

Deracemisation of Mandelic Acid to Optically Pure Non-Natural L-Phenylglycine *via* a Redox-Neutral Biocatalytic Cascade

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Received: December 22, 2009; Published online: April 7, 2010

Abstract: A biocatalytic redox-neutral reaction cascade was designed for the deracemisation of racemic mandelic acid to yield optically pure L-phenylglycine employing three enzymes. The cascade consisted of three steps: a racemisation, an enantioselective oxidation and a stereoselective reductive amination. The enantioselective oxidation of D-mandelic acid to the corresponding oxo acid was coupled with the stereoselective reductive amination of the latter; thus the oxidation as well as the reduction reactions were performed simultaneously. The formal hydrogen abstracted in the first step – the oxidation – was consumed in the reductive amination allowing a redox-neutral cascade due to a cascade-internal cofactor recycling. The enantiomers of the starting material were interconverted by a racemase (mandelate racemase) ensuring that in theory 100% of the starting material can be transformed. Using this set-up racemic mandelic acid was transformed to optically pure L-phenylglycine (*ee* > 97%) at 94% conversion without the requirement of any additional redox reagents in stoichiometric amounts.

Keywords: amino acids; deracemisation; domino reaction; enzyme catalysis; oxidation; reduction

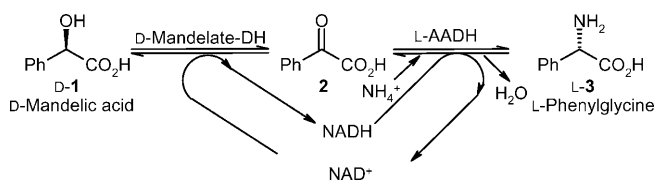
Catalytic tandem reactions^[1–5] are processes where different catalysts operate concurrently in one pot to overcome the limitations and disadvantages of a multistep synthesis such as time-consuming or yield-reducing isolation and purification of the intermediates.^[6] Although the concept of cascade reactions is very elegant, there are still a lot of challenges that need to be addressed. Especially a cascade involving a concurrent reduction and an oxidation step renders the system even more complex due to the diverging reaction conditions.^[7] Since in living cells oxidation and reduction processes are working simultaneously,

enzymes might be the perfect catalysts to set up a similar system for synthetic applications. Combinations of simultaneous biocatalytic reduction and oxidation have already been reported for the deracemisation of *sec*-alcohols.^[8,9]

Enantiomeric pure α -amino acids are becoming more and more important as building blocks and starting materials, especially in the pharmaceutical and fine chemicals industry.^[10] Particularly non-natural α -amino acids are in the focus of interest. For example, D-phenylglycine or D-4-hydroxyphenylglycine are used as building blocks for semi-synthetic broad-spectrum antibiotics like Ampicillin and Amoxicillin.^[11] Other non-natural amino acids are needed as templates in asymmetric synthesis.^[12] Various methods for the preparation of enantiopure non-natural α -amino acids have been reported.^[11]

The aim of the concept presented in this paper is to transform the α -hydroxy acid mandelic acid **1** into the corresponding optically pure non-natural α -amino acid. In a first step the α -hydroxy acid should be oxidised to the corresponding α -oxo acid (Scheme 1); the latter should concurrently be transformed to the corresponding α -amino acid *via* an asymmetric reductive amination, whereby the formal hydrogen ‘abstracted’ in the oxidation is used in the reduction; therefore both reactions – the enantioselective oxidation as well as the stereoselective reduction – have to run simultaneously.

Such a concept of ‘re-using’ the hydrogen has been referred to as ‘borrowing hydrogen’ in metal cataly-



Scheme 1. Concurrent redox-neutral cascade for the transformation of an α -hydroxy acid to the corresponding α -amino acid. L-AADH: L-amino acid dehydrogenase.

sis.^[13] In biocatalysis only few related examples of redox-neutral cascades with internal cofactor recycling have been mentioned,^[14–16] where the nicotinamide cofactors NAD(P)⁺ are reduced in the first step of a cascade and the NAD(P)H formed is then consumed in a second step, whereby oxidation and reduction mediated by the NAD(P)-cofactor occur at the same molecule. This approach of internal cofactor recycling avoids the additional use of other enzymes as well as reagents for the recycling of the cofactors, therefore leading to a redox-neutral cascade.

