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Biosynthesis of unusual monocyclic alkenes by the diatom *Rhizosolenia setigera* (Brightwell)

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Abstract

Novel, polyunsaturated monocyclic sester- and triterpenes isolated from the diatom *Rhizosolenia setigera* (Brightwell), are biosynthesised mainly via the mevalonate pathway. The experiments involved incubation of the alga with [1-^{13}C]acetate, isolation of the alkenes by extraction and silver ion HPLC, followed by determination of the labelling pattern of one of the monocyclic triterpenes by ^{13}C-NMR spectroscopy. In addition, the extent of ^{13}C incorporation was also measured by mass spectrometry which revealed that the involvement of the mevalonate route in the biosynthesis of these cyclic compounds was less than for the co-occurring acyclic highly branched isoprenoid alkenes.

Keywords: *Rhizosolenia setigera*; Bacillariophyceae; Diatoms; Isoprenoid alkenes; mevalonate; biosynthesis; monocyclics

1. Introduction

The widespread planktonic diatom *Rhizosolenia setigera* (Brightwell), biosynthesises a number of straight-chain (Sinninghe Damsté et al., 1999, 2000; Rowland et al., 2001a), highly branched isoprenoid (HBI; Volkman et al., 1994, 1998; Belt et al., 2001; Rowland et al., 2001a) and monocyclic alkenes (Belt et al., 2003). Representative structures of these three groups of compounds are shown in Fig. 1. In culture, factors such as salinity, temperature, algal strain and position in the life cycle all influence the HBI and cyclic alkene distributions, and these have been described in detail elsewhere (Rowland et al., 2001a; Belt et al., 2002). Biosynthesis of the HBI alkenes, which have unknown function in the alga, has been studied by a series of experiments involving selective pathway inhibition, ^{13}C and ^{2}H isotopic incorporation from labelled precursors, determination of labelling patterns by NMR spectroscopy, and measurement of the ^{13}C/^{12}C isotope ratios of HBIs isolated from algae grown under natural conditions (Massé et al., 2004). The HBIs and sterols are biosynthesised under these conditions, mainly by the well-known mevalonate (MVA) pathway (e.g. Rohmer, 1999). This is in contrast to plastidic phytol (from chlorophyll *a*) which is produced by the methylerythritol (MEP) route. This divergence of pathways for presumed cytosolic and known plastidic isoprenoids is similar to that observed in some other diatoms (Cvejić and Rohmer, 2000). However, it was noted previously that the (then unknown) cyclic alkenes produced by *Rhizosolenia setigera* had ^{13}C/^{12}C ratios which were somewhat isotopically distinct from both the HBI alkenes and from phytol under all culture conditions (Rowland et al., 2001a). For example, compared with phytol, the HBI and cyclic alkenes were consistently 2‰ and 1‰ depleted in ^{13}C respectively.

Subsequently the cyclic alkenes were identified as V–VII by isolation and characterisation by NMR spectroscopy (Belt et al., 2003). The structures of the cyclic alkenes are thus unrelated to the HBI alkenes. The former may reasonably be assumed to be biosynthesised from coupling of a geranyl (C_{10}) moiety to either a farnesyl (C_{15}) or geranylgeranyl unit (C_{20}) at C-7 (Fig. 2) to yield C_{25} and C_{30} compounds respectively. By contrast, the HBI alkenes are likely biosynthesised via coupling of geranyl and farnesyl or two farnesyl units at C-6 (Fig. 2).

