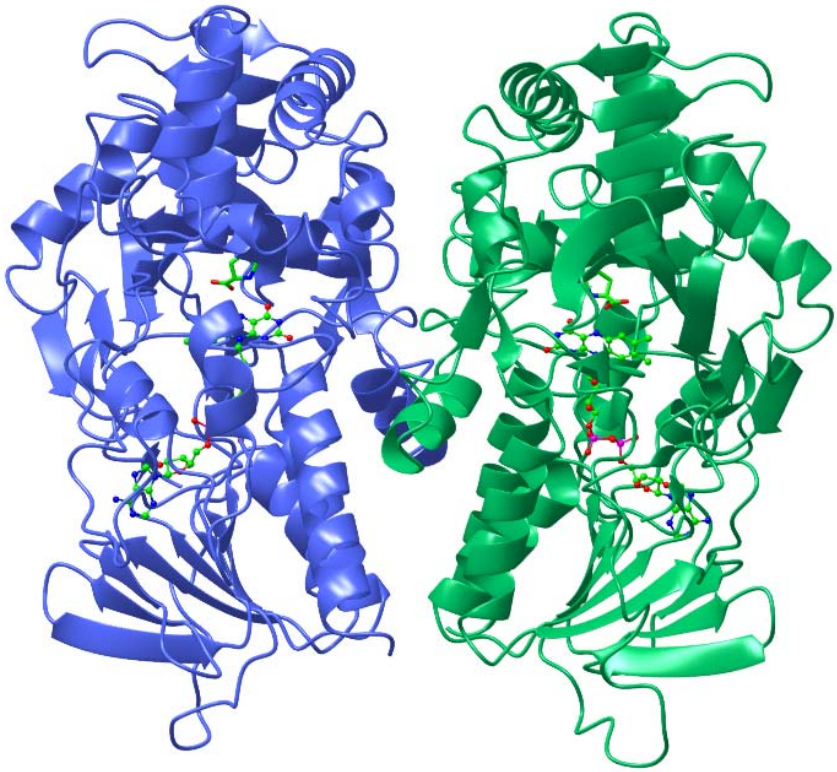
ChemComm

This article is part of the

Enzymes & Proteins web themed issue

This issue showcases high quality research in the field of enzymes and proteins.

Please visit the website to access the other articles in this issue:-
http://www.rsc.org/chemcomm/enzymesandproteins



MhyADH catalysed Michael addition of water and *in situ* oxidation†

Jianfeng Jin,[ab] Philip C. Oskam,[a] Sanjib K. Karmee,[a] Adrie J. J. Straathof[b] and Ulf Hanefeld*[a]

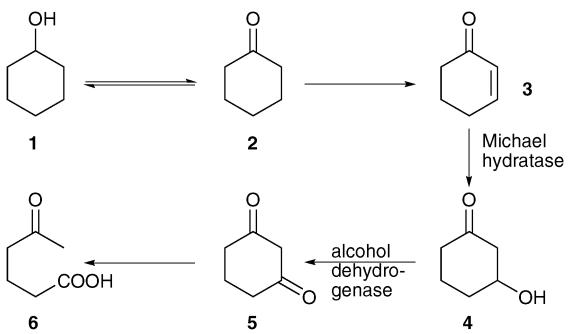
Received 13th August 2010, Accepted 9th September 2010
DOI: 10.1039/c0cc03229h

The Michael addition of water is a major challenge. Here an enzymatic approach is described. Interestingly, the enzyme (MhyADH) does not only catalyse the Michael addition of water but also the *in situ* oxidation of the product.

The β-hydroxy carbonyl connectivity is widely spread in nature, since it is part of the fatty acid metabolism and the polyketide biosynthesis.[1–3] Synthetic approaches are normally based on Claisen-condensations or Aldol-type chemistry—both in nature and in the laboratory.[4,5] The Michael addition of water to conjugated carbonyl compounds is little known.[6–8] Only one enantioselective Michael addition of water has been described, albeit indirect, proceeding *via* a hydroboration.[9] This is surprising, all the more so since it would enable an entirely new route towards β-hydroxy carbonyl compounds.[9]

In our search for an enzyme that would catalyse the Michael addition of water to α,β-unsaturated carbonyl compounds and more specifically α,β-unsaturated ketones such as **3**, it was noted that *Alicycliphilus denitrificans* DSMZ 14773 can anaerobically degrade cyclohexanol (**1**).[10–12] In the degradation pathway a Michael hydratase was postulated and indirect evidence for its existence was presented (Scheme 1).[10] Here we describe the enrichment and catalytic activity of this unique enzyme. To date only very limited knowledge about the equivalent chemical reaction is available.[6–9]

