



LIPOXYGENASE-MEDIATED FORMATION OF HYDROCARBONS AND UNSATURATED ALDEHYDES IN FRESHWATER DIATOMS

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Abstract—Upon initiation of lipoxygenase reactions one group of freshwater diatoms synthesized unsaturated acyclic and alicyclic hydrocarbons (ectocarpene, dictyopterene and fucoserratene); another group synthesized polyunsaturated aldehydes. Among others (2*E*),(4*Z*),(7*Z*)-decatrinal is described as a new lipoxygenase cleavage product in *Melosira*. The patterns of the volatile organic compounds were typical for particular species of diatoms, indicating different specificities of the enzymes involved. The formation of unsaturated hydrocarbons and aldehydes was fully inhibited by strict exclusion of dioxygen, but could be regained by subsequent addition of dioxygen. The application of radical scavengers or chelating agents resulted in a high percentage inhibition of hydrocarbon and aldehyde formation.

INTRODUCTION

Odorous compounds are frequently observed in running waters and lakes and are the result of rich cyanobacterial and algal growth [1]. Among these compounds, acyclic (fucoserratene) and alicyclic hydrocarbons (ectocarpene and dictyopterene) have been found in both lake water [2] and river water [3]. Although the structural elements of these compounds pointed to a biogenic origin, the sources remained unknown. Candidates were various diatoms, which are the most abundant algae in epilithic biofilms of rivers and brooks and are an important but variable part of the phytoplankton in lakes.

Since the production of volatile organic compounds (VOC) in freshwater diatoms has, with the exception with *Asterionella formosa* [4], not been studied, we analysed some commonly growing diatoms and describe the lipoxygenase-mediated formation of unsaturated acyclic and alicyclic hydrocarbons and polyunsaturated aldehydes by these organisms. The pathways of hydrocarbon formation observed for these compounds differ essentially from those of straight-chain alkenes that are constituents of nearly any cyanobacterium and alga.

RESULTS

Activation of lipoxygenase reactions

The concentration of odorous compounds of diatom cultures can be easily and essentially increased when

a lipoxygenase reaction is initiated, e.g. by osmotic stress. Addition of solid NaCl to the algal suspension to give a final concentration of 20% (w/v) disintegrates the cells, and the lipoxygenases of the cells become fully active. Adding solid NaCl or brine to initiate the lipoxygenase reaction was superior to other techniques, such as drying the cells on filters, freezing and thawing of the cells, and mechanical disintegration, because the first procedure can easily be conducted in a closed reaction vessel under strictly anoxic conditions. This was necessary to study the effects of dioxygen on the reactions. During normal growth, the concentrations of the lipoxygenase reaction products in the medium were rather low. Spent medium that was separated by centrifugation from a culture of *Gomphonema* contained not more than 3% of the VOC concentration found after salt treatment.

Identification of volatile lipoxygenase reaction products

The VOC liberated by diatoms after initiation of the lipoxygenase reactions by NaCl treatment differed essentially from one species to another. *Gomphonema*, *Amphora* and *Phaeodactylum* synthesized several alicyclic alkenes, which were identified as ectocarpene [(*S*)-6-(1(*Z*)-butenyl)-cyclohepta-1,4-diene], dictyoptere A {(1*R*,2*R*)-1-[(1*E*)-1-hexenyl]-2-vinylcyclopropane} and dictyoptere C' [(*R*)-6-butylcyclohepta-1,4-diene]. As previously described [4], *Asterionella* produced only the acyclic alkenes 1,3-octadiene and 1,(3*E*),(5*Z*)-octatriene (fucoserratene). The other diatoms studied followed the general rule that unsaturated aldehydes were liberated. The VOC that have been positively identified and

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Table 1. VOC of diatoms after activation of the lipoxygenase reaction with NaCl

Species	Strain*	Compound
<i>Gomphonema parvulum</i>	SAG 1032-1	Dictyopterene A† Ectocarpene† Dictyopterene C'†
<i>Amphora veneta</i>	UTEX 2305	Ectocarpene† Dictyopterene C'
<i>Phaeodactylum tricornutum</i>	UTEX 640	Ectocarpene† Dictyopterene C'
<i>Asterionella formosa</i>	ISF	1,3-Octadiene Fucoserratene†
<i>Fragilaria</i> sp.	MPI	(2E),(4Z)-Heptadienal† (2E),(4Z)-Octadienal† (2E),(4Z)-Decadienal
<i>Melosira varians</i>	MPI	(2E),(4Z)-Heptadienal (2E),(4Z)-Octadienal† (2E),(4Z),(7Z)-Decatrienal† (2E),(4E),(7Z)-Decatrienal

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†Major compound.

verified by reference compounds are listed in Table 1. The *cis/trans* stereochemistry of 1,3-octadiene could not be established. The (2E),(4Z)-dienals of *Melosira varians* and *Fragilaria* spp. were accompanied by minor amounts of the (2E),(4E)-isomers.

