# Engineering Membrane-Bound Alkane Monooxygenase from *Marinobacter* sp. for Increased Activity in the Selective ω-Hydroxylation of Linear and Branched Aliphatic Esters

*Jelena Spasic‡[a], Andrea Nigl‡[a], Huijin Cheon‡[b], Christine L. Kaiserer[a], Stela Galušić[a], Elske van der Pol[a,c], Lenny Malihan Yap[a], Jin-Byung Park[b]*, Robert Kourist[a]**

[a] Graz University of Technology, Institute of Molecular Biotechnology, Petersgasse 14, 8010 Graz, Austria

[b] Department of Food Science and Biotechnology, Ewha Womans University, Seoul 03760, Republic of Korea

[c] Graz University of Technology, Institute of Organic Chemistry, Stremayrgasse 9, 8010, Graz, Austria.

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## ABSTRACT

The regio- and stereoselective hydroxylation of unactivated C($sp^3$)-H bonds is an important reaction in organic synthesis. While bacterial alkane monooxygenase AlkB catalyzes the terminal hydroxylation of aliphatic esters with excellent regioselectivity, the molecular principles of substrate recognition and selectivity of this integral membrane enzyme are still poorly understood.

In this study, we investigated the substrate scope and engineered the medium-chain alkane monooxygenase from *Marinobacter* sp. (M_AlkB) for the terminal hydroxylation of linear and branched esters of fatty acids and alcohols. For the first time, we demonstrated the stereoselectivity of AlkB towards prochiral substrates containing terminal *gem*-dimethyl groups, leading to the corresponding chiral *β*-methyl primary alcohols in good optical purity (51-79% *ee*). The hydroxylation products can be further derivatized to chiral diols and lactones. Substitution of the highly conserved active site residue F169 to leucine increased the activity towards short and medium-chain esters up to two-fold. While the wildtype enzyme does not accept long-chain substrates, activity towards *n*-dodecyl acetate could be unlocked by reducing the size of the tryptophan residue 60 situated in the putative substrate tunnel. Substitution of the peripheral I238 with valine increased activity regardless of the chain length of the substrate. Our results lay the groundwork for the establishment of a whole-cell process for the regio- and stereoselective hydroxylation of linear and branched esters, leading to valuable bifunctionalized products. The insights gained from mutating key residues and the substrate acceptance of AlkB will guide future protein engineering campaigns.

## INTRODUCTION

The selective oxyfunctionalization of inherently inert C($sp^3$)–H bonds into more reactive C–O bonds is a highly important reaction in organic chemistry. It enables the synthesis of value-added compounds such as alcohols, epoxides, aldehydes, ketones, and carboxylic acids,[1–5] which can also be further functionalized to more complex molecules.[6–10] Challenges associated with the direct oxygenation of aliphatic C–H bonds include regio-, chemo-, and stereoselectivity, product yields, and sustainability.[1–5] Nature has evolved numerous enzymes, typically relying on metal cofactors, that are capable of selectively oxyfunctionalizing C($sp^3$)–H bonds of various substrates.[1,5,11,12] Many heme-dependent cytochrome P450s (CYP450) target subterminal ω-1 and ω-2 positions, with only a few being specific for the terminal position.[13–19] In contrast, the diiron non-heme alkane monooxygenase AlkB exhibits exceptional regioselectivity for the terminal hydroxylation of linear aliphatic compounds. AlkB and its electron transfer accessory proteins were first isolated from the soil bacterium *Pseudomonas putida* GPo1 (PpAlkB; recently renamed to *Ectopseudomonas oleovorans,* formerly known as *P. oleovorans*).[20–22] Since then, various homologs from other soil and marine bacteria have been identified.[23–30] AlkB is part of the *alk*-operon that enables bacteria to use *n*-alkanes as their sole energy and carbon source. AlkB catalyzes the initial ω-hydroxylation of *n*-alkanes to 1-alkanols, with two electrons provided by rubredoxin AlkG, which is in turn reduced by reductase AlkT (Figure 1A).[31–33] In addition to linear medium-chain hydrocarbons ($C_6$ to $C_{12}$), AlkB also acts on branched, cyclic, and aromatic compounds. Terminal olefin groups are typically converted to epoxides.[34–37] Recently, the dehalogenation of ω-fluorinated hydrocarbons to aldehydes by AlkB has been reported.[38,39] The well-studied PpAlkB has been used to hydroxylate esters of fatty acids (FAcE) and fatty alcohols (FAlE) for the production of polyester precursors.[40–45] The terminal oxyfunctionalization in conjunction with amination yields precursors

for polyamides.[46–48] The α,ω-oxyfunctionalized products also find applications in the pharmaceutical,[49] cosmetic[50] and agricultural industry.[51]

The challenges associated with the kinetic characterization of membrane-bound enzymes and the elucidation of their structures have hampered their engineering. The emergence of cryogenic electron microscopy (cryo-EM)[52–54] and *de novo* structure prediction tools[55,56] have significantly increased our structural understanding of membrane-embedded biocatalysts, allowing their engineering.[57] In the case of AlkB, only recently, the first structures of two homologs from *Fontimonas thermophila* (FtAlkB) were determined by cryo-EM.[52,53] However, the absence of high-throughput screening (HTS) assays for unnatural substrates has limited its engineering to random mutagenesis and selection based on the utilization of natural *n*-alkane substrates.[58,59]

In a recent study, we engineered PpAlkB to catalyze the crucial terminal hydroxylation of isoprenyl acetate in a multi-enzyme cascade towards the highly interesting polymer precursor tulipalin A.[60] AlkB exclusively acted on the terminal methyl group while leaving the adjacent *exo*-olefin intact. The insights from engineering PpAlkB could be transferred to AlkB from *Marinobacter* sp. (M_AlkB), which displayed the highest hydroxylation activity among several homologues.[60]

Although AlkB is known to hydroxylate (ω-1)-methyl-substituted alkanes, it prefers the linear terminus.[34] We assumed that the hydroxylation activity could be directed towards the prochiral methyl groups of the alkyl chain by protecting the linear end with a small ester group. The stereoselective hydroxylation of prochiral esters by AlkB leads to optically pure primary alcohols, thereby opening new pathways towards chiral hydroxy acids, diols, and lactones. These chiral compounds present interesting building blocks, not only for polymer production,[61–64] but also for pharmaceuticals.[65,66]

In this work, we aimed to shed light on the substrate scope, activity, and stereoselectivity of M_AlkB. Therefore, we investigated the effect of selected point mutations in the active site, substrate tunnel, and membrane-cytosol interface on the reactivity of M_AlkB towards a set of linear and branched esters of fatty acids and alcohols varying in the length of the alkyl chain (Figure 1B).

