学 位 論 文 内 容 の 要 約/Dissertation Summary

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学 位 論 文 題 名/Title of Dissertation

Enzyme catalysis for algal secondary metabolites: Studies on halogenated compounds from *Laurencia* spp. and cyanobacterial homotyrosine
（藻類の二次代謝における酵素反応：ソゾ類のハロゲン化合物および藍藻のホモチロシンに関する研究）

The study of biosynthesis is a fundamental aspect of natural product research. Deciphering biosynthetic pathways not only provides deeper insights into the underlying mechanisms of life but also establishes methodologies for the sustainable utilization of natural products as well as the development of novel compounds.

Algae play a crucial role in aquatic ecosystems. As primary producers, they carry out photosynthesis, create habitats for other organisms, and produce a variety of secondary metabolites with a wide range of bioactivity. These metabolites are primarily divided into four categories: terpenoids, nonribosomal peptides (NRPs), polyketides, and ribosomally synthesized and post-translationally modified peptides (RiPPs).

In Chapter 1, cyanobacteria *Microcystis aeruginosa* NIES-4285 was chosen for the investigation as this strain produced some novel compounds that belonged to anabaenopeptin and microginin. Particularly these compounds contained a rare non-proteinogenic amino acid as their building block, 2-amino-5-(4’-hydroxylphenyl) pentanoic acid, (doubly homologated tyrosine, di-hTyr). Because it remained unknown that how the organism produce di-hTyr, its biosynthetic mechanism was studied in this chapter.

To determine the corresponding genes, the genome of *M. aeruginosa* NIES-4285 was subjected to antiSMASH for analysis. a gene cluster comprising *MahphA*, *MahphB*, and *MahphCD* was identified. This gene cluster together with aminotransferase

MahphE is supposed to produce di-Tyr. Among them, *MahphA* belonged to Isopropylmalate synthase, *MahphB* was Isopropylmalate dehydrogenase, while *MahphCD* was Isopropylmalate dehydratase.

After the PCR (polymerase chain reaction) and constructed the genes into the plasmids, *in vitro assay* was conducted to determine the catalysis. L-Tyrosine (L-Tyr) was incubated with the *E. coli* BL21 (DE3) strain that contained *MahphABCDE*. The supernatant was subjected to LC-MS and the products were identified by comparing with standards including hTyr and di-hTyr as well as tri-hTyr. The results suggested that hTyr as well as di-hTyr can be detected from the supernatant in 24 hours incubation. When the incubation time was extended to 72 hours, tri-hTyr can be detected from the supernatant. Similarly, when hTyr was incubated with the *E. coli* strain, di-hTyr can be detected in 24 hours and tri-hTyr can be detected in 72 hours. Furthermore, the promiscuity of the gene cluster was texted by incubating L-phenylalanine with the *E. coli* strain. Followed by the LC-MS analysis, hPhe as well as di-hPhe was determined from the supernatant by comparing with the standards. These experiments demonstrated that this cluster *MahphABCDE* was capable of converting tyrosine and phenylalanine into their homologated and doubly homologated derivatives. Additionally, the doubly homologated form of tyrosine was further transformed into its triply homologated derivative.

The putative pathway of di-hTyr can be outlined as follows: Tyr is converted into 4-hydroxyphenyl-pyruvic acid by the action of MaHphE. MaHphA then catalyzes the condensation of acetyl-CoA with 4-hydroxyphenyl-pyruvic acid, leading to the formation of 2-(4-hydroxybenzyl)-malic acid. Subsequently, MaHphCD functions as an isomerase to convert 2-(4-hydroxybenzyl)-malic acid into 3-(4-hydroxybenzyl)-malic acid. Decarboxylation and oxidation of 3-(4-hydroxybenzyl)-malic acid by MaHphB result in the formation of 2-oxo-4-(4-hydroxybenzyl) butanoic acid. This product is then subjected to the catalytic activity of MaHphE, yielding hTyr. hTyr is further transformed to generate di-hTyr and tri-hTyr. Following a similar process for Phe to produce hPhe

and di-hPhe.

This gene cluster represents the first reported instance of transforming two distinct amino acids into their doubly homologated forms, to the best of our knowledge.

As the gene cluster can produce several homologated amino acids, an effort was made to identify the gate keeper that recognize di-hTyr. However, this effort failed due to the insolubility of the candidate protein.

