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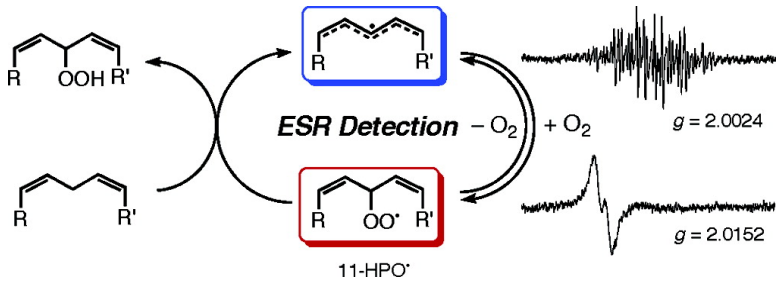
J. Am. Chem. Soc., **2005**, 127 (18), 6605-6609• DOI: 10.1021/ja044345j • Publication Date (Web): 12 April 2005



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Direct ESR Detection of Pentadienyl Radicals and Peroxyl Radicals in Lipid Peroxidation: Mechanistic Insight into Regioselective Oxygenation in Lipoxygenases

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Abstract: Well-resolved ESR spectra of free pentadienyl radicals have been observed under photoirradiation of di-*tert*-butylperoxide (Bu^tOOBu^t) and polyunsaturated fatty acids in the absence of O_2, allowing us to determine the *hfc* values. The *hfc* values of linoleyl radical indicate that the spin density is the largest at the C-11 position. The linoleyl radical is readily trapped by O_2 to produce the peroxyl radical (11-HPO$^\bullet$) in which O_2 is added mainly at the C-11 position of the pentadienyl radical as indicated by the comparison of the ESR spectra of peroxyl radicals derived from linoleic acid and [11,11-$^2H_2$]linoleic acid. The peroxyl radical (13-HPO$^\bullet$), which is initially formed by the hydrogen abstraction from 13-(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPOD) by $Bu^tO^\bullet$, is found to isomerize to 11-HPO$^\bullet$ via removal of O_2 from 13-HPO$^\bullet$ and addition of O_2 to linoleyl radical to produce 11-HPO$^\bullet$. This finding supports an idea of O_2 entering via a specific protein channel, which determines the stereo- and regiochemistry of the biradical combination between O_2 and linoleyl radical in lipoxygenases.

Introduction

Lipid peroxidation plays a pivotal role in the oxidative stress associated with several diseases.[1−5] Lipid hydroperoxides are the primary products of lipid peroxidation. Lipid peroxidation occurs through nonenzymatic (autoxidative or photooxidative) or enzymatic processes.[6] In the latter case, lipid hydroperoxides are generated by the action of lipoxygenases (LO).[7,8] The most widely accepted mechanism of LO involves hydrogen abstraction from the unsaturated fatty acid substrate with concomitant reduction of the ferric-hydoxide reaction center and formation of the pentadienyl radical.[9,10] The trapping of the radical by O_2 to produce the peroxyl radical, followed by oxidation of the ferrous−water complex by the peroxyl radical, completes the catalytic cycle. Alternatively, an organoiron intermediate has been proposed to be involved, and the product is formed by σ-bond insertion of O_2 to the organoiron intermediate. In this hypothesis, the role of the Fe^{3+} ion is to assist the deprotonation of the diene moiety by coordinating the resulting carbanion. Insertion of O_2 into the iron−carbon bond followed by protolytic iron−oxygen bond cleavage would yield the product. This mechanism can elegantly explain the regio- and stereoselectivity of LO without resorting to a postulate of steric hindrance of the approach of O_2 to one face or end of the radical intermediate.[11,12] However, the possibility of such an organo-iron intermediate formation has been ruled out by the theoretical studies.[13] While the C−H bond activation of the *cis,cis*-1,4-pentadiene subunit has been well established in LO activity, the resulting substrate pentadienyl radical or peroxyl radical has yet to be well characterized because of very short lifetimes of the highly reactive radical species. Although carbon-centered radicals and peroxyl radicals have been detected by ESR in the LO system as well as in the nonenzymatic system, the poorly resolved ESR spectra have precluded to obtain the definitive structural information.[14−16] On the other hand, a convenient and versatile technique for detecting the ESR spectra of a variety of short-lived free radicals in solution has been established by