A. denitrificans DSMZ 14773 was grown, both aerobically and anaerobically, with **1** as the sole carbon source. Cell extracts from both cultures were submitted to the earlier described indirect assay for the Michael hydratase activity.[10] In this coupled assay **3** is utilised as a substrate and compound **4** once generated is oxidised *in situ* to **5**. The oxidation is catalysed by an alcohol dehydrogenase also present in *A. denitrificans* DSMZ 14773 (Scheme 1). Methylene blue or dichlorophenol indophenol (DCPIP) as electron acceptor allows this oxidation of **4** to **5** to occur while its reduction can be followed UV-spectroscopically. The anaerobically grown *A. denitrificans* DSMZ 14773 displayed higher activity in this assay and from here on anaerobic growth conditions were applied.‡



Scheme 1 Anaerobic degradation of cyclohexanol by *Alicycliphilus denitrificans* DSMZ 14773.

To confirm the presence of the Michael hydratase it was essential to develop analytical tools that would allow us to follow the formation of **4** directly. The racemic reference compound **4** was obtained selectively by a straightforward cobalt-catalysed oxidation of 1,3-cyclohexanediol.[13] With this in hand a GC method for analysing the reaction was established. However, the extremely high solubility of **4** in water and its volatility made it difficult to isolate **4** or to derivatise it for analysis.

As a direct test for the existence of the Michael hydratase, cell free extract of *A. denitrificans* DSMZ 14773 was incubated with **3**. Extensive extraction and GC analysis revealed the conversion of **3** to **4**. This was further validated by GC-MS and by co-injection of the extract with a synthetic sample of **4**. The yield of **4** reached approximately 15% after 1.5 h and no further conversion was observed (Fig. 1).

To enhance the search for the Michael hydratase *via* the coupled assay we first purified 3-hydroxycyclohexanone dehydrogenase. To our surprise methylene blue or DCPIP was still reduced when **3** was added to this purified alcohol dehydrogenase. This result indicates that the hydration of **3** and the oxidation of **4** are catalysed by the same enzyme.

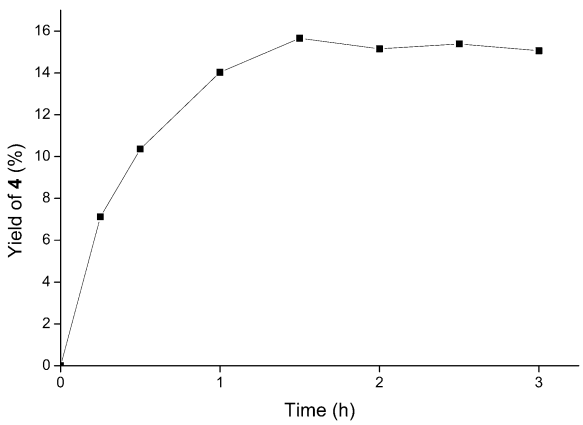


Fig. 1 The conversion of 2-cyclohexenone (**3**) to 3-hydroxy-cyclohexanone (**4**) by MhyADH without the addition of an oxidising reagent.

[a] *Gebouw voor Scheikunde, Afdeling Biotechnologie, Technische Universiteit Delft, Julianalaan 136, 2628 BL Delft, The Netherlands. E-mail: u.hanefeld@tudelft.nl; Fax: +31 (0)15-2781415*
[b] *Bioseparation Technology, Afdeling Biotechnologie, Technische Universiteit Delft, Julianalaan 67, 2628 BC Delft, The Netherlands. E-mail: A.J.J.Straathof@tudelft.nl*

† This article is part of the 'Enzymes and Proteins' web-theme issue for ChemComm.