In Chapter 2 and 3, red alga *Laurencia nipponica* and *L. saitoi* were investigated. As *Laurencia* spp. is widely recognized as a prolific source of secondary metabolites. To date, over 1,000 compounds have been identified from this genus, as cataloged in the MarinLit database (marinlit.rsc.org), many of which are halogenated compounds, including terpenoids and acetogenins. And many of the compounds contain halogen atom, such as Cl, Br and I. It is suggested that the halogenated compounds are catalyzed by VBPOs (vanadium-dependent bromoperoxidases), however, the biosynthetic pathway of many natural halogenated compounds still remain unknown. Therefore, it is necessary to study the pathway mediated by VBPOs.

In Chapter 2, the biosythetic of laurencin was investigated. Laurencin, an eight-membered cyclic ether, has attracted significant attention due to its intriguing structure and notable antifouling activity. Since its isolation, its biosynthetic pathway has been a topic of ongoing investigation. While laurediol has been proposed as the natural precursor and deacetyllaurencin has been regarded as a key intermediate, definitive evidence has remained elusive.

To study this, four vanadium bromoperoxidases (VBPOs), including LnVBPO1, LnVBPO2 from *L. nipponica*, and LsVBPO1, LsVBPO2 from *L. saitoi*, were employed in enzymatic assays to investigate the role of VBPOs in the production of acetogenins. *L. nipponica* produces laurencin, while *L. saitoi* produces thyrsiferol. The four VBPOs were constructed into the plasmid with SUMO-tag and His-tag for heterologous expression in *E. coli* BL21 (DE3). Laurencin was extracted from *L. nipponica*, followed by converting it into deacetyllaurencin, TMS-deacetyllaurencin and TMS-capped (3*E*,

6*R*, 7*R*)-laurediol via organic chemistry. Each standard was checked by LC-MS and NMR. Once the standards were prepared, VBPOs were purified from *E. coli* by a nickel column. Thereafter, *in vitro* assay was conducted, followed by transforming the supernatant to LC-MS for analysis. In the small scale assay, four VBPOs were able to produce brominated compounds by using TMS-laurediol as substrate. Through comparing and co-injecting with TMS-deacetyllaurencin standard, one of the products showed the same retention time as well as mass pattern as that of TMS-deacetyllaurencin. Therefore, this product was marked as product **1** (*m/z* 385, 387) and was purified via HPLC from the large scale assay. Finally, 0.8 mg product **1** was obtained, and its ^{1}H NMR almost superimposed on that of deacetyllaurencin, except for the terminal alkyne proton. As the terminal alkyne proton in deacetyllaurencin was replaced by trimethylsilyl group in TMS-deacetyllaurencin. While the carbon chemical shift almost matched with that of deacetyllaurencin and TMS-deacetyllaurencin via HSQC. These results suggested that deacetyllaurencin can be converted from laurediol by the four VBPOs.

Then, 1 mg/mL standard sample of TMS-deacetyllaurencin was prepared, by utilizing this sample, a linear equation was obtained via injecting different volume of the sample into the LC-MS. Finally, the equation was y = 51506x - 104449 (R^2 = 0.9745). According to this equation, the yield of TMS-deacetyllaurencin by each VBPO was calculated, which showed as follow: LnVBPO1: 76%, LnVBPO2: 64%, LsVBPO1: 78% and LsVBPO2: 90%.

Considering that not only mono-brominated compounds were uncovered from *Laurencia* spp., but also doubly brominated compounds were determined, such as laureoxanyne. Thus, deacetyllaurencin was further incubated with each VBPO for *in vitro* assay. Based on the small scale assay, doubly brominated compounds were detected from each VBPO, analyzed by LC-MS. Although the yield of LsVBPO2 was quite low. To purify the enough amount for NMR determination, large scale assay was conducted. Finally, four doubly brominated compounds were purified successfully, they

were marked as compound **2** (*m/z* 390, 392, 394), **3** (*m/z* 409, 411, 413), **4** (*m/z* 409, 411, 413) and **5** (*m/z* 390, 392, 394), respectively. After analyzing the NMR, compound **2** was identified as laureoxanyne. While bromine was attached to the C9 and C10 in compound **3** and **4**, respectively. In compound **5**, bromine was suspected to attached to the terminal alkyne, as its terminal alkyne proton (δ_H = 2.8 ppm) disappeared. Moreover, HMBC indicated that δ_H = 5.5 ppm correlated to δ_C = 201 ppm and δ_C = 74 ppm. Also, δ_H = 6.1 ppm correlated to δ_C = 201 ppm and δ_C = 103 ppm. Additionally, C5 showed the correlation with C4 and C6, these correlations in COSY. These chemical shifts indicated that the partial structure of compound **5** should be bromoallene unit. Bromoallene unit was further confirmed by specific rotation with JASCO P2200. The specific rotation of **5** was turned out to be $[\alpha]^{20}_D$= +147.43° (*c* = 0.1g/dL, $CHCl_3$). Nevertheless, more evidence on the total structure of compound **5** need to be involved.

