Formation of a Substituted 1,N^6-Etheno-2′-deoxyadenosine Adduct by Lipid Hydroperoxide-Mediated Generation of 4-Oxo-2-nonenal

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Analysis of the reaction between 2′-deoxyadenosine and 13-hydroperoxylinoleic acid by liquid chromatography/constant neutral loss mass spectrometry revealed the presence of two major products (adducts **A** and **B**). Adduct **A** was shown to be a mixture of two isomers ($\mathbf{A}_1$ and $\mathbf{A}_2$) that each decomposed with the loss of water to form adduct **B**. The mass spectral characteristics of adduct **B** were consistent with the substituted 1,N^6-etheno-2′-deoxyadensoine adduct 1″-[3-(2′-deoxy-β-D-*erythro*-pentafuranosyl)-3*H*-imidazo[2,1-*i*]purin-7-yl]heptan-2″-one. Adducts $\mathbf{A}_1$, $\mathbf{A}_2$, and **B** were formed when 2′-deoxyadenosine was treated with synthetic 4-oxo-2-nonenal, which suggested that it was formed by the breakdown of 13-hydroperoxylinoleic acid. A substantial increase in the rate of formation of adducts $\mathbf{A}_1$, $\mathbf{A}_2$, and **B** was observed when 13-hydroperoxylinoleic acid and 2′-deoxyadenosine were incubated in the presence of Fe^{II}. Thus, 4-oxo-2-nonenal was most likely formed by a homolytic process. Although adducts $\mathbf{A}_1$, $\mathbf{A}_2$, and **B** were formed in the reaction between 4-hydroxy-2-nonenal and 2′-deoxyadenosine, a number of additional products were observed. This suggested that 4-hydroxy-2-nonenal was not a precursor in the formation of 4-oxo-2-nonenal from 13-hydroperoxylinoleic acid. This study has provided additional evidence which shows that 4-oxo-2-nonenal is a major product of lipid peroxidation and that it reacts efficiently with DNA to form substituted etheno adducts.

Introduction

Free radical-mediated lipid peroxidation results in the formation of lipid hydroperoxides that readily decompose to the corresponding alkoxy radicals (*1*). The alkoxy radicals can either chain terminate by the abstraction of a hydrogen atom from a suitable donor or undergo various secondary reactions which lead to the formation of highly reactive unsaturated aldehydes (*2*, *3*). Lipid peroxidation has been implicated as a contributing factor in the etiology of a number of disease states (*4*−*7*). Several lipid peroxidation end products have been shown to covalently modify nucleic acids to form mutagenic lesions in DNA (*8*). Malondialdehyde (MDA, β-hydroxyacrolein) is a well-characterized α,β-unsaturated aldehyde, which arises from both free radical (*9*−*11*) and enzymatic (*12*, *13*) peroxidation of polyunsaturated fatty acids. MDA modifies both purine bases to generate a tricyclic adduct with guanine (*14*), which has been detected in rat (*15*) and human liver DNA (*16*). It also forms an acyclic adduct with adenine (*17*, *18*). The degradation of lipid hydroperoxides results in the production of 4-hydroxy-2-nonenal, another well-studied lipid peroxidation product (*2*, *3*). 4-Hydroxy-2-nonenal is a bifunctional electrophile, which reacts directly with 2′-deoxyguanosine (dGuo) to produce a tricyclic substituted propano adduct (*19*, *20*). It is also readily oxidized to the epoxide derivative which can covalently modify both dGuo and dAdo to form etheno adducts (*21*, *22*).

We have suggested that 4-oxo-2-nonenal is a novel product of lipid peroxides, which covalently modifies dGuo either as the free nucleoside or when present in DNA (*23*). The adducts that arise from the reaction of 4-oxo-2-nonenal with dAdo have recently been characterized (*24*). A detailed analysis of the reaction between dAdo and linoleic acid hydroperoxide {13-hydroperoxy-[*S*-(*Z*,*E*)]-9,11-octadecadienoic acid (13-HPODE[1])}, a representative ω − 6 lipid hydroperoxide has now been performed. The decomposition product of 13-HPODE responsible for covalent modifications to dAdo was unequivocally identified as 4-oxo-2-nonenal. Therefore, this study provides additional insight into the mechanism by which lipid hydroperoxides can initiate the formation of DNA adducts (*25*).

Materials and Methods

Materials. 2′-Deoxyadenosine, soybean lipoxidase (type V), and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO). 9,11-Octadecadienoic acid, 13-hydroperoxy-[*S*-(*Z*,*E*)], was obtained from Cayman Chemical Co. (Ann Arbor,

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[1] Abbreviations: CID, collision-induced dissociation; CNL, constant neutral loss; ESI, electrospray ionization; 13-HPODE, 13-hydroperoxy-[*S*-(*Z*,*E*)]-9,11-octadecadienoic acid; LC/MS, liquid chromatography/mass spectrometry; LOX, lipoxygenase; MH^+, protonated molecular ion; MS^n, multiple tandem mass spectrometry; PBS, phosphate-buffered saline; SRM, selected reaction monitoring; TIC, total ion current.

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MI). 4-Hydroxy-2-nonenal was purchased from Sigma. HPLC grade water was obtained from Fisher Scientific Co. (Fair Lawn, NJ). HPLC grade methanol was purchased from Burdick and Jackson (Muskegon, MI). Gases were supplied by BOC Gases (Lebanon, NJ).

