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Algal Pheromone Biosynthesis: Stereochemical Analysis and Mechanistic Implications in Gametes of *Ectocarpus siliculosus*

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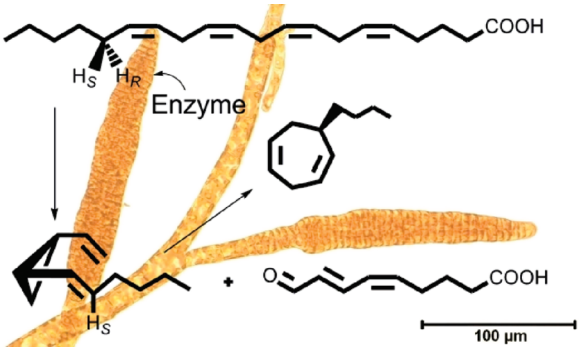

During sexual reproduction, female gametes or eggs of brown algae release pheromones to attract their male mating partners. The biologically active compounds comprise linear or alicyclic unsaturated hydrocarbons derived from the aliphatic terminus of C_{20} polyunsaturated fatty acids (PUFAs) by oxidative cleavage. The current study addresses the stereochemical course of the pheromone biosynthesis using female gametes of the marine brown alga *E. siliculosus* and chiral deuterium-labeled arachidonic acids. The biosynthetic sequence is likely to proceed via an intermediary 9-hydroperoxyarachidonic acid, which is cleaved with loss of the C(16)-H_R into the C_{11}-hydrocarbon dictyopterene C and 9-oxonona-(5*Z*,7*E*)-dienoic acid.

Introduction

Brown algae live on marine coasts worldwide.[1] They are distantly related to other eukaryotic groups such as plants and fungi, and they provide a valuable source of novel polysaccharides and lipids.[2] Their lipid metabolites comprise not only complex and bioactive oxylipins[3,4] but also C_{11} hydrocarbons deriving from the transformation of C_{20} polyunsaturated fatty acids (PUFAs).[5] During sexual reproduction, mediated by eggs or sessile female gametes and flagellated male gametes, these compounds direct the motile male gametes to the signaling female. The relevant compounds are linear or alicyclic unsaturated C_8-, C_{11}-, or oxygenated C_{11}-hydrocarbons[6] (selected examples are shown in Figure 1).

The structurally different C_{11} hydrocarbons **1**−**8** derive from the aliphatic terminus of polyunsaturated fatty acids (PUFAs) such as arachidonic acid (**9**) and eicosapentaenoic acid, both of which are abundant in the female gametes.[7] The mechanistic details of this biosynthetic pathway have been elucidated with certain diatoms, as these unicellular organisms can be cultivated easily and produce the same compounds. Cell homogenates of the diatom *Gomphonema parvulum* synthesize hydrocarbons dictyopterene A (**7**) and hormosirene (**2**) from arachidonic and eicosapentaenoic acid.[8]

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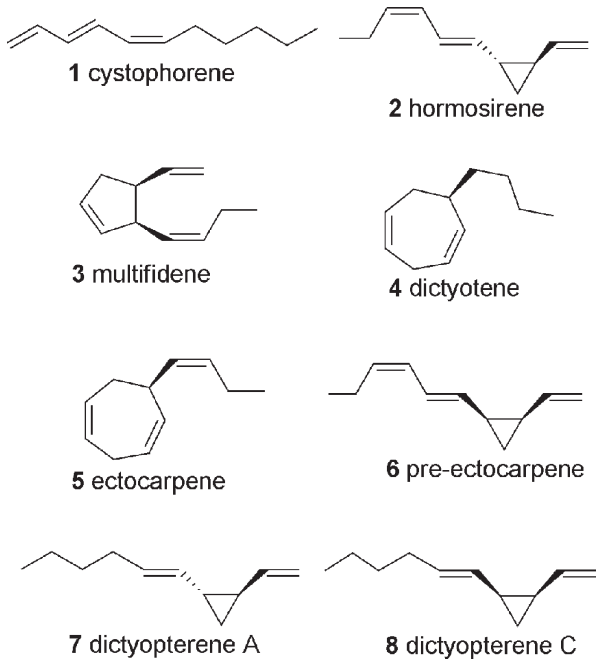
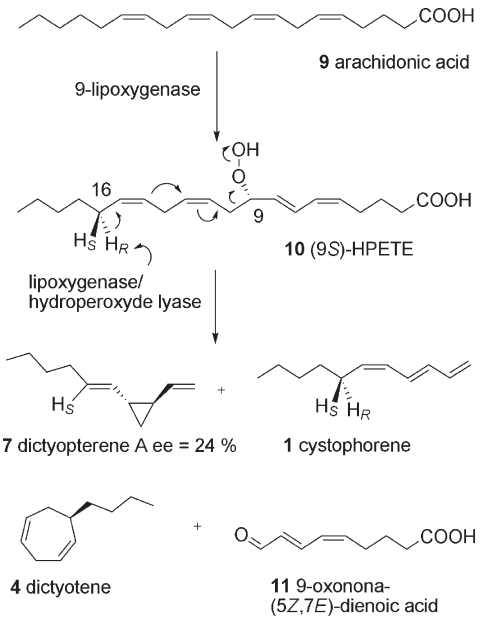


FIGURE 1. Structures of selected brown algal pheromones.

