Alkyl Formate Ester Synthesis by a Fungal Baeyer–Villiger Monooxygenase

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We investigated Baeyer–Villiger monooxygenase (BVMO)-mediated synthesis of alkyl formate esters, which are important flavor and fragrance products. A recombinant fungal BVMO from *Aspergillus flavus* was found to transform a selection of aliphatic aldehydes into alkyl formates with high regioselectivity. Near complete conversion of 10 mm octanal was achieved within 8 h with a regiomeric excess of ~80%. Substrate concentration was found to affect specific activity and regioselectivity of the BVMO, as well as the rate of product autohydrolysis to the primary alcohol. More than 80% conversion of 50 mm octanal was reached after 72 h (TTN nearly 20 000). Biotransformation on a 200 mL scale under unoptimized conditions gave a space-time yield (STY) of 4.2 g L^{-1} d^{-1} (3.4 g L^{-1} d^{-1} extracted product).

Alkyl formate esters are valuable compounds for the flavor and fragrance industry, and alkyl formates such as heptyl formate and octyl formate possess fruity flavors.[1] More recently they have attracted attention for use as the organic phase in two-liquid phase systems in oxidoreductase biocatalysis,[2, 3] as they can at the same time serve as substrates for co-factor regeneration by formate dehydrogenase.[4] Typically, alkyl formates are chemically synthesized through esterification of formic acid and primary alcohols, or through reacting alkyl halides with formamide.[1] Lipases have been used to perform similar transesterification,[5] specifically of ethyl formate with 1-octanol to produce the corresponding formic acid ester.[3] Because of the equilibrium constant of the transesterification reaction, yields of less than 50% octyl formate are obtained. The yield could be improved to 90% (relative to the alkanol), although only through the use of excess ethyl formate.

Baeyer–Villiger monooxygenases (BVMOs) are flavoproteins that convert ketones and cyclic ketones into esters and lactones, respectively, by using molecular oxygen and NAD(P)H under mild conditions.[6–9] In addition, BVMOs have been shown to catalyze heteroatom oxidation,[10–12] epoxidation of alkenes,[13] and oxidation of aldehydes.[14–16] Conversion of the aromatic aldehydes benzaldehyde and 4-hydroxybenzaldehyde results in carbon migration to form the corresponding ester, with autohydrolysis to the phenol product.[17] The distance between the aldehyde group and the benzene ring also affects

carbon versus hydrogen migration, thus forming either the acid or the ester product, respectively. Phenylacetaldehyde is converted with low selectivity for the acid product by cyclohexanone monooxygenase (CHMO) and BVMO4 from *Dietzia* sp. D5,[14, 15] whereas BVMO4 converted 3-phenylpropionaldehyde exclusively to the acid product.[15] Substitutions on either the benzene ring or alkyl chain do however affect the regioselectivity.[16, 18, 19] It was recently demonstrated through mutational studies on CHMO that regioselectivity is mediated by substrate positioning in the active site due to steric constraints.[20]

However, to the best of our knowledge, all BVMOs tested with aliphatic aldehydes yielded the corresponding carboxylic acids.[14, 15] We therefore decided to test our library of recombinant fungal BVMOs from *Aspergillus flavus* expressed in *Escherichia coli* (Figure S2 in the Supporting Information) against aliphatic aldehydes with octanal as the test substrate (Figure S3). Whole-cell and cell-free extract (CFE) biotransformations of aldehydes are complicated because of the high background activity of endogenous *E. coli* (host) enzymes. Aldehydes such as octanal are rapidly oxidized by aldehyde dehydrogenases/oxidases to their corresponding fatty acids. Nevertheless, CFE screening of the recombinant fungal BVMOs revealed five BVMOs able to produce heptyl formate from octanal. In aqueous medium, the alkyl formate undergo spontaneous decarbonylation to an alcohol through hydrolysis by a water molecule, which adds to the carbonyl group of the ester with small amounts of heptan-1-ol therefore detected. Of the five BVMOs, BVMO$_{AFL838}$ produced the highest concentration of heptan-1-ol. BVMO$_{AFL838}$ has previously been shown to catalyze the conversion of various 2-alkanones with absolute regioselectivity to the corresponding alkyl acetate products.[21, 22]

In order to investigate the substrate specificity and selectivity of BVMO$_{AFL838}$ towards aldehydes, the enzyme was purified to homogeneity, and biotransformations were performed with *Bacillus megaterium* glucose dehydrogenase (*Bm*GDH) for co-factor regeneration. Gratifyingly, all the aldehydes tested (Scheme 1) yielded the corresponding formate esters (Table 1).

