

Enzyme-Catalyzed Synthesis of Esters in Water

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DOI

[10.1002/cctc.201801991](https://doi.org/10.1002/cctc.201801991)

Publication date

2019

Document Version

Final published version

Published in

ChemCatChem

Citation (APA)

Mestrom, L., Claessen, J. G. R., & Hanefeld, U. (2019). Enzyme-Catalyzed Synthesis of Esters in Water. *ChemCatChem*, 11(7), 2004-2010. <https://doi.org/10.1002/cctc.201801991>

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10th Enzyme-Catalyzed Synthesis of Esters in WaterLuuk Mestrom,^[a] Jord G. R. Claessen,^[a] and Ulf Hanefeld*^[a]

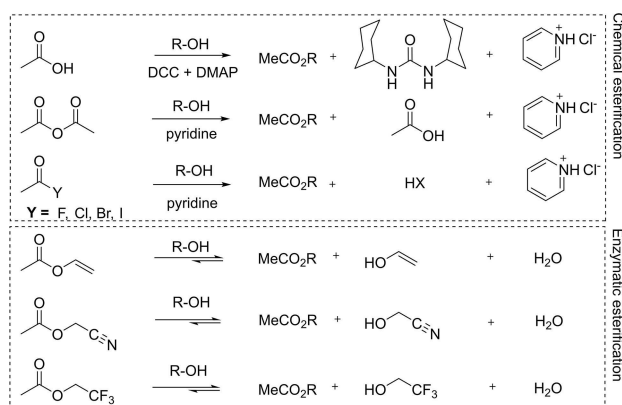
MsAcT catalyzes the esterification of primary alcohols in water. When utilizing acid and alcohol as starting materials low yields dictated by thermodynamics were observed. However, with activated esters such as ethyl acetate and vinyl acetate very high yields of the desired ester can be achieved in combination with the appropriate alcohol. This study investigated both the intrinsic kinetic properties of MsAcT for the hydrolysis and

transesterification of esters in water as well as the thermodynamics of the reaction. In comparison to the chemical or enzymatic ester synthesis using either toxic reagent, and harsh organic solvents, the MsAcT-catalyzed synthesis of esters of primary alcohols can be achieved efficiently in water without neutralization steps.

Introduction

The synthesis of esters is textbook-knowledge and is performed according to standard protocols that are taught unaltered already for many decades.^[1] An activated acid, i.e. an acid chloride or anhydride, reacts with an alcohol in the presence of a base, typically pyridine, often catalyzed by DMAP, in an organic solvent. Alternatively, the acid is activated *in situ* with reagents such as DCC. In all cases considerable amounts of waste are generated: the solvent and stoichiometric amounts of salt from neutralization steps. Furthermore, the activation, *in situ* or not, also generates considerable amounts of waste. The direct esterification catalyzed by an acid requires azeotropic removal of water to shift the equilibrium towards ester formation and the reaction conditions are so drastic that it is limited to stable starting materials and products. Moreover, it still leaves the problem of the organic solvent and the neutralization steps generating salt waste.^[1a-c] Similarly the enzyme catalyzed synthesis of esters is performed in dry organic solvents with activated acids (acyl donors), often generating much waste which needs to be removed in additional work up steps (Scheme 1).^[2] Since the activated acids in the enzyme catalyzed ester synthesis are esters themselves, these reactions are transesterifications.

To address the problem the acyltransferase from *Mycobacterium smegmatis* (MsAcT) was employed as catalyst.^[3] This enzyme was described to enable the synthesis the esters in water. With MsAcT it should thus be possible to avoid the use of organic solvents and to eradicate the need for neutralization



Scheme 1. Textbook chemical and enzymatic synthesis strategies for esters.

steps commonly employed to remove the base utilized in ester syntheses. In all cases described to date, the ester synthesis catalyzed by MsAcT is strictly speaking a transesterification since the reactions all utilize ethyl acetate or more reactive acid derivatives.^[3] While this is similar to the classical ester synthesis which utilizes acid chlorides or anhydrides it raises the question whether MsAcT catalyzes the direct synthesis of esters, too. This has been described for lipases and other catalysts,^[4] but in all those examples the yield was determined by the thermodynamic stability of the product under reaction conditions. Therefore, the direct esterification and transesterification of alcohols catalyzed by MsAcT in water was studied in parallel. As a catalyst MsAcT should accelerate the reaction but not alter its overall equilibrium.^[5] High transesterification yields with MsAcT can only be obtained under kinetic control using activated acids.

MsAcT is known to be an excellent catalyst for the transesterification of primary alcohols and amines with a variety of acyl donors.^[3d,6] Benzyl alcohol and isobutyl alcohol were chosen for this study in combination with acetic acid in the form of potassium acetate buffer or ethyl-, vinyl- and phenyl-acetate as activated acids. The kinetic properties of MsAcT were investigated for both the hydrolysis and transesterification rate of vinyl acetate. This acyl donor irreversibly tautomerizes upon

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Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cctc.201801991>

This manuscript is part of the Anniversary Issue in celebration of 10 years of ChemCatChem.