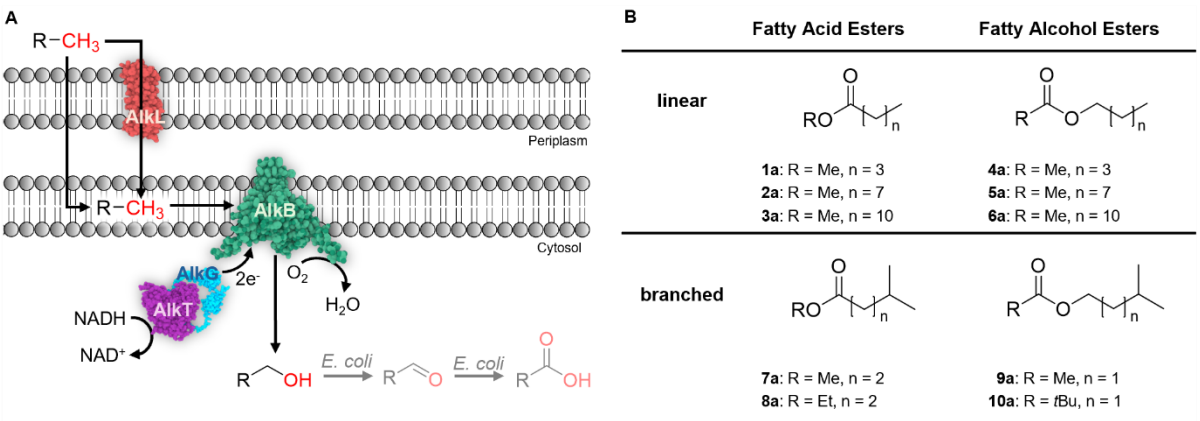


**Figure 1.** A) Terminal hydroxylation of aliphatic compounds by alkane monooxygenases AlkB and potential oxidation of the resulting alcohol to the aldehyde and carboxylic acid by the host background. B) Linear and branched esters investigated in this study.

## MATERIALS AND METHODS

### Chemicals

If not stated otherwise, all commercially available chemicals and compounds used in this work were purchased either from Merck/Sigma-Aldrich, TCI chemicals, or Carl Roth, usually in the highest purity available. The standard methyl 9-hydroxynonanoate was purchased from Ambeed (Arlington Heights, IL, USA). 5-methyltetrahydropyran-2-one and (5*R*)-tetrahydro-5-methyl-2H-pyran-2-one were purchased from Angene Chemical (Nacharam, India), 2-methylbutane-1, 4-diol

was purchased from Enamine Ltd. (Kyiv, Ukraine), and (2*R*)-2-methylbutane-1, 4-diol was purchased from ABCR (Karlsruhe, Germany).

**Cloning of the *alk*-operon**

The plasmid for the expression of *M_alkB* from *Marinobacter* sp. (M_AlkB; NCBI protein: MAB50652.1) was previously prepared by cloning synthetic DNA fragments from IDT into the *pPpalkBFGTL* vector via Gibson Assembly, replacing the *alkB*, while leaving the electron transfer system *alkFGT* from *P. putida* GPo1 intact.[60] Single and combinatorial mutants of *M_alkB* were either available in-house[60] or generated by site-directed mutagenesis (SDM) (Table S8). Successful mutagenesis was verified by Sanger sequencing (Microsynth, Vienna, Austria). Table S7 lists all the strains used in this study.

**Heterologous protein production and whole-cell biotransformations**

*E. coli* BL21(DE3) cells were transformed with the p*M_alkB*(mutant)*FGTL* expression constructs based on the pCom10 backbone, where the expression of the *alkBFGTL* operon is under the control of the natural alkane-inducible promoter $P_{alkB}$.[67] All substrates were tested in whole-cell biotransformation, using *E. coli* BL21(DE3) expressing *M_alkB*(mutant)*FGTL*, similar to previous work.[60] Briefly, a single colony of freshly plated *E. coli* containing the respective construct was used to inoculate Lennox lysogeny broth (LB; 5 mL) and incubated overnight at 37 °C with constant agitation (120 rpm). Adapted M9 minimal media (M9MM; 20 mL)[60] was inoculated with seed culture (200 µL) and incubated at 30 °C for 18 to 20 h. For heterologous gene expression, M9MM (200 mL) was inoculated to an $OD_{600}$ of 0.15 with the respective preculture, grown at 30 °C until an $OD_{600}$ of 0.4 to 0.5, and induced by adding 0.05% (v/v) dicyclopropyl ketone (DCPK). After four hours of expression at 30 °C, the cells were harvested (4,400 x *g*, 4 °C,

15 min) and resuspended in resting cell (RC) buffer (50 mM KPi, pH 7.4, 2 mM MgSO4, 1% glucose) to an $OD_{600}$ of 3.2 or 10 (corresponding to 1 $g_{CDW}$/L, and 3.1 $g_{CDW}$/L, respectively). The reaction was initiated by adding 2 mM of substrate (from an 80 mM stock solution in EtOH or DMSO) to the RCs and performed at 25 °C, 180 rpm, with a total volume of either 300 µL in 1.5 mL or 1 mL in 20 mL tightly sealed glass vials. For each sampling point, a separate reaction was prepared from a master mix. Samples (250 µL) were taken and quenched by adding 2 M HCl (25 µL) and stored at -20 °C until further analysis.

**Gas chromatography (GC) analysis**

To analyze biotransformation samples, the quenched samples (275 µL) were extracted with EtOAc (250 µL) containing 1 mM methyl benzoate as internal standard (ISTD) by vigorous shaking for 1 min. After phase separation by centrifugation (16,000 x *g*, 4 °C, 5 min), the organic layer was dried over $Na_2SO_4$, and the extract (200 µL) was directly subjected to GC analysis. To obtain the lactone **9e**, the biotransformation sample (300 µL) was quenched with 2 M HCl (30 µL), centrifuged (16,000 x *g*, 4 °C, 5 min), and the supernatant was incubated for 1 h at 80 °C, 200 rpm in a thermoshaker. 275 µL was then extracted with EtOAc (250 µL) containing ISTD as described. Chiral diols were analyzed by extracting non-quenched biotransformation samples (500 µL) with MTBE (250 µL). After the phases were separated by centrifugation (16,000 x *g*, 4 °C, 5 min), the organic phase was mixed with 10 M NaOH (2.5 µL), and the sample was incubated at 40 °C and 900 rpm for 24 h in a thermoshaker. The sample was dried over $Na_2SO_4$ and directly analyzed by GC.