To provide a plausible reason on why the yield of doubly brominated compounds from LsVBPO2 was quite low, docking was conducted between the four VBPOs and laurediol and deacetyllaurencin, separately. First of all, Autodock Vina was employed for the analysis. The results suggested that there was no hydrogen bond between deacetyllaurencin and LsVBPO2. Whereas, when other methods were applied, such as SeamDock and DiffDock, almost no difference among all the samples. Therefore, convincing explanation still remains lacking.

To deepen our understanding of VHPOs (vanadium dependent haloperoxidases) characteristics, a phylogenetic tree was conducted and analyzed the domains of each gene with thirty VHPOs along with two phosphatases as outgroups. The results revealed that all VBPOs clustered together, exhibiting the presence of the PAP2_haloperoxidase domain at the C-terminal region. In contrast, VCPOs not only featured the PAP2 domain but also harbored the VCPO_N domain at the N-terminal, a feature that is absent in both VBPOs and VIPOs.

In this chapter, the pathway from laurediol to deacetyllaurencin and further to laureoxanyne has been comfirmed completely. The final step for laurencin is supposed

to be catalyzed by an acetyltransferase. Since two substrates were administrated to the four VBPOs, these VBPOs produced the overlapped products based on the LC-MS results. This result indicated that the four VBPOs are promiscuous as they can accept more than one substrate, and multiproducts as one precursor can be transformed into multiple different products. Hence, it can be inferred that the compounds with functional groups such as alkene and alkyne that can undergo electrophilic reaction, then the compounds can be catalyzed by VBPO.

In Chapter3, the role of VBPOs in the biosynthesis of polyether triterpenoids was investigated. As *L. saitoi* produces magireol-A, -B and -C as well as thyrsiferol. These compounds garner the attention due to their bioactivity and intriguing structure. However, the biosynthetic pathway remains unclear. It is hypothesized that these halogenated polyethers triterpeniod came from a precursor, named as squalene epoxide. According to their structure, thyrsiferol is suspected to be derivative from squalene tetraepoxide. Nevertheless, it is arduous to prepare squalene tetraepoxide, therefore, simpler compounds were synthesized as the model substrate to study the mechanism of cyclic ether. Furthermore, robust evidence was provided that VBPOs can not only produce brominated compounds but also bromo-chloro compounds by using *trans*-nerolidol.

Firstly, monoepoxide 154 was employed as the model substrate. It was incubated with LsVBPO1 or 2, under the different pH (pH 7 and pH 6), followed by LC-MS analysis with APCI positive mode. The results suggested that a brominated compound was produced with *m/z* 233, 235. Thus, a large scale assay was conducted for 3 days and 5 days. The product was checked by LC-MS, followed by HPLC. However, the product was not detected at 210 nm, indicating that no sp^2 hybridization. Therefore, the molecular formulate was suspected as $C_{10}H_{19}BrO_2$, and the mass was supposed to be $[M - H_2O + H]^+$: *m/z* 233. Also, the product disappeared after the vacuum drying. Thus, it can be confirmed that the product is volatile, leading to the difficulty in accumulating. To make it nonvolatile, MTPA-Cl esterification was applied to the crude extract. The

fraction was subjected to LC-MS, in which displayed a product of *m/z* 467 was observed. Unfortunately, the compound was failed to accumulated the enough amount for NMR check due to the low yield.

Sequentially, the other starting material, monoepoxide 222, was utilized for the *in vitro* assay. The enzymatic products were purified via HPLC and the structure were determined by NMR.

Monoepoxide 222 was incubated with the four VBPOs, respectively, for small scale assay, followed by LC-MS analysis. The LC-MS showed that the four enzymes produced brominated compounds from monoepoxide 222. Thus, the large scale assay was conducted, followed by HPLC purification. The first compound was purified, denoting as compound **7** (*m/z* 319, 321). In the NMR of compound **7**, the δ_H=5.17 (1H) ppm indicated a double bond within the structure while the other carbon should be a quaternary carbon as the integrity suggested there was only one proton. However, the epoxide group did not changed in the structure, therefore, the double bond between C2 and C3 should react with HOBr. And quaternary carbon δ_C=72.5 ppm was supposed to attach to an oxygen, while δ_C=70.67 ppm with δ_H=3.97 ppm is supposed to be the position connecting to bromine. Finally, based on the HMBC and COSY, the structure of compound **7** was determined as a linear structure.

