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Structural Characterisation and the Diagenetic Pathways of C25 Highly Branched Isoprenoid Hydrocarbons

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Structural Characterisation and the Diagenetic Pathways of C_{25} Highly Branched Isoprenoid Hydrocarbons.

by

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TO MY PARENTS

STRUCTURAL CHARACTERISATION AND THE DIAGENETIC PATHWAYS OF C_{25} HIGHLY BRANCHED ISOPRENOID HYDROCARBONS

by

Lesley Johns

ABSTRACT-

Highly branched isoprenoid (HBI) hydrocarbons have been widely reported in recent sediments. It is known that two species of diatomaceous algae, the diatoms *Haslea ostrearia* and *Rhizosolenia setigera* are able to biosynthesise C_{25} HBI alkenes. Large scale cultures of *H. ostrearia* studied previously, allowed isolation and characterisation of five HBI polyenes. The stereochemistry of the chiral centres was not reported however. The concentrations of HBIs often decrease with depth in sediment cores but the reasons for this are unknown.

In the present study, two previously unidentified C_{25} HBIs have been isolated from *H. ostrearia* and the structures unambiguously assigned by nuclear magnetic resonance spectroscopy (NMR) and mass spectral analysis. They have been identified as 2,10,14-trimethyl-6-methylene-7-(3-methylpent-4-enyl)pentadecane and the triene 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadeca-5,9-diene.

A different C_{25} triene from the diatom *R. setigera* has been isolated and tentatively identified by ^{1}H NMR spectroscopy as 3,9,13-trimethyl-6-(1,5-dimethylhexyl)tetradeca-1,5,8-triene. This compound appears to be common in sediments, judging from published retention indices and mass spectra.

The partial stereochemical configurations of C_{25} HBI alkenes have been established, for the first time. A combination of NMR spectroscopy studies of the alkenes with chiral shift reagents in conjunction with soluble silver B-diketonate complexes and enantioselective gas chromatography of oxidation products of the HBIs was used. Differences in HBI isomer configurations were observed between different laboratory cultures of *H. ostrearia*.

A C_{25} diene identified in Antarctic sea-ice diatoms and surface layer sediments from Antarctica was identified as 2,10,14-trimethyl-6-methylene-7-(3-methylpent-4- enyl)pentadecane. The identification was made by comparison of mass spectra and GC retention indices on both polar and apolar GC stationary phases with those of the diene isolated from *H. ostrearia*. Stereochemical studies identified the C_{25} diene from sea-ice diatoms as 2,10S,14-trimethyl-6-methylene-7-(3S-methylpent-4- enyl)pentadecane whilst the structurally similar diene from the sediments was identified as 2,10S,14-trimethyl-6-methylene-7-(3RS-methylpent-4- enyl)pentadecane.

Individual HBI alkenes reacted rapidly under mild acid-catalysed conditions (i.e. K-10 montmorillonite clay or TSOH-HOAc). For the dienes, double bond migration and geometric isomerisation was observed as the only reactions. In contrast, the trienes underwent both isomerisation and rapid cyclisation reactions to yield substituted cyclopentenes and cyclohexenes. A compound with similar mass spectrum and GC retention indices was identified in sapropels from the Eastern Mediterranean Sea.

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Publications:

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Introduction

Chapter One Introduction

1.1 C_{25} highly branched isoprenoid hydrocarbons

C_{20}, C_{25}, C_{30} and recently C_{35} hydrocarbons (Hoefs *et al.*, 1995) with highly branched structures have been of interest to chemists and geochemists since they were first discovered in 1976 (Gearing *et al.*, 1976). The occurrence of these compounds in algae, their widespread distribution in many recent sediments and their unusual structures with varying degree of unsaturation are important factors when considering their potential as biomarkers (review Rowland and Robson, 1990).

Biomarkers are defined as 'any organic compound detected in the geo-sphere whose basic skeleton suggests an unambiguous link with known contemporary products' (Mackenzie, 1984). They contain information pertinent to the history of organic matter in sediments and oils and this information may be used to elucidate the palaeo-environment of deposition, conditions of deposition, burial (diagenesis), thermal maturation (catagenesis), the degree of biodegradation, some aspects of source rock mineralogy (lithology), age, oil/source rock correlations and oil/oil correlations.

However, prior to 1994 advancement in the use of C_{25} highly branched isoprenoids (HBIs) as biomarkers was limited. There was no known biological source of these compounds although the occurrence of the alkenes in seawater particulate matter and sediment trap samples (Prahl *et al.*, 1980; Volkman *et al.*, 1983) implied that the origin was from certain species of phytoplankton. This was substantiated by the identification of a C_{25} HBI diene in sea ice diatoms and the isolation of a different C_{25} diene from benthic microbial communities dominated by diatoms (Nichols *et al.*, 1988; Summons *et al.*, 1993).

Additionally the structural characterisation of sedimentary C_{25} HBIs was confined to the parent structure which had been unambiguously identified by synthesis (Robson and Rowland, 1986; Figure 1.1) and a few HBI alkenes which had been tentatively

characterised using GC, GC-MS, ozonolysis, epoxidation, and less frequently NMR (Dunlop and Jefferies, 1985; Robson, 1987; Rowland and Robson, 1990; Yruela *et al.*, 1990; Hird *et al.*, 1992; Summons *et al.*, 1993).

The limited knowledge on the structures of sedimentary C_{25} HBIs made it difficult to establish whether some HBI isomers, reported by different authors were actually the same. However, comparison of retention indices (gas chromatography) and/or mass spectral data allowed Hird (1992) to establish that there were at least twenty six structurally different C_{25} HBIs reported within the literature (Table 1.1). Some of these C_{25} HBIs were considered geometric isomers. For example, Barrick *et al.* (1980) proposed that the trienes ($C_{25:3}$* 2044, $C_{25:3}$* $2090_{SP\text{-}2100}$) and tetraenes ($C_{25:4}$* RI 2078 $C_{25:4}$* RI $2124_{SP\text{-}2100}$) were geometric isomers, based on the conclusion that mass spectra of the corresponding pairs were identical. Moreover, the difference in retention indices of the two trienes was identical to that between the two tetraenes on both SP-2100 ($\Delta I_{Sp\text{-}2100}$=46) and SP-1000 ($\Delta I_{SP\text{-}1000}$=58). Similarly Porte *et al.* (1990) assigned eight of the C_{25} HBIs identified in bivalves from Todos Bay, Brazil as four pairs of geometric isomers, based on the identity of their mass spectra ($C_{25:3}$* RI 2044, $C_{25:3}$* RI 2091; $C_{25:4}$* RI 2079, $C_{25:4}$* RI 2126; $C_{25:4}$**, RI 2086, $C_{25:4}$** 2133 and $C_{25:5}$* RI 2144, $C_{25:5}$* RI 2169_{DB5}; Table 1.1).

In 1994, a family of C_{25} HBIs polyenes was identified in a non-axenic culture of the diatom, *Haslea ostrearia* (Volkman *et al.*, 1994) and large scale cultures of this alga allowed isolation and characterisation of five HBI polyenes, a $C_{25:3}$ [II], a $C_{25:4}$ [III], two $C_{25:5}$ [IV], [V] and a $C_{25:6}$ [VI] (Belt *et al.*, 1996; Wraige *et al.*, 1997; Figure 1.1). It was noted that the five HBIs were structurally very similar, differing only in the position of the double bond at C6 and the number of tri-substituted double bonds.

* and ** denote geometric isomers

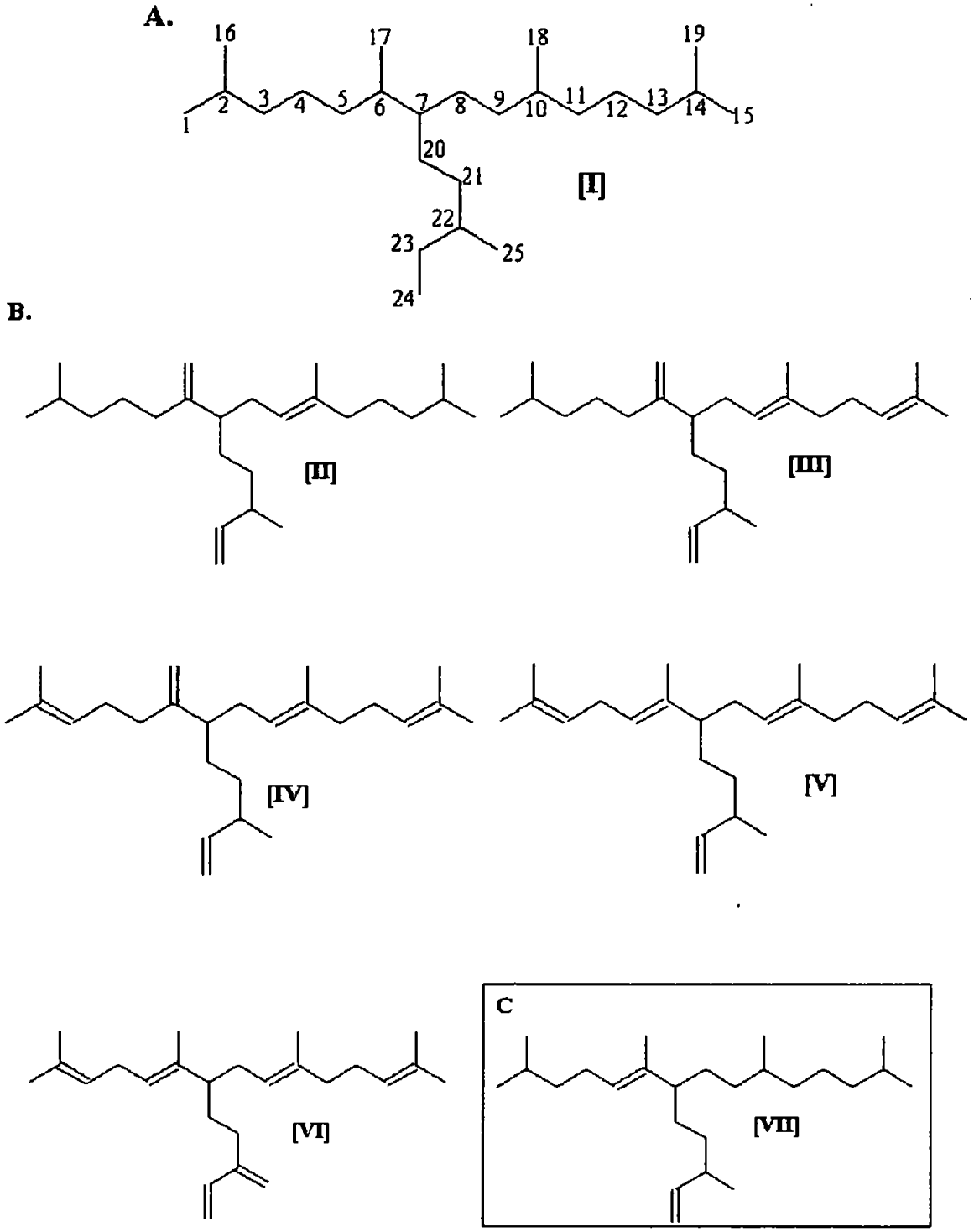


Figure 1.1 a: $C_{25:0}$ alkane (Robson and Rowland, 1986)

b: C_{25} HBI alkenes identified in cultures of *Haslea ostrearia* (Belt *et al.*, 1996, Wraige *et al.*, 1997)

c: $C_{25:2}$ HBI isolated from Caspian Sea sediment (Belt *et al.*, 1994)

Table 1.1 Reported occurrences of C_{25} HBI hydrocarbons in sediments and biota where GC retention indices and/or mass spectra are cited (updated from Cooke, 1995).

HBI	Average RI	Example of Source /Location	References (list in table 1.1a)
$C_{25:0}$	2110 (n=2)	Immature oil/Siberia	21, 34, 35, 41
$C_{25:1}$	2106 (n=1)	Recent sediment/Southern California	7
$C_{25:1}$	2112 (n=1)	Recent sediment/Shark Bay, Australia	19, 29
$C_{25:1}$	2074 (n=2)	Recent Sediment/SW England	24, 38
$C_{25:1}$	2091 (n=1)	Recent Sediment/SW England	24
$C_{25:2}$	2071 ± 4 (n=8)	Recent Sediment/Peru Coast	1, 4, 12, 13, 16, 23, 31, 32, 38
$C_{25:2}$	2082 ± 2 (n =12)	Recent Sediment/Antarctic	3, 11, 13, 14, 16, 17, 20, 23, 25, 26, 29, 30, 36, 38
$C_{25:2}$	2085 (n=1)	Recent sediment/Spain	33
$C_{25:2}$	2088 ± 0 (n=4)	Sea ice diatoms/ Antarctic	13, 17, 27, 30, 31
$C_{25:2}$	2139 (n=1)	Recent Sediment/Scotland	3, 12, 16
$C_{25:3}$*	2044 ± 1 (n=10)	Recent sediment/ USA	4, 6, 11, 12, 17, 22, 27, 31, 32, 38
$C_{25:3}$*	2091 ± 1 (n=4)	Recent sediment/Puget Sound	5, 6, 7, 10, 11, 12, 13, 16, 17, 18, 22, 31, 32, 38
$C_{25:3}$	2104 (n=2)	Recent sediment/Spain	11, 17
$C_{25:3}$	**2107 ± 1 (n=8)**	***H. ostrearia* culture/recent sediment Antarctica**	**11, 14, 17, 27, 31, 37, 38, 40**
$C_{25:3}$	2119 (n=1)	Recent sediment/Spain	13
$C_{25:3}$	2156 (n=1)	Bivalves/Todos Bay, Brazil	31
$C_{25:4}$	2055 (n=1)	Recent sediment/Alaska	9
$C_{25:4}$*	2079 ± 1 (n=7)	Sediment traps/North Pacific	2, 4, 6, 12, 22, 31, 32
$C_{25:4}$**	2086 (n=1)	Bivalves/Todos Bay, Brazil	31
$C_{25:4}$	2098 ± 4 (n=4)	Sediment traps/	1, 8, 16, 32,
$C_{25:4}$*	2127 ± 2 (n=5)	Recent Sediment/SW England	6, 12, 31, 32, 38
$C_{25:4}$**	**2134 (n=2)**	***H. ostrearia* culture/bivalves Todos Bay, Brazil**	**31, 37**
$C_{25:4}$	**2144 (n=2)**	***Haslea ostrearia* culture**	**37, 40**
$C_{25:4}$	2175 (n=1)	Recent sediment/SW England	38
$C_{25:5}$	2125 (n=1)	Bivalves/Todos Bay, Brazil	31
$C_{25:5}$*	2144 (n=1)	Bivalves/Todos Bay, Brazil	31
$C_{25:5}$*	2170 (n=2)	*Rhizosolenia setigera* culture	31, 43
$C_{25:5}$	2183 (n=2)	Recent sediment/Tamar Estuary	31, 38
$C_{25:5}$	**2190 (n=2)**	***Haslea ostrearia* culture**	**37, 40**
$C_{25:5}$	**2201 (n=2)**	***Haslea ostrearia* culture**	**37, 42**
$C_{25:6}$	**2248 (n=1)**	***Haslea ostrearia* culture**	**42**

* & ** Denotes pair of geometric isomers

Bold Denotes C_{25} HBIs isolated from *Haslea ostrearia* cultures (Belt *et al.*, 1996; Wraige *et al.*, 1997).

Table 1.1a List of References

No.	Reference	Sampling Location
1	Farrington (1977)	North Western Atlantic coastal sediments
2	Boehm and Quinn (1978)	Rhode Island, USA
3	Blanchard (1979)	Kelp bed sediments Loch Creran Scotland
4	Crisps *et al.* (1979)	Southern California, U.S.A
5	Prahl *et al.* (1980)	Dabob Bay, Washington State, U.S.A.
6	Barrick *et al.* (1980)	Puget Sound U.S.A.
7	Venkatesan *et al.* (1980)	Southern California Bight. U.S.A.
8	Barrick *et al.* (1981)	Puget Sound U.S.A.
9	Venkatesan and Kaplan (1982)	Alaskan Outer Continental Shelf
10	Osterroht *et al.* (1983)	Kiel Bight, Baltic Sea
11	Requejo and Quinn (1983)	Narrangansett Bay estuary, U.S.A.
12	Volkman *et al.* (1983)	Peru upwelling region, Peru
13	Albaiges *et al.* (1984a)	Erbo Delta, Spain
14	Albaiges *et al.* (1984b)	Alfacs and Fangar Bays, Erbo Delta, Spain
15	Prahl and Carpenter (1984)	Washington State, U.S.A.
16	Requejo and Quinn (1984)	Pettaquamscutt river, Rhode island, USA
17	Requejo and Quinn (1985)	New England Salt Marsh, USA
18	Shaw *et al.* (1985)	Port Valdez, Alaska, USA
19	Dunlop and Jefferies (1985)	Shark Bay, Western Australia
20	Rowland *et al.* (1985)	Field sample of *Enteromorpha prolifera,* S.Wales
21	Sinninghe Damsté *et al.* (1985)	Messinian, Upper Miocene; Italy
22	Matsueda *et al.* (1986)	Sediment traps in the eastern North Pacific
23	Voudrais *et al.* (1986)	Eastern Virginia estuarine creeks, USA
24	Robson (1987)	Tamar estuary, SW England
25	Venkatesan and Kaplan (1987)	Bransfield Strait, Antarctica
26	Brault and Simoneit (1988)	Bransfield Strait, Antarctica,
27	Venkatesan (1988)	McMurdo sound, Antarctica
28	Nichols *et al.* (1988)	Sea ice diatoms, Antarctica
29	Kenig *et al.* (1989)	Abu Dhabi
30	Pihlaja *et al.* (1990)	Baltic Sea
31	Porte *et al.* (1990)	Bivalves, Todos Bay, Brazil
32	Wakeham (1990)	Cariaco Trench
33	Yruela *et al.* (1990)	Guadalquivir Delta, Spain
34	Bazhenova *et al.* (1990)	Immature oil, Siberia
35	Ten haven *et al.* (1993)	Carpathian Foredeep and Overthrust, Poland
36	Belt *et al.* (1994)	Caspian Sea sediment
37	Volkman *et al.* (1994)	*Haslea ostrearia* diatom
38	Hird and Rowland (1995)	Tamar Estuary, SW England
39	Cripps (1995)	Antarctic
40	Belt *et al.* (1996)	*Haslea ostrearia* diatom
41	Koopmans *et al.* (1997)	Immature oil, Jordan
42	Wraige *et al.* (1997)	*Haslea ostrearia* diatom
43	Sinninghe Damsté *et al.* (1999)	*Rhizoselenia setigera* diatom

However, both Cooke (1995) and Volkman *et al.* (1994) reported that the distributions of C_{25} HBI isomers found in laboratory cultures of *Haslea ostrearia* were different from those previously reported in marine sediments and seawater even though the alkenes possess the same unusual carbon skeleton. Indeed, four of the five C_{25} HBI alkenes, $C_{25:4}$ (RI 2144), $C_{25:5}$ (RI 2191 and RI 2201) and $C_{25:6}$ (RI 2248) have not been detected in sediments at all and the $C_{25:3}$ (RI 2107) is not predominant in sediments (Table 1.1).

In addition, it is clear from the Table 1.1 that C_{25} dienes and trienes are by far the most abundant of the HBIs reported in the environment i.e. at least 70% of HBIs reported are either $C_{25:2}$ or $C_{25:3}$ isomers. In contrast, for two of the three cultures of *Haslea ostrearia* analysed to date, the dominant HBI was the $C_{25:4}$ (RI 2144) i.e. 78.5% and 70% of the total HBI alkenes in the culture reported by Volkman *et al.* (1994) and Belt *et al.* (1996) respectively.

1.2 Other potential sources of C_{25} HBIs

Haslea ostrearia, although reported in many coastal sediments (e.g. Hustedt and Aleem, 1951; Simonsen, 1974; Neville and Daste, 1978; Robert, 1986; Ricard, 1987) may not be the dominate source of C_{25} HBIs in the marine environment.

$C_{25:5}$ (RI $2170_{CP\ Sil\text{-}5}$) was recently identified in a North Atlantic strain of the marine diatom *Rhizosolenia setigera* (Sinninghe Damsté *et al.*, 1999), thus suggesting to the authors that a suite of eight C_{25} HBI alkenes detected in plankton samples of Newfoundland coastal waters and related sediments (Bieger *et al.*, 1997) originated from *R setigera*. *Rhizosolenia setigera* was a known source of the C_{30} HBIs (Volkman *et al.*, 1994, 1998). However, this was the first identification of a C_{25} HBI in this alga. The observed variability in lipid biosynthesis was attributed to differences between the strains of *R. setigera* (Sinninghe Damsté *et al.*, 1999).

It has been demonstrated that highly branched polyprenylated polyprenols, can possibly be produced in sediments by abiotic processes. Nagano *et al.* (1999) reported that

dimerisation of geraniol and farnesol occurs in the presence of unactivated clay (K 10 montmorillonite). For example, the direct condensation of (2E, 6E)-farnesol VIII on montmorillonite K 10 followed by acetylation gave a mixture of acetates. One was shown by NMR to be 10-farnesyl-substituted farnesyl acetate [IX] which shares a similar backbone structure to that of the C_{25} HBI diene isolated from Shark Bay sediments (Summons *et al.*, 1990). Another was shown to be 6-farnesyl-substituted farnesyl acetate [X] which shares the same backbone structure to that of the biosynthetic C_{25} HBIs (Figure 1.2). HBIs can reasonably be derived from these corresponding polyprenylated polyprenols although they have yet to been identified in sediments and/or biota (Ourisson and Nakatani., 1994).

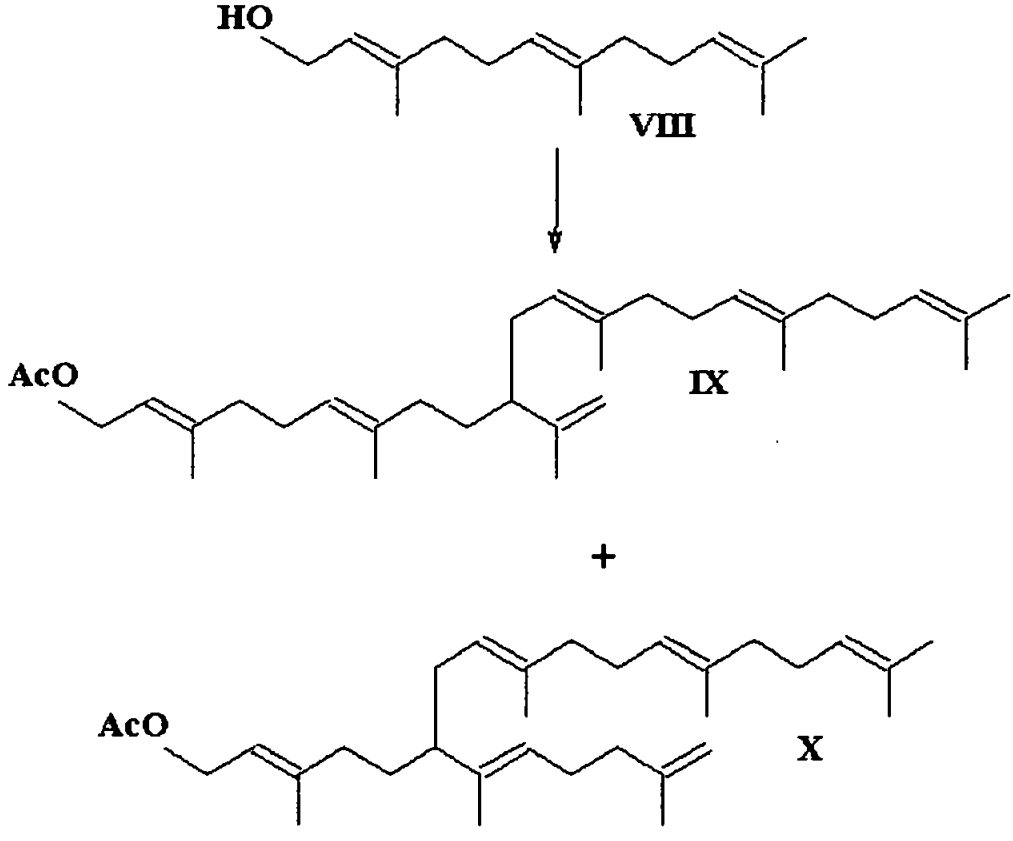


Figure 1.2 Formation of 10-farnesyl-substituted farnesyl acetate **IX** and 6-farnesyl-substituted farnesyl acetate **X** by condensation of (2E, 6E) – farnesol **VIII** on K 10 montmorillonite followed by acetylation (Nagano *et al.*, 1999).

1.3 Diagenetic fate of C_{25} HBIs

Another possible explanation for the differences in the distribution between that of the HBIs identified in *Haslea ostrearia* and those previously reported in sediments is that the biogeneic HBIs undergo molecular transformations upon entering the aquatic environment, (i.e. water column, sediment–water interface and the underlying sediment).

HBI composition of sinking particles in the water column has been shown to change with increased sampling depth (e.g. Matsueda and Handa, 1986ab; Wakeham, 1990). Matsueda and Handa (1986a) showed that the vertical flux of the sum of C_{25} HBI tri and tetraenes ($C_{25:3}$, 2047, $C_{25:4}$ 2083 and $C_{25:3}$ 2092) decreased with depth throughout the stations sampled in the Eastern North Pacific Ocean. Additionally, Wakeham *et al.* (1990) found that the concentration of a $C_{25:3}$ decreased rapidly with depth but also that the hydrocarbon distribution in the sediment floc (the upper 2-3 mm of flocculant material at the sediment-water interface) was markedly different from those of the suspended particles from the water column. The most abundant compound in the C_{25} HBI alkene group was another tetraene $C_{25:4}$ (RI 2097). This compound was only a minor constituent of the suspended particles in the water column. The enrichment of a $C_{25:2}$ (RI unreported) was also noted. This enrichment was explained by the suggestion that HBIs had an anaerobic microbial origin. However, another plausible explanation is that they are diagenetic products from other more labile C_{25} HBI alkenes e.g. by isomerisation or partial hydrogenation of double bonds. Interestingly the same tetraene (RI 2097) was reported to have a sub-surface maxima in cores removed from Narragansett Bay, USA (Requejo and Quinn, 1983).

Numerous studies of sediment cores have shown that the concentration of C_{25} HBI alkenes decreases rapidly with depth (Rowland *et al.*, 1990 and references cited therein). However, there is some evidence that C_{25} HBI alkenes with two double bonds and less are removed from the hydrocarbon fraction at a less rapid rate than the higher polyenes (Requejo and Quinn, 1983; 1984; Dunlop and Jefferies, 1985). A possible explanation for this is that the higher unsaturated HBI alkenes are more susceptible to biodegradation than

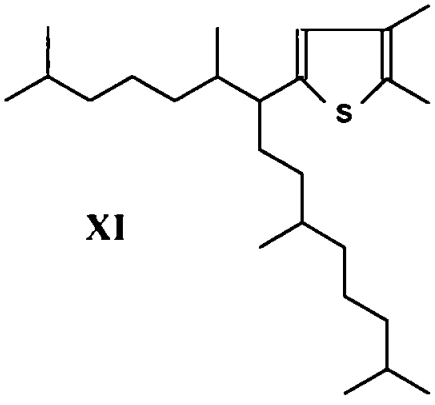
those with two double bonds or less. Laboratory based studies by Robson and Rowland (1988) and Gough *et al.* (1992) showed that the parent alkane I and a mixture of related monoenes, were more resistant to aerobic biodegradation than *n*-alkanes, *n*-alkenes, and other branched alkanes of the same molecular weight.

An alternative mechanism for the depletion of the HBI alkenes is that sulphur is incorporated into HBIs during early diagenesis to form Highly Branched Isoprenoid Thiophenes (HBIT; reviewed by Sinninghe Damsté and de Leeuw, 1990; Kohnen *et al.*, 1992). The C_{25} HBITs XI and XII have been identified in sediments and immature oils (Sinninghe Damsté *et al.*, 1989; Figure 1.3; table 1.2). It is proposed that the formation of these HBIT compounds is initiated by addition of hydrogen sulphide (or polysulphides) to a double bond followed by intramolecular cyclization to form thiolanes which undergo dehydrogenation to form thiophenes. This only occurs when two double bonds are separated by fewer than four sp^3-hybridised carbon atoms (Sinninghe Damsté *et al.*, 1989). Therefore the formation of the widespread occurring C_{25} HBIT XI could be explained by the sulphur incorporation into a C_{25} HBI diene with double bond positions at C21 – C25 of the carbon skeleton (Sinninghe Damsté *et al.*, 1989; Figure 1.4). However, none of the HBI dienes characterised to date contain these double bond positions (Belt *et al.*, 1994; Summons *et al.*, 1993; Yruela *et al.*, 1990).

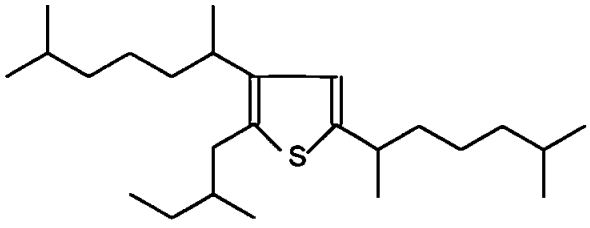
Table 1.2 Occurrence of C_{25} HBIT in sediments and oils

Sample	Age	Compounds
	Sediments	
Gulf of California	Pleistocene	XI
Monterey Shale	Miocene	XI
Jurf ed Darawish oil Shale	Cretaceous	XI, XII
Northern Apennines Marl	Miocene	XI
	Oils	
Rozel Point oil seep	Miocene	XI
West Rozel point Oil	Miocene	XI
Sicily Seep Oils	Miocene	XI
Jianghan oils	Palaogene	XI

Source: Sinninghe Damsté *et al.*, 1989.

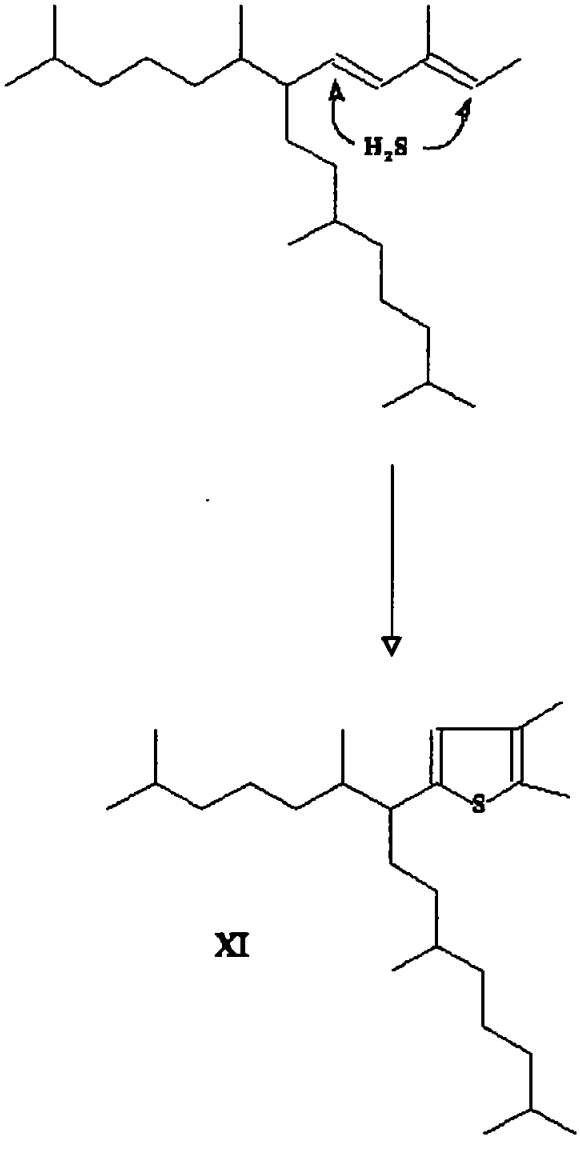


XI



XII

Figure 1.3 2,3-dimethyl-5-(7'-(2',6',10',14'-tetramethylpentadecyl)) thiophene [XI] and 2-(2'-methylbutyl)-3,5-di-(2'-(6'-methylheptyl)) thiophene [XII]



XI

Figure 1.4 A $C_{25:2}$ amendable for intramolecular sulphur incorporation to produce XI

There is some evidence that the double bonds undergo rearrangement in the sediments, producing isomers with different GC retention characteristics. For example the $C_{25:5}$ [V] and $C_{25:6}$ [VI] have common structural similarities to that of a HBI diene isolated and characterised from Caspian sea sediment i.e. a vinyl group at C23-C24 and a tri-substituted double bond at C5-C6 (Belt *et al.*, 1994; Figure 1.1c). This suggested to the authors that a C6-C17 double bond migration to the C5-C6 position may be a facile process in sediments and may also be occurring on storage as the $C_{25:5}$ [V] and $C_{25:6}$ [VI] were isolated from a culture that had been stored for over a year prior to analysis (Wraige *et al.*, 1997).

1.4 Objectives

It is fundamental that the structures, sources and short-term fate of HBI alkenes are fully understood before they can be utilised as molecular indicators of paleoenvironments and this is the general objective of the research described in this thesis.

Structures – Chapter two

- To identify and establish the structures of uncharacterised sedimentary HBIs and/or new compounds from further cultures of the diatom *Haslea ostrearia*.

- To identify and characterise C_{25} HBIs in cultures of the diatom *Rhizosolenia setigera*, a known source of the C_{30} HBIs (Volkman *et al.*, 1994). Sinninghe Damsté *et al.* (1999) recently identified a $C_{25:5}$ HBI in a North Atlantic strain of the diatom.

- To establish the stereochemistry of the isolated and characterised C_{25} HBIs and hence gain better knowledge on the biosynthesis of these compounds.

Establish the link between the C_{25} HBIs identified in *Haslea ostrearia* and those found in sediments– Chapter three

- Compare the mass spectra and retention indices, on both polar and apolar GC stationary phases of sedimentary C_{25} HBIs with those of the authenicated C_{25} HBIs (Chapter two and Belt *et al.*, 1994, 1996).

Early diagenetic reactions – Chapter four

- To subject pure C_{25} HBIs previously isolated from *Haslea ostrearia* (Chapter two, Belt *et al.*, 1994, 1996) to laboratory simulation of early diagenetic reactions, thereby gaining a better understanding of why there appears to be a rapid decrease in concentration of the higher polyunsaturated C_{25} alkenes ($> C_{25:2}$) with increasing sediment depth.

Structural Characterisation of C_{25} HBI Hydrocarbons

2.1 Introduction

2.1.1 Primary Structural Characterisation

It has already been described that in order that C_{25} HBIs may be candidates as biomarkers and/or palaeoenvironmental markers, so it is fundamental that the structures of the sedimentary C_{25} HBIs are known unambiguously. However, the concentration of the HBIs in sediments is often low, e.g. 230 ng/g of dry sediment (Requejo and Quinn, 1983), which prevents thorough structural elucidation. Historically, characterisation has often been confined to the determination of the parent structure by hydrogenation of the alkenes to the alkane ($C_{25:0}$) which has been unambiguously identified by synthesis (Robson and Rowland, 1986) and the degree of unsaturation for each sedimentary HBI determined by mass spectrometry. Until 1994, relatively few full structural studies of sedimentary C_{25} HBIs had been reported (Dunlop and Jefferies, 1985; Yruela *et al.*, 1992 and Summons *et al.*, 1993; Figure 2.1).

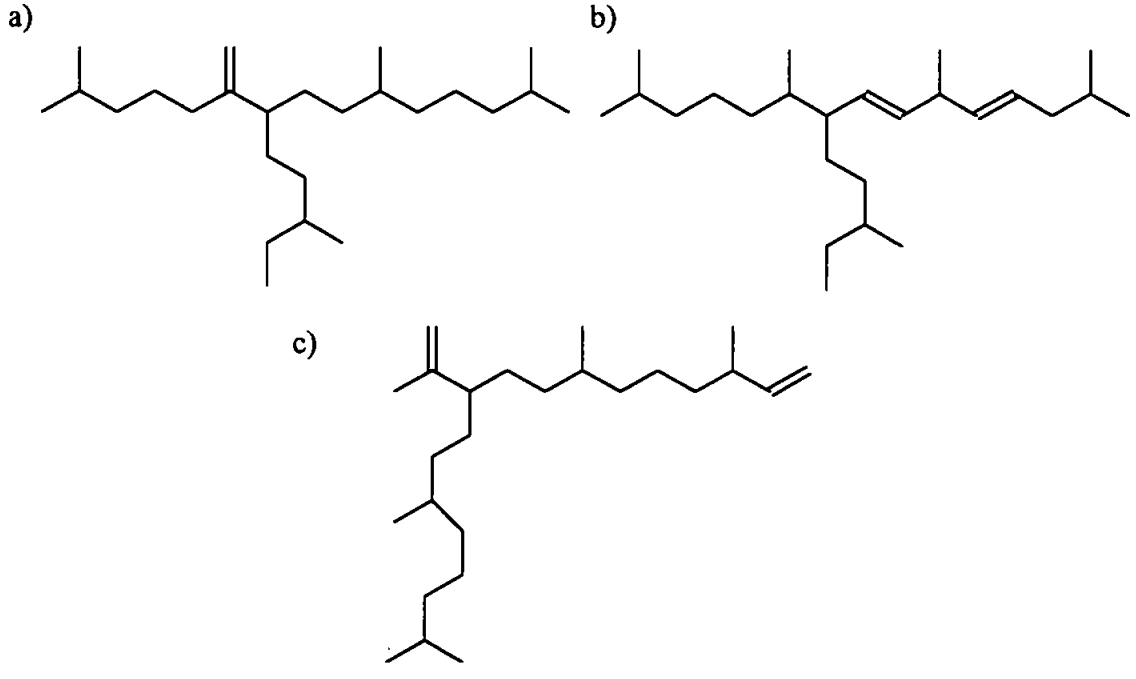
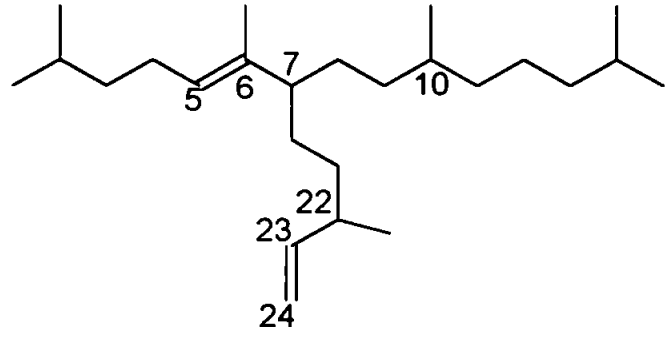


Figure 2.1 Reported structures of C_{25} HBIs prior to 1994

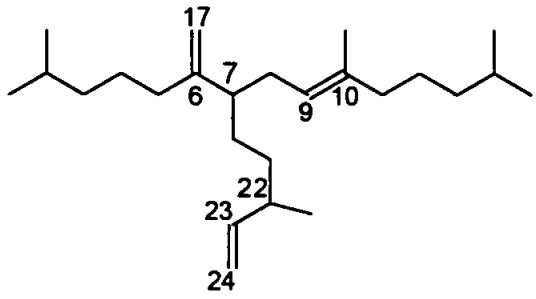
a) $C_{25:1}$, $RI_{(HP1)}$ 2110, from Shark Bay, Australia (Dunlop and Jefferies, 1985).
b) $C_{25:2}$, $RI_{(CP\text{-}Sil\ 8\ CB)}$ 2085 Mesohaline lagoon, Guadalquivir Delta,Spain (Yruela *et al.*, 1992).
c) $C_{25:2}$, RI (not reported) Benthic microbial communities Shark Bay, Australia (Summons *et al.*, 1993).

In 1994, Belt *et al.*, extracted several milligrams of a $C_{25:2}$ [I] from Caspian sea sediments for unequivocal characterisation by gas chromatography (GC), gas chromatography mass spectrometry (GC-MS) and NMR (Belt *et al.*, 1994: Figure 2.2). In the same year, a biological source of the C_{25} HBIs was identified in the diatomaceous alga, *Haslea ostrearia* (Volkman *et al.*, 1994). Large scale cultures of this alga allowed isolation and characterisation of five C_{25} HBI polyenes from 40-80g of wet alga (Belt *et al.*, 1996; Wraige *et al.*, 1997). A $C_{25:3}$ [II], a $C_{25:4}$ [III] and a $C_{25:5}$ [IV] were isolated from a culture harvested in June 1995 (Figure 2.3a). It was noted that these three HBI alkenes had common structural features: i) a vinyl group (C23-C24) identified by an ABMX spin system in the ^{1}H NMR spectrum (δ= 5.65 ppm) ii) a methylenic double bond (C6-C17) and iii) a tri-substituted double bond (C9-C10). Another culture of *Haslea ostrearia*, harvested in September 1988 contained a structurally different $C_{25:5}$ [V] and a $C_{25:6}$ [VI] (Figure 2.3b). These HBIs were also found to possess the vinyl group (C23-C24) and the tri-substituted double bond (C9-C10) but the methylenic double bond (C6-C17) was replaced by a tri-substituted double bond at C5-C6. This structural feature and the vinyl group were also observed in the $C_{25:2}$ [I] isolated from the Caspian Sea sediments.

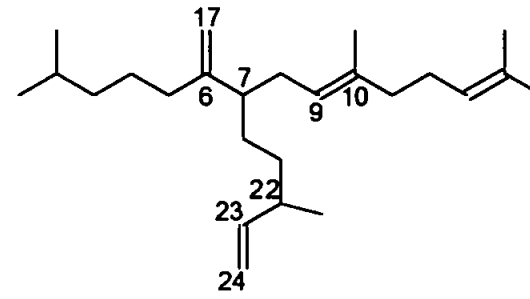


[I]

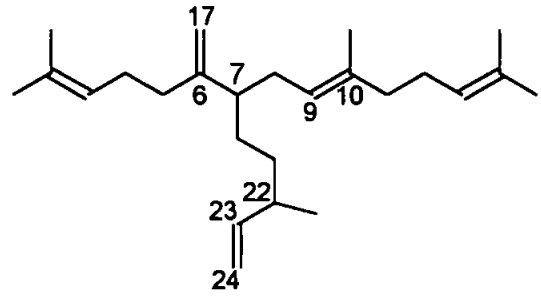
Figure 2.2 $C_{25:2}$, $RI_{(HP1)}$ 2079
Source: Caspian Sea sediment.
Ref: Belt *et al.*, 1994



[II]

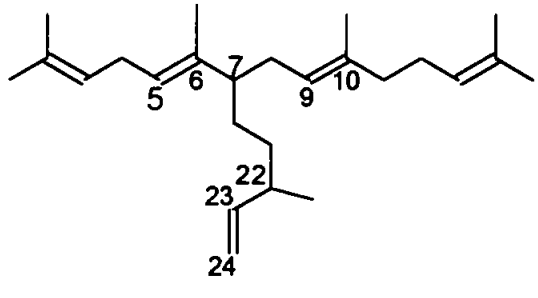


[III]

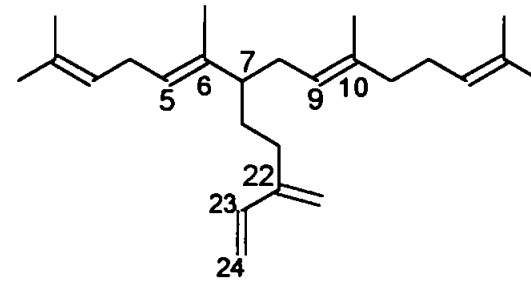


[IV]

Figure 2.3a C_{25} HBI alkenes identified in cultures of *Haslea ostrearia* (harvested in June 1995; Belt *et al.*, 1996)



[V]



[VI]

Figure 2.3b C_{25} HBI alkenes identified in cultures of *Haslea ostrearia* (harvested in September 1988; Wraige *et al.*, 1997)

2.1.2 Stereoisomerism of HBIs

The HBI $C_{25:3}$ [II], $C_{25:4}$ [III], $C_{25:5}$ [IV], $C_{25:5}$ [V] all have two chiral centres, at C7 and C22 (Figure 2.3a/b), whilst $C_{25:2}$ [I] has an additional chiral centre at C10 (Figure 2.2). It is possible therefore for the HBIs to exist in several stereoisomeric forms. NMR as an achiral tool, is incapable of distinguishing enantiomers but can distinguish between diastereoisomers, providing the difference in chemical shift of the diastereoisomeric carbon nuclei are greater than the resolution limit. This was found to be the case for [II], [III] and [IV] where Belt *et al.* (1996) observed that ten of the carbon resonances (C5, C6, C8, C17, C20, C22, C23, C24, C25) 'doubled-up' in appearance (Figure 2.4a). This feature was also observed for the vinylic proton (H23) in ^{1}H spectra (Figure 2.4b). However, this phenomenon is not always observed, and in some cases e.g. $C_{25:2}$ [I], $C_{25:5}$ [V] a single isomer was present (Figure 2.4c).

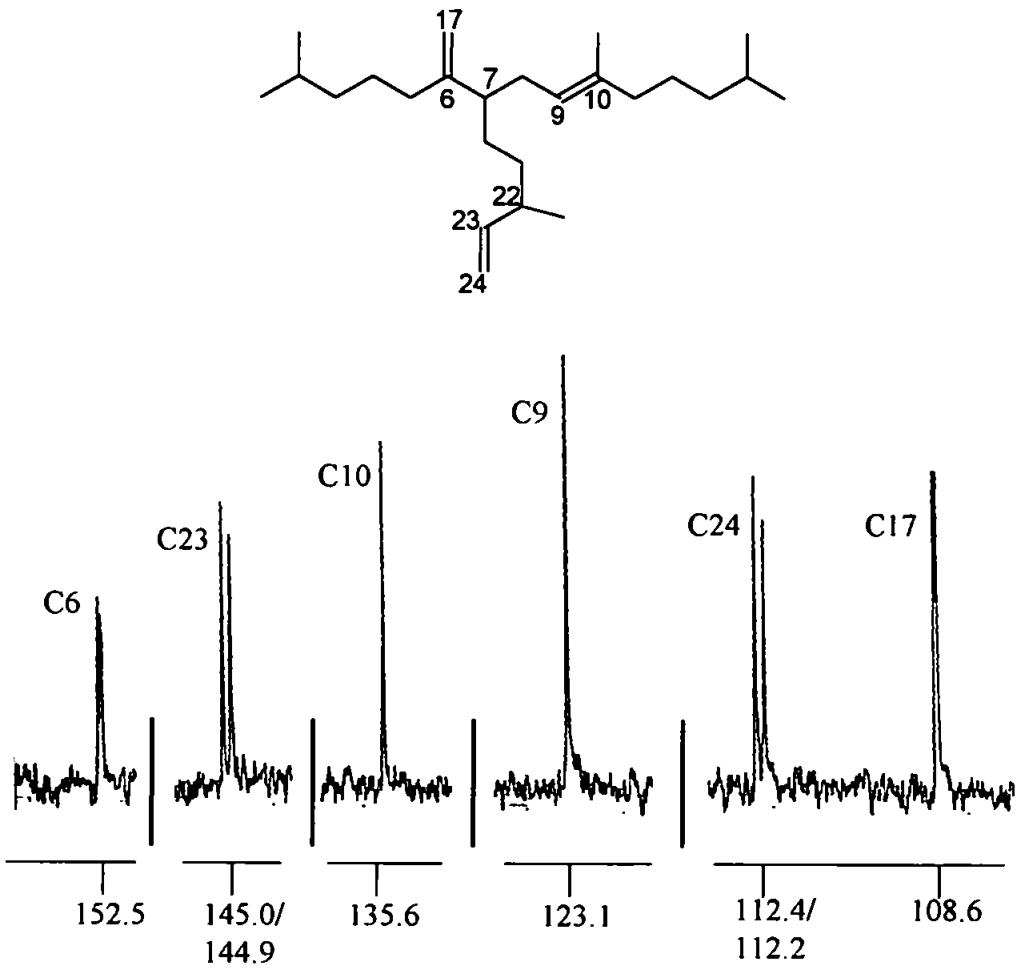


Figure 2.4a Partial ^{13}C NMR spectrum of the alkenic carbons of $C_{25:3}$ [II]

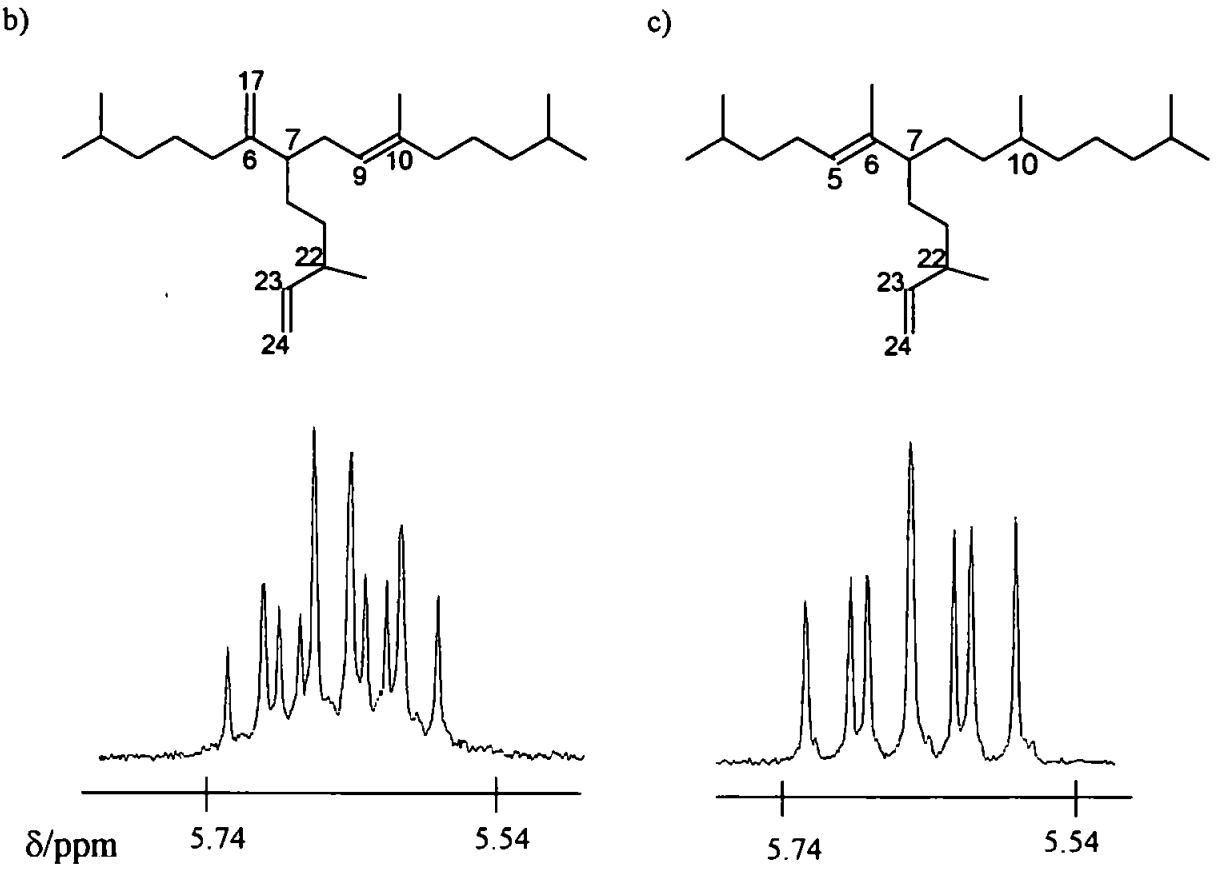


Figure 2.4b Partial ^1H spectrum showing the H23 resonance for $C_{25:3}$ [II]
2.4c Partial ^1H spectrum showing the H23 resonance for $C_{25:2}$ [I]

E/Z isomerism about C9-C10 could be an alternative explanation for these observations but this possibility was rejected since the resonances due to C9, C10 and C18 do not appear to 'double-up' as would be expected for geometrical isomers (Figure 2.4a).

