Multi-enzyme cascade synthesis of the most odorous stereoisomers of the commercial odorant Muguesia®

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1. Introduction

The phenomenon of the enantioselectivity in odour perception has been intensively investigated in the last years [1]. Human olfac-tory receptors are chiral and can discriminate the two enantiomers of a chiral odorous molecule [2]. The most common situation is the one in which both enantiomers possess qualitatively similar odours of quantitatively different intensities. It is also possible that the two enantiomers are not differentiated by the receptors, so both of them elicit a similar, weak odour. In the case of very potent odorants, which are nearly totally complementary to the binding pocket of the olfactory receptor proteins, the odour of the molecule is most likely due to one enantiomer only, whereas the other one is weak to odourless. The rarest case is when both enantiomers of an odorant are mainly complementary to different receptors, thus they possess completely different odour profiles.

The evaluation of the olfactory properties of the enantiomers of chiral odorants can lead to novel ways of assessing and enhanc-ing product performance. As a matter of fact, the use of the most potent stereoisomers allows the content of chemicals in perfumery products to be decreased, with a consequent beneficial reduction of their environmental load. Furthermore, stereoisomers may exhibit different biodegradation properties [3] that impact their registration and use. The importance of the chiral switch procedure in fragrance chemistry has been established, and some commercial odorants are now being sold as single enantiomers (*e.g.* Paradisone®, Dextro Norlimbanol®, Thesaron®, Citronellyl Nitrile®, L-Laurinal®). However, the possibility to promote the use of enantiomerically enriched odorous ingredients is strictly related to the viability of a high yield enantioselective procedure to the desired enantiomer, showing advantageous price-performance ratio in comparison with the racemic material.

Nearly ten years ago we prepared [4] all the four stereoisomers of the chiral odorant Muguesia® (**1**) (Scheme 1), according to a procedure based on the use of lipase-mediated kinetic resolution. The synthetic sequence had been devised to obtain all the stereoisomers in enantiopure form, in order to submit them to odour evaluation.

The configuration of the carbon atom in position 3 was found to be important in establishing the odour properties of Muguesia®: the (3*R*) stereoisomers **1c** and **1d** were described as weak and com-pletely devoid of odour in the dry down note; the (3*S*) stereoisomers **1a** and **1b** were found to be the effective odour vectors of the com-mercial odorant, with floral and lily of the valley notes.

We report now on an efficient biocatalysed procedure for the stereoselective synthesis of the odorous (3*S*)-stereoisomers of Muguesia®, based on a multi-enzyme cascade reaction which combines the reduction of the activated carbon carbon double bond of (*E*)-3-methyl-4-phenylbut-3-en-2-one (**2**), mediated by an ene-reductase (ER), with the reduction of the carbonyl group catalyzed by an alcohol dehydrogenase (ADH).

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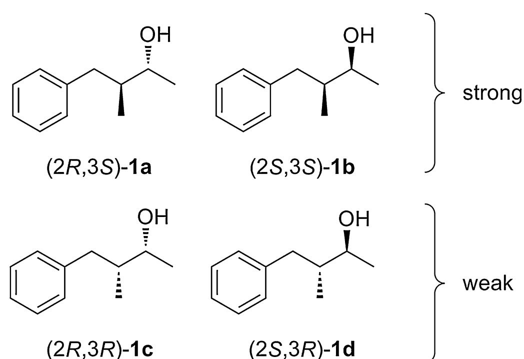


Scheme 1. The four stereoisomers of Muguesia®.

2. Experimental

2.1. General methods

TLC analyses were performed on Merck Kieselgel 60 F254 plates. All the chromatographic separations were carried out on silica gel columns. ^{1}H and ^{13}C NMR spectra were recorded on a 400 or 500 MHz spectrometer. The chemical shift scale was based on internal tetramethylsilane. GC/MS (EI) analyses were performed using a HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent). The following temperature programme was employed: 60 °C (1 min)/6 °C min^{-1}/150 °C (1 min)/12 °C min^{-1}/280 °C (5 min). Chiral GC analyses of Muguesia® isomers were performed on a Chirasil DEX CB column (25 m × 0.25 mm, Chrompack). The following temperature programme was employed: 50 °C (3 min)/2 °C min^{-1}/120 °C/30°/min/180 °C; t_R acetate of (2*S*,3*S*)-**1b** = 24.11 min, t_R acetate of (2*R*,3*R*)-**1c** = 26.20 min, t_R acetate of (2*S*,3*R*)-**1d** = 24.37 min, t_R acetate of (2*R*,3*S*)-**1a** = 26.17 min

