Advanced Synthesis & Catalysis

Accepted Article

Title: Enzyme Cascade Reactions for the Biosynthesis of Long Chain Aliphatic Amines from Renewable Fatty Acids

Authors: Da-Som Lee, Ji-Won Song, moritz Voß, Eva Schuiten, Ravi Kumar A, Yong-Uk Kwon, Uwe Bornscheuer, and Jin-Byung Park

To be cited as: *Adv. Synth. Catal.* 10.1002/adsc.201801501

Link to VoR: http://dx.doi.org/10.1002/adsc.201801501

10.1002/adsc.201801501

FULL PAPER

DOI: 10.1002/adsc.201((will be filled in by the editorial staff))

Enzyme Cascade Reactions for the Biosynthesis of Long Chain Aliphatic Amines from Renewable Fatty Acids

Da-Som Lee[a], Ji-Won Song[a], Moritz Voß[b], Eva Schuiten[b], Ravi Kumar Akula[c], Yong-Uk Kwon[c], Uwe Bornscheuer[b] and Jin-Byung Park[a]*

[a] Department of Food Science & Engineering, Ewha Womans University, Seoul 03760, Republic of Korea
Fax: (+82)-2-3277-4213; phone: (+82)-2-3277-4509; e-mail: jbpark06@ewha.ac.kr
[b] Institute of Biochemistry, Department of Biotechnology & Enzyme Catalysis, Greifswald University, 17487 Greifswald, Germany
[c] Department of Chemistry and Nanoscience, Ewha Womans University, Seoul 03760, Republic of Korea

Received: ((will be filled in by the editorial staff))

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201######.((Please delete if not appropriate))

Abstract. Enzyme cascade reactions for the synthesis of long chain aliphatic amines such as (*Z*)-12-aminooctadec-9-enoic acid, 10- or 12-aminooctadecanoic acid, and 10-amino-12-hydroxyoctadecanoic acid from renewable fatty acids were investigated. (*Z*)-12-aminooctadec-9-enoic acid was produced from ricinoleic acid ((*Z*)-12-hydroxyoctadec-9-enoic acid) via (*Z*)-12-ketooctadec-9-enoic acid with a conversion of 71% by a two-step in vivo biotransformation involving a long chain secondary alcohol dehydrogenase (SADH) from *Micrococcus luteus* and a variant of the amine transaminase (ATA) from *Vibrio fluvialis*. 10-Aminooctadecanoic acid was prepared from oleic acid ((*Z*)-octadec-9-enoic acid) via 10-hydroxyoctadecanoic acid and 10-ketooctadecanoic acid by an in vivo three-step biocatalysis reaction involving not only the SADH and ATA variants, but also a fatty acid double bond hydratase (OhyA) from *Stenotrophomonas maltophilia*. 10-Aminooctadecanoic acid was produced at a total rate of 4.4 U/g dry cells with a conversion of 87% by recombinant *Escherichia coli* expressing the SADH and ATA variants, and OhyA simultaneously. In addition, bulky aliphatic amines could also be produced by the isolated enzymes (i.e., the SADH, the ATA variants, and a nicotinamide adenine dinucleotide (NADH) oxidase from *Lactobacillus brevis*) with methylbenzylamine or benzylamine as amino donor. This study thus contributes to the biosynthesis of long chain aliphatic amines having two large substituents next to the amine functionality.

Keywords: enzyme cascade reactions, long chain aliphatic amines, amine transaminase, whole cell biotransformation, *Escherichia coli*

Introduction

Biocatalysis has been experiencing sustainable growth due to fast and continuous developments in enzyme engineering, metabolic engineering, synthetic biology, and systems biocatalysis.[1] In particular, multi-step biotransformations with self-sufficient redox recycling and utilization of whole-cell biocatalysis are expanding the product range, as well as enhancing efficiency of the biocatalysis.[2] For instance, chiral amines and lactones were produced in a high yield from alcohols through enzyme cascade reactions involving alcohol dehydrogenases (ADHs) and amine transaminases (ATAs) with redox-recycling ADHs (e.g., lactate dehydrogenases and amine dehydrogenases) and Baeyer-Villiger monooxygenases.[3] Another example is the production of 12-aminododecanoic acid methyl ester from dodecanoic acid methyl ester by recombinant *Escherichia coli* expressing an alkane hydroxylase, ω-transaminase, and alanine dehydrogenase.[4] The nicotinamide cofactors were provided by cellular carbon metabolism, while the amino donor (i.e., alanine) was regenerated by the alanine dehydrogenase inside cells.

Amines are versatile building blocks for the pharmaceutical and agrochemical industries. However, bulky aliphatic amines are difficult to produce by either enzymatic or chemical means.[5] There are only a few reports where aromatic β- and γ-amino acids were prepared by kinetic resolution or asymmetric synthesis using ω-transaminases from *Polaromonas* sp., *Burkholderia graminis*, and *Sphaerobacter thermophilus*.[6] Aromatic amines with bulky branched chains (e.g., 2,2-dimethyl-1-phenylpropan-1-amine) were produced by variants of ATA from *Vibrio fluvialis* (VF-ATA)[7] and from *Ruegeria* sp. TM1040 (Rsp-ATA),[8] of which the active site has been engineered to accept bulky ketones. Reshaping of the substrate-binding cavity of amine dehydrogenases has also allowed asymmetric synthesis of bulky aliphatic amines (e.g., (*R*)-2-heptanamine).[9]

In this study, we have investigated enzyme cascade reactions to provide bulky aliphatic amines from renewable fatty acids (Schemes 1 and 2). One example is the synthesis of (*Z*)-12-aminooctadec-9-enoic acid (**3**), which is a precursor of antibacterial lipoamino acid derivatives (e.g., (*Z*)-methyl-12-(2-aminoacetamido)octadec-9-enoate) and phenolipids (e.g., (*Z*)-methyl-12-cinnamamidooctadec-9-enoate and (*Z*)-methyl-12-(4-hydroxybenzamido)octadec-9-enoate).[10] The long chain aliphatic amine **3** was produced from ricinoleic acid ((*Z*)-12-hydroxyoctadec-9-enoic acid, **1**) via (*Z*)-12-ketooctadec-9-enoic acid **2** (Scheme 1) with a high conversion by recombinant *E. coli* expressing a long-chain secondary alcohol dehydrogenase (SADH) from *Micrococcus luteus*[11] and VF-ATA variants.[7] The amine product **3** was also produced using isolated enzymes (i.e., the SADH, the VF-ATA variants, and the NADH oxidase (NOX) from *Lactobacillus brevis*[12] with methylbenzylamine or benzylamine as amino donor (Scheme 1).

