Autooxidation of docosahexaenoic acid: analysis of ten isomers of hydroxydocosahexaenoate

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Abstract Docosahexaenoic acid, an n-3 essential fatty acid, was recently shown to be enzymically converted by platelets, basophils, and liver microsomes into metabolites containing conjugated dienes with allylic hydroxyl groups. To help identify these metabolites, standards were prepared by autooxidation of docosahexaenoic acid. After isolation by reverse phase and normal phase high performance chromatography (HPLC), ten hydroxy isomers of docosahexaenoic acid were identified by capillary gas–liquid chromatography, ultraviolet spectroscopy, and mass spectrometry. From these studies and reported elution orders for similar metabolites derived from linoleic, linolenic, and arachidonic acids, two basic HPLC elution patterns became apparent. Under reverse phase chromatography conditions, the distance of the *trans*-double bond from the carboxyl group was the critical parameter in determining the elution order. Under silicic acid chromatography conditions, the distance of the hydroxyl from the carbomethoxy group seemed to determine the elution order. The dramatic difference in selectivity between reverse and normal phase HPLC of the hydroxy acids provides critical information useful for identifying endogenous metabolites.—**VanRollins, M., and R. C. Murphy.** Autooxidation of docosahexaenoic acid: analysis of ten isomers of hydroxydocosahexaenoate. *J. Lipid Res.* 1984. **25:** 507–517.

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Most mammalian tissues examined to date can enzymically oxidize polyunsaturated fatty acids to conjugated dienes with allylic hydroperoxy groups. Almost immediately the hydroperoxides are reduced in vivo by peroxidases to allylic alcohols. Commonly studied reduction products, hydroxyoctadecadienoic acids (HODDs) and hydroxyeicosatetraenoic acids (HETEs), are formed from the n-6 essential fatty acids, linoleic and arachidonic acids, respectively. The HODDs, first discovered in sheep vesicular gland (1), are the primary endogenous products from octadecadienoic acids of peritoneal (2), VX_2 carcinoma (3), and perhaps testes cells (4). The HETEs, originally discovered in platelets, have been found in neutrophils, lymphocytes, and mast cells (reviewed in reference 5). Recently, HETEs have been observed in reticulocytes (6), basophils (7), eosinophils (8, 9) as well as in alveolar (10) and peritoneal (11) macrophages. Solid organs are also believed to be sources of HETEs although frequently HETE contributions from circulating cells have not been carefully assessed. HETEs may be present in seminal vesicle (12, 13), testis (4), blood vessel (14), lung (5), peritoneum (2), spleen (5), liver (15, 16), pancreas (17), kidney (18), pituitary (19), brain (20), VX_2 carcinoma cells (3), and in skin (5).

The biosyntheses of HETEs and HODDs are catalyzed by lipoxygenases (20), P-450 monooxygenases (15), or cyclooxygenases (1–3, 12, 13, 21), each of which operates through an autooxidation-like mechanism (15, 20). The rate-limiting step, abstraction of bis-allylic hydrogen atoms (22), would suggest that only two positional isomers of HODDs (9- and 13-) and six positional isomers of HETEs (5-, 9-, 8-, 12-, 11-, and 15-) will be found biologically (20). In fact, both the 9- and 13-HODDs are usually produced together in tissues (1–3), whereas from one (5) to six (8, 11) positional isomers of HETEs may be produced in a single cell. However, it is the 5(S)-, 12(S)-, or 15(S)-HETEs which are the predominant biological products in various cells. Thus it seems that the actual spectrum of hydroxylated fatty acids observed for any particular biological sample will depend upon both the type of the fatty acid precursor and the tissue examined.

The biological actions of these metabolites of n-6 essential fatty acids have primarily been tested with leukocytes using HETEs or their hydroperoxy precursors, HPETEs. Addition of the relatively stable HETEs to leukocyte populations can result in chemokinesis (5), chemotaxis (5), aggregation (23), degranulation (24), sensitization of complement receptors (5), and immunosuppression (25). Moreover, HETEs can enhance leukocyte uptake of hexoses (26) and calcium (27). Such effects suggest that HETEs may be important modulators

Abbreviations: n, methyl terminus of a fatty acid; 22:6, docosahexaenoic acid; HODD, hydroxyoctadecadienoic acid; HODT, hydroxyoctadecatrienoic acid; HETE, hydroxyeicosatetraenoic acid; HDHE, hydroxydocosahexaenoic acid; HPLC, high performance liquid chromatography; ODS, octadecylsilane; GLC, gas–liquid chromatography; MS, mass spectrometry; ECL, equivalent chain length.

of inflammatory and immunological reactions. HPETEs are usually more potent than their HETE products in eliciting many of the biological properties (5). HPETEs, unlike HETEs, can inhibit platelet cyclooxygenase, thromboxane synthase, and diglyceride lipase (28). HPETEs can also inhibit prostacyclin synthase, activate guanylate cyclase, and are potent producers of pain (29).

Recently docosahexaenoic acid, an n-3 essential acid, has been shown to undergo similar enzymic reactions to form 11- and 14-hydroxydocosahexaenoic acid (HDHE) in platelets (30), 4- and 7-HDHE in basophils (31) and 17-, 16-, 14-, 13-, 11-, 10-, 8-, and 7-HDHE in liver.[1] The biological properties of these metabolites of docosahexaenoic acid have yet to be examined. In order to elucidate the biological functions of these compounds, the availability of highly purified standards in sufficient quantities is essential. This report describes the synthesis and characterization of all ten theoretically predicted racemic hydroxy isomers of docosahexaenoic acid.

METHODS

Preparation of hydroxydocosahexaenoic acid (HEHE) isomers

An autooxidation procedure (32) coupled with chemical reduction was used to generate HDHE isomers. Short incubation times were employed in order to minimize secondary *trans, trans-* isomerizations of *cis, trans*-conjugated dienes. The presence of *trans, trans-* isomers will increase the complexity of autooxidation mixtures. *Trans, trans*-conjugated diene isomers have longer retention times during normal phase and reverse phase high pressure liquid chromatography (HPLC) and 3–4 nm lower UV absorption maxima than their *cis, trans* counterparts (6, 32–34).

