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Biosynthesis of dictyopterene A: stereoselectivity of a lipoxygenase/hydroperoxide lyase from *Gomphonema parvulum* (Bacillariophyceae)

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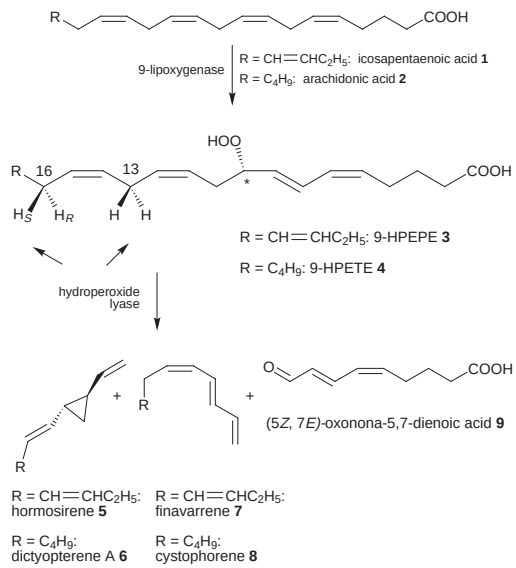
(9*S*)-Hydroperoxyicosatetraenoic (9*S*-HPETE) acid is shown to be an intermediate in the biosynthesis of dictyopterene A in the freshwater diatom *Gomphonema parvulum*; the stereochemistry of (9*S*)-HPETE and the position of the hydrogen atom at C(16) lost during fatty acid cyclisation and oxidative cleavage of the hydroperoxide were investigated using trapping experiments and chirally deuterium labelled fatty acids.

Fatty acid derived C_8 and C_{11} hydrocarbons like finavarrene **7**, hormosirene **5** or dictyopterene A **6** act as pheromones in marine brown algae (Scheme 1).[1] The same compounds occur in heterocontophytic diatoms[2,3] and in higher plants,[4] however here, their biological function is unknown. Brown algal derived cyclopropanes and cycloheptadienes are secreted as mixtures of enantiomers with often well defined compositions that depend on species and habitat. Hormosirene **5** is a particularly interesting example. Both enantiomers are known and, in general, the ee of the secreted mixtures (52–92% ee) is characteristic for the species, sometimes even for the habitat.[1] This raises questions about how the stereochemistry of the cyclopropane is established and how the ratio of enantiomers is controlled in the biosynthetic pathway.

In earlier work we demonstrated that both brown algae[5] and diatoms[2] exploit the pool of unsaturated C_{20} fatty acids for the biosynthesis of C_8 and C_{11} hydrocarbons. Cell free extracts of the diatom *G. parvulum* were shown to oxidatively cleave deuterium labelled arachidonic acid into two isotopically labelled fragments, namely **6** and 9-oxononadienoic acid **9**. Performing the transformation in the presence of $^{18}O_2$ resulted in incorporation of one ^{18}O in the C(9)-aldehyde group. This suggests an initial functionalisation of the fatty acid to



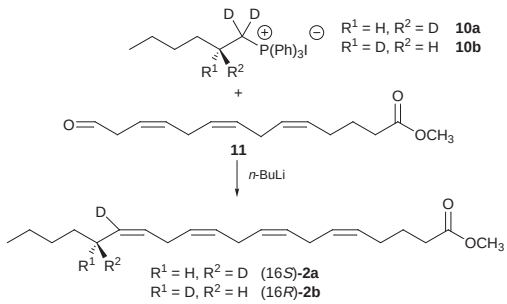
Scheme 1 Biosynthesis of C_9 and C_{11} hydrocarbons in the diatom *G. parvulum*.

9-hydroperoxyicosatetraenoic acid (9-HPETE) **4** followed by oxidative cleavage of the reactive intermediate into the bifunctional **9** and the olefin **6**. By analogy, the more highly unsaturated icosapentaenoic acid **1** could serve as the precursor for **5** (Scheme 1)

In principle, the configuration and ee of cyclopropanes **5** and **6** could be controlled either by the ee of the 9-OOH group directing all subsequent steps, by selective abstraction of one enantiotopic hydrogen atom at the methylene group of C(16), or by the folding of the fatty acids within the enzyme active site. To evaluate the influence of the two different stereogenic centres on the reaction course, we approached the absolute configuration and ee of the postulated **4** intermediate by a trapping experiment yielding 9-hydroxyicosatetraenoic acid (9-HETE). The hydrogen atom eliminated from C(16) was identified by using chiral, deuterium labelled arachidonic acid precursors and MS analysis of the metabolites after GLC separation on chiral stationary phases.

