A Bi-Enzymatic Cascade Pathway towards Optically Pure FAHFAs**

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Recently discovered endogenous mammalian lipids, fatty acid esters of hydroxy fatty acids (FAHFAs), have been proved to have anti-inflammatory and anti-diabetic effects. Due to their extremely low abundancies *in vivo*, forging a feasible scenario for FAHFA synthesis is critical for their use in uncovering biological mechanisms or in clinical trials. Here, we showcase a fully enzymatic approach, a novel *in vitro* bi-enzymatic cascade system, enabling an effective conversion of nature-abundant fatty acids into FAHFAs. Two hydratases from *Lactobacillus acidophilus* were used for converting unsaturated fatty acids to various enantiomeric hydroxy fatty acids, followed by esterification with another fatty acid catalyzed by *Candida antarctica* lipase A (CALA). Various FAHFAs were synthesized in a semi-preparative scale using this bi-enzymatic approach in a one-pot two-step operation mode. In all, we demonstrate that the hydratase-CALA system offers a promising route for the synthesis of optically pure structure-diverse FAHFAs.

Introduction

Diabetes mellitus is a metabolic disorder caused by insulin deficiency or insulin receptor resistance and characterized by high blood glucose levels. It was estimated that more than 400 million people are suffering from diabetes worldwide.[1] Diabetes gives rise to several complications such as cardiovascular diseases, eye disease, renal and neurological problems, resulting in higher risk of morbidity and mortality.[2] Thus, diabetes has become a global disease with high economic burden on health systems.[3] The traditional drugs used for diabetes treatment often have various side effects, like xerostomia or burning sensation in the mouth.[4] Therefore, effective prevention and treatments using molecules of natural origin, with low production cost and minimal side effects, are desired. Recently, a new class of endogenous mammalian lipids i.e. fatty acid esters of hydroxy fatty acids (FAHFAs), discovered by Kahn and coworkers in 2014, have shown promising effects for potential treatment of diabetes and inflammatory diseases.[5] Based on the structure of acyl chains and the ester bond position, FAHFAs are grouped in over 20 FAHFA families, which have been found in organisms ranging from plants to humans, especially in mammalian adipose tissue.[5a,6] As shown in Figure 1, we illustrate the structures of several representative FAHFAs and FAHFA-containing triacylglycerols (FAHFA-TGs) that have been identified in mammalian tissues. Among them, palmitic acid esters of hydroxy stearic acids (PAHSAs) are representing one of the most abundant FAHFA families. To date, eight different regioisomers of PAHSAs (5-, 7-, 8-, 9-, 10-, 11-, 12-, and 13-) have been identified, potentially with significant variation in biological function and regulation. For instance, through oral administration to mice, 9-PAHSA has been demonstrated to display effective anti-diabetic effects via promoting insulin secretion and glucose transport, as well as prominent anti-inflammatory effects in dendritic cells. However, 5-PAHSA is only correlative with insulin sensitivity, i.e. anti-diabetic effects.[5a,7]

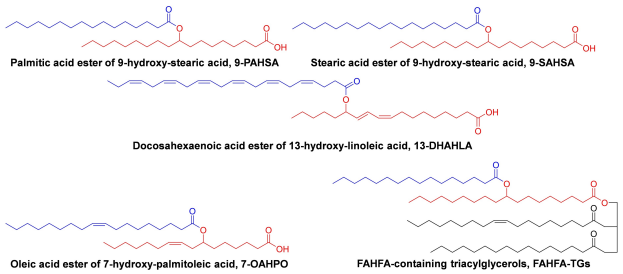

Figure 1. Structures of representative FAHFA families and lipid classes that have been identified in mammalian cells and tissues. 9-PAHSA displays potent anti-diabetic and anti-inflammatory effects.[5a,7] Docosahexaenoic acid-derived FAHFAs (e.g. 13-DHAHLA) have also been shown to exert anti-inflammatory effects in both mice and humans.[6c] FAHFA-TGs play key roles in the regulation of inflammation and metabolic health.[20]

Due to the therapeutic potential toward diabetes and inflammation, the stereospecific and environmentally friendly synthesis of FAHFAs has received substantial attention and remains of high interest (Scheme 1). However, studies on enzymes that synthesize FAHFAs *in vivo* are limited and the abundancies of FAHFAs in biological samples are extremely low and always naturally occurring as isomer mixtures, which make it highly challenging to enrich and isolate the FAHFA isomers from nature.[8] In order to identify and characterize the biological properties of FAHFAs, several chemical synthesis strategies have been developed recently. The first synthetic case of FAHFAs was

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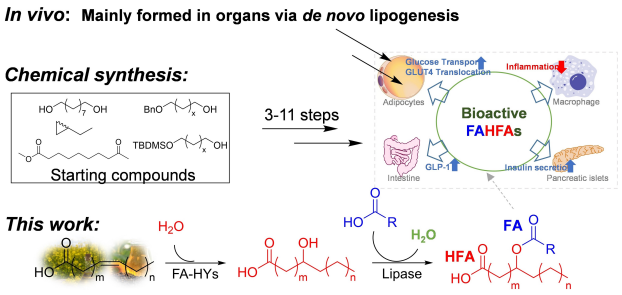


Scheme 1. Overview of the synthesis strategies for FAHFA preparation. *In vivo*: biosynthesis in *de novo* lipogenesis organs, like adipose tissue, liver and kidney; *Chemical synthesis*: starting from 1,9-nonanediol, *S*/*R*-(+)-epichlorohydrin, methyl 9-oxononanoate or monoprotected α,ω-diols; *This work*: bi-enzymatic cascade reaction by combination of hydratase and lipase for producing FAHFAs from renewable biomass.

