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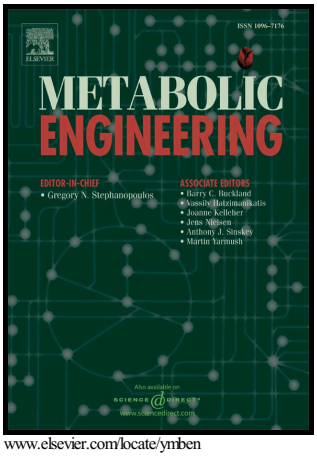

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Biocatalytic, One-pot Diterminal Oxidation and Esterification of n-Alkanes for Production of α,ω-Diol and α,ω-Dicarboxylic Acid Esters

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Keywords

Alkanes, whole-cell biocatalysis, α,ω-diols, α,ω-dicarboxylic acids, monooxygenases

1 Abstract

Direct and selective terminal oxidation of medium-chain n-alkanes is a major challenge in chemistry. Efforts to achieve this have so far resulted in low specificity and overoxidized products. Biocatalytic oxidation of medium-chain n-alkanes – with for example the alkane monooxygenase AlkB from *P. putida* GPo1- on the other hand is highly selective. However, it also results in overoxidation. Moreover, diterminal oxidation of medium-chain n-alkanes is inefficient. Hence, α,ω-bifunctional monomers are mostly produced from olefins using energy intensive, multi-step processes.

By combining biocatalytic oxidation with esterification we drastically increased diterminal oxidation upto 92 mol % and reduced overoxidation to 3 % for n-hexane. This methodology allowed us to convert medium-chain n-alkanes into α,ω-diacetoxyalkanes and esterified α,ω-dicarboxylic acids. We achieved this in a one-pot reaction with resting-cell suspensions of genetically engineered *Escherichia coli*.

The combination of terminal oxidation and esterification constitutes a versatile toolbox to produce α,ω-bifunctional monomers from n-alkanes.

2 Introduction

Diterminal oxidation of abundantly available, inexpensive medium-chain n-alkanes to valuable medium-chain α,ω-diols and α,ω-dicarboxylic acids (DCAs) is highly desirable. Many medium-chain α,ω-oxygenated products are important commodities, such as 1,4-butanediol and adipic acid, with annual production volumes of nearly 2 and 3 million tons, respectively(1, 2). These α,ω-oxygenated compounds are used for the manufacture of many products, such as plastics, fibers and coatings. Currently, they are produced in multistep, energy intensive processes. Adipic acid for example, is produced from benzene. Benzene is first hydrogenated to cyclohexane, after which cyclohexane is oxidized to yield a mixture of cyclohexanone and cyclohexanol. This mixture is then further oxidized with nitric acid to yield adipic acid (3). Hydrogenation of adipic acid yields 1,6-hexanediol (4). For 1,4-butanediol, acetylene is mostly used as precursor. Acetylene reacts with 2 equivalents of formaldehyde to yield 1,4-butynediol. The latter is hydrogenated to give 1,4-butanediol (5). Both adipic acid and 1,4-butanediol can also be produced from carbohydrates (6–8), only the 1,4-butanediol process has been commercialized so far. Direct, diterminal oxidation of n-alkanes could circumvent these multistep processes and create a paradigm shift, resulting in a more sustainable petrochemistry (9). There are several chemical means to terminally oxidize

alkanes, *via* organometallic C-H activation or by heterogeneous catalysis (10). However, due to the relative inertness of terminal C-H bonds, alkane oxidation results in subterminal oxidation (11, 12). Great improvements have been made on targeting the terminal C-H bonds, but this has not yet resulted in commercial applications (10). Diterminal oxidation of alkanes is even more complicated. Molecular sieves have been successfully applied for diterminal oxidation, but this strategy results in a large share of subterminally oxidized products (13). Synthesis of alkanols or alkanediols from alkanes poses another challenge, because the formed alcohols are easily overoxidized. α,ω-Diols were produced from medium-chain alkanes with low product titers (at most 0.2 g/L) by biocatalysis (14–16), whereas organometallic routes still seem restricted to ethane as substrate for selective production of (esterified) α,ω-diols (10, 17, 18).

Monooxygenases such as CYP52 from *Candida tropicalis* have been applied for long-chain DCA and ω-hydroxy fatty acid production from long-chain alkanes with 100% selectivity and high conversion efficiencies (19–21). This has resulted in production of high titers of α,ω-bifunctional monomers *via* whole-cell biocatalysis. CYP52 monooxygenases are however not active on medium-chain alkanes. Terminal oxidation of medium-chain n-alkanes is possible with the AlkB monooxygenase from *Pseudomonas putida* GPo1, but this results in overoxidation of the substrate to carboxylic acids (22, 23). Furthermore, diterminal oxidation of n-alkanes by AlkB has not been reported. Ω-oxidation of medium-chain fatty acids by monooxygenases is possible but only to a limited extent (8, 24–26).

Ω-oxidation by AlkB is however efficient with esterified fatty acids as substrates (27, 28). We realized that combining the ω-oxidation of medium-chain n-alkanes to acids with the subsequent *in vivo* esterification of the acids to ethyl esters could improve diterminal oxidation. In a similar fashion, esterifying fatty alcohols *in vivo* with acetate could be used to

enable their ω-oxidation. Moreover, esterified alcohols and alkanediols would be protected against overoxidation.

In vivo esterification of alcohols and acids has especially been studied for the production of ethylated fatty acids for biodiesel applications(29, 30). It requires alcohol acetyltransferases (AATs) that transesterify acyl-CoAs with alcohols. AATs typically used are Atf1 and Eeb1 from *Saccharomyces cerevisiae* and AtfA from *Acinetobacter baylyi*(31, 32). Atf1 can utilize endogenously produced acetyl-CoA, whereas AtfA and Eeb1 utilize longer-chain acyl-CoAs. Atf1 and AtfA can accept a wide range of alcohols, whereas Eeb1 seems restricted to short-chain alcohols(33–35).

We investigated whether combining terminal methyl group oxidation by AlkB with *in vivo* esterification using *E. coli* as chassis could enable diterminal oxidation of alkanes and reduce overoxidation. The final products we aimed for were acetoxy esters of α,ω-alkanediols and ethyl esters of DCAs.

3 Materials and methods

3.1 Strains and plasmids

Plasmids and strains are listed in Table 1. The construction of the plasmids is described in the supplementary info.

