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Rational Biosynthetic Engineering for Optimization of Geldanamycin Analogues

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A rational biosynthetic engineering approach was applied to the optimization of the pharmacological properties of the benzoquinone ansamycin, geldanamycin. Geldanamycin and its natural or semisynthetic derivatives have the potential to serve as anticancer chemotherapeutic agents. However, these first-generation Hsp90 inhibitors share an unfavorable structural feature that causes both reduced efficacy and toxicity during clinical evaluation. We report the rationally designed biosynthesis of C15 hydroxylated non-quinone geldanamycin analogues by site-directed mutagenesis of the geldanamycin polyketide synthase (PKS), together with a combination of post-PKS tailoring genes. A 15-hydroxyl-17-demethoxy non-quinone analogue, DHQ3, exhibited stronger inhibition of Hsp90 ATPase activity (4.6-fold) than geldanamycin. Taken together, the results of the present study indicate that rational biosynthetic engineering allows the generation of derivatives of geldanamycin with superior pharmacological properties.

Introduction

Geldanamycin and its closely related natural analogues, herbimycin and macbecin, are benzoquinone ansamacrolide antibiotics.[1–3] Geldanamycin competes with ATP at the nucleotide binding site in the N-terminal domain of heat shock protein 90 (Hsp90), and thereby acts as a Hsp90 inhibitor.[4–6] Hsp90 is a molecular chaperone the main function of which is to ensure the stability and proper folding of its client proteins.[7] Because Hsp90 client proteins are crucial in maintaining the transformed phenotype of cancer cells as well as increasing their survival and growth potential, geldanamycin and its natural or semisynthetic derivatives could serve as anticancer chemotherapeutic agents.[8,9] Although geldanamycin has shown effective preclinical anticancer activity, its hepatotoxic effects have limited its further clinical development.[10] Instead, a C17 positional derivative of this compound, 17-allylamino-17-demethoxygeldanamycin (17-AAG), is now being tested in phase I and II clinical trials.[11] A phase I clinical trial of another geldanamycin derivative, 17-(2-dimethylamino)ethyl-amino-17-demethoxygeldanamycin (17-DMAG), showed promising results, but due to unacceptable side effects further development was terminated.

The biosynthesis of geldanamycin is initiated by the priming of 3-amino-5-hydroxy benzoic acid (AHBA).[12,13] Nascent polyketide assembly is catalyzed by three modular polyketide synthases (PKS), which are encoded by the *gelA–C* PKS genes; these genes are very similar to the *gdmA1–A3* genes[12] from the other geldanamycin producing strain, *Streptomyces hygroscopicus* NRRL3602. In most modular polyketides, there is a convincing correlation between the predicted constituent domains and the chemical structure of the corresponding polyketide chain.[14] The variety of polyketide structures largely arises from the presence or absence of ketoreductase (KR), dehydratase (DH) or enoylreductase (ER) domains. After the discovery of the modular architecture of certain PKSs, several reports have highlighted the functional versatility of these multienzyme assemblies with experiments involving domain inactivation, substitution or addition.[15–17] The boundaries of this approach to creating novel polyketide antibiotics have been extensively studied in several polyketide-producing strains.[18–21] However, the yields of such engineered analogues were often not sufficient to permit their isolation or elucidation of their chemical structures, or even for primary biological assays to be conduct.[18,22] The reasons for the lower productivity are poorly understood, but could include structural instability of the engineered protein, suboptimal chemistry within the altered module, or inefficient processing of the unnatural polyketide

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intermediates by downstream modules. Nevertheless, the genetic manipulation of PKSs, which is also known as combinatorial biosynthesis, has proven to be a versatile technology in increasing the chemical diversity of "unnatural" natural products.[23–26]

Based on the interactions observed between geldanamycin and Hsp90, Stebbins et al. postulated that the benzoquinone group of geldanamycin binds near the entrance of the ATP binding pocket and the carbamate group forms a water-bridged hydrogen bond with Hsp90.[5,27] Other molecular modeling studies with 17-DMAG and macbecin have also shown similar results to that of the geldanamycin–Hsp90 interaction.[28,29] Interestingly, macbecin forms one extra hydrogen bond with Hsp90 through the C15 methoxy moiety.[29] Meanwhile, it has been shown that geldanamycin exhibits high hepatotoxicity and is therefore unsuitable for use in humans.[10] 17-AAG and 17-DMAG exhibit lower hepatotoxicity and retain their in vivo anticancer activity; however, hepatotoxicity still remains a dose-limiting side effect with both these agents. The dose-limiting toxicity of geldanamycin and its derivatives might be partly due to "off target" effects and could be attributable to the high chemical reactivity of its benzoquinone group, and not as a direct consequence of Hsp90 inhibition.[30,31] For these reasons, a second generation of geldanamycin derivatives that lack a quinone moiety was proposed as alternative compounds that might have fewer undesirable toxic effects.[31] Recently, non-quinone compounds created by biosynthetic modifications of macbecin showed significantly improved binding affinities for Hsp90, as well as reduced toxicity profiles.[32] Taken together, a non-quinone geldanamycin analogue with an additional functional residue at the C15 position could have reduced side effects and improved binding properties to Hsp90.

Here, we report the rationally designed biosynthesis of C15-modified geldanamycin analogues by site-directed mutagenesis of the DH1 domain of the geldanamycin PKS. The non-quinone type 15-hydroxylgeldanamycin analogue was produced through combinatorial genetic inactivation of the DH1 domain along with the monooxygenase, Gel7, which is responsible for the formation of the quinone. In addition, the 15-hydroxyl-17-demethoxy non-quinone analogue showed an improved inhibitory effect on the ATPase activity of Hsp90.

