Identification of Novel Autoxidation Products of the ω-3 Fatty Acid Eicosapentaenoic Acid in Vitro and in Vivo*

Received for publication, April 12, 2007, and in revised form, August 20, 2007 Published, JBC Papers in Press, August 21, 2007, DOI 10.1074/jbc.M703108200

Huiyong Yin‡§, Joshua D. Brooks‡, Ling Gao‡‡, Ned A. Porter‡, and Jason D. Morrow‡†

From the Departments of ‡Medical and Pharmacology and ‡Chemistry, Vanderbilt University, Nashville, Tennessee 37232

Increased intake of fish oil rich in the ω-3 fatty acids eicosapentaenoic acid (EPA, C20:5 ω-3) and docosahexaenoic acid (DHA, C22:6 ω-3) reduces the incidence of human disorders such as atherosclerotic cardiovascular disease. However, mechanisms that contribute to the beneficial effects of fish oil consumption are poorly understood. Mounting evidence suggests that oxidation products of EPA and DHA may be responsible, at least in part, for these benefits. Previously, we have defined the free radical-induced oxidation of arachidonic acid in vitro and in vivo and have proposed a unified mechanism for its peroxidation. We hypothesize that the oxidation of EPA can be rationally defined but would be predicted to be significantly more complex than arachidonate because of the fact that EPA contains an additional carbon-carbon double bond. Herein, we present, for the first time, a unified mechanism for the peroxidation of EPA. Novel oxidation products were identified employing state-of-the-art mass spectrometric techniques including Ag⁺ coordination ionspray and atmospheric pressure chemical ionization mass spectrometry. Predicted compounds detected both in vitro and in vivo included monocyclic peroxides, serial cyclic peroxides, bicyclic endoperoxides, and dioxygen-endoperoxides. Systematic study of the peroxidation of EPA provides the basis to examine the role of specific oxidation products as mediators of the biological effects of fish oil.

Eicosapentaenoic acid (C20:5 ω-3; EPA)³ and docosahexaenoic acid (C22:6, ω-3; DHA) are two major polyunsaturated fatty acids (PUFAs) in fish oil (Scheme 1). Experiments in animals, as well as human epidemiological studies and recent clinical intervention trials, suggest that fish consumption or dietary supplementation with fish oil reduces the incidence of important human disorders including atherosclerotic cardiovascular disease, cardiac sudden death, stroke, and asthma, among others (1–3). Dietary supplementation with fish oil also shows promise for slowing the progression of neurodegenerative diseases, psychiatric disorders, and AIDS (4–9). Identifying the mechanism(s) responsible for the beneficial effects of ω-3 fatty acids is of significant importance although it is not currently understood. One hypothesis to account for the anti-atherogenic and anti-inflammatory properties of ω-3 PUFAs is via interference with the arachidonic acid cascade that generates pro-inflammatory eicosanoids. EPA, a structurally close analogue of arachidonic acid, not only can replace arachidonic acid in phospholipid bilayers but is also a substrate for the cyclooxygenase (COX) and lipoxygenase enzymes, generating 3-series prostaglandins (PGs) and thromboxane and 5-series leukotrienes (10). In addition, EPA reduces the production of 2-series PGs and thromboxane and 4-series leukotrienes from arachidonate. These latter compounds are known to possess potent pro-inflammatory activities (11, 12). On the other hand, eicosanoids derived from EPA are either less biologically active or inactive compared with those from arachidonate and are thus considered to exert effects that are less inflammatory (13). Of particular interest, Serhan et al. (14) have more recently reported that lipid mediators derived from EPA via COX-2 after aspirin treatment show potent anti-inflammatory actions. These compounds are termed Resolvins because they were first identified in resolving inflammatory exudates (15).

Several studies have also reported that ω-3 PUFAs can be oxidized non-enzymatically and that the products are both anti-inflammatory and anti-proliferative (16, 17). However, structural characterization of these biologically active peroxidation products of EPA and DHA has never been undertaken. EPA and DHA are readily oxidized under free radical conditions because they have additional carbon-carbon double bonds compared with arachidonate and other PUFAs (18–20). Thus, studies to rationally define the peroxidation of EPA and DHA may help to identify those products that contribute to the biology of ω-3 PUFA consumption.

Free radical-induced oxidation of arachidonic acid has been well studied and a unified mechanism that leads to the

*f This work was supported by National Institutes of Health Grants ES13125, ES000267, GM15431, ES31125, RR00096, DK48831, and CA77839. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Current address: Pharmaceutical Candidate Optimization, Discovery Metabolism, and Pharmacokinetik, Bristol-Myers Squibb, Route 206 and Provinceline Rd., Princeton, NJ 08540.

2 To whom correspondence should be addressed: Division of Clinical Pharmacology, Dept. of Medicine and Pharmacology, Vanderbilt University School of Medicine, 352 RRB, 23rd and Pierce Ave., Nashville, TN 37232-6602. Tel.: 615-343-1124; Fax: 615-322-3669; E-mail: Jason.morrow@Vanderbilt.edu.

3 The abbreviations used are: EPA, eicosapentaenoic acid; AIN-93, American Institute of Nutrition-93; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; COX, cyclooxygenase; PG, prostaglandin; IsoP, isoprostane; PFB, pentfluorobenzyl; DTBN, di-tert-butyl hyponitrite; BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; DEIA, disopropylethylamine; HPLC, high pressure liquid chromatography; APCL, atmospheric pressure chemical ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMHBA, N-methyl benzohydroxamic acid; IPA, isopropyl alcohol; PIP, isoprostane; TLC, thin layer chromatography; CIS, coordination ionspray; HpEPA, hydroperoxyeicosapentaenoic acid; HEPA, hydroxyeicosapentaenoic acid; TMS, trimethylsilyl; CID, collision-induced dissociation; SRM, selective reaction monitoring; SIM selective ion monitoring; PPh₃, triphenylphosphine.
formation of various oxidation products has been proposed (Scheme 2) (21–23). The oxidation proceeds via hydrogen atom abstraction at one of three bis-allylic positions at carbon 7, 10, and 13 to form a pentadienyl radical that can be trapped by molecular oxygen to form a peroxyl radical. A regiosomeric mixture of peroxyl radicals can be formed and the 11-peroxyl radical \(2b\) is taken as an example. This peroxyl radical can lead to the formation of 11-hyperoxyeicosatetraenoic acid (HpETE), \(2c\) and the corresponding alcohol 11-hydroxyeicosatetraenoic acid (11-HETE), \(2d\). On the other hand, further cyclization of the peroxyl radical \(2b\) will generate bicyclic endoperoxides \(2g\) via two consecutive steps of \(5\)-exo cyclization. Reduction of \(2g\) gives rise to one of the major classes of oxidation products, F2-isoprostanes (IsoPs) (24, 25). These compounds are analogues of PGs but are formed non-enzymatically from the autooxidation of arachidonic acid. Quantification of F2-IsoPs by gas chromatography/mass spectrometry (GC-MS) has been shown to be one of the most reliable markers to assess oxidant stress status and lipid peroxidation in vivo (26–28). In addition to the IsoPs, monomeric peroxides \(2j\), serial cyclic peroxides \(2k\), and dioxolane-isoprostane peroxides \(2l\) have also been identified. Thus, the major oxidation products from arachidonate can be predicted based on a unified free radical mechanism and this mechanism has been confirmed experimentally.

