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C_{25} highly branched isoprenoid alkenes from the marine benthic diatom *Pleurosigma strigosum*

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Abstract

The hydrocarbon composition of the marine diatom *Pleurosigma strigosum* isolated from coastal Mediterranean sediments is described. A suite of five C_{25} highly branched isoprenoid (HBI) alkenes with 2–5 double bonds were detected together with *n*-$C_{21:4}$ and *n*-$C_{21:5}$ alkenes and squalene. The analysis by ^{1}H and ^{13}C NMR spectroscopy of two isolated HBI alkenes allowed the structural identification of a novel C_{25} HBI triene (2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadeca-5*E*,13-diene) and the first identification in diatom cells of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5*E*-ene, an HBI previously detected in marine sediments and particulate matter. The other minor C_{25} HBIs detected were a tetraene and a pentaene that have been previously identified in other diatoms from the genera *Haslea* and *Rhizosolenia*, and one other C_{25} tetraene that could not be structurally identified. The structures of the HBI alkenes of *P. strigosum* were compared with those of C_{25} homologues previously identified in three other *Pleurosigma* sp. (*Pleurosigma intermedium*, *Pleurosigma planktonicum* and *Pleurosigma* sp.). Unlike most structures previously reported, none of the HBI alkenes produced by *P. strigosum* showed an unsaturation at C7–C20, or *E*/*Z* isomerism of the trisubstituted double bond at C9–C10 (whenever present).

Keywords: *Pleurosigma strigosum*; Diatoms; Bacillariophyceae; Highly branched isoprenoid alkenes; Structural identification; NMR spectroscopy; Mass spectrometry

1. Introduction

Highly branched isoprenoid (HBI) hydrocarbons are widespread components in modern marine sediments (Rowland and Robson, 1990; Belt et al., 2000a). During the last decades, the identification of C_{25} HBI alkenes in natural populations of diatoms (Nichols et al., 1988; Summons et al., 1993; Johns et al., 1999) and of C_{25} and C_{30} HBI alkenes in laboratory cultures of isolated diatoms (e.g. Volkman et al., 1994, 1998; Wraige et al., 1997) clearly established that diatoms are the major source of HBI alkenes in sediments.

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To date, HBI alkenes present in marine sediments appear to originate from four genera of marine diatoms (Class Bacillariophyceae), namely: *Rhizosolenia*, *Haslea*, *Navicula* and *Pleurosigma*. C_{30} HBI alkenes with 4–6 double bonds have been detected in cultures of various *Rhizosolenia* sp. (Volkman et al., 1994, 1998; Rowland et al., 2001; Massé et al., 2004; Sinninghe Damsté et al., 2004), whereas numerous C_{25} HBI dienes through hexaenes have been characterised in cultures of seven species of *Haslea* (Volkman et al., 1994; Wraige et al., 1997, 1999; Allard et al., 2001; Sinninghe Damsté et al., 2004), three species of *Navicula* (Belt et al., 2001b; Sinninghe Damsté et al., 2004), various *Rhizosolenia* sp. (Sinninghe Damsté et al., 1999a, 2004; Rowland et al., 2001) and three species of *Pleurosigma* (i.e. *Pleurosigma intermedium*, *Pleurosigma planktonicum* and *Pleurosigma* sp.; Belt et al., 2000a, 2001a).

From large scale cultures of these diatoms, many individual C_{25} HBI alkenes could be isolated and unambiguously identified (i.e. position and stereochemistry of the double bonds) by nuclear magnetic resonance (NMR) spectroscopy (e.g. Wraige et al., 1997; Sinninghe Damsté et al., 1999b; Belt et al., 2000a). The comparison of the gas chromatographic retention indices (RI) and mass spectra (MS) of these compounds with those of alkenes present in sediments allowed the structure and putative origin of many sedimentary HBIs to be assigned (e.g. Belt et al., 2000a). However, there are still a number of reports of HBI alkenes present in the marine environment whose origin and/or exact structure remains to be determined. For example, the biological source of the well characterised C_{25} HBI diene **2** isolated from sediments of the Caspian Sea (Belt et al., 1994) is still not known. Also, the origin and structures of some of the C_{25} HBIs polyenes detected by Porte et al. (1990) in bivalves from the Todos os Santos Bay (Brazil) are unknown although some isomers have subsequently been detected in *P. intermedium* and fully characterised (Belt et al., 2000a). It is thus evident that other species or genera of diatoms, in addition to those already described, also contribute to the widespread distribution of HBI compounds in the marine environment. Detailed studies of the hydrocarbon composition of other species of diatoms thus remain relevant for a more comprehensive account of HBI sources and a better interpretation of the sedimentary biomarker record.

