

Insights into the Biosynthesis of the Benzoquinone Ansamycins Geldanamycin and Herbimycin, Obtained by Gene Sequencing and Disruption†

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Geldanamycin and the closely related herbimycins A, B, and C were the first benzoquinone ansamycins to be extensively studied for their antitumor properties as small-molecule inhibitors of the Hsp90 protein chaperone complex. These compounds are produced by two different *Streptomyces hygroscopicus* strains and have the same modular polyketide synthase (PKS)-derived carbon skeleton but different substitution patterns at C-11, C-15, and C-17. To set the stage for structural modification by genetic engineering, we previously identified the gene cluster responsible for geldanamycin biosynthesis. We have now cloned and sequenced a 115-kb segment of the herbimycin biosynthetic gene cluster from *S. hygroscopicus* AM 3672, including the genes for the PKS and most of the post-PKS tailoring enzymes. The similarities and differences between the gene clusters and biosynthetic pathways for these closely related ansamycins are interpreted with support from the results of gene inactivation experiments. In addition, the organization and functions of genes involved in the biosynthesis of the 3-amino-5-hydroxybenzoic acid (AHBA) starter unit and the post-PKS modifications of progeldanamycin were assessed by inactivating the subclusters of AHBA biosynthetic genes and two oxygenase genes (*gdmM* and *gdmL*) that were proposed to be involved in formation of the geldanamycin benzoquinoid system. A resulting novel geldanamycin analog, KOS-1806, was isolated and characterized.

The herbimycins (19) and macbecins (18), belonging to the group of benzoquinone ansamycins (24), are closely related to geldanamycin (7) in structure and bioactivity. *Streptomyces hygroscopicus* NRRL 3602 produces geldanamycin, whereas *S. hygroscopicus* AM 3672 makes herbimycin A, a C-11,15 dimethoxy-17-desmethoxy analog of geldanamycin, as its major natural product.

Like geldanamycin, the herbimycins were discovered in the late 1970s and found to have weak antibacterial and antifungal properties and also to show plant growth-inhibitory effects (13, 19). Both compounds were later discovered to have potent antitumor activity at nanomolar concentrations (26, 33). Although initially identified as a tyrosine kinase inhibitor, geldanamycin was subsequently found to bind to members of the heat shock protein 90 (Hsp90) family. Interest in these compounds greatly increased when Neckers and coworkers (35) showed that Hsp90, an abundant protein chaperone in mammalian cells (23), is the primary cellular target of benzoquinone ansamycins and the basis of their antitumor properties. These findings led to a new therapeutic concept for anticancer research because Hsp90 plays a central role in various signal transduction pathways involved in tumorigenesis (23). Because of their potential to affect multiple targets simultaneously, benzoquinoid ansamycins are believed to be superior to agents, such as Herceptin and Gleevec, whose therapeutic value is limited to certain tumor types and can be compromised by the development of drug resistance.

Because of its nanomolar potency and apparent specificity for tumor cells, geldanamycin was the first small-molecule inhibitor of Hsp90 to undergo development as an anticancer drug. However, severe hepatotoxicity (30) led to its withdrawal from clinical trials in 1995. Nonetheless, during the 1990s, considerable interest in this class of anticancer compound led to the development of geldanamycin analogs with improved pharmacological profiles. 17-Allylamino-17-demethoxygeldanamycin and 17-(2'-dimethylamino)ethylamino-17-demethoxygeldanamycin are currently undergoing clinical trials (12, 27). Some pharmacological disadvantages of these compounds have yet to be overcome, and therefore more analogs of this class of compounds may be required if drug development is to succeed.

Both herbimycin and geldanamycin are produced by *S. hygroscopicus* strains and are assembled by modular polyketide synthases, large polypeptides composed of sets of active sites called modules. Each module contains the catalytic domains required for a single round of polyketide chain elongation by substrate condensation and various reductions or dehydrations; sequential action of the set of modules as a protein “assembly line” produces the nascent polyketide (9, 28). In most cases, dedicated post-PKS enzymes introduce additional modifications of the polyketide, such as glycosylation and oxidation, with important effects on biological activity.

As is typical of all ansamycins, the carbon skeleton of geldanamycin is assembled from the 3-amino-5-hydroxybenzoic acid (AHBA) starter unit (2, 15) by successive condensation of two-carbon building blocks in seven chain-elongation steps that use one malonyl, four methylmalonyl, and two methoxymalonyl extender units (Fig. 1). We have proposed (21) that C-21 oxidation, C-17 oxidation/O-methylation, C-7 carbamoylation, and C-4,5 dehydrogenation occur after poly-

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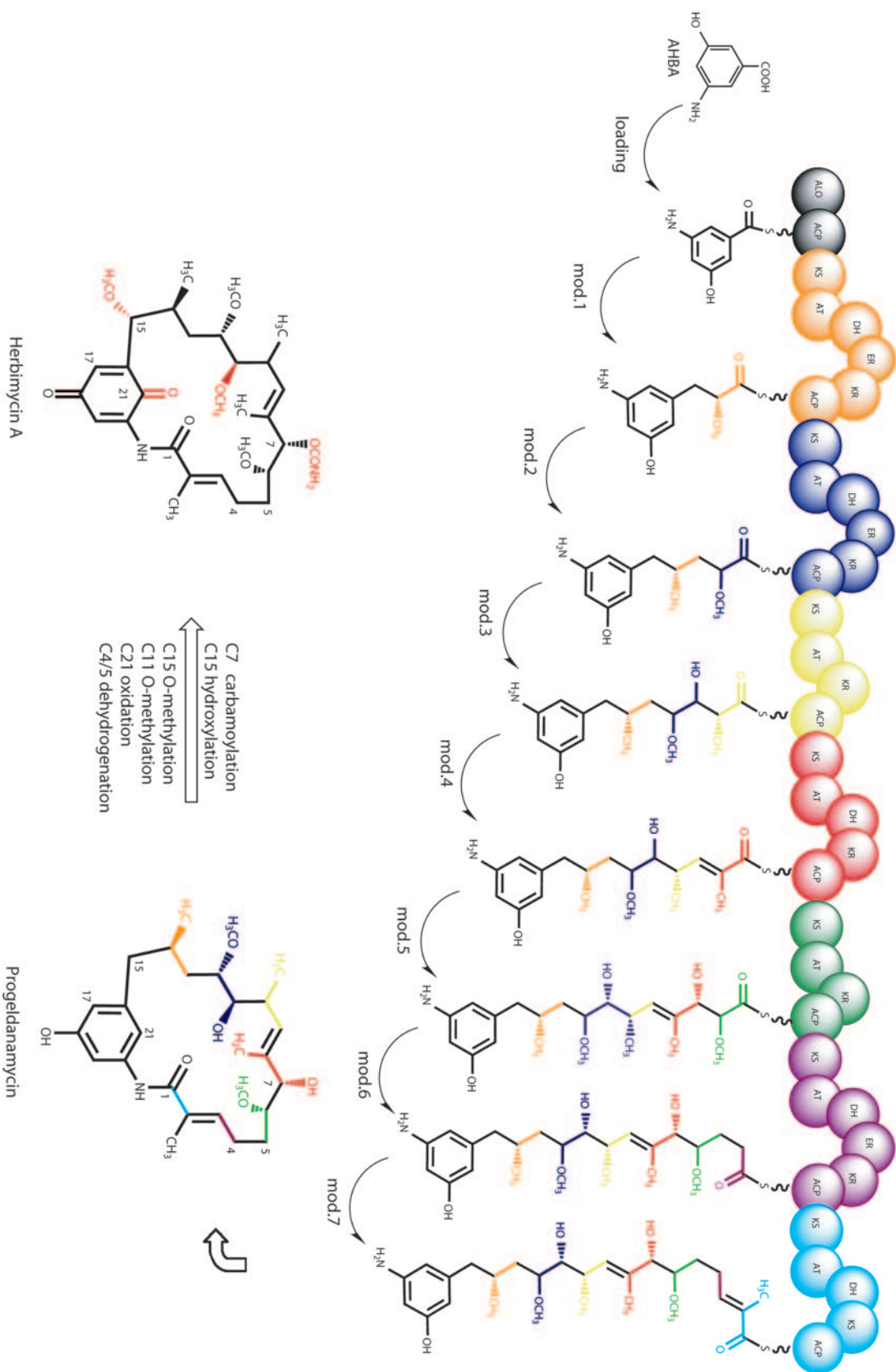


