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An update on products and mechanisms of lipid peroxidation

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The free radical reaction of polyunsaturated fatty acids with molecular oxygen leads to hydroperoxides as the first stable products. From linoleic acid the two conjugated diene hydroperoxides at carbons 9 and 13 were considered the only primary products until the recent discovery of the *bis*-allylic 11-hydroperoxide. The 11-carbon is the site of the initial hydrogen abstraction, and the 11-hydroperoxide is formed without isomerization of the 9,10 and 12,13 *cis* double bonds. In the autoxidation reaction, *bis*-allylic hydroperoxides are obtained only in the presence of an efficient antioxidant, for example, α-tocopherol. The antioxidant functions as a hydrogen atom donor, necessary to trap the fleeting *bis*-allylic peroxyl radical intermediate as the hydroperoxide. Understanding of the mechanism of formation of *bis*-allylic hydroperoxides has led to increased appreciation of the central role of the intermediate peroxyl radical in determining the outcome of lipid peroxidation.

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1 Introduction

The mechanism of the autoxidation of polyunsaturated fatty acids as a radical chain reaction was established more than half a century ago (*e.g.* refs. [1–4]). Soon after followed the elucidation of the role of antioxidants as agents that break the radical chain [5], and the identification of secondary transformation products of the primary hydroperoxides through enzymatic and non-enzymatic transformations, the latter an active area of research that continues to unravel novel enzymes and products (*e.g.* refs [6–10]).

Within the process of lipid peroxidation, three partially overlapping phases of radical reactions can be distinguished: initiation, propagation, and termination (Fig. 1). In the initiation phase, reactions prevail that form and expand the pool of radicals. During the propagation phase, the chain reaction between fatty acid radicals and molecular oxygen leads to the formation and accumulation of the primary hydroperoxide products. Reactions between radicals leading to non-radical products dominate during the termination phase.

In this review, an emphasis will be put on the discovery and mechanism of formation of the long sought-after *bis*-allylic 11-hydroperoxide of linoleic acid autoxidation. For consistency of numbering of the carbon atoms, the chemical reactions will be explained using linoleic acid as the model (9Z,12Z-octadecadienoic acid, C18.2) in the text and figures. Similar reactions occur with higher unsaturated fatty acids like linolenic acid (C18.3) and arachidonic acid (C20.4), although it is important to keep in mind that the additional double bond(s) enable types of reactions that are not possible in linoleic acid.

The formation of 9-, 11-, and 13-hydroperoxides is expected based on the three mesomeric structures for the pentadienyl radical of linoleic acid that implicate localization (and therefore reactivity with O_2) of the radical at carbons 9, 11, and 13 (Fig. 2). In contrast to the two well-known 9- and 13-hydroperoxides that are easily identified in autoxidation reactions, the 11-hydroperoxide as the third presumptive primary product had defied isolation and proven elusive for decades [11]. It turned out that the instability of the intermediate *bis*-allylic peroxyl radical is the crucial factor why this hydroperoxide has proven so difficult to identify and isolate. Therefore, in order to be able to better explain the mechanistic basis for formation of the *bis*-allylic hydroperoxide, the concept of radical reactions as

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Abbreviations: HPODE, hydroperoxyoctadecadienoic acid; **LOX**, lipoxygenase

