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A synthetic biology approach for the transformation of L-α-amino acids to the corresponding enantiopure (*R*)- or (*S*)-α-hydroxy acids

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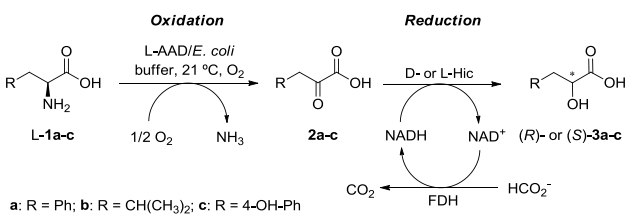
Combinatorial assembly and variation of promoters on a single expression plasmid allowed to balance the catalytic steps of a three enzyme (L-AAD, HIC, FDH) cascade in *E. coli*. The designer cell catalyst quantitatively transformed L-amino acids to the corresponding optically pure (*R*)- and (*S*)-α-hydroxy acids at up to 200 mM substrate concentration.

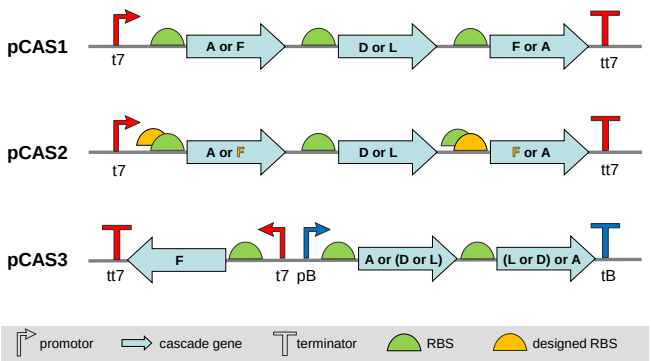
The co-expression of multiple enzymes to set up an artificial reaction cascade in *E. coli* has become a powerful method to generate highly efficient designer cell catalysts.[1] The conversion by such multi-enzyme cascades is optimal if the individual catalytic steps are balanced.[1d,j]

α-Hydroxy acids are highly relevant organic molecules present in numerous natural products as well as in pharmaceuticals[2] and particularly in depsipeptides where they mimic the natural amino acids.[3] We recently reported a redox cascade for the transformation of L-amino acids to (*R*)- or (*S*)-hydroxy acids employing three individual enzyme preparations (Scheme 1).[4] The redox cascade encompassed the oxidation of the L-amino acid **1** to the corresponding keto acid **2** by an L-amino acid deaminase (L-AAD from *Proteus myxofaciens*)[5] followed by the enantioselective reduction of the keto acid catalysed by an 2-hydroxyisocaproate dehydrogenase (L-Hic from *Lactobacillus confusus* DSM 20196[6] or D-Hic from *Lactobacillus paracasei* DSM 20008).[7] L-Hic gave access to the (*S*)-hydroxy acid (*S*)-**3**, while D-Hic produced the (*R*)-enantiomer (*R*)-**3**. A formate dehydrogenase (FDH) from *Candida boidinii* (UniProtKB ID O13437) was used for the regeneration of the cofactor NADH. The sequences of all cascade enzymes are given in the ESI.



Scheme 1 Redox cascade for the preparation of (*R*)- or (*S*)-hydroxy acids from L-amino acids.

To implement this cascade in a designer cell catalyst fine tuning of the expression levels of the cascade enzymes was required. We achieved this by varying the promoters and ribosome binding sites (RBS) as well as by systematically varying the order of the gene sequences (Scheme 2, for a detailed explanation see ESI, Figure S1). For maximum flexibility in the design of the individual cascade expression constructs a combinatorial assembly approach was chosen.[8] A combination of overlap-extension PCR (OE-PCR) and Gibson isothermal assembly was used to generate a combinatorial library of the α-hydroxy acid cascade genes. The custom made pCAS vector (Figure S1) was used as the backbone for all desired cascade constructs.



