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Whole-Cell Biocatalysts for Stereoselective C–H Amination Reactions

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Abstract: *Enantiomerically pure chiral amines are ubiquitous chemical building blocks in bioactive pharmaceutical products and their synthesis from simple starting materials is of great interest. One of the most attractive strategies is the stereoselective installation of a chiral amine through C–H amination, which is a challenging chemical transformation. Herein we report the application of a multienzyme cascade, generated in a single bacterial whole-cell system, which is able to catalyze stereoselective benzylic aminations with ee values of 97.5 %. The cascade uses four heterologously expressed recombinant enzymes with cofactors provided by the host cell and isopropyl amine added as the amine donor. The cascade presents the first example of the successful de novo design of a single whole-cell biocatalyst for formal stereoselective C–H amination.*

Stereoselective C–H amination is a very attractive strategy for the conversion of simple low-cost chemical starting materials to high-value chiral amine building blocks, and has therefore attracted much interest in organic chemistry. The most successful chemical strategies reported so far have involved intramolecular and transition-metal-catalyzed reactions[1] with a number of elegant methods using both chemical[2] and enzymatic[3,4] catalysts. We were interested in constructing de novo biosynthetic multienzyme cascades that are guided in design and built by retrosynthetic considerations,[5] which led us to the design of a single whole-cell system that can catalyze the stereoselective benzylic C–H amination of simple non-functionalized organic compounds using molecular oxygen and isopropyl amine (Figure 1).

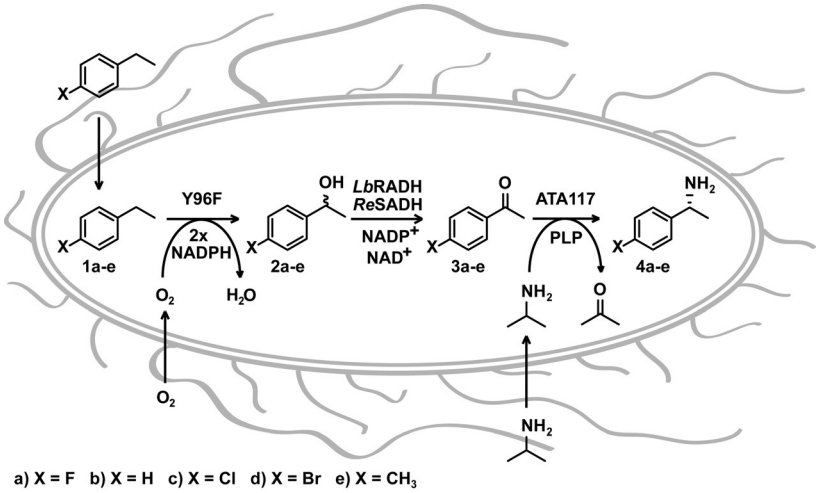

Figure 1. *E. coli* BL21(DE3) cells harboring a monooxygenase [Y96F], *R*- and *S*-selective alcohol dehydrogenases [*Lb*RADH and *Re*SADH], and an ω-transaminase [ATA117] capable of converting 4-substituted ethylbenzenes **1a–e** into alcohols **2a–e**, ketones **3a–e**, and finally amines **4a–e.**

Based upon previous work by us and others[4,6–13] we designed the target cascade to include four enzymes as shown in Figure 1. We envisaged initial C–H activation by a self-sufficient cytochrome P450 monooxygenase to generate the benzylic alcohol, which could then be oxidized to the ketone using two alcohol dehydrogenases (ADH) with complementary stereoselectivity. Two ADHs were deemed necessary, since C–H activation is not always stereoselective and thus we would be able to maximize product yields. Finally, a transaminase (ATA) would be used for generating the amine with the desired stereoselectivity. To our knowledge, such a system represents the first example of a four-enzyme cascade and poses the question whether these enzymes are compatible in a single whole-cell system, given the complex demand for cofactors, transport of substrate and product through the bacterial cell wall, and metabolic stability and potential toxicity of intermediates and products.