We hypothesize that the oxidation of EPA can also be rationally defined even though the oxidation product profile would be predicted to be significantly more complex than that of arachidonic acid. We report, for the first time, systematic identification of the oxidation products derived from EPA using a variety of mass spectrometric techniques. It is our contention that formation of these novel oxidation products may be responsible for some of the beneficial effects of fish oil consumption in animals and humans.

**EXPERIMENTAL PROCEDURES**

**Materials**—Eicosapentaenoic acid methyl ester was purchased from Nu Chek Prep (Elysian, MN) and was of the highest purity (>99%). \([2H_4]15-F_2t-IsoP (8-iso-PGF_2\alpha)\) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Pentfluorobenzyl (PFB) bromide and diisopropylethylamine (DIEA) were from Sigma. N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) was from Supelco (Bellefonte, PA).

**Detection and Separation of Lipid Oxidation Products**—Reactions involving hydroperoxides were visualized by thin layer chromatography (TLC) using a stain of 1.5 g of \(N,N\)-dimethyl-p-phenylenediamine dihydrochloride/25 ml \(H_2O/125\) ml of MeOH/1 ml of acetic acid. Hydroperoxides yield an immediate pink color, while cyclic peroxides exhibit a pink color after mild charring. General TLC staining was accomplished by use of a phosphomolybdic acid stain prepared as a 20% (w:v) solution in ethanol. In general, hydroperoxides were stored as dilute solutions with 1 mol% BHT in either hexanes or benzene at \(-78^\circ C\) and were never exposed to temperatures \(>40^\circ C\). Flash column chromatography was performed using silica gel 60 (EMD, NJ) and silica Sep-Pak cartridges were purchased from Waters Associates (Milford, MA). HPLC grade solvents, methanol, chloroform, hexane, isopropyl alcohol, and ethanol are purchased from Burdick and Jackson brand (VWR Scientific Inc., McGraw Park, IL). Other chemicals were from Aldrich (Milwaukee, WI) and were of the highest purity.

Detection and Separation of Lipid Oxidation Products: Reactions involving hydroperoxides were visualized by thin layer chromatography (TLC) using a stain of 1.5 g of \(N,N\)-dimethyl-p-phenylenediamine dihydrochloride/25 ml \(H_2O/125\) ml of MeOH/1 ml of acetic acid. Hydroperoxides yield an immediate pink color, while cyclic peroxides exhibit a pink color after mild charring. General TLC staining was accomplished by use of a phosphomolybdic acid stain prepared as a 20% (w:v) solution in ethanol. In general, hydroperoxides were stored as dilute solutions with 1 mol% BHT in either hexanes or benzene at \(-78^\circ C\) and were never exposed to temperatures \(>40^\circ C\). Flash column chromatography was performed using silica gel 60 (EMD, NJ) and eluted with hexaneethyl acetate (90:10). TLC was performed using 0.2-mm layer thickness silica gel-coated aluminum (60 F_254\,) in a millimetres, EM Industries, Industry, CA), and TLC plates
were analyzed using UV light (254 nm) with a Mineralight UVSL–25 hand lamp.

Analytical HPLC was performed using a Waters Model 600E pump with a Waters 996 photodiode array detector. Millennium32 chromatography software (Waters Corp., Milford, MA) was utilized to control the array detector and to collect and process data. Semi-preparative HPLC was carried out using a Dynamax-60 Å 8 µm (83–121-C) silica column (21.4 mm × 25 cm) with a flow rate of 10 ml/min. For reverse phase HPLC analysis of PFB esters, a Phenomenex Ultracarb ODS 5 µm (25 cm) column (Torrrence, CA) was used at 1 ml/min flow rate with a gradient starting with 50% solvent A (95:5, water: MeOH) and 50% solvent B (5:95, water : MeOH), holding for 2 min and increasing to 100% B in 20 min and holding for 10 min. PFB esters of F3-IsoPs were also analyzed by normal phase HPLC using 12% IPA in hexanes and a analytical Beckman UltraspHERE Ultra 5 µm (4.6 mm × 25 cm) silica column or narrowbore Beckman UltraspHERE 5 µm (2.0 mm × 25 cm) Si column (the latter column coupled to mass spectrometry). A flow rate of 1 ml/min was used for analytical normal phase HPLC whereas 0.2 ml/min was employed for narrowbore columns.

Mass Spectrometry—On-line reverse phase liquid chromatography (LC) was carried out using the ThermoFinnigan Surveyor MS Pump 2.0 equipped with the columns mentioned above. Samples were analyzed using a ThermoFinnigan TSQ Quantum Ultra instrument. The atmospheric pressure chemical ionization (APCI) source was fitted with a deactivated fused silica capillary (100 µm i.d.). The mass spectrometer was operated in negative ion mode with a capillary temperature of 300 °C, vaporizer temperature of 300 °C. The spray voltage, ion transfer tube voltage, and skimmer voltage were optimized to achieve maximal response. Nitrogen was used as both the sheath gas and the auxiliary gas. For tandem mass spectrometry (MS/MS) experiments, collision energies were optimized under 1.5 mTorr of argon. Data acquisition and analysis were performed using Xcaliber software, version 2.0 Service Release-2 (SR-2). Samples were introduced either by direct liquid infusion (DLI) or by HPLC. For normal phase HPLC, samples were introduced by a Hewlett-Packard 1090 HPLC system.

