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Biochemistry

Docosahexaenoic acid is a strong inhibitor of prostaglandin but not leukotriene biosynthesis

(fish lipid/cardiovascular protective effect/chemical purification)

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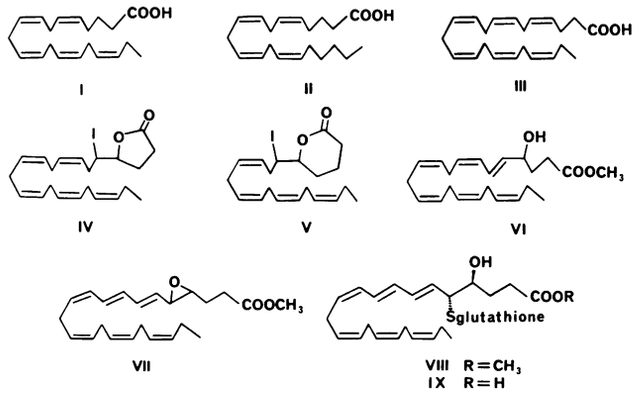
ABSTRACT **Docosahexaenoic acid (DCHA), a major polyunsaturated acid component of fish lipid, is not a substrate for prostaglandin synthetase from ram seminal vesicles but is a strong competitive inhibitor (K_i, 0.36 μM) of the conversion by this enzyme of arachidonate (K_m, 5.9 μM) to prostaglandins. In contrast, DCHA exhibits little interference to the conversion of arachidonate to metabolites on the leukotriene pathway. DCHA is a very poor substrate for the leukotriene-synthesizing system from RBL-1 cells and no formation of the C_{22} analog of leukotriene B could be detected from it. The C_{22} analog of leukotriene C_4 was produced by chemical synthesis from DCHA and found to be less than 1/10,000th as active as leukotriene C_4 in contracting guinea pig ileum. The DCHA used in this work was obtained in >99.8% purity by a practical process developed for its separation from fish lipid. The cardiovascular protective effects ascribed to dietary intake of fish lipid by certain populations may be due in part to the biological action of DCHA, including the inhibition of prostaglandin biosynthesis.**

Considerable evidence suggests that human populations whose dietary protein is derived mainly from fish (e.g., Greenland Eskimos) are at low risk with regard to cardiovascular disease as compared with populations consuming diets based on meat or dairy products (1–6). It is believed that an important factor underlying this dichotomy is the very different composition of the lipids of fish and those of meat or dairy products, especially with reference to the essential polyunsaturated fatty acids that can serve as prostaglandin (PG) precursors (2–5, 7, 8). Fish lipid



contains large amounts of icosapentaenoic acid (**I**) relative to arachidonic acid (**II**) whereas icosapentaenoic acid is only a very minor component of meat and dairy lipid. For this reason, attention has been drawn to the different biological properties of the PGs derived from arachidonate (PG_2s) and those from icosapentaenoate (PG_3s) (2, 5, 7, 8). Evidence has been presented that PGH_3 and thromboxane A_3 have considerably less activity than PGH_2 and thromboxane A_2 in causing platelet aggregation and also that icosapentaenoic acid is a somewhat poorer substrate for PG biosynthesis than arachidonate (5, 7, 8). These results are consistent with observations of long bleeding times and decreased platelet aggregation of Eskimos and other groups on a high fish diet. As a result of this accumulated information, there has been increasing interest in the use of icosapentaenoic acid as a cardioprotective agent (9, 10) and also for other medical applications (11–13).

Docosahexaenoic acid (**III**; DCHA), also known as clupanodonic acid, occurs in fish lipid in amounts comparable with icosapentaenoic acid (10) yet little is known about its biological action or roles, apart from the recent report of a prostanoid derived from DCHA (a $PGF_{4\alpha}$) in fish gill tissue (14). The present study, which has been focused on DCHA in contrast to previous work, has shown that (*i*) DCHA is not a substrate for a mammalian PG synthetase, (*ii*) DCHA is a potent competitive inhibitor of this mammalian prostaglandin synthetase, (*iii*) DCHA is only a weak competitive inhibitor of leukotriene (LT) biosynthesis from arachidonate, (*iv*) rat basophilic leukemia (RBL-1) cells that generate LTB_4 from arachidonate (15) do not convert DCHA to a C_{22} analog of this LT, and (*v*) a synthetic C_{22} analog of LTC_4 derived from DCHA shows essentially none of the spasmogenic activity characteristic of LTC_4, LTD_4, and LTE_4. In addition, a chemical method has been developed for obtaining very pure DCHA from fish oil that is suitable for a much larger scale of operation than the complex chromatographic processes previously used (16).