Results

Site-directed mutagenesis of the DH1 domain of GelA

In the geldanamycin producer, *S. hygroscopicus* JCM4427, the geldanamycin PKS consists of seven modules encoded by three genes, *gelA–gelC*. Each module contains a suitable reductive domain that is entirely consistent with the structure of the polyketide backbone, except for module 6, which contains a functional ER domain that reduces the C4/5 double bond.[33] The first module of GelA, which is made up of a full set of domains, is responsible for the C15 functional group variety in the geldanamycin polyketide backbone. Accordingly, the activity of DH in module 1 is required for the reduction of the keto residue on C15 of the geldanamycin polyketide. The first step in the preparation of a C15-modified geldanamycin derivative was the gene disruption of the DH1 domain by insertion of an antibiotic resistance gene (Figure 1). The *gelA* gene disruption mutant, *S. hyg*-ΔDH, was constructed by insertion of a kanamycin resistance gene (*aphII*) in the DH1 domain by homologous recombination. To confirm the insertion of the *aphII* gene in the DH1 domain, genomic DNA from wild-type and *S. hyg*-ΔDH cells was used as template for PCR amplification with the relevant primer set. A 1.4 kb PCR product was detected from the wild-type strain and a 2.4 kb PCR product was obtained from *S. hyg*-ΔDH (see the Supporting Information). Also, the growth of the *S. hyg*-ΔDH mutant in YEME medium containing kanamycin was normal and comparable to the growth of the wild-type strain, but the mutant completely lost its ability to produce geldanamycin and 17-*O*-demethylgeldanamycin, which

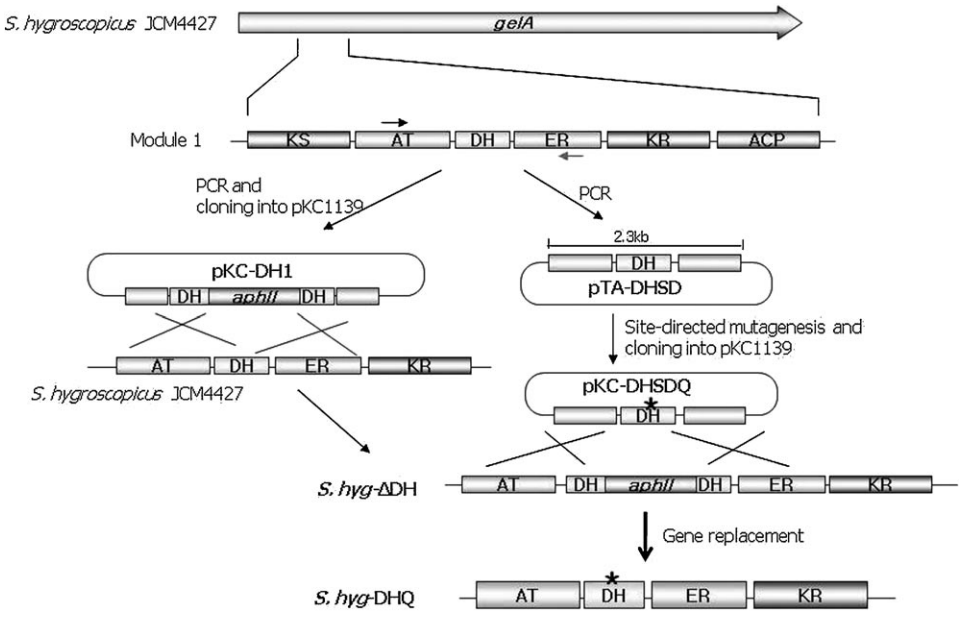


Figure 1. Strategy for the site-directed mutagenesis of the dehydratase domain (DH) in the geldanamycin PKS gene cluster. The *S. hyg*-ΔDH mutant resulted from a double crossover to produce an apramycin resistant strain in which module 1 of the *gelA* gene was disrupted. This resulted in a strain that did not produce geldanamycin. The site-directed gene replacement vector, pKC-DHSDQ, was introduced into the *S. hyg*-ΔDH mutant and a number of transformants were isolated from apramycin- and kanamycin-containing plates. The final gene replacement mutant, *S. hyg*-DHQ, was selected and confirmed by sequencing. The *aphII* gene is an apramycin resistance gene.

are the two major metabolites of the wild-type strain, *S. hygroscopicus* JCM4427 (see the Supporting Information).

The DH1 domain has the apparent NADP binding motif $H(X_3)G(X_4)P$ (where X denotes any amino acid), which is a well conserved active-site sequence in the functional DH domain in polyketide and fatty acid biosynthesis (Figure 2).[34,35] A single amino acid (His878) mutation in the active site of the rat fatty acid synthase eliminates DH activity.[35,36] Mutation of the essential histidine residue (His2041) in the conserved sequence of the GelA DH1 was expected to result in a dehydratase-deficient mutant. The active site His2041 of the DH1 domain in GelA was substituted with glutamine (H2041Q) by PCR-based site-directed mutagenesis with the designed primer sets (as described in the Experimental Section). The final site-directed gene replacement vector, pKC-DHSDQ, was introduced into the *S. hyg*-ΔDH mutant and a number of transformants were obtained from apramycin- and kanamycin-containing plates. These transformants were then cultured at 37 °C to cure the temperature-sensitive delivery plasmid and induce homologous recombination between the disrupted region and the mutated DH1 fragment. The final selected gene replacement mutant, *S. hyg*-DHQ, showed sensitivity to both kanamycin and apramycin. Site-directed mutagenesis of the DH domain was expected to cause a malfunction of the dehydration step in a manner that minimized perturbation to the tertiary structure of GelA.

