

Oxidized derivatives of ω -3 fatty acids: identification of IPF_{3 α} -VI in human urine¹

John A. Lawson,* Seongjin Kim,[†] William S. Powell,[§] Garret A. FitzGerald,* and Joshua Rokach^{2,†}

Institute for Translational Medicine and Therapeutics and Department of Pharmacology,* The University of Pennsylvania, Philadelphia, PA 19104; Claude Pepper Institute and Department of Chemistry,[†] Florida Institute of Technology, Melbourne, FL 32901; and Meakins-Christie Laboratories, Department of Medicine,[§] McGill University, Montreal, Quebec, Canada H2X 2P2

Abstract Isoprostanes (iPs) are prostaglandin-like molecules derived from autoxidation of polyunsaturated fatty acids (PUFAs). Urinary iP levels have been used as indices of in vivo lipid peroxidation. Thus far, it has only been possible to measure iPs derived from arachidonic acid in urine, because levels of iPs/neuroprostanes (nPs) derived from ω -3-PUFAs have been found to be below detection limits of available assays. Because of the interest in ω -3-PUFA dietary supplementation, we developed specific methods to measure nPF_{4 α} -VI and iPF_{3 α} -VI [derived from 4,7,10,13,16,19-docosahexaenoic acid (DHA) and 5,8,11,14,17-eicosapentaenoic acid (EPA)] using a combination of chemical synthesis, gas chromatography/mass spectrometry (GC/MS), and liquid chromatography tandem mass spectrometry (LC/MS/MS). Although nPF_{4 α} -VI was below the detection limit of the assay, we conclusively identified iPF_{3 α} -VI in human urine by GC/MS and LC/MS/MS. The mean levels in 26 subjects were \sim 300 pg/mg creatinine. Our failure to detect nPF_{4 α} -VI may have been due to its rapid metabolism by β -oxidation to iPF_{3 α} -VI, which we showed to occur in rat liver homogenates. In contrast, iPF_{3 α} -VI is highly resistant to β -oxidation in vitro. Thus iPF_{3 α} -VI can be formed by two mechanisms: *i*) direct autoxidation of EPA, and *ii*) β -oxidation of nPF_{4 α} -VI, formed by autoxidation of DHA. This iP may therefore serve as an excellent marker for the combined in vivo peroxidation of EPA and DHA.—Lawson, J. A., S. Kim, W. S. Powell, G. A. FitzGerald, and J. Rokach. Oxidized derivatives of ω -3 fatty acids: identification of IPF_{3 α} -VI in human urine. *J. Lipid Res.* 2006. 47: 2515–2524.

Supplementary key words isoprostanes • arachidonic acid • eicosapentaenoic acid • docosahexaenoic acid • neuroprostaglandin F_{4 α} -VI • mass spectrometry • lipid peroxidation • ω -3-polyunsaturated fatty acids

Isoprostanes (iPs) are a class of natural products generated by the action of free radicals on polyunsaturated fatty acids (PUFAs) (1, 2). We have previously reported on the total synthesis of iPs and metabolites derived from free radical peroxidation of arachidonic acid (AA) (3–7).

These synthetic markers have been used to discover and characterize four classes of iPs in biological fluids (2, 8, 9). The availability of these standards permitted the development of gas chromatography/mass spectrometry (GC/MS) and liquid chromatography tandem mass spectrometry (LC/MS/MS) methodologies to examine the distribution of iPs within each of these classes (10, 11). These methods have been used to measure iPs, in particular Groups III and VI, in biological fluids, and have revealed a correlation between their levels and the severity of diseases such as atherosclerosis (12) and Alzheimer's disease (AD) (13).

Although iPs can be formed from many PUFAs, research to date has focused almost exclusively on F₂-iPs derived from AA. 5,8,11,14,17-Eicosapentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA) are two other naturally occurring PUFAs that constitute an important and sizeable component of cellular phospholipids, especially in the case of DHA, which is found in high levels in the brain (14). Because of the increased absorption of EPA and DHA by individuals whose diets include substantial amounts of fatty fish, iPs and neuroprostanes (nPs) derived from these ω -3-PUFAs may be more abundant than those derived from ω -6-PUFAs in these subjects. Moreover, because of their health benefits, ω -3-PUFAs are increasingly being used as dietary supplements and have been the subject of various clinical trials (15). For these reasons, it has become very important to be able to assess the extent of peroxidation of these highly unsaturated fatty acids.

Abbreviations: AA, arachidonic acid; AD, Alzheimer's disease; BDS, Base Deactivated Silica; DHA, 4,7,10,13,16,19-docosahexaenoic acid; ECNI, electron capture/negative ion; EPA, 5,8,11,14,17-eicosapentaenoic acid; iP, isoprostane; i.s., internal standard; LC, liquid chromatography; MS/MS, tandem mass spectrometry; nP, neuroprostaglandin; PFB, pentafluorobenzyl; SPE, solid-phase extraction; TMS, trimethylsilyl.

¹The iP nomenclature used throughout this manuscript was previously reported (Rokach, J., S. P. Khanapure, S. W. Hwang, M. Adiyaman, J. A. Lawson, and G. A. FitzGerald. 1997. *Prostaglandins*. 54: 853–873). Another nomenclature is also in use (Taber, D. F., J. D. Morrow, and L. J. Roberts II. 1997. *Prostaglandins*. 53: 63–67).

²To whom correspondence should be addressed.

e-mail: jrokach@fit.edu

Manuscript received 21 July 2006 and in revised form 25 August 2006.

Published, JLR Papers in Press, August 30, 2006.

DOI 10.1194/jlr.M600327-JLR200

Copyright © 2006 by the American Society for Biochemistry and Molecular Biology, Inc.

This article is available online at <http://www.jlr.org>

Journal of Lipid Research Volume 47, 2006 2515

However, attempts to measure ω 3-derived iPs/nPs have so far met with only limited success, especially with noninvasively collected samples such as urine. Although F_3 -iPs are formed following chemical peroxidation of EPA (16), a recently published study failed to identify these substances in tissues from rodents and humans on chow and typical North American diets, respectively (17). In rodents, it was only possible to detect tissue F_3 -iPs following dietary supplementation with EPA (17). F_4 -nPs are formed by chemical oxidation of DHA (18, 19) and are found in human and rodent brain tissue (19). However, it has thus far not been possible to detect these substances in urine from humans, including subjects with AD (20).

Further investigation into the biological occurrence of ω 3-iPs and nPs and the identification of selective markers for these substances is therefore of great importance. We have previously shown that $iPF_{2\alpha}$ -VI is a more abundant marker of endogenous AA peroxidation than the more commonly measured $iPF_{2\alpha}$ -III (8-*iso*-PGF $_{2\alpha}$) (Fig. 1) (2, 5). The objective of the current study was to determine whether the analogous EPA- and DHA-derived oxidation products, $iPF_{3\alpha}$ -VI and $nPF_{4\alpha}$ -VI, could serve as markers for peroxidation of ω 3-PUFAs. We succeeded in detecting for the first time high levels of $iPF_{3\alpha}$ -VI in human urine, but were unable to detect $nPF_{4\alpha}$ -VI. This is probably due to the rapid β -oxidation of this substance; we found that it is readily converted to $iPF_{3\alpha}$ -VI by rat liver homogenates. Thus, $iPF_{3\alpha}$ -VI could serve as an excellent *in vivo* marker for ω 3-PUFAs, because it can be formed as a result of peroxidation of both EPA and DHA.

METHODS

Synthesis of $iPF_{3\alpha}$ -VI

Details of the total synthesis of $iPF_{3\alpha}$ -VI **3** and its deuterated analog are shown in Figs. 2 and 3. Synthon **7** was prepared from commercially available methyl-5-chloro-5-oxo-valerate (21). The synthesis of the aldehyde **6** was performed in 10 steps from **5** as described by us previously for the synthesis of $nPF_{4\alpha}$ -VI (21). The structure of $iPF_{3\alpha}$ -VI was confirmed by 1H NMR ($CDCl_3$): δ 5.67–5.25 (m, 6H), 4.12–3.98 (m, 3H), 2.82 (m, 3H), 2.31 (m, 2H), 2.12–1.90 (m, 6H), 1.54 (m, 3H), 1.23 (m, 2H), 0.89 (t, 3H), and by electron impact MS [methyl ester, trimethylsilyl (TMS) deriv]: m/z 582(M^+), 567(M-15), 492(M-90) base peak, 403(M-179), 391(M-191).

