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Bio-based α,ω-functionalized hydrocarbons from multi-step reaction sequences with bio- and metallo-catalysts based on the fatty acid decarboxylase $OleT_{JE}$

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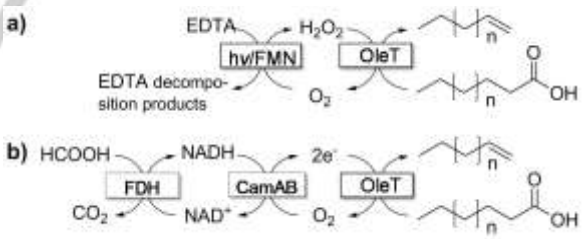
Abstract: OleT from *Jeotgalicoccus* sp. ATCC 8456 catalyzes the decarboxylation of *ω*-functionalized fatty acids to the corresponding alkenols, which can themselves serve as starting material for the synthesis of polymers and fine chemicals. To show the versatility of possible reactions, we developed a series of *in vitro* reaction cascades where an alkenol produced by the decarboxylation of *ω*-hydroxy fatty acids can be further converted into alkenylamines and diols. By coupling OleT with an alcohol dehydrogenase or alcohol oxidase as well as an amino-transaminase, an oxidative decarboxylation followed by the oxidation of the terminal alcohol and a subsequent reductive transamination could be carried out. By using different cofactors or electron sources, the reactions could be performed sequentially or simultaneously. In addition, the combination of enzymatic decarboxylation with a ruthenium catalyst in a chemo-enzymatic cascade provides a novel way to synthesize long-chain diols.

Introduction

As a result of the rapid depletion of fossil resources and continuously increasing environmental concerns, the interest to use renewable raw materials for the chemical industry has increased in the last decade.[1,2] Nowadays, alkenes form the basis for the production of plasticizers, lubricants and surfactants.[3] Oils and fats are regarded as one of the most important pools for the extraction of raw materials from renewable resources.[4] The use of enzymes in these first-stage valorization processes can facilitate the use of renewable resources for the production of fine chemicals.

Four specific enzymes, OleT, UndA, UndB and recently characterized CYP-Sm46, directly catalyze the enzymatic conversion of fatty acids to terminal alkenes.[5,6,7] The fatty acid decarboxylase cytochrome P450 OleT (CYP152L1) from *Jeotgalicoccus* sp. ATCC 8456 possesses the unique ability to convert saturated and (*E*)-unsaturated long-chain fatty acids to the corresponding 1-alkenes at neutral pH and room temperature.[8–12] Besides being active as a terminal decarboxylase, OleT also catalyzes the hydroxylation of fatty acids in *α*-, *β*- and *γ*-position.[11,12,19] It might be that this secondary reaction is related to the natural role of this enzyme since it is known that *β*-hydroxy fatty acid esters have regulatory functions in bacteria.[20] In contrast to other alkene producing enzymes, OleT acts predominantly as H_2O_2-dependent peroxygenase.[13,14] To minimize heme destruction by excess of H_2O_2,[14] the amount of H_2O_2 present in the reaction mixture needs to be carefully controlled.[15] For this purpose, we developed a system for the *in situ* formation of H_2O_2 by photocatalytic reduction of O_2 (h*ν*/FMN/H_2O_2 system).[12] Different mutagenesis analyses revealed the specific residues in the active site of the enzyme that are crucial for the activity of OleT as peroxygenase in the decarboxylation of fatty acids.[13,16,17] By using OleT as peroxygenase, we can circumvent the complicated multi-step electron transport chain as well as the recycling of expensive cofactors like NAD(P)H, which are usually required for this type of reaction (Scheme 1).[8,10,18]



Scheme 1. Oxidative decarboxylation of fatty acids to 1-alkene by OleT as peroxygenase or monooxygenase. **a)** Photocatalytic *in situ* generation of H_2O_2 to promote OleT catalyzed oxidative decarboxylation of fatty acids.[12] **b)** Enzymatic redox cascade process for the decarboxylation of fatty acids using the CamAB system.[8]

While OleT efficiently decarboxylates saturated and (*E*)-unsaturated long-chain fatty acids, its activity drops with decreasing chain length. Medium-chain (8C-14C) fatty acids are not or only slowly decarboxylated.[21] Faber and co-workers could still recently show that OleT is an efficient catalyst for the decarboxylation of short-chain (4C-5C) organic acids with NADH as electron donor, giving rise to gaseous 1-alkenes.[8] Furthermore, medium-chain fatty acids could also be decarboxylated with this system.

As the enzymatic decarboxylation proceeds under very mild reaction conditions and can be expected to have a high

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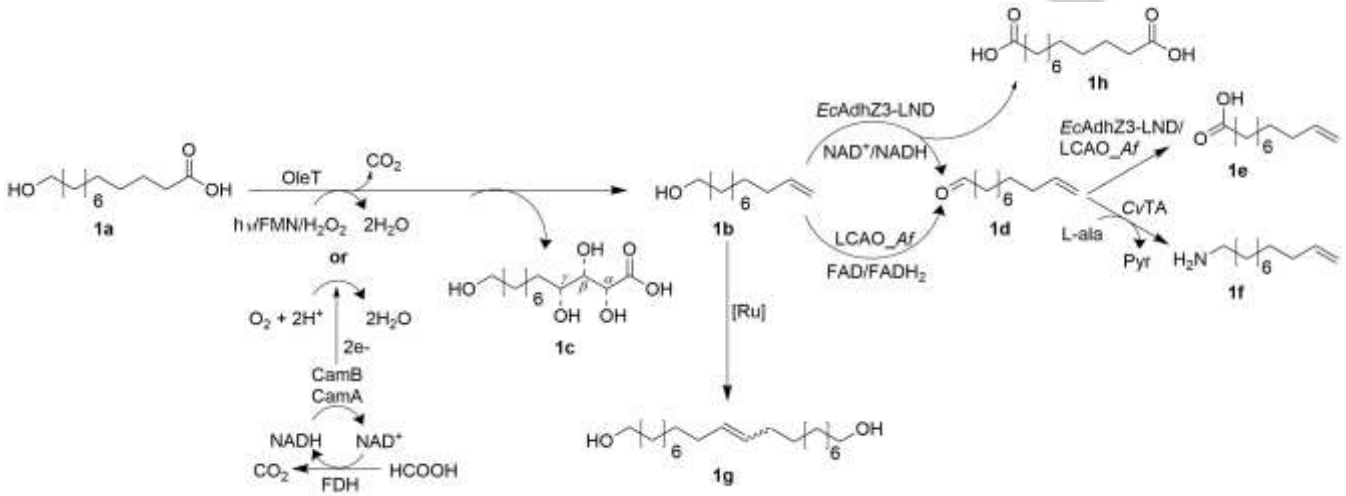
functional-group tolerance, we envisioned that OleT should be a suitable catalyst for the synthesis of *ω*-alkenols and other olefins from the corresponding *ω*-functionalized fatty acids. *ω*-Hydroxylauric acid (**1a**) can be obtained from the inexpensive, renewable raw material palm kernel oil.[22] In addition, the terminal oxidation of fatty acids by CYP450 monooxygenases to terminally oxidized hydroxy fatty acids has recently been established and demonstrated with **1a**.[23] Accordingly, this fatty acid is an industrially interesting substrate which can be converted into further substances like *ω*-alkenols. *ω*-Alkenols can serve as starting materials for the synthesis of functionalized long-chain amines, acids or even diols. In this work, we present a concept for the valorization of *ω*-alkenols like undec-10-en-1-ol (**1b**) by enzymatic and chemo-enzymatic reaction sequences.



