Biocatalysed Concurrent Production of Enantioenriched Compounds through Parallel Interconnected Kinetic Asymmetric Transformations

Ana Rioz-Martínez,[a] *Fabricio R. Bisogno,*[a] *Cristina Rodríguez,*[a] *Gonzalo de Gonzalo,*[a] *Iván Lavandera,*[a] *Daniel E. Torres Pazmiño,*[b] *Marco W. Fraaije,*[b] *and Vicente Gotor**[a]

[a] Departamento de Química Orgánica e Inorgánica
Instituto Universitario de Biotecnología de Asturias, Universidad de Oviedo
c/ Julián Clavería 8, 33006 Oviedo, Spain
Fax: (+) 34 985 103448
E-mail: vgs@fq.uniovi.es

[b] Laboratory of Biochemistry
Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen
Nijenborgh 4, 9747 AG Groningen, The Netherlands

Electronic Supplementary Information

Contents

1. General

Recombinant histidine-tagged phenylacetone monooxigenase and 4-hydroxyacetophenone monooxigenase were overexpressed and purified according to previously described methods.[1] The oxidation reactions were performed using purified enzymes. One unit (U) of Baeyer-Villiger monooxygenase oxidises 1.0 μM of 2-phenylpentan-3-one **1a** to 1-phenylethyl propionate **2a** per minute at pH 8.0 and 20ºC in the presence of NADPH (Sigma-Aldrich). Cell-free extract from overexpressed HAPMO on *E. coli* TOP10 has been obtained following a similar procedure as previously described.[2] Terrific Broth (TB), containing 50 μg mL^{-1} ampicillin and 0.02% L-arabinose, was inoculated with 1% of an overnight preculture of recombinant *E. coli* TOP10 overexpressing HAPMO. The culture was incubated at 200 rpm at 28ºC in an orbital shaker for 24 hours. Cells were harvested by centrifugation (6000 rpm for 10 minutes, 4ºC, A614 rotor), washed and resuspended in 10 mL of 50 mM Tris/HCl pH 7.5. A crude extract was prepared by ultrasonication (70% amplitude, 5 min, 2 sec on/off, 4ºC). Cell debris were removed by centrifugation (10000 rpm for 30 min, 4ºC) resulting in the cell-free extract. The latter was stored at -20ºC before use. Protein concentration of cell free extract was determined by Bradford method using bovine serum albumin (BSA) as standard for the calibration curve.[3]

Alcohol dehydrogenases from *Lactobacillus brevis* (LBADH) and *Thermoanaerobacter* sp. (ADH-T), and glucose 6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides* were purchased from commercial sources. The amount of ADH used in the different assays was calculated according to the activity data provided by the supplier. (ADH-T 780 U mL^{-1}, LBADH 2320 U mL^{-1}, G6PDH 1000 U mL^{-1}).

Racemic alcohols 2-octanol (±)-**3a**, 2-undecanol (±)-**3b**, sulcatol (±)-**3c**, and β-tetralol (±)-**3d**, ketones 2-octanone (**4a**), 2-undecanone (**4b**), sulcatone (**4c**), and β-tetralone (**4d**), methyl phenyl sulfide (**6**), methyl phenyl sulfoxide (**7**), as well as other starting compounds, reagents and solvents were of the highest quality grade available, supplied by Sigma-Aldrich-Fluka. Racemic ketones 2-phenylpentan-3-one (±)-**1a**, 4-phenylhexan-3-one (±)-**1b**, and 2-phenylheptan-3-one (±)-**1c**, were prepared according to the literature, starting from 1-phenylbutan-2-one or 1-phenylhexan-2-one and using the corresponding alkyl iodide and NaOH in a biphasic medium (46-60% yields).[4] Racemic esters (±)-**2a**-**2c** were prepared by acylation of commercial 1-phenylethanol or 1-phenylpropanol using propionic or valeric anhydride (yields higher than 80%).

Compounds **5a-5c** were prepared by acetylation of hexan-1-ol, nonan-1-ol and 4-methyl-3-penten-1-ol, respectively, using acetic anhydride and 4-DMAP (80-90% yields). Ester **5d** was prepared by oxidation of 2-tetralone using *m*-CPBA in CH_2Cl_2 (15% yield). All the synthesised compounds exhibit physical and spectral data in agreement with those reported.[5]

2. Determination of absolute configurations

Absolute configurations of ketone **1**, ester **2**, alcohol **3**, and sulfoxide **7** were determined by comparison of retention times on GC and HPLC with published data.[6]

3. Experimental procedures

3.1. Study of the stability of substrate (R)-**1a** *at pH 7.5.*

In order to demonstrate that substrate **1a** was not able to racemise at pH 7.5, it was dissolved in Tris-HCl buffer pH 7.5 as follows: (*R*)-**1a** (10 mM) was added to Tris-HCl buffer (50 mM, pH 7.5, 0.2 mM NADPH, 500 μL). Then, 2-propanol (5% v v^{-1}) was added to the mixture in order to reproduce the reaction conditions. This mixture was shaken at 20ºC and 250 rpm for 48 h and then stopped by extraction with ethyl acetate (2 x 0.5 mL). The organic layer was separated by centrifugation and dried over Na_2SO_4. Enantiomeric excess of (*R*)-**1a** was determined by GC analysis.