2.2. Enzymes

OYE3 from *Saccharomyces cerevisiae* BY4741 and GDH from *Bacillus megaterium* DSM509 were overexpressed in *E. coli* BL21(DE3) strains harbouring specific plasmids (pET30a-OYE3 and pKTS-GDH, respectively) prepared according to standard molecular biology techniques [5]. The enzymes were produced and purified as described in Section 2.3.

The ADHs employed were obtained from commercial sources: CPADH and READH were purchased from Jülich; BYADH, HLADH, TBADH, KRED, PLADH and DRADH were purchased from Sigma–Aldrich.

Specific activities were measured as follows:

OYE3: 1 U equals to the amount of enzyme that reduces 1 μmol of α-methylcinnamaldehyde per minute, as measured by GC analysis (biotransformation conditions: 5 mM substrate, 1% v/v DMSO, 0.1 mM $NADP^+$, an aliquot of OYE solution, 4 U GDH, 50 mM potassium phosphate buffer pH 7.0, total volume 1 mL, 160 rpm, 30 °C, 30 min).

ADHs: according to the data provided by the manufacturers (related to different substrates and conditions).

GDH: 1 U equals to the amount of enzyme that reduces 1 μmol of $NADP^+$ per minute, as measured spectrophotometrically at 340 nm (reaction mixture: 20 mM D-glucose, 0.2 mM $NADP^+$, an aliquot of GDH solution, 50 mM potassium phosphate buffer pH 7.0, total volume 1 mL).

Protein concentrations were determined according to Bradford, using bovine serum albumin (BSA) as a standard.

2.3. Overexpression of OYE3 and GDH in E. coli BL21(DE3)

LB medium (5 mL) containing the appropriate antibiotic (50 μg mL^{-1} kanamycin for pET-30a, 100 μg mL^{-1} ampicillin for pKTS) was inoculated with a single colony from a fresh plate and grown for 8 h at 37 °C and 220 rpm This starter culture was used to inoculate 200 mL medium, which was incubated for 8 h at the same conditions and used to inoculate 1.5 L medium. The latter culture was shaken at 37 °C and 220 rpm until OD_{600} reached 0.4–0.5, then enzyme expression was induced by the addition of 0.1 mM IPTG (50 ng mL^{-1} anhydrotetracycline was also added in the case of the pKTS-GDH plasmid). After 5–6 h the cells were harvested by centrifugation (5000 × *g*, 20 min, 4 °C) resuspended in 50 mL of lysis buffer (20 mM potassium phosphate buffer pH 7.0, 300 mM NaCl, 10 mM imidazole) and disrupted by sonication (Omni Ruptor 250 ultrasonic homogenizer, five sonication cycles, 15 s each, 50% duty). The cell-free extract, after centrifugation (20,000 × *g*, 20 min, 4 °C), was chromatographed on IMAC stationary phase (Ni-Sepharose Fast Flow, GE Healthcare) with a mobile phase composed of 20 mM potassium phosphate buffer pH 7.0, 300 mM NaCl and a 10–300 mM imidazole gradient. Protein elution was monitored at 280 nm, the fractions were collected according to the chromatogram and dialysed twice against 1.0 L of 50 mM potassium phosphate buffer pH 7.0 (12 h each, 4 °C) to remove imidazole and salts. Purified protein aliquots were stored frozen at −80 °C.

