

Biocatalysis

Enantioselective Michael Addition of Water

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Abstract: The enantioselective Michael addition using water as both nucleophile and solvent has to date proved beyond the ability of synthetic chemists. Herein, the direct, enantioselective Michael addition of water in water to prepare important β -hydroxy carbonyl compounds using whole cells of

Rhodococcus strains is described. Good yields and excellent enantioselectivities were achieved with this method. Deuterium labeling studies demonstrate that a Michael hydratase catalyzes the water addition exclusively with *anti*-stereochemistry.

Introduction

The direct addition of water to C=C bonds is a highly attractive transformation, yielding (chiral) alcohols.^[1] However, the enantioselective addition of water to α,β -unsaturated carbonyl (Michael) acceptors still represents a chemically very challenging reaction,^[2] due to the poor nucleophilicity of water and its small size, which make regio- and stereoselection difficult. Equally, the often unfavorable equilibrium of water-addition reactions remains to be solved. Although this reaction benefits from its simplicity and excellent atom economy, no protocol with broad applicability has to date been developed. Indirect methods^[3] using complex catalysts^[4] or strong alternative nucleophiles^[5] have been employed. Some of the described methods require either cumbersome catalyst preparation or reductive/oxidative follow-up chemistry. Selective direct methods have been reported by Roelfes and co-workers, applying DNA-based Cu^{II} catalysts^[6] or the use of a protein as chiral ligand.^[7] However, they are limited to α,β -unsaturated 2-acyl imidazoles as substrates and yield the corresponding alcohols in moderate enantiomeric purities. The only chemocatalytic process run on industrial scale was the addition of water to acrolein.^[1d] Nevertheless, due to its poor selectivity and productivity, even this seemingly straightforward reaction has been replaced by a fermentative process.^[1d,8]

In contrast, enzymes such as fumarase, malease, citraconase, aconitase, and enoyl-CoA hydratase have been successfully used on industrial scale, and their excellent (enantio-) selectivities are highly valued.^[1d,9] Unfortunately, most hydratases are part of the primary metabolism where perfect substrate specificity is required. This very high substrate selectivity severely limits their practical applicability in organic synthesis.^[2a] A recent report on an asymmetric hydration of hydroxystyrene-type substrates catalyzed by phenolic acid decarboxylases showed that a broader flexibility in the substrate spectrum for hydratases is possible.^[10] In order to broaden the biocatalytic toolbox of hydratases, the work represented herein is dedicated to the search for a Michael hydratase with a more relaxed substrate specificity.

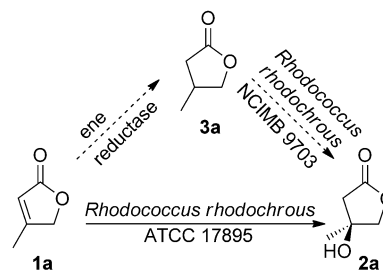
In our search for a straightforward approach for the preparation of β -hydroxy carbonyl compounds via the direct Michael addition of water, it was noted that whole cells of *Rhodococcus rhodochrous* ATCC 17895 convert 3-methylfuran-2(5H)-one **1a** into (*S*)-3-hydroxy-3-methylfuranone **2a**; as briefly described in 1998.^[11] Neither substrate **1a** nor product **2a** are part of the primary metabolism indicating the involvement of a putative Michael hydratase with possibly a broader substrate scope. Since whole cells were used in this transformation, the hydratase activity needed to be critically evaluated.^[11–14] Instead of a direct addition of water, the conversion of **1a** to **2a** could also occur via a two-step approach (Scheme 1). Indeed, the

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Scheme 1. Biotransformation of **1a** to **2a** by *R. rhodochrous* by Michael addition of water or alternatively by a reduction–oxidation stepwise approach.^[11,15]

enantioselective hydroxylation of a range of THF and THP derivatives was reported for *R. rhodochrous* strains.^[15] Therefore, it is of high interest to probe whether the conversion of **1a** to **2a** is actually a Michael addition of water and how broadly it is applicable.

Herein we report the results of screening several *Rhodococcus* strains as promising biocatalysts for the enantioselective Michael addition of water to a variety of α,β -unsaturated carbonyl compounds.

Results and Discussion

Optimization

To fully assess the potential of the putative Michael addition of water, the previously reported conversion of 3-methylfuran-2(5*H*)-one (**1a**)^[11] was repeated and optimized. **1a** was synthesized using a modified literature procedure (see the Supporting Information, S3).^[16] Whole cells of *R. rhodochrous* ATCC 17895 were used in two different concentrations (100 mg mL⁻¹ and 330 mg mL⁻¹ of wet cells; Table 1). The reaction with 100 mg mL⁻¹ cells gave a maximum conversion of 69% after 17 h and, even after a prolonged reaction time (4 days), no further increase in conversion was observed. Furthermore, an *ee* of 91% was determined, which is in agreement with the previously reported study.^[11] An increase of the cell concentration to 330 mg mL⁻¹ of wet cells resulted in full conversion after 17 h, while *ee* values remained unchanged (90%). When using

3a as substrate under aerobic conditions (Table 1, control 1), no conversion to **2a** was detected, indicating that no oxidation occurs. In previous studies^[2b,14] we were able to show that a chemically catalyzed addition reaction occurs when 2-cyclohexenone (**1h**) is used as a substrate. Therefore, any undesired background reaction needed to be ruled out. Heat-denatured cell preparations in control experiments (Table 1, control 2) clearly showed that there is no chemically catalyzed reaction taking place; thus the reaction is effected by the active enzyme.

Encouraged by the complete conversion after 17 h, we evaluated the rate of the reaction with 330 mg mL⁻¹ of wet cells. This revealed an almost linear increase in product formation during the first 6 h of the reaction and **2a** was formed in 75% yield (Figure 1 A). Complete conversion based on the consump-

Table 1. Influence of the catalyst concentration on the conversion.

	Catalyst	Catalyst conc. (wet cells)	Substrate	Conversion ^[a] of 1a [%]	Yield ^[a] of 2a ^[b] [%]	<i>ee</i> ^[a] of 2a [%]
this study	resting cells	100 mg mL ⁻¹	1a	69	57	91
	resting cells	330 mg mL ⁻¹	1a	99	87	90
ref. [11]	resting cells	100 mg mL ⁻¹	1a	55	55	95
control 1	resting cells ^[c]	330 mg mL ⁻¹	3a	–	<3	n.d. ^[f]
control 2	denatured cells ^[d]	330 mg mL ⁻¹	1a	12 ^[e]	<3	n.d. ^[f]

[a] Conversion, yield, and *ee* values were determined by GC; [b] absolute configuration of **2a** has been established by converting **2a** into the corresponding methyl ester [methyl *S*-(-)-3,4-dihydroxy-3-methylbutanoate];^[12,13] [c] reaction with **3a** was carried out to rule out possible oxidation; [d] reaction with heat-denatured cells was carried out to ensure no background reaction is taking place; [e] conversion is caused by the ring opening of lactone **1a**, no water addition product (**2a**) was detected; [f] n.d. = not determined.