Synthesis of $[19,19,20,20-^2H]iPF_{3\alpha}$ -VI

$[19,19,20,20-^2H]iPF_{3\alpha}$ -VI **21**, the tetradeuterated analog of $iPF_{3\alpha}$ -VI, was prepared as shown in Fig. 3. The deuterated synthon **19**, which was used to complete the bottom side chain,

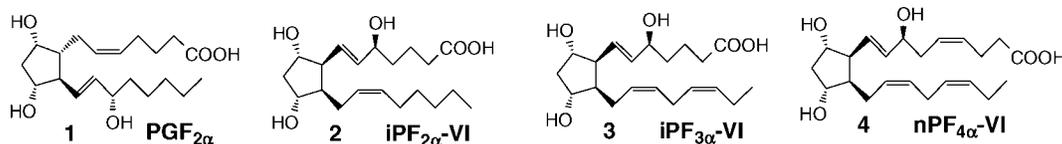


Fig. 1. Structures of PGF $_{2\alpha}$, $iPF_{2\alpha}$ -VI, $iPF_{3\alpha}$ -VI, and $nPF_{4\alpha}$ -VI.

was prepared from propargyl alcohol in five steps (21). We have recently reported the transformation of **11** to **14** in connection with the total synthesis of $[21,21,22,22-^2H]nPF_{4\alpha}$ -VI (21). The structure of d_4 - $iPF_{3\alpha}$ -VI was confirmed by 1H NMR (CD_3OD): δ 5.52–5.25 (m, 6H), 3.98 (q, 1H, $J = 4.3$ and 10.9), 3.92 (q, 1H, $J = 7$ and 11.8), 3.83 (q, 1H, $J = 6.8$ and 11.2), 2.74 (m, 2H), 2.63 (m, 2H), 2.43 (m, 1H), 2.25 (m, 2H, $J = 6.8$), 2.02 (m, 2H), 1.58 (m, 3H), 1.46 (m, 2H), 1.21 (s, 1H).

β -Oxidation of $nPF_{4\alpha}$ -VI and $iPF_{3\alpha}$ -VI by rat liver homogenates

Liver from Brown Norway rats was minced in ice-cold 0.25 M sucrose and then diluted with this medium to give a final ratio of 5 ml/g tissue. The suspension was homogenized by hand using six to eight strokes in a Potter-Elvehjem homogenizer in an ice bath, and the homogenate was centrifuged at 600 *g* for 10 min. To aliquots (1 ml) of the supernatant fraction were added 1 ml of 100 mM phosphate buffer, pH 7.4, containing 50 mM KCl, 7.2 mM $MgCl_2$, 8 mM ATP, 1 mM CoA, 4 mM L-carnitine, and 4 mM NAD^+ . Either $nPF_{4\alpha}$ -VI [final concentration, 20 μ M; synthesized as described previously (21)] or $iPF_{3\alpha}$ -VI (final concentration, 20 μ M) was added, and the mixture was incubated for 90 min at 37°C. The incubations were stopped by the addition of 1 vol of MeOH and cooled on ice. Water was then added to give a final concentration of 15% MeOH, and the mixture was centrifuged. The supernatant was subjected to solid-phase extraction (SPE) as described previously (22) using a C_{18} SepPak cartridge (Waters Associates, Milford, MA) that had been pre-treated with MeOH and water. The cartridge was eluted successively with 15% MeOH, water, and petroleum ether prior to elution of eicosanoids with methyl formate. The methyl formate was then removed using a rotary evaporator, and the residue was dissolved in a small volume of ethanol prior to further analysis.

Processing of urine samples for MS analysis

Urine was collected from 20 healthy individuals, evenly divided between males and females. Samples were immediately frozen at $-80^\circ C$ until further processing. After thawing, 10 ng $[^2H_4]nPF_{4\alpha}$ -VI [synthesized as described previously (21)] was added to 2 ml aliquots, which were mixed and allowed to equilibrate for 15 min at room temperature. The sample was extracted by SPE on a StrataX cartridge, (Phenomenex, Torrance, CA), which had been conditioned with 1 ml of acetonitrile (MeCN) followed by 1 ml H_2O . The sample was then applied to the cartridge, which was washed successively with H_2O (1 ml) and 5% MeCN in H_2O (1 ml), dried by applying a vacuum for 15 min, and eluted with 1 ml of 5% MeCN in EtOAc. The eluate was dried under a gentle stream of nitrogen, and the sample was stored in 40 μ l MeCN at $-80^\circ C$ until analysis, at which time H_2O (160 μ l) was added and the sample was filtered in a Costar Spin-X HPLC filter (0.2 μ m nylon; Corning Inc., Corning, NY).

$iPF_{3\alpha}$ -VI (Group I)

Urine was obtained from six healthy individuals, three males and three females. Samples were immediately frozen at $-80^\circ C$

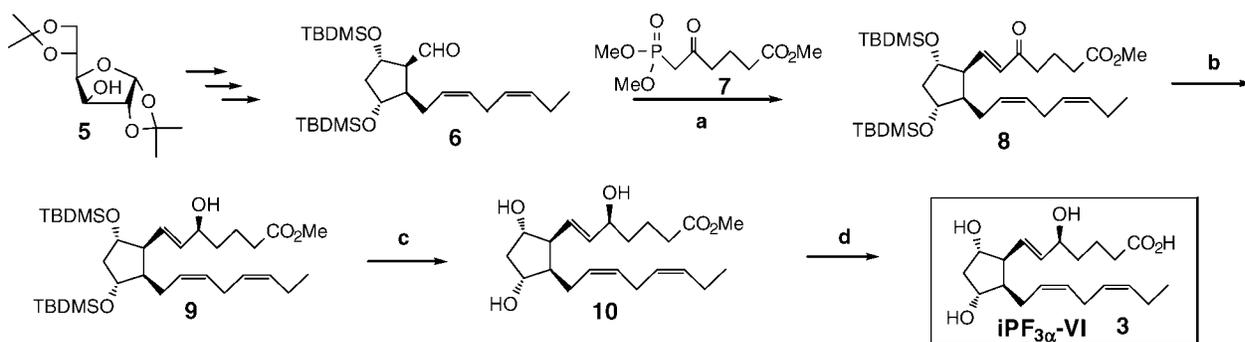


Fig. 2. Synthesis of $iPF_{3\alpha}\text{-VI}$. Reagents and conditions: **7**, LiHMDS, THF, **6**, -78°C , 3 h, 68% (a); S-BINAL-H, THF, -100°C , 4 h, 94% (b); 6:3:1 = THF:formic acid:H₂O, room temperature, 3 h, 74% (c); and 5% KOH, THF, room temperature, 2 h, 97% (d).

until further processing. After thawing, 2 ml aliquots were taken, and to each was added 6.9 ng of [²H₄] $iPF_{3\alpha}\text{-VI}$ and 10 ng of the methoxime derivative of 2,3-dinor-6-keto-PGF_{1 α} (d6k-PGF_{1 α} -MO). The samples were mixed, allowed to equilibrate for 15 min at room temperature, and extracted by SPE on StrataX cartridges as described above. The eluate was dried under a gentle stream of nitrogen, dissolved in 1 ml 75% EtOAc in hexane, and applied to a diol SPE cartridge (Bond Elut 2OH; Varian, Palo Alto, CA), which was washed with 1 ml of 75% EtOAc in hexane and eluted with 1 ml of 5% MeCN in EtOAc containing 0.1% acetic acid. Samples were then dried under nitrogen and dissolved in 100 μl of 10% MeCN in water for injection onto a 150 \times 2 mm HyperClone 5 μm C18 Base Deactivated Silica (BDS) column (Phenomenex, Torrance, CA) using a linear gradient between 10% and 50% solvent B over 20 min at a flow rate of 200 $\mu\text{l}/\text{min}$. Solvent A was water-acetic acid (100:0.005), adjusted to pH 5.7 with ammonium hydroxide, whereas solvent B was MeCN-MeOH-acetic acid (95:5:0.005). The transition monitored was m/z 370 \rightarrow 150 (d6k-PGF_{1 α} -MO). The elution time of d6k-PGF_{1 α} -MO served to predict the elution time of $iPF_{3\alpha}\text{-VI}$. One minute after the apex

of the d6k-PGF_{1 α} -MO peak, the column eluate was diverted from the mass spectrometer and collected for a period of 0.6 min. Samples were split into two equal portions for quantitation of $iPF_{3\alpha}\text{-VI}$ by GC/MS and LC/MS/MS as described below.

$iPF_{3\alpha}\text{-VI}$ (Group II)

Urine from 20 healthy individuals (see above) was thawed, 1 ml aliquots were removed, and to each was added 7.5 ng of [²H₄] $iPF_{3\alpha}\text{-VI}$. $iPF_{3\alpha}\text{-VI}$ was then extracted by SPE as described above for $nPF_{4\alpha}\text{-VI}$, and the extract was dissolved in MeCN (20 μl) and stored at -80°C until analysis by LC/MS/MS as described below.