In brief, 10 mg (30.5 μmol) of docosahexaenoic acid (22:6, greater than 99% purity; Nu-chek, Elysian, MN) was transferred to a silanized 100-ml glass tube (3 (i.d.) × 12 cm) using 5–15 ml of tetrahydrofuran (HPLC quality, Burdick and Jackson, Muskegon, MI). A film of 22:6 was generated by rotating the solution in vacuo and keeping the container horizontal. After bringing the sample to ambient pressure with 99.9% oxygen (3.6 mmol), the tube was sealed and rotated overnight at room temperature. The resulting mixture of hydroperoxides was transferred in CH_2Cl_2 to a 50-ml centrifuge tube, concentrated under a stream of N_2, and rapidly dissolved in 5 ml of cold (−20° C) methanol. The hydroperoxides were immediately reduced (35, 36) by adding 10 mg of $NaBH_4$ and incubating on ice for 15 min and then at room temperature for 10 min. After 5 ml of H_2O was added and the pH was adjusted to 3 with HCl, HDHE isomers were partitioned into 30 ml of CH_2CL_2. Combined CH_2Cl_2 extracts were then back-washed with 20 ml of H_2O to remove residual HCl. The entire sample, after concentrating in vacuo, was dissolved in 1 ml of CH_3OH.

Purification of HDHE isomers

The first step in isolating HDHE isomers involved a modification of a reverse phase HPLC procedure used to resolve natural mixtures of prostaglandins (37) and free fatty acids (38). This method is particularly suitable for separating fatty acids which differ in positions or geometries of double bonds. The columns used (0.46 (i.d.) × 25 cm or 1.0 (i.d.) × 25 cm) contained 5 μm octadecylsilane (ODS) particles (Ultrasphere-ODS, Beckman, Irvine, CA). HPLC grade acetonitrile and phosphoric acid were obtained from Fisher Scientific Company, Pittsburgh, PA. Solvents were filtered (0.22 μm, GVMP; Millipore Corporation, Bedford, MA) just prior to use. Appropriate aliquots (20 or 200 μl) of sample were injected onto the analytical or semipreparative columns and developed (1.75 or 6.6 ml/min) isocratically in a mobile phase of acetonitrile–aqueous phosphoric acid 43:57 (pH 2.3). Individual fractions were collected from the ascending or descending portions of peaks absorbing at 229 nm (detector Model 160, Beckman). After combining comparable fractions from four or five runs, each fraction was neutralized with excess $NaHCO_3$ and acetonitrile was removed in vacuo. The aqueous residue was then acidified to pH 3 with HCl and four volumes of CH_2Cl_2 were added. After centrifugation, the isolated organic extracts were concentrated in vacuo and methylated by reacting with methanol–ethereal diazomethane 1:9 for 10 min.

The final step in purification of HDHE isomers involved normal phase HPLC. Methyl esters of isolates from reverse phase HPLC were concentrated under a stream of nitrogen and dissolved in 50 μl of isopropanol–hexane 0.5:100. Samples were injected and chromatographed (1.0 ml/min) using a 5-μm particle, silicic acid guard (0.46 (i.d.) × 5 cm; Apex, Jones Chromatography, Columbus, OH) and an analytical (0.46 (i.d.) × 25 cm; Supelco, Bellefonte, PA) column directly connected to each other, i.e., without intervening tubing. The mobile phase was isopropanol–hexane 0.21:100 and fractions absorbing at 229 nm were collected.

Characterization of HDHE isomers

The purified methyl esters of HDHE isomers were characterized using UV spectroscopy, capillary gas–liquid chromatography (GLC), and mass spectrometry (MS). The

[1] VanRollins, M., R. C. Baker, H. W. Sprecher, and R. C. Murphy. 1984. Oxidation of docosahexaenoic acid by rat liver microsomes. *J. Biol. Chem.* In press.

UV spectra were determined using a Cary 14 spectrophotometer equipped with a Hamamatsu (Type R 456 HA) photomultiplier tube.

The methyl esters, isolated by normal phase HPLC, were directly silylated or catalytically hydrogenated with 5% rhodium on alumina[2] and then silylated for GLC. Silylation was done by heating the sample at 60°C for 45 min after it was dissolved in 50 μl of acetonitrile and 50 μl of bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Company, Rockford, IL). Since fatty acid methyl esters that contain conjugated double bonds with allylic trimethylsilyl ether groups appeared to be thermally labile (5, 39), GLC separations of unsaturated derivatives were performed using relatively low temperatures and short columns with large amounts of stationary phase. For saturated derivatives, no thermal decomposition products were evident and capillary columns were employed to give optimal resolution. The equivalent chain length (ECL) of each derivative was determined by relating the log of its retention time to the log retention time of 22:0, 23:0, 24:0, 26:0, and 28:0 fatty acid methyl esters on packed and capillary columns.

GLC–MS

Electron impact (70 eV) studies were conducted using a quadrupole mass spectrometer (Model 3200, Finnigan, Sunnyvale, CA) equipped with a Technivent data system (St. Louis, MO). Glass GLC columns (2.5 mm (i.d.) × 90 cm) were silanized and then packed either with 3% OV-101 on 80/100 Supelcoport or with SP-2330 on 100/120 Chromosorb (Supelco). The injection temperature was set at 215°C and column temperatures were maintained at 210°C (OV-101), or at 200°C (SP-2330). The flow rate of helium carrier gas was 30 ml/min.

