DOI: 10.1002/adsc.200800354

Efficient Biocatalytic Synthesis of (*R*)-Pantolactone

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Received: June 6, 2008; Published online: August 19, 2008

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.200800354.

Abstract: Screening for stereoselective cyanohydrin synthesis in 96-well plates was employed in the development of an efficient, pH-stable hydroxynitrile lyase for the conversion of sterically hindered aliphatic aldehydes. Site-saturation mutagenesis (SSM) resulted in a powerful catalyst for the stereoselective conversion of hydroxypivalaldehyde and pivalaldehyde to their corresponding (*R*)-cyanohydrins (*ee* >97%) which are used as chiral building blocks (e.g., for pantothenic acid production). Furthermore, redesigning the *Pa*HNL5 gene and improving its expression by *Pichia pastoris* with the help of a new P_{AOX1} promoter variant and the helper protein PDI (protein disulfide isomerase) led to elevated amounts of today's most efficient biocatalyst for vitamin B_5 synthesis.

Keywords: biocatalysis; cyanohydrin; enzyme engineering; hydroxynitrile lyase; pantolactone

(*R*)-Pantolactone is the most important chiral building block for the synthesis of (*R*)-pantothenic acid, also called vitamin B_5 – a constituent of coenzyme A, and its provitamin (*R*)-pantothenol. Since the first successful synthesis in 1940,[1] great efforts have been made to develop efficient techniques for the technical preparation of the (*R*)-enantiomer [(*R*)-**3**, Scheme 1].[2–10]

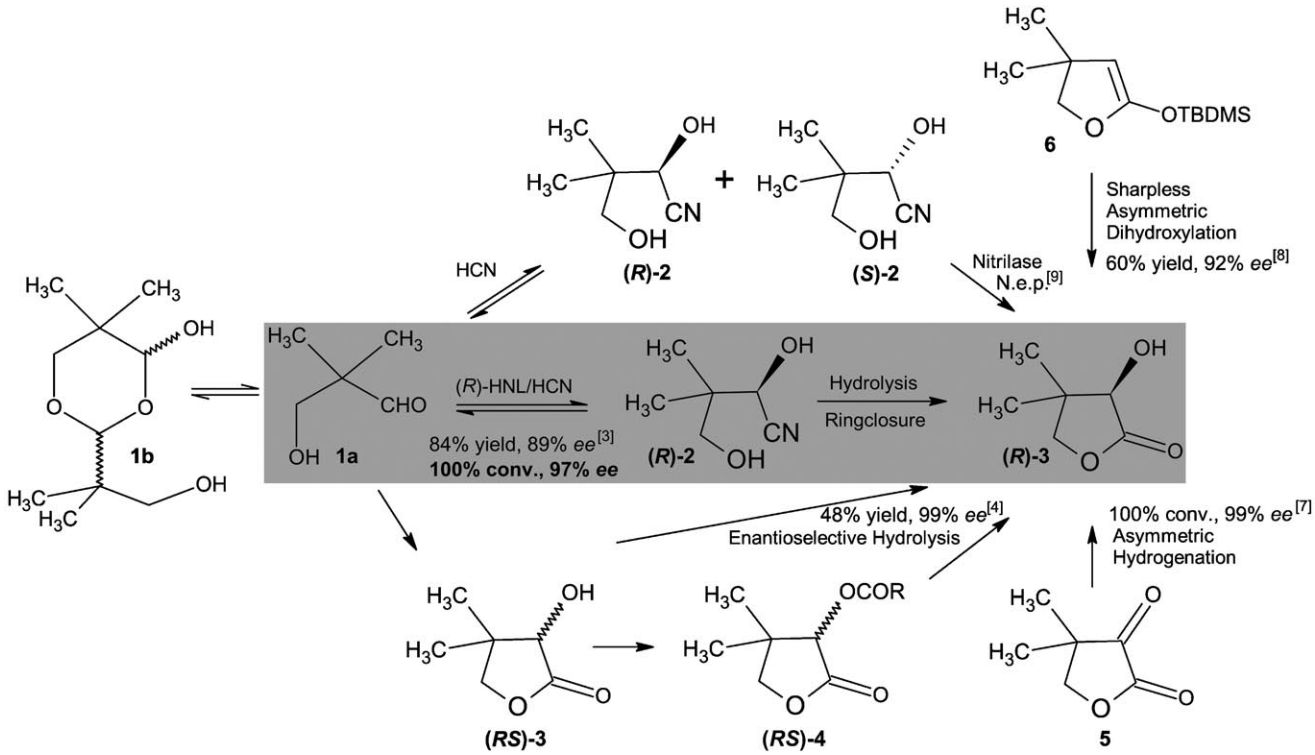
Starting with hydroxypivalaldehyde (**1a**), common stereoselective synthesis routes depend on either additional process steps (e.g., enzymatic kinetic resolution) or the employment of highly complex metal catalysts (selective reduction) which are often not desirable for the production of food and feed, cosmetic and pharmaceutical ingredients. The (*R*)-selective hydroxynitrile lyase [(*R*)-HNL] based synthesis of hydroxypivalaldehyde cyanohydrin [(*R*)-**2**],[2,3] followed by acid-catalyzed hydrolysis, provides a highly attractive alternative.