The urine samples were obtained with the informed consent of the donors.

GC/MS

GC/MS was done on a Hewlett-Packard 5973 mass spectrometer equipped with a chemical ionization source coupled to an

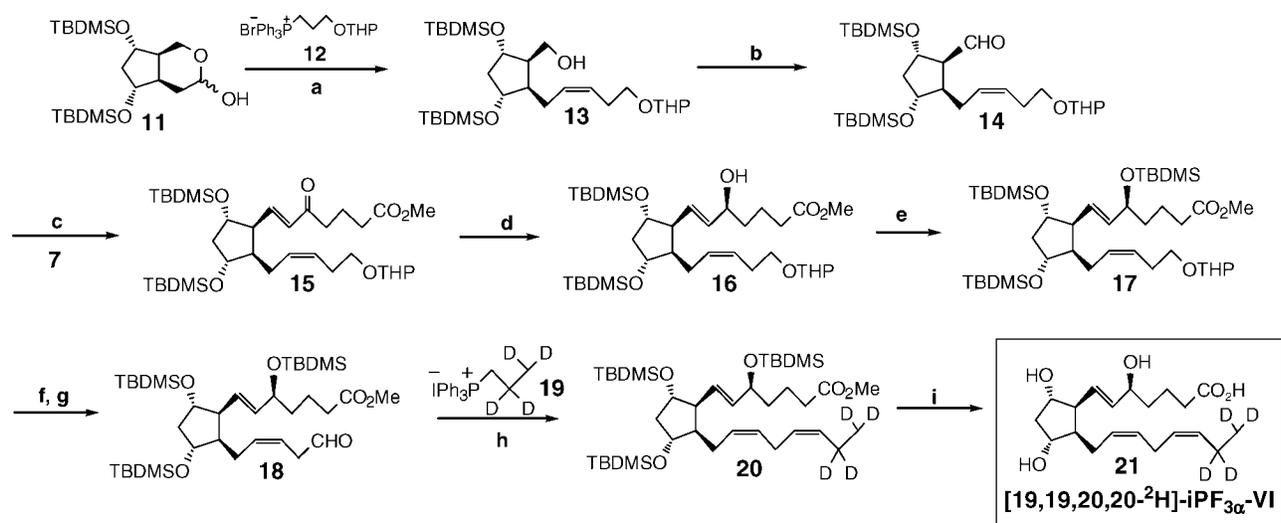


Fig. 3. Synthesis of [^{19,19,20,20-2}H] $iPF_{3\alpha}\text{-VI}$. Reagents and conditions: t-BuOK, THF, -20°C , 5 h, 82% (a); periodinane, CH₂Cl₂, room temperature, 2 h, 97% (b); **7**, LiHMDS, THF, **14**, -78°C , 14 h, 76% (c); S-BINAL-H, THF, -100°C , 5 h, 97% (d); TBDMSCl, Im, CH₂Cl₂, room temperature, 8 h, 75% (e); Me₂AlCl, CH₂Cl₂, -20°C to room temperature, 3 h, 72% (f); periodinane, t-BuOH, CH₂Cl₂, room temperature, 5 h, 95% (g); **19**, LiHMDS, HMPA, THF, **18**, -78°C , 3 h, 68% (h); 6:3:1 = THF:formic acid:H₂O, room temperature, 3 h, and KOH, THF, room temperature, 1.5 h, 85%, two steps (i).

HP 6890 gas chromatograph. The instrument was operated in the electron capture/negative ion (ECNI) mode, using ammonia as the moderating gas. The stationary phase was a column of $60\text{ m} \times 0.25\text{ mm i.d. DB-5MS}$ ($0.25\text{ }\mu\text{m}$; Agilent, Palo Alto, CA). The split/splitless injector was operated at 260°C and the interface at 280°C . The temperature program consisted of 1 min at 190°C followed by a linear increase to 300°C at a rate of $30^\circ\text{C}/\text{min}$.

LC/MS/MS

All LC/MS/MS was performed using a Thermo-Finnigan TSQ Quantum Ultra AM (Thermo Electron Corp.), except for the Group I study on $i\text{PF}_{3\alpha}\text{-VI}$, which was performed using a Micromass Ultima instrument (Waters). In both cases, the mass spectrometer was interfaced with two Shimadzu LC-10ADvp pumps (Shimadzu, Columbia, MD), a Shimadzu SCL-10Avp system controller, and a CTC Analytics HTC PAL autosampler (LEAP Technologies, Carrboro, NC). All LC/MS/MS analyses were performed in the negative-ion electrospray mode using Argon, 1.5 mTorr, as the collision gas. Quantitation was by peak area ratios. Mobile phases for HPLC were as described above. The flow rate was $0.2\text{ ml}/\text{min}$. All LC conditions and MS/MS tuning parameters were optimized by injection or infusion of authentic synthetic standards.

LC/MS/MS quantitation of $n\text{PF}_{4\alpha}\text{-VI}$ from urine. Filtered samples (see above) were injected onto a $150 \times 2\text{ mm}$ HyperClone $3\text{ }\mu\text{m}$ C18 BDS column (Phenomenex). The mobile phase was a gradient between 20% and 32% B over 20 min. The transitions monitored were $m/z\ 377 \rightarrow 113$ for endogenous $n\text{PF}_{4\alpha}\text{-VI}$ and $m/z\ 381 \rightarrow 113$ for $d_4\text{-nPF}_{4\alpha}\text{-VI}$. Collision energy was 21 V. Source collision-induced dissociation was 10 eV. Capillary temperature was 400°C , and spray voltage was 4 kV.

LC/MS/MS quantitation of $i\text{PF}_{3\alpha}\text{-VI}$ from urine (Group I). Filtered urine samples were injected onto a $150 \times 2\text{ mm}$ Luna $5\text{ }\mu\text{m}$ C18(2) column (Phenomenex). The mobile phase was 23% B at a flow rate of $0.2\text{ ml}/\text{min}$. The transitions monitored were $m/z\ 351 \rightarrow 115$ for endogenous $i\text{PF}_{3\alpha}\text{-VI}$ and $m/z\ 355 \rightarrow 115$ for the tetradeuterated analog. Collision energy was 21 V. Cone potential was -81 V , source temperature was 70°C , desolvation temperature was 200°C , and capillary potential was 3 kV (Fig. 7a).

LC/MS/MS quantitation of $i\text{PF}_{3\alpha}\text{-VI}$ from urine (Group II). Filtered samples were injected onto a $150 \times 2\text{ mm}$ HyperClone $3\text{ }\mu\text{m}$ C18 BDS column. The mobile phase was a gradient between 20% and 32% B over 20 min at a flow rate of $0.2\text{ ml}/\text{min}$. The transitions monitored were $m/z\ 351 \rightarrow 115$ for endogenous $i\text{PF}_{3\alpha}\text{-VI}$ and $m/z\ 355 \rightarrow 115$ for the tetradeuterated analog. MS conditions were as described above for $n\text{PF}_{4\alpha}\text{-VI}$. These conditions were also used for the analysis of β -oxidation products of $n\text{PF}_{4\alpha}\text{-VI}$.

GC/MS analysis of $n\text{PF}_{4\alpha}\text{-VI}$ metabolites. Samples were converted to the pentafluorobenzyl (PFB) ester by adding $10\text{ }\mu\text{l}$ *N,N*-diisopropylethylamine (Sigma Chemical Co., St. Louis, MO) and $20\text{ }\mu\text{l}$ 10% PFB Br (Sigma) in MeCN. After 10 min at room temperature, the solvent was removed under a stream of nitrogen and the TMS ether derivative was formed by treatment with $10\text{ }\mu\text{l}$ pyridine (Sigma) and $10\text{ }\mu\text{l}$ bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA) for 10 min. Samples were then dried under nitrogen and dissolved in dodecane (Sigma) for injection into the GC/MS. Partial mass spectra were obtained by scanning the mass range from $m/z\ 300$ to $m/z\ 600$ for 0.4 s. Selected ion monitoring (SIM) techniques were also used, focusing on $m/z\ 567$ for $i\text{PF}_{3\alpha}\text{-VI}$ and $m/z\ 571$ for the tetradeuterated analog.

GC/MS quantitation of $i\text{PF}_{3\alpha}\text{-VI}$ from urine. Samples were dried under nitrogen and the PFB ester, TMS ether derivatives were prepared as described above. SIM was used, monitoring $m/z\ 567$ for $i\text{PF}_{3\alpha}\text{-VI}$ and $m/z\ 571$ for the tetradeuterated analog. Quantitation was by peak area ratios.

