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12-LIPOXYGENASE ACTIVITY IN THE RED MARINE ALGA *GRACILARIOPSIS LEMANEIFORMIS*

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Key Word Index—*Gracilariopsis lemaneiformis*; Rhodophyta; algae; 12-lipoxygenase; icosanoids; 12-hydroxyicosatetraenoic acid; 12-HETE.

Abstract—The temperate red alga *Gracilariopsis lemaneiformis* is a rich source of polyunsaturated fatty acids that are regio-specifically oxidized at C-12. In this *in vitro* study, we demonstrate the existence of a highly active lipoxygenase-type activity in *Gracilariopsis*. GC-MS of the methyl ester, trimethylsilyl ether derivatives of the crude reaction mixture identified the production of 12-hydroxyicosatetraenoic acid, a 12-lipoxygenase product previously isolated from this alga. This is the first report of 12-lipoxygenase-type activity from the plant kingdom.

INTRODUCTION

Recently, we have demonstrated the widespread occurrence of a variety of pharmacologically active hydroxyicosanoids in red algae [1–7]. This includes 12-hydroxyicosatetraenoic acid (**1**, 12-HETE) [4], 12-hydroxyicosapentaenoic acid (**2**, 12-HEPE) [3], 6-*trans*-leukotriene B_4, ethyl ester (**3**, 6-E-LTB_4, ethyl ester) [Bernart, M. and Gerwick, W. H., unpublished results], hepoxilin B_3 (**4**) [6], and 12*R*,13*S*-dihydroxyicosatetraenoic acid [**5**, 12(*R*),13(*S*)-diHETE] [5, 7], all of which are oxidized at C-12. In mammals, icosanoids of this general type arise most commonly by metabolic action of 12-lipoxygenase on arachidonic acid (**6**, AA) or its congeners, although their formation also occurs to a lessor extent via a cytochrome P-450 pathway [8]. Metabolites of the 12-lipoxygenase pathway are not only involved in many normal metabolic functions in humans, but also are central to the etiology of numerous disease states, including psoriasis, asthma and disorders involving the immune system. Hence, description of these enzyme systems in other life forms may aid our understanding of the fine elements of the enzymology and biosynthesis of these mammalian immunohormones as well as giving an insight into evolutionary trends and molecular recognition phenomena in these classes of enzymes.

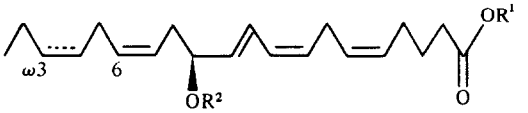
Further biosynthetic manipulation of the initial product of 12-lipoxygenase, 12-hydroperoxyicosatetraenoic acid (**7**, 12-HPETE), ultimately gives rise to several physiologically important mammalian autacoids, including the HETE's, hepoxilins and leukotrienes [9–11]. Interestingly, while algae [13], higher plants [12] and animals all contain lipoxygenase, only animals contain lipoxygenase with a regiospecificity for C-12 of the fatty acid chain.

Although red algae are now a recognized source of hydroxyicosanoids, the lipoxygenase enzyme system presumably responsible for their formation has not yet been reported. Our recent chemical work with a red alga from the Oregon coast, *Gracilariopsis lemaneiformis*, showed it to be a rich source of AA and EPA derivatives oxidized at C-12, including 12-HETE (**1**), 12-HEPE (**2**), and 12(*R*),13(*S*)-dihydroxyicosapentaenoic acid [8, 12(*R*), 13(*S*)-diHEPE] [7, Jiang, Z.D., and Gerwick, W. H., unpublished results]. It was thus a logical choice for our initial efforts in the evaluation of red algae for 12-lipoxygenase-type activity.

