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Characterization of Tailoring Genes Involved in the Modification of Geldanamycin Polyketide in *Streptomyces hygroscopicus* JCM4427

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Geldanamycin and its analogs are important anticancer agents that inhibit the newly targeted heat-shock protein (Hsp) 90, which is a chaperone protein in eukaryotic cells. To resolve which geldanamycin biosynthetic genes are responsible for particular post-polyketide synthase (PKS) processing steps and in which order the reactions occur, we individually inactivated candidate genes in *Streptomyces hygroscopicus* subsp. *duamyceticus* JCM4427 and isolated and elucidated the structures of intermediates from each mutant. The results indicated that *gel7* governs at least one of the benzoquinone ring oxidation steps. The *gel16* was found to be involved in double-bond formation between C-4 and C-5 of 4,5-dihydrogeldanamycin, which confirmed our previous findings that this double bond is reduced during the post-PKS modification of the polyketide assembly. In addition, pro-geldanamycin, which does not possess a double bond at C-4/5, was purified from the *gel7* and *gel8* double-gene-inactivated mutant.

Keywords: Geldanamycin, biosynthesis, *Streptomyces*, polyketide

Geldanamycin is a well-known inhibitor of heat-shock protein 90 (Hsp90), a molecular chaperone with essential ATPase activity, which is required for the maturation and activation of a number of client proteins, many of which are involved in carcinogenesis and/or tumor development [22]. Accordingly, several geldanamycin analogs, including 17-allyamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), are at various stages of clinical trials as novel antitumor agents [20].

The biosynthetic pathway of geldanamycin, which is a member of an essential polyketide family named "ansamycins," was partly revealed by classical isotopic tracer experiments and by using genetic approaches [12, 13, 15, 18, 24]. The biosynthesis of the polyketide backbone of geldanamycin is initiated by loading the 3-amino-5-hydroxy benzoic acid starter unit [2], and this is subsequently followed by seven steps of condensation with extender units in a chain-elongation stage, involving one malonyl-, four methylmalonyl-, and two methoxymalonyl-CoA units. The nascent polyketide assembly is catalyzed by three modular polyketide synthases, which are large polypeptides composed of sets of active sites called modules [16, 24]. The linear product so produced is released from the PKS complex and cyclized by amide synthase to form a macrocyclic intermediate *via* intramolecular amide formation [1]. The nascent PKS product is then converted to geldanamycin by the post-PKS modification steps, which include C-17 hydroxylation, C-17 *O*-methylation, C-21 oxidation, C-7 carbamoylation, and C-4,5 oxidation [13, 18, 23, 24].

Recently, we reported that the carbamoylation step is catalyzed by the *gel8* gene product, and decarbamoyl geldanamycin compounds were found as the main product in a culture of carbamoyltransferase gene-inactivated strain of *S. hygroscopicus* JCM4427 [13]. Furthermore, we previously suggested that C-4,5 oxidation is the final biosynthetic step of the geldanamycin biosynthetic pathway. Also, Rascher *et al.* [23] reported that *gdmM* governs at least one of the post-PKS oxidation steps in *S. hygroscopicus* NRRL3602.

Herein, we describe the characterization of post-PKS modification genes, *gel1*, *gel7*, and *gel16*, of the geldanamycin

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biosynthetic gene cluster in *S. hygroscopicus* JCM4427. The results present that *gel7* governs at least one of the benzoquinone ring oxidation steps and *gel16* is involved in double-bond formation between C-4 and C-5 of 4,5-dihydrogeldanamycin. In addition, pro-geldanamycin was purified from a *gel7* and *8* double-gene-inactivated mutant and its structure was elucidated.

Materials and Methods

Bacterial Strains, Media, and Plasmids

Wild-type geldanamycin-producing strain *S. hygroscopicus* subsp. *duamyceticus* JCM4427 was obtained from the Japanese Culture Collection of Microorganisms. *Streptomyces lividans* 1326 was used for complementation experiments. These strains were grown in YEME to obtain mycelia for chromosomal DNA isolation and metabolite extraction [14]. *Escherichia coli* strain XL1-blue was used as the general cloning host, and *E. coli* ET12567 (pUZ8002) was used as the donor strain for intergeneric conjugation [4]. *E. coli* strains were grown in Luria-Bertani (LB) medium or on LB agar supplemented with appropriate antibiotics. Apramycin (50 mg/ml), chloramphenicol (25 mg/ml), or kanamycin (50 mg/ml) were added to growth media as required. Gene disruption experiments were carried out using the pKC1139 vector and by inserting the kanamycin resistance gene from the pFDneo-S vector as a selective marker [6].

General Genetic Manipulation

General molecular biological methods were performed as described by Sambrook *et al.* [25]. All restriction and modifying enzymes were purchased from Takara (Shiga, Japan) and New England Biolabs (Beverly, U.S.A.) and were used as recommended by the suppliers. The PCR reactions were carried out using Ex-*Taq* from Takara, according to the manufacturer's instructions, except that 5% dimethyl sulfoxide was also added to the reaction. DNA was sequenced on an automated DNA sequencer (GenoTech, Daejeon, Korea). The sequence data for the genes displayed in Fig. 2 have been deposited with GenBank under the accession number DQ249341.

