Biochemical Pharmacology, Vol. 55, pp. 405–411, 1998.



ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00497-8

# α- and β- Alkyl-Substituted Eicosapentaenoic Acids

## Incorporation into Phospholipids and Effects on Prostaglandin H Synthase and 5-Lipoxygenase

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**ABSTRACT.** α-ethyl-, α-methyl- and β-methyl eicosapentaenoic acid (EPA) were prepared and their incorporation into cell lipids and effects on eicosanoid synthesis compared with EPA and docosahexaenoic acid (DHA). α- and β-methyl EPA were incorporated into hepatocyte triacylglycerols as efficiently as EPA, whereas lesser amounts were found in phospholipids. α-ethyl EPA was not incorporated into phospholipids but small amounts were detected in triacylglycerol. All derivatives inhibited the synthesis of arachidonic acid, although less efficiently than EPA and DHA. The derivatives were poor substrates of prostaglandin H (PGH) synthase and 5-lipoxygenase, and they all inactivated PGH synthase. In isolated platelets, α-methyl EPA was a stronger inhibitor of $TxB_2$ production than EPA, α-ethyl- and β-methyl EPA. All derivatives were stronger inducers of peroxisomal β-oxidation than EPA and DHA. This increased induction probably is a consequence of the blocked mitochondrial β-oxidation of the derivatives. BIOCHEM PHARMACOL 55;4:405–411, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** EPA derivatives; phospholipids; triacylglycerols; eicosanoids; β-oxidation

It is believed that the effects of fish oils (eicosapentaenoic acid (EPA)§, docosahexaenoic acid (DHA)) on cardiovascular diseases [1] are connected with the incorporation of the n-3 fatty acids into the phospholipids, at the expense of arachidonic acid. When liberated from phospholipids, EPA and DHA may inhibit the conversion of arachidonic acid into eicosanoids or be converted to different eicosanoids with reduced effects. The n-3 double bond of these fatty acids is important for the fatty acid effects on eicosanoid synthesis [2, 3].

Recently, we have shown that heneicosa-6,9,12,15,18-pentaenoic acid (EPA elongated with a methylene group at the carboxyl end) is incorporated into phospholipids to a similar extent as EPA and DHA but is almost inactive as substrate for PGH (prostaglandin H) synthase and 5-lipoxygenase.

In the present study we introduced a methyl or an ethyl group in the α-/β-position of EPA. This is sufficient to prevent its β-oxidation in mitochondria [4], leading to peroxisomal and presumably slower oxidation of these fatty acids. This may change or increase their metabolic effects. Thus, α-methyl EPA has a stronger hypolipemic effect than EPA in rats [5].

We studied the incorporation of these derivatives into phospholipids and triacylglycerols, and their ability to act as substrates and/or inhibitors of PGH synthase and 5-lipoxygenase.

Since fatty acids which are poorly metabolized by mitochondria often induce peroxisomal β-oxidation [6], we measured the ability of the α- and β-substituted EPA derivatives to induce peroxisomal acyl-CoA oxidase.

## MATERIALS AND METHODS

### *Materials*

[1-$^{14}$C]arachidonic acid (50–60 mCi/mmol) and [1-$^{14}$C]EPA (58 μCi/μmol) were obtained from Amersham Laboratories. Arachidonic acid as well as reduced glutathione, hydroquinone, prostaglandin-/leukotriene standards, phenylmethylsulfonyl fluoride (PMSF), trypsin inhibitor Type II-S: Soybean, ATP, and Tris were all purchased from Sigma. The ethyl esters of EPA and DHA were gifts from Norsk Hydro. 2,7 dichlorofluorescein diacetate was obtained from Eastman Kodak Company, and Picofluor™ 40 from Packard Instrument Company, Inc. Dulbecco's minimum essential medium (DMEM) w 74500 mg/L, glucose, sodium pyruvate, ultroser and gentamicin, anti PPLO, fungizone, penicillin, streptomycin and Ham's F10 medium were from Gibco. Plastic culture dishes and bottles were

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§ *Abbreviations*: AA, arachidonic acid, C20:4, n-6; DHA, docosahexaenoic acid, C22:6, n-3; 5,12 DiHETE, 5,12 dihydroxy-eicosatetraenoic acid ($LTB_4$); αe C20:5, α-ethyl EPA; αm C20:5, α-methyl EPA; βm C20:5 (3-$CH_3$ C20:5), β-methyl EPA; EPA, eicosapentaenoic acid, C20:5, n-3; GSH, reduced glutathione; 5-HETE, 5-hydroxyeicosa-tetraenoic acid; 5-HPETE, 5-hydroperoxy-eicosatetraenoic acid; 1-DCF, leucodichlorofluorescein-diacetate; LT, leukotriene; PG, prostaglandin; RP-HPLC, reversed phase high performance liquid chromatography.

Received 18 February 1997; accepted 30 July 1997.

obtained from Costar. The $TxB_2$ EIA kit was obtained from The Cayman Chemical Company.