A GC coupled to a mass spectrometer (GC-MS) was used for qualitative analysis of the reactions and product identification. The measurements were performed on a Shimadzu GC-MS-QP2010

SE (Shimadzu, Kyoto, Japan) equipped with a Zebron ZB-5Plus GC column (30 m × 0.25 mm × 0.25 µm, Phenomenex (Torrance, CA, USA)), using the method presented in Table S9. For quantitative analysis, the extracted reaction samples were analyzed on a GC with a flame ionization detector (GC-FID) from Shimadzu Nexis GC-2030 equipped with a Zebron ZB-5 capillary column (30 m × 0.25 mm × 0.25 µm) from Phenomenex (Torrance, CA, USA). Details on the GC-FID methods used for achiral and chiral analysis are provided in Tables S10-12. Due to the lack of authentic standards for **1b** and **3b**, their hydroxy products and overoxidized products were only tentatively identified by GC-MS. The concentrations of the analytes were calculated by external calibration curves generated by measuring samples with known concentrations of the pure compounds extracted from the RC buffer and treated equally to reaction samples. Enzymatic activities ($U/g_{CDW}$) were obtained from biological triplicates and calculated as µmol of product(s) formed per min and $g_{CDW}$. The data is represented as the arithmetic mean ± standard deviation (SD).

Chiral compounds were analyzed by GC-FID (Shimadzu Nexis GC-2030) using either a Hydrodex βTBDAc (50 m × 0.25 mm × 0.25 µm, Macherey-Nagel, GmbH & Co. KG, Düren, Germany) or a Hydrodex βTBDM (25 m × 0.25 mm × 0.25 µm, Macherey-Nagel, GmbH & Co. KG, Düren, Germany) column. Tables S11 and S12 provide detailed descriptions of the methods used.

## Results and Discussion

### Characterization of the alkane monooxygenase M_AlkB from *Marinobacter* sp.

Petroleum-contaminated areas show an enrichment of *alkB* genes and prove to be a vast source of *alkB* sequences.[68–70] Bacteria of the genus *Marinobacter* are highly abundant in such areas, and

are known for their ability to grow on alkanes, with most strains showing a preference for medium chain *n*-alkanes ranging from $C_6$ to $C_8$.[69,71,72] Here we characterize an AlkB from *Marinobacter* sp., which is derived from a metagenome sample,[73] and was shown to have higher hydroxylation activity compared to the well-described PpAlkB towards *n*-octane and isoprenyl acetate.[60] M_AlkB shares 78% sequence similarity with PpAlkB[22,33,74] and 66% and 51% similarity with the AlkBs from *Fontimonas thermophila* FtAlkB[53] and FtAlkBG,[52] respectively, whose structures have been recently solved by cryo-EM (Figure S2). *M_alkB* was expressed in *E. coli* BL21 (DE3) under the control of the natural alkane-inducible $P_{alkB}$ promoter encoded on the pCom10 plasmid[67] together with the redox partners *alkFGT* and the transporter *alkL* from *P. putida* GPo1 (Figure S5).[61] An initial substrate screening showed that M_AlkB converted esters of different chain lengths (**1a**-**6a**) to their respective hydroxy products (**1b**-**6b**; Figure S9).The mass balances of the biotransformations were not fully closed, which could be attributed to substrate or product volatility, hydrolysis, or losses during extraction steps. We observed that the fatty acid methyl esters (FAcME) **1a**-**3a** were partially hydrolyzed to the respective acids **1**-**3** (Figure S9A-C), which prevented efficient quantification of the enzymatic activity for **1a** and **3a**. FAcME hydrolysis was previously reported and attributed to endogenous esterases from *E. coli*.[41,43,46,75] In whole-cell reactions with the acetate esters **4a**-**6a** less hydrolysis was observed (Figure S9D-E). The conversion by the wildtype (WT) PpAlkB and M_AlkB of the $C_{12}$ chain esters **3a** and **6a** was very low. This follows the previously reported substrate preference for PpAlkB.[34,40,43] The substrate chain length accepted by M_AlkB fits in the range of medium-chain monooxygenases, which is in line with the reported activity towards *n*-octane and isoprenyl acetate.[60]

We determined the whole-cell activity for the nonanoic acid methyl ester **2a**,[43] and three fatty alcohol acetates (FAlAc) with varying chain lengths (**4a**, **5a**, and **6a**). M_AlkB showed comparable

activity to PpAlkB towards **2a** (5.8 U/$g_{CDW}$), whereas the activity towards medium-chain acetates was up to 8-fold higher, reaching 2.7 U/$g_{CDW}$ and 4.7 U/$g_{CDW}$ for **4a** and **5a**, respectively (Figure 2B-E). For **6a**, we observed very low conversions with both enzymes (Figure S9F), limiting activity calculations. Interestingly, when comparing the whole-cell oxygenation rates of methyl nonanoate **2a** with the nonyl acetate **5a** by PpAlkB, the conversion of **2a** was significantly faster than that of **5a** (Figure 2CE). In reactions with M_AlkB such a notable difference was not observed. Even though **2a** and **5a** have the same alkyl chain length and are sterically very similar, their hydrophobicity differs (Table S6). The logP value of **2a** and **5a** is 4.32 and 3.95, respectively. We assumed that hydrophobicity and partition coefficient of the substrates could influence the whole-cell hydroxylation rates, especially when considering that the substrate is expected to enter the substrate tunnel leading to the enzyme active site via the lipid bilayer (Figure 1A).[76] A potential reason why the two enzymes behave so differently with the structurally similar substrates **2a** and **5a**, could be due to the disparate residues in the four transmembrane helices lining the predicted substrate tunnels (Figure S3D).