Gene Inactivation

All gene disruptions were performed in a similar manner using plasmid pKC1139 to deliver the corresponding kanamycin resistance gene cassettes (Table 1). A 1.1-kb DNA fragment from pFDneo-S carrying the *aph*II gene responsible for kanamycin resistance was routinely used as the selective marker for the constructions of the gene disruption vector. These cassettes consisted of two PCR-derived flanking regions, in which suitable restriction sites were introduced. Detailed primer information for each construction is summarized in Table 1. All of the constructs, pKC-gel1, -gel7, -gel16, and -gel7&8, were delivered into *S. hygroscopicus* JCM4427 by conjugation with *E. coli* ET12567 (pUZ8002). Intergeneric conjugation between *E. coli* and *Streptomyces* was performed as described previously, with minor modification [4]. The transformants obtained possessed resistance to both apramycin and kanamycin, and were grown in fresh YEME/kanamycin liquid medium at 37°C for 4 days in order to force integration between chromosome DNA and cassette DNA of gene disruption vectors. The resulting gene disruption mutants were selected on R2YE/kanamycin medium and confirmed by PCR, with the relevant primer set designed using the total genomic DNA of each mutant as template.

Gene Complementation Experiments

To confirm the direct involvement of *gel7* or *gel16* in geldanamycin biosynthesis, complementation experiments were carried out by

Table 1. Design of gene disruption cassettes and oligonucleotides used.

Target	PCR primer (5'-3')		Cloning design (Name of vector)
	Name	Sequence	
gel1	g1-1-1	CGAGATCGCCGCGTCGGAGATCG	EcoRI-LF-SalI-*aphII*-SalI-RF-EcoRI
	g1-1-2	GTCGACAGCATGGACCAGTAGCCGCGC	(pKC-gel1)
	g1-2-1	TCCCGCCACGAGTCGACCTCGTC	
	g1-2-2	GTGGACGTGGAGGGCGACCGGGCGACGG	
gel7	g7-1-1	ATGAGCGGGAAGGAGGCGGCGGTGG	EcoRI-LF-SalI-*aphII*-SalI-RF-EcoRI
	g7-1-2	GTGTCGACCGCGTGCCGGTCCAGAACG	(pKC-gel7)
	g7-2-1	GGTCGACACCTTCCAGGCCCGCTGGGC	
	g7-2-2	GCAGGGGAGCCTTACGAAGGAGTGG	
	gelC-1	AAGCTTGGTCCGCTCGGCCCAGTCCG	Pair with gel7-2-2 pair (pKC-C7)
gel8	ct-1	CTCGACGCGGTGGGCTACTACTTTCC	EcoRI-LF-SalI-*aphII*-SalI-RF-EcoRI
	ct2-sal	CAGTCGACCAACCGTGCCCTGATCTG	(pKC-gel7&8)
gel16	g16-1-1	GAATTCGCGGGCTGCTGCATGCCCGTTC	EcoRI-LF-SalI-*aphII*-SalI-RF-EcoRI
	g16-1-2	GTCAGCTCCTGGTCGACGATGAGCCG	(pKC-gel16)
	g16-2-1	TGGTGGACTACGTCGCCCGGATGTGC	
	g16-2-2	GCCAGGTCGTCGAACGGGACCTCCAG	
	gel16-N1	AGATCTGACGAGATACGCGACTACCCCGAATC	EcoRI-*ermE**-BglII-*gel16*-EcoRI
	gel15-3	GAGCGTCACACTGGCCGAGATCTGGTC	(pKC-E16)
*ermE**	ermE-5	GGAATTCCCGACCCGAGACGCGCCGGCACG	
	ermE-3-1	AGATCTCTCACCGCTGGATCCTACCAACCG	

introducing the individual gene cloned from the wild-type strain into the corresponding gene-inactivated mutants using replicating vector derived from plasmid pKC1139. To clone the *gel7* gene region containing the complete open reading frame of *gelD* and *gel7* and the partial sequence of *gelA3*, a 3.8-kb fragment in total was amplified using the gelC-1 and g7-2-2 primer pairs. The 3.8-kb fragment was confirmed by sequence analysis and cloned into pKC1139, generating pKC-C7. To clone the *gel16* region containing *gel16Fd*, the primer pairs g16-N1 and gel15-3 were used in amplification of this region. To enhance the efficiency of gene expression, a promoter of *ermE** from *Saccharopolyspora erythraea* was introduced in front of the cloned 2.1-kb *gel16* region [3, 21]. The locations of cloned gene regions for complementation are shown as filled bars in Fig. 2. The complementation vectors (pKC-C7 and pKC-E16) were transformed into each gene disruption mutant by the conjugational method, as described above.

Analysis of Metabolites

Wild-type strain, gene inactivation mutants, and its complementation strains were grown in 50 ml of liquid YEME or R2YE medium and incubated at 28°C for 7 days. Cultured broths were extracted with EtOAc twice and extracts were filtered through a fritted funnel *in vacuo* to remove insolubles. Extracts were analyzed by liquid chromatography using a Dionex HPLC system [YMC J'sphere ODS-H80, 150×4.6 mm i.d., CH_3CN-H_2O (0.05% TFA) 20:80 to 100:0 over 25 min, 1 ml/min]. The extracts from geldanamycin-producing strains had the same HPLC profiles, *i.e.*, retention time and UV pattern, as those of authentic geldanamycin (Sigma, St. Louis, MO, U.S.A.). Thin-layer chromatography (TLC) analysis was performed as follows; extracts were developed on Silica gel 60 F_{254} (Merck) using chloroform:methanol (9:1 v/v). The photograph of TLC plates in Fig. 3 was taken at a wavelength of 350 nm. LC-MS was performed using a Finnigan LCQ Advantage MAX ion-trap mass spectrometer (Thermo Electron Co., U.S.A.) equipped with an electrospray ionization (ESI) source. HPLC separations were performed using a Finnigan Surveyor Modular HPLC System unit (Thermo Electron Co., U.S.A.) using a YMC Hydrosphere C_{18} column (5 μm, 50×2.0 mm, Japan) and a BetaBasic-18 guard column (10×2.1 mm, Thermo, U.S.A.) [17, 18].