### *Preparation of α-Methyl EPA and α-Ethyl EPA*

**2-METHYLEICOSA-5,8,11,14,17-PENTAENOIC ACID (α-METHYL EPA).** Butyllitium (0.34 mL, 0.54 mmol, 1.6 M in hexane) was added dropwise to a stirred solution of diisopropylamine (0.080 mL, 0.57 mmol) in dry THF (0.60 mL) under nitrogen at −20°. The mixture was stirred at −75° for 45 min before dropwise addition of EPA ethyl ester (150 mg, 0.46 mmol) in dry THF (2.5 mL). After stirring for 30 min at −75°, methyl iodide (0.045 mL, 0.72 mmol) was added. The mixture was stirred at 0° for 30 min. before being poured into water (2.0 mL). The water phase was separated and extracted with hexane (2 × 2.0 mL). The organic solution was washed with 2M HCl (3.0 mL) and water (2 × 3.0 mL) and dried ($MgSO_4$). Filtration and evaporation gave 120 mg (77%) of α-methyl-EPA-ethyl ester.

$^1H$ NMR (200 MHz, $CDCl_3$): δ 0.94 (t, 3H, *J*7.5), 1.13 (d, 3H, *J*7.0), 1.23 (t, *J*7.1), 1.33–1.51 (m, 1H), 1.64–1.83 (m, 1H), 1.97–2.12 (m, 4H), 2.33–2.49 (m, 1H), 2.75–2.84 (m, 8H), 4.1 (q, 2H, *J*7.1), 5.22–5.43 (m, 10H)

$^{13}C$ NMR (50 MHz, $CDCl_3$): δ 14.6, 17.4, 20.8, 25.2, 25.9, 33.8, 39.2, 60.2, 126.7, 127.5, 127.7, 127.9, 128.1, 128.2, 128.9, 131.6

A solution of LiOH (110 mg, 2.6 mmol) in water (1.5 mL) was added to a stirred solution of α-methyl-EPA-ethyl ester (120 mg, 0.35 mmol) in ethanol (1.5 mL) at room temperature. The mixture was left stirring overnight. Water and pet.ether were added and the organic phase (containing unreacted ester) was collected. The water phase was acidified with 5% HCl to pH 2 and extracted with pet.ether (3×). The organic solution was washed with water and $NaCl_{aq}$ and dried ($MgSO_4$). Filtration and evaporation gave 80 mg (73%) of α-methyl EPA.

$^1H$ NMR (200 MHz, $CDCl_3$): δ 0.95 (t, 3H, *J*7.5), 1.18 (d, 3H, *J*7.5), 1.35–1.59 (m, 1H), 1.65–1.88 (m, 1H), 1.98–2.19 (m, 4H), 2.35–2.58 (m, 1H), 2.68–2.95 (m, 8H), 5.22–5.51 (m, 10H)

$^{13}C$ NMR (50 MHz, $CDCl_3$): δ 14.6, 17.4, 20.8, 25.2, 25.9, 33.8, 39.2, 60.2, 126.7, 127.5, 127.7, 127.9, 128.1, 128.2, 128.9, 131.6.

[$^{14}CH_3$]-α-methyl EPA was prepared as described above.

**2-ETHYLEICOSA-5,8,11,14,17-PENTAENOIC ACID (α-ETHYL EPA).** The α-ethyl eicosapentaenoic acid was prepared from EPA ethyl ester and ethyl iodide by the above procedure.

$^1H$ NMR (200 MHz, $CDCl_3$): δ 0.93 (t, 3H, *J*7.3), 0.96 (t, 3H, *J*7.5) 1.39–1.85 (m, 4H), 1.95–2.19 (m, 4H), 2.22–2.42 (m, 1H), 2.68–2.95 (m, 8H), 5.21–5.52 (m, 10H).

$^{13}C$ NMR (50 MHz, $CDCl_3$): δ 12.4, 15.0, 21.2, 25.6, 25.7, 26.1, 26.2, 31.9, 46.8, 126.4, 127.2, 127.5, 127.6, 127.9, 128.0, 128.4, 131.3, 181.6.

β-methyl EPA was prepared through a multistep synthesis.*

Synthesis of α- and β-branched EPA derivatives resulted in racemic mixtures of S and R forms. Metabolism of the racemic mixture of α-methyl EPA is ensured by the α-methylacyl-CoA racemase [7].

### *Methods*

Methods are essentially as described earlier [8].

### *7800 C1 Morris Hepatoma Cells*

The cells were cultivated as monolayers in 60 × 15 mm culture dishes, and grown in Ham's F 10 medium supplemented with 10% horse serum and 3% calf serum as described by Richardson *et al.* [9]. The medium was supplemented with penicillin (50 U/mL), streptomycin (50 μg/mL), fungizone (2.5 μg/mL) and anti PPLO (pleuropneumonia-like organisms) (60 μg/mL), and was changed every 48 hr. The cells were incubated at 37° in humidified atmosphere of 5% $CO_2$ and 95% air and grown to confluence before use in experiments. The cells were harvested in 1–2 mL PBS, by using a rubber policeman.

### *RBL-1 Cells*

RBL-1 cells were grown essentially as described by Kulczycki *et al.* [10] in suspension culture, in minimum essential medium with Earle's salts and 2 mM L-glutamine. The medium was supplemented with 15% inactivated foetal calf serum, penicillin (50 U/mL), streptomycin (50 μg/mL), fungizone (2.5 μg/mL) and anti PPLO (60 μg/mL) and was changed every 48 hr. Cells were harvested and crude 5-lipoxygenase extracted as described by Haurand and Flohe [11].