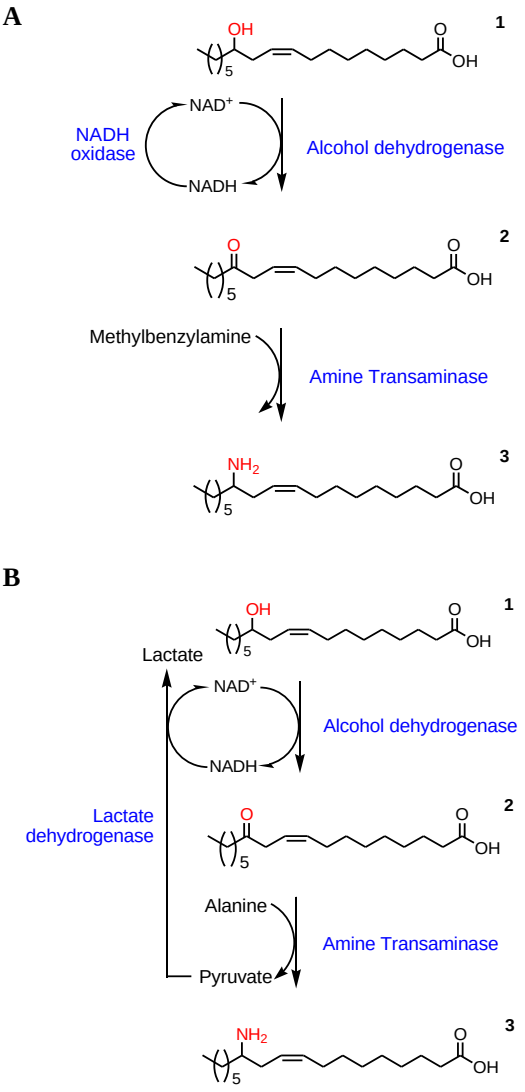
Results and Discussion

Enzyme Screening for the Synthesis of (*Z*)-12-Aminooctadec-9-enoic Acid from Ricinoleic Acid

Hydroxy fatty acids (e.g., ricinoleic acid) can be converted into amino fatty acids via keto fatty acids by enzyme cascade reactions involving a long chain secondary alcohol dehydrogenase (SADH) and an amine transaminase (ATA) (Scheme 1). A SADH from *M. luteus* was reported to catalyze oxidation of hydroxy fatty acids into the corresponding keto fatty acids[11] and was therefore included in the cascade.

ATAs are versatile enzymes that synthesize a variety of amines, but most do not accept ketones with two large substituents next to the carbonyl functionality.[13] ATAs which have been reported to have catalytic activity with sterically hindered ketones, were thus considered as enzyme candidates. These candidates included the variants of *V. fluvialis* ATA (VF-ATA)[7] and the variants of *Ruegeria* sp. TM1040 ATA (Rsp-ATA).[8] A variant of *Arthrobacter* sp. ATA, which had been developed to accept the prositagliptin ketone,[14] was also chosen for initial studies (Table S1 and S2).

The biocatalytic activity of the ATAs was evaluated by measuring acetophenone formation with thin layer chromatography (TLC).[15] After reaction in 50 mM HEPES buffer (pH 8.0) containing 1 mM 10-ketooctadecanoic acid, 10 mM racemic methylbenzylamine (MBA), 0.1 mM pyridoxal phosphate (PLP), and 10% dimethyl sulfoxide (DMSO), the resulting ketone product acetophenone was analyzed by TLC. The acetophenone spot was clearly visible from the VF-ATA and Rsp-ATA mutants as well as the positive control (Fig. S1).



Scheme 1. Designed biotransformation pathway 1. Ricinoleic acid (**1**) is converted into (*Z*)-12-aminooctadec-9-enoic acid (**3**) via (*Z*)-12-ketooctadec-9-enoic acid (**2**). The NADH oxidase (NOX) was included for regeneration of NAD^+, which is required for the SADH reaction (A). The lactate dehydrogenase was included for removal of pyruvate and regeneration of NAD^+ (B).

Biotransformation of Ricinoleic Acid into (*Z*)-12-Aminooctadec-9-enoic Acid

The biocatalytic activity of the VF-ATA variants was quantitatively estimated by conducting the biotransformation of ricinoleic acid **1** into (*Z*)-12-aminooctadec-9-enoic acid **3** (Scheme 1). The biotransformation was initiated by adding ricinoleic acid and NAD^+ into the reaction medium containing SADH, ATAs, and NADH oxidase (NOX) from *L. brevis*[12] (Fig. 1). The NOX was included for regeneration of NAD^+, which is required for the SADH reaction (Scheme 1A). The amino donor (i.e., *rac*-MBA) was added into the reaction medium at t = 0, just after ricinoleic acid (**1**) was transformed into (*Z*)-12-ketooctadec-9-enoic acid (**2**).

Notably, the H3_RA and H1_R variants of VF-ATA, which had been designed to accept the bulky aliphatic ketone,[7] converted (*Z*)-12-ketooctadec-9-enoic acid (**2**) into the amine product (**3**) at a rate of over 200 U/L with over 100 U/g proteins at t < 0.25 h (Fig. 1AB and Table 1). The conversion, which was determined based on the product concentration measured by GC/MS at t = 1 h, reached approximately 90% (Fig. S2A). In contrast, another VF-ATA variant, H3_RAV, showed only little bioconversion (Fig. 1C).

The isolated yield of the amine product (**3**) from the reaction medium was over 70%; (*Z*)-12-aminooctadec-9-enoic acid (**3**) (54 mg , purity > 90%) was obtained as a liquid form from 50 mL reaction medium containing 70 mg amine (Fig. S2B). The structure of **3** was confirmed by GC/MS and NMR analysis (Fig. S2B and S3). This indicates that the VF-ATA variants H3_RA and H1_R are able to catalyze transamination of the bulky aliphatic ketone (**2**) into the bulky aliphatic amine (**3**). Chirality of the amine product remained to be investigated.

