

COX-2 Inhibitory Effects of Naturally Occurring and Modified Fatty Acids

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In the search for new cyclooxygenase-2 (COX-2) selective inhibitors, the inhibitory effects of naturally occurring fatty acids and some of their structural derivatives on COX-2-catalyzed prostaglandin biosynthesis were investigated. Among these fatty acids, linoleic acid (LA), α -linolenic acid (α -LNA), myristic acid, and palmitic acid were isolated from a CH_2Cl_2 extract of the plant *Plantago major* by bioassay-guided fractionation. Inhibitory effects of other natural, structurally related fatty acids were also investigated: stearic acid, oleic acid, pentadecanoic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Further, the inhibitory effects of these compounds on COX-2- and COX-1-catalyzed prostaglandin biosynthesis was compared with the inhibition of some synthesized analogues of EPA and DHA with ether or thioether functions. The most potent COX-2-catalyzed prostaglandin biosynthesis inhibitor was all-(*Z*)-5-thia-8,11,14,17-eicosatetraenoic acid (**2**), followed by EPA, DHA, α -LNA, LA, (7*E*,11*Z*,14*Z*,17*Z*)-5-thiaeicosa-7,11,14,17-tetraenoic acid, all-(*Z*)-3-thia-6,9,12,15-octadecatetraenoic acid, and (5*E*,9*Z*,12*Z*,15*Z*,18*Z*)-3-oxaheneicosa-5,9,12,15,18-pentaenoic acid, with IC_{50} values ranging from 3.9 to 180 μM . The modified compound **2** and α -LNA were most selective toward COX-2, with COX-2/COX-1 ratios of 0.2 and 0.1, respectively. This study shows that several of the natural fatty acids as well as all of the semisynthetic thioether-containing fatty acids inhibited COX-2-catalyzed prostaglandin biosynthesis, where α -LNA and compound **2** showed selectivity toward COX-2.

Cyclooxygenase-2 (COX-2, prostaglandin H synthase-2, PGHS-2) is normally unexpressed in most cells or tissues, but elevated levels are observed at inflammatory events.¹ The other cyclooxygenase isoform, COX-1, is constitutively expressed in many tissues and predominates, for example, in gastric mucosa and in the kidney. Inhibition of COX-1, which reduces the basal production of cytoprotective PGE_2 and PGI_2 in the stomach, may contribute to gastric ulceration. Since COX-2 is generally expressed only in cells where prostaglandins are upregulated (e.g., inflamed tissues), compounds that selectively inhibit COX-2 are proposed to possess fewer side effects.²

X-ray crystallography of the 3-D structure of COX-1 and COX-2 shows that these two enzymes have very similar active sites, each consisting of a long, narrow hydrophobic channel. A single amino acid difference between the two enzymes is generally critical for the selectivity of many COX inhibitory drugs: at position 523, COX-1 has an isoleucine molecule, and COX-2 a valine.² This difference results in access to a wider side pocket in the COX-2 enzyme, which is thought to be the binding site of many selective drugs. Another key active site of both COX-2 and COX-1 is Arg-120, which serves as a counterion for the carboxylate group of arachidonic acid and NSAIDs.³

The COX enzymes catalyze the conversion of arachidonic acid (AA) to prostaglandins in a two-step process.¹ In the first step, hydrogen is abstracted from C13 of AA, and then two molecules of oxygen are added by the cyclooxygenase, resulting in PGG_2 . In the second step, PGG_2 is reduced to PGH_2 by the peroxidase activity of the enzymes. The synthesized PGH_2 is rapidly converted to prostaglandins (PGD_2 , PGE_2 , $\text{PGF}_{2\alpha}$), prostacyclin (PGI_2), or thromboxane A_2 (TxA_2) by tissue-specific isomerases.¹

As part of a search for new COX-2 selective inhibitors, bioassay-guided fractionation of a hexane extract from the

plant *Plantago major* L. (Plantaginaceae) resulted in isolation of ursolic acid as a COX-2 inhibitory compound.⁴ The structural triterpenoid isomers oleanolic acid and glycyrrhetic acid were also examined, where only oleanolic acid inhibited COX-2- and COX-1-catalyzed prostaglandin biosynthesis.