Scheme 2. Enzymatic decarboxylation of *ω*-functionalized fatty acids and potential follow-up reactions. *ω*-Hydroxylauric acid **1a** is decarboxylated by OleT to give *ω*-alkenol **1b**, which can be further converted by additional enzymatic reactions to **1e**, **1f** or even **1g** by using ruthenium-based metathesis catalysts. As electron supply system for OleT, CamAB and the *in situ* light driven H_2O_2 generation system was used.

A series of *in vitro* reaction cascades combines the de-functionalization by enzymatic decarboxylation to a re-functionalization of the *ω*-hydroxy group or the double bonds (Scheme 2). The first and central step of all valorization chains is the decarboxylation of **1a** by OleT to form **1b**. The further oxidation of **1b** to undec-10-en-1-al (**1d**) can be carried out by a mutated NAD^+ dependent alcohol dehydrogenase from *Escherichia coli* (*Ec*AdhZ3-LND)[24] or a FAD^+ dependent alcohol oxidase from *Aspergillus fumigatus* (LCAO_*Af*).[25] A further oxidation of **1d** by either of the used enzymes can lead to the formation of undec-10-en-1-oic acid (**1e**). The reductive amination of the aldehyde **1d** by the amine-transaminase from *Chromobacterium violaceum* (*Cv*TA)[25] results in the formation of undec-10-en-1-amine (**1f**). Furthermore, we can produce icos-10-ene-1,20-diol (**1g**) from **1b** in a chemo-catalytic olefin metathesis reaction, using ruthenium-based metathesis catalysts. A similar approach with hydroxystilbene-based intermediates has recently led to the establishment of an effective chemo-enzymatic cascade using a phenolic acid decarboxylase with ruthenium catalysts.[26,27] The comparison of two different electron donor systems will show whether the *in situ* generation of H_2O_2[12] or the recyclization of NADH via the CamAB[8] system from *Pseudomonas putida* is more proficient with OleT. With this approach we can obtain **1e**, **1f** and **1g** which are interesting building blocks for the chemical industry.[2,28,29]

Results and Discussion

Enzymatic decarboxylation of ω-hydroxy fatty acids.

OleT was expressed in a constructed *E. coli* JW5020 expression strain with inactivated acyl-coenzyme A dehydrogenase (FadE) to avoid the non-desired degradation of fatty acids by enzymes present in the crude cellular extract (CE). OleT was either used as cell-free extract or in purified form. An *in situ* light-driven H_2O_2 generation system (h*ν*/FMN/H_2O_2) was used to fuel the oxidative decarboxylation of hydroxy fatty acids to the corresponding *ω*-alkenols. While we expected that OleT would be capable of decarboxylating long-chain *ω*-hydroxy fatty acids like *ω*-hydroxypentadecanoic acid (**2a**, 15C) and *ω*-hydroxyhexadecanoic acid (**3a**, 16C), it was difficult to anticipate whether the shorter hydroxylauric acid (**1a**, 12C) would be converted. We were pleased observe decarboxylation of fatty acids of different chain lengths (12C-OH (**1a**), 15C-OH (**2a**) and 16C-OH (**3a**)) to give the corresponding terminal alkenols with a chain length of one carbon atom less (**1b**-**3b**) took place. Formation of *ω*-alkenols and hydroxy fatty acids was determined by gas chromatography with MS and FID detection after derivatization with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). In addition, formation of the terminal olefins was confirmed by the identification of the characteristic 1H-NMR signals of the terminal double bonds in the crude extract of small-scale biocatalytic reactions. We also observed the formation of side-products. OleT is known to convert stearic acid to 1-heptadecene and β-hydroxystearic acid with two additional side-products that were tentatively assigned to be the α- and γ-isomer, respectively. Similarly we assume that it is likely that OleT converts **1a**-**3a** to the corresponding

hydroxyacids (**1c-3c**).[11] For each substrate, three different tentative hydroxylation products, the *α*-, *β*-, and *γ*-hydroxy fatty acids, were identified. However, due to the low amounts formed, confirmation by NMR was not possible. GC-MS-analysis indicated the *β*-hydroxy isomer as the most prominent form (Figures 1 and S 5).[11] While **1b** was identified by comparison with an authentic standard, the homologs **2b** and **3b** were identified by a tentative assignment based on their mass spectra (Figure S 2 – Figure S 4). MS analysis also suggested that the side-products stem from a hydroxylation of the substrate (**1c-3c**).

To optimize the reaction conditions to favor the decarboxylation by OleT, we investigated the influence of different buffer systems, the pH value and of co-solvents on the ratio between decarboxylation and hydroxylation of **1a** to give **1b** and **1c**. The ratio between decarboxylation and hydroxylation of fatty acids depends on the chain length.[12] While for long-chain fatty acids the decarboxylation is the dominant reaction, the hydroxylation is more pronounced with decreasing chain length. Studies on the crystal structure of OleT indicate that the exact accommodation of the carboxylic group towards the heme function in the enzyme's active site decides the outcome of the reaction.[30] To determine the decarboxylation *vs.* hydroxylation ratio, the signals of the three tentative hydroxylation products were considered in their sum (Figure 1).

The selectivity of OleT between decarboxylation and hydroxylation of **1a** differed under the influence of various co-solvents and their concentrations as well as the buffer system used. The ratio of the signal intensities between *ω*-alkenols and hydroxylation products was about 0.3 under initial reaction conditions with **1a** (0.5 mM), DMSO (5% (v/v)) and Tris buffer (50 mM at pH 7.5). From analyzing the influence of different buffers on the selectivity of OleT, the Britton-Robinson buffer showed the best results for *ω*-alkenol production with a ratio of