Mass Spectrometry. The data presented were acquired on either a Finnigan TSQ 7000 or a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with a Finnigan electrospray source. The mass spectrometers were operated in the positive ion mode with a potential of 4.25 kV applied to the electrospray needle. Nitrogen was used as the sheath and auxiliary gas to assist with nebulization. The capillary temperature was held at 200 °C. Full scanning analyses were performed in the range of *m*/*z* 100−900. Helium was used as the collision gas.

Liquid Chromatography. Chromatography was performed using a Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA). Gradient elutions were all performed in the linear mode. Gradient systems 1 and 2 employed a C_{18} ODS-AQ column (250 mm × 2.0 mm i.d., 3 μm; YMC, Inc., Wilmington, NC) at a flow rate of 170 μL/min. Solvent A was 5 mM aqueous ammonium acetate and 0.01% trifluoroacetic acid, and solvent B was 5 mM methanolic ammonium acetate and 0.01% trifluoroacetic acid. The gradient conditions for system 1 were as follows: 0% B at 0 min, 100% B at 15 min, and 0% B at 20 min, followed by an equilibration time of 20 min. The gradient conditions for system 2 were as follows: 30% B at 0 min, 30% B at 5 min, 100% B at 16 min, 100% B at 26 min, and 30% B at 31 min, followed by an equilibration time of 19 min. Gradient system 3 employed a YMC C_{18} ODS-AQ column (250 mm × 4.6 mm i.d., 5 μm; YMC, Inc.) at a flow rate of 1 mL/min. The gradient conditions were as follows: 30% B at 0 min, 30% B at 5 min, 100% B at 16 min, 100% B at 24 min, and 30% B at 26 min, followed by an equilibration time of 4 min.

Reaction of dAdo with 13-HPODE. dAdo (44 μg, 0.18 μmol) in H_2O (40 μL) was added together with 13-HPODE (500 μg, 1.6 μmol) in ethanol (120 μL) to PBS (210 μL). The final pH was 7.0. Reaction mixtures were incubated at 37 °C for 36 h, after which they were placed on ice prior to LC/MS analysis using gradient system 1 or 2.

Reaction of dAdo with 13-HPODE/Fe^{II}. 13-HPODE (100 μg, 0.32 μmol) in ethanol (20 μL) was added together with 50 μL of an aqueous solution of $FeSO_4 \cdot 7H_2O$ (0.89 mg/mL, 0.16 μmol of Fe^{II}) and dAdo (40 μg, 0.16 μmol) in water (45 μL) to water (135 μL). The reaction mixture (total volume of 250 μL; 640 μM Fe^{II}) was incubated at 37 °C for 30 min. Samples were placed on ice prior to LC/MS analysis using gradient system 2.

Preparation of [$^{13}C_5$]dAdo. [$^{13}C_5$]Ade was prepared as described previously (*26*) and was used to generate [$^{13}C_5$]dAdo via the procedure described by Chapeau and Marnett (*27*).

Reaction of dAdo with 13-HPODE from Lipoxygenase (LOX)/Linoleic Acid. Linoleic acid (450 μg, 1.6 μmol) or [$^{13}C_{18}$]-linoleic acid (475 μg, 1.6 μmol) in ethanol (2 μL) was added to soybean LOX (1100 units, type V) in PBS (300 μL, pH 7.4). The samples were incubated at 37 °C for 24 h. They were then cooled to room temperature, and dAdo (40 μg, 0.16 μmol) or [$^{13}C_5$]dAdo (42 μg, 0.16 μmol) in H_2O (40 μL) was added. The incubations were continued for a further 12 h at 37 °C. After being cooled on ice, the samples were filtered through 0.2 μm CoStar filter cartridges prior to LC/MS analysis using gradient system 3.

Reaction of dAdo with 4-Hydroxy-2-nonenal. dAdo (45 μg, 0.18 μmol) in H_2O (35 μL) was added together with 4-hydroxy-2-nonenal (280 μg, 1.8 μmol) in hexane (26 μL) to PBS (289 μL, pH 7.4) or 25 mM KH_2PO_4 (289 μL, pH 7.4). The slightly turbid solutions were incubated at 37 °C for 37 h and then placed on ice prior to analysis by LC/MS using gradient system 2.

Reaction of dAdo with 2,3-Epoxy-4-hydroxynonanal. A stirred aqueous solution of *tert*-butylhydroperoxide (246 μL of a 70% solution) was adjusted to pH 8 with 0.01 N NaOH. This was followed by the addition of 4-hydroxy-2-nonenal (100 mg, 0.64 mmol) in methanol (500 μL). The reaction mixture was stirred for 30 min in an ice/water bath followed by 3 h at room temperature. The reaction mixture was then extracted with chloroform (3 × 1 mL); the chloroform extracts were dried over sodium sulfate and filtered, and the chloroform was evaporated under nitrogen. The 2,3-epoxy-4-hydroxynonanal was redissolved in ethanol (800 μL). dAdo (43 mg, 0.17 mmol) in a 1:1 PBS/H_2O solution (3 mL of PBS/3 mL of H_2O) was added to the stirred solution of 2,3-epoxy-4-hydroxynonanal in ethanol (400 μL) and incubated at 37 °C for 60 h. The reaction mixture was placed in an ice bath prior to analysis by LC/MS using gradient system 2.