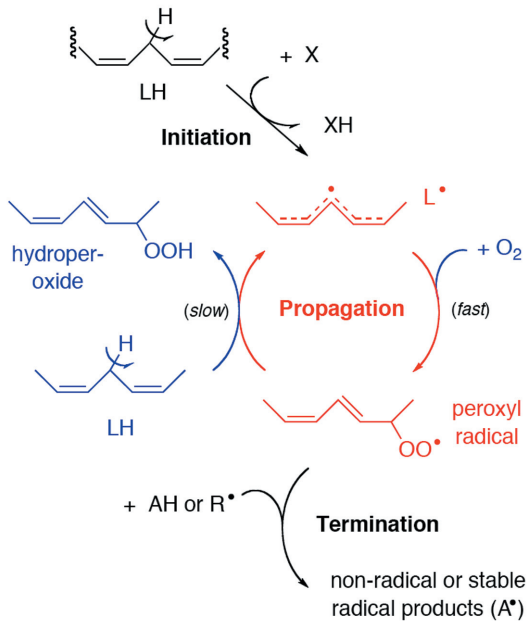

Figure 1. Three phases of the autoxidation process. Following the initial hydrogen abstraction (initiation), the radical cycles between the fatty acyl (pentadienyl) radical L• and the peroxyl radical (red). As the net result of the propagation phase, oxygen and the fatty acid are fed into the cycle to give the hydroperoxide as the product (blue). Note that the peroxyl radical (red) goes on to give the hydroperoxide (blue) upon hydrogen atom transfer from a new molecule of fatty acid (LH, blue) which in turn becomes a pentadienyl radical (L•, red), ready to pick up oxygen to form the next peroxyl radical. Termination of the process occurs when either two radicals react giving non-radical products, or when an antioxidant (AH) reduces the peroxyl to a hydroperoxide while being transformed into a stable radical (A•). For sake of simplicity, only the reacting pentadiene is shown in this and the following figures.

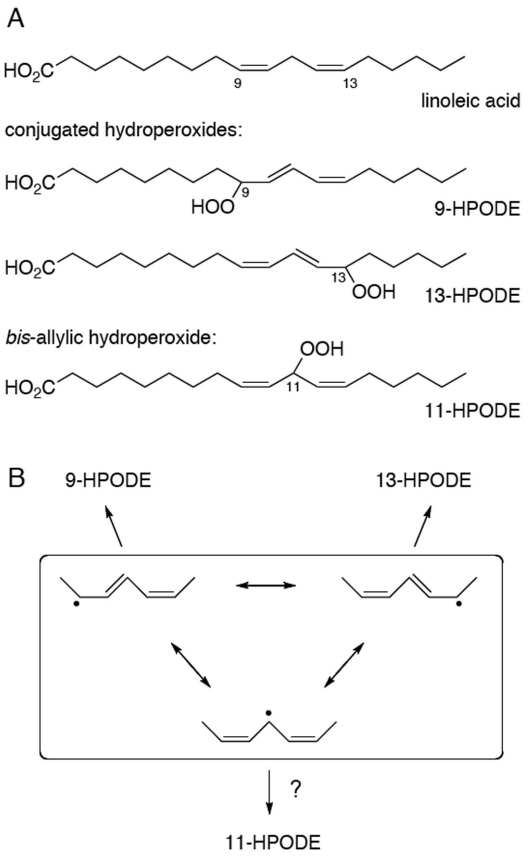

Figure 2. Conjugated and *bis*-allylic hydroperoxides. (A) The 9-HPODE and 13-HPODE are the well-known conjugated hydroperoxides formed in the autoxidation of linoleic acid. Formation of 11-HPODE was presumed based on the mesomeric structures of the intermediate pentadienyl radical (B) but remained an elusive product until its first isolation in 2000 [25].

reversible and competing reactions will be introduced in the following Section. This will set the stage for understanding of how the rate constants of different and competing reactions are a determining factor for the product outcome during lipid autoxidation.

2 Role of the peroxyl radical in the autoxidation of fatty acids

The initial hydrogen abstraction from the pentadiene is essentially irreversible and commits the fatty acid to a reaction with oxygen one-way or other. In the case of linoleic acid, hydrogen abstraction occurs at the methylene group (C11) in between the 9,10*cis* and 12,13*cis* double bonds and gives a fatty acid radical (pentadienyl radical) that is delocalized over carbons 9 through 13 (Fig. 3, step 1) [12]. Addition of oxygen to the carbon-centered radical is "diffusion-controlled", meaning that it is exceedingly fast, and the rate of reaction is only limited by how fast oxygen can get to the fatty acid radical; but there is essentially no energy barrier for formation of the peroxyl radical (step 2) [13]. [This is true for addition of oxygen to all three carbons 9, 11, and 13, but in this Section, we will only consider formation of the conjugated hydroperoxides 9-hydroperoxyoctadecadienoic acid (HPODE) and 13-HPODE.] The reaction with oxygen is reversible, so that the peroxyl radical can lose O_2 and revert to the carbon radical. This reaction of the peroxyl radical is called β-fragmentation because the bond that breaks is in β-position respective to the location of the radical [14]. Still, depending on the reaction conditions, peroxyl radicals have a considerable half-life, which ranges from milliseconds to seconds, opening up the possibility to follow different reaction pathways [15]. Competing with β-fragmentation is the trapping of the peroxyl radical as the hydroperoxide product by transfer of a hydrogen atom, *i.e.* the peroxyl radical abstracts a hydrogen atom from an available *bis*-allylic methylene group (step 3). This is the rate limiting (*i.e.* slowest) step of the radical reactions during autoxidation [12]. Nevertheless, it is the dominating reaction for the peroxyl radical during the early stages of

