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1 Novel tri- and tetra-unsaturated highly branched isoprenoid (HBI) alkenes  
2 from the marine diatom *Pleurosigma intermedium*.

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11

12 ABSTRACT

13 Two previously uncharacterised C<sub>25</sub> highly branched isoprenoid (HBI) alkenes  
14 have been identified in a laboratory culture of the diatom *Pleurosigma*  
15 *intermedium* and their structures determined using a combination of NMR  
16 spectroscopy and gas chromatography–mass spectrometry (GC–MS). These  
17 new tri- and tetra-unsaturated HBI alkenes are stereoisomers of previously  
18 characterised HBIs, with a *Z* stereochemistry for the C<sub>5</sub>–C<sub>6</sub> double bond in  
19 each case. HBIs with an isomeric (*E/Z*) C<sub>5</sub>–C<sub>6</sub> double bond and with 1–4  
20 double bonds, in total, have extremely similar mass spectra and a consistent  
21 difference in retention index ( $\Delta$ RI 28), with a *Z* before *E* elution order in all  
22 cases.

23 **Keywords:** highly branched isoprenoid; alkene; diatom; *Pleurosigma*

24 *intermedium*

## 25 1. Introduction

26 C<sub>25</sub> highly branched isoprenoid (HBI) alkenes are common components  
27 of marine and lacustrine sediments worldwide and are biosynthesised by  
28 certain diatoms mainly belonging to the genera *Haslea*, *Rhizosolenia*,  
29 *Pleurosigma* and *Berkeleya* (Volkman et al., 1994; Belt et al., 1996, 2000;  
30 Sinninghe Damsté et al., 1999; Grossi et al., 2004; Brown et al., 2014). C<sub>25</sub>  
31 HBIs contain between one and six double bonds (DB) and occur in a number of  
32 regio- and stereo-isomeric forms. Individual structures have been established  
33 mainly through diatom culturing and analysis of purified extracts using NMR  
34 spectroscopy (e.g. Belt et al., 1996, 2000; Sinninghe Damsté et al., 1999; Grossi  
35 et al., 2004; Brown et al., 2014). Here, we identify two novel C<sub>25</sub> HBIs isolated  
36 from a culture of the diatom *Pleurosigma intermedium* and report their  
37 structures following analysis of partially purified extracts using NMR  
38 spectroscopy and gas chromatography–mass spectrometry (GC–MS).

39

## 40 2. Experimental

41 *P. intermedium* (RA120910 P1B3) was obtained from the Roscoff  
42 Culture Collection (<http://roscoff-culture-collection.org/>) and cultured in the  
43 laboratory (15°C, 100 μmol m<sup>-2</sup> s<sup>-1</sup>, 14/10 L/D cycle in f/2 enriched water). After  
44 21 days, cells were filtered (GF/F), sonicated (DCM; 5 ml), re-filtered through a  
45 small plug cotton wool, dried (N<sub>2</sub>), then partially purified using column  
46 chromatography (SiO<sub>2</sub>). The hydrocarbon fractions (hexane; 5 ml) were  
47 analysed by GC–MS using a Hewlett-Packard 5890 Series II gas  
48 chromatograph, fitted with a 30 m fused silica HP<sub>5ms</sub> column (0.25 mm i.d.,

49 0.25  $\mu\text{m}$  film) coupled to a 5970 Series Mass Selective Detector (MSD) (Brown  
50 et al., 2014). For further separation of HBIs we used Ag-ion chromatography  
51 (Supelco discovery® Ag-Ion; 0.1 g) to obtain saturated hydrocarbons (hexane; 3  
52 ml), HBI I (acetone/DCM; 50/50; 5 ml) and HBI III (acetone; 5 ml). NMR data  
53 were obtained using a JEOL ECP-400 NMR spectrometer with chemical shifts  
54 measured relative to those of  $\text{CDCl}_3$  ( $^1\text{H}$ : 7.24 ppm;  $^{13}\text{C}$ : 77.0 ppm).

55

### 56 3. Results and discussion

57 Extraction of a culture of *P. intermedium*, followed by a two-stage  
58 purification (see Experimental) yielded sufficient quantities of two HBI  
59 alkenes for analysis by GC–MS and NMR spectroscopy. These HBIs were  
60 identified as tri- and tetra-unsaturated  $\text{C}_{25}$  HBIs on the basis of the  $\text{M}^+$  ( $m/z$   
61 346 and 344, respectively) in their mass spectra (Fig. 1).

