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# Occurrence and biosynthesis of 11(*R*)-hydroxy-eicosatetraenoic acid (11-*R*-HETE) in the Caribbean soft coral *Plexaurella dichotoma*

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**Abstract.** Gorgonian soft corals from the Caribbean Sea are known to contain prostaglandin-like compounds as well as other products of arachidonic acid lipoxygenation, and the formation of the latter has been suggested to represent the first step in prostaglandin biosynthesis. Here we present evidence for the presence of 11-*R*-hydroxy-5*Z*,8*Z*,12*E*,14*Z*-eicosatetraenoic acid (11-*R*-HETE), as well as of the enzyme responsible for its biosynthesis, in the Caribbean gorgonian *Plexaurella dichotoma*. Lipid extracts from *P. dichotoma* were purified by conventional $SiO_2$ column chromatography followed by reversed phase high-pressure liquid chromatography (HPLC). These yielded a component having chromatographic and spectroscopic properties identical to synthetic 11-HETE. Electron impact mass spectrometric analysis of the acetoxy-, methyl ester derivative of the compound confirmed its identity as 11-HETE, while chiral phase HPLC of the methyl ester derivative showed that the stereochemistry of the alcoholic carbon atom was *R*. Enzymatically active homogenates from *P. dichotoma* were able to convert both unlabelled and [$^3$H]arachidonic acid into 11-HETE. In vitro biosynthesis of the latter metabolite was also observed with homogenates of the Mediterranean gorgonian *Paramuricea clavata*, another non-prostaglandin-containing soft coral, thus suggesting that 11-*R*-HETE production is not necessarily accompanied by prostaglandin formation in gorgonian corals.

**Key words.** Marine eicosanoids; polyunsaturated fatty acid derivatives; arachidonic acid; soft corals; lipoxygenase.

Among lipidic signalling molecules, arachidonic acid derivatives, known as eicosanoids, are a class of compounds with a particularly wide spectrum of chemical structures, metabolic pathways and biological actions. Eicosanoids are widespread in both vertebrate and invertebrate phyla, and by acting either as paracrine, autocrine or second messengers, they mediate several physiopathological responses ranging from control of reproduction, development, homeostasis, osmosis and body temperature to modulation of neural transmission, long-term synaptic potentiation, and cell proliferation, motility and differentiation[1]. A landmark in eicosanoid research, the discovery in the 1970s of high amounts of prostaglandin (PG)-like metabolites in the gorgonian soft coral *Plexaura homomalla*[2] opened the way for the synthesis of large amounts of these physiologically important eicosanoids and subsequently raised the question whether PG biosynthesis in invertebrates might follow a route different from that shown in mammals (ref. 3; for recent reviews see refs 4 and 5). It was suggested that the pathway to PG formation in *Plexaura* species did not pass through the intermediacy of PG endoperoxides, like in mammals, and that an unstable allene oxide, formed from arachidonic acid via a lipoxygenase-mediated pathway, was instead a key intermediate for the synthesis of PGs[3-5]. This hypothesis, however, has not found unambiguous experimental support, due mainly to the inability of coral enzymatic preparations to produce chiral prostaglandins from either arachidonic acid or allene oxides in vitro. Moreover, the possibility that corals do in fact use PG endoperoxides such as $PGG_2$ as the first step in PG biosynthesis has gained support from two recent reports[6,7] showing formation of this metabolite as well as its nonenzymatic conversion to optically active PGs in homogenates of the white sea coral *Gersemia fruticosa*. The enzyme responsible for arachidonate transformation into $PGG_2$ is located in microsomal fractions of the coelenterate and, unlike mammalian PG endoperoxide synthase, is not sensitive to indomethacin and lacks a PG hydroperoxidase activity. This latter property prevents the formation of $PGH_2$ from $PGG_2$ in *G. fruticosa*.