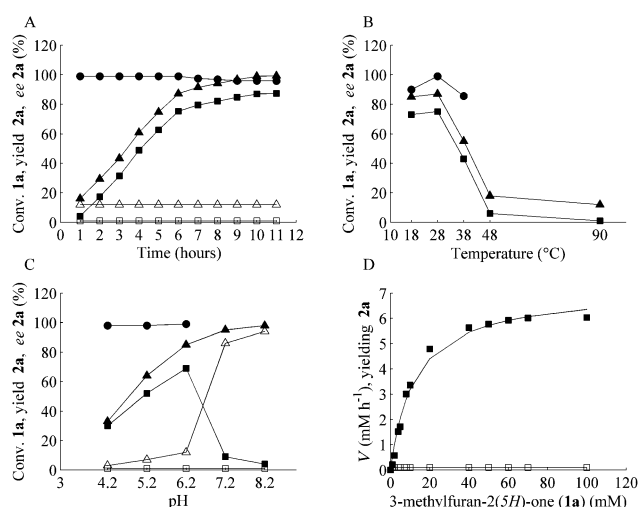


Figure 1. Time course (A), temperature profile at reaction time 6 h (B), pH profile at reaction time 6 h (C) and Michaelis–Menten kinetics (D, based on the yield of **2a**) of the putative Michael addition catalyzed using whole cells of *R. rhodochrous* ATCC 17895. For reaction conditions, see the Experimental Section. Conversion, yield, and *ee* values were determined by GC. Filled circles represent *ee* of **2a**. Filled triangles represent consumption of **1a**. Filled squares represent yield of **2a**. Empty triangles represent consumption of **1a** in blank reactions. Empty squares represent yield of **2a** in blank reactions (in A and D, blank reaction was carried out with heat-denatured cells; in C, blank reaction was carried out without the addition of cells).

tion of **1a** was obtained after 9 h. No significant changes of the product *ee* within the first 9 h were observed (from 99% to 95%; see the Supporting Information, S11 for GC chromatographs). It should be noted that, since the desired Michael addition products (**2a**) are highly soluble in water, the choice of the organic solvent for extraction is crucial. For example, using ethyl acetate or dichloromethane as the extraction solvent only allowed recovery of 30% of the product (data not shown). In extraction studies, isoamyl alcohol gave the best result for extraction of (*S*)-3-hydroxy-3-methylfuranone (**2a**). However, due to the similar polarity of isoamyl alcohol and water-addition product **2a**, severe problems, such as separation issues during purification, arose. Therefore, for preparative-scale experiments, reaction mixtures were always continu-

ously extracted overnight using a liquid–liquid extractor and ethyl acetate as the organic solvent (see the Supporting Information, S9). This procedure had no influence on the *ee* values of the product (data not shown).

The temperature profile of the reaction was evaluated as well. Temperatures ranging from 18 °C to 48 °C were tested. Conversions and *ee* values at different temperatures are summarized in Figure 1B. When increasing the temperature above 28 °C a decrease in enzyme activity was observed. At 48 °C, a yield of only 5% was detected (an additional 12% was brought about by ring opening of lactone **1a**). Due to the low amount of product at 48 °C, no reliable *ee* determination was possible. Taking both the conversion and enantioselectivity into account, the best results were achieved at 28 °C. These results are in agreement with the reported optimal growth temperature of 26 °C for *R. rhodochrous*.^[17]

Since water serves not only as the reaction medium but also as a substrate, the pH needs to be considered as a very important parameter. To quantify this effect, the reaction system was tested at different pH values using potassium phosphate buffer (pH 5.2–8.2) and citrate/phosphate buffer (pH 4.2) to control the pH of the reaction medium. The results from this study clearly show the dependence on pH. The conversion increased with increasing pH (Figure 1C, filled triangles), as expected from our previous study,^[2b] demonstrating that the hydration reaction is generally base-catalyzed. However, at neutral and slightly basic conditions (pH 7.2 and pH 8.2), significant ring opening of lactone **1a** took place (Figure 1C, empty triangles), which explains the rather poor product yield (Figure 1C, filled squares). This effect can be explained by the spontaneous hydrolysis of the lactone in basic aqueous medium, which is an often observed phenomenon.^[18] To confirm this, the blank reaction mixtures at pH 7.2 and 8.2 were acidified with conc. HCl to pH 1. This leads to complete recovery of the substrate **1a**, validating our hypothesis. No desired enantioselective Michael addition product **2a** was detected in the blank reactions (Figure 1C, empty squares) indicating that chemical/base-catalyzed Michael addition does not occur within the measured pH ranges. Therefore, the conversion in the blank reactions (which is based on the decrease in amount of substrate) is caused by the hydrolysis of **1a**. Moreover, product **2a** showed good stability under strongly acidic conditions and only 10% yield was lost overnight. Comparing the mass balance and reaction rate, slightly acidic conditions (pH 6.2) represented the optimal pH for this substrate.

Control experiments (Table 1 and Figure 1A–C) confirmed that the formation of **2a** is based on an enzymatic reaction with high enantioselectivity and that no chemical background reaction occurred. Therefore, the kinetic parameters K_m , V_{max} , and V_{max}/K_m were determined with the optimized conditions. The Michaelis–Menten Plot (Figure 1D) allowed calculation of the affinity constant K_m as 1.7×10^{-2} M and V_{max} as $6.9 \text{ nmol s}^{-1} \text{ g}^{-1}$ (wet cells), providing further support for one enzymatic reaction, rather than a sequence of reactions (Scheme 1).

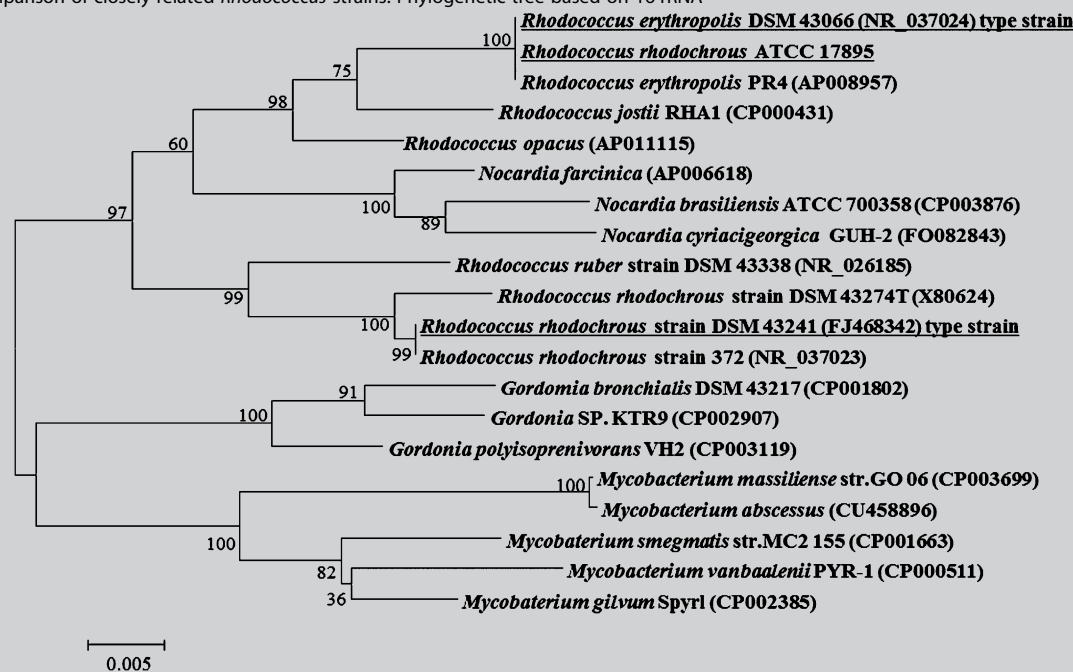
To establish the distribution of the enzymatic activity over different organisms, we proceeded with testing different close-

