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Highly branched C_{25} isoprenoids in axenic cultures of *Haslea ostrearia*

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Abstract

The hydrocarbon compositions of axenic cultures of the diatom *Haslea ostrearia* grown in the presence of penicillin, streptomycin and kanamycin were examined at lag, exponential and stationary growth phases. The production of highly branched isoprenoid (HBI) C_{25} trienes to pentaenes with the 2,6,10,14-tetramethyl-7-(3-methylpentyl)pentadecane carbon skeleton was demonstrated at all three phases (2300–7000 fg cell^{-1}). Of the ten HBI trienes to hexaenes reported previously from non-axenic cultures of *H. ostrearia*, four were present in the axenic samples. In addition, two novel trienes and a pentaene were found. The most abundant of the new trienes was isolated from a larger, non-axenic batch culture and identified from ^{13}C- and ^{1}H-NMR data as 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadec-5,9-diene. Interesting differences in HBI isomer distributions were observed among the three growth phases. For example, the newly identified, non-methylenic triene above, only occurred in the exponential growth phase. As a primary producer of these alkenes, several of which have demonstrated cytostatic activity, *Haslea ostrearia*, and perhaps related *Haslea* species, is clearly worthy of further study.

Keywords: *Haslea ostrearia*; Bacillariophyceae; Microalgae; Diatoms; Isoprenoid alkenes; Highly branched isoprenoids; C_{25} alkenes; Axenic cultures

1. Introduction

Recently, a family of highly branched isoprenoid (HBI) C_{25} polyenes was reported in a non-axenic culture of the diatom, *Haslea ostrearia* (Gaillon) Simonsen (Volkman, Barrett, & Dunstan, 1994). Hence a primary source was suggested for some of the HBI hydrocarbons which have been found in contemporary marine environments worldwide (reviewed in Robson & Rowland, 1986; Rowland & Robson, 1990), but for which no source was known previously. The compounds are also important in a number of other respects. The highly branched structures have led to the suggestion that the phosphate esters of such compounds may have been important in the formation of vesicles during the early evolution of life on Earth (Ourisson & Nakatani, 1994) and it has recently been demonstrated that polyunsaturated HBI compounds can be produced in the presence of unactivated clay by abiotic dimerisation of geraniol and farnesol (Nagano & Nakanishi, 1998).

Isolation of individual HBI alkenes from non-axenic, large scale batch cultures of *H. ostrearia* and characterisation by NMR and mass spectral methods has confirmed the highly branched structures and revealed the positions and stereochemistry of the double bonds (Belt, Cooke, Robert, & Rowland, 1996). Cytostatic tests on some of these pure alkenes have shown that some are active in slowing the growth of lung cancer cell lines in vitro (Patent GB-9708934.6).

Clearly, given the non-axenic nature of the algal cultures studied to date and the possibility of HBI formation in sediments by abiotic processes, it is important that the role of *H. ostrearia* as a primary producer be confirmed, particularly if the bioactivity of the compounds is to be investigated further (cf. Gerwick & Roberts, 1994; Borowitzka, 1995). This makes the demonstration of HBI biosynthesis from an axenic algal culture imperative. In the present study we report the production of HBI trienes to pentaenes in the three principal growth stages of *H. ostrearia* grown axenically in the presence of three antibiotics. These growth phases (lag, exponential, station-

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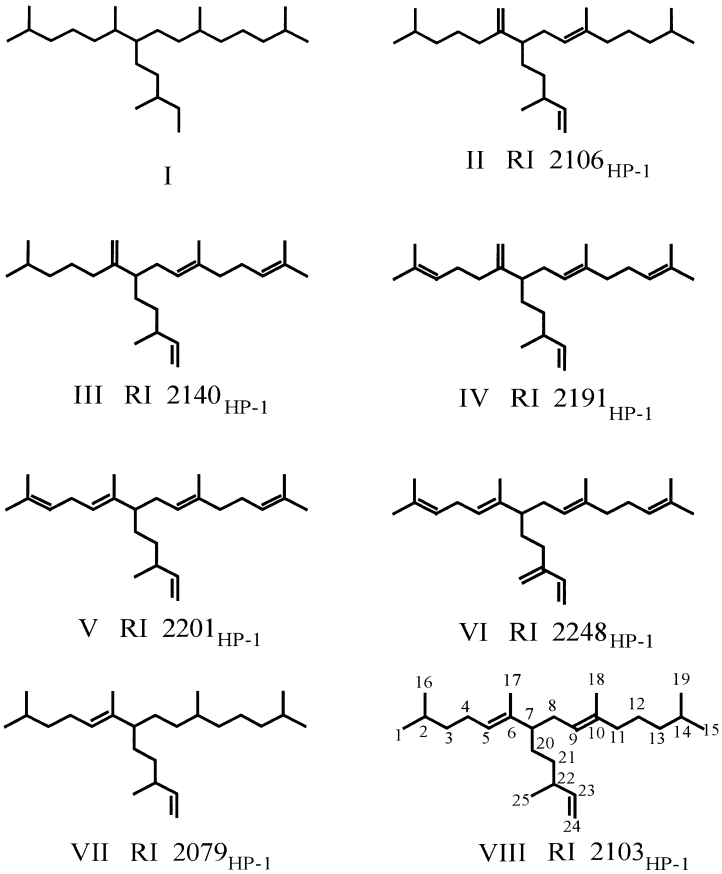
ary) have been shown by previous studies (Robert, 1983, 1984) to be typified by, yellow colouration (lag phase), the production of chlorophyll (green, exponential phase) and the blue pigment marennine (blue, stationary phase) and the algae for our experiments were harvested at each of these stages. The range of alkenes reported includes four of those identified in non-axenic cultures plus three not found in previous studies. The importance of *H. ostrearia* as a producer of HBI alkenes is thus confirmed.