2.4. Synthesis of (E)-3-methyl-4-phenyl-3-buten-2-one (2)

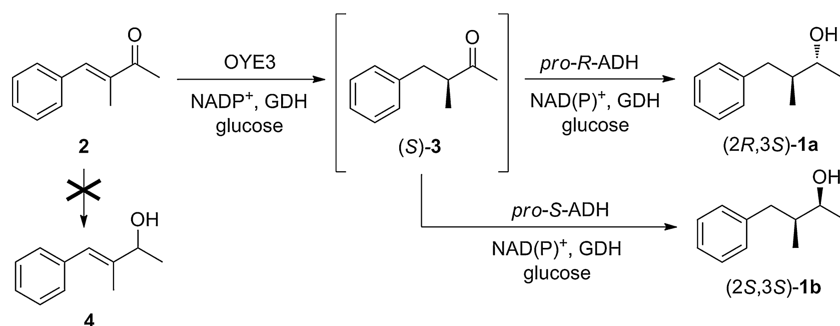
Benzaldehyde (4.24 g, 40 mmol) and 2-butanone (2.88 g, 50 mmol) were dissolved in AcOH (80 mL) under magnetic stirring and conc. H_2SO_4 (4 mL) was added dropwise to the solution. The mixture was stirred at r.t. for 24 h, then it was poured in ice water (100 mL), neutralized with aq. NaOH (20% *w*/*v*) and extracted with EtOAc (3 × 100 mL). The combined organic phase was washed with water (100 mL) and brine (100 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the crude residue was purified by column chromatography using *n*-hexane/EtOAc (9:1) as eluent, affording the unsaturated ketone **2** (4.42 g, 69%): ^{1}H NMR [6] (400 MHz, CDCl3): *δ* = 7.51 (q, 1 H, *J* = 1.2 Hz, C = CH), 7.36–7.44 (m, 5 H, aromatic hydrogens), 2.44 (s, 3 H, $COCH_3$), 2.04 (d, 3 H, *J* = 1.5 Hz, CH_3) ppm; ^{13}C NMR (100.6 MHz, CDCl3): *δ* = 199.9, 139.4, 137.7, 135.8, 129.5, 128.4, 128.3, 25.6, 12.8 ppm; GC/MS: t_R = 16.40 min, *m*/*z* (%) = 160 (M^+, 70), 159 (100), 145 (35), 117 (80), 115 (85).

2.5. General procedure for ADH-mediated biotransformations of an equimolar mixture of (E)-3-methyl-4-phenylbut-3-en-2-one (2) and 3-methyl-4-phenylbutan-2-one (3) (ADH chemoselectivity screening)

A solution of the equimolar mixture of ketones **2** and **3** in DMSO (10 μL, total concentration 500 mM) was added to a potassium phosphate buffer solution (1.0 mL, 50 mM, pH 7.0) containing glucose (20 μmol), $NADP^+$ (0.1 μmol) or NAD^+ (0.1 μmol) (according to the ADH preference), GDH (4 U) and the required ADH (200 μg mL^{-1}). The mixture was incubated for 24 h in an orbital shaker (160 rpm, 30 °C). The solution was extracted with EtOAc (2 × 250 μL), centrifuging after each extraction (15,000 × *g*, 1.5 min), and the combined organic solutions were dried over anhydrous Na_2SO_4.

2.6. General procedure for ADH-mediated biotransformations of (S)-3-methyl-4-phenylbutan-2-one ((S)-3) (ADH stereoselectivity screening)

A solution of ketone (*S*)-**3** [7] in DMSO (10 μ L, 500 mM) was added to a potassium phosphate buffer solution (1.0 mL, 50 mM, pH 7.0) containing glucose (20 μmol), $NADP^+$ (0.1 μmol) or NAD^+ (0.1 μmol) (according to the ADH preference), GDH (4 U) and the required ADH (200 μg mL^{-1}). The mixture was incubated for 24 h in



Scheme 2. Biocatalysed cascade reaction for the synthesis of the best stereoisomers of Muguesia®.

an orbital shaker (160 rpm, 30 °C). The solution was extracted with EtOAc (2 × 250 μL), centrifuging after each extraction (15,000 × g, 1.5 min), and the combined organic solutions were dried over anhydrous Na_2SO_4.

