1995

STRUCTURAL CHARACTERISATION OF HIGHLY BRANCHED ISOPRENOID ALKENES FROM SEDIMENTS AND ALGAE

COOKE, DAVID ALAN

STRUCTURAL CHARACTERISATION OF HIGHLY BRANCHED ISOPRENOID ALKENES FROM SEDIMENTS AND ALGAE

DAVID ALAN COOKE B.Sc. (Hons) M.Sc.

A thesis submitted to the University of Plymouth

in partial fulfilment of the requirements for admittance

to the degree of:

DOCTOR OF PHILOSOPHY

University of Plymouth,

Department of Environmental Sciences,

Plymouth, Devon,

PL4 8AA, U.K.

and

Plymouth Marine Laboratory,

Prospect Place, The Hoe,

Plymouth, Devon,

PL1 3DH, U.K.

Submitted December 1995

TO MY PARENTS

Structural characterisation of highly branched isoprenoid alkenes from sediment and algae

David Alan Cooke

ABSTRACT

At least fourteen C_{25} highly branched isoprenoid (HBI) alkenes have been previously reported in estuarine and coastal sediments from locations worldwide. The parent alkane structure has been proven but only a few of the double bond positions established previously. A wide body of evidence suggests that the compounds have a diatomaceous origin, with one report in the laboratory cultured diatom *Haslea ostrearia*. Alkenes with more than two double bonds appear to be rapidly removed from the hydrocarbon fraction of most sediments. There is evidence that some of the alkenes react rapidly with sulphur to form S-containing analogues. HBIs, both as hydrocarbons and S-containing analogues, may prove useful environmental indicators once the sources and exact structures have been established.

A study of the temporal variations of HBI and carotenoid biomarker concentrations in Tamar sediments was conducted during 1994. The concentrations of C_{25} HBI alkenes exhibited strong negative correlations with those of n-$C_{21:6}$ and fucoxanthin, common diatom markers. The strong inverse variance suggests that HBI concentrations are related to diatom populations in the Tamar estuary.

Six C_{25} HBI alkenes ranging from a diene to hexaene have been isolated from 'bulk' cultures of *Haslea ostrearia* and Caspian Sea sediments. The occurrence of HBIs in *Haslea ostrearia* is thus confirmed. Furthermore, the structures were unambiguously assigned by detailed nuclear magnetic resonance spectroscopy and mass spectral analysis, as well as by chemical degradation. The hydrocarbons are 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadec-5-ene; 2,10,14-trimethyl-6-methylene-7-(3-methylpent-4-enyl)pentadec-9-ene; 2,10,14-trimethyl-6-methylene-7-(3-methylpent-4-enyl)pentadec-9,13-ene; 2,10,14-trimethyl-6-methylene-7-(3-methylpent-4-enyl)pentadec-2,9,13-ene; 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadec-2,5,9,13-ene and 2,6,10,14-tetramethyl-7-(3-methylenepent-4-enyl)pentadec-2,5,9,13-ene. Structural elucidation of the HBI alkenes produced by *Haslea ostrearia* and comparison with sedimentary distributions suggests that the latter may arise from a facile double bond isomerisation of the biosynthetic compounds. The evidence presented suggests that inputs from *Haslea ostrearia* may account for at least eight of the dominant sedimentary HBI alkenes.

The unusual behaviour exhibited by HBI alkenes during solid phase chromatography suggests that an incomplete record exists for HBI alkenes reported in the environment.

Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was financed with the aid of a studentship from the University of Plymouth and carried out in collaboration with Plymouth Marine Laboratory.

A programme of advanced study was carried out, with relevant scientific seminars and conferences attended at which work was often presented; external institutions were consulted.

Publications:

Belt, S.T., **Cooke, D.A.**, Hird, S.J. and Rowland, S.J. (1994). Structural determination of a highly branched C_{25} sedimentary isoprenoid biomarker by NMR and mass spectrometry. *J. Chem. Soc., Chem Commun.*, 2077-2078.

Oral Presentations:

Cooke, D.A. and Rowland, S.J. The application of highly branched isoprenoid alkenes as palaeoenvironmental indicators. British Organic Geochemistry Society meeting 1993 (Plymouth, UK).

Cooke, D.A., Belt, S.T. and Rowland, S.J. The structural characterisation of a C_{25} highly branched isoprenoid alkene. British Organic Geochemistry Society meeting 1994 (Aberdeen, UK).

Cooke, D.A., Belt, S.T. and Rowland, S.J. The structural characterisation of highly branched isoprenoid polyenes. The Geochemistry Society meeting 1994 (Kingston, UK).

Rowland, S.J., Belt, S.T., **Cooke, D.A.**, Hird, S.J. and Robert, J-M. (1995). Structural characterisation of saturated through heptaunsaturated C_{25} highly branched isoprenoids. In *Organic Geochemistry: Developments and applications to energy, climate, environment and human history* 1995 (eds. J.O. Grimalt and C. Dorronsoro).

Signed 15/12/95

Date

ACKNOWLEDGEMENTS

I would like to thank first and foremost Prof. Steve Rowland for his supervision of this work. I offer my most sincere thanks and appreciation for his constant help, encouragement, patience and continuing support.

I am also grateful for the assistance of the following people and organisations who provided funding, samples, loaned equipment or performed specialist analyses, they are:

The University of Plymouth for the award of a Research Studentship.

Prof. J-M. Robert (Universite de Nantes) for providing samples of *Haslea ostrearia*.

Dr. R. Barlow (Plymouth Marine Laboratory) for helping with the HPLC analyses of Tamar sediments, and informative discussions.

Dr. J. Green (Plymouth Marine Laboratory) for his assistance in culturing of diatoms and many helpful suggestions.

Dr. T. Peakman (O.G.U., University of Bristol) for obtaining 400 Mhz ^{1}H & ^{13}C NMR spectra.

Dr. A. Douglas (NRG, University of Newcastle upon Tyne) for the loan of a micro-ozoniser.

Dr. A. Lewis for being a veritable mine of information !

Drs. Simon Belt and Pete Sutton for their expert assistance in the field of NMR.

The technical staff of the Department of Environmental Sciences, University of Plymouth, most notably Ian Doidge, who was always ready with a helping hand. Friends and colleagues in Plymouth.

I would also like to thank Dr M. Jones (retired, NRG), Dr. D. Manning (University of Manchester) and Dr. P. Farrimond (NRG) who all motivated my interest in geology / geochemistry during the previous stages of my academic career.

Finally, a special "thankyou" to Zac and Angus.

PREFACE

This thesis is presented in six chapters. Each chapter is divided into subsections (*e.g.* 1.1, 1.2 ... *etc.*). Further subdivisions are similarly numbered sequentially and, where necessary by the use of bold text. Compound structures are assigned unique numbers (*e.g.* **I**, **II**, **III** ... *etc.*), generally in chronological order of appearance in the text.

Chapter 1 provides an introduction and general background to the research described herein. Chapters 2, 3, 4 describe the research into characterisation, distribution, and fate of highly branched isoprenoid (HBI) hydrocarbons. Experimental and analytical procedures used are given in Chapter 5, and the conclusions and suggestions for further work are presented in Chapter 6.

ABBREVIATIONS USED IN TEXT

HBI	highly branched isoprenoid
DCM	dichloromethane
Na_2SO_4	sodium sulphate
NaOH	sodium hydroxide
$AgNO_3$	silver nitrate
TsOH	toluene-ρ-sulphonic acid
TLC	thin layer chromatography
HPLC	high performance liquid chromatography
GC	gas chromatography
MS	mass spectrometry
RI	retention index
NMR	nuclear magnetic resonance
$PtO_2.H_2O$	platinum (IV) oxide monohydrate
THE	total hexane extract
TOE	total organic extract

TABLE OF CONTENTS

CHAPTER ONE

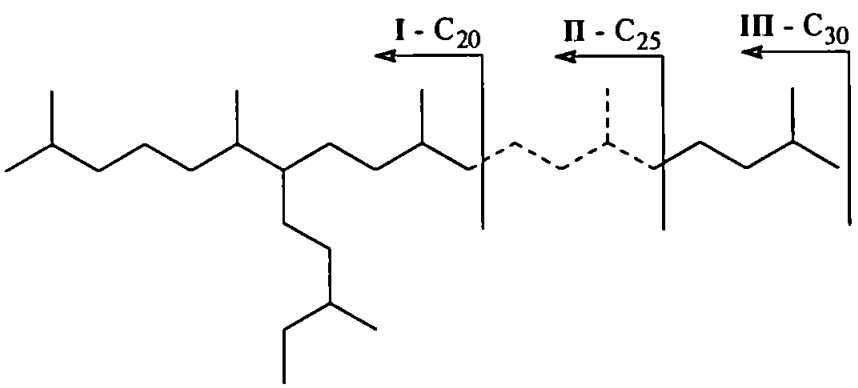
Introduction

The occurrence of widely distributed C_{25} highly branched isoprenoid alkenes in sediments and biota is reviewed. These compounds occur as alkanes and alkenes with one to five double bonds in young aquatic (marine) sediments from many parts of the globe (e.g. Peru, Antarctica, North Sea and Caspian Sea). Sometimes found in high concentrations in surface sediments (e.g. 60 $\mu g\ g^{-1}$), the compounds rapidly disappear in older sediments, possibly due to reaction with sedimentary sulphur. The source of these C_{25} hydrocarbons is thought to be diatoms, and there is one report of a suite of highly branched isoprenoid polyenes biosynthesised by the diatom, Haslea ostrearia.

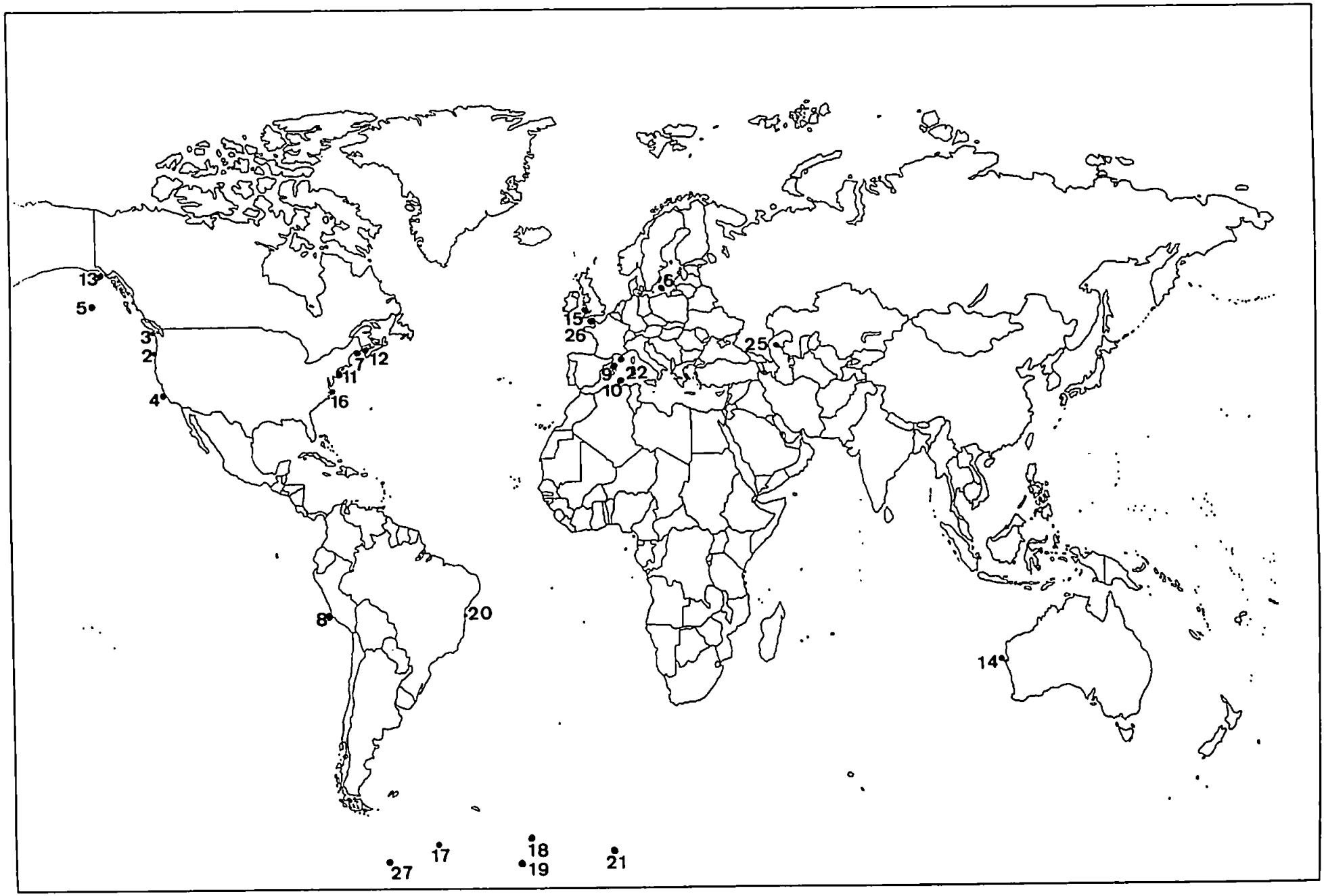
1.1 Highly Branched Isoprenoid Hydrocarbons

Highly branched isoprenoid (HBI) alkanes and alkenes are widely distributed, and often abundant, natural hydrocarbons found in coastal marine sediments across the globe (reviewed by Rowland and Robson 1990; Hird, 1992). They have been reported in regions as diverse as Puget Sound, west coast USA (Barrick *et al.*, 1980), coastal Peru (Volkman *et al.*, 1983), Antarctica (Nichols *et al.*, 1988; Cripps *et al.*, 1995), S.W. England (Hird *et al.*, 1992) and the Caspian Sea (Belt *et al.*, 1994), (Figure 1.1). The C_{20} (**I**), C_{25} (**II**) and C_{30} (**III**) alkanes have been unambiguously identified by synthesis (Yon *et al.*, 1982; Robson and Rowland, 1986, 1988), but the unsaturated counterparts with one to six double bonds, which are much more common, have been less well studied.

Figure 1.2 Structures of the parent C_{20}, C_{25} and C_{30} carbon skeletons



Only monoenes of C_{20} HBIs have been reported, whereas C_{25} HBI alkenes possess one to five double bonds (Table 1.1) and the C_{30} analogues five or six. The



Numbers indicate references listed in Table 1.1a

Table 1.1 Sedimentary occurrences of C_{25} HBI hydrocarbons where GC retention indices are cited (updated from Hird, 1992)

Stationary phase	OV1	OV1	OV1	DB1	DB1	HP1	HP1	SE30	SE30	SE30	SE30	SE30	DB5	DB5	DB5	DB5	DB5	DB5	SP2100	SP2100	SP2100	SP2100	SP2100	OV101	MS
Compound																									
C25:0 (II)		2112		2110				2111					2105						2109	2109					2112
C25:1																					2106				
C25:1																									2112
C25:2			2067					2070	2072		2068		2068			2068	2070								
C25:2	2080	2082	2082		2079			2084		2082	2084	2084	2082	2082			2083								
C25:2																		2085							
C25:2						2088						2088	2088		2088	2088									
C25:3								2044	2044			2044			2046	2044	2044			2044					
C25:3								2091	2092		2091	2091	2091			2091	2090		2090	2090	2094		2089	2092	
C25:3								2104				2104													
C25:3							2106	2106		2107		2106			2110	2107	2107								
C25:3													2119												
C25:3																2156									
C25:4									2082							2079				2078					
C25:4																2086									
C25:4																						2055			
C25:4									2129							2126	2128			2124					
C25:4							2134									2133									
C25:4							2144																		
C25:4																	2175								
C25:5																2125									
C25:5																2144									
C25:5									2170							2169									
C25:5							2173																		
C25:5									2183							2183	2182								
C25:5							2191		2188																
C25:5							2200																		
References	*1*	*15*	*16*	*23*	*25*	*22*	*24*	*7*	*8*	*9*	*11*	*12*	*10*	*17*	*18*	*20*	*26*	*21*	*2*	*3*	*4*	*5*	*6*	*13*	*14*

Key : Table 1.1a

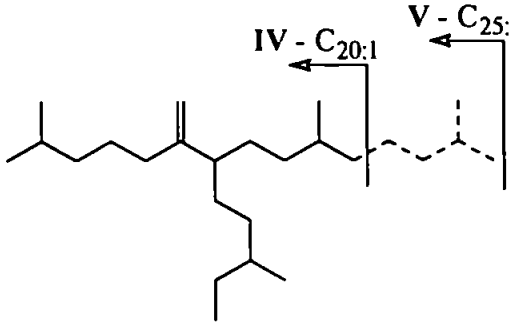
Table 1.1a

Key : Numerical values stated in Table 1.1 are GC retention indices (GC RI). Italic numbers relate to the references listed below:

(1) Blanchard (1979)
(2) Prahl *et al.*, (1980)
(3) Barrick *et al.*, (1980)
(4) Venkatesan *et al.*, (1980)
(5) Venkatesan and Kaplan (1982)
(6) Osterroht *et al.*, (1983)
(7) Requejo and Quinn (1983)
(8) Volkman *et al.*, (1983)
(9) Albaiges *et al.*, (1984a)
(10) Albaiges *et al.*, (1984b)
(11) Requejo *et al.*, (1984)
(12) Requejo and Quinn (1985)
(13) Shaw *et al.*, (1985)
(14) Dunlop and Jefferies (1985)
(15) Rowland *et al.*, (1985)
(16) Voudrias *et al.*, (1986)
(17) Venkatesan and Kaplan (1987)
(18) Venkatesan (1988)
(19) Nichols *et al.*, (1988)
(20) Porte *et al.*, (1990)
(21) Yruela *et al.*, (1990)
(22) Green *et al.*, (1992)
(23) Ten Haven *et al.*, (1993)
(24) Volkman *et al.*, (1994)
(25) Belt *et al.*, (1994)
(26) Hird and Rowland (1995)
(27) Cripps (1995)

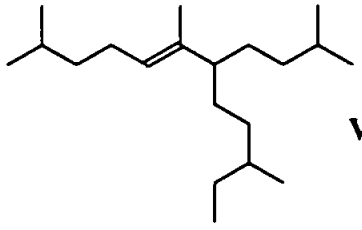
unusual highly branched structures, variation in degree of unsaturation and widespread distribution has led to the proposal that HBIs may be useful as palaeoenvironmental indicators or biomarkers (Kenig *et al.*, 1990; Kohnen *et al.*, 1991b; 1992a). However, it would seem to be premature to use HBIs as palaeoenvironmental indicators before they have been rigorously identified and their biological sources have been established.

A complete structural analysis of the alkenes has been limited to a relatively few examples. Dunlop and Jefferies (1985) observed a C_{20} monoene (gas chromatographic retention index, GC RI 1703) and a C_{25} monoene (GC RI 2112) in the hypersaline waters of Shark Bay, Western Australia. Ozonolysis of the $C_{20:1}$ and $C_{25:1}$ isolates yielded a C_{19} ketone molecular weight, M.W. 282 and a C_{24} ketone M.W. 352 respectively, indicating in both cases an olefinic methylene bond. Mass spectral analysis of the ozonolysis products suggested structures (**IV**) and (**V**) for the alkenes.



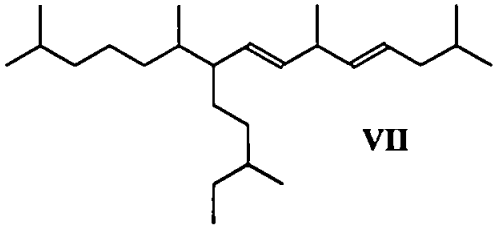
Hird *et al.*, (1992) characterised four HBI monoenes by a combination of gas chromatographic (GC), spectroscopic (GC-MS, in one case NMR) and degradative (ozonolysis) analyses of pure isolates. A C_{20} monoene from sediments of the Tamar estuary (UK) and a C_{25} monoene (hydrogenation product of a diene) from McMurdo Sound (Antarctica) both contained methylene double bonds, identical to those reported

by Dunlop and Jefferies (1985), (**IV**) and (**V**). The structure of a sedimentary C_{20} monoene from Gluss Voe (Shetland Isles) was tentatively assigned by comparing its RI and mass spectrum with that of a synthetic C_{20} monoene (**VI**).



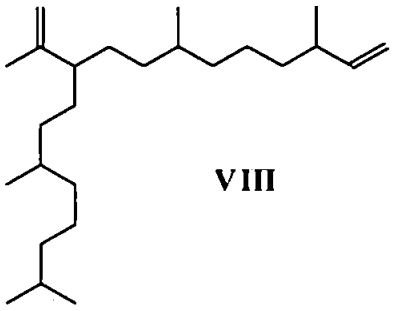
VI

Hird *et al.*, (1992) also concluded that the double bonds for two C_{25} monoenes from the Tamar estuary were probably in non-methylenic positions by comparison with other synthetic C_{25} monoenes (Robson, 1987). The double bond positions in a HBI diene have also been established. Thus, Yruela *et al.*, (1990) used epoxide formation with *m*-chloroperoxybenzoic acid to assign tentatively, the double bond positions (not geometry) in a C_{25} diene (RI 2085) (**VII**), isolated from mesohaline sediments (Guadalquivir delta, S.W. Spain).



VII

This derivatisation process succeeded where alkylthiolation, oxymercuration and treatment with osmium tetroxide, failed (reviewed by Robson, 1987; Rowland and Robson, 1990; Nichols *et al.*, 1988). The double bond positions in another C_{25} diene (**VIII**) were unambiguously assigned *via* NMR spectroscopy and ozonolysis (Summons *et al.*, 1993). However, this is the only report of a C_{25} alkene with this parent structure.



VIII

In view of the potential geochemical importance of HBI compounds it is essential that the structures and biological origin(s) are established and the factors controlling HBI production are investigated. Evidence to date strongly indicates that certain diatomaceous algae are a source of these unusual compounds. Nichols *et al.*, (1988) reported a C_{25} HBI alkadiene as a major hydrocarbon in natural populations of Antarctic sea-ice diatom communities and suggested that the earlier report of the compound in *Enteromorpha prolifera* was also due to epiphytic diatoms. Summons *et al.*, (1993) also isolated the C_{25} HBI alkadiene (**VIII**) from a diatomaceous benthic microbial community. These findings indicate a source from diatoms, but the possibility that associated marine bacteria actually produce the HBI alkenes (Requejo and Quinn, 1983; Robson and

Rowland, 1986) was not refuted until the first isolation of a suite of HBI hydrocarbons from two laboratory grown diatom species. Volkman *et al.*, (1994) identified C_{25} HBI alkenes with three to five double bonds and C_{30} HBI alkenes with five and six double bonds in axenic cultures of the diatoms *Haslea ostrearia* and *Rhizosolenia setigera* respectively. These results probably explain why the HBI alkenes are abundant in marine environments where diatoms are the major constituents of the benthic and planktonic algal communities (e.g. upwelling areas off Peru, productive coastal regions, algal mats and sea-ice diatoms). However, HBIs have not been reported in other diatoms (reviewed by Rowland and Robson, 1990) and virtually nothing is known about the conditions under which diatoms produce HBIs. Furthermore, none of the alkenes in the cultured or field diatoms have been rigorously characterised, their parent structures having simply been established by hydrogenation to the alkane, for which full spectral data are available (Robson, 1987; Robson and Rowland, 1986, 1988). No specific source has yet been reported for the C_{20} HBIs. Rowland *et al.*, (1985) isolated a C_{20} HBI alkane and monoene from the macroscopic green alga *Enteromorpha prolifera*, but this was reattributed to the presence of epiphytic diatoms, by Volkman *et al.*, (1994) and Nichols *et al.*, (1988), particularly since n-$C_{21:6}$ was also observed in the hydrocarbon fraction and this alkene is typical of diatoms.

1.2 Sedimentary occurrence of C_{25} HBI alkene isomers

Twenty six apparently different C_{25} HBI alkenes have been reported to occur in the marine environment (Table 1.1). Previous authors have tentatively identified some isomers by a combination of gas chromatographic retention indices (GC RI), spectroscopic features (MS, NMR), degradative analysis (ozonolysis, epoxidation) and more rarely by co-injection with synthetic compounds (Dunlop and Jefferies, 1985; Rowland and Robson, 1990; Yruela *et al.*, 1990; Hird *et al.*, 1992; Summons *et al.*, 1993). However, the majority of HBI alkenes have yet to be fully characterised and without this detailed knowledge it is difficult to establish whether some HBI isomers, reported by different authors, are actually the same. Hird (1992) showed that a number of C_{20} monoenes had similar GC RIs, yet had different double bond positions, emphasizing the care needed in making assignments by GC RI alone. The classification, by Hird (1992), of HBI isomers from different reports (Table 1.1) was based on a comparison of GC RI and mass spectral data. However, a detailed comparison of the GC RI and mass spectra, coupled with the frequency of occurrence and relative abundance of all the isomers reported in the literature (Table 1.1a), suggested to the present author that there are, in fact, only fourteen dominant C_{25} alkene isomers that are abundant and/or commonly occurring in the marine environment (Table 1.2).

Barrick and Hedges (1980) and Porte *et al.*, (1990) both reported that the mass spectra of two trienes (GC RI 2044 & 2091) and two tetraenes (GC RI 2080 & 2127) identified in their studies were identical (*e.g.* Figure 1.3). Moreover, the difference in GC RI (ΔGC RI = 47) between the two trienes was identical to the ΔGC RI between the two tetraenes. The explanation suggested for this, in both reports, was that all four molecules

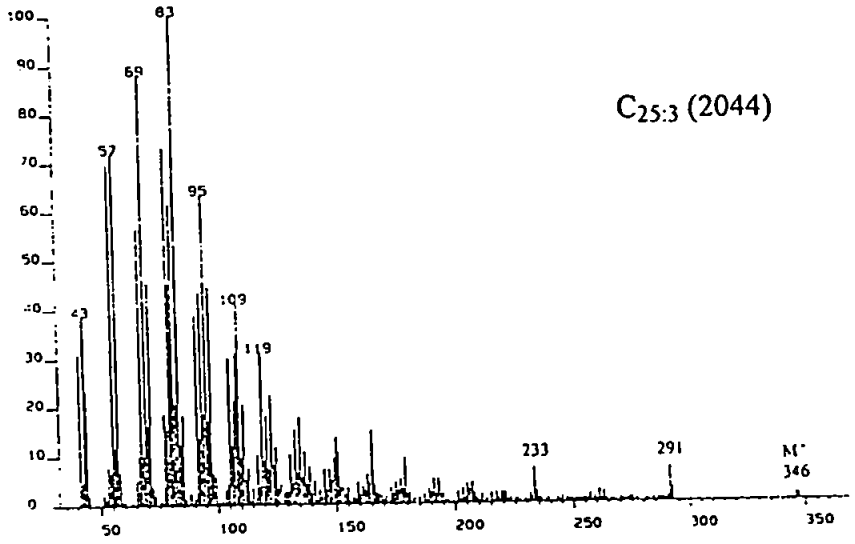
Table 1.2 Sedimentary occurrences of major C_{25} HBI alkenes (adapted from Table 1.1)

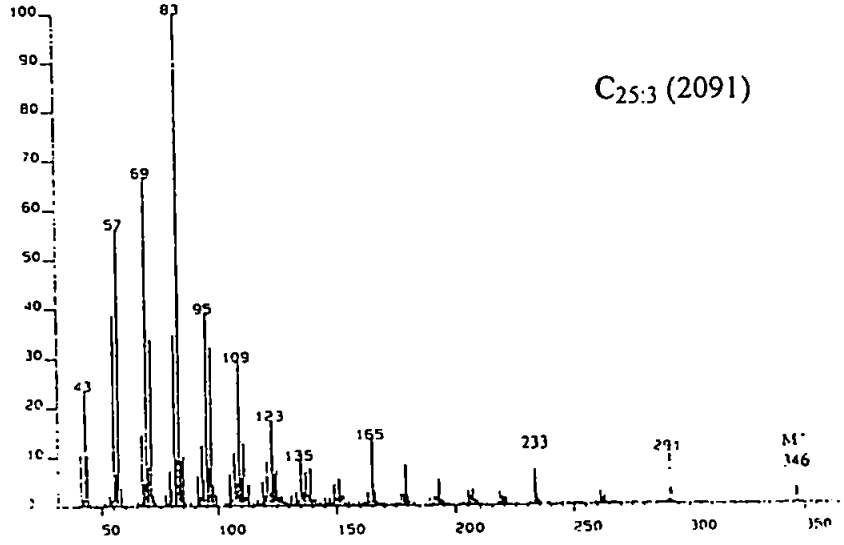
Reference	*1*	*15*	*16*	*25*	*7*	*8*	*9*	*11*	*12*	*10*	*18*	*20*	*26*	*2*	*3*	*4*	*6*	*19*		**Average RI for**	
Major Alkene																				**major alkenes**	
C25:2			2067		2070	2072		2068		2068		2068	2070							**C25:2**	**2069**
C25:2	2080	2082	2082	2079	2084		2082	2084	2084	2082			2083							**C25:2**	**2082**
C25:2									2088	2088	2088	2088						2088		**C25:2**	**2088**
C25:3					2044	2044			2044		2046	2044	2044		2044					**C25:3**	**2044**
C25:3					2091	2092		2091	2091	2091		2091	2090	2090	2090	2094	2089			**C25:3**	**2091**
C25:3					2106		2107		2106		2110	2107	2107							**C25:3**	**2107**
C25:4						2082						2079			2078					**C25:4**	**2080**
C25:4												2086								**C25:4**	**2086**
C25:4						2129						2126	2128		2124					**C25:4**	**2127**
C25:4												2133								**C25:4**	**2133**
C25:5												2125								**C25:5**	**2125**
C25:5												2144								**C25:5**	**2144**
C25:5						2183						2183	2182							**C25:5**	**2183**
C25:5												2191								**C25:5**	**2191**

Key : Numerical values are GC retention indices

Italics indicates references listed in Table 1.1a

Figure 1.3 Representative mass spectra of $C_{25:3}$ (2044) and $C_{25:3}$ (2091)





were structurally identical except for geometric isomerism about one double bond and the presence of an additional double bond at a 'remote site' in the $C_{25:4}$ compounds. The two trienes and tetraenes reported by Barrick and Hedges (1980) and Porte *et al.*, (1990) are the dominant isomers in many reports (Table 1.2), but several other pairs of the major sedimentary alkenes in Table 1.2 also exhibit ΔGC RI of 47. For example, one pair of pentaene isomers also has ΔGC RI = 47, (GC RI 2144 & 2191), and these occur in high concentrations in the marine environment (Volkman *et al.*, 1983; Porte *et al.*, 1990).

Although none of the C_{25} dienes exhibit ΔGC RI = 47, Venkatesan (1988) noted that the mass spectrum of a $C_{25:2}$ (GC RI 2088) from Antarctica was virtually identical in both fragmentation and fragment abundance to that of a C_{25} diene (GC RI 2082), previously reported from sediments (Requejo and Quinn, 1983; Requejo *et al.*, 1984; Venkatesan and Kaplan, 1987a) and particulates (Albaiges *et al.*, 1984b). Co-injection of two aliphatic fractions (Venkatesan, 1988), one isolated from Bransfield Strait sediments (Venkatesan and Kaplan, 1987a) and the other isolated from McMurdo Sound sediments (Venkatesan, 1988), containing a $C_{25:2}$ GC RI 2080 and a $C_{25:2}$ GC RI 2088 respectively, showed that the two alkenes were not the same and led the author to suggest that they were geometric isomers.

These data tend to corroborate the earlier suggestion that several of the C_{25} HBI alkenes are geometric isomers and that some HBIs with three, four and five degrees of unsaturation may contain some double bonds in common positions, but this requires verification by full structural characterisation.

1.3 Diagenetic fate of C_{25} HBI hydrocarbons

In common with many organic geochemical studies of biological marker compounds, considerable interest has been shown, not only in the origins and structures of the HBI hydrocarbons, but also in their long term geological fate. Although detailed depth related studies have been hampered by the incomplete structural characterisation of most of the HBI compounds, several studies have attempted to follow their fate in a general way, both in the sediments and water column (reviewed by Rowland and Robson, 1990; Hird, 1992).

Numerous studies have shown that high concentrations of C_{25} HBI alkenes are typically only present in surface sediments and decrease rapidly with increasing sediment depth (Farrington *et al.*, 1977; Barrick *et al.*, 1980; Volkman *et al.*, 1983). Few explanations have been given for the rapid removal of C_{25} alkenes from the hydrocarbon fraction. Laboratory based studies by Robson and Rowland (1988b) and Gough *et al.*, (1992) showed the parent alkane **II** 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, the alkane **II** being most resistant. Volkman *et al.*, (1983) observed a slight increase in the concentration of *n*-alkanes with depth, in sharp contrast with profile of HBI alkenes, were concentrations decreased. This suggested that biodegradation was not a major mechanism in the removal of HBI compounds from the hydrocarbon fraction and Volkman *et al.*, (1983) postulated that the trends observed may be due to incorporation of HBIs into accreting humic substances.

An alternative mechanism for the depletion of the HBI alkenes comes from analysis of related organic sulphur compounds (reviewed by Sinninghe Damste and de

Leeuw, 1990; Kohnen *et al.*, 1992). Sinninghe Damste *et al.*, (1989b) proposed that sulphur is abiotically incorporated into a variety of unsaturated lipid precursors, including HBIs, during the early stages of diagenesis. This sulphur enrichment would remove the labile functionalised precursors from the geochemical record, while still preserving their carbon skeletons and information on the sites of their functionality. This could be significant for HBI alkenes, as it suggests that double bond positions might be preserved through the incorporation of sedimentary sulphur into the structures. Additionally, the potential use of HBI alkenes as biomarkers would depend not only on a rigorous determination of their structures, but also for related organic sulphur compounds and an understanding of the relevant diagenetic mechanisms.

1.4 Summary

- C_{25} HBI alkenes are widely distributed, and often abundant, natural hydrocarbons found in coastal marine sediments across the globe.

- A wide body of evidence suggests the C_{25} compounds are biogenic in origin, and one common diatom species, *Haslea ostrearia* is known to produce triene, tetraene and pentaene HBIs in culture. Other diatoms (> 50 spp.) have been shown to not produce them.

- The parent alkane structures **I, II & III** have been proved by synthesis, but only a few of the double bond positions have been established in the alkenes, comprising several monoenes and a diene (**IV - VIII**).

- Of the C_{25} HBI alkenes reported (Table 1.1), only fourteen of these are dominant in the marine environment (Table 1.2).

- Mass spectral and ΔGC RI (Table 1.2) data for some of the alkenes suggests that they may be structurally related in the geometry and location of the double bonds.

- HBI alkenes with two or more double bonds appear to be rapidly removed from the hydrocarbon fraction of most sediments, possibly due to the incorporation of sulphur.

- HBI compounds, both as hydrocarbons and S-containing analogues, may prove useful environmental indicators once the exact structures have been established.

CHAPTER TWO

Investigations into the sedimentary occurrence and biological sources of C_{25} HBI hydrocarbons

This chapter describes the distribution of C_{25} HBI hydrocarbons in recent estuarine sediments. The temporal distribution of HBI hydrocarbons and carotenoid biomarkers in sediments is reported and the implications discussed. The results suggest that diatoms are a source of the sedimentary HBI hydrocarbons.

2.1 Introduction

Ideally, before HBI alkenes are applied as environmental and/or palaeoenvironmental indicators, their biological sources should be established. Prior to the recent report of C_{25} HBIs in the common coastal diatom *Haslea ostrearia* (Volkman *et al.*, 1994), the origins of these compounds had remained obscure, with numerous authors tentatively suggesting diatomaceous or bacterial sources (e.g. Barrick and Hedges, 1981; Volkman *et al.*, 1983; Rowland and Robson, 1990). In an attempt to establish the temporal variations of HBIs in sediments and thereby to establish possible biological sources, Hird and Rowland (1995) examined the concentrations of HBIs in sediments of the Tamar estuary, at monthly intervals throughout 1990. C_{25} HBI alkenes with two to five double bonds were present at various times of the year and the concentrations of these co-varied with the corresponding C_{20} HBI alkane and monoene concentrations. However the concentrations of HBI alkenes did not co-vary directly with those of another well known biochemical produced by diatoms (viz. the alkene, *n*-$C_{21:6}$, heneicosahexaene), but maximum concentrations preceded those of *n*-$C_{21:6}$ by 1-2 months at times in 1990. The authors suggested that this may be due to production of HBIs by diatoms, such as *Haslea ostrearia*, earlier in their growth phase than production of *n*-$C_{21:6}$, but they also acknowledged the possibility that HBIs were produced by diatoms other than those, such as *Haslea ostrearia*, which produced *n*-$C_{21:6}$. Obviously then, further research is needed to investigate these possibilities.

Although *n*-$C_{21:6}$ is produced by many diatoms, including *Haslea ostrearia*, it does not necessarily reflect total diatom population growth throughout the year. To

establish a less ambiguous link with diatom populations, additional and perhaps better, diatom markers are required. Carotenoids are likely candidates for this purpose since to date, over 500 carotenoid pigments have been reported in the marine environment. Interest in using carotenoids as chemotaxonomic indicators has resulted in a systematic and thorough investigation of the carotenoids of most major classes of marine organisms (e.g. Jeffrey, 1976; Liaaen-Jensen, 1978; 1979; Jeffrey and Hallegraeff 1987; Barlow *et al.*, 1993a). Indeed, all marine phytoplankton, many bacteria, fungi, zooplankton, and higher heterotrophs synthesise characteristic suites of pigments which can serve as biomarkers. These carotenoids often incorporate unique structural components which allow assignment of the source organism at the genus or family level of taxonomic classification (Table 2.1).

The marked increase in the use of photosynthetic pigments as markers for identifying different algal classes in the marine environment is mainly attributable to advances in analytical techniques, particularly the development of HPLC (reviewed by Roy, 1987; Wright *et al.*, 1991) which has replaced two-dimensional thin-layer chromatography (Jeffrey, 1974) as the method of choice for routine screening of samples. Pigment chromatography has proven especially valuable for identifying contributions from the nanoplankton. For example, the presence of green algae in marine waters was suggested from the identification of chlorophyll *b* (Jeffrey, 1976; Gieskes *et al.*, 1978; Klein and Sournia, 1987) and Cryptophytes were identified from the presence of alloxanthin (Klein and Sournia, 1987).

Although marker pigments have been used to provide qualitative descriptions of phytoplankton communities (Hallegraeff, 1981; Gieskes and Kraay, 1984, 1986; Jeffrey

Table 2.1 Summary of major signature pigments for algal types in the ocean (adapted from Wright *et al*., 1991).

Pigment	Algal type	Reference
Chlorophyll *a*	All photosynthetic microalgae, except prochlorophytes	1, 2
Chlorophyll *b*	Chlorophytes, prasinophytes, euglenophytes, prochlorophytes	1, 2
Chlorophyll *c* family	Chromophyte algae	3, 18
c_1	Most diatoms, some prymnesiophytes, some chysophytes	3, 4, 5
c_2	Most marine chromophyte algae (except some chysphytes)	3, 4, 5
c_3	Some prymnesiophytes, chrysophytes, diatoms	3, 6, 7
Fucoxanthin	Diatoms, some prymnesiophytes, chrysophytes, a few raphidophytes	1, 8, 18
19'-hexanoyloxyfucoxanthin	Prymnesiophytes	7, 8, 9, 10, 18
19'-butanoyloxyfucoxanthin	Prymnesiophytes and chrysophytes	7, 8, 9, 10, 18
Peridinin	Most photosynthetic dinoflagellates	11, 12, 18
Zeaxantin	Cyanobacteria (blue-green algae), prochlorophytes	13, 14, 18
Fucoxanthin and Violaxanthin	Some chrysophytes	8, 15
Alloxanthin	Cryptomonads	16,18
Prasinoxantin	Some prasinophytes	17, 21
Lutein	Green algae (chlorophytes and some prasinophytes)	1, 13

References: 1, Jeffrey, (1974); 2, Chisholm *et al*., (1988); 3, Jeffrey, (1989); 4, Stauber *et al*., (1988); 5, Andersen and Mulkey, (1983); 6, Jeffrey and Wright, (1987); 7, Vesk and Jeffrey, (1987); 8, Bjornland, (1987); 9, Wright and Jeffrey, (1987); 10, Bjornland *et al*., (1989); 11, Jeffrey *et al*., (1975); 12, Johansen *et al*., (1974); 13, Guillard *et al*., (1985); 14, Gieskes *et al*., (1988); 15, Withers *et al*., (1981); 16, Pennington *et al*., (1985); 17, Foss *et al*., (1984); 18, Barlow *et al*., (1993).

and Hallegraeff, 1980, 1987; Wright, 1987), it is only recently that there have been attempts to estimate the quantitative contribution to biomass of algal groups from the proportions of marker pigments. Gieskes *et al.*, (1988) used HPLC analysis of carotenoid and chlorophyll pigments to estimate the abundances of algal groups in the Banda Sea, near Indonesia. They calculated the contribution of each algal class to total Chl *a* using multiple linear regression. This technique makes no assumptions about the Chl *a* / marker pigment ratios in particular algal groups, other than that they are constant in each sample group. This procedure was shown to work best with a large data set and the analysis was restricted to the major pigments. The contributions of minor groups can be lost in the variance of the data and the technique fails when the concentrations of all pigments co-vary, which can be a fairly common situation, for example, when several types of algae respond to a localised nutrient enrichment. The most popular current approach is rather to assume that certain Chl *a* / marker pigment ratios apply to each of the algal groups and to use these directly to calculate the contribution of Chl *a* from each group. This method uses more of the marker pigments, including minor components, and allows multiple contributions of a particular pigment to be included and quantified. This technique has been applied successfully in numerous studies of the marine environment (e.g. Gieskes *et al.*, 1988; Roy, 1989; Wright *et al.*, 1990; Bidigare *et al.*, 1990b; Barlow *et al.*, 1990, 1993a, 1993b, 1995; Latasa, 1992). Some pigments are produced by more than one algal class and this must be taken into consideration. Most diatoms have very similar pigment compositions, and Stauber and Jeffrey (1988) analysed 51 diatom species and found that all but one contained fucoxanthin, diadinoxanthin and diatoxanthin. A few prymnesiophytes, chrysophytes and raphidophytes are also known to produce fucoxanthin

(Arpin *et al.*, 1976; Wright and Jeffrey, 1987). However, Everitt *et al.*, (1990) and Barlow *et al.*, (1993a) report that where diatoms are the major constituents of phytoplankton communities (as in the Tamar estuary) it is possible to use fucoxanthin as a direct marker for diatoms. Similarly 19'-hexanoyloxyfucoxanthin is a marker for prymnesiophytes, since only one diatom and a few dinoflagellates are known to produce this pigment (Vesk and Jeffrey, 1987; Wright and Jeffrey, 1987). Chlorophyll *b* was thought to be an accurate marker for green algae (Chlorophyceae and Prasinophyceae; e.g. Jeffrey, 1976), but prokaryotic prochlorophytes are now recognised as significant contributors (Gieskes *et al.*, 1988). However, it is even possible to distinguish between these two sources since prochlorophytes also contain high concentrations of zeaxanthin (Chisholm *et al.*, 1988).

Hence, many of the sources of organic matter in the marine water column can be identified to a high degree of specificity from a knowledge of the carotenoid distributions. In a few cases this approach has been used to assign organic inputs to young sediments (e.g. Repeta and Simpson, 1991), but carotenoids are labile compounds and undergo a variety of transformations in sediments and in laboratory procedures, particularly if care is not taken with their isolation and analysis (Repeta and Gagosian, 1981; 1984; 1987). Such transformations may have the effect of obscuring or complicating the original pigment distributions and may also influence the extractability of the carotenoids. Therefore in the current study, the extractability of carotenoids from recent sediments in the Tamar estuary was investigated and suitable analytical and sampling methods for the labile carotenoids developed. The multiple carotenoid approach (e.g. Barlow *et al.*, 1995) was then applied to marker pigment distributions in the Tamar estuary during 1993/4 and

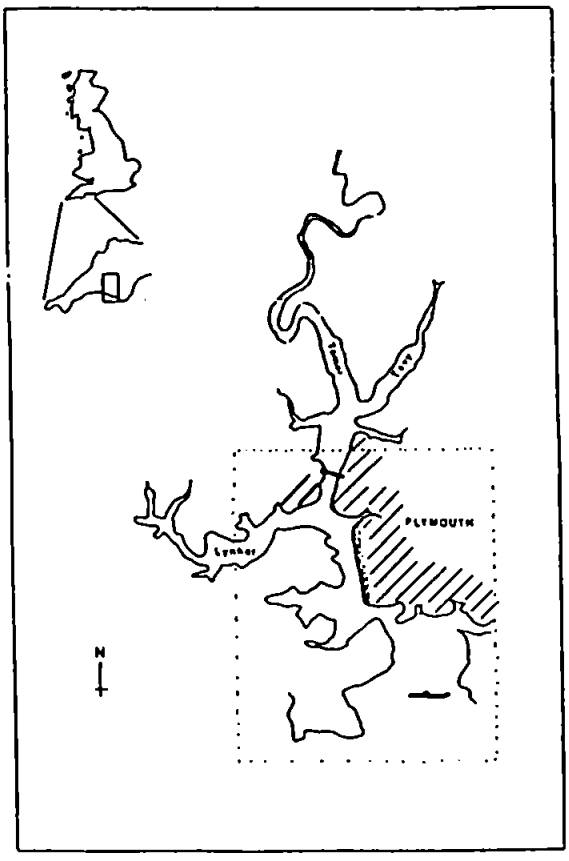
these compared to concentrations of HBIs and other biochemicals produced by diatoms in a study similar to that of Hird and Rowland (1995) but with increased numbers of samples collected during periods of diatom blooms. The use of carotenoid pigments as markers for algae also allowed the variation of HBIs with other algal classes to be investigated.

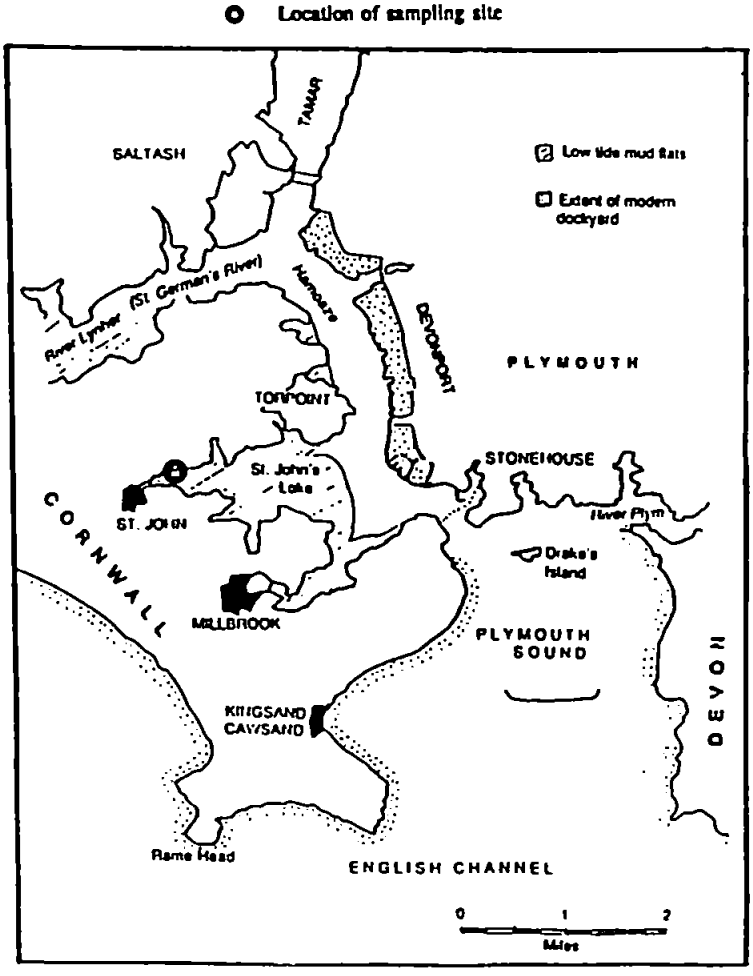
2.2 Results and Discussion

2.2.1 Environmental setting of Tamar estuary

Surface (0-1 cm) sediment samples were collected from St. Johns Lake (Tamar estuary), located in south-west England, (Figure 2.1). This location was primarily chosen due to the abundant and consistent production of HBIs (Robson, 1987; Hird and Rowland, 1995), the very low input of anthropogenic organic matter (Robson, 1987; Hird, 1992) and also because its physical, chemical and biological processes are well documented (e.g. Morris *et al.*, 1978; 1981; 1982; Loring *et al.*, 1982; 1985; Reeves and Preston, 1989). It is characterised by extensive mud flats, with tidal water depths reaching 4-5 m and water temperatures varying between ~20°C in August to ~4°C in February (Morris *et al.*, 1982). The intertidal sediments are chemically homogenous and generally black in colour but the latter are overlain by a thin light-brown oxidised layer (~2-5 mm). The sediments are relatively stable with an annual sedimentation rate of ~1 cm (Clifton and Hamilton, 1979), although small perturbations of the uppermost 1 cm of the sediment column occur in response to tidal oscillations (Bale *et al.*, 1985).

Figure 2.1 Map of Tamar estuary showing location of sediment sampling site at St. Johns Lake





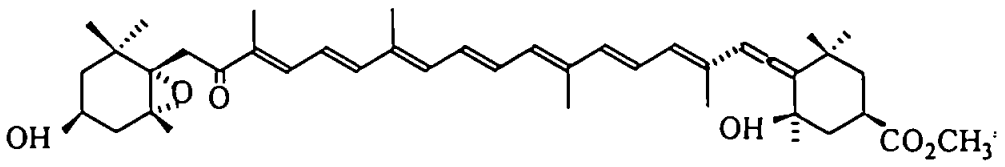
2.2.2 Extractability of carotenoid pigments from Tamar sediments

The complex mixtures of carotenoids found in most marine environments and the lability of these compounds in sediments (Repeta and Gagosian, 1981; 1984; 1987) can make their quantitative determination in sediments very difficult (Wright *et al.*, 1991). To date, little or no research has been published on the extractability and recovery of different pigments from sediments. Jeffrey *et al.*, (1995) have reviewed the various solvent systems applied to extraction of pigments from algae in a *magnum opus* of carotenoid chemistry which is soon to be published, but such data do not necessarily relate to the efficiency of extraction of carotenoids from sediments. In the absence of such data a limited number of experiments were conducted in the present study to ascertain whether selectivity in recoveries of carotenoid pigments of different algal classes could be expected. Obviously such selectivity might negate or restrict the use of carotenoids as quantitative markers of algal inputs to sediments.

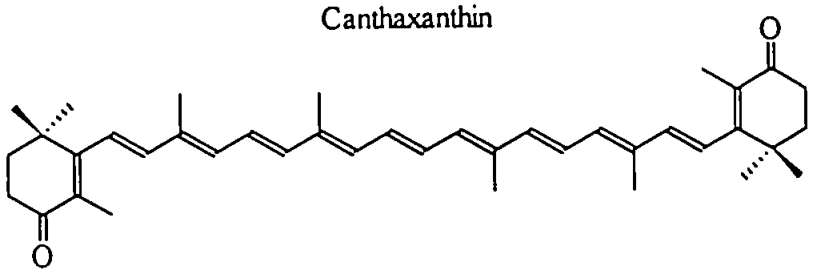
Two clays (montmorillonite and kaolinite, 10 g each, >99.8% purity; Aldrich Chemicals) and a pre-extracted Tamar sediment, (repeated Soxhlet extraction, DCM / MeOH, until no extractable organic matter remained, checked by GC and UV-Vis. spectroscopy), were spiked with equal amounts (~1 mg) of three carotenoids and chlorophyll *a*, (canthaxanthin, bixin, β-carotene and chlorophyll *a*, >99% purity, Natural Products). These carotenoids were chosen on the basis that they have very different polarities (Fig 2.2) and are available commercially in pure state. Whilst these carotenoids are not all found in algae, most algal carotenoids are not available in pure form in large quantities and are therefore not very suitable for method development.

Figure 2.2 Structures of carotenoids

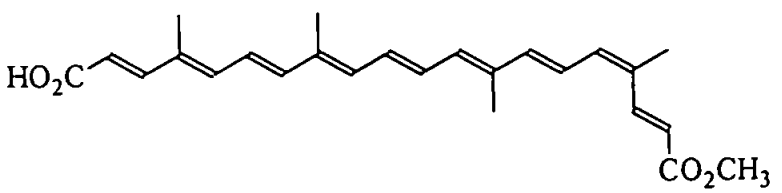
Fucoxanthin



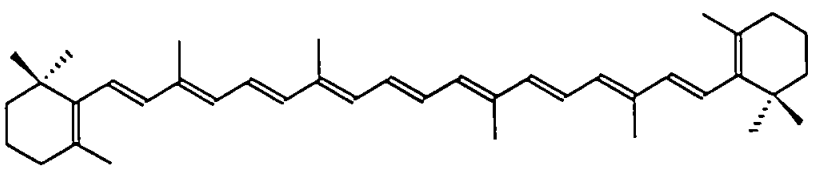
Canthaxanthin



Bixin *(9Z)-form*



β-Carotene



The spiked clays and sediments were extracted, (90% acetone / 10% MeOH, ultrasonication 15 minutes, repeated ×10), by the method of Barlow *et al.*, (1990) and the results are shown in Table 2.2 (samples analysed via UV/vis spectroscopy).

Table 2.2 Recovery of carotenoids for extractability experiments, (values indicate the percentage of each carotenoid extracted, n=1).

Pigment	Montmorillonite	Kaolinite	Sediment (Tamar)
Canthaxanthin	21	29	25
Bixin	29	26	32
β-Carotene	29	37	23
Chlorophyll a	31	34	27

Whilst recoveries were low (~ 30%) there was no significant preferential extraction of any of the carotenoids, despite the differences in their chemistries. Thus the suggestion that the selectivity of the extraction might negate the use of carotenoids as algal biomarkers in sediments (Barlow, personal communication) appears to be groundless, at least judging from these experiments, for these minerals and sediments.

2.2.3. Phytoplankton and HBI distributions at St. Johns Lake, Tamar estuary

Forty-six pigments and pigment degradation products, were extracted (using the validated method described in 2.2.2), resolved and measured (HPLC-UV / fluorescence) in sediments from the Tamar estuary collected during 1993 / 1994. Sediment samples were packed in dry ice at the sampling site for transportation to laboratory and then stored in liquid nitrogen prior to analysis. These procedures greatly reduced degradation of the pigments between collection and analysis, as reflected by the low abundance of degradation products such as *cis*-carotenoids, 5,8-epoxides, and phaeopigments (Figure

2.3). Major pigments were identified by their relative retention times (cf. Barlow *et al.*, 1993) and minor peaks in the chromatograms were not assigned (Figure 2.4).

The sediments were dominated by seven major pigments, indicative of the relative composition of the major classes of algae. The estimated contributions of each algal class to total Chl *a* provide an indication of their relative contributions to total algal biomass and were calculated by multiplying concentrations of marker pigments by an appropriate Chl *a* / pigment ratio (Table 2.3), as advocated by numerous authors (e.g. Gieskes *et al.*, 1988; Roy, 1989; Wright *et al.*, 1990; Bidigare *et al.*, 1990b; Barlow *et al.*, 1990, 1993a, 1993b, 1995; Latasa, 1992).

Table 2.3 Marker pigment ratios used to estimate the contribution of individual phytoplankton classes to total chlorophyll *a* from carotenoid concentrations

Class	Pigment ratio	Ratio	Reference
Diatoms	Chl a : fuc	1.24	1
Dinoflagellates	Chl a : pdn	2.58	1
Cryptomonads	Chl a : all	2.01	2
Green algae	Chl a : Chl b	1.13	1
	zea : Chl b	0.019	3
Prochlorophytes	Chl b : Chl a	0.93	4
	zea : Chl b	0.16	4
Cyanobacteria	Chl a : zea	1.71	2
Prymnesiophytes	Chl a : hex	0.69	1

Chl, chlorophyll; fuc, fucoxanthin; all, alloxanthin; pdn, peridinin;
zea, zeaxanthin; hex, 19'-hexanoyloxyfucoxanthin.
References : (1) Barlow *et al.*, (1993); (2) Hager and Stransky (1970); (3) Wright and Jeffery (1987); (4) Chisholm *et al.*, (1988)

Figure 2.3 Representative HPLC chromatogram (fluoresence, 405 nm) of phaeopigments in Tamar sediments

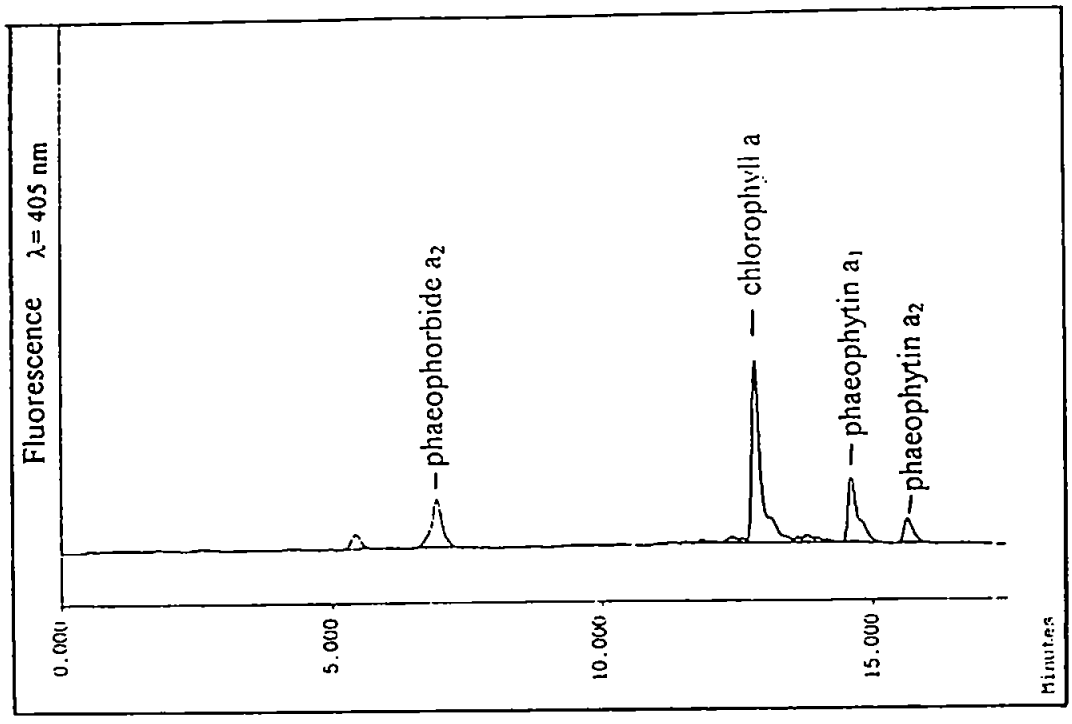
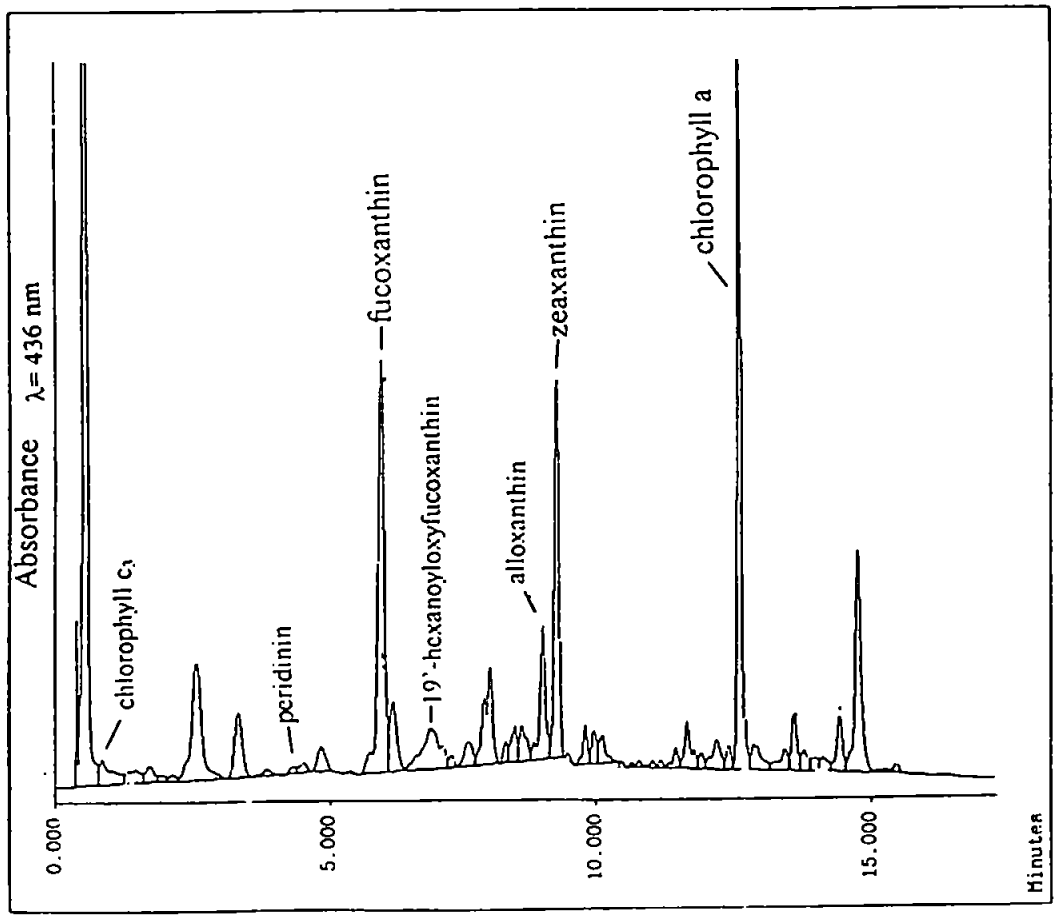


Figure 2.4 Representative HPLC chromatogram (absorbance, 436 nm) of carotenoids in Tamar sediments



The pigment biomarker data (Table 2.4) indicates that there are variations in algal community composition and total biomass in the Tamar estuary at St. Johns Lake during the year. Diatoms are major components of the phytoplankton throughout the year (29-58% of total biomass), whilst prymnesiophytes, green algae, and cryptomonads (each algal class ranging between 5-25% of total biomass), are important secondary components of the community. Cyanobacteria, prochlorophytes and dinoflagellates all comprise below 6%, reflecting relatively small contributions to the total biomass.

