論文の内容の要旨

応用生命工学専攻
平成 30 年度博士課程入学
氏　　名　張　倢
指導教員名　葛山智久

Studies on the biosynthesis of dialkylbenzene-containing natural products produced by *Streptomyces*
（放線菌の生産するジアルキルベンゼン含有天然化合物の生合成に関する研究）

Introduction

Polyketide synthases (PKSs) are multifunctional enzymes that produce polyketides, which have diverse structures and impressive biological activities. It is well known that the vast majority of aromatic natural polyketides are synthesized by type II PKSs. In general, the most distinctive feature of type II PKSs is that they contain a chain length factor (CLF) that forms a heterodimer with its ketosynthase (KS) partner. This KS-CLF heterodimer determines the number of condensation reactions during polyketide chain elongation, which is always catalyzed on the acyl carrier protein (ACP). The resulting poly-β-keto intermediate is subsequently cyclized by modification enzymes to diversify the product structures. Recently, another noteworthy type II PKS named highly reducing (HR) type II PKS has been identified and characterized *in vitro* as a linear polyene structure synthesized by a KS-CLF heterodimer, a ketoreductase (KR), and a dehydratase (DH), which together catalyze the repeating cycles of chain elongation, β-ketoreduction, and dehydration, respectively.

Two natural products **1** and **2** (**Figure 1**) have been isolated and identified from a rifampicin resistant mutant TW-R50-13 derived from *Streptomyces* sp. SANK 60404 in our laboratory. Both compounds contain a very rare *o*-tolyl group among natural products, and in addition, **1** has a relatively long polyene moiety. Interestingly, feeding experiments using ^{13}C-labeled acetate have suggested that the backbone including the intact tolyl group of **1** is synthesized by a PKS system. A small family of natural products containing *o*-dialkylbenzene motif have also been isolated, which exhibit a variety of biological activities, including antibacterial, antifungal, antitumor, and anti-angiogenic activities. Bioinformatics analysis suggests that bacteria producing these natural products encode HR type II PKS genes in their genomes. It has been speculated that the *o*-dialkylated benzene moiety is formed from polyene precursor via an intramolecular 6π-electrocyclic reaction, followed by dehydrogenation. However, the biosynthetic mechanism of *o*-dialkylated benzene has remained elusive, and such an enzyme, if it actually exists, has never been documented.

Chapter 1. Gene inactivation experiments and bioinformatics analysis of the *mbg* cluster

The compounds **1** and **2** biosynthetic gene cluster containing 31 discrete open reading frames (ORFs), which encodes HR type II PKS, have been identified in our laboratory. This gene cluster was named *mbg*. Since the *mbg*

cluster features eight KSs, three ACPs, and three DHs but only one KR, it is difficult to elucidate how *o*-tolyl polyene moieties of **1** and **2** were synthesized. Therefore, *mbg* genes associated with HR type II PKS were individually inactivated. The results showed that the backbone polyenes of **1** and **2** would share part of the biosynthetic pathway by using a common set of HR type II PKS. When each gene *mbg8* (ACP), *mbg7* (KS), *mbg6* (KS) or *mbg25* (KR) was destroyed, the production of both **1** and **2** were abolished. The biosynthesis of intact backbone polyene of **1** also required extra HR type II PKS genes *mbg21* (KS) and *mbg9* (DH), because the deletion of these genes resulted in impossible to detect **1**. On the other hand, *mbg2* was annotated as an isomerase showing significant homology to glutathione-dependent 2-hydroxychromene-2-carboxylate isomerase, which is conserved in all BGCs of containing *o*-dialkylbenzene natural compounds mentioned above. Disruption of *mbg2* also resulted in the complete disappearance of **1** and **2** production. These results indicate that after the *o*-tolyl moiety linked to an ACP was synthesized by a set of HR type II PKS and Mbg2, this intermediate would be catalyzed by other set of HR type II PKSs for the further chain elongation.

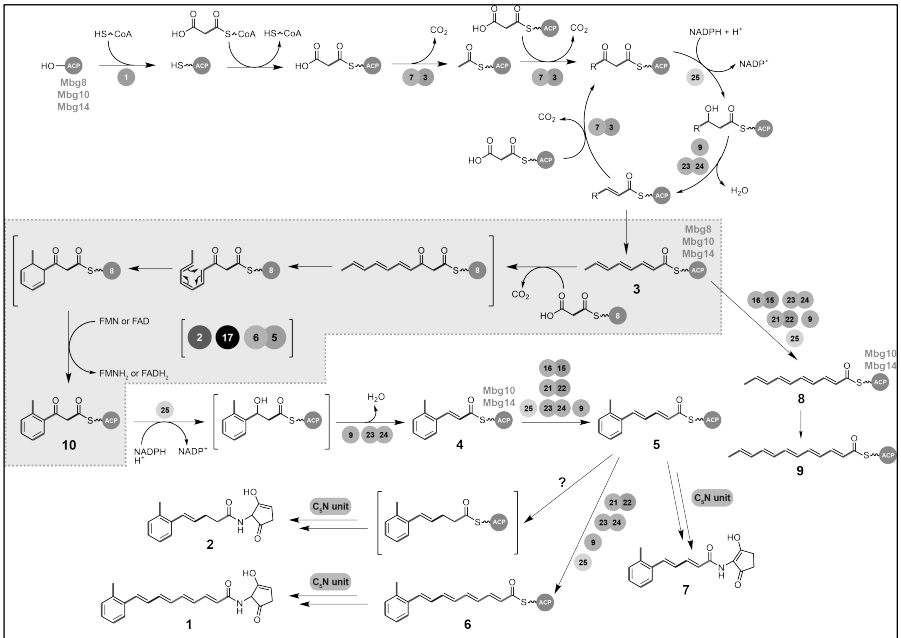

Figure 1. Proposed biosynthetic pathway of compounds **1** and **2**.

