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Understanding FAHFAs: From structure to metabolic regulation

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ABSTRACT

The discovery of branched fatty acid esters of hydroxy fatty acids (FAHFAs) in humans draw attention of many researches to their biological effects. Although FAHFAs were originally discovered in insects and plants, their introduction into the mammalian realm opened new horizons in bioactive lipid research. Hundreds of isomers from different families have been identified so far and their role in (patho) physiological processes is currently being explored.

The family of palmitic acid esters of hydroxy stearic acids (PAHSAs), especially 5-PAHSA and 9-PAHSA regioisomers, stands out in the crowd of other FAHFAs for their anti-inflammatory and anti-diabetic effects. Beneficial effects of PAHSAs have been linked to metabolic disorders such as type 1 and type 2 diabetes, colitis, and chronic inflammation. Besides PAHSAs, a growing family of polyunsaturated FAHFAs exerts mainly immunomodulatory effects and biological roles of many other FAHFAs remain currently unknown. Therefore, FAHFAs represent unique lipid messengers capable of affecting many immunometabolic processes.

The objective of this review is to summarize the knowledge concerning the diversity of FAHFAs, nomenclature, and their analysis and detection. Special attention is paid to the total syntheses of FAHFAs, optimal strategies, and to the formation of the stereocenter required for optically active molecules. Biosynthetic pathways of saturated and polyunsaturated FAHFAs in mammals and plants are reviewed together with their metabolism and degradation. Moreover, an overview of biological effects of branched FAHFAs is provided and many unanswered questions regarding FAHFAs are discussed.

1. Introduction

Lipids are a diverse class of compounds that range from simple fatty acids to complex multichain structures. Lipids serve as structural components of membranes, energy storage and signaling molecules that contribute to the regulation of many metabolic and immune processes. The metabolic pathways of lipids and carbohydrates are closely associated, and research on the role of glucose transporter 4 (GLUT4) in the pathogenesis of insulin resistance in mice has revealed favorable

metabolic effects of *de novo* lipogenesis (DNL) in white adipose tissue (WAT) [2,3]. Mice overexpressing Glut4 in adipocytes exhibit elevated DNL and increased glucose tolerance despite being obese [3]. In 2014, a lipidomic analysis of WAT revealed the existence of branched fatty acid esters of hydroxy fatty acids (FAHFAs) that have anti-inflammatory and anti-diabetic properties [3]. This discovery opened a new horizon in bioactive lipid research, and this new realm of FAHFAs appears to be at least as heterogeneous as eicosanoids and docosanoids [4].

Although Yore et al. coined the term “FAHFAs” for the bioactive

Abbreviations: ADTRP, androgen-dependent TFPI regulating protein; AIG1, androgen-induced gene 1; ATGL, adipose triglyceride lipase; BINAP, 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl; BINOL, 1,1'-Bi-2-naphthol; cat., catalytic; Bn, benzyl; CEL, carboxyl ester lipase; CES3, carboxylesterase 3; CGI-58, comparative gene identification-58; DAG, diacylglycerol; DGAT, diacylglycerol O-acyltransferase; DNL, de novo lipogenesis; EDCI, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; FA, fatty acid; FAHFA, fatty acid ester of hydroxy fatty acid; FAH12, Δ -12 fatty acid hydroxylase; FFAR, free fatty acid receptor; GLP-1, glucagon-like peptide 1; GLUT4, glucose transporter 4; HALA, hydroxy- α -linolenic acid; HFA, hydroxy fatty acid; HFD, high fat diet; HSL, hormone-sensitive lipase; MCPBA, meta-chloroperbenzoic acid; [Ox.], oxidation; PAHSA, palmitic acid ester of hydroxy stearic acid; PUFA, polyunsaturated FA; TAG, triacylglycerol; TBAF, Tetra-n-butylammonium fluoride; TBS, *tert*-butyldimethylsilyl; TEMPO, 2,2,6,6-Tetramethylpiperidin-1-yl)oxyl; WAT, white adipose tissue; The abbreviations used in the schemes are explained in the ACS list of abbreviations (2020), Common abbreviations for fatty acids are used for the most abundant structures [1] see also Fig. 2

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lipids they uncovered in mammalian WAT, many other endogenous or naturally occurring molecules contain a hydroxylated fatty acid (HFA) esterified by a fatty acid (FA). The first biologically active branched FAHFAs were discovered in larvae of the European cabbage butterfly and named mayolenes in honor of May Berenbaum, an American entomologist [5]. This review primarily focuses on the endogenous branched FAHFAs present in humans. For ease of reading the term “branched” in “branched FAHFA” will therefore be omitted herein. Structurally related molecules present in insects, plants, bacteria as well as “linear” (ω -hydroxylated) FAHFAs in mammals are discussed in Section 5.3.

Prior to a discussion of the occurrence of FAHFAs and current knowledge on their biosynthesis, metabolism, biological relevance, this review will present FAHFA nomenclatures and an overview of their chemical synthesis.

2. FAHFA structure and nomenclature

The lipid nomenclature used throughout this text is based on general literature recommendations and have been customized to describe FAHFA-containing structures [1,3]. FAHFAs belong to a family of estolides that are defined as oligomers of fatty acids. An estolide is characterized by the number of estolide linkages per molecule (estolide number, number of oligomers). Therefore, a FAHFA is a monoestolide (Fig. 1). Common (trivial) nomenclature of FAHFAs is used for the most studied compounds, such as 9-PAHSA, where “9” defines the ester position relative to carboxylic acid, PAHSA stands for palmitic acid ester of hydroxy stearic acid. Common abbreviations for fatty acids are used for the most abundant structures [1], and stereochemistry is eventually defined (e.g., 9(*R*)-PAHSA). However, because this simplification does not reflect the real position of double bonds, it does not distinguish between isomers. For example, hydroxy derivatives of palmitoleic acid (C16:1 ω -7) and sapienic acid (C16:1 ω -10) are both abbreviated as hydroxy palmitoleic acid (HPO) according to the naturally prevailing isomer in mammals.

Alternatively, to avoid abbreviations for each fatty acid, the generalized nomenclature X1:Y1-(z-O-X2:Y2) is used [1,6]. The notation

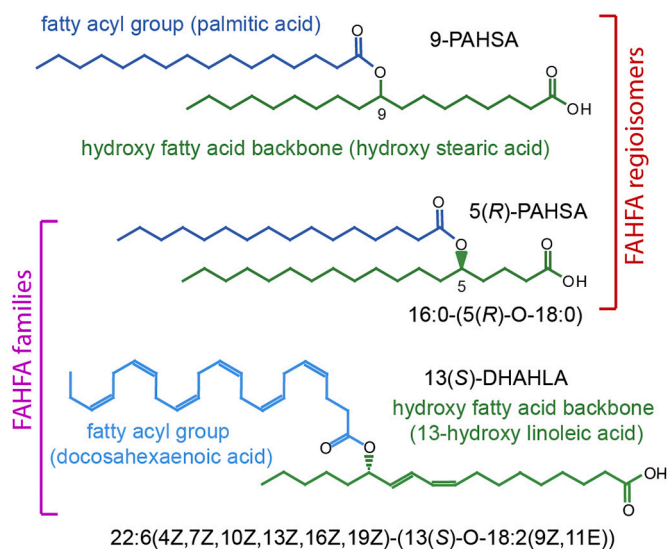


Fig. 1. Examples of FAHFA structures. 9-PAHSA (formal name 9-[(1-oxohexadecyl)oxy]-octadecanoic acid; CAS Number 1481636–31-0) and 5-PAHSA are regioisomers from the same family. 5-PAHSA and 13-DHAHLA belong to different families. The (*R*) and (*S*)-notation defines the absolute configuration of the acylated stereocenter, according to the Cahn-Ingold-Prelog priority rules. (Note that a similar spatial drawing (away from the viewer for instance) can represent both an (*R*)- or an (*S*)-stereocenter depending on the nature and length of the chains).

X:Y refers to a fatty acid chain, where X is the total number of carbon atoms, Y is the number of double bonds, and z is the position of the branched acylated hydroxyl function. For example, 16:0-(9-O-18:0) represents the 9-PAHSA isomer. For unsaturated FAHFAs, double bond positions and configurations need to be specified [e.g., 22:6(4Z,7Z,10Z,13Z,16Z,19Z)-(13(*S*)-O-18:2(9Z,11E)) for 13(*S*)-DHAHLA] (Fig. 1).

3. Synthetic FAHFAs as chemical tools

Access to pure FAHFA compounds is of great importance for the future research of FAHFAs. Synthetic FAHFAs are required as liquid chromatography-mass spectrometry standards (for monitoring the retention time, finding suitable chromatographic conditions to avoid the overlap of many regioisomers, calibration curves, etc.). Synthetic FAHFAs can also be used to obtain a better understanding of their biological role and properties, biosynthesis and metabolism, including in diet supplementation experiments. Thus, the pioneering work of organic chemists is crucial, as only several FAHFAs are commercially available at present.

A review on the chemical synthesis of FAHFAs has recently been published [7]. The present section is organized differently, highlighting the differences in the strategies instead of the chronological order. As the FAHFA field grew considerable over the past four years, many novel total syntheses have been reported, increasing the number of FAHFAs available for biological experiments and improving the efficiency of the strategies. Special attention has been paid to the formation of the stereocenter required for optically active FAHFAs and the demanding total synthesis of polyunsaturated FAHFAs. The case of fatty acid esters of ω -hydroxylated fatty acid [8,9] (“linear” FAHFAs, see Section 5.3) is not discussed in the present section.

3.1. Many FAHFA preparation strategies have been developed

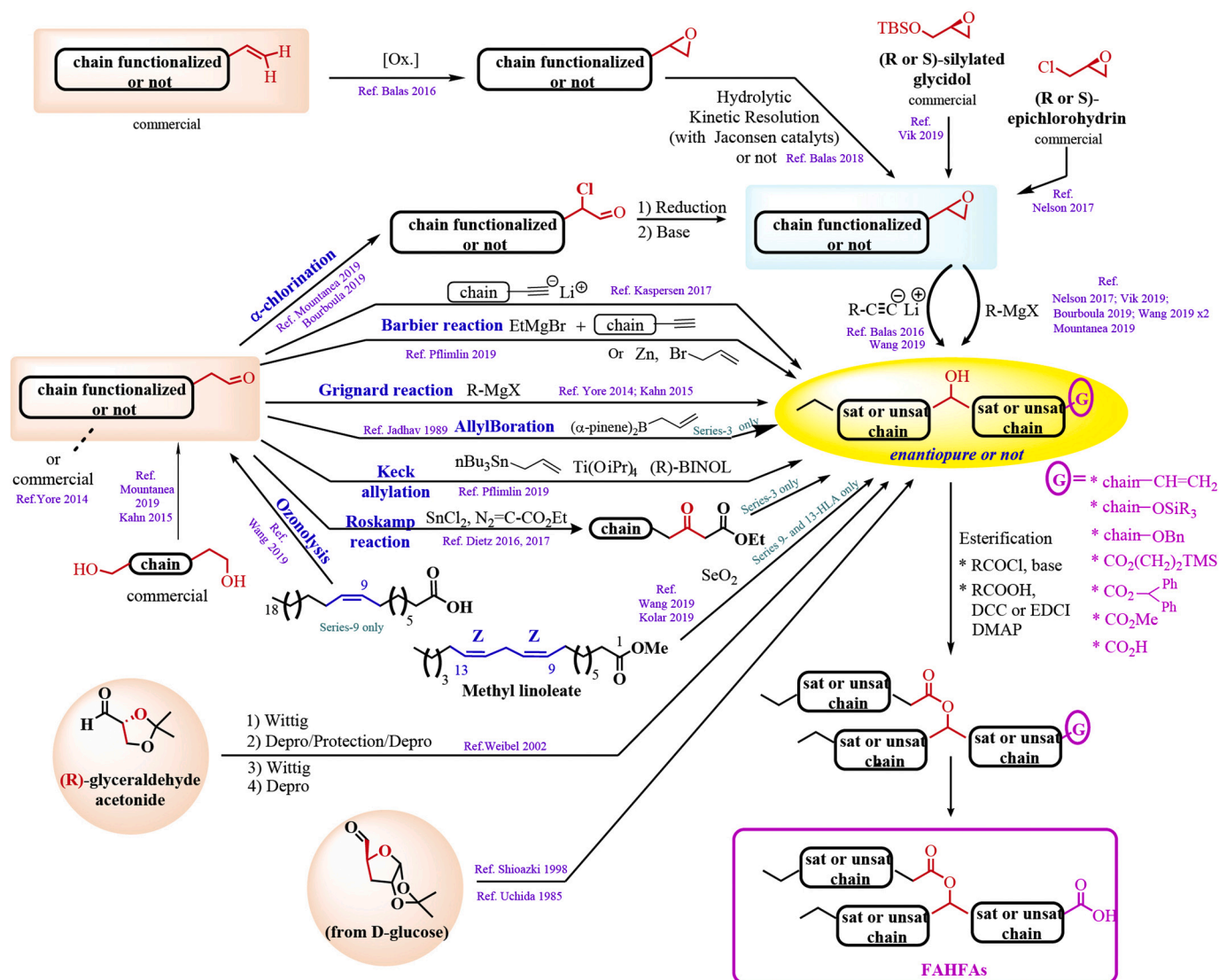
In this section, an overview of all the strategies developed for the preparation of FAHFAs is discussed and depicted in Scheme 1. Most reported strategies are flexible and can therefore be applied to the synthesis of many FAHFA families and/or many regioisomers, since in most cases, the selection of the appropriate chain lengths of the precursors allows the introduction of the hydroxyl functionality at any position along the long fatty chains.

The FA chain is commonly introduced by esterification of the obtained secondary alcohol (Scheme 1, in yellow) using fatty acid chlorides in the presence of an amine or through Steglich esterification. Yields are good to excellent, with saturated or unsaturated chains for both the FA and the hydroxylated chains.

Regardless the racemic or asymmetric procedures, the presence of two carboxylated functions in FAHFAs dictates some reaction sequences that may differentiate both groups or postpone the formation of the final free carboxylic acid. Thus, the various strategies for FAHFA synthesis may be divided in two groups: A) strategies that introduce the carboxylated group of the hydroxylated chain after the acylation of the secondary alcohol in the late stage of synthesis (Scheme 1, group G represents a chemical function that is different from a COOR group); and B) strategies that introduce the carboxylated group early in the synthesis, before acylation with an FA, thereby requiring a chemoselective deprotection of the HFA ester without affecting the branched fatty ester (Scheme 1, G represents an ester group).

