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Peroxygenase-Promoted Enzymatic Cascades for the Valorisation of Fatty Acids

Yinqi Wu,[a] Caroline E. Paul,[a] Thomas Hilberath,[a] Ewald P. J. Jongkind,[a] Wuyuan Zhang,[b] Miguel Alcalde,[c] and Frank Hollmann*[a]

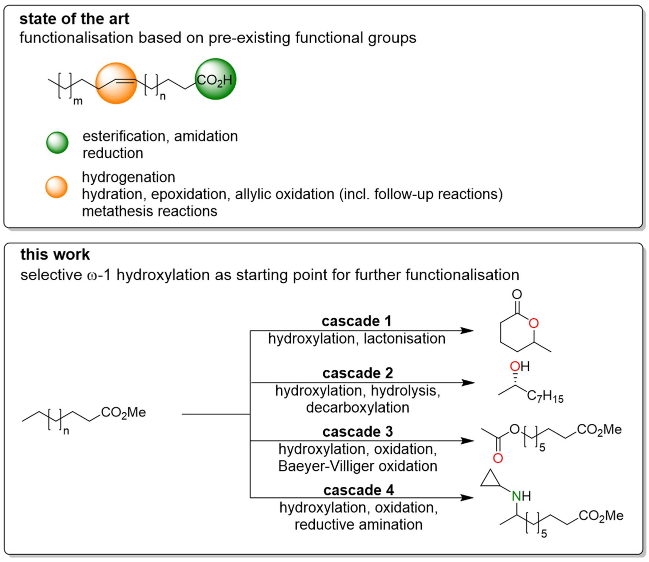
Utilisation of fatty acids generally relies on pre-existing functional groups such as the carboxylate group or C=C-double bonds. Addition of new functionalities into the hydrocarbon part opens up new possibilities for fatty acid valorisation. In this contribution we demonstrate the synthetic potential of a peroxygenase mutant *Aae*UPO–Fett for selective fatty acid oxyfunctionalisation. The ω-1 hydroxy fatty acid (esters) produced are further transformed into lactones, alcohols, esters and amines via multi-enzyme cascades thereby paving the way for new fatty acid valorisation pathways.

Introduction

En route to a circular economy, renewable feedstock are increasingly recognised as starting materials for the synthesis of intermediates and consumer products.[1] Fatty acids as well as their glycerides are already now used for the synthesis of a range of products such as polymer building blocks, paints, coatings, surfactants and lubricants.[2]

Biocatalytic transformations of the carboxylate group are very well established enabling (trans)esterification, amidation and reduction reactions (Scheme 1).[3] The conversion of unsaturated, i.e. functionalised fatty acids is also straightforward comprising epoxidation[2b] and hydration[4] of the C=C-double bond or allylic hydroperoxidation.[5]

Less well-established are biocatalytic transformations of saturated, non-activated fatty acids. Obviously, this can be attributed to the challenging character of C–H activation chemistry.[2c] Nevertheless, adding functional groups to the alkane part of fatty acids would broaden their potential as starting material for a bio-based chemistry. Recently, P450 enzymes exhibiting α-,[6] β-,[7] δ-[8] or ω-selectivity[9] have been reported.[10] Also, fungal peroxygenases[11] are capable of hydroxylating non-activated C–H-bonds in fatty acids. While peroxygenase-catalysed hydroxylations benefit from the intrinsically much simpler reaction mechanism compared to P450 monooxygenases, their practical usefulness is hampered by their generally low selectivity towards fatty acids.[12]


state of the art
functionalisation based on pre-existing functional groups
m
n
CO2H
esterification, amidation
reduction
hydrogenation
hydration, epoxidation, allylic oxidation (incl. follow-up reactions)
metathesis reactions
this work
selective ω-1 hydroxylation as starting point for further functionalisation
n
CO2Me
cascade 1
hydroxylation, lactonisation
cascade 2
hydroxylation, hydrolysis, decarboxylation
OH
C7H15
cascade 3
hydroxylation, oxidation, Baeyer-Villiger oxidation
CO2Me
5
cascade 4
hydroxylation, oxidation, reductive amination
NH
5
CO2Me

Scheme 1. Established fatty acid valorisation approaches utilise existing functionalities such as the carboxylate group or pre-existing C=C-double bonds. In this contribution we add functionality via selective, peroxygenase-catalysed hydroxylation enabling further transformations.