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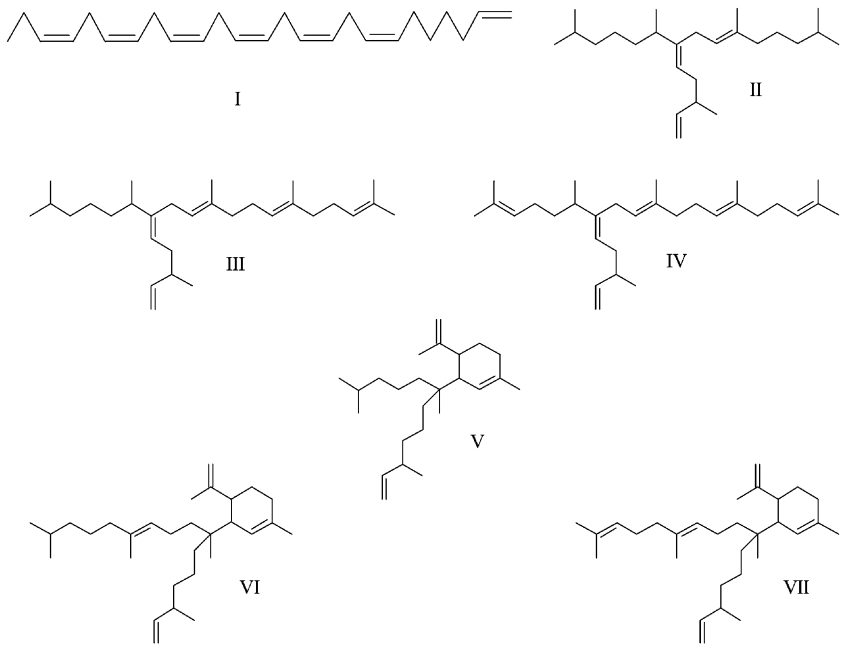

Fig. 1. Representative structures of straight chain (I), HBI alkenes (II–IV) and monocyclic alkenes (V–VII) characterised previously from *R. setigera*.

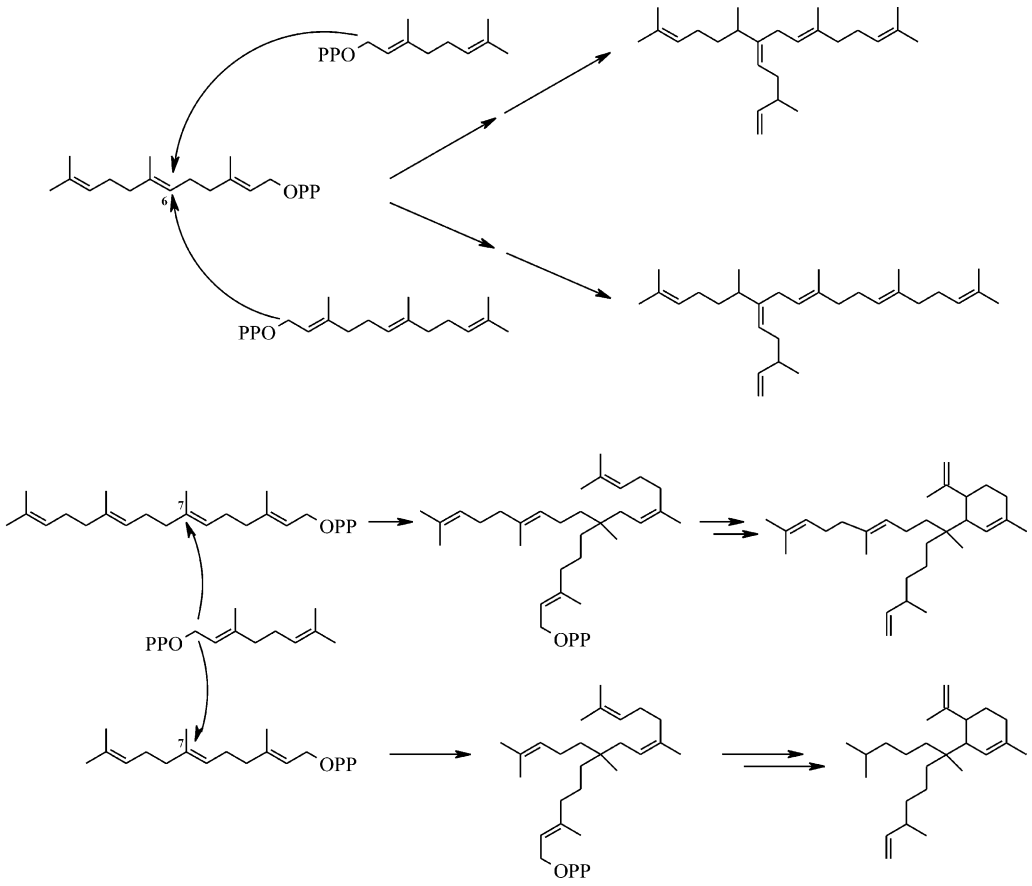

Fig. 2. Simplified representation of a hypothetical biosynthetic pathway for the formation of (a) highly branched isoprenoid alkenes and (b) monocyclic isoprenoid alkenes in *R. setigera*.