MATERIALS AND METHODS

Conversion of a Mixture of Icosapentaenoic Acid and DCHA to Iodolactone (IV). A mixture (1.278 g) of icosapentaenoic acid and DCHA [3:7 (mol/mol)], obtained from tuna fish oil (15), in tetrahydrofuran (4 ml) was added to sodium bicarbonate (1.36 g) in water (4 ml) and the solution was treated with aqueous sodium iodide (410 mg)/iodine (693 mg) at 23°C for 8.3 hr, by which time, iodine (initially equivalent to the DCHA in the mixture) had been consumed. The iodolactones **IV** and **V** were isolated at a ratio of 14:1 (1.475 g) by pentane extraction and separated by rapid chromatography on a silica gel column using methylene chloride for elution. The liquid iodo-γ-lactone **IV** (1.022 g), which eluted first, had the following properties: IR (CCl_4) 1,795 cm^{-1} (C = O) and R_f (TLC on silica gel, methylene chloride), 0.51. The iodo-δ-lactone **V** (32.5 mg eluted subsequently from the column) had the properties IR max (CCl_4) 1,755 cm^{-1} and R_f, 0.42. The structures of **IV** and **V** were confirmed

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Abbreviations: DCHA, docosahexaenoic acid; PG, prostaglandin; LT, leukotriene; RSVM, ram seminal vesicle microsomes; RBL, rat basophilic leukemia; HETE, hydroxyicosatetraenoic acid.

by their mass spectra (molecular ions at 454 and 428, respectively) and 270-MHz NMR spectra. In larger scale experiments, 50 g of a mixture of icosapentaenoic acid and DCHA gave pure **IV** and **V** with comparable efficiency using the same procedure with ordinary laboratory glassware.

Acidification and extraction of the aqueous layer from the iodolactonization process gave a mixture of unreacted DCHA and icosapentaenoic acid enriched in the latter to the degree calculated from the iodolactonization results.

The iodo-γ-lactone **IV** was converted to the methyl ester of DCHA in 90.2% overall yield and >99.8% purity (gas chromatographic analysis) by the sequence (*i*) reaction with triethylamine in methanol at reflux under argon for 7 hr to give the 4,5-epoxide of DCHA methyl ester (98.6% yield), (*ii*) reaction with excess sodium thiocyanate in methanol at reflux for 40 hr under argon to give the 4,5-episulfide of DCHA methyl ester (93.6%), and (*iii*) reaction with trimethyl phosphite at 70°C for 4 hr (97.8% yield).

Pure icosapentaenoic acid methyl ester and arachidonic acid methyl ester were obtained by the same process in >90% overall yield from the corresponding iodo-δ-lactones.

The free polyunsaturated acids were obtained from the methyl esters by saponification with 0.5 M lithium hydroxide in water/tetrahydrofuran (1:1) at 20°C for 16 hr under argon followed by acidification and extraction with ether/pentane (1:1) and stored under argon in frozen benzene at −45°C.

