATCCGGCTGGCGATTGCAG CTGCAATCGCCAGCCG<u>CAA</u>TTCCCGGAGCAGCCGG CCGGCTGCTCCGGGAATTGCGGCTGGCGATTGCAG CTGCAATCGCCAGCCG<u>GAA</u>TTCCCGGAGCAGCCGG CCGGCTGCTCCGGGAATTCCGGCTGGCGATTGCAG CTGCAATCGCCAGCCG<u>TAT</u>TTCCCGGAGCAGCCGG CCGGCTGCTCCGGGAAAATACGGCTGGCGATTGCAG GCGCACGCCAGCCGGCTTTCCCGGAGCAGCCGGAT ATCCGGCTGCTCCGGGAAAGCCGGCTGGCGTGCGC GGTTGATGAGCTTT<u>GAC</u>TGC<u>GAC</u>CGCCAGCCGCATT AATGCGGCTGGCG<u>GTC</u>GCA<u>GTC</u>AAAGCTCATCAACC GGTTGATGAGCTTT<u>GAC</u>TGC<u>AGC</u>CGCCAGCCGCATT AATGCGGCTGGCG<u>GCT</u>GCA<u>GTC</u>AAAGCTCATCAACC

## **Recombinant protein expression**

OhyA was recombinantly expressed in E. coli. First, a pre-culture was inoculated with E. coli BL21 Star (DE3) cells harboring pMS470-HISTEV-OhyA wild type enzyme or variants, and grown in LB supplemented with 100 µg mL-1 ampicillin at 28°C and 130 rpm overnight. Main cultures were inoculated to an OD600 of 0.1 in auto induction medium (AIM) - Terrific Broth Base including Trace elements (Formedium, UK) containing 100 µg mL-1 ampicillin. Recombinant protein was expressed at 28°C and 130 rpm for 22 h. Cells were harvested by centrifugation for 10 min at 4,400 x g and 22°C, and were instantly used for whole cell biotransformations or frozen at -20°C until preparation of cell-free lysates.

## In vitro conversion of OA and OA derivatives with cell-free lysate

For preparation of cell-free lysates containing recombinant hydratase enzyme, thawed cell pellets were resuspended in 50 mM HEPES, pH 7.4. Cells were lysed by ultrasonication for 4 min with a Sonifier® 250 (Branson, Danbury, CT) setting the duty cycle to 80% and the output control to level 8. Cell-free lysate was separated from the total lysate by centrifugation for 35 min at 48,300 x g and 4°C. In vitro activity assays were performed with 2 mg of *E. coli* cell-free lysate in Pyrex® glass culture tubes (Corning, NY). Cell-free lysate was incubated with 2 mM substrates **1a–1j** in 1 mL of 50 mM HEPES, pH 6.0, and 2% (v/v) of ethanol. Assays were shaken over night at 25°C and 150 rpm in the presence of 1 mM *n*-pentadecanoic acid as internal standard. After conversion, assays were quenched either by acidification to pH 2.0 with 0.12 M HCl and extraction with 2 × 2 mL of ethyl acetate (in case of **1a** and **1c–1j**), or only by extraction with 2 × 2 mL of ethyl acetate (in case of **1b**) while agitating on a Vibrax VXR basic shaker (IKA, Germany) for 30 min. The suspension was centrifuged for 5 min at 2,900 × g and 22°C to improve separation of the phases. Combined organic phases were concentrated under a N<sub>2</sub> stream. Fatty acid derivatives were silylated with 10 µL of pyridine and 50 µL of *N*, *O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). After incubation for 30 min at 500 rpm, the reaction mixtures were diluted with 200 µL of ethyl acetate and analyzed by GC-MS or GC-FID.

#### Whole cell bioconversions

Bioconversion assays of **1a–j** were performed with *E. coli* BL21 Star (DE3) cells immediately after expression of recombinant OhyA. Fifty OD<sub>600</sub> units, which are corresponding to a cell dry weight of 50 mg, were resuspended in 50 mM HEPES, pH 6.0, supplemented with 100 mM glucose and 0.2 mM FAD in Pyrex® glass culture tubes (Corning, NY). Biotransformations at 1 mL scale were started by adding substrate to a final concentration of 2 mM from an ethanolic stock solution (100 mM). *n*-pentadecanoic acid (1 mM) was used as internal standard. The reactions were conducted in the presence of 2% (v/v) of ethanol as co-solvent at 30°C and shaking at 150 rpm at a defined angle of the Pyrex® tubes (55°). Biotransformations were performed for 22 h or 96 h.

Whole cell bioconversions of OA esters **1f** and **1g** with alcohol additives were performed with 50 mg of *E. coli* BL21 Star (DE3) after recombinant expression of OhyA Gln265Ala/Thr436Ala/Asn438Ala. Biotransformations of **1f** were coincubated with equimolar concentrations of the substrate and either ethanol or *i*-propanol, and biotransformations of **1g** were co-incubated with equimolar concentrations of the substrate and either methanol or *i*-propanol. Otherwise, assay conditions were maintained as described above.

# **GC-MS** analyses

Free fatty acids and derivatives thereof were initially analyzed and identified by gas chromatography-mass spectrometry (GC-MS). A HP-5 column (crosslinked 5% Ph-Me Polysiloxane; 30 m length, 0.25 mm in diameter and 0.25  $\mu$ m film thickness) on a Hewlett-Packard 6890 Series II GC equipped with a mass selective detector was used. Sample aliquots of 1  $\mu$ L were injected in split mode (split ratio 30:1) at 240°C injector temperature and 290°C detector temperature with N<sub>2</sub> as carrier at a flow rate set to 36 cm s<sup>-1</sup> in constant flow mode. The temperature program was as follows: 100°C for 1 min, 15°C min<sup>-1</sup> to 300°C, hold for 5 min. The total run time was 19.33 min. The mass selective detector was operated in a mass range of 50-400 amu at an electron multiplier voltage of 1765 V. Results were evaluated with the GC-MS Data Analysis software (Agilent Technologies, Austria).

## **GC-FID** analyses

Product formation was quantified by GC after derivatization of extracted samples with BSTFA. A Shimadzu GC-2010 Plus instrument equipped with a flame ionization detector and a Phenomenex Zebron ZB-5 column (crosslinked 5% Ph-Me Polysiloxane; 30 m length, 0.32 mm in diameter and 0.25  $\mu$ m film thickness) was used. Sample aliquots of 1  $\mu$ L were injected in split mode (split ratio 10:1) at 240°C injector temperature and 320°C detector temperature. N<sub>2</sub> was used as carrier gas at a flow rate set to 20 cm s<sup>-1</sup> in constant flow mode. The oven temperature program was as follows: 70°C for 4 min, 35°C min<sup>-1</sup> to 300°C, hold for 5 min. The total run time was 15.57 min.

# **Preparation of OA derivatives**

Oleamide (1c) was obtained via a literature procedure<sup>[5]</sup> and the material was purified through recrystallization from acetone<sup>[6]</sup> to a purity of 95% as checked by rp-HPLC at 210 nm. *n*-propyl (1i) and *n*-butyl oleate (1j) were synthesized from OA (1a) via Fischer esterification as described in the literature<sup>[7]</sup> and purified via flash chromatography on silica gel using cyclohexane/ethyl acetate 20:1 as eluent.

n-propyl oleate (1i):

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.88 (3H, t, <sup>3</sup>J(H,H) = 6.7 Hz, Me), 0.94 (3H, t, <sup>3</sup>J(H,H) = 7.4 Hz, -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 1.15-1.45 (20H, m, 10 CH<sub>2</sub>), 1.52-1.70 (4H, m, 2 CH<sub>2</sub>), 1.92-2.10 (4H, m, 2 CH<sub>2</sub>), 2.29 (2H, t, <sup>3</sup>J(H,H) = 7.5 Hz, -C<u>H<sub>2</sub>-CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me)</u>, 4.02 (2H, t, <sup>3</sup>J(H,H) = 6.7 Hz, -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 5.34 (2H, m, -C<u>H</u>=C<u>H</u>-).

<sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>): δ = 10.54, 14.25, 22.17, 22.83, 25.17, 27.32, 27.37, 29.26, 29.29, 29.32, 29.47 (2×C), 29.67, 29.84, 29.92, 32.06, 34.54, 65.97, 129.91, 130.15, 174.13.

## *n*-butyl oleate (1j):

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.88 (3H, t, <sup>3</sup>*J*(H,H) = 6.7 Hz, Me), 0.93 (3H, t, <sup>3</sup>*J*(H,H) = 7.4 Hz, -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 1.15-1.45 (22H, m, 11 CH<sub>2</sub>), 1.52-1.70 (4H, m, 2 CH<sub>2</sub>), 1.92-2.10 (4H, m, 2 CH<sub>2</sub>), 2.29 (2H, t, <sup>3</sup>*J*(H,H) = 7.5 Hz, -C<u>H<sub>2</sub>-CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 4.07 (2H, t, <sup>3</sup>*J*(H,H) = 6.6 Hz, -CO<sub>2</sub>C<u>H<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 5.34 (2H, m, -C<u>H</u>=C<u>H</u>-). <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>):  $\delta$  = 13.85, 14.25, 19.30, 22.83, 25.17, 27.32, 27.37, 29.26, 29.29, 29.31, 29.47 (2xC), 29.67, 29.84, 29.92, 30.87, 32.06, 34.55, 64.25, 129.91, 130.15, 174.13.</u></u>

#### Preparative-scale hydration of OA derivatives

OA derivatives 1c-1j were hydrated to 2c-2j in a semi-preparative scale. Twenty to 150 mg of non-physiological substrates were converted in 1 mL scale whole cell bioconversions. Each reaction contained 200 mg of *E. coli* cells in Pyrex® glass tubes, after over-expression of OhyA Gln265Ala/Thr436Ala/Asn438Ala, resuspended in 50 mM HEPES, pH 6.0, containing 100 mM glucose and 0.2 mM FAD. Biotransformations were incubated for 96 h at 30°C and 150 rpm at a defined angle of the Pyrex® tubes (55°). After quenching by acidification to pH 2.0 with 0.12 M HCl, the suspensions were extracted with ethyl acetate (3 × 2 mL for 30 min) with intermittent centrifugation for 5 min at 2,900 × g and 22°C to improve phase separation. The organic phases were quantitatively collected and concentrated under a stream of N<sub>2</sub>.

#### Purification of crude reaction products

The products extracted with ethyl acetate were purified via flash chromatography (9.5 g silica gel,  $20 \times 1$  cm column size) using eluent mixtures of cyclohexane/ethyl acetate in ratios from 10:1 to 1:1 (v/v) dependent on the polarity of the respective derivative. All fractions containing the desired product were pooled and evaporated to dryness.

# NMR analysis of reaction products

<sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Bruker AVANCE III 300 spectrometer (<sup>1</sup>H: 300.36 MHz; <sup>13</sup>C: 75.53 MHz) or a Varian INOVA 500 (<sup>1</sup>H: 499.88 MHz; <sup>13</sup>C: 125.71 MHz). Chemical shifts were referenced to residual protonated solvent signals as internal standard. Chemical shift values are reported in parts per million, and coupling constants (*J* values) are given in Hertz. Abbreviations for <sup>1</sup>H-NMR signals are as follows: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; and m, multiplet.

# Modeling of OA and derivatives to the OhyA 3D structure

Docking of OA to the OhyA 3D structure was performed using AutoDock implemented in YASARA structure as described previously.<sup>[2]</sup> Briefly, receptor (chain A of OhyA; PDB code: 4uir) and ligand (OA; formal charge of -1) were prepared and energy minimized with the Schrodinger package. The receptor was kept rigid, and the ligand had full

conformational flexibility around each single bond. The docking box (x=25 Å, y=27 Å, z= 25 Å) was set to be close to the flavin cofactor covering the elongated active site cavity. After 50 individual runs, the best docking modes were sorted by binding energies and chemical plausibility and were finally clustered according to a maximum root-mean-square deviation (r.m.s.d.) of the heavy atoms of 2.0 Å.

OA derivatives 1c-1j were prepared using YASARA and AM1 charges were applied accordingly. The derivatives 1c-1j were docked to the 3D structure model of OhyA after introducing amino acid exchanges that resulted in the best activity on each substrate. The binding mode with the lowest docking energy was visually inspected. Amino acid exchanges were introduced in silico using YASARA. Docking was performed using VINA<sup>[8]</sup> implemented in YASARA structure in analogy to OA after substitution of the carboxylate for the different head groups of 1c-1j using 20 independent docking runs and a 2.0 Å r.m.s.d cluster deviation.