As illustrated in Scheme 1, first a lipoxygenase activates the fatty acid to the (9*S*)-hydroperoxide **10** which in turn suffers an oxidative cleavage to the respective hydrocarbon and the polar fragment 9-oxonona-5(*Z*),7(*E*-)dienoic acid (**11**). In the diatom, the abstraction of the *pro-R* hydrogen from C(16) of **10** is the rate-determining step of the cleavage reaction, which generates an enantiomeric mixture of *trans*-divinylcyclopropane (dictyopterene A) (**7**) along with a small amount of the linear hydrocarbon cystophorene (**1**). In the latter case, the oxidative cleavage involves the loss of a hydrogen atom from C(13).[9] Mechanistic studies have also been conducted with the asteraceae *Senecio isatidaeus*, which produces C_{11} hydrocarbons by decarboxylation of a C_{12} fatty acid ultimately derived from linolenic acid.[10] Previous studies with female gametes of *E. siliculosus* established the origin of the algal pheromones form arachidonic and eicosapentaenoic acid, but mechanistic details have not been addressed.[11] The chemotactic system of the brown alga *E. siliculosus* is particularly interesting since the genuine pheromone pre-ectocarpene (**6**) is inactivated after a short time by a Cope rearrangement to ectocarpene (**5**).[12] While the thermolabile pre-ectocarpene (**6**) ($t_{1/2}$ = 21 min at 18 °C)[12] attracts male gametes down to 5 pmol L^{-1}, the rearranged ectocarpene (**5**) requires 2000-fold higher concentrations for attraction.[13] To investigate the stereochemical and mechanistic aspects of algal pheromone biosynthesis, we administered chirally labeled deuterated arachidonic acids as metabolic probes to suspensions of female gametes of *E. siliculosus* and analyzed their transformation products by GLC−MS. The results

SCHEME 1. Biosynthesis of C_{11} Hydrocarbons in the Diatom *G. parvulum*[a]



[a]HPETE: hydroperoxyeicosatetraenoic acid.

allowed to reconstruct the biosynthetic sequence from the fatty acid substrate to the genuine pheromone.

Results and Discussion

In diatoms, the C_{11} hydrocarbons of the type dictyopterene A (**7**), pre-ectocarpene (**6**), as well as dictyotene (**4**), and ectocarpene (**5**) are produced from PUFAs by consecutive action of a lipoxygenase and a lyase. As illustrated in Scheme 1, the lipoxygenase generates a (9*S*)-hydroperoxide that is cleaved by a lyase activity into a hydrocarbon and 9-oxonona-5*Z*,7*E*-dienoic acid (**11**). In diatoms, the mechanistic details were obtained by administration of labeled arachidonic acid to growing cultures of *G. parvulum*. In the present study, we followed the previous strategy and first developed a novel route to chirally labeled (16*R*)- and (16*S*)-[16,19,20-$^2H_3$]-arachidonic acids. Two additional deuterium atoms at C(19) and at C(20) of the probe were introduced to warrant a safe detection of the metabolites even after removal of the deuterium atom at C(16).

As illustrated in Scheme 2, the scaffold of the chiral, deuterated arachidonic acid (16*R*)-[$^2H_3$]-**9** was envisaged to be constructed by double-Wittig olefination with the bis-ylide of (Z)-hex-3-enyl-1,6-bis(triphenylphosphonium bromide) **12** and two easily accessible aldehydes.[14,15] This strategy allows the simultaneous generation of a segment with three methylene-interrupted *Z*-double bonds which is typical for highly unsaturated fatty acids. The deuterium-labeled hexanal **13** was synthesized as outlined in Scheme 3. Jacobsen's hydrolytic kinetic resolution of benzylglycidyl ether **14**[16] provided the chiral building block (2*S*)-**14** at a gram scale. Regioselective

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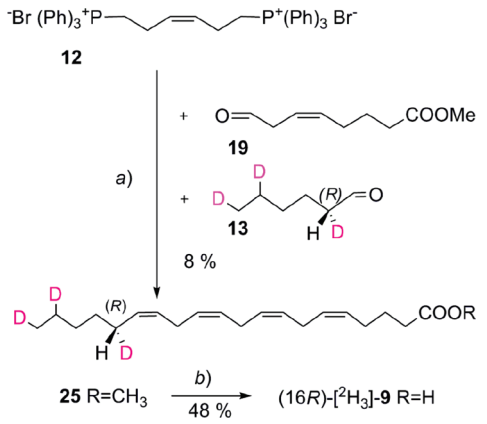
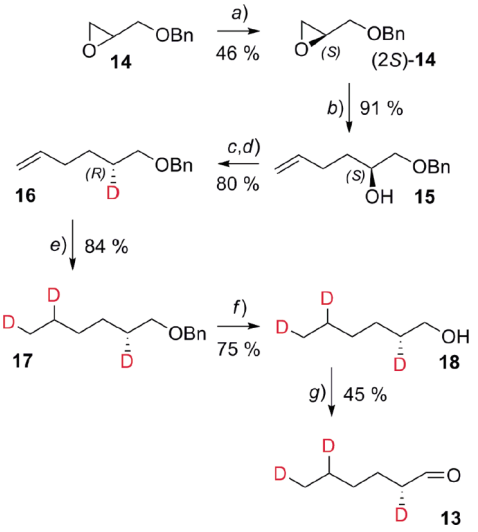
SCHEME 2. Synthesis of (16*R*)-[16,19,20-$^2H_3$]-Arachidonic Acid (16*R*)-[$^2H_3$]-9[a]



[a]Reagents and conditions: (a) (1) KN[Si(CH$_3$)$_3$]$_2$, THF, −78 °C, (2) oxoester **19**, (3) aldehyde **13**; (b) LiOH, THF, H_2O.

