The effects of auxosporulation on the distributions of C_{25} and C_{30} highly branched isoprenoid (HBI) alkenes in the diatom *Rhizosolenia setigera*

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Introduction

C_{25} and C_{30} highly branched isoprenoid (HBI) alkenes are unusual secondary metabolites that are derived from diatoms and commonly used as biological markers in sediments and other geochemical environments (Robson & Rowland, 1986; Rowland & Robson, 1990). 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 isomers) following isolation from large scale diatom cultures and analysis by NMR spectroscopy. Some representative structures of C_{25} (haslenes) and C_{30} (rhizenes) HBIs are shown in Fig. 1. However, although an account of the C_{25} HBIs produced by *Haslea species* and *P. intermedium* would appear to be well defined, the situation with *R. setigera* is less clear. The following observations have been reported :

- **Volkman *et al.* (1994) : *R. setigera* contained three C_{30} pentaenes ($C_{30:5}$) and two C_{30} hexaenes ($C_{30:6}$) with no detection of any C_{25} alkenes.**
- **Sinninghe Damsté *et al.* (1999) : C_{25} pentaene (previously reported in *Haslea* spp.) in addition to two novel *n*-alkenes, with no C_{30} homologues.**
- **Rowland *et al.* (2001) :**
  - ***R. setigera* strain CS 389/A contained three $C_{30:5}$, three $C_{30:6}$ and two C_{25} trienes previously reported in *Pleurosigma intermedium*).**
  - ***R. setigera* strains CCMP 1330 and 1820 produced same C_{25} HBI and n-alkenes previously reported by Sinninghe Damsté *et al.***
  - ***R setigera* strains Nantes 1999 and 2000 contained two C_{30} pentaenes ($C_{30:5}$) and two C_{30} hexaenes ($C_{30:6}$) with C_{25} alkenes (Two trienes, a tetraene and traces of a pentaene)**
- **Belt *et al.* (unpublished) : -Sample 1: two C_{30} pentaenes ($C_{30:5}$) and two C_{30} hexaenes ($C_{30:6}$) with no detection of any C_{25} alkenes**
  - **-Sample 2 : detection of both C_{25} and C_{30} HBI alkenes**
  - **-Sample 1 and 2 were obtained from the *same* strain (RS Nantes 1999).**

Here, we describe an investigation into the distribution of C_{25} and C_{30} HBI alkenes biosynthesised by *R. setigera* as a function of the position of the cells through their life cycle. Our observations reveal a relationship between cell size and HBI content including a dramatic change in the distribution of alkenes during the regeneration of their original size through a sexual cycle (auxosporulation).

Methods

- **Isolation :** *R. setigera* was isolated from Etel, France
- **Identification :** *R. setigera* was 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) for a number of consecutive cycles (Figure 3).
- **Cell counts and volume determination :** cell counting and detection of auxospores was performed via light microscopy and cell volumes determined using a Coulter-counter.
- **HBI Analysis :** Hexane extracts of the filtered diatoms analysed by GC-MS. Identification of individual HBIs was achieved by comparison of retention indices and mass spectra with authentic standards.

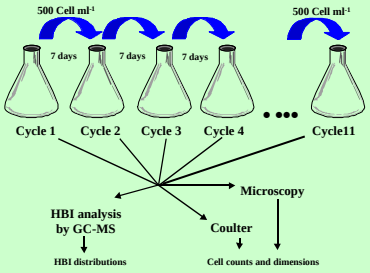

Figure 3 : General experimental protocol

Results

How is the position of the diatom in its life cycle estimated?

The position of a diatom within its life cycle can be estimated by analysis of its cell dimensions : Diatoms undergo a progressive reduction in size as a result of cell division until a critical point is reached. At this point, sexual reproduction (auxosporulation) is induced, with subsequent regeneration of cells with their initial (large) size.

- **Pre-auxosporulation (Cycles 1-2)**
  - 100% mother cells (Figure 2 A, B).
  - Cell volumes were small and constant (125 µm³)
- **Auxosporulation (Cycles 3-4)**
  - Auxospores detected in Cycles 3 and 4 (Figure 2 C).
  - The proportion of Daughter (Figure 2 D, E) cells increased from 14% (Cycle 3) to 61% (Cycle 4).
  - The average cell volume increased dramatically due to the higher proportion of Daughter cells : 373 µm³ (Cycle 3) and 1379 µm³ (Cycle 4).
- **Post-auxosporulation (Cycle 5-11)**
  - The proportion of Daughter cells increased from 93% (Cycle 5) to 100% (Cycle 6-11)
  - The cell volumes were large and almost constant from Cycle 6 to Cycle 11 (mean = 2290 µm³)

Cell volumes increased approximately 20 fold as a result of auxosporulation.