3.2. Group A strategies: formation of the HFA ester group after acylation with FA

Several G groups have been reported. Terminal olefins (G is CH=CH₂) were converted [10–12] to the desired free carboxylic acid group by ozonolysis and subsequent Pinnick oxidation. This two-step sequence is readily performed and efficient, but it requires an ozone



Scheme 1. Schematic overview of all the strategies developed for FAHFA synthesis. FAHFAs are chemically synthesized from a fatty secondary alcohol (in the yellow box), which, in turn, may be prepared from an epoxide (blue box) obtained from a commercially available epoxide or from a terminal alkene (cream color box, top left corner) or an aldehyde (long chains or derived from sugar derivatives; cream color boxes).

generator and cannot be used for polyunsaturated FAHFAs.

In the synthesis of 5-PAHSA and 9-PAHSA, Pflimlin et al. [13] employed a terminal homoallylic functional group (G is CH=CH₂) for later extension of the carbon chain length with concomitant introduction of the carboxylated head via an olefin metathesis cross-coupling reaction in the presence of benzyl hept-6-enoate (54–70%) or benzylacrylate (49%). Subsequently, Pd/C-catalyzed hydrogenation simultaneously reduced the resulting double bond and deprotected the benzyl group with good yields.

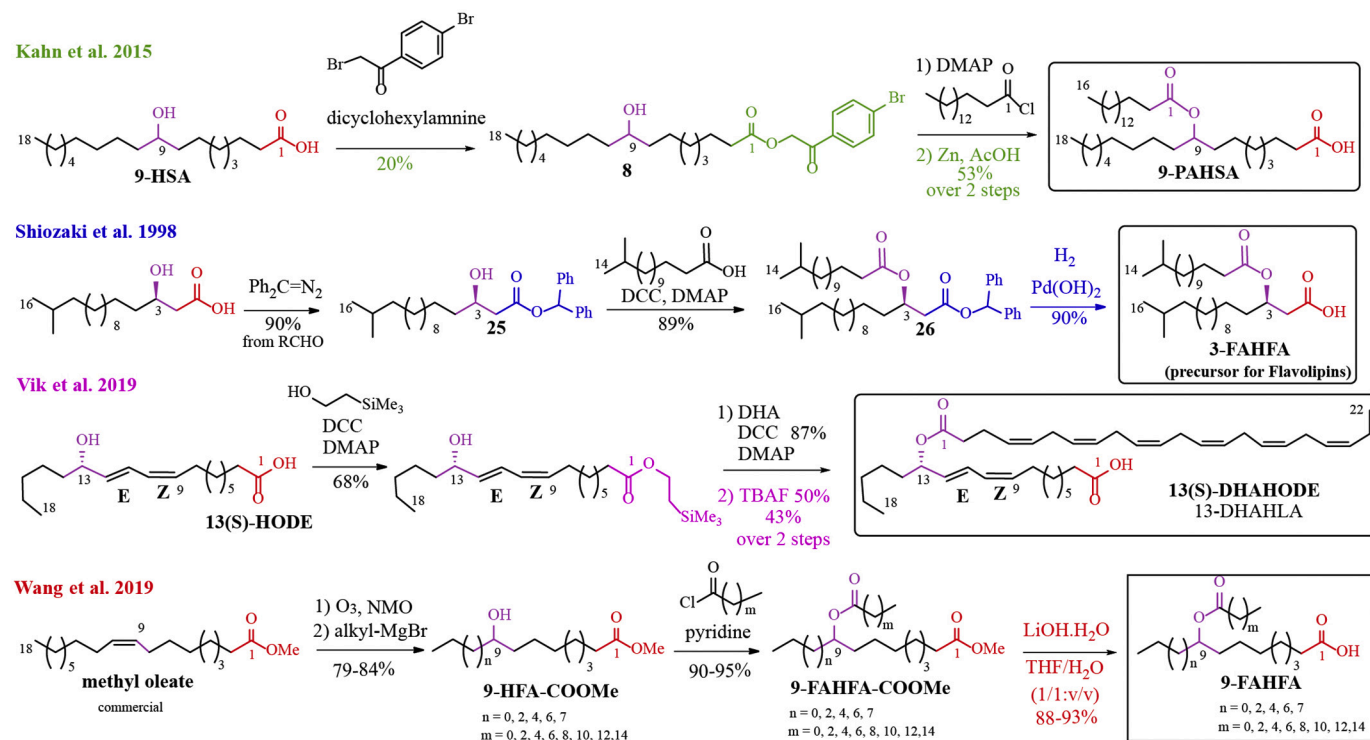
Protected primary alcohols (G is an OTBS [14] or OBn [13,15] group) have been deprotected using fluoride anions (TBAF) followed by Jones oxidation, or hydrogenolysis with Pd/C, respectively. Although highly efficient, an obvious limitation of this two-step conversion is the use of a toxic chromium reagent that will be deleterious for pharmaceutical use. Alternative oxidation reactions may be performed if needed, as recently reported by Pflimlin et al. [13], using a TEMPO/NaClO/NaClO₂ oxidation system. However, this two-step sequence is less efficient (41%) than the Jones reaction (71–94%) [14,15].

3.3. Group B strategies: formation of the HFA ester group before acylation with FA

In 2002, direct Steglich esterification of free HFAs (G is COOH) was conducted and produced a modest yield (59%), suggesting the occurrence of competitive side-reactions and subsequent difficulties in the purification step. As the free HFA is derived from a costly and lengthy strategy (9 steps from (R)-glyceraldehyde acetonide), esterification of HFAs without protection of their carboxylic acids is not appealing [5,16].

Several synthetic routes [17–21] carried out the acylation reaction from hydroxy-fatty acids protected as a methyl ester (G = COOMe). Thus, saponification to the expected FAHFA target (G = COOH) requires mild conditions (LiOH, THF/MeOH: 1/1) to favor the selective reactivity at the methyl ester group rather than at the bulkier branched ester functions. Experimentally, the yields are unexpectedly good (88–93%) [17], although rather inconsistent (21–91%), with FA release sometimes observed [22], making purification difficult.

This strategy represents a much more straightforward and more efficient approach compared to the zinc-mediated chemoselective cleavage of 2-(4-bromophenyl)-2-oxoethyl ester in the first synthesis of



Scheme 2. Comparison of four chemoselective cleavages of FAHFA-esters.

9-PAHFA developed by Kahn et al. [11]. In the course of the total synthesis of flavolipins and dipeptide-FAHFAs, Shiozaki [23] and Uchida [24,25] suggested the protection of 3-HFA as a diphenylmethyl ester using diazodiphenylmethane and hydrogenolysis over Pearlman's catalyst after Steglich acylation.

To facilitate the final deprotection of the carboxyl function, Vik et al. [26] recently converted the methyl ester of the HFA precursor to a fluoride-sensitive trimethylsilyl ethyl ester ($G = \text{CO}_2\text{CH}_2\text{CH}_2\text{SiMe}_3$, 54%) prior to a Steglich esterification into FAHFA. However, TBAF-deprotection only yielded the desired free carboxylic function in modest yields (50%, with branched ester containing a sensitive conjugated E,Z-diene).

A comparison of the four reported carboxylated G groups is outlined in Scheme 2.

3.4. Construction of the secondary alcohol function (Scheme 1)

Whatever the choice of the group A or B strategies described above, the primary challenge in FAHFA synthesis is the preparation of the free secondary alcohol precursor (yellow box, in Scheme 1).

Regardless the stereo-outcomes, most of the free secondary alcohol functions have been generated by carbon-carbon bond formation using Grignard reagent additions to aldehydes [3,11,17] or through epoxide ring-opening with lithium acetylides [14,20] or organomagnesium halides [15,20,26,27].

In the total synthesis of mayolenes, polyunsaturated FAHFAs biosynthesized by caterpillars, the secondary alcohol function arose from the starting material [i.e., (R)-glyceraldehyde acetonide, its chirality being unchanged throughout the overall steps (depicted in Section 3.7)] [5,16].

In the case of 3-series FAHFAs, the secondary alcohol function was obtained with good yields via sodium borohydride ketone reduction or asymmetric BINAP-ruthenium-mediated hydrogenation of the ketone of a β -ketoester [28,29] that was prepared from a SnCl_2 -catalyzed Roskamp reaction in the presence of ethyl diazoacetate. Saturated 3-HFAs were also synthesized (Scheme 1 and outlined in Section 3.7) from

alpha-D-ribofuranose derivatives via sodium periodate mediated-oxidative cleavage of 1,2-diol function [23–25] or via reductive ring opening of the hemiacetal group [23].

Since all these multistep synthetic routes are long, Wang et al. [19,20] recently suggested a one-step sequence for the preparation of a monohydroxylated metabolite of methyl linoleate based on a selenium oxide-mediated allylic oxidation. The (Z,Z)-bis-allylic moiety of linoleic acid is converted into the desired conjugated (E,Z)-dienyl-alcohols. This reaction will be discussed in more detail in Section 3.8 (Scheme 12) for polyunsaturated FAHFAs.

3.5. Strategies based on epoxide ring opening

While some short-chain epoxides such as epichlorohydrins [20,27] and glycidols [26] are commercially available, long chain epoxides have been synthesized by meta-chloroperoxybenzoic acid (MCPBA) oxidation of terminal alkenes [14] or via the alpha-chlorination of aldehydes followed by sodium borohydride (NaBH_4) reduction and rearrangement under basic conditions [10,15]. The corresponding approaches leading to optically active FAHFAs are discussed in Section 3.7.

3.6. Strategies based on aldehydes

Starting from aldehydes, the well-known Grignard reaction is broadly exemplified [3,11,12,17] in FAHFA syntheses. Both alkyl and monosaturated chains have been used, resulting in modest to good yields. As mentioned above, aldehydes have also served as starting materials for the synthesis of epoxides via alpha-chlorination [10,15] and for Roskamp beta-ketoester formation [28,29].

When not commercially available, the aldehydes were prepared via a large panel [11,15,17,23,29] of oxidation reactions of primary alcohols (or selective protection of 1, ω -diols and subsequent oxidation).

Furthermore, in the synthesis of a library of 9-FAHFAs with various short saturated chains, Wang et al. [17] carried out the ozonolysis of methyl oleate into 9-oxononanoate (93%), which was subsequently

subjected to Grignard reactions. Although poor atom economy and expensive starting material may be a limitation, a noteworthy point is the conciseness of this approach by Wang to generate the 9-HFA methyl ester (2 steps only, high yields of 79–84%) due to the amazing selectivity of the organomagnesium halide addition in favor of the aldehyde function over the ester group (less electrophilic due to pi-donation).

Avoiding the preparation of organomagnesium halides, Pflimlin et al. [13] conducted Barbier-type reactions with allylbromides in the presence of zinc dust as well as with omega-O-benzyl functionalized terminal alkyne in the presence of ethylmagnesium bromide, providing secondary homoallylic alcohols with good yields. In addition, enantioenriched derivatives were obtained using the Keck-asymmetric allylation (more details in Section 3.7).

Jadhav et al. [30] also performed an allylation reaction using an asymmetric allyl boronate derived from alpha-pinene (75% yield after H₂O₂-mediated oxidation of the borinate intermediate).

In addition, Kaspersen et al. also recently reported [31] a concise approach (2 or 3 steps) to access 6–11-series HFAs by the addition of doubly deprotonated omega-alkynoic acids to aldehydes, which was followed by hydrogenation over platinum on carbon.

3.7. Optically active FAHFAs

Several chemical syntheses providing (*R*) and (*S*)-enantiopure or enantio-enriched FAHFAs have been developed, providing new ways to explore ligand-receptor interactions.

Before the name FAHFA was coined by Kahn and colleagues for a hydroxylated fatty acid esterified by a fatty acid, (*R*)-glyceraldehyde acetonide and alpha-D-ribofuranose derivatives (from D-glucose) were utilized as chiral pools for several chemically related structures, such as mayolenes (11(*R*)-SAHALA and 11(*R*)-PAHALA from caterpillars) [16] and flavolipins (chiral dipeptide FAHFAs) [23,24], respectively. Schemes 3 and 4 briefly outline these total syntheses. However, as a recent publication by Balas et al. [7] reviewed these total syntheses, we will not discuss them herein.

To gain a better understanding of FAHFA metabolism and biosynthesis, Nelson et al. [27] performed syntheses of saturated and optically active FAHFAs, yielding both enantiopure 9-PAHSA enantiomers. These synthetic molecules helped to provide a better understanding of FAHFA metabolism (see Section 8).

The approach used by Nelson et al. is based on an epoxide ring opening with an alkyl Grignard reagent using (*R*)- or (*S*)-epichlorohydrin as starting material (Scheme 5). The six-step strategy resulted in a 55% overall yield. Although the starting material is expensive, the use of chiral-pool starting materials ensured the enantiopurity of the final targets.

Wang et al. [20] also based their strategy on the functionalization of optically active epichlorohydrins that involved an initial lithium acetylide addition to the epoxide. Then, rearrangement under basic conditions to a newly formed epoxide and the nucleophile addition of ethylmagnesium bromide afforded the desired homoallylic alcohol with excellent enantiopurity and good yields. This Wang approach is briefly outlined in Scheme 6 and it is further discussed in Section 3.8 for polyunsaturated FAHFA structures.