As compound **7** was not a cyclic compound, the process of purification was continuously conducted. The second product was denoted as compound **8** (*m/z* 337, 339).

The ^{1}H NMR and ^{13}C NMR of **8** indicated that major NMR data superimposed as those of **7**. However, except the quaternary carbon connected with the terminal hydroxyl group (δ_c = 72.58 ppm), additional two carbons also connected to an oxygen. Finally, it can be inferred that the epoxide group was converted into two hydroxyl groups. Thus, **8** is still a linear compound.

The third product was named as compound **9** with *m/z* 319, 321. Ultimately, the amount was 2.8 mg derivative from 30 mg monoepoxide 222. The ^{1}H NMR was similar

to those of **7** and **8**, indicating a common structure among the three compounds. However, the ^{13}C NMR of δ_C = 78.63 ppm indicated the carbon in a cyclic structure connecting to an oxygen. Moreover, the disappearance of the epoxide signals further provided a possibility of a cyclic structure within **9**. Finally, product **9** was identified by 2D NMR, especially δ_H = 3.97 ppm showed a HMBC signal to δ_C = 78.63 ppm, which indicated there was a cyclic structure.

Therefore, it is demonstrated that epoxide group can be converted into cyclic ether by VBPOs. This result laid the foundation for the biosynthesis of polyether triterpenoids.

During structure determination, it is observed that compound **7** closely resembled haloneroliol. Additionally, no vanadium-dependent chloroperoxidases (VCPOs) have been identified from *Laurencia* spp., providing an opportunity to test whether VBPOs are capable of producing bromo-chloro compounds.

In this assay, (*E*)-3*S*-nerolidol was employed as a substrate for *in vitro* assay. Considering that the average Cl^-concentration in the ocean is around 600 mM, we designed eight test groups with Cl^-concentrations ranging from 50 mM to 800 mM. These small-scale assays (100 μg substrate with 20 μg enzyme) were conducted using the four VBPOs.

Following LC-MS analysis, extracted ion chromatograms (EIC) were used to detect different halogenated compounds: EIC at *m/z* 300-330 for brominated compounds, EIC at *m/z* 250-280 for chlorinated compounds, and EIC at *m/z* 335-360 for bromo-chloro compounds. The results showed that all four VBPOs produced brominated compounds, but no chlorinated compounds were detected. Notably, a possible bromo-chloro compound (*m/z* 335, 337, 339 in a 2:3:1 ratio) was observed. Hence, a large-scale assay was subsequently performed using LsVBPO1 for product purification. The crude extract was subjected to silica gel chromatography, and each fraction was analyzed by LC-MS. In total, twelve halogenated compounds were detected, labeled from **10** to **21** (**Figure 3.2-35**). Among these, seven were brominated compounds, and five were bromo-chloro

compounds.

Two bromo-chloro compounds — **10** and **11**— were successfully purified for NMR identification. Notably, compound **10** was determined as halonerolidol. For **11**, Br^+ and Cl^-were attached to C6 and C7, respectively. Regarding compound **11**, the HMBC clearly demonstrated that the dimethyl allyl group existed. Also, the chemical shift from C1 to C4 did not changed from (*E*)-nerolidol, therefore, it suggested that double bond between C6 and C7 reacted with HOBr. Furthermore, a brominated proton δ_H=4.05 ppm is connected to C6 of 63.77 ppm, while C7 showed 75.86 ppm. Thus, it suggested that Br was connected to C6 while Cl was connected to C7.

Here, it is the first time to prove that VBPOs are able to produce bromo-chloro compounds. The mechanism is likely to rise from the quench of Cl^- during the bromonium cation, in other words, the $Cl^-$is passively added to the substrate to produce bromo-chloro compound.

Two additional brominated compounds, **19** and **20** were also purified. Compound **19** had a structure similar to **7** and **10**, while **20** resembled **11**. These findings suggest again that anions in the environment can attack the molecule during bromonium cation formation, indicating a non-enzymatic reaction.

Regarding compound **19**, its ^{1}H NMR and ^{13}C NMR suggested that two double bond were within the structure. Furthermore, HMBC of two methyl correlated to δ_C=72.62 ppm indicted that the terminal double bond reacted with HOBr as well as H_2O. Based on the 1H NMR (δ_H=3.96 ppm) and ^{13}C NMR (δ_C=70.96 ppm and δ_C=72.62 ppm), it can be inferred that Br attached to C10 while OH attached to C11.

