Pergamon

Tetrahedron, Vol. 52, No. 30, pp. 10073-10082, 1996

0040-4020/96 $15.00 + 0.00

PII: S0040-4020(96)00548-0

Biosynthesis of the Algal Pheromone Hormosirene by the Freshwater Diatom *Gomphonema parvulum* (Bacillariophyceae)

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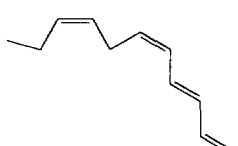
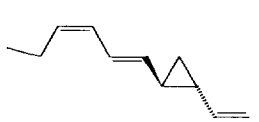
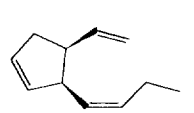
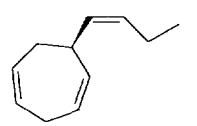
Abstract: The fresh water diatom *Gomphonema parvulum* (Bacillariophyceae) produces the algal pheromones hormosirene **2** ($C_{11}H_{16}$) and dictyopterene A **16** ($C_{11}H_{18}$) from highly unsaturated eicosanoic acids like 20:5 (ω-3) **21** and 20:4 (ω-6) **14**. The oxidative degradation is believed to proceed by cleavage of an intermediate 9-hydroperoxide like **6** by a hydroperoxide-lyase to yield 9-oxo-nona-(5*Z*,7*E*)-dienoic acid **8** and the corresponding C_{11} hydrocarbon. The biosynthetic sequence is confirmed by identification and synthesis of the novel polar fatty acid fragment **8**. Copyright © 1996 Elsevier Science Ltd

Fertile female gametes of many species of marine brown algae attract conspecific males using chemical signals[1,2,3] like, for example, **1** to **4**. Females from the advanced orders Laminariales, Desmarestiales and Sporochnales produce similar compounds which first induce spermatozoid release and then provide a gradient of attractant leading to the signalling female.[4,5] Release occurs within only 8-12 seconds of males receiving the signal and represents one of the fastest signal-responses known in the Plant Kingdom. Some of these compounds, e.g. **1** and **4**, are also produced by higher plants,[3,6] especially fruits.[7,8]
Ectocarpene **4** has also been isolated from blooms of undefined micro-organisms from the Mediterranean Sea,[9,10] and the biosynthetically related C_8 hydrocarbon fucoserratene was reported as a major volatile from laboratory cultures of certain freshwater diatoms.[11] However, in each of these cases the biological significance of the compounds is unknown.