RESULTS

Synthesis of $i\text{PF}_{3\alpha}\text{-VI}$ and $[19,19,20,20\text{-}^2\text{H}]i\text{PF}_{3\alpha}\text{-VI}$

The first total and stereospecific syntheses of $i\text{PF}_{3\alpha}\text{-VI}$ 3 and its deuterated analog 21 were accomplished as shown in Figs. 2 and 3. It is interesting to note that in the synthesis of $[19,19,20,20\text{-}^2\text{H}]i\text{PF}_{3\alpha}\text{-VI}$ 21, the completion of the bottom side chain was done differently from that of the nondeuterated analog, as can be seen in Fig. 3 (transformation 18 to 20). To spare the deuterated synthon 19, the introduction of the deuterium piece was effected at the last stage. The structures of both compounds were confirmed by NMR. An analysis of the deuterium content of the deuterated analog expressed as the ratio of $^2\text{H}_0/^2\text{H}_4$, determined by GC/MS, was 0.0009.

Measurement of $n\text{PF}_{4\alpha}\text{-VI}$ in urine

We attempted to measure the levels of $n\text{PF}_{4\alpha}\text{-VI}$ in urine samples from healthy subjects by LC/MS/MS, but were unable to detect this nP in any of the 20 samples tested (Fig. 4A). In contrast, we were easily able to detect a small amount ($100\text{ pg}/\text{ml}$) of exogenous synthetic $n\text{PF}_{4\alpha}\text{-VI}$ that had been added to the same sample (Fig. 4B). As expected, the tetradeuterated analog had a retention time slightly

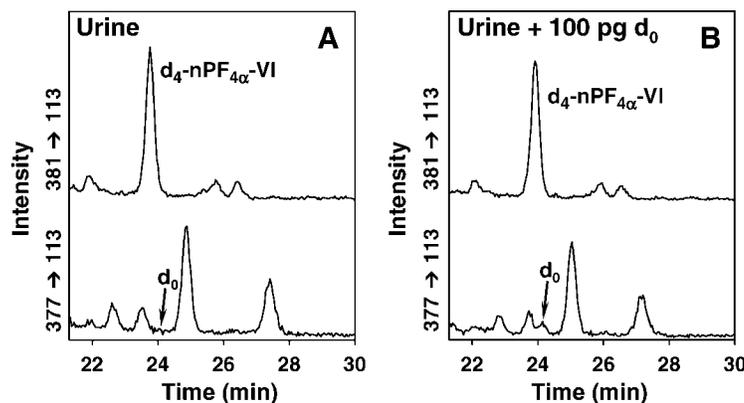


Fig. 4. $i\text{PF}_{4\alpha}\text{-VI}$ is not present in detectable amounts in human urine. Urine from a healthy individual was extracted and analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS), monitoring the transitions $m/z\ 377 \rightarrow 113$ (unlabeled $n\text{PF}_{4\alpha}\text{-VI}$) and $381 \rightarrow 113$ ($d_4\text{-nPF}_{4\alpha}\text{-VI}$). A: Mass chromatogram of an extract from a urine sample to which the internal standard $d_4\text{-nPF}_{4\alpha}\text{-VI}$ (10 ng) was added. B: Mass chromatogram of an identical sample that also contained chemically synthesized unlabeled $n\text{PF}_{4\alpha}\text{-VI}$ (100 pg). As shown by the arrows, $n\text{PF}_{4\alpha}\text{-VI}$ can only be detected in the sample containing the exogenous material.

shorter than that of the unlabeled compound. The retention times of both the labeled and unlabeled compounds were confirmed by separate analysis of chemically synthesized standards. Note that some of the samples exhibited a small peak near the retention time of endogenous nPF_{4α}-VI. When two other ion transitions characteristic of the fragmentation of nPF_{4α}-VI were monitored (m/z 377 → 199 and m/z 377 → 359), it was clear that this peak did not originate from authentic nPF_{4α}-VI.

Conversion of nPF_{4α}-VI to iPF_{3α}-VI by β-oxidation

In view of the presence of DHA and nPs in the brain, we were surprised to find nPF_{4α}-VI to be undetectable in urine. We speculated that this could be due to its metabolism by β-oxidation, and to test this hypothesis, we investigated its metabolism by a rat liver homogenate in the presence of ATP, CoA, L-carnitine, and NAD⁺. Incubation of nPF_{4α}-VI (20 μM) with this preparation for 90 min led to the formation of a single metabolite, amounting to 19% of the substrate. Analysis by LC/MS revealed the presence of an intense ion with an m/z of 351 (M-1; data not shown). This indicates a loss of 26 mass units from the substrate nPF_{4α}-VI, which exhibited an intense ion at m/z 377 (M-1), suggesting that it had been converted by β-oxidation to iPF_{3α}-VI. Collision-induced dissociation of the ion at m/z 351 gave rise to a series of daughter ions with m/z values of 297, 289, 271, 253, 245, 217, 191, and 115 (base peak) (Fig. 5A), which was

virtually identical to the mass spectrum obtained from synthetic iPF_{3α}-VI (Fig. 5B).

The chromatographic profile of nPF_{4α}-VI metabolites with the properties of iPF_{3α}-VI (i.e., intense ion at m/z 351 giving rise to a daughter ion at m/z 115) is shown in the lower panel of Fig. 6A. The retention time (t_R) of the major peak (10.8 min) is nearly identical to that of synthetic d₄-iPF_{3α}-VI (10.6 min), shown for comparison in the top panel of Fig. 6A. As expected, the deuterated analog has a very slightly shorter retention time due to its shorter C-²H bonds. A small peak, presumably an isomer of iPF_{3α}-VI, was also detected. To provide further confirmation of the identity of the major nPF_{4α}-VI metabolite, we examined its properties by GC/MS after conversion to its PFB ester, TMS ether derivative. One major metabolite with an intense ion at m/z 567 (M-PFB) and a t_R of 17.17 min, only slightly longer than that of authentic d₄-iPF_{3α}-VI (m/z 571; t_R , 17.10 min), was observed (Fig. 6B).

In contrast to nPF_{4α}-VI, iPF_{3α}-VI was quite resistant to metabolism by β-oxidation. Following incubation with a rat liver homogenate under conditions identical to those described above and analysis by LC/MS/MS, the only major component observed was the unmetabolized substrate. A very small amount (~0.06% of the substrate) of a substance with the properties expected for 2,3-dinor-iPF_{3α}-VI was detected, but the amount was insufficient for positive identification.

Identification of iPF_{3α}-VI in urine

We conducted an initial study with six subjects (Group I) using a rigorous purification protocol, involving an independent LC purification step prior to mass spectrometric analysis to demonstrate conclusively the presence of iPF_{3α}-VI in human urine. d6k-PGF_{1α}-MO was added to the urine along with the internal standard d₄-iPF_{3α}-VI to act as a marker to define the elution position of iPF_{3α}-VI. Samples were then purified by two steps of SPE using reversed-phase and diol stationary phases, followed by LC using MS/MS to detect the d6k-PGF_{1α}-MO marker. The material eluting immediately after the elution marker was then analyzed by both LC/MS/MS and GC/MS.

Analysis by LC/MS/MS revealed a constituent with an intense ion at m/z 351 (M-1), which gave rise to a daughter ion at m/z 115 (Fig. 7A, lower panel). We previously found that this daughter ion is characteristic of Group VI iPs and does not occur to an appreciable extent with iPs of other groups (10). A single major peak was observed with a t_R of 26.1 min, slightly longer than that of the corresponding ion (355 → 115) from the tetradeuterated internal standard (t_R , 25.7 min; Fig. 7A, upper panel), as expected for iPF_{3α}-VI. The sample was also analyzed by GC/MS following conversion of iPs to their PFB ester, TMS ether derivatives, which would be expected to give rise to a derivative with a major fragment ion at m/z 567 (M-PFB). When this ion was monitored, a major peak was observed with a t_R of 13.5 min, slightly longer than that of the internal standard (t_R , 13.45 min; Fig. 7B, upper panel), confirming the presence of iPF_{3α}-VI.