62 The  $^1\text{H}$  NMR spectrum of the HBI triene (I; Fig. 1) exhibits multiplets  
63 at 5.63 and 4.89 ppm (Table 1) characteristic of a vinyl moiety at C23–C24, a  
64 structural feature found in virtually all previously reported HBI alkenes (Belt  
65 et al., 1996, 2000; Sinninghe Damsté et al., 1999; Grossi et al., 2004; Brown et  
66 al., 2014). Two further alkene protons (H5 and H9) could also be identified  
67 from their distinctive chemical shifts and multiplicities (Table 1). Alternative  
68 positions for the two tri-substituted DB at C2–C3 or C13–C14 could be  
69 discounted due to the observation of two isopropyl groups at 0.85 and 0.84 ppm,  
70 totalling 12H (Table 1). The  $^{13}\text{C}$  NMR spectrum of triene I was also consistent  
71 with the presence of a vinyl group (C23–C24), two tri-substituted DB (C5–C6,  
72 C9–C10) two isopropyl  $\text{CH}_3$  (C1&C16, C15&C19) and three further  $\text{CH}_3$  groups

73 (C17, C18, C25) (see Table 1 for details). However, since a C<sub>25</sub> triene with DB  
74 at C5–C6, C9–C10 and C23–C24 has been characterised previously (Wraige et  
75 al., 1999), but with a different retention index (RI) to that of the triene  
76 investigated here, we believed that the latter likely represented a stereoisomer  
77 of the former, which has known *E* stereochemistry for both DB (structure II;  
78 Fig. 1; Wraige et al., 1999). Confirmation of this, and identification of the  
79 stereochemistry for each DB, was achieved by further inspection of the <sup>1</sup>H and  
80 <sup>13</sup>C NMR data. Specifically, it has been shown previously that the <sup>13</sup>C chemical  
81 shifts for C11 and C18 in other C<sub>25</sub> HBI trienes are particularly characteristic  
82 of the stereochemistry at C9–C10, with C11 occurring at ca. 40 ppm and 32  
83 ppm for the *E* and *Z* isomers, respectively, while C18 appears at 15.7 ppm (*E*)  
84 and 23.5 ppm (*Z*) (Belt et al., 2000). Since C11 for the triene in our *P.*  
85 *intermedium* culture appears at 40.1 ppm, with a further resonance at 16.4  
86 ppm attributed to C18, the stereochemistry of the C9–C10 DB (*E*) could be  
87 deduced, and this was confirmed by identification of a singlet at 1.55 ppm in  
88 the <sup>1</sup>H spectrum due to H18 (c.f. 1.69 ppm when the C9–C10 DB is *Z*; Belt et  
89 al., 2000). In contrast, the resonance attributed to C17 (18.3 ppm) is shifted to  
90 lower field compared to that of its counterpart with known (*E*) stereochemistry  
91 (11.8 ppm; Wraige et al., 1999), while the corresponding <sup>1</sup>H resonance (H17) is  
92 also low field shifted (1.49 ppm) relative to the *E* isomer (1.43 ppm; Wraige et  
93 al., 1999). Finally, the resonance for the alkenic proton H5 in I (5.14 ppm) is  
94 low field shifted compared to the corresponding proton in II and to H9 in both  
95 (5.02 ppm). As such, the NMR data indicate that the stereochemistry of the  
96 C5–C6 DB is *Z* and we also provide chromatographic and mass spectrometric

97 data in support of this assignment. Belt and Cabedo-Sanz (2015) demonstrated  
98 that geometric isomers of C<sub>25</sub> HBI monoenes and dienes with C5–C6 DB are  
99 well resolved by GC–MS, with *Z* isomers eluting before their *E* counterparts  
100 and a difference in RI ( $\Delta$ RI) of ca. 28. Significantly, therefore, the HBI triene  
101 identified here (I; RI<sub>HP5ms</sub> 2075), elutes before the corresponding *E* isomer  
102 (RI<sub>HP5ms</sub> 2103) with a  $\Delta$ RI of 28. Finally, the mass spectrum of I is extremely  
103 similar to that of II (Fig. 1), also consistent with observations made previously  
104 for other HBI geometric isomers (Belt et al., 2000; Belt and Cabedo Sanz,  
105 2015).