With hydrozoans, the phylum Cnidaria provided even more intriguing questions regarding eicosanoid biosynthesis. Species belonging to the freshwater genus *Hydra*, as well as the marine *Hydractinia echinata*, although unable to synthesize PGs, contain high levels of enantioselective lipoxygenases whose catalytic action leads to the formation of hydroxyeicosatetraenoic and hydroxy-

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octadecatrienoic acids (HETEs and HOTrEs) with multiple roles in hydrozoan development and behaviour[5,8-11]. In particular, an unprecedented $\omega$10-($R$)-lipoxygenase has been partially characterized from *Hydra vulgaris*, where it is responsible for the production of 11-$R$-hydroperoxy-5$Z$,8$Z$,12$E$,14$Z$-eicosatetraenoic acid (11-$R$-HPETE) and of 9-$R$-hydroperoxy-10$E$,12$Z$,15$Z$-octadecatrienoic acid (9-$R$-HPOTrE) and, consequently, of their corresponding hydroxides, 11-$R$-HETE and 9-$R$-HOTrE[8-10]. This enzyme might also be involved in 11-$R$-HETE biosynthesis in *H. magnipapillata*[9].

In this study we show that 11-$R$-HETE, unlike prostaglandins, is present in lipid extracts from the Caribbean soft coral *P. dichotoma*, and that homogenates of this species and of the Mediterranean coral *Paramuricea clavata* contain an enzymatic activity capable of converting exogenous arachidonic acid into this eicosanoid. Optically pure $R$-11-, -8- and -15-HETEs are markers for lipoxygenase activity in living organisms, and have previously been found in gorgonian corals only in the presence of PGs. The finding that, in soft corals, the occurrence of this metabolite does not appear any longer to be strictly accompanied by PG biosynthesis, together with the data previously described for *G. fruticosa*[6,7], encourages the hypothesis that PG production and lipoxygenase activation are not necessarily related metabolic events in coelenterates.

## Materials and methods

*P. dichotoma* was taken from the sea off Puerto Morelos, Mexico, whereas *P. clavata* was fished in the Gulf of Naples. One branch of *P. dichotoma* (170 g dry weight) was extracted with acetone, and the acetone extract was dried down to yield a water suspension which was extracted three times with ethyl ether. The ethereal extract was crudely purified by column chromatography on $SiO_2$ (70–230 mesh, Merck, Germany) eluted with increasing concentrations of methanol (from 1 to 10%) in chloroform. Fractions were pooled according to the ratio front (Rfs) of their components on thin-layer chromatography (TLC) analytical plates developed with chloroform/methanol 95/5 v/v, and excess solvent evaporated under vacuum. Dry fractions were then dissolved in $CDCl_3$ and transferred to nuclear magnetic resonance (NMR) tubes for $^1$H NMR analysis on a Brucker 500 MHz apparatus. A fraction weighing about 200 µg and whose components exhibited $0.35 < Rf < 0.40$ was purified further by reversed phase high-pressure liquid chromatography (HPLC) carried out by using an analytical Spherisorb ODS-2 column (5 µm, 25 cm × 4.6 mm) eluted with a linear 60 min gradient from 80 to 95% methanol in water at a flow rate of 1 ml/min. Ultraviolet (UV) absorbance of the eluate was monitored simultaneously at 205, 235 and 280 nm using a Waters multiwavelength 490E detector. The major component, which had maximal absorbance at 235 nm and was eluted after 24 min, was collected, dried down and dissolved in $CDCl_3$ for $^1$H NMR analysis. After acquisition of NMR data, the sample was methylated by reaction with diazomethane for 15 min at room temperature in ethyl ether. The methylated compound was partly analyzed by chiral phase HPLC and partly acetylated by reaction with acetic anhydride/pyridine 1/4 v/v at 60 °C for 40 min under a nitrogen atmosphere. Chiral phase HPLC was conducted on a Chiralcel OB column (Daicel Chemical Industry) eluted with $n$-hexane/propan-2-ol (99/1 v/v) at a flow rate of 1.5 ml/min, and monitoring the UV absorbance at 235 nm. The acetoxy-, methyl ester derivative of the metabolite was analyzed by electron impact mass spectrometry (EIMS) using a Tri-O-2000 apparatus equipped with a VG quadrupole mass spectrometer.

In vitro biosynthesis experiments were conducted by incubating unlabelled (1 mg/ml) and/or 0.1 µCi/ml [$^3$H]arachidonic acid (NEN, Dupont de Nemours Gmbh, 60 Ci/mmol) with either *P. dichotoma* or *P. clavata* homogenates at 37 °C for 1 h. Whole homogenates were prepared by homogenizing either coral species in Tris-HCl, pH 7.4, as previously described[8-11]. Control incubations were carried out in homogenates inactivated either by lowering the pH or by heating. After 60 min, incubation was terminated by adding 6 N HCl and by lowering the temperature to 4 °C, the incubates were centrifuged for 15 min at 10,000$g$, and the supernatants were loaded onto Sep-pak C-18 cartridges (Millipore) primed and eluted as described previously[8-11]. Sep-pak eluates were dried down, and the residues were dissolved in methanol to be analyzed by reversed phase HPLC carried out as described above, but using an isocratic elution with 75% methanol in water, which ensures a higher resolution of HETEs. Radioactivity contained in 1-ml HPLC fractions was assessed by adding 4 ml of scintillation liquid and by counting $\beta$ emission by means of a liquid scintillation counter (Packard).