Table 1. Plasmids and strains used in this study

Name	Description	Source
Plasmids		
pUC57-*atfAcodopt*	Codon optimized *atfA* in pUC57	This study
pET-Duet-*eeb1*	*eeb1* (*Saccharomyces cerevisiae* NCYC2926) in pET-Duet	This study

pGEc47	*alkBFGHJKL* and *alkST* in pLAFR1	(36)
pCOM10-*alkL*	Contains P_{alkB}:*alkL* in pCOM10, a broad host range alkane responsive vector	(37)
pSTL	*alkT* and P_{alkB}:*alkL* in pCOM10	(28)
pBGTL	P_{alkB}:*alkBFGL* and *alkST* in pCOM10	(28)
pET9a:Atf1-opt	Codon optimized *atf1* in pET9a	Provided by A. Kruis, dept. of Microbiology, Wageningen UR. Codon optimized *atf1* according to (33)
pSKL-*atf1*	P_{alkB}:*alkKL*, P_{alkB}:codon optimized *atf1* in pCOM10	This study
pBGTL-*atf1*	P_{alkB}:*alkBFGL*, P_{alkB}:codon optimized *atf1* and *alkST*, in pCOM10	This study
pE	P_{alkB}:*alkKL*, P_{alkB}:codon optimized *atfA* in pCOM10	This study
pE-II	P_{alkB}:*alkKL*, P_{alkB}:codon optimized *atfA* in pCOM10 with pBR322 ori, Amp^R	This study
pBGTHJL	P_{alkB}:*alkBFGHJL* and *alkST* in pCOM10	(38)
pBGTHJKL-*atfA*	P_{alkB}:*alkBFGHJKL*; P_{alkB}:*atfA* and *alkST* in pCOM10	This study
pBGTHJKL-*eeb1*	P_{alkB}:*alkBFGHJKL*; P_{alkB}:*eeb1* and *alkST* in pCOM10	This study
***E. coli* strain**		
NEBT7	*fhuA2 lacZ::T7 gene1* [*lon*] *ompT gal sulA11 R(mcr-73::miniTn10*--Tet^S*)2* [*dcm*] *R(zgb-210::Tn10*--Tet^S*) endA1* Δ*(mcrC-mrr)114::IS10*	New England Biolabs®
M	NEBT7 pBGTL	(28)
ME1	NEBT7 pBGTL-*atf1*	This study
MD	NEBT7 pBGTHJL	(38)
MDE2	NEBT7 pBGTHJKL-*atfA*	This study
MDE2*	NEBT7 pBGTHJL + pE-II	This study
MDE3	NEBT7 pBGTHJKL-*eeb1*	This study

3.2 Cultivation and gene expression

E. coli NEBT7 (a BL21 derivative) was used for conversion studies. For strains containing a single plasmid 50 µg/mL kanamycin was added to the medium. For strains with two plasmids 25 µg/mL kanamycin and 50 µg/mL ampicillin was added to the medium. Strains were inoculated from glycerol stocks stored at -80 °C in LB containing the appropriate antibiotic(s) and incubated overnight at 30 °C in a rotary shaker set to 250 rpm. The overnight culture was diluted 100 times in M9 medium containing 0.5 % glucose and 1 mL/L trace elements US^{Fe} (39). This culture was again incubated overnight. The next day, this second preculture was used to inoculate M9 mineral medium to an OD_{600nm} of 0.167. This culture was directly induced with 0.025 % v/v dicyclopropylketone to induce recombinant gene expression. After 4 h induction at 30 °C, 250 rpm, the cells were harvested by centrifugation for 10 min. at 4255 x g.

3.3 Conversions

The cell pellet was resuspended in resting cell buffer (1 g_{cdw}/L), which contained 1 % glucose, 2 mM $MgSO_4$, and 50 mM KPi pH 7.4. Of this resting cell suspension, 0.5 mL was transferred to a pyrex tube. After addition of substrate or organic phase containing substrate, tubes were tightly capped with a PTFE cap and transferred to a rotary shaker, set to 30 °C, 250 rpm. Reactions were stopped by addition of 1 % v/v of concentrated phosphoric acid and immediate transfer of the reaction to ice.

3.4 Analytical methods

All compounds were analyzed with GC, except butyrate and valerate, which were analyzed with HPLC. Reactions were extracted with $CHCl_3$ containing 0.2 mM tetradecane or 0.2 mM dodecane as internal standard. The extraction was done for ~5 min. with a rotator (Labinco,

The Netherlands, maximum speed). For conversions containing a carrier solvent or bulk n-alkane phase, the organic phase was sampled directly and diluted 20-40 times in $CHCl_3$ + internal standard. The samples were derivatized with 10% v/v of a 0.2 M trimethylsulfoniumhydroxide solution in MeOH. Qualitative analysis was done with GC-MS, quantitative analysis with GC-FID.

GC-MS

GC-MS analysis was done with a Trace GC Ultra coupled to a DSQII mass spectrometer. 1 µL sample was injected in splitless mode, with the inlet set at 350 °C. The temperature program was as follows: 50 °C hold 3 min.,7.5 °C/min ramp to 350 °C, hold for 10 min.

GC-FID

GC-FID analysis was done with a 7890A (Agilent). 1 µL sample was injected in splitless mode, with the following temperature program: 50 °C hold 1 min., 15 °C/min to 180 °C, 7 °C/min to 230 °C, 30 °C/min to 350 °C hold 3 min. Quantification was done by using available standards. If standards were not commercially available, quantification was done on basis of structurally related compounds with similar numbers of carbon/oxygen atoms. For example, mono-hexyl adipate was quantified on the basis of mono-ethyl sebacate.

HPLC

HPLC analysis was done with an Agilent 1260 Infinity UPLC, equipped with a 30 cm Rezex ROA column (Phenomenex), operated at a flowrate of 0.5 mL/min with 5 mM H_2SO_4 as running buffer. The RI detector was used for quantification of analytes.

4 Results

Testing overoxidation and diterminal oxidation by AlkB

E. coli strain M was used to assess the level of overoxidation and diterminal oxidation of n-alkanes by AlkB. It possesses the monooxygenation module, consisting of AlkBGTL from *Pseudomonas putida* GPo1 (Figure 1). AlkB is a non-heme di-iron monooxygenase, responsible for the hydroxylation of terminal methyl groups (36). AlkB overoxidizes this alcohol to the aldehyde and acid. AlkG and AlkT are involved in the transfer of electrons from NADH to AlkB. AlkL, an outer membrane protein, improves uptake of n-alkanes (Figure 1a) (23).

