Heterogeneous & Homogeneous & Bio- & Nano-

CHEMCATCHEM

CATALYSIS

Accepted Article

Title: Efficient Stereoselective Synthesis of Structurally Diverse γ- and δ-Lactones Using an Engineered Carbonyl Reductase

Authors: Meng Chen, Xiao-Yan Zhang, Chen-Guang Xing, Chao Zhang, Yu-Cong Zheng, Jiang Pan, Jian-He Xu, and Yunpeng Bai

To be cited as: *ChemCatChem* 10.1002/cctc.201900382

Link to VoR: http://dx.doi.org/10.1002/cctc.201900382

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chemcatchem.org

10.1002/cctc.201900382

WILEY-VCH

COMMUNICATION

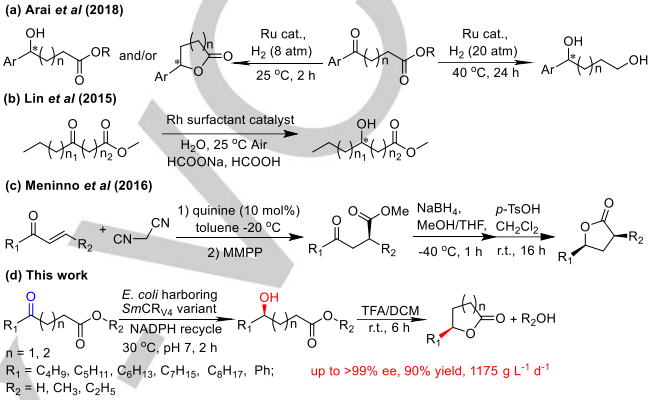
Efficient Stereoselective Synthesis of Structurally Diverse γ- and δ-Lactones Using an Engineered Carbonyl Reductase

Meng Chen,[a] Xiao-Yan Zhang,[a] Chen-Guang Xing,[b] Chao Zhang,[a] Yu-Cong Zheng,[a] Jiang Pan,[a] Jian-He Xu*[a] and Yun-Peng Bai*[a]

Abstract: Structurally diverse γ- and δ-lactones were efficiently synthesized stereoselectively using an engineered carbonyl reductase from *Serratia marcescens* (*Sm*CR$_{V4}$). *Sm*CR$_{V4}$ exhibited improved activity (up to 500-fold) and thermostability toward 14 γ-/δ-keto acids and esters, compared with the wild-type enzyme, with 110-fold enhancement in catalytic efficiency (k_{cat}/K_m) toward methyl 4-oxodecanoate. The preparative synthesis of alkyl and aromatic γ- and δ-lactones with 95%–99% ee and 78%-90% yields was demonstrated. The highest space-time yield, 1175 g L^{-1} d^{-1}, was achieved for (*R*)-γ-decalactone.

Chiral γ- and δ-hydroxy acid derivatives are of importance in synthetic chemistry as key building blocks for constructing natural products and pharmaceutically active compounds.[1] The asymmetric reduction of γ- and δ-keto acids and esters is regarded as an atom-economic method to prepare such active compounds. However, the position of the γ-/δ-carbonyls in the carbon chain is distant from the terminal carboxyl group, which decreases the reactivity, and makes it difficult to accurately control the stereoconfiguration of the hydroxy group.

The use of ruthenium catalyts to catalyze the asymmetric hydrogenation of keto esters to chiral hydroxy esters is well-established.[2] Yang and co-workers have studied the use of iridium complexes for the synthesis of chiral diols.[3] Recently, Arai *et al.* have developed a flexible method to prepare either chiral lactones or diols depending on the reaction conditions (Scheme 1a).[4] Zhao *et al.* have demonstrated the potential of this method for keto amide hydrogenation.[5a] Lin *et al.* have developed a surfactant-type rhodium complex to catalyze the asymmetric reduction of long-chain aliphatic keto esters (Scheme 1b).[5b] In addition to using the organometallic catalyst, Meninno and co-workers have developed a quinine-catalyzed route for preparing chiral keto esters (Scheme 1c).[6] Although noble metals and organocatalysts are efficient for the stereoselective synthesis of hydroxy esters, either high pressure of H_2 (normally 8–70 atm)[3,4] or low temperature (–20 °C)[6] are required.



Scheme 1 Stereoselective synthesis of optically pure γ- and δ-lactones.

In particular, an environmentally friendly preparation process is imperative for the synthesis of chiral γ- and δ-lactones, which are used for a variety of artificial flavors in the food and cosmetic industries.[7] To date, the whole-cell biotransformation of γ- and δ-keto acids has been studied using wild-type yeasts, such as *Saccharomyces cerevisiae* and *Yarrowia lipolytica*.[8] However, in this method a large amount of fermentation by-products are produced, and the volumetric productivity is low because of the toxicity of the lactones to the growth of yeast cells.[9] To solve these problems, it is necessary to find and engineer the functional enzymes responsible for the highly enantioselective reduction of γ- and δ-keto acids in yeast. Unfortunately, although many carbonyl reductases have been found in *Saccharomyces cerevisiae*[10a], these enzymes are still unknown up to now.[10]

