New Biosynthetic pathways to Highly Branched Isoprenoid (HBI) alkenes in diatoms

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Introduction

C_{25} and C_{30} highly branched isoprenoid (HBI) alkenes are unusual secondary metabolites that are derived from diatoms. Volkman and co-workers were the first to determine biological sources of these isoprenoids, namely the marine diatoms *Haslea ostrearia* (C_{25}) and *Rhizosolenia setigera* (C_{30}) (Volkman *et al*., 1994). Since this initial report, we have reported on a further species of diatom capable of biosynthesising the C_{25} HBIs (viz. *Pleurosigma intermedium*) and elucidated the structures of numerous C_{25} and C_{30} HBIs (including the most widespread and abundant sedimentary isomers) following isolation from large scale diatom cultures and analysis by NMR spectroscopy (Belt *et al*., 2000ab ; Belt *et al*., 2001). Some representative structures of C_{25} (haslenes) and C_{30} (rhizenes) HBIs are shown in Fig. 1.

Whilst investigations into the biosynthesis of isoprenoids have seen a dramatic resurgence over the past few years, following the discovery of the so-called non-mevalonate pathway (Schwender *et al*., 1996 ; Rohmer, 1999), only one study of isoprenoid biosynthesis in diatoms has been made (Cvejic and Rohmer, 2000).

Here, we describe an investigation into the biosynthesis of phytol (C_{20}), and both C_{25} and C_{30} HBI alkenes by two different diatom species : *Rhizosolenia setigera*, a large ubiquitous planktonic diatom and *Haslea ostrearia*, a benthic pennate diatom (Fig. 2) using a combined approach integrating pathway specific blocking experiments and stable isotope incorporation (NMR and MS analysis). Our observations provide evidence for the co-occurrence of both mevalonate (MVA) and non-mevalonate (MEP) routes and reveal a dependence of the utilised pathways on the species under investigation.

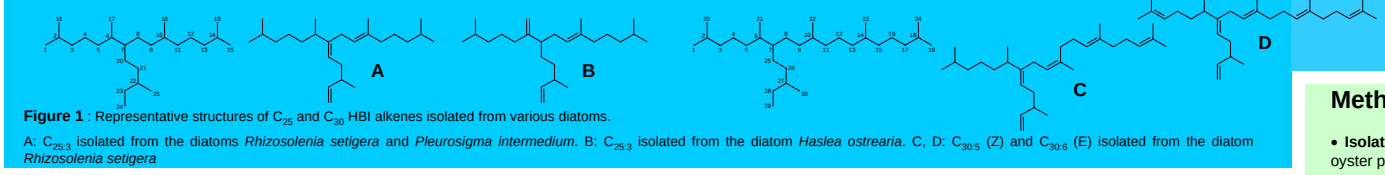


Figure 1 : Representative structures of C_{25} and C_{30} HBI alkenes isolated from various diatoms.

A: $C_{25:3}$ isolated from the diatoms *Rhizosolenia setigera* and *Pleurosigma intermedium*. B: $C_{25:3}$ isolated from the diatom *Haslea ostrearia*. C, D: $C_{30:5}$ (Z) and $C_{30:6}$ (E) isolated from the diatom *Rhizosolenia setigera*

Methods

- **Isolation :** *Rhizosolenia setigera* was isolated from Etel, France while *Haslea ostrearia* was isolated from oyster ponds in the bay of Bourgneuf, France
- **Identification :** Both species were identified *via* light and scanning electron microscopy techniques (Figure 2).
- **Culture :** Cells were grown on F/2 Guillard medium under standard controlled conditions (14°C, 14/10 Light/Dark cycle) in the presence of various labelled or unlabelled substrates (e.g. [1-^{13}C] acetate, [2-^{13}C] acetate, [$^2H_3$] acetate, $^{13}CO_2$) or in the presence of pathway specific inhibitors (mevinolin, fosmidomycin) (Figure 3).
- **HBI Analysis :** Hexane extracts of the filtered diatoms were analysed by GC-MS. Identification of individual HBIs was achieved by comparison of retention indices and mass spectra with authentic standards. Each individual HBI was purified using preparative Ag^+-HPLC techniques, Phytol was purified using open column chromatography. ^{13}C incorporation was then monitored using NMR spectroscopy.

