SOURCES AND STRUCTURES OF COMMONLY OCCURRING HIGHLY BRANCHED ISOPRENOID ALKENES

by

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ABSTRACT

Highly branched isoprenoid (HBI) alkenes are ubiquitous lipids which have been identified in numerous geochemical samples, ranging from recent sediments to ancient oils. At the outset of the current investigation, the diatomaceous algae *Haslea ostrearia* (C_{25} alkenes) and *Rhizosolenia setigera* (C_{25} or C_{30} alkenes) were the only reported biological sources of these compounds. However, there remained a poor correlation between isomers found in diatoms and those commonly reported in sediments and water column particles.

In the present study, the structures of fourteen novel C_{25} HBI trienes, tetraenes and pentaenes, and four C_{30} HBI pentaenes and hexaenes have been rigorously characterised *via* gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy following isolation from diatoms. The GC-MS characteristics of the four novel C_{30} HBIs and eight of the novel C_{25} HBIs characterised herein show an excellent correlation with those of the HBIs commonly reported in sediments and water column particles. In contrast to the HBIs characterised previously, which all possess a saturated branch point and fixed double bond stereochemistry, the common isomers (C_{25} and C_{30}) possess an unsaturated branch point and exhibit *E/Z* isomerism about a trisubstituted double bond.

Three diatoms belonging to the *Pleurosigma* genus have been identified as HBI producers. Of these, the benthic species *P. intermedium* and the planktonic species *Pleurosigma sp.* have been found to biosynthesise the common C_{25} HBI isomers. *P. planktonicum* has also been identified as a producer of C_{25} HBIs possessing a novel structural type.

The HBI distributions in five distinct strains of *R. setigera* have been investigated, and these were found to be highly variable. Two strains isolated from the northwest Atlantic were found to produce a single, uncommon C_{25} HBI pentaene, which has also been reported in *H. ostrearia*. In contrast, *R. setigera* isolated from the Arabian Sea was found to produce C_{30} HBIs only, whilst two strains isolated from southern Brittany were found to co-produce the common C_{25} and C_{30} HBI isomers.

Four diatom species belonging to the *Haslea* genus have also been newly identified as producers of C_{25} HBI alkenes. The HBI distributions in *H. salstonica, H. crucigera, H. pseudostrearia* and *Haslea sp.* were examined, and HBI production appears to be widespread within the *Haslea* genus. All of the HBIs identified in these *Haslea* spp. were of the structural type previously observed in *H. ostrearia*, and thus do not correspond to the HBI isomers most commonly reported in sediments and particles.

Hydrocarbon extracts isolated from sediments and particulates from the Arabian Sea, Cariaco Trench, Peru upwelling region and the Black Sea were examined by GC-MS, and the HBI isomers in these samples were identified.

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G. Allard, G. Gubbin, J. Rintatalo, S. Belt, S. Rowland, G. Massé, J. M. Robert. Sources, Structures and Diagenesis of C_{25} and C_{30} Highly Branched Isoprenoid (HBI) Hydrocarbons. 11th annual Meeting of the British Organic Geochemistry Society 1999 (York, U.K.).

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Date..23/7/02.....

CHAPTER ONE

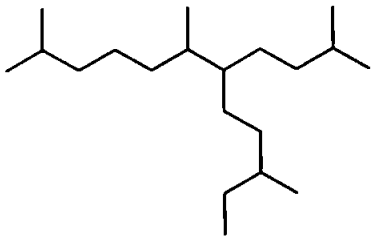
Introduction

1.1 Highly branched isoprenoid hydrocarbons

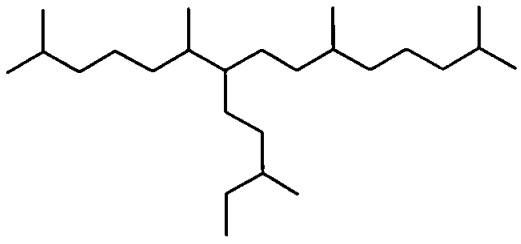
Since the first reports of C_{20}, C_{25} and C_{30} highly branched isoprenoid (HBI) hydrocarbons in sediments (e.g. Gearing *et al.*, 1976; Farrington *et al.*, 1977), many studies have described the occurrence of a large number of different HBI isomers with 0-6 degrees of unsaturation in a wide range of geochemical settings (reviewed by Rowland and Robson, 1990) including sediments (e.g. Barrick *et al.*, 1980), sedimenting particles (e.g. Albaiges *et al.*, 1984), phytoplankton communities (e.g. Nichols *et al.*, 1988) and crude oil (Yon *et al.*, 1982). The determination of the parent carbon skeletons of the C_{20} (I Figure 1.1; Yon *et al.*, 1982), C_{25} (II Figure 1.1; Robson and Rowland, 1986) and C_{30} (III Figure 1.1; Robson and Rowland, 1988) HBIs was achieved by synthesis. The distinctive structures of the HBIs led to their proposed use as 'biological marker' compounds (see Peters and Moldowan, 1993 for a general review of biomarkers).

1.2 The identification of diatoms as sources of HBIs

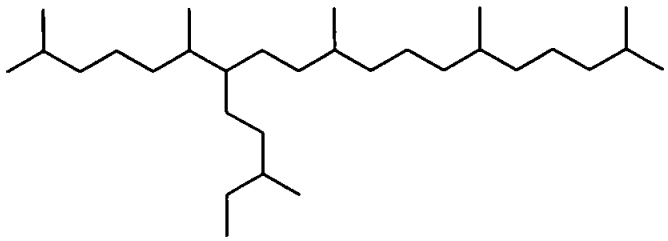
An investigation into the hydrocarbon distributions of fifteen diatom species led to the identification of the marine diatoms *Rhizosolenia setigera* and *Haslea ostrearia* as biological producers of HBI alkenes (Volkman *et al.*, 1994). The authors therein identified a group of HBIs possessing the C_{25} parent skeleton (II, Figure 1.1) and exhibiting between three and five degrees of unsaturation in *H. ostrearia* strain CS-250, and several alkenes possessing the C_{30} parent skeleton (III, Fig 1.1) containing five and six double bonds in *R. setigera* strain CS-62. Studies of the hydrocarbons in two further strains of *R. setigera* showed substantial



I
$C_{20:0}$
Yon *et al.*, 1982



II
$C_{25:0}$
Robson and Rowland, 1986



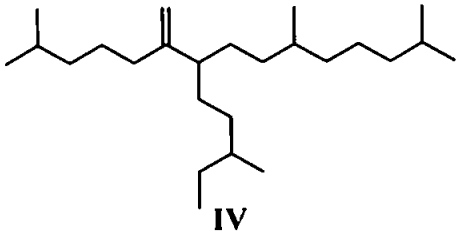
III
$C_{30:0}$
Robson and Rowland, 1988

Figure 1.1 Structures of the HBI alkanes determined by synthesis.

variation in the HBI isomers produced, which ranged from C_{30} HBI penta- and hexaenes in strain CS-389/1 (Volkman *et al.*, 1998) to a single C_{25} HBI pentaene in strain CCMP 1330 (Sinninghe Damsté *et al.*, 1999a). Since the initial report by Volkman *et al.* (1994), the role of *H. ostrearia* as a producer of C_{25} HBIs has also been the subject of further study. The identification of C_{25} HBIs in cultures of *H. ostrearia* grown under axenic conditions (Wraige *et al.*, 1999) confirmed the role of *H. ostrearia* as a biological source of HBI alkenes, and the isolation of pure quantities of HBIs from cultures of *H. ostrearia* has allowed the structures of a number of C_{25} HBIs to be fully characterised (Belt *et al.*, 1996; Wraige *et al.*, 1997; Wraige *et al.*, 1999; Johns *et al.*, 1999). Although biological sources of C_{25} and C_{30} HBIs have been identified (Volkman *et al.*, 1994), the source of the related C_{20} HBIs remains unknown, although it seems likely that they too are a product of biosynthesis by diatoms.

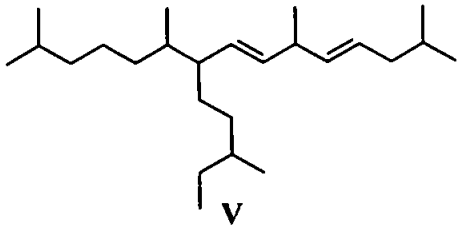
1.3 Structural characterisation of HBI alkenes

Although HBIs are of widespread occurrence in sediments and biota from both marine and lacustrine environments (reviewed by Rowland and Robson, 1990), the low concentrations (e.g. 230 ng/g dry sediment; Requejo and Quinn, 1983) of these compounds in environmental matrices has hindered the structural characterisation of these alkenes by e.g. NMR spectroscopy. Typically, identification of HBIs in such samples has been limited to the determination of the carbon skeleton *via* hydrogenation to the parent alkane, and assignment of the number of double bonds by mass spectrometry. However, the determination of double bond positions and/or stereochemistry of HBIs isolated directly from environmental matrices has been achieved for a small number of C_{25} HBI alkenes. Dunlop and Jefferies (1985) isolated a C_{25} monoene (IV; Figure 1.2) from sediments of Shark Bay (Western Australia) and determined the double bond position *via* ozonolysis and GC-MS. In a similar study, Yruela *et al.* (1990) used epoxidation followed by GC-MS to identify a C_{25} HBI diene (V)



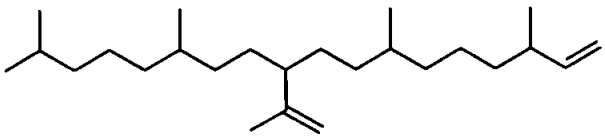
IV

Dunlop and Jefferies, 1985



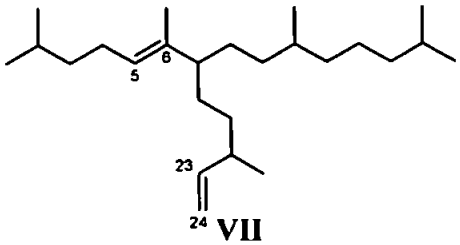
V

Yruela *et al.*, 1990



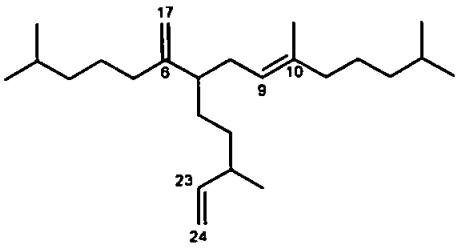
VI

Summons *et al.*, 1993



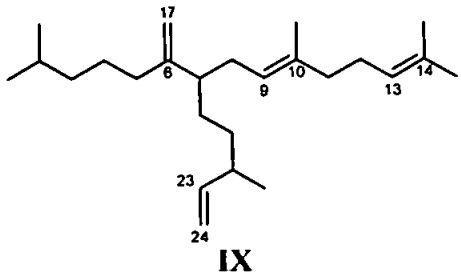
VII

Belt *et al.*, 1994



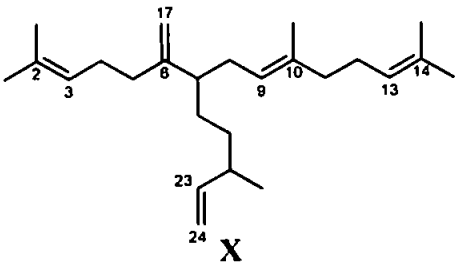
VIII

Belt *et al.*, 1996



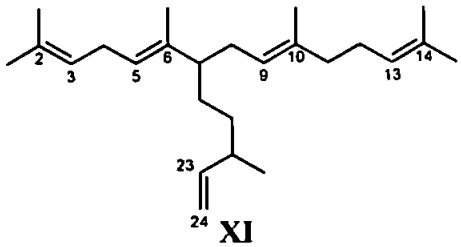
IX

Belt *et al.*, 1996



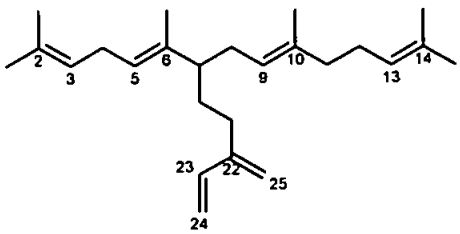
X

Belt *et al.*, 1996



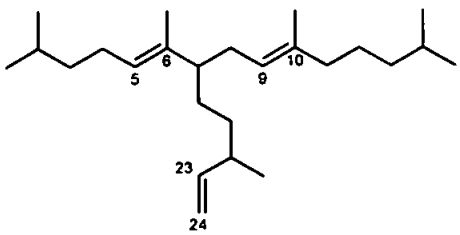
XI

Wraige *et al.*, 1997



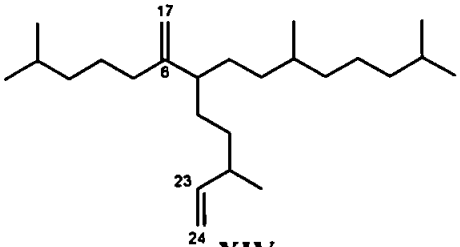
XII

Wraige *et al.*, 1997



XIII

Wraige *et al.*, 1999



XIV

Johns *et al.*, 1999

Figure 1.2 Structures of C_{25} HBI alkenes characterised to date.

which was isolated from sediments of the Guadalquivir Delta (Spain). Summons *et al.* (1993) isolated an unusual highly branched C_{25} alkadiene (VI) with a novel carbon skeleton, from a diatomaceous benthic microbial community of Hamelin Pool (Western Australia) and determined the structure *via* NMR, MS and chemical degradation. The first unequivocal characterisation of a C_{25} HBI alkene possessing the parent skeleton (II) was achieved by Belt *et al.* (1994), who isolated milligram quantities of the C_{25} HBI diene (VII) from sediments of the Caspian Sea, and determined the structure using a combination of GC-MS and NMR spectroscopy.

The identification of HBI alkenes in cultures of the diatoms *H. ostrearia* and *R. setigera* (Volkman *et al.*, 1994) provided a convenient new source of HBIs which allowed isolation of sufficient quantities of pure alkenes for rigorous characterisation by NMR and GC-MS. Thus, Belt *et al.* (1996) isolated the HBI triene (VIII), tetraene (IX) and pentaene (X) from a single batch culture (500 L) of *H. ostrearia* and determined their structures by NMR spectroscopy. Wraige *et al.* (1997) determined the structures of the HBI pentaene (XI) and hexaene (XII) by NMR spectroscopy and epoxidation following isolation of the pure alkenes from a further batch culture (440 L) of *H. ostrearia*. In a different study, the pentaene (XI) was also isolated from a laboratory culture of *R. setigera* (Sinninghe Damsté *et al.*, 1999b) and the structure was confirmed by NMR analysis. Similar studies using cultures of *H. ostrearia* allowed the structural characterisation of the HBI triene (XIII; Wraige *et al.*, 1999) and diene (XIV; Johns *et al.*, 1999).

In contrast to these advances in C_{25} HBI structural characterisation, the situation with C_{30} compounds has remained more constrained. Despite the identification of C_{30} HBIs in sediments and particulates (reviewed by Rowland and Robson, 1990), identification of the C_{30} HBIs from environmental samples and diatom cultures (Volkman *et al.*, 1994) has to

date been confined to the determination of carbon skeleton and the degree of unsaturation. At the outset of the present study, the double bond positions and stereochemistries for these compounds were unknown.

1.4 Structural features of C_{25} HBI alkenes isolated from diatoms

It can be seen from Figure 1.2 that the C_{25} HBI alkenes (VII – XIV) which have been identified in cultures of *H. ostrearia* (Volkman *et al.*, 1994; Belt *et al.*, 1996; Wraige *et al.*, 1997; Wraige *et al.*, 1999; Johns *et al.*, 1999) all possess a vinyl group at C23-C24, characterised by an ABMX spin system in the ^{1}H NMR spectrum (H-23, δ=5.65 ppm), and a saturated branch point at C-7. In addition to these structural features, the HBIs can be divided into two distinct groups with either a tri-substituted double bond at C5-C6 (e.g. VII, XI, XII, XIII) or a methylenic double bond at C6-C17 (e.g. VIII, IX, X, XIV). HBIs with further degrees of unsaturation contain additional double bonds which appear to follow sequentially in the order C9-C10 ($C_{25:3}$; VIII, XIII), C13-C14 ($C_{25:4}$; IX), C2-C3 ($C_{25:5}$; X, XI) and finally C22-C25 ($C_{25:6}$; XII). In all cases, the tri-substituted double bonds (e.g. C5-C6) were found to have an *E* configuration, with no evidence of *Z* isomers.

Each of the C_{25} HBIs observed previously in cultures of *H. ostrearia* (VII – XIV) contain two chiral centres, at C7 and C22 (VII and XIV have an additional chiral centre at C10), and thus have the potential for stereoisomerism (Figure 1.3 a). Belt *et al.* (1996) observed that several resonances appeared as doublets in the ^{13}C NMR spectra of VIII, IX and X (notably C5-C8 and C20-C25), and also observed an analogous feature for the ^{1}H NMR resonance for the vinylic proton H-23. This was attributed to the presence of diastereomeric mixtures of the HBIs (Belt *et al.*, 1996). However, the resonances for VII (Belt *et al.*, 1994) and XI (Wraige

et al., 1997) did not appear as doublets, suggesting that the HBIs were homochiral. In all cases, the HBIs chromatographed as single peaks on two GC phases (HP-1, DB-5).

Johns *et al.* (2000) investigated the stereochemistry of the diene (XIV) and triene (VIII) isolated from several cultures of *H. ostrearia*, using oxidative degradation of the HBIs followed by analysis *via* enantioselective GC in combination with NMR spectroscopy. It was found that the stereochemistry at C7 was fixed (but unknown) in all cases studied, and that the stereochemistry at C22 varied from either 100% *S* (Figure 1.3 b), 100% *R* (Figure 1.3 c), or as a 1:1 *R/S* diastereomeric mixture (Figure 1.3 d). Additionally, Johns *et al.* (2000) observed that the diastereoisomers of the HBI triene VIII could be resolved by GC using an apolar (carbowax) stationary phase, and determined the elution order as 22*S* before 22*R*.

1.5 Discrepancies between HBIs in the geosphere and those identified in diatoms

When Volkman *et al.* (1994) reported the presence of several C_{25} HBI alkenes in a culture of the benthic diatom *Haslea ostrearia*, it seemed likely that a source of the sedimentary compounds had been found, though the authors noted that the mass spectra and retention indices of the HBIs in the alga were different from those in many sediments and seawater. Following detailed studies on the structures of C_{25} HBIs isolated from diatom cultures (Belt *et al.*, 1996; Volkman *et al.*, 1998; Wraige *et al.*, 1997, 1999; Johns *et al.*, 1999; Sinninghe Damsté *et al.*, 1999a,b), it became apparent that there were indeed often discrepancies between the chromatographic (RI) and mass spectral properties of many of the sedimentary isomers and those found in *H. ostrearia* and *R. setigera*.

Work was conducted in an attempt to determine whether such discrepancies between the HBIs observed in sediments and those observed in diatoms could be due to the effects of growth conditions such as salinity and temperature. Wraige *et al.* (1998) observed that

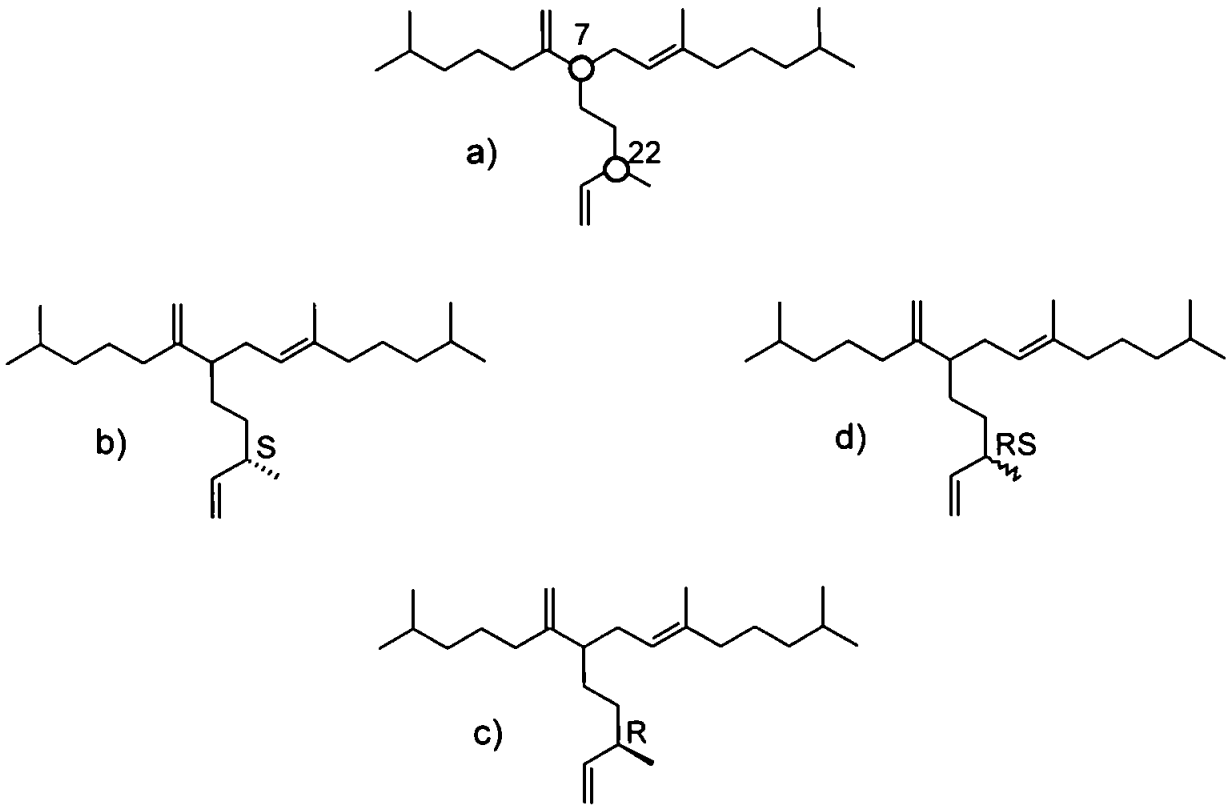


Figure 1.3 Stereochemistry of C_{25} HBIs isolated from *H. ostrearia* (Johns *et al.*, 2000).

salinity of the growth medium did not have a pronounced effect on the HBI distributions in *H. ostrearia*. Rowland *et al.* (2001) observed that growth temperature did have a significant effect on the HBI distributions produced by *H. ostrearia*, and a correlation was observed between the growth temperature and the degree of unsaturation of the HBI isomers, suggesting that HBIs could have potential for palaeoclimatic reconstruction. However, despite these observations, the variations in HBIs reported by Rowland *et al.* (2001) could not account for the common sedimentary isomers.

This led to the consideration of whether diagenetic processes could be responsible for the conversion of the C_{25} HBI isomers produced by diatoms into those observed in the geosphere. Belt *et al.* (2000) conducted experiments investigating the simulated diagenesis of the C_{25} HBI diene (XIV) and triene (XIII), and found that isomerisation of the HBIs was facile under mildly acidic (tosic acid or montmorillonite clay) conditions. The methylenic (C6-C17) double bond of the diene (XIV) was found to be labile, and isomerisation of this double bond led to the formation of a pair of geometrically isomeric dienes (VII; Figure 1.4). Under the same reaction conditions, the triene (XIII) was found to undergo rapid cyclisation to yield a novel substituted cyclohexane (XV; Figure 1.5). Once again, this investigation failed to produce the most common sedimentary isomers, although it indicated that HBI alkenes are susceptible to diagenetic changes under relatively mild conditions. It therefore seemed likely that the sedimentary HBI isomers may originate from diatom sources not yet studied.

The rearrangement of HBI alkenes under mildly acidic conditions as demonstrated by Belt *et al.* (2000) raises the question as to whether the range of HBI isomers reported in diatom cultures (e.g. Volkman *et al.*, 1994; Belt *et al.*, 1998; Wraige *et al.*, 1999) were a result of the rearrangement of HBI alkenes during work-up procedures involving elevated temperatures

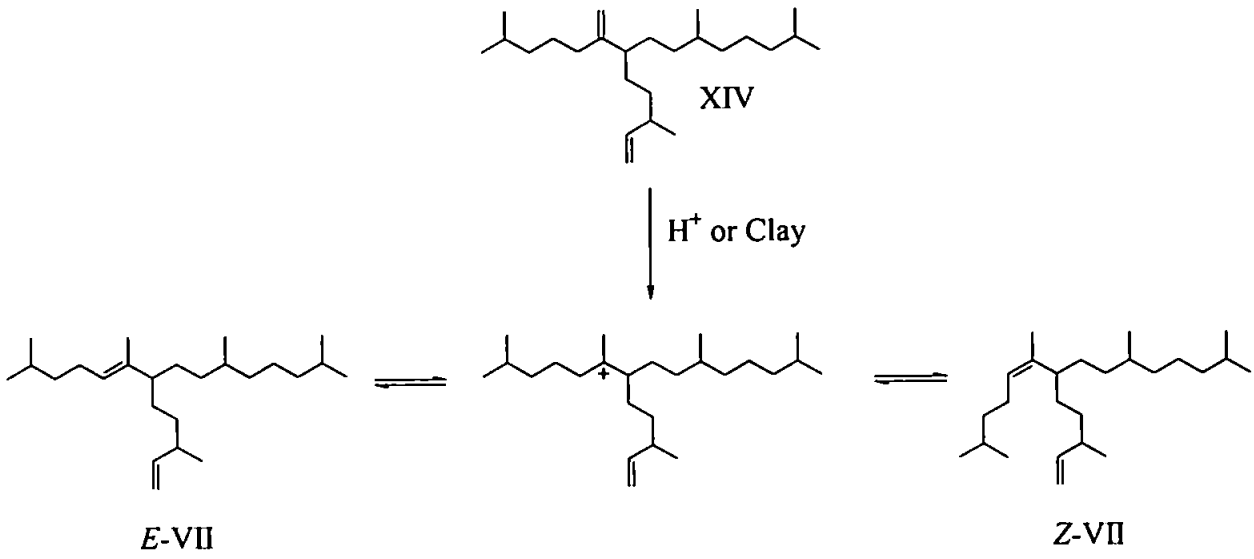


Figure 1.4 Isomerisation of $C_{25:2}$ (XIV) under mildly acidic conditions (Belt *et al.*, 2000).

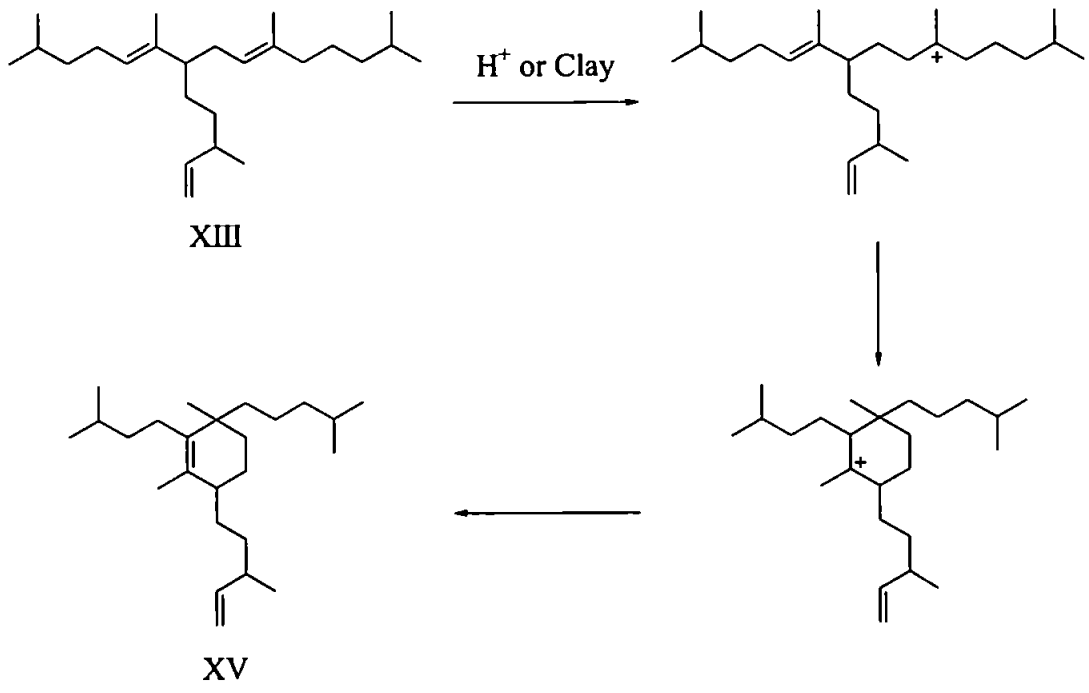


Figure 1.5 Cyclisation of $C_{25:3}$ (XIII) under mildly acidic conditions (Belt *et al.*, 2000)

such as saponification, or purification using acidic media such as silica. However, as HBI isomers have been identified in both the aqueous culture medium (Johns, 1999; Massé, personal communication) and the total hexane extracts of *H. ostrearia* (Wraige *et al.*, 1999) obtained *via* mild solvent extraction, it is likely that these isomers are genuine diatom lipids and not the products of rearrangements.

1.6 The present study

At the outset of the present study, it was clear that although the structures of a number of C_{25} HBIs had been determined (IV – XIV), the structures of the most common and abundant sedimentary HBIs remained unknown. Since the HBIs produced by *H. ostrearia* did not represent the most common HBIs found in environmental matrices (reviewed by Rowland and Robson, 1990), it seemed likely that other diatom species were important contributors to global HBI distributions. In addition, whilst the production of C_{25} HBIs by *H. ostrearia* had been extensively studied, HBI production by *R. setigera* (the other known HBI producer) had not received the same level of attention, and the structures of the C_{30} HBIs produced by this diatom were unknown. Therefore the main aims of this study were to:

(i) Identify new diatom species capable of HBI biosynthesis.

(ii) Identify the HBI isomers that are common in the geosphere.

(iii) Determine the structures of the C_{30} HBIs.

The results of this combined investigation are the main subject of this thesis and are described as follows:

Chapter 2 describes the identification of three diatoms of the *Pleurosigma* genus (*P. intermedium*, *P. planktonicum* and *Pleurosigma sp.*) as biological producers of C_{25} HBI alkenes. The HBI distributions in these diatoms have been investigated, and ten novel C_{25}

HBI trienes, tetraenes and pentaenes have been rigorously characterised by GC-MS and NMR spectroscopy following isolation from these new HBI producers. The chromatographic and mass spectral properties of several of these novel HBI alkenes show an excellent correlation with those HBIs most commonly reported in sediments. Thus, the common sedimentary isomers have at last been identified.

Chapter 3 describes for the first time the structural characterisation of C_{30} HBI alkenes. Four C_{30} HBI penta- and hexaenes were isolated from the diatom *R. setigera* and their structures were characterised by GC-MS and NMR spectroscopy. The HBI distributions of five different *R. setigera* strains were investigated, and HBI production by these strains was found to be highly variable. Additionally, certain strains of *R. setigera* were found to produce the C_{25} and C_{30} HBI isomers most commonly reported in sediments and particulates.

Chapter 4 describes the production of HBIs by members of the *Haslea* genus, and four *Haslea* species (*H. pseudostrearia, H. salstonica, H. crucigera* and *Haslea* sp.) have been identified as biological producers of HBI alkenes. Four novel C_{25} HBIs have been characterised by GC-MS and NMR spectroscopy following isolation from cultures of *H. ostrearia, H. pseudostrearia* and *Haslea* sp.

Chapter 5 describes the HBI distributions in sediments and particulates from a range of marine environments, namely the Arabian Sea, Cariaco Trench, Black Sea and Peru upwelling region. The novel HBIs characterised in chapters 2 and 3 have allowed the unambiguous identification of the most abundant HBI isomers in these environmental samples.

(n.b. Individual C_{25} and C_{30} HBI isomers are numbered on a chapter-by-chapter basis.)

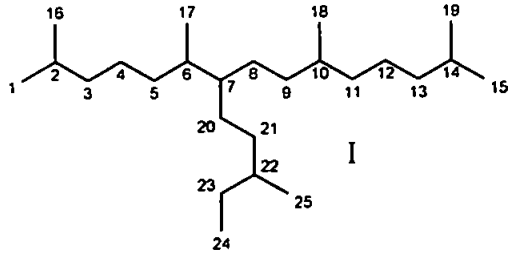
CHAPTER TWO

HBIs in diatoms of the *Pleurosigma* genus

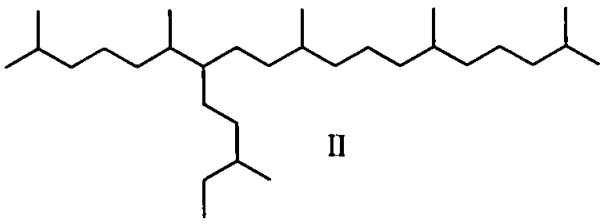
2.1 Introduction

Since the first reports of C_{25} highly branched isoprenoid (HBI) hydrocarbons in sediments (e.g. Gearing *et al.*, 1976; Farrington *et al.*, 1977) and the determination of the parent carbon skeleton (I, Figure 2.1) by synthesis (Robson and Rowland, 1986) many studies have described the occurrence of a large number of different HBI isomers with 0-6 degrees of unsaturation, in a wide range of geochemical settings (reviewed by Rowland and Robson, 1990). When Volkman *et al.* (1994) reported the presence of several C_{25} HBI alkenes in a culture of the benthic diatom *Haslea ostrearia*, it seemed likely that a source of the sedimentary compounds had been found, though the authors noted that the mass spectra and retention indices of the HBIs in the alga were different from those in many sediments and seawater.

Determinations of the structures, including double bond positions and stereochemistry, and in some cases, either the relative or absolute stereochemistries of the asymmetric centres (Johns *et al.*, 2000) of a number of dienes through hexaenes (III-X, Figure 2.1) from large scale cultures of *H. ostrearia* and recently from the diatom *Rhizosolenia setigera,* have succeeded the initial findings (Belt *et al.*, 1996; Volkman *et al.*, 1998; Wraige *et al.*, 1997, 1999; Johns *et al.*, 1999; Sinninghe Damsté *et al.*, 1999a,b). From detailed studies on the structures of C_{25} HBIs, it became apparent that there were indeed often discrepancies between the chromatographic (RI) and mass spectral properties of many of the sedimentary isomers and those found in *H. ostrearia* and *R. setigera* (c.f. Volkman *et al.*, 1994). This led to consideration of whether the biological HBI distributions were affected by algal growth



I



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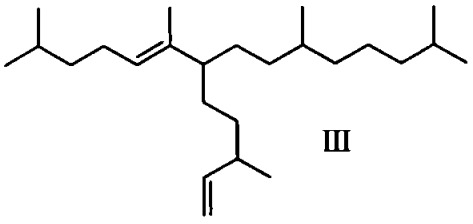

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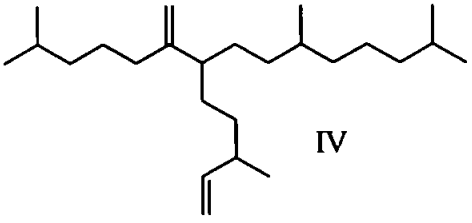

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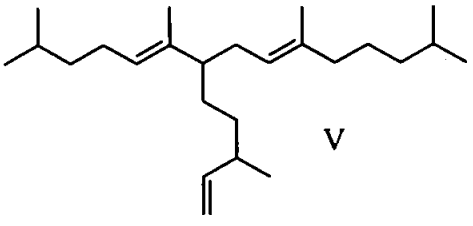

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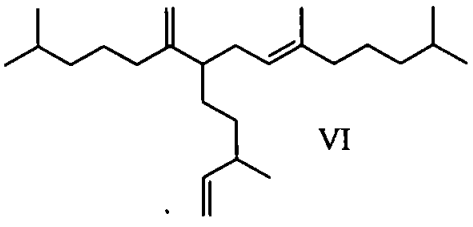

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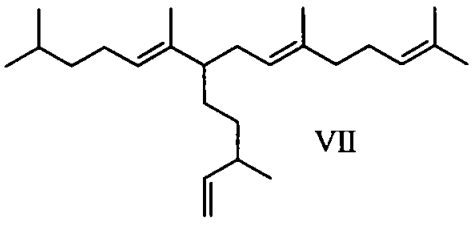
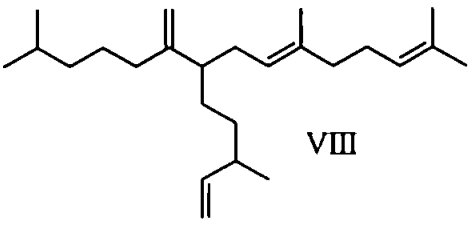
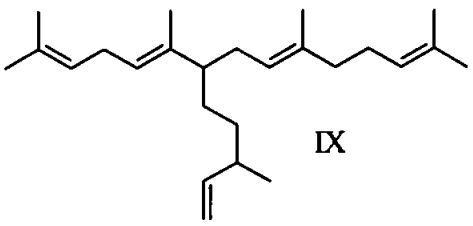


VII



VIII



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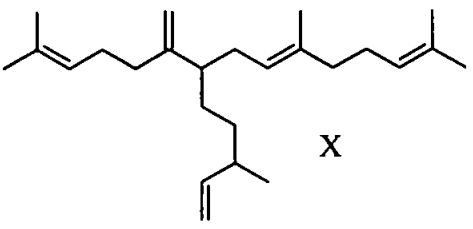


X

Figure 2.1 C_{25} and C_{30} parent structures and typical HBI alkenes from sediments and diatoms.

conditions such as salinity or temperature (Wraige *et al.*, 1997, 1999) and/or if HBIs produced by diatoms such as *H. ostrearia* might undergo rapid diagenetic changes in sediments to yield isomeric forms of the biogenic compounds (Belt *et al.*, 2000). Although both phenotypic and diagenetic variables did alter HBI isomer distributions, there was often still a poor correspondence of the resulting distributions with sedimentary HBIs. For example, whereas HBI dienes and trienes isolated from *H. ostrearia* underwent facile isomerisation and cyclisation reactions under mild acid conditions including Montmorillonite clay, most of the products were not those widely reported in sediments to date (Belt *et al.*, 2000). This suggested that the common and abundant sedimentary HBI isomers may originate from diatom sources not previously studied.

The isolation and characterisation by NMR spectroscopy, of six new HBI alkenes from several large scale cultures of the common benthic diatom, *Pleurosigma intermedium* is reported herein. In contrast to the structures of HBIs characterised previously from diatoms, the chromatographic and mass spectral properties of these compounds show an excellent correlation with those HBIs most commonly reported in sediments (Robson and Rowland, 1990). Additionally two planktonic members of the *Pleurosigma* genus; *P. planktonicum* and *Pleurosigma* sp. have been identified as producers of HBI alkenes and the structures and distributions are reported.

2.2 Experimental

2.2.1 Algal cultures

P. intermedium was isolated from oyster ponds of the Bay of Bourgneuf (France). Batch cultures of the diatom were grown in Nantes in an outdoor culture facility (9/7 - 21/7 1999; Culture 1 (PI-1) and 7/9 – 14/9 1999; Culture 2 (PI-2)) or in an indoor laboratory (21/10 –

5/11 1999; Culture 3 (PI-3), 11/2 – 25/2 2000; Culture 4 (PI-4) and 15/2 – 9/3 2000; Culture 5 (PI-5)). *P. planktonicum and Pleurosigma* sp. were isolated from surface waters at Le Croisic (France). Cultures were grown in seawater obtained from an underground supply in Nantes at constant salinity (31 per mil). Samples were collected during the stationary phase of the growth cycle and filtered to produce a concentrate of algal cells.

2.2.2 Hydrocarbon extraction and isolation

Isolation and purification of the NSLs obtained from freeze dried algal concentrates was achieved using open column chromatography (SiO_2/ hexane). Fractions thus obtained were analysed by GC-MS and combined where appropriate (i.e. HBIs with the same degree of unsaturation). Separation of individual isomers was not achieved using the chromatographic methods used. Fractions suitable for analysis by NMR spectroscopy (> 95% (GC)) were obtained from PI-1, P-2 and PI-3 in the following amounts (mg). PI-1: $C_{25:3}$ (5.6); $C_{25:4}$ (17.0); $C_{25:5}$ (44.0). PI-2: $C_{25:3}$ (29.0); $C_{25:4}$ (46.0); $C_{25:5}$ (39.0). PI-3: $C_{25:3}$ (1.6); $C_{25:4}$ (12.5); $C_{25:5}$ (13.0).

2.2.3 Stereochemical analysis of HBIs

Following hydrogenation of the individual HBIs to the parent C_{25} alkane ($PtO_2.2H_2O$/ H_2), oxidation was achieved using CrO_3 in acetic acid as previously described (Johns *et al.*, 2000). Following methylation (BF_3 / methanol complex) the stereochemistries of the methyl esters were determined via co-chromatography with authenticated chiral standards using enentioselective GC (König *et al.*, 1988).

2.3 Identification and characterisation of HBIs in *P. intermedium*

2.3.1 Chromatographic and mass spectral analysis of HBIs in P. intermedium

Analysis of GC-MS total ion chromatograms of the non-saponifiable lipid fractions from several cultures of *P. intermedium* typically showed the presence of eight compounds that had chromatographic (RI) and mass spectral properties consistent with HBI alkenes. Figure 2.2 shows examples of partial total ion current chromatograms of hydrocarbon fractions obtained from 2 cultures (PI-1 and PI-4) of *P. intermedium*. The presence of *n*-$C_{21:5}$, *n*-$C_{21:6}$ as additional components together with significant variability in HBI distributions can also be noted. Hydrogenation (H_2 / $PtO_2.2H_2O$) of aliquots of solutions containing these HBIs resulted in the formation of a compound which co-chromatographed with, and had an identical mass spectrum to that obtained for authentic $C_{25:0}$ (Robson and Rowland, 1986). Thus, the parent carbon skeleton (I) for these eight compounds is verified. Examination of the mass spectral data reveals that this suite of HBIs can be grouped into two trienes ($C_{25:3}$, $M^{+\cdot}$ 346), four tetraenes ($C_{25:4}$, $M^{+\cdot}$ 344) and two pentaenes ($C_{25:5}$, $M^{+\cdot}$ 342). For the trienes and the pentaenes, the mass spectral fragmentation patterns and the ion distributions are extremely similar for each compound with the same degree of unsaturation (see Figure 2.3) suggesting the occurrence of geometric or configurational isomers rather than positional isomers. Certainly, there is evidence from previously characterised HBIs that positional isomerisation (for a given degree of unsaturation) results in significant differences in fragmentation pathways and relative ion intensities. For example, HBIs V and VI which have been isolated from cultures of *H. ostrearia* (Belt *et al.*, 1996; Wraige *et al.*, 1997, 1999), have mass spectra (Figure 2.4) that show major differences particularly in the relative intensities of ions with *m/z* 261, 233 (and different base peaks), despite the similarity in their structures and RIs (2103 and 2106 for V and VI respectively). Similarly, the pseudo-

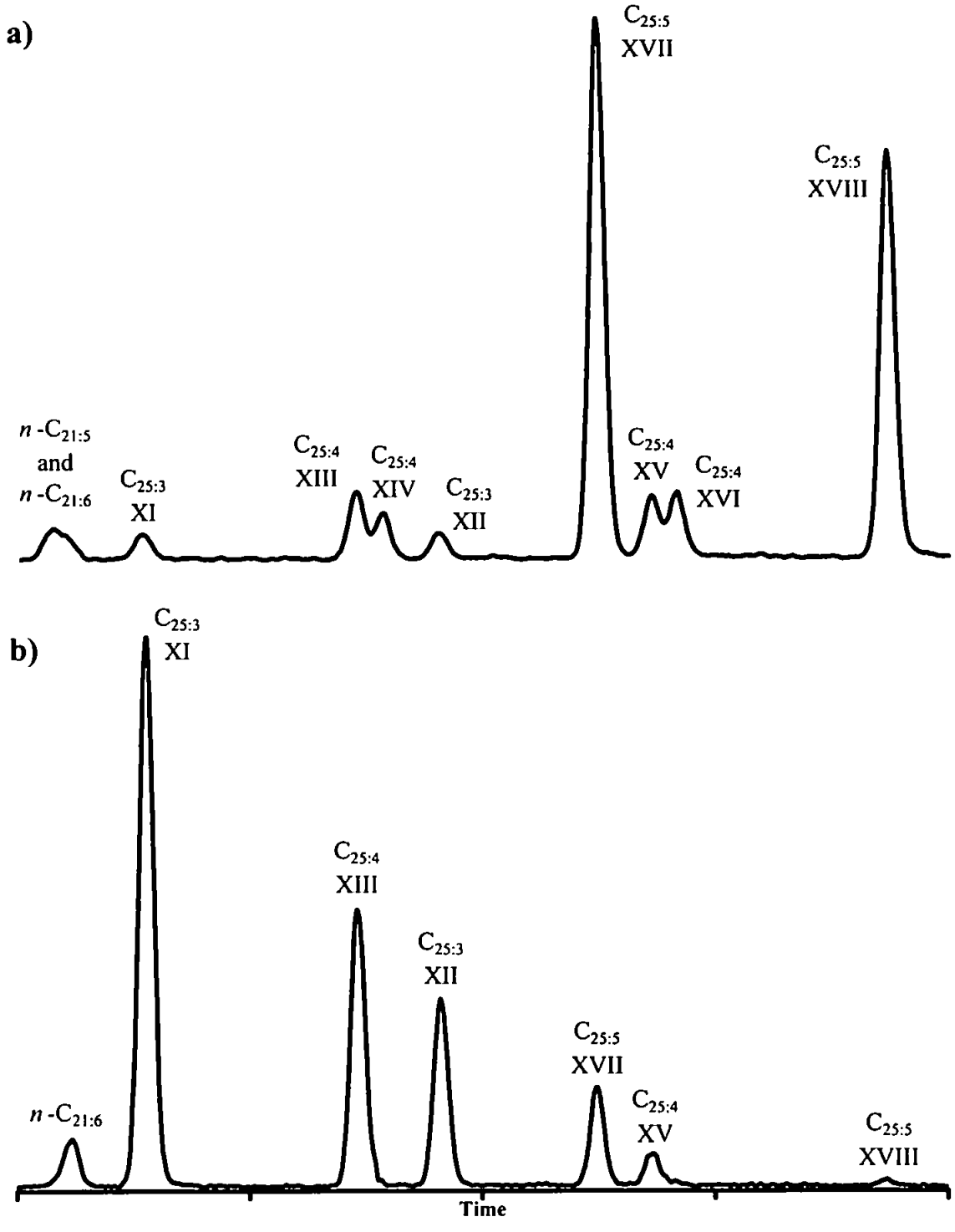


Figure 2.2 Partial TIC chromatograms of the non-saponifiable lipids from 2 cultures of *P. intermedium* (a) PI-1 (b) PI-4.