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(16) The ESR spin trapping technique has been widely used to detect unstable free radicals. However, the ESR spectra of the spin-trapped radicals have provided no direct structural information. See: (a) Janzen, E. G. *Acc. Chem. Res*. **1971**, *4*, 31. (b) Qian, S. Y.; Tomer, K. B.; Yue, G.-H.; Guo, Q.; Kadiiska, M. B.; Mason, R. P. *Free Radical Biol. Med*. **2002**, *33*, 998.

Kochi and Krusic.[17] Radicals ($R^{\bullet}$) are generated by ultraviolet irradiation of a static solution of di-*tert*-butyl peroxide (Bu^tOOBu^t) in the presence of a hydrogen donor (RH), and the generated radicals under photoirradiation can be detected by ESR at low temperature.[17,18] Although this method is particularly useful for the study of alkyl and other organic free radicals in nonaqueous systems,[17−19] where the conventional flow techniques are difficult to apply, it has never been applied for detection of pentadienyl radicals and subsequent peroxyl radicals in lipid peroxidation.

We report herein the first well-resolved ESR spectra of free pentadienyl radicals and subsequent peroxyl radicals detected in lipid peroxidation under photoirradiation of Bu^tOOBu^t and polyunsaturated fatty acids in the absence and the presence of O_2, respectively. The ESR spectra provide valuable mechanistic insight into the position of oxygen addition in relation to the LO mechanism.

Experimental Section

Materials. Di-*tert*-butyl peroxide (Bu^tOOBu^t) was purchased from Nacalai Tesque Co., Ltd., and was purified by chromatography through alumina, which removes traces of the hydroperoxide. Linoleic acid, linolenic acid, and oleic acid were otained from Aldrich Co., Ltd. Methyl linoleate was purchased from Wako Pure Chemical Industries, Ltd. 13-(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPOD) was synthesized by addition of 100 mg of linoleic acid in ethanol to 100 mL of oxygen-saturated buffer (0.10 M Tris-HCl, pH 8.5) containing 20 mg of purified soybean lipoxygenase-1 which was obtained from Nacalai Tesque Co., Ltd. [11,11-$^2H_2$]Linoleic acid was synthesized according to the literature,[20] but an alternate procedure was used to convert 2-octyn-1-ol to the corresponding bromide.[21] 9-Decynoic acid used in this procedure was synthesized according to the literature.[22] [11-^{2}H]-13-HPOD was synthesized by the peroxidation of [11,11-$^2H_2$]-linoleic acid with soybean lipoxygenase-1.

ESR Measurements. The ESR spectra were performed on a JEOL X-band ESR spectrometer (JES-ME-LX) at 253−273 or 143 K. A quartz ESR tube (internal diameter: 1.5 mm) containing a sample solution at 253−273 K was irradiated in the cavity of the ESR spectrometer with the focused light of a 1000-W high-pressure Hg lamp (Ushio-USH1005D) through an aqueous filter. A quartz ESR tube (internal diameter: 5.5 mm) containing a deaerated sample frozen solution at 143 K was irradiated under the same experimental conditions. The internal diameter of the ESR tube is 5.5 mm, which is small enough to fill the ESR cavity but large enough to obtain good signal-to-noise ratios during the ESR measurements under photoirradiation at low temperatures (at 143 K). The photoirradiation of a deaerated neat solution of Bu^tOOBu^t containing linoleic acid, [11,11-$^2H_2$]linoleic acid, methyl linoleate, linolenic acid, and oleic acid (1.3 M) with a 1000 W high-pressure Hg lamp results in the formation of the pentadienyl radical and allyl radical which could be detected at 273 K. The photoirradiation of an oxygen-saturated neat solution of Bu^tOOBu^t containing linoleic acid, [11,11-$^2H_2$]linoleic acid (1.3 M), at 253 K resulted in the formation of peroxyl radical. The photoirradiation of a deaerated neat solution of Bu^tOOBu^t containing 13-HPOD (1.3 M) and [11-^{2}H]-13-HPOD (0.3 M) at 253 K resulted in the same formation of peroxyl radical. The photoirradiation of a deaerated neat frozen solution of Bu^tOOBu^t containing 13-HPOD (1.3 M) at 143 K resulted in the magnetic anisotropy. The ESR spectra were measured under nonsaturating microwave power conditions. The amplitude of modulation was chosen to optimize the resolution and the signal-to-noise (S/N) ratio of the observed spectra: typically 0.6 G for linoneyl and oleyl radicals and 1.0 G for peroxyl radicals. The *g* values were calibrated with a Mn^{2+} marker, and the hyperfine coupling (*hfc*) constants were determined by computer simulation using Calleo ESR Version 1.2 program coded by Calleo Scientific on an Apple Macintosh personal computer.