reported in 2014, where 9-PAHSA was produced starting from 1,9-nonanediol in eleven steps, however, synthesis of the other analogs of 9-PAHSA were not achieved with this strategy.[5a] In another method, several branched FAHFAs have been totally synthesized from terminal alkenes and alkynes in seven steps, including epoxide ring opening with acetylide carbanions mediated by boron trifluoride and hydrogenation of the alkyne function.[9] Subsequently, several similar strategies using Grignard reagents have also been developed starting from non-racemic epichlorohydrin and monoprotected α,ω-diols to accomplish the introduction of the hydroxy group at different positions of the long chain, followed by esterification with another fatty acid molecule for enantioselective synthesis of FAHFAs.[10]

The key step of FAHFA synthesis is the introduction of the hydroxy group at different positions of the fatty acids (FAs) to produce hydroxy fatty acids (HFAs). Thus, the complexity of chemical synthetic strategies are caused by the challenge of hydroxylation, which involve harsh reaction conditions like high temperatures, hazardous organic solvents and elaborate group protecting steps.[11] Many chemical methods have low selectivity and do not render the biologically active enantiomer.[10a] However, long-term biological studies as well as potential medical treatments and dietary supplements require a sufficient supply of the desired FAHFAs. In this respect, enzymatic methods have great potential for high-scale production of desired FAHFAs with excellent selectivity in a green manner. In contrast to chemical catalysts, Nature has developed an extensive list of enzymes to generate HFAs from FAs, including lipoxygenases, cytochrome P450s and fatty acid hydratases.[12] Among them, fatty acid hydratases (FAHs) are able to perform asymmetric synthesis of enantiomerically pure HFAs from abundant renewable unsaturated fatty acids. FAHs use water as a substrate and catalyze the hydration without the need for continuous supply of reducing cofactors (e.g. NAD(P)H), which present an attractive green pathway for the production of HFAs.[13] Although, until recently, low substrate promiscuity and limited regioselectivity of FAHs had been the main hurdle for their wider applicability, we demonstrated earlier that wild-type and rationally engineered variants of two FAHs from *Lactobacillus acidophilus* greatly expanded substrate scope and regioselectivity of fatty acid hydration leading to generation of various HFAs with different –OH positions (Table 1).[14] Moreover, we have shown semi-preparative scale enzymatic synthesis of a number of HFA products, with excellent regio- and stereoselectivity, using these wild-type and engineered FAHs (Table S1). Inspired by our diversiform FAH toolbox enabling the synthesis of various HFAs, we envisioned that we could use lipases in tandem with FAHs for the efficient synthesis of various FAHFAs, through a novel bi-enzymatic cascade pathway as shown in Scheme 1. Thus, on the basis of FAH driven conversion of FAs to HFAs, we executed the following work strategy: enabling the biotransformation of HFA to FAHFA, and then assembling the two conversion steps in tandem in one reaction system, affording a fully enzymatic synthesis of FAHFAs.

Results and Discussion

Biotransformation of HFA to FAHFA

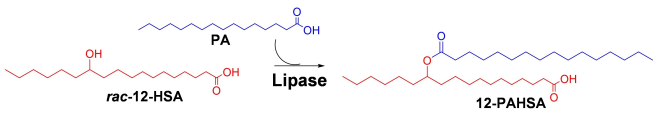
In a first round of experiments, we screened 21 commercially available lipases to explore their esterification activity toward palmitic acid (PA) and *rac*-12-hydroxystearic acid (*rac*-12-HSA), selected as a model reaction for HFA to FAHFA conversion. Small-scale (1 mL) activity assays were performed at 60 °C using anhydrous toluene (< 0.001 % water) as solvent. To avoid the self-esterification of *rac*-12-HSA, the concentration of PA was excessive (2-fold) over *rac*-12-HSA. After 24 h, the reactions were analyzed by GC-FID (see Supporting Information for method), and half of the tested lipases exhibited activity as shown in Table 2 (column 2). Among them, five candidates have shown high to excellent activities, with > 55 % conversion. The active lipases can be categorized into two sources of strains: *Candida antarctica A* and *Candida rugosa* (*C. cylindracea* is a former name of *C. rugosa*); even though they may be from different producers of different activities with/without genetic modification. However, the structures and substrate binding pockets of these lipases are highly similar with tunnel-like binding sites.[15] Considering the compatibility of FAH and a robust lipase, we decreased the screening reaction temperature to 30 °C in the second round of experiments. Interestingly, only CALA retained high conversion rate under lower temperature (85.3 % at 30 °C vs. 91.9 % at 60 °C, Table 2), which indicated that CALA displays a prominent activity and a broad temperature range for the synthesis of FAHFAs. Actually, the substrate specificity of CALA had been explored in many studies, which demonstrated that CALA exhibited a relatively high activity towards straight-chained primary alcohols and carboxylic acids, as well as sterically hindered secondary and tertiary alcohols.[16] However, low or no activity was observed towards very short-chain acids.[16] In addition, CALA was also used for enzymatic synthesis of fatty acid polyesters (estolide) from 12-hydroxy-9-*cis*-octadecenoic acid (ricinoleic acid).[17] These results indicate that CALA has a unique applicability for the esterification of secondary alcohols (like HFAs) and long-chain FAs, which is an excellent candidate for production of FAHFAs. Thus, CALA was chosen for further investigation in our case.

