Doctorate Thesis (abridged)

Studies on the Biosynthesis of Highly Branched Isoprenoids in the Marine Diatom *Rhizosolenia setigera* Brightwell

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Supervisor
Shigeru Okada

Victor Marco Emmanuel Nuestro Ferriols
ビクター　マルコ　エマニュエル　ヌエストロ　フェリオルス

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List of Abbreviations

HBI – highly branched isoprenoid

IPP – isopentenyl pyrophosphate

DMAPP – dimethylallyl pyrophosphate

GPP – geranyl pyrophosphate

FPP – farnesyl pyrophosphate

FPPS – farnesyl pyrophosphate synthase

FOH – farnesol

PPPS – prenyl pyrophosphate synthase

TPS – terpene synthase

TPT – *trans*-prenyl transferase

CPT – *cis*-prenyl transferase

Tris – Tris(hydroxymethyl)aminomethane

DTT – Dithiothreitol

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Introduction

The thesis presented herein describes a series of studies aimed at understanding how the marine diatom *Rhizosolenia seitgera* biosynthesizes the unique highly branched isoprenoid (HBI) hydrocarbons that have been previously characterized from this and other species of diatoms. In particular, the studies focus on determining the possible biological functions of these HBIs, elucidating the potential precursors and enzymes involved in HBI biosynthesis, and defining further the underlying mechanisms for HBI biosynthesis. Overviews on terpenoids, the enzymes involved in their biosynthesis, and previous studies on *R. setigera* and the HBIs they produce will provide a background for the overall aims and scope of the current study.

Terpenoids

Among all known and characterized natural products, it has been estimated that terpenoids make up an astounding 60% (Firn, 2010). This group of natural products structurally consist of distinctive C_5 isoprene units that occur in variable yet defined multiples. It is because of this feature that terpenoids are also commonly referred to as isoprenoids and the two terms are used interchangeably within this study.

Terpenoids occur universally in all living organisms where they play a myriad of physiological and ecological functions (Gershenzon & Dudareva, 2007). Due to the diverse natural functions and physical properties that terpenoids exhibit, humans have also found a variety of applications for these compounds dating as far back as the earliest of civilizations (Firn, 2010). In more recent history, a significant amount of focus has been given on their potential applications in pharmaceuticals and renewable energy (Gross & Konig, 2006;

Bohlmann & Keeling, 2008; Melis, 2012). The rapidly expanding applications for terpenoids now push the envelope for understanding how these compounds are biosynthesized and harnessing this information to provide innovations in fields such as biotechnology and synthetic biology.

Terpenoid biosynthesis

The amazing structural diversity among terpenoids stems from the wide variety of enzymes involved in their biosynthesis. Despite this diversity, all terpenoids originate from the fundamental C_5 phosphorylated isoprene unit isopentenyl pyrophosphate (IPP), and its isomer dimethylallyl pyrophosphate (DMAPP). These C_5 isoprenoid units are derived from either of two distinct biosynthetic pathways: the mevalonate (MVA) pathway and the alternative methylerythritol 4-phosphate (MEP) pathway. The MVA pathway is widely present in eukaryotes, archaea, and some bacteria (Buhaescu & Izzedine, 2007) while the MEP pathway is present only in plants, prokaryotes, and some protozoa (Rohmer, 1999; Wiemer *et al*., 2010). In terms of localization within the cell in plants, the MVA pathway occurs in the cytosol while the MEP pathway occurs in plastids. It has generally been accepted that the cytosolic pool of IPP derived from the MVA pathway is used for the biosynthesis of sesqui- (C_{15}), tri- (C_{30}), and polyterpenes (C_{45} and higher) while MEP-derived IPP is used for the biosynthesis of mono- (C_{10}), di- (C_{20}), and tetraterpenes (C_{40}) (Bohlmann *et al*., 1998) but more recent studies have revealed the possibility of the two pathways interacting with each other (Dudareva *et al*., 2005).

Downstream from the MVA and MEP pathways, the formation of larger (C_{10} and higher) terpenoids requires the head to tail condensation of IPP and DMAPP into longer chain prenyl pyrophosphates such as geranyl pyrophosphate (C_{10}, GPP), farnesyl pyrophosphate (C_{15}, FPP), and geranylgeranyl pyrophosphate (C_{20}, GGPP). These linear polyprenyl

pyrophosphates are subsequently used as direct precursors for terpenoid biosynthesis through enzymes broadly classified as terpene synthases (TPSs) which generally catalyse condensation, cyclization, or more complex modification reactions (Christianson, 2008).

The enzymes responsible for the formation of these precursor polyprenyl pyrophosphates are broadly classified as prenyltransferases and are also known as prenyl pyrophosphate synthases (PPPSs). These PPPSs could be further categorized as either *trans*- or *cis*-prenyltransferases and are classified as such based on the resulting geometric orientation of the constituent IPP units (Takahashi & Koyama, 2006) although both typically catalyze chain elongation reactions. This chain elongation reaction is carried out by PPPSs via the initial formation of an allylic carbocation (i.e. from DMAPP) through the dissociation of the pyrophosphate group and the subsequent addition of IPP via the stereospecific elimination of a proton at C-2 of the IPP moiety (Christianson, 2008).