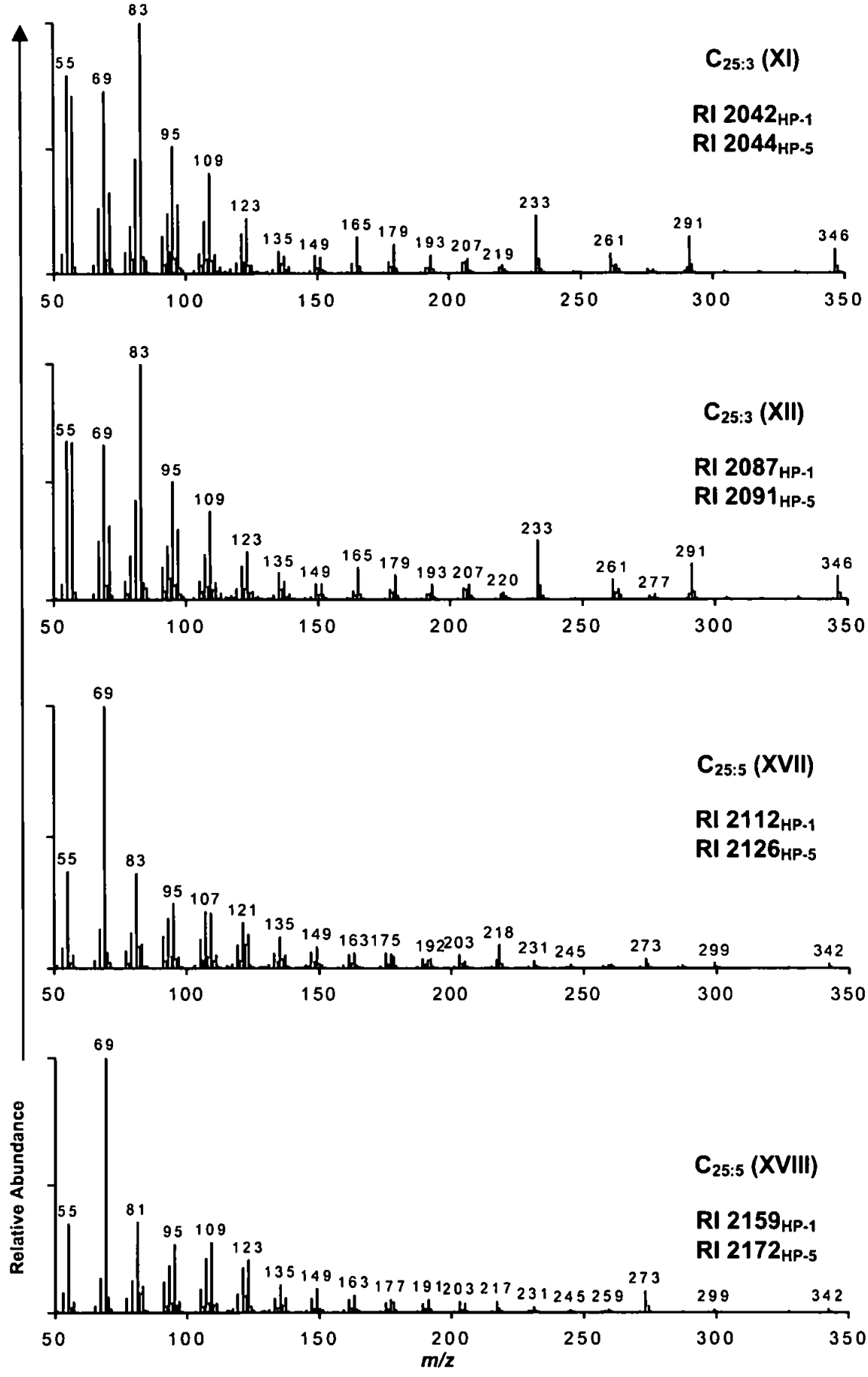


Figure 2.3 Mass spectra of HBI trienes ($C_{25:3}$) and pentaenes ($C_{25:5}$) isolated from *P. intermedium*.

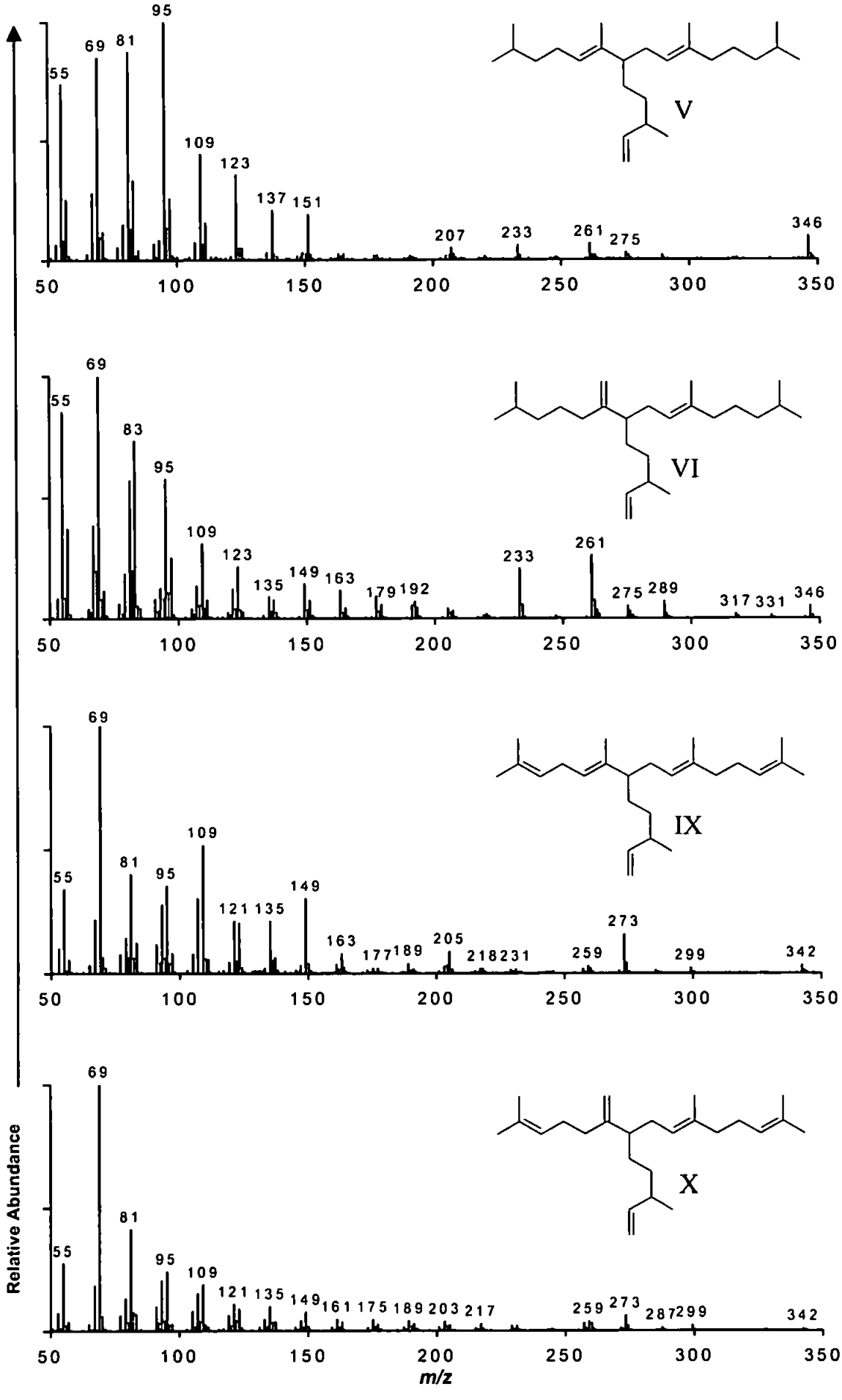


Figure 2.4 Mass spectra of HBI trienes and pentaenes isolated from *H. ostrearia*.

homologous C_{25} pentaenes IX and X isolated from *R. setigera* (IX) and *H. ostrearia* (IX, X), have substantially different mass spectra (Figure 2.4) despite the minor differences in structure.

In contrast to the trienes and pentaenes, the mass spectra of the four C_{25} tetraenes isolated from *P. intermedium* do have noticeable differences between them, though qualitatively they can be grouped into two pairs. Thus, tetraenes with RI 2074 and 2121 $_{HP\text{-}1}$ (Figure 2.5) have major fragments at *m/z* 289 and 177, while the related tetraenes with RI 2078 and 2124 $_{HP\text{-}1}$ have characteristic ions at *m/z* 301 and 180. In some cases, there are ions that are common to all 4 tetraenes, though the relative intensities are different and fall into 2 pairs (e.g. *m/z* 259, 149; Figure 2.5). These observations are consistent with the presence of a pair of positional isomers, each of which exists as a pair of geometric isomers. The position of one or more of the double bonds presumably precludes this from occurring with the related trienes and pentaenes (*vide infra*).

Subsequent structural characterisation of the HBI alkenes from *P. intermedium* by NMR spectroscopy (described in detail in sections 2.3.2 – 2.3.5) revealed the structures to be XI – XVIII (Figure 2.6).

2.3.2 Characterisation of HBI alkenes using NMR spectroscopy

The NMR analysis of HBI alkenes (e.g. Wraige *et al.*, 1999; Sinninghe Damsté, 1999b) is aided by spectroscopic features readily attributable to distinct structural types. The ^{1}H NMR spectra of these compounds typically exhibit several characteristic low-field resonances (*ca* 4.8 – 5.8 ppm) that can be assigned to alkenic protons, with distinct resonances attributable to the protons of vinylic (-CH=CH_2) and tri-substituted double bonds. Allylic and di-allylic protons can be identified by resonances at *ca* 2.0 and 2.5 ppm respectively.

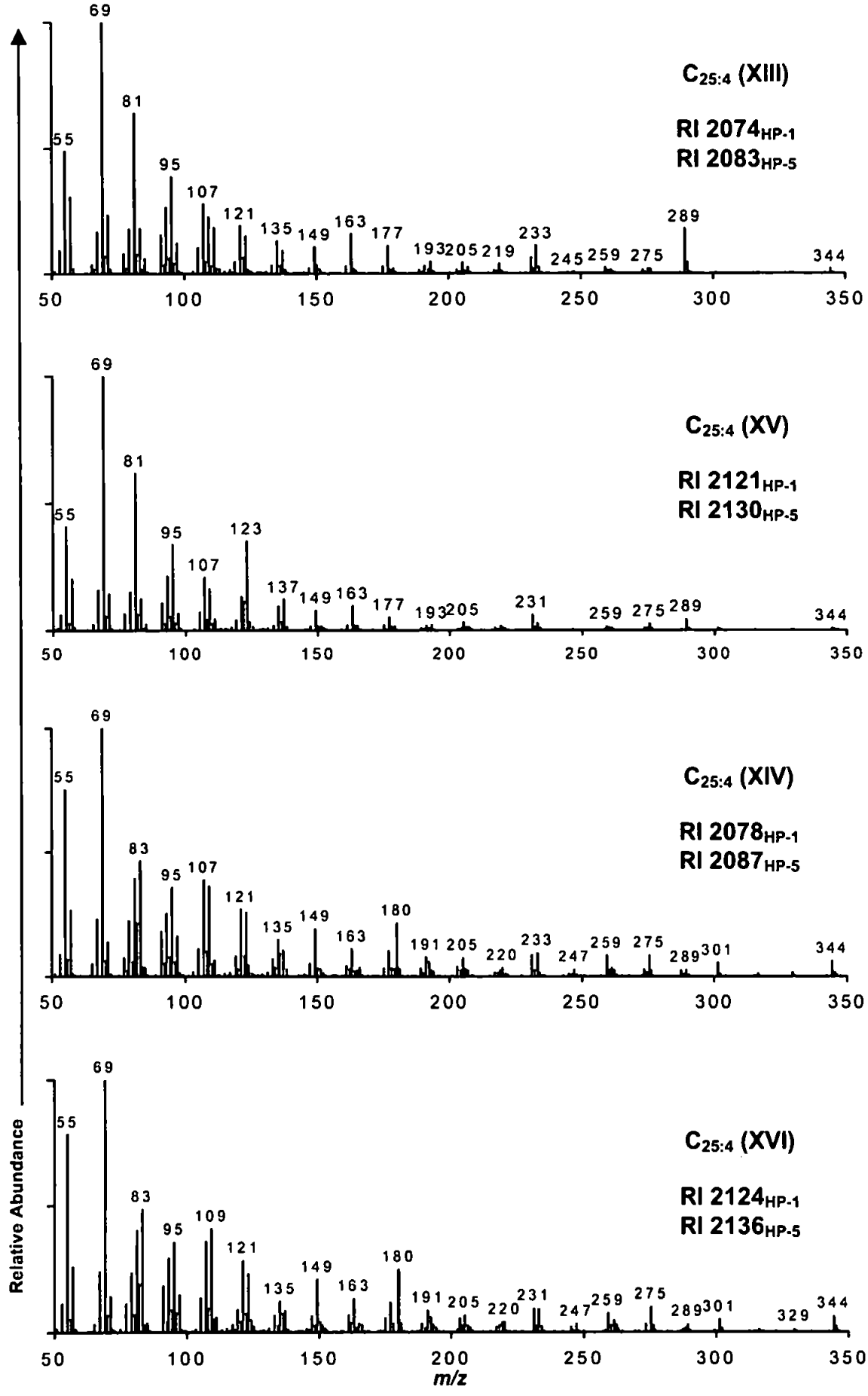


Figure 2.5 Mass spectra of HBI tetraenes ($C_{25:4}$) isolated from *P. intermedium*

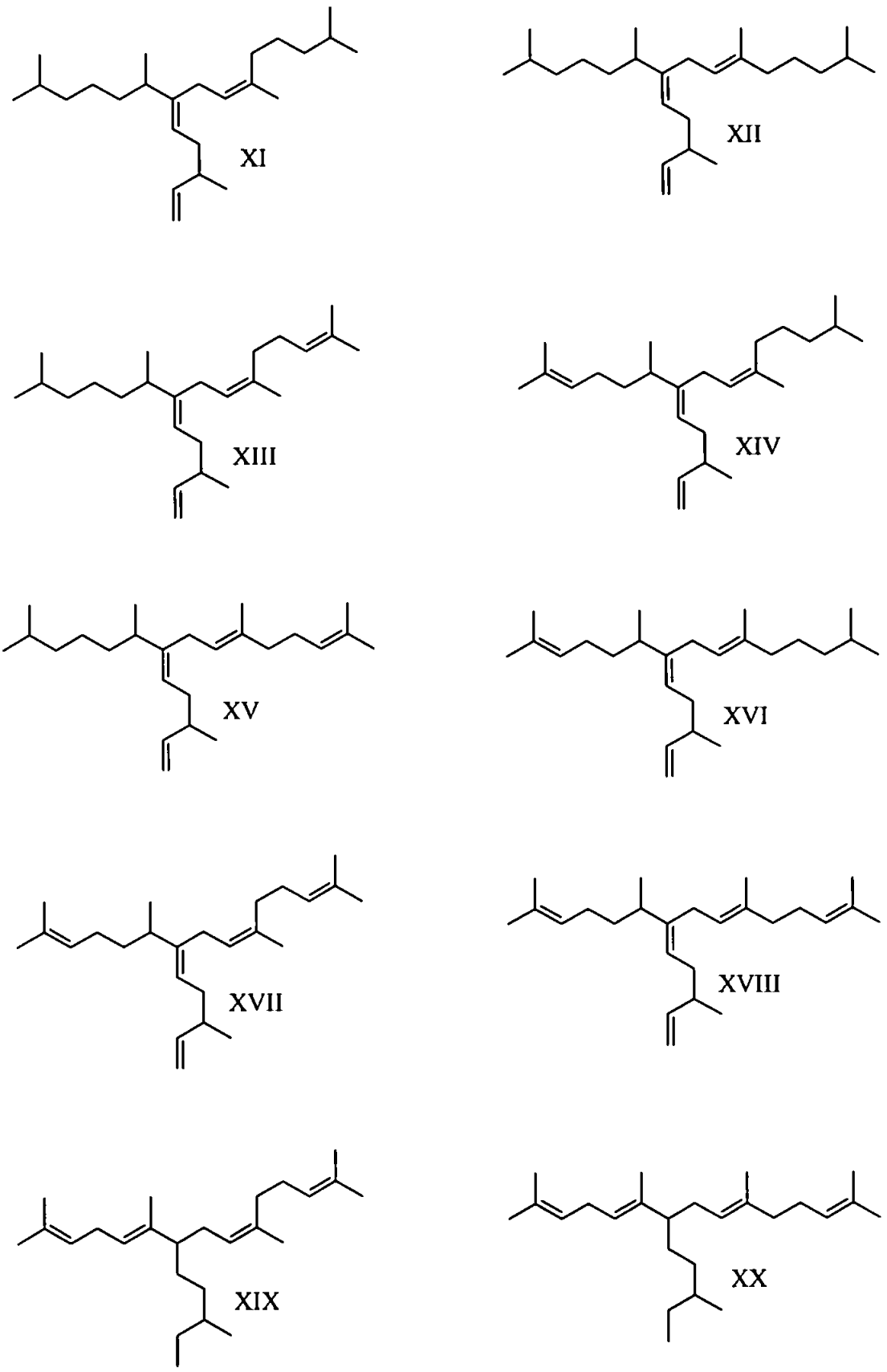


Figure 2.6 Structures of HBIs isolated from *P. intermedium* (XI – XVIII) and *P. planktonicum* (XIX and XX).

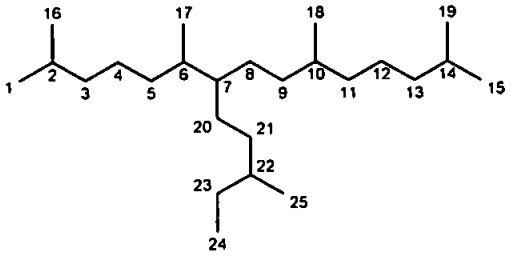
Isopropyl($(CH_3)_2CH$-), isoprenyl ($(CH_3)_2C$=) and isolated methyl groups can also be readily distinguished. ^{13}C techniques provide additional information, allowing the identification of carbon hybridisation and double bond configuration.

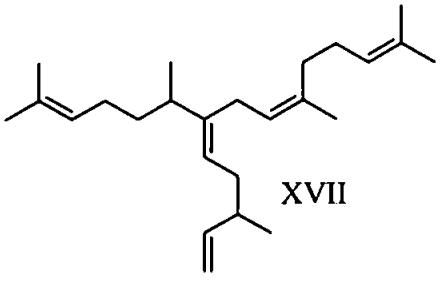
2.3.3 Characterisation of (9E, 6E/Z)-2,6,10,14-tetramethyl-9-(3-methylpent-4-enylidene)-pentadeca-2,6,13-triene (XVII, XVIII)

Examination of the ^{1}H (Table 2.1) and ^{13}C (Table 2.2) NMR spectra of the pentaene fraction (39 mg) isolated from culture PI2 revealed the presence of 4 tri-substituted alkene moieties, together with a vinyl group, a structural feature common to all known polyunsaturated HBIs. For all of these previously reported HBIs, the main branch point is at C-7, probably as the result of a biosynthetic coupling of geranyl and farnesyl type precursors (Johns *et al.*, 1999). However, for the new sesterterpenoids isolated from *Pleurosigma intermedium*, C-7 is unsaturated with a double bond between C-7 and C-20 (n.b. the numbering scheme for NMR assignments is for parent alkane (I)). The determination of this double bond position (C7-C20) and of the other tri-substituted double bonds was established using 2-D NMR methods (COSY, HMQC, HMBC). Of particular note is the absence of any ^{13}C resonances in the 40-50 ppm region indicative of saturated, branched positions (C-7) observed for all previously reported HBIs (Belt *et al.*, 1996; Wraige *et al.*, 1997; Wraige *et al.*, 1998; Wraige *et al.*, 1999; Sinninghe Damsté *et al.*, 1999b; Johns *et al.*, 1999; Johns *et al.*, 2000). Instead, alkenic C-7 resonates at 142.8 and 142.4 ppm for XVII and XVIII respectively. Separation of the two $C_{25:5}$ isomers using further chromatography, was not achieved. However, both spectroscopic (^{13}C NMR) and chromatographic (GC) separations of these compounds are most consistent with the presence of two geometric isomers (C9-C10). The mixed double bond stereochemistry of C9-C10 (and not C7-C20) was determined by careful examination of the NMR data. Specifically, unique ^{1}H and ^{13}C (CH_3) resonances at 1.55, 1.69 ppm (H-18)

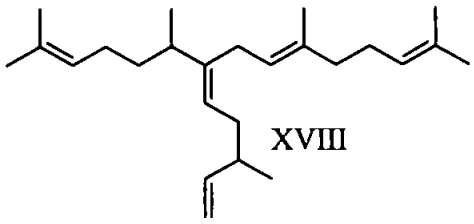
Table 2.1 ^{1}H NMR data for C_{25} pentaenes XVII and XVIII

Chemical shift (ppm)	Assignment XVII	Assignment XVIII	Multiplicity (Coupling constant, Integration)
5.74	23		ddd (J = 17.5, 10.0, 7.0 Hz, 1H)
5.1	3, 9, 13, 20		m (4H)
4.93	24		m (2H)
2.58	6, 8		m (3H)
2.01	4, 11, 12, 21, 22		m (9H)
1.69	18		s (3H)
1.65	1, 15		s (6H)
1.58	16/19		s (3H)
1.55	16/19	18	s (6H)
0.95	25		d (J = 6.5 Hz, 3H)
0.94, 0.93	17		2 x d (J = 6.5 Hz,3H)





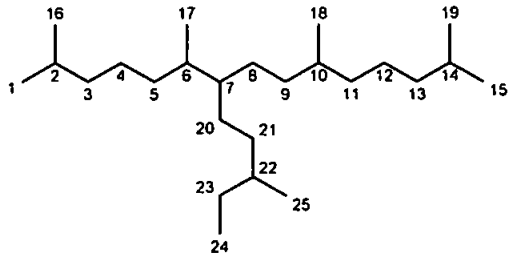
XVII

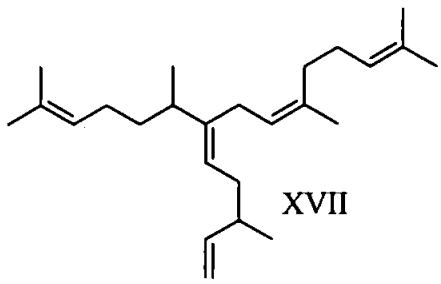


XVIII

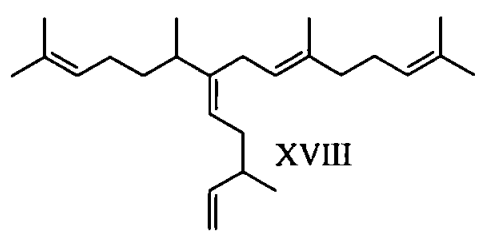
Table 2.2 ^{13}C NMR data for C_{25} pentaenes XVII and XVIII

Chemical shift (ppm)	Multiplicity (DEPT)	Assignment	
		XVII	XVIII
144.5	CH	23	
142.8	C	7	
142.4	C		7
135.6	C	10	
131.4, 131.2, 131.1	C	2, 14	
124.9, 124.4, 123.9, 123.3, 123.0, 122.9	CH	3, 9, 13, 20	
112.1	CH_2	24	
39.8	CH_2		11
38.2	CH	22	
35.2	CH	5	
34.4	CH_2	21	
33.9	CH	6	
31.8	CH_2	11	
29.2	CH_2		8
28.9	CH_2	8	
26.7, 26.6, 26.4	CH_2	4, 12	
25.7	CH_3	1, 15	
23.4	CH_3	18	
19.5	CH_3	25	
19.4	CH_3	17	
17.6	CH_3	16, 19	
15.8	CH_3		18



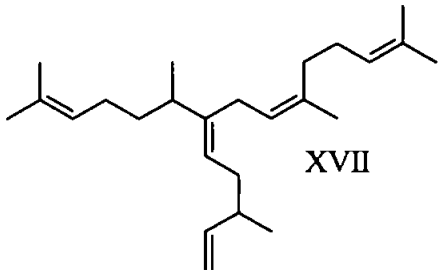


XVII

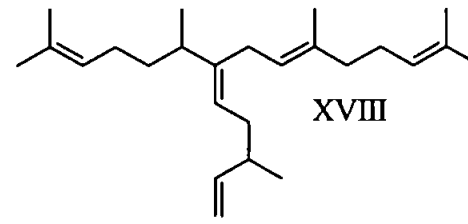


XVIII

and 15.8, 23.4 ppm (C-18) were observed for XVII and XVIII (with XVII being the major isomer). Two further ^{13}C resonances for C-11, each of which correlated with the corresponding H-18 protons in the HMBC spectrum were also observed. Since the alternative position for geometric isomerism (C7-C20) does not possess a CH_3 substituent, the assignment of E/Z isomerism must be restricted to C9-C10. In order to determine the stereochemistry of the C7-C20 double bond, nOe data was obtained for XVII and XVIII. Significantly, nOes were observed between H-6 and H-20, indicating an *E* configuration about the C7-C20 double bond. The stereochemistry of the C9-C10 double bond for XIII and XIV was determined most conveniently by analysis of the chemical shifts for H/C-18 and C-11 obtained from solutions containing different, but known (GC) relative concentrations of the two isomers (Table 2.3). This in turn enabled the GC elution order of the 2 isomers to be determined as *Z* (XVII) before *E* (XVIII).



XVII



XVIII

2.3.4 Characterisation of (6E/Z)-2,6,10,14-tetramethyl-9-(3-methylpent-4-enylidene)-pentadec-6-ene (XI, XII)

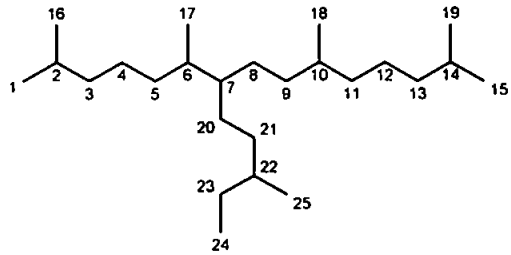
The ^{1}H (Table 2.4) and ^{13}C NMR (Table 2.5) spectra of a mixture of the two trienes isolated from culture PI-1 revealed the presence of a vinyl moiety (C23-C24) together with two trisubstituted double bonds (C9-C10, C7-C20), six allylic (H-6, 11, 21, 22) and two doubly allylic (H-8) protons, twelve (CH_3)$_2$CH protons (H-1, 15, 16, 19), six (CH_3)CH protons (H-

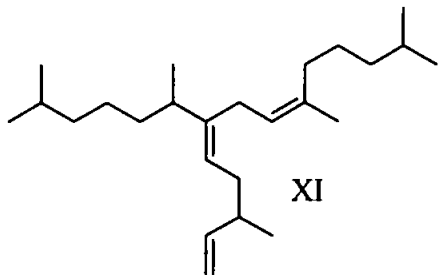
Table 2.3 Relative concentrations of HBI trienes ($C_{25:3}$), tetraenes ($C_{25:4}$) and pentaenes ($C_{25:5}$) together with geometric isomer ratios following extraction of HBIs from five bulk cultures of *P. intermedium*.

HBI	$C_{25:3}$	$C_{25:3}$	$C_{25:4}$	$C_{25:4}$	$C_{25:4}$	$C_{25:4}$	$C_{25:5}$	$C_{25:5}$
RI HP-1	2042	2087	2074	2121	2078	2124	2112	2159
RI HP-5	2044	2091	2083	2130	2087	2136	2126	2172
Structure	XI	XII	XIII	XV	XIV	XVI	XVII	XVIII
Culture 1 (PI-1)								
Relative Amount	1.0	0.78	2.0	1.8	1.3	1.8	15	11
Z/E Ratio	1.3		1.1		0.72		1.4	
Culture 2 (PI-2)								
Relative Amount	1.0	0.48	0.79	0.40	0.21	0.23	0.94	0.58
Z/E Ratio	2.1		2.0		0.91		1.6	
Culture 3 (PI-3)								
Relative Amount	1.0	0.3	8.0	2.3	-	-	9.6	1.8
Z/E Ratio	3.0		3.5		-		5.3	
Culture 4 (PI-4)								
Relative Amount	1.0	0.35	0.54	0.076	-	-	0.18	0.023
Z/E Ratio	2.9		7.1		-		7.7	
Culture 5 (PI-5)								
Relative Amount	1.0	0.54	1.1	0.52	-	-	0.39	0.16
Z/E Ratio	1.9		2.1		-		2.4	

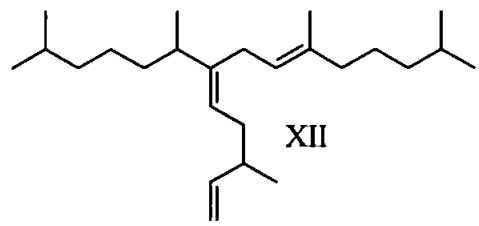
Table 2.4 ^{1}H NMR data for C_{25} trienes XI and XII

Chemical shift (ppm)	Assignment XI	Assignment XII	Multiplicity (Coupling constant, Integration)
5.74	23		ddd (J = 17.1, 10.2, 6.6 Hz, 1H)
5.11	9, 20		m (2H)
4.92	24		d (J = 17.2 Hz, 1H)
4.88	24		d (J = 10.2 Hz, 1H)
2.62	6		m (1H)
2.56	8		m (2H)
2.13	22		m (1H)
1.97	11, 21		m (4H)
1.69	18		s (3H)
1.54		18	s (3H)
1.52	2, 14		m (2H)
1.28	5		m (2H)
1.2	4, 12		m (4H)
1.14	3, 13		m (4H)
0.93	17		d (J = 6.6 Hz, 3H)
0.85, 0.84	1, 15, 16, 19		2 x d (J = 6.6 Hz, 12H)





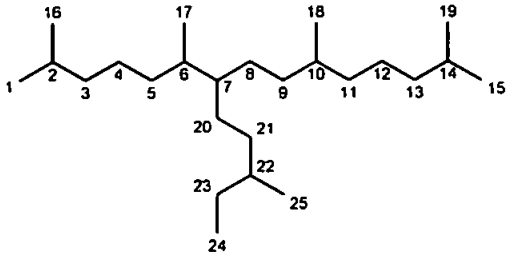
XI

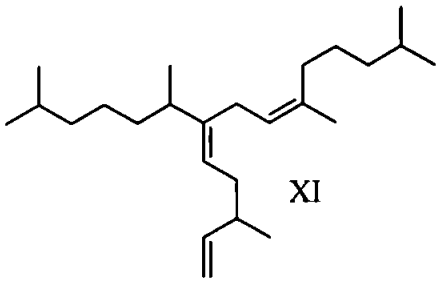


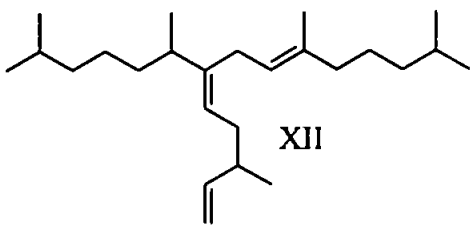
XII

Table 2.5 ^{13}C NMR data for C_{25} trienes XI and XII

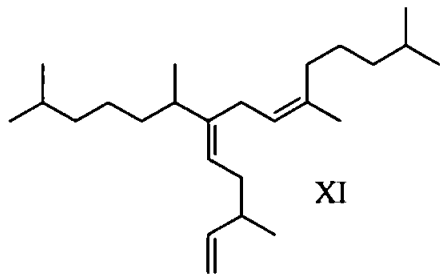
Chemical shift (ppm)	Multiplicity (DEPT)	Assignment XI	Assignment XII
144.6	CH	23	
143.0	C	7	
142.8	C		7
136.1	C	10	
136.0	C		10
123.7	CH	9	
123.2	CH		9
122.9	CH	20	
122.7	CH		20
112.1	CH_2	24	
40.0	CH_2		11
39.3	CH_2	3	
39.0	CH_2	13	
38.7	CH_2		13
38.2	CH	22	
35.3	CH_2	5	
34.4	CH_2	21	
34.3	CH	6	
31.8	CH_2	11	
29.3	CH_2		8
29.0	CH_2	8	
27.9	CH	2, 14	
25.8	CH_2	12	
25.7	CH_2	4	
23.5	CH_3	18	
22.6	CH_3	1, 15, 16, 19	
19.6, 19.5	CH_3	17, 25	
15.7	CH_3		18



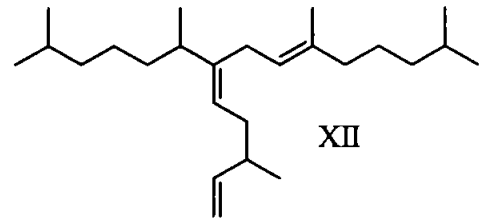




17, 25) and an isolated CH_3 group (H-18). Quaternary (DEPT) alkene ^{13}C resonances were observed for C-7 for each triene consistent with the observations made previously for XVII and XVIII (Section 2.3.3). Additionally, the ^{1}H NMR spectrum of the two trienes revealed two CH_3 singlets (1.54 and 1.69 ppm; H-18) due to the presence of both *E* and *Z* isomers arising from geometric isomerism about the C9-C10 double bond, as observed for the pentaenes (XVII and XVIII). An analogous pair of resonances was observed in the ^{13}C spectrum (23.5 and 15.7 ppm for XI and XII respectively). Thus, the three double bonds were located at C-9(10), C-7(20) and C-23(24). Comparison of the intensities of the resonances for H/C-18 and C-11 in solutions with different but known relative concentrations (GC) of the two trienes (Table 2.3) allowed the GC elution order to be determined as *Z* (XI) before *E* (XII).



XI



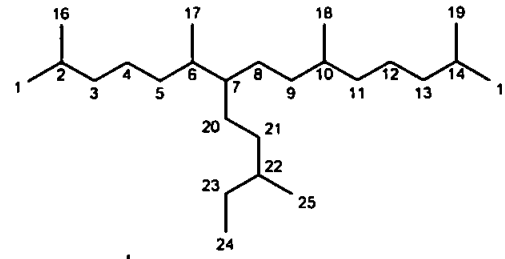
XII

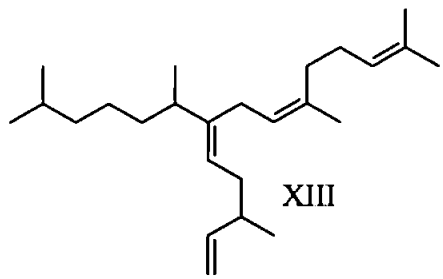
2.3.5 Characterisation of (6E/Z)-2,6,10,14-tetramethyl-9-(3-methylpent-4-enylidene)-pentadeca-2,6-diene (XIII, XV) and (9E/Z)-2,6,10,14-tetramethyl-7-(3-methylpent-4-enylidene)-pentadeca-2,9-diene (XIV, XVI)

The characterisation of the tetraenes isolated from *P. intermedium* was complicated by the presence of four isomeric compounds compared to two for the related trienes (XI, XII) and pentaenes (XVII, XVIII). However, the ^{1}H (Table 2.6) and ^{13}C NMR (Table 2.7) spectra of mixtures of tetraenes from all 5 cultures exhibited features which could be attributable to

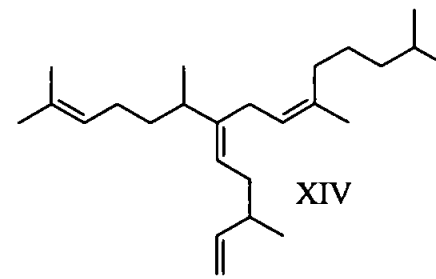
Table 2.6 ^{1}H NMR data for C_{25} tetraenes XIII, XIV, XV and XVI

Chemical shift (ppm)	Assignment				Multiplicity (Coupling constant, Integration)
	XIII	XIV	XV	XVI	
5.73	23	23	23	23	ddd (J = 17.1, 10.2, 6.6 Hz, 1H)
5.11	9, 13, 20	3, 9, 20	9, 13, 20	3, 9, 20	m (3H)
4.92	24	24	24	24	d (J = 17.1 Hz, 1H)
4.88	24	24	24	24	d (J = 10.2 Hz, 1H)
2.62	6	6	6	6	m (1H)
2.56	8	8	8	8	m (2H)
2.13	22	22	22	22	m (1H)
1.99	11, 12, 21	4, 11, 21	11, 12, 21	4, 11, 21	m (6H)
1.53 - 1.69	15, 19, 18	1, 16, 18	15, 19, 18	1, 16, 18	7 x s (9H)
1.52	2	14	2	14	m (1H)
1.28	5	5	5	5	m (2H)
1.2	4	12	4	12	m (2H)
1.14	3	13	3	13	m (2H)
0.95	17, 25	17, 25	17, 25	17, 25	m (6H)
0.86	1, 16	15, 19	1, 16	15, 19	m (6H)

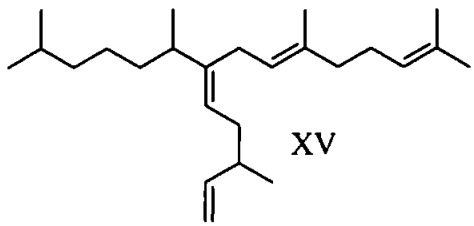




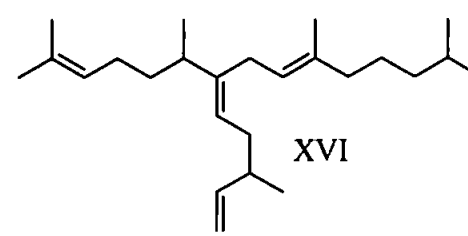
XIII



XIV



XV



XVI

Table 2.7 ^{13}C NMR data for C_{25} tetraenes XIII, XIV, XV and XVI

Chemical shift (ppm)	Multiplicity (DEPT)	Assignment			
		XIII	XIV	XV	XVI
144.6	CH	23	23	23	23
143.0	C	7			
142.8	C		7		
142.7	C			7	
142.5	C				7
136.2, 136.1	C		10		10
135.6	C	10		10	
131.4	C	14			
131.3	C		2		(2)
131.1	C		(2)	14	2
124.4	CH	13			
124.0	CH	9			
122.9	CH	20			
124.9, 124.5, 123.6, 123.4, 123.0, 122.8, 122.7	CH		9, 20	9, 13, 20	9, 20
112.1	CH_2	24	24	24	24
40.0	CH_2				11
39.8	CH_2			11	
39.3	CH_2	3		3	
38.9	CH_2		13		
38.6	CH_2				13
38.2	CH	22	22	22	22
35.2	CH_2	5	5	5	5
34.4	CH_2	21	21	21	21
34.3	CH	6		6	
34.0	CH		6		6
31.9	CH_2	11			
31.8	CH_2		11		
29.3, 29.2	CH_2		8	8	8
28.9	CH_2	8			
27.9	CH	2	14	2	14
26.7	CH_2	12		12	
26.4	CH_2		4		4
25.8	CH_2		12		12
25.7	CH_2	4		4	
25.7	CH_3	15	1	15	1
23.5	CH_3	18	18		
22.6	CH_3	1, 16	15, 19	1, 16	15, 19
19.6, 19.5, 19.4	CH_3	17, 25	17, 25	17, 25	17, 25
17.7, 17.6	CH_3	19	16	19	16
15.8, 15.7	CH_3			18	18

double bonds in each of the 3 positions identified for the trienes XI and XII (viz. C9-C10, C7-C20 and C23-C24) together with *E/Z* isomerisation at C9-C10. In addition, the ^{1}H and ^{13}C data also demonstrate that all solutions of tetraenes contained an equal number of isopropyl ($(CH_3)_2CH$-) and isoprenyl ($(CH_3)_2C$=) moieties irrespective of the composition of the mixture. Therefore, the appearance of 4 tetraenes could be rationalised in terms of 2 structurally isomeric forms, each containing a terminal, trisubstituted double bond at C2-C3 or C13-C14 together with a pair of geometric isomers (C9-C10) for each of these (XIII - XVI).

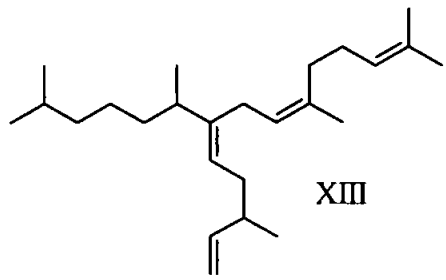
In order to characterise the compounds individually and to determine their GC elution order, examination was made of solutions containing varying proportions of the different tetraenes in a manner similar to that performed for the trienes XI and XII and pentaenes XVII and XVIII (*vide infra*). GC and GC-MS analysis of the tetraene fraction from culture 3 (PI-3; Table 2.3) showed that the major isomer (RI 2074 $_{HP\text{-}1}$) was the first GC-eluting compound observed in PI-1, and that two of the other three tetraenes (RI 2078, 2124 $_{HP\text{-}1}$) were virtually absent. Examination of the ^{1}H and ^{13}C NMR data for this major isomer revealed that the stereochemistry at C9-C10 is *Z* (1.70 ppm (H-18); 23.5 ppm (C-18)).

To determine which terminal position of the main carbon chain was unsaturated (C2-C3 or C13-C14) for the major isomer, the dependence of the ^{13}C chemical shift for C-10 on the nature of C13-C14 observed for the corresponding trienes (XI and XII) and pentaenes (XVII and XVIII) was used. Thus, the ^{13}C resonances for C-10 appeared at 136.1 and 136.0 ppm for the *Z* and *E* isomers XI and XII respectively, with the spectral order and chemical shift separation being the same in all cases studied. In contrast, the corresponding resonances for the related pentaenes XVII and XVIII both appeared at 135.6 ppm due to co-resonance for the two isomers. Therefore, the chemical shift for C-10 is strongly dependent on whether

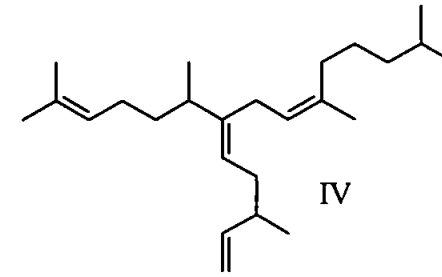
C13-C14 is saturated (e.g. trienes XI and XII) or unsaturated (e.g. pentaenes XVII and XVIII). Importantly, the C-10 resonances for the tetraene fractions from PI-1 and PI-2 exhibited three peaks due to the presence of compounds containing unsaturation at C2-C3 (136.1 and 136.0 ppm) and C13-C14 (135.6 ppm) which verified that the observations made for the C-10 chemical shift from the trienes (XI and XII) and the pentaenes (XVII and XVIII) could be translated to the tetraenes (XIII-XVI). The resonance for the major tetraene isomer from PI-3 appeared as a single peak at 135.6 ppm, which established that C13-C14 must be unsaturated (c.f. pentaenes XVII and XVIII). Since the *Z* configuration (C9-C10) for this isomer was also known, this compound could be assigned to structure XIII (Figure 2.6).

The other tetraene component (RI 2121 $_{HP\text{-}1}$) from this culture (PI-3) could be identified as XV due to the observations of corresponding ^{1}H and ^{13}C resonances for H/C-18 together with the similarity between the mass spectral data obtained for these two isomers (Figure 2.5). Other spectroscopic data for XV also lend further support for unsaturation at the C13-C14 position. Chemical shifts for C-11 (*E* isomer only) for the previously characterised triene XII and pentaene XVIII appeared at 40.0 and 39.8 ppm respectively, while spectra of mixtures containing significant concentrations of all four tetraenes (i.e. PI-1 and PI-2) exhibited two resonances coincident with these. This is consistent with the presence of tetraene isomers possessing unsaturation at both C2-C3 and C13-C14 positions. In the case of PI-3 which contained predominantly XV (i.e. *Z* isomer), there was sufficient quantity of the related *E* isomer to observe a single resonance for C-11 at 39.8 ppm which is most consistent with unsaturation at C13-C14 (c.f. chemical shift of pentaene XVIII *vide infra*). Finally, since the ^{13}C NMR spectra obtained for mixtures of all 4 tetraenes demonstrated that the remaining 2 isomers (RI 2078, 2124 $_{HP\text{-}1}$) had a double bond in the C2-C3 position, and that for each of the triene, tetraene and pentaene pairs (XI and XII, XIII and XV, XVII and XVIII) the GC elution order was *Z* before *E*, it can be inferred that the tetraenes with RIs

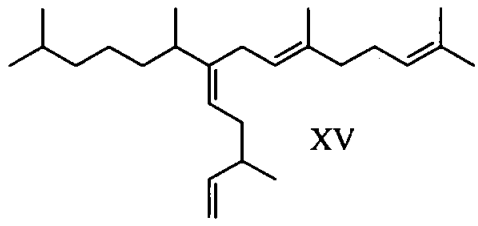
2078 and 2124 $_{HP\text{-}1}$ could be assigned to XIV and XVI respectively (n.b. the mass spectra for these two compounds were also very similar (Figure 2.5) which supported these assignments). Therefore, the structures of the four tetraenes identified in cultures of *P. intermedium* were determined as XIII – XVI.