The target enzymes for the cascade were carefully chosen based upon their known complementarity with respect to substrate recognition. The first enzyme is an engineered chimeric self-sufficient P450 monooxygenase (Y96F)[9] consisting of the P450camY96F catalytic domain fused to a reductase domain from *Rhodococcus* sp. (RhFRed). The enzyme produces scalemic mixtures of alcohols with a general preference for the *R*-enantiomer.[10] Two alcohol dehydrogenases with complementary stereoselectivity were selected for the next step. *Lb*RADH from *Lactobacillus brevis* is known to oxidize *R*-alcohols using $NADP^+$,[11] whereas *Re*SADH from *Rhodococcus erythropolis* oxidizes *S*-alcohols using NAD^+ as the cofactor.[8,12] The final step for the stereoselective formation of the chiral amine is catalyzed by ω-transaminase ATA117 from *Arthrobacter* sp. requiring an amine donor and pyridoxal 5′-phosphate (PLP).[13]

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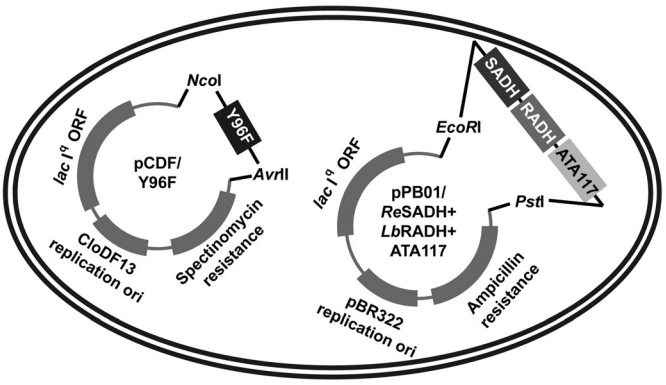

Figure 2. *E. coli* BL21(DE3) cell cotransformed with two plasmids: one harboring a monooxygenase [Y96F], the other harboring *R*- and *S*-selective alcohol dehydrogenases [*Lb*RADH and *Re*SADH], and an ω-transaminase [ATA117].

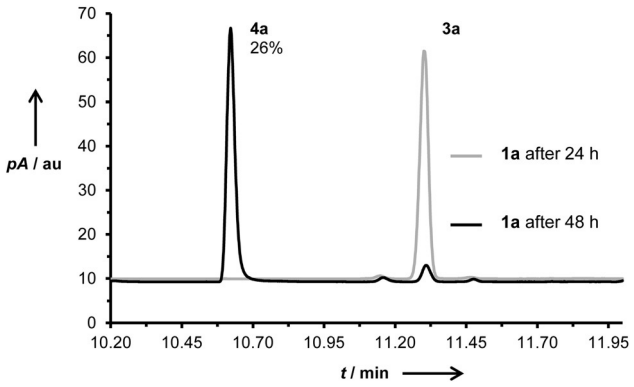

Figure 3. Gas chromatogram of the optimized whole-cell biotransformation using 300 mg mL^{-1} cells and 1 mM of substrate **1a** in 50 mM sodium phosphate buffer pH 7.4 after 24 h and 48 h with the addition of isopropylamine to a final concentration of 200 mM at 24 h (incubated at 20 °C and 220 rpm).

The pathway was introduced into *Escherichia coli* (Figure 1) using a two-plasmid system as shown in Figure 2. In order to avoid possible problems with inefficient transcription and/or translation we opted for a monocistronic design. This ensures independent regulation of each gene via its own operon (Figure S1). For the majority of the cascade we constructed a new plasmid, pPB01, which was designed to be void of regulatory elements adjacent to the *Eco*RI and *Pst*I restriction sites. The plasmid was derived from the pTrcHis plasmid (Lifetechnologies) and is composed of the pBR322 ori, the gene for ampicillin resistance, and the gene for the *lac* repressor, and thus it can be used with any operons containing the *lac* operator for expression regulation. pPB01 allows the insertion of operons in the BioBrick format for fast cloning strategies.[14] Using this method the final construct pPB01/*Re*SADH + *Lb*RADH + ATA117 bearing three new genes was generated (Figure S2) and inserted into *E. coli* together with plasmid pCDF/Y96F bearing the P450 gene. This two-plasmid strategy was designed to ensure better control over P450 gene expression, which can be toxic to bacterial cells.

Analysis of the *E. coli* BL21(DE3) cells harboring both plasmids after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) by protein gel electrophoresis and western blotting of the soluble fraction of cell lysates showed bands at molecular weights expected for enzymes of the cascade (Figure S3). The whole-cell system was then tested for conversion of substrate **1a** to **4a** thus measuring activity of the cascade encoded by plasmids pCDF/Y96F and pPB01/*Re*SADH + *Lb*SADH + ATA117, respectively. It was gratifying to see that product **4a** could clearly be detected in all samples using GC analysis and comparison to authentic, commercially available standard amine **4a** (Figure 3 and Figure S9).

The effect of a number of parameters on the conversion of **1a** to **4a** was investigated in order to optimize the reaction conditions. A number of variables for cell growth were investigated and the following found to produce the highest conversions: 16 h expression of protein in BL21(DE3) cells at 20 °C after induction with 0.4 mM IPTG at $OD_{600} = 0.6$ in M9 minimal media supplemented with 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, and 3 mM $FeCl_3$, and with the addition of 0.5 mM 5-aminolevulinic acid (ALA) upon induction (for more details see Section 1D in the Supporting Information).