Given the distinctive structures and $^{13}C/^{12}C$ ratios of V–VII, it seemed desirable to establish the route of biosynthesis of the cyclic alkenes. Specifically, we wished to investigate (a) whether the differences in $^{13}C/^{12}C$ ratios of the cyclics V–VII reflect biosynthesis from non-MVA routes [e.g. the MEP route used by other diatoms for HBI biosynthesis (Massé et al., 2003) or leucine catabolism or incorporation pathways (cf Ginger et al., 2001 and references therein)], (b) if the ratios reflect biosynthesis from a mix of pathways, or (c) whether they simply reflect the different extents of isotopic fractionation occurring in the MVA route during the prenylation and cyclisation steps leading to cyclic alkene formation compared with the acyclic HBIs. Whilst one might expect similar classes of chemicals (e.g. alkenes) to be biosynthesised by a common route, surprisingly, different diatom genera actually biosynthesise closely related HBI alkenes by different routes (Massé et al., 2003). There is also some evidence to suggest that some plants are able to use alternative pathways for isoprenoid biosynthesis when other pathways are inhibited (so-called 'crosstalk' between the MEP and MVA pathways; Hemmerlin et al., 2003 and references therein). A study of cyclic isoprenoid biosynthesis such as that conducted herein might add to existing knowledge of pathway 'crosstalk'. Such biosynthetic information might also be important, if, like some HBI alkenes (Rowland et al., 2001b), the cyclic alkenes were found to possess cytostatic activity against human lung cancer cells, since then it may be desirable to optimise biosynthetic production of the chemicals.

2. Results and discussion

2.1. NMR studies of labelling patterns

Previously cultured strains of *R. setigera* have shown quite varied hydrocarbon distributions. Neither strains RS 99 or RS 00 isolated from French waters nor strains CCMP 1330 and 1820 isolated from the North Atlantic, produced the cyclic alkenes V–VII when grown at 16–18 °C in Guillard's medium, whereas strains CS389/A, CS 389/1 and CS 62 from Tasmania (Volkman et al., 1994, 1998), grown at temperatures ranging from 10 to 25 °C in FE or FE/2-1 medium all produced the cyclic compounds (Rowland et al., 2001a). The latter strains were not available to us for a study of the biosynthesis of the cyclic alkenes. However, we noted that strain RS 99 did produce V–VII at certain points in the life cycle, notably between auxosporulation phases (Massé, 2003; Belt et al., 2002). Culturing of RS 99 cells post auxosporulation in the presence of 0.25 g l^{-1} 20% enriched [1-^{13}C]acetate herein, followed by extraction of the freeze-dried filtered or centrifuged cells (CH_2Cl_2/CH_3OH) and open column chromatography, yielded a fraction containing both cyclic and acyclic alkenes from which V–VII were isolated by silver ion HPLC (Massé, 2003). The amounts of V and VII were too small for NMR studies but ^{13}C NMR analysis of VI (0.7 mg) was achievable and yielded the data listed in Table 1. These could be compared with a reference spectrum obtained for VI isolated from *R. setigera* grown in the absence of labelled acetate (Table 1). Each resonance in the spectrum of VI could be assigned to each of the thirty carbon atoms in VI (cf Belt et al., 2003). Since the intensity of a ^{13}C NMR signal from a carbon atom is dependent on its chemical environment, each resonance signal intensity was normalised to the response due to C-22 (which was given an arbitrary value of 1.0) in VI (Table 1; cf Schwender et al., 1996).

Incorporation of [1-^{13}C]acetate into VI via the mevalonate pathway would be expected to lead to isotopic enrichment (labelling) of C-1 and C-3 of isopentenyl diphosphate (IDP; Fig. 3, VIIIa) and thus to labelling of C-2,4,6,8,10,12,14,16,21,23,25 and 27 of alkene VI (Fig. 3). Thus, relative to the value for the corresponding

Table 1
^{13}C NMR data for monocyclic alkene VI obtained from *R. setigera* grown in the absence (reference) and presence of [1-^{13}C]acetate. The amplitudes of the individual resonances have been normalised to C-22 = 1.0 for ease of comparison