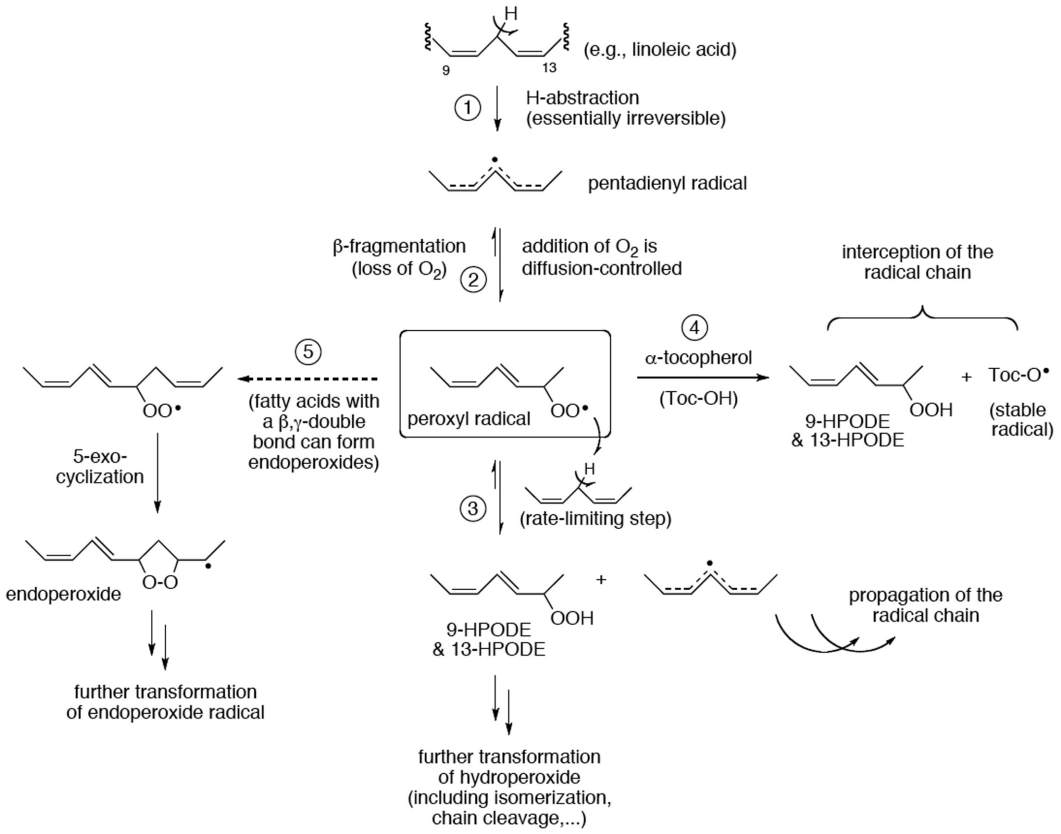

Figure 3. A central role for the peroxyl radical in fatty acid autoxidation. The peroxyl radical is a rather stable entity with a considerable half-life (milliseconds to seconds), and accordingly, it has several possibilities for further reaction; see the main text for further explanations. Of interest for the formation of *bis*-allylic hydroperoxides is the competition between β-fragmentation of the peroxyl and its reduction to the hydroperoxide, see Fig. 4.

autoxidation, and it is the step that propagates the chain by passing the radical on to the next molecule of fatty acid.