The concentrations of HBI isomers in Tamar sediments for 1994 are listed in Table 2.5. Figure 2.5 shows a gas chromatogram of the 'aliphatic' hydrocarbon extract from St. Johns Lake. This is similar to those presented by both Robson and Rowland (1986) and Hird (1992). The major chromatographic peaks of interest elute between GC RI 2000-2200.

Figure 2.5 Gas chromatogram of the 'aliphatic' hydrocarbons isolated from sediments at St. Johns Lake

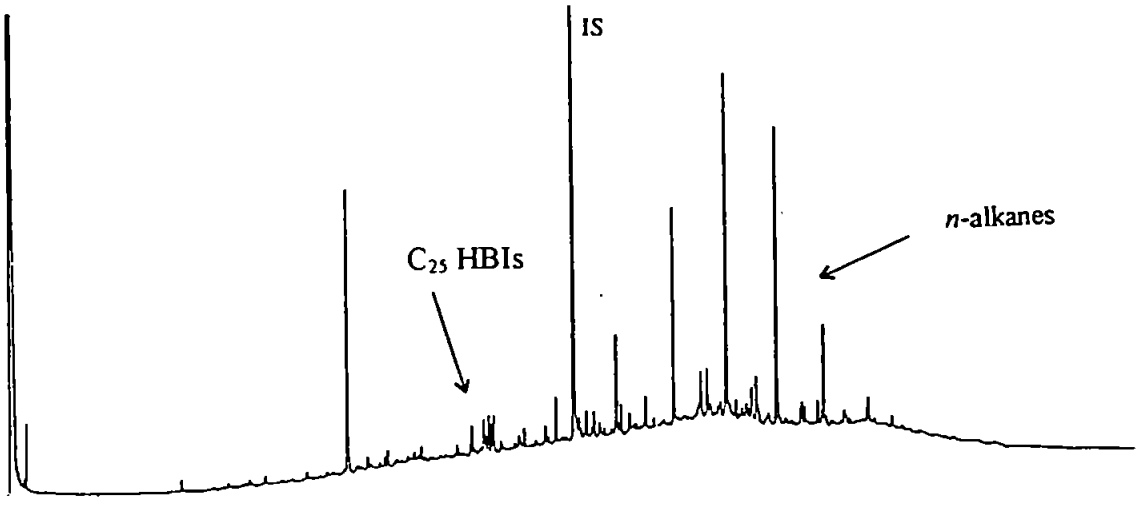


Table 2.4 Concentrations of pigments in Tamar sediments, for 1993 / 1994 (ng/g dry wt)

Signature pigment	Fucoxanthin	Peridinin	19'-hexano	19'-butano	Alloxanthin	Zeaxanthin	Chl b	Chl a
Date								
15.09.93	495.09	11.33	353.57	70.71	61.82	431.67	257.75	1189.62
23.10.93	403.23	7.41	105.99	13.44	67.5	263.64	192.44	1238.12
28.11.93	807.24	49.81	403.62	44.84	50.79	668.9	404.91	2170.79
19.12.93	483.59	46.52	176.4	69.87	76.38	395.25	238.17	1706.4
13.01.94	477.09	39.01	357.21	83.26	128.25	157.24	193.21	1510.56
17.02.94	433.63	37.99	213.12	68.41	90.48	309.43	268.79	1829.38
16.03.94	930.24	31.8	199.59	20.66	107.62	761.98	426.05	2358.61
11.04.94	1074.19	21.86	294.3	143.22	143.1	418.14	451.26	2282.15
07.05.94	1240.52	20.65	215.19	84.58	343.85	497.57	339.21	3163.62
22.05.94	1165.33	25.06	248.32	74.63	285.85	368.08	303.82	3282.87
05.06.94	911.12	54.05	260.62	24.96	192.78	245.79	337.39	2772.44
22.06.94	920.9	61.66	396.12	95.87	135.25	323.43	378.56	2739.36
02.07.94	1159.64	17.97	511.8	110.96	88.28	296.13	271.39	2754.77
19.07.94	1433.29	32.46	587.03	122.64	132.88	398.13	336.5	2846.97
28.07.94	1602.15	38.49	572.75	111.38	96.2	346.37	348.18	3368.84
08.08.94	1698.27	35.71	585.83	141.52	106.73	549.79	587.71	3595.49
23.08.94	1489.03	49.78	508.26	126.22	84.31	468.17	492.94	3163.19
31.08.94	1089.11	33.34	406.02	118.8	91.97	429.76	313.99	2191.23

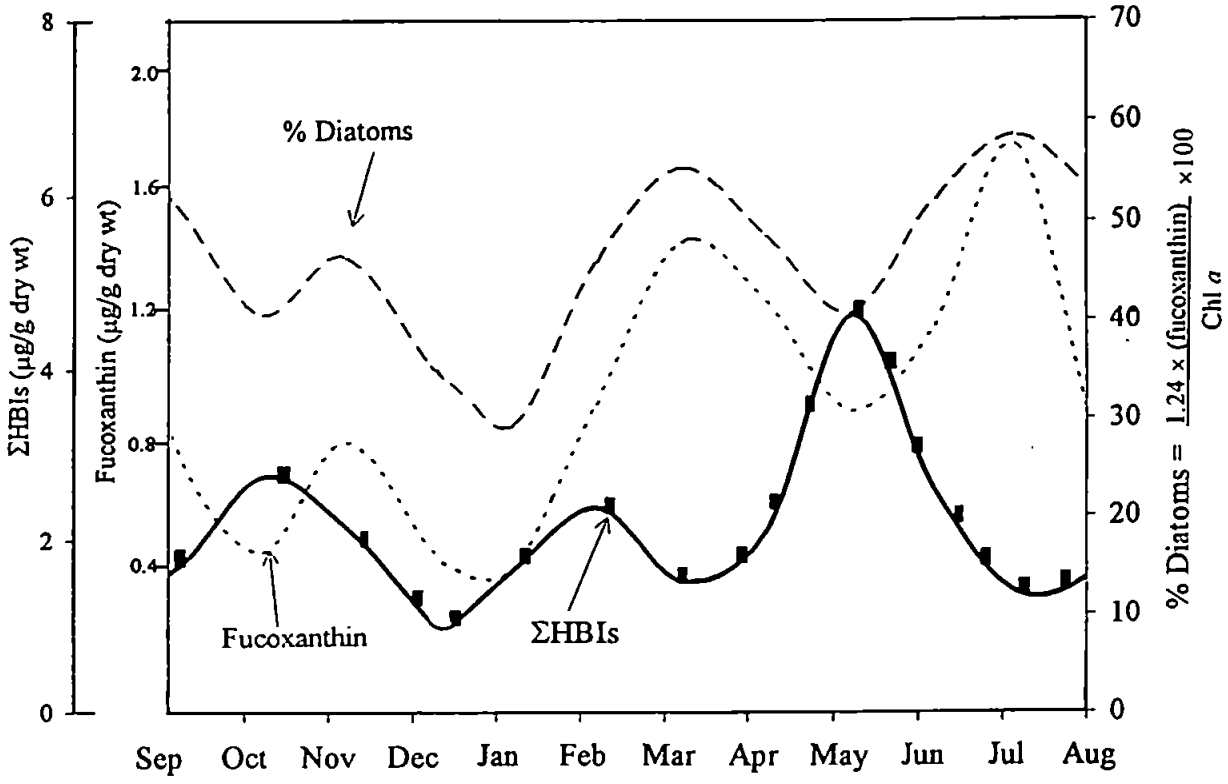
Key : 19'-hexano, 19'-hexanoyloxyfucoxanthin; 19'-butano, 19'-butanoyloxyfucoxanthin; Chl b, chlorophyll b; Chl a, chlorophyll a

Table 2.5 Concentrations of individual HBI isomers in Tamar sediments, for 1993 / 1994 (ng/g dry wt)

	Compound (GC RI $_{OV-1}$)							
	C25:2 (2069)	C25:2 (2079)	C25:3 (2044)	C25:3 (2091)	C25:3 (2107)	C25:4 (2128)	C25:4 (2175)	C25:5 (2128)
Date								
15.09.93	155	151	200	788	232	90	0	164
23.10.93	226	195	261	880	177	0	0	941
28.11.93	76	25	240	415	60	0	0	1134
19.12.93	111	63	149	527	73	195	82	160
13.01.94	0	76	56	449	53	192	148	0
17.02.94	26	159	63	946	253	0	353	0
16.03.94	565	211	172	383	288	330	384	89
11.04.94	93	80	361	779	29	103	57	0
07.05.94	64	371	89	1060	219	39	0	0
22.05.94	124	536	175	1226	182	161	0	0
05.06.94	285	357	194	1763	333	422	96	140
22.06.94	196	549	390	2219	460	506	81	54
02.07.94	264	102	487	1597	1245	96	0	209
19.07.94	193	64	236	1285	728	150	0	294
28.07.94	75	106	159	1252	309	16	37	238
08.08.94	188	229	451	677	108	0	0	43
23.08.94	136	219	290	85	365	78	92	117
31.08.94	172	135	448	105	291	153	0	130

The relationship between the diatom biomarker fucoxanthin, the relative abundance of diatoms (as indicated by the ratio of fucoxanthin to Chl *a*) and the HBI concentrations , are shown in Figure 2.6.

Figure 2.6 Variation of HBI concentrations, fucoxanthin, and % diatoms $= \left(\frac{1.24 \times (\text{fucoxanthin})}{\text{Chl } a} \times 100 \right)$ in Tamar sediments in 1993/1994. Data points indicate collection date for sediments, Table 2.3



There appears to be a strong negative correlation between ΣHBI concentrations and fucoxanthin. The HBI concentration is at a minimum during the spring (April) and summer (August) when fucoxanthin concentrations maximise, and HBI maxima during June and October coincide with fucoxanthin minima.

The hydrocarbon, *n*-heneicosahexaene (n-$C_{21:6}$), is abundant in many algae, including marine phytoplankton, benthic and epipelic algae (Lee and Loeblich, 1977). It is

produced by many diatoms, including those species which produce C_{25} and C_{30} HBIs (Nichols *et al.*, 1988; Volkman *et al.*, 1994). The variation in n-$C_{21:6}$, ΣHBIs and fucoxanthin concentrations is shown in Fig 2.7. The maximum HBI concentration (June) precedes the n-$C_{21:6}$ maxima by 4 weeks and n-$C_{21:6}$, in turn, precedes the fucoxanthin maxima by ~ 3 weeks. The other, less marked HBI maxima (March and October), show less clear and consistent ordering of the peaks.

These data are consistent with the findings of Hird and Rowland, (1995), who reported that in 1990 C_{20} and C_{25} HBI concentrations in the Tamar sediments maximised 1-2 months before n-$C_{21:6}$ maxima, (Figure 2.8).

These strong negative correlations between HBIs, n-$C_{21:6}$ (1990 and 1994) and fucoxanthin (1994) suggest that :

1. the major n-$C_{21:6}$ and fucoxanthin-producing diatoms do not produce HBIs,

2. those diatoms producing HBIs are at maximum growth earlier than the main diatom population or,

3. HBIs are produced earlier in the growth cycles of the diatoms than n-$C_{21:6}$ and fucoxanthin.

All these factors probably contribute. The suggestion that HBI-producing diatoms are maximising earlier than the main diatom population is supported by the fact that all the HBI maxima generally precede the n-$C_{21:6}$ and fucoxanthin maxima by similar periods. The growth phase dependence is supported by the observation that in laboratory growth phase experiments (Chapter 4) various biochemicals, including n-$C_{21:6}$ and carotenoids are indeed produced by the diatoms, such as *Haslea ostrearia*, at different growth stages.

Figure 2.7 Variation of HBI concentrations with % diatoms and *n*-$C_{21:6}$ in Tamar estuary sediments

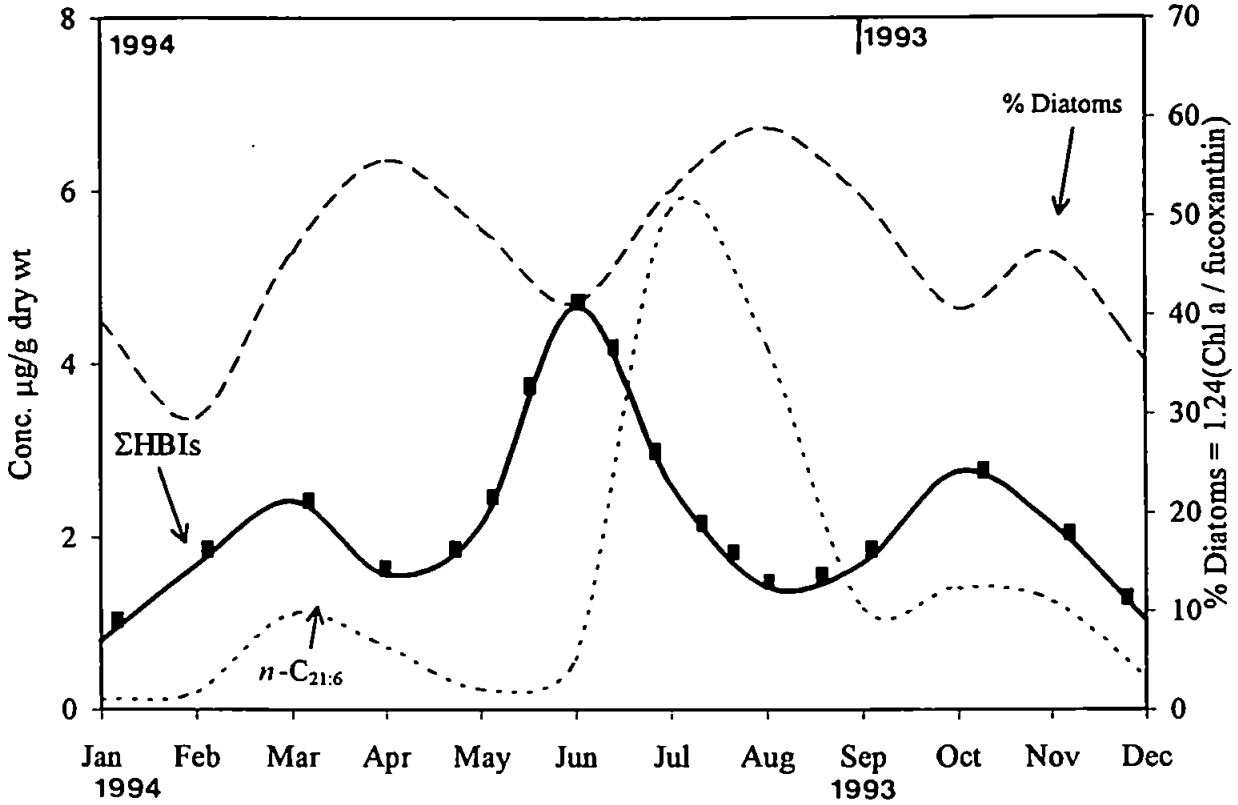
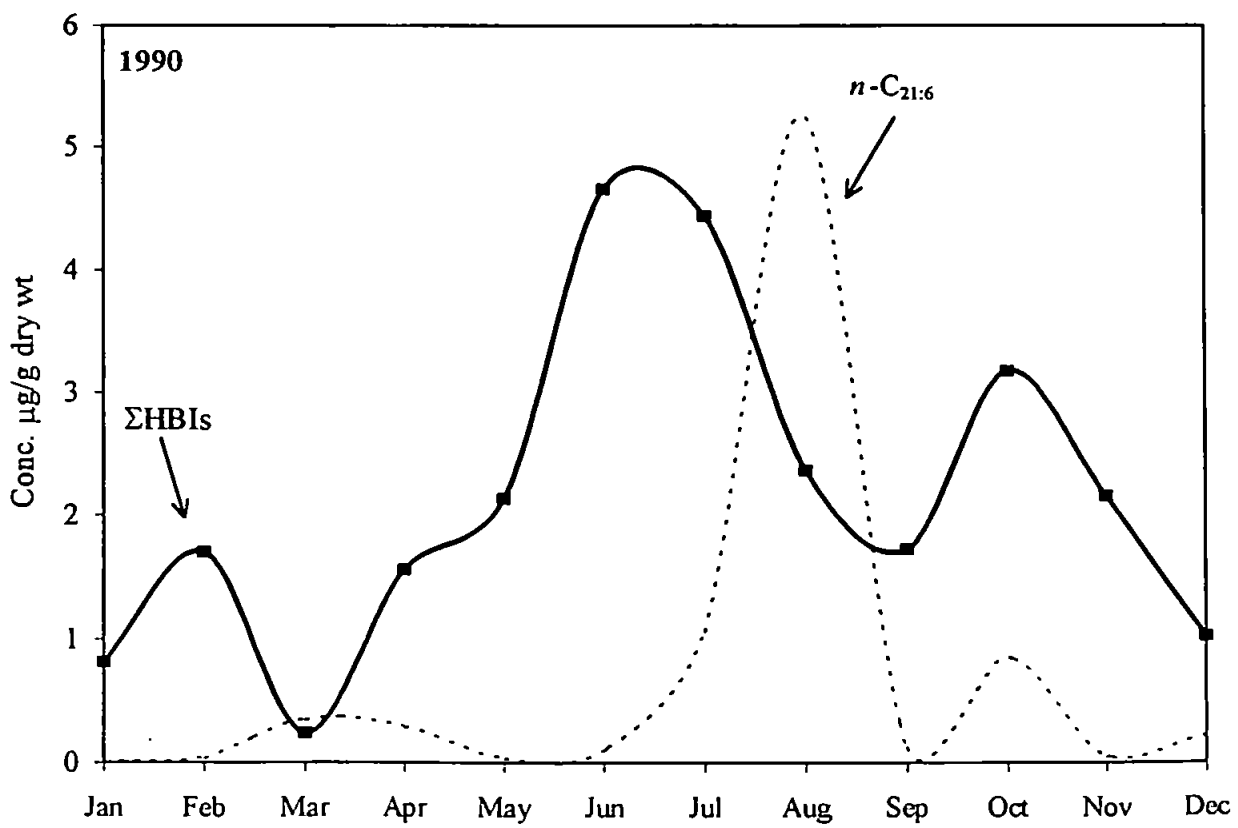


Figure 2.8 Variation of HBI and *n*-$C_{21:6}$ concentrations in Tamar sediments (Hird and Rowland, 1995).



Of course, it is also possible that other organisms are producing HBIs in the sediments, but there has been no report of these compounds in laboratory cultures of various algal classes (e.g. Nichols *et al.*, 1988; Hird, 1992) and furthermore, when the concentrations of ΣHBIs are plotted against the carotenoids indicative of other algal classes found in the Tamar estuary (Figures 2.9, 2.10 and 2.11), there appears to be little or no correlation with any of these classes, suggesting that for this location, diatoms are the main producer of HBI alkenes.

Figure 2.9 Variation of cryptomonads and green algae with HBIs (ref. Table 2.2 for chl *a* / carotenoid ratios)

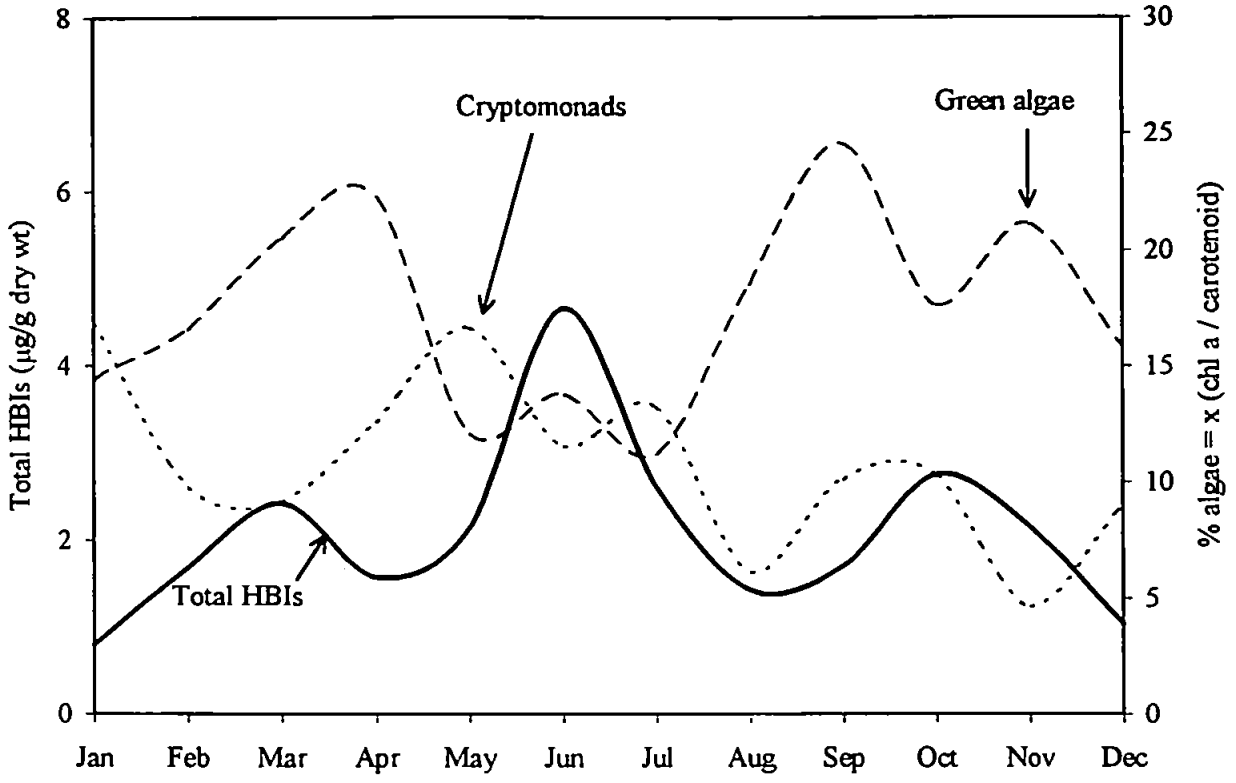


Figure 2.10 Variation of prymnesiophytes and cyanobacteria with HBIs (ref. Table 2.2 for chl *a* / carotenoid ratios)

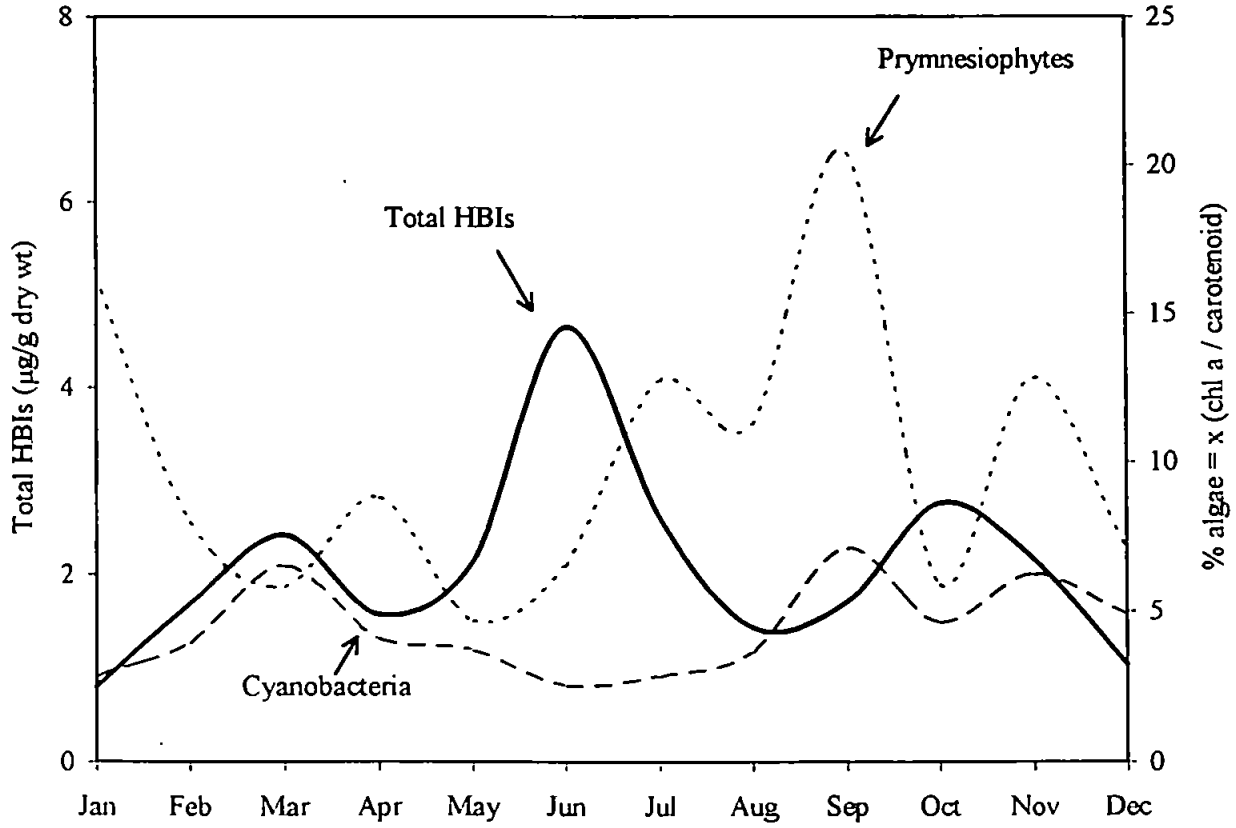
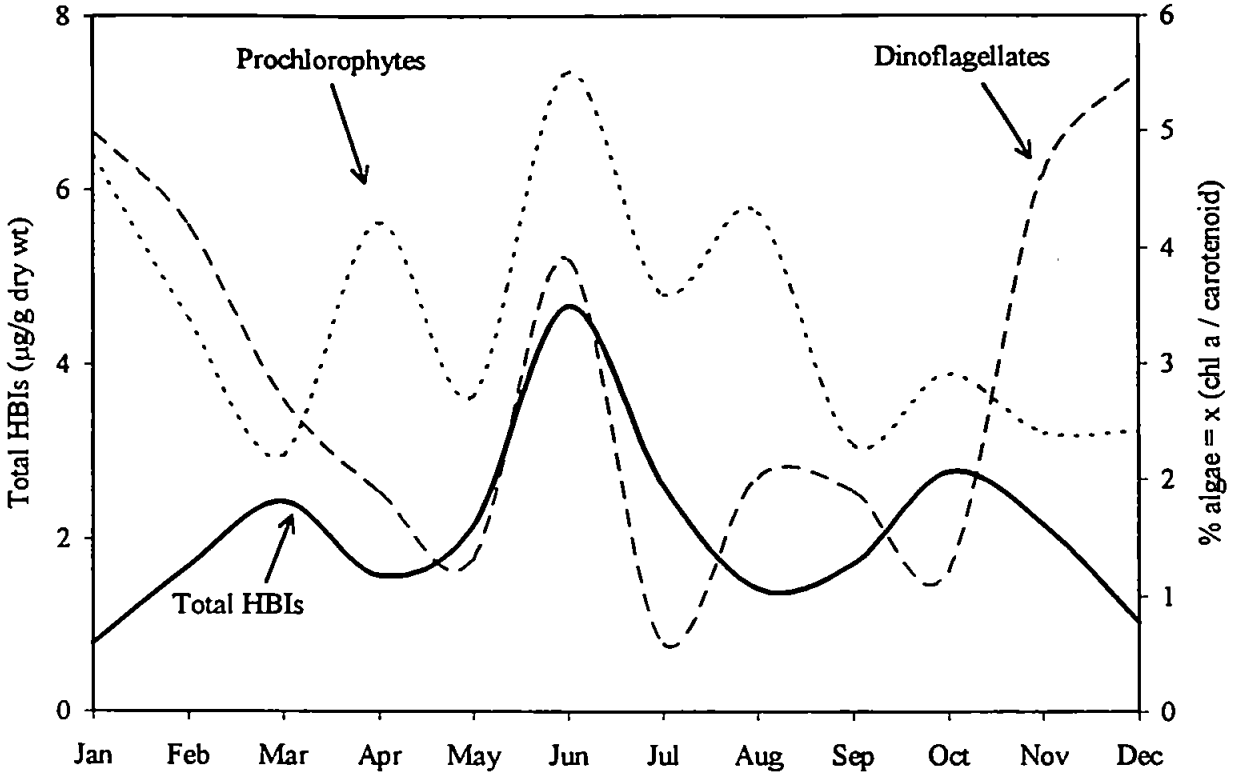


Figure 2.11 Variation of dinoflagellates and prochlorophytes with HBIs (ref. Table 2.2 for chl *a* / carotenoid ratios)



2.3 Summary

- When sufficient care is taken over sampling and preservation conditions, the relative concentrations of carotenoid pigments in surficial sediments can be quantitatively determined and the ratios used to establish algal sources. There is no evidence of preferential extraction of carotenoids of any one algal class under the conditions employed herein.

- The concentrations of C_{25} HBI alkenes, in Tamar sediments in 1994, exhibited strong negative correlations with those of *n*-$C_{21:6}$ and fucoxanthin. This is consistent with the observations of other workers for *n*-$C_{21:6}$ and HBIs in 1990.

- The strong inverse variance suggests to the present author that HBI concentrations are nonetheless related to diatom populations in the Tamar estuary, since the maxima in concentrations of the various diatom markers are offset by consistent intervals of 2 - 4 (*n*-$C_{21:6}$) and 6 - 7 (fucoxanthin) weeks.

- The results suggest that either the HBI producing diatoms are maximising earlier than the main diatom population, and/or that HBIs are produced earlier in the growth cycle of diatoms than *n*-$C_{21:6}$ and fucoxanthin.

- At this location the concentration of carotenoids indicative of algae other than diatoms did not strongly covary (or inversely vary) with the concentration of HBIs.

CHAPTER THREE

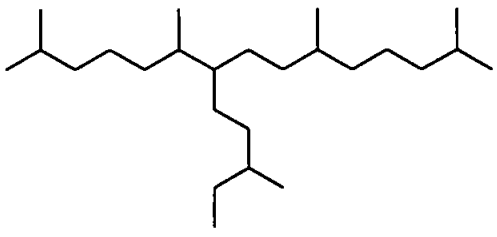
Characterisation of highly branched isoprenoid polyenes

This chapter details the first unambiguous structural assignments of a suite of HBI polyenes, dienes to hexaenes. Haslea ostrearia and Caspian Sea sediments provided sufficient quantities of isolate for six different HBI alkenes to be structurally assigned via nuclear magnetic resonance spectroscopy and derivatisation techniques (epoxidation and ozonolysis). Epoxidation proved a valuable tool in the structural elucidation of HBI compounds, particularly for micro-scale reactions.

3.1 Introduction

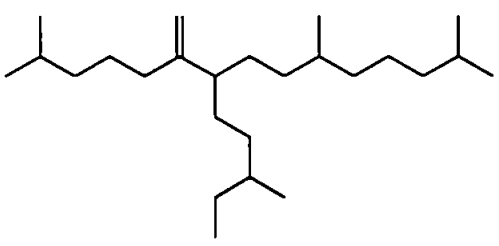
The C_{25} highly branched isoprenoid (HBI) hydrocarbons discussed in this thesis with carbon skeleton **II**, commonly occur as polyenes, with two to five double bonds (Table 1.1).

II

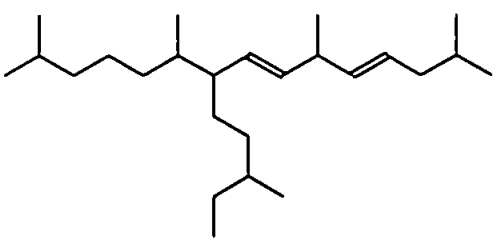


However, to date their structural elucidation has been confined to one monoene, identified by ozonolysis (**V**, Dunlop and Jefferies, 1985; Hird, 1992) and one diene (**VII**), by Yruela *et al.*, 1990, who used epoxide formation with *m*-chloroperoxybenzoic acid to tentatively assign the double bond positions.

V



VII



The epoxide derivatisation process for **VII** succeeded where alkylthiolation, methoxymercuration and treatment with osmium tetroxide have failed in other studies (reviewed by Robson, 1987; Rowland and Robson, 1990; Nichols *et al.*, 1988).

For the majority of HBI alkenes, rigorous spectroscopic characterisation has not been possible since these hydrocarbons generally occur at low concentrations in complex mixtures in sediments. The low natural abundances have precluded isolation in sufficient quantities for detailed analysis by ^{1}H and ^{13}C NMR spectroscopy. Taking a typical example, the maximum concentration of $C_{25:2}$ 2069, recorded in Tamar sediments for 1993/1994, was 285 ng g^{-1} dry weight. A yield of ~5.0 mg of isolate (amount required for complete NMR analysis) would require extraction of a minimum of 20 kg of dry surface sediment. Therefore the utility of epoxidation and ozonolysis as methods for assignment of double bond positions, in alkenes isolated from sediments in low quantities (~ 100 μg), becomes apparent. Analysis *via* GC and GC-MS allows these derivatisation experiments to be conducted and analysed on microgram quantities of isolate, particularly as the reactions do not need to be quantitative.

In the present chapter, the double bond positions in six C_{25} HBI alkenes are reported. Five HBI compounds were isolated from large scale cultures of the diatom *Haslea ostrearia* and one sedimentary alkene was isolated from Caspian Sea sediments. The unusually high concentrations of a C_{25} diene (GC RI 2079) (≤ 60 μg g^{-1} dry wt) found in the Caspian Sea sediments provided sufficient quantities of isolate (~5 mg) for a full characterisation of the structure *via* NMR spectroscopy.

Since *Haslea ostrearia* biosynthesises a suite of C_{25} HBI alkenes it provided a unique source of these compounds for characterisation. With concentrations as high as 0.46 mg g^{-1} dry wt ($C_{25:4}$ 2144) there was a substantial reduction in the quantity of material required for extraction. Bulk cultures (40 g and 90 g wet wt.) were kindly provided by Prof. J-M Robert, at the research institute ISOMer, University of Nantes.

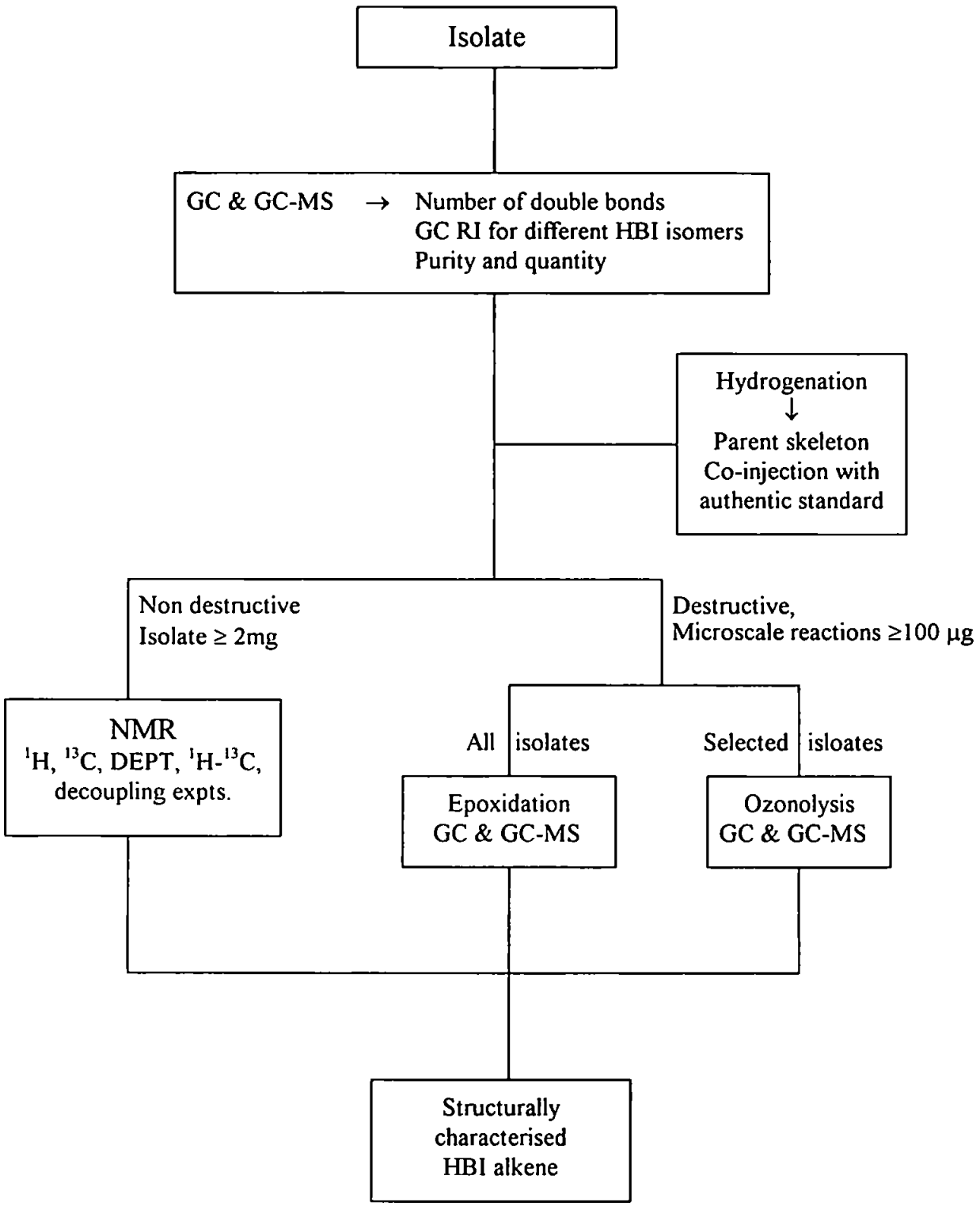
The two cultures contained different suites of HBI alkenes; one cultured during September 1988, contained $C_{25:5}$ (GC RI 2201) and $C_{25:6}$ (GC RI 2248) and the other a growth phase experiment (see chapter 4 for further details) contained $C_{25:3}$ (GC RI 2106), $C_{25:4}$ (GC RI 2144) and $C_{25:5}$ (GC RI 2191). These 'batch' cultures of *Haslea ostrearia* contained sufficient quantities of five different HBI alkenes for isolation and characterisation by NMR spectroscopy and epoxide derivatisation.

3.1.1 General procedure for the characterisation of HBI isolates

The general protocol used for the full characterisation of isolated C_{25} HBI alkenes is illustrated in Figure 3.1. Preliminary analysis (GC & GC-MS) of the aliphatic fraction (or total hexane extract, THE, for algae) indicated the number of HBI alkenes, the number of double bonds in each alkene and an indication of the specific alkene isomers present from the retention index (GC RI). These HBIs were then isolated (Section 5.4), and purity determined and identity checked by GC & GC-MS.

Confirmation of the highly branched isoprenoid parent structure was achieved by hydrogenation (PtO_2 . H_2O) to the alkane (**II**), followed by GC-MS analysis and co-injection with synthetic **II** (Robson, 1987). Mass spectrometry usually revealed the molecular ion of the product of hydrogenated alkenes as either *m/z* 350 (M^+-2), or M^+ 352. The *m/z* 350 ion has been reported by numerous authors (reviewed by Rowland and Robson, 1990; Hird, 1992; Summons *et al.*, 1993) and may be an ion source-dependent artefact, as suggested by Robson and Rowland (1988).

Figure 3.1 Structural determination of HBI alkene isolates



3.1.2 Characterisation methods

3.1.2.1 Microscale ozonolysis

Historically, ozonolysis has been found to be a particularly useful technique for the location of double bond positions of organic compounds (*e.g.* Davison and Dutton, 1966; Nickell and Privett, 1966). Its development as a microchemical technique owes much to the work of Beroza and Bierl (1966, 1967). The ozone generator and equipment used during the present study was similar to that described by Beroza and Bierl (1969) and the apparatus is illustrated in Figure 3.2.

Although a large amount of work has been accomplished concerning the mechanism of ozonization (formation of ozonides: see review by Criegee, 1975; March, 1985) not all the details are known. However, it has been established that when compounds containing double bonds are treated with ozone, they are converted to compounds called ozonides.

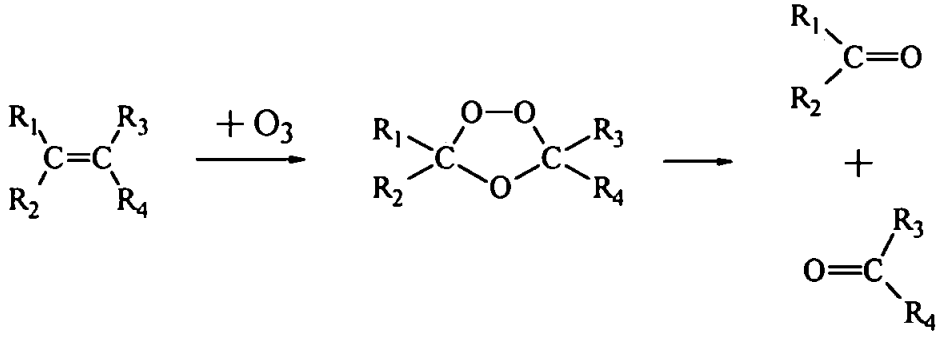
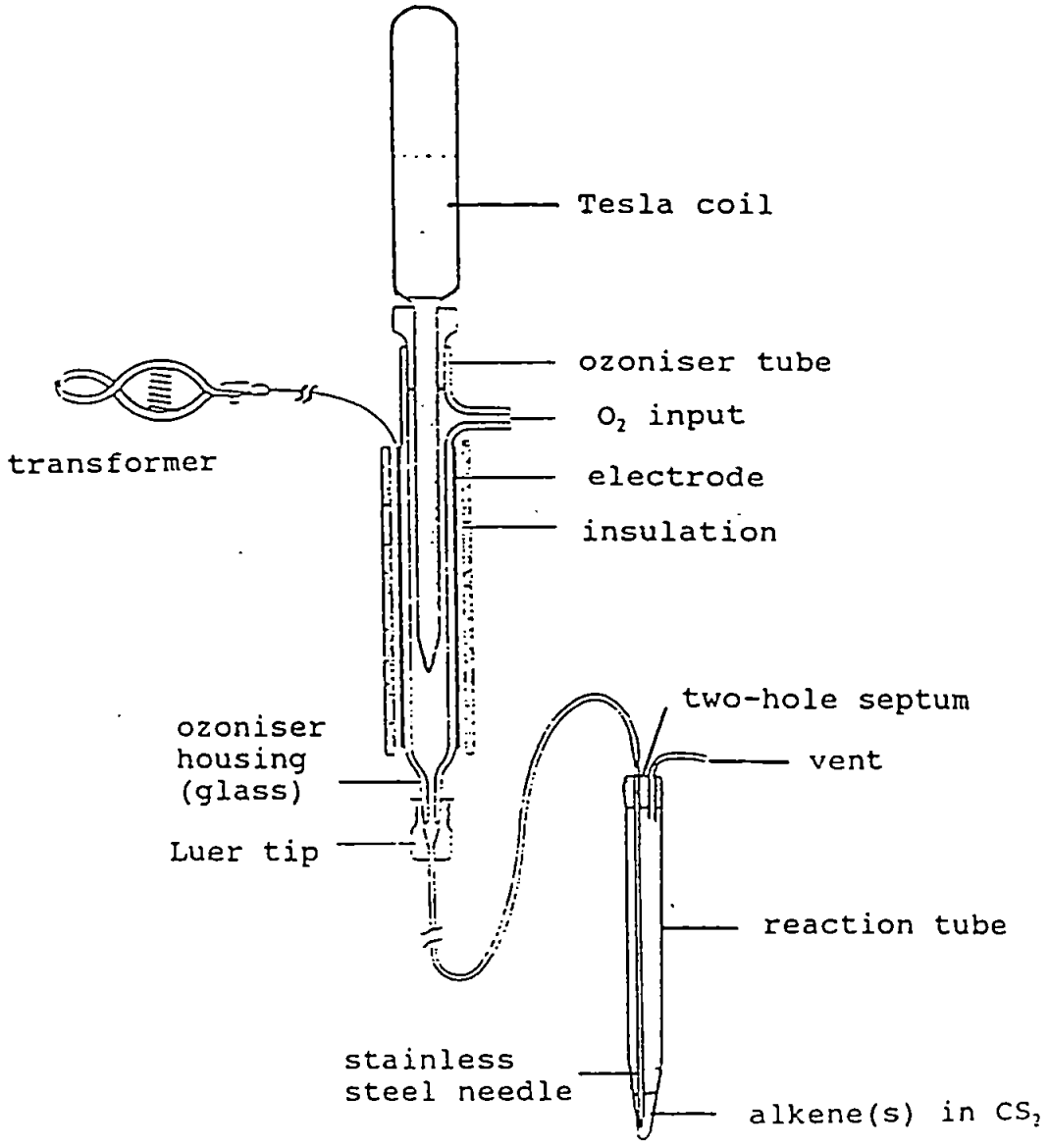


Figure 3.2 Apparatus for micro-scale ozonolysis



These compounds are unstable and decompose to give two moles of aldehyde, two moles of ketone or one mole of each, depending on the groups attached to the alkene.

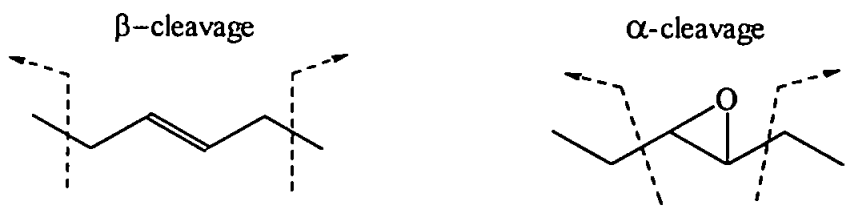
The method used in this study was that of Hird *et al.*, (1992) and the technique proved successful in the structural elucidation of a diene (GC RI 2079). The main drawback to this technique was the very low yield of products, (for example, $C_{25:2}$ GC RI 2079 yielded oxo aldehyde products of only 4.8%). The methodology applied in this study did not involve an oxygen acceptor (*e.g.* triphenylphosphine, Ph_3P). It is the authors opinion that exclusion of Ph_3P from the reaction was responsible for the low yield of products and this is substantiated in a study by Summons *et al.*, (1993) who, with the addition of Ph_3P, gained a 62.5% yield of a oxo aldehyde after ozonolysis of a structurally similar C_{25} diene (**VIII**). Another drawback to this technique is the increasing number of fragments that would be produced with more unsaturated compounds. The volatility of some of these fragments would make analysis more difficult (see Section 3.3.1).

3.1.2.2 Microscale conversion of alkenes to epoxides

Perhaps the most common method for the epoxidation of alkenes is their reaction with peroxy acids (RCO_3H), which is often referred to as the Prilezhaev reaction (Prilezhaev, 1909; Swern, 1949). This reaction does not require transition metal catalysis, and the yield of epoxide is often high. Several peroxy acids are commercially available, including peroxyformic, peroxybenzoic, trifluoroperoxyacetic, and *meta*-chloroperoxybenzoic (*m*-CPBA) acids. *m*-Chloroperoxybenzoic acid was the reagent

used in the following experiments, as opposed to other peroxy acids which are highly reactive and decompose readily, whereas *m*-CPBA is stable, crystalline, and an easily handled material. Epoxide formation with *m*-CPBA has also been applied successfully to natural products (*e.g.* Bierl *et al.*, 1980), but more significantly to a C_{25} HBI diene (Yruela *et al.*, 1990).

The advantage of epoxide formation, when locating double bond positions, is that the reactions can be carried out on microgram quantitities of isolate and the products analysed directly *via* GC and GC-MS. As opposed to allylic cleavage of the alkenes, the epoxides have preferential cleavage *alpha* to the epoxy group. This produces identifiable fragments that indicate the position of the epoxy group and thus the position of the double bonds in the original compound, as shown below (Bierl *et al.*, 1980).

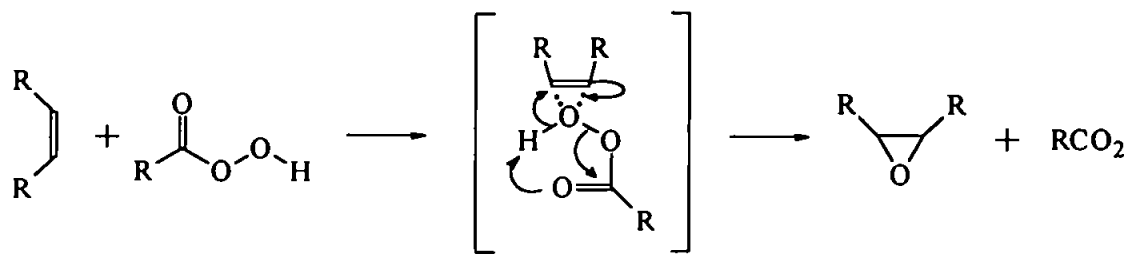


3.1.2.2.1 Mechanism of epoxide formation

Although the processes of epoxide formation are not fully understood, the reaction probably proceeds *via* a concerted mechanism (Bartlett, 1950; Carey, 1992) in which the alkene and the electrophilic oxygen co-ordinate, with concomitant explusion of the carboxylic acid and release of the epoxide (Figure 3.3). The geometry of the transition

state facilitates loss of the RCO_2H by-product and also dictates a *syn* delivery of oxygen, without changing the geometry of the alkene.

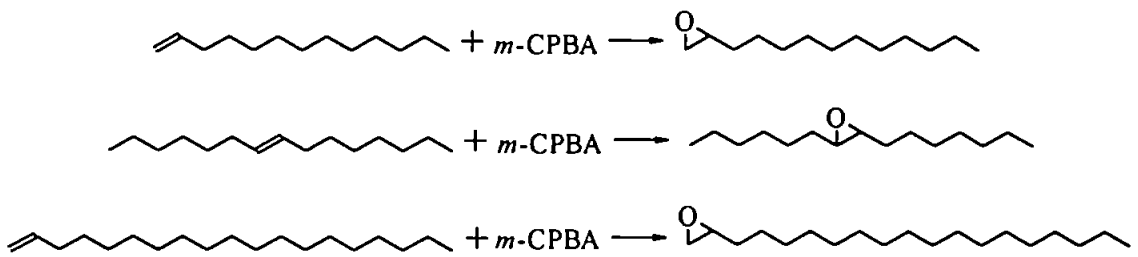
Figure 3.3 Probable reaction mechanism for epoxide formation (Carey, 1992)



More highly substituted alkenes are electron rich and tend to react faster with the peroxy acid than do less substituted alkenes (Swern, 1947). According to Lewis, (1969) the relative rate of epoxidation increases with the nucleophilic character of the alkene: $CH_2=CH_2$ has a relative rate of 1, $RCH=CH_2$ 24, RCH=CHR 500, $R_2C=CH_2$ 500, $R_2C=CHR$ 6500, and $R_2C=CR_2 \geq 6500$. If a molecule contains two alkene moieties, the more highly substituted alkene is epoxidised faster. *m*-Chloroperoxybenzoic acid is a widely applied peroxyacid and has been shown to be as effective as much stronger peroxy acids, such as trifluoroperoxyacetic acid (Lewis, 1969). Even highly hindered alkenes can epoxidised with only a modicom of difficulty (Abruscato *et al.*, 1972; Swindell *et al.*, 1986).

3.1.2.2.2 Method development for epoxidation reactions

Having established that epoxide derivatisation of alkenes was a potential method for the location of double bond positions, the technique was first validated on a set of standard compounds. The *n*-alkenes (dodec-1-ene, tetradec-7-ene & octadec-1-ene) were primarily chosen as standards because their epoxide counterparts (1,2 epoxydodecane, 7,8 epoxytetradecane & 1,2 epoxyoctadecane) were commercially available.



This facilitated early method development as the reaction could be monitored (GC and GC-MS) to a known point of completion. The effects of peroxy acid concentration and time for reaction were optimised to give the highest yields of epoxide for the n-alkenes (≥95%). The technique was then applied to a synthesised C_{30} HBI monoene (Robson, 1987) of known structure and again the reaction conditions optimised. Details of this are given in Appendix I.

From the preliminary experiments it was found that changing the concentration of peroxy acid had little or no effect on the rate of reaction (*m*-CPBA was varied from equimolar to an excess of ×60). More significantly, the complete formation of an epoxide,

was found to be strongly dependent on the length of time of the reaction. Although epoxide formation was observed after a few hours, it was found that the reactions could continue for as long as 96 hours before complete conversion of alkene to epoxide. Having successfully established the reaction conditions for standards the method was then applied to HBI isolates of unknown structure.

3.1.2.3 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is perhaps the most valuable spectroscopic technique available in organic chemistry. The information derived from ^{1}H and ^{13}C NMR spectroscopy is extraordinarily useful for structure determination, providing a 'map' of the carbon-hydrogen framework.

The interpreted data collected from NMR analysis is presented in tables and only salient spectroscopic features are illustrated for selected HBI alkenes in this chapter. All NMR spectra recorded are presented in Appendix II.

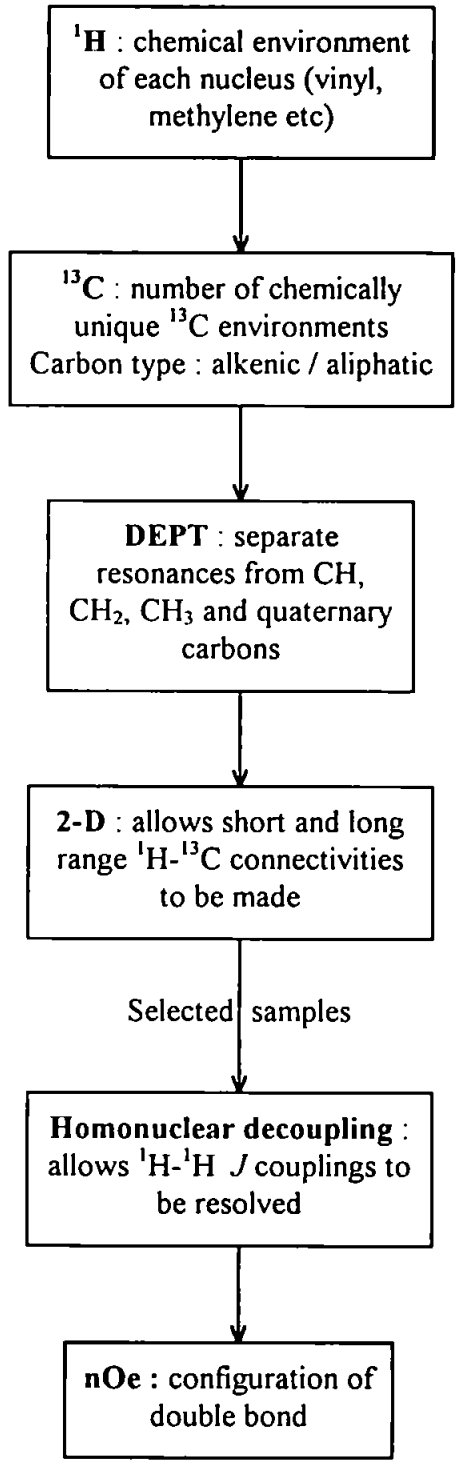
3.1.2.3.1 NMR experiments

For an unambiguous structural assignment a combination of experimental techniques was applied to each isolate and the general protocol is illustrated in Figure 3.4.

^{1}H and ^{13}C NMR spectra provides the following information:

- Number of NMR resonances. Each nonequivalent (^{1}H, ^{13}C) nucleus gives rise to a separate resonance, indicating the number of different environments in the molecule.