Complete or near-complete conversions of valeraldehyde (**1 a**) and hexanal (**2 a**) and more than 80% conversion of octanal (**3 a**) were obtained within 2 h. These products were slowly hydrolyzed to primary alcohols, with the shorter chain-length alkyl formates giving more of the hydrolyzed alcohol products. This could be attributed to the higher solubility of the shorter-chain alkyl formates in the aqueous phase or simply to the slower reaction rates of the longer-chain aldehydes. Extractions were performed without acidification, thus yielding higher effective regiomeric excesses because of the lower solubility of

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Supporting information for this article can be found under:
http://dx.doi.org/10.1002/cbic.201600684.
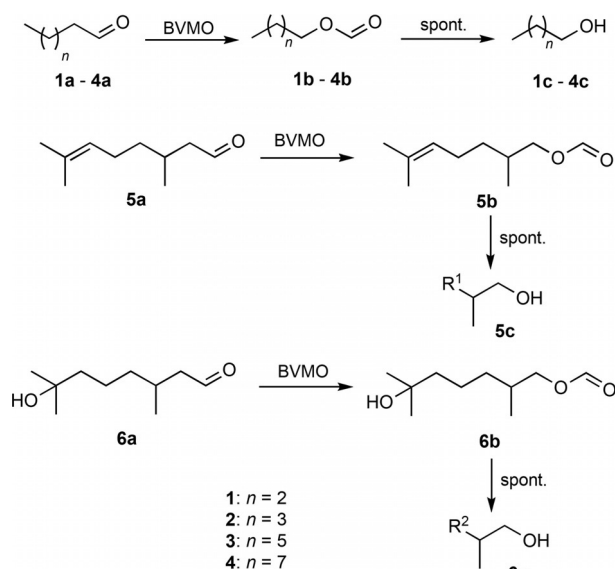
Scheme 1. Biotransformations of aliphatic aldehydes (**a**) into alkyl formate esters (**b**) and their spontaneous hydrolysis to alcohols (**c**). Detailed reaction scheme given in Scheme S1.

Table 1. Conversion of aliphatic aldehydes (**a**, 10 mM) to alkyl formate esters (**b**) and their spontaneous hydrolysis to alcohols (**c**) by using purified $BVMO_{AFL838}$ (2 μM) at 20 °C and BmGDH for co-factor regeneration.

Substrate	Conversions [%]		RE[a] [%]
	2 h	8 h	
1a	>99 (11% **1c**)	>99 (37% **1c**)	95
2a	98 (11% **2c**)	99 (37% **2c**)	93
3a	84 (5% **3c**)	97 (15% **3c**)	82
4a	69 (1% **4c**)	93 (2% **4c**)	66
5a	98 (1% **5c**)	>99 (4% **5c**)	99
6a	64 (12% **6c**)	76 (42% **6c**)	99

[a] Regiomeric excess (RE) in organic phase = (GC areas of ester product + alcohol from autohydrolysis − acid product)/(total GC areas of oxygenated products).

the fatty acid anions at high pH in organic solvents (Table 1). The effective regioselectivity decreased with increasing chain length, with decanoic acid constituting approximately 18% of the total oxygenated products from decanal (**4a**). Branched-chain aldehydes citronellal (**5a**) and 7-hydroxycitronellal (**6a**) were converted exclusively into the corresponding formate esters. The autohydrolysis rate of **6b** was significantly higher than that of **5b**, although the specific activity was lower, thus supporting the argument that hydrolysis rate correlates with water solubility.

Steady-state kinetic studies with octanal revealed maximum reaction rates lower than those observed for octan-2-one and octan-3-one, but its affinity (K_M) was between those of octan-2-one and octan-3-one.[21] Substrate inhibition was also less pronounced for octanal than octan-2-one (Figure S4).

In order to investigate the usefulness of $BVMO_{AFL838}$, we performed time-course reactions with increasing substrate concentration. Overall, the reaction rates decreased significantly

after 8 h, but activity was still observed after 24 h. Turnover frequencies (TOFs) decreased with increasing substrate concentration, but approximately 80% conversion was still achieved from 50 mM octanal after 72 h (Figure 1), with extracted yields of 20 mM (2.8 g L^{-1}) heptyl formate (calculated to represent 24 mM or 3.5 g L^{-1} in the biotransformation reaction mixture (BRM)). Total turnover number (TTN) exceeded 12 000 with respect to total oxygenated products extracted, and nearly 20 000 with respect to products in BRM.
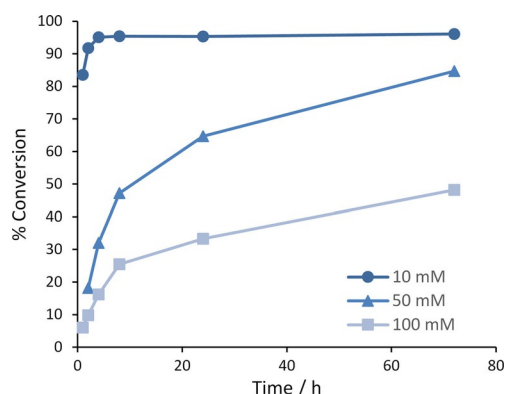

Figure 1. Conversion (sum of total oxygenated products) of octanal. Reaction conditions: 1 mL reaction volumes, containing 2 μM BVMO, 1 U BmGDH, 100 mM glucose, 0.3 mM NADP$^+$, 10–100 mM octanal, 1–2% (v/v) methanol as co-solvent, 100 mM Tris·HCl (pH 8), 20 °C, 200 rpm.

