Free and esterified 13(R,S)-hydroxyoctadecadienoic acids: principal oxygenase products in psoriatic skin scales

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Abstract Characterization of the chemical form and stereospecificity of the fatty acid derivatives of arachidonic and linoleic acid in psoriatic epidermis is needed to define the enzymatic origin of these compounds and their possible role in pathogenesis. In an analysis of psoriatic skin scales, both free and esterified 13-hydroxyoctadecadienoic acids were the principal fatty acid derivatives, present in mean concentrations of 115 and 17 ng/mg scales, respectively. The analysis included reversed-phase high performance liquid chromatography of the free acid and of its methyl ester, gas chromatography, gas–liquid chromatography–mass spectrometry of the methyl ester derivatives, and chiral separation. The free and esterified 13-hydroxyoctadecadienoic acids isolated from the psoriatic scales contained a mixture of the S/R stereoisomers, averaging 1.9:1 for free 13- hydroxyoctadecadienoic acid. These findings are not compatible with the strict S-stereospecificity for oxygen insertion exhibited by mammalian lipoxygenase but rather could point to the action of a cyclooxygenase. The demonstration that a hydroxylated fatty acid derivative is esterifed in vivo in psoriatic keratinocytes suggests that the physiology of these cells may be altered early in the process of keratinization. —**Baer, A. N., P. B. Costello, and F. A. Green.** Free and esterified 13(R,S)-hydroxyoctadecadienoic acids: principal oxygenase products in psoriatic skin scales. *J. Lipid Res.* 1990. **31:** 125–130.

Supplementary key words cyclooxygenase • lipoxygenase • keratinocytes

A number of eicosanoids have been detected in psoriatic skin lesions, some of which are present in biologically active concentrations. Hammarstrom et al. (1) first reported a marked increase in endogenous levels of free 12-hydroxy-5,8,10,14-eicosatetraenoic (12-HETE) acid in keratome strips of psoriatic epidermis. The 5- and 15-lipoxygenase products, leukotriene B_4 (LTB_4) and 15-hydroxyeicosatetraenoic acid (15-HETE), respectively, have been described more recently in keratome specimens of psoriatic skin lesions (2) and in exudates from abraded psoriatic plaques (3). Chemokinetic activity has been detected in association with extracted material co-eluting with LTB_4 and 12-HETE (4). Smaller amounts of 8-, 9-, and 11-hydroxyeicosatetraenoic acids have also been described in extracts of psoriatic skin scales (4). The eicosanoids described to date in psoriatic lesions are free (nonesterified). The possibility that these hydroxylated fatty acids are re-esterified into cellular lipids of the keratinocyte has not been examined.

Some evidence for the presence in psoriatic lesions of the monohydroxylated fatty acid derivatives of linoleic acid, 13-hydroxyoctadecadienoic acid (13-HODD) and 9-hydroxyoctadecadienoic acid (9-HODD), has also been provided (4). The concentration of 13-HODD in keratome specimens of psoriatic lesions and in psoriatic scales has been reported to be equivalent to or greater than that of 12-HETE (4, 5). A specific pathogenetic role in psoriasis for the hydroxylated derivatives of arachidonic and linoleic acids has not been defined.

The cellular origin of the hydroxylated fatty acids in psoriatic epidermis is uncertain, and the effects of the procedure used to remove tissue on oxygenase activation have not been completely assessed. Both 12-HETE and 15-HETE may be synthesized by dermis and epidermis (6, 7), and 15-HETE may be produced in damaged neutrophils (8), possibly those infiltrating the psoriatic epidermis. There is also uncertainty as to the type of oxygenases responsible for generating these monohydroxylated fatty acids. 13-HODD may be generated from linoleic acid by the action of either a lipoxygenase or a cyclooxygenase as well as by autooxidation. The lipox-

Abbreviations: 12-HETE, 12(s)-hydroxy-5,8,10,14-eicosatetraenoic acid; LTB_4, leukotriene B_4; 15-HETE, 15-hydroxyeicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; 13-HODD, 13-hydroxyoctadecadienoic acid; 9-HODD, 9-hydroxyoctadecadienoic acid; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; LC, liquid chromatography; RT, retention time; UV, ultraviolet; GLC–MS, gas–liquid chromatography–mass spectrometry.