### *Statistics*

The Student's *t*-test was used for statistical calculations.

## RESULTS

### *Incorporation of EPA, DHA and EPA Derivatives into Phospholipids and Triacylglycerols*

Since most of the substrates for PGH synthase and 5-lipoxygenase are fatty acids liberated from phospholipids, we compared the incorporation of the derivatives into hepatocyte phospholipids and triacylglycerols with those of EPA and DHA, all at a concentration of 80 μM in the medium (fatty acid/albumin ratio 2.5/1). At this concentration, no visible toxic effects were seen. Table 1 shows that EPA and DHA were incorporated partly at the expense of oleic acid, the only other fatty acid which

* Rikenes OJ, Flock S and Skattebøl L, unpublished work.

**TABLE 1. Incorporation of EPA, DHA and α- and β-substituted eicosapentaenoic acid into hepatocyte phospholipid and triacylglycerol**

| Fatty acid added | Percent of fatty acids 18:1 | 20:4 | 20:5 | 22:6 | Derivative |
|---|---|---|---|---|---|
| A: Phospholipids | | | | | |
| 0 | 8.1 ± 1.5 | 25.7 ± 4.0 | ND | 3.9 ± 1.2 | |
| 20:5 | 5.5 ± 1.1* | 24.3 ± 1.7 | **10.3 ± 2.6** | 3.7 ± 1.1 | |
| 22:6 | 5.5 ± 1.2* | 29.9 ± 4.5 | 1.4 ± 1.8 | **9.6 ± 1.1** | |
| αm C20:5 | 7.0 ± 1.2 | 26.5 ± 2.1 | ND | 4.1 ± 1.6 | **2.8 ± 0.5** |
| αe C20:5 | 7.8 ± 2.1 | 26.9 ± 2.0 | ND | 4.8 ± 1.7 | ND |
| βm C20:5 | 6.9 ± 1.4 | 25.5 ± 2.5 | ND | 4.0 ± 1.8 | **5.5 ± 2.8** |
| B: Triacylglycerol | | | | | |
| 0 | 25.9 ± 2.7 | 3.7 ± 2.6 | ND | 1.4 ± 0.8 | |
| 20:5 | 18.2 ± 2.2* | 5.7 ± 1.9 | **14.3 ± 9.3** | 2.2 ± 0.9 | |
| 22:6 | 17.3 ± 1.9** | 5.1 ± 1.8 | 2.3 ± 0.9 | **18.1 ± 5.9** | |
| αm C20:5 | 18.3 ± 2.7** | 6.5 ± 2.6 | ND | 1.6 ± 1.2 | **14.0 ± 3.7** |
| αe C20:5 | 17.9 ± 0.6** | 6.2 ± 3.5 | ND | 1.8 ± 1.2 | **5.2 ± 2.1**[a] |
| βm C20:5 | 14.7 ± 1.7** | 4.5 ± 2.5 | 0.1 ± 0.2 | 1.4 ± 0.9 | **27.6 ± 6.8** |

Plated rat hepatocytes (1–1.5 mg cell-protein per dish) were incubated in medium without or with 80 μM (final concentration) of EPA, DHA or EPA derivatives for 24 hr with renewal of the medium after 8 hr. BSA was added to a final concentration of 32 μM, giving a fatty acid:BSA ratio of 2.5:1. Phospholipids and triacylglycerol were separated by anion-exchange chromatography on bonded phase columns [12]. Fatty acid composition was analysed with G.C., with results presented as percent of total amount of fatty acid in A: phospholipids/B: triacylglycerol. Results are shown as means of three experiments, each with 2–3 parallels ± SD. Bold numbers indicate incorporation of the added fatty acid/derivative.

ND: not detectable.

*$P < 0.005$, **$P < 0.001$ vs control; a: $P < 0.001$ vs α-methyl EPA.

showed significant change. Only small amounts of α-methyl EPA were detected in the phospholipids after 24 hr (2.8% of the fatty acids). β-methyl EPA was more easily incorporated than α-methyl EPA, particularly into triacylglycerol. No incorporation of α-ethyl EPA was detected. No decrease in arachidonic acid was observed. In triacylglycerols, α-methyl EPA was incorporated to a similar extent as EPA and DHA, again mainly at the expense of oleic acid. Significant incorporation of α-ethyl EPA was also observed. The relative content of other fatty acids in the phospholipids and triacylglycerols showed no significant changes in concentration and are therefore not included.

EPA, DHA, α-methyl, β-methyl EPA and α-ethyl EPA were incorporated into hepatoma cell lipids in a similar manner as in liver cell lipids, mainly at the expense of arachidonic acid (not shown). Again α-ethyl EPA was less incorporated than the α-methyl analogue. Fractionation and gas chromatography analyses of lipids extracted from hepatoma cells showed that the β-methyl EPA was incorporated mainly into the triacylglycerols (not shown), as in the hepatocytes. Table 2 shows a comparison of the metabolism of [1-$^{14}$C]AA, [1-$^{14}$C]EPA and [$^{14}CH_3$]α-methyl EPA in hepatoma cells. Here, α-methyl EPA showed the slowest rate of oxidation to acid-soluble products and the lowest incorporation into phospholipids, but showed the highest incorporation into triacylglycerol ($CO_2$ was not measured). Pilot experiments showed that α-methyl EPA was less incorporated than β-methyl EPA into the lipids of mouse macrophages, while α-ethyl EPA was not incorporated.