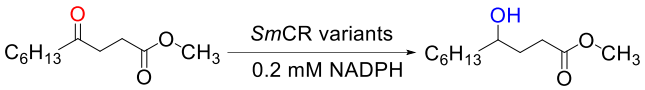
Although several enzymatic processes have been exploited for the synthesis of chiral γ- and δ-lactones,[11] the stereoselectivity and productivity of these processes are not yet satisfactory. Lavandera and co-workers[1a] made a breakthrough in enantioselective synthesis of short-chain γ- or δ-alkyl and aromatic hydroxy esters and lactones. Recently, the first carbonyl reductase, which could convert linear long-chain γ/δ-keto acids into the corresponding γ-/δ-lactones with high enantioselectivity up to >99% ee, such as (*R*)-γ-/δ-decalactones (**3c**, **3d**), was discovered from *Serratia marcescens* (*Sm*CR).[12] *Sm*CR displayed catalytic activity toward long-chain γ-/δ-keto acids and esters, in contrast to the majority of reported ketoreductases, which are mainly active toward the α- or β-carbonyls in short-chain alkyl and aromatic keto acids.[13] However, the activity of *Sm*CR was too low for practical application. In this report we describe the engineering of *Sm*CR for improved catalytic performance. The effectiveness of this enzyme was demonstrated in the synthesis of structurally diverse γ- and δ-

[a] M. Chen, Dr. X.-Y. Zhang, C. Zhang, Y.-C. Zheng, Dr. J. Pan, Prof. Dr. J.-H. Xu, Assoc. Prof. Dr. Y.-P. Bai
State Key Laboratory of Bioreactor Engineering,
East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, P.R. China.
E-mail: jianhexu@ecust.edu.cn; ybai@ecust.edu.cn.
[b] C.-G. Xing
Xiamen Oamic Biotech. Co. Ltd, 36 Longmen Road, Xiamen 361026, P.R. China
Supporting information for this article is given via a link at the end of the document.

10.1002/cctc.201900382

COMMUNICATION

Table 1 Kinetic parameters of purified *Sm*CR$_{wt}$ and engineered proteins toward substrate methyl 4-oxodecanoate



Entry	Evolutionary strategy	Enzyme	Mutation	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1}mM^{-1})	Fold[a]
1	starting enzyme	*Sm*CR$_{wt}$	-	5.4 ± 0.3	2.38 ± 0.34	2.3 ± 0.3	1.0
2	*ep*PCR	*Sm*CR$_{V1}$	R123C	7.5 ± 0.5	1.59 ± 0.34	4.5 ± 1.0	2.0
3	*ep*PCR	*Sm*CR$_{V2}$	R123C/L209P	88.0 ± 5.3	0.87 ± 0.15	101 ± 19	44.8
4	ISM	*Sm*CR$_{V3}$	R123C/L209P/F183Y	153 ± 5.0	1.35 ± 0.12	114 ± 11	62.3
5	ISM	*Sm*CR$_{V4}$	R123C/L209P/F183Y/V61K	197 ± 14.0	0.79 ± 0.16	249 ± 53	110

[a] Fold change enhancement in k_{cat}/K_m compared to the wild-type enzyme

lactones with space-time yields up to 1175 g L^{-1} d^{-1} and *ee*'s up to >99% (Scheme 1d).

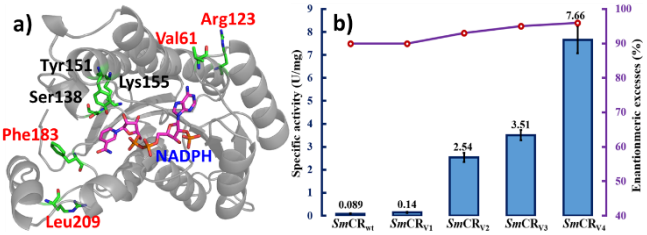


Figure 1. Directed evolution of *Sm*CR$_{wt}$. (**a**) Homology model of *Sm*CR$_{wt}$ binding with NADPH in the active site. Ser138, Tyr151 and Lys155 are catalytic triad. (**b**) The specific activity and stereoselectivity of *Sm*CR variants toward **1g**.

Initially, *Sm*CR displayed a specific activity of 3.3 and 89.0 mU/mg$_{protein}$ toward 4-oxodecanoic acid (**1c**) and methyl 4-oxodecanoate (**1g**) with >99% and 90% *ee*, respectively. To improve the activity, we performed directed evolution of the wild-type enzyme, with **1g** as a model substrate. The homology model of *Sm*CR$_{wt}$ was constructed based on the known structure of a β-ketoacyl-acyl carrier protein reductase (PDB ID: 1Q7B) in complex with NADPH form *Escherichia coli* using the Modeller program (Figure 1a). The template protein possesses 2.05 Å resolution with 87.3% sequence identity of *Sm*CR$_{wt}$. First, error-prone polymerase chain reaction (*ep*PCR) was used to construct a library of variants covering random changes in the whole nucleotide sequence of the gene for the enzyme.[14] After first-round mutagenesis and screening, the variant *Sm*CR$_{V1}$ R123C with 1.6-fold increased activity was identified (Figure 1b and Table S1). The catalytic efficiency (k_{cat}/K_m) was also improved by 2-fold (Table 1). Using *Sm*CR$_{V1}$ R123C as the parent, we performed an additional round of directed evolution, and, out of 6000 clones, obtained the variant *Sm*CR$_{V2}$ R123C/L209P with a dramatic 28.4-fold increase in the specific activity (Figure 1b and Table S2). Compared with the R123C mutation which occurred at a distant helix in the homology model of *Sm*CR$_{wt}$, the model suggests that mutation at residue 209 is near to the active site, which is essential for increased activity (Figure 1a). Thus, further mutations at this position were investigated by site-saturation mutagenesis. The activity assays for the mutants showed that proline was indeed the best residue (Figure S1). Due to this mutation, k_{cat}/K_m was improved dramatically by 44.8-fold, which can be attributed to the smaller K_m and larger k_{cat} (Table 1). Notably, the stereoselectivity of *Sm*CR$_{V2}$ was also improved as the ee value slightly increased from 91% to 93%.