SCHEME 3. Synthesis of Chiral, Deuterium-Labeled Hexanal 13[a]



[a]Reagents and conditions: (a) Jacobsen's (*R*,*R*) catalyst, H_2O; (b) CH_2=$CHCH_2MgBr$, CuI, THF, −40 °C; (c) Et_3N, MsCl, CH_2Cl_2, −78 °C; (d) LiAl$^2H_4$, THF, −78 °C; (e) Wilkinson's catalyst,$^2H_2$, benzene; (f) Pd−C, H_2, CH_2Cl_2; (g) IBX, CH_2Cl_2, reflux.

opening of the epoxide at C(3) with allylmagnesium bromide in the presence of CuI at low temperatures[17] provided (2*S*)-1-(benzyloxy)hex-5-en-2-ol **15**. The enantiomeric purity of **15** (er = 96.3 ± 0.1) was determined by GLC−MS of diastereoisomeric carbamates formed by **15** with 1-phenylethyl isocyanate.[18] Stereospecific introduction of the deuterium label was achieved by a two-step mesylation/reduction procedure using LiAl$^2H_4$ without isolating the reactive mesylate.[19,20] The displacement of the mesylate of a secondary alcohol by a deuteride from LiAl$^2H_4$ proceeds with complete inversion of configuration at the stereocenter.[20] To confirm inversion at the stereocenter also in the mesylate of the benzyloxy alcohol **15**, the absolute configuration of deuterated hexanol **18** was determined by ^{1}H NMR after oxidation[21] to deuterated hexanoic acid and esterification with methyl (*S*)-mandelate.[22] If a chiral α-deuterated carboxylic acid is esterified with (*S*)-mandelate, the acid *pro-S* proton resonates at high fields of the *pro-R* proton.[22] ^{1}H NMR of the methyl (*S*)-mandelate diester of [2,5,6-$^2H_3$]-hexanoic acid displays a major signal at 2.20−2.10 ppm and a small signal at 2.30−2.30 ppm, confirming the (*R*)-configuration of **18**. The optical purity of **18** cannot be determined with this method because the procedure for the oxidation to hexanoic acid promotes enolization of the labeled aldehyde intermediate.

The additional two deuterium atoms were introduced in the chiral, deuterated benzyl ether **16** by deuteration with Wilkinson's catalyst[23] to avoid deuterium scrambling.[24] The benzyl group of 1-((2*R*)-[2,5,6-$^2H_3$]-hexyloxy)methylbenzene (**17**) was removed by hydrogenolysis with Pd(C) yielding (2*R*)-[2,5,6-$^2H_3$]-hexanol (**18**) without concomitant scrambling. Oxidation with IBX[25] in CH_2Cl_2 afforded the labeled hexanal **13** and allowed to isolate the volatile aldehyde in moderate yield. The second carbonyl component, namely methyl 8-oxooct-(5*Z*)-enoate (**19**), was obtained from commercially available δ-valerolactone **20** by a one-pot methanolysis and oxidation (Scheme 4, see the Supporting Information). Methyl 5-oxopentanoate **21** was olefinated with the C_3-homologating agent 2-(1,3-dioxan-2-yl)ethyltriphenylphosphonium bromide (**22**),[26] resulting in methyl 7-(1,3-dioxan-2-yl)hept-(5*Z*)-enoate (**23**). Methanolysis gave the corresponding dimethyl acetal **24**, and hydrolysis in pentane/formic acid resulted in the target β,γ-unsaturated 8-oxoester **19** with the correct stereochemistry (*Z*/*E* ratio >95:5, GLC−MS). The sequential addition[14] of carbonyl **19** and **13** to a solution of the bis-ylide of **12** gave the labeled fatty acid methyl ester **25** after HPLC purification. The low yield of the double olefination could be possibly due to the instability of the homoconjugated ester ylide intermediate formed by the bis-ylide of **12** and β,γ-unsaturated oxoester **19**, as preliminary experiments with non labeled, saturated carbonyls gave higher yields. Methyl ester **25** was hydrolyzed in small batches shortly before the incubation experiments in order to avoid autoxidation, and resulted in (16*R*)-[16,19,20-$^2H_3$]-arachidonic acid (16*R*)-[$^2H_3$]-**9**.

The metabolic probe (16*R*)-[$^2H_3$]-**9** was obtained with good isotopic and stereoisomeric purity. The isotopic purity (d_2:d_3:d_4 = 1:98:1) of the fatty acid methyl ester **25** was determined by GLC−MS comparing the molecular ion traces of the d_1−d_5 isotopomers corrected for the contribution of the natural abundance of ^{13}C.

Transformation of (16*R*)- and (16*S*)-[16,19,20-$^2H_3$]-Arachidonic Acids in Gametes of *Ectocarpus siliculosus*. The typical volatiles released from suspensions of female gametes of *E. siliculosus* were ectocarpene (>95%), dictyotene

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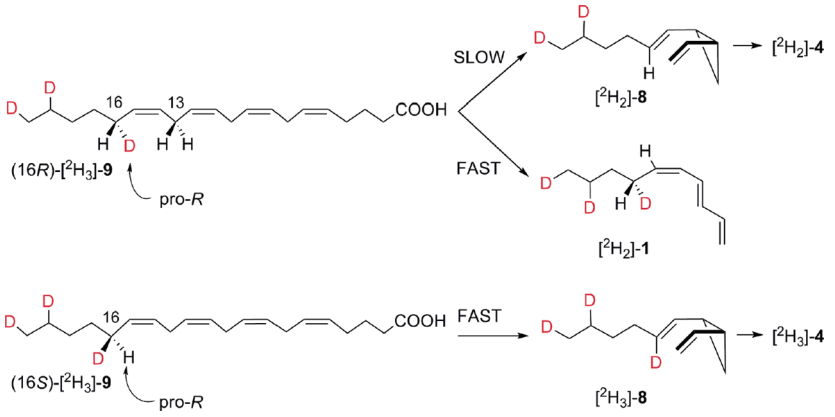
SCHEME 4. Reaction Pathways and Kinetic Isotope Effect (KIE) in *E. siliculosus*