## Results and discussion

Lipids extracted from *P. dichotoma* were purified by $SiO_2$ column chromatography, and the fractions were analyzed by $^1$H NMR. None of these fractions was found to contain signals, such as those previously reported[2,12-14] or those observed for synthetic standards of PGE, PGA and PGF derivatives, with chemical shifts typical of the cyclopentyl ring of PG-like metabolites. Conversely, a fraction containing a mixture of compounds with $0.35 < Rf < 0.40$ (on TLC developed with chloroform/methanol 95/5) displayed signals that could

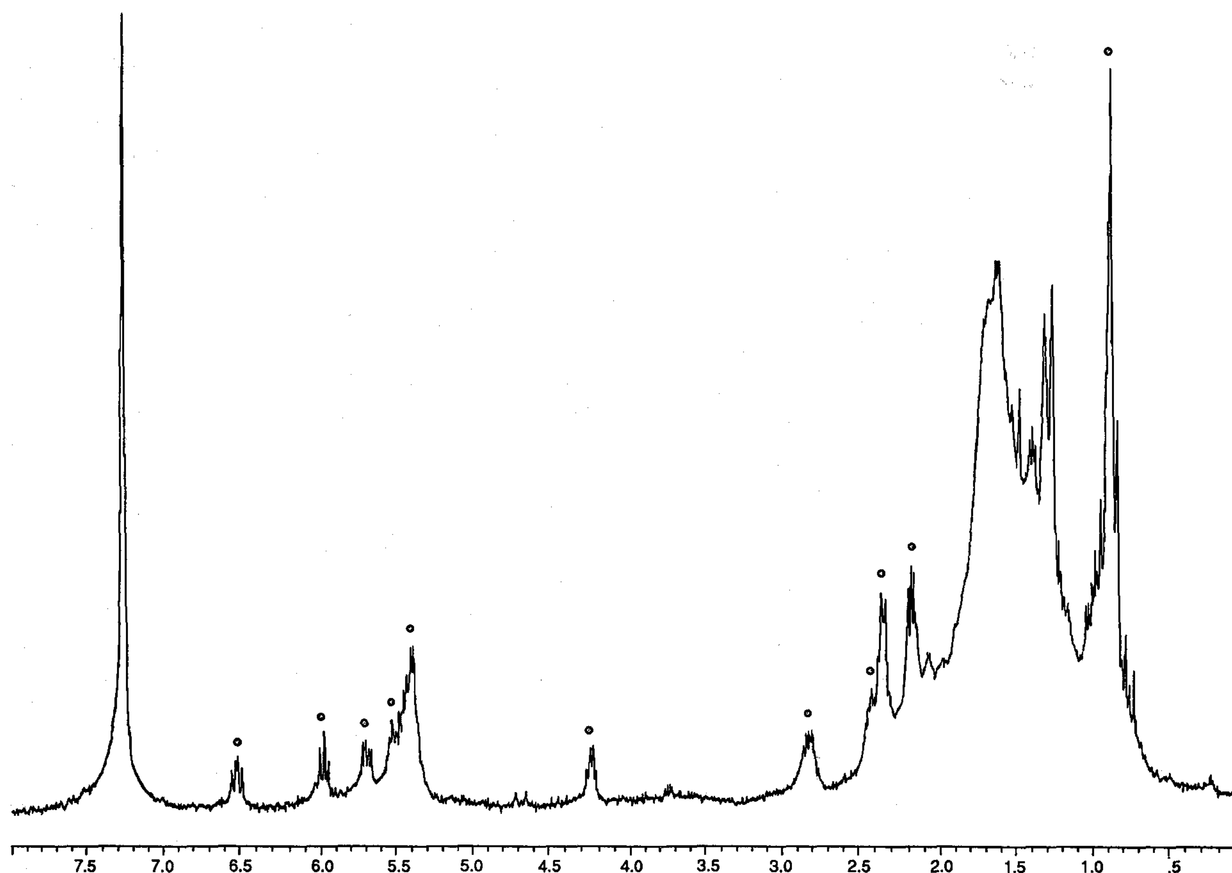


Figure 1. $^1$H NMR spectrum ($CDCl_3$, 500 MHz) of 11-HETE (about 60 μg) purified from lipid extracts of *P. dichotoma*. Signals assigned to the metabolite by comparison with spectra run with either natural or synthetic 11-HETE are indicated by dots. The signals at $\delta = 7.26$ and 1.50 are due to $CHCl_3$ and $H_2O$ impurities, respectively.

