DOI: 10.1002/ejoc.201100107

Asymmetric Reduction of α-Keto Esters with *Thermus thermophilus* NADH-Dependent Carbonyl Reductase using Glucose Dehydrogenase and Alcohol Dehydrogenase for Cofactor Regeneration

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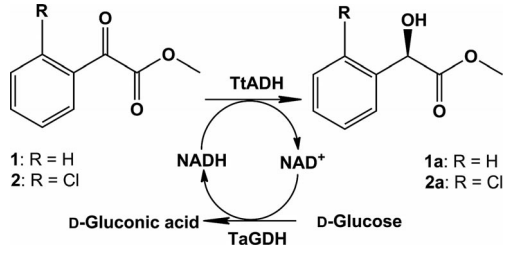
Keywords: Asymmetric catalysis / Enzymes / Biotransformations / Ketones / Reduction

The enantioselective synthesis of methyl (*R*)-mandelate and methyl (*R*)-*o*-chloromandelate was investigated using an NADH-dependent carbonyl reductase from *Thermus thermophilus* (TtADH) and, separately, archaeal glucose dehydrogenase and *Bacillus stearothermophilus* alcohol dehydrogenase (BsADH) for NADH regeneration. Optimal reaction times and substrate concentrations in the absence and presence of organic solvents were determined. The enantiofacial selectivity of TtADH was shown to be inversely proportional to the hydrophobicity of the short-chain linear alcohols employed as co-substrates of the bacillar ADH. The bioreduction of methyl benzoylformate yielded the (*R*)-alcohol with a 77 % yield (*ee* = 96 %) using glucose dehydrogenase and glucose, and 81 % yield (*ee* = 94 %) applying BsADH and ethanol. The bioreduction of methyl *o*-chlorobenzoylformate yielded the halogenated (*R*)-alcohol with 95 % and 92 % *ee*, and 62 % and 78 % yield using glucose dehydrogenase and BsADH, respectively.

Introduction

Optically active hydroxy esters provide very versatile building blocks widely used as chiral intermediates for the synthesis of pharmaceuticals and other fine chemicals.[1] Among them, methyl (*R*)-mandelate (**1a**) and methyl (*R*)-*o*-chloromandelate (**2a**) are valuable synthons used in organic synthesis (Scheme 1). *O*-protected **1a** is used as an intermediate for the synthesis of pharmaceuticals[2] and **2a** is an intermediate for the *anti*-thrombotic agent, (*S*)-clopidogrel, commercialized under the brand name Plavix (clopidogrel sulfate).[3] Compound **1a** has been obtained by reduction of methyl benzoylformate (**1**) using resting cells of *Rhodotorula* sp. AS2.2241[4a] and more recently on lab-scale using *Saccharomyces cerevisiae* AS2.1392 whole cells with 85.8 % isolated yield and *ee* = 96.6 %.[4b] However, several methods for the preparation of the key enantiomer for clopidogrel, **2a** have been developed, including (i) preparation of the corresponding (*R*)-carboxylic acid through fractional crystallization after diastereomeric salt formation of (*R*,*S*)-*o*-chloromandelic acid,[3] (ii) asymmetric reduction of the corresponding α-keto acid,[5] (iii) asymmetric hydrocyanation of the corresponding *o*-chlorobenzaldehyde using (*R*)-selective oxynitrilase,[6] and (iv) direct reduction of methyl *o*-chlorobenzoylformate (**2**) by Ru-catalyzed asymmetric hydrogenation.[7] A process has also been reported for resolution of racemic methyl *o*-chloromandelate using *Candida antarctica* lipase A-mediated transesterification which produces **2a** in 99 % *ee* and 41 % yield.[8] More recently, Ema and co-workers[9] reported the efficient and environmentally friendly chemoenzymatic synthesis of **2a** from **2** in >99 % *ee* on a 15-g scale using recombinant *Escherichia coli* overproducing a carbonyl reductase from baker's yeast. Moreover, Jeong and co-workers have also reported the reduction of **2a** from **2** in 96.1 % *ee* and 100 % conversion using whole cells of baker's yeast.[10]



Scheme 1. Reduction of methyl benzoylformate (**1**) and methyl *o*-chlorobenzoylformate (**2**) catalyzed by *T. thermophilus* ADH (TtADH) coupled with *T. acidophilum* glucose dehydrogenase (TaGDH) for NADH regeneration.