Figure 3.4 General protocol for structural assignment *via* NMR spectroscopy



- Chemical shifts. The exact position of an NMR resonance gives information relating to the chemical environment of a nucleus (^{1}H, ^{13}C).

- Integration of NMR resonances (^{1}H). Electronic integrations of the area under a peak yields the number of ^{1}H nuclei giving rise to a resonance.

- Spin-spin splitting (^{1}H). The splitting of resonances into multiplets by coupling of neighbouring nuclear spins provides information about the number and geometry of J coupled nuclei.

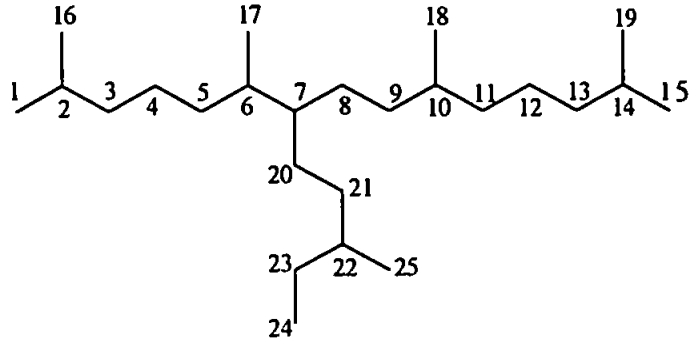
- A DEPT experiment produces separate carbon sub-spectra for methyl, methylene and methine carbons. Quaternary carbons are absent from the spectra.

- Heteronuclear correlations: Allows individual ^{1}H-^{13}C connectivities to be made. These experiments

3.2 Characterised C_{25} HBI alkenes

All structural assignments relate to the numbering system as applied to the parent alkane shown below, Figure 3.5.

Figure 3.5 Numbering scheme used for C_{25} HBI alkenes

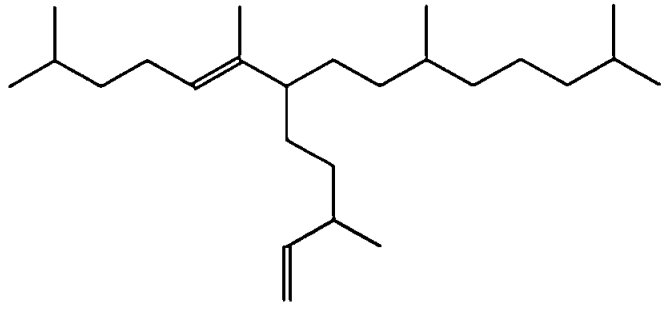


3.2.1 $C_{25:2}$ (GC RI 2079) **IX**, Belt *et al.*, (1994)

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

Sufficient isolate (~ 5 mg) of **IX** was obtained from a bulk sediment extraction (Caspian Sea; Belt *et al.*, 1994) for analysis *via* ^{1}H and ^{13}C NMR spectroscopy, epoxide derivatisation and ozonolysis. The double bond positions within the structure have been unambiguously assigned *via* NMR spectroscopy and additionally confirmed by derivatisation.

IX



The C_{25} HBI (IX) exhibited a molecular ion M^+ 348, indicating two degrees of unsaturation and the mass spectrum (Figure 3.7), shows characteristic β cleavage allylic to the double bonds with diagnostic fragment ions *m/z* 207, *m/z* 235, *m/z* 266 and *m/z* 291. Mass spectrometry of the hydrogenated ($Pt_2O.H_2O$) alkene displayed a molecular ion M^+ 352, with the diagnostic ions *m/z* 210, *m/z* 238 and *m/z* 266. The alkane co-eluted (GC) with a synthesised C_{25} alkane 2,6,10,14-tetramethyl-7-(3-methylpentyl) pentadecane (Robson, 1987) on three different stationary phases (DB1, DB5 & DBWAX).

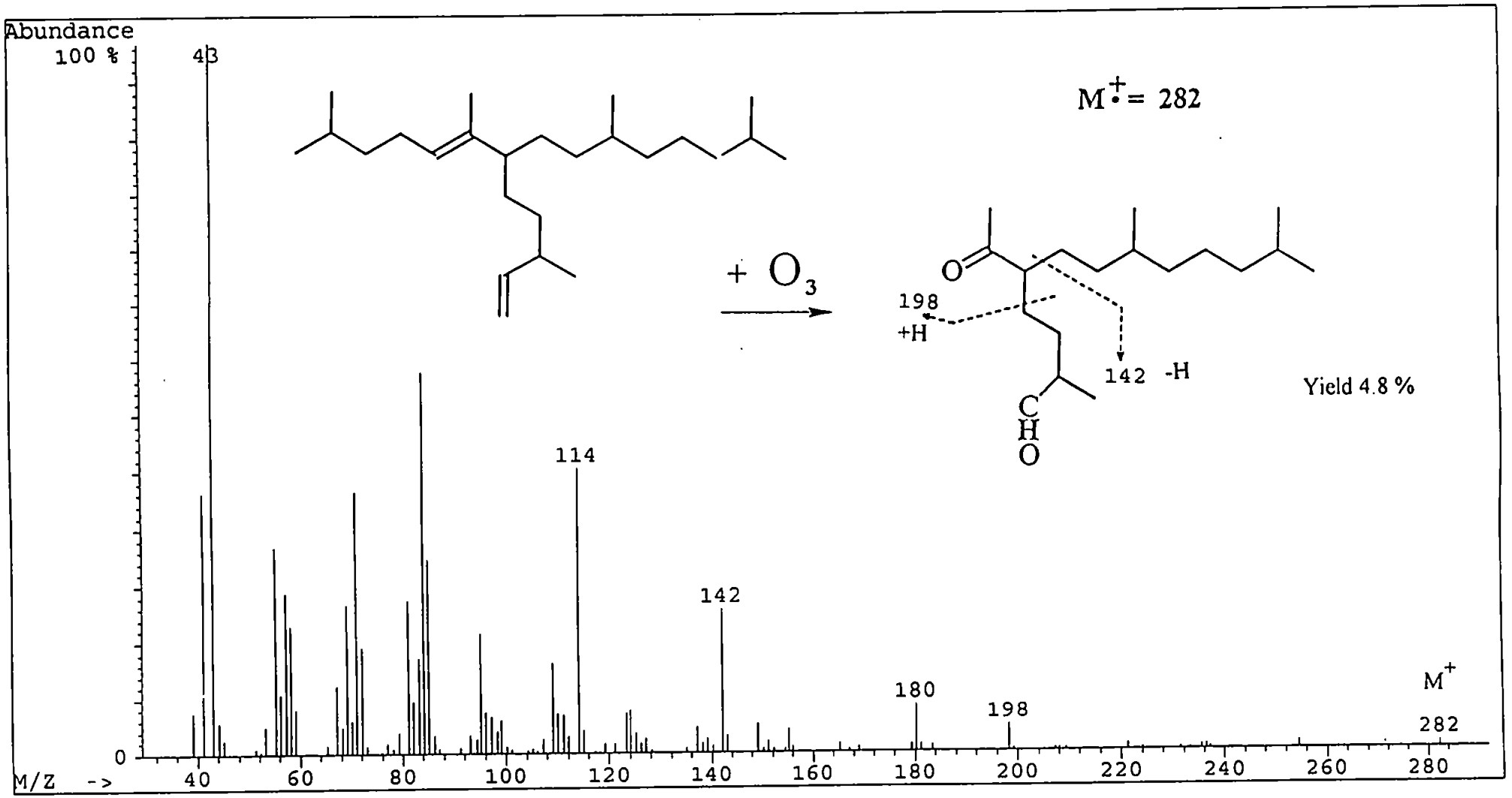
3.2.1.1 Ozonolysis of $C_{25:2}$ (GC RI 2079)

Ozonolysis of the C_{25} diene yielded a C_{18} oxo aldehyde, which was identified by GC-MS (Figure 3.6). The mass spectrum exhibits a M^+ 282, with diagnostic ions at *m/z* 198 (with *m/z* 180 due to loss of H_2O) and *m/z* 142, (with *m/z* 114 due to loss of CO). The two other expected products (C_6 ketone and formaldehyde) were not recorded by GC-MS.

3.2.1.2 Epoxidation of $C_{25:2}$ (GC RI 2079)

The reaction conditions that were applied to the standard compounds did not produce a *bis*-epoxide product, either by varying the concentration of *m*-CPBA (up to a sixty fold excess) or over a prolonged period of time (168 hours). The reaction would not progress past the formation of a *mono*-epoxy monoene (identified by GC-MS) and it was the author's belief that one of the double bonds was probably located in a hindered position to the *m*-CPBA. The reaction was then conducted at different temperatures (from 25°C - 80°C, increasing at 5°C increments) to monitor the effects of temperature

Figure 3.6 Mass spectrum of ozonolysis products of $C_{25:2}$ GC RI 2079



on the reaction. The diene was successfully converted to a C_{25} *bis*-epoxide, but only at reaction temperatures ≥ 55°C. Above 55°C, *bis*-epoxide formation was also accompanied by further reaction of the epoxide to yield unidentifiable compounds and the formation of an unresolved complex mixture.

The reaction of the diene with *m*-CPBA commenced with the formation of a *mono*-epoxide after 6.5 hours (identified from ion *m/z* 43, after 6.5 hours) and formation of a *bis*-epoxide after 90 hours. The epoxides have mass spectral fragmentation pathways characterised by cleavage *alpha* to the epoxide ring and the mass spectrum of the *bis*-epoxide (Figure 3.7) displays a M^+ 380, with particularly diagnostic ions *m/z* 43, *m/z* 239, *m/z* 253, *m/z* 281 and *m/z* 309. The ion *m/z* 43 can only be produced if an epoxide is located in the C23-24 position and the second epoxide is confined to the C5-6 position by the diagnostic ions *m/z* 309 and *m/z* 253. It is clear from Figure 3.7 that the epoxides can be located readily *via* GC-MS and from this the original double bond positions located.

3.2.1.3 NMR of $C_{25:2}$ (GC RI 2079)

The NMR data for the diene is presented in Table 3.1. The ^1H spectrum consists of resonances associated with alkenic, allylic and alkyl protons. The most conspicuous of these are three sets of resonances at δ 5.65, 4.93 and 4.89, which arise from a vinyl (-C_2H_3) functionality (Figure 3.8). Given the established C_{25} isoprenoid skeleton, it is clear that this double bond must be located at C23-24. Proton H-23 appears as a low field seven line multiplet due to a *trans* coupling to H-24a (17 Hz), a *cis* coupling to H-24b (10 Hz) and an additional vicinal coupling to H-22 (8 Hz). Further confirmation of this double bond position was achieved by a decoupling experiment. Irradiation of H-22 (δ

Figure 3.7 Mass spectrum of $C_{25:2}$ GC RI 2079 (top) and *bis*-epoxide product (bottom)

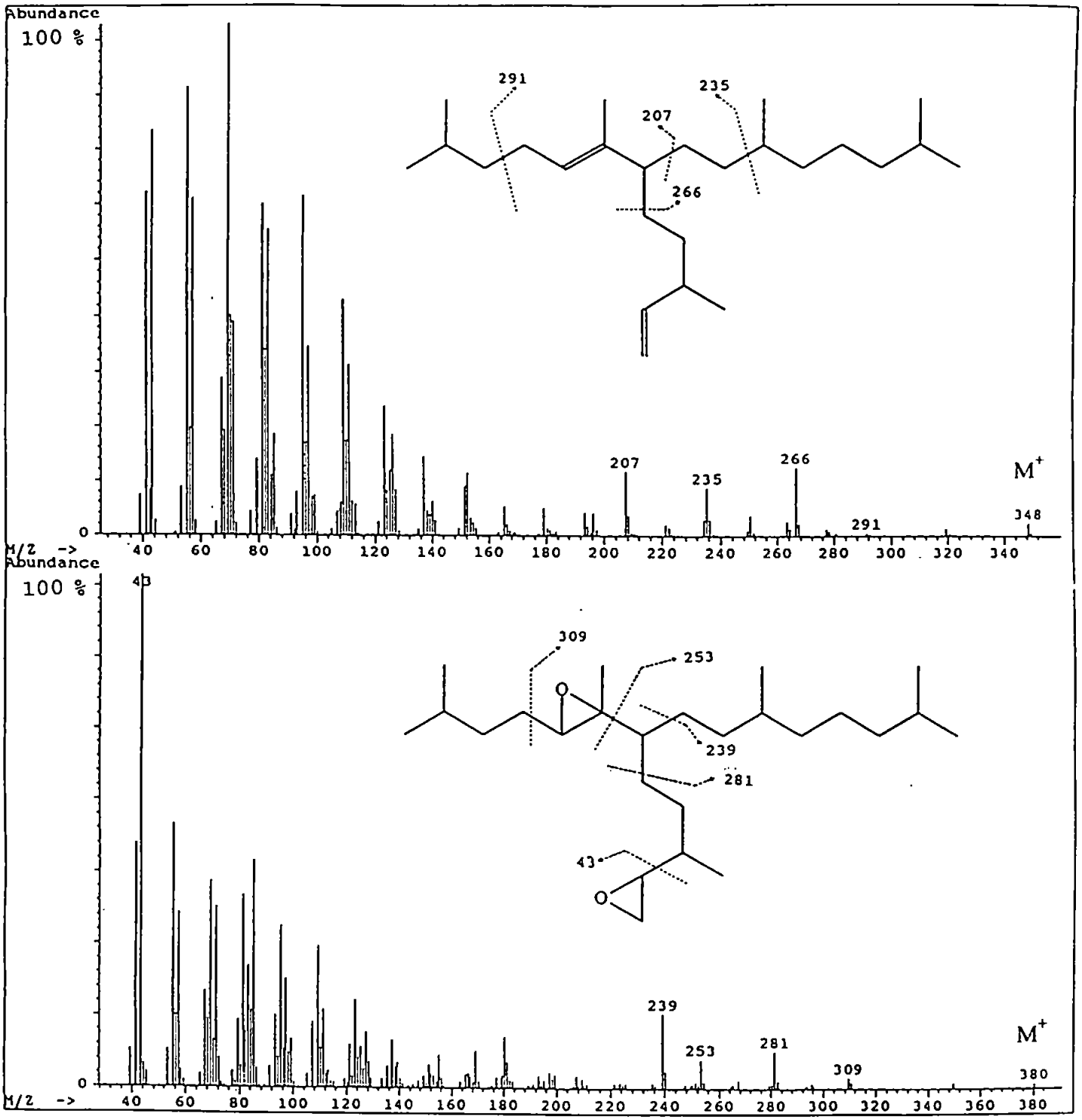
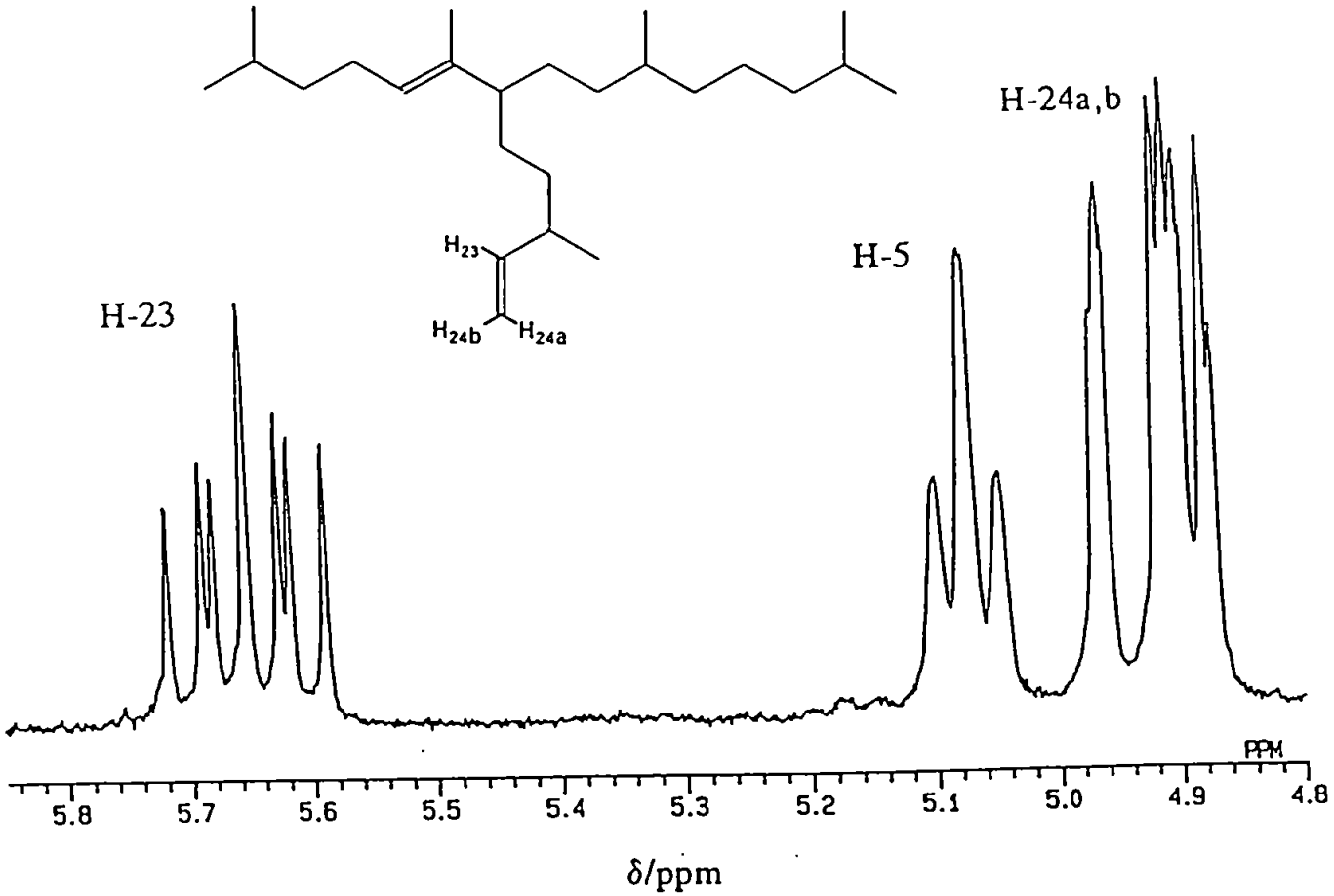
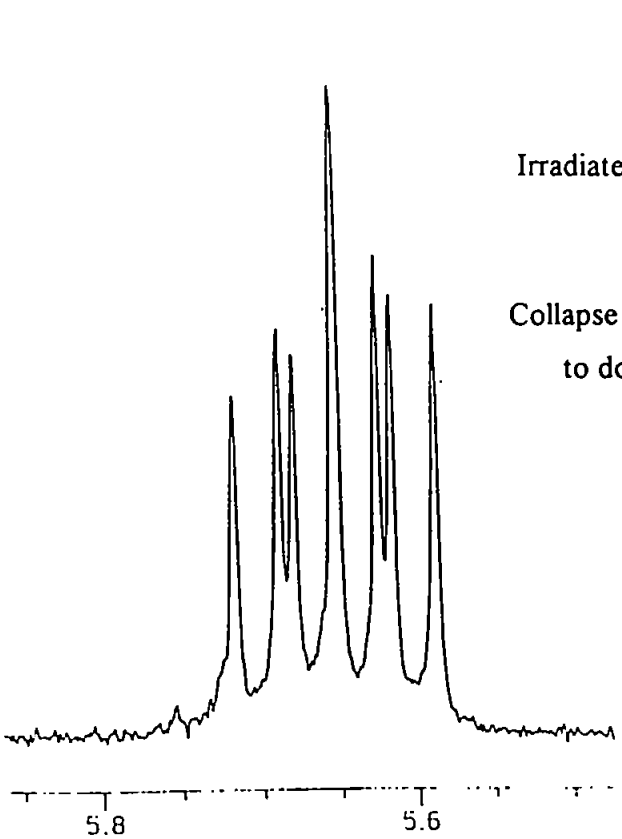


Figure 3.8 Alkenic region of ^1H spectrum for $C_{25:2}$ GC RI 2079 (top) and the collapse of the seven line multiplet (H-23) to a doublet of doublets upon irradiation of H-22 (bottom)





Irradiate at δ 2.08 (H-22)

Collapse of seven line multiplet to doublet of doublets

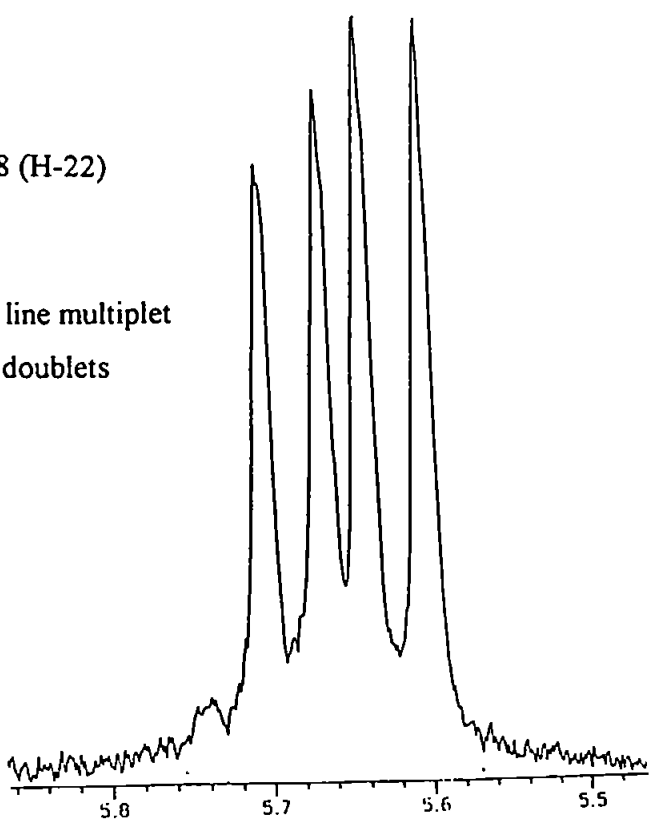
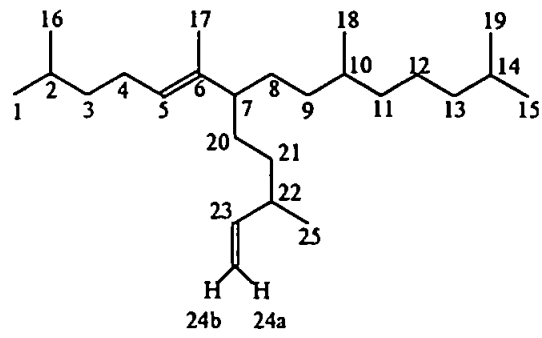


Table 3.1 NMR spectroscopic data for $C_{25:2}$ GC RI 2079, (Caspian Sea)

$C_{25:2}$ GC RI 2079 (DB1) Caspian Sea	
Purity : >98% (by GC) Quantity : 5.0 mg Solvent : $CDCl_3$	

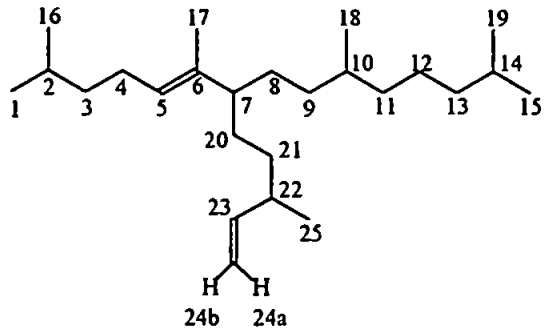
Proton (^1H) chemical shifts (multiplicities, integration)

	1, 16	**4**	**5**
δ(ppm)	0.88 (d, 6H)	1.99 (q, 2H)	5.08 (t, 1H)
	7	**15, 19**	**17**
δ(ppm)	1.80 (m, 1H)	0.85 (d, 6H)	1.42 (s, 3H)
	18	**22**	**23**
δ(ppm)	0.82 (d, 3H)	2.08 (m, 1H)	5.65 (ddd, 1H)
	24a	**24b**	**25**
δ(ppm)	4.93 (dd, 1H)	4.89 (dd, 1H)	0.95 (s, 3H)

Proton coupling constants

	$J_{H(1, 16) - H2}$	$J_{H4 - H5}$	$J_{H5 - H9}$	$J_{H(15, 19) - H14}$	$J_{H18 - H10}$
J(Hz)	6	7	7	6	6.5
	$J_{H23trans}$	J_{H23cis}	J_{H23vic}	$J_{H24a - H24b}$	$J_{H25 - H22}$
J(Hz)	17	10	8	2	6.5

Table 3.1 cont.



Carbon (^{13}C) chemical shifts

	1	2	4	5	6
δ(ppm)	22.6	27.9	25.5	126.3	135.3
	7	**15**	**16**	**17**	**18**
δ(ppm)	49.3	22.6	22.6	11.3	19.9
	19	**22**	**23**	**24**	**25**
δ(ppm)	22.6	37.8	145.0	112.3	20.5

Not assigned : 3, 8, 9, 10, 11, 12, 13, 14, 20 & 21

^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively). Numbering shown in displayed formulae.

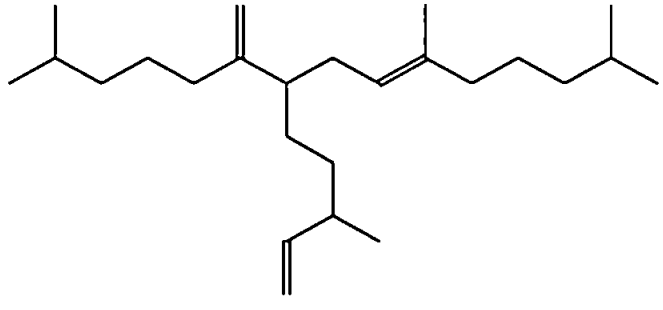
2.08) resulted in the collapse of the seven line multiplet associated with H-23 to a doublet of doublets due to the H-24a (*trans*) and H-24b (*cis*) couplings (Figure 3.8).

3.2.2 $C_{25:3}$ (GC RI 2106) **X**

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

6 mg of **X** was obtained from a bulk extraction of *Haslea ostrearia* for ^{1}H and ^{13}C NMR spectroscopy and epoxide derivatisation. The double bond positions within the structure have been unambiguously assigned *via* NMR spectroscopy and additionally confirmed by epoxide derivatisation.

X



The C_{25} triene (**X**) exhibits a molecular ion M^+ 346, indicating three degrees of unsaturation. The mass spectrum (Figure 3.9) shows characteristic β cleavage allylic to the double bonds with diagnostic fragment ions *m/z* 233, *m/z* 261, *m/z* 275, *m/z* 289 and *m/z* 331 (due to loss of CH_3). Mass spectrometry of the hydrogenated ($Pt_2O.H_2O$) alkene displayed a molecular ion M^+ 350 (M-2), with the diagnostic ions *m/z* 210, *m/z* 238 and

m/z 266. The alkane co-eluted (GC) with a synthesised C_{25} alkane (Robson, 1987) on three different stationary phases (DB1, DB5 & DBWAX).

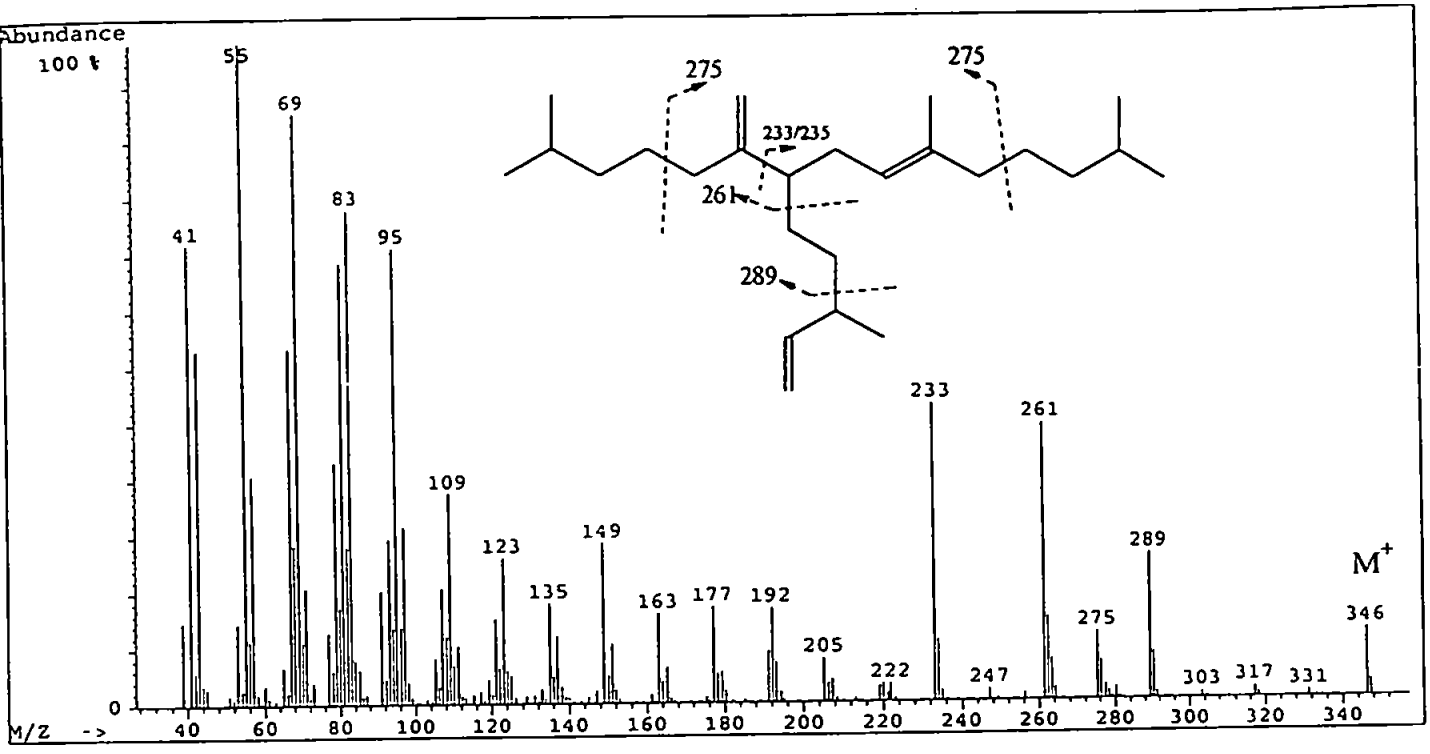
3.2.2.1 Epoxidation of $C_{25:3}$ (GC RI 2106)

Reaction of the triene with *m*-CPBA was conducted at 55°C, resulting in the formation of a *tris*-epoxide (Figure 3.9). A *mono*-epoxide formed after 7 hours and a *tris*-epoxide after 72 hours. The mass spectrum of the *tris*-epoxide displays a M^+ 394, with loss of CH_3 (M-15, *m/z* 379). The presence of vinyl double bond is indicated by the diagnostic ion *m/z* 43, confining the epoxide to the C23-24 location. The second epoxide group is confined by ions *m/z* 309 and *m/z* 267 (C6-17) and the third by *m/z* 253 and *m/z* 309 (C-9). As seen in Figure 3.9 structures of the epoxides (and hence original double bond positions) can clearly be inferred from mass spectrometry.

3.2.2.2 NMR of $C_{25:3}$ (GC RI 2106)

The NMR data are presented in Table 3.2 and assignments relate to Figures 3.5 & 3.10. The ^{13}C and DEPT spectra (Figure 3.10) clearly illustrate the information that can be derived from the simplest of NMR experiments. The fully proton decoupled ^{13}C spectrum shows a total of 25 resonances with 6 alkenic and 19 aliphatic type carbons. Analysis *via* the DEPT sequence reveals the presence of six methyl, eleven methylene, six methine and two quaternary carbons (C-6 and C-10). The observation of two methylene carbons in the alkenic region (δ 108.6 & δ 112.3) confirms the presence of two double bonds in terminal positions (C-17 and C-24).

Figure 3.9 Mass spectrum of $C_{25:3}$ GC RI 2106 (top) and *tris*-epoxide product (bottom)



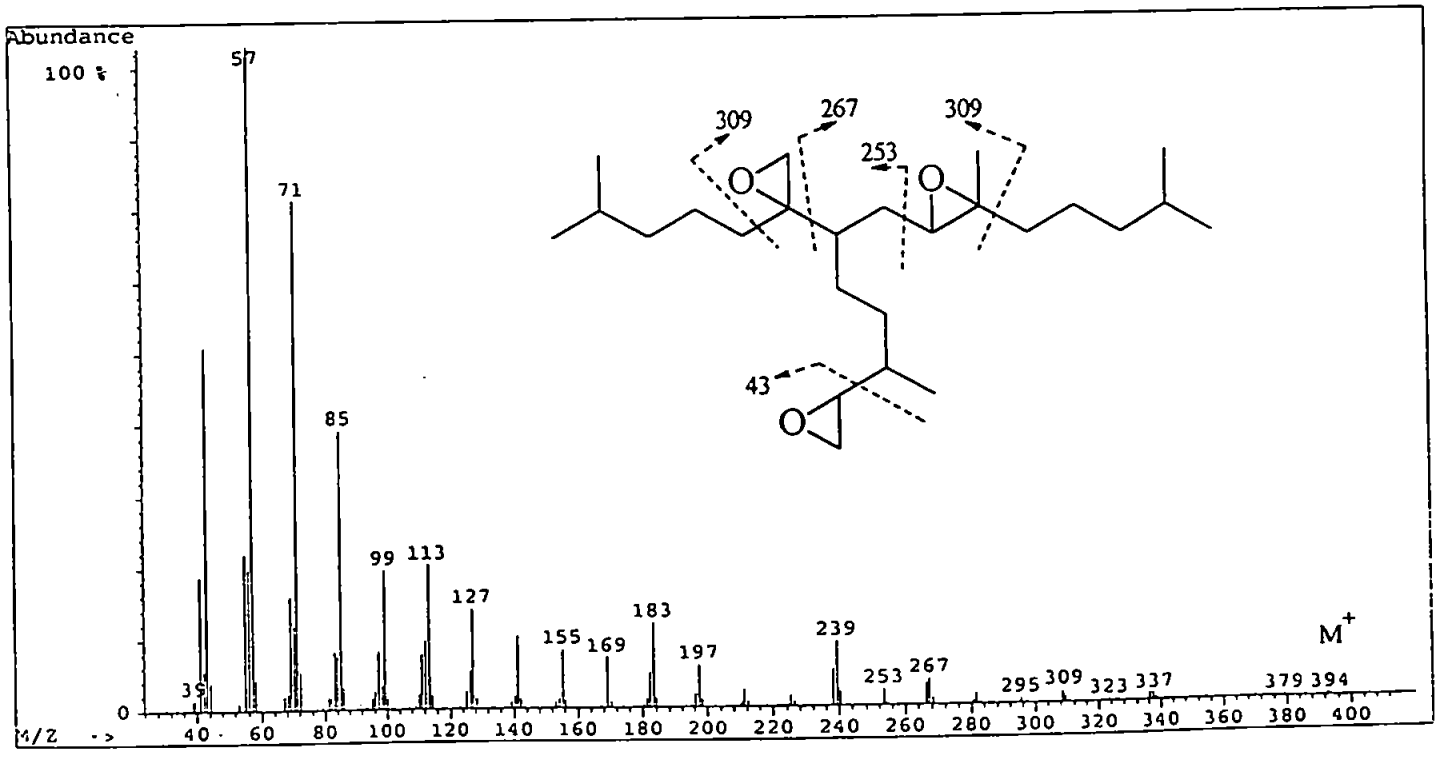
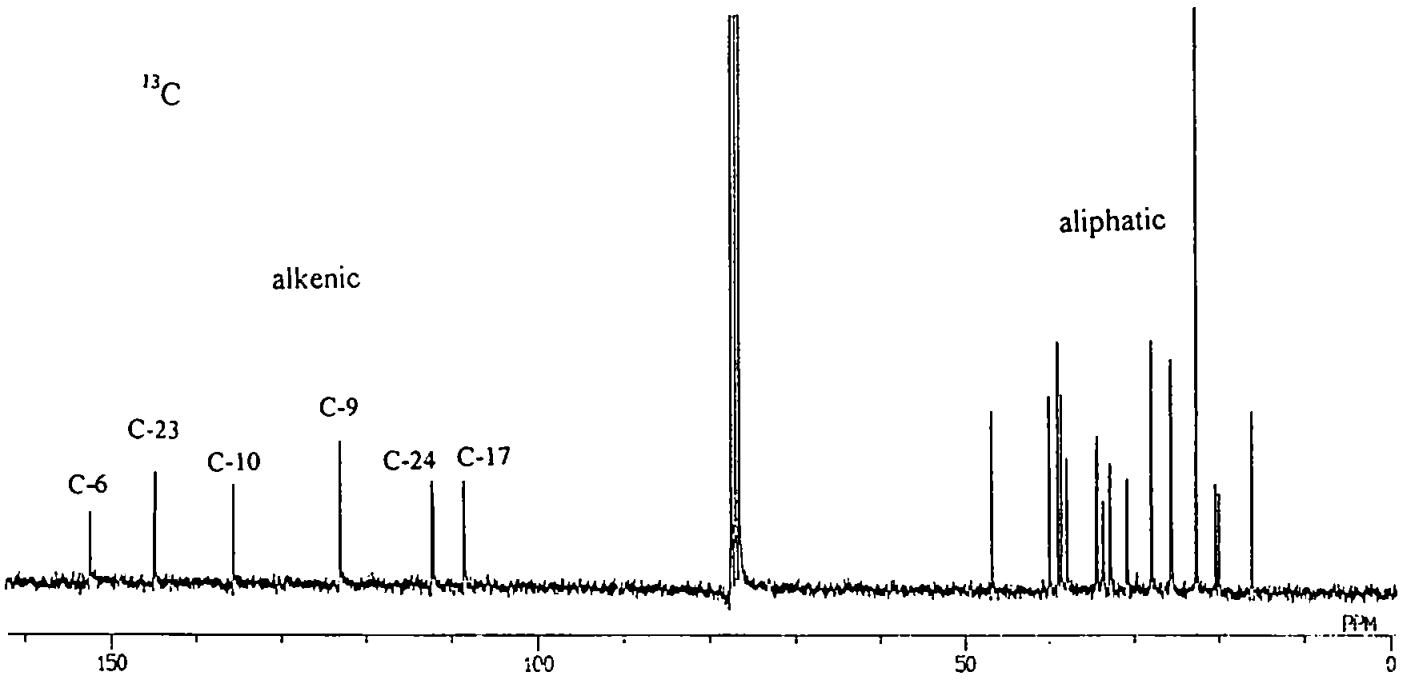


Figure 3.10 ^{13}C spectrum of $C_{25:3}$ GC RI 2106 (top) and DEPT spectrum (bottom)



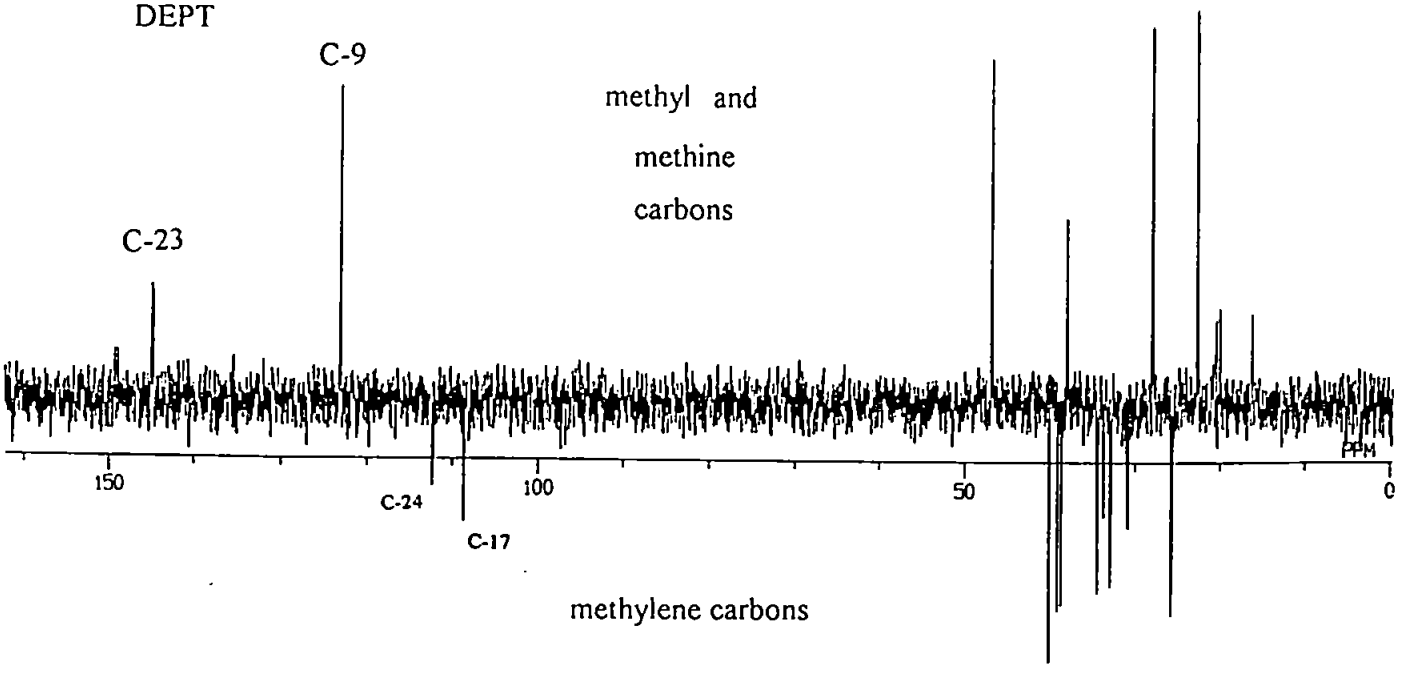
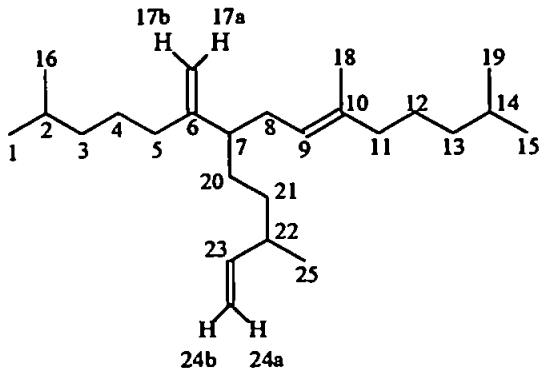


Table 3.2 NMR spectroscopic data for $C_{25:3}$ GC RI 2106, *Haslea ostrearia*

$C_{25:3}$ GC RI 2106(DB1) *Haslea ostrearia*	
Purity : >98% (by GC) Quantity : 5.1 mg Solvent : $CDCl_3$	

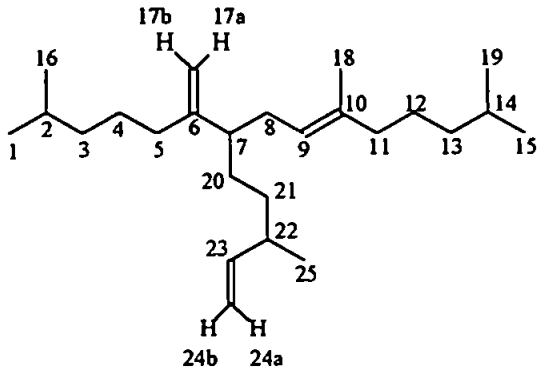
Proton (^{1}H) chemical shifts (multiplicities, integration)

	1, 16	**5, 7, 11**	**8, 22**
δ(ppm)	0.86 (d, 6H)	1.88 (m, 5H)	2.02 (m, 3H)
	9	**15, 19**	**17a**
δ(ppm)	5.05 (t, 1H)	0.86 (d, 6H)	4.70 (br, s, 1H)
	17b	**18**	**23**
δ(ppm)	4.74 (br, d, 1H)	1.56 (s, 3H)	5.67, 5.65* (ddd, 1H)
	24a	**24b**	**25**
δ(ppm)	4.92 (br, d, 1H)	4.88 (br, d, 1H)	0.95 (d, 3H)

Proton coupling constants

	1, 16	**9**	**15, 19**
J(Hz)	6.6	6.6	6.6

Table 3.2 cont.



Carbon (^{13}C) chemical shifts

	1	**2**	**3**	**4**	**5**
δ(ppm)	22.6	27.9	39.0	25.6	33.7, 33.6*
	6	**7**	**8**	**9**	**10**
δ(ppm)	152.5*	46.8	32.9, 32.8*	123.1	135.6
	11	**12**	**13**	**14**	**15**
δ(ppm)	40.0	25.7	38.6	28.0	22.6
	16	**17**	**18**	**19**	**20**
δ(ppm)	22.6	108.6*	16.1	22.6	30.8*
	21	**22**		**23**	
δ(ppm)	34.4	38.0, 37.9*		145.0, 144.9*	
	24		**25**		
δ(ppm)	112.4, 112.2*		20.4, 20.0*		

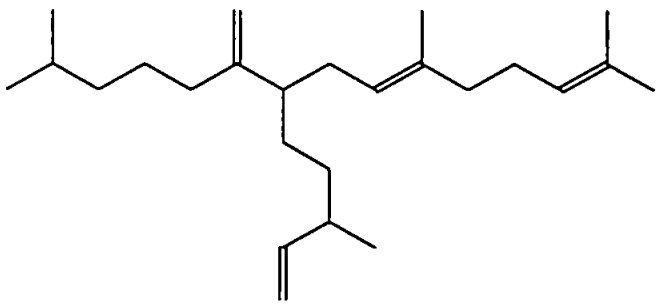
^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively.
Numbering shown in displayed formulae.
Chemical shifts labelled * are due to the presence of diastereoisomers.

3.2.3 $C_{25:4}$ (GC RI 2144) **XI**

2,10,14-trimethyl-6-methylene-7-(3-methylpent-4-enyl)pentadec-9,13-ene

19 mg of **XI** was obtained from an extraction of *Haslea ostrearia* for ^{1}H and ^{13}C NMR spectroscopy and epoxide derivatisation. The double bond positions within the structure have been unambiguously assigned *via* NMR spectroscopy and additionally confirmed by derivatisation.

XI



The C_{25} tetraene **XI** exhibits a molecular ion M^{+} 344, indicating four degrees of unsaturation, with diagnostic fragment ions *m/z* 259, *m/z* 275, *m/z* 287, *m/z* 301 and loss of CH_3 to give *m/z* 329 (Figure 3.11). Mass spectrometry of the hydrogenated ($Pt_2O.H_2O$) alkene displayed the diagnostic ions *m/z* 210, *m/z* 238 and *m/z* 266, but no molecular ion was recorded. The alkane co-eluted (GC) with a synthesised C_{25} alkane (Robson, 1987) on three different stationary phases (DB1, DB5 & DBWAX).

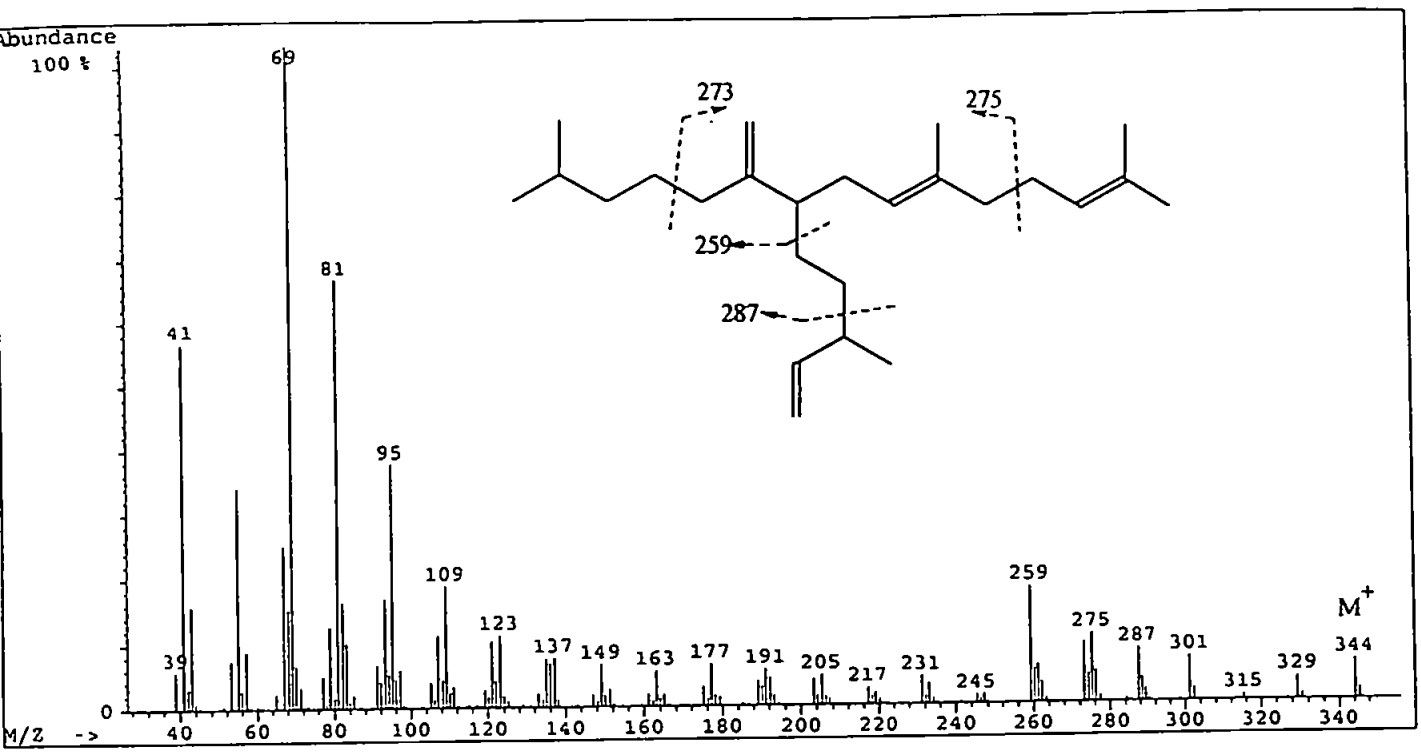
3.2.3.1 Epoxidation of $C_{25:4}$ (GC RI 2144)

Reaction of the tetraene with *m*-CPBA was conducted at 55°C with the formation of a *tetrakis*-epoxide after 72 hours. The mass spectrum of the epoxide (Figure 3.11) displays a M^+ 408, with loss of CH_3 (M-15, *m/z* 393) and diagnostic ions *m/z* 43, *m/z* 239, *m/z* 253, *m/z* 281, *m/z* 309, *m/z* 323 and *m/z* 337. The position of the vinyl double bond is indicated by the diagnostic ion *m/z* 43, confining the epoxide to this location, C23-24. The second epoxide group is confined by ions *m/z* 323 and *m/z* 281 (C6-17) and the third by *m/z* 253 and *m/z* 309 (C9-10). The fourth epoxide group has a diagnostic fragmentation ion *m/z* 337, indicating that it must be located at either the C2-3 or C13-14 positions. From the epoxidation results it is clear that the fourth double bond can only be in one of these two positions (C2-3 or C13-14) and NMR analysis was required to distinguish between the two (double bond located at C13-14).

3.2.3.2 NMR of $C_{25:4}$ (GC RI 2144)

The NMR data are presented in Table 3.3 and assignments relate to Figures 3.5 & 3.12. The ^1H-^{13}C technique is very effective in the structural elucidation of compounds providing unequivocal results, as seen in Figure 3.12, correlation of the ^{13}C shifts in one direction with the ^1H shift in the other, *via* one-bond CH coupling, can be made. This technique, in conjunction with two / three-bond CH couplings (COLOC), facilitates the complete assignment of the structures.

Figure 3.11 Mass spectrum of $C_{25:4}$ GC RI 2144 (top) and *tetrakis*-epoxide product (bottom)



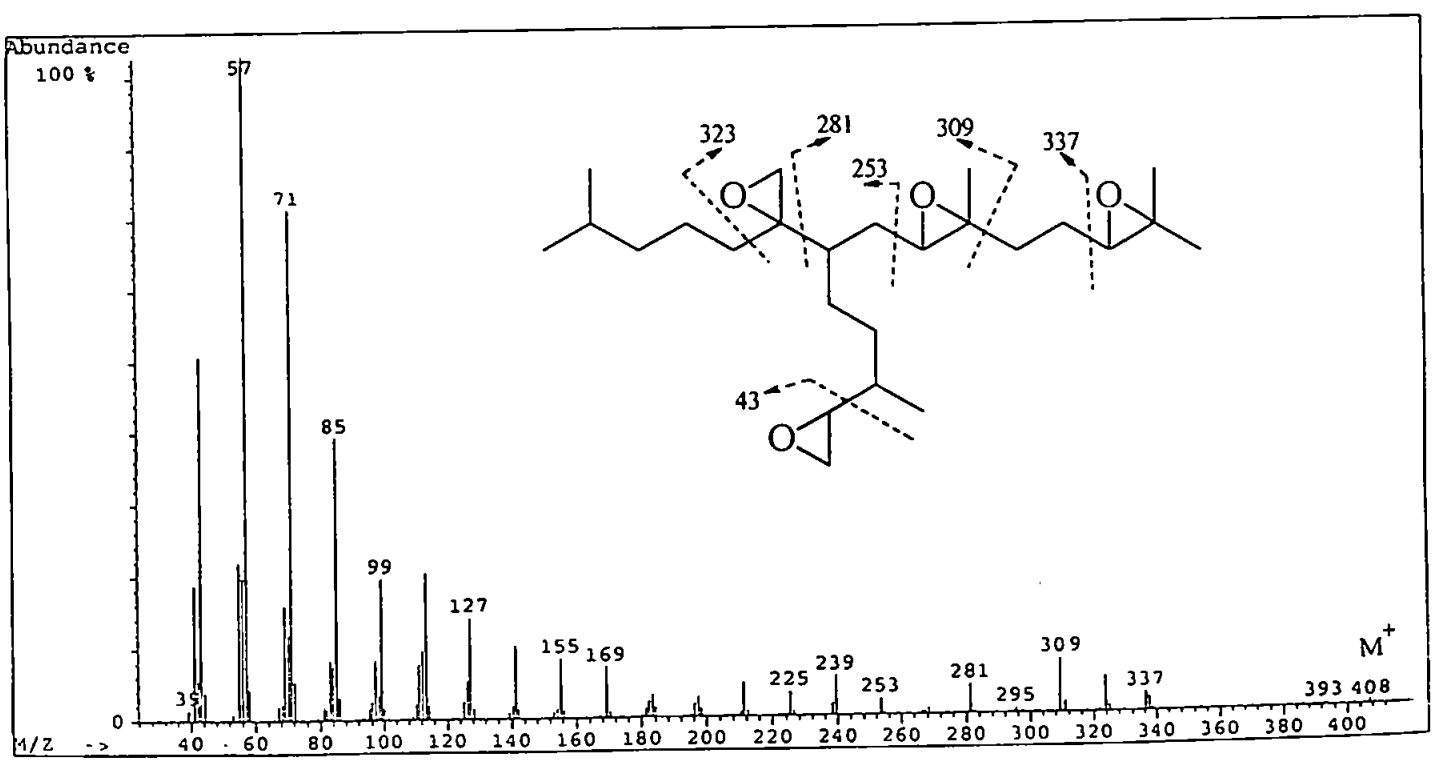
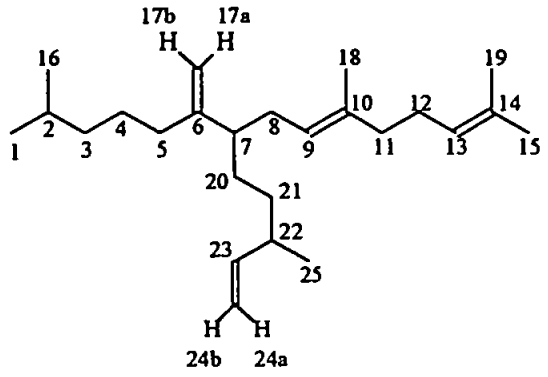


Table 3.3 NMR spectroscopic data for $C_{25:4}$ GC RI 2144, *Haslea ostrearia*

$C_{25:4}$ GC RI 2144 (DB1)
Haslea ostrearia



Purity : >98% (by GC)
Quantity : 18.9 mg
Solvent : $CDCl_3$

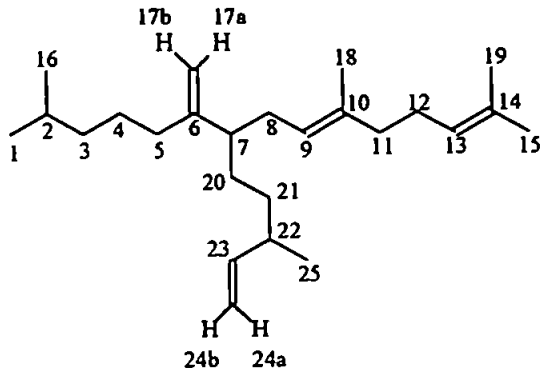
Proton (^{1}H) chemical shifts (multiplicities, integration)

	1, 16	**5**	**7, 11**
δ(ppm)	0.87 (d, 6H)	1.87 (t, 2H)	1.96 (m, 3H)
	8, 12, 22	**9, 13**	**15**
δ(ppm)	2.04 (m, 5H)	5.07, 5.08 (br, m, 2H)	1.67 (s, 3H)
	17a	**17b**	**18**
δ(ppm)	4.70 (br, s, 1H)	4.74 (br, d, 1H)	1.57 (s, 3H)
	19	**23**	**24a**
δ(ppm)	1.59 (s, 3H)	5.67, 5.65* (ddd, 1H)	4.92 (br, d, 1H)
	24b	**25**	
δ(ppm)	4.88 (br, d, 1H)	0.95 (d, 3H)	

Proton coupling constants

	1, 16	**5**		**23**	**25**
J(Hz)	6.6	7.5	J_{trans}	= 17.5	6.9
			J_{cis}	= 11.5	
			J_{vic}	= 7.5	

Table 3.3 cont.