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hydrolysis, simplifying the reaction kinetics of the transesterification.

Results and Discussion

To establish the thermodynamic equilibrium of the reaction and to probe whether MsAcT had the potential to overcome the macroscopic thermodynamic forces of the reaction^[3b] the direct esterification of benzyl alcohol with acetic acid at pH 7.5 in potassium phosphate buffer (KPi) was studied. Investigation of both the synthesis (10 mM benzyl alcohol) and hydrolysis (10 mM benzyl acetate) reaction catalyzed by MsAcT revealed a final concentration of benzyl acetate of 0.12 mM. A K_{eq} of 0.00012 could thus be established. The uncatalyzed control reactions yielded essentially no product. MsAcT is thus a classical catalyst, catalyzing a reaction but not altering it.

To prepare the desired ester it is therefore necessary to employ activated acids. As was demonstrated earlier this can be simple ethyl acetate, a benign solvent that can be used monophasic, i.e. dissolved in water, or as a separate layer.^[3] Here, all studies were performed under monophasic conditions with the acyl donor dissolved in the pH 7.5 KPi buffer. In order to characterize the affinity and activity of activated acyl donor ethyl, vinyl and phenyl acetate to MsAcT, the optimal enzyme concentration was investigated. At less than 50 ng mL⁻¹ MsAcT the conversion of benzyl alcohol to benzyl acetate was not complete. In line with earlier observations,^[3d] a significant amount of benzyl acetate was hydrolyzed again at high enzyme concentrations (Figure 1). The trend in conversion of vinyl

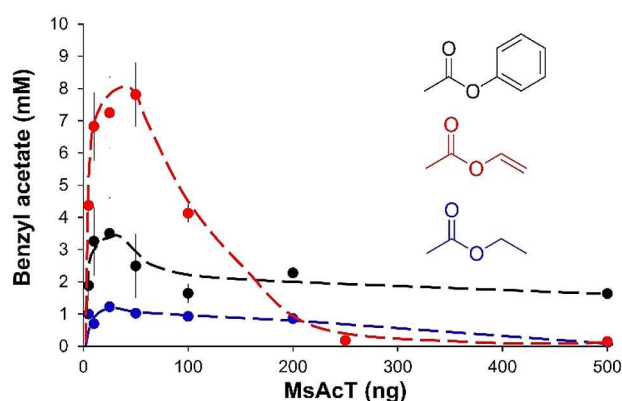


Figure 1. Transesterification of benzyl alcohol with the acyl donor vinyl acetate (red), phenyl acetate (black), and ethyl acetate (blue) using different MsAcT concentrations. **Reaction conditions:** KPi buffer (200 mM, pH 7.5), 10 mM benzyl alcohol, 100 mM acyl donor, 5–500 ng MsAcT, 1 mL, 1000 rpm, 1 hour, 21 °C. The experiment was performed in triplicates and the error bars show the standard deviation.

acetate > phenyl > ethyl acetate after one hour of reaction time was in line with the reactivity of the acyl donor.^[2a,b] Vinyl acetate clearly gave the highest conversion for ester synthesis. Moreover, it is synthesized via an atom efficient catalytic process from acetic acid and ethylene, making its production environ-

mentally benign.^[7] Additionally it is very readily available because it is a monomer for polymer synthesis. Also, the side product acetaldehyde does not require special work up steps in laboratory scale. On industrial scale measures to handle it are well established.^[8] Therefore, this activated acyl donor is relatively environmentally benign. It does not require neutralization steps in down-stream processing nor are toxic compounds produced at a large scale. The characterization of this acyl donor was pursued in more detail in water to also avoid organic solvents necessary when used in combination with lipases.

To firmly establish all possible products and side products, such as hemiacetal esters, *in-situ* ¹H-NMR analysis was performed. In a previous study, we have shown that MsAcT catalyzes the transesterification of isobutanol in water.^[3d] Based on those results and since transesterification of benzyl alcohol and vinyl acetate could not be followed via *in-situ* ¹H-NMR due to overlapping NMR signals, the acylation of 11 mM isobutanol with 212 mM vinyl acetate with less than a $\mu\text{g mL}^{-1}$ of MsAcT was studied. Full conversion of isobutanol toward isobutyl acetate was observed^[3d] and no ester of the *gem*-diol was observed (Figure S6–S9). Next, the substrate concentration was increased to 97 mM isobutanol and 20-fold more MsAcT was added. Under these conditions the starting materials are all soluble, but the product will be insoluble in water. During the course of the reaction a second organic phase appeared due to the insolubility of isobutyl acetate (Figure 2, Figure S10, and the