ly related *Rhodococcus* strains. The selection was based on phylogenetic analysis (Table 2). The previously reported strain *R. rhodochrous* ATCC 17895 was shown to be much more closely related to *R. erythropolis* than to *R. rhodochrous*.^[17] For this reason, strains *R. erythropolis* DSM 43296, *R. erythropolis* CCM 2595, *R. erythropolis* NBRC 100887, and *R. erythropolis* DSM 43066 were evaluated (Table 2). Experiments for comparing the different organisms were carried out under conditions optimized for *R. rhodochrous* ATCC 17895. Gratifyingly, in each case, 3-methylfuran-2(5*H*)-one (**1a**) was converted into (*S*)-3-hydroxy-3-methylfuranone (**2a**) with good yields and excellent enantioselectivities (see the Supporting Information, S12 for GC chromatographs). Encouraged by these results, the less closely related strain *R. rhodochrous* DSM 43241 was also tested for water addition activity. Interestingly, the enantiomerically enriched water-addition product (*S*)-3-hydroxy-3-methylfuranone **2a** was also obtained in 75% yield and with an 86% *ee*, which was slightly lower than that with *R. erythropolis* strains. All the results suggest that this promising hydratase activity is not limited to *R. rhodochrous* ATCC 17895 but may be a general feature in several *Rhodococcus* strains. Taking the conversion, enantioselectivity, and available genome sequence into account, we decided to continue to use strain *R. rhodochrous* ATCC 17895 for all further studies.

Substrate scope and limitations

Since the very limited substrate scope of the known hydratases is one of the challenging factors for their broad application, we were interested in the scope of the Michael hydratase from *R. rhodochrous* ATCC 17895. Neither substrate **1a** nor product **2a** are known to be part of primary metabolic pathways, therefore the substrate scope of the hydratase from *R. rhodochrous* ATCC 17895 might be more relaxed than that for other known hydratases. Hence we tested a set of different substrates to evaluate the limitations of the enzyme (Table 3). For α,β -unsaturated lactones ($X=O$; Table 3, entries 1–3) with substituents in the β -positions, the reaction proceeded smoothly in all cases to yield the corresponding hydration products, whereas for $R^1=H$ ($X=O$; Table 3, entries 4 and 5), no water addition product was obtained. This result is surprising, as the tertiary alcohols obtained are sterically much more demanding than the secondary alcohols, and suggests that substituents in the β -position might play an important role for proper orientation of the lactones in the enzyme's active site. However, the enzyme did not accept substrates with substituents in both β - and γ -positions, such as **1f** (Table 3, entry 6), which is probably due to its more bulky structure. Products **2a** and **2b** are tertiary alcohols, representing a class of compounds that are difficult to prepare by chemical methods, to date only accessible via this route.^[11] The enantioselectivity was measured using a chiral Ivadex 7/PS086 GC column and, in parallel, the *ee* was confirmed by analysis of ^1H and ^{19}F NMR spectra of their corresponding Mosher esters (see the Supporting Information, S4, S5, and S27–S29). In both cases, results from ^1H and ^{19}F NMR spectra of the Mosher esters and chiral GC analysis of the alcohols were comparable, showing excel-

Table 2. Comparison of closely related *Rhodococcus* strains. Phylogenetic tree based on 16 rRNA



Entry ^[a]	Catalysts	Conversion ^[b] of 1a [%]	Yield ^[b] of 2a [%]	ee ^[b] of 2a [%]	Genome sequence
1	<i>R. rhodochrous</i> ATCC 17895	87	75	95	+
2	<i>R. erythropolis</i> DSM 43296	82	70	93	–
3	<i>R. erythropolis</i> CCM 2595	88	76	95	+
4	<i>R. erythropolis</i> NBRC100887	80	68	93	+
5	<i>R. erythropolis</i> DSM 43066	90	78	95	–
6	<i>R. rhodochrous</i> DSM 43241	87	75	86	–
7	90 °C heat-denatured cells of <i>R. rhodochrous</i> ATCC 17895	12	< 3	n.d.	

[a] List of entries comparing activities using different organisms. [b] Conversion, yield, and ee values were determined by GC.

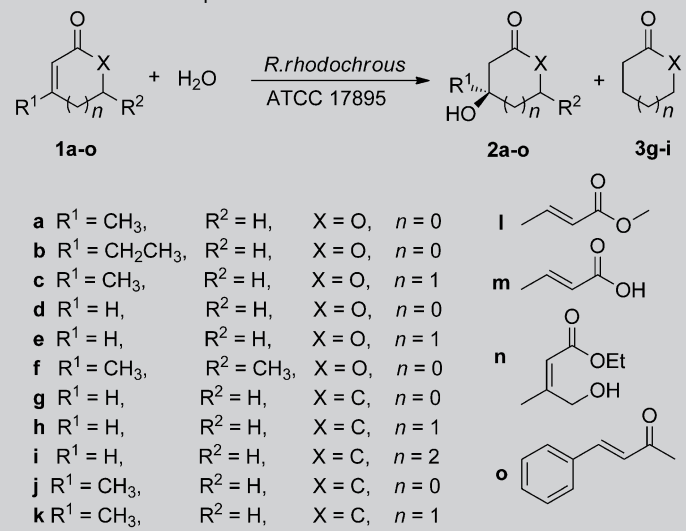
lent enantioselectivities. The absolute configuration of the product was established by converting **2a** into the corresponding methyl ester [methyl *S*-(–)-3,4-dihydroxy-3-methylbutanoate].^[12,13]

Interestingly, the hydration of substrate **1c** (Table 3, entry 3) gave access to the natural product mevalonolactone **2c**, the salt form of which represents an intermediate in the pathway leading to terpenoids.^[19] Absolute configuration of (*R*)-**2c** was determined by comparison with previously reported optical rotation data.^[20] Mevalonate is a product of acetate metabolism and thus a key building block in secondary metabolism.^[21] To identify whether the putative Michael hydratase is a promiscuous enzyme of the mevalonate pathway, bioinformatics studies were performed. We have sequenced and annotated the genome of *R. rhodochrous* ATCC 17895 in a previous study.^[17] Looking for annotated hydratases in this genome only showed known hydratases with their narrow substrate specificity, emphasizing that the hydratase of this study has not been described before. This enzyme could therefore be one of the many unknown gene functions in the genome, or a promiscuous activity of a known enzyme. Screening through all three sequenced *Rhodococci* genomes (Table 2, entry 1, 3 and 4), we unexpectedly found that most of the typical enzymes from the mevalonate pathway were missing. Instead the full deoxyxylu-

lose phosphate (mevalonate-independent) pathway for terpenoid biosynthesis was present.