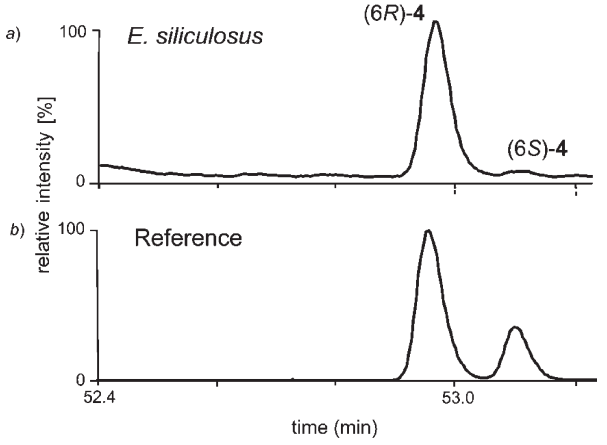
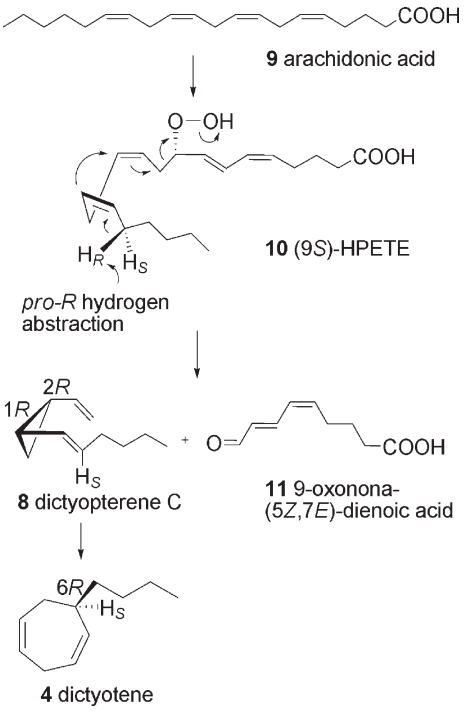


FIGURE 2. GLC separation of (6*R*)- and (6*S*)-dictyotene. GLC-separation of dictyotene enantiomers was achieved on Hydrodex-*β*-6-TBDMS: (a) dictyotene from gametes of *E. siliculosus*; (b) dictyotene reference from *G. parvulum*.

SCHEME 5. Biosynthesis of Dictyotene (4) in Gametes of *E. siliculosus*



(ca. 3−4%), and trace amounts of the linear hydrocarbon cystophorene (< 1%). Dictyotene **4** is a direct metabolite of arachidonic acid and proved to be of high er (97.9 ± 0.3), which demonstrates that its biosynthesis from arachidonic acid proceeds with high stereoselectivity (Figure 2). The main product ectocarpene (**5**), a metabolite of the higher unsaturated eicosapentaenoic acid, was not resolved on this column.

The overall stereochemical course (Scheme 5) of the transformation of arachidonic acid into dictyotene (**4**) and the linear hydrocarbon cystophorene **1** was investigated by incubating algal gametes with (16*S*)- and (16*R*)-[$^2H_3$]-**9**. The resulting C_{11} hydrocarbons were collected by SPME and analyzed by GLC−MS.

Figure 3 (lane a) illustrates the result for endogenous, nonlabeled arachidonic acid **9**, which yielded the two $C_{11}H_{16}$ hydrocarbons dictyotene (**4**) (*m*/*z* 150) and a small amount of the linear hydrocarbon cystophorene (**1**) (*m*/*z* 150; Figure 3, lane a). Administration of (16*R*)-[$^2H_3$]-**9** resulted in the release of [$^2H_2$]-**4** as the major metabolite, demonstrating that a single deuterium atom had been removed from the terminal segment of the precursor acid (Figure 3, m/*z* 152, lane b). Moreover, the genuine product ratio of dictyotene (**4**) to cystophorene (**1**) (Figure 3, lane a) changed dramatically in favor of cystophorene (**1**) (Figure 3, lane c). On the contrary, administration of (16*S*)-[$^2H_3$]-**9** resulted in the formation of [$^2H_3$]-**4** and did not affect the original product distribution of dictyotene and cystophorene (not shown). Accordingly, the product shift was due to the presence of a *pro-R* deuterium atom at C(16) of the precursor acid.

These observations are perfectly in line with the stereochemical and mechanistic course of the biosynthesis of dictyotene (**4**) in the diatom *G. parvulum*[9] and of ectocarpene (**5**) in the higher plant *S. isatidaeus*.[10] In all these organisms, the C_{11} hydrocarbons were generated from the aliphatic part of unsaturated fatty acids via thermolabile *cis*-divinylcyclopropane intermediates (Scheme 5). The latter rearrange in a highly concerted manner to the thermally stable dictyotenes (**4**).[8,10,13] Moreover, in diatoms the isotopic substitution of the abstracted hydrogen atom by a deuterium atom led to a significant shift in the product spectrum.[9] This is consistent

