論 文 の 内 容 の 要 旨

水圏生物科学専攻
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論文題目: Studies on the Biosynthesis of Highly Branched Isoprenoids in the Marine Diatom *Rhizosolenia setigera* Brightwell
(海産珪藻 *Rhizosolenia setigera* Brightwell の高分岐イソプレノイド生合成に関する研究)

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 while C_{25} and C_{30} HBI alkenes being ubiquitously present in seawater and marine sediments. The relatively wide distribution of C_{25} and C_{30} HBIs has made them ideal geochemical markers for the marine environment since it was further established that the biological source of these HBIs were diatoms. Aside from their role as geochemical markers, some isomers of C_{25} HBIs have also been found to have cytostatic activity against certain lung cancer cell lines thus giving them the potential for applications in medicine as well. Moreover, structural similarities between HBIs and botryococcenes from the green microalga *Botryococcus braunii*, which has been touted as a renewable source for energy, also reflect the potential of these compounds as a form of biofuel.

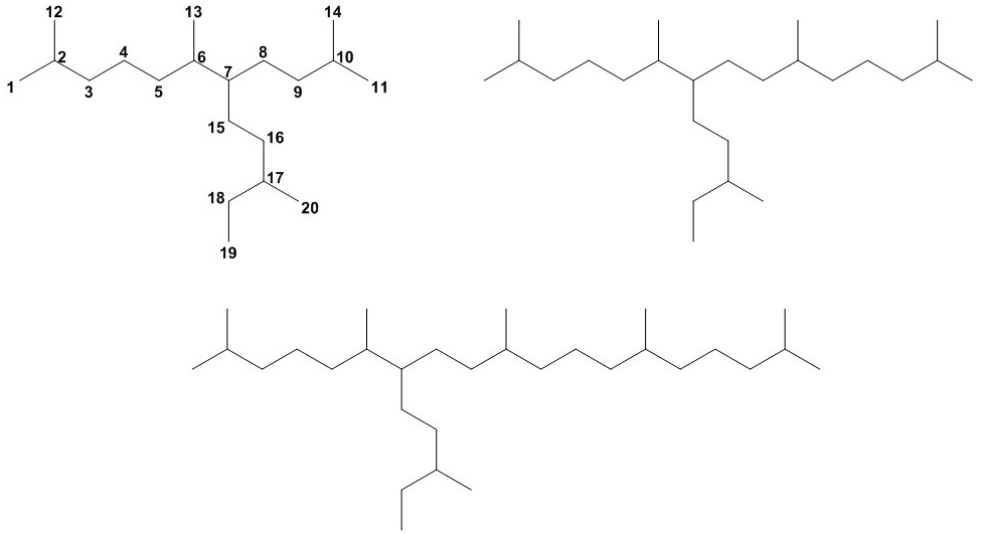

Figure 1: Representative saturated carbon skeletons of C_{20}, C_{25}, and C_{30} HBIs isolated from crude oils, seawater, marine sediments, and diatom cultures. Numbering of carbon atoms were based on structures described by Wraige *et al*. (1999) and Masse *et al*. (2004)

Diatoms are one of the most diverse groups of marine microalgae with estimates of as high as 100,000 probable species. 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 pathway. Based on this and other structural studies on HBIs, it has been proposed that the distinctive branched structure in these HBIs are 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). Although sufficient evidence has 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.

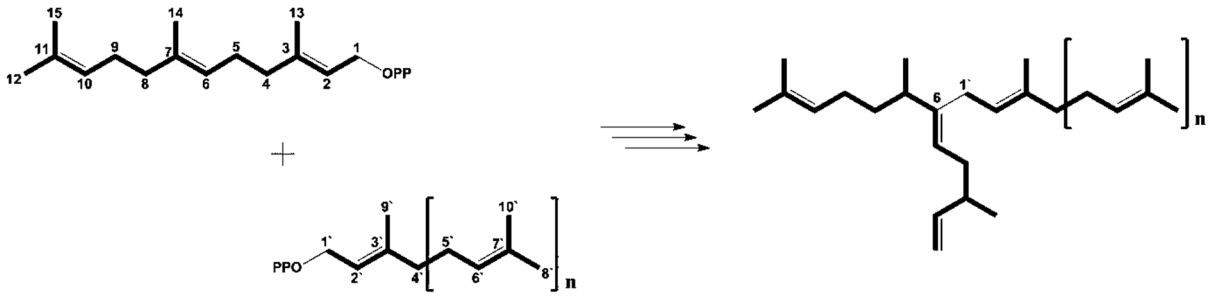

Figure 2: Simplified hypothetical biosynthetic pathway for the formation of representative C_{25} and C_{30} highly branched isoprenoids produced by *R. setigera* (n = 1 or 2).