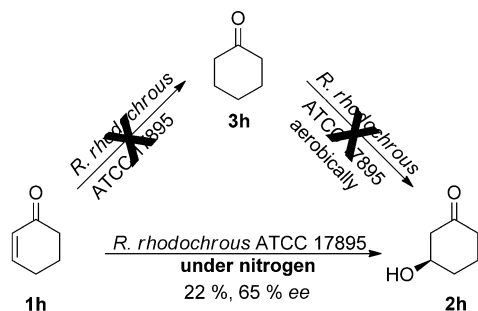
For α,β -unsaturated ketones ($X=C$), substrates without substituent in the β -position were surprisingly accepted by the putative Michael hydratase (Table 3, entries 7–9) but no activity towards the β -substituted 3-methylcyclohex-2-enone and 3-methylcyclopent-2-enone was found (Table 3, entries 10 and 11). This might lead to the conclusion that β -substituted α,β -unsaturated ketones may be challenging for Michael addition of water using *R. rhodochrous*, although the opposite is true for α,β -unsaturated lactones. The α,β -unsaturated ketones **1g–i**, were mostly reduced into ketones **3g–i** (75%, 76%, and 80% yields, respectively), which explains the rather poor yield of the water-addition reaction (Table 3, entries 7–9). Experiments to rule out the reduction–oxidation as a possible reaction pathway were performed for these cyclic ketones (Scheme 2). Reaction using **1h** as substrate was performed under a nitrogen atmosphere to exclude air as a potential oxygen source. Even so, 22% yield of **2h** was obtained with 65% ee, ruling out the involvement of O_2 as an active species in the reaction. Furthermore, when **3h** was used as a substrate directly under aerobic conditions, no product **2h** was detected. These two control experiments demonstrate that the alcohol **2h** was the result of the enantioselective Michael addition of water to **1h**. The com-

Table 3. Substrate scope for the enantioselective Michael addition of water.



Entry	Substrate	Product	Conversion ^[a] of 1 [%]	Yield ^[a] of 2 [%]	ee of 2 [%]	Enantio- preference	Equilibrium yield of 2 [%]
1	1a	2a	87	75	95 ^[c,d]	S ^[e]	> 97 ^[i]
2	1b	2b	80	68	94 ^[d]	S	— ^[i]
3 ^[b]	1c	2c	75	62	73 ^[c]	R ^[f]	— ^[i]
4	1d	2d	12	<3	n.d.	n.d.	— ^[i]
5	1e	2e	32	<3	n.d.	n.d.	— ^[i]
6	rac-1f	2f	12	<3	n.d.	n.d.	— ^[i]
7	1g	2g	93	18	22 ^[c]	R ^[g]	— ^[i]
8	1h	2h	98	22	65 ^[c]	R ^[g]	25 ^[k]
9	1i	2i	95	15	20 ^[c]	R ^[h]	— ^[i]
10	1j	2j	<3	<3	n.d.	n.d.	— ^[i]
11	1k	2k	<3	<3	n.d.	n.d.	— ^[i]
12	1l	2l	42	40	48 ^[c]	R ^[f]	— ^[i]
13	1m	2m	<3	<3	n.d.	n.d.	— ^[i]
14	1n	2n	<3	<3	n.d.	n.d.	— ^[i]
15	1o	2o	<3	<3	n.d.	n.d.	— ^[i]

[a] Conversion and yield were determined by GC; [b] reaction was performed at pH 5.2 to suppress ring open of lactone **1c** at pH 6.2; [c] ee was determined by GC; [d] ee was determined by ¹H and ¹⁹F NMR of the corresponding Mosher ester; [e] changing CIP priorities; [f] (*R*)-enantiomers commercially available; [g] absolute stereochemistry was determined by converting them into literature-known derivatives, following a procedure established earlier in our laboratory;^[24] [h] absolute configuration was determined by comparison of the retention times using the same GC column with a reported method;^[25] [i] reverse reaction with **2a** as substrate was performed, analysis of this sample showed no dehydration and no decrease of the ee; [j] no literature values available; [k] see references^[2b,23].



Scheme 2. Control experiments to confirm that **2h** was formed by enzymatic water addition, rather than a reduction–oxidation sequence.

peting reduction reaction to **3g**, **3h**, and **3i** is most likely due to an ene reductase also present in the *Rhodococcus* cells.

To further probe whether the putative Michael hydratase also accepts acyclic α,β -unsaturated carbonyl compounds, methyl crotonate (**1l**), crotonic acid (**1m**), (*Z*)-ethyl-4-hydroxy-3-methylbut-2-enoate (**1n**), and benzylideneacetone (**1o**) were subjected to the resting cell suspensions (Table 3, entries 12–15). Gratifyingly, the enzyme readily accepted acyclic α,β -unsaturated ester (**1l**), although no activity was observed for acyclic α,β -unsaturated carboxylic acid **1m**, ester **1n**, or ketone **1o**. Notably, in many water-addition reactions to carbon–carbon double bonds, the equilibrium can be on the side of the starting material although the reaction is performed in water.^[1d,6c,22] The unfavorable equilibrium might impede the Michael addition of water; for example, the equilibrium yield of 3-hydroxycyclohexanone (**2h**) was determined to be 25% (Table 3, entry 8),^[2b,23] corresponding with the measured yield of 22%.

Finally we tested the scalability of the developed reaction system. Therefore the reaction was scaled to gram scale using **1a** (2 g, 20 mmol, 200 g of wet cells) to give **2a** in 69% isolated yield after column chromatography and an ee of 90% was determined.

Recyclability and enzyme investigation

One of the most important characteristics of a catalyst is its operational stability and reusability over an extended period of time, to ensure a practical application.^[26] From the viewpoint of process economics, the higher the number of cycles that an enzyme remains stable, the more efficiently a process can be run. Experiments were performed to examine this recyclability of the whole cells of strain *R. rhodochrous* ATCC 17895 for the Michael addition of water to 3-methylfuran-2(5*H*)-one (**1a**). Based on the results summarized in Table 1, every reaction was carried out in a 50 mL Erlenmeyer flask at 28 °C with 180 rpm for 23 h. At the end of the reaction, the cells were centrifuged, washed twice with potassium phosphate buffer (100 mM, pH 6.2), and then reused for the next cycle. Whole cells showed high activity and complete conversion for 4 cycles (Figure 2). Only a slight decrease was observed in cycle 5, whereas 27% lower conversion was detected in the cycle 6. However, even after 9 consecutive cycles, the whole cells retained 20% of the initial activity. Notably, no significant changes in enantioselectivities of the water-addition reactions were detected during the 9 cycles (see the Supporting Information, S12 for GC chromatography).