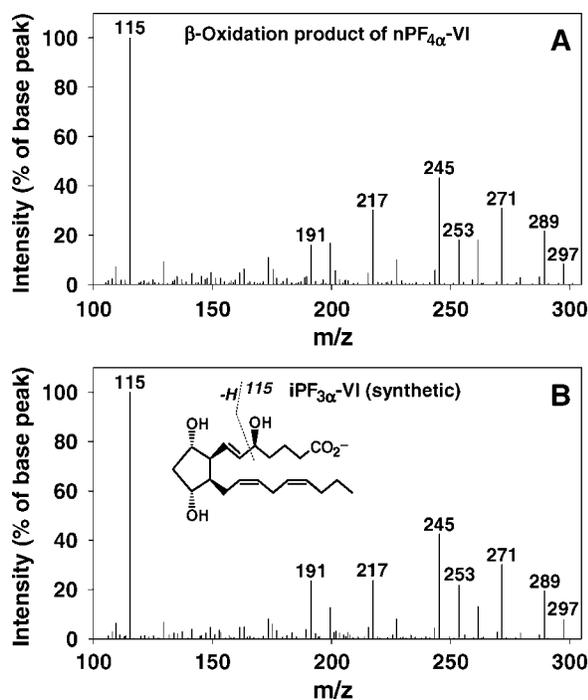


Fig. 5. Mass spectrum of the β-oxidation product of nPF_{4α}-VI. A: The mass spectrum of the major product formed during incubation of nPF_{4α}-VI with a rat liver homogenate in the presence of ATP, CoA, L-carnitine, and NAD⁺, as described in Methods. B: The mass spectrum of chemically synthesized iPF_{3α}-VI.

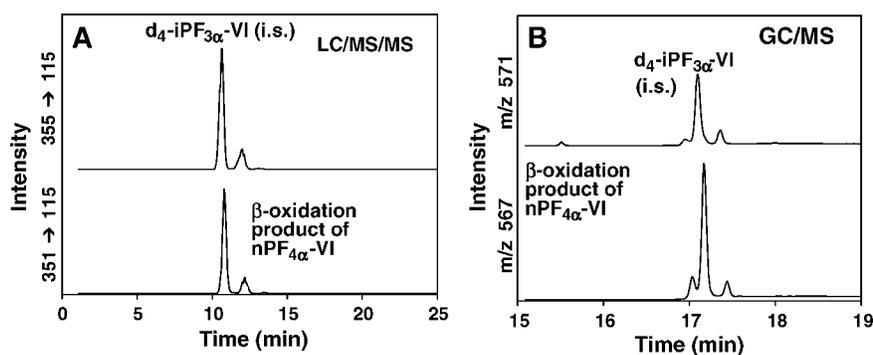


Fig. 6. Identification of the β -oxidation product of nPF_{4 α} -VI as iPF_{3 α} -VI by LC/MS/MS and gas chromatography (GC)/MS. A: LC/MS/MS electrospray-selected reaction monitoring analysis of an extract of a rat liver homogenate incubated with nPF_{4 α} -VI as described in Methods. d_4 -iPF_{3 α} -VI was added as an internal standard (i.s.). The transition m/z 355 \rightarrow 115 (d_4 -iPF_{3 α} -VI) is shown in the upper panel, whereas the transition m/z 351 \rightarrow 115 is shown in the lower panel. B: Electron capture/negative ion (ECNI) GC/MS of the same extract shown in A, after conversion of iPs to their pentafluorobenzyl (PFB) ester, and trimethylsilyl (TMS) ether derivatives. The upper panel shows the mass chromatogram for m/z 571 (d_4 -iPF_{3 α} -VI), whereas the lower panel shows m/z 567 (iPF_{3 α} -VI).

Urinary levels of iPF_{3 α} -VI in healthy subjects

In an initial study, the urinary levels of iPF_{3 α} -VI were measured by both the GC/MS and LC/MS/MS methods in six healthy individuals (Group I), including three males and three females. The levels of iPF_{3 α} -VI were between approximately 200 and 300 pg/ml creatinine for five out of the six subjects, with excellent agreement between the two methods (Fig. 8). However, one subject had substantially higher levels of iPF_{3 α} -VI with both methods, and in this case, the level as determined by LC/MS/MS was nearly twice as high as that found using the GC/MS assay (Fig. 8, inset). This could be due to either overestimation of the internal standard in the GC/MS sample or overestimation of the endogenous signal in the LC/MS/MS sample. Because the internal standard is added at a relatively high level, it is a single, symmetrical peak in all samples, and not likely to be artifactually elevated. Considering the endog-

enous LC/MS/MS traces (i.e., m/z 351 \rightarrow 115), five of the six samples exhibited a single peak with a retention time identical to that of authentic iPF_{3 α} -VI. Only the chromatogram of the outlier sample had an additional peak, raising the possibility that the peak coeluting with the internal standard may be somewhat elevated due to the coelution of an interfering compound that was not removed by the sample prepurification.

A second study was performed in which the urinary levels of iPF_{3 α} -VI were measured by LC/MS/MS in an additional 20 subjects, including 10 males and 10 females (Group II). The average age was 34 ± 13.6 years, and the average body mass index (weight/height²) was 22.3 ± 2.6 . The mean urinary level of iPF_{3 α} -VI among these subjects was 274 ± 201 pg/mg creatinine (mean \pm SD). There were no significant differences between males and females (Fig. 9) and no correlation of iPF_{3 α} -VI levels with body mass

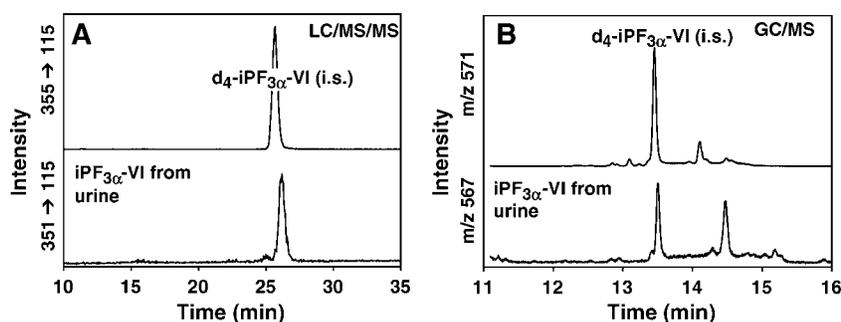


Fig. 7. Identification of iPF_{3 α} -VI in urine by LC/MS/MS and GC/MS. iPs were extracted from a urine sample from a subject in Group I to which d_4 -iPF_{3 α} -VI had been added as an internal standard (i.s.). iPF_{3 α} -VI was purified by HPLC using d6k-PGF_{1 α} -MO as a marker for its elution position as described in Methods (initial study). A: LC/MS/MS electrospray-selected reaction monitoring analysis of iPF_{3 α} -VI. Transitions characteristic of d_4 -iPF_{3 α} -VI (m/z 355 \rightarrow 115) and iPF_{3 α} -VI (m/z 351 \rightarrow 115) are shown in the upper and lower panels, respectively. B: ECNI GC/MS analysis of iPF_{3 α} -VI in the above urinary extract following conversion to its PFB ester, TMS ether derivative. The ions due to loss of PFB are shown in the upper (m/z 571, d_4 -iPF_{3 α} -VI) and lower (m/z 567, iPF_{3 α} -VI) panels, respectively.

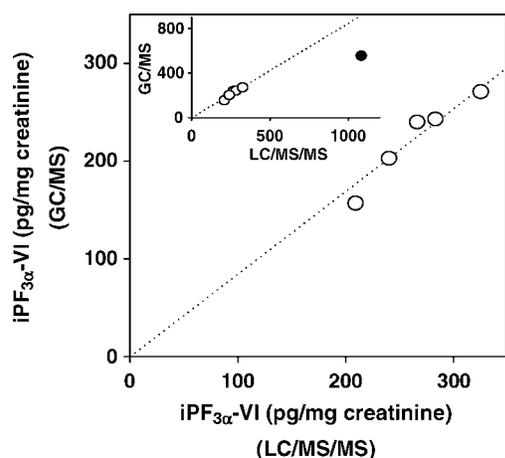


Fig. 8. Correlation between LC/MS/MS and GC/MS assays for $iPF_{3\alpha}\text{-VI}$. Following addition of the internal standards, urine samples from the six subjects in Group I were extracted and $iPF_{3\alpha}\text{-VI}$ was purified by HPLC prior to analysis by mass spectrometry, as described in Methods. Five (open circles) out of six individuals had levels of $iPF_{3\alpha}\text{-VI}$ at or below 325 pg/mg creatinine, whereas the sixth individual had considerably higher levels (closed circle, inset).

index (data not shown). There was a tendency for older subjects to have higher levels of $iPF_{3\alpha}\text{-VI}$, but this was not statistically significant (Fig. 9).