Although these diastereomers were observed readily by NMR, no chromatographic separation (GC) on either of two different phases (HP1 and DB5) was observed.

2.1.3 Objectives

The previously characterised C_{25} HBIs isolated from *Haslea ostrearia* (Belt *et al.*, 1996; Wraige *et al.*, 1997) had similar retention indices and mass spectra to some, but not all, of the previously reported HBIs in sediments. In the present study, a further twelve cultures of *Haslea ostrearia* were analysed for the further uncharacterised sedimentary HBIs and/or new compounds. In addition to establishing the structures of previously unidentified HBIs, the structural characteristics of a $C_{25:2}$ and $C_{25:1}$, resulting from a controlled partial hydrogenation of $C_{25:3}$ [II] are described

The structure of a $C_{25:3}$ HBI isolated from the alga *Rhizosolenia setigera* is also described. *Rhizosolenia setigera* is known to be a source of the C_{30} HBIs (Volkman *et al.*, 1994) and recently Sinninghe Damsté *et al.* (1999) also identified a $C_{25:5}$ HBI in a North Atlantic strain of the diatom.

An investigation into the stereochemistry of the $C_{25:3}$ [II] and that of a previously unidentified $C_{25:2}$ [VII], a key feature in understanding the biosynthesis and diagenetic fate of the C_{25} HBIs is also described

2.2 Experimental

2.2.1 Algal Pastes

Twelve algal cultures, (Table 2.1) were provided by the Université de Nantes, France as centrifuged algal pastes. The cultures were grown as previously reported (Wraige *et al.*, 1997). Briefly, *Haslea ostrearia* was isolated from oyster ponds of the Bay of Bourgneuf, France and the cultures grown in an outdoor culture facility (440 L) in Nantes at ambient temperatures. Seawater for the cultures was obtained from an underground supply in Nantes at constant salinity. At the end of growth (*ca* 17 days) as monitored by cell counts (Wraige *et al.*, 1997), the algae were concentrated to 40-110g wet weight by centrifugation.

Table 2.1. Culture dates and wet/dry weights of the *Haslea ostrearia* algal paste.

Sample	Date of culture	Wet weight	Dry weight
Sodexal 18	November 1989	-	5g
BP 003	February 1993	3 x 50g	-
Soproma 19	June 1994	65g	21.12g
Soproma 22	July 1994	27g	7.81g
Soproma 24	April 1994	90g	21.46g
Soproma 26	October 1994	46g	14.98g
Soproma 34	December 1994	50g	16.34g
Soproma 37	April 1995	84g	83.95g
Soproma 43	May 1995	87g	51.37g
Soproma 44	May 1995	110g	32.30g
Soproma 46	June 1995	89g	19.40g
Soproma 49	June 1995	87g	29.45g

The thick algal pastes were frozen prior to transport to the University of Plymouth. All the samples, with the exception of BP003, were freeze-dried at Plymouth and stored at 0°C until needed.

2.2.2 Extraction of Algal Pastes

The freeze-dried samples, with the exception of Soproma 22 and 26, were soxhlet extracted in hexane (500 mL, 24 hr). Preliminary experiments showed that this method gave the best recovery of HBIs in comparison to ultrasonication (3 x 20 mins; Ultrasonic bath). The total hexane extract (THE) was dried (anhydrous Na_2SO_4), solvent removed (Buchi, N_2) and the THE of each algal paste sample was weighed and examined by gas chromatography-mass spectrometry (GC-MS) and ^1H NMR spectroscopy. HBIs were identified by comparison of the mass spectra and retention indices (RI) with those of previously authenticated HBIs (Belt *et al.*, 1996; Wraige *et al.*, 1997). Previously unidentified HBIs were noted and the samples were subjected to further examination as outlined in section 2.2.6.

2.2.3 Total Lipid Extracts (TLE)

Total lipid extracts were obtained from 5 g (dry weight) of Soproma 22 and Soproma 26 by the modified one phase $CHCl_3$-MeOH extraction, (Bligh and Dyer, 1959; White *et al.*, 1979; Nichols *et al.*, 1988). Briefly, the pastes were extracted in $CHCl_3$-MeOH (2:1) by ultrasonication (20 min, ultrasonic bath). Extraction was repeated until the extract was colourless (5 x 10 mL). The combined extracts were washed with water (Millipore, 10 mL) and the lipids recovered in $CHCl_3$. The TLE was dried (Na_2SO_4) and solvent was removed (Buchi, N_2). A sample (5 mg) of the TLE was examined by TLC-FID (Volkman *et al.*, 1986; Nichols *et al.*, 1988)[1]

[1] Analysis made by CSIRO Division of Marine Research, Hobart, Tasmania.

2.2.4 THE of the Exudate.

The aqueous exudate of a *Haslea ostrearia* culture, harvested in September 1989 was concentrated to 153 g, frozen and transported to the University of Plymouth for analysis. The sample was allowed to thaw and the THE obtained by liquid extraction in hexane (8 x 100 mL; separating funnel). The combined hexane extracts were dried (Na_2SO_4) and the solvent removed (Buchi). The THE was weighed and examined by GC-MS.

2.2.5 Rhizosolenia setigera

Rhizosolenia setigera strain was isolated from Huon Estuary, Tasmania, and cultured in a continuous culture facility (10 L; 18 °C). The alga was harvested, concentrated to a wet paste by centrifugation and filtered. Wet paste was not dried further but transferred to a Schott bottle (500 mL) with $CHCl_3$-MeOH (2:1; 200 mL) and left to stand overnight. The crude extract was decanted through glass fibre paper to remove particulates (pre-washed in $CHCl_3$) into round bottom flask (500 mL). The crude extract was washed with additional $CHCl_3$ (4 x 50 mL) and the combined solvent was removed (rotary evaporator; 40°C). The total chloroform extract (TCE; 0.20 g) was dried (Na_2SO_4) and immediately prior to GC-MS analysis, was derivatised with BSTFA (10 mL, 70°C/10 min). The TCE was then subjected to further examination as outlined in section 2.2.6.

2.2.6 Isolation of uncharacterised HBIs

Previously unidentified HBIs were isolated from the THE of Soproma 34 (901 mg), Sodexal 18 (103 mg), BP003 (1000 mg), exudate (2 mg) and the TCE (197 mg) of *Rhizosolenia setigera*. The THE or TCE of each sample was applied to a column (20 cm x 1.0 cm i.d.) containing 5% deactivated silica and eluted with hexane (300 mL), DCM (100 mL) and 1:1 DCM/methanol (100 mL). The hexane elutant was collected in 10 mL vials and the separation of the alkenes was monitored by GC-MS and the respective fractions

combined. The DCM fractions were collected in one vessel, solvent removed (Buchi, N_2) and immediately prior to analysis by GC-MS was derivatised with BSTFA (10 µl, 70°C/10 min). The THE of the exudate was applied to a shorter column (7 cm x 0.5 cm i.d) containing 5% deactivated silica and eluted with hexane (20 mL) and DCM (20 mL). The hexane eluant was collected in 2 mL vials and monitored as described previously.

After removal of solvent, the extracts were weighed and the identity of HBI alkenes determined by GC-MS, hydrogenation ($PtO_2.H_2O$, H_2; hexane; 3hr) to the alkane, ($C_{25:0}$) and by ^1H and ^{13}C NMR spectroscopy.

2.2.7 Partial hydrogenation of $C_{25:3}$ [II]

Catalyst (<10 mg; $PtO_2.H_2O$; Aldrich) was activated (hexane, 10 mL; 30 min). The $C_{25:3}$ [II] (2 mg) was dissolved in hexane (2 mL) and added to the catalyst. Hydrogen gas was gently bubbled through the solution. The reaction was halted at regular intervals (30 mins; for up to three hours) filtered and the solvent removed (N_2) and analysed by GC-MS and NMR. Following analysis the sample was re-dissolved in hexane (2 mL) and the reaction was continued.

2.2.8 Preparation of stereoisomeric acids for configurational elucidation of HBIs

<u>3 (S)-methylpentanoic acid (MP)</u>

3 (S)-methylpentanoic acid was synthesised by the Arndt-Eistert chain extension of 2-methylbutanoic acid. Thionyl chloride (5 mL) was added 2 (S) methylbutanoic acid (100 mg, Aldrich) and refluxed for 30 mins. After allowing to cool, excess solvent was removed under a gentle stream of nitrogen, until near dryness. The acid chloride was dissolved in diethyl ether (2 mL) and a diethyl ether solution of diazomethane (*ca.* 100 mg mL^{-1}) was slowly added. The solution was left to stand overnight. The yellow solution was carefully distilled to remove excess diazomethane and ether. The diazoketone was dissolved in hot

anhydrous methanol (20 mL) and a suspension of Ag0 (0.5g) in methanol (10 mL) was added dropwise with continuous stirring. The mixture was refluxed (2 hr), allowed to cool and the methanol gently decanted and the Ag washed with ether (5 mL x 3). The washings were combined with the methanol and the solvent removed (Buchi, N_2). The acid was esterified with BF_3MeOH (reflux; 15 min), extracted into DCM (3 x 10 mL), dried (Na_2SO_4) and examined by GC-MS and GC on 25m x 0.25mm fused silica column coated with octakis(6-O–methyl-2,3-di-O-pentyl)-γ-cylcodextrin, (hydrogen carrier, 0.5bar inlet pressure, 50°C).

4 (RS)-methylhexanoic acid

4(RS)-methylhexanoic acid was synthesised by the Ardnt-Eistert chain extension of 3(R/S)-methylpentanoic acid using the procedure described previously.

3 (RS), 7-dimethyloctanoic acid

3 (RS), 7-dimethyloctanoic acid were synthesised by hydrogenation of 3 (RS), 7 dimethyloct-6-enoic acid (Aldrich). $PtO_2.H_20$ (10 mg) was activated in hexane (30 min; H_2). 3 (RS), 7-dimethyloctenoic acid (100 mg) was added and hydrogen gas bubbled through gently for one hour. The acid were examined by GC on 25m x 0.25mm fused silica column coated with 2,6-dimethyl-3-pentyl-β-cyclodextrin (50% in polysiloxane OV 1701, w/w; hydrogen carrier, 0.5 bar inlet pressure, 95°C).

3 (R), 7-dimethyloctanoic acid

3(R), 7-dimethyloctanoic acid was synthesised by hydrogenation of 3 (R), 7-dimethyloct-6-enoic acid (Aldrich), using the procedure described for 3 (RS), 7-dimethyloctanoic acid.

2.2.9 Hydrogenation

Catalyst (<10 mg; $PtO_2.H_20$, Aldrich) was activated (hexane, 10 mL; 30 min). The HBI alkene (2 mg) was dissolved in hexane (2 mL) and added to the catalyst. Hydrogen gas was gently bubbled through the solution for three hours

2.2.10 Oxidation of $C_{25:0}$ HBI

The $C_{25:0}$ alkane obtained from the hydrogenation of each C_{25} HBI alkene was oxidised by the modified chromium trioxide oxidation previously reported (Brooks *et al.*, 1977; Patience *et al.*, 1978; Gough, 1989). The alkane (2 mg) was added to glacial acetic acid (10 mL) and the solution was heated (70 °C, waterbath) with stirring (5 min). CrO_3 was added at a 10:1 molar ratio of oxidant to substrate. The solution was maintained at 70 °C for two hours, cooled and extracted with DCM. The DCM extract was hydrolysed using methanolic KOH (10% KOH:Methanol w/v) under reflux (30 min). After cooling, the hydrolysed material was acidified to pH 1(conc HCL) and extracted into DCM. The DCM extracts were dried and methylated using BF_3/methanol complex (reflux, 15 min). The oxidised products were extracted into DCM (3 x 10 mL), washed with water (MilliQ; 3 x 10 mL) and dried (Na_2SO_4). The solvent was gently removed (Buchi; N_2) and the oxidised products were examined by GC-MS and GC on 25m x 0.25mm fused silica columns coated with octakis (6-O-methyl-2,3-di-O-pentyl)-γ-cylcodextrin and 2,6-dimethyl-3-pentyl-β-cyclodextrin (50% in polysiloxane OV 1701, w/w).

2.2.11 NMR + Chiral Shift Reagents

NMR studies of the HBI alkenes [$C_{25:3}$ [II (a)] and $C_{25:3}$ [II (c)] with a lanthanide chiral shift reagent and a soluble silver auxiliary compound was adapted from the method of Wenzel and Sievers (1981; 1982). Briefly, equal amounts of the lanthanide shift reagent, $Pr(hfbc)_3$ (0.24 mol., Aldrich) and Sievers' Ag(fod) (0.24 mol.) were dissolved in 1 mL of $CDCl_3$

(Fluka) and shaken for 5 minutes (mechanical shaker). The solution was centrifuged (2000 r.p.m.; 10 min) and the clear green supernatant was decanted from the grey precipitate and added to a NMR tube containing 0.08 mol. of the substrate. During these procedures, the solutions were kept covered with aluminium foil. It was noted that when the substrate was added to the solution prior to centrifuging, the concentration of the substrate, shown *via* NMR, was reduced considerably probably due to the substrate adhering to the grey precipitate. On completion of the NMR studies, the alkene was recovered by removal of the solvent under a gentle stream of nitrogen, and applied to a short column (10 cm x 0.5cm i.d.) containing 5% deactivated silica and eluted with 30 mls of hexane. Recovery 50 %, 99% purity.

Preparation of (6,6,7,7,8,8,8-Heptafluoro-2,2-dimethyl-3,5-octanedionato) silver (I), Ag(fod).

Ag(fod) was synthesised as previously reported by Sievers *et al.*, (1981; 1982). A solution of 0.96g of H(fod) [Aldrich] in 0.5 mL of CH_3OH was neutralised with 0.8 mL of 4M aqueous NaOH, and this solution was added to a stirred solution of 0.55g of $AgNO_3$ in 7.5 mL of H_20. The white precipitate was collected by filtration, washed (MilliQ; 2 x 3 mL) and dried (vacuum dessicator, 16hr) in the dark. Light exposure was minimised by use of aluminium foil.

2.3 Results and Discussion

2.3.1 Characterisation of HBIs: Algal Pastes

Twelve large-scale cultures of *Haslea ostrearia* were provided by the Université de Nantes, France. The total hexane extract (THE) was obtained from ten cultures and examined for HBIs by ^{1}H NMR spectroscopy and GC-MS. The amount of HBIs in the THE varied from 7–46% (Table 2.2) with free fatty acids being the major lipids. Few other hydrocarbons

were detected and this is consistent with the TLC-FID analysis of the total lipid extracts for Soproma 22 and Soproma 26 (Figure 2.5) where the C_{25} HBIs dominated the hydrocarbon fraction, comprising 2.9% ± 0.2 and 4.5% ± 0.4 (n=3) of each total lipid extract.

Table 2.2: Total HBIs as a percentage in the THE of different strains of *Haslea ostrearia* and exudate.

Sample	Date of culture	Dry weight/g	THE Weight/mg	% of Total HBIs in THE
Exudant	September 1996	153g*	2	39
Sodexal 18	November 1989	5g	103	24
BP 003	February 1993	132g*	1000	17
Soproma 19	June 1994	21.12g	497	46
Soproma 24	April 1994	21.46g	1206	14
Soproma 34	December 1994	16.34g	901	21
Soproma 37	April 1995	83.95g	1886	7
Soproma 43	May 1995	51.37g	2884	17
Soproma 44	May 1995	32.30g	8978	12
Soproma 46	June 1995	19.40g	780	15
Soproma 49	June 1995	29.45g	950	35

* Wet weight

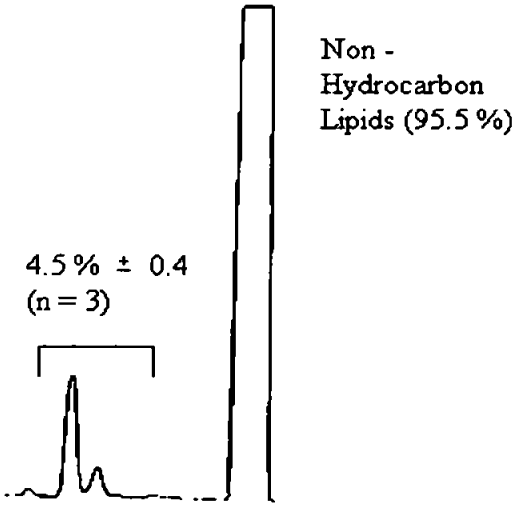


Figure 2.5 TLC-FID chromatogram of lipid extract from *Haslea ostrearia*, developed in hexane, silica chromarods.

The THE of small scale cultures of *Haslea ostrearia* were also found to have an abundance of HBI alkenes where *n*-henicosahexane (n-$C_{21:6}$) was the only non-HBI hydrocarbon (Wraige *et al.*, 1997).

Twelve different C_{25} HBIs with two to five double bonds were detected in the cultures (Table 2.3). Three of these HBIs could be identified unambiguously (II, III, IV) by comparison of the gas chromatographic retention indices and mass spectra with those of authenicated HBIs (Belt *et al.*, 1996). Previously unidentified diene [VII] and triene[VIII], were isolated from Soproma 34 and BP003 respectively, in sufficient amounts for characterisation by ^{13}C and ^{1}H NMR spectroscopy (section 2.3.2). The other seven alkenes were all tetraenes and identified by GC-MS only (insufficient quantities of NMR analysis). The retention indices and mass spectra of two of the tetraenes ($RI_{(DB5)}$ 2135 and 2158; Table 2.4) are very similar to the tetraenes identified in laboratory cultures of *Halsea ostrearia* by Volkman (1994), *viz* RI 2134_{HP1}, RI 2158_{HP1}. The remaining tetraenes ($RI_{(DB5)}$ 2088, 2092, 2166, 2186, 2216) detected only in the Sodexal 18 sample, appear not to share similar RI and mass spectra with those reported previously in either sediments or biota. The mass spectral data of each of these unknown $C_{25:4}$ HBIs is presented in Table 2.4.

The triene [II] was the dominant HBI (52-100% of total HBIs) in 9 of the 10 cultures. This is consistent with recent well-controlled temperature experiments where [II] is the major HBI when *Haslea ostrearia* is cultured at 15°C (Rowland *et al.*, 1999 in preparation). Culture BP003 was dominated by triene [VIII] (67%). It is possible that [II] isomerised to [VIII] during storage (three years). However filters from small scale cultures stored for *ca.* 1 month have also been found to contain [VIII] and [II] (Wraige *et al.*, 1999).

Table 2.3: Individual HBIs as a percentage of total HBIs in different strains of *Haslea ostrearia* and exudate.

Sample	Date of culture	Individual HBIs as a % of total HBI							Diastereo-isomeric*
		$C_{25:2}$ [VI]	$C_{25:3}$ [VII]	$C_{25:3}$ [II]	Other $C_{25:3}$	$C_{25:4}$ [III]	Other $C_{25:4}$	$C_{25:5}$ [IV]	
Retention Indices (DB5)		2084	2103	2106	2121	2144		$2191_{(HP1)}$	
Exudant	September 1996	23		56	9	7	5 (2146)		No
Sodexal 18	November 1989	12		52		4	32 (2088, 2092, 2166,2186, 2216)		No
BP 003	February 1993		67				33 (RI 2135, 2158 only)		No
Soproma 19	June 1994			96		4			Yes
Soproma 24	April 1994			26		28		46	Yes
Soproma 34	December 1994	9		77		14			Yes
Soproma 37	April 1995			100					Yes
Soproma 43	May 1995			84		13		3	Yes
Soproma 44	May 1995			86		14			Yes
Soproma 46	June 1995			74		15		11	No
Soproma 49	June 1995			54		36		10	Yes

* refers to "doubling up" of resonances in ^{1}H and ^{13}C NMR spectra (*cf* Belt et al., 1996)

Table 2.4: Mass spectra and retention indices of HBI alkenes, structure unknown, identified herein in cultures of *H.ostrearia* and exudate

HBI	Source	RI_{DB5}	Mass spectral ions m/z (rel. int) 70 eV
$C_{25:3}$	Exudate	2121	55(46), 81(44), 95(38), 109(68), 123(17), 149(10), 205(10), 233/235(4), 261(5), 303 (7), 346(5)
$C_{25:4}$	Sodexal 18	2088	55(100), 69(90), 81(75), 93(64), 107(55), 121(45), 135(28), 149(21), 163(23), 175(24), 189(38), 205(6), 231(13), 259(47), 273(5), 287(4), 301(2), 315(3), 344(2)
$C_{25:4}$	Sodexal 18	2092	55(100), 69(79), 81(72), 95(54), 109(40), 119(26), 135(19), 149(17), 163(18), 175(13), 191(13), 205(9), 231(8), 259(25), 273(8), 287(5), 344(2)
$C_{25:4}$	BP003	2135	55(74), 69(100), 81(90), 95(88), 109(32), 123(20), 137(12), 151(11), 165(3), 207(5), 222(2), 239(1), 259(1), 275(6), 310(2), 329(0.5), 344(2)
$C_{25:4}$	BP003	2158	55(100), 69(96), 81(63), 93(89), 95(38), 107(57), 121(26), 135(19), 149(37), 163(10), 175(3), 191(8), 231(7), 259(8), 275(9), 301(0.5), 329(5), 344(2).
$C_{25:4}$	Sodexal 18	2166	55(100), 69(86), 81(76), 95(63), 107(46), 121(33), 135(15), 149(20), 163(2), 175(13), 191(17), 205(7), 231(11), 259(32), 277(24), 287(5), 344(3)
$C_{25:4}$	Sodexal 18	2186	55(91), 69(100), 81(75), 95(97), 109(54), 121/123(28), 135/137(18), 149(24), 163(18), 189(14), 203(10), 219(12), 233(8), 259(33), 265(2), 277(33), 329(1), 344((1)
$C_{25:4}$	Sodexal 18	2216	55(67), 69(100), 81(49), 95(49), 109(28), 121(16), 137(7), 149(21), 163(6), 177(6), 193/195(5), 207(3), 221(5), 233(2), 259(4), 263(1), 277/279(7), 344(0.5)

2.3.2. Characterisation of HBIs: *Haslea ostrearia* exudate

Five HBI alkenes were detected in the THE (Table 2.3). Two were identified as triene [II] and tetraene [III] by comparison of the gas chromatographic retention indices and mass spectra of those of authenicated HBIs (Belt et al., 1996). Triene [II] was dominant (52%). The second most dominant alkene was the diene [VII] (23%). This was identified as the same diene detected in Sodexal 18 and Soproma 34 by gas-chromatography co-elution on two stationary phases (HP1 and DB5). The other HBI alkenes were a triene (RI 2121) which contributed 7 % to the total HBI content and a tetraene (RI 2146) which was less than 5 % of the total HBIs. The triene (RI 2121) had a similar RI and mass spectrum (Table 2.4) to that of a triene (RI 2120) identified in axenic cultures of *Haslea ostrearia* (Wraige *et al.*, 1999).

2.3.3 Characterisation of C_{25} HBIs*: Rhizosolenia setigera.*

The total chloroform extract of *Rhizosolenia setigera* contained two $C_{25:3}$ HBI alkenes (RI 2044, 2090 $_{HP1}$). *Rhizosolenia setigera* is a known source of the C_{30} HBIs (Volkman *et al*, 1994). However this is the first time a $C_{25:3}$ has been identified in this alga. Recently a $C_{25:5}$ HBI (RI 2170 $_{CP\ Sil\text{-}5}$) was detected in a North Atlantic strain of *Rhizosolenia setigera* (Sinninghe Damsté *et al.*, 1999). Column chromatography of the TCE of the current sample produced a sufficient amount of the $C_{25:3}$ (RI 2090) to allow partial characterisation by ^{1}H NMR spectroscopy (section 2.3.7). In addition, some of the fractions contained two tetraenes (RI 2072,2121 $_{(HP1)}$) which were minor constituents of the TCE of the alga. The retention indices and mass spectra of all the HBI alkenes found in *Rhizosolenia setigera* are presented in Figure 2.6.

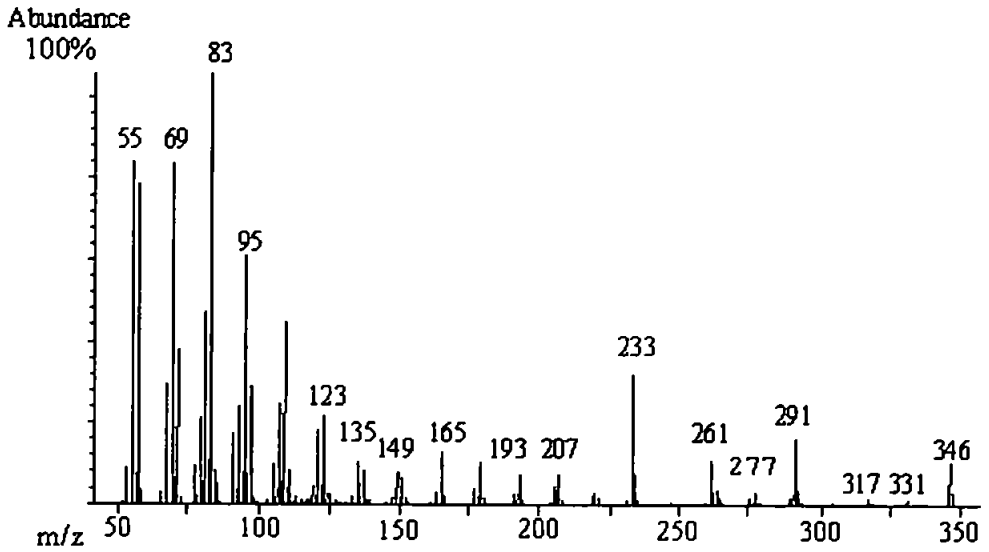


Figure 2.4a Mass spectrum of $C_{25:3}$, RI $2044_{(HP1)}$, $2050_{(Carbowax)}$

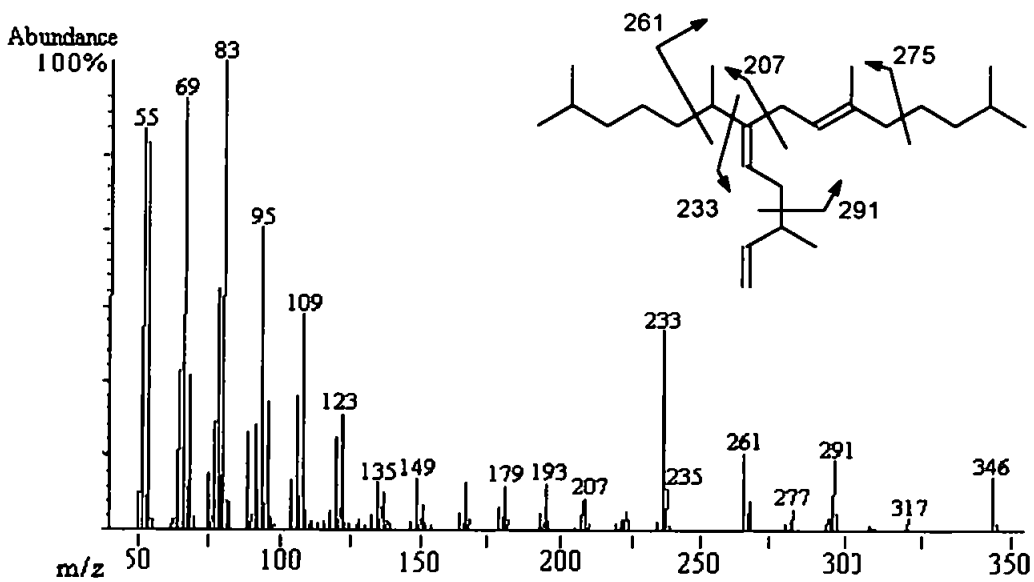


Figure 2.4b Mass spectrum of $C_{25:3}$, RI $2090_{(HP1)}$, $2142_{(Carbowax)}$

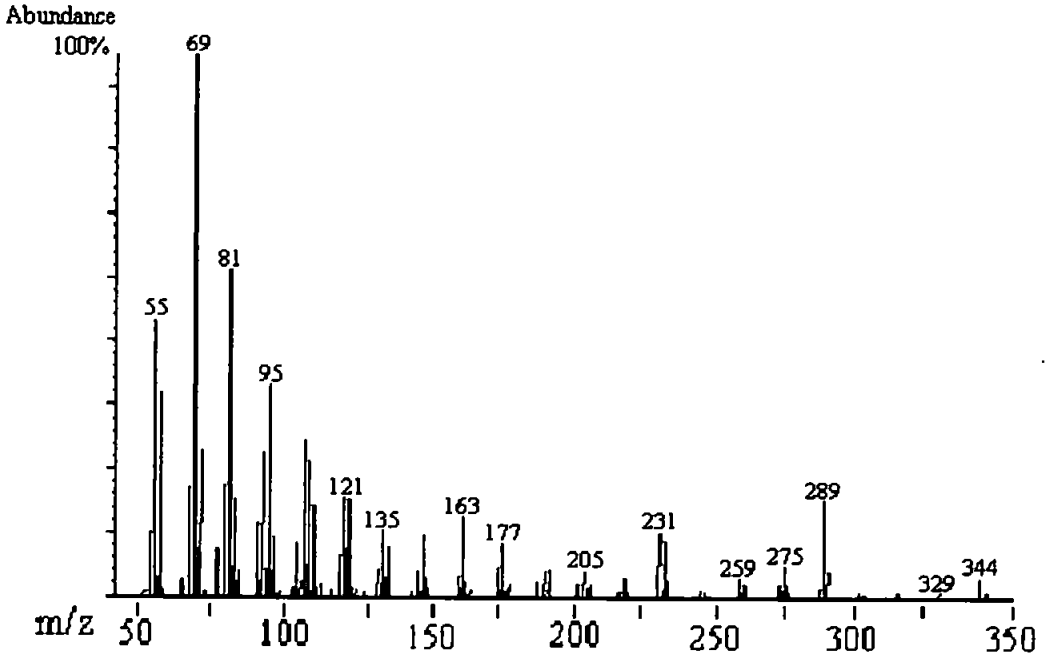


Figure 2.4c Mass spectrum of $C_{25:4}$ RI 2078 $_{(HP1)}$, 2108$_{(Carbowax)}$

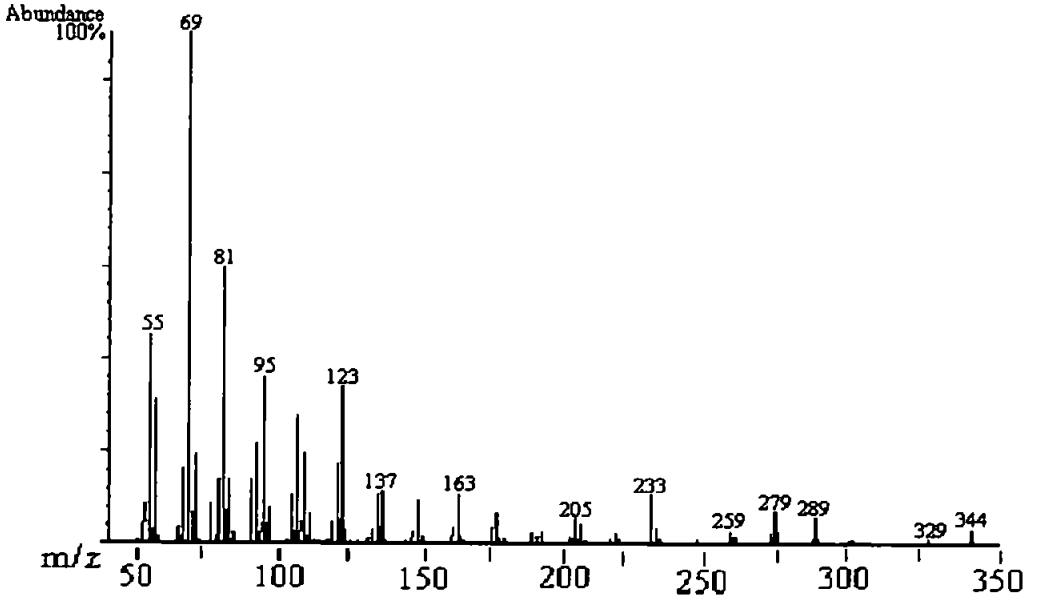


Figure 2.4d Mass spectrum of $C_{25:4}$ RI 2124$_{(HP1)}$, 2199$_{(Carbowax)}$

It can be observed that the mass spectra of the two trienes (RI 2044 & 2090) are similar and likewise for the two tetraenes (RI 2078 & 2124). Futhermore, the difference in RI between the two trienes (Δ RI = 46 $_{HP1}$) is identical to that of the two tetraenes (Δ RI = 46 $_{HP1}$). It is therefore reasonable to suggest that the two trienes and tetraenes are pairs of geometric (E/Z) isomers. Many reported sedimentary C_{25} HBIs share similar RI and mass spectra to those now identified in *Rhizosolenia setigera* for the first time (e.g. *cf* Barrick and Hedges, 1980; Porte *et al.*, 1990; reviewed by Rowland and Robson, 1990; Cooke, 1995).

2.3.4 Isolation and characterisation of previously unidentified C_{25} HBI alkenes from *Haslea ostrearia* and *Rhizosolenia setigera* cultures and exudate.

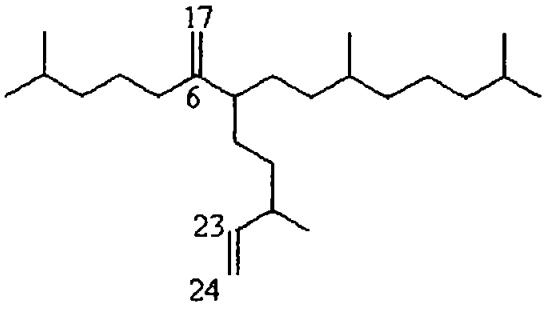
Three C_{25} HBI alkenes were isolated from the diatoms by column chromatography and characterised using NMR spectroscopy. Thus, $C_{25:2}$ (RI 2084 $_{HP1}$) was isolated from the THE of Soproma 34 (901 mg), Sodexal 18 (103 mg) and the exudate (2 mg). A $C_{25:3}$ (RI 2103_{HP1}) was isolated from the THE of BP003 (1000 mg) and another $C_{25:3}$ (2090_{HP1}) isolated from the TCE of a laboratory culture of *Rhizosolenia setigera* (197 mg) to yield < 1 to *ca* 60 mg of each compound. Table 2.5 shows the isolated yields, purity, and retention indices on three phases for each of the isolates.

Table 2.5 Retention Indices ($_{HP1,\ DB5,\ Carbowax}$) isolated yields and purity of the HBI alkenes isolated from algal pastes and exudate (*Haslea ostrearia* unless otherwise stated).

HBI	Source	Mass	Purity	Retention Indices		
				HP1	DB5	Carbowax
$C_{25:2}$	Exudate	0.3mg	64%	2084	2084	not detected
	Sodexal 18	1.5mg	57%	2084	2084	2084
	Soproma 34	3.1mg	< 99%	2084	2084	2080, 2084
$C_{25:3}$	BP003	63.1mg	< 99%	2103	2103	2124
$C_{25:3}$	*Rhizosolenia setigera*	0.5 mg	98%*	2090	not detected	2142

* contains 18% of $C_{25:3}$ (RI 2044 $_{HP1}$)

2.3.5 Characterisation of 2,10,14-trimethyl-6-methylene-7-(3'-methylpent-4-enyl)pentadecane [VII]



[VII]

The diene was shown to possess the $C_{25:0}$ carbon skeleton by co-elution of the fully hydrogenated $C_{25:2}$ with that of synthetic $C_{25:0}$ (Robson and Rowland, 1986). Diene [VII] (RI $2084_{HP1,\ DB5,\ Carbowax}$) did not co-elute with the previously identified $C_{25:2}$[I] (RI 2079_{HP1} 2080_{DB5}, $2062_{Carbowax}$), although the mass spectrum was very similar (Figure 2.7). ^{1}H and ^{13}C NMR spectroscopy was carried out on the 3.0 mg isolated from Soproma 34. The alkenic region (Figure 2.8) showed the characteristic ABMX spin system at δ=5.65 and 4.94 ppm due to the presence of a vinyl group at C23-C24. In addition, there were two broad singlets (δ= 4.67 and 4.72 ppm) with an integral of 2 with nearly identical chemical shifts to that of the methylenic protons, H17 in $C_{25:3}$, $C_{25:4}$, $C_{25:5}$ (Belt *et al.*, 1996). Further evidence for the methylenic double bond at C6-C17 was the total number of 4 allylic protons (δ= 1.82 ppm and 2.02 ppm; Table 2.6). This structure is further supported by analysis of the ^{13}C NMR data, presented in Table 2.7 where the presence of a monosubstituted double bond (δ= 144.89 (CH), 112.0 ppm (CH_2)) and a 1,1-disubstituted double bond (δ= 152 ppm (C), 133 (CH_2)) were established using the DEPT technique.

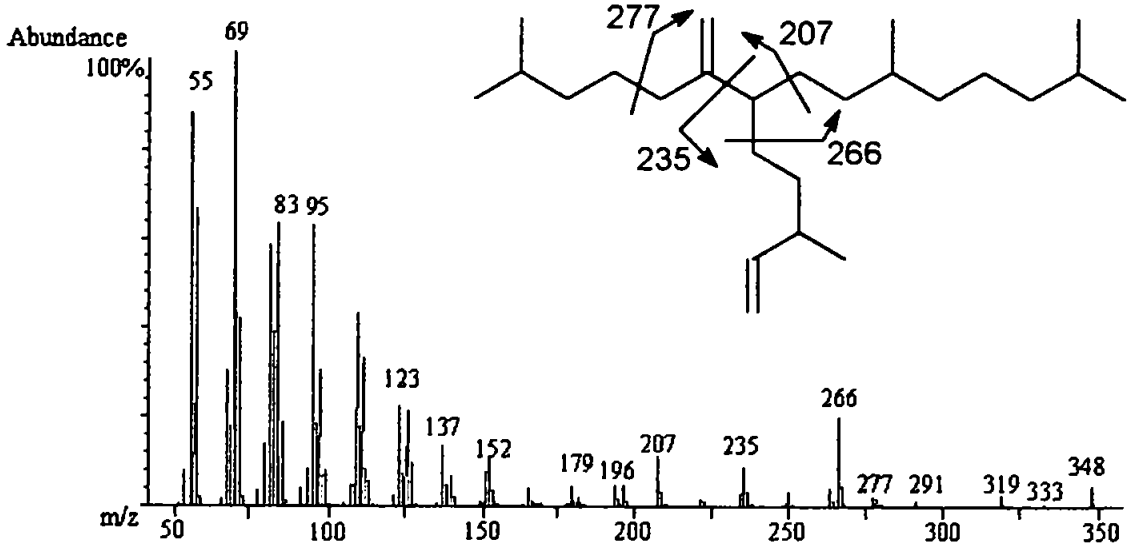


Figure 2.7 Mass spectrum and fragmentation pathway of $C_{25:2}$ RI 2084

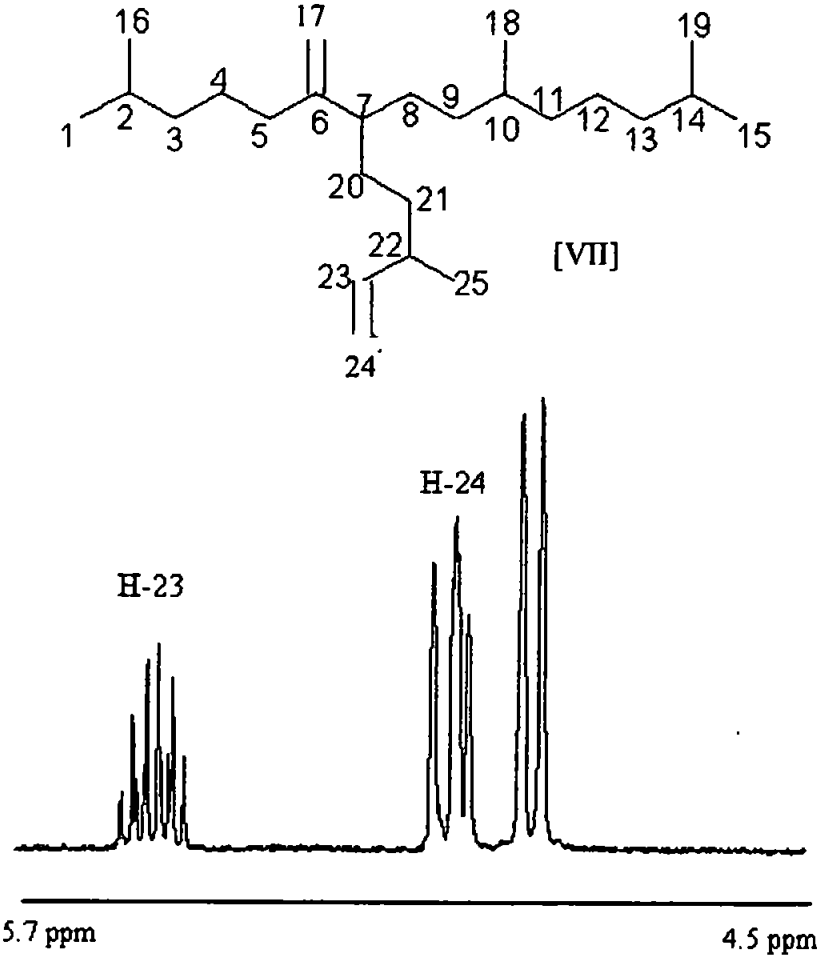


Figure 2.8 Partial alkenic ^{1}H spectrum of $C_{25:2}$ [VII] isolated from Soproma 34

Table 2.6 ^{1}H chemical shifts, multiplicities and integration for $C_{25:2}$ [VII]

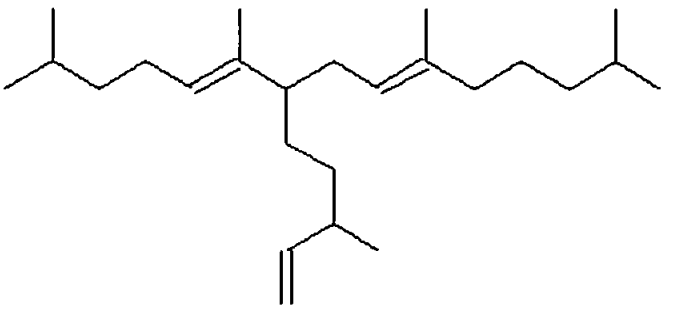
Chemical shift (ppm)	Assignment	Multiplicity (Coupling constant, Integration)
0.82	18	d (, J= 6.9Hz, 3H)
0.85, 0.86	1, 15,16,19	d (J= 6.5Hz, 12H)
0.94	25	d (6.8Hz, 3H)
1.03-1.60	2, 10, 14, 3, 4, 8, 9, 11, 12, 13, 20, 21	m (21H)
1.82	7, 5	m (3H)
2.03	22	m (1H)
4.67	17	br,s (1H))
4.72		br,s (1H)
4.90	24	m (2H)
5.64	23	ddd (J= 17.5, 10, 8 Hz, 1H)

Table 2.7 ^{13}C chemical shifts for [VII] isolated from Soproma 34

Chemical Shift	Carbon Group via DEPT	Assignment
19.8	CH_3	18
20.0, 20.4 *	CH_3	25
22.6, 22.7	CH_3	1,15,16,19
24.7	CH_2	
25.5	CH_2	
28.0	CH	2,14
29.7	CH_2	
31.4, 31.5 *	CH_2	
32.7 *	CH_2	
33.0	CH	10
34.4, 34.5 *	CH_2	
34.8	CH_2	
37.0	CH_2	
38.0	CH	22
39.0	CH_2	
39.3	CH_2	
47.2 *	CH	7
108.7	CH_2	17
112.2, 112.4 *	CH_2	24
144.9, 145.0 *	CH	23
152.5	C	6

* indicates the presence of diastereomers

2.3.6 Characterisation of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadeca-5,9-diene [VIII].



[VIII]

The triene, isolated from culture BP003 was again shown to possess the $C_{25:0}$ carbon skeleton by co-elution of the hydrogenated $C_{25:3}$ with that of the synthetic $C_{25:0}$ (Robson and Rowland, 1986). $C_{25:3}$[VIII] (RI $2103_{HP1,\ DB5}$, $2124_{Carbowax}$) did not co-elute with the previously identified $C_{25:3}$[II] (RI 2106_{HP1} $2108_{DB\text{-}5}$, $2142,2146_{Carbowax}$). The mass spectrum of $C_{25:3}$ [VIII] (Figure 2.9) shows subtle differences to the mass spectrum produced by $C_{25:3}$ [II] (Cooke, 1995). Notably, the fragment ions 233 and 261 are less abundant for [VIII] (6% and 9% respectively) than for [II] (24% and 29%). It may also be observed that the ratio of the 149/151 ion for [II] is 1.2:1 which is the same ratio observed for the fragment ions 135/137 and 205/207 whilst for [VIII] the ratio for these three pairs of ions is 1:5.

^{1}H, ^{13}C and 2D NMR spectroscopy carried out on 20.0 mg of the isolate confirmed the structure to be that of [VIII]. The alkenic region of the ^{1}H spectrum (Figure 2.10; Table 2.8) shows the characteristic ABMX spin system (δ=5.65 and 4.94 ppm) due to the presence of a vinyl group at C23-C24 and a multiplet with an integral of two (δ= 5.02 ppm) produced by two trisubstituted double bonds at C5-C6 and C9-C10. These positions were confirmed by 2D analysis where a long range coupling ($^{3}J_{CH}$) was observed between C5 and H17, and C9 and H18 (Table 2.9).