FIG. 1. Proposed pathway for herbimycin biosynthesis, starting from the ansamycin starter unit AHBA, followed by PKS processing through seven chain extension units (modules [mod.1 to 7]) to give the hypothetical intermediate progeldanamycin, followed by formation of herbimycin A by PKS tailoring enzymes.

TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Properties or product	Source or reference
Strains		
<i>S. hygroscopicus</i> NRRL 3602	Wild type	NRRL ^a
<i>S. hygroscopicus</i> AM 3672	Wild type	S. Omura, Kitasato Institute (Japan)
<i>S. lividans</i> TK24	SLP1 ⁻ SLP2 ⁻ ; <i>str</i>	11
<i>S. hygroscopicus</i> K390-76-1	<i>ahba-B::neo</i>	This study
<i>S. hygroscopicus</i> K390-76-2	<i>ahba-N::neo</i>	This study
<i>S. hygroscopicus</i> K313-115-1	<i>gdmA1::neo</i>	This study
<i>S. hygroscopicus</i> K390-48-1	<i>hbmA1::neo</i>	This study
<i>S. hygroscopicus</i> K390-13-1	<i>gdmM::neo</i>	This study
<i>S. hygroscopicus</i> K390-33	<i>gdmL::neo</i>	This study
Plasmid and phage vectors		
pHP45	<i>aac(3)IV</i>	5
KC1139	Bifunctional <i>oriT</i> RK2 vector	4
pFDneoS	pUC19 containing <i>aphII</i> gene	8
KC515	c ⁺ $\Delta att-int::tsr::vph$	14
pKOS305-117A	c ⁺ $\Delta att-int::apr::vph$	KC515
pKOS256-166-2	LDD PCR fragment of <i>hbm</i>	This study
pKOS279-78-17	<i>hbm</i> PKS	This study
pKOS279-78-04	<i>hbm</i> PKS	This study
pKOS205-110-12	<i>hbm</i> PKS	This study
pKOS206-116-10	<i>ahba-B</i> cluster	This study

^a NRRL, Northern Regional Research Laboratory of the Agricultural Service.

ketide chain assembly. Because the polyketide backbone of herbimycin is identical to that of geldanamycin, PKS activity is expected to be very similar between the two pathways, with the structural differences in the products being introduced post-PKS.

As geldanamycin does not show herbicidal effects such as those caused by herbimycin (13), we expect that these properties are acquired through methoxy substitution at C-11 and

C-15. This raises the possibility that similar alterations of the geldanamycin moiety could result in derivatives with different bioactivity. To enable assessment of such structural alterations by genetic engineering, the corresponding herbimycin biosynthetic genes were needed.

We describe here the cloning and characterization of a major portion of the herbimycin (*hbm*) biosynthetic gene cluster containing the PKS genes and some other genes involved in

TABLE 2. Design of gene disruption cassettes and oligonucleotides used

Target	PCR primer (3'-5')		Cassette design
	Name	Sequence	
<i>gdmA1</i>	LFfw	CGGGATCCGACGACCTGGGCCCTGGACGAAAT	BamHI-LF-XhoI- <i>neo</i> -XbaI-RF-NsiI
	LFbw	CCGCTGGAGGTGGTGGCCACCACGAACGTCGT	
	RFfw	GCTCTAGAAGTGATACGGCAGGCGCTGGCGAA	
	RFbw	TCGATGCATACTGCACGGTTTGGCGGAGGTGG	
<i>hbmA1</i>	LFfw	CGCGGATCCTGTCTGTCTGGGCTACGGACACCAC	BamHI-LF-XhoI- <i>neo</i> -XbaI-RF-NsiI
	LFbw	CCGCTCGAG AGTCCGTAGCATGCCGCCACACCT	
	RFfw	TGCTCTAGATTCCGGCTGGACTGATTCCTCCGTC	
	RFbw	TGCATGCATCTTACCCGACAGCATCCACGGCAC	
AHBA-B	LFfw	CGCGGATCCAGACCTCGACCACCGGTGTCTGGA	BamHI-LF-XhoI- <i>neo</i> -XbaI-RF-NsiI
	LFbw	CCGCTCGAGCACGATTTCCAGCGCATGGCCCA	
	RFfw	TGCTCTAGACTCACCCGCTCGCCTTCGTCAT	
	RFbw	TGCATGCATTGAGCCACCACGGCGTGTGACA	
AHBA-N	LFfw	CGCGGATCCTGCCAGTGGTGCATCTGCCACCA	BamHI-LF-XhoI- <i>neo</i> -XbaI-RF-NsiI
	LFbw	CCGCTCGAGTCAACCCGATGGGGTTGGCGAGTA	
	RFfw	TGCTCTAGATTGCCAGGCAGGGGCCGAATTGCT	
	RFbw	TGCATGCATCAGCGGAGCTACAGAAGCGGTAC	
<i>gdmM</i>	LFfw	CGGGATCCGACGACCTGGGCCCTGGACGAAAT	BamHI-LF-XhoI- <i>neo</i> -XbaI-RF-NsiI
	LFbw	ACGACGTTCTGGTGGCCACCACCTCGAGCGG	
	RFfw	GCTCTAGA AGTGATACGGCAGGCGCTGGCGAA	
	RFbw	CAACCTCCGCC AAACCGTGCAGTATGCATGCA	
<i>gdmL</i>	LFfw	CCCAAGCTTTCCGTCACTCTGCCGACGCTCTGA	HindIII-LF-XbaI- <i>neo</i> -KpnI-RF-StuI
	LFbw	GCTCTAGAGGTCTGTCTCTCCGTCAACACCGT	
	RFfw	GGGGTACCAGGGTGCCTGAGCCAGGTGACAA	
	RFbw	TAGGCTCGAACGCGATGAAGCCCTTCGGCA	
LDD	LDfw	GAYGASCCSGCSTGGATGYTSTA	
	LDbw	CCRTCSGTSCKGTACCASCRTC	

post-PKS modifications, and we provide information about the AHBA biosynthesis genes that are essential for the formation of geldanamycin, as well as herbimycin.