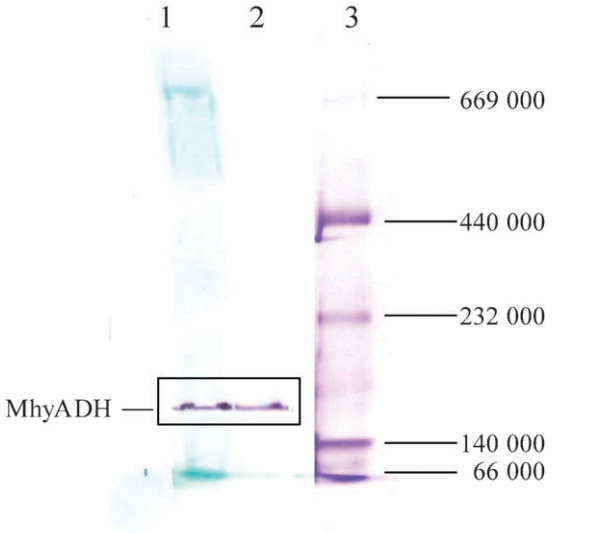
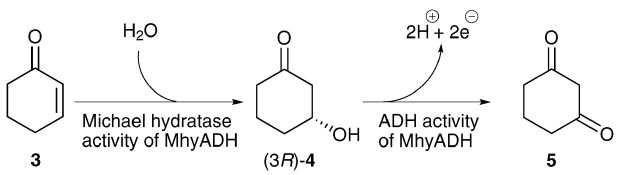


Fig. 2 The activity staining of MhyADH in the native protein gel. MhyADH stained with methylene blue (60 μM), nitro blue tetrazolium chloride (0.3 M), 2-cyclohexenone **3** (1 mM) (lane 1) or 3-hydroxy-cyclo-hexanone **4** (1 mM) (lane 2). Lane 3 are protein markers: albumin (66 000), lactate dehydrogenase (140 000), catalase (232 000), ferritin (440 000), thyroglobulin (669 000).

Enriching these activities 43 times by a two-step purification (DEAE Sepharose and Mono Q) yielded a single protein band at ~200 kD which did indeed contain both activities (Fig. 2). This bifunctional enzyme first adds water to the C=C bond of **3** (Michael hydratase activity), resulting in **4** and then oxidises this hydration product to **5** (alcohol dehydrogenase activity). We therefore abbreviate it as MhyADH. Again, without added oxidation reagent, MhyADH catalysed only the Michael addition of water as described above (Fig. 1); however the enzyme was not stable under reaction conditions and deactivation was observed. When methylene blue or DCPIP was added, MhyADH catalysed both reactions (Scheme 2).

To probe the enantioselectivity of MhyADH enantiopure (3*R*)-**4** was prepared *via* kinetic resolution of *rac*-**4** by Porcine pancreas lipase-catalysed acylation in freshly distilled vinyl acetate. The (3*R*)-acetate (ee = 92%) was deprotected by ethanolysis catalysed by CALB (Novozym 435) in dry MTBE yielding (3*R*)-**4**. The absolute stereochemistry of (3*R*)-**4** was determined by converting it into the known TBDMS ether.[9,14]

MhyADH was incubated with *rac*-**4** and (3*R*)-**4**. While the 3*R*-alcohol was rapidly oxidised the racemic mixture was converted much more sluggishly (Fig. 3). Indeed, it can be clearly seen that the oxidation is inhibited by the *S*-enantiomer. MhyADH thus displays *R*-selectivity in the oxidation step.



Scheme 2 MhyADH catalyses the Michael addition of water and the subsequent enantioselective oxidation of the alcohol. In the absence of an oxidation reagent the Michael product might be isolated.

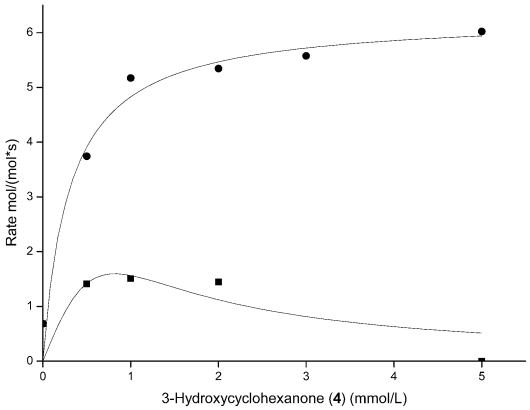


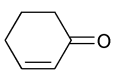
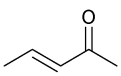
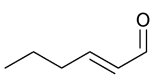
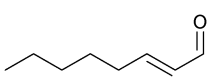
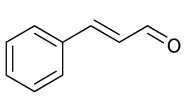
Fig. 3 The activity of MhyADH towards (3*R*)-**4** (●) and racemic **4** (■).

Given the high water-solubility and volatility of **4** it proved to be impossible to derivatise the analytical quantities of **4** obtained from the MhyADH catalysed addition of water to **3**.

The substrate specificity of MhyADH was investigated by screening a variety of α,β-unsaturated carbonyl compounds. Both aldehydes and ketones proved to be substrates for MhyADH (Table 1), although cyclohexenone **3** clearly was the best substrate. No activity towards α,β-unsaturated carboxylic acids was observed.

