A novel tri-unsaturated highly branched isoprenoid (HBI) alkene from the marine diatom *Navicula salinicola*

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ABSTRACT

A novel tri-unsaturated C_{25} highly branched isoprenoid (HBI) alkene has been identified in a laboratory culture of the diatom *Navicula salinicola* and its structure determined using a combination of NMR spectroscopy and gas chromatography–mass spectrometry (GC–MS). This represents the first report of a C_{25} HBI in a marine diatom from the *Navicula* genus, although a different tri-unsaturated C_{25} HBI has been reported previously in the freshwater species *N. sclesvicensis* and unspecified HBIs have been identified in the brackish *N. phyllepta*. The newly characterised HBI contains a relatively unusual conjugated diene sub-unit, a structural feature

only previously reported in some HBIs biosynthesised by a further marine diatom, *Haslea ostrearia*.

Keywords: highly branched isoprenoid; alkene; diatom; *Navicula salinicola*

1. Introduction

C_{25} highly branched isoprenoid (HBI) alkenes are common components of marine and lacustrine sediments worldwide and are biosynthesised by certain diatoms mainly belonging to the genera *Haslea*, *Navicula*, *Rhizosolenia*, *Pleurosigma, Berkeleya* and *Pseudosolenia* (Volkman et al., 1994; Belt et al., 1996, 2000, 2001; Sinninghe Damsté et al., 1999; Grossi et al., 2004; Brown et al., 2014; Kaiser et al., 2016). A single tri-unsaturated C_{25} HBI (Structure 6; Fig. 1) has been identified in the freshwater diatom *Navicula sclesvicensis* (Belt et al., 2001), and the mainly brackish species *N. phyllepta* has also been reported as an HBI-producing diatom (Sinninghe Damsté et al., 2004), although no structures were given. In contrast, no HBIs have as yet been reported in any marine species within the *Navicula* genus. Here, we identify a novel tri-unsaturated C_{25} HBI isolated from a laboratory culture of the marine diatom *N. salinicola* and report its structure based on analysis by NMR spectroscopy and gas chromatography–mass spectrometry (GC–MS).

2. Experimental

The benthic diatom *N. salinicola* was collected from a coastal marine environment (M. Kulikovskiy, June 2016 at Nha Trang, Vietnam, 12°13'14.5"N 109°12'18.3"E) and kept as strain BTD1 in the Laboratory of

Molecular Taxonomy of Aquatic Plants Institute of Plant Physiology (RAS). (Further taxonomic information can be found in the Supplementary Information). Initially, *N. salinicola* was cultured at 15 °C, 150 µmol m^{-2} s^{-1} continuous light in small flasks (150ml). Samples were harvested by filtration using MF-Millipore™ membrane filters (25 mm diameter, 0.3 µm pore size) during the exponential and stationary growth phases (Fig. 2). Large-scale cultures were then set up in several 2 l conical flasks under the same growth conditions. 80 l of such cultures were harvested by centrifugation (4000 rpm, 10 mins) during the stationary growth phase. For the small- and large-scale culture experiments, we used enriched f/2 medium (Guillard and Ryther 1962), along with the following nutrient concentrations: 2646 µM $NaNO_3$, 318 µM Na_2SiO_3, 108 µM NaH_2PO_4. The filtered and centrifuged biomass was freeze dried and the resulting dry material extracted via sonication using hexane (3 ml (small-scale cultures); 25 ml (large-scale culture)). The total hexane extract (THE) was then concentrated by removing hexane under a stream of nitrogen and partially purified using column chromatography (SiO_2). The hydrocarbon fraction (hexane) was analysed by GC–MS using an Agilent 7890 gas chromatograph equipped with a HP_{5MS} fused-silica column (30 m; 0.25 µm film thickness; 0.25 mm internal diameter) coupled to an Agilent 5975 series Mass Selective Detector (MSD). NMR data were obtained using a JEOL ECP-400 NMR spectrometer with chemical shifts measured relative to those of $CDCl_3$ (^1H: 7.24 ppm; ^{13}C: 77.0 ppm). NMR data were collected on the THE

obtained from the large-scale culture. The purity of the newly reported $C_{25:3}$ HBI (see Section 3) in this THE was estimated to be ca. 90% based on its relative peak area (GC–MS; Supplementary Information) and by the relative integration values of H-23 (Fig. 1) versus the alkenic protons of the co-occurring polyunsaturated linear alkenes (δ = ca. 5.3 ppm) in the ^{1}H NMR spectrum.).