2. Results

The total hexane extract of each of the three axenic cultures contained a series of eight alkenes including the non-HBI, *n*-henicosahexaene (*n*-$C_{21:6}$) and seven C_{25} HBIs with three to five double bonds. The latter were shown to possess the 2,6,10,14-tetramethyl-7-(3-methylpentyl) pentadecane (I) carbon skeleton by comparison of the GC retention indices on two stationary phases and mass spectra with those of HBIs authenticated in our earlier studies by NMR spectroscopy and derivatisation techniques (e.g. Belt et al., 1996). Only three of these HBIs could be identified unambiguously (II, III, VIII); triene II and tetraene III were assigned by comparison with our previous studies (Belt et al., 1996) and triene VIII by isolation of sufficient pure material from a large-scale, non-axenic culture, for characterisation by ^{13}C- and ^{1}H-NMR spectroscopy. The other alkenes were identified by GC–MS only. The salient mass spectral (GC–MS) features of the new alkenes are given in Table 1. The compositions and concentrations of the alkenes were measured in each of the growth stages (Table 2).

3. Discussion

H. ostrearia is a large, pennate diatom which has proven difficult to classify, having been assigned to four different genera since 1820 (Simonsen, 1974). Routinely classified in the major genus *Navicula* until 1984 (Robert, 1984), it is most easily identified by the production of an unidentified blue pigment during stages of nutrient-limited growth, i.e. during the stationary growth phase in batch culture (Robert, 1983). At other times it is yellow (lag phase) to green (exponential phase) and contains the characteristic carotenoids and chlorophylls of diatoms generally (Robert, 1983). It is a fairly common member of the marine epipelon and is also planktonic in warm waters (Round, Crawford, & Mann, 1990). The genus includes several other species, the hydrocarbons of which have not been reported, including sea-ice diatoms com-



Scheme 1.

Table 1
Mass spectral features and GC retention indices of previously unreported or incompletely reported HBI alkenes identified in axenic cultures of *H. ostrearia*

HBI alkene	RI_{HP-1}	RI_{DB-5}	*m/z* (rel. int) 70 eV
$C_{25:3}$	2117	2120	55 (59), 69 (100), 81 (51), 95 (31), 109 (57), 233 (11), 261 (14), 278 (4), 346 (6)
$C_{25:4}$	2159	2165	55 (100), 69 (89), 83 (58), 95 (54), 107 (28), 231 (22), 259 (49), 275 (9), 287 (4), 344 (10)
$C_{25:4}$	2177	2188	55 (100), 69 (78), 81 (61), 95 (47), 107 (33), 231 (20), 259 (32), 277 (14), 287 (6), 344 (7)
$C_{25:5}$	2175	2185	55 (28), 69 (100), 81 (43), 93 (24), 107 (23), 231 (6), 259/7 (7), 273 (16), 287 (6), 299 (13), 327 (4), 342 (4)

mon in the Arctic and Antarctic (*H. crucigeroides*, *H. kjellmanii*, *H. vitrea* in the Arctic and *H. trompei* in the Antarctic (von Stosch, 1985; Medlin & Priddle, 1990)) and tropical species such as *H. gigantea,* which are planktonic in warm waters of the Indian and Pacific oceans and especially of the South China sea and the Gulf of Carpentaria, Australia and the Gulf of Mexico (von Stosch, 1985). *H. ostrearia* has been reported in coastal sediments from France, Norway, UK, north Australia and from the Indian Ocean (e.g. Hustedt & Aleem, 1951; Simonsen, 1974; Neuville & Daste, 1978; Robert, 1986; Ricard, 1987). Thus the genus is probably an important source of the widespread C_{25} HBI alkenes in sediments (reviewed in Rowland & Robson, 1990).