3.2. Selection of the appropriate substrates for the whole system.

Since in this type of system both processes should not interfere, we first tested if the ketone employed in this study could be reduced by ADH-T. In principle, no interference was expected as the substrate profile of these enzymes is significantly different; PAMO efficiently oxidises aromatic ethyl compounds while the ADH-T is primarily active on aromatic/aliphatic methyl alcohols/ketones. Control experiments demonstrated that ketone **1a** was not reduced by ADH-T.

Procedure: ADH-T (2 U) was added to Tris-HCl buffer (50 mM, pH 7.5, 0.2 mM NADPH, 500 μL). Then, **1a** (10 mM) and 2-propanol (5% v v^{-1}) were added to the mixture. Reactions were shaken at 20ºC and 250 rpm for 24 h and then stopped by extraction with ethyl acetate (2 x 0.5 mL). The organic layer was separated by centrifugation and dried over Na_2SO_4. Conversions were determined by GC analysis.

On the other hand, ketone **4a** was tested with PAMO to study if this substrate could be oxidised by the BVMO. In this case, after 24 h it was observed some amount of ester **5a** (around 40%). However, it became clear that reaction proceeded much slower than the oxidation of ketone **1a**.[6a]

Procedure: Ketone **4a** (10 mM) was added to Tris-HCl buffer (50 mM, pH 7.5, 500 μL), containing glucose-6-phosphate (1.5 equiv), glucose-6-phosphate dehydrogenase (10.0 U), NADPH (0.2 mM) and 2 U of PAMO. The mixture was shaken at 250 rpm in a rotatory shaker at 20ºC. The reactions were stopped after 24 h by extraction with ethyl acetate (2 x 0.5 mL) and the organic layer was dried over Na_2SO_4. Conversions were determined by GC analysis.

*3.3. Time-dependent study with ketone (±)-**1a** and alcohol (±)-**3a** using PAMO and ADH-T.*

Ketone **1a** (11.3 mM) was added to Tris-HCl buffer (50 mM, pH 7.5, 0.5 mL). Then, NADPH (0.2 mM), ADH-T (2 U), PAMO (2 U), and racemic alcohol **3a** (11.3 mM) were added. The mixture was shaken at 20 ºC and 250 rpm for the selected times. Then, the reactions were stopped by extraction with ethyl acetate (2 x 0.5 mL) and the organic layer was dried over Na_2SO_4. Conversions and enantiomeric excesses of compounds **1a**-**5a** were determined by GC analysis, as shown in Table S1.

Table S1. Time course of the reaction between *(±)*-**1** and *(±)*-**3** employing PAMO and ADH-T.

t (h)	**2a** (%)[a]	**4a** (%)[a]	**5a** (%)[a]	*ee* **1a** (%)[a]	*ee* **3a** (%)[b]
0.5	23	23	--	30	30
1	31	30	1	47	40
2	42	41	2	72	71
6	50	49	2	≥ 99	95
24	56	54	13	≥ 99	≥ 99

[a] Measured by GC. [b] Determined by GC after acetylation.

4. Kinetic data

BVMOs concentration were measured photometrically by monitoring the absorption of the FAD-cofactor at 450 nm (ε_{450} = 11.3 mM^{-1} cm^{-1}) after treating the enzyme with 0.1% SDS. Commercial LBADH concentration was determined as described by Bradford[3] employing BSA as standard.

Steady-state kinetics.

The enzymatic activity of PAMO and HAPMO for the oxidation of ketones (±)-**1a-b** and **4a-c**, the activity of M446G PAMO for the oxidation of ketone **4a** and the activity of HAPMO and M446G PAMO for sulfide **6** were determined spectrophotometrically by monitoring NADPH consumption at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}). The enzymatic activity showed by LBADH in the oxidation of (±)-alcohol **3** was measured by monitoring NADPH formation at 340 nm.

Stock solutions of substrates (1 M) were made in dimethyl sulfoxide (DMSO). A reaction mixture (1 mL) usually contained Tris-HCl (50 mM, pH 7.5 for PAMO or HAPMO, pH 9.0 for M446G PAMO), 100 μM NADPH, 1% (v v^{-1}) DMSO, and 0.05-0.10 μM BVMO.

When analyzing the oxidation of (±)-**3a** by means of LBADH, a reaction mixture (1 mL) contained Tris-HCl (50 mM, pH 7.5), 100 μM $NADP^+$, 1% (v v^{-1}) DMSO, and 0.02 μM LBADH.

5. GC and HPLC analyses

5.1. Chiral GC analyses

The following columns were used for the determination of enantiomeric excesses: Column A: RT-BetaDEXe (30 m x 0.25 mm x 0.25 μm, 12 psi N_2); column B: Chiralsil Dex CB (25 m x 0.25 mm x 0.25 μm, 12.2 psi N_2). For all the analyses, the injector temperature was 225ºC and the FID temperature was 250ºC.