Theoretical Calculations. Density-functional theory (DFT) calculations were performed on a COMPAQ DS20E computer. Geometry optimizations were carried out using the Becke3LYP and 6-311G** basis set for the pentadienyl radical, and Becke3LYP functional and 3-21G* basis set for the peroxyl radicals[23,24] with the unrestricted Hartree−Fock (UHF) formalism as implemented in the Gaussian 98 program.[25] Intrinsic reaction coordinate (IRC) calculations were carried out using the Becke3LYP and 6-31G* basis set with UHF formalism, where geometry optimization was carried out for the peroxyl radical in which the alkyl chains at the position of C1−C7 and C16−C18 were removed. The graphical output of the computational results was generated with the Cerius[2] software program developed by Molecular Simulations Inc.

Results and Discussion

ESR Spectra of Carbon-Centered Lipid Radicals. The photoirradiation of a deaerated neat solution of Bu^tOOBu^t containing linoleic acid (1.3 M) at 273 K with a 1000 W high-pressure Hg lamp results in the formation of linoleyl radical, which is clearly detected by ESR as shown in Figure 1a. The pentadienyl radical is formed via hydrogen abstraction from linoleic acid by *tert*-butoxyl radical ($Bu^tO^{\bullet}$) generated by the homolytic cleavage of the O−O bond of Bu^tOOBu^t.[17−19] The *g* value (2.0024) of the ESR signal in Figure 1a, which is close to the free spin value (2.0023), is typical for a carbon-centered radical.[26] The amount of linoleyl radical observed under the steady-state photoirradiation was determined as 2.5×10^{-5} M using stable galvinoxyl radical as a reference (see Experimental Section). The well-resolved ESR spectrum in Figure 1a allows us to determine the hyperfine coupling constants (*hfc*) due to one proton at the C-11 position (a(H11) = 11.3 G), two equivalent protons at the C-9 and C-13 positions (a(H9) = a(H13) = 9.90 G), two equivalent protons at the C-10 and C-12 positions (a(H10) = a(H12) = 3.30 G), and four equivalent methylene protons at the C-8 and C-14 positions (a(H8) = a(H14) = 8.25 G). The computer simulation spectrum using these *hfc* values (Figure 1b) agrees well with the observed ESR spectrum as indicated by the dotted lines. The deuterium substitution of two hydrogen atoms at the C-11 position of linoleic acid ([11,11-$^2H_2$]linoleic acid) confirmed the *hfc* assignment in Figure 1b, because the observed ESR spectrum agrees with the computer simulation spectrum using the same *hfc* values except for the value of the deuterium (I = 1) at the C-11 position, which is reduced by the magnetogyric ratio of proton to deuterium (0.153); see Supporting Information S1.[26] The S/N ratio of the ESR signal is significantly worse than that