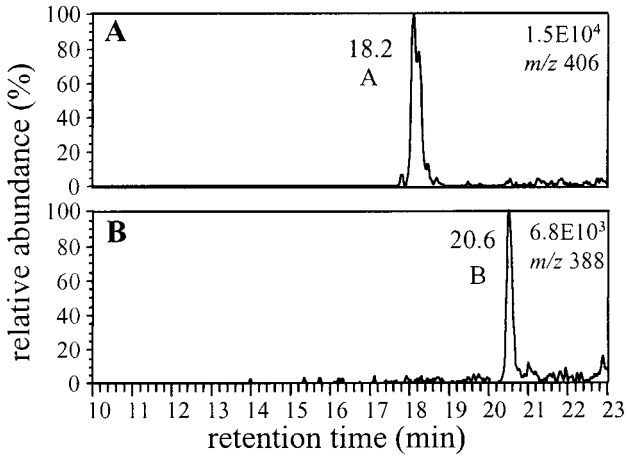


Figure 1. Analysis of the reaction between dAdo and 13-HPODE after 36 h at 37 °C by LC/MS with CNL analysis using gradient system 1. (A) Selected ion chromatogram for the MH^+ of adduct **A** (*m*/*z* 406). (B) Selected ion chromatogram for the MH^+ of adduct **B** (*m*/*z* 388).

Synthesis of 4-Oxo-2-nonenal. 4-Hydroxy-2-nonenal diethyl acetal was oxidized with activated MnO_2 as described by Esterbauer and Weger (*28*). The resulting 4-oxo-2-nonenal diethyl acetal was then hydrolyzed by citric acid/HCl as described previously (*23*).

Reaction of dAdo with 4-Oxo-2-nonenal. A solution of 4-oxo-2-nonenal (1300 μg, 8.4 μmol) in ethanol (15 μL) was added to dAdo (1200 μg, 4.8 μmol) in water (250 μL). The reaction mixture was sonicated for 15 min at room temperature and incubated at 37 or 60 °C for 24 h, after which it was placed on ice. An aliquot of the sample (20 μL) was diluted to 100 μL with a water/methanol mixture (7:3 v/v) and filtered through a 0.2 μm CoStar cartridge prior to analysis of a portion of the sample (10 μL) by LC/MS using gradient system 2.

Results

Reaction of dAdo with 13-HPODE. LC/MS analysis was conducted using gradient system 1 coupled with constant neutral loss (CNL) scanning to screen the reaction mixture for compounds undergoing the loss of 116 amu under collision-induced dissociation (CID) conditions (*29*). This fragmentation (which is characteristic of 2′-deoxynucleosides) revealed the presence of adduct **A** and adduct **B** with MH^+ ions at *m*/*z* 406 and 388, respectively (Figure 1). Adduct **B** appeared to be a dehydration product of adduct **A** because of its mass spectral characteristics and its longer retention time under reversed-phase conditions. The response in the *m*/*z* 406 channel suggested the presence of two components, and this was confirmed when the reaction mixture was analyzed using modified gradient system 2 (Figure 2). The reconstructed mass chromatogram for *m*/*z* 406 showed the presence of two isomeric compounds (adduct $\mathbf{A}_1$ and adduct $\mathbf{A}_2$) which both possessed major aglycone product ions at *m*/*z* 290. A single response was observed

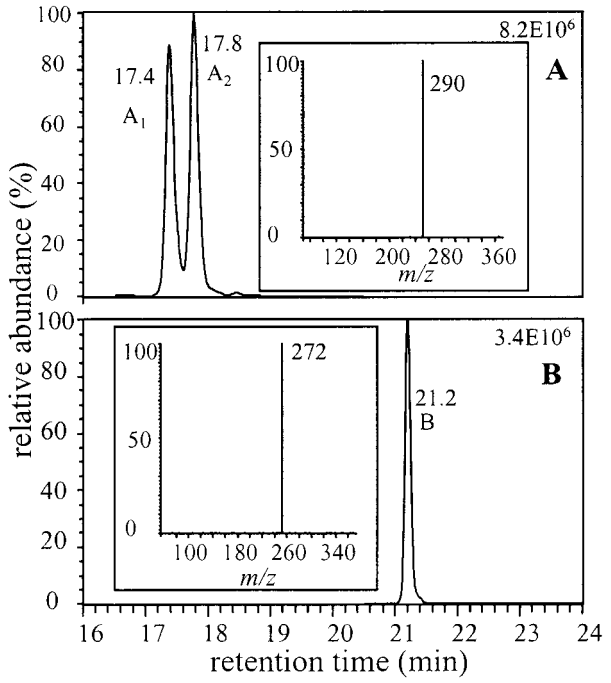


Figure 2. Analysis of the reaction between dAdo and 13-HPODE after 36 h at 37 °C by LC/MS^2 using gradient system 2. Product ion spectra are shown in the insets. The spectrum for adduct $\mathbf{A}_1$ was identical with that for adduct $\mathbf{A}_2$. (A) TIC chromatogram for adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ (*m/z* 406 →). (B) TIC chromatogram for adduct **B** (*m/z* 388 →).

for the *m/z* 388 product (adduct **B**) with the expected aglycone ion detected at *m/z* 272.