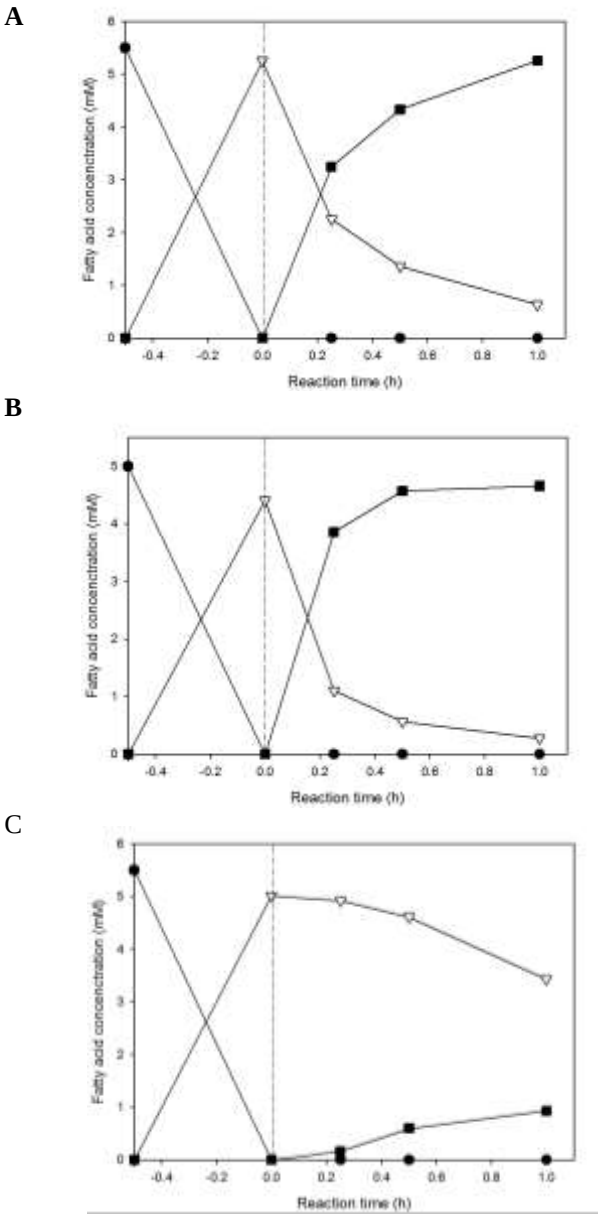


Figure 1. Time course of the biotransformation of ricinoleic acid (**1**) into (*Z*)-12-aminooctadec-9-enoic acid (**3**). The biotransformation was initiated by adding ricinoleic acid and NAD^+ into the reaction medium containing the cascade enzymes (i.e., secondary alcohol dehydrogenase (SADH), amine transaminase (ATA), and NADH oxidase (NOX)). The amino donor (i.e., methylbenzylamine (MBA)) was added into the reaction medium at t = 0, just after ricinoleic acid (**1**) was transformed into (*Z*)-12-ketooctadec-9-enoic acid (**2**). The *V. fluvialis* ATA (VF-ATA) variants (i.e., H1_R (triple mutant of VF-ATA (L56V, W57F, F85V)) (A), H3_RA (triple mutant of VF-ATA (L56V, W57C, V153A)) (B), and H3_RAV (quadruple mutant of VF-ATA (L56V, W57C, F85V, V153A)) (C) were used for amination of (*Z*)-12-ketooctadec-9-enoic acid (**2**). Symbols indicate the concentrations of ricinoleic acid (**1**) (●), (*Z*)-12-ketooctadec-9-enoic acid (**2**) (▽), (*Z*)-12-aminooctadec-9-enoic acid (**3**) (■).

Biosynthesis of (*Z*)-12-Aminooctadec-9-enoic Acid with Benzylamine and Alanine as Amino Donor

Benzylamine is one of the cheapest amino donors for ATAs.[16] Thus, the ricinoleic acid biotransformation into (*Z*)-12-aminooctadec-9-enoic acid (**3**) was conducted with benzylamine (Fig. 2A). All reaction conditions were equal to those for the biotransformations with MBA (Fig. 1B), except that octane was added into the reaction medium up to 20% (v/v) to in situ remove possible ATA inhibitors (e.g., benzaldehyde).[1c, 16] (*Z*)-12-Ketooctadec-9-enoic acid (**2**) was transformed into the amine product (**3**) by H3_RA at a rate of 83 U/L and 42 U/g proteins at t < 0.5 h (Fig. 2A and Table 1). The conversion reached approximately 80%. Thereby, it was confirmed that benzylamine can be used as amino donor for the transamination of bulky aliphatic ketones.

Alanine has been widely used as an amino donor and therefore, the biosynthesis of (*Z*)-12-aminooctadec-9-enoic acid (**3**) was investigated with alanine as amino donor (Fig. 2B). The biotransformation of ricinoleic acid (**1**) into (*Z*)-12-aminooctadec-9-enoic acid (**3**) was initiated by adding 6 mM ricinoleic acid and 50 mM alanine into the reaction medium containing the enzymes, SADH, H3_RA, and lactate dehydrogenase (LDH) from porcine heart, and 1 mM NAD^+ (Scheme 1B). The LDH was included for regeneration of NAD^+, which is required for the SADH reaction and for removal of pyruvate, which is one of the ATA inhibitors.[1c] Ricinoleic acid was converted into the amine product (**3**) via (*Z*)-12-ketooctadec-9-enoic acid (**2**) at a rate of 79 U/L at t < 0.5 h (Table 1). The bioconversion yield reached approximately 65%. This result indicates that alanine can also be used as amino donor for transamination of the long chain aliphatic ketones.