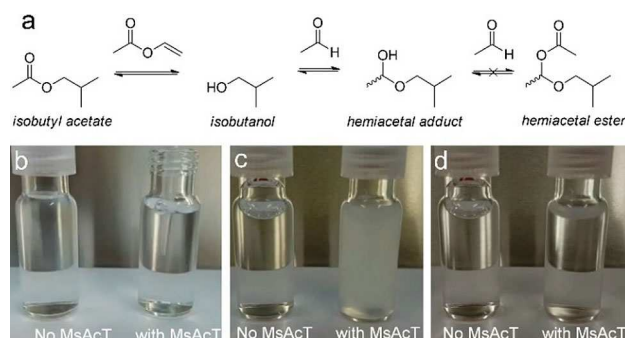
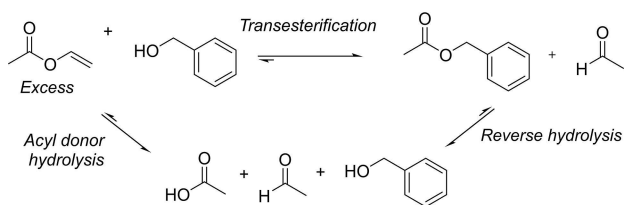


Figure 2. Transesterification of isobutyl alcohol with MsAcT resulting in the reversible formation of an emulsion. a) desired reaction and potential side reaction; b) initial reaction mixture at $t=0$; c) reaction mixture at $t=5$ min; d) reaction mixture at $t=60$ min.

video will be uploaded together with the proofs). No hemiacetal ester was observed. After prolonged reaction time the reaction mixture became clear again demonstrating the hydrolysis of isobutyl acetate. This occurred after all acyl donor had been consumed, indeed no isobutyl acetate was detectable after 18 hours of reaction time at this stage of the reaction. MsAcT catalyzed only the hydrolysis of the product ester, leading to the final reaction equilibrium; that of acid, alcohol, ester and water.

Having ruled out hemiacetal esters as side product and established the transient formation of the desired ester, optimal



Scheme 2. MsAcT catalyzed transesterification of benzyl alcohol with vinyl acetate.

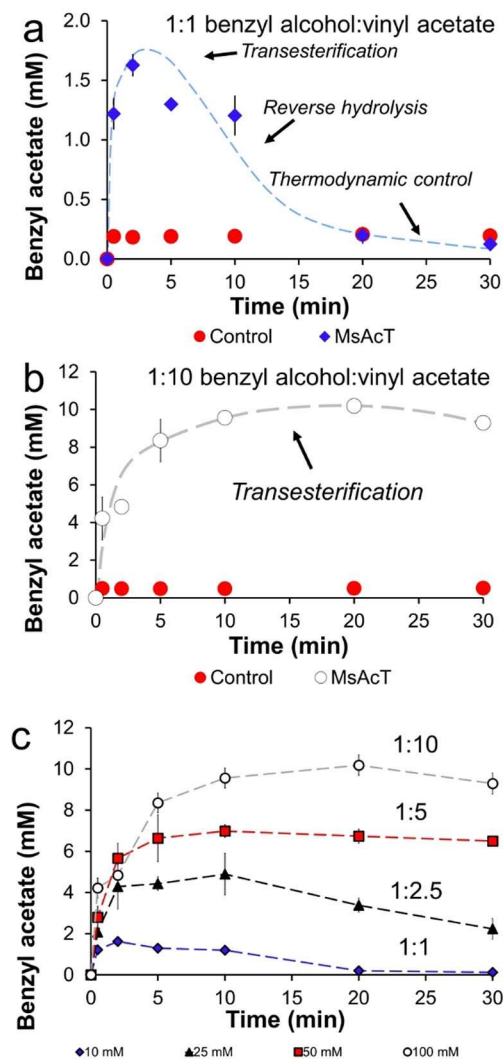


Figure 3. The synthesis of an ester in water. In a) the synthesis of an ester under kinetic control followed by reverse hydrolysis into the thermodynamic equilibrium; b) solely kinetic control is demonstrated by a 10-fold excess of vinyl acetate; c) one can observe the decrease in reverse hydrolysis by 1, 2.5-, 5-, and 10-fold excess of acyl donor. Reaction conditions: KPi buffer (200 mM, pH 7.5), 10 mM benzyl alcohol, 0–100 mM acyl donor, 50 ng MsAcT, 1 mL, 1000 rpm, 21 °C. The error bars show the standard deviation of triplicates.

reaction conditions were established. The concentration of vinyl acetate was varied at constant benzyl alcohol and MsAcT concentration (Scheme 2 and Figure 3c). At a 1:1 ratio of benzyl alcohol:vinyl acetate the benzyl ester is quickly formed and

hydrolyzed, full conversion is never reached (Figure 3a). However, at 1:10 benzyl alcohol:vinyl acetate excellent conversions are obtained with a prolonged stability of the synthesized ester (Figure 3b). Indeed, by increasing the acyl donor concentration the yield is increased and hydrolysis is outcompeted until all acyl donor is consumed.