finavarrene **1**


hormosirene **2**

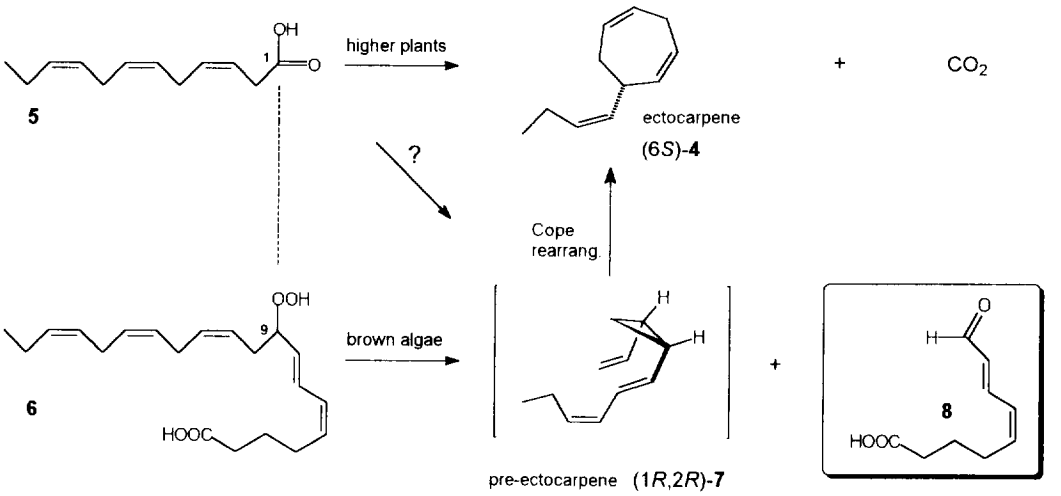

multifidene **3**


ectocarpene **4**

Interestingly, the higher and lower plants have developed different routes to the C_{11} hydrocarbons.[3,12,13] In higher plants, for example in the South African plant *Senecio isatideus* (Asteraceae) dodeca-3,6,9-trienoic acid **5** is the immediate precursor of **1** and **4**.[13] In contrast, brown algae utilise a C_{20} precursor, namely eicosa-5,8,11,14,17-pentaenoic acid **21** for the biosynthesis of the same hydrocarbons.[14] Since the eicosanoids are not catabolised to dodeca-(3*Z*,6*Z*,9*Z*)-trienoic acid **5** prior to the olefin formation, the postulate of direct cleavage of an activated intermediate, like the 9-hydroperoxy acid **6**, appears reasonable. Scheme 1 illustrates the structural similarities of hydroperoxide **6** and C_{12} acid **5** which support such a suggestion. Heterolytic cleavage of the hydroperoxide **6** (by a novel type of a hydroperoxide-lyase, vide infra) is assumed to generate

a reactive intermediate which first yields the cyclopropane (1*R*,2*R*)-7. This compound is thermolabile and rapidly rearranges ($t_{0.5}$ = 18 min at 18 °C)) to (6*S*)-**4**. The unstable cyclopropane 7 has recently been confirmed as an intermediate in the biosynthesis of **4** by female gametes of the brown alga *Ectocarpus siliculosus*.[15] However, the existence of 9-oxo-nona-(5*Z*,7*E*)-dienoic acid **8** (see Scheme 1) remains uncertain. In the case of higher plants, oxidative decarboxylation of acid **5** followed by rearrangement may also yield the cyclopropane 7 *en route* to ectocarpene **4**, although this is yet to be established.

Scheme 1



Since direct identification of the dicarbonyl compound **8** as the second fragment from an eicosanoid precursor is essential for the understanding of the mechanistic basis of the hitherto unknown type of hydroperoxide-lyase[16] activity (outlined in Scheme 1), we screened various diatoms (bacillariophyceae) for their aptitude as test organisms for C_{11} hydrocarbon biosynthesis. Many different species of these micro-organisms are available,[17] they are rather easy to cultivate, and for some of them the production of C_{11}- and/or C_8 hydrocarbons has been already reported.[9,10,11,12]

We report here that the freshwater diatom *Gomphonema parvulum* synthesises the $C_{11}H_{16}$ hydrocarbon **2** (56% *e.e.*) as a major product. Small amounts of 1,2-*trans*-1-hex-1-enyl-2-vinylcyclopropane **16** (51% *e.e.*), a metabolite of arachidonic acid **14** were also observed. Treatment of a crude, cell-free extract of *G. parvulum* with deuterium labelled arachidonic acid **14** gave the labelled hydrocarbon **16** together with deuterium labelled 9-oxo-nona-5*Z*,7*E*-dienoic acid **8**. When the experiment was conducted in the presence of $^{18}O_2$ gas, the oxygen isotope was incorporated into the aldehyde function of **8**, supporting the anticipated functionalisation of the fatty acid at C(9) (Schemes 1 and 4).

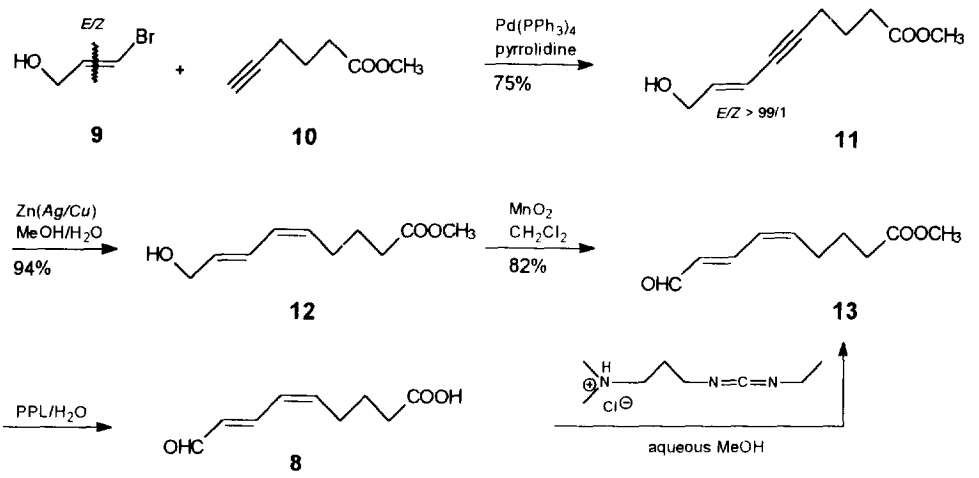
Synthesis of 9-Oxo-nona-(5*Z*,7*E*)-dienoic Acid (8)