Focusing on process and reaction engineering, Effenberger et al.[3] succeeded in achieving the highest *ee* (89%) and yield (84%) reported so far, employing large amounts of highly purified and immobilized native *Pa*HNL. Screening for new natural enzymes did not result in any further improvements.[10,11] Herein, we communicate the semi-rational redesign of acid-stable almond (*R*)-HNL isoenzyme 5 (*Pa*HNL5)[12] to realize high selectivity and yield in one of the most challenging biocatalytic routes for today's fine chemical industry.

Hydroxypivalaldehyde (**1a**), exists as an equilibrium (Scheme 1)[13] between the dimer and the monomer with the exposed aldehyde functionality for HCN addition. Heating at 65 °C in THF[14] or without solvent at elevated temperatures (≥100 °C)[15] are common procedures to shift the equilibrium to the monomer. As reported previously,[16] we observed unwanted by-product formation *via* an 'anomalous Tishchenko reaction' when the aldehyde was stirred at elevated temperatures.

Alternatively, acid-catalyzed hemi-acetal cleavage of **1b** (Figure 1) can be used for efficient substrate monomerization. Thus, only gentle substrate heating was necessary prior to the biocatalytic reaction. A pH value ≤2.5 provided the preferred compromise between substrate monomerization, enzyme stability/activity and stability of the chiral cyanohydrin [(*R*)-**2**].

Recently, *Pa*HNL5 was successfully engineered for superior conversion of non-natural aromatic substrates following a structure-guided, rational approach.[12,17] Thereupon, we expanded into the chal-



Scheme 1. Asymmetric synthesis of (*R*)-pantolactone ((*R*)-**3**) from hydroxypivalaldehyde (**1a**) by the (*R*)-HNL route[2,3,10] (gray background). Other methods comprise the chemical resolution or enantioselective hydrolysis of (*RS*)-pantolactone [(*RS*)-**3**] or (*RS*)-pantolactone esters [(*RS*)-**4**],[4,5] asymmetric hydrogenation of ketopantolactone (**5**),[6,7] Sharpless asymmetric dihydroxylation of **6**[8] and the proposed enantioselective hydrolysis of the corresponding α-hydroxynitrile using nitrilases,[9] respectively, as key step for the introduction of chirality. (N.e.p. = no example provided)

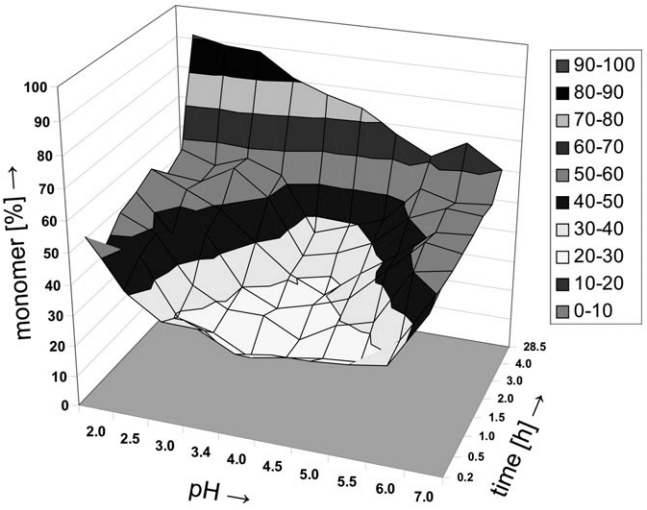


Figure 1. Investigation of the amount of monomer available after incubation in buffered aqueous solution at different pH values. A pH $\leq$2.5 is advantageous for efficient substrate monomerization prior to the stereoselective, enzymatic hydrocyanation of **1a** to (*R*)-**2**. At neutral or basic pH values, the desired cyanohydrin (*R*)-**2** would racemize again and non-selective chemical addition of HCN to the aldehyde would be favored [180 mg gently liquefied **1b** were dissolved in 5 mL of a 30 mM potassium phosphate buffer (pH 2.0–7.0)]