A number of carbonyl reductases from different sources have been described which catalyze the direct asymmetric reduction of **1** to **1a** with high yields and high *ee*, such as NADH-dependent alcohol dehydrogenase (ADH) from *Pyrococcus furiosus*,[11] NADH-dependent α-keto ester reductase from the actinomycete *Streptomices coelicolor* A3(2),[12] and NADH-dependent ADH from *Leifsonia* sp. S749.[13]

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejoc.201100107.

More recently, an NADH-dependent, highly enantioselective ADH (TtADH), identified from the thermophilic, halotolerant gram-negative eubacterium *Thermus thermophilus* HB27, has been purified and characterized in our laboratory.[14] The thermophilic enzyme catalyses the reduction of α-methyl and α-ethyl benzoylformate to methyl (*R*)-mandelate (*ee* = 91%) and ethyl (*R*)-mandelate (*ee* = 95%), respectively, by way of an in situ NADH-recycling system involving 2-propanol and *Bacillus stearothermophilus* ADH (BsADH).[14]

This paper reports the enzymatic synthesis of **1a** and **2a** by TtADH and the evaluation of glucose dehydrogenase as well as BsADH for achieving cofactor regeneration. The work describes the determination of the optimal reaction time and substrate concentration in the absence and presence of organic solvents and the choice of the alcohol which is most suitable co-substrate for the TtADH/BsADH system.

Results and Discussion

Process Development using Glucose Dehydrogenase for Cofactor Regeneration

Previous studies showed that the *E. coli* expression system developed for TtADH is quite efficient, producing ca. 30 mg protein per litre of culture, and that this enzyme shows potential for applications involving bioconversions of substituted acetophenones and aromatic α-keto esters such as **1**.[14] Kinetic studies showed that this latter compound was a better substrate when halogenated at the *ortho* position of the benzene ring. The k_{cat}, K_m and k_{cat}/K_m values determined for the reduction of **1** were $38.1 \pm 3.7\ s^{-1}$, 2.7 ± 0.6 mM, and $14.1\ s^{-1}\ mM^{-1}$, respectively and those for **2** were $7.1 \pm 0.2\ s^{-1}$, 0.32 ± 0.1 mM, and $22.2\ s^{-1}\ mM^{-1}$, respectively. This indicates that the electronic properties of the halogen rather than its steric effects determine the efficiency of the reaction.

Glucose dehydrogenase from *Thermoplasma acidophilum* (TaGDH), a thermophilic and stable enzyme possessing dual cofactor-specificity,[15] was chosen for the in situ NADH-regeneration system utilizing glucose as the hydride source (Scheme 1). Experimental conditions including buffer, pH, temperature, reaction time and organic solvent were considered in order to optimise bioconversion of **1**. The optimal pH for the reduction reaction catalyzed by TtADH is approximately 6.0[14] and that for glucose oxidation catalysed by TaGDH is 7.0.[16] Due to the instability of the reduced cofactor under acidic conditions, neutral pH was chosen as a compromise taking into account cofactor stability and TtADH activity at suboptimal pHs. Nevertheless, TaGDH retains full catalytic activity after 9 h at 55 °C,[17] while TtADH is highly efficient and selective at 50 °C.[14] To establish the optimal reaction time the conversion of **1** was carried out at 50 °C, at low substrate concentration ($1\ g\,L^{-1}$, 6.2 mM) in aqueous solution and by letting the reactions proceed for 1, 3, 6 and 24 h. The conversion of **1** proceeded in 99% after 6 h, affording the (*R*)-α-hydroxy ester **1a** with 95% *ee*; the yield and *ee* obtained using BsADH were 99% and 91%, respectively (see the Supporting Information).[14] The degree of conversion and *ee* of the biotransformation was unchanged at reaction times as long as 24 h, as already observed for the BsADH/2-propanol system.[14] The high conversion is noteworthy, considering that TaGDH has a marked preference for NADP(H) over NAD(H).[15]