Very recently, we reported a protein engineering study on the prototype evolved peroxygenase from *Agrocybe aegerita* (*Aae*UPO–PaDa–I)[13] to increase the regioselectivity of fatty acid hydroxylation.[14] Due to a dramatic narrowed substrate access channel, the new mutant (referred to as *Aae*UPO–Fett) enabled higher regioselectivity (almost exclusively located at ω-1) as compared with its parent *Aae*UPO–PaDa–I mutant. Therefore, we reasoned that *Aae*UPO–Fett may be a useful biocatalyst for the valorisation of fatty acid (methyl esters) (FA(ME)s) into

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value-added chemical building blocks, such as esters, lactones, alcohols and amines.

Results and Discussion

In a first set of experiments, we systematically investigated the substrate scope of *Aae*UPO–Fett in comparison with its parent *Aae*UPO–PaDa–I (Figure 1). Both *Aae*UPO variants exhibited a more relaxed chain length spectrum when converting FAMEs as compared to free FAs. While in the last case, the C14 fatty acid (myristic acid) was the best converted substrate, both variants oxidised a much broader range of FAMEs ranging from C5 to C14.

As expected, *Aae*UPO–Fett-catalysed FA- or FAME–hydroxylations occurred with much higher regioselectivity when compared with the same reactions mediated by *Aae*UPO–PaDa–I. With the latter, the regioselectivity for ω-1 was generally lower than 60 %, while for *Aae*UPO–Fett the ω-1 product was formed at least at 92 % selectivity.

Having a highly regioselective hydroxylation catalyst at hand, we further investigated the synthetic possibilities with *Aae*UPO–Fett. Selective ω-1 hydroxylation of medium-chain FAMEs such as methyl hexanoate (**1 a**) should yield a chiral, lactonisable product potentially useful as polymer building block or flavour and fragrance ingredient. The hydroxylation of **1 a** itself went smoothly to approx. 90 % conversion within the first 6 h and neither the undesired ω-2 hydroxylation product nor any overoxidation product (methyl 5-ketohexanoate) were observed (Figure 2). To our surprise, the envisaged lactone was formed only in traces during the reaction. It could, however, be obtained quantitatively by subsequent acid treatment of the primary hydroxyl ester in 77 % *ee* (Figure S10).

Next, we investigated the *Aae*UPO–Fett-catalysed hydroxylation of longer chain FAMEs such as methyl decanoate (**2 a**). Particularly, we were interested in the effect of different H_2O_2 addition rates on the robustness of the hydroxylation reaction (Figure 3). Under monophasic reaction conditions (i. e. the starting material being completely dissolved in the aqueous reaction medium) the robustness decreased with increasing feeding H_2O_2 rates with product accumulation ceasing after 2 to 24 h, depending on the H_2O_2 feed rate (Figure 3a). Furthermore, a pronounced overoxidation of the primary product to the corresponding ketone (**2 c**) was observed accounting to up to 23 % of the overall product. We reasoned that a two liquid phase system (2LPS)[15] with **2 a** as water insoluble organic phase serving as substrate reservoir and product sink (particularly for the desired **2 b**) may circumvent the overoxidation issue and repeated the experiments in the presence of 20 % (v/v) of **2 a** (forming a second phase). Indeed, under 2LPS conditions the overoxidation was suppressed to less than 5 % of the overall product formed (Figure 3b). Surprisingly, the reaction robustness was also increased significantly in the presence of **2 a** as an organic phase. For example, dosing H_2O_2 at 2 $mM_{final} \times h^{-1}$ the product formation time increased from 6 h (under monophasic conditions) to 72 h in the 2LPS approach. As a result, the product concentration increased from 4.3 mM to more than