Oxidation of EPA Methyl Ester to Primary Hydroperoxides and More Complex Oxidation Products—EPA methyl ester (500 mg, 1.59 mmol) was dissolved in 1.5 ml of acetonitrile to make a solution of 1.06 M. To the mixture were added 44 mg of N-methyl benzohydoxamic acid (NMBHA), 2.92 mmol and 0.05 equivalent in benzene of free radical initiator di-tert-butyl hyponitrite (DTBN) (29). The reaction was stirred under oxygen at 37 °C for 24 h. The reaction was quenched by adding 2 mg of BHT and NMBHA and DTBN were removed by flash chromatography on silica gel using hexane:ethyl acetate (90:10). The hydroperoxides were analyzed and separated by analytical and semi-preparative HPLC. The separated fractions of primary hydroperoxides were converted to more highly oxidized peroxides according to our previous procedure (30).

Ag⁺ Coordination Ionspray (CIS)-MS Analysis of Oxidized EPA Methyl Esters—The Ag⁺ CIS-MS experiments were carried out following the methods reported previously (21, 30–33). A narrowbore C18 column was used with a flow rate of 0.2 ml/min employing 0.15 mM AgBF₄ in a mixture of isopropyl alcohol (50%) and water (50%).

Analysis of Oxidized EPA by LC/APCI-MS—Oxidized lipids derived from ~100 µg of lipid hydroperoxides were reduced by adding an excess amount of triphenylphosphine (PPh₃). The reduced lipid was subjected to basic hydrolysis using 1.0 M KOH for 60 min at 37 °C. After adjusting to pH 3, the mixture was loaded onto a C18 Sep-Pak cartridge that was preconditioned with 5 ml of methanol and then 5 ml of water. The column was washed with 5 ml of water and 5 ml of heptane. The eluent of 10 ml of ethyl acetate was collected. The solution was dried over Na₂SO₄. After evaporation of the solvent, the residue was dissolved in 20 µl of CH₂CN. To the resulting solution was added 20 µl of 10% (v/v) PFB bromide in acetonitrile and 10 µl of 10% (v/v) N,N-disopropylethylamine (DIEA) in acetonitrile and the mixture was kept at room temperature for 30 min. The reagent was dried under nitrogen, and the residue was subjected to TLC separation (60ALK6D plates, Whatman, Maidstone UK). The PFB esters, after TLC separation, were either directly analyzed by LC/APCI-MS or were further derivatized to TMS derivatives. For the latter, after evaporation of the ethyl acetate, 20 µl of BSTFA and 10 µl of dimethylformamide were added to the residue, and the mixture was incubated at 37 °C for 20 min.

Identification of Oxidation Products of EPA in Mice Tissue—Mice (strain C57/BL/6(JB6)) were fed with a rodent American Institute of Nutrition (AIN)-93 M (maintenance) (Dyets Inc., Bethlehem, PA) diet supplemented with either 0% (control diet) or 0.56% EPA (EPA diet) (by weight). The formulation of AIN-93 diets is an improvement to replace the previous version of AIN-76 (34). The EPA in this modified AIN-93 diet is stable employing the feeding conditions used in the studies as noted under "Results." In particular, experiments were carried out to ensure that complex oxidation products of EPA were present at minimal levels in the diet when freshly obtained, at the end of the study, and after being stored at room temperature in the same conditions used in the feeding studies. After 8 weeks of feeding, mice were administered CCl₄ (2 ml/kg in corn oil). After 2 h, the animals were anesthetized with pentobarbital (60 mg/kg) intraperitoneally, sacrificed, and the livers were removed. Approximately 1.0 g of tissue was immediately homogenized and extracted in 20 ml of Folch solution (MeOH: CHCl₃, CHCl₃: MeOH: CHCl₃: CHCl₃: H₂O, 2:1:1:1:1) and 4 ml of 0.9% NaCl solution was added to obtain a crude phospholipid extract (35). The aqueous layer was not acidified prior to the extraction. It should be noted that we have previously shown that this extraction approach does not lead to oxidation of arachidonate or EPA ex vivo (19, 25). Basic hydrolysis was carried out in 0.5 ml of MeOH and 0.5 ml of 15% KOH at 37 °C for 30 min. The pH of the solution was adjusted to 3 by addition of 1 N HCl 10 µl of 0.1 ng/µl internal standard 15-F₂₂–Isop–d₄ ethanol was added to the mixture to correct the sample loss. Then the mixture was further purified by C18 as mentioned above and an additional silica Sep-Pak solid phase extraction was carried out. The silica Sep-Pak was preconditioned with 5 ml of ethyl acetate. Afterward, it was washed with 5 ml of ethyl acetate and the samples were eluted with 5 ml of 1:1 (ethyl acetate: methanol). PFB esterification was carried out, and TLC was performed using chloroform: ethanol (93:7). A wide TLC cut was chosen to include
all the possible oxidation products. After extraction of the compounds from the scraped silica gel by ethyl acetate, the TMS derivatives were obtained by the procedures described above. LC/APCI-MS analyses were carried out using a narrowbore reverse phase column using methanol as a solvent. Analysis of F3-IsoPs as PFB esters was performed using the same protocol as above employing normal phase LC with a solvent system of 12% isopropyl alcohol in hexanes (32). For quantitative studies involving the measurement of F3-IsoPs in mouse tissues, a highly accurate gas chromatography/MS assay using stable isotope dilution methods was employed as described (19).

