Re-Investigation of Hydration Potential of Rhodococcus Whole-Cell Biocatalysts towards Michael Acceptors

Busch, Hanna; Alvarenga, Natália; Abdelraheem, Eman; Hoek, Max; Hagedoorn, Peter Leon; Hanefeld, Ulf

DOI
10.1002/cctc.201901606

Publication date
2019

Document Version
Final published version

Published in
ChemCatChem

ChemPubSoc Europe

DOI: 10.1002/cctc.201901606

CHEMCATCHEM Full Papers

Re-Investigation of Hydration Potential of *Rhodococcus* Whole-Cell Biocatalysts towards Michael Acceptors

Hanna Busch[+],[a] Natália Alvarenga[+],[a] Eman Abdelraheem[+],[a] Max Hoek,[a] Peter-Leon Hagedoorn,[a] and Ulf Hanefeld*[a]

The implementation of a stereoselective Michael addition with water as substrate is still a major challenge by classical, chemical means. Inspired by nature's ability to carry out this attractive reaction with both high selectivity and efficiency, the interest in hydratases (EC 4.2.1.x) to accomplish a selective water addition is steadily rising. The gram-positive bacterial genus *Rhodococcus* is known as biocatalytic powerhouse and has been reported to hydrate various Michael acceptors leading to chiral alcohols. This study aimed at the in-depth re-investigation of the hydration potential of *Rhodococcus* whole-cells towards Michael acceptors. Here, two concurrent effects responsible for the hydration reaction were found: while the majority of substrates was hydrated in an oxygen-independent manner by amino-acid catalysis, an enzyme-catalysed water addition to (*E*)-4-hydroxy-3-methylbut-2-enoic acid was proven to be oxygen-dependent. $^{18}O_2$-labelling studies showed that no $^{18}O_2$ was incorporated in the product. Therefore, a novel O_2-dependent hydratase distinct from all characterised hydratases so far was found.

Introduction

The selective addition of water to (un)-activated double bonds is known to be a very appealing yet chemically challenging reaction. Though water as a reactant provides benefits regarding sustainability and atom-efficiency, it is both a poor electro- and nucleophile and therefore often first requires activation by *e.g.* strong acids. Additionally, unfavourable reaction equilibria often impede a profitable reaction.[1–3] While chemists still struggle to find activating conditions without diminishing the stereoselectivity, nature developed a way to circumvent these problems by the use of enzymes. Their unique 3-dimensional structures provide ways to activate the water molecule as well as stabilise transition states during the reaction.[1,4] Therefore, using microbial activities or applying purified enzymes in biocatalytic reactions to achieve a selective water addition to double bonds is nowadays often seen as advantageous.[5–7] Hydratases (EC 4.2.1.x) catalyse the water addition to activated as well as isolated double bonds. However, especially enzymes from the primary metabolism exhibit a narrow substrate scope which confines the applicability of these enzymes to their natural substrates.[4] While the use of hydratases to convert natural substrates already offers a huge advantage over classical chemical routes and has already been applied in industry,[8–11] the identification of hydratases with a broader substrate scope is highly desirable. A biocatalytic hydration of two non-natural, α,β-unsaturated substrates using whole-cells of *Rhodococcus rhodochrous* ATCC 17895 was first reported in 1998.[12] Further research to develop a straightforward approach to produce β-hydroxy carbonyl compounds catalysed by a presumed 'Michael hydratase' followed in 2015. It was described that whole-cells of *Rhodococcus* additionally add water to small ring-closed organic molecules like *c*-hexenone or *c*-pentenone thereby improving future applications of an attractive water addition system.[13]

Rhodococcus is a genus of a gram-positive bacteria that has been described to host a variety of enzymes and degradation pathways with high potential for the use in industrial processes.[8,14] Its metabolic diversity often stems from large, linear plasmids that carry the protein-encoding genes for degradative enzyme systems.[15] Abundant horizontal gene-transfer events occurring *via* these linear plasmids as well as a high multiplicity of catabolic enzymes contributed to the catabolic versatility of *Rhodococcus* genus members.[15–17]

To study the described water addition in-depth, the identification and isolation of the responsible enzyme were first aimed for. Due to complications in the protein isolation, whole-cell systems were further investigated to explore the substrate range. During this process inconsistencies and contradictions within the earlier reports became evident.[12,13] Therefore, the aim of this study was to assess and extend those reports. In addition to a re-evaluation of described substrate structures, the main focus was on the clarification of the reaction type. Labelling studies with D_2O and $^{18}O_2$ with subsequent high resolution liquid chromatography-mass spectrometry (HRLC-MS) analysis were used to identify whether the net microbial

[a] *H. Busch,[+] Dr. N. Alvarenga,[+] Dr. E. Abdelraheem,[+] M. Hoek, Dr. P.-L. Hagedoorn, Prof. U. Hanefeld*
Department of Biotechnology
Delft University of Technology
Van der Maasweg 9
Delft 2629 HZ (The Netherlands)
E-mail: U.Hanefeld@tudelft.nl

[+] *Authors contributed equally to this work.*

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cctc.201901606

This publication is part of a joint Special Collection with ChemBioChem on "Excellence in Biocatalysis Research". Please follow the link for more articles in the collection.

hydration activity involves a true water addition or an oxidative process.