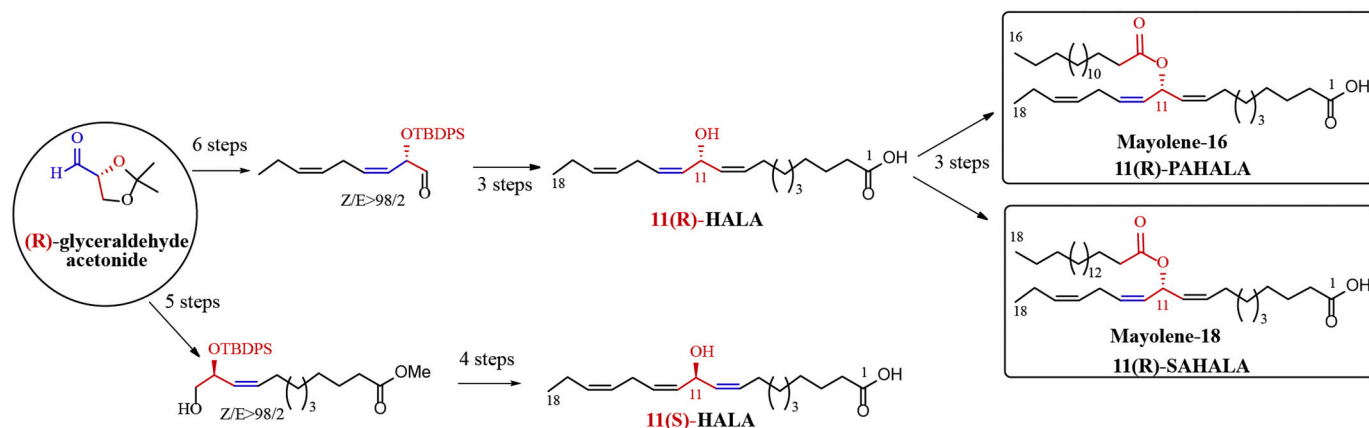
Vik et al. [26] also debuted the synthesis with a commercially available enantiopure epoxide using optically active O-silylated glycidols. Grignard addition and subsequent functionalization to a (*E*)-vinyl iodide for a Sonogashira cross-coupling reaction are key steps towards the synthesis of 13(*S*)- and 13(*R*)-DHAHLA enantiomers. Scheme 7 summarizes the formation of the desired chiral center in optically active DHAHLA. Further discussion is provided in Section 3.8 for the synthesis of polyunsaturated FAHFAs.

Instead of using commercially available enantiopure chiral pool (expensive) starting materials, Balas et al. [14] performed a hydrolytic kinetic resolution of the racemic epoxides (obtained by MCPBA oxidation) using (*R*)- or (*S*)-Jacobsen catalysts (yield 94%). Although there was no steric strain in the starting epoxide, the enantioselectivity is good (ee = 93–96%). However, half of the racemic epoxides is wasted as diols, which is a noticeable disadvantage for this enantioselective approach.

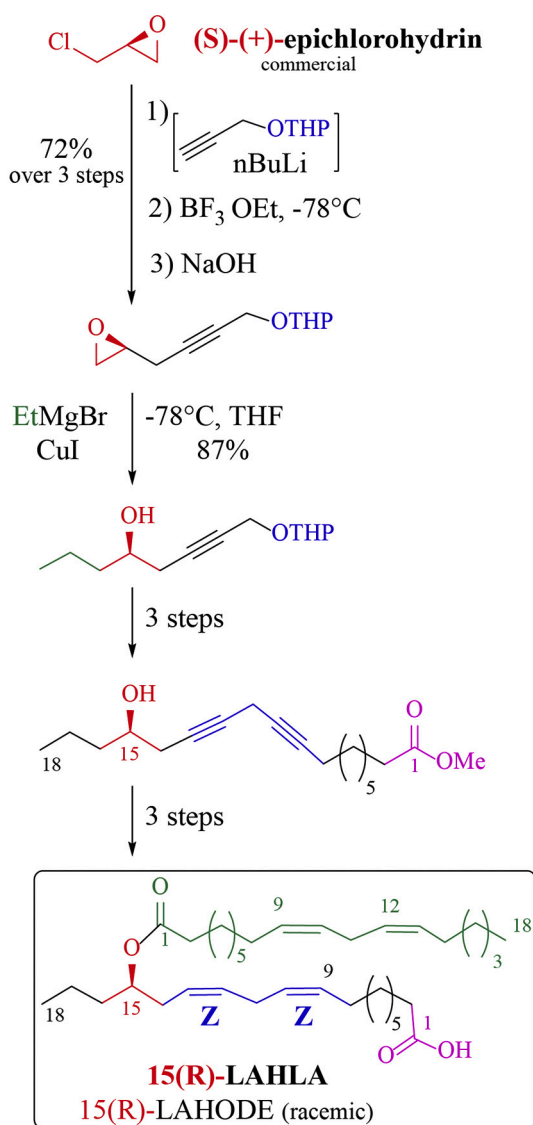
Mountanea et al. [15] also developed a method for the synthesis of 9(*S*)-PAHSA through the ring opening of terminal optically active epoxides by alkyl Grignard reagents. The desired chirality is introduced by an enantioselective asymmetric Jorgensen epoxidation (93–98% ee, Scheme 8) from enamine-based chlorination [32] of long chain aldehydes with McMillan's third generation imidazolidinone organocatalyst and 2,3,4,5,6,6-hexachlorocyclohexa-2,4-dien-1-one as the chlorination agent. Subsequent reduction with NaBH₄, which was followed by treatment with KOH, led to the formation of the desired enantioenriched epoxides in a one-pot procedure.

This strategy was applied to the synthesis of 9(*S*)-PAHPA and 9(*S*)-SAHPA, as well as the synthesis of 5(*S*)-SAHPA and 5(*S*)-PAHSA. In addition, since the hydrogenation step required for the deprotection of the O-benzyl group precludes the application of the strategy for FAHFAs containing unsaturated chains, the strategy was also performed starting from an OTBS-monoprotected 1,ω-diol, resulting in a similar overall yield and number of steps, with the silylated protection being removed by fluoride anions. This sequence is exemplified by the synthesis of 9(*S*)-OAHSA.

Starting from non-functionalized aldehydes (instead of omega-O-protected aldehydes) and replacing alkylmagnesium halide with vinylmagnesium bromide led to several 3-HFAs after a two-step oxidation of the terminal double bond [10]. However, its use for the synthesis of



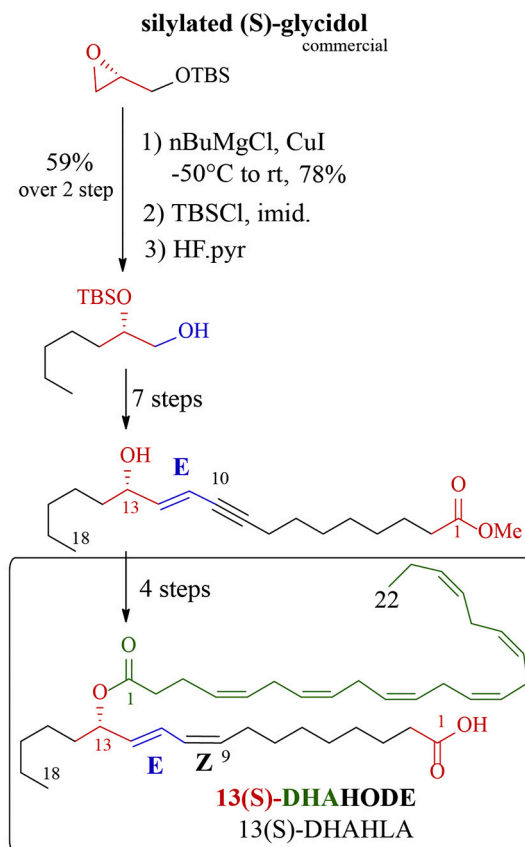
Scheme 3. Total synthesis of mayolene-16 and 18, palmitate and stearate of 11(*R*)-hydroxy- α -linolenic acid (HALA), respectively (Weibel et al. 2002) [16].



Scheme 6. Synthesis of 15(R)-LAHLA from optically active epichlorohydrin (Wang 2019) [20].

acylation of 13(S)-HLA (also known as 13(S)-HODE), which came from soybean lipoxygenase-mediated oxidation of LA (yields not measured). Vik et al. [26] recently performed a chemical synthesis of 13(S)- and 13-(R)-DHAHLA in 15 steps and obtained excellent yields given the demanding chemo-, regio- and stereo-selectivities required. The Vik synthesis requires the preparation of two terminal alkynes (via a Corey-Fuch olefination) and a subsequent hydrozirconation-iodination reaction of the alkyne with the enantiopure alcohol function (obtained from a Grignard reaction from enantiopure glycidol as abovementioned in Section 3.7). The conjugated *E,Z*-diene is generated via a Sonogashira cross-coupling reaction followed by a stereo- and chemo-selective zinc-mediated reduction of the (*E*)-enyne.

Interest in the polyunsaturated FAHLA family was further piqued by an analysis of oat oil [36], which revealed that 15-LAHLA is embedded in digalactosyldiacylglycerol structures (i.e., glycosylated DAG- mono-, di-, or tri- estolides). In 2019, Siegel and colleagues [20] achieved the total synthesis of 15(R)-LAHLA in 9 steps (plus 3 steps aside from the longest linear sequence) and obtained excellent yields, albeit sensitive and demanding reactions were required (Scheme 11). Based on the Nelson approach using optically active epichlorohydrin (see Section 3.7) as a starting material, the Wang strategy featured two epoxide ring openings (first with a lithium acetylide followed by a basic

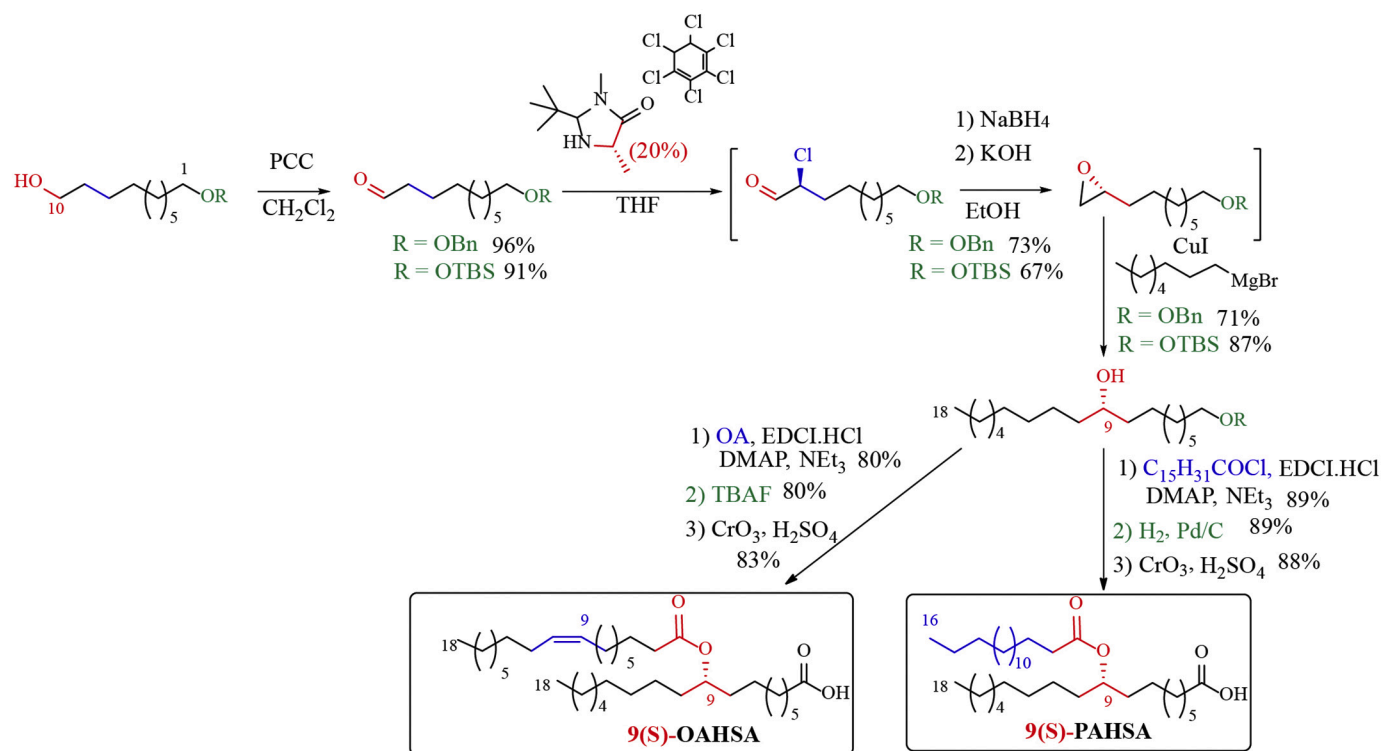


Scheme 7. Synthesis of the 13(S)-DHAHLA (Vik 2019) [26].

rearrangement with a commercial and saturated Grignard reagent), which settled the tail chain with the enantiopure free secondary alcohol (98% ee). The (*Z*)-double bonds were installed via a chemo- and stereo-selective Brown reduction of a skipped diyne, which was generated by a Caruso coupling reaction from a terminal alkynoate ester and a propargylic bromide carrying the enantiopure alcohol function. A modified Steglich acylation followed by a chemoselective saponification of the methyl ester group afforded the desired target 15(R)-LAHLA without affecting the FAHFA ester linkage.

Given the complexity and great number of steps required for the total synthesis of these polyunsaturated FAHFAs, Siegel and colleagues carried out [19,20] a direct and very concise strategy at the expense of yield, avoiding numerous steps such protection-deprotection reactions and carbon-carbon bond formation, which allowed time to be saved. Thus, the Siegel approach (Scheme 12) featured a selenium oxide-mediated allylic oxidation of methyl alpha-linoleate, a modified Steglich esterification and a chemoselective deprotection of the methyl ester over the FAHFA junction ester. Unfortunately, the allylation reaction is not regioselective, producing hydroxylation at both positions 9 and 13 (in approximately equimolar amounts) with extremely poor yields (ca. 10% each). In addition, the allylation step is not stereo-selective, producing some conjugated *E,E*-diene stereoisomers along with the desired conjugated *E,Z*-diene regioisomers. With such a mixture, the subsequent purification is certainly difficult, requiring a special preparative chromatography device.

Although costly and resulting in sacrificed yields, compared to the demanding efforts (time and skills) and prices for the many chemicals required for the challenging long total synthesis of polyunsaturated HFA, this short non selective approach is an interesting alternative to perform a synthesis in a much shorter period of time. A primary disadvantage of this method is the lack of enantioselectivity, affording each regioisomer as a racemic mixture (not a problem for LC-MS



Scheme 8. Organocatalyzed-enantioselective synthesis of 9(S)-PAHSA and 9(S)-OAHA (Mountanea 2019) [15].

standards).