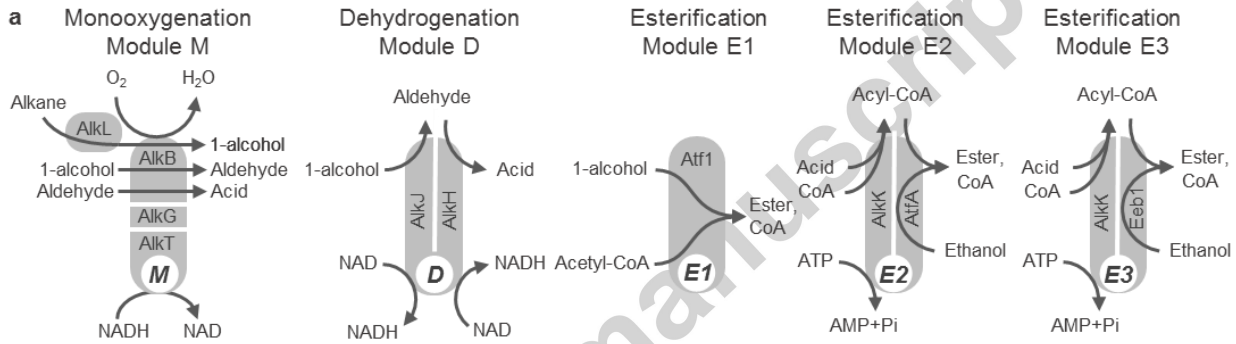

Figure 1. Function of the different modules applied in this study. The monooxygenation module (abbreviated M), consists of AlkBGTL. AlkL is an outer membrane protein that improves transport of hydrophobic molecules. The dehydrogenation module (D), consists of AlkJ and AlkH. The esterification module 1 (E1) consists of Atf1, esterification module E2/E2* of AlkK and AtfA, esterification module E3 of AlkK and Eeb1.

For the structures of the chemicals involved, see Figure 2. The fate of alkanes converted by monooxygenation module M is depicted in Figure 3a.

Tests were done in tightly capped tubes, with 1 g_{cdw}/L resting, induced *E. coli* M cells fed with 1 % v/v of n-alkanes of different chain lengths, corresponding to a range of 51.3 mM for n-decane to 76.0 mM for n-hexane. This experimental set-up did not allow the addition of smaller alkanes due to their low boiling points. Therefore, we added 0.30 % v/v 1-butanol (32.8 mM) and 1-pentanol (27.7 mM) instead. Glucose served as an energy source. Samples were taken after 19 hours incubation. At that time glucose was still present and the absence of fermentation products indicated that no oxygen limitation occurred (data not shown).

Overoxidized products - fatty acids and ω-hydroxy fatty acids - formed at least 95 % of the total products (Figure 3c). Diterminal oxidation efficiency was below 16 % for the C4 to C8 alkanes. It increased to 64 % for n-decane but at very low concentrations (Figure 3e).

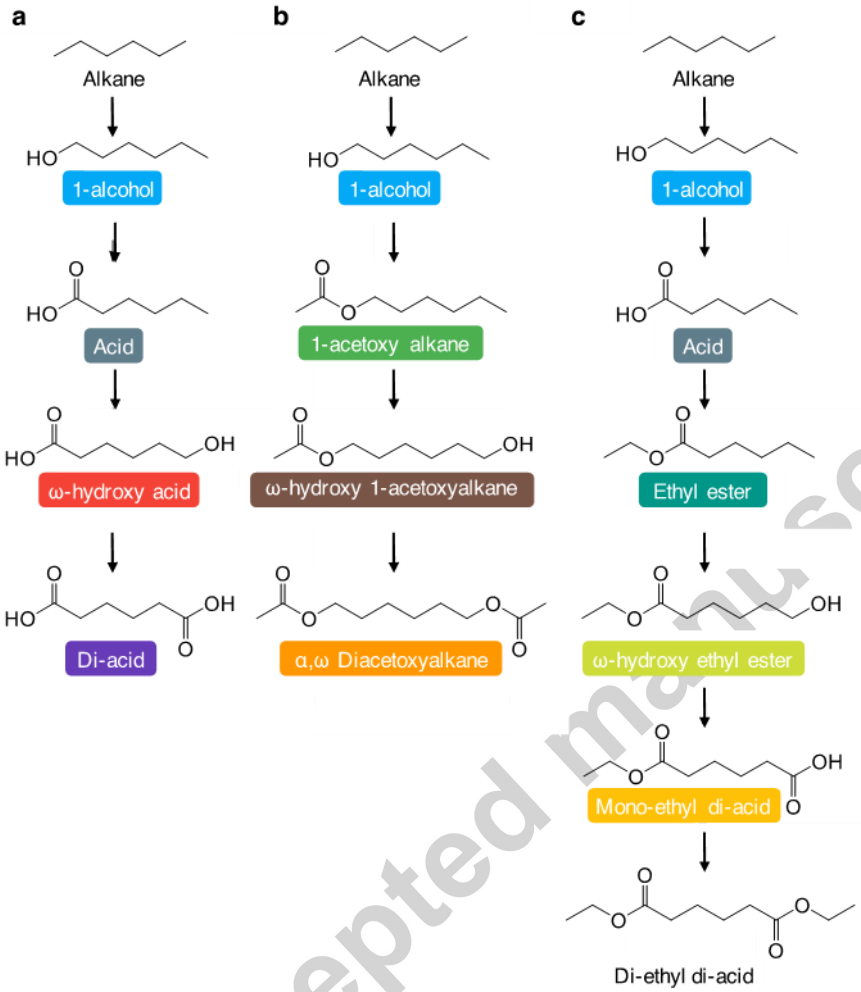

Figure 2. Chemical structures of substrates used and products formed in the experiments. (**a**) oxidation of alkanes to dicarboxylic acids. (**b**) Conversion of alkanes into α,ω-diacetoxyalkanes. (**c**) Conversion of alkanes into di-ethyl dicarboxylic acids. C6 compounds were used as example for the chemical structures. The color codes of the names of the chemicals are the same as used in the rest of the document.