The LCQ instrument was used to further study the fragmentation behavior of the aglycone ions of adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ (*m/z* 406) and adduct **B** (*m/z* 388). Each ion, namely, *m/z* 290 (adducts $\mathbf{A}_1$ and $\mathbf{A}_2$) and 272 (adduct **B**), was isolated and CID conducted. The aglycone ions at *m/z* 290 from adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ each generated two identical product ions at *m/z* 272 and 136 (Figure 3A). This confirmed that they were isomeric dAdo adducts. The ion at *m/z* 272 arose through the loss of a water molecule, while the ion at *m/z* 136 was protonated adenine. The aglycone ion at *m/z* 272 from adduct **B** generated one major product ion at *m/z* 174 ($-C_5H_{10}CO$) (Figure 3B).

Reaction of dAdo with 13-HPODE in the Presence of Fe^{II}. Small amounts of adducts $\mathbf{A}_1$, $\mathbf{A}_2$, and **B** could be observed in the LC/MS chromatogram after reaction for only 30 min between 13-HPODE and dAdo (Figure 4A,C). However, there was a large increase in the signal intensity for $\mathbf{A}_1$, $\mathbf{A}_2$, and **B** when Fe^{II} (640 μM) was included in the incubation mixture. After incubation for 30 min, the magnitude of the *m/z* 406 signals (adducts $\mathbf{A}_1$ and $\mathbf{A}_2$) exhibited an increase of more than 2 orders of magnitude (8.4×10^6; Figure 4B) when compared with the magnitude of the same signals in the absence of Fe^{II} (1.9×10^4; Figure 4A). The signal corresponding to adduct B in the *m/z* 388 channel increased almost 20-fold (1.7×10^5; Figure 4D) when compared to the signal in the absence of Fe^{II} (9.2×10^3; Figure 4C).

Reaction of dAdo with LOX/Linoleic Acid-Derived 13-HPODE. Linoleic acid is converted primarily to 13-HPODE by soybean LOX (*30*). When 13-HPODE was generated enzymatically by soybean LOX, the reaction products observed with dAdo were identical to those formed in the reaction of synthetic 13-HPODE with dAdo

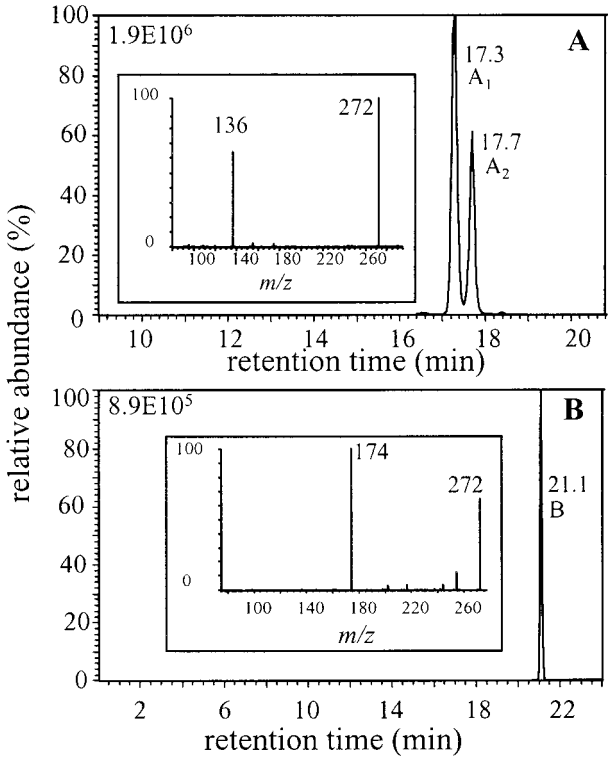


Figure 3. Analysis of the reaction between dAdo and 13-HPODE after 36 h at 37 °C by LC/MS^3 using gradient system 2. Product ion spectra are shown in the insets. The spectrum for adduct $\mathbf{A}_1$ was identical with that for adduct $\mathbf{A}_2$. (A) TIC chromatograms for adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ (*m/z* 406 → 290 →). (B) TIC chromatogram for adduct **B** (*m/z* 388 → 272 →).

(Figure 5). The reaction was repeated using [$^{13}C_{18}$]linoleic acid as substrate, and the products were compared with those obtained with the unlabeled fatty acid. Adducts $\mathbf{A}_1$, $\mathbf{A}_2$, and **B** each displayed a 9 Da increase in MH^+, which confirmed the incorporation of a nine-carbon fragment from linoleic acid into the three adducts. These experiments were also conducted using [$^{13}C_5$]dAdo, and the expected 5 Da mass shifts in MH^+ were observed.

Reaction of dAdo with 4-Hydroxy-2-nonenal. A targeted CID analysis was conducted on the reaction mixture focusing on the product ions derived from the aglycone ion (*m/z* 290) of *m/z* 406 from adducts $\mathbf{A}_1$ and $\mathbf{A}_2$. A number of compounds were formed in this reaction (Figure 6). It should be noted that there was no clear evidence for the formation of the Michael addition product, which would generate an MH^+ ion at *m/z* 408 (data not shown). The mass spectrometric behavior of the two earliest eluting compounds at 17.2 and 17.6 min was identical to that of adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ generated in the dAdo/13-HPODE incubation; i.e., major product ions were observed at *m/z* 272 (loss of water) and 136 (Ade + H). Each compound eluting between 18.0 and 20.0 min exhibited a major product ion at *m/z* 160. This product ion is known to arise by CID of the MH^+ ion (*m/z* 276) of 1,N^6-etheno-dAdo, which suggested that these compounds were substituted etheno adducts such as those described by Sodum and Chung (*21*). The later two eluting compounds (20.3 and 20.8 min) did not fragment readily under the conditions of the analysis, although the elimination of water was observed.