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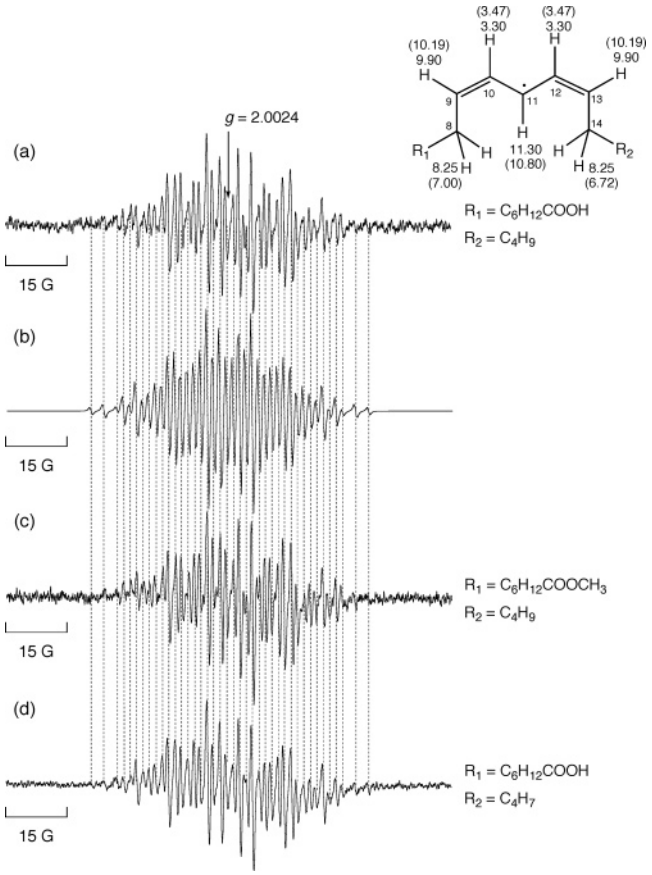


Figure 1. (a) ESR spectrum of linoleyl radical observed under photoirradiation of a deaerated Bu^{t}OOBu^t (neat) solution containing linoleic acid (1.3 M) with a high-pressure Hg lamp at 273 K. (b) Computer simulation spectrum of pentadienyl radical derived from linoleic acid with the *hfc* values. Values in parentheses were obtained from the DFT calculation (B3LYP/6-311G** basis set). (c) ESR spectrum of linoleyl radical derived from linoleic acid ester under the same experimental conditions. (d) ESR spectrum of linolenyl radical derived from linolenic acid under the same experimental conditions.

in Figure 1a. The amount of the radical derived from [11,11-$^2H_2$]linoleic acid was determined as 3.8×10^{-6} M, which is only 15% of the radical derived from nondeuterated linoleic acid (vide supra). The smaller radical amount derived from [11,11-$^2H_2$]linoleic acid may result from the deuterium kinetic isotope effect on the hydrogen abstraction from linoleic acid by Bu^{t}O$^\bullet$.

The same ESR spectra are obtained from linoleic acid ester and linolenic acid, the latter of which has one more double bond, as shown in Figure 1c and d, respectively. The complete agreement of the ESR spectra with that from linoleic acid in Figure 1a and the computer simulation spectrum in Figure 1b is clearly demonstrated by the dotted lines that connect each line in Figure 1a−d. The *hfc* values indicate that the spin density is the largest at the C-11 position of the pentadienyl radical. The *hfc* assignment is also supported by the DFT calculation (see the calculated *hfc* values in parentheses in Figure 1).

Thus, the hydrogen abstraction of lipids containing the *cis,cis*-1,4-pentadiene subunit by Bu^{t}O$^\bullet$, which is produced by the photocleavage of the O−O bond of Bu^{t}OOBu^t, produces the pentadienyl radical in which the spin density is the largest at the C-11 position as shown in Scheme 1.

An allyl type radical has also been successfully detected by ESR under photoirradiation of a deaerated neat solution of