MATERIALS AND METHODS

Media and growth conditions, bacterial strains, plasmids, and phages. DNA manipulations were performed in *Escherichia coli* XL1-Blue (Stratagene) by standard procedures (25). For routine subcloning we used pLitmus 28 (New England Biolabs) and the pBluescript II plasmid vector series (Stratagene). The *Streptomyces* strains, phage, and plasmid vectors used in the present study are listed in Table 1. Lysogens were selected on solid R5 medium (14) by using a 2-ml overlay of 1 mg ml⁻¹ neomycin (Sigma). *Streptomyces lividans* TK24, used as host strain for propagation of the ϕ C31-phage derived vectors KC515 and pKOS-117A, was grown at 30°C on SFM solid medium (14) for sporulation and in liquid yeast extract-malt extract for preparation of protoplasts (14). Phage propagation was performed as described by Kieser et al. (14).

YPD seed medium was prepared according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Fermentation medium contained (per liter) 2.5 g of peptone (Difco), 2.5 g of tryptone (Difco), 2.5 g of yeast extract (Difco), 5 g of baby oatmeal (Gerber), and 10 g of beet molasses (Minn-dak). The medium was sterilized for 30 min at 121°C. For shake-flask fermentation, the geldanamycin- and herbimycin-producing strains, as well as their mutant derivatives, were grown under the conditions described previously (21). For fermentation of *gdmM* knockout mutant *S. hygroscopicus* K309-27-1 to produce compound KOS-1806, spores were seeded into 50 ml of YPD medium in a 250-ml flask. The culture was grown for 24 h at 30°C. An aliquot of 25 ml was transferred into 500 ml of YPD medium in a 2.8-liter Fernbach flask, and the culture was incubated at 30°C for 1 day and then used to inoculate a 20-liter bioreactor with a 12-liter working volume (New Brunswick Scientific BioFlo IV) at 4% (vol/vol) to produce KOS-1806. The temperature was maintained at 30°C. The pH was initially controlled at 6.5 with 2.5 N sulfuric acid or 2.5 N sodium hydroxide. Two days after inoculation, the pH was reduced to 6.0 for the rest of the process. Dissolved oxygen was controlled above 30% with agitation (200 to 400 rpm). The overall pressure in the bioreactors was manually set at 6 lb/in², and airflow was controlled at 1 volume/volume per min (vvm). Foam was controlled by automatic addition of 50% (vol/vol) Antifoam B (JT Baker). The fermentations were harvested 4 days after inoculation.

Isolation and in vitro manipulation of DNA. Manipulation and transformation of DNA in *E. coli* was performed according to standard procedures (25) and by using a QIAprep spin miniprep kit (QIAGEN) according to the supplier's protocols. Phage DNA was isolated by using a combination of the QIAGEN lambda minikit (QIAGEN) for polyethylene glycol-mediated DNA precipitation in combination with the ethanol precipitation method described by Kieser et al. (14). Restriction endonuclease digestions and ligations were performed by standard techniques (25). DNA fragments for labeling and subcloning were isolated with the QIAGEN gel extraction kit. The conditions for phage DNA transfection were as described by Kieser et al. (14). Genomic DNA preparation from *Streptomyces* for PCR and Southern blot hybridization was performed by using standard procedures (14).

For DNA amplification by PCR a standard protocol with initial sample denaturation at 94°C for 5 min was used. One amplification cycle consisted of sample denaturation (40 s), a primer variable melting temperature (T_m), and polymerization at 72°C. To estimate suitable polymerization intervals, we calculated 1 min/kb for *Taq* polymerase (Roche) and 1.5 min/kb for *Pfu* Ultra (Stratagene). The PCR mix was used as recommended by the manufacturer of the DNA polymerase, and amplification was performed with a thermocycler PCT-200 (MJ Research). For degenerate PCR, the optimal T_m was determined by T_m variation with a temperature gradient of 50 to 70°C.

For Southern blot hybridization analysis, *Streptomyces* chromosomal DNA was digested with suitable restriction enzymes overnight and electrophoresed in a 0.6% agarose gel for 8 h in TAE buffer (25). Gels were blotted on nitrocellulose membranes by using the neutral transfer method of the rapid transfer system (Schleicher & Schuell) as recommended by the manufacturer. For radioactive [³²P]DNA probe labeling, we used [³²P]dCTP (Amersham) with the random-primed labeling kit (Roche) to label 40 ng of probe DNA according to the manufacturer's instructions. Labeled probes were purified with the ProbeQuant G-50 spin column (Amersham). Hybridizations were performed by using the formamide hybridization protocol (25) under high-stringency conditions. For nonradioactive, colorimetric DIG-labeling we used the DIG Easy Hyb protocol (Roche) as recommended by the manufacturer.

TABLE 3. NMR assignments for KOS-1806^a

Carbon no.	δ C	δ H	COSY	HMBC
25	12.6	1.33, 3H, s	H-9	C-7,8,9
22	14.3	1.69, 3H, s	H-3	C-1,2,3
26	17.9	0.89, 3H, d, J = 6 Hz	H-10	C-9,10,11,23
28	18.7	0.69, 3H, d, J = 4.8 Hz	H-14	C-13,14,15
4	19.2	2.02, 1H, m	H-4,5	
		2.13, 1H, m	H-3,4,5	
5	30.5	1.09, 1H, m	H-5,6	
		1.21, 1H, m		
14	31.2	1.78, 1H, m	H-13,28	
13	34.4	1.05, 1H, m	H-13,14	
		1.49, 1H, m	H-13,15	
10	35.1	2.30, 1H, m	H-9	
15	43.6	2.27, 1H, dd	H-15	C-16,17
		2.53, 1H, dd	H-14,15	C-16,17,21
27	57.2	3.19, 3H, s	H-7	C-12
23	59.3	3.29, 3H, s		C-6
11	73.9	3.39, 1H,	H-10,11-OH	C-27
6	80.1	3.19, 1H,	H-5	C-7,23
12	81.4	2.95, 1H, m	H-11,13,27	C-14
7	81.8	4.81, 1H, d, J = 10	H-6	C-5,6,8,9,24,25
19	106.8	6.56, 1H, bs		C-17
17	113.8	6.23, 1H, s		C-15,18,19,21
21	117.2	6.16, 1H, s		C-17,19
8	130.8			
2	132.6			
9	133.9	5.19, 1H, d, J = 8.8	H-10,25	C-7,25
3	134.9	5.65, 1H, s	H-4,22	
20	141.0			
16	141.9			
24	157.1			
18	158.3			
1	172.1			
11-OH		4.34, 1H, s	H-11	
1-NH		9.30, 1H, bs		C-2,17,19

^a COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation.