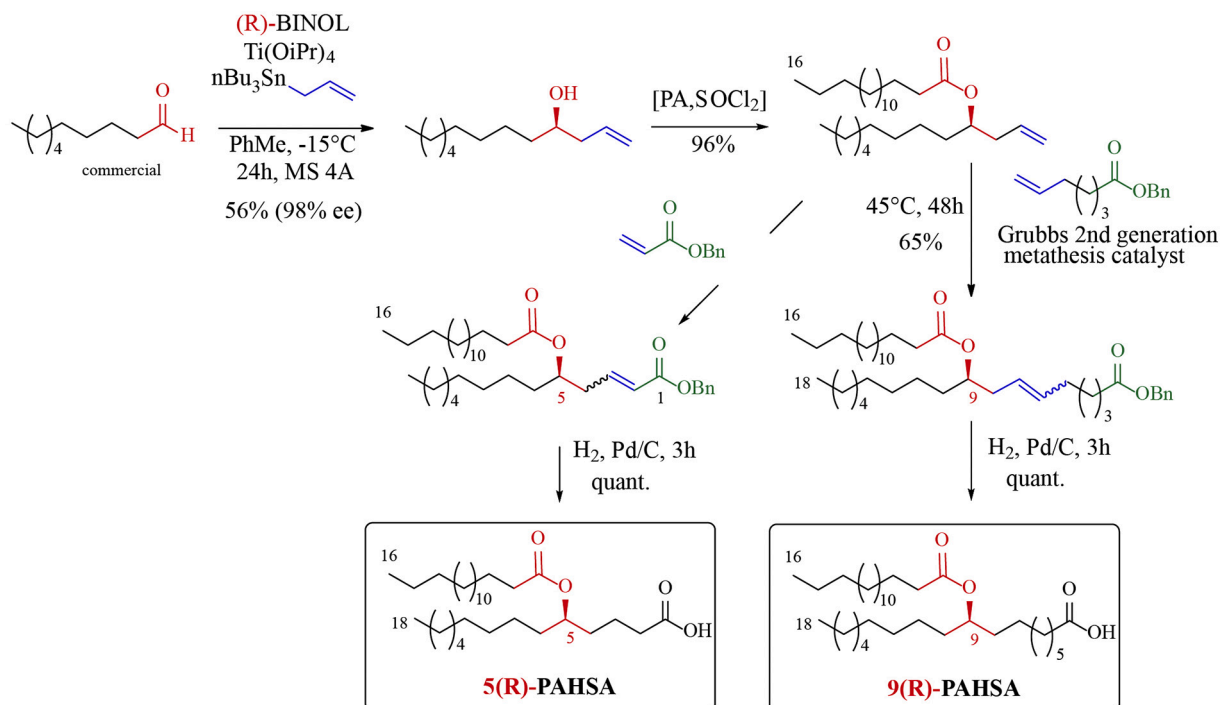
3.9. Deuterated analogues

While many commercially available deuterated FAHFAs have been synthesized from the corresponding deuterated FAs (for instance, 5-, 9-, 10- and 12-PAHSA (²H₃₁-PA) and 9-PAHSA (²H₄-PA) from Cayman Chemicals), the deuterium atoms were also incorporated in the HFA

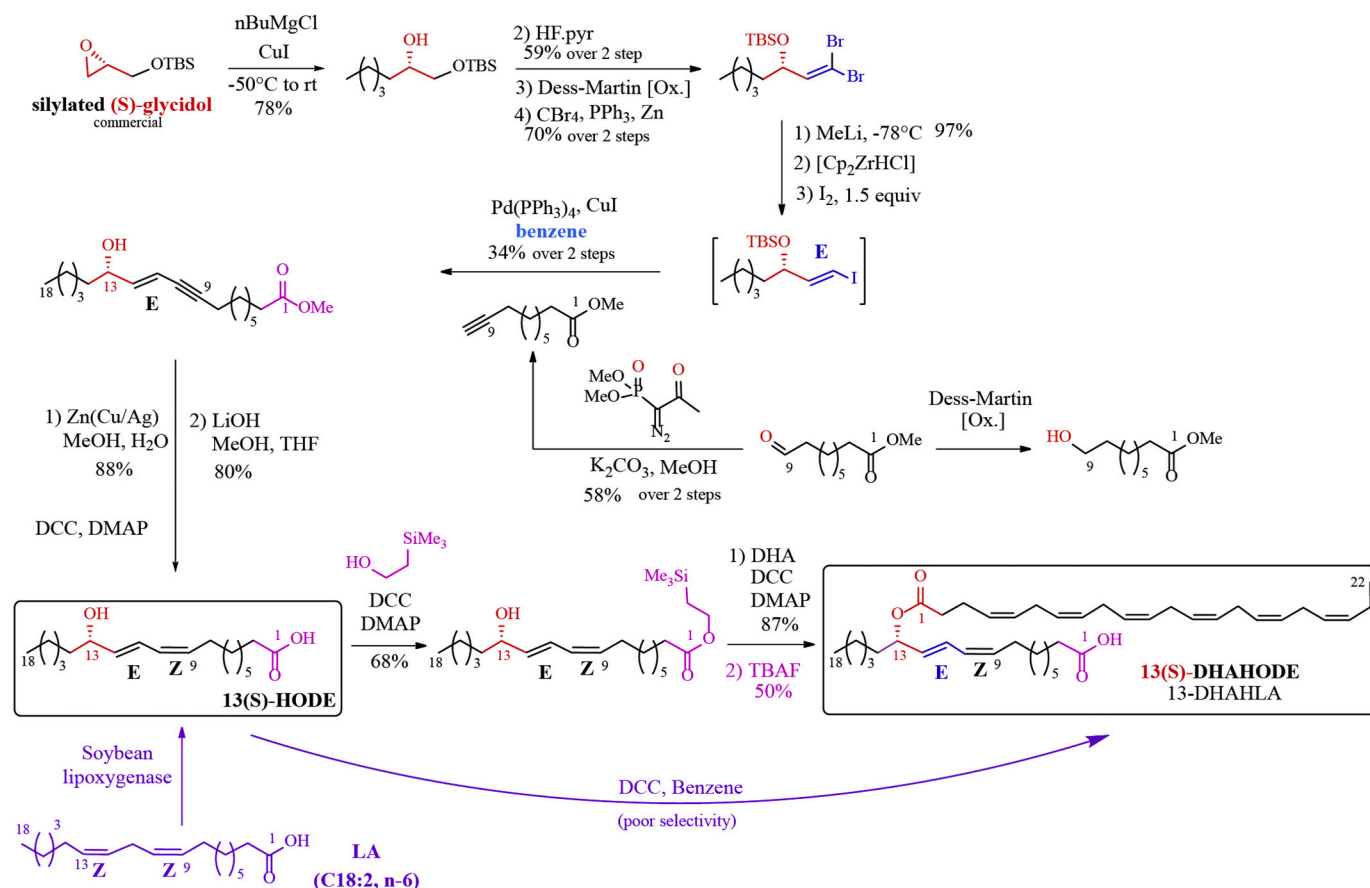
chain by reduction of a homopropargylic alcohol with deuterium gas [37] or a Grignard reaction with a fully deuterated organomagnesium [28] bromide. Scheme 13 summarizes this latter strategy.

More recently, based on the fact that replacement of a hydrogen atom by a deuterium atom may inhibit the metabolism of drug molecules [38], Mountanea et al. [15] incorporated a deuterium atom at the carbon that joins FA and HFA (Scheme 14).

It seems unlikely that the deuterium protects the FAHFA ester



Scheme 9. Enantioselective synthesis of 9-PAHSA and 5-PAHSA (Pflimlin 2019) [13].



Scheme 10. Total synthesis of 13(S)-DHAHLA (Vik 2019) [26] and enzymatic synthesis (Kuda 2016, in purple) [35]. Red color follows the (S)-configuration, blue color highlights formation of the E double bond, and pink color marks the formation of carboxylated group.

linkage, as the modified C–H bond is not involved in the ester hydrolysis mechanism. In addition, as the carboxyl ester lipase (CEL) enzyme was shown to be sensitive to the configuration of the secondary alcohol [27], the enzymes implicated in FAHFA biosynthesis and metabolism may be sensitive to deuterium isotopic effects. However, although the authors have developed an efficient enantioselective chemical synthesis of FAHFA (Scheme 8, Section 3.7), they surprisingly applied their strategy with a racemic organocatalyst, using (D/L)-proline as catalyst instead of the optically active McMillan catalyst (using N-chlorosuccinimide as source of chlorine instead of the hexachlorocyclohexadiene-one derivative) [10,15]. From the perspective of mass spectrometry measurements, at least four deuterium atoms per molecule are needed to avoid isotopic profile overlap, but increasing number of deuterium atoms also affects the hydrogen bond formation and interaction with the stationary (reverse) phase [18].

The authors efficiently labeled FAHFAs by simply stirring the starting aldehyde with deuterium water in triethylamine (88%, with 97% of incorporation of deuterium atom). However, the deuterium isotopic effect seriously affected the course of the epoxidation reaction (performed via alpha-chlorination of the deuterated aldehyde), greatly lowering the yield (30% instead of 73% with nondeuterated starting aldehyde). The next three steps are similar to the steps described in Scheme 8 (Section 3.7, similar yields).

For FAHFA quantification in biological samples from several (patho) physiological conditions, Hu et al. [39] synthesized a tetradeuterated 12-PAHSA, branching $^2\text{H}_4$ -palmitic acid on a 12-HSA precursor, which was prepared according to Balas procedures [14]. Kuda and colleagues also used a $^2\text{H}_4$ -5-PAHSA, where the four deuterium atoms were located in the HSA substructure. This synthesis is based on Balas approach [7,14], replacing the hydrogen gas with deuterium gas in the

Brown reduction of the homopropargylic precursor.

3.10. Synthesis TAG-FAHFAs, also called TAG (mono) estolides

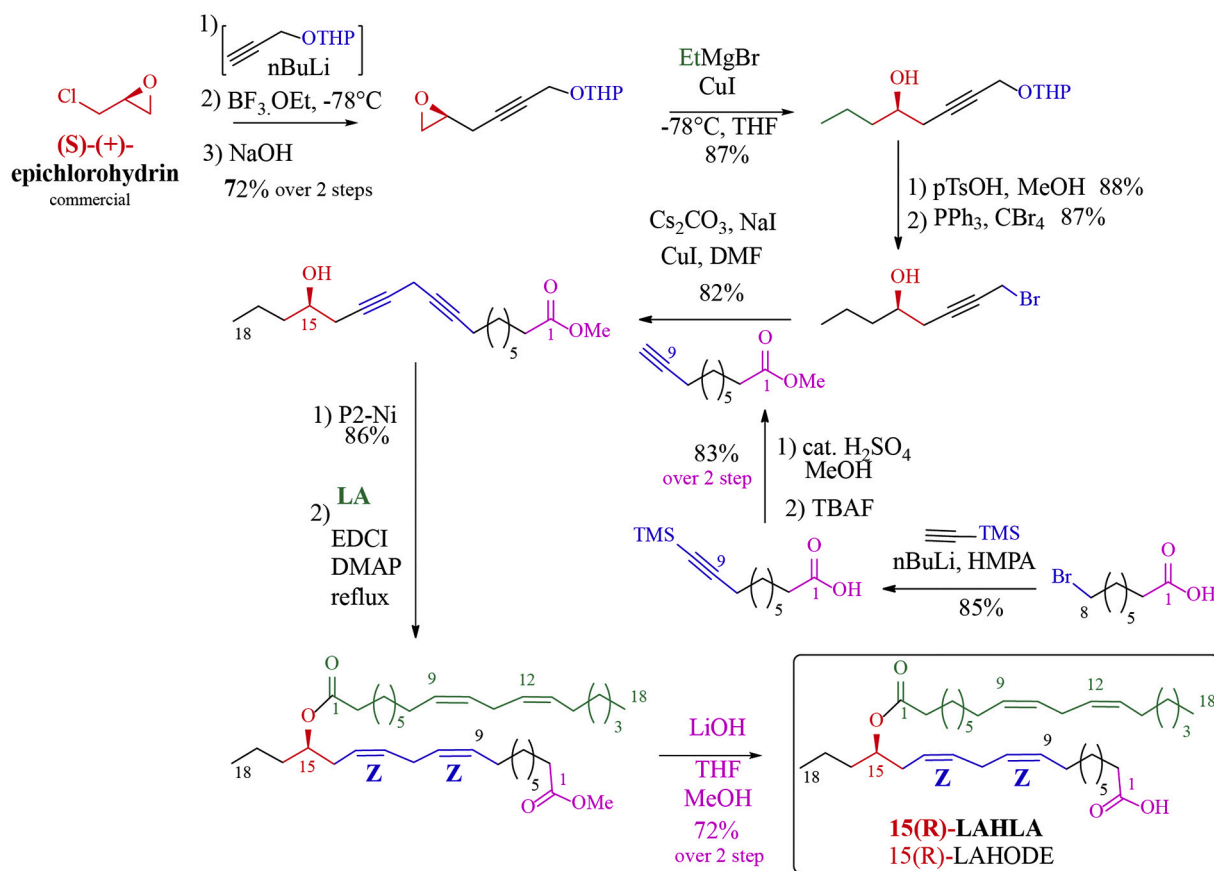
A review of the literature [40] was very recently published on fatty acid estolides. However, this review primarily reported on the types of catalysts used for the oligomerization of FA (i.e., more than two FAHFA chains) and their physical properties. As the synthesis of TAG-FAHFA is based on the “common” synthesis of TAGs, only a brief update is given below with recent examples.

In most cases, the FAHFA is esterified with a DAG containing two similar fatty chains [37,41]. Tan et al. [41] recently synthesized several TAG (mono) estolides, including TAG with three different chains, by modified Steglich esterification reactions without any protection of the glycerol (Scheme 15). The first esterification proceeded with a modest yield, wasting half of the FAHFA. In addition, the introduction of the OA chain at the free primary alcohol was also poorly selective, providing a byproduct with the OA chain at position 2 of glycerol (34%).

To study the lubrication properties and thermal degradation of castor oil, Romsdahl et al. recently esterified [42] the castor oil with palmitoyl chloride in pyridine (Scheme 16). Thus, ricinolein, the major constituent of castor oil (a TAG with three free hydroxyl functions from the three ricinoleate chains), was converted to a TAG tri-estolide. Interestingly, the tribological properties (coefficient of friction, wear, thermal resistance, etc.) were nicely improved compared to the non-esterified castor oil [42].

