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Characterization of Geldanamycin Biosynthetic Genes from *Streptomyces hygroscopicus*

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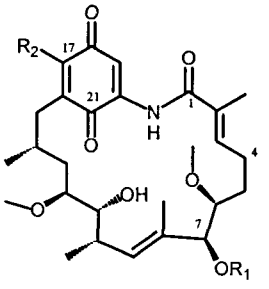
Geldanamycin (**1**) is a naturally occurring antitumor antibiotic produced by several *Streptomyces hygroscopicus*.[1] Geldanamycin binds to the N-terminal domain ATP binding site of heat-shock protein (Hsp) 90, inhibiting the chaperone activity of the protein, and its analogs have an unusual and novel mechanism of action that appears to sensitize cancer cells to other anticancer agents.[2] One semi-synthetic analog of these compounds, 17-allylamino-17-demethoxygeldanamycin, demonstrated antitumor activity *in vivo* and subjected to Phase II clinical trials.[3]



1 R_1=$CONH_2$, R_2=OCH_3, $\Delta^{4(5)}$
2 R_1=$CONH_2$, R_2=OH, $\Delta^{4(5)}$
3 R_1=H, R_2=OCH_3
4 R_1=H, R_2=OH
5 R_1=$CONH_2$, R_2=OCH_3
6 R_1=$CONH_2$, R_2=H, $\Delta^{4(5)}$

Geldanamycin biosynthesis is of interest as a means of introducing structural diversity by reprogramming biosynthetic assembly lines to create novel antibiotics with optimal properties. Geldanamycin is a 19-membered macrocyclic lactam and is related to ansamycin antibiotics, such as rifamycins and ansamitocins.[4] The biosynthesis of this class of compounds involves the assembly of 3-amino-5-hydroxybenzoic acid (AHBA), as a starter unit, followed by the sequential addition of extender units such as acetate, propionate and glycolate, to form a polyketide backbone, which then undergoes further downstream processing. The genes required for geldanamycin biosynthesis, including a set of type-I polyketide synthase (PKS) gene, have been cloned, sequenced, and analyzed from several streptomycetes, independently.[5] Based on predictions from sequence homology and the results of feeding experiment with ^{14}C-labeled precursor, it was proposed that the successful production of geldanamycin requires the modification of several steps, which including the O-carbamoylation, hydroxylation, O-methylation and oxidation of the initial polyketide synthase product. However, beyond determining sequences and deducing putative functions from sequence homologies, little had been learned about the post-PKS modification genes and the tailoring processes leading from initial polyketide to geldanamycin.[7]

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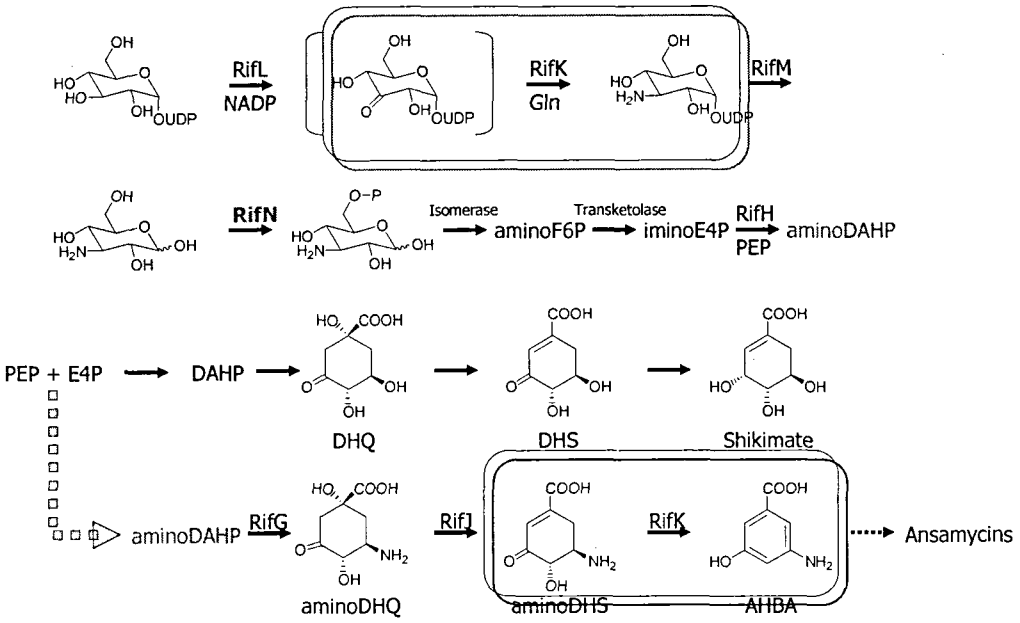
Early Biosynthetic Steps