Reaction of dAdo with 2,3-Epoxy-4-hydroxynonanal. Two major products were observed with retention times of 20.3 and 20.8 min (data not shown). Their mass

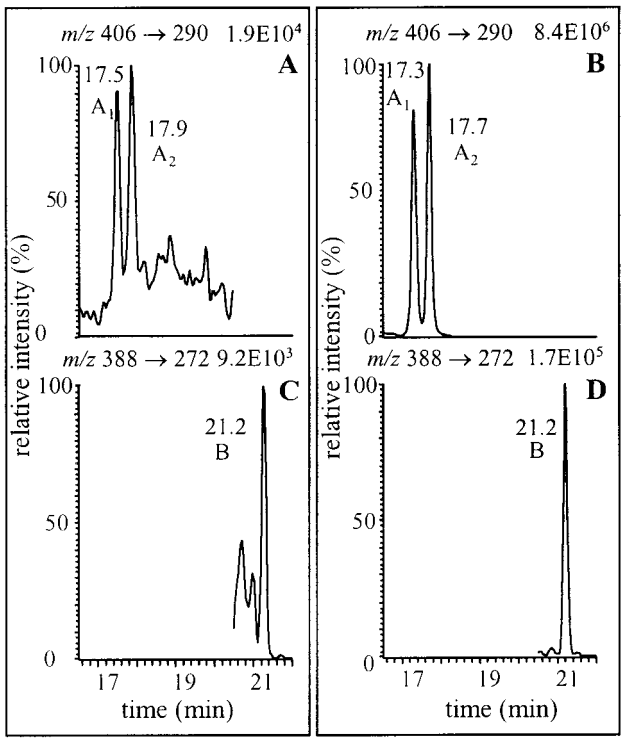


Figure 4. Analysis of the reaction between dAdo and 13-HPODE after 30 min at 37 °C by LC/MS^2 using gradient system 2. (A) SRM chromatogram for adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ (*m/z* 406 → 290) in the absence of Fe^{II}. (B) SRM chromatogram for adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ (*m/z* 406 → 290) in the presence of Fe^{II} (640 μM). (C) SRM chromatogram for adduct **B** (*m/z* 388 → 272) in the absence of Fe^{II}. (D) SRM chromatogram for adduct **B** (*m/z* 388 → 272) in the presence of Fe^{II} (640 μM).

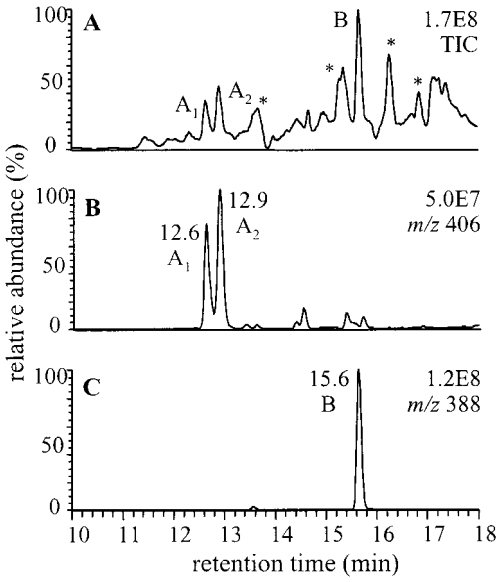


Figure 5. Analysis of the reaction between dAdo and LOX/linoleic acid-derived 13-HPODE after 12 h at 37 °C by LC/MS using gradient system 3. (A) TIC chromatogram. Asterisks denote signals observed in a control incubation with no dAdo added. (B) Selected ion chromatogram for adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ (*m/z* 406). (C) Selected ion chromatogram for adduct **B** (*m/z* 388).

spectral properties were identical with those of the later eluting compounds observed at 20.3 and 20.8 min in the chromatogram from the dAdo/4-hydroxy-2-nonenal reaction (Figure 6).

Reaction of dAdo with 4-Oxo-2-nonenal. A targeted CID analysis of the products from the reaction of

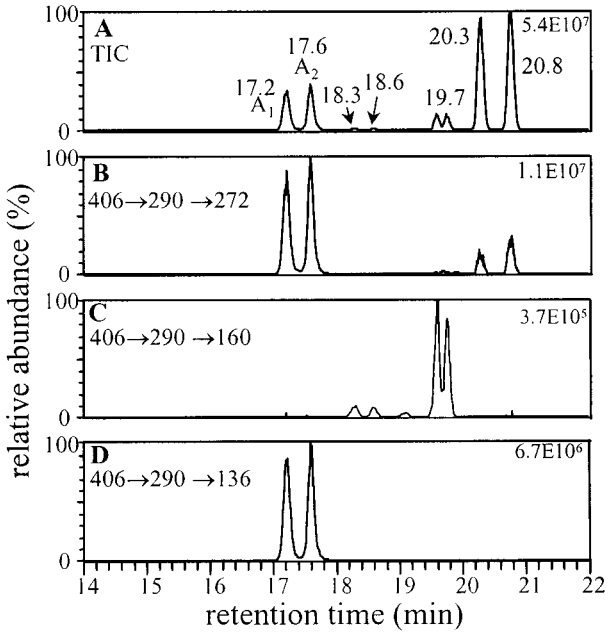


Figure 6. Analysis of the reaction between dAdo and 4-hydroxy-2-nonenal for 36 h at 37 °C by LC/MS^3 using gradient system 2. (A) TIC. (B) SRM chromatogram for adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ (*m/z* 406 → 290 → 272). (C) SRM chromatogram for *m/z* 406 → 290 → 160. (D) SRM chromatogram for *m/z* 406 → 290 → 136.