Scheme 2 General overview of the flexible design of different expression constructs used in this study.
Legend: A: L-AAD; D: D-Hic; L: L-Hic; F: FDH; t7: IPTG-inducible T7/lacO promoter; tt7: T7 terminator; pB: arabinose-inducible promoter pBAD; tB: terminator rrnBT2.

The position of a gene in a polycistronic operon has an impact on its expression as it is lower the closer the gene is to the end of the operon.[9] Consequently, the first and the last positions in the polycistron would have the most severe impact on the expression of the genes. Therefore, we decided to place the FDH due to its known low specific activity[10] in the first position to enable an efficient co-factor recycling. L-AAD was placed at the end since high amounts

of L-AAD might be toxic for the cells. The three cascade genes were assembled first under the control of a strong inducible promoter (T7/*lacO*) and all genes carried the same RBS originating from the pET21a(+) vector. Moreover, to confirm the impact of the first and last position in the polycystronic operon the positions of L-AAD and FDH were varied in a second construct (pCAS1-series, Table 1, Scheme 2).

Table 1 Overview of pCAS1 constructs.

pCAS1 construct	Constituted cascade[a]	Abbreviation
pCAS1.1	t7[-A-D-F]tt7	ADF
pCAS1.2	t7[-F-D-A]tt7	FDA
pCAS1.3	t7[-A-L-F]tt7	ALF
pCAS1.4	t7[-F-L-A]tt7	FLA

[a] Meaning of abbreviations: A: L-AAD; D: D-Hic; L: L-Hic; F: FDH; t7: IPTG-inducible T7/*lacO* promoter; tt7: T7 terminator; -: RBS from pET21a(+); []: expressed under control of the promoter preceding the square bracket and with the ensuing terminator.

The enzymes were co-expressed under two different conditions (4 h at 37 °C and 24 h at 28 °C, ESI, Figure S2). Since initial results showed a better balance between the oxidation and reduction steps for 24 h at 28 °C, the catalysts were prepared using these conditions and used for the transformation of L-**1a** to (*S*)- and (*R*)-**3a**, respectively. As the catalyst *E. coli*/pCAS1.1-4 cells were employed lyophilized in a small volume of phosphate buffered saline (PBS, see ESI for composition). Lyophilized cell catalysts performed better when prepared with PBS than lyophilized with phosphate buffer (see ESI, Figures S5-S7). All *E. coli*/pCAS1 catalysts transformed 100 mM L-Phe L-**1a** to the corresponding α-hydroxy acids (*R*)- or (*S*)-**3a** (Figure 1) whereby the hydroxy acids were obtained in optically pure form (*ee* >99%). Already within one hour more than 30% of the substrate was converted. Construct pCAS1.3 allowed a good balance between the oxidation and the reduction step since the amount of keto acid **2a** was kept below 5%. However, with pCAS1.1-2 or 1.4 the intermediate keto acid **2a** accumulated in the reaction indicating a limiting reduction step. Minimizing keto acid **2a** is also desirable to avoid side reactions. Surprisingly, the highest FDH activity was obtained using pCAS1.3 (see also Table S3), here the FDH gene was in third position in the operon where a low expression level and activity was expected. According to the experimental data, the regulation based on position in the operon was not the most determining factor on both expression and activity levels.