Culture broth from the *S. hyg*-DHQ mutant grown in production media was analyzed by LC/MS. A 15-hydroxygeldanamycin peak (*m/z* 575 $[M-H]^-$) was detected in trace amounts at a retention time (t_R) of 7.2 min. The peak showed MS^n fragmentation patterns that resembled that of geldanamycin, which exhibits a decarbamoylated peak, 575 $[M-H]^- \rightarrow 532$ $[M-CONH_2]^-$, but the yields were not sufficient to allow full structural characterization. Also, a 15-hydroxy-4,5-dihydrogeldanamycin peak (*m/z* 577 $[M-H]^- \rightarrow 534$ $[M-CONH_2]^-$) was detected in trace amounts at a retention time of 11.6 min (Figure 3). In contrast, two peaks with retention times of between 12.7 and 13.2 min, which showed similar MS^n fragmentation patterns to geldanamycin, were detected in amounts adequate for purification (see the Supporting Information).[37]

DHQ1 and DHQ2 were thus purified and structural elucidations were conducted (Scheme 1). DHQ1 did not possess a double bond at the C4 and C5 positions. The C4,5-olefin structure of DHQ2 was attributed to the action of Gel16, which is a post-PKS modification enzyme. Both compounds were assumed to contain the hydroxyl residue on the C15 position within the polyketide backbone. However, in both DHQ1 and DHQ2 the C15-OH had apparently participated in a C11/C15 dehydration reaction to form an overall tricyclic structure. DHQ2 showed the same structure as that of a minor tricyclic 15-hydoxylgeldanamycin analogue (KOSN-1633), which was produced by bioconversion in a herbimycin-producing strain.[38] The yields of the DHQ1 and DHQ2 analogues from the *S. hyg*-DHQ mutant were lower (>tenfold) than the amount of geldanamycin typically produced by the parent wild-type strain. These results show that the *S. hyg*-DHQ mutant was capable of producing geldanamycin analogues upon substitution of His2041 in the active site of the DH1 domain in GelA. In addition, they indicate that the single amino acid exchange in the active site of the DH domain does not cause the GelA megasynthase complex to fold incorrectly or lose a necessary activity (Figure 4). This result contrasts with another report in which mutation of the dehydratase active site in an erythromycin-producing PKS disabled the overall assembly line.[39]

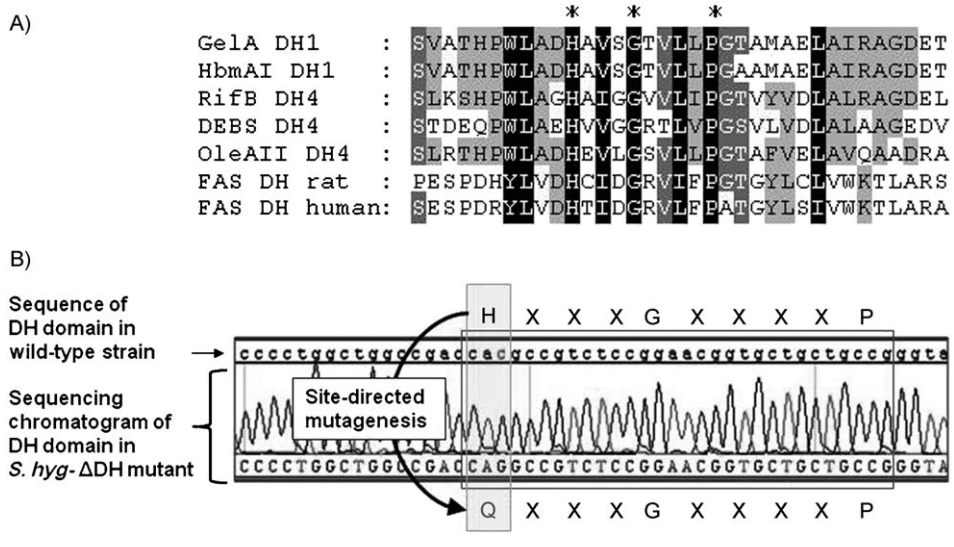


Figure 2. A) Amino acid alignments of the putative active site region of several dehydratases. GelA: geldanamycin PKS; OleAII: oleandomycin PKS; Hbm: herbimycin PKS; RifB: rifamycin PKS; DEBS: erythromycin PKS. Asterisks indicate highly conserved amino acid residues. B) Verification of the histidine-to-glutamine mutation by DNA sequencing. The sequence of the active site at the DH domain was compared to the sequence obtained from the *S. hyg*-DHQ mutant. The wild-type codon CAC (histidine) was altered to CAG (glutamine) in the mutant, as indicated.

Combinatorial biosynthesis with mutation of post-PKS modification genes

The quinone biosynthetic pathway for geldanamycin, that is, hydroxylation of a phenol precursor followed by autoxidation by molecular oxygen, was demonstrated in previous reports.[40] The *gel7* gene disruption in the wild-type strain produced a non-benzoquinoid geldanamycin analogue with a monophenolic structure.[40] In addition, we found that the *gel7* and *gel8* double mutant produced non-quinone decarbamoyl geldanamycin, which did not show any post-PKS modification of the polyketide backbone. Based on these results, we attempted an additional post-PKS modification gene disruption with the *S. hyg*-