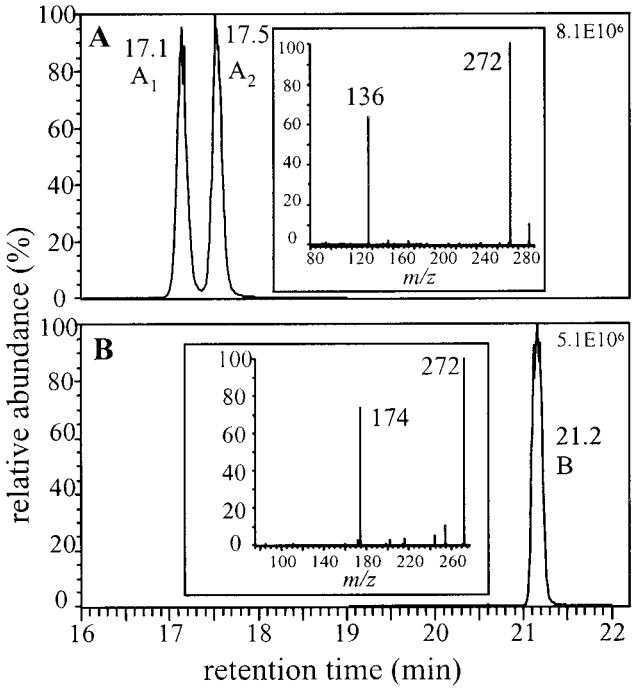


Figure 7. Analysis of the reaction between 4-oxo-2-nonenal and dAdo for 36 h at 37 °C by LC/MS^3 using gradient system 2. The product ion spectra are shown in the insets. The spectrum for adduct $\mathbf{A}_1$ was identical with that for adduct $\mathbf{A}_2$. (A) TIC chromatogram for adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ (*m/z* 406 → 290 →). (B) TIC chromatogram for adduct **B** (*m/z* 388 → 272 →).

dAdo with 4-oxo-2-nonenal revealed the presence of three major compounds (Figure 7). Their retention times and mass spectrometric behavior were identical when compared to those of adducts $\mathbf{A}_1$, $\mathbf{A}_2$, and **B** observed in the reaction of 13-HPODE and dAdo. None of the later eluting *m/z* 406 isomers (18.0−21.0 min) observed in the chromatograms from reactions of dAdo with 4-hydroxy-2-nonenal or 2,3-epoxy-4-hydroxynonanal were detected.

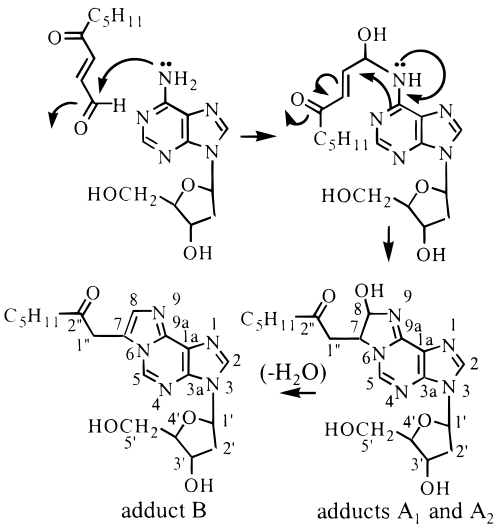
Discussion

Previous studies have investigated the covalent modifications that can occur to DNA bases from electrophilic breakdown products of peroxidized methyl linoleate (*31*, *32*). Characteristic fluorescent adducts were observed only in reactions with adenine-containing compounds. Structures for the reactive secondary lipid oxidation products responsible for adduct formation were inferred from the adduct structures. Thus, one of the adducts was suggested to contain a substituted 1,N^6-propano adduct from the reaction between 3-nonenal and adenine. The adduct structure was only tentatively assigned, and it is difficult to envisage a reasonable pathway for the generation of this adduction product.

MDA, a well-studied lipid peroxidation breakdown product, generates an acyclic N^6-oxopropenyl adduct when reacted with dAdo (*17*, *18*). This adduct is thought to result from initial 1,4-addition to the β-hydroxyacrolein form of MDA followed by dehydration. 4-Hydroxy-2-nonenal, another characteristic lipid breakdown product, does not appear to react very efficiently with dAdo. However, the epoxide derivative, 2,3-epoxy-4-hydroxynonanal, has been shown to form substituted and unsubstituted etheno adducts with dAdo (*21*, *25*, *33*). The proposed mechanism for formation of the etheno adducts involves nucleophilic attack by the exocyclic N^6 amino group of dAdo at the C1 aldehyde of 2,3-epoxy-4-hydroxynonanal followed by addition of N1 to C2 of the epoxide to generate an intermediate ethano adduct.