Results and Discussion

Attempted Isolation of Michael Hydratase

Whole-cells of *Rhodococcus rhodochrous* ATCC 17895 were described to catalyse the Michael-addition of water to several substrates.[12,13] Therefore, the isolation of the responsible enzyme was aimed for, but unfortunately, no sequence data was available nor any comparable protein has been described earlier. Subcellular fractionation of *Rhodococcus* cells led to the conclusion that the desired protein is membrane-associated. However, numerous attempts to solubilise the protein using classical detergents as well as a nanodisc approach using styrene-maleic acid (SMA) copolymers[18,19] did not lead to an isolated enzyme activity (data not shown). On the contrary, the attempted solubilisation of the membrane protein led to a complete loss of activity which could be a consequence of protein instability or the requirement of different subunits or components for the hydratase activity. As advanced comparative genomic analyses as well as membrane proteomics did not lead to the successful identification of the responsible enzyme, isolation efforts were not further pursued.

Instead, whole-cells as well as isolated membranes from a number of *Rhodococcus* strains were used to investigate the substrate range. Whole-cells were shown to also catalyse a number of unwanted side-reactions due to the presence of alcohol dehydrogenases and ene-reductases which also act on the small organic molecules (Scheme 1).[20]

In comparison, isolated membranes offered a way to decrease the number of side-products formed, because soluble enzymes that may catalyse competing reactions were removed earlier (data not shown). Unfortunately, isolated membranes lack stability, demand a time-consuming preparation and resulted in a lower recovered activity.[21] For these reasons it was decided to use whole-cells for further substrate investigations.

Substrate Re-Evaluation

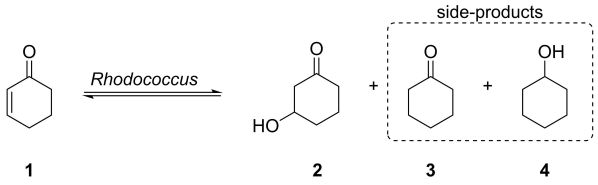
In the original report Holland *et al.* stated that 4-methyl-furan (*5H*)-one (**7**) is hydrated by whole-cells from *Rhodococcus* to give the respective 4-hydroxy-4-methyldihydrofuran-2(*3H*)-one (**6**) (Scheme 2).[12] However, bioconversions with commercially available **7** showed no expected product formation. Upon closer investigation of the chemical structure, it became evident that previous reports[12,13] assumed a wrong substrate structure.

In these reports, the synthesis of the biotransformation substrate was carried out as a two-step procedure involving a Wittig-reaction followed by a basic hydrolysis.[12,13] After repeating this protocol, however, 1- and 2-dimensional NMR spectroscopy of the product revealed no ring-closed structure **7**, but the open-chain (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) which was in fact used as the substrate for bioconversions with *Rhodococcus* cells (Scheme S1, Figures S1–S4). Attempts to obtain the corresponding open-chain (*Z*)-isomer through basic hydrolysis of commercially available 4-methyl-furan(*5H*)-one (**7**) were unsuccessful.

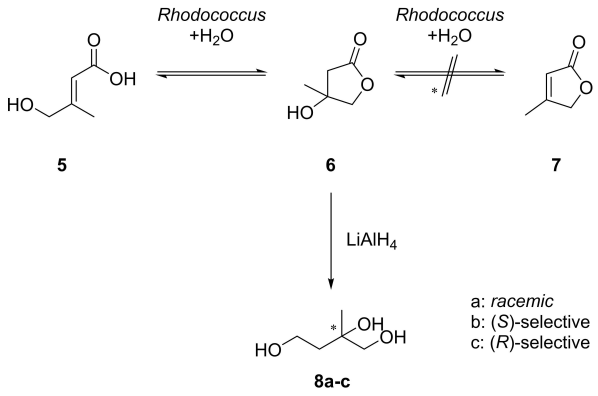
Previous reports were contradictory in assigning the stereochemistry of final lactone **6**: (*R*)-configurated in the first report and the opposite (*S*)-configuration in the second study, both based on optical rotation.[12,13] To ultimately confirm one of the two hypotheses, the stereochemistry of reduced triol **8** (Scheme 2) was compared to reference compounds using chiral gas chromatography: (*S*)-triol **8b** was obtained *via* a three-step chemical route (Scheme S2, Figure S5–S7): (*S*)-citramalic acid (**14**) was prepared from (*R*)-oxetanone **13** and was subsequently esterified with thionyl chloride to yield dimethyl-(*S*)-2-hydroxy-2-methyl-succinate (**15**, 60% yield).[22,23] Treatment of the ester **15** with an excess of $LiAlH_4$ in tetrahydrofuran gave (*S*)-2-methylbutane-1,2,4-triol (**8b**).[22]

While racemic triol **8a** was commercially available, lactone **6** was obtained from a large-scale biotransformation with *R. pyridinivorans* DSM 20415 and afterwards reduced with $LiAlH_4$ to give triol **8**. Comparative chiral gas chromatography analysis of all three triol samples (**8a–c**) revealed that the triol obtained from a large-scale biotransformation with *R. pyridinivorans* DSM 20415 was (*R*)-configurated (**8c**).