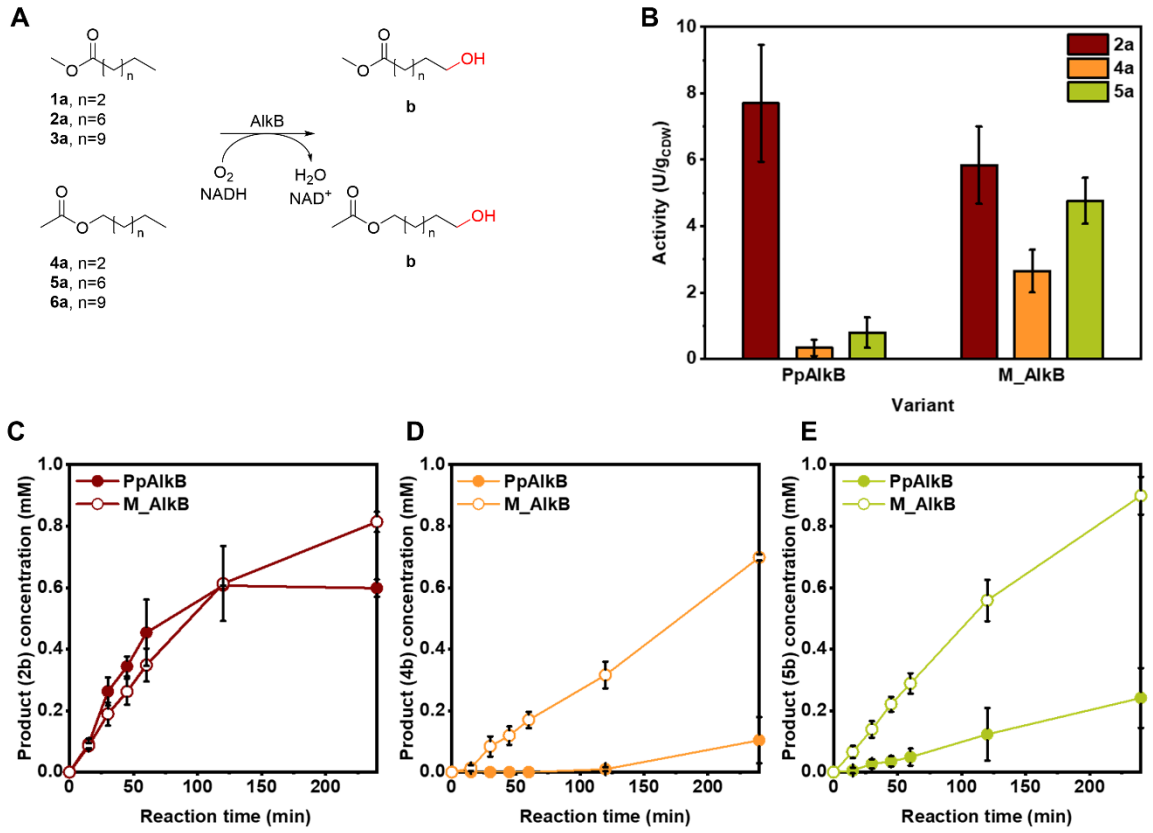


**Figure 2.** Biotransformation of linear FAcME and FAlAc by PpAlkB and M_AlkB. A) Tested substrates and expected products; only residual conversion of substrates **1a**, **3a**, and **6a** was observed. B) Activity of PpAlkB and M_AlkB towards FAcME **2a** and FAlAc **4a** and **5a**. Time-course of product formation for C) **2b**, D) **4b**, and E) **5b** by PpAlkB (filled circles) and M_AlkB (empty circles). The reactions were performed at 1 $g_{CDW}$/L (mean ± SD; $n$ = 3).

**Enzyme engineering for increased activity towards linear esters**

The growth assay developed by van Beilen[58,59] is not applicable for the esters **1a**-**6a** since they can be hydrolyzed by the cells and directly fed into the β–oxidation pathway, rendering their terminal hydroxylation unnecessary for their utilization as a carbon source. Therefore, we investigated the effect of point mutations of residues in the active site, the substrate tunnel, and the

periphery. Figure 3 highlights the residues targeted by mutagenesis in the 3D structure of M_AlkB generated by AlphaFold 3.[56] The putative substrate tunnel was predicted using CAVER Web 2.0 (Figure 3A).[77] The modeled enzyme consists of six transmembrane helices arranged in a slightly cone-like shape, opening towards the cytosol. Helices 2, 4, and 6 are close to the predicted substrate tunnel, similar to those described for FtAlkB[53] and FtAlkBG.[52] The predicted active site cavity is formed between the transmembrane helices and resembles a typical AlkB active site with the nine distinct, highly conserved histidines coordinating the two iron atoms essential for catalytic activity.[52,53,58] The distance between two iron atoms in the active site is 5.9 Å and is in accordance with distances previously observed in the cryo-EM structures of two homologues (5.4 Å for FtAlkB and 6.1 Å for FtAlkBG).[52,53] Interestingly, the two iron atoms are unusually far apart (>5 Å) with no apparent bridging ligand between them, still raising questions regarding the reaction mechanism.[52,53,76,78–81] By comparing the predicted structures of M_AlkB and PpAlkB, we observed several different amino acid (AA) residues close to the entrance tunnel (Figure S3D) that could explain the higher activities of M_AlkB towards medium-chain acetates **4a** and **5a** compared to PpAlkB (Figure 2).

