Journal of Chromatography, 305 (1984) 3–12
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1901

PREPARATION OF PENTAFLUOROBENZYL ESTERS OF ARACHIDONIC ACID LIPOXYGENASE METABOLITES

ANALYSIS BY GAS CHROMATOGRAPHY AND NEGATIVE-ION CHEMICAL IONIZATION MASS SPECTROMETRY*

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(First received May 16th, 1983; revised manuscript received August 16th, 1983)

SUMMARY

5-Hydroxyeicosatetraenoic acid (5-HETE) and leukotriene B_4 have recently been shown to possess potent chemotactic and chemokinetic properties. Because of the very low concentrations found in certain biological systems, negative-ion chemical ionization mass spectrometry has been investigated as a potential assay method for detecting these compounds. A facile derivatization to form the pentafluorobenzyl esters, and clean up are reported for these compounds at the 15-ng level. Gas chromatographic properties, negative-ion chemical ionization mass spectra, and positive-ion electron impact spectra are reported for the pentafluorobenzyl ester, trimethylsilyl ether derivatives of 5-HETE and leukotriene B_4 isomers.

INTRODUCTION

The metabolism of arachidonic acid by lipoxygenase pathways has been appreciated for several years [1]; however, the description of the 5-lipoxygenase pathway, which in certain cells leads to the leukotrienes, has provided further insight into the complex biochemical events mediated by arachidonic acid metabolites [2]. The recent description of the potent chemotactic and chemokinetic properties of 5-hydroxyeicosatetraenoic acid (5-HETE) [3] and leukotriene B_4 (LTB_4) towards the polymorphonuclear leukocyte [4] has resulted in considerable interest in detecting and measuring

*Presented in part at the 31st annual meeting of the American Society for Mass Spectrometry and Allied Topics, Boston, MA, 1983.

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these compounds in biological samples. While bioassays for these chemotactic properties are reasonably sensitive, they lack specificity, quantitative accuracy, and are somewhat time-consuming. Recently, techniques based on high-performance liquid chromatographic (HPLC) methods using UV detection of the conjugated double bonds in these lipoxygenase metabolites have been described, which are sensitive from 1 to 10 ng injected on the HPLC column [5, 6]. A specific assay by gas chromatography—mass spectrometry (GC—MS) using a deuterated internal standard has been described for 5-HETE [7] as the methyl ester, trimethylsilyl ether derivative. Larrue et al. [8] have also reported a specific assay for monohydroxyeicosanoids using 12-hydroxystearic acid as internal standard and the methyl ester, trimethylsilyl derivatives. We report herein a facile procedure for the derivatization of monohydroxyeicosanoids and LTB_4 isomers into the pentafluorobenzyl (PFB) ester, trimethylsilyl ester species. These derivatives have excellent properties in negative-ion chemical ionization (CI) MS which should permit detection of these eicosanoids from biological extracts by selected-ion recording techniques.

MATERIALS AND METHODS

Instrumentation

The MS was performed on a VG Micromass 7070H mass spectrometer (Altrincham, U.K.) interfaced to a gas chromatograph (Hewlett-Packard Model 7625A, Palo Alto, CA, U.S.A.) modified for capillary column operation with a glass falling needle injector [9] (R.H. Allen, Boulder, CO, U.S.A.). Chromatography was carried out on a 5-μm DB-1 capillary column (J & W Scientific, Rancho Cordova, CA, U.S.A.) threaded directly into the ion source of the mass spectrometer. The ion source conditions were 4 kV accelerating potential, electron energy 70 eV (electron impact, EI) or 60 eV (negative-ion CI) with methane gas pressure adjusted to give maximum signal for the PFB esters. An ion source exit slit of 0.0372 mm was constructed for negative-ion CI operation. HPLC was carried out with a 5-μm Nucleosil C_{18} column (250 mm $\times$ 4.6 mm) using methanol—water—acetic acid (67:33:0.02), adjusted to pH 5.7 with concentrated ammonium hydroxide. Silica gel thin-layer plates (LK-6D, Whatman, Clifton Park, NJ, U.S.A.) were scanned for radioactivity using a Nuclear Chicago Actigraph III (Des Plains, IL, U.S.A.). Solvents were distilled in glass or HPLC grade (Fisher, Springfield, NJ, U.S.A.) and all reagents used were of the highest quality commercially available.