106         Although a much lower abundance in the purified fraction prevented us  
107 from obtaining satisfactory <sup>13</sup>C NMR data, the structure of the co-occurring  
108 HBI tetraene (III) can be proposed on the basis of related <sup>1</sup>H NMR and GC–  
109 MS data to those found for triene I. Most notably, the <sup>1</sup>H NMR spectrum of  
110 tetraene III confirmed the presence of only one isopropyl moiety (0.85 ppm),  
111 two singlets due to H15 (1.65 ppm) and H19 (1.58 ppm), and two additional  
112 allylic protons (compared to triene I) at ca. 2 ppm. The *Z* stereochemistry of  
113 the C5–C6 DB was again evident with characteristic resonances for H17 (1.49  
114 ppm) and H5 (5.15 ppm). An additional (compared to triene I) alkene proton  
115 resonance provided evidence for a tri-substituted DB at C13–C14, while an  
116 alternative location for this additional DB at C2–C3 can be discounted on the  
117 basis of an absence of any double-allylic protons (i.e. H4) that would resonate  
118 at ca. 2.8 ppm (e.g. Sinninghe Damsté et al., 1999). Further, the mass spectral  
119 similarity between III (this study) and IV (Allard et al., 2001) (Fig. 1) and a

120  $\Delta$ RI of 28 are consistent with other HBI isomers with *E/Z* isomerism at C5–C6  
121 as described here and by others (Belt and Cabedo-Sanz, 2015).

122 Previously, HBIs containing C9-C10 DB with *Z* stereochemistry have  
123 been identified in cultures of a different strain of *P. intermedium* (Belt et al.,  
124 2000), and several other HBIs exhibiting *E/Z* isomerism have been reported in  
125 sediments and in laboratory rearrangement reactions (Belt and Cabedo-Sanz,  
126 2015). Our new findings, however, represent the first example of biogenic  
127 HBIs possessing a C5–C6 DB with *Z* stereochemistry. Finally, the organic  
128 carbon content of *P. intermedium* (14%) and intra-cellular concentrations of  
129 HBI I (0.5 pg cell<sup>-1</sup>) and III (1 pg cell<sup>-1</sup>) are similar to those reported for other  
130 HBI-producing diatoms (Volkman et al., 1994; Belt et al., 1996; Grossi et al.,  
131 2004; Brown et al., 2014).

132

#### 133 **4. Conclusions**

134 The structures of two previously uncharacterised C<sub>25</sub> HBI alkenes have  
135 been identified following laboratory culturing of the diatom *P. intermedium*  
136 and analysis of partially purified hydrocarbon extracts using NMR  
137 spectroscopy and GC–MS. This study adds to the existing suite of structurally  
138 characterised HBI alkenes and it will be interesting, in the future, to see if the  
139 same biomarkers can be identified in environmental or geochemical samples.

140

#### 141 **Acknowledgments**

142 We thank the University of Plymouth for financial support and to Joe Curiale  
143 for supportive feedback on the original manuscript.

144

145 **References**

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## 185 **Figures and Tables**

186 **Figure 1.** (a) Partial GC–MS chromatogram of the hydrocarbon fraction  
187 obtained from an extract of *P. intermedium*. (b) Structures and mass spectra of  
188 HBI alkenes described in the text.