*2.7. General procedure for OYE3-ADH-mediated synthesis of (2R,3S)-3-methyl-4-phenylbutan-2-ol (anti-**1a**) and (2S,3S)-3-methyl-4-phenylbutan-2-ol (syn-**1b**)*

A solution of unsaturated ketone **2** in DMSO (0.60 mL, 500 mM, 48 mg) was added to a potassium phosphate buffer solution (9.40 mL, 50 mM, pH 7.0) containing OYE3 (0.6 U mL^{-1}, 0.350 mg mL^{-1}), the required ADH [PLADH 3 U mL^{-1} (0.170 mg mL^{-1}) or READH 2.5 U mL^{-1} (0.750 mg mL^{-1})], GDH (4 U mL^{-1}), glucose (1.0 mmol, 180 mg) and $NADP^+$ (2.5 μmol, 1.9 mg, for PLADH) or NAD^+ (1.5 μmol, 1.0 mg, for READH). The reaction mixture was shaken at 30 °C and 130 rpm for 48 h, and it was monitored by GC/MS. The mixture was then extracted with EtOAc (3 × 10 mL) and submitted to purification by bulb-to-bulb distillation (135 °C, 20 mmHg).

*2.7.1. Data of (2R,3S)-3-methyl-4-phenylbutan-2-ol (anti-**1a**)*

From unsaturated ketone **2** by cascade reaction with OYE3 and PLADH, after purification by distillation (38.4 mg, 78%): 98% purity by GC/MS, de = 99% (GC/MS); ee = 99% (chiral GC of the corresponding acetate derivative) [4]; $[\alpha]_D$ = +29.0 (c 0.75, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ = 7.30–7.10 (m, 5H, aromatic hydrogens), 3.70 (apparent quintet, 1H, J = 6.1 Hz, CH_3CHOH), 2.87 (dd, 1H, J = 13.6, 4.9 Hz, H-C(4)), 2.34 (dd, 1H, J = 13.6, 9.6 Hz, H-C(4)), 1.82 (m, 1H, CHC(3)), 1.20 (d, 3H, J = 6.3 Hz, CH_3CHOH), 0.83 (d, 3H, J = 7.0 Hz, $CH_3C(3)$) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 140.9, 128.9, 128.1, 125.4, 71.0, 42.2, 38.8, 19.7,14.6 ppm; GC/MS t_R = 14.80 min, m/z (%) 164 (M^+, 10), 146 (35), 131 (70), 91 (100).

*2.7.2. Data of (2S,3S)-3-methyl-4-phenylbutan-2-ol (syn-**1b**)*

From unsaturated ketone **2** by cascade reaction with OYE3 and READH, after purification by distillation (36.9 mg, 75%): 95% purity by GC/MS, de = 99% (GC/MS); ee = 99% (chiral GC of the corresponding acetate derivative) [4]; $[\alpha]_D$ = −8.0 (c 0.75, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.30–7.14 (m, 5H, aromatic hydrogens), 3.75 (dq, 1H, J = 3.9, 6.2 Hz, CH_3CHOH), 2.83 (dd, 1H, J = 13.5, 5.9 Hz, H-C(4)), 2.40 (dd, 1H, J = 13.5, 8.9 Hz, H-C(4)), 1.78 (m, 1H, CHC(3)), 1.20 (d, 3H, J = 6.2 Hz, CH_3CHOH), 0.87 (d, 3H, J = 6.7 Hz, $CH_3C(3)$) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 140.9, 129.1, 128.1, 125.6, 70.4, 41.8, 39.3, 20.6, 13.4 ppm; GC/MS t_R = 14.61 min, m/z (%) 164 (M^+, 5), 146 (35), 131 (43), 91 (100).

3. Results and discussion

We have recently investigated the use of ene-reductases (ERs) belonging to the superfamily of Old Yellow Enzymes for the enantioselective reduction of suitably substituted activated alkenes to prepare enantiopure building blocks [8]. We have also shown the possibility to perform cascade reactions on α ,β-unsaturated-α-substituted aldehydes by combining a OYE-mediated reaction with the reduction of the carbonyl group by means of an alcohol dehydrogenase (ADH), in the aim of avoiding the for-mation of the configurationally unstable α -substituted chiral aldehydes [9].

In this work the concomitant action of the two types of enzymes (ER and ADH) is exploited (Scheme 2) for the one-pot conversion of the α,β-unsaturated ketone **2** into the enantiopure stereoisomers of the secondary alcohol **1**, with the creation of two stereogenic centres in 1,2 relative position under high stereochemical control. All the enzymes are added to the reaction mixture from the beginning and no isolation of the intermediate saturated ketone (*S*)-**3** has to be carried out. This is made possible by the use of ADHs showing high preference for the reduction of the carbonyl group of the saturated intermediate ketone **3**, with no concomitant formation of the allylic alcohol **4**.