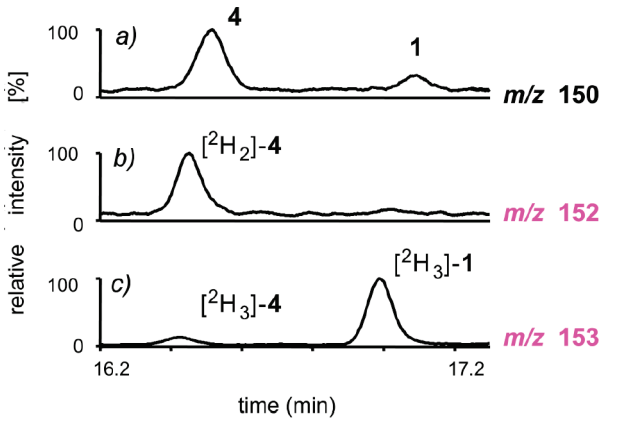


FIGURE 3. GLC−MS analysis of labeled $C_{11}H_{16}$ metabolites. Lane a: natural ratio of dictyotene (**4**) and cystophorene (**1**) in female gametes of *E. siliculosus*. Lane b: selective monitoring of the ion trace *m*/*z* 152 representing the major metabolite of (16*R*)-[$^2H_3$]-**9**. Lane c: selective monitoring of the ion trace *m*/*z* 153 demonstrating the shift toward the linear hydrocarbon cystophorene (**1**) after administration of (16*R*)-[$^2H_3$]-**9**.

TABLE 1. Calculation of the Kinetic Isotope Effect (KIE) on Dictyotene C (4) Formation

	relative ratio	KIE (k_H/k_D)
(16*S*)-[$^2H_3$]-**9**/(16*R*)-[$^2H_3$]-**9**	0.039 ± 0.001	5.4 ± 0.6
[$^2H_3$]-**4**/[$^2H_2$]-**4**	0.21 ± 0.02	

with a strong primary kinetic isotope effect (KIE) associated with the removal of the hydrogen isotope. In the present study, the KIE was calculated from the ratio of the peak areas (corrected for the natural abundance of ^{13}C) of [$^2H_3$]-**4** and [$^2H_2$]-**4** obtained after administration of (16*R*)-[$^2H_3$]-**9** (Table 1). The ratio of the two precursors (16*S*)-[$^2H_3$]-**9** and (16*R*)-[$^2H_3$]-**9** was calculated from the er (96.3 ± 0.1) of the metabolic probe (16*R*)-[$^2H_3$]-**9**.[19] The procedure implies that the secondary isotope effect caused by presence of a (16*S*) deuterium atom in the precursor (16*S*)-[$^2H_3$]-**9** can be neglected[27] for the abstraction of the C(16) *pro-R* hydrogen atom. Moreover, the rate of the subsequent cyclization of [$^2H_3$]-**8** to [$^2H_3$]-**4** is largely unaffected by deuterium substitution at the double bonds.[28]

The product shift observed after administration of (16*R*)-[$^2H_3$]-**9** is caused by the rather strong KIE associated with the abstraction of the hydrogen atom from C(16). If the *pro-R* position is occupied by a deuterium atom, abstraction of a hydrogen atom from C(13) is the preferred reaction (no KIE) and generates cystophorene (**1**) instead of dictyotene (**4**) (Scheme 5). The order of the KIE (5.4 ± 0.6) is in line with previous data for the same reaction in the diatom G. *parvulum*[9] and confirms that the hydrogen abstraction is generally the rate-limiting step in the enzymatic transformation of the precursor acid to algal pheromones.

Identification of the Polar Metabolite of the Oxidative Cleavage of Arachidonic Acid. In the diatom, *G. parvulum* 9-oxononadienoic acid **11** was identified as the polar fragment of the oxidative cleavage of arachidonic acid en route to dictyotene (**4**). In algal gametes or higher plants the polar fragment has not been observed as yet. Addition of

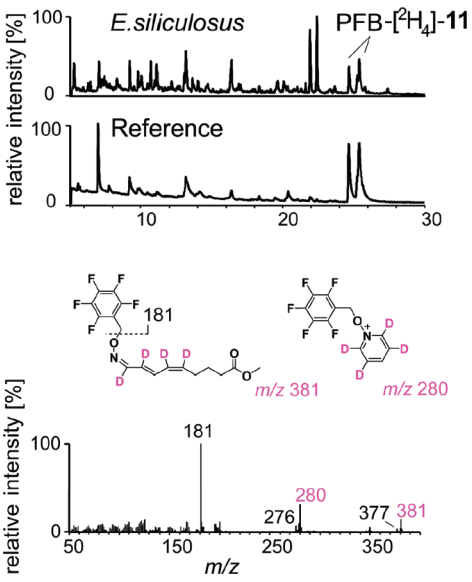


FIGURE 4. Identification of 9-oxononadienoic acid **11** as the polar metabolite.

pentafluorobenzylhydroxylamine (PFBHA) to the incubation medium containing [$^2H_8$]-arachidonic acid and female gametes of *E. siliculosus* allowed to trap the reactive aldehyde as the PFB-oxime (Figure 4). After esterification (CH_2N_2), the polar metabolite was identified by GLC−MS using an authentic reference.[8] The mass spectrum showed the molecular ion of the *O*-(2,3,4,5,6)-pentafluorobenzyl oxime (PFB-oxime) of [$^2H_4$]-**11** at *m*/*z* 381, the PFB fragment ion at *m*/*z* 181, and a pyridinium ion at *m*/*z* 280, which is characteristic for α,*β*,*γ*,*δ*-unsaturated aldehydes.[29] Since the fragment ions are shifted by four mass units compared to the unlabeled reference (*m*/*z* 377, 276) the origin from the administered precursor [$^2H_8$]-arachidonic acid is obvious. Using chemical ionization (CI) in the negative mode confirmed the presence of [$^2H_4$]-**11** by intense $[M - HF]^{\bullet -}$ signals of the PFB-oximes (Supporting Information).