G. parvulum produces icosapentaenoic acid **1** derived **5** as the major volatile product (56% ee)[2] and small amounts of the arachidonic acid metabolites, **6** (24% ee) and cystophorene **8**. Addition of (labelled) arachidonic acid to a cell free extract of *G. parvulum* resulted in an 800-fold increase in the amount of **6** and **8** and the extracted metabolites could be analysed by MS without significant interference from a background of unlabelled, natural products. When the experiment was carried out in the presence of excess glutathione and glutathione peroxidase,† a system known to reduce hydroperoxy fatty acids rapidly and universally,[6] 9-HETE could be isolated together with small amounts of various autooxidation products. In the absence of glutathione and glutathione peroxidase no free **4** or 9-HETE could be detected. Independent experiments with synthetic 9-HETE excuded this compound as biosynthetic intermediate of **6** and confirmed the involvement of **4** *en route* to **6, 8** and **9** (Scheme 1). Following RP-HPLC purification and esterification of 9-HETE with *N*-(dimethylaminopropyl)-*N*′-ethylcarbodiimide–hydrochloride in aqueous MeOH,[7] the ee of the resulting ester was determined by HPLC on cyclodextrin as the chiral stationary phase.‡ On comparison with authentic compounds (SIGMA, Deisenhofen, Germany), the configuration of the hydroxy acid was established as (9*S*)-HETE (71% ee), reflecting the configuration and the ee of the unstable precursor **4**. The ee of (9*S*)-HPETE formed by the intact system should be even higher, since significant autooxidation of **2** occurred during the experiment with the crude enzyme preparation. Comparison of this value with the very low ee of the product (1*S*,2*R*)-**6** (24% ee, GLC on γ-cyclodextrin), clearly demonstrated that the HOO group of **4** at C(9) is not controlling the product stereochemistry.

To identify which hydrogen atom at C(16) is lost during olefin formation, the enzyme preparation was incubated with deuterium labelled (16*S*)-[15,16-$^2H_2$]-**2a** or (16*R*)-[15,16-$^2H_2$]-**2b**. The chiral precursors were synthesised from chiral epoxyheptanol-derived Wittig reagents of type **10** and 14-oxotetradecatrienoic acid methyl ester[8,9] **11** in 96 and 95% ee respectively[10] (Scheme 2).



Scheme 2 Synthesis of deuterated (16*S*)-[15,16-$^2H_2$]-**2a** and (16*R*)-[15,16-$^2H_2$]-**2b**.

The olefinic metabolites of **2a**,**b** were extracted by solid-phase microextraction (SPME)[2] and analysed by GC-MS, using a chiral stationary phase for separation of the enantiomers (*vide supra*). Administration of (16*S*)-[15,16-$^2H_2$]-arachidonic acid **2a** yielded [1′,2′-$^2H_2$]dictyopterene A **6** with a molecular ion at 152 Da, confirming the presence of two deuterium atoms in each of the two enantiomers (Fig. 1A). Incubation of (16*R*)-[15,16-$^2H_2$]-arachidonic acid **2b** afforded labelled **6** showing, for both enantiomers, the same pattern of molecular ions at *m/z* 152 (45%) and 151 (55%). Thus, due to a kinetic isotope effect (KIE), the expected C(16)-H_R was not exclusively attacked, but also the C(16)-H_S generating a mixture of [1′,2′-$^2H_2$]-**6** and [1′-^{2}H]-**6** from (16*R*)-**2b**. In addition, larger amounts of [7,7-$^2H_2$]-**8**, the product of a hydrogen atom loss from C(13), were produced (Fig. 1B). Both dideuterated compounds apparently resulted from reaction paths which avoided the abstraction of the kinetically unfavoured C(16)-^2H_R of **2b**.