The postulated metabolite **8** was synthesised from hex-5-ynoic acid methyl ester **10** and 1-bromopropen-3-ol **9** (*E*:*Z*, 25:75) according to the protocol outlined in Scheme 2. The alkynylation of **9** was achieved in benzene/pyrrolidine in the presence of $Pd(Ph_3)_4$ (5 mol %), following the protocol of Linstrumelle et al.[18] The choice of the solvent was essential to warrant a high stereoselectivity of the coupling reaction (7*E*/7*Z*, ≥99:1). In pyrrolidine solvent (*E*)-**9** reacted significantly faster than the (*Z*)-isomer,[18,19] thus, kinetic resolution of the

isomers (monitored by GLC) was possible. Reduction of the conjugated triple bond was achieved using activated Zn(Cu/Ag)[20,21] in aqueous methanol and furnished the (5*Z*)-isomer stereospecifically.

Brief treatment with MnO_2 afforded the 9-oxo-ester **13** which could be converted into the labile (with respect to integrity of stereochemistry) free acid upon treatment with porcine pancreas lipase (PPL) under hydrolytic conditions.

Scheme 2



Incubation Experiments with Intact and Broken Cells of *Gomphonema parvulum* (Bacillariophyceae)

In contrast to female gametes of marine brown algae diatoms are readily available in mass cultures. Moreover, previous reports on the occurrence of some typical algal C_8- and C_{11} hydrocarbons in marine and fresh water diatoms[9-12] suggested these micro-organisms may be ideal model systems for mechanistic and enzymatic studies of algal pheromone biosynthesis. Table 1 compiles a selection of several species which were screened for their ability to produce volatile C_8- and C_{11} hydrocarbons.

Table 1 Occurrence of C_8 and C_{11} hydrocarbon in selected diatoms.

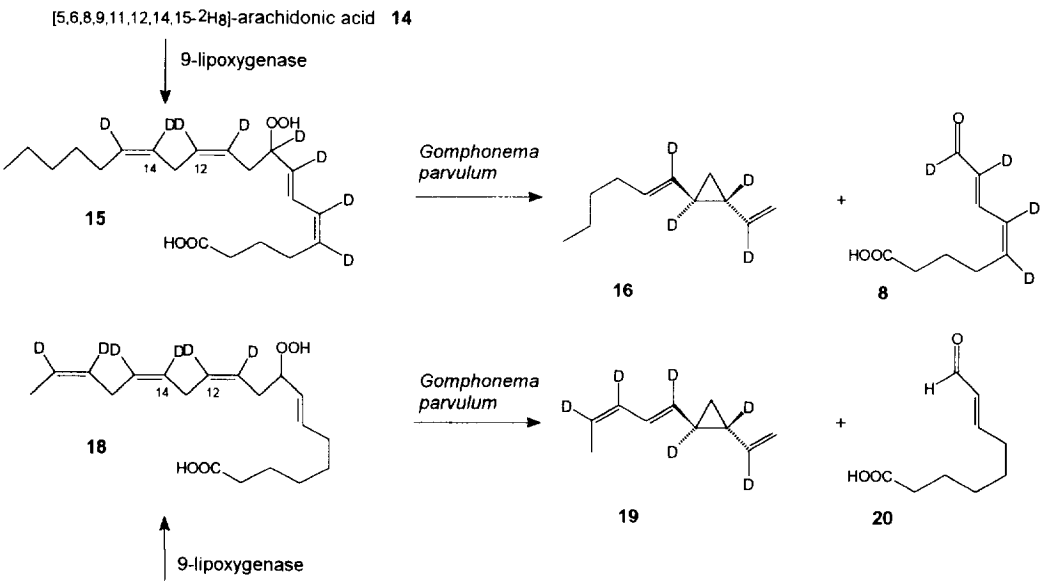
species	type	C_8 hydrocarbons	C_{11} hydrocarbons	ref.
Amphiprora paludosa Smith	freshwater	-	-	17
Asterionella formosa Hassall	marine	+	-	17,12
Fragilaria crotonensis Kitton	freshwater	-	-	17
Gomphonema parvulum Kützing	freshwater	-	+	17
Pinnularia nobilis Ehrenberg	freshwater	-	-	17
Stephanodiscus minutulus Kützing	freshwater	-	-	17
Thalassiosira fluviatilis Hustedt	marine	-	-	17

Except of the freshwater diatom *G. parvulum* none of the species from Table 1 was found to produce significant amounts of C_{11} hydrocarbons. The major volatile from *G. parvulum* was identified as hormosirene[22] **2**