Mechanistic Aspects and Stereochemistry of Algal Pheromone Biosynthesis. The identification of 9-oxononadienoic acid **11** as the second fragment of arachidonic acid en route to the $C_{11}H_{16}$ hydrocarbon dictyotene (**4**) demonstrates the principal similarity of this transformation in brown algae and diatoms. In the diatom *G. parvulum*, arachidonic acid (**9**) is first activated by a lipoxygenase to the (9*S*)-hydroperoxide **10** (Scheme 5). Protonation and loss of water could induce a Hock−Criegee-type rearrangement[30,31] yielding the polar fragment **11** along with the thermolabile dictyopterene C (**8**). The *cis*-bisalkenylcyclopropane **8** rearranges spontaneously in a highly coordinated fashion and with complete chirality-transfer to the cycloheptadiene **4**.[32] Since the involved enzymes are still unknown, the initial step of the oxidative fragmentation of the precursor **10**, namely the rate-limiting removal of the hydrogen atom or the activation of the hydroperoxy group, remains to be established. The high

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er of dictyotene (**4**) supports an arrangement of the arachidonic acid precursor as outlined in Scheme 5.

The U-shaped orientation of the precursor can be easily adopted by other fatty acids, for example, by eicosa-5,8,11,14,17-pentaenoic acid or eicosa-5,8,11,14,17,19-hexaenoic acid both of which are known to occur in brown algae.[33] Eicosapentaenoic acid is the precursor of ectocarpene (**5**), and the eicosahexaenoic acid is the logic precursor of desmarestene (6-buta-1,3-dienylcyclohepta-1,4-diene), which acts as a potent gamete-releasing and gamete-attracting pheromone in the brown alga *Desmarestia aculeata*.[34] Evolutionary modifications of the active center may have created environments that favor other conformations of the precursor acid which could generate the families of *trans*-bisalkenylcycloporpanes, cyclopentene, and cyclohexene hydrocarbons[35] according to the same principal mechanism. The simultaneous formation of a pheromone and 9-oxononadienoic acid (**11**) from a single precursor such as arachidonic acid represents an exiting example of "atom-economy"[36] in nature, since dictyotene (**4**) acts as a gamete attractant while 9-oxononadienoic acid **11** was shown to act as a strong deterrent[37] and toxin for marine copepods that feed on algal resources.

Experimental Section

1-((2*R*)-[2-^{2}H]-Hex-5-enyloxy)methylbenzene (16). Mesyl chloride (2.52 g, 22.0 mmol) in CH_2Cl_2 was added dropwise to a stirred solution of (*S*)-1-(benzyloxy)hex-5-en-2-ol (**15**) (2.52 g, 12.2 mmol) and triethylamine (3.70 g, 36.6 mmol) in 50 mL of CH_2Cl_2 at −78 °C. After the mixture was allowed to warm to 0 °C and stirred for 30 min, the excess base was neutralized with 2 N HCl. The solution was washed with brine and extracted with ether, and the combined organic layers were dried (Na_2SO_4) and evaporated. The resulting oil was dissolved in 3 mL of dry diethyl ether in a flame-dried flask, and the solution was cooled to −78 °C under argon to exclude humidity. After addition of $LiAl^2H_4$ (1.54 g, 36.6 mmol), the suspension was stirred for 30 min and allowed to come slowly to 20 °C overnight. After careful hydrolysis with water and 1 M sulfuric acid, the mixture was extracted with diethyl ether. The organic phase was washed with brine, dried (Na_2SO_4), and evaporated at reduced pressure to give a colorless liquid (1.87 g, 80%). ^{1}H NMR ($CDCl_3$) δ: 7.38−7.28 (m, 5H; aromatics); 5.86−5.76 (m, 1H, C(5)); 5.12 (m, 1H, C(6)); 4.95 (m, 1H, C(6)); 4.51 (s, 2H, C(1′)); 3.48 (d, J = 8 Hz; 2H, C(1)); 2.12−2.04 (m, 2H, C(4)); 1.62 (m, 1H, C(2)); 1.49 (m, 2H, C(3)). ^{13}C NMR ($CDCl_3$) δ: 138.8 (C(5)); 138.7(C(2′)); (128.4, (C(4′)); (127.6 (C(5′)); 127.5 (C(3′); 114.5, (C(6)); 72.9 (C(1′)); 70.2 (C(1)); 33.6 (C(4)); 28.9 (t, ^{CD}J = 19 Hz, C(2)); 25.4, (C (3)). MS m/z: 191 ($M^{+\bullet}$, 1), 162 (2), 107 (16), 91 (100), 82 (11), 65 (8). IR (cm^{-1}): 3070, 3031, 2925, 2156 (C−D), 2000−1700, 1640, 1605, 1452. HRMS (EI): calcd for $C_{13}H_{17}DO$ 191.142042, found 191.142712.

1-((2*R*)-[2,5,6-$^2H_3$]-Hexyloxy)methylbenzene (17). Dry benzene was degassed using the freeze−thaw method, and then 40 mL was transferred with a syringe in a flask with a septum and cooled to 0 °C; the headspace was purged with argon to exclude oxygen. After Wilkinson's catalyst $Rh[P(Ph)_3]_3Cl$ (445 mg, 0.48 mmol, 0.05 equiv) and **19** (1.84 g, 9.6 mmol) were added to the solvent, a balloon filled with deuterium, connected with a Pasteur pipet to ensure tightness, was applied through the septum. The mixture was purged with deuterium for 1 min, warmed to 18 °C, and stirred. After 45 min, the flask turned an intense dark red color, and after 8 h the gas balloon was removed. The solution was concentrated at reduced pressure; the catalyst was precipitated with hexane and filtered over Celite. The solvent was evaporated under reduced pressure, and the residue was dissolved in hexane, filtered through a silica pad, and concentrated under reduced pressure to give a colorless liquid (1.41 g, 75%).