Three major products (adducts $\mathbf{A}_1$, $\mathbf{A}_2$, and **B**) were detected in the reaction between dAdo and 13-HPODE after 36 h (Figures 2 and 3). The reaction was then performed for 30 min using a 2-fold excess of 13-HPODE and 8% ethanol rather than a 10-fold excess of 13-HPODE and 32% ethanol. The same three products were observed, although in much smaller amounts (Figure 4A,C). A dramatic increase in the extent of adduct formation was observed when the reaction was performed for 30 min in the presence of Fe^{II} (Figure 4B,D). This indicated that the adducts were formed through a homolytic process (*34*). The MS fragmentation patterns of these adducts were not consistent with the direct involvement of either 4-hydroxy-2-nonenal or 2,3-epoxy-4-hydroxynonanal in their formation. For example, the product of direct reaction between 4-hydroxy-2-nonenal and dAdo would generate an MH^+ ion at *m*/*z* 408. This ion was not observed in any reaction mixture containing dAdo and 13-HPODE. The substituted 1,N^6-etheno-dAdo adduct derived from the reaction of 2,3-epoxy-4-hydroxynonanal and dAdo would exhibit an MH^+ ion at *m*/*z* 406 (*21*). However, LC/MS^n analysis of the MH^+ ions at *m*/*z* 406 derived from adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ shown in Figures 2 and 3 did not support this structure. LC/MS^2 analysis revealed that both adduct $\mathbf{A}_1$ and adduct $\mathbf{A}_2$ formed intense aglycone product ions at *m*/*z* 290. CID analysis of these aglycone ions showed two major product ions at *m*/*z* 272 ($-H_2O$) and 136 (Ade + H) (Figure 3A). The aglycone ion (*m*/*z* 272) of adduct B generated a product ion at *m*/*z* 174 ($-C_5H_{10}CO$) (Figure 3B). Although these data were consistent with the addition of a nine-carbon unit from 13-HPODE to dAdo, the fragmentation pathways could not be reconciled with any of the known dAdo adducts derived from C_9 products of lipid peroxidation.

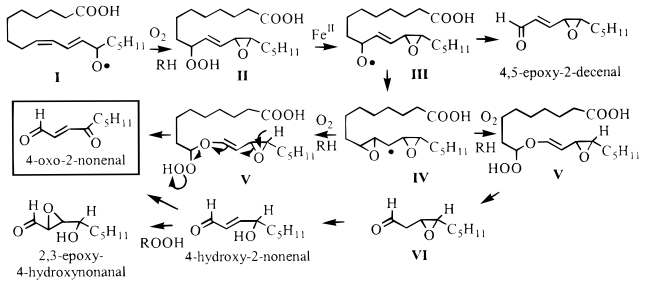
Scheme 1. Proposed Mechanism for the Reaction between 4-Oxo-2-nonenal and DAdo



We have proposed that 4-oxo-2-nonenal is a previously unrecognized product of lipid peroxidation (*23*). Furthermore, using NMR and LC/MS, it was demonstrated that 4-oxo-2-nonenal reacts with dAdo to give two isomeric substituted ethano adducts (adducts $\mathbf{A}_1$ and $\mathbf{A}_2$), which dehydrate to give the etheno adduct **B**, 1″-[3-(2′-deoxy-β-D-*erythro*-pentafuranosyl)-3*H*-imidazo[2,1-*i*]purin-7-yl]-heptan-2″-one (*24*). In the study presented here, we have compared the products of the reaction between dAdo and 13-HPODE with those derived from synthetic 4-oxo-2-nonenal. The reaction was conducted at a higher concentration of dAdo than the reaction between 13-HPODE and dAdo, and the concentration of ethanol was lower. However, the three dAdo adducts were identical, and no other adducts were detected. This suggested that the 13-HPODE had decomposed to 4-oxo-2-nonenal and that this had then reacted with the dAdo to form adducts $\mathbf{A}_1$, $\mathbf{A}_2$, and **B**. Formation of these adducts is thought to arise by a mechanism similar to that described above for 2,3-epoxy-4-hydroxynonanal (*21*). Thus, initial nucleophilic addition of N^6 to the C1 aldehyde of 4-oxo-2-nonenal occurs. This is followed by reaction of N1 at C2 of the resulting α,β-unsaturated ketone to generate adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ as a mixture of diastereomers. Subsequent dehydration of adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ results in the formation of adduct B (Scheme 1).

We examined the reaction of 4-hydroxy-2-nonenal using conditions similar to those of the reaction between 13-HPODE and dAdo except that 7% hexane was used to dissolve the lipid instead of 32% ethanol. As noted in the Results, LC/MS analysis of the reaction between 4-hydroxy-2-nonenal and dAdo revealed the absence of a signal from the direct Michael addition product (*m*/*z* 408). However, there was a series of signals arising from MH^+ ions at *m*/*z* 406. Adducts of dAdo with a molecular weight of 405 are most likely derived from a two-electron oxidation (−2H) of 4-hydroxy-2-nonenal or initial epoxidation (+O) followed by dehydration ($-H_2O$). Using targeted LC/MS^3 analysis (406 → 290 →), the early eluting peaks (17.2 and 17.6 min) were shown to be identical with adducts $\mathbf{A}_1$ and $\mathbf{A}_2$, respectively (Figure 6). The later eluting peaks (20.3 and 20.8 min) were identical with the products derived from the reaction between 2,3-epoxy-4-hydroxynonanal and dAdo (Figure