Table S3. Determination of *ee* values by chiral GC.

compound	program[a]	column	retention times (min)
1a	70/5/1/110/0/3/150/0	A	48.3 (*R*); 49.2 (*S*)
1c	90/45/2/120/0	B	61.3 (*R*); 63.2 (*S*)
2a	70/5/1/110/0/3/150/0	A	50.2 (*S*); 51.5 (*R*)
2b	90/30/3/200/0	A	47.2 (*S*); 47.8 (*R*)
2c	90/45/2/120/0	B	65.8 (*S*); 68.3 (*R*)
3a[b]	90/5/2.5/105/5/130/2/20/180/3	A	14.6 (*S*); 16.4 (*R*)
3b[b]	90/5/2.5/105/20/140/2.5/160/20/180/0	B	17.2 (*S*); 17.9 (*R*)
3c	70/5/1/110/0/3/150/0	A	28.0 (*S*); 28.9(*R*)
3d[b]	90/5/2.5/105/5/180/5	A	24.8 (*S*); 25.8 (*R*)

[a] Program: initial T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/ time (min).
[b] Alcohol was derivatised into the corresponding acetate derivative.

5.2. Chiral HPLC analyses

The following conditions were used to determine the enantiomeric excesses of compound **7**: Chiralcel OD column (0.46 cm x 25 cm); isocratic eluent: *n*-hexane / *i*-propanol (90:10), 20°C, flow 1 mL min^{-1}. t_R 11.2 min (*R*); t_R 14.2 min (*S*).

5.3. GC analyses to measure conversions

The following columns were used for the determination of conversions: column A: RT-BetaDEXe (30 m x 0.25 mm x 0.25 μm, 12 psi N_2); column B: HP-1 (crosslinked methyl siloxane, 30 m x 0.25 mm 0.25 μm, 1.0 bar N_2).

Table S4. Determination of conversion values by GC.

compound	program[a]	column	retention times (min)
1b	90/30/3/200/0	A	45.2
3a	70/5/1/110/0/3/150/0	A	28.9
3b	70/5/1/110/0/3/150/0	A	55.5
3d	70/5/1/110/0/3/150/0/10/200/0	A	61.4
4a	70/5/1/110/0/3/150/0	A	22.9
4b	70/5/1/110/0/3/150/0	A	53.0
4c	70/5/1/110/0/3/150/0	A	22.5
4d	70/5/1/110/0/3/150/0	A	62.5
5a	70/5/1/110/0/3/150/0	A	25.1
5b	70/5/1/110/0/3/150/0	A	53.5
5c	70/5/1/110/0/3/150/0	A	23.9
6	70/4//20/150/3	B	4.8
7	70/4//20/150/3	B	7.6

[a] Program: initial T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/ time (min).

6. References

1. a) D. E. Torres Pazmiño, R. Snajdrova, D. V. Rial, M. D. Mihovilovic and M. W. Fraaije, *Adv. Synth. Catal.*, 2007, **349**, 1361; b) M. W. Fraaije, J. Wu, D. P. H. M. Heuts, E. W. van Hellemond, J. H. Lutje Spelberg and D. B. Janssen, *Appl. Microbiol. Biotechnol.*, 2005, **66**, 393; c) N. M. Kamerbeek, M. J. H. Moonen, J. G. M. van der Ven, W. J. H. van Berkel, M. W. Fraaije and D. B. Janssen, *Eur. J. Biochem.*, 2001, **268**, 2547.

2. D. E. Torres Pazmiño, R. Snajdrova, B.-J. Baas, M. Ghobrial, M. D. Mihovilovic and M. W. Fraaije, *Angew. Chem. Int. Ed.*, 2008, **47**, 2275.

3. M. M. Bradford, *Anal. Biochem*., 1976, **72**, 248.

4. A. J. Fry and J. P. Bujanauskas, *J. Org. Chem*., 1978, **43**, 3157.

5. See: a) A. Berkessel, M. L. Sebastian-Ibarz and T. N. Mueller, *Angew. Chem. Int. Ed*., 2006, **45**, 6567; b) V. B. Birman and H. Jiang, *Org. Lett*., 2005, **7**, 3445; c) K. Sørbye, C. Tautermann, P. Carlsen and A. Fiksdahl, *Tetrahedron: Asymmetry*, 1998, **9**, 681; d) M. W. Rahtke and D. Vogiazoglou, *J. Org. Chem*., 1987, **52**, 3697; e) D. Enders, H. Eichenauer, U. Baus, H. Schubert and K. A. M. Kremer, *Tetrahedron*, 1984, **40**, 1345.

6. a) C. Rodríguez, G. de Gonzalo, M. W. Fraaije and V. Gotor, *Tetrahedron: Asymmetry*, 2007, **18**, 1338; b) G. de Gonzalo, I. Lavandera, K. Faber and W. Kroutil, *Org. Lett.*, 2007, **9**, 2163; c) G. de Gonzalo, D. E. Torres Pazmiño, G. Ottolina, M. W. Fraaije and G. Carrea, *Tetrahedron: Asymmetry*, 2006, **17**, 130.