Carbon number	Chemical shift (ppm)	Carbon atom of IDP	Reference	[1-^{13}C]acetate (20%)
27	148.1	3	0.4	1.4
15	145.1	2	0.7	0.8
23	136.0	3	0.5	1.9
6	135.2	3	0.3	1.5
22[a]	126.0	2	1.0	1.0
7	124.1	2	0.8	1.5
16	112.3	1	0.9	2.4
28	109.3	4	1.1	1.3
21	52.2	1	1.1	1.9
26	51.9	2	1.2	1.1
10	46.6	3	0.6	1.3
11	42.6	2	1.0	1.3
24	40.3	4	1.1	1.2
5	40.1	4	1.0	1.0
3	38.8	2	1.2	1.0
13	38.1	4	1.0	1.1
9,14	37.9	4,3	0.6	1.5[b]
25	28.8	1	1.0	1.8
2	28.0	3	0.7	1.9
8	26.7	1	1.2	1.7
4	25.9	1	0.9	2.2
1,17	22.8	4,5	0.8	1.3
12	22.6	1	0.9	2.5
19	21.1	5	0.9	1.4
20	20.3	5	1.0	1.2
29	20.0	5	0.9	0.9
30	16.7	5	0.8	0.9
18	16.0	5	0.6	0.9

[a] Assigned a value of 1.0 against which other resonances are normalised.

[b] The enhancement corresponds to C-14 only.

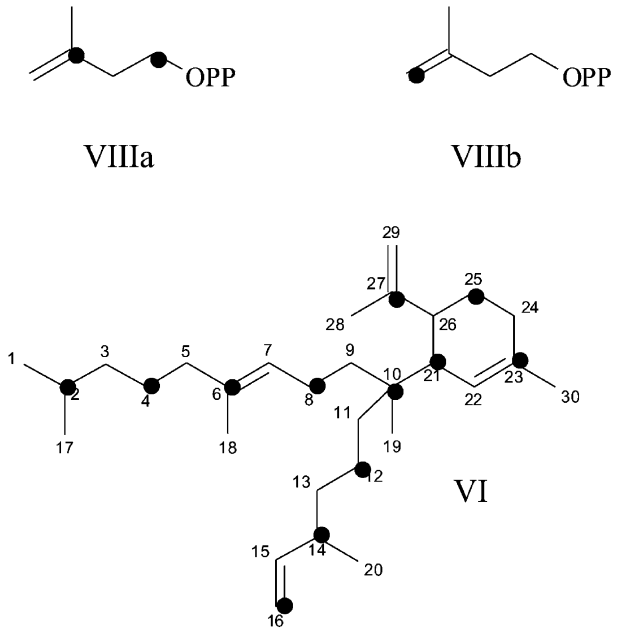

Fig. 3. Structures and numbering systems for IDP labelled from [1-^{13}C]acetate according to the MVA (VIIIa) and MEP-glyoxylate shunt (VIIIb) pathways, and subsequent labelling of VI following coupling of IDP units derived from the MVA route.

resonance in the reference compound, the integrated signals for C-2,4,6,8,10,12,14,16,21,23,25 and 27 would be expected to be enhanced (Table 1). By contrast, operation of the MEP pathway would lead to no labelling of IDP unless there were co-involvement of the glyoxylate shunt to produce [1-^{13}C]glyceraldehyde-3-phosphate from [1-^{13}C]acetate, which would lead to some labelling of C- 4 of IDP (VIIIb) and thus of C-1 (17), C-5 (18), C-9 (19), C-13 (20), C-24 (30) and C-28 (29) in VI via the MEP pathway. This has sometimes been observed in other algae (Schwender et al., 1996; Yang and Orihara, 2002).

As can be seen from the ^{13}C NMR data in Table 1, only the resonances due to C-2,4,6,8,10,12,14,16,21,23,25 and 27 were enhanced relative to those in the reference compound. This implies that C-1 and C-3 of IDP were enriched, as expected from operation of the MVA route. The mean enhancements ($\pm\sigma$, $n=6$) of C-1 and C-3 of IDP resulting from uptake of [1-^{13}C]acetate by *Rhizosolenia setigera* RS 99 are shown in Table 2. These are appreciably higher than those of C-2, 4 and C-5 and are enhanced relative to those of the reference compound. Thus, the mean enhancement of C-1 (2.08 ± 0.33, $n=6$) was not equal ($P=0.02$) to those of C-2 (1.12 ± 0.25, $n=6$), C-4 (1.17 ± 0.20, $n=6$) or C-5 (1.13 ± 0.19, $n=6$). Similarly, the mean enhancement of C-3 (1.58 ± 0.26, $n=6$) was not equal ($P=0.10$) to those of C-2, C-4 or C-5, but was equal to that of C-1 (the null hypothesis that the means are equal was acceptable at all confidence levels). The non-labelling of atoms originating from C-4