lenging group of sterically hindered aliphatic substrates. However, as the modeled *Pa*HNL5/(*R*)-**2** and (*S*)-**2** complexes did not display any clearly unfavorable interactions, all hydrophobic residues of the substrate binding pocket, namely F72, V113, V317, V329, L331, L343 and V360, were considered as targets for saturation mutagenesis combined with screening for cyanohydrin synthesis.

For saturation library generation, *Pichia pastoris* was transformed with linear expression cassettes made by overlap extension PCR employing the *Pa*HNL5 gene ligated into the plasmid pGAPZA as a template. Therefore, the basic method established by Liu et al.[18] was adapted for site-saturation mutagenesis. For each of the 7 targeted sites, $\geq$200 transformants were assayed for improved conversion of **1a**. Following the first law of directed evolution 'you get what you screen for'[19] – whose consequences we had to encounter quite recently when engineering *Pa*HNL5 for improved (*R*)-2-chloromandelonitrile synthesis[18] – we searched for a method which for the first time provided the possibility to screen specifically for the cyanohydrin synthesis reaction, namely the conversion of **1a** to **2** at pH 2.4 in a 96-well format. For detection, a colorimetric high-throughput screening method from Bornscheuer and co-workers[20] was

Table 1. Comparison of V317A and V317G with the unmodified *Pa*HNL5 for the hydrocyanation of **1a**.

Entry	Enzyme	Reaction time (conv. [%]/*ee* [%])[a]			TOF[b] [s^{-1}]
		2.5 h	5.0 h	20.5 h	
1	Blank	5.6/*rac*[c]	8.4/*rac*[c]	37.8/*rac*[c]	–
2	*Pa*HNL5	14.9/22.2	n.d./n.d.	64.9/22.2	0.83 ± 0.02
3	V317G	15.1/21.8	24.4/25.9	53.5/10.6	n.d.[d]
4	V317A	42.4/87.1	59.2/86.7	86.5/81.8	11.08 ± 0.19

[a] *Reaction conditions:* 1.17 mmol of **1a** and 1 mg of enzyme after 2-L shake flask cultivation (0.85 mg enzyme $mmol^{-1}$ substrate), 4 °C, pH 2.4.
[b] *Reaction conditions:* 1.17 mmol of substrate and necessary amount of purified enzyme after production in a 5-L bioreactor so that conversion of **1a** was in a linear range during the first 30 min.
[c] *rac*: racemic.
[d] n.d.: not determined since *ee* was not improved.

adapted for lyases and an aqueous system. All variants were screened for improved conversion (see Experimental Section). Subsequently, a 96-well plate-based GC method was used to determine the enantiomeric excess of preselected variants. Rescreening of the best clones led to the identification of one significantly improved variant with the amino acid exchange V317A (GTA→GCT).

This was surprising, because mutation V317G caused negative effects for the synthesis of another bulky compound [i.e., (*R*)-2-chloromandelonitrile].[12] However, mutation V317A seemed to be beneficial for sterically hindered aliphatic aldehydes. Employing 1 mg of protein after shake-flask cultivation resulted in elevated conversion and almost four-fold improved enantiopurity (Table 1). At the same time, the introduction of alanine at position 317 did not negatively influence the enzyme's stability at low pH. This is especially important for (*R*)-**2** production. In contrast to previous results with the wild-type enzyme,[11] the mutant V317A even retained stability at the very low pH 2.0 where the unselective chemical background reaction is repressed in a large part (Figure 2).