DISCUSSION

EPA and DHA are the main PUFAs in fish and fish oil (23) and are popular food supplements, sold in pharmacies alone or in combination with vitamins and minerals. The reason for the interest in these ω 3-PUFAs stems from observations that populations that consume large amounts of fatty fish, such as the Inuit (24), have a lower incidence of myocardial infarction than populations that consume a Western diet high in ω 6-PUFAs. The potentially beneficial effects of EPA and DHA have been supported by a variety of epidemiological and interventional studies of fish consumption (15, 25). However, it remains unclear whether the reported benefit reflects a decrease in saturated fat consumption or a beneficial effect of ω -3 fatty acids. A quantitative marker of ω -3 fatty acid bioavailability that could be related to clinical outcome would assist in the prospective, randomized, controlled trials necessary to evaluate the clinical efficacy of dietary supplements.

Multiple potentially beneficial effects have been ascribed to ω -3 fatty acids, including a reduction in blood pressure (26), modulation of the response to endogenous and exogenous thrombolytic agents (27), antiarrhythmic actions (28), inhibition of platelet activation (29), and modulation of inflammation (30), triglycerides, and VLDL (31). Some of these effects are, at least in part, mediated by alterations in the biosynthesis and effects of eicosanoids (25, 32). ω 3-PUFAs, in particular DHA, are present in high levels in the brain, and may play a role in cognitive de-

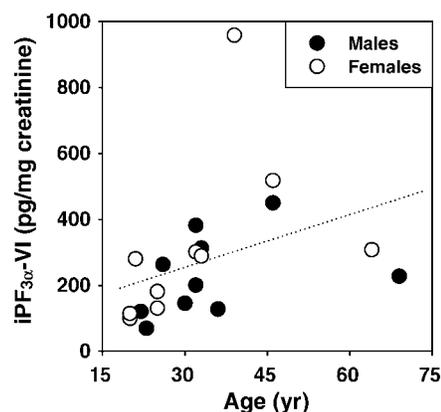


Fig. 9. Relationship between gender, age, and urinary $iPF_{3\alpha}\text{-VI}$ levels in healthy human subjects. $iPF_{3\alpha}\text{-VI}$ was quantitated by LC/MS/MS in urine samples from 20 individuals (Group II) as described in Methods. The group consisted of 10 males (closed circles) and 10 females (open circles).

velopment (33). There is also evidence that there is an inverse relationship between the content of EPA/DHA in the diet and the incidence of depression (25). Like AA, EPA and DHA can be converted into a series of iPs, which, in the case of DHA-derived iPs, have been termed nPs (34). However, prior to the present study, no assays to measure the in vivo production of F_3 -iPs derived from EPA through the measurement of urinary levels were available. Although nPs can be measured in brain tissue, an attempt to measure F_4 -nPs by GC/MS in the urine of patients suffering from AD found them to be undetectable (20). We selected Group VI F_3 -iPs and F_4 -nPs as our initial targets for detection in biological fluids because we had previously observed that among AA-derived iPs, Group VI iPs such as $iPF_{2\alpha}\text{-VI}$ 2 and 8,12-*iso*- $iPF_{2\alpha}\text{-VI}$ are the most abundant in human urine (2, 8). Because of the close structural analogy of $iPF_{3\alpha}\text{-VI}$ 3 and $nPF_{4\alpha}\text{-VI}$ to $iPF_{2\alpha}\text{-VI}$ 2 (Fig. 1), it would seem likely that this would also be the case for Group VI F_3 -iPs and F_4 -nPs.

Because DHA is the most abundant PUFA in the brain, we initially sought to measure urinary $nPF_{4\alpha}\text{-VI}$ levels as an in vivo indicator of its peroxidation. Such biomarkers might be used to titrate the response to ω -3s in interventional studies to assess their value in the assessment of cognitive function. For this reason, we recently developed a method for the total synthesis of $nPF_{4\alpha}\text{-VI}$ and its tetradeuterio analog (21) to permit its quantitation in biological fluids. However, in the present study, we were unable to detect $nPF_{4\alpha}\text{-VI}$ in urine by LC/MS/MS. One potential reason for this could be that it was rapidly metabolized in vivo. Comparison of the structures of $iPF_{2\alpha}\text{-VI}$ and $iPF_{3\alpha}\text{-VI}$ (Fig. 1) reveals that the carboxyl side chains of these two molecules are identical, whereas the carboxyl side chain of $nPF_{4\alpha}\text{-VI}$ is elongated and contains an addition double bond at C_4 . Others as well as ourselves have reported that the presence of a hydroxyl group in the 5-position of the carboxyl side chain of leukotrienes and iPs can have a marked effect on their metabolic fates (9, 35,

36). 5-Hydroxyeicosanoids are resistant to β -oxidation, probably because the 5-hydroxyl group interacts with the carboxyl group, thus limiting access of the enzymes involved in this process. It was this assumption that 5-hydroxy-iPs would be resistant to β -oxidation that led us to focus originally on AA-derived Group VI iPs, because we suspected that they would be more abundant in urine than the more commonly measured Group III iPs and hence easier to discover and measure. We therefore reasoned that the greater distance between this hydroxyl group and the C₁ carboxylic acid group in nPF_{4 α} -VI could result in less protection against β -oxidation compared with iPF_{2 α} -VI and iPF_{3 α} -VI. We compared the extent of β -oxidation of iPF_{3 α} -VI and nPF_{4 α} -VI in vitro by rat liver homogenates to test this hypothesis. In contrast to iPF_{3 α} -VI, which was highly resistant to β -oxidation, nPF_{4 α} -VI was a good substrate for this process, being converted to iPF_{3 α} -VI (i.e., 2,3-dinor-4,5-dihydro-nPF_{4 α} -VI) to the extent of 19%. The identity of this metabolite was established by GC/MS and LC/MS/MS comparison with chemically synthesized standards.

The extent of β -oxidation of nPF_{4 α} -VI was over three times greater than that of [17,18,19,20-²H₄]iPF_{2 α} -III, which has a 7 carbon carboxylic acid side chain without a hydroxyl group. Under identical reaction conditions, only 3.6% of iPF_{2 α} -III was converted to the dinor metabolite, along with an additional 2.2% to the dihydro-dinor compound (data not shown). This suggests that nPF_{4 α} -VI is particularly prone to β -oxidation at C₁, providing a possible explanation for our inability to identify this substance in human urine. Others, too, have been unable to measure nP formation in the urine of AD patients (20). The metabolism argument presented here is strengthened by the finding that iPF_{2 α} -III is metabolized in vivo, with a major (29%) urinary metabolite being the dinor dihydro derivative (37). We and others have also studied this metabolic step (38–40). Because nPF_{4 α} -VI is a much better substrate than iPF_{2 α} -III for β -oxidation, we would expect its rate of in vivo metabolism to be several-fold higher, hence explaining the lack of nPF_{4 α} -VI in urine. We were unable to identify any dinor-nPF_{4 α} -VI in incubations with rat liver homogenates, indicating that the 4,5-double bond of nPF_{4 α} -VI was completely reduced. This is presumably due to the formation of the $\Delta^{2,4}$ -conjugated diene **22**, followed by reduction to the Δ^3 metabolite **23** and β -oxidation (Fig. 10) (41). A similar situation has previously been encountered in the metabo-

lism of LTE₄ (34, 35, 42), which is metabolized by ω -oxidation, followed by two cycles of β -oxidation coupled with reduction of the 14,15-double bond.

The facile metabolism of nPF_{4 α} -VI to iPF_{3 α} -VI suggests that the formation of iPF_{3 α} -VI could not only be an indicator of the production of EPA-derived iPs, but also, to a considerable extent, could reflect the endogenous formation of nPF_{4 α} -VI. For this reason, we prepared iPF_{3 α} -VI and [19,19,20,20-²H]iPF_{3 α} -VI by total synthesis to allow the measurement of the former compound in urine by MS. We used two independent assays based on GC/ECNI-MS and LC/MS/MS to conclusively identify iPF_{3 α} -VI in urine. The requirement for conversion of iPF_{3 α} -VI to a volatile derivative for GC/ECNI-MS analysis adds an additional level of complexity to this method. Thus, with GC/ECNI-MS of the PFB ester, TMS ether derivatives are separated in the gas phase and ionized by the gentle capture of thermal electrons by the electrophilic PFB group, leading to cleavage of the bond between the PFB and carboxyl group, leaving the intact quasi-molecular anion at *m/z* 567 for MS analysis. The underivatized compounds are chromatographed with LC/MS/MS on a reversed-phase column at a pH that ensures their anionic form. The highly selective nature of the selected reaction monitoring technique screens out all ions other than those possessing *m/z* ratios equivalent to that of the analyte (*m/z* 351 for iPF_{3 α} -VI), fragments them by collision with argon, and finally, filters a preselected product ion (*m/z* 115 for iPF_{3 α} -VI). Despite the differing principles of separation and detection between the GC and LC methods described above, there was very good agreement in the urinary levels of iPF_{3 α} -VI between the two methods, providing conclusive evidence for the identification of this iP.