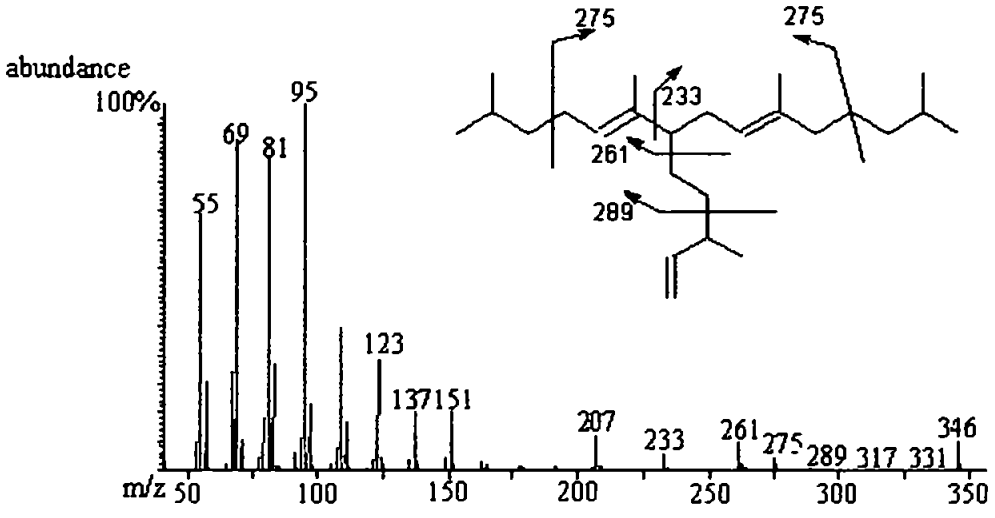


Figure 2.9 Mass Spectrum and fragmentation pathway of $C_{25:3}$ (VIII RI $2103_{(HP-1)}$)

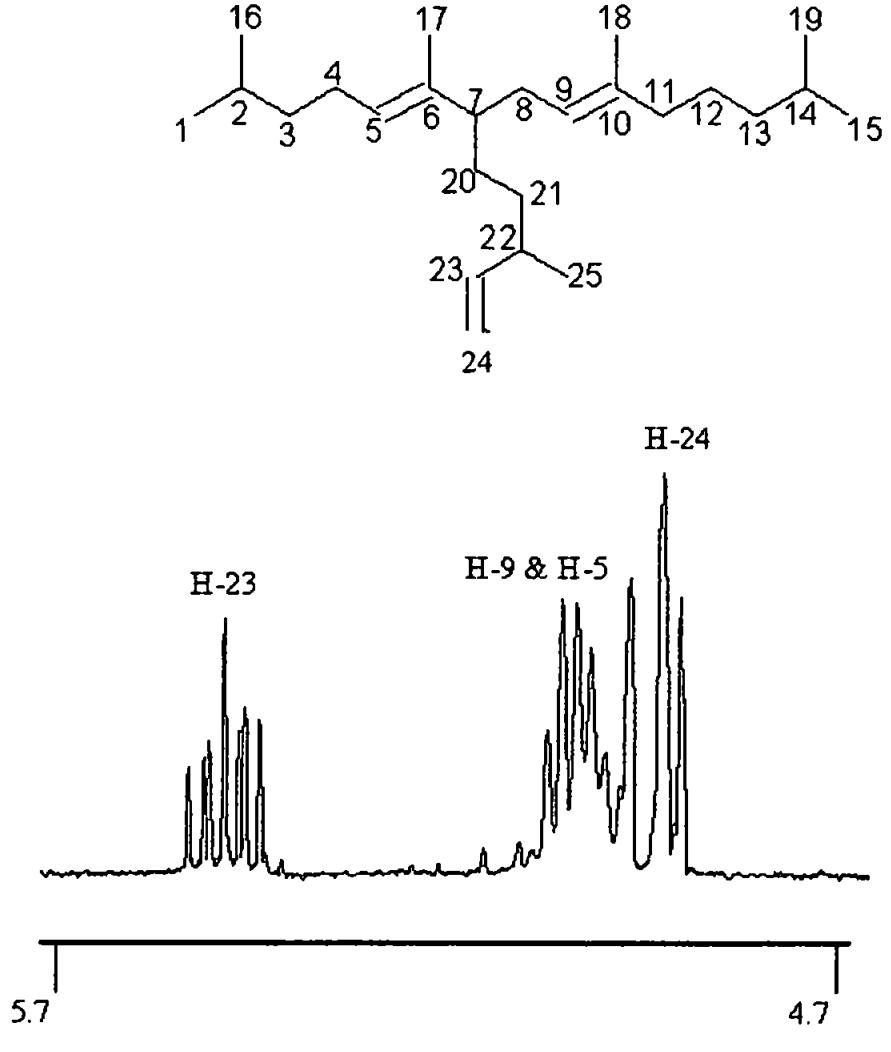


Figure 2.10 Partial alkenic ^{1}H NMR spectrum of $C_{25:3}$ [VIII] isolated from culture BP003

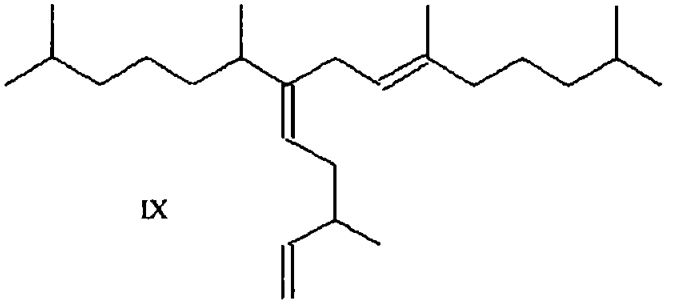
Table 2.8 ^{1}H chemical shifts, multiplicities and integration for $C_{25:3}$ [VIII]

Chemical shift (ppm)	Assignment	Multiplicity (Coupling constant, Integration)
0.84	15, 19	d (J = 6.6Hz, 6H)
0.85	1, 16	d (J = 6.6Hz, 6H)
0.93	25	d (J = 6.6Hz, 3H)
1.04-1.38, 1.47-1.50	2, 14, 12, 13, 20, 21	m (12H)
1.43	17	s (3H)
1.54	18	s (3H)
1.86-2.08	4, 8, 11, 7, 22	m (8H)
4.85	24	m (2H)
4.94		
5.04	5, 9	2 x t (2H)
5.67	23	ddd (J = 7, 10.5, 17.5 Hz, 1H)

Table 2.9 ^{13}C and 2D NMR data for $C_{25:3}$ [VIII] isolated from culture BP003

Chemical Shift (ppm)	Carbon group via DEPT	Long range coupling	Assignment
11.8	CH_3	$^{1}J_{CH}$ (H17), $^{3}J_{CH}$ (H7)	17
16.0	CH_3	$^{1}J_{CH}$ (H18)	18
19.9	CH_3	$^{1}J_{CH}$ (H25)	25
22.6	CH_3	$^{1}J_{CH}$ (H1,15)	1,15
22.7	CH_3	$^{1}J_{CH}$ (H16,19)	16,19
25.5	CH_2	$^{2}J_{CH}$ (H3) $^{1}J_{CH}$ (H12)	12
25.7	CH_2	$^{2}J_{CH}$ (H11) $^{1}J_{CH}$ (H4)	4
27.5	CH		14
27.9	CH		2
30.4	CH_2	$^{1}J_{CH}$ (H20)	20
32.2	CH_2	$^{1}J_{CH}$ (H8)	8
34.5	CH_2	$^{1}J_{CH}$ (H21) $^{2}J_{CH}$ (H20)	21
37.8	CH	$^{1}J_{CH}$ (H22), $^{2}J_{CH}$ (H25)	22
38.6	CH_2	$^{1}J_{CH}$(H13), $^{2}J_{CH}$ (H15,19)	13
39.1	CH_2	$^{1}J_{CH}$ (H3), $^{2}J_{CH}$ (H1,16)	3
40.0	CH_2	$^{1}J_{CH}$ (H11), $^{2}J_{CH}$ (H18)	11
49.5	CH	$^{1}J_{CH}$ (H7), $^{2}J_{CH}$ (H17)	7
112.0	CH_2	$^{1}J_{CH}$ (H24)	24
123.3	CH	$^{1}J_{CH}$ (H9), $^{2}J_{CH}$ (H18)	9
126.2	CH	$^{1}J_{CH}$ (H5), $^{2}J_{CH}$ (H17)	5
135.2	C	$^{2}J_{CH}$ (H18)	10
136.2	C	$^{2}J_{CH}$ (H17)	6
145.2	CH	$^{1}J_{CH}$ (H23)	23

2.3.7 Characterisation of 3, 9,13 trimethyl-6-(1,5 dimethylhexyl)tetradec-1,5,8 –triene [IX]



IX

A mixture of two $C_{25:3}$ HBIs (0.5 mg) was isolated from the total chloroform extract (197 mg) of a laboratory culture of *Rhizosolenia setigera*; 79% of this mixture was the $C_{25:3}$ (IX; RI 2090_{HP1}) while 18% was another $C_{25:3}$ (RI 2044_{HP1}). The triene (RI 2044_{HP1}) mass spectrum was very similar to that of [IX] (Figure 2.6) and is believed to be a Z isomer of [IX]. The $C_{25:3}$ [IX] was shown to possess the $C_{25:0}$ carbon skeleton by co-elution of the hydrogenated $C_{25:3}$ with that of the synthetic $C_{25:0}$ (Robson and Rowland, 1986). ^{1}H NMR data presented in Table 2.9 shows the presence of the vinyl group at C1-2 (δ = 5.73 ppm) and two tri-substituted double bonds at C5-C6 and C8-C9 (δ = 5.09 ppm). Examination of the ^{1}H spectrum upfield from that of the alkenic protons shows two distinct multiplets with an integral of 3:5 (δ=1.0-2.7 ppm; Figure 2.11). This is different from what would be expected i.e. 2:6, due to the protons C4 being diastereotopic and are therefore magnetically different and hence the two resonances at δ=2.61 and δ=2.12 ppm. Since this work more of [IX] has been isolated and the structure confirmed by ^{13}C and 2D NMR (Allard, 1999; personal communication).

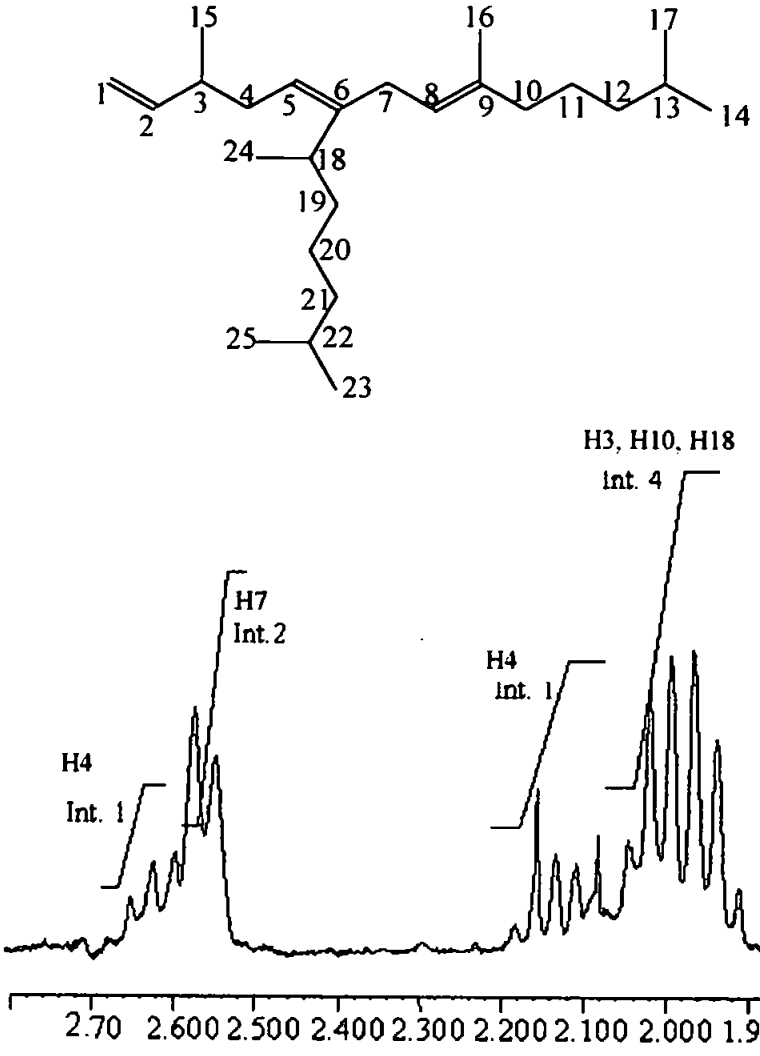


Figure 2.11 Partial ^1H spectrum of $C_{25:3}$ HBI triene IX, isolated from *Rhizosolenia setigera*

Table 2.10 ^1H chemical shifts, multiplicities and integration for IX

Chemical shift (ppm)	Assignment	Multiplicity (Coupling constant, Intergration)
0.83	14, 17, 23, 25	d (J=6.6Hz, 6H)
0.85		d (J=6.6Hz, 6H)
0.93	24	d (J=6.9Hz, 3H)
0.96	15	d (J=6.6Hz, 3H)
1.51	16	s (3H)
1.90-2.04	3, 10, 18	m (4H)
2.08-2.15	4	m (1H)
2.55	7	br, d (J=6.9Hz, 2H)
2.59-2.65	4	m (1H)
4.86	1	m (1H)
4.95		m (1H)
5.09	5, 8	m (2H)
5.73	2	ddd (J= 8 Hz, 10.5 Hz, 17.5 Hz, 1H)

2.3.8 Characterisation of a $C_{25:2}$ [X] and a $C_{25:1}$ [XI] by partial hydrogenation of $C_{25:3}$ [II]

The previously characterised $C_{25:3}$ II (Belt *et al.*, 1996) was partially hydrogenated (1 hr) to produce a reaction mixture which was shown by GC to contain four components, a $C_{25:2}$ (RI 2144 $_{HP1}$, 2125 $_{Carbowax}$; 69 %), a $C_{25:1}$ (RI 2159 $_{HP1}$, 2096 $_{Carbowax}$; 12 %), $C_{25:0}$ (RI 2110 $_{HP1}$, 2032 $_{Carbowax}$; 4 %) and the starting material $C_{25:3}$ (RI 2105 $_{HP1}$, RI 2142, 2146 $_{Carbowax}$; 15%). Examination of the reaction mixture by ^{1}H NMR showed that the vinyl group at C23-24 (δ =5.73 and 4.90 ppm) was absent whilst the methylenic double bond at C17 (δ=4.70, 4.74 ppm) and the trisubstituted double bond at C9-10 remained (δ=5.05 ppm), thereby confirming the structure of $C_{25:2}$ to be X. Further hydrogenation (2 hr) produced a reaction mixture, which was now shown by GC to contain three of the four above components, $C_{25:2}$ (30 %), $C_{25:1}$ (45 %) and $C_{25:0}$ (25 %). Examination of the reaction mixture by ^{1}H NMR showed that the integral of the methylenic double bond at C17 had decreased with respect to the integral of the trisubstituted double bond at C9-10 (H17, 2→ 0.8; H9, 1 → 1). This thereby suggests that the structure of the $C_{25:1}$ is XI (Figure 2.12). Neither the $C_{25:2}$ nor the $C_{25:1}$ appear to share similar RI and mass spectra with those reported previously in either sediments or biota (Figure 2.13).

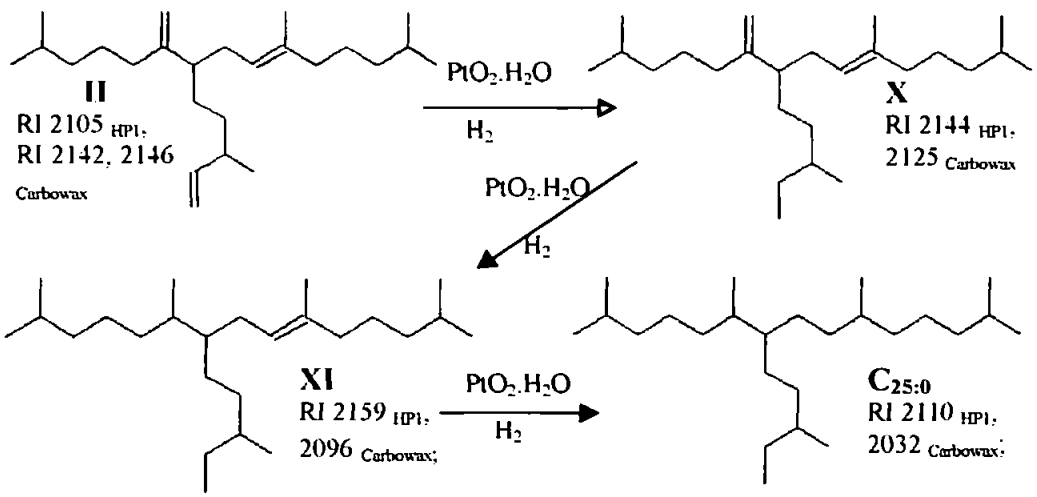


Figure 2.12 Partial hydrogenation of $C_{25:3}$ [II] to produce a $C_{25:2}$ [IX] and a $C_{25:1}$ [X]

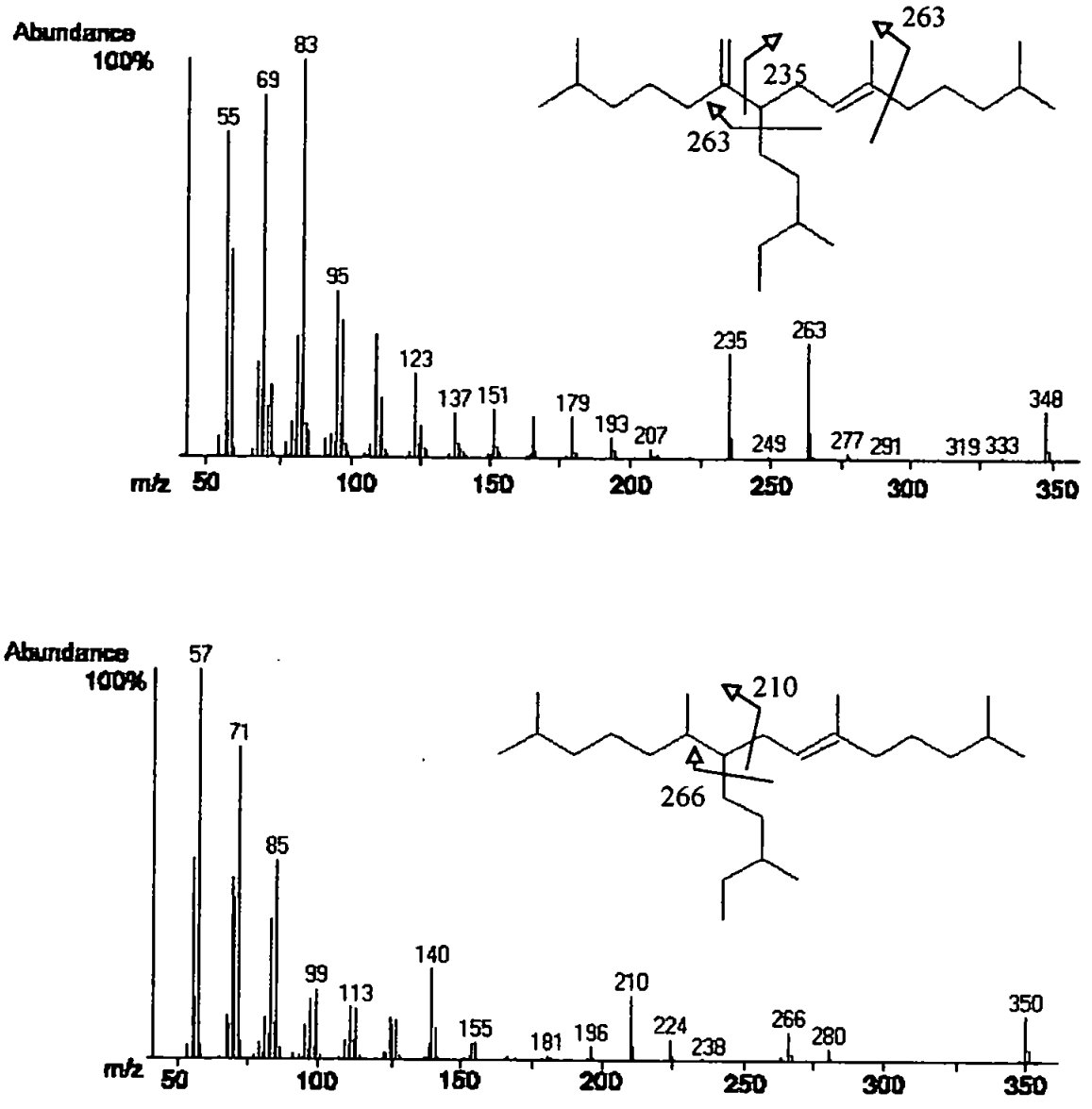


Figure 2.13 Mass Spectrum and fragmentation pathway of

a: $C_{25:2}$ (X RI 2144 $_{HP1}$, 2125 $_{Carbowax}$)

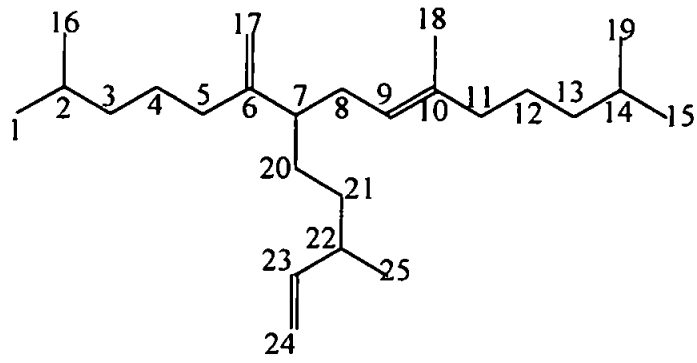
b: $C_{25:1}$ (XI RI 2159 $_{HP1}$, 2096 $_{Carbowax}$)

2.3.9 Stereoisomerism of HBIs

It has already been discussed that HBI alkenes isolated from *Haslea ostrearia* by Belt *et al.* (1996) were shown by ^{1}H and ^{13}C NMR spectroscopy to exist as diastereoisomers. Examination of the diagnostic H-23 resonance in the crude THE of the algal pastes by ^{1}H NMR spectroscopy showed that in seven of the pastes, the HBI alkenes existed as diastereomers, while in three, (Sodexal 18, Soproma 46 and BP003) the presence of a single isomer or racemate was observed. (Table 2.3). The ^{13}C chemical shifts for the $C_{25:3}$ [II] isolated from Soproma 34, Sodexal 18 and Soproma 46 are presented in Table 2.11. The 'doubling-up' of nine of the ^{13}C resonances can clearly be observed for [II (a)] isolated from Soproma 34. This 'doubling-up' effect was absent for the [II (b)] and [II (c)] isolated from Soproma 46 and Sodexal 18. Hence, it appears from comparison of the ^{13}C chemical shift data that [II (c)] from Sodexal 18 is a diastereomer of [II (b)] from Soproma 46.

For the first time, the diastereomers of [II (a)] were successfully separated by gas-chromatography using 'Carbowax' stationary phase. Thereby [II (a)], isolated from Soproma 34 gave a doublet with a RI of 2142 and 2146 (Figure 2.14a). While the proposed single isomer of [II (b)], isolated from Soproma 46 gave a single peak which co-eluted with the first peak of the doublet (RI 2142; Figure 2.14b) and [II (c)] from Sodexal 18, co-eluted with the second peak (RI 2146; Figure 2.14c). This verifies that [II (c)] from Sodexal 18 is a diastereoisomer of the [II (b)] from Soproma 46.

For [II (a)], isolated from Soproma 34 to exist as diastereoisomers, both chiral centres (C7 and C22) cannot be in a fixed configuration, consequently the configuration at one or both chiral centres is racemic. Similarly, [II (b)] from Soproma 46 must have a different configuration at one of the chiral centres to that of the [II (c)] isolated from Sodexal 18.



[II]

Table 2.11 ^{13}C chemical shifts (δ/ppm; $CDCl_3$) for [II] isolated from Soproma 34, Sodexal 18 and Soproma 46.

δ = [II (a)], Soproma 34	δ = [II, (b)], Soproma 46	δ = [II, (c)], Sodexal 18	Assignment
16.1	16.1	16.1	18
20.4/20.0	20.4	20.0	25
22.6	22.6	22.6	1,15,16,19
25.6	25.6	25.6	4
25.7	25.7	25.7	12
27.9	27.9	27.9	2
28.0	28.0	28.0	14
30.9/30.8	30.8	30.9	20
32.9/32.8	32.9	32.8	8
33.7/33.6	33.6	33.7	5
34.4	34.4	34.4	21
38.0/37.9	38.0	37.9	22
38.6	38.6	38.6	13
39.0	39.0	39.0	3
40.0	40.0	40.0	11
46.8	46.8	46.8	7
108.6/108.6	108.6	108.6	24
112.3/112.2	112.3	112.2	17
123.1	123.1	123.1	9
135.6	135.6	135.6	10
145.0/144.9	144.9	145.0	23
152.6/152.5	152.5	152.6	6

Isolation of sufficient amounts of the pure triene [II] from Sodexal 18, Soproma 46 and Soproma 34 cultures permitted a more detailed configurational analysis to be made. Additionally, the configuration of the $C_{25:2}$ [VII] was also investigated.

Seven of the ^{13}C resonances (Table 2.7) 'double-up' for [VII (a)], isolated from Soproma 34. In addition, GC on 'Carbowax' stationary phase produced a doublet (RI 2080, 2084) while $C_{25:2}$ [II (b)], isolated from Sodexal 18 gave a single peak which co-eluted with the second peak of the doublet (RI 2084). Similarly to the arguments evoked for the $C_{25:3}$ [II], for the $C_{25:2}$ [VII] to exist as diastereomers not all of the three chiral centres (C7, C10 and C22) can have a fixed configuration. To investigate this further, a sufficient quantity (3.1mg) of the $C_{25:2}$ [VII (a)] was obtained from Soproma 34.

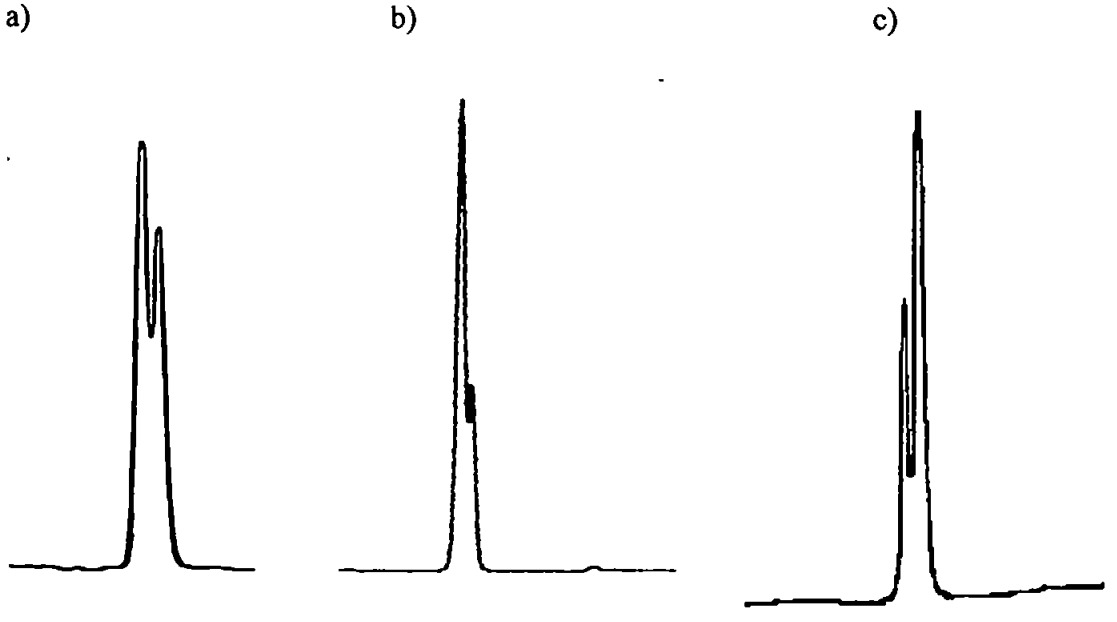


Figure 2.14: Partial GC chromatograms of [II], (30 m x 0.25 mm Carbowax fused silica GC column, 50-200°C at 6°C per min)
a) [II (a)], isolated from Soproma 34
b) [II (a)] and [II (b)] isolated from Soproma 46.
c) [II (a)] and [II (c)] isolated from Sodexal 18.

An established method for determining the configurations of chiral acyclic isoprenoid alkenes is *via* the oxidation to a series of branched acids which retain the configuration of the alkanes (Brooks *et al.*, 1977; Patience *et al.*, 1978). Preparation of (-) menthyl esters

produces diastereomers which can be separated and compared to authentic standards by gas chromatography (Ackman *et al.*, 1972). The absolute configuration at C22 for $C_{25:3}$ isolated from Soproma 34 and Soproma 46 and that of C10 for the $C_{25:2}$ from Soproma 34 was determined using an adaptation of this method, where the acids were converted to methyl esters and then examined by enantioselective GC on cyclodextrin stationary phases (König *et al.*, 1988, Armstrong, 1991). A schematic of the oxidation process and the desired products is presented in Figure 2.15.

Oxidation of the $C_{25:0}$ alkane (2mg), produced by hydrogenating the respective trienes yielded the low molecular acids; 3-methylpentanoic acid (3-MP; *ca* 1% of the total oxidation products) and 4-methyl hexanoic acid (4-MH; *ca* 1% of the total oxidation products). Both these acids retain the configuration originally at C22 in the triene. A racemic mixture of 3-MP, available commerically and 4-MH, synthesised by the Arndt-Eistert chain extension of 3-MP, was successfully separated as methyl esters using octakis (6-O-methyl-2,3-di-O-pentyl)-γ-cyclodextrin as the enantioselective stationary phase (Figure 2.16a). Co-chromatography of 3RS-MP with the S isomer synthesised by chain extending commercially available 2S-methyl butanoic acid established that the elution order as R followed by S (Figure 2.16b). Examination of the oxidised products from the alkane derived from the $C_{25:3}$ isolated from Soproma 34, yielded two peaks for 3-MP and 4-MH (Figure 2.17) which co-eluted with the authentic standards. It could be concluded therefore that the configuration at C22 for the $C_{25:3}$ [II (a)] isolated from Soproma 34 was a mixture of R and S. By analogy this is also true for $C_{25:2}$ [VII (a)], which was also isolated from Soproma 34 and shown to exist as diastereomers. However, 3-MP and 4-MH produced by oxidation of the alkane from $C_{25:3}$ [II (b)], isolated from Soproma 46 each gave a single peak (Figure 2.18a).

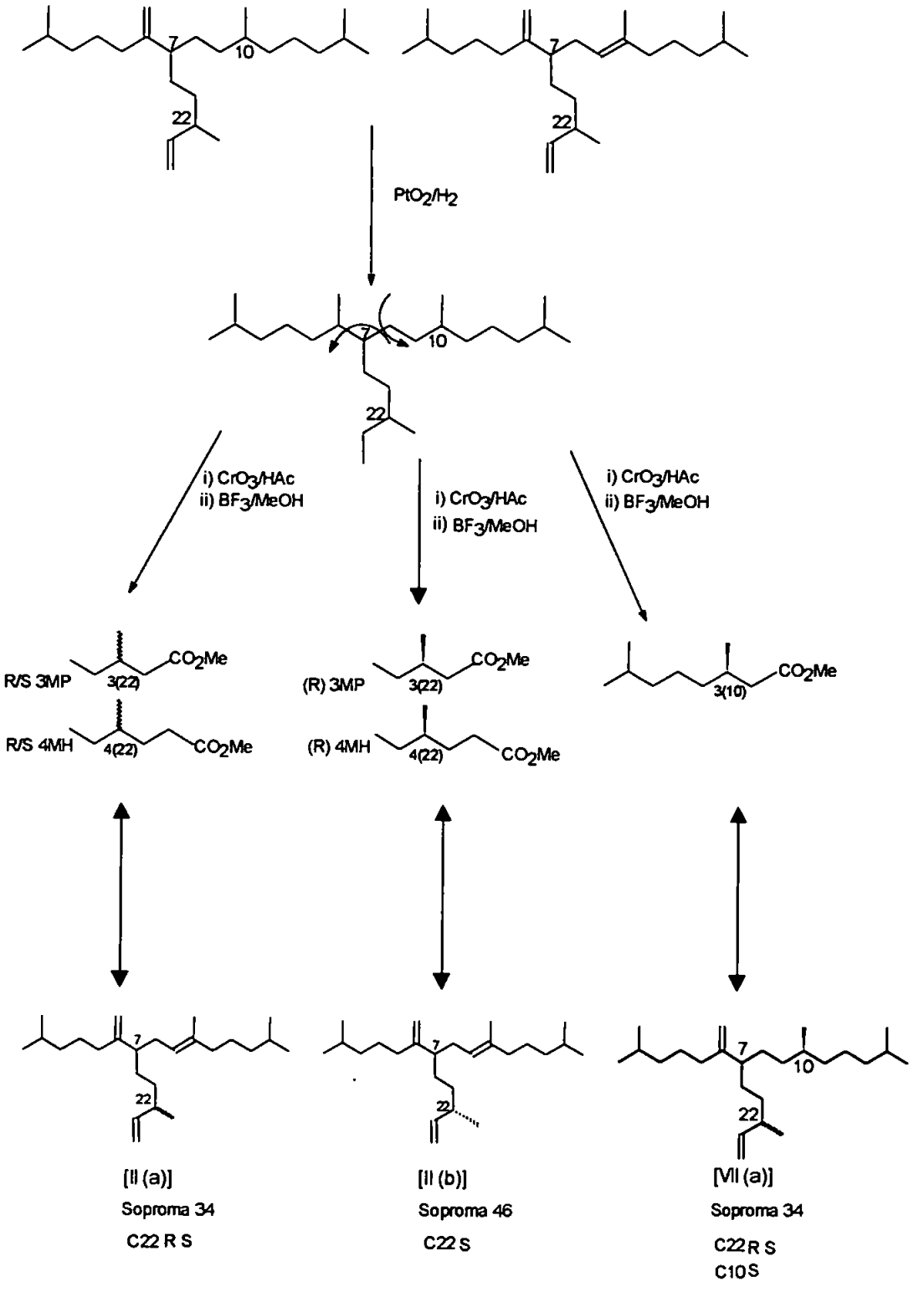


Figure 2.15 Oxidation scheme for $C_{25:3}$ [II (a)], [II (b)] and $C_{25:2}$ [VII (a)].

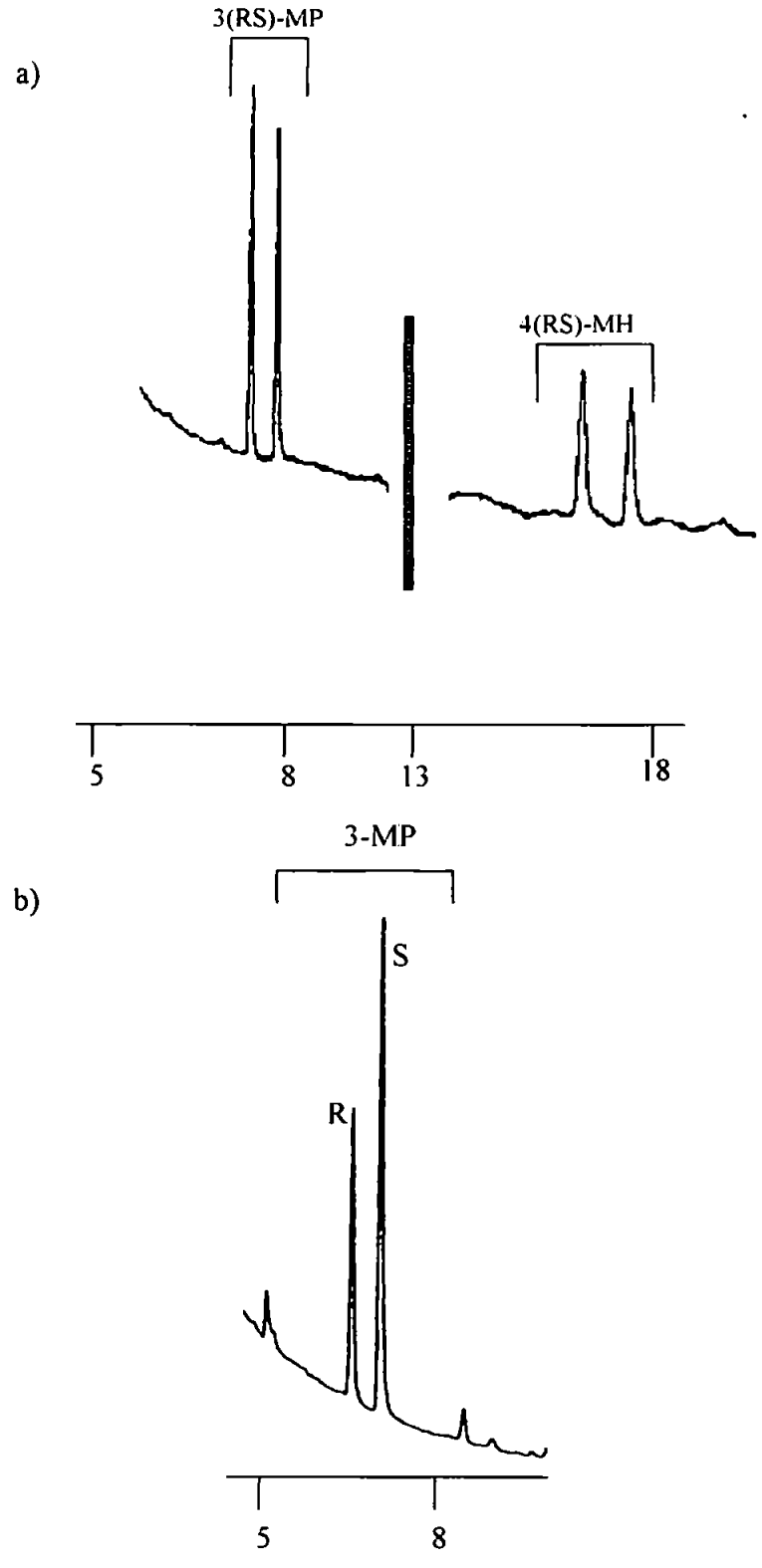


Figure 2.16 Partial GC chromatogram a) Enantiomeric separatioon of 3(RS)-MP and 4(RS)-MH and b) Co-injection of 3(RS)-MP with 3(S)-MP (Octakis (6-O-methyl-2, 3-di-O-pentyl)-γ-cyclodextrin (50% in polysiloxane OV 1701, w/w) at 50°C isothermal. Hydrogen carrier gas at 0.5 bar inlet pressure.

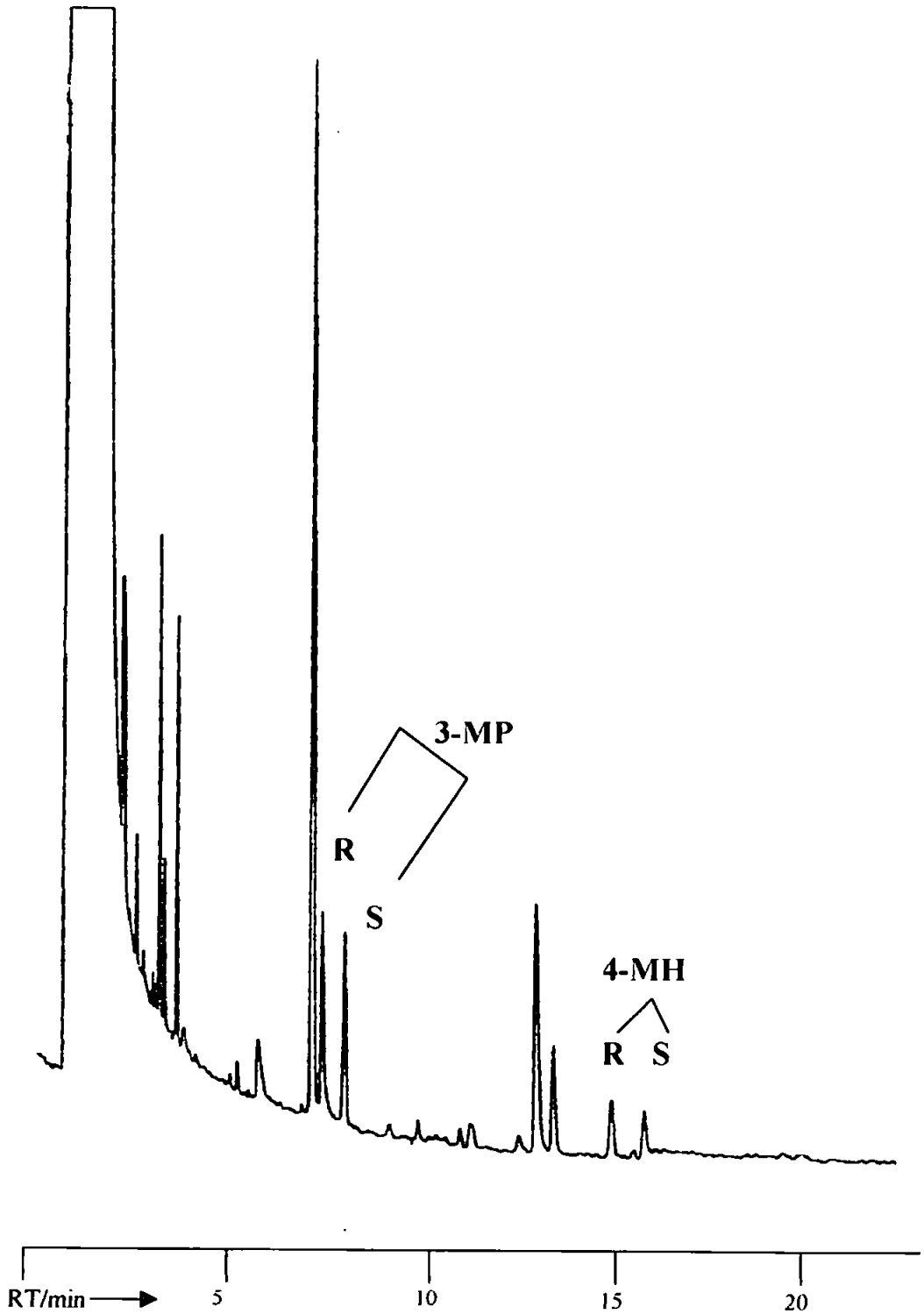


Figure 2.17 Partial GC chromatogram a) Oxidation products of $C_{25:3}$ [II (a)], isolated from Soproma 34 (Octakis (6-O-methyl-2,3-di-O-pentyl)-γ-cyclodextrin (50% in polysiloxane OV 1701, w/w) at 50°C, isothermal. hydrogen carrier gas at 0.5 bar inlet pressure.

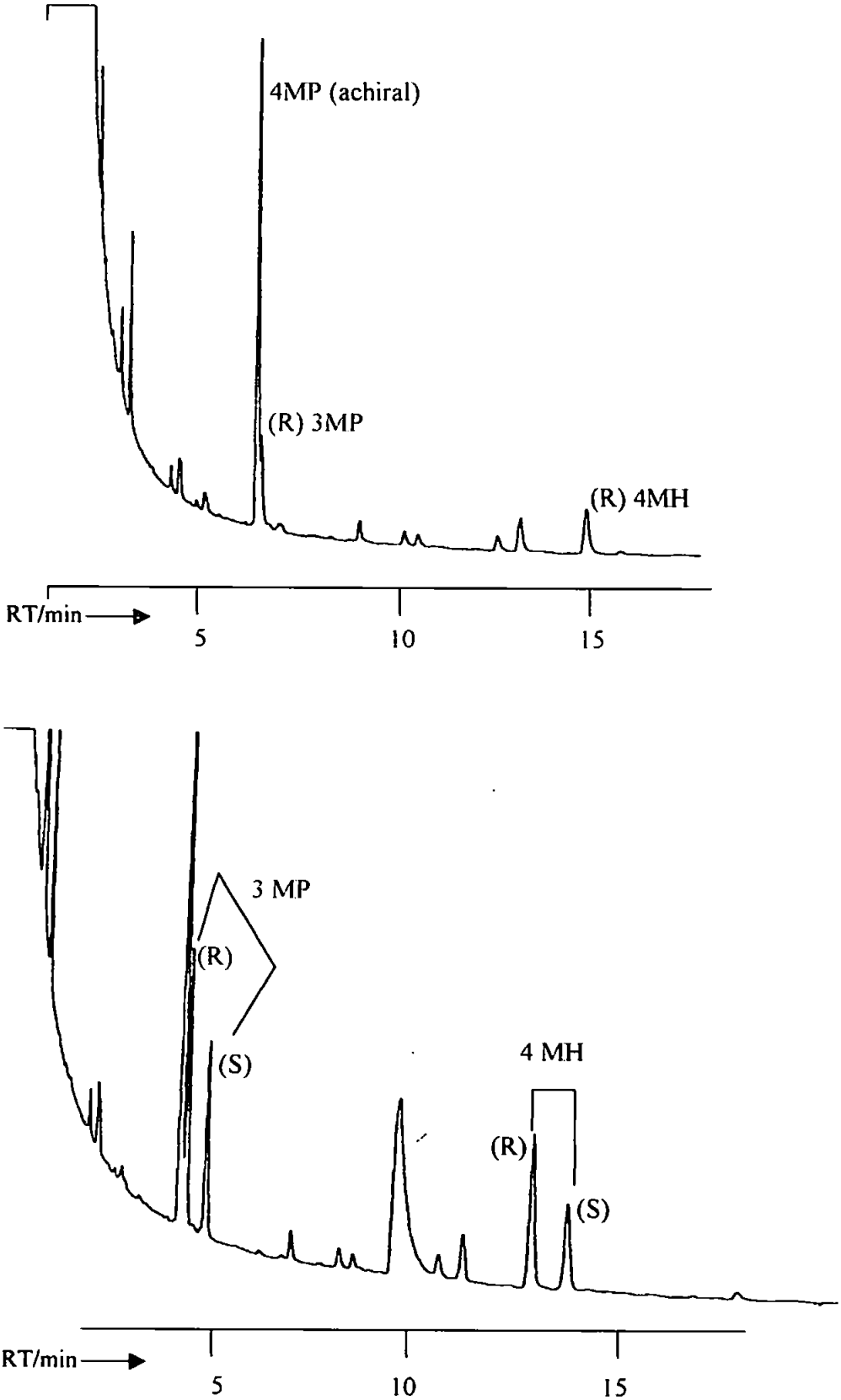


Figure 2.18 Partial GC chromatogram a) Oxidation products of $C_{25:3}$ [II (b)], isolated from Soproma 46 and b) Co-injection of the R/S standards with the oxidation products of [II (b)] (Octakis (6-O-methyl-2,3-di-O-pentyl)-γ-cyclodextrin (50% in polysiloxane OV 1701, w/w) at 50°C, isothermal. hydrogen carrier gas at 0.5 bar inlet pressure

Co-injection of these acids, as methyl esters with that of the racemic 3-MP and 4-MH standards showed a clear enhancement of the first peak (Figure 2.18b). Therefore it can be concluded that the configuration of C22 for the respective acids is R, hence it is S for the $C_{25:3}$ [II (b)] isolated from Soproma 46.

The oxidation method was also applied to the $C_{25:2}$ [VII (a)] isolated from Soproma 34 to obtain the configuration at C10. The acid of interest is 3,7 Dimethyloctanoic acid (DMO) where the configuration originally at C10 is preserved in position 3 of DMO; 3 RS-DMO, obtained by hydrogenation of commercial 3RS-citronellic acid (3RS, 7-dimethyloct-6-enoic acid) was successfully separated in two peaks on a chiral column using 2,6-dimethyl-3-pentyl-β-cyclodextrin as the stationary phase (Figure 2.19a). The elution order of the acid was R before S. This was established by co-chromatography of the RS mixture with 3R-DMO, obtained from hydrogenation of commercial 3R-citronellic acid (Figure 2.19b). Chiral GC of the products formed upon oxidising $C_{25:2}$ [VII] gave pre-dominantly a single peak for 3,7 DMO (*ca* 0.5% of the total oxidised products; Figure 2.20a). Co-elution with the RS standard showed enhancement of the second peak, the S isomer (Figure 2.20b). Therefore it can be concluded that the configuration at C10 is predominantly S.

The remaining chiral centre in [II] and [VII] is at C7, which cannot be determined by the foregoing oxidative approach. However, this could be delimited by examination of the [II (a)] sample, where C22 is now known to be a mixture of R and S, by ^{13}C NMR spectroscopy, using chiral shift reagents (CSR) in conjunction with soluble silver β-diketonate complexes (Wenzel *et al.*, 1980).