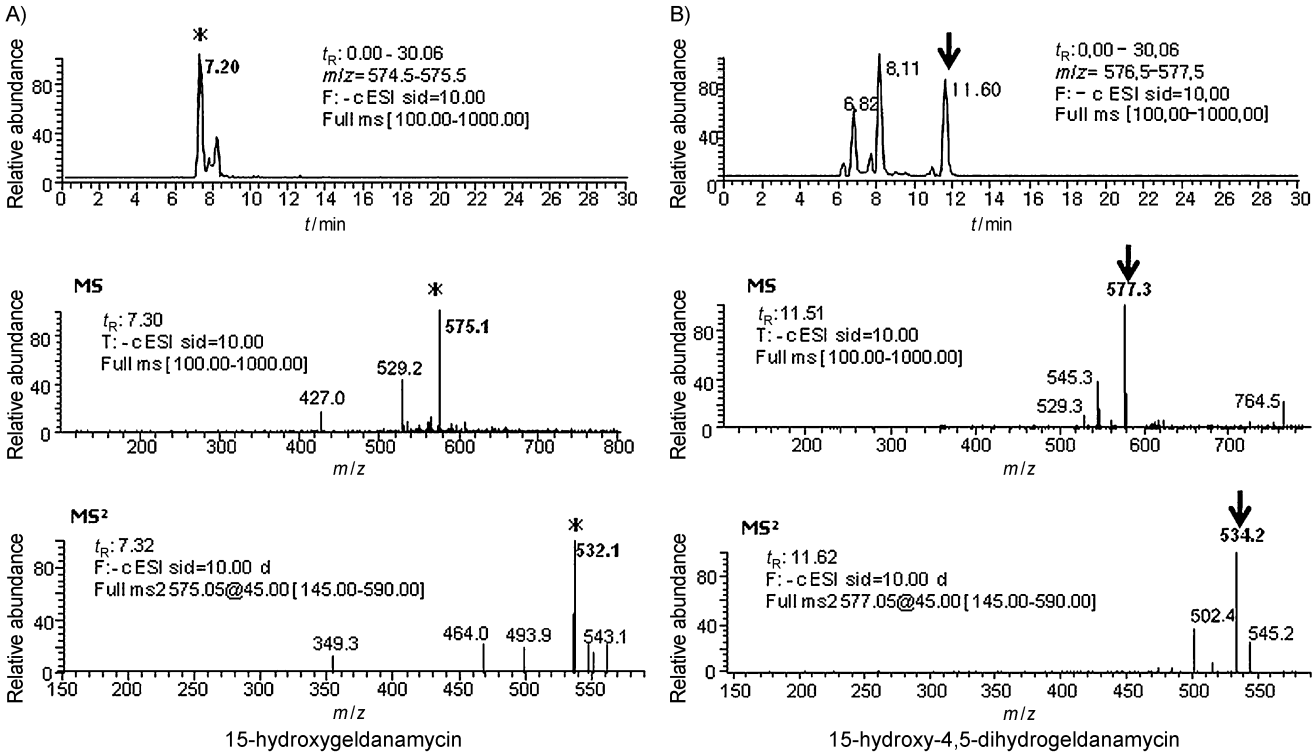
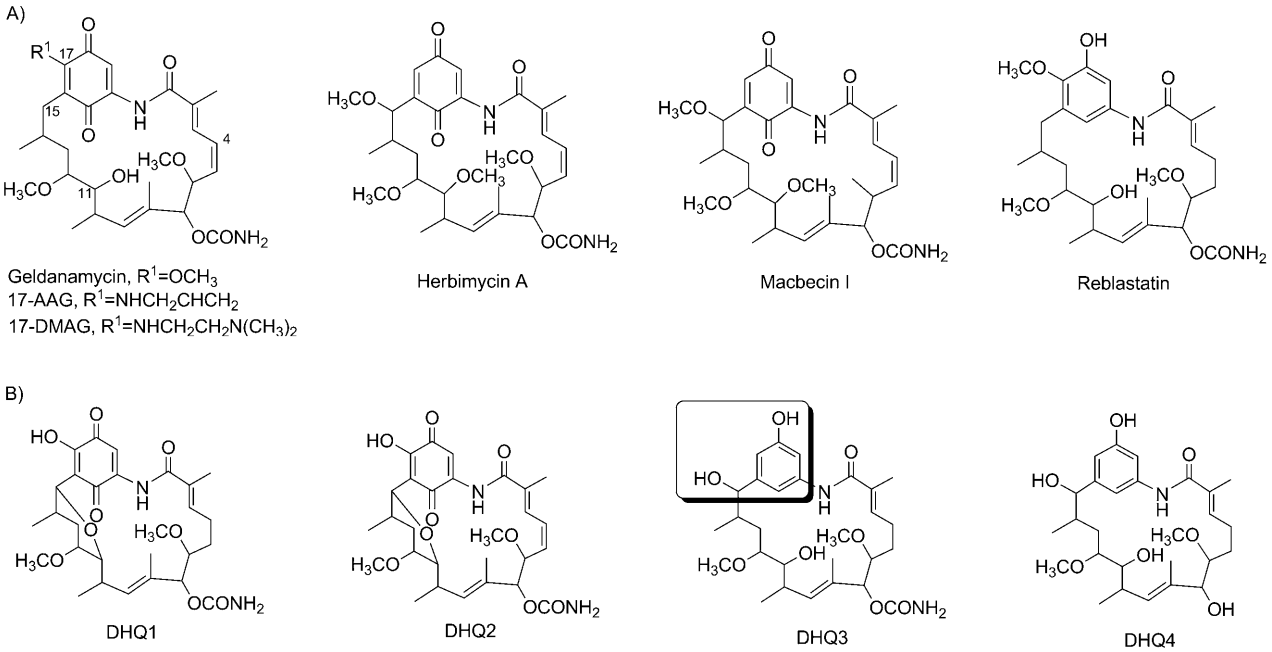


Figure 3. LC/MS analysis of 15-hydroxygeldanamycin and 15-hydroxy-4,5-dihydrogeldanamycin production in the *S. hyg*-DHQ mutant. MS^n spectra of predicted A) 15-hydroxygeldanamycin and B) 15-hydroxy-4,5-dihydrogeldanamycin were recorded. 15-Hydroxygeldanamycin (*m/z* 575 $[M-H]^- \rightarrow 532$ $[M-CONH_2]^-$) and 15-hydroxy-4,5-dihydrogeldanamycin peaks (*m/z* 577 $[M-H]^- \rightarrow 534$ $[M-CONH_2]^-$) are shown. The two top panels represent the extracted ion scan with *m/z* 575 and 577 (A and B, respectively). The asterisks and arrows indicate the peaks for 15-hydroxygeldanamycin and 15-hydroxy-4,5-dihydrogeldanamycin, respectively.