Table 1. Hydroxy fatty acids that can be synthesized from fatty acid precursors by two FAHs from *L. acidophilus* and their variants as reported in previous studies.[14]

Enzyme	Fatty acid	Hydroxy fatty acid[a]	Conv. %[a]
FA-HY2	Palmitoleic acid (16:1$^{\Delta 9}$)	10-Hydroxy-16:0	89.3
FA-HY2	Oleic acid (18:1$^{\Delta 9}$)[b]	10-Hydroxy-18:0	96.9
FA-HY2	Linoleic acid (18:2$^{\Delta 9,\Delta 12}$)[b]	(*S*)-10-Hydroxy-*cis*-12-18:1[a]	40.9
FA-HY2	α-Linolenic acid (18:$^{3\Delta 9,\Delta 12,\Delta 15}$)[b]	10-Hydroxy-*cis*-12,*cis*-15-18:2	60.8
FA-HY2	Arachidonic acid (20:4$^{\Delta 5,\Delta 8,\Delta 11,\Delta 14}$)	12-Hydroxy-*cis*-5,*cis*-8,*cis*-14-20:3	1.7
FA-HY1	*cis*-Vaccenic acid (18:1$^{\Delta 11}$)[b]	12-Hydroxy-18:0	58.8
FA-HY1	Linoleic acid (18:2$^{\Delta 9,\Delta 12}$)[b]	(*R*)-13-Hydroxy-*cis*-9-18:1	48.1
FA-HY1	Pinolenic acid (18:3$^{\Delta 5,\Delta 9,\Delta 12}$)	13-Hydroxy-*cis*-5,*cis*-9-18:2	57.0
FA-HY1	γ-Linolenic acid (18:3$^{\Delta 6,\Delta 9,\Delta 12}$)	13-Hydroxy-*cis*-6,*cis*-9-18:2	56.6
FA-HY1	α-Linolenic acid (18:3$^{\Delta 9,\Delta 12,\Delta 15}$)[b]	(*S*)-13-Hydroxy-*cis*-9,*cis*-15-18:2	53.8
FA-HY1	Stearidonic acid (18:4$^{\Delta 6,\Delta 9,\Delta 12,\Delta 15}$)	13-Hydroxy-*cis*-6,*cis*-9,*cis*-15-18:3	12.1
FA-HY1	Eicosadienoic acid (20:2$^{\Delta 11,\Delta 14}$)	15-Hydroxy-*cis*-11-20:1	9.2
FA-HY1	Mead acid (20:3$^{\Delta 5,\Delta 8,\Delta 11}$)	12-Hydroxy-*cis*-5,*cis*-8-20:2	18.7
FA-HY1	Sciadonic acid (20:3$^{\Delta 5,\Delta 11,\Delta 14}$)	15-Hydroxy-*cis*-5,*cis*-11-20:2	51.7
FA-HY1	Dihomo-γ-linolenic acid (20:3$^{\Delta 8,\Delta 11,\Delta 14}$)	15-Hydroxy-*cis*-8,*cis*-11-20:2	14.1
		12-hydroxy-*cis*-8,*cis*-14-20:2	4.7
FA-HY1	Eicosatrienoic acid (20:3$^{\Delta 11,\Delta 14,\Delta 17}$)	12-Hydroxy-*cis*-14,*cis*-17-20:2	0.4
		15-Hydroxy-*cis*-11,*cis*-17-20:2	0.2
FA-HY1	Arachidonic acid (20:4$^{\Delta 5,\Delta 8,\Delta 11,\Delta 14}$)	15-Hydroxy-*cis*-5,*cis*-8,*cis*-11-20:3	45.7
FA-HY1	Eicosapentaenoic acid (20:5$^{\Delta 5,\Delta 8,\Delta 11,\Delta 14,\Delta 17}$)	12-Hydroxy-*cis*-5,*cis*-8,*cis*-14,*cis*-17-20:4	10.3
		15-Hydroxy-*cis*-5,*cis*-8,*cis*-11,*cis*-17-20:4	6.0
FA-HY1	Docosahexaenoic acid (22:6$^{\Delta 4,\Delta 7,\Delta 10,\Delta 13,\Delta 16,\Delta 19}$)	14-Hydroxy-*cis*-4,*cis*-7,*cis*-10,*cis*-16,*cis*-19-22:5	0.6
FA-HY2-T391S[c]	γ-Linolenic acid (18:3$^{\Delta 6,\Delta 9,\Delta 12}$)	10-Hydroxy-*cis*-6,*cis*-12-18:2	35.6
FA-HY2-H393S	Docosahexaenoic acid (22:6$^{\Delta 4,\Delta 7,\Delta 10,\Delta 13,\Delta 16,\Delta 19}$)	14-Hydroxy-*cis*-4,*cis*-7,*cis*-10,*cis*-16,*cis*-19-22:5	2.8
FA-HY2-T391S/H393S/I378P	Dihomo-γ-linolenic acid (20:3$^{\Delta 8,\Delta 11,\Delta 14}$)	(*S*)-12-Hydroxy-*cis*-8,*cis*-14-20:2	92.1
FA-HY2-T391S/H393S/I378P	Eicosapentaenoic acid (20:5$^{\Delta 5,\Delta 8,\Delta 11,\Delta 14,\Delta 17}$)	(*S*)-12-Hydroxy-*cis*-5, *cis*-8, *cis*-14,*cis*-17-20:4	69.4

[a] The conversions and absolute configurations of the hydroxy fatty acids were reported in previous studies.[14] [b] Indicates that the hydration of these substrates were used to verify the cascade reaction in a semi-preparative scale in this study. [c] Only the variants that exhibited the highest conversion were displayed.