One of the most studied examples of *trans*-prenyltranserases (TPTs) is farnesyl pyrophosphate synthase (FPPS), which catalyses the formation of farnesyl pyrophosphate (FPP), the primary precursor for sequi- and triterpenes. In brief, FPPS initially catalyses the head to tail condensation of DMAPP and IPP after which, another IPP molecule is added to form a C_{15} prenyl pyrophosphate in an all-*trans*-configuration (Reed & Rilling, 1975). All TPTs share a high degree of homology and are characterized by the presence of two conserved aspartate-rich motifs (DDXX(XX)D) that serve as binding sites for IPP and DMAPP (Christianson, 2008). The length of the resulting polyprenyl pyrophohsphate products are determined by amino acid residues found upstream of the first aspartate-rich motif (Ohnuma *et al*., 1996; Tarshis *et al*., 1996).

Unlike the more well studied TPTs, *cis*-prenyltransferases (CPTs) have only recently gained considerable research interest in part due to the fact that most terpenoids characterized

so far arise from polyprenyl pyrophosphate precursors produced by TPTs. As their name suggests, CPTs carry out the chain elongation of allylic prenyl pyrophosphates by the subsequent addition of IPP molecules in the *cis*-configuration. Although both *cis*- and *trans*-prenyltransferases typically carry out chain elongation reactions involving similar substrates, they share very little in terms of amino acid sequence homology and in fact have widely different three-dimensional structures (Takahashi & Koyama, 2006). One of the most studied CPTs is undecaprenyl pyrophosphate synthase (UPPS) which carries out the formation of the C_{55} undecaprenyl pyrophosphate (UPP) by the addition of 8 more IPP molecules in the *cis*-configuration to an FPP molecule (Allen, 1985). Unlike the well-defined aspartate-rich motifs in TPTs, substrate binding in CPTs appears to be mediated by a flexible P-loop located in the conserved Region III (Ko *et al*., 2001) and binding of allylic substrates leads to a conformational change in the enzyme (Guo *et al*., 2005). Product chain length in CPTs has been postulated to be determined by a series of charged residues found at the hinge of helix-3 (Takahashi & Koyama, 2006).

Until recently, nearly all TPTs and CPTs that have been isolated and characterized only catalyze the formation of linear polyprenyl pyrophosphates but studies conducted just within the last decade or so have uncovered examples of TPTs and CPTs that do not catalyze chain elongation reactions but instead carry out unconventional branching and cyclopropanation reactions. Hemmerlin and co-workers (2003) were able to isolate the cDNA of an FPPS homolog in *Artemisia tridentata* (which they denoted as FDS-5) that catalysed the formation of both chrysanthemyl pyrophosphate (CPP) and lavandulyl pyrophosphate (LPP) as its main products. More recently, Demissie and co-workers (2013) were able to isolate the gene that encoded for a CPT homolog, lavandulyl pyrophosphate synthase (LPPS) from the lavender plant. Furthermore, Ozaki and co-workers (2014) discovered the gene for a unique CPT homolog that catalyzed the formation of cyclolavandulyl pyrophosphate. It is interesting to

note that all the enzymes characterized in these studies required only DMAPP as the sole substrate. Such enzymes, especially those that carry out branching reactions, served as interesting precedents for the current study since the branched structure in lavandulyl pyrophosphate closely resembles the branched conjugation found in marine-derived highly branched isoprenoid hydrocarbons.

Highly branched isoprenoids and their occurrence in R. setigera

Highly branched isoprenoid (HBI) hydrocarbons are a unique group of C_{20}, C_{25}, and C_{30} isoprenoids that contain a distinctive branched conjugation at C-7 of their parent carbon skeletons (Figure 1). Interest in these HBIs stems mainly from their widespread occurrence in the marine environment, with C_{20} HBI alkanes being a major component in some crude oils (Yon *et al*., 1982), and C_{25} and C_{30} HBI alkenes being ubiquitously present in seawater and marine sediments (Rowland & Robson, 1990). The relatively wide distribution of C_{25} and C_{30} HBIs has made them ideal geochemical markers for a marine environment since it was further established that the biological source of these HBIs were diatoms (Volkman *et al*., 1994, Wraige *et al*., 1999). Aside from their role as geochemical markers, some isomers of C_{25} HBIs have also been found to exhibit cytostatic activity against certain lung cancer cell lines thus giving them the potential for applications in medicine as well (Rowland *et al*., 2001a).