Clearly, the identification (Tables 1–2) of seven HBI trienes through pentaenes in the three cultures of *H. ostrearia* grown in this study under axenic conditions, is strong support for their biosynthesis by the diatom.

To our knowledge, the only previously confirmed structures of C_{25} HBI hydrocarbons (from non-axenic *H. ostrearia*) are five trienes through hexaenes II–VI (Belt et al., 1996; Wraige et al., 1997). In addition, the GC–MS data only for another five tetraenes or pentaenes have been reported (Volkman et al., 1994). Two further dienes have been identified in sediments (e.g. VII; Yruela, Barbe, & Grimalt, 1990; Belt, Cooke, Hird, & Rowland, 1994) and GC–MS data for at least twenty monoenes through pentaenes have been obtained (reviewed in Cooke, 1995). Compared with these the axenic cultures in the present study produced known triene II and tetraene III. In addition, the trienes RI $2103_{HP-1, DB-5}$ and 2117_{HP-1} (2120_{DB-5}), two tetraenes (RI 2159, 2177_{HP-1}) and a pentaene (RI 2175_{HP-1}) were present (Table 1). We have now characterised the former triene (VIII). The trienes VIII (RI $2103_{HP-1, DB-5}$) and unknown RI 2120_{DB-5} have not been reported in earlier studies of *H. ostrearia* but both have been found in sediments or sedimenting particles judging from similar GC retention indices (RI 2104_{SE-30} (Requejo & Quinn, 1983, 1985); RI 2119_{DB-5} (Albaiges et al., 1984)). The degree of unsaturation in two of the remaining HBI alkenes noted in the axenic cultures was not assigned previously in non-axenic cultures due to weak mass spectra (Volkman et al., 1994). Judging from the similarities in retention index and mass spectra, the unknowns of Volkman et al. (1994), viz. RI 2158_{HP-1}, RI 2173_{HP-1}, are one of the tetraenes (RI 2159_{HP-1}) and the pentaene identified in our axenic cultures (RI 2175_{HP-1}). The remaining tetraene (RI 2177_{HP-1}) in our samples appears not to have been reported previously in either sediments or biota. None of the dienes reported in sediments (Yruela et al., 1990; Belt et al., 1994), nor the known pentaene and hexaene (V, VI), were present in the axenic cultures.

Whilst HBI production was demonstrated at each

Table 2
Concentrations of HBI alkenes in different growth phases of axenic *H. ostrearia* cultures

Alkene	RI_{HP-1}	RI_{DB-5}	Concentration (fg/cell), yellow phase[a]	Concentration (fg/cell), green phase[b]	Concentration (fg/cell), blue phase[c]	Structure
n-$C_{21:6}$	2044	–	200	200	1200	–
$C_{25:3}$	2103	2103	n.d.	600	n.d.	VIII
$C_{25:3}$	2106–9	2108	300	1700	4800	II
$C_{25:3}$	2117	2120	200	n.d.	n.d.	unknown
$C_{25:4}$	2140–2	2144–7	1260	200	600	III
$C_{25:4}$	2159	2165	n.d.	200	200	unknown
$C_{25:5}$	2175	2185	350	n.d.	200	unknown
$C_{25:4}$	2177	2188	n.d.	100	n.d.	unknown

[a] 42,000 cell/ml.
[b] 92,000 cell/ml.
[c] 102,000 cell/ml.

phase of axenic growth, maximum production occurred at the stationary phase (Table 2). HBI production in earlier non-axenic cultures, at somewhat higher temperatures maximised either in the exponential phase at about the same concentration of total HBIs or no clear maximum was observed and HBI production was lower (Wraige et al., 1997). The reasons for these differences will require further carefully controlled experiments at specific temperatures and other conditions. As with one of our previous experiments with non-axenic cultures (Wraige et al., 1997), HBI production also differed at the different stages of algal growth (Table 2). For example the triene VIII ($RI = 2103_{HP\text{-}1,DB\text{-}5}$), identified for the first time herein, was only present in the exponential phase of growth whereas the known methylenic triene II ($RI = 2106_{HP\text{-}1}$) was most abundant at stationary phase.

In addition to the effects of phenotypic variables such as temperature and salinity, the influence of bacteria might also be important in controlling the biochemistry of the alga. Thus, the relative proportions of the different HBIs of axenic cultures of *H. ostrearia* may be different from those of non-axenic cultures, as suggested by the present results.

Such parameters are also likely to be important if production of the bioactive HBI alkenes during culturing is to be optimised (cf. Gerwick & Roberts, 1994; Borowitzka, 1995). The latter will also probably require investigations of the biosynthesis of the HBI alkenes, about which nothing is presently known. The recent discovery of a pathway for algal isoprenoid biosynthesis via pyruvate/glyceraldehyde-3-phosphate (Schwender, Seemann, Lichtenthaler, & Rohmer, 1996) opens up the intriguing question as to whether these unusual and potentially useful compounds originate from an established or novel biosynthetic route.