Glassware was routinely cleaned in chromic acid, rinsed thoroughly with doubly distilled water, and where indicated, silylated for 20 min at room temperature with 10% dimethyldichlorosilane in toluene, followed by a methanol wash.

Eicosanoids

12-Hydroxyeicosatetraenoic acid (12-HETE) was prepared from platelets by the method of Sun [10]. Racemic leukotriene A_4 (LTA_4) methyl ester was a kind gift from Dr. J. Rokach (Merck-Frosst, Montreal, Canada); [^{3}H]LTA_4 and [^{3}H]12-HETE were obtained from New England Nuclear (Boston, MA, U.S.A.). The Δ^6-*trans*-LTB_4 isomers (5,12-di-HETEs) were synthesized by

hydrolysis of LTA_4 methyl ester (350 μg) for 5 min at room temperature in 1 ml of tetrahydrofuran–0.01 *M* hydrochloric acid (1:1). Conversion to the free acid was accomplished by alkaline hydrolysis (tetrahydrofuran—0.2 *M* lithium hydroxide, 1:1) for 1 h at room temperature. The yield was 91% based on UV absorbance. The synthesis of labeled Δ^6-*trans*-LTB_4 was essentially the same, but started with 300 ng of LTA_4 methyl ester and 5 μCi of $[^3H]LTA_4$ methyl ester. This synthesis was carried out in silylated glassware. The synthetic products were purified by reversed-phase HPLC [5] and the diastereoisomeric pairs were collected separately.

Derivatization

Straight-chain fatty acids (C_{19}—C_{23}) were converted to their PFB esters essentially by the method of Min et al. [11], modified by Blair et al. [12] with 30 μl of acetonitrile, 10 μl of 35% PFB bromide in acetonitrile and diisopropylethylamine (10 μl). Most excess reagent was removed by evaporation under a stream of nitrogen. Any underivatized free fatty acids were methylated by diazomethane treatment for 10 min in diethyl ether—methylene chloride. After evaporation the remaining residue was dissolved in 100 μl of methylene chloride, and applied to a short column of silicic acid (0.5 g Silicar CC-4, Mallinckrodt, St. Louis, MO, U.S.A.) in a disposable pipet. The saturated fatty acid derivatives were quantitatively eluted with 2 ml of methylene chloride. The solvents were rapidly concentrated at 40°C by a stream of nitrogen. Reaction times and temperature using the derivatizing reagents were altered as described below.

The eicosanoids were reacted with 35% PFB bromide in acetonitrile (10 μl), diisopropylethylamine (10 μl), and 30 μl of acetonitrile for 10 min at ambient temperature (23—25°C). Clean up of the PFB ester was carried out using column chromatography (Silicar CC-4) after dissolving the dried residue with 100 μl of 50% ethyl acetate in methylene chloride. The monohydroxy PFB esters were eluted with 2-ml volumes of various solvents as described in Table I.

TABLE I

ELUTION OF TRITIUM-LABELED 12-HETE (15 ng) FROM SILICIC ACID AFTER REACTION WITH PFB BROMIDE AT ROOM TEMPERATURE

Percent ethyl acetate*	Percent total radioactivity eluted from silicic acid**,*** (cpm)	
	Free acid	Reaction
0	3.1	69.8
5	1.2	17.9
10	5.4	3.8
15	29.6	2.4
25	40.1	2.6
50	15.8	2.4
100	4.8	1.1

*In methylene chloride, 2 ml per fraction.
**Sample was dissolved in ethyl acetate—methylene chloride (1:1), 100 μl and added to the short silicic acid column.
***Total recovery based on counting of standard aliquots = 90%.

The extent of derivatization of [^{3}H]12-HETE (15 ng carrier) was further evaluated by thin-layer chromatographic (TLC) separation of the combined methylene chloride and 5% ethyl acetate eluates (Table I) using hexane—ethyl acetate—acetic acid (1:1:0.05) as developing solvent. The Δ^6-*trans*-LTB_4 esters were isolated by further step elution of the Silicar CC-4 column with 2 ml each of 10%, 20% and 30% ethyl acetate in methylene chloride. Trimethylsilylation was carried out by reaction of the dried eluates with 50 μl of bis(trimethylsilyl)trifluoroacetamide—acetonitrile (1:1) at 60°C for 15 min.