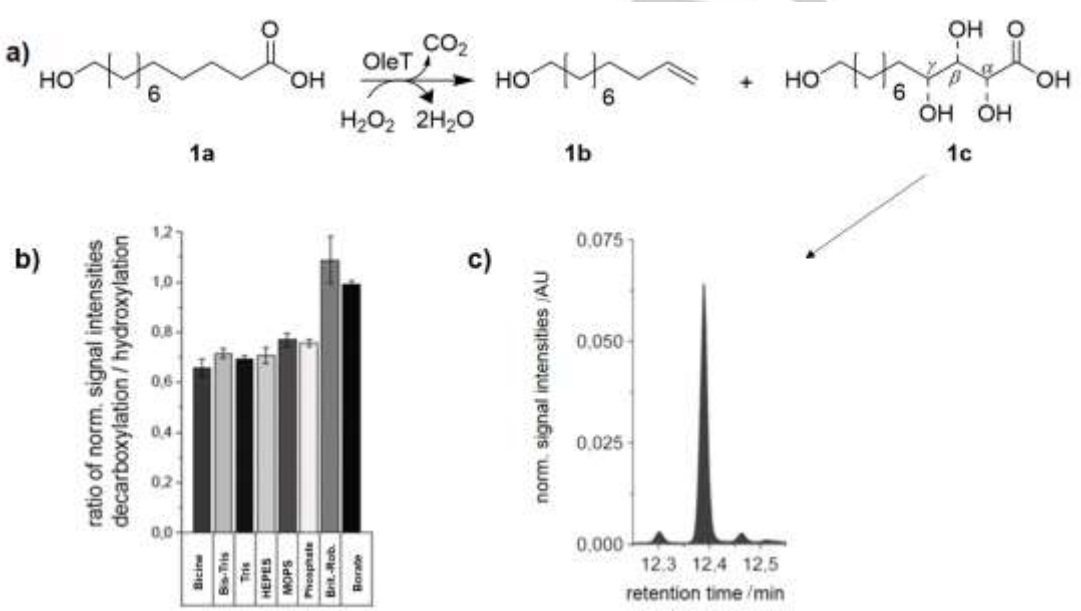


Figure 1. Influence of different buffers (50 mM each, with pH 7) on the activity of OleT in the reaction with **1a**. **a**) decarboxylation and hydroxylation reaction of **1a** by OleT.[11] **b**) Ratios of the normalized signal intensities of the decarboxylation *vs*. hydroxylation products in the reaction of 0.5 mM **1a**, catalyzed by cell-free extract of 60 mg (cdw) Containing $OleT_{JE}$ for 60 minutes in different buffer systems: bicine, bis-Tris Methane (Bis-Tris), tris(hydroxymethyl)aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(N-morpholino)propanesulfonic acid (MOPS), Phosphate, Britton–Robinson (Brit.-Rob.) and borate **c)** Formation of three side-products was observed. As it is known that OleT hydroxylates stearic acids, the production of hydroxyfatty acids (*α*-, *β*-, or *γ*-position) would be a possible explanation. GC-MS and GC-FID chromatograms showed a typical pattern for hydroxy-fatty acids (see also Figure S 3 and Figure S 4).

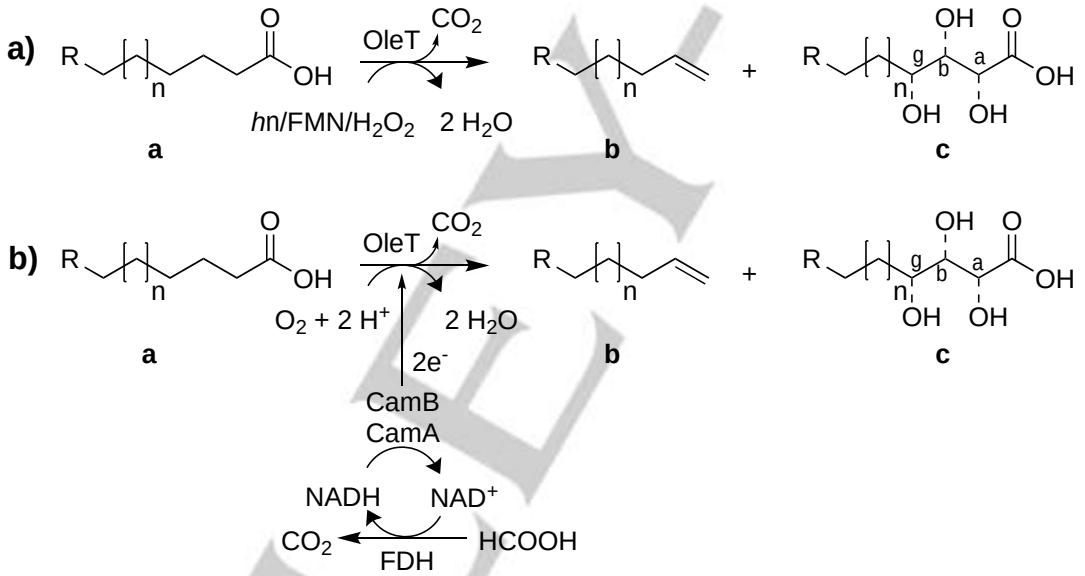
1.1 (using 5% DMSO, Figure 1b), which means that the hydroxylation of **1a** could be suppressed by a proper choice of reaction buffer.

The pH activity profile regarding the decarboxylation/hydroxylation ratio was investigated within a range of pH 3–9, where at pH 7 the best results were obtained in favor of decarboxylation (Figure S 6). Therefore, for further reactions, Britton-Robinson buffer at pH 7 and 10% (v/v) DMSO were selected.

Due to its low solubility in water, fatty acids need to be dissolved in a suitable co-solvent, which itself can influence the reaction. To verify this, different co-solvents like 1,4-dioxane, tetrahydrofuran (THF), 1,1-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) in concentrations of 5 and 30% (v/v) were tested. In presence of 10% (v/v) DMSO, the highest overall conversion could be observed (Figure S 7). The use of 30% (v/v) DMSO led to an increase of the ratio from 0.43 with 5% DMSO to about 0.7, indicating a growing preference for decarboxylation in presence of this co-solvent (Figure S 8). This shift of the ratio between decarboxylation and hydroxylation with increasing DMSO concentration can be influenced by several factors. We assume that a high concentration of DMSO leads to more

soluble substrate in the reaction and therefore more product formation by OleT. Crystallization studies on monooxygenase P450 BM3 with DMSO demonstrated that DMSO can coordinate to heme-bound iron as axial ligand,[31] thereby closing the coordination sphere around the central metal - which can significantly affect the enzymatic activity. When using OleT as a monooxygenase, the presence of DMSO decreases the overall activity of OleT.[32] Overall, to minimize a negative effect on other enzymes in the cascades by using a high concentration of DMSO (Scheme 2), 10% (v/v) was used for all following experiments.

Preliminary experiments with 0.5 mM **1a** and $OleT_{CE}$ in the h*v*/FMN/H_2O_2 system allowed us to achieve full conversion within 4 h (Table S 1, entry 6). When the concentration of **1a** was increased to 2 mM, the conversion did not exceed 60% even after 24 h (i.e., 60% of **1a** were consumed). In addition, by using $OleT_{CE}$, the formed **1b** was over-oxidized to undec-11-enoic acid (**1e**) and the remaining substrate **1a** into 1,12-dodecandioic acid (**1h**), presumably by enzymatic background reactions from the *E. coli* metabolism (Figure S 9b). To avoid these undesirable background reactions, OleT was purified. After the conversion of several *ω*-functionalized fatty acids with OleT (Scheme 3a), different isomers of hydroxy fatty acids were identified. GC-MS data indicated that the *β*-hydroxy isomer (75% relative abundance of all detected hydroxy fatty acids) was again the most prominent form. No over-oxidation reactions of synthetized *ω*-alkenols or remaining substrate were observed (Scheme 3a). Moreover, we recognized that with reduced chain length, the overall conversion of the substrate reduced and the ratio of decarboxylation and hydroxylation shifted to favor the hydroxylation reaction (*cf.* above and Table 1, entries 1-3). This means that the decarboxylating activity of OleT in the h*v*/FMN/H_2O_2 system increases with growing chain length, thus showing a similar trend to the one previously observed with saturated fatty acids.[12] Long-chain fatty acids are assumed to interact with hydrophobic amino acid residues in the substrate channel of the active site, whereas smaller substrates are accommodated entirely in the binding pocket, as shown for the related enzyme $CYP_{BSβ}$.[8,33] This would imply that medium-chain fatty acids have less capacity to bind efficiently and form