Given that the biological role of these HBIs has yet to be determined, initial investigations comprising Chapter 1 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 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 could be divided into four phases, namely the lag, exponential, stationary, and death phases. The duration of this entire cycle varies from organism to organism and for *R. setigera*, a typical culture cycle up until the start of the death phase lasts for around 15 days with the lag phase lasting for 1 to 2 days after inoculation followed by an exponential growth phase of 7 to 9 days, and a stationary phase of around 5 to 7 days after which the death phase follows. Determination of the total amount of HBIs per cell at 3-day intervals throughout one culture cycle revealed that there was a significant decrease from the 1st day of culture, up until the start of the exponential growth phase. During the exponential phase though, total HBI amount per cell remained relatively constant with only a slight increase upon the onset of the stationary phase. This type of oscillation in the total amount of HBIs per cell was repeatedly observed for three consecutive culture cycles. This indicates that the production of HBIs throughout a single culture cycle is concomitant with growth and hints at the possibility of HBIs having membrane associated functions as previously hypothesized.

The unique morphology of diatoms such as *R. setigera* lies in the presence of a rigid outer silica shell 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. 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 been observed that the HBI profile of *R. setigera* changes as well with variations in the predominance of either C_{25} or C_{30} HBIs throughout their life cycle. In the *R. setigera* strain CCMP 1694 used throughout this study, it was observed that C_{30} HBIs were the more dominant HBIs in culture cycles directly after an auxosporulation event. As culture cycles progress further from an auxosporulation event, the amount of C_{25} HBIs gradually increased, and from around 15-20 culture cycles after auxosporulation, C_{25} HBIs were more dominant than their C_{30} counterparts in cells. This phenomenon was observed in two complete life cycles of *R. setigera* CCMP 1694 which included three auxosporulation events. The observations made in this study had notable differences from those made by Masse and co-workers (2002) using a different strain although some general similarities noted in terms of shifts in relative abundance of HBI homologues could further support the hypothesis that HBIs have membrane associated functions.

In line with the previously hypothesized biosynthetic route for the formation of HBIs, Chapter 2 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} prenyl pyrophosphate 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 and phosphorylated into FPP which is then utilized in the biosynthesis of other more complex isoprenoids. When unlabeled FOH was fed to cultures of *R. setigera* and their HBI profiles were analyzed by GCMS, it was observed that new peaks corresponding to C_{25} pentaenes were detected compared to control cultures. These C_{25} pentaenes are believed to be pecursors of the more dominant C_{25} tetra- and trienes generally observed in the cultures of *R. setigera*. Although increasing concentrations of FOH had a negative effect on the growth of *R. setigera*, a significant increase in total HBI content per cell was observed. In feeding experiments using ^{14}C-labelled FOH, considerable incorporation of radioactivity was also observed in both C_{25} and C_{30} HBIs and it is presumed that this incorporation takes place via the successive phosphorylation of FOH to form FPP prior to HBI biosynthesis. Variations in incorporation rate between C_{25} and C_{30} HBIs were also noted depending on the life cycle stage of the cells used.

Armed with the assumption that FPP serves as a precursor for the biosynthesis of HBIs, Chapter 3 focuses on efforts that were made to isolate and clone a cDNA encoding a putative FPP synthase (FPPS) from *R. setigera* and characterize the coding enzyme through heterologous expression in *Escherichia coli*. Mining a *de novo* assembled transcriptome database from RNASeq data of *R. setigera*, a single hit for a contig spanning 1545 bp with an open reading frame of 1299 bp (432 amino acid residues) was isolated and designated as Rs*FPPS*. Cloning and subsequent sequence analysis revealed a high degree of similarity with other known FPPSs. Assays using the purified RsFPPS enzyme heterologously expressed in *E. coli* showed considerable FPPS activity, thus confirming its function. Along with this, expression analysis of Rs*FPPS* using qRT-PCR was done to try to correlate Rs*FPPS* expression levels with the type of HBIs observed at various life cycle stages of *R. setigera*. Although Rs*FPPS* expression did not follow the expectation that higher expression levels would be observed in stages wherein C_{30} HBIs were predominant, expression levels did reflect a positive correlation with relative growth rates. Furthermore, culture experiments using the specific FPPS inhibitor risedronate also revealed that inhibition of FPPS

significantly reduced the amount of HBIs produced by *R. setigera*, thus demonstrating the important role of FPPS in supplying precursors for HBI biosynthesis.

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 4 of this study. Cells of *R. setigera* from large scale cultures were harvested by filtration and homogenized in an assay buffer system with components similar to those used in prenyltransferase and terpene synthase assays. Initial experiments using ^{3}H-FPP as the sole substrate revealed the time-dependent formation of C_{30} HBIs over a 48-hour incubation period at 20 °C. The formation of C_{25} HBIs on the other hand was minimal to negligible. To demonstrate that the enzymatic reaction responsible for the formation of HBIs is similar to the activity of prenyltransferases and terpene synthases that have an absolute requirement of a metal ion for substrate binding, assays were done in the absence of magnesium (Mg^{2+}) or with the addition of EDTA. The results of these experiments showed an absolute requirement for a divalent metal cation, in this case Mg^{2+}, and that the HBI synthase activity could be similar to that of prenyltransferases and terpene synthases. This is the first instance that HBI synthase activity from *R. setigera* or any other diatom has been demonstrated *in vitro* and 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.

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.