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ygenases in mammalian platelets, reticulocytes, and leukocytes that have been analyzed to date all demonstrate strict (S) chirality at the point of oxygen insertion in unsaturated fatty acids (9, 10). It has thus been assumed that S-stereospecificity would also be found for lipoxygenases in other cells and tissues. Woollard (11), however, has provided evidence that 12-HETE extracted from psoriatic scales has the R-stereospecificity.

We report herein an analysis of the hydroxylated fatty acid profile of psoriatic skin scales, using a transesterification method to extract both free and esterified fatty acid derivatives. A stereochemical analysis of the major hydroxylated fatty acid in these samples, 13-hydroxyoctadecadienoic acid, was performed in order to shed light on the type of oxygenase involved in its generation in vivo.

MATERIALS AND METHODS

Reagents

Arachidonic acid and linoleic acid were obtained from Nu-Chek-Prep, Inc. (Elysian, MN). Synthetic eicosanoids and 13(s)-hydroxyoctadeca-9Z,11E-dienoic acid were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). All solvents were of HPLC grade.

Subjects

Psoriatic skin scales were obtained by gentle removal from the skin of patients with psoriasis vulgaris. Skin scales were also obtained from patients with erythroderma secondary to pityriasis rubra pilaris, gold-induced exfoliative dermatitis, and sun-induced erythema. A sample of heel stratum corneum was obtained from two normal male volunteers by gentle abrasion with a file.

Sample preparation

The tissue samples, 30–130 mg, were treated by the anhydrous methanolic NaOH method of Kates (12) to quantitatively transesterify all esterified fatty acids and their derivatives to their respective methyl esters while preserving free fatty acids and their derivatives intact. A single modification was made in order to partition nonesterified fatty acids and their hydroxylated derivatives into the organic layer: after the addition of water to separate the phases, 100 μl of 2 M ammonium formate buffer, pH 3.2, was added. After washing with methanol–water 10:9 saturated with chloroform, the lower phase was reconstituted into a mobile phase consisting of methanol–water–acetic acid 80:20:0.1. The average recovery of fatty acids with this extraction procedure was 65%. High performance liquid chromatography (HPLC) was performed isocratically on a Hewlett-Packard 1090 liquid chromatograph (LC) using Hewlett-Packard ODS-Hypersil columns, 20 cm in length × 4.6 mm at a flow rate of 0.4 ml/min. Since this instrument is equipped with a diode array spectrophotometer and a computer for on-line display and storage of ultraviolet spectra, precise measurement of the λ_{max} of each peak could be performed after the run was completed. Hydroxy fatty acids in concentrations of 10 ng or more can be clearly identified with this instrument. Free hydroxylated fatty acids collected during the LC runs were methylated with ethereal diazomethane and rechromatographed on the same LC columns (delayed retention time of approximately 15 min). In some experiments, the methylated fatty acids were collected during the LC run, and rechromatographed on two 25 cm Bakerbond chiral phase LC columns in series (dinitrobenzoylphenyl glycine coupled ionically over aminopropyl residues; J. T. Baker Research Products). The mobile phase mixture for this straight phase HPLC consisted of hexane–isopropanol 1000:15. Chiral phase separations were carried out on esters that were either generated in vitro by diazomethane or in vivo followed by transesterification to methyl esters in vitro. Mixing experiments with synthetic standards were carried out to confirm exact retention times in the chiral phase HPLC studies. All the LC studies were monitored at a wavelength of 236 nm but recall was available at 270 nm and 301 nm.

In the figures showing chromatographic results, the ordinate scale is given in milliabsorption units and all chromatograms are reproduced directly from the LC computer plots. Software programs for quantitation using published molar extinction coefficients and molecular weights were used to measure concentrations (13).

In some experiments, the fatty acids were analyzed by gas–liquid chromatography. After the methylation of the free hydroxylated fatty acid with ethereal diazomethane, the trimethylsilyl ether derivative was prepared by the addition of 20 μl of pyridine and 40 μl of N,O,-bis(trimethylsilyl)-trifluoroacetamide. With this procedure, the fatty acids could be clearly separated by GLC on an HP 3940 gas chromatograph using a Supelco 2330 semi-polar column and flame ionization detector. Under 10 lbs of head pressure and using isothermal elution (oven temperature 200°C), the retention times were: linoleic acid, min; 13-hydroxyoctadecadienoic acid, 4.9 min; arachidonic acid, 6.1 min; 12-HETE, 7.9 min; 15-HETE, 8.1 min; and 5-HETE, 8.2 min.