Subsequently, the active pocket of *Sm*CR$_{V2}$ was finely tuned to further enhance the catalytic efficiency by iterative saturation mutagenesis.[15] Using the homologous model, we targeted 19 potential residues lining the substrate binding pocket of *Sm*CR$_{V2}$ and screened the library constructed by the combinatorial saturation mutagenesis at these sites (Figure S3). This endeavor led to the discovery of the important residues F183 and V61. The introduction of the mutation F183Y yielded *Sm*CR$_{V3}$ with an activity of 3.51 ± 0.22 U/mg$_{protein}$, affording methyl (*R*)-4-hydroxydecanoate (**2g)** with 95% ee (Figure 1b and Table S3). The further incorporation of V61K into *Sm*CR$_{V3}$ led to the mutant *Sm*CR$_{V4}$, which showed the highest specific activity of all the engineered enzymes (7.66 ± 0.92 U/mg$_{protein}$), an 86-fold increase compared with *Sm*CR$_{wt}$ (Table S3). Reaction kinetics analysis was performed for the wild-type enzyme and the four variants (Table 1). Clearly, directed evolution increased the binding affinity of the substrate with the enzyme as the K_m of *Sm*CR$_{V4}$ toward **1g** was decreased to 0.79 ± 0.16 mM compared with 2.38 ± 0.34 mM for *Sm*CR$_{wt}$. In particular, the turnover number k_{cat} was gradually increased by the iterative accumulation of beneficial mutations, with the k_{cat} value for *Sm*CR$_{V4}$ reaching 197 ± 14 min^{-1}, which was 36.5-fold higher than that of *Sm*CR$_{wt}$ (5.4 ± 0.3 min^{-1}). The total catalytic efficiency (k_{cat}/K_m) of *Sm*CR$_{V4}$ was increased by 110-fold compared with *Sm*CR$_{wt}$. Notably, the stereoselectivity of *Sm*CR$_{V4}$ was also improved from 91% for the wild-type enzyme to 97% (Figure 1b).

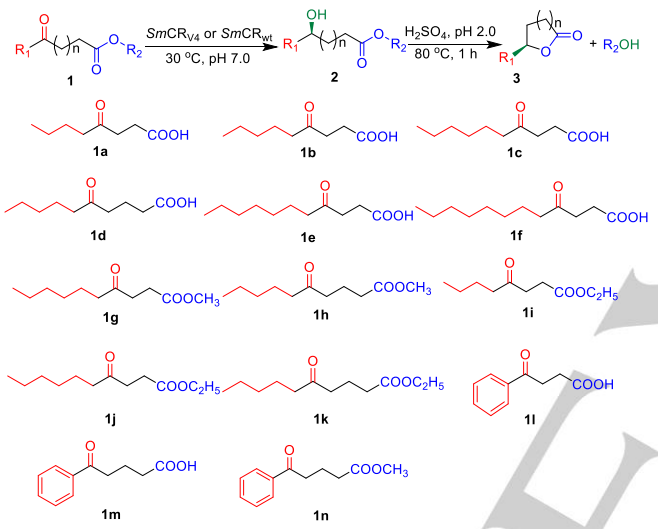
*Sm*CR$_{V4}$ could be heterologously expressed in a soluble form in *Escherichia coli* cells and was purified for characterization (Figure S4). *Sm*CR$_{V4}$ displayed the highest activity at 45 °C and pH 7.0 in a phosphate buffer (Figure S5 and S6). The enzyme activity was unchanged, or enhanced slightly, in most frequently used organic solvents except THF, acetonitrile, and isopropanol (Figure S7). *Sm*CR$_{V4}$ showed enhanced thermostability with half-lives of 124 and 72 h at 30 and 40 °C (Figure S8 and Table S4), respectively, which were higher than 83 h (30 °C) and 52 (40 °C) h for *Sm*CR$_{wt}$ (Figure S9 and Table S5). To investigate the substrate scope of *Sm*CR$_{V4}$, the specific activity and stereoselectivity of the purified enzyme toward a panel of structurally diverse aliphatic and aromatic keto acids and esters were measured (Table 2). *Sm*CR$_{wt}$ showed only marginal activity toward the majority of the substrates tested (**1a–1n**). The activity of *Sm*CR$_{V4}$ toward all the substrates was significantly enhanced by directed evolution with improvements from 3.8-fold (**1l**) to 500-