Scheme 3. Oxidative decarboxylation and hydroxylation reactions of fatty acids to 1-alkenes with OleT by different electron supply systems. **a)** Decarboxylation of saturated and functionalized fatty acids using the *in situ* light driven H_2O_2 generation system. **b)** Enzymatic redox cascade process for the decarboxylation of fatty acids using the CamAB system.[8]

a productive binding mode.[8] Indeed, Faber and coworkers could show the decarboxylation of short-chain carboxylic acids with 99% alkene production in the CamAB system.[8] For *ω*-amino lauric acid (**4a**) no conversion could be observed using the light-driven system (Table 1, entry 7). We could furthermore not detect any conversion with dodecandioic acid (**5a**),[8] even though OleT activity towards this substrate has been demonstrated before (Table 1, entry 8).[32]

Since our aim was to produce **1b** from the bio-based precursor **1a**, further reactions were carried out with this hydroxy fatty acid as a substrate. Results presented above describe the optimal conditions for *ω*-alkenol synthesis in the h*v*/FMN/H_2O_2 system. To determine the influence of different electron donor systems on the decarboxylation reaction with **1a** as a substrate, two methods were compared: h*v*/FMN/H_2O_2 (using H_2O_2 as electron donor) and CamAB (using NADH as electron donor) (Scheme 3). Interestingly, by using the CamAB electron supply system, a full conversion of 2 mM of **1a** could be achieved (Table 1, entry 6). Furthermore, the formation of hydroxylated by-products **1c** was significantly reduced (Figure S 10).

Taken together, the ratio of decarboxylation to hydroxylation shows a strong dependence on the chain length of the substrate and the electron supply system used. Moreover, we could show that OleT converts *ω*-hydroxy functionalized substrates (Table 1). With the optimized h*v*/FMN/H_2O_2 system we could mainly produce alkenols from the long-chain substrates **2a** and **3a**. Similar results were observed earlier in the CamAB system with hexadecanoic acid (16C) as substrate.[8] However, carboxylic

acids with short- to medium-chain lengths are rather poor substrates for the decarboxylation with OleT by using the h*v*/FMN/H_2O_2 system. Nevertheless, the light-driven decarboxylation is less complex in its application and therefore offers more possibilities for application in multi-catalyst approaches, especially due to the use of less regenerating enzymes which might interfere with further enzymatic reactions. We therefore used the light-driven electron supply system in all following cascade approaches.

Synthesis of undec-10 en-1-oic acid

Once the decarboxylation reaction of the *ω*-hydroxy fatty acid **1a** was completed, the oxidation of the terminal alcohol **1b** to a carboxylic group to produce **1e** via the aldehyde **1d** was attempted. Two enzymes were tested for this task: an enzyme variant of the NAD^+-dependent alcohol dehydrogenase from *E. coli* (*Ec*AdhZ3-LND) where the SSN (serine, serine, asparagine) motif was substituted by a LND (leucine, asparagine, aspartic acid) motif in the nucleotide-binding region,[24] and a putative flavin-dependent long-chain alcohol oxidase from *Aspergillus fumigatus* (LCAO_*Af*).[25]

To determine the influence of the conditions established for the decarboxylation reaction on the other enzymes of the cascade (Scheme 2), we investigated different buffers and co-solvents. Analyzing the influence of different buffers (Tris-HCl, Bis-Tris, Britton-Robinson and boric acid buffers) on **1e** formation, the speed of the reaction with *Ec*AdhZ3-LND reduces in Britton-Robinson buffer as compared to the results in Tris or Bis-Tris buffer (Figure S 11a). In the case of LCAO_*Af*, the reaction rate was identical in all buffers tested (Figure S 11b). When comparing the influence of 10% *vs.* 30% (v/v) DMSO used as co-solvent, it was found that 30% DMSO had a negative effect on the activity of *Ec*AdhZ3-LND, whereas in LCAO_*Af* reactions, no difference was observed. According to reported studies, the efficiency of the LCAO_*Af* reaction continuously increases from pH 6 to a maximum pH 10,[25] whereas *Ec*AdhZ3-LND has an optimal pH between 5.5 and 6 for using NAD^+ as cofactor.[24] A pH of 7 appeared to offer the most favorable conditions for both LCAO_*Af* and OleT.

To analyze the specific activity of both hydroxy group oxidizing enzymes, the alcohol **1b** and the corresponding aldehyde **1d** were used as substrates. *Ec*AdhZ3-LND showed a specific activity of 0.083 mU mg^{-1} towards **1b** and 3.8 mU mg^{-1} for **1d**. The higher activity towards **1d** explains the strong preference of *Ec*AdhZ3-LND to convert aldehydes and therefore the preferred synthesis of **1e**, which was also observed with endogenous alcohol dehydrogenases present in the crude extract of *E. coli*. In contrast, LCAO_*Af* showed an overall lower activity against **1b** (0.023 mU mg^{-1}) and **1d** (1.58 mU mg^{-1}).

Table 1. Substrate scope of the OleT in *in situ* light driven H_2O_2 generation system (h*v*/FMN/H_2O_2) and CamAB cascade process under individually optimized conditions:

Entry		Substrate	Conc. [mM]	OleT [mg mL^{-1}]	Cofactor supply	Conversion [%]	Ratio Decarboxylation/ Hydroxylation
1[a]	**3a**	*ω*-OH-C_{16}	2	0.3	h*v*/FMN/H_2O_2	99	4.2
2[a]	**2a**	*ω*-OH-C_{15}	2	0.3	h*v*/FMN/H_2O_2	78	2.1
3[a]	**1a**	*ω*-OH-C_{12}	2	0.3	h*v*/FMN/H_2O_2	25	0.14
4[b]	**1a**	*ω*-OH-C_{12}	2	0.3	h*v*/FMN/H_2O_2	6	0.5
5[b]	**1a**	*ω*-OH-C_{12}	2	1.0	h*v*/FMN/H_2O_2	28	0.8
6[c]	**1a**	*ω*-OH-C_{12}	2	0.3	CamAB	100	19
7[d]	**4a**	*ω*-NH_2-C_{12}	0.5	0.1	h*v*/FMN/H_2O_2	n.c.	-
8[d]	**5a**	*ω*-COOH-C_{11}	0.5	0.1	h*v*/FMN/H_2O_2	n.c.	-

Reaction conditions: [a] substrate, OleT, DMSO (10% v/v), FMN (10 µM) and EDTA (50 mM), Britton-Robinson buffer (50 mM, pH 7), at 25°C for 20 h. [b] substrate, OleT, DMSO (10% v/v), FMN (10 µM) and EDTA (50 mM), KPi buffer (100 mM, pH 7.5), at 25°C for 20 h. [c] substrate, OleT, EtOH (10% v/v), CamAB (0.05 U mL^{-1}), catalase (1200 U mL^{-1}), FDH (2 U mL^{-1}), ammonium formate (100 mM), and NAD^+ (200 µM), KPi buffer (100 mM, pH 7.5) at 25°C for 20 h: [d] substrate, OleT, DMSO (5% v/v) FMN (10 µM) and EDTA (50 mM), Tris-HCl (50 mM Tris, 200 mM NaCl, pH 7.5), at 25 °C for 2 h.[12] For all reactions purified OleT was used. Conversion was determined by: sum (c(all products))·100% / c(substrate). Detection by GC-FID and GC-MS analysis. n.c. = no conversion.

This was most probably due to a low soluble expression of LCAO_*Af* (2.16 mg L^{-1} expression yield in comparison to *Ec*AdhZ3-LND with 166 mg L^{-1}) which was attributed to inclusion body formation.