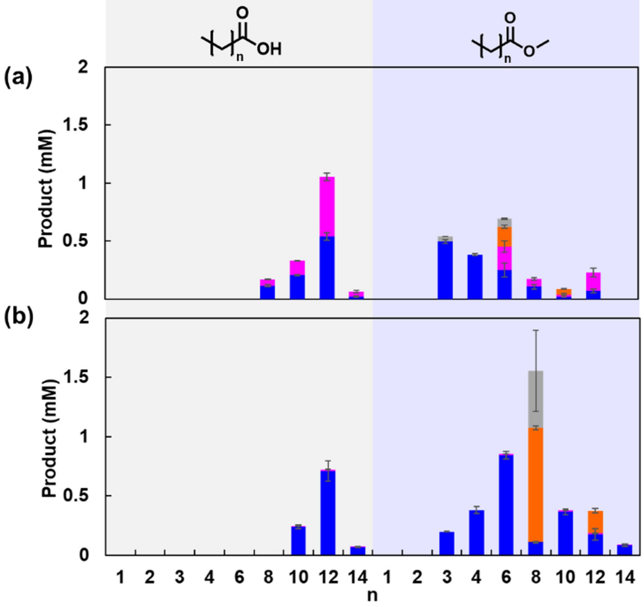

Figure 1. Comparison of fatty acid (ester) hydroxylations catalysed by *Aae*UPO–PaDa–I (a) and *Aae*UPO–Fett (b) by using different substrates. ω-1 hydroxylation products (■); ω-2 hydroxylation products (■); ω-1 ketone products (■); unknown products to be determined (■). Reaction conditions: [*Aae*UPO] = 1 μM (for both PaDa–I and Fett), [substrate] = 5 mM, [MeCN] = 10 % (v/v), $[H_2O_2] = 2\ mM \times h^{-1}$, 50 mM KPi (pH 7.0), 600 rpm, 25 °C, reaction time = 4 h. Values represent the average of duplicates (n = 2). Error bars indicate the standard deviation.

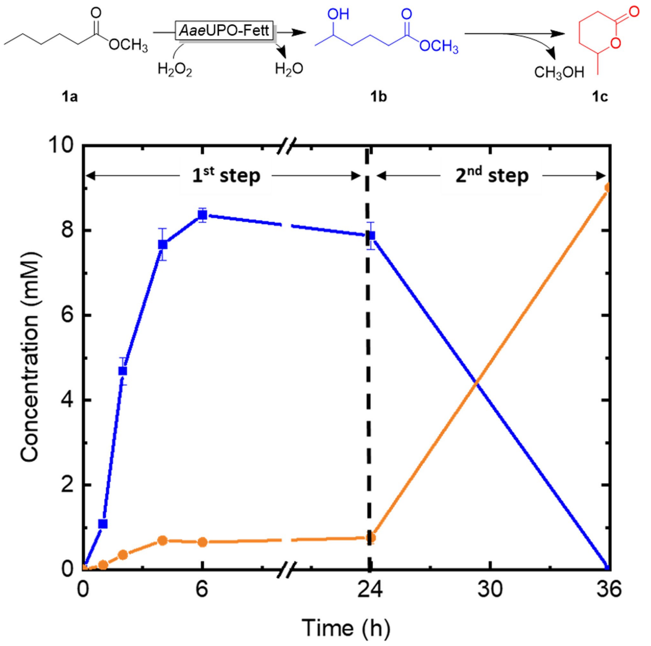

Figure 2. Hydroxylation reaction of methyl hexanoate catalysed by *Aae*UPO–Fett for the production of methyl 5-hydroxyhexanoate (■) and δ-hexalactone (●). Reaction conditions: First step: [*Aae*UPO–Fett] = 2.5 μM, [methyl hexanoate] = 10 mM, [MeCN] = 10 % (v/v), $[H_2O_2] = 5\ mM \times h^{-1}$, 50 mM KPi (pH 7.0), 600 rpm, 25 °C, 10 mL scale; second step: CH_2Cl_2 ($5\ mL \times g^{-1}$ lactone), trifluoroacetic acid ($0.04\ mL \times g^{-1}$ lactone), 300 rpm, 25 °C. Values represent the average of duplicates (n = 2). Error bars indicate the standard deviation.