10.1002/cctc.201900382

COMMUNICATION

fold (**1f**). Specifically, *Sm*CR$_{V4}$ showed similar activity toward linear medium- and long-chain γ/δ-keto acids (8–12 carbons, **1a**–**1f**). In particular, the stereoselectivity toward these substrates was high, with ee values for the corresponding (*R*)-hydroxy acids >99% for **1c**, **1e**, and **1f**. *Sm*CR$_{V4}$ was more active toward γ-/δ-keto esters than acids. For example, the activity toward **1g** (7.66 U/mg$_{protein}$) and methyl 5-oxodecanoate (**1h**, 2.51 U/mg$_{protein}$) was higher than toward **1c** and **1d**, respectively. The position of the carbonyl group in the aliphatic keto acids and esters also affected the activity of *Sm*CR$_{V4}$. Normally, *Sm*CR$_{V4}$ showed higher activity toward γ- (**1c**, **1g**, and **1j**) compared with δ-keto acids and esters (**1d**, **1h**, and **1k**). In addition to the aliphatic keto acids, *Sm*CR$_{V4}$ also showed measurable activity and high stereoselectivity toward the aromatic γ/δ-keto acids, **1l** and **1m**, which are two important chiral building blocks for the synthesis of various pharmaceuticals.

Table 2 Substrate scope of *Sm*CR$_{wt}$ and *Sm*CR$_{V4}$



Entry	Substrate	Specific activity (mU/mg)			ee$_p$ (%)	
		*Sm*CR$_{wt}$	*Sm*CR$_{V4}$	Fold[a]	*Sm*CR$_{wt}$	*Sm*CR$_{V4}$
1	**1a**	3.7	210	56.8	61 (*R*)	96 (*R*)
2	**1b**	17.0	86.0	5.1	97 (*R*)	99 (*R*)
3	**1c**	3.3	640	194	>99 (*R*)	>99 (*R*)
4	**1d**	2.1	330	157	95[c] (*R*)	95[c] (*R*)
5	**1e**	2.2	410	186	>99 (*R*)	>99 (*R*)
6	**1f**	0.8	400	500	>99 (*R*)	>99 (*R*)
7	**1g**	89.0	7660	86.1	91 (*R*)	97 (*R*)
8	**1h**	51.0	2510	49.2	31[c] (*S*)	70[c] (*S*)
9	**1i**	12.0	1510	126	n.d.[b]	7 (*R*)
10	**1j**	69.0	3380	49	75 (*R*)	80 (*R*)
11	**1k**	51.0	2510	49.2	40[c] (*S*)	71[c] (*S*)
12	**1l**	5.3	20	3.8	n.d.	98 (*S*)
13	**1m**	1.7	130	76.5	57 (*S*)	97 (*S*)
14	**1n**	9.4	510	54.3	n.d.	45 (*S*)

[a] Fold change improvement in the activity of *Sm*CR$_{V4}$ over *Sm*CR$_{wt}$. [b] n.d. = not detected. [c] These numbers are estimated values.

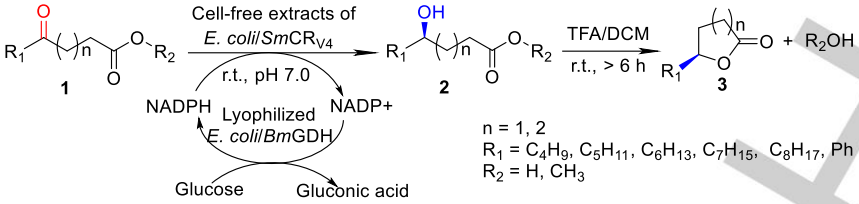
With the best variant *Sm*CR$_{V4}$ in hand, we investigated the synthesis of a series of valuable optically pure γ-/δ-lactones with diverse structures at a preparative scale. In general, the substrates were dissolved in a sodium phosphate buffer (100 mM, pH 7.0) containing 0.2 mM NADP$^+$, 5% v/v DMSO, and the necessary amount of *E. coli* cells expressing *Sm*CR$_{V4}$. The reaction was performed at 30 °C with the addition of glucose and cells containing glucose dehydrogenase from *Bacillus megaterium* (*Bm*GDH) to recycle of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH). Under these conditions, the time-course for the asymmetric reduction of **1c** and **1d** by *Sm*CR$_{wt}$ and *Sm*CR$_{V4}$ was explored (Figure S10). Ten mM **1c** was completely converted in 3 h by the variant, while only 10% was converted by the wild-type enzyme. For **1d**, 10 mM substrate was completely converted by *Sm*CR$_{V4}$ after 10 h, which was superior to the conversion using the wild-type enzyme. Next, we prepared eight optically pure γ-/δ-lactones (Table 3).[16] *Sm*CR$_{V4}$ displayed a powerful ability for the synthesis of (*R*)-γ-decalactone (**3c**). After work-up, using 50 mM **1c** as the substrate gave 99% conversion after 2 h, yielding **3c** (82%) with >99% ee and a specific rotation of $[\alpha]_D^{30}$+45.0° (Table S7). The space-time yield (STY) reached 83 g L^{-1}d^{-1}, which was nearly 10-fold greater than the previously reported value (8.8 g L^{-1}d^{-1}). The STY was increased up to 1175 g L^{-1}d^{-1} for **3c**, the highest value reported to date, when 1.0 M **1g** was 98% converted after 3 h, yielding **3c** (88%) with an ee of 97%. *Sm*CR$_{V4}$ was also very effective in the synthesis of (*R*)-δ-decalactone (**3d**). After 3 h, 99% of 50 mM **1d** was converted, affording an 83% isolated yield of **3d** with the same ee value of 95% (*R*) as previously reported; however, the catalyst consumption was greatly reduced from 150 to 20 g L^{-1} and the STY was increased to 56 g L^{-1} d^{-1}. (*R*)-γ-undecalactone (**3e**) and (*R*)-γ-dodecalactone (**3f**) are difficult to synthesize because of the long carbon chains (11 and 12 carbons, respectively) that decrease the reactivity of aliphatic keto acids. Herein, both **3e** and **3f** were obtained with >99% ee in yields of 87% and 83%, respectively. The reactions were completed with 99% and 98% conversion with STY values of 24 and 12 g L^{-1} d^{-1}, respectively. Additionally, (*R*)-γ-lactones with medium chain lengths, such as (*R*)-γ-octalactone (**3a**) and (*R*)-γ-nonalactone (**3b**), were obtained with good efficiency. Furthermore, *Sm*CR$_{V4}$ was effective in the asymmetric synthesis of **3l** and **3m**, which are used for preparing many pharmaceuticals. Previously, **3l** and **3m** have been prepared using Ru complexes at high pressures of H_2 (8–70 atm).[3,4] Herein, *Sm*CR$_{V4}$ also showed high stereoselectivity for aromatic γ- and δ-keto esters and efficiency comparable with the Ru-catalyzed asymmetric hydrogenation. In 10 or 6 h, 50 mM substrates were >98% converted giving **3l** and **3m** in 85% and 78% yield, respectively. In particular, the reactions proceeded at room temperature and normal pressure.