Scheme 1. A) Structures of geldanamycin, herbimycin A, macbecin I, reblastatin, and B) four biosynthetically engineered analogues. DHQ2 showed the same structure as KOSN-1633.[38]

DHQ mutant; this was predicted to result in various C15 hydroxyl geldanamycin analogues without the benzoquinone ring. These gene disruption vectors, which had already been constructed in previous experiments,[40] were introduced into

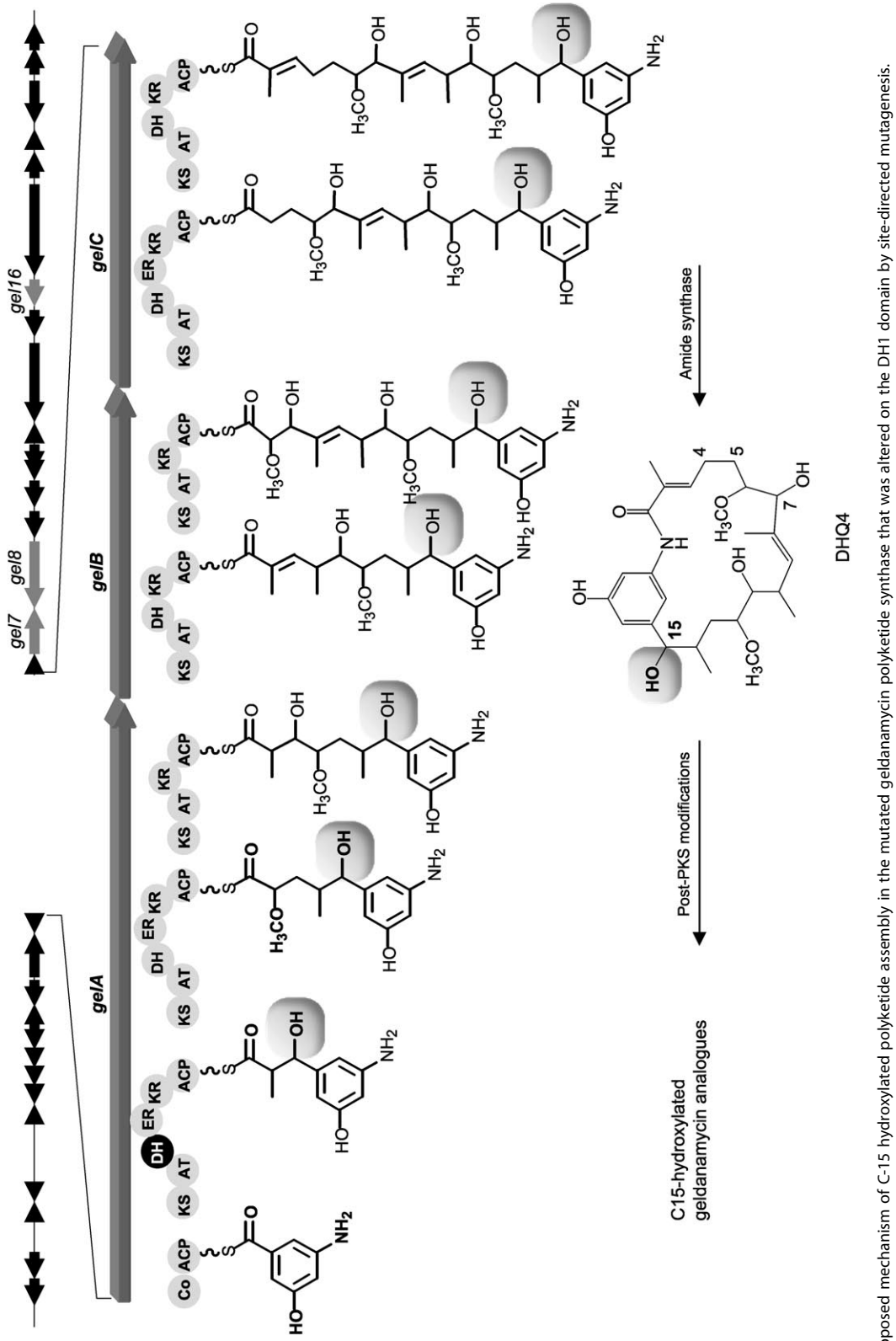


Figure 4. Proposed mechanism of C-15 hydroxylated polyketide assembly in the mutated geldanamycin polyketide synthase that was altered on the DH1 domain by site-directed mutagenesis.

the *S. hyg*-DHQ mutant, and each mutant strain was selected on kanamycin plates.