The role of an antioxidant during lipid peroxidation is to reduce the peroxyl radical to the hydroperoxide before it can propagate the radical chain (Fig. 3, step 4) [5, 16, 17]. Good antioxidants like α-tocopherol (vitamin E) are efficient hydrogen donors and are transformed into rather stable radicals that do not initiate or propagate a radical chain reaction (although there are exceptions where the tocopheroxyl radical can become a pro-oxidant [18 – 20]). Note that antioxidants do not react directly with molecular oxygen or with fatty acid radicals because reaction of the carbon radical with molecular oxygen is several orders of magnitude faster than hydrogen transfer from the antioxidant is.

Another possibility for reaction of peroxyl radicals ensues if the autoxidizing fatty acid contains three or more double bonds (Fig. 3, step 5). In these fatty acids, the peroxyl radical can react with the additional double bond and form an internal or cyclic peroxide (endoperoxide) [6, 21]. Endoperoxide formation occurs non-enzymatically in the formation of isoprostanes, a class of autoxidation products derived from arachidonic acid that is being used as a reliable marker for lipid peroxidation and oxidant stress in biological systems [22]. A similar reaction is catalyzed by the enzyme cyclooxygenase during prostaglandin synthesis [23].

It is important to understand that the peroxyl radical can perform additional reactions that compete with formation of the primary hydroperoxide product. Whichever reaction proceeds with the highest rate constant, will be the preferred reaction (albeit not exclusive) and will dominate product formation. The rate constants for the particular reactions depend on the reaction conditions (*e.g.* temperature, solvent or lipid film), available reagents (antioxidant, double bonds, concentration of radicals), and, as will be highlighted further below, the structure of the peroxyl radical itself [24].

3 Discovery of the *bis*-allylic 11-hydroperoxide

The first isolation of the *bis*-allylic 11-hydroperoxide (11-HPODE) from an autoxidation of linoleic acid methyl ester was reported in 2000 [25]. The autoxidation reaction fol-

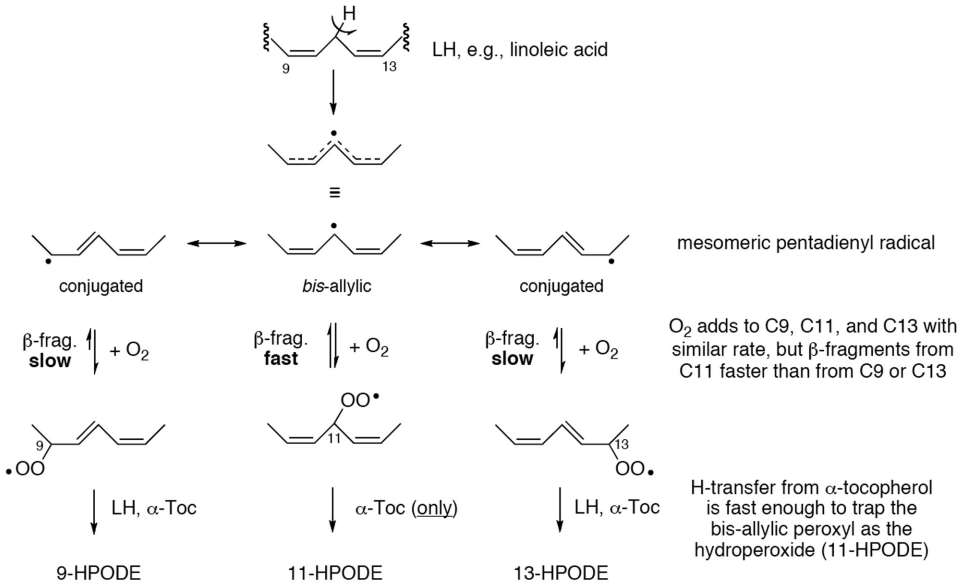

Figure 4. Mechanistic basis for the formation of conjugated and *bis*-allylic hydroperoxides. Following hydrogen abstraction from the *bis*-allylic methylene oxygen adds to all three reactive positions (9, 11, and 13) of the mesomeric pentadienyl radical to form the corresponding peroxyl radicals. However, loss of oxygen (β-fragmentation) from the *bis*-allylic peroxyl (C11) is much faster than from the conjugated peroxyls (C9 and C13). Therefore, the *bis*-allylic hydroperoxide (11-HPODE) can only be formed if a very good H-donor (like α-tocopherol) is included in the autoxidation reaction, whereas 9-HPDOE and 13-HPODE can be formed upon hydrogen abstraction from a fatty acid *bis*-allylic methylene group.