Bioconversions were then carried out using increasingly higher concentrations of **1** for a reaction time of 24 h. The conversion proceeded with >99% at 1 to $10\ g\,L^{-1}$ **1**, but decreased to ca. 10% and ca. 1% yields at 30 and $50\ g\,L^{-1}$ substrate, respectively. However, the *ee* of **1a** was 95% over the whole range of concentrations for **1** that were examined (see Supporting Information for more details). The decrease in conversion could be due to a decrease in solubility of the substrate, enzyme inactivation by substrate or product, or both factors. The next step was to develop a suitable solvent system to improve bioconversions at higher substrate concentrations. TtADH possesses a remarkable tolerance to common organic solvents. Indeed, it even showed a significant increase in activity in the presence of various organic solvents, reaching 150% and 180% of the initial value with 5% v/v acetonitrile and 10% v/v 2-propanol, respectively, after 24 h of incubation at 50 °C and 182% with 10% v/v hexane after 65 h at 25 °C.[14] The stability of the archaeabacterial glucose dehydrogenase in the presence of organic solvents was investigated to evaluate its tolerance to organic solvents (see the Supporting Information). TaGDH was inactivated by 34% and 28% following 24 h incubation at 50 °C, in the absence and presence of 20% v/v hexane, respectively. Moreover, the enzyme was inactivated by over 50% in the presence of 5% v/v and 10% v/v DMSO, 10% v/v acetonitrile or 10% v/v methanol. However, incubation in the presence of 5% and 10% v/v 2-propanol, 5% v/v methanol or 5% v/v acetonitrile resulted in activities that were similar to those measured in aqueous buffer after 6 h and 24 h. Therefore, bioconversions were carried out at different concentrations of **1** in the presence of acetonitrile or hexane at concentrations of 5% and 20% v/v, respectively; these concentrations fulfilled the majority of the criteria for a solvent system in which both enzymes retained activity. As shown in the Supporting Information, the presence of water-miscible or immiscible organic solvents did not improve conversion at concentrations of **1** higher than $10\ g\,L^{-1}$, since the profiles of the conversion with acetonitrile and hexane were similar to those obtained in aqueous buffer (Figure S2). Nevertheless, the good levels of conversion obtained between 1 and $10\ g\,L^{-1}$substrate was accompanied by a slight decrease in enantioselectivity in the presence of acetonitrile (*ee* = 85%–89%) as well as in the presence of hexane (*ee* = 92–93%) compared to the 95% *ee* obtained in aqueous buffer. The decrease in enantioselectivity could be related to the solvent-mediated enhancement of catalysis as a result of increased flexibility of the enzyme active site.[14] However, an increase in *ee* from 37% in phosphate buffer up to 43% in the presence of ace-

tonitrile was observed for the 2-butanone to (*R*)-2-butanol reduction catalyzed by *L. brevis* ADH.[18]

In light of our findings related to enantioselectivity and concentration, optimum conditions for the bioconversion of **1** as well as **2** were determined to involve a temperature of 50 °C, substrate concentrations up to 10 g L^{-1}, in the presence of 1 mM NAD^+ in pH 7.0 aqueous buffer. Table 1 summarizes the results of a study carried out for the two α-hydroxy esters. The bioreduction of 100 mg of **1** at 50 °C for 24 h gave 74 mg of **1a** with *ee* = 95% (Table 1, Entry 1). The same reaction carried out in 6 h with a 5-fold lower substrate and 2-fold higher TtADH concentrations yielded slightly better results (Table 1, Entry 2). However, the conversion and optical purity of **2a** were somewhat lower when the concentration of **2** was 10 g L^{-1} (Table 1, Entry 3). Nevertheless, the bioreduction of 100 mg of **2** gave 86 and 62 mg of **2a** with similar *ee* (Table 1, Entries 4 and 5) at substrate concentrations of 2 and 5 g L^{-1}, respectively. This suggests that greater insolubility of the halogenated keto ester and/or enzyme inactivation alters the efficiency of the biotransformation. It is noteworthy that, although the affinities of the two substrates are quite different, the presence of the halogen in substrate **2** did not affect TtADH enantioselectivity.

Table 1. Reduction of **1** and **2** to **1a** and **2a** with TtADH by the TaGDH/glucose-NADH regeneration system.[a]

Entry	**1** [mg]	[**1**] [g L^{-1}]	TtADH [mg mL^{-1}]	TaGDH [mg mL^{-1}]	Conv.[b] [%]	*ee*[b] [%]
1	100	10	0.05	0.010	100 (74) [d]	95
2[c]	100	2	0.10	0.010	78 (77) [d]	96
	2 [mg]	[**2a**] [g L^{-1}]				
3	100	10	0.125	0.0275	28 (n.d.) [d]	88
4	100	2	0.100	0.020	96 (86) [d]	93
5	100	5	0.125	0.0275	100 (62) [d]	95

[a] Reactions were carried out for 24 h at 50 °C. [b] Determined by HPLC {Chiralcel OD-H, hexane/*i*PrOH [(9:1)]}. [c] The reaction was carried out in 6 h. [d] Isolated yield in parentheses; n.d.: not determined.