The docking with the lowest energy for **1i** and **1j** did not result in a productive binding mode, even in the triple variant (visual inspection). This may be due to the rigid receptor docking, which resulted in higher docking energies for the larger derivatives in the productive binding mode. By overlaying of **1i** and **1j** with the docked OA and by performing an energy minimization step (Force Field: Amber03), the ligands **1i** and **1j** fitted quite well into the larger pocket introduced by the mutations. It can be assumed that small changes in the structure on the site of the mutations, although not optimal, do also provide enough space for the larger derivatives **1i** and **1j**.

# **Results and Discussion**

# **Nucleotide information**

Open reading frame of the codon-optimized *E. meningoseptica* oleate hydratase gene (OhyA, GenBank: ACT54545.1) used in this study:

5' - ATGCATCACCATCACCATCACCATCACCACCAACCCAATCACCAGCAAATTCGACAAAGTCCTGAACGCATCC AGCGAGTACGGCCACGTTAATCACGAACCGGATAGCAGCAAAGAGCAGCAACGCAACACCCCGCAGAAGTCCATG CCATTTAGCGATCAAATCGGCAACTATCAACGTAACAAAGGTATTCCGGTTCAGAGCTATGATAATTCGAAGATTTAC ATCATTGGTTCTGGTATTGCGGGTATGTCGGCTGCGTACTACTTCATCCGTGACGGTCACGTTCCGGCGAAGAACA CCGTGGTGGTCGTGAAATGGATATGACCTACGAGAACCTGTGGGATATGTTCCAGGATATTCCCGGCGCTGGAGATG CCGGCACCGTATAGCGTTCTGGATGAATATCGTCTGATTAATGACAACGATAGCAATTACAGCAAAGCACGTCTGAT CAACAATAAGGGCGAAATCAAGGACTTCAGCAAGTTTGGTCTGAATAAGATGGACCAGCTGGCCATCATCCGTCTG TTGCTGAAAAACAAAGAAGAGCTGGACGACTTGACCATTGAGGACTACTTCTCTGAGAGCCTTTCTGAAAAGCAATTT CTGGACGTTTTGGCGCACGATGTTCGCGTTTGAGAACTGGCATAGCCTGTTGGAACTGAAGCTGTACATGCACCGC CACGCCGCTGCGTAAATTCCTGCAAGAAAAGGGTGTTAACATCCACTTGAATACCTTGGTCAAGGATCTGGATATTC ACATCAATACCGAGGGTAAAGTCGTCGAGGGCATCATTACCGAGCAAGACGGTAAAGAGGTAAAGATTCCGGTGG GTAAGAATGACTATGTTATCGTGACGACCGGTTCCATGACCGAGGACACGTTTTACGGTAACAACAAAACCGCACC CGAAATCTTCGGCAAGCCGGAGAAATTCTGTAGCAATATTGAGAAATCCGCGTGGGAAAGCGCGACCCTGACGTGT AAACCTTCCGCGTTGATCGACAAACTGAAAGAATATTCGGTCAACGACCCGTACAGCGGTAAGACCGTGACCGGCG GTATCATTACTATCACCGATAGCAACTGGTTGATGAGCTTTACCTGCAATCGCCAGCCGCATTTCCCGGAGCAGCC GGATGACGTCCTGGTGCTGTGGGTGTATGCGCTGTTTATGGATAAAGAAGGTAACTACATTAAGAAAACCATGCTG GAGTGCACCGGTGATGAGATTTTGGCGGAGCTGTGTTACCATCTGGGCATTGAAGATCAGCTGGAAAATGTGCAGA AGAATACGATTGTTCGCACCGCATTCATGCCGTATATCACGAGCATGTTTATGCCACGTGCCAAAGGTGACCGCCC ACGATGGAATCTAGCGTTCGCACGGCCCGTATTGCGGTGTACAAGTTGCTGAATCTGAACAAGCAGGTGCCGGACA TTAATCCGCTGCAATACGATATTCGCCATCTGCTGAAAGCGGCAAAGACCCTGAATGATGACAAACCGTTCGTGGG AGAGCATGAGAGCTTCATTGCGGAACATGTTAACAAGTTCCGTGAGTGGGTCAAGGGTATCCGTGGCTAATAA - 3'

# GC-MS monitoring of the hydration of OA derivatives

Bioconversions of **1a–1j** were performed with whole *E. coli* cells. Representative GC-chromatograms of technical triplicates of an authentic substrate standard, an OhyA-free *E. coli* strain (empty vector control, EVC) and a biotransformation with cells after over-expression of OhyA are overlaid (Figure S1–Figure **S10**). In case a substrate was not converted with the wild type enzyme (**1h** and **1j**), a representative chromatogram of the OhyA Gln265Ala/Thr436Ala/Asn438Ala bioconversion is shown. The OA-derived *N*-hydroxy oleamide (**1d**) and the hydrated reaction product (**2d**) were both detected as the respective isocyanates after a Lossen rearrangement occurring under

GC-MS analysis conditions.<sup>[9]</sup> Moreover, conversion of **1d** with the *E. coli* EVC and the strain expressing OhyA led to the unexpected formation of oleamide (**1c**), with a subsequent hydration to 10-hydroxy octadecanamide (**2c**) only in OhyA biotransformations. Since *N*-hydroxy oleamide (**1d**) was initially oleamide-free, one must assume that the oleamide (**1c**) was formed by degradation of *N*-hydroxy oleamide (**1d**) in *E. coli*.<sup>[10]</sup>



Figure S1. Bioconversion of OA (1a) to 10-hydroxy stearic acid (2a) with *E. coli* whole cells over-expressing OhyA wild type enzyme. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic 1a standard and biotransformations of 1a with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA wild type. Retention times of the TMS-derivatives of the internal standard *n*-pentadecanoic acid (10.40 min), 1a (12.12 min) and 2a (13.24 min) are highlighted. b) Mass spectrum of the peak at 12.12 min, corresponding to the TMS-derivative of 1a. c) Mass spectrum of the peak at 13.24 min, corresponding to the TMS-derivative of 2a.



Figure S2. Bioconversion of oleyl amine (1b) with *E. coli* whole cells over-expressing OhyA wild type enzyme. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic 1b standard and biotransformations of 1b with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA wild type. Retention times of the TMS-derivatives of the internal standard *n*-pentadecanoic acid (10.40 min) and 1b (11.07 min) are highlighted. b) Mass spectrum of the peak at 11.07 min, corresponding to the TMS-derivative of 1b.



**Figure S3.** Bioconversion of oleamide (**1c**) to **2c** with *E. coli* whole cells over-expressing OhyA wild type enzyme. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic **1c** standard and biotransformations of **1c** with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA wild type. Retention times of the TMS-derivatives of the internal standard *n*-pentadecanoic acid (10.59 min), **1c** (13.44 min) and **2c** (14.48 min) are highlighted. b) Mass spectrum of the peak at 13.44 min, corresponding to the TMS-derivative of **1c**. c)



**Figure S4.** Bioconversion of *N*-hydroxy oleamide (**1d**) to 2d with *E. coli* whole cells over-expressing OhyA wild type enzyme. Due to thermally induced Lossen rearrangement, only the isocyanates were detected in the GC-MS. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic **1d** standard and biotransformations of **1d** with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA wild type. Retention times of the TMS-derivatives of the internal standard *n*-pentadecanoic acid (10.59 min), **1d** (11.39 min) and **2d** (12.71 min) are highlighted. The latter two compounds may exist in the Lossen-rearrangement expression by Lossen rearrangement. c) Mass spectrum of the peak at 12.71 min, corresponding to the TMS-derivative of 1-isocyanatoheptadecan-9-ol after Lossen rearrangement of silylated **2d**.



Figure S5. Bioconversion of oleyl alcohol (1e) to 2e with *E. coli* whole cells over-expressing OhyA wild type enzyme. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic 1e standard and biotransformations of 1e with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA wild type. Retention times of the TMS-derivatives of the internal standard *n*-pentadecanoic acid (10.40 min), 1e (11.59 min) and 2e (12.77 min) are highlighted. b) Mass spectrum of the peak at 11.59 min, corresponding to the TMS-derivative of **1e**. c) Mass spectrum of the peak at 12.77 min, corresponding to the TMS-derivative of **2e**.



**Figure S6.** Bioconversion of methyl oleate (1f) to **2f** with *E. coli* whole cells over-expressing OhyA wild type enzyme. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic **1f** standard and biotransformations of **1f** with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA wild type. Retention times of the TMS-derivative of the internal standard *n*-pentadecanoic acid (10.40 min), **1f** (11.43 min) and the TMS-derivative of **2f** (12.67 min) are highlighted. b) Mass spectrum of the peak at 11.43 min, corresponding to **1f**. c) Mass spectrum of the peak at 12.67 min, corresponding to the TMS-derivative of **2f**.



**Figure S7.** Bioconversion of ethyl oleate (**1g**) to **2g** with *E. coli* whole cells over-expressing OhyA wild type enzyme. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic **1g** standard and biotransformations of **1g** with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA wild type. Retention times of the TMS-derivative of the internal standard *n*-pentadecanoic acid (10.40 min), **1g** (11.84 min) and the TMS-derivative of **2g** (13.02 min) are highlighted. b) Mass spectrum of the peak at 11.84 min, corresponding to **1g**.



**Figure 88.** Bioconversion of *i*-propyl oleate (**1h**) to **2h** with *E. coli* whole cells over-expressing OhyA Gln265Ala/Thr436Ala/Asn438Ala. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic **1h** standard and biotransformations of **1h** with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA Gln265Ala/Thr436Ala/Asn438Ala. Retention times of the TMS-derivative of the internal standard *n*-pentadecanoic acid (10.40 min), **1h** (12.00 min) and the TMS-derivative of **2h** (13.18 min) are highlighted. b) Mass spectrum of the peak at 12.00 min, corresponding to **1h**. c) Mass spectrum of the peak at 13.18 min, corresponding to the TMS-derivative of **2h**.



**Figure S9.** Bioconversion of *n*-propyl oleate (**1i**) to **2i** with *E. coli* whole cells over-expressing OhyA wild type enzyme. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic **1i** standard and biotransformations of **1i** with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA wild type. Retention times of the TMS-derivative of the internal standard *n*-pentadecanoic acid (10.58 min) and the TMS-derivative of **2i** (13.72 min) are highlighted. b) Mass spectrum of the peak at 13.72 min, corresponding to the TMS-derivative of **2i**.



**Figure S10.** Bioconversion of *n*-butyl oleate (**1**) to 2j with *E. coli* whole cells over-expressing OhyA Gln265Ala/Thr436Ala/Asn438Ala. a) Overlay of representative GC-MS chromatograms from technical triplicates of an authentic **1** istandard and biotransformations of **1** with an *E. coli* empty vector control (EVC) and an *E. coli* strain over-expressing OhyA Gln265Ala/Thr436Ala. Retention times of the TMS-derivative of the internal standard *n*-pentadecanoic acid (10.58 min), **1j** (13.13 min) and the TMS-derivative of **2j** (14.21 min) are highlighted. b) Mass spectrum of the peak at 13.13 min, corresponding to **1j**. c) Mass spectrum of the peak at 14.21 min, corresponding to the TMS-derivative of **2j**.

#### Spectroscopic and optical data of purified reaction products

Compound **2a**: (*R*)-10-hydroxy stearic acid (analyzed as methyl ester and after esterification of the 10-hydroxy group with (*S*)-(+)-*O*-acetylmandelic acid) (see lit.<sup>[2,11]</sup>).

<sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.87 (3H, t, <sup>3</sup>*J*(H,H) = 7.2 Hz, H-18), 0.99-1.33 (24H, m, 12 CH<sub>2</sub>], 1.33-1.40 (2H, dd, <sup>3</sup>*J*(H,H) = 14.8 Hz, 7.0 Hz, H-9 or H-11), 1.58-1.65 (2H, m, H-3 or H-4), 2.19 (3H, s, CH<sub>3</sub>CO), 2.30 (2H, t, <sup>3</sup>*J*(H,H) = 7.5 Hz, H-2), 3.6646 (3H, s, OCH<sub>3</sub>), 4.87 (1H, p, <sup>3</sup>*J*(H,H) = 6.2 Hz, H-10), 5.87 (1H, s, H-2'), 7.33-7.39 (3H, m, H-3", H-4", H-5"), 7.47 (2H, dd, <sup>3</sup>*J*(H,H) = 7.2 Hz, <sup>4</sup>*J*(H,H) = 2.0 Hz, H-2", H-6").

<sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>): δ = 14.24, 20.87, 22.78, 24.88, 25.09, 25.26, 29.26 (3C), 29.40, 29.42, 29.49, 29.51, 31.99, 34.07, 34.26, 34.29, 51.55, 74.92, 76.14, 127.75, 128.77, 129.22, 134.35, 168.85, 170.36, 174.44.

#### Compound 2c: 10-hydroxy octadecanamide

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.88 (3H, t, <sup>3</sup>J(H,H) = 6.6 Hz, Me), 1.15-1.45 (23H, m, 11 CH<sub>2</sub>, -CH-O<u>H</u>), 1.45-1.72 (6H, m, -C<u>H<sub>2</sub></u>-CH(OH)-C<u>H<sub>2</sub></u>-, -C<u>H<sub>2</sub></u>-CH<sub>2</sub>-CO<sub>2</sub>NH<sub>2</sub>), 2.22 (2H, t, <sup>3</sup>J(H,H) = 7.5 Hz, -C<u>H<sub>2</sub></u>-CO<sub>2</sub>NH<sub>2</sub>), 3.58 (1H, m, >C<u>H</u>-OH), 5.35 (2H, br, -CO<sub>2</sub>N<u>H<sub>2</sub></u>).

<sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>): δ = 14.25, 22.82, 25.63, 25.74, 25.82, 29.32, 29.36, 29.43, 29.53, 29.74 (2C), 29.87, 32.03, 36.03, 37.60, 37.69, 72.16, 175.56.

 $[\alpha]_D^{25} = -4.0$  ° (c = 0.15 in CHCl<sub>3</sub>)

## Compound **2d**: *N*,10-dihydroxyoctadecanamide

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.88 (3H, t, <sup>3</sup>*J*(H,H) = 6.7 Hz, Me), 1.05-1.40 (22H, m, 11 CH<sub>2</sub>), 1.40-1.53 (4H, m, -C<u>H<sub>2</sub>-CH(OH)-CH<sub>2</sub>-</u>), 1.56-1.72 (3H, m, -C<u>H<sub>2</sub>-CH<sub>2</sub>-CO<sub>2</sub>NHOH, -CH-OH</u>), 2.17 (2H, t, <sup>3</sup>*J*(H,H) = 7.1 Hz, -C<u>H<sub>2</sub>-CO<sub>2</sub>NHOH</u>), 3.58 (1H, m, >C<u>H</u>-OH), 4.37 (2H, d, <sup>3</sup>*J*(H,H) = 7.0 Hz, -CO<sub>2</sub>N<u>HOH</u>).

<sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>): δ = 14.25, 22.82, 25.24, 25.54, 25.81, 28.98, 29.21, 29.41, 29.49, 29.71, 29.73, 29.86, 32.04, 35.99, 37.46, 37.68, 72.25, 175.41.

 $[\alpha]_D^{25} = -7.9 \circ (c = 0.1 \text{ in CHCl}_3)$ 

Compound 2e: 1,10-octadecanediol

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.88 (3H, t, <sup>3</sup>*J*(H,H) = 6.8 Hz, Me), 1.15-1.50 (30H, m, 12 CH<sub>2</sub>, -CH<sub>2</sub>-CH(OH)-CH<sub>2</sub>-, 2× OH), 1.56 (2H, m, -CH<sub>2</sub>-CH<sub>2</sub>OH), 3.58 (1H, m, >CH-OH), 3.64 (2H, t, <sup>3</sup>*J*(H,H) = 6.6 Hz, -CH<sub>2</sub>OH).

<sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>): δ = 14.25, 22.82, 25.79, 25.81, 25.87, 29.43, 29.55, 29.68 (2C), 29.75, 29.83, 29.87, 32.03, 32.95, 37.63, 37.67, 63.23, 72.18.

 $[\alpha]_D^{25} = -0.5 \circ (c = 0.3 \text{ in CHCl}_3)$ 

Compound 2f: 10-hydroxy stearic acid acid methyl ester

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.87 (3H, t, <sup>3</sup>J(H,H) = 6.3 Hz, Me), 1.15-1.39 (22H, m, 11 CH<sub>2</sub>), 1.39-1.52 (4H, m, -C<u>H<sub>2</sub>-CH(OH)-CH<sub>2</sub>-</u>), 1.52-1.69 (3H, t+m, <sup>3</sup>J(H,H) = 7.2 Hz, -C<u>H<sub>2</sub>-CH<sub>2</sub>-COOMe</u>, -CH-O<u>H</u>), 2.30 (2H, t, <sup>3</sup>J(H,H) = 7.5 Hz, -C<u>H<sub>2</sub>-CO<sub>2</sub>Me</u>), 3.58 (1H, m, >C<u>H</u>-OH), 3.66 (3H, s, -CO<sub>2</sub>Me).

 $^{13}\text{C-NMR}$  (75 MHz, CDCl<sub>3</sub>):  $\delta$  = 14.25, 22.82, 25.09, 25.76, 25.81, 29.27, 29.33, 29.43, 29.55, 29.75, 29.76, 29.87, 32.04, 34.26, 37.62, 37.67, 51.59, 72.16, 174.47.

 $[\alpha]_D^{25} = -1.0$  ° (c = 0.35 in CHCl<sub>3</sub>)

Compound 2g: 10-hydroxy stearic acid acid ethyl ester

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.87 (3H, t, <sup>3</sup>*J*(H,H) = 6.0 Hz, Me), 1.15-1.39 (25H, m, -CO<sub>2</sub>CH<sub>2</sub>-Me, 11 CH<sub>2</sub>), 1.40-1.50 (4H, m, -CH<sub>2</sub>-CH(OH)-CH<sub>2</sub>-), 1.51-1.68 (3H, t+m, <sup>3</sup>*J*(H,H) = 6.8 Hz, -CH<sub>2</sub>-CH<sub>2</sub>-CO<sub>2</sub>Et, -CH-OH), 2.28 (2H, t, <sup>3</sup>*J*(H,H) = 7.5 Hz, -CH<sub>2</sub>-CO<sub>2</sub>Et), 3.58 (1H, m, >CH-OH), 4.12 (2H, t, <sup>3</sup>*J*(H,H) = 7.1 Hz, -CO<sub>2</sub>CH<sub>2</sub>-Me).

<sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>): δ = 14.24, 14.40, 22.82, 25.11, 25.76, 25.81, 29.26, 29.34, 29.43, 29.55, 29.74, 29.77, 29.87, 32.03, 34.53, 37.62, 37.67, 60.30, 72.15, 174.04.

 $[\alpha]_D^{25} = -0.7 \circ (c = 0.3 \text{ in CHCl}_3)$ 

Compound 2h: 10-hydroxy stearic acid acid *i*-propyl ester

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.88 (3H, t, <sup>3</sup>J(H,H) = 6.6 Hz, Me), 1.12-1.37 (28H, m, 11 CH<sub>2</sub>, -CO<sub>2</sub>CH<u>Me<sub>2</sub></u>), 1.37-1.48 (4H, m, -C<u>H</u><sub>2</sub>-CH(OH)-C<u>H</u><sub>2</sub>-), 1.50-1.67 (3H, m, -C<u>H</u><sub>2</sub>-CH<sub>2</sub>-CO<sub>2</sub>CHMe<sub>2</sub>, -CH-O<u>H</u>), 2.25 (2H, t, <sup>3</sup>J(H,H) = 7.4 Hz, -C<u>H</u><sub>2</sub>-CO<sub>2</sub>CHMe<sub>2</sub>), 3.58 (1H, m, >C<u>H</u>-OH), 5.00 (1H, p, <sup>3</sup>J(H,H) = 6.4 Hz, -CO<sub>2</sub>CHMe<sub>2</sub>).

<sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>): δ = 14.25, 22.01 (-CO<sub>2</sub>CH<u>Me</u><sub>2</sub>), 22.82, 25.18, 25.77, 25.81, 29.24, 29.35, 29.43, 29.57, 29.75, 29.78, 29.87, 32.04, 34.87, 37.63, 37.67, 67.48, 72.17, 173.59.

 $[\alpha]_D^{25} = -3.1 \circ (c = 0.1 \text{ in CHCl}_3)$ 

Compound 2i: 10-hydroxy stearic acid acid n-propyl ester

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.88 (3H, t, <sup>3</sup>*J*(H,H) = 6.3 Hz, Me), 0.94 (3H, t, <sup>3</sup>*J*(H,H) = 7.4 Hz, -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 1.15-1.38 (22H, m, 11 CH<sub>2</sub>), 1.38-1.48 (4H, m, -C<u>H<sub>2</sub>-CH(OH)-CH<sub>2</sub>-)</u>, 1.48-1.67 (3H, m, -C<u>H<sub>2</sub>-CH<sub>2</sub>-CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me, -CH-O<u>H</u>), 1.65 (2H, sextet, <sup>3</sup>*J*(H,H) = 7.1 Hz, -CO<sub>2</sub>CH<sub>2</sub>C<u>H<sub>2</sub>Me</u>), 2.29 (2H, t, <sup>3</sup>*J*(H,H) = 7.5 Hz, -C<u>H<sub>2</sub>-CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me</u>), 3.58 (1H, m, >C<u>H</u>-OH), 4.02 (2H, t, <sup>3</sup>*J*(H,H) = 6.6 Hz, -CO<sub>2</sub>C<u>H<sub>2</sub>CH<sub>2</sub>Me</u>).</u>

<sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>): δ = 10.55, 14.25, 22.17, 22.82, 25.16, 25.77, 25.81, 29.28, 29.35, 29.43, 29.56, 29.75, 29.77, 29.87, 32.04, 34.54, 37.62, 37.67, 65.98, 72.17, 174.16.

 $[\alpha]_D^{25} = -1.5 \circ (c = 0.15 \text{ in CHCl}_3)$ 

Compound **2j**: 10-hydroxy stearic acid acid *n*-butyl ester

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.88 (3H, t, <sup>3</sup>*J*(H,H) = 6.6 Hz, Me), 0.93 (3H, t, <sup>3</sup>*J*(H,H) = 7.4 Hz, -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 1.18-1.36 (22H, m, 11 CH<sub>2</sub>), 1.36-1.47 (6H, m, -C<u>H<sub>2</sub></u>-CH(OH)-C<u>H<sub>2</sub></u>-, -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 1.50-1.67 (5H, m, -C<u>H<sub>2</sub></u>-CH<sub>2</sub>-CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 1.50-1.67 (5H, m, -C<u>H<sub>2</sub></u>-CH<sub>2</sub>-CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me, -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me, -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 2.29 (2H, t, <sup>3</sup>*J*(H,H) = 7.4 Hz, -C<u>H<sub>2</sub></u>-CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me), 3.58 (1H, m, >C<u>H</u>-OH), 4.07 (2H, t, <sup>3</sup>*J*(H,H) = 6.5 Hz, -CO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>Me).

 $^{13}\text{C-NMR}$  (75 MHz, CDCl<sub>3</sub>):  $\delta$  = 13.86, 14.25, 19.31, 22.83, 25.16, 25.77, 25.81, 29.28, 29.35, 29.43, 29.56, 29.75, 29.78, 29.87, 30.87, 32.04, 34.55, 37.62, 37.67, 64.26, 72.17, 174.15.

 $[\alpha]_D^{25} = -5.1$  (c = 0.1 in CHCl<sub>3</sub>)



# NMR spectra of reaction products

Figure S11. <sup>1</sup>H-NMR (500MHz, CDCI<sub>3</sub>) of 2a as methyl ester and after esterification of the 10-hydroxy group with (S)-(+)-O-acetylmandelic acid.