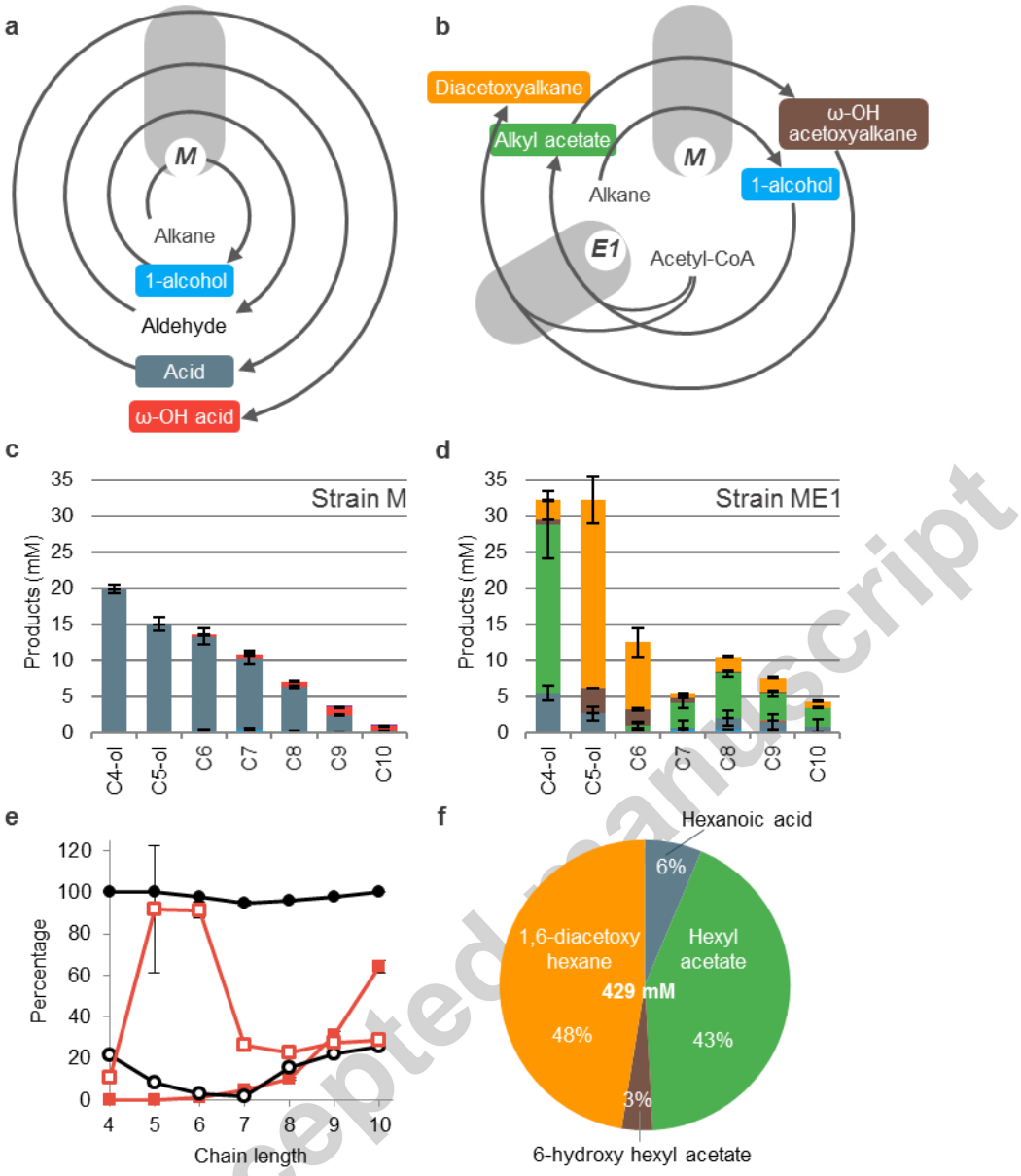

Figure 3. n-Alkane conversions by resting cells of *E. coli* strains M and ME1 (1 g_{cdw}/L). The colors of the products correspond to Figure 2. (**a**) Fate of n-alkane conversion by the monooxygenation module M only. (**b**) Fate of n-alkane conversion with *both monooxygenation module M and esterification module* E1. (**c**) Products of 19 h conversions of 1 % pure n-alkane of C6-C10 chain lengths or 0.3% n-butanol/n-pentanol by *E. coli* M. (**d**) Products of 19 h conversions of 1 % pure n-alkane of C6-C10 chain lengths or 0.3% n-butanol/n-pentanol by *E. coli* ME1. (**e**) Percentage of total product that was overoxidized (black lines), percentage of total product that was diterminally oxidized (red lines). Filled symbols correspond to strain M, open symbols to strain ME1. (**f**) Conversion of n-hexane added as 10 % v/v solution in bis(2-ethylhexyl) phthalate. The ratio of organic phase to aqueous phase was 1:10, conversions lasted 31 h.

Converting n-alkanes into acetate esters of α,ω-diols

For production of acetate esters of α,ω-diols, we added esterification module E1(Figure 1). This module consisted of the alcohol acetyltransferase (AAT) Atf1, which can convert the formed 1-alcohol together with acetyl-CoA, derived from central carbon metabolism, to an acetoxyalkane.

In the resulting strain ME1, alkanes can be converted via the monooxygenation module M only (Figure 3a), and via the combination of the monooxygenation module M and the esterification module E1 (Figure 3b).

Strain ME1 produced mainly acetoxyalkanes from 1-butanol, and n-heptane to n-decane. These alkyl acetates were products of transesterification of the 1-alcohols - generated by AlkB - and acetyl-CoA. All alkyl acetates produced from the different alkane chain lengths were ω-oxidized, as in most samples ω-hydroxy acetoxyalkanes were detected. Although it is known that AlkB accepts a wide range of aliphatic substrates (40), no reports have been made that it can ω-hydroxylate alkyl acetates. Surprisingly, these ω-hydroxy alkyl acetates were again transesterified with acetyl-CoA to yield α,ω-diacetoxyalkanes, ensuring that overoxidation was prevented at both ends. Both ω-hydroxy acetoxyalkanes and α,ω-diacetoxyalkanes can serve as precursor for α,ω-diols.

Overoxidation only occurred to a limited extent, due to the presence of the esterification module (Figure 3e); 1-alcohols were overoxidized to fatty acids, and also ω-hydroxy acetoxyalkanes were overoxidized to ω-acetoxy acids (maximally 0.51 mM for n-decane, data not shown). The highest percentage of overoxidized products was 26 mol % in reactions using n-decane, while experiments performed with n-hexane resulted in only 3 mol % overoxidized products. Thus, converting the products into acetate esters efficiently protects the molecule from overoxidation. The observed levels of overoxidation are higher in 1-butanol and 1-pentanol conversions. The reason for this is probably that there were no alkanes competing

with the alcohol for the active site of AlkB. This causes AlkB to further oxidize the alcohols to acids. For n-alkanes <C9, also more diterminal oxidation occurred, with up to 92 mol % of diterminally oxidized product in case of n-hexane.

Diacetoxyalkanes were detected for all chain lengths investigated. With n-hexane as substrate, 1,6-diacetoxyhexane was the major product after 19 h incubation, with a concentration of 9.20 mM. Furthermore, 2.23 mM 6-hydroxy hexyl acetate accumulated. So in total, 91 mol % contained two terminal hydroxy groups.