Process Development using BsADH for Cofactor Regeneration

Early bioconversion processes performed to establish TtADH enantioselectivity were carried out on analytical scale by way of an in situ NADH-recycling system involving a second thermophilic NAD-dependent ADH, the recombinant ADH from *Bacillus stearothermophilus* (BsADH).[14] The bacillary enzyme is active with 2-propanol which was therefore used as both co-solvent and substrate. The enzyme is inactive on aliphatic and aromatic ketones, including the carbonyl substrates of TtADH and the corresponding alcohols. However, TtADH does not accept 2-propanol or acetone as substrates. These features allowed the reaction to proceed almost to completion for reduction of 3 mg of **1** to **1a** with 99% conversion and *ee* = 91%, in 24 h at 50 °C, and in the presence of 2% v/v 2-propanol.[14] The scaled-up bioreduction of **1** involving 100 mg of substrate was performed by increasing the amount of 2-propanol to 4% v/v and using different enzyme concentrations. The data summarized in Table 2 indicate that the improved conversion was obtained either in a short reaction time (6 h) using higher amounts of the two enzymes (Table 2, Entry 3) or using lower amounts of enzymes at a reaction time which was four times longer (Table 2, Entry 2). Notably, the yields of 96–97% were similar to those obtained from bioreductions using 3 mg substrate,[14] but with a lower optical purity (86–87% *ee* relative to 91% *ee*), suggesting that the change in TtADH selectivity could be related to 2-propanol concentrations.

Table 2. Asymmetric reduction of **1** with TtADH by the BsADH/2-propanol-NADH regeneration system.[a]

Entry	TtADH [mg mL^{-1}]	BsADH [mg mL^{-1}]	Time [h]	2-Propanol (% v/v)	Conv.[b] [%]	*ee* [b] [%]
1	0.050	0.010	6	4	81	84
2	0.050	0.010	24	4	97	86
3	0.265	0.024	6	4	96	87

[a] Conditions: concentration of **1**: 10 g L^{-1}; TtADH and BsADH at the indicated concentrations; 1 mM NAD^+; buffer, 0.1 M sodium phosphate, pH 7.0, 0.1 M KCl, 5 mM 2-mercaptoethanol. Reaction volume, 10 mL; T = 50 °C. [b] Determined by HPLC {Chiralcel OD-H, hexane/*i*PrOH [(9:1)]}.

In addition to 2-propanol (k_{cat}/K_m = 9 s^{-1} mM^{-1}), BsADH oxidizes other alcohols with even greater efficiency. Examples include ethanol (k_{cat}/K_m = 64 s^{-1} mM^{-1}), 1-propanol (286 s^{-1} mM^{-1}), 1-butanol (437 s^{-1} mM^{-1}), 1-pentanol (64 s^{-1} mM^{-1}), and 1-hexanol (64 s^{-1} mM^{-1}) (Raia, unpublished data) and these were therefore tested as alternative hydride sources for NADH recycling. Moreover, these alcohols and their respective aldehydes are not substrates of TtADH.[14] Thus, only the cofactor is the co-substrate of TtADH and BsADH.

Bioreductions of 100 mg of **1** were carried out in the presence of each alcohol at a concentration of 4% v/v, which corresponds to 700 mM for ethanol down to 310 mM for 1-hexanol. All values were over-saturating for the bacillar ADH. The data obtained were plotted against the respective log P values to correlate conversions and the TtADH enantioselectivity with the hydrophobicity of the alcohol added (see Supporting Information). The TtADH enantioselectivity decreased as the hydrophobicity of the medium increased. However, the level of conversion remained high and near constant for all of the alcohols tested, emphasizing the versatility and high efficiency of the BsADH/alcohol substrate system in recycling the reduced cofactor. An increase in conversion rate with increasing log P with no effect on enantioselectivity was recently reported for the 2-octanone to (*R*)-2-octanol reduction with *Oenococcu oeni* cells in a biphasic system.[19] Moreover, *Lactobacillus brevis* ADH enantioselectivity was recently shown not to be altered by the presence of an organic phase.[20] Instead, *Thermoanaerobium brockii* ADH enantioselectivity was seen to increase proportionally to increasing

Table 3. Asymmetric reduction of **1** with the TtADH/BsADH system at different ethanol and cofactor concentrations.[a]