The transesterification of alcohols with activated acids catalyzed by MsAcT follows overall a synthesis/hydrolysis pattern as is well established for the amide bond synthesis.^[9] Initially the alcohol is the preferred substrate of MsAcT and the desired ester, the kinetic product, is formed. In parallel both the activated acid and the ester are also subject to hydrolysis. Once all acyl donor is consumed the hydrolysis reaction leads to the final reaction equilibrium and thermodynamics dictate the ester yield (Scheme 2 and Figure 3a).

After the synthesis of esters in water with different acyl donors or enzyme concentrations the kinetic parameters of MsAcT catalyzed transesterification in water was investigated. To firmly establish the transesterification of benzyl alcohol with vinyl acetate the ester formation was monitored using GC analysis for the determination of the Michaelis-Menten constants of two-substrate reactions.^[10] The MsAcT-catalyzed transesterification of benzyl alcohol with vinyl acetate gave for benzyl alcohol a K_m of $9.1 \text{ mM} \pm 2.8 \text{ mM}$ and k_{cat} of $3.16 \times 10^3 \text{ s}^{-1}$, demonstrating high transesterification efficiencies. More interestingly, the transesterification was revealing a K_m and k_{cat} of $30.1 \text{ mM} \pm 6.3 \text{ mM}$ and $2.49 \times 10^3 \text{ s}^{-1}$ respectively.

The formation of acetaldehyde via the hydrolysis of vinyl acetate was monitored using a coupled spectrophotometric activity assay, as is shown in Table 1 (Figure S11–S13). The

Table 1. Kinetic parameters of MsAcT catalyzed hydrolysis and esterification in water

Compound ^[a]	K_i (mM)	K_m (mM)	k_{cat} (s^{-1}) [†]
Vinyl acetate ^[a]	n.a.*	0.0123 ± 0.0012	182 ± 2.5
Benzyl acetate ^[b]	2.28 ± 0.42	0.0308 ± 0.0087	52.7 ± 3.5
Benzyl alcohol ^[c]	n.a.*	13.6 ± 1.7	2586 ± 2.4

[†] k_{cat} is based on the molecular weight of MsAcT (23.3 kDa).

* n.a.: not applicable

[a] Assay conditions: Vinyl acetate (0–10 mM), NADH (0.3 mM), KPi (200 mM, pH 7.5), MsAcT (24 ng mL⁻¹), ScADH (50 U mL⁻¹); [b] Assay conditions: Benzyl alcohol (0–100 mM), vinyl acetate (10 mM), NADH (0.3 mM), KPi (200 mM, pH 7.5), MsAcT (24 ng mL⁻¹), ScADH (50 U mL⁻¹); [c] Assay conditions: Benzyl acetate (0.00–9.45 mM), NAD (5 mM), KPi (200 mM, pH 7.5), MsAcT (24 ng mL⁻¹), HLADH-E (5 U mL⁻¹).

hydrolysis of vinyl acetate with MsAcT showed high affinity to the acyl donor vinyl acetate, with moderate catalytic efficiency. The transesterification of benzyl alcohol with vinyl acetate was repeated using the spectrophotometric assay to ensure saturation conditions for two-substrate reactions of vinyl acetate with initial rate conditions,^[11] where a > 100-fold higher k_{cat} was observed for the transesterification over hydrolysis. The ratio between the catalytic rate constants of the hydrolysis and synthesis has been utilized to classify acyltransferase efficiencies,^[12] indicating that MsAcT classifies as an efficient

acyltransferase in water. The affinity for vinyl acetate is high during the hydrolysis towards acetaldehyde, but during transesterification in the presence of benzyl alcohol the apparent affinity is significantly reduced indicating that competitive inhibition occurs. Taken together, MsAcT favors transesterification over hydrolysis towards benzyl acetate under kinetic control while hydrolyzing benzyl acetate to its alcohol under thermodynamic control.

More interestingly, using a coupled spectrophotometric assay the hydrolysis of benzyl acetate was measured demonstrating high affinity and uncompetitive inhibition for MsAcT. When benzyl acetate was added to the hydrolysis of vinyl acetate the apparent affinity towards vinyl acetate was significantly lower representing competitive inhibition (Figure 4). Recently, a computational study suggested that benzyl

parameters for MsAcT demonstrated a high synthesis to hydrolysis ratio. If the reaction is performed at high concentrations of benzyl alcohol, the product will form a separate layer leading to *in-situ* product removal essential for reducing enzyme inhibition under synthesis conditions. From a reaction engineering perspective, since the side product acetaldehyde is volatile, the laboratory scale reaction is straightforward to perform. On a larger scale standard measures to prevent acetaldehyde from escaping into the environment need to be taken.^[8] These are well-established for industrial scale. Overall, organic solvents and wasteful neutralization steps can be avoided.