3. Results and discussion

Following extraction of several small-scale cultures of *N. salinicola* from the exponential and stationary growth phases, analysis of partially purified THEs by GC–MS revealed the presence of a suite of closely eluting polyunsaturated linear alkenes (e.g. heneicosa-3,6,9,12,15,18-hexaene; *n*-$C_{21:6}$), trace amounts of a di-unsaturated HBI (**2**; Fig. 1) and a further compound exhibiting similar mass spectral properties to a range of C_{25} HBIs characterised previously. However, although the retention index (RI) of this component (RI_{HP5ms} = 2141) did not match that of any previously reported C_{25} HBIs, hydrogenation of an aliquot of one THE resulted in the formation of the parent HBI alkane $C_{25:0}$, thus confirming the C_{25} carbon skeleton. Interestingly, this new HBI was only detected in cultures harvested during the stationary phase and was identified as tri-unsaturated on the basis of its molecular ion ($M^{+\cdot}$ 346; Fig. 3). At this point, it is not clear why this new HBI and the co-occurring diene **2** were not detected during the exponential growth phase, although we note that some variability in cellular HBI concentrations and distributions have been reported in a small number of

previous studies (e.g. Wraige et al., 1997,1998,1999; Brown et al., 2020). From the large-scale culture of *N. salinicola*, we obtained ca 0.2 mg of the partially purified HBI triene (**3**; Fig. 1), which enabled full structural characterisation using ^{1}H and ^{13}C NMR spectroscopy. A conjugated diene sub-structure (C22–C25; C23–C24; Fig 1) could be readily identified through a particularly characteristic low field resonance in the ^{1}H NMR spectrum due to H-23 (Wraige et al., 1997; Allard et al., 2001), together with further low field resonances that could be attributed to alkenic methylene protons at C24 and C25 (Table 1). The third double bond could also be identified from its methylene protons at C17. Alternative positions for this third double bond at C1–C2 or C14–C15 can be discounted due to the observation of two isopropyl groups in the ^{1}H and ^{13}C NMR spectra (Table 1). Further, a double bond at C10–C18 leaves a solitary methyl group at C17 whose ^{13}C chemical shift would be at ca. 15.5 ppm, by comparison with related compounds (e.g. **1**; Belt et al., 2012). In contrast, isolated methyl groups at C18 in previously characterised HBIs resonate at ca. 19–20 ppm (e.g. 19.8 ppm for HBIs **1** and **2**; Fig. 1) (Johns et al., 1999; Belt et al., 2012), consistent with that observed for HBI **3** (i.e. 19.9 ppm; Table 1). The ^{13}C NMR spectrum of HBI **3** also contained individual resonances due to the six magnetically inequivalent alkenic carbon nuclei (Table 1) and complete ^{13}C resonance assignments could be proposed by comparison with structurally similar HBIs characterised previously (Fig. 1; Belt et al., 1996, 2012; Wraige et al., 1997; Allard et al., 2001), some of which contain a conjugated diene

115 sub-unit (i.e. **7**–**8**). The GC RI of HBI **3** (RI_{HP5ms} = 2141) is substantially
116 higher than those of some other HBI trienes (e.g. RI_{HP5ms} = 2114 (**4**); 2109
117 (**5**); 2090 (**6**)) but is consistent with that reported for the structurally related
118 tetraene **7** ($RI_{HP\text{-}5}$ = 2159; Allard et al., 2001).