Capillary-GLC

A nonpolar (DB-1, J & W, Rancho Cordova, CA) column (0.25 mm (i.d.) × 30 m with 0.25 μm film thickness) and a "falling needle" all-glass injector (R. A. Allen Company, Boulder, CO) were employed to measure the equivalent chain lengths of hydrogenated derivatives. The helium carrier gas was adjusted to a linear velocity of 20 cm/sec for the isothermal (230° C) determinations.

RESULTS

HPLC of autooxidation products

Compounds derived from autooxidation of 22:6 were separated by reverse phase HPLC using acetonitrile–aqueous phosphoric acid 43:57 and visualized by monitoring absorption at 229 nm. As seen in **Fig. 1A,** nine peaks were apparent with compounds V and VI (vide infra) exactly coeluting in one of them. An incomplete separation of Compounds II and III or VII and VIII was also evident. Resolution was not improved with the use of methanol–aqueous phosphoric acid 75:25 nor with prior methylation of the sample mixture and development in acetonitrile–water 55:73 or methanol–water 75:81.

Ten peaks were seen upon normal phase HPLC of the methylated sample mixture (**Fig. 2A**). Methylation was essential for optimal resolution of all ten components in the autooxidation mixture. Compounds I and VI were baseline-separated with low (0.21%) isopropanol concentrations. However, critical pairs III and V or IX and X were not completely separated at any of the isopropanol concentrations (0.2–0.5%) tried. Nevertheless, because different problem pairs were found for reverse phase HPLC than with normal phase HPLC, a combination of reverse phase and normal phase HPLC provided complete resolution of all ten compounds. Moreover, because components of each problem pair from reverse phase HPLC were so widely separated during normal phase HPLC, they could be collected together during reverse phase HPLC and then easily resolved as free fatty acids using normal phase HPLC and hexane–isopropanol–glacial acetic acid 100:0.3:0.05 for the mobile phase.

Yields of hydroxy fatty acids were estimated assuming an extinction coefficient of 27,000 (43). The total yield for Compound I was 96 μg while the relative yields, based on peak height analyses, for Compounds I to X were 1.00, 0.34, 0.29, 0.33, 0.40, 0.27, 0.27, 0.26, 0.29, and 0.24, respectively. The relative HETE yields obtained using identical autooxidation conditions were: 15-, 1.00; 11-, 0.50; 12- plus 8-, 0.46; 9-, 0.41; and 5-HETE, 0.07. Identification of the arachidonic acid autooxidation products was done using reverse phase and normal phase HPLC retention times obtained for HETE standards (Figs. 1B and 2B).

Ultraviolet and GLC characteristics of autooxidation products of docosahexaenoic acid

Ultraviolet spectra (in hexane) were determined for each of the methylated isolates recovered after reverse phase and normal phase HPLC. The individual absorption maxima ranged from 233–237 nm (**Table 1**), indicating the presence of a *cis, trans*-conjugated diene in each compound (6, 32).

Nonhydrogenated Compounds I to X were poorly resolved from each other when chromatographed as trimethylsilyl ether, methyl ester derivatives on either polar or nonpolar stationary phases (Table 1). Nevertheless, the ECL for all the HDHEs were three units higher on polar phases than on nonpolar phases, suggesting the presence of multiple double bonds in the HDHEs. The

[2] VanRollins, M., and R. C. Murphy. Unpublished experiments.

