

A bi-enzymatic cascade pathway towards FAHFAs

Yan Zhang, Bekir Engin Eser* and Zheng Guo*

Abstract: Recently discovered endogenous mammalian lipids fatty acid esters of hydroxy fatty acids (FAHFAs), 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 mechanism or 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 acid into FAHFAs. Two hydratases from *L. acidophilus* were used for converting unsaturated fatty acids to various stereospecific hydroxy fatty acids, followed by esterification with another fatty acid catalyzed by *C. antarctica* lipase A (CALA). Various FAHFAs were synthesized in a preparative scale using this bi-enzymatic approach in a one-pot two-step operation mode. In all, we demonstrated that hydratase-CALA system promises a sustainable solution to the synthesis of structure-diverse stereospecific 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 effect 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) is one of the first reported and most abundant FAHFA family. 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]

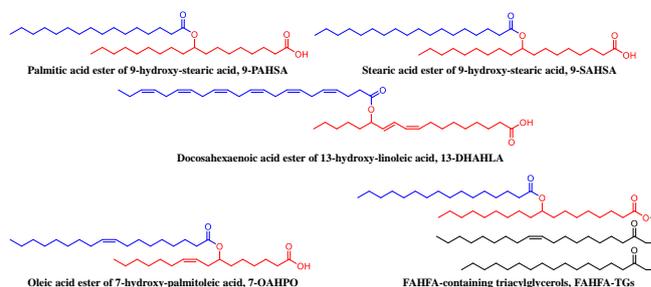
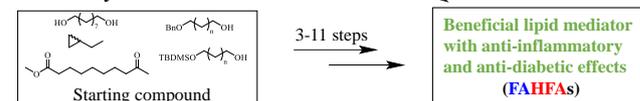


Figure 1. Structures of representative FAHFA families and lipid classes have been identified in mammalian cells and tissues. 9-PAHSA displays potent anti-diabetic and anti-inflammatory.^[5a, 7] Docosahexaenoic acid-derived FAHFAs (e.g. 13-DHAHLA) has also been shown to exert anti-inflammatory effects in both mice and humans.^[6c] FAHFA-TGs play key roles in regulation of inflammation and metabolic health.^[2d]

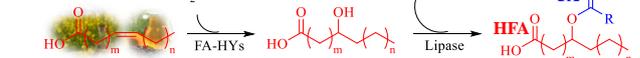
Due to the therapeutic potential toward diabetes and inflammation, the stereospecific and environmental friendly synthesis of FAHFAs has received substantial attention and remains of high interest (**Scheme 1**). However, studies on enzymes that synthesize FAHFAs *in vivo* are limited and the abundancies 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 reported in 2014, where 9-PAHSA was produced starting from 1,9-nonanediol in eleven steps, however, 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]

In vivo: Mainly formed in organs via *de novo* lipogenesis

Chemical synthesis:



This work:



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.

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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 sufficient supply of 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 its own chemical solutions to add hydroxyl group along carbon chain, which is an extensive list of enzymes, 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 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, including 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

In a first round of experiments, we screened 21 commercially available lipases to explore their esterification activity toward palmitic acid (PA) and 12-hydroxystearic acid (12-HSA), selected as a model reaction for HFA to FAHFA conversion. Small-scale (1 mL) activity assays were performed at 60 °C using toluene as solvent. To avoid the self-esterification of 12-HSA, the concentration of PA was excessive (2-fold) over 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, their structures and substrate binding pockets 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