Since in such a sequence all transformations are reversible the favoured product formed depends on thermodynamics. Calculation^[17] of the Gibbs free energy ΔG for the interconversion of lactic acid and alanine showed that the thermodynamic equilibrium is on the side of the amino acid ($-5.7 \text{ kcal mol}^{-1}$); thus an α -hydroxy acid should be preferentially converted to the amino acid, which encouraged us to perform further investigations on this subject.

Since the enantioselective oxidation of D-mandelic acid by mandelate dehydrogenase is well described^[18] we tested first various commercial amino acid dehydrogenases (AADHs) exclusively for the reductive amination of phenyl glyoxylate **2** employing glucose and glucose dehydrogenase for the recycling of the cofactor NADH (Table 1).

Interestingly L- as well as D-AADHs led to the formation of the L-amino acid, although in case of D-AADHs at rather low conversion. Probably these D-AADHs are only stereospecific for aliphatic amino acids as stated by the provider.^[19]

Before the D-mandelate dehydrogenase (D-MDH) was coupled with an AADH the activity of the D-MDH as well L-AADH (L-AADH-101) was determined by a UV assay, following the formation/consumption of NADH. The units of the enzymes employed for the cascade were chosen in such a way that the reductive amination step is six times faster than the oxidation step.

Employing this set-up it is ensured that NADH is quickly consumed in the second step providing NAD⁺ for the thermodynamically unfavoured first step, the asymmetric oxidation.

Having now chosen possible suitable reaction conditions, enantiopure D-mandelic acid was transformed in the presence of D-MDH, L-AADH, cofactor and ammonium to yield indeed the desired L-phenylglycine. At a substrate concentration of 10 g L^{-1} a conversion of up to 76% was reached, whereby the amount of oxo acid intermediate was below 0.3%. Interestingly, longer reaction times did not lead to higher conversion. Additional experiments showed that the achieved concentration of L-phenylglycine caused inhibition of D-MDH. Reducing the substrate concentration to 5 g L^{-1} circumvented inhibition. Nevertheless, the successful transformation proved the in-

Table 1. Reductive amination of phenyl glyoxylate **2** yielding **3**.

Entry	AADH ^[a]	Conv. ^[b,c] [%]	ee ^[d] [%]
1	L-AADH-101	>97	>97 (L)
2	L-AADH-102	<1	n.d. ^[e]
3	L-AADH-103	5	52 (L)
4	L-AADH-104	>97	>97 (L)
5	L-AADH-105	>97	>97 (L)
6	L-AADH-106	>97	>97 (L)
7	L-AADH-107	>97	>97 (L)
8	L-AADH-108	>97	>97 (L)
9	L-AADH-109	4	51 (L)
10	L-AADH-110	5	48 (L)
11	L-AADH-111	>97	>97 (L)
12	L-AADH-112	4	>97 (L)
13	L-AADH-113	<1	n.d.
14	L-AADH-114	>97	>97 (L)
15	L-AADH-115	>97	>97 (L)
16	L-AADH-116	4	>97 (L)
17	L-AADH-117	5	>97 (L)
18	L-AADH-118	>97	>97 (L)
19	D-AADH-101	4	54 (L)
20	D-AADH-102	5	51 (L)
21	D-AADH-103	3	>97 (L)
22	D-AADH-104	4	>97 (L)
23	D-AADH-105	4	>97 (L)
24	D-AADH-106	3	>97 (L)

^[a] Amino acid dehydrogenase, the numbers refer to the enzyme kit of Codexis.

^[b] Reaction conditions: phenylglyoxylic acid **2** (10 mg, 0.07 mmol), amino acid dehydrogenase (2 mg, L-AADH-101–118, D-AADH-101–105), glucose (30 mg, 0.17 mmol), glucose dehydrogenase (10 μL , 5 U), NADH (1 mg, 0.002 mmol), buffer (1 mL, pH 9.5, sodium bicarbonate 100 mM, NH_4Cl 200 mM), shaking at 30 °C with 120 rpm for 22 h.

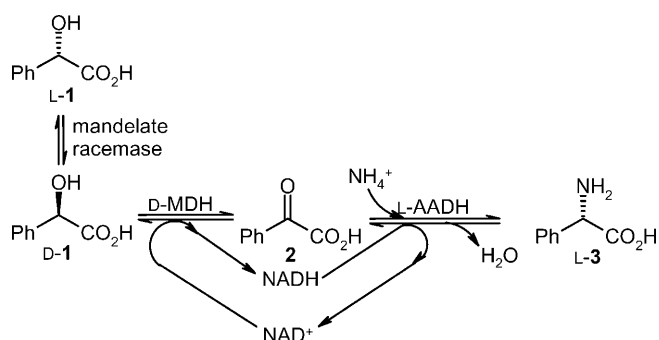
^[c] Conversions were measured by HPLC-DAD analysis on an achiral phase. The measured values were corrected using a calibration curve.