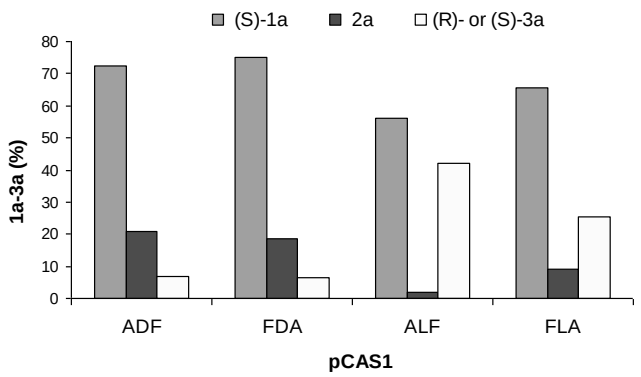


Figure 1 Percentage of **1a**-**3a** of the transformation according to scheme 1 employing lyophilized *E. coli* cells harbouring constructs pCAS1.1-4. The abbreviations for the different cascades are defined in Table 1. Reaction conditions (*S*)-**1a** 100 mM, O_2 1 bar, *E. coli*/CAS1.1-4 (20 mg), NAD^+ (1 mM), HCO_2NH_4 (300 mM), KP_i (100 mM, pH 7), 1 h, 21 °C, 170 rpm. Experiments were conducted in duplicate.

The unbalance between the oxidation and the reduction steps for pCAS1.1-2 and 1.4 was pinned down to the insufficient activity of FDH in these catalyst preparations resulting in limited NADH recycling. This was proven by supplementation of the reaction with additional FDH which substantially improved the balance between the oxidation and the reduction steps. The additional NADH recycling activity avoided the accumulation of the intermediate keto acid **2** (see ESI, Figure S3).

To improve the NADH recycling activity of the cell catalysts a second series of cascade expression vectors was designed. The pCAS2 constructs were as the pCAS1 constructs except that a very strong RBS sequence[11] was designed for the FDH gene to elevate its expression level relative to the other cascade genes (Scheme 2 and Table 2).

Table 2 Overview of pCAS2 constructs.

pCAS2 construct	Constituted cascade[a]	Abbreviation
pCAS2.1	t7[-A-D*F]tt7	AD**F**
pCAS2.2	t7[*F-D-A]tt7	**F**DA
pCAS2.3	t7[-A-L*F]tt7	AL**F**
pCAS2.4	t7[*F-L-A]tt7	**F**LA

[a] Meaning of abbreviations: A: L-AAD; D: D-Hic; L: L-Hic; F: FDH; t7: IPTG-inducible T7/*lacO* promoter; tt7: T7 terminator; -: RBS from pET21a(+); *: designed RBS; []: expressed under control of the promoter preceding the square bracket and with the ensuing terminator.

Although all *E. coli*/pCAS2 catalysts were active (see ESI, Figure S4), in all cases accumulation of the keto acid **2a** was found due to insufficient activity of the FDH. Measuring activities by spectrophotometry of both Hic and FDH showed that the FDH activities were below the detection limit of the assay for all pCAS2 constructs (see ESI, Table S3).

Consequently, a third alternative construct (pCAS3, Scheme 2) was designed using two different promoters for the cascade genes: the stronger T7/*lacO* promoter was employed to drive the expression of the co-factor recycling enzyme FDH while the two other cascade enzymes were under the control of the weaker pBAD promoter (Table 3).[12]

Table 3 Overview of pCAS3 constructs.

pCAS3 construct	Constituted cascade[a]	Abbreviation
pCAS3.1	t7[-F]tt7; pB[-A-D]tB	AD F
pCAS3.2	t7[-F]tt7; pB[D-A]tB	DA F
pCAS3.3	t7[-F]tt7; pB[-A-L]tB	AL F
pCAS3.4	t7[-F]tt7; pB[-L-A]tB	LA F

[a] Meaning of abbreviations: A: L-AAD; D: D-Hic; L: L-Hic; F: FDH; t7: IPTG-inducible T7/*lacO* promoter; tt7: T7 terminator; -: RBS from pET21a(+); pB: arabinose-inducible promoter pBAD; tB: terminator *rrnBT2*; []: expressed under control of the promoter preceding the square bracket and with the ensuing terminator.