Reconsidering the modeled enzyme-cyanohydrin complexes in detail, we recognized three possible interactions between valine 317 and a methyl group of the (*S*)-enantiomer but only one hydrophobic interaction with the (*R*)-enantiomer (Figure 3). Thus, exchanging the isopropyl moiety of V317 for a methyl group most probably accounts for a loss of stabilizing interactions with (*S*)-**2**. Wanting to confirm the impact of this qualitative structural analysis, we used the program Amber to elucidate differences between the native *Pa*HNL5 and mutein V317A. Complexes of the native enzyme resulted in comparable energies for the binding of both enantiomers, whereas mutein V317A provided a more favorable binding site for (*R*)-**2** with a (potential) energy difference of 2 kcal

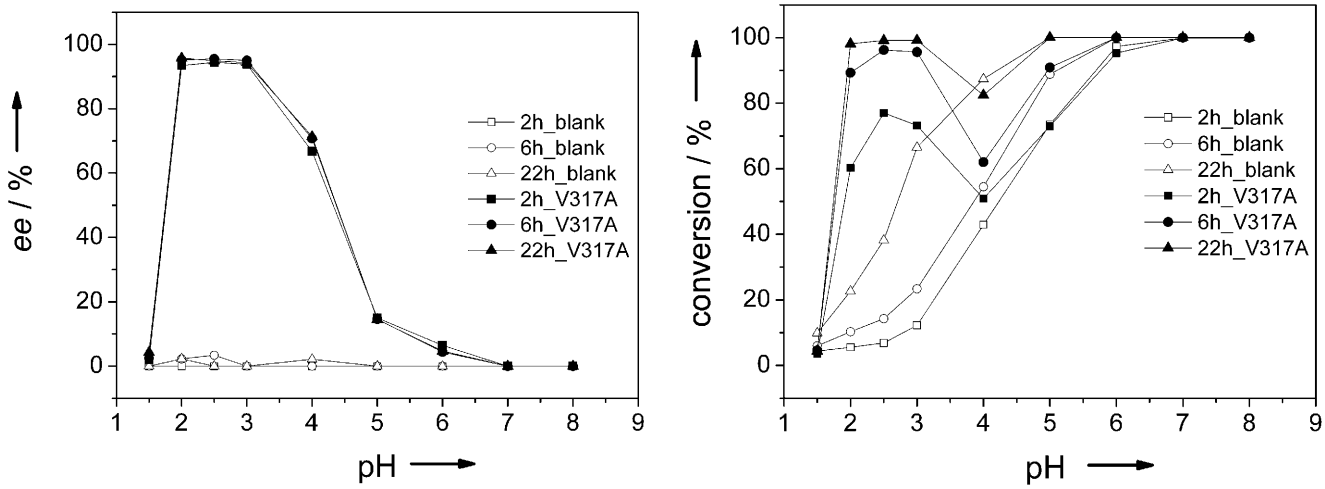


Figure 2. Testing different pH values for the synthesis of (*R*)-**2** employing *Pa*HNL5 V317A in buffered aqueous solution at pH 1.5 to 8.0. The *Pa*HNL5-variant V317A retained stability even at pH 2.0 where the unselective chemical background reaction is repressed in a large part. *Reaction conditions:* 1.17 mmol of **1a**, 170 U of enzyme *Pa*HNL5 V317A (~1.7 mg $mmol^{-1}$ substrate) and 100 μL of anhydrous HCN were diluted in 5 mL of 2 M potassium phosphate buffer at pH 1.5–8.0.