Following the revision of the substrate structure, the Mhy activity was (re)-evaluated on a number of other Michael acceptors that have been reported previously (Table S1). The



Scheme 1. Biotransformation of *c*-hexenone with *Rhodococcus* whole-cells leads to desired water addition product and unwanted side-reactions.



Scheme 2. Bioconversions using whole-cells from *Rhodococcus* using substrates (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**, confirmed substrate, left side) and 4-methyl-furan(*5H*)-one (**7**, not converted, right side). A subsequent reduction of lactone **6** led to triol **8** to confirm the stereochemistry.

conversion of *c*-pentenone (**18**), *c*-hexenone (**1**) and *c*-heptenone (**19**) yielded hydroxylated product in low yields while methyl crotonate was not accepted.

Oxygen-Dependency

Whole-cell optimisation experiments with substrate **5** surprisingly showed an improved product yield with an increasing headspace to reaction-mixture ratio (Figure 1). These findings indicate an oxygen-dependency which was not reported earlier. On the contrary, previous reports stated that both the reaction of (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) and *c*-hexenone (**1**) were as efficiently catalysed under N_2-atmosphere as under air. With this experiment the possibility of an oxidative process was previously excluded.[13] To resolve these apparently conflicting findings, the oxygen-dependency of the water addition reaction was re-investigated. Here, numerous experiments using substrate **5** under N_2-atmosphere did not lead to any product formation while *c*-hexenone (**1**) was still converted and gave similar results as in aerobic trials (Figure 1). The contradicting results of water addition activity under anaerobic conditions depending on the substrate class led to the question whether both substrate classes are converted by the same reaction mechanism and biocatalyst. Consequently, control reactions with whole-cells of *Escherichia coli* TOP 10 containing an empty pBADHisA expression vector were carried out. No product formation in the case of (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) was observed while *c*-hexenone (**1**) showed comparable product formations with the *E. coli* control system.

These results indicate the presence of two different effects acting on the two substrate classes. One possible explanation for the latter observation is a water addition catalysed by amino acids. In the past, amino acids were already shown to add water to Michael acceptors like *c*-hexenone or *c*-pentenone without any oxygen requirement. L-lysine obtained the highest formation of *rac*-3-hydroxy-*c*-hexanone with a yield of 21%.[24] It is therefore likely that the amino acids present in both *E. coli* and *Rhodococcus* catalyse the reaction on *c*-hexenone regardless of the presence or absence of oxygen in our system as well.

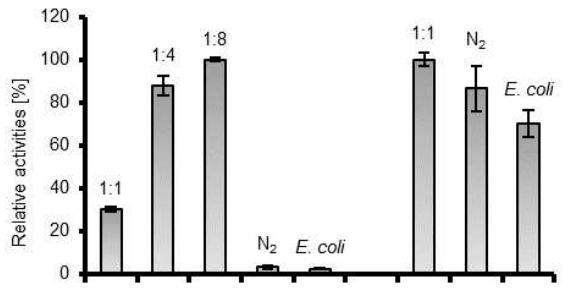


Figure 1. Investigation of oxygen-dependency of bioconversions with substrates (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**, left) and *c*-hexenone (**1**, right) using different headspace-reaction mixture ratios [1:1, 1:4, 1:8 (reaction mixture : air)], N_2 and *E. coli* reactions. Relative activities normalised on highest activity achieved (100% with ratio 1:8 for substrate **5** and 100% for the ratio 1:1 for substrate **1**).

Oxidation or Water Addition?

