ChemPubSoc Europe

DOI: 10.1002/cctc.201701229

CHEMCATCHEM Communications

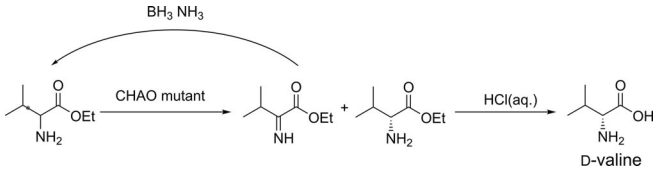
Accessing D-Valine Synthesis by Improved Variants of Bacterial Cyclohexylamine Oxidase

Rui Gong,[a, b] Peiyuan Yao,*[a] Xi Chen,[a] Jinhui Feng,[a] Qiaqing Wu,*[a] Peter C. K. Lau,*[a] and Dunming Zhu[a]

Chemoenzymatic deracemization was applied to prepare D-valine from racemic valine ethyl ester or L-valine ethyl ester in high yield (up to 95%) with excellent optical purity (>99% *ee*) by employing a newly evolved cyclohexylamine oxidase (CHAO) variant Y321I/M226T exhibiting catalytic efficiency that was 30 times higher than that of the wildtype CHAO. Interestingly, CHAO and its variants showed opposite enantioselectivity for valine ethyl ester and phenylalanine ethyl ester.

D-Amino acids are important building blocks in pharmaceuticals, agrochemicals, and food additives.[1] D-Valine, in particular, exists widely in a variety of agricultural pesticides, semisynthetic veterinary antibiotics, and pharmaceutical drugs, such as fluvalinate, valnemulin, penicillamine, actinomycin D, fungisporin, and valinomycin.[2] It can also selectively inhibit the proliferation of fibroblasts in cell culture.[3] Therefore, the investigation of efficient methods for the synthesis of D-valine is of high importance. The current state-of-the-art approaches for the synthesis of D-valine include chemical resolution, chemical asymmetric synthesis, and enzymatic transformation, each with its shortcomings.[2] Chemical resolution is commonly used in industry,[4] but it requires expensive chiral-resolving agents and complex experimental processes. Chiral auxiliaries, such as (*R*)- and (*S*)-4-phenyl-2-oxazolidinone, in chemical asymmetric synthesis are expensive.[5] Unlike chemical methods, the enzymatic preparation of D-valine is an environmentally friendly process with high stereoselectivity and mild reaction conditions. To date, many enzymatic methods have been developed, for example, asymmetric degradation of D/L-valine by L-amino acid oxidase,[6] stereoselective hydrolysis by D-stereospecific amidohydrolase,[7] specific hydrolysis of D/L-5-isopropylhydantoin by D-hydrantoinase coupled with *N*-carbamoyl-D-amino acid amidohydrolase,[8] and asymmetric synthesis from 2-oxo-3-methylbutyric acid by D-amino acid aminotransferase[9] or D-amino acid dehydrogenase.[10]

Turner et al. reported a deracemization strategy for the preparation of primary,[11] secondary,[12] and tertiary amines[13] as well as substituted pyrrolidines[14] by employing the monoamine oxidase from *Aspergillus niger* (MAO-N) mutants. Similarly, on the basis of the substrate profile and crystal-structure analysis of the cyclohexylamine oxidase (CHAO) from *Brevibacterium oxydans* strain IH-35A by Lau et al.,[15] we extended the substrate scope of CHAO to more bulky amines by protein engineering and examined the biocatalytic potential of these CHAO mutants.[16] Although this chemoenzymatic deracemization strategy was demonstrated to be an effective approach for the preparation of optically pure amines, it has not been applied to the deracemization of racemic amino acid esters for the preparation of D-amino acids such as D-valine. Herein, we explored the feasibility of preparing D-valine from racemic valine ethyl ester or L-valine ethyl ester by employing the wildtype CHAO (wt CHAO) and its genetic variants (Scheme 1). The improved CHAO mutants were also tested toward other amino-acid derivatives to determine their activity and enantioselectivity.