Figure S12. <sup>13</sup>C-NMR (125MHz, CDCl<sub>3</sub>) of 2a as methyl ester and after esterification of the 10-hydroxy group with (S)-(+)-O-acetylmandelic acid.









































# Amino acid sequence alignment

To support our selection of amino acid residues involved in substrate binding in OhyA, we performed alignments of the OhyA protein sequence with all sequences of HFam11 in the HyED (Figure S29).<sup>[3]</sup> The high degree of conservation of Gln265, Thr436, Asn438 and His442 is highlighted by the red boxes, and perfectly in line with our docking and sitedirected mutagenesis analyses.<sup>[2]</sup> a)

Consensus	a l v F p k Y n (	ydt fv+p	I + n h L k e k G \	ki q f d t r v k d l dn	h i ng dg kytg t ktytg	i i tevdggdgke	- kevkipygpnd
	274 278 280 28	2 284 286 288 2	0 292 294 296 298	300 302 304 306 308 310 31	2 314 316 318 320 322 324 326 32	8 330 332 334 336 338 340 34	344 346 348 350 352 35
D 238 Elizabethkingiameningoseptica ?8113 taxonID 238 Elizabethkingiameningosept	257 SLVFPKYNC 257 SLVFPKYNC	2 DTFVKP	RNFLKEKG	TIELNTLVKDLDI	HVNTEG KVVEG	I I T E Q D G	- KEVKIPVREND
38 Elizabethkingiameningoseptica	257 SLVFPKYNC	DTEVTP	RKFLQEKG	NIHLNTLVKDLDI	HINTEG KVVEG	IITEQDG	- KEVKIPVGKND
408 Methylobacteriumextorquens	249 ALVFPKYNC	DSFVRP	VNHLMARG	TVRFGVRAYDLAN	ISVDGDA RTVTG	I H V K T - K G	- KEDVIPVESRD
408 Methylobacteriumextorquens	249 ALVEPKYNC 249 ALVEPKYNC	2 D S F V R P	VNHLMARG\	TVRFGVRAYDLAN	ISVDGDA RTVTG	I H V K T - K G	- KEDVIPVESRD
408 Methylobacteriumextorquens	249 ALVEPKYNC	DSFVRP	VNHLMARG	TVRFGVRAYDLAN	ISVDGDA RTVTG	I H V K T - K G	- KEDVIPVESRD
374 Bradyrhizobium	250 A L V F P K Y N C	DSFVRP	MTHLRERG	K V Q F D T R A Y D L D N	I T T D G E A R T V T G	I K A K V - G G	- A D T T I A V G P K D
40324 Stenotrophomonasmaltophilia	253 ALVEPKYNC	Q E S F V V P	SRMLRAQG	N V Q F D T R V H D L E N	IAVDGQSRTVTA	LRCRV-AG	- NETTLPVAAGD
13690 Sphingobiumyanoikuyae	255 ALVEPKYNC	POSEVRP	SKLLRDKG	O I R F D T Q V R D M R M	IR LEGDR RTVTE	LICRI-DG	- REDSTATGPRD
9 Acinetobacter	257 ALVFPKYNC	DTFVKP	LGQYLKKKGV	TLQLNTLVKDVE1	E Q K G Q E S I A K S	L L I E K D G	- KEQKIDLTEND
13690 Sphingobiumyanoikuyae	255 ALVFPKYNC	DSFVRP	SKLLRDKG	QIRFDTQVRDMRN	IRLEGDR RTVTE	LLCRI-GG	- QEDSIAIGPRD
1355//Idiomarinaloiniensis 89801Acinetobacterurringii	255 V L L F P K Y P C	TELEVIT		THOLNTIVED VE1	EOKGKE STAKS		- EEQYIEVGKND
1230476 Bradyrhizobiumsp.DFCI-1	248 ALIFPKYNC	DSFVRP	VTHLRERGI	KVQFDTRAYDLDI	KVEGAT RTVTA	IKAKV - NG	- ADSTISIGPKD
553151 Pseudomonaspelagia	255 V L V F P K Y N C	DTFIKP	ANMLRDKG	SVQLGTRVYDLDM	ITLSAGQ KTVTG	L R C V V A G	- E E Q R I D V A D G D
216778 Stenotrophomonasrhizophila	253 ALVEPKYNC	2 E S F V V P	. A R M L R A Q G \	QIQFDTRVHDLEN	AVDGDA RTVTA	IRCKV-GG	- REDRIAVGEGD
7453101Sphingomonassp.MM-1	247 ALVEPKYNC	DIFVRP	. I KM L REQGV	KVRFDTRGYDLDI	AVAGEQ	I K I K E - G G	- KDAAIPVGPKD
1144307 Sphingobiumsp.AP49	253 ALVEPKYNC	DSFVRP	VDHLKARG	KVQFGTRAYDLAN	RVAGDT RIVTG	I H A R I - D G	- I D Q V M P V A E K D
1260624 Idiomarinasp.28-8	255 V L V F P K Y I C	2 F D T F I K P	ANMLRDQG\	KFQLETRVYDLEN	1 A E S S D K K T V T G	I L C A V A G	- E E Q R I D V A D G D
366616 Haematobactermissouriensis	251 ALVFPKYNC	DTFVVP	SRHLRGLG	QIRLGVSVQDLDM	IEEEDGQ RRVTG	LRCTV-EG	- GEQVIPVGPKD
117645 Elizabethkinglaanophelis 107618 hodonseudomonaspalustris	257 SLVFPKYNC	2 DTFVTP	L R K F L Q E K G \	K V O E G T P A Y D I D I	HINTEG KVVEG	I I TEQDG	- KEVKIPVGKND
370622 Aureimonasaltamirensis	248 ALVEPKYNC	DSFVVP	LTRLLKEKG	KVOFDTRAYDLDN	I GEADK RTVTA	IRCKV-GG	- KDETIALGPDD
8980 Acinetobacterursingii	257 ALVFPKYNC	DTFVKP	GQYLKKKG	TLKLNTLVKDVEI	E Q K G Q N S F A K S	LLIEKDG	- KEQKIDLTEND
648885 Methylobacteriumsp. MB200	249 A L V F P K Y N C	DSFVRP	VSHLQSHG	TVRFGVRAYDLTM	SVDGET RTVTG	I R T R T - D G	- KDEVIPVEAED
228 Pseudoalteromonashaloplanktis	247 LLVFPKYNC	DTFIKP	V R H L K D R C V	K F Q F D T C V H D L D M	INFTDDK KTVAG	LQAITKG	- Q K Q Y I A I E P K D
265IParacoccus	249 ALVEPKYNC	NDGFIKP	LVRHLKDRGV	TEREGTRVTDMS	DEKDGARTVTA	LHAVV-DG	- TPOOIAVGPRD
1525716 Paracoccussp.10990	249 ALVFPKYNC	DGFIKP	VRHLKDRG	TFRFGTRVTDMSF	DEKDGARTVTA	L H A V V - D G	- TPQEIAVGPRD
1076 Rhodopseudomonaspalustris	252 A L V F P K Y N C	DSFVVP	TRLLKDKG	KVQFGTRAYDLDM	I V E E A G R R T V T A	I R C K K - H G	- KDDSIAVGPKD
34003 Paracoccusaminophilus	245 CLVFPKYNC	DTFVKP	VDHLKKLG\	QVQFATRVSDLEN	TEDAGK RSVTG	I L A S V - N G	- QEHRIPVDEKD
29440 prauyrnizobiumeikanii 29448 Bradyrhizobiumelkanii	248 ALVEPKYNC 248 ALVEPKYNC	FDSFVRP	VIHLKERG\	KVQFDIRVHDLDI KVOFDTRAHDIDI	KADGDARTVT/	. I H A K V - G G	- ADTTIPVGPKD
29448 Bradyrhizobiumelkanii	248 ALVEPKYNC	FDSFVRP	VTHLRERGY	KVOFDTRVHDLDI	KTDGDA RTVTA	I H A K V - G G	- ADTTIPVGPKD
29448 Bradyrhizobiumelkanii	248 A L V F P K Y N	DSFVRP	VTHLRERG	KVQFDTRAHDLDI	K V D G D A R T V T A	I H A K V - G G	- A D T T I P V G P K D
356 Rhizobiales	252 ALVFPKYNC	DSFVVP	IRLLKEKG	KVQFDTRAYDLDM	I T E A E G K R T V T A	I R C K V - D G	- R D E V I D L G P D D
1208321 Marinomonasprofundimaris	255 ALVERKYNC	2 DTFIKP	LANLLREKG	T F Q F G T R V H D L D I	NFSGDKKTVIG	IHAKVTK-DGIE	- DAKHIPVGLGD
207949 (bermanellamarisrubri 1076) Rhodonseudomonaspalustris	250 ALVEPKTIC	2 D S F V V P	TRIIKEKGV	KIOFGVRAYDIDA	UVEKAGR RTVT4	IRCKK-AG	- ONDSIAVGEND
257440 Pleomorphomonaskoreensis	248 ALVEPKYNC	DSFVRP	IDHLRQRG	KVQFNTRVYDLEN	RVAGDT YTVTA	I R A E I - G G	- KDETITVGPND
285 Comamonastestosteroni	254 A L V F P K Y I C	DSFVVP	ARMLQEQG\	RVQFDTRAHELDN	IREDEGS RTVTA	I R C K V - A G	- REESITVGSDD
285 Comamonastestosteroni	254 ALVFPKYNC	DSFVVP	ARMLQEQG	RVQFDTRAHELDN	REDEGSRTVTA	I R C K V - A G	- REESITVGSDD
285 Comamonastestosteroni 1317124 Thioclayasp 1302W-2	254 ALVEPRYNC		VNWIKARGI	N FRYDTRYTDI FN		1 H 4 F V - D G	- REESITVGSDD
5324 Enhydrobacteraerosaccus ab EEV22910.1	255 T L L F A R Y N C	DSFVVP.	AOKFLINKG	KLOSDTLVTAVD	EOOGDK KIVKG	LTAI00G	- OOVHIPVRDND
1280946 Hyphomonasberingensis	255 A L V F P K Y I C	DTFIKP	ADMLRQKG	RFQFDTRVQDLEI	SEADGR RTVTG	IVCTVSG	- Q P Q R L E V G D T D
D 1434011 Komagataeibacter	252 TLVFPKYNC	DSFVVP	ARLLQDKG	KIQFNTRVHDVDI	QIRGDE KTVTG	L R A M V - D G	- QETTIPVHPND
D 442 Gluconobacteroxydans 1055192 Comamonasso B=9	247 ALVERKYNC	2 D S F V V P	TRMIKDKG	KIQFNIRVHDVDI	Q I RGDE KI VIG	I R C K S . A G	- QETTIPVHPND
601969 taxonID 1052004 Granulibacterbethesa	269 ALVEPKYNC	DTFVRP	VAHLAERG	RMQLRTRVYDLEN	ITEEGDT RTVTA	I K A K I - D G	- KESILPVGAKD
81029 Hyphomonasadhaerens	255 A L V F P K Y I C	DTFIKP	ADMLRQKG\	RFQFDTRVQDLEI	SEADGR KTVTG	I V C T V S G	- Q P Q R L E V G D T D
7835204 taxonID 400668 Marinomonassp.MWYL	255 ALVFPKYPC	<b>CDTFIKP</b>	ADMLREKG	TFQFGTRVEDLDI	NFSGDKKTVTG	IRAVVIK-EGKE	- EAKHIHVGLGD
643451 Sobingobacteriumsp. 4g1	257 CLVFPKYNC 257 SLVFPKYNC		RKHLESKGV	VQIQENIEVKDEDI INTRENVIVKDIDI	OSNTDGKVVEG	IVIEQDG	- KEVRIPIGKDD - KEVKIPVGKED
6345\Flavobacteriumpsychrophilum	257 CLIFPKYNC	DTFVKP	TEHLKSKG	KIQFDTLVKDLDI	QINSEG KIVKG	I I T KQ N N	- KEVVIPVTKND
21 Sulfurospirillummultivorans	260 ALVFPKYNC	DTFIAP	RKLLQEKG	QFQFDTLVEDLEI	TMTHNEKIVEN	IVTIHNE	- TSSKIAVGRDD
6345 [Flavobacteriumpsychrophilum 222950] Chorceobacteriumps 1M1	257 CLIFPKYNC		LTEHLKSKG	CLOENTLVKDLD	QINSEGKIVKG	I I T KQ N N	- KEVVIPVTKND
99141 Halomonassp. TD01	262 SLVFPKYNC	FDSFVRP	MSWLKDOG	KVEYDTVVENLDN	IETLEGG RRTVTT	IOCHGSN	- GGKTIPVGRED
91 Flavobacteriumhydatis	257 SLVFPKYNC	DTEVTP	RKFLQEKG	SIQFNTLINDLDI	HINTDG KVVKG	I I T E K D G	- KEVTIPVEKDD
88908 Oleispiraantarctica	237 GLVFPKYNC	DSFVLP	LLNWLKNKG\	KVQTGTIISDVDM	ISIH-DDAMTSTA	IRCRTQD	- G D Y D I P L E P K D
731540 (taxon1D) 698738 (OleispiraantarcticaRB- 49295 [Elauthum/bacterpetaceur	262 GLVFPKYNC	2 D S F V L P		KVQTGTIISDVDN	ISIH-DDAMTSTA	I R C R T Q D	- GDYDIPLEPKD
782201Chrvseobacteriumsp.StRB126	257 CLVFPKYNC	DTEVTP	LKNFLVEKG	OIOFDTLVKDLD	HINTEG KTVEG	IITEONG	- EEVRIPISKDD
32346 Halomonasalkaliantarctica	262 SLVFPKYNC	DSFVRP	MSWLKDQG	KIEYDTVVENLGN	IETQEGG SRTVTT	IQCHGSN	- G G K A I T V G P R D
6022 Flavobacteriumsp.JRM	257 SLVFPKYNC	2 D T F V T P	RKFLQEKG\	SIQFNTLINDLDI	HINTDG KVVKG	I I Т Е К D G	- KEVIIPVEKDD
47 Empedobacterbrevis 42824 Empedobacterfalcenii	257 SLVFPKYNC		LG KWLKEKG	KIQLNTLVKDLD1	LVNTEGKVVKG	I I TEQDG	- KDIIIPVTPND
13554 Halomonascampaniensis	262 SLVFPKYNC	DSFVRP	LMNWLODOG	NIQYDTVVENIDA	IDTQDG CRTVT4	LQCRGSD	- GDKSIPIGTRD
183151 Myroidesinjenensis	256 ALVFPKYNC	DTEVKP	LQNYLKSKG	KVQFDTLVKDIDI	HVDTSGKVVEG	I Î T E Q N G	- KDVKIAVGQDD
288027 Arcticibactersvalbardensis	257 CLVFPKYNC	2 D T Y V T P	RKFLQSKG	KIELNTIVRDLDI	H I D T E G K V V E G	I F A E R K G	- KELKIAVGKDD
38 Elizabethkinglameningoseptica	257 SLVFPKYNC	2 DTFVTP	RKFLQEKG	NIHLNTLVKDLDI	HINTEGKVVEG	IITEQDG	- KEVKIPVGKND
241913306770361pdb1401K(A,B)[taxon1D]238[E 6023]Flavobacteriumsp.KMS	257 SLVFPKYNC 257 SLVFPKYNC		L K K F L Q E K G \ L R K F L O F K G \	SIOFNTIINDUDI	HINTDG KVVEG	IITEKDG	- KEVKIPVGKND
117645 Elizabethkingiaanophelis	257 SLVFPKYNC	DTEVTP	LRKFLQEKGV	NIHLNTLVKDLDI	HINTEG KVVEG	IITEQDG · · · · · ·	- KEVKIPVGKND
22877 Elizabethkingiasp.BM10	257 SLVFPKYNC	Q D T F V T P	RKFLQEKGV	NIHLNTLVKDLDI	HINTEG KVVEG	IITEQDG · · · · · ·	- K E V K I P V G K N D
/2045 (Elizabethkingiamiricola 117645 (Elizabethkingiaanonholic	257 SLVFPKYNC	2 DTEVTP	RKFLQEKG	INTHUNTUVKDLD	HINTEGKVVEG	IITEQDG	- KEVKIPVGKND
117645 Elizabethkingiaanophelis	257 SLVFPKYNC	DTEVTP	LRKFLOEKGV	NIHLNTLVKDIDI	HINTEG KVVFG	IITEQDG	- KEVKIPVGKND
500282 Chryseobacteriumsp. CF365	257 CLVFPKYNC	DTYVTP	KNFLVEKG	QIRFNTLVKDLDI	HINTEG KTVEG	I I T E Q N G	- EEVRIPIGKDD
50 Chryseobacteriumgleum	257 C L V F P K Y N C	2 D T Y V T P	KNFLVEKG	QIQFNTLVKDLDI	H I N T E G K T V E G	I I T E Q N G	- EEVKIPISKED
385985 Sphingobacteriumpaucimobilis	257 ALVEPKYNC	DTEVTP	GRFLKDKG	GIQFKTLVKDLDI	HIDTEGKTVKG	IITEQNG	- KEVTIPVREQD
150997 [marinobactermanganoxydans 01630]taxonID[1298607]Psychrobacterso_ICM1	252 SLVFPKYNC 253 ALVFPRYNC	EDSEVRP	L M N W L K D Q G \ L O N Y L K D K G \	OFOYDTIVTDLDN	E FEHADKVTGTKKVV/	IUCHGAD	- GUKIENVGARD
470 taxonID 1298610 Psychrobactersp. JCM189	253 ALVEPRYNC	FDSFVKP	LQNYLKDKG	Q FQYDTLVTNLD1	EFEHADKVTGTKKVKA	IITEKAN	- EQTTIPMGSND
63331 Chryseobacteriumtaiwanense	257 CLVFPKYNC	DTYITP	KNYLVSKG	KIEFNTLVKDLDI	HIDTEG KTVES	I I T E Q N G	- EEVKIPVGKED
479237 Marinobactersp.HL-58	262 S L V F P K Y N C	DTFVRP	MNWLKEQG	NIQYDTVVENLEN	1 E T S - G S T R T V T A	IQCHGSE	- G D K T F N V G E R D
7029[Psychroflexustorquis 45961[Chargeobacterium=="	257 CLVFPKYNC	DSEVKP	T D H L K S K G V	KIQFDTLVKDLDI	QINTEGKVVRG	IIITQQKD	- KEVKIPVTEND
+55611Cnryseobacteriumsoli 764701Olivibactersitiensis	257 SLIFPKYNC		L N F L I E K G \ L G R W L K F K G \	(KIOLNTIVKDID)	ILINADG KTVEG	IITEODG	- KEVKIPVGKED
9600 Cellulophagaalgicola	257 CLVFPKYNC	DTEVKP	TDHLKSKG	KIQFNTFVKDLEV	QINTEG KVVKG	IITQQED	- KEVTIAVTEND
5831 Myroides	257 A L V F P R Y N C	DTEVAP	RNHLTELG	Q I R L D T L V H D V D I	. H S T T A G K L V K G	L L V N Q G G	- Q E T R I E M N E Q D
480 Chryseobacteriumvrystaatense	257 CLVFPKYNC	DTYVTP	KNFLVEKG	QIEFNTLVKDLDI	HINTEG KTVEG	IITEQDG	- KEVKIPIGKED
ooseynyrolaesoaoratimimus 521Flavobacteriumhibernum	257 CLVFPRYNC		L K K T L Q E L G \ L R K F L O S K C \	O I E L N T I V K D L D I	Q 3 N A G G K I V H A	ILINRDG	- IEIKIPITAND
570 Marinobacterexcellens	262 SLVFPKYNC	DGFVRP	LMSWLKDOG	NIQYDTVVENLEN	ITTS-GDTRTVT4	IQCHGSE	- GDKTFNVGDRD
02113 Novosphingobiumsp. PP1Y	255 TLVFPKYC	DSFVRP	SKMLRDKG	KVRYDVQVRNLRI	EIVGDK KTVTG	LVCRTREG	- VEEVTDVDARD
076 Rhodopseudomonaspalustris	250 A L V F P K Y N C	DSFVVP	VRMLKDKG\	KVQFDTRAYDLDN	IVEQDGERIVTA	I R C K V - G G	- N S E S I A V G E N D
52021  gi 735022192  taxonID 1206458  Novosp	252 ALVFPKYNC	DSFVVP	INLLKAKG	KVQFNTRAYDLAN	TEGDGE RTVTE	I L C K V - G D	- KDETIPLGPDD
/42 (Marinobactersantoriniensis 90759 (Marinobactersp. BSs20148	262 SLVFPKYNC 262 ALVFPKYNC	DSEVEP	MGWLIACCY	INVOYDTVVSDLEN	IHIE-NGRRTVTG	LICRTTD	- GUKTIRVGKND
87493 Thalassolituusoleivorans	255 ALVEPKYNC	DTFIKP	LTNFLREKGV	VEKENTCIHDIDI	KFSSEK KVVT4	IHGVVTIKKGKEDE	KEDLRIDVEAFD
1475 Frateuriaaurantia	251 ALVFPKYNC	DSFVVP	MRMLEQEG	RTQFDTHVRDLAF	SEVGGQ RTVTA	IHCKL-QG	- RDEVIRLGKDD
71254 Ochrobactrumrhizosphaerae	252 A L V F P K Y N C	DSFVVP	TRLLKÉKG	Q V R F G T R A Y D L D N	ISESAGK RTVTG	I R C K V - Å G	- E D E M I A L G P D D
0324 Stenotrophomonasmaltophilia	250 ALVFPKYNC	ESFVKP	VKMLREQG	QVTFGTRVYDLDM	RVDGET RTVTG	L R C R V - E G	- NDTLLPVAQGD
uszenotropnomonasmaltophilia 364761Marinomonasnosidonica	243 A L V F P K Y N C	2 ESFVKP	SDYLPOOCY	Q VIEGIRVYDLDN O FO FAT PVVDIDN	INVEGEI RTVIG	I C A M V R E N D 0	- NUILLPVAQGD
85 Comamonastestosteroni	246 ALVEPKYNC	DSFVVP	LARMLQEQG	RVQFDTRAHELDN	IREEEGS RTVTA	IRCKV-AS	- REETITVGSDD
076 Rhodopseudomonaspalustris	252 A L V F P K Y N C	DSFVVP	LTRLLKEKG\	KVQFGTRAYDLAN	I V E E G G R R T V T #	I R C K K - D G	- K D D S I A I G P K D
08 Methylobacteriumextorquens	249 A L V F P K Y I C	PDSFVRP	L V N H L M A R G \	TVRFGVRAYDLAN	ISVDGDA RTVTG	I H V K T - K G	- KEDVIPVENRD