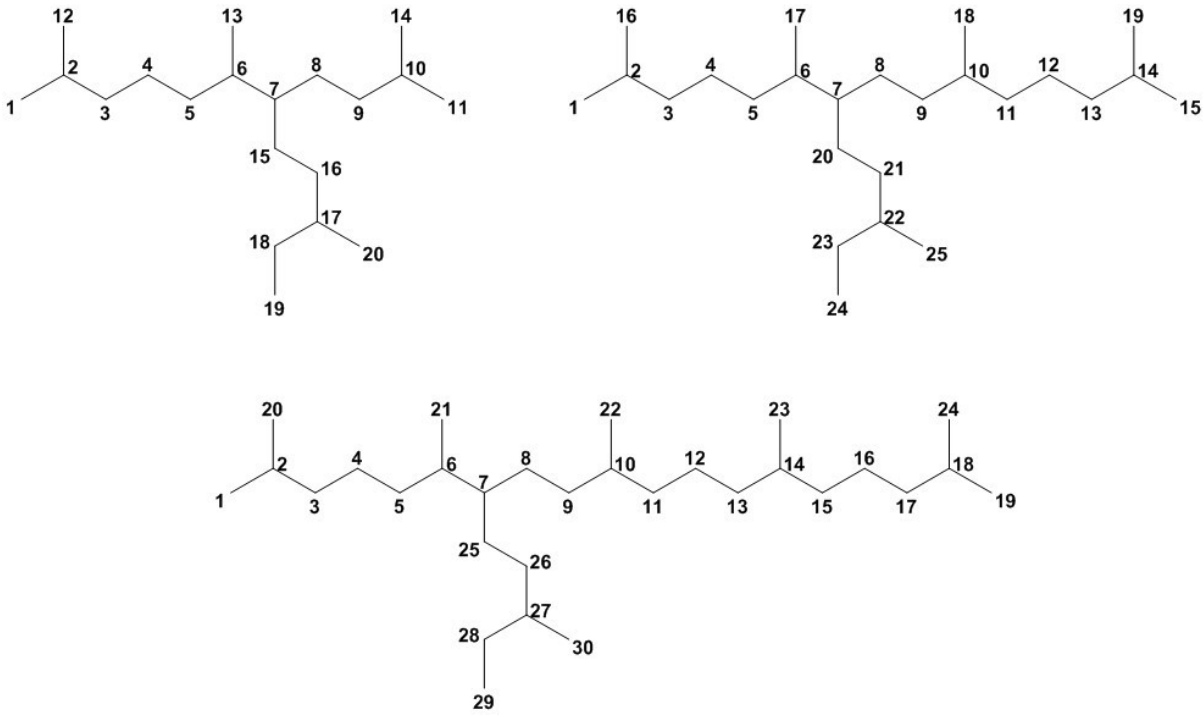

Figure 1: Representative saturated carbon skeletons of C_{20}, C_{25}, and C_{30} HBIs isolated from crude oils, seawater, marine sediments, and diatom cultures. Numbering of carbon atoms was based on structures described by Wraige *et al*. (1999) and Masse *et al*. (2004a)

Diatoms are one of the most diverse groups of marine microalgae with estimates of as high as 100,000 probable species (Mann & Vanormelingen, 2013). Within this extensively diverse group, only a handful are known to produce structurally unique HBI hydrocarbons and among these, the marine diatom *Rhizosolenia setigera* is the only one documented to produce both C_{25} and C_{30} HBIs. Experiments using stable isotope labelling and specific inhibitors for biosynthetic pathways of isoprenoid precursors have revealed that HBIs produced by *R. setigera* are biosynthesized via the mevalonate-based isoprenoid pathway (Masse *et al*., 2004a). Based on this and other structural studies, it has been proposed that the distinctive branched structure in these HBIs is formed by the coupling of either a C_{10} or C_{15} prenyl chain at C-6 of another C_{15} prenyl chain to give rise to C_{25} or C_{30} HBIs, respectively (Figure 2) (Masse *et al*., 2004b; Belt *et al*., 2006). Although sufficient evidence have already been presented regarding the involvement of the mevalonate pathway in producing the basic C_5 isoprenoid unit

that is eventually incorporated into these HBIs, more downstream processes such as the actual coupling reaction for the distinctive branched conjugation and the direct precursors involved in such a reaction still remain to be elucidated. The purpose therefore of the current endeavor was to focus on the intermediate and final steps in the biosynthesis of these HBIs in order to help complete the picture of a potentially unique biosynthetic mechanism.