Table 2. Lipase screening for fatty acid and hydroxy fatty acid esterification.[a]



Commercial lipase	Conv. % (60 °C)	Conv. % (30 °C)
CALA (Immobilized *Candida antarctica* lipase A from Codexis)	91.9	85.3
CALB (Liquid/Unimmobilized *Candida antarctica* lipase B)	4.0	ND[b]
Novozyme 435 FG (*Candida antarctica* lipase B immobilized on a macroporous acrylic resin)	6.6	ND
Lipozyme 435 (*Candida antarctica* lipase B immobilized on silica)	27.5	ND
Lipozyme TL IM (Lipase from *Thermomyces lanuginosus* immobilized on silica)	1.7	ND
Lipozyme RM IM (Lipase from *Rhezomucor miehei* immobilized on a macroporous ion-exchange resin)	ND	ND
NS-40042 (Genetically modified lipase from *Thermomyces lanuginosus*)	4.1	ND
NS-40079 (Genetically modified lipase from *Thermomyces lanuginosus*)	10.2	ND
Lipase A "Amano" 12 (From *Aspergillus niger*)	ND	ND
Lipase D "Amano" 350 (From *Rhizopus delemar*)	ND	ND
Lipase G "Amano" 50 (From *Penicillium camemberti*)	ND	ND
Lipase M "Amano" 10 (From *Mucor javanicus*)	ND	ND
Lipase OF (From *Candida cylindracea* (*C. rugosa*))	56.5	5.1
Lipase MY (From *Candida cylindracea* (*C. rugosa*))	81.5	19.5
Lipase F-AP15 (From *Rhizopus oryzae*)	ND	ND
Lipase PS–D "Amano" I (From *Burkholderia cepacia*)	ND	ND
Lipase from *Candida rugosa* (Sigma, powder)	63.4	21.1
Lipase from *Candida cylindracea* (Sigma, powder)	69.7	9.5
Lipase from porcine pancreas (Sigma, powder)	1.2	ND
Lipase from *Candida rugosa* immobilized on Immobead 150 (Sigma, powder)	3.6	ND
Lipase AK from *Pseudomonas fluorescens* immobilized on Immobead 150 (Sigma, powder)	ND	ND

[a] Reaction system: Lipase-10 mg, 10 mg/ml PA, 5 mg/ml *rac*-12-HSA, Toluene, 30 or 60 °C, 250 rpm, 24 h. [b] ND: not detected.

Subsequently, the reaction was scaled up to 20 mL for product preparation and identification (palmitic acid esters of 12-hydroxystearic acids, 12-PAHSA) under the same conditions. From time-course analysis (Figure S6), it was obvious that there was no significant difference when reactions were performed at 30 °C or 60 °C (final conversion was slightly higher at 60 °C), which was

consistent with the small scale screening result. The reaction rate started decreasing after 1.5–2.0 h, and conversion levels reached 86.8% or 90.5% at 8 h for 30 °C or 60 °C, respectively. Less than complete conversion is probably due to the generation of water as the by-product, driving the equilibrium towards reactant side.[18] Afterwards, purification of the product was carried out on preparative TLC plate, giving 78.1% isolated molar yield and 90.1% purity. The structure of isolated product was confirmed by ^{1}H and ^{13}C NMR (Figure S18–19).

Preliminary optimization of the two conversion steps

We also simply compared the catalytic capacity of CALA under different conditions, in terms of reaction solvents and the ratio of PA/*rac*-12-HSA (Table S2). As anticipated, the esterification activity of CALA was completely lost in single aqueous phase (Kpi, 100 mM potassium phosphate buffer, pH 6.0). When toluene was included, the conversion rates achieved were 43.3% and 69.9% in Kpi/Toluene (1:1 v/v) and pure toluene, respectively (Table S2, entry 1–3). We next investigated the effect of the PA/*rac*-12-HSA ratio on the production of 12-PAHSA. The yield of 12-PAHSA improved when a higher PA/*rac*-12-HSA ratio was reached. By increasing the PA concentration to 10 mg/mL and lowering the *rac*-12-HSA concentration to 1 mg/mL, we were able to achieve the highest conversion at 95.1% (Table S2, entry 3–5). Moreover, we performed a control reaction without PA substrate (only involved *rac*-12-HSA). As expected there was no peak observed for 12-PAHSA, however, a new peak was observed with less than 10% conversion which might be caused by the self-esterification of *rac*-12-HSA (Figure S10). Interestingly, this product was not observed when PA was present in the reaction system, indicating a lower reactivity of the *rac*-12-HSA as an acyl donor.

After demonstrating efficient enzymatic conversion of a HFA to FAHFA, we next explored a bi-enzymatic cascade reaction where FAH and CALA are combined for direct conversion of renewable FAs into FAHFAs. As described in the literature, one of the hurdles in the biotransformation of plant oils is the low fatty acid transport rate across the membrane of the cells from or into the media.[19] Since we initially aimed at using whole cells expressing hydratase as the biocatalyst in the first step of our cascade, we firstly investigated FA and HFA transport through the membrane of *E. coli* cells. After performing the hydration of linoleic acid (LA) using the *E. coli* whole-cells (containing FA-HY1) as catalyst, both LA and 13-hydroxy-*cis*-octadec-9-enoic acid (13-HODA) were observed at higher concentration in the cells (over 4-fold than the supernatant, Figure S7), which indicated that the HFA product was trapped in majority inside the cells. To ensure a sufficient HFA interaction with the second enzyme CALA, we chose to use cell lysates of *E. coli* expressing FAHs in the cascade reaction, facilitating FAHFAs preparation.