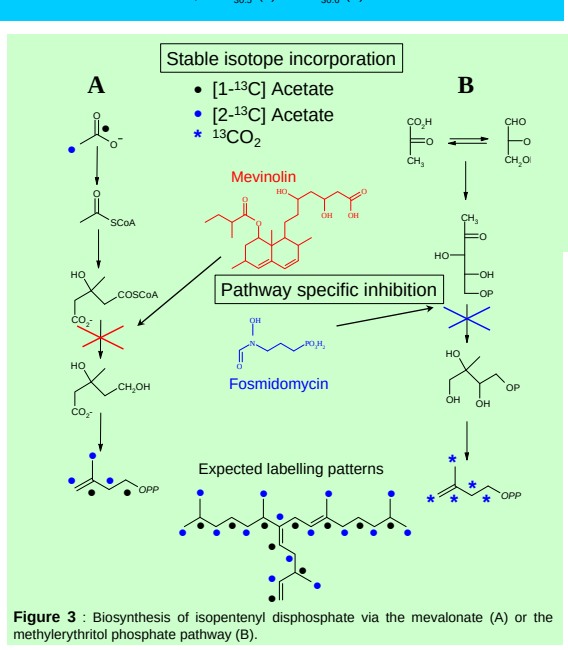


Figure 3 : Biosynthesis of isopentenyl disphosphate via the mevalonate (A) or the methylerythritol phosphate pathway (B).

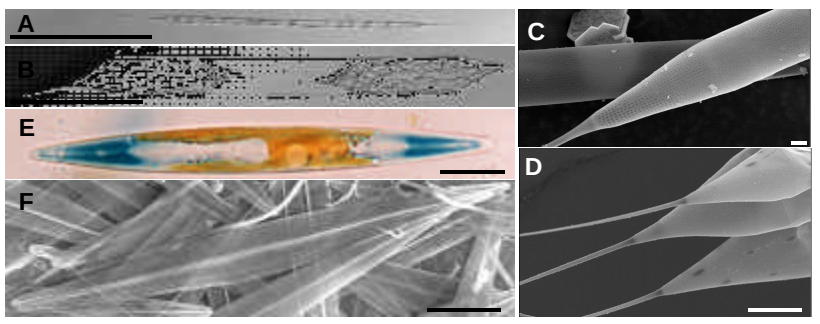


Figure 2 : A-D Photographs of the diatom *Rhizosolenia setigera* obtained using light and scanning electron microscopy. E & F Photographs of the diatom *Haslea ostrearia* obtained using light and scanning electron microscopy. Scale bars = figs. A & B: 100 µm; fig. C : 1µm; fig. D-F: 10µm.

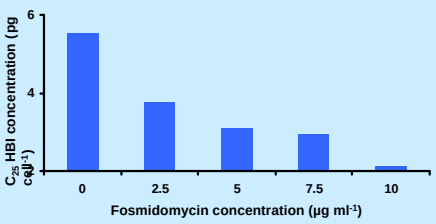
Results

- **Pathway specific inhibition experiment:**

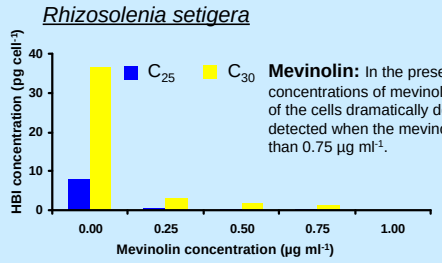
Haslea ostrearia

Mevinolin: In the presence of increasing concentrations of mevinolin, the C_{25} content of the cells increased by *ca*. x2 compared with their initial concentration.

Fosmidomycin: In the presence of increasing concentrations of fosmidomycin, the C_{25} content of the cells progressively decreased.

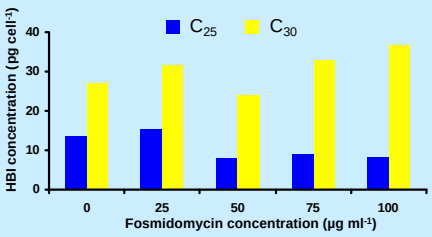


Rhizosolenia setigera



Mevinolin: In the presence of increasing concentrations of mevinolin, the C_{25} and C_{30} HBI content of the cells dramatically decreased. HBIs were not detected when the mevinolin concentration was higher than 0.75 µg ml^{-1}.

Fosmidomycin: In the presence of increasing concentrations of fosmidomycin, the C_{25} content of the cells progressively decreased whereas the C_{30} content gradually increased.