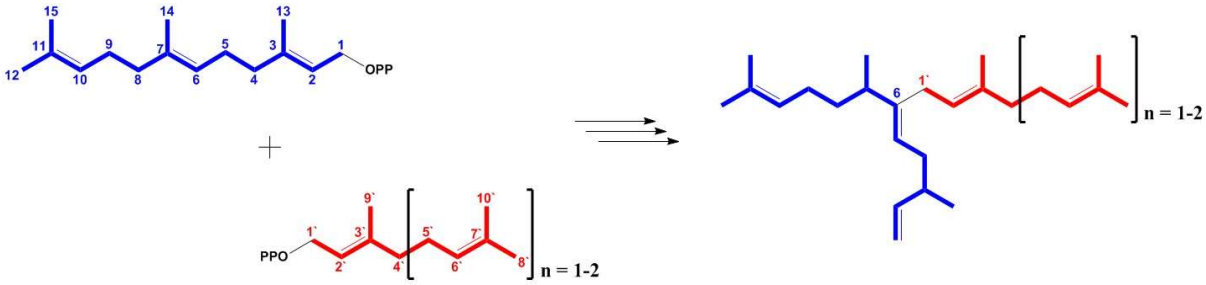

Figure 2: Simplified hypothetical biosynthetic pathway for the formation of representative C_{25} and C_{30} HBIs produced by *R. setigera*.

The current study

Given that the biological role of these HBIs has yet to be determined, initial investigations comprising Chapter I of this study were conducted to shed light on the production rates of these compounds throughout a single culture cycle, and to verify previous observations (Belt *et al*., 2002; Masse, 2003) regarding the predominance of either C_{25} or C_{30} HBIs in relation to the diatom's stage in its life cycle.

Like all other unicellular organisms, a diatom's growth cycle in batch cultures can be divided into four phases, namely the lag, exponential, stationary, and death phases (Figure 3). Preliminary studies were done in order to determine the duration of each phase in the context

of *R. setigera* and to further correlate these phases with the amount of total HBIs present per cell at pre-determined intervals.

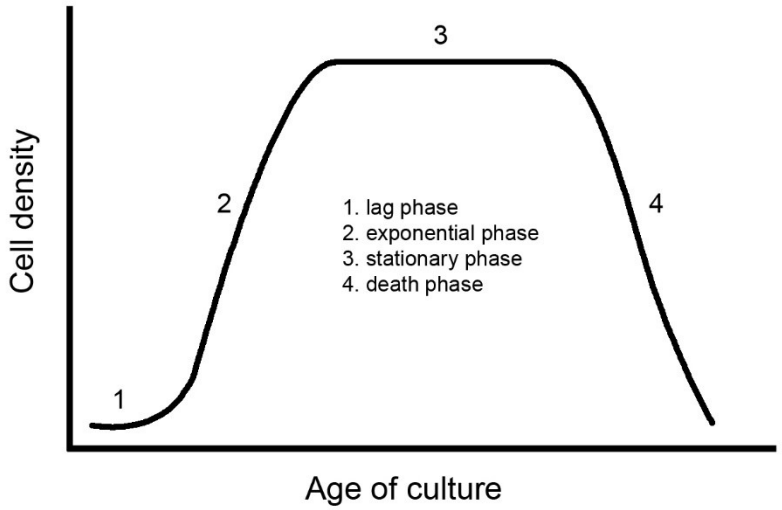

Figure 3: Typical growth curve of microalgae in a single batch culture cycle (adopted from Coutteau, 1996).

The unique morphology of diatoms such as *R. setigera* lies in the presence of a rigid outer silica shell or frustule composed of two overlapping valves with one smaller than the other. With every round of cell division, the two valves are divided among the resulting daughter cells and new valves are subsequently biosynthesized to complete a new silica shell (Figure 4). Throughout this process, one of the resulting daughter cells will be a fraction smaller up until they reach a critical minimum size. Upon reaching this critical minimum size, cells undergo auxosporulation – a type of sexual reproduction specific for diatoms – in order to produce daughter cells that are of their original size. Based on this life cycle, it has also been observed that the HBI profile of *R. setigera* changes, with variations in the predominance of either C_{25} or C_{30} HBIs throughout the life cycle. To verify this previous observation in a different strain, *R. setigera* CCMP 1694 was continuously cultured over 55 culture cycles spanning a period of approximately 2 years and the HBI profile of these were analyzed at the

end of each culture cycle. Results of these studies have thus provided a glimpse of the dynamics of HBI biosynthesis in *R. setigera* that hint at the possible biological role of these unique compounds.