GLC–MS was carried out using a Kratos MS80 RFA mass spectrometer at 50-70 eV.

RESULTS

The chromatographic separation of extracts from psoriatic skin scales of six patients each yielded a major peak at 21.5 min (compound I), with an ultraviolet spectrum showing a λ_{max} at 234 nm and a retention time identical to that of authentic 13-hydroxyoctadeca-9Z,11E-dienoic

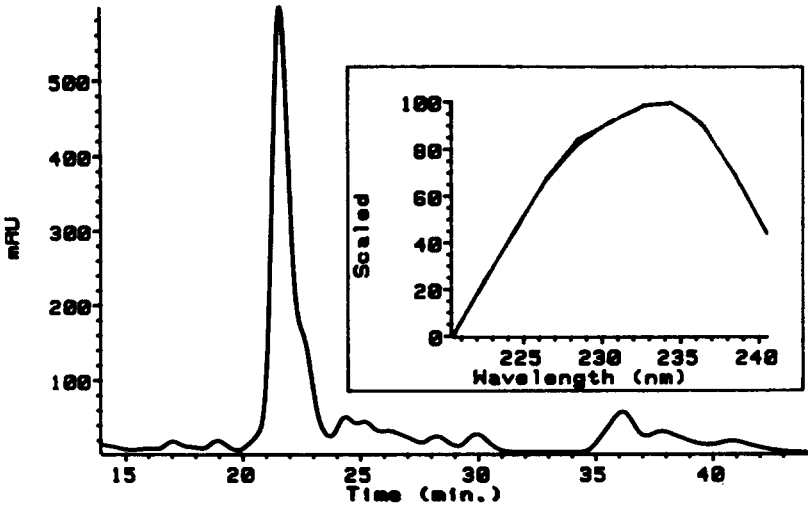
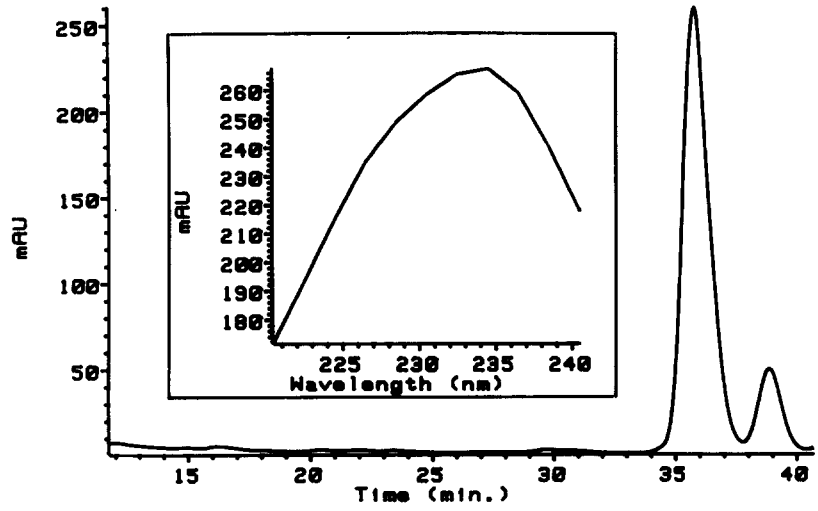


Fig. 1. Reversed phase HPLC chromatogram of the monohydroxylated fatty acids extracted after chemical transesterification of psoriatic scales. The peak at 21.5 min (compound I in text) comigrates with synthetic 13-hydroxyoctadecadienoic acid (13-HODD) and the peak at 36 min comigrates with the methyl ester of 13-HODD (compound II in text). The UV spectra of peaks I and II are shown superimposed in the inset, with their ordinate axes scaled. Both spectra show a λ_{max} at 234 nm.