DNA sequencing and analysis. BAC and cosmid DNA was sequenced by using standard shotgun procedures (16). DNA sequencing was performed on an ABI 3730 capillary sequencer by using reagents and protocols provided by the manufacturer. DNA and corresponding protein sequences were analyzed with SEQUENCHER 4.2 (Gene Codes) and MAC VECTOR 7.1 (Accelrys) and compared to sequences in the public databases by using CLUSTAL W (32) and BLAST (1).

Isolation of the herbimycin PKS gene cluster. A cosmid library was prepared by using a pSET152-based vector, pKOS97-64C (Z. Hu, unpublished data), with *S. hygroscopicus* AM 3672 total DNA partially digested with Sau3A that was ligated and introduced into *E. coli* by using the Gigapack III XL (Stratagene) in vitro packaging kit. DIG-labeled probes encompassing the PKS loading didomain and the carbamoyl transferase gene were used jointly in an approach similar to that described for detection of the geldanamycin PKS genes (21), using degenerate PCR to generate 100% matching probes from the genome. We amplified a 650-bp fragment for the carbamoyl transferase gene and a 690-bp fragment for the PKS loading didomain by using the primers listed in Table 2. The DNA fragments were subcloned with the pCR2.1-TOPO TA cloning kit (Invitrogen) to give pKOS256-166-2, the corresponding insert was confirmed by sequence analysis. The library was screened with this probe DNA by colony hybridization. Colonies that hybridized were further analyzed by DNA end-sequencing and restriction enzyme analysis, which revealed three overlapping clones, pKOS279-78-17, pKOS279-78-04 and pKOS205-110-12. These clones together cover a 115-kb region, including the herbimycin PKS genes.

Gene disruptions. All gene disruptions were performed with a similar design with phages KC515 and pKOS305-117A (M. Piagentini, unpublished work) or plasmid pKC1139 (4) to deliver the corresponding neomycin resistance gene disruption cassettes. These cassettes consisted of two PCR-derived flanking regions (1 to 1.5 kb), where suitable restriction sites were introduced. The resulting DNA fragments were cloned, together with the neomycin resistance gene from pFDneoS (8). Detailed cloning information for each construct is summarized in Table 2.

For inactivation of the *gdmA1* PKS and *gdmM* genes, the corresponding

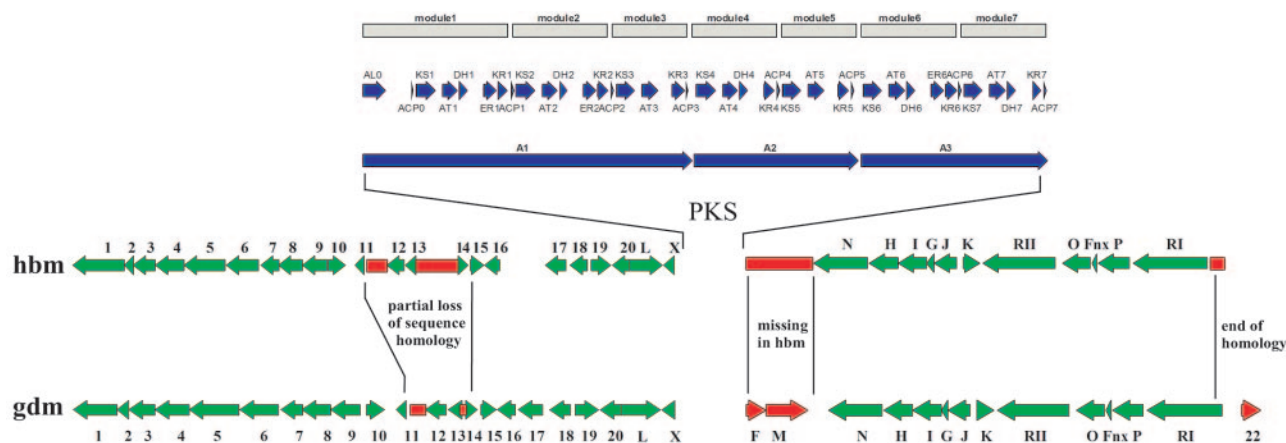


FIG. 2. Comparison of a 90-kb section of the herbimycin biosynthetic gene cluster with the genes responsible for geldanamycin production. PKS genes (*A1*, *A2*, and *A3*) and the PKS domains for both the *hbm* and *gdm* cluster are given in blue, with the modular organization indicated above each PKS gene by broad arrows. PKS tailoring genes are in green, and differences in gene organization or homology are indicated in red. “No homology” or “end of homology” indicates that these DNA regions are present but have <20% similarity; “missing in *hbm*” indicates that this region of DNA is absent in the herbimycin-producing gene cluster, whereas the comparable region contains the *gdmF* and *gdmM* genes in the geldanamycin producer.

neomycin cassettes were cloned into phage KC515 at the BamHI-PstI sites (the thio streptomycin resistance marker in KC515 [14] could not be used because both *Streptomyces* strains are resistant to this antibiotic). The resulting disruption mutants were selected on neomycin and confirmed by Southern blot hybridization. The cassettes for the disruption of *hbmA1* and the *ahba-B* and *ahba-N* clusters were cloned into phage KOS305-117A carrying the apramycin resistance gene *aac(3)IV* from pHP45 (5) at the BamHI-BglII sites to replace the thio streptomycin resistance gene. This phage construct was prepared to circumvent the thio streptomycin resistance of *S. hygroscopicus* NRRL 3602 and the partial neomycin resistance we discovered for the herbimycin producer.

***S. hygroscopicus* AM 3672.** After transfection, the lysogens were grown under neomycin selection until sporulation occurred and screened for immediate double-crossover events, indicated either by loss of the second marker or by Southern analysis. Because of inappropriate restriction sites for *gdmL*, we used the temperature-sensitive plasmid pKC1139 (4) as a delivery system instead of phage KC515, using the same methodology as previously described for disruption of the *gdmH* gene (21). Genotypes of the disruption mutants were confirmed by Southern analysis with the neomycin resistance gene as the DNA probe. Phenotypic characterization was performed by metabolite production analysis.