Bioinformatics analysis and co-expression experiments indicated that the interaction of KS and CLF were Mbg7-Mbg3, Mbg6-Mbg5, Mbg16-Mbg15, and Mbg21-Mbg22. Based on BLAST analysis and sequence alignment, the Mbg9 (DH) has a double hot dog motif, and its N-terminal side and C-terminal side show high homology to Mbg23 (DH) and Mbg24 (DH), respectively. Further functional analysis demonstrated that Mbg9 solely exhibits DH activity, while Mbg23 and Mbg24 form a heterodimer to exhibit DH activity. The *mbg* cluster has three ACPs, Mbg8, Mbg10 and Mbg14. Of these ACPs, Mbg10 and Mbg14 show extremely high homology with each other. Mbg17 is annotated as a 4-hydroxybenzoyl-CoA thioesterase (4-HBT) with a hot dog motif. Confusingly, sequence alignment with 4-HBT homologues shows that this hot dog motif has a very low homology and only located in the C-terminus of Mbg17, while the function of the N-terminus (including about 200 residues) is unknown.

Chapter 2. Reconstitution of the biosynthesis of compounds 1 and 2 *in vitro*.

To reconstitute the biosynthesis of *o*-tolyl polyene moiety of **1** and **2** *in vitro*, 19 recombinant proteins Mbg0 (Malonyl-CoA:ACP transacylase), Mbg1 (ACP synthase), Mbg2 (isomerase), Mbg7-Mbg3 (KS-CLF), Mbg6-Mbg5 (KS-CLF), Mbg16-Mbg15 (KS-CLF), Mbg21-Mbg22 (KS-CLF), Mbg8 (ACP), Mbg10 (ACP), Mbg14

(ACP), Mbg17 (4-HBT like), Mbg9 (DH), Mbg23-Mbg24 (DH), Mbg25 (KR) were prepared and incubated with appropriate substrates at 30°C for 1 h (Reaction mixture 1). As a result, compounds **3**–**6**, where **5** and **6** are C_5N unit-less precursors of compounds **1** and **2**, were accumulated. Further incubation of **5** and **6** with the enzymes and substrates required for C_5N unit formation at 30 °C for 2 h (Reaction mixture 2), compound **1** and a tiny amount of **7**, which is the oxidized precursor of compound **2,** were detected. On the other hand, compounds **3**–**6** were undetectable when the hydrolysis step was skipped, suggesting that **3**–**6** remain to be tethered to ACPs during the biosynthesis and that formation of the *o*-tolyl group is carried out in an ACP-bound state.

Chapter 3. Elucidation of the biosynthetic pathway of compounds 5 and 6.

First, the types of ACP (Mbg8, Mbg10, and Mbg14) required for the formation of compounds **5** and **6** in Reaction mixture 1 were investigated. As expected, removal of either Mbg10 or Mbg14 did not affect the reaction to produce both **5** and **6** since they are 99% identical. Therefore, only Mbg8 and Mbg10 (Reaction mixture 3) were used for further *in vitro* experiments described in the present study. When Mbg10 was omitted from Reaction mixture 3, compounds **5** and **6** were disappeared but compound **4** was still produced. On the other hand, enzymatic reaction products containing the *o*-tolyl moiety were all disappeared in the absence of Mbg8 while new compounds **8** and **9** were accumulated. Compounds **8** and **9** are linear polyenoic acids that contain quadruple and quintuple double bonds, respectively. These results indicate that Mbg8, not Mgb10 (and Mbg14), is responsible for the *o*-tolyl group formation and the resulting intermediate could be transferred to Mbg10 for further chain elongation.

Subsequently, the types of KS-CLF responsible for the biosynthesis of compounds **5** and **6** were investigated. When Mbg16-Mbg15 was omitted from Reaction mixture 3, production of both **5** and **6** was not affected. On the other hand, the removal of Mbg21-Mbg22 caused disappearance of **6** but not significantly affected the production of **5**. Since compound **4** was the major product in the absence of Mbg16-Mbg15 and Mbg21-Mbg22, it is speculated that compound **4** (C_{10}) attached to Mbg10 is elongated by Mbg21-Mbg22 to produce compound **6** (C_{16}), while elongated by Mbg16-Mbg15 or Mbg21-Mbg22 to produce compound **5** (C_{12}), which agrees with *in vivo* knockout data mentioned above. Compounds **8** and **9** were observed when either Mbg8**,** Mbg7-Mbg3 or Mbg6-Mbg5 was omitted from Reaction mixture 3, which indicates that Mbg7-Mbg3 and Mbg6-Mbg5 together synthesize the *o*-tolyl group on Mbg8.