4. Analytical techniques for FAHFA detection and quantitation

Given the large theoretical number of FAHFA structures (> 3000



Scheme 11. Synthesis of 15(R)-LAHLA from optically active epichlorohydrin (Wang 2019) [20].

structures, families and isobaric isomers; see Section 5.1), the expected complexity of the lipid extracts promoted the development of efficient analytical techniques.

FAHFA physiological levels in mice and humans range from 0.5 to 500 nmol/L in plasma or serum and from 1 to 200 pmol/g in tissues [3]. Therefore, sensitive liquid chromatography-mass spectrometry systems are needed to analyze FAHFA regioisomers. Liquid-liquid extraction of total lipids based on modified Bligh Dyer protocol is widely used by several laboratories (Table 1). Acidification of the polar phase by the citrate buffer facilitates protonation of FAHFAs that can be quantitatively collected within the nonpolar phase. Internal standards labeled with ^{13}C atoms are preferred over ^2H -labeled standards for quantitative analysis, because the retention time of ^2H -labeled standards is shifted according to the number of deuterium atoms per molecule.

Subsequently, solid phase extraction has to be used to separate FAHFAs from other lipid classes and to concentrate the sample. This is a crucial step that also removes ceramide contaminants within the FAHFA fraction [18,43] and prevents faulty results [44,45]. Extracted FAHFAs are then analyzed using LC-MS. Reverse-phase C18 or C30 columns are used to separate FAHFA regioisomers, and a combination of multiple reaction monitoring (MRM) transitions is used to target FAHFAs [18]. Alternatively, parallel reaction monitoring (PRM) [37], full scan [3,37], neutral loss [39], or MS^3 scan [19,35,43] modes can be applied for structural studies. Derivatization of the carboxylic function may increase the sensitivity of the analysis [46] or introduce a characteristic fragment for shotgun lipidomic approaches [39]. The fragmentation mechanisms and product ion structures of hydroxy fatty acid have been explored using gas-phase ozonolysis [6,47].

The stereochemistry of PAHSAs, LAHLAs and 13-DHAHLA was explored using LC-MS and chiral columns with cellulose tris(4-methylbenzoate) and amylose tris(3,5-dimethylphenylcarbamate) selectors, respectively [19,20,27,43,52]. With the orthogonal separation power of

ion mobility spectrometry, PAHSA regioisomers can be resolved, but due to the technical complications and sensitivity, this approach has only a limited use [43,56].

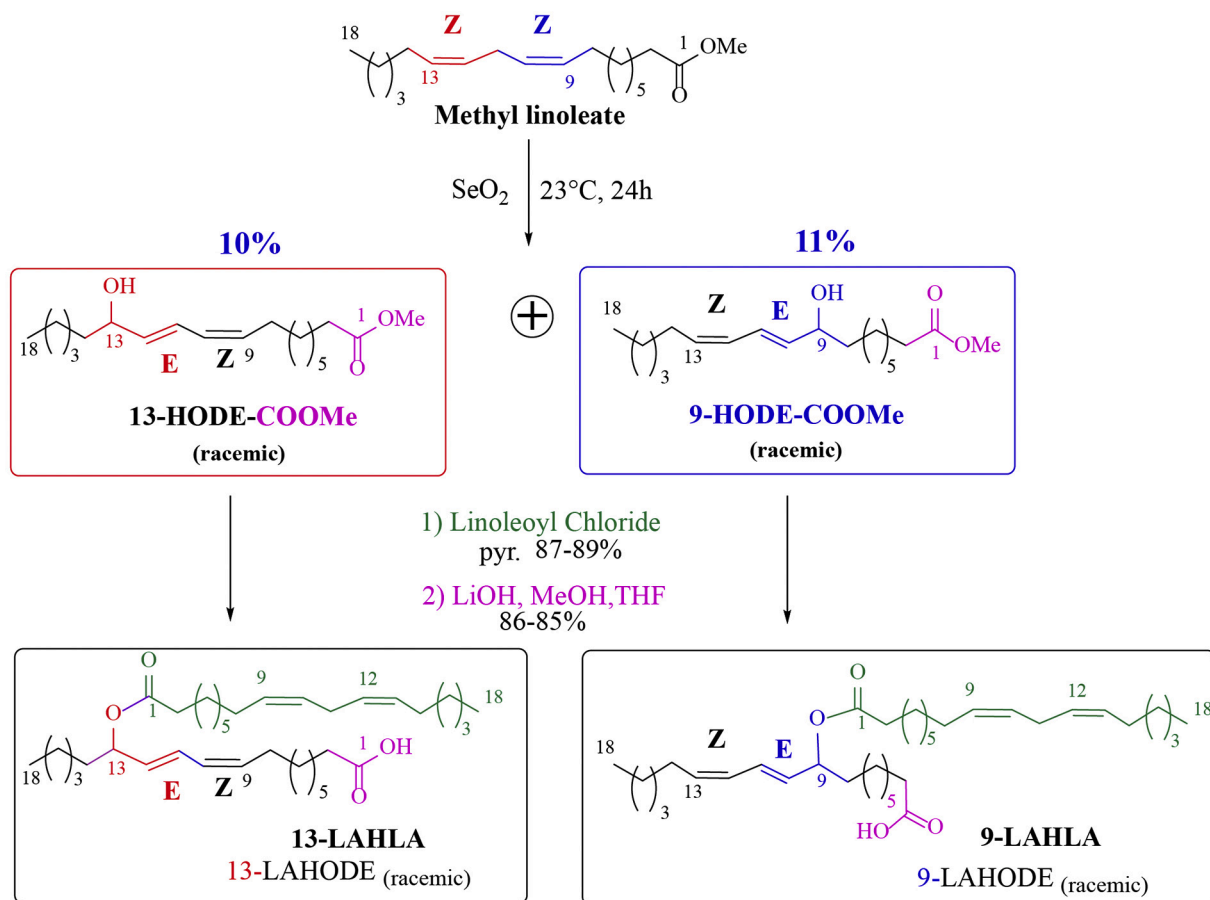
Each FAHFA regioisomer is a unique molecule with specific effects, and changes in their levels are independent of the other regioisomers in this family. Therefore, only methods capable of regioisomer separation are useful for the analysis of biological mechanisms [18,35,46]. The separation of enantiomers is a highly targeted approach used to test hypotheses such as enzyme specificity [27]. The current state-of-the-art technique for FAHFA analysis involves a combination of liquid-liquid and solid-phase extractions, ^{13}C -labeled internal standards and LC-MS (sensitive triple quadrupole) with qualitative and quantitative MRM transitions [18,44,45].

5. Diversity of endogenous FAHFA families

5.1. Families and isomers

Ma et al. calculated that 1089 theoretical FAHFAs exist when 33 common fatty acids are permuted [1]. When we consider that each FAHFA contains a chiral center and that both enantiomers are typically present [19,27,43], this number increases to 2178 FAHFAs. Furthermore, we also have to include the diverse possibilities for the position of the ester bond in the HFA chains defined by the branching position (e.g., 9 regioisomers for (R) and 9 regioisomers for (S) PAHSAs), but the number of regioisomers is highly dependent on the length and unsaturation of the hydroxy fatty acid.

We previously prepared a library of theoretical MRM transitions [49] according to published guidelines [1,48] and explored the presence of potential FAHFAs in human subcutaneous adipose tissue samples. Pooled samples and backup aliquots acquired within previous projects were used [35,57]. Briefly, samples were collected from both



Scheme 12. Racemic allylic oxidation of methyl linoleate (Wang 2019) [20].

healthy volunteers and patients with type 2 diabetes, where both men and women were or were not provided omega-3 PUFA supplements for several months. This exploratory work identified 583 FAHFA regioisomers from 21 FAHFA families and represents a compilation of analytes from multiple sources (Fig. 2 and Supplementary Fig. 1). Because the composition of FAHFAs was dependent on the nutrition (bioavailability of fatty acid precursors), omega-3 PUFA-related FAHFAs were only detected in samples from patients supplemented with marine concentrates. Relatively high number of regioisomers could be attributed to the fact that a portion of the samples were acquired from elderly women aged 60–85 years and that the FAHFA concentration and the number of regioisomers increases with age [50]. Zhu et al. explored FAHFA diversity in mouse visceral fat during their lifespan and observed a maximum of 284 regioisomers within 46 FAHFA families in aged mice [50]. Interestingly, their analytical approach using a derivatization tag yielded up to 13 regioisomers of PAHSAs, while other laboratories analyzing underivatized FAHFAs have only detected 9 PAHSA regioisomers (branching at carbon atoms 5 to 13) [18,49,50].

Analysis of FAHFA regioisomer compositions revealed several structural patterns. The diversity of hydroxy fatty acid backbones was lower than that of the esterified fatty acids. Saturated hydroxy fatty acids have the highest number of regioisomers, and the number of branching position decreases with the number of double bonds (Table 2). Fatty acids with 18 carbons belong to the most populated FAHFA family. Interestingly, almost no FAHFAs with 20 and 22 carbon backbones are present despite the number of potential eicosanoid and docosanoid precursors (see Section 6.3). Although not all regioisomers were successfully identified using the MS/MS/MS technique (Supplementary Fig. 1), the general pattern suggests that the hydroxylation of saturated acyl chains occurs approximately in the middle of the chain

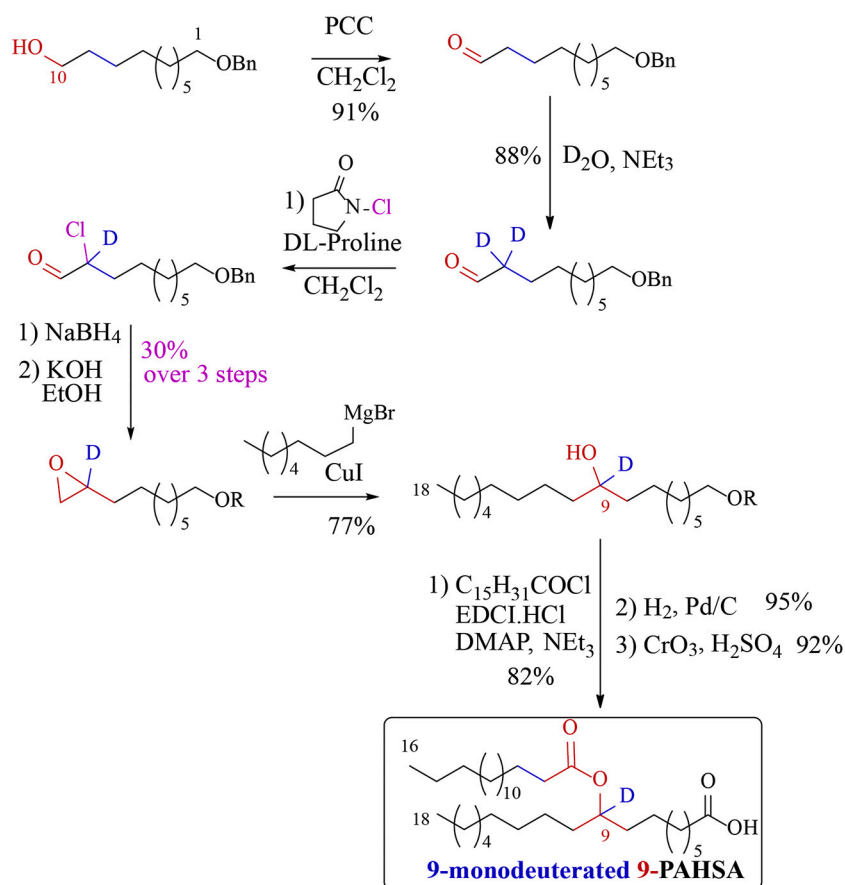
(e.g., 9-PAHSA) or between carbons 5 and 13.

In contrast, the composition of fatty acids esterified on the hydroxy fatty acid backbone is broad. The only observed limitation is the general availability of a fatty acids resulting from the composition of a diet (e.g., polyunsaturated fatty acid-rich diet results in higher content of polyunsaturated FAHFAs [35,52,58]).

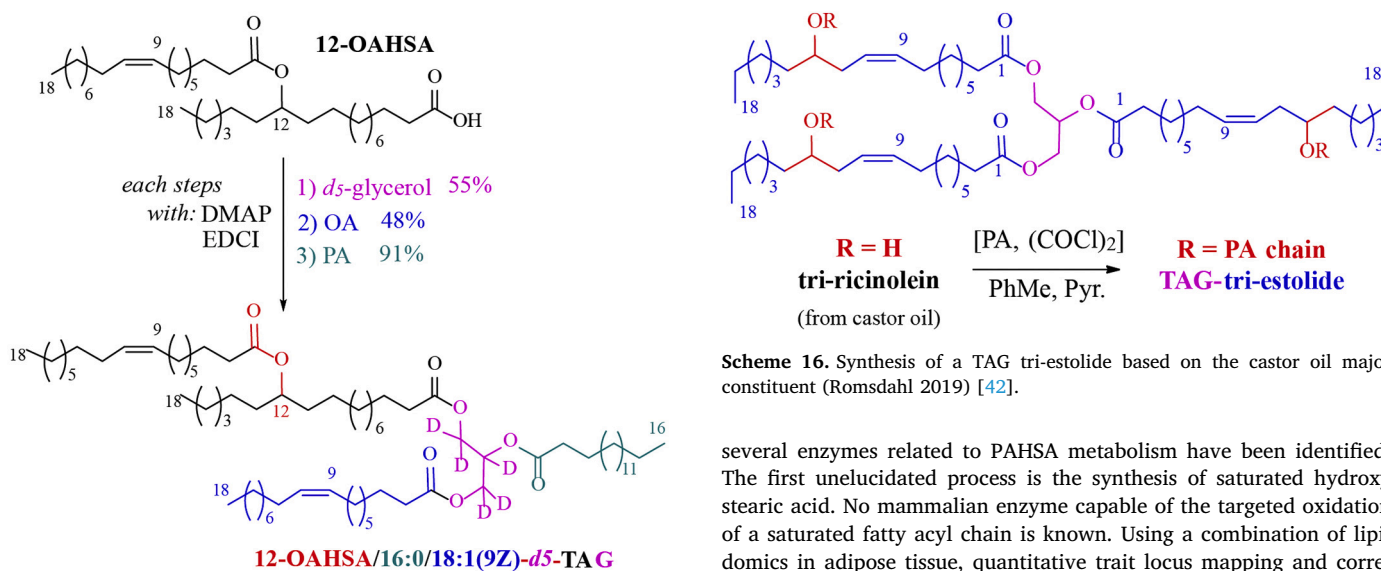
5.2. Dietary sources and concentrations

FAHFAs are endogenously synthesized in mammals, but dietary sources are also of interest. Yore et al., showed that ~100 pmol/g of 9-PAHSA and other PAHSAs were present in chow diet (Lab Diet, #5008), while only ~12 pmol/g of 9-PAHSA was present in a high fat diet (Harlan Teklad, TD.93075) [3]. Given the components of the chow diet (corn oil, soybean, porcine fat, etc.), it is obvious that FAHFAs are also present in oils and grains. Olive oils contains ~1 μmol/L of PAHSA regioisomers and ~5 μmol/L of OAHSA regioisomers, while oat oil contains ~0.5 pmol/mg of PAHSAs and high levels (~300 pmol/mg) of LAHSA regioisomers [19,45]. Therefore, natural oils are not suitable vehicles for FAHFA administration [44,45] but could be great sources of bioactive lipid nutrients.