- **Stable isotope incorporation experiments:**

Haslea ostrearia

- *Significant incorporation of ^{13}C in both phytol and C_{25} HBIs when grown in the presence of $^{13}CO_2$ (20% isotopic abundance)*
- *Low (and random) incorporation of ^{13}C in both phytol and C_{25} HBIs when the diatom is grown in the presence of [^{13}C-1]acetate (20% isotopic abundance)*
- *No incorporation of any ^2H in both phytol and C_{25} HBIs when the diatom is grown in the presence of [$^2H_3$]acetate (20% isotopic abundance)*

Rhizosolenia setigera

- *Large incorporation of ^{13}C in both phytol and C_{25} – C_{30} HBIs when grown in the presence of $^{13}CO_2$ (20% isotopic abundance)*
- *No incorporation of ^{13}C into phytol when grown in the presence of [^{13}C-1]acetate or or [^{13}C-2]acetate (20-100% isotopic abundance)*
- *Increasing incorporation of ^{13}C in both C_{25} and C_{30} HBIs when grown in the presence of increasing concentrations of [^{13}C-1]acetate or [^{13}C-2]acetate (20-100% isotopic abundance)*
- *Incorporation of ^2H in both C_{25} and C_{30} HBIs when grown in the presence of [$^2H_3$]acetate (20% isotopic abundance)*

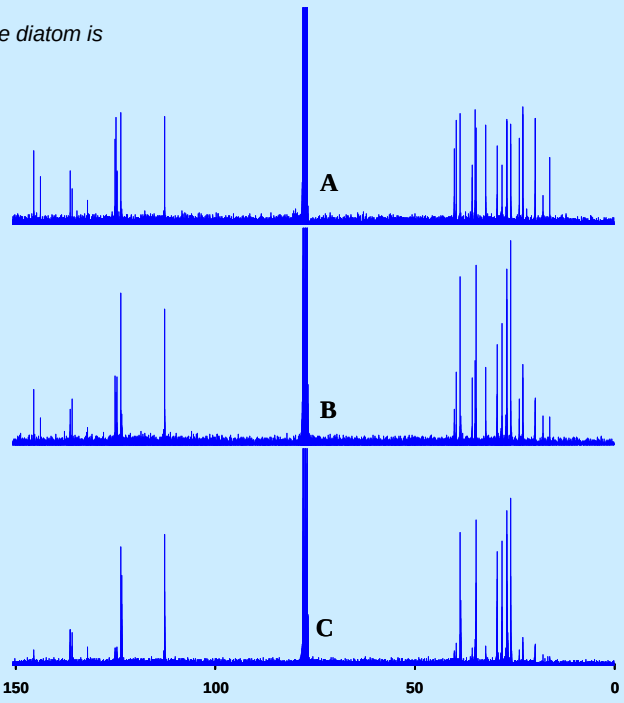


Figure 4 : ^{13}C NMR spectra of $C_{30:5}$ (Z) isolated from *R. setigera* cells cultured in the presence of A: unlabelled acetate. B: [1-^{13}C] acetate (250 mg l^{-1}, 20% isotopic abundance). C: [1-^{13}C] acetate (250 mg l^{-1}, 100% isotopic abundance).

Conclusion

We have demonstrated that the biosynthesis of Phytol or C_{25} and C_{30} HBI alkenes is strongly dependent on the studied diatom.

The major observations can be summarised as follows:

- **Both diatoms use exclusively the non-mevalonate pathway to synthesise phytol.**
- **C_{25} HBIs (haslenes) from *Haslea ostrearia* are biosynthesised exclusively using the non-mevalonate (MEP) pathway.**
- **C_{25} and C_{30} HBIs from the diatom *Rhizosolenia setigera* are biosynthesised mainly using the mevalonate pathway, though there would also appear to a contribution from the MEP route.**
- **The relative contributions of the mevalonate and MEP pathways towards HBI biosynthesis in *Rhizosolenia setigera* appears to be *dynamic*, dependent on the culture and/or physiological conditions.**

Other considerations

Using GC-irm-MS techniques, Jux *et al*. (2001) have elegantly shown that the simultaneous operation of the two pathways within a single organism can be demonstrated using stable isotope fractionation methods. Significantly, this methodology can be used to provide evidences for intracellular interchanges of the two pathways and allows for biosynthetic investigations to be carried out under 'natural conditions'. Recently, we have demonstrated that C_{25} and C_{30} distributions in *Rhizosolenia setigera* depend on the life cycle of the species. Further, in a preliminary investigation on this species, we have observed that the isotopic fractionation of carbon ($\delta^{13}C$) within individual isoprenoids can undergo substantial change within the life cycle. Since Jux and co-workers have correlated different biosynthetic pathways with the degree of isotopic fractionation, the hypothesis to be tested is concerned with whether diatoms are able to modify their biosynthetic pathways concomitant with their evolution.

Acknowledgments

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