In the present study, we describe the HBI composition of the marine diatom *P. strigosum* isolated from coastal Mediterranean sediments. The analysis by NMR spectroscopy of two isolated isomers led to the structural identification of a major C_{25} HBI triene **3** that has not been previously characterised, and to the first identification of the HBI diene **2** in diatoms.

2. Results and discussion

2.1. Taxonomy and occurrence of *P. strigosum*

P. strigosum W. Smith is a littoral marine species of benthic pennate diatom, which has been described in detail by Sterrenburg (1991, 2003). The observation of *P. strigosum* in phytoplanktonic populations from the North Sea and Wadden Sea (The Netherlands), the Gulf of Oman, Mauritania and Spain (Sterrenburg, 2003), together with the present report, attest to the widespread presence of this species in coastal marine sediments. Structural differences between *Pleurosigma* sp. can be difficult to evaluate by light microscopy, so electron microscopy is therefore necessary for unambiguous identification of strains. This is probably why *P. strigosum* has been repeatedly regarded as a variety of *Pleurosigma angulatum*, although fundamental differences exist between these two species (Sterrenburg, 2003).

2.2. Hydrocarbon composition of *P. strigosum*

GC-MS analysis of the hydrocarbon fraction of *P. strigosum* typically showed the presence of five components with RI (Kováts factors calculated according to Kissin et al. (1986)) and MS characteristic of HBI alkene structures and accounting in total for ca. 77% of the total hydrocarbons (Fig. 1 and Table 1). Squalene was also present in substantial quantities (ca. 20%) together with smaller amounts of *n*-$C_{21:4}$ and *n*-$C_{21:5}$ (ca. 3% in total alkenes). These two latter alkenes are rarely reported in phytoplankton (Volkman et al., 1994, 1998) and their presence without the co-occurrence of the *n*-$C_{21:6}$ is unusual. The *n*-$C_{21:5}$ alkene is probably formed by decarboxylation of the *n*-$C_{22:5}$ fatty acid (Volkman et al., 1994, 1998) which is present in the acid fraction of *P. strigosum* (data not shown).

Hydrogenation of the total hydrocarbons with Adam's catalyst (PtO_2) produced *n*-eicosane, squalane

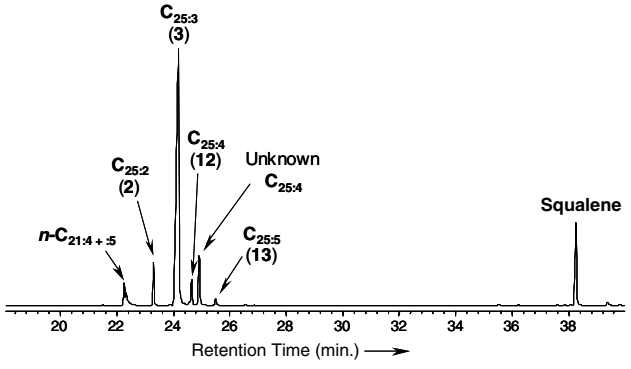


Fig. 1. Total ion current (TIC) chromatogram of the hydrocarbon fraction of the extract of *P. strigosum* isolated from coastal Mediterranean sediments.