Experimental Section

Materials and Methods

Ampicillin (Sigma-Aldrich), benzyl acetate (Sigma-Aldrich), benzyl alcohol (Acros Organics), bovine serum albumin (ThermoFisher), diethyl ether (VWR), DNase I (bovine pancreas, Sigma-Aldrich), dodecane (Janssen), ethyl acetate (VWR), imidazole (VWR), isopropyl β -D-1-thiogalactopyranoside (ThermoFisher), lysozyme (chicken egg white, Sigma-Aldrich), magnesium sulfate (VWR), neopentyl glycol (Sigma-Aldrich), phenyl acetate (Sigma-Aldrich), potassium acetate (Sigma-Aldrich), potassium phosphate dibasic (Honeywell), potassium phosphate monobasic (Merck), sodium sulfate (Merck), vinyl acetate (Sigma-Aldrich). LB-medium consists of 1.0% (w/w) tryptone, 0.5% (w/w) yeast extract, 1.0% NaCl, and autoclaved at 121 °C for 20 minutes. All media was supplemented with 100 $\mu\text{g mL}^{-1}$ ampicillin before use. A multimode spectrophotometer plate reader (Synergy 2, BioTek) was used for spectrophotometric measurements. The pH electrode (Metrohm) was calibrated before use with standards of pH 4.00 and pH 7.00 with $R^2 > 0.98$. ^1H and ^{13}C NMR spectra were recorded on a Varian 400 (400 MHz) spectrometer in CDCl_3 (buffer for the NMR-monitored reaction). The chemical shifts are given in ppm relative to the solvent signal (^1H : δ (CHCl_3) = 7.26 ppm) and (^{13}C : δ (CDCl_3) = 77.16 ppm, for centerline of CDCl_3 triplet).

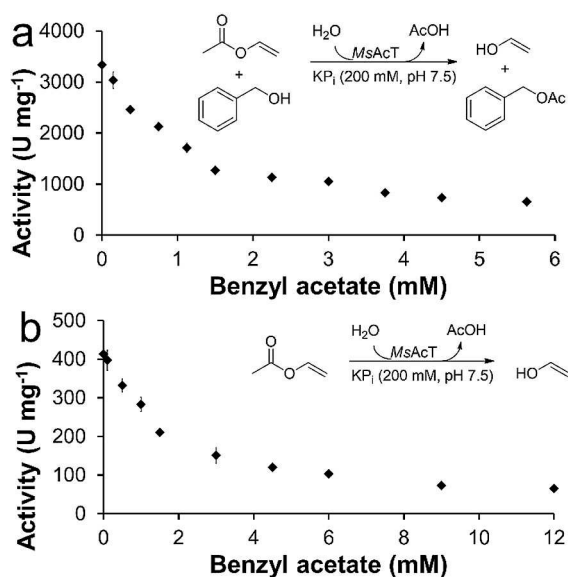


Figure 4. Product inhibition of the synthesis of benzyl acetate shown in a), while the competitive inhibition of the hydrolysis of vinyl acetate is shown in b). Reaction conditions: (i) product inhibition: benzyl acetate (0.0–12.0 mM), vinyl acetate (5.4 mM), benzyl alcohol (80 mM), NADH (0.25 mM), KPi (200 mM, pH 7.5), MsAcT (24 ng mL^{-1}), ScADH (50 U mL^{-1}); (ii) competitive hydrolysis: Benzyl acetate (0.00–5.6 mM), vinyl acetate (5.4 mM), NADH (0.25 mM), KPi (200 mM, pH 7.5), MsAcT (24 ng mL^{-1}), ScADH (50 U mL^{-1}). The error bars show the standard deviation of triplicates.

acetate would have high affinity towards the aromatic active site.^[6b] In this study, we demonstrated that the kinetics of the enzymatic reaction are severely inhibited by the addition of benzyl acetate indicating that ligand exchange severely lowers the reaction rates.