The use of *P. major* in traditional medicine is quite extensive, most notably for the wound-healing effect. A comprehensive review of the traditional use, chemical constituents, and biological activities of *P. major* was published by Samuelsen.⁵ The plant extracts have been investigated for several biological activities. Antiinflammatory and analgesic effects were observed on carrageenan-induced rat paw edema, on exudate and leucocyte mobilization, and also on exudate inhibition in the air pouch after oral treatment with plant extracts. Dextran-induced edemas were not inhibited by the extract.

The plant *P. major* is known to contain compounds from various substance-classes, e.g., carbohydrates, fatty acids, alkaloids, caffeic acid derivatives, flavonoids, iridoid glycosides, and triterpenoids.⁵ Except for the triterpenoids already mentioned, compounds reported to have antiinflammatory effects include the caffeic acid derivatives plantamajoside and acetoside, the flavonoids baicalein and hispidulin, and the iridoid glycoside aucubin.⁵

Several naturally occurring compounds have been investigated for COX-2 inhibition, either for effects on enzymatic activity or for expression of COX-2 mRNA and protein.⁶ For instance, caffeic acid phenethyl ester and several flavonoids have suppressed COX-2 enzymatic activity and PGE_2 production. However, although about 500 plant extracts have been tested for COX-2 inhibition, less than 10% have been reported active.⁶

In this study, further bioassay-guided separation of a CH_2Cl_2 extract from *P. major* resulted in isolation of a number of fatty acids: linoleic acid (LA, 18:2 ω -6), α -linolenic acid (α -LNA, 18:3 ω -3), myristic acid (MA, 14:0), and palmitic acid (PA, 16:0). Since fatty acids commonly occur

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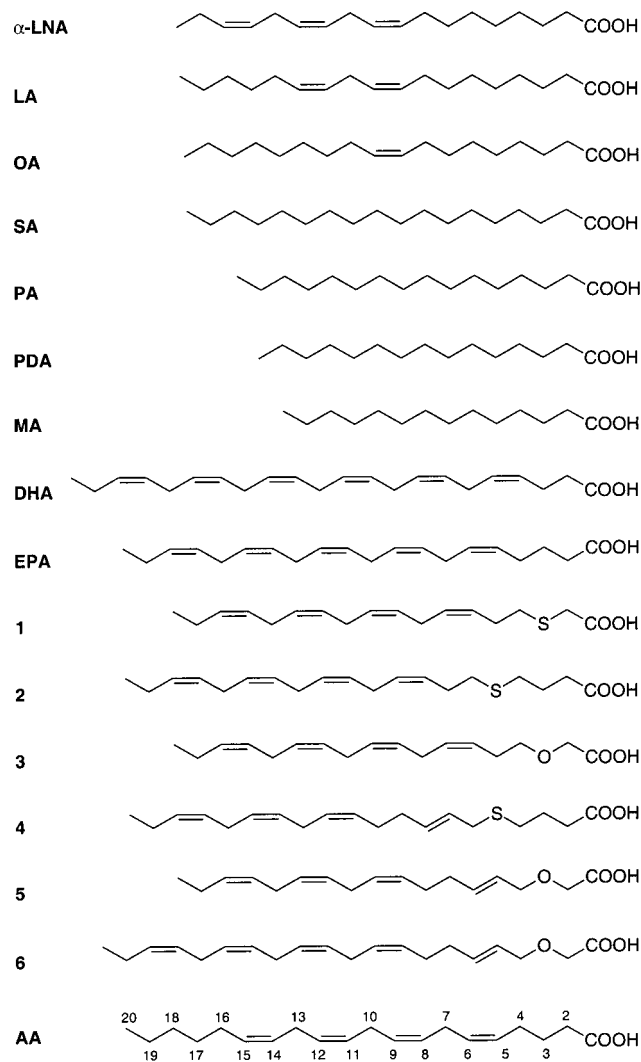


Figure 1. Structures of natural and synthetic fatty acids, tested on COX-2- and COX-1-catalyzed prostaglandin biosynthesis.³⁴