Scheme 1. Deracemization of racemic ethyl valinate by employing the CHAO mutant and NH_3·BH_3.

Previously, the application of CHAO in biocatalysis was focused on the deracemization of primary and secondary amines to produce chiral amine building blocks in high yields with high enantiomeric excess (*ee*) values.[16] In this work, we initially found that L-valine ethyl ester was a better substrate than L-valine, but the activity of the wt CHAO was not high (0.9 U mg^{-1}).

We thus sought CHAO variants that may have improved activity by modeling L-valine ethyl ester into the active site of CHAO.[15a] Effectively, 11 amino-acid residues (F88, T198, L199, M226, Q233, Y321, F351, L353, F368, P422, and Y459) lining the binding pocket of CHAO were selected and mutated to six

[a] R. Gong, Prof. P. Yao, Prof. X. Chen, Prof. J. Feng, Prof. Q. Wu, Prof. P. C. K. Lau, Prof. D. Zhu
National Engineering Laboratory for Industrial Enzymes, Tianjin Engineering Research Center of Biocatalytic Technology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences
32 Xi Qi Dao, Tianjin Airport Economic Area, Tianjin 300308 (P.R. China)
Fax: (+86) 22–24828703
E-mail: yao_py@tib.cas.cn
wu_qq@tib.cas.cn
peter.lau@tib.cas.cn

[b] R. Gong
University of Chinese Academy of Sciences
No.19(A) Yuquan Road, Shijingshan District, Beijing, 100049 (P.R. China)

Supporting Information and the ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/cctc.201701229.

typical amino acids (Ala, Ile, Phe, Trp, Thr, and Tyr) by site-specific mutagenesis (Figure S1 in the Supporting Information). The resultant mutants were assayed against L-valine ethyl ester.[17] As a result, four substitutions (T198I, L199F, M226T, and Y321I) were found to possess catalytic activity that was significantly improved relative to that of the wt CHAO enzyme (Table S1). To obtain better mutants toward L-valine ethyl ester, iterative mutagenesis was performed on the best mutant, Y321I. Consequently, three double mutants, Y321I/T198I, Y321I/L199F, and Y321I/M226T, were created and assayed. The best mutant, Y321I/M226T, was selected as a template for the last round of mutagenesis, which resulted in Y321I/M226T/T198I, Y321I/M226T/L199F, and Y321I/M226T/T198I/L199F.

The kinetic parameters of the wt CHAO and its variants against the L-valine ethyl ester substrate showed that three of the single amino-acid substitutions (T198I, L199F, and Y321I) displayed improved catalytic efficiency (2–13 fold), whereas the catalytic efficiency of M226T was marginal relative to that of the wt CHAO (Table 1). According to the structure of CHAO,[15a] M226 is located at the entrance of the tunnel distal to the active site. Hence, the M226T mutation may have a low influence on the activity. Interestingly, T198I, L199F, and Y321I all showed high turnover number (k_{cat}) values but their high Michaelis constant (K_m) values in the 5 mM range resulted in a sharp decline in catalytic efficiency. In contrast, the catalytic efficiency of the double mutant Y321I/M226T was 30-fold higher than that of the wt CHAO. Interestingly, iterative mutagenesis of M226T and Y321I yielded triple (Y321I/M226T/T198I) and quadruple mutants (Y321I/M226T/T198I/L199F) that possessed higher k_{cat} values than the other mutants, but their high K_m values resulted in lower catalytic efficiencies.

Table 1. Kinetic parameters of the wt CHAO and its mutants toward L-valine ethyl ester.