Conclusions

The synthesis of esters in high yields can be achieved in water with activated acids using MsAcT. The transesterification in water proceeds under kinetic control and careful monitoring of the reaction is necessary to avoid reverse hydrolysis. The kinetic

Expression and Purification of MsAcT

A previous expression protocol was followed with slight modifications.^[3d] A synthetic gene of MsAcT (GenBank accession: ABK70783) from *Mycobacterium smegmatis* MC2 155 was cloned in pET16b-MsAcT containing a C-terminal HisTag and expressed in *E. coli* BL21(DE3). A baffled 5 L shake flask containing 1 liter of TB-media was inoculated with 10 mL preculture and shaken until the OD₆₀₀ reached 0.5 (25 °C, 180 rpm). Expression was induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and the culture was shaken overnight (25 °C, 180 rpm). The cells were harvested with centrifugation (10 min, 10 000 rpm, 4 °C, Sorvall RC 6+) and the supernatant was discarded. The cell pellet was washed with KPi buffer (20 mM, pH 7.5) and the supernatant was discarded again. The cell pellet was suspended in KPi buffer (20 mM, pH 7.5) containing 20 mM imidazole, lysozyme (0.5 mg mL^{-1} , chicken egg white, Sigma-Aldrich), DNase I (0.1 mg mL^{-1} , bovine pancreas, Sigma-Aldrich) were incubated at 0 °C for 30 minutes. The cells were lysed by cell disruption (Constant Cell disruption systems) for three consecutive rounds (1.35 kbar). The cell-free extract was obtained after centrifugation (10 min, 10000 rpm, 4 °C, Sorvall RC 6+). The cell-free extract was filtered using a syringe filter (0.45 μm) before sample application on Ni Sepharose 6Fast Flow resin (12 mL,

GE Healthcare) equipped in a XK 16/20 adapter (GE Healthcare) connected to an NGC system (Bio-Rad). Before sample application the system and resin were flushed with 20% ethanol, deionized water, and equilibration buffer containing KPi (20 mM, pH 7.5) with 20 mM imidazole. The protein was flushed through the column until all MsAcT protein was bound and eluted with a gradient of elution buffer KPi (20 mM, pH 7.5) and 500 mM imidazole. The eluted fractions containing MsAcT were desalted with a PD-10 desalting column and flash frozen with liquid nitrogen prior to storage at -80°C .

SDS-PAGE Analysis

The purity of MsAcT (24 kDa) was analyzed with SDS-PAGE analysis. The protein samples were denatured using a XT Sample Buffer (Bio-Rad) and XT Reducing Agent (Bio-Rad) at 95°C for 5 to 10 minutes. A Criterion XT Bis-Tris MOPS 4–12% precast gel (Bio-Rad) equipped with a Precision Plus Protein Unstained Standard (Bio-Rad) and denatured protein samples was run at 180 V in MES buffer (Bio-Rad) for 40 minutes. The gel was stained with SimplyBlue SafeStain (ThermoFisher).

BCA Assay

Protein content was determined with the bicinchoninic acid (BCA) protein quantitation kit (Thermo Scientific, Carlsbad, USA). Standard curves were prepared with bovine serum albumin (BSA) in the range of 0.003 to 1.38 mg mL^{-1} in a (poly)styrene 96-well plate. Samples were measured in triplicate and monitored at 562 nm utilizing a microtiter plate spectrophotometer (Synergy 2, BioTek).

MsAcT Activity Assay with Neopentyl Glycol (NPG)

The activity assay was started with the addition of 25 μL from a solution of MsAcT (0.02 mg mL^{-1}) dissolved in KPi buffer (200 mM, pH 8) to 975 μL ethyl acetate containing the external standard dodecane (10 mM) and NPG (100 mM). The biphasic reaction mixtures were shaken for 30 seconds at 2500 rpm at room temperature. Reactions were quenched after 0, 2, 4, 6 and 8 minutes by addition of excess sodium sulfate. 50 μL of each reaction mixture was pipetted into a GC vial and 950 μL ethyl acetate was added prior to GC analysis. The amount of NPG monoacetate was quantified using external calibration curve, as is shown in Figure S5. Activity is reported as micromoles of NPG monoacetate produced per minute per milligram of purified MsAcT.

MsAcT Activity assay with *p*-Nitrophenylbutyrate (pNPB)

The activity assay was started by adding 20 μL of MsAcT to 180 μL buffer containing KPi buffer (50 mM, pH 7.5) in transparent 96-well polystyrene plates. The rate of hydrolysis was monitored at 37°C at 405 nm using a multimode spectrophotometer plate reader (Synergy 2, BioTek). Buffer and 96-well plate were pre-heated to 37°C before the start of the reaction. An external calibration curve of *p*-nitrophenol was constructed between 0.00–1.00 mM.