Table 1
GC retention indices (RI), concentrations and relative distribution of alkenes in *P. strigosum*

Alkene	$RI_{Solgel-1}$	RI_{HP-5MS}	Concentration (pg/cell)	Total alkenes (%)
n-$C_{21:4}$	2033	2040	0.9	1.5
n-$C_{21:5}$	2036	2046	1.0	1.6
$C_{25:2}$ (**2**)	2072	2074	4.3	7.0
$C_{25:3}$ (**3**)	2108	2114	36.9	60.2
$C_{25:4}$ (**12**)	2132	2139	2.0	3.3
Unknown $C_{25:4}$	2146	2152	3.5	5.7
$C_{25:5}$ (**13**)	2168	2181	0.6	1.0
Squalene	2817	2828	12.1	19.7

and a single C_{25} HBI alkane indicating that all HBI alkenes had the same carbon skeleton. No C_{20} or C_{30} HBI isomers were detected. The mass spectrum of the C_{25} HBI alkane obtained after hydrogenation was identical to that previously published by Rowland and Robson (1990) for 2,6,10,14-pentamethyl-7-(3-methylpentyl)-pentadecane (**1**). The molecular ions present in the mass spectra of the five HBI alkenes (Fig. 2) allowed the number of double bonds to be assigned. This suite of compounds consisted of one major triene ($C_{25:3}$, M^+ 346), one diene ($C_{25:2}$, M^+ 348), two tetraenes ($C_{25:4}$, M^+ 344) and one pentaene ($C_{25:5}$, M^+ 342), present in lower proportions (Table 1). The comparison of the chromatographic and mass spectral properties of these compounds with those of C_{25} HBI alkenes characterised previously from diatoms (Sinninghe Damsté et al., 1999a,b; Wraige et al., 1997, 1999; Rowland et al., 2001) only permitted one tetraene (**12**) and the pentaene **13** to be identified as 2,6,10,14-tetramethyl-7-(3-methylpentyl-4-enyl)-pentadeca-5,9,13-triene and 2,6,10,14-tetramethyl-7-(3-methylpentyl-4-enyl)-pentadeca-2,5,9,13-tetraene, respectively.

The accumulation of sufficient amounts of hydrocarbons from several cultures grown under identical conditions allowed their full structural identification. HPLC of the hydrocarbon fraction of *P. strigosum* resulted in the isolation (purity > 95% by GC) of the predominant triene **3** (ca. 1.5 mg) and of the diene **2** (ca. 0.5 mg). Analysis by high field ^1H and ^{13}C NMR of the triene **3** led to complete assignment of proton and carbon chemical shifts (Table 2). The ^1H NMR spectrum revealed the presence of 5 olefinic H, 6 allylic H, 3 olefinic CH_3 and 4 aliphatic CH_3. Carbon multiplicities were established by an APT spectrum and revealed that the triene contains 25 carbon atoms with 2 olefinic C, 3 olefinic and 4 aliphatic CH, 1 olefinic and 8 aliphatic CH_2 and 7 CH_3 units. Homonuclear (COSY) and heteronuclear (HMQC, HMBC) two-dimentional NMR spectra (Table 3) were used to assign chemical

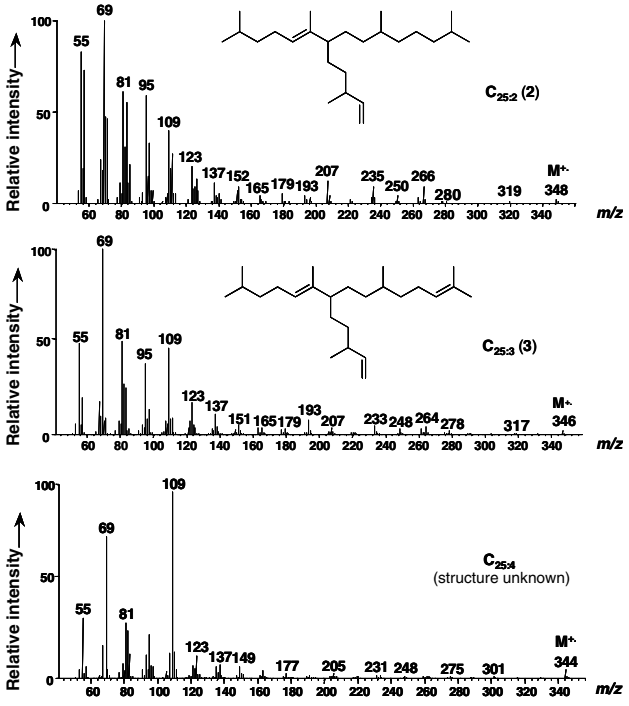


Fig. 2. EI Mass spectra of the C_{25} HBI diene, triene and unknown tetraene detected in *P. strigosum* isolated from coastal Mediterranean sediments.