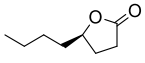
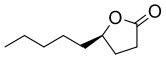
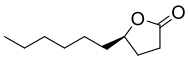
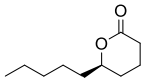
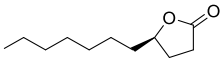
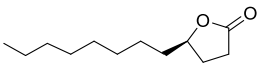
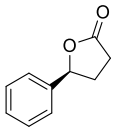
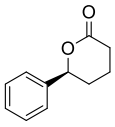
In conclusion, the efficient enantioselective synthesis of diverse aliphatic and aromatic γ- and δ-lactones using the engineered carbonyl reductase *Sm*CR$_{V4}$ was achieved. The variant was identified using directed evolution and showed enhanced activity, stability, and stereoselectivity toward a panel of γ-/δ-keto acids and esters, compared with the wild-type enzyme and other variants. Under the optimized conditions, the γ-/δ-keto acids and esters were efficiently reduced to the corresponding γ- and δ-hydroxy acids and esters, followed by simple intramolecular cyclization yielding γ- and δ-lactones with *ee* values up to >99%. The volumetric productivity for the preparation of the (*R*)-γ/δ-decalactones was the highest reported to date, and lactones

10.1002/cctc.201900382

COMMUNICATION

Table 3 Preparative synthesis of optically pure γ- and δ-lactones



Entry	Product	Substrate (mM)	Catalyst (g L^{-1})	Time (h)	Conv. (%)	Isolated yield (%)	STY (g L^{-1} d^{-1})	ee (%)	Ref.
1	 (**3a**)	50	20[b]	4	98	82	34	96 (*R*)	this work
2	 (**3b**)	50	20[b]	12	>99	90	13	99 (*R*)	this work
3		50	150[a]	16	95	73	8.8	>99 (*R*)	12
4	 (**3c**)	50	20[b]	2	>99	82	83	>99 (*R*)	this work
5		1000	75[c]	3	98	88	1175	97 (*R*)	this work
6	 (**3d**)	50	150[a]	16	92	75	8.8	95 (*R*)	12
7		50	20[b]	3	>99	83	56	95 (*R*)	this work
8	 (**3e**)	50	20[b]	8	>99	87	24	>99 (*R*)	this work
9	 (**3f**)	50	20[b]	16	98	83	12	>99 (*R*)	this work
10	 (**3l**)	50	20[b]	10	>99	85	18	98 (*S*)	this work
11	 (**3m**)	50	20[b]	6	98	78	25	97 (*S*)	this work

[a] Glucose (5.0 equiv), NADP$^+$ (0.5 mM), lyophilized *E. coli*/*Bm*GDH (10 g L^{-1}). [b] Glucose (1.5 equiv), NADP$^+$ (0.2 mM), lyophilized *E. coli*/*Bm*GDH (2 g L^{-1}). [c] Glucose (1.5 equiv), NADP$^+$ (0.2 mM), wet cells of *E. coli*/*Sm*CR$_{V4}$ (75 g L^{-1}), lyophilized *E. coli*/*Bm*GDH (10 g L^{-1}). [d] Space-time yield. [e] Determined by chiral GC.

substituted with alkyl chains of 11 and 12 carbons were also prepared for the first time using this enzyme-catalyzed route. This enzyme-catalyzed synthesis exhibited high efficiency and was environmentally friendly, thus making this a promising method for the industrial preparation of optically pure lactones for various purposes.

Experimental Section

General information: All chemicals were purchased from TCI (Japan), Aladdin and Shaoyuan (Shanghai, China) without further treatment unless otherwise indicated. NADPH and NADP$^+$ were purchased from Bontac Bioengineering (Shenzhen, China). Luria-Bertani media was used for the culture of *Escherichia coli* cells. A Shimadzu GC-2014 gas chromatography was used for GC analysis with a CP-Chirasil-Dex CB column (25 m × 0.25 mm × 0.39 mm, Varian) and a flame ionization detector (FID). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were all obtained in $CDCl_3$. High resolution mass spectra (HRMS) were obtained by electro spray ionization (ESI) and the errors between observed and theoretical monoisotopic molecular masses were less than 5 ppm. Column chromatography was carried out by using silica gel of the selected particle size of 200-300 mesh. n-Dodecane was used as internal standard. A Rudolph Research Analytical Autopol I automatic polarimeter was used for optical rotation measurement.