# b)

Consensus	ç l ş v Ņ d P y s g k t y t Ģ Ģ i i t f t D Ş n W l r	ns f T	c N R Q P	P h	To de participation de la construcción de la const
238 Elizabethkingiameningoseptica	438 442 444 446 448 450 452 454 455 458 460 462 46 407 EYSVNDPYSGKTVTGGIITITDSNWLI	4 466 48	N R O F	2 4 H	FPEOPDDILVLWVYALFMDKEGNYIKKTMPECTGDEILAELCHHLG48
8113  taxonID   238   Elizabethkingiameningosepti	407 EYSVNDPYSGKTVTGGIITITDSNWLM	1 S I T	NRQI	н	P E Q P D D V L V L W V Y A L F M D K E G N Y I K K T M P E C T G D E I L A E L C Y H L G 48
8 Elizabethkingiameningoseptica 08 Methylobacteriumextorquens	407 E Y S V N D P Y S G K T V T G G I I T I T D S N W L P 399 E L A V N D P Y S G K T V T G G I I T F T D S N W V	ISIT SVT	NRQF	н Н	F P E Q P D D V L V L W V Y A L F M D K E G N Y I K K T M P E C T G D E I L A E L C Y H L G 48 F P E Q P Q D V L V L W A Y A L L M D K D G N H V R K P M P A C T G R E I L S E L C Y H L G 48
08 Methylobacteriumextorquens	399 E L A V N D P Y S G K T V T G G I I T F T D S N W V I	. S V T	NRQF	н	F P E Q P K D V L V L W A Y A L L M D K D G N H V R K P M P A C T G R E I L S E L C Y H L G 48
08 Methylobacteriumextorquens 08 Methylobacteriumextorquens	399 E L A V N D P Y S G K T V T G G I I T F T D S N W V 399 F L A V N D P Y S G K T V T G G I I T F T D S N W V	SVT.	NRQE	н	F P E Q P K D V L V L W A Y A L L M D K D G N H V R K P M P A C T G R E I L S E L C Y H L G 48 F P E O P K D V L V L W A Y A L L M D K D G N H V R K P M P A C T G R E I L S E L C Y H L G 48
74 Bradyrhizobium	400 ELSVNDPYSGKTVTGGIITFTDSNWMM	ISIT	NRQE	н	F P T Q P K D V L V V W V Y A L L M D K P G N Y V K K P M P A C T G R E I L A E L C H H L G 48
0324 Stenotrophomonasmaltophilia	403 T L S V N D P Y S G R T V T G G V I T I T D S N W V I 405 O L S V N D P Y S G P T V T G G V I T F T D S N W V I	SIT	NRQF	н	V D Q P K D V L V V W V Y A L L M D Q D G N H I K K P M P A C T G R E V L A E L C H H L G 48
3690 Sphingobiumyanoikuyae	405 Q L S V N D P Y S G R T V T G G V I T F T D S N W V	1 S I T	NRQE	н	F P D Q P G D V L V L W V Y A L L M D K D G N H V K K P M P A C T G R E I L A E L C Y H L G 48
Acinetobacter	407 EYAVNDPYSGKTVTGGIITITDSNWLM	ISIT	NRQE	н	F P D Q P D D V L V L W V Y A L F M D K Q G N Y V K K T M P E C T G N E I L A E L C Y H L G48
3590 Springobiumyanoikuyae 35577 Idiomarinaloihiensis	405 Q L S V N D P Y S G R T V T G G V I T F T D S N W V 405 T L S V N N P Y S G R T V T G G I V T F T D S N W L	. S V T	NROF	н	F P D Q P G D V L V L W V Y A L L M D K D G N H V K K P M P A C I G K E I L A E L C Y H L G 48 F P D O P D D T L V L W V Y G L L M D K O G N R I N K T M P E C T G K E I L T E L C H H L G 48
980 Acinetobacterursingii	407 EYAVNDPYSGKTVTGGIITITDSNWLM	1 S I T	NRQF	н	F P E Q P D D V L V L W V Y A L F M D K Q G N Y V K K T M P E C T G N E I L A E L C Y H L G 48
230476 Bradyrhizobiumsp.DFCI-1 53151 Pseudomonaspelagia	398 E L S V N D P Y S G K T V T G G I I T F T D S N W V 405 F I S V N D P Y S G R T V T G G I I T F T D S N W F	S I T	NRQE	н	F P T Q P K D V L V V W V Y A L L M D K E G N Y V K K P M P A C T G R E I I A E L C Y H L G48 F P E O P D D T I V I W V Y G I I M D K D G N F V K K P M P O C T G K E I I A E I C H H I G48
16778 Stenotrophomonasrhizophila	403 S L S V N D P Y S G R T V T G G V I T I T D S N W V	SIT	NRQE	н	FVDQPRDVLVVWVYALLMDQDGNHIKKPMPACTGREILAELCHHLG48
94867 Sphingomonassp.ATCC31555	406 A L A V N D P Y S G R T V T G G I I T F T D S K W L I	SVT	NRQE	н	P D Q P E D V L V L W A Y A L R M D V A G D K V A K A M P D C T G R E I L E E L C H H L G 48
144307 Sphingobiumsp. AP49	403 Q L S V N D P Y S G R T V T G G I I T F T D S N W V	SVT	NRQE	н	FPDQPKDVLVLWVYALLMDKDGNRVAKPMPACTGREILEELCHHLG4/
260624 Idiomarinasp.28-8	405 ELSVNDPYSGRTVTGGIITFTDSNWF	SVT	NRQF	н	F P G Q P E D T L V V W V Y G L L M D K D G N F V K K P M P Q C T G K E I L A E L C H H L G 48
66616 Haematobactermissouriensis	401 E L S V N D P Y S G K T V T G G V I T F T D S N W V	SIT	NRQE	н	F P D Q P K D V L V L W V Y A L L M D K D G N H I K K P M P Q C T G R E V L A E M C Y H L G 48
076 Rhodopseudomonaspalustris	402 Q L S V N D P Y S G K T A T G G I I T F T D S N W L	SIT	NRQE	н	F P T Q P D D V L V L W V Y A L L M D K D G N H V K K P M P A C T G R E I L A E L C Y H L G 48
70622 Aureimonasaltamirensis	398 E V S V N D P Y S G K T V T G G V I T F T D S N W V I	SIT	NRQF	н	F P N Q P D D V L V L W V Y A L L M D K E G N H V K K P M P A C T G R E I L A E L C Y H L G48
980 Acinetobacterursingii 488851 Methylobacteriumsp. MB200	407 E Y A V N D P Y S G K T V T G G I I T I T D S N W L 399 E L A V N D P Y S G R T V T G G I I T F T D S N W V	SVT	NRQE	н	F P N Q P D D V L V L W V Y A L F M D K Q G N Y V K K T M P E C T G N E I L A E L C Y H L G 48 F P G O P R D V L V L W A Y A L L M D R D G N H V R K P M P A C T G R E I L S E L C Y H L G 48
28 Pseudoalteromonashaloplanktis	397 T L S V N D P Y S G R S V T G G I I T I T D S N W L	SVT	NRQI	н	PEQPDDILVLWVYGLLMDKKGNKIEKTMAECTGKEILTELCYHLN47
525717 Paracoccussp.5503	399 D L S V N D P Y S G R T A T G G I I T F T D S S W V	SVT	NRQF	н	P D Q P K D T L V V W V Y A L L M D K P G D K V K K T M P E C T G R E I L S E L C H H L G 48
525716 Paracoccussp.10990	399 D L S V N D P Y S G R T A T G G I I T F T D S S W V	SVT	NROF	н	FPDOPKDTLVVWVTALLMDKPGDKVKKTMPECTGREILSELCHHLG48
076 Rhodopseudomonaspalustris	402 E L S V N D P Y S G K T A T G G I I T F T D S N W V I	. S I T	NRQF	н	F P T Q P D D V L V L W V Y A L L M D K D G N Y V K K P M P A C T G R E I L A E L C Y H L G 48
14003 Paracoccusaminophilus	395 E L S V N D P Y S G K T V T G G I I T F T D S N W V	1SVT	NRQF	H	ELG Q P K D V L V L W V Y A L L M D K D G N K V K K P M P A C T G R E I L A E L C H H L G 47
9448 Bradyrhizobiumelkanii	398 E L S V N D P Y S G K T V T G G I I T F T D S N W V	SIT	NRQE	н	PTQPKDVLVVWVYALLMDKEGNYVKKPMPACTGREILAELCYHLG48
9448 Bradyrhizobiumelkanii	398 E L S V N D P Y S G K T V T G G I I T F T D S N W V I	SIT	NRQF	н	P T Q P K D V L V V W V Y A L L M D K E G N Y I K K P M P A C T G R Q I L A E L C Y H L G 48
9448 Bradyrhizobiumelkanii 1561 Rhizobiales	398 E L S V N D P Y S G K T V T G G I I T F T D S N W V I 402 F I S V N D P Y S G K T V T G G I I T F T D S N W V	S I T	NRQE	н	F P T Q P K D V L V V W V Y A L L M D K E G N Y V K K P M P A C T G R E I L A E L C Y H L G48 F P D O P G D V L V L W V Y A L L M D K D G N Y V K K P M P A C N G R E V L T E L C Y H L G48
208321 Marinomonasprofundimaris	409 T L S V N D P S S G R S V T G G I V T I T D S N W L	SVT	NRQF	н	F P D Q P D D T L V L W V Y G L L M D K P G N R I Q K T M AQ C T G K E I V T E L C Y H L G 49
07949 Bermanellamarisrubri	397 SLSVNDPFSGKTATGGICTITDSNWLM	1 S C T	NRQF	н	FRNQPDDVLVVWVYALLMDKQGNKVHKTMPECTGKEILEELCHHLG47
0/6 Rhodopseudomonaspaiustris 57440 Pleomorphomonaskoreensis	402 ELSVNDPASGKTVTGGVITFTDSNWV 398 ELSVNDPYSGKTVTGGIITFTDSNWVI	1 S I T	NRQI	н	F P N Q P D D V L V L W V Y A L L M D K D G N Y V Q K P M P A C T G R E I L A E L C Y H L G 48 F P N O P D D V L V V W L Y A L L M R E D G N Y I K K P M P A C T G R E I I A E L C Y H L G 48
85 Comamonastestosteroni	404 E L A V N D P Y S G K T V T G G I I T F T D S N W V N	1 S I T	NRQI	н	F P D Q P D D V L V L
85 Comamonastestosteroni	404 E L A V N D P Y S G K T V T G G I I T F T D S N W V N	ISIT	NRQF	H	P D Q P D D V L V L W V Y A L L M D K E G N H I R K P M P A C T G R E I L A E L C Y H L G 48
317124 Thioclavasp.13D2W-2	400 E L S V N D P Y S G R T A T G G V I T F T D S N W V	1511	NROF	н	FRDOPDDVLVLWAYGLDMDGVGDVTGKTMADCTGREILAELCHHLG48
324 Enhydrobacteraerosaccus gb EEV22910.1	406 KYCVNPPLSGKTVTGGIVTITDSNWLM	1 S I T	NRQI	Q	FLDQPKDVVVIWVYGLLMDKKGNYVPKTMPECTGHEIVTELLYHLD48
280946 Hyphomonasberingensis	405 T L S V N D P Y S G R T V T G G I I T F T D S N W L I 402 E L S V N D P Y S G K T V T G G V I T F T D S N W V	SIT	NRQP	н	F P D Q P E D T L V L W V Y G L L M D Q P G N H V S K T M P E C T G R E I L S E L C H H L G48
0 442 Gluconobacteroxydans	402 E L S V N D P Y S G K T V T G G V I T F T D S N W V	SIT	NRQF	н	F P T Q P D D V L V I W V Y A L L M D K E G N Y V K K P M P A C T G R E I L A E L C Y H L G 48
055192 Comamonassp.B-9	397 E L S V N D P Y S G K T V T G G I I T F T D S N W V M	ISIT	NRQE	н	P D Q P S D V L V L W V Y A L L M D K K G N H V A K T M P E C T G R E I L A E L C Y H L G 47
81029Hyphomonasadhaerens	419 ELSINDPYSGRIVIGGVIIFIDSNWI 405 TLSVNDPYSGRTVTGGIITFTDSNWL	NVT	NROF	н	F P D Q P G D V L V L W A Y A L L M D K N G N K V A K P M P A C T G K E I L Q E L C Y H L G 50 F R D O P E D T L V L W V Y G L L M D O P G N H V G K T M S E C T G R E I L S E L C Y H L G 48