Entry	Ethanol (% v/v)	NAD^+ [mM]	TtADH [mg mL^{-1}]	BsADH [mg mL^{-1}]	Conversion [%]	*ee* [%]	Isolated yield [%]
1	0.6	1 (2)	0.05	0.01	60 (89)	90 (90)	n.d.
2	1	1 (2)	0.05	0.01	79 (98)	89 (92)	n.d.
3	2	1 (2)	0.05	0.01	92 (98)	90 (93)	n.d.
4	3	1 (2)	0.05	0.01	91 (98)	92 (92)	n.d.
5	4	1 (2)	0.05	0.01	91 (99)	91 (93)	n.d.
6	4	2	0.10	0.02	99	94	81

[a] Conditions reaction and chiral analysis as in Table 1. Amount of **1**: 100 mg. **1** concentration, 10 g L^{-1}, except for #6: 2 g L^{-1}. Values in parentheses refer to data obtained with 2 mM NAD^+. n.d.: not determined.

water composition.[21] In general terms, the stereoselectivity of enzymes decreased as solvent hydrophobicity increased[22] and enantiofacial selectivity was also significantly affected by the reaction medium.[23] Although the examples mentioned describe biphasic systems, including high concentrations of an apolar solvent, the effect of relatively low concentrations of polar protic solvents on the TtADH enantiofacial selectivity is rather remarkable. Docking calculations using the TtADH structure explain the selective formation of the methyl (*R*)-mandelate **1a**[24] by showing that the methyl benzoylformate molecule assumes the lowest energy orientation by fitting the phenyl ring into a hydrophobic pocket, with the two carbonyl groups staggered by about 78° and the methoxy group pointing toward the carboxyamide group of the cofactor. Moreover, the docking analysis showed that the keto ester molecule can also assume another conformation, less energetically favourable compared to that described above, which has the opposite face of the carbonyl group directed to the nicotinamide ring, and therefore leading to the (*S*)-enantiomer of the alcohol product. Importantly, there is no major steric constraint preventing the positioning of this alternative conformation in the enzyme active site.[24] A change in polarity occurring in the active site may induce a structural rearrangement that facilitates the positioning of this alternative conformation.

Since the TtADH/BsADH system showed higher enantioselectivity with ethanol than with 2-propanol, the next step was to examine the synthesis of **1a** using concentrations of ethanol lower than 4% v/v (Table 3). The keto ester reduction was limited by the cofactor recycling rate when the ethanol percentage was 0.6% or 1% v/v and the cofactor concentration was 1 mM (Table 3, Entries 1 and 2). This concentration is much lower than the cofactor K_m (13 ± 2 mM) and is therefore far below that required for saturation of the BsADH active sites. However, by doubling the cofactor concentration, the conversion increased from 60 to 89% (Table 3, Entry 1) and from 79 to 98% (Table 3, Entry 2) in the presence of 0.6% and 1% v/v ethanol, respectively. Conditions employing 4% v/v ethanol and 2 mM NAD (Table 3, Entries 5 and 6) and a substrate concentration of 2 g L^{-1} (as in Table 1, Entry 2) were shown to be ideal for achieving asymmetric reduction of 100 mg of **1** with 81% isolated yield and *ee* = 94% (Table 3, Entry 6). The bioconversion of **2** to **2a** occurred with the same enantioselectivity as the non-halogenated compound and with good productivity (Table 4). Using relatively low substrate concentrations 78 mg of **2a** (*ee* = 92%) was obtained from 100 mg of **2** in 24 h at 50 °C (Table 3, Entry 3). As in the case for the TtADH/TaGDH system (Table 1, Entry 3) the substrate concentration of 2 g L^{-1} was found to be optimal for scaling up production of **2a**. However, the formation of the more reactive acetaldehyde could limit the use of ethanol as a regeneration reductant. Nevertheless, new applications for ethanol as a tunable nicotinamide reductant under four-electron redox conditions for the chemoenzymatic synthesis of important synthons,[25a] as well as solvent and reductant in the enantioselective synthesis of (*S*)-profens using *Sulfolobus solfataricus* ADH[25b] and (2*S*)-2-arylpropanols using horse liver and yeast ADHs[25c] have been recently reported.

Table 4. Reduction of **2** to **2a** with TtADH by the BsADH/ethanol-NADH regeneration system.[a]

Entry	**2** [mg]	[**2**] [g L^{-1}]	TtADH [mg mL^{-1}]	BsADH [mg mL^{-1}]	Conv.[b] [%]	*ee*[b] [%]
1	1	1	0.05	0.01	100	91
2	2	2	0.05	0.01	100	92
3	100	2	0.10	0.02	98 (78)[c]	92

[a] The reactions were carried out for 24 h at 50 °C. Ethanol, 4% v/v. [b] Determined by HPLC {Chiralcel OD-H, hexane/*i*PrOH [(9:1)]}. [c] Isolated yield in parentheses.