The addition of a CSR effectively converts all enantiomers into diastereoisomers which are theoretically distinguishable by ^{13}C NMR. For example, if the chiral centre at C7 is epimeric for 22RS $C_{25:3}$ it will exist as two pairs of enantiomers. The addition of the CSR will convert each individual enantiomer into a unique diastereoisomer. Therefore, up to four resonances will be observed for each carbon.

a)

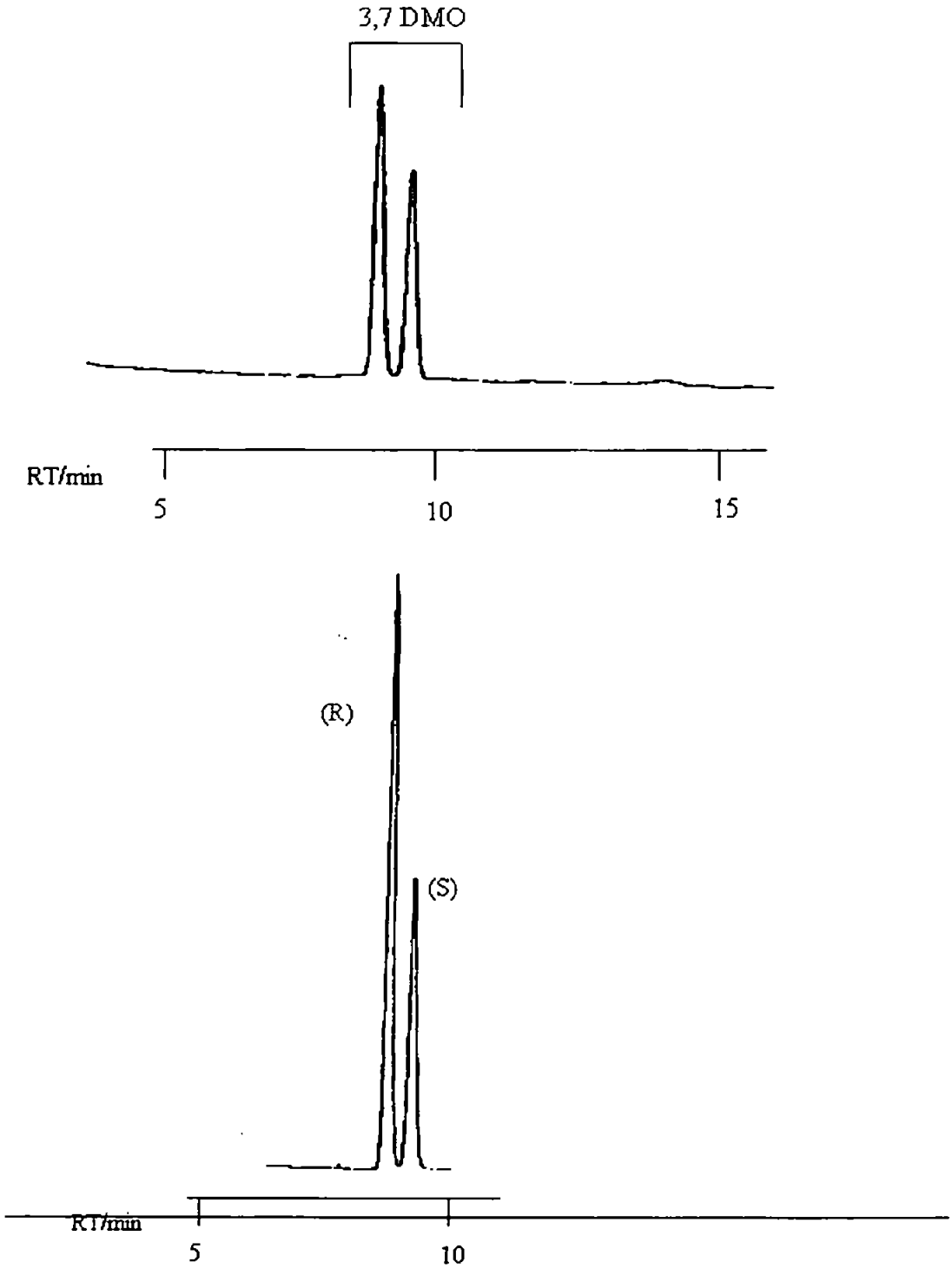


Figure 2.19 Partial GC chromatogram of a) 3,7 R/S Dimethyloctanoic acid (methyl ester) and b) Co-injection of 3,7 R/S DMO with 3,7 R DMO. (2,6-dimethyl-3-pentyl-β-cyclodextrin, Hydrogen carrier, 0.5bar inlet pressure, 95°C)

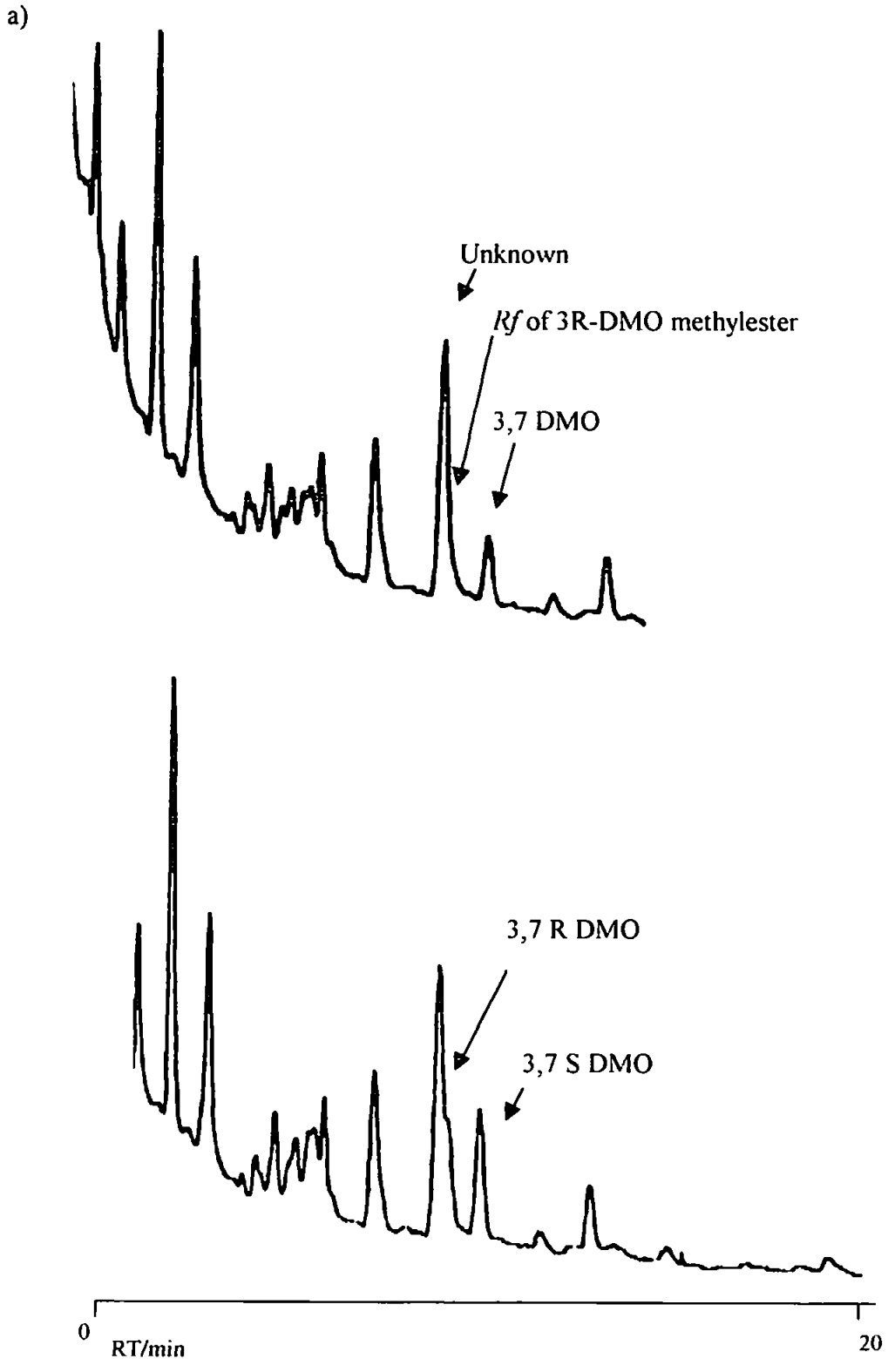


Figure 2.20 Partial GC chromatogram a) Oxidation products of $C_{25:2}$ [VII (a)], isolated from Soproma 34 and b) Co-injection of the R/S standard of 3,7 DMO with the oxidation products of [VII (a)] (2,6-dimethyl-3-pentyl-β-cyclodextrin, Hydrogen carrier, 0.5bar inlet pressure, 95°C)

Similarly, if the configuration at C7 is fixed for the 22RS $C_{25:3}$, no further splitting of the ^{13}C resonances would be observed upon the addition of a CSR since only two diastereoisomers would be present. This is summarised in Table 2.12. However, CSRs do not interact with HBI alkenes directly due to lack of functionality in the single alkene, so it was necessary to use a silver β-diketonate complex as an intermediate bridging agent (Wenzel *et al.*, 1982).

Table 2.12 Representative combinations of configurations at C7 where C22 is known to be R* or RS respectively.

Option	Absolute configuration	NMR distinguishable isomers	Isomers + CSR [R]	NMR distinguishable isomers
Option 1	7R 22R	2	[R]RR	2
	7S 22R		[R]SR	
Option 2	7R 22R	2	[R]RR	4
	7S 22R		[R]SR	
	7R 22S		[R]RS	
	7S 22S		[R]SS	

* or S

The mechanism of interaction between the silver [I] complex, CSR and substrate is unclear although it is known that the addition of a silver(I) β-diketonate to a lanthanide (III) tris β-diketonate (CSR) in solution leads to the formation of a lanthanide (III) tetrakis β-diketonate anion to which the silver is ion paired and the substrate binds to the silver in this pair (Eqn. 1&2; Wenzel and Sievers 1980).

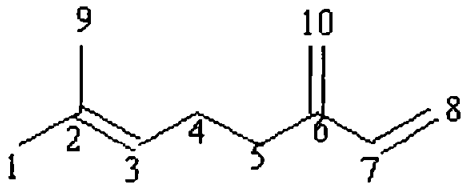
$Ln(\beta\text{-dik})_3 + Ag(\beta\text{-dik}) \rightleftharpoons Ag(Ln(\beta\text{-dik})_4]$ Eqn. 1

$Ag[Ln(\beta\text{-dik})_4] + Substrate \rightleftharpoons (Substrate)Ag[Ln(\beta\text{-dik})_4]$ Eqn. 2

Owing to the existence of at least two equilibria, the use of shift reagents does not follow a standard procedure. Therefore it was necessary to validate the method using two model compounds, myrcene and 3-methyl-1-pentene.

All the individual ^{13}C resonances of myrcene [XII], which contains mono, di- and tri-substitiuted alkene moieties of (*cf* (II)), were shifted ($\Delta\ \delta$ = -11.2-1.3 ppm, Table 2.13) upon addition of praseodymium tris[3-heptafluoropropylhydroxymethylene) (+)-camphorate/ silver (I) 6,6,7,7,8,8,-heptafluoro-2,2-dimethyl-3,5-octanedionate ($Pr(hfbc)_3$)/ Ag(fod) to a solution of myrcene in $CDCl_3$. This verified that co-ordination of the shift reagent to all three double bonds *via* the silver β-diketonate had taken place. In the absence of Ag(fod), no resonance shifts were observed.

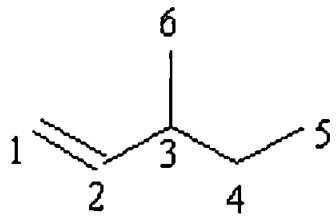
Upon the addition of $Pr(hfbc)_3$/Ag(fod) to 3RS-methylpent-1-ene [XIII] (0.24M:0.08M), all the ^{13}C resonances shifted upfield ($\Delta\ \delta$ =16.5-0.8 ppm; Table 2.14). Furthermore, the influence of a chiral shift reagent resulted in the spectroscopic separation of the two enantiomers of 3-MP ($\Delta\ \delta$ (RS) = 0.1 ppm). Thus, both the dynamic co-ordination of the shift reagent to a tri-unsaturated alkene and separation of a pair of alkene enantiomers was verified.



[XII]

Table 2.13 ^{13}C chemical shifts (δ/ppm) of 0.1M myrcene and 0.1M myrcene + $Pr(hfbc)_3$/Ag(fod)

^{13}C Assignment	Mycrene δ/ppm	Mycrene + $Pr(hfbc)_3$/Ag(fod) δ/ppm	Δ δ /ppm
6	146.1	143.7	-2.4
7	139	134.6	-4.4
2	131.7	133	1.3
3	124.2	121.1	-3.1
8	115.6	114.5	-1.1
10	113	101.8	-11.2
5	31.4	29.3	-2.1
4	26.7	25.5	-1.2
1	25.6	25.2	-0.4
9	17.6	17.2	-0.4



[XIII]

Table 2.14 ^{13}C chemical shifts ($CDCl_3$; ppm) for 3RS-MP (0.08M) and 3RS-MP (0.08M)+ $Pr(hfbc)_3$/Ag(fod).(0.24M)

^{13}C Assignment	3RS-MP δ/ppm	3RS-MP+ $Pr(hfbc)_3$/Ag(fod) δ/ppm	Δ δ /ppm	Δ RS ppm
1	112.4	95.9	16.5	0.1
		95.8	16.6	
2	144.7	141.7	3	
3	39.4	37.5	1.9	0.1
		37.4	2.0	
4	29.3	28.5	0.8	0.1
		28.4	0.9	
5	11.6	10.6	1.0	0.1
		10.5	1.1	
6	19.7	18	1.7	0.1
		17.9	1.8	

When this method was applied to the 22-RS sample of $C_{25:3}$ a shift was observed for all the ^{13}C resonances ($\Delta\ \delta$ = -10.4-1.9ppm; Table 2.15). This confirmed that an interaction between the shift reagent and the $C_{25:3}$ had taken place. Furthermore, the distance between the pair of ^{13}C resonances due to the existence of diastereoisomers for C5, C22 and C25 had increased by a further 0.1 ppm upon the addition of the CSR (Table 2.15). However, no additional splitting was observed of any other ^{13}C resonances. It is possible that the difference in the chemical shift of the diastereoisomeric carbon atoms formed upon the addition of the CSR is smaller than that of the resolution limit. A possible way of increasing the chemical shift difference between the diastereomeric carbon atoms is to induce further complexation of the substrate to the CSR. This is made possible by reducing the temperature at which the ^{13}C spectrum is obtained for the complex. Table 2.15 presents the results achieved when the ^{13}C NMR spectrum for the $C_{25:3}$ and CSR complex was obtained at 10°C. A slightly increased chemical shift change for all the ^{13}C resonances was observed upon the reduction of the temperature ($\Delta\ \delta$ = -10.5-2.1ppm) confirming that additional complexation had taken place. However, no more than two ^{13}C resonances were observed for each carbon nucleus, suggesting that $C_{25:3}$ from Soproma 34 exists as only two isomers (i.e. that there is a fixed configuration at C7).

The experiment was repeated using a different chiral shift reagent, yterbium $(hfbc)_3$/Ag(fod) to substantiate the above findings. Upon addition of $Yb(hfbc)_3$/Ag(fod) to 3 RS-methylpent-1-ene, a change in chemical shift was observed for all ^{13}C resonances ($\Delta\ \delta$ = -9.4- 3.63 ppm; Table 2.16). A spectroscopic separation of the enantiomers was also observed using this CSR ($\Delta\ \delta$ = 0.1-0.2 ppm; Table 2.16).

Table 2.15 ^{13}C chemical shifts (δ/ppm; $CDCl_3$) of 0.08M $C_{25:3}$ [II], 0.08M $C_{25:3}$ + 0.24M $Pr(hfbc)_3$/Ag(fod) at 30°C and 0.08M $C_{25:3}$ [II] + 0.24 M $Pr(hfbc)_3$/Ag(fod) at 10°C

^{13}C assignment	$C_{25:3}$ [II] δ ppm 30 °C & 10 ° C **A**	$C_{25:3}$ [II] + $Pr(hfbc)_3$/Ag(fod) δ ppm for 30°C **B**	Δ δ (ppm) **B-A**	$C_{25:3}$ [II] + $Pr(hfbc)_3$/Ag(fod) δ ppm 10°C **C**	Δ δ (ppm) **C-A**
6	152.6/152.5	153.6	+ 1.1	153.6	+ 1.1
23	145.0/144.9	142.7/142.6	- 2.3	142.5/142.3	- 2.5/-2.6
10	135.6	137.5	+ 1.9	137.7/137.7	+ 2.1
9	123.1	121.0	- 2.1	120.8	- 2.3
24	112.3/112.2	101.9	- 10.4/10.3	101.8	-10.5
17	108.6/108.6	106.2	- 2.4	106.1	- 2.5
7	46.8	46.9	+ 0.1	47.0/46.9	+0.2/0.1
11	40.0	39.9	- 0.1	40.0/39.9	0/-0.1
3	39.0	38.8	- 0.2	38.7	-0.3
13	38.6	38.5	- 0.1	38.4	-0.4
22	38.0/37.9	36.9/36.7	-1.1/1.2	36.9/36.6	-1.1/1.3
21	34.4	33.8	- 0.6	33.8	- 0.6
5	33.7/33.6	33.4/33.1	-0.3/0.2	32.9/32.8	- 0.8
8	32.9/32.8	32.7	- 0.2/0.1	32.7/32.7	- 0.2/0.1
20	30.9/30.8	30.3	- 0.6/0.5	30.2	- 0.7/0.6
14	28.0	27.8	-0.2	27.8	-0.2
2	27.9	27.8	-0.1	27.8	-0.1
12	25.7	25.8	+ 0.1	25.8	+ 0.1
4	25.6	25.4	- 0.2	25.3	- 0.3
1,16,15,19	22.6	22.6/22.5	0/-0.1	22.6/22.4	0/-0.2
25	20.4/20.0	19.4/18.9	-1.0/-1.1	19.6/19.0	-0.8/-1.0
18	16.1	16.0	- 0.1	16.0	-0.1

Table 2.16 ^{13}C chemical shifts (δ/ppm; $CDCl_3$) of 0.08M 3 RS-MP and 0.08M 3RS-MP + $Yb(hfbc)_3$/Ag(fod)

^{13}C Assignment	3 R/S MP δ/ppm	3 RS-MP+ $Yb(hfbc)_3$/Ag(fod) δ/ppm	Δ δ /ppm	Δ RS /ppm
1	112.4	103.0	-9.4	
2	144.7	148.3	+3.63	
3	39.4	41.6	+2.2	0.1
		41.5	+2.1	
4	29.3	31.9	+2.6	0.2
		31.7	+2.4	
5	11.6	12.6	+1.0	0.1
		12.7	+ 1.1	
6	19.7	22.4	+2.7	

Upon addition of $Yb(hfbc)_3$/Ag(fod) to 22 RS $C_{25:3}$ [II (a)] most ^{13}C resonances were observed to shift (Δ δ = -7.3-3.9 ppm; Table 2.17) and an increase in the distance between the ^{13}C resonances owing to the diatereoisomeric carbon atoms was observed of C23 (Δ δ= 0.4 ppm), C9 (Δ δ = 0.2 ppm), C17 (Δ δ = 0.1 ppm), C7 (Δ δ = 0.4 ppm), C11 (Δ δ = 0.1 ppm), C3 (Δ δ = 0.1 ppm), C13 (Δ δ = 0.1 ppm), C22 (Δ δ=0.5 ppm) and C5 (Δ δ= 0.2 ppm) but only a maximum of two resonances were observed for each carbon atom. Therefore, the evidence strongly suggests that the configuration at C7 is fixed for 22 RS $C_{25:3}$ [II (a)].

The $C_{25:3}$ [II (C)], isolated from Sodexal 18 was suggested by conventional NMR to exist as a single isomer or pair of enantiomers. The homochirality of $C_{25:3}$ [II (c)], isolated from Sodexal 18 was confirmed using the aforementioned method. Upon addition of $Pr(hfbc)_3$/Ag(fod) to [II (c)], a change in difference in chemical shift was observed for all ^{13}C resonances (Δ δ = -14.5-4.7 ppm; Table 2.18). However, only one ^{13}C resonance was observed for each carbon nucleus thereby confirming the [II (c)] is homochiral and that both centres, C7 and C22 have a fixed configuration. Futhermore, use of the oxidative approach had established that $C_{25:3}$ [II (b)], isolated from Soproma 46 has a S configuration at C22,

and co-chromatography, on a Carbowax stationary phase had established that $C_{25:3}$ [II (b)] is a diastereoisomer of $C_{25:3}$ [II (c)]. Therefore, from this evidence, it can be concluded that the homochiral $C_{25:3}$ [II (c)] has the R configuration at C22. This also suggests that the $C_{25:2}$ [VII (b)], isolated from Sodexal 18 also has a R configuration at C22, by analogy with that of $C_{25:3}$ [II (c)].

Table 2.17 ^{13}C chemical shifts (δ/ppm) of 0.08M $C_{25:3}$ [II] and 0.08M $C_{25:3}$ [II] + 0.24M $Yb(hfbc)_3$/Ag(fod).

^{13}C Assignment	$C_{25:3}$ [II (a)] δ ppm **A**	$C_{25:3}$ [II] + $Yb(hfbc)_3$/Ag(fod). δ ppm 30°C **D**	Δ δ/ppm **D-A**
6	152.6/152.5 (0.1)	154.9	+ 2.4
23	145.0/144.9 (0.1)	148.9/149.4	+3.9 (0.5)
10	135.6	138.7	+3.1
9	123.1	122.4/122.2	- 0.7 (0.2)
24	112.3/112.2 (0.1)	105.0	- 7.3
17	108.6/108.6 (< 0.1)	107.0/106.9	-1.6 (0.1)
7	46.8	48.7/48.3	+ 1.9 (0.4)
11	40.0	40.9/40.8	+ 0.9 (0.1)
3	39.0	39.5/39.4	+ 0.5 (0.1)
13	38.6	38.8/38.7	+ 0.2 (0.1)
22	38.0/37.9 (0.1)	37.1/36.5	+ 0.1 (0.5)
21	34.4	34.4	
5	33.7/33.6 (0.1)	34.2/34.0	+ 0.5 (0.2)
8	32.9/32.8 (0.1)	32.1	- 0.8
20	30.9/30.8 (0.1)	31.8	+ 0.9
14	28.0	28.0	
2	27.9	27.9	
12	25.7	26.4	+ 0.7
4	25.6	26.3	+ 0.7
1,16,15,19	22.6	22.8/22.6	(0.2)
25	20.4/20.0 (0.4)	17.1/17.0	- 3.3 (0.1)
18	16.1	14.0	2.1

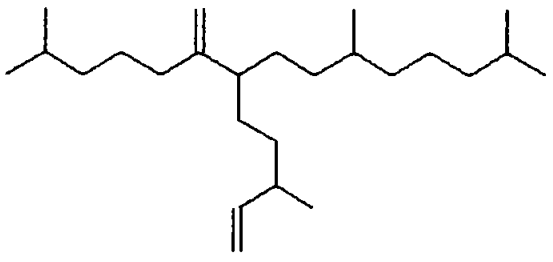
Table 2.18 ^{13}C chemical shifts (δ/ppm) of 0.08M $C_{25:3}$ [II (c)] isolated from Sodexal 18 and 0.08M $C_{25:3}$ [II (c)] + 0.24M $Pr(hfbc)_3/Ag(fod)$

^{13}C Assignment	$C_{25:3}$ [II (c)] δ/ppm	$C_{25:3}$ [II (c)] + $Pr(hfbc)_3/Ag(fod)$ δ/ppm	Δδ/ppm
6	152.6	155.7	+ 3.1
23	145.0	142.9	– 2.1
10	135.6	140.3	+ 4.7
9	123.1	116.5	– 6.6
24	112.2	97.7	– 14.5
17	108.6	99.1	– 9.5
7	46.8	48.5	+ 1.7
11	40.0	40.3	+ 0.3
3	39.0	38.9	+ 0.1
13	38.6	38.5	– 0.1
22	37.9	36.7	– 1.2
21	34.4	34.1	– 0.3
5	33.7	33.1	– 0.6
8	32.8	32.7	– 0.1
20	30.9	31.1	+ 0.2
14	28.0	27.8	– 0.2
2	27.9	27.7	– 0.2
12	25.7	26.3	+ 0.6
4	25.6	25.7	+ 0.1
1,16,15,19	22.6	22.5/22.4	–0.1/0.2
25	20.0	19.2	– 0.8
18	16.1	16.8	+ 0.7

2.4 Conclusion

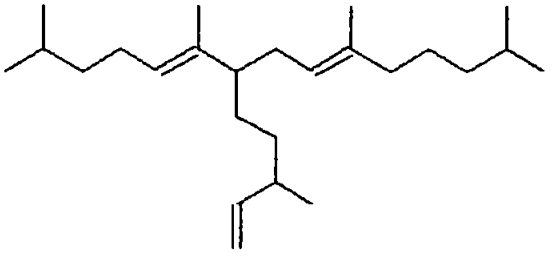
Two previously unidentified C_{25} HBIs have been isolated and characterised from laboratory cultures of *Haslea ostrearia*. They have been identified as 2,10,14-trimethyl-6-methylene-7-(3-methylpent-4-enyl)pentadecane [VII] and 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadeca-5,9-diene [VIII].

For the first time, a $C_{25:3}$ HBI was detected in laboratory culture of *Rhizosolenia setigera*. It was identified as 3,9,13-trimethyl-6-(1,5-dimethylhexyl)tetradeca-1,5,8-triene [IX].



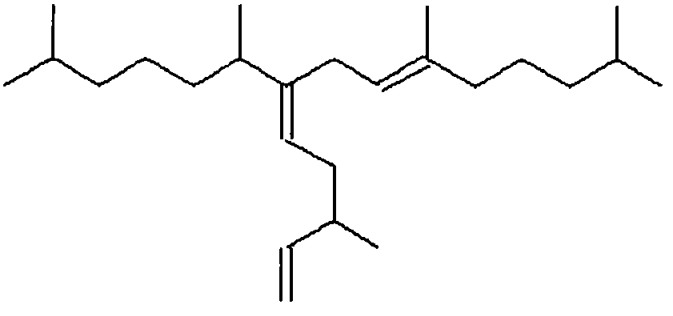
VII

Source: *Haslea ostrearia*
Soproma 34, Sodexal 18, Exudate
RI 2084 $_{(HP1,\ DB5,\ Carbowax)}$



VIII

Haslea ostrearia
Culture BP003
RI $2103_{(HP1,\ DB5)}$ $2124_{(Carbowax)}$

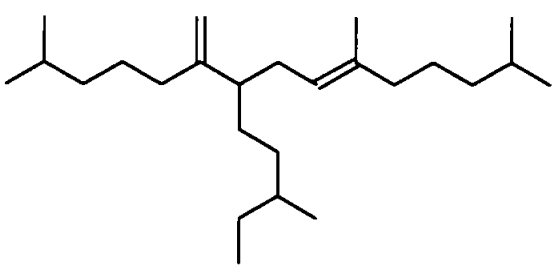


IX

Source: *Rhizosolenia setigera*
RI $2090_{(HP1)}$ $2142_{(Carbowax)}$

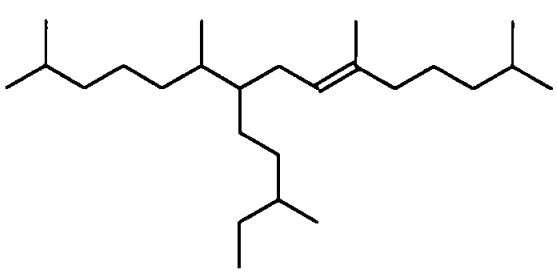
An additional $C_{25:3}$ (RI 2121_{DB5}) and eight $C_{25:4}$ HBIs (RI 2088, 2092, 2135, 2146, 2158, 2166, 2186, 2216_{DB5}) were detected in cultures of *Haslea ostrearia* and exudate and a $C_{25:3}$ (RI 2044_{HP1}) and two $C_{25:4}$ alkenes (RI 2078, 2124_{HP1}) were detected in cultures of *Rhizosolenia setigera*. Low concentrations of those C_{25} HBIs allowed identification by GC retention indices and mass spectra only. Two of the $C_{25:4}$ (RI 2135_{DB5} and 2158_{DB5}) have similar RIs and mass spectra to the C_{25} HBIs previously reported by Volkman *et al.* (1994) from *Haslea ostrearia*. The $C_{25:3}$ (RI 2044 and 2190_{HP1}) and $C_{25:4}$ (RI 2078 and 2124_{HP1}) HBIs detected in *Rhizosolenia setigera* have similar RIs and mass spectra to many reported sedimentary C_{25} HBIs (Prahl *et al.*, 1980, Barrick *et al.*, 1980, Venkatesan *et al.*, 1980, Osterroht *et al.*, 1983, Requejo and Quinn, 1983, Volkman *et al.*, 1983, Albaiges *et al.*, 1984a, Requejo and Quinn, 1984, Requejo and Quinn, 1985, Shaw *et al.*, 1985, Matsueda *et al.*, 1986, Porte *et al.*, 1990, Wakeham, 1990, Hird and Rowland, 1995).

Partial hydrogenation of the previously characterised $C_{25:3}$ [II] (Belt *et al.*, 1996) resulted in the assignment of structures for $C_{25:2}$ X (RI 2144_{HP1}, $2125_{Carbowax}$) and $C_{25:1}$ XI (RI 2159_{HP1}, $2096_{Carbowax}$). Neither the $C_{25:2}$ or the $C_{25:1}$ appear to share similar RI and mass spectra with those reported previously in either sediments or biota.



[X]

RI 2144_{HP1} $2125_{Carbowax}$



[XI]

RI 2159_{HP1}, $2096_{Carbowax}$

Source: Partial hydrogenation of $C_{25:3}$ [II]

Detailed analysis involving ^{13}C NMR with a chiral shift reagent, chemical degradation to chiral acids followed by enantioselective GC of the esters and direct GC of the alkenes on Carbowax stationary phase established that;

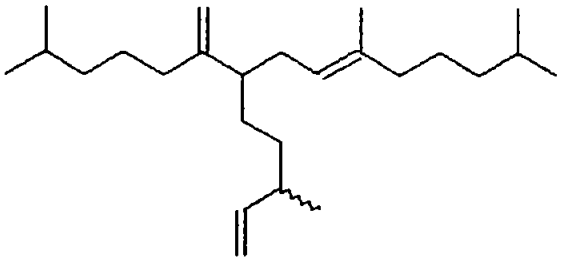
1. $C_{25:3}$ [II (a)], isolated from Soproma 34 has a RS configuration at C22 and a fixed but unknown configuration at C7 (figure 2.21a)

2. $C_{25:3}$ [II (b)], isolated from Soproma 46 has a S configuration at C22 and a fixed but unknown configuration at C7 (figure 2.21b)

3. $C_{25:3}$ [II (c)], isolated from Sodexal 18 has a R configuration at C22 and a fixed but unknown configuration at C7 (figure 2.21c)

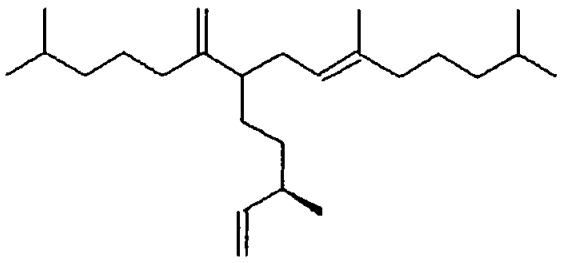
4. $C_{25:2}$ [VI (a)], isolated from Soproma 34 has a S configuration at C10 and RS at C22 (figure 2.21d)

5. $C_{25:2}$ [VI (b)], isolated from Sodexal 18 has a R configuration at C22 (figure 2.21e)



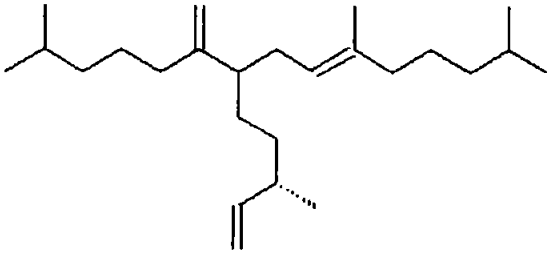
a) 2,10,14-trimethyl-6-methylene-7-(3RS-methylpent-4-enyl)pentadec-9-ene

Soproma 34



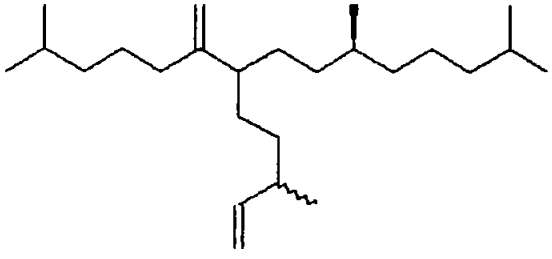
b) 2,10,14-trimethyl-6-methylene-7-(3S-methylpent-4-enyl)pentadec-9-ene

Soproma 46



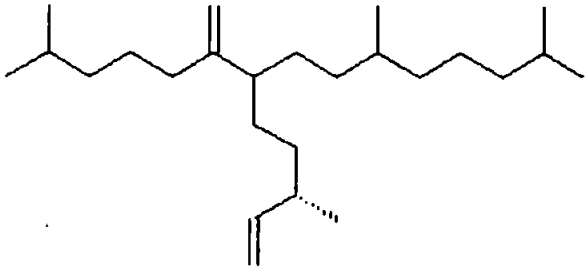
c) 2,10,14-trimethyl-6-methylene-7-(3R-methylpent-4-enyl)pentadec-9-ene

Sodexal 18



d) 2,10S,14-trimethyl-6-methylene-7-(3RS-methylpent-4-enyl)pentadecane

Soproma 34



e) 2,10,14-trimethyl-6-methylene-7-(3R-methylpent-4-enyl)pentadecane

Sodexal 18

Figure 2.21

Identification of C_{25} Highly Branched Isoprenoid (HBI) Alkenes in Sediments, Sea-Ice Diatoms and Laboratory Cultures of Diatoms

Identification of C_{25} Highly Branched Isoprenoid (HBI) Alkenes in Sediments, Sea-ice Diatoms and Laboratory Cultures of Diatoms.

3.1 Introduction

It has been established that the diatom *Haslea ostrearia*, in laboratory cultures biosynthesises a suite of C_{25} HBI alkenes from a triene through to a hexaene (Wraige *et al.*, 1997; Belt *et al.*, 1996; Cooke, 1995 and Volkman *et al.*, 1994). However, what is not clear is how these HBI alkenes compare to those reported in many recent sediments. It is known that the $C_{25:3}$ (RI 2106; Belt *et al*; 1996) has a similar retention index to some of the reported sedimentary HBIs (Table 3.1). However, only rarely have the mass spectra been included in literature reports (Albaiges *et al.*, 1984; Ventatesan, 1988; Porte *et al.*, 1990).

Table 3.1: Reported sedimentary $C_{25:3}$ with similar RI and/or mass spectral data to $C_{25:3}$ (RI 2106) isolated from *Haslea ostrearia* (Belt *et al.*, 1996)

RI (stationary phase)	Location	Reference	Mass spectral data
$2106_{(SE-30)}$	Narrangansett Bay Estuary, U.S.A.	Requejo and Quinn (1983)	No
$2107_{(SE-30)}$	Ebro Delta, Spain	Albaiges et al. (1984)	No
$2107_{(SE-30)}$	Hypersaline pond, Los Monegros, Spain.	Albaiges et al. (1984)	Yes
$2106_{(SE-30)}$	Salt marsh, Narrangansett Bay, U.S.A.	Requejo and Quinn (1985)	No
$2107_{(DB5)}$	Tamar Estuary. S.W. England	Hird and Rowland (1995)	No
$2107_{(DB5)}$	Todos os Santos, Bahia, Brazil	Porte et al. (1990)	Yes
$2110_{(DB5)}$	McMurdo Sound, Ross Sea, Antarctic.	Venkatesan (1988)	Yes

Therefore, although it can be suggested that these sedimentary HBI trienes have the same structure as that of the biosynthesised $C_{25:3}$ (RI 2106) it is erroneous to firmly conclude this by the use of retention index data only. For example, the $C_{25:3}$ identified in sediments from

McMurdo Sound, Antarctic is reported to have a RI of 2110 $_{DB5}$. On this evidence this compound would not be considered similar to that of $C_{25:3}$ (RI $2106_{HP1}2107_{DB5}$). However the mass spectra are very similar to that of the biosynthetic triene.

This chapter describes how fully characterised HBI alkenes, isolated from the diatom *Halsea ostrearia* were used to more firmly identify the dienes and a triene previously reported, but not fully identified, in sea-ice diatoms and sediments from Antarctica (Nichols *et al.*, 1998, 1989, 1993; Venkatesan, 1988; Venkatesan and Kaplan, 1987).

In addition, studies of the stereochemical configuations of these sedimentary HBI alkenes, which have not previously reported are given herein. NMR evidence that some HBIs exist as diastereomers has only recently been reported (inferred from a 'doubling-up' of some ^{1}H and ^{13}C resonances; Belt *et al.*, 1996). This was not observed for the sedimentary diene isolated from the Caspian Sea nor the diene isolated from Shark Bay sediments, Australia (Belt *et al.*, 1994; Summons *et al.*, 1993). The successful separation herein of sedimentary and algal HBI diastereomers by GC using a polar stationary phase (Carbowax) has allowed the stereochemical configurations to be determined.

3.2 Experimental

3.2.1 Large-scale laboratory cultures.

The isolation and characterisation of the individual HBIs from the alga, *Haslea ostrearia* is described fully in Chapter two. Briefly, centrifuged algal paste of different laboratory cultures was extracted with hexane (soxhlet extraction, 24 hrs), followed by column chromatography on silica and elution with hexane. Previously unidentified HBIs were characterised by hydrogenation, GC-MS, ^{1}H and ^{13}C NMR. The stereochemical configurations of the HBI isolates were determined by two complementary methods; oxidation of the HBI alkene (CrO_3/glacial acetic acid) and examination of the low

molecular weight acids by enantioselective GC and ^{13}C NMR experiments with a bridging ion (Ag^{+}) and chiral shift reagents.

3.2.2 Small scale laboratory cultures

H. ostrearia was isolated from oyster ponds of the Bay of Bourgneuf (France). The cultures were grown in 250 mL Erlenmeyer flasks at 5°C and 15°C with illumination provided by cool-white fluorescent tubes in a 14/10 hr light/dark cycle. They were incubated in a modified ES 1/3 Provasoli medium at salinities of *ca* 32 per mil under 100 µmol photons m^{-2} s^{-1} irradiance. Samples were harvested by filtration in triplicate after 2,5,6,8,11,16,21 and 26 days growth. Hydrocarbon extraction and analysis on a HP1 column was performed by a co-worker. Briefly, the filters were extracted by ultrasonication in hexane, the total hexane extract was dried (anhydrous Na_2SO_4) and solvent removed under nitrogen. The samples were re-examined on a 30m x 0.25 mm Carbowax fused silica GC column. The GC oven temperature was programmed from 50 – 200°C at 6°C per min.

3.1.3 Sea –ice diatoms

Samples of freeze-dried sea-ice diatoms collected from McMurdo Sound, Antarctica and described previously (Nichols *et al.*, 1989) were extracted by ultrasonication in hexane (5 mL, 3 x 20mins, Kerry Pulsatron HB172) and the total hexane extract (THE) dried (anhydrous Na_2SO_4). Solvent was removed under nitrogen and the THE examined by GC-MS on two stationary different phases, a 12m x 0.2mm HP1 fused silica column fused silica column, operating at an oven temperature programme of 40-300°C at 5°C per min and a carbowax column as above.

3.1.4 Antarctic sediments

Aliphatic hydrocarbon fractions isolated previously from Antarctic sediments (0-2cm sub-bottom depth) collected from McMurdo Sound Antarctica (Venkatesan, 1988) were re-examined by GC-MS as described above.

3.3 Results and Discussion

3.3.1 Authenticated alkenes.

The authenticated alkenes described in chapter two were used in the present study to identify HBI alkenes from small-scale cultures of *Haslea ostrearia* and those found in sea-ice diatoms and sediments from the Antarctic. It is therefore appropriate to summarise the evidence for elucidation of these HBIs.

Many of the ^{1}H NMR spectra of isolated HBI alkenes from *Haslea ostrearia* were characterised by a septet of resonances at δ 5.67 ppm, attributed to the ABMX coupling of H23 to H24, H22 (ddd; Figure 2.4c; Chapter two). However, in some isolates, HBIs exhibited spectra where these and other resonances, affectively "doubled-up" in appearance (Figure 2.4b; Chapter two). This was attributed, in the trienes, tetraenes and pentaenes to the existence of diastereomers at C7 and/or C22. NMR experiments with a bridging ion (Ag+) and chiral shift reagents showed that the configuration in such mixtures was fixed at one centre (C7 or C22) and epimeric at the other. Finally, oxidation of a diastereomeric mixture of an HBI triene from *Haslea ostrearia*, culture Soproma 34, and examination of the configuration of the resulting acids by chiral GC, established that the epimeric centre was C22. It is therefore reasonable to assume that the diastereomeric diene (NMR) from the same culture also has an epimeric centre at C22. Oxidation of a non-diastereomeric mixture (NMR) of the same triene from culture Soproma 46 established the configuration at C22 as S. The 22 RS triene from Soproma 34 produced a doublet (RI

2142, 2146; Figure 3.3a) when analysed by GC-MS on a polar stationary phase (Carbowax) whereas the 22S triene from Soproma 46 produced a singlet (RI 2142; Figure 3.3c). Co-injection established that the first peak of the doublet corresponded to a triene with a 22S configuration. A homochiral triene (NMR) isolated from Sodexal 18 also produced a singlet (RI 2146; Figure 3.3b) when analysed on a carbowax stationary phase. However, this triene co-eluted with the second peak of the doublet and hence has a 22R configuration. A non-diastereomeric diene (NMR) was also isolated from Sodexal 18 and it is reasonable to assume that it shares the same configuration as that of the triene i.e. 7 R (or S) 22R. This is consistent with GC analysis where the singlet produced by the diene from Sodexal 18 (RI 2084; Figure 3.2b) co-eluted with the second peak of the doublet produced by the diastereomeric diene from Soproma 34 (2080; 2084; Figure 3.2a).

GC analysis of a diastereomeric tetraene (NMR) from Soproma 34 produced a doublet (2234, 2238; Figure 3.4a) which is consistent with the diene and triene from the same culture. Therefore it is reasonable to assume that the C22 chiral centre is epimeric. Similarly the non-diastereomeric tetraene (NMR) from Soproma 46 produced a singlet (RI 2134; Figure 3.4b) which co-eluted with the first peak of the doublet and therefore is thought to have an S configuration at C22. Table 3.2 summaries the structures and their corresponding retention indices.

3.1.2 HBI alkenes in *Haslea ostrearia*-small batch cultures

Although the large cultures described in chapter two were very valuable for provision of sufficient HBIs for isolation and NMR study, the conditions of growth of these cultures were not well-controlled (non-constant temperature and light intensity). Therefore, smaller scale (*ca* 250 ml), well-controlled culture experiments were devised. The cultures were grown at two different temperatures, 5°C and 15°C. The sole HBIs produced in the 5°C culture were C_{25} dienes. GC-MS on apolar HP1 phase revealed two components with

Table 3.2: Summary of stereochemical configurations of authenicated HBI alkenes with their corresponding retention indices (HP1 and Carbowax).

HBI	Culture or sediment source	Retention Index		Known Structure	Inferred Structure
		Carbowax	HP1		
$C_{25:2}$	Soproma 34	2080 2084	2084	Diastereoisomeric at C7 or C22, C10S	C7R (or S), C10S, C22RS[I]
$C_{25:3}$	Soproma 34	2142 2146	2106	C7R (or S), C22 RS[V]	
$C_{25:4}$	Soproma 34	2234 2238	2144	Diastereoisomeric at C7 or C22	C7R (or S); C22RS [IX]
$C_{25:3}$	Soproma 46	2142	2106	Homochiral; C7R (S); C22 (S) [VI]	
$C_{25:4}$	Soproma 46	2234	2144	Homochiral Unknown C7, C10 or C22	C7R (or S), C22S [X]
$C_{25:2}$	Sodexal 18	2080	2084	Homochiral unknown C7, C10 or C22	C7R (or S), C22R and C10S [II]
$C_{25:3}$	Sodexal 18	2142	2106	Homochiral unknown C7 or C22	C7R (or S), C22R [VII]
$C_{25:2}$	Caspian Sea	2063	2079	Homochiral C7, C10 or C22 unknown [IV]	
$C_{25:3}$	BP003	2130	2103	Homochiral C7 and C22 unknown [VIII]	

Note: Structures are presented in Figure 3.1.

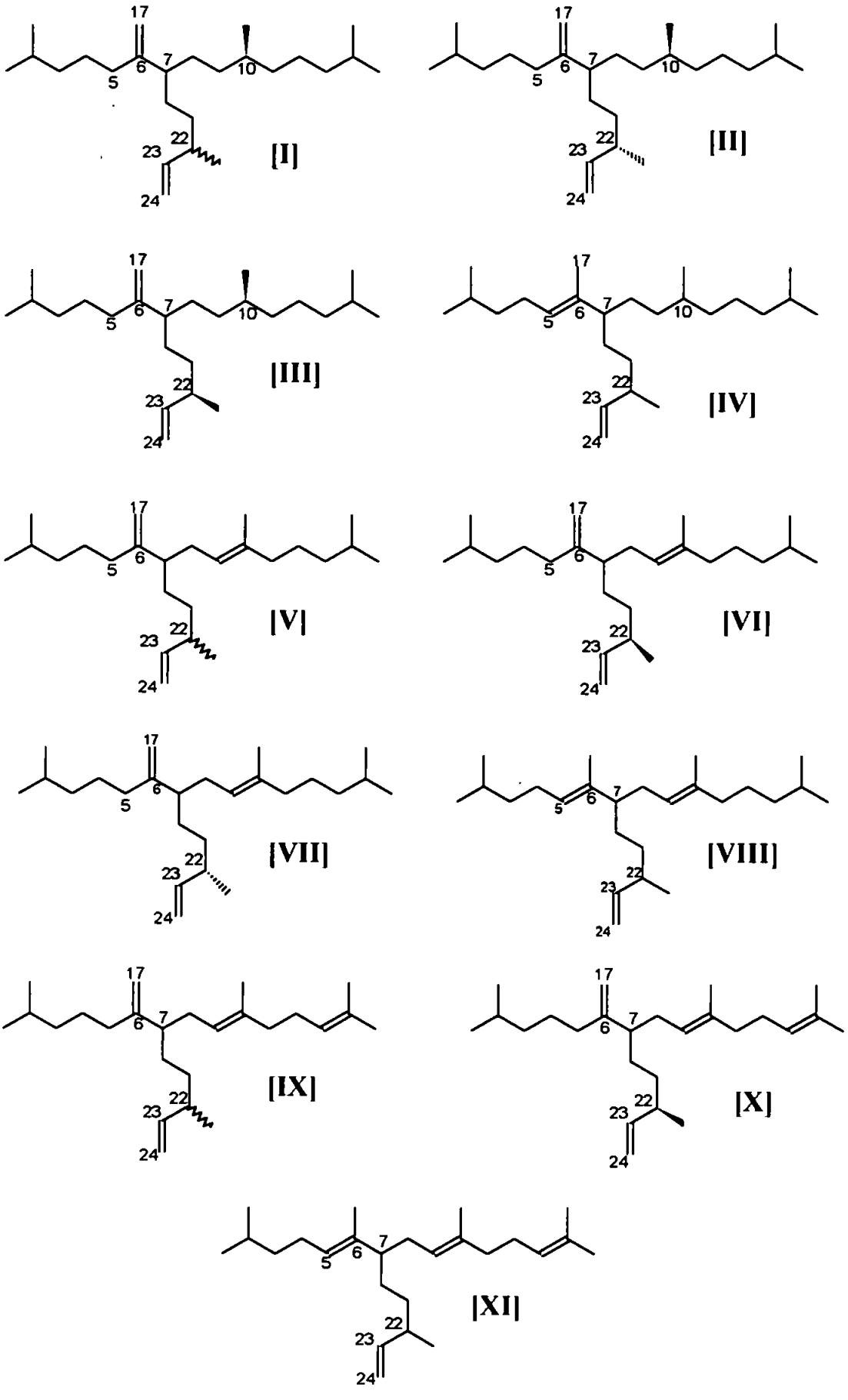


Figure 3.1: Structures of HBI alkenes

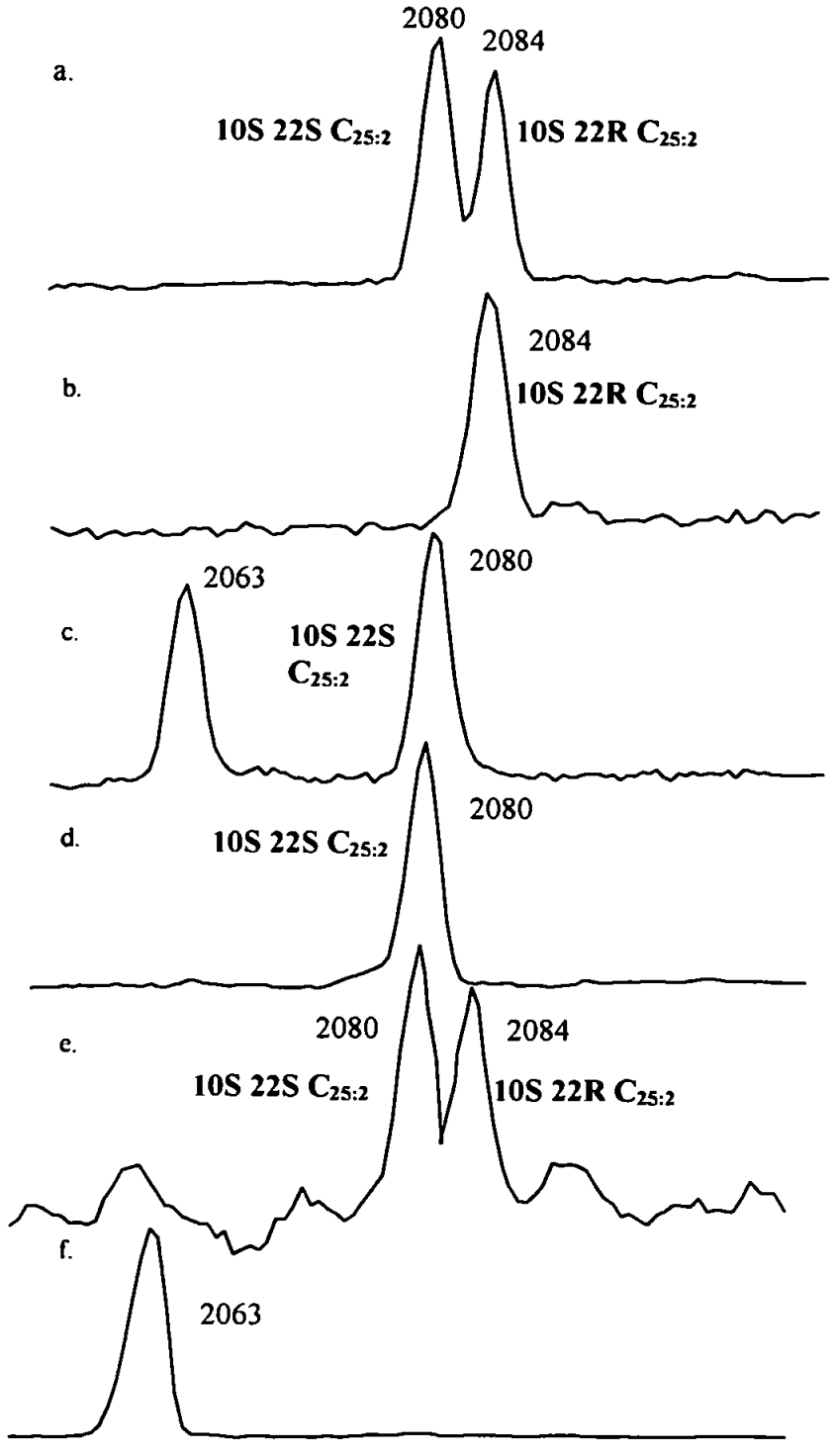


Figure 3.2: Partial GC-MS total ion current chromatograms on Carbowax stationary phase of 2,10,14-trimethyl-6-methylene-7(3'-methtylpent-4-enyl)pentadecane isolated from a) Large-scale culture Soproma 34 of the diatom *Haslea ostrearia*, b) Large scale culture Sodexal 18 of the diatom *Haslea ostrearia*, c)Small scale culture of the diatom Haslea ostrearia cultured at 5°C. d) Mixed sea-ice diatoms form McMurdo Sound, Antatctica. e) McMurdo Sound sediment (0-2cm), Antarctica (cf Venkatesan, 1988) and f) 2,6,10,14-trimethyl-6-methylene-7(3'methylpent-4-enyl)pentadec-5-ene from Caspian Sea sediment (cf Belt *et al.*, 1994)

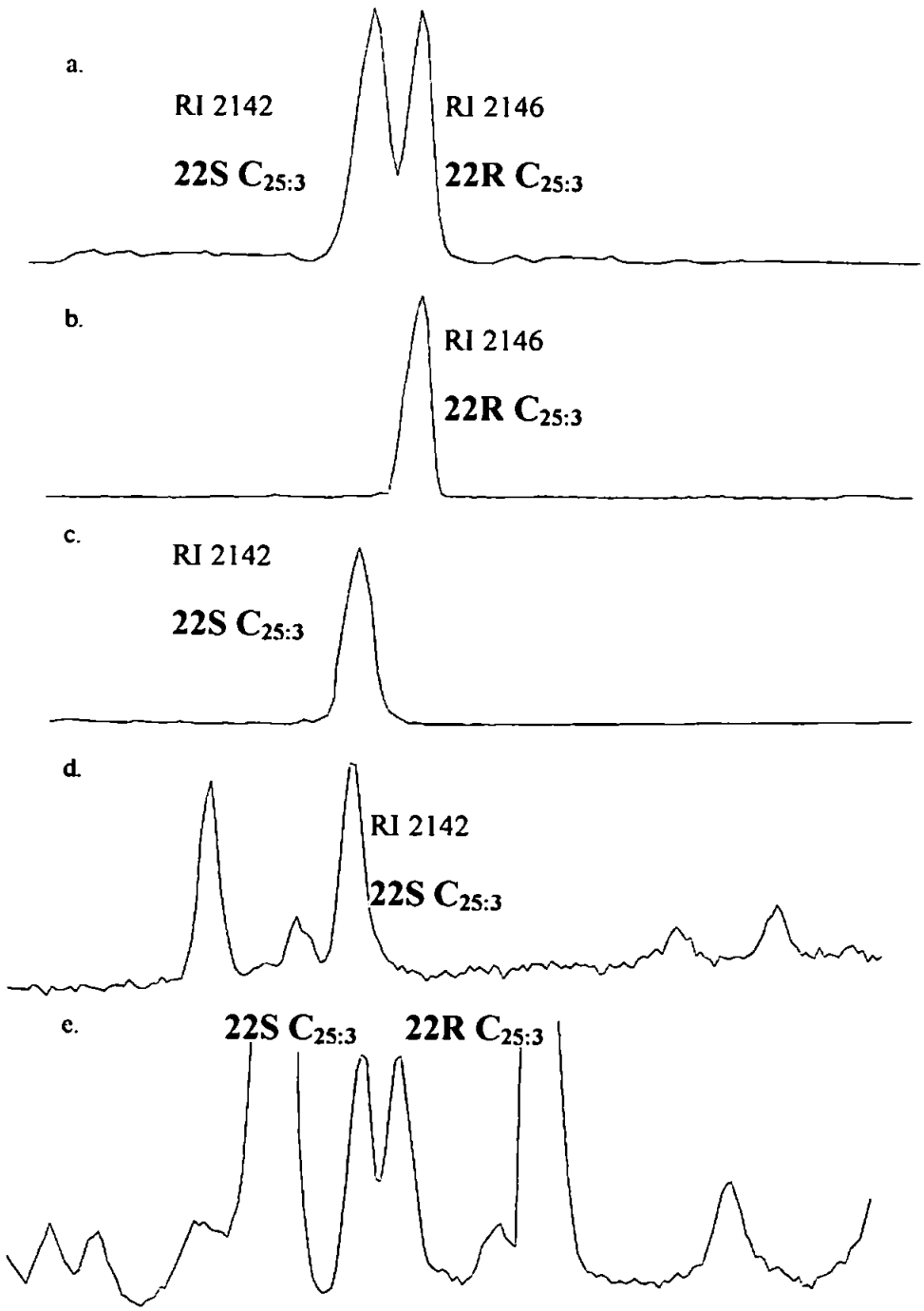


Figure 3.3: Partial GC-MS total ion current chromatograms on Carbowax stationary phase of 2,10,14-trimethyl-6-methylene-7(3'-methylpent-4-enyl)pentadec-9-ene isolated from a) Large-scale culture Soproma 34 of the diatom *Haslea ostrearia*, b) Large scale culture Sodexal 18 of the diatom *Haslea ostrearia*, c) Large scale culture Soproma 46 of the diatom *Haslea ostrearia*; d) Small scale culture of the diatom *Haslea ostrearia* cultured at 15°C and e) McMurdo Sound sediment (0-2cm), Antarctica (cf Venkatesan, 1988).