Assembling and optimization of the bi-enzymatic cascade

As described above, no esterification reaction takes place between PA and *rac*-12-HSA when CALA was subjected to single-phase aqueous buffer. Thus, in order to identify the optimal solvent system for the cascade, we evaluated the conversion rate of FA-HY1 and CALA in different biphasic systems (Figure 2a). Several organic solvents with the *log* P value varying from 2.5 to 5.8 were selected, as well as three versatile eco-friendly solvents (2-methyltetrahydrofuran, 2-MeTHF; cyclopentyl methyl ether, CPME; and γ-valerolactone, GVL), which have been shown as promising bio-solvents in lipase-catalyzed reactions.[20] Obviously, due to the poor organic solvent tolerance and low stability, the conversion rate of LA by FA-HY1 in nine of the biphasic systems were lower than 10% compared with 74.5% in single Kpi buffer. CALA displayed similar performance, with less than 30% esterification in most of the biphasic systems. However, 90.4% conversion was observed in Kpi/Toluene system, which seems to be optimum for the bi-enzymatic cascade. The three green solvents are not suitable for either hydratase or CALA in our case.

We further compared two different operation modes in Kpi/Toluene biphasic system; one-pot one-step mode where all enzymes and substrates were added at the start of the reaction; and one-pot two-step mode in which the hydration reaction of unsaturated fatty acid was performed first (in single Kpi buffer), followed by the addition of CALA, toluene and another fatty acid. As shown in Figure 2c, different Kpi/Toluene ratios were evaluated for both reaction modes. The highest overall conversion was achieved at 65% with one-pot two-step mode

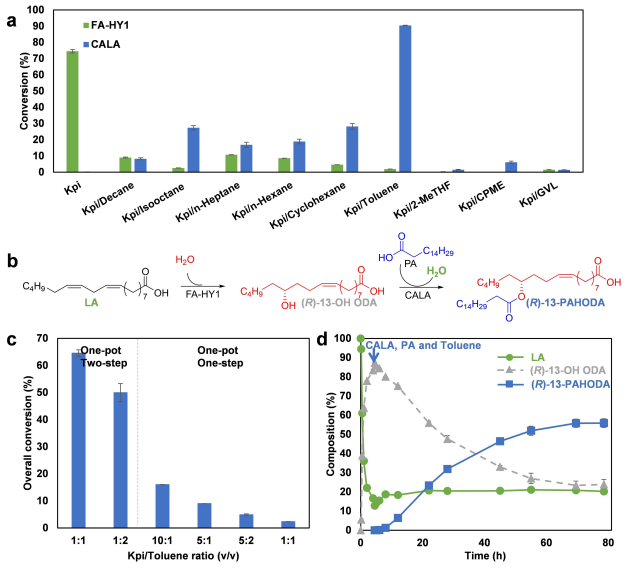


Figure 2. Cascades for the transformation of LA into (*R*)-13-PAHODA. a) Organic solvent tolerance of FA-HY1 and CALA. Kpi means only Kpi buffer without organic solvent, the volume ratio of Kpi/organic solvent for other reactions is 1:1. b) Reaction scheme for the synthesis of (*R*)-13-PAHODA from LA with the bi-enzymatic cascade. c) The influence of reaction mode and solvent ratio to the cascade reaction. d) Time dependent one-pot two-step bi-enzymatic cascade for biotransformation of LA and PA into (*R*)-13-PAHODA.

using 1:1 Kpi/Toluene ratio. With one-pot one-step mode, all of the tested solvent ratios resulted in less than 20% overall conversion, which can be attributed to the instability of FAHs in the presence of organic solvent. Therefore, for all further experiments, a one-pot two-step operation was followed. Under the optimized one-pot two-step conditions, the enzymatic synthesis of (*R*)-palmitic acid ester of 13-hydroxy-*cis*-octadec-9-enoic acid ((*R*)-13-PAHODA) from LA and PA was scaled up to 100 mL in a semi-preparative scale. FA-HY1 almost completely converted LA into its 13-OH product ((*R*)-13-HODA) in 8 h (Figure 2d). The second step took significantly longer after adding CALA, PA and toluene to the sample pot. The final composition of (*R*)-13-PAHODA amounted up to 56% at plateau stage. In other words, the cascade reaction reaches an equilibrium, which might be caused by a reversible balance between hydrolysis and esterification.