Table 2
Mean ^{13}C enhancements ($\pm\sigma$, $n=6$) of individual IDP carbon atoms in monocyclic alkene VI obtained from *R. setigera* grown in the presence of [1-^{13}C]acetate. Single values for VI isolated from the diatom grown in the absence of added acetate (reference) are also given

Carbon atom of IDP	Reference	[1-^{13}C]acetate (20%)
C-1	1.0	2.08 ± 0.33
C-2	1.0	1.12 ± 0.25
C-3	0.5	1.58 ± 0.26
C-4	0.9	1.17 ± 0.20
C-5	0.8	1.13 ± 0.19

of the IDP unit indicates that under these conditions, the MEP pathway did not operate via the glyoxylate shunt in *R. setigera*.

2.2. Monitoring of isotope incorporation by GC–MS: isotopic enrichment factors

In addition to the labelling pattern of the cyclic and HBI alkenes established by NMR studies of the products of culturing of *R. setigera* with [1-^{13}C]acetate (described above and previously; Massé et al., 2003), the extent of ^{13}C incorporation into the acyclic and cyclic alkenes could also be estimated using mass spectrometry (MS). In a study on the biosynthesis of 2-methyl-3-buten-2-ol (MBO) from pine needles, Zeidler and Lichtenthaler (2001) estimated the degree of isotopic labelling in MBO after incubation of the needles with labelled precursors using a simple ratio of mass spectral peak intensities. However, in contrast to MBO, the higher molecular masses together with the variable structures of the cyclic terpenoids synthesised by the diatom studied here, means that such ratios would not allow for accurate measures of the degree of labelling to be made. Instead, ^{13}C "isotopic enrichment factors" (IEFs) were calculated for each compound according to Eq. (1),

$$\text{Isotopic Enrichment Factor} = \frac{100}{n} \times \left\{ \frac{(M^{*}I_M) + (M+1^{*}I_{M+1}) + \ldots + (M+X^{*}I_{M+X})}{I_M + I_{M+1} + \ldots + I_{M+X}} - M \right\} \quad (1)$$

where M, M + 1, etc are the values of the molecular ions for various isotopomers, I_M is the intensity of the molecular ion, I_{M+1} is the intensity of the M + 1 peak, I_{M+X} is the intensity of the highest mass ion (quantifiable), and n is the number of carbon atoms in the molecule. Comparison of IEFs from compounds obtained from cells cultured in the presence of isotopically labelled acetate with those obtained from control cultures thus revealed the level of ^{13}C incorporation within the individual compounds. If, for example, the contribution of

Table 3
Isotope Enrichment Factors (IEF; described in the text) for incorporation of 20% and 100% enriched [1-^{13}C]acetate by *R. setigera* into HBI (II–IV) and cyclic (VI–VII) alkenes

Compound	Control	[1-^{13}C]acetate (20%)	[1-^{13}C]acetate (100%)
$C_{25:3}$ (II)	1.1	1.7	4.8
$C_{30:5}$ (III)	1.0	1.7	4.6
$C_{30:6}$ (IV)	1.0	1.7	4.7
$C_{30:4:1}$ (VI)	1.1	1.5	3.9
$C_{30:5:1}$ (VII)	1.2	1.5	4

the MVA pathway to the biosynthesis of the cyclic compounds were less than that for the formation of the HBI alkenes, or if there were some partial 'switching' from the MVA to the MEP pathway ('crosstalk'; Hemmerlin et al., 2003), then the extent of isotope incorporation in the cyclic compounds would be expected to be less than that of the HBI alkenes.

The IEFs of the HBIs and cyclic alkenes are shown in Table 3. When the cells were grown in the presence of 20% [1-^{13}C]acetate, a small, but measurable difference between the isotopic enrichment factors of the HBIs (1.7) and the cyclic compounds (1.5) was indeed observed. This difference was enhanced further in the presence of 100% [1-^{13}C]acetate, where the IEFs for the HBIs and the cyclic compounds were found to be 4.7 and 3.9 respectively.