### *Conversion of $^{14}$C-Linoleic Acid into Arachidonic Acid*

EPA and DHA are known to decrease the delta 6 desaturation of linoleic acid and its conversion to arachidonic acid [13, 14]. This was confirmed by Fig. 1, where EPA and DHA decreased arachidonic acid formation to less than 1/3 of control. The effect of α-methyl EPA and particularly that of α-ethyl EPA was significantly less than that of EPA. In addition, when the methyl group was moved into the β-position of EPA, the inhibitory effect decreased (results not shown).

### *PGH Synthase*

We used two methods to measure the substrate specificity of PGH synthase.

Table 3 shows that the initial rate of oxygen consump-

**TABLE 2. Uptake and metabolism of [1-$^{14}$C]AA, [1-$^{14}$C]-EPA and [$^{14}CH_3$]-α-methyl EPA into hepatoma cells**

| | AA | EPA | α-m | EPA |
|---|---|---|---|---|
| Total lipids | 268.9 ± 16.2 | 197.5 ± 8.8 | 299.9 | 341.8 |
| Triacylglycerols | 156.6 ± 11.01 | 121.0 ± 6.2 | 243.3 | 242.7 |
| Phospholipids | 93.6 ± 4.5 | 57.5 ± 5.9 | 31.1 | 35.0 |
| Oxidized* | 110.9 ± 3.7 | 135.6 ± 7.0 | 76.5 | 79.1 |

Confluent cells were incubated with 80 μM fatty acid for 3 days with change of medium on the second day. Cells were harvested and lipids were extracted with butanol and run on thin layer chromatography to separate phospholipids and triacylglycerols. The amounts of acid-soluble products in the medium were measured by the addition of perchlor acid, centrifugation and counting radioactivity in the supernatant. Results are presented as nmol ± SD and are the means of 3 parallels (for AA and EPA) or 2 parallels (α-methyl EPA). *Acid-soluble radioactivity in the medium.

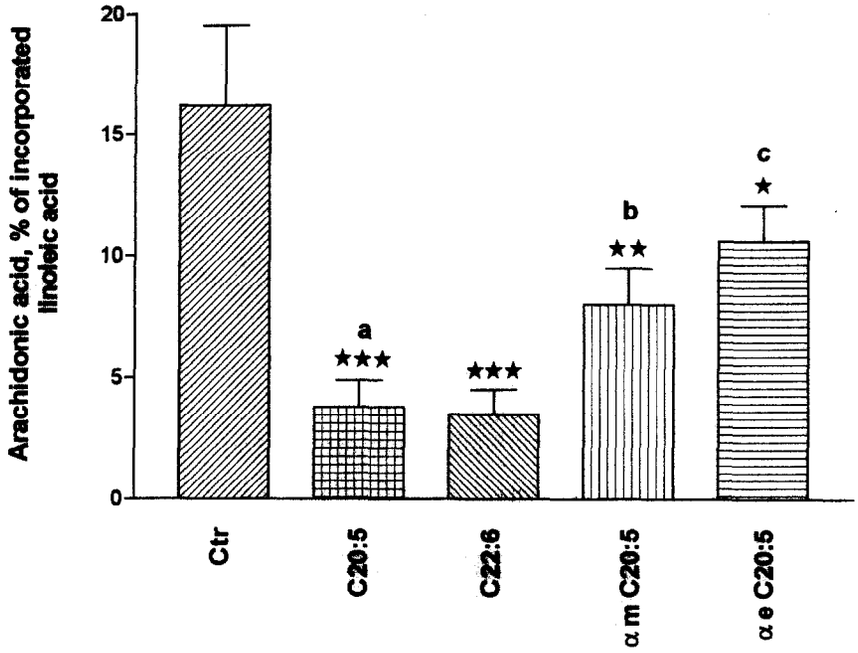


FIG. 1. Inhibition of arachidonic acid synthesis in confluent hepatoma cell cultures. Hepatoma cells were incubated for three days with [1-$^{14}$C]linoleic acid (50 μM) and with EPA, DHA or EPA derivatives (50 μM). The medium with fatty acids was renewed after 48 hr. After cell harvesting, lipids were extracted, methylated and run on HPLC. The added n-3 fatty acids had no effect on the incorporation of $^{14}$C-Linoleic acid into the cells. Results are presented as % of incorporated linoleic acid converted into arachidonic acid. Each bar indicates the mean of two experiments, each with 2–3 parallels ± SD. *P < 0.01, **P < 0.001, ***P < 0.0001 vs Control; a is statistically different from b and c (P < 0.05).

tion with EPA as substrate was 1/4–1/2 as fast as with arachidonic acid. DHA and the α- and β-alkyl derivatives were all poor substrates.

Figure 2A represents one of many similar experiments where the oxidation of the dye 1-DCF as reducing cosubstrate was measured. EPA again gave a reaction rate that is slower than that of arachidonic acid but much faster than DHA, the latter being a very poor substrate. Although all derivatives were poor substrates, α-methyl EPA and β-methyl EPA were better substrates than DHA and α-ethyl EPA.