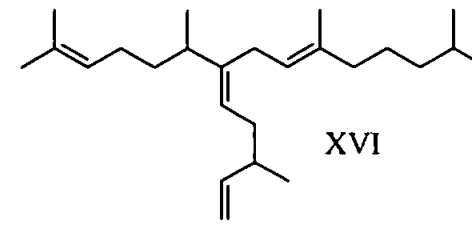
XIII



IV



XV



XVI

2.3.6 Determination of the stereochemical configuration of HBIs from P. intermedium

The C_{25} HBIs isolated and characterised from *P. intermedium* (XI – XVIII) all contain chiral centres at C-6 and C-22. In order to determine the absolute stereochemical configuration of the chiral centre at C-22, the HBIs from cultures PI-2 and PI-4 were hydrogenated (PtO_2. $.2H_2O$/hexane) to the C_{25} alkane (I), then oxidised (CrO_3/AcOH) to yield mixtures containing 3-methylpentanoic (3MP) and 4-methylhexanoic acids (4MH; Figure 2.7). 3MP derives from oxidation at C-20 and 4MH from oxidation at C-7 of the HBI alkane (I). In each of these acids, the stereochemistry at one of the original chiral centres (C-22) is

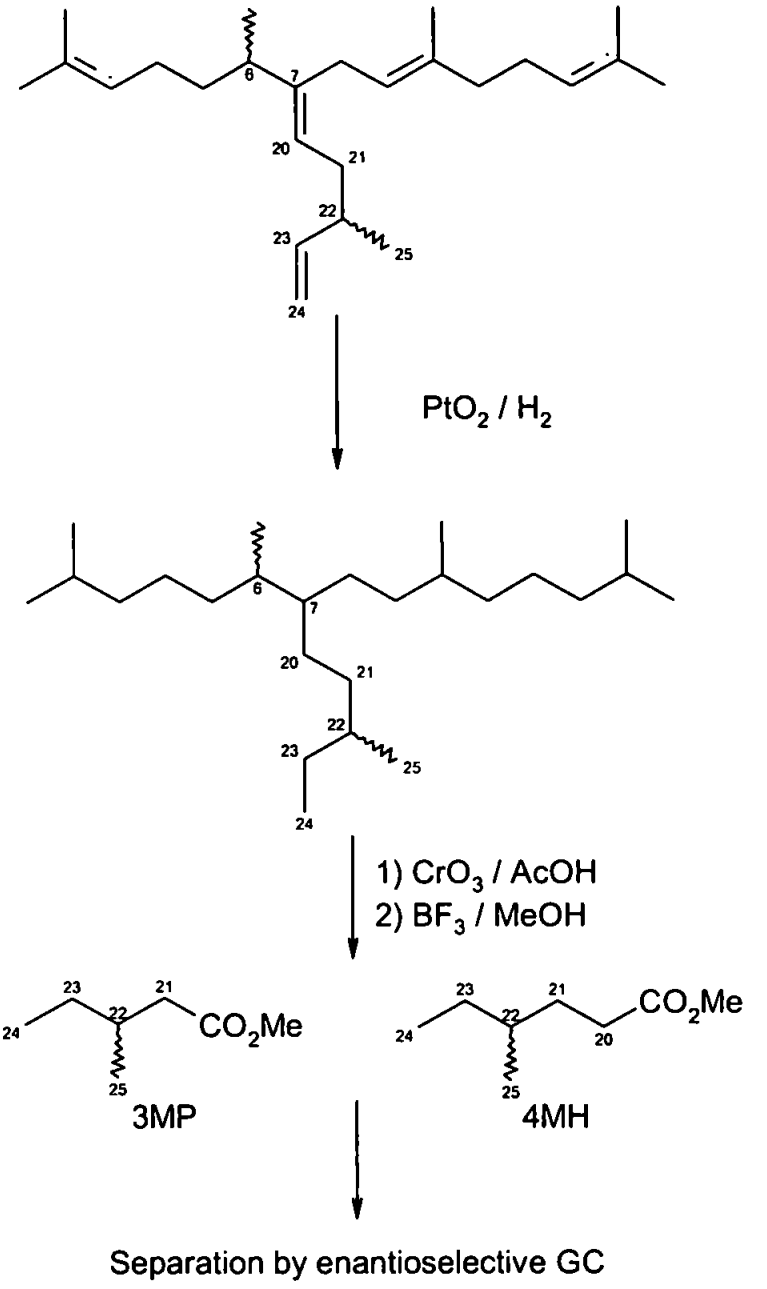


Figure 2.7 Determination of the stereochemical configuration of HBIs. Numbering shown for 3MP and 4MH is that of the original HBI.

preserved (in C-3 and C-4 of 3MP and 4MH respectively). Methylation of the chiral acids (3MP and 4MH; BF_3/methanol complex) allowed the identification of the absolute stereochemical configuration at the original C-22 chiral centre *via* co-chromatography with authenticated chiral standards using enantioselective GC (König *et al.*, 1988; Johns *et al.*, 2000). For each of the HBIs investigated, the acids produced were predominantly (>90%) homochiral with a S configuration at C-3 and C-4 for 3MP and 4MH respectively. This corresponds to a 22S configuration in alkane (I), and thus an R configuration at C-22 for the HBI alkenes (XI – XVIII).

Although it was not possible to determine the absolute configuration at C-6 (the remaining chiral centre) due to the absence in the oxidation products of acids such as 2,6-dimethylheptanoic acid which would incorporate this carbon, the NMR data reported here suggests a fixed configuration (either R or S) at C-6 for the HBIs from each of the cultures studied. Diastereomeric mixtures of C_{25} HBIs typically exhibit a characteristic 'doubling up' of resonances in the ^{13}C NMR spectrum (Belt *et al.*, 1996; Johns *et al.*, 1999), and C_{25} HBIs from *H. ostrearia* (which have chiral centres at C-7 and C-22) have been identified with a fixed configuration at C-7 and a variable configuration at C-22 (Johns *et al.*, 2000). The absence of any such 'doubling up' of resonances in the ^{13}C NMR spectra of the HBIs from *P. intermedium* suggests a fixed stereochemistry at both C-6 and C-22 for the cultures studied here.

2.4 Identification and characterisation of C_{25} HBIs in other members of the *Pleurosigma* genus

Whilst the identification of HBIs in the benthic diatom *P. intermedium* can account for the occurrence of C_{25} HBIs in sediments, there are also numerous reports of these compounds in

water column particles (e.g. Prahl *et al.*, 1980; Osterroht *et al.*, 1983; Volkman *et al.*, 1983; Albaiges *et al.*, 1984; Bates *et al.*, 1984; Matsueda and Handa, 1986; Wakeham, 1990) which could be better accounted for by a planktonic, HBI producing diatom. Whilst *R. setigera* is such a diatom, to date the only reported compounds from this organism are an uncommon C_{25} pentaene (Sinninghé Damste *et al.*, 1999a,b) or a group of C_{30} penta- and hexaenes (Volkman *et al.*, 1994, 1998). Thus, *R. setigera* is probably not the sole source of C_{25} HBIs in the deep sea. Therefore, it was decided to investigate the production of HBIs by planktonic species of the *Pleurosigma* genus. The diatoms *Pleurosigma sp.* and *P. planktonicum* were chosen for this purpose.

2.4.1 Chromatographic and mass spectral analysis of HBIs in Pleurosigma sp.

Two small scale cultures of *Pleurosigma.* sp., isolated from phytoplankton in surface waters at Le Croisic (France), contained C_{25} HBI tetraenes (XIII, XIV, XV and XVI) and pentaenes (XVII and XVIII) as described above for the related *P. intermedium*. Assignment of HBI isomers was made by co-chromatography and comparison of MS with the authenticated compounds isolated from *P. intermedium*. Thus, in the two different cultures, tetraenes IV and V and pentaenes VI and VII were identified in the non-saponifiable lipid fractions (Figure 2.8 a, b). Interestingly, the trienes XI and XII were absent from the cultures studied here.

2.4.2 Chromatographic and mass spectral analysis of HBIs in P. planktonicum.

Following isolation from the phytoplankton in surface waters from Le Croisic (France), *P. planktonicum* was cultured on a large scale (400 l). Extraction and purification of the non-saponifiable lipids (NSLs) obtained from this culture yielded 6 mg of a mixture of three compounds whose chromatographic (RI 2163 and 2198 $_{HP\text{-}1}$) and mass spectral properties

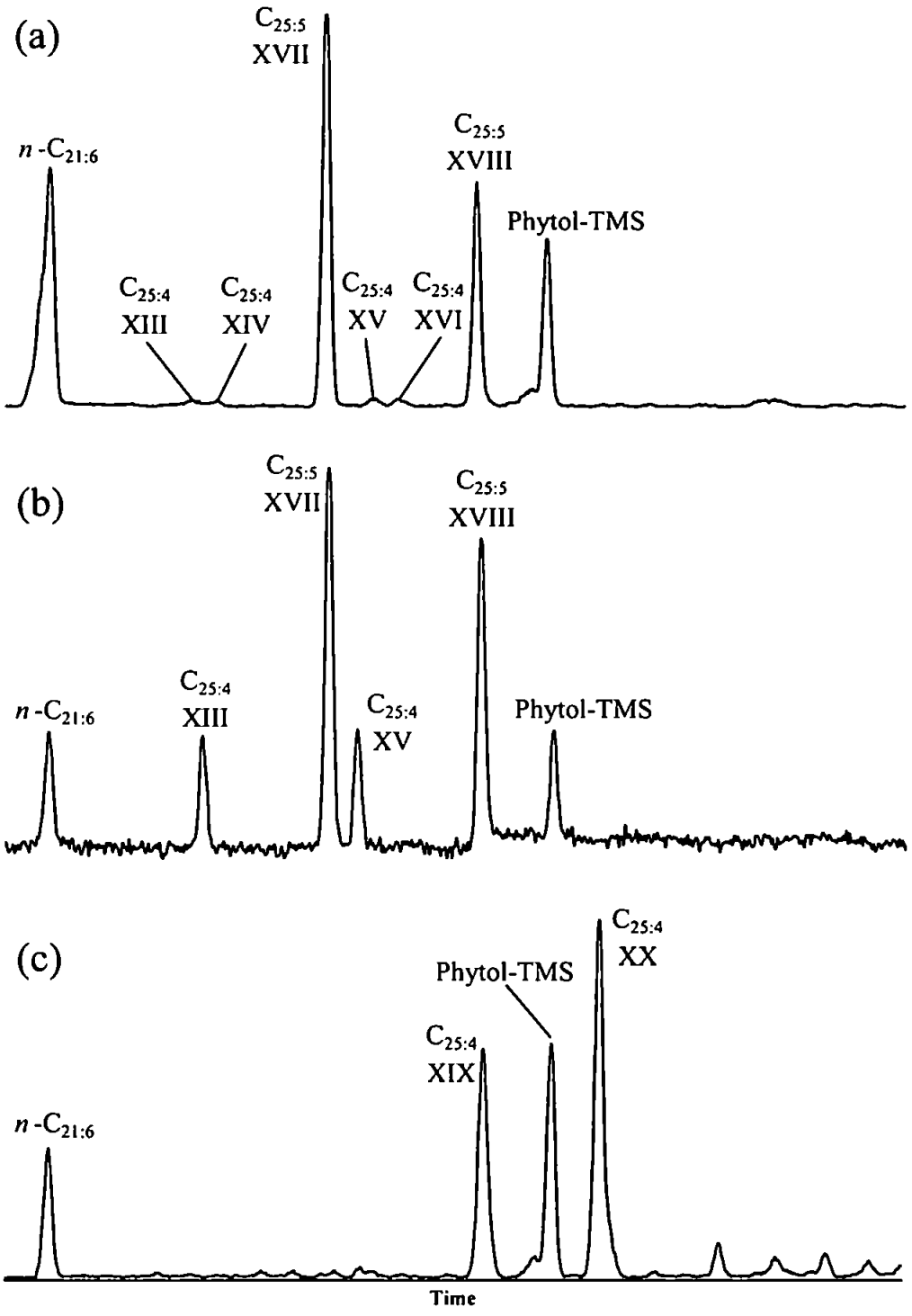


Figure 2.8 Partial TIC chromatograms of the non-saponifiable lipid fractions obtained from cultures of *Pleurosigma* sp. (a,b) and *P. planktonicum* (c).

($M^{+\cdot}$ 344) were consistent with the presence of two isomeric C_{25} HBI tetraenes together with a small quantity of *n*-$C_{21:6}$ (Figure 2.8 c). The mass spectra of the two HBI isomers XIX and XX (Figure 2.9) were indistinguishable, indicating stereo- rather than positional isomerism. Hydrogenation of an aliquot of this mixture ($PtO_2.2H_2O$/hexane/H_2) resulted in the formation of a compound, identified as the parent hydrocarbon $C_{25:0}$ (I) by comparison of its GC retention index (RI 2110 $_{HP\text{-}1}$) and mass spectrum with that of an authentic standard.

2.4.3 Characterisation of (9E/Z)-2,6,10,14-tetramethyl-7-(3-methylpentyl)pentadeca-2,5,9,13-tetraene (XIX, XX)

^{1}H NMR analysis (Table 2.8) of the two HBI tetraenes revealed the presence of tri-substituted double bonds (δ 5.06 ppm), but surprisingly, the absence of any resonances associated with a vinyl moiety, a structural feature which is common to all other HBI alkenes that have been rigorously characterised by NMR spectroscopy (Belt *et al.*, 1996; Sinninghe Damsté *et al.*, 1999b; Wraige *et al.*, 1997) including those identified in *P. intermedium*. ^{13}C NMR analysis (Table 2.9) confirmed this observation with exclusive detection of resonances attributable to tri-substituted alkenes. Further, both ^{1}H (Table 2.8) and ^{13}C NMR (Table 2.9) spectra verified the presence of an ethyl moiety as the terminal group of the main side chain. The absence of any isopropyl groups enabled two double bonds to be positioned at C2-C3 and C13-C14, while the positions of the other two double bonds could be located by detailed analysis of the NMR spectra and comparison of published data for compounds with related structures.

The appearance of two closely related compounds was explained by the presence of two geometric isomers (C9-C10), as described in sections 2.3.3 – 2.3.5 above. This included the observation of characteristic resonances (^{1}H and ^{13}C) associated with (*E* and *Z*) H/C-18. In

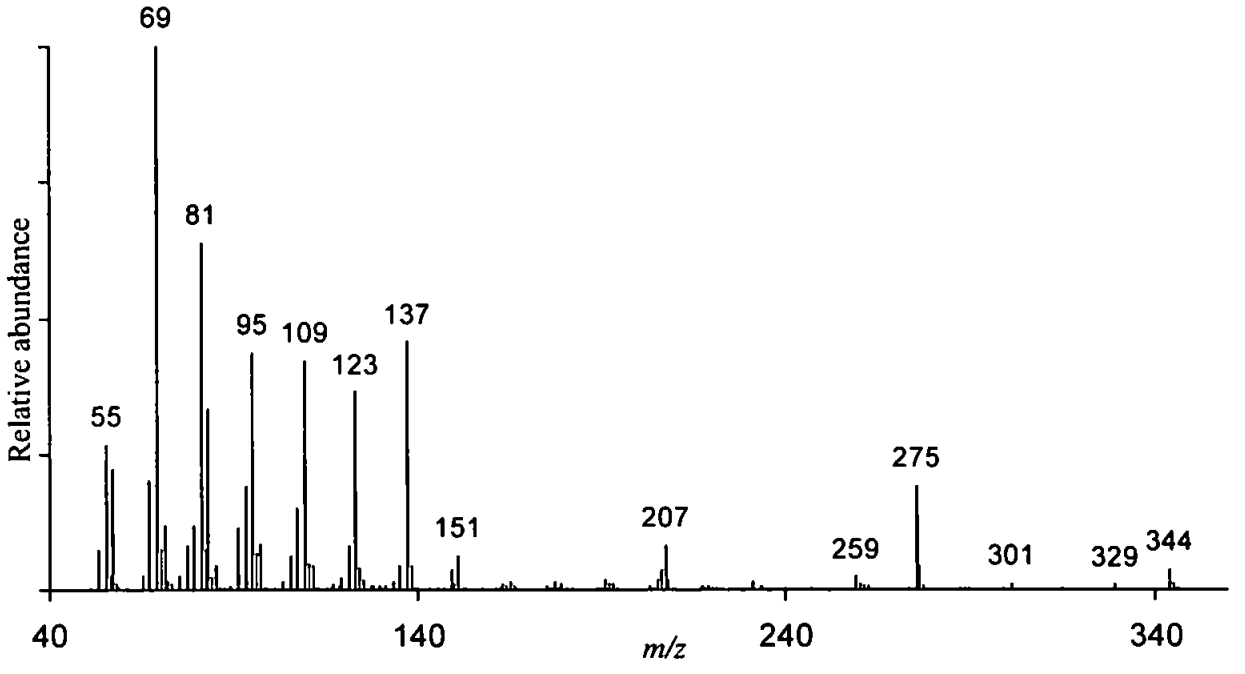
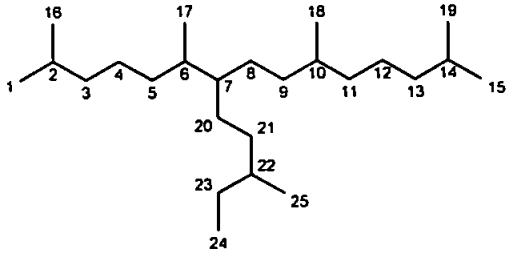
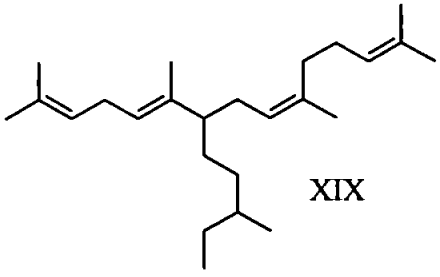


Figure 2.9 Mass spectrum of C_{25} HBI tetraene XIX (n.b. the spectrum of XX is the same as that shown for XIX)

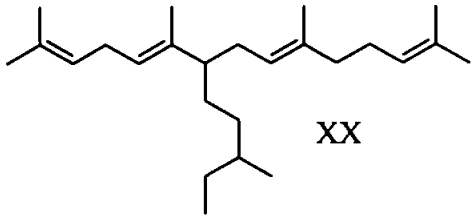
Table 2.8 ^{1}H NMR data for C_{25} tetraenes XIX and XX

Chemical shift (ppm)	Assignment XIX	Assignment XX	Multiplicity (Coupling constant, Integration)
5.06	3, 5, 9, 13		m (4H)
2.66	4		t (J = 7 Hz, 2H)
1.99	7, 8, 11, 12		m (7H)
1.67	1, 15, 18	1, 15	s
1.60, 1.58, 1.55	16, 19		s
1.54		18	s
1.46	17		s (3H)
1.04 - 1.35	20, 21, 22, 23		m (7H)
0.85	24, 25		m (6H)





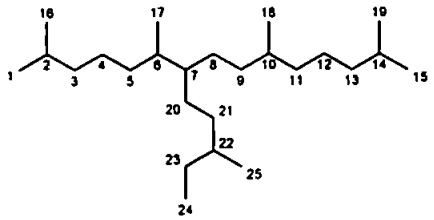
XIX

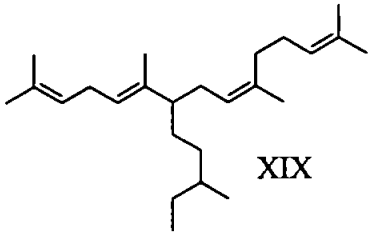


XX

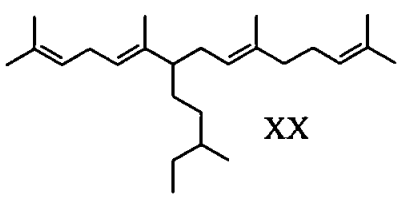
Table 2.9 ^{13}C NMR data for C_{25} tetraenes XIX and XX

Chemical shift (ppm)	Multiplicity (DEPT)	Assignment	
		XIX	XX
136.7			6
136.6		6	
135.0		10	
134.8			10
131.4		2	
131.2		14	
124.4, 124.3, 124.2		3, 5, 13	
123.6			9
123.5		9	
49.4		7	
49.2			7
39.8			11
34.3		22	
34.2		21	
32.4			8
32.2		8	
31.9		11	
30.2		20	
30.0		23	
26.7, 26.8		4, 12	
25.6		1, 15	
23.4		18	
19.1		25	
17.7		16, 19	
16.1			18
11.9			17
11.8		17	
11.5		24	



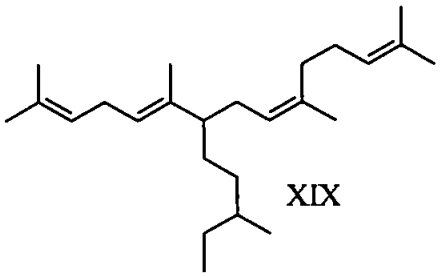


XIX

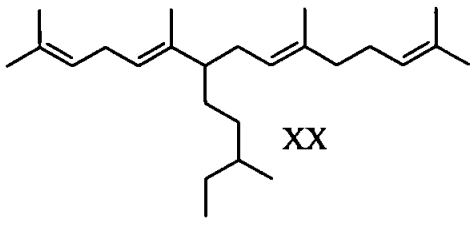


XX

addition, since the two isomers were present in a *ca.* 2:1 ratio (*E*:*Z*), the GC elution order was also established (*viz.* Z before E). This elution order is the same as that found for other HBIs possessing geometric isomerism in the C9-C10 position. Thus, the structures of the two previously uncharacterised C_{25} HBI tetraenes isolated from *P. planktonicum* have been identified as XIX and XX.



XIX



XX

2.5 Discussion

P. intermedium, *P. planktonicum* and *Pleurosigma sp.* are demonstrably producers of a range of HBI alkenes, as are *H. ostrearia* and *R. setigera* (e.g. Volkman *et al.*, 1994). Whilst the cultures examined herein were not axenic (cf. Wraige *et al.*, 1999), the quantities of HBIs produced argue against a bacterial source.

2.5.1 Taxonomy and distribution of Pleurosigma species.

The genus *Pleurosigma* belongs to the same Class (Bacillariophyceae) as the other known producers of HBIs (*viz H. ostrearia* and *R. setigera*). However, the taxonomy of *Pleurosigma* lies closer to *H. ostrearia* than to *R. setigera* since they share the same Order (Pennales) and Family (Naviculaceae) (cf. Order Centrales for *R. setigera* (Simonsen,

1974)). The taxonomy of *P. intermedium* and *P. planktonicum* have been described in detail elsewhere (Peragallo, 1890-1891; Peragallo and Peragallo, 1897-1908; Hendey, 1964; Cardinal *et al.*, 1986, 1989; Simonsen, 1974; Boalch and Harbour, 1977). The species identity of *Pleurosigma* sp. has not been confirmed, although it is certainly a planktonic member of the *Pleurosigma* genus (Massé, personal communication).

P. intermedium has been reported in UK (Smith, 1853; Hendey, 1964), French (Peragallo, 1890-1891; Peragallo and Peragallo, 1897-1908) and Canadian (Cardinal *et al.*, 1986, 1989) coastal sediments and it has also been observed in Arctic regions (Cleve and Grunow, 1880). Peragallo (1890-1891) concludes that *P. intermedium* has a widespread global occurrence. Nichols *et al.* (1988) described the presence of an unknown *Pleurosigma* species in mixed communities of Antarctic sea-ice diatoms. Interestingly, the hydrocarbons of this mixture contained a C_{25} HBI diene.

2.5.2 Structural features of HBIs from P. intermedium and Pleurosigma sp.

The results described herein have identified new source diatom species (*P. intermedium*, *Pleurosigma* sp. and *P. planktonicum*) for C_{25} HBI trienes, tetraenes and pentaenes. The structural characterisation of these previously uncharacterised compounds was achieved using NMR spectroscopy and mass spectrometry. The RI and MS data for these compounds allows comparison with other geochemical reports of HBIs. The structures of the eight new HBIs identified in *P. intermedium* and *Pleurosigma* sp. show similarities but important differences to those reported previously from *H. ostrearia* (Belt *et al.*, 1996; Johns *et al.*, 1999; Wraige *et al.*, 1997, 1999) and *R. setigera* (Sinninghe Damsté *et al.*, 1999b). Thus, while the new trienes from *P. intermedium* (XI and XII) have 2 double bonds whose positions are common with trienes from *H. ostrearia* (viz. C9-C10 and C23-C24 positions),

the third double bond is located at the C7-C20 position, resulting in an unsaturated branch point. This is in contrast to previously reported HBIs from *H. ostrearia* (Belt *et al.*, 1996; Johns *et al.*, 1999; Wraige *et al.*, 1997, 1999) and *R. setigera* (Sinninghe Damsté *et al.*, 1999b), in which C-7 is saturated (sp^3 hybridised). Further similarities are noted for the related tetraenes (XIII-XVI) which contain an additional double bond in either the C2-C3 (XV and XVI) or C13-C14 (XIII and XIV) positions compared with trienes XI and XII. (n.b. only tetraenes with a double bond in the C13-C14 position have been observed to date from *H. ostrearia* (Belt *et al.*, 1996)). A similar structural correlation can be made with the pentaenes XVII and XVIII since these contain double bonds in both C2-C3 and C13-C14 positions.

Of additional note are the stereochemical features of these new compounds, which include the presence of both *E* and *Z* isomers at position C9-C10, with the relative concentrations of these dependent on the cultures from which they have been isolated (Table 2.3). In contrast, mixtures of related stereoisomers due to isomerisation at C7-C20 are not observed. This type of geometric isomerisation has not been reported for HBIs from other diatoms although structural isomerism is common (Belt *et al.*, 1996; Johns *et al.*, 1999; Sinninghe Damsté *et al.*, 1999b; Wraige *et al.*, 1997, 1999). Further, all eight newly characterised HBIs from *P. intermedium* have two asymmetric centres at C-6 and C-22 with consequential potential for further stereoisomerism. However, both NMR and chromatographic evidence for XI – XVIII indicates a fixed configuration at each of these two chiral centres and that each compound exists as a single enantiomer. Thus, each HBI elutes as a single peak on both HP-1 and HP-5 GC stationary phases, while in solution (NMR), there is no doubling of ^{1}H or ^{13}C resonances, a feature that is common for mixtures of HBI diastereoisomers (Belt *et al.*, 1996), and determination of the absolute stereochemical configuration *via* oxidation indicated a fixed R configuration at C-22 for all HBI alkenes studied from *P. intermedium*. This apparent

enantioselectivity in the biosynthesis of HBIs by *P. intermedium* is in contrast to that found for many other HBIs, where the presence of configurational diastereoisomerism has been reported (Belt *et al.*, 1996; Wraige *et al.*, 1999; Johns *et al.*, 2000). In summary, all HBIs found in *P. intermedium* and *Pleurosigma* sp. have double bonds that are common to all eight compounds characterised (viz. C7-C20, C9-C10 and C23-C24) with the tetraenes (x4) and pentaenes (x2) containing an additional 1 and 2 isoprenyl moieties respectively.

2.5.3 Structural features of HBIs from P. planktonicum

The C_{25} tetraenes (XIX and XX) isolated from *P. planktonicum* share structural similarities with HBIs from *H. ostrearia, R. setigera, P. intermedium* and *Pleurosigma sp.* The presence of a saturated branch point (C-7) and a C-5(6) double bond is a common feature of the HBIs previously characterised from *H. ostrearia* and *R. setigera* (Belt *et al.*, 1996; Johns *et al.*, 1999; Sinninghe Damsté *et al.*, 1999b; Wraige *et al.*, 1997, 1999). However, while *E/Z* isomerism (C9-10) is a structural feature of HBIs from *P. intermedium* and *Pleurosigma* sp., the absence of a vinyl moiety (C-23(24)) has not been reported previously for any polyunsaturated HBI alkene. Although stereoisomerism about the C-9(10) double bond appears to be a characteristic of the HBIs produced by several *Pleurosigma* spp., in *P. intermedium* and *Pleurosigma* sp., such stereoisomerism is observed in conjunction with an unsaturated (C-7) major branch point, whilst the tetraenes from *P. planktonicum* are saturated at C-7.

2.5.4 HBIs found in sediments, particles and biota

P. intermedium and *Pleurosigma* sp. have proved to be a valuable source of a range of novel HBIs as described here and these authenticated compounds have allowed for the first time, identification of most of the previously unidentified HBIs in sediments, biota and

sedimentary particles. Thus, the RI and MS properties for these HBI trienes, tetraenes and pentaenes (XI – XVIII) are all in excellent agreement with those described by other authors who have reported HBIs in geochemical samples. Barrick *et al.* (1980) identified two C_{25} HBI trienes and tetraenes in sediments from Puget Sound, USA whose RIs ($C_{25:3}$ 2044, 2090 $_{SP\text{-}2100}$; $C_{25:4}$ 2078, 2124 $_{SP\text{-}2100}$) are very similar to those found for XI, XII, XIII and XV (Table 2.10). Further, a comparison of the mass spectra for XI or XII with those reported by Barrick *et al.* (1980) for the Puget Sound trienes shows an excellent correlation between common ions (e.g. *m/z* 346, 291, 233, 165, 83 and 69) and their relative intensities. A similar correlation can be made between XIII or XV and the two tetraenes reported in the same sediments (Barrick *et al.*, 1980). Thus, the HBIs reported by Barrick *et al.* (1980) can be identified as being XI, XII, XIII and XV. C_{25} HBI trienes and tetraenes have also been reported in sediment traps collected from Puget Sound (Bates *et al.*, 1984) and from neighbouring Washington coastal sediments (Prahl and Carpenter, 1984), though the RI data of the latter were not reported, precluding confirmation of their identities here.

Interestingly, on the basis of identical differences in RIs between the trienes and tetraenes found in Pugent Sound sediments ($\Delta RI = 46$ $_{SP\text{-}2100}$), Barrick *et al.* (1980) suggested that each triene and tetraene existed as a geometric pair due to (E/Z) isomerism about one double bond. This was neither confirmed nor the position of the appropriate double bond identified. Volkman *et al.* (1983) also reported a suite of four C_{25} HBIs in sediments and sediment traps from the coast of Peru (Table 2.10) and noted that their chromatographic and mass spectral features were consistent with the same suite of compounds described by Barrick *et al.* (1980). A similar report of C_{25} trienes and tetraenes was made by Smith *et al.* following GC analysis (OV-1) of sediments from the Peru upwelling region (Smith *et al.*, 1983). In a later report, Porte *et al.* (1990) identified C_{25} HBI trienes and tetraenes in bivalves collected from the Todos os Santos Bay (Brazil) and noted similar RI differences ($\Delta RI = 47$ $_{DB\text{-}5}$) associated

Table 2.10 Correlation between the HBIs isolated from *P. intermedium* with sedimentary reports. Assignments are made on the basis of both GC (RI) and MS data.

Reference	This Report		Barrick *et al.* 1980	Volkman *et al.* 1983	Porte *et al.* 1990	Prahl *et al.* 1980	Requejo *et al.* 1984	Requejo and Quinn 1983; 1985	Albaiges *et al.* 1984	Osterroht *et al.* 1983	Shaw *et al.* 1985	Voudrais and Smith 1986	Vankatasen 1988	Matsueda and Handa 1986[a,b]
Column Phase	HP-1	HP-5	SP-2100	SP-2100	DB-5	SP2100	SE-30	SE-30	DB-5	SP2100	OV101	SE-52	DB-5	SE-52
Compound														
$C_{25:3}$ XI	2042	2044	2044	2044	2044			2044					2046	2047
$C_{25:3}$ XII	2087	2091	2090	2090	2091	2090	2091	2091	2091	2089	2092	2091		2092
$C_{25:4}$ XIII	2074	2083	2078	2078	2079									2083
$C_{25:4}$ XIV	2078	2087			2086									
$C_{25:4}$ XV	2121	2130	2124	2124	2126									
$C_{25:4}$ XVI	2124	2136			2133									
$C_{25:5}$ XVII	2112	2126			2124									
$C_{25:5}$ XVIII	2159	2172			2169									

with pairs of HBIs possessing identical mass spectra (Table 2.10). These authors described the presence of two trienes (RI 2044, 2091 $_{DB-5}$) and two tetraenes (RI 2079, 2126 $_{DB-5}$) whose chromatographic and mass spectral properties were very similar to those found by Barrick *et al.* for the HBIs found in Puget Sound sediments (Barrick *et al.*, 1980). In addition, Porte *et al.* reported two, previously unreported tetraenes whose mass spectra were identical to each other yet different from the other pair of tetraenes found in the same bivalves. For all three pairs of HBIs (two tetraenes and one triene pair) the ΔRI was found to be 47, lending further support to a E/Z geometric isomerism assignment. Comparison of the RIs and mass spectra for the two pairs of tetraenes and the triene pair reported by Porte *et al.* (1990) with those obtained for the HBIs described here from *Pleurosigma intermedium* and *Pleurosigma* sp. indicates that they are the same compounds. Thus, as a result of the structural, chromatographic and mass spectral characteristion of XI - XVIII, the proposal by Barrick *et al.* (1980) and Porte *et al.* (1990) that HBI trienes and tetraenes may exist as mixtures of geometric isomers has now been verified and, importantly, the positions of the double bonds resulting in the geometric isomerism have been identified.

Porte *et al.* (1990) also noted that bivalves analysed from the Todos os Santos Bay contained four C_{25} pentaenes (Table 2.10) with two of these (RI 2144, 2169 $_{DB-5}$) possessing identical mass spectra. On the basis of matching RI and mass spectral data, it is proposed that two of these pentaenes (RI 2124, 2169 $_{DB-5}$) correspond to the HBIs XVII and XVIII (RI 2126, 2172 $_{HP-5}$). It is also possible that the two other pentaenes described in the bivalves from Todos os Santos Bay may have been formed by rearrangement of XVII and XVIII, as the isomerisation of the double bonds of other HBI alkenes has been found to be facile under mildly acidic conditions (Belt *et al.*, 2000).

There are a number of other reports of sedimentary HBIs that can reasonably be assigned to those characterised and described here *via* combined analyses of GC (RI) and mass spectral data. Prahl *et al.* (1980) reported the occurrence of a compound associated with sedimentary particulates from Dabob Bay, Washington whose RI (2090 $_{SP2100}$) and mass spectrum, together with those of the hydrogenation product, were found to be identical to one of the trienes reported by Barrick *et al.* (1980) and can therefore be assigned as XII. Evidence for three additional HBIs in the Dabob Bay sediment samples analysed by Prahl *et al.* (1980) was presented by Volkman *et al.* (1983) following comparison of the gas chromatographic data with that obtained by these authors from Peruvian sediments (*vide infra*); these corresponding to compounds XI, XIII and XV. Requejo *et al.* (1984) reported the identification of several C_{25} HBIs in sediment cores taken from the Pettaquamscutt River, Rhode Island including three dienes and a triene. Although the mass spectrum of the triene was not reported, the RI (2091 $_{SE-30}$) is consistent with triene XII. In further studies, Requejo and Quinn (1983; 1985) reported high concentrations of acyclic and cyclic isoprenoids in sediments from Narrangansett Bay, Rhode Island and a neighbouring salt marsh including two C_{25} acyclic trienes. The RIs of these (2044, 2091 SE-30) are in close agreement with those found for XI and XII respectively (Table 2.10).

By further use of RI and mass spectral data, compound XII can be assigned to HBI trienes found in particulate fractions from the Ebro Delta, Spain (RI 2091 $_{DB-5}$; Albaigés *et al.*, 1984), particulates from the Kiel Bight (Germany; RI 2089 $_{SP2100}$; Osterroht *et al.*, 1983), sediments from the Port Valdez (Alaska, USA; RI 2092 $_{OV101}$; Shaw *et al.*, 1985) and hydrocarbon extracts from 3 Eastern Virginia estuarine creeks (RI 2091 $_{SE-52}$; Voudrias and Smith, 1986). Similarly, reports of a compound from M^{c}Murdo Sound, identified by mass spectrometry as $C_{25}H_{46}$ (Antarctica; RI 2046 $_{DB-5}$; Venkatesan, 1988) can be assigned as the isomeric triene XI. Trienes XI and XII (RI 2047, 2092 $_{SE-52}$) together with one of the

tetraenes (XIII, RI 2083 $_{SE-52}$) have been reported in zooplankton faecal pellets in sediment traps from the eastern North Pacific Ocean (Matsueda and Handa, 1986 a, b). These assignments are summarised in Table 2.10.

The geochemical significance of the isomeric tetraenes (XIX and XX) produced by *P. planktonicum* is unknown at this stage. It has not been possible to find any reports of these isomers in sediments or in the water column, suggesting that *P. planktonicum* is not a major contributor of HBIs in the geosphere. It is possible that *P. planktonicum* is not a sufficiently abundant diatom species in marine plankton communities. Alternatively, the isomers produced by *P. planktonicum* may be more susceptible to diagenetic or degradative processes, or the unusually high retention indices (for C_{25} HBIs) associated with these compounds (RI 2163 and 2198 $_{HP-1}$) may mean that they have not been identified as HBIs in previous analyses.

2.5.5 Potential early diagenesis of HBIs from P. intermedium and *Pleurosigma sp.*

In addition to the determination of the structures of common sedimentary HBIs, the compounds described here may provide an insight into the potential sources of diagenetic products of HBIs in sediments. These include sulphurised derivatives of HBIs (viz. HBI thiolanes and thiophenes, HBITs) which have been reported in a variety of geochemical samples (see e.g. Kohnen *et al.*, 1990; Sinninghe Damsté and de Leeuw, 1990; Sinninghe Damsté *et al.*, 1989). Further, it is also known that sulphur –rich macromolecular aggregates are important geochemical sinks for HBI alkenes (Bosch *et al.*, 1998). In the cases of well characterised HBITs (e.g. XXI, XXIII-XXIV, Figure 2.10), the positions of the thiolane and thiophene moieties are in poor agreement with the double bond positions in HBIs from diatoms such as *H. ostrearia* and *R. setigera* (Belt *et al.*, 1996; Johns *et al.*, 1999; Sinninghe

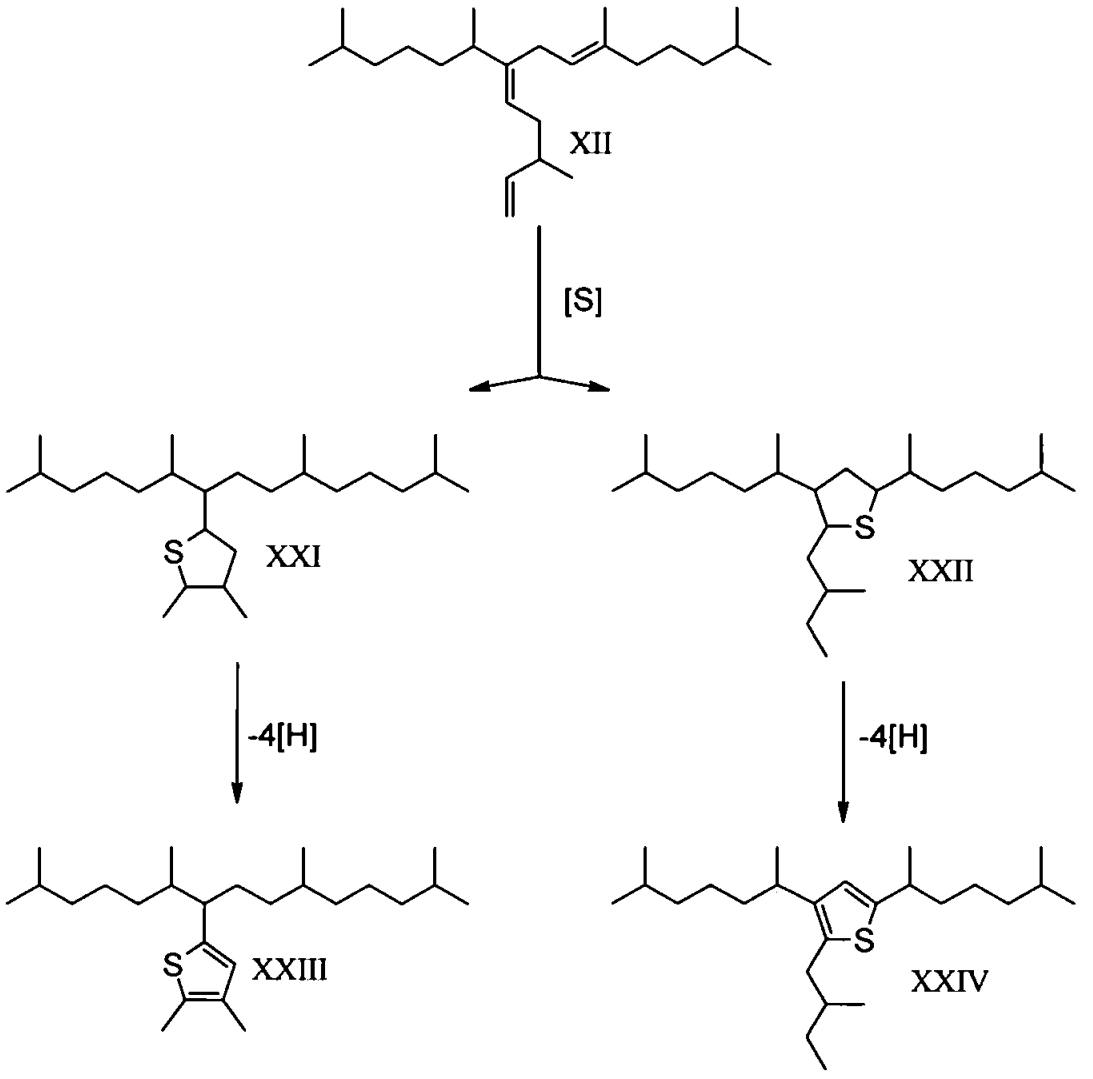


Figure 2.10 Potential route for diagenetic incorporation of sulphur into HBI trienes to yield HBI thiolanes and thiophenes (HBITs).

Damsté *et al.*, 1999b; Wraige *et al.*, 1997, 1999) and so incorporation of sulphur into these HBI isomers to yield these HBITs seems unlikely. In contrast, the presence of an unsaturated branch point (C7-C20) together with double bonds in the C9-C10 and C23-C24 positions for HBIs XI-XVIII isolated from *P. intermedium*, provides the possibility for sulphur incorporation to yield (Figure 2.10) HBITs that have been reported and structurally verified (Sinninghe Damsté *et al.*, 1989; Kohnen *et al.*, 1990). Thus, HBI alkenes from *P. intermedium* and *Pleurosigma sp.* are realistic precursors to sedimentary HBITs. Laboratory experiments to simulate these transformations should be conducted in the future.

2.6 Conclusion

The structures of ten C_{25} alkenes (XI – XX) have been rigorously characterised. The diatoms *P. intermedium*, *P. planktonicum* and *Pleurosigma* sp. have been identified as new diatom sources for HBI alkenes. Eight of the HBIs characterised herein (XI – XVIII) are widespread in the geosphere, and the identification of the most common and abundant HBIs, first reported by Gearing *et al.* (1976) has now been accomplished. Additionally, viable precursors for geochemically significant C_{25} HBI thiophenes and thiolanes have been identified, and investigations into the incorporation of sulphur by HBI alkenes should be conducted. It is clear (Figure 2.2; Figure 2.8; Table 2.3) that there is a significant variation in the distributions of the HBIs that are biosynthesised by *Pleurosigma spp.*, both between different cultures, and between different species, and the reasons for this variation need to be investigated further.

The production of HBIs by a benthic diatom such as *P. intermedium* can clearly contribute towards the sedimentary occurrences of HBIs. The identification of HBIs in the water column (Prahl *et al.*, 1980; Osterroht *et al.*, 1983; Volkman *et al.*, 1983; Albaigés *et al.*,

1984; Bates *et al.*, 1984; Matsueda and Handa, 1986a,b) suggests a significant contribution from planktonic diatoms such as *Pleurosigma sp.* and *P. planktonicum.*

CHAPTER THREE

The Structures and Distributions of HBI alkenes in the diatom *Rhizosolenia setigera*

3.1 Introduction

An investigation into the hydrocarbon distributions of fifteen diatom species led to the identification of the marine diatoms *Rhizosolenia setigera* and *Haslea ostrearia* as biological producers of HBI alkenes (Volkman *et al.*, 1994). The authors therein identified a group of HBIs possessing the C_{25} parent skeleton (I, Figure 3.1) with between three and five degrees of unsaturation in *H. ostrearia,* and several C_{30} HBIs possessing the C_{30} parent skeleton (II, Fig 3.1) with five and six double bonds in *R. setigera.* Since the initial report by Volkman *et al.* (1994), the role of *H. ostrearia* as a producer of C_{25} HBIs has been confirmed by the identification of these compounds in cultures of *H. ostrearia* grown under axenic conditions (Wraige *et al.*, 1999), and the structures of a number of C_{25} HBIs isolated from *H. ostrearia* have been fully characterised (Belt *et al.*, 1996; Wraige *et al.*, 1997; Wraige *et al.*, 1999; Johns *et al.*, 1999).