also be found in $^1$H NMR spectra of HETEs[8,11]. Reversed phase HPLC analysis of this fraction yielded as the major component (about 68 μg, 0.4 μg/g coral dry weight) a peak with a retention time (24 min) identical to that of authentic standards of 11-HETE, and a maximum of UV absorbance at 235 nm, typical of conjugated dienes. The $^1$H NMR ($CDCl_3$, 500 MHz) spectrum of this metabolite (fig. 1) could be superimposed on that obtained from either natural[8,11] or synthetic 11-HETE. In particular, it displayed signals at $\delta$: 2.80 (2H, C-7), typical of methylene protons between two isolated double bonds, 4.26 (1H, double triplet, C-11), assigned to the alcoholic proton, 5.51 (1H, C-15), 5.70 (1H, double doublet, C-12), 5.99 (1H, double doublet, C-14) and 6.52 (1H, double triplet, C-13), all typical of olefinic protons of a conjugated diene group, 5.35–5.45 (4H, multiplet, C-5, C-6, C-8, C-9), assigned to isolated olefinic protons, 2.15 (4H, multiplet, C-4, C-16), due to allylic methylene protons, 2.35 (2H, multiplet, C-2), assigned to the methylene protons in α to the carboxylic group, 2.40 (2H, multiplet, C-10), typical of methylene protons between a double bond and an alcohol group, and 0.89 (3H, triplet, C-20), due to the terminal methyl group.

The structure of the *P. dichotoma* metabolite was confirmed as that of 11-HETE by low-resolution EIMS analysis of the acetoxy-, methyl ester derivative, which yielded fragments at $m/z$: 316 (3.7%, loss of acetic acid from the molecular ion 376), 259 (66.7%, loss of $-CH_2-CH_2-CH_2-CH_3$ from the fragment at $m/z =$ 316), 195 and the twin fragment at 181 (17.7% and 43.8%, fragmentation between C-10 and C-11, with loss of $-(CH_2-CH{=}CH)_2-(CH_2)_3-COOCH_3$), 167 and the twin fragment at 149 (50% and 38.5%, fragmentation between C-9 and C-10 of the fragment ion at $m/z =$ 316, with loss of $-CH{=}CH-CH_2-CH{=}CH-(CH_2)_3-COOCH_3$), and 153 (100%, loss of an acetyl group from the fragment at $m/z = 195$). These EIMS data were necessary to establish the structure of the metabolite, since $^1$H NMR does not distinguish between 11- and 9-HETE[8,11].

The stereochemistry of the alcoholic carbon atom was established by chiral phase HPLC analysis of the methyl ester derivative carried out in comparison with synthetic standards of 11-*R*- and 11-*S*-HETE methyl esters. The natural product was eluted after 25 min exactly like the *R* enantiomer of 11-HETE (the *S* enantiomer was eluted after 17 min). The optical purity of the metabo-

lite strongly suggested that 11-HETE was not produced by spontaneous, nonenzymatic oxidation of arachidonic acid during the *P. dichotoma* storage and extraction procedure. This suggestion was supported by the outcome of the in vitro biosynthesis experiments described below.