To increase the concentration of soluble LCAO_*Af* in the crude extract, different expression media such as TB, LB, TYGPN and autoinduction media were tested, with the best result (11.75 mg L^{-1}) obtained by using autoinduction medium (Figure S 1). Additionally, commercially purchased AO from *Pichia sp.* was also tested but did not convert **1b**.

Figure 2 shows a comparison of both enzymes in the oxidation of **1b** to **1d**, which is then further oxidized to form **1e**. While the LCAO_*Af* system shows very little activity in the oxidation of the terminal alcohol, the *Ec*AdhZ3-LND system achieved 100% conversion of the substrate into the desired carboxylic acid **1e**, with the intermediate aldehyde not being detectable at any time point.

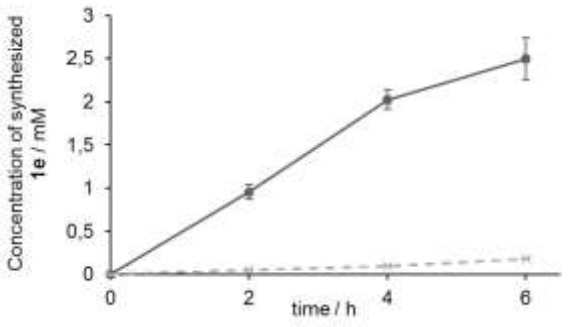


Figure 2. Comparison between *Ec*AdhZ3-LND and LCAO_*Af* in the synthesis of **1e**. General conditions: **1b** (2.5 mM), DMSO (10%), NAD^+ (2 mM) or FAD^+ (0.25 mM), Britton-Robinson buffer (50 mM, 300 mM NaCl, pH 7.0) and *Ec*AdhZ3-LND (full line) or LCAO_*Af* (dashed line) (CE of 90 mg cdw), reaction volume (3 mL). To have the same conditions as with **1a** as starting material in the h*v*/FMN/H_2O_2 system, EDTA (50 mM) and FMN (10 µM) were also added to the reactions. Samples were derivatized by MSTFA and analyzed by GC-FID. For this reaction, we used commercially purchased **1b**.

To analyze the capability of a cascade approach for the direct synthesis of **1e** from **1a**, a sequential enzyme cascade of conversions by $OleT_{CE}$ in the h*v*/FMN/H_2O_2 system and subsequent oxidation by *Ec*AdhZ3-LND was performed to avoid undesired attack of *Ec*AdhZ3-LND on **1a**. The light-driven biocatalysis (4 h reaction time) resulted in conversion of **1a** to a mixture of **1b** (17%) and **1h** as by-product (43%). We were pleased to find a complete conversion of the intermediary **1b** to **1e** by *Ec*AdhZ3-LND within 4 additional hours (Figure S 12).

Synthesis of 10-undecen-1-amine

By combining the oxidation of the terminal alcohol to an aldehyde with a subsequent enzymatic reductive amination, **1b** can be used as starting material for the synthesis of undec-10-en-1-amine (**1f**). Amine formation from alcohol **1b** was tested by combining *Ec*AdhZ3-LND and an amine transaminase (*Cv*TA) from *Chromobacterium violaceum.* This approach resulted in significant oxidation to **1e** (80.7%) with only 15% formation of the desired **1f** (Figure S 13). In contrast, a one-pot reaction with LCAO_*Af* and *Cv*TA achieved 85% of **1f** (Figure 3). The lower activity of LCAO_*Af* towards forming the intermediary aldehyde increases its availability for the amination reaction. When the concentration of **1b** was increased (1.5 mM), the conversion slightly decreased and achieved 58% amine (Figure S 14).

The challenge in asymmetric reductive amination, especially when using L-alanine as amine donor, is to shift the equilibrium to the product side. Therefore, to improve the amination step, pyruvate must be removed during the reaction by using additional enzymes like alanine dehydrogenase (AlaDH) or its formation circumvented by using other amine donors.[34,35] Furthermore, the incomplete transamination may not only be due to the chemical equilibrium, but also to a poor solubility of **1b** in the buffer medium.[36] Nevertheless, with undec-10-en-1-ol as substrate, Pickl *et al.* showed only 16% conversion to the respective amine; Significant amine formation was only observed with 6C-8C alcohols.[25]

To further extend the cascade and to directly obtain **1f** from **1a**, a sequential enzyme cascade using $OleT_{CE}$ in the h*v*/FMN/H_2O_2 system, LCAO_*Af* as well as *Cv*TA was applied. Only a low production of **1f** (8%) in a one-pot reaction was achieved (Figure S 15), indicating that the use of a two pot-two step reaction with intermediary extraction produce better results in this case. The decrease in amine formation in the one-pot reaction was caused by substantial over-oxidation to **1e**. In the amination of alcohols via intermediary aldehydes overoxidation is a significant risk.[37,38,39]

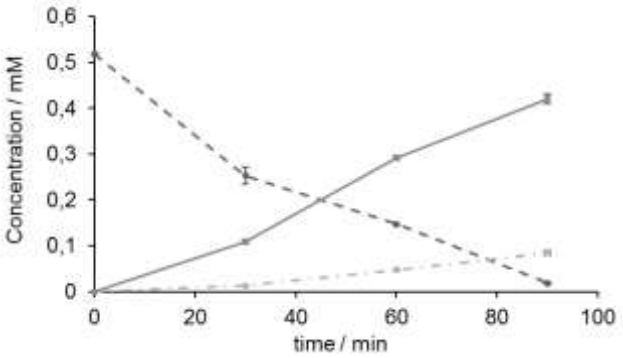


Figure 3. Amine formation from alcohol 1b in a one-pot reaction with LCAO_*Af* and *Cv*TA. General conditions: Britton-Robinson buffer (50 mM, 300 mM NaCl, pH 7.0), 1b (0.5 mM), DMSO (10%), FAD^+ (0.25 mM), catalase (1700 U), L-alanine (20 mM), PLP (1 mM), LCAO_*Af* (CE of 60 mg cdw) and *Cv*TA (CE of 120 mg of cdw) at 25°C for 90 min. Samples were derivatized by MSTFA and analyzed by GC-FID. For this reaction, we used commercially purchased **1b**. 1b = 10 undec-1-enol (dashed line); 1e = undec-11-enoic acid (chain line); 1f = 10-undecen-1-amine (full line).

Synthesis of a long-chain terminal diol by olefin metathesis

The terminal alkenes from enzymatic decarboxylation of fatty acids can also be used to produce long-chain terminal diols with an internal double bond by ruthenium-catalyzed olefin metathesis (Scheme 1). To achieve an efficient substrate conversion, the choice of the proper catalyst is crucial. Apart of its effectivity to convert the desired substrate, the reaction conditions required are critical for the development of a chemo-enzymatic cascade. Therefore, in a first step to establish the olefin metathesis, different commercially available ruthenium-based metathesis catalysts were tested for the conversion of **1b** in selected organic solvents. As a result, three catalysts **C1**-**C3** (Figure 4) were identified that could convert **1b** to **1g** in the presence of water and oxygen. GC-FID and GC-MS analyses revealed several peaks corresponding to the desired product and degradation products, possibly due to thermal decomposition during GC analytics (Figures S 16 – S 18). Therefore, the performance of the catalysts was assessed by substrate consumption rather than by product formation (Table S 3, Figure 5).