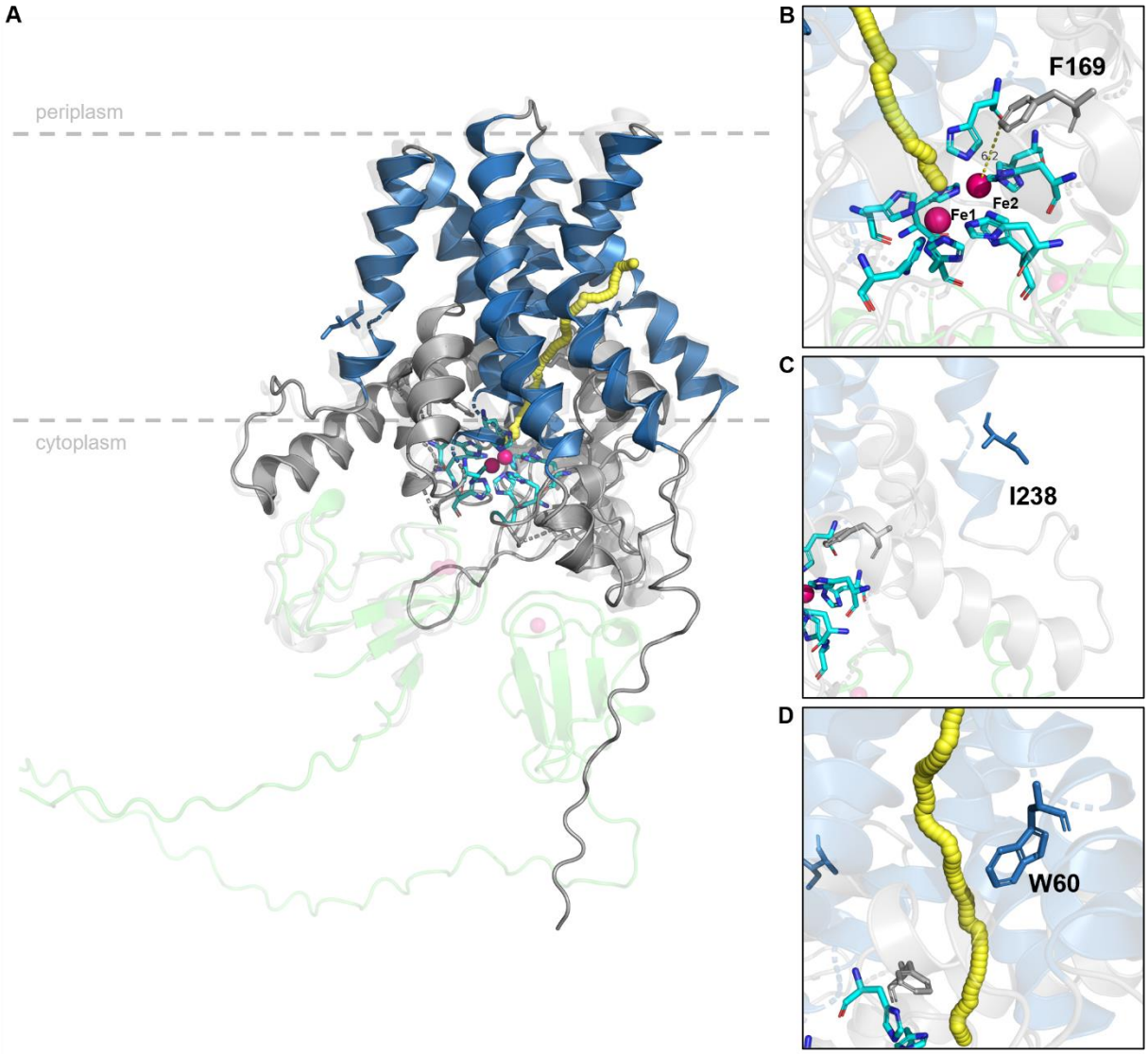


**Figure 3.** A) AlphaFold 3 predicted structure of M_AlkB (dark grey) with highlighted transmembrane domains (dark blue), active site histidine residues (cyano) coordinating two iron atoms (pink), and substrate tunnel (yellow). The interaction with PpAlkG (UniProt P00272, light green) was predicted by AlphaFold 3. The predicted structure of the protein complex was aligned with FtAlkBG (PDB 8f6t, light grey). AA residues targeted for site-directed mutagenesis: B) I238 in the transmembrane domain facing the lipid bilayer, C) F169 close to the active site, and D) W60 at the entrance of the predicted substrate tunnel.

To improve the enzyme's activity, several point mutations were tested (Figure 3B-D). Residue I238 is located close to the periplasm in the fifth predicted transmembrane helix, distant from the active site (Figure 3C). The analog substitution of I233 to valine in PpAlkB was identified in a random mutagenesis-based directed evolution as part of a triple mutant, which showed improved activity towards butane and pentane.[59] M_AlkB I238V showed higher activity towards *n*-octane and isoprenyl acetate.[60] As shown in Figure 4A, at a cell density of 1 $g_{CDW}$/L, the mutation I238V increased the hydroxylation activity towards **2a**, **4a**, and **5a**, resulting in 9.7, 3.5, and 5.6 U/$g_{CDW}$, respectively. Increasing the cell density to 3.1 $g_{CDW}$/L still led to higher activity compared to the wildtype enzyme, reaching 6.7, 6.4, and 7.4 U/$g_{CDW}$ for **2a**, **4a**, and **5a**, respectively (Figure 4B). Overall, this M_AlkB variant led to the highest product yields obtained after 2 h, resulting in 0.92 mM **2b** (Table S18). These results indicate that the I238V mutation indeed increases activity of AlkB towards linear esters in the whole-cell biocatalysts, independent of cell density and substrate chain length, which confirms results from previous engineering studies.[59,60]

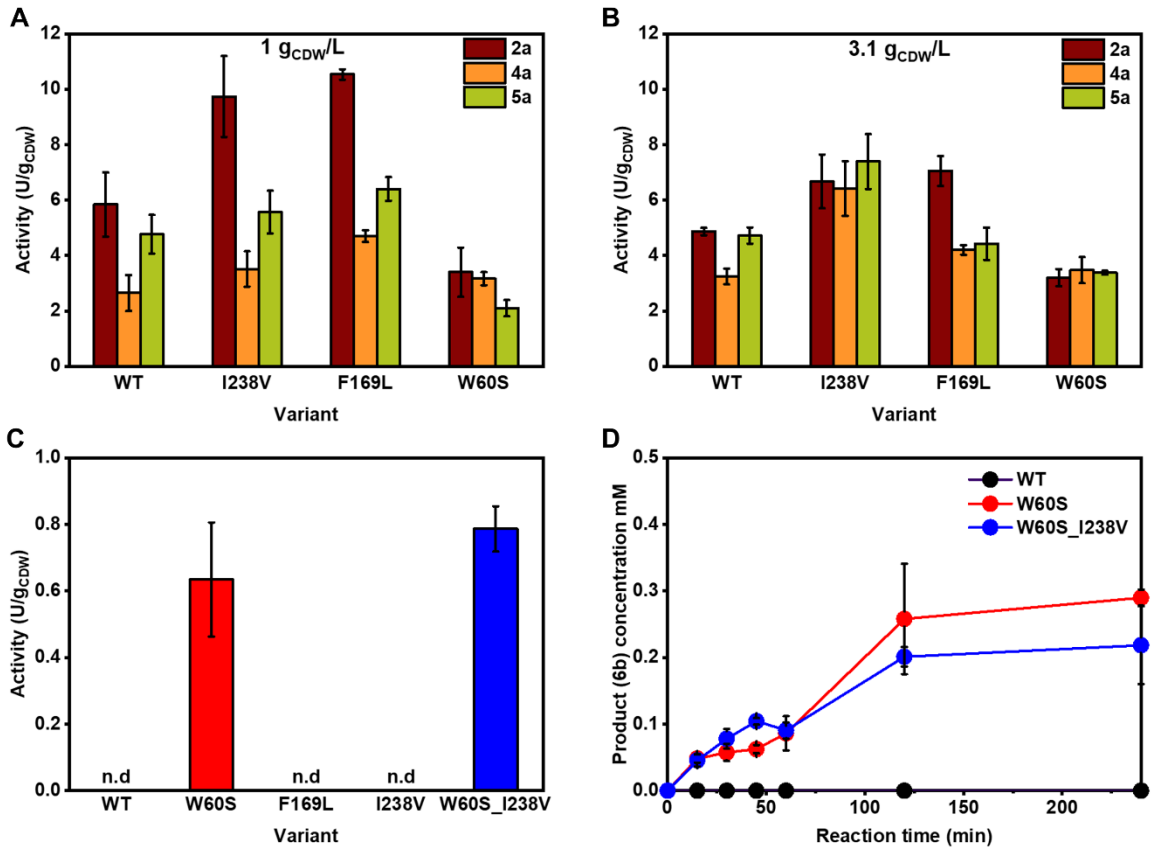


**Figure 4.** Hydroxylation activities of M_AlkB wildtype (WT) and variants using linear esters **2a**, **4a**, and **5a** at A) 1 $g_{CDW}$/L and B) 3.1 $g_{CDW}$/L. C) Activities and D) time-course of M_AlkB wildtype (WT) and W60S and W60S_I238V with **6a** as substrate at 3.1 $g_{CDW}$/L. (mean ± SD; $n$ = 3). n.d. not determined.

The residue F169 is situated in the active site, being 6.2 Å distant from the second Fe atom (Fe2 in Figure 3B). Its substitution to leucine was recently reported to improve the activity towards isoprenyl acetate.[60] This residue is highly conserved among homologs (99% F, Table S1). It is, therefore, interesting that a change to leucine led to improved activity for **2a**, with up to a 1.8-fold increase reaching 10.5 U/$g_{CDW}$. In contrast to I238V, the activity of F169L strongly depended on the cell density, with a pronounced activity increase at lower cell density (1 $g_{CDW}$/L).