Inhibition of PG Synthetase by DCHA. The PG synthetase used for these studies was derived from ram seminal vesicle microsomes (RSVM) (17, 18). To 1.5 mg of RSVM in a 15 × 125 mm test tube with a Teflon stirring bar was added 3.0 ml of aqueous glutathione [3.0 μmol, 1.0 mM in 0.1 M Tris buffer (pH 8.3)] and 33 μl (150 nmol) of 4.54 mM aqueous hydroquinone and the suspension was stirred at 23°C for 10 min. Then, a 1.0-ml aliquot was transferred to a 10 × 75 mm culture tube with a Teflon stirring bar for the control run, 1.0 ml was placed in an identical tube for the inhibition run, and 1.0 ml was placed in a third tube for the blank run. A solution of DCHA (2.73 μl, 0.9146 mM, 2.5 nmol) was added to the inhibition reaction tube, and arachidonic acid [6.29 μl, 10 nmol, 1.5894 mM in 0.1 M Tris buffer (pH 8.3)] was then added to the control and to the inhibition reactions. The mixtures were stirred at 23°C in air, and 100-μl aliquots were withdrawn from each tube after 5, 10, 20, 40, 80, and 160 min and added to 6 × 50 mm culture tubes containing 32 μl (0.11 mmol) of 3.5 M aqueous KOH in 0.1 ml of methanol. This mixture was stirred at 55°C for 15 min to effect conversion of any PGE_2 to PGB_2 for UV analysis (19). Upon cooling, 12 μl (0.21 mmol) of acetic acid and 12 μl [*ca.* 0.6 nmol, 50 μM in 0.01 M phosphate buffer (pH 7.0)] of 4-phenylbenzoic acid as internal standard were added. This solution was filtered through glass wool and analyzed for PGB_2 [UV max 278 nm (ε 27,000)] (19) by reversed-phase HPLC using methanol/water/acetic acid (65:35:0.01) buffered to pH 5.6 with ammonia on a DuPont Zorbax Sil (C_{18}-silanized silica) column. In a typical run with 10 μM arachidonic acid, the ratio of rates of PGE_2 biosynthesis with no DCHA present and with 2.5 μM DCHA was 1:0.08 (i.e., 92% inhibition). Incubation of PG synthetase with DCHA at 23°C showed no conversion (<2%) to enzymic products even after 3 hr; all DCHA was recovered unchanged on extractive workup. In a control run, the same enzyme preparation converted arachidonate to PGE_2 (measured as PGB_2) in *ca.* 90% yield after 80 min.

The synthesis of PGE_2 by the RSVM PG synthetase was studied as a function of arachidonate concentration over the range 1.0 to 50 μM using the analytical procedure measuring PGB_2 described above. The results are summarized in Fig. 1. Data on the inhibition of PGE_2 synthesis in the presence of 0.40 and 1.0 μM DCHA were gathered as a function of arachidonate and are summarized in Fig. 2.

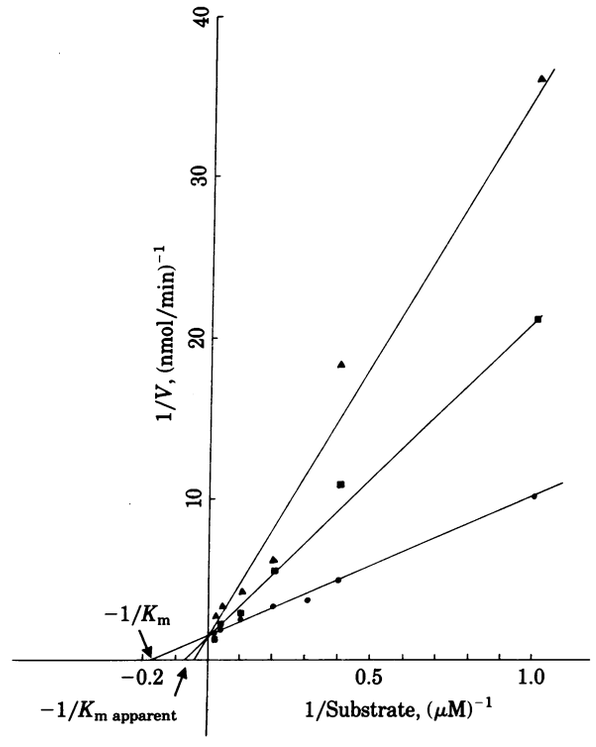

FIG. 1. Kinetic study of inhibition by DCHA of the enzymic conversion of arachidonate to PGE_2 by PG synthetase from RSVM. Lineweaver–Burk plot for DCHA at 0 (●), 0.4 (■), and 1.0 (▲) μM at pH 8.3, 20°C. $K_m = 5.88 \times 10^{-6}$ M.

Inhibition of the Conversion of Arachidonate to Products of the LT Pathway by DCHA. Enzymic conversion of arachidonate to the LT products (5*S*)-hydroxy-(6*E*)-(8,11,14*Z*)-icosatetraenoic acid (5-HETE) and (5*S*, 12*R*)-dihydroxy-(6,14*Z*)-(8,10*E*)-icosatetraenoic acid (LTB_4) was investigated using enzyme derived from RBL-1 cells that had been broken using a tissue disrupter, further homogenized in a blender, and centrifuged at 10,000 × *g*, all at 0°C. The supernatant containing 5-lipoxygenase and LTB_4 synthetase was stored at −78°C until needed in containers containing sufficient enzyme for a day's needs.