RESULTS

After derivatization of the mixture of C_{19}—C_{23} straight-chain fatty acids with PFB bromide at elevated temperatures (40°C) by the method of Blair et al. [12], a brown, viscous residue was always obtained. When the fatty acid mixture was derivatized with PFB bromide in acetonitrile at room temperature for 10 min, no such residue was obtained. The extent of the derivatization under these mild conditions was evaluated by subsequent methylation of any remaining free fatty acids before GC analysis. As seen in Fig. 1, the room temperature derivatization of the fatty acids was approximately 90% complete after 2 min. In data not shown, room temperature derivatization for 10 min resulted in greater than 95% conversion of the PFB esters.

The room temperature derivatization condition and use of diisopropylethylamine was evaluated for PFB ester formation of hydroxyeicosanoids.

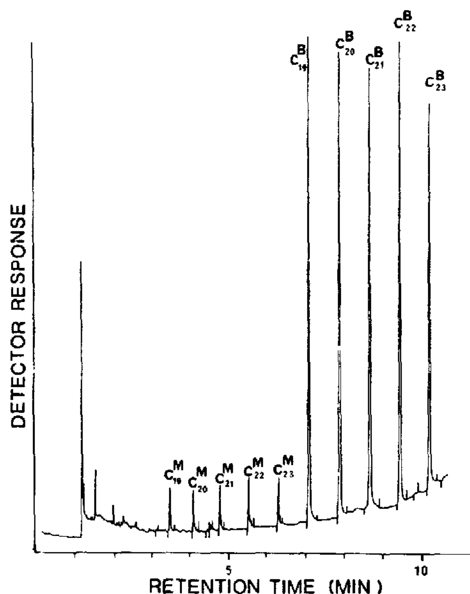

Fig. 1. Capillary gas chromatogram of methyl (M) and pentafluorobenzyl (B) esters, after reaction of straight-chain free fatty acids (5 μg) with PFB bromide, 2 min at room temperature, followed by diazomethane treatment. Column 30 m, DB-1, flame ionization detector, temperature program 220°C up at 8°C/min. Carrier gas helium at approximately 1.5 ml/min at 200°C (0.5% of sample on column).

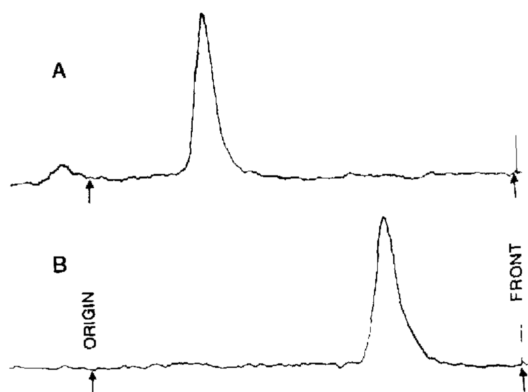

Fig. 2. Radiochromatograms of the TLC separation of standard aliquots of (A) 12-HETE, (B) 12-HETE after reaction with PFB bromide for 10 min and CC-4 mini-column clean up. Standard aliquots from a stock solution contain 15 ng 12-HETE with 0.1 μCi [^{3}H]12-HETE. The developing solvent was hexane—ethyl acetate—acetic acid (1:1:0.005).

Since [^{3}H]12-HETE was readily available, it was used as a model compound to evaluate PFB ester formation, and elution properties of HETE-PFB esters on CC-4. Likewise, [^{3}H]Δ^6-*trans*-LTB_4 isomers were readily synthesized from commercially available [^{3}H]LTA_4 methyl ester and served as a model for LTB_4. The extent of reaction was followed by column chromatography and TLC. Fig. 2 shows the TLC analysis of [^{3}H]12-HETE (15 ng) and that after treatment with PFB bromide at room temperature for 10 min and CC-4 clean up. Approximately 90% of the 12-HETE was recovered as a single, less polar compound (Fig. 2B) which was identified by MS following trimethylsilylation as the TMS ether, PFB ester of 12-HETE. The non-reactivity of the hydroxyls towards PFB bromide under these conditions was further verified by the failure of tricosanol to undergo any reaction with PFB bromide (data not shown).