Table 3 and Fig. 2A demonstrate a reasonably good agreement between the two assay methods with regard to the relative reaction rates with the different substrates.

**TABLE 3. Substrate specificity of cyclooxygenase and 5-lipoxygenase**

| | $O_2$ uptake (nmol/min) | | | |
|---|---|---|---|---|
| | Cyclooxygenase | | 5-Lipoxygenase | |
| Fatty acid | 30 μM | 60 μM | 100 μM | |
| C20:4 | 439.6 ± 26.7 | 563.9 ± 33.5 | 138 | 62 |
| C20:5 | 109.5 ± 2.5 | 260.2 ± 29.4 | 147 | 64 |
| αm C20:5 | 29.7 ± 2.8 | 49.7 ± 8.5 | 23 | 3 |
| αe C20:5 | 16.4 ± 6.7 | 7.8 ± 1.9 | 8 | 3 |
| βm C20:5 | 29.2 ± 2.2 | 30.2 ± 1.6 | 9 | |
| C22:6 | 28.3 ± 3 | 42.8 ± 5.4 | 24 | 4 |

Crude PGH synthase (63 μg protein) was incubated with 30 or 60 nmol/mL fatty acid/derivative, 0.33 mM glutathione, 0.28 mM hydroquinone (or 0.5 mM phenol) in 0.1 M Tris at 30°. Oxygen consumption was monitored for ca. 2 min, and the initial reaction rate was estimated. Results are presented as means of three parallels ± SD. Crude 5-lipoxygenase ($5 \times 10^7$ cell equivalents) was incubated with 100 nmol/mL fatty acid/derivative, 8 mM ATP, 3 mM $CaCl_2$ and 3 mM GSH in 50 mM Tris (pH 7.5). Oxygen consumption was followed for 2–3 min. The initial reaction rate was estimated from the initial slope of the oxygraph curve. The results represent two independent experiments. Oxygen concentration in the buffer was assumed to be 221 nmol $O_2$/mL [15].

However, the spectrophotometric assay was far more sensitive and was used with much smaller amounts of enzyme and lower substrate concentrations. Figure 2A shows that PGH synthase rapidly lost activity upon addition of substrate.

This autoinactivation of the enzyme with the different derivatives was tested with both assay methods by preincubating the enzyme with arachidonic acid, EPA, DHA, or derivatives. Figure 2B shows that by preincubation with 30 μM fatty acid or derivative for 1 min before the addition of 60 μM arachidonic acid, EPA, DHA, α-methyl-, β-methyl- and α-ethyl EPA inactivated the enzyme almost as rapidly as arachidonic acid itself. EPA was the only fatty acid which inactivated the enzyme to a greater extent than AA ($P < 0.0001$). This inactivation took place in the presence of reducing cosubstrates (GSH + phenol). In the absence of reducing cosubstrates the inactivation was much more rapid [16]. We confirmed this in experiments with the more sensitive spectrophotometric assay, where the reducing cosubstrates were added along with the arachidonic acid *after* the preincubation period.

### *Thromboxane Synthesis*

Arachidonic acid is a well-known inducer of platelet aggregation [17, 18]. By adding EPA, DHA or the α- and β-substituted EPA derivatives to the platelet suspension 30 sec before addition of equal amounts of arachidonic acid, the inhibitory effect of these fatty acids and derivatives on arachidonic acid-induced thromboxane production was measured. The control represent thromboxane formation after AA addition to platelets not preincubated with any fatty acid. Figure 2C shows that α-methyl EPA was a stronger inhibitor than EPA and DHA. α-ethyl EPA and