Incubations were carried out in 13 × 100 mm culture tubes suspended in a 22°C water bath-metabolic shaker apparatus. The reaction mixture consisted of 25 μl of $CaCl_2$ (20 mM), 25

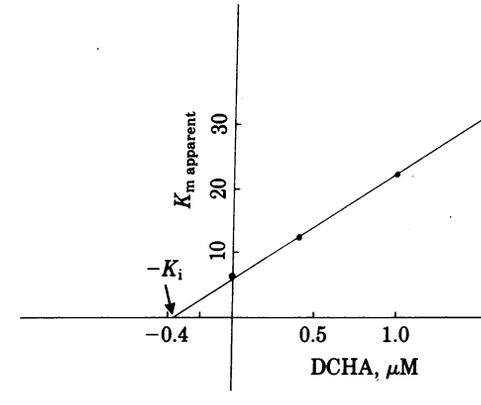

FIG. 2. Relationship between DCHA concentration and $K_{m\ apparent}$ for the enzymic conversion of arachidonate to PGE_2 by the PG synthetase from RSVM. $K_i = 0.36 \times 10^{-6}$ M.

μl of indomethacin (1 mM), 25 μl of ethanolic arachidonic acid (3.2 mCi/mmol; 1 Ci = 37 GBq), 25 μl of either DCHA or phosphate buffer (pH 7), 50 μl of 5-lipoxygenase (3.0 mg/ml), and 100 μl of 50 μM phosphate buffer (pH 7). Reactions were initiated by addition of [^{3}H]arachidonic acid to the previously equilibrated enzyme solution and was quenched at the appropriate time by the addition of 0.25 ml of 4 mM methanolic trimethylphosphite to reduce hydroperoxide. The mixture was spun on a Vortex and centrifuged, and the supernatant was decanted, acidified, and partitioned between ether and saturated brine. The dried ethereal extracts were combined and methylated with ethereal diazomethane. The methyl ester mixture was applied to the loading zone of a Whatman LK5DF TLC plate previously co-spotted with authentic methyl esters of 5-HETE, 15-HETE, LTB_4, and arachidonic acid. After air drying, the plate was developed in ethyl ether/hexane/acetic acid/methanol (50:50:1:0.1). The following bands (R_f) were scraped into scintillation vials for assay: arachidonic acid (0.77), 15-HETE (0.51), 5-HETE (0.37), and LTB_4 (0.15); a Beckman LS-2000 scintillation counter was used. Each vial contained seven drops of methanol and 12 ml of Packard Scint A toluene-based scintillation cocktail. The counting efficiency was 46.6% as judged by the addition of a known amount of [^{3}H]toluene.

DCHA was a weak competitive inhibitor of the conversion of arachidonate to 5-HETE and LTB_4; at 30 μM arachidonate and 300 μM DCHA, the formation of 5-HETE and LTB_4 was reduced by 37% and 48%, respectively. Lineweaver–Burk analysis of runs using varying concentrations of arachidonate and 300 μM DCHA at 22°C with an incubation time of 1 min showed competitive inhibition. K_m values for 5-HETE and LTB_4 formation in the absence of inhibitor were 10.87 and 9.76×10^{-6} M, respectively; in the presence of 300 μM DCHA, the corresponding values were 12.9 and 12.0×10^{-6} M. Dixon analysis (20) showed a K_i value for inhibition of 5-HETE of 390×10^{-6} M and a K_i for inhibition of LTB_4 of 410×10^{-6} M.