119 The identification of a C_{25} HBI in *N. salinicola* represents the first
120 example of HBI production within a marine *Navicula* species despite the
121 near-ubiquity of this genus within natural diatom populations. Since the
122 *Navicula* and *Haslea* genera are quite similar, phylogenetically, with the
123 latter well-known as an HBI-producing genus, the new finding is probably
124 not surprising; however *Navicula* is a far more common genus, potentially
125 making it a more important source of some HBIs in marine sediments. In
126 terms of its structure, the conjugated diene sub-structure (C22–C25; C23-
127 C24; Fig. 1) is somewhat unusual, although there is some previous
128 precedent for such a feature in other HBIs isolated from a small number of
129 cultures of the marine diatom *Haslea ostrearia* (Wraige et al., 1997; Allard
130 et al., 2001). In fact, HBI **3** is a close structural analogue of HBI **7** identified
131 previously in *H. ostrearia*, albeit as a minor component. Since the retention
132 index of HBI **3** (RI_{HP5ms} = 2141) is similar to two HBI tetraenes identified in
133 some cultures of *H. ostrearia* (*viz.* $RI_{HP\text{-}5}$ = 2143–2146; Allard et al., 2001),
134 one of which also contained HBI **7**, it possible that HBI **3** may also have
135 been present in the corresponding lipid extracts, but not identified due to co-
136 elution. We are unware of any geochemical reports of HBI **3** although its
137 characterisation described herein may, in the future, lead to its positive

identification in sedimentary archives, an outcome that may potentially add to the use of HBIs as palaeoenvironmental indicators (c.f. HBIs **1** and **2** for Arctic and Antarctic sea ice; see Belt, 2018 for a review).

4. Conclusions

We report the structural identification of a novel C_{25} HBI biomarker in the marine diatom *N. salinicola*, the first example of HBI production within a marine *Navicula* species, thus expanding the potential number of sources of HBIs in the environment. Further studies into *N. salinicola* and related species may prove valuable in the use of HBIs as palaeoenvironmental proxies.

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221 **Figure legends**

222 **Figure 1.** Structures of C_{25} HBIs referred to in the text. HBIs are numbered
223 in order of increasing unsaturation.

224 **Figure 2.** Growth curve of the small-scale culture of the marine diatom *N.*
225 *salinicola*. Cell densities were estimated by measuring in vivo fluorescence
226 between 460 nm to 670 nm using the SpectraMax® iD3 Multi-Mode
227 Microplate Reader. Cultures were harvested during the exponential (empty
228 arrow) and/or stationary (solid arrow) growth phases (n=1).

229 **Figure 3.** Structure and mass spectrum of HBI **3**.

Figure 1.

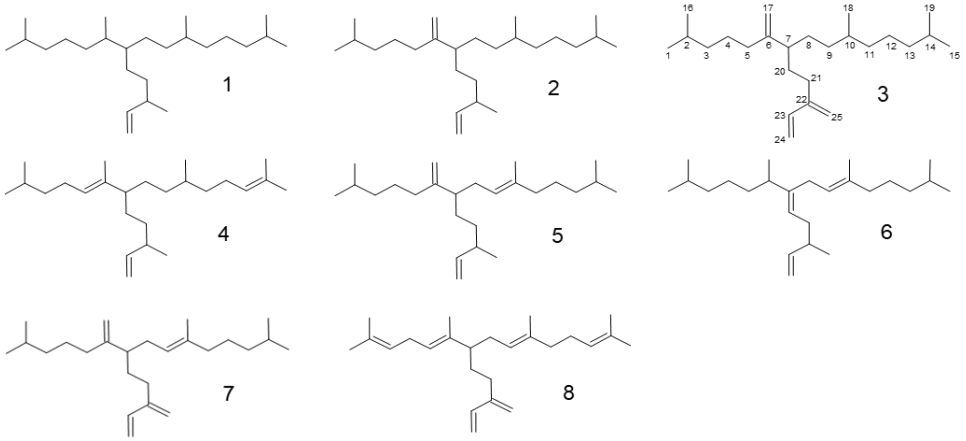


Figure 2.