lowed a standard protocol including 5% α-tocopherol in order to suppress secondary transformation of the primary hydroperoxide products [26]. Under these conditions, 11-HPODE accounted for about 5 – 10% of the hydroperoxide products. The 11-HPODE was isolated using a combination of RP- and SP-HPLC and characterized by UV, GC-MS, and ^{1}H-NMR spectroscopy [25]. The *bis*-allylic hydroperoxide does not share the typical diene chromophore of the conjugated 9- and 13-hydroperoxides (λmax around 235 nm) but instead it has prominent end absorbance around 205 nm. Treatment of 11-HPODE with mild acid induces quantitative transformation to the conjugated 9- and 13-HPODE, enabling quantification of the ensuing diene chromophore using UV spectroscopy.

The antioxidant was shown to be a critical reagent in the autoxidation reaction, as the *bis*-allylic hydroperoxide was only formed in the presence of α-tocopherol and was undetectable in its absence [25]. Subsequently, it was shown that with increasing concentrations of the antioxidant 11-HPODE becomes equally abundant as 9-HPODE or 13-HPODE, respectively [27]. Besides the strict requirement of a strong antioxidant for its formation, the facile isomerization into the conjugated hydroperoxides together with a less prominent UV absorbance and co-elution with other products on HPLC are features of 11-HPODE that may have contributed to its elusive character [25]. In addition to autoxidative transformation of polyunsaturated fatty acids in the presence of α-tocopherol, *bis*-allylic hydroperoxides can also be formed by total chemical synthesis or photosensitized (singlet) oxidation of polyunsaturated fatty acids [28 – 30].

4 How is the *bis*-allylic 11-hydroperoxide formed?

Why is the *bis*-allylic 11-hydroperoxide formed only when the antioxidant α-tocopherol is present in the autoxidation reactions? The answer to this question relates directly to the stability of the intermediate *bis*-allylic peroxyl radical (Fig. 4). As explained above, one of the possibilities for reaction of the peroxyl radical is β-fragmentation, the loss of O_2 and reversion back to the pentadienyl radical. It turned out that the rate of β-fragmentation of *bis*-allylic peroxyl radicals is more than three orders of magnitude faster than for conjugated peroxyls [24, 27]. Therefore, the corresponding *bis*-allylic hydroperoxide can only be formed if trapping of the peroxyl radical as the hydroperoxide is faster than its β-fragmentation. In reactions without α-tocopherol, trapping of peroxyl radicals occurs through hydrogen abstraction from a methylene, but this reaction is rather slow (see Fig. 1). Still, it is fast enough to compete with β-fragmentation of the slower fragmenting conjugated peroxyl radicals, whereas β-fragmentation of the *bis*-allylic peroxyl by far outcompetes the hydrogen abstraction from the fatty acid methylene. However, if a good hydrogen donor like α-tocopherol is present in the reaction, the antioxidant can transfer its hydrogen atom fast enough to the

bis-allylic peroxyl radical so that the corresponding hydroperoxide product is formed. In fact, the more antioxidant is present in the autoxidation reaction, the more prominent the *bis*-allylic hydroperoxide becomes as a product [27].

The rate constants for the reactions involved are as follows: β-Fragmentation of the bis-allylic peroxyl radical occurs at $1.9 \times 10^6\ s^{-1}$, whereas the same fragmentation of the conjugated peroxyls occurs at $30\ s^{-1}$ or $400\ s^{-1}$, respectively, depending on whether a *cis*,*cis*- or a *cis*,*trans*-pentadienyl radical results [27]. Hydrogen abstraction from the fatty acid methylene by the peroxyl radical (*i.e.* the propagation of the autoxidation chain) occurs at about $100\ M^{-1}\ s^{-1}$ whereas the antioxidant reaction with α-tocopherol as the hydrogen donor has a rate constant of $3.8 \times 10^6\ M^{-1}\ s^{-1}$ [19].