10.1002/adsc.201801501

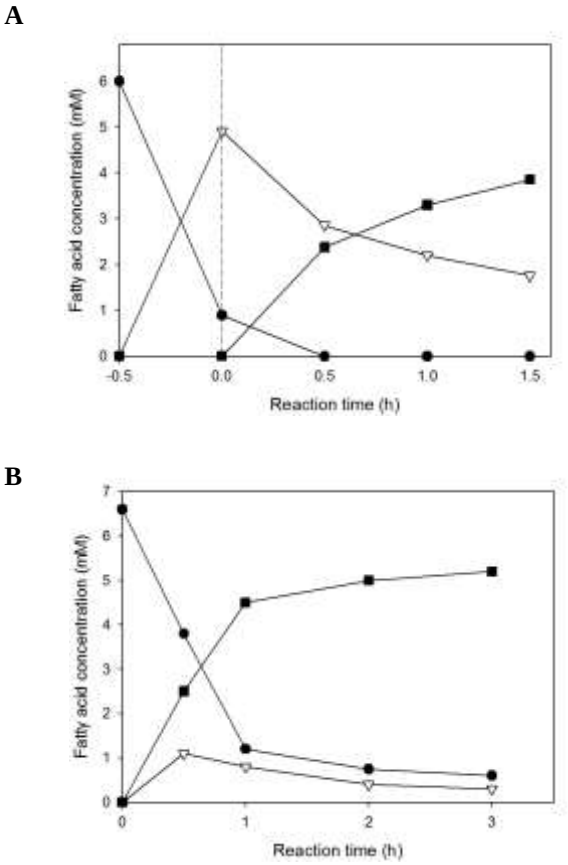


Figure 2. Time course of the biotransformation of ricinoleic acid (**1**) into (*Z*)-12-aminooctadec-9-enoic acid (**3**). The biotransformation conditions were identical to the experiments shown in Fig. 1B, except for the amino donor. Benzylamine (A) and alanine (B) was used instead of MBA. In case of Fig. 2A, octane was added into the reaction medium to 20% (v/v) to in situ remove one of the ATA inhibitors (e.g., benzaldehyde). Symbols indicate the concentrations of ricinoleic acid (**1**) (●), (*Z*)-12-keto-octadec-9-enoic acid (**2**) (▽), (*Z*)-12-aminooctadec-9-enoic acid (**3**) (■).

Whole-cell Biotransformation of Ricinoleic Acid into (*Z*)-12-Aminooctadec-9-enoic Acid

Next, the cascade described above using isolated enzymes (Scheme 1A) was introduced into *E. coli* by expressing the SADH and one of the different VF-ATA variants simultaneously. NOX was not overexpressed because the NAD^+ cofactor could be provided through cellular carbon metabolism. The fatty acid transporter FadL was overexpressed in the outer membrane of the *E. coli* to facilitate substrate transport into the cell.[17] The two-step biotransformation was initiated by adding ricinoleic acid as substrate and (*S*)-MBA as amino donor into the reaction medium containing 7.4 g dry cells/L of the recombinant *E. coli*.

The target product (**3**) was produced at a rate of 24 U/L at t < 1 h by recombinant *E. coli* expressing the SADH and H3_RA (Fig. 3A). The specific biotransformation rate was determined to be 3.5 U/g dry cells. This corresponds to approximately 42 U/g ATAs, as estimated from the SDS-PAGE where the H3_RA band in the soluble fraction accounted for approximately 15% of the total amount of proteins (Fig. S4). As compared to the biotransformation by isolated enzymes (Fig. 1B and Table 1), the specific enzyme activity in the recombinant *E. coli* was ca. 40% of the isolated ATA. Thereby, it could be concluded that the enzyme cascade (Scheme 1) was efficiently working even in a whole-cell system.

The biotransformation activity of the H1_R expressed in the recombinant *E. coli* was comparable to that of the H3_RA (Fig. 3B). On the other hand, H3_RAV expressed in the recombinant *E. coli* was markedly lower than that of H3_RA and H1_R (Fig. 3C), as shown in the biotransformation with isolated enzymes (Table 1).

Table 1. Biotransformation activity of the *V. fluvialis* amine transferase variants

	Enzymes involved	Amino donor	Initial Bioconversion rate (U/L)[a]	Initial bioconversion rate (U/g proteins)[a]	Conversion (%)[b]
Biotrans 1	SADH/H1_R/ NOX	MBA	210	108	88
Biotrans 2	SADH/H3_RA/NOX	MBA	230	115	90
Biotrans 3	SADH/H3_RAV/NOX	MBA	20	10	17
Biotrans 4	SADH/H3_RA/NOX	Benzylamine	83	42	79
Biotrans 5	SADH/H3_RA/LDH	Alanine	79	40	65

[a] The initial biotransformation rates were determined based on the product concentrations, which were measured at t = 0.25 or 0.5 h by gas chromatography/mass spectrometry (GC/MS)

[b] The conversion was calculated based on the substrate and product concentrations determined by GC/MS.