Oxidation of DCHA by Enzymes Extracted from RBL-1 Cells. In a typical run, 2 ml of enzyme (1.75 mg of total protein) was incubated aerobically at 22°C with 1.53 mg of DCHA in phosphate buffer, pH 7/4 mM $CaCl_2$/20 μM indomethacin for 1 hr. The product was analyzed by extraction of the quenched and acidified incubation mixture with ether followed by esterification with diazomethane and HPLC [DuPont Zorbax silica gel with hexane/tetrahydrofuran (15:1) as eluent] with UV detection at 235 nm. The enzymatic reaction was only 1/1000th as fast as with arachidonate. Two products were detected as methyl esters by HPLC: (*i*) A, retention vol, 32.3 ml; UV max 236 nm and (*ii*) B, retention vol, 36.0 ml; UV max 236 nm. A was identified as a 4-lipoxygenation product, methyl 4-hydroxy-(5*E*,7,10,13,16,19Z)-docosahexaenoate (**VI**) both by mass spectral analysis as the trimethylsilyl ether derivative and the corresponding saturated (dodecahydro) ester and by comparison (mass spectra and HPLC) with a synthetic reference sample. Mass spectra were determined using a Kratos MS-50 spectrometer by both ammonia chemical ionization and electron impact techniques. The chemical ionization spectrum of A showed MNH_4^+ at 448, $MH^+ - C_4H_7O_2$ at 344, and $MNH_4^+ - C_4H_7O_2$ at 361. The electron impact spectrum of the saturated trimethylsilyl ether of A showed $M^+ - OCH_3$ at 411 and $M^+ - C_4H_7O_2$ at 355. Synthetic A (**VI**) was prepared by reaction of iodolactone **IV** with diazabicyclo[5,3,0]undecene followed by saponification and methylation with diazomethane in ether (21, 22).

The chemical ionization mass spectrum of the trimethylsilyl derivative of B showed MNH_4^+ at 448, MH^+ at 431, and $MH^+ - C_7H_{11}O_2$ at 304 and the electron impact spectrum showed a peak for $MH^+ - OSiC_3H_9$ at 342. The corresponding saturated compound showed major peaks in the electron impact spectrum at 313 ($M^+ - C_7H_{13}O_2$) and 129 ($C_7H_{13}O_2^+$). The data on product B strongly suggest that it is the 7-lipoxygenation product, methyl 7-hydroxy-(4,10,13,16,19Z,8*E*)-docosahexaenoate.

4*S*-Hydroxy-(5*R*)-*S*-Glutathionyl-(6,8*E*)-(10,13,16,19Z)-Docosahexaenoic Acid (VIII) and Its 4,5-Diastereomer. The hydroxy methyl ester **VI** was converted via the 4-methanesulfonate ester to the corresponding 4-hydroperoxide (*ca.* 28% yield) using the procedure previously reported for the synthesis of 5-hydroperoxyicosatetraenoic acid methyl ester (21). The 4-hydroperoxide was then transformed via the triflate ester to the docosahexaenoate analog of LTA_4 methyl ester (**VII**) by the reported procedure (23). Pure **VII**, UV max 270, 279.5, and 290 nm, was isolated by preparative normal-phase HPLC on silica gel using hexane/ethyl acetate/triethylamine (100:0.6:0.6) for elution in a yield of 40% from the 4-hydroperoxide. Reaction of **VII** with excess glutathione and triethylamine in concentrated solution in methanol afforded two diastereomeric C (5) conjugates (**VIII** and the 4,5-diastereomer), which were separated by reversed-phase HPLC using a DuPont Zorbax Sil column (4.6 mm × 25 cm) using methanol/water/acetic acid, 65:35:0.01 (pH 5.6), for elution. The 4,5-diastereomers were eluted (flow rate, 2 ml/min) at 21 min (33% of mixture), UV max (CH_3OH) 271, 280, 290 nm, and at 26.6 min (67% of mixture), UV max (CH_3OH) 270.5, 280, 290.5 nm. Each of these diastereomers was hydrolyzed with aqueous lithium hydroxide in methanol/water to form the free C (1) carboxylic acid and was assayed for contractile activity on guinea pig ileum test strips (courtesy of Robert Lewis, Harvard Medical School) and found to be less than 1/10,000th as active as LTC_4.

RESULTS AND DISCUSSION

The most distinctive characteristic of fish lipid is the relative abundance of the two polyunsaturated fatty acids icosapentaenoic acid (**I**) and DCHA (**III**). Because of the importance of the C_{20} family consisting of PGs, LTs, and other metabolites of arachidonate (**II**) as bioregulators, attention has been directed toward the C_{20} fatty acid icosapentaenoic acid and its effect on biological systems rather than toward the C_{22} acid DCHA. A major result of the present study is the discovery that DCHA is a powerful though strictly competitive inhibitor of PG biosynthesis.