Different foods contain various FAHFAs at various levels. Oat, clementine, garlic and pineapple were identified as vegetables and fruits with the highest FAHFA levels (~56–94 ng/g) [59], with up to 71 FAHFA regioisomers were identified in rice [46]; FAHFAs based on hydroxy oleic acid were observed in egg yolk [1]; caribou meat was shown to be a potential source of polyunsaturated FAHFAs [58] and breast milk also contains several FAHFAs [43]. Although there are many FAHFA-rich dietary sources, it is unclear whether the free FAHFAs can survive the digestive process in the intestine, as pancreatic



Scheme 14. Synthesis of a 9-mono-deuterated 9-PAHSA (Mountanea 2019) [15].

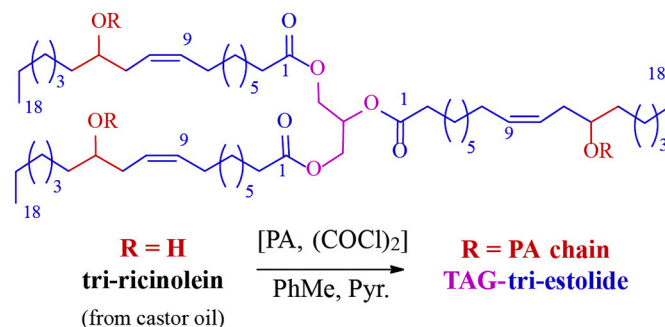


Scheme 15. Synthesis of a deuterated TAG estolide with two different fatty acyl chains (Tan 2019) [41].

FA appear to be different for saturated and unsaturated chains. In addition, the many regioisomers within a FAHFA family may result from different biological mechanisms.

6.2. Saturated FAHFAs

The biosynthetic pathway of saturated FAHFAs is unknown, but



Scheme 16. Synthesis of a TAG tri-estolide based on the castor oil major constituent (Romsdahl 2019) [42].

several enzymes related to PAHSA metabolism have been identified. The first unelucidated process is the synthesis of saturated hydroxy stearic acid. No mammalian enzyme capable of the targeted oxidation of a saturated fatty acyl chain is known. Using a combination of lipidomics in adipose tissue, quantitative trait locus mapping and correlation analyses in rat recombinant inbred strains, as well as response to oxidative stress in murine models, we elucidated the putative pathway for the biosynthesis of saturated hydroxy fatty acids [49]. A family of flavin containing monooxygenases and several enzymes from antioxidant defense system, especially peroxiredoxin 6, can synthesize a hydroxy stearic acid precursor from (per)oxidized membrane phospholipids. Peroxiredoxin 6 has peroxidase activity using phospholipid hydroperoxides as substrates and possesses phospholipase A2 and lysophosphatidylcholine acyltransferase activities [73]. This enzyme can thus produce a phospholipid with a hydroxy fatty acid or even cleave this hydroxy fatty acid in the case of peroxiredoxin 6 (Fig. 3A). Several

Table 1

AMPP, *N*-(4-aminomethylphenyl)pyridinium; DMED, *N,N*-dimethylethylenediamine; MRM, multiple reaction monitoring; MTBE, methyl *tert*-butyl ether extraction protocol; SPE, solid-phase extraction; TOF, time of flight; UHPLC, ultra-high-performance liquid chromatography; QQQ, triple quadrupole.

Lipid extraction	Internal standard	SPE	Derivatization	Instrument type	Scan type	Ref.
Bligh Dyer & citrate buffer	$^{13}\text{C}_x$ - $^2\text{H}_x$ -PAHSAs	SPE silica	none	UHPLC-TOF/QQQ	Full scan/MRM	[1,3,18,48]
Bligh Dyer & citrate buffer	$^{13}\text{C}_4$ 9-PAHSA	SPE silica	none	UHPLC-QQQ	MRM/MS ³	[35,43]
Ethyl acetate & citrate buffer	$^{13}\text{C}_4$ 9-PAHSA	SPE silica	none	UHPLC-QQQ	MRM/MS ³	[49]
Bligh Dyer	None	SPE strong anion exchange	DMED and $^2\text{H}_4$ -DMED	UHPLC-QQQ	MRM	[46,50,51]
MTBE	$^{13}\text{C}_4$ 9-PAHSA	none	none	UHPLC- Q Orbitrap	Full scan/PRM	[52,53]
Acetonitrile	$^2\text{H}_{31}$ PAHSAs	none	none	UHPLC-QQQ	MRM	[13]
Bligh Dyer	$^2\text{H}_4$ 12-PAHSA	SPE silica	AMPP	QQQ	shotgun/neutral loss	[39,54]
Methanol	none	Online SPE C8	none	UHPLC-QQQ	MRM	[55]

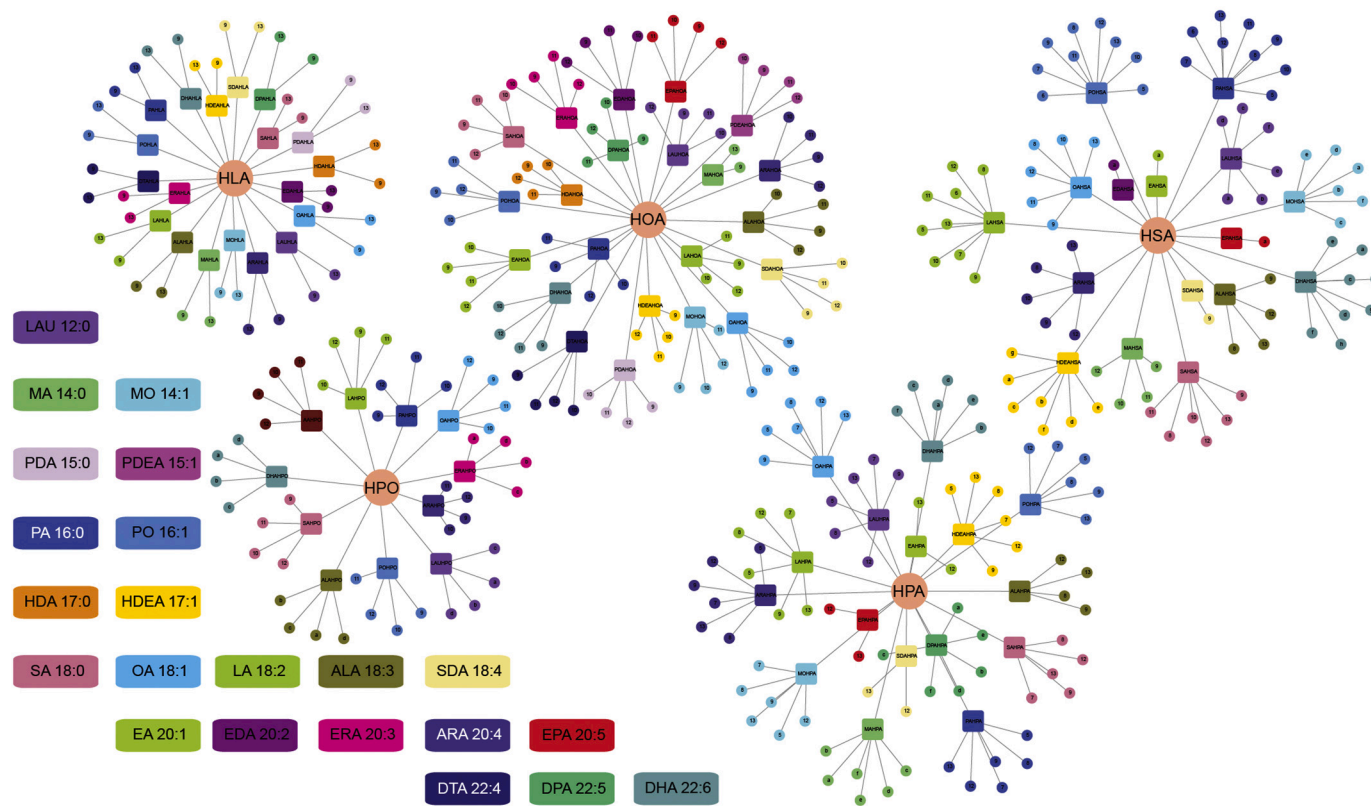


Fig. 2. Illustrative overview of the most populated FAHFA families. Individual networks are derived from a hydroxy fatty acid backbone, and esterified fatty acids are color-coded according to the legend. Distant circle nodes represent the regioisomers. The full-scale image is available as Supplementary Fig. 1. HLA, hydroxy linoleic acid; HOA, hydroxy oleic acid; HSA, hydroxy stearic acid; HPO, hydroxy palmitoleic acid; HPA, hydroxy palmitic acid. Abbreviations for fatty acids follow the nomenclature by Ma et al. [1].

phospholipids containing 9-HSA were identified in human colorectal cancer samples and are related to FAHFA synthesis [74], but an FAHFA-containing phospholipid has yet to be identified. Importantly, PAHSA synthesis has been linked to the NADPH-generating pathway, which probably supplies reduced coenzymes for both the reduction of lipid peroxides and the synthesis of fatty acids via the DNL pathway [37].

Using several independent models, it has been shown that cells supplemented with hydroxy fatty acid precursors can synthesize FAHFAs [3,27,35,74], although the putative acyltransferase involved remains unknown (Fig. 3B). With respect to the number of regioisomers of HSA and the observation that only some FAHFA regioisomers are present in various organs or cells [3,49], there are probably several position-specific acyltransferases expressed in different cell types (e.g., adipose tissue vs liver). FAHFAs can also be obtained from food, and mouse experiments have documented that bioavailability of ingested PAHSAs [13,43,72,75].

Recently, triacylglycerols containing PAHSAs were identified as a metabolic reservoir that modulates free PAHSA levels upon activation

of lipolysis and lipid remodeling in adipose tissue [37,41]. FAHFAs esterified at all positions of the glycerol backbone have been described [37,41], while only the *sn*-2 position was esterified with hydroxy fatty acid in phospholipids for the putative FAHFA precursors [74]. The involvement of adipose triglyceride lipase, hormone-sensitive lipase, and diacylglycerol O-acyltransferases 1 and 2 in the release of FAHFAs and remodeling of triacylglycerol estolides suggests a tight control of unesterified levels of these lipid messengers [37,41]. Moreover, the possible combinations of acyl chains, saturation, position of the estolide bond, and enzyme substrate preferences provide the cell with the ability to selectively regulate which FAHFAs are liberated. With respect to the structure of TAG estolides, several levels of isomerism exist, including 1) the position of a FAHFA on glycerol backbone (*sn*-1, *sn*-2, or *sn*-3), which defines the substrate specificity of lipases; 2) the FAHFA branching position, which defines the substrate affinity; and 3) the number and configuration of multiple FAHFAs on glycerol, which defines the metabolic intermediates.

Interestingly, comparative gene identification-58 (CGI-58; ABHD5)

8 weeks [35]. The existence of 14-HDHA backbone was confirmed in the epididymal adipose tissue of rats, where 14- and 17- regioisomers of hydroxy DHA-derived FAHFAs were identified [49]. Although hydroperoxy DHA is widely used precursor for protectins and resolvins [78,79], and hydroxy DHA isomers are widely present in mammals, the presence of only two FAHFA regioisomers is unexpected.

For hydroxy arachidonic acid, the building block of eicosanoid class molecules, only one regioisomer of the backbone (11-hydroxy eicosatetraenoic acid; 11-HETE) has been identified [49]. 11-HETE backbone was esterified with the common fatty acids (e.g. 16:0, 18:1, 22:6, etc.) [49]. Pham et al. explored the composition of moose and caribou meat and identified a series of polyunsaturated FAHFAs with up to 9 double bonds and various HFA chains (e.g. 18:1, 20:3, 22:3, 22:4 and 22:4) [58]. Interestingly, the prevailing (and only) estolide branching position among all families was carbon 5 (5-HETE backbone).

These lines of evidence suggest that 1) unsaturated FAHFAs are synthesized via a pathway independent of the saturated FAHFA pathway; 2) unsaturated HFA precursors are regulated by known mechanisms; and 3) polyunsaturated FAHFAs are rare due to limited sources (e.g., omega-3 PUFA) or due to a lack of enzymes specific to hydroxylated polyunsaturated chains.