RESULTS AND DISCUSSION

gel1 and *gel7* Gene Disruption and the Complementation Experiment

Analysis of the geldanamycin biosynthetic gene cluster revealed several putative oxidation genes. Among these, *gel1* and *gel7* (identical to *gdmL* and *gdmM*, respectively [24]) belonged to a group of FAD-binding monooxygenases. *In silico* analysis of the amino acid sequences of these two genes revealed that all proteins within this family contained typical nucleotide-binding motifs for flavoprotein hydroxylases and had conserved amino acid sequences;

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A
                 FAD fingerprint (1)                              Conserved
                 *  *  *                                          ** *  *
Gel1  :  10 VVVAGAGATGLMLAYELALAGVETLVLEKL : 40     RAHYLVACDGGHSTVRKLLGLPEP :174
Gel7  :  20 VLIVGYGPVGQLLSVLLAQRGRRVTVVERW : 50     TALWVVGCDGANSLVRTGVGTTMT :193
HbmL  :   8 VVVAGAGPTGLMLAYELALAGVETLVLEKL : 38     RAHYLVACDGGHSTVRKLLGLPEP :172
Rif19 :   5 VVIVGNGPIGATLSVLLAQRGWRVTVLERR : 35     SGRWVVGCDGANSFVREHLDVPVT :178
OxyS  :   4 VVIAGAGPTGLMLACELRLAGARTLVLERL : 34     ECAYLVGCDGGGSTVRKLLGIDEP :170
UbiH  :   7 VAVIGYGPVGQLMAALLAQRGWDVVAVERW : 37     VADWVVGCDGANSFVRDRLGSGFT :180
JadH  :  12 VLVVGAGPTGLMLAGERRLGGARVLVAERL : 42     RAAYVVGCDGGHSPVRKAAGFDEP :178
MhpA  :  17 VAIAGAGPVGLMMANYLGQMGIDVLVVEKL : 47     KAQWLVACDGGASFVRRTLNVPLE :189
              GxGxxG                                       DGxxSxxR

                                  FAD fingerprint (2)
                    ** *
Gel1  : 275 RQLEHYRTGRVLFAGDAAHIHPPLGAQGLNLGVQDALNLGWKLAAVLQDRAPNGLLDSYH :335
Gel7  : 285 RWAERWRTGRMVLAGDSAHLMPPFAGQGMCSGFRDAANLAWKLDLVLGGHAAPTLLDTYT :345
HbmL  : 273 RQLEHYRTGRVLFAGDAAHIHPPLGAQGLNLGVQDALNLGWKLAAVLQDRAPSGLLDSYH :333
Rif19 : 270 RWADRWRVGHVLLAGDAAHLMPPFAGQGMCSGIRDVTNLAWKLDLTLRGLAPESLLDSYG :330
OxyS  : 272 RQAERYRDGRVLLAGDACHIHLPAGGQGLNLGFQDAVNLGWKLGATIAGTAPPELLDTYE :332
UbiH  : 272 GHAERWRSGRMLLAGDAAHIMPPFAGQGMSSGFRDAANLAWKLDLVLSGSAGERIVDTYA :332
JadH  : 272 RQATEYRRGRVLLVGDAAHIHLPAGGQGLSTGVQDAANLGWKLAAVVRGTAPDGLLDTYH :332
MhpA  : 279 RLAQRFRIDRVLLAGDAAHIMPVWQGQGYNSGMRDAFNLAWKLALVIQGKARDALLDTYQ :339
                           GDxxH

B
            O2 binding                      HEME binding
                                                    *
Gel16  : ALVLLLAGHETTANMI : 244     REHVAFGFGVHQCLGQTLARVELQI : 362
HbmP   : ALVLLLAGHETTANMI : 244     REHVAFGFGVHQCLGQTLARVELQI : 362
CalE10 : AFVLLVAGHVTTSNMI : 241     RHHLAFGYGRHICPGQHLARLELDV : 359
NikF   : AVMLLIAGHQTTANMI : 257     RHHVAFGYGVHQCLGQNLARVELEI : 375
ORF10  : CRLLLVAGHGTTTSQA : 255     RRHLAFGHGMHQCLGQWLARVELEE : 373
OleP   : GVSLLIAGHETSVNQI : 253     NPHIAFGHGAHHCIGAQLGRLELQE : 372
PikC   : AHILLVAGHETTVNLI : 252     AGHLAFGHGIHFCLGAPLARLEARI : 370
PimG   : LMLLLIAGHETTANNI : 253     RHHVAFGYGVHQCLGQNLVRIEMEV : 363
RapN   : AVTLLIAGYETTANAI : 250     NPHMSLGHGAHHCMGAQLVRVEMQT : 369
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Fig. 1. A. Comparison of the FAD-binding sites from Gel1 and Gel7 with those of other monooxygenases. **B.** Oxygen-binding sequences and heme-binding pockets from *Streptomyces* P450 monooxygenases.

The consensus sequence for NADP(H)-binding is shown beneath the sequence. The asterisk shows the heme-binding cysteine residue. Numbers indicate amino acid residues from the N-terminus of the protein. Conserved amino acids are shown in bold and highlight.

