Evaluation of 5- and 6-fluoro derivatives of arachidonic acid and 5,8,14-eicosatrienoic acid as substrates and inhibitors of 5-lipoxygenase

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The 5- and 6-fluoro derivatives of arachidonic acid (5F-ETE and 6F-ETE) were evaluated as substrates of rat basophilic leukaemia cell (RBL-1) 5-lipoxygenase. 5F-ETE was found to be a poor substrate and was converted into a single product, 5-oxoeicosa-6,8,11,14-tetraenoic acid (5-oxo-ETE). 6F-ETE was a good substrate and was mainly converted into 5-hydroperoxy-6-fluoroeicosa-6,8,11,14-tetraenoic acid (5-OOH-6F-ETE) with concomitant formation of a small amount of 5-oxo-6-fluoroeicosa-6,8,11,14-tetraenoic acid (5-oxo-6F-ETE). However the formation of 5,12-dihydroxy-6-fluoroeicosa-6,8,10,14-tetraenoic acids, epimeric at C-12, was not observed. Eicosa-5(Z),8(Z),14(Z)-trienoic acid (ET), previously described as a good substrate of 5-lipoxygenase, is oxidized mainly to 5-hydroperoxyeicosa-6,8,14-trienoic acid (5-OOH-ET), which does not serve as a substrate for the leukotriene A₄ (LTA₄) synthase activity of 5-lipoxygenase [Navé, Dulery, Gaget & Ducep (1988) *Prostaglandins* **36**, 385–398]. To allow a better estimation of the effect of fluorine substitution on the rate of oxidation of the 5,8-*cis,cis*-diene moiety by 5-lipoxygenase, the 5- and 6-fluoro derivatives of ET were studied as substrates. Qualitatively, the metabolism of 5F-ET and 6F-ET was found to be similar to that observed for 5F-ETE and 6F-ETE. Quantitatively, 6F-ET proved to be a somewhat better substrate than ET, whereas 5F-ET was poorly metabolized. The relative ability of arachidonic acid, ET and the corresponding 5- and 6-fluoro derivatives to inhibit the 5-lipoxygenase-catalysed oxidation of eicosa-5(Z),8(Z)-dienoic acid (ED) was also investigated. 6F-ETE and 5F-ETE were found to be effective and about equipotent inhibitors of 5-lipoxygenase in the micromolar range. In view of their close structural similarity to arachidonic acid, these two inhibitors are expected to be important tools in the study of the 5-lipoxygenase pathway *in vivo*.

INTRODUCTION

Arachidonate 5-lipoxygenase (EC 1.13.11.34) is the first step in the cascade of enzymes converting arachidonic acid (ETE) into leukotrienes. These substances have been recognized as important mediators in a variety of diseases, including asthma, allergy, arthritis, psoriasis [1] and myocardial infarction [2]. Therefore 5-lipoxygenase inhibitors have a high potential as therapeutic agents. Several known inhibitors of this enzyme are ETE analogues [3–7]. Nevertheless, the steric conformation of these compounds differs substantially from that of ETE. Very close analogues of ETE likely to compete for the oxidative metabolism of ETE are the fluorinated derivatives. Substitution of hydrogen by fluorine produces little steric modification in molecules but important changes in electronic distribution owing to the strong electron-withdrawing effect of fluorine. Our interest in these fluorinated fatty acids was to evaluate their ability to serve as substrates or inhibitors of 5-lipoxygenase, and, if they are substrates, to identify their metabolites. Fluorinated metabolites might, for instance, behave as agonists or antagonists of leukotrienes and modulate their effects *in situ*. Another interesting characteristic of fluorinated polyunsaturated fatty acids might be their facile incorporation *in vivo* into phospholipid pools containing ETE. Recently, the syntheses of 7,7- and 10,10-difluoroarachidonic acids [8] as well as that of 5-, 12- and 15-fluoroarachidonic acids [9,10] have been described. However,

studies of the interaction of these compounds with 5-lipoxygenase have not been reported. In the present paper we describe the properties of the 5- and 6-fluoro derivatives of ETE and eicosa-5(Z),8(Z),14(Z)-trienoic acid (ET) as substrates and inhibitors of RBL-1 5-lipoxygenase.

MATERIALS AND METHODS

Chemicals

(±)-5-Hydroxyeicosa-6,8,11,14-tetraenoic acid (5-HETE), 12S-hydroxyeicosa-5,8,10,14-tetraenoic acid (12-HETE) and leukotriene B₄ (LTB₄) were obtained from Paesel G.m.b.H. (Frankfurt, Germany). 5S,12S- and 5S,12R-Dihydroxyeicosa-6,8,10,14-tetraenoic acids (5,12-diHETEs) were prepared by the method of Beckman *et al.* [11] from leukotriene A₄ (LTA₄), generously donated by Dr. C. Mioskowski (U.L.P., Strasbourg, France). ETE and prostaglandin B₂ (PGB₂) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Eicosa-5(Z),8(Z)-dienoic acid (ED) and eicosa-5(Z),8(Z),14(Z)-trienoic acid (ET) were prepared as described previously [12]. The 5- and 6-fluoro derivatives of ETE and ET were synthesized from (*E*)-2-fluorobut-2-ene-1,4-diol; details of their preparation may be obtained from the authors on request. To eliminate traces of hydroperoxides, all the polyunsaturated fatty acids were purified by silicic acid chromatography (Silicar CC₄; Mallinckrodt, KY,