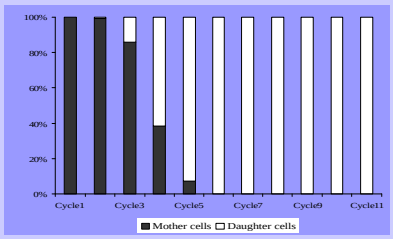

Figure 4 : Relative populations of mother and daughter cells during 11 consecutive growth cycles

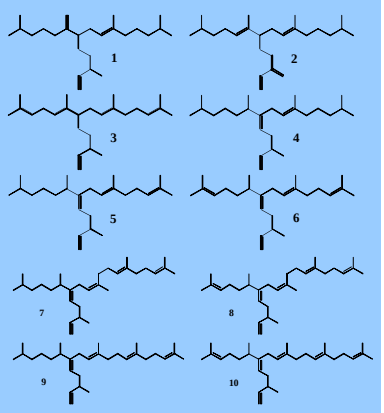

Figure 1 : Representative structures of C_{25} and C_{30} HBI alkenes isolated from various diatoms

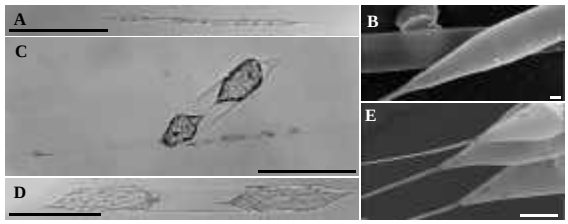

Figure 2 : Photographs of the diatom *Rhizosolenia setigera* obtained using scanning electron microscopy and light microscopy : A & B : mother cells close to the onset of auxosporulation. C : Two auxosporulating mother cells (long, thin) with the new daughter cells in formation. D & E. Two daughter cells. Scale bars = figs. A, C, D : 100 µm; fig. B : 1µm, fig. E : 10µm

Conclusion

We have demonstrated that distributions of C_{25} and C_{30} HBI alkenes biosynthesised by *R. setigera* are strongly dependent on the position of the diatom in its life cycle, with the most significant changes taking place as a result of auxosporulation.

The major observations can be summarised as follows:

- **C_{30} HBIs (rhizenes) are biosynthesised with five or six degrees of unsaturation as mixtures of E and Z isomers.**
- **Total rhizene concentrations (measured on a unit cell volume basis) remain constant during different stages of the life cycle, but the degree of unsaturation ($C_{30:5}$ / $C_{30:6}$) is highly variable especially during the period of auxosporulation (sexual reproduction).**
- **C_{25} HBIs (haslenes) are biosynthesised with between three and five degrees of unsaturation.**
- **Unlike rhizenes, haslenes are not observed during *all* phases of the life cycle. Haslene production appears to be mainly stimulated by the onset of auxosporulation, although their concentrations and unsaturation continue to increase after this phase.**
- **We have found no clear correlations between haslenes and rhizenes in terms of unsaturation, double bond stereochemistry, etc.**

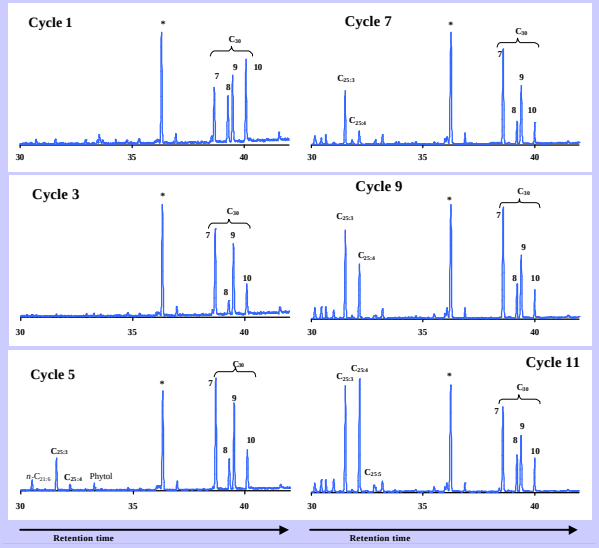

Figure 5 : Partial TIC chromatograms corresponding to alternate growth cycles of *Rhizosolenia setigera*

HBI analysis (figure 5 –6)