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

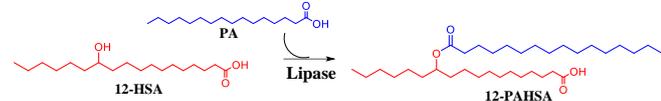
Enzyme	Fatty acid	Hydroxy fatty acid
FA-HY2	Palmitoleic acid (16:1 ^{Δ9})	10-Hydroxy-16:0
FA-HY2	Oleic acid (18:1 ^{Δ9}) ^[a]	10-Hydroxy-18:0
FA-HY2	Linoleic acid (18:2 ^{Δ9,Δ12}) ^[a]	(S)-10-Hydroxy- <i>cis</i> -12-18:1
FA-HY2	α-Linolenic acid (18:3 ^{Δ9,Δ12,Δ15}) ^[a]	10-Hydroxy- <i>cis</i> -12, <i>cis</i> -15-18:2
FA-HY2	Arachidonic acid (20:4 ^{Δ5,Δ8,Δ11,Δ14})	12-Hydroxy- <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -14-20:3
FA-HY1	<i>cis</i> -Vaccenic acid (18:1 ^{Δ11}) ^[a]	12-Hydroxy-18:0
FA-HY1	Linoleic acid (18:2 ^{Δ9,Δ12}) ^[a]	(R)-13-Hydroxy- <i>cis</i> -9-18:1
FA-HY1	Pinolenic acid (18:3 ^{Δ5,Δ9,Δ12})	13-Hydroxy- <i>cis</i> -5, <i>cis</i> -9-18:2
FA-HY1	γ-Linolenic acid (18:3 ^{Δ6,Δ9,Δ12})	13-Hydroxy- <i>cis</i> -6, <i>cis</i> -9-18:2
FA-HY1	α-Linolenic acid (18:3 ^{Δ9,Δ12,Δ15}) ^[a]	(S)-13-Hydroxy- <i>cis</i> -9, <i>cis</i> -15-18:2
FA-HY1	Stearidonic acid (18:4 ^{Δ6,Δ9,Δ12,Δ15})	13-Hydroxy- <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -15-18:3
FA-HY1	Eicosadienoic acid (20:2 ^{Δ11,Δ14})	15-Hydroxy- <i>cis</i> -11-20:1
FA-HY1	Mead acid (20:3 ^{Δ5,Δ8,Δ11})	12-Hydroxy- <i>cis</i> -5, <i>cis</i> -8-20:2
FA-HY1	Sciadonic acid (20:3 ^{Δ5,Δ11,Δ14})	15-Hydroxy- <i>cis</i> -5, <i>cis</i> -11-20:2
FA-HY1	Dihomo-γ-linolenic acid (20:3 ^{Δ8,Δ11,Δ14})	15-Hydroxy- <i>cis</i> -8, <i>cis</i> -11-20:2 ^[b]
FA-HY1	Eicosatrienoic acid (20:3 ^{Δ11,Δ14,Δ17})	12-Hydroxy- <i>cis</i> -14, <i>cis</i> -17-20:2 15-Hydroxy- <i>cis</i> -11, <i>cis</i> -17-20:2
FA-HY1	Arachidonic acid (20:4 ^{Δ5,Δ8,Δ11,Δ14})	15-Hydroxy- <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11-20:3
FA-HY1	Eicosapentaenoic acid (20:5 ^{Δ5,Δ8,Δ11,Δ14,Δ17})	12-Hydroxy- <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -14, <i>cis</i> -17-20:4 15-Hydroxy- <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -17-20:4
FA-HY1	Docosahexaenoic acid (22:6 ^{Δ4,Δ7,Δ10,Δ13,Δ16,Δ19})	14-Hydroxy- <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -16, <i>cis</i> -19-22:5
FA-HY2-T391S ^[c]	γ-Linolenic acid (18:3 ^{Δ6,Δ9,Δ12})	10-Hydroxy- <i>cis</i> -6, <i>cis</i> -12-18:2
FA-HY2-H393S	Docosahexaenoic acid (22:6 ^{Δ4,Δ7,Δ10,Δ13,Δ16,Δ19})	14-Hydroxy- <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -16, <i>cis</i> -19-22:5
FA-HY2-T391S/H393S/I378P	Dihomo-γ-linolenic acid (20:3 ^{Δ8,Δ11,Δ14})	(S)-12-Hydroxy- <i>cis</i> -8, <i>cis</i> -14-20:2
FA-HY2-T391S/H393S/I378P	Eicosapentaenoic acid (20:5 ^{Δ5,Δ8,Δ11,Δ14,Δ17})	(S)-12-Hydroxy- <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -14, <i>cis</i> -17-20:4