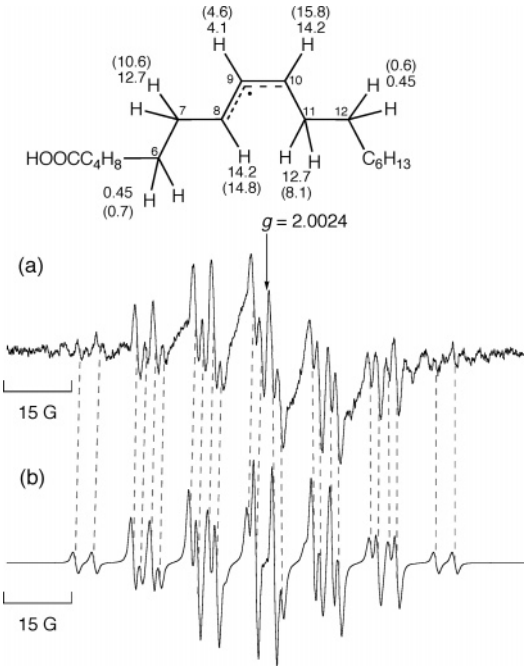
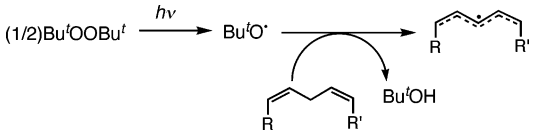
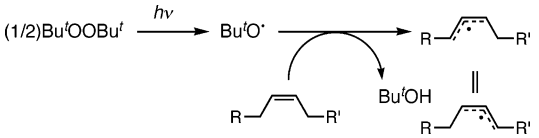


Figure 2. (a) ESR spectrum of oleyl radical observed under photoirradiation of a deaerated Bu^{t}OOBu^t (neat) solution containing oleic acid (1.3 M) with a high-pressure Hg lamp at 273 K. (b) Computer simulation spectrum of oleyl radical derived from oleic acid with the *hfc* values. Values in parentheses were obtained from the DFT calculation (B3LYP/6-311G** basis set).

Scheme 1



Scheme 2



Bu^{t}OOBu^t containing oleic acid (1.3 M) at 273 K with a 1000 W high-pressure Hg lamp as shown in Figure 2a. The computer simulation spectrum (Figure 2b) affords the *hfc* values due to one proton at the C-9 position (a(H9) = 4.1 G), two equivalent protons at the C-8 and C-10 positions (a(H8) = a(H10) = 14.2 G), four equivalent protons at the C-7 and C-11 positions (a(H7) = a(H11) = 12.7 G), and four equivalent protons at the C-6 and C-12 positions (a(H6) = a(H12) = 0.45 G). The *hfc* assignment is supported by the DFT calculation (see the calculated *hfc* values in parentheses in Figure 2). These *hfc* values are typical for an allyl radical in which the unpaired electron is mainly located at both edges of the allyl group as shown in Scheme 2.

ESR Spectra of Lipid Peroxyl Radicals. When the photoirradiation was carried out using an oxygen-saturated solution containing linoleic acid and Bu^{t}OOBu^t, no ESR signal due to the pentadienyl radical was observed, but instead a broad doublet ESR signal due to the peroxyl radical was observed at g = 2.0152 as shown in Figure 3a. The g value and the hyperfine coupling constant (a(H) = 4.06 G) are diagnostic of secondary alkylperoxyl radicals.[27,28] The observed amount of the peroxyl

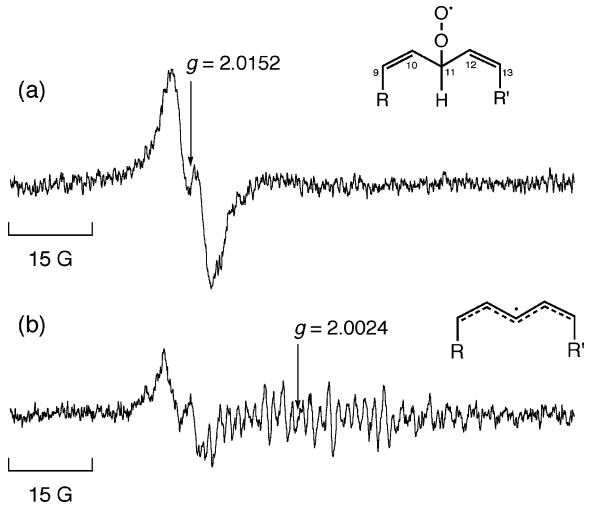


Figure 3. (a) ESR spectrum of peroxyl radical observed under photoirradiation of an O_2-saturated Bu^{t}OOBu^t (neat) solution containing linoleic acid (1.3 M) with a high-pressure Hg lamp at 253 K. (b) ESR spectrum of linoleyl radical and peroxyl radical observed under photoirradiation of an air-saturated Bu^{t}OOBu^t (neat) solution containing linoleic acid (1.3 M) with a high-pressure Hg lamp at 253 K.