The *gel7* gene disruption in the *S. hyg*-DHQ mutant produced the 15-hydroxyl-17-demethoxy derivative, DHQ3, which possesses a monophenolic structure containing a C15 hydroxyl residue (Scheme 1). When both the *gel7* and *gel8* genes were disrupted in *S. hyg*-DHQ, a decarbamoyl derivative of DHQ3, DHQ4, which was not processed by the post-PKS modification steps, was produced (Scheme 1). Both compounds showed a deficiency of the oxidation reaction C17 and C21 and the double bond at C4/5. The yield of DHQ3 (about 8–10 mg L^{-1}) and DHQ4 (about 8–15 mg L^{-1}) analogues were not significantly different to the amount of geldanamycin typically produced by the parent wild-type strain. The appearance of significant amounts of the C4,5-dihydro form confirmed our previous suggestion that this C4/5 desaturation reaction was the final post-PKS step.[40]

All these compounds contained the hydroxyl group at the C15 position of the polyketide backbone. This meant that even if the *S. hyg*-DHQ mutant mainly produced the cyclized compounds between the hydroxyl group of C15 and C11, it was the inactivity of the DH1 domain that led to the retention of the hydroxyl group at this position. The C15 hydroxyl ketide chains were well-processed in the following modules even though site-directed mutagenesis only occurred on the first module, and interfered minimally with the productivity of the nascent polyketide.

Inhibition of Hsp90 ATPase activity by the biosynthetically engineered analogues

All biosynthetically engineered analogues in this study were assessed by using a yeast Hsp90 ATPase assay (Table 1). The IC_{50} values were determined from a range of inhibitor concentrations (5 nM–50 μM). Geldanamycin gave an IC_{50} value of 3.19 μM in our ATPase assay system, which was comparable to the value obtained with a previously reported ATPase inhibition assay.[41] The inhibitory activity of DHQ3 was 4.6-fold higher than that of geldanamycin. These results indicate that the extra hydrogen bond on the C15 position of DHQ3 might offer sufficient enthalpy gain to yield slightly tighter binding interactions with Hsp90 than geldanamycin.[29] The tricyclic and decarbamoyl compounds, DHQ1, DHQ2 and DHQ4 showed no such activity. DHQ3 thus represents an attractive lead for further chemical modification.

Table 1. Potency of geldanamycin and the newly engineered 15-hydroxyl non-quinone derivatives, DHQ1–4, against yeast Hsp90; ATPase inhibition was tested by using the malachite green assay.

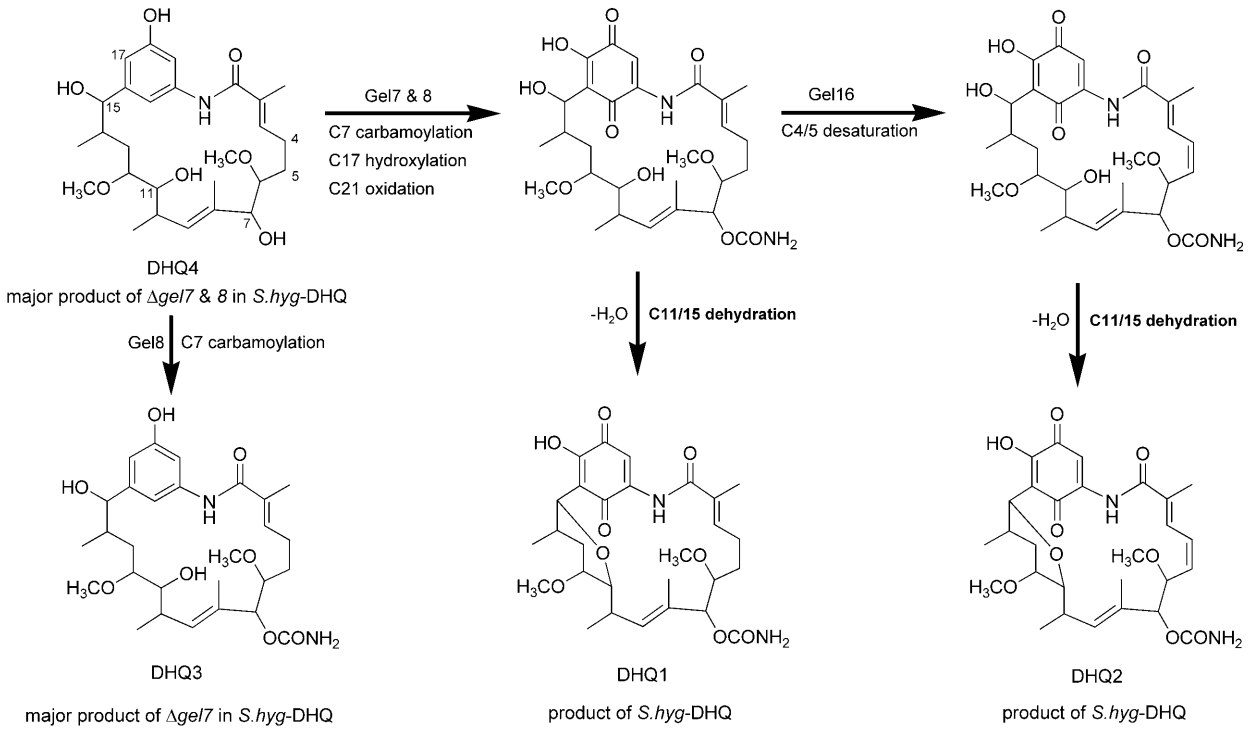
Compounds	ATPase inhibition assay IC_{50} [μM ± SD]
geldanamycin	3.19 ± 0.65
DHQ1	> 50
DHQ2	> 50
DHQ3	0.68 ± 0.29
DHQ4	> 50