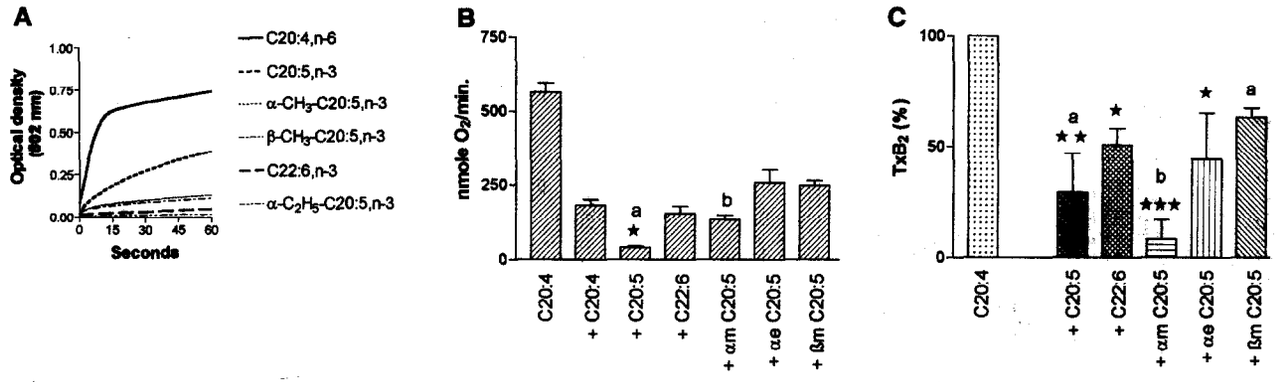


**FIG. 2. A) Substrate specificity of PGH synthase, measured as oxidation of reducing cosubstrate (1-DCF). Crude PGH synthase (40 μg protein) was incubated with EPA, DHA or EPA derivatives in a concentration of 7.5 μM. Reaction mixture: 0.4 mM phenol, 20 μM 1-DCF, 1 μM hemin, substrate and enzyme. The absorbance at 502 nm was monitored. B) Arachidonic acid, EPA, DHA, α- and β-substituted EPA derivatives as inhibitors/competing substrates of PGH synthase, measured as oxygen uptake. Crude PGH synthase (63 μg) was preincubated with 30 μM fatty acid or derivative, 0.33 mM glutathione, 0.28 mM hydroquinone or 0.5 mM phenol in 0.1 M Tris (pH 8) at 30° M for 1 min before addition of 60 μM arachidonic acid. The initial reaction rate was measured and nmoles of oxygen consumed per minute calculated. Oxygen concentration in the buffer was assumed to be 221 nmol $O_2$/mL [21]. The reaction rate with 60 μM arachidonic acid without preincubation is represented by the first column. Each bar indicates the mean of 3 parallels ± SD. *P < 0.0001 vs C20:4; a and b are statistically different (P < 0.05). C) Inhibition of $TxB_2$ production in isolated human blood platelets after preincubation with polyunsaturated fatty acids or EPA derivatives. Isolated blood platelets (3 × $10^8$) from human volunteers were preincubated in 1 mL Thyrode–Hepes buffer at 37° for 30 sec without (control) or with 10 μM EPA, DHA or α-substituted EPA derivatives, before addition of 10 nmol arachidonic acid. Production of thromboxane $B_2$ was estimated after 4 min by EIA [19]. Thromboxane $B_2$ formation is given as % of the formation in platelets not exposed to inhibitors. Each bar represents the mean of five experiments ± SD. *P < 0.05, **P < 0.005, ***P < 0.0005 vs C20:4; a and b are statistically different (P < 0.05).**

β-methyl EPA inhibit $TxB_2$ production to a lesser extent, similar to that observed for DHA.

The experiment was repeated five times with platelets from different human volunteers. The amounts of $TxB_2$ formed upon addition of arachidonic acid varied from one individual to the other. The absolute values of $TxB_2$ formed were 324–1460 pmol thromboxane $B_2$ (3 × $10^8$ platelets), and the effect of inhibition was thus calculated as percent of that obtained with arachidonic acid alone in each batch of platelets.

Feeding experiments show that α-methyl EPA inhibited the aggregation of platelets by 50–70% compared to platelets from eicosapentaenoic acid-treated rats [5]. In the feeding experiments, platelet aggregation was induced by ADP and A 23187, whereas our platelets were induced by arachidonic acid.

### *5-Lipoxygenase*

Table 3 shows, as previously reported [20], that EPA and arachidonic acid were almost equal as substrates for 5-lipoxygenase, while α-methyl EPA, β-methyl EPA, α-ethyl EPA and DHA showed very low reaction rates. α-ethyl EPA gave a barely detectable oxygen uptake. Similar results were obtained in other experiments. Because of variable activities in different preparations of 5-lipoxygenase and because of fast, spontaneous inactivation of the enzyme, no statistical treatment was possible. Figure 3 shows that α-methyl, β-methyl and α-ethyl EPA, which are poor substrates (Table 3), inhibited the conversion of radioactive arachidonic acid into 5-HETE and 5-HPETE as efficiently as AA. EPA, a very good competing substrate (Table 3), appeared to be the strongest inhibitor.

### *Induction of Acyl-CoA Oxidase*

Figure 4 demonstrates that α-methyl- and α-ethyl EPA were particularly strong inducers of acyl-CoA oxidase, while EPA and DHA were weak inducers.

## DISCUSSION

Long chain n-3 fatty acids from fish oil (EPA and DHA) have hypolipemic and antithrombotic effects. These effects are believed to be connected with their ability to compete with arachidonic acid in its reactions. It is in this context that we have studied the effects of EPA derivatives. Depending on the specificity of the different enzymes involved, the biological effects of the n-3 fatty acids might be increased or disappear. Since α-methyl EPA and probably other α-alkyl derivatives are more slowly broken down by β-oxidation [4] and as β-oxidation of β-methyl EPA is prohibited, their effect(s) might be increased if the derivatives were to retain their ability to be metabolized in (or inhibit) other enzyme systems involved.

Recently, sufficient α-methyl EPA was synthesized and tested in feeding experiments. It proved to be more hypolipemic than EPA, inducing increased peroxisomal oxidation in the liver of rats more efficiently than EPA and reducing platelet aggregation in the animals as well [5]. We have confirmed that a fatty acid with an α-methyl group is more slowly oxidized in liver cells, presumably because mitochon-