Enzyme[a]	K_m [mM]	k_{cat} [min^{-1}]	k_{cat}/K_m [min^{-1} mM^{-1}]
wt CHAO	7.5	38.5	5.1
T198I	5.0	65.2	13.0
L199F	5.2	55.3	10.6
M226T	5.1	33.3	6.5
Y321I	1.1	75.0	66.4
Y321I/ T198I	1.8	130.7	72.2
Y321I/ M226T	1.2	186.2	152.2
Y321I/ M226T/T198I	2.7	212.6	77.5
Y321I/M226T/T198I/F199F	5.8	221.4	38.2

[a] Y321I/L199F and Y321I/ M226T/L199F failed to be expressed.

Then we applied the Turner-deracemization technique[11, 13a,b,d, 18] to racemic valine ethyl ester and L-valine ethyl ester by using *E. coli* whole cells of CHAO and its double mutant Y321I/M226T in combination with a nonselective chemical reducing agent (NH_3·BH_3) at pH 6.5 and 30 °C. The time–course study (Figure 1) showed that the reactions of racemic valine ethyl ester and L-valine ethyl ester were fast and linear in the first 2 h and resulted in up to 99 and 77% *ee*, respectively, for the mutant Y321I/M226T. The deracemization process of L-valine ethyl ester plateaued in the next 1 h, with a modest increase in the *ee* to 99%. As a result, D-valine ethyl ester was isolated after 4 h and was hydrolyzed to D-valine by 2 M HCl solution. After removal of the solvent, D-valine hydrochloride was obtained in yields of 95 and 91% from racemic valine ethyl ester and L-valine ethyl ester, respectively. Although the reactions of racemic valine ethyl ester and L-valine ethyl ester were also fast and linear in the first 2 h for CHAO, resulting in 58 and 19% *ee*, respectively, the *ee* values leveled out at to lower values (62 and 64% *ee*.

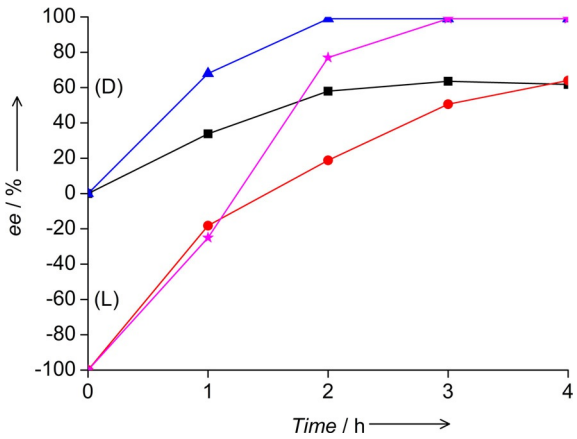


Figure 1. Time course for the deracemization of D/L-valine ethyl ester and L-valine ethyl ester by employing CHAO or its mutant Y321I/M226T and the borane–ammonia complex. D/L-Valine ethyl ester with CHAO (■) or its mutant Y321I/M226T(▲) and L-valine ethyl ester with CHAO (●) or its mutant Y321I/M226T(★).

To shed light on the possible molecular mechanism of the catalysis, we performed docking experiments. Docking D/L-valine ethyl ester to the wt CHAO generated several binding poses. In the D-docking position, Cα and NH of valine ethyl ester are 4.31 and 4.75 Å from the N5 atom of flavin adenine dinucleotide (FAD). The O4 atom of FAD forms a hydrogen-bonding interaction with the NH group of valine ethyl ester, so that the NH group moves and deviates from above the N5 atom of FAD, as shown in Figure 2a. In the L-docking position (Figure 2b), the Cα atom and NH group of valine ethyl ester are 4.22 and 4.44 Å from the N5 atom of FAD. Y321 has a hydrogen-bonding interaction with the NH group of valine ethyl ester, and this places the NH group right above the N5 atom of FAD. Therefore the L enantiomer is favored, whereas the activity toward the D enantiomer is below detection.