Substrate scope exploration

As described above, FAHFA regioisomers display differential regulation and biological activity,[5a,7] which have also been observed for FAHFA enantiomers. For example, only (*R*)-9-PAHSA enantiomer was identified as the predominant enantiomer that accumulates in adipose tissues from transgenic mice.[10a] Encouraged by the broad substrate promiscuity of our FAHs library, the substrate scope of this proof-of-concept bi-enzymatic cascade was further expanded by employing the above one-pot two-step approach for the production of different enantiomeric FAHFAs (Figure 3). The typical hydratase, FA-HY2, can only add water on Δ9 double bonds to produce 10-OH products, whereas the unique FA-HY1 preferably hydrates double bonds other than Δ9 to generate 12-OH, 13-OH, 14-OH and 15-OH products. As we reported previously, FA-HY1 and FA-HY2 are highly stereospecific enzymes that generate a single stereoisomer product with an enantiomeric excess of over 95%.[14] Therefore, several pairs of FAH-substrates that exhibited high conversion rates in our previous study,[14a] were chosen for FAHFA preparation. By using either FA-HY1 or FA-HY2, oleic acid (OA), LA, *cis*-vaccenic acid (CVA) and α-linolenic acid (ALA) were converted to corresponding HFAs with different –OH position, followed by the esterification with PA catalyzed by CALA. From the semi-preparative reactions in 100 mL media, several branched FAHFA products were synthesized, and the overall conversion of FAHFA products can be achieved at 28–67%. Since the polarity of the remaining excess fatty acid substrates and FAHFA products are quite similar, trailing phenomena of these compounds on silica gel rendered the separation of fatty acids and FAHFAs very difficult. To eliminate the trailing phenomena caused by the strong interaction between the carboxyl groups of FAHFA and silica gel for a better FAHFA isolation, methylation of reaction mixture before purification was performed to convert the carboxyl groups to corresponding methyl esters. On the other hand, methyl esters (or other esters) of FAHFAs can afford similar reactivity as free FAHFAs as acyl donors for interesterification or biological incorporation into triglycerides, which are the major form of FAHFAs in the tissues.[21] As shown in Figure S8, a better resolution of fatty acids and FAHFA products was observed on TLC plate after methylation. Then, the methylated FAHFA products were purified by preparative TLC plate. Among them, palmitic acid ester of 12-hydroxy steric acid (12-PAHSA), prepared by converting CVA to 12-HSA (catalyzed by FA-HY1) and followed by esterification with PA (catalyzed by CALA), was isolated in a highest molar yield of 42%. The majority of the purified FAHFA products had a purity of >90%, as determined by GC-FID analysis. The structures of isolated products were confirmed by ^{1}H and ^{13}C NMR (Figures S20–31). The absolute configurations of three products i.e. palmitic acid ester of 10-hydroxy-*cis*-octadec-12-enoic acid (10-PAHODA), palmitic acid ester of 13-hydroxy-*cis*-octadec-9-enoic acid (13-PAHODA) and palmitic acid ester of 13-hydroxy-*cis*-9,*cis*-15-octadecadienoic acid (13-PAHODDA) can be given as (*S*), (*R*) and (*S*), respectively, based on our previous analysis of the corresponding chiral HFAs. The enantiomeric excess (*ee*) of relevant HFA products were >95%.[14]

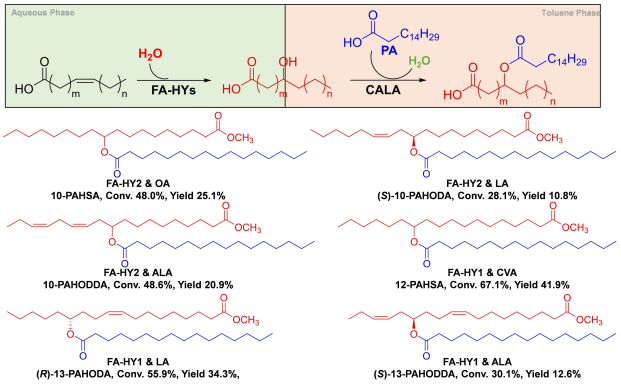


Figure 3. Substrate scope of hydratase-lipase cascade reaction. The reactions were performed in a one-pot two-step procedure as described above. Conversion = $[FAHFA]_{final}/[Unsaturated\ fatty\ acid]_{initial} \times 100\%$; determined via GC-FID as described in the Supporting Information. Yield: isolated molar yield of methylated FAHFA products.

Conclusion

In summary, we have successfully demonstrated that the medically important endogenous lipids of FAHFAs can be synthesized enzymatically from renewable fatty acids using the FAHs-CALA catalytic system, either by a two-step synthesis or by a bi-enzymatic cascade of a one-pot two-step process. To our knowledge, the only other study that reported synthesis of fatty acid estolides (including FAHFAs) by an enzymatic cascade approach utilizing hydratase and lipase is a patent,[22] where oleate hydratase (OhyA) was used for the hydration of unsaturated FAs at the Δ9-position to produce only 10-OH products. By utilizing two different FAHs from *L. acidophilus* and various fatty acids, our system greatly expands the product scope by enabling preparation of various enantiomeric structures of FAHFAs, possibly associated with differential regulatory and biological functions. Our results suggested that the two

types of lipases from *C. antarctica* A and *C. rugosa* are highly selective and active catalysts for esterification between FAs and HFAs. Lipases from both strains exhibit similar tunnel-like binding sites with ample space, which is capable to accept bulky secondary alcohol donors; thus allowing effective interaction of two reactants with large steric hindrance.[15,23] Overall, we demonstrate a promising method for enzymatic synthesis of a variety of FAHFAs from sustainable fatty acids. We admit that our approach is still in the conceptual stage, with low substrate loading and incomplete transformation, probably caused by the thermodynamic equilibrium of the second step in a biphasic reaction system. It has been suggested that esterification is a thermodynamically controlled process; the molar ratio of substrates and water content strongly affect the initial reaction rate and the final conversion, which can be enhanced by maintaining the water activity sufficiently low to push the thermodynamic equilibrium towards the formation of the product.[24] Therefore, further optimization and reaction engineering studies should enable feasible large-scale production of FAHFAs for use in pre-clinical and clinical studies as well as for future applications as medications and as dietary supplements. Moreover, due to the limited regioselectivity of FAHs as well as the limited double bond positions of natural fatty acids, the proposed method cannot achieve the synthesis of some FAHFAs, like 5-FAHFAs and 9-FAHFAs. Protein engineering efforts to further increase the activity as well as to expand the substrate scope and regiodiversity of FAHs is ongoing in our lab. Use of lipoxygenases and cytochrome P450s, which also add –OH groups on FAs with complementary selectivity and substrate scope to FAHs, can further diversify the product spectra of enzymatically synthesized FAHFAs.