Directed evolution: The recombination plasmid containing *Sm*CR$_{wt}$ gene was used as the template for random mutagenesis. 0.15 mM $MnCl_2$ was used to obtain the desired level of mutagenesis rate (about 1 to 2 amino acid substitutions). The amplified PCR products were extracted, digested with *Eco*R I and *Hin*d III, and ligated into the *Eco*R I and *Hin*d III sites of pET-28a, then transformed into chemical competent *E. coli* BL21 (DE3) cells. The recombination plasmid containing *Sm*CR$_{V2}$ gene used was amplified by PCR with NNK codon degeneracy. The resulting PCR products were digested with *Dpn* I (20 U) at 37°C for 1 h and was next transformed into the chemical competent *E. coli* BL21 (DE3) cells and plated on LB agar plate containing 50 μg/ml kanamycin. The colonies were

picked with sterile toothpicks to inoculate in 200 mL LB media containing 50 μg/mL kanamycin in 96-well plates. The cultures were grown overnight at 37°C prior to inoculate 600 mL LB in new 96-well plates. The plates were incubated at 37°C for 3 h, and protein expression was induced with the addition of IPTG (0.1 mM) at 16°C for another 24 h. Cells were lysed by adding 200 mL buffer containing lysozyme (0.75 mg/mL) and DNase I (0.01 mg/mL) at 37°C for 2 h. The plates were centrifuged at 3420 × g for 20 min at 4°C. A volume of 50 mL sample from each well was transferred to a microtiter plate, and then the reduction reaction was initiated by adding a reaction mixture of 150 mL 100 mM phosphate buffer (pH 7.0), 0.2 mM NADPH and 4 mM substrate **1g**. The activity of the mutants was determined by measuring the absorbance change of NADPH at 340 nm for 10 min at 30°C using a microplate spectrophotometer (BioTek, USA). Mutants with higher acitivity were chosen for re-screening in an 96 deep-well plate, and the top hits were further grown on a 100 mL scale. The best mutants were selected for sequencing and purified by nickel affinity chromatography using N-terminal His-tagged for further characterization.

Enzyme assay and kinetic analysis: The reductase activity was assayed at 30°C by monitoring the decrease in the absorbance of NADPH at 340 nm on a UV spectrophotometer (Beckman DU730). The standard assay mixture (1 mL) consists of 970 μL of sodium phosphate buffer (100 mM, pH 7.0), 10 μL of methyl 4-oxodecanoate(200 mM), 10 μL NADPH (10 mM), and 10 μL of pure enzyme with an appropriate concentration. One unit of enzyme activity (U) is defined as the amount of enzyme that can catalyze the oxidation of 1 μmol of NADPH per minute under above conditions. The substrates were dissolved to different final concentrations with DMSO, and then the specific activity was measured by a UV spectrophotometer. The data were processed using Origin 9.0 based on the Michaelis–Menten equation.

Stereoconfiguration assay of products: The stereoconfigurations of different products were determined in a 500 μL reaction solution. In this solution, 10 mM substrate, 20 mM glucose, 0.2 mM $NADP^+$, 10 mg *Sm*CR_{wt} (or *Sm*CR_{V4}) and 2 mg *Bm*GDH lyophilized crude enzymes were mixed and stirred at 30°C, 1000 rpm. After 24 h, the reaction was terminated by 1.0 M sulfuric acid solution at pH 2.0, and then the reaction mixture was extracted with ethyl acetate after heating at 80℃ for 1 h. The enantioselectivity was determined by chiral GC analysis.

Preparation of structurally diverse γ- and δ-lactones： The reaction mixture (50 mL) was prepared by dissolving substrates (50-1000 mmol/L), glucose (1.5 equiv of substrate), lyophilized cells extract of *Sm*CR_{V4} (0.1-10 kU/L), lyophilized cells of GDH (0.2-20 kU), 5% DMSO (v/v), and $NADP^+$ (0.2 mM) in a sodium phosphate buffer (100 mM, pH 7.0). The pH value of the mixture was maintained at 7.0 using 1 M Na_2CO_3 and the reaction was carried out at 30℃ and 180 rpm for an appropriate time depending on the reaction conditions. Then, the reaction was terminated by acidification at pH 2.0 and the mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4. Removing solvents afforded the hydroxy esters, which was dissolved in CH_2Cl_2 (5 mL g^{-1} lactones) and cooled to 0℃, followed by the addition of trifluoroacetic acid (0.04 mL g^{-1} lactones). Stirring for 6 h at room temperature to complete the lactonization, and the reaction was worked up with aqueous sodium bicarbonate. The organic layer was washed with water, and then the organic phase was separated and dried over Na_2SO_4. The residue was purified by flash column chromatography elutedg with a mixture of ethyl acetate and petroleum ether (1:20) to provide pure γ- and δ-lactones. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were all obtained in $CDCl_3$ for pure γ- and δ-lactones. High resolution mass spectra (HRMS) were also obtained by electro spray ionization (ESI) for them.