From 1-butanol, butyl acetate was produced successfully (23.2 mM). Butyl acetate served as substrate for 4-hydroxy butyl acetate (0.74 mM) and diacetoxybutane production, which accumulated to 2.7 mM. 1-pentanol was very efficiently converted into 1,5-diacetoxypentane (Figure 3d), which accumulated to 26.0 mM. Thus, with this platform also precursors for short-chain α,ω-diols can be produced. The titers of 1,5-diacetoxypentane were much higher than titers of other diacetoxyalkanes. This was probably caused by the higher solubility of the substrate 1-pentanol. Furthermore, one alcohol group is already present, which means that the monooxygenation module only has to function once. Titers of 1,4-diacetoxybutane were much lower than 1,5-diacetoxypentane titers. The intermediate butyl acetate was present in high concentrations (facilitated by the high concentration of dissolved 1-butanol), which implies that the activity of AlkB towards this substrate is low.

Among the tested n-alkanes, n-hexane yielded most diacetoxyalkane. To increase product titers we added n-hexane dissolved to 10 % in bis(2-ethylhexyl) phthalate (BEHP) as carrier solvent. BEHP has been shown before to be a solvent that is compatible with *E. coli* whole-cell conversions(41–43). The organic phase served as substrate reservoir, which reduced evaporation of the volatile n-hexane. The apolar BEHP also acted as product sink, since the products are relatively apolar. BEHP containing n-hexane was added with an organic:aqueous

phase ratio of 1:10 (Figure 3f). The overall product titers increased; 429 mM product accumulated in the organic phase. Thus, 56 % of the added n-hexane was converted into product. In conversions where n-hexane was added directly, only 16 % was converted into product. The amount of product with two alcohol moieties was 218 mM (43 g/L in the organic phase), 51 % of the total product. The selectivity of 1,6-diacetoxyhexane production was 47 % (defined as [product]/[total product]).

Converting n-alkanes into ethyl esters of diacids

For the production of esterified diacids, we added dehydrogenation module D and esterification module E2 (Figure 1). The dehydrogenation module consisted of the alcohol dehydrogenase AlkJ and the aldehyde dehydrogenase AlkH, both from *Pseudomonas putida* GPo1. Like AlkB from module M, it oxidizes alcohols to acids, but is more efficient with respect to cofactor utilization and oxygen consumption (38). We designed E2 based on acyl-CoA ligase AlkK (*P. putida* GPo1) and the AAT AtfA (from *Acinetobacter baylyi*). AlkK activates the carboxy groups with CoA, and the AATs transesterify acyl-CoAs with externally supplied ethanol.

We tested MD and MDE2 strains with 5 mM n-alkane or n-alcohols as substrate, and added 2.5 % ethanol for the esterification reaction (Figure 4). Strain MD converts alkanes via only the M module (Figure 3a), or via a combination of the M and D modules (Figure 4a). Strain MDE2 converts alkanes via module M only (Figure 3a), via a combination of the M and D modules (Figure 4a), or via all three modules (Figure 4b). Strain MD produced fatty acids from all n-alkanes. Diterminal oxidation occurred to a limited extent for C7-C10 n-alkanes, since ω-hydroxy fatty acids accumulated. In C8-C10 n-alkane conversions, only traces of DCAs ($\leq$ 0.04 mM) were formed.

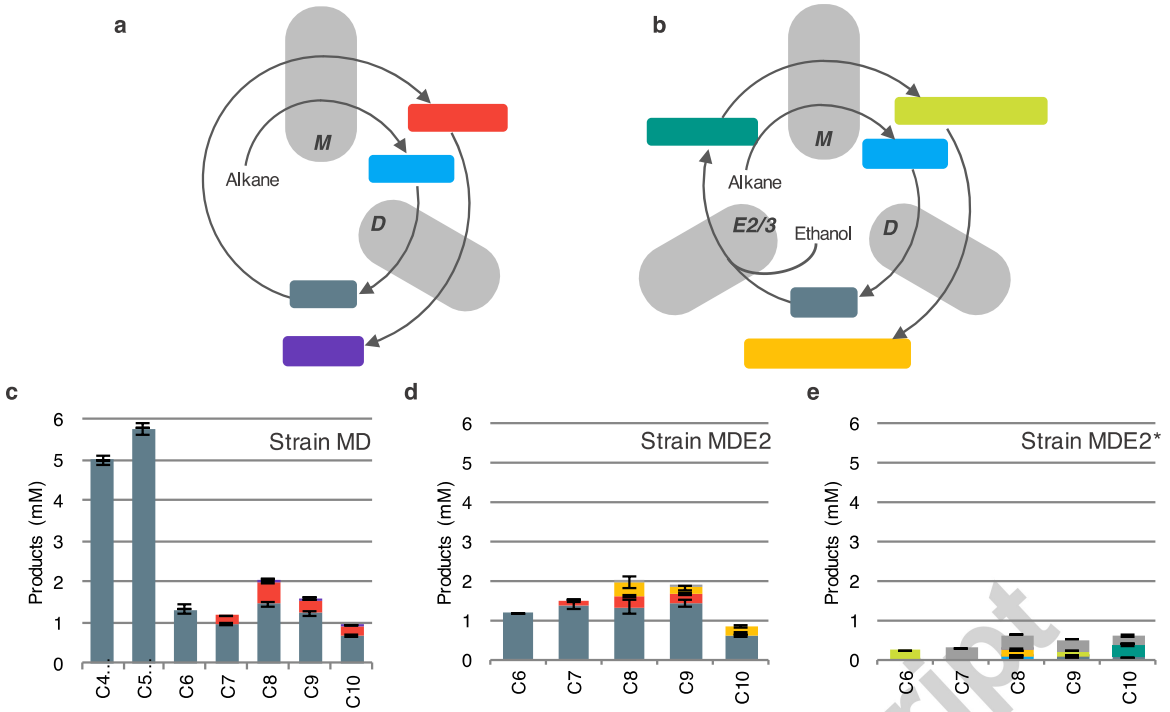

Figure 4. Conversion of 5 mM n-alkanes or 5 mM 1-alcohols by MD(E) strains (1 g_{cdw}/L), in presence of 2.5 % ethanol. The colors of the products correspond to Figure 2. (**a**) Fate of n-alkane conversion with the monooxygenation module M and the dehydrogenation module D. (**b**) Fate of n-alkane conversion with the monooxygenation module M, the dehydrogenation module D and esterification module E2, E2* or E3. (**c-e)** Products of 19 h conversions of pure n-alkane of C6 to C10 chain lengths or n-butanol/n-pentanol by *E. coli* MD (**c**), MDE2 (**d**), MDE2* (**e**).