Abbreviations used: ETE, arachidonic acid; 5F-ETE and 6F-ETE, 5-fluoro and 6-fluoro derivatives of arachidonic acid; HETE and HPETE, hydroxy and hydroperoxy derivatives of eicosatetraenoic acids; diHETEs, dihydroxyeicosatetraenoic acids; ED, eicosa-5(Z),8(Z)-dienoic acid; ET, eicosa-5(Z),8(Z),14(Z)-trienoic acid; 5-OH(OOH)-ED, 5-OH(OOH)-ET, 5-OH(OOH)-6F-ET and 5-OH(OOH)-6F-ETE, 5-hydroxy (and/or hydroperoxy)-eicosa-6,8-dienoic, -eicosa-6,8,14-trienoic, -6-fluoroeicosa-6,8,14-trienoic and -6-fluoroeicosa-6,8,11,14-tetraenoic acids; 5-oxo-ED, 5-oxo-ET, 5-oxo-6F-ET, 5-oxo-ETE and 5-oxo-6F-ETE, 5-oxo-eicosa-6,8-dienoic, -eicosa-6,8,14-trienoic, -6-fluoroeicosa-6,8,14-trienoic, -eicosa-6,8,11,14-tetraenoic and -6-fluoroeicosa-6,8,11,14-tetraenoic acids; LTA₄ and 6F-LTA₄, leukotriene A₄ and 6-fluoroleukotriene A₄; LTB₄, leukotriene B₄; PGB₂, prostaglandin B₂; g.c.-m.s., gas chromatography-mass spectrometry.

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U.S.A.). Batches (10 mg each) of the fatty acids loaded on 4 g columns were eluted with hexane/diethyl ether (9:1, v/v). Stock solutions (50 mM) in ethanol were then stored at -80°C under Ar atmosphere and titrated by g.c. as described previously [12]. All other chemicals were of the highest grade commercially available.

5-Lipoxygenase assay, h.p.l.c. analysis and quantification of products

The high-speed supernatant obtained from rat basophilic leukaemia (RBL-1) cell lysates was used as a source of 5-lipoxygenase. Preparation of the high-speed supernatant and the 5-lipoxygenase assay were performed essentially as previously described [12]. The protein concentration in the assay was set at 1.2 mg/ml. For analysis of 5-lipoxygenase products, two different h.p.l.c. programmes were employed [12]. A 38 min programme that allowed the resolution of most of the ETE metabolites was also used for analysis of products formed from the 5-fluoro and 6-fluoro derivatives of ETE (5F-ETE and 6F-ETE). The retention times of the oxygenated products of ETE, 5F-ETE and 6F-ETE were as shown in Fig. 1. A 12 min programme was used for the rapid analysis of the 5-hydroperoxy, 5-hydroxy and 5-oxo products of ET, 5F-ET and 6F-ET when the compounds were studied as substrates of 5-lipoxygenase and also for analysis of the products of ED oxygenation in the studies of inhibition. With this programme the 5-hydroperoxy and 5-hydroxy derivatives of the polyunsaturated fatty acids studied were eluted as a single peak and the retention times of 5-OH(OOH)-ED, 5-OH(OOH)-ET, 5-OH(OOH)-6F-ET, 5-oxo-ED, 5-oxo-ET and 5-oxo-6F-ET were 7.6, 6.3, 6.3, 8.3, 6.9 and 7.2 min respectively. 5-OH-ED and 5-OH-ET were characterized by g.c.-m.s. as previously described [12]. The other products of ET, 5F-ET and 6F-ET were tentatively identified, mainly on the basis of their retention times in reverse-phase h.p.l.c. and by measurement, on-line, of their u.v.-absorption spectra with a diode array detector (Hewlett-Packard 1040 A). For 5-OH(OOH)-ET, 5-oxo-ET, 5-OH(OOH)-6F-ET and 5-oxo-6F-ET, the λ_{max} values measured were 235, 281, 233 and 283 nm respectively.

For quantification, the lipoxygenase products were monitored by measurement of absorbance at 235 nm and 280 nm with a dual-wavelength u.v. detector (U.V.480; Kontron Analytic). 5-H(P)ETE, 5-OH(OOH)-6F-ETE and 12-H(P)ETE, the 12-H(P)-ETE derivatives of 5F-ETE, 5-OH(OOH)-ET, 5-OH(OOH)-6F-ET and 5-OH(OOH)-ED respectively, were quantified with the aid of a titrated 5-HETE solution ($\epsilon_{235} = 28600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [13]) as external standard and assuming that they all have the same ϵ value as 5-HETE, and PGB₂ (introduced into the samples before extraction of the fatty acids) was used to quantify the recovery of these fatty acids. 5,12-diHETEs, 5-oxo-ETE, 5-oxo-6F-ETE, 5-oxo-ET and 5-oxo-6F-ET were quantified with the aid of PGB₂ as internal standard and assuming ϵ values of $26800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (278 nm) for PGB₂ [14], $39000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (281 nm) for 5,12-diHETEs [15] and $27000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (279 nm) for the 5-oxo derivatives of the polyunsaturated fatty acids [16]. Recoveries of 5-HETE and 5-HPETE were 69% and 58% respectively.