A compound found in *Melosira* was identified as (2E),(4Z),(7Z)-decatrienal on account of the following features and reactions. The EI mass spectrum exhibited a weak molecular ion at m/z 150, and the CI mass spectrum (methane as the reactant gas) exhibited adducts at m/z 151 $[MH]^+$, m/z 179 $[M + C_2H_5]^+$ and m/z 191 $[M + C_3H_5]^+$. To characterize this compound further, a stream of air was passed through an initiated lipoxygenase reaction mixture of *Melosira* and the stripped VOC were sorbed on a C18-cartridge. The VOC containing the unknown compound were eluted with methanol and separated by HPLC. The absorption maximum of the unknown compound was identical to that of 2(E),4(Z)-dienals, indicating the conjugation of only two double bonds with the aldehyde group. The unknown compound treated with Pt/H_2 gave decanal and decan-1-ol and, upon reduction with $NaBH_4$, decatrienol. The EI mass spectrum of the compound closely fitted, with the exception of one fragment ion, to the mass fragmentation pattern of (2E),(4E),(7Z)-decatrienal published by Seifert and Buttery [5] and was fully identical to a mass spectrum obtained from an authentic compound (mass spectral library, Firmenich SA, Geneva). A reference compound of (2E),(4E),(7Z)-decatrienal synthesized according to a procedure given by Seifert and Buttery [5] was identical with a minor peak of the VOC of *Melosira*. The major peak with a similar mass fragmentation pattern eluted about 0.7 min before (2E),(4E),(7Z)-

decatrienal. The data obtained suggest the structure of (2E),(4Z),(7Z)-decatrienal for the major compound. This configuration is consistent with the FTIR gas phase spectra obtained. The synthetic (2E),(4E),(7Z)-decatrienal exhibited an absorption maximum at 989 cm^{-1} indicative of a conjugated E, E configuration, while (2E),(4Z),(7Z)-decatrienal showed an absorption maximum at 968 cm^{-1} as did (2E),(4Z)-octadienal that was another major VOC of *Melosira*.

Oxygen-dependent formation of aldehydes and hydrocarbons

It is well known that the formation of unsaturated and saturated aldehydes requires dioxygen for their formation via a lipoxygenase reaction. This could clearly be shown to be the case in experiments in which a saturated NaCl solution was added under strict exclusion of dioxygen to suspensions of different diatoms. These experiments were conducted in a specially constructed one-piece glass apparatus in which the algal suspension and the brine were in two round-bottomed flasks connected to each other via a bridge. After intense gassing of the brine and the darkened diatom suspension with helium, both were combined and the lipoxygenase reaction initiated. Under these strictly anoxic conditions not even traces of saturated and unsaturated aldehydes were synthesized. However, when the helium gas atmosphere was replaced by air an intense formation of these compounds took place. It was unexpected, however, that the alkenes (fucoserratene, ectocarpene and dictyopterene) exhibited the same behaviour, although these molecules do not contain any oxygen (Table 2). The formation of these

Table 2. Dioxygen-dependent formation of aldehydes and hydrocarbons in diatoms. Lipoxygenase activity was initiated by addition of brine under an atmosphere of air or helium

Diatom	Compound	Area (IU)*	
		Air	Helium
<i>Asterionella formosa</i>	1,3-Octadiene	2600	0
	Fucoserratene	8990	0
<i>Gomphonema parvulum</i>	Ectocarpene	1850	0
	Dictyoptere C'	500	0

*IU = integration units.

hydrocarbons in a lipoxygenase-catalysed reaction was probable and was supported by further experiments.

When air was added to a suspension of *A. formosa* kept under a helium gas atmosphere after activation of the lipoxygenase activity by NaCl treatment, the formation of 1,3-octadiene and fucoserratene was initiated, but the amounts liberated declined with the extension of the period between activation and addition of oxygen and were entirely lost after 40 min (Table 3).