To isolate the putative Michael hydratase for further investigation, we first broke the whole cells of *R. rhodochrous* ATCC 17895. The desired hydratase activity (yielding **2a**) was only found in the cell pellets, rather than in the cell-free extract, when **1a** was used as a substrate (Figure 3). Furthermore, no significant difference was found between the initial rate of whole cells and pelleted cell debris (Figure 3). Additionally,

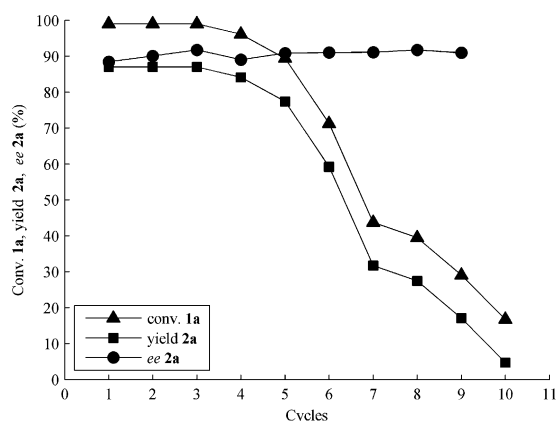


Figure 2. Repeated water-addition reactions catalyzed by whole cells of *R. rhodochrous* ATCC 17895. Conversion, yield, and ee values were determined by GC.

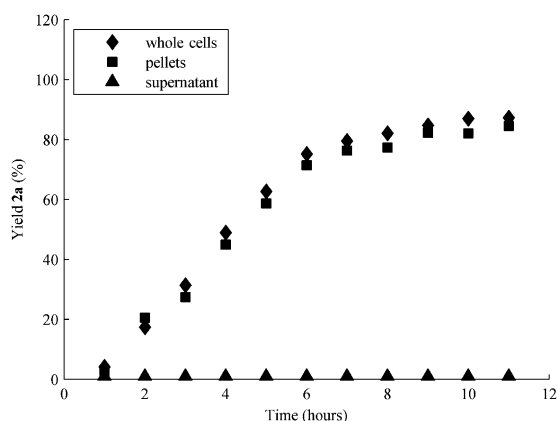


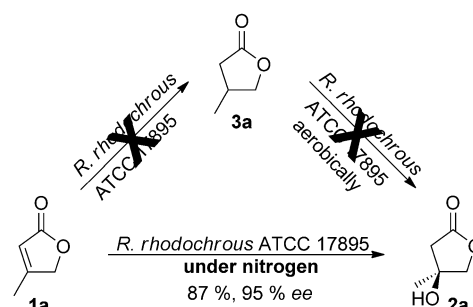
Figure 3. Michael hydratase activities in different biocatalyst preparations (whole cells, pelleted cell debris, and cell-free extract). Conversion was determined by GC.

with **1h** as a substrate, most of the ene reductase activity (Table 3, entries 7–9) was retained in the cell-free extract, whereas only minor activity was still detectable in the cell pellets. These results indicate that the putative Michael hydratase is not a soluble protein but bound to either the membrane or cell wall, whereas the reductase activity apparently resides within another enzyme that is soluble. This natural immobilization of the putative Michael hydratase explains the high reusability of the whole cells (Figure 2).

Mechanistic studies

As mentioned above, *Rhodococci* have been shown to mediate the hydroxylation of unactivated C–H bonds on selected THF and THP derivatives.^[15] To clearly rule out the possibility of detecting a hydroxylation reaction instead of a hydration, we turned our attention to the mechanism, including the stereochemical course of the reaction. The reaction was performed under a nitrogen atmosphere to exclude air as a potential oxygen source. Under these conditions, 87% conversion with

95% enantioselectivity was still obtained, ruling out that O₂ is involved as an active species in the reaction. The second approach included the use of substrate **3a**, which might be formed by reduction of **1a**. Therefore **3a** was synthesized (see the Supporting Information, S5) according to a standard procedure.^[27a] When using **3a** with a resting cell suspension, no corresponding oxidation product was detected under aerobic conditions (Scheme 3). These two control experiments demon-



Scheme 3. Control experiments to confirm that **2a** was formed by enzymatic water addition, rather than a reduction–oxidation sequence.

strated that the enantiomerically enriched tertiary alcohol **2a** was the result of the direct enantioselective Michael addition of water to **1a**.

The stereochemical course of this water-addition reaction was further evaluated by carrying out the biotransformation in D₂O using lyophilized cells as catalyst. The reaction in D₂O was found to be slower than that in H₂O, which might be due to activity loss caused by the lyophilization or an isotope effect. However, upon elongation of the reaction time to 24 h, deuterium oxide-addition product **4a** was found at a conversion of 90%. After extraction with ethyl acetate and column purification, compound **4a**, containing the optically active OD group, was exchanged back into an OH group, which is an often-observed phenomenon.^[28] NMR and GC-MS measurements showed that the obtained compound (**4b**) contained one deuterium at the α carbon (Figure 4A). In the ¹H NMR spectrum of **2a**, the geminal coupling constant between the two α protons is 17.6 Hz, whereas the ¹H NMR spectrum of **4b** showed only one α proton, which indicates one deuterium at the α carbon. Comparing with the singlet signal of **2a** in the ¹³C NMR spectrum, the triplet signal (coupling constant of 19.75 Hz) of **4b** again indicates one deuterium at the α carbon. GC-MS spectra also show **4b** to be one unit heavier than **2a** (for full spectra, see the Supporting Information, S21–S23). A control experiment was performed by shaking pure 3-hydroxy-3-methylfuranone (**2a**) in D₂O. Analysis of this sample showed that no deuterium was incorporated at the α -position; hence, the ²H-labeled product **4b** must have resulted from enzymatic water addition. This further supports a one-step hydration mechanism, via a Michael addition reaction.

According to the NMR measurements, the reaction of substrate **1a** and lyophilized *R. rhodochrous* ATCC 17895 cells in D₂O yielded monodeuterated **4b** as a sole diastereoisomer