Isolation and characterization of the 5-lipoxygenase products of ETE, 5F-ETE and 6F-ETE

Isolation of the various lipoxygenase products from large-scale incubations and measurement of their u.v.-absorption spectra were performed as previously reported [12]. The isolated products were converted into derivatives that were characterized by g.c.-m.s. analysis in a Finnigan TSQ g.c.-m.s. system coupled with an Incos data system. A 5% phenylmethyl-silicone column (12 m $\times$ 0.32 mm internal diameter) with a film thickness of

0.52 μm (Hewlett-Packard) was used. The mass spectra were recorded in the electron-impact mode at an ionization voltage of 70 eV.

Characterization or identification of the major ETE products formed in the RBL-1 high-speed supernatant was performed as reported previously [12].

5-Oxo-ETE was converted into its methoxime methyl ester. In a first step, 5-oxo-ETE, dissolved in 200 μl of a 16 mg/ml solution of methoxyamine hydrochloride in dry pyridine, was heated overnight at 65°C in a screw-capped tube fitted with a Teflon gasket. Then the solution was evaporated under N_2 and the residue dissolved in ethereal diazomethane for 2 min. After evaporation to dryness under N_2 , the sample was finally dissolved in dichloromethane/methanol (1:1, v/v). The mass spectrum showed ions at m/z 361 (M), 346 ($M-15$), 330 ($M-31$), 315 ($M-31-15$), 298 ($M-31-\text{CH}_3\text{OH}$), 260 ($M-101$, loss of $\cdot\text{CH}_2[\text{CH}_2]_2\text{CO}_2\text{CH}_3$), 210 ($M-151$, loss of $\cdot\text{CH}_2[\text{CH}=\text{CH}-\text{CH}_2]_2[\text{CH}_2]_3\text{CH}_3$), 203 ($M-158$, loss of $\text{CH}_3\text{ON}=\text{C}-[\text{CH}_2]_3\text{CO}_2\text{CH}_3$), 158 ($M-203$), 150 and 101.

Contrary to 5-HPETE and 5-HETE, which could be well separated, 5-OOH-6F-ETE and 5-OH-6F-ETE were eluted as a single peak in reverse-phase h.p.l.c. (Fig. 1b). After isolation, the mixture of the two compounds was injected on to a silicic acid h.p.l.c. column (220 mm $\times$ 4.6 mm internal diameter; Spheri 5 μm , Brownlee cartridge; Touzard Matignon, France) eluted with a mixture of hexane/propan-2-ol/acetic acid (969:30:1, by vol.) at a flow rate of 1.5 ml/min. The compounds were eluted at 5.6 and 7.6 min. The more polar compound corresponding to 5-OH-6F-ETE was further purified by reverse-phase h.p.l.c. as described above and converted into its methyl ester trimethylsilyl ether derivative before g.c.-m.s. analysis. After treatment of 5-OH-6F-ETE with ethereal diazomethane (15 min, room temperature), and evaporation of diethyl ether under N_2 , the sample was heated at 60°C for 15 min in 100 μl of a mixture of *N*-bis(trimethylsilyl)acetamide and dry pyridine (1:1, v/v). After elimination of pyridine under vacuum, the compound was dissolved in hexane. The mass spectrum showed ions at m/z 424 (M), 409 ($M-15$), 404 ($M-20$, loss of HF), 389 ($M-15-20$), 373 ($M-20-31$, loss of HF and $\cdot\text{OCH}_3$), 344 ($M-20-60$, loss of HF and $\text{CH}_3\text{CO}_2\text{H}$), 334 ($M-90$, loss of $(\text{CH}_3)_3\text{SiOH}$), 323 ($M-101$, loss of $\cdot\text{CH}_2[\text{CH}_2]_2\text{CO}_2\text{CH}_3$), 314 ($M-90-20$, loss of $(\text{CH}_3)_3\text{SiOH}$ and HF), 303 ($M-101-20$, loss of $\cdot\text{CH}_2[\text{CH}_2]_2\text{CO}_2\text{CH}_3$ and HF), 273 ($M-151$, loss of $\cdot\text{CH}_2[\text{CH}=\text{CH}-\text{CH}_2]_2[\text{CH}_2]_3\text{CH}_3$), 221 ($M-203$, loss of $(\text{CH}_3)_3\text{SiOCH}[\text{CH}_2]_3\text{CO}_2\text{CH}_3$), 203 and 150.