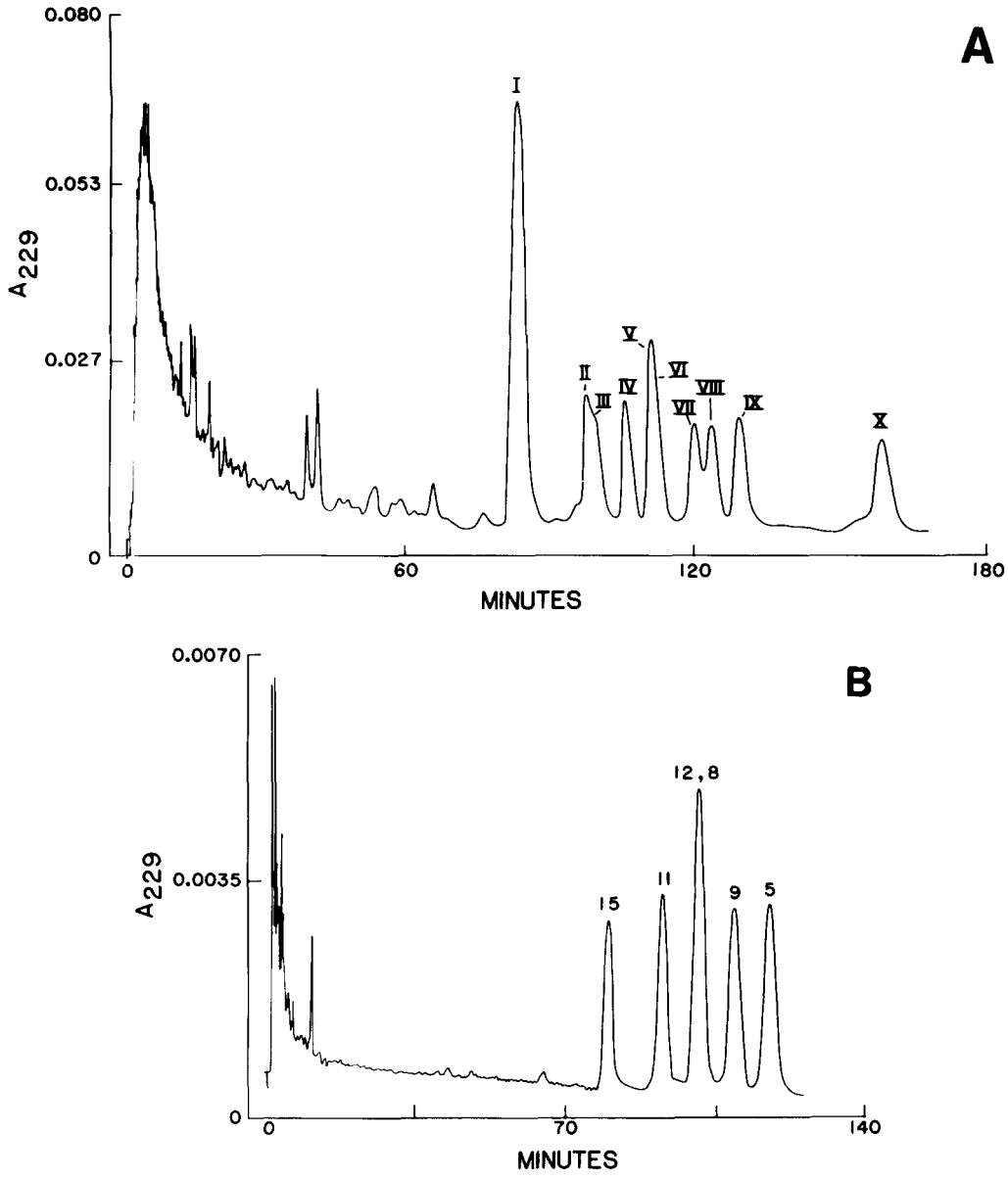

Fig. 1. Reverse phase chromatograms of hydroxy fatty acids containing conjugated dienes. A, $NaBH_4$-reduced autooxidation products of docosahexaenoic acid (200 μg) were separated using a 0.46 (i.d.) × 25 cm column, containing 5 μm ODS particles, and a mobile phase (1.75 ml/min) of 43% acetonitrile in water (pH 2.2). B, HETE standards were separated using identical chromatographic conditions. The position of the hydroxyl group substitution on the aliphatic chain is indicated by a number. 12-HETE was prepared from platelets (40), 15-HETE from soybean lipoxygenase (41) and 5-HETE by organic synthesis (42). The 8-, 9-, and 11-HETEs were standards provided by Dr. Robert Bryant of George Washington University.

observed 23.1 ECL (non-polar) was 1.8 units greater than the 21.3 value reported for HETEs (43). The magnitude of this difference is consistent with the HDHEs having two extra carbons and double bonds.

Hydrogenation of Compounds I to X improved the separations between these autooxidation products (Table 1). The resulting 0.9–1.3 increase in ECL was larger than the 0.7 unit increase reported for HETEs (43) and confirmed the high level of unsaturation in HDHEs. Since the ECLs of hydrogenated HDHEs did not match the ECL of 21- and 22-hydroxydocosanoic acids (24.8 and 25.5, respectively), it was clear that none of the HDHEs had a hydroxyl group at C-21 or C-22. As discussed below, retention times of the hydrogenated HDHEs increased as the distance between the trimethylsilyl and carbomethoxy groups increased.