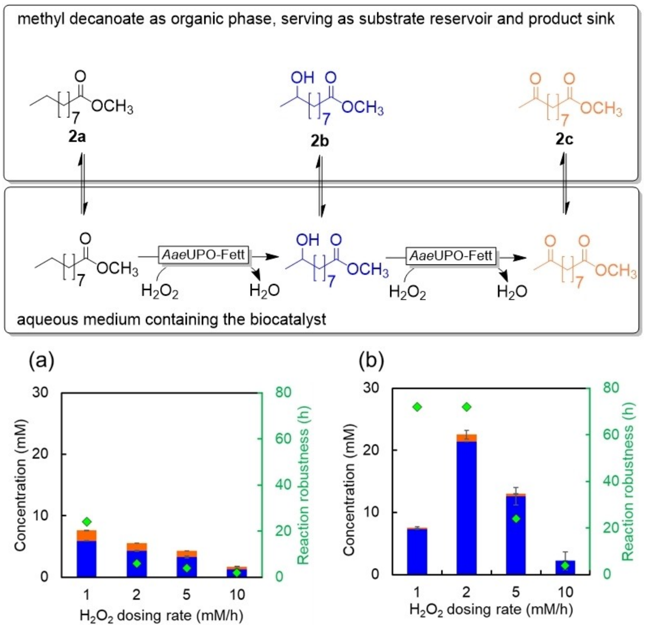

Figure 3. Hydroxylation reaction of methyl decanoate catalysed by *Aae*UPO–Fett (a) in a monophasic reaction system and (b) using a 2LPS. Methyl 9-hydroxydecanoate (■); methyl 9-oxo-decanoate (■); reaction robustness (◆). Reaction conditions: (a) [*Aae*UPO–Fett] = 1 μM, [substrate] = 10 mM, [MeCN] = 10% (v/v), 50 mM KPi (pH 7.0), H_2O_2 dosing rate = 1–10 mM × h^{-1}, 600 rpm, 25 °C, 5 mL scale; (b) [*Aae*UPO–Fett] = 1 μM, substrate/buffer = 1:4 (v/v), 50 mM KPi (pH 7.0), H_2O_2 dosing rate = 1–10 mM × h^{-1}, 1000 rpm, 25 °C, 1 mL scale.

21 mM. 29 mg of **2b** were isolated from 10 mL reaction with 79% isolation yield in more than 94% purity (Figure S3). *Aae*UPO–Fett performed respectable 22540 catalytic turnovers.

While hydroxy–FAMEs may be an interesting starting material for polyester synthesis we sought after further possible applications applying *Aae*UPO–Fett in different enzyme cascades.

Decarboxylation of the free hydroxy acids results in the corresponding (C1-shortened) 2-alkanols. We tested a cascade with methyl decanoate (**2a**) combined with a lipase-catalysed hydrolysis and photoenzymatic decarboxylation (Figure 4). Particularly, the well-known lipase B from *Candida antarctica* (CalB)[16] as well as the fatty acid photodecarboxylase from *Chlorella variabilis* (*Cv*FAP) were used.[17] To avoid undesired hydrolysis and photodecarboxylation of the starting material, the hydroxylation step was performed prior the combined hydrolysis/photodecarboxylation. Pleasingly, the desired (*S*)-2-nonanol (**3**, 80% *ee*) as sole product was achieved.