Esterification of Benzyl Alcohol with Potassium Acetate under Monophasic Reaction Conditions

For the synthesis of benzyl acetate from benzyl alcohol and potassium acetate the following reaction conditions were applied. MsAcT (12000 ng) was added to KPi buffer (200 mM, pH 7.5) containing 10 mM benzyl alcohol and 100 mM potassium acetate to yield a monophasic reaction mixture of 1 mL. For the hydrolysis

of benzyl acetate to benzyl alcohol and potassium acetate the following reaction conditions were applied. MsAcT (12000 ng) was added to KPi buffer (200 mM, pH 7.5) containing 10 mM benzyl acetate and 100 mM potassium acetate to yield a monophasic reaction mixture of 1 mL. The reaction mixture was shaken at 1000 rpm at 21°C . Samples were taken after 1, 2, 3, 4, 5 and 24 hours. Samples were quenched by addition of 500 μL diethyl ether containing dodecane (10 mM) as external standard. The mixture was rapidly vortexed for 30 seconds and centrifuged at 13 000 rpm to remove enzyme precipitates. The organic layer was collected, dried with an excess of dry MgSO_4 , and analyzed by GC. The amount of benzyl alcohol and benzyl acetate was quantified using an external calibration curve, as is shown in Figure S4.

Reversible Hydrolysis and Synthesis of Isobutyl Acetate by MsAcT

The reaction was initiated by the addition of MsAcT to a reaction solution resulting in the final concentration of 11 mM isobutanol, 212 mM vinyl acetate, KPi (200 mM, pH = 7.5), and $20\text{ }\mu\text{g mL}^{-1}$ MsAcT. The solution was resuspended vigorously during addition for 1–3 seconds and measured immediately with $^1\text{H-NMR}$ (Agilent, 400 MHz). Also, the reaction was repeated and recorded with a dual pixel 12MP OIS (F1.7) camera to show the reversible synthesis and hydrolysis. For the synthesis of isobutyl acetate from isobutanol and vinyl acetate the following reaction conditions were applied. The reaction was initiated by the addition of MsAcT to a reaction solution resulting in the final concentration of 11 mM isobutanol, 212 mM vinyl acetate, 200 mM KPi (200 mM, pH = 7.5), and $0.5\text{ }\mu\text{g mL}^{-1}$ MsAcT. The solution was resuspended vigorously during addition for 1–3 seconds and measured immediately with $^1\text{H-NMR}$ (Agilent, 400 MHz).

Optimizing MsAcT Concentration for the Acylation of Benzyl Alcohol with Vinyl-, Phenyl-, and Ethyl Acetate

MsAcT (50–12000 ng) was added to KPi buffer (200 mM, pH 7.5) containing 10 mM benzyl alcohol, and 100 mM acyl donor being either vinyl-, phenyl-, and ethyl acetate to yield a monophasic reaction mixture of 1 mL. The mixture was shaken at 1000 rpm at 21°C for 60 minutes. Product and substrate were extracted twice by addition of 500 μL diethyl ether containing dodecane (10 mM) as external standard. The mixture was rapidly vortexed for 30 seconds and centrifuged at 13 000 rpm to remove enzyme precipitates. The organic layer was collected, dried with an excess of dry MgSO_4 , and analyzed by GC. The amount of benzyl alcohol and benzyl acetate was quantified using external calibration curve (Figure S4). Initial rates were calculated from the linear slope of benzyl acetate concentration over time.

Determination Kinetic Parameters for Vinyl Acetate and Benzyl Alcohol using GC Analysis

For the kinetic parameters for benzyl alcohol the following reaction conditions were applied. MsAcT (50 ng) was added to KPi buffer (200 mM, pH 7.5) containing either 0, 1, 2.5, 5, 10, 15, and 20 mM benzyl alcohol, and 100 mM vinyl acetate to yield a monophasic reaction mixture of 1 mL. For the kinetic parameters for vinyl acetate the following reaction conditions were applied. MsAcT (50 ng) was added to KPi buffer (200 mM, pH 7.5), 10 mM benzyl alcohol, and 0, 10, 25, 50, 100, and 200 mM vinyl acetate to yield a monophasic reaction mixture of 1 mL. The mixture was shaken at 1000 rpm at 21°C for 0, 2, 5, 10, 20, and 30 minutes. Product and substrate were extracted twice by addition of 500 μL diethyl ether containing dodecane (10 mM) as external standard. The mixture

was rapidly vortexed for 30 seconds and centrifuged at 13 000 rpm to remove enzyme precipitates. The organic layer was collected, dried with an excess of dry MgSO_4 , and analyzed by GC. The amount of benzyl alcohol and benzyl acetate was quantified using external calibration curves (Figure S4) and the data is shown in Figure S14. Initial rates were calculated from the linear slope of benzyl acetate concentration over time. The curves were fitted to the Michaelis-Menten equation using the fit function of Gnuplot 5.2,^[13] as is shown in Table S1.