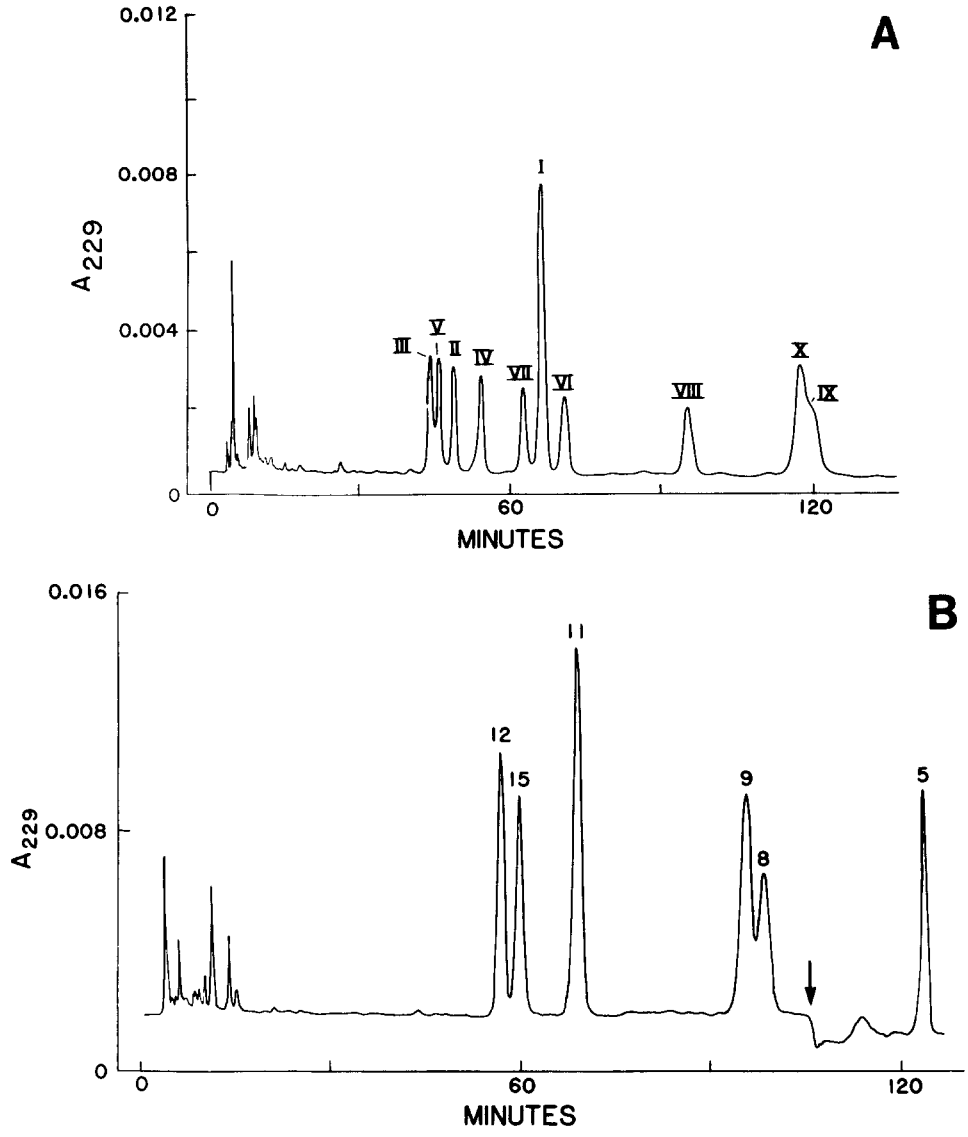

Fig. 2. Normal phase chromatograms of hydroxy fatty acids containing conjugated dienes. A, Methyl esters of $NaBH_4$-reduced autooxidation products of docosahexaenoic acid were separated using 0.46 (i.d.) × 5 cm and 0.46 (i.d.) × 25 cm columns, containing 5 μm particles of silicic acid, and a mobile phase (1.0 ml/min) of hexane–isopropanol 100:0.21. B, Methyl esters of HETE standards were resolved under identical conditions except that the flow rate was increased (arrow) to 4 ml/min at 105 min after injection of the compounds.

Mass spectra of autooxidation products of docosahexaenoic acid

Electron impact (70 eV) mass spectra were obtained for hydrogenated, trimethylsilyl ether, methyl ester derivatives of Compounds I–X. A molecular weight of 442 (**Fig. 3A, Table 2**) was apparent from the m/z values of 427 (M-CH_3), 411 (M-OCH_3), 395 (M-(CH_3 + CH_3OH)) and 320 (M-($HOSi(CH_3)_3$ + CH_3OH)). The position of each $OSi(CH_3)_3$ group was also established from the alpha cleavage (e.g., m/z 131, 413) and the "T" trimethylsilyl rearrangement ions (e.g., m/z 384) (Fig. 3A). The transfer of a remote trimethylsilyl group to the carbomethoxy moiety is a well-characterized rearrangement (44–46) typically followed by loss of RCHO and resulting in formation of odd electron species occurring at even mass to charge ratios. For example, m/z 384 (M-CH_3CH_2CHO) represents formation of $(CH_2)_{18}C(=OSi(CH_3)_3)OCH_3$ from Compound I (Fig. 3A). This T ion undergoes gamma cleavage to produce m/z 159 (CH_2=$CHC(OSi(CH_3)_3)$=OCH_3) (45).

Hydrogenation of the methyl ester of Compound X resulted in the formation of a derivative that generated ions (Table 2) at m/z 320 (5.0%), 302 (3.3%), 276 (3.0%),

TABLE 1. Ultraviolet absorption maxima and equivalent chain lengths (ECL) of Compounds I–X

Peak Number[a]	Wavelength (Max) (Hexane)	Non-polar GLC Phase[b]	Polar GLC Phase[c]
I	236	23.4 (24.7)[d]	ND
II	234	23.1 (24.1)	26.6
III	237	23.1 (24.2)	26.6
IV	233	23.1 (23.9)	26.6
V	236	23.1 (24.0)	26.5
VI	236	23.1 (24.0)	26.5
VII	237	23.1 (24.0)	26.6
VIII	236	23.1 (24.0)	26.6
IX	ND	23.1 (24.0)	26.5
X	236	23.1 (23.8)	26.5

[a] Peak numbering is described in Figs. 1A and 2A.
[b] ECL was determined on methyl ester, trimethylsilyl ether derivatives using 3% OV-101 (210°C). Numbers in parentheses represent values for corresponding hydrogenated derivatives chromatographed on DB-1 capillary columns (230°C).
[c] ECL was measured for methyl ester, trimethylsilyl ether derivatives using 10% SP-2330 (200°C).
[d] The value in parentheses was determined for the 4-lactone derivative.

and 85 (100%). Apparently, hydrogenation had resulted in a transesterification reaction in which the carbomethoxy group had been attacked by a hydroxyl group at C-4. Formation of this internal ester yielded a relatively volatile derivative (Table 1) whose hydroxyl group was unavailable for silylation. The latter point was indicated by the virtual absence of m/z 73 ($Si(CH_3)_3$), 75 ($HOSi(CH_3)_2$), 89 ($OSi(CH_3)_3$), 103 ($CH_2{=}OSi(CH_3)_3$), 117 ($CH_2\text{-}CH_2OSi(CH_3)_3$), and 129 ($CH_2{=}CHCH_2OSi(CH_3)_3$) ions.

In summary, mass spectra of hydrogenated Compounds I to X indicated the presence of hydroxyl groups at carbons 20, 16, 17, 13, 14, 10, 11, 7, 8, and 4, respectively. It was also clear that as the distance between the trimethylsilyl and carbomethoxy groups diminished, alpha cleavage on the methyl side increased while that on the carbomethoxyl side decreased (Table 2). Moreover, an increased formation of T ion accompanied the decreasing separation of the trimethylsilyl from the carbomethoxy groups.

Mass spectra of trimethylsilyl ether, methyl ester derivatives of nonhydrogenated Compounds I to X were also obtained (Fig. 3B, **Table 3**). Occasional high mass ions at m/z 415 ($M\text{-}CH_3$) and 340 ($M\text{-}HOSi(CH_3)_3$) plus the regular occurrence of ions at m/z 309 ($M\text{-}(CH_3O + HOSi(CH_3)_3)$) and 307 ($309\text{-}H_2$) indicated a molecular weight of 430 for these autooxidation products. Comparisons with the molecular weight of corresponding hydrogenated derivatives revealed the presence of six (442–430) double bonds (Tables 2 and 3). Moreover, similar comparisons for the corresponding alpha cleavage and T ions revealed the number of double bonds located on each side of the $OSi(CH_3)_3$ group. For instance, Compound I had no double bonds (131–131) on the methyl side of the $OSi(CH_3)_3$ group (Fig. 3). This was confirmed by noting the presence of six double bonds (384–372) in the T ions (Fig. 3) which arise from the carbomethoxy side. Realizing that the *cis, trans*-conjugated dienes (Table 1) are allylic to the hydroxyl groups, and assuming that all other double bonds retained their original positions, the ten autooxidation products were identified as: I, 20-hydroxy-4,7,10,13,16,18-; II, 16-hydroxy-4,7,10,13,17,19-; III, 17-hydroxy-4,7,10,13,15,19-; IV, 13-hydroxy-4,7,10,14,16,19-; V, 14-hydroxy-4,7,10,-12,16,19-; VI, 10-hydroxy-4,7,11,13,16,19- VII, 11-hydroxy-4,7,9,13,16,19-; VIII, 7-hydroxy-4,8,10,13,16,19-; IX, 8-hydroxy-4,6,10,13,16,19-; and X, 4-hydroxy-5,7,10,13,16,19-docosahexaenoic acid.