5-Oxo-6F-ETE was converted into its methoxime methyl ester derivative as described above for 5-oxo-ETE. The mass spectrum showed ions at m/z 379 (M), 364 ($M-15$), 348 ($M-31$, loss of $\cdot\text{OCH}_3$), 328 ($M-31-20$, loss of $\cdot\text{OCH}_3$ and HF), 278 ($M-101$, loss of $\cdot\text{CH}_2[\text{CH}_2]_2\text{CO}_2\text{CH}_3$), 228 ($M-151$, loss of $\cdot\text{CH}_2[\text{CH}=\text{CH}-\text{CH}_2]_2[\text{CH}_2]_3\text{CH}_3$) and 150.

Inhibition of 5-lipoxygenase

After addition of the RBL-1 cell supernatant containing 5-lipoxygenase to the assay mixture, the samples were preincubated for 1 min at 25°C . The enzyme reaction was started by addition of substrate (10 or 60 μM , final concentration) in the absence or in the presence of the potential inhibitor to be tested. The final concentration of ethanol (vehicle fluid) was 0.8% (v/v) and had no effect on 5-lipoxygenase activity. To obtain the progress curves of reaction, 4 ml incubations were performed. After different periods of time, 0.5 ml samples were withdrawn and processed as previously described [12]. To classify potential inhibitors according to their ability to decrease the extent of ED oxygenation after termination of the reaction, 0.5 ml incubations were performed and the reaction was run for 9 min. The products

Fig. 1. Reverse-phase h.p.l.c. analysis (a, b and c) and time courses of appearance (d, e and f) of products formed during incubations of ETE, 6F-ETE and 5F-ETE respectively with RBL-1 supernatant

ETE (49 μ M) (a), 6F-ETE (41 μ M) (b) and 5F-ETE (45 μ M) (c) were incubated with RBL-1 supernatant for 4 min under the conditions defined in the Materials and methods section. In similar incubations run with boiled supernatant (10 min), only peaks denoted by ● were observed (shown only in a). Peaks denoted by ★ are due to absorption at 235 nm of PGB₂, 5,12-diHETEs and ketones conjugated to a diene function. In (d), (e) and (f) for 5-HPETE, 12-HPETE, 5-OOH-6F-ETE and 5F-12-OOH-ETE, the sum of the reduced and unreduced hydroperoxide is considered.

of conversion of the substrate (ED) by 5-lipoxygenase, namely 5-hydroperoxy- and 5-hydroxyeicosa-6,8-dienoic acids (5-OOH-ED and 5-OH-ED), were extracted and analysed by h.p.l.c. as described above. In reverse-phase h.p.l.c., the oxygenated products of ED were well separated from all the oxygenated products formed from ET and ETE and from the fluorinated ET and ETE analogues.

RESULTS AND DISCUSSION

Substrate properties of the fluorinated arachidonic acids

In order to assay 5F-ETE, 6F-ETE and the corresponding 11,12-dihydro analogues as potential substrates or inhibitors of 5-lipoxygenase, the high-speed supernatant from RBL-1 cell lysates was used as a source of enzyme under optimal conditions defined previously [12].

In this preparation, ETE was converted mainly into 5-lipoxygenase products (Fig. 1a). Detected at 235 nm, 5S-hydroperoxyeicosa-6(E),8(Z),11(Z),14(Z)-tetraenoic acid (5-HPETE, t_R = 22.6 min), the product of the first reaction [17] catalysed by 5-lipoxygenase (5S-oxygenase activity; Scheme 1) was the major product (Fig. 1a). This compound was recovered in part as its reduced form (5-HETE, t_R = 22.2 min) because of the presence of glutathione peroxidase activities [18] in the enzyme preparation. At 280 nm, 5S,12S- and 5S,12R-dihydroxyeicosa-6(E),8(E),10(E),14(Z)-tetraenoic acids (5,12-diHETEs), two of the four products of LTA₄ hydrolysis [19], were identified (Fig. 1a), indicators of the LTA₄ synthase activity [17] of 5-lipoxygenase (Scheme 1). Nine other minor peaks were detected at 280 nm but

not identified. Two of these minor peaks should correspond to 5,6-dihydroxyeicosa-7(E),9(E),11(Z),14(Z)-tetraenoic acids (5,6-diHETEs), the two minor products of LTA₄ hydrolysis [19], whereas peak 1, which co-migrated with LTB₄, might correspond to a mixture [20] of LTB₄ and 5S,12S-dihydroxyeicosa-6(E),8(Z),10(E),14(Z)-tetraenoic acid (the product of double lipoxygenation of ETE by 5-lipoxygenase and 12-lipoxygenase). A significant conversion of ETE into 12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (12-HPETE) and its reduction product (12-HETE) occurred, as evidenced by the formation of peaks b and a detected at 235 nm (t_R = 21.8 and 21.4 min; Fig. 1a), in agreement with the fact that RBL-1 supernatant, besides 5-lipoxygenase, contains some contaminating 12-lipoxygenase [21].