Converting the peroxygenase-introduced hydroxyl group itself also offers further possibilities for manipulation if further oxidised to the keto group. For example, reductive amination or Baeyer-Villiger oxidations come into reach in case a keto group is present in the substrate. For this approach, we opted for an alcohol dehydrogenase-catalysed alternative (*vide infra*). We evaluated the alcohol dehydrogenase from *Sphingobium yanoikuyae* (*Sy*ADH) for the oxidation of **2b** into the corresponding ketone (**2c**). *Sy*ADH was chosen because of its known low enantioselectivity and broad substrate scope.[18] Indeed, already a preliminary test with *Sy*ADH gave near-full conversion of **2c** (Figure S16).

Next we tested a cascade comprising *Aae*UPO–Fett, *Sy*ADH and the Baeyer-Villiger monooxygenase from *Aspergillus flavus* (BVMOAfl838)[19] to convert FAMEs into the corresponding ω-hydroxy FAME acetate ester (**2d**) (Figure 5). The GCMS spectrum of the ester product showed a signal at m/z = 43 characteristic for acetyl esters (Figure S15) indicating a preference for the "normal" migration product.[19] As the ADH- and BVMO-catalysed oxidation steps were cofactor complementary, no additional cofactor regeneration system was necessary.

We then conducted the cascade as a two-pot-two-step reaction (Figure 5a) first performing the *Aae*UPO–Fett-catalysed hydroxylation for 72 h resulting in approx. 20 mM of the expected product **2b**. Afterwards, the organic phase was transferred to another aqueous reaction mixture comprising *Sy*ADH and BVMOAfl838. The conversion of **2b** into the final product (**2d**) was near complete after 5–6 h (> 90% of **2d** based on the initially applied **2b**). Nevertheless, the conversion was not complete, which may be due to the unproductive decoupling reaction (i.e. direct, aerobic oxidation of NADPH)[20] resulting in a $NADP^+$ regeneration system and causing the accumulation of 1.1 mM of the intermediate ketone. Interestingly, this uncoupling appeared to be less problematic when performing the reaction in a one-pot-one-step fashion (Figure 5b) as the intermediate products did not accumulate significantly.

Finally, we pursued reductive amination of the intermediate ketone (**2c**) by replacing BVMOAfl838 with the reductive aminase from *Aspergillus oryzae* (*Asp*RedAm), which accepts cyclopropylamine as amine donor.[21] Thus, we obtained a

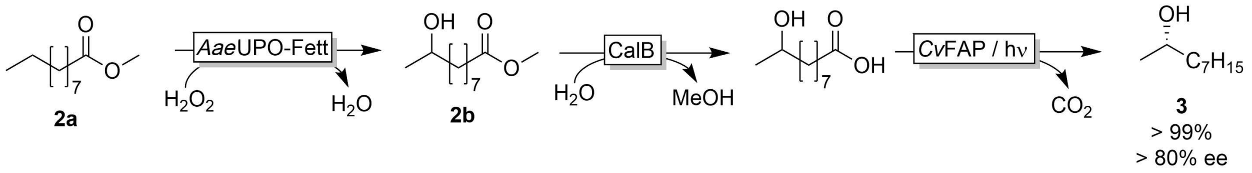

Figure 4. Multi-enzymatic cascade to convert methyl decanoate into (*S*)-2-nonanol. Reaction conditions: for the first step, [*Aae*UPO–Fett] = 1 μM, substrate/buffer = 1:4 (v/v), 50 mM KPi (pH 7.0), H_2O_2 dosing rate = 2 mM × h^{-1}, 600 rpm, 25 °C, 10 mL scale, 72 h. For the second and third steps, [methyl 9-hydroxydecanoate] = 5 mM, [CalB] = 25 U × mL^{-1}, [MeOH] = 10% (v/v), 100 mM Tris–HCl (pH 9.0), 25 °C, 1 mL scale, overnight; then added [*Cv*FAP] = 6 μM, and switched on blue light (λ_{max} = 450 nm), light intensity of blue light = 14.5 μE × L^{-1} × s^{-1}.