The biosynthetically unique aromatic element called AHBA serve as a starting unit for the assembly of a polyketide by addition of acyl units. AHBA is generated by the aminoshikimate pathway, which parallels the first three steps of the shikimate pathway, but is modified by the introduction of nitrogen in the first step to give 3,4-dideoxy-4-amino-D-arabino-heptulosonic acid 7-phosphate (aminoDAHP). Rifamycin is a typical ansamycin antibiotic which uses AHBA as a starting material. The reaction steps and roles of each enzyme for AHBA biosynthesis pathway included in rifamycin biosynthetic pathway were proposed by Floss and coworkers.[6] Rifamycin biosynthetic gene cluster required for AHBA production have seven genes, *rif*G, -H, -J, -K, -L, -M, and -N. It was reported that three genes, *rif*G, -H, and -J, encode homologous enzymes to shikimate pathway enzymes, 5-amino-5-deoxy-3-dehydroquinic acid (aminoDHQ) synthase, 5-amino-5-deoxy-3-dehydroshikimic acid (aminoDHS) synthase, aminoDHQ dehydratase, respectively. The roles of three additional gene products, RifL, RifM, and RifN, were assumed that those may have function as oxidoreductase, phosphatase and glucose kinase from their sequence homologies, respectively.[6] Geldanamycin producer also use AHBA as a starting material for geldanamycin biosynthesis and has the AHBA biosynthetic gene cluster which has comparable sequence to that of rifamycin. Geldanamycin biosynthetic gene cluster required for AHBA biosynthesis has seven genes, *nap*G, -H, -K, -L, -M, and -N. The homology of these genes compared to that of rifamycin B, so its corresponding genes were named as *nap*.

Floss and co-workers proposed model of AHBA biosynthesis pathway and suggested that AHBA synthase may interact with oxidoreductase and act as an aminotransferase which carry the amino group from amino acid to ketone group of UDP-glucose intermediate.[6] To confirm the secondary function of AHBA synthase and the interaction between AHBA synthase and oxidoreductase, NapK and NapL, which are in the AHBA synthesis pathway of geldanamycin synthesis and homologous to rifamycin AHBA



Scheme. 1 Early biosynthetic steps of the proposed pathway for the formation of AHBA

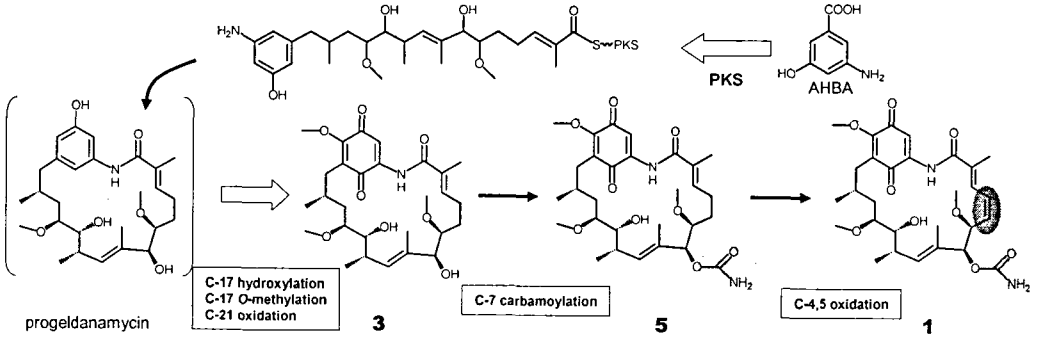
synthase (RifK) and oxidoreductase (RifL) were used. Dual cofactor specificity of NapL and binding stoichiometry between NapL and NapK was measured by NAD(P)H production. NapL prefer NADP+ three times than NAD+ as a cofactor and the activity of NapL was increased seven times when it was coexisted with NapK. Maximum activity of NapL was reached when NapL and NapK interacts each other with a 1:1 stoichiometry. The protein-protein interaction was confirmed by affinity chromatography. From these results, we suppose that the binding of NapK to NapL induces a conformational change of NapL to more active form. Secondary function of NapK as transaminase, which transfers amine from glutamine to ketone intermediate of UDP-glucose, was proved by detection of UDP-kanosamine production.