Interestingly, the metabolism of 5F-ETE by 5-lipoxygenase (Fig. 1c) was restricted to the formation of a single product (t_R = 23.5 min). This product had a λ_{max} of 279 nm and was characterized as 5-oxoeicosa-6,8,11,14-tetraenoic acid (5-oxo-ETE) after conversion into the methoxime of the methyl ester derivative and g.c.-m.s. analysis. In Scheme 2, possible pathways towards formation of 5-oxo-ETE are depicted. They postulate the formation of a transient unstable 5-hydroperoxide intermediate, which might be directly converted into 5-oxo-ETE either spontaneously and/or by catalysis by, among other agents, haem-containing proteins of the supernatant [22]. Another route might involve reduction to the corresponding 5-hydroxy analogue catalysed by the supernatant glutathione peroxidases [18], followed by rapid elimination of HF. At concentrations of the polyunsaturated fatty acid substrates in the 50–60 μ M range, 5-oxygenation of 5F-ETE occurred at a rate that was estimated in

Scheme 1. Metabolic pathways for ETE in RBL-1 supernatant

Scheme 2. Possible pathways involved in the formation of 5-oxo-ETE after reaction of 5F-ETE with RBL-1 5-lipoxygenase

three separate experiments to be 6, 10 and 11 times smaller than that of 6F-ETE. Since, under the same conditions, rates of 5-oxygenation of 6F-ETE and ETE were similar (results not shown), the results indicate that substitution of hydrogen by fluorine at C-5 of ETE produces an important decrease in the rate of oxygenation whereas fluorine substitution at C-6 has no effect. The generally accepted mechanism for the enzymic 5-lipoxygenation of penta-1,4-*cis,cis*-dienes [17] involves hydrogen abstraction at C-7 and formation of a radical delocalized on C-9, C-7 and C-5, which is trapped at C-5 by molecular O_2 . The observed low rate of oxygenation of 5F-ETE with respect to ETE agrees well with an expected decrease in the probability of formation of a radical at C-5 due to fluorine. The experimental result is also not incompatible with a mechanism involving an organoiron intermediate. Evidence for such a mechanism has recently been presented by Corey & Nagata in the case of soybean lipoxygenase [23]. If such a mechanism was occurring, the effect of fluorine would be to decrease the nucleophilic character of C-5 and, hence, to make the addition of iron unfavourable. Finally, it is worth noting that 5F-ETE was converted, albeit at a low rate, into two metabolites detected at 235 nm (peaks c and d, Fig. 1c). On the basis of their retention times in reverse-phase h.p.l.c. (t_R = 20.8 and 21.2 min), these metabolites were ten-

tatively identified as 12-hydroxy- and 12-hydroperoxy-5-fluoroeicosa-5,8,10,14-tetraenoic acids (12-OH-5F-ETE and 12-OOH-5F-ETE) formed via the contaminating 12-lipoxygenase. As shown in Fig. 1(f), the global rate of formation of these two metabolites was similar to that measured for 12-HPETE plus 12-HETE (Fig. 1d).

In comparison with the complex metabolism of ETE in RBL-1 cell supernatant, metabolism of 6F-ETE looked simpler (Fig. 1b). Only one major peak (t_R = 22.8 min) was detected at 235 nm, which, after isolation, displayed the typical absorption spectrum of HPETE and HETE analogues (λ_{max} = 235 nm). In fact, this corresponded to a mixture of two compounds easily separated by straight-phase h.p.l.c. (t_R = 5.6 and 7.6 min). After g.c.-m.s., the most polar compound (trimethylsilyl ether of the methyl ester derivative) was characterized as 5-hydroxy-6-fluoroeicosa-6,8,11,14-tetraenoic acid (5-OH-6F-ETE). Hence the less polar compound, which was not isolated, was most probably the corresponding hydroperoxide (5-OOH-6F-ETE). In spite of the synthesis of 5-OOH-6F-ETE, formation of two 5,12-dihydroxy-6-fluoroeicosa-6,8,10,14-tetraenoic acids, epimeric at C-12, was not observed in reverse-phase h.p.l.c. at 280 nm (Fig. 1b), suggesting that 5-OOH-6F-ETE was not converted into 6-fluoroleukotriene A_4 (6F- LTA_4) by the LTA_4 synthase activity of

Scheme 3. Possible radical species occurring in the course of the reaction of 6F-ETE with RBL-1 5-lipoxygenase

Fig. 2. (a) Rates of 5-lipoxygenase-catalysed synthesis of 5-OOH-6F-ET, 5-OOH-ET and 5-oxo-ET respectively for various concentrations of each substrate and (b) rates of formation of 5-oxo-ET, 5-oxo-6F-ET and 5-oxo-ET formed from ET, 6F-ET and 5F-ET respectively for various concentrations of each substrate

The rates of product formation were estimated from the steepest linear portion of the progress curves. The data are from six separate experiments. In (a), for 5-OOH-6F-ET (□) and 5-OOH-ET (■), the sum of the reduced and unreduced hydroperoxide is considered. ●, 5-Oxo-ET. In (b) ■ and ● represent 5-oxo-ET formed from ET and 5F-ET respectively. □, 5-Oxo-6F-ET formed from 6F-ET.