Acknowledgements

This work was financially sponsored by the National Key Research and Development Program of China (2016YFA0204300), National Natural Science Foundation of China (Nos. 21536004, 21776085 & 21871085), Natural Science Foundation of Shanghai (18ZR1409900 & 18DZ1112703) and the Fundamental Research Funds for the Central Universities (22221818014, 222201714026 & WF1714026).

Keywords: Asymmetric reduction• directed evolution• chiral lactones• carbonyl reductase• biocatalysis

[1] a) A. Díaz-Rodríguez, W. Borzęcka, I. Lavandera, V. Gotor, *ACS Catal*. **2014**, *4*, 386-393; b) K. Aplander, O. Hidestål, K. Katebzadeh, U. M. Lindström, *Green Chem*. **2006**, *8*, 22-24; c) M. Seitz, O. Reiser, *Curr. Opin. Chem. Biol*. **2005**, *9*, 285-292; d) R. R. A. Kitson, A. Millemaggi, R. J. K. Taylor, *Angew. Chem. Int. Ed*. **2009**, *48*, 9426-9451; e) H. K. Grover, M. R. Emmett, M. A. Kerr, *Org. Lett*. **2013**, *15*, 4838-3841.

[2] a) R. Noyori, T. Ohkuma, M. Kitamura, H. Takaya, N. Sayo, H. Kumobayashi, S. Akutagawa, *J. Am. Chem. Soc.* **1987**, *109*, 5856-5658; b) R. Noyori, *Angew. Chem. Int. Ed.* **2002**, *41*, 2008-2022.

[3] X. H. Yang, J. H. Xie, W. P. Liu, Q. L. Zhou, *Angew. Chem. Int. Ed.* **2013**, *52*, 1-5.

[4] N. Arai, T. Namba, K. Kawaguchi, Y. Matsumoto, T. Ohkuma, *Angew. Chem. Int. Ed.* **2018**, *57*, 1386-1389.

[5] a) M. M. Zhao, B. Lu, G. N. Ding, K. Ren, X. M. Xie, Z. G. Zhang, *Org. Biomol. Chem.* **2016**, *14*, 2723-2730; b) Z. C. Lin, J. H. Li, Q. F. Huang, Q. Y. Huang, Q. W. Wang, L. Tang *et al*., *J. Org. Chem.* **2015**, *80*, 4419−4429.

[6] S. Meninno, C. Volpe, A. Lattanzi, *Adv. Synth. Catal.* **2016**, *358*, 2845-2848.

[7] a) M. C. Warner, A. Nagendiran, K. Bogár, J. E. Bäckvall, *Org. Lett.* **2012**, *14*, 5094-5097; b) K. Aplander, O. Hidestål, K. Katebzadeh, U. M. Lindström, *Green Chem.* **2006**, *8*, 22–24; c) A. Yajima, *Tetrahedron Lett.* **2014**, *55*, 2773-2780; d) J. Staroń, J. M. Dąbrowski, E. Cichoń, M. Guzik, *Crit. Rev. Biotechnol.* **2017**, *2*, 1-14; e) L. Forti, S. D. Mauro, M. R. Cramarossa, S. Filippucci, B. Turchetti, P. Buzzini, *Molecules* **2015**, *20*, 10377-10398.

[8] a) G. T. Muys, B. V. D. Ven, A. P. D. Jonge, *Nature* **1962**, *194*, 995-996; b) A. Endrizzi, A. C. Awadé, J. M. Belin, *Fems. Microbiol. Lett.* **2010**, *114*, 153-159; c) M. Alchihab, J. Destain, M. Aguedo, L. Majad, H. Ghalfi, J. P. Wathelet, P. Thonart, *Appl. Biochem. Biotech.* **2009,** *158*, 41-50.

[9] G. Feron, L. Dufosse, E. Pierard, P. Bonnarme, J. L. Quere, H. Spinnler, *Appl. Environ. Microbiol.* **1996**, *62*, 2826-2831.

[10] a) I. A. Kaluzna, T. Matsuda, A. K. Sewell, J. D. Stewart, *J. Am. Chem. Soc*. **2004**, *126*, 12827-12832; b) A. Francke, *Nature* **1963**, *197*, 384-385; c) Y. Wache, M. Aguedo, A. Choquet, I. L. Gatfield, J. M. Nicaud, J. M. Belin, *Appl. Environ. Microbiol*. **2001**, *67*, 5700-5704; d) J. U. An, D. K. Oh, *Appl. Microbiol. Biotechnol*. **2013**, *97*, 8265-8272; e) C. Romeroguido, I. Belo, T. M. N. Ta, L. Caohoang, M. Alchihab, N. Gomes *et al*, *Appl. Microbiol. Biotechnol*. **2013**, *89*, 535-547.

[11] a) M. M. Kayser, G. Chen, J. D. Stewart, *J. Org. Chem.* **1998**, *63*, 7103-7106; b) S. Z. Wang, G. Chen, M. M. Kayser, H. Iwaki, P. C. K. Lau, Y. Hasegawa, *Cheminform.* **2010***, 33*, 34; c) L. Blanco, E. Guibé-Jampel, G. Rousseau, *Tetrahedron Lett.* **1988**, *29*, 1915-1918; d) M. M. Enzelberger, U. T. Bornscheuer, I. Gatfield, R. D. Schmid, *J. Biotechnol.* **1997**, *56*, 129-133; e) J. Liu, Z. Li, *ACS Catal.* **2013**, *3*, 908-911; f) J. Liu, J. Wu, Li Z, *Chem. Commum*. **2014**, *50*, 9729-9732; g) S. Kara, D. Spickermann, J. H. Schrittwieser, A. Weckbecker, C. Leggewie, I. W. C. E. Arends, F. Hollmann, *ACS Catal*. **2013**, *3*, 2436-2439. h) A. Díaz-Rodríguez, J. Iglesias-Fernández, C. Rovira, C. Gotor-Fernández, *ChemCatChem*. **2014**, *6*, 977-980.