10.1002/adsc.201801501

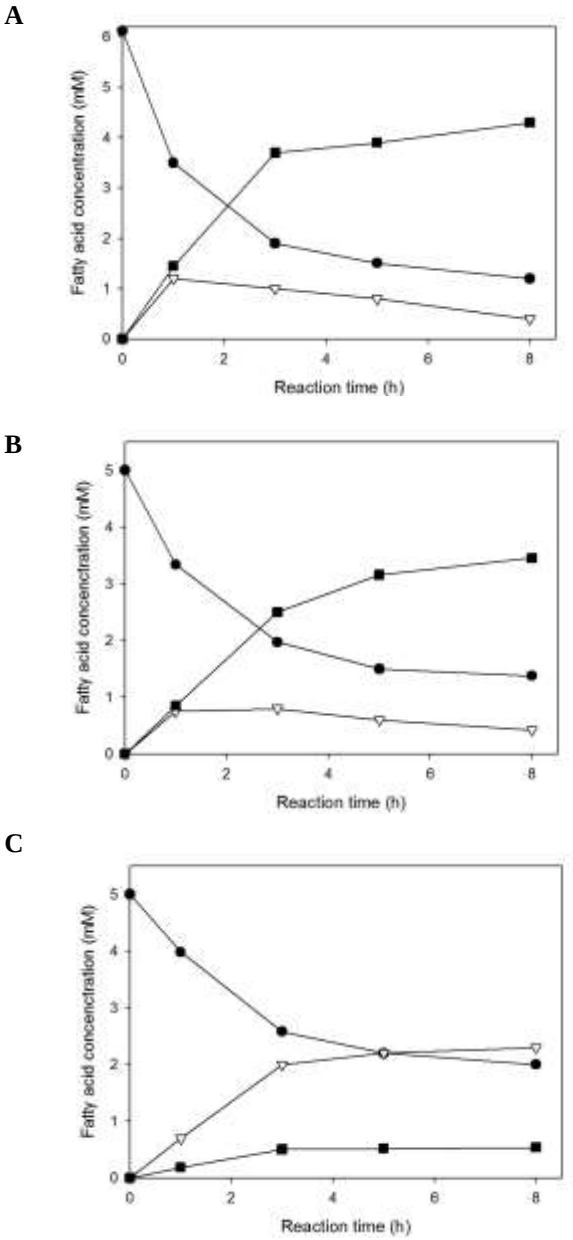


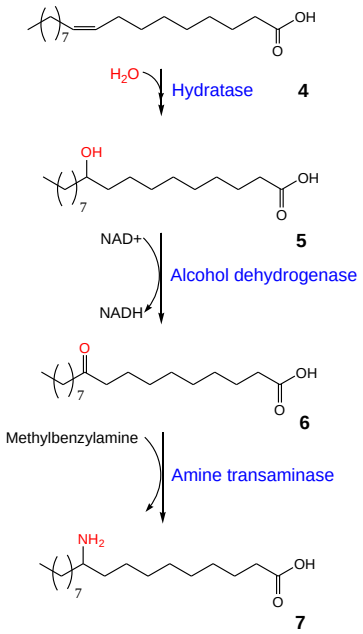
Figure 3. Time course of the biotransformation of ricinoleic acid (**1**) into (*Z*)-12-aminooctadec-9-enoic acid (**3**) by the recombinant *E. coli* BL21(DE3) expressing a secondary alcohol dehydrogenase (SADH) of *M. luteus*, the VF-ATA variants (i.e., H3_RA (A), H1_R (B), and H3_RAV (C)), and a long chain fatty acid transporter (FadL). The biotransformation was initiated by adding 5 to 6 mM ricinoleic acid and 10 mM MBA into the recombinant *E. coli* culture broth (cell density: 7.4 g dry cells/L). Symbols indicate the concentrations of ricinoleic acid (**1**) (●), (*Z*)-12-ketooctadec-9-enoic acid (**2**) (▽), (*Z*)-12-aminooctadec-9-enoic acid (**3**) (■).

Biotransformation of Other Hydroxy Fatty Acids

Whole-cell biotransformation of 10-hydroxy-octadecanoic acid, 12-hydroxyoctadecanoic acid, and 10,12-dihydroxyoctadecanoic acid, which had been produced from renewable fatty acids according to our previous studies,[11] into corresponding amino fatty acids was investigated as well. The initial biotransformation rate of 12-hydroxyoctadecanoic acid into 12-aminooctadecanoic acid by the recombinant *E. coli* expressing H3_RA was 4.0 U/g dry cells. This value was similar to that of ricinoleic acid ((*Z*)-12-hydroxyoctadec-9-enoic acid), while the reaction rate of 10-hydroxyoctadecanoic acid increased up to 9.1 U/g dry cells (Table 2). On the other hand, the biotransformation rate of 10,12-dihydroxyoctadecanoic acid into 10-amino-12-hydroxyoctadecanoic acid was significantly lower than that of ricinoleic acid. These results indicated that structure of the substrates and especially the position of the carbonyl group in the carbon skeleton of fatty acids could be one of the key factors to determine bioconversion rates. The structure of 12-aminooctadecanoic acid and 10-amino-12-hydroxyoctadecanoic acid was confirmed by GC/MS (Fig. S5 and S6).

Biosynthesis of 10-Aminooctadecanoic Acid from Oleic Acid

Finally, the recombinant biotransformation of oleic acid (**4**) into 10-aminooctadecanoic acid (**7**) was examined (Scheme 2). The recombinant *E. coli* was engineered to express not only the SADH, H3_RA and FadL, but also a fatty acid double bond hydratase (i.e., OhyA) from *S. maltophilia*.[18]
Oleic acid will be transported into the cytoplasm via FadL in the outer membrane and subjected to hydration by OhyA into 10-hydroxyoctadecanoic acid (**5**).



Scheme 2. Designed biotransformation pathway 2. Oleic acid (**4**) is converted into 10-aminooctadecanoic acid (**7**) via 10-hydroxyoctadecanoic acid (**5**) and 10-keto-octadecanoic acid (**6**).

10.1002/adsc.201801501

When the oleic acid and MBA were added at the same time into culture broth of the recombinant *E. coli*, oleic acid was not converted into 10-hydroxyoctadecanoic acid. One of the reasons might include inhibition of OhyA by the amine donor. Therefore, MBA was added after production of 10-ketooctadecanoic acid (**6**) from oleic acid (Fig. 4). Addition of MBA into culture broth of the recombinant *E. coli* at t = 0 allowed to produce 4.5 mM 10-aminooctadecanoic acid (**7**) in the medium. The conversion of oleic acid into 10-aminooctadecanoic acid reached 87%, which is comparable to that of 10-hydroxyoctadecanoic acid (Table 2). The structure of **7** was confirmed by GC/MS and NMR analysis (Fig. S7 and S8).

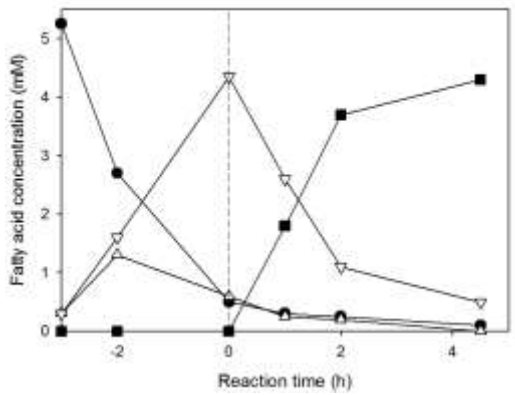


Figure 4. Time course of the biotransformation of oleic acid (**4**) into 10-aminooctadecanoic acid (**7**) by the recombinant *E. coli* BL21(DE3) expressing a fatty acid double bond hydratase (OhyA) of *S. maltophilia*, the SADH of *M. luteus*, the VF-ATA variant (i.e., H3_RA), and FadL. The biotransformation was initiated by adding 5.3 mM oleic acid into the recombinant *E. coli* culture broth (cell density: 7.4 g dry cells/L) at t = -3 h. MBA was added at t = 0. Symbols indicate the concentrations of oleic acid (**4**) (●), 10-hydroxyoctadecanoic acid (**5**) (△), 10-ketooctadecanoic acid (**6**) (▽), and 10-amino-octadecanoic acid (**7**) (■).

Comparison with Chemical Procedure

(*Z*)-Methyl-12-aminooctadec-9-enoate, a precursor of antibacterial lipoamino acid derivatives (e.g., (*Z*)-methyl-12-(2-aminoacetamido)octadec-9-enoate) and phenolipids,[10] was chemically synthesized from methyl ricinoleate by a three-step chemical procedure.[19] Methyl ricinoleate which was prepared by the esterification of ricinoleic acid was treated with methanesulfonyl chloride to give the corresponding mesylate, which was then treated with sodium azide to afford methyl-12-azidooctadec-9-enoate. The third step involved the selective reduction of azido group using Zn-$AlCl_3$·$6H_2O$ to provide the corresponding methyl-12-aminooctadec-9-enoate in 77% yield over three steps from methyl ricinoleate. As compared to the chemical procedures for the conversion of hydroxyl group to amino group, the whole-cell biocatalysis is much simpler and environmentally benign. It can be operated in a one-pot process without toxic chemicals.