Strain MDE2 produced mono-ethyl DCAs (Figure 3d), showing that esterification was successful. No fatty acid ethyl esters accumulated, indicating that they were efficiently converted to mono-ethyl dicarboxylic acids. This strain produced up to 0.39 mM mono-ethyl DCA. Strain MD produced at most 0.04 mM dicarboxylic acid. Hence, complete oxidation to the ω-acid was more efficient in presence of an esterification module. AtfA also coupled 1-alcohols, that accumulated from n-alkane hydroxylation, to acyl-CoA. This resulted in alkyl alkanoate production. See Figure 5 for the chemical structures of the alkyl alkanoates. Furthermore, ω-hydroxy fatty acids accumulated, indicating that the esterification module was bypassed. Ester formation from hexanoate was poor, probably caused by the low specificity of AtfA for shorter chain fatty acids. Therefore, we did not test 1-butanol and 1-pentanol with these strains.

Strain MDE2 produced mostly acid, indicating that the esterification module was not very effective. Therefore strain MDE2* was constructed (Figure 1b), aimed at higher expression levels of AlkK, to increase the activity of the esterification module. MDE2* produced mostly esters (Figure 3e), but it was less efficient regarding terminal oxidation. This resulted in lower product titers, and a high concentration of alkyl alkanoates. This shows that tuning the activity of the enzymes is important for module performance.

Converting n-alkanes to alkyl esters of diacids

Apparently, ethyl alkanoate production competes with alkyl alkanoate production. The availability of ethanol could be a determining factor. To investigate alkyl alkanoate production in more detail we added n-alkanes without addition of ethanol (Figure 5&6).

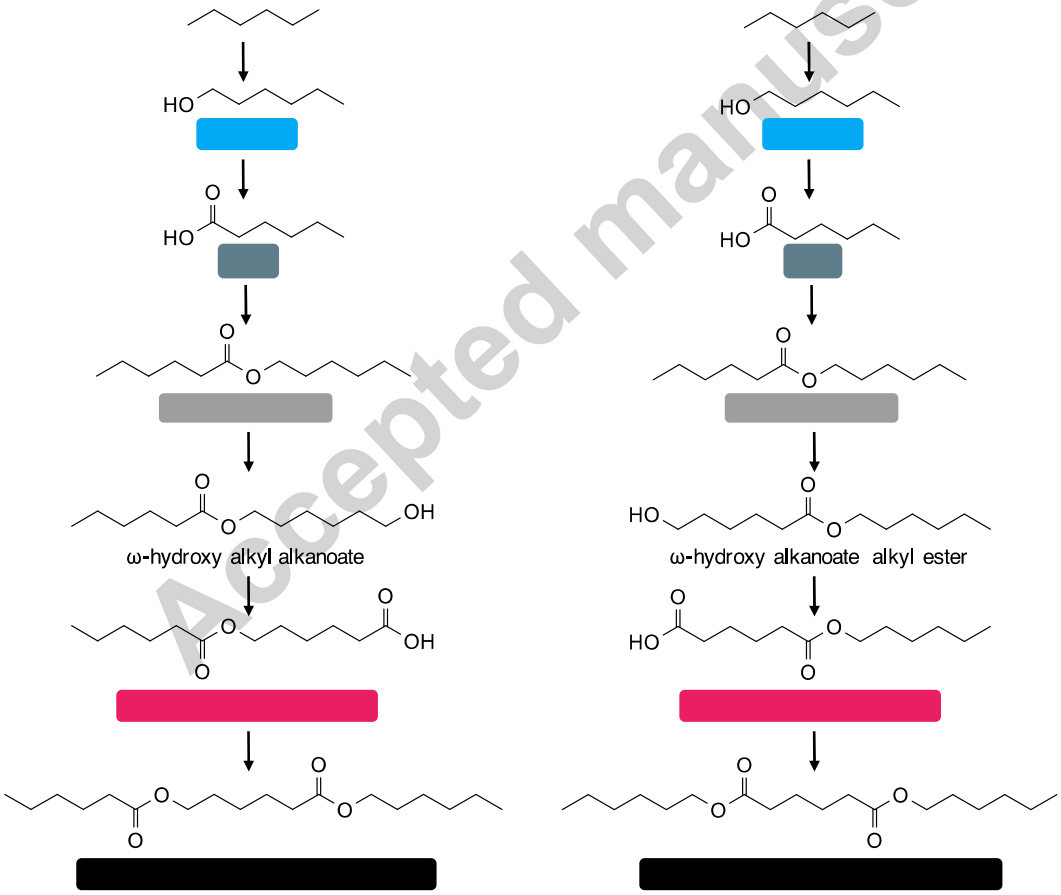

Figure 5. Chemical structures of substrates used and products formed in experiments with *E. coli* MDE2/MDE2* without added ethanol. On the left side, the scenario is depicted wherein the alkyl chain is oxidized by AlkB, on the right side the scenario wherein the acyl chain is oxidized by AlkB. C6 compounds were used as example for

the chemical structures. The color codes of the names of the chemicals is the same as used in the rest of the document.

More alkyl alkanoates were formed, maximally 1.61 mM from n-heptane. Strains MDE2 and MDE2* could terminally oxidize hexyl hexanoate. Both strains produced a mixture of hexyl 6-(hexanoyloxy)hexanoate and di-hexyl adipate (see Figure 5 & 6a,b). AlkB is thus able to oxidize both ends of hexyl hexanoate. Strain MDE2 also accumulated a mixture of mono-hexyl adipate and 6-(hexanoyloxy)hexanoic acid. These mixtures were quantified as the sum as gas chromatography did not allow sufficient separation of these compounds. However, it was possible to identify the different compounds with GC-MS. Hence, also without external alcohol addition, it is possible to di-functionalize n-hexane, yielding precursors for α,ω-dicarboxylic acids and ω-hydroxy fatty acids directly from n-hexane.