RESULTS

A Unified Mechanism That Leads to the Formation of Novel Oxidation Products from EPA and Rationale for Overall Methods Employed For Product Analysis—Based on the free radical mechanism of arachidonic acid autoxidation, the oxidation profile of EPA can be rationally defined. As shown in Scheme 3, eight regioisomeric hydroperoxides (hydroperoxyeicosapentaenoic acid, HpEPA) can be generated from the peroxidation of EPA because of the presence of an additional double bond compared with arachidonate. These eight primary hydroperoxides can be further oxidized to generate more complex peroxidation products. The same types of reactions as those observed for arachidonate peroxidation are anticipated for EPA and include 5-exo cyclization and hydrogen atom abstraction. Different precursors should give rise to different sets of oxidation products (Scheme 4). For example, oxidation of 15-HpEPA (4a) will lead to the formation of bicyclic endoperoxides 4b via two 5-exo cyclizations; these endoperoxides are precursors of IsoPs including F3-/E3-IsoPs and D3/E3-IsoPs. Monocyclic peroxides 4c, dioxolane-IsoP peroxides 4d, and bis-dioxolane IsoP 4e will also be formed. On the other hand, oxidation of 5-HpEPA gives rise to a different set of oxidation products that include monocyclic peroxides 4g, bicyclic endoperoxides 4h, bicyclic peroxides 4j, and tricyclic peroxides 4i. Overall, the oxidation profile of 8-HpEPA, 12-HpEPA, and 11-HpEPA will resemble that of 15-HpEPA whereas oxidation of 9-HpEPA, 14-HpEPA, and 18-HpEPA will generate similar types of oxidation products as those of 5-HpEPA. In order to simplify the oxidation pattern for the studies reported herein, we separated the primary hydroperoxides of EPA methyl ester by semi-preparative HPLC and oxidized the separated fractions.

A number of MS techniques were developed and applied to identify these novel oxidation products (Scheme 5). Ag⁺/H11001 CIS-MS has proven powerful to characterize the intact peroxides of PUFAs based on the unique fragmentation patterns induced by Ag⁺ coordination and was initially employed in studies discussed below. Oxidation mixtures were then reduced by PPh3 and derivatized to PFB esters and APCI-MS was employed to further confirm the structure of the oxidation products. APCI-MS was also utilized to provide additional information about EPA oxidation products after trimethylsilyl (TMS) derivatization.

Formation of Eight Regioisomeric Hydroperoxides from the Oxidation of EPA—Primary hydroperoxides of EPA can be obtained from the oxidation of EPA in the presence of good...
Identification of Novel Autoxidation Products of ω-3 Fatty Acid EPA

**Mass Spectrometry**

![Mass Spectrometry Diagram](image)

**SCHEME 5**

hydrogen atom donors such as α-tocopherol, methyl trolox, or cyclohexadiene although a catalytic system based on the hydrogen atom donor NMBHA gives much higher yield of hydroperoxides from polyunsaturated fatty acids (29). The eight hydroperoxide regioisomers of HpEPA Me were prepared using the latter reagent and separated by HPLC (Fig. 1a). Only 5- and 15-HpEPA Me can be separated from other hydroperoxides whereas 12 and 14-HpEPA, 11- and 18-HpEPA, and 8-HpEPA and 9-HpEPA are collected as mixtures. The structures of these different hydroperoxides were confirmed using Ag⁺ CIS-MS based on the specific Hock cleavage fragments of each hydroperoxide (data not shown). It is interesting that once the hydroperoxides are reduced to corresponding alcohols (hydroxyeicosapentaenoic acids, HEPA), all regioisomers can be separated as eight distinct peaks and their structures can be identified by APCI-MS after PFB esterification (Fig. 1b). The elution order of the eight alcohols is 15-, 12-, 14-, 11-, 18-, 8-, 9-, and 5-HEPA employing normal phase HPLC.

**Identification of Intact Peroxides from the Oxidation of EPA by Ag⁺ CIS-MS**—After separation, individual HpEPA regioisomers were further oxidized to generate different cyclic peroxides predicted in Scheme 4. Oxidation products of 5-HpEPA and 15-HpEPA are detailed herein to provide evidence confirming the proposed mechanism of EPA oxidation. Most of the predicted oxidation products of EPA have cyclic peroxide moieties and are unstable when analyzed by conventional ESI-MS. However, a milder ionization technique, Ag⁺ CIS-MS, has proven to be useful in identification of these intact peroxides based on characteristic fragmentation induced by Ag⁺ coordination of lipids (30, 32, 33). Furthermore, the doublet peaks in the Ag⁺ CIS spectra are characteristic of silver adducts because silver has two isotopes 107 daltons and 109 daltons with almost equal abundance. Products resulting from the oxidation mixture of 5-HpEPA analyzed by Ag⁺ CIS-MS are shown in Fig. 2. Predicted oxidation products include the starting material 5-HpEPA Me (m/z 455/457), monocyclic peroxides 4g and bicyclic endoperoxides 4h (m/z 487/489), bicyclic peroxides 4j (m/z 519/521), and tricyclic peroxide 4i (m/z 551/553). Collision-induced dissociation (CID) MS analyses were then carried out on each precursor ion to generate fragmentation that can be used to identify the structure of the predicted oxidation products (Fig. 3). The starting material 5-HpEPA Me has a precursor ion at m/z 455/457 and CID on m/z 455 gives rise to a predominant peak at m/z 325 (Fig. 3a). The formation of this fragment can be understood based on Hock fragmentation induced by Ag⁺ to generate an aldehyde in the gas phase (30). Thus, this characteristic fragment can be used to locate the hydroperoxyl functional group in the molecule. The fragmentation patterns of monocyclic peroxides 4g (Fig. 3b) and bicyclic endoperoxides 4h (Fig. 3c) are quite different even though they have the same precursor ion at m/z 487/489. Fragmentation at the peroxyl bond in the monocyclic peroxide 4g leads to an aldehyde with a product ion at m/z 291 or an epoxide at m/z 305. However, the fragmentation of silver adduct of bicyclic endoperoxides 4h readily occurs at the hydroperoxyl moiety and gives rise to m/z 357 and 369, respectively. The fragment at m/z 357 is a product derived from Hock cleavage. The bicyclic peroxide 4j (Fig. 3d) and tricyclic peroxide 4i (Fig. 3e) give quite different fragmentation upon analysis using CID. Fragmentation primarily occurs at peroxide bonds resulting in the formation of aldehydes or epoxides. For example, for both the bicyclic and...
Identification of Novel Autoxidation Products of \(\omega-3\) Fatty Acid EPA