acid. An example is shown in **Fig. 1**. A second, lesser peak with an identical λ_{max} at 234 nm eluted at 36 min (Fig. 1) in all specimens. Additional small peaks were observed at the approximate retention times of 15-HETE and 12-HETE. When compound I was collected, methylated, and re-chromatographed on the same LC columns, there was a delay in retention time of approximately 15 min (**Fig. 2**). The methylated compound eluting at 36 min had a λ_{max} of 234 nm, the same as the methyl esters of the synthetic standard, 13-HODD. From these observations, it was concluded that compound I was a free hydroxylated fatty acid, with a conjugated diene structure and that the lesser peak at 36 min on the original chromatogram was the methyl ester of compound I which was formed in vivo and was released by transesterification. With GLC, the trimethylsilyl ether derivative of the methyl ester of compound I eluted as a single peak with a retention time of 4.9 min, which was identical to that of the corresponding derivative of synthetic 13(S)-HODD. On GLC–MS, this derivative of compound I had a mass spectrum with major fragment ions at *m/z* 382 (M^+), 367 (M^+–15), 351 (M^+–31), 311 (M^+–71), 292 (M^+– 90), and 225 (M^+–157) (**Fig. 3**) indicating that compound I is a monohydroxylated C-18 fatty acid containing two double bonds. The relative intensity of the fragment at *m/z* 311 was greater than that at 225, a finding characteristic of the trimethylsilyl derivative of 13-HODD methyl ester (14, 15) and which differentiates it from the corresponding derivative of 9-HODD methyl ester. Using a molar extinction coefficient of 27,000 M^{-1}/cm^{-1}, the concentration of free 13-HODD averaged 115 ± 28 ng/mg scales (mean ± SEM) (range 49–237 ng/mg scales) in the six psoriatic skin scale specimens. Esterified 13-HODD was present in an average concentration of 17 ± 4 ng/mg scales (range 7–23 ng/mg scales) in the six specimens. A 9-HODD standard was found to be widely separated (△RT = 10

Fig. 2. Reversed phase HPLC chromatogram of the fractions comprising peak I of Fig. 1 following reaction with ethereal diazomethane. The retention time (delayed by 15 min compared to Fig. 1) and UV spectrum (inset) of the resulting compound eluting at 36 min is characteristic of the methyl ester of 13-HODD.



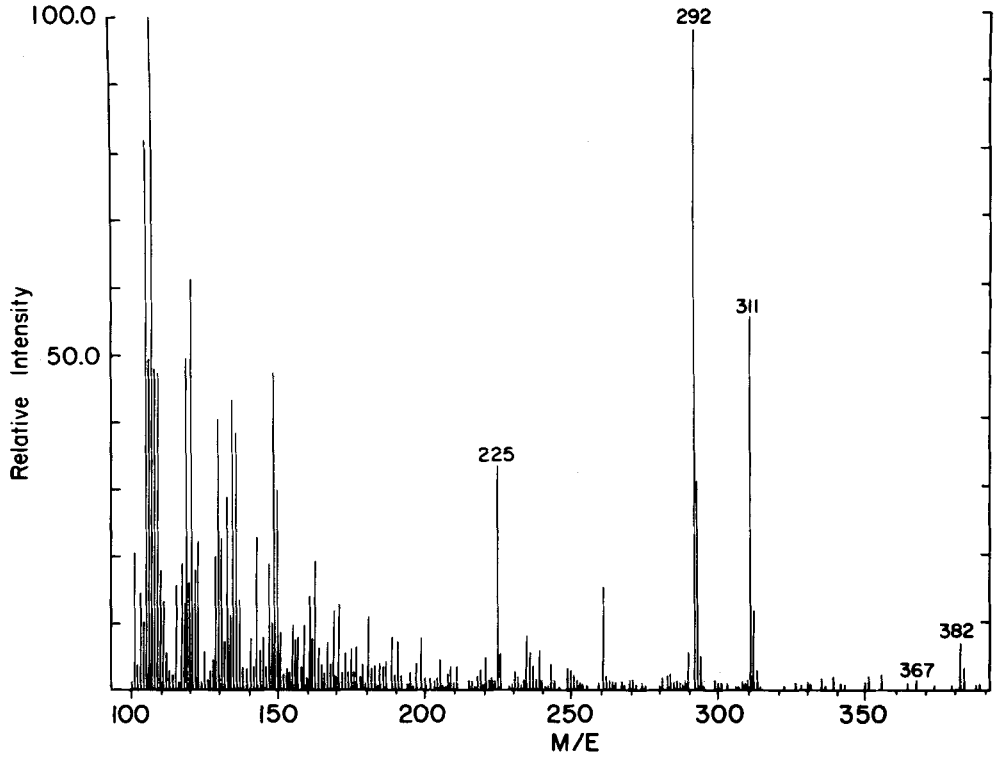


Fig. 3. GLC-MS spectrum of the trimethylsilyl ether derivative of the methyl ester of compound I eluting at 21.5 min in Fig. 1.

min) from 13-HODD on these chiral/straight phase columns, thus confirming the 13-HODD identification of the principal compound recovered.