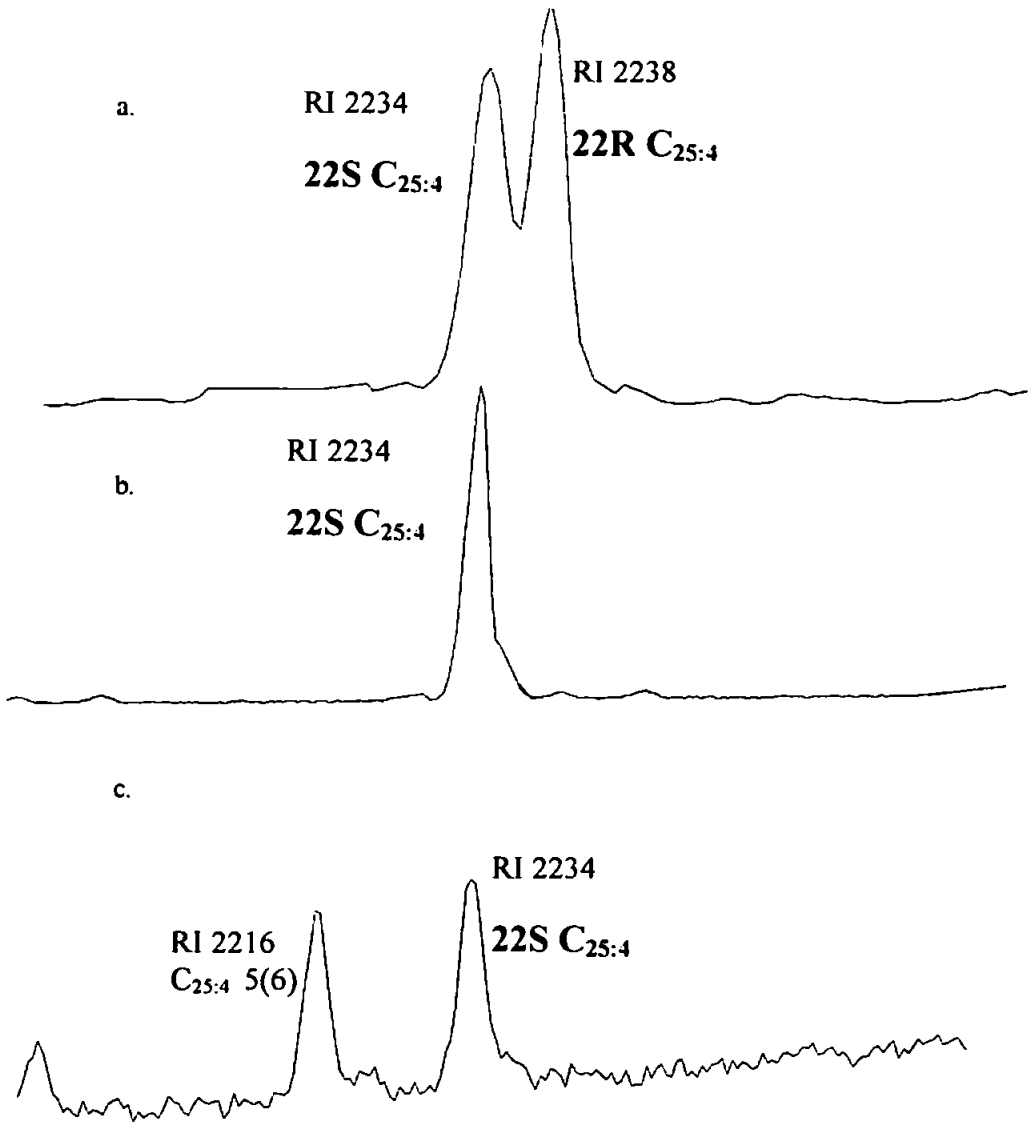


Figure 3.4: Partial GC-MS total ion current chromatograms on Carbowax stationary phase of 2,10,14-trimethyl-6-methylene-7(3'-methylpent-4-enyl)pentadec-9.13-ene isolated from a) Large-scale culture Soproma 34 of the diatom *Haslea ostrearia*, b) Large scale culture Soproma 46 of the diatom *Haslea ostrearia* and c)Small scale culture of the diatom *Haslea ostrearia* cultured at 15 °C.

retention indices of 2079 and 2084. These co-eluted with diene [IV] (*viz* the Δ5 positional isomer and the 6 (17) isomer [III] respectively. Re-analysis of these samples by GC-MS on carbowax gave well-separated singlets (RI 2063 and 2080; figure 3.2c). The singlet (RI 2080) produced by the diene [III] co-eluted with the first peak of the diastereomeric sample. It therefore has opposing stereochemistry to that of [II] from Sodexal 18 and thus can be tentatively assigned as 10S, 22S [III]. The singlet with RI of 2063 co-eluted with the singlet from the Caspian Sea diene [IV] the stereochemistry of which is unknown. However the absence of 'doubling-up' of any ^{13}C resonances which were observed for [IV], as described in chapter two, and the absence of a doublet on carbowax phase suggest that [IV] is homochiral or a racemate.

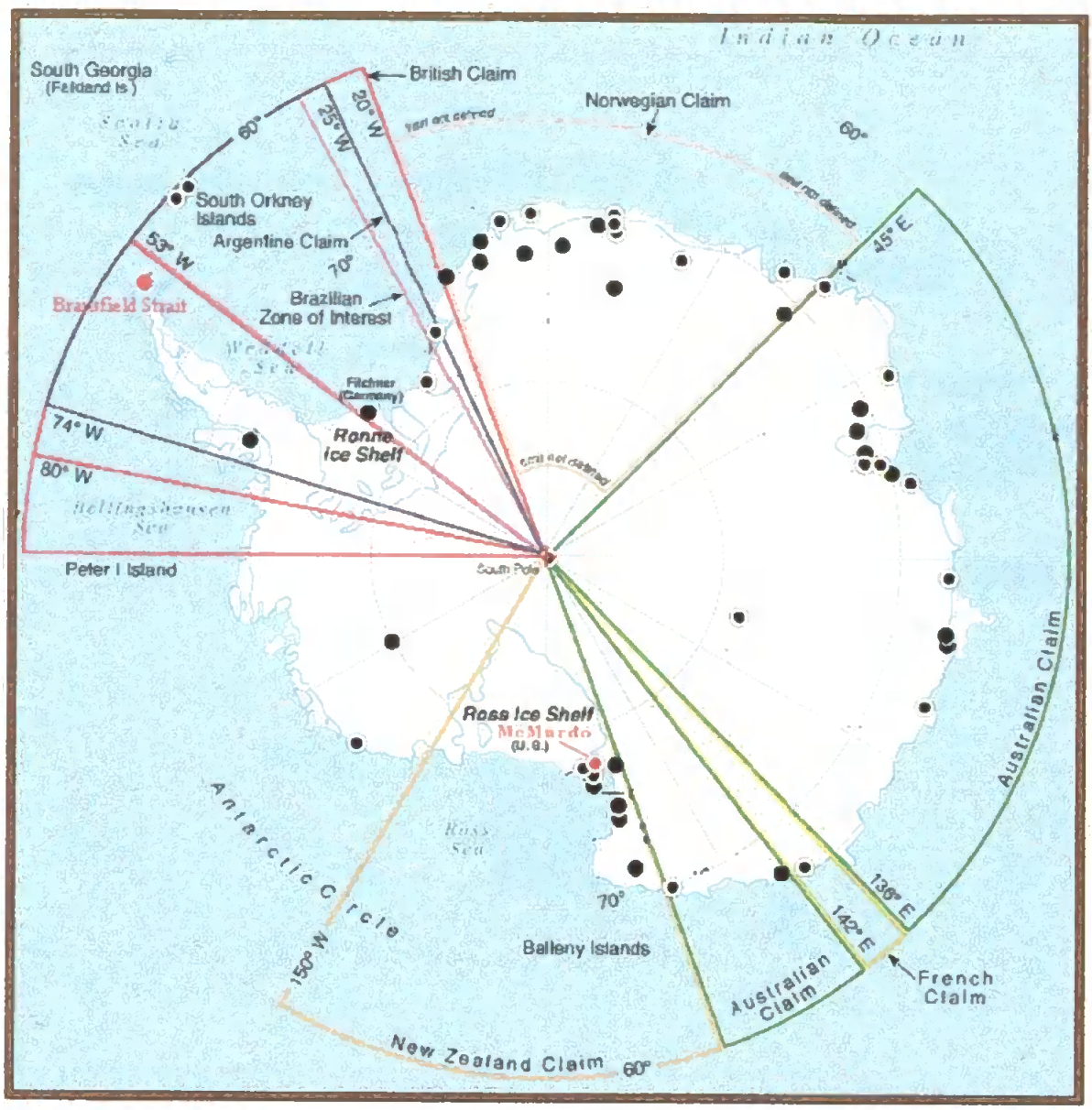
Examination of the culture grown at 15°C was observed to produce C_{25} trienes and tetraenes. GC-MS on apolar HP1 phase revealed two trienes with retention indices of 2103 and 2106. These peaks were not well resolved and this made identication difficult although the mass spectra and RI compared favourably with that of the Δ 5 triene from the BP003 culture [VIII] and the 6 (17) isomer [VI] respectively. Re-examination of the samples on a Carbowax column by GC-MS gave two well-separated singlets (RI 2124 and 2142; Figure 3.3d). The singlet for the 6 (17) isomer from this microscale sample of *H. ostrearia* co-eluted with the first peak of the diasteremeric triene. It therefore has the same stereochemistry to that of [VI] from Soproma 46 and thus can be assigned as 22S [VI]. The singlet at RI 2124 did not co-elute with that of the singlet produced by [VIII] isolated from BP003. However, it is likely to share the same structure as that of [VIII] but with an opposing configuration at C22 (or C7) owing to the identical mass spectrum and retention index on HP1. This is further substantiated by results obtained from the facile isomerisation of the 6 (17) diastereomeric triene (RI 2142, 2146) to give the Δ 5 diastereomeric triene (RI 2124, 2130) in the presence of acidic clays (chapter four).

Similarly the C_{25} tetraenes when examined on carbowax also produced two well separated singlets with retention indices of RI 2216, and 2234 (Figure 3.4a) where the singlet (RI

2234; Figure 3.4b) co-eluted with the first peak of the diastereomeric tetraene [IX] from Soproma 34. Therefore it can be observed that this tetraene (RI 2234) shares the same 22 S configuration as [X] Soproma 46 (RI 2134; Figure 3.4b). The tetraene RI 2216 is probably the Δ 5 isomer, which has not yet been isolated from *Halsea ostrearia* for structural characterisation.

3.1.3 Sea-ice diatoms

Sea-ice diatoms represent a wide array of micoalgae, mostly diatoms, found throughout the sea ice surrounding Antarctica and are thought to represent a major component of the carbon/energy flux of the polar oceans (Nichols *et al.*, 1993). The particular sample of sea-ice diatoms studied herein was from Cape Armitage within McMurdo Sound (map; Figure 3.5). The authenicated samples of [I] and [IV] were co-chromatographed (GC-MS) on two stationary phases with the hexane extract of sea-ice diatoms, the lipid compositions of which have been described previously (Nichols *et al.*, 1989). The mass spectrum of the diene from sea-ice diatoms was identical to that of [I] and [IV] and hydrogenation produced the $C_{25:0}$ alkane parent structure (Nichols *et al.*, 1989). On apolar HP1 phase the diene co-chromatographed with [I] and on the more polar carbowax, the compound co-eluted with the first peak of the doublet obtained for [I] from the Soproma 34 sample (Figure 3.2d). This confirms the identity of the diene in this diatom extract as the 6 (17) isomer with a restricted stereochemistry assigned as 10S, 22S. This configuration appears to be the same as that for [III] examined in the small scale cultures of *H. ostrearia*. The source organism of the diene in this sea-ice diatom mixture is not known (Nichols *et al.*, 1988) but *Haslea spp* such as *H. trompei* do occur in the Antarctic (von Stosch, 1985).



Bransfield Strait and McMurdo Sound (including Cape Armitage)

Research stations

Figure 3.5: Map of Antarctica showing locations of McMurdo Sound and Bransfield Strait Research Stations (source: www.ice.wizard.net)

3.1.4 HBI dienes and trienes in Antarctic sediments

Detailed studies of lipids have been carried out on previous Antarctic sediments from Bransfield Strait and McMurdo Sound (Venkatesan, 1988 Venkatesan and Kaplan, 1987). The two locations are shown on the map (Figure 3.5).

A different C_{25} diene was identified in the hydrocarbon fraction at each location, one (RI 2088_{DB5}) from M°Murdo Sound and the other from Bransfield Strait (RI 2082_{DB5}; Venkatesan, 1988; Venkatesan and Kaplan, 1987). These were later identified as HBI alkenes by hydrogenation to the parent alkane which co-chromatographed with synthetic $C_{25:0}$ (Robson and Rowland, 1986) but the double bond positions were not assigned (Rowland *et al.*, 1990). The hydrocarbons from McMurdo Sound were re-examined by GC-MS on carbowax (figure 3.2e). The diene had an identical mass spectrum to that of [I] and co-chromatographed as a doublet with [I] isolated from the Soproma 34 (figure 3.2e). The identity of the diene is therefore confirmed as the 6 (17) isomer which is consistent with the findings of Hird *et al.* (1992) who correctly assigned one of the double bonds in this diene to the 6 (17) position on the basis of ozonolysis experiments. Since the diene appeared as a doublet on the carbowax column it can be deduced that the configuration at C22 is RS. This is different to that of the sea-ice diatoms from the same region (*cf* figure 3.2d). This could be explained by epimerisation at the chiral centre, a well-known process for acyclic isoprenoid hydrocarbons in sediments (e.g. reviewed by Volkman and Maxwell, 1986). However, given the occurrence of the mixture in sediments of only 0-2cm sub-bottom depth, (*viz* presumably geologically very young) and of diastereomeric mixtures in some of the cultured algae, a more likely explanation for the sedimentary mixtures may be the variations in the stereospecificity of the saturase enzyme on the C22-C25 double bond in the corresponding $C_{25:6}$ (Wraige *et al.*, 1997) during production of the dienes *via* penta-, tetra- and trienes (Belt *et al.*, 1996; Wraige *et al.*, 1997).

Insufficient diene was left from a Bransfield Strait Antarctic sediment sample (Venkatesan and Kaplan, 1987) for chromatographic comparison with the authenicated dienes, but the alkene in these sediments seems likely to be [IV] given the very similar mass spectrum and similar RI. The occurrence of [IV] in the sediments and in the small scale algal cultures (Figure 3.2c, RI 2063) suggests that this isomer might arise from both a direct algal origin, and probably additionally from double bond migration in the biological 6 (17) isomer to the Δ5 alkene in the sediments. This theory is further substantiated by results obtained from the facile isomerisation of the 6 (17) diene to the Δ5 isomer in the presence of acidic clays (Chapter four). No isomerisation of the 6(17) to the Δ5 isomer occurs on work-up or storage as demonstrated by experiments with the pure 6(17) isomer (Allard W.G, personal communication).

HBI trienes in Antarctic sediment

A C_{25} triene (RI 2110_{DB5}) was also reported with the C_{25} diene in sediment from the McMurdo Sound. The mass spectrum is very similar to that of triene [V]; notably characteristic are the relative ratio of m/z 149/151 ions is *ca.* 1.4/1 (1.2:1 for the authentic triene Chapter 2) and the relative abundance of the high mass ions m/z 233 and 261. Examination of the hydrocarbon fraction on a carbowax column showed that the triene produced a doublet (RI 2142, 2146, figure 3.3e) which co-chromatographed with triene [V] from Soproma 34. The identity of the triene is therefore confirmed as 22RS which is consistent with the co-occurring diene.

3.4 Conclusion

- A C_{25} HBI diene and triene reported in Antarctic sediments from McMurdo Sound have been identified as 2,10S,14-trimethyl-6-methylene-7-(3RS'-methylpent-4-enyl)pentadecane [I] and 2, 10, 14-trimethyl-6-methylene-7-(3 RS'-methylpent-4-enyl)pentadec-9-ene [V]. Both these compounds are produced by *Haslea ostrearia* in culture.

- A C_{25} HBI diene produced by Antarctic sea-ice diatoms was identified as 2,10S,14-trimethyl-6-methylene-7-(3S'-methylpent-4-enyl)pentadecane [III]. This compound has also been identified in *Haslea ostrearia* cultures

- The Δ5 positional isomer of $C_{25:2}$ [I] and $C_{25:3}$ [V] i.e.2,6,10,14-tetramethyl-7-(3'-methylpent-4-enyl)pentadeca-5-ene [IV] and 2,6,10,14-tetramethyl-7-(3'-methylpent-4-enyl)pentadeca-5,9-diene [VIII] were identified in small-scale cultures of *Haslea ostrearia*. The $C_{25:2}$ [IV] has been reported previously in Caspian Sea sediment (Belt *et al.*, 1994).

- Evidence suggests that double bond isomerisation and stereoisomerism at the chiral centre C22 occurs during biosynthesis and/or in the sediments.

Acid-Catalysed Rearrangement of HBI Alkenes

Chapter Four Acid-Catalyzed Rearrangement of HBI Alkenes

4.1 Introduction

Organic molecules synthesised by living organisms are tailored for specific biological requirements and therefore are often not the most geologically stable. Upon deposition in sedimentary environments they undergo diagenesis, a process that often involves a series of sequential reactions. Understanding the diagenetic pathway of a compound allows recognition of other biological markers that can be linked to that of the biosynthesised molecule. However, the elucidation of such pathways is often complicated and is typically inferred from a combination of two techniques,

I) the sedimentary occurrence and distribution of biological marker compounds, especially in depth sequences and

II) reactions established by laboratory simulation experiments, which may involve the chemical treatment (e.g. acidic rearrangement) of a precursor compound or laboratory heating experiments, typically in evacuated sealed vessels, with chosen matrices (e.g. clay, carbonate, sulphur).

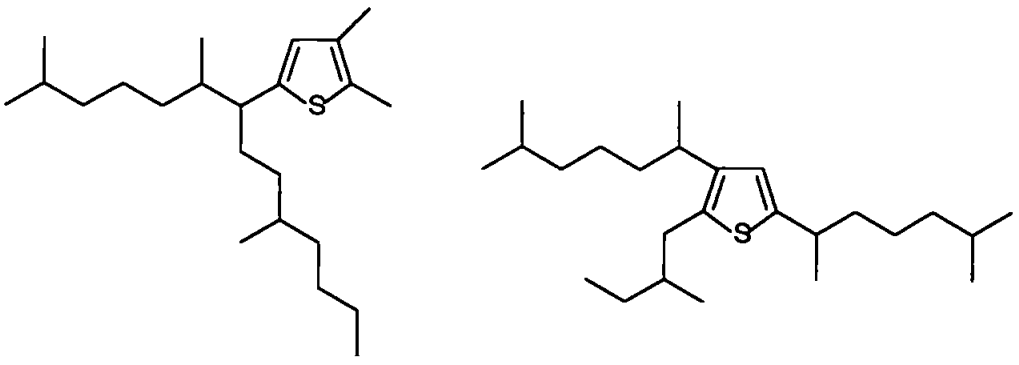
Previous research of the diagenetic fate of C_{25} HBIs has been limited. Many early reports of the distribution of the C_{25} HBI alkenes with depth were frustrated by the unknown structures of the compounds. In addition, many authors mis-assigned branched HBIs as cyclic owing to the unpredictable hydrogenation behaviour of these compounds (Requejo and Quinn, 1983; Venkatesan, 1988). Nonetheless, numerous studies have shown that the concentration of the C_{25} alkenes, dominantly tri- and tetraenes, rapidly decreased with depth (Rowland *et al.*, 1990 and references cited therein). For example, Volkman *et al.* (1983) reported that the concentrations of two C_{25} trienes (2044 $_{SE-52}$ and 2092 $_{SE-52}$) and two C_{25} tetraenes (2082 $_{SE-52}$ and 2129 $_{SE-52}$)

decreased by an order of magnitude in the first 5 cm of sediment. This contrasted strongly with the concentrations of *n*-alkanes which increased over the same interval.

There is also some evidence that C_{25} HBI alkenes with two double bonds and less are removed from the hydrocarbon fraction at a less rapid rate than the higher polyenes. For example, Requejo and Quinn (1983), noted that in cores taken from the Narragansett Bay estuary, U.S.A, the rate of decrease for a $C_{25:4}$ (*sic* $C_{25:2:2}$, 2097 $_{SE-30}$) was greater than that observed for the other co-occurring alkenes, $C_{25:2}$ (2084 $_{SE-30}$) and $C_{25:2}$ (2079 $_{SE-30}$). In a different report of sediments from a similar location, the same authors, noted that the concentration of two trienes, RI 2044_{SE-30} and 2090_{SE-30}, decreased abruptly to low levels yet the $C_{25:2}$ (2084 $_{SE-30}$) showed a much more gradual decrease with depth (Requejo and Quinn, 1984). Furthermore, Dunlop and Jefferies (1985), reported that the concentration of a $C_{25:1}$ (2112) isolated from Shark Bay showed no significant change in concentration over a depth profile of 36 cm.

The diagenetic pathways by which HBI alkenes are degraded are presently unknown. However microbial degradation, microbial oxidation and/or cross-linking polymerization reactions have all been suggested (Volkman *et al.*, 1983; Venkatesan, 1984). As described previously there is some evidence to suggest that the alkenes react rapidly with sulphur to form sedimentary thiophenes (Kohnen *et al.*, 1990, 1993; Sinninghe Damsté *et al.*, 1989, 1990, 1993). Two C_{25} highly branched thiophenes (HBIT) have been characterised, 2,3-dimethyl-5-(7'-(2',6',10',14'-tetramethylpentadecyl)) thiophene [I] and 2-(2'-methylbutyl)-3,5-di-(2'-(6'-methylheptyl)) thiophene [II] (Figure 4.1) where the precursors are thought to be C_{25} HBI alkenes with at least two double bonds Sinninghe Damsté *et al.*, 1989).

Literature on laboratory simulation experiments involving C_{25} HBIs is very limited. The main reason for this is that, prior to 1995 access to sufficient amounts of pure C_{25} HBI isolates was difficult. However, a laboratory degradation experiment was reported

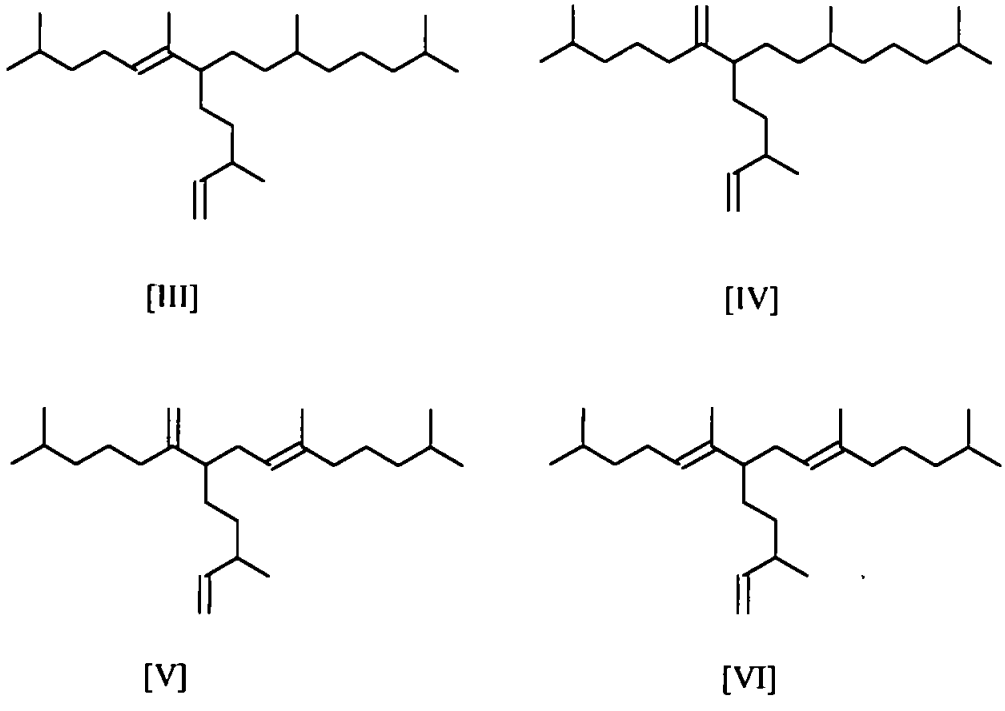


[I] [II]

Figure 4.1: 2,3-dimethyl-5-(7'-(2',6',10',14'-tetramethylpentadecyl)) thiophene [I] and 2-(2'-methylbutyl)-3,5-di-(2'-(6'-methylheptyl)) thiophene [II]

by Robson and Rowland (1987) which showed that *n*-alkanes and *n*-alkenes were biodegraded at a faster rate than that of the C_{25} HBI monoenes.

The present chapter describes the results of experiments with acid-catalysed rearrangements of three C_{25} HBIs, ([IV], [V], [VI]) isolated from *Haslea ostrearia* (Chapter two) and one sedimentary diene [III] (Belt *et al.*, 1994). The structures are presented in Figure 4.2.

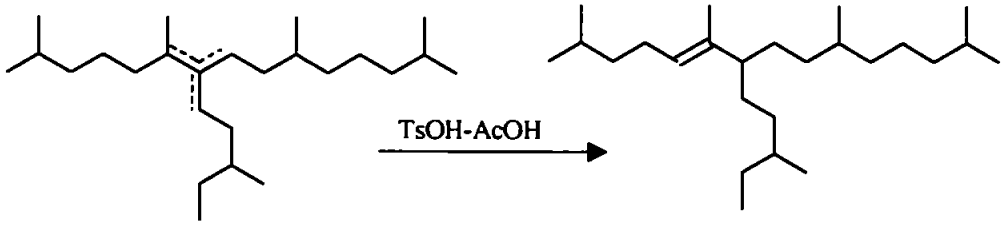


[III] [IV]

[V] [VI]

Figure 4.2: C_{25} HBI alkenes used in acid catalysed rearrangement experiments.

Two different acid catalysts were used; the clay, montmorillonite and a toluene-*p*-sulphonic-acetic acid mixture. Montmorillonite is frequently found in recent geological sediments and has a demonstrable catalytic activity towards rearrangement reactions owing to the 'active acid sites' on the mineral surface (Rubinstein *et al.*, 1975; Sieskind and Albrecht, 1985; Hauke *et al.*, 1994). The toluene-*p*-sulphonic-acetic acid mixture has been successfully used by Peakman and Maxwell (1988) for the isomerisation of sterenes and for the isomerisation of a C_{25} HBI monoene mixture of all isomer [VII] to the C_{25} monene [VIII] (Hird *et al.*, 1992).



[VII] [VIII]

4.2 Experimental

4.2.1 Isolation of HBI alkenes

Pure samples of the individual HBIs (IV, V and VI) were isolated from bulk cultures of Haslea ostrearia, by extraction of centrifuged algal with hexane (soxhlet, 24 hr) followed by column chromatography on silica and elution with hexane as described previously (Chapter 2). The Δ5 HBI diene [III] was isolated from a Caspian Sea sediment by extraction with hexane, aided by ultrasonication and purified using the same procedure as described above (Belt *et al.*, 1994). Each compound was fully characterised by NMR, GC and GC-MS (Chapter 2, Belt *et al.*, 1994, Belt *et al.*, 1996).

4.2.2 Clay-catalysed rearrangement of $C_{25:3}$ [V] and $C_{25:3}$ [VI]

HBI trienes [V] and [VI] were separately isomerised using an adaption of the method of Sieskind *et al.* (1989). A known ratio of the alkene (*ca* 1 mg) and internal standard, 7-hexylnonadecane (*ca* 300 μg), determined by GC was added to a suspension of K10 montmorillonite clay (BDH; *ca* 100 mg of clay in 10 mL of hexane), in a round bottom flask fitted with a Teflon magnetic stirrer. The solution was stirred at 25°C (water-bath). A blank experiment containing 7-hexylnondecane (300 mg) and a suspension of montmorillonite (*ca* 100 mg in 10 mL: of hexane) only was also conducted. Aliquots (*ca* 1 mL) were removed from the stirred suspension every 24 hr for up to eight days. Each hexane aliquot was filtered through glass wool (DCM rinsed), to remove any clay particles. The residual clay particles were washed with DCM (3 x 0.5 mL) and the washings combined with the hexane filtrate which was then transferred to a vial and the solvents removed (N_2). The mixture was examined by GC-MS and the retention index (RI) and mass spectrum of each isomer were recorded. The concentration of each isomer was expressed as a ratio to that of the internal standard and the recovery of the total mixture was calculated as the sum of the ratio of each individual isomer/total sum of the ratios at day 0.

4.2.3 Tosic acid-catalysed rearrangement of C_{25} HBI alkenes

Four C_{25} HBI alkenes, $C_{25:2}$ [III], $C_{25:2}$ [IV], $C_{25:3}$ [V], $C_{25:3}$ [VI] were separately isomerised by an adaptation of the method of Peakman and Maxwell (1988). Toluene-*p*-sulphonic acid (TsOH) was prepared from the monohydrate by recrystallisation from ethyl acetate and dried under vacuum (50°C). Anhydrous toluene-*p*-sulphonic acid-acetic acid (TsOH-HOAc) was prepared by heating TsOH (1 g) under reflux in HOAc (35 mL) and cyclohexane (10 mL) in a distillation apparatus until the temperature reached 117°C. The remaining solution was allowed to cool and used as required.

Anhydrous TsOH-HOAc (3 mL) was added to a known ratio of the HBI alkene (*ca* 1mg) and the internal standard, 7-hexylnonadecane (*ca* 300 μg)) in a two neck stoppered round bottom flask (25 mL). The flasks were placed in a water bath (25°C) and stirred continuously. Aliquots (200 μL) were removed from each flask over a range of time periods (every five minutes for the first hour, fifteen minutes for the next hour, thirty minutes for the next two hours then every sixty minutes for the next eight hours). Each aliquot was quenched into a vial (2 mL) containing Na_2CO_3 (200 μL). The reaction mixture was diluted with water (200 mL) and extracted in DCM (3 x 200 ml). The combined organic extracts were washed with water (3 x 200 ml), dried ($NaSO_4$) and filtered. Solvent was evaporated (N_2) and the isomeric mixtures were quantitatively examined by measuring the ratio of the area of the HBI peaks with that of the internal standard by GC-MS. The retention index (RI) of each isomer was recorded on two stationary phases, an apolar (HP1) and a polar phase (Carbowax).

4.2.4 Hydrogenation

The mixture at the end of each acid-catalysed rearrangement was dissolved in hexane (2 mL) and added to activated Adam's catalyst (< 1 mg; $PtO_2.H_2O$/hexane; 2 mL; 30min). Hydrogen gas was gently bubbled through the solution for three hours. The retention index, on an apolar (HP1) stationary phase and mass spectra of the hydrogenated products were recorded.

4.2.5 Preparation of samples for characterisation by NMR

The tosic-acid catalysed rearrangement was repeated using a larger quantity of $C_{25:3}$ [VI] for structural analysis by NMR. The triene (3 mg) was mixed with the tosic acid mixture (3 mL) for 24 hours (25°C; waterbath). The mixture was diluted with water (3 mL) and extracted in DCM (3 x 1 mL). The combined organic extracts were washed

with Na_2CO_3 (saturated solution) and dried (anhydrous Na_2SO_4). Solvent was evaporated under a stream of nitrogen gas and analysed by GC-MS and ^1H NMR.

4.2.6 Nomenclature

For clarity, br denotes branched (acyclic) HBIs whilst suspected cyclic compounds whose structures are unknown are referred by the notation used by Barrick and Hedges (1980) where for example $C_{25:2:1}$ denotes a 25 carbon cyclic compound with two double bonds and one ring.

4.3 Results and Discussion

4.3.1 Clay-catalysed rearrangement of br $_{25:3}$ [V] and br $_{25:3}$ [VI]

The action of clay on the HBI triene [V] for six days produced a mixture (78% of the starting material) which by GC was observed to contain two major components, $C_{25:2:1}$ (A) (RI 2094 $_{HP1}$) and $C_{25:2:1}$ (B) (RI 2142 $_{HP1}$) (63% & 28% of the reaction mixture respectively; Figure 4.3). Although they elute in the same region as the HBI alkenes, mass spectral analysis (Figure 4.4) showed the appearance of significant and abundant ions with masses > 100 Da which differentiate these compounds from that of the HBI alkene (*cf* Figure 2.4, Figure 2.5, Figure 2.8). For example, the abundance of ions m/z 149, m/z 261 and m/z 275 for $C_{25:2:1}$ (A) are 100%, 43% and 35% respectively whilst the abundance of the same ions for [V] are 11%, 3% and 1%. The observed mass spectra are more consistent with that of cyclic hydrocarbons (Robson and Rowland, 1994). Further evidence that the compounds are cyclic was obtained by hydrogenation ($Pt_2O.H_2O$, 3hr) of the mixture at the end of the experiment, where the same number of components in the same proportion as that prior to hydrogenation were produced each with a shift in retention index of + 20 ± 2. The mass spectrum of the hydrogenation product of $C_{25:2:1}$ (A) ($C_{25:1:1}$; RI 2114_{HP1}) is shown in Figure 4.5.

The molecular ion at m/z 348 (16%) with major ions at m/z 277 (52%), 263 (43%) and 152 (100%) are also consistent with that of a cyclic compound.

A similar result was observed when the 6(17) triene isomer [VI] was used as the starting material. The final mixture (86% of the starting material) was observed by GC to contain both $C_{25:2:1}$ (A and B; 40% & 15% of the reaction mixture respectively). However, the $C_{25:2:1}$ (C, RI 2119_{HP1}) and $C_{25:2:1}$ (D, RI 2129_{HP1}) considered as minor in the previous reaction were more significant here, with both contributing *ca.* 15% each to the final mixture. The mass spectra of the C and D are both consistent with that of a cyclic hydrocarbon. Indeed the mass spectra of C is identical to that observed for A whilst the mass spectrum of D (Figure 4.6) has a similar fragmentation pattern but differs with respect to the relative intensity of major fragment ions e.g. the ion m/z 261 has a relative intensity of 86% for $C_{25:2:1}$ (D) compared to 43% for $C_{25:2:1}$ (A).

GC analysis of the reaction mixture every 24 hours revealed the presence of another major peak (13% of the reaction mixture; day three) which had an identical RI and mass spectrum to that of the $\Delta 5$ $br_{25:3}$ isomer [V].

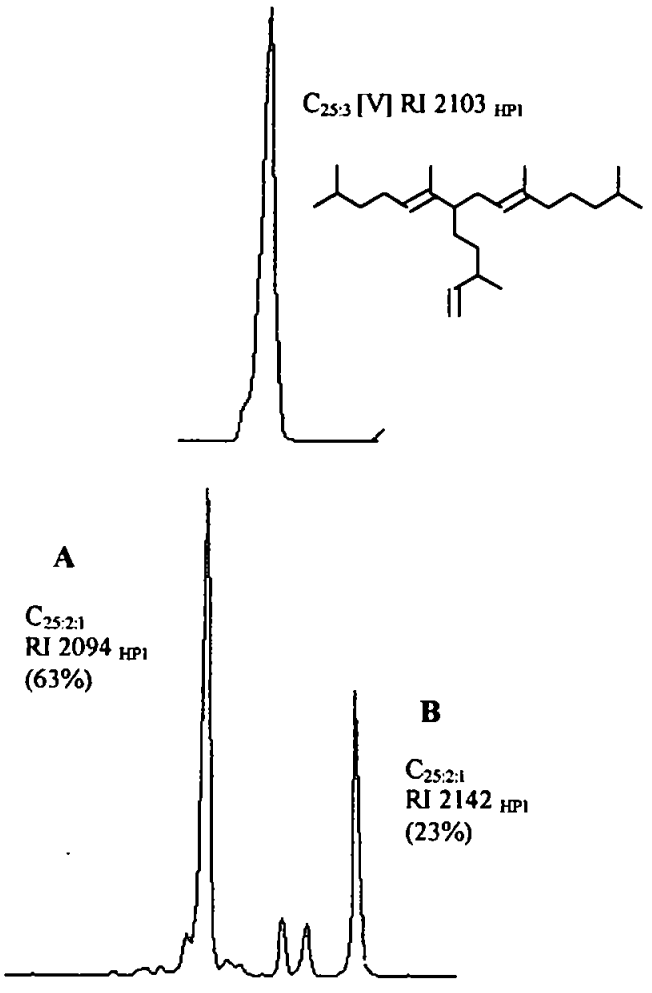


Figure 4.3: Partial Gas chromatograms of the clay-catalysed rearrangement products of the Δ5 $br_{25:3}$ isomer [V] after 0hr (upper) and 6 day (lower). GC conditions; HP1 (12m x 0.3 mm), oven temperature programme 40-300°C at 5°C/min.

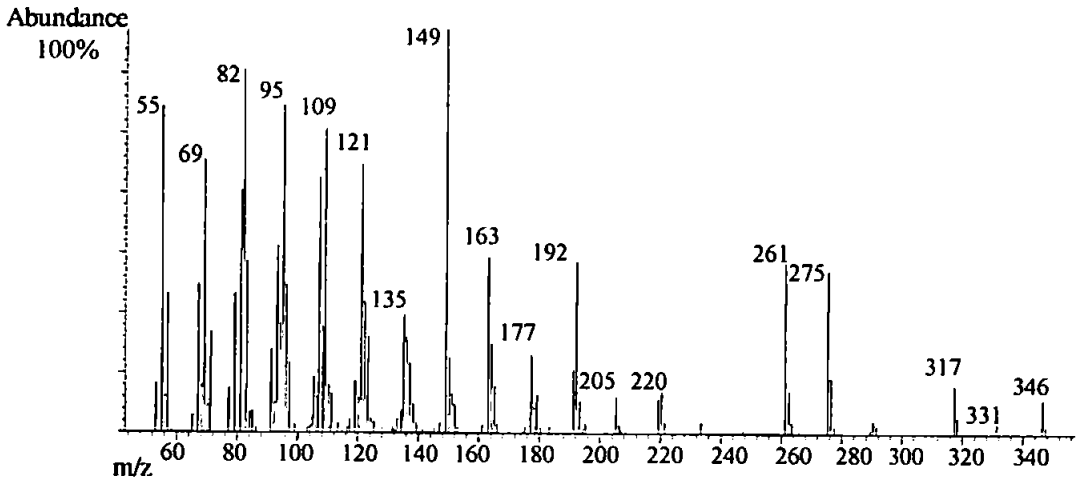


Figure 4.4a Mass spectrum (EI, 70 eV) of compound A $C_{25:2:1}$ [RI 2094]

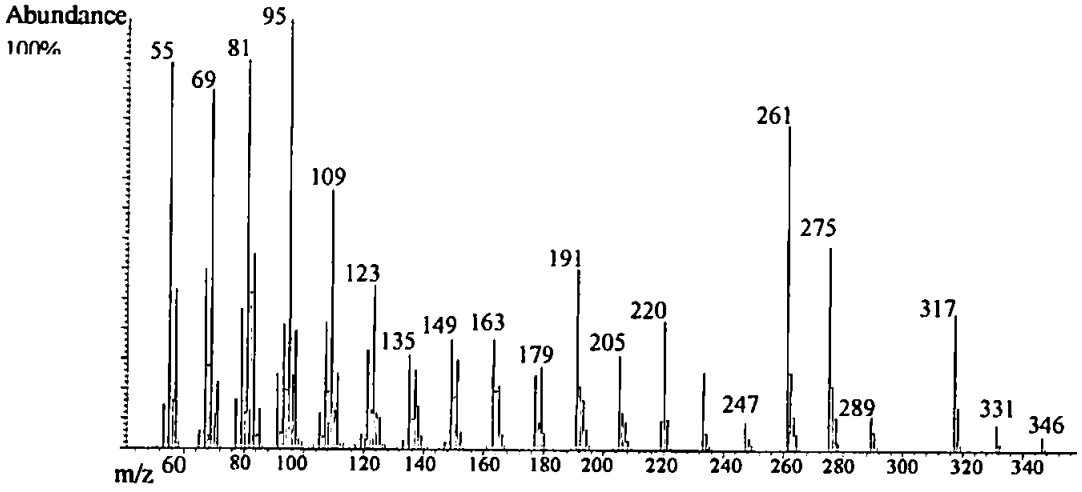


Figure 4.4b Mass spectrum (EI, 70eV) of compound B $C_{25:2:1}$ [RI 2142]

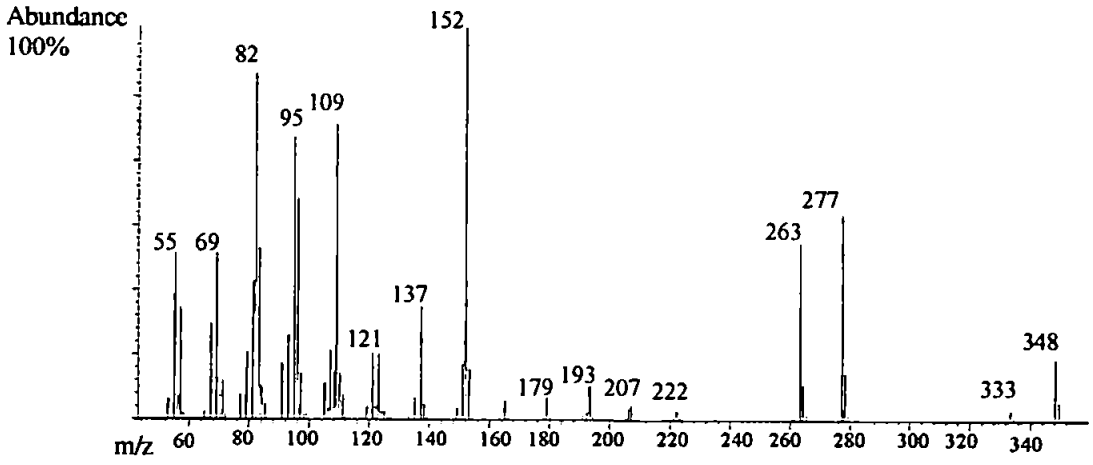


Figure 4.5 Mass spectrum (EI, 70eV) of compound A' $C_{25:1:1}$ [RI $2114_{HP\text{-}1}$]. Hydrogenated product of compound A $C_{25:2:1}$.

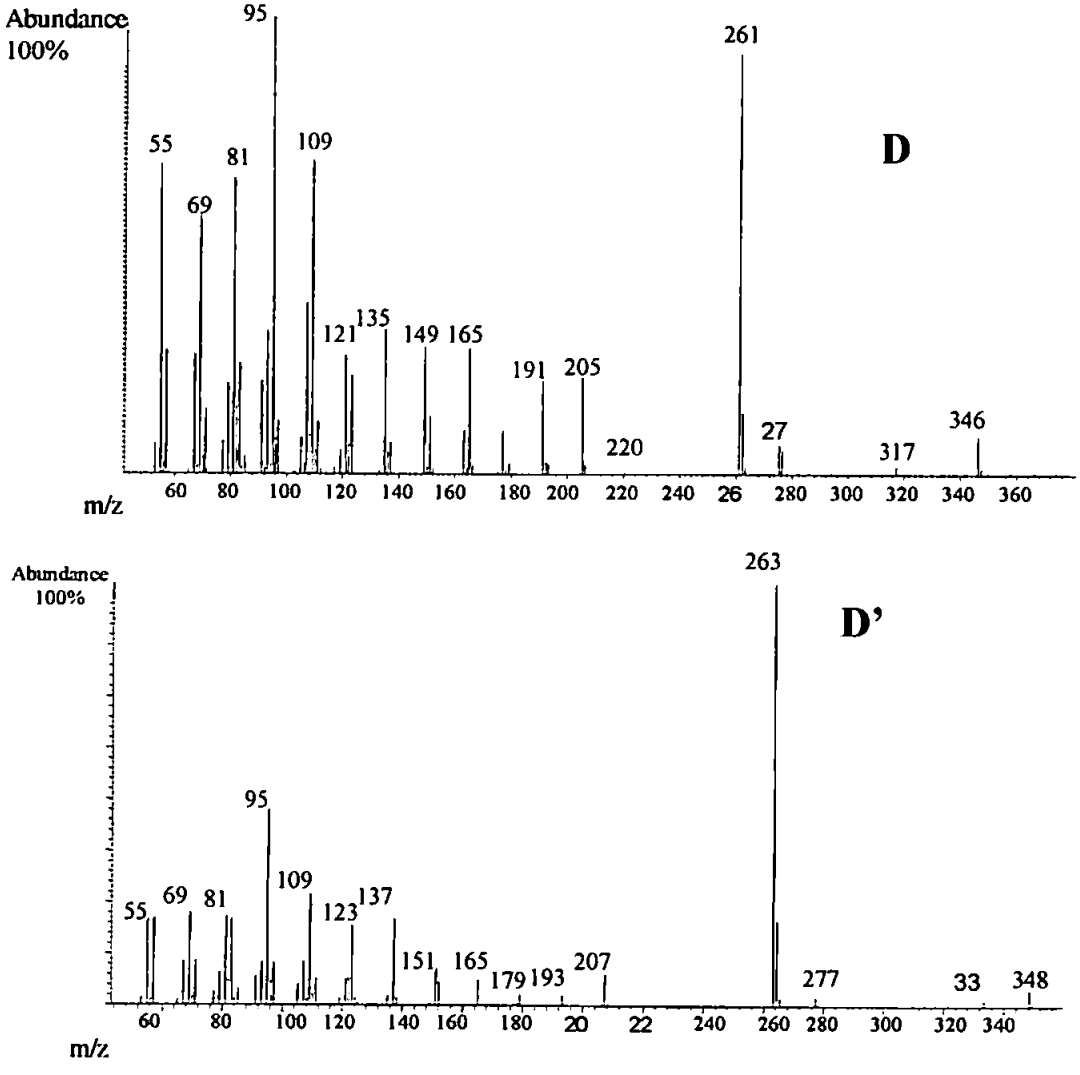


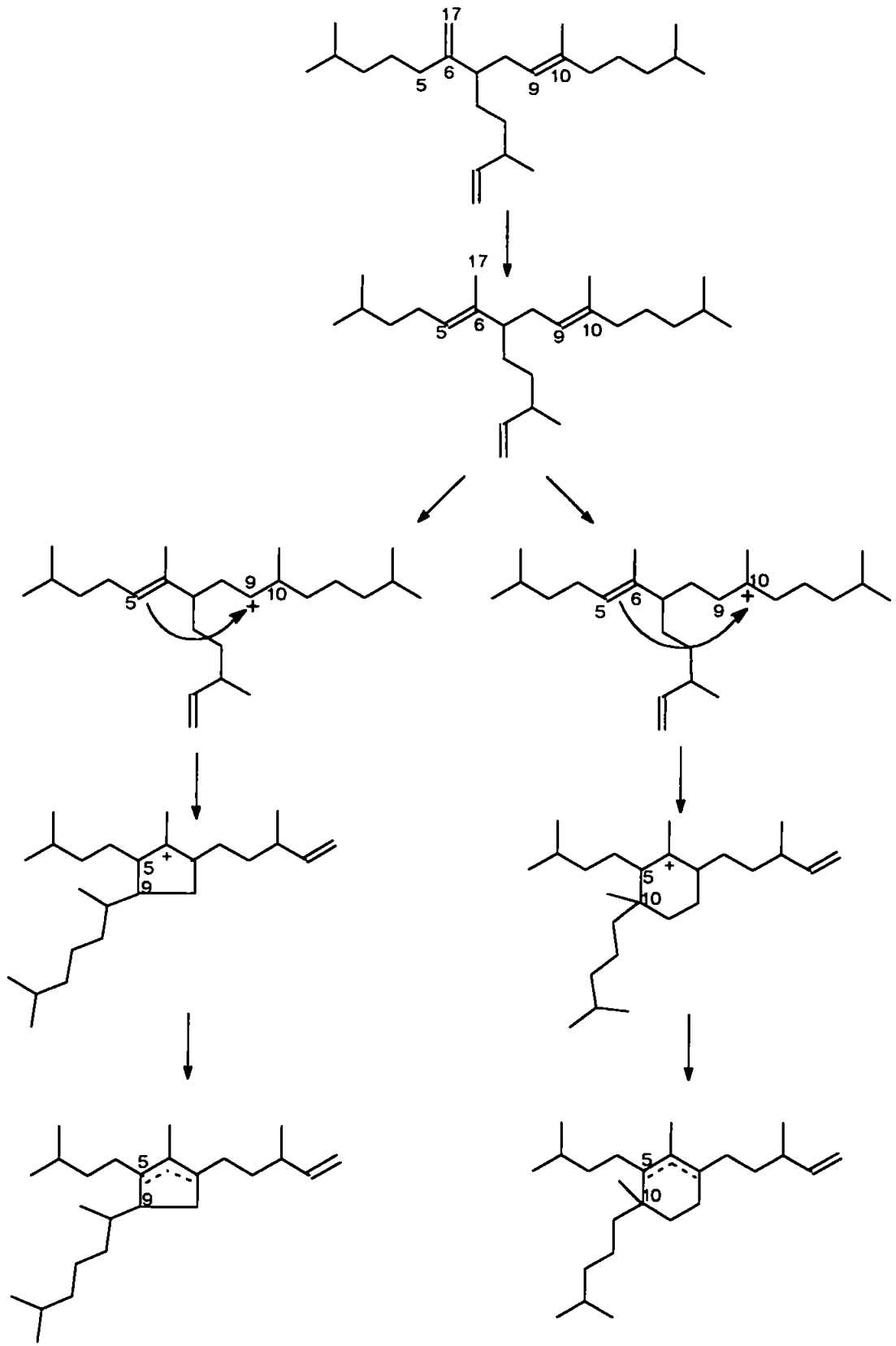
Figure 4.6 Mass spectra (EI 70eV) of compound D $C_{25:2:1}$ (RI 2129_{HP1}) and D' $C_{25:1:1}$ (RI 2125_{HP1}) the hydrogenated product of D.