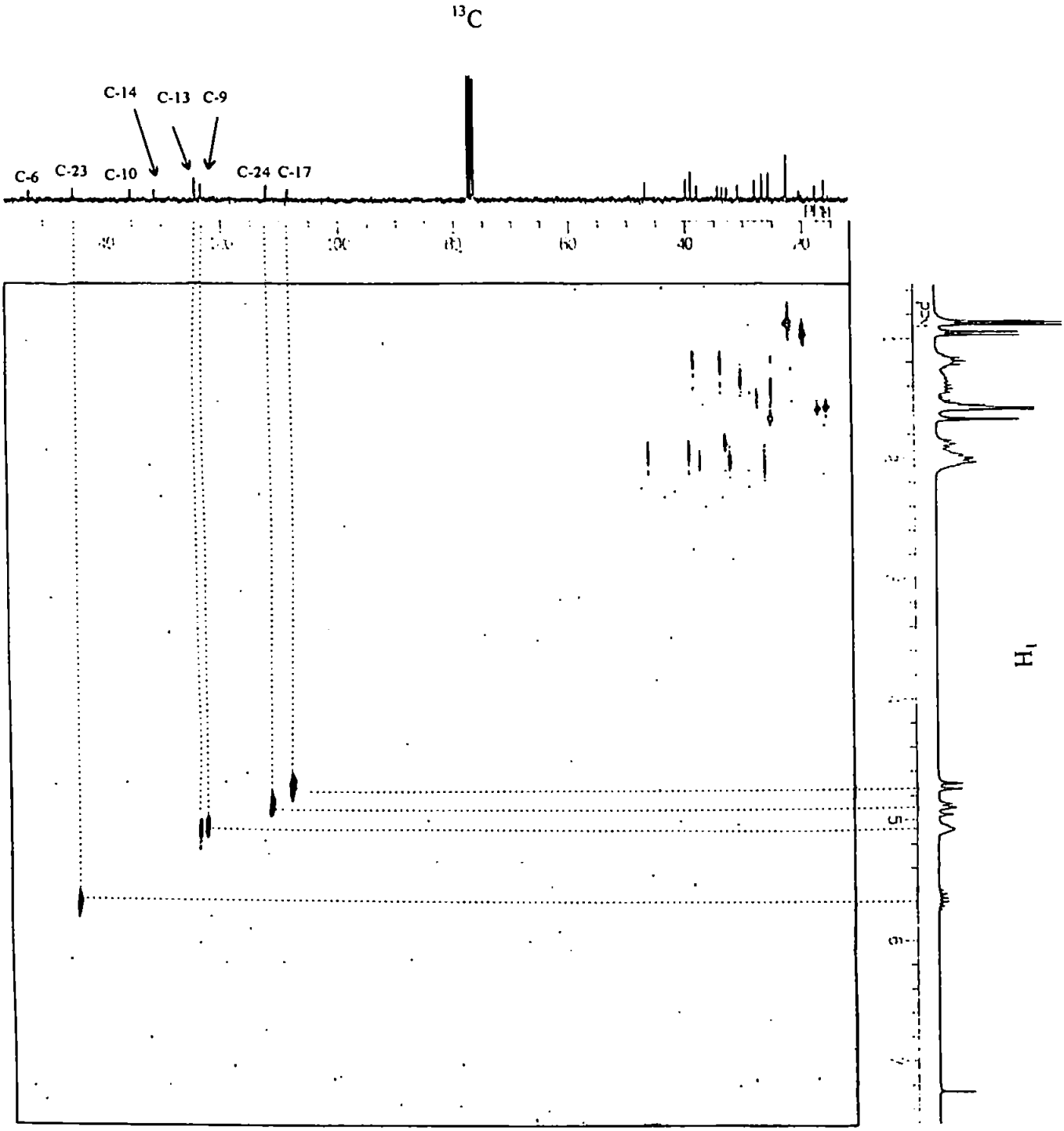


Carbon (^{13}C) chemical shifts

	1	**2**	**3**	**4**	**5**
δ(ppm)	22.6	27.9	39.0	25.6	33.7, 33.6*
	6		**7**	**8**	**9**
δ(ppm)	152.5, 152.4*		46.8	32.8, 32.7*	123.3
	10	**11**	**12**	**13**	**14**
δ(ppm)	135.2	39.8	26.7	124.4	131.2
	15	**16**	**17**	**18**	**19**
δ(ppm)	25.7	22.6	108.6*	16.2	17.7
	20		**21**		**22**
δ(ppm)	30.9, 30.8*		34.4, 34.3*		38.0, 37.9*
	23		**24**		**25**
δ(ppm)	145.0, 144.8*		112.4, 112.2*		20.4, 20.0*

^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively.
Numbering shown in displayed formulae.
Chemical shifts labelled * are due to the presence of diastereoisomers.

Figure 3.12 Correlation of ^{13}C shifts and ^{1}H shifts, *via* a one-bond coupling (HETCOR)

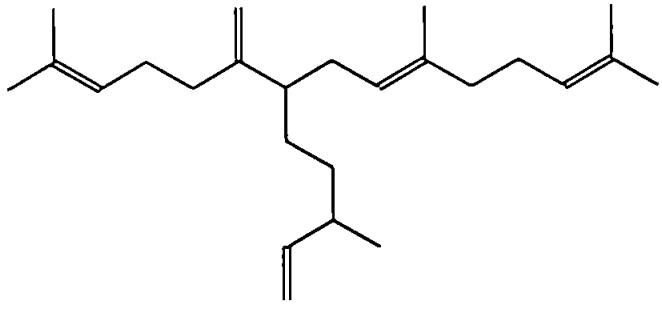


3.2.4 $C_{25:5}$ (GC RI 2191) **XII**

2,10,14-trimethyl-6-methylene-7-(3-methylpent-4-enyl)pentadec-2,9,13-ene

4.8 mg of **XII** was obtained from a bulk culture extraction of *Haslea ostrearia* for ^{1}H and ^{13}C NMR spectroscopy and epoxide derivatisation. The double bond positions within the structure have been unambiguously assigned *via* NMR spectroscopy and additionally confirmed by derivatisation.

XII



The C_{25} pentaene (**XII**) exhibits a molecular ion M^{+} 342 (indicating five degrees of unsaturation), loss of CH_3 to give *m/z* 327 and with diagnostic fragment ions *m/z* 259, *m/z* 273, *m/z* 287 and *m/z* 299. Mass spectrometry of the hydrogenated ($Pt_2O.H_2O$) alkene displayed a molecular ion M^{+} 350 (M-2), with the diagnostic ions *m/z* 210, *m/z* 238 and *m/z* 266. The alkane co-eluted (GC) with a synthesised C_{25} alkane (Robson, 1987) on three different stationary phases (DB1, DB5 & DBWAX).

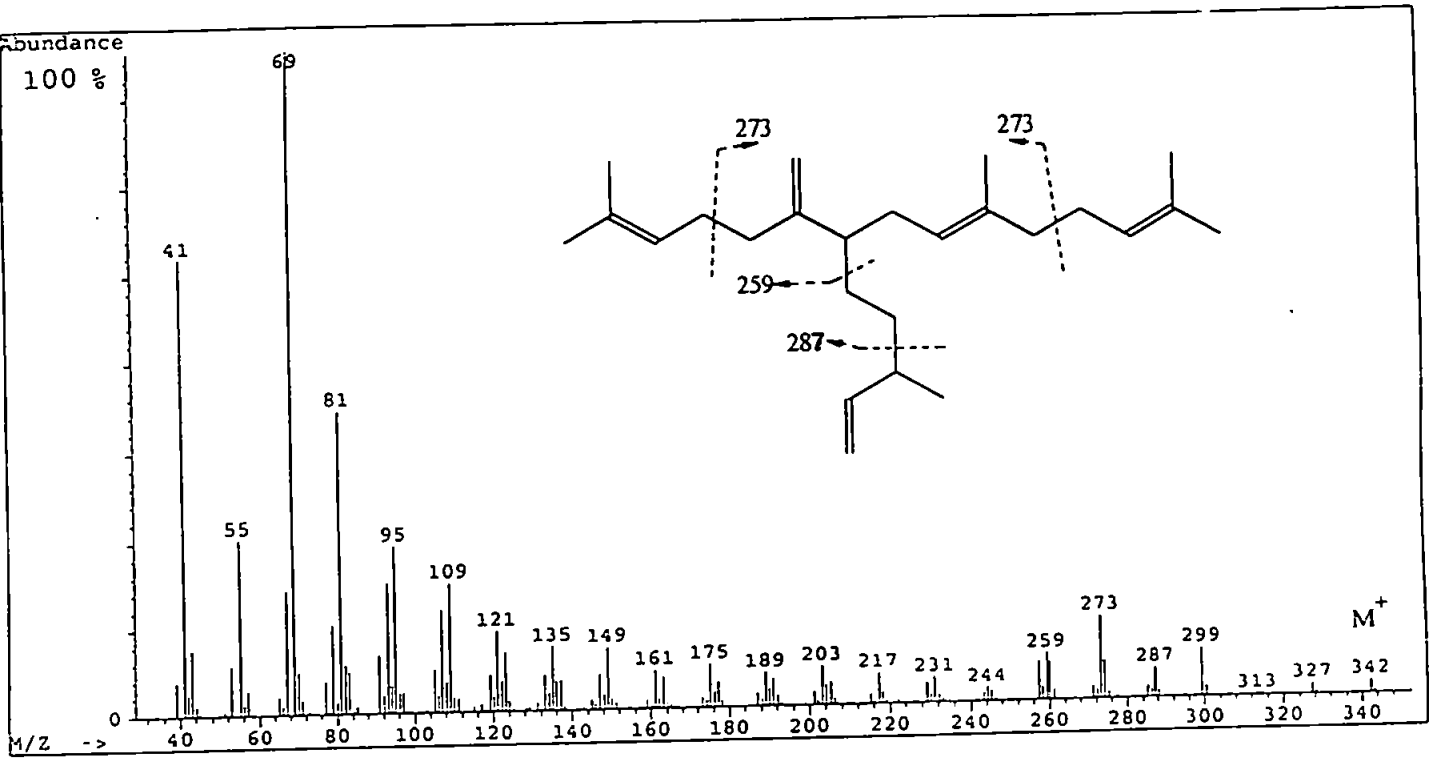
3.2.4.1 Epoxidation of $C_{25:5}$ (GC RI 2191)

Reaction of the pentaene with *m*-CPBA was conducted at 55°C with the formation of a *pentakis*-epoxide after 72 hours. The epoxide mass spectrum (Figure 3.13) displays a M^+ 422, with loss of CH_3 (M-15, *m/z* 407) and diagnostic ions *m/z* 43, *m/z* 267, *m/z* 281, *m/z* 323 and *m/z* 351. The position of the vinyl double bond is indicated by the diagnostic ion *m/z* 43, confining the epoxide to this location, C23-24. The second epoxide group is confined by ions *m/z* 323 and *m/z* 281 (C6-17) and the third by *m/z* 267 and *m/z* 323 (C9-10). The fourth and fifth epoxide groups have a diagnostic fragmentation ion *m/z* 351, indicating that these epoxides can only be located at C2-3 and C13-14 positions.

3.2.4.2 NMR of $C_{25:5}$ (GC RI 2191)

The NMR data is presented in Table 3.4. No salient features are reported for this compound.

Figure 3.13 Mass spectrum of $C_{25:5}$ GC RI 2191 (top) and *pentakis*-epoxide product (bottom)



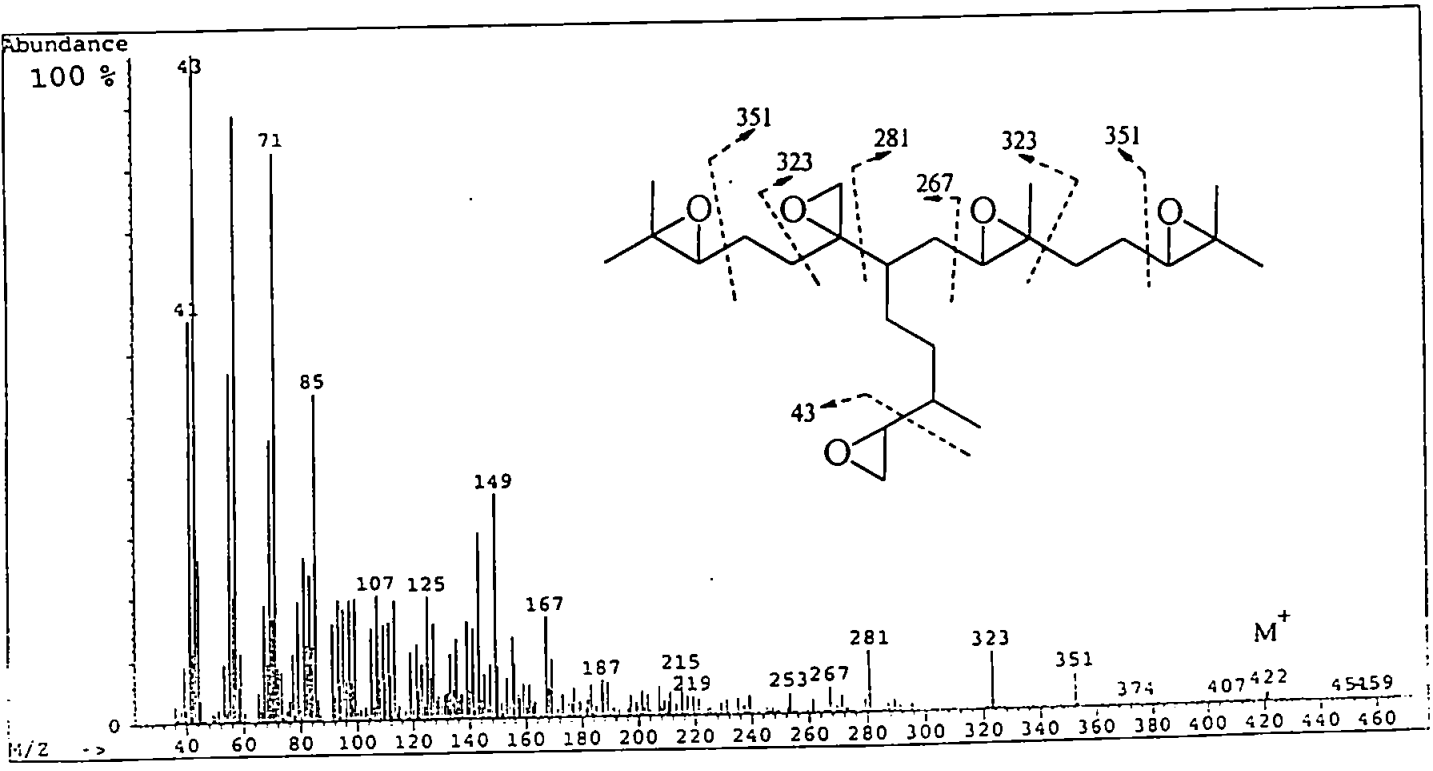
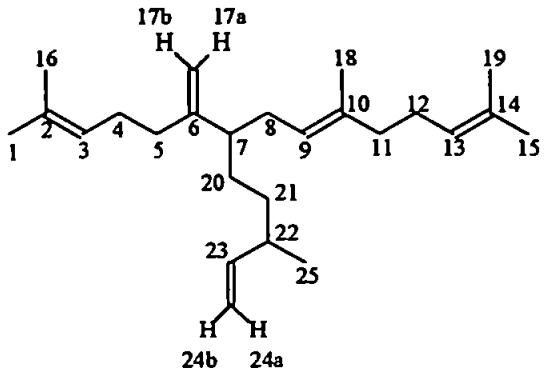


Table 3.4 NMR spectroscopic data for $C_{25:5}$ GC RI 2191, *Haslea ostrearia*

$C_{25:5}$ GC RI 2191 (DB1) *Haslea ostrearia*	
Purity : >89% (by GC) Quantity : 2.9 mg Solvent : $CDCl_3$	

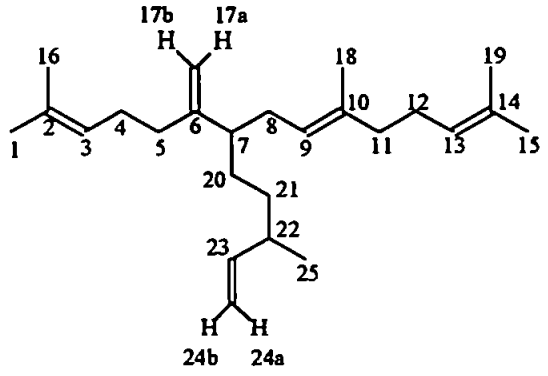
Proton (^{1}H) chemical shifts (multiplicities, integration)

	1, 15	**5**	**7, 11**
δ(ppm)	1.67 (s, 6H)	1.87 (t, 2H)	1.96 (m, 3H)
	8, 12, 22	**9, 12, 13**	**16**
δ(ppm)	2.04 (m, 5H)	5.09 (m, 4H)	1.60 (s, 3H)
	17a	**17b**	**18**
δ(ppm)	4.72 (br, s, 1H)	4.76 (br, d, 1H)	1.57 (s, 3H)
	19	**23**	**24a**
δ(ppm)	1.59 (s, 3H)	5.67, 5.65* (m, 1H)	4.92 (br, d, 1H)
	24b	**25**	
δ(ppm)	4.88 (br, d, 1H)	0.95 (d, 3H)	

Proton coupling constants

	5		**23**	**25**
J(Hz)	7.5	J_{trans}	= 17.5	6.9
		J_{cis}	= 11.5	
		J_{vic}	= 7.5	

Table 3.4 cont.



Carbon (^{13}C) chemical shifts

	1	**2**	**3**	**4**	**5**
δ(ppm)	25.7	131.2	124.4	26.6	33.7, 33.6*
	6		**7**	**8**	**9**
δ(ppm)	152.5, 152.4*		46.8	32.8, 32.7*	123.3
	10	**11**	**12**	**13**	**14**
δ(ppm)	135.2	39.8	26.7	124.4	131.2
	15	**16**	**17**	**18**	**19**
δ(ppm)	25.7	17.7	108.6*	16.2	17.7
	20		**21**		**22**
δ(ppm)	30.9, 30.8*		34.4, 34.3*		38.0, 37.9*
	23		**24**		**25**
δ(ppm)	145.0, 144.8*		112.4, 112.2*		20.4, 20.0*

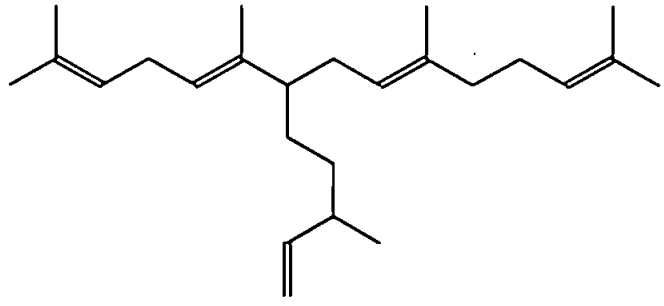
^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively.
Numbering shown in displayed formulae.
Chemical shifts labelled * are due to the presence of diastereoisomers.

3.2.5 $C_{25:5}$ (GC RI 2201) **XIII**

2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadec-2,5,9,13-ene

Insufficient isolate (<1.0 mg) of **XIII** was obtained from an extraction of *Haslea ostrearia* for ^{13}C NMR spectroscopy, and only ^{1}H analysis and epoxide derivatisation were possible for this sample. The double bond positions within the structure have been tentatively assigned *via* epoxide derivatisation and additional confirmation by comparison of ^{1}H spectrum with other characterised HBI compounds, particularly $C_{25:6}$ (GC RI 2248).

XIII



The C_{25} pentaene (**XIII**) has a molecular ion M^+ 342, indicating five degrees of unsaturation, loss of CH_3 to give *m/z* 327, with further diagnostic fragment ions *m/z* 259, *m/z* 273, *m/z* 285 and *m/z* 299. Mass spectrometry of the hydrogenated ($Pt_2O.H_2O$) alkane displayed the diagnostic ions *m/z* 210, *m/z* 238 and *m/z* 266, but no molecular ion was recorded. The alkane co-eluted (GC) with a synthesised C_{25} alkane (Robson, 1987) on two different stationary phases (DB1 & DB5).

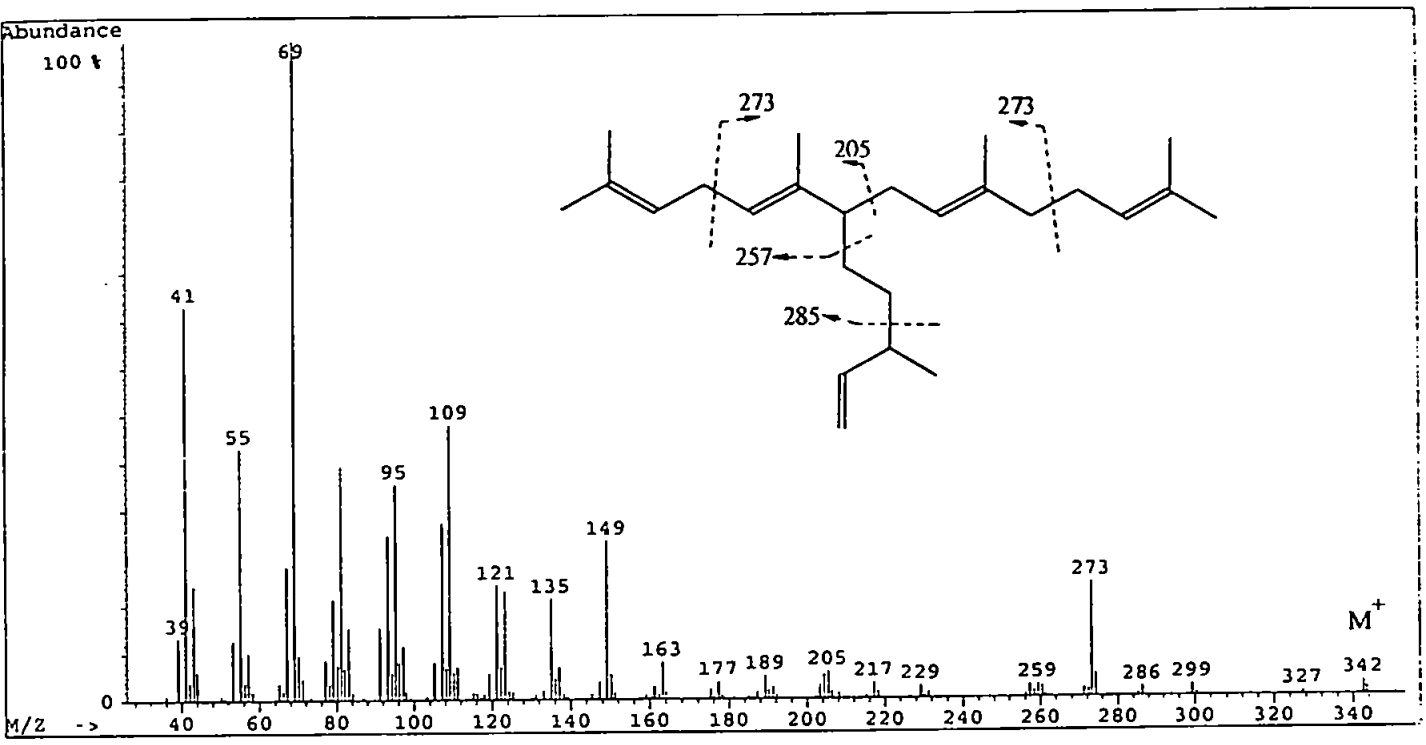
3.2.5.1 Epoxidation of $C_{25:5}$ (GC RI 2201)

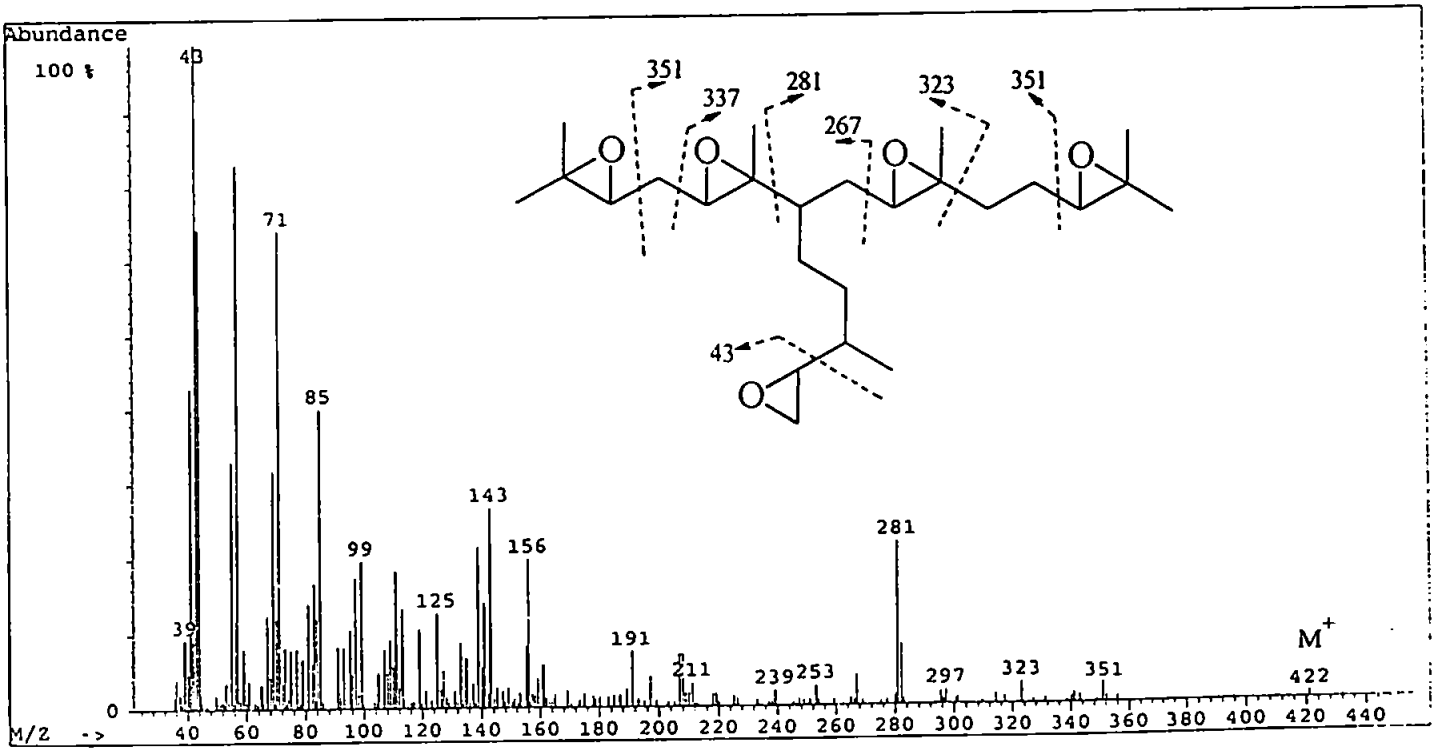
Reaction of the pentaene with *m*-CPBA was conducted at 60°C with the formation of a *pentakis*-epoxide after 72 hours. The mass spectrum of the epoxide (Figure 3.14) displays a M^+ 422, with loss of CH_3 (M-15, *m/z* 407) and diagnostic ions *m/z* 43, *m/z* 267, *m/z* 281, *m/z* 323 and *m/z* 351. The position of vinyl double bond is indicated by the diagnostic ion *m/z* 43, confining the epoxide to this location, C23-24. The second epoxide group is confined by ions *m/z* 337 and *m/z* 281 (C5-6) and the third by *m/z* 267 and *m/z* 323 (C9-10). The fourth and fifth epoxide groups have a diagnostic fragment ion *m/z* 351, indicating that these epoxides can only be located at C2-3 and C13-14 positions.

3.2.5.2 NMR of $C_{25:5}$ (GC RI 2201)

Only enough isolate for ^1H NMR analysis was obtained. The major difference between the ^1H spectrum of this alkene and $C_{25:5}$ (GC RI 2191) is the loss of methylenic ($=CH_2$, 17 Hz) signal and the appearance of a triplet at 2.70 ppm. The triplet (δ = 2.70) results from the two H-4 protons coupling equally to H-3 and H-5 and the resonance appears at relatively low field (δ = 2.70, as opposed to 1.8 - 2.0) since the H-4 protons are allylic to two double bonds.

Figure 3.14 Mass spectrum of $C_{25:5}$ GC RI 2201 (top) and *pentakis*-epoxide product (bottom)



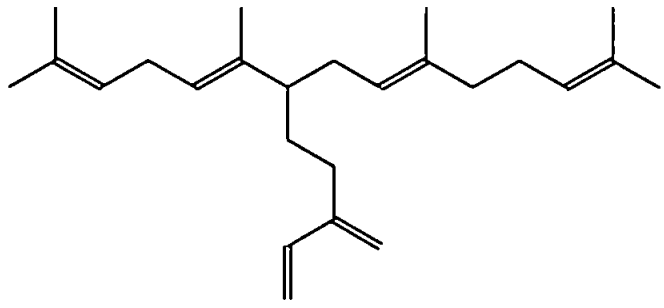


3.2.6 $C_{25:6}$ (GC RI 2248) **XIV**

2,6,10,14-tetramethyl-7-(3-methylenepent-4-enyl)pentadec-2,5,9,13-ene

Alkene **XIV** (3 mg) was obtained via extraction of *Haslea ostrearia* and was sufficient for ^{1}H and ^{13}C NMR spectroscopy and epoxide derivatisation. The double bond positions within the structure have been assigned *via* NMR spectroscopy and additionally confirmed by derivatisation.

XIV



The C_{25} hexaene (**XIV**) has a molecular ion M^{+} 340 indicating six degrees of unsaturation, loss of CH_3 to give *m/z* 325, with further diagnostic fragment ions *m/z* 255, *m/z* 271, *m/z* 283 and *m/z* 297 (Figure 3.16). Mass spectrometry of the hydrogenated ($Pt_2O.H_2O$) alkene displayed the diagnostic ions *m/z* 210, *m/z* 238 and *m/z* 266, but no molecular ion was recorded. The alkane co-eluted (GC) with a synthesised C_{25} alkane (Robson, 1987) on two different stationary phases (DB1 & DB5).

3.2.6.1 Epoxidation of $C_{25:6}$ (GC RI 2248)

Reaction of the hexaene with *m*-CPBA was conducted at 60°C and the formation of a *hexakis*-epoxide recorded after 72 hours. The progress of the reaction, from starting compound to a *hexakis*-epoxide is illustrated in Figure 3.15. The mass spectrum (Figure 3.16) displays a M^+ 436, loss of CH_3 to give *m/z* 421 and diagnostic ions *m/z* 43, *m/z 85*, *m/z* 281, *m/z* 295, *m/z* 327, *m/z* 351 and *m/z* 365. The position of the vinyl double bond is indicated by the diagnostic ion *m/z* 43, confining one epoxy group to this location, C23-24. The second epoxide group is confined by ions *m/z* 351 and *m/z* 295 (C5-6) and the third by *m/z* 281 and *m/z* 337 (C9-10). The fourth and fifth epoxide groups have a diagnostic fragment ion *m/z* 365, indicating that these epoxides can only be located at C2-3 and C13-14 positions. The sixth epoxide group was located by the diagnostic ion *m/z* 85, which suggested that it was at the C22-25 position.

3.2.6.2 NMR of $C_{25:6}$ (GC RI 2248)

The NMR data are presented in Table 3.5. The set of assignments for ^1H and ^{13}C nuclei is incomplete since limited spectroscopic data were collected. As seen in Table 3.5, the double bond located at the C22-25 position is conjugated with the double bond at C23-24. This was identified by the low field shift of the resonance H-23 from $\delta = 5.65$ (for all other HBIs characterised here), to $\delta = 6.35$. This resonance (H-23) appears as a doublet of doublets due to *cis* and *trans* couplings to H-24a and H-24b respectively.

Figure 3.15 Reaction of $C_{25:6}$ with *m*-CPBA: a, $C_{25:6}$ isolate; b, after 20 hours; c, after 72 hours. GC conditions: OV-1, 40-300°C at 5°Cmin^{-1}

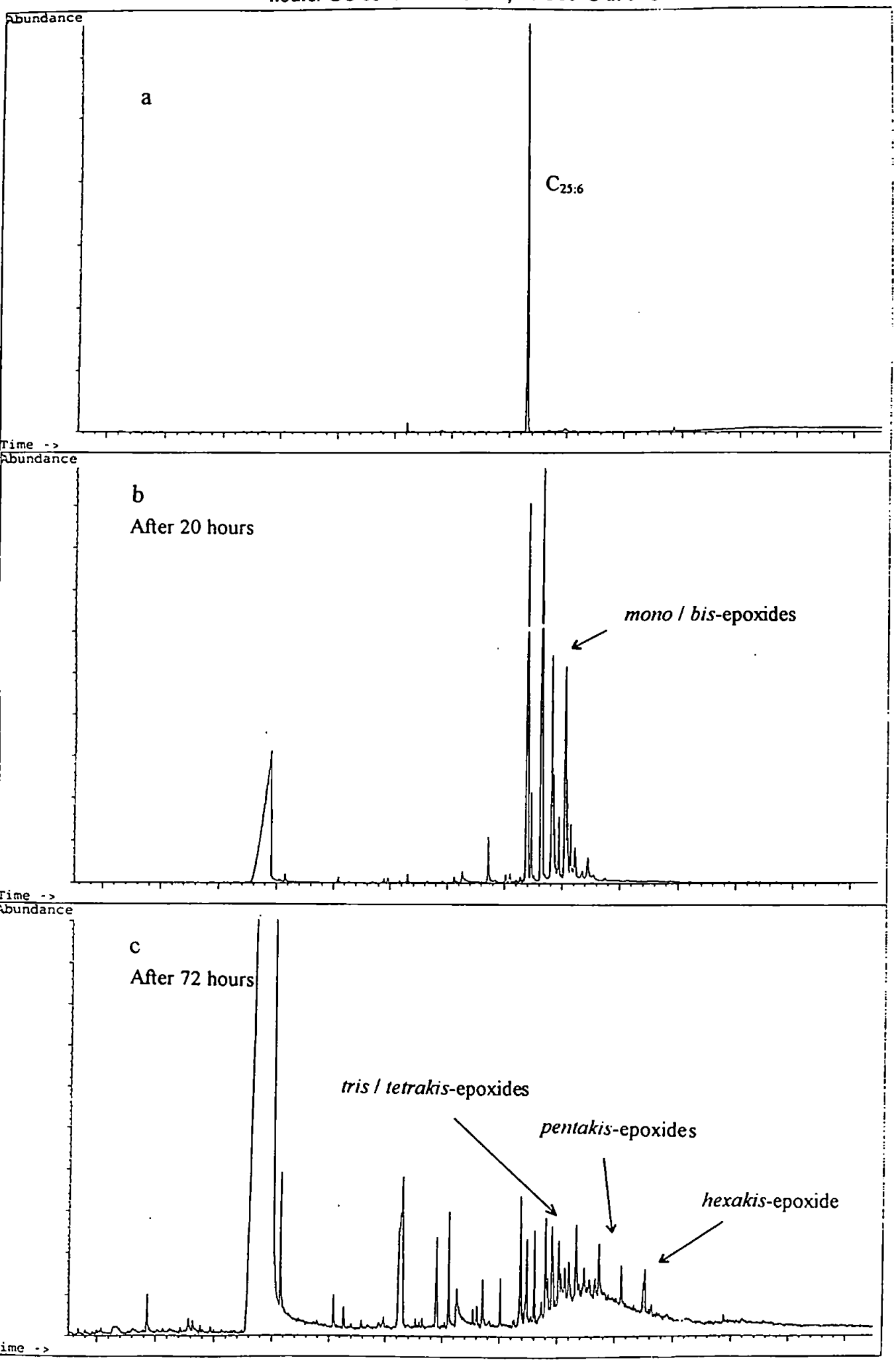
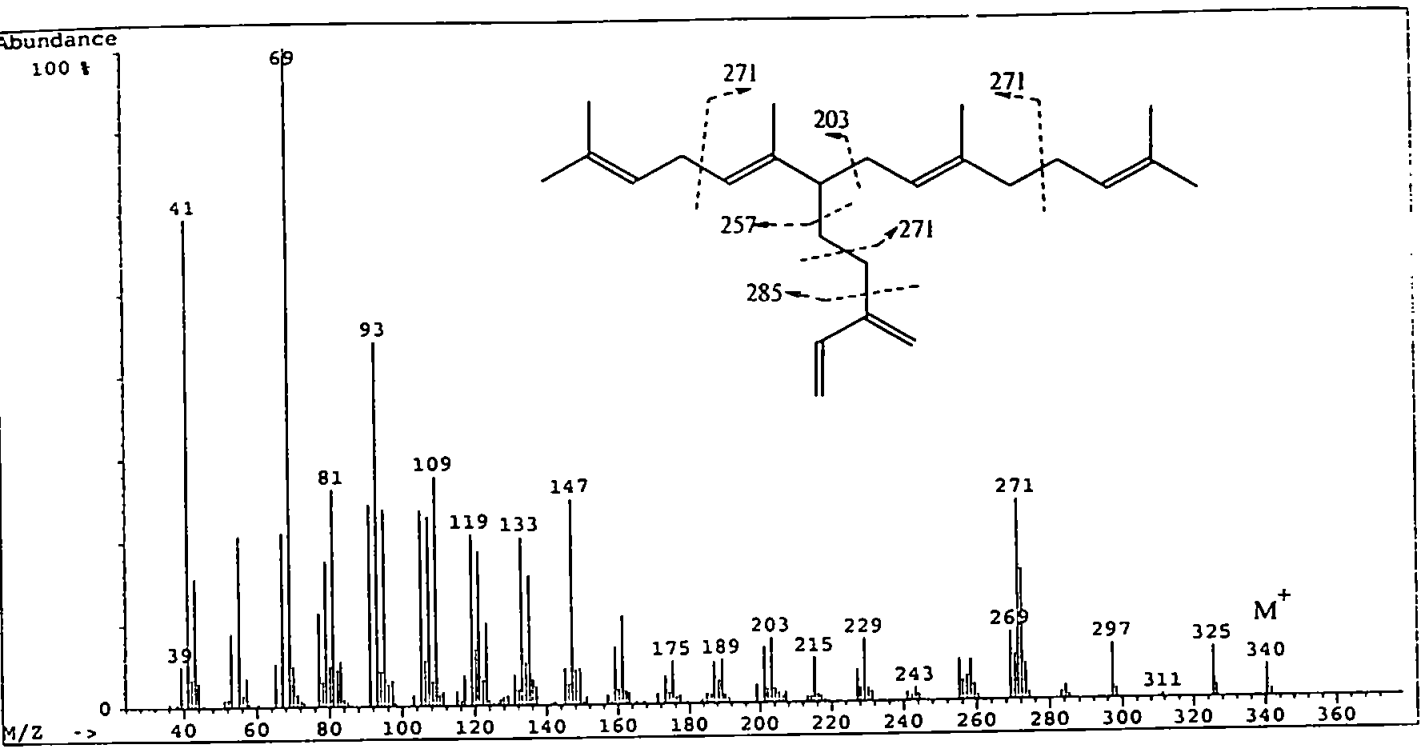


Figure 3.16 Mass spectrum of $C_{25:6}$ GC RI 2248 (top) and *hexakis*-epoxide product (bottom)



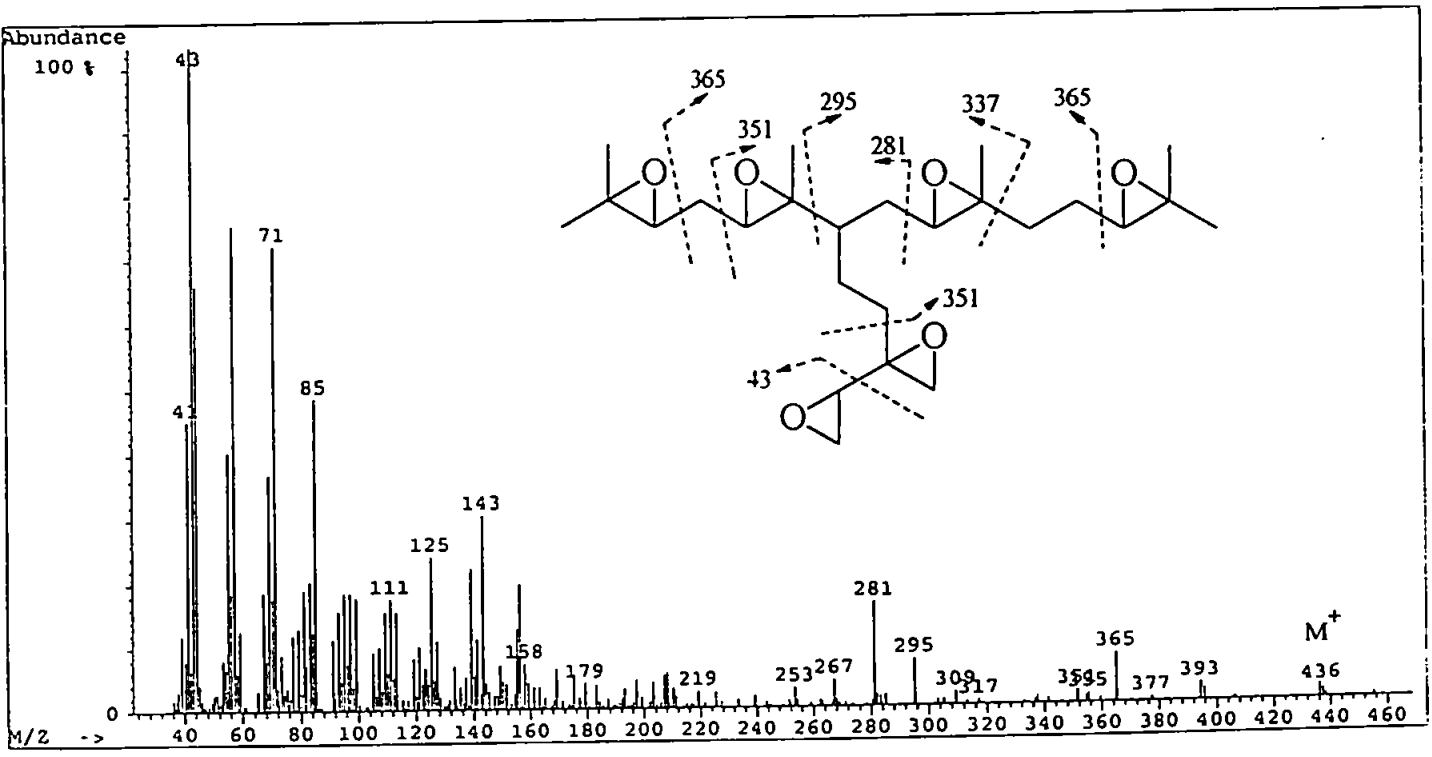
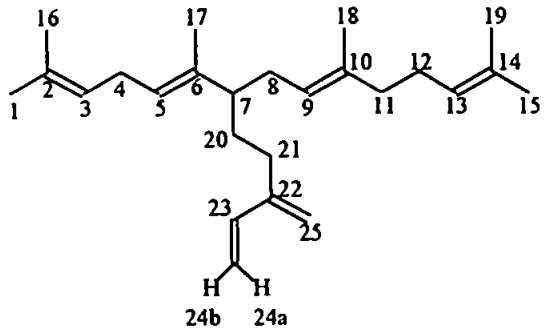


Table 3.5 NMR spectroscopic data for $C_{25:6}$ GC RI 2248, *Haslea ostrearia*

$C_{25:6}$ GC RI 2248 (DB1) *Haslea ostrearia*	
Purity : >98% (by GC) Quantity : 3.0 mg Solvent : $CDCl_3$	

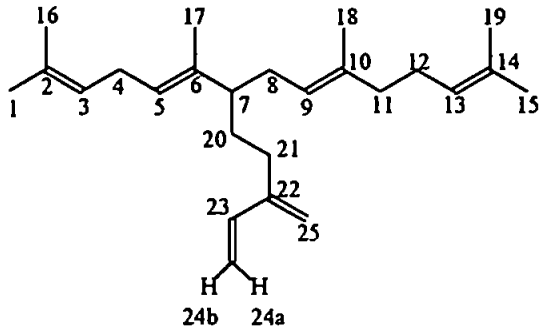
Proton (^{1}H) chemical shifts (multiplicities, integration)

	1, 15, 16, 17, 18, 19	**3, 5, 9, 13**	**4**
δ(ppm)	1.5 -1.7 (s, 18H)	5.05 - 5.13 (br, m, 2H)	2.70 (t, 2H)
	7, 8, 11, 12, 21	**9, 13**	**23**
δ(ppm)	1.96 - 2.18 (m, 9H)	5.07, 5.08 (br, m, 2H)	6.35 (dd, 1H)
	24a	**24b**	**25**
δ(ppm)	5.19 (d, 1H)	5.03 (d, 1H)	4.98 (br, s, 2H)

Proton coupling constants

	J_{H4-H3}	J_{H4-H5}	**H-23**
J(Hz)	6.8	6.8	J_{trans} = 17.5 J_{cis} = 10.9

Table 3.5 cont.



Carbon (^{13}C) chemical shifts

	1	**2**	**3**	**4**	**5**
δ(ppm)	25.6	131.2	124.3	39.8	124.3
	6	**7**	**8**	**9**	
δ(ppm)	135.1	49.1	32.2	124.3	
	10	**11**	**12**	**13**	**14**
δ(ppm)	136.3	26.7	26.7	124.3	131.2
	15	**16**	**17**	**18**	**19**
δ(ppm)	25.6	17.7	12.1	16.2	17.7
	20	**21**	**22**	**23**	**24**
δ(ppm)	32.2	32.2	146.9	139.0	113.0
	25				
δ(ppm)	115.4				

^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively). Numbering shown in displayed formulae.

3.3 Conclusions and summary

3.3.1 Applications of ozonolysis

Ozonolysis proved an effective tool for the location of double bond positions in the $C_{25:2}$ (GC RI 2079), but due to the low yield of products ($C_{25:2}$ GC RI 2079, yielded 4.8 % of product) the technique had a limited application during this study. Another possible drawback to this technique is the increasing number of fragments produced as more unsaturated compounds are treated with ozone. For example, the HBI $C_{25:5}$ (GC RI 2201) upon reaction with ozone would produce six ketone and / or aldehyde fragments, at least three of which would be volatile and could not be identified by conventional GC-MS. This technique appears more suited to HBI compounds with less than three degrees of unsaturation and is best applied in conjunction with other methods of analysis.

3.3.2 Applications of epoxidation

This technique has proved a very powerful tool in the structural elucidation of HBI compounds. The advantages of this technique are:

1. That it can be applied to microgram quantities of isolate, greatly reducing the amount of material required for extraction and isolation,

2. The technique can be applied to HBI alkenes which have at least six double bonds,

3. The resultant epoxides can be analysed directly by GC and / or GC-MS,

4. *Alpha*-cleavage of the epoxides, during GC-MS analysis, gives diagnostic fragment ions which clearly indicate the position of the epoxides and subsequently the original double bond positions,

5. HBI compounds with less than five degrees of unsaturation give relatively high yields of product.

The main disadvantages to this technique are:

1. Some epoxide groups cannot be confined to one position, with diagnostic mass spectrum fragments indicating a number of different locations,

2. Increasing the temperature to ≥ 55°C causes further oxidation of the epoxide products and the formation of an unresolved complex mixture, greatly reducing the yield of identifiable components.

3.3.3 Applications of NMR

NMR analysis is the definitive technique for structural elucidation. It is a non-destructive method of analysis and gives unambiguous structural assignments. The main disadvantage of this technique is the large amount of pure isolate required for complete analysis (≥ 5.0 mg).

3.4 Summary of HBI alkenes characterised

Table 3.7 shows the structures of the six HBI alkenes characterised and summarises the data relating to each compound.

Table 3.7 Summary of HBI alkenes characterised

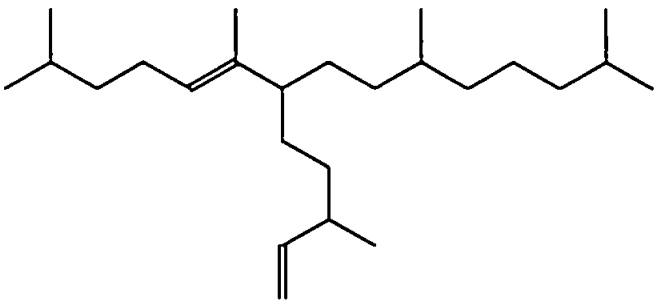
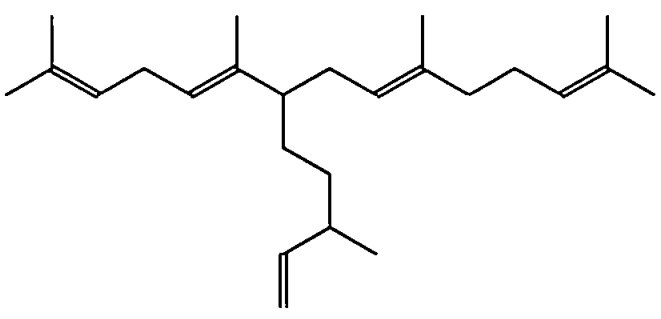
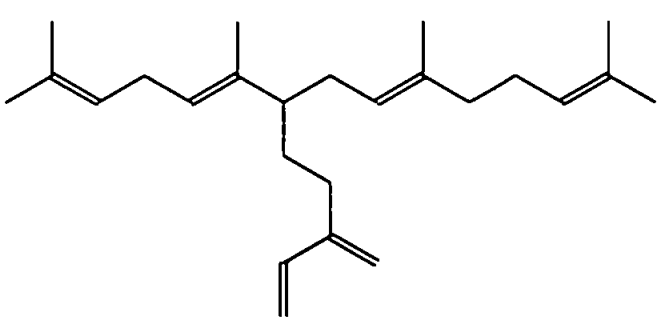
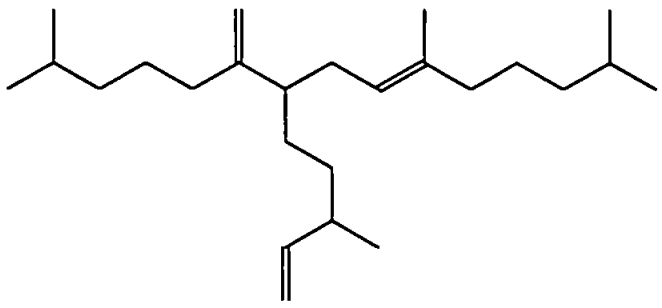
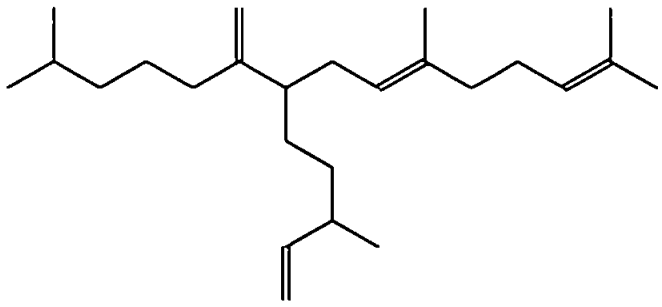
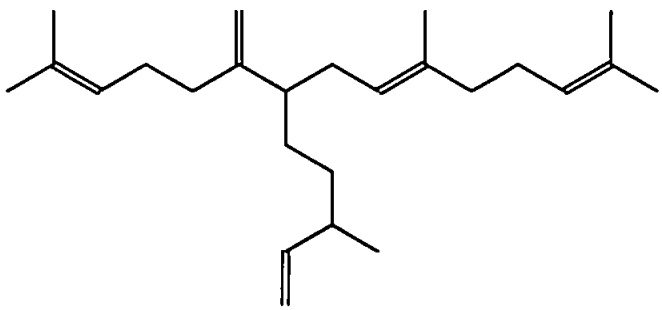
HBI : $C_{25:2}$ GC RI(DBI) : **2079** Origin : Caspian Sea Conc. : ≤ 60 μg g^{-1} (dry wt) Quantity : 5 mg Purity : 98% (by GC) Date collected : 2 / 6 / 92 Characterisation techniques, O_3, epoxidation and ^1H & ^{13}C NMR	**IX** 
HBI : $C_{25:5}$ GC RI(DBI) : **2201** Origin : *Haslea ostrearia* Conc. : 22 μg g^{-1} (dry wt) Quantity : < 1.0 mg Purity : 98% (by GC) Date collected : 18 / 10 / 88 Characterisation techniques, Epoxidation and ^1H NMR	**XIII** 
HBI : $C_{25:6}$ GC RI(DBI) : **2248** Origin : *Haslea ostrearia* Conc. : 77 μg g^{-1} (dry wt) Quantity : 6.0 mg Purity : 99% (by GC) Date collected : 18 / 10 / 88 Characterisation techniques, Epoxidation and ^1H & ^{13}C NMR	**XIV** 

Table 3.7 cont.

HBI : $C_{25:3}$ GC RI(DB1) : **2106** Origin : *Haslea ostrearia* (growth phase expt) Conc. per cell : ≤ 1.92 pg Conc. (bulk) : 210 µg g^{-1} (dry wt) Quantity : 6.1 mg Purity : 98% (by GC) Date collected : 10 / 5 / 95 Characterisation techniques, Epoxidation and ^{1}H & ^{13}C NMR	**X** 
HBI : $C_{25:4}$ GC RI(DB1) : **2144** Origin : *Haslea ostrearia* (growth phase expt) Conc. per cell : ≤ 3.66 pg Conc. (bulk) : 460 µmg g^{-1} (dry wt) Quantity : 18.9 mg Purity : 98% (by GC) Date collected : 10 / 5 / 95 Characterisation techniques, Epoxidation and ^{1}H & ^{13}C NMR	**XI** 
HBI : $C_{25:5}$ GC RI(DB1) : **2191** Origin : *Haslea ostrearia* (growth phase expt) Conc. per cell : ≤ 0.83 pg Conc. (bulk) : 92 µg g^{-1} (dry wt) Quantity : 2.8 mg Purity : 89% (by GC) Date collected : 10 / 5 / 95 Characterisation techniques, Epoxidation and ^{1}H & ^{13}C NMR	**XII** 

CHAPTER FOUR

Growth phase experiment : *Haslea ostrearia*

This chapter reports preliminary findings of a growth phase experiment conducted with Haslea ostrearia. The relationship of highly branched isoprenoid alkene production to the growth phase of the diatoms and to other parameters (e.g. carotenoids and n-$C_{21:6}$) is examined.

4.1 Introduction

The Class Bacillariophyceae, better known as diatoms, are unicellular algae, which have an opaline silica cell wall, and which contain chrysophyte-like photosynthetic pigments. Most diatoms are autotrophic and form the basis of food chains in many aqueous ecosystems. Different species occupy benthic and planktonic niches in lakes, lagoons, seas and oceans.

Pennate diatoms dominate the freshwater and epiphytic niches, but they also thrive in benthic marine habitats. Diatoms require light and are therefore limited to the photic zone of the water column (<200 m), but some epipelic species are motile and spend periods at depth in dark sediments. Each species tends to have distinct requirements for temperature, salinity, acidity, oxygen and mineral concentrations. Seasonal fluxes in these factors at high latitudes lead to spring and late summer blooms, especially amongst the planktonic forms, where diatoms may number as much as 10^9 cells per m^3 of water (Werner, 1977). Diatoms are abundant in regions of oceanic upwelling caused by current divergences, as in those of the Antarctic divergence. These waters are favoured because of their high silica, phosphate, nitrate, and iron contents. Cool waters are more dense and, together with the ascending currents, they pose the minimum of buoyancy problems for these non-motile organisms.

4.2 Genus *Haslea*

Haslea is a new genus of the family Naviculaceae. The cells have a thin membrane, spindle-shaped outline with acute ends and convex sides, rarely parallel in the

middle. The raphe are straight, with small, approximate central pores and little developed apical pores. The genus *Haslea* is interpreted as a link between *Navicula* and the genera *Gyrosigma / Pleurosigma*. Previous taxonomy studies had assigned the species within this group to numerous other genera (*Navicula, Amphipleura, Berkeleya, Gyrosigma, Pleurosigma, Schizonema, Stauroneis, Vibrio*), and it was not until 1974 that this group was separated, mainly from *Navicula*, as a genus of its own (Simonsen, 1974). These include: *ostrearia, frauenfeldii, trompii, crystallina, vitrea, kjelmanii, crucigera, sulcata, crucigeroides, gigantea, hyalinissima, wawrikae*. However, it was a further ten years before this new taxonomy was to be commonly applied (Robert, 1984). The numerous re-classifications of *Haslea ostrearia* pose considerable problems for studies of exact taxonomic history, and subsequent global distribution. Thus prior to 1984, *Haslea ostrearia* may have been classified as any of the species listed in Table 4.1, which to the author's knowledge is the most detailed listing to date.

Table 4.1 Taxonomic evolution of *Haslea ostrearia*

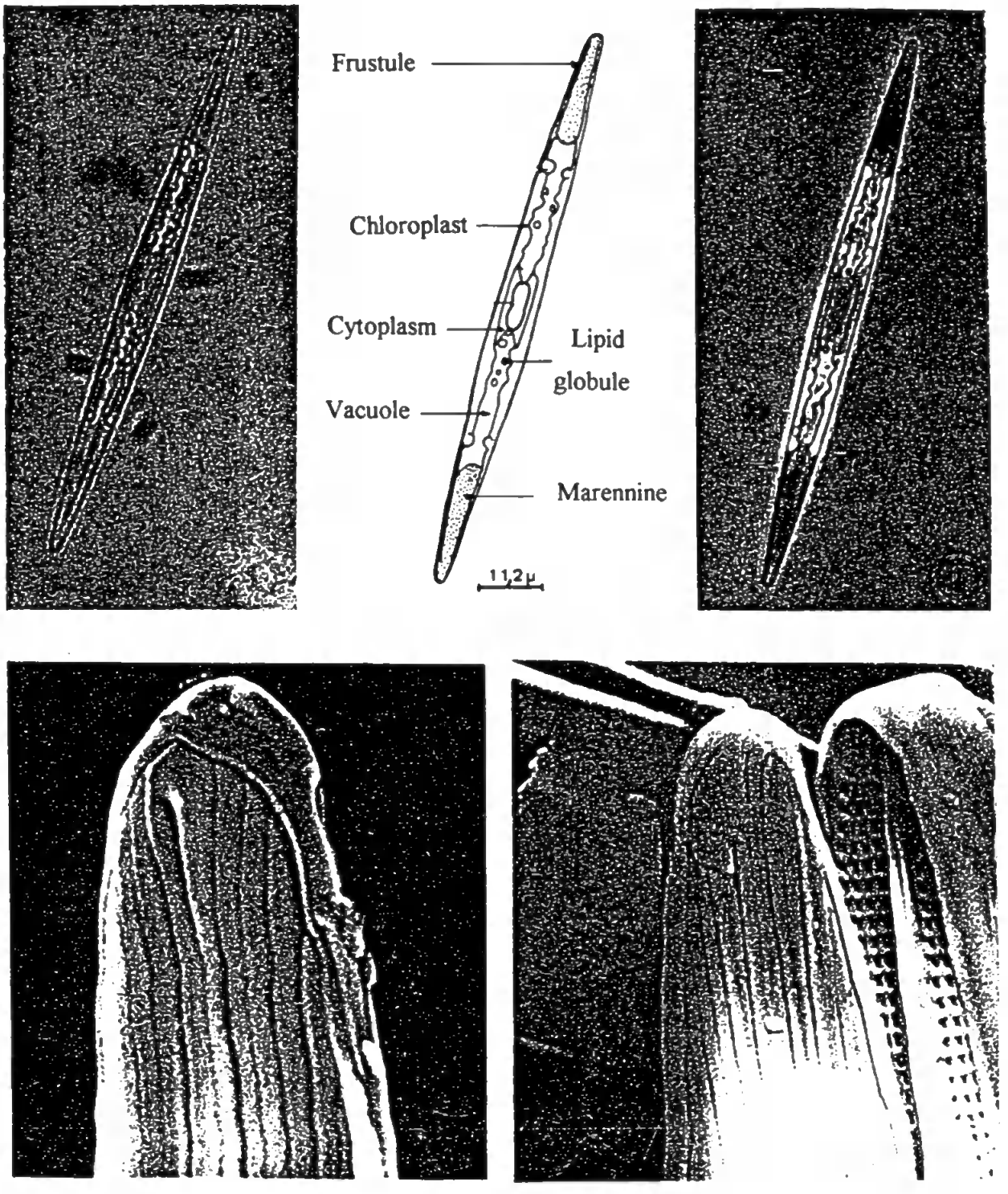
Genus	**Species**	**Author & Year**	
Vibrio	*ostrearius*	Gaillon	1820
Navicula	*ostrearia*	Bory	1827
Berkeleya	*fusiformis*	Grunow	1867
Navicula	*fusiformis*	Grunow	1877
Navicula	*fusiformes*	Cleve	1884
Navicula	*fusiformes ostrearia*	Patrick	1959
Navicula	*ostrearia*	Neville	1971
Haslea	***ostrearia***	**Simonsen**	**1974**

4.2.1 *Haslea ostrearia* : biology, morphology and identification

The morphology of the species *ostrearia*, which is of interest in the current study because of the production of HBIs (see chapters 1 and 2), is described as valves lanceolate, spindle-shaped, with acute to roundish ends, ~60-100 μm long, 6-10 μm wide, straight raphe and approximate central pores (Figure 4.1). The cell walls are extremely delicate and the internal striations (36 transapical striae in 10 μm) are therefore hardly discernible by transmitted light microscopy. For diatoms of this morphology it is the number of internal striations present (per 10 μm) that indicate the correct genus and species, and an electron microscopic examination is therefore required to reveal the fine detail.