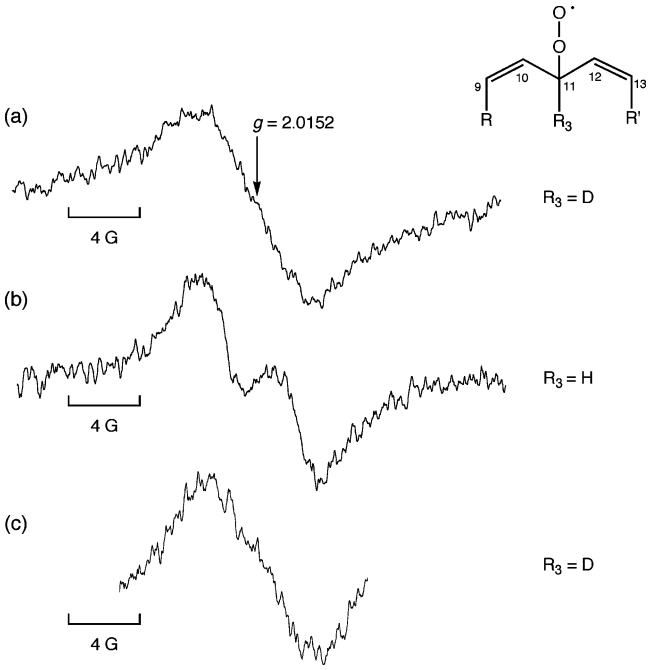


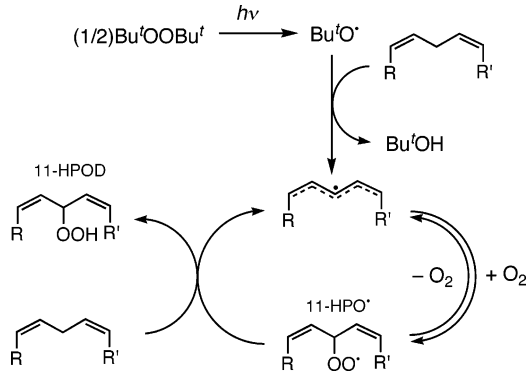
Figure 4. ESR spectra of peroxyl radicals observed under photoirradiation of (a) an O_2-saturated Bu^{t}OOBu^t (neat) solution containing [11,11-$^2H_2$]-linoleic acid (1.3 M), (b) an argon-saturated Bu^{t}OOBu^t solution containing 13-HPOD (1.3 M), and (c) an argon-saturated Bu^{t}OOBu^t solution containing [11-^{2}H]-13-HPOD (0.30 M) with a high-pressure Hg lamp at 253 K.

radical was determined as 4.8×10^{-5} M using galvinoxyl as a reference.[29] The observed a(H) value agrees with the value of 11-HPO$^\bullet$ (4.7 G) estimated by DFT calculation using B3LYP/3-21G* basis set.

When the photoirradiation was carried out using an air-saturated solution under otherwise the same experimental conditions, the ESR signal due to the pentadienyl radical (g = 2.0024) derived from linoleic acid is observed together with the peroxyl radical (g = 2.0152) as shown in Figure 3b. This indicates that the oxygen addition to pentadienyl radical derived from linoleic acid to produce the peroxyl radical is a reversible process.