4.3.2: Structural characterisation

The observations from the clay-catalysed rearrangement of both triene isomers suggests that $C_{25:2:1}$ (RI 2094) is formed by the cyclisation of the Δ5 isomer [V] and not the 6(17) isomer [VI]. Cyclisation of [V] could occur through various pathways all producing different isomers. However ^{1}H NMR and mass spectral analysis is consistent with only two of these isomers, a cyclopentene [IX] and a cyclohexene [X]. The proposed mechanism for the formation of [IX] and [X] is outlined in Figure 4.7. The protonation of C9 (from K-10 clay or TsOH-HOAc) results in the formation of a tertiary carbocation intermediate at C10. This in turn undergoes electrophilic ring closure with C5 yielding a further intermediate carbocation at C6. Elimination of a proton from C5 or C7 results in the formation of the cyclohexene X. The formation of cyclopentene IX results from the coupling of C5 with a secondary carbocation at C9.

The mass spectrum of $C_{25:2:1}$ (RI 2094; Figure 4.4) is consistent with both structures [IX] and [X]. The proposed fragmentation pathways are presented in Figure 4.8. The mass spectrum (Figure 4.5) of the hydrogenated product, $C_{25:1:1}$ is also consistent with that of the structures [IX] and [X] (Figure 4.8).

However, formation of the cyclohexene structure [X] is more likely owing to the formation of a tertiary carbocation intermediate at C10 (Figure 4.7) and the absence of the ion at m/z 233 in the mass spectrum of $C_{25:2:1}$ (RI 2094; Figure 4.4a).



[IX] [X]

Figure 4.7 Proposed mechanism for the formation of the cyclopentene [IX] and cyclohexene [X] isomers

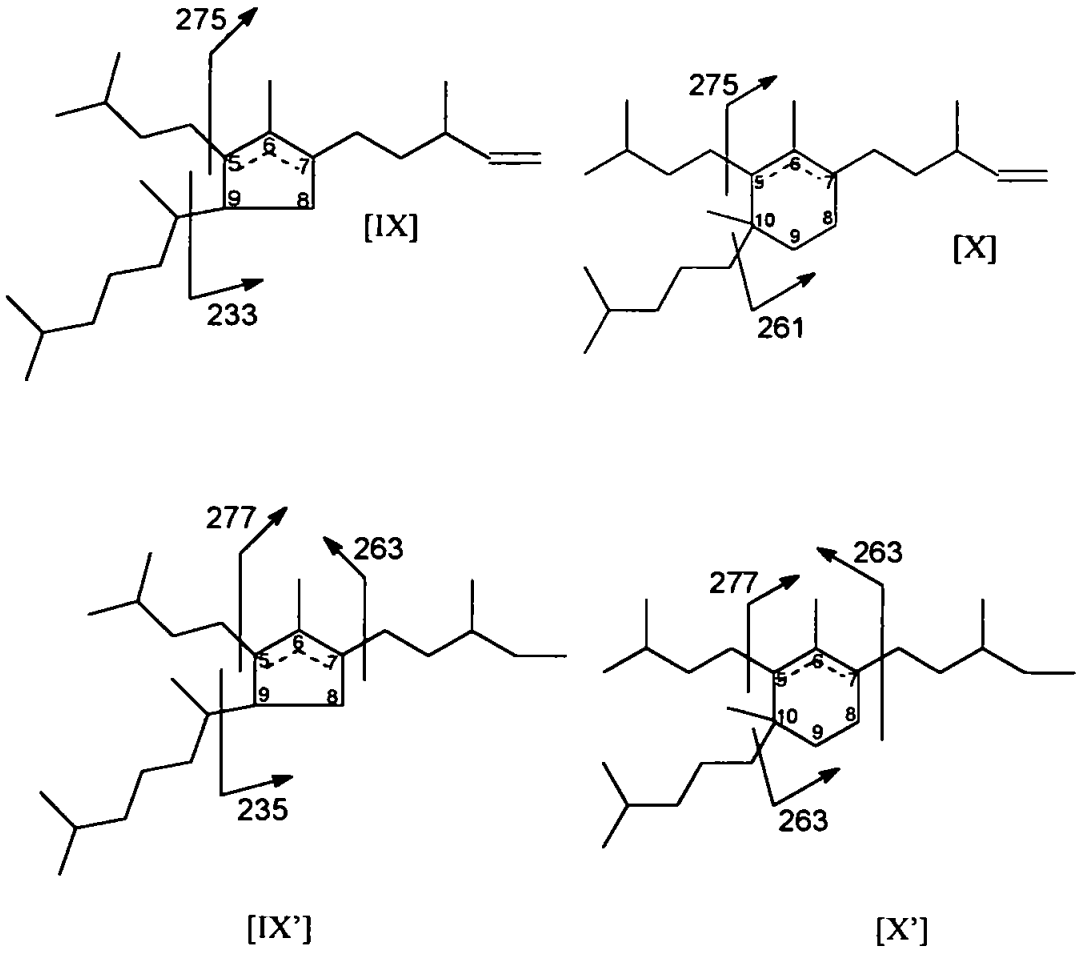


Figure 4.8: Proposed mass spectral fragmentation pathway of [IX] and [X] and the corresponding hydrogenated products [IX'] and [X'].

NMR analysis

Tosic-acid rearrangement of the 6(17) triene isomer (3 mg) afforded sufficient amounts of the reaction mixture to perform ^{1}H NMR analysis. The reaction mixture by GC contained the three monocyclic products, RI 2094 (35%), RI 2119 (15%) and RI 2129 (22%). The rest of the mixture (28%) consisted of minor components, each less than 8% of total mixture. Attempts at purifying the mixture by column chromatography were unsuccessful, hence ^{1}H NMR was carried out on the mixture.

The characteristic NMR spectroscopic features of HBI triene [VI] (Belt *et al.*, 1996) correspond to the vinyl ($-C_2H_3$) functionality (δ 5.67 ppm, H23; 4.85, 4.94 ppm, H24), a methylenic double bond (δ 4.70, 4.74 ppm H17) and a tri-substituted double bonds (δ 5.06 ppm). For the TsOH-HOAc reaction mixture, the only resonances detected in the alkene region of the ^{1}H NMR spectrum corresponded to the vinyl group (δ = 5.70 ppm, H23, 4.89, 4.96 ppm H24). This provides further evidence for the formation of a cyclic structure resulting from the coupling of 2 double bonds. No further examination of the ^{1}H spectrum was carried out owing to the impurity of the sample.

4.3.3 Tosic-acid catalysed rearrangement of HBI alkenes

The results of tosic-acid catalysed rearrangement on four authenicated HBI alkenes (chapter two, Belt *et al.*, 1994, 1996) are described below. The chosen HBI alkenes were the two dienes [III] and [IV] and the two-aforementioned trienes [V] and [VI]. GC-MS data was collected over a twelve-hour period and quantitatively analysed using 7-hexylnonadecane as an internal standard. Gas chromatgraphic analysis was carried out on apolar (HP1) and polar phases (Carbowax). The analysis of quantitative data was facilitated by using the Carbowax stationary phase owing to better separation of the observed rearranged products.

Tosic acid catalysed rearrangement of $br_{25:2}$ [III]

The results of the time course experiment for $br_{25:2}$ [III], which has a double bond in the 5,6 position, are shown in Figure 4.9. The final mixture (99 % of the starting material) was observed by GC. to contain no new isomers. However, a slow and partial isomerisation of the E 5 isomer (RI 2062 $_{Carbowax}$, 2079 $_{HP1}$) to the corresponding Z isomer (RI 2036 $_{Carbowax}$, 2052 $_{HP1}$). was observed. For example, after 12 hours of reaction between [III] and TsOH-HOAc the relative concentrations of the E and Z isomers (GC) was found to be 9 as compared with >30 at the outset of the reaction. Identification of the isomerisation product of this reaction as the Z isomer was made by comparison of the extremely similar mass spectrum (Figure 4.10) for this compound with that of the authenicated E isomer.

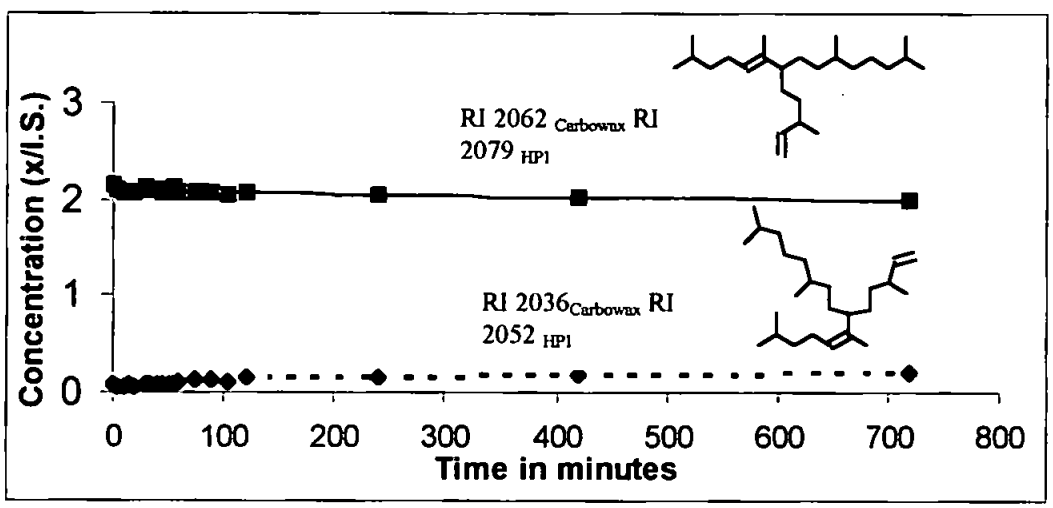


Figure 4.9 Tosic acid-catalysed rearrangement of sedimentary $br_{25:2}$ [III]

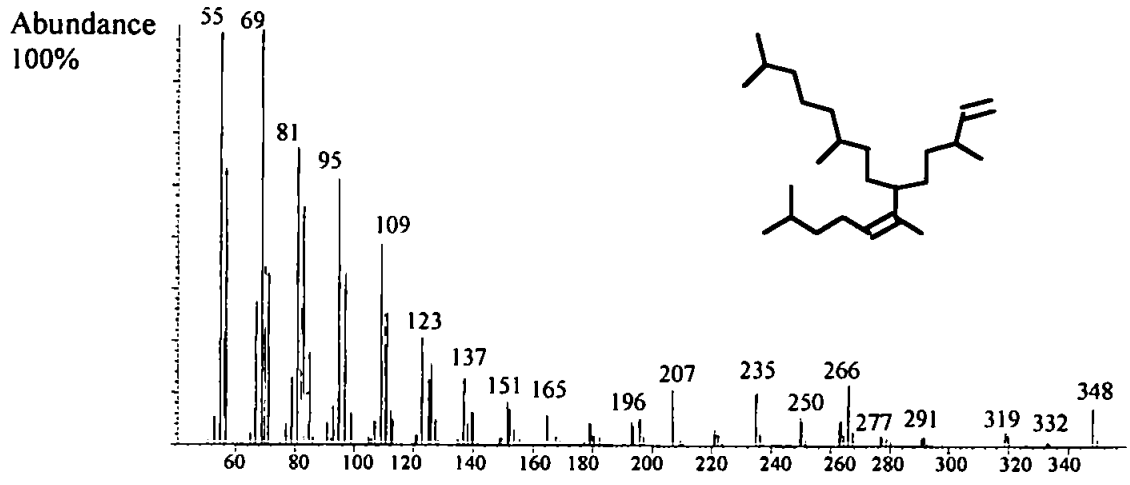


Figure 4.10 Mass spectrum (E.I. 70 eV) of proposed 5 Z isomer of [III] (RI 2036 $_{Carbowax}$ 2052$_{HP1}$)

Tosic acid catalysed rearrangement of br$_{25:2}$ [IV]

The reaction of the 6(17) $C_{25:2}$ [IV] with TsOH-AcOH resulted in the complete isomerisation to a mixture of the E and Z Δ5 $C_{25:2}$ [II] in *ca* 2 hours (Figure 4.11). Identification of the E isomer, Δ5 $C_{25:2}$ (II; RI 2062, 2067 $_{Carbowax}$, 2079 $_{HP1}$) was confirmed by co-chromatography of the mixture with the authentic Δ5 $C_{25:2}$ (III; RI 2062 $_{Carbowax}$ 2079 $_{HP1}$) on apolar and polar phases. The final mixture (88% of the starting material; 12 hr) was observed to contain mainly the E Δ5 br$_{25:2}$ [III] (77%) with minor amounts of the Z Δ5 br$_{25:2}$ [III] isomer (18%) and the starting material (5%). Therefore the final E/Z ratio is 4.3. Molecular mechanics calculations performed on these two isomers has shown that the E isomer is expected to be more stable than the Z isomer by 0.9 kcal mol^{-1}, corresponding to a thermodynamic ratio of 4.6 which is excellent agreement with the experimental result (Belt *et al.*, in preparation).

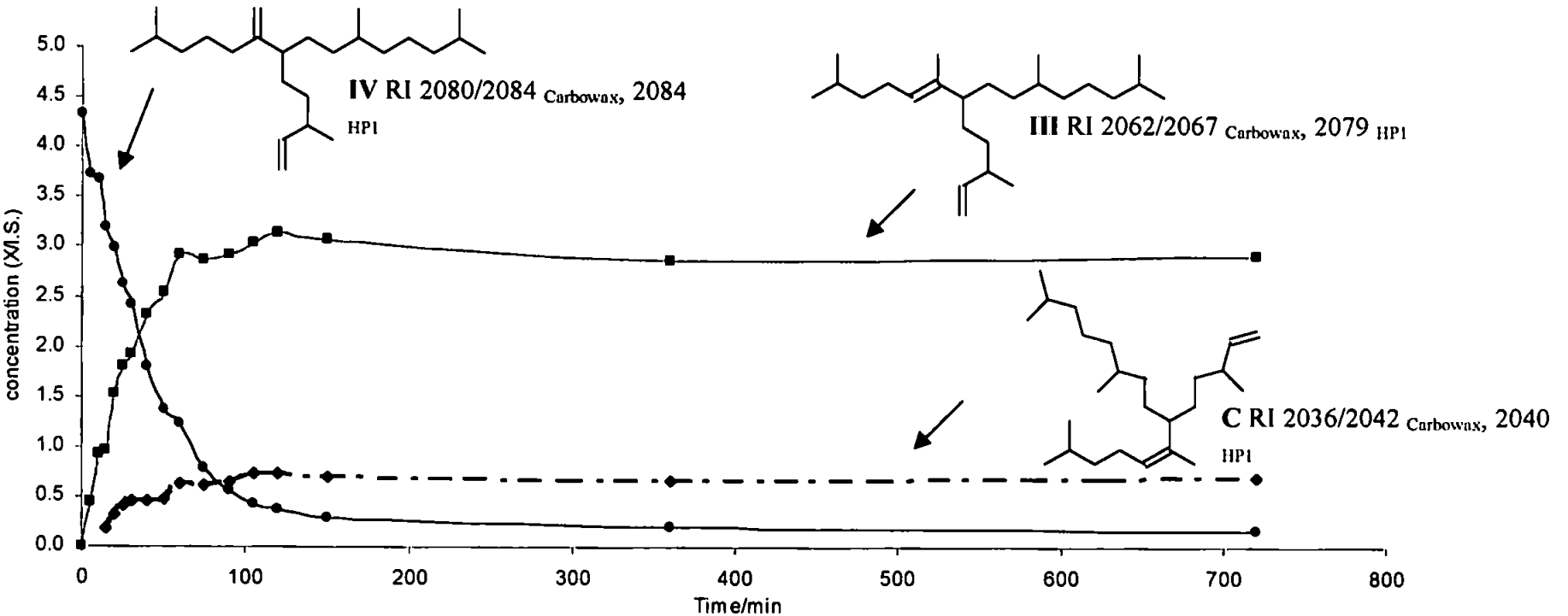


Figure 4.11 Tosic-acid catalysed rearrangement of $br_{25:2}$ [IV]

Tosic acid catalysed rearrangement of $br_{25:3}$ [V]

The results of the time course experiment of tosic acid catalysed rearrangement of $br_{25:3}$ (V; RI $2130_{Carbowax}$) are presented in Figure 4.12. For clarity there are two plots. Figure 4.12a shows the formation of the acyclic HBIs over time whilst Figure 4.12b shows the formation of the cyclic alkenes.

The graphs clearly show that the concentration of $br_{25:3}$ [V] decreases with the formation of eight new isomers. The retention indices (Carbowax and HP1) and mass spectra of each isomer are presented in Table 4.1. Seven of the eight isomers were observed in the clay catalysed rearrangement of [V] the other is only observed when analysis is undertaken on the Carbowax phase. Three of these isomers (RI 2089, 2108 and 2138 $_{Carbowax}$; Figure 4.12a) show the characteristic mass spectra of acyclic HBI trienes, with a molecular ion of M^+ 346, and diagnostic fragment ions m/z 233 and m/z 261 owing to fragmentation at the C7 branch point. Indeed the $br_{25:3}$ RI 2089 has a nearly identical mass spectrum to that of the starting material, [V]. This can clearly be shown by a plot of the percentage abundance of all the mass ions of RI 2089 against the percentage abundance of all the mass ions of $br_{25:3}$ [V] (RI 2130) which shows a good correlation (R^2 = 0.99; Figure 4.13). This similarity between the mass spectra suggests that $br_{25:3}$ (RI 2089) is a geometric isomer of Δ5E, 9E $br_{25:3}$ (V; RI 2130). The isomers RI 2089 and 2108 are very minor, (max. concentration < 10% of reaction mixture) and are represented on the graph as the sum of the two. Although the acyclic $br_{25:3}$ RI 2138 might also be considered a good candidate for a geometric isomer of br $_{25:3}$ [V] when the abundance (%) of mass ions for br $_{25:3}$ RI 2138 and br $_{25:3}$ [V] were plotted against each other a poorer correlation was observed (r^2 = 0.80).

The other five isomers (Figure 4.12b) possessed mass spectra characteristic of cyclic compounds (i.e. appearance of significant and abundant ions with masses > 100 Da).

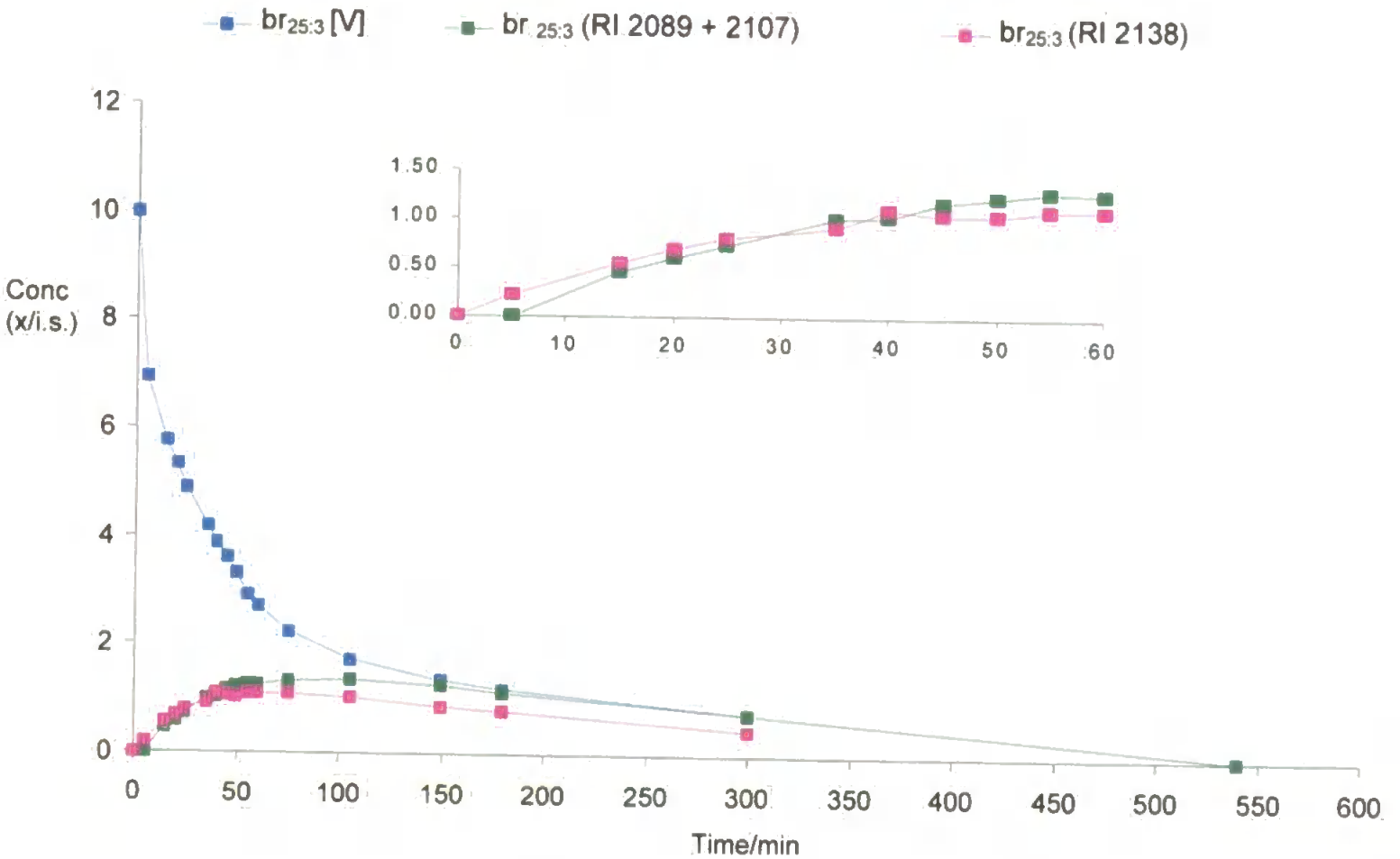


Figure 4.12a Acid catalysed rearrangement of $br_{25:3}$ [V] showing the acyclic isomers only

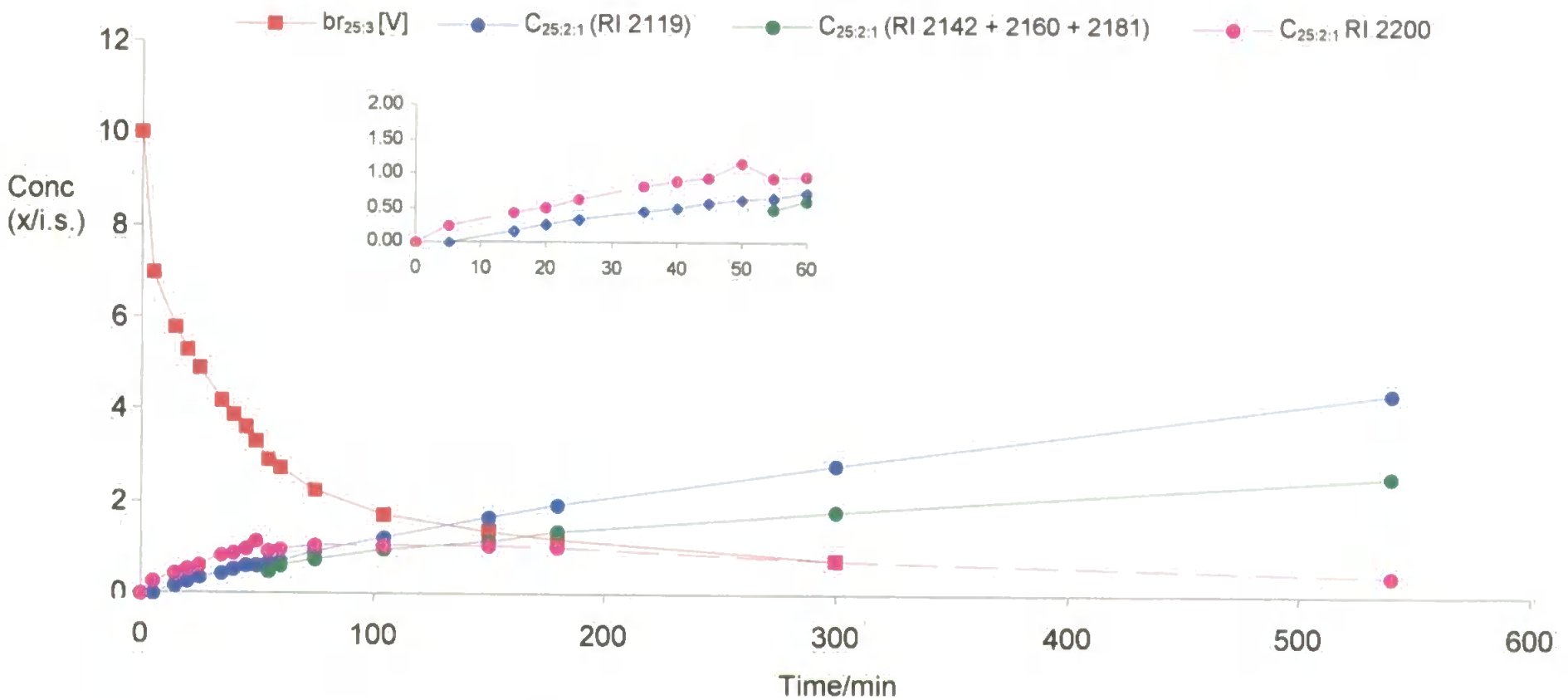


Figure 4.12b Acid catalysed rearrangement of $br_{25:3}$ [V] showing the cyclic isomers and $br_{25:3}$ [V] only

Table 4.1: Mass spectral details and retention indices of products formed by the acid –catalysed rearrangement of $br_{25:3}$ [V] and [VI]

Compound	Retention Index		Mass Spectral ions m/z (rel. int) 70eV
	HP1	Carbowax	
br $_{25:3}$	2067	2083/2089	55 (75), 69 (93), 81 (79), 95 (100), 109 (42), 123 (20), 137 (18), 151 (16), 163 (4), 178 (3), 191 (2), 207 (4), 233 (6), 261 (4), 276 (3), 289 (2), 327 (2), 346 (6).
br $_{25:3}$	2076	2101/2108	55 (97), 69 (99), 81 (89), 83 (79), 95 (100), 109 (55), 123 (10), 159 (16), 163 (4), 177 (6), 191 (2), 208 (5), 233 (4), 261 (4), 275 (2), 289 (1), 317 (1), 331 (1), 346 (4)
$C_{25:2:1}$	2094	2119	55 (85), 69 (67), 81 (73), 82 (100), 95 (90), 109 (79), 121 (59), 135 (34), 149 (87), 163 (37), 177 (21), 192 (49), 205 (11), 220 (11), 233 (2), 248 (2), 261 (42), 275 (35), 291 (1), 317 (9), 330 (2), 346 (4).
br $_{25:3}$	2103	2131/2138	55 (69), 69 (77), 81 (69), 82 (69), 95 (100), 109 (37), 123 (36), 149 (19), 192 (7), 233 (2), 261 (7), 275 (3), 346 (1).
$C_{25:2:1}$	2121	2142	55 (97), 69 (78), 82 (100), 95 (90), 109 (83), 123 (49), 135 (23), 149 (82), 163 (42), 177 (14), 192 (40), 205 (14), 220 (16), 233 (5), 261 (38), 275 (28), 290 (3), 317 (7), 335 (3), 346 (3).
$C_{25:2:1}$	2129	2161	55 (54), 69 (47), 81 (40), 89 (60), 95 (100), 103 (48), 109 (64), 123 (21), 135 (22), 149 (22), 165 (24), 177 (14), 191 (11), 205 (19), 219 (5), 261 (88), 346 (5).
$C_{25:2:1}$		2181	55 (92), 69 (95), 81 (100), 95 (93), 109 (79), 123 (34), 137 (22), 149 (26), 163 (22), 191 (36), 261 (45), 275 (19), 317 (18), 331 (3).
$C_{25:2:1}$	2142	2200	55 (97), 69 (86), 81 (92), 95 (100), 109 (60), 123 (36), 135 (18), 149 (19), 163 (21), 177 (10), 191 (26), 205 (11), 220 (12), 233 (9), 247 (3), 261 (30), 275 (19), 289 (3), 317 (14), 331 (3), 346 (2).

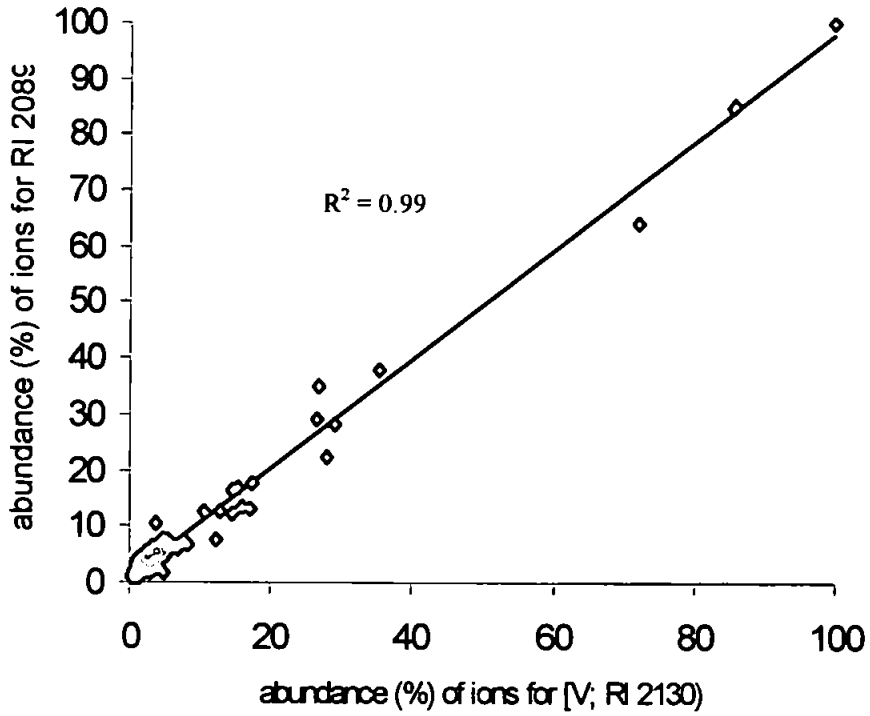


Figure 4.13 A graph showing the good correlation between the mass spectral data for $br_{25:3}$ RI 2089 and $br_{25:3}$ [V]

As observed with the clay-catalysed rearrangement in the early stages of the reaction (< 100 minutes) two monocyclic isomers are present in appreciable amounts, $C_{25:2:1}$ (A) (RI 2119 $_{Carbowax}$ 2094 $_{HP1}$), whose structures have tentatively been assigned as the cyclohexene [X] or the cyclopentene [IX] and $C_{25:2:1}$ (RI 2200 $_{Carbowax}$ 2142 $_{HP1}$). The other three monocyclic compounds are not detected until 55 minutes into the reaction (Figure 4.12b; inset). The presence of the cyclohexene/pentene alkene (RI 2119) as one of the dominant new isomers early on in the reaction (10 minutes) gives further evidence that this monocyclic compound is formed directly from the starting material [V]. In addition it can be observed that the concentrations of $br_{25:3}$ RI 2138 and $C_{25:2:1}$ RI 2200 are observed to decrease with the concurrent formation of new minor $C_{25:2:1}$ isomers RI 2142, RI 2161 and RI 2181.

Tosic acid catalysed rearrangement of $br_{25:3}$ [VI]

The results of the time course experiment of br $_{25:3}$ 6,17 [VI] under acid conditions are presented in Figure 4.14. Similar to that of the $br_{25:3}$ [V], described previously, there are two plots shown. Figure 4.14a shows the formation of the acyclic HBIs over time whilst Figure 4.14b shows the formation of the cyclic alkenes over time. Owing to the diastereomeric nature of [VI], two peaks are observed for each diastereomeric pair when monitoring the reaction on the polar phase (Carbowax) as opposed to one peak on the apolar phase (HP1; refer to Chapter two).

The decrease in concentration of the diastereomeric mixture of $br_{25:3}$ [VI] can clearly be observed with the formation of ten new isomers. Nine of these isomers were observed in the acid-catalysed rearrangement of [V], the other is a minor acyclic triene with a RI of $2094/2101_{Carbowax}$ (mass spectral data in Table 4.1). Five of these isomers (RI 2083/2089, 2094/2101, 2101/2108, 2124/2130, 2131/2138 $_{carbowax}$) are acyclic HBIs (Figure 4.14a). Three of five of these isomers are very minor, (max. concentration < 7% of reaction mixture) and are therefore represented on the graph as the sum of the three. The major acyclic isomer formed was the Δ5 isomer [V]. Indeed in the early stages (< 150 minutes) this isomer was the dominant isomer in the reaction mixture with a maximum concentration of 31% of the reaction mixture (30 minutes). The other five isomers are cyclic (RI 2119, 2142, 2160, 2181, $2200_{Carbowax}$). The plot of formation of the cyclic compounds against time is very similar to that observed with the Δ5 isomer [V] (Figure 4.12b) and the $C_{25:2:1}$ (A) (RI 2119 Carbowax) is the major isomer (68% of the reaction mixture at the end of the experiment). However it can be observed that in the early stages of the experiment (< 60 minutes, inset Figure 4.14b) the monocyclic isomer $C_{25:2:1}$ (D) RI 2160 was produced by 15 minutes compared to 55 minutes in the acid-rearrangement of the br $_{25:3}$ [V]. This could suggest that this monocyclic product be formed by cyclisation involving C6 as opposed to the proposed pathway of C5 for the formation of $C_{25:2:1}$ [A].

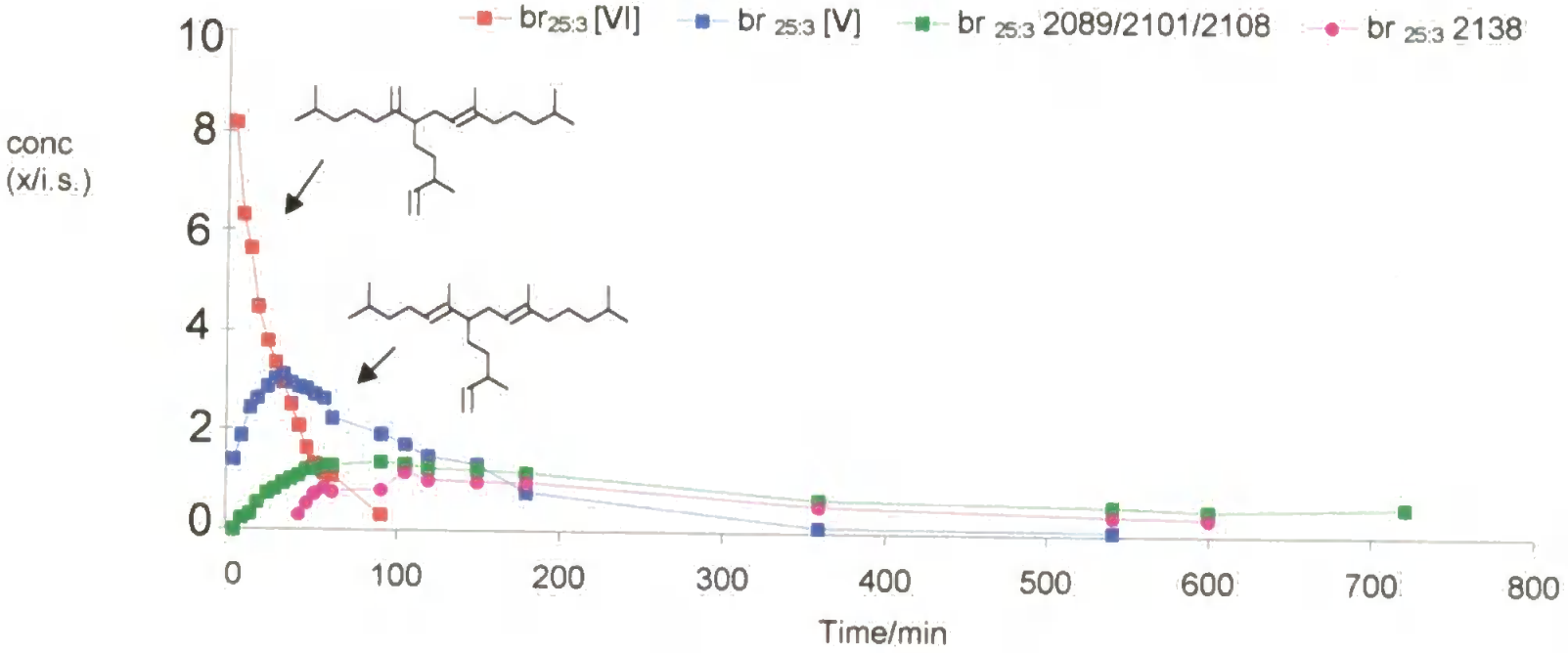


Figure 4.14a Acid catalysed rearrangement of $br_{25:3}$ [VI] showing the acyclic isomers only

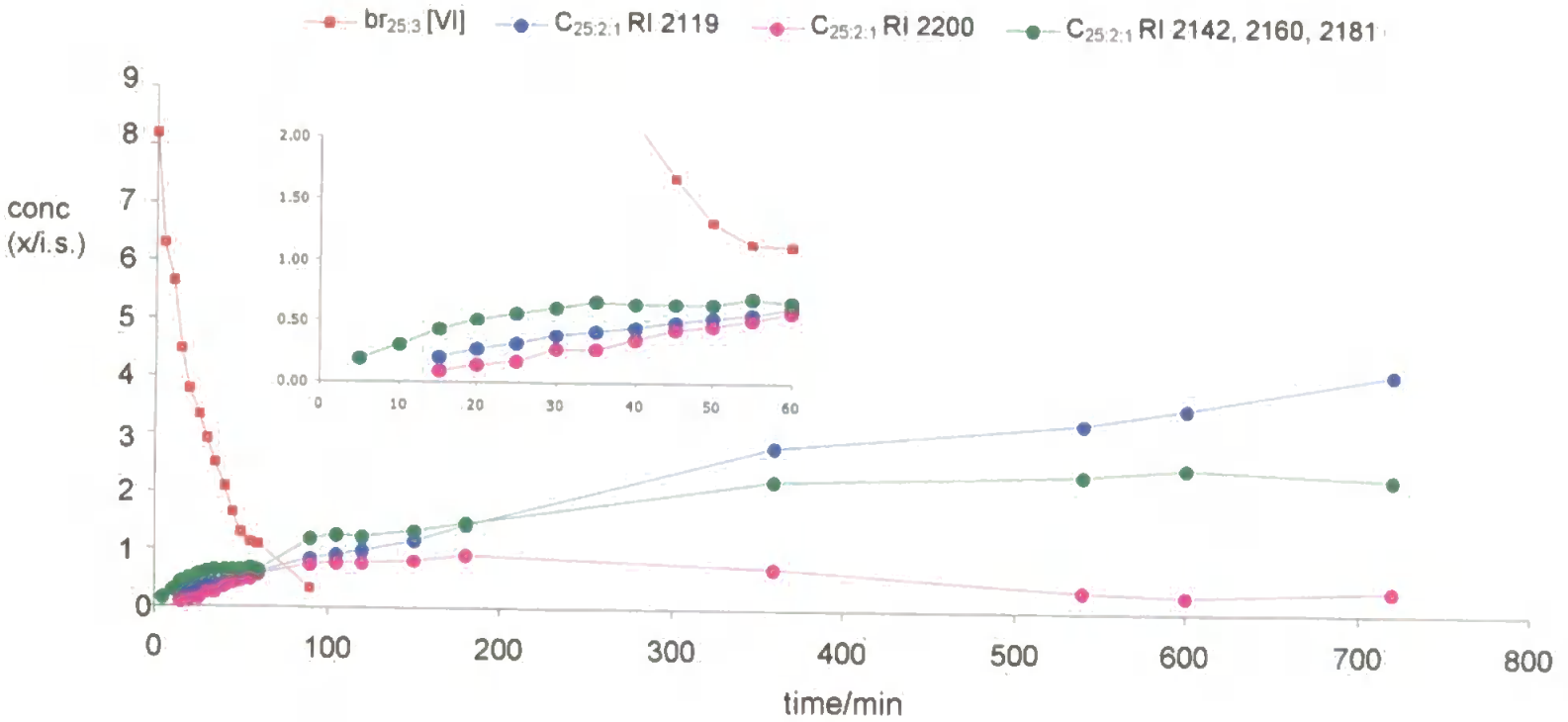


Figure 4.14b Acid catalysed rearrangement of $br_{25:3}$ [VI] showing the cyclic isomers and $br_{25:3}$ [VI] only

The results may give a partial explanation why HBI dienes are observed to be more abundant in depth profile studies (Rowland *et al.*, 1990 and references cited therein) than more unsaturated HBIs. The facile isomerisation of the 6(17) br $_{25:2}$ [III] to the Δ5 br $_{25:2}$ [II] and the resistance of [II] to further isomerisation contrasts with the rapid cyclisation of the trienes [V] and [VI].

There have been numerous reports of unidentified C_{25} cyclic alkenes in geological samples (Rowland and Robson, 1990 and reference cited therein). However, it is thought that many of these compounds have been mis-assigned owing to incomplete hydrogenation and are C_{25} HBIs (Rowland *et al.*, 1990).

Analysis herein of the nonaromatic hydrocarbon fraction of Pleistocene and Pliocene East Mediterranean sapropels[1] contained at least nine unknown compounds, some in high concentrations, eluting between RI 2100 and 2200 and with a molecular weight of 348 Da. Two main types of mass spectra were found to be common to all nine hydrocarbons. Interestingly, four of these compounds had a similar mass spectra (Figure 4.15) to one of the minor hydrogenated products from the acid-catalysed rearrangements ($C_{25:1:1}$ (D' RI $_{Carbowax}$, 2151 $_{HP1}$; Figure 4.6b) and one of the four also had a similar retention index (RI 2155 $_{HP1}$) (Rullkötter *et al.*, 1998).

The minor monocyclic compound, $C_{25:2:1}$ (D, RI 2129 $_{HP1}$, 2160 $_{Carbowax}$) is thought to be formed by cyclisation involving C6 as opposed to the proposed pathway of C5 for the formation of $C_{25:2:1}$ [A]. Therefore it is reasonable to suggest that compound D has a cyclopentene structure resulting from the formation of a tertiary carbocation intermediate at C6, which undergoes electrophilic ring closure with C10. The proposed mechanism for the formation of compound D is outlined in Figure 4.16.

[1] Donated by Rullkötter, Institut fur Chemie und Biologie des Meeres, Oldenburg, Germany (Rullkötter *et al.*,1998).

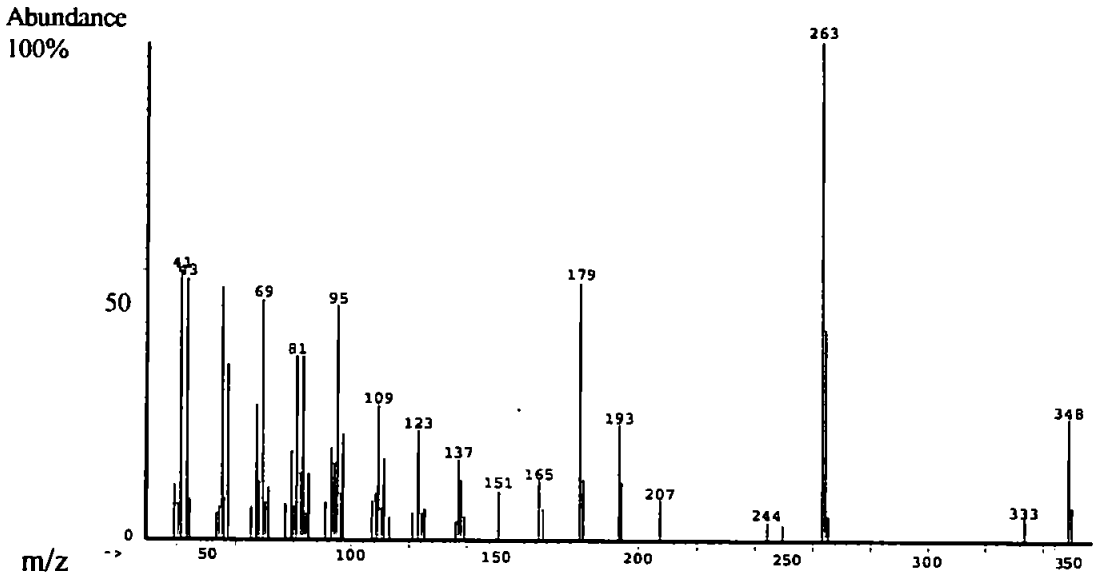


Figure 4.15 Mass spectrum (EI, 70 eV) of $C_{25:1:1}$ (RI 2155_{HP1}) in the hydrocarbon fraction of a sapropel fraction in the Ionian Basin of the Eastern Mediterranean Sea

4.4 Conclusion

Four HBI alkenes, whose structures have been fully characterised were subjected to laboratory simulation of early diagenetic reactions in sediments. Under mild acid conditions (i.e. K-10 Montmorillonite clay and TsOH-HOAc) the products of these reactions were observed to be highly dependent on the degree of unsaturation of the HBI alkenes.

<u>C_{25} dienes</u>

- A slow and partial isomerisation of the E 5 isomer (RI 2062 $_{Carbowax}$, 2079 $_{HP1}$) to the corresponding Z isomer (RI 2036 $_{Carbowax}$, 2052 $_{HP1}$) was observed as the only reaction for the Δ5 C_{25} diene III.
- Complete isomerisation to a mixture of E and Z isomers of Δ5 diene III (E/Z ratio = 4.3; 12 hr) was observed for the 6(17) C_{25} diene IV.

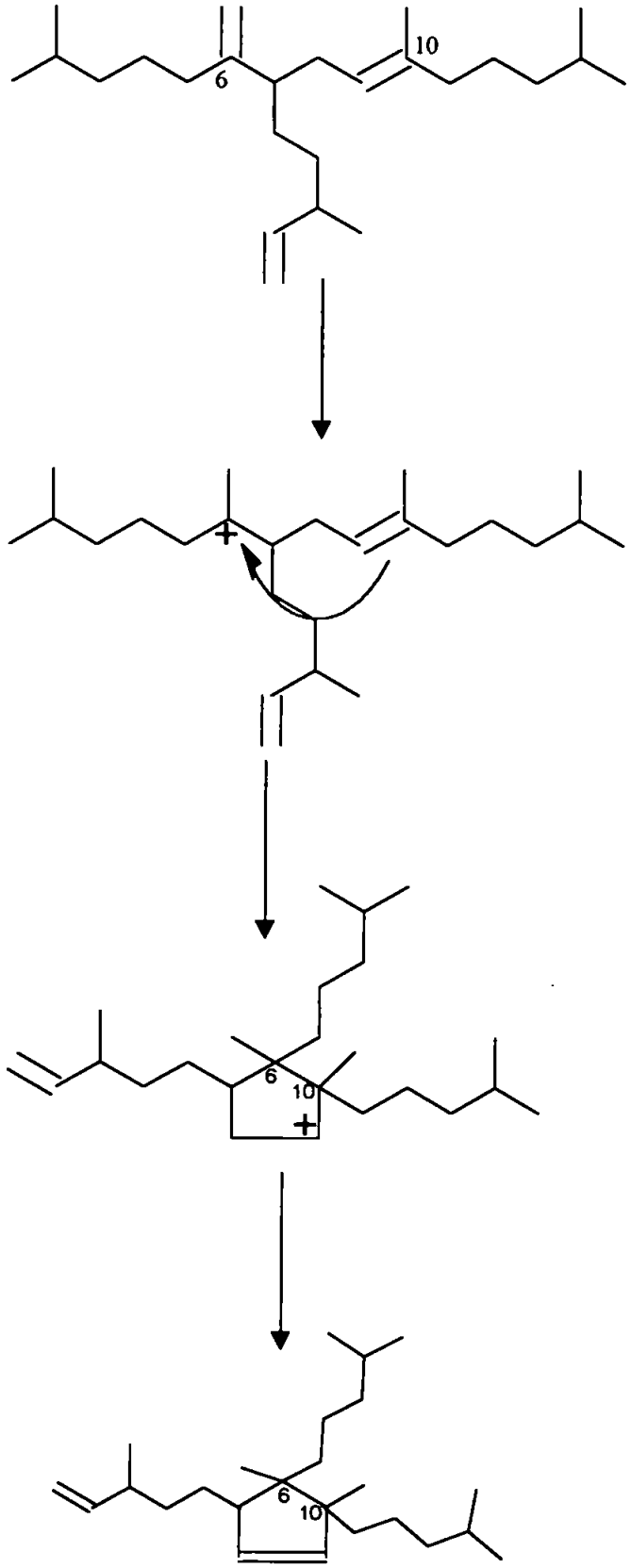


Figure 4.16 Proposed mechanism for the formation of the compound D (RI 2151_{HPI}) [XI]

C_{25} trienes

- Isomerisation and rapid cyclisation reactions were observed for the two C_{25} trienes V and VI.
- Evidence suggests that the major compound produced at the end of the acid-catalysed reaction for both trienes [V] and [VI], was a substituted cyclopentene IX or cyclohexene X (Figure 4.7).