DISCUSSION

Autooxidation of a film of docosahexaenoic acid resulted in the formation of ten hydroxylated compounds containing *cis, trans*-conjugated dienes, as identified by ultraviolet spectroscopy and mass spectrometry. The product mixture was essentially devoid of hydroxylated compounds containing *trans, trans*-conjugated dienes (32, 34) or hydroxylated compounds with only isolated double bonds (47), the latter being easily detectable during reverse phase HPLC by monitoring absorption at 192 nm.[1] The production of minimally contaminated mixtures was important since, as previously recognized (5), the principal factor preventing the isolation of large amounts of hydroxy fatty acids is the relatively low capacity of reverse phase HPLC columns. Thus, increasing the complexity of an already complex mixture reduces resolution capabilities dramatically. By scaling up the present procedure and by using preparative columns, at least quantities of each HDHE in mg amounts should be obtainable.

The production of a large series of hydroxylated fatty acids proved useful in understanding elution patterns under reverse phase conditions. The observed reverse phase HPLC elution sequence for HDHEs, 20 ➤ 16 ➤ 17 ➤ 13 ➤ 14 ➤ 10 ➤ 11 ➤ 7 ➤ 8 ➤ 4 followed the order described in **Fig. 4.** This schematic reveals that there are two homologous series of HDHEs: one in which the hydroxyl group is on the methyl side of the conjugated diene (the "M" series, underlined in the above sequence) and another in which the hydroxyl group is located on the carboxyl side of the conjugated diene ("C" series, shown above not underlined). For either series, the further the hydroxyl group is from the carboxyl moiety, the more rapidly the compound eluted from the column, presumably because there is less terminal aliphatic chain available for intercalation between the C-18 chains of the

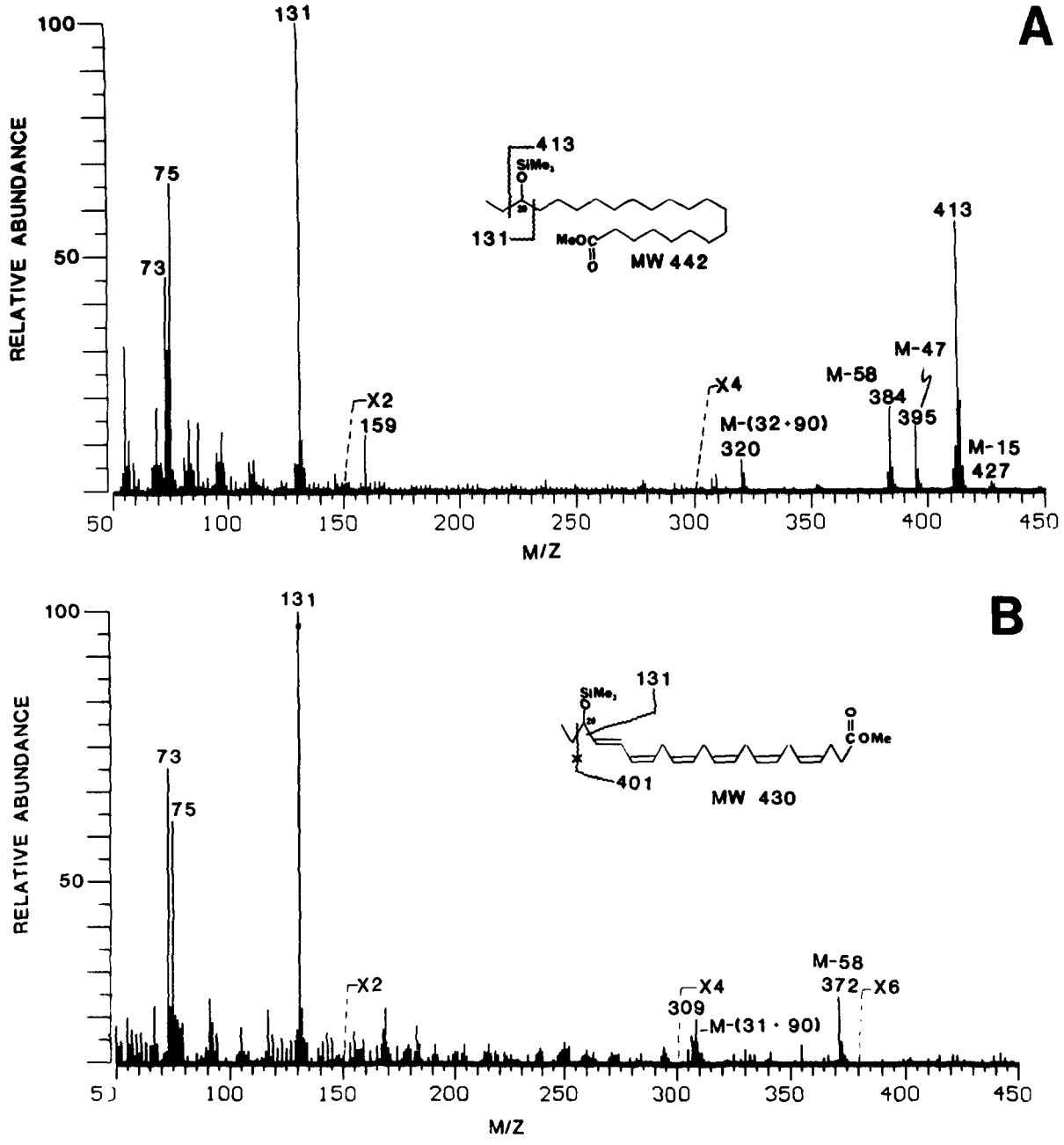

Fig. 3. Electron impact (70 eV) mass spectra of hydrogenated Compound I (A) and non-hydrogenated Compound I (B) obtained with trimethylsilyl ether, methyl ester derivatives.