Table 2
^1H (600 MHz) and ^{13}C (150 MHz) NMR data of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadeca-5*E*,13-diene (**3**) in $CDCl_3$

C-number	H-shift	C-shift
1	0.90 (3H, *d*, *J* = 6.8 Hz)	22.56 (*q*)
2	1.56 (1H, *m*)	27.51 (*d*)
3	1.22 (2H, *m*)	39.08 (*t*)
4	2.01 (2H, *q*)	25.53[a] (*t*)
5	5.08 (1H, *d*)	126.34 (*d*)
6		136.26 (*s*)
7	1.82 (1H, *m*)	49.28 (*d*)
8	0.99 (Ha, *m*), 1.20 (Hb, *m*)	30.91 (*t*)
9	0.99 (1H, *m*), 1.20 (1H, *m*)	34.83 (*t*)
10	1.36 (1H, *m*)	32.50 (*d*)
11	1.11 (Ha, *m*), 1.31 (Hb, *m*)	36.81 (*t*)
12	1.93 (Ha, *m*), 2.00 (Hb, *m*)	25.49[a] (*t*)
13	5.11 (1H, *m*)	125.11 (*d*)
14		130.91 (*s*)
15	1.70 (3H, *s*)	25.73 (*q*)
16	0.90 (3H, *d*, *J* = 6.8 Hz)	22.56 (*q*)
17	1.44 (3H, *s*)	11.24 (*q*)
18	0.85 (3H, *d*, *J* = 6.6 Hz)	19.84 (*q*)
19	1.62 (3H, *s*)	17.62 (*q*)
20	1.26 (2H, *m*)	30.91 (*t*)
21	1.17 (2H, *m*)	34.52 (*t*)
22	2.08 (1H, *m*)	37.79 (*d*)
23	5.66 (1H, *ddd*, *J* = 17, 10, 7 Hz)	144.96 (*d*)
24	4.91 (Hb, *dd*, *J* = 10, 1.5 Hz), 4.95 (Ha, *dd*, *J* = 17, 1.5 Hz)	112.29 (*t*)
25	0.98 (3H, *d*, *J* = 6.6 Hz)	20.50 (*q*)

[a] Assignments may be interchanged.

Table 3
Selected COSY and HMBC correlations of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadeca-5*E*,13-diene (**3**)

Proton(s)	COSY	HMBC
H-1 and H-16	H-2	C-1, C-2, C-3, C-16
H-3		C-1, C-2, C-4, C-5, C-16
H-4	H-3, H-5, H-17 (LR)[a]	C-2, C-3, C-5, C-6
H-5	H-4, H-17 (LR)	C-3, C-4, C-7, C-17
H-7	H-8, H-20	
H-12	H-11, H-13, H-15 (LR), H-19 (LR)	
H-13	H-12, H-15 (LR), H-19 (LR)	
H-15	H-12 (LR), H-13 (LR), H-19 (LR)	C-13, C-14, C-19
H-17	H-4 (LR), H-5 (LR)	C-5, C-6, C-7
H-18	H-10	C-9, C-10, C-11
H-19	H-12 (LR), H-13 (LR), H-15 (LR)	C-13, C-14, C-15
H-21		C-20, C-22, C-23, C-25
H-22	H-21, H-25, H-23, H-24 (LR)	C-23
H-23	H-22, H-24	C-22
H-24	H-23, H-22 (LR)	C-22, C-23
H-25	H-22	C-21, C-22, C-23

[a] LR indicates long-range allylic and homo-allylic couplings.

shifts. These assignments, in combination with the known HBI carbon skeleton of the compound established by hydrogenation, proved that the double bonds are at positions 5, 13 and 23. A NOESY experiments indicated the stereochemistry of the double bond at C-5 to be *trans* (5*E*), no NOESY interaction being observed between the protons H-5 and H-17. The stereochemistry of the chiral centres at C-7 and C-22 could not be established. The triene **3** was thus identified as 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadeca-5*E*, 13-diene. The ^{1}H NMR spectrum of the diene **2** was quite similar to that of triene **3**, but lacked the singlets at $\delta = 1.70$ and 1.62 ppm and the multiplet at $\delta = 5.11$ ppm. Accordingly, this diene was identified as 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5*E*-ene.