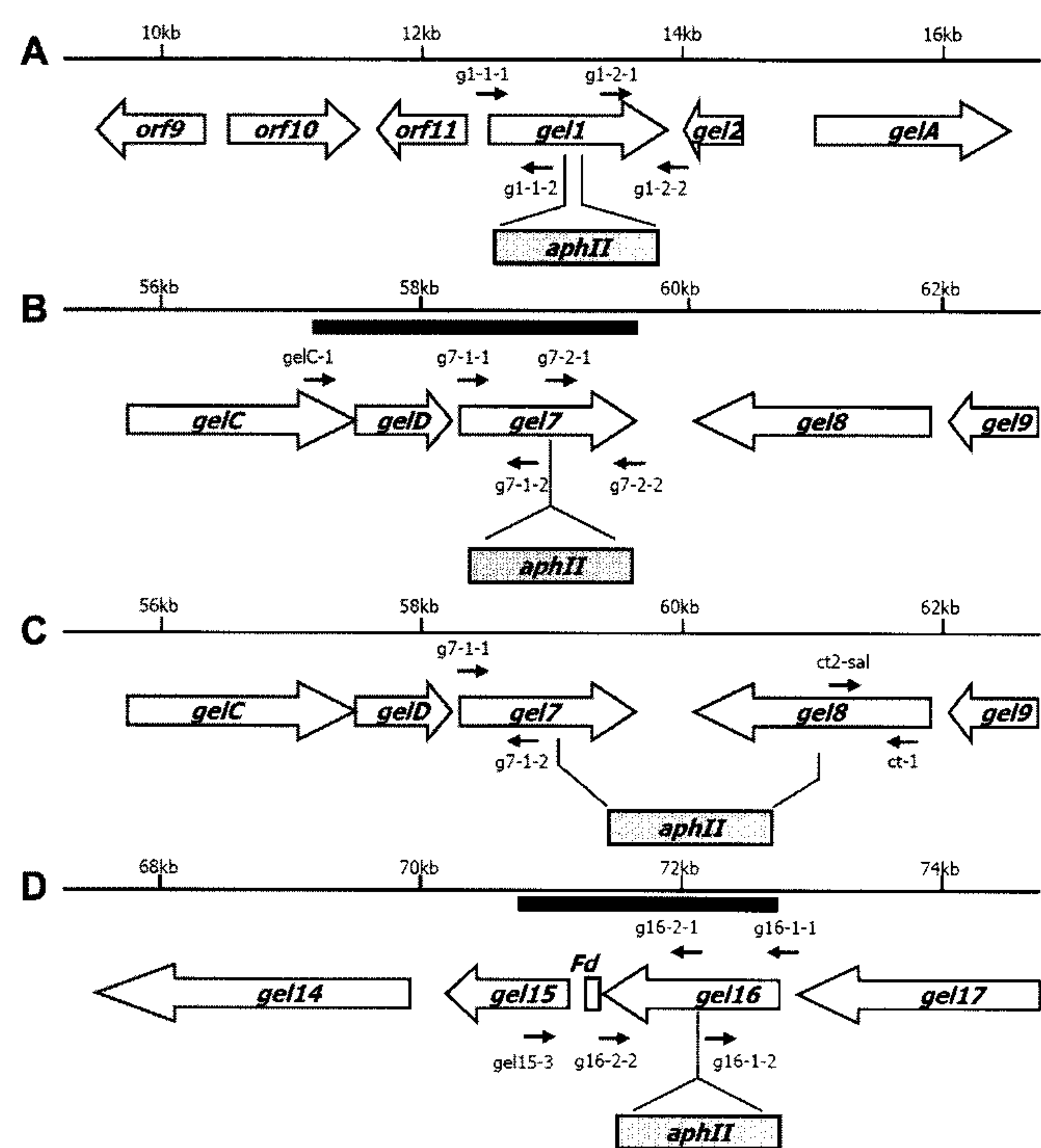

Fig. 2. Scheme representing the insertional inactivations of *gel1* (**A**), *gel7* (**B**), *gel7&gel8* (**C**), and *gel16* (**D**) in *S. hygroscopicus* JCM4427.

Transcriptional direction and the relative sizes of the predicted ORFs are indicated by arrowed boxes. The primers used to amplify the desired DNA fragments are indicated by solid thin arrows. Numbers indicate nucleotide residues from GenBank Accession No. DQ249341, and filled bars indicate the fragments used in the complementation experiment.