Derivatization of the Δ^6-*trans*-LTB_4 isomers with PFB bromide at room temperature for 10 min was also studied. Analysis of reaction products using [^{3}H]Δ^6-*trans*-LTB_4 showed that at the 15-ng level these dihydroxyeicosanoic acids were consistently esterified and recovered 76—85% based upon elution of

TABLE II

GC RETENTION CHARACTERISTICS OF PFB ESTER, TMS ETHER EICOSANOIDS AND METHYL ESTER, TMS ETHER EICOSANOIDS

	PFB*	Me
5-HETE	21.0	21.3
12-HETE	21.0	21.3
Δ^6-*trans*-LTB_4	24.7	24.8
LTB_4		23.6
5,6-di-HETE	24.2	23.9

*Equivalent chain length of corresponding ester derivative, Me = methyl ester, PFB = pentafluorobenzyl ester. Determined by capillary GC (5-m DB-1 column).

radioactivity from the CC-4 column. When microgram amounts were derivatized, quantitation by UV spectroscopy (λ_{max} 270 nm, ϵ 50,000) gave identical results for derivatization and recovery. The GC retention characteristics of these compounds are summarized in Table II.

Mass spectrometry

Fig. 3 is the positive-ion EI spectrum of the PFB derivative of tricosanoic acid. A molecular ion of low abundance was typically seen for all these straight-chain esters (Fig. 3, *m/z* 534). The most abundant ion in all cases under EI conditions was *m/z* 181 corresponding to a PFB cation, possibly expanded to a tropylium structure. All straight-chain fatty acids studied had two ions at high mass due to the loss of $C_7H_2F_5$ and a further loss of water from the carboxyl cation. This was confirmed by high-resolution MS of PFB-tricosanoic acid (Fig. 3). A series of hydrocarbon ions (*m/z* 43, 57, 71, 85 and 97) constituted the major ion current under EI conditions.

The positive-ion EI mass spectra of the TMS ether, PFB ester of 5-HETE and Δ^6-*trans*-LTB_4 are summarized in Table III. The most abundant ions observed from these molecules (*m/z* 73 and 181) corresponded to the moieties introduced by derivatization, rather than being structurally characteristic for

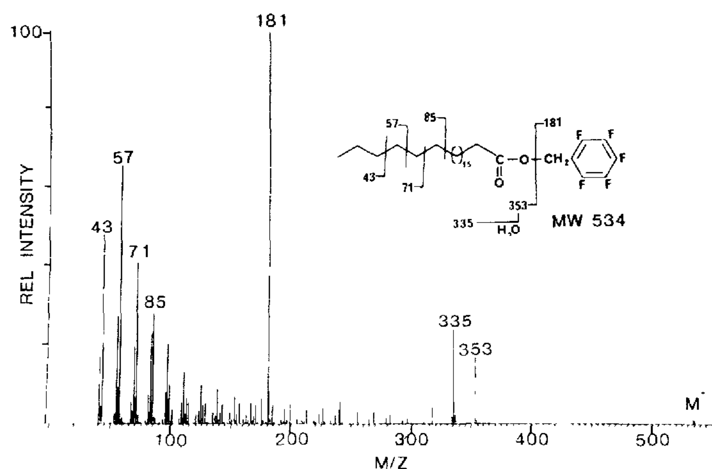

Fig. 3. Positive-ion EI (70 eV) mass spectrum of PFB tricosanoate.

TABLE III

SIGNIFICANT IONS IN THE POSITIVE ION EI MASS SPECTRA (70 eV) OF 5-HETE AND Δ^6-*TRANS*-LTB_4 AS THE TMS ETHER PFB ESTER

5-HETE	572(1), 482(1), 421(7), 369(3), 305(8), 215(4), 190(8), 181(42), 150(20), 129(16), 117(13), 105(17), 91(20), 81(32), 79(34), 75(50), 73(100)
Δ^6-*Trans*-LTB_4	645(1), 549(13), 459(12), 433(3), 407(2), 395(7), 369(12), 283(4), 217(21), 191(16), 181(41), 171(5), 167(5), 129(65), 103(7), 91(5), 75(35), 73(100)

the eicosanoid. However the 5-HETE-PFB, TMS did yield molecular ions and ions from the loss of trimethylsilanol (M—90) in low abundance. Information concerning the position of the hydroxyl substitution on the arachidonic acid backbone is provided by two ions resulting from α-cleavage to the trimethylsilyl ether moiety. In 5-HETE these ions are seen at *m/z* 305 and 369; in Δ^6-*trans*-LTB_4 similar fragmentations are observed at *m/z* 549 and 369. The origins of the abundant ions at *m/z* 129 and 217 have been previously described [13].