Conclusions

The enzymatic synthesis of methyl (*R*)-mandelate (**1a**) and methyl (*R*)-*o*-chloromandelate (**2a**), two important pharmaceutical building blocks, has been developed in a one-phase system, using a carbonyl reductase and two different dehydrogenases to recycle the NADH. The two regeneration modes gave similar yields and optical purities. BsADH displayed two distinct advantages: feasibility of purification and high efficiency at suboptimal pH. For the TaGDH method, the hydride source was glucose, which constitutes cheap biomass whereas for the BsADH method, ethanol was the cheap sacrificial substrate. Both methods are characterized by favourable thermodynamics since alcohol/aldehyde, and glucose/gluconic acid do not interfere with the synthesis reaction. Only a few examples are known for enzyme-coupled cofactor regeneration involving a second ADH[26] and the present study represents a successful application of the bacillary ADH.

Experimental Section

Chemicals: NAD(P)$^+$ and NAD(P)H were obtained from AppliChem. (Darmstadt, Germany). Methyl benzoylformate **1** and methyl (*S*)- and (*R*)-mandelate were obtained from Sigma–Aldrich. Methyl *o*-chlorobenzoylformate **2** was obtained from Ricci Chimica (Perugia, Italy). Other chemicals were A grade substances from Applichem. Solutions of NAD(P)H and NAD$^+$ were prepared as previously reported.[14] All solutions were made up with MilliQ water.

Enzymes and Kinetic Assays: Recombinant *Thermus thermophilus* ADH (TtADH) and recombinant *Bacillus stearothermophilus LLD-R strain* (BsADH) were prepared as described previously.[14] Glucose dehydrogenase from *Thermoplasma acidophilum* (TaGDH) was from Sigma, St. Luis, MO. TtADH activity was assayed spectrophotometrically at 65 °C by measuring the change in absorbance of NADH at 340 nm using a Cary 1E spectrophotometer equipped with a Peltier effect-controlled temperature cuvette holder. The kinetic parameters of TtADH for α-keto esters were determined as described previously.[14] TaGDH was assayed at 50 °C by measuring the change in absorbance of NADPH at 340 nm. The standard assay was performed by adding 0.25 μg of enzyme to 1 mL of preheated assay mixture containing 50 mM glucose and 0.4 mM NADP in 50 mM sodium phosphate, pH 7.0.

The effect of organic solvents on TaGDH was investigated by incubating 0.025 mg mL^{-1} protein in 50 mM sodium phosphate, pH 7.0, at 50 °C, in the absence and presence of organic solvents. At specific time intervals, the samples were centrifuged and small aliquots were withdrawn and assayed. The volume of solution in the tight capped test tube did not change during incubation.

Procedure for Bioreduction: Bioreduction of α-keto esters was performed at 50 °C using two NADH regeneration systems. The first consisted of TaGDH and glucose. For analytical biotransformation the reaction mixture contained 6.2 mM carbonyl compound, 1 mM NAD$^+$, 50 mM glucose, 25 μg TtADH, and 5.5 μg TaGDH in 0.2 mL of 100 mM sodium phosphate, pH 7.0. Semi-preparative reactions were performed on a 0.1 g scale in a reaction volume up to 10 mL, using 0.05 mg mL^{-1} TtADH, and 0.01 mg mL^{-1} TaGDH. During the reaction the pH was maintained at pH 6.5–7.0 with the addition of a 2 M NaOH solution.

The second NADH regeneration system consisted of BsADH and 2-propanol or different linear alcohols as described previously[14] with some modifications. The reaction mixture contained 6.2 mM carbonyl compound, 1 mM NAD$^+$, 15 μg of BsADH, 0.6 to 4% v/v alcohol substrate, and 125 μg TtADH in 1 mL of 100 mM sodium phosphate, pH 7.0, 5 mM 2-mercaptoethanol and 100 mM KCl. Semi-preparative reactions were performed on a 0.1 g scale in a reaction volume up to 10 mL, using 0.05 mg mL^{-1} TtADH, and 0.01 mg mL^{-1} BsADH. In both systems the mixtures were shaken at 160 rpm for different reaction times in a temperature-controlled water bath. Upon termination of the reaction, the mixtures were extracted twice with ethyl acetate, dried with anhydrous Na_2SO_4 and concentrated under reduced pressure. The samples were prepared in hexane/2-propanol (9:1) for HPLC analysis.