^{1}H NMR δ: 7.37−7.27 (m, 5H, aromatics); 4.51 (s, 2H, C(1′)); 3.47 (d, J = 6.6 Hz, 2H, C(1)); 1.65−1.55 (m, 1H, C(2)); 1.40−1.34 (m, 2H, C(3)); 1.32−1.25 (m, 3H, (C(4), C(5)); 0.90−0.80 (m, 2H, C(6)). ^{13}C NMR ($CDCl_3$) δ: 138.7 (C(2′)); 128.3 (C(4′)); 127.6 (C(5′)); 127.4 (^{CD}J = 19 Hz; C(5)); 13.6 (^{CD}J = 19 Hz; C(6)). MS m/z: 195 ($M^{+\bullet}$, 1); 108 (10); 92 (69); 91 (100); 65 (7). IR (cm^{-1}): 3032, 2924, 2855, 2165 (C−D), 2000−1700, 1602, 1454. HRMS (EI): calcd for $C_{13}H_{17}D_3O$ 195.170246, found 195.171005.

(2*R*)-[2,5,6-$^2H_3$]-Hexanal (13). Freshly prepared 2-idoxybenzoic acid[38] (IBX, 2.36 g, 8.4 mmol) was added to a solution of labeled hexanol **18** (290 mg, 2.8 mmol) in 15 mL of CH_2Cl_2 in a flask with cock. A reflux condenser closed by a balloon filled with argon was applied, and the suspension was stirred under reflux. Reaction control measurements were taken by ^{1}H NMR after precipitation of suspension aliquots (0.5 mL) at −20 °C, filtration, and evaporation of the solvent. After 15 h, the suspension was precipitated at −20 °C and filtered over a pad of Florisil. The solvent was removed at reduced pressure using a Vigreux column. The colorless liquid obtained contained dichloromethane, which was determined to be 36 mol % (31% w/w) by ^{1}H NMR. The product was used immediately for olefination (186 mg, 69% w/w, 45%).

^{1}H NMR δ: 9.76 (d, J = 1.8 Hz, 2H, C(1)); 2.42−2.35 (m, 1H, C(2)); 1.65−1.69 (m, 2H (C(3)); 1.33−1.22 (m, 3H, C(4), C(5)); 0.90−0.82 (m, 3H). ^{13}C NMR ($CDCl_3$) δ: 203.1 (C(1)); 43.5 (t, ^{CD}J = 19 Hz; C(2)); 31.1 (C(4)); 21.9 (^{CD}J = 19 Hz, C(5)); 21.7 (C(3)); 13.4 (^{CD}J = 19 Hz, C(6)). MS m/z : 103 ($M^{+\bullet}$, 1), 85 (21), 84 (9), 74 (35), 59 (33), 58 (93), 46 (20), 45 (100). HRMS (EI): calcd for $C_6H_9D_3O$ 103.107645, found 103.108058.

(16*R*)-[16,19,20-$^2H_3$]-Methyl Arachidonate (25). A flame-dried 50 mL flask with a cock loaded with the (*Z*)-hex-3-enyl-1,6-bis(triphenylphosphonium bromide) **12** (927 mg, 1.2 mmol) was dried under high vacuum to remove traces of water from the hygroscopic salt. After venting with argon, dry THF (20 mL) was added through a septum and the suspension was cooled to −78 °C. A solution of $KN[Si(CH_3)_3]_2$ (0.91 M, 2.6 mL, 2.4 mmol) in THF was slowly added, and the mixture was allowed to warm to 0 °C. The orange solution was stirred for 30 min and cooled again to −78 °C. A solution of the oxoester **19** (165 mg, 77% w/w, 1.2 mmol) in 2 mL of THF was added with a syringe pump over 40 min, and then the mixture was warmed slowly to 0 °C. After the suspension was cooled again to −78 °C, a solution of the aldehyde **13** (180 mg, 69% w/w, 1.2 mmol) in 1 mL of THF was added, and the mixture was allowed to warm to room temperature within 30 min. After the addition of 2 N HCl and extraction with ether, the organic phase was washed with brine until a pH of 6 was reached, dried with Na_2SO_4 in an ice bath, and evaporated at reduced pressure. The substance was purified on a silica column (PE/Et_2O = 20/1), equipped with a cooling jacket in darkness. Separation of **25** (R_f 0.32) from impurities of $Ph_3P{=}O$ and 2,6-di-*tert*-butyl-4-methylphenol (R_f 0.36) was incomplete. The mixed fractions were dried and further purified by semipreparative straight-phase HPLC under the following conditions: hexane (A)/THF (B) 2% B to 10% B in 20 min, then 90 B % for 6 min; 3 mL/min, UV detection 210 nm.