835204 taxonID 400668 Marinomonassp.MWYL	409 T L S V N D P Y S G R S V T G G I I T I T D S N W L I	SVT	NRQF	н	FPDQPEDTLVLWVYGLLMDKPGNRIHKTMAQCTGKEILTELCHHLG49
9791 Mesoniamobilis	407 EYTINDPYSGKTATGGIITITDSDWL	ISIT	NRQE	н	P N Q P D D V L V L W V Y S L Y M D K E G N Y V K K A M P Q C T G D E I L T E L C Y H L G 48
345 Flavobacteriumpsychrophilum	407 EYCVNDPYSGKTATGGIVTITDSNWLM	1 S I T	NRQF	н	PTQPDDILVVWVYALYIDKPGNYVQKTMTQCTGNEILSELCYHLG48
1 Sulfurospirillummultivorans	410 KLSVNDPYSGKTVTGGIITITDSNWL	1 S I T	NRQE	H	FIEQPDDILVIWLYALFMDKEGNYVKKPMPECSGDEILTELCYHLG49
33950 Chryseobacteriumsp.JM1	407 ELCVNDPYSGRTATGGIITITDSNWU	1 S I T	NRQF	н	PTQPDDILVVWVYSLLMDKEGNYIKKTMPECTGNEILAELCYHLG48
9141 Halomonassp. TD01	412 E L S V N D P Y S G F T A T G G I I T F T D S S W L M	1 S I T	NRQF	н	F P D Q P D D V I V L W T Y A L L M D K P G D Y V K K P M P E C T G K E V L T E M C Y H L G 49
1 Flavobacteriumhydatis 1890810/eispiraantarctica	407 EYCVNDPYSGKTATGGIITITDSNWL 387 FISVNDPYSGKTVSGGIITFADSNWL	4 S I T	NRQE	н	F P E Q P D D I L V L W V Y A L F M D K E G N Y I K K K M P Q C T G D E I L A E L C Y H T G 48 F P D O P D D V I V I W V Y A L I M D K A G N I V K K A M P F C T G K F I I T F M C Y H I G 46
31540 taxonID 698738 OleispiraantarcticaRB-	412 E L S V N D P Y S G K T V S G G I I T F A D S N W L M	1 S I T	NRQE	н	PDQPDDVIVIWVYALLMDKAGNLVKKAMPECTGKEILTEMCYHLG49
9295 Flavihumibacterpetaseus	407 ALSVNDPYSGKTVTGGIITITDSNWLM	1 S I T	NRQE	н	P T Q P D D I L V V W V Y A L Y M D K E G N H V K K P M P L C T G N E I L A E L C Y H L G 48
2220 Chryseobacteriumsp. StRB125	407 ELCVNDPYSGKTATGGIITITDSNWVP 412 ELSVNDPYSGFTATGGIITFTDSSWLI	1511 1517	NROF	н	F P T Q P D D I L V V W V Y A L L M D K E G N H I K K I M P E C I G N E I L A E L C H H L G 48 F P D O P D D V I V L W T Y A L L M D K P G D Y V K K P M P A C T G K E V L T E M C Y H L G 49
022 Flavobacteriumsp.JRM	407 EYSVNDPYSGKTVTGGIITITDSNWLM	1 S I T	NRQF	н	FPEQPDDILVLWVYALFMDKEGNYIKKKMPQCTGDEILAELCYHTG48
17 Empedobacterbrevis	407 EYSVNDPYSGKTVTGGIITITDSNWL		NRQE	н	F P T Q P D D V L V L W V Y A L F M D K E G N Y I K K T M P T C T G N E I L T E L C Y H L G 48
3554 Halomonascampaniensis	411 ELSVNDPYSGFTATGGIITFTDSSWL	1 S I T	NRQE	н	PDQPDDVIVLWTYALLMDKPGDYVKKPMPECTGKEVLTEMCYHLG49
83151 Myroidesinjenensis	406 EYAVNDPYSGRTVTGGIITITDSNWLM	1 S I T	NRQF	н	F P T Q P D D V L V L W V Y A L F M D K E G N Y I K K V M P E C T G E E I L S E L C Y H L G48
88027 Arcticibactersvalbardensis IBI Elizabethkingiameningoseptica	407 ELCVNDPYSGRIAIGGIIIIIDSNWLP 407 EYSVNDPYSGKTVTGGIITITDSNWLP	1511 1517	NRQE	н	F L N Q P E D V L V L W V Y S L F M N K E G N Y I K K I M P Q C I G N E I L A E L C Y H L G 48 F P E O P D D V L V L W V Y A L F M D K E G N Y I K K T M P E C T G D E I L A E L C Y H L G 48
4 gi 380877058 pdb 4UIR(A,B) taxonID 238 E	407 EYSVNDPYSGKTVTGGIITITDSNWL	1 S I T	NRQF	н	F P E Q P D D V L V L W V Y A L F M D K E G N Y I K K T M L E C T G D E I L A E L C Y H L G 48
125451Flavobacteriumsp.KMS	407 EYSVNDPYSGKTVTGGIITITDSNWL	ISIT ISIT	NRQF	н	F P E Q P D D I L V L W V Y A L F M D K E G N Y I K K K M P Q C T G D E I L A E L C Y H T G 48
2877 Elizabethkingiasp.BM10	407 EYSVNDPYSRKTVTGGIITVTDSNWL	ISIT	NRQI	н	FPEQPDDVLVLWVYALFMDKEGNYIKKTMPECTGDEILAELCYHLC48
2045 Elizabethkingiamiricola	407 EYSVNDPYSGKTVTGGIITITDSNWLM	1 S I T	NRQE	н	F P E Q P D D V L V L W V Y A L F M D K E G N Y I K K T M P E C T G D E I V A E L C Y H L G 48
17645 Elizabethkingiaanophelis	407 EYSVNDPYSGKTVTGGIITITDSNWL	1 S I T	NRQE	н	FPEQPDDVLVLWVYALFNDKEGNYIKKTMPECTGDEILAELCYHLG48
00282 Chryseobacteriumsp.CF365	407 ELCVNDPYSGRTATGGIITITDSNWVM	1 S I T	NRQF	н	F P T Q P D D I L V V W V Y A L L M D K E G N Y I K K P M P Q C T G N E I L A E L C Y H L G48
0 Chryseobacteriumgleum 185985 Sphingobacteriumpaucimobilis	407 ELCVNDPYSGRTATGGIITITDSNWV 407 FYAVNDPYSGKTVTGGIITVTDSNWI	ISIT	NRQE	н	F P T Q P D D I L V V W V Y A L L M D K E G N Y I K K T M P Q C T G N E I L A E L C Y H L G 48 E P E O P D D V I V I W V Y A L E M D K K G D Y V R K T M P E C T G N E I L A E L C H H I G 48
50997   Marinobactermanganoxydans	411 E L S V N D P Y S G F T A T G G I V T F T D S A W L M	1 S I T	NRQI	н	F P D Q P D D V I V L W T Y A L L M D K P G D Y V N K T M P E C T G K E V L I E L C Y H L G 49
1630 taxonID 1298607 Psychrobactersp.JCM1	408 T L S V N D P Y M G K T V T G G I I T I T D S N W L M	1 S I T	NRQF	н	P D Q P K D T L V VW L Y A L F M D K E G N Y V K K P I P E C T G K E I L S E L C H H L G 49
3331 Chryseobacteriumtaiwanense	408 TESVNDPTTGKTVTGGIITITDSNWL	1 S I T	NRQF	Ĥ	P T Q P D D I L V I W V A L L M D K E G N Y V K K T M P E C T G K E I L S E L C H L G 49
179237 Marinobactersp. HL-58	411 E L S V N D P Y S G F T A T G G I I T F T D S A W L M	1 S I T	NRQF	н	F P D Q P D D V I V L W T Y A L L M D K P G D Y V K K P M P E C T G K E V L A E M C Y H L G 49
229 Psychroflexustorquis	407 E Y C V N D P Y S G K A A T G G I V S I T D S NW LN 407 E L C V N D P Y S G K T A T G G I I T V T D S NW VI	1 S I T	NRQF	н	F P E Q H D D V L V I W V Y A L F M N K D G N Y N K K T M P Q S T G N E I L S E L C F H I G48
6470 Olivibactersitiensis	407 EYSVNDPYSGKTVTGGIITITDSNWLM	ISIT	NRQF	н	F P T Q P D D V L V L W V Y A L F M D K E G N Y I K K T M P A C T G N E I L T E L C Y H L G 48
600 Cellulophagaalgicola	407 EYCVNDPYSGKSATGGIVTITDSNWLM	ISIT	NRQE	н	F P E Q P D D I L V I W V Y A L F M D K N G N Y S K K T M P Q C T G N E V L A E L C F H I G 48
180 Chryseobacteriumvrvstaatense	407 ELCVNDPTSGKIVTGGIITITDSNWL 407 ELCVNDPYSGRTATGGIITITDSNWV	151T 151T	NROF	н	FFIQFDDILVLWVTALFMDKDGNYVKKTMPACTGNEILAELCYHLG48 FPTQPDDILVVWVYSLLMDKEGNYIKKTMPECTGNEILAFISYHIG48
832 Myroidesodoratimimus	407 GYAVNDPYSGKTVSGGIITITDSNWL	1 S I T	NRQI	н	F P T Q P D D V L V L W V Y A L F M D K K G N H I K K T M P E C T G N E L L E E L C Y H L G 48
2  Flavobacteriumhibernum 20  Marinobacterexcellers	407 ELCVNDPYSGKTATGGIVTISDSNWLM	IS IT	NRQE	н	F PEQ P D D I L V I W V Y A L F M D K E G D Y V K K T M P C T G N E I L A E L A H H L G 48
2113 Novosphingobiumsp.PP1Y	406 E L S V N D P Y S G R T V T G G I I T F T D S AW L	SNT	NRQE	H.	FPTQPSDTLVLWVYGLFMDKNGDYVKKPMPQCTGKEVLAELCYHLG49
76 Rhodopseudomonaspalustris	400 Q L S V N D P Y S G K T V T G G V I T F T D S N W V N	ISIT	NRQF	н	F P T Q P D D V L V L W V Y A L L M D K D G N Y V K K P M P A C T G R E I L A E L C Y H L G 48
52021 [gl]735022192 [taxonID]1206458 [Novosp. 242 [Marinobactersantoriniensis	402 E L S V N D P Y S G K T V T G G I I T F T D S NWVN 411 E L S V N D P Y S G F T A T G G T V T I T D S AWI /	1 S V T	NRQE	H H	P P I Q P D D V L V L W V Y A L L M D K D G N Y V K K P M P A C T G R E V L T E L C Y H L G 48 F P D O P D D V I V L W V Y A L L I D K P G D Y V K K P M P F C T G K F V V T E L C Y H L G 40
0759 Marinobactersp. BSs20148	412 E L A V N D P Y S G K T V T G G V V T F T D S A W L M	ISIT	NRQE	H	LDQPSDVIVPWVYGLFMDKPGDYVKKPMPECTGEEILTELCYHLC49
17493 Thalassolituusoleivorans 4751Frateurlaaurantia	413 T L S V N D P Y S G R T V T G G I I T F T D S NW LN	1SVT	NRQE	Н	F P D Q P D D T L V L W V Y G L L M D K Q G N C V P K T M S Q C T G K E I L A E L C Y H L G 49
1254 Ochrobactrumrhizosphaerae	402 E L A V N D P Y S G K T V T G G I I T F T D S N W V	SIT	NRQE	н	F P T Q P D D V L V L W V Y A L L M D K E G N Y I K K P M P E C T G R E I L A E L S Y H L G 48
1324 Stenotrophomonasmaltophilia	400 A L S V N D P Y S G R T V T G G V I T F T D S N W V	SIT	NRQF	н	V D Q P K D V L V W V Y A L L M D Q N G N H V A K P M P A C T G R E I L A E L C Y H L G48
152+13cenotropnomonasmaltophilla 16476 Marinomonasposidonica	409 E LA VND PYTGRTATGGIITFTD S KWI	SVT	NROF	н	F V D Q F N D V L V V W V T A L L M D Q N G N H V A K P M P A C I G R E I L A E L C Y H L G47 F P N Q P N D T L V L W V Y G L L M D Q Q G N A V K K T M P E C T G K E I I T F I C Y H I G49
5 Comamonastestosteroni	396 E L A V N D P Y S G K T V T G G I I T F T D S N W V M	1 S I T	NRQI	н	PDQPDDVLVLWVYALLMDKEGNHIRKPMPACTGREILAELCYHLG47
1/6 Knodopseudomonaspalustris 18 Methylobacteriumextorauens	402 E L S V N D P F S G K T V T G G I I T F T D S N W V 399 E L A V N D P Y S G K T V T G G I I T F T D S N W V	SIT SVT	NRQE	H H	F P I Q P ND V L V LW V Y A L LMD K D G N Y V K K P M P A C T G R E I L A E L C Y H L G 48 F P E O P K D V L V LW A Y A L LMD K D G N H I R K P M P A C T G R F I I S F I C Y H L G 40
180 Acinetobacterursingli	407 EYAVNDPYSGKTVTGGIITITDSNWL	ISIT	NRQE	н	PEQPDDVLVLWVYALFMDKQGNYVKKTMPECTGNEILAELCYHLC48
			- ·		