stationary phase. Moreover, for each pair of HDHEs arising from a common hydrogen abstraction step (Fig. 4), the M type (e.g., 20-HDHE) eluted before the C type (e.g., 16-HDHE), presumably because the M type has its hydroxyl group located further from the carboxyl moiety than the C type. However, carbon position of the hydroxyl moiety is not the only determining factor since the observed elution sequence is different from the expected order: 20 ➤ 17 ➤ 16 ➤ 14 ➤ 13 ➤ 11 ➤ 10 ➤ 8 ➤ 7 ➤ 4. Apparently the *trans* double bond which is located alpha to the carbinol group also affects the degree to which the hydroxy moiety can inhibit alkyl interactions. The importance of this *trans* double bond is evident by the striking parallelism between observed elution order and position of the *trans* double bond, labeled in parentheses: 20(18) ➤ 16(17) ➤ 17(15) ➤ 13(14) ➤ 14(12) ➤ 10(11) ➤ 11(9) ➤ 7(8) ➤ 8(6) ➤ 4(5). The importance of the *trans* double bond position also applies to

TABLE 2. Characteristic fragments (amu) in mass spectra of hydrogenated Compounds I–X[a]

Compound Number[b]	Alpha Cleavage to $OSi(CH_3)_3$: on Methyl Side	Alpha Cleavage to $OSi(CH_3)_3$: on Carboxyl Side	T Migration Ion[c]	159[d]	M-(90 + 32) 320	M-(15 + 32) 395	M-31 411	M-15 427
I	413 (14.1)	131 (100)	384 (4.3)	(5.6)	(2.9)	(3.3)		(0.4)
II	357 (35.0)	187 (87.7)	328 (7.5)	(7.5)	(3.6)	(4.3)	(1.2)	(0.5)
III	371 (23.2)	173 (100)	342 (5.2)	(7.0)	(2.2)	(2.8)	(1.4)	(0.4)
IV	315 (73.4)	229 (91.7)	286 (11.6)	(8.3)	(4.2)	(5.3)	(1.5)	(1.3)
V	329 (51.4)	215 (100)	300 (6.6)	(8.6)	(4.0)	(5.2)	(1.4)	(0.4)
VI	273 (87.3)	271 (68.6)	244 (10.3)	(5.4)	(2.1)	(3.0)	(0.9)	(1.4)
VII	287 (83.3)	257 (80.0)	258 (12.3)	(10.5)	(2.8)	(4.7)	(1.5)	(0.8)
VIII	231 (100)	313 (48.0)	202 (12.6)	(7.6)	(2.4)	(2.4)	(2.6)	(2.4)
IX	245 (100)	299 (52.6)	216 (14.6)	(9.4)	(1.6)	(2.6)	(1.4)	(1.6)
X	85 (100)				(5.0)[e]			

[a] Electron impact (70 eV) spectra were determined on methyl ester, trimethylsilyl ether derivatives.
[b] Compounds eluted as described in Fig. 1.
[c] "T" means that the formation of this rearrangement ion involved transfer of a $Si(CH_3)_3$ group to the carbomethoxy moiety.
[d] m/z 159 results from gamma cleavage of the T ion (45).
[e] m/z 320 (M-18).

the series of conjugated dienes with allylic hydroxyl groups derived from arachidonic acid (Fig. 1B; 2, 15): 15(13) ➤ 11(12) ➤ 12 + 8(10 + 9) ➤ 9(7) ➤ 5(6).

The large series of HDHEs also gave insight into normal phase HPLC elution orders for methyl esters of hydroxy fatty acids with conjugated dienes. The HDHE elution sequence was: 17 ➤ 14 ➤ 16 ➤ 13 ➤ 11 ➤ 20 ➤ 10 ➤ 7 ➤ 4 ➤ 8. For all HDHEs of the C series (not underlined) the closer the hydroxyl group was to the carbomethoxy moiety, the greater the retention time. This pattern was also generally applicable to the HDHEs of the M series (underlined) and for hydroxy isomers with conjugated dienes derived from linoleic acid (33, 48): 13 ➤ 9; from alpha (n-3) linolenic acid (34): 13 ➤ 12 ➤ 16 ➤ 9; from gamma (n-6) linolenic acid (49): 13 ➤ 9; and for HETEs (Fig. 2B; 32, 42): 12 ➤ 15 ➤ 11 ➤ 9 ➤ 8 ➤ 5. This general elution pattern suggested that if the hydroxyl group is located closer to the carbomethoxy group, chances are better that additional hydrogen bonding will arise from interactions between the hydroxyl of the eluate and silanols of the stationary phase. The three anomalies in this general elution pattern: 20-HDHE, 15-HETE, and 16-HODT all belonged to the M series (underlined above) and contained the most remote hydroxyl groups in their respective series. Upon examination of space filling molecular models, it became apparent that

TABLE 3. Characteristic fragments (amu) in mass spectra of Compounds I–X[a]