3. Conclusions

Under the culturing conditions used, it appears that *R. setigera* biosynthesises alkene VI and presumably V and VII, mainly by the MVA route. This is also the route used for the biosynthesis of the HBI alkenes and of desmosterol by *R. setigera*, whereas plastidic phytol is made via the MEP route (Massé et al., 2003). This divergence of pathways for presumed cytosolic (alkenes, desmosterol) and plastidic (phytol) lipids is similar to that observed in other diatoms. Thus, the above labelling experiments (NMR analysis and IEFs) suggest that the ca 1‰ differences in $^{13}C/^{12}C$ ratios ($\delta^{13}C$) between V–VI and HBIs observed previously in unlabelled cultures of *R. setigera* (Rowland et al., 2001a) do not reflect biosynthesis of the cyclics solely from non-MVA routes and HBIs from MVA routes. Rather, the differences in the $\delta^{13}C$ ratios are likely to reflect biosynthesis of both HBIs and cyclic compounds from the MVA pathway, but with a greater contribution from this route to the formation of the acyclic compounds. There is evidence to suggest some plants are able to use alternative pathways for isoprenoid biosynthesis when other pathways are inhibited (so-called 'crosstalk' between the MEP and MVA pathways; Hemmerlin et al., 2003 and references therein). However, a further investigation of this possibility will require studies with added inhibitors of each route (cf. Hemmerlin et al., 2003). Such studies are underway in our laboratories.

4. Experimental

4.1. Algal cultures

Rhizosolenia setigera (strain RS 99) was isolated in spring 1999 from surface waters at Le Croisic (France). Large-scale cultures were performed using 25 l glass barrels using F/2 Guillard enriched seawater and grown under controlled conditions (14 °C, 100 μmol Photon m^{-2} s^{-1}, 14/10 Light/Dark cycle). During all of the experiments, cells were harvested by filtration or centrifugation at the end of the exponential growing phase. Experiments using sodium [1-^{13}C]acetate (0.25 g l^{-1}, 20% and 100% isotopic abundance; Sigma-Aldrich, France), were performed in 250 ml flasks.

4.2. Isolation of alkenes

Cells were harvested by continuous flow centrifugation (Cepa patberg) and freeze dried prior to extraction. Centrifuged samples gave a concentrated algal paste which was then freeze dried. The paste was then extracted five times using CH_2Cl_2/MeOH (50:50, v/v,) to yield a total organic extract (TOE). Solvents were then removed under reduced pressure and the extract obtained was kept frozen (−20 °C) before purification. Isolation and purification of alkenes from the TOEs was achieved using a combination of open column chromatography (5% deactivated SiO_2/hexane) techniques and preparative high pressure chromatography techniques (Chromspher 5 lipid column). For open columns, the size varied depending on the amount of material to be separated, with a typical ratio of 50:1 SiO_2:TOE (w/w). The hydrocarbon fraction from the TOE applied onto the column was first eluted with hexane (5 column volumes).

4.3. Instrumental

Individual lipids from the hydrocarbon fraction obtained by open column chromatography were separated by silver-ion chromatography (Chromspher 5 lipid, 250× 4.6 mm internal diameter) under isocratic conditions. A Hewlett Packard 5010 HPLC system coupled to a Hewlett Packard diode array detector was used. Alkenes were monitored at 206 nm and the fractions were collected manually. The isocratic mobile phase was a mixture of hexane-isopropanol (98.5:1.5 v/v). A preliminary fractionation using a silica column was sometimes necessary prior to the separation by silver-ion chromatography to allow sufficient resolution for the separation of individual isomers.

GC–MS was performed using a Hewlett Packard 5890 series II gas chromatograph coupled to a Hewlett Packard 5970 mass selective detector fitted with a 12 m (0.2 mm i.d.) fused silica capillary column (HP-1 Ultra stationary phase). Auto-splitless injection and helium carrier gas were used. The gas chromatograph oven temperature was programmed from 40–300 °C at 5 °C min^{-1} and held at the final temperature for 10 min. Mass spectrometer operating conditions were; ion source temperature 250 °C and 70 eV ionisation energy. Spectra (35–500 Daltons) were collected using Hewlett Packard Chemstation™ software. ^{13}C and ^{1}H NMR spectroscopy was performed on a JEOL EX-270 FT-NMR spectrometer. Spectra were recorded in $CDCl_3$ using residual $CHCl_3$ (7.24 ppm) and $^{13}CDCl_3$ (77.0 ppm) as references. Data were collected using JEOL Delta software.

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