Experimental Section

Materials

Fatty acid substrates were purchased from Sigma-Aldrich (now Merck), except *cis*-vaccenic acid (CVA) which was from MP Biomedicals. All FAs had a purity of at least 98.5%. N,O-Bis (trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA +TMCS; 99:1) was purchased from Fisher Scientific. All other chemicals were commercially available and analytical grade. Immobilized *Candida antarctica* lipase A (CALA) is kindly provided by Codexis. Liquid/unimmobilized *Candida antarctica* lipase B (CALB), CALB immobilized on a macroporous acrylic resin (Novozym 435 FG), CALB immobilized on silica (Lipozyme 435), lipase from *Thermomyces lanuginosus* immobilized on silica (Lipozyme TL IM), lipase from *Rhezomucor miehei* immobilized on a macroporous ion-exchange resin (Lipozyme RM IM), genetically modified lipase from *Thermomyces lanuginosus* (NS-40042, NS-40079) are kindly provided by Novozymes (Denmark). Lipase A "Amano" 12 from *Aspergillus niger*, lipase D "Amano" 350 from *Rhizopus delemar*, lipase G "Amano" 50 from *Penicillium camemberti*, lipase M "Amano" 10 from *Mucor javanicus*, lipase OF from *Candida cylindracea* (*C. rugosa*), lipase MY from *Candida cylindracea* (*C. rugosa*), lipase F-AP15 from *Rhizopus oryzae* and lipase PS–D "Amano" I from *Burkholderia cepacia* are kindly provided by Amano Enzyme. Lipase from *Candida rugosa* (L1754-5G), lipase from *Candida cylindracea* (62316-50G), lipase from porcine pancreas (L3126-25G), lipase from *Candida rugosa* immobilized on immobead 150 (89444-10G), lipase from *Pseudomonas fluorescens* immobilized on immobead 150 (90678-10G) are purchased from Sigma-Aldrich (now Merck). *fa-hy1* (GenBank ID: LC030242.1) and *fa-hy2* (Genebank ID LC030243.1) genes from the strain *Lactobacillus acidophilus* NTV001 were received (from Prof. Jun Ogawa's group at Kyoto University) as cloned into pET-21b vector using XhoI and NdeI sites including a stop codon at the end of the genes.

Protein expression

Following transformation of *E.coli* Rosetta (DE3) cells (Merck Millipore) with pET-21b-*fa-hy1* or pET-21b-*fa-hy2* plasmids, starter cultures of 5 mL were grown overnight at 37 °C in LB media supplemented with a final ampicillin concentration of 100 μg/mL. For protein expression, 0.5 L of LB media was inoculated with 5 mL of overnight culture and the expression was induced by addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the OD_{600} reached 0.6-1.0. After IPTG addition, the expression was continued at 20 °C and 180 rpm for 16–20 h. Cells were harvested by centrifugation at 5,000 rpm and 4 °C for 30 min. After washing the cells twice with 0.85% NaCl solution, the cell pellet was stored at −80 °C. For cell lysis, the cell pellet was resuspended in an ice-cold lysis buffer of 100 mM potassium phosphate buffer (Kpi) at pH 6.0. Sonicator (Bandelin Sonopuls) was used for cell disruption on ice. After sonication, cell lysate was centrifuged at 12,000 rpm for 30 min at 4 °C and the supernatant was used for following reactions.

Small-scale reactions

Small-scale reactions were set up aerobically (we have determined that there is no significant oxidation of the unsaturated fatty acids up to 12 h, as shown in Figure S5) on 1 mL scale and were placed in 5-mL glass screw top vials. After all materials were added, the vials were then sealed with a cap and moved to a shaker. After shaking at the indicated temperature, speed and time, ethyl acetate was added for extraction. The mixture was vortexed for a few seconds, and the phases were separated by centrifugation at room temperature and 12,000 rpm for 10 min. For totally organic phase reactions, the extraction step can be escaped. The organic phase was moved to a clean Eppendorf tube, and the solvent was completely evaporated under a gentle nitrogen flow. Subsequently, 100 μL BSTFA + TMCS (99:1) was added to convert the extracted reaction mixture into their TMS derivatives at 60 °C for 30 min. After silylation, samples were moved to a 2 mL glass GC screw top vial with a glass insert and analyzed via GC-FID.

Optimization of the cascade reaction

The effect of various organic solvents, including decane, isooctane, *n*-heptane, *n*-hexane, cyclohexane, toluene, 2-MeTHF, CPME, and GVL on enzyme activity of FA-HY1 and CALA were tested to determine an optimal biphasic reaction system of the bi-enzymatic cascade reaction. For FA-HY1 reaction system, 1 mL reaction included 500 μL organic solvent, 500 μL supernatant of *E. coli* cell lysate expressing FA-HY1 (sonication of 50 mg/mL cells in 100 mM Kpi, pH 6.0) and 2 mg linoleic acid (LA). For CALA reaction system, 1 mL reaction included 500 μL organic solvent, 500 μL Kpi (100 mM, pH 6.0), 10 mg palmitic acid (PA) and 5 mg *rac*-12-hydroxystearic acid (*rac*-12-HSA). After shaking at 30 °C and 220 rpm for 12 h, reactions were extracted by adding 1 mL ethyl acetate. After evaporation and silylation, samples were analyzed by GC-FID. The optimum reaction operation mode was also determined by comparing the overall conversions in one-pot one-step (all of the starting materials and enzymes were added at the beginning) or

one-pot two-step approach (the hydration reaction of one unsaturated fatty acid was first performed in single Kpi buffer, followed by the addition of CALA, toluene and another fatty acid). For one-pot one-step reactions, all of the starting materials were mixed at the beginning, including 500–1000 μL supernatants of FA-HY1, 100–500 μL toluene, 10 mg CALA, 2 mg LA and 4 mg PA. Reactions were performed at 30 °C and 220 rpm for 24 h, then ethyl acetate was added for extraction. For one-pot two-step reactions, the hydration reaction of the LA was first performed in single Kpi buffer including 1 mL supernatants of FA-HY1 and 2 mg LA. After 12 h, then the esterification was activated by the addition of 10 mg CALA, 1–2 mL toluene and 4 mg PA. After shaking at 30 °C and 220 rpm for another 12 h, reactions were centrifuged and the toluene phases were collected. After evaporation and silylation, samples were analyzed by GC-FID.