To confirm which DH domain is responsible for the biosynthesis of compounds **5** and **6**, either Mbg9 or Mbg23-Mbg24 was omitted from Reaction mixture 3. While removal of Mbg9 did not significantly affect the production of both compounds, compound **6** was almost disappeared in the absence of Mbg23-Mbg24, which is contrary to gene disruption data. The reason is still unclear. On the other hand, compound **5** was produced in the absence of Mbg9 or Mbg23-Mbg24, which is consistent with data obtained from *in vivo* knockout experiments.

Chapter 4. Formation of the *o*-tolyl group in compound 4.

Based on data described above, a minimal reaction mixture containing Mbg0, Mbg1, Mbg2, Mbg7-Mbg3, Mbg6-Mbg5, Mbg8, Mbg10, Mbg17, Mbg23-Mbg24, and Mbg25 (Reaction mixture 4) was defined, which is sufficient for the biosynthesis of *o*-tolyl moiety, and employed to study the *o*-tolyl group biosynthesis. Considering the structures of the linear C_8 triene intermediate **3** and the C_{10} *o*-tolyl intermediate **4**, C_2 extension in **3** and

subsequent intramolecular 6π-electrocyclization would occur to form the *o*-tolyl moiety. In order to determine a KS-CLF to convert **3** to **4**, either Mbg7-Mbg3 or Mbg6-Mbg5 was omitted from Reaction mixture 4. The intermediate **3** was detected with Mbg7-Mbg3 while no product was observed with Mbg6-Mbg5 alone, suggesting that Mbg7-Mbg3 is sufficient for the biosynthesis of **3** and that Mbg6-Mbg5 catalyzes the final C_2 extension. In the absence of Mbg2 or Mbg17, the production of compound **4** was completely abolished, demonstrating that both the enzymes are responsible for the cyclization reaction. Co-expression experiments indicated that Mbg17 has an interaction with *holo*-Mbg8 only, neither *apo*-Mbg8 nor Mbg10/Mbg14. Another interesting finding is that intermediate **3** is not used as a substrate for chain elongation by Mbg6-Mbg5 in the absence of either Mbg2 or Mbg17. These data suggested that Mbg6-Mbg5 may need to form an active complex with Mbg2 and Mbg17 to elongate **3**-Mbg8 and cyclize it. In addition, the presence of coenzyme FAD/FMN is necessary to form the *o*-tolyl moiety.

To investigate the above reactions in an ACP attached manner, **3** attached with ACPs (Mbg8 and Mbg10) was first synthesized using Reaction mixture 5, where Mbg6-Mbg5, Mbg2, and Mbg17 were omitted from Reaction mixture 4. These **3**-ACPs were then purified using ultrafiltration membrane with molecular weight cut-off 30,000 NMWL and employed for further *in vitro* assay. Incubation of **3**-ACPs with Mbg0, Mbg6-Mbg5, Mbg23-Mbg24, Mbg25, Mbg2, and Mbg17 in addition to necessary substrates (Reaction mixture 6) resulted in production of **4**.

The order of the reaction using the above ACP assay was further analyzed. When the DH (Mbg23-Mbg24) and the KR (Mbg25) were omitted from Reaction mixture 6 (Reaction mixture 7), two new mass peaks (12222.8 Da and 11656.6 Da) were detected where the mass of both *holo*-Mbg8 (12062.3 Da) and *holo*-Mbg10 (11496.5 Da) were increased by 160 Da, respectively, corresponding to a putative intermediate **10** (C_{10}). This observation suggests that the cyclization reaction occurs before β-ketoreduction and dehydration, followed by a FAD/FMN-mediated desaturation to form the *o*-tolyl moiety. To further confirm the molecular structure of **10**, the ACPs were trypsinized and then analyzed by LC-ESI-HRMS analysis. The analysis revealed a divalent ion with *m/z* 531.2209, corresponding to the mass of the *holo*-Mbg8 peptide fragment ^{40}ADSLR^{44} with a phosphopantetheine arm acylated with intermediate **10** within 2.64 ppm error. Moreover, the decarboxylation product of **10** was successfully detected after alkaline hydrolysis of **10**-ACP. Subsequently, **10**-ACPs were purified and further *in vitro* reactions were performed to reconstitute the biosynthesis of **4** by adding Mbg23-Mbg24 and Mbg25 (Reaction mixture 8), confirming the order of the reaction to form the *o*-tolyl group.

Conclusion

The present work provided detailed insights into the biosynthesis of compounds **1** and **2** including the formation of *o*-tolyl group (**Figure 1**), which not only expands a catalytic repertoire of pericyclic reactions found in biological processes but also unravels the biosynthetic schemes of other natural products containing an *o*-dialkylbenzene motif. Mbg2 represents the first example of a carbocyclase that acts on a polyene structure and catalyzes an intramolecular 6π-electrocyclic reaction.