^[d] Enantiomeric excess was determined by HPLC analysis on a chiral phase. In brackets the isomer obtained in excess is given.

^[e] n.d. not determined due to too low conversion.

itial idea for internal cofactor recycling to set up a redox-neutral cascade. Since the substrate possessed the D-configuration and the product showed the L-configuration an inversion of absolute configuration was achieved resembling a Mitsunobu stereoinversion.^[20–23] In contrast to the reagent intensive Mitsunobu reaction (stoichiometric amount of, for example, PPh_3 and DEAD), the here presented system requires just two biocatalysts, catalytic amounts of cofactor and ammonium ions.

In the first experiments the substrate was optically pure D-mandelic acid. As a further challenge our aim was to start from racemic mandelic acid and to convert the racemate to the optically pure L-amino acid,



Scheme 2. Deracemisation of mandelic acid *via* a concurrent redox-neutral cascade to yield L-phenylglycine.

thus performing a deracemisation. For this purpose mandelate racemase^[24] as a third enzyme was employed in an extended system (Scheme 2) for the interconversion/racemisation of mandelic acid. Mandelate racemase has been shown to racemise a broad spectrum of substituted mandelic acid derivatives as well as heteroaromatic analogues.^[24,25] The racemase was required since D-mandelate dehydrogenase is a highly stereoselective enzyme exclusively transforming D-mandelic acid and leaving the L-enantiomer untouched. Fortunately, mandelate racemase racemises only the α -hydroxy acid **1** but not the corresponding amino acid **3**.^[24]

Consequently, racemic mandelic acid was transformed in the presence of mandelate racemase, D-MDH and various AADHs at a substrate concentration of 5 gL⁻¹ (Table 2, entries 1–10) on an analytical scale to yield L-phenylglycine L-3 in up to 86% conversion (entry 1). Performing the redox-neutral cascade at a 10-fold larger scale led to 94% conversion of L-3 (entry 11). Considering in an approximation the Gibbs free energy for the interconversion of lactate/alanine (-5.7 kcalmol⁻¹, see above), a maximum conversion of 98.6% to the L-amino acid is possible at the equilibrium under the conditions employed. Therefore, the obtained conversion of 94% of L-amino acid is rather close to the theoretical maximum. The concept for the deracemisation employed in this paper (Scheme 2) is definitely different from a dynamic kinetic resolution,^[26,27] which encompasses a racemisation step and a kinetic resolution. Scheme 2 resembles more a dynamic kinetic asymmetric transformation (DYKAT) of type II *via* a prochiral intermediate I as introduced by Trost (see Scheme 3, a).^[28–32]

However, the difference is that the here presented concept involved an additional racemisation step of the substrate enantiomers (Scheme 3, b) since the transformation of the substrate enantiomers is highly stereoselective due to the usage of intrinsic chiral enzymes.

Table 2. Transformation of racemic mandelic acid to optically pure L-phenylglycine *via* a redox-neutral cascade (see Scheme 2).^[a]

Entry	AADH ^[b]	1 ^[c] [%]	2 ^[c] [%]	3 ^[c] [%]	ee _p ^[d] [%]
1	L-AADH-101	14.0	0.2	85.8	>97
2	L-AADH-104	15.7	0.2	84.1	>97
3	L-AADH-105	14.9	0.2	84.9	>97
4	L-AADH-106	16.1	0.2	83.7	>97
5	L-AADH-107	16.7	0.2	83.1	>97
6	L-AADH-108	19.4	0.2	80.3	>97
7	L-AADH-111	14.8	0.2	85.0	>97
8	L-AADH-114	14.6	0.2	85.2	>97
9	L-AADH-115	19.7	0.3	80.0	>97
10	L-AADH-118	21.0	0.3	78.7	>97
11	L-AADH-101 ^[e]	6.30	0.1	93.6	>97

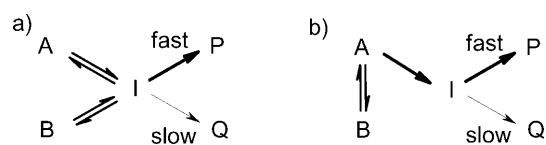
^[a] Reaction conditions: *rac*-mandelic acid **1** (5 mg, 0.03 mmol), amino acid dehydrogenase (2 mg, L-AADH-101, 104, 105, 106, 107, 108, 111, 114, 115, 118), mandelate dehydrogenase (5 μ L, 2.9 U), mandelate racemase (20 mg), NAD⁺ (1 mg, 0.002 mmol), buffer (1 mL, pH 9.5, sodium bicarbonate 100 mM, ammonium chloride 200 mM), shaking at 30 °C with 120 rpm for 22 h.