Conclusion

This study demonstrates that long chain aliphatic amines such as (*Z*)-12-aminooctadec-9-enoic acid, 10- or 12-aminooctadecanoic acid, and 10-amino-12-hydroxyoctadecanoic acid can be prepared from renewable fatty acids by multi-step biotransformations involving a fatty acid double bond hydratase (OhyA) from *S. maltophilia*, a long chain SADH from *M. luteus*, and a variant of *V. fluvialis* amine transaminase. Not only screening of an active enzyme but also tight control of putative enzyme inhibitors (e.g., amino donor, byproducts) and cofactor regeneration allows for the production of bulky aliphatic amines at a rather high rate and yield. This study will be continued by determining the chirality of the amine products and by engineering of the enzymes to improve the transamination activity.

Table 2. Substrate specificity of the *V. fluvialis* amine transferase variants

Substrates	Initial bioconversion rate (U/L)[a]	Initial bioconversion rate (U/g dry cells)[a]	Conversion (%)[b]
12-Ketooctadec-9-enoic acid	24	3.5 (42)	71
12-Ketooctadecanoic acid	28	4.0 (48)	87
10-Ketooctadecanoic acid	63	9.1 (110)	94
10-Keto-12-hydroxyoctadecanoic acid	6.0	0.9 (10)	60

[a] The initial biotransformation rates were determined based on the product concentrations, which were measured at t = 0.25 or 0.5 h by GC/MS. The values in the parenthesis indicate initial bioconversion rates per g ATAs (U/g ATAs), which were determined from the SDS-PAGE analysis (Fig. S4).
[b] The conversion was calculated based on the substrate and product concentrations determined by GC/MS.

10.1002/adsc.201801501

Experimental Section

Microbial Strains and Cultivation Media

Recombinant *E. coli* BL21(DE3) was cultivated in the Riesenberg medium, which was supplemented with 10 g/L glucose and the appropriate antibiotics for plasmid maintenance. The Riesenberg medium consisted of 4 g/L $(NH_4)_2HPO_4$, 13.5 g/L KH_2PO_4, 1.7 g/L citric acid, 1.4 g/L $MgSO_4$, and 10 mL/L trace metal solution [10 g/L $FeSO_4$, 2.25 g/L $ZnSO_4$, 1.0 g/L $CuSO_4$, 0.5 g/L $MnSO_4$, 0.23 g/L$Na_2B_4O_7$, 2.0 g/L $CaCl_2$, and 0.1 g/L $(NH_4)_6Mo_7O_{24}$]. The recombinant gene expression was induced by adding 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) an OD600 of 0.6-0.8 into the cultivation medium.

Chemicals and materials

Ricinoleic acid, *N*-methyl-*N*-(trimethylsilyl)trifluoro-acetamide (TMS), (*S*)-methy-lbenzylamine (MBA), and *rac*-MBA were obtained from TCI (Tokyo, Japan). Oleic acid was purchased from Daejung (Siheung, Korea). 10-Hydroxyoctadecanoic acid, 12-hydroxyoctadecanoic acid, and 10,12-dihidroxyoctadecanoic acid were purified in our lab as described earlier.[11] Antibiotics, trace elements for culture medium, IPTG, Tween80, L-alanine, benzylamine, nicotinamide adenine dinucleotide (NAD^+), pyridoxal 5′-phosphate (PLP), and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) were purchased from Sigma (St. Louis, MO, USA). Lactate dehydrogenase from porcine heart was obtained from MP bio (Santa Ana, California, USA).

Protein Purification

After the recombinant *E. coli* BL21(DE3) expressing the cascade enzymes were cultivated in the Riesenberg medium, the cells were harvested through centrifugation at 3,500 rpm (2,602 ×g) for 15 min and washed with 50 mM HEPES buffer. The washed cells were resuspended into lysis buffer containing 10 mM imidazole and 0.1 mM cofactor PLP or NAD^+ and subjected to the cell lysis by sonication. The cell debris was removed by centrifugation at 13,000 rpm (15,814 ×g) for 7 min at 4 °C. The supernatant was mixed with 1.0 mL per g dry cells of Ni^{2+}-NTA agarose beads solution (Quiagen, Hilden, Germany) and incubated at 4 °C, 250 rpm for 1 h. The mixture was loaded onto the column and flowed out. The column was washed twice with a wash buffer containing 20 mM imidazole. The target proteins were then eluted by increasing the imidazole concentration to 250 mM. Fractions containing the recombinant proteins were pooled and dialyzed to remove imidazole.

Acetophenone Assay

The biocatalytic activity of the ATAs was evaluated by measuring acetophenone formation with thin layer chromatography (TLC).[15] In brief, the enzyme reaction was carried out in 1 mL scale with the following conditions in the kinetic resolution mode: 1 mM 10-ketooctadecanoic acid, 10 mM *rac*-MBA, 0.1 mM PLP and 10% DMSO in 50 mM HEPES buffer pH 8.0. The reaction was incubated over night at 30 °C, 600 rpm. For product analysis, the reactions were terminated by basification with NaOH and extracting the reaction products with ethyl acetate (3x with 200 µL each). The samples were analyzed by the TLC with different staining methods and mobile phases. The mobile phase was hexane and ethyl acetate (70:30). Staining of ketones was conducted using 2,4-dinitrophenylhydrazine.

Enzymatic Transformation of Ricinoleic Acid into (*Z*)-12-Aminooctadec-9-enoic Acid (3)

Enzymatic Transformation of ricinoleic acid into (*Z*)-12-aminooctadec-9-enoic acid was performed in 50 mM HEPES buffer (pH 8.0) containing 5 mM ricinoleic acid, 2 mM PLP, 2.5 mM NAD^+, and 10 mM *rac*-MBA or 50 mM alanine. After the cascade enzymes (i.e., SADH (4 mg/mL), VF-ATA variants (2 mg/mL), and NOX (1 mg/mL) or LDH (45 U/mL) were added into the reaction media, they were incubated at 30 °C for 3 h with agitation at 200 rpm (working volume: 2 mL). Biotransformation of ricinoleic acid into (*Z*)-12-aminooctadec-9-enoic acid was also carried out with benzylamine as an amine donor. The reaction conditions were similar to the experiment with *rac*-MBA or alanine as amino donor. The difference was that 50mM Tris-HCl buffer (pH 7.0) and octane (ratio = 5:1) was used as the reaction medium.