General analytical procedures. ^1H nuclear magnetic resonance (^1H NMR) (400 MHz) and ^{13}C NMR (100 MHz) were recorded in CDCl_3 solution at 300 K with a Bruker DRX 400 spectrometer. Chemical shifts were referenced to δ 7.26 and 77.0 for ^1H and ^{13}C spectra, respectively. To determine the structure of KOS-1806, ^1H , ^{13}C , COSY, ctHMBC, and multiplicity-edited HSQC experiments were carried out. High-resolution electrospray ionization mass spectra were obtained by manual peak matching versus internal standards with an Applied Biosystems Mariner TOF spectrometer configured with a Turbo-Ionspray source in positive ion mode.

Detection, purification, and analysis of the novel geldanamycin analog KOS-1806. After fermentation of the *gdmM*-null mutant strain, the culture broth was extracted with an equal volume of methanol and the supernatant was submitted for liquid chromatography-mass spectrometry analysis. The mass spectral fragmentation pattern indicated that the new peak (m/z 541) was a sodium adduct of a geldanamycin compound. For purification, the methanol was evaporated, the residue was extracted with ethyl acetate, and the extract was concentrated to solid material. The crude, oily product was dissolved in 21 ml of methanol and diluted to 60% (vol/vol) with water. The solution was loaded onto a C_{18} column (3 by 11 cm, 80 ml). The column was eluted with 70% (vol/vol) MeOH- H_2O . Fractions (80 ml) were collected and analyzed by high-pressure liquid chromatography with 55% (vol/vol) MeOH- H_2O as the mobile phase and an Inertsil column (25 by 4.6 cm). The fractions containing the target molecule were pooled and diluted to 25% (vol/vol) MeOH- H_2O (350 ml). The solution was loaded onto a C_{18} column (3 by 11 cm, 80 ml) and eluted with 43% (vol/vol) MeOH- H_2O . Fractions (40 ml) were collected and analyzed by high-pressure liquid chromatography. Fractions 15 to 22 containing the compound of interest were pooled

and concentrated in vacuum to yield KOS-1806 (59 mg) as a white solid. High-resolution liquid chromatography-mass spectrometry data pointed to the sodium adduct of the previously detected geldanamycin analog. Electrospray ionization TOF mass spectrometry indicated an m/z of 541.2896, compared to a calculated value for $\text{C}_{28}\text{H}_{42}\text{N}_2\text{O}_7\text{Na}$ ($[\text{M} + \text{Na}]^+$) of 541.2884. This assignment was confirmed by NMR. The structure of KOS-1806 was elucidated by interpretation of the two-dimensional NMR spectra and comparison of the proton and ^{13}C NMR data with those of known geldanamycins. All spectral data of KOS-1806 were thus consistent with the structure shown in Fig. 3. ^1H and ^{13}C NMR data are shown in Table 3.

RESULTS

Nucleotide sequence of the *hbm* gene cluster. A cosmid library prepared from genomic DNA of *S. hygroscopicus* AM 3672 was screened by DNA hybridization by using labeled fragments of the *hbm* PKS loading domain gene segment and the *hbmN* carbamoyl transferase gene obtained by degenerate PCR on genomic DNA of *S. hygroscopicus* AM 3672. Three overlapping cosmids containing the *hbm* PKS gene cluster were isolated covering a 115-kb DNA segment. Sequencing revealed that the cloned DNA segment presumed to be involved in herbimycin biosynthesis contains 35 open reading frames (ORFs) with three large ORFs, *A1*, *A2* and *A3*, coding for the PKS (Fig. 2; Table 4). The sequence data have been submitted to the DDBJ, EMBL, and GenBank databases under accession number AY947889.

The *hbmA1-A3* ORFs encode the loading didomain and seven chain-extension modules of a multifunctional modular PKS that is essentially identical to the one described for geldanamycin (21). Each of the three predicted polypeptides is 95% similar to its *gdm* counterpart, supporting the expectation that the *hbm* polyketide product is identical to the hypothetical intermediate progeldanamycin and that differences between herbimycin and geldanamycin are introduced by post-PKS processing.

To probe the functions of the *hbm* and *gdm* PKS genes, each set was inactivated in its respective producer strain, which involved deletion of major parts of the *A1* ORF via insertion of

TABLE 4. Comparison of geldanamycin and herbimycin ORFs^a

Gdm ORF		Proposed function	Hbm ORF	
Boundaries	Name		Name	Boundaries
1–1652 ^b	ORF01	Putative cation-transporting ATPase; homolog of <i>S. coelicolor</i> SC0860c and <i>S. avermitillis</i> SAV617	ORF01	71–2359
1652–2083	ORF02	Putative secreted protein; homolog of <i>S. coelicolor</i> SC0861c and <i>S. avermitillis</i> SAV618	ORF02	2359–2775
2070–3053	ORF03	Homolog of PvcA (<i>P. aeruginosa</i> PA2234) and of <i>V. cholerae</i> VC1949	ORF03	2762–3745
3057–4313	ORF04	P450	ORF04	3757–5013
4326–6152	ORF05	Asparagine synthase family	ORF05	5026–6852
6187–7617	ORF06	Transmembrane efflux protein	ORF06	6887–8317
7723–8526	ORF07	Permease (Fe); homolog of FtrE, <i>S. coelicolor</i> SC0998	ORF07	8437–9240
8490–9572	ORF08	Homolog of FtrD, <i>S. coelicolor</i> SC0997	ORF08	9204–10286
9572–10648	ORF09	Lipoprotein	ORF09	10286–11362
10649–15731	ORF10–15	Functions unclear	ORF10–15	11363–18359
15732–16415	ORF16	RhtB family transporter	ORF16	18360–19043
16502–17404	ORF17	Secreted protein	ORF17	21063–21965
17676–18467	ORF18	Hydrolase	ORF18	22155–22946
18621–19505	ORF19	Transcriptional regulator (AraC family)	ORF19	23100–23984
19555–20316	ORF20	Transcriptional regulator (TetR family)	ORF20	24036–24797
20357–21796	GdmL	Flavin-dependent monooxygenase	HbmL	24781–26277
21838–22308	GdmX	Conserved JadX and MmyY homolog	HbmX	26325–26795
22939–43464	GdmAI	PKS modules 0 to 3	HbmAI	27677–48139
	Loading	AL0 X ACP0	Loading	
	Module 1	KS AT DH ER KR ACP	Module 1	
	Module 2	KS AT DH ER KR ACP	Module 2	
	Module 3	KS AT KR ACP	Module 3	
43525–53829	GdmAII	PKS modules 4 and 5	HbmAII	48197–58492
	Module 4	KS AT DH KR ACP	Module 4	
	Module 5	KS AT KR ACP	Module 5	
53859–65546	GdmAIII	PKS modules 6 and 7	HbmAIII	
	Module 6	KS AT DH ER KR ACP	Module 6	58519–70125
	Module 7	KS AT DH KR ACP	Module 7	
6558–66331	GdmF	Amide synthase	none	
66328–67962	GdmM	Flavin-dependent monooxygenase	none	
68782–70791	GdmN	Carbamoyltransferase	HbmN	70662–72719
70853–71965	GdmH	Methoxymalonyl-ACP biosynthesis pathway	HbmH	72781–73893
71962–73074	GdmI	Methoxymalonyl-ACP biosynthesis pathway	HbmI	73890–75002
73071–73346	GdmJ	ACP in methoxymalonyl-ACP biosynthesis pathway	HbmJ	74999–75274
73343–74209	GdmK	Methoxymalonyl-ACP biosynthesis pathway	HbmK	75271–76137
74453–75019	GdmG	<i>O</i> -Methyltransferase in methoxymalonyl-ACP biosynthesis	HbmG	76381–77037
75234–78014	GdmRII	LuxR-type transcriptional regulator	HbmRII	77137–79917
78289–79353	GdmO	Amino DHQ synthase	HbmO	80193–81257
	GdmFdx	Ferredoxin	HbmFdx	81334–81528
79671–80864	GdmP	P450	HbmP	81571–82764
81021–83909	GdmRI	LuxR-type transcriptional regulator	HbmRI	82921–86764
84662–85375	ORF22	Hydrolase	None	