Compound Number	Alpha Cleavage to $OSi(CH_3)_3$: on Methyl Side	Alpha Cleavage to $OSi(CH_3)_3$: on Carboxyl Side	T Migration Ion[b]	M-(90 + 31) 309	(309-H_2) 307	Other High Mass Ions
I		131 (100.0)[c]	372 (3.5)	(2.3)	(1.4)	355 (1.0)
II	349 (1.0)	183 (73.7)		(5.2)	(3.4)	415 (0.6), 340 (1.0)
III	361 (2.0)	171 (56.9)	332 (6.1)	(12.1)	(8.0)	
IV	309 (6.0)[d]	223 (13.7)		(6.0)[d]	(2.7)	
V	321 (4.5)	211 (9.9)	292 (4.0)	(2.8)	(1.3)	
VI	269 (12.9)	263 (4.7)		(4.7)	(2.7)	
VII	281 (13.9)	251 (4.5)[e]	252 (15.9)	(4.6)	(3.0)	
VIII	229 (53.2)	303 (2.4)		(5.5)	(3.4)	415 (1.4)
IX	241 (46.9)		181 (3.6)[f]	(3.1)	(1.5)	
X	189 (71.8)			(25.9)	(16.4)	

[a] Electron impact (70 eV) mass spectra were obtained on methyl ester, trimethylsilyl ether derivatives. The elution order for compounds is described in Fig. 1.
[b] A "T" rearrangement ion results from transfer of a $Si(CH_3)_3$ group to the carbomethoxy moiety (44–46).
[c] Values in parentheses represent relative intensities. Usually, m/z 75 was the base peak (100% relative intensity) except for Compounds VII and X, which had 73 as base peak, and Compound I which had 131 as base peak.
[d] In this case, m/z 309 had two likely origins: alpha cleavage to the $OSi(CH_3)_3$ group and loss of $HOSi(CH_3)_3$ plus CH_3O.
[e] The contribution of m/z 252 to 251 via loss of hydrogen is unknown.
[f] The m/z 181 probably represented M-(218 + 31).

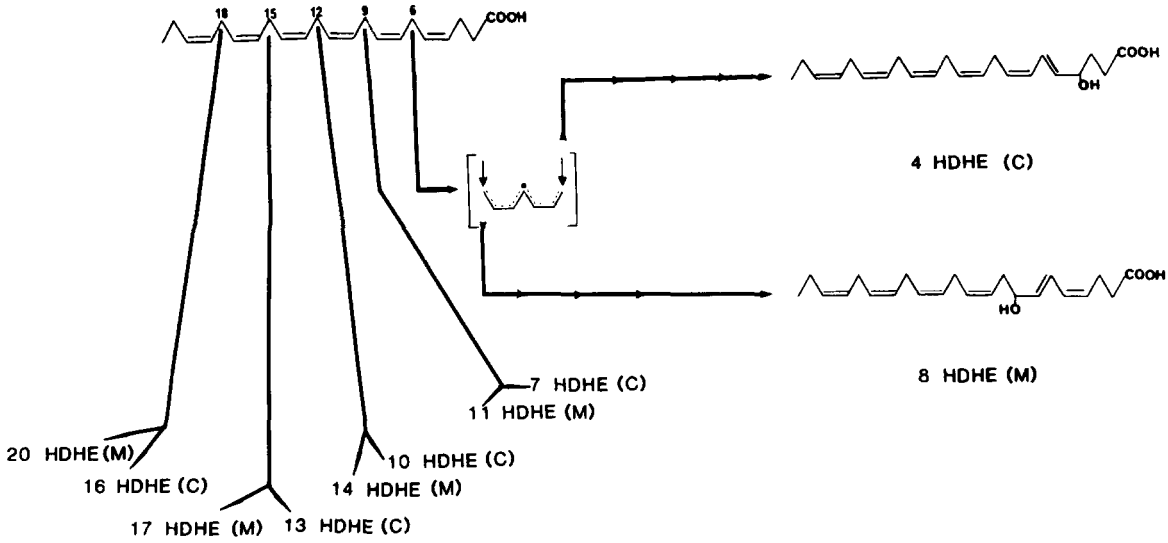

Fig. 4. Summary of hydroxydocosahexaenoic (HDHE) isomers produced by autooxidation. Abstraction of bis-allylic hydrogen atoms at five carbons (numbered) resulted in the production of five pentadiene stabilized radicals, one of which is shown on the right side of the figure. Insertion of molecular oxygen into the methyl side ("M" reaction) or into the carboxy side ("C" reaction) resulted in the formation of M or C series of hydroperoxides. These hydroperoxides were then chemically reduced to the corresponding HDHEs. Thus hydrogen abstraction at five carbons resulted in the formation of five M and five C type hydroxyl isomers.

the M type conjugated dienes, when located far enough from the carbomethoxy group, would not prevent hydroxyl interactions with the carbomethoxy group. Any formation of internal hydrogen bonds should inhibit the fatty acid from straightening out its alkyl chain and thus keep the hydroxyl group more available for hydrogen bonding with silanols. That the three anomalous eluants can form internal hydrogen bonds is supported by the reported elution sequence for hydroxy isomers of stearic acid (50): 13 ➤ 12 ➤ 15 ➤ 16 ➤ 10 ➤ 9 ➤ 8.

The ten isomers derived from autooxidation of docosahexaenoic acid should prove useful as standards for identifying and quantitating biologically produced HDHEs. For instance, they may aid in identifying hydroxyl positions and double bond configurations by establishing GLC and reverse and normal phase HPLC retention times.

It should be noted that autooxidation results in the production of racemic mixtures of HDHEs. If labeled from commercially available 22:6, these mixtures could be used to study the biochemical stereospecificity of HDHE metabolism, e.g., during use of HDHEs as substrates for ω-oxidation,[3] for lipoxygenases (51, 52) or for phospholipid acyl transferases (53, 54). Moreover, racemic mixtures of HDHEs may also be useful in studies of the effects of HDHEs upon lipoxygenases (31, 55), cyclooxygenases (31, 56), as well as on biological properties such as chemotaxis of chemokinesis.

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