The stereospecificity of both the free and esterified 13-HODD present in the psoriatic skin scales was analyzed using chiral phase HPLC columns under straight phase conditions. For these analyses, the 13-HODD methyl ester was collected during reversed phase chromatography, and re-chromatographed on chiral phase columns. An example of one such LC run is shown in **Fig. 4**. During the analysis of each patient sample, a mixing experiment was performed as illustrated in Fig. 4 to confirm the identity of the compound as 13-HODD methyl ester and to differentiate the stereoisomers. In each sample analyzed, there was a nonracemic mixture of the S- and R-stereoisomers, with the S/R ratio ranging from 1.4 to 2.3

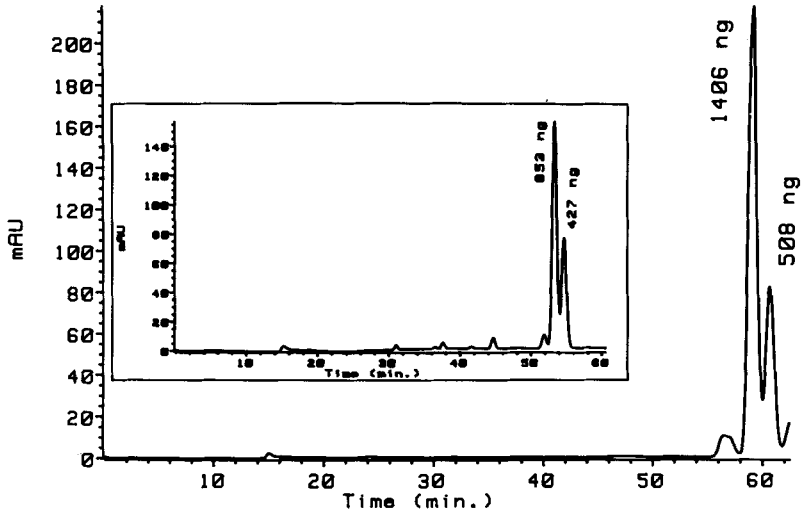


Fig. 4. Representative straight phase chiral HPLC chromatograms of the methyl ester of 13-HODD. The chromatogram shown in the inset is from peak I (Fig. 1) which was then esterified (Fig. 2). The outer chromatogram is that of the same material to which 13(S)-HODD methyl ester synthetic standard was added in order to define the chirality of the two peaks. The original mixture of stereoisomers had a S/R ratio of 2:1 (insert) whereas after addition of 360 ng of the S-stereoisomer the ratio was increased to 2.8:1.

(mean ± SEM, 1.9 ± 0.2). The chiral separation was confirmed by the finding of a single peak (with retention time of 13-HODD) when the material separating on chiral phase HPLC was collected, derivatized, and analyzed by gas–liquid chromatography. The methyl ester of 13-HODD, derived from a sample of psoriatic skin scales by transesterification (representing in vivo esterified 13-HODD) was also a nonracemic mixture on chiral phase HPLC, with a predominance of the S-stereoisomer (ratio 2.7:1).

In the analysis of skin samples from the patients with pityriasis rubra pilaris and gold-induced dermatitis, both free and esterified 13-HODD were found, although the concentration of free 13-HODD was less than that in the psoriatic scales. The free 13-HODD in the sample from the patient with gold-induced dermatitis had an S/R ratio of 5.5:1 while the in vivo esterified 13-HODD had an S/R ratio of 1.8:1. No 13-HODD, either free or esterified, was found in the sample of normal heel stratum corneum or in the sample of scales from an individual with sun-induced erythema. However, analysis of the fatty acid profile of the normal stratum corneum sample by GLC showed the presence of precursor linoleic acid in a concentration of 62 ng/mg.