in plants, especially in plant oils of the human diet, further characterization of COX-2 inhibitory effects of other naturally occurring fatty acids, with different chain-lengths and numbers of unsaturations, was performed. These were oleic acid (OA, 18:1 ω -9), pentadecanoic acid (PDA, 15:0), stearic acid (SA, 18:0), docosahexaenoic acid (DHA, 22:6 ω -3), and eicosapentaenoic acid (EPA, 20:5 ω -3) (Figure 1). The inhibition of COX-2- and COX-1-catalyzed prostaglandin biosynthesis by these naturally occurring fatty acids was compared with the effects of some semisynthetic polyunsaturated fatty acids synthesized from EPA or DHA with the aim of enhancing the biological activity of those two compounds. These synthetic fatty acids were all-(*Z*)-3-thia-6,9,12,15-octadecatetraenoic acid (**1**), all-(*Z*)-5-thia-8,11,14,17-eicosatetraenoic acid (**2**), all-(*Z*)-3-oxa-6,9,12,15-octadecatetraenoic acid (**3**), (7*E*,11*Z*,14*Z*,17*Z*)-5-thiaicosa-7,11,14,17-tetraenoic acid (**4**), (5*E*,9*Z*,12*Z*,15*Z*)-3-oxa-octa-deca-5,9,12,15-tetraenoic acid (**5**), and (5*E*,9*Z*,12*Z*,15*Z*,18*Z*)-3-oxa-heneicosa-5,9,12,15,18-pentaenoic acid (**6**) (Figure 1).⁷

Results and Discussion

The bioassay-guided fractionation of a CH_2Cl_2 extract of *P. major* led to the detection of triterpenoids and fatty acids that had COX-2 inhibitory effect. The isolation of the triterpenoids ursolic acid and oleanolic acid is consistent

Table 1. Inhibition of COX-2- and COX-1-Catalyzed Prostaglandin Biosynthesis (IC_{50} Values, μM) and COX-2/COX-1 Ratios for Natural and Modified Fatty Acids

compound	COX-2 (μM)	COX-1 (μM)	COX-2/COX-1
α -linolenic acid (α-LNA) (18:3 ω -3)	12	93	0.1
linoleic acid (LA) (18:2 ω -6)	94	170	0.6
oleic acid (OA) (18:1 ω -9)	> 500	> 500	-
stearic acid (SA) (18:0)	> 500	> 500	-
palmitic acid (PA) (16:0)	> 500	> 500	-
pentadecanoic acid (PDA) (15:0)	> 500	> 500	-
myristic acid (MA) (14:0)	> 500	> 500	-
docosahexaenoic acid (DHA) (22:6 ω -3)	9.8	15	0.7
eicosapentaenoic acid (EPA) (20:5 ω -3)	7.1	13	0.5
3-thia-6,9,12,15-octadecatetraenoic acid (1)	160	84	1.9
5-thia-8,11,14,17-eicosatetraenoic acid (2)	3.9	26	0.2
3-oxa-6,9,12,15-octadecatetraenoic acid (3)	> 500	> 500	-
5-thiaicosa-7,11,14,17-tetraenoic acid (4)	120	81	1.4
3-oxa-octa-deca-5,9,12,15-tetraenoic acid (5)	> 500	> 500	-
3-oxa-heneicosa-5,9,12,15,18-pentaenoic acid (6)	180	50	3.6

with our earlier findings that those triterpenoids inhibit COX-2.⁴ The bioassay-guided fractionation of the CH_2Cl_2 extract also led to isolation of several fatty acids, α -LNA, LA, PA, and MA.

These fatty acids, which commonly occur in plants, especially in edible plants, are an important part of the human diet.^{8,9} PA is the major saturated fatty acid in the diet, followed by SA, MA, and lauric acid, while OA is the most common monounsaturated fatty acid and LA the principal polyunsaturated.⁸ PA and OA are found in all edible fats and oils: SA, for example, in cocoa butter; LA, in vegetable oils, such as soybean, corn, and sunflower oils; and α -LNA, for instance in dark green leafy plants and in linseed, rapeseed, walnut, and black currant oils.⁸ The ω -3 fatty acids, such as EPA and DHA, found in cold-water fatty fishes, are suggested to be remarkable nutrients because of their importance in brain development and treatment of chronic cardiovascular diseases, arthritic disorders, and diabetes mellitus.^{9,10} The long-chained fatty acids, which occur at high levels in some fish oils, exert protective effects against some common cancers, including prostate, colon, and prostate cancer.^{8,10}

An association between cancer and COX-2 is proposed, since increased levels of prostaglandins as well as COX-2 mRNA and protein expression are observed, mainly in colon cancer, but also in other cancers, such as gastric carcinoma and breast cancer.¹¹⁻¹³ Noguchi et al. suggest that an effect of the ω -6 polyunsaturated fatty acids on breast cancer tumorigenesis and tumor cell proliferation is promoted both directly and indirectly through increased synthesis of COX- or LOX-catalyzed products.¹⁴ Also, several investigations on dietary supplementation of fish oils and other fats show that such oils can affect COX-2 expression in rats.⁶