Haslea ostrearia is well known in parts of France where it is fed to oysters to produce green colouration of the gills. This is due to the synthesis by *Haslea ostrearia* of marennine, a blue green pigment of unidentified chemical structure. Although *Haslea ostrearia* is reported as a common diatom along the coastal areas of France (*e.g.* Neuville and Daste, 1978; Robert, 1986), elsewhere its record of distribution appears to be limited to only three reports: the Indian Ocean (Simonsen, 1974), a north Australian coastal region (Ricard, 1987a) and one coastal location in southern England, (Hustedt and Aleem, 1951). This suggests that *Haslea ostrearia* has either a restricted distribution or that it has been incorrectly identified / classified in previous studies. Some tentative evidence exists to support the latter suggestion. Studies of microbial assemblages in coastal areas and oceans are usually based on examination of samples with a light microscope, with only a few, if any, examinations by electron microscopy. This is insufficient for correct classification of *Haslea ostrearia*. Moreover, an examination of

Figure 4.1 *Haslea ostrearia* (after Robert, 1983)



Top left	Transmitted light microscopic photograph, plane polarized
Top middle	Internal morphology
Top right	Transmitted light microscopic photograph, cross polarised
Bottom left	Electron micrograph showing external frustule (×12000)
Bottom right	Electron micrograph revealing internal frustule and striae (×20000)

literature reports of the diatoms found in areas of high HBI concentrations reveals that many classifications were only to genus level (*e.g. Navicula* sp.) and these localities often contained genera that were morphologically similar to *Haslea ostrearia*, (*e.g.* McMurdo Sound, Grossi *et al.*, 1984; North Sea, Reid *et al.*, 1990; Ebro Delta, Mir *et al.*, 1991). In other words, *Haslea ostrearia* may be much more widely distributed in recent environments than recognised heretofore. Also, because of the thin silica frustule, *Haslea ostrearia* does not survive morphologically in sediments; *Haslea ostrearia* may therefore even have been widespread in the geological past, as testified by occurrence of HBIs in Eocene (Yon *et al.*, 1982) and Oligocene (ten Haven *et al.*, 1993) oils and sediments.

4.3 Growth phase experiment : *Haslea ostrearia* and HBI concentrations

4.3.1 Introduction

Growth phase experiments are commonly employed by biologists to replicate the different environmental conditions experienced by algae, in a controlled laboratory environment. Initially these experiments are usually conducted under optimum growth conditions to establish a set of control parameters, and then under more stressed conditions (*e.g.* with variations in temperature, salinity and nutrient concentrations). This provides essential information on how the parameter concentrations may vary from optimum conditions. The parameters are usually recorded at set intervals, from the beginning of growth (stationary phase), through maximum growth (exponential phase), to the end of growth (lag phase).

Thus growth phase experiments might be expected to prove to be a powerful tool in aiding understanding of HBI production by *Haslea ostrearia*. Indeed without such data

it is possibly premature to advocate the use of HBIs as biomarkers (Kohnen *et al.*, 1992; Rowland and Robson, 1990). The degree of unsaturation, position and geometries of double bonds in HBIs may be controlled by factors such as temperature and salinity during biosynthesis. As a well known organic geochemical analogy, the number of double bonds in the so-called long chain alkenones, as reflected by the index U^{K}_{37}, is controlled by temperature (Marlowe *et al.*, 1984). These, and other possibilities can be examined by monitoring HBI production by *Haslea ostrearia* under different stressed conditions.

The growth phase experiment presented herein is the first to date to have involved measurement of HBIs, *n*-$C_{21:6}$, and carotenoid concentrations, along with conventional parameters (*e.g.* biomass, temperature, salinity and total lipids). Measurements were made every 24 hours for six days. From this experiment it is possible to draw some preliminary conclusions about the dependence of HBIs on the growth phase and to correlate HBI production with other parameters, such carotenoids and *n*-$C_{21:6}$. However, the conclusions must remain tentative, as the experiment was concluded before the culture had reached the 'lag' phase of growth and further replicate experiments are necessary.

4.3.2 Growth conditions and results

The parameters used for growth are listed in Table 4.2. The experiment was conducted at 20-26°C, pH 7.5-8.6, constant salinity (32 parts per ml), under adequate nutrient conditions. The concentrations of HBIs and *n*-$C_{21:6}$ determined are given in Table 4.3. A total ion chromatogram (TIC) of the THE of *Haslea ostrearia* (day 6) is illustrated in Figure 4.2. The THE is dominated by three HBI alkenes, $C_{25:3}$ (GC RI 2106), $C_{25:4}$

Figure 4.2 GC-MS TIC of total hexane extract of Haslea ostrearia, growth phase experiment day 6. GC conditions: OV-1, 40-260°C at 10°Cmin^{-1}

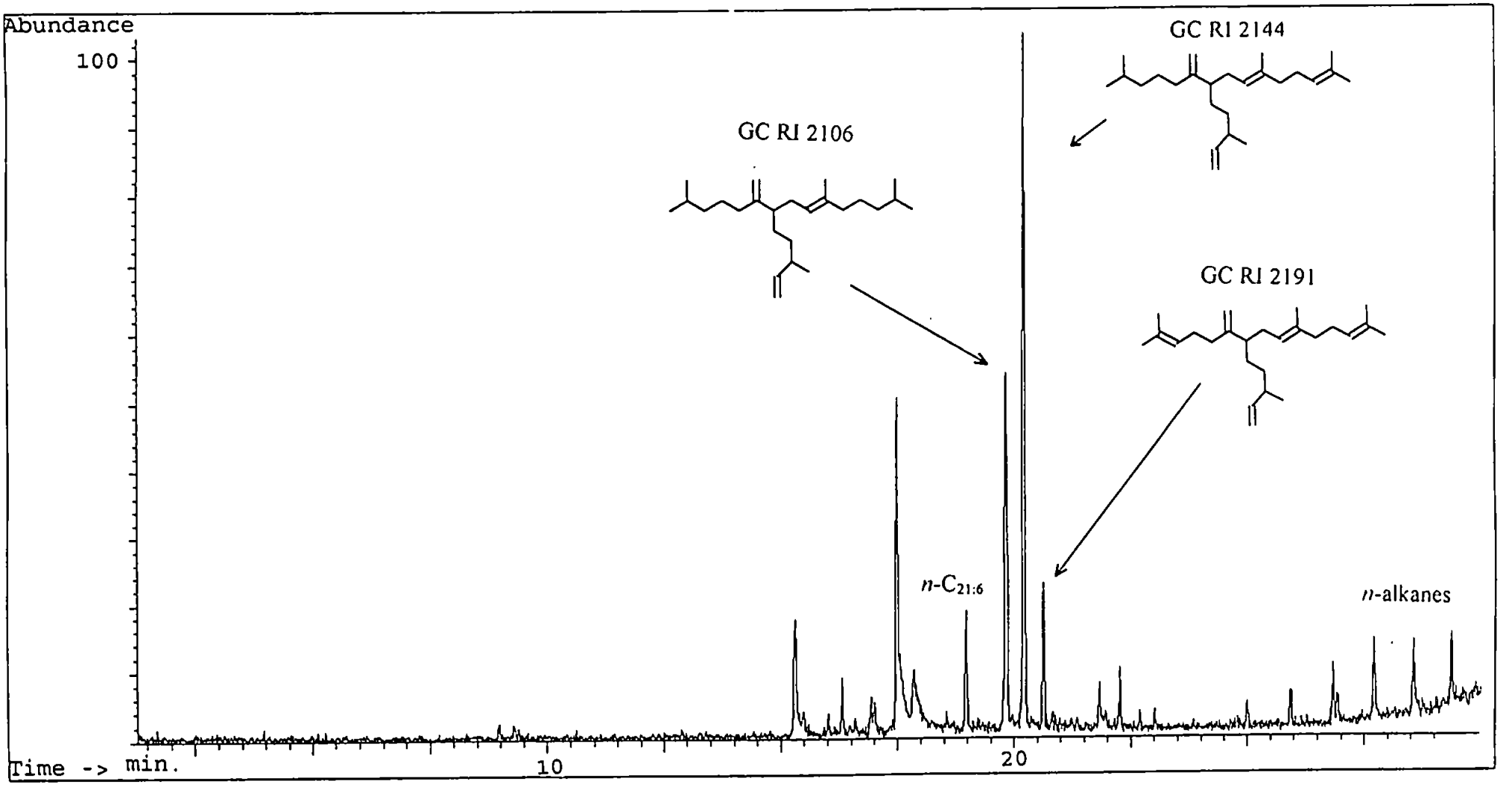


Table 4.2 Biochemical parameters recorded for growth phase experiment

Day	Cell number	Temperature	pH	Lipids mg.10^{-6}cell	Proteins mg.10^{-6}cell	Glucide mg.10^{-6}cell
0	3320	20	7.5	0.36	0.57	0.36
1	4260	21	7.6	0.61	0.47	0.35
2	9260	23	7.8	0.14	0.39	0.18
3	22400	26	8.1	0.09	0.32	0.08
4	43822	25	8.4	0.09	0.26	0.12
5	58933	20	8.6	0.06	0.23	0.04
6	76000	21	8.5	0.07	0.19	0.03

Day	Silica mg.10^{-6}cell	Chlorophyll a µg.10^{-6}cell	Carotenoids µg.10^{-6}cell	Phosphates µg.10^{-6}cell	Nitrates µM.10^{-6}cell	Nitrites µM.10^{-6}cell
0	36.2	5.3	2.2	1.7	18.4	0.051
1	11.8	9.2	0.8	3.4	13.2	0.035
2	17.4	5.2	3.7	0.6	7.7	0
3	3.2	5.1	3.9	0.2	2.1	0.014
4	2.6	7.8	5.2	0.1	0.7	0.006
5	1.7	6.4	6.5	0.05	0.3	0.007
6	1.2	6.5	4.1	0.004	0.07	0.004

Table 4.3 Concentrations of HBIs and *n*-$C_{21:6}$ recorded for growth phase experiment (fg.cell^{-1})

Day	$C_{25:3}$ GC RI 2106	$C_{25:4}$ GC RI 2144	$C_{25:5}$ GC RI 2191	*n*-$C_{21:6}$
0	1060	510	210	0
1	779	408	89	0
2	427	728	153	101
3	627	1736	414	367
4	1920	3666	834	854
5	722	1544	300	298
6	290	822	164	102

(GC RI 2144) and $C_{25:5}$ (GC RI 2191), which accounts for 50% of the hexane extract and forms a significant contribution to the total cellular lipid content, ranging between 7 and 22%. The total hydrocarbon content (day 6) is 4.0 pg per cell, which accounts for 17.8% of the total lipid content. This value is unusually high compared to those reported for other diatoms. The average, of the total hydrocarbon to total lipid content for twelve diatom species was 1.4%, with no values higher than 2%, (Dunstan *et al.*, 1994). The lipids present in *Haslea ostrearia* are visible during transmitted light microscopy and occur as discrete globules in the central vacuole, as illustrated in Figure 4.1.

4.3.3 Discussion

HBIs are indeed produced by *Haslea ostrearia* under these conditions confirming the single report of Volkman *et al.*, (1994). The HBI composition comprised a triene, tetraene and pentaene all of which have now been fully identified (Structures **X**, **XI**, **XII**; see section 3.4). From Figure 4.3 it is clear that HBIs are produced from the earliest stages of growth and the maximum concentrations occur 'early' in the exponential growth phase. The concentrations of the HBI alkenes all maximised together at day 4. After some initial fluctuations at day 0, 1, between days 2 - 6, HBI concentrations in relation to one another remained relatively constant, with the tetraene almost three times as abundant as the triene, which was almost twice as abundant as the pentaene. Independence of growth phase may be an important prerequisite if alkene ratios are to be used for palaeoenvironmental reconstruction. The absolute concentrations were similar to those reported by Volkman *et al.*, (1994), except for the tetraene, which was significantly more abundant after 13 days (Table 4.4).

Figure 4.3 Concentration of HBIs produced by *Haslea ostrearia* ($C_{25:3}$ GC RI 2106, $C_{25:4}$ GC RI 2144 and $C_{25:5}$ GC RI 2191)

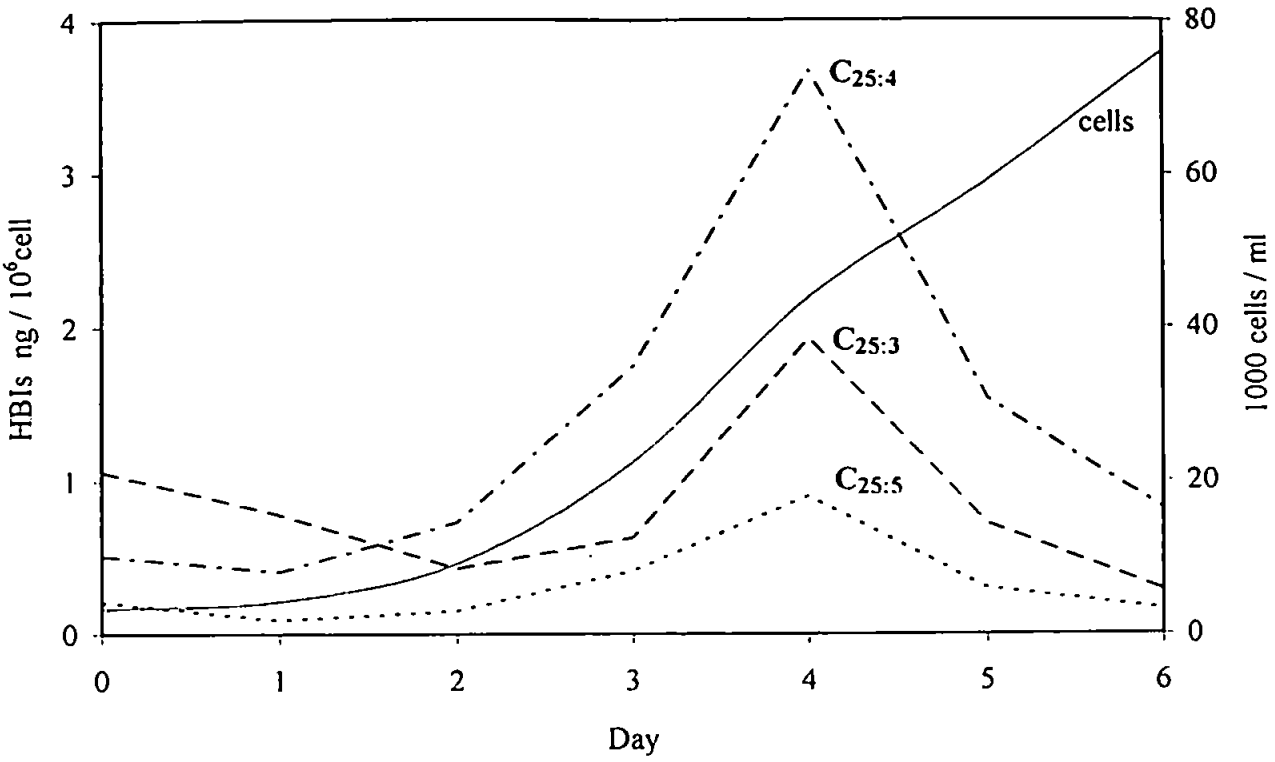


Figure 4.4 Concentration of HBIs, *n*-$C_{21:6}$ and carotenoids produced by *Haslea ostrearia*

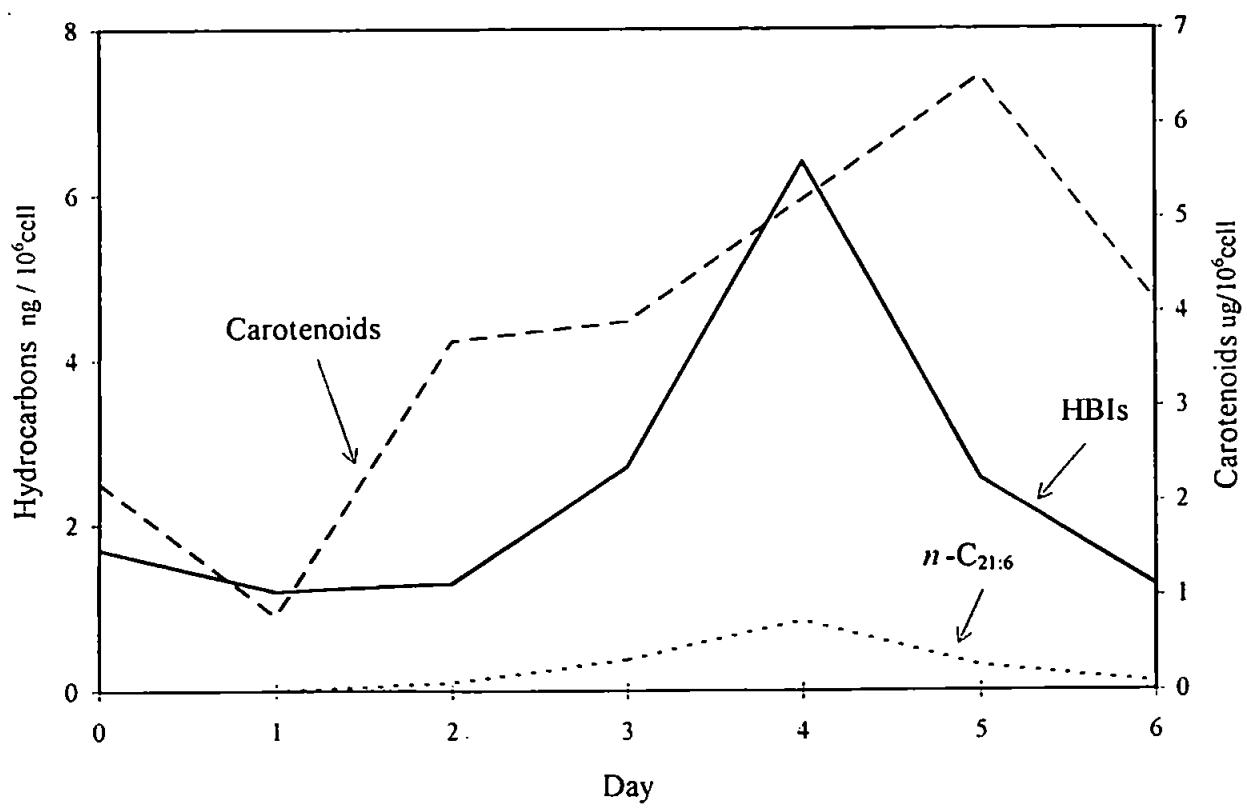


Table 4.4 HBI concentrations reported in *Haslea ostrearia*

HBI alkene	13 days (fg/cell) (Volkman *et al.*, 1994)	6 days (fg/cell) (This study)
$C_{25:3}$ (GC RI 2106)	189	290
$C_{25:4}$ (GC RI 2144)	2340	822
$C_{25:5}$ (GC RI 2191)	77	164

The relationship between the concentrations of HBIs, carotenoids (mainly fucoxanthin; see section 2.1) and *n*-$C_{21:6}$ (a common diatom hydrocarbon; reviewed by Hird and Rowland, 1995) is shown in Figure 4.4. It is interesting to note that both the HBI and *n*-$C_{21:6}$ concentrations maximise on day 4, earlier in the growth phase than the carotenoids. This may have important implications in relation to the environmental setting of HBIs, tentatively suggesting that HBIs would reach maximum concentrations before the main diatom biochemicals (commonly measured by fucoxanthin, Barlow *et al.*, 1993) as proposed in section 2.2.3, and perhaps offering a unique measure of *Haslea ostrearia* contributions to diatomaceous inputs to sediments.

Growth phase experiments may yet prove useful in establishing HBIs as a quantitative biomarker for *Haslea ostrearia*. Reproducible growth phase experiments, simulating different marine environments, would give a relatively accurate measure of the concentrations of HBIs produced per cell. From the concentration of HBIs in sediments (or the water column) it would then be possible to calculate the actual number of cells

required to produce the concentration of HBIs recorded. This would be a measure of the total number of cells that contributed to that sample.

Furthermore, this could be differentiated into HBIs from the number of living *Haslea ostrearia* in the surface sediments and those with a longer history. Evidence presented in section 6.3 suggests that one double bond rearranges in HBI alkenes soon after the death of the cells. Should this be the case, then by measuring the concentration of the 'primary' HBIs (produced by living diatoms) a direct value could be calculated for the number of living cells. This hypothesis could have important chemotaxonomic implications in the future, but a substantial amount of research, (based on growth phase experiments) would first have to be conducted.

CHAPTER FIVE

Experimental Details

This chapter describes the analytical procedures used in this study.

5.1 General Procedures

Glassware was cleaned in Decon-90, rinsed in distilled / Millipore-grade water, oven dried (150°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 concentrates (100 ml to 10 µl under vacuum). Blank requirements were as follows: on-column injections of 0.5 µl should result in chromatograms with peaks representing less than 0.2 ng in terms of their FID response. This threshold, under the above dilution factor, is equivalent to a level of artifacts below 0.25 ng g^{-1} when referenced to 50 g of dry sediment.

Silica gel (BDH; 60-120) and alumina (BDH; Grade 1; neutral) were used as adsorbents in column chromatography and were extracted with dichloromethane in a Soxhlet apparatus for 24 hours. After solvent evaporation, the silica and alumina were activated by heating to 180°C and 350°C respectively, (24 hours). Deactivated silica gel and alumina were prepared by shaking (4 hours) the absorbent with the appropriate quantity of Millipore grade water and stored (50°C and 120°C; 12 hours). Thin layer chromatography (TLC) plates were prepared on solvent-washed 20 × 20 cm or 20 × 10 cm glass plates with a coating of 0.25 mm silica gel (Merck Kiesel gel type 60G). Argentation TLC plates were prepared from slurries of silica gel made up in an aqueous solution of 10, 5, or 2% (wet wt) $AgNO_3$. Following drying (120°C; 1 hour) all plates were predeveloped in ethyl acetate and used after activation (120°C; 6 hours).

Anhydrous sodium sulphate (anhydrous Na_2SO_4), cotton wool, water (Millipore grade H_2O), hydrochloric acid (HCl), aqueous solutions of sodium chloride (NaCl; brine), potassium chloride (KCl), glacial ethanoic acid and activated copper were all extracted with dichloromethane before use to remove possible contaminants.

Activated copper, for the removal of elemental sulphur, was prepared according to the method of Blumer (1957). Copper sulphate (Aldrich) (~ 45 g) was placed in a beaker (500 ml) containing ice-cold deionised water and hydrochloric acid (2M; 25 ml). In another beaker (1 L) a thick slurry of powdered zinc (Aldrich; 15 g) in 25 ml deionised water was prepared. To aid the wetting of the zinc powder, acetone was added as a 'wetting agent'. The copper solution was then added to the rapidly stirred zinc slurry. Stirring was continued until effervescence ceased and the colour of the copper turned from a bright red to a dark red-brown. The supernatant was decanted, allowing the finer particles to be removed. The copper was washed with deionised water repeatedly, until all traces of dark particulate matter were removed. Any excess copper not used immediately was covered in ice and stored in a freezer. The activated copper was packed into columns of various dimensions and water removed by washings with acetone. After several washings the solvent was changed to hexane prior to use.

5.2 Extraction and fractionation of hydrocarbons from sediment samples

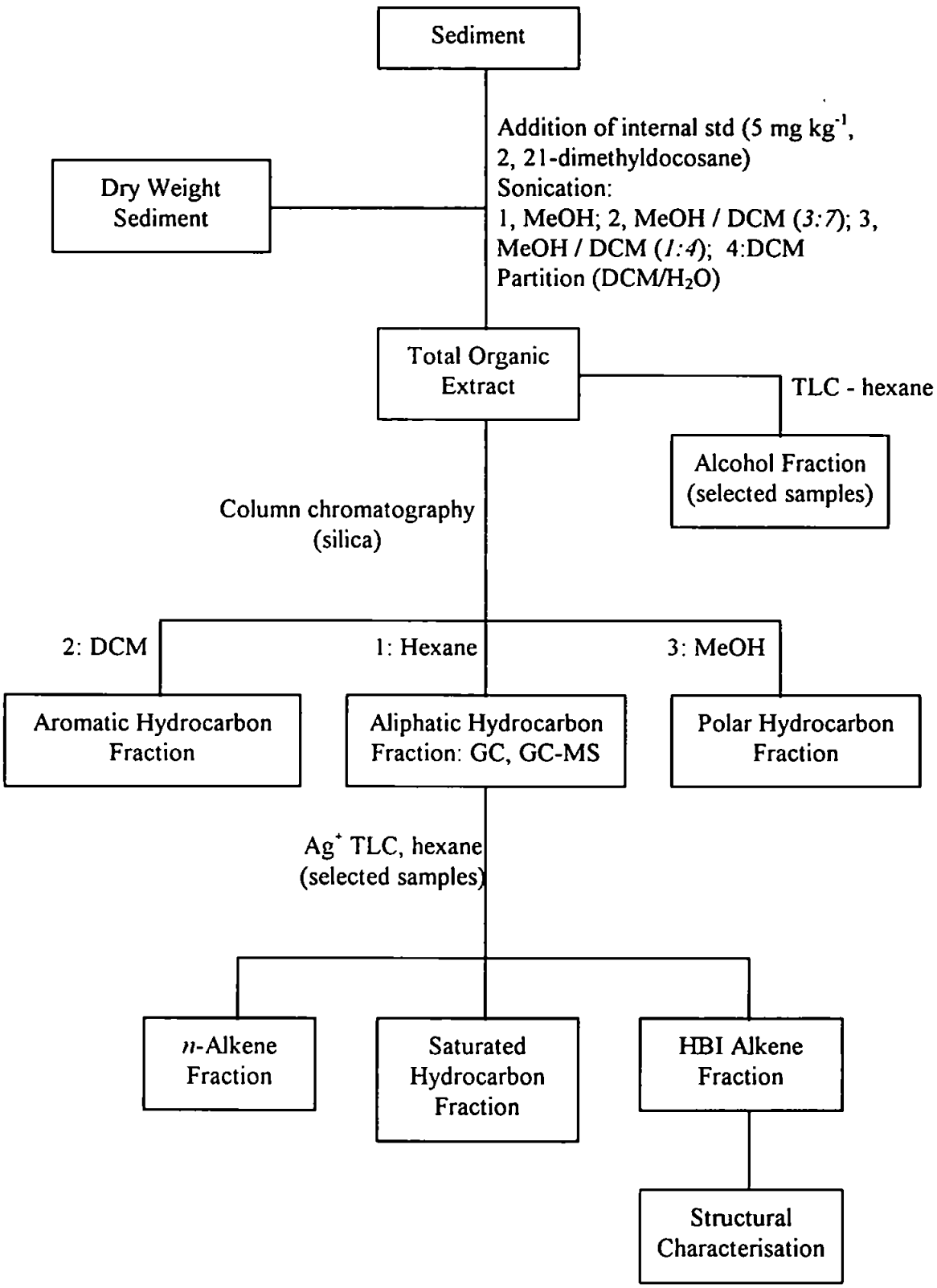
5.2.1 Sample collection and solvent extraction

The general protocol used for the isolation of hydrocarbons from sediment samples is illustrated in Figure 5.1.

For the Tamar sediments study (Chp 2) all the samples were collected at St. Johns Lake (Figure 2.1). The sediment was collected randomly (random numbers generated by Casio FX85 calculator) from within the same 20 x 20 m quadrant at each visit. The surface sediment, 0 - 5 mm depth, was gathered by metal spatula, transferred to clean aluminium cans and frozen immediately.

The thawed samples were solvent extracted using the method of Douglas *et al.*, (1981). Sediment (~40g wet weight) was extracted with methanol (40 ml) by ultrasonication (10 min.; ultrasonic bath; Kerry Pulsatron HB172). The organic extract was separated by centrifugation (15 min.; 2200 r.p.m.) and decanted. This procedure was repeated using dichloromethane / methanol (7:3 v/v; 40 ml), dichloromethane / methanol (4:1 v/v; 40 ml) and dichloromethane (40 ml). The combined extracts were shaken (separating funnel) with water (Millipore grade; 30 ml) and the lower organic layer collected, along with the dichloromethane washings (3 x 15 ml) of the aqueous layer. Solvent was removed (Buchi; 30°C) and the total organic extract transferred to a vial and weighed. If water was still present after solvent removal, dichloromethane (20 ml) was added and the mixture transferred to a small separating funnel, where the lower organic layer was carefully removed, reconcentrated, and weighed.

Figure 5.1 Isolation of aliphatic hydrocarbons from Tamar sediments



5.2.2 Fractionation of total organic extract (TOE)

The extract was pre-absorbed onto alumina (TOE, dissolved in DCM, was quantitatively transferred to a vial containing ~200 mg of alumina and solvent removed under a stream of nitrogen) and applied to a short column (20 cm x 1.0 cm i.d.) containing silica over activated copper powder (0.2 - 0.5g w/w). The column was eluted with 60 ml of hexane and the 'aliphatic' fraction collected in a round bottomed flask. The column was subsequently eluted with 60 ml of dichloromethane for the 'aromatic' fraction and finally 60 ml of methanol for the more 'polar' compounds. After removal of the solvent the extracts were weighed and stored at 4°C prior to analysis by GC and GC-MS.

5.3 Diatom cultures: *Haslea ostrearia*

5.3.1 Extraction of 'bulk' cultures of *Haslea ostrearia*

Frozen samples of bulk cultures of *Haslea ostrearia*, were provided by the Universite de Nantes. Algal paste (between 20 and 50 g wet wt.) was allowed to thaw and was then extracted with hexane (200 ml) by ultrasonication (20 min.; ultrasonic bath; Kerry Pulsatron HB172). The hexane extract was separated by centrifugation (20 min.; 2600 r.p.m.) and decanted. The extraction of the algae was repeated until no hexane soluble hydrocarbons remained (checked by GC) to yield a total hexane extract (THE). Solvent was removed (Buchi; 30°C), the extract dried (anhydrous Na_2SO_4) and the THE transferred quantitatively to a vial and weighed when dry. Preliminary experiments indicated that extraction with methanol / dichloromethane co-extracted high

concentrations of pigments, which caused significant chromatographic problems; thus extraction with hexane only, was adopted.

5.3.2 Microscale extraction of filter samples

Frozen filter samples of *Haslea ostrearia*, collected as part of a growth phase experiment (chapter 4), were provided by the Universite de Nantes. The thawed filter samples were extracted in 2 ml of hexane by ultrasonication (40 min.; ultrasonic bath; Kerry Pulsatron HB172). The hexane extract was then passed through a Pasteur pipette containing a bed of anhydrous Na_2SO_4 (~0.5 g) and collected in a 10 ml vial. The extraction of the filter was repeated once and the extracts combined in the 10 ml vial. The solvent was removed under a stream of nitrogen and the 'dried' extract then redissolved in 20 µl of DCM for analysis by GC-MS. Extraction efficiency was calculated by comparison with the response of an internal standard (2, 21-dimethyldocosane, 5 mg kg^{-1}) added prior to extraction and compared to a co-injected standard of known concentration (see section 5.6.1).

5.4 Isolation of HBI alkenes

5.4.1 Thin-Layer Chromatography (TLC)

A HBI pentaene $C_{25:5}$ (GC RI 2201) and a hexaene $C_{25:6}$ (GC RI 2248) were isolated from the THE (141 mg) of 48 g of *Haslea ostrearia* by TLC (20 x 20 cm plate, 0.25 mm silica, with hexane as the mobile phase). The plate was visualised (UV light, 365nm; 0.5% Rhodamine 6G in methanol) and two bands corresponding to R_f values

between 0.25 & 0.4 ($C_{25:6}$ GC RI 2248) and 0.4 & 0.55 ($C_{25:5}$ GC RI 2201) were removed. The alkenes were recovered from the silica gel by desorption with hexane / dichloromethane (60:40 v/v; ~5 ml) using a Pasteur pipette containing a bed of alumina and the eluates collected in vials. After removal of the solvent the extracts were weighed and stored at -18°C.

Alkene	R_f values	Amount	Purity (GC)
$C_{25:5}$	0.40 - 0.55	< 1.0 mg	>92%
$C_{25:6}$	0.25 - 0.40	~ 6.0 mg	>96%

Each fraction was analysed by GC and GC-MS to confirm the identity and purity of HBI alkenes isolated. These were then characterised *via* NMR and derivatisation techniques.

5.4.2 Column chromatography

A mixture of HBI polyenes, dienes to pentaenes, ($C_{25:2}$ GC RI 2088, $C_{25:3}$ GC RI 2106, $C_{25:4}$ GC RI 2088 and $C_{25:5}$ GC RI 2191) were separated, from the THE of 37 g of *Haslea ostrearia*, into individual isolates (purity up to 98%; checked by GC and GC-MS) using column chromatography. The hexane extract (197 mg) was adsorbed onto alumina (1 g) and applied to a column (20 cm x 1.0 cm i.d.) containing activated silica and eluted with hexane. The eluant was collected in 2 ml vials, for the first 60 ml, and 10 ml vials until all fractions had been collected. The separation of the alkenes was monitored

throughout the procedure by GC & GC-MS and respective fractions combined. The elution order of fractions was as follows:

n-alkanes	0 - 30 ml	hexane		
HBI diene	30 - 60 ml	hexane	>95% purity	< 0.1 mg
HBI triene	60 - 150 ml	hexane	>98% purity	5.1 mg
HBI tetraene	150 - 310 ml	hexane	>98% purity	18.9 mg
HBI pentaene	310 - 460 ml	DCM	~54% purity	2.8 mg

After removal of solvent the extracts were weighed and stored at -18°C. Each fraction was analysed by GC and GC-MS to confirm the identity of HBI alkenes isolated in each fraction and those of sufficient purity and quantity were further characterised *via* NMR and derivatisation techniques.

5.5 Pigment samples : collection and analysis

Surface sediment (0 - 5 mm depth) samples collected from St. Johns Lake were stored at the collection site in aluminum containers (packed in solid CO_2) until arrival at the laboratory where they were frozen in liquid N_2. Frozen sediments (~ 3 g) were extracted in 1 ml 90% acetone / 10% methanol using ultrasonication (5 min. Soniprep 150-probe) and the organic extract separated by centrifugation (10 min. at 2200 r.p.m.) and decanted (extraction repeated x 10). An aliquot (300 µl) of clarified extract was mixed with 300 µl of 1M ammonium acetate and 100 µl injected into a Shimadzu HPLC system (dual LC-6A pumps, SPD-6AV spectrophotometric detector, SCL-6B system

controller) incorporating a 3-μm Pecosphere column (3.5 x 0.45 cm; Perkin-Elmer). Pigments were separated by a modification of the reversed-phase method of Mantoura and Llewellyn, (1983): solvent A consisted of 80% methanol and 20% 1M ammonium acetate and solvent B contained 60% methanol and 40% acetone. A linear gradient from 0% B to 100% B for 10 min. followed by an isocratic hold at 100% B for 7.5 min. was used at a flow rate of 1 ml $min.^{-1}$. Chlorophylls and carotenoids were detected by absorbance at 440 nm, and detection of phaeopigments was performed with a Perkin-Elmer LS1 fluorescence detector using an excitation wavelength of 400 (± 20) nm and emission at >600 nm. Dual channel data collection and integration utilised the Philips PU6000 software on a Dell personal computer and the software calculated a concentration for each pigment.

5.6 Analysis

5.6.1 Gas Chromatography (GC)

Hydrocarbons were examined on a Carlo Erba Series 5300 Mega gas chromatograph fitted with fused silica columns (0.32 mm i.d.) of various length (25, 30 or 50 m x 0.32 i.d.) and phases (DB1 or DB5; J & W), using flame ionisation detection and on-column injection. The column oven was programmed from 40 - 300°C at 5°C $min.^{-1}$ and held at the final temperature for 10 minutes. Hydrogen was used as the carrier gas at a flow rate of 2 ml $min.^{-1}$ (set at 250°C). Some HBI isolates were also analysed using a 25 m DBWAX (J & W) column. The carrier gas was hydrogen and the oven temperature programmed from 40 - 270 at either 2.5 or 5°C $min.^{-1}$ and held at 270°C for 10 min.

Retention indices (GC RI) were calculated according to the following formula (Poole and Schuette, 1984) :

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

RI is retention index; t_R is retention time; z represents an n-alkane with z carbon atoms. A known alkane mixture was added to the hydrocarbons where appropriate.

Quantification of individual hydrocarbons was accomplished by measurements of GC peak area using a Shimadzu CR3-A recording integrator. These were then compared to the response of a co-injected standard of known concentration (*n*-alkane; usually C_{18}, C_{20} or C_{23}). An internal standard (2, 21-dimethyldocosane) was added prior to solvent extraction of sediments and algae to calculate the extraction efficiency (5 mg kg^{-1}).

5.6.2 Gas Chromatography- Mass Spectrometry (GC-MS)

Analysis of hydrocarbon extracts was performed on a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard Mass Selective Detector (MSD) 5970 Series.. A 12 m (0.2 mm i.d.) fused silica column coated with HP1 (Hewlett Packard) was introduced directly into the ion source of the mass spectrometer. Auto splitless injection and helium gas were used and the column oven programmed as for GC.

Mass spectrometer operating conditions were; ion source temperature 250°C and 70 eV ionisation energy. Spectra (40 - 550 Daltons) were collected using Chemstation software.

5.6.3 Compound identification

Where possible, individual hydrocarbons were identified by co-chromatography with authentic compounds on GC columns of different polarities and by comparison of gas chromatographic retention indices (GC RI) with literature data. Additional information was provided by GC-MS: the recognition of components from their mass spectra was made by comparison with the spectra of authentic compounds, published spectra or by spectral interpretation.

5.6.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^{1}H and ^{13}C NMR spectra of HBI isolates were recorded in $CDCl_3$ solutions, using a Jeol EX270 (270 MHz; University of Plymouth) high resolution FT-NMR spectrometer. Chemical shifts were measured (δ/ppm) using residual $CHCl_3$ in the solvent (δ= 7.25 and 77.0 for ^{1}H and ^{13}C respectively) as reference. Multiplicities for ^{1}H resonances are described as singlet (*s*), doublet (*d*), doublet of doublets (*dd*), doublet of doublet of doublets (*ddd*), triplet (*t*), doublet of triplets (*dt*), quartet (*q*) or multiplet (*m*). Multiplicities for ^{13}C resonances were achieved via the DEPT sequence. Short and long range heteronuclear ^{1}H - ^{13}C correlations were determined using HETCOR and COLOC methods.

5.6.5 Ultraviolet / visible (UV/vis) spectroscopy

UV/vis absorbance spectra of selected samples were recorded using a Perkin Elmer Lamba 7 UV/visible spectrophotometer. Both samples and solvent blanks were measured in acetone / methanol (90 : 10).

5.7 Derivatisation of HBI alkene isolates

5.7.1 Microscale hydrogenation

All HBI alkene isolates were hydrogenated using the following procedure. 100 μg of isolate, dissolved in 400 μl of DCM was added to a Reacti-vial (2 ml; Pierce) containing activated $PtO_2.H_2O$ (<1 mg) and hydrogen bubbled through the solution for 60 minutes. The resulting solution was passed through a Pasteur pipette with a cotton wool plug (to remove any $PtO_2.H_2O$) and analysed directly by GC and GC-MS.

5.7.2 Microscale ozonolysis

Ozonolysis was employed in the elucidation of the positions of double bonds in selected HBI alkenes. A 'Micro-Ozonizer" (Supelco Inc., U.S.A.; a modification of the design of Beroza and Bierl, 1969) was used to generate ozone. A sample of HBI alkene (~200 μg) dissolved in DCM (500 μl) was transferred to a Reacti-vial and sealed with a Teflon-lined cap. The reaction mixture was cooled to ~ -70°C (acetone / solid CO_2 bath) and an O_3 gas stream was passed through an inlet syringe and bubbled (~ 0.5 ml $min.^{-1}$) through the reaction mixture for 10 minutes. The solution was analysed by GC and GC-MS and possible positions of double bonds determined from *ab initio* interpretation of

mass spectra or by comparison with reference spectra (*e.g.* Hird, 1992). Prior to the analysis of HBI alkenes, the technique was validated using simple *n*-alkenes (tetradec-7-ene and heptdec-1-ene) which were successfully ozonolysed to *n*-1-heptanal and *n*-1-hexadecanal respectively.

5.7.3 Microscale Epoxidation

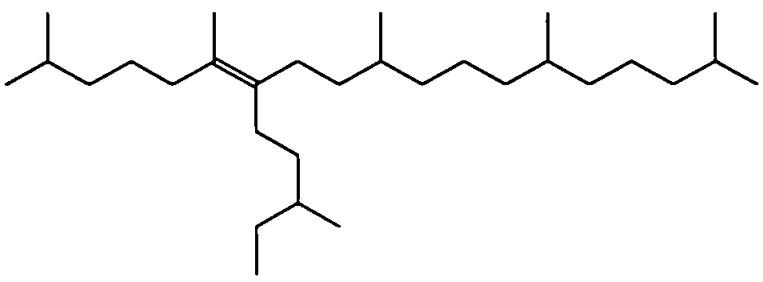
The epoxy derivatives of HBI alkenes were prepared by reaction with *m*-chloroperoxybenzoic acid (CPBA), (Aldrich Chemicals). All reactions were carried out in 2 ml Reacti-vials. The alkene isolate, dissolved in DCM, was quantitatively transferred to a Reacti-vial and solvent removed under a stream of nitrogen. The *m*-chloroperoxybenzoic acid, as a 0.16 or 0.32 molar (in DCM) solution was added in set volumes. The Reacti-vial was sealed with a Teflon-lined cap and placed in a water bath at various temperatures. The reaction was monitored by GC & GC-MS until completion.

The method was validated on three different *n*-alkenes (dodec-1-ene, octadec-1-ene and tetradec-7-ene: Aldrich Chemicals) and the resulting compounds compared (GC and GC-MS) to the respective epoxide derivatives, which were purchased from commercial sources (1,2 epoxydodecane, 1,2 epoxyoctadecane and 7,8 epoxytetradecane: Aldrich Chemicals). This method was further validated on a synthetic C_{30} HBI monoene of known structure (Robson, 1987). From these preliminary experiments, and those conducted on HBI compounds of previously unknown structure, it was found that exact reaction conditions were specific with respect to time, T°C and *m*-CPBA concentration (see sections 5.7.3.1 - 5.7.3.8).

5.7.3.1 Dodec-1-ene, octadec-1-ene and tetradec-7-ene

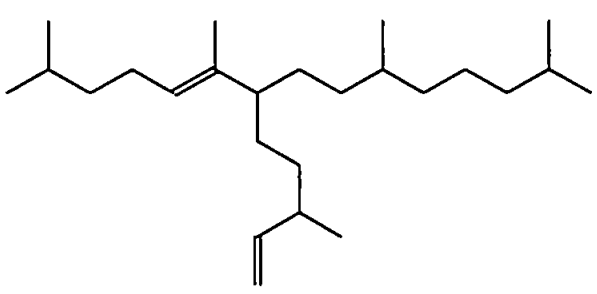
Reaction conditions:	Alkenes	$C_{12:1}$, $C_{14:1}$ and $C_{18:1}$ each 200 µg
	CPBA	0.16 molar, 300 µL
	Temp.	78 hours @ 25°C
	Yield	Each > 95%

5.7.3.2 Synthetic $C_{30:1}$ HBI (Robson, 1987; Hird, 1992)



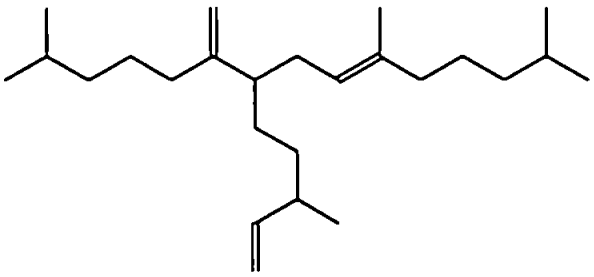
Reaction conditions:	HBI alkene	150 µg of $C_{30:1}$
	CPBA	0.16 molar, 300 µL
	Temp.	90 hours @ 25°C
	Yield	92%

5.7.3.3 $C_{25:2}$ (GC RI 2079) Caspian Sea



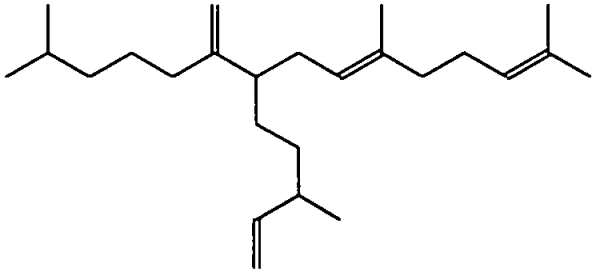
Reaction conditions:	HBI alkene	200 µg of $C_{25:2}$
	CPBA	0.16 molar, 200 µL
	Temp.	90 hours @ 25°C
	Yield	78%

5.7.3.4 $C_{25:3}$ (GC RI 2106) *Haslea ostrearia*



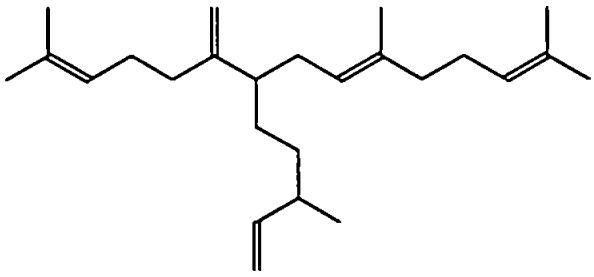
Reaction conditions:	HBI alkene	150 µg of $C_{25:3}$
	CPBA	0.16 molar, 200 µL
	Temp.	72 hours @ 50°C
	Yield	44%

5.7.3.5 $C_{25:4}$ (GC RI 2144) *Haslea ostrearia*



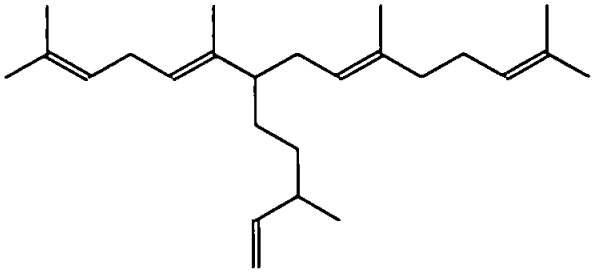
Reaction conditions:	HBI alkene	250 µg of $C_{25:4}$
	CPBA	0.32 molar, 300 µL
	Temp.	72 hours @ 55°C
	Yield	51%

5.7.3.6 $C_{25:5}$ (GC RI 2191) *Haslea ostrearia*



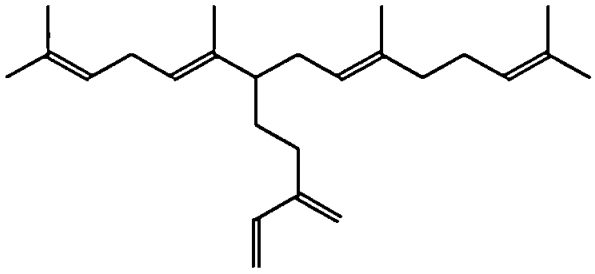
Reaction conditions:	HBI alkene	250 µg of $C_{25:5}$
	CPBA	0.32 molar, 300 µL
	Temp.	72 hours @ 55°C
	Yield	32%

5.7.3.7 $C_{25:5}$ (GC RI 2201) *Haslea ostrearia*



Reaction conditions:	HBI alkene	300 µg of $C_{25:5}$
	CPBA	0.32 molar, 300 µL
	Temp.	72 hours @ 60°C
	Yield	6.0%

5.7.3.8 $C_{25:6}$ (GC RI 2248) *Haslea ostrearia*



Reaction conditions:	HBI alkene	300 µg of $C_{25:6}$
	CPBA	0.32 molar, 300 µL
	Temp.	72 hours @ 60°C
	Yield	2.8%

CHAPTER SIX

Conclusions and future research

Two important conclusions which may have significant geochemical implications, can be drawn from the research presented within this thesis. Experimental procedures used during isolation indicate that a strong interaction exists between HBI alkenes and silica. For conventional chromatographic techniques, this interaction could result in HBIs, with three or more double bonds, being collected in the so-called 'aromatic' hydrocarbon fraction and not identified. A subsequent review of previous fractionation procedures suggests that an incomplete record exists for HBI alkenes in the environment.

The structural characterisation of HBI alkenes produced by Haslea ostrearia has enabled a direct correlation to sedimentary counterparts to be established. If, upon death of diatom cells, the C6-17 double bond positionally rearranges to C5-6, resulting in a mixture of E and Z isomers, then Haslea ostrearia may account for at least eight of the fourteen dominant HBI hydrocarbons in sediments. There is still much to be learned about HBI polyenes and some possible future approaches are suggested.

6.1 Introduction

Evidence has been presented for the important role of diatoms in controlling the sedimentary disributions of C_{25} HBI hydrocarbons.

The significant phase differences between HBI concentrations and those of the diatom biomarkers fucoxanthin and n-$C_{21:6}$ in Tamar estuary sediments throughout 1994, suggests that HBIs are either produced earlier in the growth cycle of some diatoms than fucoxanthin and n-$C_{21:6}$ or, more likely, that the principal diatom producers of HBIs are not the predominant diatom species in the sediments.

The confirmation of HBIs as the major hydrocarbons of the diatom, *Haslea ostrearia* (Volkman *et al.*, 1994) perhaps supports the latter view; whilst it is a common species in coastal environments, *Haslea ostrearia* has rarely been identified as a major component of the total diatom population in such environments. It is also worth noting that in the Tamar sediments the concentration of n-$C_{21:6}$ is approximately equal to the total HBI's, but laboratory cultures show that n-$C_{21:6}$ is found in significantly lower abundances than total HBI's, strengthening the argument for different dominant sources in Tamar sediments. The HBI concentrations in the Tamar estuary thus afford the promise of a better temporal resolution of different diatom species inputs to the sediments and may allow a better differentiation of the chemical inputs of sedimentary diatom populations generally.

The isolation of large enough quantities of individual HBI alkenes from laboratory cultures of *Haslea ostrearia* for NMR spectral analysis has allowed the structures of most of the HBIs to be firmly established. Novel and interesting though these detailed chemical

findings may be, it is fair to ask, what are the geochemical implications of the data ? As will be discussed below, the rigorous identification of HBI alkenes may have a profound influence on the environmental and geochemical conclusions which can be drawn from the sedimentary HBI distributions.

6.2 Implications for isolation and fractionation of HBI alkenes

To date there have been at least twenty five different reports of C_{25} HBI dienes in the environment and over thirty reports of trienes. This contrasts with only eleven reports of the tetraene and eight of pentaenes (Table 6.1). At face value, this suggests that sedimentary polyenes with four or more double bonds are less abundant in the environment. However, the data presented herein (Chapters 3 and 4), shows that the source organism *Haslea ostrearia* contains dienes to (at least) hexaenes. For instance, the pentaene (GC RI 2191) and triene (GC RI 2106) are both relatively abundant in *Haslea ostrearia* and appear to be biosynthesised at approximately equal rates, under the conditions investigated (Chapter 4). Why then should the triene be common in the environment, and the pentaene, rare or absent ? The data presented herein (see sections 5.4.1 and 5.4.2) suggest an explanation may be the unexpected chromatographic behaviour of the HBI alkenes on traditional adsorbents (*e.g.* silica). For example, silica column chromatographic separation of individual dienes, trienes, tetraenes and pentaenes from the total hexane extract of *Haslea ostrearia* (Chapter 5) showed that the diene was eluted with three column volumes of hexane, whereas the tetraene (structure **XI**) required fifteen column volumes. Similarly, the C_{25} HBI pentaene (GC RI 2201; structure **XIII**)

Table 6.1 Sedimentary occurrences of C_{25} HBI hydrocarbons where GC retention indices are cited (updated Table 1.1)

Stationary	DB1	OV1	OV1	DB1	DB1	HP1	HP1	SE30	SE30	SE30	SE30	SE30	DB5	DB5	DB5	DB5	DB5	DB5	SP2100	SP2100	SP2100	SP2100	SP2100	OV101	MS	MS
phase																										
Compound																										
C25:0 (II)		2112		2110				2111					2105						2109	2109					2112	
C25:1																					2106					
C25:1																									2112	
C25:2	**2069**		2067					2070	2072		2068		2068			2068	2070									
C25:2	**2079**	2082	2082		2079			2084		2082	2084	2084	2082	2082			2083									
C25:2																		2085								
C25:2	**2088**					2088						2088	2088		2088	2088										2088
C25:3	**2044**							2044	2044			2044			2046	2044	2044			2044						
C25:3	**2091**							2091	2092		2091	2091	2091			2091	2090		2090	2090	2094		2089	2092		
C25:3								2104				2104														
C25:3	**2106**						2106	2106		2107		2106			2110	2107	2107									
C25:3													2119													
C25:3																2156										
C25:4									2082							2079				2078						
C25:4																2086										
C25:4																						2055				
C25:4	**2128**								2129							2126	2128			2124						
C25:4							2134									2133										
C25:4	**2144**						2144																			
C25:4	**2175**																2175									
C25:5	**2128**															2125										
C25:5																2144										
C25:5									2170							2169										
C25:5							2173																			
C25:5									2183							2183	2182									
C25:5	**2191**						2191		2188																	
C25:5	**2201**						2200																			
C25:6	**2248**																									
References		*15*	*16*	*23*	*25*	*22*	*24*	*7*	*8*	*9*	*11*	*12*	*10*	*17*	*18*	*20*	*26*	*21*	*2*	*3*	*4*	*5*	*6*	*13*	*14*	*19*

Key : Table 1.1a (Bold text indicates C_{25} HBI alkenes reported in this thesis)

had a R_f value on silica gel TLC (see section 5.4.1) ranging from 0.40 to 0.55 (0.25 mm thickness; hexane mobile phase) whereas the hexaene (GC RI 2248; structure **XIV**) had a R_f value of 0.25 to 0.40. Thus the sorption behaviour of the polyunsaturated tetra, penta- and hexaenes to silica appears to be sufficiently different as to require careful elution with a range of solvents (column) or monitoring of different R_f values (TLC) for the quantitative recovery of all HBI alkenes.

This behaviour can be contrasted with 'normal' geochemical isolation procedures. For example, Venkatesan and Kaplan (1987) and Venkatesan (1988) reported the presence of mainly diene isomers and minor trienes in sediments and algae from Antarctica. The methodology used for fractionation of the total organic extracts in both cases was column chromatography (silica gel) and the TOE was separated into 'aliphatic' (eluted with hexane) and 'aromatic' hydrocarbons (eluted with hexane : benzene, 50/50). Considering the findings herein, it is likely that trienes, tetraenes and pentaenes, if they were present, were eluted in the 'aromatic' fraction and were not identified. Similarly, Nichols *et al.* (1988), reported the presence of a C_{25} diene in sea-ice diatom communities from Antarctica. The fractionation used column chromatography (silica) to obtain an 'aliphatic' fraction (eluted with hexane) and an 'aromatic' fraction (eluted with hexane : toluene, 50/50). The ratio of the volume of hexane eluant to silica gel was less than two column volumes (3 g of silica; 4 ml of hexane). With such a small relative volume of eluant, other polyenes, if present, would almost certainly have been collected in the 'aromatic' fraction.

Whilst it is, of course, possible that the more unsaturated compounds do not occur in the Antarctic, where the degree of unsaturation may be controlled by other factors, such as temperature or salinity, it is interesting that Cripps (1995) reported the occurrence of a suite of HBI polyenes (dienes to pentaenes) in the lipids of Antarctic marine organisms (fish and squid) when the total hydrocarbons were extracted from the digested animals with hexane and not fractionated further by solid phase chromatography.

The evidence suggests that an incomplete record exists for HBIs reported in sediments and any previous conclusions made from the global distribution patterns of C_{25} HBI alkenes would have to be treated with some care. Re-analysis of sediments and algae using appropriate fractionation procedures, calibrated with the known compounds whose structures have been established herein, is required.