Whole-Cell Biotransformations

The recombinant *E. coli* cells, expressing the SADH, VF-ATA variants, and FadL, were used for the biotransformation of hydroxy fatty acids, whereas the recombinant *E. coli* cells, expressing not only the SADH, VF-ATA variants, and FadL but also a fatty acid double bond hydratase (i.e., OhyA) were used for the biotransformation of oleic acid, based on our previous studies.[17] The recombinant *E. coli* cells were cultivated in Riesenberg medium at 30 °C and expressions of target genes were induced with 0.1 mM IPTG at 20 °C based on our earlier works.[20] When added glucose was completely consumed, the recombinant cells were harvested by centrifugation at 3,500 rpm (2,602 ×g) for 15 min at 4 °C. The biotransformation was initiated by adding 5 mM of a fatty acid, 1 mM PLP, and 10 mM (*S*)-MBA into 50 mM HEPES buffer (pH 8.0) containing 0.5 g/L Tween80 and 7.4 g/dry cells. The reaction mixture were incubated at 35 °C and 200 rpm.

Product Analysis by Gas Chromato graphy/Mass Spectrometry (GC/MS)

Concentrations of ricinoleic acid, (*Z*)-12-aminooctadec-9-enoic acid and other intermediates were measured according to the method based on our previous works.[20] The reaction medium was mixed with 2.8 times volume of ethyl acetate containing 0.5 g/L palmitic acid as an internal standard. The organic phase was harvested after vigorous vortexing and subjected to derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (TMS). The trimethylsilyl derivatives were analyzed using a Thermo Ultra Trace GC system connected to San ion trap mass detector (Thermo ITQ1100GC-ion Trap MS, Thermo Scientific, Indianapolis, IN, USA) and were separated on a non-polar capillary column (30 m length, 0.25 mm film thickness, HP-5MS, Agilent technologies, Palo Alto, CA, USA). A linear temperature gradient was programmed as 160 °C, 25 °C /min to 235 °C, 3 °C /min to 253 °C. Injection port temperature was 230 °C. Mass spectra were obtained by electron impact ionization at 70 eV. Scan spectra were obtained within the range of 100-600 *m/z*. Selected ion monitoring was used for detection and the fragmentation analysis of the reaction products.

Isolation of (*Z*)-12-Aminooctadec-9-enoic Acid (3) after Biotransformation

(*Z*)-12-Aminooctadec-9-enoic acid was isolated from the biotransformation medium by simple extraction with ethyl acetate. The combined organic layer was washed with saline, dried over anhydrous Na_2SO_4, filtered, and evaporated *in vacuo*. The crude compounds were separated and purified by silica gel column chromatography. The varying concentrations of ethyl acetate/hexane were used to remove the fatty acid impurities. The amine products were finally recovered with 10-20% methanol in dichloromethane as the eluent. The isolation/purification yield of the amine product (**3**) was over 70%, based on the concentration of the amine product after whole-cell biotransformation. The purified amine product (54 mg) was a liquid form; ^{1}H NMR (300 MHz, CD_3OD) δ 5.69–5.60 (m, 1H), 5.41–5.32 (1H, m), 3.17 (quin, *J* = 6.7 Hz, 1H), 2.42–2.31 (m, 2H), 2.19 (t, *J* = 7.5 Hz, 2H), 2.12-2.05 (m, 2H), 1.68–1.52 (m, 4H), 1.41–1.29 (m, 16H), 0.91 (t, *J* = 6.0 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 181.2 (COOH), 135.8 (CH=CH), 123.8 (CH=CH), 71.5 (CNH_2), 53.1, 37.6, 33.4, 32.7, 31.4, 30.26, 30.22, 30.21, 30.0, 28.2, 27.0, 26.2, 23.6, 14.4 (CH_3).

Acknowledgements

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2017008670) and the Marine Biomaterials Research Center grant from the Marine Biotechnology Program funded by the Ministry of Oceans and Fisheries, Republic of Korea (No. D11013214H480000100).

References

[1] a) Y. J. Yeon, J. B. Park, in : *Lipid Modification by Enzymes and Engineered Microbes* (Eds.: U. T. Bornscheuer), Elsevier, Amsterdam, **2018**, pp 139-155; b) X. Chen, C. Gao, L. Guo, G. Hu, Q. Luo, J. Liu, J. Nielsen, J. Chen, L. Liu, *Chem. Rev.* **2018**, *118*, 4–72; c) F. Guo, P. Berglund, *Green Chem*. **2017**, *19*, 333-360; d) Y. Liang, J. Wei, X. Qiu, N. Jiao, *Chem. Rev.* **2018**, *118*, 4912−4945; e) I. Slabu, J. L. Galman, R. C. Lloyd, N. J. Turner, *ACS Catal*. **2017**, *7*, 8263-8284; f) R. A. Sheldon, J. M. Woodley, *Chem. Rev.* **2018**, *118*, 801−838.

[2] a) J. Muschiol, C. Peters, N. Oberleitner, M. D. Mihovilovic, U. T. Bornscheuer, F. Rudroff, *Chem. Commun*. **2015**, *51*, 5798; b) J. H. Schrittwieser, S. Velikogne, M. Hall, W. Kroutil, *Chem. Rev*. **2018**, *118*, 270-348; c) J. M. Sperl, V. Sieber, *ACS Catal*. **2018**, *8*, 2385−2396; d) S. Wu, Z. Li, *ChemCatChem* **2018**, *10*, 2164–2178.

[3] a) J. H. Sattler, M. Fuchs, K. Tauber, F. G. Mutti, K. Faber, J. Pfeffer, T. Haas, W. Kroutil, *Angew. Chem. Int. Ed*. **2012**, *51*, 9156–9159; b) S. Schmidt, C. Scherkus, J. Muschiol, U. Menyes, T. Winkler, W. Hummel, H. Groger, A. Liese, H. G. Herz, U. T. Bornscheuer, *Angew. Chem. Int. Ed*. **2015**, *54*, 2784–2787.

[4] N. Ladkau, M. Assmann, M. Schrewe, M. K. Julsing, A. Schmid, B. Bühler, *Metab. Eng*. **2016**, *36*, 1–9.