Docking of D-valine ethyl ester to the Y321I/M226T mutant showed a result that was similar to that obtained with the wt CHAO, which gave negligible activity (Figure 2c). The best docking result was obtained for L-valine ethyl ester (Figure 2d). Here, the Cα atom and the NH group of L-valine ethyl ester are 3.75 and 3.13 Å from the N5 atom of FAD. The hydrogen-bonding interaction formed between O4, N5 of FAD, and NH of L-valine ethyl ester with other interactions pulls them closer, and these interactions anchor the L-valine ethyl ester and promote stability. These attributes evidently allow the mutant to show higher activity and affinity to the substrate with an overall 30-fold increase in catalytic efficiency.

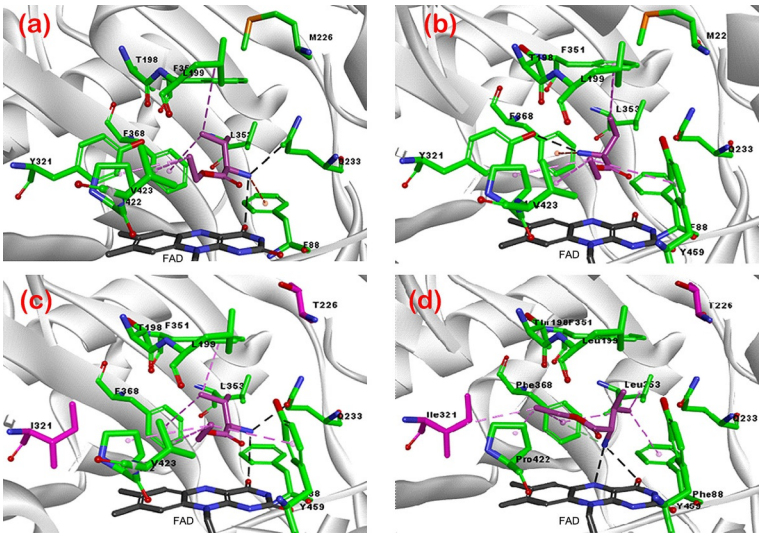


Figure 2. Docking structures of a) D-valine ethyl ester and b) L-valine ethyl ester in the active site of wt CHAO; docking structures of c) D-valine ethyl ester and d) L-valine ethyl ester in the active site of the Y321I/M226T mutant.

A series of amino-acid derivatives were examined as substrates of the wt CHAO and its mutants Y321I and Y321I/M226T. Each substrate was assayed individually at 10 mM substrate concentration with the purified enzymes (Table 2). In the case of D-phenylalanine ethyl ester, the activity of the double mutant Y321I/M226T was significantly better than those of the wt CHAO and mutant Y321I. Interestingly, wt CHAO and Y321I had inverse enantioselectivity toward tyrosine ethyl ester, but Y321I/M226T showed no selectivity. It is possible that residues lining the active site and entrance tunnel can influence the enantioselectivity. In all cases, there was no activity towards the enantiomers of prolinamide. The enantioselectivity of both mutants toward phenylalanine ethyl ester and phenylglycinamide was the D enantiomer. In contrast, the L enantiomer was the selectivity of the mutants toward alanine ethyl ester and valine ammonia amide. These results show that the enzymes display opposite enantioselectivity toward aromatic compounds.

To confirm further the enantioselectivity of the Y321I/M226T mutant toward phenylalanine ethyl ester, deracemization of racemic phenylalanine ethyl ester was performed by employing Y321I/M226T and the borane–ammonia complex in a 10 mL reaction. L-Phenylalanine was obtained in up to 99% *ee* within 8 h (Figures S6 and S7).

To elucidate the possible basis for the dramatic reversal in enantioselectivity, protein–ligand docking simulations with the structure of the Y321I/M226T mutant indicated that D-phenylalanine ethyl ester adopted a binding mode in which the benzene ring pointed out of the active site, whereas the benzene ring of L-phenylalanine ethyl ester pointed towards the active site (Figure 3). The latter state resulted in an increase in the distance between the NH of phenylalanine ethyl ester and N5 of FAD from D-phenylalanine ethyl to L-phenylalanine ethyl. Consequently, the benzene ring was postulated to exert an important influence on the enantioselectivity of the enzyme. Valinamide and 2-aminobutanamide have similar structures, but the enzyme had opposite enantioselectivity; this is an interesting result and is worthy of further research.