In other AlkB homologs, the substitution of the tryptophan located at the entrance of the substrate tunnel (Figure 3D) in the second predicted transmembrane helix (W55 in PpAlkB, W62 in FtAlkB, W60 in M_AlkB) to a smaller amino acid, such as serine or valine, shifted the substrate acceptance of the monooxygenases towards longer substrates (> $C_{12}$).[53,58] The position of this tryptophan in the predicted tunnel leading from the membrane as a possible substrate reservoir to the active site suggests that this residue might act as a gate-keeper for long-chain substrates.[53,58] For the medium-chain esters **2a**, **4a**, and **5a**, the analogous mutation W60S in *M_alkB* did not lead to improved activity, but rather to a decreased hydroxylation rate, more pronounced at a lower cell density. However, this residue was tested, knowing that the acceptance of longer chain substrates might be unlocked. The variants I238V and F169L showed only traces of product formation in the conversion of **6a** (not shown), similar to the WT. As anticipated, M_AlkB W60S converted **6a**, reaching 0.63 U/$g_{CDW}$ after 2 h of the reaction at 3.1 $g_{CDW}$/L (Figure 4C, Table S18). In further testing with the M_AlkB double variant W60S_I238V, we reached an activity of 0.79 U/$g_{CDW}$ at 3.1 $g_{CDW}$/L, yielding 0.22 mM **6b** after 2h (Figure 4D, Table S18). Although the increase in activity by the double mutation was modest (1.25-fold), this result shows that the activity of AlkB towards longer chain substrates can be increased by combining W60S with other activity-enhancing amino acid substitutions.

Our results highlight that the knowledge gained by rational engineering can be transferred among AlkB homologs and that the effect on the activity towards alkanes,[53,58–60] can also influence the activity towards esters. For instance, the mutation I238V in *M_alkB* seems to improve the catalytic efficiency of the whole-cell biocatalyst independently of the substrate. The molecular effect of the peripheral substitution I238V remains to be clarified. Nevertheless, I238 is only 8.2 Å distant from a flexible loop closely situated to R215 and E232, which potentially play a role in the binding of

AlkG.[76,78] The limitation of M_AlkB towards longer chains could be unlocked via the single mutation W60S (0.63 U/$g_{CDW}$) and further enhanced in the double variant W60S_I238V, reaching 0.79 U/$g_{CDW}$ in the hydroxylation of the C12 substrate **6a**. This underlines the potential function of W60 as a gatekeeper in the substrate access tunnel.[53,58] Overall, our results demonstrate that the protein engineering of M_AlkB increased the conversion of linear $C_5$-$C_{12}$ esters, representing a new starting point to produce ω-hydroxy acetates and diols.

**Activity and stereoselectivity of M_AlkB WT and mutants towards branched esters**

AlkB monooxygenase has been previously reported to catalyze the allylic oxidation of sterically hindered substrates (Table S19), such as isobutene[82] and isoprenyl acetate.[60] We hypothesized that structurally similar substrates with a terminal *gem*-dimethyl group should also be converted, even though the steric hindrance is higher than that of the *exo*-olefin group. Due to the prochiral nature of the *gem*-dimethyl group, hydroxylation of one of the methyl groups would give rise to a chiral primary alcohol. May et al. reported the formation of enantiomerically enriched (*R*)-epoxides from 1-alkenes by PpAlkB.[36,83] In the conversion of terminally branched dimethyl alkanes, such as 2-methyl octane (**11a**), PpAlkB strongly preferred hydroxylation of the linear terminus.[34] Indeed, the selectivity of M_AlkB for the hydroxylation of the linear over the branched end was 36-fold, leading to **11b** with only traces of chiral **11c** observed (Figure S12, Table S17). van Nuland et al. showed that PpAlkB does not act on terminal methyl ester or acetyl ester groups.[43,45] We hypothesized that in an ester composed of a germinal dimethyl end and a methyl ester or acetyl ester end, the activity of AlkB would be directed to the prochiral group, albeit with lower activity. This would allow us to determine the capacity of AlkB to discriminate between the two methyl groups (Figure 5A). To confirm our hypothesis, we investigated the hydroxylation of several prochiral esters **7a** to **10a**, varying in their chain length and the protective ester group (Figure 5A).

The terminal hydroxylation of the branched FAcME **7a** would result in the chiral hydroxy fatty acid ester **7b**, which can easily be converted to the corresponding lactone **7e**. Similarly, terminal hydroxylation of the ethyl ester **8a** would result in the chiral ω-hydroxy fatty acid ester **8b**, which can be lactonized to **7e** (Figure 5A). On the other hand, terminal hydroxylation of the FAlE **9a** and **10a** would form the corresponding monoester of 2-methyl-1,4-butanediol (**9b**) (Figure 5A). We were pleased to see the formation of the corresponding hydroxy products **7b** and **9b** starting from prochiral **7a** and **9a**, respectively (Figure S13 and S14). The formation of **7b** was further confirmed by the presence of the corresponding lactone **7e**. The hydroxylation of **9a** to **9b** was confirmed by comparison with an authentic standard produced by the esterification of the diol **9g** using a *Pseudomonas cepacia* lipase. Initial testing in 1.5 mL vials at a cell density of 1 $g_{CDW}$/L led to very low conversion of the sterically demanding substrates. Henceforth, the biotransformations were conducted at 3.1 $g_{CDW}$/L in sealed glass vials of 20 mL, leading to increased product formation. To facilitate dissolution of the hydrophobic substrates, stocks were prepared in water-miscible cosolvents, ethanol or DMSO (both 2.5% (v/v)). Interestingly, in biotransformations with DMSO as a cosolvent, we observed further oxidation of the alcohol **9b** to the corresponding aldehyde **9c** and carboxylic acid **9d**, which was further lactonized to **9e** (74% *ee*) under acidic conditions (Figure S14B and S16C-D). The overoxidation is probably caused by alcohol dehydrogenases present in *E. coli.*[44] Contrarily, no overoxidation was observed when ethanol was used as a cosolvent.

Under optimized reaction conditions, M_AlkB WT showed an activity of 0.27 U/$g_{CDW}$ and 0.15 U/$g_{CDW}$ for the hydroxylation of **7a** and **9a**, which is 1.6-fold and 2.1-fold lower compared to PpAlkB (0.42 and 0.32 U/$g_{CDW}$, respectively). Interestingly, by extending the methyl to an ethyl moiety in **8a**, the activity was higher, reaching 1.08 U/$g_{CDW}$ for M_AlkB and 0.90 U/$g_{CDW}$ for

PpAlkB. This could be attributed to the higher logP of **8a** (2.44), compared to **7a** (2.08). We also considered further increasing the size of the ester moiety as a strategy to increase the activity and selectivity. However, the ester **10a** containing a *tert*-butyl group was barely converted (< 2%) (Figure S17), indicating that M_AlkB is rather limited in the conversion of bulky substrates.