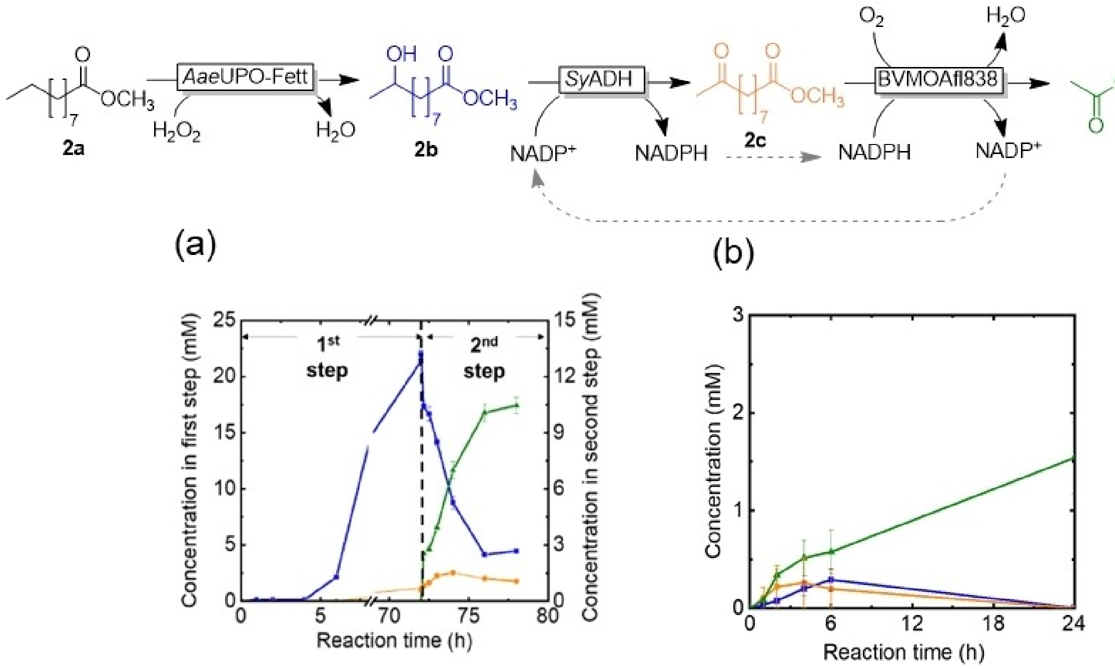

Figure 5. Proposed enzymatic cascade reaction to convert methyl decanoate into methyl 8-(acetyloxy)octanoate (▲), with the intermediate methyl 9-hydroxydecanoate (■) and methyl 9-oxo-decanoate (●) catalysed by the combination of *Aae*UPO–Fett, *Sy*ADH and BVMOAfl838. Representative time courses of the (a) two-pot-two-step and (b) one-pot-one-step cascade reaction. Reaction conditions: (a) For the first step (0 h–72 h), [*Aae*UPO–Fett] = 1 μM, methyl decanoate/buffer = 1 : 4 (v/v), 50 mM KPi buffer (pH 7.0), [H_2O_2] = 2 mM × h^{-1}, 1000 rpm, 25 °C, 1 mL scale. For the second step (72 h–78 h): diluted organic phase from first step/buffer = 1 : 4 (v/v), 100 mM Tris–HCl (pH 8.0), [*Sy*ADH cells] = 1 mg × mL^{-1}, [BVMOAfl838] = 0.5 μM, [$NADP^+$] = 1 mM, 1000 rpm, 25 °C, 1 mL scale. (b) [*Aae*UPO–Fett] = 1 μM, [*Sy*ADH cells] = 5 mg × mL^{-1}, [BVMOAfl838] = 0.5 μM, [$NADP^+$] = 1 mM, methyl decanoate/buffer = 1 : 4 (v/v), 100 mM Tris buffer (pH 8.0), [H_2O_2] = 2 mM × h^{-1}, 1000 rpm, 25 °C, 1 mL scale. Values represent the average of duplicates (n = 2). Error bars indicate the standard deviation.

moderate amount of the desired methyl 9-cyclopropylaminodecanoate (**2 e**) (Figure 6).