This is a linear biocatalysed cascade reaction [10], in which a single substrate is converted into a single product in a two-step one-pot fashion. The procedure is useful to save time and diminish waste in a multi-step synthesis, because the telescoping process reduces the number of intermediates to be submitted to purification steps.

We had already investigated the OYE-mediated reduction of substrate **2** [7], and we had established that OYE3 gave the best results affording the reduced product (*S*)-**3** showing an enantiomeric excess (ee) value of 98% in quantitative yields. The regeneration of the NADPH cofactor was performed using a glucose dehydrogenase (GDH from *Bacillus megaterium*) with glucose as a co-substrate.

As for the selection of the ADHs for the cascade procedures, first we performed a screening of some commercial recombinant and/or purified ADHs, in order to select those showing the highest chemoselectivity towards the reduction of the saturated ketone, and to avoid the formation of the allylic alcohol **4** which cannot be transformed by OYEs.

The ADH-mediated reductions were carried out on an equimolar mixture of the two ketone derivatives **2** and **3**, using the GDH/glucose system for cofactor regeneration. The results of this screening are reported in Table 1.

For the choice of the most suitable ADHs, their stereoselectivity was investigated by performing the carbonyl reduction on a sample of (*S*)-**3** (ee = 98%) obtained by OYE3-mediated reaction. The two diastereoisomers of alcohol **1** that could be obtained from (*S*)-**3**, *i.e. anti*-(2*R*,3*S*)-**1a**, and *syn*-(2*S*,3*S*)-**1b**, could be distinguished by GC/MS analysis (see Experimental). The diastereoisomeric excess (de) values obtained with the screened ADHs are reported in Table 1, together with the corresponding conversion values.

READH and PLADH were selected for their chemoselectivity, and their high conversion and stereoselectivity towards the two desired diastereoisomers.

Table 1
Screening of a set of commercial ADHs for chemo[a] and stereoselectivity[b] evaluation.

Alcohol dehydrogenase	% Reduction of 2[a,c]	% Reduction of 3[a,c]	Chemoselectivity 3 *vs* 2	de[b] (%)	c[b] (%)
CPADH (from *Candida parapsilosis*)	1.5	10	6.7	33 (*syn*)	25
READH (from *Rhodococcus erythropolis*)	42	79	1.9	99 (*syn*)	99
BYADH (from *Saccharomyces cerevisiae*)	n.r.	n.r.	–	–	–
HLADH (from horse liver)	n.r.	n.r.	–	–	–
TBADH (from *Thermoanaerobium brockii*)	n.r.	n.r.	–	–	–
KRED (ketoreductase, unspecified source)	79	99	1.3	99 (*anti*)	99
PLADH (from *Parvibaculum lavamentivorans*)	24	76	3.2	99 (*anti*)	99
DRADH (from *Deinococcus radiodurans*)	25	30	1.2	78 (*anti*)	23

[a] Experimental conditions: 0.8 g L^{-1} mixture of substrates **2** and *rac*-**3**, 200 μg mL^{-1} ADH, 4 U mL^{-1} GDH, 4 eq. glucose, 0.1 mM NAD^+,0.1 mM $NADP^+$,50 mMpotassium phosphate buffer pH 7.0, 30 °C, reaction time 24 h.
[b] Experimental conditions: 0.8 g L^{-1} (*S*)-**3**, 200 μg mL^{-1} ADH, 4 U mL^{-1} GDH, 4 eq. glucose, 0.1 mM NAD^+,0.1 mM $NADP^+$,50 mMpotassium phosphate buffer pH 7.0, 30 °C, reaction time 24 h.
[c] Calculated by using the percentage values of the GC analysis of the crude mixture after 24 h reaction time, respectively as (% compound **4**)/(% compound **4** + % compound **2**) and (% compound **1**)/(% compound **1** + % compound **3**).