When the substrate is replaced by [11,11-$^2H_2$]linoleic acid, the hyperfine splitting disappears to afford a single line ESR signal (Figure 4a). Such disappearance of the hyperfine splitting results from the decrease in the hyperfine coupling constant by the deuterium substitution at the C-11 position. The hyperfine pattern would be changed from a doublet signal of the peroxyl radical derived from linoleic acid to a triplet signal of that derived from [11,11-$^2H_2$]linoleic acid due to one deuteron splitting (I = 1) at the C-11 position, and the a(D) value should decrease by the magnetogyric ratio of proton to deuterium (0.153).[26] In such a case, the hyperfine structure would be hidden within the line width of the ESR signal as observed in Figure 4a. Thus, O_2 may be preferably added to the C-11 position of the pentadienyl radical to produce 11-HPO$^\bullet$, which can abstract a hydrogen from linoleic acid to form 11-HPOD, accompanied by regeneration of the pentadienyl radical as shown in Scheme 3. The preferable addition of O_2 at the C-11 position rather than at the C-9 and C-13 positions may result from the largest spin density of the pentadienyl radical at the C-11 position (Figure 1). Because the addition of oxygen to linoleyl radical is reversible (vide supra), the other isomer peroxyl radicals (9-HPO$^\bullet$ and 13-HPO$^\bullet$) may also contribute to the observed ESR signal to some extent in Figure 4a.

Scheme 3



An ESR spectrum that is the same as that observed in Figure 3a is obtained when 13-(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPOD) is used instead of linoleic acid for the photoirradiation as shown in Figure 4b. At a low temperature (143 K), the solution is frozen and the ESR spectrum exhibits the anisotropic signals with $g_{\|} = 2.034$ and $g_{\perp} = 2.005$ as shown in Figure 5. The average value $[(g_{\|} + 2g_{\perp})/3 = 2.015]$ agrees with the isotropic g value in solution (Figure 3a).

Hydrogen abstraction of 13-HPOD by the peroxyl radical (13-HPO$^\bullet$) in which O_2 is attached at the C-13 position must be initially produced by the hydrogen abstraction from the hydroperoxide hydrogen of 13-HPOD by Bu^{t}O$^\bullet$. If the observed ESR spectrum comes from 13-HPO$^\bullet$, the deuterium substitution of two hydrogen atoms at the C-11 position of 13-HPOD would not affect the ESR spectrum, because the hyperfine splitting results from the hydrogen at the C-13 position. However, the ESR spectrum of the peroxyl radical observed under photoirradiation of a Bu^{t}OOBu^t solution containing [11-^{2}H]-13-HPOD

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(29) The larger amount of the peroxyl radical than the liloleic acid radical may be ascribed to the slow bimolecular decay of the peroxyl radicals as compared to that of the carbon centered radical.

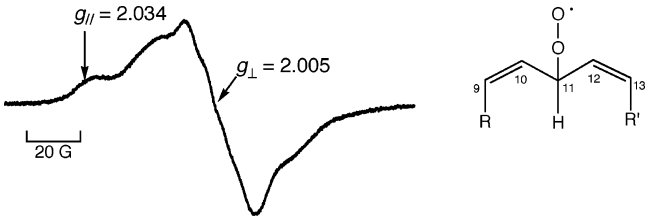
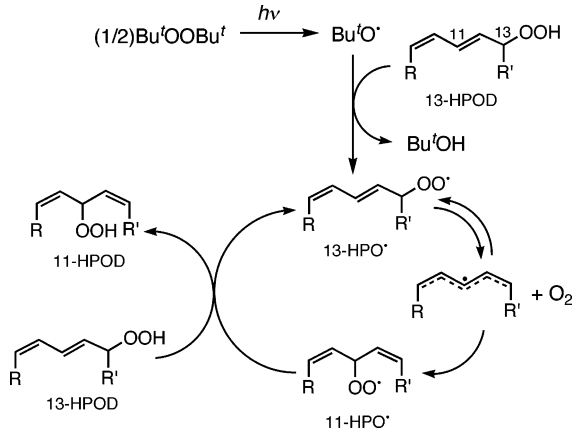


Figure 5. ESR spectrum of the peroxyl radical observed under photoirradiation of an argon-saturated Bu^tOOBu^t (neat) solution containing 13-HPOD (1.3 M) with a high-pressure Hg lamp measured at 143 K.

Scheme 4



at 253 K exhibits no hyperfine structure as shown in Figure 4c. This indicates that the peroxyl radical (13-HPO•), which is initially formed by the hydrogen abstraction from the hydroperoxide hydrogen of 13-HPOD by Bu^tO•, isomerizes at least partially to 11-HPO•, in which no hyperfine splitting is observed (see Figure 4a), via removal of O_2 from 13-HPO• and addition of O_2 to linoleyl radical to produce 11-HPO•, as shown in Scheme 4.