The use of isopropylamine (IPA) rather than alanine as the amine donor resulted in higher conversions with this whole-cell system (Table 1) and hence IPA was used in subsequent optimization experiments. However, IPA was found to inhibit the first P450-catalyzed step (loss of activity), which could be overcome by adding **1a** (substrates were added from 500 mM stock solutions in DMSO) and amine in consecutive steps to the reaction mixture: in the first step, substrate **1a** was added and the reaction mixture incubated for 24 h at 20 °C in sealed reaction vials with sufficient headspace to supply oxygen but minimize loss of volatile substrate. In the next step 200 mM IPA was added to the biotransformation, which was incubated for another 24 h at 20 °C, as reported by Koszelewski and co-workers.[13e] We also examined whether additional PLP cofactor was required; however, no improvement in yield was observed consistent with the observation that *E. coli* produces high levels of endogenous PLP.[15]

Table 1: Conversion of **1a–e** and **2a** into corresponding amines **4a–e** using the cascade of enzymes Y96F, *Lb*ADH, *Re*ADH, and ATA117. I

Substrate	Amine donor	Conversion to amines **4a–e** [%][a]
2a	IPA	45
1a	L-Ala	0
1a	IPA	26
1b	IPA	17
1c	IPA	14
1d	IPA	15
1e	IPA	5

[a] Conversion determined by GC after 48 h.

For optimized biotransformations, the effects of different components such as cell mass (Figure S5), amine donor concentration (Figure S6), and substrate concentration (Figure S7) were investigated with the best conversion obtained shown in Figure 3 and Table 1. When racemic 1-(4-fluoro-

phenyl)ethanol (**2a**) was added as a substrate instead of **1a,** conversion to the corresponding amine was 45% while there was 18% of unconverted keto intermediate **3a** with no traces of alcohol **2a** (Figure S9). Conversions for the whole cascade starting from **1a** were lower (26%), suggesting that the reaction performed by Y96F might be the limiting step of the cascade. A number of attempts were made to improve the activity, such as supplementing higher concentrations of ALA or co-expression of glucose dehydrogenase II from *Bacillus megaterium* in order to facilitate cofactor recycling; however, none of these resulted in higher yields.

The transaminase ATA117 is well known to be highly *R*-selective and formation of (*R*)-**4a** with an *ee* value of 97.5% was established when the product of the cascade was analyzed by GC chromatography using a chiral stationary phase (Figure 4). The substrate scope of the whole-cell enzyme cascade was further explored using a small panel of substrates (**1b**–**1e**) resulting in overall conversions of 5–26% from ethyl benzene derivatives **1b**–**1e** to amines **4b**–**4e**, respectively (Table 1).

In summary, we have developed a new biocatalytic cascade, based upon the co-expression of four genes within a single bacterial cell, for the conversion of ethylbenzenes **1a**–**e** to enantiopure (*R*)-1-phenylethanamines **4a**–**e**, respectively, with conversions of up to 26%. Under the present reaction conditions, no additional cofactor except for the amine donor IPA and molecular oxygen was required. None of the substrates, cofactors, intermediates, or products appeared to have significant adverse effects on the transformation. The flexible generic nature of the design of the cascade will allow for the easy substitution of individual enzymes with homologues or mutants and thereby provide a cassette-based modular approach for the design and construction of alternative cascades for the enantioselective C–H amination of other substrates.

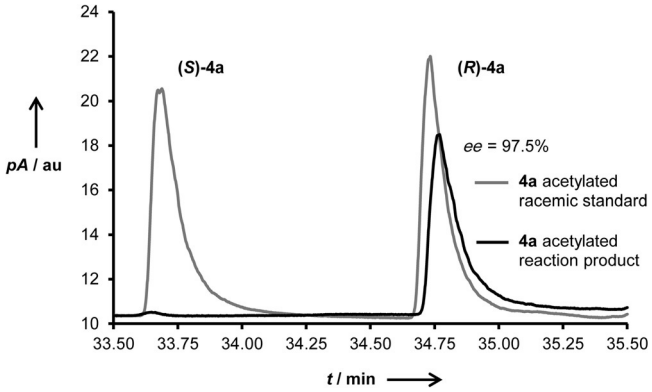

Figure 4. Chiral GC of biotransformation products leading to **4a** after acetylation in comparison with authentic standards.