6.3 Implications for occurrence and sources of C_{25} HBI alkenes

Even given the possible inferences of incomplete isolation procedures, of the twenty six apparently different C_{25} HBI alkenes reported in the marine environment, only fourteen are relatively abundant and frequently occurring (see section 1.2). The retention indices of major C_{25} HBI isomers occurring in sediments and those produced by *Haslea ostrearia* are shown in Table 6.2. It is interesting to note that four of the dominant alkenes produced by *Haslea ostrearia*, $C_{25:4}$ (GC RI 2144), $C_{25:5}$ (GC RI 2191 & GC RI 2201) and $C_{25:6}$ (GC RI 2248), are not detected in sediments at all and the triene (GC RI 2106) is not predominant in sediments. The fact that only one of the five HBIs produced by *Haslea ostrearia* correlates with sedimentary isomers might suggest either that; *Haslea ostrearia* is not the source of sedimentary C_{25} HBI alkenes; or that *Haslea*

Table 6.2 Occurrence of HBI alkenes in *Haslea ostrearia* and sediments (average GC RI for major sedimentary alkenes)

HBI isomer	*Haslea ostrearia*	Sedimentary (major alkenes)
C25:2		2069
C25:2		**2082**
C25:2	2088	2088
C25:3		2044
C25:3		2091
C25:3	**2106**	2107
C25:4		2080
C25:4		2086
C25:4		2127
C25:4		2133
C25:4	**2144**	
C25:5		2125
C25:5		2144
C25:5		2183
C25:5	**2191**	
C25:5	**2201**	
C25:6	**2248**	

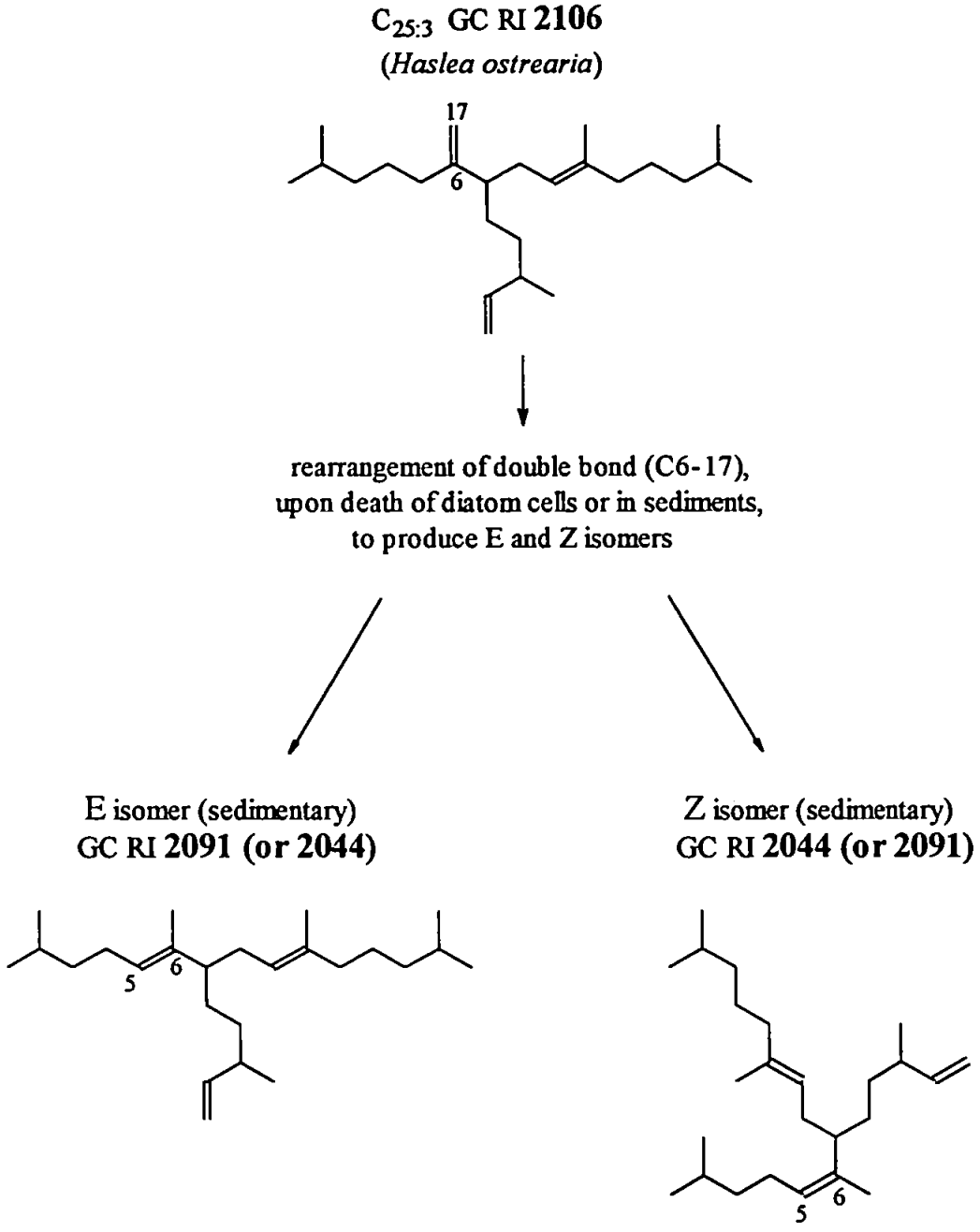
Key : Numerical values are GC retention indices
Bold text denotes characterised HBI alkenes

ostrearia produces different HBI isomers under environmental conditions to those used for the culturing the diatoms in the laboratory (see chapter 4). An alternative suggestion is that the positions and / or geometries of the double bonds of the alkenes biosynthesised by *Haslea ostrearia* are rearranged in the sediments, producing isomers with different GC retention characteristics. The painstaking structural characterisation of individual alkenes adopted herein allows this hypothesis (for which there is ample geochemical precedent in other alkenes, most notably steroids and hopanoids; *e.g.* Peakman *et al.*, 1988; de Leeuw *et al.*, 1989) to be tested. It is also interesting to note that no such reactions have been reported for the alkenones.

Previous workers have sometimes suggested that some sedimentary HBI isomers are geometric isomers. Barrick and Hedges (1980) and Porte *et al.*, (1990) both reported that the mass spectra of two trienes (GC RI 2044 & 2091) were identical. The same was true of two tetraenes (GC RI 2080 & 2127). Moreover, the difference in GC RI (ΔGC RI) between the two trienes was identical (ΔGC RI = 47) to the ΔGC RI between the two tetraenes. An examination of the data in Table 6.2 reveals that a number sedimentary alkenes exhibit ΔGC RI of 47, (*e.g.* $C_{25:4}$ GC RI 2086 & 2133; $C_{25:5}$ GC RI 2144 & 2191). Indeed, the two triene and tetraene isomers reported by Barrick and Hedges (1980) and the two pairs of isomers above are the dominant sedimentary HBI alkenes in many reports.

Comparison of the GC RI of sedimentary alkenes and those of *Haslea ostrearia*, the structures of which have now been established, suggests a positional rearrangement of one double bond in the biosynthetic products. The dominant HBI alkenes biosynthesised by *Haslea ostrearia*, $C_{25:3}$ (GC RI 2106), $C_{25:4}$ (GC RI 2144) and $C_{25:5}$ (GC RI 2191) all

Figure 6.1 Proposed rearrangement of HBI triene (*Haslea ostrearia*) to produce the two major triene isomers reported in sediments



differ by either 17 +/- 2 or 64 +/- 2 retention units to the sedimentary alkenes discussed above. For example, the *Haslea ostrearia* triene (GC RI 2106; structure **X**) exhibits ΔGC RI of -15 and -62, compared to the two major sedimentary trienes (GC RI 2044 & 2091), which Barrick and Hedges (1980) suggest are geometric isomers. Given the structure of the *Haslea ostrearia* triene (**X**), it is possible the C6-17 double bond rearranges in the sediment, to the C5-6 position, with formation of a mixture of E and Z isomers (Figure 6.1). The same rearrangement can be envisaged for the tetraene (**XI**) and pentaene (**XII**) structures produced by *Haslea ostrearia*. The tetraene, GC RI 2144 (**XI**), differs by -17 and -64 retention units from the two major sedimentary tetraenes (GC RI 2127 & 2080, Figure 6.2). Similarly, the sedimentary pentaene isomers GC RI 2173 (minor component) and GC RI 2125 which have identical mass spectra and are probably a geometric isomer 'pair' have ΔGC RI of -18 and -66 compared to the pentaene produced by *Haslea ostrearia* (GC RI 2191, **XII**; Figure 6.3).

The results of acid-catalysed (TsOH-HOAc) rearrangement experiments conducted by Hird *et al.*, (1992), on a mixture of synthetic monoene HBI isomers, resulted in the production of a new isomer with the double bond in the C5-6 position, (Figure 6.4).

Figure 6.4 Acid-catalysed rearrangement of a double bond in C_{25} monoenes (Hird *et al.*, 1992

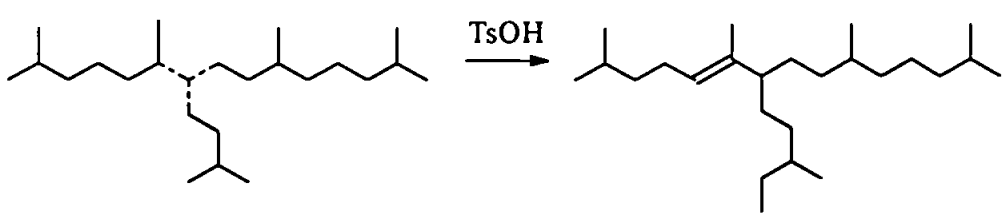


Figure 6.2 Proposed rearrangement of HBI tetraene (*Haslea ostrearia*) to produce the two major tetraene isomers reported in sediments

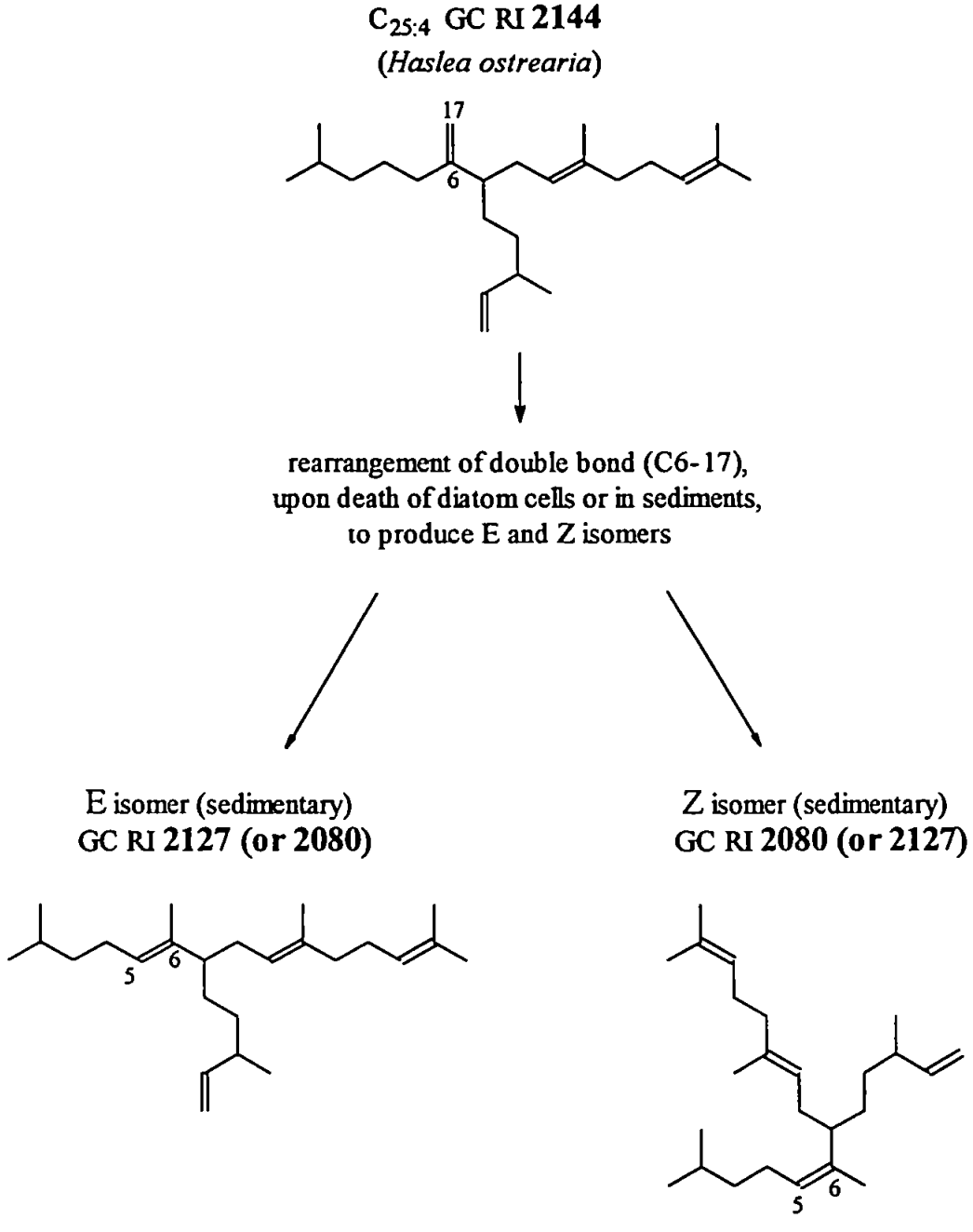
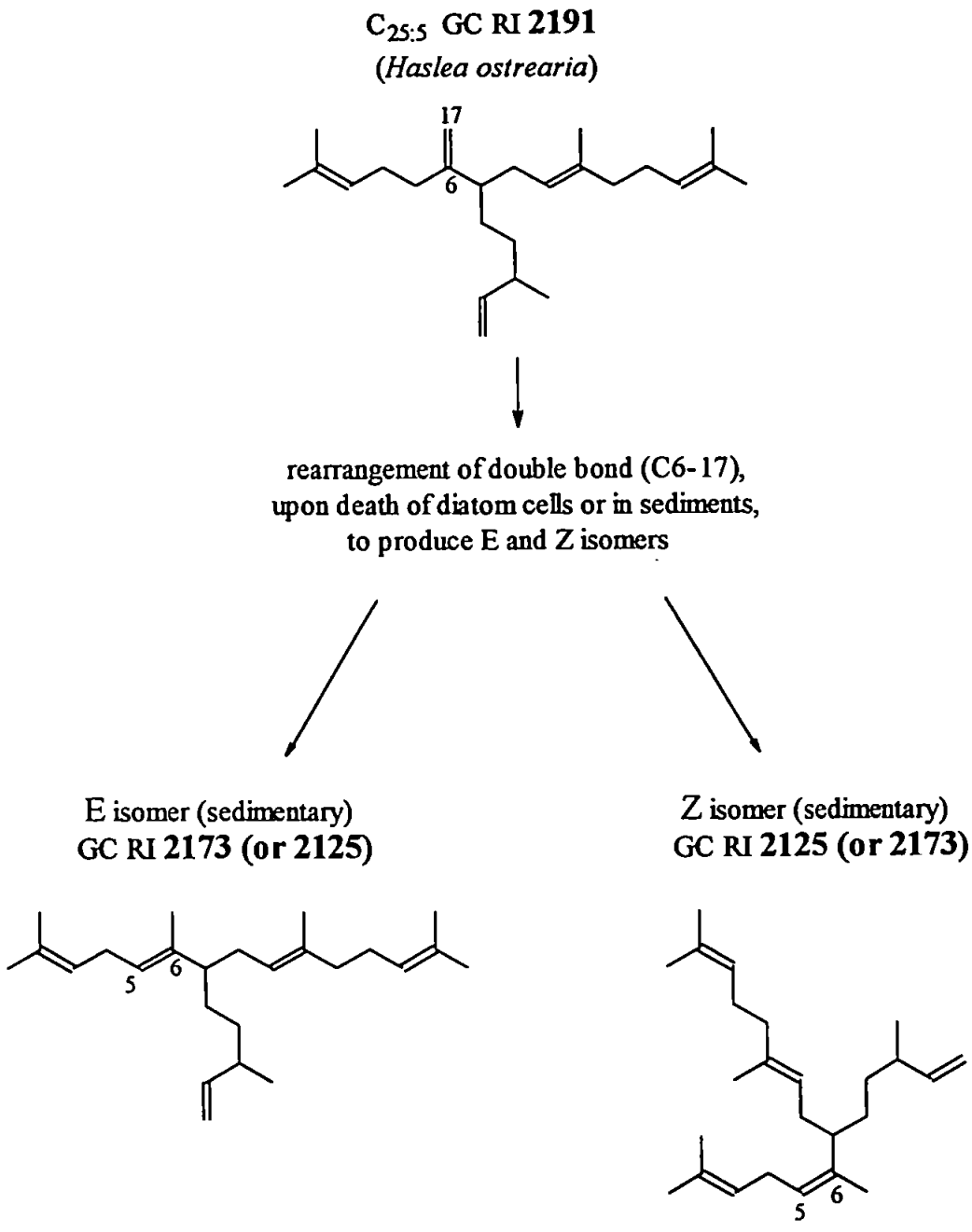


Figure 6.3 Proposed rearrangement of HBI pentaene (*Haslea ostrearia*) to produce the two pentaene isomers



seven years do not contain a double bond at the C6-17 position, but rather at the C5-6 position. It is possible that the C5-6 double bond resulted from positional rearrangements during prolonged storage. It is also noteworthy that the diene (GC RI 2079; structure **IX**), isolated from sediments had a double bond located at the C5-6 position.

Previous characterisation studies of both C_{20} and C_{25} sedimentary monoenes, indicate that the position of the double bond is only located at either the C6-17 or C5-6 position (**IV**, **V** and **VI**, section 1.1; Dunlop and Jefferies, 1985; Hird *et al.*, 1992).

If the double bond at position C6-17, primarily produced by living *Haslea ostrearia* diatoms, undergoes a positional rearrangement to the C5-6 position, upon death of the cells or in sediments, then *Haslea ostrearia* is indirectly accountable for eight of the fourteen dominant sedimentary HBI alkenes reported to date. Four of the other major HBIs reported in the sediment are two tetraenes and pentaenes. Although these have not been found in *Haslea ostrearia* to date, with the increasing number of double bonds, there is an increased possibility of rearrangement reactions.

The evidence presented strongly supports the conclusion that *Haslea ostrearia* is responsible for the majority of dominant C_{25} HBI alkenes reported in the sediments. Whilst this does not exclude the possibility that other HBIs are produced under different environmental conditions or by other diatom species, it is perhaps a vindication of the detailed approach to structural characterisation adopted in this study.

Hird *et al.*, (1992) also reported that the rearrangement of the double bond produced both E and Z isomers at a ratio of E/Z or Z/E = 1:1.6. The average relative abundance for two geometric isomer 'pairs', reported in the environment (calculated from concentrations where these were reported, or GC peak heights measured from published chromatograms) are listed in Table 6.3.

Table 6.3 Ratio of triene and tetraenes isomers reported in sediments

Alkene	Isomers	*3*	*7*	*8*	*20*	*27*	Average Ratio
				Ratios			
C25:3	2044 : 2091	2.1	1.3	2.3	1.1	n.d.	1 : 1.7
C25:4	2078 : 2124	1.6	n.d.	1.9	1.8	1.3	1 : 1.6

Numbers in italics indicate references listed in Table 1.1a

The ratios between triene and tetraene isomer 'pairs' is very similar ($C_{25:3}$ = 1 : 1.7; $C_{25:4}$ = 1 : 1.6) and these values are very close to that reported by Hird *et al.*, (1992). The modeling this reaction, would support the proposed theory.

Other tentative evidence for the positional rearrangements in HBI alkenes can be found from examining the origin of the HBI alkenes so far characterised. The three HBI alkenes $C_{25:3}$ (GC RI 2106), $C_{25:4}$ (GC RI 2144) and $C_{25:5}$ (GC RI 2191) were isolated from the 'batch' of *Haslea ostrearia* cultured for the growth phase experiment. The culture was extracted within two weeks of completion of the experiment and the three polyenes all have a double bond located at the C6-17 position. In contrast, the two HBIs ($C_{25:5}$ GC RI 2201 and $C_{25:6}$ GC RI 2248) isolated from a batch of *Haslea ostrearia* cultured over a longer period (one month, September 1988) and stored at -18°C for

6.4 Future research

For these unusual compounds to be applied as biomarkers for oil-source rock correlations and as indicators of recent and palaeoenvironments, there are important areas of research still to be examined.

To confirm the theory that there may be an incomplete record of HBIs present in the sediments it is very important to further establish the fractionation behaviour of the HBI polyenes. This should be conducted in two ways; one, by using a number of fully characterised C_{25} HBI compounds (dienes to hexaenes) for different chromatographic experiments; and where possible, re-analysis of the 'aromatic' fractions isolated from sediments, for the presence of HBI polyenes.

The chromatographic techniques established herein for HBI isolation should now be applied to a range of sediments and biota, to obtain further pure HBI alkenes (*i.e.* those currently uncharacterised), in sufficient quantity for characterisation by spectroscopy (NMR) and chemical degradation (epoxidation).

Acid-catalysed isomerisation of HBIs has proved successful in a previous study (Hird *et al.*, 1992) and to unambiguously confirm the proposed link between sedimentary HBIs and those of *Haslea ostrearia* it is essential to conduct similar experiments with known compounds. The characterised HBIs from *Haslea ostrearia* should undergo acid-catalysed isomerisation and the rearranged products that correlate directly with sedimentary counterparts can then be examined further.

The HBI compounds that have been characterised could be used as models in experiments designed to investigate the diagenetic fate of HBI hydrocarbons. These

might include the incorporation of sedimentary sulphur and anaerobic biodegradation. 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).

There are two main areas of further research that should be conducted with *Haslea ostrearia*. The first is more growth phase experiments, both under optimum conditions and with conditions that will better simulate environmental stresses on the diatoms. The stress experiments should be conducted with cultures grown at different temperatures, salinities and nutrient concentrations to note any variation in HBIs produced.

Perhaps the most intriguing area for future research involves the biosynthesis of HBIs by *Haslea ostrearia*. This could be achieved by growing large scale cultures in the presence of isotopically-labelled (^{14}C and ^{13}C) bicarbonate and carbon dioxide feedstocks. Subsamples of algae at defined growth stages (lag, exponential and stationary) could be extracted and the aliphatic hydrocarbons isolated by TLC, radio-TLC, CC, HPLC or radio-HPLC. The alkene isolates could then be analysed by NMR (^{1}H, ^{13}C), GC, radio-GC / GC-MS and GC-IRMS. Numerous other studies of isoprenoid biosynthesis have proved successful (*e.g.* Cornforth, 1975) and an examination of the biosynthetic mechanisms of HBIs may well provide a valuable insight as to why *Haslea ostrearia* is producing these unusual and interesting compounds.

REFERENCES

Ackman, R.G., Jangaard, P.M., Hoyle, R.J. and Brockerhoff, H. (1964). Origin of marine fatty acids. I. Analysis of the fatty acids produced by the diatom, *Skeletonema costatum*. *J. Fish. Res. Board Can.* **21**: 745-756.

Albaiges, J., Alaba, J. and Grimalt, J. (1984a). Extractable and bound neutral lipids in some lacustrine sediments. In *Advances in Organic Geochemistry* 1983 (eds. P. A. Schenck, J. W. de Leeuw and G. W. M. Lijmbach); *Org. Geochem.* **6**: 223-236.

Albaiges, J., Grimalt, J., Bayona, J. M., Riseborough, R. W., de Lappe, B. and Walker II, W. (1984b). Dissolved, particulate and sedimentary hydrocarbons in a deltaic environment. In *Advances in Organic Geochemistry* 1983 (eds. P. A. Schenck, J. W. de Leeuw and G. W. M. Lijmbach); *Org. Geochem.* **6**: 237-248.

Andersen, R.A. and Mullkey, T. (1983). The occurrence of chlorophylls c_1 and c_2 in the Chrysophyceae. *J. Phycol.* **19**: 289-294.

Bale, A. J., Morris, A. W. and Holland, R. J. M. (1985). Seasonal sediment movement in the Tamar estuary. *Oceanol. Acta* **8**: 1-6.

Barbe, A., Grimalt, J. O., Pueyo, J. J. and Albaiges, J. (1990). Characterisation of model evaporitic environments through the study of lipid components. In *Advances in Organic Geochemistry 1989* (eds. B. Durand and F. Behar); *Org Geochem.* **16**: 815-828

Barlow, R.G., Collos, Y., Maestrini, S. and Roy, S. (1990). Microphytobenthic pigments in a salt marsh pond determined by HPLC and spectrophotometry. *Mar. Microbial Food Webs* **4**: 117-128.

Barlow, R.G., Mantoura, R., Gough, M.A. and Fileman, T.W. (1993a). Pigment signatures of the phytoplankton composition in the northeastern Atlantic during the 1990 spring bloom. *Deep-Sea Research* **40**: 459-477.

Barlow, R.G., Mantoura, R., Gough, M.A. and Fileman, T.W. (1993b). Phaeopigment distribution in the northeastern Atlantic during the 1990 spring bloom. *Deep-Sea Research* **40**: 2229-2242.

Barlow, R.G., Mantoura, R., Peinert, R.A., Miller, A. and Fileman, T.W. (1995). The distribution, sedimentation and fate of pigment biomarkers following thermal stratification in the Western Alboran Sea. *Mar. Ecol. Prog. Ser.* (in press).

Barrick, R. C., Hedges, J. I. and Peterson, M. L. (1980). Hydrocarbon geochemistry of the Puget Sound region - I, Sedimentary acyclic hydrocarbons. *Geochim. Cosmochim.* Acta **44**: 1349-1362.

Bartlett, J. (1950). Chlorophyllase and formation of an atypical chlorophyllide in marine algae. *Plant Physiol.* **39**: 44-47.

Belt, S.T., Cooke, D.A., Hird, S.J. and Rowland, S.J. (1994). Structural determination of a highly branched C_{25} sedimentary isoprenoid biomarker by NMR spectroscopy and mass spectrometry. *J.Chem. Soc., Chem. Commum.*, 2077-2088.

Beroza, M. and Bierl, B. (1969). Ozone generator for micro-ozonolysis. *Mikrochim. Acta* **4**: 720-723.

Bidigare, R.R., Marra, J., Dickey, T.D., Baker, K., Smith, R.C. and Pak, H. (1990b). Evidence for phytoplankton succession and chromatic adaptation in the Sargasso Sea during spring 1985. *Mar. Ecol. Prog. Ser.* **60**: 113-122.

Bierl, B.A., DeVilbiss, E.D. and Plimmer, J.R. (1980). Location of double bond positions in long-chain aldehydes and acetates by mass spectral analysis of epoxide derivatives. *J. Chromatogr. Sci.* **18**: 364-371.

Bjornland, T. (1987). *Distribution patterns of carotenoids in relation to chromophyte phylogeny and systematics.* Clarendon Press, Oxford, pp. 21-37.

Bjornland, T., Liaaen-Jensen, S. and Throndsen, J. (1989). Carotenoids of the marine chyrsophyte *Pelagococcus subviridis. Phytochem.* **28**: 3347-3353.

Blanchard, R. (1979). *Contribution of Laminarian production to benthic inshore ecosystems.* Ph.D. thesis, Heriot-Watt university, Edinburgh.

Blumer, M. (1957). Removal of elemental sulphur from hydrocarbon fractions. *Anal. Chem.* **29:** 1039-1040.

Carey, I. (1992). *Organic Chemistry.* McGraw-Hill.

Chisholm, S. W., Olson, R.J., Zetter, E.R. and Waterbury, J.B. (1988). A novel, free living prochlorophyte abundant in the oceanic euphotic zone. *Nature (London)* **334**: 340-343.

Clifton, R.J. and Hamilton, E.I. (1979). Lead-210 chronology in relation to levels of elements in dated sediment core profiles. *Estuar. Coast. Mar. Sci.* **8**: 259-269.

Criegee, R. (1975). Mechanisms of ozonolysis. *Angew. Chem. Int. Ed. Engl.* **14**: 745-752.

Cripps, G. C. and Priddle, J. (1991). Hydrocarbons in the Antarctic marine environment. *Antarctic Sci.* **3**; 233-250.

Cripps, G.C. (1995). The occurrence of monounsaturated *n*-C_{21} and polyunsaturated C_{25} sedimentary hydrocarbons in the lipids of Antarctic marine organisms. *Polar Biol.* **15**: 253-259.

Davison, V.L. and Dutton, H.J. (1966). *Anal. Chem.* **38**:1302.

Douglas, A. G., Hall, P. B., Bowler, B. and Williams, P. F. V. (1981). Analysis of hydrocarbons in sediment as indicators of pollution. *Proc. R. Soc. Edinburgh.* **80B**: 113-134.

Dunlop, R. and Jefferies, P. (1985). Hydrocarbons of the hypersaline basins of Shark Bay, Western Austraila. *Org. Geochem.* **8**. 313-320.

Dunstan, G.A., Volkman, J.K., Barrett, S.M. and Jeffrey, S.W. (1994). Essential polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae). *Phytochem.* **35**: 155-161.

Everitt, D.A., Wright, S.W., Volkman, J.K. and Lindstrom (1990). Phytoplankton community compositions in the western equatorial Pacific determined from chlorophyll and carotenoid pigment distributions. *Deep-Sea Research* **37**: 975-997.

Farrington, J.W., Frew, N. and Tripp, B.W. (1977). Hydrocarbons in cores of northwestern Atlantic coastal and continental margin sediments. *Estuar. Coast Mar. Sci.* **5**: 793-808.

Foss, P., Guillard, R.R. and Liaaen-Jensen, S. (1984).Prasinoxanthin - a chemosystematic marker for algae. *Phytochem.* **23**: 1629-1633.

Gagosian, R. B., Nigrelli, G. E. and Volkman, J. K. (1983a). The use of sediment traps to determine sterol sources in coastal sediments off Peru. In *Advances in Organic Geochemistry 1981* (eds. M. Bjoroy et. al.); *Org Geochem.* 369-379.

Gieskes, W.W., Kraay, G.W. and Tussen, S.B. (1978). Chlorophylls and their degradation products in the deep pigment maximum layer of the tropical north Atlantic. *Neth. J. Sea. Research.* **12**: 195-204.

Gieskes, W.W. and Kraay, G.W. (1984). Phytoplankton, its pigments and primary production at a central North Sea station. *Neth. J. Sea. Research.* **18**: 51-70.

Gieskes, W.W. and Kraay, G.W. (1986). Analysis of phytoplankton pigments by HPLC before, during and after mass occurrence of the microflagellate *Corybellus aureus* during the spring bloom in the open North Sea. *Mar. Biol.* **92**: 45-52

Gieskes, W.W., Kraay, G. W. and Sumoto, H. (1988). Monsoonal alteration of a mixed and layered structure in the phytoplankton of the euphotic zone of the Banda Sea (Indonesia). *Neth. J. Sea. Research.* **22**: 123-137.

Guillard, R.R., Murphy, L.S., Foss, P. and Liaaen-Jensen, S. (1985). *Synechococcus* species as likely zeaxanthin-dominant ultraphytoplankton in the north Atlantic. *Limn. Oceanogr.* **30**: 412-414.

Gough, M.A., Rhead, M.M. and Rowland, S.J. (1992). Biodegradation studies of unresolved complex mixtures of hydrocarbons: model UCM hydrocarbons and the aliphatic UCM. *Org. Geochem.* **18**: 17-22.

Grossi, S.M., Kottmeier, S.T. and Sullivan, C.W. (1984). Sea ice microbial communities. III. Seasonal abundance of microalgae and associated bacteria, McMurdo Sound, Antarctica. *Microb. Ecol. Ser.* **35**: 231-242

Hager, A. and Stransky, H. (1970). The carotenoid pattern and the occurrence of the light induced xanthophyll cycle in various classes of algae. *Archiv fur Mikrobiologie.* **73**: 77-89 (in German).

Hallegraeff, G.M. (1980). Seasonal study of phytoplankton pigments and species at a coastal station of Sydney: *Mar. Biol.* **61**: 107-118.

Hallegraeff, G.M. and Jeffrey, S.W. (1987). Description of new chlorophyll *a* alteration products in marine phytoplankton. *Deep-Sea Research.* **32**: 697-705.

Hird, S.J. (1992). *Origins and short term sedimentary fate of globally distributed biological marker hydrocarbons.* PhD Thesis, University of Plymouth, UK.

Hird, S.J., Evans, R. and Rowland, S.J. (1992). Isolation and characterisation of sedimentary and synthetic highly branched C_{20} and C_{25} monoenes. *Mar. Chem.* **37**: 117-129.

Hird, S.J. and Rowland, S.J. (1995). An investigation of the sources and seasonal variations of highly branched isoprenoid hydrocarbons in intertidal sediments of the Tamar Estuary, UK. *Mar. Environ. Res.* **40**: 423-438

Hustedt, F. and Aleem, A.A. (1951). Littoral diatoms from Salstone, near Plymouth. *Journ. Mar. Biol. Assoc.* **30**: 179-197

Jeffrey, S.W. (1974). Profiles of photosynthetic pigments in the ocean using thin layer chromatography. *Mar. Biol.* **26**: 101-110.

Jeffrey, S.W. and Humphrey, G.F.(1975). New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1 and c_2. *Biochem. Physiol. Pflanz.* **167**: 191-194.

Jeffrey, S.W. and Vesk, M. (1976). Further evidence for a membrane bound endosymbiont within the dinoflagellate *Peridinium foliaceum. J. Phycol.* **12**: 450-455.

Jeffrey, S.W. and Hallegraeff, G. M. (1987). Phytoplankton pigments, species and light climate in a warm-core eddy of the east Australian Current. *Deep-Sea Research.* **34**: 649-673.

Jeffrey, S.W. and Wright, S.W. (1987). A new spectrally distinct component in the preparations of chlorophyll *c* in the micro-alga *Emiliania huxleyi* (Prymnesiophyceae). *Biochimica et Biophysica Acta.* **894**: 180-188.

Jeffrey, S.W. (1989). Chlorophyll *c* pigments and their distribution in the chromophyte algae. In Green, J.C., Leadbeater, B.S.C., Diver, W.L. (eds.) *The chromophyte algae: problems and perspectives.* Clarendon Press, Oxford, pp. 13-36.

Johansen, J. E., Svec, W.A., Liaaen-Jensen, S. and Haxo, F.T. (1974). Carotenoids of Dinophyceae. *Phytochem.* **13**: 2261-2271.

Kenig, F. and Huc, A. Y. (1990). Early sulphur incorporation in recent sedimentary organic matter within Abu Dhabi carbonate environment. In *Geochemistry of Sulphur in Fossil Fuels* (eds. W. L. Orr and C. M. White), ASC *Symposium Series* 249, pp. 170-185. *Amer. Chem. Soc.*, Washington DC.

Kenig, F., Huc, A. Y., Purser, B. H. and Oudin, J. L. (1990). Sedimentation distribution and diagenesis of organic matter in a recent carbonate environment, Abu Dhabi, U.A.E.. *Org. Geochem.* **16**: 735-748.

Klein, B. and Sournia, A. (1987). A daily study of the diatom spring bloom at Roscoff (France) in 1985. II. Phytoplankton pigment composition studied by HPLC analysis. *Mar. Ecol. Prog. Ser.* **3**: 285-294.

Kohnen, M. E. L., Sinninghe Damste, J. S., Rijpstra, W. I. C. and de Leeuw, J. W. (1991b). Alkylthiophenes as sensitive indicators of palaeoenvironmental changes: A study of Cretaceous oil shale from Jordan.In *Geochemistry of Sulphur in Fossil Fuels* (eds. W. L. Orr and C. M. White), ASC *Symposium Series* 249, pp. 444-485. *Amer. Chem. Soc.*, Washington DC.

Kohnen, M. E. L., Schouten, S., Sinninghe Damste, J. S., de Leeuw, J. W., Merrit, D. and Hayes, J. M. (1992a). The combined molecular sulphur and isotope geochemistry to assess multiple sources of palaeobiochemicals with identical carbon skeletons. In *Advances in Organic Geochemistry 1991* (eds. J. R. Maxwell); *Org. Geochem* **18**: 345-351

Latasa, M., Estrada, M. and Delgado, M. (1992). Plankton-pigment relationships in the Northwestern Mediterranean during stratification. *Mar. Ecol. Prog. Ser.* **88**: 61-73.

Lee, R.F. and Leoblich, A.R. (1971). Distribution of 21:6 hydrocarbon and its relationship to 22:5 fatty acid in algae. *Phytochem.* **10**: 593-602.

de Leeuw, J. W., Cox, H. C., van Grass G., van de Meer F. W., Peakman, T. M., Baas, M and van de Graaf, B. (1989). Limited double bond isomerisation and selective hydrogeneration of sterenes during early diagenesis. *Geochim. Cosmochim. Acta* **53**: 903-909.

Lewis, T.L. (1969). *Alkene chemistry.* Brooks and Cole.

Loring, D.H., Rantala,R., Morris, A.W. and Blake, A.J. (1982). Chemical composition of suspended particles in an estuarine turbidity maximum zone. *Can. J. Fish. Aquat. Sci.* **40**: 201-206.

Loring, D.H., Rantala,R., Morris, A.W. and Asmund, G. (1985). Nondetrital and detrital particulate metal transport in estuarine systems. In *Planetary Ecology*, pp. 137-155. Van Nostrand Reinhold, New York.

March, J. (1985). *Advanced Organic Chemistry. Reactions, Mechanisms and Structure.* 3rd edition. Wiley-Interscience.

Marlowe, I. T., Green, J. C., Neal, A. C., Brassell, S. C., Eglinton, G. and Course, P. A. (1984). Long chain alkenones in the *Prymnesiophyceae*. Distribution of alkenones and other lipids and their taxonomic significance. *Br. Phycol.* **19**: 203-216.

Mir, J., Alfonso, M. and Esteve, I. (1991). Vertical stratification and microbial assemblage in the Ebro Delta (Spain). *Microb. Ecol.* 86: 59-68.

Morris, A.W., Mantoura, R.F.C., Bale, A.J. and Howland, R. (1978). Very low salinity regions of estuaries: important sites for chemical and biological reactions. *Nature (London)* **274**: 678-680.

Morris, A.W., Bale, A.J. and Howland, R. (1981). Nutrient distributions in an estuary: evidence of chemical precipitation of dissolved silica and phosphate. *Estuar. Coast. Shelf. Sci.* **12**: 205-215.

Morris, A.W., Bale, A.J. and Howland, R. (1982). Chemical variability in the Tamar estuary, south west England. *Estuar. Coast. Shelf. Sci.* **14**: 649-661.

Neuville, D. and Daste, P. (1978). Recherches sur la determinisme de la production de marennine par la Diatomee marine *Navicula ostrearia* Bory en culture. *Rev. Gen. Bot.* **85**: 255-303.

Nichols, P. D., Volkman, J. K., Palmisano, A. C., Smith, G. A. and White, D. C. (1988). Occurrence of an isoprenoid C_{25} diunsaturated alkene and high neutral lipid content in Antarctic sea-ice diatom communities. *J. Phycol.* **24**: 90-96.

Nickell, E.C. and Privett, O.S. (1966). *Lipids* **1**: 166.

Osterroht, C., Petrick, G. and Wenck, A. (1983). Seasonal variation of particulate hydrocarbons in relation to biological parameters. *Mar. Chem.* **14**: 175-194.

Peakman, T.M. and Maxwell, J.R. (1988). Acid-catalysed rearrangement of steroid alkenes. Part 1.rearrangement of 5-cholest-7-ene. *J.Chem. Soc. Perkin Trans.* **I**: 1071-1075.

Pennington, F.C., Haxo, F.T. and Borch, G. (1985). Carotenoids of Cryptophyceae. *Biochem. Syst. Ecol.* **13**: 215-219.

Porte, C., Barcelo, D., Tavres, T. M., Rocha, V. C. and Albaiges, J. (1990). The use of Mussel Watch and molecular marker concepts in studies of hydrocarbons in a tropical bay (Todos os Santos, Bahia, Brazil). *Arch. Environ. Contam. Toxicol.* **19**: 236-274.

Prahl, F. G. Bennet, J. T. and Carpenter, R. (1980). the diagenesis of aliphatic hydrocarbons and organic matter in Dabob Bay, Washington. *Geochim. Cosmochim Acta* **52**: 2303-2310.

Reid, P.C., Lancelot, C., Gieskes, W.W.C. and Hagmeier, E. (1990). Phytoplankton of the North Sea and its dynamics. *Neth. J. Sea. Res.* **26**: 295-331.

Repeta, D.J. and Gasosian, R.B. (1981). Carotenoid transformation products in the upwelling waters off the Peruvian coast: Suspended particulate matter, sediment trap material, and zooplankton fecal pellet analyses. *Advances in Org. Geochem. 1981*: 380-388.

Repeta, D.J. and Gasosian, R.B. (1984). Transformation reactions and recycling of carotenoids and chlorins in the Peru upwelling region (15°S, 75°W). *Geochim. Cosmochim. Acta* **48**: 1265-1277.

Repeta, D.J. and Gasosian, R.B. (1987). Carotenoid diagenesis in recent marine sediments-1. The Peru continental shelf (15°S, 75°W). *Geochim. Cosmochim. Acta* **51**: 1001-1009.

Repeta, D.J. and Simpson, D.J. (1991). The distribution and recycling of chlorophyll, bacteriochlorophyll and carotenoids in the Black Sea. *Deep-Sea Research*, **38**: 969-984.

Requejo, A. G. and Quinn, J. G. (1983a). Geochemistry of C_{25} and C_{30} biogenic alkenes in sediments of the Narragansett Bay estury. *Geochim. Cosmochim. Acta* **47**: 1075-1090.

Requejo, A. G. and Quinn, J. G. (1983b). Formation of *n*-alkenes during anaerobic decomposition of a marine algal mat. *Nature (London)* **305**: 520-523.

Requejo, A. G., Quinn, J. G., Gearing, J. N. and Gearing (1984). C_{25} and C_{30} biogenic alkenes in a sediment core from the upper anoxic basin of the Pettaquamscutt River (Rhode Island, U.S.A.). *Org. Geochem.* **7**: 1-10.

Requejo, A. G. and Quinn, J. G. (1985). C_{25} and C_{30} biogenic alkenes in sediments and detritus of a New England salt marsh. *Estuar. Coast. Shelf Sci.* **20**: 281-297.

Ricard, M. (1987a). *Atlas du Phytoplankton Marin. Vol. II: Diatomophycees*, 102-103. Editions du Centre Nationale de la Recherche, Paris.

Robert, J-M. (1983). *Fertilite des eaux de claires ostreicoles et verdissement.* These Doct. Etat, Biologie, Universite de Nantes.

Robert, J-M. (1984). Greening of the oyster-ponds by the diatom *Haslea ostrearia* Bory: a model of the phenomeon. *8th Diatom Synposium 1984*, Paris: 517-523.

Robert, J-M. (1986). Variations biometriques de l'algae *Navicula ostrearia* Bory. *Bull. Soc. Phycol. Fr.*, **23**: 38-44.

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

Robson, J. N. and Rowland, S. J. (1986a). Synthesis of a highly branched isoprenoid hydrocarbons In *Advances in Organic Geochemistry 1987* (eds. L. Matavelli and L. Novelli); *Org Geochem.* **13**: 691-695.

Robson, J. N. (1987). *Synthetic and biodegradation studies of some novel, widely distributed sedimentary isoprenoid hydrocarbons.* Ph.D. thesis, CNAA, Plymouth Polytechnic.

Robson, J.N. and Rowland, S.J. (1988). Synthesis of a highly branched C_{30} sedimentary hydrocarbon. *Tetrahedron Lett.*, **29**: 3837-3840.

Rowland, S. J. (1982). *Origins and fate of sedimentary acyclic isoprenoids.* Ph.D. thesis, University of Bristol.

Rowland, S. J., Lamb, N. A., Wilkinson, C. F. and Maxwell, J. R. (1982). Confirmation of 2,6,10,15,19-pentamethyleicosane in methanogenic bacteria and sediments. *Tetrahedron Lett.* **23**: 101-104.

Rowland, S.J., Yon, D.A., Lewis, C.A. and Maxwell, J.R. (1985). Occurrence of 2,6,10-trimethyl-7-(3-methylbutyl)dodecane and related hydrocarbons in the green alga *Enteromorpha prolifera* and sediments. *Org. Geochem.* **8**: 207-213.

Rowland, S. J. and Robson, J. N. (1990). The widespread occurence of highly branched acyclic C_{20}, C_{25} and C_{30} hydrocarbons in recent sediments in biota - A review. *Mar. Environ. Res.* **30**: 191-216.

Roy, S. (1987). HPLC analysis of chloropigments. *J. Chromatog.* **391**: 19-34.

Roy, S. (1989). HPLC measured chlorophyll-type pigments during a phytoplankton spring bloom in Bedford Basin (Canada). *Mar. Ecol. Prog. Ser.* **55**: 279-290.

Shaw, D. G., Hogan, T. E. and McIntosh, D. J. (1985). Hydrocarbons in the intertidal environment of Kachemak Bay, Alaska. Mar. *Pollut. Bull.* **11**: 297-300.

Simonsen, S. (1974). "*Meteor*", Forschungsergebnisse, Berlin.

Sinninghe Damste, J.S., Rijpstra, W., Hock-van Dalen, A.C., de Leeuw, J.W. and Schenck, P.A. (1989b). Quenching of labile functional lipids by inorganic sulphur species: Evidence for the formation of sedimentary organic sulphur compounds at the early stage of diagenesis. *Geochim. Cosmochim. Acta.* **53**: 1443-1455.

Sinninghe Damste, J.S. and de Leeuw, J.W. (1990). Analysis, structure and geochemical significance of organically-bound sulphur in the geosphere: State of the art and future research. In *Advances in Organic Geochemistry 1989* (eds. B. Durand and F. Behar); *Org. Geochem.* **16**: 1077-1101.

Stauber, J.L. and Jeffrey, S.W. (1988). Photosynthetic pigments in 51 species of marine diatoms. *J. Phycol.* **24**: 158-172.

Summons, R.E., Barrow, R.A., Capon, R.J. and Stranger, C. (1993). The structure of a new C_{25} isoprenoid alkene biomarker from diatomaceous microbial communities. *Aust. J. Chem.*, **46**: 907-915.

ten Haven, H.L., Lafargue, E. and Kotarba, M. (1993). Oil/oil and oil/source rock correlations in the Carpathian Foredeep and Overthrust, south-east Poland. *Org. Geochem.* **20**: 935-959.

Venkatesan, M. I., Brenner, S., Ruth, E., Bonilla, J. and Kaplan, I. R. (1980). Hydrocarbons in age-dated sediment cores from two basins in the Southern California Bight. *Geochim. Cosmochim. Acta.* **44**: 789-802.

Venkatesan, M. I. and Kaplan, I. R. (1982). Distribution and transport of hydrocarbons in surface sediments of the Alaskan Outer continental shelf. *Geochim. Cosmochim. Acta* **46**: 2135-2149.

Venkatesan, M. I. and Kaplan, I. R. (1987). Organic geochemistry of Antarctic marine sediments, Part 1. Bransfield Strait. *Mar. Chem.* **21**: 347-375.

Venkatesan, M. I. (1988). Organic geochemistry of marine sediments in the Antarctic region. Marine lipids in McMurdo Sound. *Org. Geochem.* **12**: 13-27.

Vesk, M. and Jeffrey S.W. (1987). Ultrastructure and pigments of two strains of the picoplanktonic alga, *Pelagococcus subviridis* (Chrysophyceae). *J. Phycol.* **23**: 322-336.

Volkman, J. K., Farrington, J. W., Gagosian, R. B. and Wakeham, S. G. (1983). Lipid composition of coastal marine sediments from the Peru upwelling region. In *Advances in Organic geochemistry* 1981 (eds. M. Bjoroy *et al.*), pp. 228-240. Wiley, Chichester.

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

Voudrais, E.A. and Smith, C.L. (1986). Hydrocarbon pollution from marinas in estuarine sediments. *Estuar. Coast. Shelf. Sci.* **22**: 271-284.

Werner, D. (1977)(ed.). *The biology of diatoms.* Oxford: Blackwell Scientific Publications.

Withers, N. W., Fiksdhal, A. and Tuttle, R.C. (1981). Carotenoids of the Chrysophyceae. *Comp. Biochem. Physiol.* **68B**: 345-349.

Wright, S.W. and Jeffrey, S.W. (1987). Fucoxanthin pigment markers of marine phytoplankton analysed by HPLC and HPTLC. *Mar. Ecol. Prog. Ser.* **38**: 259-266.

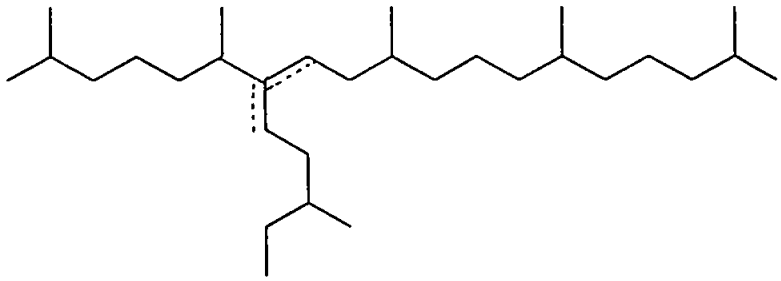
Wright, S.W., Jeffrey, S.W., Mantoura, R.F.C., Llewellyn, C.A., Repeta, D. and Welshmeyer, N. (1991). Improved HPLC method for the analysis of chlorophylls from marine phytoplankton. *Mar. Ecol. Prog. Ser.* **77**: 183-196.

Yon, D.A., Ryback, G. and Maxwell, J.R. (1982). 2,6,10-trimethyl-7-(3-methylbutyl)dodecane, a novel sedimentary biological marker compound. *Tetrahedron Lett.* **23**: 2143-2146.

Yruela, I., Barbe, A. and Grimalt, J.O. (1990). Determination of double bond position and geometry in linear and highly branched hydrocarbons from GC-MS of epoxides. *J. Chromatogr. Sci.* **28**: 421-427.

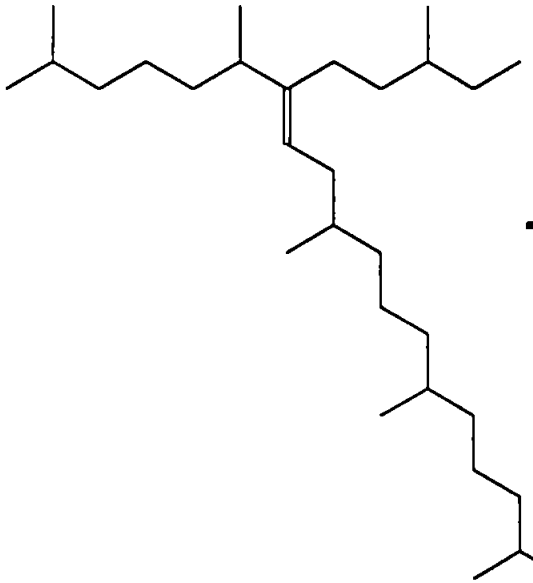
APPENDIX I

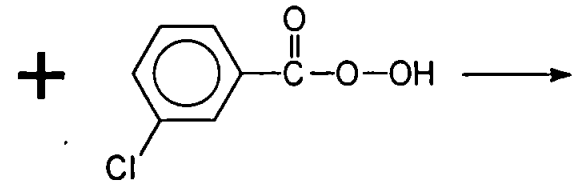
Synthetic $C_{30:1}$ HBI (Robson, 1987; Hird, 1992)

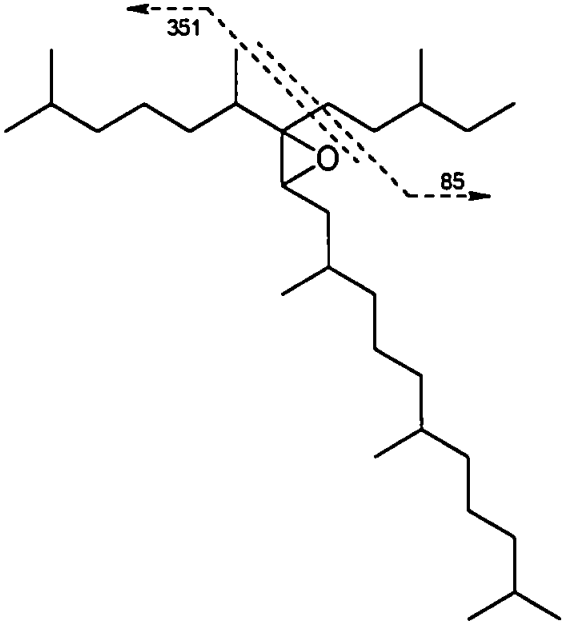


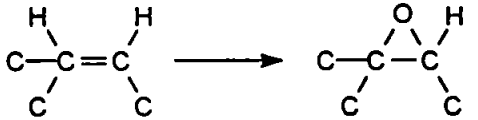
Reaction conditions:	HBI alkene	150 μg of $C_{30:1}$
	CPBA	0.16 molar, 300 μL
	Temp.	90 hours @ 25°C
	Yield	92%

ISOMER 1

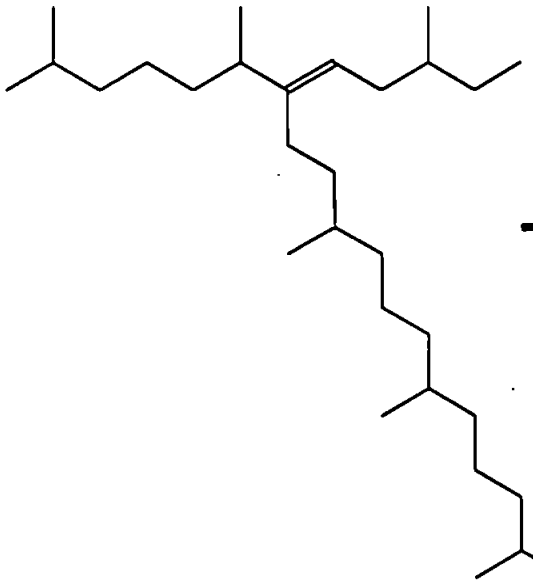


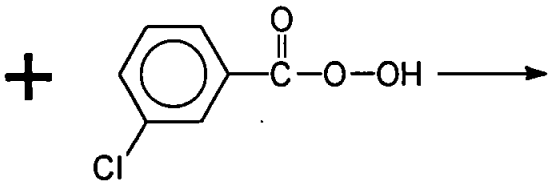




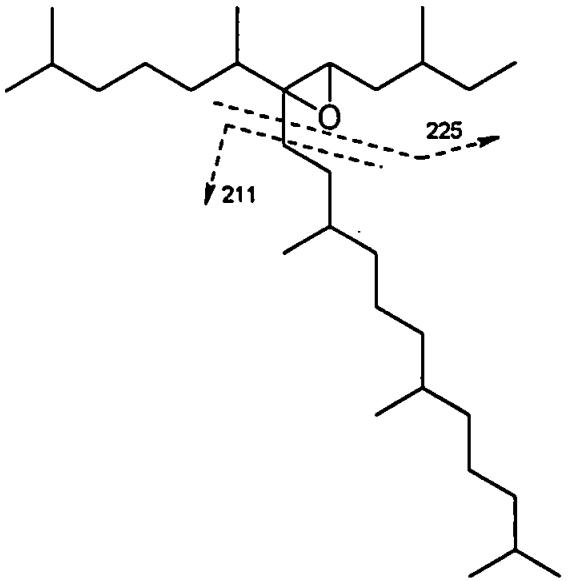


ISOMER 2

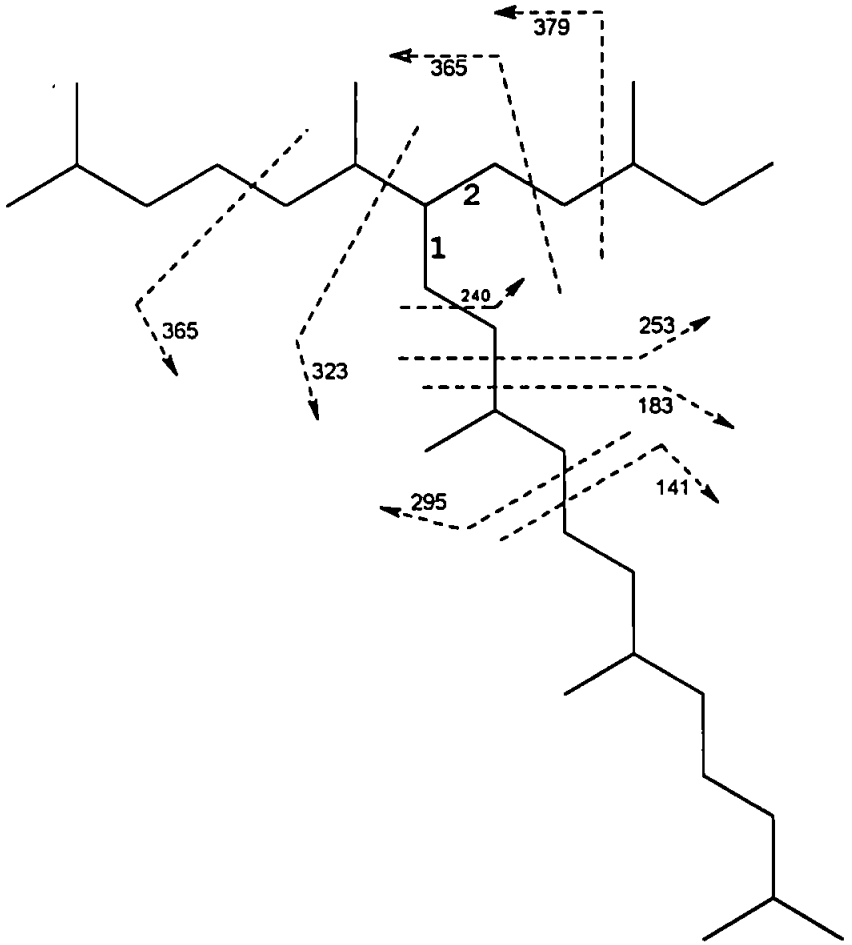




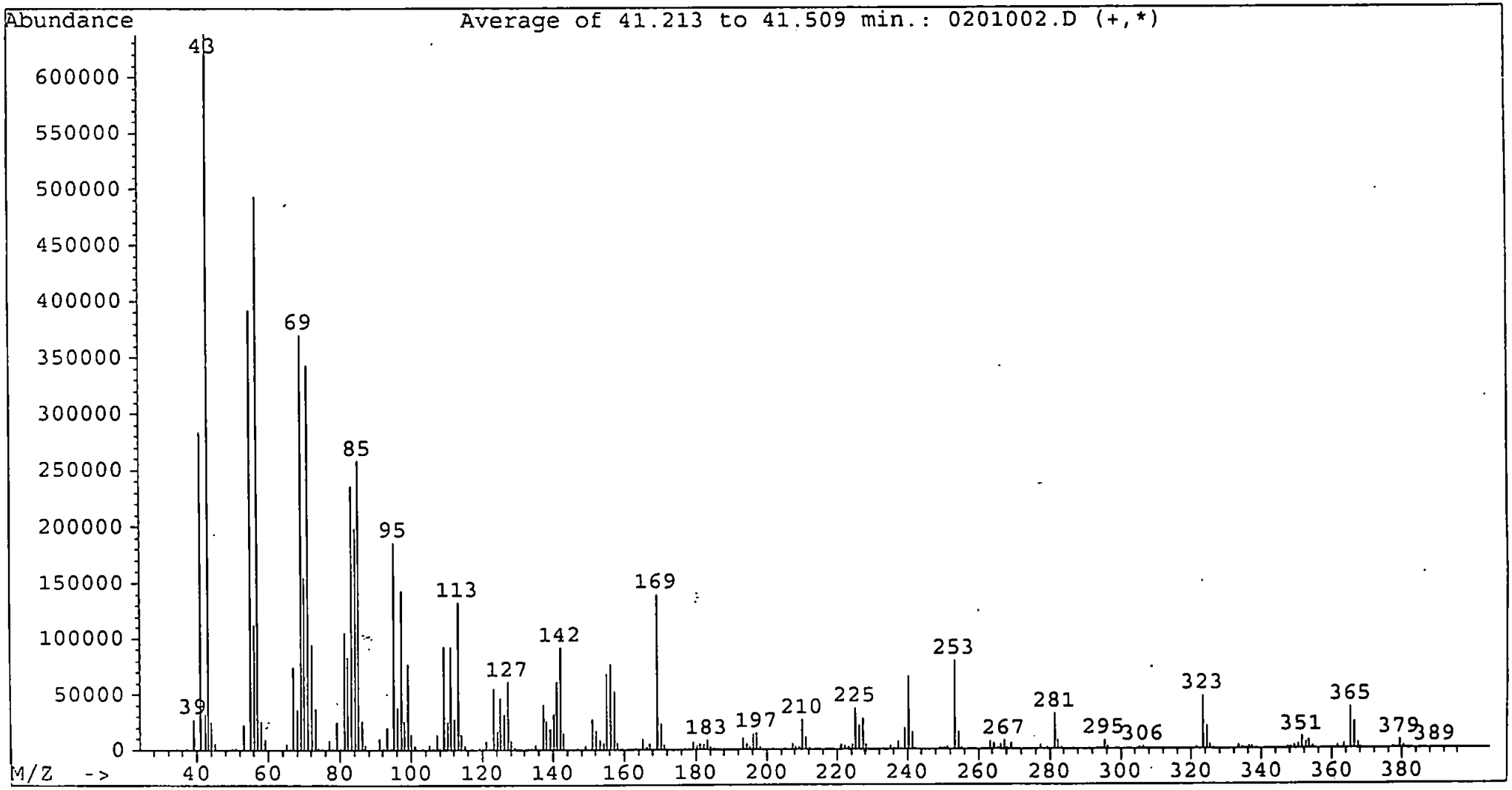
DIAGNOSTIC IONS FOR ISOMER 2



CHARACTERISTIC IONS FOR $C_{30:1}$: FOR THE EPOXIDES OF ISOMERS 1 & 2



File: C:\CHEMPC\DATA\DA8\0201002.D
Operator: David Cooke
Date Acquired: 18 Mar 94 12:08 pm
Method File: DAC1.M
Sample Name: C30:1 Epoxidation 12 Hours Sample SH888A
Misc Info:
ALS vial: 2