Discussion

The genetic alteration of the catalytic domains of PKSs by inactivation, substitution, addition or deletion, can yield predicted structural alterations of the final product.[18,21] Hence, engineered biosynthetic processes provide the opportunity to create an expanded chemical diversity of the appropriate compound. However, the genetic engineering of PKSs also involves various problems from an industrial point of view.[42,43] Firstly, the randomly increased number of compounds produced in this manner is not always an answer to complex biological issues. Likewise, even the ability to perform genetic manipulations in actinomycetes or other host organisms harboring the PKS gene cluster does not promise the development of biologically improved analogues.[22] Secondly, the engineered strain often exhibits lower productivity, which could be due to structural disagreement between the engineered protein and its intermediates.[44] Minimally invasive approaches, such as site-directed mutagenesis, which leave the highly complex modules intact, seem to offer the best chances of avoiding the perturbation of the overall PKS architecture. The single amino acid exchanged mutant, *S. hyg*-DHQ, produced detectable amounts of 15-hydroxylated geldanamycin as determined by LC/MS, but mainly produced tricyclic compounds formed by cyclization between C15-OH and C11-OH. However, combinational mutation with post-PKS genes in the *S. hyg*-DHQ mutant clearly produced the C15-hydroxlyated geldanamycin analogues. Therefore, single mutation of the histidine residue in the conserved region ($H(X_3)G(X_4)P$) within the DH1 domain, which is deduced to be responsible for NAD binding, eliminated the dehydratase activity in the GelA PKS protein. The abolished function of the DH domain did not hamper further polyketide elongation as the modified ketide chain was successfully processed by the later modules and modified by post-PKS tailoring reactions.

DHQ3, which does not carry a double bond at the C4 and C5 positions, was detected as the major product in *S. hyg*-DHQ, having the *gel7* gene mutation. However, the presence of both the saturated (DHQ1) and unsaturated compounds (DHQ2) at the C4/5 position in the *S. hyg*-DHQ mutant indicate that the intramolecular cyclization was unaffected by the C4/5 desaturation reaction. In addition, 15-hydroxygeldanamycin was detected in trace amounts. This suggests that the nascent final product, 15-hydroxygeldanamycin possibly underwent an unpredicted modification process in the cell; the dehydration step on C11/15 is anticipated to be one of these processes (Scheme 2).

The significant ATPase inhibitory activity of DHQ3 implies that the addition of the 15-hydroxy residue allowed for the formation of one more hydrogen bond with Hsp90. The extra hydrogen bond might offer sufficient enthalpy gain to yield slightly tighter binding in the ATP binding pocket of Hsp90. However, Hu et al. reported that 15-hydroxygeldanamycin, produced by bioconversion experiments with herbimycin-producing strains, might be unfavorable for cellular activity.[38] Therefore, although the C15 hydroxyl derivative is advantageous for in vitro activity, it could show weak solubility and/or membrane permeability.



Scheme 2. Proposed biosynthetic pathway for the tricyclic ring formation of C15 hydroxylgeldanamycin analogues in the *S. hyg*-DHQ mutant. The C15-OH of 17-*O*-demethylated 15-hydroxygeldanamycins had apparently participated in a C11/C15 dehydration reaction to form a tricyclic structure.

Meanwhile, geldanamycin and its benzoquinone derivatives have been shown to react chemically with the thiol group of glutathione (GSH); this results in stable drug–GSH adducts.[31] The chemical reactivity of the benzoquinone group has been associated with dose-limiting hepatotoxicity.[30,31] Also, expression of P-glycoprotein and loss or mutation of the NAD(P)H/quinone oxidoreductase 1 (*NQO1*) gene, which is required for the reduction of the quinone to the more potent hydroquinone, have been proposed as mechanisms of de novo or acquired resistance to the quinone form.[45–47] Therefore, Hsp90 inhibitors that are not substrates for P-glycoprotein and that do not require NQO1 metabolism could be more effective clinical agents than the quinone form. For these reasons, a second generation of Hsp90 inhibitors that lack a quinone moiety might be more efficacious and less toxic than the front-runner compound, 17-AAG. DHQ3 showed better ATPase inhibition than the original geldanamycin, which contained the benzoquinone moiety. Hence, non-quinone geldanamycin analogues with an extra functional C15 residue could be an ideal strategy to improve the pharmacological properties of the compound.

Conclusions

Combinatorial biosynthetic methods involving inactivation, substitution, addition or deletion of functional domains in modular PKSs, can cause structural instabilities in proteins. In the present study, we present a useful alternate method of combinatorial biosynthetic engineering through a minor tuning in the target domain of the PKS. The function of dehydratase domains on module 1 of the geldanamycin PKS, which is responsible for C15 dehydration on the polyketide, was successfully removed. In addition, the combinatorial genetic inactivation of the monooxygenase (*gel7*) gene, which is responsible for the formation of the quinone, resulted in the production of C15 hydroxylated non-quinone geldanamycin analogues. Hence, our strategy, which is based on modification of the benzoquinone type geldanamycin, demonstrates a successful example of the rational alteration of complex polyketide structures.

Experimental Section

Bacterial strains, media and plasmids: *Escherichia coli* XL1-Blue strain was used as the general host for cloning, and *E. coli* ET12567 (pUZ8002) was used as the donor strain in intergeneric conjugation.[48] *E. coli* strains were grown either in LB medium or on LB agar supplemented with appropriate antibiotics. Apramycin (50 μg mL^{-1}), chloramphenicol (25 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) were added to growth media as required.[49] Wild-type geldanamycin producing strain *Streptomyces hygroscopicus* subsp. *duamyceticus* JCM4427 was obtained from the Japanese collection of microorganisms. Wild-type and mutant strains were grown in yeast extract/malt extract medium (YEME) to obtain mycelia, and chromosomal DNA isolation and metabolite extraction was carried out with these cultures.[50] PCR products were cloned into the pCR2.1-TOPO cloning vector. Gene disruption experiments were carried out by using the vector pKC1139, and insertion of the kanamycin resistance gene from pFD-neoS was used as a selection marker.[51]