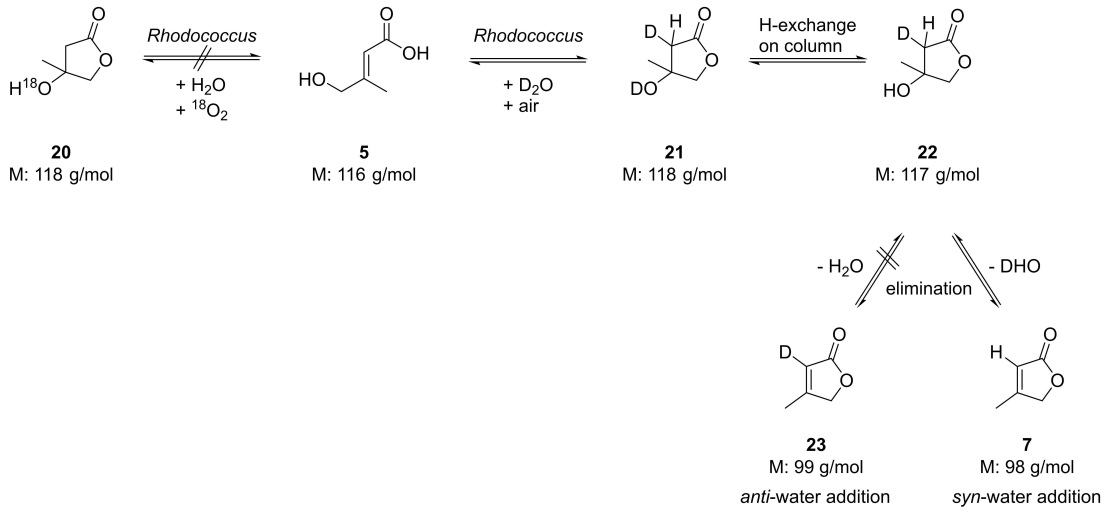
To exclude the possibility of an oxidative process instead of a water addition reaction, a number of labelling experiments were carried out. The conversion of **1** and **5** was catalysed both by cells of *R. rhodochrous* ATCC 17895 and *R. pyridinivorans* DSM 20415 under $^{18}O_2$ atmosphere in H_2O as well as under air in both D_2O and H_2O. The results were subsequently analysed with high resolution liquid chromatography-mass spectrometry (HRLC-MS). In case of an oxidative process, the product will show a higher mass due to the incorporated ^{18}O-atom (**20**) (Scheme 3). On the other hand, a water addition will lead to the incorporation of two deuterium-atoms (**21**) of which one is readily exchangeable thus **22** will be detected. A subsequent chemical elimination step will reveal whether the deuterated water was added in *syn*- (**7**) or *anti*-fashion (**23**) as the chemical E2-elimination selectively takes place in *anti*-fashion.

Biotransformations with both cells and both substrates **1** and **5** did not incorporate any labelled ^{18}O-atom thereby excluding any oxidative process (Figures S10 and S11). Comparative HRLC-MS analysis of reactions run under air with D_2O and H_2O showed a clear formation of compound **22** for substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) and the respective deuterated product for *c*-hexenone indicating the incorporation of D_2O and thereby confirming a water addition for both *Rhodococcus* strains. Control reactions without cells did not show any significant water addition activity.

To review the course of the water addition (*syn- versus anti*-addition) purified deuterated compound **22** was obtained in a large-scale biotransformation with *Rhodococcus rhodochrous* ATCC 17895 whole-cells. A subsequent chemical *anti*-elimination was performed yielding exclusively the undeuterated product **7** (Figures S12–S14). This experiment thus confirms earlier results,[13] but due to the revised substrate structure which is an open-chain rather than a ring-closed molecule, the water addition catalysed by *Rhodococcus* whole-cells consequently occurs in *syn*-fashion.

Biotransformations carried out under nitrogen atmosphere proved that the presence of oxygen is required for the conversion of **5** to take place. This was further sustained by the fact that more lactone **6** was produced under pure $^{18}O_2$-atmosphere than under air-conditions with less oxygen present (Figure S10). Additional experiments with D_2O under nitrogen atmosphere revealed no product formation.

The difference to the conversion of *c*-hexenone (**1**), however, is the remaining activity in the absence of oxygen as well as comparable activities achieved with *E. coli* cells. The obtained results from the labelling study further confirm the earlier theory that this conversion is an amino-acid catalysed process for ring-closed α,β-unsaturated carbonyl compounds **1**, **18** and **19**. This leaves compound **5** and its ethyl-derivative (*E*)-4-hydroxy-3-ethylbut-2-enoic acid (**16**)[12] to be the sole substrates requiring oxygen for a water addition. Due to high



Scheme 3. Schematic labelling studies with $^{18}O_2$ and D_2O on (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) with a subsequent elimination step including the molecular weights of respective intermediates.

enantiomeric excess values of the final lactones which were shown in earlier studies,[13] the high substrate specificity and the fact that the activity is associated with the membrane fraction, we propose an enzyme-catalysed process rather than a metal-assisted or amino-acid catalysed water addition.

The described O_2-dependent behaviour of a presumed hydratase is, however, surprising and atypical compared to other identified hydratases as they are either known to be oxygen-independent or negatively affected by trace amounts of oxygen. The latter enzymes often hold metal-containing cofactors or cysteine residues that lead to the sensitivity.[2] One example of this group is the linalool (de)hydratase-isomerase which catalyses the water addition and isomerisation to unactivated monoterpenes like (*S*)-(+)-linalool to yield β-myrcene and geraniol.[2,25,26] Interestingly, *Rhodococcus erythropolis* MLT1 cells were shown to catalyse a similar reaction converting β-myrcene to geraniol.[2,27] In this whole-cell system, however, oxygen is required for the reaction and anaerobic conditions lead to no product formation.[27] This phenomenon is similar to our findings and, to the best of our knowledge, the only other report of an oxygen-dependent formal hydration reaction. The substrates, however, differ as our substrates belong to the group of activated Michael acceptors while in β-myrcene an unactivated double bond is hydrated. Nonetheless, it is remarkable that this phenomenon was observed only twice and only in *Rhodococcus* cells.