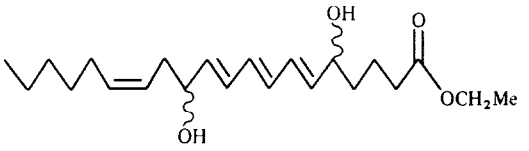
RESULTS AND DISCUSSION

An acetone powder extract (AP) of fresh-frozen *G. lemaneiformis*, collected from the Central Oregon Coast, was incubated with AA in an air-saturated buffer solution under three experimental conditions: (1) AA + AP, (2) AA + boiled AP and (3) AP alone. An additional control consisted of AA alone in air-saturated buffer. Following an 80 min incubation, all samples were similarly extracted for their lipids and these were then analysed by TLC. On the same chromatogram, aliquots were applied from all of the above experimental conditions as well as AA standard and the acetone extracted lipids used in preparing the enzyme extract.

Although the chloroform–methanol (2:1) extract of this collection of *G. lemaneiformis* showed a rich assortment of icosanoid-type natural products, the acetone extract did not show major amounts of these compounds (UV-active spots charring blue with cupric acetate). This may have been due to the 'swamping effect' of other compounds and pigments in this acetone extract which overlapped the icosanoid R_f region. Analysis of the TLC of the extract from the 'AP alone' treatment (without added AA) demonstrated the absence of detectable amounts of endogenous icosanoids or substrates in the 'AP' preparations. The materials extracted from the 'AA + buffer' treatment showed the presence of mainly AA (R_f 0.5) along with minute quantities of more polar materials which represented non-enzymatic oxidation products of AA. These faint spots also appeared in the TLC of the AA standard and represented minor impurities. The 'AA + AP' treatment showed only a small quantity of unmetabolized AA along with major quant-



1 ω6; $R^1 = R^2 = H$
2 ω3; $R^1 = R^2 = H$
7 ω6; $R^1 = H$, $R^2 = OH$
10 ω6; $R^1 = Me$, $R^2 = TMSi$



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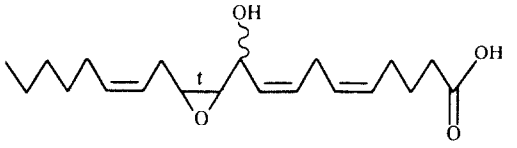

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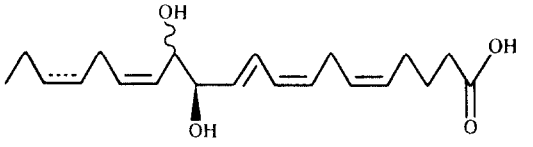
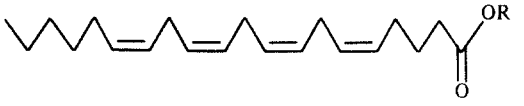


5 ω6
8 ω3



6 R = H
9 R = Me

ities of a number of UV-active, blue-charring compounds with R_f values 0–0.3. In contrast, the 'AA + boiled AP' preparation once again yielded major quantities of AA and none of the metabolites obtained with the active enzyme preparation, suggesting that these products are formed as a result of heat labile, enzyme mediated reactions.

The lipids obtained from the 'AA + AP' treatment were checked for the presence of hydroperoxides (see Experimental) because these are the expected intermediates between AA and its hydroxylated metabolites; however, none were found. This is probably due to a high conversion rate of hydroperoxides to other products by hydroperoxidase and perhaps other enzymes in the crude AP preparation. Alternatively, the mono-oxygenase enzyme, cytochrome P-450, could be responsible for the production of these hydroxy metabolites since this route does not normally involve hydroperoxide intermediates [8]. However, our recent isolation of hepoxilin-type natural products from red algae [6] suggests a hydroperoxide intermediate [14] which is consistent with the lipoxygenase pathway.

The crude mixture of the AA metabolites obtained from the 'AA + AP' treatment was subjected to analysis by GC-MS following appropriate derivatization (see Experimental). Two compounds from this mixture were identified, one as derivatized starting material (**9**, AA methyl ester) and the other as 12-HETE TMSi ether, methyl ester (**10**). A key fragment ion in the CIMS of derivative **10** was observed at *m/z* 295 (9%). This is diagnostic for the cleavage α to the carbon bearing the silylated alcohol (C-12/C-13) [9], and thus, identifies C-12 as the point of oxidation. The ion at *m/z* 193 reinforces this structural assignment as it arises from the alternate cleavage α to the carbon bearing the silylated alcohol (C-11/C-12). Although this latter cleavage has not been previously reported for this derivative of 12-HETE [9], it has precedence by analogy to this same derivative in other hydroxyicosanoids [10].