Table 2. Specific activity of wt CHAO and its mutants.

Substrate	Specific activity [U m^{-1}][a]		
	CHAO	Y321I	Y321I/M226T
D-tyrosine ethyl ester	trace	0.014	0.019
L-tyrosine ethyl ester	0.029	ND	0.016
D-phenylalanine ethyl ester	0.188	0.705	1.043
L-phenylalanine ethyl ester	0.018	0.013	ND
D-alanine ethyl ester	0.022	0.012	ND
L-alanine ethyl ester	0.117	0.106	0.141
D-prolinamide	ND	ND	ND
L-prolinamide	ND	ND	ND
D-valine ammonia amide	ND	trace	ND
L-valine ammonia amide	0.011	0.016	0.03
D-phenylglycinamide	0.023	0.021	0.034
L-phenylglycinamide	trace	trace	0.012
L-2- aminobutanamide	ND	ND	ND
D-2-aminobutanamide	0.016	0.016	0.022

[a] One enzyme unit (U) was defined as the amount of enzyme that produced 1 μmol of hydrogen peroxide per minute; ND: not detected; trace: activity below 0.01 U mg^{-1}.

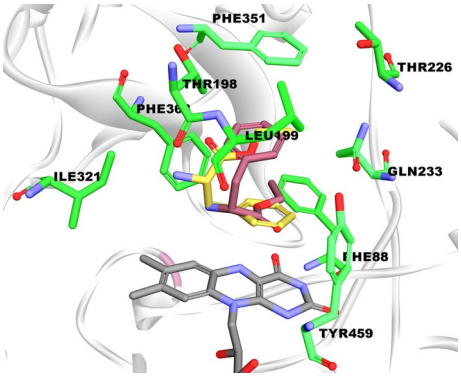


Figure 3. Docked structures of D-phenylalanine ethyl ester (dull red) and L-phenylalanine ethyl ester (yellow).

In summary, a new enzyme variant of the flavoprotein cyclohexylamine oxidase (CHAO) was found useful for the synthesis of D-valine in high yield in enantiomerically pure form through the deracemization and stereoinversion of valine ethyl ester, properties that are otherwise difficult to obtain by previously reported enzymatic methods. In addition, CHAO and its variants showed opposite enantioselectivity for valine ethyl ester and phenylalanine ethyl ester. Further protein engineering of CHAO is underway in our laboratory to improve its enantioselectivity and activity towards other amino-acid derivatives.

Acknowledgements

This work was financially supported by the Youth Innovation Promotion Association of the Chinese Academy of Sciences (Grant No. 2016166) and National Natural Science Foundation of China (Grant No. 21302215). Support by Tianjin "Thousand Talents" Program for Senior International Scientists to P.C.K.L. is gratefully acknowledged.

Conflict of interest

The authors declare no conflict of interest.

Keywords: amino acids · chirality · deracemization · enzymes · stereoinversion

[1] a) M. Yagasaki, A. Ozaki, *J. Mol. Catal. B* **1998**, *4*, 1 – 11; b) M. Friedman, *J. Agric. Food Chem.* **1999**, *47*, 3457 – 3479; c) M. Wakayama, K. Yoshimune, Y. Hirose, M. Moriguchi, *J. Mol. Catal. B* **2003**, *23*, 71 – 85; d) S. A. Fuchs, R. Berger, L. W. J. Klomp, T. J. de Koning, *Mol. Genet. Metab.* **2005**, *85*, 168 – 180; e) S. Martínez-Rodríguez, A. I. Martinez-Gomez, F. Rodriguez-Vico, J. M. Clemente-Jimenez, F. J. L. Heras-Vazquez, *Chem. Biodiversity* **2010**, *7*, 1531 – 1548; f) X. Z. Gao, Q. Y. Ma, H. L. Zhu, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 3341 – 3349; g) M. Melchionna, K. E. Styan, S. Marchesan, *Curr. Top. Med. Chem.* **2016**, *16*, 2009 – 2018.