The resistance of C_{25} diene III to further short-term changes could partially explain the widespread occurrence of the HBI dienes and the observation that they appear to be more abundant in depth profile studies than more unsaturated HBIs.

A previously unknown compound detected in the sapropel fraction of the Eastern Mediterranean Sea (Rullkötter *et al.*, 1998) has been tentatively identified as a substituted cyclopentene (Figure 4.16).

Experimental Details

Chapter Five Experimental

This chapter describes the general procedures and instrumentation used throughout the project. Experimental details are described within each chapter.

5.1 General Procedures

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

Silica gel (BDH; 60-120) was used as absorbents in column chromatography and was solvent extracted (soxhlet; DCM; 24hr). Silica was activated by heating to 180 ^{0}C (24 hr). Deactivated silica gel was prepared by shaking (4 hours) the absorbent with the appropriate quantity of Millipore grade water and stored under vacuum.

Anhydrous sodium sulphate, cotton wool, Montmorillonite K-10 clay, water (MilliQ-grade), hydrochloric acid and glacial acetic acid were all extracted with DCM before use to remove trace organic impurities.

5.2 Column Chromatography

The HBI alkenes were separated from the total hexane extract (THE) of different cultures of *Haslea ostrearia* (i.e. Soproma 34 (901 mg), Sodexal 18 (103 mg), BP003 (1000 mg) and the total chloroform extract (TCE; 197 mg) of *Rhizosolenia setigera* using column chromatography. The THE of Soproma 34 and BP003 were further divided into four approximately equal parts. These and the THE and TCE of the other samples were adsorbed

onto alumina (1 g) and applied to a column (20 cm x 1.0 cm i.d.) containing activated silica (5 %) and eluted with hexane (300 mL), DCM (100 mL) and 1:1 DCM/methanol (100 mL). The hexane elutant was collected in 10 mL vials and the separation of the alkenes was monitored by GC-MS and the respective fractions combined. The elution order of fractions for each sample were as follows:

Soproma 34	THE	4 x *ca* 225 mg	Total weight = 901 mg	
Compound	$RI_{(HP1)}$	volume of eluant	purity	Weight/mg
HBI diene	2084	30 - 60 mL	> 99 % purity	3.1
HBI triene	2106	60 - 90 mL	> 99 % purity	27
HBI tetraene	2144	100 - 200 mL	> 95 % purity	4.8

BP003	THE	4 x ca 250 mg	Total weight = 1000 mg	
Compound	$RI_{(HP1)}$	volume of eluant	purity	Weight/mg
HBI triene	2103	60 – 100 mL	> 99 % purity	63.1
HBI tetraene	2135 & 2158	110 – 250 mL	> 75 % purity	15

Sodexal 18 THE 103 mg

Compound	RI $_{(HP1)}$	volume of eluant	purity	Weight/mg
HBI diene	2084	30 – 50 mL	> 57 % purity	1.5
HBI triene	2106	60-100 mL	> 98 % purity	9.1
HBI tetraene	$2088_{(DB5)}$, $2092_{(DB5)}$, 2144, $2166_{(DB5)}$, $2186_{(DB5}$ $2216_{(DB5)}$	110 – 250 mL		4

Rhizosolenia setigera TCE 197 mg

Compound	RI $_{(HP1)}$	volume of eluant	purity	Weight/mg
HBI triene	2040 and 2090	40-60 mL	98 %	0.5
HBI tetraene	2078 and 2124	70-120 mL	98 %	< 0.1

5.3 Analytical Methods

5.3.1 Gas chromatography-mass spectroscopy

Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard Mass Selective Detector (MSD) 5970 Series fitted with either a 12 m (0.2 mm i.d.) fused silica capillary column (HP1 Ultra stationary phase) or a 30 m (0.25 mm i.d.) Carbowax fused silica capillary column (0.25 μm film thickness). For the HP1 column, the GC oven temperature was programmed from 40-300^{0}C at 5^{0}C min^{-1} and held at the final temperature for 10 min. The corresponding temperature programme for the Carbowax column was 50-200 ^{0}C at 6 ^{0}C min^{-1}.

In both cases the mass spectrometer conditions were ion source temperature 250 ^{0}C and 70 eV ionisation energy. Spectra (40-500 Da) were collected using Hewlett Packard Chemstation™ software.

Retention Indices (GC RI) were calculated according to the following formula (Lee *et al.*, 1979):

$$RI = 100z + 100 \frac{t_R(\text{unknown}) - t_R(z)}{t_R(z+1) - t_R(z)}$$

RI is retention index; t_R is retention time; z represents an *n*-alkane with z carbon atoms.

The *n*-alkane mixture was analysed immediately prior to analysis of the sample.

5.3.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions, using a Jeol EX-270 (270 MHz) high resolution FT-NMR spectrometer. Chemical shifts were measured (δ/ppm) using residual $CHCl_3$ in the solvent (δ= 7.24 and 77.0 for ^{1}H and ^{13}C respectively) as reference. Short and long range ^{1}H –^{13}C correlations were determined using HETCOR and COLOC methods.

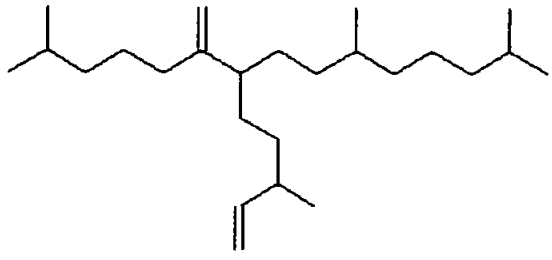
Conclusion and Future Work

Chapter 6 Conclusion and Future Work

6.1 Conclusion

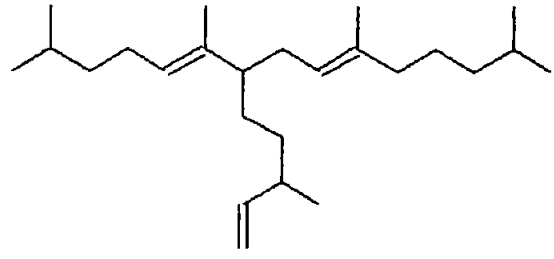
Two previously unidentified C_{25} HBIs have been isolated and characterised from laboratory cultures of *Haslea ostrearia*. They have been identified as 2,10,14-trimethyl-6-methylene-7-(3-methylpent-4-enyl)pentadecane I and 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadeca-5,9-diene II (Figure 6.1).

In addition, a $C_{25:3}$ HBI isolated from a laboratory culture of *Rhizosolenia setigera* was tentatively identified as 3,9,13-trimethyl-6-(1,5-dimethylhexyl)tetradeca-1,5,8-triene III (Figure 6.1).



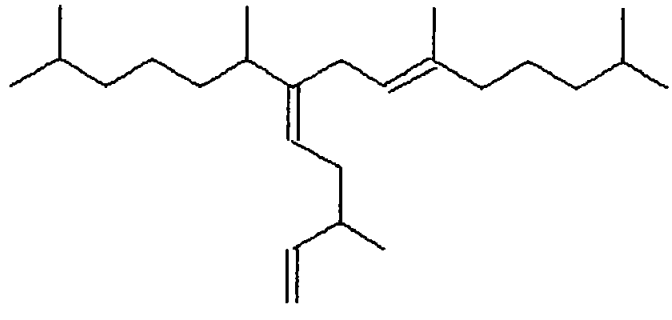
I

Source: *Haslea ostrearia*
RI 2084 (HP1, DB5, Carbowax)



II

Haslea ostrearia
RI $2103_{(HP1, DB5)}$ $2124_{(Carbowax)}$



III

Source: *Rhizosolenia setigera*
RI $2090_{(HP1)}$ $2142_{(Carbowax)}$

Figure 6.1 C_{25} HBI alkenes identified in cultures of *Haslea ostrearia* and *Rhizosolenia setigera*.

For the first time, the partial configurations of C_{25} isoprenoid alkenes were established. Detailed analysis involving;

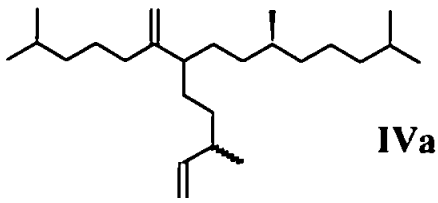
- ^{13}C NMR with a chiral shift reagent, together with
- chemical degradation to short chain chiral acids followed by enantioselective GC of the esters and direct GC of the alkenes on Carbowax stationary phase has established the partial configuration of:

- $C_{25:2}$ [IVa], isolated from *Haslea ostrearia* culture, Soproma 34 as 2,10S,14-trimethyl-6-methylene-7-(3RS-methylpent-4-enyl)pentadecane
- $C_{25:2}$ [IVb], isolated from *Haslea ostrearia* culture, Sodexal 18 as 2,10S,14-trimethyl-6-methylene-7-(3R-methylpent-4-enyl)pentadecane
- $C_{25:3}$ [VI (a)], isolated from *Haslea ostrearia* culture, Soproma 34 as 2,10,14-trimethyl-6-methylene-7-(3RS-methylpent-4-enyl)pentadec-9-ene
- $C_{25:3}$ [VI (b)], isolated from *Haslea ostrearia* culture, Soproma 46 as 2,10,14-trimethyl-6-methylene-7-(3S-methylpent-4-enyl)pentadec-9-ene
- $C_{25:3}$ [VI (c)], isolated from *Haslea ostrearia* culture, Sodexal 18 as 2,10,14-trimethyl-6-methylene-7-(3R-methylpent-4-enyl)pentadec-9-ene

The structures are shown in Figure 6.2.

The $C_{25:2}$ diene previously reported in Antarctic sediments and in sea-ice diatoms from McMurdo Sound (Nichols *et al.*, 1998, 1989, 1993; Venkatesan, 1988; Venkatesan and Kaplan, 1987) was identified as 2,10,14-trimethyl-6-methylene-7-(3-methylpent-4-enyl)pentadecane. Differences in the configurations were revealed between the two samples i.e. 22S configuration for the $C_{25:2}$ identified in sea-ice diatoms [IVc], 22RS configuration for the same $C_{25:2}$ identified in the sediments [IVa].

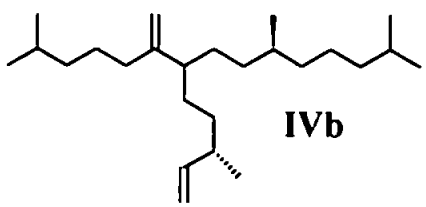
2, 10, 14-trimethyl-6-methylene-7-(3RS-methylpent-4-enyl)pentadec-9-ene [VIa] was also identified in sediments from McMurdo Sound.



IVa

2,10S,14-trimethyl-6-methylene-7-(3RS-methylpent-4-enyl)pentadecane

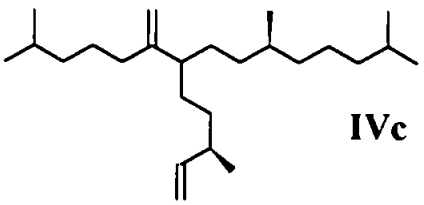
Haslea ostrearia culture, Soproma 34
Antarctic Sediments



IVb

2,10S,14-trimethyl-6-methylene-7-(3R-methylpent-4-enyl)pentadecane

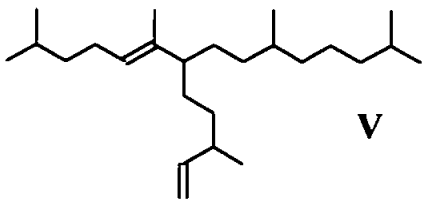
Sodexal 18



IVc

2,10S,14-trimethyl-6-methylene-7-(3S-methylpent-4-enyl)pentadecane

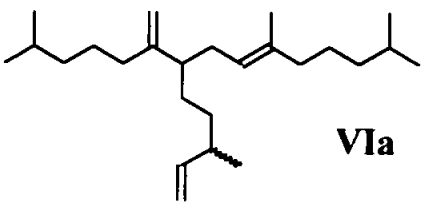
Sea-ice diatoms, Antarctic



V

2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadec-5-ene

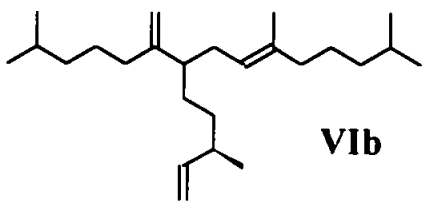
Haslea ostrearia cultures



VIa

2,10,14-trimethyl-6-methylene-7-(3RS-methylpent-4-enyl)pentadec-9-ene

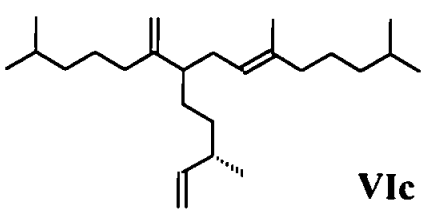
Haslea ostrearia culture, Soproma 34
Antarctic sediments



VIb

2,10,14-trimethyl-6-methylene-7-(3S-methylpent-4-enyl)pentadec-9-ene

Haslea ostrearia culture, Soproma 46



VIc

2,10,14-trimethyl-6-methylene-7-(3R-methylpent-4-enyl)pentadec-9-ene

Haslea ostrearia culture, Sodexal 18

Figure 6.2 The partial configurations of C_{25} HBI alkenes isolated from Haslea ostrearia cultures and sediments.

The Δ5 positional isomers of $C_{25:2}$ [IV] and $C_{25:3}$ [VI] i.e.2,6,10,14-tetramethyl-7-(3'-methylpent-4-enyl)pentadeca-5-ene [V] and 2,6,10,14-tetramethyl-7-(3'-methylpent-4-enyl)pentadeca-5,9-diene [II] were identified in small-scale cultures of *Haslea ostrearia*. The $C_{25:2}$ [V] has been reported previously in Caspian Sea sediments (Belt *et al.*, 1994).

The structures of twelve C_{25} HBIs, isolated from sediments and biota are now known (present study, Wraige *et al.*, 1997, Belt *et al.*, 1996, Belt *et al.*, 1994, Summons *et al.*, 1993, Yruela *et al.*, 1990, Dunlop and Jefferies, 1985). This accounts for approximately 50% of the C_{25} HBIs reported since they were first discovered in 1976.

$C_{25:3}$ (III, RI 2090 $_{HP1}$) isolated from a *Rhizosolenia setigera* culture shares similar retention indices and mass spectra to those of many sedimentary HBIs (Prahl *et al.*, 1980, Barrick *et al.*, 1980, Venkatesan *et al.*, 1980, Osterroht *et al.*, 1983, Requejo and Quinn, 1983, Volkman *et al.*, 1983, Albaiges *et al.*, 1984a, Requejo and Quinn, 1984, Requejo and Quinn, 1985, Shaw *et al.*, 1985, Matsueda *et al.*, 1986, Porte *et al.*, 1990, Wakeham, 1990, Hird and Rowland, 1995).

HBI alkenes isolated from *Haslea ostrearia* cultures have been shown to possess variability in the configuration at C22, with S, R and RS configurations being produced. The stereochemical studies may aid elucidation of the biosynthetic pathways to the HBIs. For instance, others have suggested (Ourisson and Nakatani, 1994) that a plausible precursor to the alkenes is the diphosphate ester of a C_{25} pentaunsaturated alcohol. The loss of diphosphate from the alcohol or other similar esters, would produce a hexaene [VIII] and progressive saturation would yield $C_{25:5}$ to $C_{25:2}$, all of which have been identified in *Haslea ostrearia*. Non-stereospecific saturation at C22 would give rise to 22RS, S or R isomers whereas reduction of the 9(10) bond in the $C_{25:3}$ to $C_{25:2}$ would involve a stereospecific step leading to the reported 10S configuration (Figure 6.3).

Laboratory simulations of early diagenetic reactions in sediments showed that under mild acid conditions, the HBI alkenes undergo reactions which are highly dependent on the degree of unsaturation of the HBI alkene. For the two dienes [I] and [V], double bond migration and geometric isomerisation were observed as the only reactions. For example;

- a slow and partial isomerisation of the E 5 isomer to the corresponding Z isomer was observed as the only reaction for the Δ5 C_{25} diene [V] and
- a complete isomerisation to a mixture of E and Z isomers of Δ5 diene was observed for the 6(17) isomer C_{25} diene [I].

In contrast, the two trienes [II] and [VI] underwent both isomerisation and rapid cyclisation reactions to yield substituted cyclopentenes and/or cyclohexenes. A substituted cyclopentene with a similar structure to [IX] was identified in the sapropel fraction of the Eastern Mediterranean Sea (Figure 6.4).

The resistance of the Δ5 C_{25} diene to further short-term changes could partially explain the observation that C_{25} HBI alkenes with two double bonds and less are removed from the hydrocarbon fraction at a less rapid rate than the higher polyenes (Requejo and Quinn, 1983, 1984; Dunlop and Jefferies, 1985). Other possible explanations for the removal of C_{25} HBIs with depth include, microbial degradation, microbial oxidation, cross-linking polymerization reactions and sulphur incorporation (Volkman *et al.*, 1983; Venkatesan, 1984, Kohnen *et al.*, 1990, 1993; Sinninghé Damste *et al.*, 1989, 1990, 1993).

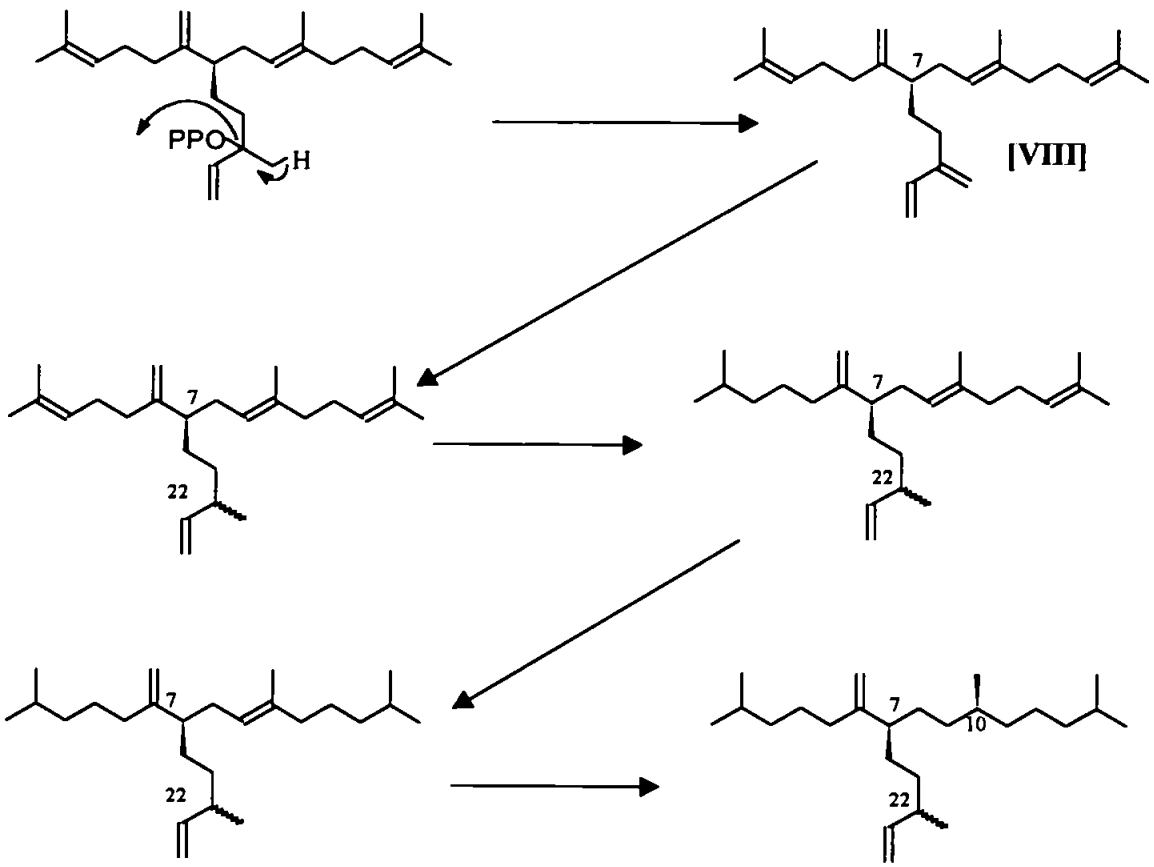
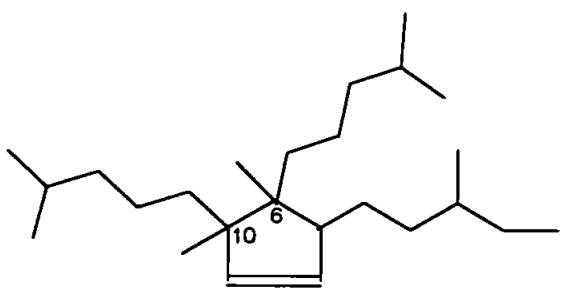


Figure 6.3 Proposed biosynthetic pathway for the HBI alkenes



IX

Figure 6.4 Proposed structure for the cyclopentene identified in the sapropel fraction of the Eastern Mediterranean Sea.

6.2 Future work

There are still many reported HBIs whose structures have not been unambiguously assigned. The present study has demonstrated that *Rhizosolenia setigera* is a probable source for previously uncharacterised compounds and therefore large scale cultures of this alga should be grown to obtain sufficient quantities of pure isolates for structural characterisation.

It seems reasonable to suggest that *Haslea ostrearia* and *Rhizosolenia setigera* are not the only biogenic source of HBIs. Indeed, the identification of the $C_{25:2}$ [I] in sea-ice diatoms suggests that other *Haslea spp* such as *H. trompei* may biosynthesise structurally similar HBIs. This should be investigated further by culturing different species of diatoms and examining them for HBIs.

The fully characterised HBI compounds should continue to be used for identification of sedimentary HBIs and as models in laboratory simulation of early diagenetic changes. Further studies should include repeating the acid-catalysed reactions with other C_{25} HBIs e.g. $C_{25:3}$ isolated from *Rhizosolenia setigera* followed by full characterisation of the products. The sedimentary occurrence and distribution of these cyclic compounds with that of the C_{25} HBIs especially in depth sequences should then be examined.

It is possible that the formation of the widespread C_{25} HBITs IX and X (reviewed by Sinninghe Damsté and de Leeuw, 1990; Kohnen *et al.*, 1992) occurs by sulphur incorporation into the C_{25} HBI triene, isolated from *Rhizosolenia setigera* followed by reduction of a double bond. Some authors have proposed that the formation of these HBIT compounds is initiated by addition of hydrogen sulphide (or polysulphides) to a double bond followed by intramolecular cyclization to form thiolanes which undergo dehydrogenation to form thiophenes. This only occurs when two double bonds are separated by fewer than four sp^3-hybridised carbon atoms (Sinninghe Damsté *et al.*, 1989; Figure 6.5). The HBI compounds could again be used as models to investigate this hypothesis. Sulphur incorporation experiments should be conducted under the low

temperature simulated sedimentary conditions which have proved useful in previous studies (e.g. Rowland *et al.*, 1993).

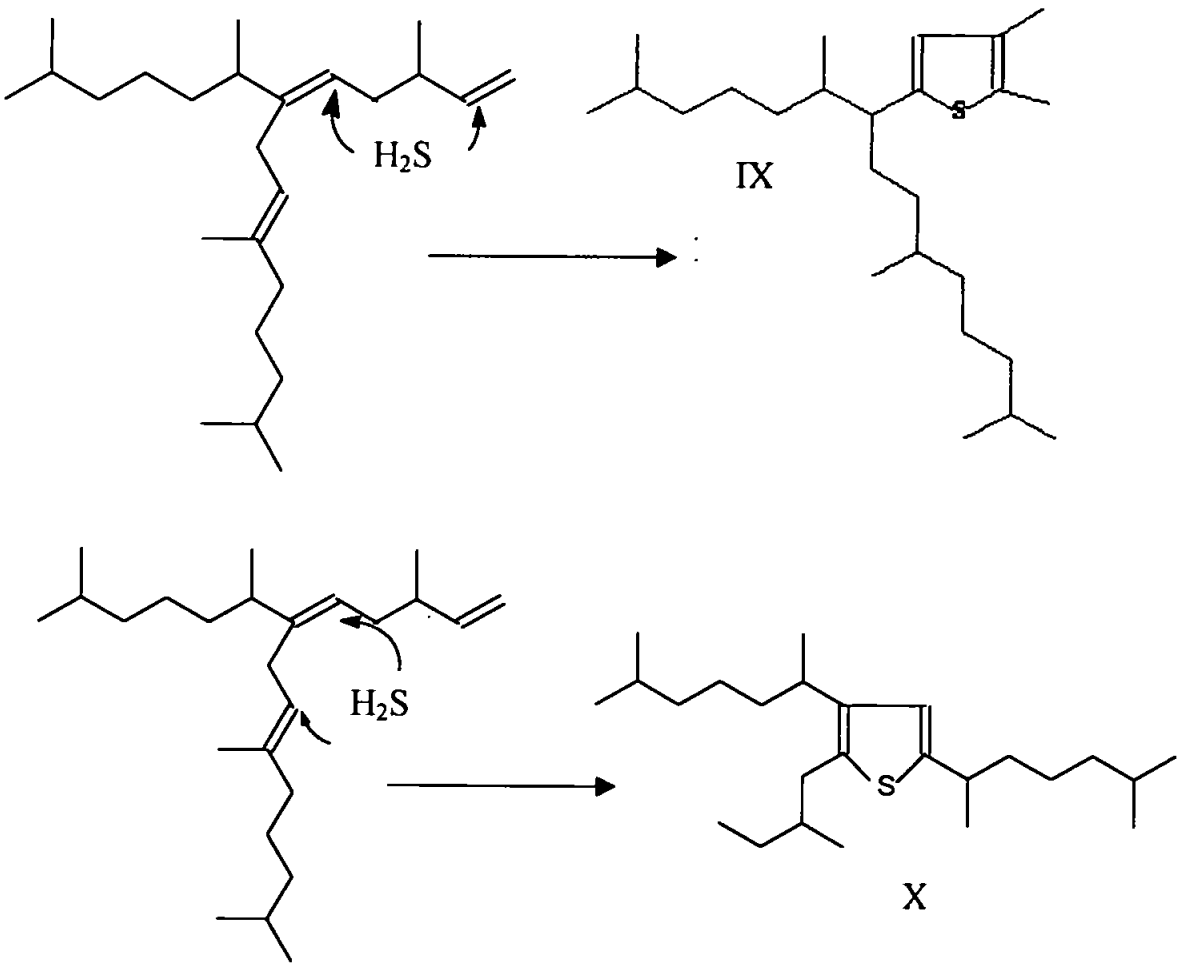


Figure 6.6 Proposed intramolecular sulphur incorporation of $C_{25:3}$, isolated from *Rhizosolenia setigera* to produce the thiophenes X and XI.

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

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Abstract

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

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

1. Introduction

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

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

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

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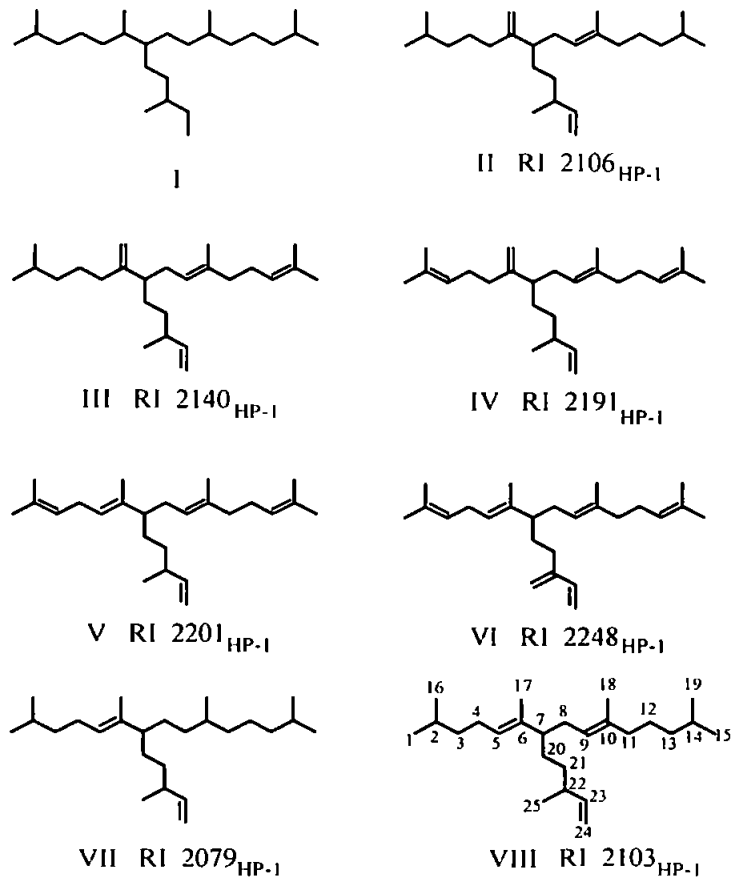
ary) have been shown by previous studies (Robert, 1983, 1984) to be typified by, yellow colouration (lag phase), the production of chlorophyll (green, exponential phase) and the blue pigment marennine (blue, stationary phase) and the algae for our experiments were harvested at each of these stages. The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

2. Results

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

3. Discussion

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



Scheme 1.

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

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

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

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

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

Whilst HBI production was demonstrated at each

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

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

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

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

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

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

4. Experimental

4.1. Algal cultures and axenic strains

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

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

4.2. Hydrocarbon extraction and isolation

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

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

4.3. NMR spectroscopy

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

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

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

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

4.4. Chromatography

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

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22. BIOLOGICAL MARKER SIGNIFICANCE OF ORGANIC MATTER ORIGIN AND TRANSFORMATION IN SAPROPELS FROM THE PISANO PLATEAU, SITE 964[1]

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ABSTRACT

The organic matter in a series of 39 sapropel samples from Hole 964D drilled during Ocean Drilling Program Leg 160 in the Ionian Basin of the Eastern Mediterranean Sea has been characterized. Organic carbon contents exceed 20% in many sapropels of Pliocene to early Pleistocene age, but are conspicuously lower in those of the late Pleistocene and Holocene. The organic matter is predominantly of marine origin, with varying admixtures of terrigenous organic matter. The most abundant molecular algal markers are long-chain alkenones, alkandiols, and alkanolones (ketools) as well as a significant proportion of sterols. Terrigenous markers include long-chain alkanes, *n*-alcohols, and fatty acids. Although strong alteration of the organic matter by sulfate-reducing bacteria is inferred from the high total sulfur contents of the sapropels, there was little direct molecular evidence of a bacterial biomass contribution. High C_{org}:N ratios, exceeding values of 20, particularly in the most organic-carbon-rich sapropels, have to be interpreted as the result of partial degradation of the sinking organic matter with selective remineralization of nitrogen-bearing compounds.

INTRODUCTION

Organic-carbon-rich, dark-colored strata (sapropels) are common in post-Messinian sediments of the Mediterranean Sea. Bradley (1938) first proposed that periods of stagnation occurred in the Mediterranean during the Quaternary and caused the formation of sediment layers that contain high proportions of organic matter. This hypothesis was supported by data from sediment cores that were recovered by a Swedish deep-sea expedition to the Mediterranean in 1947/48 (Kullenberg, 1952). Their work initiated a multitude of investigations of the Mediterranean seafloor (e.g., Stanley, 1972; Ryan, Hsü, et al., 1973; Hsü, Montadert, et al., 1978; Kastens, Mascle, Auroux, et al., 1987) devoted to determining the occurrence of sapropels, the reasons for their formation, and the origin of the organic matter preserved in them.

Kidd et al. (1978) defined sapropels in the Mediterranean Sea as discrete, more than 1-cm-thick layers in open-marine (pelagic) deposits, with more than 2% organic carbon. A "sapropelic layer," according to the classification of the same authors, in sediments of the same type contains between 0.5% and 2% organic carbon. This strict distinction was not, however, used by many later investigators, and it was not adopted by the Leg 160 Shipboard Scientific Party, partly because it does not take into consideration the occurrence of sapropels altered by secondary processes ("burnt-out" sapropels; see Shipboard Scientific Party, 1996b), and phenomena related to sapropel formation according to a broad spectrum of characteristic element concentrations, but not represented by organic matter enrichment (see Wehausen and Brumsack, Chap. 17, this volume).

For a long time, sapropels were considered a special phenomenon of the Eastern Mediterranean Sea, but recent drilling campaigns have demonstrated that sedimentary layers with high contents of organic matter also occur in the western basins (Cita et al., 1973; Kidd et al., 1978). In many instances, the organic carbon contents were below 2%, however, and so these layers were not considered real sapropels. Ocean Drilling Program (ODP) Leg 107 revealed organic-carbon-rich sapropels also in the western Mediterranean Sea (Kastens, Mascle, Auroux, et al., 1987; Brosse and Herbin, 1990; Emeis et al., 1991).

Organic Geochemical Studies

The type of organic matter in Mediterranean sapropels is still a matter of debate. Some authors favor a predominantly terrigenous (Deroo et al., 1978; Sigl et al., 1978; Hahn-Weinheimer et al., 1978) or marine (e.g., Nesteroff, 1973; Cita and Grignani, 1982) origin of the organic matter. A dominance of amorphous organic matter and high concentrations of C_1 to C_{12} compounds were taken as evidence to support the latter view. Low C:N ratios of late Pleistocene sapropels indicate a marine origin (Calvert, 1979, cited by Thunell et al., 1984), while high C:N ratios in early Pleistocene sapropels are more consistent with a considerable proportion of terrigenous material (Sigl et al., 1978). C:N and $\delta^{13}C$ ratios of organic matter in the youngest sapropel layer (S_1) are in accordance with a marine organic matter origin (Sutherland et al., 1984).

Comprehensive organic geochemical investigations on a molecular level are too scarce so far to resolve the aforementioned discrepancies. According to the results of Comet (1984) on late Pliocene and early Pleistocene sapropels from DSDP holes, the organic matter is of a predominantly marine origin. The youngest sapropel layer (S_1: ≈9–7 ka) contains a mixture of marine and a significant proportion of terrigenous organic matter (Smith, 1984). High concentrations of dinosterol and long-chain alkenones in sapropels S_1 and S_7 were taken as evidence of an intense bloom of coccolithophorides and dinoflagellates at the time of sapropel formation and, thus, to support the high-productivity model for sapropels from the Eastern Mediterranean Sea (Smith et al., 1986; ten Haven, 1986; ten Haven et al., 1986, 1987).

This study provides preliminary information on the origin and preservation of the organic matter in the sapropels from ODP Site 964 on the Pisano Plateau (Fig. 1) by combining shipboard measurements with data on the composition of the extractable organic matter, particularly the polar low-molecular-weight lipid fractions, analyzed by gas chromatography (GC) and combined gas chromatography-

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mass spectrometry (GC-MS) as an initial step for paleoenvironmental assessment.

ANALYTICAL METHODS

We investigated a total of 39 sapropel core samples from Hole 964D (Table 1). After freeze-drying and grinding, the sediments were analyzed for total carbon (TC) and total sulfur (TS) contents by combustion in a LECO CS-444 instrument. Carbonate contents were determined after acidification as carbon dioxide using a UIC-Coulometrics CM 5012 device. Total organic carbon (TOC) content was calculated as the difference between total and inorganic carbon contents (Shipboard Scientific Party, 1996a).

Dry sediment (1 to 3 g) was solvent extracted (dichloromethane/1% methanol (v/v): 1 × 50 mL, 3 × 30 mL) in an ultrasonic bath. The decanted combined extracts were concentrated by rotary evaporation (2 mL) and dried by a stream of nitrogen at mild temperature (30°–35°C). Extract yields were determined gravimetrically.

Separation into fractions of different polarities was performed after addition of internal standards (squalane, anthracene-d_{10}, 5α-androstan-17-one, 5α-androstan-3β-ol and erucic acid [n-$C_{22:1}$ fatty acid]). Prior to column chromatographic separation, the n-hexane-insoluble fraction (asphaltenes) was precipitated. The n-hexane-soluble portion was separated by medium-pressure liquid chromatography (MPLC; Radke et al., 1980) into fractions of nonaromatic hydrocarbons, aromatic hydrocarbons and heterocompounds (NSO fraction). Elemental sulfur was removed with activated copper filings from the nonaromatic hydrocarbon fraction.

Subsequently, the ketones, esters, and n-alcohols (>C_{20}) were separated from the NSO-fraction by flash chromatography (Still et al., 1978) with a moderate overpressure of nitrogen. For this purpose, a 10-mm × 200-mm column was filled with 5 g silica gel 60 (40–63 μm, deactivated with 5% by weight of water) and washed with 50 mL dichloromethane. The unpolar fraction of ketones, esters, and n-alcohols (>C_{20}) was eluted with 60 mL dichloromethane. The other compounds were removed from the column with 100 mL of a mixture of dichloromethane and methanol (10% by volume). The polar portion of the NSO fraction was separated into an acid fraction and a steroid alcohol fraction using a column filled with KOH-impregnated silica gel (McCarthy and Duthie, 1962; modified according to Hinrichs et al., 1995). All fractions were concentrated as described for the whole extracts and weighed. For analysis of the molecular constituents, the heterocompound fractions were trimethylsilylated with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA).

Gas chromatography was performed on a Hewlett-Packard 5890 Series II instrument equipped with a temperature-programmed cold-injection system (Gerstel KAS 3) and a fused-silica capillary column (J&W DB-5, length = 30 m, inner diameter = 0.25 mm, film thickness = 0.25 μm). Helium was used as the carrier gas, and the temperature of the oven was programmed from 60°C (1 min isothermal) to 305°C (50 min isothermal) at a rate of 3°C/min.

GC/MS studies were performed with the same type of gas chromatograph (helium as carrier gas) and a temperature program from 60°C (1 min isothermal) to 300°C (50 min isothermal) at a rate of 3°C/min. The gas chromatograph was coupled to a Finnigan SSQ 710B mass spectrometer operated at 70 eV. Structural assignments of individual compounds are based on comparison of relative gas chromatographic retention times and mass spectra with those reported in the literature. Quantification was performed relative to the amount of internal standards with mass correction for trimethylsilylated compounds.

The determination of Kovats retention indices was carried out using two different gas chromatographic columns. The first column was fitted into a Hewlett Packard 5890 series II gas chromatograph coupled to a Hewlett Packard 5970 series mass selective detector. The sample was transferred on a 12 m (0.2 mm i.d.) fused silica column coated with HP1 by auto-splitless injection. The temperature was programmed from 40° to 300°C at 5°C/min with a hold time of 10 min at the final temperature. The carrier gas was helium. The second analysis was performed on a Finnigan Mat gas chromatograph coupled to a Finnigan Mat mass selective detector. Auto-splitless injection was made on a 30 m (0.25 mm i.d.) fused silica column coated with DB 5. The temperature program was the same as before. The carrier gas was helium at a linear velocity of 40 cm/s. C_{20}, C_{21}, and C_{22} n-alkane standards (Aldrich) were co-injected with the sample. In addition, two different isomers of a highly branched isoprenoid alkadiene were used as internal standards. $C_{25:2}$ (I) was from a diatom culture of *Haslea osfrearia*, $C_{25:2}$ (II) was from a Caspian Sea plankton catch. The Kovats retention indices were calculated according to the formula published by Braiswaithe and Smith (1996):

$$RI_C = 100\,Z + 100\,[(\log t'_{RC} - \log t_{RZ})/(\log t'_{R(Z+1)} - \log t'_{RZ})].$$

RESULTS AND DISCUSSION

Elemental Composition of Organic Matter

The results of elemental analysis for total organic carbon (TOC), carbonate, and total sulfur contents of the Hole 964D sapropels are

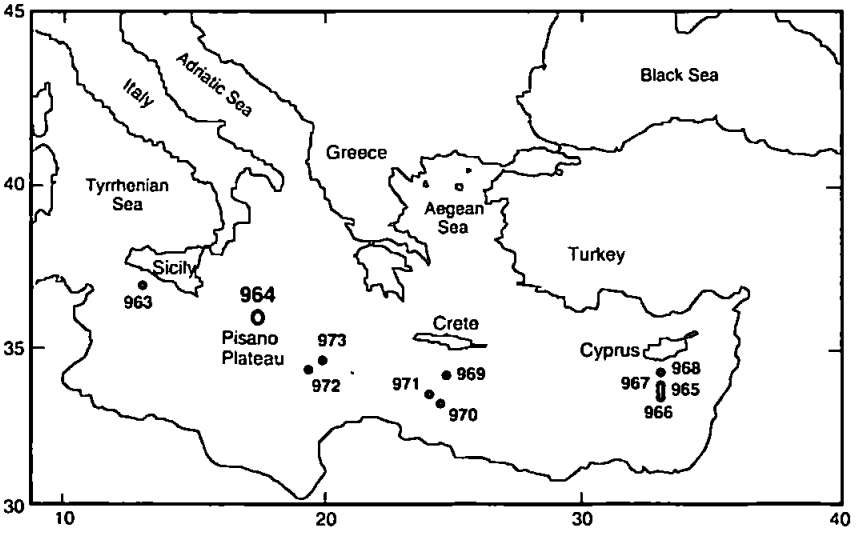

Figure 1. Drilling location of Site 964 in the Ionian Basin (Eastern Mediterranean Sea) and other ODP Leg 160 sites (after Shipboard Scientific Party, 1996a).

Table 1. Elemental data, alkenone ratios, and paleo–sea-surface temperatures for sapropels from Holes 964A (Scientific Shipboard Party, 1996b) and 964D.

Core, section, interval (cm)	Depth (mbsf)	Sapropel number	Organic carbon* (%)	$CaCO_3$** (%)	Sulfur** (%)	$U^{K'}_{37}$/SST (–/°C)
160-964A-						
1H-1, 70–71	0.70	1	3.14	37.15	1.73	
2H-1, 94–95	7.74	3	5.78	27.41	2.18	
2H-1, 103–104	7.83	3	2.87	29.40	2.15	
2H-3, 122–123	11.02	4	3.50	37.98	2.99	
2H-3, 130–131	11.10	4	2.97	33.57	3.36	
2H-4, 69–70	11.99	5	7.30	12.66	3.93	
2H-4, 73–74	12.03	5	3.57	33.40	4.89	
2H-4, 133–134	12.63	6	3.15	31.49	2.29	
2H-4, 143–144	12.73	6	2.18	37.32	1.98	
3H-2, 108–109	18.88	8	2.43	36.99	2.73	
3H-4, 104–105	21.84	9	3.09	46.56	6.01	
3H-5, 92–93	23.22	10	2.59	48.40	2.36	
3H-6, 113–114	24.93	12	5.56	34.07	1.97	
4H-4, 38–39	30.68	16	4.95	43.98	3.24	
4H-4, 103–104	31.33	17	4.25	56.14	1.66	
4H-5, 56–57	32.36	18	16.38	2.33	3.94	
4H-6, 2–3	33.32	18	4.35	59.81	1.62	
4H-7, 16–17	34.96	19	2.69	43.48	2.29	
5H-1, 114–115	36.44	20	3.96	57.56	1.25	
5H-3, 29–30	38.59	21	13.22	1.67	2.34	
5H-3, 30–31	38.60	21	18.21	1.33	0.94	
5H-3, 31–32	38.61	21	14.22	3.42	4.67	
5H-3, 32–33	38.62	21	12.44	12.66	4.70	
5H-3, 33–34	38.63	21	17.19	1.83	5.80	
5H-3, 34–35	38.64	21	3.55	42.57	3.01	
5H-4, 102–103	40.82	22	13.11	3.83	4.19	
5H-5, 46–47	41.76	23	3.52	34.57	2.57	
5H-5, 100–101	42.30	24	20.10	2.25	8.70	
6H-1, 86–87	45.66	26	7.36	30.74	4.88	
6H-1, 87–88	45.67	26	1.98	58.48	2.54	
6H-3, 41–42	48.21	27	16.26	5.50	7.47	
6H-3, 44–45	48.24	27	2.25	50.90	4.58	
6H-4, 32–33	49.62	30	11.29	4.17	8.89	
6H-4, 38–39	49.68	30	7.11	37.40	8.00	
6H-5, 14–15	50.94	32	24.34	2.33	7.85	
6H-6, 83–84	53.13	35	9.28	39.40	6.10	
6H-7, 18–19	53.98	37	18.69	2.67	9.80	
7H-4, 107–108	59.87	39	8.84	29.65	5.48	
8H-1, 69–70	64.49	42	11.14	26.57	8.69	
8H-6, 22–23	71.52	48	4.79	59.14	4.70	
9H-3, 42–43	76.72	50	7.73	34.07	1.50	
9H-5, 35–36	79.65		13.51	14.49	0.77	
9H-5, 89–90	80.19	54	20.25	1.17	1.14	
9H-6, 14–15	80.94	55	14.42	3.00	18.24	
9H-6, 18–19	80.98	55	8.17	25.16	13.93	
9H-7, 4–5	82.34	57	3.85	12.41	2.76	
9H-CC, 25–27	83.11	58	24.62	1.17	9.66	
160-964D-						
1H-1, 72–74	0.72	1	2.80	39.0	1.75	0.55/16.8
1H-1, 74–76	0.74	1	2.85	38.5	1.79	0.62/18.7
2H-3, 67–69	7.77	2	2.79	40.0	2.20	0.46/14.4
2H-3, 69–71	7.79	3	2.99	37.5	4.68	
2H-3, 81–83	7.91	4	5.22	31.3	2.61	0.48/14.9
2H-3, 83–85	7.93	4	4.44	27.9	2.56	
2H-4, 23–25	8.83	5	5.07	34.4	4.54	
2H-4, 25–27	8.85	5	3.87	34.3	3.16	
2H-4, 84–86	9.44	6	2.66	34.6	2.01	
2H-4, 86–88	9.46	6	3.68	31.8	2.78	
2H-4, 92–94	9.52	6	2.44	35.9	2.37	0.54/16.4
2H-4, 94–96	9.54	6	2.97	35.1	2.50	
3H-2, 83–85	15.93	8	2.35	28.0	3.14	0.62/18.6
3H-4, 47–49	18.57	9	2.90	47.0	1.14	0.73/21.6
3H-5, 29–31	19.89	10	2.40	45.5	1.45	
3H-5, 31–33	19.91	10	2.40	47.0	2.62	
4H-5, 66–68	29.76	15	4.99	33.4	2.20	0.66/19.8
4H-5, 141–143	30.51	16	5.33	49.2	2.45	
4H-6, 92–94	31.52	17	17.5	2.10	4.01	
4H-7, 35–37	32.45	18	5.04	58.0	2.00	0.74/21.8
5H-4, 54–56	37.64	23	4.33	17.4	3.03	
5H-4, 56–58	37.66	23	4.17	28.0	3.44	
5H-4, 58–60	37.68	23	3.09	36.4	2.92	
5H-4, 60–62	37.70	23	2.93	37.8	2.71	
5H-4, 137–139	38.47	24	20.1	2.08	4.31	0.71/21.1
5H-4, 139–141	38.49	24	14.2	9.58	5.47	
6H-2, 70–72	44.30	27	7.75	38.8	3.10	
6H-3, 113–115	46.23	29	18.3	2.40	4.10	0.76/22.3
6H-5, 58–60	48.68	32	14.4	10.3	5.05	0.82/24.0
6H-7, 49–51	51.59	37	10.6	21.3	5.05	0.81/23.7
7H-1, 25–27	51.85	38	6.80	40.8	1.54	0.79/23.3
7H-6, 98–100	60.08	41	8.75	42.1	4.58	0.74/21.9
7H-CC, 01–03	61.13	43	19.5	5.08	8.87	
7H-CC, 03–05	61.15	43	13.4	21.2	5.84	0.85/24.9
9H-2, 149–150	73.59	48	4.01	38.1	1.95	
9H-3, 03–04	73.63	48	3.38	62.7	3.86	
10H-2, 01–03	81.61	54	15.0	3.92	5.57	0.83/24.4
10H-2, 62–64	82.22	55	20.0	2.75	10.2	
10H-3, 68–70	83.78	58	11.6	30.0	7.03	0.84/23.5

Notes: Sapropel numbers after Shipboard Scientific Party (1996b). * = calculated difference between total and carbonate carbon contents. ** = average of two measurements. $U^{K'}_{37}$ = alkenone index (see Prahl and Wakeham, 1987); SST = sea-surface temperature (calibration after Prahl and Wakeham, 1987).

compiled in Table 1. Together with the shipboard results on Hole 964A sapropels, the TOC data are also plotted vs. biostratigraphic age (Shipboard Scientific Party, 1996b) in Figure 2. The organic carbon contents in the sapropels vary, on a high level, by more than one order of magnitude. They range between about 2% and 5% for both investigated holes in the upper Pleistocene and show strong variations between 3% and 25% in the other time sections. The values are covariant for the upper sapropels (<1 Ma) in both holes. This correlation is not so pronounced in Figure 2 for the deeper part of the cores because sapropels selected from both cores do not match in all cases and because of some variation of organic carbon contents within a given sapropel particularly in the case of the extremely organic-carbon-rich sapropels (Shipboard Scientific Party, 1996b; Table 1). For several sapropels, however, there is a good match of organic carbon contents for samples from Holes 964A and 964D.