To determine the isolated yield of the semi-preparative reactions carried out using the two NADH regeneration systems, the ethyl acetate extracts were dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by silica gel column chromatography (hexane/ethyl acetate, 9:1) gave **1a** or **2a** as colourless oils; yield: 77 mg (77% isolated yield; conversion: 78%) and 81 mg (81%; 99%) of **1a** produced by the TtADH/TaGDH and TtADH/BsADH system, respectively; 86 mg (86% isolated yield; conversion: 96%) and 78 mg (78%; 98%) of **2a** produced by TtADH/TaGDH and TtADH/BsADH system, respectively. For details on the NMR and optical rotation analyses, see the Supporting Information.

The degree of conversion and enantiomeric purity of the products were determined on the basis of the peak areas of ketone substrates and alcohol products separated and visualized by HPLC, on a Chiralcel OD-H column (Daicel Chemical Industries, Ltd., Osaka, Japan). The absolute configuration of product alcohols was determined by comparing the HPLC data with standard samples. Products were analyzed with isocratic elution under the following conditions: hexane/2-propanol (9:1) (mobile phase), flow rate of 1 mL min^{-1}, detection for bioconversions of **1** and **2** at 210 nm. At this wavelength, both **1** and **1a** as well as **2** and **2a** have the same molar extinction coefficient values, so that areas of substrates and products are equally proportional to concentrations. Retention times were as follows: 6.14, 8.82 and 14.15 min for **1**, methyl (*S*)-mandelate and methyl (*R*)-mandelate, respectively; 6.92, 10.12 and 17.16 min for **2**, methyl (*S*)-*o*-chloromandelate and methyl (*R*)-*o*-chloromandelate, respectively. The absolute stereochemistry of the two halogenated alcohol enantiomers were assigned by comparison to the values described in the literature for methyl *o*-chloromandelates.[9] The log *P* values were obtained from Laane and coworkers.[27]

Supporting Information (see footnote on the first page of this article): Time course of the bioreduction of **1** by TtADH. Production of methyl (*R*)-mandelate **1a** at different concentrations of **1**. Effects of organic solvents on TaGDH. Production of methyl (*R*)-mandelate (**1a**) at different concentrations of **1** in the presence of the water-miscible or immiscible organic solvents. Effect of the BsADH alcohol substrates on the enantioselectivity and efficiency of the reduction catalysed by TtADH. NMR spectra and spectroscopic analyses. Optical rotation analyses.

Acknowledgments

This work was funded by Fondo per gli Investimenti della Ricerca di Base (FIRB) n. RBNE034XSW, and by the Agenzia Spaziale Italiana (ASI), project "From Molecules to Man" (MoMan), 1/014/06/0. We thank Prof. Tadashi Ema for the generous gift of methyl *o*-chlorobenzoylformate at an early stage of the work.

[1] a) D. Zhu, Y. Yang, L. Hua, *J. Org. Chem.* **2006**, *71*, 4202–4205; b) T. Ema, H. Yagasaki, N. Okita, K. Nishikawa, T. Korenaga, T. Sakai, *Tetrahedron: Asymmetry* **2005**, *16*, 1075–1078; c) C. He, D. Chang, J. Zhang, *Tetrahedron: Asymmetry* **2008**, *19*, 1347–1351; d) R. Kratzer, M. Pukl, S. Egger, B. Nidetzky, *Microb. Cell Fact.* **2008**, *7*, 37; e) K. Nakamura, R. Yamanaka, T. Matsuda, T. Harada, *Tetrahedron: Asymmetry* **2003**, *14*, 2659–2681.

[2] a) Y. Kobayashi, Y. Takemoto, Y. Ito, S. Terashima, *Tetrahedron Lett.* **1990**, *31*, 3031–3034; b) C. Pousset, M. Haddad, M. Larchevêque, *Tetrahedron* **2001**, *57*, 7163–7167.

[3] A. Bousquet, A. Musolino, PCT Int. Appl., WO9918110 A1; *Chem. Abstr.* **1999**, *130*, 296510e.

[4] a) W. Yang, J.-H. Xu, Y. Xie, Y. Xu, Zh. Gang, G.-Q. Lin, *Tetrahedron: Asymmetry* **2006**, *17*, 1769–1774; b) J. L. Guo, X. Q. Mu, Y. Xu, *Bioprocess Biosyst. Eng.* **2010**, *33*, 797–804.