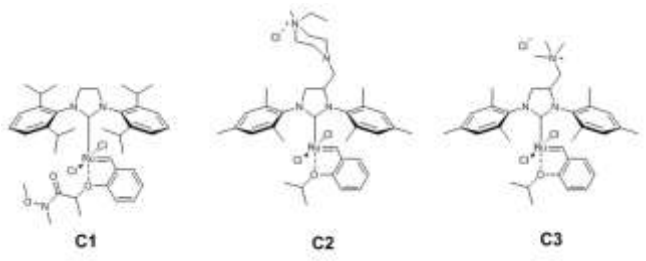


Figure 4. Cross metathesis catalysts that were used for the olefin metathesis reaction of **1b**. **C1** = Dichloro [1,3-bis (2,6-isopropylphenyl)- 2-imidazolidinylidene] -2-[[1-(methoxy(methyl) amino)-1-oxopropan- 2yl]oxy] benzylideneruthenium (VI); **C2** = Dichloro[1,3-bis (2,4,6-trimethylphenyl)-4- [(4-ethyl-4-methyl piperazinyl)methyl]-2-imidazolidinylidene] -(2 isopropoxy benzylidene) ruthenium(VI) chloride and **C3** = Dichloro [1,3-bis(2,4,6-trimethylphenyl)-4-[(trimethylammonio)methyl]-2-imidazolidinylidene]- (2-isopropoxybenzylidene)-ruthenium(VI)chloride.

Based on these data, the reaction was then performed in a biphasic system consisting of Tris buffer and isooctane. It was found that only catalyst **C1** catalyzes the conversion of **1b** to **1g** in presence of an aqueous phase, with a conversion of 89% in 18 h reaction time while using a 5 mol-% catalyst loading (Figure S 18).

In parallel, the metathesis was performed in isooctane with **C1** as catalyst in a semi-preparative scale. **C1** converted 0.5 mmol **1b** in 18 h and produced the desired **1g**, which was isolated after preparative TLC with 43% isolated yield.

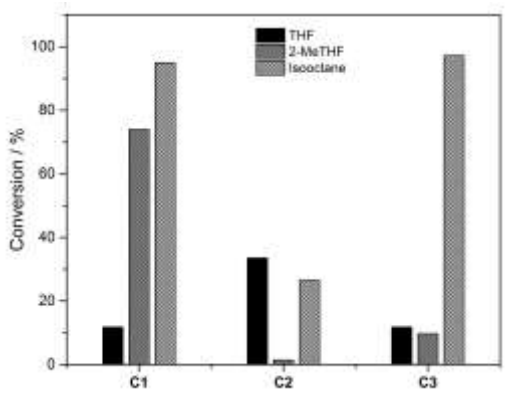


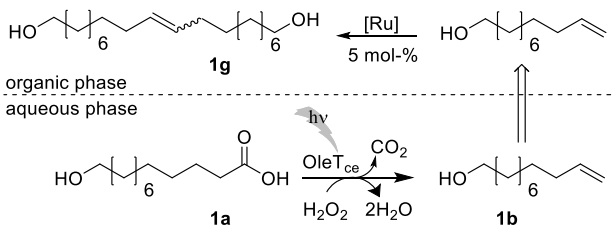
Figure 5. Substrate conversion rates for the three selected catalysts in the screened solvents. Conversion was determined by GC-FID as substrate consumption.

Once the metathesis reaction of **1b** was established, the practicability of the combination of enzymatic and chemical catalysis was tested. Different approaches, applying sequential and simultaneous one- and two-pot conditions were tested. Working in a biphasic system brings great advantages for the planned chemo-enzymatic cascade reaction, since the enzymatic decarboxylation can take place in aqueous buffer, and the intermediately formed *α-ω* functionalized alkenol, being less polar than the *ω*-hydroxy fatty acid, can immediately be extracted into the organic phase where metathesis to form the long-chain alkenediol is performed.

Firstly, the reaction was tested in a sequential two-pot mode, with the decarboxylation by OleT run in purely aqueous buffer, followed by extraction of the reaction products in ethyl acetate (EtOAc). After evaporation of the solvent, the intermediates were re-dissolved in isooctane and the metathesis performed in pure organic solvent. Three different *ω*-hydroxy fatty acids (**1a**, **2a** and **3a**) served as substrates and were successfully converted to the respective long-chain alkenediols (icos-10-ene-1,20-diol **1g**, Hexacos-13-en-1,26-diol (**2g**), Octacos-14-en-1,28-diol (**3g**)) within 3 hours of reaction, as judged by GC-MS measurements (Figures S 16 and S 17). This sequential two-pot reaction can be considered as the starting point, with both reactions being run under their respective ideal conditions. Nevertheless, the requirement of an intermediary extraction step makes this reaction economically unfavorable.

Secondly, the reaction was performed in a sequential one-pot mode, with the enzymatic decarboxylation taking place in purely aqueous buffer or already in presence of isooctane as the second phase. Afterwards, with the addition of the catalyst **C1** (and isooctane in the case of biocatalysis in pure buffer), the metathesis was performed. In both cases, an enzymatic conversion of ca. 90% **1a** was estimated from GC-based investigation of the aqueous phase. In the organic phase, only the intermediate **1b** and the desired metathesis product **1g** were found. These results indicate that OleT is active even in the presence of isooctane as organic solvent; an important prerequisite for the final one-pot reaction.

Finally, a simultaneous one-pot metathesis reaction was performed. In this case, the reaction was conducted in the bi-phasic buffer/isooctane system with both catalysts present from beginning on. To protect the ruthenium compound from potential light-induced degradation, the upper organic phase was shielded from light during the course of the reaction. Even in this case, the formation of the desired product **1g** could be observed by GC-MS measurements. However, conversions did not exceed 20%, which can be explained with the incompatibility of cell-free extracts with the metathesis catalyst. Due to the high cost of using purified enzyme, this option was not considered for the cascade reaction. The most practical approach is therefore the sequential cascade approach, with the *in situ* extraction of the intermediate into the organic phase, followed by an efficient metathesis reaction (Scheme 4).



Scheme 1. Sequential chemo-enzymatic cascade to produce long-chain alkendiol **1g** from bio-based fatty acid **1a**. In a first step, the light-driven decarboxylation takes place in presence of an organic solvent (isooctane) to aid the *in situ* extraction of the intermediate **1b** into the organic phase. After addition of the ruthenium catalyst **C1**, the metathesis takes place in presence of water.

Conclusion

The conversion of ω-hydroxy fatty acids by the fatty acid decarboxylase OleT gives rise to ω-alkenols, which can be further converted to ω-unsaturated fatty acids, alkenylamines and long-chain diols. We could show here the capacity of OleT to produce terminal alkenes and the possibility to combine this decarboxylase with various other enzymes and even with metal catalysts in a number of different reaction sequences. On the way to synthetic applications, however, several challenges remain to be solved. In all examples, the extraction of the intermediate product without purification turned out to be more successful than cascade approaches. This underlines the complexity of cascades,[40] and the requirement for a detailed optimization of these multi-catalytic one pot reactions. The formation of side-products by OleT and incomplete conversions in cascade reactions must be overcome in order to facilitate the downstream-processing. It should be also noted that current applications of OleT rely on the use of purified enzyme.[8,9] The total turnover numbers with cell-free extracts are considerably lower.[11] Applications in whole cells in bacteria and yeast do not exceed volumetric yield 100 mg L^{-1} or 10 mg L^{-1},[10] respectively. The low solubility of fatty acids in water is another serious problem. While the capacity of OleT to cleave non-activated C-C-bonds under very mild reactions is of high synthetic interest, the current state of OleT is far from concrete applications. Despite these current limitations, the unique reaction catalyzed by OleT is an important addition of the biocatalytic toolbox for the valorization of bio-based resources.