As previously noted, the oxidation profile of different hydroperoxyl radical precursors will vary depending on the starting compound. Formation of dioxolane-IsoP peroxides and bis-dioxolane-IsoP peroxides is predicted from 15-HpEPA and generation of these compounds is believed to be responsible for the regioisomeric selectivity of different \(F_3\)-IsoPs. Identification of these novel IsoPs from EPA oxidation would confirm this hypothesis. Thus, 15-HpEPA Me was oxidized under free radical conditions and the oxidation mixture was examined in a manner similar to that for 5-HpEPA. The CID spectra of \(m/z\) 519 (dioxolane-IsoP) and 551 (bis-dioxolane-IsoP) are shown in Fig. 5. In addition to dehydration from the precursor ion that leads to the formation of \(m/z\) 501, there are characteristic fragments that are useful to identify the presence of dioxolane-IsoP peroxide 4d (Fig. 5a). The peak with \(m/z\) 305 is consistent with the cleavage of a dioxolane moiety to generate an aldehyde and dehydration from the hydroperoxide. The other half of the fragment with an epoxide will have an \(m/z\) 303. The cleavage of the cyclic peroxide bond to form an aldehyde toward the end of the molecule can give rise to a fragment with \(m/z\) 289. Fragments derived from the cleavage of the hydroperoxide will generate aldehydes \(m/z\) 305 and 361 respectively. Overall, the major fragments derived from the CID of \(m/z\) 519 from 15-HpEPA Me oxidation are consistent with the presence of the dioxolane-IsoP peroxides 4d.

Further cyclization of the peroxy radical from 4d will lead to the formation of the bis-dioxolane-IsoP peroxide 4e. The CID of \(m/z\) 551 is shown in Fig. 5b. Some similar fragments are observed as those from 4d, for example, \(m/z\) 289, 303, and 361 but there are some unique fragments that are not present in 4d. Peak \(m/z\) 433 and 237 may be derived from the cleavage of hydroperoxide at carbon C5.

Identification of Novel Oxidation Products from EPA Oxidation by LC/APCI-MS after Derivatization—\(Ag^+\) CIS-MS has been successfully employed to identify the intact novel oxidation products from EPA as predicted based on free radical mechanisms. Besides the hydroperoxides, HpEPAs and corresponding alcohols HEPAs, monocyclic peroxides, bicyclic endoperoxides (precursor to 3-series IsoPs), bicyclic peroxide, tricyclic peroxides, dioxolane-IsoP peroxides and bis-dioxolane-IsoP peroxides have also been identified from the oxidation of EPA. To further confirm the identification of these novel products, we employed various MS methods after compounds were reduced to alcohols with PPh₃ and/or derivatized to PFB esters/TMS ether derivatives. The protocols and MS methods that were used to confirm the structures are summarized in

tricyclic peroxide, \(m/z\) 289 is an aldehyde generated from the fragmentation of the first cyclic peroxide bond whereas \(m/z\) 303 is consistent with an epoxide structure. Additional fragments are also observed that confirm the presence of 4j and 4i in the mixture of compounds resulting from the oxidation of 5-HpEPA.

The oxidation of individual HpEPAs results in the formation of a number of stereoisomerically distinct compounds since the mechanism is non-enzymatic. Selective reaction monitoring (SRM) employing CIS-MS was then used to characterize the different diastereoisomers from the oxidation of 5-HpEPA. These experiments were conducted by selecting the precursor ions of interest in the first MS quadrupole, fragmenting each of them in the second quadrupole, and monitoring the characteristic fragments for individual precursor ion. The results are shown in Fig. 4. The starting material 5-HpEPA Me is shown in Fig. 4a as a single peak when the transition from \(m/z\) 455 to 325 is monitored. Bicyclic endoperoxides 4h, monocyclic peroxides 4g are shown in Fig. 4, b and c, respectively. The two compounds can be separated by SRM because they generate different product ions even though they have the same precursor ion \(m/z\). Significantly more peaks representing more diastereomers are observed for bicyclic endoperoxides 4h as opposed to the monocyclic peroxides because five stereogenic centers are present in 4h whereas three stereogenic centers are present in the monocyclic peroxide 4g. The bicyclic peroxides 4j and tricyclic peroxide 4i can also be studied by monitoring their SRM fragments induced by \(Ag^+\) coordination (Fig. 4, d and e). Of note, bicyclic and tricyclic peroxides each can generate fragment ions \(m/z\) 303 and \(m/z\) 289 (Fig. 3, d and e). They can, however, be differentiated in Fig. 4 using SRM since they possess precursor ions with different \(m/z\) values.

FIGURE 2. Direct liquid infusion analysis of an oxidized mixture of 5-HpEPA Me using \(Ag^+\) CIS-MS and the representative structures of predicted oxidation products. The oxidation mixture was dissolved in 50 ng/\(\mu l\) AgBF₄ in IPA and a syringe pump was used to deliver 10 \(\mu l\)/min of the mixture. A mass range of \(m/z\) 100 to 1000 was monitored in the scan mode.
Scheme 5. The dioxolane-IsoP peroxide 4d is taken as an example. PPh₃ reduces the bicyclic endoperoxide to a 1,3-diol on the cyclopentane ring and the hydroperoxide to alcohol whereas the dioxolane moiety is not reduced. After basic hydrolysis, the free acid can be derivatized to the PFB ester which makes it amenable to APCI-MS analysis. TMS derivatization caps the hydroxyl groups on the molecule and makes them amenable for further APCI-MS analysis.

These methods were utilized to analyze the peroxidation of each HpEPA and oxidation product mixture of 5-HpEPA is taken as an example and the results are shown in Fig. 6. The PFB esters of the oxidation products are analyzed by LC/APCI-MS (Fig. 6a). Selective ion monitoring was carried out to detect the ions of the predicted oxidation products and the expected compounds are all detected by this method including monocyclic peroxides (m/z 349), F₃-IsoPs (m/z 351), bicyclic peroxides (m/z 381), and tricyclic peroxides (m/z 413). The same mixture was further derivatized by treating with BSTFA to protect the hydroxyl groups in the molecules. All the corresponding oxidation product derivatives were subsequently detected using LC/APCI-MS (Fig. 6b). Oxidation products from other regioisomers of HpEPA have also been studied by these methods, and the predicted structures have been confirmed (data not shown).
Identification of Novel Oxidation Products from EPA in Vivo—

After identification of novel EPA oxidation products in vitro using various MS methods, we undertook studies to detect these compounds in vivo in mice.