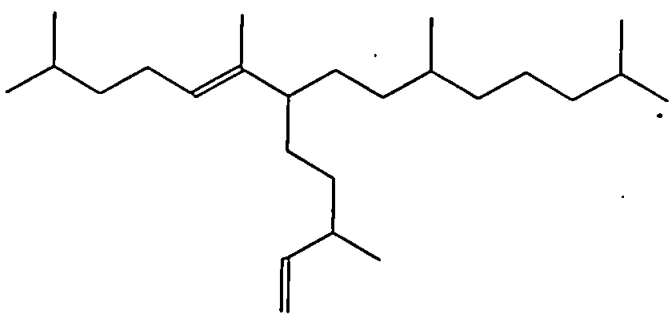
APPENDIX II

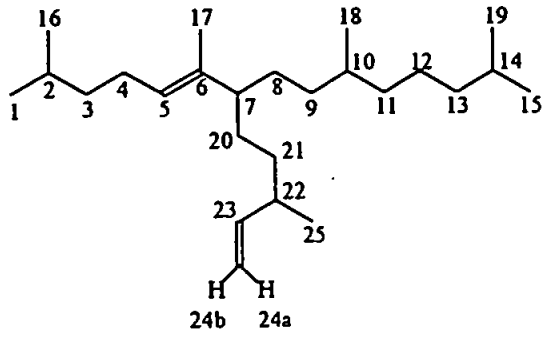
NMR spectra for HBI alkenes reported in Chapter 3

HBI	: $C_{25:2}$
GC $RI_{(DB1)}$	: 2079
Origin	: Caspian Sea
Conc.	: ≤ 60 μg g^{-1} (dry wt)
Quantity	: 5 mg
Purity	: 98% (by GC)
Date collected	: 2 / 6 / 92

Characterisation techniques,
O_3, epoxidation and ^{1}H & ^{13}C NMR

IX



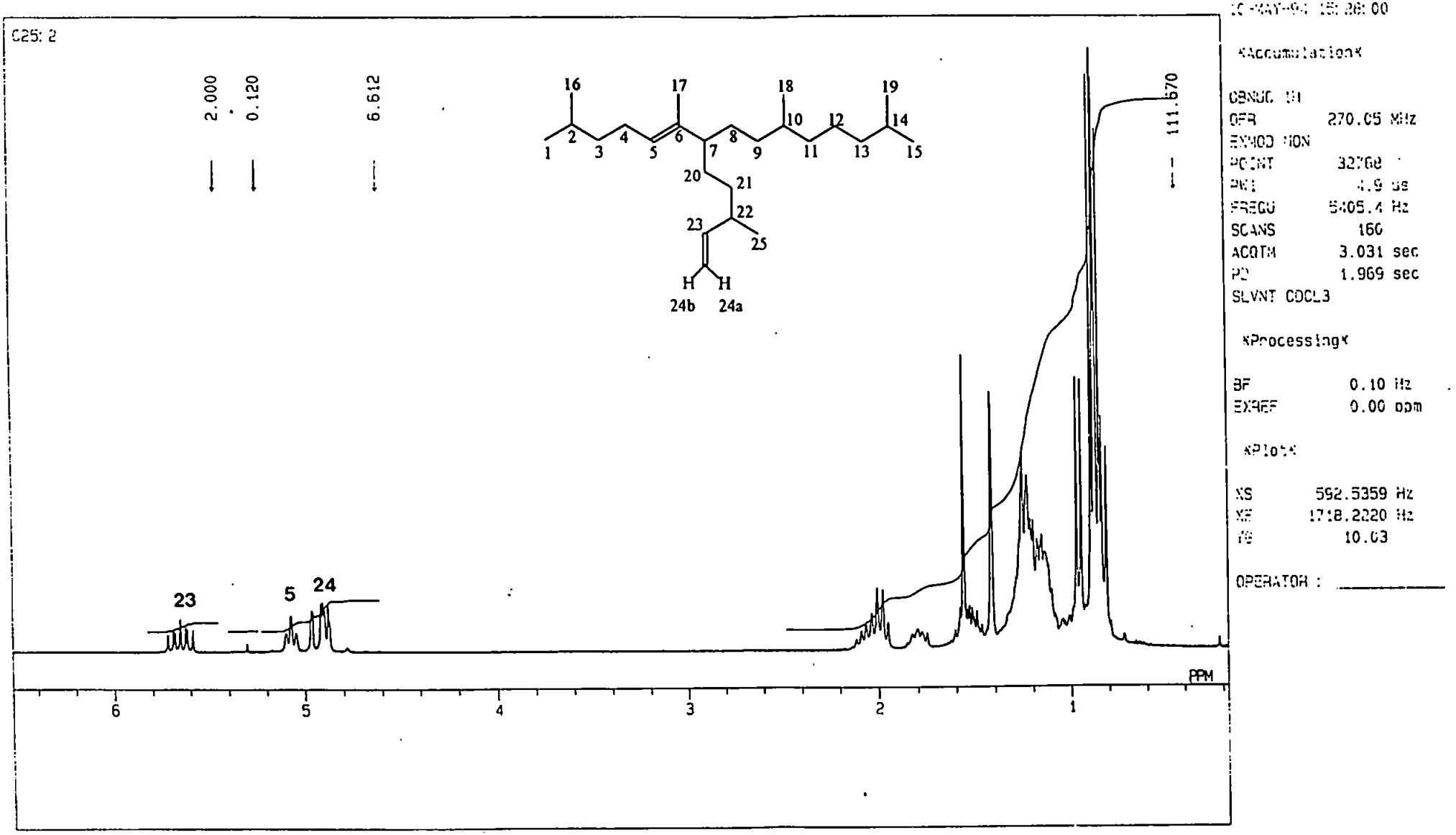
$C_{25:2}$ GC RI 2079 (DB1) Caspian Sea	
Purity : >98% (by GC) Quantity : 5.0 mg Solvent : $CDCl_3$	

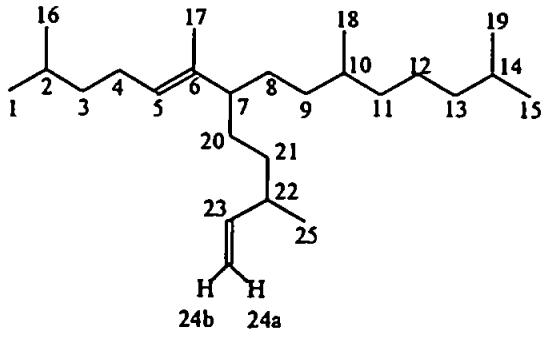
Proton (^1H) chemical shifts (multiplicities, integration)

	1, 16	**4**	**5**
δ(ppm)	0.88 (d, 6H)	1.99 (q, 2H)	5.08 (t, 1H)
	7	**15, 19**	**17**
δ(ppm)	1.80 (m, 1H)	0.85 (d, 6H)	1.42 (s, 3H)
	18	**22**	**23**
δ(ppm)	0.82 (d, 3H)	2.08 (m, 1H)	5.65 (ddd, 1H)
	24a	**24b**	**25**
δ(ppm)	4.93 (dd, 1H)	4.89 (dd, 1H)	0.95 (s, 3H)

Proton coupling constants

	$J_{H(1, 16) - H2}$	$J_{H4 - H5}$	$J_{H5 - H9}$	$J_{H(15, 19) - H14}$	$J_{H18 - H10}$
J(Hz)	6	7	7	6	6.5
	$J_{H23trans}$	J_{H23cis}	J_{H23vic}	$J_{H24a - H24b}$	$J_{H25 - H22}$
J(Hz)	17	10	8	2	6.5


C25: 2
2.000
0.120
6.612
111.670
16
17
18
19
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
20
21
22
23
25
H
H
24b
24a
23
5
24
PPM
6
5
4
3
2
1
Accumulation
OBNUC 1H
OFR 270.05 MHz
EXMOD 1ON
POINT 32768
PW1 4.9 us
FREQU 5405.4 Hz
SCANS 160
ACQTM 3.031 sec
PD 1.969 sec
SLVNT CDCL3
Processing
BF 0.10 Hz
EXREF 0.00 ppm
Plot
XS 592.5359 Hz
XF 1718.2220 Hz
YS 10.03
OPERATOR :

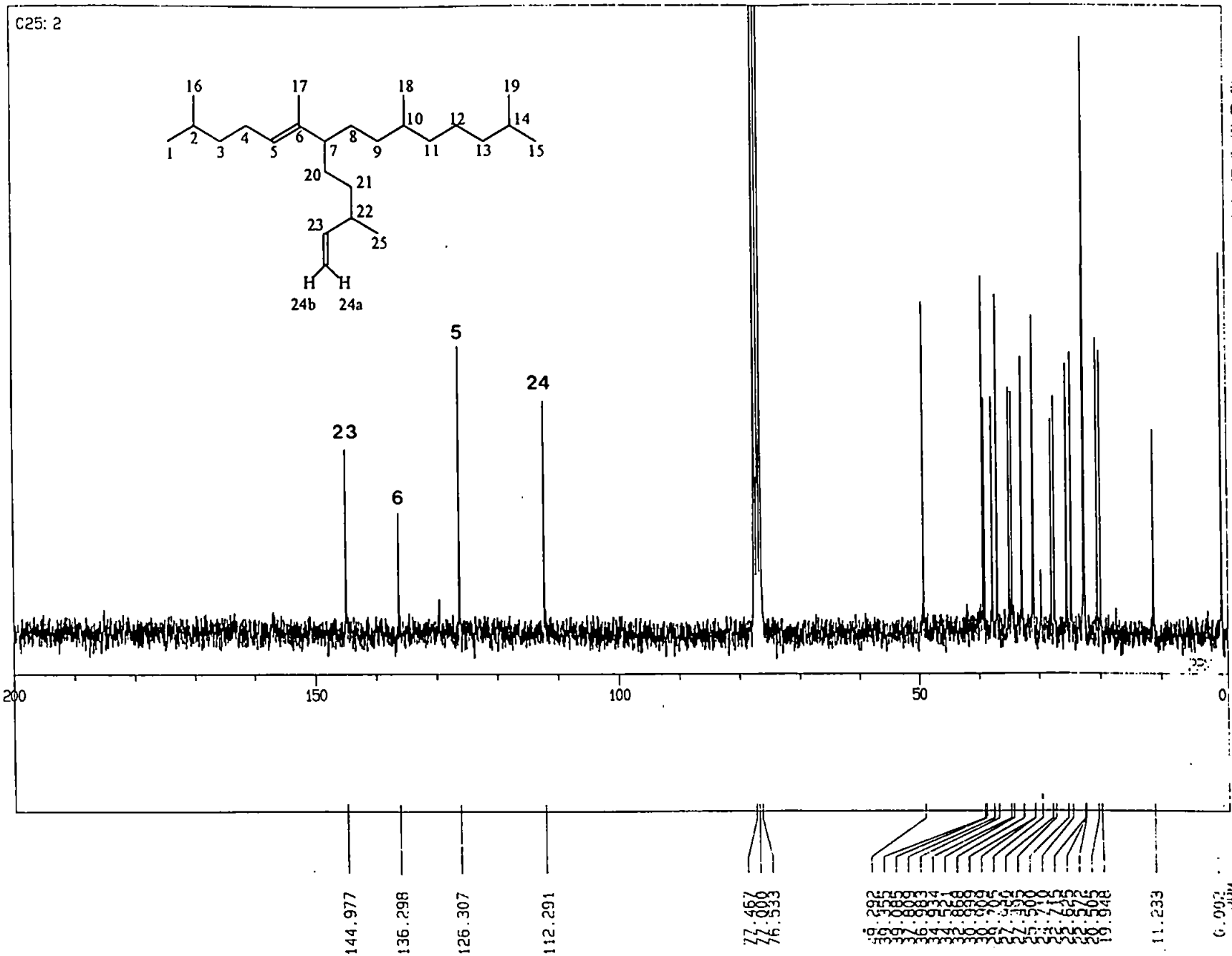


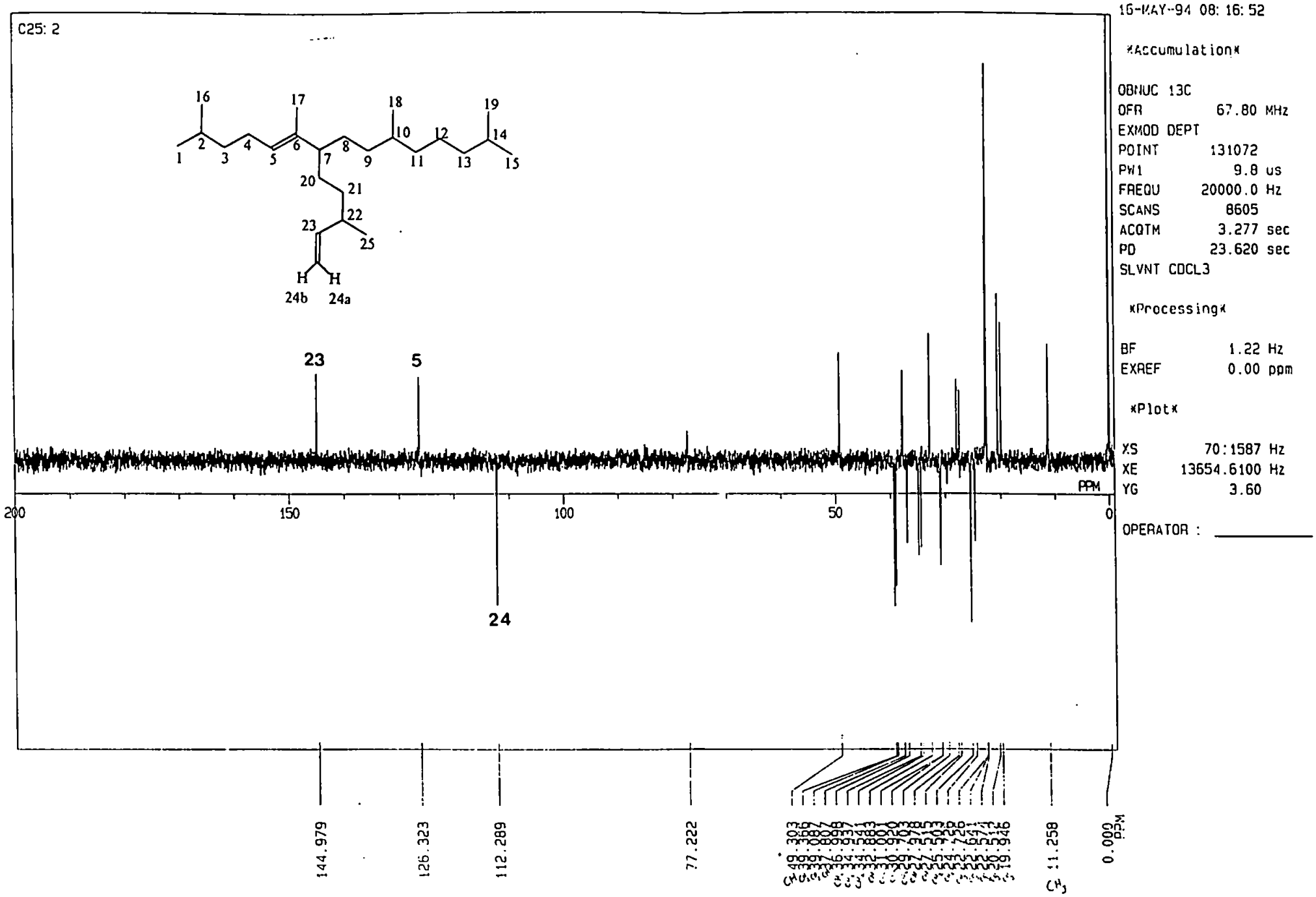
Carbon (^{13}C) chemical shifts

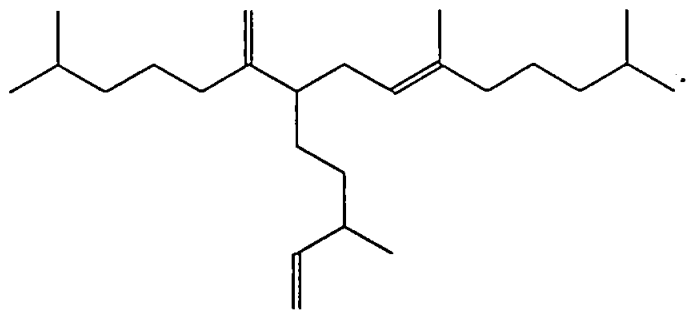
	1	**2**	**4**	**5**	**6**
δ(ppm)	22.6	27.9	25.5	126.3	135.3
	7	**15**	**16**	**17**	**18**
δ(ppm)	49.3	22.6	22.6	11.3	19.9
	19	**22**	**23**	**24**	**25**
δ(ppm)	22.6	37.8	145.0	112.3	20.5

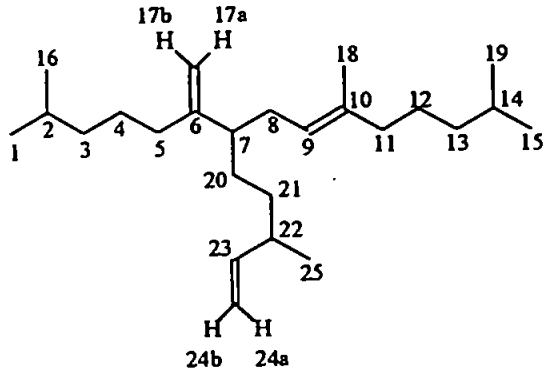
Not assigned : 3, 8, 9, 10, 11, 12, 13, 14, 20 & 21

^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively). Numbering shown in displayed formulae.


C25: 2
16
17
18
19
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
20
21
22
23
25
H
H
24b
24a
5
24
23
6
200
150
100
50
0
144.977
136.298
126.307
112.291
77.466
76.933
11.233
11-MAY-94 08:48:57
%Accumulation%
OBNUC 13C
OFR 67.80 MHz
EXMOD BCM
POINT 32768
PW1 5.0 us
FREQU 20000.0 Hz
SCANS 30780
ACQTM 0.819 sec
PD 1.181 sec
SLVNT CDCL3
%Processing%
BF 1.22 Hz
EXREF 77.00 ppm
%Plot%
XS 69.3989 Hz
XE 13654.6100 Hz
YG 11.16
OPERATOR :


C25: 2
16-MAY-94 08: 16: 52
Accumulation
OBNUC 13C
OFR 67.80 MHz
EXMOD DEPT
POINT 131072
PW1 9.8 us
FREQU 20000.0 Hz
SCANS 8605
ACQTM 3.277 sec
PD 23.620 sec
SLVNT CDCL3
Processing
BF 1.22 Hz
EXREF 0.00 ppm
Plot
XS 70:1587 Hz
XE 13654.6100 Hz
YG 3.60
OPERATOR :
23
5
24
PPM
200
150
100
50
0
144.979
126.323
112.289
77.222
11.258
0.000
PPM
CH3

HBI : $C_{25:3}$ GC $RI_{(DB1)}$: 2106 Origin : *Haslea ostrearia* (growth phase expt) Conc. per cell : ≤ 1.92 pg Conc. (bulk) : 210 μg g^{-1} (dry wt) Quantity : 6.1 mg Purity : 98% (by GC) Date collected : 10 / 5 / 95 Characterisation techniques, Epoxidation and ^{1}H & ^{13}C NMR	**X** 

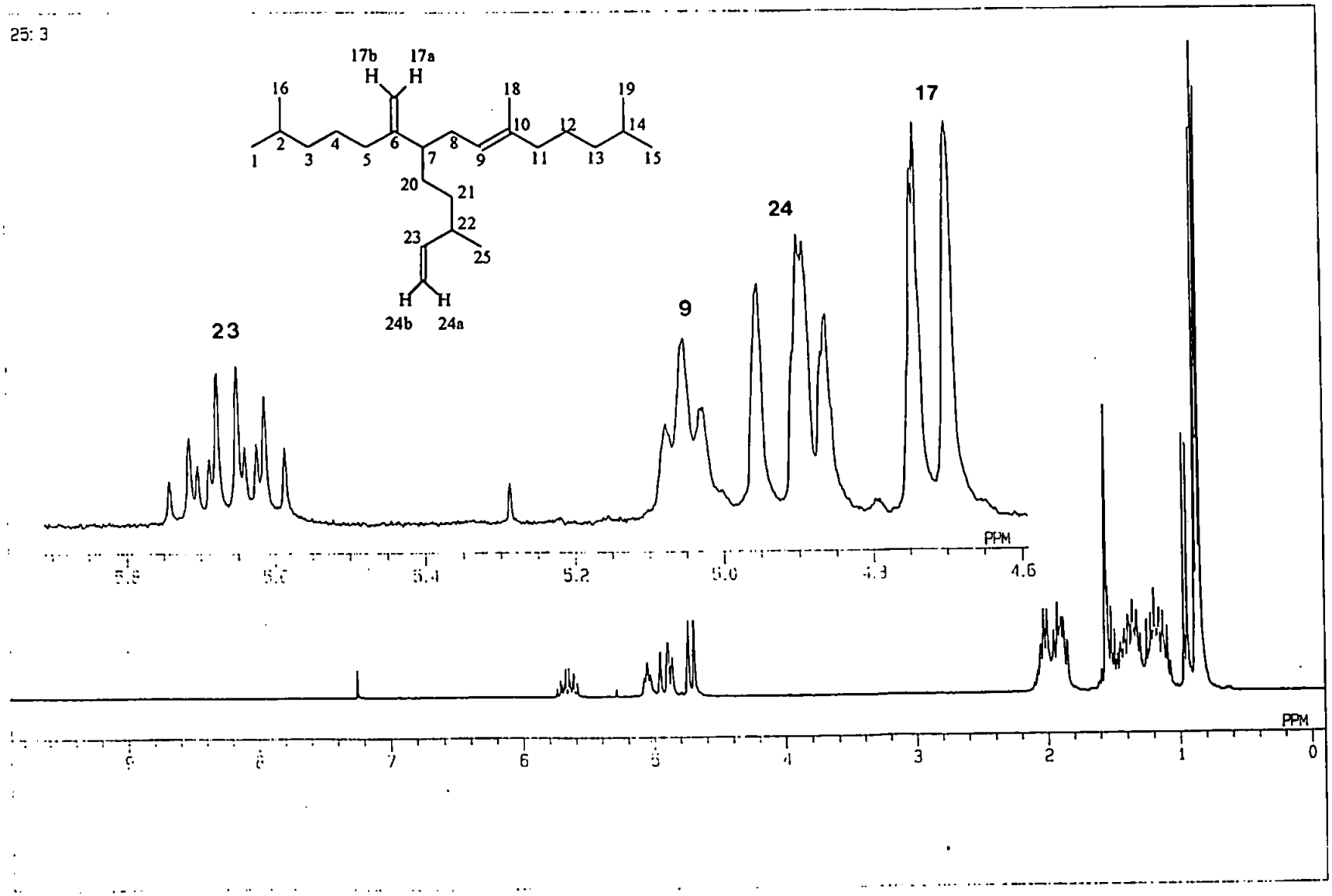
$C_{25:3}$ GC RI 2106(DB1) *Haslea ostrearia*	
Purity : >98% (by GC) Quantity : 5.1 mg Solvent : $CDCl_3$	

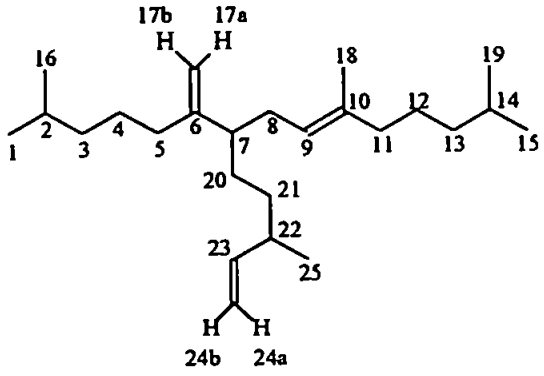
Proton (^1H) chemical shifts (multiplicities, integration)

	1, 16	**5, 7, 11**	**8, 22**
δ(ppm)	0.86 (d, 6H)	1.88 (m, 5H)	2.02 (m, 3H)
	9	**15, 19**	**17a**
δ(ppm)	5.05 (t, 1H)	0.86 (d, 6H)	4.70 (br, s, 1H)
	17b	**18**	**23**
δ(ppm)	4.74 (br, d, 1H)	1.56 (s, 3H)	5.67, 5.65* (ddd, 1H)
	24a	**24b**	**25**
δ(ppm)	4.92 (br, d, 1H)	4.88 (br, d, 1H)	0.95 (d, 3H)

Proton coupling constants

	1, 16	**9**	**15, 19**
J(Hz)	6.6	6.6	6.6


25: 3
17b
17a
H
H
16
18
19
17
10
12
14
2
6
8
1
3
4
5
7
9
11
13
15
20
21
24
22
23
25
H
H
24b
24a
23
9
PPM
5.6
5.4
5.2
5.0
4.8
4.6
PPM
9
8
7
6
5
4
3
2
1
0

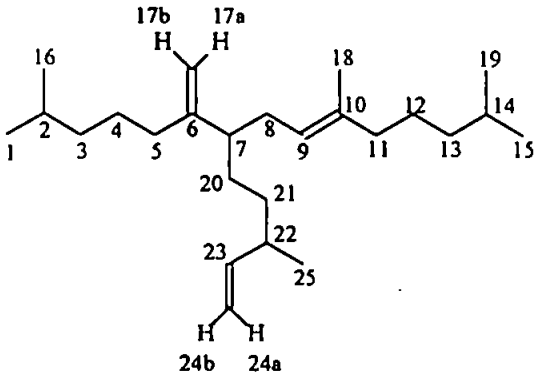


Carbon (^{13}C) chemical shifts

	1	**2**	**3**	**4**	**5**
δ(ppm)	22.6	27.9	39.0	25.6	33.7, 33.6*
	6	**7**	**8**	**9**	**10**
δ(ppm)	152.5*	46.8	32.9, 32.8*	123.1	135.6
	11	**12**	**13**	**14**	**15**
δ(ppm)	40.0	25.7	38.6	28.0	22.6
	16	**17**	**18**	**19**	**20**
δ(ppm)	22.6	108.6*	16.1	22.6	30.8*
	21	**22**		**23**	
δ(ppm)	34.4	38.0, 37.9*		145.0, 144.9*	
	24		**25**		
δ(ppm)	112.4, 112.2*		20.4, 20.0*		

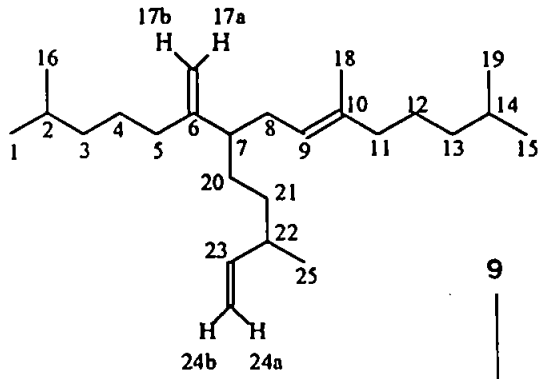
^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively.
Numbering shown in displayed formulae.
Chemical shifts labelled * are due to the presence of diastereoisomers.

25: 3

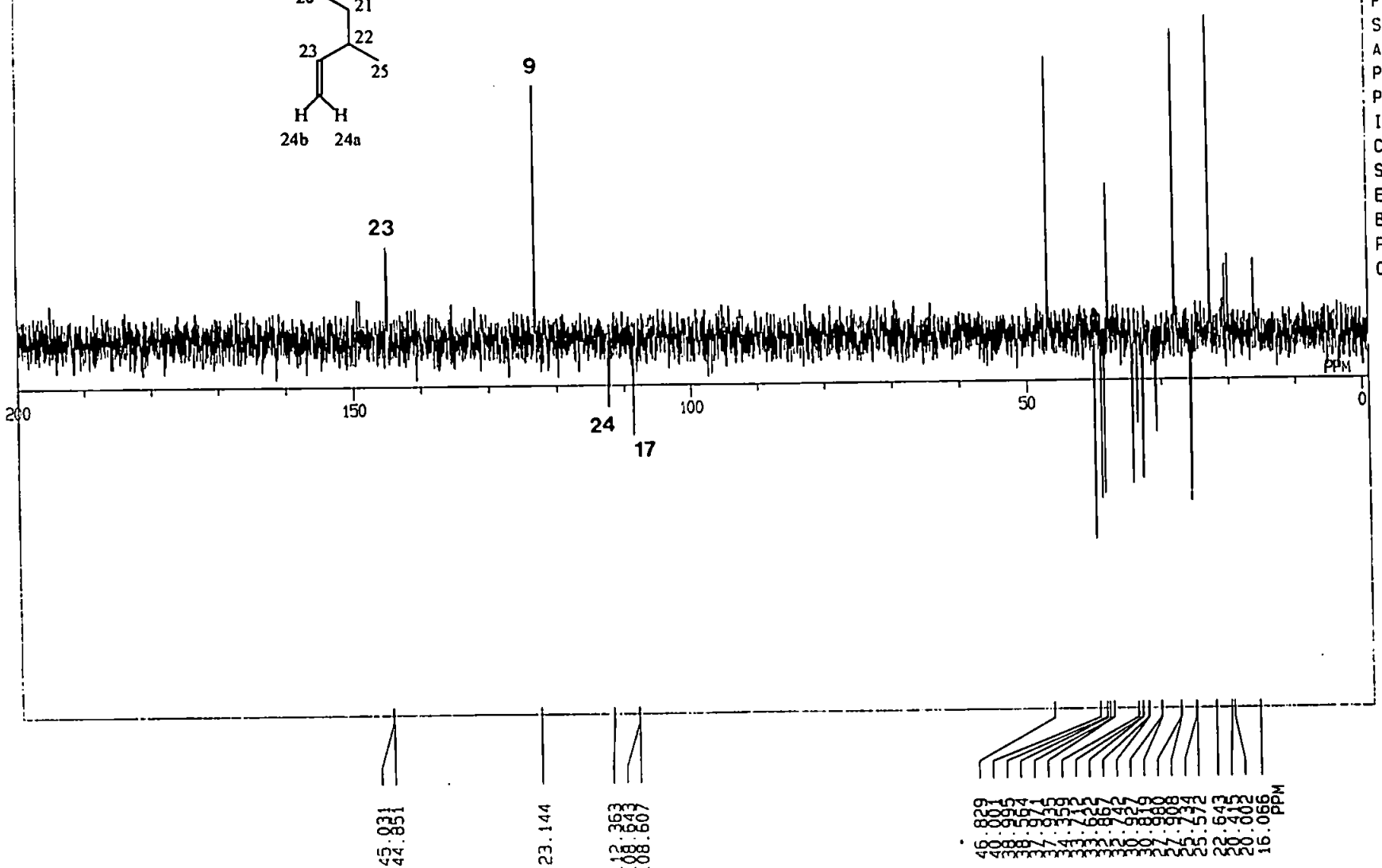


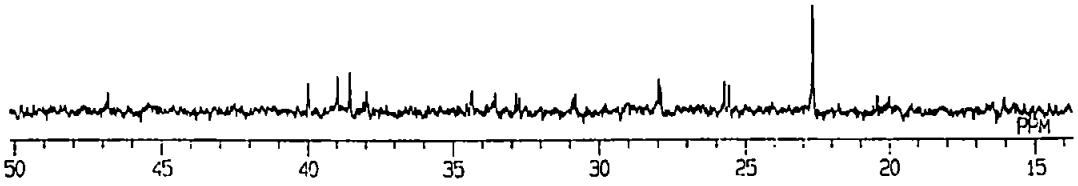
29-JUN-95 08: 02: 46
DFILE C0027
OBNUC 13C
EXMOD BCM
OFR 67.80 MHz
OBSET 135.00 kHz
OBFIN 5200.0 Hz
POINT 32768
FREQU 20000.0 Hz
SCANS 19259
ACQTM 0.819 sec
PD 2.181 sec
PW1 3.9 us
IRNUC 1H
CTEMP 27.0 c
SLVNT CDCL3
EXREF 77.00 ppm
BF 1.22 Hz
RGAIN 26
OPERATOR : _____

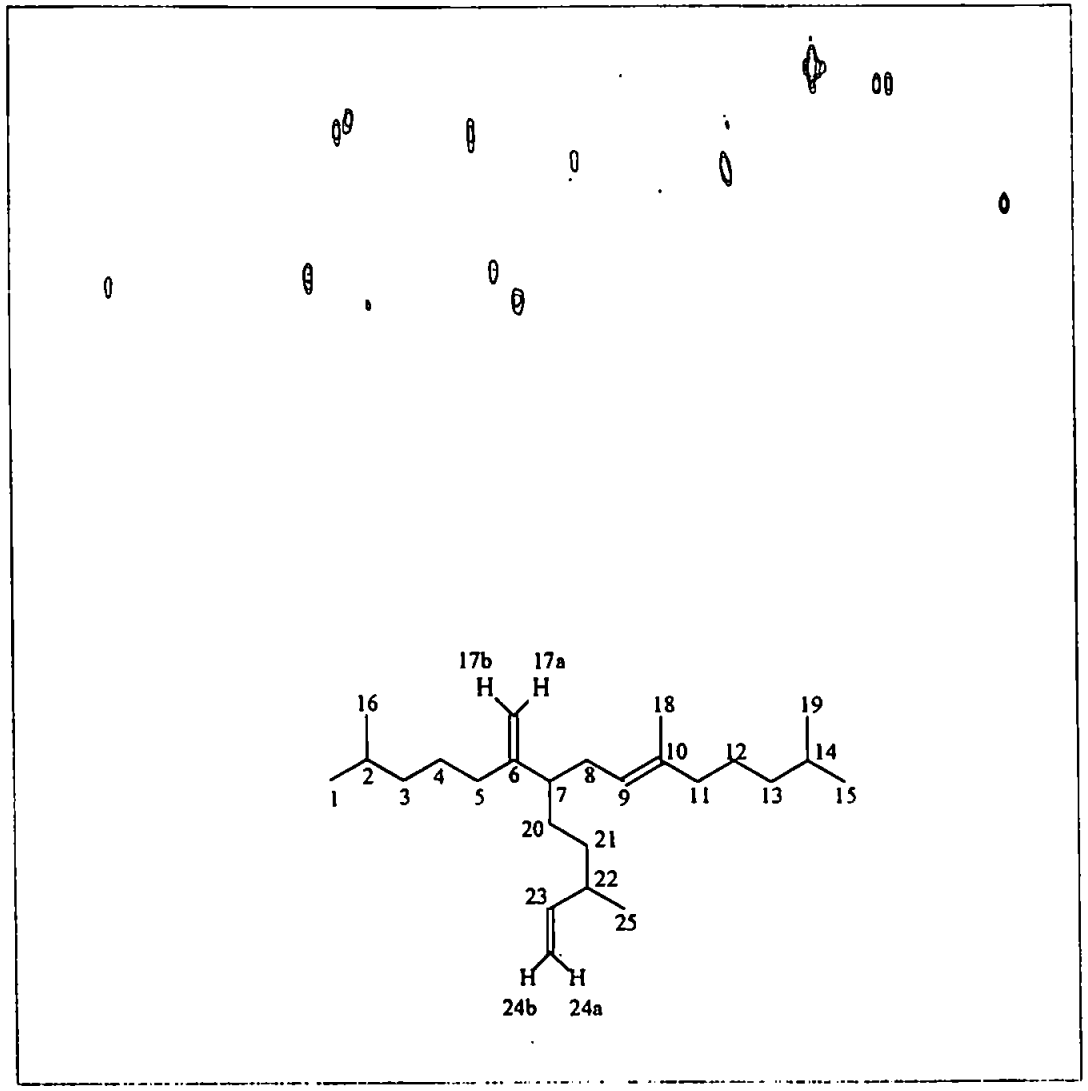
25: 3

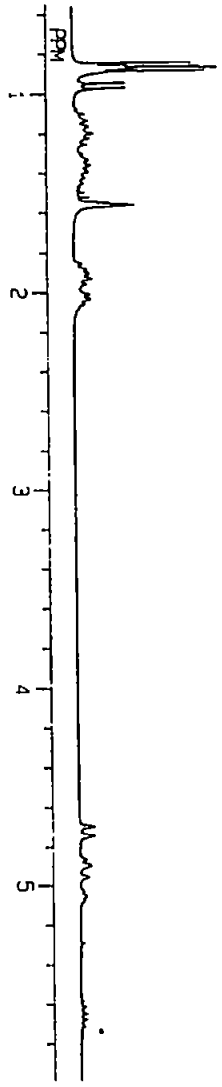


29-JUN-95 10: 04: 47
DFILE C0028
OBNUC 13C
EXMOD DEPT
OFR 67.80 MHz
OBSET 135.00 kHz
OBFIN 5200.0 Hz
POINT 32768
FREQU 20000.0 Hz
SCANS 2500
ACQTM 0.819 sec
PD 1.181 sec
PW1 7.8 us
IRNUC 1H
CTEMP 27.4 c
SLVNT CDCL3
EXREF 22.64 ppm
BF 1.22 Hz
RGAIN 26
OPERATOR : _____

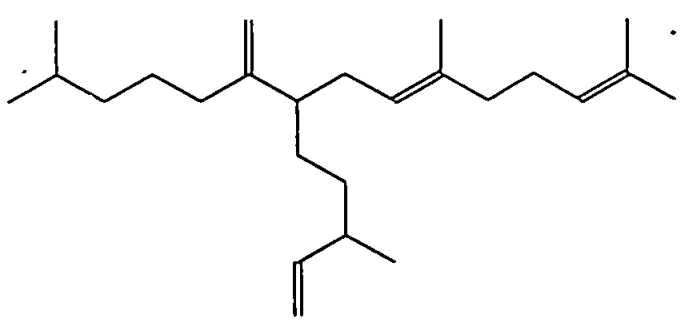




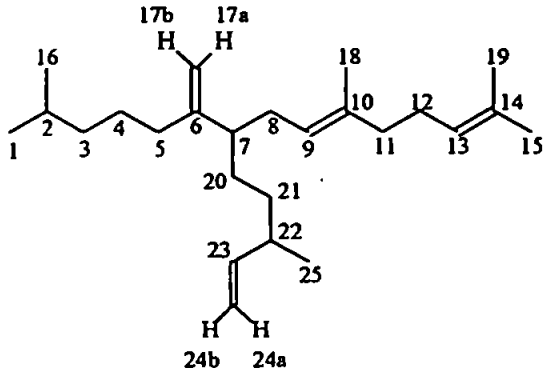




28-JUL-95 12: 16: 45
DFILE BEL64
COMNT C25: 3
EXMOD VCHSHF
OBNUC 13C
OBFIN 536.9 Hz
POINT 1024
FREQU 2481.4 Hz
CLPNT 256
TODAT 128
CLFRQ 1474.9 Hz
SCANS 1000
ACQTM 0.103 sec
PD 0.587 sec
PW1 7.8 us
PW2 15.6 us
PW3 19.6 us
PI1 3.571 ms
PI2 0.339 ms
PI3 1.786 ms
IRATN 511
OBATN 511
CTEMP 25.7 c
CSPED 14 Hz
SLVNT CDCL3
LOOP1 1
XS -2.4232 Hz
CXS -2.8807 Hz
XE 2476.5430 Hz
CXE 1469.1390 Hz
TH 3500.00000
DET 236.1 us
DELAY 160.0 us
OPERATOR : _____

HBI : $C_{25:4}$ GC RI(DB1) : 2144 Origin : *Haslea ostrearia* (growth phase expt) Conc. per cell : ≤ 3.66 pg Conc. (bulk) : 460 μmg g^{-1} (dry wt) Quantity : 18.9 mg Purity : 98% (by GC) Date collected : 10 / 5 / 95 Characterisation techniques, Epoxidation and ^{1}H & ^{13}C NMR	**XI** 

$C_{25:4}$ GC RI 2144 (DBI)
Haslea ostrearia



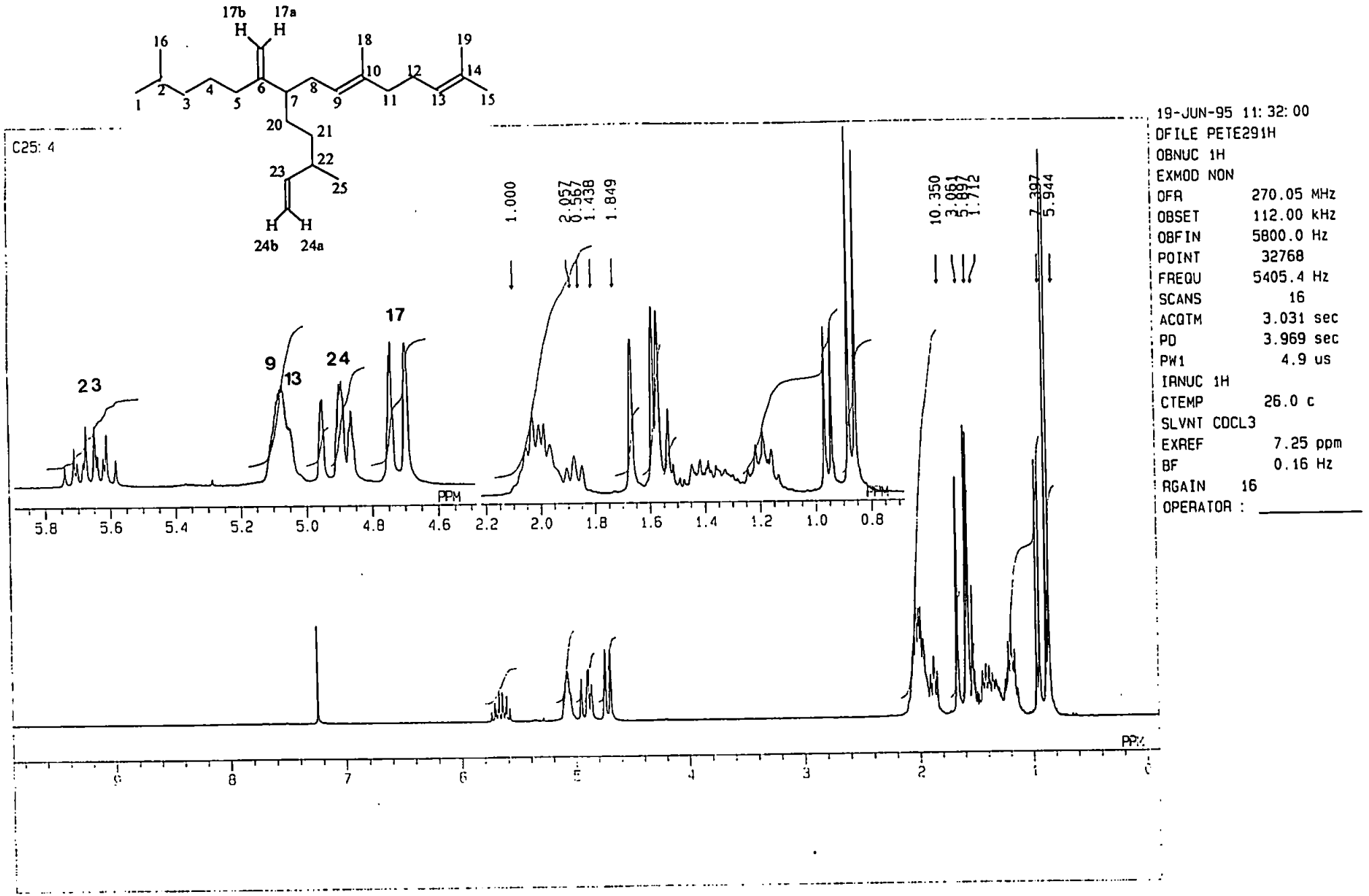
Purity : >98% (by GC)
Quantity : 18.9 mg
Solvent : $CDCl_3$

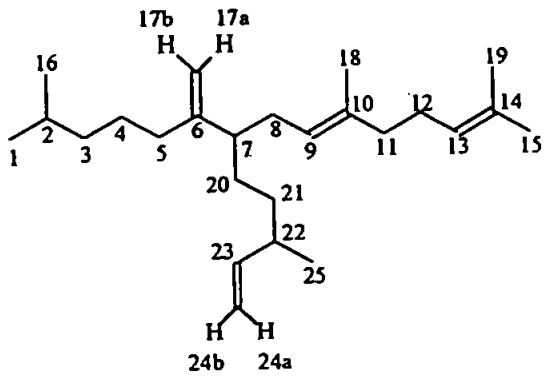
Proton (^{1}H) chemical shifts (multiplicities, integration)

	1, 16	**5**	**7, 11**
δ(ppm)	0.87 (d, 6H)	1.87 (t, 2H)	1.96 (m, 3H)
	8, 12, 22	**9, 13**	**15**
δ(ppm)	2.04 (m, 5H)	5.07, 5.08 (br, m, 2H)	1.67 (s, 3H)
	17a	**17b**	**18**
δ(ppm)	4.70 (br, s, 1H)	4.74 (br, d, 1H)	1.57 (s, 3H)
	19	**23**	**24a**
δ(ppm)	1.59 (s, 3H)	5.67, 5.65* (ddd, 1H)	4.92 (br, d, 1H)
	24b	**25**	
δ(ppm)	4.88 (br, d, 1H)	0.95 (d, 3H)	

Proton coupling constants

	1, 16	**5**	**23**	**25**
J(Hz)	6.6	7.5	J_{trans} = 17.5	6.9
			J_{cis} = 11.5	
			J_{vic} = 7.5	

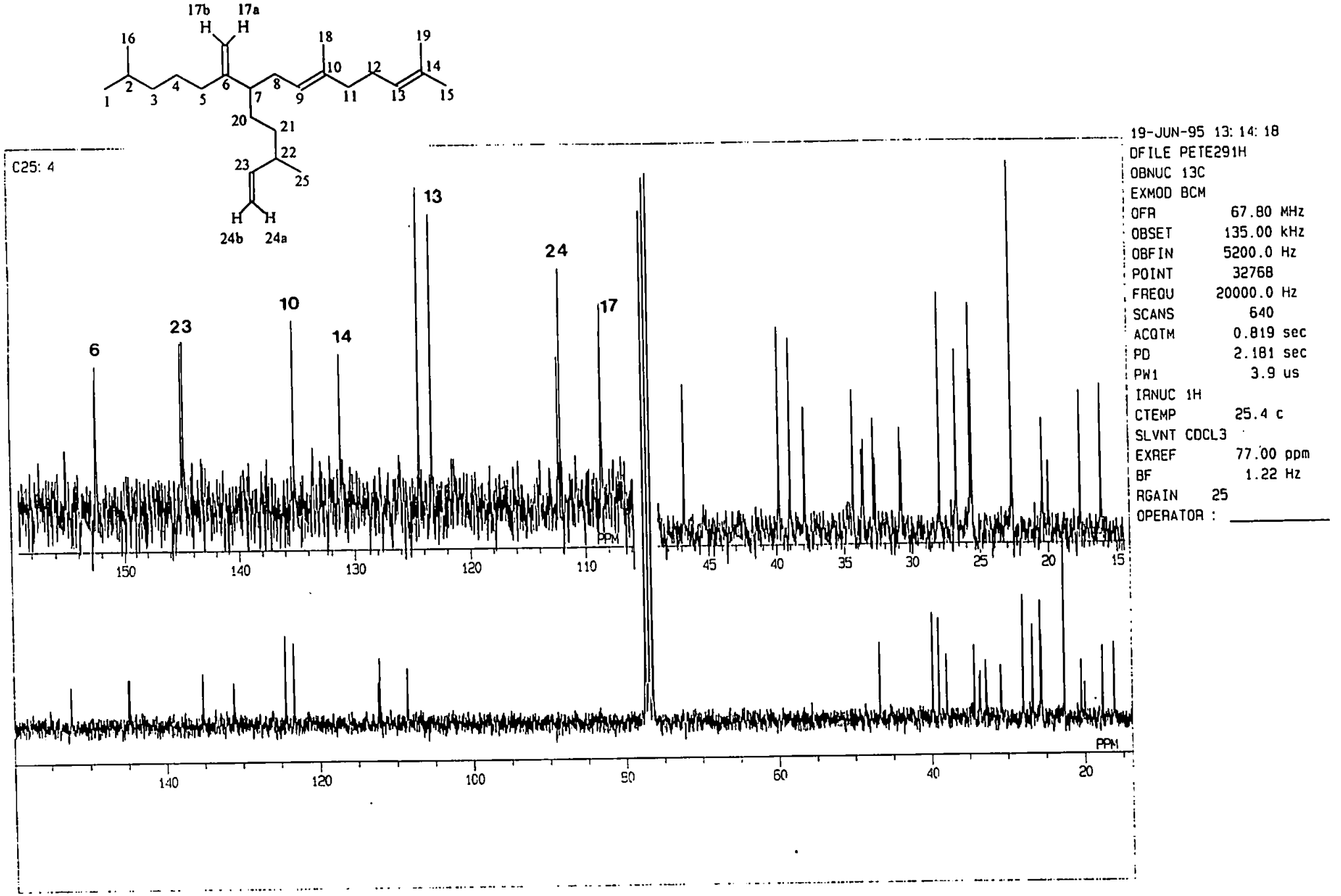

17b 17a
16 18 19
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
20 21 22 23 25
24b 24a
C25: 4
23
9
13
24
17
1.000
2.057
6.567
1.438
1.849
10.350
3.881
5.892
1.712
7.397
5.944
PPM
5.8 5.6 5.4 5.2 5.0 4.8 4.6
2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8
8 7 6 5 4 3 2 1 0
19-JUN-95 11:32:00
OFILE PETE291H
OBNUC 1H
EXMOD NON
OFR 270.05 MHz
OBSET 112.00 kHz
OBFIN 5800.0 Hz
POINT 32768
FREQU 5405.4 Hz
SCANS 16
ACQTM 3.031 sec
PD 3.969 sec
PW1 4.9 us
IRNUC 1H
CTEMP 26.0 c
SLVNT CDCL3
EXREF 7.25 ppm
BF 0.16 Hz
RGAIN 16
OPERATOR :

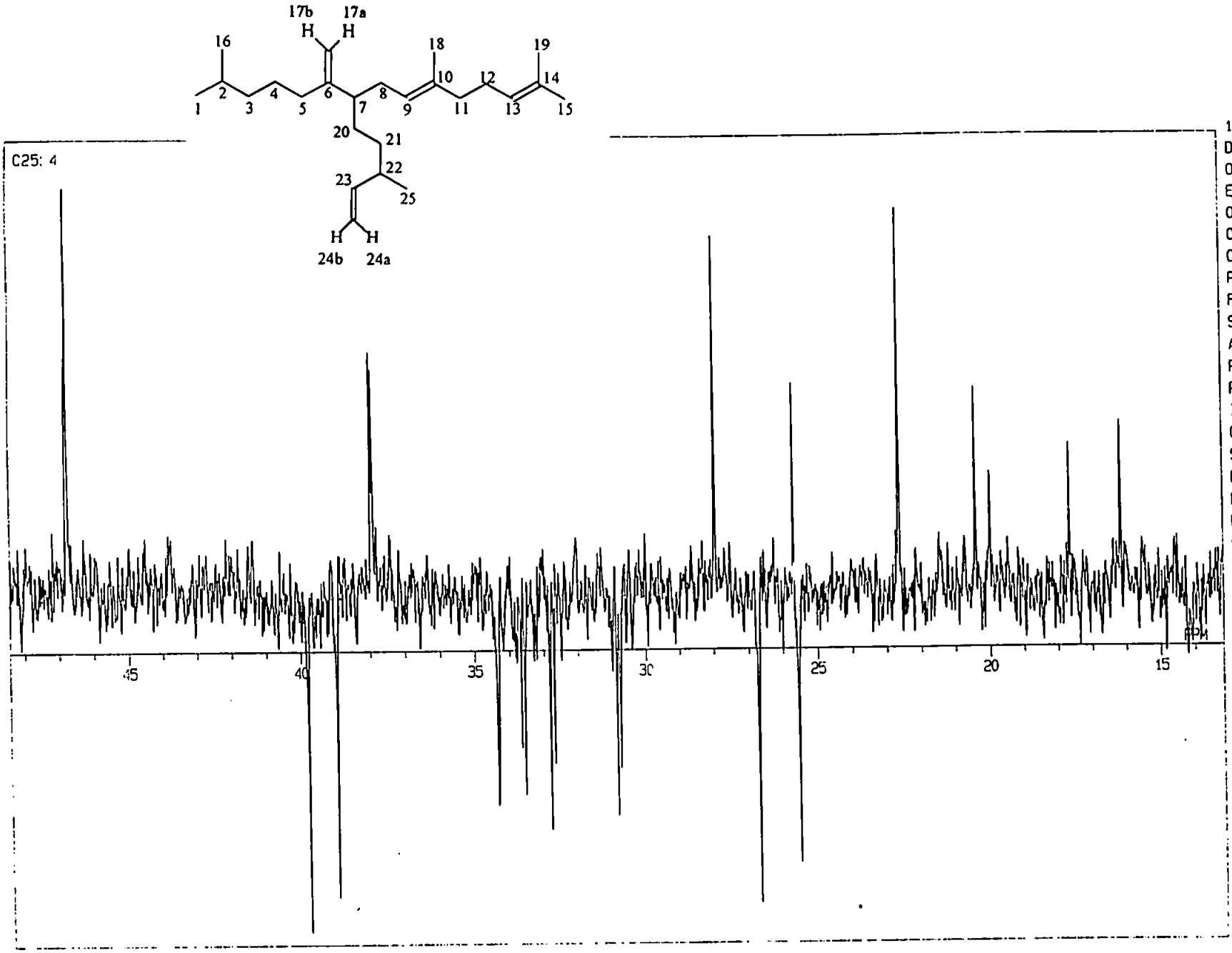


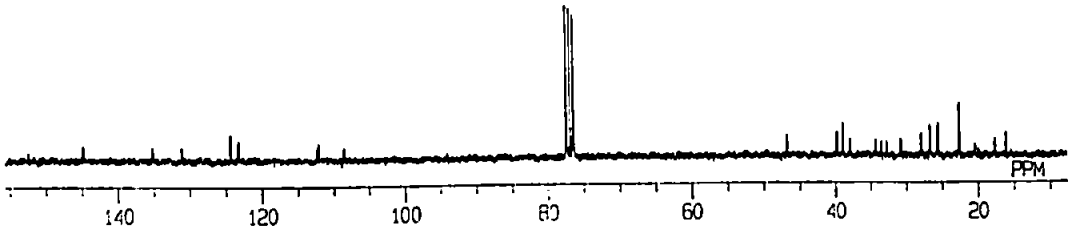
Carbon (^{13}C) chemical shifts

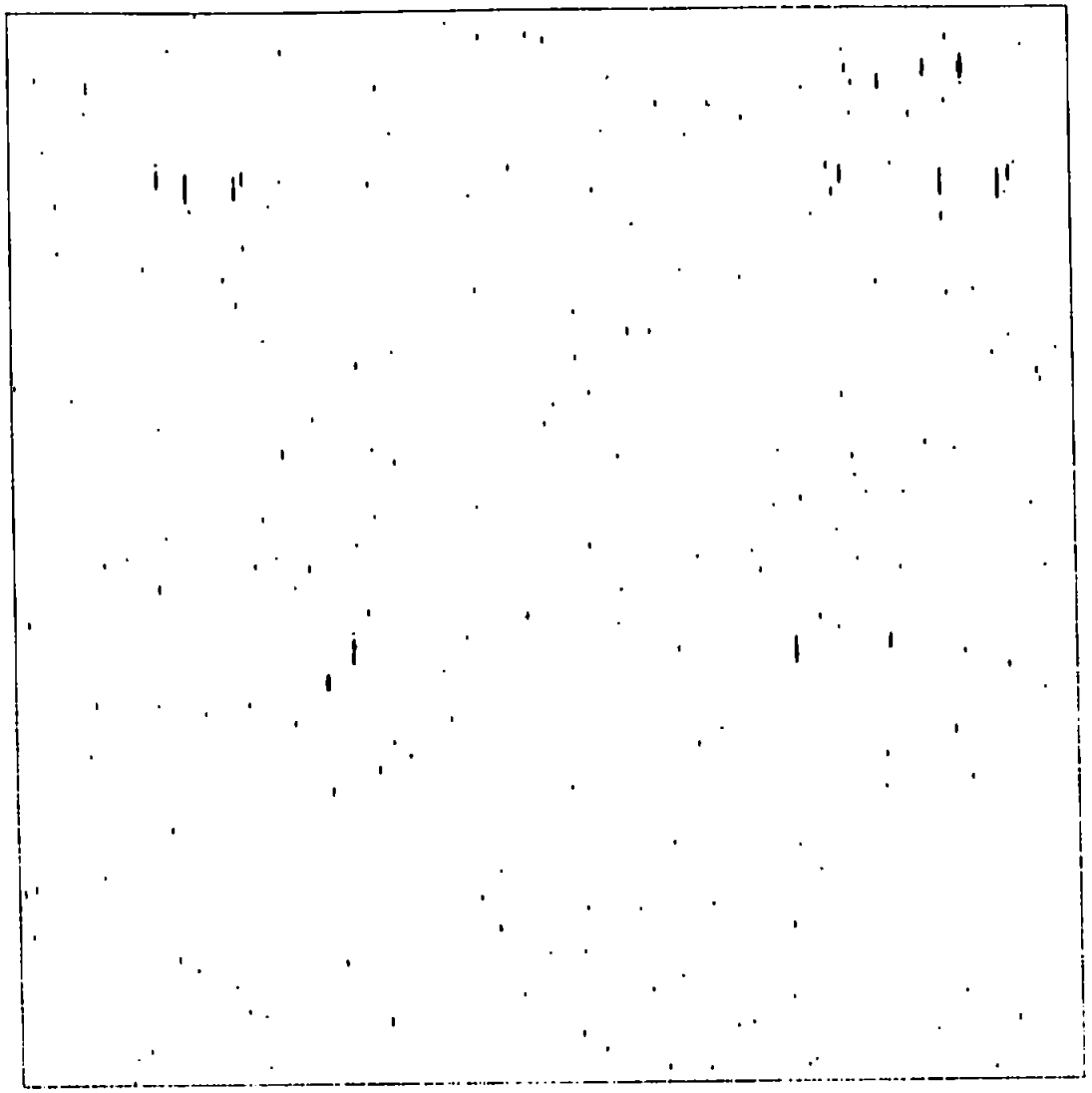
	1	**2**	**3**	**4**	**5**
δ(ppm)	22.6	27.9	39.0	25.6	33.7, 33.6*.
	6		**7**	**8**	**9**
δ(ppm)	152.5, 152.4*		46.8	32.8, 32.7*	123.3
	10	**11**	**12**	**13**	**14**
δ(ppm)	135.2	39.8	26.7	124.4	131.2
	15	**16**	**17**	**18**	**19**
δ(ppm)	25.7	22.6	108.6*	16.2	17.7
	20		**21**		**22**
δ(ppm)	30.9, 30.8*		34.4, 34.3*		38.0, 37.9*
	23		**24**		**25**
δ(ppm)	145.0, 144.8*		112.4, 112.2*		20.4, 20.0*

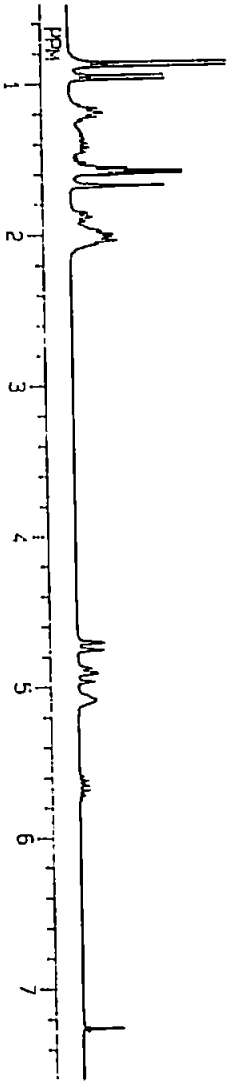
^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively.
Numbering shown in displayed formulae.
Chemical shifts labelled * are due to the presence of diastereoisomers.