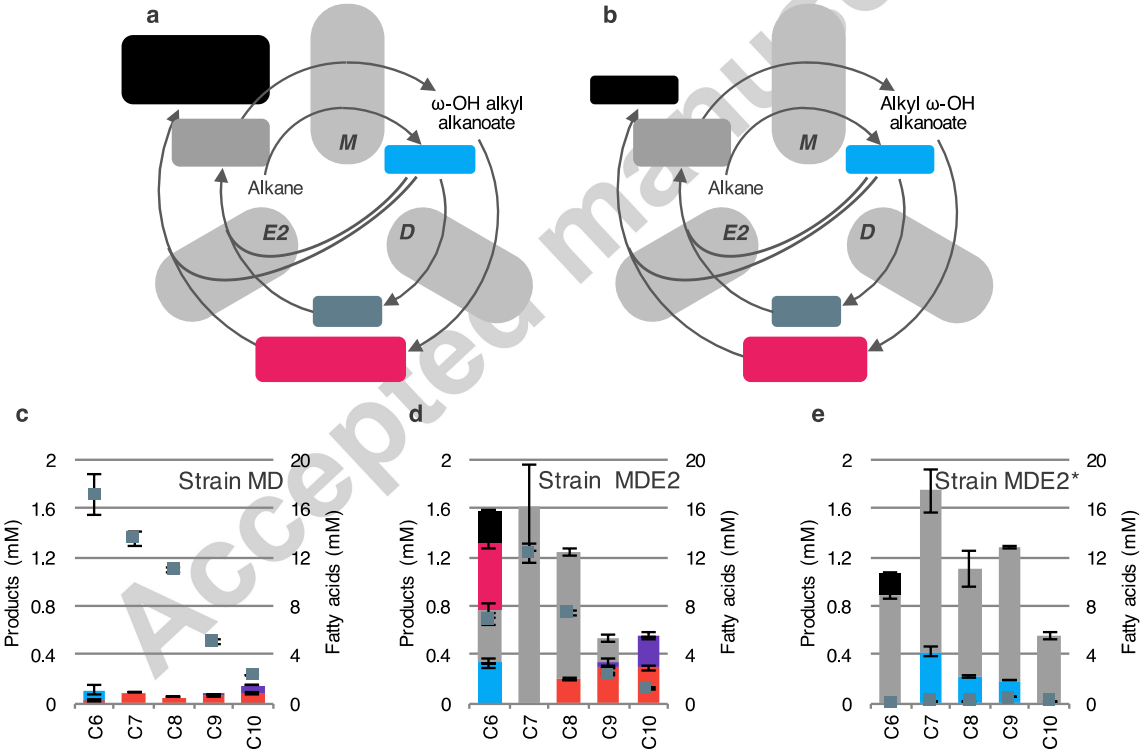

Figure 6. Conversion of n-alkanes, in absence of ethanol. The colors of the products correspond to Figure 5. (**a**) Fate of n-alkane conversion with the monooxygenation module M, the dehydrogenation module D and the esterification module E2 or E2*, when AlkB oxidizes the terminal position of the alkyl chain. (**b**) Fate of n-alkane conversion by monooxygenation module M, the dehydrogenation module D and the esterification module E2 or E2*, when AlkB oxidizes the terminal position of the acyl chain. (**c**) Products of 19 h conversion with 1 % n-alkanes or 0.26% n-butanol/n-pentanol by *E. coli* MD (see Figure 4a for pathway). (**d**) Products of 19 h conversion with 1 % n-alkanes by *E. coli* MDE2. (**e**) Products of 19 h conversion with 1 % n-alkanes by *E. coli* MDE2*.

To avoid alkyl alkanoate formation, we created strain MDE3. This strain expresses Eeb1 (from *Saccharomyces cerevisiae*), an AAT specific for short alcohols (33, 34). As a result MDE3 did not produce alkyl alkanoates (Figure 7a). Ethyl ester synthesis was more efficient, and as a consequence more mono-ethyl DCA accumulated, up to 0.87 mM from n-octane. Titers of products that contain two carboxylate moieties thus improved ~22-fold. This strain also produced 0.36 mM mono-ethyl adipate from n-hexane, a product which was not produced by MDE2/2*. We also tested MD and MDE3 with 1-pentanol and 1-butanol as substrate. MD only produced fatty acids. MDE3 produced 0.65 mM mono-ethyl glutarate from 1-pentanol, and trace amounts of mono-ethyl succinate (0.08 mM). This indicates that ethyl butyrate and ethyl valerate were formed and subsequently ω-oxidized to add another carboxyl group.

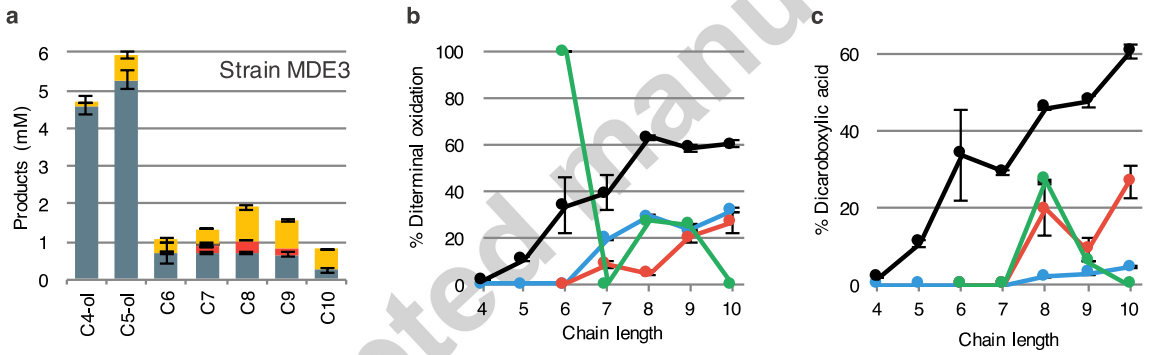

Figure 7. (**a**) Conversion of 5 mM n-alkanes or 5 mM 1-alcohols by strain MDE3 (1 g_{cdw}/L), in presence of 2.5 % ethanol. The colors of the products correspond to Figure 2, in Figure 4b is shown how the products are formed (**b**) Percentage of total product that was diterminally oxidized by MD(E) strains. Blue line: strain MD. Red line: MDE2. Green line: MDE2*. Black line: MDE3. (**c**) Percentage of total product that contained two terminal carboxylic functionalities. Blue line: strain MD. Red line: MDE2. Green line: MDE2*. Black line: MDE3.

Strain MDE3 produced more diterminally functionalized product than strain MD for all tested chain lengths (Figure 7b). In most cases, MDE3 also performed better than MDE2 and MDE2* with respect to diterminal functionalization. MD produced small amounts of DCA, indicating that AlkJ and AlkH are poorly or not accepting ω-hydroxy fatty acids as substrate. Esterification improved the conversion towards two carboxylic acid functionalities, since the

mole percentage of (mono-ethyl) DCA increased from maximally 5 % with MD to 60 % with MDE3 (Figure 7c).

Diterminal oxidation preferentially occurred after esterification, since 1-butanol, 1-pentanol and n-hexane were diterminally oxidized by MDE3, but not by MD. Furthermore, MDE3 produced more diterminally oxidized product than MD. We did not detect any products that were shortened by β-oxidation. The β-oxidation system of *E. coli* is not induced by medium-chain fatty acids (44). Moreover, we used resting cells grown on glucose, which are probably unable to perform β-oxidation.