As noted, the majority of the predicted oxidation products from EPA are initially formed as peroxides and the stability and metabolic fate of these compounds in vivo are completely unknown. Thus, to identify these compounds in vivo, we developed protocols to maximize our ability to detect them (Scheme 6). In particular, we utilized APCI-MS to identify oxidation products after derivatization to PFB ester/TMS ether derivatives because of the sensitivity of this methodology. In addition, because levels of EPA in humans and animals on a western diet are relatively low, we supplemented the diet of mice with this PUFA. Thus, for these studies, mice were fed an AIN-93 diet supplemented with 0.56% EPA for 8 weeks which resulted in significant incorporation of EPA into all tissues. Although the various oxidation products can be detected in mice supplemented with EPA without an additional oxidant stress, CCl4 was administered intraperitoneally to enhance the generation of these oxidation products. The mice were then sacrificed and liver tissue harvested. Lipids were extracted by the Folch method and hydrolyzed under basic conditions. The hydrolyzed free fatty acid oxidation products were further separated by solid phase extraction using reverse phase and silica Sep-Pak cartridges. The purified fractions were derivatized to PFB/TMS derivatives and analyzed by APCI-MS employing SRM. The results are summarized in Fig. 7. As is evident, in addition to alcohols (HEPAs), dihydroxy-EPA products, F3-IsoPs, monocyclic peroxide and bicyclic peroxide, dioxolane-IsoPs, and bis-dioxolane-IsoPs have also been detected in the liver tissue of mice. Because of the instability of some of these oxidation products, accurate quantification of these compounds is extremely difficult except for a few products, such as HEPAs and F3-IsoPs. In addition, internal standards do not exist for many of the oxidation products. Nonetheless, the approximate rank order for the abundance of different types oxidation products is HEPAs > dihydroxy-EPA products > F3-IsoPs > Bicyclic Peroxides > Dioxolane-IsoPs > Monocyclic peroxides > bis-Dioxolane peroxides based on the relative intensities shown in Fig. 7. Of note, these compounds are not observed in measurable amounts in mice not supplemented with EPA.

As noted, the above experiments consisted of feeding mice a diet supplemented with 0.56% EPA for 8 weeks and inducing an oxidant stress with the administration of CCl4 as described. In the course of performing such experiments, we also undertook...
studies to confirm the stability of EPA in the diet fed to mice to ensure that the source of oxidized EPA oxidation products was not the diet and that they had been generated in vivo. For these experiments, the AIN-93 diet containing EPA was quantified for F3-IsoPs at the beginning of the study and after its completion using the methods we have previously employed to quantify these compounds in animal tissues employing a stable isotope dilution gas chromatography MS assay (19). In addition, the diet was allowed to remain at room temperature under the same conditions as present in mouse cages for up to 7 days and F3-IsoPs measured. Levels of F3-IsoPs were unchanged in the diet at the end of the 8-week feeding period and also after remaining at room temperature for 1 week in comparison to levels in the freshly prepared diet. Levels quantified in fresh diets were 0.41 ± 0.05 ng/mg EPA. In contrast, levels of F3-IsoPs in liver tissue of EPA-fed animals were 86 ± 11 ng/mg EPA at baseline, a 200-fold difference compared with that present in the diet per mg EPA. In addition, administration of CCl4 to mice increased liver levels of F3-IsoPs to 311 ± 61 ng/mg EPA Fig. 8 (19). Together, these data support the contention that complex oxidation products of EPA such as F3-IsoPs are generated in vivo after supplementation and are not merely derived from the diet.

Regioselective Formation of Different F3-IsoPs from EPA in Vitro and in Vivo—Recently we identified the formation of large amounts of F3-IsoPs from the oxidation of EPA in vitro and in vivo (19). Not only do these compounds serve as potential biomarkers for oxidation of EPA but they also appear to have biological properties that contrast with arachidonate-derived F2-IsoPs suggesting that unlike F2-IsoPs, F3-IsoPs may be anti-inflammatory (36). As would be predicted based on our unified mechanism of EPA oxidation, six series of F3-IsoPs are formed from EPA whereas only four series of F2-IsoPs are generated from arachidonic acid. We have previously shown that 5- and 15-series F2-IsoPs are formed in greater abundance than 8- and 12-series F2-IsoPs because the precursors that lead to 8- and 12-series F2-IsoPs can further cyclized to form dioxolane-IsoPs (33). Similarly, we would predict that the oxidation of EPA will lead to the preferential generation of 5- and 18-series F3-IsoPs compared with 8-, 12-, 11-, and 15-series compounds because the precursors of the latter F3-IsoPs may undergo further oxidation to form dioxolane-IsoPs and bis-dioxolane-IsoPs. Fig. 9 provides evidence to support this hypothesis. Fig. 9,
and b show the regioselectivity of EPA-derived F3-IsoP formation in vitro and in vivo, respectively. As is evident, 5- and 18-series F3-IsoPs are indeed formed in far greater abundance than other series of F3-IsoPs both in vitro and in vivo. It is also striking that the chromatographic patterns for each regioisomer series are nearly identical in vitro and in vivo although the relative abundance of some of the peaks differs. These results clearly support our proposed hypothesis to account for the regioselectivity of F3-IsoP formation in vitro and in vivo. It is also noteworthy to point out that we have previously synthesized a 15-F3-IsoP standard and confirmed its structure by MS and NMR approaches. The MS fragmentation pattern and HPLC retention time of synthetic 15-F3-IsoP are identical with puta-
Identification of Novel Autoxidation Products of ω-3 Fatty Acid EPA

DISCUSSION

Based on a unifying hypothesis to explain the oxidation of EPA in vitro and in vivo, we have identified predicted novel oxidation products of this PUFA using various MS methods. Besides the formation of bicyclic endoperoxides (precursors of F3-IsoPs), monocyclic peroxide, bicyclic peroxides, and tricyclic peroxides, dioxolane-IsoPs and bis-dioxolane-IsoPs have also been characterized. The observation that similar oxidation products are formed both in vitro and in vivo suggests that the same free radical mechanisms occur in biological systems even though the stability and metabolic fates of compounds generated may differ.