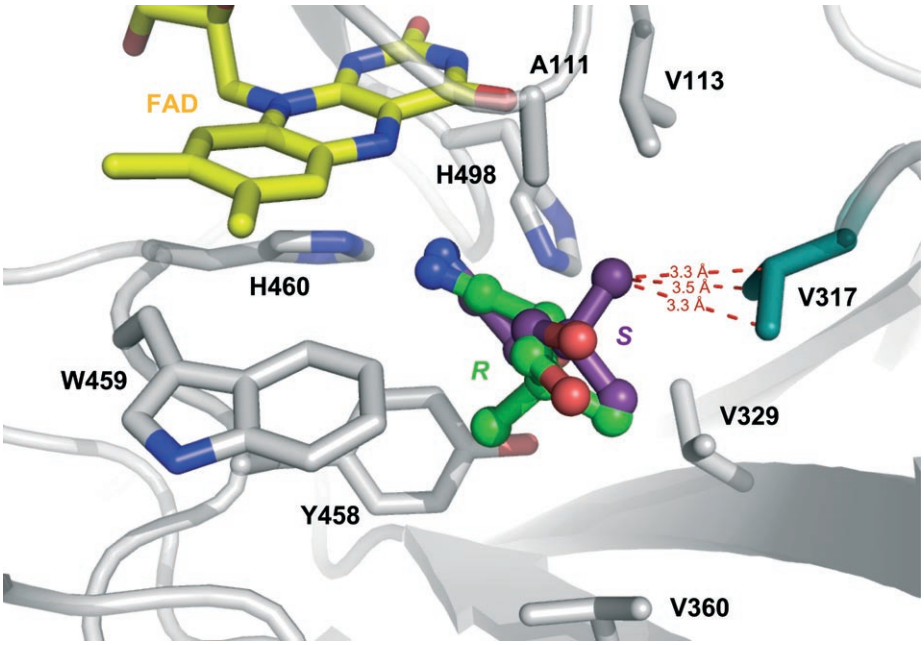


Figure 3. Superposition of the modelled complexes with the substituents (*R*)-**2** (green) and (*S*)-**2** (purple) at the active site of *Pa*HNL5 provides an insight into the substrate/product binding pocket at the active site and indicates possible interactions of V317 with a methyl group of (*S*)-**2**. Amino acid side chains are depicted in gray, the FAD cofactor in yellow and N and O atoms in blue and red, respectively. V317 is shown in cyan. The image was created using the software PyMol v0.99 (http://www.pymol.org).

mol^{-1} compared to (*S*)-**2**. Calculations thus supported our interpretation, but again did not exclude additional hydrophobic residues to be possible engineering targets. In contrast to our previous studies with other substrates,[12,17] not the removal of steric hindrance but the reduction of hydrophobic interactions with the wrong enantiomer resulted in increased stereoselectivity. As in the case of (*R*)-2-chloromandelonitrile synthesis,[12] the variant V317G, providing even more space at this position, resulted in decreased enantiomeric excess and conversion (Table 1).

To explore turnover rates, purified enzyme produced with a 5-L bioreactor was employed. Compared to the unmodified *Pa*HNL5, mutein V317A accelerated the conversion of **1a** approximately 13-fold. In this case, modeling results did not provide further insight. Then, we examined V317A's performance in different reaction systems (Table 2). Using Celite® as enzyme support in a microaqueous, organic solvent system also resulted in high enantiopurities but compared to a pure aqueous reaction solution, conversion was low. The buffered aqueous system also surpassed a biphasic (H_2O/TBME) reaction system. Finally, the ability

Table 2. Synthesis of (*R*)-**2** employing *Pa*HNL5 V317A in different reaction systems.

Entry	Conditions	pH	Enzyme amount [U/mmol **1a**]	Reaction time (conv. [%]/*ee* [%])		
				2 h	6 h	22 h
1[a]	H_2O	2.5	140	77.0/94.4	96.2/95.5	99.1/94.9
2[a]		2.5	310	87.9/94.5	100/95.0	100/94.9
3[a]		2.5	1340	99.8/95.5	100/97.0	100/97.5
					4 h	24 h
4[b]	H_2O/TBME	3.4	100		16/60.8	29/58.2
5[b]	H_2O	3.4	100		31/86.6	54/87.5
				2 h	6 h	20 h
6[c]	Celite®/DIPE	3.4	100	22/94.0	26/95.5	37/96.9

[a] Buffered aqueous system.
[b] Comparison of biphasic system (H_2O/TBME) with aqueous system.
[c] V317A immobilized on Celite®: microaqueous, organic (DIPE) system.

for scale-up of the aqueous system was investigated. 1.2 g of **1a** were converted employing only 218 U V317A mmol^{-1} **1a** in a 100-mL reactor at 4 °C. After 20 h the reaction was completed and subsequent work-up supplied the crude product with 88% yield and 96% *ee*.

Furthermore, variant V317A proved to be beneficial for another bulky aliphatic substrate, namely pivalaldehyde, resulting in quantitative conversion with an approximately 15% elevated enantiopurity (97.3% *ee* compared to 82.5% *ee* for *Pa*HNL5, employing 1.1 mg protein per mmol of pivalaldehyde).