^a Gdm, geldanamycin; Hbm, herbimycin.

^b N terminus only.

a neomycin resistance gene. Delivery of the corresponding disruption cassettes was performed by homologous recombination using in the *Streptomyces* phages KC515 (*gdmA1*) and pKOS305-117A (*hbmA1*), respectively. The corresponding null mutants (*gdmA1*, K313-115-1) and (*hbmA1*, K390-48-1) were unable to produce herbimycin or geldanamycin when grown under standard fermentation conditions. Their genotypes were confirmed by Southern analysis with the neomycin resistance gene as probe. Detailed experimental procedures are described in the supplemental material.

Comparison of *gdm* and *hbm* tailoring genes. As in the *gdm* biosynthetic gene cluster, the *hbm* PKS genes are flanked by sets of presumed biosynthetic genes (Fig. 2), some of which are

believed to contribute to post-PKS modification by converting the hypothetical PKS intermediate, progeldanamycin (Fig. 1), into herbimycins A (Fig. 1), B, and C. The herbimycins and geldanamycin differ in the substitution pattern of their progeldanamycin backbone in three ways. First, the benzoquinoid system of all herbimycins lacks the methoxy group at the C-17 position that is present in geldanamycin. Second, an additional methoxy group is introduced at polyketide backbone position 15 in herbimycins A and C, where no such substitution is present in herbimycin B or has been reported for any geldanamycin analog. Third, the C-11 hydroxyl group of herbimycin A is *O* methylated, whereas herbimycin B and C remain hydroxylated at this position, like geldanamycin.

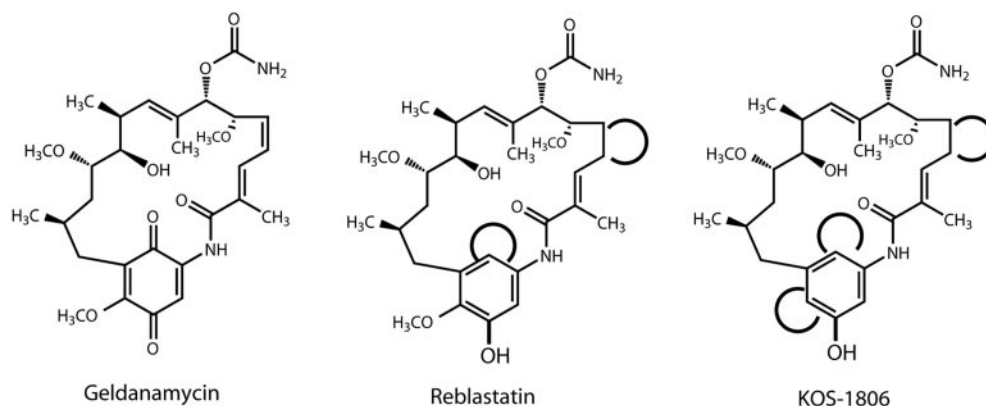


FIG. 3. Structures of geldanamycin and monophenolic analogs. Distinctions from the geldanamycin structure are highlighted, and missing functions are indicated by open circles.

Comparison of a continuous 90-kb segment including the *hbm* PKS and tailoring genes with the corresponding segment of the *gdm* biosynthetic genes is shown in (Fig. 2), which is based on BLAST (1) analysis. The organization of genes flanking the *hbm* PKS is largely consistent with that observed for the *gdm* gene cluster. Pairwise comparisons of the corresponding ORFs revealed sequence similarities of 90 to 97%. However, there are two major differences, as highlighted in red. The first is a 4-kb segment upstream of the PKS genes between ORFs 11 and 14 where the homology between the clusters is partially lost. We could not deduce functions for ORFs 11 to 13 by sequence comparison with the databases. Only a part of ORF14 found in the *hbm* cluster is present in the *gdm* cluster. More interestingly, downstream of the PKS genes, the *hbm* cluster lacks homologs of *gdmF* and *gdmM*, and BLAST analysis of all ORFs identified in the 115-kb sequenced region failed to reveal a *gdmF* or *gdmM* homolog. Their absence was confirmed by a PCR analysis of total DNA from the herbimycin producer with nondegenerate primers to each end of the DNA flanking *gdmF* and *gdmM* in the region of the *hbm* gene cluster corresponding to the *gdm* genes (data not shown). These two genes are thought to be involved in post-PKS modifications of progeldanamycin. The lack of a *gdmF* homolog, an amide synthase believed to be responsible for cyclization of all proansamycin PKS products, raises the intriguing question of how cyclization of the nascent PKS product is achieved for herbimycin and whether a *gdmF* paralog is responsible. Furthermore, it should be noted that there are two structural features in both geldanamycin and herbimycin that are not consistent with the analyzed sequences. One is the formation

of the C4/5 *cis* double bond, and another is the apparent absence of *O*-methyltransferases, which are required for formation of the methoxy residues at C-17 for geldanamycin and C-15 for herbimycin, respectively.