The regioselectivity of $BVMO_{AFL838}$ was also negatively affected by increased substrate concentration, with octanoic acid constituting approximately 20 and 30% of the total extracted oxygenated products from 50 and 100 mM octanal, respectively. Interestingly, the regioselectivity increased during the reaction as the effective concentration of substrate decreased (Figure S7). Bisagni and co-workers similarly found that both substrate concentration and enzyme concentration influence the regioselectivity of BVMO-mediated oxygenation of 2-phenyl-propionaldehyde.[15] Surprisingly, the rate of autohydrolysis of the heptyl formate decreased with increasing substrate concentration: whereas nearly 90% of the heptyl formate was converted into heptan-1-ol after 72 h during the biotransformation of 10 mM octanal, only approximately 4% of the heptyl formate in the 50 mM reactions (<1% in 100 mM) was hydrolyzed to heptan-1-ol (Figures 2 and S5–S7).

Encouraged by these results, we performed a 200 mL scale synthesis of heptyl formate under these unoptimized conditions. Biocatalyst concentration was increased from 2 to 5 μM, with 50 mM octanal added neat. Protein precipitation was already noticeable within the first few hours of the biotransformation. Nevertheless, the heptyl formate product constituted approximately 60% of the extracted reaction mixture after 24 h (Figure S8): 0.42 g heptyl formate in BRM, with a space–time yield (STY) of 4.2 g L^{-1} d^{-1} (3.4 g L^{-1} d^{-1} extracted product).

In summary, we have, for the first time, demonstrated BVMO-mediated synthesis of alkyl formate esters from aldehydes. Activity, selectivity, and autohydrolysis of the product
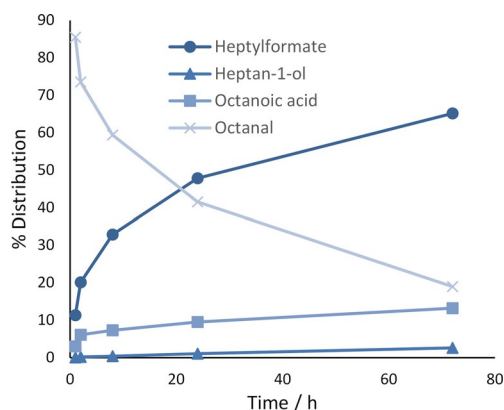
Figure 2. Time-resolved distribution of octanal and oxygenated products extracted during biotransformation of 50 mm octanal.

depend on substrate concentration. We are currently investigating two-phase liquid systems, as we anticipate this will decrease the effective concentration of the substrate to the enzyme for improved selectivity, as well as serve as an organic reservoir for the product to circumvent product hydrolysis. Alternatively, improved product hydrolysis could serve as a route to natural uneven chain-length primary alcohols, similar to studies combining BVMOs with esterases to yield primary alcohols from ketones.[23, 24]

Experimental Section

All substrates were obtained from Sigma–Aldrich and used without any further purification.

BVMOs were expressed from pET22b(+) (Novagen) in *E. coli* BL21-Gold (DE3) (Stratagene/Agilent Technologies) and purified as previously described.[22] Glucose dehydrogenase from *B. megaterium* (pET28:*Bm*GDH) was similarly expressed and purified to homogeneity by Ni-affinity chromatography and desalted on a PD10 column (GE Healthcare).[22] For *Bm*GDH 1 U is defined as the amount of enzyme that converts 1 µmol of $NADP^+$ in 1 min at 20 °C (in Tris·HCl (100 mm pH 8) with glucose (100 mm)).

Biotransformations (1 mL) were performed in 40 mL capped amber glass vials. The mixtures comprised purified BVMO (2 µm, 0.13 mg mL^{-1}), *Bm*GDH (1 U), glucose (100 mm), $NADP^+$ (0.3 mm), octanal (10–100 mm), and methanol (1–2%, *v/v*; co-solvent) in Tris·HCl (100 mm, pH 8). Reactions were performed at 20 °C, and the content was extracted by using equal volumes of ethyl acetate containing 1-undecanol (2 mm) as an internal standard. The 200 mL scale synthesis was performed in a capped 500 mL Schott bottle. The mixtures comprised purified BVMO (5 µm, 0.32 mg mL^{-1}), *Bm*GDH (1 U mL^{-1}), glucose (100 mm), $NADP^+$ (0.3 mm), and octanal (50 mm) in Tris·HCl (100 mm, pH 8). Reactions were performed for 24 h and extracted twice with ethyl acetate (50 mL) containing 1-undecanol (2 mm) as an internal standard. The organic phases were combined, and the extracted products were quantified against authentic standards. GC-FID and GC-MS were carried out on a TRACE GC Ultra (Thermo Fisher Scientific)

with a FactorFour VF-5ms column (60 m×0.32 mm×0.25 µm, Varian).

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