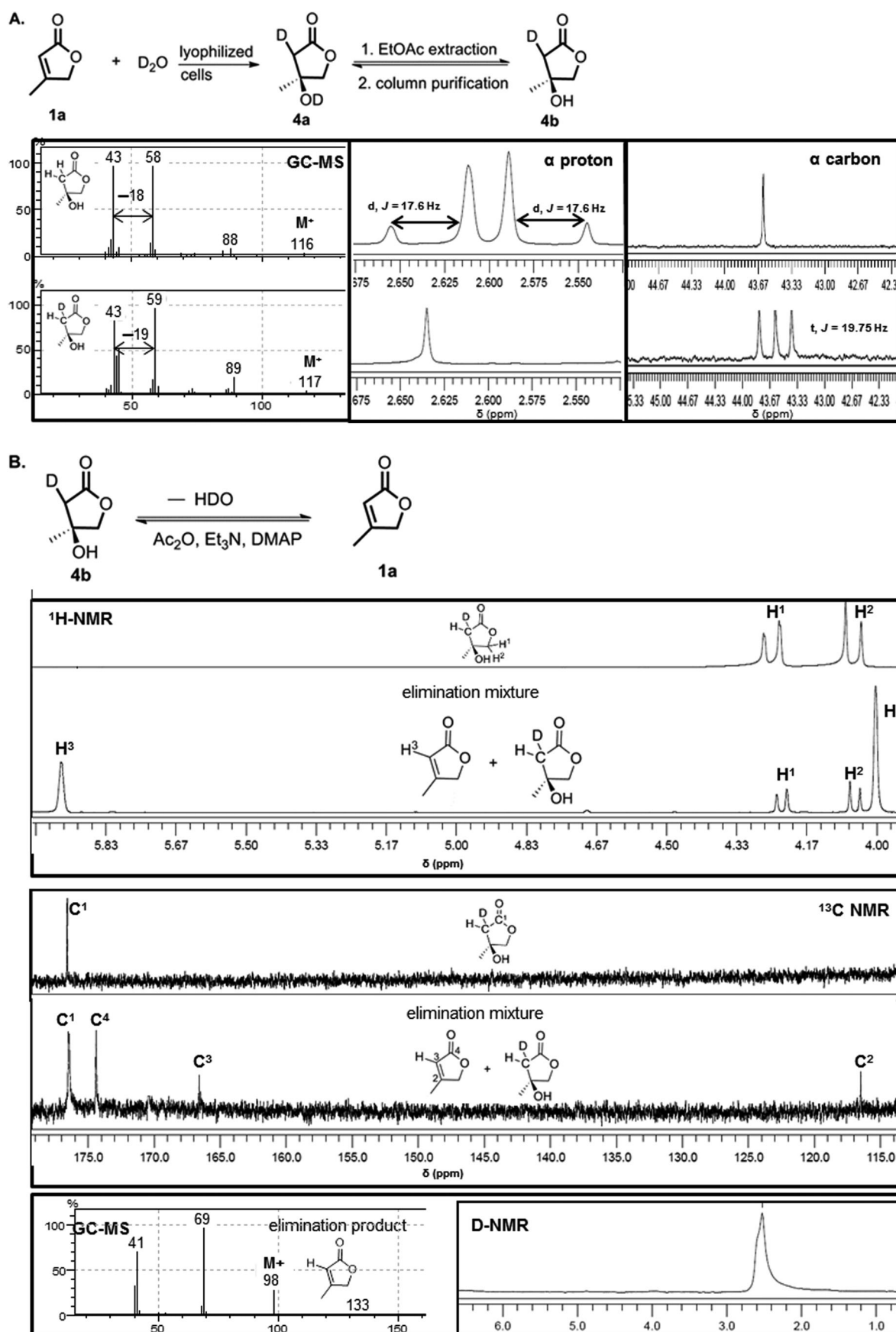


Figure 4. Diastereoselective Michael addition of water catalyzed by lyophilized cells of *R. rhodochrous* ATCC 17895.

(Figure 4 A; for full spectra see the Supporting Information, S21 and S22). The observation brought us to investigate the diastereospecificity of the Michael addition of water, which until now had been reported to show, depending on the enzyme, either *syn* or *anti* preference.^[29a] For example, in the case of enoyl-CoA hydratase, selectivity towards *syn*-addition was observed,^[29b] whereas enzymes belonging to the aspartase/fumarase superfamily, such as fumarase, aconitase, or enolase, showed *anti* preference.^[29c]

Nuclear Overhauser effect (NOE) experiments unfortunately did not give conclusive results on the stereochemical course of the water addition (see the Supporting Information, S32 and S33). To further probe the stereoselectivity of the addition of water, an *anti* E2 elimination of the deuterium oxide addition product **4b** was performed.^[30] The reaction was accomplished with acetic anhydride/triethylamine in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP) and the corresponding product was measured directly by NMR and GC-MS without further purification. The results showed **1a** as the only elimination product, HDO being expelled during the elimination process (Figure 4B). ¹H NMR spectroscopy showed the appearance of signal for α proton at 5.91 ppm, indicating that HDO was eliminated via an *anti* E2 elimination. The appearance of a singlet (which was visible as a triplet at 43.6 ppm previously in **4b**) at 116.2 ppm for the α carbon in the ¹³C NMR spectrum also proved the loss of molecular HDO. The D-NMR spectrum shows only one peak at 2.60 ppm, which belongs to the unconverted **4b**, but no peak at 5.91 ppm, again proving that HDO was eliminated. This elimination was further confirmed by GC-MS analysis (for full spectra, see the Supporting Information, S24 and S25). Since the E2 elimination always occurs exclusively in *anti* fashion, and removed α -D and β -OH groups, the enzymatic D₂O addition must have proceeded with exclusive *anti* stereochemistry. Our results are supported by the findings of Mohrig et al.,^[29a,d] who described that the stereopreference of water addition depends on the position of the abstracted proton: If the proton is in the α -position to the carboxylate group, as in the case of our studies, *anti*-selectivity is observed; abstracted protons that are in the α -position to the carbonyl group of the thioester lead to *syn*-selectivity.^[29a,b]

Conclusion

β -Hydroxy carbonyl compounds represent an important class of compounds that is often found as a structural motif in natural products. Although the molecules themselves look rather simple, their synthesis can be challenging. A straightforward route for the preparation of chiral β -hydroxy carbonyl compounds was established, employing whole cells from several *Rhodococcus* strains harboring a Michael hydratase. They catalyzed the enantioselective Michael addition of water in water with good yields and excellent enantioselectivities. Compared to the very narrow substrate scope of known hydratases, the particularly intriguing feature and advantage of this new hydratase is its broad substrate range; α,β -unsaturated lactones with substituents in β -position (**1a**, **1b** and **1c**), α,β -unsaturated cyclic ketones with no substituent in β -position (**1g**, **1h**

and **1i**), and an α,β -unsaturated ester (**1l**). A series of control experiments and deuterium labeling studies demonstrate that the reaction is diastereospecific, with only the *anti* hydration product formed. The biocatalytic reaction system was carefully optimized for gram-scale synthesis, resulting in good conversions and excellent enantioselectivities. Under the optimized conditions, whole cells could be reused for 4 cycles without significant loss of activity while maintaining up to 90% *ee*. Our study suggests that this promising Michael hydratase is not soluble but membrane-bound or cell wall-associated. In summary, whole cells from *Rhodococcus* strains are able to catalyze the enantioselective Michael addition of water to several different substrates using water as both solvent and substrate under mild conditions. This opens up an entirely new approach to the synthesis of chiral β -hydroxy carbonyl compounds.

Experimental Section

Material and Methods

All chemicals were purchased from Sigma-Aldrich (Schnellendorf, Germany) and were used without further purification unless otherwise specified. The culture media components were obtained from BD (Becton, Dickinson and Company, Germany).

¹H, ²H, ¹³C, and ¹⁹F NMR spectra were recorded with Bruker Avance 400 or Varian 300 (400 MHz, 61.4 MHz, 100 MHz and 376.33 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=doublet, t=triplet, q=quartet, m=multiplet), integration, coupling constant (Hz) and assignment. Data for ¹³C NMR and ¹⁹F NMR are reported in terms of chemical shift. Optical rotations were obtained at 20 °C with a PerkinElmer 241 polarimeter (sodium D line). Column chromatography was carried out with silica gel (0.060–0.200 mm, pore diameter ca. 6 nm) and with mixtures of petroleum ether (PE) and ethyl acetate (EtOAc) as solvents. Thin-layer chromatography (TLC) was performed on 0.20 mm silica gel 60-F plates. Organic solutions were concentrated under reduced pressure with a rotary evaporator.

Conversion of substrates and yield of products were quantified by GC using calibration lines with dodecane as an internal standard (specifications and temperature programs given in the Supporting Information, S2) and the optical purity of the products [excepted for **2b**] were determined using chiral GC (specifications and temperature programs given in the Supporting Information, S2). The enantiomeric excess (*ee*) of **2b** was determined by ¹H and ¹⁹F NMR spectroscopy of the corresponding Mosher ester (see the Supporting Information, S4, S5, and S27–S29).