The COX-2 inhibitory effects of the tested compounds are summarized in Table 1. The most potent COX-2 inhibitory compound is **2**, followed by EPA, DHA, α -LNA, LA, **4**, **1**, and **6**, with COX-2 IC_{50} values of 3.9, 7.1, 9.8, 12, 94, 120, 160, and 180 μM , respectively. The same compounds also inhibit COX-1 with IC_{50} values of 26, 13, 15, 93, 170, 81, 84, and 50 μM . Although inhibitors of both

COX-2- and COX-1-catalyzed prostaglandin biosynthesis, compound **2**, EPA, DHA, α -LNA, and LA showed selectivity toward COX-2 with COX-2/COX-1 ratios of 0.2, 0.5, 0.7, 0.1, and 0.6, respectively. However, compounds **4**, **1**, and **6** inhibit COX-1 more than COX-2 with COX-2/COX-1 ratios of 1.4, 1.9, and 3.6, respectively. Compounds MA, OA, PA, PDA, and SA as well as **3** and **5** were found inactive toward the enzymes at concentrations up to 500 μ M.

Only compound **2**, EPA, DHA, and α -LNA are more potent inhibitors of COX-2 than the COX-2 selective reference compound NS-398 (IC₅₀ of 53 μ M, calculated with linear regression¹⁵). Furthermore all compounds are less potent inhibitors of COX-1-catalyzed prostaglandin biosynthesis than indomethacin, a COX-1 inhibitor used as reference compound (IC₅₀ of 1.3 μ M).

Except for the triterpenoids isolated from *P. major*, the fatty acids LA and α -LNA seem to be responsible for the COX-2 and COX-1 inhibitory effects in the plant extract, since PA and MA did not inhibit any of the enzymes at concentrations up to 500 μ M. The high fatty acid content in the herbal extract is probably due to the seeds it contained.⁵

Both LA and α -LNA are less potent inhibitors than EPA and DHA, but of all the compounds tested, α -LNA is the most COX-2 selective, with a COX-2/COX-1 ratio of 0.1. At concentrations up to 500 μ M, the natural fatty acids OA, PDA, and SA showed no effect on any of the enzymes.

Of the semisynthetic fatty acids, compound **2** was the most potent COX-2 and COX-1 inhibitor, as well as most COX-2 selective. Also, of all substances tested, only the semisynthetic compound **2** inhibited COX-2 more than its origin, EPA. Compound **4** was found to be a significantly less potent inhibitor of both enzymes, although the structural difference between compounds **4** and **2** is the position and configuration of the double bond at C7 and C8. Also the third thioether-containing fatty acid, **1**, inhibited COX-2 and COX-1 at about the same level as **4**. Of the synthetic fatty acids that contain an ether function, only **6** inhibited the enzymes. Interestingly this compound, having the longest chain (21 atoms), was found most COX-1 selective of all compounds. The importance of the thioether moiety is apparent, since compounds **3** and **5**, which contain an ether function, inhibited none of the enzymes at doses up to 500 μ M.

When comparing the chain lengths of the fatty acids, those with more than 20 atoms were inhibitors and those with less than 16 atoms, inactive. Among the compounds consisting of 18 atoms, some were active and some were not, which might correlate with less unsaturations (SA and OA) or with an ether function (**3** and **5**). The active fatty acids consisting of more than 18 atoms are probably accepted as substrates by the enzymes.

The fatty acids DHA, eicosadienoic acid (EDA, 20:2 ω -6), eicosatrienoic acid (ETA, 20:6 ω -9), EPA, LA, γ -LNA, LNA, and mead acid (20:3, ω -9) are reported to compete with AA (20-carbon) as substrates for the COX enzymes.^{3,16–19} However, Corey et al. propose that DHA is resistant to COX-1 enzymatic oxidation and therefore functions as an inhibitor and not as a substrate.²⁰ An inhibition observed as a consequence of a diet consisting of ω -3 fatty acids can also be a result of displacement of AA, since ω -3 fatty acids will be metabolized instead of LA by the desaturases and elongases.^{10,19}

Although LA is a metabolic precursor of AA, the conversion of LA results in production of hydroxyoctadecadienoic acids (HODE), especially 9-HODE and 13-HODE, where