The difference in the thermodynamic stability between 13-HPO• and 11-HPO• is estimated by the DFT calculation (UB3LYP/6-31G* basis set, see Experimental Section). The calculations suggest that 13-HPO• is by 6.2 kcal mol^{-1} more stable than 11-HPO•. In such a case, the isomerization of 13-HPO• to 11-HPO• may occur by the kinetic control rather than the thermodynamic control. The reversible addition and removal of O_2 from the peroxyl radical mentioned above is supported by low activation barriers (6 kcal mol^{-1} for 11-HPO• and 13 kcal mol^{-1} for 13-HPO),[30] which were calculated for the C−O bond cleavage in 11-HPO• and 13-HPO• using the DFT method (B3LYP/6-31G* basis set; see Experimental Section and Supporting Information S2). Thus, the C−O bond cleavage in 13-HPO• results in the formation of linoleyl radical to which O_2 may be added mainly at the C-11 position rather than at the C-9 and C-13 positions, because the spin density of linoleyl radical is the largest at the C-11 position (Scheme 4).

Mechanistic Insight into Lipoxygenase. As described above, we have successfully detected pentadienyl radicals and subsequent peroxyl radicals in lipid peroxidation by ESR, demonstrating the preferable addition of O_2 at the C-11 position of free linoleyl radical derived from linoleic acid, where the spin density is the largest. This finding provides valuable insight into the mechanism of the regioselective oxygenation of linoleic acid by LO (vide infra). If O_2 has free access to the pentadienyl radical derived from linoleic acid, O_2 would add mainly to the C-11 position to produce 11-HPO• radical. Even if 13-HPO• were formed, it would be readily isomerized to 11-HPO• provided that the peroxyl radical can move freely. Thus, the regio- and stereoselective addition of O_2 at the C-13 position in LO may result from the enzyme protein environment, which limits O_2 access to the pentadienyl radical, which is bound to the enzyme. The X-ray crystal structure of soybean lipoxygenase-1 (SLO) reveals a side channel intersecting the substrate pocket near the reactive C-11 of linoleic acid,[31−33] which is proposed to be the O_2 access channel.[34] This channel is constricted at the Fe^{3+}−OH by the side chains of Leu^{546} and Leu^{754}. Klinman et al.[9] have reported that steady-state kinetics and product distribution data from single-point mutants of SLO, which show that Leu^{546} and Leu^{754} grant selectivity for 13-(*S*)-HPOD by blocking O_2 access to C-9 of linoleic acid and that O_2 enters the active site via the postulated side channel. The reactivity of O_2 with SLO has also been examined using a range of kinetic proves to rule out diffusional encounter of O_2 with protein, an outer-sphere electron transfer to O_2, and proton transfer as rate-limiting steps.[35] Either a radical combination of O_2 with linoleyl radical or a subsequent slow conformational change is suggested to be the rate-determining step.[35] The primary role of the Fe^{3+} cofactor is therefore to generate an enzyme-bound radical, while the protein controls the stereo- and regiochemistry of O_2 encounter with this radical.[35] Our study supports such an idea of O_2 entering via a specific protein channel, which determines the stereo- and regiochemistry of the biradical combination between O_2 and linoleyl radical.

Acknowledgment. This work was partially supported by a Grant-in-Aid (No. 16205020) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Supporting Information Available: ESR spectrum of linoleyl radical observed under photoirradiation of a deaerated Bu^tOOBu^t (neat) solution containing [11,11-$^2H_2$]linoleic acid (1.3 M) with a high-pressure Hg lamp at 273 K (S1), plots of energy versus C−O bond length of 11-HPO• and 13-HPO• obtained from the intrinsic reaction coordinate calculation using the UB3LYP/6-31G* basis set (S2), and the full list of authors for ref 25 (S3). This material is available free of charge via the Internet at http://pubs.acs.org.

JA044345J

(30) The calculations were performed for the peroxyl radical in which the alkyl chains at the positions of C1−C7 and C16−C18 were removed.
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