Conclusions

Michael additions with the unreactive water-molecule as substrate are highly attractive yet chemically very challenging reactions. *Rhodococcus* cells were shown to have microbial activity towards a number of Michael acceptors leading to β-hydroxy carbonyl compounds. This study serves to expand the knowledge about these water addition reactions and a presumed 'Michael hydratase' was investigated in detail.

Though trials to isolate or identify the responsible hydratase failed, whole-cell and membrane systems were employed to examine the substrate scope. Here, previously described substrates were re-visited and corrected regarding their chemical structure using 1- and 2D-NMR analysis. Experiments under nitrogen atmosphere revealed two different effects being responsible for the water addition depending on the substrate used. The main group of substrates was proposed to be amino-acid catalysed as the same hydration activity was found in the presence and absence of oxygen as well as with *E. coli* cells. (*E*)-4-hydroxy-3-methylbut-2-enoic acid, however, was shown to be oxygen-dependently converted only by *Rhodococcus* cells and presumed to be enzyme-catalysed. Complete absence of oxygen led to no hydration. Labelling studies with D_2O and $^{18}O_2$ exposed a true water addition in *syn*-fashion and excluded any oxidative process. The described microbial hydration activity therefore remains highly attractive yet still elusive. A complete understanding of the oxygen dependency, a probable reaction mechanism, the finding of its natural substrate as well as an expansion of its substrate scope for future applications will only be possible upon identification of the responsible membrane protein (-complex) and successful heterologous expression in a suitable host system.

Experimental Section

Chemicals

Unless stated otherwise, all commercial chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) and used without further purification. Petroleum ether was purchased from VWR International (Amsterdam, The Netherlands) and distilled before utilisation. Hydroxyacetone was purchased from Alfa Aesar (Kandel,

Germany) and *rac*-2-methyl-1,2,4-triol was purchased from Chemspace (Riga, Latvia).

Chemical Synthesis Procedures

Compounds (*E*)-ethyl-4-hydroxy-3-methylbut-2-enoate (**11**) and (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) were synthesised as described earlier and results are in accordance with literature (Figures S1–S2).[28,29] *Rac*-3-hydroxy-*c*-hexanone was synthesised following described protocol and data is in accordance with literature (Figures S9).[24]

(*S*)-Citramalic Acid (14)

A solution of sodium hydroxide (10 M, 3 ml) was added dropwise to a suspension of (*R*)-oxetanone **13** (1.017 g, 5 mmol) in water (5 ml) at 5 °C. The mixture was stirred at room temperature for 15 h, acidified to pH 1 with concentrated hydrochloric acid (10 M) and evaporated under reduced pressure. The crude acid was extracted from the residue with warm ethyl acetate (4×5 mL) and the crude product (yellow oil) was used without further purification (67%, 0.5 g, 3.35 mmol). ^{1}H-NMR (400 MHz, Methanol-d_4) δ: 2.74 (d, J= 16.1 Hz, 1H), 2.44 (d, J=16.2 Hz, 1H), 1.25 (s, 3H). ^{13}C-NMR (101 MHz, Methanol-d_4) δ: 177.3, 172.7, 71.9, 43.7, 25.3. NMR data is in accordance with literature.[23,30]

Dimethyl-(*S*)-2-hydroxy-2-methylsuccinate (15)

(*S*)-citramalic acid (**14**) (296 mg, 2 mmol) was dissolved in methanol (1 mL) and thionyl chloride (2 equiv.) was added dropwise under cooling. The reaction mixture was stirred at room temperature for 3 h and followed by TLC. The solvent was evaporated and the crude product (yellow oil) was used without further purification (97%, 340 mg, 1.94 mmol). ^{1}H-NMR (400 MHz, Methanol-d_4) δ: 3.74 (s, 3H), 3.64 (s, 3H), 2.92 (d, J=15.7 Hz, 1H), 2.66 (d, J=15.7 Hz, 1H), 1.41 (s, 3H). ^{13}C-NMR (101 MHz, Methanol-d_4) δ: 176.9, 172.3, 73.8, 52.9, 52.1, 45.4, 26.6. NMR data is in accordance with literature.[22]

(*S*)- and (*R*)-2-Methylbutane-1,2,4-triol (8b+8c)