Figure S29. A segment of the multiple sequence alignment of the 116 amino acid sequences from HFam11 collected in the hydratase engineering database (HyED), highlighting the conserved residues involved in binding of a carboxylate (red boxes). a) Gln265 is conserved among all members of HFam11. b) Thr436 and Asn438 are conserved among all members of HFam11, while His442 is conserved among all but one member, where it is substituted with a Gln.

# Conversion of OA derivatives with OhyA wild type and substrate binding variants

We compared the activity of OhyA wild type and all solubly expressed variants for hydration of **1a–1j** by performing bioconversions with whole *E. coli* cells for 22 h (Figure S32 - Figure S39).

Head	Entry	OhyA variants applied in conversions						
group	Single variants		Double variants	Triple variants	Quadruple variants			
		GIn265Glu; GIn265Ser						
Amine	1h	Thr436Asp	Gln265Glu/Thr436Asp					
Amme	15	Asn438Asp; Asn438Ser	Gln265Glu/Asn438Asp					
		His442Asp; His442Glu; His442Tyr						
			Gln265Glu/Thr436Asp					
		GIn265Glu; GIn265Ser	Gln265Glu/Asn438Asp					
		Thr436Asp	GIn265Ser/Asn438Asp	Gln265Glu/Thr436Asp/Asn438Asp				
Amide	1C	Asn438Asp; Asn438Ser	Gln265Ser/Asn438Ser	Gln265Ser/Thr436Asp/Asn438Asp				
		His442Asp; His442Glu; His442Tyr	Thr436Asp/Asn438Asp					
			Thr436Asp/Asn438Ser					
		GIn265Ala; GIn265Lys	GIn265Ala/Asn438Ala					
Amide 1c Hydroxamic acid ( <i>N</i> -hydroxy oleamide)		Thr436Ala; Thr436Asn; Thr436Lys	Gln265Lys/Thr436Lys					
( <i>N</i> -hydroxy oleamide)	1d A:	Asn438Ala; Asn438Arg; Asn438Lys	Gln265Lys/Asn438Lys	GIN265AIa/I nr436AIa/Asn438AIa				
oloamiaoy		His442Ala; His442Asn; His442GIn	Thr436Ala/Asn438Ala					
		GIn265Ala; GIn265Lys	<b>.</b>					
		Thr436Ala; Thr436Asn; Thr436Lys	Gin265Ala/Asn438Ala					
Alcohol	1e	Asn438Ala; Asn438Arg; Asn438Lys	Gin265Lys/Thr436Lys Gin265Lys/Asn438Lys	GIn265Ala/Thr436Ala/Asn438Ala				
		His442Ala; His442Asn; His442GIn						
			Thr436Ala/Asn438Ala					

Table S1. OhyA variants used for conversion of OA derivatives 1b-1j. The enzymes tested with the different substrates were selected on basis of favoring the interaction between head groups and the substrate binding residues.