It has been reported that in the modular biosynthesis of fatty acids, polyketides and nonribosomal peptides, large multifunctional enzymes such as fatty acid synthase (FAS), polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) are required to produce such primary and secondary metabolites.[15] These enzymes contain discrete catalytic domains which are responsible for each step of the biosynthesis and together they produce the corresponding natural product. The bifunctionality of the MhyADH described here might therefore be less uncommon in nature

Table 1 The substrate specificity of MhyADH as determined with the coupled assay‡

Substrate	Relative activity (%)
	100
	60.0
	28.1
	56.3
	34.4
	10.6

For experimental detail see footnote ‡.

than one may assume at first glance. The functions it combines, however are exceptional. An enzyme that effectively catalyses the unusual hydration of cyclic and acyclic α,β-unsaturated ketones and aldehydes, *i.e.* the Michael addition was to date unknown.[16] Its ability to enantioselectively oxidise (in the presence of electron acceptors) the Michael products only expands the applicability of this enzyme. To summarise, because of its bifunctional nature, MhyADH opens up a new route to 3-hydroxy carbonyl and 1,3-dicarbonyl compounds, both essential 1,3-difunctional compounds.

Financial support for part of this project by B-Basic is gratefully acknowledged. Permission to publish has been given for the results obtained with B-Basic support.

Notes and references

‡ Chemicals were purchased from Sigma-Aldrich; 2-cyclohexenone, 1,3-cyclohexanediol, 2-buten-2-one, 3-penten-2-one, cinnamaldehyde, 2-hexenal and 2-octenal were distilled prior to use. *A. denitrificans* DSMZ 14773 was purchased from DSMZ (Germany).

Cultivation of *A. denitrificans* DSMZ 14773 and enzyme purification: the cells were cultivated in LB medium and minimal medium containing cyclohexanol (**1**, 100 μg mL^{-1}). The minimal medium used for aerobic cultivation contained (1 L distilled water): $Na_2HPO_4{\cdot}2H_2O$ 3.5 g, KH_2PO_4 1.0 g, $(NH_4)_2SO_4$ 0.5 g, $MgCl_2{\cdot}6H_2O$ 0.1 g, $Ca(NO_3)_2{\cdot}4H_2O$ 0.05 g, 1.0 mL trace element solution SL-4. The trace element solution SL-4 contained (1 L distilled water): EDTA 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.2 g, trace element solution SL-6 100 mL.[10] The trace element solution SL-6 contained (1 L distilled water): $ZnSO_4{\cdot}7H_2O$ 0.10 g, $MnCl_2{\cdot}4H_2O$ 0.03 g, H_3BO_3 0.30 g, $CoCl_2{\cdot}6H_2O$ 0.20 g, $CuCl_2{\cdot}2H_2O$ 0.01 g, $NiCl_2{\cdot}6H_2O$ 0.02 g, $Na_2MoO_4{\cdot}2H_2O$ 0.03 g. The final pH was 7.2–7.4.[10]

The minimal medium used for anaerobic cultivation contained (1 L distilled water): KH_2PO_4 0.816 g, K_2HPO_4 5.92 g, NH_4Cl 0.53 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, KNO_3 2.0 g, $CaSO_4{\cdot}7H_2O$ 0.025 g, cyclohexanol (**1**) 0.1 g, 10 mL trace element solution SL-10 and 5 mL vitamin solution.[10] The final pH was adjusted to 7.2. The cultures were grown on a rotary shaker at 180 rpm and 30 °C.

For the preparation of cell extracts, 1.5 g cells (wet weight) were suspended in 10 mL Tris–HCl buffer (100 mM, pH 7.8). Cells were disrupted by passage through a cooled French pressure cell with a pressure difference of 139 MPa. Unbroken cells and debris were removed by centrifugation at 100 000 × *g* for 1 h. The cell free extract of *A. denitrificans* was applied to a 28 mL DEAE Sepharose column previously equilibrated with buffer A (20 mM Tris–HCl, pH 7.8). The elution was performed with buffer B (20 mM Tris–HCl, 1 M NaCl, pH 7.8) from 0–50% at a flow rate of 5 mL min^{-1}. Fractions showing MhyADH activity were pooled and concentrated using Centricon YM30 centrifugal filter device (Millipore) in a centrifuge at 3000 × *g*. After desalting with a PD-10 column, pooled fractions containing MhyADH activity were applied to a MonoQ 5/50 GL column (1 mL) (GE Healthcare) equilibrated with buffer A. The bound MhyADH activity was eluted by a linear gradient from 0–50% of buffer B at a flow rate of 0.4 mL min^{-1}. Active fractions were pooled and concentrated using Centrion YM30 filter devices. The purified protein was stored at −20 °C. The protein content was determined using a Pierce® BCA protein assay kit (Pierce Biotechnology).