In the biotransformation of L-**1a** to **3a** using pCAS3.1-4 no accumulation of intermediate **2a** was observed for CAS3.1, 3.3 or 3.4 and negligible quantities for CAS3.2 (Figure 2). Additionally, the pCAS3 constructs allowed to reach best conversions at 100 mM substrate concentration within one hour. For instance, construct pCAS3.1 (AD F) led to highest conversion for the formation of (*R*)-**3a**, while pCAS3.3 (AL F) was the best catalyst to prepare (*S*)-**3a**.

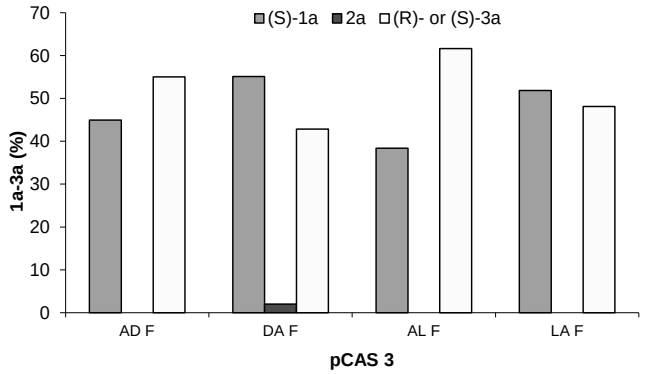


Figure 2 Percentage of **1a**-**3a** after the transformation employing lyophilized *E. coli*/CAS3.1-4. Reaction conditions (*S*)-**1** 100 mM, O_2 1 bar, *E. coli*/CAS3 (20 mg), NAD^+ (1 mM), HCO_2NH_4 (300 mM), KP_i (100 mM, pH 7), 1 h, 21 °C, 170 rpm.

The analysis of total protein extracts of the *E. coli*/pCAS1-3 preparations on Coomassie stained SDS gels (see ESI, Figure S2) confirmed the observations from the activity assays performed by spectrophotometry (ESI): It revealed that the use of the two-promoter system tended to balance protein expression as indicated by clearly visible bands after expression at 28 °C for 24 h for all three cascade enzymes. Balanced expression occurred under the single strong T7/*lacO* promoter on pCAS1 as well. The 'ultrastrong' designed RBS did not enhance FDH expression from the pCAS2 constructs.

The results obtained for the conversion (Figure 2) can be rationalized by comparing the rate of the overall biotransformation of the cascade with the individual activities of the oxidation and the reduction steps involved. The accumulation of the intermediate keto acid **2a** was observed if the oxidation step was faster than the reduction, e.g, with pCAS1.1 (ADF, Figure 3). In this case the overall rate is determined by the reduction step. On the other hand, the formation of keto acid **2a** was negligible when the reduction step was faster than the oxidation as for instance found for pCAS 3.1 or pCAS 3.3 (AD F or AL F, respectively). Here, the overall reaction rate was determined by the oxidation step.