Attempts to isolate the unidentified tetraene from *P. strigosum* using HPLC were unsuccessful, this component always co-eluted with tetraene **12** under the conditions used. Thus, this compound could not be fully structurally identified. However, the structural similarities between the co-occurring triene **3**, tetraene **12** and pentaene **13** suggest that the unidentified tetraene also has three double bonds located at C-5, C-13 and C-23. The substantial differences between the mass spectra of the unknown tetraene (Fig. 2) and that of tetraene **12** (Rowland et al., 2001) suggest the fourth double bond is in another position than in **12** and is not a stereoisomer of **12** (Belt et al., 2000a).

2.3. Diversity of HBI alkenes produced by the *Pleurosigma* genus

The identification of C_{25} HBI alkenes in *P. strigosum* increases the number of diatom species known to synthesize these specific biomarkers. Within the *Pleurosigma* genus, two planktonic species (*P. planktonicum* and *Pleurosigma* sp. tentatively identified as *Pleurosigma subhyalinum*, Belt et al., 2001a) and two benthic species (*P. intermedium* and *P. strigosum*) are now clearly established as HBI producers, and fourteen distinct C_{25} HBI dienes through pentaenes have been fully characterised (Table 4). Although some of these compounds have been shown to be produced by other genera of diatoms, eight isomers have been detected so far exclusively in *Pleurosigma* sp. and even sometimes in only one species (Table 4). This is the case of the diene **2** and the triene **3** detected in *P. strigosum*. These differences in HBI production might perhaps be regarded as characteristic of specific *Pleurosigma* sp. However, it has been shown that different cultures of the same HBI-producing microalga (including *P. intermedium*) can exhibit substantial variations in HBI distributions likely depending on growth conditions (e.g. Belt

Table 4
Identified C_{25} HBI alkenes produced by *Pleurosigma* sp. and occurrence in other HBI-producing diatoms

Compound[a]	*Pleurosigma* producer	Presence in other diatoms
2	*P. strigosum*	Not reported
3	*P. strigosum*	Not reported
4	*P. intermedium*[b]	*R. setigera*[e]
5	*P. intermedium*[b]	*R. setigera*[e], *N. sclesvicensis*[f]
6	*P. intermedium*[b], *Pleurosigma* sp.[c,d]	*R. setigera*[e]
7	*P. intermedium*[b], *Pleurosigma* sp.[c,d]	*R. setigera*[e]
8	*P. intermedium*[b], *Pleurosigma* sp.[c,d]	Not reported
9	*P. intermedium*[b], *Pleurosigma* sp.[c,d]	Not reported
10	*P. planktonicum*[d]	Not reported
11	*P. planktonicum*[d]	Not reported
12	*P. strigosum*	*H. pseudostrearia*[g]
13	*P. strigosum*	*H. ostrearia*[h], *H. pseudostrearia*[g], *R. setigera*[i]
14	*P. intermedium*[b], *Pleurosigma* sp.[c,d]	Not reported
15	*P. intermedium*[b], *Pleurosigma* sp.[c,d]	Not reported

[a] See formulae for structure assignments.
[b] From Belt et al. (2000a,b).
[c] Tentatively identified as *P. subhyalinum*.
[d] From Belt et al. (2001a)
[e] From Rowland et al. (2001).
[f] From Belt et al. (2001b).
[g] From Allard et al. (2001).
[h] From Wraige et al. (1997).
[i] From Sinninghe Damsté et al. (1999b).

et al., 2000a; Allard et al., 2001; Rowland et al., 2001), and the same may thus hold true for *P. strigosum*.

It is also interesting to note that C_{25} HBI alkenes have not been detected in *P. angulatum* (Belt et al., 2001b). *P. strigosum* and *P. angulatum* are two species often misidentified because of their common features (Sterrenburg, 2003). Thus, the present report suggests that the analysis of the hydrocarbon composition of diatom cells may support microscopy observations to help distinguish species with close phenotypes, although this is undoubtedly less powerful then molecular phylogeny (Sinninghe Damsté et al., 2004).