6.4. Synthesis in plants

While the complete biosynthetic FAHFA pathway in mammals remains unclear, much more is known with respect to unusual fatty acids (e.g., hydroxy, epoxy, branched, etc.) in plants used in the oleochemical industry [80]. The most studied of these is ricinoleic acid (18:1-OH; (9Z,12R)-12-hydroxyoctadec-9-enoic acid), which is present in castor (*Ricinus communis*) seed oil, where it is esterified in TAG estolides [61,81]. Ricinoleic acid is synthesized from oleic acid by Δ -12 fatty acid hydroxylase (FAH12) on the *sn*-2 position of phosphatidylcholine in the endoplasmic reticulum [80]. FAH12 is also involved in the production of lesquerolic acid (20:1-OH; (11Z, 14R)-14-hydroxyicos-11-enoic acid) in *Lesquerella fendleri*. Genetic studies comparing *Arabidopsis thaliana* and *L. fendleri*, two genetically closely related plants that differ in the accumulation of hydroxy fatty acids, revealed multiple lipid biosynthetic genes related to those involved in the metabolism of hydroxy fatty acids [82]. Transcriptomic analysis suggests that a wide set of coevolved mechanisms and adaptations exists and that the *Lesquerella* optimized these mechanisms to drive hydroxy fatty acids away from membranes to TAGs. FAH12 creates 18:1-OH esterified to membrane phospholipids, but only traces of HFA were detected in the phosphatidylcholine fraction during seed development, while 20:1-OH in the TAG fraction increased with time [83]. Horn et al. identified a small group of phospholipases that may remove HFAs from phosphatidylcholines and channel them to desaturases, elongases and acyltransferases associated with TAG synthesis [82]. Although the HFA in phosphatidylcholines is present at the *sn*-2 position, the majority of TAG and DAG species have HFA in the *sn*-1/3 position [84]. Furthermore, as the 18:1-OH is only elongated to 20:1-OH as an acyl-CoA, it has to be cleaved from the phospholipid before esterification into TAGs.

We may find inspiration in these biochemical pathways for the putative synthesis of PAHSAs (Fig. 1 in Lee et al., [80]). Similar to plants, oxidized phospholipid acyl chains have to be excluded from membranes (e.g., by peroxiredoxin 6 [49]), which primarily exist esterified to the *sn*-2 position, and the intermediates are difficult to detect [74]. Furthermore, TAG estolides with PAHSAs bound to both *sn*-2 and *sn*-1/3 have been observed [41]. These lines of evidence suggest that HFA has to be cleaved from the putative intermediate before it is esterified to an FAHFA or further incorporated into a TAG estolide.

6.5. Degradation pathway: serine and threonine hydrolases

Several hydrolases [carboxyl ester lipase (CEL), carboxylesterase 3, androgen-induced gene 1 (AIG1) protein and androgen-dependent TFPI

regulating protein (ADTRP)] have been identified as FAHFA hydrolases that cleave the FAHFA estolide bond with a stereospecific preference [60,85–87]. Both AIG1 and ADTRP were recently confirmed as endogenous FAHFA hydrolases in mice [87]. ADTRP plays a major role in vascular development and function, most likely through its expression in endothelial cells [88]. Levels of Adtrp mRNA were observed to be several fold lower in adipose tissue than in the liver [35], suggesting that it may regulate circulating FAHFA levels in target organs. AIG1 is primarily expressed in the liver, adipose tissue, lung and heart and is involved in the control of intracellular Ca^{2+} concentrations in the endoplasmic reticulum [35,89]. CEL preferentially hydrolyzes FAHFAs with the ester bond further away from the carboxylate (12-PAHSA > 9-PAHSA \gg 5-PAHSA), and unsaturated FAHFAs are degraded faster than saturated species [60].

Both ADTRP and AIG1 are atypical hydrolases that use catalytic threonines instead of serines to degrade free FAHFAs [87]. In contrast, the hydrolytic activity of ATGL and HSL, which are involved in TAG estolide remodeling [37,41], depends on a catalytic serine [90]. Additional studies on lipid-remodeling enzymes will be needed to describe the TAG estolide metabolism in detail.

7. Biological effects

Since the discovery of FAHFA in WAT, many beneficial biological effects have been reported.

7.1. Anti-diabetic and anti-inflammatory effects of PAHSAs

There are many effects that FAHFAs exert on target tissues, specific cell types or metabolic pathways some of which are summarized in Tables 4 and 5. One FAHFA family, the PAHSAs, has attracted a great deal of attention due to it being closely associated with insulin sensitivity in humans. PAHSA serum and adipose tissue levels are decreased in insulin-resistant humans, with their levels correlating with insulin sensitivity [3,91], and they also enhance glucagon-like peptide-1 (GLP-1) production and increase glucose-stimulated insulin secretion [92,93]. Oral gavages of 5- and 9-PAHSA have been shown to improve glucose tolerance in aged mice and mice fed high-fat diet [3,45,75].

Anti-inflammatory effects are the second most important properties of PAHSAs. 9-PAHSA blocks lipopolysaccharide-stimulated dendritic cell and macrophage activation [3,35,75] via free fatty acid receptor 4 (FFAR4, GPR120) and modulates both innate and adaptive immune responses in a mouse colitis model [94] and type 1 diabetes [93]. 5- and 9-PAHSAs have been used as standards to compare anti-inflammatory effects of new FAHFAs [19,20,35]. Therefore, PAHSAs have beneficial effects on both metabolism and immune system, and could possibly be used to target autoimmune metabolic diseases such as type 1 diabetes [93].

Following the original publication by Yore et al. [3], several studies have demonstrated the positive impact of PAHSAs on glucose metabolism (Table 4). Pflimlin et al. reported that the supplementation of 5- or 9-PAHSA did not improve glucose homeostasis in mice fed a high fat diet [13]. However, several methodological and conceptual problems were identified in this study, including an inappropriate vehicle, the dosage of PAHSAs, the composition of high fat diets, unsuitable biochemical tests and an improper analytical approach [44,45]. Syed et al. [45] repeated some of the original experiments by Yore et al. [3] and retested the results published by Pflimlin et al. [13], confirming the beneficial effects of PAHSAs. This situation highlighted the need for standardized techniques and methodological papers [18] that should make basic research less prone to incorrect results and interpretations.

7.2. PAHSAs and signal transduction

The first identified receptor for PAHSAs was FFAR4 (alternatively GPR120) which regulates GLUT4 translocation in adipocytes [3].

Table 4

Selected biological effects of PAHSAs excerpted from the literature. db/db, mutation in the leptin receptor gene; DNL, de novo lipogenesis; GLP-1, glucagon-like peptide-1; HFD, high fat diet; ob/ob, mutation in leptin gene; TAG, triacylglycerols; WAT, white adipose tissue.

Refs	FAHFA	Model	Dose	Effect
[3]	5-PAHSA	C57BL/6 J HFD	45 mg/kg, single gavage	↑ glucose tolerance
	9-PAHSA	C57BL/6 J HFD	45 mg/kg, single gavage	↑ glucose tolerance
	5-PAHSA	C57BL/6 J aged chow	30 mg/kg, single gavage	↑ glucose tolerance
	5-PAHSA	pancreatic islets from humans	20 μM for 45 m	↑ insulin secretion
	5-PAHSA	STC-1 enteroendocrine cells	30 μM for 1 h	↑ GLP-1 secretion
	9-PAHSA	STC-1 enteroendocrine cells	30 μM for 1 h	↑ GLP-1 secretion
	9-PAHSA or 5-PAHSA	3 T3-L1 adipocytes	20 μM for 6 d	↑ glucose uptake and GLUT4 translocation
	9-PAHSA	dendritic cells C57BL/6 J	2–80 μM for 10 m	↓ dendritic cell maturation and cytokine production
	9-PAHSA	C57CL/6 J mice chow	30 mg/kg for 3 d	↓ WAT inflammation
	9-PAHSA	C57CL/6 J mice HFD	45 mg/kg for 3 d	↓ WAT inflammation
[91]	5-PAHSA or 9-PAHSA	human preadipocytes	20–40 μM for 1–8 d	↑ adipocyte differentiation
[72]	9-PAHSA	3 T3-L1 adipocytes	20 μM for 20 h	↑ glucose uptake
[37]	5-PAHSA	C57BL/6 J chow	45 mg/kg for 4 d	↑ DNL and TAG remodeling
	5-PAHSA	3 T3-L1 adipocytes	40 μM for 3 d	↑ lipolysis and TAG remodeling
				↑ glucose uptake
				↑ metabolic rearrangements and DNL
				↑ lipolysis
[95]	5-PAHSA	adipocytes C57BL/6 J	40 μM for 30 m	↑ lipolysis
	9-PAHSA	3 T3-L1 adipocytes	5–30 μM for 6 d	↑ adipocyte browning
	9-PAHSA	3 T3-L1 adipocytes	30 μM for 1 h	↓ activation of NF-κB
	9-PAHSA	C57BL/6 J chow ob/ob	50 mg/kg for 4 w	↑ subcutaneous WAT browning
[75]	5-PAHSA and 9-PAHSA	C57BL/6 J chow	2 mg/kg for 18 w	↑ insulin sensitivity and glucose tolerance
				↑ glucose-stimulated insulin secretion
				↓ WAT inflammation
				↑ glucose-stimulated insulin secretion
				→ weight, fat mass, food intake
				↑ insulin sensitivity and glucose tolerance
				→ glucose-stimulated insulin secretion
				↓ WAT inflammation
[92]	5-PAHSA	pancreatic islets from CD1 mice	20–80 μM for 20 m	↑ glucose-stimulated insulin secretion
	5-PAHSA	pancreatic islets from humans	40 μM for 2 d	↑ glucose-stimulated insulin secretion
[96]	5- and 9-PAHSA	C57BL/6 J chow	30 mg/kg, single gavage	↓ glycemia pyruvate tolerance test
	9-PAHSA	C57BL/6 J chow	9 μg/h for 3 h	↓ endogenous glucose production
	9-PAHSA	C57BL/6 J HFD	18 μg/h for 3 h	↓ endogenous glucose production
	5- and 9-PAHSA	C57BL/6 J chow	0.1 mg/d and 0.4 mg/d for 13 w	↑ systemic insulin sensitivity
	5- and 9-PAHSA	C57BL/6 J HFD	0.1 mg/d and 0.4 mg/d for 13 w	↑ systemic insulin sensitivity
				↑ hepatic insulin sensitivity
	9-PAHSA	WAT explants C57BL/6 J chow	40 μM for 30 m	↓ lipolysis
	9-PAHSA	hepatocytes C57BL/6 J chow	40 μM for 30 m	↓ endogenous glucose production
[97]	9-PAHSA	C57BLKS/J chow db/db	50 mg/kg for 4 w	↓ cognitive impairment
[12]	5-PAHSA	3 T3-L1 adipocytes	20 μM for 2 d	↑ glucose uptake
	5-PAHSA	HepG2 hepatocytes	20 μM for 2 d	↑ glucose uptake
				↓ lipid synthesis, inflammation
	5-PAHSA	C57BL/6 J HFD	50 mg/kg for 30 d	↑ glucose tolerance
	5-PAHSA	C57BL/6 J HFD	50 and 150 mg/kg for 10 and 30 d	→ glucose tolerance
	5-PAHSA	C57BL/6 J HFD db/db	50 and 150 mg/kg for 10 and 30 d	→ glucose tolerance
				↑ liver steatosis
				↑ hepatic inflammation
[35]	9-PAHSA	RAW 264.7 macrophages	10 μM for 18 h	↓ cytokine production
[98]	5-PAHSA	THP-1 macrophages	30 nmol/well	↓ cytokine production
[17]	9-PAHSA	RAW 264.7 macrophages	25 μM for 20 h	↓ cytokine production
[94]	5-PAHSA and 9-PAHSA	C57BL/6 J chow	5–10 mg/kg for 1–9 d	↓ weight loss due to colitis
			5–10 mg/kg for 28 d	↓ colonic inflammation
		Paneth cells C57BL/6 J	20 μM for 30 m	↑ Paneth cell bactericidal function
		dendritic cells C57BL/6 J	20 μM for 30 m	↓ inflammatory response
[74]	9-PAHSA	HTC-116 colon cancer cells	1–400 μM for 24 h	→ no apoptosis
[13]	5-PAHSA and 9-PAHSA	C57BL/6 J HFD	45 mg/kg, single gavage	→ glucose tolerance
				→ GLP-1 secretion
				→ systemic insulin sensitivity

Subsequently, FFAR1 (GPR40) and FFAR4 were identified as mediators of the anti-inflammatory effects [75,93–95]. Studies on the liver and WAT crosstalk suggest that multiple G protein-coupled receptors (families) could be involved in signal transduction [96].

Sialic acid-binding immunoglobulin-like lectins (Siglecs) are a family of cell-surface immune receptors on macrophages, monocytes, and neutrophils that bind to sialic acid at the terminal glycan residues. 5-PAHSA binds Siglec5 and Siglec14 and suppresses interleukin-8 (IL-8) production in human monocytic cells [98].

7.3. PAHSAs and adipose tissue

Adipose tissue is the primary source of PAHSAs and is also the first target tissue of their effects. 5- and 9-PAHSA regulate glucose uptake and GLUT4 translocation at submaximal and maximal insulin concentrations in adipocytes via FFAR4 signaling [3]. Assays involving hyperinsulinemic-euglycemic clamps in mice revealed that 5- and 9-PAHSA suppress lipolysis in adipose tissue and hepatic glucose production through modulation of free fatty acid levels in circulation [96]. Although 9-PAHSA was shown to suppress isoproterenol-induced lipolysis in perigonadal but not in subcutaneous WAT [96], 5-PAHSA

Table 5

Selected biological effects of FAHFAs excerpted from literature. ALT, alanine aminotransferase; AST, aspartate aminotransferase; PBMC, peripheral blood mononuclear cells.