Despite the observation of C_{25} and C_{30} HBIs in a range of geochemical settings (reviewed by Robson and Rowland, 1990), the role of *R. setigera* as a producer of C_{30} HBIs has received less attention than C_{25} HBI production by *H. ostrearia.* The HBIs of three strains of *R. setigera* have been reported previously (Sinninghe Damsté et al., 1999a; Volkman *et al.*, 1994,1998). The different HBI distributions observed between these strains varied from a group of C_{30} HBI alkenes possessing between five and six double bonds, in two Australian strains CS-62 and CS-389/1 grown at 20 and 18.5 °C and 70-80 $\mu E\ m^{-2}\ s^{-1}$ white light and 28 psu salinity (Volkman et al., 1994,1998), to a single C_{25} HBI pentaene (III, Figure 3.1) only, in strain CCMP1330 grown at 4, 12 and 20°C in North Atlantic seawater (Sinninghe

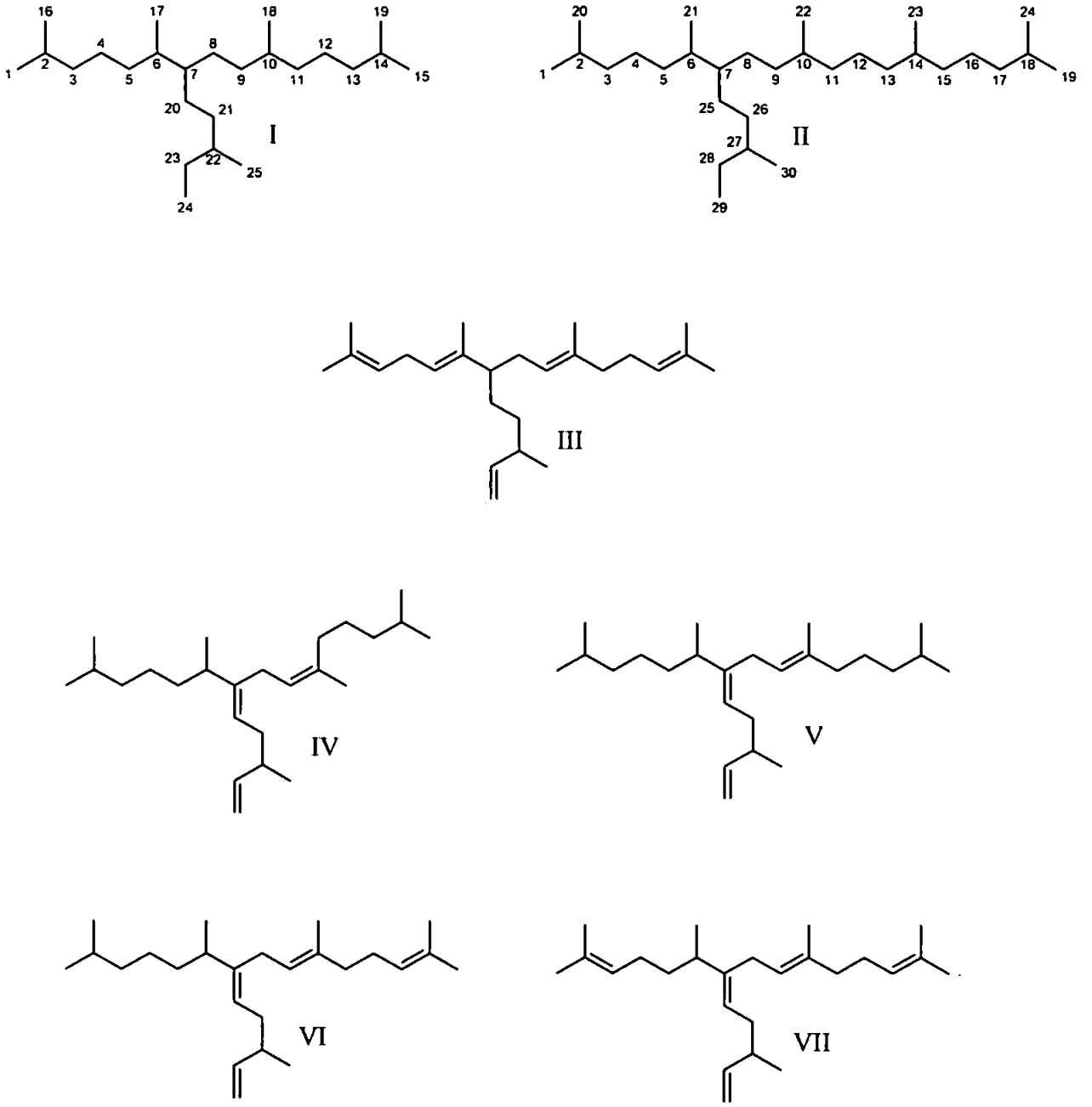


Figure 3.1 Structures of HBIs found in *R. setigera.*

Damsté et al., 1999a). The single C_{25} HBI pentaene (III) identified thus far in *R. setigera*, has been characterised by NMR spectroscopy and comparison with data for the same compound in *H. ostrearia* (Sinninghe Damsté et al., 1999b; Wraige et al., 1997). There have been no reports of the structures of the C_{30} HBI alkenes other than the determination of unsaturation and carbon skeleton by GCMS and hydrogenation to the parent alkane (II) (Volkman et al., 1994).

It was therefore decided to determine the structures of the C_{30} HBIs reported previously *via* isolation from *R. setigera* followed by characterisation by GC-MS and NMR. Since the HBI distributions in the previous reports were rather different from one another, it was also decided to examine further the hydrocarbon distributions in several strains of *R. setigera*.

3.2 Experimental

3.2.1 Algal cultures

Two *R. setigera* strains (Nantes 99 and Nantes 00) were isolated one year apart (1999 and 2000) from the surface waters at Le Croisic, France. Strains CCMP 1330, CCMP 1694 and CCMP 1820 were purchased from the Guillard Collection USA. Cultures were grown in Guillards medium (f/2, 150 ml, 0.2 ml L^{-1}) at 14°C and were illuminated with 100μE m^{-2} s^{-1} and a 14/10 Light/Dark cycle.

Large scale cultures of *R. setigera* strains (Nantes 99 and Nantes 00) were cultured in 4 x 60 L tanks containing underground saltwater enriched with $NaNO_3$ (8 mg mL^{-1}) and Guillard's medium (f/2, 0.2 ml L^{-1}) at 16-18 °C.

3.2.2 Determination of HBIs in R. setigera strains

Detection of HBIs was achieved using filtered aliquots (25 to 50 ml) of algal cultures. Following extraction into hexane, aided by sonication, the total hexane extract (THE) was then saponified (KOH/ MeOH/ H_2O) and the non-saponifiable lipids (NSLs) were re-extracted into hexane, and examined by GC-MS. Identification of HBIs was achieved by comparison of GC RIs and mass spectral data with authenticated standards where possible, followed by hydrogenation ($PtO_2.2H_2O$ / hexane) to the parent alkanes (I and II).

3.2.3 Isolation and characterisation of HBIs

Centrifugation of large scale cultures of *R. setigera* gave algal pastes which were freeze dried (Nantes 99: 15.5 g wet, 2.0 g dry; Nantes 00: 18.0 g wet, 3.54 g dry) and extracted with hexane (Soxhlet; 24 hr). The total hexane extracts (Nantes 99: 225 mg; Nantes 00: 475 mg) thus obtained were combined (600 mg), saponified (5% KOH / MeOH/ H_2O) and re-extracted into hexane to give the non-saponifiable lipids (NSLs; 54 mg). The NSLs were purified using open column chromatography (SiO_2 / hexane), and fractions were examined by GC-MS and combined where appropriate (i.e. C_{30} HBIs with the same degree of unsaturation). Repeated fractionation yielded two $C_{30:5}$ (21 mg) and two $C_{30:6}$ (10 mg) alkenes which were suitable for analysis by NMR spectroscopy.

3.3 Identification and characterisation of C_{30} HBI alkenes from *R. setigera*

3.3.1 Chromatographic and mass spectral analysis of HBIs in R. setigera strains Nantes 99 and Nantes 00

Analysis of the GC-MS total ion current (TIC) chromatograms of the non-saponifiable lipid fractions from *R. setigera* strains Nantes 99 and Nantes 00 revealed the presence of both C_{25} and C_{30} HBI alkenes along with small amounts of *n*-$C_{21:6}$ and phytol. Figure 3.2 shows the partial TIC chromatograms obtained for the two strains. The C_{25} HBI isomers consisted of two trienes (IV and V Figure 3.1), a single tetraene (VI) and pentaene (VII), each of which possess Δ7(20) unsaturation. Identification of the C_{25} HBI isomers was achieved *via* comparison of their GC-MS properties with those of authenticated compounds from *P. intermedium* (Chapter 2).

Examination of the mass spectra obtained for the four C_{30} compounds revealed the presence of two pentaenes ($C_{30:5}$; M^+ 412; RI 2505, 2558 $_{HP\text{-}1}$, 2519, 2574 $_{HP\text{-}5}$) and two hexaenes ($C_{30:6}$; M^+ 410; RI 2545, 2596 $_{HP\text{-}1}$, 2565, 2617 $_{HP\text{-}5}$). Hydrogenation ($H_2/PtO_2.2H_2O$) resulted in the formation of a compound which co-chromatographed with, and had an identical mass spectrum to that obtained for synthetic $C_{30:0}$ (Robson and Rowland, 1988), thereby confirming the parent carbon skeleton (II) for these four compounds. The mass spectra of the two pentaenes (Figure 3.3 a, b) were very similar, and gave enhanced ions with *m/z* 231, 299 and 357, with the second eluting pentaene (RI 2558 $_{HP\text{-}1}$) exhibiting an enhanced intensity of *m/z* 191 when compared with the first eluting isomer (RI 2505 $_{HP\text{-}1}$). The mass spectra of the two hexaenes (Figure 3.3 c, d) were also similar to each other, although the first eluting hexaene (RI 2545 $_{HP\text{-}1}$) gave enhanced fragment ions with *m/z* 286 and 313 whilst the spectrum of the second eluting hexaene (RI $2565_{HP\text{-}1}$) showed an enhancement of the *m/z* 273 ion.

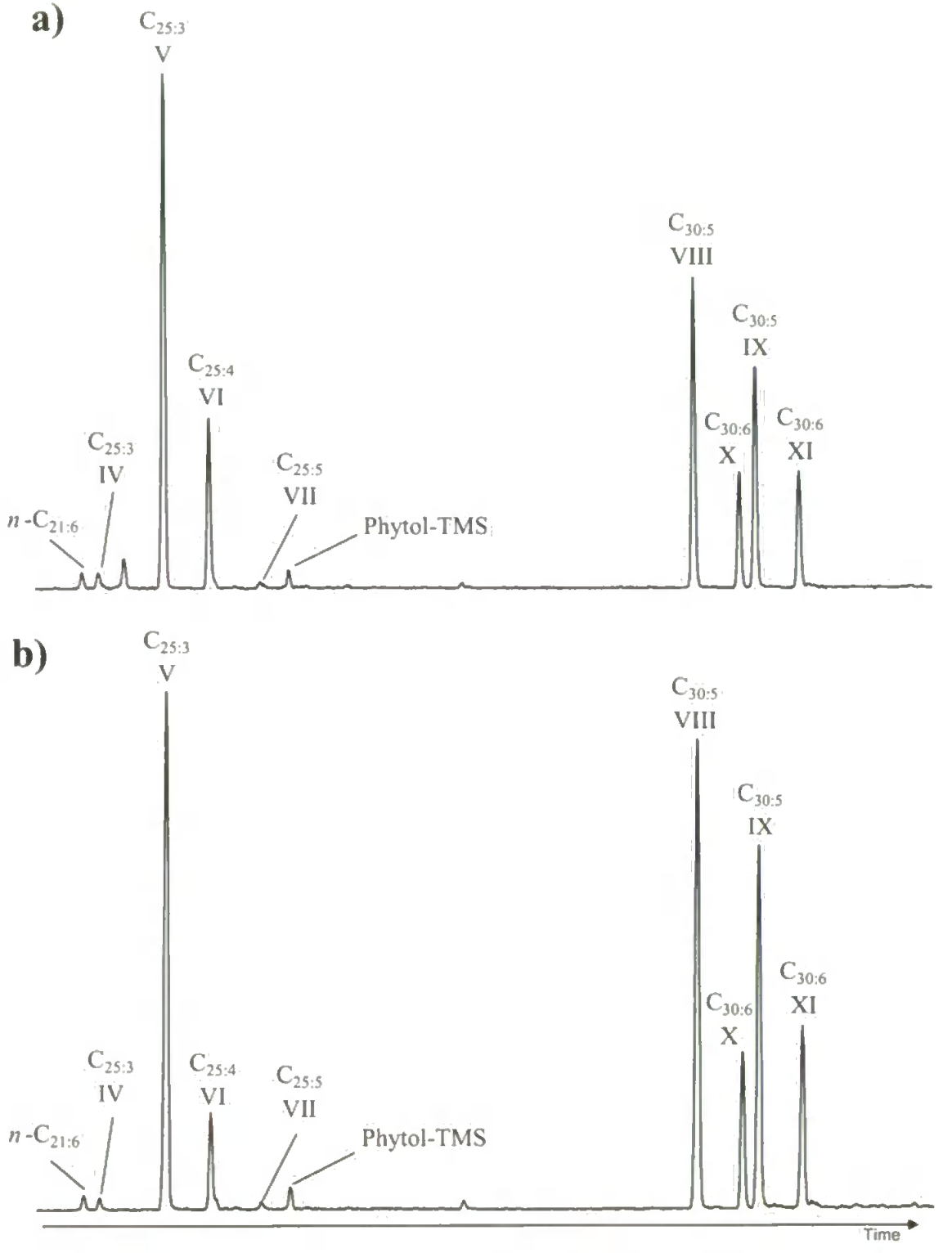


Figure 3.2 Partial TIC chromatograms showing the hydrocarbon distributions in *R.setigera* strains; (a) Nantes 99 and (b) Nantes 00.

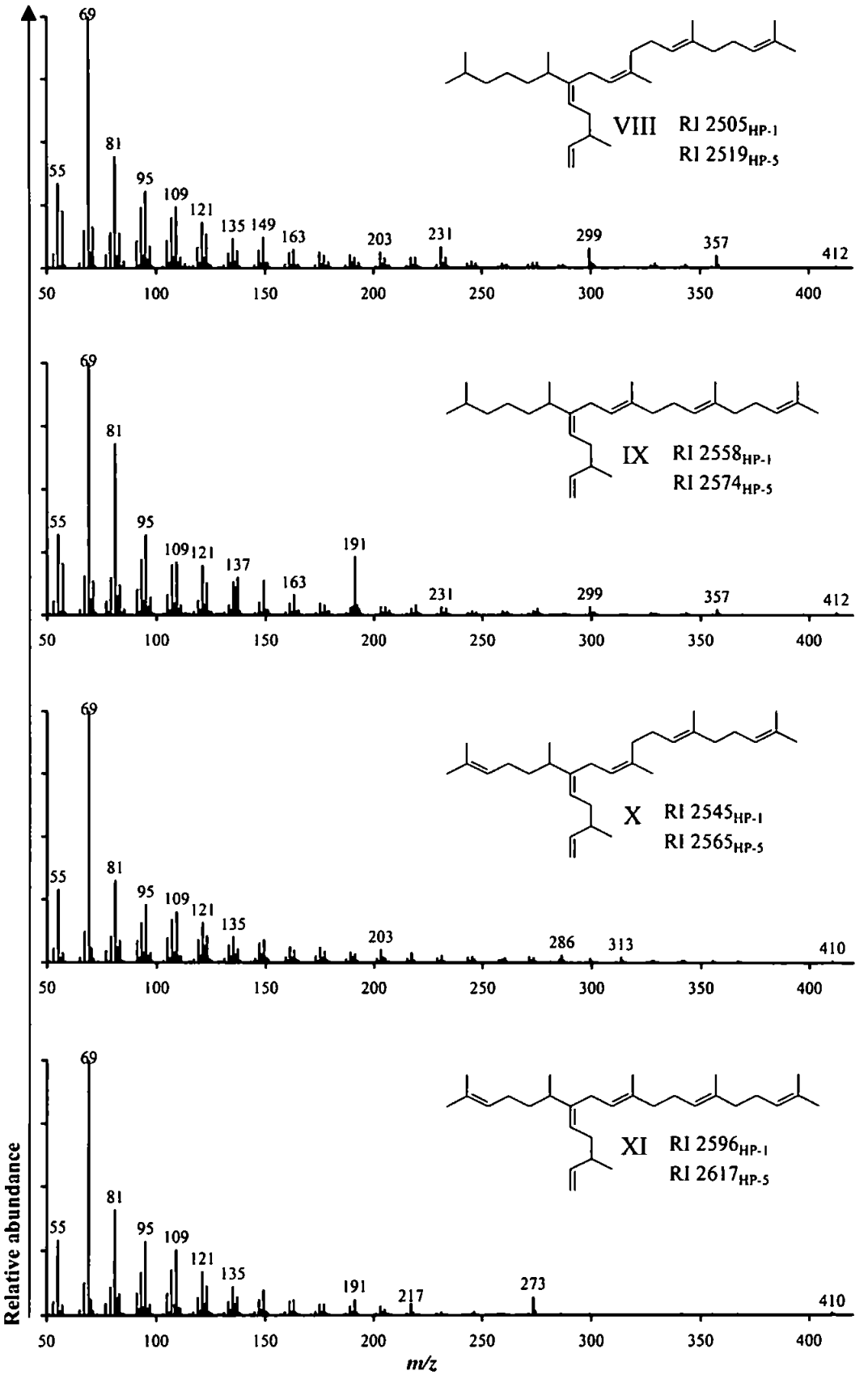


Figure 3.3 Mass spectra of the C_{30} HBIs from *R. setigera* strains Nantes 99 and Nantes 00

The subsequent characterisation of the HBI alkenes from *R. setigera* strains Nantes 99 and Nantes 00 by NMR spectroscopy (described in detail in sections 3.3.2 – 3.3.3) revealed the structures of the four C_{30} HBI penta- and hexaenes to be VIII – XI (Figure 3.4).

3.3.2 *Structural characterisation of (6 E/Z)-2,6,10,14,18-pentamethyl-13-(3-methylpent-4-enylidine)-nonadeca-2,6,10,17-tetraene (X and XI)*

Examination of the ^{1}H (Table 3.1), ^{13}C and DEPT NMR (Table 3.2) spectra of the two $C_{30:6}$ HBI alkenes (10 mg) isolated from *R. setigera* strains Nantes 99 and Nantes 00 revealed the presence of a vinyl moiety (C28 – C29) and five tri-substituted double bonds. Two of the tri-substituted double bonds could be located at the terminal positions of the main alkyl chain (C2-C3 and C17-C18) as ^{1}H resonances due to isopropyl groups (*ca* 0.85 ppm) were absent. A further double bond could be located at the C-7 branch point due to characteristic ^{13}C resonances for this quaternary carbon (δ 142.6 and 142.3 ppm) and the absence of any peak at ca. 45-50 ppm which is diagnostic when HBI alkene isomers are saturated at this position (e.g. Wraige *et al.*, 1999). The remaining two tri-substituted double bonds were positioned at C9-C10 and C13-C14 since the H-8 protons (and only H-8) were found to be di-allylic (δ 2.6 ppm). No evidence could be found for the presence of positional isomers, a feature which is commonly observed for C_{25} HBI alkenes from *Haslea* spp. (e.g. Belt *et al.*, 1996; Chapter 4). However, the presence of two hexaenes could be explained in terms of geometric (and not configurational) isomerism. Thus, unique ^{1}H and ^{13}C resonances were detected for the methyl group at C-22, indicating that the geometric isomerism exists at C9-C10 in an analogous manner to that observed for the pseudo-homologous C_{25} tri-, tetra- and pentaenes isolated from *P. intermedium* (Chapter 2). Pairs of resonances with significant differences in

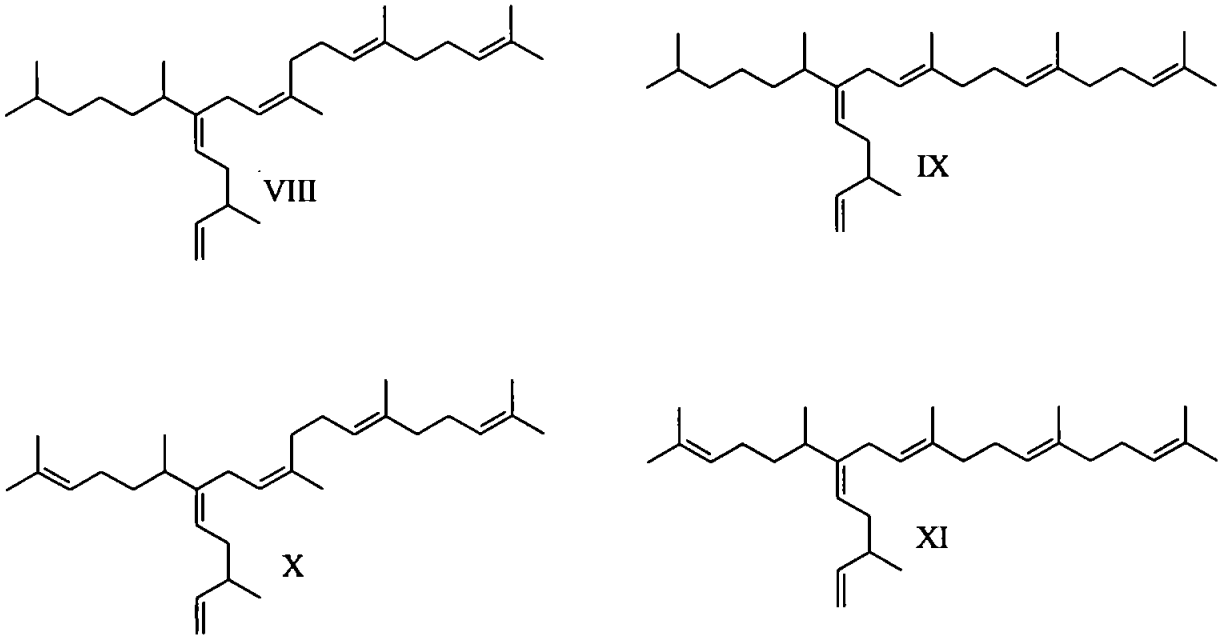
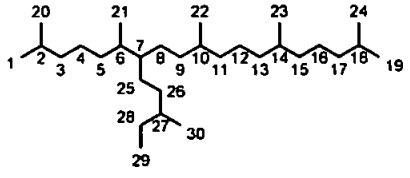
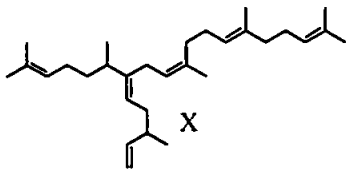


Figure 3.4 Structures of C_{30} HBIs isolated and characterised from *R. setigera* strains Nantes 99 and Nantes 00.

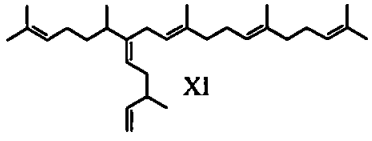
Table 3.1 ^{1}H NMR data for C_{30} hexaenes X and XI

Chemical shift (ppm)	Assignment X	Assignment XI	Multiplicity (Coupling constant, Integration)
5.71	28		m (1H)
5.07	3,9,13,17,25		m (5H)
4.92, 4.88	29		2 x m (2H)
2.6	6, 8		m (3H)
1.8 - 2.2	4, 11, 12, 15, 16, 26, 27		m (13H)
1.7	22		s
1.66	1, 19		s (6H)
1.57, 1.54	20, 23, 24	20, 22, 23, 24	2 x s
1.3	5		m (2H)
0.95	21, 30		m (6H)





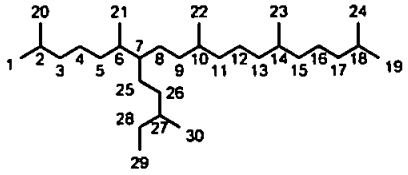
X

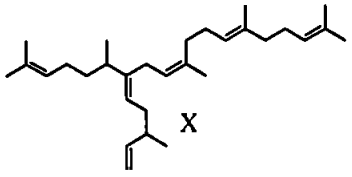


XI

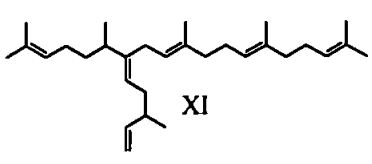
Table 3.2 ^{13}C NMR data for C_{30} hexaenes X and XI

Chemical shift (ppm)	Multiplicity (DEPT)	Assignment	
		X	XI
144.5	CH	28	
142.6	C	7	
142.3	C		7
135.7	C	10	
135.1	C	14	
134.9	C		14
131.3, 131.2	C	2, 18	
124.9, 124.3, 124.2, 124.1	CH	3, 13, 17	
123.8	CH	9	
123.1	CH		9
123.0, 122.9	CH	25	
112.20	CH_2	29	
39.8, 39.7	CH_2	15	11, 15
38.22, 38.17	CH	27	
35.1	CH_2	5	
34.3	CH_2	26	
33.9	CH	6	
31.8	CH_2	11	
29.1	CH_2		8
28.8	CH_2	8	
26.7, 26.5, 26.4	CH_2	4, 12, 16	
25.7	CH_3	1, 19	
23.5	CH_3	22	
19.5, 19.4	CH_3	21, 30	
17.68, 17.65	CH_3	20, 24	
15.96, 15.92	CH_3	23	
15.8	CH_3		22



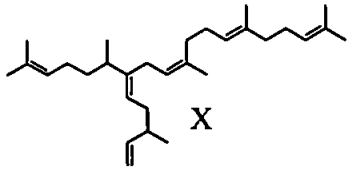


X

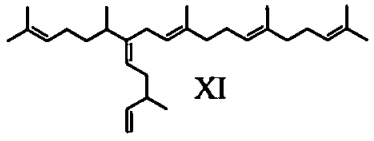


XI

frequency could be assigned to C-7, C-8 and C-9 verifying that this isomerism was at C9-C10 rather than at C13-C14. Further, by comparison of the relative intensities of the ^{1}H and ^{13}C resonances with those of the peaks corresponding to the two $C_{30:6}$ HBIs in the total ion current (TIC) chromatogram, the GC elution order could be determined (*viz. Z* (X) before *E* (XI)) which is the same as that observed for the related C_{25} alkenes (Chapter 2). The structures of the previously uncharacterised C_{30} hexaenes, isolated and characterised from *R. setigera* were therefore determined as X and XI.



X



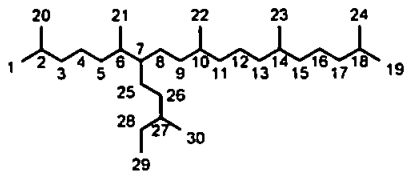
XI

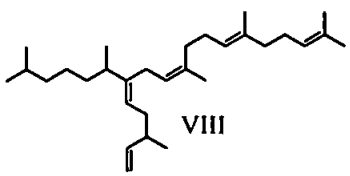
3.3.3 Structural characterisation of (6 E/Z)-2,6,10,14,18-pentamethyl-13-(3-methylpent-4-enylidine)-nonadeca-2,6,10,-triene (VIII and IX)

The structures of the two pentaenes from *R. setigera* ($C_{30:5}$; 10 mg) were examined by ^{1}H (Table 3.3) and ^{13}C (Table 3.4) NMR spectroscopy and not surprisingly, many of the spectral features were extremely similar to those found for the $C_{30:6}$ alkenes X and XI. Thus, resonances were consistent with a vinyl moiety and four tri-substituted double bonds with one of these occurring at the C7-C20 branch point. *E/Z* isomerism was also verified as being present at C9-C10, and the major isomer was found to be *Z* (*Z* / *E* = 1.5 c.f. 0.9 for X/XI). The only significant spectral differences consisted of resonances attributable to a single isopropyl group confirming that one end of the main hydrocarbon chain was saturated. Despite the extreme structural similarities and expected spectroscopic features for isomers

Table 3.3 ^{1}H NMR data for C_{30} pentaenes VIII and IX

Chemical shift (ppm)	Assignment VIII	Assignment IX	Multiplicity (Coupling constant, Integration)
5.73	28		m (1H)
5.09	9, 13, 17, 25		m (4H)
4.93, 4.88	29		2 x m (2H)
2.6	6, 8		m (3H)
1.9 - 2.2	11, 12, 15, 16, 26, 27		m (11H)
1.71	22		s
1.66	19		s (3H)
1.58, 1.56, 1.53	23, 24	22, 23, 24	3 x s
1.5	2		m (1H)
1.04 - 1.3	3, 4, 5		m (6H)
0.96, 0.94	21, 30		2 x d (J = 6.6 Hz, 6H)
0.84	1, 20		d (J = 6.6 Hz, 6H)




VIII

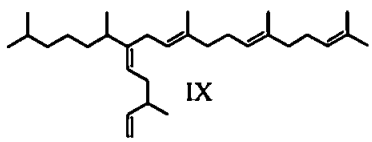
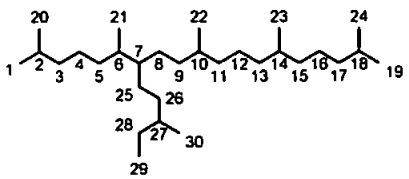
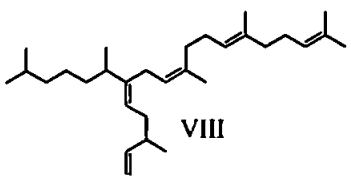
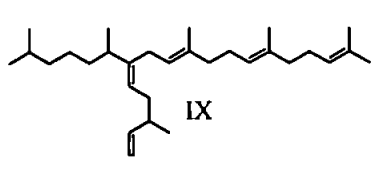

IX

Table 3.4 ^{13}C NMR data for C_{30} pentaenes VIII and IX

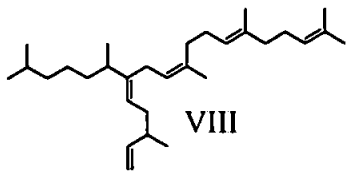
Chemical shift (ppm)	Multiplicity (DEPT)	Assignment	
		VIII	IX
144.6	CH	28	
143.0	C	7	
142.7	C		7
135.6	C	10	
135.1	C	14	
134.9	C		14
131.3	C	18	
124.4, 124.3, 124.2	CH	13, 17	
124.0	CH	9	
123.3	CH		9
122.9	CH	25	
122.7	CH		25
112.1	CH_2	29	
39.8	CH_2		11
39.7	CH_2	15	
39.3	CH_2	3	
38.2	CH	27	
35.3	CH_2	5	
34.4	CH_2	26	
34.3	CH	6	
31.8	CH_2	11	
29.2	CH_2		8
28.9	CH_2	8	
27.9	CH	2	
26.8, 26.7, 26.6	CH_2	12, 16	
25.7	CH_3	19	
25.7	CH_2	4	
23.5	CH_3	22	
22.6	CH_3	1, 20	
19.6, 19.5	CH_3	21, 30	
17.7	CH_3	24	
15.9	CH_3	23	
15.8	CH_3		22



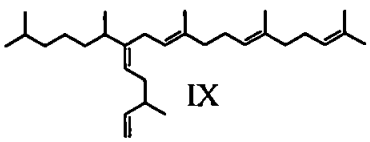




possessing unsaturation at either end of the principal carbon chain (*viz.* C2-C3 and C17-C18), an unambiguous assignment could be made by careful examination of the ^{13}C spectrum. For example, for the pseudo-homologous $C_{25:3}$, $C_{25:4}$ and $C_{25:5}$ alkenes (eight isomers due to positional and geometric isomerism), the frequency for C-6 is always significantly higher ($\Delta\delta$ = 0.3-0.4 ppm) when C2-C3 is saturated (ca. δ 34.3 ppm; Chapter 2). Since C-6 was found to resonate at 34.3 ppm, with the corresponding resonance for the C_{30} hexaenes (which is unsaturated at C2-C3) occurring at 33.9 ppm, C2-C3 must be saturated at this position. An analogous trend was observed for C-7 with chemical shifts to higher frequency when C2-C3 is saturated (irrespective of whether C9-C10 is *E* or *Z*). The structures of the previously uncharacterised C_{30} pentaenes, isolated and characterised from *R. setigera* were therefore determined as VIII and IX.



VIII



IX

3.4 HBI distributions in different strains of *R. setigera*

Analysis of the NSLs obtained from small-scale (150 ml) cultures of *R. setigera* strains CCMP 1330, CCMP 1694, CCMP 1820, Nantes 99 and Nantes 00 revealed the presence of HBI alkenes with varying distributions. Figure 3.5 shows representative partial total ion current chromatograms (TICs) of the NSLs obtained from the five individual strains of *R. setigera*. In all cases, the NSL fractions contained significant quantities of HBIs, with

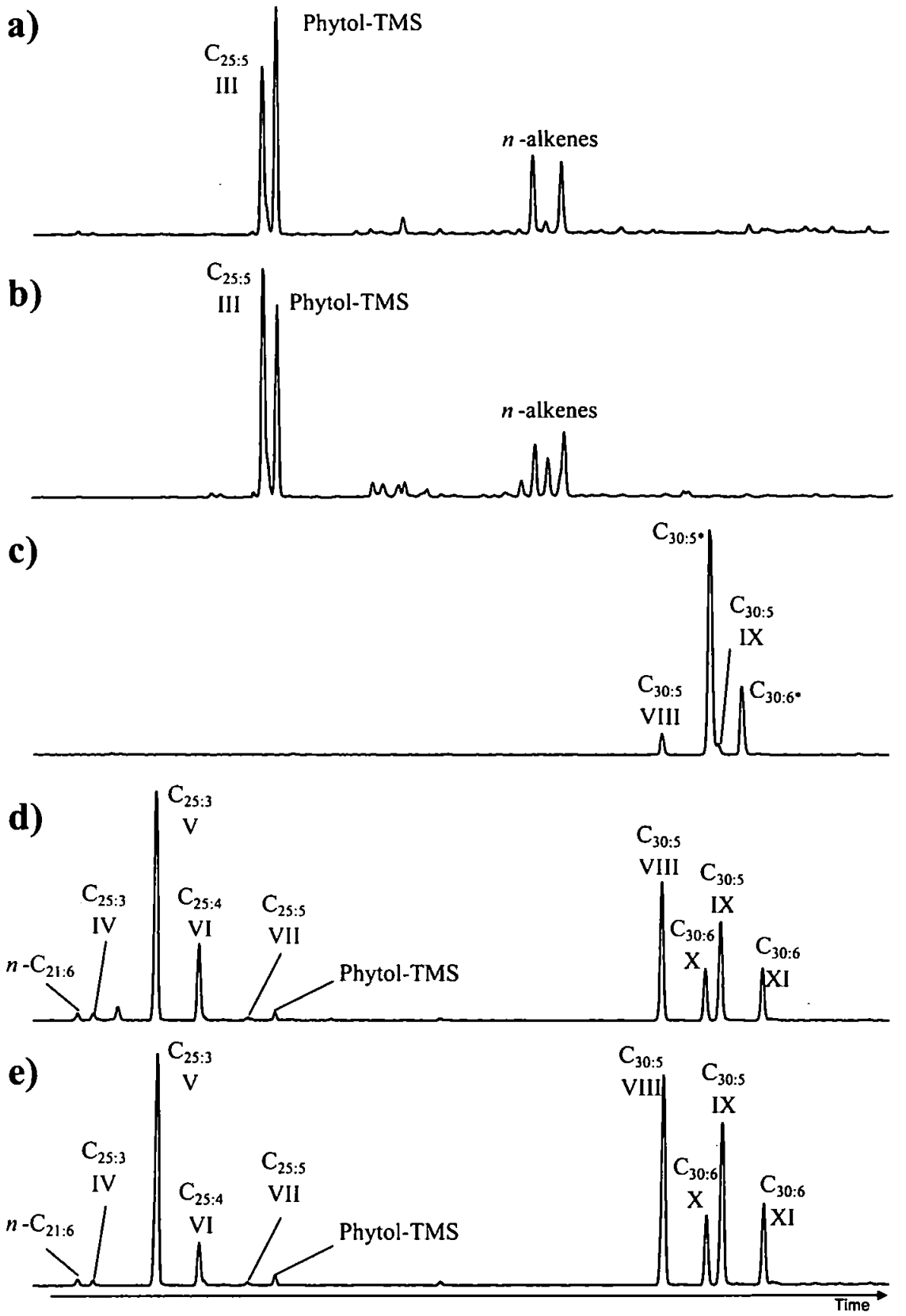


Figure 3.5 Partial TIC chromatograms showing the hydrocarbon distributions in *R.setigera* strains; (a) CCMP 1330, (b) CCMP 1820, (c) CCMP 1694, (d) Nantes 99, (e) Nantes 00.

distributions that varied between strains. The GC retention indices for the HBI alkenes observed in the different strains are summarised in Table 3.5.

3.4.1 HBI alkenes in R. setigera strain CCMP 1330

R. setigera strain CCMP 1330 produced a single C_{25} HBI pentaene (III), phytol, and a group of *n*-C_{27} polyenes (Figure 3.5a). Previous cultures of this strain (Sinninghe Damsté et al., 1999a, 2000) produced similar hydrocarbon distributions consisting of the C_{25} HBI pentaene (III) and several *n*-C_{27} linear polyenes.

3.4.2 HBI alkenes in R. setigera strain CCMP 1820

The HBIs of this strain have not been reported previously but were found herein (Figure 3.5b) to comprise the C_{25} HBI pentaene (III) as the only HBI in a culture grown at 15°C. Again, phytol and the *n*-C_{27} polyenes observed in strain CCMP 1330 were also present (Figure 3.5b).

3.4.3 HBI alkenes in R. setigera strain CCMP 1694

The HBIs of this strain have not been reported previously, and were found to consist of four previously uncharacterised C_{30} compounds with 5 and 6 degrees of unsaturation (Figure 3.5c). The GC-MS characteristics of these compounds were consistent with those observed previously in two strains of *R. setigera* (Volkman *et al.*, 1994, 1998). Subsequent characterisation of C_{30} HBIs from a different strain of *R. setigera* (section 3.3.2) revealed that they corresponded to the minor components (VIII and IX, Figure 3.4). The dominant $C_{30:5•}$ (RI $2548_{HP\text{-}1}$) and the less abundant $C_{30:6•}$ (RI $2579_{HP\text{-}1}$) gave mass spectra (Figure 3.6 a,b) similar to compounds also observed previously in sediments from Puget Sound (Barrick and Hedges, 1981) and Dabob Bay (Prahl *et al.*, 1980), for which bicyclic structures with

Table 3.5 Retention indices for HBIs observed in *R. setigera* strains

HBI Alkene	Structure	Retention Index HP-1	HP-5
$C_{25:5}$	I	2175	2185
$C_{25:3}$	IV	2042	2044
$C_{25:3}$	V	2087	2091
$C_{25:4}$	VI	2121	2130
$C_{25:5}$	VII	2159	2172
$C_{30:5}$	VIII	2505	2519
$C_{30:5}$	IX	2558	2574
$C_{30:6}$	X	2545	2565
$C_{30:6}$	XI	2596	2617
$C_{30:5\bullet}$	unknown	2548	2568
$C_{30:6\bullet}$	unknown	2579	2605

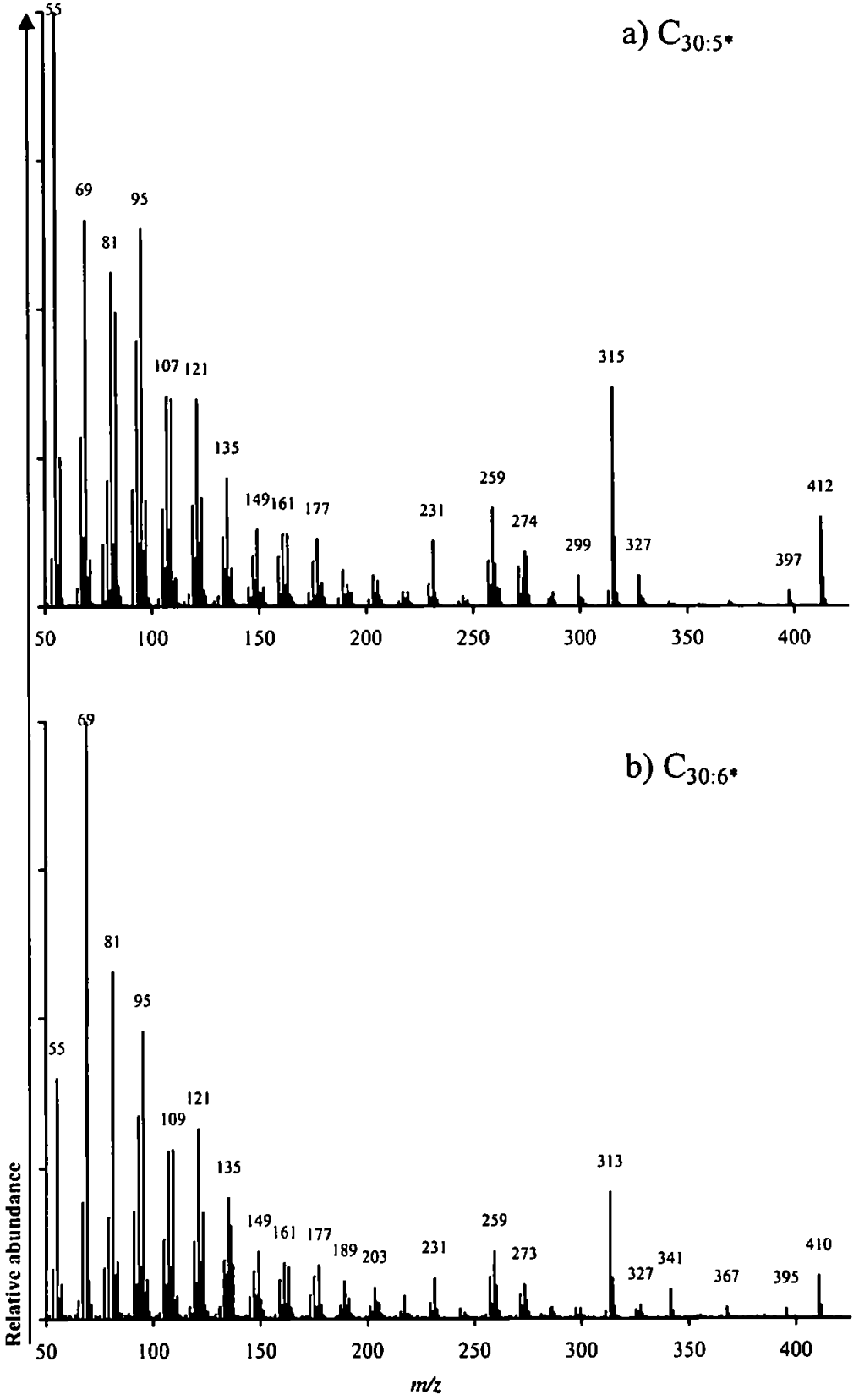


Figure 3.6 Mass spectra of uncharacterised $C_{30:5*}$ (a) and $C_{30:6*}$ (b) observed in *R. setigera* strain CCMP 1694.

three and four double bonds were proposed on the basis of hydrogenation behaviour (M^+ 418).

Hydrogenation ($PtO_2.2H_2O$ /hexane; 4h) of the C_{30} alkenes reported here from *R. setigera* (CCMP 1694) produced a mixture of compounds (Figure 3.7 a) containing the $C_{30:0}$ HBI alkane (II) which exhibits a characteristic fragment ion at *m/z* 308, identified by comparison of RI and MS data with that of authentic II (Robson and Rowland, 1988), plus a C_{30} compound with one degree of unsaturation (Figure 3.7 c; $C_{30:1}$•; M^+ 420; RI 2532 $_{HP\text{-}1}$) and a C_{30} compound with two degrees of unsaturation (Figure 3.7 d; $C_{30:2}$•; M^+ 418; RI 2549 $_{HP\text{-}1}$).

Extraction of the ion intensities for *m/z* 308, 418 and 420 from the total ion current chromatogram of the hydrogenation products mass chromatograms (Figure 3.7 b) which revealed the presence of an additional $C_{30:1}$• compound (Figure 3.7 e; $C_{30:1}$; M^+ 420; RI 2546 $_{HP\text{-}1}$) which co-eluted with the $C_{30:2}$• component. The mass spectra of both $C_{30:1}$• compounds (Figure 3.7 c,e) were extremely similar.

Hydrogenation of the mixture for a further six hours resulted in an increase in the amounts of the two $C_{30:1}$• compounds relative to the $C_{30:2}$• compound. The amount of alkane (II) remained unchanged relative to the other isomers. Figure 3.8 shows partial TIC and mass chromatograms illustrating the hydrogenation behaviour of the hydrocarbons from *R. setigera* strain CCMP 1694.

The hydrogenation behaviour of the C_{30} alkenes from *R. setigera* strain CCMP 1694 suggests that the C_{30} HBI pentaenes (VIII and IX) readily hydrogenated to the alkane (II). However, hydrogenation of the unidentified $C_{30:5}$• and $C_{30:6}$• compounds produced a $C_{30:2}$• alkene for which one degree of unsaturation could be attributed to a double bond in a position fairly resistant to hydrogenation. Exhaustive hydrogenation of this (hindered) double bond lead to the formation of a pair of isomeric C_{30} compounds with one degree of

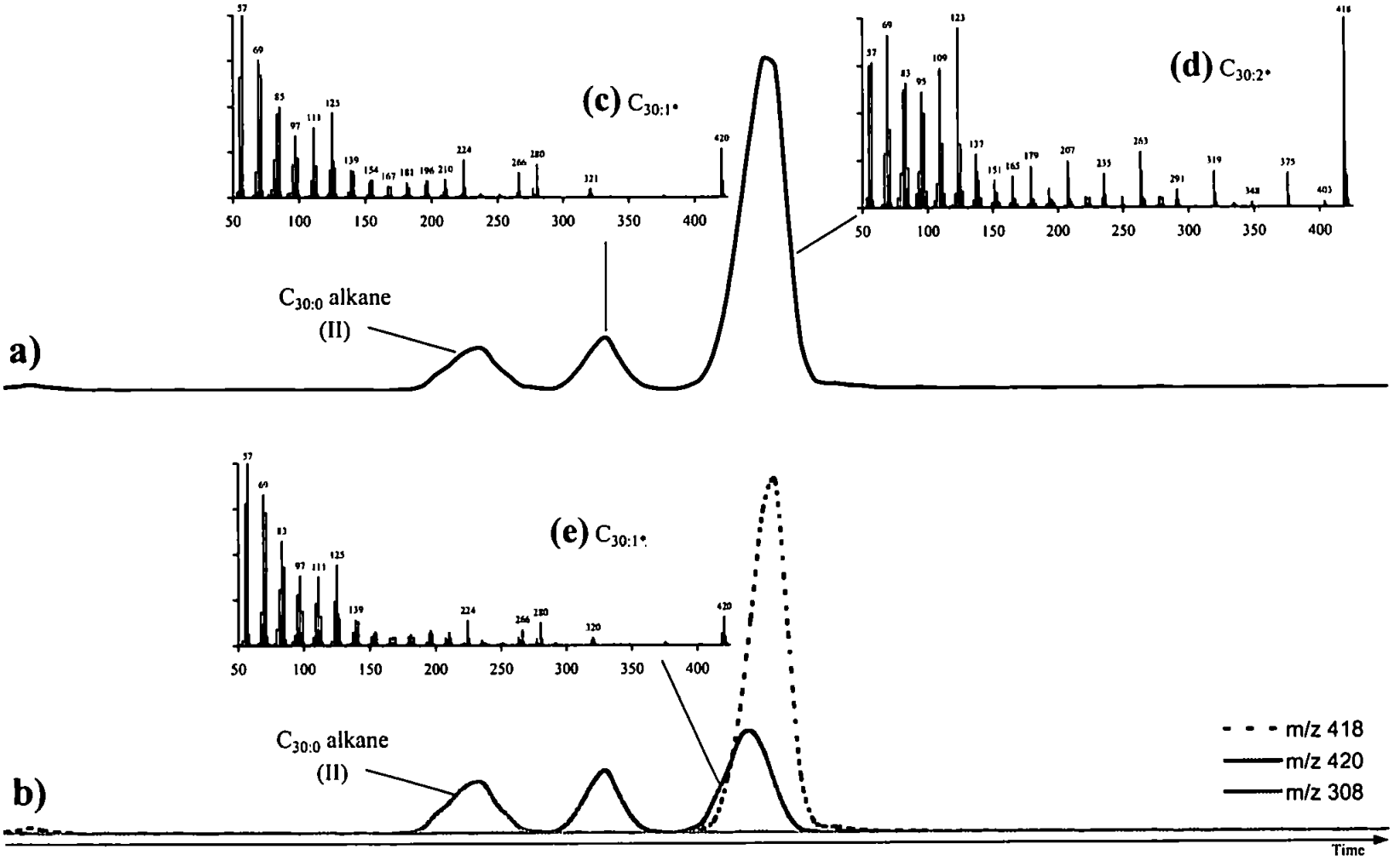


Figure 3.7 Partial TIC (a) and mass (b) chromatograms showing the products obtained from hydrogenation of the C_{30} alkenes from *R. setigera* strain CCMP 1694. Mass spectra of the hydrogenation products are also given (c,d,e).