Late Biosynthetic Steps

Analysis of the geldanamycin biosynthetic gene cluster revealed a series of putative post-PKS modifying genes. Of these, *gel*8 encodes a protein highly homologous to carbamoyltransferases, which carry out the O-carbamoylation step in the biosynthesis of novobiocin, ansamitocin, and cephamycin. Therefore, the putative function of *gel*8 could be to encode carbamoyltransferase.

To verify the assumption that *gel*8 gene is responsible for the carbamoylation step in the geldanamycin biosynthesis, we inactivated the *gel*8 gene in *Streptomyces hygroscopicus* subsp. *duamyceticus* JCM4427, a geldanamycin producer. The mutated gene with an insertion of a kanamycin resistance gene was introduced into *S. hygroscopicus* JCM4427 to replace the wild-type gene by a sequential homologous recombination. The *gel*8 gene-inactivated mutant grew normally in YEME medium containing kanamycin, but completely lost its ability to produce geldanamycin (**1**) and 17-O-demethylgeldanamycin (**2**), which are the two major metabolites of the wild-type strain. Instead, two major metabolites, **3** and **4** (m/z 519 and 505, respectively) were detected and isolated from the gene-inactivated mutant.

The accumulation of descarbamoylated compounds in the mutant lacking a functional Gel8 confirmed that the *gel*8 encodes a carbamoyltransferase. Interestingly, **3** and **4** do not contain the *cis*-double bond between C-4 and -5. This result suggests that module 6 of geldanamycin PKS contains a functional enoylreductase (ER) domain that reduces the double bond during polyketide assembly, as could be expected by sequence comparison of the ER domain in module 6 with other functional ER domains. Putative



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Scheme. 2 Proposed Geldanamycin Biosynthetic Pathway

NADPH binding sites, GxGxxAxxxA, of the ER domains in animal fatty acid synthase and erythromycin PKS are well conserved in the corresponding ER domains of module1, 2, and 6 in the geldanamycin PKS. Taken together, these results indicate that C-17 hydroxylation, 17-O-methylation and C-21 oxidation occur prior to the carbamoyltransferase reaction, and the hypothetical progeldanamycin does not posses a double bond at the C-4 and 5 positions (Scheme 2). However, they do not provide information on the nature of the last step between carbamoylation and C-4,5 oxidation.

In order to determine whether **3** is a direct intermediate in the biosynthesis of **1**, a bioconversion experiment was performed using a geldanamycin PKS gene-inactivated mutant.[6,7] This mutant cannot produce **1** and **2**, due to the inactivation of the loading domain in the PKS gene by the insertion of the kanamycin resistance gene, however, has a full complement of post-PKS processing genes. The mutant converted **3** to **1** effectively, confirming not only that **3** is an intermediate in the main pathway, but also that C-4,5 oxidation is a post-PKS modification step. In addition, a prospective intermediate, 4,5-dihydrogeldanamycin (**5**) was detected in the bioconversion experiment. The presence of **5**, but no detection of 7-O-descarbamoyl-7-hydroxygeldanamycin indicates that C-4,5 oxidation is likely to be the final post-PKS modification step in the biosynthesis of geldanamycin.[7] Many steps in the biosynthesis of geldanamycin are still unclear, however, based on the present results, we were able to refine the post modification steps in the biosynthesis of geldanamycin, such as that O-carbamoylation occurs prior to the C-4,5 *cis*-double bond formation and that the hypothetical progeldanamycin does not carry a double bond at the C-4 and -5 positions (Scheme 2).[7]

Acknowledgments

This work was supported by the 21C Frontier Microbial Genomics and Applications Center, the Ministry of Science and Technology (Grant MG05-0303-2-0 to Y.-S.H), Republic of Korea and by a grant from the KRIBB Research Initiative Program.

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