One-hour incubation of *P. dichotoma* homogenates with both unlabelled and [$^3$H]arachidonic acid, followed by reversed phase HPLC analysis of the Sep-pak eluate, resulted in the formation of a UV visible and radioactive peak with retention time (15 min) identical to that of 11-HETE standard (fig. 2B). This peak was absent from the extracts of control incubations (where the homogenate had been inactivated either by heating or by lowering the pH to 2.5) (fig. 2A), and was also present in homogenates from *P. clavata* incubated with [$^3$H]arachidonic acid (fig. 2C). This finding suggests that an enzymatic activity is present in both gorgonian species, capable of converting arachidonic acid into 11-HETE, possibly via hydroperoxidation of the fatty acid, as would be expected from an enantioselective lipoxygenase enzyme[5]. Thus, as previously shown for soft corals belonging to the *Plexaura*, *Clavularia* and *Gersemia* genera[4–6], the genus *Plexaurella* also appears to contain a species capable of synthesizing a putative lipoxygenase product. Unlike the other gorgonian corals mentioned above, however, *P. dichotoma* does not produce detectable amounts of PG-like metabolites, thus behaving like the antipatharian genus *Leiopathes* (Hexacorallia), for which the occurrence of the $\omega$13-hydroxylation products of both arachidonic and eicosapentaenoic acids but not of the corresponding PGs of the 2 and 3 series has been reported[15]. The conversion of arachidonic acid into 11-HETE by homogenates of the Mediterranean soft coral *P. clavata*, which again does not contain PG-like compounds, confirms that enzymatic lipoxygenation of polyunsaturated fatty acids is a general metabolic pathway common to soft coral species living in completely different environments, as well as to hydrozoans[5,8–11], and can occur independently from PG biosynthesis.

In conclusion, in the present study the occurrence of 11-*R*-HETE has been reported for the first time for a *Plexaurella* species. Evidence has also been presented that this species, as well as another non-prostaglandin-containing soft coral, *P. clavata*, contains the enzymatic activity leading to 11-HETE formation. The separation of HETE (and, therefore, HPETE) formation from PG synthesis in these two gorgonian corals is in accordance with the current hypothesis[6,7] that, in these coelenterates, the latter pathway does not occur necessarily through the intermediacy of lipoxygenase-derived hydroxy and hydroperoxy derivatives of arachidonic acid.

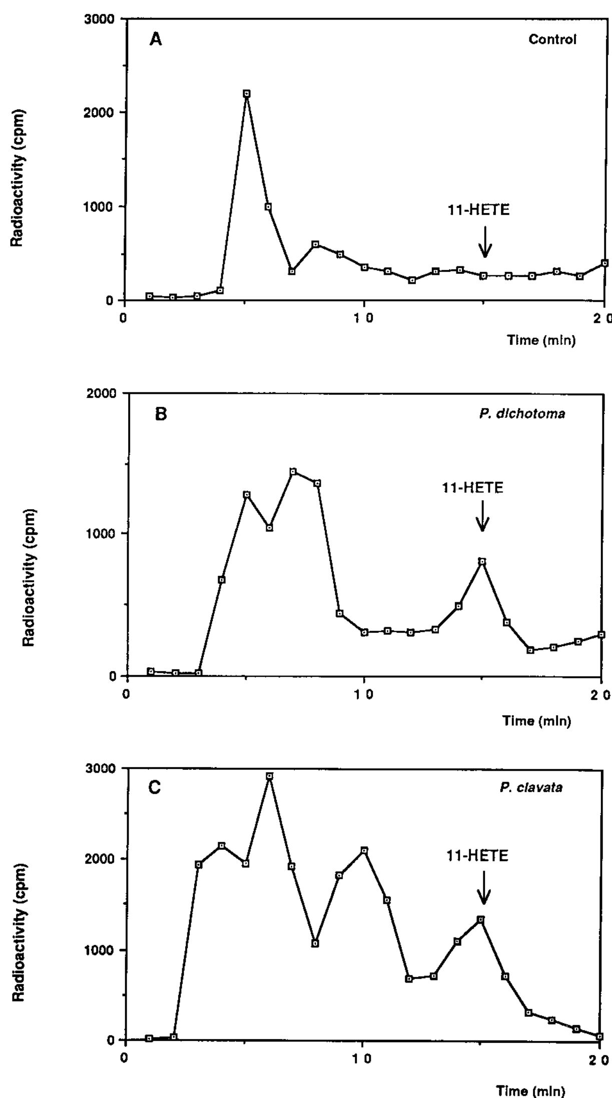


Figure 2. Radioactivity (cpm/1 ml fraction) elution profiles of reversed phase HPLC analyses of samples from incubations of homogenates from *P. dichotoma* (*A* and *B*) and *P. clavata* (*C*) with either [$^3$H]arachidonic acid plus unlabelled arachidonic acid (*A* and *B*) or [$^3$H]arachidonic acid only (*C*). Incubation and HPLC conditions are described in the experimental section. Arrows show the retention time of a synthetic standard of 11-HETE.

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