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The pure product obtained was a colorless liquid (31 mg, 8%). The isotopic purity was calculated from the molecular ion m/z 321 of the ester **25** and the molecular ions m/z 320 and 322, respectively, of the [$^2H_2$]- and [$^2H_4$]-analogues. Extracted ion chromatograms were obtained by GLC−MS with sector field analyzer, and the peak areas were corrected for the contribution of the natural isotopic abundance of ^{13}C. The resulting areas at m/z 320, 321, 322, attributed, respectively, to d_2-, d_3-, and d_4-methyl arachidonate, were used for calculating isotopic purity. ^1H NMR δ: 5.45−5.30 (m, 8H, olefinic protons, C(5/6/8/9/11/12/14/15)), 3.67 (s, 3H, C(1′)), 2.90−2.75 (broad, 6H, C(7/10/13)), 2.35 (t, J = 7.6 Hz, 2H, C(2)), 2.14−2.08 (m, 2H, C(4)), 2.07−2.01 (m, 1H, C(16)), 1.71 (quintet, J = 7.6 Hz, 2H, C(3)), 1.4−1.2 (broad, 5H, C(17/18/19)), 0.9−0.8 (broad, 2H, C(20)). ^{13}C NMR δ: 174.03 (C(1)), 130.45 (C(15)), 128.92 (C(5), 128.87 (C(6)), 128.58 (C(12)), 128.20(C(8)), 128.16 (C(14)), 127.86 (C(11)), 127.55 (C(9)), 51.45 (C(1′)), 33.44 (C(2)), 31.35 (C(18)), 29.20 (C(17)), 26.86 (t, ^{CD}J = 19 Hz, C(16)), 26.55 (C(4)), 25.63, 25.62, 25.60 (C(7), (C(10)), (C(13)), 24.78 (C(3)), 22.08 (t, ^{CD}J = 19 Hz, C(19)), 13.63 (t, ^{CD}J = 19 Hz, C(20)). MS m/z: 321 ($M^{+\bullet}$, 5), 180 (27), 153 (35), 120 (22), 119 (28), 106 (52), 106 (52), 105 (42), 93 (56), 91 (69), 80 (85), 79 (100). IR (cm^{-1}): 3012, 2923, 2854, 2168 (C−D), 1741, 1656, 1438, 1365, 1156. HRMS (EI): calcd for $C_{21}H_{31}D_3O_2$ 321.274711, found 321.275711. Isotopic purity: d_2:d_3:d_4 =1:98:1; the sum of d_0, d_1, and d_5 was less than 1%.

Incubation with (16*R*)-[$^2H_3$]-9 and Analysis of Volatiles. Suspensions of female gametes of *E. siliculosus* with typical concentration 1×10^7 cells/mL were prepared (see the Supporting Information). Aliquots of 2 mL were transferred into 4 mL screw-cap vials and incubated with 30 μg of labeled arachidonic acid (16*R*)-[$^2H_3$]-**9** dissolved in 1 μL of DMSO. After gentle agitation, the suspension was left for 5 h in a growth chamber at 16 °C to allow complete cell settling, and at the fifth hour solid-phase microextraction (SPME) was carried out for 60 min. For the analysis of total fatty acids after a test incubation with (16*R*)-[$^2H_3$]-**9**, a modified protocol for the extraction and transesterification of fatty acid was used.[39] The gamete suspension was centrifuged and the pellet was transferred into a sealed hydrolysis vial and homogenized in 0.5 mL of methanol. Then 0.5 mL of a mixture of acetyl chloride/methanol 1:9 and 0.5 mL of hexane were added together with 2 μg of d_{27}-myristic acid as an internal standard. Heating at 100 °C for 10 min caused the formation of a single phase. After cooling on ice and addition of 1 mL of water, the upper phase was collected and dried (Na_2SO_4), and the solvent was evaporated. After the residue was dissolved in 40 μL of hexane, GLC−MS analysis and external calibration using methyl arachidonate were carried out. The amount of (16*R*)-[$^2H_3$]-**9** in the cell suspension was determined as ca. 22 μg.

Incubation with [$^2H_8$]-9 and Analysis of Oxylipins. Diatom cells were harvested (see the Supporting Information) and incubated with a solution containing 6 μg/μL of [5,6,8,9,11,12,14,15-$^2H_8$]-arachidonic acid ([$^2H_8$]-**9**). After SPME, a water solution of PFBHA (0.75 mL; 25 mmol/L) containing 2,6-di-*tert*-butyl-4-methylphenol (BHT, 10 μg/mL) was added to the homogenate and mixed. After 30 min, it was carefully acidified to pH 3 with diluted H_2SO_4 and extracted three times with 0.5 mL of dichloromethane containing BHT (10 μg/mL) in Eppendorf tubes. After centrifugation, the combined lower phases were dried (Na_2SO_4), transferred to a GLC vial, concentrated, and treated with diazomethane. After evaporation of the solvent, the residue was dissolved in dichloromethane, transferred in a microvial, concentrated, and dissolved in 40 μL of the same solvent. For the incubation of *E. siliculosus,* 2 mL aliquots of gamete suspension were transferred into 4 mL screw-cap vials and incubated with 30 μg of [$^2H_8$]-**9** dissolved in 1 μL of DMSO. After gentle agitation, the suspension was left for 5 h in a growth chamber at 16 °C to allow complete cell settling, and at the fifth hour SPME was carried out for 60 min. After volatile sampling, 0.75 mL of a water solution of PFBHA (25 mM) containing BHT (10 μg/mL) was added, and the suspension was sonicated in an ice bath with high power for 30 s with a 0.5 s pulse. The homogenate was acidified, extracted, and derivatized as described for *G. parvulum* (see above). After concentration, the residue was dissolved in a microvial with 40 mL of dichloromethane.

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Supporting Information Available: Synthetic procedures and characterization data for all compounds (except **13**, **16**, **17**, and **25**, reported here), copies of NMR spectra for all compounds, cultivation of algae, and additional graphics. This material is available free of charge via the Internet at http://pubs.acs.org.

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