Scheme 2. Proposed Mechanism for the Formation of 4-Oxo-2-nonenal from 13-HPODE



6). Adduct B was also observed (data not shown). It is unlikely that adducts $\mathbf{A}_1$ and $\mathbf{A}_2$ observed in the reaction between 4-hydroxy-2-nonenal and dAdo are different from those observed with 4-oxo-2-nonenal because they have the same retention time, the same parent ion, and the same product ions. These data suggested that both 2,3-epoxy-4-hydroxynonanal and 4-oxo-2-nonenal were formed by oxidation of 4-hydroxy-2-nonenal under the reaction conditions. It is noteworthy that 4-oxo-2-nonenal-derived adducts were observed when 4-hydroxy-2-nonenal was reacted with *n*-butylamine in the presence of oxygen (*35*).

There are two possible explanations for the formation of 4-oxo-2-nonenal from 13-HPODE. The 13-HPODE could have decomposed to 4-hydroxy-2-nonenal, which was then oxidized by excess 13-HPODE to 4-oxo-2-nonenal. Alternatively, the 13-HPODE could have decomposed directly to 4-oxo-2-nonenal without the intermediate formation of 4-hydroxy-2-nonenal (Scheme 2). The former possibility was considered less likely for three reasons. First, Chen and Chung (*36*) have demonstrated that 4-hydroxy-2-nonenal is oxidized to 2,3-epoxy-4-hydroxynonanal rather than 4-oxo-2-nonenal by lipid hydroperoxides. Second, we were unable to detect any products of reaction between dAdo and 2,3-epoxy-4-hydroxynonanal (*21*, *22*) in the reaction between 13-HPODE and dAdo (Figures 3 and 5). Third, LC/MS analysis of products from the reaction of 13-HPODE and dAdo (Figure 3) revealed a different profile when compared to the profile of the products of the reaction between 4-hydroxy-2-nonenal and dAdo (Figure 6).

Pryor and Porter have proposed a mechanism to account for the formation of 4-hydroxy-2-nonenal from 13-HPODE (*3*). Initial Fe^{II}-mediated one-electron reduction of the hydroperoxide to generate alkoxy radical **I** (*37*) is followed by epoxide formation and concomitant addition of oxygen to give 12,13-epoxy-9-hydroperoxy-10-octadecenoic acid (**II**) (*38*, *39*). Another Fe^{II}-mediated one-electron reduction of the hydroperoxide results in the formation of alkoxy radical intermediate **III** (*39*). β-Scission of this alkoxy radical would lead to the formation of the known lipid peroxidation end product 4,5-epoxy-2-decenal (*39*, *40*). Alkoxy radical **III** was proposed to form a bis-epoxy carbon-centered radical (intermediate **IV**) that rearranges to intermediate **V** with the concomitant addition of oxygen (*3*). Loss of a C_9 aldehyde moiety then provides intermediate **VI**, which rearranges to 4-hydroxy-2-nonenal. Hydroperoxide-mediated oxidation of 4-hydroxy-2-nonenal would provide 2,3-epoxy-4-hydroxynonanal (*22*) (Scheme 2). As we were unable to detect any dAdo adducts derived from either 4-hydroxy-2-nonenal or 2,3-epoxy-4-hydroxynonanal, we propose that 4-oxo-2-nonenal is formed directly from intermediate **V** through the concerted loss of water and a C_9 aldehyde (Scheme 2). However, the possibility that 4-oxo-2-nonenal is formed from 4-hydroxy-2-nonenal as described by Xu and Sayre or by some alternative pathway cannot be ruled out (*35*).

Interestingly, 4-oxo-2-nonenal has been observed in the oxidation of 3(*Z*)-nonenal by soybean LOX when hydroperoxide concentrations are low (*41*). Therefore, it is conceivable that 4-oxo-2-nonenal-derived dAdo adducts observed in the soybean LOX reaction may have been a consequence of the hydroperoxide lyase activity that is also present. The hydroperoxide lyase activity could have converted 13-HPODE to 3(*Z*)-nonenal (*42*). LOX-mediated oxidation of the 3(*Z*)-nonenal to 4-hydroperoxy-2-nonenal (*43*) followed by Fe^{II}-mediated generation of the 4-alkoxy radical and a β-scission (similar to that postulated for intermediate **III**) would have resulted in the formation of 4-oxo-2-nonenal (*41*). However, the observation that 4-oxo-2-nonenal-derived dAdo adducts were also formed from 13-HPODE in the absence of soybean LOX suggests that this pathway is not of major importance.

In summary, we have provided further evidence that 4-oxo-2-nonenal is a novel product of lipid peroxidation and that it can form substituted etheno adducts with DNA bases. The role of etheno DNA adducts in mutagenesis and carcinogenesis is currently under intense study (*8*). The ability to quantify 4-oxo-2-nonenal-derived DNA adducts will help to further our understanding of the role that this novel bifunctional electrophile plays in lipid hydroperoxide-mediated carcinogenesis. Therefore, our current studies are focused on the development of LC/MS assay methodology for the quantitation of 4-oxo-2-nonenal-derived etheno-DNA adducts in tissues and biological fluids.

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