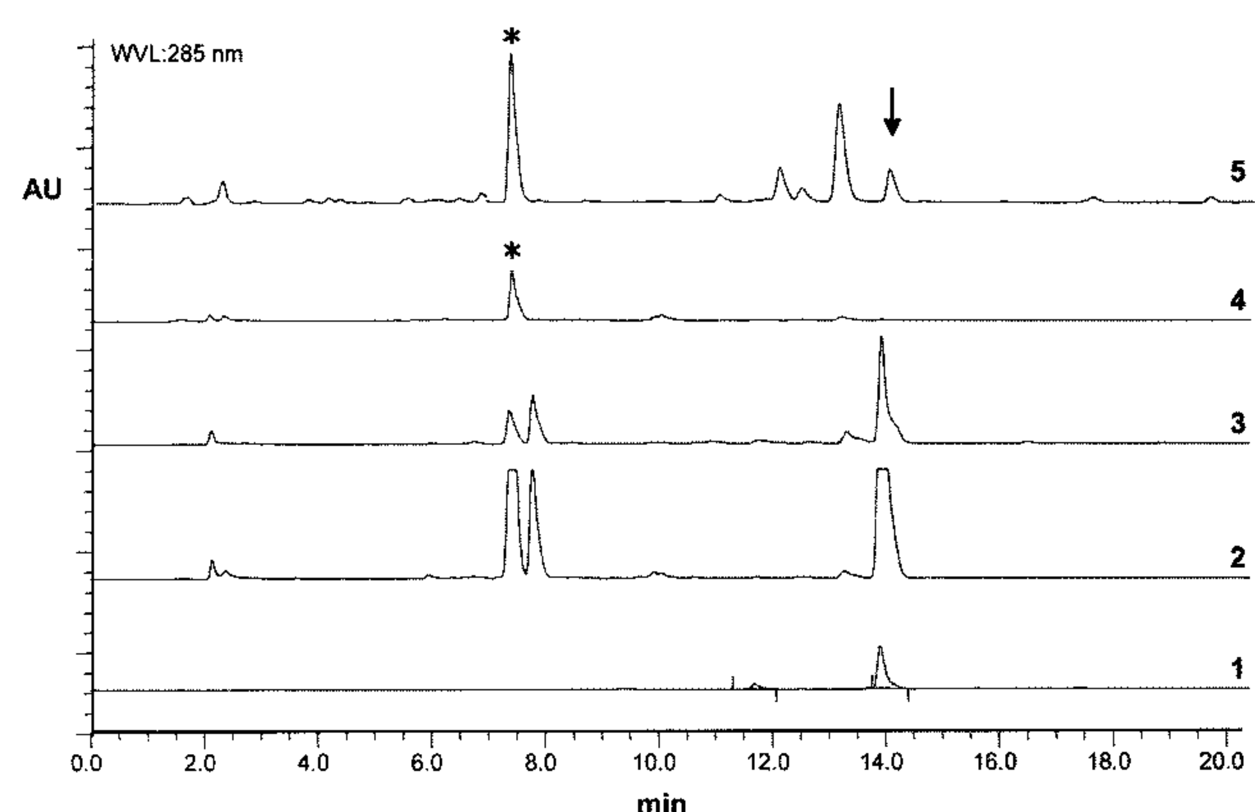

Fig. 3. Comparison of HPLC analysis of ethylacetate-extracted broths.

Trace 1, geldanamycin standard; trace 2, extract of culture broth from *S. hygroscopicus* JCM4427; trace 3, ΔgeI1 mutant; trace 4, Δgel7 mutant; trace 5, pKC-C7 harboring Δgel7 mutant. The arrow and asterisk indicate the position of geldanamycin and 17-demethoxy-reblastatin, respectively.

i.e., GxGxxG, DGxxSxxR, and GDxxH (Fig. 1) [7, 8]. Genes similar to *gel1* have been reported to be involved in a number of secondary metabolite biosynthesis, such as that of oxytetracycline (OxyS: 43.3% identity) from *Streptomyces rimosus* (GenBank Accession No. DQ143963) and jadomycin (JadH: 38.5% identity) from *Streptomyces venezuelae* (GenBank No. AY773079). Additionally, genes similar to *gel7* have been reported to be involved in the hydroxylation of phenolic structures; *e.g.*, MhpA (27.1% identity) from *Rhodococcus globerulus* (GenBank No. BAA13052), Rif19 (50.2% identity) from *Amycolatopsis mediterranei* S699 (GenBank No. AF040570), and UbiH (52.9% identity) from *Saccharopolyspora erythraea* NRRL 2338 (EMBL No. AM420293). Interestingly, Gel7 showed high identity with Rif19 protein, which is directly involved in naphthalene ring formation during the biosynthesis of the ansamycin antibiotic rifamycin [26]. Taken together, Gel7 appears to play an important role in oxidation of the aromatic ring of pro-geldanamycin at positions C-17 and/or C-21.

To confirm the role of the functions of Gel1 and/or Gel7 in geldanamycin biosynthesis, gene-knockout experiments were carried out as described in Materials and Methods. These cassettes consisted of two PCR-derived flanking regions, in which suitable restriction sites were introduced (Fig. 2). The recombinants (Δgel1 or Δgel7 mutants), which were resistant to kanamycin but sensitive to apramycin, were selected from replica plates containing apramycin or kanamycin. Recombinants carrying the disrupted gene were confirmed by PCR and sequencing analysis using their total genomic DNA as template (data not shown). Culture extracts from the wild-type strain and from the Δgel1 and Δgel7 mutants were analyzed by HPLC (Fig. 3). Inactivation of the *gel1* gene did not show any remarkable effect on the geldanamycin profile by metabolite analysis, but geldanamycin production was completely abolished in the Δgel7 mutant (Fig. 3). However, a new peak with a different retention time showed in the Δgel7 mutant, and the peak was identified as 17-demethoxy-reblastatin by LC-MS ($[M-H]^-$ at *m/z* 517) and NMR analysis (data not shown). 17-Demethoxy-reblastatin is a nonbenzoquinoid geldanamycin analog with a monophenolic structure, as described by Rascher *et al.* [23]. This finding demonstrates that *gel7* (identical to *gdmM*) governs at least one of the benzoquinone ring oxidation steps and also confirms that *gel1* (identical to *gdmL*) is a nonfunctional pseudogene in geldanamycin biosynthesis.