17b
17a
H
H
16
18
19
10
12
14
2
4
6
8
1
3
5
7
9
11
13
15
20
21
22
23
25
H
H
24b
24a
C25: 4
6
23
10
14
13
24
17
PPM
150
140
130
120
110
45
40
35
30
25
20
15
140
120
100
80
60
40
20
PPM
19-JUN-95 13: 14: 18
DFILE PETE291H
OBNUC 13C
EXMOD BCM
OFR 67.80 MHz
OBSET 135.00 kHz
OBFIN 5200.0 Hz
POINT 32768
FREQU 20000.0 Hz
SCANS 640
ACQTM 0.819 sec
PD 2.181 sec
PW1 3.9 us
IRNUC 1H
CTEMP 25.4 c
SLVNT CDCL3
EXREF 77.00 ppm
BF 1.22 Hz
RGAIN 25
OPERATOR :

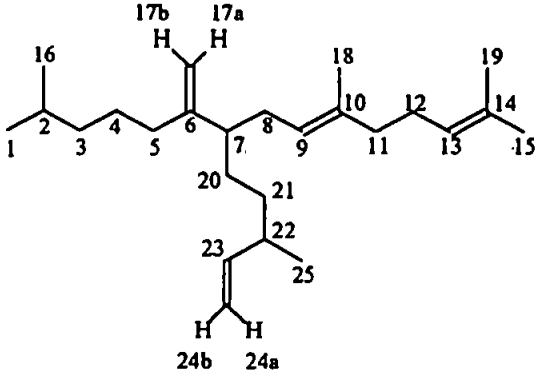

17b
17a
H
H
16
18
19
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
20
21
22
23
25
H
H
24b
24a
C25: 4
45
40
35
30
25
20
15
ppm
19-JUN-95 14: 46: 50
DFILE PETE290E
OBNUC 13C
EXMOD DEPT
OFR 67.80 MHz
OBSET 135.00 kHz
OBFIN 5200.0 Hz
POINT 32768
FREQU 20000.0 Hz
SCANS 320
ACQTM 0.819 sec
PD 1.181 sec
PW1 7.8 us
IRNUC 1H
CTEMP 25.1 c
SLVNT CDCL3
EXREF 77.00 ppm
BF 1.22 Hz
RGAIN 26
OPERATOR :

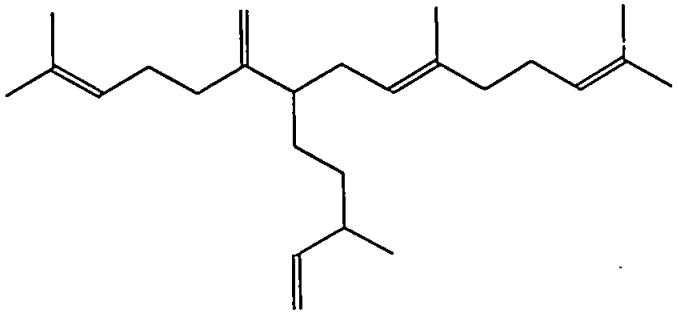




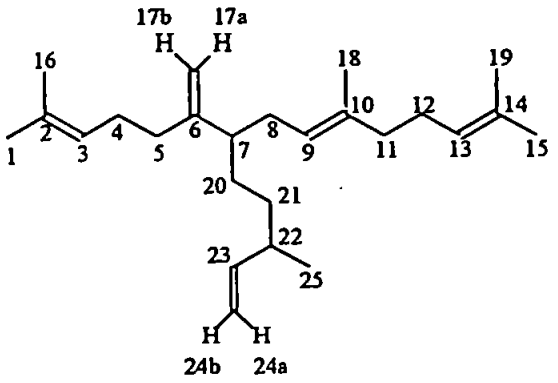



```
22-JUN-95 05: 19: 09
OFILE BEL60
COMNT C25: 4
EXMOD VCOLOC
OBNUC 13C
OBFIN       3915.8 Hz
POINT         4096
FREQU      10060.4 Hz
CLPNT          256
TODAT          128
CLFRQ       1930.5 Hz
SCANS          320
ACQTM        0.102 sec
PD           0.898 sec
PW1            7.8 us
PW2           15.6 us
PW3           19.6 us
PI1         62.500 ms
PI2          0.259 ms
PI3         31.250 ms
IRATN          511
OBATN          511
CTEMP        20.9 c
CSPED          15 Hz
SLVNT CDCL3
LOOP1             1
XS         -2.4561 Hz
CXS        -3.7705 Hz
XE      10055.4500 Hz
CXE      1922.9590 Hz
TH      5250.00000
DET           55.5 us
DELAY         39.6 us
```



HBI : $C_{25:5}$ GC RI(DB1) : 2191 Origin : *Haslea ostrearia* (growth phase expt) Conc. per cell : ≤ 0.83 pg Conc. (bulk) : 92 μg g^{-1} (dry wt) Quantity : 2.8 mg Purity : 89% (by GC) Date collected : 10 / 5 / 95 Characterisation techniques, Epoxidation and ^{1}H & ^{13}C NMR	**XII** 

$C_{25:5}$ GC RI 2191 (DB1)
Haslea ostrearia



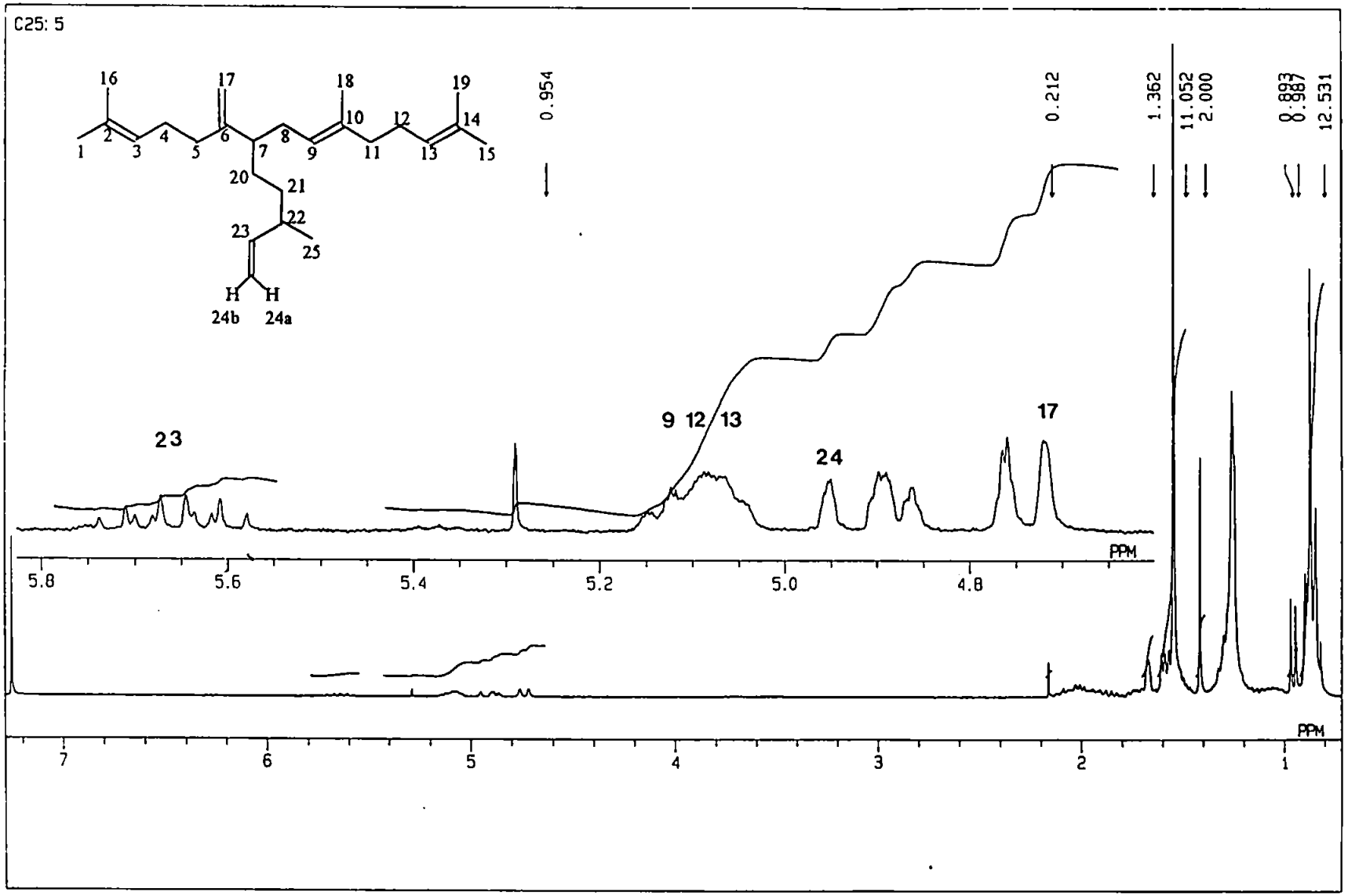
Purity : >89% (by GC)
Quantity : 2.9 mg
Solvent : $CDCl_3$

Proton (^1H) chemical shifts (multiplicities, integration)

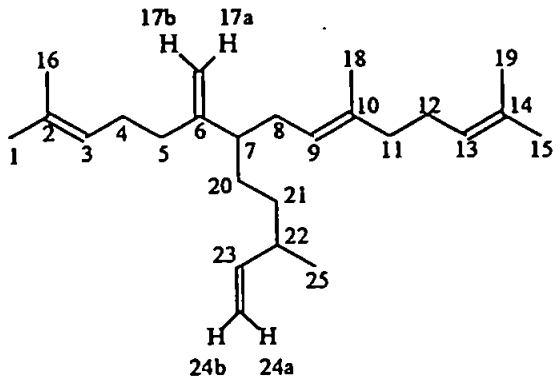
	1, 15	**5**	**7, 11**
δ(ppm)	1.67 (s, 6H)	1.87 (t, 2H)	1.96 (m, 3H)
	8, 12, 22	**9, 12, 13**	**16**
δ(ppm)	2.04 (m, 5H)	5.09 (m, 4H)	1.60 (s, 3H)
	17a	**17b**	**18**
δ(ppm)	4.72 (br, s, 1H)	4.76 (br, d, 1H)	1.57 (s, 3H)
	19	**23**	**24a**
δ(ppm)	1.59 (s, 3H)	5.67, 5.65* (m, 1H)	4.92 (br, d, 1H)
	24b	**25**	
δ(ppm)	4.88 (br, d, 1H)	0.95 (d, 3H)	

Proton coupling constants

	5		**23**	**25**
J(Hz)	7.5	J_{trans}	= 17.5	6.9
		J_{cis}	= 11.5	
		J_{vic}	= 7.5	



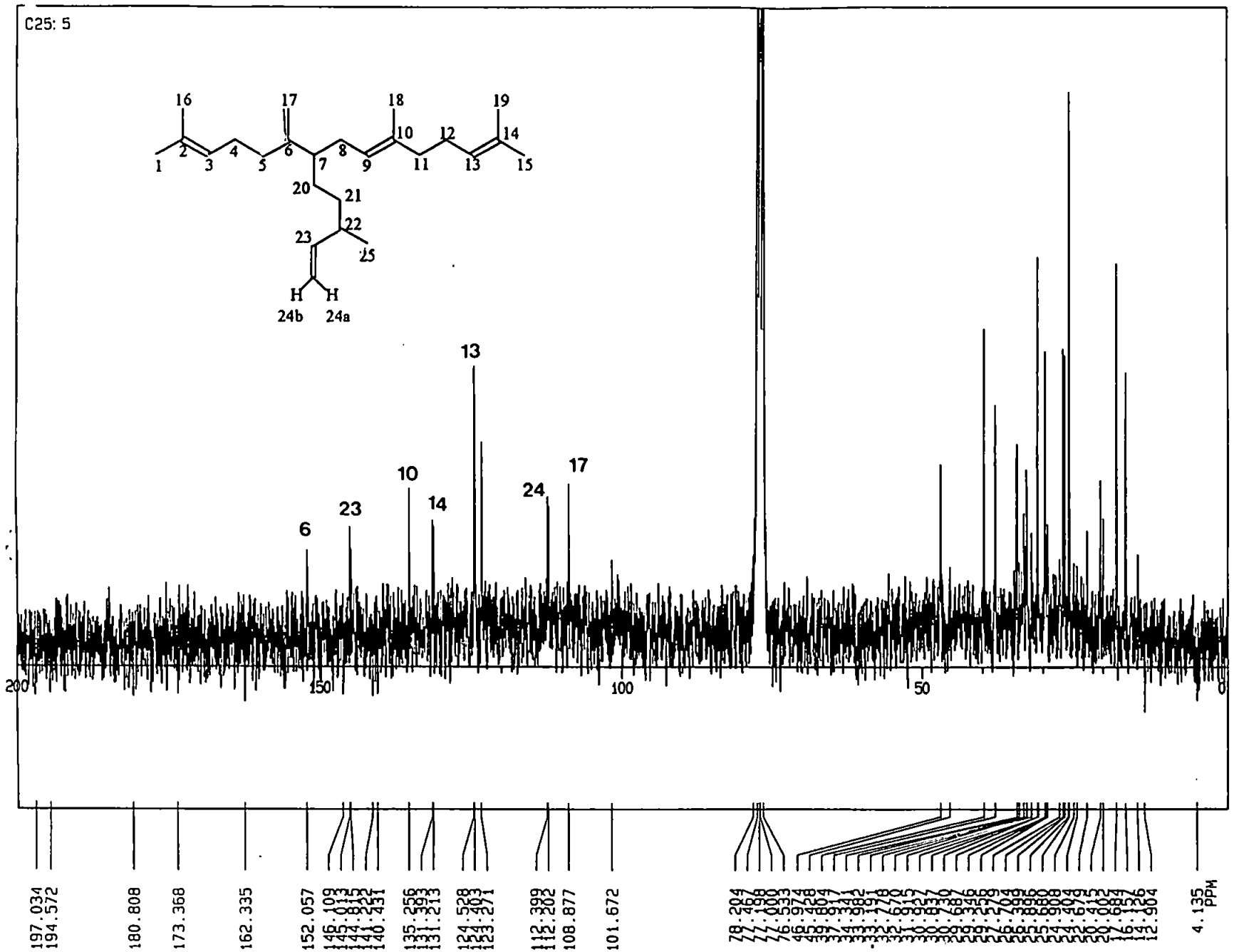
25-SEP-95 17: 28: 49
DFILE BEL70
OBNUC 1H
EXMOD NON
OFR 270.05 MHz
OBSET 112.00 kHz
OBFIN 5800.0 Hz
POINT 32768
FREQU 5405.4 Hz
SCANS 640
ACQTM 3.031 sec
PD 3.969 sec
PW1 4.9 us
IRNUC 1H
CTEMP 23.8 c
SLVNT CDCL3
EXREF 5.54 ppm
BF 0.16 Hz
RGAIN 22
OPERATOR : _____



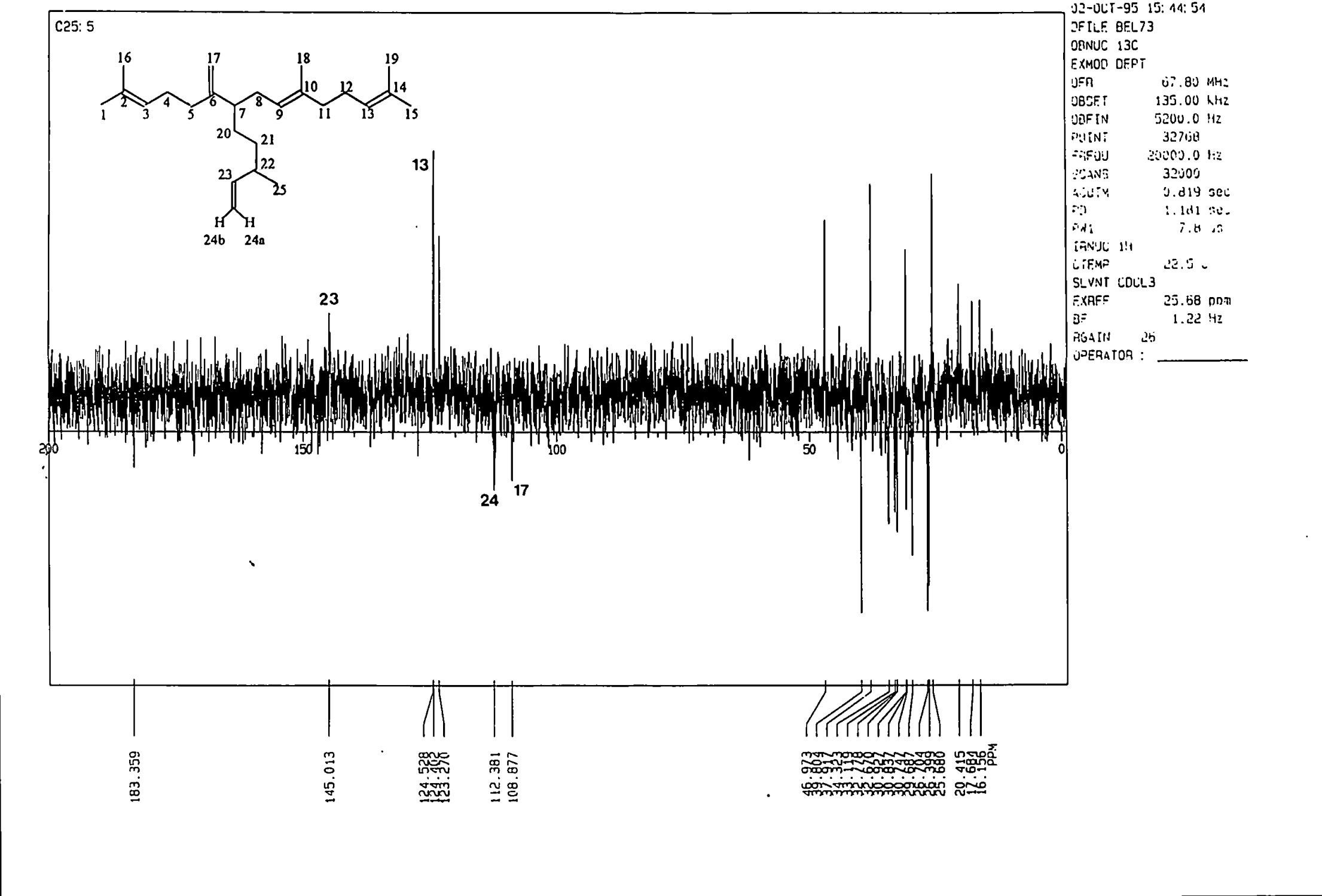
Carbon (^{13}C) chemical shifts

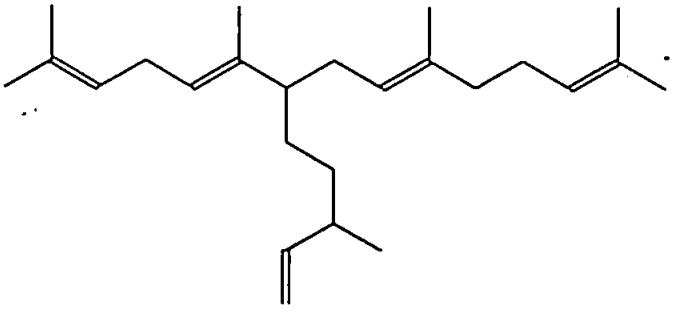
	1	**2**	**3**	**4**	**5**
δ(ppm)	25.7	131.2	124.4	26.6	33.7, 33.6*
	6		**7**	**8**	**9**
δ(ppm)	152.5, 152.4*		46.8	32.8, 32.7*	123.3
	10	**11**	**12**	**13**	**14**
δ(ppm)	135.2	39.8	26.7	124.4	131.2
	15	**16**	**17**	**18**	**19**
δ(ppm)	25.7	17.7	108.6*	16.2	17.7
	20		**21**		**22**
δ(ppm)	30.9, 30.8*		34.4, 34.3*		38.0, 37.9*
	23		**24**		**25**
δ(ppm)	145.0, 144.8*		112.4, 112.2*		20.4, 20.0*

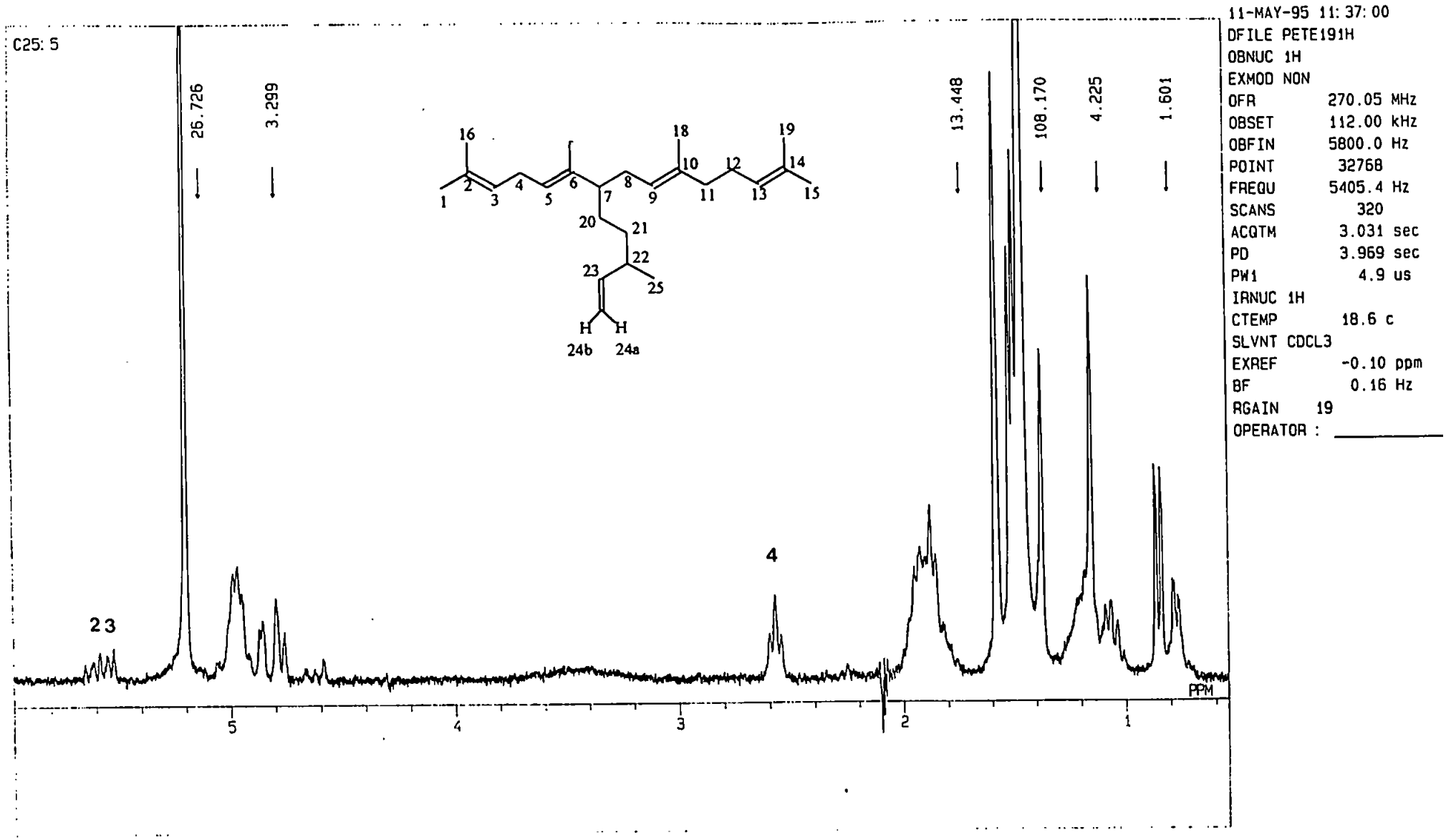
^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively.
Numbering shown in displayed formulae.
Chemical shifts labelled * are due to the presence of diastereoisomers.



04-OCT-95 13:50:23
DFILE BEL74
OBNUC 13C
EXMOD BCM
OFR 67.80 MHz
OBSET 135.00 kHz
OBFIN 5200.0 Hz
POINT 32768
FREQU 20000.0 Hz
SCANS 55000
ACQTM 0.819 sec
PD 2.181 sec
PW1 3.9 us
IRNUC 1H
CTEMP 23.1 c
SLVNT CDCL3
EXREF 77.00 ppm
BF 1.22 Hz
RGAIN 26
OPERATOR : _____


C25: 5
02-OCT-95 15:44:54
DFILE BEL73
OBNUC 13C
EXMOD DEPT
OFR 67.80 MHz
OBSET 135.00 kHz
OBFIN 5200.0 Hz
POINT 32768
FREQU 20000.0 Hz
SCANS 32000
ACQTM 0.819 sec
PD 1.181 sec
PW1 7.8 us
IRNUC 1H
CTEMP 22.5 C
SLVNT CDCL3
EXREF 25.68 ppm
BF 1.22 Hz
RGAIN 26
OPERATOR :
13
23
24
17
200
150
100
50
0
183.359
145.013
124.528
124.366
123.270
112.381
108.877
46.973
39.804
37.811
37.328
33.118
32.270
25.680
20.415
17.684
16.156
ppm

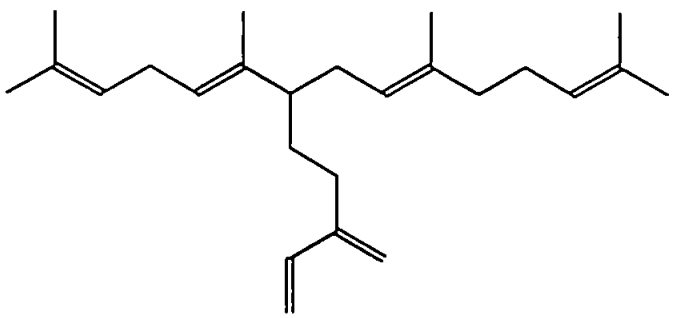
HBI : $C_{25:5}$ GC $RI_{(DB1)}$: 2201 Origin : *Haslea ostrearia* Conc. : 22 µg g^{-1} (dry wt) Quantity : < 1.0 mg Purity : 98% (by GC) Date collected : 18 / 10 / 88 Characterisation techniques, Epoxidation and ^{1}H NMR	**XIII** 


C25: 5
26.726
3.299
13.448
108.170
4.225
1.601
16
18
19
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
20
21
22
23
25
H
H
24b
24a
23
4
5
4
3
2
1
PPM
11-MAY-95 11: 37: 00
DFILE PETE191H
OBNUC 1H
EXMOD NON
OFR 270.05 MHz
OBSET 112.00 kHz
OBFIN 5800.0 Hz
POINT 32768
FREQU 5405.4 Hz
SCANS 320
ACQTM 3.031 sec
PD 3.969 sec
PW1 4.9 us
IRNUC 1H
CTEMP 18.6 c
SLVNT CDCL3
EXREF -0.10 ppm
BF 0.16 Hz
RGAIN 19
OPERATOR :

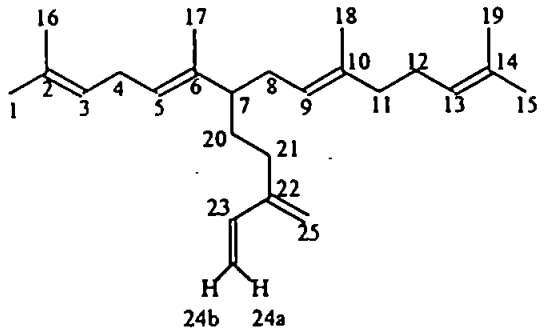
HBI	: $C_{25:6}$
GC $RI_{(DB1)}$	: 2248
Origin	: *Haslea ostrearia*
Conc.	: 77 µg g^{-1} (dry wt)
Quantity	: 6.0 mg
Purity	: 99% (by GC)
Date collected	: 18 / 10 / 88

Characterisation techniques,
Epoxidation and ^{1}H & ^{13}C NMR

XIV



$C_{25:6}$ GC RI 2248 (DB1)
Haslea ostrearia



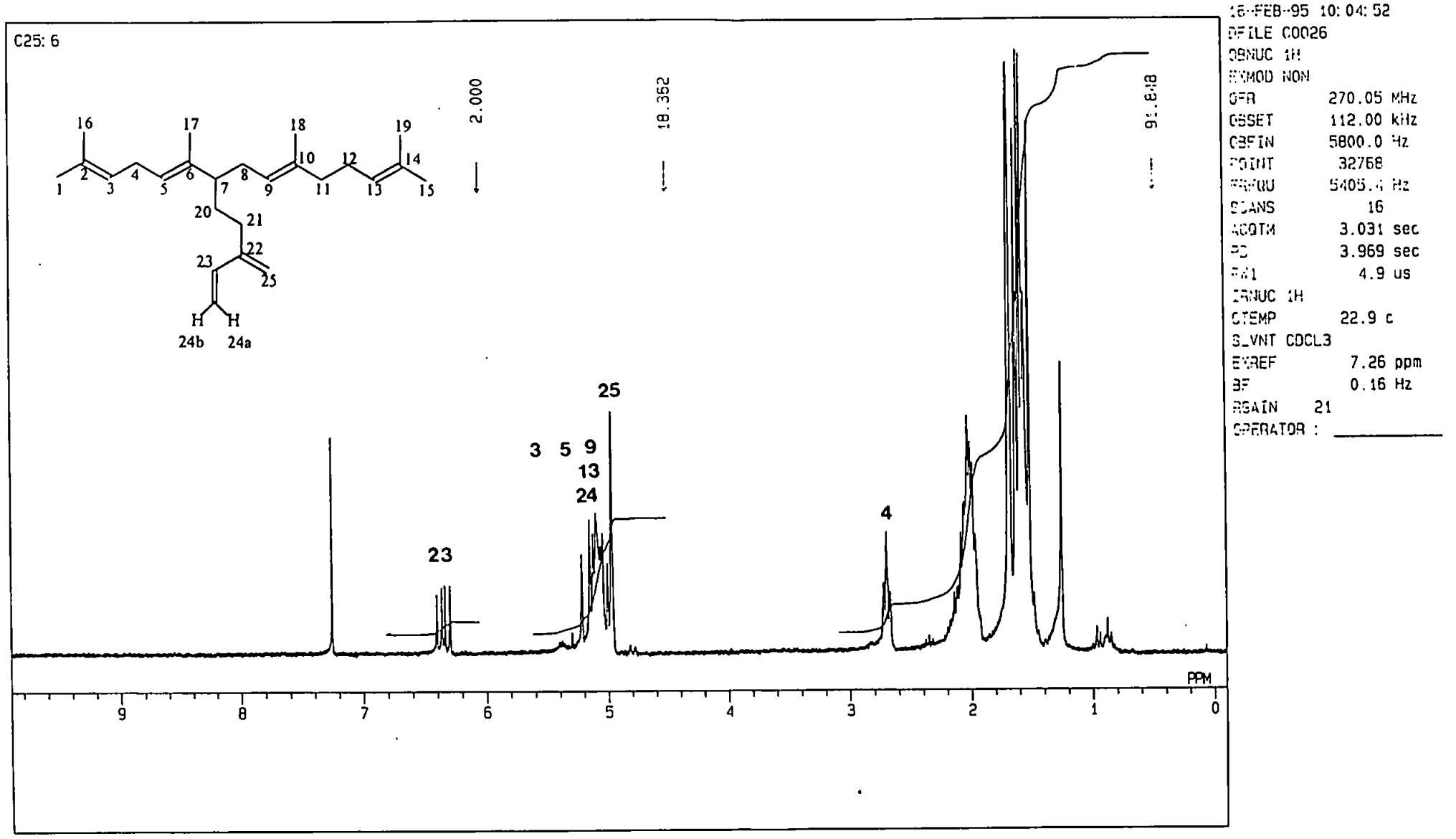
Purity : >98% (by GC)
Quantity : 3.0 mg
Solvent : $CDCl_3$

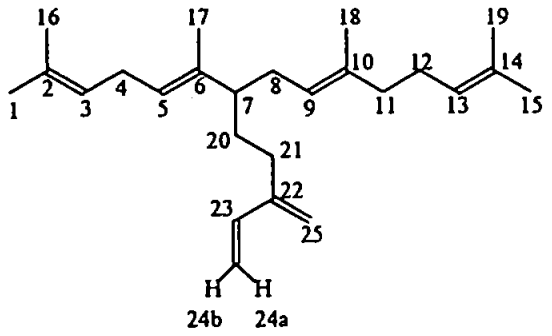
Proton (^1H) chemical shifts (multiplicities, integration)

	1, 15, 16, 17, 18, 19	**3, 5, 9, 13**	**4**
δ(ppm)	1.5 -1.7 (s, 18H)	5.05 - 5.13 (br, m, 2H)	2.70 (t, 2H)
	7, 8, 11, 12, 21	**9, 13**	**23**
δ(ppm)	1.96 - 2.18 (m, 9H)	5.07, 5.08 (br, m, 2H)	6.35 (dd, 1H)
	24a	**24b**	**25**
δ(ppm)	5.19 (d, 1H)	5.03 (d, 1H)	4.98 (br, s, 2H)

Proton coupling constants

	J_{H4-H3}	J_{H4-H5}	**H-23**
J(Hz)	6.8	6.8	J_{trans} = 17.5
			J_{cis} = 10.9

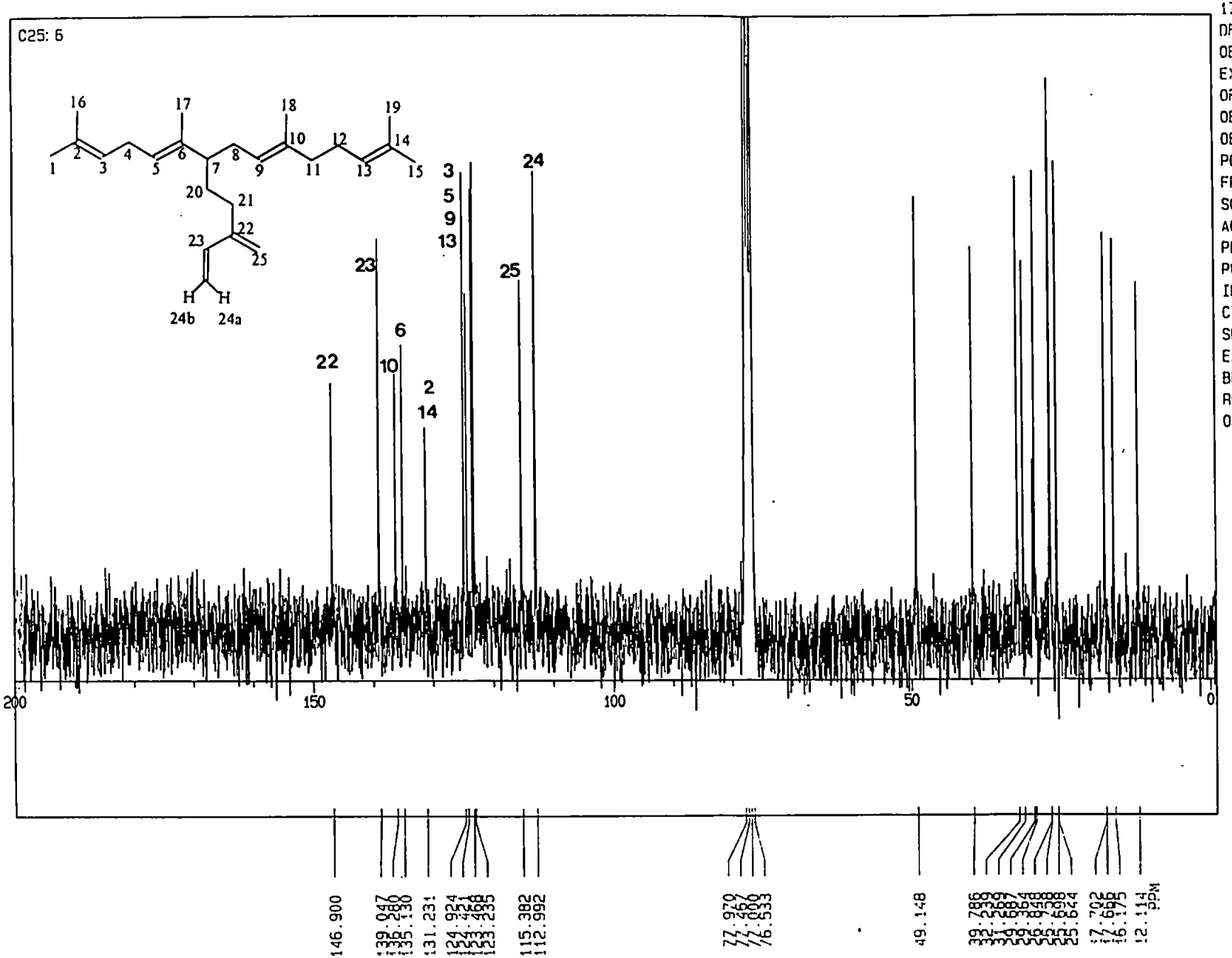

C25: 6
2.000
18.362
91.848
16
17
18
19
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
20
21
22
23
25
H
H
24b
24a
25
3 5 9
13
24
4
23
PPM
9
8
7
6
5
4
3
2
1
0
16-FEB-95 10:04:52
FILE C0026
OBNUC 1H
EXMOD NON
OFR 270.05 MHz
OBSET 112.00 kHz
OBFIN 5800.0 Hz
POINT 32768
FREQU 5405.4 Hz
SCANS 16
ACQTM 3.031 sec
PD 3.969 sec
PW1 4.9 us
IRNUC 1H
CTEMP 22.9 c
SLVNT CDCL3
EXREF 7.26 ppm
BF 0.16 Hz
RGAIN 21
OPERATOR :



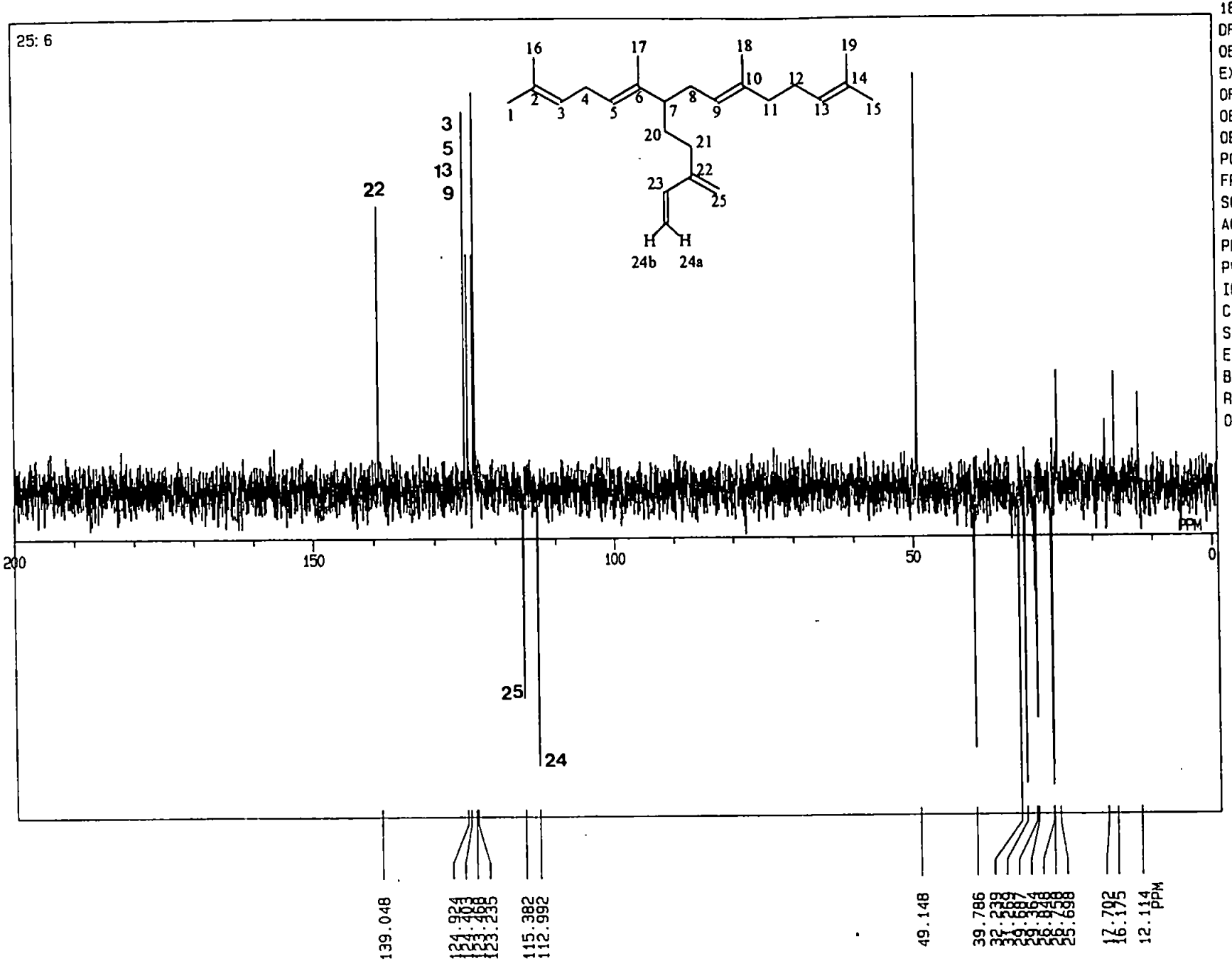
Carbon (^{13}C) chemical shifts

	1	**2**	**3**	**4**	**5**
δ(ppm)	25.6	131.2	124.3	39.8	124.3
	6	**7**	**8**	**9**	
δ(ppm)	135.1	49.1	32.2	124.3	
	10	**11**	**12**	**13**	**14**
δ(ppm)	136.3	26.7	26.7	124.3	131.2
	15	**16**	**17**	**18**	**19**
δ(ppm)	25.6	17.7	12.1	16.2	17.7
	20	**21**	**22**	**23**	**24**
δ(ppm)	32.2	32.2	146.9	139.0	113.0
	25				
δ(ppm)	115.4				

^{1}H and ^{13}C chemical shifts are in ppm using residual $CHCl_3$ as an internal reference (7.25 and 77.0 ppm for ^{1}H and ^{13}C respectively. Numbering shown in displayed formulae.



17-FEB-95 08:38:07
DFILE C0027
OBNUC 13C
EXMOD BCM
OFR 67.80 MHz
OBSET 135.00 kHz
OBFIN 5200.0 Hz
POINT 32768
FREQU 20000.0 Hz
SCANS 26721
ACQTM 0.819 sec
PD 2.181 sec
PW1 3.9 us
IRNUC 1H
CTEMP 20.8 c
SLVNT CDCL3
EXREF 77.00 ppm
BF 1.22 Hz
RGAIN 26
OPERATOR : _____



18-FEB-95 03: 56: 10
DFILE C0028
OBNUC 13C
EXMOD DEPT
OFR 67.80 MHz
OBSET 135.00 kHz
OBFIN 5200.0 Hz
POINT 32768
FREQU 20000.0 Hz
SCANS 25000
ACQTM 0.819 sec
PD 1.181 sec
PW1 7.8 us
IRNUC 1H
CTEMP 20.7 c
SLVNT CDCL3
EXREF 49.15 ppm
BF 1.22 Hz
RGAIN 26
OPERATOR : _____

PUBLICATION

Structural Determination of a Highly Branched C_{25} Sedimentary Isoprenoid Biomarker by NMR Spectroscopy and Mass Spectrometry

Simon T. Belt, David A. Cooke, Simon J. Hird and Steve Rowland

Petroleum and Environmental Geochemistry Group, Department of Environmental Sciences, University of Plymouth, Drake Circus, Plymouth, Devon, UK PL4 8AA

The C_{25} diene 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)pentadec-5-ene has been isolated from benthic sediments and characterised by ^1H and ^{13}C NMR spectroscopy together with mass spectrometry.

Polyunsaturated lipids found in sediments and sedimentary rocks have proved to be extremely valuable tools for the determination of palaeoseawater surface temperatures and thus for palaeoclimatic reconstruction.[1-3] Despite this, a complete structural analysis (*i.e.* beyond the parent skeleton)[5-7] of the abundant and widespread polyunsaturated highly branched isoprenoid hydrocarbons found in sediments[4] has been confined to a relatively few examples.[8-10] The recent discovery that these highly branched isoprenoids are biosynthesised by a restricted number of diatomaceous algae[11] is likely to increase their value as 'biomarker' compounds.[12] In this communication, we describe the detailed structural characterisation of a widely occurring highly branched isoprenoid C_{25} diene **1** using NMR spectroscopy and mass spectrometry. Compound **1** is a colourless oil that was isolated from recent benthic sediments of the Caspian Sea by solvent extraction, column chromatography and Argentation TLC.[9] Initial identification of a highly branched C_{25} diene structure came from the EI mass spectrum ($M^+ = 348$), which showed two degrees of unsaturation, together with its low RI 2079 (DB1). Further, the position of the T branch in the isoprenoid chain was located by hydrogenation of **1** to a C_{25} alkane which co-chromatographed (GC) with previously synthesised 2,6,10,14-tetramethyl-7-(3-methylpentyl)pentadecane[6] on three phases (DB1, DB5 and DB-wax). Having established the parent structure, the locations of the two double bonds needed to be established.

Sufficient **1** (*ca.* 5 mg) was obtained from a bulk sediment extraction for ^1H and ^{13}C NMR spectroscopic analysis.† The fully proton decoupled ^{13}C spectrum shows a total of 25 resonances with four alkenic and 21 aliphatic type carbons as expected. Analysis *via* the DEPT sequence reveals the presence of seven methyl, ten methylene, seven methine and a single quaternary carbon (C-6) consistent with structure **1** (amongst others), while the observation of a methylene carbon in the alkenic region (δ 112.3) confirms the presence of a double bond in a terminal position. The ^1H spectrum of **1** consists of resonances associated with alkenic, allylic and aliphatic protons. The most conspicuous of these are the three sets of resonances at δ 5.65, 4.93 and 4.89, which arise from a vinyl ($-C_2H_3$) functionality (Fig. 1). Given the established C_{25} isoprenoid skeleton, it is clear that this double bond must be located at C-24. Proton H-23 appears as a low field seven line multiplet due to a *trans* coupling to H-24a (17 Hz), a *cis* coupling to H-24b (10 Hz) and an additional vicinal coupling to H-22 (8 Hz). This latter coupling to a single allylic H-22 proton is further verification of the assignment of the first double bond. The allylic H-22 proton itself appears as a multiplet at δ 2.08 due to couplings to H-23, H-21 and H-25. This assignment was based upon the following decoupling experiments. Primarily, when the resonance at δ 2.08 was irradiated, the seven line multiplet associated with H-23 collapsed to a doublet of doublets due to the H-24a and H-24b couplings. Similarly, when a reciprocal irradiation was carried out at δ 5.65, one of the couplings at δ 2.08 was eliminated. The irradiation of H-22 also resulted in the transformation of the doublet at δ 0.95 to a singlet, allowing assignment of H-25. The only singlet in the spectrum is found at δ 1.42 and arises due to H-17. The remaining methyl protons [H-18, H-(1,16), H-(15,19)] show couplings to the neighbouring methine protons and appear as doublets in the region δ 0.8–0.9.

Further analysis of the ^1H spectrum demonstrates the presence of an additional alkenic proton (H-5) which appears as a triplet due to an allylic methylene coupling (H-4). The absence of a proton on the remaining alkenic carbon (C-6) is consistent with the observation of a single quaternary carbon in the ^{13}C spectrum. The assignment of the H-4 allylic protons was made by irradiating H-5 and observing the change in multiplicity at δ 1.99 from a quartet to a triplet. Similarly, irradiation at δ 1.99 resulted in the observation of a singlet for the H-5 resonance. At this stage, the structural data described for the second double bond position was consistent with six

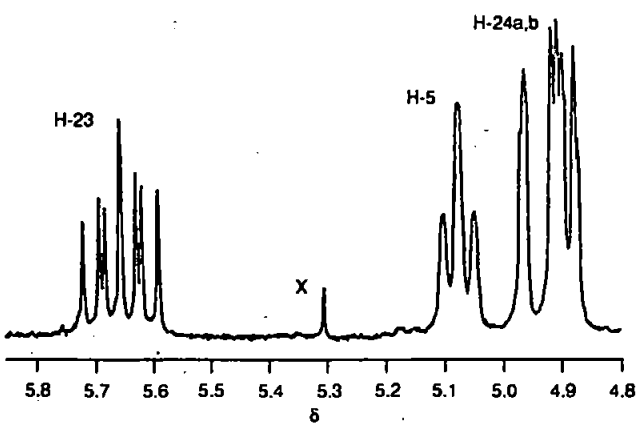


Fig. 1 ^1H spectrum showing the resonances due to the 2 alkenic functions in **1**. The peak marked X is due to an impurity.

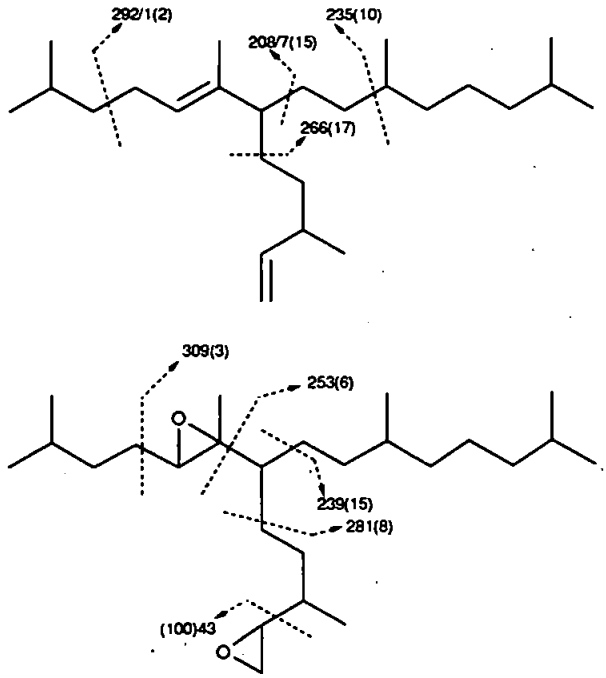
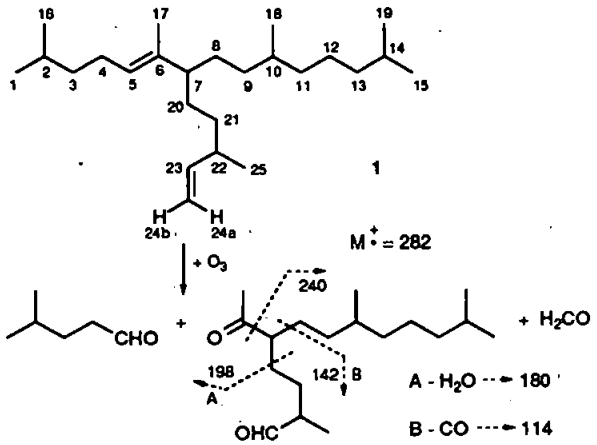


Fig. 2 Fragmentation pathways observed from the mass spectra of **1** and its bis-epoxidation product.



Scheme 1 *Reagents and conditions*: 0.5 mmol dm^{-3} (CH_2Cl_2), O_3 flow rate 5 cm^3 min^{-1}, −70 °C

other C_{25} diene structural isomers. However, by inspection of these, it could be seen that structure **1** was the only isomer that contained a total of four allylic methylene or methine protons (H-22, H-4, H-7) in agreement with the integration of these proton resonances. From this, it is possible to assign a single structure for compound **1**. The allylic proton located at the T-branch (H-7) occurs as a multiplet at δ 1.8 with couplings to H-20 and H-8. The corresponding carbon resonance is shifted to significantly higher frequency (at least 10 ppm, identified *via* ^1H–^{13}C COSY compared with the other sp^3 hybridised carbons.

Additional confirmation of structure **1** has been achieved *via* ozonolysis and epoxidation in conjunction with mass spectrometry. Ozonolysis of **1** yields the aldehyde and ketone products as shown in Scheme 1, while Fig. 2 demonstrates how epoxidation of the parent diene can be monitored conveniently by mass spectrometry.

We acknowledge Dr Roger Evens for obtaining the NMR spectra, Mr R. Srodzinski for help with the MS and M-Scan Ltd for provision of the sediment samples.

Received, 20th June 1994; Com. 4/03737E

Footnotes

† *Selected NMR data* for **1** at 270 MHz for ^1H in $CDCl_3$. ^1H and ^{13}C. Numbering shown in displayed formula. Missing integrations due to overlap. ^1H: δ 5.65 (ddd, 1H, $J_{H23-H24a}$ 17, $J_{H23-H24b}$ 10, $J_{H23-H22}$ 8 Hz, H-23) 4.93 (dd, $J_{H24a-H24b}$ 2 Hz, H-24a), 4.89 (dd, H-24b), 5.08 (t, 1H, J_{H5-H9} 7 Hz, H-5), 2.08 (m, H-22), 1.99 (q, $J_{H4-H5} = J_{H-H3}$ = 7 Hz, H-4), 1.80 (m, 1H, H-7), 1.42 (s, 3H, H-17), 0.95 [d, 3H, $J_{H25-H22}$ = 6.5 Hz, H-25], 0.88 [d, 6H, $J_{H(1,16)-H2}$ = 4.5 Hz, H(1,16)], 0.85 [d, 6H, $J_{H15,19-H14}$ = 4.5 Hz, H(15,19)], 0.82 (d, 3H, $J_{H18-H10}$ = 6.5 Hz, H-18); $^{13}C\{^1H\}$, DEPT and ^1H–^{13}C COSY: δ 145.0 (C-23), 136.3 (C-6), 126.3 (C-5), 112.3 (C-24), 49.3 (C-7), 37.8 (C-22), 25.5 (C-4), 22.5–22.7 (C-(1,16,15,19)), 20.5 (C-25), 19.9 (C-18), 11.3 (C-17).

‡ *Conditions for epoxidation*: 37.5 μmol MCPBA, 6.25 μmol **1** in 0.25 cm^3 CH_2Cl_2, 25 °C, 90 h.

References

1 J. P. Jasper and J. M. Hayes, *Nature*, 1990, **347**, 462.
2 M. Lyle, *Nature*, 1992, **356**, 385.
3 G. Eglinton, S. A. Bradshaw, A. Rosell, M. Sarnthein, U. Pflaumann and R. Tiedemann, *Nature*, 1992, **356**, 423.
4 S. J. Rowland and J. N. Robson, *Mar. Env. Res.*, 1990, **30**, 191.
5 D. A. Yon, G. Ryback and J. R. Maxwell, *Tetrahedron Lett.*, 1982, **23**, 2143.
6 J. N. Robson and S. J. Rowland, *Nature*, 1986, **324**, 561.
7 J. N. Robson and S. J. Rowland, *Tetrahedron Lett.*, 1988, **29**, 3837.
8 I. Yruela, A. Barbe and J. O. Grimalt, *J. Chromatogr. Sci.*, 1990, **28**, 421.
9 S. J. Hird, R. Evens and S. J. Rowland, *Mar. Chem.*, 1992, **37**, 117.
10 R. E. Summons, R. A. Barrow, R. J. Capon, J. M. Hope and C. Stranger, *Aust. J. Chem.*, 1993, **46**, 907.
11 J. K. Volkman, S. M. Barrett and G. A. Dunstan, *Org. Geochem.*, 1994, **21**, 407.
12 K. E. Peters and J. M. Moldowan, *The Biomarker Guide*, Prentice Hall, UK, 1993.