Pure DCHA is resistant to enzymic oxidation with an unpurified preparation of PG synthetase derived from RSVM and thus appears not to be a precursor of prostanoids in this system, in contrast to icosapentaenoic acid, which is enzymically transformed into members of the PG_3 series (24, 25). Although not a substrate for the RSVM PG synthetase, DCHA is a remarkably potent competitive inhibitor (Fig. 1). The K_i value determined for the competitive inhibition by DCHA of the conversion of arachidonate to PGE_2 by RSVM enzyme was 0.36 μM (Fig. 2). This value is about 6% of the K_m value for arachidonate as substrate (5.88 μM) with the same enzyme. Thus, for an *in vivo* situation in which free arachidonate and DCHA might be present at equal concentrations, PG biosynthesis would be expected to be only 5% of that occurring in the absence of DCHA. In such a circumstance, there would be associated with DCHA an antiinflammatory activity, analogous to that of known nonsteroidal antiinflammatory agents, such as indomethacin, that inhibit the conversion of arachidonate to PGs. In addition, an antithrombotic effect of DCHA would be a logical possibility because of reduced biosynthesis of PGG_2, PGH_2, and thromboxane A_2, all of which are powerful inducers of platelet aggregation.

In contrast to the potentially important effect of DCHA on prostanoid biosynthesis, the influence of DCHA on the LT

pathway (15, 26) appears to be minimal. First of all, DCHA is only a weak competitive inhibitor of the conversion of arachidonate to 5-HETE and LTB_4 by RBL-1 enzymes; at equal concentrations of DCHA and arachidonate, the enzymic synthesis of 5-HETE and LTB_4 is reduced by less than 5%. Further, incubation of DCHA with a RBL-1 enzyme preparation that converts arachidonate to 5-HETE and LTB_4 afforded only miniscule conversion to products, specifically the 4-lipoxygenation product, 4-hydroxy-(5*E*,7,10,13,16,19Z)-docosahexaenoate (0.05% yield), and the 7-lipoxygenation product, 7-hydroxy-(4,10,13,16,19Z,8*E*)-docosahexaenoate (0.5% yield). These monohydroxy acids are not known to possess biological activity and, though deserving of further biological scrutiny, are unlikely to manifest LT-like actions. Although the DCHA analogs of LTC, LTD, and LTE have not been detected as biotransformation products of DCHA, the LTC_4 analog **IX** has been synthesized in this work and tested for activity on smooth muscle. The finding of little or no spasmogenic activity indicates that even if **IX** were formed biologically from DCHA, it would not function as a spasmogenic LT.

The results reported here argue strongly that DCHA may be a biologically important fatty acid in a regulatory sense and thus is deserving of careful study. Substantial amounts of DCHA can be expected in body tissues, depending on dietary intake. Dogs on a diet in which 25% of calories were derived from fish oil were found to have approximately equal concentrations of arachidonate and DCHA in the free fatty acid component of blood serum (10). Eskimos in northwestern Greenland whose diet is rich in fish ingest much more DCHA (5.9% of total fatty acids) than arachidonate (0.4% of total fatty acids) (3).

Careful study needs to be made with regard to the effect of DCHA on prostaglandin synthesis in various body cells and tissues and the question of whether the cardiovascular protective effect of a fish diet may be due to a considerable extent to DCHA. The claim (27) that DCHA sensitizes heart muscle to catecholamines, perhaps leading to sudden death, points up the need to investigate a range of biological effects of DCHA and other key unsaturated components of fish lipid one at a time using pure fatty acids or esters, and it emphasizes the importance of fundamental biochemical and physiological studies, rather than the nutritional approach via dietary supplementation.

The availability of pure DCHA by the process outlined herein should accelerate research on its biological actions. Reaction of the sodium salt of DCHA in aqueous solution with iodine/sodium iodide reagent converts DCHA preferentially to the γ-lactone **IV** rather than to the isomeric δ-lactone, in accord with expectations based on the known greater stability and faster rates of formation of γ- over δ-lactones. Similarly, the rate of iodolactonization of DCHA to form **IV** is considerably faster than the iodolactonization of icosapentaenoic acid, which leads to δ-lactone **V**. Thus selective iodolactonization of DCHA in a mixture of fatty acids, extractive separation of neutral iodolactones from fatty acid salts, chromatographic purification of **IV**, and elimination to regenerate the 4,5-Z olefinic linkage provides a practical process for generating >99.8% pure DCHA in substantial quantities.

The chemical synthesis of various oxidative metabolites from pure DCHA by using the selective processes already developed for arachidonic acid and totally synthetic procedures can make available a range of structures that may have significant biological function. One such structure is the marine-derived C_{22} prostanoid reported recently (14).

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