5 Discussion

Mild, direct conversion of medium-chain n-alkanes into α,ω-diols and α,ω-dicarboxylic acids is of longstanding interest. Poor (di)terminal selectivity and overoxidation have impeded this type of conversions. The terminal selectivity can be overcome by using alkane monooxygenases, but application of these enzymes also results in overoxidation. Moreover, they are not or poorly able to oxidize both ends of the n-alkanes (14, 15, 23). In this paper we have shown that by combining the specific oxidation of alkane monooxygenase AlkB with an esterification reaction it is possible to control both the level of oxidation and to enable diterminal oxidation.

Diterminal oxidation of longer n-alkanes (>C10) with whole cells has been shown before using alkane monooxygenases like CYP450 (19). With whole cells expressing AlkB, medium-chain n-alkanes were only monofunctionalized (45). It seems that the shorter distance between the first hydrophilic terminally oxidized group and the other terminus, prevents a proper docking of the latter in the catalytic site of the enzyme. We reasoned that

converting the oxidized end into a less hydrophilic ester group could enable diterminal oxidation. To realise this we combined the action of AlkB with the alcohol acetyltransferase Atf1. This enzyme is able to esterify C4-C14 alcohols with acetyl-CoA, forming acetoxyalkanes (33). The combined action of AlkB and Atf1 indeed resulted in the oxidation of the ω-end of these acetoxyalkanes, forming ω-hydroxy acetoxyalkanes and even α,ω-diacetoxyalkanes. In this way diterminal oxidation of alkanes to (esters of) α,ω-alkanediols was established. The combination of terminal oxidation with esterification enhanced diterminal oxidation and enabled accumulation of up to 92 mol % diterminally oxidized product, under non-optimized conditions with a low biomass concentration. N-butanol and n-pentanol were also converted into esters of diols and dicarboxylic acids, which highlights the versatility of this technology. Diterminal oxidation and esterification have been combined recently, starting from ethane to yield 1,2-diacetoxyethane based on organometallic C-H activation(17). This route was highly selective with the substrate ethane, but with propane mainly subterminal oxidation occurred. With our technology only terminal oxygenates were produced, which once more demonstrates the excellent terminal selectivity of AlkB.

We gained control over the oxidation level, by the application of Atf1 for the esterification module. Without this module, AlkB overoxidized nearly all the formed product towards fatty acids and/or ω-hydroxy fatty acids. With esterification by Atf1, only 3 % of the product was overoxidized with n-hexane as substrate, and at most 26 % with n-decane as substrate. The longer the n-alkane, the more overoxidation occurred. This is likely due to a lower specificity of Atf1 for longer 1-alcohols. Atf1 also esterified the formed ω-hydroxy alkyl acetates, which prevented overoxidation also on the second terminus. These results also show that AlkB apparently releases its product after every oxidation step. If it did not, the hydroxygroup would not have been available to form an ester with acetyl-CoA, but would have been overoxidized.

AlkB is clearly able to oxidize the omega methyl group of esters. We reasoned that esterification of fatty acids produced by AlkB with ethanol, using the AATs AtfA or Eeb1, may allow the formation (of ethyl esters) of dicarboxylic acids. Overoxidation is desired for the production of (esterified) dicarboxylic acids, and this was achieved with the incorporation of the dehydrogenation module. Esterification with module E2, harbouring AtfA, did not always result in more diterminal oxidation, but the oxidation of terminal alcohols towards acids was in general more efficient. ω-Hydroxy fatty acid ethyl esters barely accumulated. This implies that AlkJ and AlkH do not or poorly accept ω-hydroxy fatty acids as substrate, but do accept ω-hydroxy fatty acid ethyl esters.

Module E2 also coupled the acyl-CoAs produced from the added n-alkanes to 1-alcohols formed from n-alkane oxidation by AlkB. Omitting ethanol as substrate increased this conversion. With n-hexane as substrate, this yielded hexyl hexanoate, which was again terminally oxidized by AlkB. AlkB acted on either the alkyl or acyl chain, resulting in a mixture of products. These products were again esterified with an alcohol, yielding di-esters. The longer alkyl alkanoates were too long to be accepted by AlkB. Alkyl alkanoate production could be reduced by using module E3, harbouring Eeb1, an AAT more specific for short chain alcohols. Esterification with module E3 improved diterminal oxidation roughly 2-fold, and the majority of products were mono-ethyl DCAs.

Mono-ethyl DCA titers were lower than diacetoxyalkane titers. This is probably caused by the low intrinsic activities of AtfA and Eeb1, which are in the range of 0.003 – 0.3 U/mg (34, 46). This is clearly less than the 190 U/mg reported for Atf1 (47). Before any process conditions are optimized, this issue has to be addressed, e.g. by protein engineering or screening enzymes with higher alcohol acyltransferase activity.

The results show as proof-of-principle that C4 to C10 n-alkanes or related molecules can be converted into (esters of) diols and diacids. This required the concerted action of 5 to 8 proteins in relation to the concentration of substrates, products and cofactors. Many permutations are possible to optimize the conversion. We have seen that increasing the expression of AlkK in module E2 had a drastic effect on product formation. It is also clear that using AATs with different specificities – AtfA and Eeb1 – has a significant effect on the efficiency of the conversion. Furthermore, AlkB displayed low activity on butyl acetate and ethyl butyrate. Hence, for diterminal oxyfunctionalization of shorter alkanes, engineered AlkB with a higher activity on short alkanes should be tested (48, 49). For diacetoxyalkane production from the n-alkanes with longer chain lengths, more overoxidation occurred. The esterification also has to be optimized here to prevent this.

The conversion process involves gaseous or water-immiscible substrates and products. This opens possibilities to recover the products easily in a two-liquid phase setup. By adding the substrate n-hexane dissolved in carrier solvent BEHP, more n-hexane was converted into products. The products accumulated in the organic phase, showing that this is a promising strategy for *in situ* product removal.

The final products made are diacetoxyalkanes and mono-ethyl dicarboxylic acids. These can be easily converted into the desired α,ω-diols and α,ω-DCAs by conventional chemical methods. The remaining ethanol and acetic acid can be recovered. They can be recycled in the process, or sold as by-products.

This study shows that the combination of biocatalytic terminal oxidation and esterification holds great promise for the production of an array of functional chemicals, such as adipic acid and 1,4-butanediol, without the need for energy intensive processes such as cracking.

6 Conflict of interest statement

The authors declare no conflict of interest.

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Highlights

- N-alkanes were converted to esters of α,ω-diols and α,ω-dicarboxylic acids
- Combining ω-oxidation and esterification limits overoxidation of n-alkanes
- The combination also enables efficient diterminal oxidation of n-alkanes by AlkB
- This toolbox provides a new route to commodities, such as adipic acid and 1,4-BDO
- It provides a mild alternative to current energy-intensive and polluting processes