In order to boost the biocatalytic system from another side, in parallel, a synthetic *Pahnl5 V317A* gene was designed and expressed with the help of the improved promoter variant $P_{AOX1\Delta1}$[21,22] and the co-expression of PDI.[23] Thus, a further 4-fold improved enzyme activity per volume of yeast culture was obtained.

Stepwise redesign of *Pa*HNL5 gene and enzyme thus led to elevated amounts of today's most efficient biocatalyst for the vitamin B_5 synthesis route and seemingly, for the conversion of sterically hindered aliphatic aldehydes in general. Screening for cyanohydrin synthesis at low pH, resulted in a highly stable biocatalyst for the synthesis of the chiral key intermediate for (*R*)-pantolactone synthesis with *ee* > 97% *via* the efficient HNL route. Furthermore, the aqueous system was shown to be superior to alternatives employing organic solvents.

Experimental Section

Site-saturation mutagenesis libraries (F72X, V113X, V317X, V329X, L331X, L343X, V360X) were created by overlap extension PCR (oePCR) using template plasmid pGAPZA *Pa*HNL5[24] and partly complementary mutagenic primers containing a degenerated codon [N N (G/T)] at the desired site. The resulting oePCR products were purified and directly employed for *Pichia pastoris* X33 transformation. Zeocin was used as selection marker.

The resultant *P. pastoris* X33 transformants were cultivated in 96-well deep well plates and screened for improved conversion of **1a**, also on the 96-well deep well plate scale. Therefore, 400 μL of the culture supernatant were mixed with 150 μL of a 3 M citrate phosphate buffer (pH 2.4) and 200 μL of a **1a**-stock solution [1.2 mL of **1a** were added in 46.8 mL of a 3 M citrate phosphate buffer (pH 2.4)]. 22 μL of a 12 M NaCN solution were added to start the reaction. To prevent HCN evaporation, deep well plates were finally covered with a SILVERseal™ sealer aluminium foil (Greiner Bio-One GmbH, Germany). To agitate the reaction solution, a magnetic stirrer for 96-well deep-well plates (Alligator Tumble Stirrer from V&P Scientific) was used together with one PTFE encapsulated stir stick (VP 734–2) per well. After one hour, the reaction was stopped by removing the silver seal foil and adding a 50% (v/v) solution of H_2SO_4 in H_2O. The addition of the acidic solution shifted the pH to < 1.0 removing the remaining HCN and preventing a continuing enzymatic reaction. All steps including the possible evaporation of HCN were carried out under a well ventilated hood.

In order to screen for conversion, a fluorimetric assay was carried out in 96-well PP-microplates. 4-Hydrazino-7-nitrobenzofurazane (NBDH) was employed to detect the remaining amount of aldehyde in the reaction solution. The increase in the strongly fluorescent hydrazone was tracked and the slope of this chemical reaction was evaluated. It could be directly correlated to the amount of aldehyde **1a** remaining in the reaction solution after a distinct reaction time.

Subsequently, preselected variants were rescreened employing a 96-well plate based GC method in order to determine enantiomeric excess. After the hydrocyanation reaction in 96-well deep well plates, **1a** and the corresponding cyanohydrins (*R*)-**2** and (*S*)-**2** were extracted with *tert*-butyl methyl ether (TBME). Aliquots of the organic phase were transferred to a new 96-well PP-microplate (V-bottom, clear) and after derivatization with acetic anhydride in the presence of pyridine and dichloromethane the enantiomeric purities were analyzed using a Hewlett–Packard 6890 instrument equipped with an FID and a Chirasil-DEX CB column.

Biotransformations for the characterization of PaHNL5 V317A were performed on a 5-mL reaction scale. Varying amounts of enzyme were diluted to 5 mL using a citrate phosphate or potassium phosphate buffer at different pH values (pH 1.5–8.0). Then, for example, 120 mg of **1a** and, for example, 100 μL of anhydrous HCN were added. Reactions were performed at 4 °C and 600 rpm applying the magnetic stirrer Variomag Electronicrührer Poly 15. After the scheduled time, samples were taken, extracted with TBME and derivatized for chiral GC analysis.

Further experimental details are available in the Supporting Information.

Acknowledgements

This research was supported by DSM, the FFG, the Province of Styria, the SFG and the City of Graz. We want to thank Prof. Manfred Reetz for providing valuable information about robotized high-throughput screening instruments. We acknowledge H. Mandl and B. Krenn for excellent technical support.

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