[2] M. Chen, C. Shi, J. Zhao, Z. Gao, C. Zhang, *World J. Microbiol. Biotechnol.* **2016**, *32*, 171 – 178.

[3] a) S. F. Gilbert, B. R. Migeon, *Cell* **1975**, *5*, 11 – 17; b) J. Hongpaisan, *Cell Biol. Int.* **2000**, *24*, 1 – 7.

[4] a) D. F. Holmes, R. Adams, *J. Am. Chem. Soc.* **1934**, *56*, 2093 – 2094; b) T. Shiraiwa, A. Ikawa, K. Sakaguchi, H. Kurokawa, *Chem. Lett.* **1984**, *13*, 113 – 114; c) K. Harada, T. Okawara, *J. Org. Chem.* **1973**, *38*, 707 – 710; d) Y. Nian, J. Wang, S. B. Zhou, W. H. Dai, S. N. Wang, H. Moriwaki, A. Kawashima, V. A. Soloshonok, H. Liu, *J. Org. Chem.* **2016**, *81*, 3501 – 3508; e) R. Yoshioka, O. Ohtsuki, T. Date, K. Okamura, M. Senuma, *Bull. Chem. Soc. Jpn.* **1994**, *67*, 3012 – 3020.

[5] a) S. Sabelle, D. Lucet, T. L. Gall, C. Mioskowski, *Tetrahedron Lett.* **1998**, *39*, 2111 – 2114; b) D. Lucet, S. Sabelle, O. Kostelitz, T. Le Gall, C. Mioskowski, *Eur. J. Org. Chem.* **1999**, 2583 – 2591.

[6] a) C. H. Zhang, W. T. Xin, M. Chen, Y. Bi, Z. Q. Gao, J. Zhang, *Lett. Appl. Microbiol.* **2015**, *61*, 453 – 459; b) E. Takahashi, M. Furui, T. Shibatani, *Biotechnol. Tech.* **1997**, *11*, 913 – 916.

[7] a) S. Kumagai, M. Kobayashi, S. Yamaguchi, T. Kanaya, R. Motohashi, K. Isobe, *J. Mol. Catal. B* **2004**, *30*, 159 – 165; b) H. Komeda, Y. Asano, *Enzyme Microb. Technol.* **2008**, *43*, 276 – 283; c) S. Yano, H. Haruta, T. Ikeda, T. Kikuchi, M. Murakami, M. Moriguchi, M. Wakayama, *J. Chromatogr. B J. Chromatogr. B* **2011**, *879*, 3247 – 3252.

[8] a) M. J. Rodríguez-Alonso, J. M. Clemente-Jiménez, F. Rodríguez-Vico, *Biochem. Eng. J.* **2015**, *101*, 68 – 76; b) M. Battilotti, U. Barberini, *J. Mol. Catal.* **1988**, *43*, 343 – 352; c) G. C. Xu, L. Li, R. Z. Han, J. J. Dong, Y. Ni, *Appl. Biochem. Biotechnol.* **2016**, *179*, 1 – 15.

[9] J. Kobayashi, Y. Shimizu, Y. Mutaguchi, K. Doi, T. Ohshima, *J. Mol. Catal. B* **2013**, *94*, 15 – 22.

[10] a) H. Akita, H. Suzuki, K. Doi, T. Ohshima, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1135 – 1143; b) X. Z. Gao, X. Chen, W. D. Liu, J. H. Feng, Q. Q. Wu, L. Hua, D. M. Zhu, *Appl. Environ. Microbiol.* **2012**, *78*, 8595 – 8600.