[12] C. Zhang, J. Pan, C. X. Li, Y. P. Bai, J. H. Xu, *Catal. Commun*. **2017**, *102*. 35-39.

[13] a) K. Nakamura, K. Inoue, K. Ushio, S. Oka, A. Ohno, *J. Org. Chem*. **1988**, *53*, 2589-2593; b) K. Ishihara, *N.* Nakajima, *Biosci. Biotechnol. Biochem*. **2006**, *70*, 3077-3080; c) T. Ema, H. Moriya, T. Kofukuda, T. Ishida, K. Maehara, M. Utaka, T. Sakai, *J. Org. Chem*. **2001**, *66*, 8682-8684; d) H.-M. Ma, L.-L. Yang, Y. Ni, J. Zhang, C.-X. Li, G.-W. Zheng, H.-Y. Yang, J.-H. Xua, *Adv. Synth. Catal*. **2012**, *354*, 1765-1772; e) N.-D. Shen, Y. Ni, H.-M. Ma, L.-J. Wang, C.-X. Li, G.-W. Zheng, J. Zhang, J.-H. Xu, *Org. Lett*. **2012**, *14*, 1982-1985; f) T. Ema, H. Yagasaki, N. Okita, M. Takeda, T. Sakai, *Tetrahedron*. **2006**, *62*, 6143-6149; g) A. L. Gutman, K. Zuobi, T. Bravdo, *J. Org. Chem*. **1990**, *55*, 3546-3552; h) C. Liang, Y. Nie, X. Q. Mu, Y. Xu, *Bioresour. Bioprocess*. **2018**, *5*, 1-8; i) P. Wei, Y. H. Cui, M. H. Zong, P. Xu, J. Zhou, W. Y. Lou, *Bioresour. Bioprocess*. **2017**, *4*, 1-12; j) Y. Yan, H. L. Yu, J. Pan, J. H. Xu, *Journal of East China University of Science and Technology (Natural Science Edition)*.

[14] a) J. N. Copp, P. Hanson-Manful, D. F. Ackerley, W. M. Patrick, *Methods in Molecular Biology* **2014**, *1179*, 3-22; b) E. Henke, U. T. Bornscheuer, *J. Biol. Chem.* **1999**, *380*, 1029-1033; c) N. Tokuriki, C. J. Jackson, L. Afriat-Jurnou, K. T. Wyganowski, D. S. Tawfik, *Nat. Commun*. **2012**, *3*, 1257.

[15] a) J. B. Wang, G. Li, M. T. Reetz, *Chem. Commun*. **2017**, *53*, 3916-3928; b) G. C. Xu, Y. P. Shang, H. L. Yu, J. H. Xu, *Chem. Commun.* **2015**, 51, 15728-15731; c) Z. N. You, Q. Chen, S. C. Shi, M. M. Zheng, J. Pan, X. L. Qian, C. X. Li, J. H. Xu, *ACS Catal.* **2019**, 9, 466−473.

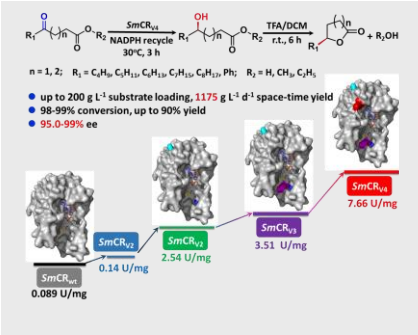
[16] a) Y. Su, Y. Q. Tu, P. M. Gu, *Org. Lett.* **2014**, *16*, 4204-4207; b) P. V. Ramachandran, H. C. Brown, S. Pitre, *Org. Lett.* **2001**, 3, 17-18.

10.1002/cctc.201900382

WILEY-VCH

COMMUNICATION

In this work, structurally diverse γ- and δ-lactones were efficiently stereoselectively synthesized using an engineered carbonyl reductase from *Serratia marcescens* (*Sm*CR$_{V4}$). *Sm*CR$_{V4}$ exhibited improved activity (up to 500-fold) and thermostability toward 14 γ-/δ-keto acids and esters compared with the wild-type enzyme, with 110-fold enhancement in catalytic efficiency (k_{cat}/K_m) toward methyl 4-oxodecanoate. The preparative synthesis of alkyl and aromatic γ- and δ-lactones with 95.0%–99.9% ee was demonstrated. The highest space-time yield, 1175 g L^{-1} d^{-1}, was achieved for (*R*)-γ-decalactone.



Meng Chen, Xiao-Yan Zhang, Chen-Guang Xing, Chao Zhang, Yu-Cong Zheng, Jiang Pan, Jian-He Xu and Yun-Peng Bai**

Page No. – Page No.

Efficient Stereoselective Synthesis of Structurally Diverse γ- and δ-Lactones Using an Engineered Carbonyl Reductase