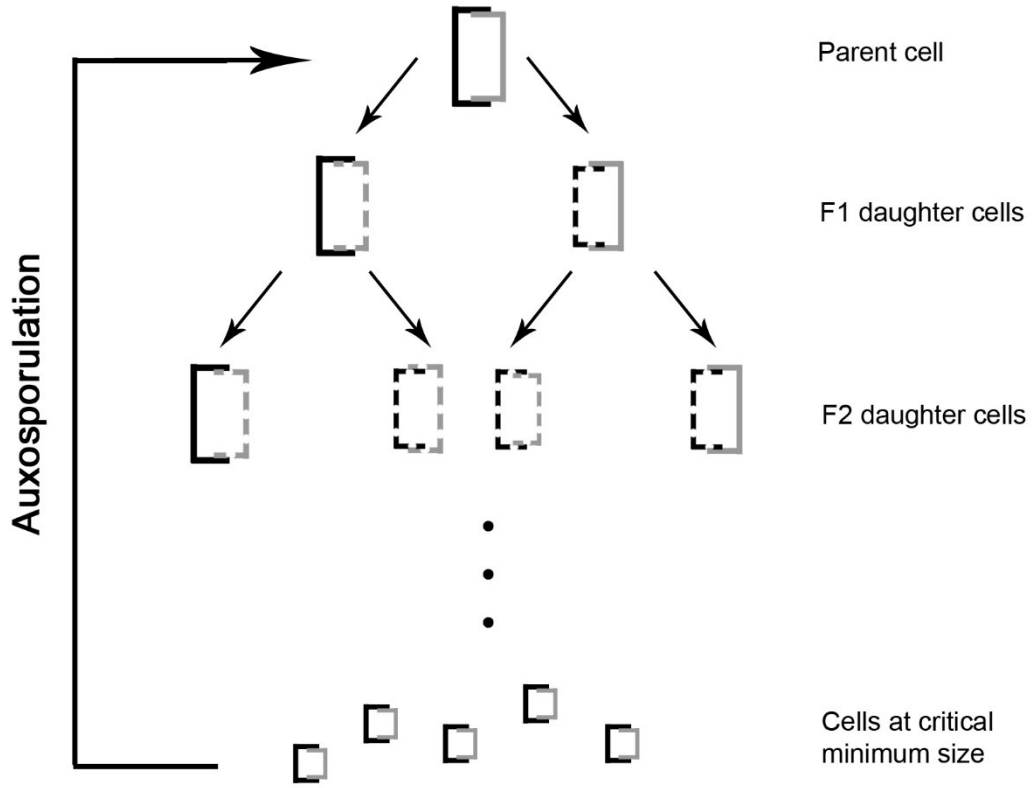

Figure 4: Simplified representation of how diatom size decreases with each successive generation of daughter cells.

In line with the previously hypothesized biosynthetic route for the formation of HBIs, Chapter II of this study revolves around a series of *in vivo* feeding experiments using labelled and unlabeled farnesol (FOH), an alcohol derivative of the C_{15} isoprenoid unit farnesyl pyrophosphate (FPP) in order to investigate its effects on HBI production in *R. setigera*. Previous studies in both higher plants and green algae have demonstrated that FOH can be taken up by cells and phosphorylated into FPP, which is then utilized in the biosynthesis of

other more complex isoprenoids (Thai *et al*., 1999; Inoue *et al*., 1994). In general, the current feeding experiments revealed that treatments with FOH increased the overall HBI content of *R. setigera* cells and indicated that this most likely occurs through the incorporation of FOH via the same pathway that has been previously demonstrated for other plants. It therefore directed the course of the succeeding studies towards determining the role of FPP in HBI biosynthesis.

Armed with the assumption that FPP serves as a precursor for the biosynthesis of HBIs, Chapter III focuses on efforts that were made to isolate and clone a cDNA encoding a putative FPP synthase (FPPS) from *R. setigera* and to characterize the coded enzyme through heterologous expression in *Escherichia coli*. *In vitro* enzyme assays using the purified RsFPPS enzyme revealed that it was a functional FPPS. A series of *in vivo* inhibition experiments using risedronate further suggested that RsFPPS provided substrates for HBI biosynthesis. Expression levels of Rs*FPPS* were also monitored using qRT-PCR in a bid to determine whether this enzyme played a role in the regulation of the types of HBIs biosynthesized by *R. setigera*.

In order to determine the final steps in HBI biosynthesis in *R. setigera* and confirm the role of FPP as a precursor to these unique compounds, a series of cell-free enzyme assays using radiolabeled substrates were conducted and comprise Chapter IV of this study. Crude cell homogenates were used to characterize HBI synthesis activity in terms of the time-dependent formation of products, the possible involvement of other prenyl pyrophosphate precursors aside from FPP, and the tentative requirement for a divalent metal cation for enzyme activity. In all the assays conducted, the formation of HBIs from labeled precursors were observed and this was the first instance that HBI synthesis activity from *R. setigera* or any other diatom has been demonstrated *in vitro*. As such, the findings of these experiments can be considered a major turning point in research regarding HBI biosynthesis. The possible mechanisms for the final

steps in the formation of HBIs deduced from these studies thus help in postulating a new biosynthetic pathway for these unique compounds.

The final section of this thesis summarizes all these results and provides possible directions for future work on HBI biosynthesis.

Chapter I

As the contents of this chapter are anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published in 4 years.

Chapter II

As the contents of this chapter are anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published in 4 years.

Chapter III

The contents of this chapter have been published in a jointly authored paper in a scholarly journal and as the permission of all collaborating authors has not been granted, they cannot be published online. The contents of this chapter are detailed in Scientific Reports Vol. 5, Article number 10246.