Negative-ion CI mass spectra of each of the PFB esters in this study yielded abundant ions due to the loss of the PFB radical to give a stabilized carboxylate anion. Fig. 4 shows the negative-ion CI mass spectrum of the derivatized tricosanoic acid. This loss is characteristic for all molecules studied thus far [11, 12, 14]. The TMS ether, PFB ester derivative of 5-HETE (Fig. 5) and 12-HETE (data not shown) also yielded an abundant ion for further loss of the elements of trimethylsilanol (90 daltons) to produce *m/z* 301. The negative-ion CI mass spectra of 5- and 12-monohydroxyeicosanoids were

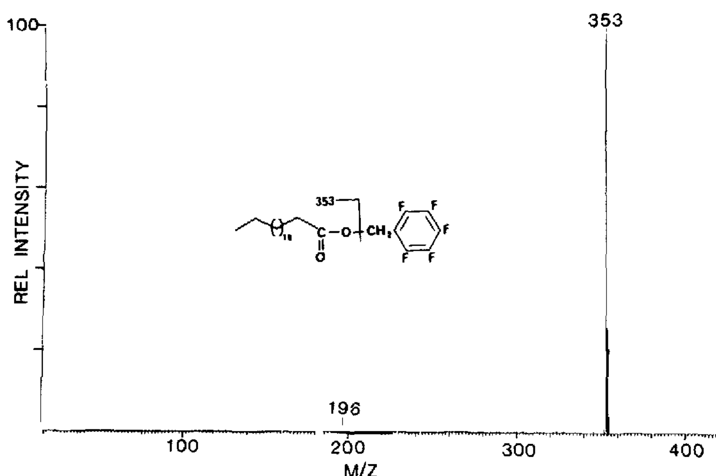

Fig. 4. Negative-ion CI (CH_4) mass spectrum of PFB tricosanoate.

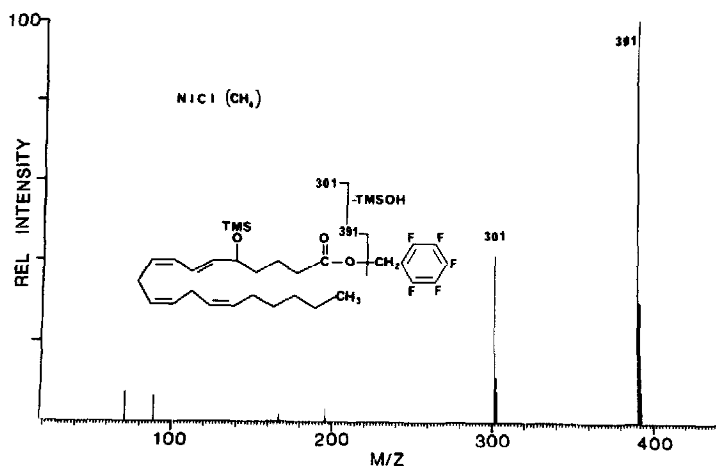

Fig. 5. Negative-ion CI (CH_4) mass spectrum of 5-HETE, PFB ester, TMS ether.

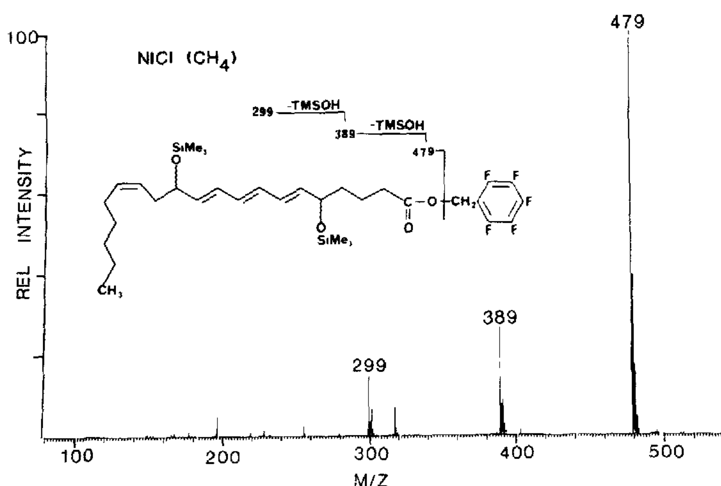

Fig. 6. Negative-ion CI (CH_4) mass spectrum of Δ^6-*trans*-LTB_4, PFB ester, di-TMS ether.

essentially indistinguishable from each other and these compounds had identical GC retention times.