^[a] Indicates the hydration of these substrates were used to verify the cascade reaction in a preparative scale in this study. ^[b] Trace amount of 12-hydroxy-*cis*-8,*cis*-14-20:2 product has also been detected. ^[c] Only the variants that exhibited the highest conversion were displayed.

prominent activity and a broad temperature range for synthesis of FAHFAs. Actually, the substrate specificity of CALA had been explored in many studies, which demonstrated that CALA exhibited a relative high activity towards straight-chained primary alcohols and carboxylic acids, as well as sterically hindered secondary and tertiary alcohols. 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 2. Lipase screening for fatty acid and hydroxy fatty acid esterification^[a].



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

^[a] Reaction system: Lipase-10 mg, 10 mg/ml PA, 5 mg/ml 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 reached

86.8% or 90.5% conversion levels at 8 h for 30 °C or 60 °C, respectively. Then, 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 ¹H and ¹³C NMR (Figure S18-19).

We also simply compared the catalytic capacity of CALA under different conditions, in terms of reaction solvents and the ratio of PA/12-HSA (Table S2). As anticipated, the esterification activity of CALA was completely lost in aqueous phase. The conversion rate improved along with the increasing organic solvent ratio (Table S2, entry 1-3), and achieved the yield of 43.3% and 69.9% in Kpi/Toluene (1:1 v/v) and pure toluene, respectively. We next investigated the effect of PA/12-HSA ratio on the production of 12-PAHSA. The yield of 12-PAHSA improved when higher PA/12-HSA ratio was reached. By increasing the PA concentration to 10 mg/mL and lowering the 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 12-HSA). As expected there was no peak observed for 12-PAHSA, however, a new peak (Figure S10) was observed with less than 10% conversion which might be caused by the self-esterification of 12-HSA. Interestingly, this product was not observed when PA was present in the reaction system, indicating lower reactivity of 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.^[18] Since we initially aimed 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 in 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.

As described above, no esterification reaction takes place between PA and 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-MeTHF, CPME and GVL), which have been shown as promising bio-solvents in lipase-catalyzed reactions.^[19] 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. Three green solvents are not suitable for either hydratase or CALA in our case.

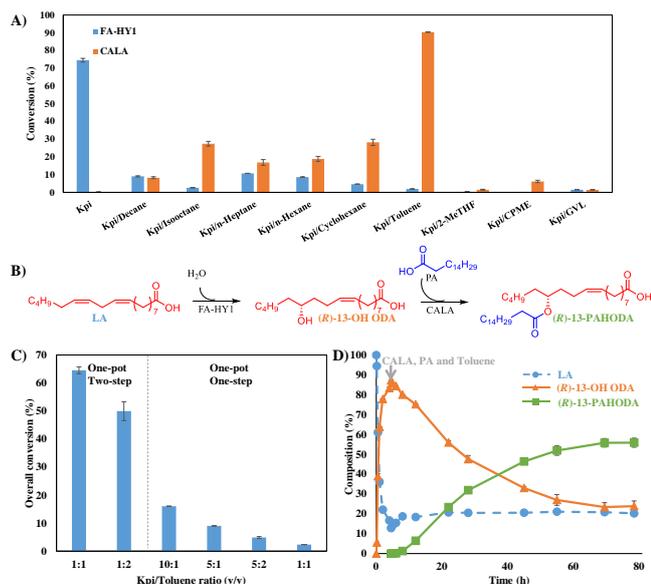


Figure 2. Cascades for the transformation of LA into 13-PAHODA. A) Organic solvent tolerance of FA-HY1 and CALA. Kpi: 100 mM potassium phosphate buffer at pH 6.0; 2-MeTHF: 2-methyltetrahydrofuran; CPME: cyclopentyl methyl ether; GVL: γ -valerolactone. The volume ratio of Kpi/organic solvent is 1:1. B) Reaction scheme for the 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 13-PAHODA. The first hydration of LA step was performed in single aqueous condition, including 50 mL supernatant of cell lysate (ultrasonication of 50 mg/mL cells in Kpi buffer) and 100 mg LA; the second step was started by the addition of 500 mg CALA, 200 mg PA and 50 mL toluene.

