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Direct Terminal Alkylamino-Functionalization *via* Multistep Biocatalysis in One Recombinant Whole-Cell Catalyst

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Abstract: Direct and regiospecific amino-functionalization of non-activated carbon could be achieved using one recombinant microbial catalyst. The presented proof of concept shows that heterologous pathway engineering allowed the construction of a whole-cell biocatalyst catalyzing the terminal amino-functionalization of fatty acid methyl esters (e.g., dodecanoic acid methyl ester) and alkanes (e.g., octane). By coupling oxygenase and transaminase catalysis *in vivo*, both substrates are converted with absolute regiospecificity to the terminal amine *via* two sequential oxidation reactions followed by an amination step. Such demanding chemical three-step reactions achieved with a single catalyst demonstrate the tremendous potential of whole-cell biocatalysts for the production of industrially relevant building blocks.

Keywords: amination; C–H activation; multicomponent reactions; terminal functionalization; whole-cell biocatalysis

Direct and selective functionalization of saturated hydrocarbons remains a major challenge in organic chemistry.[1,2] Direct activation of sp^3 C–H bonds can be achieved with solid metal catalysts and non-heme iron complexes.[3,4] However, the application of such catalysts usually suffers from low efficiency and poor selectivity.[5] Despite the general inertness of unactivated C–H bonds in alkanes, the reactivity even varies among primary, secondary, and tertiary sites. The primary site is least reactive making specific terminal functionalization most demanding.[2,4,6] Transition metal-boryl complexes catalyze the functionalization of alkanes specifically at the terminal position.[7] In an additional reaction step, the resulting borane products can be converted to products with different terminal functionalities, e.g., alcohols or amines.[8] As an alternative, oxygenases can be applied for the direct and regioselective oxyfunctionalization of sp^3 coordinated C–H bonds.[9] Besides alcohol formation, further oxidation is described for various oxygenases resulting in the accumulation of aldehydes and/or acids.[10,11,12] Terminal amines can be obtained from aldehydes for example, *via* ω-transaminase (ω-TA) catalysis. Omega-transaminases catalyze the reversible amino group transfer from a donor to an acceptor, whereby one of the two compounds can be a non-activated aldehyde, ketone, or amine. Transaminases are also versatile enzymes for the synthesis of chiral amines.[13] Here, combining oxygenase and transaminase catalysis, we report the development of a biocatalyst catalyzing directly the specific amino-functionalization of an unactivated, terminal C–H bond. The renewable educt dodecanoic acid methyl ester (**1**) was chosen as model substrate giving 12-aminododecanoic acid methyl ester (**4**) as product (Figure 1). As a bifunctional molecule, **4** constitutes a building block for polymer production.[14] The synthesis of **4** was realized *via* a consecutive three-step reaction in one recombi-

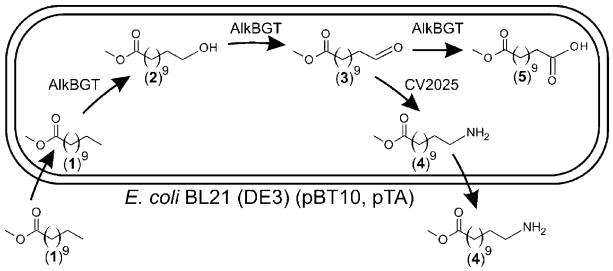

Figure 1. Terminal amino-functionalization of dodecanoic acid methyl ester (**1**) *via* 12-hydroxydodecanoic acid methyl ester (**2**) and 12-oxododecanoic acid methyl ester (**3**) to 12-aminododecanoic acid methyl ester (**4**) with *E. coli* BL21 (DE3) (pBT10, pTA) containing alkane monooxygenase AlkBGT and ω-transaminase CV2025. See the Supporting Information for the detailed reaction scheme.

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nant microbe expressing the genes for alkane monooxygenase AlkBGT from *Pseudomonas putida* GPo1 and ω-TA CV2025 from *Chromobacterium violaceum*. AlkBGT catalyzes the NADH-dependent terminal oxyfunctionalization of medium-chain length alkanes and fatty acids[15,16] and was recently shown to form terminal alcohols and the corresponding aldehydes from fatty acid methyl esters.[17] The ω-TA CV2025 accepts a wide range of amino donors and acceptors[18] with pyridoxal 5′-phosphate (PLP) as the typical coenzyme of TAs enabling reversible amino group transfer.[19]

In a single whole-cell catalyst based on *Escherichia coli* BL21 (DE3), the oxygenase and transaminase reactions are coupled *via* heterologous pathway engineering. Thereby, **1** is converted by AlkBGT in a two-step reaction *via* 12-hydroxydodecanoic acid methyl ester (**2**) to 12-oxododecanoic acid methyl ester (**3**), which is further converted to **4** by CV2025 with L-alanine serving as the amino donor. Additionally, the same concept for terminal amino-functionalization is applied for the synthesis of octylamine from octane.