Gene inactivation: All gene disruptions were performed with a similar design by using the plasmid pKC1139 to deliver the corre-

sponding kanamycin resistance gene cassettes.[40] A 1.1 kb DNA fragment from pFD-neoS carrying the *aphII* gene responsible for kanamycin resistance was routinely used as a selective marker for construction of gene disruption vectors. The DH domain of the *gelA* gene was amplified by PCR from total genomic DNA. The 1.5 kb 5′ region of the DH domain was amplified by PCR with the forward primer 5′-CGG AAT TCA CGC CAA CCC GGT CGA TGT GGG-3′ (EcoRI site underlined) and the reverse primer 5′-AAC TGC AGA CCG TCT TCG GGC AGT GTC ATC-3′ (PstI site underlined). The other half of the 1.1 kb DH domain was amplified by PCR with the forward primer 5′-GGG GTA CCG AGG GGT GCG GAT CTA CTC TC-3′ (KpnI site underlined) and the reverse primer 5′-CCC AAG CTT AGA CGA GGC ACC CAC AGC AGC CCA-3′ (HindIII site underlined). The cassette consisted of two PCR-derived flanking regions, in which suitable restriction sites were introduced. The disruption construct, pKC-DH1 was introduced 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,[48] and gene disruption was confirmed with PCR analysis by using a previously reported method.[40] The other disruption vectors related to the post-PKS modification genes (*gel7* and *gel7/gel8*) were constructed by the same methods described in our previous report,[32,38] and were introduced into the DH1 gene mutant strain.

Site-directed mutagenesis and gene replacement: Site-directed mutants were generated by using the QuickChange (Stratagene) PCR-based method. A 2 kb fragment from the DH domain region was amplified with the forward primer 5′-CCG AAT TCG CCG TCG TCG TCT CCC TCG CCG CA-3′ (EcoRI site underlined) and reverse primer 5′-CCC AAG CTT CTC CGA GAC GTC GAG GTA CCA GTG-3′ (HindIII site underlined). The PCR product was cloned into the pCR-TOPO2.1 vector to obtain pTA-DHSD, which was used as the template for site-directed mutagenesis. The histidine residue (H2041) within the putative active-site region in the DH1 domain was substituted with glutamine by using the following primer pairs: forward primer 5′-CCG TGG CTG GCC GAC CAG GCC GTC TCC GGA ACG G-3′ and reverse primer 5′-CCG TTC CGG AGA CGG CCT GGT CGG CCA GCC ACG G-3′ (mutation site underlined). The construct, pTA-DHSDQ, was sequenced in order to verify the mutation and was ligated into pKC1139. The final gene replacement vector, pKC-DHSDQ, was introduced into *S. hygroscopicus* JCM4427 by conjugation with *E. coli* ET12567 (pUZ8002). The transformants were resistant against both apramycin and kanamycin and were grown in fresh YEME liquid medium without antibiotics at 37 °C for four days in order to induce the integration of the fragment with the single nucleotide mutation in the gene replacement vector into the chromosomal DNA. This resulting gene replacement mutant did not contain any detectable free plasmid DNA and showed an antibiotics-sensitive phenotype on R2YE plates containing kanamycin and apramycin. This mutant was confirmed by PCR and sequencing with each designed primer set.

Metabolite analysis and structure elucidation: Each mutant strain was grown in liquid YEME medium and incubated at 28 °C for five days. The cultured broth was extracted with EtOAc twice and these extracts were filtered through a fritted funnel in vacuo to remove insolubles. The volume of the eluant was reduced in vacuo and then partitioned between EtOAc and H_2O to give the organic extracts. Fractionation of the extracts was initiated by Si gel chromatography by using a $CHCl_3$/MeOH gradient as the mobile phase; the fractions were then pooled based on HPLC and LC/MS analysis. All NMR spectroscopy experiments were performed in $CDCl_3$ and pyridine either by using a Bruker DMX 600 NMR spectrometer or a Varian Unity 400 NMR spectrometer. LC/MS was performed by using a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co., Waltham, USA) equipped with an electrospray ionization (ESI) source.[13] HPLC was carried out by using a Dionex Summit or a Waters Delta Prep 3000 system.

Colorimetric determination of ATPase activity: Histidine-tagged yeast Hsp90 was purified by metal affinity.[52] The protein was used after dialysis with the assay buffer. The assay procedure was based on a previously described method.[41] On the day of use, the malachite green reagent was prepared by mixing malachite green (0.0812%, *w/v*), polyvinyl alcohol (2.32%, *w/v*), ammonium molybdate (5.72% in 6 M HCl, *w/v*) and water in the ratio 2:1:1:2. The reagent was initially dark brown, but on incubation for ~2 h at room temperature it changed to a golden yellow and was then ready for use. The assay buffer was Tris-HCl (100 mM), KCl (20 mM), $MgCl_2$ (6 mM) at pH 7.4. Each compound was dissolved in DMSO at a concentration of 10 mM and the stock solutions were diluted as needed. ATP was dissolved in the assay buffer to give a stock concentration of 2.5 mM. Hsp90 protein was thawed on ice and diluted in chilled assay buffer to give a stock solution of 0.3 mg mL^{-1}. The compound solution (1 μL), Hsp90 (10 μL) and assay buffer (4 μL) were added together into each well and preincubated for 1 h at 37 °C. ATP (10 μL of 2.5 mM solution) was then added to each well and the plate was shaken for 1 min and was incubated for 3 h at 37 °C. To stop the reaction, malachite green reagent (80 μL) was added to each well and the plate was shaken again. Following the addition of sodium citrate (10 μL of 34% solution) to each well, the plate was shaken once more and incubated at room temperature for about 15 min, and then the absorbance was measured at 620 nm.

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