Triols **8b** and **8c** were independently synthesised following the same reaction conditions but using different starting materials: for the synthesis of (*S*)-**8b** a solution of dimethyl-(*S*)-2-hydroxy-2-methylsuccinate (**15**) (120 mg, 0.7 mmol) in tetrahydrofuran (0.5 ml) was prepared and for synthesis of (*R*)-**8c** a solution of bioconversion product 4-hydroxy-4-methyldihydrofuran-2(*3H*)-one (**6**) (90 mg, 0.77 mmol) in tetrahydrofuran (0.5 ml) was prepared. The solutions were each added dropwise to a solution of lithium aluminium hydride (85.1 mg, 2.3 mmol) in tetrahydrofuran (3.5 mL). After formation of hydrogen gas stopped, the mixtures were heated at reflux for 5 h, cooled to 0 °C and the excess reducing agent was neutralised by the successive addition of water (2 mL) and aqueous sodium hydroxide (2.5 M, 2 mL). After stirring for 1 h at room temperature, the mixtures were filtered and the filter cakes were washed with tetrahydrofuran (3 mL) and absolute ethanol (3 mL). The filtrate and washings were combined and evaporated. The residues were passed through a short column of silica gel with DCM:Methanol (1:9) as eluent. (*S*)-selective triol **8b** was obtained as yellow oil with a yield of 70% (59 mg, 0.49 mmol) while (*R*)-selective triol **8c** was obtained as yellow oil in 55% yield (58 mg, 0.42 mmol). ^{1}H-NMR for (*S*)-**8b** (400 MHz, Methanol-d_4) δ: 3.72 (m, 2H), 3.37 (d, J=4.9 Hz, 2H), 1.84–1.64 (m, J=6.9 Hz, 2H), 1.17 (s, 3H). ^{13}C-NMR (101 MHz, Methanol-d_4) δ: 73.4, 70.5, 59.2, 41.5, 24.4. ^{1}H-NMR for (*R*)-**8b** (400 MHz, Methanol-d_4) δ: 3.79–3.66 (m, 2H), 3.37 (d, J=4.7 Hz, 2H), 1.82–1.65 (m, 2H), 1.17 (s, 3H). ^{13}C-NMR (101 MHz, Methanol-d_4) δ: 73.4, 70.5, 59.2, 41.5, 24.3. NMR data are in accordance with literature.[22]

Labelling Studies

Whole-cells of *R. pyridinivorans* DSM 20415 and *R. rhodochrous* ATCC 17895 were resuspended to a final cell content of 100 mg/mL in either deuterated (100 mM, pD 6.2~pH 6.6) or standard (100 mM, pH 6.2) KPi buffer. Substrates (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) and *c*-hexenone (**1**) were added to a final concentration of 10 mM. Reactions were either carried out under $^{18}O_2$-atmosphere or under air (headspace-reaction mixture ratio 3:1). Reactions were run overnight at 28 °C and the supernatant was analysed by high resolution liquid chromatography-mass spectrometry (HRLC-MS).

Elimination Studies

Whole-cells of *R. rhodochrous* ATCC 17895 were resuspended to a final cell content of 100 mg/mL in deuterated KPi buffer (100 mM, pD 6.2~pH 6.6). Substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) was added to a final concentration of 15 mM. The reaction was run for 48 hours at 28 °C. The whole-cell mixture was extracted with EtOAc and concentrated upon flash column purification (eluent EtOAc:heptane 1:1, 99 mg, 0.85 mmol, 19%). ^{1}H-NMR for (*R*)-**6** (400 MHz, Chloroform-*d*) δ: 4.24 (d, J=9.7 Hz, 1H), 4.11 (d, J= 9.7 Hz, 1H), 3.21 (s, 1H), 2.59 (m, 1H), 1.46 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 176.7, 79.8, 74.5, 43.2, 24.8. 30 mg of purified 4-hydroxy-4-methyldihydrofuran-2(*3H*)-one-3-*d* (**22**) was dissolved in 1 mL EtOAc and 35 μL acetic anhydride and 120 μL trimethlamine were slowly added to the solution followed by 50 μL 4-dimethylaminopyridine (DMAP, 3 mg/mL solution in EtOAc). The reaction was stirred at room temperature for 24 hours. Afterwards, 1 mL H_2O was added and the organic layer was separated and dried over $MgSO_4$. The crude mixture was measured by NMR-spectroscopy (Figures S12–S14).

Bacterial Strains and Microorganisms

R. pyridinivorans DSM 20415 was purchased from the German Collection of Microorganisms and Cell Culture (Leibniz Institute DMSZ) while *R. rhodochrous* ATCC 17895 was bought from the American Type Culture Collection (Manassas, Virginia, US).

Microbiological Protocols

The organisms were maintained at 4 °C on a nutrient agar plate (3 g beef extract, 5 g peptone, 15 g agar dissolved 1 L de-ionised water and autoclaved at 121 °C) and were regularly sub-cultured. The growth medium used consisted of 1 L de-ionised water with 6.59 g glucose, 9.2 g peptone, 1.84 g yeast extract, 7 mM K_2HPO_4 (1.2 g), 3 mM KH_2PO_4 (0.4 g) with a final pH of 6.8 and was autoclaved at 110 °C. A preculture was inoculated with a single colony and grown overnight at 28 °C. The preculture (2 mL) was used to inoculate 1 L of culture medium grown in 5 L flasks. The culture was incubated for 72 hours at 28 °C with 180 rpm orbital shaking. The cells were harvested by centrifugation (17.696 x*g*, 15 min, 4 °C). The cells were washed with potassium phosphate buffer (100 mM, pH 6.2) and stored at −20 °C.