In a recent study, recombinant *E. coli* W3110 containing the *alkBGT* expression vector pBT10 was shown to produce **2** and **3** from **1**.[17] Here, *E. coli* BL21 (DE3) was transformed with the same plasmid enabling an initial hydroxylation activity of 2.2 $U\,g_{CDW}^{-1}$ (1 U = 1 μmol product formed per min, CDW = cell dry weight) (Table 1), which is in the same range as observed for *E. coli* W3110 (pBT10) (1.9 $U\,g_{CDW}^{-1}$).[17] Besides the expected products **2** and **3**, accumulation of dodecanedioic acid monomethyl ester (**5**) was observed after 15 min (Figure 2) which was not seen in the short-term assays performed for 5 min.[17] After 60 min, the acid concentration exceeded alcohol and aldehyde concentrations. Since acid formation from **3** was almost absent with *E. coli* BL21

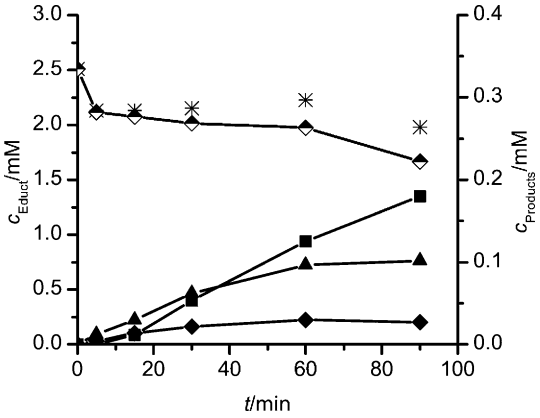

Figure 2. Oxyfunctionalization of **1** with *E. coli* BL21 (DE3) (pBT10) in KPi buffer (pH 7.4) containing 1% (w/v) glucose; biomass conc.: 1.5 $g_{CDW}\,L^{-1}$. Half diamonds: **1**, triangles: alcohol, diamonds: aldehyde, squares: acid, asterisks: sum of educt and products. Alcohol and acid refer to the sums of 12-hydroxydodecanoic acid methyl ester (**2**) and 12-hydroxydodecanoic acid and of dodecanedioic acid monomethyl ester (**5**) and dodecanedioic acid, respectively. See the Supporting Information for details on ester hydrolysis. Aldehyde is 12-oxododecanoic acid methyl ester (**3**).

(DE3) carrying the *cv2025* expression vector pTA, but lacking AlkBGT (Figure 3, a) and in control experiments with *E. coli* BL21 (DE3) (Figure 3, b), the alkane monooxygenase can be considered as the main cause for aldehyde oxidation. Overoxidation of the substrate is a characteristic often observed for monooxygenases[10] and three subsequent oxidation steps catalyzed by one enzyme are also described for xylene monooxygenase XylM from *P. putida* mt-2 showing 25% amino acid sequence homology with AlkB.[11,20] Aiming at an intracellular coupling of oxygenase and transaminase catalysis allowing production

Table 1. Maximal specific net oxygenation and transamination rates achieved with *E. coli* BL21 (DE3) containing alkane monooxygenase AlkBGT and/or ω-transaminase CV2025.

Plasmid	Substrate	Alcohol formation rate[a]	Aldehyde formation rate[a]	Acid formation rate[a]	Amine formation rate[a]
–[b]	**3**	16[f]	–	0.6 (0)	–
pTA[c]	**3**	17[f]	–	0.8 (0)	82
pBT10[c]	**1**	3.7 (2.2)	2.2 (0.5)	1.9 (0)	–
pBT10, pTA[d]	**1**	2.9 (2.7)	2.4 (0.8)	1.4 (0)	1.5 (0.5)
pBT10, pTA[e]	Octane	26	12	2.6 (1.6)	10 (8.6)