In contrast to the extensive amount of information available on the biological activities of the enzymatically derived prostaglandins and leukotrienes, relatively little is known about the bioactivities of iPs. Although a limited number of studies have been performed on certain iPs derived from AA, it is not yet known whether iPs derived from EPA and DHA have biological activities. We have shown that exogenous iPF_{2 α} -III can activate the thromboxane receptor in platelets and the vasculature in vivo (43) and that 8,12-*iso*-iPF_{2 α} -III can activate the receptor for PGF_{2 α} in vitro (44, 45). However, it is unknown whether these iPs function as endogenous receptor ligands at the

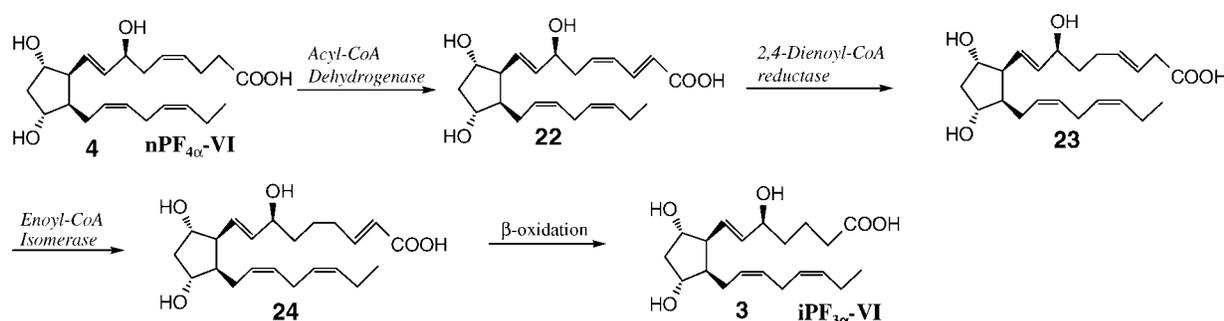


Fig. 10. Proposed metabolism pathway (β -oxidation) of nPF_{4 α} -VI.

concentrations formed *in vivo*. It would be of interest to determine whether iPs derived from EPA and DHA are also biologically active and to compare their effects with those of AA-derived iPs.

The present data raise several important questions. Does the measurement of iPF_{3α}-VI in urine of normal volunteers reflect two sources of iPs, namely, unmetabolized iPF_{3α}-VI derived from EPA and the dinor dihydro metabolite of nPF_{4α}-VI? If this is true, measurement of iPF_{3α}-VI could be an excellent index of the combined endogenous autooxidation of EPA and DHA, and might reflect a process of relevance to disease progression in syndromes of neurodegeneration, such as AD. Recently, F₄-nPs have been detected in brain tissue (19, 46). However, the measurement of changes in nP levels in urine as an index of AD has not been successful (20). On the basis of the present results, it would seem likely that this could be explained by the β-oxidation of F₄-nPs to F₃-iPs. A number of studies have demonstrated elevated levels of urinary F₂-iPs in AD (47), but because of the high levels of DHA in brain lipids (14), it may be more relevant to measure iPs derived from this PUFA, particularly because they could be relatively selective markers of lipid peroxidation in the brain. Because of the rapid β-oxidation of nPF_{4α}-VI to iPF_{3α}-VI, measurement of the latter substance in urine may provide important mechanistic clues and could be of diagnostic value in the investigation of neurodegenerative diseases such as AD. **■**

The authors are grateful to Sylvie Gravel for assistance with the β-oxidation experiments. This work was supported by National Institutes of Health Grants HL-81873 (J.R.), HL-69835 (J.R.), and HL-70128 (G.A.F.), Canadian Institutes of Health Research Grant MOP-6254 (W.S.P.), the Heart and Stroke Foundation of Quebec, and the J.T. Costello Memorial Research Fund. J.R. also wishes to acknowledge the National Science Foundation for a Bruker 400 MHz NMR instrument (Grant CHE-03 42251).

REFERENCES

- Morrow, J. D., K. E. Hill, R. F. Burk, T. M. Nammour, K. F. Badr, and L. J. Roberts II. 1990. A series of prostaglandin F₂-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. USA.* **87**: 9383–9387.
- Praticò, D., O. P. Barry, J. A. Lawson, M. Adiyaman, S. W. Hwang, S. P. Khanapure, L. Iuliano, J. Rokach, and G. A. FitzGerald. 1998. iPF_{2α}-I: An index of lipid peroxidation in humans. *Proc. Natl. Acad. Sci. USA.* **95**: 3449–3454.
- Hwang, S. W., M. Adiyaman, S. Khanapure, L. Schio, and J. Rokach. 1994. Total synthesis of 8-*epi*-PGF_{2α}. A novel strategy for the synthesis of isoprostanes. *J. Am. Chem. Soc.* **116**: 10829–10830.
- Adiyaman, M., J. A. Lawson, S. W. Hwang, S. P. Khanapure, G. A. FitzGerald, and J. Rokach. 1996. Total synthesis of a novel isoprostane iPF_{2α}-I and its identification in biological fluids. *Tetrahedron Lett.* **37**: 4849–4852.
- Adiyaman, M., H. Li, J. A. Lawson, S. W. Hwang, S. P. Khanapure, G. A. FitzGerald, and J. Rokach. 1997. First total synthesis of isoprostane iPF_{2α}-III. *Tetrahedron Lett.* **38**: 3339–3342.
- Pudukulathan, Z., S. Manna, S. W. Hwang, S. P. Khanapure, J. A. Lawson, G. A. FitzGerald, and J. Rokach. 1998. Diels-Alder approach to isoprostanes. Total synthesis of iPF_{2α}-V. *J. Am. Chem. Soc.* **120**: 11953–11961.
- Kim, S., Y. Jung, J. A. Lawson, G. A. FitzGerald, and J. Rokach. 2001. iPF_{2α}-III metabolism. First total synthesis of 2,3-dinor iPF_{2α}-III, a primary β-oxidation metabolite. *Tetrahedron Lett.* **42**: 8277–8280.
- Lawson, J. A., H. Li, J. Rokach, M. Adiyaman, S. W. Hwang, S. P. Khanapure, and G. A. FitzGerald. 1998. Identification of two major F₂ isoprostanes, 8,12-*iso*- and 5-*epi*-8,12-*iso*-isoprostane F_{2α}-VI, in human urine. *J. Biol. Chem.* **273**: 29295–29301.
- Adiyaman, M., J. A. Lawson, S. P. Khanapure, G. A. FitzGerald, and J. Rokach. 1998. Total synthesis of 17,17,18,18-d₄-iPF_{3α}-VI and quantification of iPF_{2α}-VI in human urine by gas chromatography/mass spectrometry. *Anal. Biochem.* **262**: 45–56.
- Li, H., J. A. Lawson, M. Reilly, M. Adiyaman, S. W. Hwang, J. Rokach, and G. A. FitzGerald. 1999. Quantitative high performance liquid chromatography/tandem mass spectrometric analysis of the four classes of F₂-isoprostanes in human urine. *Proc. Natl. Acad. Sci. USA.* **96**: 13381–13386.
- Lawson, J. A., J. Rokach, and G. A. FitzGerald. 1999. Isoprostanes: formation, analysis and use as indices of lipid peroxidation *in vivo*. *J. Biol. Chem.* **274**: 24441–24444.
- Praticò, D., R. K. Tangirala, D. J. Rader, J. Rokach, and G. A. FitzGerald. 1998. Vitamin E suppresses isoprostane generation *in vivo* and reduces atherosclerosis in ApoE-deficient mice. *Nat. Med.* **4**: 1189–1192.
- Praticò, D., J. Rokach, J. Lawson, and G. A. FitzGerald. 2004. F₂-isoprostanes as indices of lipid peroxidation in inflammatory diseases. *Chem. Phys. Lipids.* **128**: 165–171.
- Sastry, P. S. 1985. Lipids of nervous tissue: composition and metabolism. *Prog. Lipid Res.* **24**: 69–176.
- Kris-Etherton, P. M., W. S. Harris, L. J. Appel, and for the AHA Nutrition Committee. 2003. Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. *Arterioscler. Thromb. Vasc. Biol.* **23**: 151–152.
- Nourooz-Zadeh, J., B. Halliwell, and E. E. Anggard. 1997. Evidence for the formation of F₃-isoprostanes during peroxidation of eicosapentaenoic acid. *Biochem. Biophys. Res. Commun.* **236**: 467–472.
- Gao, L., H. Yin, G. L. Milne, N. A. Porter, and J. D. Morrow. 2006. Formation of F-ring isoprostane-like compounds (F₃-isoprostanes) *in vivo* from eicosapentaenoic acid. *J. Biol. Chem.* **281**: 14092–14099.
- Fam, S. S., L. J. Murphey, E. S. Terry, W. E. Zackert, Y. Chen, L. Gao, S. Pandalai, G. L. Milne, L. J. Roberts II, N. A. Porter, et al. 2002. Formation of highly reactive A-ring and J-ring isoprostane-like compounds (A₄/J₄-neuroprostanes) *in vivo* from docosahexaenoic acid. *J. Biol. Chem.* **277**: 36076–36084.
- Musiek, E. S., J. K. Cha, H. Yin, W. E. Zackert, E. S. Terry, N. A. Porter, T. J. Montine, and J. D. Morrow. 2004. Quantification of F-ring isoprostane-like compounds (F₄-neuroprostanes) derived from docosahexaenoic acid *in vivo* in humans by a stable isotope dilution mass spectrometric assay. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **799**: 95–102.
- Montine, T. J., J. F. Quinn, D. Milatovic, L. C. Silbert, T. Dang, S. Sanchez, E. Terry, L. J. Roberts II, J. A. Kaye, and J. D. Morrow. 2002. Peripheral F₂-isoprostanes and F₄-neuroprostanes are not increased in Alzheimer's Disease. *Ann. Neurol.* **52**: 175–179.
- Kim, S., J. A. Lawson, D. Praticò, G. A. FitzGerald, and J. Rokach. 2002. The first total synthesis of iPF_{4α}-VI and its deuterated analog. *Tetrahedron Lett.* **43**: 2801–2805.
- Powell, W. S. 1980. Rapid extraction of oxygenated metabolites of arachidonic acid from biological samples using octadecylsilyl silica. *Prostaglandins.* **20**: 947–957.
- Goodnight, S. H., Jr., W. S. Harris, W. E. Connor, and D. R. Illingworth. 1982. Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. *Arteriosclerosis.* **2**: 87–113.
- Dyerberg, J., and H. O. Bang. 1979. Hemostatic function and platelet unsaturated fatty acids in Eskimos. *Lancet.* **2**: 433–435.
- Smith, W. L. 2005. Cyclooxygenases, peroxide tone and the allure of fish oil. *Curr. Opin. Cell Biol.* **17**: 174–182.
- Knapp, H. R., and G. A. FitzGerald. 1989. The antihypertensive effects of fish oil. A controlled study of polyunsaturated fatty acid supplements in essential hypertension. *N. Engl. J. Med.* **320**: 1037–1043.
- Braden, G. A., H. R. Knapp, D. J. Fitzgerald, and G. A. FitzGerald. 1990. Dietary fish oil accelerates the response to coronary thrombolysis with tissue-type plasminogen activator. *Circulation.* **82**: 178–187.
- Brouwer, I. A., A. Geelen, and M. B. Katan. 2006. *n*-3 Fatty acids, cardiac arrhythmia and fatal coronary heart disease. *Prog. Lipid Res.* **45**: 357–367.