Experimental Section

General

Unless otherwise stated, all chemicals and compounds used during this work were purchased from Sigma Aldrich (Steinheim, Germany), TCI chemicals (Eschborn, Germany), VWR international (Langenfeld/Rheinland, Germany), Alfa Aesar (Karlsruhe, Germany), or Carl Roth (Karlsruhe, Germany) in the highest purity degree available. Ruthenium catalysts were purchased from Apeiron Catalysts (Wrocław, Poland).

Syntheses were carried out under standard air-exposed atmosphere unless otherwise stated. Isolation of the reaction products was conducted via column chromatography on silica gel type 60 (Carl Roth, Karlsruhe, Germany), or preparative silica gel GF TLC 20x20 cm plates (Analtech, Newark, USA). NMR spectra were recorded on a Bruker DPX200 NMR spectrometer. Chemical shifts δ (in ppm) are given relative to TMS and referenced to the undeuterated residues of deuterated chloroform as internal standard.

Preparation of OleT for biocatalysis

OleT expression and purification was performed depending on the used electron supply system.[8,12] Biocatalyses and analytics with the CamAB system were performed according to an earlier established protocol.[8] Before using the crude extract of OleT for biocatalyses, small molecules which might interfere with hydrogen peroxide formation or electron transfer were removed by ultracentrifugation through a centrifuge filter unit with a 10 kDa cut-off membrane (3 mL, 4,000 × g at 4 °C, twice). The remaining protein was resuspended in Britton-Robinson buffer (3 mL of 50 mM pH 7, 200 mM NaCl).

For biocatalyses with purified OleT, protein concentration was determined by Bradford assay. The assay was carried out in 96-well plates. 50 µL of diluted protein samples (1:10, 1:100, 1:200) and BSA standards (0, 20, 30, 40, 50, 60, 80 and 100 µg mL^{-1}) were mixed with 200 µL Bradford reagent (Coomassie Brilliant Blue G-250). After 15 min incubation time, the absorbance was measured at 595 nm using a FLUOstar Omega UV/Vis spectrophotometer (BMG Labtech). Samples for standard curves as well as protein samples were always prepared in triplicates and used for the calculation of protein concentrations.

Cloning, expression and purification of EcAdhZ3-LND, LCAO_Af and CvTA

A pET28a vector with NAD^+-dependent alcohol dehydrogenase (*Ec*AdhZ3-LND) from *Escherichia coli*[24] was kindly provided by the group of Volker Sieber (Wissenschaftszentrum Straubing, Germany). Transaminase from *Chromobacterium violaceum* (*Cv*TA*)* was kindly supplied by Karim Cassimjee (KTH Stockholm, Sweden). The alcohol oxidase from *Aspergillus fumingatus* (LCAO_*Af*) (XP_753079.1) was cloned into the pET28a vector with an N-terminal His-tag.[25] For that, the codon-optimized LCAO_*Af* was synthetized at GeneArt life technology (Regensburg, Germany) with corresponding restriction sites *Nde*I (N-terminus) and *Xho*I (C-terminus). After cloning into the pET28a vector, *E. coli* XL blue was transformed for the amplification of a constructed plasmid.

Chemically competent *E. coli* BL21 (DE3) was used as expression strain for *Ec*AdhZ3-LND, LCAO_*Af* and *Cv*TA. For efficient protein expression enzymes were overexpressed in Terrific Broth (TB)[41] medium (250 mL) with kanamycin (100 µg mL^{-1}) by addition of Isopropyl *β*-D-1-thiogalactopyranoside (IPTG 0.5 mM) and incubated overnight at 20 °C. The cells were harvested by centrifugation (12,000 × g, 20 min, 4 °C) and washed with phosphate-puffer (20 mM pH 7, 200 mM NaCl). After sonication (3 min, 50% pulse cycle, 60% amplitude, 15 sec sonication / 15 sec pause) on ice, the cell debris was removed by centrifugation (Beckman Coulter, Avanti J-26S XP – 10,000 × g, 20 min, 4°C). To improve the amount of soluble LCAO_*Af*, different media such TB, Lysogeny Broth medium (LB),[42] TYGPN (medium broth from AMRESCO) and autoinduction medium (media components: 12 g L^{-1} Tryptone, 24 g L^{-1} yeast extract in 795 mL; buffer component: 1M KPi buffer pH 7 (90 mL); induction components: 50 g L^{-1} glucose (10 mL), 20 g L^{-1} lactose (100 mL), 5 mL glycerin) in 50 mL volume were tested, where the best result with autoinduction medium were obtained (Figure S 1).

Light-driven biotransformations

Assays for the light-driven biocatalysis were carried out in clear glass tubes placed in a water bath with LED as a light source.[12] The influence of different reaction conditions on the decarboxylation as well as hydroxylation was analyzed in 3 mL reaction volume, mixed with a magnetic stirrer at 100 rpm and incubated at 25 °C. The standard biocatalytic reaction with OleT was carried out with different substrate concentrations (0.5 to 2 mM), ethylenediaminetetraacetate disodium salt (EDTA, 50 mM), flavinmononucleotide (FMN, 10 µM), dimethylsulfoxide (DMSO, 5-10%) and $OleT_{CE}$ (400 µL) or purified OleT (0.3 or 1.0 mg mL^{-1}) in Britton-Robinson buffer (50 mM pH 7, 200 mM NaCl). The reaction was started by addition of FMN and crude extract (60 mg of cell dry

weight, cdw) or purified enzyme. Biocatalysis reactions were run for 4 h or overnight. For analysis, samples (400 µL) were withdrawn at the desired times and the reaction was stopped with hydrochloric acid (40 µL, 37% w/v). Myristic acid (0.3 mM) was added as internal standard and the sample further processed for GC-FID or GC-MS analysis (cf. below).

To investigate the influence of the co-solvent on OleT activity and stability, *N*,*N*-dimethylformamide (DMF), tetrahydrofuran (THF) and 1,4-dioxane were additionally used as alternative additives to DMSO. The investigation of optimum pH, Britton-Robinson buffer (H_3BO_3, H_3PO_4, CH_3COOH, each 50 mM) with high buffering capacity in the pH range of 2 to 12 was selected. Other buffers (50 mM of Bicine/ Bis-Tris/ Borate/ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/ 3-(*N*-morpholino) propanesulfonic acid (MOPS)/ Phosphate/ Tris-HCl, or Imidazole) were used at a pH of 7. All reactions were performed as duplicates.

Alkenylamine synthesis

For the synthesis of a terminal alkenylamine from ω-hydroxy fatty acids in a sequential one-pot enzyme cascade, OleT, *Ec*AdhZ3-LND or LCAO_*Af* and a *Cv*TA were used. All enzymes were used as crude extract. First, light-driven decarboxylation by $OleT_{CE}$ was performed for 4 or 20 hours. Subsequently, the illumination was interrupted and the other enzymes, NAD^+ and L-alanine as amino donor were added. The biocatalysis reactions were carried out with substrate (1 mM), EDTA (50 mM), FMN (10 µM), DMSO (10%), L-alanine (10-50 mM), FAD (0.25 mM), PLP (1 mM), enzymes $OleT_{CE}$ and *Cv*TA (400 µL) respectively, as well as LCAO_*Af* (200 µL) in Britton-Robinson buffer (a long range buffer consisting in a mixture of phosphoric, boric and acetic acids) (50 mM pH 7, 200 mM NaCl) with a total volume of 3 mL. The reactions were incubated at 25 °C for 20 h with 100 rpm. For analysis, samples were processed as described above. All reactions were performed as triplicates.