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Keywords: aminations · biocatalysis · chiral amines · enzyme cascades · whole-cell biotransformation

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[1] M.-L. Louillat, F. W. Patureau, *Chem. Soc. Rev.* **2014**, *43*, 901.
[2] R. A. Green, J. F. Hartwig, *Angew. Chem. Int. Ed.* **2015**, *54*, 3768; *Angew. Chem.* **2015**, *127*, 3839.
[3] a) C. C. Farwell, R. K. Zhang, J. A. McIntosh, T. K. Hyster, F. H. Arnold, *ACS Cent. Sci.* **2015**, *1*, 89; b) R. Singh, J. N. Kolev, P. A. Sutera, R. Fasan, *ACS Catal.* **2015**, *5*, 1685.
[4] M. Schrewe, N. Ladkau, B. Bühler, A. Schmid, *Adv. Synth. Catal.* **2013**, *355*, 1693.
[5] N. J. Turner, E. O'Reilly, *Nat. Chem. Biol.* **2013**, *9*, 285.
[6] K. Tauber, M. Fusch, J. H. Sattler, J. Pitzer, D. Pressnitz, D. Koszelewski, K. Faber, J. Pfeffer, T. Haas, W. Kroutil, *Chem. Eur. J.* **2013**, *19*, 4030.
[7] S. Klatte, V. F. Wendisch, *Bioorg. Med. Chem.* **2014**, *22*, 5578.
[8] C. A. Müller, A. Denning, T. Welters, T. Winkler, A. J. Ruff, W. Hummel, H. Gröger, U. Schwaneberg, *J. Biotechnol.* **2014**, *191*, 196.
[9] a) A. Robin, V. Köhler, A. Jones, A. Ali, P. P. Kelly, E. O'Reilly, N. J. Turner, S. L. Flitsch, *Beilstein J. Org. Chem.* **2011**, *7*, 1494; b) E. O'Reilly, V. Köhler, N. J. Turner, S. L. Flitsch, *Chem. Commun.* **2011**, *47*, 2490; c) E. J. Basom, J. W. Spearman, M. C. Thielges, *J. Phys. Chem. B* **2015**, *119*, 6620.
[10] P. P. Kelly, A. Eichler, S. Herter, D. C. Kranz, N. J. Turner, S. L. Flitsch, *Beilstein J. Org. Chem.* **2015**, *11*, 1713.
[11] a) K. Niefind, J. Müller, B. Riebel, W. Hummel, D. Schomburg, *J. Mol. Biol.* **2003**, *327*, 317; b) M. Reuter, A. Prokoph, J. Kasprzak, K. Becker, K. Baronian, R. Bode, G. Kunze, H. Vorbrodt, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 4723; c) V. Erdmann, U. Mackfeld, D. Rother, A. Jakoblinnert, *J. Biotechnol.* **2014**, *191*, 106; d) B. Li et al., *Sci. Rep.* **2014**, *4*, 6750; e) F. G. Mutti, T. Knaus, N. S. Scrutton, M. Breuer, N. J. Turner, *Science* **2015**, *349*, 1525.
[12] a) K. Abokitse, W. Hummel, *Appl. Microbiol. Biotechnol.* **2003**, *62*, 380; b) C. A. Müller, B. Akkapurathu, T. Winkler, S. Staudt, W. Hummel, H. Grögen, U. Schwaneberg, *Adv. Synth. Catal.* **2013**, *355*, 1787.
[13] a) M. D. Truppo, N. J. Turner, J. D. Rozzell, *Chem. Commun.* **2009**, *16*, 2127; b) M. D. Truppo, N. J. Turner, *Org. Biomol. Chem.* **2010**, *8*, 1280; c) E. O'Reilly, C. Iglesias, D. Ghislieri, J. Hopwood, J. L. Galman, R. C. Lloyd, N. J. Turner, *Angew. Chem. Int. Ed.* **2014**, *53*, 2447; *Angew. Chem.* **2014**, *126*, 2479; d) A. P. Green, N. J. Turner, E. O'Reilly, *Angew. Chem. Int. Ed.* **2014**, *53*, 10714; *Angew. Chem.* **2014**, *126*, 10890; e) D. Koszelewski, I. Lavandera, D. Clay, G. M. Guebitz, D. Rozzell, W. Kroutil, *Angew. Chem. Int. Ed.* **2008**, *47*, 9337; *Angew. Chem.* **2008**, *120*, 9477.
[14] a) G. Røkke, E. Korvald, J. Pahr, O. Oyås, R. Lale, *Methods Mol. Biol.* **2014**, *1116*, 1; b) BioBrick is a trademark of The BioBricks Foundation (http://www.biobricks.org).
[15] J. Kim, J. P. Kershner, Y. Novikov, R. K. Shoemaker, S. D. Copley, *Mol. Syst. Biol.* **2010**, *6*, 436.

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