The derivatized Δ^6-*trans*-LTB_4 fragmented under CI conditions to yield the negative-ion mass spectrum in Fig. 6. The most abundant ion corresponded to loss of the PFB radical moiety and formation of the carboxylate anion (*m/z* 479). Two sequential losses of trimethylsilanol yielded the next most abundant ions (*m/z* 389 and 299). In all molecules studied the ion currents carried by the carboxylate anion (M—181) were quite high being 75%, 43%, and 29% of the total ionization for the fatty acids, mono- and dihydroxyeicosanoids, respectively. Negative-ion production under CI conditions was approximately 20% greater than positive-ion production optimized for electron impact. Comparing the abundance of structurally significant ions at high mass (e.g. *m/z* 369 from Table III and *m/z* 391 from Fig. 5 for derivatized 5-HETE) and their percentages of total ionization suggests that a 130-fold increase in sensitivity can be achieved by utilizing negative-ion CI of these derivatives.

DISCUSSION

The previous procedures for the derivatization of carboxylic acids with PFB bromide have employed relatively high temperatures and long reaction times [12, 14]. As a consequence, substantial residues (often yellow-brown) from the reagents themselves are encountered which to some extent limit the total quantity which can be injected onto the gas chromatograph. Many different conditions have been used to effect this esterification [12, 14, 15—21]. Kawahara [15, 16] was perhaps the first to use this derivative for analytical purposes to enhance the measurement of mercaptans, phenols and acids by GC with electron-capture detection (ECD). However, it was not until 1973 that Wickramasinghe and co-workers [17, 18] reported useful conditions for utilizing this derivative to analyze nanogram amounts of prostaglandins. In a systematic fashion, optimal conditions were evolved for prostaglandin $F2_{\alpha}$ to maximize signal while minimizing background in GC—ECD analysis. Waddell

et al. [14] have recently reported reaction times as long as 30 min at 40°C and at large excess of catalytic amine. The esterification yield obtained under the various conditions at the nanogram level has not been clearly established in any of these reports. In general, 15% at most of the final derivatized sample [2, 4] and as little as 0.1% [3], is actually introduced into the analytical system. In cases where a substantial amount of analyte is isolated, total sample introduction is not important. However, when the amount of isolable material is small, as with leukotrienes which are present in physiological solutions, it becomes important to introduce as much of the biological extract as possible, when the signal measured is not background limited.

We do not feel that the less than quantitative recovery of these derivatives is due to loss of radioactive impurities. Labelled starting materials were analyzed by HPLC and TLC. The average recoveries of 80% and greater for the various derivatized products were quite suitable in light of the small quantities being handled. LTB_4 and its isomers are also susceptible to oxidative destruction, some of the loss may result from this.

The EI mass spectra of the PFB ester, TMS ethers of the eicosanoids are typified by abundant ion currents corresponding to the TMS (*m/z* 73) and PFB (*m/z* 181) moieties. Much less abundant but structurally significant ions were present at high mass, and the position of the hydroxyl group was suggested by ions due to α-cleavage of the TMS ether moiety.

Negative-ion CI mass spectra of the TMS ether, PFB ester derivatives of eicosanoids are typified by the large ion currents carried mainly by the resonance stabilized carboxylate anion [11, 12, 14]. The abundance of this ion was striking. Under negative-ion CI with other perfluoronated derivatives such as those containing perfluoroalkyl moieties, the majority of the ion current is carried by small fragments from the derivatizing moiety. One possible explanation of this phenomenon is that the PFB anion is not as stable as the carboxylate anion. The stability of the benzyl cation in positive-ion MS has been widely appreciated. Indeed there have been numerous studies of the gas phase structure of this species and its rearrangement to the more stable tropylium cation. In contrast, the number of π electrons in a tropylium anion would not fit the $(4n + 2)$ π electron rule and it would also be anti-aromatic [22]. Benzyl anions are not stabilized by such structures. In part this may provide a rationale for the absence of such a species in these mass spectra. Nevertheless, the structurally significant carboxylate anions are produced in high yield with little subsequent fragmentation, which should enable sensitive detection and quantitation of mono- and dihydroxyeicosanoids using stable isotope dilution and GC—MS techniques.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the National Institutes of Health (HL 25785).

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