Coupled Spectrophotometric Activity Assay with ScADH of Vinyl Acetate Hydrolysis with MsACT

The assay was performed in polyacrylate 1 cm cuvettes by monitoring the conversion of 0.25 mM NADH at 340 nm and 20 °C with an extinction coefficient of $6.221 \text{ mM}^{-1} \text{ cm}^{-1}$. Before the addition of MsACT, the reaction was monitored until stable. Enzymatic reactions were started by the addition of MsACT with a final concentration of vinyl acetate (0–10 mM) in KPi (200 mM, pH 7.5), ScADH (50 U mL^{-1}), MsACT (24 $\text{ng } \mu\text{L}^{-1}$). All measurements were performed in triplicates. The addition of additional ScADH did not result in a higher response for indirect acetaldehyde detection. The Michaelis-Menten curve is shown in Figure S11. The curves were fitted to the Michaelis-Menten equation using the fit function of Gnuplot 5.2,^[13] as is shown in Table S1.

Coupled Spectrophotometric Activity Assay with ScADH of Benzyl Acetate Synthesis with MsACT

The assay was performed in polyacrylate 1 cm cuvettes by monitoring the conversion of 0.25 mM NADH at 340 nm and 20 °C by using an extinction coefficient of $6.221 \text{ mM}^{-1} \text{ cm}^{-1}$. Before the addition of MsACT, the reaction was monitored until stable. Enzymatic reactions were started by the addition of MsACT with a final concentration of benzyl alcohol (1–100 mM), vinyl acetate (10 mM) in KPi (200 mM, pH 7.5), ScADH (50 U mL^{-1}), MsACT (0.24 $\text{ng } \mu\text{L}^{-1}$). All measurements were performed in triplicates. The addition of additional ScADH did not result in a higher response for indirect acetaldehyde detection. The Michaelis-Menten curve is shown in Figure S12. The curves were fitted to the Michaelis-Menten equation using the fit function of Gnuplot 5.2,^[13] as is shown in Table S1.

Coupled Spectrophotometric Activity Assay with HLADH-E of Benzyl Acetate Hydrolysis with MsACT

The assay was performed in polyacrylate 1 cm cuvettes by monitoring the conversion of 0.25 mM NAD^+ at 340 nm and 20 °C by using an extinction coefficient of $6.221 \text{ mM}^{-1} \text{ cm}^{-1}$. Before the addition of MsACT, the reaction was monitored until stable. Enzymatic reactions were started by the addition of enzyme with a final concentration of benzyl acetate (1–10 mM), in KPi (200 mM, pH 7.5), HLADH-E (5 U mL^{-1}), MsACT (0.24 $\text{ng } \mu\text{L}^{-1}$). All measurements were performed in triplicates. The addition of additional HLADH-E did not result in a higher response for indirect benzyl alcohol detection. The Michaelis-Menten curve with non-competitive substrate inhibition is shown in Figure S13. The curves were fitted to the non-competitive Michaelis-Menten equation using the fit function of Gnuplot 5.2.^[13]

Coupled Spectrophotometric Activity Assay with ScADH of Competitive Vinyl- and Benzyl Acetate Hydrolysis with MsACT

The assay was performed in polyacrylate 1 cm cuvettes by monitoring the conversion of 0.25 mM NADH at 340 nm and 20 °C by using an extinction coefficient of $6.221 \text{ mM}^{-1} \text{ cm}^{-1}$. Before the addition of MsACT, the reaction was monitored until no chemical background hydrolysis occurred. Enzymatic reactions were started by the addition of MsACT with a final concentration of benzyl acetate (0.00–5.6 mM), vinyl acetate (5.4 mM), NADH (0.25 mM), KPi (200 mM, pH 7.5), MsACT (24 ng mL^{-1}), ScADH (50 U mL^{-1}). The error bars show the standard deviation of triplicates. All measurements were performed in triplicates. The addition of additional ScADH did not result in a higher response for indirect acetaldehyde detection.

Coupled Spectrophotometric Activity Assay with ScADH of Benzyl Acetate Synthesis with MsACT in the Presence of Varying Amounts of Product

The assay was performed in polyacrylate 1 cm cuvettes by monitoring the conversion of 0.25 mM NADH at 340 nm and 20 °C by using an extinction coefficient of $6.221 \text{ mM}^{-1} \text{ cm}^{-1}$. Before the addition of MsACT, the reaction was monitored until no chemical background hydrolysis occurred. Enzymatic reactions were started by the addition of MsACT with a final concentration of benzyl acetate (0.0–12.0 mM), vinyl acetate (5.4 mM), benzyl alcohol (80 mM), NADH (0.25 mM), KPi (200 mM, pH 7.5), MsACT (24 ng mL^{-1}), ScADH (50 U mL^{-1}). All measurements were performed in triplicates. The addition of additional ScADH did not result in a higher response for indirect acetaldehyde detection.

Acknowledgements

We thank Carolin Bisterfeld, Nicolas de Leeuw, and Laura van der Weel for fruitful discussions and support.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: acyl transferase · transesterification · water · *Mycobacterium smegmatis* · thermodynamic equilibrium · kinetic control

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Manuscript received: December 6, 2018
 Revised manuscript received: February 14, 2019
 Version of record online: March 18, 2019