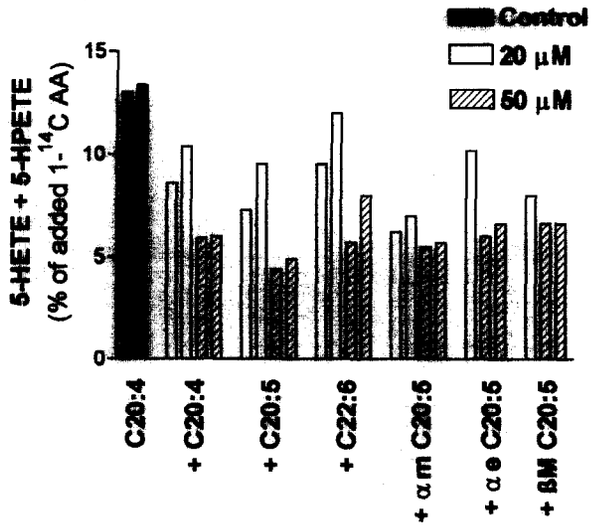


FIG. 3. EPA, DHA and EPA derivatives as inhibitors/competing substrates of 5-lipoxygenase. Crude 5-lipoxygenase, 2.5 × $10^6$ cell equivalents, was incubated for 10 sec with 20 μM [1-$^{14}$C]arachidonic acid without (control) and with EPA, DHA or EPA derivatives, 20 or 50 μM. The reaction was stopped by adding 500 μL acetone + 2 drops of HCOOH, and the products were extracted with $CHCl_3$. $CHCl_3$ was evaporated under a stream of $N_2$, and the residues were dissolved in methanol:water (3:1) and analyzed by reversed phase-HPLC [21]. 5-HETE and 5-HPETE formation after incubation of radioactive arachidonic acid ± fatty acid/derivative is shown as % of total amount of radioactive arachidonic acid added. Unlabelled arachidonic acid (20 and 50 μM) was also added as "inhibitor" (dilution of radioactive substrate) for comparison. Black bars: 5-HETE + 5-HPETE produced from radioactive arachidonic acid without added fatty acids/derivatives. Open bars: Effect of adding 20 μM of fatty acid/derivative. Hatched bars: Effect of adding 50 μM of fatty acid/derivative. Each bar indicates one sample. We also registered some production of leukotriene $B_4$, but the results were less consistent than for 5-HETE and 5-HPETE.

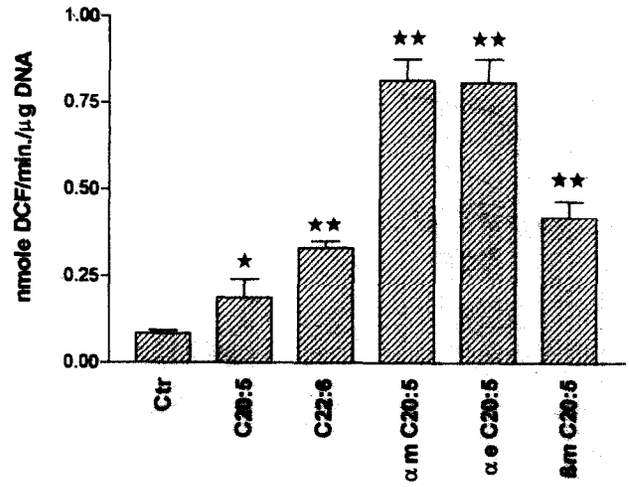


FIG. 4. EPA, DHA and EPA derivatives as inducers of acyl-CoA oxidase in 7800 $C_1$ hepatoma cells. The cells were treated with 80 μM fatty acids/derivatives for 3 days with renewal of the medium on the second day. Cells were harvested, sonicated and the acyl-CoA oxidase was measured with palmitoyl-CoA as substrate [22]. The presented results are the means of three parallells ± SD. Enzyme activity is expressed as nmol of 1DCF oxidized per min and per μg DNA. *P < 0.05, **P < 0.005 vs Control.

drial β-oxidation is prevented [4]. In agreement with this, we found α-methyl-, β-methyl- and α-ethyl EPA to induce peroxisomal oxidation in cultivated hepatoma cells. Previously, we had found that saturated 3-thia fatty acids, which are prevented from β-oxidation, have this effect [23, 24].

It is striking that the introduction of an α-methyl group or β-methyl group in EPA still permits some incorporation into phospholipids, and particularly into triacylglycerol, while an ethyl group virtually blocks both incorporations. The α-methyl group still permits inhibition of AA synthesis from linoleic acid, but less than that of EPA. This metabolic pattern suggests that α- and β-methyl EPA may be retained in body triacylglycerols (depot effect) without disturbing cell membrane structure significantly.

The small methyl group at the α-position also permits some reactivity and inactivation of the key enzymes PGH synthase and 5-lipoxygenase in eicosanoid synthesis, while α-ethyl EPA is completely or almost completely inert. It is obvious that the position of the methyl group is important, since β-methyl EPA is a poorer inactivator than α-methyl EPA. Evidently, the cyclooxygenase makes the PGG derivative of α-methyl EPA at a sufficient rate to permit self-inactivation of the enzyme. This may be the reason why α-methyl EPA is a better inhibitor of $TXB_2$ production in blood platelets than both EPA and DHA, and may explain why α-methyl EPA also gave a pronounced reduction in blood platelet aggregation [5]. Altogether, our studies suggest that the hypolipemic α-methyl EPA may also have antithrombotic effects.