- **Pre-auxosporulation (Cycles 1-2)**
  - Detection of four C_{30} HBIs (geometric (E/Z) isomers of $C_{30:5}$ and $C_{30:6}$)
  - $C_{30:6}$ are slightly more abundant than the $C_{30:5}$ isomers ($C_{30:5}/C_{30:6}$ = 0.89)
  - C_{25} HBIs are absent
- **Auxosporulation (Cycles 3-4)**
  - Presence of the same four C_{30} HBIs as cycles 1-2
  - C_{30} pentaenes are significantly more abundant than C_{30} hexaenes ($C_{30:5}/C_{30:6}$ = 4)
  - $C_{25:3}$ **(4)** detected in cycle 4 for the first time
- **Post-auxosporulation (Cycle 5-11)**
  - Detection of four C_{30} HBIs in all cycles
  - $C_{30:5}/C_{30:6}$ ratio slightly decreases with consecutive cycles (Fig. 6).
  - $C_{25:4}$ **(5)** detected from cycle 5.
  - $C_{25:3}$ and $C_{25:4}$ concentration (per cell) continues to increase with cycle number.
  - $C_{25:5}$ **(6)** detected in cycle 11

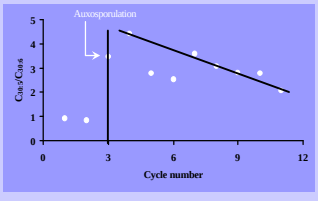

Figure 6 : Variation of $C_{30:5}$ / $C_{30:6}$ ratio during the life cycle

Other considerations

While these results can be used in part to explain the large variations in C_{25} and C_{30} HBI distributions observed in other cultures of *R. setigera*, it is not clear at this stage if similar or related effects apply to other lipid classes. Relationships between carbon content or chlorophyll *a* levels as functions of cell dimensions (including volume) have been reported (Mullin *et al.* 1966; Durbin, 1977), though to our knowledge, such studies have not been carried out on individual lipids. However, it has been reported recently that cell size (amongst other factors) in phytoplankton can contribute to carbon isotope fractionation and therefore cell dimensions and growth rates should be considered if stable carbon isotope compositions of organic material are to be used in determining e.g. paleo-[CO_2(aq)] levels (Popp *et al.*, 1998). It has also been shown that variations in carbon and hydrogen isotopes of individual compounds within the same and different organisms can occur, and this may result from different and competing biosynthetic pathways, isotopic enrichment in biosynthetic precursors or substrates involved in biosynthesis (e.g. NADPH) (Rowland *et al.*, 2001; Session *et al.*, 1999). Clearly then, changes in distributions of individual lipids may cause variations in measured isotopic fractionation. The fact that changes in distributions may also be associated with cell dimensions as shown here suggests that the measurement of isotopic fractionation of individual lipids as a function of cell size or dimensio

Acknowledgments

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References

Robson, J. N. & Rowland, S. J. (1986) Identification of novel widely distributed sedimentary acyclic sesterterpenoids. *Nature* **324**, 561-563

Rowland, S. J. & Robson, J. N. (1990) The widespread occurrence of highly branched acyclic C_{29}, C_{25} and C_{30} hydrocarbons in recent sediments and biota - a review. M*ar. Environ. Res.* **30**, 191-216.

Volkman, J. K., Barratt, S. M. & Dunstan, G. A. (1994) C_{25} and C_{30} highly branched isoprenoid alkenes in laboratory cultures of two marine diatoms. *Org. Geochem.* **21**, 407-414.

Sinninghe Damsté, J. S., Rijpstra, W. I. C., Schouten, S., Peletier, H., van der Maarel, M. J. E. C. & Gieskes, W. W. C. (1999) A C_{25} highly branched isoprenoid alkene and C_{25} and C_{27} *n*-polyenes in the marine diatom *Rhizosolenia setigera*. *Org. Geochem.* **30**, 95-100.

Rowland, S. J., Allard, W. G., Belt, S. T., Massé, G., Robert, J. –M., Blackburn, S, Frampton, D., Revill, A. T. & Volkman, J. K. (2001) Factors effecting the distributions of polyunsaturated terpenoids in the diatom, *Rhizosolenia setigera*. *Phytochemistry* in press.

Mullin, M. M., Sloan, P. R. & Eppley. (1966) Relationship between carbon content, cell volume, and area in phytoplankton. *Limnol. Oceanogr.* **11**, 307-311.

Durbin, E. G. (1977) Studies on the autecology of the marine diatom *Thalassiosira nordenskioeldii*. II. The influence of cell size on growth rate, and carbon, nitrogen, chlorophyll *a* and silica content. *J. Phycol.* **13**, 150-155.

Popp, B. N., Laws, E. A., Bidigare, R. R., Dore, J. E., Hanson, K. L. & Wakeham, S. G. (1998) Effect of phytoplankton cell geometry on carbon isotopic fractionation. *Geochim. Cosmochim. Acta* **62**, 69-77.

Sessions, A. L., Burgoyne, T. W., Schimmelmann, A. & Hayes, J. M. (1999) Fractionation of hydrogen isotopes in lipid biosynthesis. *Org. Geochem.* **30**, 1193-1200.