2.4. Environmental occurrence of HBI alkenes produced by P. strigosum

Two of the five C_{25} HBI alkenes produced by *P. strigosum* (tetraene **12** and pentaene **13**) were previously shown to be produced by other diatoms. The tetraene **12** was identified in *Haslea pseudostrearia* by Allard et al. (2001). Surprisingly, this compound was reported to resolve into two peaks (possible diastereoisomers) on three different GC phases whereas, in the present study, it solely resolved into one GC peak on both phases used (e.g. Fig. 1). The pentaene **13** was previously detected in cultures of *Haslea ostrearia* (Wraige et al., 1997, 1999) and *Rhizosolenia setigera* (Sinninghe Damsté et al., 1999b) as well as in particulate matter from the North Sea (Sinninghe Damsté et al., 1999a).

The diene **2** has been previously isolated from sediments of the Caspian Sea and characterised by NMR spectroscopy (Belt et al., 1994). It was recently reported to occur also in sediments from the Arabian Sea and in particulate matter and sediments from the Black Sea (Massé et al., 2004). Although this HBI alkene is currently assumed to be produced by *H. ostrearia*, we have not been able to find any reports of this diene in diatoms. The benthic diatom *P. strigosum* is thus a potential contributor of diene **2** in marine coastal sediments. The presence of this latter compound in particulate matter (Massé et al., 2004) may suggest, however, an alternative planktonic source. The structure of the triene **3** is reported here for the first time. This triene as well as the unidentified tetraene (Fig. 2) have apparently never been reported in sediments or in water column particles.

At this stage, it seems difficult to specify diatom inputs to sediment or water column particulate matter based on structural differences between C_{25} HBI alkenes produced by distinct diatom species. Indeed, common structural features of HBI alkenes produced by *Pleurosigma* sp. are the presence of an unsaturation at C7–C20 and the occurrence of *E*/*Z* isomerism of the trisubstituted double bond at C9–C10 (Belt et al., 2000a, 2001a; Table 4). However, none of these structural criteria were observed for the HBI alkenes of *P. strigosum*, whereas these characteristics were observed for some C_{25} HBI alkenes produced by some strains of *R. setigera* (Rowland et al., 2001). These structural properties can only be envisaged to eventually distinguish between the genera *Pleurosigma* and *Rhizosolenia* on one hand, and the genus *Haslea* on the other, as the numerous HBI alkenes produced by this latter genus show a systematic absence of an unsaturation at C-7 and apparently no *E*/*Z* isomerism of the C9–C10 double bond (Wraige et al., 1997, 1999; Belt et al., 2001a; Allard et al., 2001). Moreover, several C_{25} HBI alkenes from *Haslea* sp. exhibit a double bond at C6–C17, which has never been observed in HBI alkenes produced by *Pleurosigma* and *Rhizosolenia* strains. The possible clay catalysed isomerisation of HBIs double bonds in sediments (Belt et al., 2000b) may, however, lead to a further complication in the assignment of HBI alkenes to specific biological sources.

3. Conclusions

C_{25} HBI alkenes were detected in *P. strigosum*, a benthic diatom isolated from Mediterranean coastal sediments. The major HBI alkene was the triene **3** whose structure is reported for the first time. *P. strigosum* was also found to contain the diene **2**, a compound commonly detected in the marine environment but whose presence in diatoms had up to now not been shown. The structural differences between the HBIs of *P. strigosum* and those detected previously in other *Pleurosigma* sp. indicate the wide variety in HBI biosynthetic pathways by this genus and limit the use of structural features (such as double bond positions) of C_{25} HBIs as indicators of specific diatom contribution to particulate and sedimentary organic matter.

4. Experimental

4.1. Algal cultures

Pleurosigma strigosum W. Smith (Sterrenburg, 1991, 2003) was isolated from the phytoplanktonic community growing at the surface of the sediment (5 m depth) from the Anse des cuivres (Gulf of Marseille, France). The description of *P. strigosum* in the literature is detailed, allowing an unambiguous characterisation using light and electron microscopy (Sterrenburg, 1991, 2003). *P. strigosum* was grown non-axenically to the stationary phase in fifty roux flat flasks containing 100 ml of f/2 medium (Guillard and Ryther, 1962). The cultures were grown at room temperature (20–25 °C) under 50 μEin m^{-2} s^{-1} (PAR) of fluorescent light with a 12 h light/12 h dark regime. After 15 days, cells were harvested on GF/F filters.