*This work was supported by the Norwegian Research Council for Science and the Pronova, subsidiary of Norsk Hydro, Oslo. We wish to thank Ellen Dahl and Arnfrid Adler for skillful technical assistance.*

## References

1. Hornstra G, Influence of dietary fish oil on arterial thrombosis and atherosclerosis in animal models and in man. *J Int Med* **225**: 53–59, 1989.
2. Marshall PJ and Kulmacz RJ, Prostaglandin H synthase: Distinct binding sites for cyclooxygenase and peroxidase substrates. *Arch Biochem Biophys* **266**: 162–170, 1988.
3. Struijk CB, Beerthuis RK, Pabon HJJ and van Dorp DA, Specificity in the enzymic conversion of polyunsaturated fatty acids into prostaglandins. *Rec Trav Chim* **85**: 1233–1251, 1966.
4. Singh H, Beckman K and Poulos, A peroxisomal β-oxidation of branched chain fatty acids in rat liver. *J Biol Chem* **269**: 9514–9520, 1994.
5. Willumsen N, Mechanisms for the hypolipidemic effect of poorly oxidizable fatty acids in rats. Thesis, University of Bergen, Bergen, 1996.
6. Christiansen RZ, Christiansen EN and Bremer J, The stimulation of erucate metabolism in isolated rat hepatocytes by rapeseed oil and hydrogenated marine oil-containing diets. *Biochim Biophys Acta* **573**: 417–429, 1979.
7. Schmitz W, Fingerhut R and Conzelmann E, Purification and

properties of an alpha-methylacyl-CoA racemase from rat liver. *Eur J Biochem* **222:** 313–323, 1994.
8. Larsen LN, Hoevik K, Bremer J, Myhren F and Boerretzen B, Heneicosapentaenoate (C21:5, n-3): Its incorporation into lipids and its effects on arachidonic acid- and eicosanoid-synthesis. *Lipids* **32:** 707–714, 1997.
9. Richardson UI, Snodgrass PJ, Nuzum CT and Tashjian AMJ, Establishment of a clonal strain of hepatoma cells which maintain in culture the five enzymes of urea cycle. *J Cell Physiol* **83:** 141–150, 1973.
10. Kulczycki AJ, Isersky C and Metzger H, The interaction of IgE with rat basophilic leukemia cells. *J Exp Med* **139:** 600–616, 1974.
11. Haurand M and Flohe L, Kinetic studies on arachidonate 5-lipoxygenase from rat basophilic leukemia cells. *Biol Chem Hoppe Seyler* **369:** 133–142, 1988.
12. Kaluzny MA, Duncan LA, Merritt MV and Epps DE, Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J Lipid Res* **26:** 135–140, 1985.
13. Christiansen EN, Lund JS, Rørtveit T and Rustan AC, Effect of dietary n-3 and n-6 fatty acids on fatty acid desaturation in rat liver. *Biochim Biophys Acta* **1082:** 57–62, 1991.
14. Grønn M, Christensen E, Hagve TA and Christophersen BO, Effects of dietary purified eicosapentaenoic acid (20:5(n-3)) and docosahexaenoic acid (22:6(n-3)) on fatty acid desaturation and oxidation in isolated rat liver cells. *Biochim Biophys Acta* **1125:** 35–43, 1992.
15. Reynafarje B, Costa LE and Lehninger AL, $O_2$ solubility in aqueous media determined by a kinetic method. *Anal Biochem* **145:** 406–418, 1985.
16. Markey CM, Alward A, Weller PE and Marnett LJ, Quantitative studies of hydroperoxide reduction by prostaglandin H synthase. Reducing substrate specificity and the relationship of peroxidase to cyclooxygenase activities. *J Biol Chem* **262:** 6266–6279, 1987.
17. O'Keefe SF, Lagarde M, Grandgirard A and Sebedio JL, Trans n-3 eicosapentaenoic and docosahexaenoic acid isomers exhibit different inhibitory effects on arachidonic acid metabolism in human platelets compared to respective *cis* fatty acids. *J Lipid Res* **31:** 1241–1246, 1990.
18. Gundu HRR, Physiology of blood platelet activation. *Indian J Physiol Pharmacol* **37:** 263–275, 1993.
19. Pradelles P, Grassi J and Maclouf J, Enzyme immunoassays of eicosanoids using acetylcholine esterase as label: An alternative to radioimmunoassays. *Anal Chem* **57:** 1170–1173, 1985.
20. Levine L and Worth N, Eicosapentaenoic acid: its effects on arachidonic acid metabolism by cells in culture. *J Allergy Clin Immunol* **74:** 430–436, 1984.
21. Henke DC, Kouzan S and Eling TE, Analysis of leukotrienes, prostaglandins, and other oxygenated metabolites of arachidonic acid by high-performance liquid chromatography. *Anal Biochem* **140:** 87–94, 1984.
22. Small GM, Burdett K and Connock MJ, A sensitive spectrophotometric assay for peroxisomal acyl-CoA oxidase. *Biochem J* **227:** 205–210, 1985.
23. Spydevold O and Bremer J, Induction of peroxisomal β-oxidation in 7800 C1 Morris hepatoma cells in steady state by fatty acids and fatty acid analogues. *Biochim Biophys Acta* **1003:** 72–79, 1989.
24. Berge RK, Aarsland A, Bremer J, Kryvi H and Aarsæther N, Alkylthioacetic acid (3-thia fatty acids)—a new group of non-beta-oxidizable, peroxisome-inducing fatty acid analogues. 1. A study on the structural requirements for proliferation of peroxisomes and mitochondria in rat liver. *Biochim Biophys Acta* **1004:** 345–356, 1989.