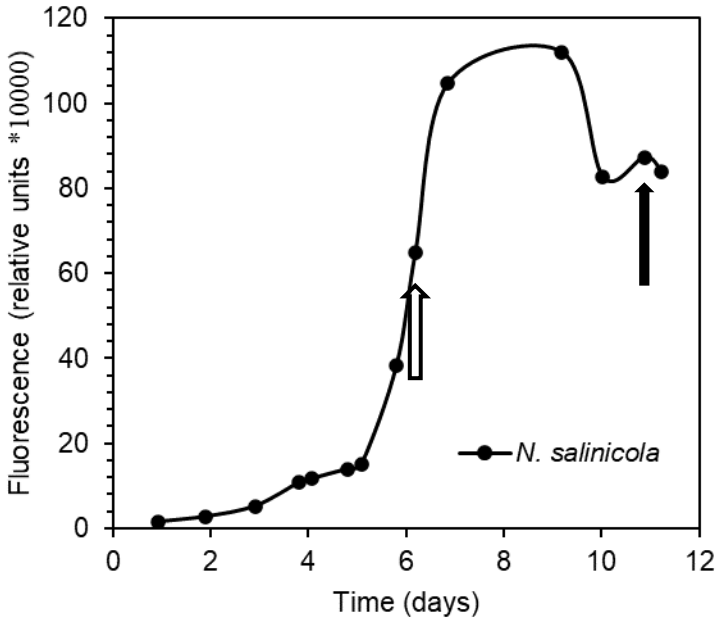
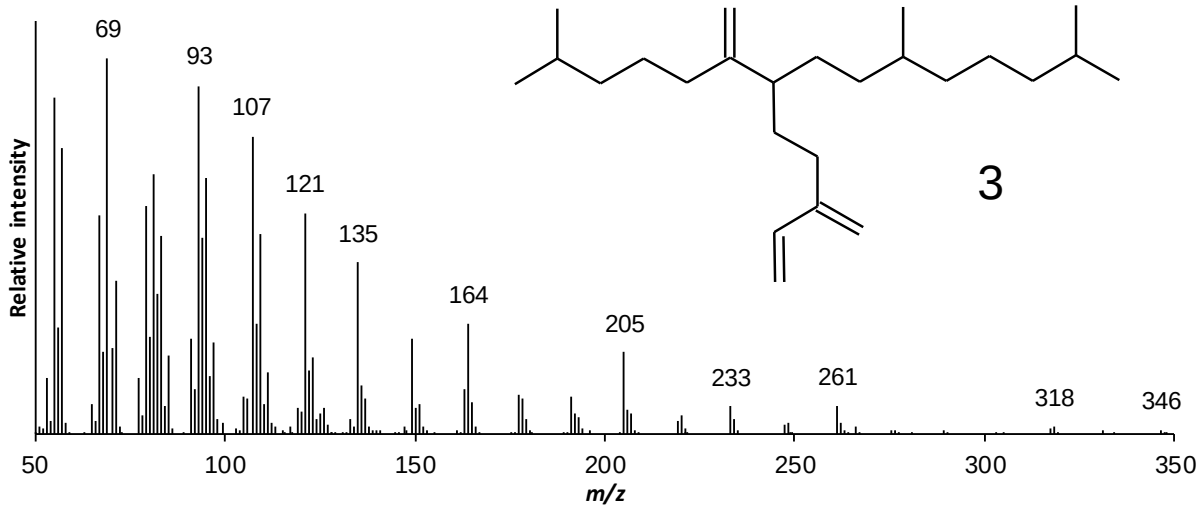


Figure 3.



249

250 Table 1. Key NMR data for HBI **3**.

	Carbon shift (δ/ppm)	Proton Number	Proton shift (δ/ppm)
1,16*	22.8, 22.7*	1,15,16,19	0.85 (12H, m)
2	28.0	5,7,21	2.05 (5H, m)
3	39.1	17	4.77, 4.72 (2H, 2 x s, br)
4	25.6	18	0.82 (3H, t, J=6.9 Hz)
5	33.0	23	6.33 (1H, dd, J=17.6, 11.0 Hz)
6	152.3	24a	5.01 (1H, d, J=11.0 Hz)
7	47.1	24b	5.16 (1H, d, J=17.6 Hz)
8	29.8	25	4.96 (2H, m, br)
9	34.9		
10	33.0		
11	37.1		
12	24.8		
13	39.4		
14	28.0		
15,19*	22.8, 22.7*		
17	109.0		
18	19.9		
20	29.4		
21	32.0		
22	146.9		
23	139.0		
24	113.1		
25	115.5		

251 *Assignments may be interchanged