We attempted to identify the function of *gel7* on the benzoquinone ring oxidation steps by performing complementation experiments of *gel7* in a Δgel7 mutant and in *S. lividans* 1326. For this purpose, the whole *gel7* gene region containing the partial *gelC* and *gelD* genes, was cloned into the pKC1139 vector for foreign gene expression. The resulting *gel7* expression vector, pKC-C7, was introduced into the Δgel7 mutant and into *S. lividans* 1326 by conjugal transformation. Culture extracts of the Δgel7 mutant with/without the *gel7* expression vector pKC-C7 were analyzed by HPLC and compared with the wild-type product spectrum.

This transformant effectively produced geldanamycin, which was compared with an authentic reference substrate (Fig. 3). On the other hand, the work of Rascher *et al.* [23] shows that the absence of the *gel7* homologous gene in the herbimycin gene cluster suggested that *gel7* is likely to be involved in C-17 hydroxylation. However, when 17-demethoxy-reblastatin was fed into the culture broth of pKC-C7 harboring *S. lividans* 1326, the extract showed no conversion of feed compound by HPLC or LC-MS (unpublished result) analysis. For this reason, we still cannot be sure as to whether the *gel7* gene was involved in the C-21 oxidation or C-17 hydroxylation steps of benzoquinone formation during geldanamycin biosynthesis.

A Double-Gene (*gel7* and *gel8*) Disruption

The *gel8* gene is highly similar to *novN* from *Streptomyces spheroides* and *asm21* from *Actinosynnema pretiosum*, which encode *O*-carbamoyltransferase [9, 27]. The functional assignment of *gel8* as a carbamoyltransferase that attaches a hydroxy group to C-7 of geldanamycin was verified by inactivating the *gel8* gene by inserting the kanamycin resistance gene [13]. The *gel8*-inactivated mutant produced a major compound, which was identified as 4,5-dihydro-7-*O*-decarbomyl-7-hydroxygeldanamycin, indicating that C-17 hydroxylation, 17-*O*-methylation, and C-21 oxidation preceded *O*-carbamoylation and that the hypothetical pro-geldanamycin did not carry a double bond at the C-4/5 positions. Moreover, these findings indicate that *O*-carbamoylation occurred prior to C-4,5 *cis*-double-bond formation during geldanamycin biosynthesis. Therefore, a mutant with *gel7* and *gel8* dual gene-inactivation appeared to produce the predictive pro-geldanamycin, which was proposed in our previous report [13]. To confirm this, a *gel7* and *gel8* double-gene-knockout mutant was constructed by deleting half of the 3' terminal in the *gel7* and *gel8* genes, respectively, and then inserting the kanamycin resistance gene between both the chopped genes. The partial deletion of the *gel7* and *gel8* genes reduced the length of the predicted PCR product while using total genomic DNA of the Δgel7&8 mutant as the template in the PCR reaction. The size of the total fragment constructed in the disrupted vector designed to replace *gel7* and *gel8* was 2.8 kb, whereas the sum sizes of *gel7* and *gel8* was 3.4 kb in wild-type chromosomal DNA. The Δgel7&8 mutant produced a novel nonbenzoquinoid geldanamycin analog (pro-geldanamycin; $[M-H]^-$ at *m/z* 503), which does not carry a double bond at the C-4/5 and had no aromatic modification and carbamoylation at C-7. The pro-geldanamycin compound had an ESIMS pattern and 1D and 2D NMR spectra similar to those of 17-demethoxy-reblastatin and geldanamycin (data not shown). This result also confirms the finding in our previous report that module 6 of geldanamycin PKS contains a functional enoylreductase (ER) domain, which reduces the C-4/5 double bond during polyketide assembly.

***gel16* Gene Disruption and the Complementation Experiment**

After finding that Gel7 monooxygenase is directly involved in benzoquinone ring formation, we focused on *gel16* (identical to *gdmP* [24]), which encodes a protein homologous to cytochrome P450 monooxygenase. Amino-acid sequence comparisons revealed a strong similarity between Gel16 and cytochrome P450 monooxygenases (Fig. 1), and >40% sequence identity along its full length with *Micromonospora echinospora* P450 (ClaE10), which is involved in calicheamicin biosynthesis (GenBank No. AF497482), and with *Streptomyces tendae* P450 (NikF), which is involved in nikkomycin biosynthesis (GenBank No. Y18574). Gel16 also shows high similarities with a P450 involved in clavulanic acid (GenBank No. U87786), OleP involved in oleandomycin (GenBank No. L37200), PikC involved in pikromycin (GenBank No. AF079139) [19], and PimG involved in pimaricin (GenBank No. AJ278573) biosytheses. These proteins all contain a part of the O_2-binding site and a part of the heme-binding pocket, including the invariant Cys residue that coordinates with heme [5]. In addition, *gel16* has been found in an operon with electron transport components, like several genes encoding prokaryotic P450 monooxygenase, and the *gel16Fd* gene immediately downstream of *gel16* encodes a small acidic protein (64 aa) with convincing similarity to ferredoxins containing [3Fe-4S] clusters.