[5] K. Mitsuhashi, H. Yamamoto, *Eur. Pat. Appl.* **2003**, EP1316613 A2.

[6] L. M. van Langen, R. P. Selassa, F. van Rantwijk, R. A. Sheldon, *Org. Lett.* **2005**, *7*, 327–329.

[7] a) Y. Sun, X. Wan, J. Wang, Q. Meng, H. Zhang, I. Jiang, Z. Zhang, *Org. Lett.* **2005**, *7*, 5425–5427; b) L. Yin, W. Shan, X.

Jia, X. Li, A. S. C. Chan, *J. Organomet. Chem.* **2009**, *694*, 2092–2095.
[8] K.-N. Uhm, S.-J. Lee, H.-K. Kim, H.-Y. Kang, Y. Lee, *J. Mol. Catal. B* **2007**, *45*, 34–38.
[9] T. Ema, S. Ide, N. Okita, T. Sakai, *Adv. Synth. Catal.* **2008**, *350*, 2039–2044.
[10] M. Jeong, Y. M. Lee, S. H. Hong, S. Y. Park, I. K. Yoo, M. J. Han, *Biotechnol. Lett.* **2010**, *32*, 1529–1531.
[11] D. Zhu, H. T. Malik, L. Hua, *Tetrahedron: Asymmetry* **2006**, *17*, 3010–3014.
[12] K. Ishihara, H. Yamaguchi, T. Omori, T. Uemura, N. Nakajima, N. Esaki, *Biosci. Biotechnol. Biochem.* **2004**, *68*, 2120–2127.
[13] K. Inoue, Y. Makino, N. Itoh, *Tetrahedron: Asymmetry* **2005**, *16*, 2539–2549.
[14] A. Pennacchio, B. Pucci, F. Secundo, F. La Cara, M. Rossi, C. A. Raia, *Appl. Environ. Microbiol.* **2008**, *74*, 3949–3958.
[15] J. John, S. J. Crennell, D. W. Hough, M. J. Danson, G. L. Taylor, *Structure* **1994**, *2*, 385–393.
[16] J. Woodward, K. A. Cordray, R. J. Edmonston, M. Blanco-Rivera, S. M. Mattingly, B. R. Evans, *Energy Fuels* **2000**, *14*, 197–201.
[17] L. D. Smith, N. Budgen, S. J. Bungard, M. J. Danson, D. W. Hough, *Biochem. J.* **1989**, *261*, 973–977.
[18] J. Shumacher, M. Eckstein, U. Kragl, *Biotechnol. J.* **2006**, *1*, 574–581.
[19] J. Hu, Y. Xu, *Biotechnol. Lett.* **2006**, *28*, 1115–1119.
[20] M. Villela Filho, T. Stilliger, M. Müller, A. Liese, C. Wandrey, *Angew. Chem. Int. Ed.* **2003**, *4*, 2993–2996.
[21] Å. Jönsson, E. Wehtje, P. Adlercreutz, B. Mattiasson, *Biochim. Biophys. Acta* **1999**, *1430*, 313–322.
[22] G. Carrea, S. Riva, *Angew. Chem.* **2000**, *39*, 2226–2254.
[23] A. M. Klibanov, *Nature* **2001**, *409*, 241–246.
[24] A. Pennacchio, A. Giordano, L. Esposito, E. Langella, M. Rossi, C. A. Raia, *Protein Pept. Lett.* **2010**, *17*, 437–444.
[25] a) S. Broussy, R. W. Cheloha, D. B. Berkowitz, *Org. Lett.* **2009**, *11*, 305–308; b) J. A. Friest, Y. Maezato, S. Broussy, P. Blum, D. B. Berkowitz, *J. Am. Chem. Soc.* **2010**, *132*, 5930–5931; c) P. Galletti, E. Emer, G. Gucciardo, A. Quintavalla, M. Pori, D. Giacomini, *Org. Biomol. Chem.* **2010**, *8*, 4117–4123.
[26] K. Goldberg, K. Schroer, S. Lütz, A. Liese, *Appl. Microbiol. Biotechnol.* **2007**, *76*, 237–248.
[27] C. Laane, S. Boeren, R. Hilhorst, C. Veeger, *Optimization of Biocatalysis in Organic Media*, in: *Biocatalysis in Organic Media* (Eds.: C. Laane, J. Tramper, M. D. Lilly), Elsevier, Amsterdam, **1987**, vol. 29, pp. 65–84.

Received: January 25, 2011
Published Online: June 1, 2011