Inhibition of hydrocarbon and aldehyde formation

When the enzymic reactions in diatoms that lead to the formation of hydrocarbons and unsaturated aldehydes were initiated after addition of inhibitors, which are usually applied to inhibit the lipoxygenase-catalysed reaction sequence, strong effects were observed (Table 4). The radical scavengers BHA (3- *tert*-butyl-4-hydroxyanisole) and propyl gallate were very effective in reducing the

Table 3. Formation of hydrocarbons in *Asterionella formosa*. The incubation period under a helium gas atmosphere between activation of the lipoxygenase reaction and addition of dioxygen was varied

Incubation period (min)	Area (IU)*	
	1,3-Octadiene	Fucoserratene
0.0	2600	8990
2.5	1700	5870
5.0	250	3320
10.0	—	1920
20.0	—	1630
30.0	—	1760
40.0	—	—

*IU = integration units.

formation of fucoserratene in *Asterionella* and that of various unsaturated aldehydes in *Melosira*. BHA was, however, less effective in reducing the formation of cyclic alkenes (dictyopterenes and ectocarpene) in *Gomphonema*, while propyl gallate inhibited the reactions so strongly that VOC could no longer be detected by GC-mass spectrometry. The chelator 4-nitrocatechol reduced the formation of fucoserratene and two cyclic alkenes completely, and that of ectocarpene, the dienals and the trienal by a high percentage.

DISCUSSION

The data obtained indicate that freshwater diatoms liberate numerous VOC when exposed to stress conditions.

Table 4. Inhibitory effects of BHA (3- *tert*-butyl-4-hydroxyanisole), propyl gallate and 4-nitrocatechol (each 200 nM) on the formation of hydrocarbons and aldehydes in *Asterionella formosa*, *Gomphonema parvulum* and *Melosira varians*. An untreated part of the same algal suspension served as the reference

Inhibitor	Organism	Compound	Inhibition (%)
BHA	<i>Asterionella</i>	Fucoserratene	100
Propyl gallate		Fucoserratene	92
4-Nitrocatechol		Fucoserratene	100
BHA	<i>Gomphonema</i>	Dictyoptere A	13
		Ectocarpene	40
		Dictyoptere C'	20
Propyl gallate		Dictyoptere A	100
		Ectocarpene	100
	<i>Melosira</i>	Dictyoptere C'	100
4-Nitrocatechol		Dictyoptere A	100
		Ectocarpene	98
		Dictyoptere C'	100
BHA		(2E),(4Z)-Octadienal	100
		(2E),(4Z),(7Z)-Decatrienal	100
Propyl gallate		(2E),(4Z)-Octadienal	85
		(2E),(4Z),(7Z)-Decatrienal	85
4-Nitrocatechol		(2E),(4Z)-Octadienal	80
		(2E),(4Z),(7Z)-Decatrienal	74

Rather unexpected was the occurrence of ectocarpene, dictyopterene A and dictyopterene C', which contribute to the odour bouquet of several marine Phaeophyceae. Dictyopterene A and C' were originally described as constituents of the essential oils of *Dictyopteris* species [6,7] and ectocarpene as a pheromone that is released from attached gynogametes to attract male gametes [8,9] of *Ectocarpus siliculosus*. Later, Derenbach and Pesando [10] observed the occurrence of ectocarpene in cultures of the marine *Skeletonema costatum* and reported indications that *Lithodesmium undulatum* was also able to generate this compound. The phylogenetic relationship between diatoms and Phaeophyceae, expressed in many biochemical features such as chlorophylls, carotenoids, fatty acids and hydrocarbons, can also be observed with these VOC. Another example of similarities is fucoserratene, which has previously been detected in *A. formosa* [4], was confirmed in this study and has also been found in the Phaeophyceae. Intense studies with *Fucus serratus* and *F. vesiculosus* have shown that this compound is a pheromone liberated by mature eggs to attract spermatozooids [11].

Remarkable is the observation that those strains (the genera *Gomphonema*, *Amphora*, *Phaeodactylum* and *Asterionella*) that produce volatile hydrocarbons do not generate aldehydes like *Fragilaria* and *Melosira*, and vice versa. Both of the latter genera synthesized several dienals, which are well known because of their intense offensive odour. In *Melosira* 2(E), 4(Z), 7(Z)-decatrienal was a major unsaturated aldehyde that has not been observed before in microorganisms, but has been described as a degradation product during storage of unsaturated fatty acids [5].