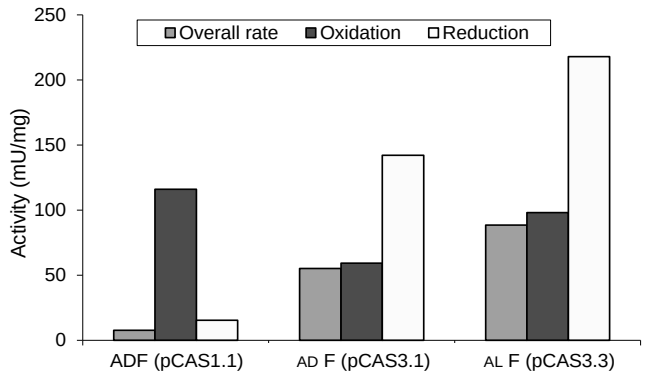


Figure 3. Initial rates (mU/mg lyophilized cells) for the overall cascade, and the individual oxidation and reduction steps using *E. coli*/pCAS1.1, pCAS3.1 and pCAS3.3. Reaction conditions: **Overall rate**: L-Phe (100 mM), *E.coli*/pCAS (5 mg), 21 °C, 1 bar O_2, NAD^+ (1 mM), HCO_2NH_4 (300 mM), 170 rpm in KPi (pH 7, 100 mM, 1 mL). **Oxidation**: L-Phe (100 mM), HCO_2NH_4 (300 mM), *E.coli*/pCAS (1 mg), 21 °C, 1 bar O_2, 170 rpm in KPi (pH 7, 100 mM, 1 mL). **Reduction**: Phenylpyruvic acid (5 mM), HCO_2NH_4 (300 mM), *E.coli*/pCAS (0.5 mg), 21 °C, 170 rpm in KP_i (pH 7, 100 mM, 1 mL).

In addition to the characterisation of the different cell catalysts, the transformation of L-**1a** to **3a** was followed over time for the best performing catalysts *E. coli*/pCAS3.1 and 3.3 (see Figure 4 for pCAS3.1 and otherwise ESI, Figure S8). For both constructs the reaction went to completion within 4 h while the concentration of the intermediate keto acid **2a** remained below the detection limit throughout the reaction.

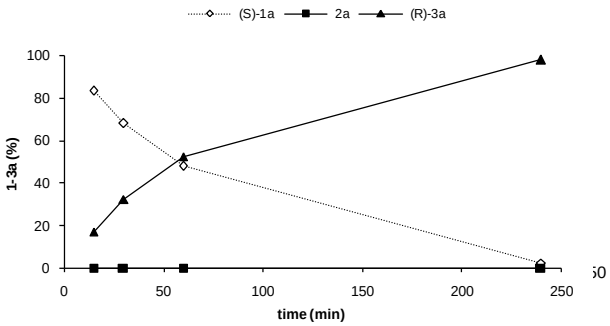


Figure 4. Time course of the transformation of L-**1a** employing lyophilized *E. coli*/pCAS3.1 cells. Reaction conditions (*S*)-**1a** 100 mM, O_2 1 bar, *E. coli*/pCAS3.1 (20 mg), NAD^+ (1 mM), HCO_2NH_4 (300 mM), KP_i (100 mM, pH 7), 21 °C, 170 rpm.

Preparative biotransformation of L-**1a** to (*R*)- or (*S*)-**3a** was achieved by using lyophilized *E. coli*/pCAS preparations (1.3 or 3.3 for L-Hic; 3.1 for D-Hic) at 100-200 mM substrate concentration affording (*R*)- or (*S*)-**3a** with complete conversion and in enantiopure form (Table 4, entries 1-6). The catalyst was also successfully applied for the aliphatic amino acid leucine **1b** at 100 mM substrate concentration (entry 7). Finally, by using this bioretention/bioinversion cascade the oxidation sensitive amino acid tyrosine **1c** was efficiently converted to (*S*)-*p*-hydroxyphenyl lactic acid (*S*)-**3c**, a precursor of biologically active compounds[13] and pharmaceuticals as the antidiabetic Saroglitazar[14] (entry 9). Notably, the hydroxy acids **3a-c** were isolated in high yields (71-86%) and chemical purities after a simple liquid-liquid extraction without requiring any additional purification step.

Table 4. Preparative synthesis of (*R*)-or (*S*)-**3** using constructs pCAS1.3, 3.1 and 3.3.[a]

entry	Construct pCAS[b]	**1** [mM]	conv. [%][c]	**2** [%][c]	**3** [%][d]	*ee* **3** [%][e]
1	1.3 (ALF)	100 (**1a**)	>99	<1	>99 (71)	>99 (*S*)
2	1.3 (ALF)	200 (**1a**)	>99	<1	>99 (80)	>99 (*S*)
3	3.1 (AD F)	100 (**1a**)	>99	<1	>99 (85)	>99 (*R*)
4	3.1 (AD F)	200 (**1a**)	>99	<1	>99 (77)	>99 (*R*)
5	3.3 (AL F)	100 (**1a**)	>99	<1	>99 (86)	>99 (*S*)
6	3.3 (AL F)	200 (**1a**)	>99	<1	>99 (81)	>99 (*S*)
7	3.1 (AD F)	100 (**1b**)	>99	<1	>99 (78)	>99 (*R*)
8	3.1 (AD F)	100 (**1c**)	>99	<1	>99 (75)	98 (*R*)
9	3.3 (AL F)	200 (**1c**)	>99	<1	>99 (86)	99 (*S*)