5-lipoxygenase. To account for the apparent absence of reaction of 5-OOH-6F-ETE with 5-lipoxygenase, several tentative explanations can be proposed. If one considers the generally accepted radical mechanism for the 5-lipoxygenase reaction and the known stereoselectivity of hydrogen abstraction and oxygen addition

[17], the intermediates that might be involved in the successive formation of 5-OOH-6F-ETE and a hypothetical 6F-LTA₄ are depicted in Scheme 3. Firstly, a free radical delocalized on C-9, C-7 and C-5 would be formed, which would then be trapped at C-5 by reaction with molecular O₂. Theoretically, the rate of

Table 1. Inhibition of the extent of ED oxygenation by ET, ETE and their 5- and 6-fluoro derivatives

The experiments were performed as detailed in the Materials and methods section. In Expts. 1, 2 and 3 the amounts of ED oxygenation products formed in controls without inhibitor were respectively 1.6, 1.1 and 0.6 nmol/9 min per assay at low concentration of ED and 10.7, 10.6 and 6.6 nmol/9 min per assay at high concentration. Abbreviation: N.D., not determined.

Expt. no.	Inhibitor	[I] (μM)	Inhibition or activation (*) (%)		
			[S] = 10 μM	[S] = 59 μM	
1	ET	13	26 (*)	7 (*)	
		40	29 (*)	7	
	5F-ET	12	40	25	
		36	57	48	
	6F-ET	10	6 (*)	2 (*)	
		30	7	14	
			[S] = 12 μM	[S] = 69 μM	
2	5F-ET	5	6	5	
		10	30	21	
		29	48	44	
	ETE	5	9	1	
		10	11	8	
		30	32	n.d.	
	5F-ETE	5	53	34	
		10	62	52	
		30	83	78	
				[S] = 10 μM	[S] = 60 μM
	3	ETE	2	9	11
			10	4	3
30			31	59	
5F-ETE		2	44	25	
		9	61	45	
		28	61	73	
6F-ETE		1.5	48	49	
		8	64	63	
		24	50	85	

formation and the stability of this radical should not significantly differ from that of the non-fluorinated analogue. This would agree with the observed similar rate of oxygenation of ETE and 6F-ETE (results not shown). Turnover of 5-OOH-6F-ETE by LTA₄ synthase would involve a free radical delocalized on C-12, C-10, C-8 and C-6, which should react as detailed in Scheme 3 to yield 6F-LTA₄. The presence of fluorine at C-6 is expected to decrease the overall stability of the radical, but also the probability of reaction at C-6 with the hydroperoxide moiety. However, free-radical formation at a carbon bearing fluorine may occur, as evidenced by the fact that 5F-ETE was converted by 5-lipoxygenase, albeit inefficiently, into 5-oxo-ETE. Hence the formation of a 6F-LTA₄ derivative is plausible. Considering the very rapid hydrolysis of α -fluoro-4-nitrostyrene oxide in slightly alkaline buffer [24] compared with the high stability of 4-nitrostyrene oxide [25], one might expect a 6F-LTA₄ derivative to be even more reactive than LTA₄ with respect to nucleophiles. Therefore, if 6F-LTA₄ was formed, it might react with an active-site residue and alkylate the enzyme. This might explain the apparent absence of 6F-LTA₄ synthesis. Alternatively, a mech-

anism involving organoiron intermediates during the 5S-oxygenase and LTA₄ synthase reactions, as recently proposed by Corey and co-workers [23,26], would also agree with our experimental results. Besides formation of 5-OOH-6F-ETE and 5-OH-6F-ETE from 6F-ETE, the formation of another metabolite (t_R = 24.4 min) was detected at 280 nm (Fig. 1b). This had a λ_{max} of 283 nm and was characterized as 5-oxo-6-fluoroeicosa-6,8,11,14-tetraenoic acid (5-oxo-6F-ETE) after g.c.-m.s. of the methoxime of the methyl ester derivative. This ketone might arise from the interaction of 5-OOH-6F-ETE with haemoproteins contained in the supernatant, as previously observed for other fatty acid hydroperoxides such as 12-HPETE and 15-HPETE [22]. Finally, in contrast with ETE and 5F-ETE, there was no evidence for conversion of 6F-ETE into 12-oxygenated fatty acid derivatives by the contaminating 12-lipoxygenase.