Chapter IV

As the contents of this chapter are anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published in 4 years.

Summary

The marine diatom *Rhizosolenia setigera* presents itself as a fascinating subject for research due to its ability to produce structurally unique C_{25} and C_{30} highly branched isoprenoids. Aside from the captivating structure of these HBIs, the potential ecological and pharmacological applications of these compounds serve as sufficient incentives to invest efforts in elucidating their biosynthetic pathway. This thesis therefore endeavored to provide new insights into how HBIs are biosynthesized through a series of biological, biochemical, and genetic experiments.

In a bid to determine the possible biological function of these HBIs, a study on the relative abundance of HBIs per cell revealed that the rate of HBI biosynthesis appeared to be constant during phases of exponential growth. Analysis of changes in HBI composition throughout *R. setigera*'s life cycle further showed that the onset of auxosporulation had a dramatic effect in the shift between C_{25} and C_{30} HBIs. The dynamics observed for HBIs in these experiments followed similar patterns of membrane lipids in other organisms and thus points to the possibility of HBIs having membrane associated functions.

As a preliminary step to picture out possible precursors for HBI biosynthesis, feeding experiments with FOH were conducted. Experiments using unlabeled FOH showed the accumulation of the more unsaturated C_{25} HBI isomers. Analysis of total HBI content per cell further revealed a dose dependent increase of HBIs hinting that FOH could be contributing to HBI biosynthesis. Using ^{14}C-FOH confirmed the incorporation of a C_{15} prenyl chain into HBIs and the incorporation of ^{14}C-FOH into squalene implied that ^{14}C-FOH is initially converted into ^{14}C-FPP before subsequently being used for further terpenoid biosynthesis.

With FPP designated as a tentative substrate for HBI biosynthesis, efforts were made to isolate a cDNA encoding for FPPS. A putatitve FPPS cDNA was successfully cloned and

characterized and revealed that it did encode for a functional FPPS. Analysis of relative expression levels for Rs*FPPS* showed no correlation with the changes in HBI profile throughout *R. setigera*'s life cycle although expression levels coincided with relative growth rates. Inhibition of RsFPPS using risedronate showed a dose-dependent decrease in HBI content per cell indicating that this enzyme most likely provides precursors for HBI biosynthesis.

Finally, cell-free enzyme assays were able to demonstrate HBI synthesis activity *in vitro* for the first time ever. Using combinations of radiolabeled substrates further revealed that DMAPP may be directly involved in HBI biosynthesis through the formation of the distinctive branched conjugation found in HBIs and through a potentially novel and unprecedented mechanism for prenyl chain elongation. The results of these experiments thus provided new information in order to revise the previously hypothesized biosynthetic route for HBI formation.

Taken together, the lines of evidence presented in this study provide additional information to support the hypothesis on the biological importance of these HBIs, reveals the integral role of FPP in their biosynthesis, and offers a more defined picture of how these unique compounds are biosynthesized. From the information gathered in this study, future work on *R. setigera* and overall understanding of HBI biosynthesis should now be focused on areas such as elucidating the actual localization of HBIs in cells and isolating and characterizing the specific genes involved in the final steps in HBI biosynthesis.

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Appendix

Table S1: ^{13}C NMR analysis of C_{25} triene (VI) from *R. setigera* compared to a C_{25} triene characterized by Belt *et al*. (2000) from *Pleurosigma intermedium*.

Carbon number	^{13}C (δ ppm)	
	VI (this study)	XIII* (Belt *et al*., 2000)
23	144.6	144.6
7	143.0	143.0
10	136.1	136.1
9	123.7	123.7
20	122.8	122.9
24	112.1	112.1
3	39.3	39.3
13	39.0	39.0
22	38.2	38.2
5	35.3	35.3
21	34.4	34.4
6	34.3	34.3
11	31.8	31.8
8	29.0	29.0
2, 14	27.9	27.9
12	25.8	25.8
4	25.7	25.7
18	23.5	23.5
1, 15, 16, 19	22.6	22.6
17	19.6	19.6
25	19.5	19.5

* Structure number is based on cited paper.