Microorganisms and culture conditions

Rhodococcus rhodochrous ATCC 17895 was purchased from ATCC (American Type Culture Collection, Manassas, USA). *Rhodococcus erythropolis* DSM 43296, *Rhodococcus erythropolis* DSM 43060, *Rhodococcus erythropolis* DSM 43066, and *Rhodococcus rhodochrous* DSM 43241 were purchased from DSMZ (Germany). *Rhodococcus erythropolis* PR4 NBRC 100887 was purchased from NBRC (Biological Resource Centre, Chiba, Japan). The organisms were maintained on agar plates at 4 °C and these were subcultured at regular intervals. The medium used for cultivation^[11] contained Solution A

(980 mL) with potassium dihydrogen phosphate (0.4 g), dipotassium hydrogen phosphate (1.2 g), peptone (5 g), yeast extract (1 g), glucose (15 g), final pH 7.2, sterilized at 110 °C in an autoclave; Solution B (10 mL) with magnesium sulphate (0.5 g), filter-sterilized; Solution C (10 mL) with iron(II) sulphate (0.3 g), filter sterilized. Solutions were mixed before inoculation to make 1 L medium with a buffer concentration of 3 mM. A loop of bacteria was used to inoculate 1 L medium in a 2 L Erlenmeyer flask. This culture was shaken reciprocally at 28 °C for about 72 h to an optical density (OD_{600}) of around 6.3. The cells of *Rhodococcus* strains were harvested by centrifugation at 10000 rpm and at 4 °C for 20 min. The supernatant was removed and the cells were rinsed with potassium phosphate buffer (100 mM, pH 6.2) and centrifuged again. The supernatant was discarded and the pellets were stored at -20 °C. When needed, the wet pellets were freeze dried overnight and collected as lyophilized cells.

General biotransformation procedure for catalyst concentration study

Cells in the culture age of $OD_{600}=6.3$ were harvested by centrifugation, washed twice with 100 mM potassium phosphate buffer (pH 6.2). Around 100 mg mL⁻¹ or 330 mg mL⁻¹ of the cells were re-suspended in the same buffer (15 mL) containing 33 mM 3-methylfuran-2(5*H*)-one (**1a**; 50 mg, 0.51 mmol). The resting cell reactions were carried out in screw-capped Erlenmeyer flasks. Reactions were shaken at 28 °C overnight (17 h). For the blank reaction the setup was the same but heat-denatured cells (90 °C, 30 min) were used. For workup, the cells were removed by centrifugation and 1 mL of the supernatant was saturated with NaCl followed by extraction with 2×0.5 mL of isoamyl alcohol (containing internal standard) by shaking for 5 min. The combined organic layer was dried over Na₂SO₄ and measured by GC for conversion, yield, and *ee* (Table 1).

General biotransformation procedure for rate measurement

The reaction setup for rate determination was the same as for the catalyst concentration study. Duplicate experiments were performed respectively in potassium phosphate buffer (100 mM, 90 mL, pH 6.2) containing 3-methylfuran-2(5*H*)-one (**1a**; 33 mM, 300 mg, 3.06 mmol) and resting cells (330 mg mL⁻¹). For the blank reaction, the setup was the same but heat-denatured cells (90 °C, 30 min) were used. Reactions were allowed to proceed at 28 °C. Every 1 hour, a 1.5 mL sample was taken from the reaction mixture. Cells were removed by centrifugation and then 1 mL of the supernatant was saturated with NaCl followed by extraction with 2×0.5 mL of isoamyl alcohol (containing internal standard) by shaking for 5 min. The combined organic layer was dried over Na₂SO₄ and measured with GC for conversion, yield, and *ee* (Figure 1A).

Reaction temperature study

The reaction setup for the temperature study was the same as for the rate determination. Reactions were performed in potassium phosphate buffer (100 mM, 15 mL, pH 6.2) containing **1a** (50 mg, 0.51 mmol) and wet cells (330 mg mL⁻¹) at given temperatures for 6 h. Workup and analysis were as described above in **General biotransformation procedure for rate measurement** (Figure 1B).

pH study

The reaction setup for the pH profile was the same as for the rate determination. Reactions were performed in buffer (15 mL) con-

taining **1a** (50 mg, 0.51 mmol) and wet cells (330 mg mL⁻¹) at given pH values (pH 5.2–8.2 were prepared as potassium phosphate buffers and pH 4.2 was prepared as citrate/phosphate buffer, all at a buffer strength of 100 mM) at 28 °C for 6 h. For the blank reactions the setup was the same but without the addition of whole cells. For substrate recovery studies, experiments were performed by dissolving 3-methylfuran-2(5*H*)-one (**1a**; 6.5 mg, 0.07 mmol) in buffer (pH 7.2 or pH 8.2, 2 mL, 100 mM) and shaken at 28 °C for 6 h (the same conditions as for the reaction), then 1 mL of the mixture was extracted directly while the remaining 1 mL was acidified with HCl to pH 1.0 before extraction. Workup and analysis were as described in **General biotransformation procedure for rate measurement** (Figure 1C).

Enzyme kinetic study

The reaction setup for the enzyme kinetic study was the same as for the rate determination. Reactions were performed in potassium phosphate buffer (100 mM, pH 6.2) at 28 °C for 2 h with various substrate concentrations (1, 2, 4, 5, 8, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100 mM) of **1a** and with of wet cells (330 mg mL⁻¹). For the blank reaction the setup was the same but heat-denatured cells (90 °C, 30 min) were used. Workup and analysis were as described in **General biotransformation procedure for rate measurement** (Figure 1D).

General procedure for organisms activity screening

The reaction setup for organisms activity screening was the same as for the rate determination. Reactions were performed in 30 mL of potassium phosphate buffer (100 mM, pH 6.2) containing **1a** (100 mg, 1.02 mmol) with whole cells of given organisms at 28 °C for 6 h. For the blank reaction the setup was the same but heat-denatured cells (90 °C, 30 min) were used. Workup and analysis were as described in **General biotransformation procedure for rate measurement** (Table 2).

General procedure for substrate screening

Reactions were carried out as described in the General biotransformation procedure for rate measurement using the same concentration for each substrate. After extraction with isoamyl alcohol (2×0.5 mL) samples were dried over Na₂SO₄ and crude samples were analyzed by GC when product reference material was available or GC-MS (Varian FactorFour VF-1 ms column [25 m×0.25 mm×0.4 μm] and He as carrier gas) when product reference material was not commercially available (Table 3).

General procedure for recyclability

Reactions were carried out with substrate **1a** (50 mg, 0.51 mmol) in 15 mL of potassium phosphate buffer (100 mM, pH 6.2) and 330 mg mL⁻¹ of wet cells, shaken at 28 °C for 23 h. At the end of the reaction, cells were centrifuged at 4000 rpm for 20 min to be separated from the reaction mixture, then washed by potassium phosphate buffer (100 mM, pH 6.2), and resuspended in 15 mL of the same buffer containing the same substrates. The reaction mixture (1 mL of supernatant separated from cells) was saturated with NaCl and then extracted with 2×0.5 mL of isoamyl alcohol (containing internal standard) by shaking for 5 min. The combined organic phase were dried over Na₂SO₄ and crude samples were analyzed by GC (Figure 2).