[11] a) M. Alexeeva, A. Enright, M. J. Dawson, M. Mahmoudian, N. J. Turner, *Angew. Chem. Int. Ed.* **2002**, *41*, 3177 – 3180; *Angew. Chem.* **2002**, *114*, 3309 – 3312; b) R. Carr, M. Alexeeva, A. Enright, T. S. C. Eve, M. J. Dawson, N. J. Turner, *Angew. Chem. Int. Ed.* **2003**, *42*, 4807 – 4810; *Angew. Chem.* **2003**, *115*, 4955 – 4958.

[12] a) R. Carr, M. Alexeeva, M. J. Dawson, V. Gotor-Fernandez, C. E. Humphrey, N. J. Turner, *ChemBioChem* **2005**, *6*, 637 – 639; b) D. Ghislieri, D. Houghton, A. P. Green, S. C. Willies, N. J. Turner, *ACS Catal.* **2013**, *3*, 2869 – 2872.

[13] a) J. H. Schrittwieser, B. Groenendaal, S. C. Willies, D. Ghislieri, I. Rowles, V. Resch, J. H. Sattler, E.-M. Fischereder, B. Grischek, W.-D. Lienhart, N. J. Turner, W. Kroutil, *Catal. Sci. Technol.* **2014**, *4*, 3657 – 3664; b) D. Ghislieri, A. P. Green, M. Pontini, S. C. Willies, I. Rowles, A. Frank, G. Grogan, N. J. Turner, *J. Am. Chem. Soc.* **2013**, *135*, 10863 – 10869; c) C. J. Dunsmore, R. Carr, T. Fleming, N. J. Turner, *J. Am. Chem. Soc.* **2006**, *128*, 2224 – 2225; d) K. R. Bailey, A. J. Ellis, R. Reiss, T. J. Snape, N. J. Turner, *Chem. Commun.* **2007**, 3640 – 3642; e) I. Rowles, K. J. Malone, L. L. Etchells, S. C. Willies, N. J. Turner, *ChemCatChem* **2012**, *4*, 1259 – 1261.

[14] V. Köhler, K. R. Bailey, A. Znabet, J. Raftery, M. Helliwell, N. J. Turner, *Angew. Chem. Int. Ed.* **2010**, *49*, 2182 – 2184; *Angew. Chem.* **2010**, *122*, 2228 – 2230.

[15] a) I. A. Mirza, D. L. Burk, B. Xiong, H. Iwaki, Y. Hasegawa, S. Grosse, P. C. K. Lau, A. M. Berghuis, *PLoS One* **2013**, *8*, e60072; b) H. Leisch, S. Grosse, H. Iwaki, Y. Hasegawa, P. C. K. Lau, *Can. J. Chem.* **2012**, *90*, 39 – 45.

[16] a) G. Li, J. Ren, H. Iwaki, D. Zhang, Y. Hasegawa, Q. Wu, J. Feng, P. C. K. Lau, D. Zhu, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1681 – 1689; b) G. Li, J. Ren, P. Yao, Y. Duan, H. Zhang, Q. Wu, J. Feng, P. C. K. Lau, D. Zhu, *ACS Catal.* **2014**, *4*, 903 – 908; c) G. Li, P. Yao, P. Cong, J. Ren, L. Wang, J. Feng, P. C. K. Lau, Q. Wu, D. Zhu, *Sci. Rep.* **2016**, *6*, 24973.

[17] M. Braun, J. M. Kim, R. D. Schmid, *Appl. Microbiol. Biotechnol.* **1992**, *37*, 594 – 598.

[18] a) T. M. Beard, N. J. Turner, *Chem. Commun.* **2002**, 246 – 247; b) A. Enright, F. R. Alexandre, G. Roff, I. G. Fotheringham, M. J. Dawson, N. J. Turner, *Chem. Commun.* **2003**, 2636 – 2637.

Manuscript received: July 27, 2017
Revised manuscript received: September 1, 2017
Accepted manuscript online: September 4, 2017
Version of record online: December 11, 2017