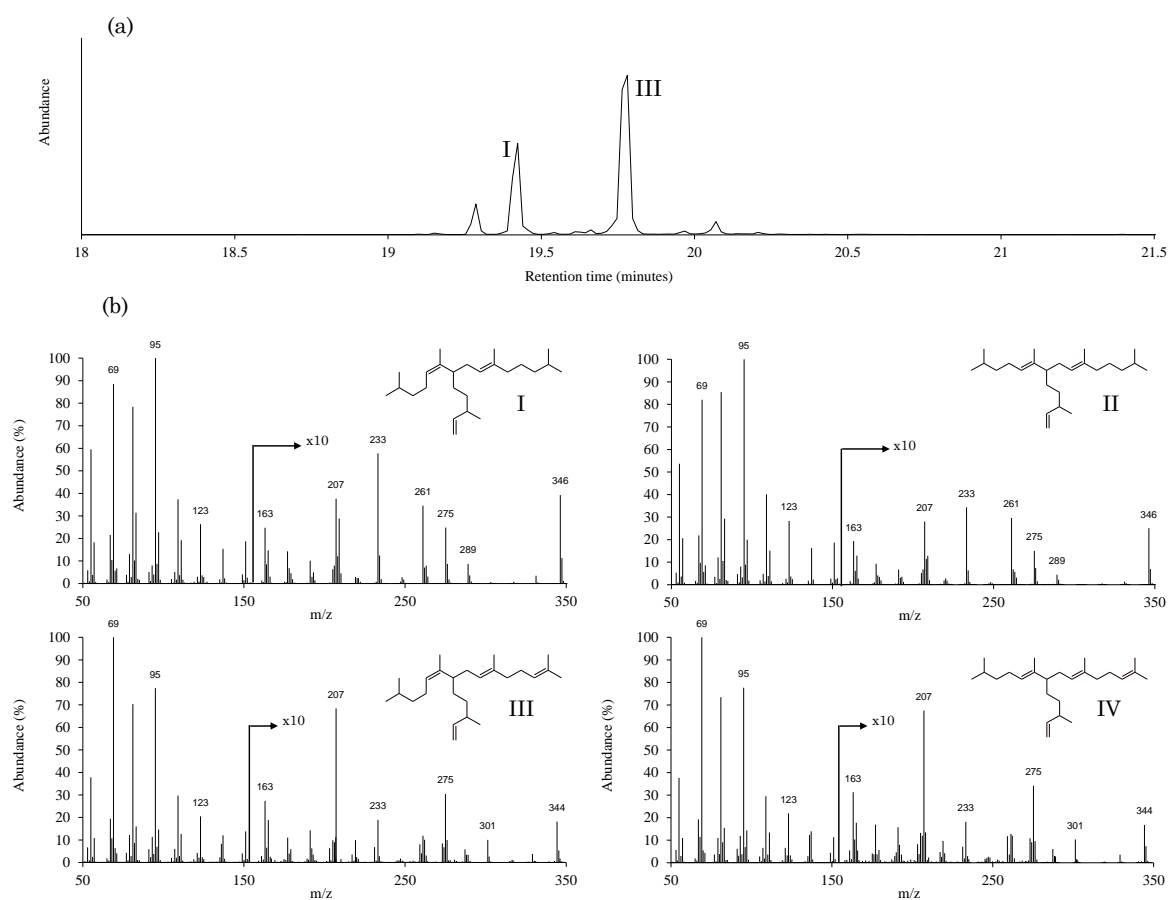
189

190 **Table 1.** NMR data for HBI I and III. (Note: <sup>13</sup>C NMR data for HBI I only)



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Figure 1



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Carbon number	Carbon shift ( $\delta$ /ppm)	Proton number	Proton shift ( $\delta$ /ppm)
1	22.6	<b>C<sub>25:3</sub> (HBI I)</b>	
2	27.7	1,15,16,19	0.84 and 0.85 (12H, 2 x d, $J = 7$ Hz)
3	38.6	25	0.94 (3H, d, $J = 7$ Hz)
4 <sup>a</sup>	25.4	2,3,12,13,14,20,21	1.05-1.37 (12H, m)
5	127.1	17	1.49 (3H, s)
6	136.8	18	1.55 (3H, s)
7	46.6	4,7,8,11,22	1.87-2.08 (8H, m)
8	32.2	24	4.89 (2H, m)
9	123.2	9	5.02 (1H, d, $J = 7$ Hz)
10	134.3	5	5.14 (1H, d, $J = 7$ Hz)
11	40.1	23	5.63 (1H, ddd, $J = 17, 10, 7$ Hz)
12 <sup>a</sup>	25.7		
13	38.6	<b>C<sub>25:4</sub> (HBI III)</b>	
14	27.7	1,16	0.85 (6H, d, $J = 7$ Hz)
15	22.6	25	0.94 (3H, d, $J = 7$ Hz)
16	22.6	2,3,20,21	1.1-1.4 (7H, m)
17	18.3	17	1.49 (3H, s)
18	16.4	18	1.56 (3H, s)
19	22.6	19	1.58 (3H, s)
20	29.9	15	1.65 (3H, s)
21	34.6	4,7,8,11,12,22	1.86-2.06 (10H, m)
22	38.6	24	4.89 (2H, m)
23	144.9	9,13	5.05 (2H, m)
24	112.5	5	5.14 (1H, t, $J = 7$ Hz)
25	20.5	23	5.63 (1H, ddd, $J = 17, 10, 7$ Hz)

<sup>a</sup> Resonances may be interchanged

Table 1

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Dr John Volkman  
Editor, Organic Geochemistry

November 2015

Dear John

Please find attached a revised manuscript: “Novel tri- and tetra-unsaturated highly branched isoprenoid (HBI) alkenes from the marine diatom *Pleurosigma intermedium*” that addresses (accepts) all of the relatively small number of editorial changes requested by the reviewer (Joe Curiale, now acknowledged). Exceptionally, we have opted to remove the ‘See Table 1’ addition from the Figure 1 legend as these represent different data.

Regards,

Simon

Professor Simon Belt  
University of Plymouth