Activities comparison using pelleted cell debris and cell free extract

15 g of cells in the culture age of $OD_{600}=6.3$ were harvested by centrifugation, washed twice with 100 mM of potassium phosphate buffer (pH 6.2) and resuspended in the same buffer (45 mL). The cells were incubated first with lysozyme (1 mg mL^{-1} , 4°C , 1 h) and subsequently disrupted using a French press (2.05 kBar, 2 shots). Cell-free extract and cell debris were separated by centrifugation for 40 min at 10000 rpm at 4°C . Substrate **1a** (150 mg, 1.53 mmol) was added to the supernatant (cell-free extract) and shaken at 28°C (reaction A). Cell debris was resuspended in potassium phosphate buffer (45 mL, 100 mM, pH 6.2) containing the same concentration of substrates (reaction B). Every 1 hour, a 1.5 mL sample was taken from both reaction A and B. For workup, the cells were removed by centrifugation and 1 mL of the supernatant was saturated with NaCl followed by extraction with 2×0.5 mL of isoamyl alcohol (containing internal standard) by shaking for 5 min. The combined organic layer was dried over Na_2SO_4 and measured with GC for conversion, yield and ee (Figure 3).

(S)-3-hydroxy-3-methylfuranone (2a); preparative scale

For isolation and characterization of the Michael addition product, the reaction was carried out on preparative scale. Pelleted cells from 20 L medium were resuspended in potassium phosphate buffer (600 mL, 100 mM, pH 6.2), and substrate 3-methylfuran-2(5*H*)-one (**1a**; 2 g, 20.38 mmol) was added. Reaction was incubated at 28°C and shaken at 180 rpm for 24 h. Then the cells were removed by centrifugation and the supernatant was saturated with NaCl. Due to the high solubility of the resulting alcohols in water, continuous extraction with ethyl acetate was performed overnight. The extract was then concentrated under reduced pressure and purified by flash column chromatography on silica gel (eluent: PE/EtOAc 1:1) to yield **2a** (1.63 g, 14.06 mmol, 69%) as a colorless oil; $[\alpha]_D^{20} = +46.6$ (c 0.96 in CHCl_3)^[11] $[\alpha]_D = +53.92$ (c 0.96 in CHCl_3); $^1\text{H NMR}$ (400 MHz, CDCl_3): $\delta = 1.48$ (s, 3H), 2.57 (d, $J = 17.6$ Hz, 1H), 2.63 (d, $J = 17.6$ Hz, 1H), 3.71 (s, 1H), 4.14 (d, $J = 9.6$ Hz, 1H), 4.27 ppm (d, $J = 9.6$ Hz, 1H); $^{13}\text{C NMR}$ (100 MHz, CDCl_3): $\delta = 24.94$, 43.06, 74.70, 79.82, 176.27 ppm (in accordance with literature^[11]).

(S)-3-hydroxy-3-ethylfuranone (2b; preparative scale)

For isolation and characterization of the Michael addition product, the reaction was carried out on preparative scale. Using the biotransformation procedure described for **2a**, reaction of 3-ethylfuran-2(5*H*)-one (**1b**; 1 g, 8.92 mmol) gave **2b** (0.75 g, 5.80 mmol, 65%) as a colorless oil; $[\alpha]_D^{20} = +49.6$ (c 0.75 in CHCl_3)^[11] $[\alpha]_D = +48.9$ (c 0.72 in CHCl_3); $^1\text{H NMR}$ (400 MHz, CDCl_3): $\delta = 0.98$ (t, $J = 7.6$ Hz, 3H), 1.72 (q, $J = 7.4$ Hz, 2H), 2.49 (s, 1H), 2.53 (s, 2H), 4.13 (d, $J = 9.6$ Hz, 1H), 4.21 (d, $J = 9.6$ Hz, 1H); $^{13}\text{C NMR}$ (100 MHz, CDCl_3): $\delta = 8.70$, 31.69, 42.36, 78.05, 79.26, 176.82 ppm (in accordance with literature^[11]).

[2-D]-3-hydroxy-3-methylfuranone (4b)

Lyophilized cells (3 g) were resuspended in D_2O (100 mL) containing 4 drops of potassium hydroxide solution (100 mM; final pD 6.5, corresponds to pH 6.1). **1a** (330 mg, 3.40 mmol) was added. The reaction mixture was shaken at 180 rpm, 28°C for 24 h, then centrifuged and the supernatant was saturated with NaCl and continuously extracted with ethyl acetate (200 mL) overnight. The extract was dried over Na_2SO_4 and evaporated under reduced pressure. The crude product mixture was purified using flash chromatogra-

phy on silica gel (eluent: PE/EtOAc 1:1) yielding deuterium oxide-addition product (S)-[2-D]-3-hydroxy-3-methylfuranone **4b** (265 mg, 2.28 mmol, 67%) as a colorless oil. $^1\text{H NMR}$ (400 MHz, CDCl_3): $\delta = 1.51$ (s, 3H), 2.64 (s, 1H), 2.96 (s, 1H), 4.15 (d, $J = 9.6$ Hz, 1H), 4.28 ppm (d, $J = 9.6$ Hz, 1H); $^{13}\text{C NMR}$ (100 MHz, CDCl_3): $\delta = 24.96$, 43.29 (t, $^1J_{\text{CD}} = 19.8$ Hz, CD), 74.61, 79.80, 176.49 ppm; m/z : 117 (M^+ , 2), 89 (5), 86 (4), 74 (3), 60 (9), 59 (100), 58 (20), 57 (4), 44 (36), 43 (87), 42 (10), 41 (4), 40 (7).

Dehydration of deuterium oxide addition product (4b)

To a solution of alcohol **4b** (30 mg, 0.26 mmol) in EtOAc (2 mL) was slowly added acetic anhydride (35 μL) and triethylamine (60 μL), followed by 4-dimethylaminopyridine (50 μL of 3 mg mL^{-1} solution in EtOAc). The reaction was allowed to proceed for 30 min at room temperature and was stopped by the addition of 0.5 mL of water. The phases were separated and the organic layer was dried over Na_2SO_4 and evaporated. The crude product was measured by NMR and GC-MS which showed the elimination product was 3-methylfuran-2(5*H*)-one (**1a**). $^1\text{H NMR}$ (400 MHz, CDCl_3): $\delta = 1.96$ (s, 3H), 4.00 (s, 2H), 5.94 ppm (s, 1H); $^{13}\text{C NMR}$ (100 MHz, CDCl_3): $\delta = 14.02$, 73.86, 116.23, 166.32, 174.1 ppm (in accordance with literature^[27]); m/z : 98 (M^+) (26), 71 (16), 70 (62), 69 (100), 68 (13), 67 (3), 55 (3), 54 (3), 53 (6), 52 (3), 51 (3), 50 (6), 45 (10), 44 (5), 43 (19), 42 (56), 41 (98), 40 (71).

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Keywords: biocatalysis • enantioselectivity • hydratases • Michael addition • water

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