# Table S2. (continued)

OhyA variants applied in conversions							
GIn265Ala/Thr436Ala/Asn438Ala/His442Ala							
GIn265Ala/Thr436Ala/Asn438Ala/His442Ala							
	is						



Figure S30. Expression analysis of OhyA wild type enzyme and variants harboring rational amino acid exchanges of substrate binding residues. The level of recombinant hydratase present in *E. coli* cell lysate before (lanes indicated with 'T' for total lysate) and after (lanes indicated with 'C' for cell-free lysate) separation of insoluble proteins was analyzed. Two  $\mu$ L of lysate was loaded in each lane. To allow for easier comparison of the protein amounts, cell lysate containing the wild type enzyme was loaded on each gel (a–h).



Figure S31. Conversion of 1a by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC).



Figure S32. Conversion of 1b by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC). No hydration of 1b was obtained.



Figure S33. Conversion of 1c by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC).



Figure S34. Conversion of 1d by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC).



Figure S35. Conversion of 1e by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC).



Figure S36. Conversion of 1f by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC).



Figure S37. Conversion of 1g by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC).



Figure S38. Conversion of 1h by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC).



Figure S39. Conversion of 1i by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC).



Figure S40. Conversion of 1j by OhyA wild type and the amino acid exchange variants as whole cell *E. coli* biocatalysts after over-expression of the enzymes. Control reactions contained either the substrate added to the reaction buffer without cells or the substrate added to an *E. coli* empty vector control (EVC).

# Stereoscopic imaging of modeling studies





Figure S41. Stereoscopic representation of the in silico docking of oleic acid (1a) and oleic acid derivatives 1c-1j to the OhyA 3D structure after mutagenesis of substrate binding residues (amino acid positions 265, 436, 438 and 442). The enzyme variant – substrate combinations resulting in the highest conversion are shown in the panels. The hydrophobicity of the enzyme cavity is represented by a color gradient from red (hydrophobic) to blue (hydrophilic). Co-crystallized FAD (yellow) and the substrates in the best docking mode are shown in stick representation. Substrate binding residues and catalytic Glu122 and Tyr241 are highlighted. a) Docking of (1a) to the 3D structure of OhyA wild type enzyme. b) Docking of oleamide (1c) to OhyA Q265A/T436A/N438A. d) Docking of oleyl alcohol (1e) to OhyA Q265A/T436A/N438A. e) Docking of methyl (1f), ethyl (1g) and *n*-propyl (1h) and *n*-butyl (1j) oleate to OhyA Q265A/T436A/N438A.

# Determination of the enantiomeric excess of reaction products by <sup>1</sup>H-NMR analysis

All products from the enzyme-catalyzed hydration reactions were O-acylated with (S)-(+)-O-acetylmandelic acid using a known procedure and purified by flash chromatography on silica gel using cyclohexane/ethyl acetate mixtures.<sup>[2]</sup> A reference material was obtained from methyl *rac*-10-hydroxy stearic acid for comparison in the <sup>1</sup>H-NMR analyses.<sup>[2]</sup>



Figure S42. <sup>1</sup>H-NMR analysis (CDCl<sub>3</sub>, 500 MHz) of (S)-O-acetylmandelic acid derivatized 2f ((10R,2'S)- vs. (10RS,2'S)-diastereomers). The material derived from racemic methyl 10-hydroxy stearic acid shows a broader signal at 5.88 ppm as consequence of incomplete resolution of the 2'-H protons and a partly resolved methyl ester signal at 3.67 ppm. At both sections of the spectrum, the signals derived from derivatized methyl (*R*)-10-hydroxy stearic acid (**1a** converted to the corresponding methyl ester **1f**) appear sharper and are on the side of lower chemical shifts.









5.98 5.96 5.94 5.92 5.90 5.88 5.86 5.84 Figure S49. <sup>1</sup>H-NMR analysis (CDCl<sub>3</sub>, 500 MHz) of (*S*)-*O*-acetylmandelic acid derivatized compound 2j.

Based on the clear NMR proof of (*R*)-selectivity in case of the hydrated compounds **2a** and **2f**, the strict enzymatic reaction mechanism involved and the fact that all other compounds **2c–2e** and **2g–2j** show a similarly shaped sharp NMR signal at 5.90 ppm, we conclude that the reaction proceeds in all cases with high stereoselectivity ( $ee \ge 95$  %) and that the products are the expected (*R*)-10-alcohols.

5.82

ppm

# Determination of OhyA wild type and variant conversions by GC-FID

Hydration reactions of **1a–1j** were quantified via GC on a Shimadzu GC-2010 Plus instrument equipped with a flame ionization detector and a Phenomenex Zebron ZB-5 column under the conditions described in the Experimental Procedures section of the Supporting Information. The improvement in catalytic activity is illustrated by representative chromatograms from wild type and the best variant conversions of each OA derivative (Figure S49–S56). Integration results are shown as peak area values for each substrate and product, respectively.



Figure S50. Overlay of GC-FID chromatograms from bioconversions of oleamide (1c) to 2c with *E. coli* whole cells over-expression OhyA wild type and OhyA GIn265Ser/Asn438Asp. Retention times and peak area values for the TMS-derivative of the internal standard *n*-pentadecanoic acid (11.21 min), 1c (12.90 min) and 2c (13.78 min) are highlighted.



Figure S51. Overlay of GC-FID chromatograms from bioconversions of *N*-hydroxy oleamide (1d) to 2d with *E. coli* whole cells over-expressing OhyA wild type and OhyA Gln265Ala/Thr436Ala/Asn438Ala. Retention times and peak area values for the TMS-derivative of the internal standard *n*-pentadecanoic acid (11.21 min), 1d (11.65 min) and 2d (12.36 min) are highlighted.



Figure S52. Overlay of GC-FID chromatograms from bioconversions of oleyl alcohol (1e) to 2e with *E. coli* whole cells over-expressing OhyA wild type and OhyA Gln265Ala/Thr436Ala/Asn438Ala. Retention times and peak area values for the TMS-derivative of the internal standard *n*-pentadecanoic acid (11.21 min), 1e (11.83 min) and 2e (12.51 min) are highlighted.



Figure S53. Overlay of GC-FID chromatograms from bioconversions of methyl oleate (1f) to 2f with *E. coli* whole cells over-expressing OhyA wild type and OhyA Gln265Ala/Thr436Ala/Asn438Ala. Retention times and peak area values for the TMS-derivative of the internal standard *n*-pentadecanoic acid (11.21 min), 1f (11.75 min) and 2f (12.45 min) are highlighted.



Figure S54. Overlay of GC-FID chromatograms from bioconversions of ethyl oleate (1g) to 2g with *E. coli* whole cells over-expressing OhyA wild type and OhyA Gln265Ala/Thr436Ala/Asn438Ala. Retention times and peak area values for the TMS-derivative of the internal standard *n*-pentadecanoic acid (11.21 min), 1g (11.96 min) and 2g (12.68 min) are highlighted.



Figure S55. Overlay of GC-FID chromatograms from bioconversions of *i*-propyl oleate (1h) to 2h with *E. coli* whole cells over-expressing OhyA wild type and OhyA Gln265Ala/Thr436Ala/Asn438Ala. Retention times and peak area values for the TMS-derivative of the internal standard *n*-pentadecanoic acid (11.21 min), 1h (12.06 min) and 2h (12.77 min) are highlighted. The insert shows a zoom-in to the section in which the reaction product 2h is eluting.



Figure S56. Overlay of GC-FID chromatograms from bioconversions of *n*-propyl oleate (1i) to 2i with *E. coli* whole cells over-expressing OhyA wild type and OhyA Gln265Ala/Thr436Ala/Asn438Ala. Retention times and peak area values for the TMS-derivative of the internal standard *n*-pentadecanoic acid (11.21 min), 1i (12.30 min) and 2i (13.07 min) are highlighted. The insert shows a zoom-in to the section in which the reaction product 2i is eluting



Figure S57. Overlay of GC-FID chromatograms from bioconversions of *n*-butyl oleate (1j) to 2j with *E. coli* whole cells over-expressing OhyA wild type and OhyA Gln265Ala/Thr436Ala/Asn438Ala. Retention times and peak area values for the TMS-derivative of the internal standard *n*-pentadecanoic acid (11.21 min), 1j (12.66 min) and 2j (13.50 min) are highlighted. The insert shows a zoom-in to section in which the reaction product 2j is eluting.

#### **Control reactions with OA esters**

In vivo hydrolysis of fatty acid esters is inherent to essentially all microbes, including *E. coli*.<sup>[12]</sup> To exclude any side reactions, in particular hydration of the corresponding free fatty acids after ester cleavage and re-esterification with available alcohols, respectively, we co-incubated **1f** (Figure S57) and **1g** (Figure S58) with methanol, ethanol or *i*-propanol. For experimental details,

please refer to the section 'Whole cell bioconversions' in the Experimental Procedures part of the Supporting Information. For this control experiment, we applied only the OhyA triple variant Gln265Ala/Thr436Ala/Asn438Ala, since this enzyme variant showed the highest activity towards **1f** and **1g**. Owing to the non-formation of 10-hydroxy stearic acid acid ethyl and *i*-propyl esters in biotransformations of **1f**, as well as the absence of any 10-hydroxy stearic acid acid methyl and *i*-propyl esters in biotransformations of **1g**, generation of free acid-derived hydration products was highly unlikely.



Figure S58. Bioconversion of methyl oleate (1f) to 2f with *E. coli* whole cells over-expressing OhyA Gln265Ala/Thr436Ala/Asn438Ala. Assays were co-incubated with alcohol additives ethanol or *i*-propanol for monitoring of potential side reactions from hydration of 1f after its possible hydrolysis to 1a by *E. coli*-endogenous hydrolases and subsequent re-esterification with available alcohols. a) Overlay of representative GC-MS chromatograms from technical triplicates of 1f biotransformations without alcohol additives and with addition of either ethanol or *i*-propanol. Retention times of the TMS-derivative of the internal standard *n*-pentadecanoic acid (10.58 min), 1f (11.60 min) and the TMS-derivative of 2f (12.87 min) are highlighted. b) Mass spectrum of 2f in biotransformations with out alcohol additive. c) Mass spectrum of 2f in biotransformations with ethanol as alcohol additive. d) Mass spectrum of 2f in biotransformations with *i*-propanol as alcohol additive. Based on the absence of additional peaks and the identical mass spectra in b) – d), we can exclude any of the aforementioned side reactions of 1f in whole *E. coli* cells.



Figure S59. Bioconversion of ethyl oleate (1g) to 2g with *E. coli* whole cells over-expressing OhyA Gln265Ala/Thr436Ala/Asn438Ala. Assays were co-incubated with alcohol additives methanol or *i*-propanol for monitoring of potential side reactions from hydration of 1g after its possible hydrolysis by *E. coli*-endogenous hydrolases and subsequent re-esterification with available alcohols. a) Overlay of representative GC-MS chromatograms from technical triplicates of 1g biotransformations without alcohol additives and with addition of either methanol or *i*-propanol. Retention times of the TMS-derivative of the internal standard *n*-pentadecanoic acid (10.58 min), 1g (12.00 min) and the TMS-derivative of 2g (13.21 min) are highlighted. b) Mass spectrum of 2g at 13.21 min in biotransformations with out alcohol additives. c) Mass spectrum of 2g in biotransformations with methanol as alcohol additive. Based on the absence of additional peaks and the identical mass spectra in b) – d), we can exclude any of the aforementioned side reactions of 1g in whole *E. coli* cells.

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