Inactivation of *gdmM* and *gdmL*. We previously proposed that two oxidative post-PKS modifications, at positions C-17 and C-21, take place on the aromatic ring of geldanamycin (21). This hypothesis is supported by the discovery of reblastatin (Fig. 3), a nonbenzoquinoid geldanamycin analog with antitumor activity (29), isolated from a geldanamycin-producing strain (31). Reblastatin lacks the 21-hydroxyl (as well as the 4,5 double bond) and is therefore likely to be a shunt product of the normal biosynthetic pathway, resulting from the lack of aromatic ring oxidation and desaturation of the 4,5 position. In the *gdm* cluster we found two ORFs (ORF4 and *gdmP*) encoding proteins with sequence homology to CYP450 monooxygenases and two other ORFs flanking the PKS that also are candidates for the oxidations: *gdmM*, a homolog of *rif19* (3), and *gdmL*. The deduced products of the latter two genes resemble FAD-dependent monooxygenases (21). Because the *hbm* cluster lacks a *gdmM* homolog in the sequenced segment and the C-17 position of herbimycin is not oxidized, we hypothesized that the monooxygenases encoded by *gdmM* and *gdmL* would play an important role in oxidation of the aromatic ring in progeldanamycin at positions C-17 or C-21 or both.

We were interested in engineering nonbenzoquinoid, reblastatin-like geldanamycin analogs to assess their effects on the pharmacological profile of this drug. Therefore, we attempted to inactivate the *gdmM* and *gdmL* genes to produce such analogs. Each gene was disrupted by insertion of a neomycin resistance gene and simultaneously deleting major parts of the corresponding ORFs. Interestingly, *gdmL* inactivation (mutant K390-33) had no discernible effect on the metabolite profile, even though its presence in the *gdm* and *hbm* clusters suggests that it could be involved in their biosynthesis. Disruption of *gdmM* (mutant K390-13-1), on the other hand, resulted in formation of a novel nonbenzoquinoid geldanamycin analog (KOS-1806) with a monophenolic structure (Fig. 3). As with reblastatin, post-PKS processing is interrupted and fails to form the C-4,5 *cis* double bond as well as the benzoquinone.

TABLE 5. *ahba-B* gene designations and functions

ORF	Predicted function	Gene (reference)	Identity (%)	Size (aa) ^a
<i>ahba-1a</i>	Oxidoreductase (DAHP synthesis)	<i>ansG</i> (6)	64	360
<i>ahba-1b</i>	Phosphatase (DAHP synthesis)	<i>ansH</i> (6)	73	231
<i>ahba-1c</i>	Kinase (DAHP synthesis)	<i>asm22</i> (36)	63	265
<i>gdmO</i>	Aminodehydroquinone synthase	<i>asm47</i> (36)	76	354
<i>ahba-3</i>	Aminodehydroquinone synthase	<i>mitP</i> (17)	74	349
<i>ahba-4</i>	Aminodehydroquinone dehydratase	<i>asm23</i> (36)	75	149
<i>ahba-B</i>	AHBA-synthase	<i>ansF</i> (6)	79	388

^a aa, amino acids.

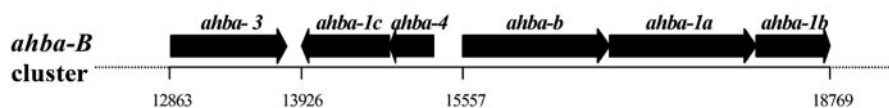


FIG. 4. *ahba-B* biosynthetic gene cluster in *S. hygroscopicus* AM 3602.

However, like herbimycin, KOS-1806 remains unsubstituted at C-17. This result establishes that *gdmM* governs at least one of the post-PKS oxidation steps and also shows that *gdmL* is dispensable or that loss of its function is compensated for by a paralogous gene that is located outside of the sequenced *gdm* segment.

Inactivation of the genes required for AHBA formation. We previously reported that neither the *hbm* nor the *gdm* gene cluster contains all of the genes required for formation of the ansamycin starter unit, AHBA (Table 5). A similar situation has been reported for the biosynthesis of ansamitocin (36), where the AHBA biosynthetic genes were identified in two subclusters separated by more than 30 kb. This contrasts with the situation for rifamycin where a complete ABHA biosynthetic gene set is found as part of the biosynthetic gene cluster (3).

The geldanamycin producer has two different AHBA clusters (*ahba-B* and *ahba-N*) (21), whereas the herbimycin producer revealed only a *ahba-B* homolog (*ahba-H*) when its genomic DNA was analyzed by the same PCR method used for the NRRL 3062 DNA. Pairwise comparison of these deduced protein sequences to corresponding ones from other ansamycin gene clusters—ansamitocin (36), rifamycin (3), ansatrienin (6), and mitomycin C (17)—showed that the polypeptides encoded by the *ahba-B* and *ahba-H* loci are most similar to AHBA synthases involved in the biosynthesis of benzoquinone ansamycins and that the *ahba-N* proteins are most similar to those for the biosynthesis of naphthoquinone ansamycins. Because geldanamycin and herbimycin are benzoquinone ansamycins, we concluded that the product of *ahba-B* and not *ahba-N* was most likely to be involved in geldanamycin biosynthesis. Sequence analysis of a *ahba-B*-containing clone, pKOS256-116-10, which we detected in the initial screen for the *gdm* PKS genes (21), revealed six ORFs (Fig. 4) more than 30 kb apart from the *gdm* PKS cluster with sequence homology to other sets of AHBA biosynthetic genes reported for the previously mentioned ansamycins (Table 5). The sequence data have been submitted to the DDBJ, EMBL, and GenBank databases under accession number AY952143.

Interestingly, a paralog of *gdmO* encoding a dehydroquinase synthase and the only *ahba*-related gene found close to the *gdm* PKS was also detected within the *ahba-B* group. As for the ABHA biosynthetic genes in the ansamitocin producer (36), the *rifH* homolog (amino-DHAP-synthase) required for early-stage AHBA biosynthesis (37) is missing from this group. We also identified a similar set of genes for the *ahba-N* cluster by end sequencing of various BAC clones; however, this cluster was not fully sequenced.

Two gene disruptions were carried out to test the essential role of the *ahba-B* cluster in geldanamycin biosynthesis. With the data we obtained for the *ahba-B* genes and end-sequencing data for the *ahba-N* cluster, we could use phage KC515 to

delete the full set of either group of AHBA biosynthetic genes coincident with insertion of the neomycin resistance marker. The deletion mutants of the *ahba-B* (K390-76-1) genes consistently failed to produce geldanamycin, whereas deletion of the *ahba-N* group (K390-76-2) had no discernible effect on the production profile. Feeding of AHBA to a representative *ahba-B* disruption mutant (K390-76-1), fully restored geldanamycin production.

DISCUSSION

In this study we have shown that the structural similarities between herbimycin and geldanamycin are reflected in the organization of their corresponding gene clusters and hence in the predicted biosynthetic proteins. The strongest similarities were observed at the catalytic sites, with more variability seen around the interdomain linker regions. We propose that these two benzoquinone antibiotics are made from the same hypothetical polyketide intermediate, progeldanamycin, and that their structural differentiation therefore occurs through post-PKS biosynthetic steps.