COX-2 is thought to be the main enzyme involved in the conversion.^{21,22} The fatty acid α -LNA is metabolized to longer fatty acids, first to EPA and then to DHA.¹⁰ This results in production of prostaglandins and leukotrienes of the 3-series (e.g., PGE₃), which are often less biologically active and found in smaller amounts than prostaglandins of the 2-series (e.g., PGE₂).¹⁰ Since the major oxidation product of α -LNA is 12-hydroxy-(9*Z*,13*E*,15*Z*)-octadecatrienoic acid, Laneuville et al. suggest that α -LNA is positioned in the cyclooxygenase active site just like AA, but with a distortion of the carbon chain such that hydrogen abstraction occurs from C-14 rather than from C-13, as is the case for AA.¹⁶ Rieke et al. concluded that the Arg-120 is important for oxygenation of EPA and EDA and that each fatty acid substrate depends on a unique set of interactions within the COX active site of human COX-2 for binding and oxygenation.³

Several compounds have been investigated for COX-2 inhibitory effect, and many structure–activity studies have been performed.²³ Modification of the COX-1 selective inhibitor ETYA (5,8,11,14-eicosatetraynoic acid) with a 3-phenylpropyl amide and a 3-(4-methoxyphenyl)propylamide resulted in inhibitors with modest COX-2 selectivity.²⁴ Especially, additions of a sulfonamide moiety to, among others, diarylheterocycles, acidic sulfonamide derivatives, and di-*tert*-butylphenols look promising.²⁵ For example, a specific COX-2 inhibitor is afforded after exchanging the carboxylate moiety of aspirin with an alkyl sulfide functionality.²⁶ Recently, rofecoxib and celecoxib, two COX-2 selective drugs, have entered the market, for indications such as osteoarthritis and rheumatoid arthritis. Both of these new drugs are diarylheterocycles containing a sulfur moiety.

Naturally occurring sulfur-containing fatty acids are rare, although some diisothiocyanates with carbon chains between 12 and 20 (diisothiocyanatododecadiene to diisothiocyanatoeicosene) have been isolated from the marine sponge *Pseudaxinyssa* sp.^{27,28} and the biologically active sulfonoglycolipids have been isolated from plants, e.g., *Polypodium decumanum* and *Byrsonima crassifolia*.^{29,30} Although not a fatty acid, the thioester ajoene, isolated from *Allium sativum* L., has shown COX-2 inhibitory effects.³¹ Ajoene inhibited LPS-induced PGE₂-release in RAW 264.7 macrophages, an effect that was found to be due to inhibition of the COX-2 enzymatic activity. Considering the enhanced COX-2 inhibition of the thioether-containing fatty acids, further exploration of sulfur-containing compounds, e.g., glycolipids and fatty acids, should be of interest in the search for COX-2 selective compounds.

In conclusion, linoleic acid and α -linolenic acid seem to be the fatty acids responsible for some of the inhibition of COX-2- and COX-1-catalyzed prostaglandin biosynthesis in *Plantago major*, while palmitic acid and myristic acid apparently do not inhibit any of the enzymes. Obviously, other compounds that inhibit COX-2 and COX-1 are also present in *P. major*.

Further, both eicosapentaenoic acid and docosahexaenoic acid were more active on COX-2 than LA and LNA, while neither oleic acid, pentadecanoic acid, nor stearic acid inhibited COX-2- or COX-1-catalyzed prostaglandin biosynthesis. Hence, a thioether moiety seems to improve the COX-2 inhibitory effect, because all of the thioethers were active, and in addition, one of them (all-(*Z*)-5-thia-8,11-, 14,17-eicosatetraenoic acid) was even more active than its natural origin EPA.

Experimental Section

Plant Material. Entire plants of *Plantago major* L. (Plantaginaceae) were collected in September 1998 in the surroundings of Uppsala and identified by Dr. Anders Backlund. A voucher specimen, coded AIP98006, was deposited at the Division of Pharmacognosy, Uppsala.

General Experimental Procedures. All solvents were of P.A. grade and distilled before use. Solid-phase extraction (SPE) was performed on Isolute columns C18 (EC, 5g, 100 mL). Preparative HPLC was done on a Shimadzu HPLC system comprised of an LC-10 AD chromatograph, an SPD-M10A photodiode array detector, a communication bus module CBM-10A, and an eluent degassing unit GT-104. A 250 × 10 mm (i.d.) ACE column (C18, 5 μm, batch 2502) was used for injections of 500 μL (10 mg/mL). The column was eluted with 81% EtOH (0–20 min), with a linear gradient to 95% EtOH (20–30 min) and with linear gradient back to 81% (42–47 min). The eluted peaks were online-monitored at 217 and 440 nm.