Refs	FAHFA	Model	Dose	Effect
[35]	9-PAHSA	RAW 264.7 macrophages	10 μ M for 18 h	↓ cytokine production
	13-DHAHLA	RAW 264.7 macrophages	10 μ M for 18 h	↓ cytokine production
	13-DHAHLA	macrophages C57BL/6 J	10 μ M for 18 h	↓ macrophage activation
	13-DHAHLA	human PBMC	1 μ M for 30 m	↓ inflammation
[52]	13-DHAHLA	mast cells C57BL/6 N	1 μ M for 15 m	↓ chemotactic response
	13-DHAHLA	mast cells C57BL/6 N	4 μ M for 15 m	↓ degranulation response
[20]	9-LAHLA	RAW 264.7 macrophages	25 μ M for 20 h	↓ inflammation
	13-LAHLA	RAW 264.7 macrophages	25 μ M for 20 h	↓ inflammation
	15-LAHLA	RAW 264.7 macrophages	25 μ M for 20 h	↓ inflammation
[19]	9-PAHSA	RAW 264.7 macrophages	10 μ M for 20 h	↓ cytokine expression
	13-LAHLA	RAW 264.7 macrophages	10 μ M for 20 h	↓ cytokine production
[53]	9-PAHPA	C57BL/6 J chow	30 μ mol/kg for 12 w	↑ insulin sensitivity 3/10 liver fibrosis
	9-OAHPA	C57BL/6 J chow	30 μ mol/kg for 12 w	↑ insulin sensitivity 1/10 liver fibrosis ↑ respiratory exchange ratio
[103]	9-PAHSA	C57BL/6 J chow	13 mg/kg for 3 d	→ AST and ALT activity
	9-OAHPA	C57BL/6 J chow	13 mg/kg for 3 d	↑ AST and ALT activity
[21]	12-EPAHSA	HepG2/C3A cells	62 μ M for 24 h	↑ Nrf2 activation

increased lipolysis in isolated perigonadal and 3T3-L1 adipocytes [37]. It is possible that different PAHSA regioisomers activate different receptors with different biological outputs. 5-PAHSA not only stimulates lipolysis, but was also shown to stimulate DNL and utilize two concurrently overlapping mechanisms: 1) futile (energy wasting) TAG/FA cycling [99], which builds and breaks TAGs completely; and 2) lipid remodeling of the TAG acyls, including FAHFAs and TAG estolides [37]. Our laboratory investigated the metabolic effects of 5-PAHSA in adipocytes using a dynamic metabolic flux analysis [37]. Experiments with ^{13}C - and ^2H -labeled glucose in adipocytes revealed that 5-PAHSA stimulates glucose uptake, but this glucose is not used for glycerol 3-phosphate production and esterification of fatty acids. The three-carbon glycolytic intermediates are channeled towards the Krebs cycle and DNL, and this is the difference between the activity of 5-PAHSA and insulin [37]. Therefore, with respect to the metabolic rearrangements that are associated with obesity, 5-PAHSA positively affects adipocyte metabolism via multiple mechanisms.

PAHSAs promote adipocyte differentiation independently of peroxisome proliferator-activated receptor γ signaling, and adipocyte hypertrophy is associated with reduced adipose PAHSA levels in humans [91]. However, PAHSAs enhance glucose tolerance in mice without affecting adiposity [3,72,75]. Furthermore, browning of cultured adipocytes was only observed to be induced by 9-PAHSA through activation of FFAR4 and inhibition of inflammatory cytokine secretion [95]. Immune cells are an important part of WAT, and PAHSAs exert their anti-inflammatory effects on both the innate and adaptive branches of immune system. Chronic PAHSA treatment was shown to reduce WAT inflammation and improve insulin sensitivity and glucose tolerance in chow- and high fat diet-fed mice [75] and reduce pro-inflammatory cytokine production from adipose tissue macrophages [3]. Another immunometabolic link in adipose tissue is B cell lymphoma 6 (BCL6), a crucial transcriptional regulator of immunity. Ablation of adipocyte Bcl6 enhances subcutaneous adipocyte lipogenesis and increases the levels of adiponectin and FAHFAs [54].

Mammary glands, which are transformed adipose tissue, produce FAHFAs, and a positive correlation between weight gain during pregnancy and FAHFA levels in breast milk has been observed [43]. In particular, 5-PAHSA levels in milk were observed to be reduced in samples from obese compared to lean nursing mothers [43].

7.4. PAHSAs and the pancreas

5- and 9-PAHSA selectively activate free fatty acid receptor 1

(FFAR1; GPR40) in pancreatic β -cells, stimulate Ca^{2+} flux and augment insulin and glucagon-like peptide 1 (GLP-1) secretion [3,75]. This effect was confirmed in human islets exposed to 5-PAHSA, which increased glucose-stimulated insulin secretion [92,93]. PAHSAs also prevented the onset of type 1 diabetes, increased β -cell viability and attenuated the immune cell response in the pancreas via the GLP-1 receptor, endoplasmic reticulum stress and the MAPK signaling pathway [93]. Such anti-inflammatory effects, which are also seen in a model of colitis and asthma/mast cell activation [52,94], could potentially be effective in the treatment of autoimmune diseases. Of note, CEL is produced in pancreatic acinar cells as a highly specific hydrolase for PAHSAs [60].

7.5. PAHSAs and the gut

A role for PAHSAs in the gut was previously demonstrated through observations of the stimulation of GLP-1 secretion from the enteroendocrine cell line STC-1 [3] and the regulation of both innate and adaptive immune responses via a FFAR4-dependent pathway in a model of colitis [94]. CEL, a pancreatic enzyme that hydrolyzes dietary lipids [60], is also produced in mammary glands and is secreted into milk, where it helps to hydrolyze dietary fats contained in human breast milk fat globules before the newborn pancreas matures [100]. High concentrations of 5(R)-PAHSA were observed in human breast milk, which we speculated may stimulate GLP-1 secretion and β -cell maturation [43]. In addition, meconium, the earliest stool with digested milk, contains more complex FAHFA profiles than could be detected in the breast milk [43]. Additional evidence that the gut actively metabolizes FAHFAs came from colon carcinoma cells that synthesize FAHFAs from 9-HSA to escape the apoptotic pathway [74] and an observation that an increase of unspecified PAHLA in serum was selected as a potential biomarker for colorectal cancer patients [101]. On the contrary, parenteral nutrition is associated with increased content of several FAHFAs, which might contribute to positive effect of this intervention over enteral nutrition [102]. We observed that gavaged triacylglycerol-based marine oils are superior to marine phospholipids and wax esters in their ability to increase the levels of 13-DHAHLA in circulation [52]. This result raises intriguing questions regarding lipid class-specific effects, the intestinal metabolism of FAHFAs and the role of TAG estolides in the bioavailability of FAHFAs.

7.6. PAHSAs and the liver

Zhou et al. performed acute (oral gavages) and chronic (osmotic

minipumps) administration of PAHSAs to explore hepatic glucose metabolism in chow- and high fat-fed mice [96]. The enhancement of hepatic insulin sensitivity and a reduction of endogenous glucose production was linked to liver-adipose tissue communication. PAHSAs directly inhibit both basal and glucagon-stimulated endogenous glucose production in hepatocytes via a cAMP-dependent mechanism and suppresses lipolysis in adipose tissue [96]. This study also highlighted the complex roles of PAHSAs, different receptors and inter-organ communication in systemic glucose homeostasis. Chronic PAHSA treatment also did not alter liver TAG levels in chow-fed mice, suggesting that no propagation of steatosis occurred [96]. However, liver histology in db/db mice fed a high-fat diet showed that 5-PAHSA administration resulted in a steatotic pattern independent of the dose [12]. Interestingly, no effect on liver mitochondrial bioenergetics was observed after three days of 9-PAHSA gavage in chow-fed mice [103].

7.7. PAHSAs and the muscles

Hyperinsulinemic-euglycemic clamp studies in chow- and high fat diet-fed mice showed that 5- and 9-PAHSAs increase glucose uptake into glycolytic muscle extensor digitorum longus and the heart via Akt/PKB signaling, but not into the anterior tibialis, soleus and gastrocnemius muscle [96]. This effect might be important for whole body insulin sensitivity, as the muscle mass is a significant sink for glucose. A clinical study with elderly women who started supervised exercise training revealed that increase fitness was associated with elevated PAHSA levels in serum and subcutaneous adipose tissue [57]. Glucose disposal rate, which also mirrors the muscle fitness and insulin sensitivity, correlated positively with markers of DNL and a TAG estolide containing a PAHSA [57]. Although no muscle biopsies were taken, PAHSA could contribute to the beneficial effect of exercise. Another study using mice reported that exercise training alters some FAHFA species in subcutaneous WAT, but no individual regioisomers were analyzed [104].

7.8. Polyunsaturated FAHFAs

In addition to PAHSAs, only a limited number of studies have focused on other FAHFAs (Table 5). Our laboratory showed that 13-DHAHLA exerts anti-inflammatory and proresolving effects while reducing macrophage activation by lipopolysaccharides, reducing pro-inflammatory cytokines and enhancing the phagocytosis of zymosan particles in human peripheral blood mononuclear cells and murine macrophages [35]. Both 13(S)- and 13(R)-DHAHLA inhibited antigen- and PGE₂-induced chemotaxis and the degranulation of mast cells [52]. 13-LAHLA suppresses the lipopolysaccharide-stimulated secretion of cytokines and the expression of pro-inflammatory genes in RAW 264.7 macrophages [19]. The results of these studies suggest that polyunsaturated FAHFAs have more potent anti-inflammatory properties than PAHSAs and no documented metabolic effects. Therefore, their biology may be more related to resolvins and protectins. Eicosapentaenoic acid (EPA)-containing FAHFAs, namely 12-EPAHSA, increased the expression of Nrf2-dependent antioxidant enzymes in hepatocellular carcinoma cells [21].

7.9. Other bioactive FAHFAs

Benlebna et al. explored the impact of 9-PAHHPA or 9-OAHPA on the metabolism of mice [53]. After 12 weeks of dietary intervention, 9-PAHHPA or 9-OAHPA increased basal metabolism, increased insulin sensitivity but did not modify glucose tolerance. Importantly, there were signs of liver steatosis and fibrosis in some mice [53]. When C57BL/6 J mice were gavaged with 9-OAHSA or 9-PAHSA, increased plasma activities of both ALT and AST were observed in the 9-OAHSA group, while 9-PAHSA-gavaged mice tended to have lower glycemia [103]. This result suggests that high doses of insulin-sensitizing

compounds could stimulate unwanted DNL in the liver when administered to healthy mice fed a chow diet and that this negative effect is absent in case of chronic PAHSA administration [75,93,103].

Recently, several new short chain FAHFAs derived from acetic acid or propanoic acid and long chain hydroxy fatty acids were identified in the large intestine and cecum of lean and obese mice, but the involvement of gut microbiota in their synthesis remains unknown [105].

Mass fragmentation patterns and retention times of FAHFAs have been integrated in many lipidomic software programs [1]. Therefore, FAHFAs have become reported more often within lipidomic datasets and their presence or levels associated with a biological effect (e.g., unspecified FAHFA regioisomers were upregulated in synovial tissue during the treatment of knee osteoarthritis in rats [106], unspecified PAHLA was selected as a biomarker [101], and unspecified FAHFA(2:0/16:2) associated with mitochondrial stress [107]). It is very important to note that FAHFA regioisomers may have different effects and that reporting FAHFAs as a lipid class has very limited biological value.

8. Future perspectives

In this review, we have summarized the literature on FAHFAs with respect to their synthesis, metabolism and endocrine effects. It has been six years since the discovery of FAHFAs as endogenous anti-inflammatory and anti-diabetic lipids in mice and humans. During this time, the biological effects of several FAHFAs have been described, strategies for the organic synthesis of FAHFAs have been developed and analytical methods have improved, but many missing pieces of this puzzle remain to be discovered.

With respect to FAHFA biosynthesis, there are still several pathways to explore. The putative missing link (when compared to plants) is the phospholipid FAHFA intermediate. The origins of different hydroxyl groups on saturated FAHFA backbones are unknown, and multiple (enzymatic) pathways will most likely be involved. The general patterns of regioisomers (Figs. 2 and S1) indicate that some carbon positions are hydroxylated more often regardless of the length of the acyl group and that a position-specific hydroxylation may exist. Unidentified, untested, alternative or moonlighting activities of (un)known enzymes remain to be explored. Furthermore, the TAG estolide reservoir plays an important role in FAHFA regulation, and their synthesis and remodeling may occur within different cellular compartments (e.g., the endoplasmic reticulum and lipid droplets) using a different subset of enzymes and their isoforms (e.g., DGAT1, DGAT2, ATGL, etc.), which may affect the position of an FAHFA on glycerol.

New analytical methods with higher sensitivity are needed to measure FAHFAs in limited amounts in samples such as human biopsies. It is crucial to separate FAHFA regioisomers and not to report pooled FAHFA class values, because individual regioisomers have different biological effects [37,96]. Furthermore, some regioisomers may simply be structural lipids or precursors without any particular biological effect.

Although no negative side effects of the (chronic) administration of PAHSAs have been documented, experiments with 9-PAHHPA, 9-OAHPA, and 9-OAHSA showed a trend towards negative effects on the liver in healthy chow-fed mice [53,103]. PAHSAs are very promising compounds, but they are not a cure-all. It has been shown that under very unfavorable metabolic conditions (e.g., db/db mice on a high-fat diet), 5-PAHSA is not strong enough to revert the negative conditions in the liver [12]. This result suggests that while the stimulation of DNL in WAT [37] is beneficial for insulin sensitivity on a whole-body level, elevated DNL in the liver can lead to steatosis and fibrosis. Thus, it will be essential to determine the structure-activity relationship of PAHSA insulin-sensitizing effects to promote the development of useful drugs. In parallel, the anti-inflammatory properties of polyunsaturated FAHFAs may contribute to the beneficial effects of omega-3 PUFAs.

There is a lack of insulin-sensitizing drugs for patients with insulin resistance and type 2 diabetes. If metformin monotherapy fails,

additional agents such as sulfonylureas, GLP-1 receptor agonists (liraglutide, semaglutide, dulaglutide) or sodium-glucose transport protein 2 inhibitors (empagliflozin or canagliflozin) are reasonable choices, but no insulin-sensitizing drugs are available besides thiazolidinediones, which have adverse effects [108]. To date, PAHSAs have been tested for biological effects either alone or as a combination of 5- and 9-PAHSAs at a physiological ratio and concentration in mice. Given the differences in effects, even among the regioisomers of PAHSAs, such an approach may be used to titrate the optimal mixture. Importantly, PAHSA derivatives with a longer biological half-life or specifically targeted compounds following structure-activity relationship between PAHSAs and their receptors are needed. The first study on truncated 9-PAHSA demonstrated that a new molecule with an enhanced ability to attenuate IL-6 production in macrophages can be designed [17]. In the future, new FAHFA analogs or formulations have to be developed for the efficient translation of mouse results to humans.

Author contributions

Conceptualization: O.K.; Data curation: V.P., L.B. and M.B.; Formal analysis: O.K.; Funding acquisition: T.D. and O.K.; Investigation: V.P. and M.B.; Methodology: V.P., L.B. and M.B.; Project administration: T.D. and O.K.; Resources: O.K.; Software: O.K.; Supervision: O.K.; Validation: O.K.; Visualization: L.B. and O.K.; Writing - original draft: O.K.; Writing - review & editing: K.B., V.P., L.B., M.B., T.D. and O.K.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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