5 The concept of kinetic and thermodynamic products

Another mechanistically significant reaction inherent to the reactivity of peroxyl radicals is the rearrangement of the fatty acid carbon chain. As would be expected, the carbon chain arrangement of the starting fatty acid is preserved in the first hydroperoxide products that are isolated form linoleic acid autoxidation, *i.e.* the conjugated HPODE have the double bonds in the 10*trans*,12*cis* (9-HPODE) or 9*cis*,11-*trans* (13-HPODE) configuration; the *bis*-allylic 11-HPODE is 9*cis*,12*cis* (cf. Fig. 2A). Since the *cis*,*trans* hydroperoxides are the immediate products, they are considered to be formed under kinetic control, *i.e.* before isomerization of the carbon chain can occur. However, with prolonged autoxidation, and especially in the absence of α-tocopherol, the double-bond configuration of the conjugated hydroperoxides rearranges from *cis*,*trans* to *trans*,-*trans* [31, 32]. The apparent driving force for this transformation is the greater thermodynamic stability of the *trans*,-*trans*-conjugated double bond in comparison to the *cis*,*trans* double bond. The *trans*,*trans* hydroperoxides are formed at the expense of the *cis*,*trans* hydroperoxides, and therefore, these products are considered to be formed under thermodynamic control.

β-Fragmentation is an essential step in the isomerization of the double-bond geometry of the primary *cis*,*trans*-conjugated diene hydroperoxide to a *trans*,*trans* diene [31, 32]. Isomerization of the double bond is initiated by reversion of the hydroperoxide back to the peroxyl radical followed by β-fragmentation of the peroxyl to the pentadienyl radical; this can occur with the conformation of the carbon chain unchanged (*cisoid*) or rearranged (*transoid*) (Fig. 5). The rate of fragmentation into a pentadienyl radical with *transoid* conformation is about 16 times faster than fragmentation into a *cisoid* pentadienyl radical (430 versus $27\ s^{-1}$ [12]). Thus, following β-fragmentation, the original *cis*,*cis* pentadiene is preferentially transformed into a *cis*,*trans* pentadienyl radical. If oxygen adds at the other end of this pentadienyl radical the resulting peroxyl radical (and the corresponding hydroperoxide) will have the *trans*,*trans*-configuration (Fig. 5). Double-bond isomerization via β-fragmentation of the peroxyl radical can be suppressed by the presence of an antioxidant (*e.g.* α-tocopherol) in the reaction because it prevents the hydroperoxide from reverting to the peroxyl radical [24].

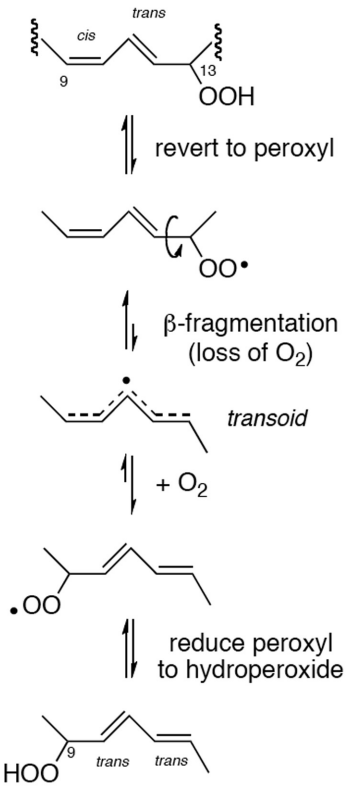

Figure 5. Proposed mechanism for the isomerization of conjugated hydroperoxides. Loss of a hydrogen atom reverts the hydroperoxide back to the peroxyl radical, illustrated here for 13-HPODE. β-Fragmentation of the peroxyl radical gives the delocalized pentadiene radical; the rate of β-fragmentation into the *cis*,*trans* configurated pentadienyl radical is about 16-times faster than fragmentation into the *cis*,*cis*-pentadienyl radical [12]. Therefore, isomerization of the 12,13 bond into the *trans* configuration is preferred during β-fragmentation. Subsequent addition of molecular oxygen at the other end of the pentadiene gives the 9-hydroperoxide and locks the 10,11 as well as the 12,13 double bonds as *trans*. Loss of oxygen from the intermediate peroxyl radical (β-fragmentation) and re-oxygenation are essential steps in the isomerization of *cis*,-*trans*-hydroperoxides to *trans*,*trans*-hydroperoxides.