EXPERIMENTAL

Algal material. *Ca* 150 g (fr. wt) of *G. lemaneiformis* (Bory) Dawson, Acleto et Foldvik was obtained from an intertidal pool at Cape Perpetua, Oregon on 5 June 1989. Voucher specimens of this alga are deposited at the herbarium of the Department of Botany, University of California, Berkeley. Half of the collected material was maintained in cool sea H_2O for transport to the laboratory and used in the enzyme prepn assay on the same day while the other half was frozen on site using dry ice and then stored at −70° for extraction at a later date.

Enzyme extraction and preparation. *Ca* 75 g (fr. wt) of either fr. or frozen *Gracilariopsis* was repeatedly extracted with a total of 450 ml Me_2CO–dry ice. Solvent was removed from algal tissue by filtration using a 10–20 μm sintered glass funnel. The final residue was rinsed with an additional 30 ml of Me_2CO and dried under N_2 [15]. The dry Me_2CO powder (7 g) was divided into two *ca* equal portions. From fr. *G. lemaneiformis*, these portions were used for 'AA + AP' and 'AP alone' treatments while fr.-frozen *G. lemaneiformis* portions were used in 'AA + AP' and 'AA + boiled AP' treatments. Each of these were then dissolved in 125 ml Erlenmeyer flasks with 60 ml of 0.1 M K_2HPO_4 buffer, pH 7.4. Boiling of the crude enzyme prepn was accomplished by heating with occasional stirring (98–100°, 10 min). At the end the temp. inside the flask had reached 90° and the crude enzyme prepn had turned green, in contrast to fr. material which was pinkish-brown. All flasks were chilled in ice for *ca* 15 min before incubation.

Incubation. Arachidonic acid (AA) was dissolved in EtOH to a final concn of 0.1 mg μl^{-1}. Treatments 'AA + AP' and 'AA + boiled AP' each received 5 mg of AA in 50 μl EtOH while the control 'AA + buffer' treatment received 2 mg of AA in 20 μl EtOH. All flasks were incubated at 6° for 80 min with continuous slow bubbling of air.

Enzyme product extraction. Following the incubation period, enzyme prepns were filtered through cheese cloth. The pH was adjusted to 4 with 5% HCl, a few ml of satd brine added and lipids repetitively extracted with 4 × 10 ml of EtOAc. The solvent of the combined extractions was reduced *in vacuo* and the recovered lipids dissolved in Et_2O for storage.

Analysis of enzyme products. TLC of the Me_2CO ext. and various enzyme incubation treatments was carried out on silica gel 60 F_{254} using Et_2O–hexane–HOAc (59:40:1). The developed TLC was then sprayed with a soln of Cu $(OAc)_2$ to

produce a characteristic blue colour with most icosanoids upon heating.

Hydroperoxide detection was performed by spraying the TLC with I_2 followed by a starch soln [16]. In addition, the crude enzyme product mixt. resulting from 'AA + AP' treatment was reduced using $SnCl_2$ [12] and compared by TLC for any changes relative to the starting mixt.

LRMS of AA metabolites resulting from the 'AA + AP' treatment were obtained using a quadropole instrument after methylation (CH_2N_2–Et_2O, 0.5 hr) and silylation (trimethysilylimidazole) of the crude mixt. For **10**: LR GC-CIMS (CH_4) obs. $[M]^+$ *m/z* 406 (45%), 315 $[M-(TMSiOH+H)]^+$, (33%), 295 $[M-C_8H_{15}]^+$ (9%), 193 $[C_{12}H_{17}O_2]^+$ (100%), 91 $[TMSiOH+H]^+$ (6%), 69 (9%).

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