(56% *e.e.* according to GLC), accompanied by small amount of the corresponding $C_{11}H_{18}$ homologue, 1,2-*trans*-hex-1-enyl-2-vinylcyclopropane, also known as dictyopterene A[23] **16** (Scheme 3). Their identity was unequivocally established by GLC-MS at two different injection port temperatures. Injection at 110 °C showed the two cyclopropanes hormosirene **2** and dictyopterene A **16**, while at 250 °C the emergence of a novel signal indicated the thermal rearrangement of the cyclopropane **2** to ectocarpene **4**. Dictyopterene A **16** proved to be more stable and rearranged only partly under these conditions. Interestingly, *G. parvulum* synthesises enantiomeric mixtures of the two cyclopropanes **2** and **16** as is generally the case in brown algae.[24,3]

According to the number and position of the unsaturations hormosirene **2**, like ectocarpene **4**, is a metabolite of eicosa-5,8,11,14,17-pentaenoic acid **21**.[14] As depicted in Scheme 1, the unsaturated precursor should be first attacked by a 9-lipoxygenase to give the hydroperoxide **6**. Subsequent cleavage of **6** was expected to yield hormosirene **2** together with 9-oxo-nona-5*Z*,7*E*-dienoic acid **8** from the polar head of **6**. Thus, when deuterium labelled arachidonic acid **14** was added to growing cultures of *G. parvulum*, the production of deuterium labelled dictyopterene A **16** (51% *e.e.*) was observed. The efficiency of this transformation was very high, since the *de novo* synthesis of labelled **16** exceeded the level of the unlabelled natural **16** by ca. 800% (calculated from the relative abundance of the molecular ions at m/z 150 and 154 *Da* of the co-eluting hydrocarbons).[14] Moreover, due to the positions of the double bonds in the precursor **14** and the preservation of only four deuterium atoms in the hydrocarbon **16**, the aliphatic segment C(10)-C(20) of **14** had been incorporated into **16**. The volatile metabolites were isolated by „headspace-trapping" as described previously[25] or, more efficiently, by using the recently developed Solid-Phase-Micro-Extraction (SPME).[26,27]

Scheme 3



The C_{19} acid **17**, designed as a nor-analogue of eicosa-5,8,11,14,17-pentaenoic acid **21**,[14] was transformed by broken cells of *G. parvulum* into deuterium labelled nor-hormosirene **19**. Owing to the well defined ms fragmentation pattern of compounds like **19**, the position of the deuterium atoms of the labelled metabolite

could be unequivocally determined by mass spectrometry and supported a ring closure between C(12) and C(14) of the precursor **17**, analogous to the biosynthesis of ectocarpene **4** in female gametes of brown algae[14,28] (Scheme 1).

To facilitate the isolation and characterisation of the polar fragment **8**, ca. 10^7-10^8 cells of *G. parvulum* were sonicated, centrifuged, and the supernatant crude enzyme preparation was treated with labelled arachidonic acid **14**. Again, a rapid transformation of **14** to dictyopterene A **16** (isolated and analysed by SPME and GLC-MS) was observed. The reaction was accompanied by an increase in UV absorption at 277 nm as to be expected for the formation of a conjugated oxo-diene like **8**. Comparative HPLC of synthetic **8** and the polar metabolite from the cell free extracts proved the two compounds to be identical with respect to retention volume and UV-absorption. The presence of a conjugated aldehyde was supported by the facile reduction of the compound with $NaBH_4$ resulting in a UV inactive compound. Owing to the high polarity and water solubility of the dicarbonyl compound **8**, all attempts to extract the metabolite remained unsuccessful. However, after removal of water and chromatography of the metabolite on RP 18 using MeOH for elution, the addition of *N*-dimethylamino-propyl-*N'*-ethylcarbodiimide hydrochloride[29] (see Scheme 2) facilitated a smooth esterification of the free acid **8** yielding the labelled methyl ester **13**.