Interestingly, while the starting material for the second reaction (methyl 9-hydroxydecanoate) was continuously consumed, the final product did not accumulate to more than approx. 1 mM. Comparative experiments, however, demonstrated that the final product itself was not stable under the reaction conditions (Figure S20). Currently, we are lacking a fully plausible explanation of this observation. Possibly, poly condensation of the amino ester product to non-extractable oligomers may account for this.

Conclusion

In conclusion, with this contribution we have extended the synthetic potential of non-functionalised fatty acids as starting materials for value-added products and building blocks. Peroxygenases, especially if engineered for regioselectivity such as *Aae*UPO–Fett, are promising catalysts to add functionality to the alkyl chain. The primary products (i.e. hydroxyl fatty acids (esters)) are interesting building blocks for lactone synthesis and for polyesters. If oxidised to the corresponding keto acids (esters), further synthetic possibilities arise as demonstrated by the Baeyer-Villiger oxidation and the reductive amination reactions demonstrated here.

Experimental Section

Enzymes: The enzymes used in this study have been obtained from either commercial sources (BVMOAfl838 from Gecco Biotech, Groningen, The Netherlands and CalB from Sigma-Aldrich, Zwijndrecht, The Netherlands) or have been produced following literature procedures (*Aae*UPO–Fett,[14] *Sy*ADH,[18] *Cv*FAP[17] and *Asp*RedAm[19]).

General procedure: A typical hydroxylation reaction mixture contained 50 mM potassium phosphate (KPi) buffer pH 7 at 25 °C supplemented with *Aae*UPO–Fett (1 μM) and 10 mM of the starting material. H_2O_2 was added continuously via a syringe pump while gently shaking the reaction mixture. For analysis, aliquots were taken, extracted with ethyl acetate (containing 5 mM of an internal standard) and subjected to GC-analysis. A detailed description of the experimental and analytical procedures can be found in the Supporting Information. Furthermore all Figure captions contain the reaction conditions.

Supporting Information

Additional references cited within the Supporting Information.[13b,21–22]