[a] Reaction conditions: L-**1a-c** (100-200 mM, 4 mL scale), KP_i buffer (100 mM, pH 7.0), NAD^+ (1.0 mM), NH_4HCOO (300 mM for 100 mM substrate and 600 mM for 200 mM substrate), lyophilized *E. coli*/pCAS cells (80 mg for 100 mM and 120 mg for 200 mM), 21 °C, 1 bar of O_2, 6 h; [b] Expression conditions: 24 h at 28 °C. [c] determined via HPLC on an achiral stationary phase; [d] isolated yields in brackets; [e] determined

via HPLC or GC on a chiral stationary phase, see supporting information for details.

Conclusions

To generate optimal *E. coli* designer cell catalysts co-expressing the three enzymes L-AAD, L- or D-Hic and FDH, a library of expression constructs was designed and constituted using a combination of OE-PCR and Gibson assembly. Promoters, RBS strengths as well as the order of the genes were optimized to balance the expression of the three enzymes and to avoid the accumulation of the reaction intermediate keto acid **2**. Preparative transformations of L-amino acids **1a-c** were performed employing freeze dried *E. coli* cells at (100-200 mM) substrate concentration providing the target (*R*)- or (*S*)-hydroxy acids **3a-c** in enantiopure form (98->99% *ee*) and high yield (71-86%) without requiring chromatographic purification.

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Notes and references

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1 (*a*) J. Nielsen, M. Fussenegger, J. Keasling, S. Y. Lee, J. C. Liao, K. Prather and B. Palsson, *Nat. Chem. Biol.* 2014, **10**, 319-322; (*b*) S. Klatte, E. Lorenz and V. F. Wendisch, *Bioengineered* 2014, **5**, 56-62; (*c*) R. Agudo and M. T. Reetz, *Chem. Commun.* 2013, **49**, 10914-10916; (*d*) X. Chen, C. Zhang, R. Zou, K. Zhou, G. Stephanopoulos and H. P. Too, *PLoS One* 2013, **8**, e79650; (*e*) N. Richter, M. Neumann, A. Liese, R. Wohlgemuth, A. Weckbecker, T. Eggert and W. Hummel, *Biotechnol. Bioeng.* 2010, **106**, 541-552; (*f*) T. Ema, S. Ide, N. Okita and T. Sakai, *Adv. Synth. Catal.* 2008, **350**, 2039-2044; (*g*) H. Gröger, C. Rollmann, F. Chamouleau, I. Sebastien, O. May, W. Wienand and K. Drauz, *Adv. Synth. Catal.* 2007, **349**, 709-712; (*h*) N. H. Tolia and L. Joshua-Tor, *Nat. Methods* 2006, **3**, 55-64; (*i*) H. Gröger, F. Chamouleau, N. Orologas, C. Rollmann, K. Drauz, W. Hummel, A. Weckbecker and O. May, *Angew. Chem. Int. Ed.* 2006, **45**, 5677-5681; (*j*). A. Menzel, H. Werner, J. Altenbuchner and H. Gröger, *Eng. Life Sci.* 2004, **4**, 573-576.