Further on, we investigated the influence on the activity and selectivity of several M_AlkB mutants, which increased the enzymatic activity with linear esters (Figure 4). The M_AlkB variant I238V was slightly less active towards **7a**, reaching 0.17 U/$g_{CDW}$, and was more active towards **8a**, reaching 1.29 U/$g_{CDW}$. For **9a**, it displayed comparable activity with a WT of 0.19 U/$g_{CDW}$. Interestingly, the variant M_AlkB F169L, which showed higher activity with the sterically demanding isoprenyl acetate[60] and linear esters, showed only 0.06 U/$g_{CDW}$ for **7a**, while it reached 0.64 U/$g_{CDW}$ for **8a**, still less than the wildtype enzyme. While the phenylalanine to leucin substitution changes size and hydrophobicity,[81] this observed effect on the activity on the prochiral substrates is difficult to rationalize. Overall, the activity of M_AlkB and its variants was about 20-fold lower towards the sterically hindered esters compared to the conversion of linear esters of the same length, with activities ranging from 0.02 to 1.29 U/$g_{CDW}$. Although activities for all tested AlkB variants were low, using PpAlkB, we produced 0.54 mM of lactone **7e** (starting from 2 mM **7a**) after 24 h (Table 1).

PpAlkB and M_AlkB displayed similar % *ee* (51 and 47, respectively) with both preferentially forming (*R*)-**7e** lactone (Figure S15 and Table 1). The enantiomeric excess of the lactone **7e** formed by the M_AlkB variants I238V and F169L was comparable with 47% and 36% *ee*, respectively. We were wondering whether prolonging the methyl ester to an ethyl group affects the enantioselectivity. Reactions with **8a** still resulted in the preferred formation of the (*R*)-enantiomer. However, compared to the methyl ester **7a**, the *ee* decreased to 35% for the WT, while for the

F169L variant, the *ee* increased to 51% (Table 1). Higher stereoselectivity was observed for M_AlkB-catalyzed hydroxylation of the branched FAlAc **9a**, yielding 79% *ee* of (*R*)-**9b**. Base-catalyzed hydrolysis of **9b** to **9g** slightly reduced the *ee* to 74%, which is similar to results obtained with PpAlkB (81% *ee* (*R*)-**9b** and 74% *ee* (*R*)-**9g**). All tested variants showed similar stereoselectivity in the hydroxylation of **9a** (73-78% *ee*) compared to the WT. Table 1 and Table 2 provide a detailed overview of the activity and stereoselectivity of PpAlkB and M_AlkB wildtype and variants with the branched esters **7a**, **8a**, and **9a**.

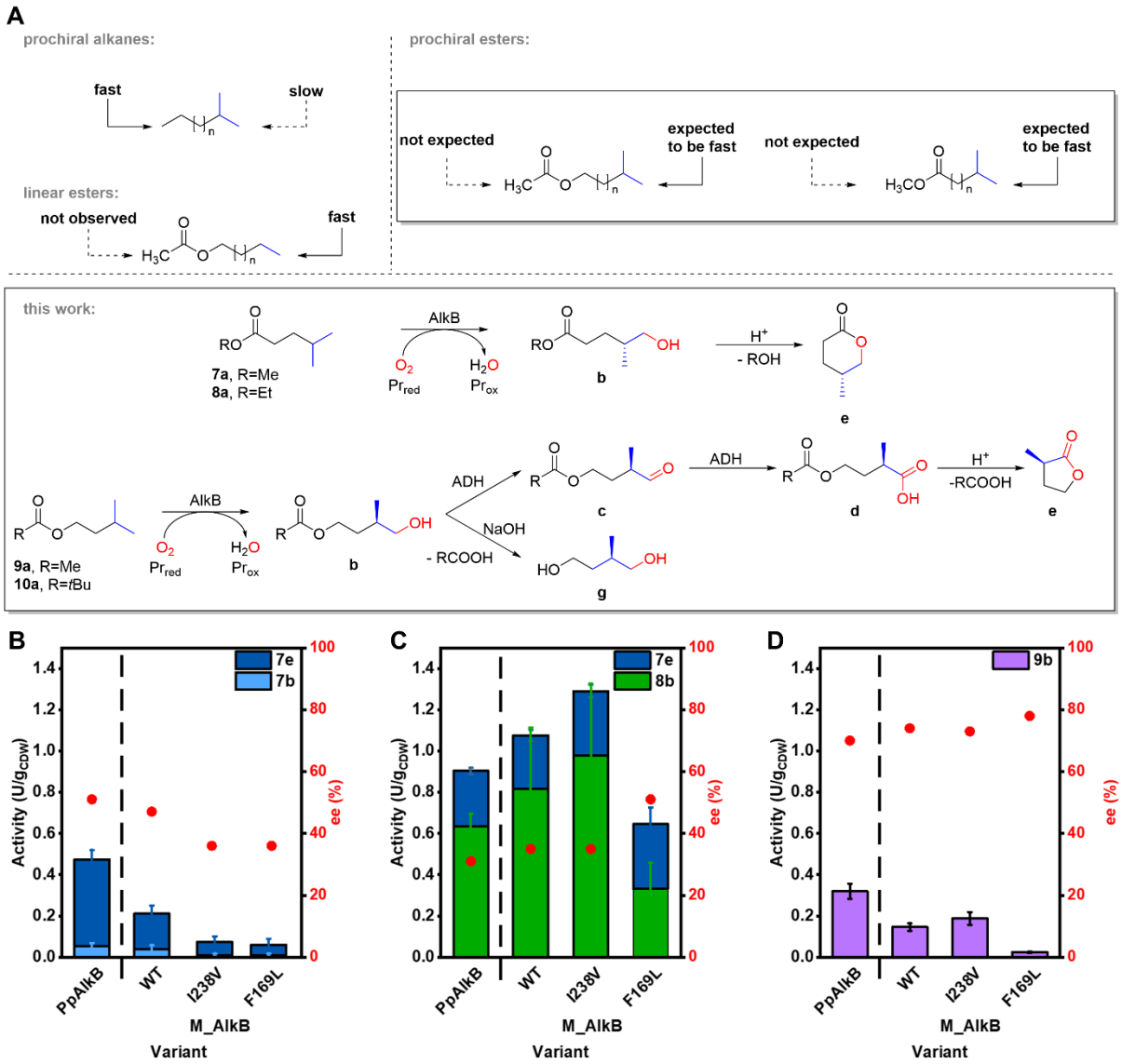

**Figure 5.** A) Rationale behind the route towards optically pure hydroxy acids, diols, and lactones via protection of the linear terminus with an ester group leading to stereoselective terminal hydroxylation of the prochiral *gem*-dimethyl group by AlkB. Hydroxylation activity and stereoselectivity of PpAlkB and M_AlkB variants performed at 3.1 $g_{CDW}$/L towards the branched esters B) **7a**, C) **8a**, and D) **9a**. Data represented as arithmetic mean ± SD ($n$ = 3).