We identified two separate sets of ABHA biosynthetic genes, *ahba-B* and *ahba-N*, in the geldanamycin producer, whereas only an *ahba-B* homolog (the *ahba-H* cluster) was found in the herbimycin producer but not completely characterized. Note that the AHBA genes are also found in two separate regions in the ansamitocin-producing organism (36). In the geldanamycin producer, we found that the *ahba-N* cluster is closely associated with a group of type I modular PKS genes (unpublished results), whereas the *ahba-B* cluster is not. Sequence analysis of the AHBA synthesis enzymes from both clusters was used to establish that the *ahba-B* set encodes proteins most similar to enzymes known to be involved in the biosynthesis of benzoquinone-type ansamycins, whereas the *ahba-N* set encodes enzymes that are more similar to those from producers of naphthoquinone-type ansamycins (data not shown). The *ahba-B* gene products are very similar to those reported for ansatrienin biosynthesis in *S. collinus* Tü 1892 (6). Inactivation of each ABHA biosynthetic gene set showed that only the *ahba-B* locus is required for formation of geldanamycin, unlike the case for ansamitocin, where both ABHA biosynthetic gene loci have genes that are essential for ansamitocin biosynthesis (36).

One feature of the biosynthesis of herbimycin and geldanamycin that cannot be explained by the gene sequence data is the formation of the C-4,5 *cis* double bond. PKS module 6, responsible for the corresponding chain-elongation step, contains an enoyl reductase (ER) domain and therefore should catalyze formation of a saturated C-4,5 carbon bond in progeldanamycin. Several observations support this idea. First, sequence comparison of ER6 with other functional ER domains in modular PKSs revealed no obvious active site muta-

tions, and therefore there is no compelling evidence that ER6 is inactive in either PKS cluster. Second, given the possibility of an inactive ER6, recent findings about the stereoselectivity of ketoreductase (KR) domains (22) suggest that the nature of the corresponding *hbm/gdm* KR6 would not support the formation of a *cis*-unsaturated system at this position. Sequence comparisons of various KR domains have revealed that a particular Asp residue is present in all KRs that catalyze the formation of β -hydroxy products with the D absolute configuration. A group of KRs that support the formation of L- β -hydroxy products lack this residue, which in combination with a functional dehydratase domain, would result in a "cis" unsaturated polyketide product (22). This particular Asp residue is present in the *gdm* and *hbm* KR6 domain, which therefore qualifies it for the formation of a *trans*, not a *cis*, α,β -unsaturated system. More importantly, there are at least four geldanamycin analogs where the C-4,5 position remains saturated. One is reblastatin; another is the product of *gdmM* inactivation, KOS-1806, a reblastatin analog with a saturated 4,5 position; and a third is the C-4,5 saturated shunt product produced by a *gdmA3* PKS acyltransferase 7 exchange mutant (20). Finally, Hong et al. (10) have recently reported that several post-PKS products with a saturated 4,5 double bond are present in a *gdmN*-null mutant. They show through bioconversion experiments that one of these compounds, 4,5-dihydro-7-descarbamoyl-7-hydroxygeldanamycin is converted to geldanamycin. A compound whose mass spectral data were consistent with the latter intermediate had previously been isolated from a *gdmH*-null mutant in which *gdmN* was partially inactivated (21). The accumulated data therefore support the belief that desaturation of the 4,5 position is the final step in geldanamycin biosynthesis (10).

Progeldanamycin therefore is very likely to have a C-4,5 saturated ansamycin system, which leads to the intriguing question of how the *cis* double bond is introduced. Catalysis by a fortuitous desaturase, utilizing a mechanism similar to that involved in the oxidative desaturation of fatty acids (34), is one possibility. However, this type of biochemical mechanism has not been reported for the biosynthesis of polyketide secondary metabolites to our knowledge. Oxidative processing by post-PKS enzymes that are unrelated to the typical fatty acid desaturases, such as the *gdmP* gene encoding a CYP450 enzyme, is a more plausible alternative.

Post-PKS processing steps distinguish the biosynthesis of herbimycin and geldanamycin. In contrast to the other ansamycin PKSs, the *hbm* PKS tailoring genes lack an amide synthase homolog in the region sequenced, raising the question of whether such a gene is located elsewhere in the genome or is not required for closure of the ansamycin ring system. If the putative *hbmF* gene is in fact absent, cyclization would have to be achieved through another mechanism. Overexpression of the *gdmF* gene in the herbimycin producer would be a way to address this question if that were to enhance the amount of herbimycin produced. Another intriguing observation is the apparent absence of *O*-methyltransferase genes (other than those required for methoxymalonate biosynthesis) in the *gdm* and *hbm* gene clusters, because formation of both of these benzoquinone antibiotics requires *O* methylation at C-17 for geldanamycin and at C-15 or C-11 for herbimycin.

We have not yet established the exact sequence of the post-

PKS oxidations necessary to form the benzoquinone system of geldanamycin or herbimycin. However, the results obtained to date by us and others (10) provide the following insights. Inactivation of *gdmH* or *gdmN* does not interfere with the required post-PKS oxidations at C-17 and C-21, but C-4,5 desaturation is affected (10, 21), probably because the resulting intermediates are poor substrates for the putative C-4,5-desaturase. Inactivation of *gdmM* results in failure to perform any of the post-PKS oxidation steps and also blocks formation of the C-4,5 *cis* double bond. Similarly, a *gdmA3* acyltransferase 7 swap mutant produces a compound that has not undergone the aromatic ring oxidations and C-4,5 desaturation. On the other hand, the work of Hong et al. (10) shows that C-21 oxidation to the benzoquinone and hydroxylation plus *O* methylation of C-17 can take place before 7-*O* carbamoylation and C-4,5 desaturation. On the basis of these observations, it seems that formation of the C-4,5 *cis*-unsaturated system is a late event in post-PKS processing that is preceded by C-21 oxidation and C-7 carbamoylation. The apparent absence of a *gdmM* homolog in the *hbm* gene cluster, together with the results from the corresponding gene disruption in the *gdm* gene cluster, suggest that *gdmM* is likely to be involved in C-17 oxidation. Furthermore, evidence from reblastatin, where C-17 is fully substituted but C-21 remains unoxidized, suggests that the C-17 and C-21 oxidations are two separate catalytic events. Yet neither C-21 oxidation nor substitution of C-17 are obligatory steps prior to 7-*O* carbamoylation. Because inactivation of *gdmL* did not affect the production of geldanamycin, other putative oxygenase genes in the *gdm* cluster, such as *gdmO4* and *gdmP*, are likely to be involved in formation of the benzoquinoid and 4,5 double bond found in geldanamycin and herbimycin.

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