The *gel16* gene is therefore likely to be involved in double-bond formation at the C-4/5 position of geldanamycin. In order to provide experimental proof for this suggestion, the cytochrome P450 monooxygenase gene *gel16* was inactivated. The double cross-over mutant (Δgel16) was screened using replica plates and antibiotics, and confirmed by PCR, and its metabolites produced by culture in YEME liquid medium for 7 days were analyzed by TLC and LC/MS (Fig. 4). Δgel16 mutants produced only 4,5-dihydrogeldanamycin but no geldanamycin. As reported previously [18], the retention times of geldanamycin and 4,5-dihydrogeldanamycin were very similar by HPLC. However, 4,5-dihydrogeldanamycin was easily detected as a main product of the Δgel16 mutant on TLC and further confirmed by LC-MS (Fig. 4). Moreover, the ESIMS/MS profile of 4,5-dihydrogeldanamycin (561 [M-H]⟶518 [M-$CONH_2$]–) was comparable to that of geldanamycin (559 [M-H]→516 [M-$CONH_2$]). Moreover, a complementation experiment was carried out by introducing the *ermE** promoter-fused whole *gel16* gene region into Δgel16 mutant strains. Culture extracts of Δgel16 mutants with and without *gel16* expression vector pKC-E16 were analyzed by TLC and LC-MS and compared with wild-type extracts. pKC-E16 harboring the Δgel16 mutant effectively produced geldanamycin, which was verified versus an authentic reference substrate (Fig. 4). Accordingly, it means that *gel16* is involved in formation of the C-4/5 double-bond in geldanamycin. Although desaturation was a well-established process for the so-called fatty acid desaturases,

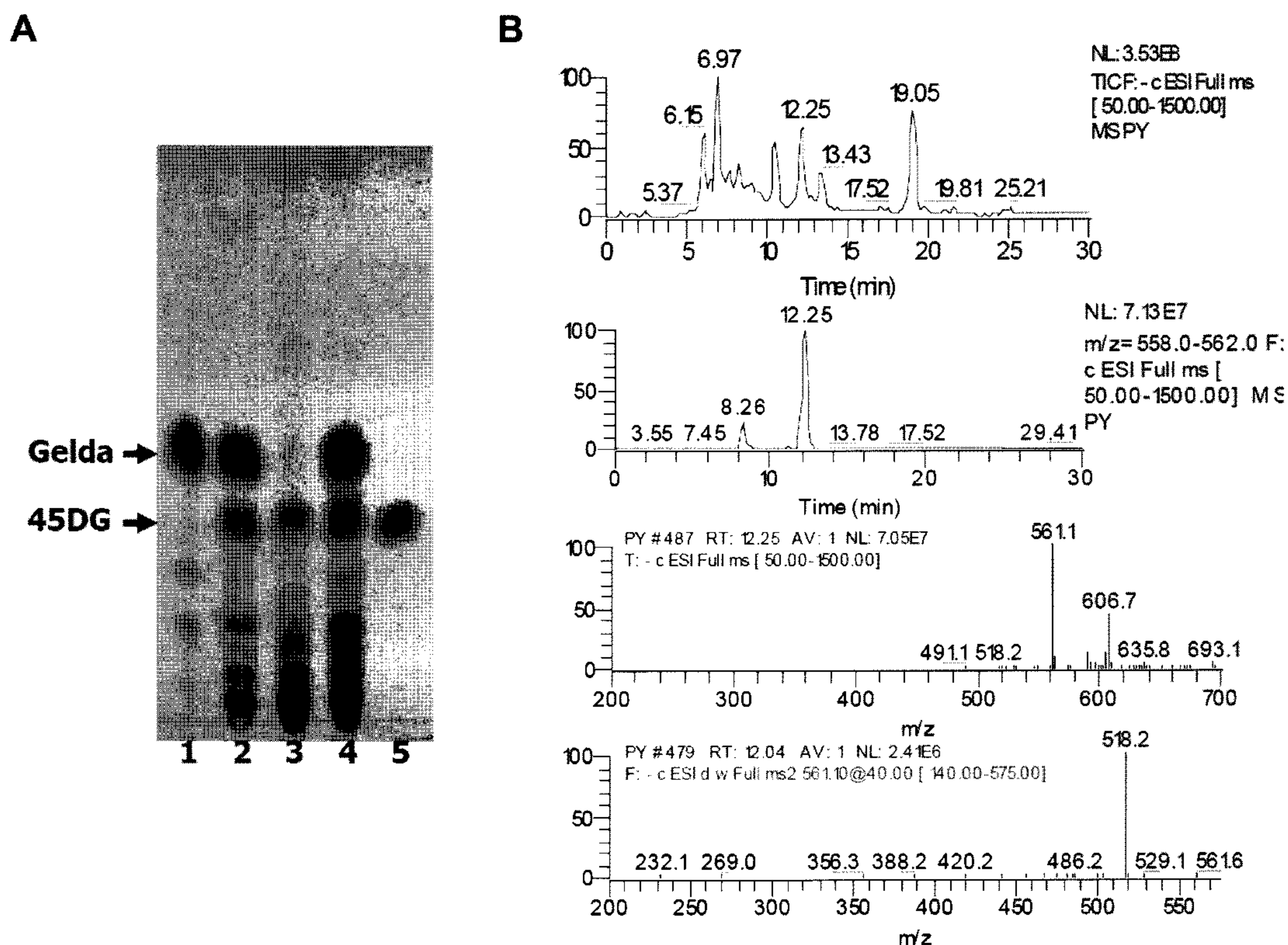

Fig. 4. TLC (**A**) and LC/MS (**B**) analyses of 4,5-dihydrogeldanamycin production in the Δgel16 mutant.
Trace 1, geldanamycin standard; trace 2, extract of culture broth from *S. hygroscopicus* JCM4427; trace 3, Δgel16 mutant; trace 4, pKC-E16 harboring Δgel16 mutant; trace 5, 4,5-dihydrogeldanamycin standard. The arrows indicate the position of geldanamycin (Gelda) and 4,5-dihydrogeldanamycin (45DG), respectively. **B.** MS and MS/MS spectra of 4,5-dihydrogeldanamycin (561[M-H]-→518[M-$CONH_2$]-) produced by the Δgel16 mutant.