We further investigated the operation mode in Kpi/Toluene biphasic system 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). 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 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 palmitic acid ester of 13-hydroxy-*cis*-octadec-9-enoic acid (13-PAHODA) from LA and PA was scaled up to 100 mL in a preparative scale. FA-HY1 almost completely converted LA into its 13-OH product (13-HODA) in 8 h (**Figure 2D**). The second esterification step took significantly longer after adding CALA, PA and toluene to the sample pot. The final composition of 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.

Considering differential regulation and biological activity of FAHFA regioisomers and 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 production of different FAHFAs (**Figure 3**). The typical hydratase, FA-HY2, can only add water on $\Delta 9$ double bonds to produce 10-OH products, whereas the unique FA-HY1 preferably hydrates double bonds

other than $\Delta 9$ to generate 12-OH, 13-OH, 14-OH and 15-OH products.^[14] Therefore, several pairs of FAH-substrate, that exhibited high conversion rate 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 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 transesterification or biological incorporation into triglycerides, which are the major form of FAHFAs in the tissues.^[20] 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), 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 ¹H and ¹³C NMR (**Figure 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 (*R*), (*S*) and (*R*), respectively, based on our previous analysis of the corresponding chiral HFAs.^[14]

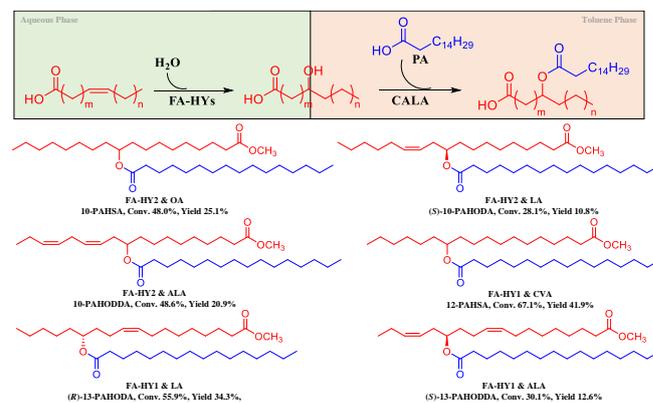


Figure 3. Substrate scope of hydratase-lipase cascade reaction. The reactions were performed in a one-pot two-step procedure as described above. Conversion = [FAHFA]_{final}/[Unsaturated fatty acid]_{initial} × 100%; determined via GC-FID as described in the Supporting Information. Yield: isolated molar 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 FAHs-CALA catalytic system, either by a two-step synthesis or by a bi-enzymatic cascade of one-pot two-step process. To our

knowledge, the only other study that reported synthesis of fatty acid estolides (including FAHFAs) by enzymatic cascade approach utilizing hydratase and lipase is a patent,^[21] where oleate hydratase (OhyA) was used for hydration of unsaturated FAs at $\Delta 9$ position to produce only 10-OH products. By utilizing two different FAHs from *L. acidophilus* and various fatty acids, the product profile of our system was greatly expanded to prepare various stereospecific isomers of FAHFAs corresponding to possibly differential regulation and biological functions. Our results suggested two types of lipases from *C. antarctica* A and *C. Rugosa* are highly selective and active catalyst 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 donor; thus allowing effective interaction of two reactants with large steric hindrance.^[15, 22] Our approach paves the way for enzymatic synthesis of a variety of FAHFAs from sustainable fatty acids in a large scale, enabling their production for use in pre-clinical and clinical studies as well as for applications in medications and as dietary supplements. We honestly admit this approach is still in the conceptual stage due to the low substrate loading and incomplete transformation. Protein engineering to further increase the activity and to expand the substrate diversity of FAHs, as well as reaction optimization studies are ongoing in our lab to improve the product yield and broaden product spectra. Moreover, lipoxigenases 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.

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Conflict of interest

The authors declare no conflicts of interest.

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

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Entry for the Table of Contents

Fully 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 values.