2 G. M. Coppola, H. F. Schuster, α-Hydroxy Acids in Enantioselective Synthesis, Wiley-VCH, Weinheim, **1997**.

3 See for example: (*a*) J. Scherkenbeck, S. Lütetenberg, M. Ludwig, K. Brücher and A. Kotthaus *Eur. J. Org. Chem.*, 2012, 1546-1553; (*b*) S. Stolze and M. Kaiser, *Synthesis* 2012, **44**, 1755-1777; (*c*) M. Lemmens-Gruber, R. Kamyar and R. Dornetshuber, *Curr. Med. Chem.* 2009, **16**, 1122-1137; (*d*) S. Bunyajetpong, W. M. Yoshida, N. Sitachitta and K. Kaya *J. Nat. Prod.* 2006, **69**, 1539-1542.

4 E. Busto, N. Richter, B. Grischek and W. Kroutil *Chem. Eur. J.* 2014, **20**, 11225-11228.

5 (*a*) F. R. Alexandre, D. P. Pantaleone, P. P. Taylor, I. G. Fotheringham, D. J. Ager and N. J. Turner, *Tetrahedron Lett.* 2002, **43**, 707-710; (*b*) D. P. Pantaleone, A. M. Geller and P. P. Taylor, *J. Mol. Catal. B: Enzym.* 2001, **11**, 795-803.

6 H. Schütte, W. Hummel and M.-R. Kula, *Appl. Microbiol. Biotechnol.* 1984, **19**, 167-176.

7 W. Hummel, H. Schütte and M.-R. Kula, *Appl. Microbiol. Biotechnol.* 1985, **21**, 7-15.

8 (*a*) B. Kim, J. Du, D. T. Eriksen and H. Zhao, *Appl. Environ. Microbiol.*, 2013, **79,** 931-941; (*b*) Y. Li, Q. Gu, Z. Lin, Z. Wang, T. Chen and X. Zhao, *ACS Synth. Biol.*, 2013, **2**, 651-661; (*c*) P. K. Ajikumar, W.-H. Xiao, K. E. J. Tyo, Y. Wang, F. Simeon, E. Leonard, O. Mucha, T. H. Phon, B. Pfeiffer and G. Stephanopoulos *Science* 2010, **330**, 70-74; (*d*) J. Du, Y. Yuan, T. Si, J. Lian and H. Zhao, *Nucleic Acids Res.* 2012 **40**, e142-e142; (*e*) C. N. S. Santos and G. Stephanopoulos, *Curr. Opin. Chem. Biol.* 2008, **12**, 168-176.

9 H. N. Lim, Y. Lee and R. Hussein, *Proc. Natl. Acad. Sci. U. S. A.* 2011, **108**, 10626-10631.

10 R. Kratzer, M. Pukl, S. Egger, B. Nidetzky, *Microb. Cell Fact.* 2008, **7**, 37-48.

11 H. M. Salis, E. A. Mirsky and C. A. Voigt, *Nat. Biotechnol.* 2009, **27**, 946-950.

12 K. Terpe, *Appl. Microbiol. Biotechnol*. 2006, **72**, 211-222

13 (*a*) N. Valls, M. López-Canet, M. Vallribera and J. Bonjoch, *Chem. Eur. J.* 2001, **7**, 3446-3460; (*b*) C. Parmenon, J. Guillard, D.-H. Caignard, N. Hennuyer, B. Staels, V. Audinot-Bouchez, C. Dacquet, A. Ktorza and M.-C. Viaud-Masuard, *Bioorg. Med. Chem. Lett.* 2008, **18**, 1617-1622; (*c*) J. A. Akins, M. Haurez, J. R. Rizzo, J.-P. van Hoeck, W. Brione, J.-P. Kestmont, C. Stevens, X. Lemair, G. A. Stephenson, E. Marlot, M. Forst and I. N. Houpis, *J. Org. Chem.* 2005, **70**, 4695-4705; (*d*) S. Diethelm, C. S. Schindler and E. M. Carreira, *Org. Lett.* 2010, **12**, 3950-3953; (*e*) N. Valls, M. Vallribera, M. López-Canet and J. Bonjoch, *J. Org. Chem.* 2002, **67**, 4945-4950.

14 R. Agarwal *Curr. Drug Targets* 2014, **15**, 151-155.