Preparative-scale reactions and product purification

All preparative-scale reactions were set at a scale of 20 mL (esterification of PA and *rac*-12-HSA by CALA) or 100 mL (bi-enzymatic cascade) total reaction volume in Erlenmeyer flasks under aerobic condition. Reaction mixtures were incubated at 30 °C with shaking at 220 rpm. For esterification of PA and *rac*-12-HSA, 100 mg *rac*-12-HSA, 200 mg PA, 200 mg CALA and 20 mL toluene were mixed and reactions were performed at 30 and 60 °C, 200 rpm, respectively. The bi-enzymatic cascade reactions started with an unsaturated FA substrate (100 mg) and 50 mL supernatants of *E. coli* cells expressing the appropriate hydratase (50 mg/mL) in 100 mM Kpi buffer, pH 6.0 in first hydration step. The second step was started by the addition of CALA (500 mg), PA (200 mg) and 50 mL toluene. Extraction of the lipid components from the reaction mixture was performed by using ethyl acetate, similar to that for the 1 mL scale assays, except the volumes of solvents used for extraction were increased 100-fold and the extraction was performed in several 50 mL centrifuge tubes. The upper organic phase was recovered, the solvent was evaporated by a Buchi rotary evaporator. The reaction mixture after evaporation was methylated by following steps: add 2 mL of BF_3-methanol and 0.6 mL of hydroquinone; heat at 80 °C on the heating block for 2 min, shake once in a while, cool to room temperature; add 0.4 mL of salt solution, shake for 10 sec; add 2 mL of heptane, shake for 10 sec; centrifuge at 4,000 rpm for 5 min; take the supernatant for evaporation. Then, the methylated mixture was re-dissolved in ethyl acetate (1–2 mL) for purification of FAHFA products on silica gel by preparative TLC plates (L×W, 20 cm×20 cm, Merck). The solvent path was 18 cm and the development system was hexane/acetone (20:1, v/v). A Hanessian's stain was used to stain the TLC plates. The bands with the Rf value at 0.41, which belonged to the FAHFA products, were scraped out of the TLC plates. The products in silica gel powder were extracted with ethyl acetate and evaporated by a Buchi rotary evaporator. The amount of dried products was measured for yield calculation. Chemical structure identification of FAHFA products were recorded on a Bruker Avance III spectrometer (400 MHz).

GC-FID analysis

All samples analyzed via GC-FID were treated by silylation reagent to convert the reaction mixture into their TMS derivatives. GC-FID analysis was performed using Scion 436-GC (Bruker, Billerica, MA) system equipped with a flame ionization detector (FID) and a non-polar fused silica capillary Zebron ZB-5HT column (20 m length× 0.18 mm I.D.×0.18 μm film thickness; Phenomenex). The oven program was a starting temperature of 150 °C, a ramp of 4 °C min^{-1} was settled to 310 °C, followed by a hold for 20 min at 310 °C. Injector and detector temperature was set at 320 °C and the column flow was maintained at a constant pressure of 22 Psi throughout the analysis with helium as the carrier gas. Conversion was calculated by the standard curves of palmitic acid (PA), 12-hydroxystearic acid (*rac*-12-HSA) and palmitic acid ester of 9-hydroxystearic acid (9-PAHSA).

Table 3. List of abbreviations

Abbreviation	Explanation
FAHFAs	Fatty acid esters of hydroxy fatty acids
CALA	*C. antarctica* lipase A
FAHFA-TGs	FAHFA-containing triacylglycerols
PAHSA	Palmitic acid esters of hydroxy stearic acid
FAs	Fatty acids
HFAs	Hydroxy fatty acids
FAHs	Fatty acid hydratases
PA	Palmitic acid
rac-12-HSA	*rac*-12-Hydroxystearic acid
LA	Linoleic acid
HODA	Hydroxy-octadec-enoic acid
OA	Oleic acid
CVA	*cis*-Vaccenic acid
ALA	α-Linolenic acid
HODDA	Hydroxy-octadecadienoic acid
2-MeTHF	2-Methyltetrahydrofuran
CPME	Cyclopentyl methyl ether
GVL	γ-Valerolactone
Kpi	Potassium phosphate buffer

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: bioactive FAHFAs · enzymatic synthesis · fatty acid hydratase · lipase · renewable biomass

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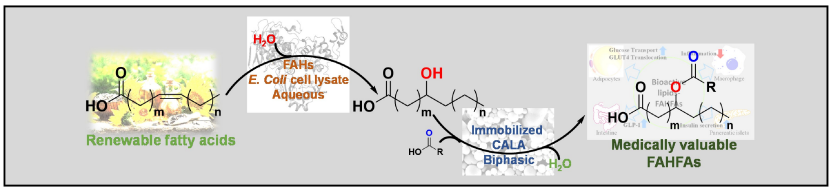

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Full enzymatic synthesis of FAHFAs: Various hydroxy fatty acids with different –OH positions can be synthesized from renewable fatty acids by wild-type and engineered fatty acid hydratases, which can be cascaded with lipase to prepare value-added FAHFAs with potential medical value.