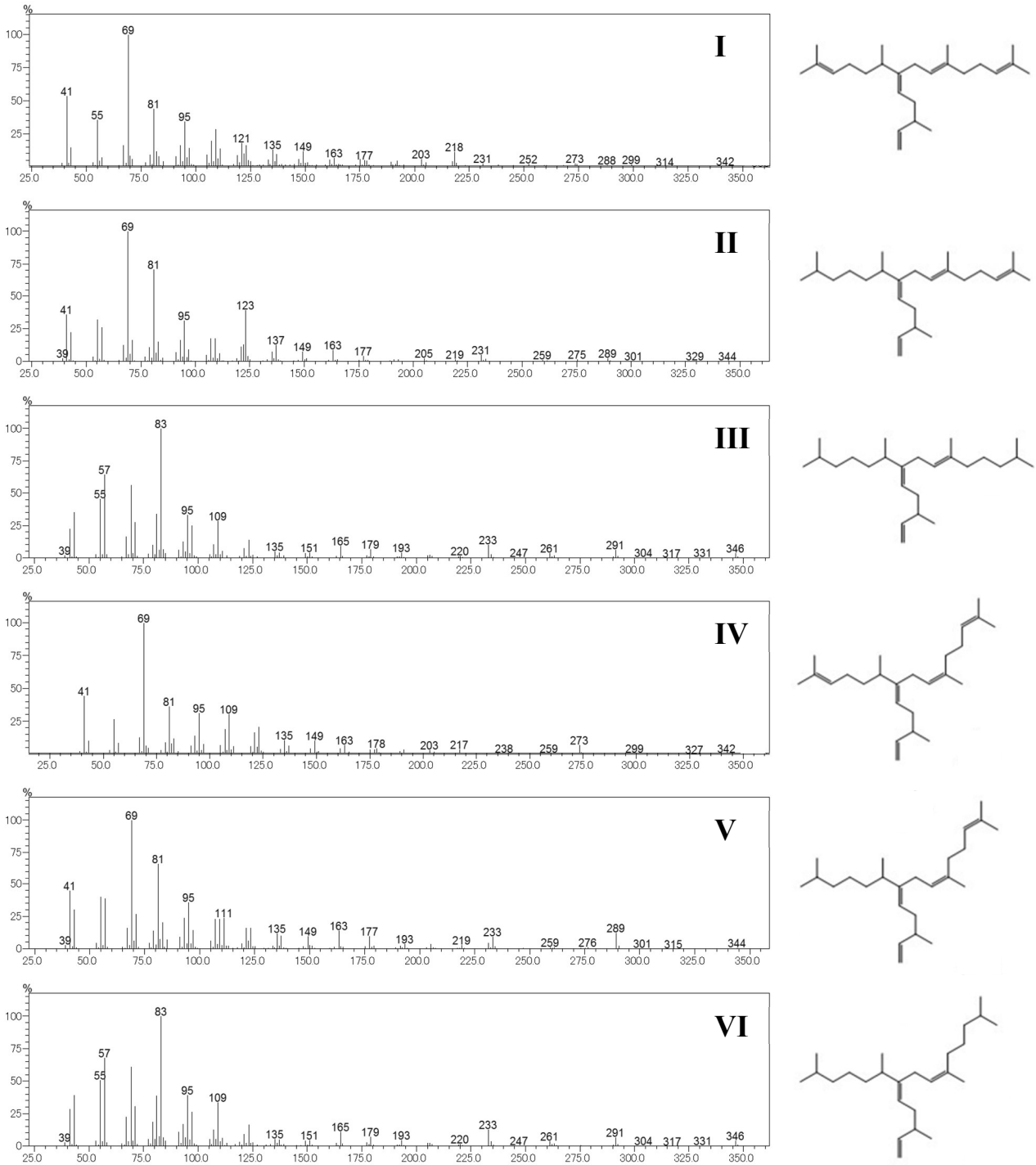

Figure S1: Mass spectra of C_{25} and C_{30} HBIs isolated from *R. setigera* CCMP 1694.