The systematic identification of novel oxidation products from EPA is of potential biological importance. A number of studies have shown that supplementation of humans with fish oil rich in EPA and DHA has beneficial effects on the prevention of important human diseases such as atherosclerosis and sudden death, neurodegeneration, and various inflammatory disorders (2, 3, 6–8, 37). A recent Japanese study on effects of EPA on major coronary events in hypercholesterolemia (JELIS) showed a 19% reduction in major coronary events after 4.6 years of EPA intervention in 18,645 hypercholesterolemic patients (3). Although the mechanism(s) by which these beneficial effects occur is unknown, a potentially important aspect related to the biological activity of ω-3 PUFAs is their ability to interfere with the arachidonic acid cascade that generates pro-inflammatory eicosanoids (2, 38). EPA not only can replace arachidonic acid in phospholipid bilayers but is also a competitive inhibitor of COX, reducing the production of 2-series PGs and thromboxane, in addition to the 4-series leukotrienes (11, 12). Recent studies have also shown that 3- and 5-series eicosanoids derived from EPA are either less biologically active or inactive compared with the former products and are thus considered to exert effects that are less inflammatory (10, 13, 39, 40). Furthermore, Serhan (14, 15, 39, 41, 42) and co-workers have described a group of polyoxygenated DHA and EPA derivatives termed Resolvins that are produced enzymatically in various tissues. These compounds inhibit cytokine expression and other inflammatory responses in murine, skin cells, and other cell types.

There has also been significant interest in the biological activities of non-enzymatic free radical-initiated peroxidation products of ω-3 PUFAs (18–20, 43). Sethi et al. (16, 17) recently reported that EPA oxidized in the presence of Cu2+, but not native EPA, significantly inhibits human neutrophil and monocyte adhesion to endothelial cells, a process linked to the development of atherosclerosis and other inflammatory disorders. This effect was induced via inhibition of endothelial adhesion receptor expression and was modulated by the activation of the peroxisome proliferator-activated receptor-α (PPAR-α) by EPA oxidation products. In addition, oxidized EPA markedly reduced leukocyte rolling and adhesion to venular endothelium of lipopolysaccharide-treated mice in vivo and the effect was not observed in PPAR-α-deficient mice. These studies suggest that the beneficial effect of ω-3 fatty acids may be mediated, in part, by the anti-inflammatory effects of oxidized EPA. Similarly, Vallve et al. (44) have shown that various non-enzymatically generated aldehyde oxidation products of EPA and DHA decrease the expression of the CD36 receptor in human macrophages. Up-regulation of this receptor has been linked to atherosclerosis. Additional recent reports have suggested that other related biological effects of EPA and DHA, such as modulation of endothelial inflammatory molecules, are related to their peroxidation products. Arita et al. (45) have also shown that non-enzymatically oxidized EPA enhances apoptosis in HL-60 leukemia cells supporting the contention that oxidized ω-3 PUFAs are both anti-proliferative and anti-inflammatory. In virtually none of these reports, however, have the specific peroxidation products responsible for these effects been identified.

Our studies reported herein have systematically defined the oxidation of EPA in vitro and in vivo for the first time. The vast majority of oxidation products are peroxides and because of their stability, detection is an important issue for any analytical technique employed. As reported, Ag+ CIS-MS has proven powerful to identify these intact peroxides generated in vitro. The unique fragmentation patterns induced by silver ion coordination were employed to characterize these novel compounds. However, the poor sensitivity of this technique makes it difficult to be used to study these oxidation products in vivo (46). Furthermore, the stability of these peroxides in vivo is virtually unknown. Other MS techniques such as APCI-MS afford significant sensitivity and can thus be complimentary to silver ion CIS-MS even though compound derivatization is needed. We successfully utilized this latter approach to indeed show that the oxidation of EPA can be rationally defined both in vitro and in vivo. Nonetheless, it is noteworthy that accurate quantification of EPA oxidation products in vivo is difficult because the stability and metabolism of these compounds are unknown. Furthermore, internal standards for each class of oxidation products are lacking (except for F3-IsoPs), which poses another challenge to accurately quantifying these compounds.

Systematic identification of the oxidation products of EPA and other PUFAs provides the rationale for studying the biology of these compounds. For example, we reported recently that a novel class of J3-IsoPs derived from EPA activates the master transcription factor, Nrf2 by destabilizing the association between Keap1 and Cullin 3 (18). Nrf2 activation leads to antioxidant gene expression that regulates detoxification of reactive oxygen species. This pathway represents a novel and potentially beneficial aspect of fish oil supplementation and studies are further ongoing to examine the role of EPA oxidation products to regulate this response to oxidant stress in vivo.

Another rationale for undertaking these studies was based on the hypothesis that oxidation products contribute to the beneficial biological effects of EPA and fish oil supplementation in that they exert biological activities that are less pro-inflammatory than oxidation products derived from arachidonic acid. Indeed, as noted, one limited report noted that the EPA-derived IsoP, 15-F3t-IsoP, possesses activity that is significantly different from 15-F2t-IsoP in that it does not affect human platelet shape change or aggregation (36). 15-F2t-IsoP is a ligand for the
Identification of Novel Autoxidation Products of \(\omega-3\) Fatty Acid EPA

Tx receptor and induces platelet shape change and also causes vasoconstriction. The lack of activity of 15-\(F_3\)-IsoP is consistent with observations regarding EPA-derived PGs in that these latter compounds exert either weaker agonist or no effects in comparison to arachidonate-derived PGs. Future studies will need to further explore these observations.

In summary, we report, for the first time, systematic identification of novel oxidation products of EPA by various MS techniques. The same oxidation products generated in vitro can be detected in vivo. We also observed a strikingly similar pattern of product distribution of \(F_3\)-IsoPs in vivo and in vitro, suggesting that the same free radical mechanisms operative in vitro occur in vivo. Further understanding of the biological consequences of the formation of these novel compounds and factors influencing their formation and metabolism will provide valuable insights into the role of EPA in human physiology and pathophysiology.