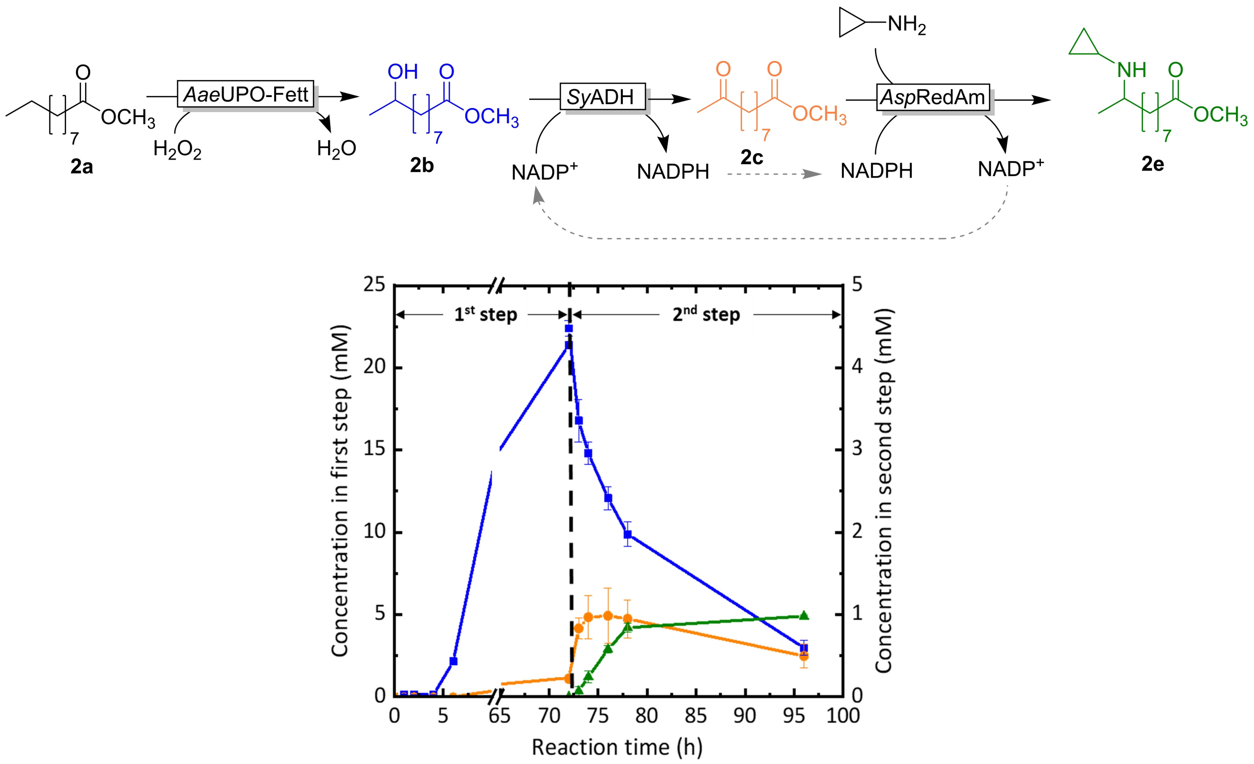

Figure 6. Proposed enzymatic cascade reaction to convert methyl decanoate into methyl 9-cyclopropylaminodecanoate (▲), with the intermediate methyl 9-hydroxydecanoate (■) and methyl 9-oxo-decanoate (●) catalysed by *Aae*UPO–Fett, *Sy*ADH and *Asp*RedAm. Reaction conditions: for the first step (0–72 h), [*Aae*UPO–Fett] = 1 μM, methyl decanoate/buffer = 1 : 4 (v/v), 50 mM KPi buffer (pH 7.0), [H_2O_2] = 2 mM × h^{-1}, 1000 rpm, 25 °C, 1 mL scale. For the second step (72 h–96 h), [methyl 9-hydroxydecanoate] = 5 mM, [*Sy*ADH cells] = 0.5 mg × mL^{-1}, [*Asp*RedAm$_{\text{purified enzyme}}$] = 0.8 mg × mL^{-1}, [DMSO] = 2 % (v/v), [$NADP^+$] = 1 mM, [cyclopropylamine] = 40 mM, 100 mM Tris–HCl (pH 9.0), 300 rpm, 25 °C, 1 mL scale. Values represent the average of duplicates (n = 2). Error bars indicate the standard deviation.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: fatty acid valorisation · peroxygenase · selective oxyfunctionalisation · multi-enzyme cascades

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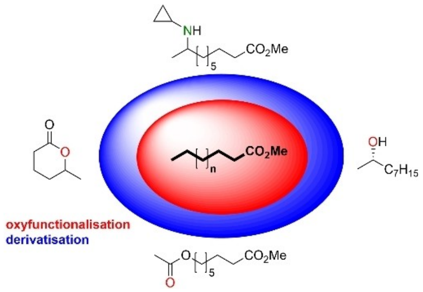
Multi-enzyme cascade: Peroxygenases enable facile functionalisation of fatty acids. The resulting hydroxy fatty acids can be further derivatised to new building blocks for chemical synthesis.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*Y. Wu, Dr. C. E. Paul, Dr. T. Hilberath, E. P. J. Jongkind, Prof. Dr. W. Zhang, Prof. Dr. M. Alcalde, Prof. Dr. F. Hollmann**

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Peroxygenase-Promoted Enzymatic Cascades for the Valorisation of Fatty Acids