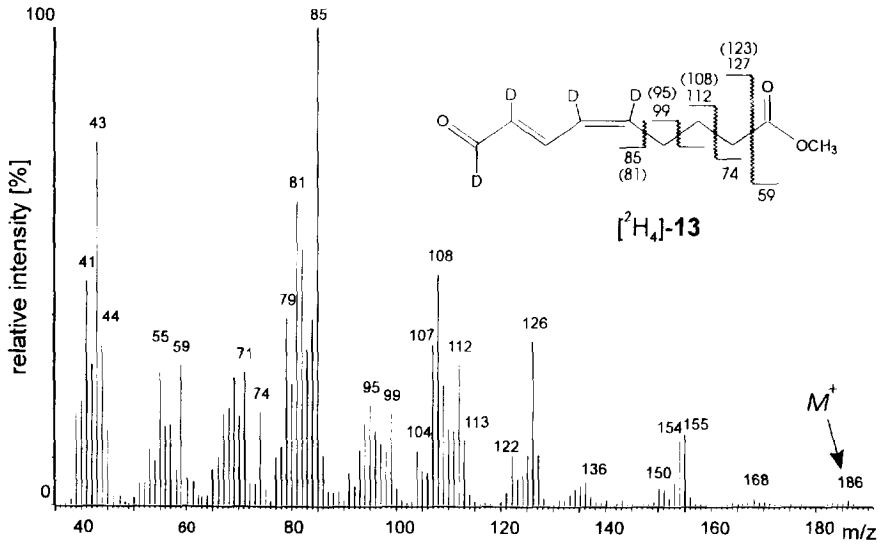
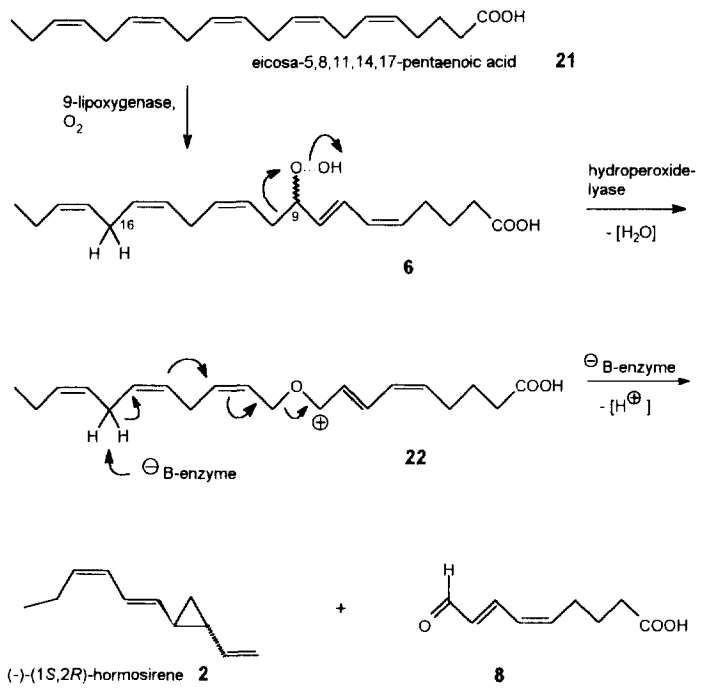


Figure 1 Mass spectrum of [$^2H_4$]-**13** resulting from the biotransformation of labelled arachidonic acid [$^2H_8$]-**14** by broken cells of *Gomphonema parvulum*. Values in brackets correspond to unlabelled **13**.

Owing to the molecular ion of the 9-oxo-ester **13** at m/z 186, the C_9 fragment of [$^2H_8$]-arachidonic acid **14** carried four deuterium atoms which were located in the oxodiene segment of **13** (Scheme 3). Some representative fragments (α-,ß- and γ-cleavage) supporting the position of the isotopes are indicated in Figure 1. The origin of the oxygen atom of the 9-oxo group of **13** was demonstrated by repeating the experiment in the presence of $^{18}O_2$ gas. After esterification the metabolite exhibited a molecular ion at m/z 188 and a base peak at m/z 87 indicating that the ^{18}O-isotope was incorporated into [$^2H_8$]-**14** yielding [$^2H_4$-^{18}O]-**8** after bond cleavage. The specific labelling pattern of the two eicosanoid fragments, together with the incorporation of molecular oxygen, strongly supports the current concept of algal pheromone biosynthesis (Schemes 1 and 4).

Thus, the two $C_{11}H_{16}$ hydrocarbons hormosirene **16** and ectocarpene **4** are produced from eicosa-5,8,11,14,-17-pentaenoic acid **21** *via* an C(9)-oxygenated intermediate, presumably the hydroperoxide **6**. It appears reasonable to assume that the subsequent degradation of the hydroperoxide **6** by a hydroperoxide-lyase may proceed as an ionic process as illustrated in Scheme 4.