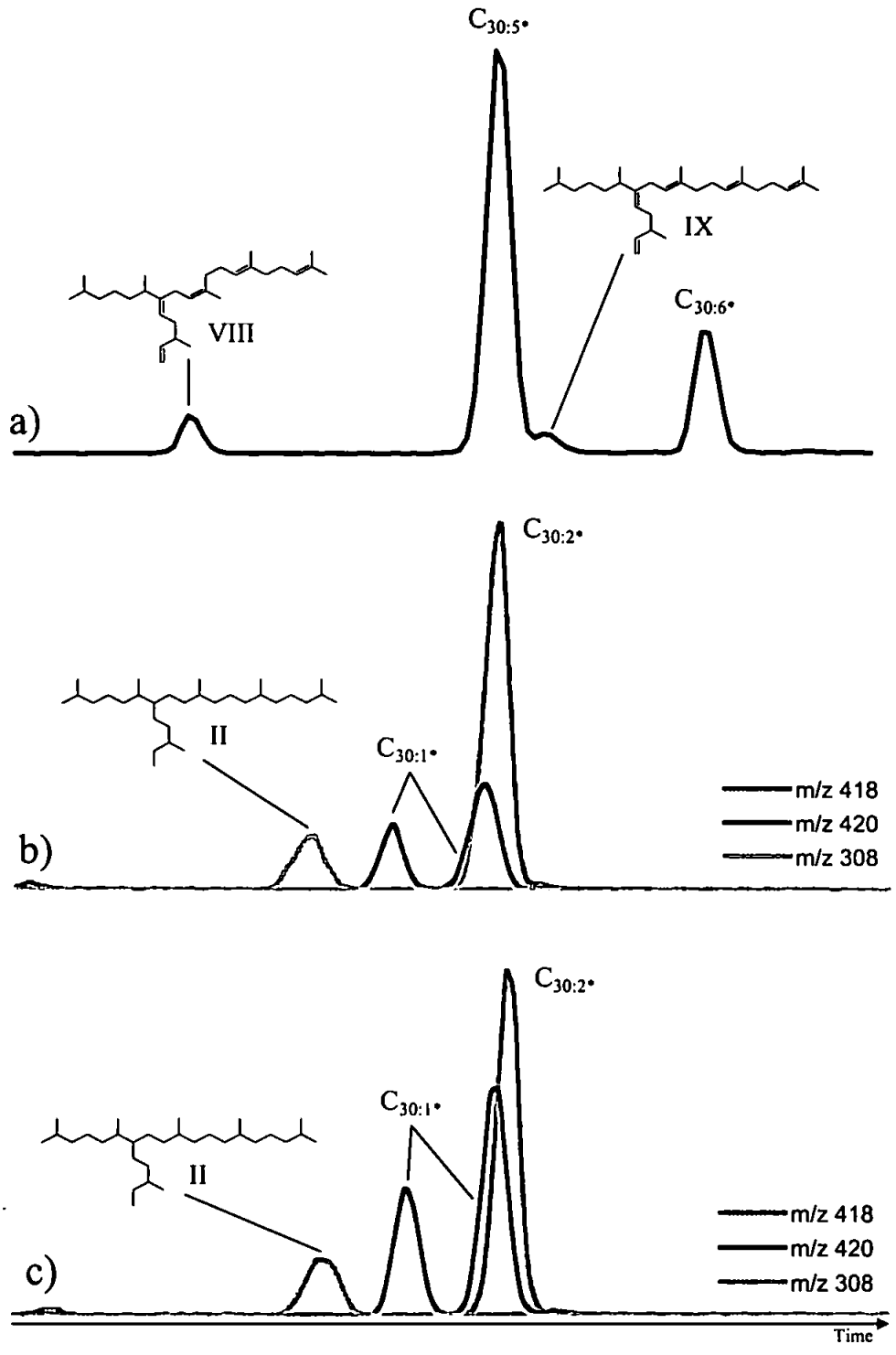
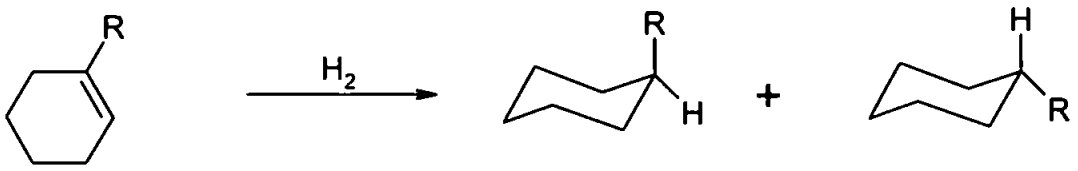


Figure 3.8 Partial TIC chromatogram of the C_{30} alkenes from *R. setigera* strain CCMP 1694 (a). Partial mass chromatograms showing the products formed after 4h (b) and 10 h (c) hydrogenation.

unsaturation ($C_{30:1}$•) which had very similar mass spectra (Figure 3.7 c,e). As discussed in Chapter 2, such similarity between mass spectra suggests that the two $C_{30:1}$• compounds could be a pair of geometrical or other stereoisomers. Either a cyclic skeleton, or a double bond that is extremely resistant to hydrogenation could account for the remaining degree of unsaturation. A cyclic skeleton could also explain the presence of two stereoisomers, since hydrogenation of a substituted double bond located within a ring has the potential to produce cyclic alkanes with axial/equatorial isomerism, e.g.



3.4.4 HBI alkenes in R. setigera strains Nantes 99 and Nantes 00

The HBI distributions in these strains are described in detail in section 3.3.1. Briefly, *R. setigera* strains Nantes 99 and Nantes 00, produced complex HBI distributions consisting of both C_{25} and C_{30} HBIs. The co-production of C_{25} and C_{30} HBIs has not been reported previously. The distributions of HBI isomers, which were very similar in both strains, are shown in Figs 3.5d and e. The C_{25} HBI isomers included trienes (IV and V) with Δ7(20) unsaturation, a related tetraene (VI) and traces of the analogous Δ7(20) pentaene (VII) which have been characterised following isolation from *P. intermedium* (Chapter 2). The C_{30} HBIs consisted of pentaenes (VIII, IX) and hexaenes (X, XI).

3.5 Discussion

3.5.1 Structural features of C_{30} HBIs isolated and characterised from R. setigera

R. setigera has proved to be a valuable source of novel C_{30} HBI pentaenes and hexaenes (and also of C_{25} trienes, tetraenes and pentaenes), and the isolation and characterisation of four C_{30} HBIs (VIII, IX, X and XI) has been achieved. The structures of the four new C_{30} HBIs show important similarities to the C_{25} tri-, tetra-, and penta-enes isolated and characterised herein from laboratory cultures of *P. intermedium* (Chapter 2) and important differences to C_{25} HBIs characterised previously from cultures of *H. ostrearia* (Belt *et al.*, 1996; Johns *et al.*, 1999; Wraige *et al.*, 1997, 1999) and *R. setigera* (Sinninghe Damsté *et al.*, 1999b). All of the four new C_{30} hexaenes and pentaenes (VIII – XI) possess an unsaturated branch point at C7, and exist as pairs of isomers due to *E/Z* geometrical isomerism about the C9-C10 double bond. Unsaturation at C7 and isomerism about the C9-C10 double bond are features observed for all of the C_{25} HBIs currently identified in *P. intermedium* (Chapter 2), but have not been observed previously for HBIs isolated from other sources (Belt *et al.*, 1996; Johns *et al.*, 1999; Wraige *et al.*, 1997, 1999; Sinninghe Damsté *et al.*, 1999b). The vinyl moiety (C28-C29) appears to be a feature common to many of the polyunsaturated HBIs reported to date (e.g. Belt *et al.*, 1996; Johns *et al.*, 1999; Wraige *et al.*, 1997, 1999), although this was notably absent from the C_{25} HBI tetraenes produced by *P. planktonicum* (Chapter 2).

3.5.2 C_{30} alkenes in sediments, particles and biota

The structural characterisation of the four novel C_{30} HBIs isolated from *R. setigera* allows for the identification of C_{30} HBIs reported previously in sediments, sedimenting particles and diatom cultures *via* comparison of the GC (RI) and MS properties of the compounds characterised herein (VIII – XI) with those of previous reports. Volkman *et al.* (1994, 1998)

reported a suite of C_{30} HBIs with five and six degrees of unsaturation in two different strains of *R. setigera* (strains CS-62 and CS-389/1) grown under laboratory conditions. Three of the HBIs reported by Volkman *et al.* (1994, 1998) had RIs ($C_{30:5}$ 2507, 2560 $_{HP-1}$; $C_{30:6}$ 2600 $_{HP-1}$) in close agreement to those of VIII, IX and XI (Table 3.6). A comparison of the mass spectra for VIII and IX with those reported by Volkman *et al.* (1994) for the C_{30} HBI pentaenes R_1 (RI 2507_{HP-1}) and R_3 (RI 2560_{HP-1}) shows an excellent agreement between common ions (e.g. *m/z* 412, 357, 299, 231, 191) and their relative intensities. A similar correlation can be made between XI and a further C_{30} hexaene (R_5; RI 2600_{HP-1}) reported in *R. setigera* strains CS-62 and CS-389/1 (Volkman *et al.*, 1994, 1998). Thus, the C_{30} pentaenes R_1 and R_3 and hexaene R_5 reported by Volkman *et al.* (1994, 1998) can now be identified as being VIII, IX and XI (Table 3.6). The remaining two C_{30} alkenes reported by Volkman *et al.* (1994, 1998) consisting of a C_{30} pentaene (R_2; RI 2550_{HP-1}) and a C_{30} hexaene (R_4; RI 2581_{HP-1}) gave mass spectra different to those obtained for VIII, IX, X and XI, but which were very similar to those obtained for the uncharacterised $C_{30:5\bullet}$ (RI 2548_{HP-1}) and $C_{30:6\bullet}$ (RI 2579_{HP-1}) reported herein in *R. setigera* strain CCMP 1694 (Table 3.6).

Comparison between C_{30} HBIs, reported here from *R setigera* and those reported in geochemical samples are hindered by incomplete published mass spectral data, non-standardisation of GC phases, and incorrect assignment of the carbon skeleton due to incomplete hydrogenation of the C_{30} alkenes (reviewed by Robson, 1987). However, Prahl *et al.* (1980) reported four C_{30} alkenes (RI 2509, 2563, 2558 and 2590_{SP2100}) in sediments and sediment trap particles from Dabob Bay, USA. A monocyclic structure containing four double bonds was proposed for RI 2509_{SP2100} ($C_{30:4:1}$), and a bicyclic structure with three double bonds was proposed for RI 2558_{SP2100} ($C_{30:3:2}$) on the basis of their MS characteristics and hydrogenation behaviour (Prahl *et al.*, 1980). Examination of the mass spectrum reported by Prahl *et al.* (1980) for the compound with RI 2509_{SP2100} showed characteristic

Table 3.6 Correlation between the C_{30} alkenes isolated from *R. setigera* with compounds reported previously in diatoms, sediments and particulates. Assignments are made on the basis of both GC (RI) and MS data.

Reference	This Report		Volkman *et al.* 1994, 1998	Barrick & Hedges 1981	Prahl *et al.* 1980	Osterroht *et al.* 1983
Column Phase	HP-1	HP-5	HP-1	SP2100	SP2100	SE52
Compound						
$C_{30:5}$ VIII	2505	2519	2507	2509	2509	
$C_{30:5}$ IX	2558	2574	2560	2563		
$C_{30:6}$ X	2545	2565				
$C_{30:6}$ XI	2596	2617	2600			
$C_{30:5}$•	2548	2568	2550	2558	2558	2548
$C_{30:6}$•	2579	2605	2581	2590		2580

ions (*m/z* 412, 357, 299 and 231) and ion intensities which were in good agreement with those observed herein for the C_{30} HBI pentaene VIII (Table 3.6). The mass spectrum of RI 2558_{SP2100} reported by Prahl *et al.* (1980) was different to the C_{30} penta- and hexaenes characterised herein (VIII – XI) from *R. setigera* strains (Nantes 99 and Nantes 00), but exhibited characteristic ions (*m/z* 412, 315, 259) in common with the uncharacterised $C_{30:5}$• (RI $2548_{HP\text{-}1}$) observed herein in *R. setigera* strain CCMP 1694 (Table 3.6). No MS data were reported by Prahl *et al.* (1980) for the remaining two C_{30} compounds from Dabob Bay (RI 2563, 2590_{SP2100}), preventing further identification herein.

Barrick and Hedges (1981) also observed a suite of four C_{30} alkenes (RI 2509, 2563, 2558 and 2590_{SP2100}) in sediment cores from Puget Sound, USA, of which two (RI 2509, 2558_{SP2100}) were reported to be the same compounds as those observed by Prahl *et al.* (1980) in sediments and particulates from Puget Sound (Table 3.6). The C_{30} alkene RI 2563_{SP2100} observed in sediments from Puget Sound (Barrick and Hedges, 1981) was assigned as a monocyclic tetraene ($C_{30:4:1}$) on the basis of its hydrogenation behaviour (M^+ 420). However, examination of the mass spectrum reported by Barrick and Hedges (1981) for this compound (RI 2563_{SP2100}) reveals characteristic ions (*m/z* 412, 357, 299, 191) and intensities in agreement with the mass spectrum of the C_{30} pentaene (IX) characterised herein. The mass spectrum of the remaining C_{30} alkene (RI 2590_{SP2100}) reported by Barrick and Hedges (1981) in Puget Sound sediments showed characteristic ions (*m/z* 410, 313, 259) and intensities very similar to the mass spectrum of the uncharacterised $C_{30:6}$• (RI $2579_{HP\text{-}1}$) observed herein in *R. setigera* strain CCMP 1694 (Table 3.6).

Osterroht *et al.* (1983) observed a pair of C_{30} alkenes with five and six degrees of unsaturation in particulates from Kiel Bight. Comparison of the mass spectra and retention indices of the C_{30} alkenes reported by Osterroht *et al.* (1983) indicated that they are different

to the C_{30} HBI alkenes (VIII – XI), but were in good agreement with the RI and MS data obtained for the uncharacterised $C_{30:5}$• (RI $2548_{HP\text{-}1}$) and $C_{30:6}$• (RI $2579_{HP\text{-}1}$) identified in *R. setigera* strain CCMP 1694 (Table 3.6). Thus, many of the C_{30} branched alkenes in observed in sediments and particulate material have been found in various strains of *R. setigera*, and several of these compounds have now been fully characterised.

3.5.3 *Variations in HBI distributions between R. setigera strains*

Perhaps surprisingly, the HBIs produced by *R. setigera* showed significant variations between strains (Figure 3.5). The C_{25} HBI pentaene (I) with a saturated (C-7) branch point reported previously in cultures of *R. setigera* (Sinninghe Damsté *et al.*, 1999a,b) and *H. ostrearia* (Wraige *et al.*, 1997) was the sole HBI in strains CCMP 1330 and 1820. Indeed, saturation at C-7 is a structural feature common to a large number of HBIs isolated and characterised previously from cultures of *H. ostrearia* (e.g. Belt *et al.*, 1996; Wraige *et al.*, 1997). In contrast, the CCMP 1694 strain produced only C_{30} branched alkenes. Similar distributions consisting solely of C_{30} compounds have been reported previously in laboratory cultures of two different *R. setigera* strains (Volkman *et al.*, 1994, 1998). Of the four isomers present in strain CCMP 1694, two have been identified as the isomeric pentaenes VIII and IX, which contain an unsaturated branch point (C7), whilst the remaining two C_{30} compounds have not yet been characterised. Strains Nantes 99 and 00 produced both C_{25} and C_{30} HBI alkenes. The co-occurrence of C_{25} and C_{30} HBIs in the same culture has not been reported previously, although this co-occurrence has been observed in sediments (Barrick and Hedges, 1981) and sedimenting particulates (Prahl *et al.*, 1980). Both the C_{25} and the C_{30} HBIs observed herein for these strains possess an unsaturated branch point (C-7) and exhibit geometrical isomerism about the C-9(10) double bond. The C_{25} HBIs present in Nantes 99 and 00 have also been observed in the diatom *P. intermedium* (Chapter 2), and the trienes

(IV and V) are common and abundant in numerous sediments (reviewed by Rowland & Robson, 1990).

The reasons for the variability in HBI distributions between different *R. setigera* strains are unclear, and factors influencing the HBIs produced by *R. setigera* need to be investigated further. Previous studies on the effects of salinity on HBI distributions in the diatom *H. ostrearia* did not reveal a strong variation in the HBI distributions for this species (Wraige *et al.*, 1998) due to the salinity of the growth medium. Variations in growth temperature have been found to directly affect the degree of unsaturation of the HBIs produced by *H. ostrearia* (Rowland *et al.*, 2001), but another study investigating the effect of temperature on HBI production by *R. setigera* (Sinninghe Damsté *et al.*, 1999a) did not show similar effects. In the present study, each of the small scale cultures of *R. setigera* investigated herein were grown under identical, controlled conditions, suggesting that the observed variations in HBI distributions were not as a direct response to culture conditions. Recent experiments investigating the HBI distributions of *R. setigera* over an extended time period suggest that the HBI distributions can vary significantly throughout the life cycle of the organism (Massé, personal communication).

It is interesting to note that the observed variations in HBI distributions between the *R. setigera* strains analysed herein can, to some extent, be grouped according to the geographical location from which the diatom strains were originally isolated. Thus, the two strains isolated from waters of the northwest Atlantic (CCMP 1820 and CCMP 1330) both contained identical HBI distributions consisting of a single C_{25} HBI pentaene. In contrast, Strain CCMP 1694, which was originally isolated from the Arabian Sea contained only C_{30} HBI penta- and hexaenes, whilst the two strains isolated from the waters off southern Brittany (Nantes 99 and Nantes 00) contained very similar HBI distributions consisting of

both C_{25} and C_{30} HBIs. Additionally, two strains of *R. setigera* isolated from Australian waters (Volkman *et al.*, 1994, 1998) contained HBI distributions consisting solely of C_{30} HBIs. This suggests the possibility that these major variations (e.g. the production of HBIs with different structural types) are a product of the recent evolution of these strains in locations isolated from one another. Thus, these variations may perhaps be a long-term response to environmental factors such as salinity, temperature and light (both quality and quantity).

It is also possible that some misidentification of the individual *R. setigera* strains has taken place (Massé, personal communication), since the taxonomy of the genus is somewhat uncertain (Round *et al.*, 1990; Volkman *et al.*, 1994 and references therein). The classification of the *Rhizosolenia* genus has been revised by Sundström (1986) and has also been subject to several other modifications, including the transfer of the only two freshwater *Rhizosolenia* species to a new genus *Urosolenia* (Round *et al.*, 1990).

3.5.4 Uncharacterised C_{30} compounds – acyclic or cyclic?

R. setigera strain CCMP 1694 produced two C_{30} alkenes ($C_{30:5•}$ and $C_{30:6•}$) which were different to the C_{30} HBIs VIII, IX, X and XI isolated and characterised from *R. setigera* strains Nantes 99 and Nantes 00. Both $C_{30:5•}$ and $C_{30:6•}$ have RI and MS (Figure 3.5; Table 3.6) characteristics in common with abundant compounds observed previously in strains of *R. setigera* (Volkman *et al.*, 1994, 1998), and in sediments and particles from Dabob Bay (Prahl *et al.*, 1980), Puget Sound (Barrick and Hedges, 1981) and Kiel Bight (Osterroht *et al.*, 1983.

Initial (4h) hydrogenation of the $C_{30:5\bullet}$ and $C_{30:6\bullet}$ HBIs produced a single C_{30} alkene with two degrees of unsaturation ($C_{30:2\bullet}$), which had a mass spectrum very similar to that reported for the hydrogenation of the $C_{30:5}$ RI 2558_{SP2100} from Dabob Bay (Prahl *et al.*, 1980) and the $C_{30:6}$ RI 2590_{SP2100} from Puget Sound (Barrick and Hedges, 1981). Both Prahl *et al.* (1980) and Barrick and Hedges (1981) proposed that these alkenes were bicyclic on account of the remaining two degrees of unsaturation observed for the hydrogenation product. However, more exhaustive hydrogenation herein of the C_{30} alkenes from *R. setigera* strain CCMP 1694 produced a pair of isomeric compounds with one degree of unsaturation ($C_{30:1\bullet}$) which had identical mass spectra. After extensive hydrogenation (10h)the $C_{30:1\bullet}$ compound failed to hydrogenate further. Thus, this hydrogenation behaviour is consistent with the parent compounds possessing cyclic structures, as opposed to the C_{30} HBI alkane skeleton (II). The presence of a compound with skeleton II containing a double bond which is extremely resistant to hydrogenation cannot be ruled out, though this seems unlikely on the basis of the hydrogenation behaviour of all other previously characterised C_{25} and C_{30} acyclic HBI alkenes.

Volkman *et al.* (1994, 1998) also reported compounds with identical MS and RIs to $C_{30:5\bullet}$ and $C_{30:6\bullet}$ in different strains of *R. setigera,* but concluded that they did possess the HBI alkane skeleton (II). Interestingly, Volkman *et al.* (1994) also reported the presence of two monounsaturated C_{30} alkenes ($C_{30:1\bullet}$) in the hydrogenation products of the C_{30} alkenes from *R. setigera* strain CS-62, which the authors attributed to a residual double bond that was resistant to hydrogenation.

Clearly, further work is needed before the identities of $C_{30:5\bullet}$ and $C_{30:6\bullet}$ can be determined, and it would be too speculative to assign structures to these compounds on the basis of the current, limited information. However, the occurrence of these alkenes in both algal cultures

and geochemical settings makes them worthy of further study. Large scale culturing of *R. setigera* strain CCMP 1694 is currently in progress which should yield sufficient quantities of these alkenes for a detailed structural analysis by e.g. NMR spectroscopy.

3.6 Conclusions

The structures of four novel C_{30} HBI alkenes (VIII, IX, X, XI) have been rigorously characterised following isolation from *R. setigera*, and several C_{30} HBIs previously observed in sediments and particulates have been identified. Other C_{30} alkenes produced by *R. setigera,* which have yet to be characterised, appear also to have been observed previously in geochemical samples. The production of HBIs by different *R. setigera* strains showed significant variations between strains, and *R. setigera* is clearly capable of biosynthesising a wide range of C_{25} and C_{30} HBIs including those common in the geosphere.

The observation of both C_{25} and C_{30} HBIs in a planktonic diatom such as *R. setigera* probably explains (to some degree) the reports of these compounds in both sediments and water column particles (e.g. Prahl *et al.*, 1980; Osterroht *et al.*, 1983; Volkman *et al.*, 1983; Albaiges *et al.*, 1984; Bates *et al.*, 1984; Matsueda and Handa, 1986; Wakeham, 1990).

CHAPTER FOUR

The occurrence of HBI alkenes in diatoms of the genus, *Haslea*

4.1 Introduction

At the outset of the current investigation, the only known producers of C_{25} HBI alkenes were the diatoms *Haslea ostrearia* (Volkman *et al.*, 1994) and *Rhizosolenia setigera* (Volkman *et al.*, 1994; Sinninghe Damsté *et al.*, 1999a,b). Whilst the only C_{25} HBI reported in *R. setigera* was a single pentaene (V; Figure 4.1), a wide range of di- through hexa-unsaturated C_{25} HBIs have been observed in *H. ostrearia*, and the role of *H. ostrearia* as a primary producer of HBI alkenes has been extensively studied (e.g. Belt *et al.*, 1996; Johns *et al.*, 1999, 2000; Wraige *et al.*, 1997, 1999).

Following the identification of a range of HBI isomers in three *Pleurosigma* species (Chapter 2) and several different strains of *R. setigera* (Chapter 3) it was decided to investigate the production of HBIs by different *Haslea* species. The species chosen for this study were the benthic diatoms *H. crucigera* (Wm. Smith) Simonsen, and the previously unclassified species *H. pseudostrearia* Massé *et al.* and *H. salstonica* Massé *et al.* Samples of *H. ostrearia,* including a non marennine-producing ('non-blue') strain were also examined. Additionally, an unidentified diatom, which appears to be a sigmoidal *Haslea* species (Massé, personal communication), was analysed (*Haslea sp.*). Since all of these species are benthic, a planktonic species, *H. wawrikae* was also studied for comparison.

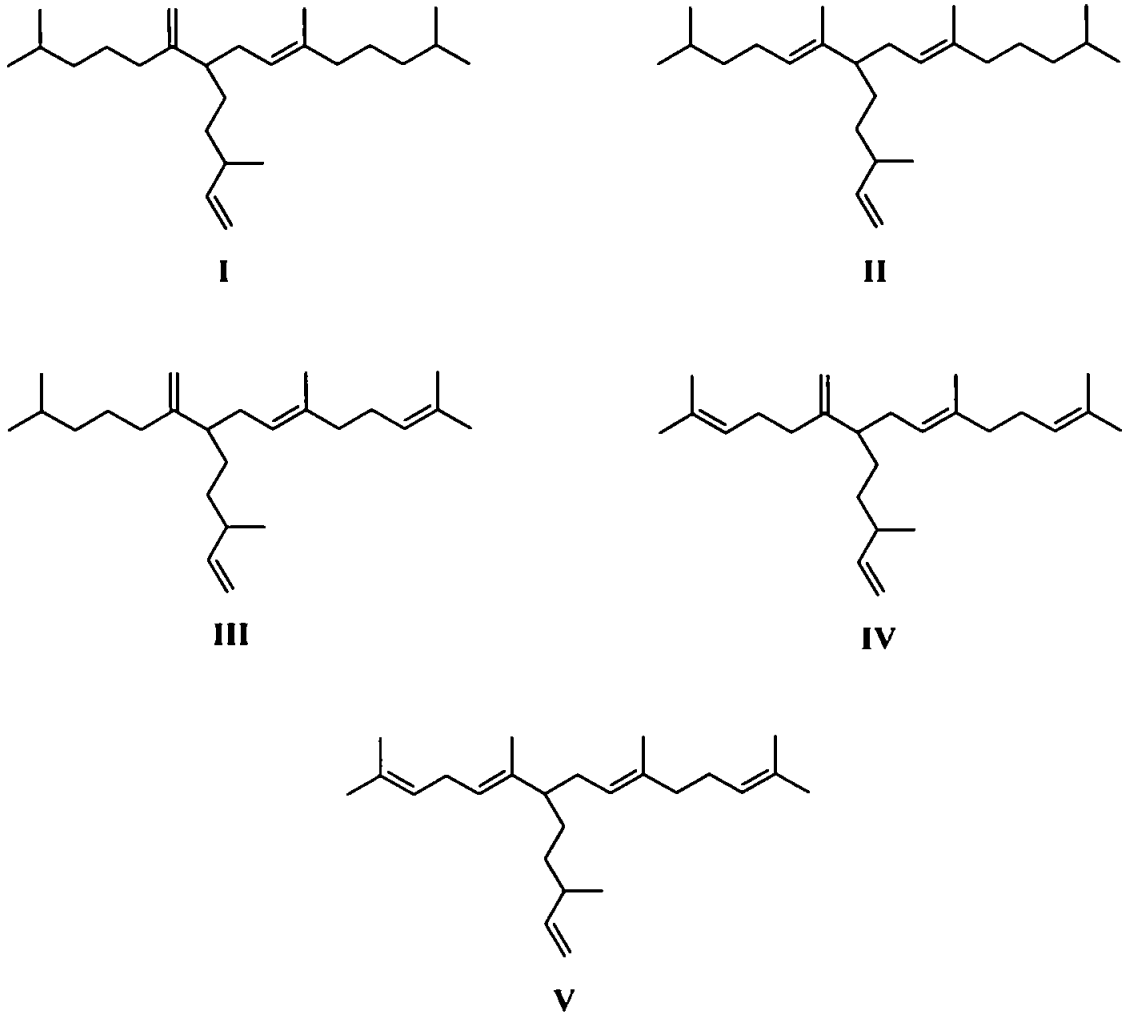


Figure 4.1: Structures of previously characterised C_{25} HBI alkenes observed in the *Haslea* species described in this study.

4.2 Experimental

4.2.1 Algal cultures

H. ostrearia, *H. crucigera* and *Haslea sp.* were isolated from the Bay of Bourgneuf (France) while *H. pseudostrearia* and *H. salstonica* were isolated from the Kingsbridge estuary (Devon, UK). *H. wawrikae* was isolated from the Bay of Brest (France), and the non-blue variety of *H. ostrearia* was isolated from the Bay of Marennes-Oléron (France). *H. ostrearia* cultures were grown in bulk between April 1991 and June 1998. A single large scale culture of *Haslea sp.* and a small scale culture of *H. wawrikae* were grown in an indoor culture facility between March and July 2000. Large scale cultures (440 L) of *H. crucigera, H. salstonica and H. pseudostrearia* were grown in an outdoor facility during September and October 1999.

4.2.2 Determination of HBIs in diatom species

Detection of HBIs in the different diatom species was achieved using filtered aliquots (25 to 50 ml) of algal cultures. Following extraction into hexane, aided by sonication, the total hexane extract (THE) was then saponified (5% KOH/MeOH/H_2O) and the non-saponifiable lipids (NSLs) were re-extracted into hexane, and examined by GC-MS. Identification of HBIs was achieved by comparison of GC RIs and mass spectral data with that of authenticated standards, followed by hydrogenation to the parent alkane.

4.2.3 Isolation and purification of HBIs

Each of the large scale cultures of the diatoms were centrifuged, freeze dried and extracted (Soxhlet) with hexane. The THEs thus obtained were saponified (5% KOH/MeOH/H_2O) and the non-saponifiable lipids re-extracted into hexane and purified by column chromatography (SiO_2 / hexane). Fractions containing >90% (GC) of individual HBIs were combined and analysed by ^1H and ^{13}C NMR spectroscopy. In all cases, the yield of purified HBIs from each culture corresponded to ca. 1 mg per 1 g wet centrifuged algal paste. Small aliquots of each isolated HBI were hydrogenated ($PtO_2.2H_2O$ / hexane) in order to confirm the carbon skeleton.

4.3 Results

4.3.1 *Identification of HBIs in Haslea spp.*

GC-MS analysis of the NSLs obtained from the small-scale screening experiments revealed the presence of several HBI alkenes with varying distributions. Figure 4.2 shows representative partial total ion current chromatograms (TICs) of the derivatised NSLs obtained from *H. ostrearia* ('blue' and 'non-blue' varieties), *H pseudostrearia, H. crucigera, H. salstonica* and *Haslea sp.*. It can be seen that in these cases, the NSL fractions contained HBIs, phytol, and in certain cases *n*-heneicosa-3,6,9,12,15,18-hexaene (*n*-$C_{21:6}$). These chromatograms clearly illustrate the presence of different HBI distributions in these species. No particular importance is attributed at this point to the differences between these distributions, since analysis of a number of different cultures of *H. ostrearia* revealed substantial variations in HBI structure(s) (Belt *et al.*, 1996; Johns *et al.*, 1999, 2000; Wraige *et al.*, 1997, 1999) and the same may well be true for the other species studied here.

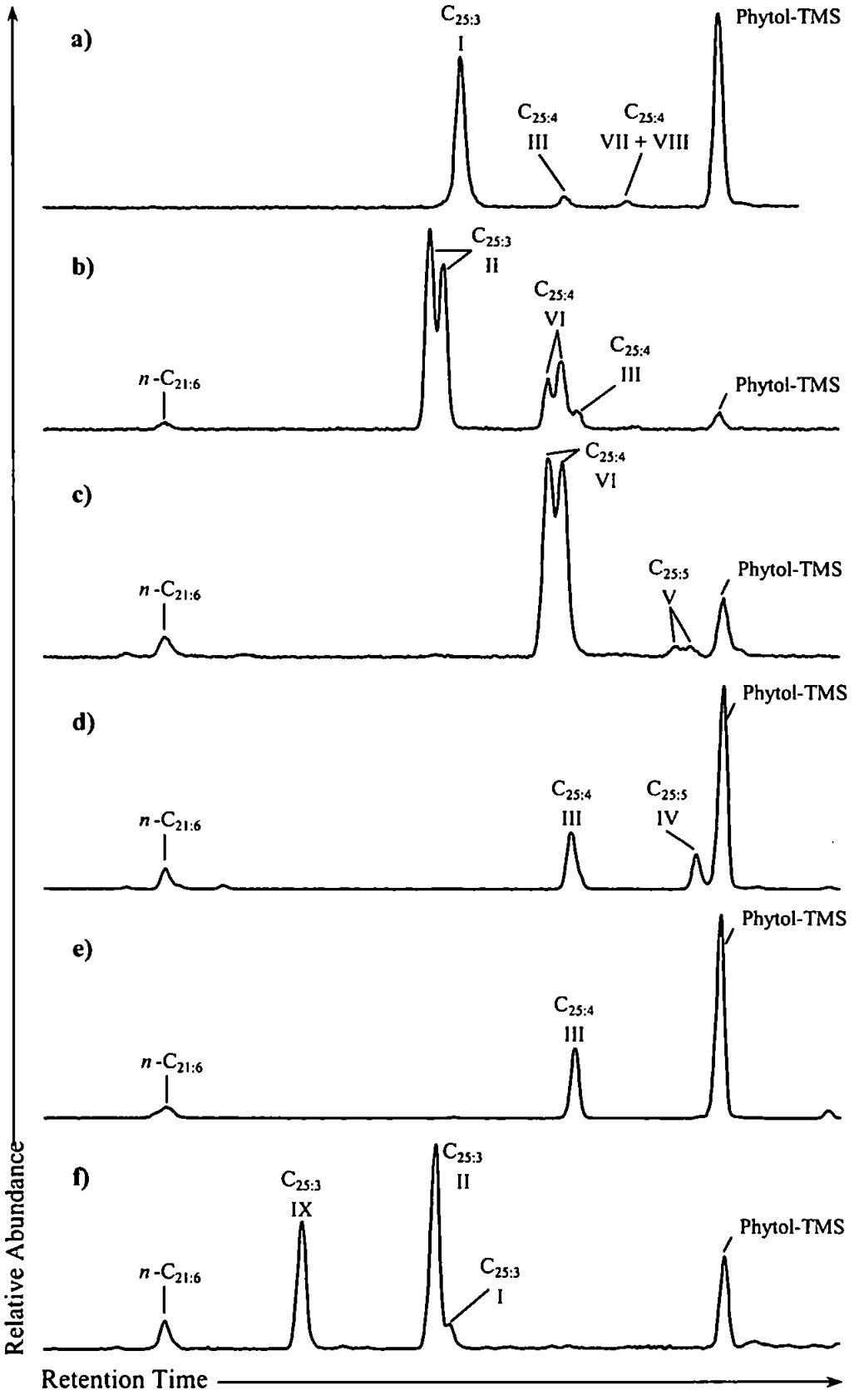


Figure 4.2: Representative partial GC (HP-1) total ion current chromatograms (TICs) of non-saponifiable hexane extracts from (a) *H. ostrearia* (b) *H. ostrearia 'non-blue'* (c) *H. pseudostrearia* (d) *H. crucigera* (e) *H. salstonica* (f) *Haslea sp.*

However, the analyses do suggest that each species can be considered to be a primary producer of these sesterterpenoid lipids. Closer examination of the TICs reveals that they consist of both HBIs reported previously (Figure 4.1) and new, uncharacterised HBI compounds (Figure 4.3).

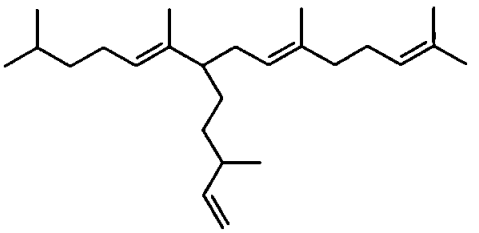
The overall HBI distributions of the various diatoms are summarised below. Previously unreported HBIs were isolated, and their structures determined by NMR as described in detail in section 4.3.2

Haslea ostrearia

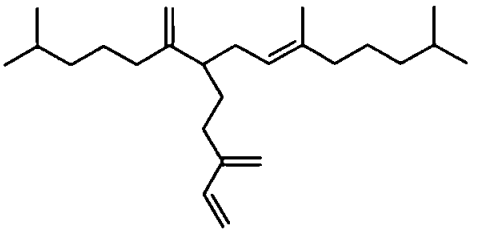
Of the three large scale cultures of *H. ostrearia* analysed in this present study, one culture (HO-1; Figure 4.2a) was found to contain mainly triene I (*ca* 90% of total HBIs) and a trace amount of the tetraene IV characterised from previous cultures (Wraige *et al.*, 1997), together with two previously unreported tetraenes (VII, VIII; table 4.1). The two other cultures of *H. ostrearia* consisted mainly of known HBI triene V with trace quantities of the pseudo-homologues; tetraene (III) and pentaene (IV). The new tetraenes (VII and VIII) found in HO-1 (*vide infra*) were also present, though their concentrations represented less than 10% of the total HBIs for all three cultures. The 'non blue' variety of *H. ostrearia* contained a range of C_{25} HBI alkenes (Figure 4.2b), including the known triene II (Wraige *et al.*, 1999) (80%; resolves as 2 GC peaks), and a trace amount of the tetraene III. This culture also contained a previously uncharacterised tetraene VI (20%; resolves as 2 GC peaks).

Haslea crucigera

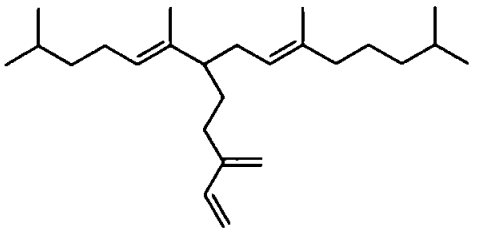
The single large scale culture of *H. crucigera* was found to yield C_{25} tetraene III (45%) and pentaene IV (22%). No new HBI alkenes were detected (Figure 4.2d). Following further fractionation of tetraene III and pentaene IV (SiO_2 / hexane), NMR analysis of these individual components demonstrated that they were present as single stereoisomers rather



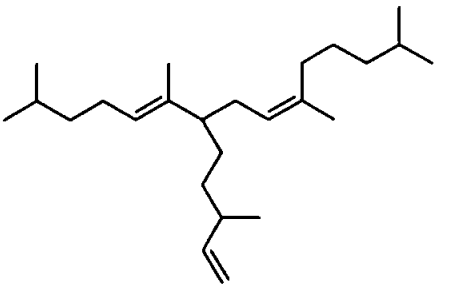
VI



VII



VIII



IX

Figure 4.3: Structures of the novel C_{25} HBI alkenes described in this study.

Table 4.1: Retention indices for the HBIs observed in diatoms of the *Haslea* genus.

HBI Alkene	Retention Index HP-1	HP-5	DB-WAX
$C_{25:3}$ I	2099, 2103	2103, 2106	-
$C_{25:3}$ IX	2065	-	-
$C_{25:4}$ III	2140	2145	2232, 2238
$C_{25:4}$ VI	2134, 2138	2143, 2146	2221, 2227
$C_{25:4}$ VIII	2157	2159	2267
$C_{25:4}$ VII	2157	2159	2277

than mixtures of diastereoisomers, even though diastereomeric mixtures of HBIs have been observed previously in cultures of *H. ostrearia* (Belt *et al.*, 1996; Johns *et al.*, 1999).

Haslea pseudostrearia

The large scale culture of *H. pseudostrearia*, contained predominantly a new C_{25} HBI tetraene (VI; resolves as 2 GC peaks), together with a trace amount of pentaene IV (Figure 4.2c).

Haslea salstonica

The bulk culture of *H. salstonica* yielded only one stereoisomeric C_{25} HBI (Figure 4.2e), identified as tetraene III (Belt *et al.*, 1996).

Haslea sp.

This unidentified *Haslea sp.* contained three C_{25} HBI trienes (Figure 4.2f); Triene I (trace) triene II (60%) and a previously uncharacterised triene IX (40%).

Haslea wawrikae

The planktonic species, *H. wawrikae* did not yield any HBI alkenes detectable by GC-MS.

4.3.2 Characterisation of 2,6,10,14-tetramethyl-9-(3-methylpent-4-enyl)pentadeca-2,6,10-triene (VI)

C_{25} tetraene (VI, 81mg; 94% purity, GC) was isolated from a single large scale culture of *H. pseudostrearia* (Massé *et al.*, 2001) by extraction (hexane) and column chromatography (SiO_2 / hexane). GC analysis revealed that it resolves into two peaks on three stationary phases (e.g. Figure 4.2c) and that the GC RIs of both peaks were different to those obtained for the previously characterised HBI tetraene III (n.b. diastereomeric HBI III only resolves

into two peaks on DB-WAX, Table 4.1). In addition, while the mass spectra of the two tetraenes in this fraction were identical to each other, they were different to that of tetraene III (Figure 4.4). In particular, the mass spectrum of tetraene III exhibited a relatively large number of fragment ions at *m/z* > 200 with the most abundant ion at *m/z* 259, whereas the same spectral region for VI is dominated by two ions at *m/z* 207 and 275 (Figure 4.4). The intensity of ion *m/z* 95 in the spectrum of III was also significantly different to that of the new alkenes. Hydrogenation of VI gave a single compound which co-eluted with, and had an identical mass spectrum to that of synthetic $C_{25:0}$ (Robson and Rowland, 1986), thereby confirming the structure of the carbon skeleton.

Examination of the ^{1}H NMR spectrum of VI (Table 4.2) demonstrated the presence of a vinyl moiety (-CH=CH_2), three trisubstituted double bonds, and a 1:1 ratio of isopropyl ((CH_3)$_2$CH-) and isoprenyl ((CH_3)$_2$C=)groups. The ^{13}C NMR spectrum (Table 4.3) revealed six CH_3, eight CH_2, seven CH and three quaternary ^{13}C resonances, thereby confirming the substitution and multiplicity of the double bonds. The appearance of a resonance at *ca.* 49 ppm confirmed that the major branch point of the carbon chain (C-7) was saturated, consistent with that of other HBIs from *H. ostrearia* (Belt *et al.*, 1996; Wraige *et al.*, 1997; Johns *et al.*, 1999). Since the presence of a vinyl group and three trisubstituted double bonds were readily identified from the ^{1}H and ^{13}C NMR data, the four double bonds must be positioned at C23-C24 (vinyl), C5-C6, C9-C10 and either C2-C3 or C13-C14. Double bonds in both of the latter positions could be ruled out from the observation of an equal number of isopropyl and isoprenyl groups.

In order to determine which end of the carbon chain was unsaturated, the allylic region of the ^{1}H NMR spectrum was examined in more detail. Methylene (CH_2) protons that are allylic to two double bonds resonate (δ 2.6-2.8 ppm) to higher frequency compared to those which are

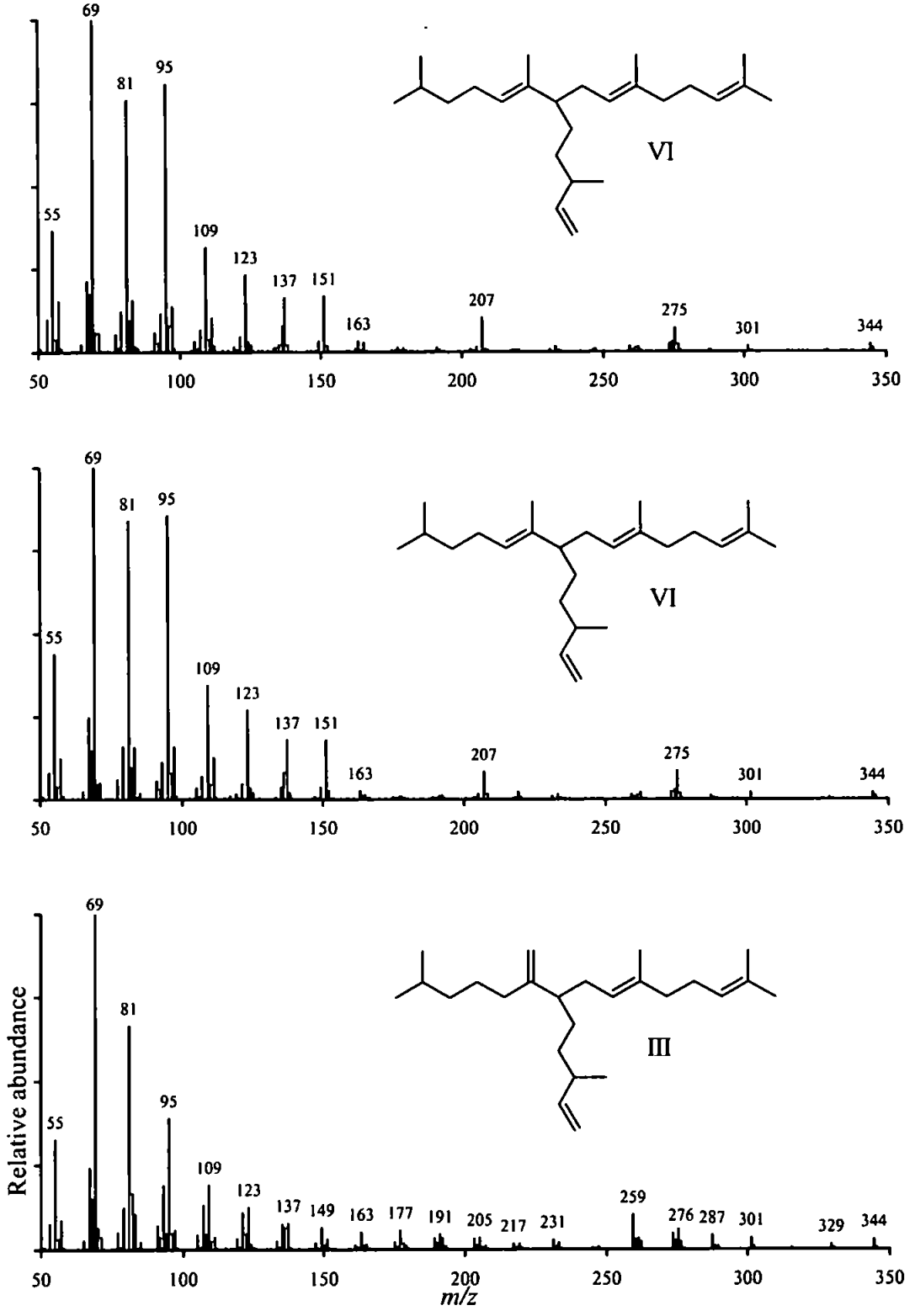


Figure 4.4: Comparison of mass spectra for the new tetraenes (VI) identified in *H. pseudostrearia* with tetraene (III) identified previously in cultures of *H. ostrearia*.