Analytical methods: GC analysis of the reactions were performed with a Shimadzu GC 2014 equipped with a CP-Wax 52 CB column (2.0 μm × 50 m × 0.5 mm) using N_2 as the carrier gas. The retention time of **3** and **4** are 28.5 min and 36.6 min, respectively. Mass spectra were determined with a Shimadzu GC-2010 Gas Chromatograph coupled to a Shimadzu GCMS-QP2010S Gas Chromatographic Mass Spectrometer.

The hydration reaction was performed in 10 mL of Tris–HCl buffer (100 mM, pH 7.8) containing 0.1 mmol 2-cyclohexenone (**3**) and 3.1 U MhyADH activity. 0.5 mL samples were taken from the reaction mixture every 30 min and extracted with 1.0 mL of ethyl acetate.

The coupled activity assay of 2-cyclohexenone hydratase and 3-hydroxycyclohexanone dehydrogenase (MhyADH) was performed at room temperature in a quartz cuvette containing 0.9 mL reaction mixture containing 1 mM substrate and 60 μM methylene blue or DCPIP in 100 mM Tris–HCl (pH 7.8). The reaction was initiated by adding 0.1 mL cell extract or enzyme solution. The spectra of the reaction mixture were recorded on a Hewlett-Packard 8452A diode array spectrophotometer at an interval of 2 s and absorbance change at 578 nm (methylene blue, ε = 20.7 cm^{-1} mol^{-1}; DCPIP, ε = 16.8 cm^{-1} mol^{-1}) was determined.[10] One unit is defined as the amount of enzyme that reduces 1 μmol of DCPIP at pH 7.8 and 25 °C in one minute.

1 J. M. Berg, J. L. Tymoczko and L. Stryer, *Biochemistry*, W. H. Freeman and Company, New York, 3rd edn, 1988, pp. 469–493.
2 P. A. Frey and A. D. Hegeman, *Enzymatic Reaction Mechanisms*, Oxford University Press, New York, 2007, pp. 433–456.
3 C. Hertweck, *Angew. Chem., Int. Ed.*, 2009, **48**, 4688–4716 (*Angew. Chem.*, 2009, **121**, 4782–4811).
4 P. Wyatt and S. Warren, *Organic Synthesis, Strategy and Control*, Wiley, Chichester, 2007, pp. 277–305.
5 J. Clayden, N. Greeves, S. Warren and P. Wothers, *Organic Chemistry*, Oxford University Press, Oxford, 2001, pp. 227–241.
6 K. Riedel and H. Krekeler, DE 2205225, 1973. AN 1973:515215.
7 X. Wang, D. Sui, M. Huang and Y. Jiang, *Polym. Adv. Technol.*, 2006, **17**, 163–167.
8 J. E. Fellmann, R. J. Saxton and P. Tung, DE 3708851 A1, 1987. AN 1988:55649.
9 X. Feng and J. Yun, *Chem. Commun.*, 2009, 6577–6579.
10 W. Dangel, A. Tschech and G. Fuchs, *Arch. Microbiol.*, 1988, **150**, 358–362.
11 W. Dangel, A. Tschech and G. Fuchs, *Arch. Microbiol.*, 1989, **152**, 273–279.
12 T. Mechichi, E. Stackebrandt and G. Fuchs, *Int. J. Syst. Evol. Microbiol.*, 2003, **53**, 147–152.
13 T. Iwahama, Y. Yoshino, T. Keitoku, S. Sakaguchi and Y. Ishii, *J. Org. Chem.*, 2000, **65**, 6502–6507.
14 M. A. Arai, R. Tsutsumi, H. Hara, T. C. Chen, T. Sakaki, N. Urushino, K. Inouye and A. Kittaka, *Heterocycles*, 2005, **66**, 469–479.
15 J. L. Meier and M. D. Burkart, *Chem. Soc. Rev.*, 2009, **38**, 2012–2045.
16 M. Wubbolts, in *Enzyme Catalysis in Organic Synthesis*, ed. K. Drauz and H. Waldmann, Wiley-VCH-Verlag, Weinheim, 2002, pp. 686–697.