4.2. Extraction and isolation of HBI alkenes

The wet cells were extracted ultrasonically with Me_2CO (×2) and *n*-hexane (×4). Extracts were combined and concentrated by means of rotary evaporation. The total hydrocarbons were isolated by column chromatography over a wet packed (*n*-hexane) column of silica gel and eluted with *n*-hexane and *n*-hexane/toluene (1/1, v/v).

Individual C_{25} HBI isomers were isolated by HPLC using a reversed phase column (Bio-Sil C_{18}; 150×4.6 mm, 3 μm) and a mobile phase of MeOH delivered at 1 ml min^{-1}. The separation was monitored by UV detection at 210 nm.

4.3. Catalytic hydrogenation

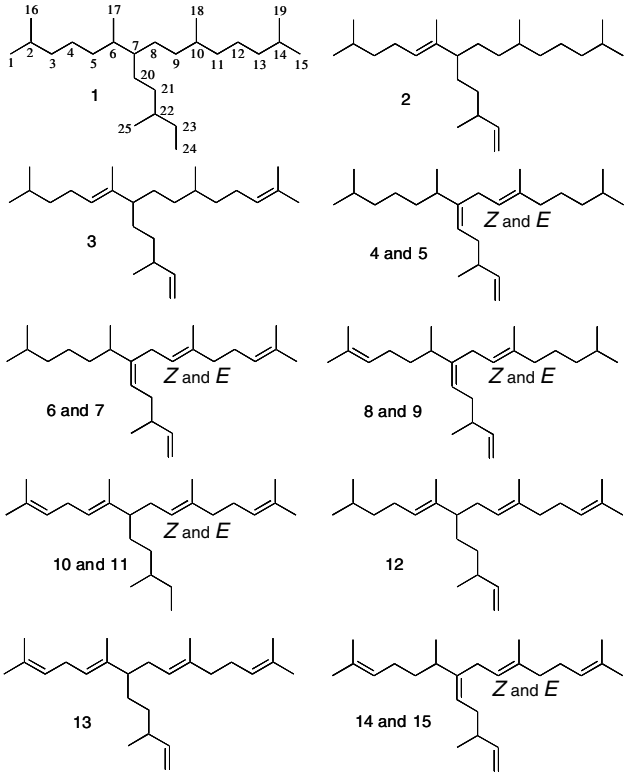
An aliquot of the total hydrocarbon fraction was suspended in EtOAc containing one drop of HOAc and stirred (12 h) under an atmosphere of H_2 in the presence of PtO_2/H_2O.

4.4. Gas chromatography–mass spectrometry

EI GC–MS analyses were performed on a HP 5890 series II plus gas chromatograph coupled to a HP 5972 mass spectrometer operated at 70 eV. Compounds were injected on-column and separated on non-polar column phases using either a Solgel-1 (SGE) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) or a HP-5MS (Hewlett–Packard) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The oven temperature was programmed from 60 to 130 °C at 30 °C min^{-1} and then at 4 °C min^{-1} to 300 °C at which it was held for 10 min. The carrier gas (He) was maintained at 1.04 bar until the end of the temperature program and then programmed from 1.04 to 1.5 bar at 0.04 bar min^{-1}.

4.5. NMR spectroscopy

NMR spectroscopy was performed on a Varian Unity Inova 500 and a Bruker DRX600 spectrometer equipped with an SWBB probe and an inverse TBI-Z probe with a pulsed field gradient (PFG) accessory, respectively. All experiments were recorded at 300 K in $CDCl_3$. Proton and carbon chemical shifts were eferenced to internal $CDCl_3$ (7.24/77.0 ppm). In the two-dimensional ^1H–^{13}C COSY, the number of complex points and sweep widths were 2 *K* points/6 ppm for ^1H and 512 points/150 ppm for ^{13}C. In the two-dimensional ^1H–^1H COSY, the number of complex points and sweep widths were 2 *K* points/5.5 ppm. Quadrature detection in the indirect dimension was achieved with the time-proportional-phase-incrementation (TTPI) method. The data were processed with the NMRSuite software package. After apodization with a 90 shifted sinebell, zero filling to 512 real points were applied for the indirect dimensions. For the direct dimensions zero filling to 4 *K* real points, Lorentz transformations were used.



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