Table 4.2 ^{1}H NMR chemical shifts, multiplicities and integrations for $C_{25:4}$ (VI)

Chemical shift (ppm)	Assignment	Multiplicity (Coupling constant, Integration)
5.66	23	ddd (J = 7, 10.5, 17.5 Hz, 1H)
5.07	13	m (3H)
4.91	24	m (2H)
2.01	4, 7, 8, 11, 12, 22	m (10H)
1.67	15	s (3H)
1.59	19	s (3H)
1.57	18	s (3H)
1.53	2	m (1H)
1.45	17	s (3H)
1.24	3, 20, 21	m (6H)
0.96	25	d (J = 6.9 Hz, 3H)
0.88	1, 16	d (J = 6.6 Hz, 6H)

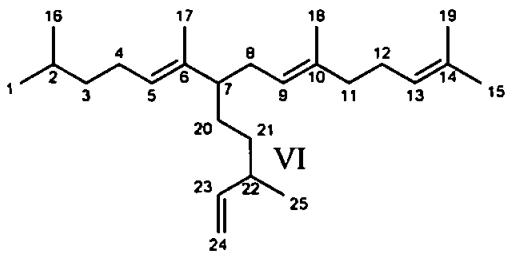
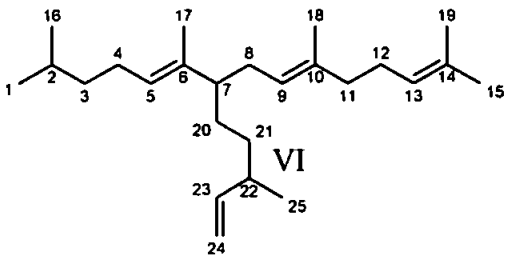


Table 4.3 ^{13}C NMR chemical shifts for $C_{25:4}$ (VI)

Chemical shift (ppm)	Multiplicity (DEPT)	Assignment
145.1, 144.8*	CH	23
136.3	C	6
134.8	C	10
131.1	C	14
126.2, 126.1*	CH	5
124.5	CH	13
123.5	CH	9
112.4, 112.1*	CH_2	24
49.4, 49.3*	CH	7
39.8	CH_2	11
39.1	CH_2	3
37.8, 37.7*	CH	22
34.6, 34.5*	CH_2	21
32.4, 32.3*	CH_2	8
30.4, 30.3*	CH_2	20
27.5	CH	2
26.8	CH_2	12
25.7	CH_3	15
25.5	CH_2	4
22.6	CH_3	1, 16
20.5, 19.9*	CH_3	25
17.7	CH_3	19
16.1	CH_3	18
11.8, 11.7*	CH_3	17

* indicates the presence of diastereomers



mono-allylic (δ 1.8-2.1 ppm). Thus, the chemical shift for H-4 in pentaene V (Sinninghe Damsté *et al.*, 1999b) appears at 2.8 ppm. The ^{1}H NMR spectrum of the tetraenes isolated from *H. pseudostrearia* did not have any resonances in this region, and integration of the mono-allylic region (10H) was consistent with a terminal double bond in the C13-C14 position. The low frequency chemical shifts for C-17 (11.8 ppm) and C-18 (16.1 ppm) confirmed that the C5-C6 and C9-C10 double bonds were *E* in both cases. The structure of the C_{25} HBI tetraene can thus be confirmed as VI (Figure 4.3). Finally, since most of the individual resonances in the ^{13}C spectrum appeared as doublets, it was concluded that the tetraene existed as a mixture of diastereoisomers, a feature that has been observed for other HBIs from *H. ostrearia* (Belt *et al.*, 1996; Johns *et al.*, 1999). In these cases, C-7 has been shown to have a fixed but unknown configuration, with variable stereochemistry at C-22 (Johns *et al.*, 2000). The existence of a pair of diastereoisomers can also explain the appearance of two compounds in the gas chromatogram which exhibit identical mass spectra.

4.3.3 Characterisation of 2,6,10,14-tetramethyl-7-(3-methylene-pent-4-enyl)pentadeca-5,9-diene (VIII) and 2,6,14-trimethyl-10-methylene-9-(3-methylene-pent-4-enyl)pentadec-6-ene (VII)

In virtually all of the cultures of *H. ostrearia* studied herein, two additional HBI tetraenes were detected as minor components (<10% of total HBIs). These had different GC (Table 4.1) and mass spectral (Figure 4.5) properties to known tetraenes III or VI. Following repeated open column chromatographic fractionation (SiO_2 / hexane), these two new tetraenes were obtained from several cultures of *H. ostrearia* in sufficient quantities and purity for structural analysis by GC-MS and NMR spectroscopy.

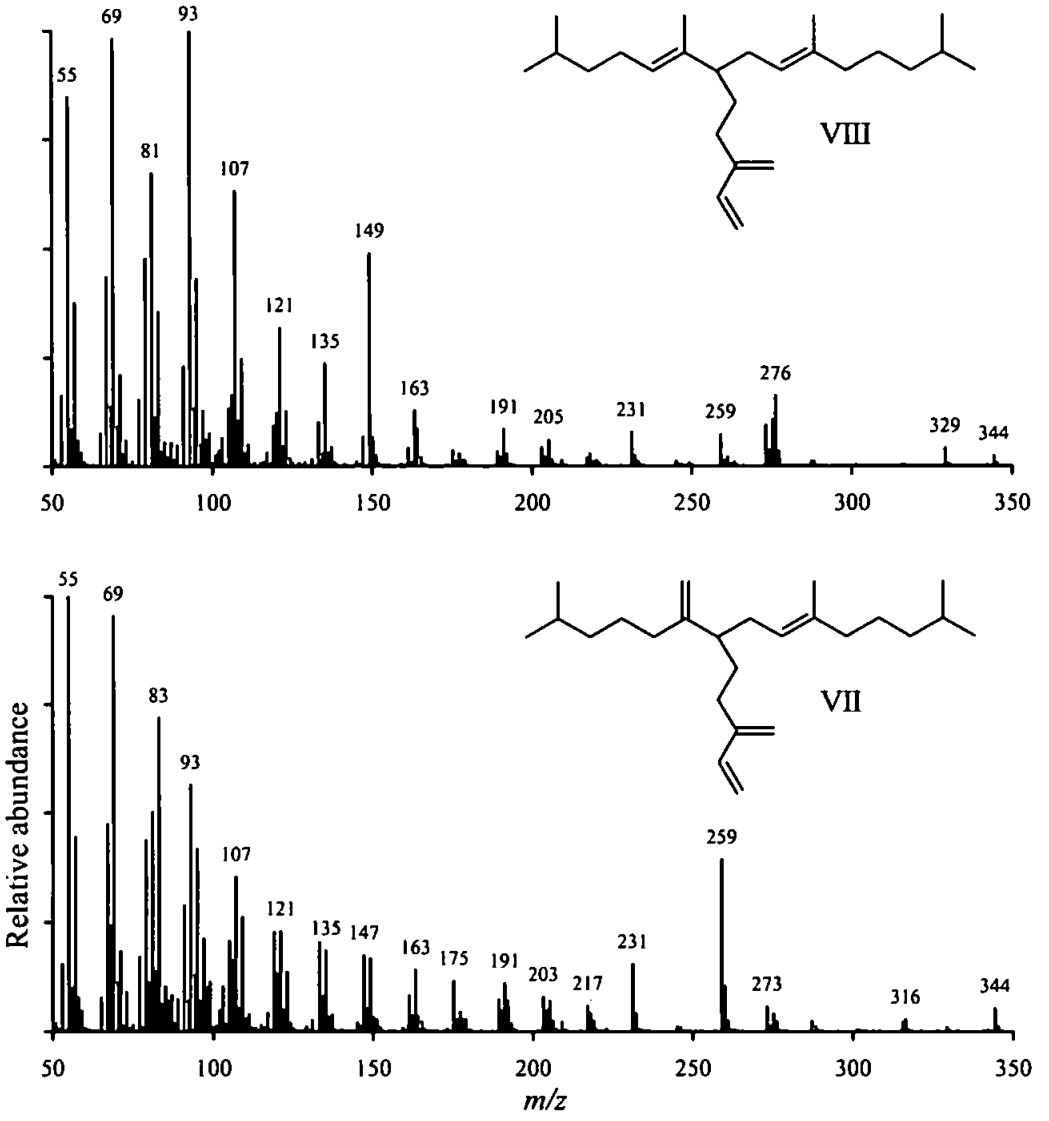


Figure 4.5: Mass spectra of the new tetraenes VII and VIII isolated from *H. ostrearia*.

The ^{1}H NMR spectra of both compounds contained a resonance due to the vinylic proton H-23, which had a significantly higher frequency chemical shift (δ 6.33 ppm) than the analogous resonance in spectra of III and VI, and appeared as a dd due to coupling to H-24_{cis} and H-24_{trans} only. Further methylene (alkene) proton resonances were observed at 4.95 ppm. These observations are consistent with double bond bonds located at C23-C24 and C22-C25. This arrangement of conjugated double bonds is unusual for HBI alkenes and has only been reported previously in a hexaene isolated from *H. ostrearia* (Wraige *et al.*, 1997). Additional ^{1}H and ^{13}C NMR spectral analysis of these two tetraenes confirmed their structures as VIII and VII (Figure 4.3). HBI VIII contains 2 trisubstituted double bonds while VII has one methylene and one trisubstituted double bond. Both compounds are saturated (isopropyl) at each end of the carbon chain and the stereochemistries of the trisubstituted double bonds (C5-C6 and C9-C10 (VIII); C9-C10 (VII)) were found to be *E* by analysis of ^{13}C data for C-17 and C-18. The ^{1}H and ^{13}C NMR data for VIII are summarised in Tables 4.4 and 4.5 respectively. Tables 4.6 and 4.7 summarise the equivalent data from VII.

4.3.4 Characterisation of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadeca-5E,9Z-diene (IX)

A mixture of two C_{25} trienes (3.0 mg; RI 2065, $2103_{HP\text{-}1}$, ratio 1:0.7, 98% purity, GC) was isolated from a single large scale culture of *Haslea sp.* by extraction (hexane) and column chromatography (SiO_2 /hexane). Further separation of the individual compounds was not achieved. Analysis of the mixture by GC-MS revealed the presence of the previously characterised C_{25} triene II, RI $2103_{HP\text{-}1}$ (Wraige *et al.*, 1999) as the minor component. The major component of this mixture had a mass spectrum which was virtually identical to that of authenticated II (Figure 4.6), but had a GC RI ($2065_{HP\text{-}1}$) which did not correlate with any

Table 4.4 ^{1}H NMR chemical shifts, multiplicities and integrations for $C_{25:4}$ (VIII)

Chemical shift (ppm)	Assignment	Multiplicity (Coupling constant, Integration)
6.33	23	dd (J = 10.7, 17.7 Hz, 1H)
5.2 - 4.99	5, 9, 24	m (4H)
4.95	25	br (s, 2H)
2.2 - 1.9	4, 7, 8, 11, 21	m (9H)
1.6 - 1.1	2, 3, 12, 13, 14, 20	m (10H)
1.54	18	s (3H)
1.47	17	s (3H)
0.86, 0.84	1, 15, 16, 19	2 x d (J = 6.6 Hz, 12H)

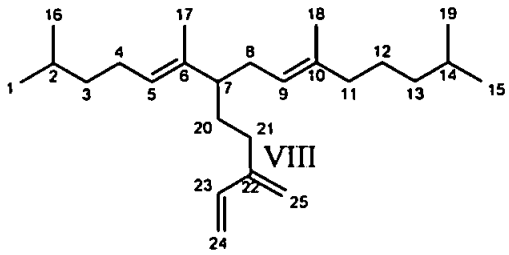


Table 4.5 ^{13}C NMR chemical shifts for $C_{25:4}$ (VIII)

Chemical shift (ppm)	Multiplicity (DEPT)	Assignment
146.9	C	22
139.1	CH	23
135.9	C	10
135.4	C	6
126.6	CH	5
123.1	CH	9
115.4	CH_2	25
113.1	CH_2	24
49.4	CH	7
39.9	CH_2	11
39.0	CH_2	3
38.5	CH_2	13
32.2	CH_2	8
31.3	CH_2	21
29.4	CH_2	20
27.9 and 27.5	CH	2, 14
25.7 and 25.5	CH_2	12, 4
22.7 and 22.6	CH_3	1, 15, 16, 19
16.0	CH_3	18
11.9	CH_3	17

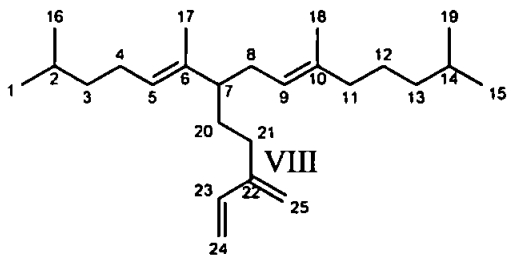


Table 4.6 ^{1}H NMR chemical shifts, multiplicities and integrations for $C_{25:4}$ (VII)

Chemical shift (ppm)	Assignment	Multiplicity (Coupling constant, Integration)
6.33	23	dd (J=10.7, 17.7 Hz, 1H)
5.20 - 4.99	9, 24	m (3H)
4.95	25	br, s (2H)
4.78	17a	br, d (1H)
4.74	17b	br, s (1H)
2.2 - 1.9	5, 7, 8, 11, 21	m (9H)
1.6 - 1.1	2, 3, 4, 12, 13, 14, 20	m (12H)
1.54	18	s (3H)
0.86, 0.84	1, 15, 16, 19	2 x d (J = 6.6 Hz, 12H)

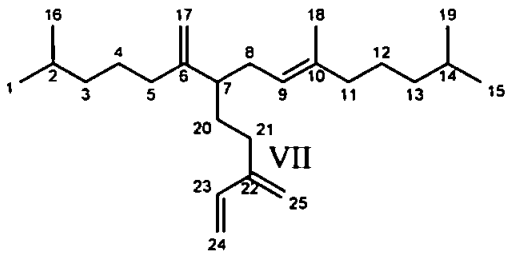
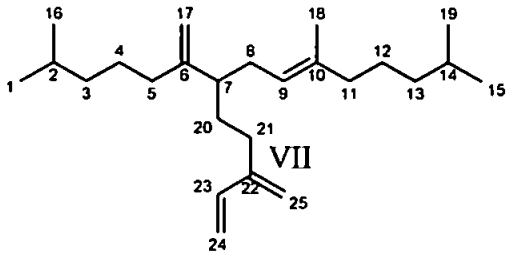


Table 4.7 ^{13}C NMR chemical shifts for $C_{25:4}$ (VII)

Chemical shift (ppm)	Multiplicity (DEPT)	Assignment
152.2	C	6
146.8	C	22
139	CH	23
135.8	C	10
122.9	CH	9
115.3	CH_2	25
113	CH_2	24
108.9	CH_2	17
46.6	CH	7
39.9	CH_2	11
38.9	CH_2	3
38.5	CH_2	13
33.8	CH_2	5
32.8	CH_2	8
31.8	CH_2	21
29.2	CH_2	20
28.0 and 27.9	CH	2, 14
25.7 and 25.5	CH_2	12, 4
22.7 and 22.6	CH_3	1, 15, 16, 19
16	CH_3	18



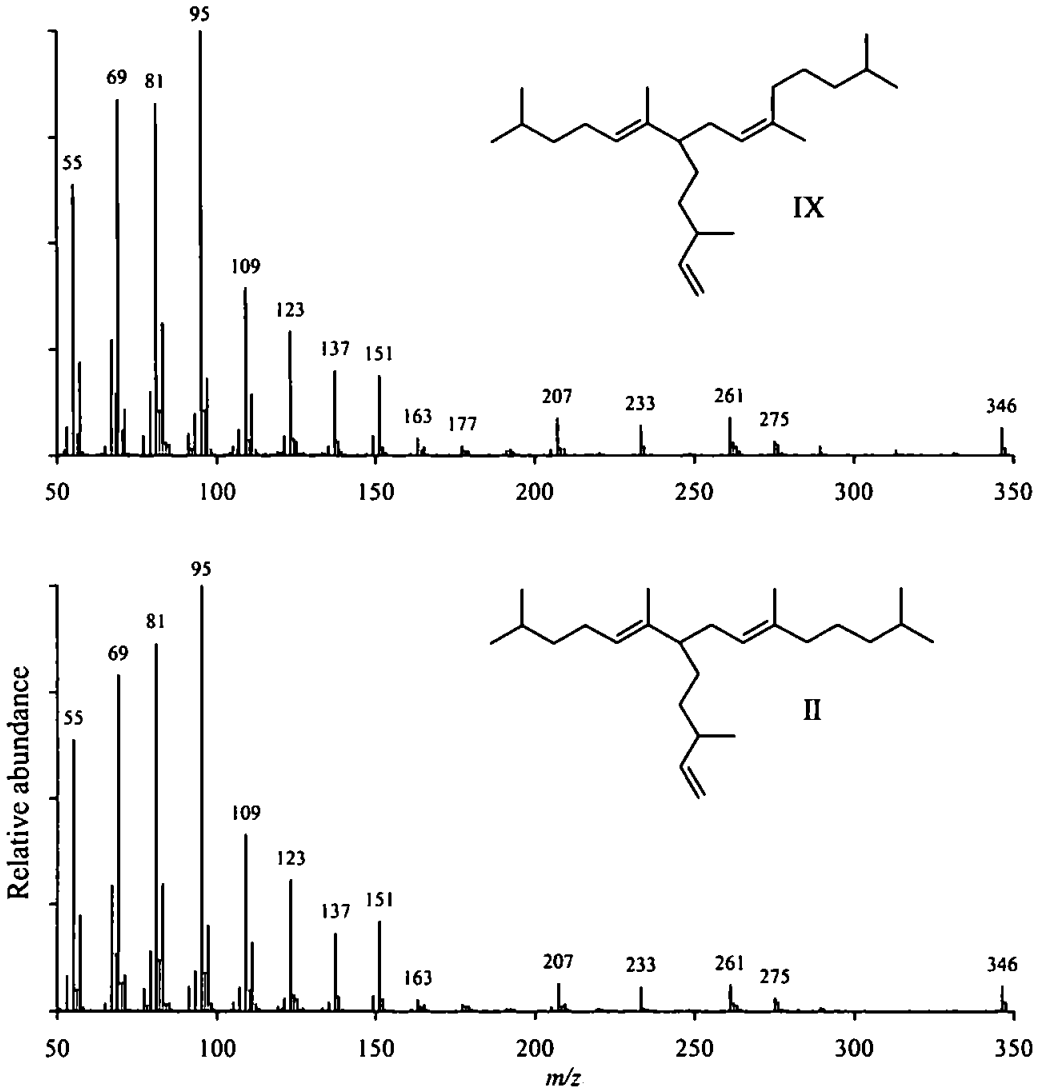


Figure 4.6: Mass spectra for the HBI trienes isolated from *Haslea* sp. (new triene IX; authenticated triene II).

previously reported C_{25} trienes (Table 4.1). Hydrogenation of this mixture yielded a single compound which co-eluted with, and had an identical mass spectrum to that of synthetic $C_{25:0}$ (Robson and Rowland, 1986), confirming the carbon skeleton.

The ^{1}H NMR spectrum of the mixture was almost identical to that obtained for pure II, but an additional resonance (δ 1.62 ppm) was also observed. This was consistent with H-18 being present in two configurations due to *E/Z* isomerism (C9-C10), a feature which has been observed for HBI alkenes from *Pleurosigma intermedium* (Chapter 2). In the ^{13}C NMR spectrum of the mixture, many resonances appeared as closely spaced doublets, the peaks of which had a relative intensity of approximately 0.7:1, consistent with the composition of the mixture, and suggesting that the structures of the two compounds were similar but not identical (e.g. that they were stereoisomers). The resonances corresponding to C-18 (CH_3) and C-11 (CH_2) (δ 16.03 and 32.1 ppm respectively) for triene II (Wraige *et al.*, 1999) were single peaks, and an extra methyl (CH_3) resonance (δ 23.4 ppm) and methylene (CH_2) resonance (δ 32.1 ppm) were also observed. This observation can be explained if the mixture contained a pair of geometric isomers, exhibiting *E/Z* isomerism about the C9-C10 double bond as observed for the C_{25} HBI isomers identified and characterised from cultures of *P. intermedium* (Chapter 2). Therefore, the structure of the new C_{25} triene can be confirmed as IX (Figure 4.3). The ^{1}H and ^{13}C NMR data for IX is summarised in Tables 4.8 and 4.9 respectively.

Table 4.8 ^{1}H NMR chemical shifts, multiplicities and integrations for $C_{25:3}$ (IX)

Chemical shift (ppm)	Assignment	Multiplicity (Coupling constant, Integration)
5.63	23	ddd (J = 7.4, 10.1, 17.4 Hz, 1H)
5.03	5, 9	m (2H)
4.9	24	m (2H)
1.83 - 2.07	4, 7, 8, 11, 22	m (8H)
1.62	18	s (3H)
1.42	17	s (3H)
1.05 - 1.39	2, 3, 12, 13, 14, 20, 21	m (12H)
0.93	25	d (J = 6.9 Hz, 3H)
0.85 and 0.84	1, 15, 16, 19	2 x d (J = 6.6 Hz, 12H)

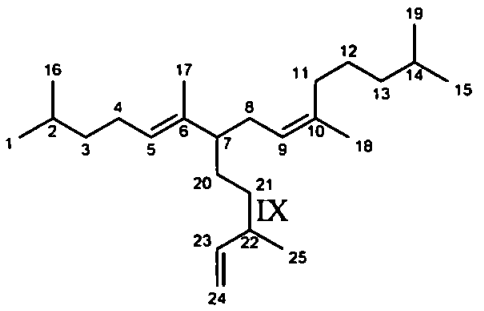
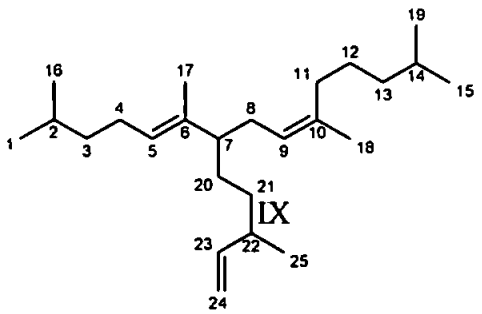


Table 4.9 ^{13}C NMR chemical shifts for $C_{25:3}$ (IX)

Chemical shift (ppm)	Multiplicity (DEPT)	Assignment
144.9	CH	23
136.2	C	6
135.4	C	10
126.3	CH	5
124.0	CH	9
112.3	CH_2	24
49.5	CH	7
39.03 and 38.96	CH_2	3, 13
37.8	CH	22
34.5	CH_2	21
32.13	CH_2	8
32.10	CH_2	11
30.3	CH_2	20
27.9	CH	2
27.4	CH	14
25.7	CH_2	4
25.5	CH_2	12
23.4	CH_3	18
22.6	CH_3	1, 15, 16, 19
20.5	CH_3	25
11.7	CH_3	17



4.4 Discussion

Several important points are made clear by the present study. First, production of HBIs is widespread within the *Haslea* genus (at least 5 spp.) and is not a unique characteristic of *H. ostrearia*. One of the species studied, *H. wawrikae*, did not produce any HBI alkenes. Thus, HBI production cannot be considered to be a feature of the entire genus. However, it should be noted that *H. wawrikae* is a planktonic diatom whilst the other species studied herein are benthic, and it is possible therefore that HBI production is limited to benthic *Haslea* species only. Further study of other planktonic *Haslea* species such as *H. gigantea* (Simonsen, 1974) is needed before such a hypothesis can be confirmed.

Secondly, the new HBI structures characterised in this study share common features with other polyunsaturated HBI alkenes previously observed in *H. ostrearia* (Belt *et al.*, 1996; Johns *et al.*, 1999, 2000; Wraige *et al.*, 1997, 1999). In all cases, the major branch point at C7 is saturated, all possess a vinyl moiety at C23-C24, and a double bond is present in either the C5-C6 or C6-C17 positions. HBIs possessing a double bond in the C7-C20 position, as observed in *P. intermedium* (Chapter 2) and *R. setigera* (Chapter 3) appear not to be produced by *Haslea* species, and this may be of taxonomic significance.

Third, the chromatographic behaviour of the diastereomeric tetraenes (VI) and trienes (II) show characteristics that have not been previously reported. Triene (II) observed in previous cultures of *H. ostrearia* (Wraige *et al.*, 1999) was present as a single enantiomer. However, in the present study, the tetraene (VI) and triene (II) were observed as pairs of diastereoisomers in the cultures of *H. pseudostrearia* (VI only) and *H. ostrearia (non-blue)* (II and VI), and resolved into two peaks by GC (Figure 4.2, Table 4.1) on all phases used. The isomeric tetraene (III) and triene (I) have been observed in *H. ostrearia* as mixtures of diastereoisomers (Johns *et al.*, 2000; Belt *et al.*, 1996), but these diastereoisomers are only

resolved by GC on apolar phases (Table 4.1). Since this study demonstrates that members of the *Haslea* genus produce different structural isomers (e.g. trienes II and IX) and variability in the stereochemistry of HBIs from *H. ostrearia* has been reported (Johns *et al.*, 2000), care should be taken when assigning the stereochemistry of HBIs by GC alone.

Finally, whilst several C_{25} HBIs are clearly produced by a number of *Haslea* species, the chromatographic (RI) and mass spectral properties of the most abundant HBI alkenes observed in sediments do not correlate with those of HBIs produced by members of the *Haslea* genus (see chapter 2). This implies that either sedimentary inputs of HBIs from *Haslea* spp. are not significant compared to the input from other diatoms, or that HBIs of the type produced by *Haslea* species are more prone to degradation and therefore do not persist in sediments, or that diagenetic reactions are responsible for the conversion of the *Haslea* type HBI isomers into those commonly found in sediments (e.g. Belt *et al.*, 2000).

CHAPTER FIVE

Identification of HBI Isomers in Sediments and Suspended Particulates from Marine Environments

5.1 Introduction

As discussed in Chapter 1, C_{25} and C_{30} HBI alkenes have been observed in sediments and biota from many marine and lacustrine environments (Reviewed by Rowland and Robson, 1990). Prior to the current study, the identification of HBIs from environmental matrices was largely limited to identification of the carbon skeleton *via* hydrogenation to the parent alkane, and assignment of the number of double bonds using mass spectrometry. Determination of double bond positions and/or stereochemical conformations of HBIs isolated from samples other than algae has been achieved in a very limited number of cases. Thus, Yruela *et al.* (1990) isolated the HBI diene (I, Figure 5.1) from sediments of the Guadalquivir Delta (Spain) and assigned double bond positions *via* epoxidation and GC-MS. An unusual highly branched C_{25} alkadiene (II, Figure 5.1) was isolated from a diatomaceous benthic microbial community of Hamelin Pool (Western Australia), and was characterised by NMR and MS (Summons *et al.*, 1993). Belt *et al.* (1994) isolated the HBI diene (III, Figure 5.1) from sediments of the Caspian Sea, and characterised it using ozonolysis and epoxidation in addition to NMR spectroscopy and MS. For the most part, such identifications require a rather painstaking isolation of the pure HBIs before spectroscopic characterisation by NMR and other methods. This is rarely possible given the low concentrations of these compounds in sediments (e.g. 230 ng/g dry sediment; Requejo and Quinn, 1983) and the small amounts of samples available from environments such as the deep sea and other locations, where accessibility is limited or where samples are expensive to obtain.

However, many of the HBIs previously reported but not fully identified in small scale environmental samples such as oceanic sedimenting particles can now be more firmly assigned by comparing their GC (RI) and MS characteristics with data reported for authenticated compounds such as those isolated and characterised from diatom cultures (e.g. Chapter 2, Chapter 3, Chapter 4, Belt *et al.*, 1996). Variations in RIs and MS characteristics due to factors such as GC column phase, temperature program and MS operating conditions make co-chromatography with authenticated compounds desirable, preferably on more than one GC stationary phase. For example, this was accomplished by Johns *et al.* (1999), who identified the HBI diene IV (Figure 5.1) in Antarctic sediments, *via* co-injection on 2 phases with authentic IV isolated and characterised from laboratory cultures of *H. ostrearia*.

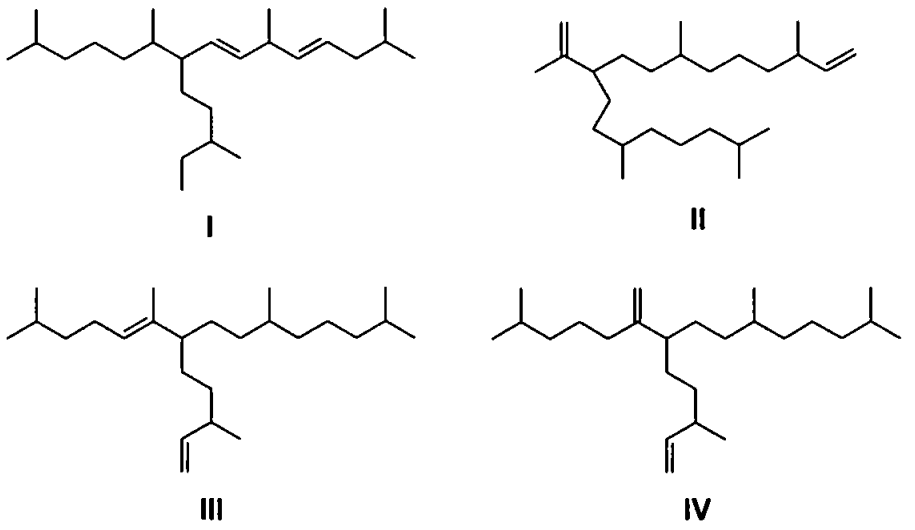


Figure 5.1 C_{25} HBI alkenes identified previously in environmental matrices.

The isolation and characterisation of HBIs from *P. intermedium, R. setigera* and *Haslea spp.* reported herein, combined with previous work in this laboratory has resulted in a library of

authenticated HBI alkenes. These can now facilitate greatly the identification of HBI isomers in sediments and suspended particulate matter (SPM) from a diverse range of marine environments.

Thus in the present study, hydrocarbon fractions isolated previously from sediments and SPM from the Cariaco Trench (Wakeham, 1990), the Peru Upwelling region (Pancost *et al.*, 1997), the Black Sea (Wakeham and Beier, 1991) and the Arabian Sea (Wakeham, 2001) were provided by Dr. S. Wakeham (Skidaway Institute of Oceanography, USA). These fractions were re-examined by GC-MS, and the HBIs identified by co-chromatography on two stationary phases with authenticated HBI standards. Additional structural information was obtained *via* hydrogenation of the hydrocarbon fractions. Such unambiguous identifications of the HBI isomers in environmental samples are important if the palaeo-environmental and geochemical signature of the HBI distributions is to be fully understood.

5.2 Sampling locations and previous findings

5.2.1 Cariaco Trench

The Cariaco Trench is a relatively small, 1400 m deep anoxic open-ocean depression, surrounded by a 150 m deep sill located on an oxygenated continental shelf to the North of Venezuela. A C_{25} HBI diene (b25:2), two trienes (b25:3 and b25:3') and three tetraenes (RI b25:4, b25:4* and b25:4') were reported previously (Wakeham, 1990) in the sediment and particulate material from this region, although no mass spectral or retention data was reported for these compounds. It was noted that the triene (b25:3 and b25:3') and tetraene (b25:4 and b25:4') pairs were abundant in the surface water particles of the Cariaco Trench, and that their concentrations decreased rapidly with depth, suggesting their production by phytoplankton (Wakeham, 1990).

5.2.2 Peru Upwelling region

The off-shore waters of Peru are an area of high primary productivity supported by the upwelling of nutrient-rich waters. Two C_{25} HBI trienes (RI 2044_{SE-52} and 2092_{SE-52}) and two HBI tetraenes (RI 2082_{SE-52} and 2129_{SE-52}) have been reported previously in the sediments of this region (Volkman *et al.*, 1983). Volkman *et al.* (1983) also suggested that the HBIs observed in the sediments of the Peru upwelling region originated from a diatom source. It was also noted by Volkman *et al.* (1983) that the concentrations of the HBI alkenes in the sediments of this area decreased rapidly with depth, and it was suggested that this was a result of biodegradation, as the authors observed a large bacterial biomass in these sediments. Volkman *et al.* (1983) also proposed that the removal of HBIs from the sediments could also be due to their irreversible incorporation into accreting polymeric material *via* cross-linking reactions involving the double bonds.

5.2.3 Black Sea

C_{25} dienes, trienes and tetraenes have been observed previously (Freeman *et al.*, 1994; Wakeham *et al.*, 1995) in the sediments from this land-locked anoxic marine basin, although no RI or MS data were given in either report. Wakeham *et al.* (1995) also observed that the HBI concentrations within the sediments decreased rapidly with depth. C_{25} HBI thiolanes were also identified in the sediments from this region, and it was proposed that these were produced *via* intramolecular incorporation of sulphur into the HBI alkenes (Wakeham *et al.*, 1995).

5.2.4 Arabian Sea

Wakeham *et al.* (2001) observed three C_{25} HBI trienes, three tetraenes and three pentaenes in the Suspended particulate matter (SPM) and sediments of this region, although no retention indices or MS data were given for these compounds. Wakeham *et al.* (2001) observed that

HBI concentrations in water column particulates of the Arabian sea decreased with depth, and that more than 99% of the total HBIs were removed from the particulate material before incorporation into the sediment. Wakeham *et al.* (2001) also noted that a C_{25} HBI triene (b25:3) accounted for the majority of the HBI flux during the southwest monsoon (SWM) season, whilst a C_{25} HBI tetraene (b25:4') was the major HBI alkene during the rest of the year.

5.3 Results

5.3.1 General observations

Analysis of the hydrocarbon fractions of each of the SPM and sediment samples by GC-MS revealed that all of the samples contained HBI alkenes. Figure 5.2 shows the structures and GC RIs of the C_{25} HBIs identified by co-chromatography of extracts from the sediment and SPM samples with authentic standards obtained from various diatoms. Significant variations in the HBI distributions were observed in samples from different locations, and also between SPM and sediment samples from the same sampling area. As many of the samples analysed herein were composites of material obtained from several sites within the same sampling area, care must be taken when drawing conclusions from the relative abundances of the individual compounds detected herein.

Samples from all four locations contained significant quantities of C_{25} HBIs. C_{30} and C_{20} HBIs were less common than the C_{25} HBIs. C_{30} HBIs were detected only in the samples from the Arabian Sea, where they were present in trace amounts in the SPM, but were significantly more abundant in the sediment. A single C_{20} monoene was detected in the sediment from the Peru upwelling region, and had an abundance comparable to that of the

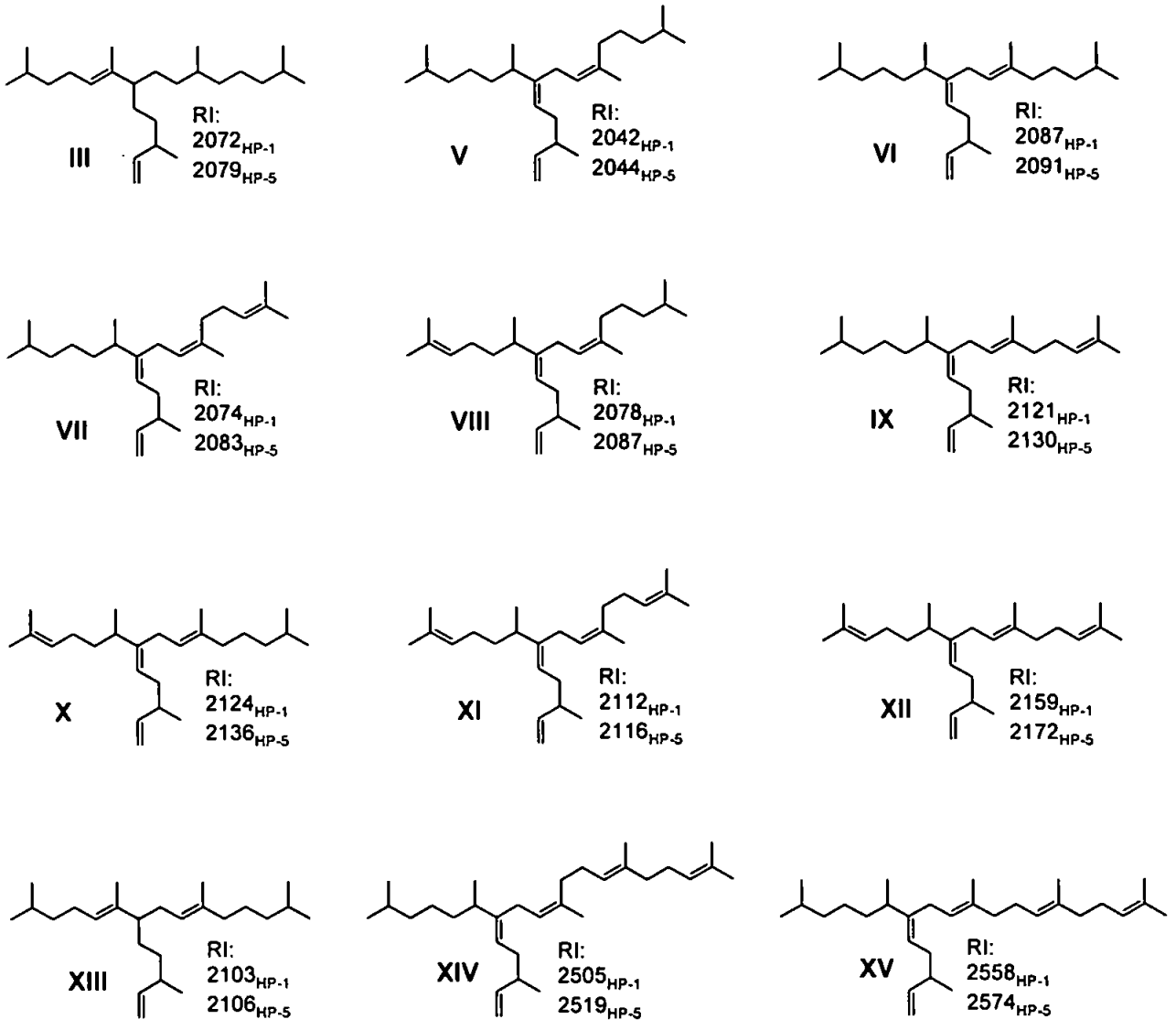


Figure 5.2 Structures of HBIs identified in sediment and particulate matter. Authentic **III** used for co-chromatography was isolated from Caspian Sea sediments (Belt *et al.*, 1994), **V to XII** from *P. intermedium* cultures (Chapter 2), **XIII** from *H. ostrearia* cultures (Wraige *et al.*, 1999), and **XIV** and **XV** from *R. setigera* (Chapter 3).

C_{25} isomers. The suite of C_{25} HBIs possessing Δ 7(20) unsaturation characterised in Chapter 2, and concluded to be the most abundant isomers found globally, were the predominant HBI isomers in the SPM and sediments from the Arabian sea, Cariaco trench and Peru upwelling region, but perhaps surprisingly, they were absent from the Black Sea. Additionally, C_{25} HBIs with Δ5(6) unsaturation, previously characterised from cultures of *H. ostrearia*, were present in many samples, and were the only HBIs observed in samples from the Black Sea. The HBI distributions in the sediment and SPM fractions from the four locations are summarised in Table 5.1.

5.3.2 HBI alkenes in hydrocarbon extracts from the Arabian Sea

Complex distributions of C_{25} HBI alkenes were present in both the SPM and sediment extracts from the Arabian Sea. The TIC chromatogram of the SPM extract (Figure 5.3a) was dominated by these compounds, and they were also abundant in the sediment extract (Figure 5.3b). C_{30} HBI alkenes were detected in both the sediment and SPM fractions, and were present in far greater abundance in the sediment than the SPM, whilst C_{20} HBIs were not detected in either sample.

Close examination of the C_{25} HBIs in the SPM extract (Figure 5.4a) revealed the presence of at least ten different C_{25} HBI isomers possessing between two and five degrees of unsaturation. The predominant HBIs were of the structural type produced by the diatom *P. intermedium*, and possessed an unsaturated major branch point (*viz* 7(20) unsaturation). Indeed, the entire suite of eight C_{25} HBIs characterised following isolation from the diatom *P. intermedium* (Chapter 2), consisting of a pair of trienes (V, and VI), four tetraenes (VII, VIII, IX and X) and two pentaenes (XI and XII), were present in the SPM. There was also a small amount of a triene (XIII) possessing a saturated major branch point (*viz* 5(6)

Table 5.1 Summary of HBI occurrence in the SPM and sediment exracts from the Arabian Sea, Black Sea, Cariaco Trench and Peru Upwelling region.

		RI		Arabian Sea		Black Sea		Cariaco Trench		Peru Upwelling	
HBI	Structure	HP-1	HP-5	SPM	Sediment	SPM	Sediment	SPM	Sediment	SPM	Sediment
$C_{25:3}$	V	2042	2044	✓	✓	-	-	✓	✓	✓	✓
$C_{25:2•}$	unknown	2068	2071	✓	✓	-	-	✓	✓	✓	✓
$C_{25:2}$	III	2072	2079	-	✓	✓	✓	-	-	-	-
$C_{25:4}$	VII	2074	2083	✓	✓	-	-	✓	-	-	✓
$C_{25:4}$	VIII	2078	2087	✓	✓	-	-	-	-	-	-
$C_{25:3}$	VI	2087	2091	✓	✓	-	-	✓	✓	✓	✓
$C_{25:4•}$	unknown	2089	2102	✓	✓	-	-	✓	✓	✓	✓
$C_{25:3}$	XIII	2103	2106	✓	✓	✓	✓	-	✓	-	-
$C_{25:5}$	XI	2112	2116	✓	✓	-	-	-	-	-	-
$C_{25:4}$	IX	2121	2130	✓	✓	-	-	✓	-	-	✓
$C_{25:4}$	X	2124	2136	✓	✓	-	-	-	-	-	-
$C_{25:5}$	XII	2159	2172	✓	✓	-	-	-	-	-	-
$C_{30:4•}$	unknown	2494	2503	✓	✓	-	-	-	-	-	-
$C_{30:5}$	XIV	2505	2519	-	✓	-	-	-	-	-	-
$C_{30:5•}$	unknown	2548	2568	✓	✓	-	-	-	-	-	-
$C_{30:5}$	XV	2558	2574	-	✓	-	-	-	-	-	-

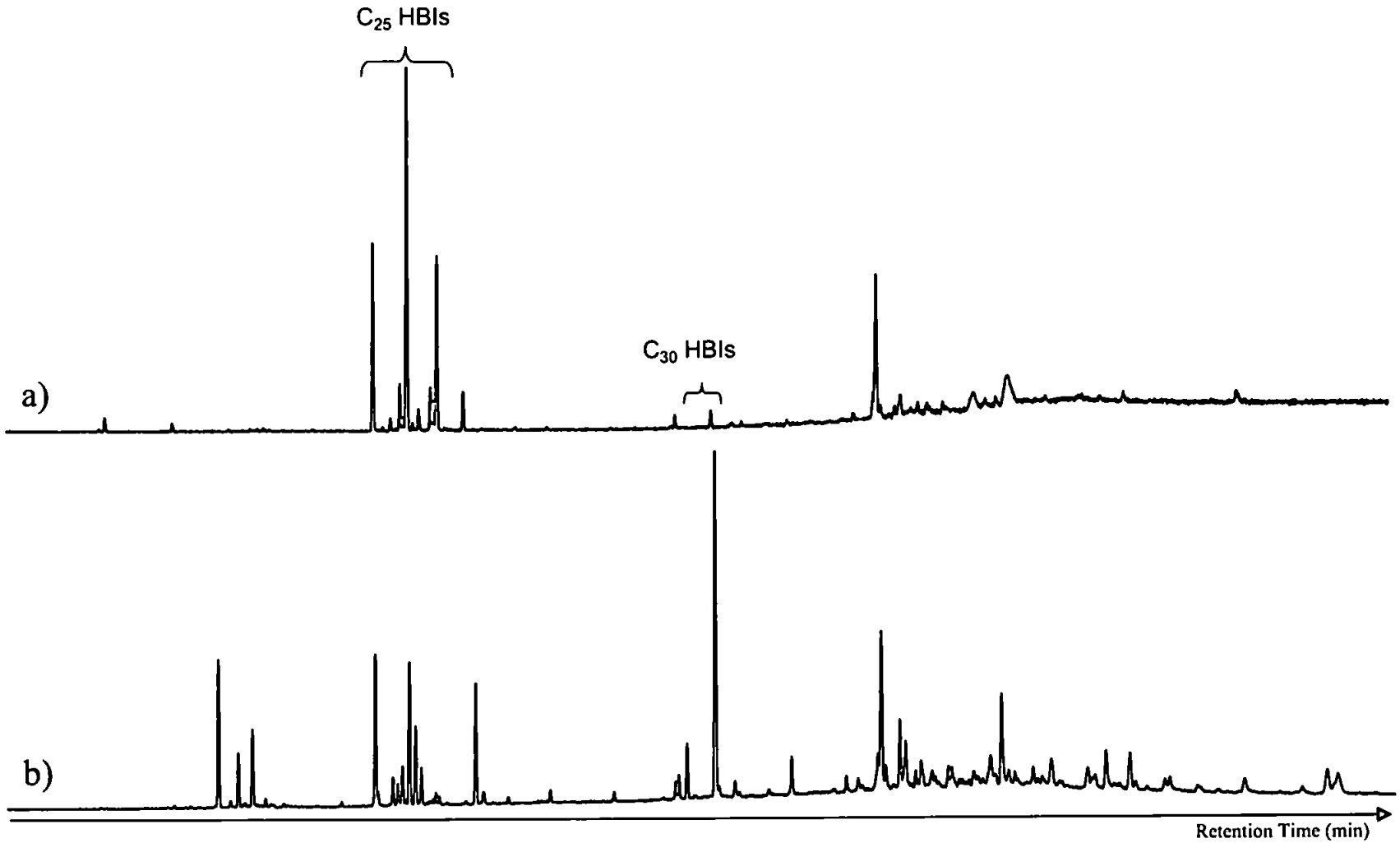


Figure 5.3 TIC chromatograms of hydrocarbon extracts of (a) SPM and (b) sediment from the Arabian Sea.