For GC/MS analyses of fatty acids, a HP 5972 GC/MS instrument, equipped with a HP-5, 30 m × 0.25 mm i.d. capillary column with a film thickness of 0.25 μm, was used. Injector and detector temperatures were 300 °C. Initially, the temperature program was 190 °C for 15 min and then was increased by a gradient (10 °C/min) to 290 °C, which was maintained for 20 min. The split ratio was 1:100. Helium was used as carrier gas.

Negative ion ESIMS spectra were measured on a LCQ, Finnigan MAT ion trap mass spectrometer in infusion mode. The flow rate was 8 μL/min, and the samples were applied in 95% EtOH (10 μg/mL). The fatty acids used as reference compounds were of 99% purity and manufactured by Larodan, Sweden.

¹H NMR spectra were recorded in CDCl₃ or CD₃OD/CDCl₃ on a JEOL NMR instrument (270 MHz) with TMS as internal standard. The ursolic and oleanolic acid reference compounds were obtained from Sigma, Sweden, and were of 90% and 97% purity, respectively.

For gas chromatography-flame ionization detection (GC-FID) analyses of the triterpenoids, a Shimadzu GC-9AM chromatograph, equipped with a DB-5, 30 m × 0.32 mm i.d. capillary column with a 0.25 μm film thickness, was used. The chromatographic conditions were the same as for the GC/MS analyses.

Extraction and Isolation. The dried and powdered plant material (85 g) was extracted for 6 h in a Soxhlet apparatus with 1.0 L of CH₂Cl₂, yielding a total of 1.5 g of extract. The dried CH₂Cl₂ extract (500 mg) was dissolved in 50 mL of EtOH by sonication (30 min). The sample was applied on a pre-conditioned Isolute column and successively eluted with EtOH (10 mL) and *n*-heptane (50 mL). Two fractions were obtained, EtOH and *n*-heptane, which inhibited COX-2 by 58% ± 2 and 34% ± 2, respectively. COX-1 was inhibited by 47% ± 1 and 67% ± 1, respectively.

The EtOH fraction was applied on HPLC, yielding fraction A (0–25 min) and fraction B (25–50 min). Injections were repeated to obtain appropriate amounts of sample for investigation of the inhibitory effect on COX-2- and COX-1-catalyzed prostaglandin biosynthesis. Fraction A provided 78% ± 1 inhibition of COX-2 and 62% ± 2 of COX-1, while fraction B provided 12% ± 2 of COX-2 and 23% ± 4 of COX-1. Fraction A was further fractionated to obtain 4 fractions: A:1 (0–7 min), A:2 (7–11 min), A:3 (11–14 min), and A:4 (14–25 min), which inhibited COX-2 by 0% ± 7, 79% ± 4, 94% ± 4, and 87% ± 0.4, and COX-1 by 11% ± 8, 66% ± 1, 103% ± 0.4, and 98% ± 1.

To identify the contents of fractions A:2, A:3, and A:4, the triterpenoids were analyzed by ¹H NMR and GC-FID and the fatty acids with GC/MS and ESIMS. Ursolic acid, oleanolic acid, and α-LNA were identified in fraction A:2; PA and LA were identified in fraction A:3; and MA was identified in fraction A:4. The fragmentation patterns and relative retention times were in accordance with corresponding fragmentation patterns and relative retention times for reference compounds.

Methylinoate (LA): *t*_R 16.01 min; GC/MS (70 eV) *m/z* 294 [M⁺] (2), 150 (2), 135 (1), 123 (1), 109 (16), 95 (52), 81 (75), 67 (100), 55 (67), 55 (43), 41 (57).

Methyl-α-linolenate (α-LNA): *t*_R 16.15 min; GC/MS (70 eV) *m/z* 292 [M⁺] (2), 261 (1), 236 (2), 163 (2), 137 (5), 135 (10), 121 (14), 108 (36), 95 (52), 79 (100), 67 (70), 55 (51), 43 (26), 41 (59).