4. Experimental

4.1. Algal cultures and axenic strains

Haslea ostrearia was isolated from oyster ponds of the Bay of Bourgneuf (France). The three strains were grown in 250 ml Erlenmeyer flasks at 16°C with illumination provided by cool-white fluorescent tubes in a 14/10 h light/dark cycle. They were incubated in a modified Provasoli medium (Robert, 1983) under 100 μmol photons $m^{-2}\,s^{-1}$.

Briefly, the original inoculum was subcultured three times (i.e. cells from the original inoculum were grown, subsampled, re-grown, subsampled and re-grown). The latter culture was then grown in the presence of penicillin, 5.05 μg ml^{-1}, streptomycin, 2.025 μg ml^{-1} and kanamycin, 2.025 μg ml^{-1}. The axenicity was tested in FAG and FG liquid media (Berland, Bonin, Cornu, Maestrini, & Marino, 1972). The culture was then re-grown in the absence of the antibiotics and tested for axenicity once more. When axenicity was found at this stage, the culture was re-grown for several generations in the absence of antibiotics. The algae were then sampled at the three growth phases represented by the yellow to green to blue colouration. In parallel to these harvested samples, a further test for axenicity was made and finally at the end of growth, a further test for axenicity was performed. In one of the cultures, the yellow phase was harvested after 6 days growth (40,000 cell ml^{-1}), whereas in a second culture, greening had occurred by this point (92,000 cell ml^{-1}). The blue stage required growth of a further culture for 13 days (102,000 cell ml^{-1}). Samples for hydrocarbon analysis were obtained by filtration or centrifugation (Wraige et al., 1997).

4.2. Hydrocarbon extraction and isolation

Immediately prior to extraction, 2,21-dimethyldocosane (0.5 μg in 50 μl hexane) was added to each filter. Filters were then extracted by ultrasonication in *n*-hexane (3 ml, 45 min, Kerry Pulsatron HB172) and the total hexane extract (THE) dried (anhydrous Na_2SO_4). Solvent was removed under a gentle stream of nitrogen and the THE examined by GC–MS.

Large amounts of alkene VIII were isolated from a non-axenic culture by extraction of centrifuged algal paste with hexane, aided by ultrasonication (Cooke, 1995; Belt et al., 1996), followed by CC on silica and elution with hexane.

4.3. NMR spectroscopy

NMR spectra were recorded in $CDCl_3$ using a JEOL EX 270 spectrometer. Chemical shifts (δ) are referenced to residual $CHCl_3$ (7.24 ppm) and $CDCl_3$ (77.0 ppm) for ^1H and ^{13}C, respectively.

4.3.1. 2,6,10,14-Tetramethyl-7-(3-methylpent-4-enyl) pentadec-5,9-diene (VIII)

4.3.1.1. ^1H *NMR (270 MHz).* δ 5.67 (ddd, $J = 7$, 10.5, 17.5 Hz, H-23), 5.06 (m, H-5, H-9), 4.88 (m, H-24), 1.83–2.06 (m, H-4, H-7, H-11, H-8, H-22), 1.54 (s, H-18), 1.43 (s, H-17), 1.37 (m, H-2, H-14), 1.05–1.3 (m, H-3, H-12, H-13, H-20, H-21), 0.93 (d, $J = 6.6$ Hz, H-25), 0.85 and 0.84 (d, $J = 6.6$ Hz, H-1, H-15, H-16, H-17).

4.3.1.2. ^{13}C *NMR (67.8 MHz).* δ 145.2 (C-23), 136.3 (C-6), 135.2 (C-10), 126.2 (C-5), 123.3 (C-9), 112.0 (C-24), 49.4 (C-7), 39.9 (C-11), 39.0 and 38.6 (C-3 and C-13), 37.8 (C-22), 34.5 (C-21), 32.2 (C-8), 30.3 (C-20), 27.9 and 27.5 (C-2 and C-14), 25.7 (C-12), 25.5 (C-4), 22.7 and 22.6 (C-1, C-15, C-16, C-19), 19.9 (C-25), 16.0 (C-18), 11.8 (C-17).

4.4. Chromatography

GC–MS was performed using a Hewlett Packard 5890 series II gas chromatograph coupled to a Hewlett Packard 5970 mass selective detector fitted with a 12 m (0.2 mm i.d.) fused silica capillary column (HP-1 Ultra stationary phase). Auto-splitless injection and helium carrier gas were used. The GC oven temperature was programmed from 40–300°C at 5°C min^{-1} and held at the final temperature for 10 min. Mass spectrometer operating conditions were; ion source temperature 250°C and 70 eV ionisation energy. Spectra (35–500 Da) were collected using Hewlett Packard Chemstation™ software.

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