Substrate properties of the fluorinated ET analogues

To have a better insight into the effect of fluorine on the rate of oxygenation of the 5,8-*cis,cis*-diene moiety by 5-lipoxygenase, ET and the corresponding 5-fluoro and 6-fluoro analogues (5F-ET and 6F-ET) were studied as substrates. ET is a good substrate of 5-lipoxygenase [12]. Moreover, the lack of the Δ -11 bond has a dual effect. First, it prevents metabolism by 12-lipoxygenase. Then, it also prevents 5-OOH-ET, the product of 5S-oxygenase, from being converted by the LTA₄ synthase activity of 5-lipoxygenase [12]. On the basis of retention times in reverse-phase h.p.l.c. and λ_{max} obtained with a diode array detector, the only products of conversion of ET and 6F-ET in RBL-1 supernatant were identified as the respective 5-hydroperoxy-, 5-hydroxy- and 5-oxoeicosa-6,8,14-trienoic acid derivatives. 5F-ET was metabolized to a single product, identified as 5-oxoeicosa-6,8,14-trienoic acid. The rates of product formation were estimated from the steepest linear portion of progress curves, which displayed the typical sigmoidal shape of a lipoxygenase-catalysed reaction. The rates of formation of 5-OOH-ET and 5-OOH-6F-ET (i.e. the sums of the 5-hydroxy- and unreduced 5-hydroperoxy-fatty acids) from ET and 6F-ET respectively were compared with the rate of formation of 5-oxo-ET from 5F-ET at various concentrations of the unsaturated fatty acids. 6F-ET was found to be a better substrate of 5-lipoxygenase than was ET (Fig. 2a). In contrast, the rate of conversion of 5F-ET, at 50 μM , was about 23 times lower than that of 6F-ET (Fig. 2a). Incubations of ET and 6F-ET with 5-lipoxygenase also produced 5-oxo-ET and 5-oxo-6F-ET, but at rates about 67 and 35 times lower than the global rate of oxygenation of the respective fatty acids (Figs. 2a and 2b).

This study with ET analogues confirms the preliminary observations made with ETE analogues. Substitution of hydrogen by fluorine at C-6 of the penta-5,8-*cis,cis*-diene moiety rather favours oxygenation by 5-lipoxygenase, whereas substitution at C-5 significantly lowers the rate of reaction. Such differences in reactivity may be either related to the strong electron-withdrawing effect of fluorine or/and to hydrogen-bond formation with fluorine in the active site [27]. Such interactions might alter the affinity of the substrate for the enzyme or lead to binding in catalytically unfavourable conformations.

Inhibitory activities of the fluorinated ET and ETE analogues

Although the fluorinated analogues of ETE and ET all displayed properties of substrates, they were also tested as potential inhibitors of 5-lipoxygenase. Turnover of substrate by 5-lipoxygenase results in a suicide-type time course of reaction, which has been observed with the enzymes from various sources [17,28] and with various 5-lipoxygenase substrates [29]. 5-HPETE, the major product resulting from ETE oxygenation by 5-lipoxygenase, has been reported to be responsible for the

inactivation of the enzyme [30]. However, inactivation by free radicals formed during catalysis is an alternative possibility. To assess the relative potency of the various fluorinated and non-fluorinated substrates of 5-lipoxygenase as inhibitors, their effects on the progress curve of ED oxygenation were investigated.

ED has been shown to be a good substrate of RBL-1 5-lipoxygenase [12]. If the progress curve of ED oxygenation is measured in the presence of another substrate (potential inhibitor), one can expect to observe two effects. The first is a decrease in the maximal rate, which might reflect a combination of competitive inhibition and inactivation of 5-lipoxygenase during turnover by the added substrate. A second effect would be a decrease in the amount of products of ED oxygenation formed after termination of the reaction. This might reflect a more rapid inactivation of 5-lipoxygenase during catalysis, compared with the control.

Such competition experiments were performed at 60 μ M-ED and increasing concentrations of the potential competitors ET, 5F-ET and 6F-ET (results not shown). In each case, an increase in the concentration of the competitor resulted in a significant decrease in both the maximal rate and the extent of ED oxygenation after completion of the reaction (9 min). At 45 μ M-5F-ET, the most effective inhibitor, the rate of ED oxygenation decreased by 59% and the extent by 48%.

An accurate determination of the rate being difficult with the discontinuous h.p.l.c. method, the various potential competitors were classified according to their ability to decrease the extent of ED oxygenation after termination of the reaction. The results of three separate experiments are summarized in Table 1. Each experiment was performed at two concentrations of ED around 10 and 60 μ M and at several concentrations of competitors. In Expt. 1, ET and its 5- and 6-fluoro analogues were compared as inhibitors. At 10 μ M-ED, addition of ET increased the extent of ED oxygenation. 6F-ET produced only marginal effects on the reaction. In contrast with ET and 6F-ET, 5F-ET was an efficient inhibitor, since at 12 μ M it decreased the extent of ED oxygenation by 40%. When the experiment was run with 59 μ M-ED, ET and 6F-ET were almost ineffective, but 5F-ET produced a 25% inhibition at 12 μ M. In Expt. 2 (Table 1), ETE, 5F-ET and 5F-ETE were compared as inhibitors. At a low concentration of ED, the concentrations of these three fatty acids required to inhibit the extent of ED oxygenation by 50% were respectively > 30, 29 and 5 μ M. The data obtained at high concentration of ED confirmed this order of potency. In Expt. 3, the higher potency of 5F-ETE with respect to ETE was confirmed. 6F-ETE proved as potent as 5F-ETE when low concentrations of ED were used, and even more potent at high ED concentration.