Scheme 4



Following peroxidation of eicosapentaenoic acid **21** by a 9-lipoxygenase to yield the 9-hydroperoxide **6**, a hydroperoxide-lyase could first induce a Hock-Criegee-type rearrangement[30,31] resulting in the Hock-oxycarbenium ion **22** after C-C-bond cleavage and rearrangement. However, in contrast to the well known lyase reactions leading to enolethers and carbonyl compounds, in the present case transfer of a proton from C(16) to a basic center of the enzyme is expected to cleave the molecule into a divinylcyclopropane like **2** and an ω-oxo-acid like **8**. Depending on the folding of the substrate at the active site of the hydroperoxide-lyase, different spatial arrangements of double bonds and reactive intermediates towards each other will result, and, hence, the individual enzymes could yield different C_{11} hydrocarbons, like for example hormosirene **2** or preectocarpene **7** from a single precursor **6**. The incorporation of molecular oxygen into the carbonyl group of **6** is in accord with such a mechanism and corresponds to earlier findings concerning the vinyl ether oxygens from fatty acid hydroperoxides.[32]

While the 9-oxo acid **6** is an, as yet, unknown compound, the homologous 13-oxo-trideca-9*Z*,11*E*-dienoic acid is known as a common degradation product of 13-hydroperoxylinole(n)ic acid under aerobic and anaerobic conditions.[33] The compound is weakly fungitoxic,[34] and is believed to be the precursor of mucondialdehyde which, for example, inhibits the growth of *Cercospora beticola*, a pathogen of *Beta vulgaris*.[34] Whether or not the polar 9-oxo acid **6** has a similar protective role for *G. parvulum* and other C_{11} hydrocarbon producing diatoms is unknown and remains to be established.

Experimental

General: Reactions were performed under argon, solvents were dried according to standard methods. IR: Perkin-Elmer Series 1600 FTIR Spectrophotometer. UV: Perking Elmer Lambda 2, thermostatised cell holder, connected to a Haake Cryostat. ^{1}H- and ^{13}C-NMR: Bruker AC 250 or Bruker AC 400 Spectrometer, $CDCl_3$ as solvent. Chemical shifts of ^{1}H- and ^{13}C-NMR are given in ppm (δ) downfield relative to TMS. GC-MS (70eV): Finnigan MAT 90 or Finnigan ITD 800 coupled with a Carlo Erba GC 6000, Model Vega. HR-MS: Kratos MS 50. GLC: Carlo Erba, Series 4100, equipped with fused silica capillaries coated with SE 30 (10m x 0.31 mm) or 6-methyl-2,3-di-O-pentyl-γ-cyclodextrin (50m x 0.31mm) from Macherey & Nagel (Düren, Germany). HPLC: Barspec Chrom-A-Scope rapid scanning photometer, combined with a Kontron HPLC pump 420 and a column filled with Merck LiChrospher 100 RP-18. Silica gel, Si 60 (0.200-0.063 mm, E. Merck, Darmstadt, Germany) was used for chromatography. Thin layer chromatography was performed with silica gel plates Polygram Sil G_{F254}, from Merck. The Solid-Phase-Microextractor (SPME) was purchased from Supelco, Bellefonte, USA. Deuterium labelled arachidonic acid [$^2H_8$]-**14** was from Campro Scientific, D-46422 Emmerich, Germany.

Methyl 9-Hydroxy-non-7*E*-en-5-ynoate (11)

3-Bromopropenol **9** (0.25 g, 1.84 mmol) was dissolved in benzene (82.0 ml) and $Pd(PPh_3)_4$ (0.22 mg, 0.19 mmol) was added. After 5 min at rt. piperidine (0.74 ml, 7.52 mmol), hexynoic acid methyl ester **10** (0.26 g, 2.24 mmol) and CuI (0.07 g, 0.38 mmol) were added with vigorous stirring. The progress of the reaction (configurational purity) was monitored by GLC. After about 4 h at 30 °C the stereoselectivity decreased, and the mixture was poured into a chilled solution of sat. aq. NH_4Cl. Extractive work-up (Et_2O) and chromatography on silica gel (pentane/ether 50/50) yielded the hydroxy ester **11** as a colorless liquid. Yield: 0.25 g 75% (*E*:*Z* > 99:1). IR (KBr, neat) 3424 (br), 3029, 2952, 2870, 2249, 1730, 1438, 1371, 1225, 1155, 1097, 1017, 912, 733 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 6.23-6.11 (dt, 1H-C(7)); 5.76-5.65 (dquint, 1H-C(8)); 4.18 (dd, 2H-C(9)); 3.67 (s, 3H-COOC<u>H</u>$_3$); 2.49-2.33 (m, 2H-C(2), 2H-C(4)); 1.85 (quint, 2H-C(3)); 1.59 (s, 1H-O<u>H</u>). ^{13}C NMR ($CDCl_3$, 250 MHz) δ: 140.76, 110.98, 89.79, 79.32, 63.04, 51.66, 32.94, 23.94, 18.92. MS (70 eV) 182($M^{+\bullet}$, 0.4); 164(55); 149(29); 122(19); 105(72); 95(93), 79(100); 65(38); 55(40). HR-MS *m z* calcd. for $C_{10}H_{14}O_3$ 182.0943, found 182.0943.