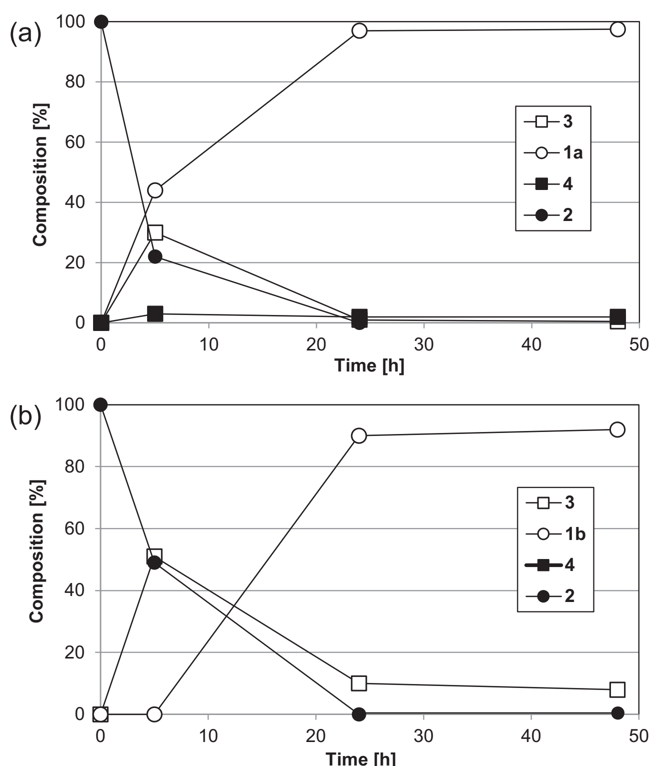


Fig. 1. Time course of (a) the OYE3-PLADH-mediated cascade reaction to (2*R*,3*S*)-**1a** and (b) the OYE3-READH-mediated cascade reaction to (2*S*,3*S*)-**1b**.

The cascade reaction conditions were optimized by combining the OYE3-mediated reduction of the carbon carbon double bond of ketone **2**, and the carbonyl reduction catalyzed either by READH or PLADH, by using GDH and glucose to recycle the cofactors for both reductions.

Surprisingly, the chemoselectivity of the two ADHs was found to be higher in the cascade reactions, in spite of the presence of the unsaturated ketone as a starting material. For example, when substrate **2** (0.8 mg mL^{-1}) was treated with OYE3 (0.3 U mL^{-1}) and either PLADH or READH (4 U mL^{-1}), in the presence of the cofactor regeneration system, stereoisomers (2*R*,3*S*)-**1a** and (2*S*,3*S*)-**1b** were obtained in quantitative yields in 24 h reaction time without any trace of the allylic alcohols.

The reaction conditions were refined, in order to develop a preparative synthesis of the two stereoisomers **1a** and **1b**. Fig. 1 shows the time course of the OYE3-PLADH- and OYE3-READH-mediated cascades, performed by using a 5 g L^{-1} substrate solution, in the presence of OYE3 (0.6 U mL^{-1} in both cases), PLADH (3 U mL^{-1}), and READH (2.5 U mL^{-1}), respectively. After 48 h reaction time, the conversion to the corresponding alcohol was nearly complete with productivity values of 4.8 and 4.4 g L^{-1} d^{-1}, respectively. Only in the reaction with PLADH traces of the allylic alcohol were detected (2% by GC/MS).

4. Conclusions

The high selectivity of biocatalysed reactions can be exploited to develop effective and sustainable manufacturing processes to those chiral fragrances for which the influence of absolute configuration on odour properties could justify the commercialization in enan-tiopure form. In the same reaction medium two enzymes, which are able to catalyze two distinct and subsequent reductions without substrate competition and with high stereoselectivity, are com-bined for the one-pot conversion of the starting unsaturated ketone into chiral compounds in enantiospecific and totally diastereose-lective way.

This procedure represents a further demonstration of the synthetic potential of enzyme-mediated reactions: the high chemo and stereoselectivity that enzymes can achieve are key requisites for the optimization of one-pot processes, which are now extensively investigated for the synthesis of valuable compounds [11]. The diminished number of isolation steps and the high specificity of biocatalysis, which minimizes undesired side-reactions, greatly simplify existing manufacturing processes, with reduction of purifi-cation costs and increase of sustainability.

Acknowledgement

Prof. Neil C. Bruce (Department of Biology, University of York) is kindly acknowledged for the gift of plasmid pT7-OYE1. Prof. Sven Panke and Christian Femmer (Department of Biosystems Science and Engineering, ETH, Basel) are kindly acknowledged for the help provided in the preparation of the plasmids and the overexpressing strains.

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