[5] a) A. Gomm, E. O'Reilly, *Curr. Opin. Chem. Biol*. **2018**, *43*, 106-112; b) F. Guo, P. Berglund, *Green Chem*. **2017**, *19*, 333-360; b) I. Slabu, J. L. Galman, R. C. Lloyd, N. J. Turner, *ACS Catal*. **2017**, *7*, 8263-8284.

[6] a) S. Mathew, S. P. Nadarajan, T. Chung, H. H. Park, H. Yun, *Enz. Microb. Technol*. **2016**, *87–88*, 52–60; b) M. Shon, R. Shanmugavel, G. Shin, S. Mathew, S. H. Lee, H. Yun, *Chem. Commun.* **2014**, *50*, 12680-12683.

[7] M. Genz, O. Melse, S. Schmidt, C. Vickers, M. Dörr, T. van den Bergh, H. J. Joosten, U. T. Bornscheuer, *ChemCatChem* **2016**, *8*, 3199-3202.

[8] a) M. S. Weiß, I. V. Pavlidis, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding, U. T. Bornscheuer, *Org. Biomol. Chem.* **2016**, *14*, 10249-10254; b) M. S. Weiß, I. V. Pavlidis, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding, U. T. Bornscheuer, *ChemBioChem* **2017**, *18*, 1022-1026; c) I. V. Pavlidis, M. S. Weiß, M. Genz, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding, U. T. Bornscheuer, *Nat. Chem.* **2016**, *8*, 1076-1082.

[9] F. F. Chen, G. W. Zheng, L. Liu, H. Li, Q. Chen, F. L. Li, C. X. Li, J. H. Xu, *ACS Catal*. **2018**, *8*, 2622−2628.

[10] a) Y. Mohini, R. B. N. Prasad, M. S. L. Karuna, Y. Poornachandra, C. Ganesh Kumar, *Bioorg. Med. Chem. Lett*. **2016**, *26*, 5198–5202; b) M. Yarra, S. S. Kaki, R. B. N. Prasad, K. S. L. Mallampall, P. Yedla, G. K. Chityala, *Eur. J. Lipid Sci. Technol*. **2016**, *118*, 622–630.

[11] a) H. J. Cha, E. J. Seo, J. W. Song, H. J. Jo, A. R. Kumar, J. B. Park, *Adv. Synth. Catal.* **2018**, *360*, 696-703; b) H. J. Oh, S. U. Kim, J. W. Song, J. H. Lee, W. R. Kang, Y. S. Jo, K. R. Kim, U. T. Bornscheuer, D. K. Oh, J. B. Park, *Adv. Synth. Catal.* **2015**, *357*, 408–416; c) J. W. Song, E. Y. Jeon, D. H. Song, H. Y. Jang, U. T. Bornscheuer, D. K. Oh, J. B. Park, *Ang. Chem. Int. Ed*. **2013**, *52*, 2534-2537.

[12] G. Rehn, A. T. Pedersen, J. M. Woodley, *J. Mol. Catal. B: Enz*. **2016**, *134*, 331–339.

[13] a) S. Calvelage, M. Dçrr, M. Hçhne, U. T. Bornscheuer, *Adv. Synth. Catal*. **2017**, *359*, 4235-4243; b) F. Steffen-Munsberg, C. Vickers, H. Kohls, H. Land, H. Mallin, A. Nobili, L. Skalden, T. van den Bergh, H. J. Joosten, P. Berglund, M. Hchne, U. T. Bornscheuer, *Biotechnol. Adv*. **2015**, *33*, 566-604.

[14] C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman, G. J. Hughes, *Science* **2010**, *329*, 305-309.

[15] S. Schätzle, M. Höhne, E. Redestad, K. Robins, U. T. Bornscheuer, *Anal. Chem.* **2009**, *81*, 8244-8248.

[16] J. S. Shin, B. G. Kim, *Biotechnol. Lett.* **2009**, *31*, 1595−1599; b) S. Sung, H. Jeon, S. Sarak, M. Murshidul Ahsan, M. D. Patil, W. Kroutil, B. G. Kim, H. Yun, *Green Chem.* **2018**, *20*, 4591–4595.

10.1002/adsc.201801501

[17] a) E. Y. Jeon, J. H. Seo, W. R. Kang, M. J. Kim, J. H. Lee, D. K. Oh, J. B. Park, *ACS Catal*. **2016**, *6*, 7547-7553; b) E. Y. Jeon, J. W. Song, H. J. Cha, S. M. Lee, J. Lee, J. B. Park, *J. Biotech.* **2018**, *281*, 161-167.

[18] a) E. Y. Jeon, J. H. Lee, K. M. Yang, Y. C. Joo, D. K. Oh, J. B. Park, *Process Biochem.* **2012**, *47*, 941-947; b) Y. C. Joo, E. S. Seo, Y. S. Kim, K. R. Kim, J. B. Park, D. K. Oh, *J. Biotechnol.* **2012**, *158*, 17-23; c) E. J. Seo, Y. J. Yeon, J. H. Seo, J. H. Lee, J. P. Boñgol, Y. Oh, J. M. Park, S. M. Lim, C. G. Lee, J. B. Park, *Biores. Technol*. **2018**, *251*, 288–294.

[19] Y. Mohini, R. B. N. Prasad, M. S. L. Karuna, Y. Poornachandra, C. Ganesh Kumar, *Bioorg. Med. Chem. Lett*. **2014**, *24*, 5224–5227.

[20] a) S. Koppireddi, J. H. Seo, E. Y. Jeon, P. S. Chowdhury, H. Y. Jang, J. B. Park, Y. U. Kwon, *Adv. Synth. Catal*. **2016**, *358*, 3084-3092; b) E. J. Seo, Y. J. Yeon, J. H. Seo, J. H. Lee, J. P. Boñgol, Y. Oh, J. M. Park, S. M. Lim, C. G. Lee, J. B. Park, *Biores. Technol.* **2018**, *251*, 288-294; c) J. M. Woo, E. Y. Jeon, E. J. Seo, J. H. Seo, D. Y. Lee, Y. J. Yeon, J. B. Park, *Sci. Rep*. **2018**, *8*, 10280.

10.1002/adsc.201801501

FULL PAPER

Enzyme cascade reactions for the biosynthesis of long chain aliphatic amines from renewable fatty acids

Adv. Synth. Catal. **Year**, *Volume*, Page – Page

Da-Som Lee, Ji-Won Song, Moritz Voß, Eva Schuiten, Ravi Kumar Akula, Yong-Uk Kwon, Uwe Bornscheuer and Jin-Byung Park*