Methyl 9-Hydroxy-nona-5Z,7*E*-dienoate (12)

A soln. of the acetylenic ester **11** (0.22 g, 1.21 mmol) in methanol/water (80 ml, 1:1, v/v) was stirred at rt. with activated Zn(Cu/Ag) (12.0 g). The progress of the reduction was followed by GLC. After complete reduction (ca. 40 h), the solids were removed by filtration and carefully washed with methanol (2 x 20 ml) and ether (6 x 20 ml). Ca. 80% of the solvent was removed, and the aq. suspension was again extracted with ether (4 x 50 ml). Removal of solvents in vacuo and chromatography on silica gel (pentane/ether, 40/60) afforded the configurationally pure dienoic ester **12** in high yield. Yield: 0.21 g, 94%. IR (KBr, neat) 3421 (br), 3007, 2952, 2865, 1736, 1438, 1416, 1368, 1317, 1245, 1241, 1163, 1119, 1087, 989, 952, 917, 863, 815, 733 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 6.47-6.38 (dd, 1H-C(7)), 5.96 (t, 1H-C(6)); 5.8-5.72 (m, 1H-C(8)); 5.35 (quart, 1H-C(5)); 4.14 (d, 2H-C(9)); 3.6 (s, 3H-COOC<u>H</u>$_3$); 2.26 (t, 2H-C(2)); 2.16 (quart, 2H-C(4)); 2.01 (s, 1H-O<u>H</u>); 1.67 (quint, 2H-C(3)); ^{13}C NMR ($CDCl_3$, 250 MHz) δ: 174.14, 132.39, 131.13, 128.8,

126.24, 63.3, 51.56, 33.31, 26.94, 24.71. MS (70 eV) 184($M^{+\bullet}$; 0.2), 166(64), 151(8), 135(13), 106(34), 92(100), 79(78), 74(55), 67(39), 55(33). HR-MS *m z* calcd. for $C_{10}H_{16}O_3$ 184.1093, found 184.1099.

Methyl 9-Oxo-nona-5Z,7*E*-dienoate (13)

A suspension of the alcohol **12** (0.09 g, 0.48 mmol) in dichloromethane (10 ml) was stirred rapidly and active MnO_2 (1.2 g) was added. After complete conversion (ca. 90 sec) the oxidant was immediately filtered off over a pad of Na_2SO_4. The solids were washed with ether (4 x 25 ml), and the combined organic layers were evaporated. Yield: 0.072 g, 82%. IR (KBr, neat) 3020, 2952, 2870, 2814, 2723, 1734, 1682, 1634, 1437, 1368, 1250, 1209, 1165, 1136, 1105, 1013, 991, 962, 866, 654 cm^{-1}. ^1H NMR ($CDCl_3$, 250 MHz) δ: 9.58 (d, 1H-CHO); 7.45-7.33 (dd, 1H-C(7)); 6.27 (t, 1H-C(6)); 6.17-6.07 (dd, 1H-C(8)); 5.99-5.87 (m, 1H-C(5)); 3.64 (s, 3H-COOC$\underline{H}_3$); 2.42-2.29 (m, 2H-C(2), 2H-C(4)); 1.77 (quint, 2H-C(3)); ^{13}C NMR ($CDCl_3$, 250 MHz) δ: 193.89, 173.59, 146.31, 142.07, 132.26, 127.71, 65.88, 33.16, 24.36, 15.29. MS (70 eV) 182($M^{+\bullet}$; 3), 164(2), 150(27), 122(42), 104(54), 91(24), 81(100), 67(23). HR-MS *m z* calcd. for $C_{10}H_{14}O_3$ 182.0943, found 182.0945.