Whole-cells of *Rhodococcus* were resuspended in 100 mM KPi buffer (pH 6.2) to a final cell content of 100 mg/mL. Substrates (10 mM) were added to the reaction volume of 500 μL and incubated 24 hours at 28 °C at 1000 rpm. Small scale samples were extracted

twice with EtOAc (2×250 μL), dried with Na_2SO_4 and analysed on GC-FID.

NMR-Spectroscopy

^1H, ^{13}C-NMR and NOESY spectra were recorded on an Agilent (400 MHz and 101 MHz, respectively) instrument and were internally referenced to residual solvent signals. Data for ^1H-NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = duplet, dd = double duplet t = triplet, q = quartet, m = multiplet), coupling constant, integration. Data for ^{13}C-NMR are reported in terms of chemical shift.

Gas-Chromatography (GC)

Achiral GC-FID analysis was performed with a Shimadzu type GC-2010 Plus equipped with a CP Wax 52 CB column (50 m × 0.53 mm × 2.0 μm) using N_2 as carrier gas. The following conditions were used for the separation using direct injection: injector 280 °C, detector (FID) 280 °C, column flow rate 1.43 mL/min: (*i*) (*E*)-4-hydroxy-3-methylbut-2-enoic acid temperature programme: start at 90 °C, hold time 3 min, rate 5 °C/min to 250 °C hold time 1 min; (*ii*) c-hexenone temperature programme: start at 80 °C, hold time 2 min, rate 10 °C/min to 125 °C, hold time 5 min, 20 °C/min to 250 °C, hold time 1 min. Aliphatic, unsaturated aldehydes were measured on the same GC equipment with using a split-injection: split ratio 30:1, column flow rate: 0.96 mL/min. Temperature programme: start at 80 °C, hold time 3.5 min, rate 10 °C/min to 110 °C, hold time 1 min, rate 10 °C/min to 125 °C, hold time 1 min, rate 10 °C/min to 175 °C, hold time 1 min, rate 10 °C/min to 205 °C, hold time 1 min, rate 20 °C/min to 250 °C, hold time 3 min.

Chiral GC-FID analysis was performed with a Shimadzu type GC-2010 Plus equipped with a Chirasil Dex CB column (25 m × 0.32 mm × 0.25 μm) using He as carrier gas. The following conditions were used for the separation using a split-injection: injector 250 °C, detector (FID) 275 °C, column flow rate 1.14 mL/min: (*rac/R/S*)-2-methylbutane-1,2,4-triol temperature programme: start at 120 °C, hold time 40 min, rate 25 °C/min to 230 °C hold time 3 min.

High Resolution Liquid Chromatography – Mass Spectrometry (HRLC-MS)

HRLC-MS data was obtained using a Waters® acquity UPLC system and a Waters® Q-Tof Premier™ mass spectrometer. Supernatants of the samples from labelling studies were directly used for injection. A mobile phase of acetonitrile/water with 0.1% formic acid was used following a gradient method. All spectra were obtained in positive ion mode.

Acknowledgements

This work is part of the Open Technology Programme with project number 14170, which is (partly) financed by the Netherlands Organisation for Scientific Research (NWO). The authors are thanking Dr. Stephen Eustace and Dr. Carolin Bisterfeld for productive discussions.

Conflict of Interest

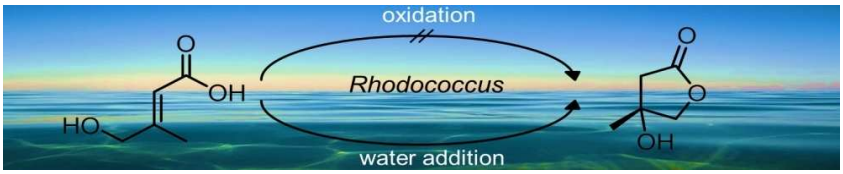
The authors declare no conflict of interest.

Keywords: alcohols · biotransformation · hydration · *Rhodococcus* · labelling studies