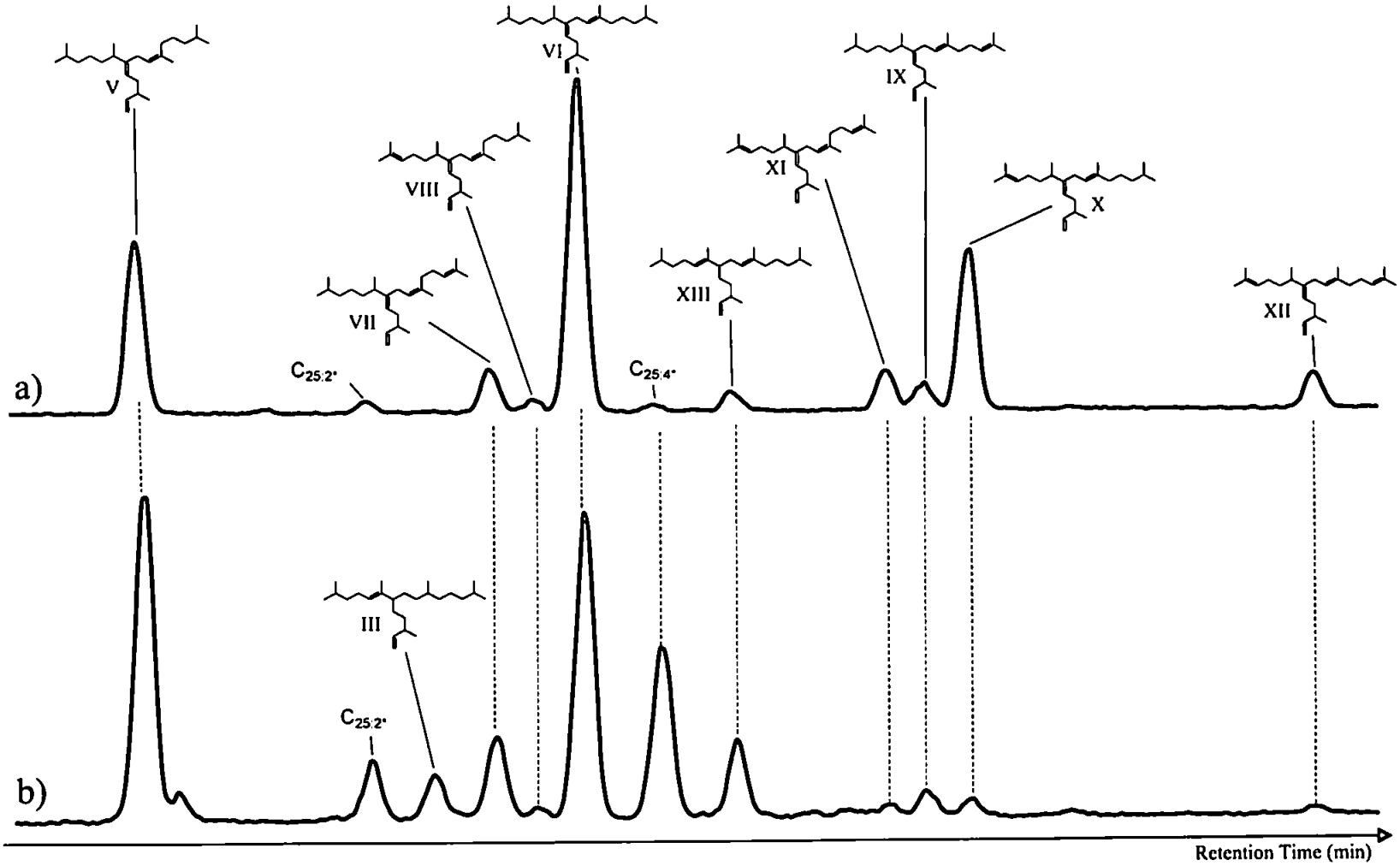


Figure 5.4 Partial TIC chromatograms showing the C_{25} HBI distributions in hydrocarbon extracts of (a) SPM and (b) sediment from the Arabian Sea. The structural identification of HBI isomers was achieved *via* co-chromatography with authenticated compounds.

unsaturation), previously characterised from *H. ostrearia* (Wraige *et al.*, 1999). An unidentified C_{25} alkene, RI $2071_{HP\text{-}5}$, $2068_{HP\text{-}1}$ possessing two degrees of unsaturation ($C_{25:2}$• Figure 5.4a), and a C_{25} alkene, RI $2102_{HP\text{-}5}$, $2089_{HP\text{-}1}$ possessing four degrees of unsaturation ($C_{25:4}$• Figure 5.4a), were also present in low abundance.

The C_{25} HBIs of the sediment extract (Figure 5.4b) also consisted of those described above, plus an additional HBI diene (III). The unidentified compounds $C_{25:2}$• and $C_{25:4}$• were present in higher relative abundances than in the SPM. The abundance of the more saturated HBI isomers (i.e. di- and trienes) appeared to be slightly higher in the sediment than the SPM when compared to the more unsaturated isomers (tetra- and pentaenes). This apparent increase in the abundance of the more saturated HBI isomers within the sediment will be discussed later.

In addition to the C_{25} HBIs, C_{30} HBIs were also detected in both the sediment and SPM extracts, and the abundances of the C_{30} compounds varied significantly between the two samples. The SPM fraction (Figure 5.5 a) contained trace amounts of an unidentified C_{30} compound RI $2503_{HP\text{-}5}$, $2494_{HP\text{-}1}$, possessing four degrees of unsaturation ($C_{30:4}$•; M^+ 414; Figure 5.5a) plus an unidentified C_{30} compound, RI $2568_{HP\text{-}5}$, $2548_{HP\text{-}1}$ possessing five degrees of unsaturation ($C_{30:5}$•; M^+ 412; Figure 5.5a). The sediment (Figure 5.5 b) contained a greater number of C_{30} compounds, including relatively low amounts of two C_{30} pentaenes with Δ 7(20) unsaturation (XIV, XV Figure 5.5b), identified by comparison with authenticated compounds isolated and characterised herein from the diatom *R. setigera* (Chapter 3). The unidentified $C_{30:4}$• and $C_{30:5}$• observed in the SPM were also present in the sediment, where the $C_{30:5}$• was the most abundant hydrocarbon. The uncharacterised $C_{30:5}$• has also been found to be the most abundant hydrocarbon in certain cultures of the diatom *R. setigera* (e.g. Figure 3.5 c), and this will be discussed more fully later.

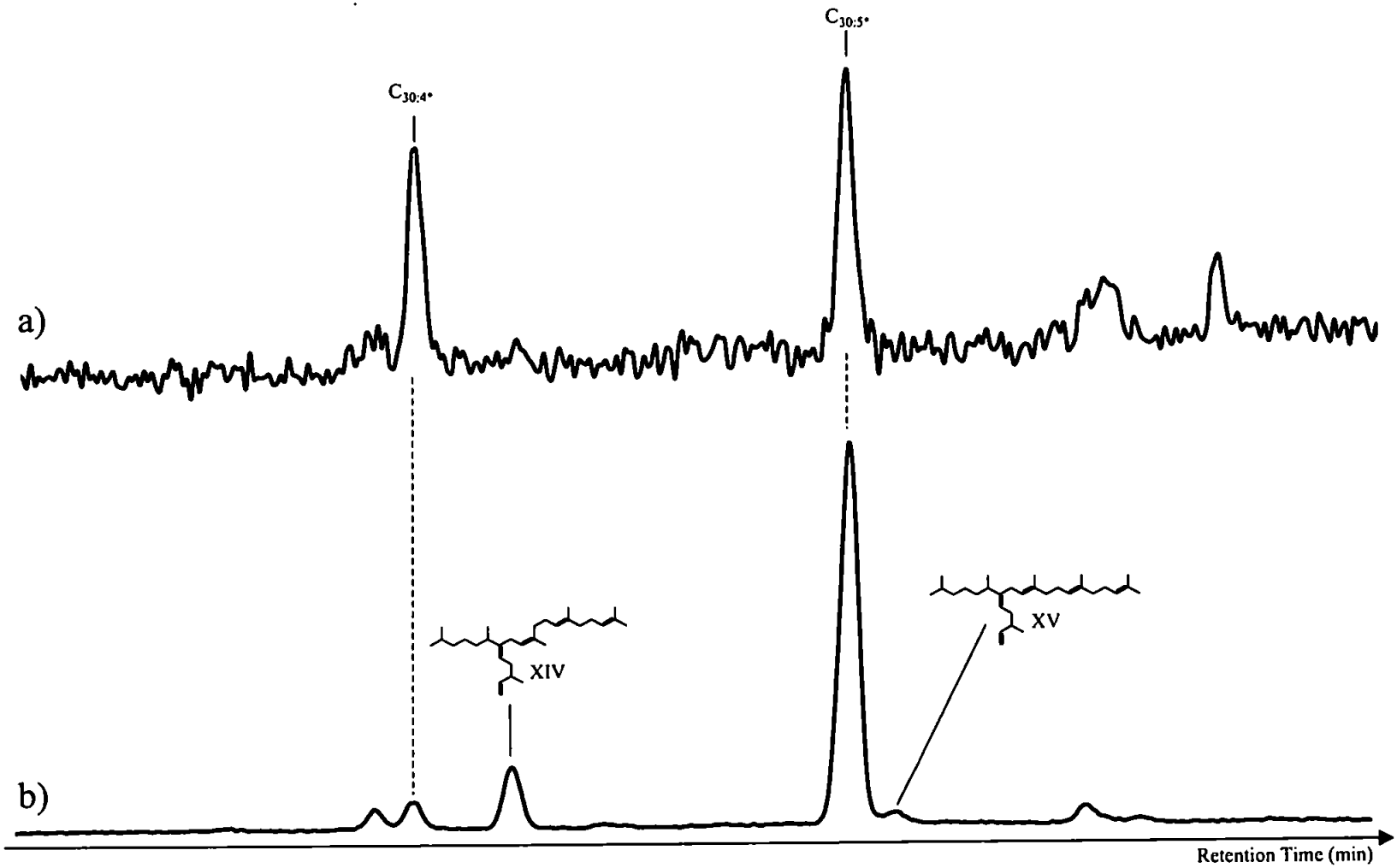


Figure 5.5 Partial TIC chromatograms showing the C_{30} HBI distributions in hydrocarbon extracts of (a) SPM and (b) sediment from the Arabian Sea.The structural identification of HBI isomers was achieved *via* co-chromatography with authenticated compounds.

The C_{25} HBI trienes (25:3' and 25:3), tetraenes (25:4 and 25:4') and pentaenes (25:5 and 25:5') reported in the hydrocarbon extracts of sediment and SPM from the Arabian Sea by Wakeham (2001) can now be identified as the trienes V and VI, tetraenes VII and X, and pentaenes XI and XII respectively.

5.3.3 HBI alkenes in hydrocarbon extracts from the Black Sea

In contrast to the complex HBI distributions observed in the samples from the Arabian Sea, the hydrocarbon extracts of both the sediment (Figure 5.6 a) and SPM (Figure 5.6 b) from the Black Sea contained very simple HBI distributions which consisted solely of two C_{25} HBI isomers. C_{20} or C_{30} HBIs were not detected in either the sediment or SPM extracts. The HBI diene (III) and triene (XIII), possessing Δ 5(6) unsaturation were the only confirmed HBI isomers observed in the both the sediment and SPM extracts from the Black Sea (Figure 5.7). HBIs possessing Δ 7(20) unsaturation, which were the most abundant isomers in the Arabian Sea extracts were not detected in either sample from the Black Sea.

In common with the Arabian Sea extracts, there appears to be an increase in the relative abundance of the more saturated HBI isomers in the sediment when compared with the SPM. Thus, the abundance of the more saturated HBI diene (III) in the sediment extract appeared to be increased in relation to that of the less saturated triene (XIII) when compared with the SPM.

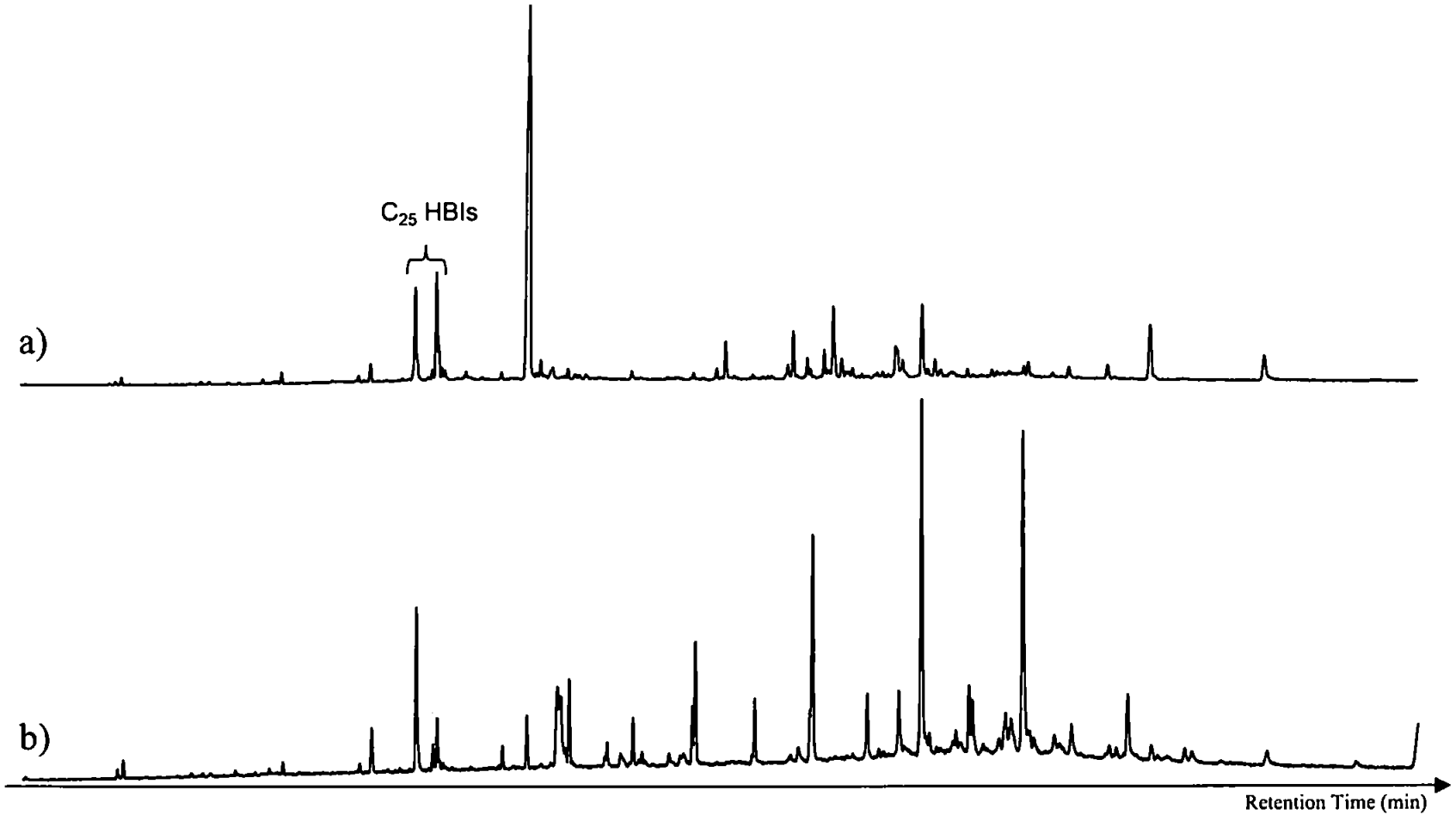


Figure 5.6 TIC chromatograms of hydrocarbon extracts of (a) SPM and (b) sediment from the Black Sea.

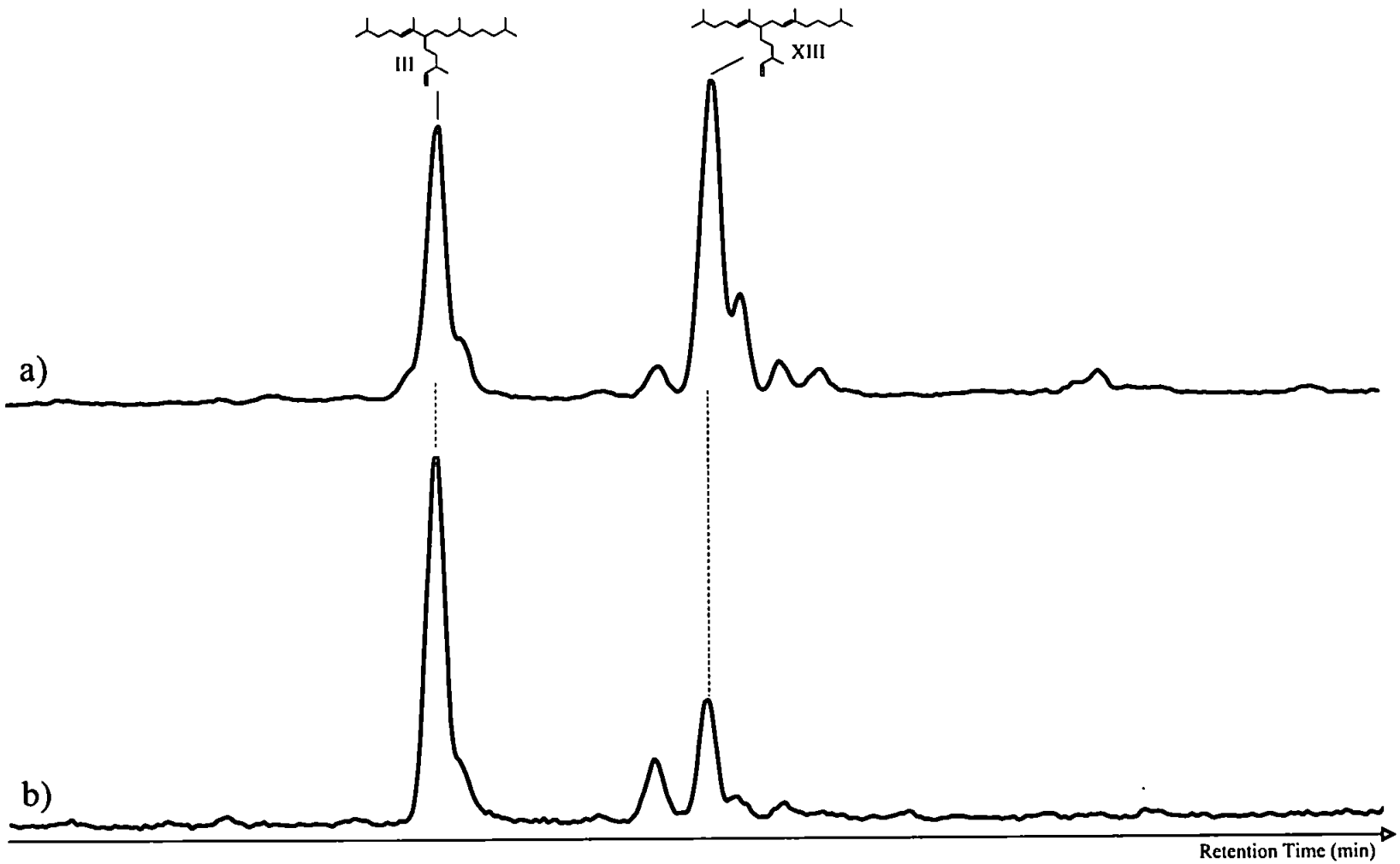


Figure 5.7 Partial TIC chromatograms showing the C_{25} HBI distributions in hydrocarbon extracts of (a) SPM and (b) sediment from the *via* co-chromatography with authenticated compounds.

5.3.4 HBI alkenes in hydrocarbon extracts from the Cariaco Trench

C_{25} HBIs were abundant hydrocarbons in both the sediment (Figure 5.8a) and SPM (Figure 5.8b) from the Cariaco Trench, whilst C_{20} and C_{30} HBIs were not observed in either sample. Like the HBIs identified in the Arabian Sea extracts, C_{25} HBIs possessing Δ 7(20) unsaturation were the most abundant HBI isomers in both the SPM and sediment extracts.

The HBIs that could be unambiguously identified in the SPM (Figure 5.9a) all contained Δ 7(20) unsaturation. The trienes V and VI were the most abundant isomers, with the tetraenes VII and IX present in much lower abundance. In contrast to the Arabian Sea SPM extract, the related tetraenes VIII and X and pentaenes XI and XII were not detected in the SPM extract from the Cariaco trench. The unidentified $C_{25:2}$• and $C_{25:4}$•, observed in the hydrocarbon fractions of the Arabian Sea SPM and sediment were also present.

The sediment extract (Figure 5.9b) also contained the trienes V and VI, but the tetraenes VII and IX identified in the SPM were not detected in the sediment extract. An additional triene (XIII), possessing Δ 5(6) unsaturation was present in low abundance. The unidentified $C_{25:2}$• and $C_{25:4}$• identified in the SPM and sediment extracts from the Arabian Sea were also present in the sediment extract from the Cariaco trench. Indeed, the uncharacterised $C_{25:4}$• isomer was the most abundant compound in the C_{25} HBI retention time region of the gas chromatogram (Figure 5.9b).

The C_{25} HBI trienes (b25:3 and b25:3'), tetraenes (25:4 and 25:4') reported in the hydrocarbon extracts of sediment and SPM from the Arabian Sea by Wakeham (2001) can now be identified as the trienes V and VI, and tetraenes VII and X respectively.

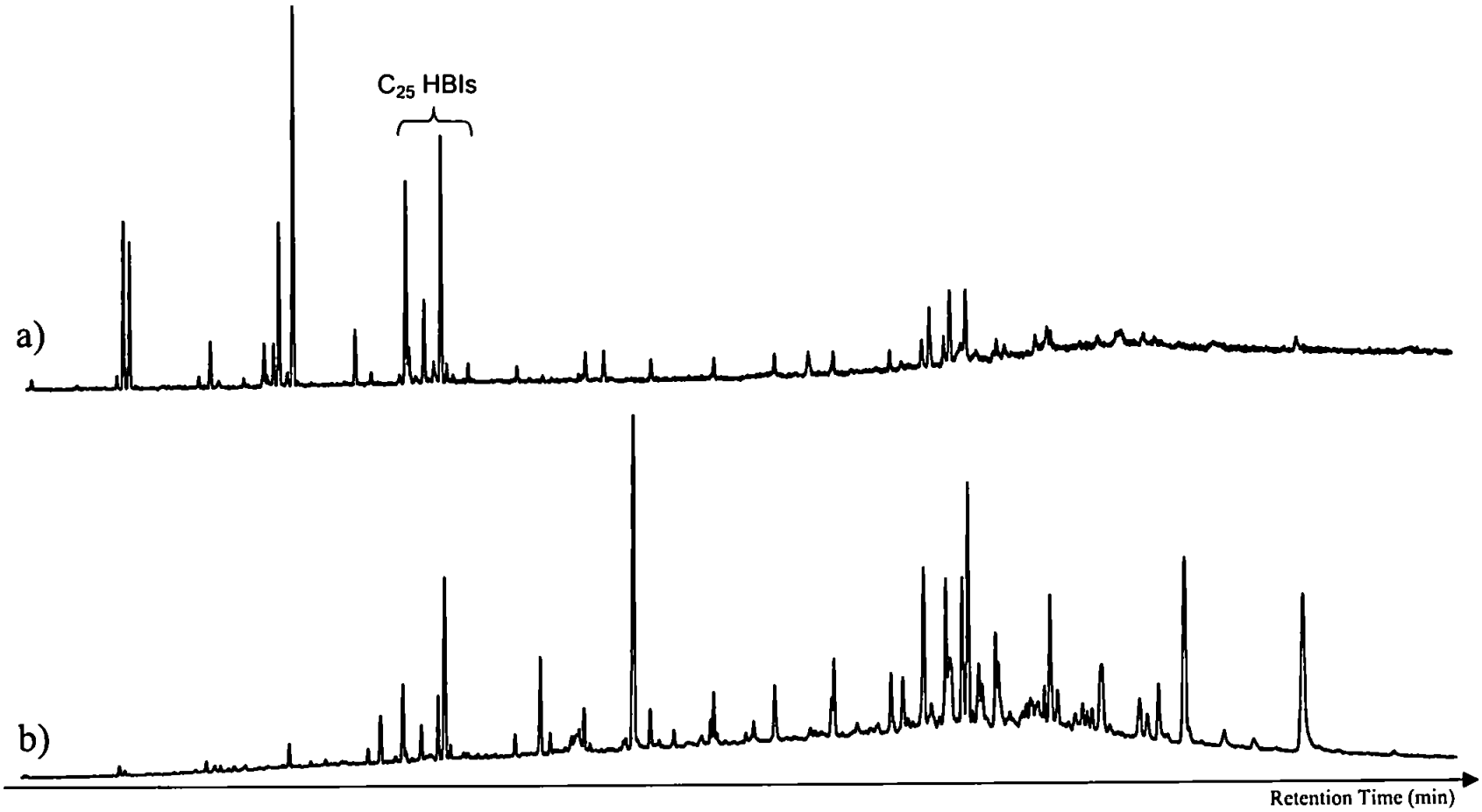


Figure 5.8 TIC chromatograms of hydrocarbon extracts of (a) SPM and (b) sediment from the Cariaco Trench.

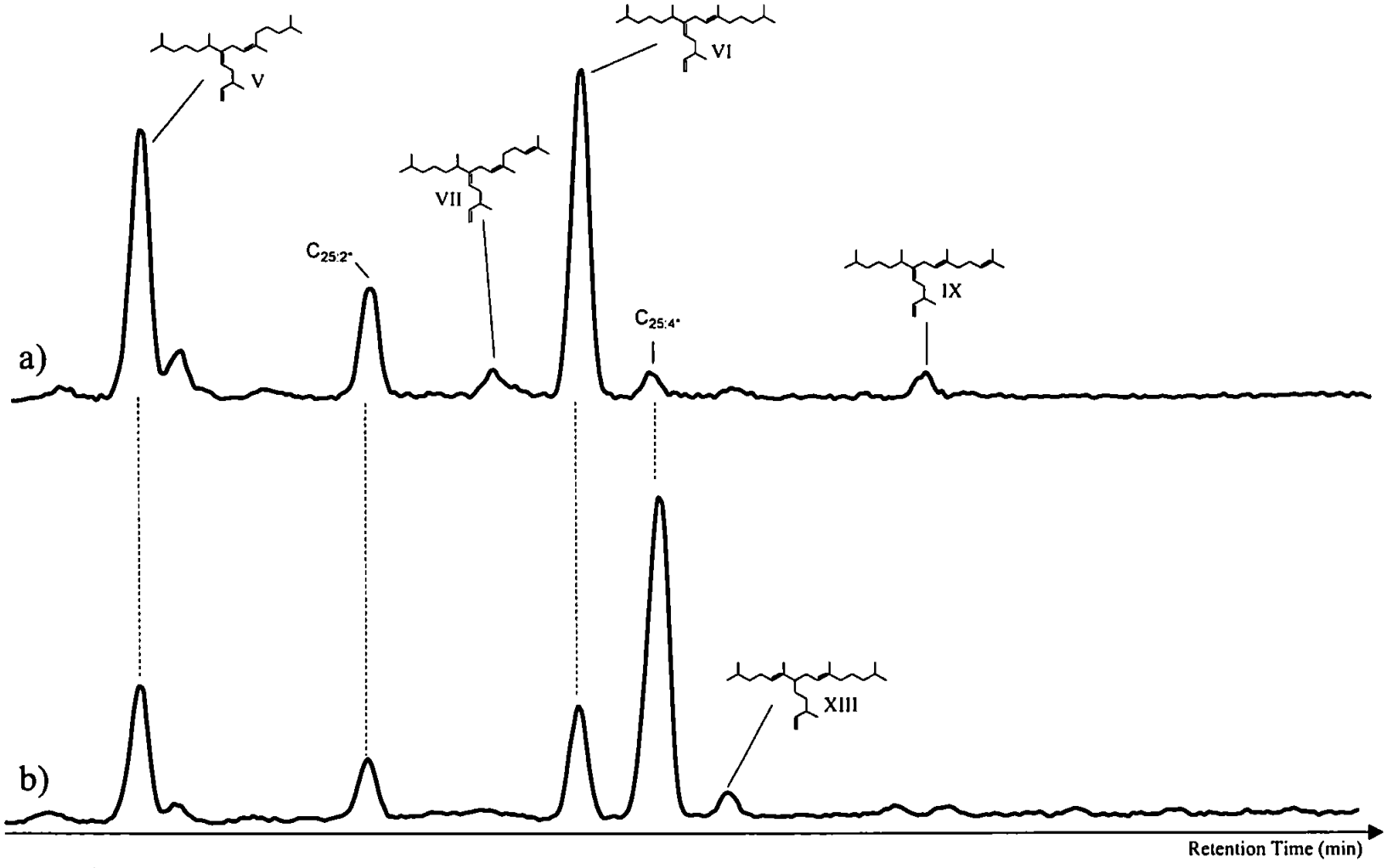


Figure 5.9 Partial TIC chromatograms showing the C_{25} HBI distributions in hydrocarbon extracts of (a) SPM and (b) sediment from the Cariaco Trench.The structural identification of HBI isomers was achieved *via* co-chromatography with authenticated compounds.

5.3.5 HBI alkenes in hydrocarbon extracts from the Peru Upwelling region

C_{25} HBI alkenes were observed in both the SPM (Figure 5.10a) and sediment (Figure 5.10b) from the Peru upwelling region. C_{30} HBIs were not observed in either sample from this location. A single C_{20} HBI monoene (XVIII) previously characterised by Dunlop and Jefferies (1985) *via* ozonolysis following isolation from sediments of Shark Bay, Western Australia, was present in the sediment, but was not detected in the SPM extract. As no authentic standard for XVIII was available at the time of this study, identification of this compound was made by comparison of its GC (RI) and MS characteristics with those reported for XVIII (Dunlop and Jefferies, 1985).

In contrast to the SPM and sediment extracts from the Arabian Sea and the Cariaco trench, the HBI distributions of the SPM (Figure 5.11a) and sediment (Figure 5.11b) extracts from the Peru upwelling region were very similar. The SPM contained two C_{25} HBI trienes with Δ 7(20) unsaturation (V, VI), but the related tetraenes (VII, VIII, IX and X) and pentaenes (XI and XII) were not identified in this sample. The unidentified $C_{25:2}$• and $C_{25:4}$• which were also observed in the samples from the Arabian Sea and Cariaco trench were also present in the SPM extract from the Peru upwelling region.

The sediment also contained the C_{25} trienes V and VI and the two unidentified compounds ($C_{25:2}$• and $C_{25:4}$•) observed in the SPM. A pair of C_{25} tetraenes (VII, IX) possessing Δ 7(20) unsaturation, which were detected in the Arabian Sea SPM and sediment, and also in the SPM extract from the Cariaco trench, were also present in the sediment extract from the Peru upwelling region, although they were present in relatively low abundance. The related tetraenes (VIII and X) and pentaenes (XI and XII) identified in the SPM and sediment samples from the Arabian Sea were not detected in the samples from the Peru upwelling

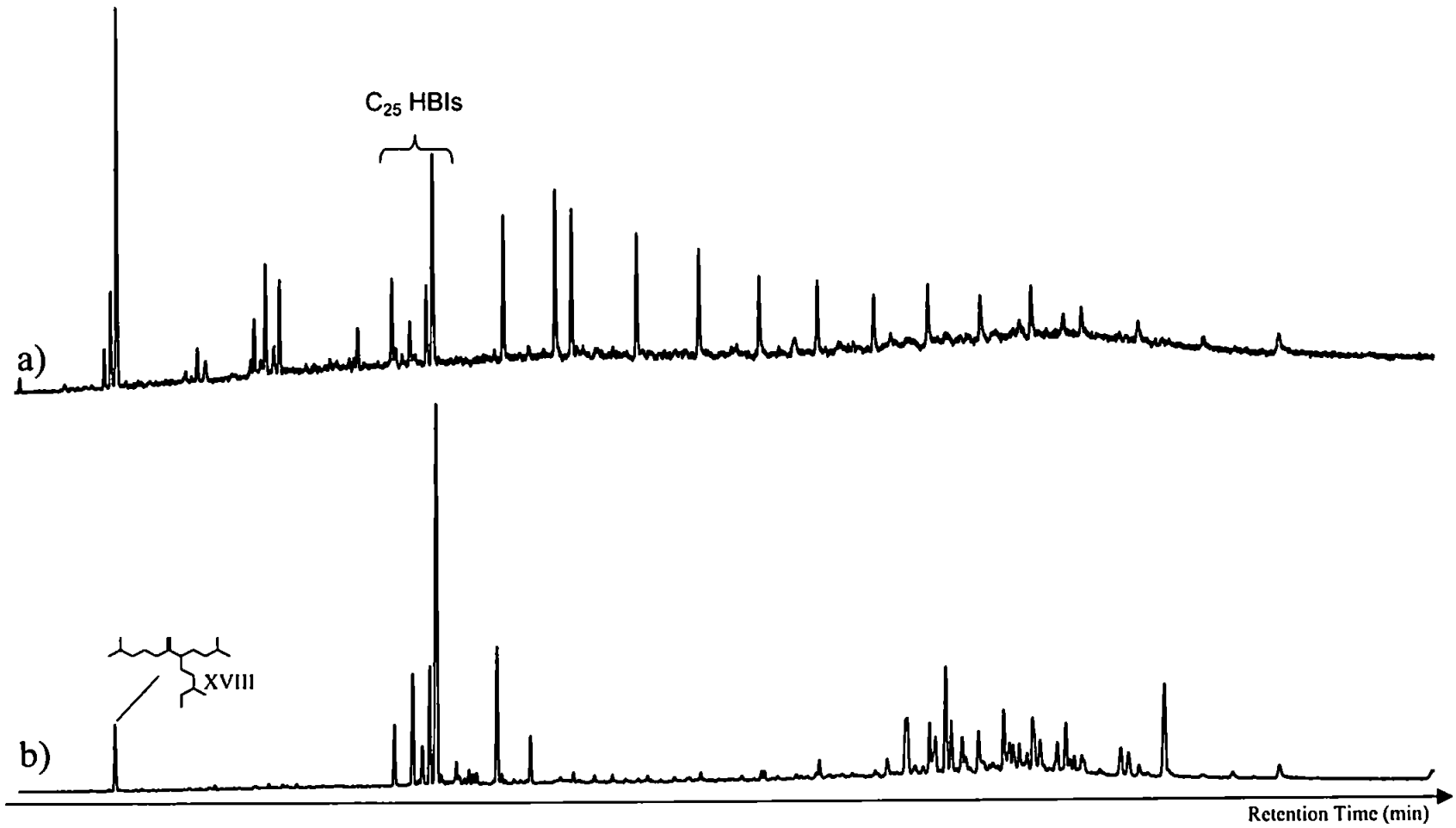


Figure 5.10 TIC chromatograms of hydrocarbon extracts of (a) SPM and (b) sediment from the Peru Upwelling region.

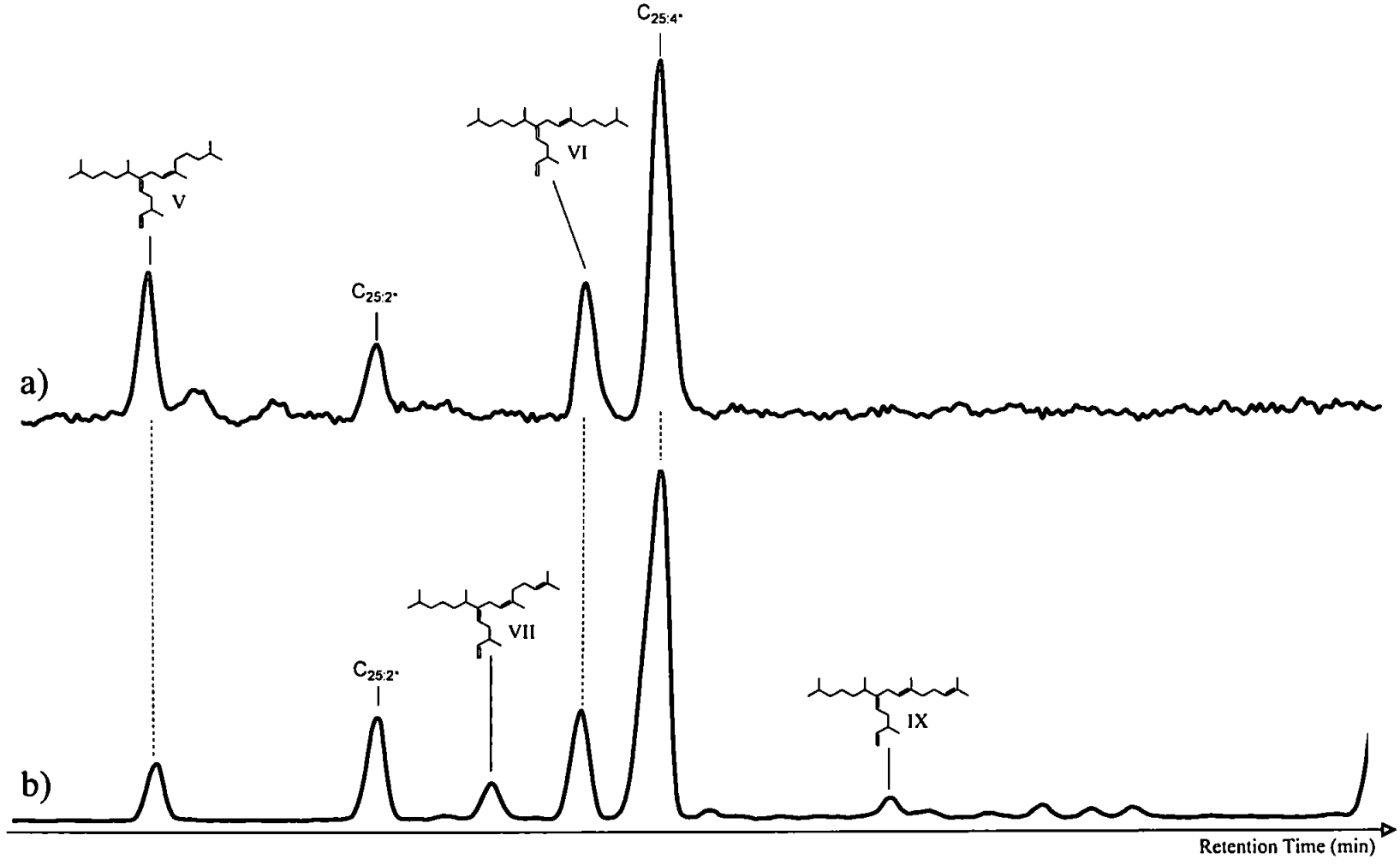


Figure 5.11 Partial TIC chromatograms showing the C_{25} HBI distributions in hydrocarbon extracts of (a) SPM and (b) sediment from the Peru Upwelling region.The structural identification of HBI isomers was achieved *via* co-chromatography with authenticated compounds.

region. Additionally, C_{25} HBIs possessing a saturated branch point (Δ 5(6) unsaturation) were not detected in either the SPM or sediment from this location.

5.4 Discussion

5.4.1 HBI isomers in sediments and SPM

It is clear that the isolation and complete structural characterisation of HBIs from diatoms (e.g. Wraige *et al.*, 1999, and Chapters 2, 3 and 4) has allowed for the identification of the majority of HBI alkenes in the sediments and SPM fractions analysed herein. Of the fifteen isomers detected in the SPM and sediment samples (Table 5.1), twelve could be unambiguously identified by comparison of GC-MS and RI characteristics with those of authenticated compounds (Figure 5.2). Two of these compounds (III and XIII) were those characterised previously (Belt *et al.*, 1994; Wraige *et al.*, 1999) whilst the remaining ten HBIs identified (V – XII, XIV, XV) were the novel C_{25} and C_{30} HBIs with Δ 7(20) unsaturation, characterised herein following isolation from cultures of *P. intermedium* (Chapter 2) and *R. setigera* (Chapter 3).

The HBI distributions in the sediment and SPM samples from the Arabian sea, Cariaco Trench and the Peru upwelling region were all dominated by HBI isomers with Δ 7(20) unsaturation (Table 5.1), and it is now known that the planktonic diatoms *R. setigera* and *Pleurosigma* sp., and the benthic *P. intermedium* are able to biosynthesise these abundant compounds (Chapters 2 and 3). In the study of the hydrocarbons of the Arabian Sea from which the fractions analysed herein were obtained, Wakeham *et al.* (2001) noted that a C_{25} HBI triene accounted for the majority of the HBI flux during the southwest monsoon (SWM) season, whilst a C_{25} HBI tetraene was the major HBI alkene during the rest of the year.

Wakeham *et al.* (2001) also observed an abundance of *Rhizosolenia* spp. in the diatom blooms associated with the SWM. Since the triene and tetraene have now been identified as VI and X respectively, it suggests that the C_{25} triene (VI) has the potential for use as a proxy of the SWM in the Arabian Sea, and also as an indicator for specific diatom species. The absence of HBIs with Δ7(20) unsaturation in the sediment and SPM from the Black Sea (Table 5.1) suggests a very different diatom community structure in this environment, compared to the other locations.

5.4.2 Unidentified C_{25} and C_{30} alkenes

Although the majority of the HBI alkenes present in the SPM and sediment extracts analysed herein could be unambiguously identified by comparison of their GC-MS characteristics with those of authenticated HBIs, several compounds that eluted (GC) with these HBIs in many of the samples did not correspond to any of the HBIs for which structures are currently known.

The SPM and sediment samples from the Arabian Sea contained varying distributions of C_{30} compounds, of which the most abundant isomer, $C_{30:5•}$ (RI $2548_{HP\text{-}1}$) had an identical mass spectrum (Figure 5.12 a) and RI to an unknown $C_{30:5•}$ observed in the non saponifiable lipids of the diatom *R. setigera* strain CCMP 1694 (Chapter 3). Indeed, the correlation between the C_{30} branched alkenes present in *R. setigera* strain CCMP 1694, which was originally isolated from the Arabian Sea, bear a striking similarity to the C_{30} alkene distribution observed in the Arabian Sea sediment (Figure 5.13). This unidentified $C_{30:5•}$ compound has also been reported previously in sediments from Dabob Bay (Prahl *et al.*, 1980), Puget Sound (Barrick and Hedges, 1981) and laboratory cultures of *R. setigera* (Volkman *et al.*, 1994). Although

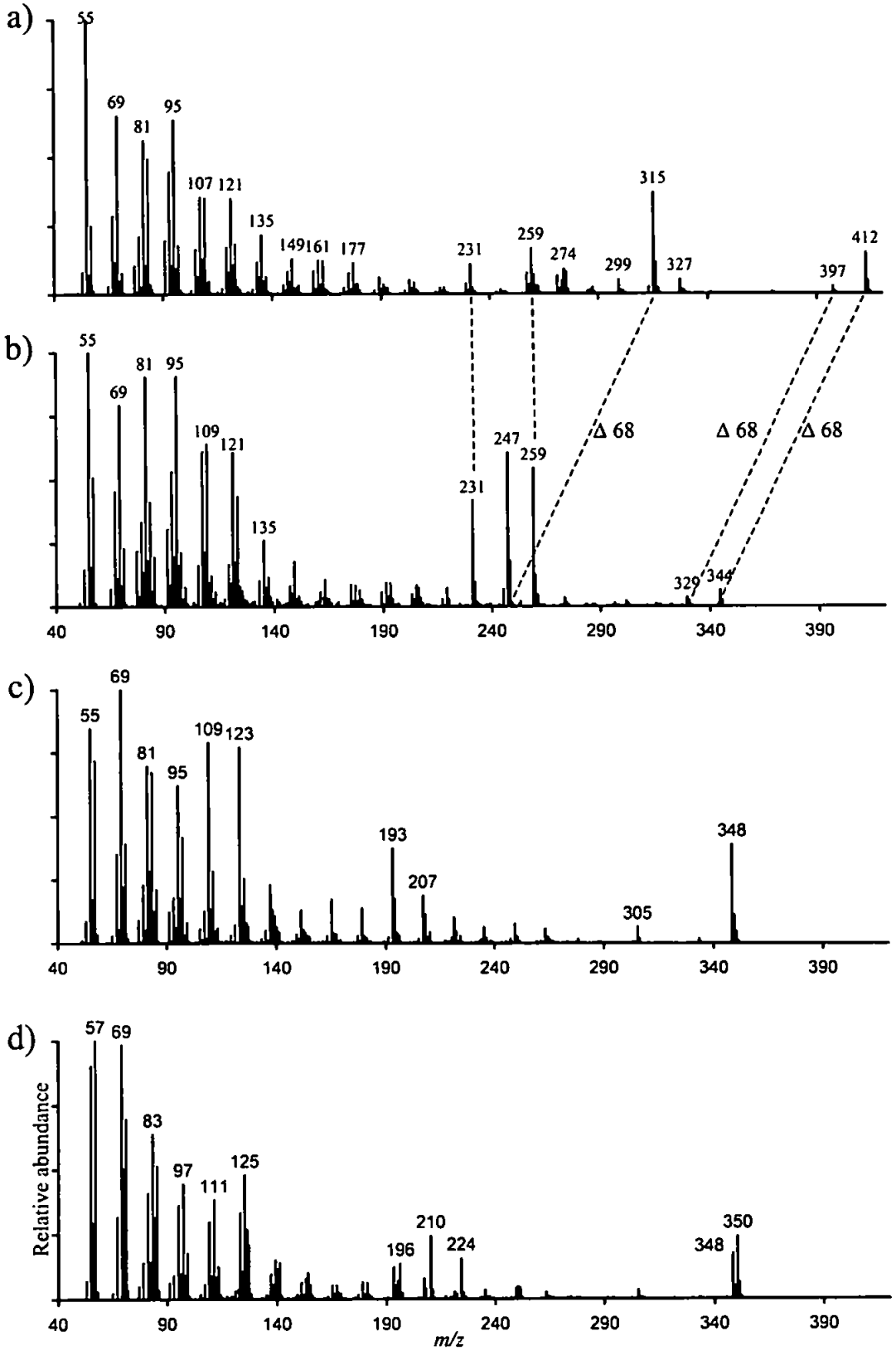


Figure 5.12 Mass spectra of unidentified $C_{30:5}$• (a) and $C_{25:4}$• (b) with compounds produced after 2 h (c) and 8 h (d) hydrogenation of $C_{25:4}$• (b).