Carbonate contents are in the 30%–50% range in the upper part of the hole, but show large fluctuations in the section below about 30 mbsf (Table 1; Shipboard Scientific Party, 1996b). As an overall observation, carbonate contents are lowest in sapropels with very high TOC values. Carbonate and TOC contents are negatively correlated with each other (Hole 964A: R = 0.84, n = 48; Hole 964D: R = 0.83, n = 39). Carbonate dissolution in the organic-carbon-rich sapropels may be due to organic acids formed during diagenesis (e.g., by hydrolysis of esters), and this effect may have been stronger in the older, particularly organic-carbon-rich sapropels. This is consistent with the high proportions of free organic acids found in the polar extractable organic matter fractions of the sapropels (see "Molecular Investigations").

Sulfur contents, as a general trend, are higher in the particularly organic-carbon-rich sapropels (Table 2; Shipboard Scientific Party, 1996b), but there is no clear relationship with TOC data (Hole 964A: R = 0.46, n = 48; Hole 964D: R = 0.77, n = 39). Most TOC:S ratios are lower in the sapropels than in present-day noneuxinic sediments, for which an average ratio of 2.8 was reported by Berner and Raiswell (1983), and thus indicate anoxic bottom-water conditions.

Shipboard analyses of sapropels by Rock-Eval pyrolysis (Shipboard Scientific Party, 1996b) formally revealed marine kerogen type II organic matter in many of the Hole 964A sapropels with hydrogen indices (HI) between 350 mg hydrocarbons (hc)/g TOC and slightly more than 500 mg hc/g TOC, whereas more than 50% of the sapropels revealed lower HI values and higher oxygen indices (OI) in the mixed kerogen type II/III range as is evident from Figure 3. This is not uncommon for black shales deposited in the deep ocean even at high levels of organic carbon (e.g., Herbin et al., 1986a, 1986b) and does not necessarily imply a strong contribution of terrigenous organic matter to the sediments. Actually, the terrigenous component in many cases was found to be low in many deep-sea sediments by organic petrography (e.g., between 2% and 14% along the continental margins of Peru and Oman; Lückge et al., 1996), and the explanation for the low hydrogen indices was microbial alteration of the organic

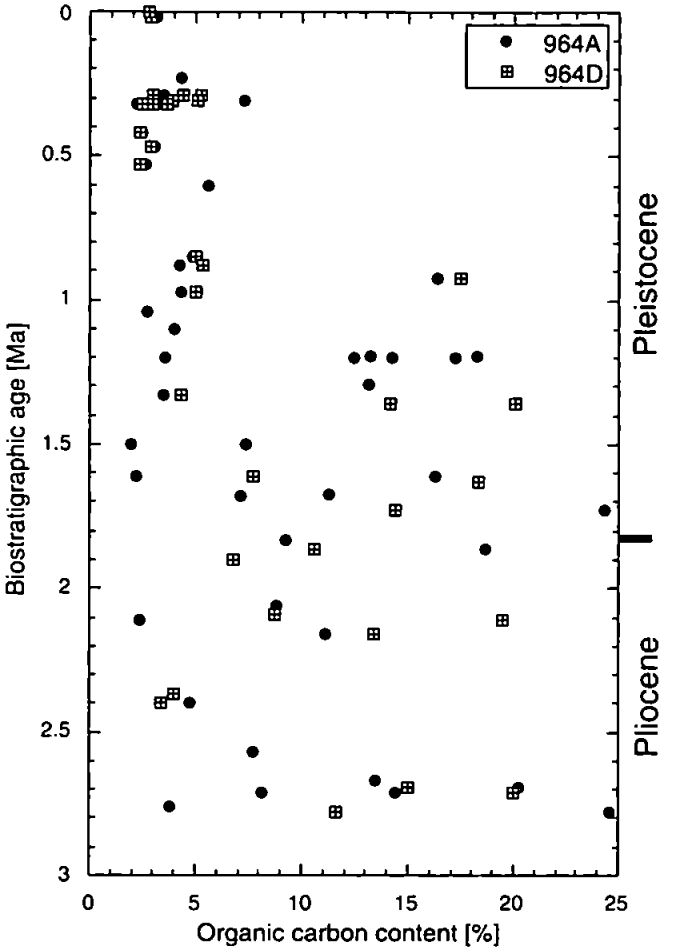

Figure 2. Organic carbon contents as a function of sediment age for sapropels from Holes 964A (shipboard data; Shipboard Scientific Party, 1996b) and 964D.

Table 2. Retention times and Kovats retention indices of unknown non-aromatic hydrocarbons and comparison with two highly branched isoprenoids.

Compound (see Fig. 5B)	Retention time (min)	Kovats retention index HP1	Kovats retention index DB5	Mass spectral type
a	53.04			II
b	53.25	2107	2096	II
c	53.87			(II)
d	54.02			I
e	54.40			I
f	54.55	2139	2116	I
g	54.73			I
h	55.10	2155	2151	II
i	55.74	2176	2161	II
HBI $C_{25:2}$ *(Haslea osfrarcia)*		2084	2063	
HBI $C_{25:2}$ *(Caspian Sea)*		2078	2059	

matter in the water column or in the upper sediment layers particularly by sulfate-reducing bacteria (Littke et al., 1991; Vetö et al., 1994). This type of alteration is largely dependent on the total organic carbon content as indicated by the TOC vs. HI diagram for Site 964 (Fig. 4A) and the other sites in the Eastern Mediterranean Sea occupied during Leg 160 (Fig. 4B). HI values increase with increasing organic-carbon content, indicating enhanced preservation of (labile) marine organic matter with increased organic-matter accumulation up to an

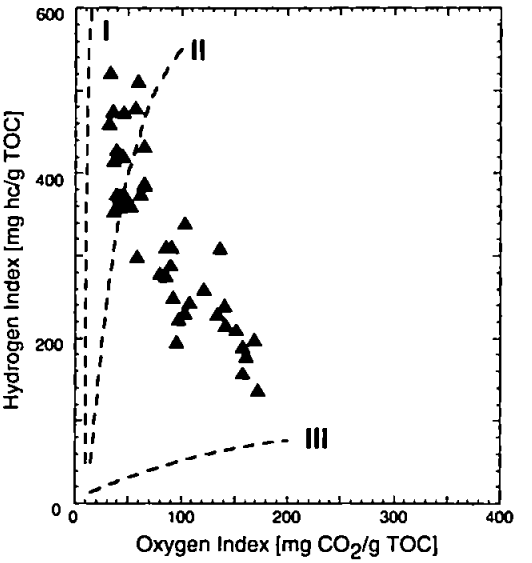

Figure 3. Van Krevelen–type diagram of hydrogen and oxygen index values for sapropels from Hole 964A (data taken from Shipboard Scientific Party, 1996b).

organic carbon content of about 10%. Hydrogen indices of sapropels richer in organic carbon are "uniform" in the range of about 400–500 mg hc/gTOC.

Shipboard measurements of C_{org}:N ratios by themselves did not provide a definite indication as to the origin and extent of preservation of the organic matter in the sapropels. The C_{org}:N ratios for all sapropels exceeded a value of 10; the carbonate-rich sapropels had ratios between 10 and 15 and the carbonate-poor sapropels ratios between 15 and 22 (Shipboard Scientific Party, 1996b). As the high C_{org}:N ratios in many cases coincide with high hydrogen index values from Rock-Eval pyrolysis, early diagenetic alteration processes appear to have preferentially removed nitrogen-bearing compounds from the organic matter of marine biomass. This is independent of the presence of some terrigenous organic matter in all sapropels studied, but the C_{org}:N ratios certainly are not a measure of the terrigenous organic matter fraction in these sapropels. A recent study demonstrated the strong effect of molecular oxygen on the C_{org}:N ratios (Cowie et al., 1995) and suggested higher C_{org}:N ratios as the result of selective organic-matter degradation in anoxic sediments. For the formation of sapropels in the Eastern Mediterranean Sea, anoxic conditions are obvious by low TOC:S ratios, so in this case the high C_{org}:N must be interpreted as reflecting diagenetic alteration of organic matter.

Molecular Investigations

Extract yields, after removal of elemental sulfur, range between 15 mg/g TOC and 70 mg/g TOC. Despite some uncertainty due to the very low absolute amounts of extract (small sample size) and low accuracy of gravimetric determination in these cases, the extract yields are higher than in other deep-sea sediments with immature organic matter (e.g., Rullkötter et al., 1981) and may indicate degradation of biomacromolecules into soluble compounds by high bacterial activity and/or an exceptionally good preservation of labile low-molecular-weight compounds. The relative distributions of gross chromatographic fractions are dominated by asphaltenes and NSO compounds (each about 35% to 50% by weight). The main portion of the fractionated NSO compounds are the green- (in the younger sapropels) to brownish-colored acid fractions with a relative percentage of more than 50%. Nonaromatic and aromatic hydrocarbons in all sapropels represent only a minor amount of the total extract.

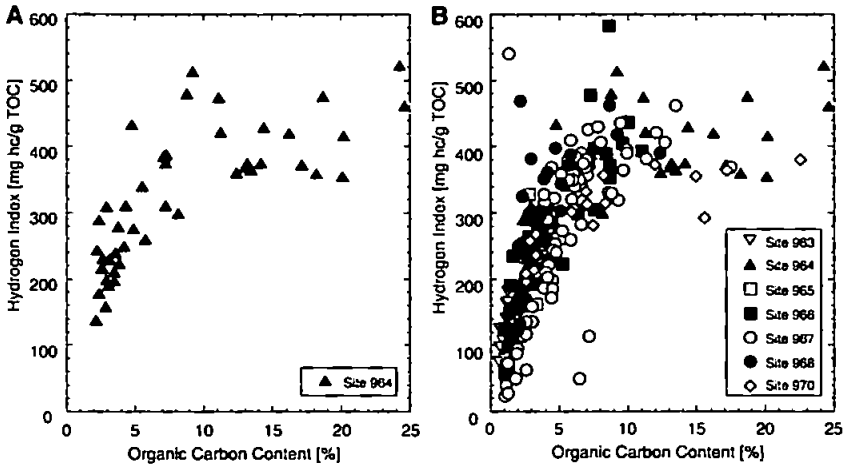

Figure 4. A. Hydrogen index values vs. organic carbon contents for sapropels from Hole 964A (data taken from Shipboard Scientific Party, 1996b). B. Hydrogen index values vs. organic carbon contents for sapropels from several holes in the Eastern Mediterranean Sea drilled during Leg 160 (data taken from Emeis, Robertson, Richter, et al., 1996).

Nonaromatic Hydrocarbons

Figure 5 shows gas chromatograms of the nonaromatic hydrocarbon fractions of Samples 160-964D-1H-1, 72–74 cm, and 10H-3, 68–70 cm, the shallowest and the deepest samples investigated, respectively. The *n*-alkane distribution patterns are very similar for all sapropel extracts studied and are typical of an origin of these compounds from cuticular waxes of higher land plants (Eglinton et al., 1962). The *n*-alkanes maximize at n-$C_{31}H_{64}$ in all samples, and Carbon Preference Index (CPI) values (Bray and Evans, 1961; corrected by Hunt, 1979) of *n*-alkanes of carbon numbers 25 to 33, as expected for a terrigenous source, consistently exceed a value of 2. At retention times between 90 min and 100 min, straight-chain C_{37} and C_{38} alkadienes (molecular weights 516 and 530, respectively; mass spectral base peak at *m/z* 96) elute from the GC column. They resemble the alkatrienes described by Volkman et al. (1980; and references therein) and, according to their carbon numbers, are most likely related to the long-chain alkenones (e.g., Volkman et al., 1980) that are present in the sapropels in high abundance. Phytane and pristane are absent or only detected in trace amounts (≈1 µg/g TOC), and thus cannot be used as redox indicators.

In samples buried more deeply than 35 m, branched and cyclic (steroid and triterpenoid) hydrocarbons increase in abundance relative to the *n*-alkanes due to a progress in diagenetic transformation of functionalized lipid compounds into nonaromatic (mostly olefinic) hydrocarbons. Among the pentacyclic triterpene hydrocarbons, oleanenes and ursenes together with the related des-A-triterpenoids, common in most of the samples, are further indicators of the terrigenous organic matter fraction in the sapropels but are outranged in abundance by sterenes of most likely marine origin. Even in the most deeply buried sapropel studied, diagenesis has not proceeded very far as is indicated by the exclusive presence of ster-2-enes and the absence of corresponding (thermodynamically more stable) ster-4- and -5-enes indicating a cool geothermal regime (Dastillung and Albrecht, 1977; Gagosian and Farrington, 1978; ten Haven et al., 1989). This is corroborated by the exclusive presence of hopanes with their unaltered biogenic 17β(H),21β(H)-22R sterical configuration in the triterpenoid series (Ensminger et al., 1977).

In the retention time range between 52 min and 58 min (Fig. 5B) the nonaromatic hydrocarbon fractions contain at least nine unknown compounds, partly in high concentrations, with a molecular weight of 348 u, corresponding to an elemental composition of $C_{25}H_{48}$ and indicating the presence of two degrees of unsaturation (double bond equivalents). The hydrocarbons exhibit two main types of mass spectra of which representative examples for two of the most abundant isomers are shown in Figure 6; the other isomers have mass spectra differing only slightly from those shown in relative intensity of major fragment ions. These compounds of unknown structure have retention times and Kovats indices that are slightly higher than those of C_{25} highly branched isoprenoids (HBIs) with two double bonds (Table 2) as reported from living organisms and sediments so far, and the unknown compounds also differ in their mass spectral fragmentation patterns from the C_{25} HBIs (Requejo and Quinn, 1983; Rowland et al., 1990; Belt et al., 1994). Any attempts to hydrogenate the unknown compounds, even under drastic conditions with an activated Adams catalyst, failed, so the novel compounds are likely to contain two rings instead of double bonds. Neither C_{20} nor C_{25} or C_{30} HBI pseudohomologs were detected in significant concentrations, indicating a low contribution of diatoms during times of sapropel formation consistent with the absence of diatom frustules in the sediments (Shipboard Scientific Party, 1996b). On the other hand, the carbon numbers of the novel bicyclic compounds suggest a relationship to isoprenoid biosynthesis and support speculations that the bicyclics may still be unknown biosynthetic products of diatoms or diagenetic transformation products of precursors from diatoms, whereas the absence of diatom frustules may be explained by post-depositional silicate dissolution. As reported by Bouloubassi et al. (Chap. 21, this volume) the C_{25} bicyclics dominate some of the nonaromatic hydrocarbon distributions of sapropels from sites farther to the east in the Mediterranean Sea. Structure elucidation and molecular isotope analysis are under way to solve the question of the origin of the novel bicyclic compounds.

n-Alcohols

n-Alcohols >C_{20} are the most abundant components in the least polar of the heterocompound fractions ("ketone fraction") (Fig. 7). *n*-Alcohols <C_{20} and phytol elute in the steroid alcohol fraction, and typically are present only in minor amounts (<1 µg/g TOC). In most samples, the distributions of long-chain *n*-alcohols have a marked preference of even-carbon-atom-number homologs, with a maximum at n-$C_{28}H_{57}OH$, which is a distribution typical of land-derived organic matter (Eglinton and Hamilton, 1963). The C_{20} to C_{30} *n*-alcohols with a strong even-over-odd carbon number predominance in the sapropels occur together with a similar range of fatty acids (C_{20}–C_{30}; see below) and a series of long-chain *n*-alkanes (C_{23}–C_{35}) with an odd-over-even carbon number predominance. All these compounds are related to a terrigenous organic matter supply (de Leeuw, 1986).

In four samples from the lower part of Hole 964D (Samples 160-964D-1H-1, 72–74 cm; 2H-3, 67–69 cm; 2H-3, 81–83 cm; and 2H-4, 92–94 cm), however, this pattern is overprinted by a series of straight-chain alcohols with an unusual odd-over-even carbon number predominance and a maximum at n-$C_{25}H_{51}OH$, which is of unknown origin and significance (Fig. 7A). The relatively high concentrations of monounsaturated *n*-alcohols of even-carbon-numbered homologs are also notable, because the $C_{30:1}$ compound in all samples

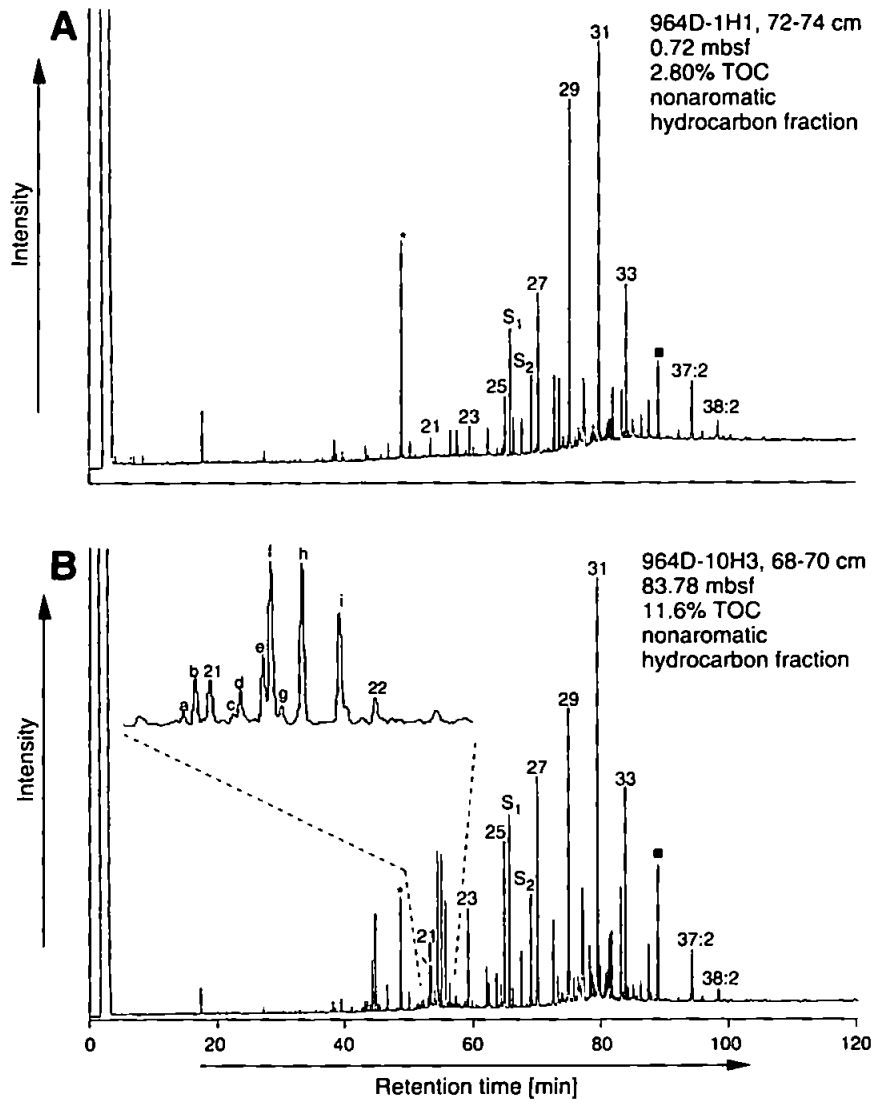

Figure 5. Gas chromatograms of the nonaromatic hydrocarbon fractions of the shallowest (A) and the deepest (B) sapropel sample from Hole 964D. The increase of branched and cyclic hydrocarbons relative to *n*-alkanes illustrates the difference in the extent of diagenetic transformation. Numbers refer to chain length of *n*-alkanes and alkadienes, respectively. S_1 = InjSTD (behenic acid methyl ester), S_2 = ISTD (squalane), square = tetraterpene, inset (expanded) = unknown bicyclic C_{25} compounds (molecular weight 348; cf. Table 2), * = contaminant.

exceeds the concentration of the related saturated analog (Fig. 7). This compound has been reported to occur in microalgae of the class *Eustigmatophyceae* (Volkman et al., 1992), which are also thought to biosynthesize the alkan-1,n-diols found in sediments (discussed separately).

Long-Chain Alkenones

Long-chain alkenones (C_{37}–C_{39}) are common in moderate to high abundances in the least polar heterocompound fractions (Fig. 7). These compounds are derived from marine prymnesiophyte species (Volkman et al., 1980). They are not restricted to sapropels younger than 268 ka, the age of the first occurrence of *Emiliania huxleyi* (Thierstein et al., 1977), or even younger than 70 ka, the beginning of the *E. huxleyi* acme zone (Gartner, 1977), respectively. They have apparently also been biosynthesized by their phylogenetic ancestors and related *Gephyrocapsaceae* species (Marlowe et al., 1990). The concentrations of total C_{37}–C_{39} alkenones vary between 125 µg/g TOC and 1876 µg/g TOC with no correlation to sediment sub-bottom depth or TOC content, but generally illustrate the strong marine influence on the organic matter in the sapropels.

Sterols

In Figure 8, the gas chromatogram shows the elution range of sterols for Sample 160-964D-5H-4, 137–139 cm. Compound identification is based on relative retention times and comparison with published mass spectra (e.g., Budzikiewicz, 1972; Brassell, 1980; McEvoy, 1983; cf. Table 3 and Appendix). The sterol distributions are complex, indicating a variety of primarily marine sources. The major compounds are the C_{27} to C_{29} sterols, although C_{30} sterols are present in significant concentrations; C_{26} sterols were only detected in trace amounts. The relative concentrations of C_{27} to C_{29} sterols are similar to each other, but with a slight predominance of C_{29} compounds, which varies among the samples (Fig. 9). This may be related to slight variations in the supply of terrigenous organic matter, even though most haptophyte algae also contain 24-ethylcholest-5-en-3β-ol, a sterol commonly assigned to a terrigenous higher plant source (Volkman, 1986). Similar sterol concentrations and distribution patterns with a predominance of C_{29} compounds were also found in modern sediments from the Peru upwelling region (Volkman et al., 1987). In that study it was concluded that an unreflected interpretation of steroid carbon number distribution using the Huang-Meinschein diagram in Figure 9 would lead to an overestimate of land-derived organic matter. Furthermore, a major portion of the sterols, and among them particularly those of marine sources, in the youngest sapropel is known to be bound as esters or sulfates due to the low progress in diagenesis (ten Haven, 1986). Thus, the distribution pattern in Figure 8 and the carbon number distributions in Figure 9 may not represent the total steroids present because bound components were not liberated prior to extraction in this study. But there is no correlation between the sterol carbon number distribution (or relative

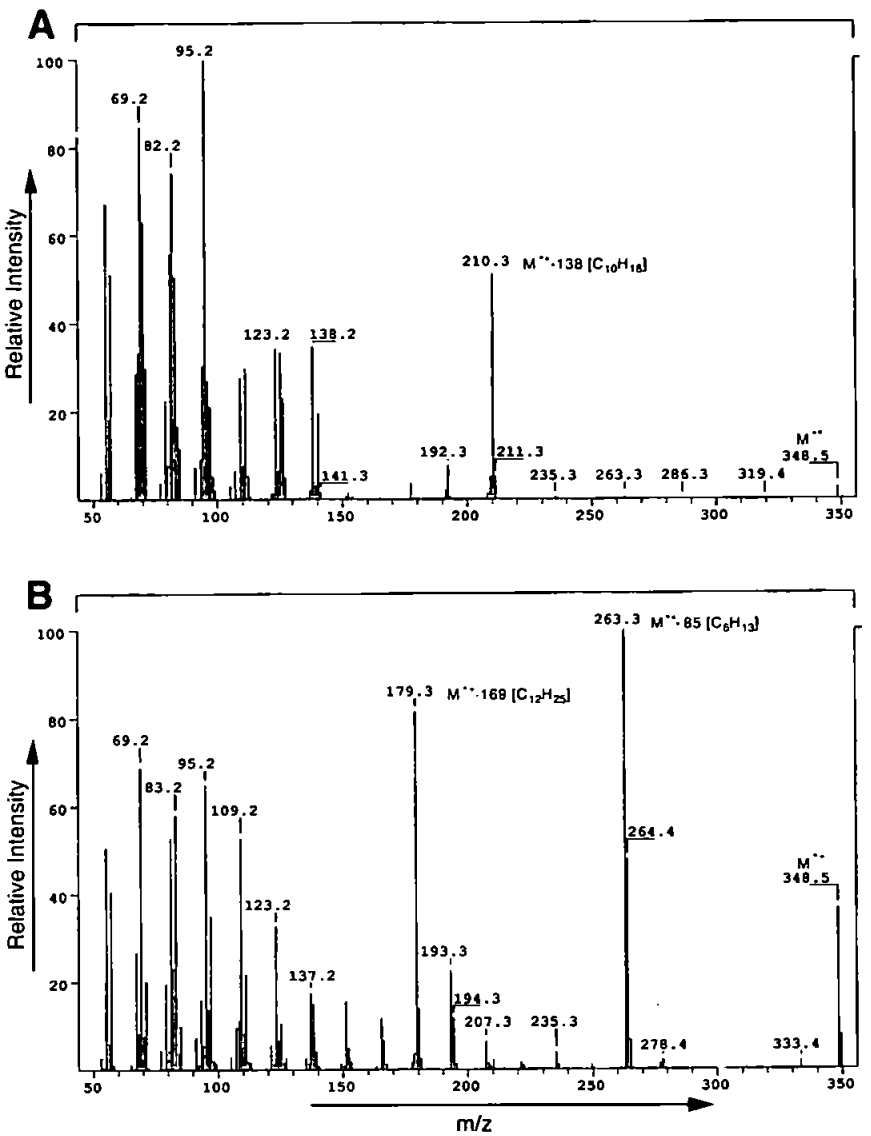

Figure 6. Representative mass spectra of unknown bicyclic C_{25} compounds in the hydrocarbon fraction of Sample 160-964D-10H-3, 68–70 cm (cf. Table 2).

proportion of C_{29} sterols; Fig. 9) with either organic carbon content or depth/age, that is, the type of sterols present does not depend on the preservation conditions but may rather reflect variations (mainly) of the phytoplanktonic community at a given time of sapropel deposition. On the other hand, the total free sterol concentrations correlate well (R = 0.87) with the total organic carbon contents (Fig. 10). This again may indicate enhanced preservation of labile organic matter in the particularly TOC-rich sapropels.

Long-Chain Alkan-1,n-diols and Alkan-1-ol-n-ones

C_{30}, C_{31}, and C_{32} alkan-1,15-diols and alkan-15-on-1-ols were initially reported to occur in immature Black Sea sediments (de Leeuw et al., 1981). Since then, these compounds and homologs with different chain lengths and positions of the midchain functionality have been found in various marine and lacustrine sediments (e.g., Morris and Brassell, 1988). *n*-Alkan-1,n-diols are now thought to be derived from eustigmatophyte algae (Volkman et al., 1992). Note that in our liquid chromatographic separation scheme, the alkandiols elute in the fatty acid fraction and the corresponding ketools in the sterol fraction, respectively.

In all investigated samples, the most abundant alkandiol (Fig. 8), and most abundant single compound in general with only one exception, is the C_{30}-1,15-diol (with coeluting isomers representing different positions of the midchain hydroxyl group). C_{28}- to C_{34}-diols with different positional isomers for the midchain functionality are also common and have a strong predominance of even-chain-length homologs. In Sample 160-964D-6H-3, 113–115 cm, a compound tentatively identified as C_{29}-1,14-diol is dominant and is accompanied by an admixture of the 1,12-isomer. The corresponding alkan-1-ol-n-ones are present in concentrations lower by a factor of 2 to 5 in all samples. Altogether, these compounds provide a strong marine molecular signal for the sapropels.

n-*Fatty Acids*

Saturated *n*-fatty acid distributions in the sapropels ranged in carbon number from 14 to 30 and contained a strong predominance of even-carbon-number homologs. The distributions were bimodal in all samples. One of the maxima occurs at C_{16} and the other one in the range of the terrigenous long-chain fatty acids (Kolattukudy, 1976). Most samples are dominated by the long-chain homologs, but a direct correlation between compound distribution pattern and depth or TOC content is not evident.

Mono- and diunsaturated fatty acids were detected in the range from C_{14} to C_{18} with a maximum at C_{18} and with a strong predominance of even-carbon-number homologs. The concentrations of the positional isomers of monounsaturated octadecenoic acids are only

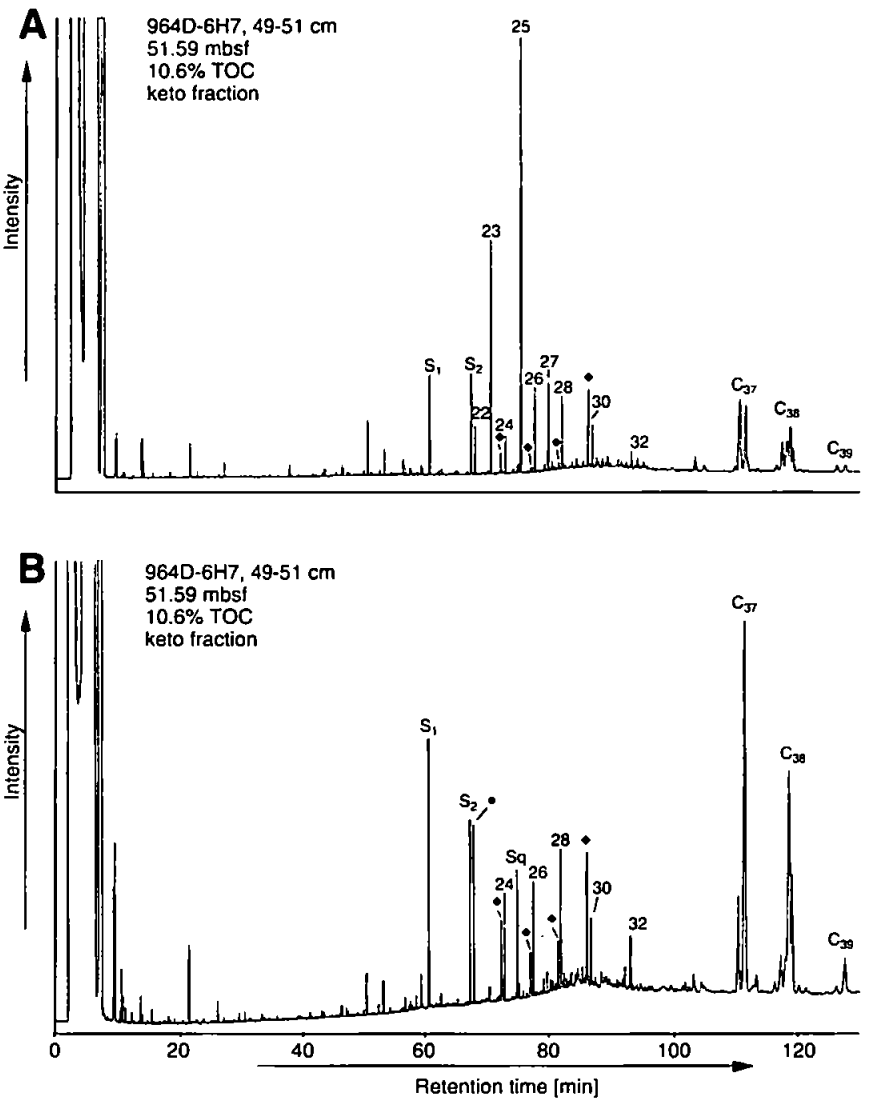

Figure 7. Gas chromatograms of two "ketone fractions" as discussed in the text. Note the differences in abundance of *n*-alcohols and long-chain alkenones. Chain lengths of *n*-alcohols are indicated by numbers, monounsaturated *n*-alcohols by diamonds. C_{37}, C_{38}, and C_{39} are long-chain alkenones. S_1 = ISTD (androstan-17-one), S_2 = InjSTD (behenic acid methyl ester), * = contaminant and C_{22} *n*-alcohol (trace).

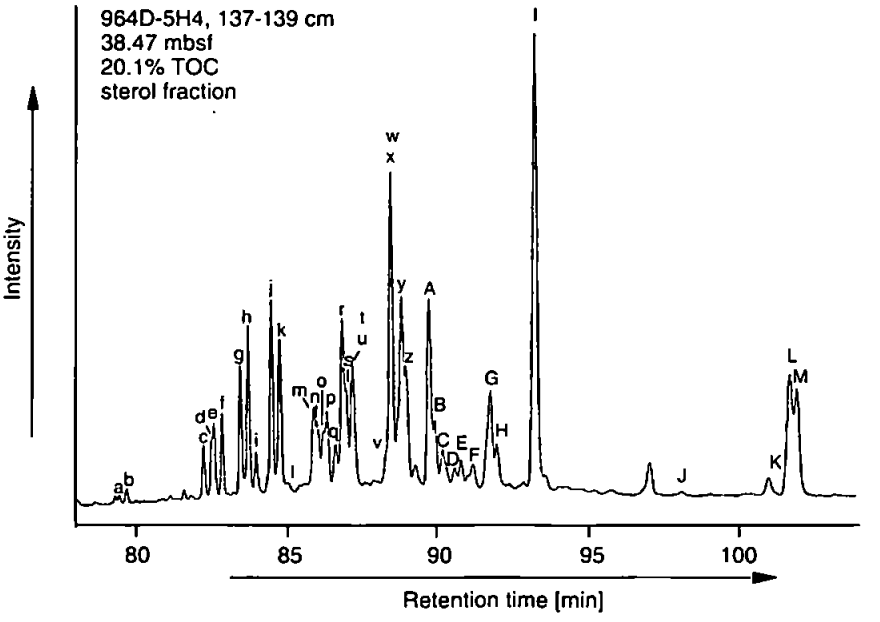

Figure 8. Gas chromatogram of the elution range of sterols extracted from Sample 160-964D-5H-4, 137–139 cm (see Table 3 for compound identification).

Table 3. Sterols and alkan-1-ol-n-ones in the sterol fractions (cf. Fig. 8).

Symbol	Compound	Structure (see Appendix)
a	24-*nor*-cholesta-5,22(E)-dien-3β-ol	b1
b	24-*nor*-5α-cholest-22(E)-en-3β-ol	a1
c	27-*nor*-24-methylcholesta-5,22(E)-dien-3β-ol	b4
d	27-*nor*-24-methyl-5α-cholest-22(E)-en-3β-ol	a4
e	cholesta-5,22(E)-dien-3β-ol	b2
f	5α-cholest-22(E)-en-3β-ol	a2
g	cholest-5-en-3β-ol	b3
h	5α-cholestan-3β-ol	a3
i	27-*nor*-24-methyl-5α-cholestan-3β-ol	a5
j	24-methylcholesta-5,22(E)-dien-3β-ol	b7
k	24-methyl-5α-cholest-22(E)-en-3β-ol	a7
l	5α-cholest-7-en-3β-ol	c3
m	C_{29}-steradienol	—
n	24-methylcholest-5-en-3β-ol	b6
o	24-methylcholesta-5,24(28)-dien-3β-ol	b8
p	24-methyl-5α-cholestan-3β-ol	a6
q	23,24-dimethylcholesta-5,22(E)-dien-3β-ol	b13
r	24-ethylcholesta-5,22(E)-dien-3β-ol	b10
s	23,24-dimethyl-5α-cholest-22(E)-en-3β-ol	a13
t	24-ethyl-5α-cholest-22(E)-en-3β-ol	a10
u	4α,24-dimethyl-5α-cholest-22(E)-en-3β-ol	d7
v	23,24-dimethylcholest-5-en-3β-ol	b12
w	24-ethylcholest-5-en-3β-ol	b9
x	+ 23,24-dimethyl-5α-cholestan-3β-ol (tr)	a12
y	24-ethyl-5α-cholestan-3β-ol	a9
z	+ 24-ethylcholesta-5,24(28)-dien-3β-ol	b11
A	4α,23,24-trimethyl-5α-cholest-22-en-3β-ol	d13
B	C_{30}-cholest-22-en-3β-ol	—
C	C_{30}-cholest-?en-3β-ol	—
D	4α,23,24-trimethyl-5α-cholest-7-en-3β-ol	e14
E	C_{30}-cholest-7-en-3β-ol	—
F	C_{30}-cholest-5-en-3β-ol	—
G	C_{30}-stanol	—
H	C_{30}-stanol	—
I	C_{30}-keto-1-ol	—
J	C_{31}-keto-1-ol	—
K	$C_{32:1}$-keto-1-ol	—
L	C_{32}-keto-1-ol	—
M	C_{32}-keto-1-ol (isomer of L)	—

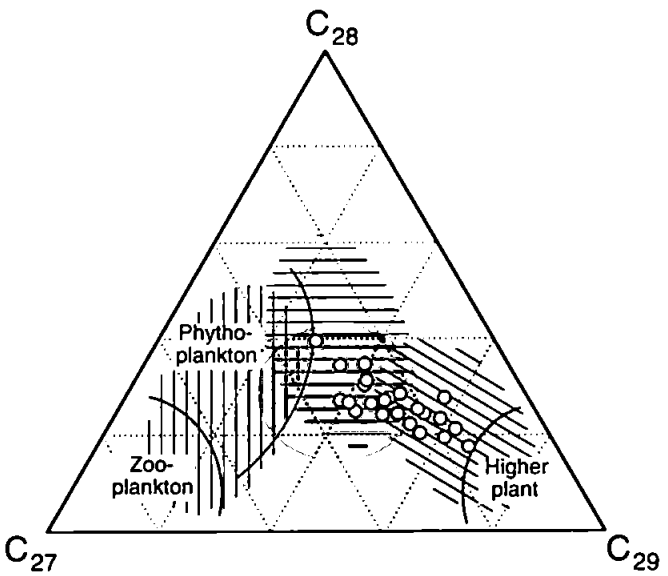

Figure 9. Triangular diagram of carbon number distributions of steroid alcohols in sapropels from Hole 964D analyzed in this study.

slightly lower than that of the saturated compound, while the monounsaturated hexadecenoic acids make up only 5%–10% of the concentration of the corresponding saturated fatty acid. Monounsaturated short-chain fatty acids are well known in many marine organisms. In microalgae the $C_{18:1}$ ω9-fatty acid (oleic acid) is in many cases more abundant than the saturated homolog, whereas this ratio is well

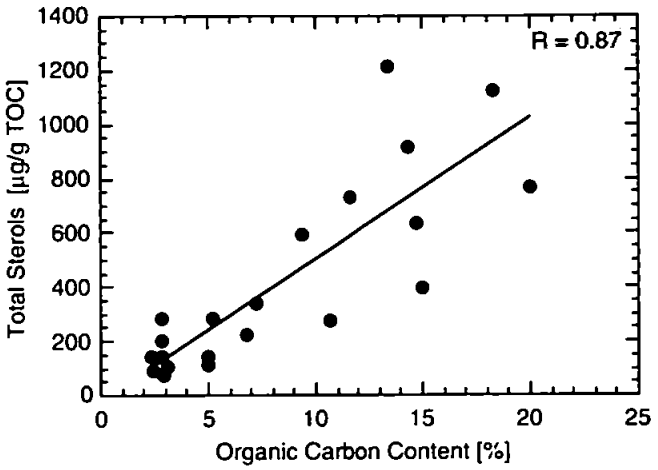

Figure 10. Linear regression of total sterol concentrations and organic carbon contents for sapropels from Hole 964D.

balanced between the $C_{16:0}$ and the $C_{16:1}$ ω7-fatty acids (Cobelas and Lechado, 1989). The $C_{16:1}$ ω7 compound is a major fatty acid in diatoms (Smith et al., 1983; Volkman et al., 1989). This finding supports again the low contribution of siliceous organisms to the organic matter and contradicts speculations of a diatom origin of the novel C_{25} bicyclic hydrocarbons.

Alkenone-Based Sea-Surface Temperatures and Average Chain Lengths of n*-Alkanes as Climatic Indicators*

In Figure 11 the TOC values of 24 sapropels are plotted against the alkenone-derived paleo-sea-surface temperatures of the same samples. With one exception (160-964D-5H-4, 137–139 cm), the TOC contents of Pleistocene sapropels are in the range between 2% and 6% and thus distinctly lower than those of Pliocene sapropels (7%–20%). While the former were deposited under sea-surface temperature (SST) conditions with a relatively large fluctuation of 8°C between 14°C and 22°C, the Pliocene sapropels formed under more uniform and higher SSTs between 22°C and 25°C; the transition from the Pliocene to the Pleistocene reflects the global cooling at that time. Formation of (extremely) TOC-rich sapropels during times of elevated SSTs can be seen either as an effect of more sluggish circulation during the warmer Pliocene times or as a consequence of more stable stratification of the water column as a result of an enhanced freshwater inflow due to a higher humidity on the surrounding continents than during later times of sapropel formation in the Pleistocene. Although SSTs are not entirely uniform in a single sapropel (Table 1; Emeis et al., Chap. 26, this volume), the measurements appear to be largely representative of a given sapropel. This is supported by the fact that the $U^{K'}_{37}$ values ($U^{K'}_{37}$ = [37:3]/([37:3] + [37:2]) = 0.037 · SST − 0.07; Table 1; Prahl and Wakeham, 1987), determined using the chromatographically separated keto fractions for the Hole 964D samples and total extracts for the Hole 964A samples during the Leg 160 cruise (Shipboard Scientific Party, 1996b), respectively, are in agreement with each other.

The lowest SST was determined for the S_2 sapropel, which is known to have been deposited in a cool climate. In contrast to this, deposition of the youngest sapropel (S_1) started between 8.8 ka in shallow water depth and 8.2 ka in a water depth corresponding to that of the Site 964 location on the Pisano Plateau (Strohle and Krom, 1997), and thus the SST data correspond to the warmer present-day (Holocene) conditions. In fact, the SST data determined based on the alkenones in the S_1 sapropel (Table 1) match the present-day spring (April) SSTs in the Site 964 area (Anonymous, 1976). Likewise, the

climate was warmer at the time of S_9 deposition (≈242 ka) than at the time of S_8 deposition (≈220 ka; e.g., Rossignol-Strick, 1985), and this is matched by a difference of 3°C in alkenone-based SST data in Table 1.

The carbon number distributions of terrestrial long-chain *n*-alkanes depend on the type of vegetation and, thus, on the terrestrial climatic conditions (Simoneit et al., 1977). A shift to higher chain-lengths can reflect (1) higher continental temperatures of the borderlands in general (Simoneit et al., 1977) or (2) an origin of the organic matter from vegetation zones with higher temperatures (Gagosian et al., 1981, 1987). For the Mediterranean Sea the origin of the land-derived organic matter may be sensitive to the strength of the wind systems, especially of the monsoonal southwesterlies, and the precipitation rate and area, respectively. As a proxy for the varying chain lengths of *n*-alkanes, Poynter (1989) has introduced the ACL_{27-31} index and demonstrated a correlation between the pollen record and ACL_{27-31} values for the West African coast (ODP Holes 658A and 658B) during the last 24 k.y., indicating varying origins of terrestrial material brought into the deep sea by eolian and fluviatile transport. In Poynter's (1989) study, the values range between 29.65 and 30.05.

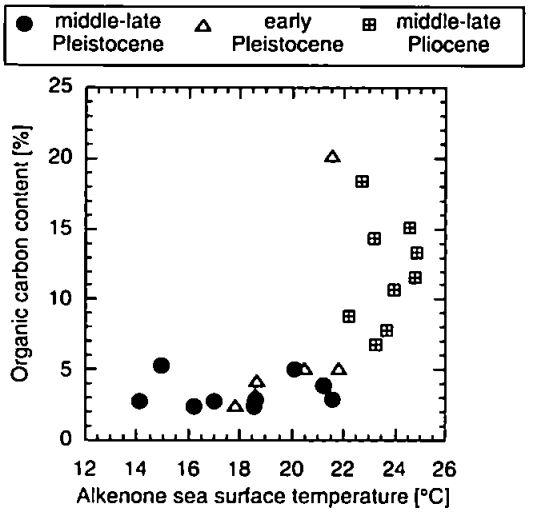

Figure 11. Organic carbon content vs. alkenone sea-surface temperatures for sapropels from Hole 964D. Biostratigraphic age (after Shipboard Scientific Party, 1996b) is indicated by different symbols.

The ACL_{27-31} values of the investigated sapropels from the Pisano Plateau are lower (29.40–29.75) than for the West African coast and indicate lower growth temperatures for the higher land plants. The corresponding ACL_{27-33} (Hinrichs, 1997) values correlate with other organic geochemical data of the Mediterranean sapropels in the following way (Fig. 12): (1) alkenone-derived SST values increase with increasing ACL values (R = 0.73) and (2) extremely TOC-rich sapropels were deposited at times when ACL values were high (R = 0.71). The relationship between ACL and continental floral assemblage is supported by the lowest measured value of the S_6 sapropel, deposited under a cold and dry land climate (e.g., Rossignol-Strick, 1985). These data suggest higher temperatures and more humid conditions during the deposition of middle–late Pliocene sapropels and can partly explain higher TOC contents as the result of elevated fresh-water inflow and thus, most probably, elevated nutrient supply and enhanced productivity.

CONCLUSIONS

Sapropels from ODP Site 964 at the Pisano Plateau (early Pliocene to Holocene) were investigated for bulk elemental composition and the distributions of solvent-extractable lipids. The bulk elemental data reflect the particular environmental conditions of sapropel formation in the Mediterranean Sea with enrichments of organic matter and sulfur otherwise only found in black shales of the Mesozoic oceans. TOC:S ratios indicate anoxic bottom water conditions.

The extractable lipids make up only a minor part of the total organic matter present, and their composition indicates a low level of diagenetic alteration. The molecular compositions of the sapropel extracts (with the exception of the fatty acids) are largely similar in all investigated samples, despite the large variation of TOC concentrations. They are dominated by marine-derived compounds (*n*-alkan-1,n-diols, *n*-alkan-1-ol-n-ones, sterols, and long-chain alkenones) with varying subordinate admixtures of terrigenous organic matter (long-chain *n*-alkanes, *n*-alkanols, and fatty acids) and less significant contributions from microbial sources. Lipid compositions are in general similar to those in sediments from modern upwelling areas (Farrimond et al., 1990; Poynter et al., 1989); a slightly enhanced proportion of land-derived organic matter may relate to a delicate balance between climatic changes, terrigenous supply, and sapropel formation. The correlation between SSTs and average chain length of *n*-alkanes and the relationship between TOC contents and average chain length of *n*-alkanes support land-climate-controlled conditions for sapropel formation.

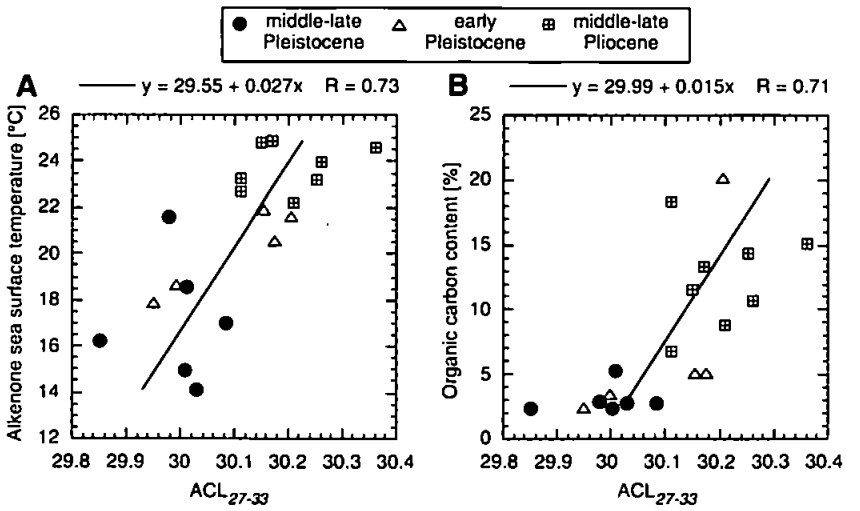

Figure 12. A. Alkenone sea-surface temperatures vs. average chain length (ACL) of *n*-alkanes for sapropels from Hole 964D. Biostratigraphic age (after Shipboard Scientific Party, 1996b) is indicated by different symbols. B. Organic carbon content vs. average chain length of *n*-alkanes for sapropels from Hole 964D. Biostratigraphic age (after Shipboard Scientific Party, 1996b) is indicated by different symbols.

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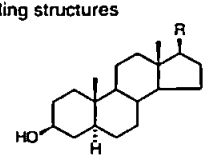
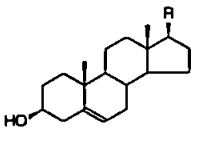
APPENDIX

Structures of sterols

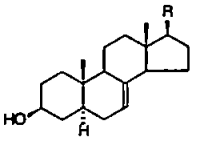
Ring structures



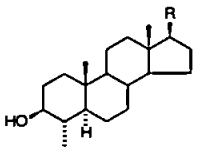
a / 5α-Cholestan-3β-ol series



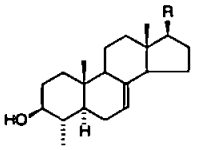
b / Cholest-5-en-3β-ol series



c / 5α-Cholest-7-en-3β-ol series

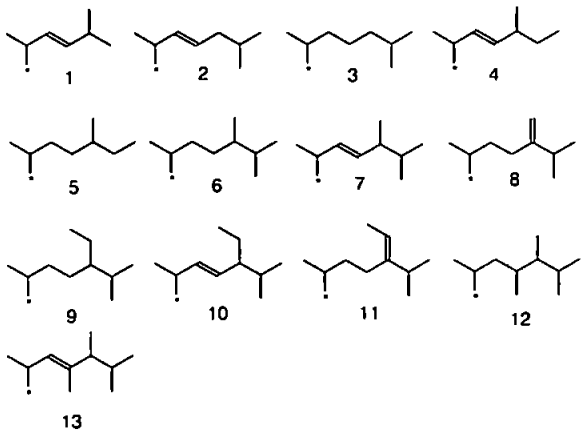


d / 4α-Methyl-5α-cholestan-3β-ol series



e / 4α-Methyl-5α-cholest-7-en-3β-ol series

side-chain structures



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