Structural similarities of the observed VOC with unsaturated fatty acids that exhibit a particularly wide spectrum in diatoms make the latter important precursors. The strict requirement of dioxygen and the efficiency of lipoxygenase inhibitors to prevent the formation of the observed aldehydes and hydrocarbons indicate the action of lipoxygenases and explain the initial reactions of the formation of C₈ and C₁₁ hydrocarbons from unsaturated fatty acids. This assumption is consistent with labelling experiments conducted by Stratmann *et al.* [12] with gynogametes of *E. siliculosus*. If a hydroperoxy formation in the ω 12 position of a multi-unsaturated fatty acid is assumed, followed by a homolytic cleavage and cyclization reaction, deuterium atoms introduced at the double bonds as shown by the experiments of Stratmann *et al.* [13] would be retained. Since eicosapentadecenoic acid is a major component of the diatoms studied (ca 23% of the total fatty acids of *Gomphonema parvulum*), the most likely formation of ectocarpene and dictyopterene C' is via a 9-hydroperoxyeicosapentaenoic and 9-hydroperoxyeicosatetraenoic acid (arachidonic acid), respectively, followed by a cleavage reaction of a homolytic hydroperoxylyase [14]. Homolytic cleavages have been found to be normal for mushrooms [15], chlorophytes [16] and cyanobacteria [17,18], and simple low-molecular-mass hydrocarbons such as pentane and pentenes have been detected. The cleavage of

the hydroperoxy fatty acid in *Gomphonema*, *Amphora* and *Phaeodactylum* is accompanied with a cyclization reaction that forms three- and seven-membered rings of unique structure. Such a ring formation is uncommon for hydrocarbons, and the enzymes responsible for this reaction are as yet unknown. These enzymes exhibit high specificity for the formation of three- and seven-membered cyclic compounds because both are not always observed in the same organism.

In green tissues of higher plants, lipoxygenases primarily lead to the formation of 9- and 13-hydroperoxy fatty acids [19,20]. After heterolytic cleavage of hydroperoxy fatty acids such as linoleic and linolenic acid, C₆ aldehydes and an oxo acid can be observed. This homogeneity of C₆ aldehydes is not found with diatoms that show a broad pattern of different lipoxygenase and hydroperoxy fatty acid cleavage enzyme specificities. Since diatoms produce the VOC in high amounts and in limited number, these organisms are superior to higher plants for studying these reactions.

EXPERIMENTAL

Origin and cultivation of diatoms. Cultures of diatoms were supplied by Sammlung von Algenkulturen, Göttingen, Germany, the Culture Collection of Algae at the University of Texas at Austin, U.S.A. and Culture Collection of Algae and Protozoa, U.K. The diatoms were cultivated in batch cultures using 300-ml or 1-l conical flasks at 16° in a Chu 11 based medium as modified in ref. [21].

Analysis of VOC. This followed essentially the procedure described in ref. [22]. To 50 ml algal suspension in a round-bottomed flask heated to 24°, 15 g NaCl was added and the VOC stripped in a closed-loop stripping device for 1 hr. The VOC were adsorbed on Tenax TA and subsequently transferred to a capillary column (DB-1301, J&W Scientific) of a GC-MS by thermodesorption. He was used as transfer and carrier gas. In the temp. programme applied, the oven was held at 0° for 4 min, then heated at 5° min⁻¹ to 280°. Mass spectra were recorded in the EI and CI mode using CH₄ as reactant gas. Gas phase FTIR spectra were obtained on a Hewlett Packard GC-FTIR (model 5965A). Reference compounds used for the definite determination of the observed compounds were obtained as previously described [1,23].

(2E),(4Z),(7Z)-Decatrienal. FTIR $\nu_{\max}^{\text{gas phase}}$ cm⁻¹: 3024, 2975, 1707, 1632, 1091, 968; EIMS m/z (rel. int.): 150 [M]⁺ (2), 121 (32), 93 (26), 92 (48), 91 (50), 81 (83), 79 (100), 77 (70), 68 (36), 67 (62), 55 (49), 53 (40), 41 (70), 39 (89).

(2E),(4E),(7Z)-Decatrienal (synthetic). FTIR $\nu_{\max}^{\text{gas phase}}$ cm⁻¹: 3024, 2975, 1707, 1642, 1150, 1103, 989; EIMS m/z (rel. int.): 150 [M]⁺ (3), 121 (28), 93 (24), 92 (21), 91 (42), 81 (100), 79 (83), 77 (61), 67 (61), 55 (42), 41 (70), 39 (85).

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