^[b] Amino acid dehydrogenase, the numbers refer to the enzyme kit of Codexis.

^[c] Composition was measured by HPLC-DAD analysis on an achiral phase. The measured values were corrected using a calibration curve.

^[d] Enantiomeric excess of L-3 was determined by HPLC analysis on a chiral phase.

^[e] Cascade performed on 50-mg scale. For details see Experimental Section.



Scheme 3. Dynamic kinetic asymmetric transformation (DYKAT): a) type II as introduced by Trost; b) the general concept applied in this paper for deracemisation. A,B: substrate enantiomers, I: prochiral intermediate P,Q: product enantiomers.

In summary, a biocatalytic, redox-neutral cascade was designed for the deracemisation of *rac*-mandelic acid to L-phenylglycine by employing three enzymes. The substrate enantiomers were interconverted by the enzyme mandelate racemase. In the first redox step D-mandelic acid was oxidised by the D-selective mandelate dehydrogenase to give the corresponding α -oxo acid consuming NAD⁺ and giving NADH. In the second step, the reductive amination, an L-selective amino acid dehydrogenase (EC 1.4.1.5) converted the oxo acid to the corresponding α -amino acids. For this step NADH is required, which is provided by the first

reaction, the oxidative step; thus in summary a cascade internal redox-recycling was achieved.

Experimental Section

Materials

Amino acid dehydrogenase kits containing different L- and D-selective enzyme preparations (LAADH-18000, DAADH-6000), NAD⁺ free acid, as well as the mandelate dehydrogenase (Ord. No.: 39.10) were obtained from Codexis. Mandelate racemase from *Pseudomonas* sp. ATCC 12633 was prepared as previously reported.^[33] L-Mandelic acid, D-mandelic acid, phenylglyoxylic acid, L-phenylglycine and D-phenylglycine were purchased from Sigma Aldrich.

Experimental Procedure

The reactions were carried out in 1.5-mL Eppendorf tubes. Each reaction mixture contained the crude commercial preparation of amino acid dehydrogenase (2 mg), D-mandelate dehydrogenase (5 µL, 2.9 U), mandelate racemase preparation (20 mg) and buffer (0.5 mL, 100 mM NaHCO₃, 200 mM NH₄Cl, pH 9.5). After rehydration of the enzymes for 10 min at 30°C and 120 rpm, *rac*-mandelic acid (5 mg, 0.03 mmol) and NAD⁺ (1 mg, 0.002 mmol) were added and the reaction was filled up to a total volume of 1 mL with buffer.

For the upscaling, the 10-fold amounts were applied of each ingredient. The upscaling was performed with amino acid dehydrogenase L-AADH-101.

The conversion was followed with HPLC using a Shimadzu CBM-20 A UV-detector and an anion exchange column (Shodex IEC QA-825, 75 × 8 mm) at 254 nm. Gradient elution programme: NaCl (0.5 M) 80%, water bidest 5%, buffer (NaHCO₃, 0.2 M, pH 10) 10%, acetonitrile 5% holding for 2 min. The concentrations of buffer and acetonitrile were kept constant. The NaCl concentration was decreased within 10 min from 80% to 5% and kept constant for 2 min. Flow: 0.6 mL min⁻¹. Retention times: phenylglycine: 6.1 min; mandelic acid: 6.9 min; phenylglyoxylic acid: 11.2 min.

The chiral analytics were performed using the same HPLC system and a C-18 column (Chirobiotic T, 150 × 4.6 mm) at a wavelength of 254 nm. The mobile phase was a mixture of 80% methanol and 20% water bidest containing 1% of TFA. Flow: 0.5 mL min⁻¹. Retention times of phenylglycine: 4.5 min (L), 7.0 min (D).

Acknowledgements

Codexis is thanked for providing amino acid dehydrogenase kits. Evonik, the European Commission ["BIOTRAINS" (FP7-PEOPLE-ITN-2008-238531) and "AmBioCas" (KBBE-2009-3-3-02, grant: 245144)] as well as the COST Action CM0701 "Cascade Chemoenzymatic Processes – New Synergies Between Chemistry and Biochemistry" are acknowledged for financial support.

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