6 Bioformation of *bis*-allylic hydroperoxides

It is a remarkable coincidence that around the time when 11-HPODE was discovered as a product of the autoxidation of

linoleic acid, it was also first described as an enzymatic product. The wheat crop fungus *Gaeumannomyces graminis* harbors an unusual lipoxygenase (LOX) that contains manganese as its active site metal [33] whereas a non-heme iron as the catalytic center is the rule in all other known LOX enzymes [34]. The fungal LOX also has unprecedented catalytic activity: it forms a mixture of 13*R*-HPODE and 11*S*-HPODE when reacting with linoleic acid as substrate. Biosynthesis of both products is initiated by abstraction of the pro-*S* hydrogen at C-11 of linoleic acid [35]. When 11*S*-HPODE was used as a substrate, it was converted to 13*R*-HPODE through β-fragmentation to a pentadienyl radical followed by oxygen rebound; oxygen was subject to partial exchange prior to its reincorporation [35, 36].

The question of whether other, iron-containing LOX enzymes can form *bis*-allylic hydroperoxides has been investigated only for soybean LOX-1 and the pathogen-inducible LOX from rice leaves [37]. Upon careful examination of the products, it was found that the rice LOX formed about 0.4% of 11-HPODE in addition to the main product, 13*S*-HPODE, whereas the soybean enzyme did not form any *bis*-allylic hydroperoxide. More promising candidates for formation of *bis*-allylic hydroperoxides could be the so-called type-II LOX enzymes from soybean seeds [34]. These enzymes have been shown to form varying mixtures of 9-HPODE and 13-HPODE with partial lack of stereocontrol [38]. The possibility that these nonspecific products could have arisen via isomerization of a *bis*-allylic hydroperoxide has not yet been investigated.

It is likely that the earlier discovered *bis*-allylic hydroxide 11*R*-hydroxy-9*Z*,12*Z*-octadecadienoic acid (11*R*-HODE) in the red alga *Lithothamnion corallioides* is biosynthesized by a LOX enzyme, too, followed by reduction of the presumed hydroperoxide intermediate to the hydroxide [39]. Whether the *bis*-allylic 11-hydroperoxide is subject to further enzymatic transformation besides reduction has not yet been described.

It has not been established whether *bis*-allylic hydroperoxides are formed under the conditions of biological autoxidation, *e.g.* in the LDL particle in human blood. A particle of LDL contains about 6 – 12 molecules of α-tocopherol on average [18], invoking the possibility for formation of *bis*-allylic oxygenation products *in vivo*. The inherent instability of the *bis*-allylic hydroperoxides and their corresponding hydroxy derivatives will make their identification in biological samples a challenging task for further investigation.

7 Summary

The *bis*-allylic 11-hydroperoxide is the third primary product of the autoxidation of linoleic acid. In contrast to the well-known conjugated diene hydroperoxides, *bis*-allylic hydroperoxides are only formed under autoxidation conditions where an efficient hydrogen atom donor is present. The antioxidant, for example α-tocopherol, is necessary to trap the extremely short-lived *bis*-allylic peroxyl radical as the hydroperoxide before it reverts to the carbon radical and molecular oxygen.

The discovery of the *bis*-allylic hydroperoxide and its mechanism of formation have highlighted the critical role occupied by the peroxyl radical during autoxidation. It is the central branching point from where reaction pathways diverge. Which of these pathways prevails is dependent on the reactions conditions, on additional reagents present, and on the structure of the fatty acid carbon chain that carries the peroxyl radical.

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