which were known to be mixed-function oxidases, the roles of P450s in desaturation were not clearly recognized. In some cases, olefins were already characterized as a stable product by a purified P450 [10, 11]. Among them, in most known examples of P450-catalyzed desaturation, C-hydroxylation is also observed and is usually the major

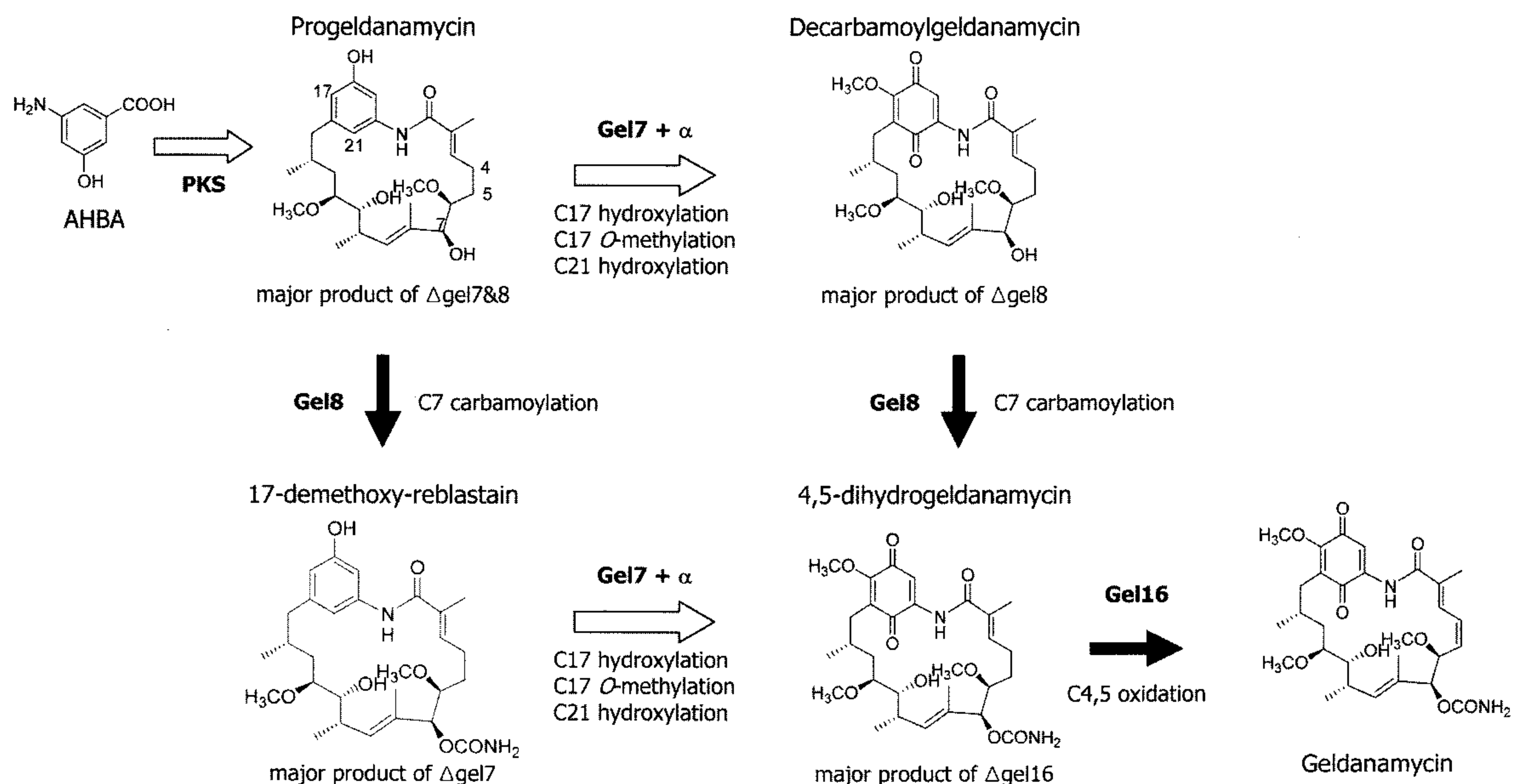

Fig. 5. Proposed post-PKS processing steps during the biosynthesis of geldanamycin.

course of the reaction. However, we couldn't find any hydroxylated-intermediates from LC/MS analysis. Therefore, it is not clear whether Gel16 is able to make double-bond formation between C-4/5 by itself, or another enzyme for additional hydroxylation is necessary.

The mechanism of benzoquinone formation during the biosynthesis of geldanamycin remains unclear, but our results indicate that C-17 hydroxylation, 17-*O*-methylation, C-21 oxidation, and *O*-carbamoylation precede C-4/5 desaturation. This means that these steps of benzoquinone modification and carbamoylation occur simultaneously (Fig. 5). The pro-geldanamycin, which did not carry a double bond at C-4/5, was purified and its structure elucidated. In addition, we determined that the *gel16* gene encodes a cytochrome P450 monooxygenase, which catalyzes C-4/5 double-bond formation as a final biosynthetic step (Fig. 5). This work opens the way for a detailed analysis of geldanamycin biosynthesis at the genetic level, and sets the stage for the structural modification of chemically inaccessible geldanamycin analogs.

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