REFERENCES

1. Barnham, K. J., Masters, C. L., and Bush, A. I. (2004) Nat. Rev. Drug Discov. 3, 205–214
2. Calder, P. C. (2006) Am. J. Clin. Nutr. 83, 1517–1522
3. Yokoyama, M., Origasa, H., Matsuuzaki, M., Matsuuzawa, Y., Saito, Y., Ishikawa, Y., Oikawa, S., Sasaki, J., Hishida, H., Itakura, H., Kita, T., Kitabatake, A., Nakaya, N., Sakata, T., Shimada, K., and Shirato, K. (2007) The Lancet 369, 1090–1098
4. Wong, K. W. (2005) J. Am. Diet. Assoc. 105, 98–105
5. Lukis, W. J., Cui, J.-G., Marcheselli, V. L., Bodker, M., Botkjaer, A., Gottlinger, K., Serhan, C. N., and Bazan, N. G. (2005) J. Clin. Invest. 115, 2774–2783
6. Connor, S., and Connor, W. (1997) Am. J. Clin. Nutr. 66, 1020S–1031
7. Kris-Etherton, P. M., Harris, W. S., Appel, L. J., and the Nutrition Committee (2002) Circulation 106, 2747–2757
8. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509
9. Pratico, D., Smyth, E. M., Violi, F., and FitzGerald, G. A. (1996) J. Biol. Chem. 271, 14916–14924
10. Brouwer, I. A., Geelen, A., and Katzen, M. B. (2006) Prog. Lipid Res. 45, 357–367
11. Arita, K., Yamamoto, Y., Takehara, Y., Utsumi, T., Kanno, T., Miyaguchi, M., Kita, T., Kita, R., Thiell, D. H. V., Wellner, D., Walter, P. B., Tome, K. B., Mason, R. P., and Barrett, J. C. (2005) Free Radic. Biol. Med. 38, 698–710
12. Davis, T. A., Gao, L., Yin, H., Morrow, J. D., and Porter, N. A. (2006) J. Am. Chem. Soc. 128, 14897–14904
13. Yin, H., Havrilla, C. M., Gao, L., Morrow, J. D., and Porter, N. A. (2003) J. Biol. Chem. 278, 16720–16725
14. Porter, N. A., Caldwell, S. E., and Mills, K. A. (1995) Lipids 30, 277–290
15. Serhan, C. N., Harris, T. M., and Roberts, L. J., Jr. (1990) Anal. Biochem. 184, 1–10
16. Serhan, C. N., Clish, C. B., Brannon, J., Chiang, N., Moreau, J., and Serhan, C. N. (2003) J. Biol. Chem. 278, 19330–19336
17. Serhan, C. N., Gotlinger, K., Hong, S., and Arita, M. (2004) J. Biol. Chem. 279, 22254–22266
18. Laneuville, O., Breuer, D. K., Xu, N., Huang, Z. H., Gage, D. A., Watson, J. T., Lagarde, M., DeWitt, D. L., and Smith, W. L. (1995) J. Biol. Chem. 270, 19330–19336
19. Achard, F., Gilbert, M., Benistant, C., Slama, S. B., Dewitt, D. L., Smith, W. L., and Lagarde, M. (1997) Biochem. Biophys. Res. Commun. 241, 513–518
20. Bagga, D., Wang, L., Farias-Eisner, R., Glaspy, J. A., and Reddy, S. T. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1751–1756
21. Serhan, C. N., Clish, C. B., Brannon, J., Colgan, S. P., Chiang, N., and Gronert, K. (2000) J. Exp. Med. 192, 1197–1204
22. Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R., Mirick, G., and Moussigian, R.-L. (2002) J. Exp. Med. 196, 1025–1037
23. Sethi, S., Liouzenkova, O., H. N., Wagner, A. D., Plutzky, J., and Mayadas, T. N. (2002) Blood 100, 1340–1346
24. Sethi, S. (2002) Redox Rep. 7, 369–378
25. Gao, L., Wang, J., Sekhar, K. R., Yin, H., Yared, N. F., Schneider, S. N., Sasi, S., Dalton, T. P., Anderson, M. E., Chan, J. Y., Morrow, J. D., and Freeman, M. L. (2007) J. Biol. Chem. 282, 2529–2537
26. Davis, T. A., Gao, L., Yin, H., Morrow, J. D., and Porter, N. A. (2006) J. Am. Chem. Soc. 128, 14897–14904
27. Yin, H., Havrilla, C. M., Gao, L., Morrow, J. D., and Porter, N. A. (2003) J. Biol. Chem. 278, 16720–16725
28. Porter, N. A., Caldwell, S. E., and Mills, K. A. (1995) Lipids 30, 277–290
29. Morrow, J. D., Harris, T. M., and Roberts, L. J., Jr. (1990) Anal. Biochem. 184, 1–10
30. Morrow, J. D., Hill, E., Burk, R. F., Nammour, T. M., Badr, K. F., and Roberts, L. J., Jr. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9383–9387
31. Morrow, J. D. (2005) J. Am. Soc. Mass Spectrom. 12, 449–455
32. Kadiiska, M. B., Gladen, B. C., Baird, D. G., Geraci, M. A., Gage, D. A., Watson, J. T., Lagarde, M., DeWitt, D. L., and Smith, W. L. (2007) J. Biol. Chem. 282, 2266–22666
33. Reves, P. G. (1997) J. Nutr. 127, 8385–8415
34. Solomon, P. S., Kaduce, T. L., Hu, S., Raj Gopal, V., Falck, J. R., and Spector, A. A. (2006) Proc. Leukot. Ess. 75, 169–177
35. Sun, Y.-P., Oh, S. F., Uddin, J. Y., Gotlinger, K., Campbell, E., Colgan, S. P., Petasis, N. A., and Serhan, C. N. (2007) J. Biol. Chem. 282, 9323–9334
36. Sheng, K., Chan, D., Felix, E., Cartwright, C., Menger, D. G., Madden, T., Klein, R. D., Fischer, S. M., and Newman, R. A. (2004) J. Lipid Res. 45, 1030–1039
37. Serhan, C. N., Gottlinger, K., Hong, S., and Arita, M. (2004) Proc. Oth. Lipid Res. 73, 155–172
38. Serhan, C. N., Gottlinger, K., Devchand, P. R., Moussigian, R.-L., and Serhan, C. N. (2003) J. Biol. Chem. 278, 14677–14687
39. Yin, H., Musiek, E. S., Gao, L., Porter, N. A., and Morrow, J. D. (2005) J. Biol. Chem. 280, 26600–26611
40. Valle, J.-C., Oulaf, K., Iwata, T., Cabre, A., Riba, J., Hervas, M., and Masana, L. (2002) Atherosclerosis 164, 45–56
41. Arita, K., Yamamoto, Y., Takehara, Y., Utsumi, T., Kanno, T., Miyaguchi, C., Akiyama, J., Yoshioka, T., and Utsumi, K. (2003) Free Radic. Biol. Med. 35, 189–199
42. Seal, J. R., Havrilla, C. M., Porter, N. A., and Hachey, D. L. (2003) J. Am. Soc. Mass Spectrom. 14, 872–880