**Table 1.** Summary of screening of AlkB WT and variants with the prochiral ester **7a** and **8a** at 3.1 $g_{CDW}$/L (mean ± SD; $n$ = 3).

| Substrate | Enzyme | Variant | Activity (U/$g_{CDW}$) | **7e** (mM)[a] | **7e** *ee* (%) |
|---|---|---|---|---|---|
| **7a** | PpAlkB | WT | 0.05 ± 0.01[b]<br>0.42 ± 0.05[c] | 0.54 ± 0.07 | 51 (*R*) |
| | M_AlkB | WT | 0.031 ± 0.001<br>0.27 ± 0.06 | 0.37 ± 0.04 | 47 (*R*) |
| | | I238V | 0.04 ± 0.02<br>0.17 ± 0.04 | 0.18 ± 0.03 | 47 (*R*) |
| | | F169L | 0.010 ± 0.007<br>0.06 ± 0.03 | 0.04 ± 0.001 | 36 (*R*) |
| **8a** | PpAlkB | WT | 0.63 ± 0.06[d]<br>0.27 ± 0.02[c] | 0.28 ± 0.05 | 31 (*R*) |
| | M_AlkB | WT | 0.82 ± 0.30<br>0.26 ± 0.03 | 0.31 ± 0.03 | 35 (*R*) |
| | | I238V | 0.98 ± 0.35<br>0.31 ± 0.03 | 0.27 ± 0.01 | 35 (*R*) |
| | | F169L | 0.33 ± 0.12<br>0.31 ± 0.08 | 0.25 ± 0.03 | 51 (*R*) |

[a]obtained after 24h, [b]product formation rate of **7b**, [c]product formation rate of **7e**, [d]product formation rate of **8b**.

**Table 2.** Summary of screening of AlkB WT and variants with the prochiral ester **9a** at 3.1 $g_{CDW}$/L (mean ± SD; $n$ = 3).

| Substrate | Enzyme | Variant | Activity ($U/g_{CDW}$) | **9b** (mM)[a] | **9b** *ee* (%) | **9g** *ee* (%) |
|---|---|---|---|---|---|---|
| **9a** | PpAlkB | WT | 0.32 ± 0.04 | 0.59 ± 0.02 | 81 (*R*) | 74 (*R*) |
| | M_AlkB | WT | 0.15 ± 0.02 | 0.22 ± 0.02 | 79 (*R*) | 74 (*R*) |
| | | I238V | 0.19 ± 0.03 | 0.34 ± 0.01 | 79 (*R*) | 73 (*R*) |
| | | F169L | 0.02 ± 0.01 | 0.04 ± 0.01 | 67 (*R*) | 78 (*R*) |

[a]obtained after 24h.

Stereoselectivity of PpAlkB was previously shown only for epoxidation of terminal alkenes with *pro-R* stereoselectivity[36,83] whereas its capacity to discriminate between different alkyl groups of branched hydrocarbons remained unknown. By using a protection strategy, we suppressed the competing hydroxylation of the linear terminus of the substrate and directed the hydroxylating activity towards the prochiral group. PpAlkB and M_AlkB are *pro-R* selective in the hydroxylation of the *gem*-dimethyl group, leading to the production of chiral lactone **7e** and chiral diol **9g**. The chiral synthon **7e** is used for the synthesis of active enantiomers of the pheromones of pine sawflies, important for pesticide control.[51] Interestingly, a previously reported chemosynthetic synthesis of (*R*)-**7e** was based on functionalizing common natural monoterpenoid L-(-)-menthol.[84] Our results demonstrate a new biocatalytic route starting from cheap branched esters towards chiral diols and lactones that can be further derivatized into a vast number of chiral building blocks. Future efforts will be directed to the identification of amino acid residues directing activity and selectivity towards these substrates and further improving the activity of the monooxygenase by enzyme engineering.

## Conclusion

Alkane monooxygenases show unique selectivity in the terminal hydroxylation of a wide range of substrates. The lack of accurate structural models has so far hampered the engineering of these interesting biocatalysts. Herein, we demonstrated the rational engineering of M_AlkB, isolated from a metagenomic dataset, to improve enzymatic activity towards the synthesis of valuable hydroxylated aliphatic esters. With these results, we gained insight into the substrate preference and stereoselectivity of M_AlkB, an enzyme that appears to be particularly interesting for the conversion of short to medium-chain esters. Using a protection strategy enabled us, for the first time, to characterize the stereoselectivity of alkane monooxygenase towards prochiral terminal *gem*-dimethyl group. This provided a new enzymatic reaction pathway for the synthesis of chiral lactones, hydroxy acids, and diols in good optical purity. We expect that the herein presented results on the substrate scope and mutagenesis of M_AlkB will guide further enzyme engineering studies of bacterial membrane-bound alkane monooxygenases.

## AUTHOR INFORMATION

### Corresponding Authors

*kourist@tugraz.at

*jbpark06@ewha.ac.kr

### Author Contributions

J.S., A.N. and H.J. devised the study and contributed experiments, C.L.K., S.G., E.v.P., L. M-Y. conducted experiments, J.-B. P. and R.K. devised the study. The manuscript was written through

the contributions of all authors. All authors have approved the final version of the manuscript. ‡These authors contributed equally.

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**Supporting information**

Includes additional experimental details, data on modeling and activities, as well as supporting GC chromatograms.

**ABBREVIATIONS**

**AA** amino acid
**CDW** cell dry weight
**cryo-EM** cryogenic electron microscopy
**CYP450** cytochrome P450
**DCM** dichloromethane
**DCPK** dicyclopropyl ketone
**DMAP** 4-dimethylaminopyridine

**DMSO** dimethyl sulfoxide

***E. coli*** *Escherichia coli*

**EtOAc** ethyl acetate

**EtOH** ethanol

**FAcE** esters of fatty acids

**FAcMe** fatty acid methyl ester

**FAlAc** fatty alcohol acetate

**FAlE** esters of fatty alcohols

**FID** flame ionization detector

**FtAlkB** AlkB from *Fontimonas thermophila*

**FtAlkBG** fused AlkB AlkG from *Fontimonas thermophila*

**GC** gas chromatography

**HTS** high-throughput screening

**ISTD** internal standard

**LB** Lennox lysogeny broth

**LPS** *Pseudomonas cepacia* lipase

**M_AlkB** AlkB from *Marinobacter* sp.

**M9MM** adapted M9 minimal media

**MS** mass spectrometer

**NADH** nicotinamide adenine dinucleotide

**NMR** nuclear Magnetic Resonance

**$OD_{600}$** optical density at 600 nm

**PpAlkB** AlkB from *Pseudomonas putida* GPo1

**RC** resting cell

**ROP** ring opening polymerization

**Rt** retention time

**SD** standard deviation

**SDM** site-directed mutagenesis

**WT** wildtype

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