[1] V. Resch, U. Hanefeld, *Catal. Sci. Technol.* **2015**, *5*, 1385–1399.
[2] M. Engleder, H. Pichler, *Appl. Biochem. Biotechnol.* **2018**, *102*, 5841–5858.
[3] R. M. Demming, M. P. Fischer, J. Schmid, B. Hauer, *Curr. Opin. Chem. Biol.* **2018**, *43*, 43–50.
[4] G. Torrelo, F. Hollmann, U. Hanefeld, in *Catalysis* (Eds.: U. Hanefeld, L. Lefferts), Wiley-VCH Verlag GmbH & Co., Weinheim,Germany, **2018**, pp. 127–190.
[5] H. Gröger, *Angew. Chem. Int. Ed.* **2014**, *53*, 3067–3069.
[6] R. A. Sheldon, D. Brady, *ChemSusChem* **2019**, *12*, 2859-2881..
[7] D. Vasic-Racki, in *Ind. Biotransformations* (Eds.: A. Liese, K. Seelbach, C. Wandrey), Wiley-VCH Verlag GmbH & Co., Weinheim,Germany, **2006**, pp. 1–36.
[8] A. Liese, K. Seelbach, A. Buchholz, J. Haberland, in *Ind. Biotransformations* (Eds.: A. Liese, K. Seelbach, C. Wandrey), Wiley-VCH Verlag GmbH & Co., Weinheim,Germany, **2006**, pp. 147–514.
[9] H. J. Daneel, R. Geiger, (Amino GmbH), DE 4424664 C1, **1994**.
[10] H. J. Daneel, M. Busse, R. Faurie, *Meded. Fac. Landbouwwet., Univ. Gent* **1995**, *4a*, 2093–2096.
[11] H. Yamada, Y. Tani, (NITTO Chemical Industry Co LTD), EP 0093782 B1, **1987**.
[12] H. L. Holland, J. X. Gu, *Biotechnol. Lett.* **1998**, *20*, 1125–1126.
[13] B.-S. Chen, V. Resch, L. G. Otten, U. Hanefeld, *Chem. Eur. J.* **2015**, *21*, 3020–3030.
[14] S. M. Thomas, R. DiCosimo, V. Nagarajan, *Trends Biotechnol.* **2002**, *20*, 238–242.
[15] D. Kim, K. Y. Choi, M. Yoo, G. J. Zylstra, E. Kim, *J. Microbiol. Biotechnol.* **2018**, *28*, 1037–1051.
[16] H. M. Alvarez, *Biology of Rhodococcus*, Springer Nature Switzerland AG, Cham, Switzerland, **2010**.
[17] R. van der Geize, L. Dijkhuizen, *Curr. Opin. Microbiol.* **2004**, *7*, 255–261.
[18] M. Overduin, B. Klumperman, *Eur. Polym. J.* **2019**, *110*, 63–68.
[19] K. S. Simon, N. L. Pollock, S. C. Lee, *Biochem. Soc. Trans.* **2018**, *46*, 1495–1504.
[20] B.-S. Chen, R. Médici, M. P. van der Helm, Y. van Zwet, L. Gjonaj, R. van der Geest, L. G. Otten, U. Hanefeld, *Appl. Microbiol. Biotechnol.* **2018**, *102*, 5545–5556.
[21] H. Ahmed, in *Princ. React. Protein Extr. Purif. Charact.* (Ed.: H. Ahmed), CRC Press LLC, Boca Raton London New York Washington, D.C., **2005**, pp. 1–33.
[22] M. Gill, M. F. Harte, A. Ten, *Aust. J. Chem.* **2000**, *53*, 245–256.
[23] E. G. J. Staring, H. Moorlag, H. Wynberg, *Recl. Trav. Chim. Pays-Bas* **1986**, *105*, 374-375.
[24] V. Resch, C. Seidler, B. S. Chen, I. Degeling, U. Hanefeld, *Eur. J. Org. Chem.* **2013**, 7697–7704.
[25] D. Brodkorb, M. Gottschall, R. Marmulla, F. Lüddeke, J. Harder, *J. Biol. Chem.* **2010**, *285*, 30436–30442.
[26] S. Weidenweber, R. Marmulla, U. Ermler, J. Harder, *FEBS Lett.* **2016**, *590*, 1375–1383.
[27] M. L. Thompson, R. Marriott, A. Dowle, G. Grogan, *Appl. Microbiol. Biotechnol.* **2010**, *85*, 721–730.
[28] J. J. Bourguignon, A. Schoenfelder, M. Schmitt, C. G. Wermuth, V. Hechler, B. Charlier, M. Maitre, *J. Med. Chem.* **1988**, *31*, 893–897.
[29] G. E. Magoulas, S. E. Bariamis, C. M. Athanassopoulos, A. Haskopoulos, P. G. Dedes, M. G. Krokidis, N. K. Karamanos, D. Kletsas, D. Papaioannou, G. Maroulis, *Eur. J. Med. Chem.* **2011**, *46*, 721–737.
[30] R. Eck, H. Simon, *Tetrahedron* **1994**, *50*, 13641–13654.

Manuscript received: August 27, 2019
Revised manuscript received: September 24, 2019
Accepted manuscript online: September 24, 2019
Version of record online: ■■, ■■■

FULL PAPERS



Whole-Cell Biocatalysis: The net microbial water addition to Michael acceptors catalysed by Rhodococcus whole-cells was re-evaluated. $^{18}O_2$- and D_2O labelling studies excluded an oxidative process while confirming a true water addition. Depending on the substrate, the water addition is amino acid-catalysed or performed by an oxygen-dependent novel hydratase.