DISCUSSION

These results confirm the findings of Camp et al. (4) that, although small amounts of other eicosanoids and 9-HODD may be present, 13-HODD is the principal hydroxylated fatty acid in human psoriatic skin scales. Fogh et al. (2) and Duell, Ellis, and Voorhees (5) have also noted large amounts of "13-HODD-like" material in keratome specimens of psoriatic lesions, but have not characterized this material further. The finding of the methyl ester of 13-HODD after chemical transesterification of these specimens indicates for the first time that a portion of the 13-HODD generated in the psoriatic lesion had been re-esterified into cellular lipids some time during the rapid passage of the keratinocyte through the psoriatic epidermis. The large abundance of 13-HODD strongly points to the keratinocyte as the cell of origin of this product. The capacity of the human keratinocyte to esterify HETEs requires an intact cell (16) implying that this esterification takes place early rather than late in the keratinization process. This may indicate a potential for a regulatory role of the hydroxylated fatty acid such as has been demonstrated for the products of arachidonate 15-lipoxygenase in rabbit reticulocytes (17).

In an analysis of samples from two other inflammatory skin disorders, the findings were similar to those for psoriasis, with both free and esterified 13-HODD constituting the major hydroxylated fatty acid components. The significance of these observations with respect to the pathogenesis of psoriasis and other inflammatory skin diseases remains to be determined. Normal heel stratum corneum contained no measurable 13-HODD but did have large amounts of its linoleic acid precursor. This would indicate that an activated oxygenase was present in the inflammatory skin lesions.

The stereospecificity of the 13-HODD derived from psoriatic skin scales had not been reported previously. Autooxidation of linoleic acid leads to a mixture of positional isomers (13-HODD and 9-HODD) and of different *cis/trans* isomers as well as a racemic mixture of the stereoisomers of these compounds (18, 19). Since the 13-HODD extracted from the psoriatic scales in the present study was a nonracemic mixture (S/R ratio, 1.4–2.3), the 13-HODD probably had an enzymatic origin. Hydroxylation of fatty acids may be mediated by lipoxygenases, fatty acid cyclooxygenases, and cytochrome P-450 monooxygenases. The stereospecificity of the lipoxygenase- and cyclooxygenase-mediated reactions has been examined. The mammalian lipoxygenases studied to date all have strict S-stereospecificity (9, 10). It has been demonstrated that the stereospecificity of the various soybean lipoxygenases is altered as a function of pH; the possibility that physicochemical conditions in the epidermis might alter epidermal lipoxygenases cannot be excluded (20, 21), but is unlikely. Hamberg and Samuelsson (22) reported that cyclooxygenase derived from sheep vasicular gland converted linoleic acid to a 77:23 mixture of 9-HODD and 13-HODD with 78% of the 13-HODD being the S-stereoisomer. The present studies of the chirality of 13-HODD from psoriatic scales appear to be incompatible with lipoxygenase activity but suggest that another enzyme, possibly cyclooxygenase, is involved. This is very surprising since 15-lipoxygenase is prominent in normal human keratinocytes (16, 23, 24) and this enzyme oxygenates linoleic acid at C-13 (n–6 specificity).

The metabolic effect of free or esterified 13-HODD in psoriatic epidermis needs to be defined. The presence of 13-HODD in scales may be closely associated with the altered process of keratinization in psoriasis. The immediate precursor of 13-HODD, 13-hydroperoxyoctadecadienoic acid, is known to activate guanylate cyclase at concentrations in the 2–3 μm range (25). The concentration of this reactive precursor of 13-HODD is psoriatic epidermis could thus be sufficient to modulate guanylate cyclase activity and thus contribute to the altered levels of cyclic 3',5'-guanosine monophosphate which have been observed in psoriatic epidermis (26).

Note added in proof: We have recently also identified both 9-hydroxyoctadecadienoic acid (9-HODD) and 12-hydroxyeicosatetraenoic acid (12-HETE) in psoriatic skin scales. The amounts relative to 13-HODD in three samples each were 41 ± 8% (mean ± SEM) for 9-HODD and 20 ± 3% for 12-HETE. The (R) stereoisomer of each compound predominated, with the R/S ratio being 2.4 ± 0.1/1 for 9-HODD and 5.7 ± 0.1/1 for the 12-HETE.

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