29. Knapp, H. R., I. A. Reilly, P. Alessandrini, and G. A. FitzGerald. 1986. *In vivo* indexes of platelet and vascular function during fish-oil administration in patients with atherosclerosis. *N. Engl. J. Med.* **314**: 937–942.
30. Basu, A., S. Devaraj, and I. Jialal. 2006. Dietary factors that promote or retard inflammation. *Arterioscler. Thromb. Vasc. Biol.* **26**: 995–1001.
31. Connor, W. E. 1988. Effects of omega-3 fatty acids in hypertriglyceridemic states. *Thromb. Haemost.* **14**: 271–284.
32. Braden, G. A., H. R. Knapp, and G. A. FitzGerald. 1991. Suppression of eicosanoid biosynthesis during coronary angioplasty by fish oil and aspirin. *Circulation.* **84**: 679–685.
33. Wainwright, P. E. 2002. Dietary essential fatty acids and brain function: a developmental perspective on mechanisms. *Proc. Nutr. Soc.* **61**: 61–69.
34. Roberts II, L. J., T. J. Montine, W. R. Markesbery, A. R. Tapper, P. Hardy, S. Chemtob, W. D. Dettbarn, and J. D. Morrow. 1998. Formation of isoprostane-like compounds (neuroprostanes) *in vivo* from docosahexaenoic acid. *J. Biol. Chem.* **273**: 13605–13612.
35. Murphy, R. C., and P. Wheelan. 1997. Metabolism of leukotrienes *in vitro* and *in vivo*. In *SRS-A to Leukotrienes: The Dawning of a New Treatment*. S. Holgate and S. E. Dahlen, editors. Blackwell Science Ltd., London. 101–120.
36. Keppler, D., M. Huber, W. Hagmann, H. A. Ball, A. Guhlmann, and S. Kastner. 1988. Metabolism and analysis of endogenous cysteinyl leukotrienes. *Ann. N. Y. Acad. Sci.* **524**: 68–74.
37. Roberts II, L. J., K. P. Moore, W. E. Zackert, J. A. Oates, and J. D. Morrow. 1996. Identification of the major urinary metabolite of the F_2 -isoprostane 8-*iso*-prostaglandin $F_{2\alpha}$ in humans. *J. Biol. Chem.* **271**: 20617–20620.
38. Burke, A., J. A. Lawson, E. A. Meagher, J. Rokach, and G. A. FitzGerald. 2000. Specific analysis in plasma and urine of 2,3-dinor-5,6-dihydro-isoprostane $F_{2\alpha}$ -III, a metabolite of isoprostane $F_{2\alpha}$ -III and an oxidation product of γ -linolenic acid. *J. Biol. Chem.* **275**: 2499–2504.
39. Kim, S., W. S. Powell, J. A. Lawson, S. H. Jacobo, D. Praticò, G. A. FitzGerald, K. Maxey, and J. Rokach. 2005. $iPF_{2\alpha}$ -III-17,18,19,20- d_4 : total synthesis and metabolism. *Bioorg. Med. Chem. Lett.* **15**: 1613–1617.
40. Chiabrando, C., A. Valagussa, C. Rivalta, T. Durand, A. Guy, E. Zuccato, P. Villa, J.-C. Rossi, and R. Fanelli. 1999. Identification and measurement of endogenous β -oxidation metabolites of 8-*epi*-prostaglandin $F_{2\alpha}$. *J. Biol. Chem.* **274**: 1313–1319.
41. Schulz, H., and W. H. Kunau. 1987. Beta-oxidation of unsaturated fatty acids: a revised pathway. *Trends Biochem. Sci.* **12**: 403–406.
42. Delorme, D., A. Foster, Y. Girard, and J. Rokach. 1988. Synthesis of β -oxidation products as potential leukotriene metabolites and their detection in bile of anesthetized rat. *Prostaglandins.* **36**: 291–302.
43. Audoly, L. P., B. Rocca, J. E. Fabre, B. H. Koller, D. Thomas, A. L. Loeb, T. M. Coffman, and G. A. FitzGerald. 2000. Cardiovascular responses to the isoprostanes $iPF_{2\alpha}$ -III and iPE_2 -III are mediated via the thromboxane A_2 receptor *in vivo*. *Circulation.* **101**: 2833–2840.
44. Kunapuli, P., J. A. Lawson, J. Rokach, and G. A. FitzGerald. 1997. Functional characterization of the ocular prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor. Activation by the isoprostane, 12-*iso*- $PGF_{2\alpha}$. *J. Biol. Chem.* **272**: 27147–27154.
45. Kunapuli, P., J. A. Lawson, J. Rokach, J. L. Meinkoth, and G. A. FitzGerald. 1998. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and the isoprostane, 8,12-*iso*-isoprostane $F_{2\alpha}$ -III, induce cardiomyocyte hypertrophy. Differential activation of downstream signaling pathways. *J. Biol. Chem.* **273**: 22442–22452.
46. Nourooz-Zadeh, J., E. H. C. Liu, B. Yhlen, E. E. Anggard, and B. Halliwell. 1999. F_4 -isoprostanes as specific marker of docosahexaenoic acid peroxidation in Alzheimer's Disease. *J. Neurochem.* **72**: 734–740.
47. Praticò, D., C. M. Clark, V. M. Y. Lee, J. Q. Trojanowski, J. Rokach, and G. A. FitzGerald. 2000. Increased 8,12-*iso*- $iPF_{2\alpha}$ -VI in Alzheimer's disease: correlation of a noninvasive index of lipid peroxidation with disease severity. *Ann. Neurol.* **48**: 809–812.