Production of Icos-10-en-1,20-diol

The olefin metathesis was performed using standard Schlenk technique under nitrogen atmosphere. Commercially available **1b** (1.2 mmol, 200 mg) and catalyst **C1** (Dichloro[1,3-bis(2,6-isopropylphenyl)-2-imidazolidinylidene]-2-[[1-(methoxy(methyl)amino)-1-oxopropan-2yl]o'xy] benzylideneruthenium (VI)) (GreenCat) (4 mol-%) were mixed in anhydrous dichloromethane (DCM) for 18 h under nitrogen atmosphere at reflux conditions (40 °C). After cooling down to room temperature, vinyl ether (30 mg) was added in order facilitate the separation of the product from the catalyst. For purification, preparative silica gel thin layer chromatography was performed in five cycles with light petroleum:EtOAc (9:1(v/v) as mobile phase. Isolated **1g** (99 mg, 51% yield) was obtained as brown wax.

1b: ^{1}H NMR (200 MHz, chloroform-d) δ 5.98 – 5.64 (m, 1H), 5.09 – 4.77 (m, 2H), 3.74 – 3.49 (m, 2H), 2.24 – 1.88 (m, 2H), 1.71 – 1.47 (m, 2H), 1.47 – 1.08 (m, 12H); ^{13}C NMR (200 MHz, chloroform-d) δ 25.74, 28.93, 29.12, 29.42, 29.55, 32.81, 33.81, 63.08, 114.11, 139.22.

2b: ^{1}H NMR (200 MHz, chloroform-d) δ 5.87-5.61 (m, 1H), 5.00-4.78 (m, 2H), 3.65-3.47 (t, J = 6.0, 2H), 2.06-1.87 (m, 2H), 1.36-1.06 (m).

3b: ^{1}H NMR (200 MHz, chloroform-d) δ5.81-5.62 (m, 1H), 5.00-4.77 (m, 2H), 3.69-3.44 (t, J = 6.0, 2H), 2.05-1.87 (m, 2H), 1.34-1.13 (m).

1g: ^{1}H NMR (200 MHz, chloroform-d) δ 5.54 – 5.31 (m, 2H), 3.63 (t, J = 6.5 Hz, 4H), 2.07 – 1.88 (m, 4H), 1.77 – 1.49 (m, 4H), 1.44 – 1.19 (m, 28H); ^{13}C NMR (200 MHz, chloroform-d) δ 25.75, 27.19, 29.05, 29.10, 29.30, 29.44, 29.59, 29.73, 32.56, 32.77, 62.97, 129.88.

General procedure: Solvent and catalyst screening for metathesis conditions

Olefin metathesis reaction of **1b** was performed using 1.5 mL closed glass vials under air-exposed conditions in 1 mL solvent. For the reaction, 20 µmol substrate was used in combination with 5 mol-% catalyst **C1**, **C2** (Dichloro[1,3-bis (2,4,6-trimethylphenyl)-4- [(4-ethyl-4-methylpiperazinyl) methyl]- 2-imidazolidinylidene]- (2-isopropoxybenzylidene) ruthenium(VI) chloride), AquaMet) and **C3** (Dichloro [1,3-bis(2,4,6-trimethylphenyl) -4-[(trimethylammonio)methyl] -2-imidazolidinylidene]- (2-isopropoxybenzylidene)-ruthenium(VI)chloride, StickyCat). The synthesis was conducted in tetrahydrofuran (THF), 2-methyl-tetrahydrofuran (2-MeTHF), and 2,2,4-trimethylpentane (isooctane) at 50 °C. After the reaction, the mixtures were cooled down to room temperature. 100 µL samples were taken and centrifuged (2 min, 17 000 × g). Subsequently, the supernatant was analyzed by GC-FID and GC-MS measurements.

Analytics

For the analysis of the reactions by GC-MS or GC-FID samples were derivatized by N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) in order to lower their boiling points and make them easier to separate. For this, samples from the bioconversion (400 µL) were extracted with ethyl acetate (700 µL). After centrifugation (12,000× g for 1 min) the organic phase was removed. The extraction was repeated once and the combined supernatants evaporated. A derivatization was then carried out with MSTFA (25 µL) for 20 minutes at 65 °C. Subsequently, ethyl acetate (150 µL) was added and the samples were transferred into GC vials.

Substrate and product identification and quantification was performed by GC-MS using a DSQII TraceGC Ultradevice (Thermo Fisher Scientific) using a Zebron ZB-1MS (30 m × 0.25 mm × 0.25 µm, Phenomenex) column. 7 µL sample volumes were injected at a split ratio of 1:10 and a temperature of 280 °C using helium as carrier gas with a flow rate of 1.2 mL min^{-1}. The analytes were separated according to the temperature programs shown in Figure S 22. The ion source of the mass spectrometer was heated to 280 °C and ions were detected in the positive mode in the range of m/z = 50-650. Signals were recorded from 4 minutes after injection to avoid detector overloading. Ions were detected with a detector gain of 3×10^5 (multiplier voltage 1390 V) and 8,000 scans sec^{-1}.

Alternatively, samples were analyzed by GC-FID using a Shimadzu GC-2010 plus (Shimadzu, Duisburg) using a CP-Sil 5 CB (30 m × 0.25 mm × 0.25 µm, Agilent) column for separation. In contrast to GC-MS measurements, this method has a much broader linear measuring range and generally smaller errors in the quantification. 7 µL sample volumes were injected at a split ratio of 1:10 and a temperature of 300 °C. A flow rate of 40 mL min^{-1} was used for the carrier gas (synthetic air). The analytes were separated using the temperature program shown in Figure S 23. The detector was heated to 350 °C.

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Keywords: Biocatalysis; Renewable resources; Decarboxylase; Olefin Metathesis; Cascade reaction.

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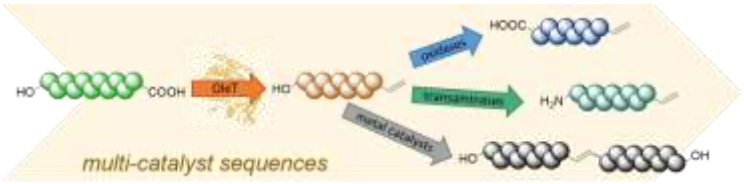

OleT from *Jeotgalicoccus* sp. ATCC 8456 holds great industrial potential for using natural fats as renewable feedstock. Different cascades with OleT to produce high value products by enzymatic and chemo-enzymatic reaction sequences are presented.

*Samiro Bojarra, Dennis Reicher, Marius Grote, Álvaro Gomez Baraibar, Alexander Dennig, Bernd Nidetzky, Carolin Mügge, and Robert Kourist[]**

Page No. – Page No.

Bio-based *α,ω*-functionalized hydrocarbons from multi-step reaction sequences with bio- and metallo-catalysts based on the fatty acid decarboxylase $OleT_{JE}$.

[1] R. Kourist, S. Schmidt, K. Castiglione, *Chemistry* **2017**, DOI: 10.1002/chem.201703353.