Regarding compound **20**, two double bonds existed based on the ^{1}H NMR and ^{13}C NMR. As the double bond between C1 and C2 did not change. While the HMBC showed the dimethyl allyl group, thus, it was the double bond between C6 and C7 that reacted with HOBr. According to COSY and HMBC, compound **20** was determined as linear structure. And δ_C=70.37 ppm and δ_H=4.00 ppm suggested the position was attached by Br.

Finally, the proposed pathway for those compounds are as follow. *trans*-nerolidol can be formed into different bromonium cation, each intermediate can be attacked by either H_2O or $Cl^-$to produce different products. From *trans*-nerolidol, the bromonium cation can form between either C10 and C11 or C6 and C7, leading to the two cation intermediate. When they were quenched by H_2O, **19** and **20** were produced, while when quenched by Cl^-, **10** and **11** were produced.

Collectively, Compounds **7** and **8** as well as **9** were analyzed by LC-MS APCI positive mode derivative from *Ls*VBPO1-monoepoxide 222. Furthermore, as **9** is a seven member cyclic ether, this result led to the determination for the role that VBPOs can convert epoxide into cyclic ether. Additionally, bromo-chloro compounds such as halonerolidol were produced by incubating (*E*)-3*S*-nerolidol with VBPOs in the solution containing Br^- and Cl^-. This result led to the confirmation that VBPOs can produce not only brominated compounds but also bromo-chloro compounds.

This study combined organic synthesis with molecular biology to investigate the biosynthetic pathways for homologated amino acids in cyanobacteria, and for acetogenin and cyclic terpenoid ether in *Laurencia* species.

A gene cluster from *Microcystis aeruginosa* NIES-4285, consisting of *MahphA*, *MahphB*, and *MahphCD*, not only produces homotyrosine and homophenylalanine but also converts these homologated amino acids into doubly homologated amino acids, specifically 2-amino-5-phenylpentanoic acid (di-hPhe) and 2-amino-5-(4'-hydroxyphenyl) pentanoic acid (di-hTyr). The pathway then leads to a triply homologated amino acid, 2-amino-6-(4'-hydroxyphenyl)hexanoic acid (tri-hTyr).

Homologated amino acids serve not only as essential building blocks of natural products but also as key intermediates in the synthesis of pharmaceutical compounds. For instance, homophenylalanine is utilized in the synthesis of Benazepril and Enalapril, which are angiotensin-converting enzyme (ACE) inhibitors. Similarly, homoalanine is a structural component of levetiracetam, a drug used to treat seizures. Furthermore,

homotyrosine has demonstrated inhibitory activity against *Pseudomonas aeruginosa* and *Streptococcus pyogenes*.

Regarding deacetyllaurencin, this is the first study to present NMR data for the enzymatic product and to compare it with an authentic standard via LC-MS. These findings confirm the complete biosynthetic pathway from laurediol to deacetyllaurencin, mediated by VBPOs. Additionally, four derivatives were isolated from an enzymatic assay applying deacetyllaurencin to LsVBPO1, marking the first time that full NMR data has been obtained for laurenoxayne, a natural compound identified in *L. nipponica*. The other three derivatives have not yet been identified in *Laurencia* spp. The study also explored the promiscuity of four VBPOs, revealing identical LC-MS profiles when administrated the identical substrate. And a single VBPO can convert a precursor into different intermediate and finally form into the products. Hence, the four VBPOs are of mutiproducts. However, these products are somehow overlapped, therefore, the four VBPOs are also of redundancy.

The final chapter delved into the mechanism of VBPO-mediated epoxide cyclization using monoepoxide 154 and monoepoxide 222. Identification of products **7**, **8**, and **9** confirmed the VBPO-mediated cyclic terpenoid ether reaction. This study represents the first use of epoxide with VBPO and the subsequent determination of products via NMR. It offers evidence for the hypothesis that polyether triterpenoids derived from epoxide precursors. Furthermore, the catalytic ability of VBPOs has been extended to produce bromo-chloro compounds, rather than brominated compounds only.

Overall, this study examined three types of secondary metabolites, including homo amino acids in the cyanobacterium *M. aeruginosa* NIES-4285, acetogenins, and brominated cyclic terpenoid ethers in *Laurencia* spp. These findings not only shed light on the biosynthetic pathways, establishing the foundation for the VBPO catalysis model, but also provide evidence for the biogenesis of halogenated cyclic terpenoid polyethers.

Furthermore, these enzymes hold significant potential for development as catalysts in chemoenzymatic synthesis and/or synthetic biology.