[a] Formation rates are given as specific activities in $U\,g_{CDW}^{-1}$.
[b] Biomass conc.: 0.9 $g_{CDW}\,L^{-1}$, substrate conc.: 0.5 mM.
[c] Biomass conc.: 1.5 $g_{CDW}\,L^{-1}$, substrate conc.: 2.5 mM.
[d] biomass conc.: 1.4 $g_{CDW}\,L^{-1}$, substrate conc.: 2.9 mM.
[e] Biomass conc.: 1.3 $g_{CDW}\,L^{-1}$, substrate conc.: 1.4 mM.
[f] Host intrinsic aldehyde reduction rate, in brackets: initial specific activities after 5 min of reaction; CDW: cell dry weight; $U = 1\ \mu mol\,min^{-1}$

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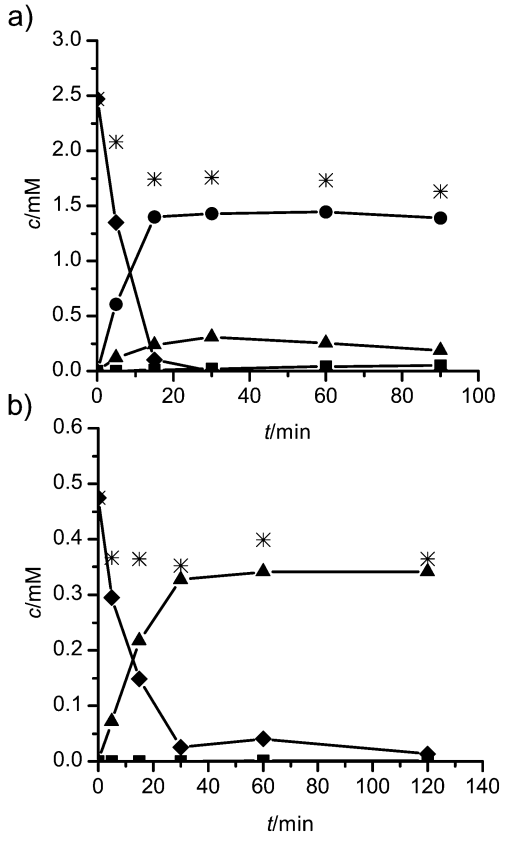

Figure 3. Whole-cell conversion of **3** with **a**) *E. coli* BL21 (DE3) (pTA) containing the transaminase CV2025 (KPi buffer (pH 7.4) containing 50 mM of L-alanine and 1% (w/v) glucose; biomass conc.: 1.5 $g_{CDW}L^{-1}$) and **b**) *E. coli* BL21 (DE3) lacking the transaminase (KPi buffer (pH 7.4) containing 1% (w/v) glucose; biomass conc.: 0.9 $g_{CDW}L^{-1}$). Circles: amine, triangles: alcohol, diamonds: aldehyde, squares: acid, asterisks: sum of educt and products. Amine, alcohol, and acid refer to the sums of 12-aminododecanoic acid methyl ester (**4**) and 12-aminododecanoic acid, of 12-hydroxydodecanoic acid (**2**) methyl ester and 12-hydroxydodecanoic acid, and of dodecanedioic acid monomethyl ester (**5**) and dodecanedioic acid, respectively. See the Supporting Information for details on ester hydrolysis.

of **4**, acid formation constitutes a competing reaction. However, the constantly increasing acid and, thus, overall product concentration showed that AlkBGT was active throughout the reaction (90 min). Therefore, terminal amine formation should be feasible if the kinetic properties of CV2025 allow an efficient conversion of **3** to **4**. As observed in all experiments with cells present, **2** and **5** were hydrolyzed to some extent to 12-hydrododecanoic acid (HDA) and dodecanedioic acid (DDA), respectively (for details of ester hydrolysis, see the Supporting Information).

Conversions of **3** were carried out with purified CV2025 in a spectrophotometric assay (see the Supporting Information) to investigate kinetic reaction parameters and the suitability of the transaminase for

Table 2. *In vitro* and apparent *in vivo* kinetic parameters for the conversion of **3** to **4** with ω-TA CV2025.

	In vitro[a]	*In vivo*[b]
V_{max}	7.1 ± 0.2 U $mg_{\omega\text{-TA}}^{-1}$	117 ± 14 U g_{CDW}^{-1}
K_M, K_S[c]	80 ± 10 μM	0.54 ± 0.13 mM
K_I	–	7.5 ± 2.7 mM

[a] Based on NADH consumption during coupled spectrophotometric assay (see the Supporting Information); 6 μg of purified ω-TA were applied.
[b] Based on product formation with resting *E. coli* BL21 (DE3) (pTA); biomass conc.: 0.1 $g_{CDW}L^{-1}$.
[c] K_S: substrate uptake constant for whole cells (equivalent to K_M for cell-free enzymes).

the desired reaction. As indicated by the high V_{max} and the low K_M (Table 2) **3** can be considered a good substrate for the ω-TA. Substrate inhibition could not be observed up to the maximum concentration applied (2.5 mM).