CONCLUSIONS

The high efficiencies of 5F-ETE and 6F-ETE as inhibitors of 5-lipoxygenase with respect to ETE are noteworthy. This property of the fluorinated ETE analogues combined with the fact that they are not metabolized into leukotriene-like metabolites makes them interesting new tools for the study of the 5-lipoxygenase pathway. Moreover, the structural similarity of these compounds to ETE may be a potential advantage with respect to other 5-lipoxygenase inhibitors. Provided that they follow the same pathways as ETE in mammals, they might be

easily incorporated into phospholipids of cell membranes and released at the same site as ETE, i.e. where synthesis of inflammatory mediators derived from ETE takes place.

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REFERENCES

1. Sirois, P. (1985) *Adv. Lipid Res.* **21**, 79–101
2. Mullane, K. M., Salmon, J. A. & Kraemer, R. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 2422–2433
3. Cohen, N., Weber, G., Banner, B. L., Welton, A. F., Hope, W. C., Crowley, H., Anderson, W. A., Simko, B. A., O'Donnel, M., Coffey, J. W., Fiedler-Nagy, C. & Batula-Bernardo, C. (1984) *Prostaglandins* **27**, 553–562
4. Nicolaou, K. C., Petasis, N. A., Li, W. S., Ladduwahetty, T., Randall, J. L., Webber, S. E. & Hernandez, P. E. (1983) *J. Org. Chem.* **48**, 5400–5403
5. Corey, E. J., Lansbury, P. T., Cashman, J. R. & Kantner, S. S. (1984) *J. Am. Chem. Soc.* **106**, 1501–1503
6. Corey, E. J., Cashman, J. R., Kantner, S. S. & Wright, S. W. (1984) *J. Am. Chem. Soc.* **106**, 1503–1504
7. Corey, E. J., Cashman, J. R., Eckrich, T. M. & Corey, D. R. (1985) *J. Am. Chem. Soc.* **107**, 713–715
8. Kwok, P., Muellner, F. W., Chen, C. & Fried, J. (1987) *J. Am. Chem. Soc.* **109**, 3684–3692
9. Kobayashi, Y. (1987) *Actual. Chim.* May issue 179–182
10. Taguchi, T., Takigawa, T., Igarashi, A., Kobayashi, Y., Tanaka, Y., Jubiz, W. & Briggs, R. G. (1987) *Chem. Pharm. Bull.* **35**, 1666–1669
11. Beckman, J. K., Gay, J. C., Brash, A. R., Lukens, J. N. & Oates, J. A. (1985) *Biochem. Biophys. Res. Commun.* **133**, 23–29
12. Navé, J.-F., Dulery, B., Gaget, C. & Ducep, J.-B. (1988) *Prostaglandins* **36**, 385–398
13. Corey, E. J., Albright, J. O., Barton, A. E. & Hashimoto, S. (1980) *J. Am. Chem. Soc.* **102**, 1435–1436
14. Bergström, S., Ryhage, R., Samuelsson, B. & Sjövall, J. (1963) *J. Biol. Chem.* **238**, 3555–3564
15. Corey, E. J., Marfat, A., Goto, G. & Brion, F. (1980) *J. Am. Chem. Soc.* **102**, 7984–7985
16. Arai, Y., Shimoji, K., Konno, M., Konishi, Y., Okuyama, S., Iguchi, S., Hayashi, M., Miyamoto, T. & Toda, M. (1983) *J. Med. Chem.* **26**, 72–78
17. Yamamoto, S. (1989) *Prostaglandins Leukotrienes Essent. Fatty Acids* **35**, 219–229
18. Reddy, C. C., Rao, M. K., Mastro, A. M. & Egan, R. W. (1984) *Biochem. Int.* **9**, 755–761
19. Samuelsson, B. (1983) *Science* **220**, 568–575
20. Jakschik, B. A. & Kuo, C. G. (1983) *Prostaglandins* **25**, 767–781
21. Hamasaki, Y. & Tai, H.-H. (1984) *Biochim. Biophys. Acta* **793**, 393–398
22. Fruteau de Lacroix, B. & Borgeat, P. (1988) *Biochim. Biophys. Acta* **958**, 424–433
23. Corey, E. J. & Nagata, R. (1987) *J. Am. Chem. Soc.* **109**, 8107–8108
24. Hanzlik, R. P. & Hilbert, J. M. (1978) *J. Org. Chem.* **43**, 610–614
25. Westkaemper, R. B. & Hanzlik, R. P. (1981) *Arch. Biochem. Biophys.* **208**, 195–204
26. Corey, E. J., Wright, S. W. & Matsuda, S. P. T. (1989) *J. Am. Chem. Soc.* **111**, 1452–1455
27. Abeles, R. H. & Alston, T. A. (1990) *J. Biol. Chem.* **265**, 16705–16708
28. Haurand, M. & Flohé, L. (1988) *Biol. Chem. Hoppe-Seyler* **369**, 133–142
29. McMillan, R. M., Masters, D. J., Vickers, V. C., Dicken, M. P. & Jacobs, V. N. (1989) *Biochim. Biophys. Acta* **1005**, 170–176
30. Aharony, D., Redkar-Brown, D. G., Hubbs, S. J. & Stein, R. L. (1987) *Prostaglandins* **33**, 85–100