Methylmyristate (MA): *t*_R 4.75 min; GC/MS (70 eV) *m/z* 242 [M⁺] (5), 211 (4), 199 (9), 185 (3), 157 (2), 143 (13), 129 (4), 115 (1), 101 (4), 87 (6), 74 (100), 55 (17), 43 (42).

Methylpalmitate (PA): *t*_R 9.39 min; GC/MS (70 eV) *m/z* 270 [M⁺] (26), 239 (11), 227 (23), 213 (2), 199 (8), 185 (8), 171 (8), 157 (2), 143 (36), 129 (13), 115 (5), 97 (16), 87 (100), 75 (71), 74 (100), 55 (80), 43 (100), 41 (79).

Oleanolic Acid (3β-Hydroxy-12-oleanen-28-oic acid, OA) and Ursolic Acid (3β-Hydroxy-12-ursen-28-oic acid, UA). ¹H NMR data of UA were in accordance with our earlier isolation.⁴ The NMR data indicated the presence of both UA and OA, which was further confirmed by GC-FID analysis of the derivatized products, *t*_R = 35.95 min (methylated and silylated OA), *t*_R = 37.01 min (methylated and silylated UA). Infusion ESIMS (4.27 kV, cap. temp 230 °C) *m/z* 455.7 [M – H][–]. At 30% CID, MS² (455.7) yielded strong signals for both compounds at 407.5 (loss of 48 u).

Methylation and Silylation of Triterpenoids. The methylation of ursolic acid and oleanolic acid was performed according to Lepage and Roy with modifications.³² Briefly it was carried out as follows: Acetyl chloride (1 mL) was added dropwise to 5 mL of Na₂SO₄-dried MeOH. Then, 300 μg of the triterpenoid acids was dissolved in 100 μL of a stock solution (prepared by dissolving 5.0 mg of 17:0 (margaric acid) in 10 mL of tetrahydrofuran). To the dissolved compound was added 200 μL of methanolic HCl, and the solution was heated for 2 h in a test tube at 50 °C. The methylated form of 17:0 was used as a reference for calculating relative retention times in the GC-FID runs. The silylation of free hydroxyl groups was performed by evaporation of the methylated triterpenoid acids with nitrogen, followed by the addition of 50 μL of BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide). The samples were then heated for 1 h at 50 °C.

Synthesis of Fatty Acids. The fatty acids were synthesized essentially as previously described.⁷ Oxidative degradation of EPA and DHA, respectively, provided the corresponding aldehydes all-(*Z*)-3,6,9,12-pentadecatetraenal and all-(*Z*)-3,6,9,12,15-octadecapentaenal in 75% overall yields. Reactions with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) converted the aldehydes quantitatively into the conjugated isomers 2*E*,6*Z*,9*Z*,12*Z*-pentadecatetraenal and 2*E*,6*Z*,9*Z*,12*Z*,15*Z*-octadecapentaenal, respectively. The four aldehydes were transformed by a sequence of reactions: reduction to alcohols, halogenation, and substitution with the appropriate mercapto esters into the corresponding sulfur-containing polyunsaturated fatty acid esters. The oxygen-containing ethyl esters were prepared from their respective alcohols by reaction with ethyl diazoacetate, catalyzed by boron trifluoride. The fatty acids were obtained by hydrolysis with lithium hydroxide in aqueous methanol.

COX-Catalyzed Prostaglandin Biosynthesis In Vitro Assay. The source of COX-1 (prostaglandin endoperoxide H synthase-1, PGHS-1) was microsomes, prepared from bovine seminal vesicles according to Noreen et al.¹⁵ Purified COX-2 (prostaglandin endoperoxide H synthase-2, PGHS-2) was obtained from sheep placental cotyledons (Cayman Chemical Company, Ann Arbor, MI). The enzyme was incubated with the test compound for 10 min before addition of [1-¹⁴C]-arachidonic acid (Amersham Pharmacia Biotech, Uppsala, Sweden). The crude extract as well as the fractions were dissolved in 20% DMSO (Sigma, Stockholm, Sweden) and tested at 100 μg/mL (final concentration). The pure compounds were dissolved in 10% EtOH. The inhibition of COX-catalyzed prostaglandin biosynthesis was calculated as the relative decrease in radioactivity (disintegrations per minute) of the samples containing the test substance as compared to the solvent vehicle. IC₅₀ values were obtained by nonlinear regression analysis³³ and represent means ± SEM of 2–5 experiments (*n* = 2–20).

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