Aiming at the *in vivo* coupling of AlkBGT and CV2025, whole-cell aminations of **3** were conducted with *E. coli* BL21 (DE3) (pTA). L-Alanine has been shown to be a preferred amino donor for CV2025.[18] In order to investigate whether the intracellular alanine synthesis allows efficient formation of **4**, the reaction was performed over 30 min applying different extracellular L-alanine and NH_4Cl concentrations. Without L-alanine or NH_4Cl, only low amine concentrations were formed (0.01 mmol g_{CDW}^{-1}) (see the Supporting Information). The addition of 187 mM NH_4Cl resulted in a 4-fold increased amine formation rate (0.04 mmol g_{CDW}^{-1}) whereas supplying 50 mM of L-alanine led to an almost 60-fold increased rate of amine formation (0.59 mmol g_{CDW}^{-1}). A further increase in L-alanine concentration only slightly enhanced amine formation. During the transamination reaction, ester hydrolysis could be detected resulting in small amounts of 12-aminododecanoic acid (ADA) (< 10% of **4**).

Next to the *in vitro* kinetics, the apparent whole-cell transamination kinetic parameters were determined. Also *in vivo*, the reaction followed Michaelis–Menten-type kinetics but showed substrate inhibition (Table 2, see the Supporting Information) at concentrations of **3** exceeding the maximum concentrations formed during oxyfunctionalization of **1** with *E. coli* BL21 (DE3) (pBT10) by a factor of more than 10 (Figure 1). Such behaviour was not unexpected, since transaminase catalysis is often impaired by inhibition at high substrate concentrations.[13] The high substrate uptake constant (K_S) observed *in vivo* as compared to the K_M observed *in vitro* indicates an uptake limitation for **3** with whole cells. In contrast, the purified TA showed a high affinity towards **3** indicated by the

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low K_M. Therefore, intracellular concentrations of **3** accumulating during AlkBGT-catalyzed oxidation of **1** might still be sufficient to enable the desired formation of **4**.

In whole-cell biotransformations, the host intrinsic enzymatic background has to be considered to learn about possible unwanted side reactions. With **3** as substrate (2.5 mM), *E. coli* BL21 (DE3) (pTA) produced 1.4 mM terminal amine in 15 min of reaction (Figure 3, a), from which 5% were hydrolyzed to ADA. After 90 min, ADA made up 30% of the total amine product (see the Supporting Information). The initial transamination activity of 82 $U\,g_{CDW}^{-1}$ (Table 1) was as expected from the apparent kinetic parameters upon addition of 2.5 mM of **3** (calculated: 76 $U\,g_{CDW}^{-1}$) (Table 2). As a result of aldehyde reduction, **2** was accumulating as side product (Figure 3 a) at a maximum rate of 17 $U\,g_{CDW}^{-1}$ (Table 1). Approximately 0.31 mM terminal alcohol was formed in 30 min of which HDA accounted for 10%. Hydrolysis of **2**, **4**, and **5** did not occur in abiotic control experiments and thus can be attributed to host intrinsic enzymes. Hydrolysis of **1** and **3** was not observed. In order to simplify downstream processing of the reaction mixture, ester hydrolysis should be avoided in future by identifying and suppressing the responsible enzyme activity.[21] Alcohol formation from **3** was also observed in control experiments with *E. coli* BL21 (DE3) indicating host intrinsic dehydrogenase activity towards the supplied substrate (Figure 3, b). Aldehyde reduction rather than oxidation catalyzed by *E. coli* dehydrogenases has been reported before.[11,22]

Since the separately investigated reactions showed promising results, *E. coli* BL21 (DE3) was transformed with both expression plasmids. Co-expression of *alkBGT* and *cv2025* was verified by SDS-PAGE analysis (not shown) and **1** (2.9 mM) was successfully converted to **4** (up to 0.13 mM) in the whole-cell reaction (Figure 4). The maximum specific amine formation rate of only 1.5 $U\,g_{CDW}^{-1}$ (Table 1) clearly indicated a severe limitation of CV2025 by the low aldehyde availability throughout the whole reaction time. It could be shown in a recent study that the hydroxylation of **1** is limited by poor mass transfer of the hydrophobic substrate over the outer cell membrane.[17,23] By enhancing the intracellular availability of **1** for AlkBGT catalysis, the aldehyde formation rates may be increased as well which in turn should improve the transamination reaction significantly. However, the amine was accumulating as main product during the first 60 min of reaction. In contrast to the decreasing CV2025 activity after 60 min, AlkBGT showed a constant activity, which resulted in similar concentrations of amine and acid after 90 min.