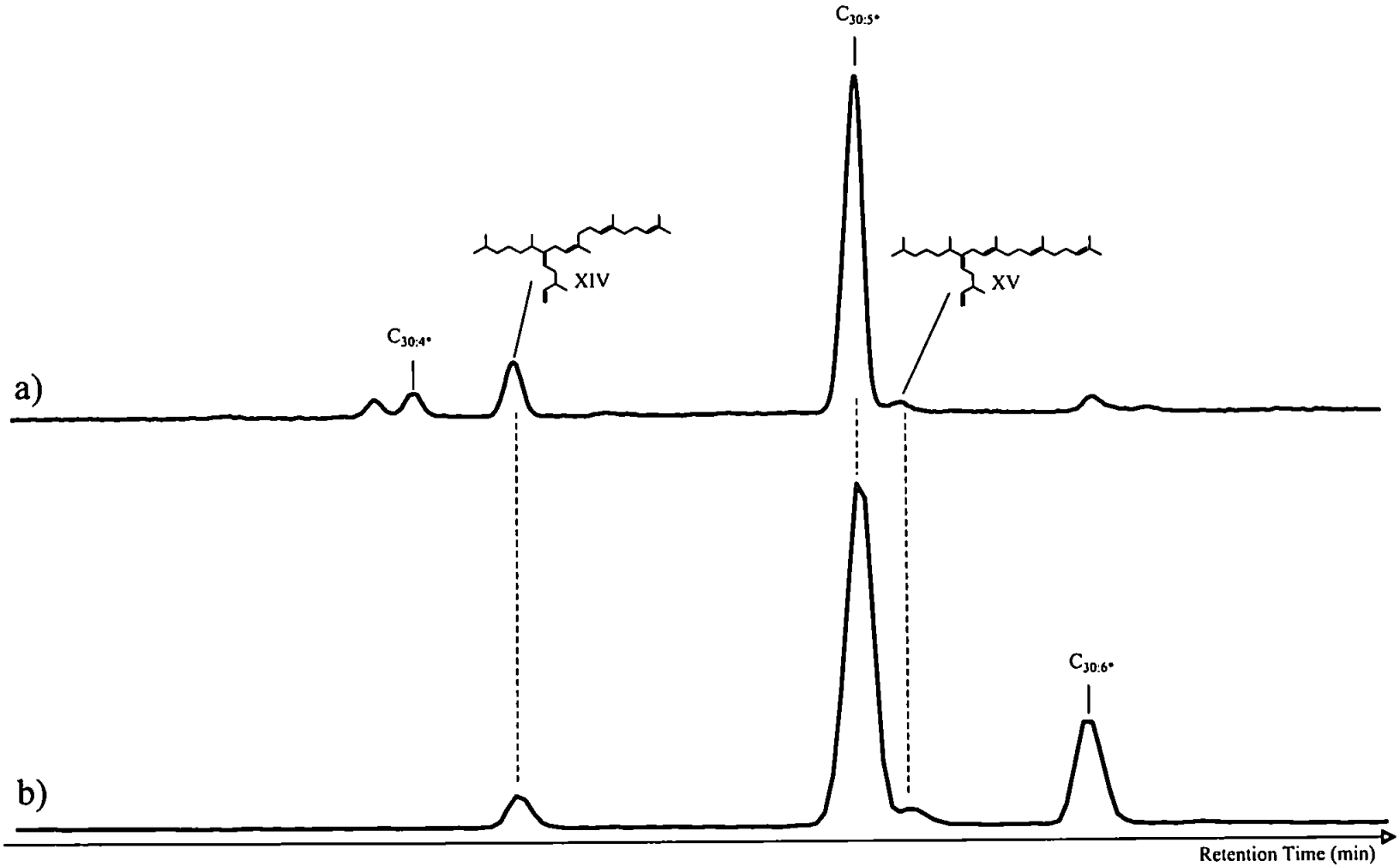


Figure 5.13 Partial TIC chromatograms showing the C_{30} branched alkene distributions of (a) Arabian Sea sediment and (b) the diatom *R. setigera* (strain CCMP 1694).

this alkene appears not to possess the C_{30} HBI skeleton (see Section 3.4.3), further work is required before a definitive structure can be assigned. To this end, culturing of *R. setigera* strain CCMP 1694 is currently in progress which should allow for the isolation of sufficient quantities of pure $C_{30:5\bullet}$ for a rigorous structural analysis.

Another unidentified compound, designated $C_{25:4\bullet}$ (GC RI $2102_{HP\text{-}5}$, $2089_{HP\text{-}1}$) eluted in the same region of the chromatogram as the C_{25} HBIs in many of the samples (Table 5.1). The mass spectrum of this unknown compound (Figure 5.12 b) had a molecular ion at *m/z* 344, suggesting a C_{25} hydrocarbon with four degrees of unsaturation. The enhanced abundances of a cluster of high molecular mass ions (*m/z* 231, 247 and 259) suggested that this compound was not acyclic. Hydrogenation (H_2 / $PtO_2.H_2O$, hexane, 2h) gave a compound with $M^{+\cdot}$ at *m/z* 348 (Figure 5.12 c) indicating that two degrees of unsaturation were still present. After further hydrogenation (H_2 / PtO2.H_2O, hexane, 6h), a compound giving a molecular ion with *m/z* 350 (Figure 5.12 d) could be detected which co-eluted with the partial hydrogenation product. This suggested the formation of a species with one degree of unsaturation following hydrogenation of a double bond, resistant to initial hydrogenation. This is similar to the hydrogenation behaviour of $C_{30:5\bullet}$, which initially hydrogenated to give a compound with two degrees of unsaturation, with subsequent formation of a pair of isomers with one degree of unsaturation under more forcing conditions (see Section 3.4.3). The mass spectrum and hydrogenation behaviour of $C_{25:4\bullet}$ showed strong similarities to those of an unidentified olefin observed in sediments from the Northwest Atlantic (Farrington *et al.*, 1977), Rhode Island Sound (Boehm and Quinn, 1978) and Puget Sound (Barrick and Hedges, 1981). A structure possessing 2 double bonds, one tri-substituted and the other vinyl, was proposed by Boehm and Quinn (1978) on the basis of IR and limited ^{1}H NMR spectroscopy. A bi-cyclic core was suggested to account for the two degrees of unsaturation observed for the initial hydrogenation product. However, Farrington *et al.* (1977) noted that

the compound could also be mono-cyclic with a double bond in a position that was resistant to hydrogenation, which is consistent with the hydrogenation behaviour reported herein. Both Boehm and Quinn (1978) and Farrington *et al.* (1977) suggest the presence of one or more branched alkyl side chains.

The mass spectra of the $C_{30:5\bullet}$ and the $C_{25:4\bullet}$ (Figure 5.12 a,b) share common features. The low mass regions of both mass spectra (Figure 5.12 a,b) have similar distributions of ions with *m/z* 69, 81, 95, 107/109, 121 and 135. Both compounds show an enhanced abundance of the ions with *m/z* 231 and 259. Additionally, the $C_{30:5\bullet}$ exhibits ions at *m/z* 315, 397 and 412, that are 68 mass units higher than the ions *m/z* 247, 329 and 344 of the $C_{25:4\bullet}$ (Figure 5.12 a,b), which corresponds to a C_5 unit containing a single double bond (C_5H_8). This was also noted by Barrick and Hedges (1981) for the $C_{30:3:2}$ and $C_{25:2:2}$ observed in the sediments of Puget Sound, and suggests that the $C_{30:5\bullet}$ is a structural homologue of the $C_{25:4\bullet}$, and that both compounds have a mono-cyclic core.

The sediment and SPM extracts from the Arabian sea also contained low concentrations of an unidentified C_{30} compound possessing four degrees of unsaturation ($C_{30:4\bullet}$; RI $2494_{HP\text{-}1}$). The mass spectrum of this compound (Figure 5.14) showed enhanced abundances of ions with *m/z* 207, 275, 301, 331, 345 and 414. Requejo and Quinn (1983) observed a C_{30} compound with four degrees of unsaturation that had a similar RI (c30:2:2; RI $2499_{SE\text{-}30}$) in sediment samples from Narragansett Bay estuary, Rhode Island. However, the mass spectrum of c30:2:2 reported by Requejo and Quinn (1983) exhibited abundant ions with *m/z* 205, 231 and 259, which suggests that it was not the same compound as the $C_{30:4\bullet}$ identified herein. Porte *et al.* (1990) also identified two tetra-unsaturated C_{30} isoprenoid alkenes, in bivalves from Todos os Santos Bay, Brazil, which were designated i-30:2:2 (RI $2444_{DB\text{-}5}$) and i-30:4 (RI $2530_{DB\text{-}5}$). Although the mass spectra of the i-30:2:2 and i-30:4 identified by

Porte *et al.* (1990) were not given, the RIs of these two compounds (RI 2444 and $2530_{DB\text{-}5}$) were different to the RI of the $C_{30:4}$• identified herein (RI $2494_{HP\text{-}1}$). Due to the low abundance of $C_{30:4}$• in the SPM and sediment extracts from the Arabian Sea, the analysis of the hydrogenation products obtained from these hydrocarbon extracts did not reveal a compound that could be determined as arising from hydrogenation of $C_{30:4}$•. Therefore, the parent skeleton of $C_{30:4}$• cannot be assigned, and it is unclear if this compound is cyclic or acylic.

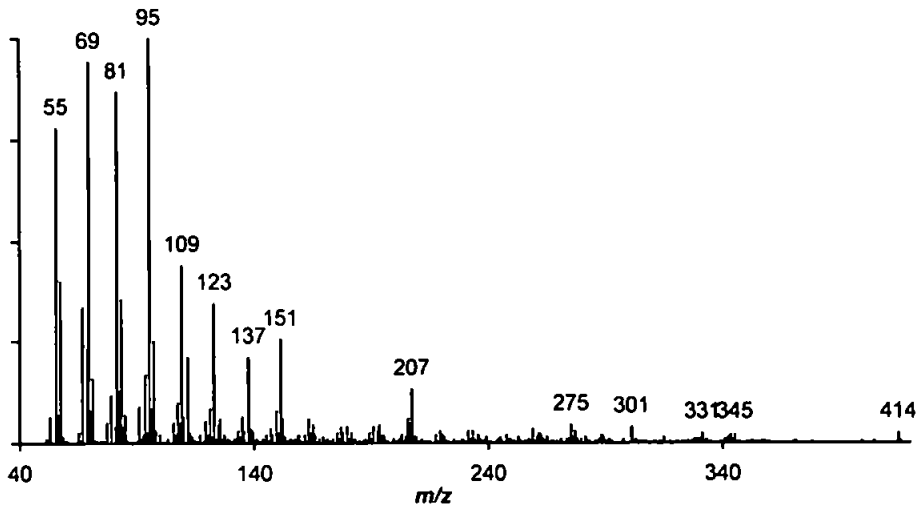


Figure 5.14 Mass spectrum of $C_{30:4}$•, RI $2494_{HP\text{-}1}$, identified in the sediment and SPM extracts from the Arabian sea.

In many samples (Table 5.1), a C_{25} HBI diene was also present ($C_{25:2}$•) which exhibited different GC (RI $2071_{HP\text{-}5}$, $2068_{HP\text{-}1}$) and MS behaviour to previously characterised C_{25} HBI dienes (eg. Figure 5.1). The mass spectrum (Figure 5.15) and retention index of this compound are consistent with an unidentified HBI diene RI 2070 observed by Hird (1992) to

be associated with species of the marine macroalga *Enteromorpha*. Albaiges *et al.* (1984) also observed a HBI diene with similar GC (RI 2068_{SE-52}) and MS characteristics in suspended particulates from Alfacs Bay (Spain). It is significant that in both of the reports by Hird (1992) and Albaiges *et al.* (1984), the HBI triene VI (RI 2091_{HP-5}), now known to possess Δ 7(20) unsaturation, was also present in association with the unidentified diene. The samples studied herein also show this co-occurrence. Indeed, in the present study, every sediment and/or SPM fraction that contained this diene also contained C_{25} HBIs with Δ 7(20) unsaturation (Table 5.1). Conversely, this compound was not detected in either of the SPM or sediment fractions isolated from the Black Sea, which contained solely HBI isomers with Δ 5(6) unsaturation, and was the only location from which haslenes with Δ 7(20) unsaturation were not observed. It is therefore possible that this unidentified diene ($C_{25:2\bullet}$) is a more saturated homologue of the Δ 7(20) HBI series, and may also share a common biological source(s). Figure 5.15 shows a postulated structure for $C_{25:2\bullet}$ and a MS fragmentation pattern consistent with the mass spectrum of the unidentified diene.

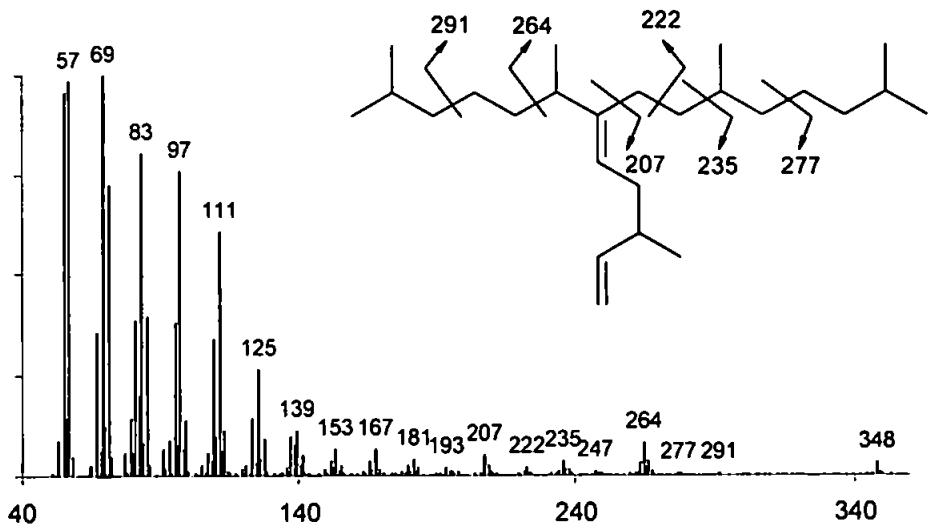


Figure 5.15 Mass spectrum of the unidentified C_{25} HBI diene, $C_{25:2\bullet}$ RI 2071_{HP-5} and a hypothetical structure for a C_{25} HBI diene with Δ 7(20) unsaturation.

5.4.3 Variations in HBI distributions

It was noted in Sections 5.3.2 and 5.3.3 that the hydrocarbon extracts isolated from the Arabian sea and Black sea sediments appeared to be enriched in the more saturated HBI isomers (e.g. dienes and trienes) when compared with the SPM fractions from the same location. It must be noted that as many of the samples were composites of material obtained from different sampling sites within the same area, it is possible that some of the observed differences are artificial. However, there are several other factors that could account for this apparent enrichment. Wakeham *et al.* (2001) observed that HBI concentrations in water column particulates of the Arabian sea decreased with depth, and that more than 99% of the total HBIs were removed from the particulate material before incorporation into the sediment. It is therefore clear that degradative processes (both biological and chemical) can affect the composition of the organic matter reaching the sediment. The more unsaturated HBI isomers may be more susceptible to such degradation than the more saturated isomers, and therefore could be preferentially removed from sedimenting particles. This would result in an increased accumulation of the more saturated HBIs than the more unsaturated HBIs in the sediment. It is also possible that degradation of the HBIs within the sediment itself results in the preferential removal of the more unsaturated isomers.

It is also possible that HBI input from benthic diatom species could alter the HBI distributions of the sediment when compared with the HBI distributions of the particulate material, although this is unlikely to be significant for the samples analysed herein, which were obtained from deep-sea environments. As the organisms currently known to produce HBI alkenes are all photosynthetic diatoms, it is therefore likely that HBI production in deep-sea environments is confined to the plankton of the surface waters where adequate light is available for photosynthesis.

CHAPTER SIX

Experimental Details

6.1 General Procedures

Glassware was cleaned in Decon-90, rinsed in distilled/Millipore-grade water, oven dried (150^0C; overnight) and finally rinsed with an appropriate solvent (e.g. hexane) immediately before use. All solvents were HPLC-grade (Rathburn) and found to be of adequate purity. The purity was checked by GC analysis of solvent concentrates (100 ml to 10 μL under vacuum).

Silica gel (BDH; 60-120 mesh) was used as the stationary phase for open column chromatography. The silica was solvent extracted (soxhlet; DCM; 24 hr) and was activated by heating (180^0C; 24 hr). When required, silica gel was deactivated immediately before use by shaking (1 h) with the appropriate quantity of Millipore grade water.

Anhydrous sodium sulphate, silver sand and cotton wool were all solvent extracted with DCM before use to remove trace organic impurities.

6.2 Hydrocarbon extraction from large scale algal cultures

Large scale diatom cultures were grown and harvested by Mr. G. Massé at the University of Nantes (France). Cultures were centrifuged to give a concentrated algal biomass (typically 20 – 150g), which was then freeze dried (~75% reduction in weight). Following extraction into hexane (Soxhlet; 24 hrs) and removal of solvent under reduced pressure, the total hexane extract (THE; <20% w/w cf. dried algal paste) was saponified (5% KOH/MeOH/H_2O; 50 mL; 70^0C; 30 min) to remove triglyceride esters of fatty acids and the

non saponifiable lipids (NSLs; ~10% of THE w/w) were re-extracted into hexane (3 x 20 mL). The hexane extract was washed with water (Milli-Q; 3 x 20 mL), dried (Na_2SO_4) and the solvent was removed.

6.3 Hydrocarbon extraction from small scale algal cultures

Small scale algal cultures were grown on F/2 media (150 mL) at a constant temperature (14°C) and light intensity (100 μmol Photons m^{-2} s^{-1}) with a 14/10 light/ dark cycle by Mr. G. Massé at the University of Nantes (France). Algal cells were isolated using filtered aliquots (25 to 50 mL) of the algal culture. The resultant algal concentrates were extracted into hexane (3 x 2 mL) aided by ultrasonication (5 min) and the total hexane extract (THE) was saponified (5% KOH/MeOH/H_2O; 2 mL; 70^{0}C; 30 min) to remove triglyceride esters of fatty acids. The non saponifiable lipids (NSLs) were extracted into hexane (3 x 2 mL), dried (Na_2SO_4) and the solvent was removed (N_2). NSLs were silylated (BSTFA:TMCS 99:1; 50 μL; 70^{0}C; 30 min) prior to analysis (GC-MS).

6.4 Isolation and purification of HBIs

Isolation and purification of HBIs from the NSLs was achieved using column chromatography (5% deactivated SiO_2/ hexane). Column size varied depending on the amount of material to be separated, with a typical ratio of 50:1 SiO_2:NSL (w/w). The volume of the fractions collected typically corresponded to approximately 0.2 column volumes (CVs). Fractions thus obtained were analysed by GC-MS and combined where appropriate (i.e. those with the same degree of unsaturation). Fractionation was repeated when necessary to improve the separation of the individual isomers. Table 6.1 summarises the amount of eluent (CV equivalents) necessary for the isolation of the C_{25} and C_{30} HBIs described in this study.

Table 6.1 Elution volumes (in CV equivalents) required for the isolation of HBIs by open column chromatography (5% deactivated SiO_2/hexane).

HBI	Eluent volume (CV equivalents)
$C_{25:3}$	1 – 2
$C_{25:4}$	1.5 – 2.5
$C_{25:5}$	2 – 3
$C_{30:5}$	2 – 3
$C_{30:6}$	2.5 – 3.5

6.5 Microscale hydrogenation

Hydrogen gas (20 cm^3 min^{-1}) was bubbled through a solution of the extract (typically < 1.0 mg) dissolved in hexane (2 – 5 ml; 20^{0}C) containing the hydrogenation catalyst ($PtO_2.2H_2O$; 0.1 g). Where necessary, aliquots were removed at regular intervals to monitor the hydrogenation progress. Following hydrogenation, samples were filtered through a short column containing anhydrous sodium sulphate, and the solvent was removed (N_2) prior to storage.

6.6 Oxidation of HBI alkanes – stereochemical studies

Following hydrogenation of HBI alkenes, the resultant alkanes were oxidised using chromium trioxide as described previously (Johns *et al.*, 2000). CrO_3 (10:1 ratio CrO_3:alkane) was added to glacial acetic acid (10 mL) and the solution was heated (70^{0}C; waterbath) with stirring (10 min). The alkane (*ca* 2 mg) was dissolved in glacial acetic acid (1 mL) and was added to the reaction mixture. The solution was maintained at 70^{0}C with stirring for 2 hours. After cooling, water was added (10 mL) and the oxidation products were extracted with DCM (2 x 10 mL), and the solvent was removed under reduced pressure. The

DCM extract thus obtained was hydrolysed with methanolic KOH (10% KOH:Methanol w/v) under reflux (30 min). Following cooling, the hydrolysate was acidified to pH 1 (conc. HCL), extracted into DCM (2 x 10 mL), dried (Na_2SO_4), and the solvent was removed under reduced pressure. Prior to analysis (GC-MS), the oxidation products were methylated (BF_3/methanol complex; reflux; 15 min) and the methyl esters were extracted into DCM (3 x 5 mL), washed with water (MilliQ; 3 x 5 mL), dried (Na_2SO_4) and the solvent was gently removed (N_2). The oxidation products were analysed by GC and GC-MS to identify the methyl esters. The stereoisomeric configurations of the methyl esters were determined by Prof. W. König (University of Hamburg, Germany) *via* enantioselective GC (König *et al.*, 1988).

6.7 Gas chromatography-mass spectrometry

GC-MS analysis was performed with a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard Mass Selective Detector (5970 series) fitted with a 12 m (0.2 mm i.d.) fused silica column (HP-1/ HP-5/ DB-Wax stationary phases). Auto splitless injection and He carrier gas were used. The gas chromatograph oven temperature was programmed from 40-300 °C at 5 °C min^{-1} and held at the final temperature for 10 min. MS operating conditions were: ion source temperature 250 °C and 70 eV ionisation energy. Spectra (50-550 Da) were collected using Chemstation software. GC-MS analysis was also performed using a Finnigan MAT GCQ™ spectrometer incorporating a quadrupole ion trap fitted with a 30 m fused silica (0.25 mm internal diameter) HP-5 column. Auto-splitless injection and He carrier gas was used. The GC temperature programme was as before. The mass spectrometer conditions were: Ion source temperature 200 °C and 70 eV ionisation energy. Spectra (50-450 Da) were collected using Finnigan MAT GCQ™ software. Methyl esters of chiral acids from stereochemical studies were examined by GC (Prof. W. König,

University of Hamburg, Germany) using a chiral octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin stationary phase (50 °C; König *et al.*, 1988).

6.8 Nuclear magnetic resonance spectroscopy

NMR analysis of purified HBIs (typically 0.5 – 20 mg) was performed using a JEOL EX-270 FT-NMR spectrometer. 1-D (^{1}H, ^{13}C and DEPT), 2-D (COSY, HMQC, HMBC) and difference nOe measurements were all recorded in $CDCl_3$ using residual $CHCl_3$ (7.24 ppm) and $^{13}CDCl_3$ (77.0 ppm) as references.

CHAPTER SEVEN

Conclusions and Future Work

The overall objective of this work was to provide new insights into the production of HBIs by diatoms and the relationships between HBI sources and occurrence in geochemical settings. At the outset of this investigation, the number of organisms known to produce HBIs was limited to only two species of diatoms (*Haslea ostrearia* and *Rhizosolenia setigera*), and although the structures of several HBIs isolated from these organisms had been determined (Figure 7.1), the structures and sources of the HBIs which are most common and abundant in geochemical samples were unknown. Therefore, it was proposed that a study of the lipid compositions of diatom species and strains that had not been investigated previously could reveal new biological sources of HBIs that would better account for the widespread occurrence of HBIs in environmental samples. The detailed structural characterisation of new HBI isomers identified by such a study, and also of HBIs that had been identified previously in diatoms but remained uncharacterised (e.g. C_{30} HBIs from *R. setigera*), would allow for comparisons to be made between the HBI distributions in diatoms and those observed in the environment. To this end, the specific aims of this study were to:

(i) Identify new diatom species capable of HBI biosynthesis.

(ii) Identify the HBI isomers that are common in the geosphere.

(iii) Determine the structures of C_{30} HBIs.

The results of this study are presented and discussed in Chapters 2-5. This final chapter provides a summary of the results obtained and a brief discussion of their significance.

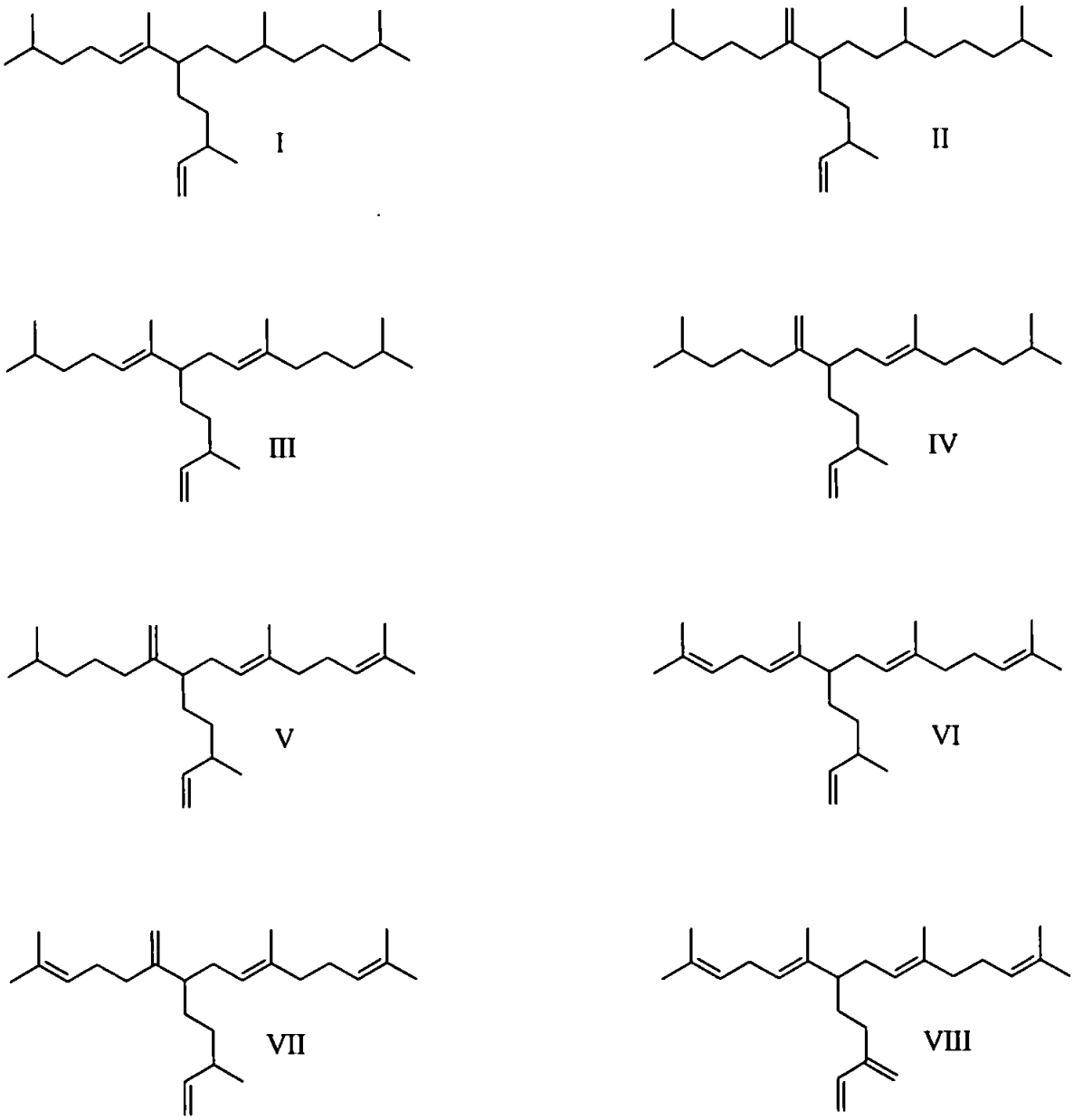


Figure 7.1 Structures of C_{25} HBI alkenes identified previously in cultured diatoms.

The first aim of this work was to identify new diatom species that are capable of HBI biosynthesis, as prior to this study, only two diatom species, *H. ostrearia* and *R. setigera*, were known to be biological producers of HBI alkenes. The present study has revealed four further diatom species belonging to the *Haslea* genus (Chapter 4) that are able to produce C_{25} HBIs (*H. salstonica, H. crucigera, H. pseudostrearia and Haslea sp.*; Table 7.1), and HBI production appears to be widespread within the *Haslea* genus (with *H. wawrikae* as a notable exception). Although the specific isomers produced by the different species varied, *Haslea* spp. appeared to produce only C_{25} HBIs (and not C_{20} or C_{30} HBIs), and the HBIs produced by *Haslea* spp. shared common structural features, with a saturated branch point (C7) and either a methylenic (C6-C17) or tri-substituted (C5-C6) double bond (e.g. I-VIII; Figure 7.1).

Furthermore, three members of the *Pleurosigma* genus (Chapter 2) have also been newly identified as HBI producers (*P. intermedium*, *P. planktonicum* and *Pleurosigma sp.*; Table 7.1). Like the *Haslea* spp., only C_{25} HBIs were identified in the *Pleurosigma* spp. and the HBI isomers also varied between species. The HBIs produced by *P. intermedium* and *Pleurosigma* sp. (IX-XVI; Figure 7.2) were structurally distinct from those produced by *Haslea* spp., and possessed an unsaturated branch point (C7) and exhibited *E/Z* isomerism about the C9-C10 double bond. *P. planktonicum* produced only C_{25} tetraenes (XIX and XX; Figure 7.2) which like the HBIs from *P. intermedium* and *Pleurosigma* sp. exhibited *E/Z* isomerism about the C9-C10 double bond. Surprisingly, the tetraenes produced by *P. planktonicum* were saturated at C7, and did not possess a vinyl moiety (C23-24), a feature common to all HBIs characterised to date following isolation from diatoms.

Additionally, HBI production by different strains of *R. setigera* (Chapter 3) was investigated. Surprisingly, the isomers produced by the different strains of this species (Table 7.1; Figure 3.5) showed extreme variability. HBI distributions ranged from a single C_{25} HBI pentaene

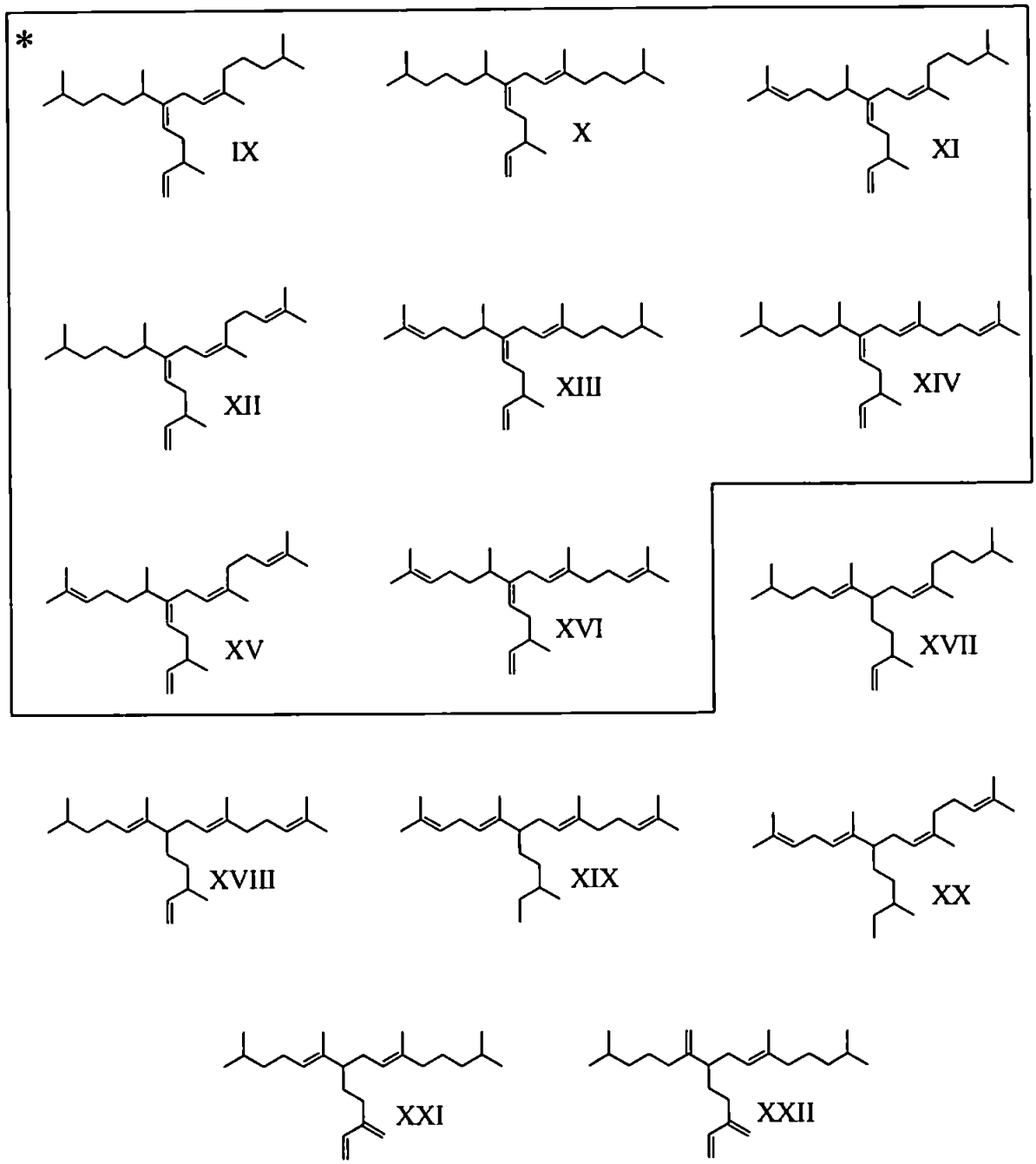


Figure 7.2 Structures of novel C_{25} HBIs isolated and characterised from cultured diatoms in this study. * Indicates the HBI isomers commonly reported in sediments and water-column particulates.

Table 7.1 A summary of HBI isomers produced by diatoms. Species names in bold indicate newly identified HBI producers, and emboldened structure numbers refer to the novel C_{25} and C_{30} HBIs characterised in this study.

Diatom species	HBI $C_{25:2}$	$C_{25:3}$	$C_{25:4}$	$C_{25:5}$	$C_{25:6}$	$C_{30:5}$	$C_{30:6}$
Haslea ostrearia	I, II	III, IV	V, **XVIII, XXI, XXII**	VI, VII	VIII		
Haslea pseudostrearia			**XVIII**	VI			
Haslea saltstonica			V				
Haslea crucigera			V	VII			
Haslea sp.		**III, XVII**					
Pleurosigma intermedium		**IX, X**	**XI, XII, XIII, XIV**	**XV, XVI**			
Pleurosigma sp.			**XI, XII, XIII, XIV**	**XV, XVI**			
Pleurosigma planktonicum			**XIX, XX**				
Rhizosolenia setigera		**IX, X**	**XIV**	VI, **XVI**		**XXIII, XXIV**	**XXV, XXVI**

with a saturated branch point in strains CCMP 1330 and CCMP 1820, only C_{30} penta and hexaenes in strain CCMP 1694, to both C_{25} and C_{30} HBIs (all with an unsaturated branch point) in strains Nantes 99 and Nantes 00.

The second aim of this work was to identify the HBI isomers that are common in the geosphere. The structures of eight HBIs identified in diatom cultures were known prior to this study (Figure 7.1), but although several of these had been observed in a number of sediments and/or suspended particulates, these HBIs were not those most common in geochemical samples. The structural characterisation herein of fourteen novel tri-, tetra- and penta-unsaturated C_{25} HBIs (Figure 7.2), following isolation from the diatom species described above, has allowed for the identification of the common sedimentary isomers (e.g. IX – XVI Figure 7.2) *via* comparison of the GC (RI) and MS characteristics of the newly characterised compounds with those of the compounds reported in sediments (Chapter 2). These common sedimentary isomers (IX - XVI) contain an unsaturated branch point (C7) and exhibited *E/Z* isomerism about the C9-C10 double bond, and both benthic (*P. intermedium*) and planktonic (*Pleurosigma sp.* and *R. setigera*) diatom species have now been identified as biological sources of these alkenes. Furthermore, the direct comparison of the HBI distributions in sediment and SPM samples from a range of marine environments with the HBIs reported herein (Chapter 5) has confirmed the geochemical significance of these novel compounds.

The third aim was to characterise the structures of the C_{30} HBIs that had been identified previously in cultures of *R. setigera* and also found in sediments and water column particulates. Thus, the structures of four novel C_{30} HBI penta- and hexaenes (Figure 7.3) were determined herein for the first time (Chapter 3), following isolation from large-scale

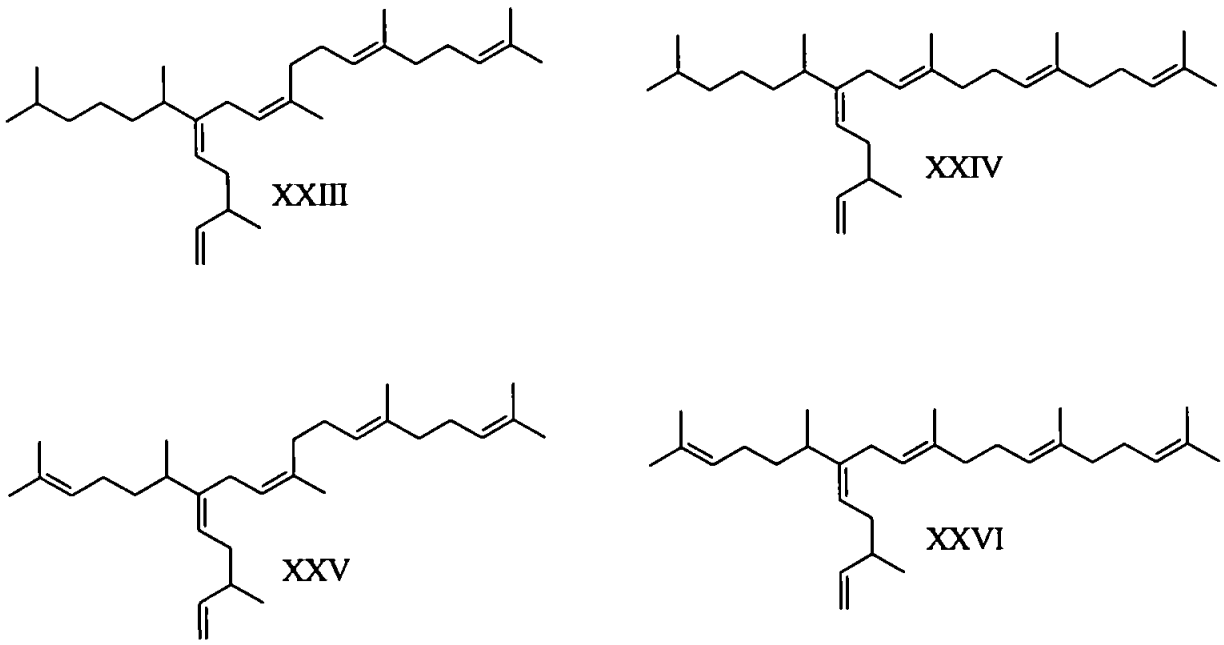


Figure 7.3 Structures of novel C_{30} HBIs characterised in this study following isolation from *R. setigera.*

cultures of *R. setigera*. This work has allowed for the structural identification of several C_{30} HBIs observed in sediments and particulates from marine environments (e.g. Chapter 5).

Table 7.1 provides a summary of the diatom species now known to produce HBIs, and lists the HBI isomers produced by each species.

Further conclusions can also be drawn from this study, with some of these providing the basis for some suggested further work. For example, although the identification of *P. intermedium* and *Pleurosigma* sp. has allowed for the structural characterisation of the HBIs most common in sediments and water column particles, it is unlikely that these diatoms are the significant sources of the most abundant HBIs in the environment. *P. intermedium* is a benthic species, and so cannot account for the occurrence of HBIs in water column particulates, and there is uncertainty as to whether *Pleurosigma* sp. is either abundant or widespread. Conversely, *R. setigera* is both a widespread and abundant diatom, and the identification of both the common C_{25} and C_{30} HBIs in this planktonic species suggests that it could be a significant source of HBIs in marine environments. Even so, it is unlikely that *R. setigera* is the only species responsible for significant HBI input into the marine environment, and thus the screening of further diatoms for HBI production would seem sensible. In addition, a detailed comparison of HBI distributions *vs.* diatom occurrence in various marine environments may reveal species that are further potential sources of HBIs, and could also allow for more detailed source/sediment relationships to be determined. Further, the continued analysis of the lipids of other diatom species may reveal the source of the C_{20} HBIs for which there is currently no known producer.

This study has also revealed significant and intriguing variations in HBI distributions from diatoms. Notably, these variations range from differences in unsaturation (e.g. trienes *vs.* tetraenes) to the production of HBIs with different structural types (e.g. saturated *vs.*

unsaturated branch point or C_{25} *vs.* C_{30} HBIs). These variations have been observed between different species of diatoms, but perhaps surprisingly, also between different cultures of the same strain and between different strains of the same species grown under identical conditions. The factors determining the HBI distributions in diatoms are therefore still unclear. Analysis of the HBI distributions of different diatom species cultured under a range of growth conditions (e.g. temperature, salinity and nutrient enrichment) could reveal if the observed variations in HBI distributions are the result of a physiological response to external factors. Alternatively, the variations may be due to differences in the biosynthetic pathways used by diatoms for HBI biosynthesis. It is not currently known whether HBI biosynthesis proceeds *via* the well known acetate/mevalonate pathway (e.g. Qureshi & Porter, 1981) or via other mevalonate-independent pathways (Rohmer, 1999), and investigations into HBI biosynthesis should be conducted. In addition, the physiological purpose of HBIs is currently unknown, although recent research shows that the phosphate esters of HBI alcohols have the potential to spontaneously form vesicles, and have been implicated in the origins of primordial life on earth (Takajo *et al.*, 2001). Thus, the biological significance of HBIs should certainly be investigated further.

The structural characterisation of the novel HBIs described herein has revealed two distinct HBI structural types. For the C_{25} HBIs these include those which are saturated at C7 (e.g. I – VIII; Figure 7.1) and those which are unsaturated at C7 (e.g. IX – XVI; Figure 7.2), with the latter corresponding to the structural type common in geochemical samples. The reason for the predominance of one structural type in environmental samples is unclear, although it may simply reflect the relative abundances of diatom species producing the different isomers. Indeed, quantatively *Rhizosolenia* spp. and *Pleurosigma* spp. are more abundant than *Haslea* spp. Alternatively, the reactivities of the two structural types may be significantly different, with one type more susceptible towards biodegradation, isomerisation (e.g. cyclisation) or

chemical modification (e.g. sulphur incorporation). Previous studies have shown that HBIs of the type produced by *Haslea* spp. (e.g. those with a saturated branch point) undergo rapid isomerisation (including cyclisation) under mildly acidic conditions (Belt *et al.*, 2000; Figures 1.4 and 1.5). HBI thiophenes and thiolanes have been identified in geochemical samples (e.g. Kohnen *et al.*, 1990), for which the novel HBIs identified herein, possessing unsaturation at C7 are likely precursors (e.g. Figure 2.10). Laboratory simulations comparing the relative susceptibilities of the two structural types to diagenetic and biodegradative processes could reveal the extent to which such transformations affect the HBI distributions observed in recent sediments.

Although the current investigation has allowed for the structural identification of the majority of the HBIs isomers observed in geochemical samples, the structures of several compounds including a C_{25} diene and tetraene identified in sediments and SPM (Chapter 5), and a C_{30} pentaene and hexaene identified in diatoms (Chapter 3) and geochemical samples (Chapter 5) remain unknown. However, since two of these, ($C_{30:5•}$ and $C_{30:6•}$; Chapter 3) have been identified in cultures of *R. setigera* (strain CCMP 1694), a large scale culture of this strain should allow for the structural characterisation of these C_{30} compounds. A biological source for the uncharacterised $C_{25:2•}$ and $C_{25:4•}$ identified in sediments and SPM (Chapter 5) has not yet been found, and thus is a further reason for the continued analysis of lipids from diatoms.

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