The isotope effect was quantified by incubating the crude enzyme preparation with excess of an equimolar mixture of **2** and [16,16-$^2H_2$]-**2**. Product analysis by GC-MS revealed that the unlabeled precursor was the preferred substrate for the formation of **6** (**6**:[2′^{2}H]-**6** = 4.5:1) and confirmed loss of the hydrogen atom as rate limiting. Moreover, in the same experiment labelled **8** was produced in excess ([7,7-$^2H_2$]-**8**:**8** = 6.5:1) indicating that a single enzyme is responsible for the production of **8** and **6**. If a second lyase, removing hydrogen atoms from C(13) of the precursor had been present in the crude enzyme preparation, a ratio of [$^2H_2$]-**8**:**8** = 1:1 should have resulted due to the absence of a KIE for this position. Thus, the dynamic product channelling, following the administration of

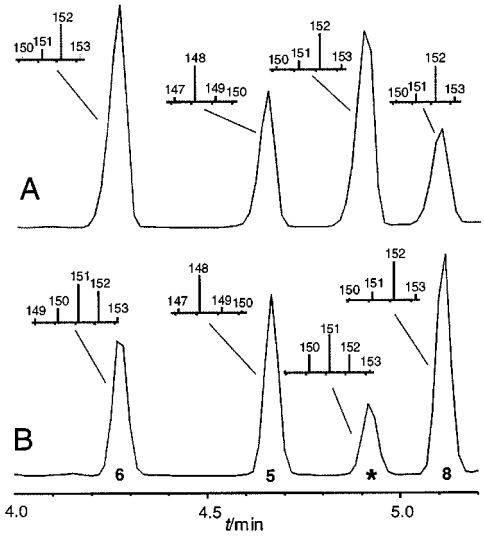
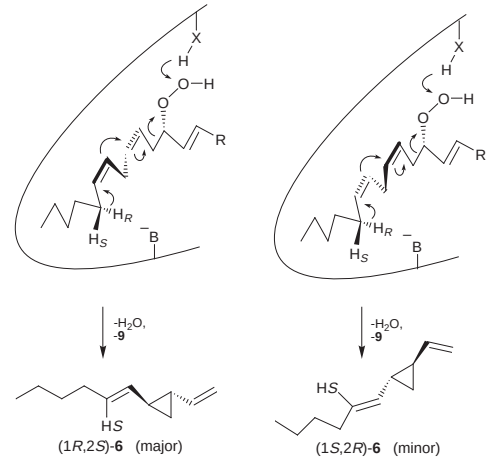


Fig. 1 GC-profile of labelled and unlabelled C_{11} hydrocarbons in *G. parvulum* with inserts of their mass spectra showing the region of the molecular ion. (A) after treatment with (16*S*)-[15,16-$^2H_2$]-**2a**. (B) after treatment with (16*R*)-[15,16-$^2H_2$]-**2b**. The large amount of 6-butylcyclohepta-1,4-diene (*) (dictyotene) results from a thermal [3.3]-sigmatropic rearrangement of **6** in the injection port of the GC and does not reflect the actual concentration of dictyotene in the hydrocarbon mixture.



Scheme 3 Proposed folding alternatives of intermediate **4** at the active centre of the *G. parvulum* hydroperoxide lyase leading to enantiomers of **6**. Whether or not the transformation proceeds *via* ionic or radical intermedates remains to be established.

labelled and unlabelled precursors, is highly indicative of a single hydroperoxide lyase exerting only a limited control on the orientation of the substrate at the active site, generating either predominately **6** or **8**, depending on the ease of hydrogen abstraction. Accordingly, the rather low ee of **6** could be also attributed to the limited control over the folding of **4** in the enzyme active site, as illustrated in Scheme 3.

Although the model of limited control over the substrate orientation at the active centre and during the catalytic process, is in accord with the experimental findings, the involvement of two enzymes, each responsible for the (predominant) production of a distinct enantiomer, cannot be ignored. Further work with isolated enzymes is required to provide a conclusive answer to the question on the origin of enantiomeric mixtures of C_{11} hydrocarbons in diatoms and marine brown algae.

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Notes and References

† *Ca.* 10^7 cells of *G. parvulum* in phosphate buffer (5 ml, 0.01 M, pH 7) were sonicated and the cell debris was removed by centrifugation. The supernatant was treated with glutathione (0.1 g, reduced form) and glutathione peroxidase (10 units) prior to addition of **2** (30 μl, 10% in EtOH). After 3 h 9-HETE was detected by RP-HPLC (Econosphere, C18, MeOH–H_2O–HAc 75:25:0.25, UV 235 nm for monitoring).

‡ The enantiomers were separated on a Chiralcel OB column[11] (Daicel Chem. Ind., hexane–propan-2-ol 99:1, 1 ml min^{-1}, baseline separation, UV 235 for monitoring).

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