To demonstrate the broad applicability of the biocatalyst, the amino-functionalization of octane (1.4 mM) was investigated. Maximal hydroxylation rates of 26 $U\,g_{CDW}^{-1}$ were in the same range as observed earlier (Table 1).[23] Within 30 min of reaction, 0.22 mM octylamine were formed as the main product with a maximum amine formation rate of 10 $U\,g_{CDW}^{-1}$. Again, acid formation was observed (Table 1), giving 0.07 mM octanoic acid in the same assay after 30 min. The higher rates achieved with octane as substrate in comparison to **1** are a result of the enhanced substrate accessibility due to the less hydrophobic character of octane.[23]

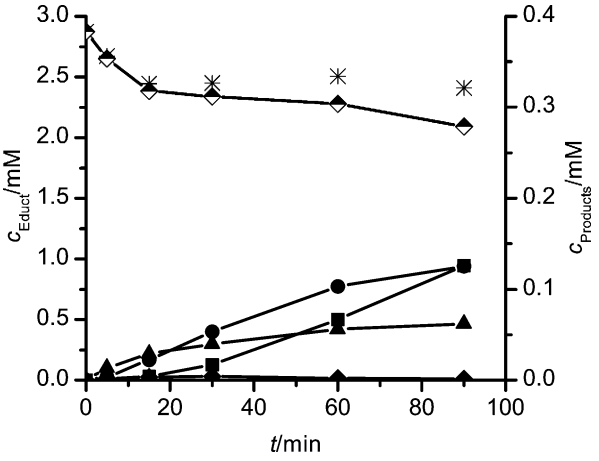

Figure 4. Amino-functionalization of **1** with *E. coli* BL21 (DE3) (pBT10, pTA) containing AlkBGT and CV2025; KPi buffer (pH 7.4) containing 50 mM of L-alanine and 1% (w/v) glucose; biomass conc.: 1.4 $g_{CDW}\,L^{-1}$. Half diamonds: **1**, circles: amine, triangles: alcohol, diamonds: aldehyde, squares: acid, asterisks: sum of educt and products. Amine, alcohol, and acid refer to the sums of 12-aminododecanoic acid methyl ester (**4**) and 12-aminododecanoic acid, of 12-hydroxydodecanoic acid methyl ester (**2**) and 12-hydroxydodecanoic acid, and of dodecanedioic acid monomethyl ester (**5**) and dodecanedioic acid, respectively. See the Supporting Information for details on ester hydrolysis. Aldehyde is 12-oxododecanoic acid methyl ester (**3**).

In conclusion, we have successfully coupled oxygenase and transaminase catalysis in one single recombinant whole-cell biocatalyst allowing for the first time the direct and specific terminal amino-functionalization of the renewable substrate **1** and of octane *via* three sequential reactions. As a proof of concept, this study shows the versatility of microbial catalysts for the specific functionalization of unactivated carbons, which is difficult to achieve by chemical means. The constructed catalyst represents an excellent starting point for the development of an efficient amino-functionalization process. Approaches in progress include the improvement of hydrophobic substrate availability by catalyst engineering[23] and application of the two-liquid phase concept to tackle substrate/product inhibition, to exploit reaction kinetics for a directed formation of the amine, and to facilitate product isolation.[24] The versatility of AlkBGT accepting aliphatic,

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alicyclic, and aromatic hydrocarbons,[25] fatty acids,[16] and fatty acid methyl esters as substrate, and the broad substrate spectrum of CV2025[18] augur well for a broad applicability of the biocatalyst for the direct amino-functionalization of unactivated terminal C–H bonds.

Experimental Section

Biotransformation Procedure

Oxidation by AlkBGT-containing *E. coli* cells (1–1.5 $g_{CDW}L^{-1}$) was performed as described before.[17] Additionally, quantification of dodecanedioic acid monomethyl ester (**5**) and dodecanedioic acid (DDA) was carried out by high performance liquid chromatography (HPLC, see the Supporting Information for details). The reaction was stopped after different reaction times by addition of 0.5 mL ice-cold acetonitrile to the reaction mixtures (1 mL). Transamination and coupled oxidation-transamination by *E. coli* cells containing CV2025 and AlkBGT/CV2025, respectively, was performed accordingly, except that the reaction buffer contained L-alanine (50 mM if not stated otherwise) (see the Supporting Information for further experimental details).

Acknowledgements

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