9-Oxo-nona-5Z,7*E*-dienoic Acid (8)

The ester **13** (20.0 mg, 0.12 mmol) was suspended in phosphate buffer (0.1 M, 6 ml, pH 7) and porcine pancreas lipase (PPL) (4.0 mg) was added to the slowly stirred mixture. After 24 h at rt. the most of the enzyme was removed by filtration. Due to the pronounced lability of the 9-oxo-acid **8**, the resulting soln. served without further purification as a reference for HPLC/UV and for GLC-MS after re-esterification.

Cultivation of Diatoms; General Procedure

The diatoms (Table 1) were cultivated as standing cultures in Petri dishes (250 ml) at 17 °C. The dishes were illuminated with a photon flux of about 70 μmol m^{-2} sec^{-1} for 14 h followed by a 10 h dark period. After seven days cells were transferred into new medium. The cells were harvested towards the end of the logarithmic growth period after 12-18 days. Fresh water media, containing $KH_2PO_4 \bullet 3H_2O$ (6.55 mg l^{-1}) were prepared according to Müller,[35] and sea water media were mixed according to Maier.[36]

Collection of Volatiles by Solid-Phase-Microextraction (SPME)

Collection of metabolites was performed according to established procedures.[26,27] Greatest sensitivity was obtained with an SPME-fibre, coated with polydimethylsiloxane (100 μm) as the stationary phase. Extraction of metabolites was achieved by simply inserting the SPME-fibre into the rapidly stirred incubation buffer containing the broken cells together with the soluble enzymes, labelled precursors and their products. Equilibration was complete after 20 min at rt. Compounds were then directly evaporated from the fibre within the injection port (250 °C) of the GLC-MS. Volatiles from living cultures were analysed by the same technique. The collection of volatiles by „headspace-trapping" was performed as described previously.[25]

Transformation of Eicosanoids by Cell Free Extracts of *Gomphonema parvulum*

For a typical incubation experiment a suspension of ca. 10^7-10^8 cells of *G. parvulum* in a phosphate buffer (10 ml, 0.1 M KH_2PO_4, pH 7) was sonicated at 0 °C (4 x 30 sec, 40 W). The suspension was centrifuged at 8000 rpm and the supernatant used for subsequent incubations. The (deuterium labelled) precursors like [$^2H_8$]-**14** or [$^2H_4$]-**18** were added (10 µl of a10% soln. in EtOH). The mixture was gently stirred at rt., and after 20 min the metabolites were extracted by SPME and analysed by GLC-MS, HPLC and UV. Before analysis by UV and HPLC the enzyme preparations were centrifuged at 12000 rpm. Incorporation of $^{18}O_2$ was carried out by analogy using a closed system with an $^{18}O_2$ atmosphere.

Isolation and Derivatisation of 9-Oxa-nona-5Z,7*E*-dienoic Acid (8)

A suspension of about 10^8 cells of *G. parvulum* was sonicated in phosphate buffer (20.0 ml, 0.1 M KH_2PO_4, pH 7) as described above, and deuterium labelled arachidonic acid **14** (40 µl of a 10% soln. in ethanol) was added. After 30 min water was removed in vacuo, the slurry was diluted with MeOH (1.0 ml) and filtered through glass wool. The solution was then prepurified by chromatography on reversed phase material in a Pasteur pipette (10 cm x 7 mm, RP-18) using MeOH (20 ml) for elution. Esterification of the free acid was achieved by addition of *N*-dimethylamino-propyl-*N'*-ethylcarbodiimide hydrochloride (10.0 mg, 1.0 µmol) to the methanol fraction. The mixture was stirred for 2 h at rt., phosphate buffer (10 ml, 0.1 M, pH 7) added, and the ester **13** extracted with ether. The extract was concentrated in vacuo and used for GLC-MS or HPLC/UV without further purification.

HPLC-Analysis of the Metabolites

The polar products of the biotransformations (100 µl per injection) were analysed by HPLC on a reverse phase column (LiChrospher 100, RP 18, 25 x 0.4 cm) using methanol:water (9:1, v:v) at 0.4 ml min^{-1} for elution. Compounds were monitored by UV (full scan, 220 nm to 360 nm). The conjugated 9-oxo-dienoic acid **8** showed a strong UV absorption at 278 nm (λ_{max}) which disappeared upon pre-treatment of the sample with $NaBH_4$.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt, is gratefully acknowledged. We also thank the BASF AG, Ludwigshafen, and the Bayer AG, Leverkusen, for generous supply with chemicals and solvents. The authors gratefully acknowledge the technical assistance of Mrs. Katja Bandemer.

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(*Received in Germany* 13 *May* 1996; *accepted* 7 *June* 1996)