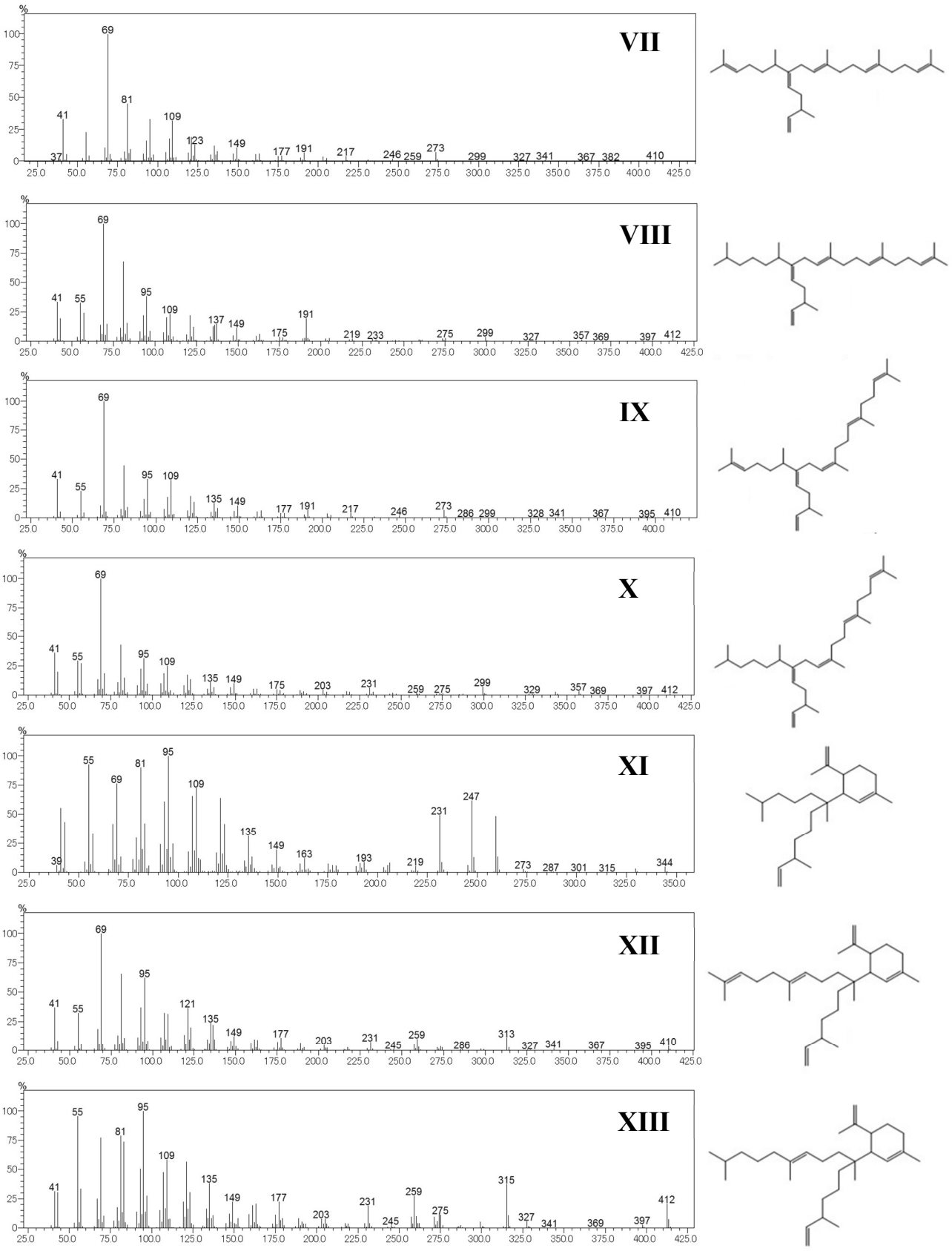

Figure S1: continued…

Table S2: Retention indices of HBIs.

Retention Index (RI)		Corresponding HBI structure[†]
This study*	Previous work[a,b,c]	
2043	2042[a]	VI
2074	2074[a]	V
2087	2087[a]	III
2089	2089[c]	XI
2113	2112[a]	IV
2120	2121[a]	II
2160	2159[a]	I
2504	2505[b]	X
2544	2545[b]	IX
2548	2548[c]	XIII
2558	2558[b]	VIII
2578	2579[c]	XII
2595	2596[b]	VII

[*] using MS-1 column
[a] Belt et al. (2000); HP-1 column
[b] Belt et al. (2001); HP-1 column
[c] Masse et al. (2004c); HP-1 column
[†] based on structures in Figure 4 and Figure S1

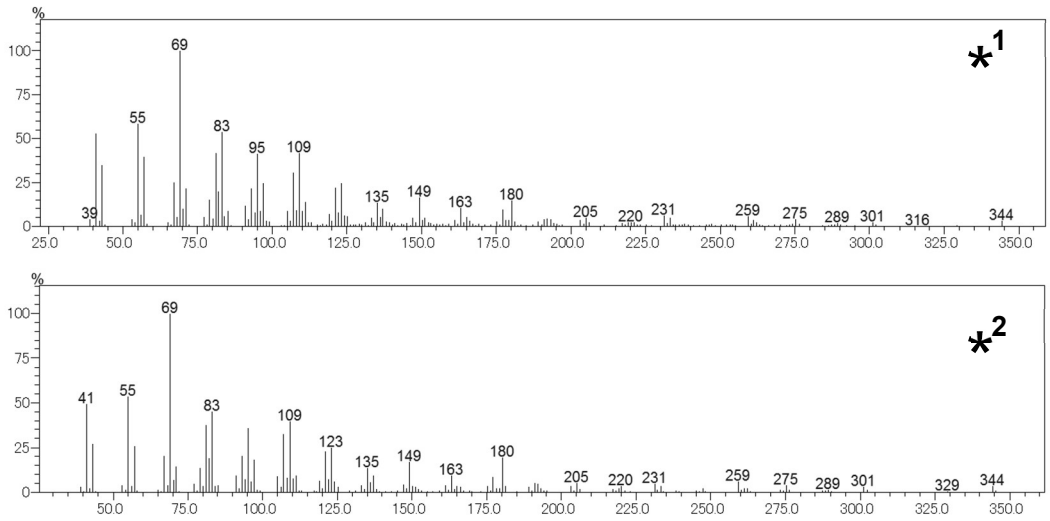

Figure S2: Mass spectra of two unidentified putative C_{25} tetraenes from *R. setigera* fed with FOH.