Production of 8-Demethylgeldanamycin and 4,5-Epoxy-8-demethylgeldanamycin from a Recombinant Strain of *Streptomyces hygroscopicus*

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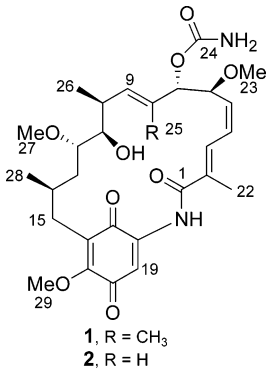
Two new geldanamycin derivatives produced by genetic engineering of *Streptomyces hygroscopicus* strain K309-27-1 were isolated and characterized. Removal of the 8-methyl group of geldanamycin was achieved by replacing the AT4 domain of the polyketide synthase with a malonyl AT domain. The resulting strain produced 8-demethyl geldanamycin (**2**) and 4,5-epoxy-8-demethylgeldanamycin (**3**). The structures of both molecules were elucidated through interpretation of 1D and 2D NMR data as well as comparison with authentic geldanamycin derivatives. Compounds **2** and **3** displayed moderate cytotoxicity against the human breast cancer cell line SK-BR-3.

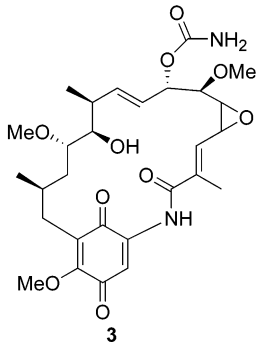
The geldanamycin family of benzoquinones has garnered attention in recent years because of their varied pharmacological properties, which include inhibition of platelet aggregation and RNA-dependent DNA polymerase (reverse transcriptase), antibacterial, antiviral, herbicidal, antiproliferative, anti-inflammatory, and antitumor activities.[1−5] Geldanamycin (**1**) is the most cytotoxic molecule of this class, with a mean IC_{50} of 13 nM when tested against the NCI cell line panel.[6] The antitumor properties of **1** are a result of its strong binding to the ATP binding domain of the chaperone heat shock protein 90 (HSP90), which leads to the inhibition of protein folding for many cancer-associated kinases and consequently cell cycle disruption.[7−9] Studies have also shown that treating cancer cells with geldanamycin (**1**) and its congeners enhances susceptibility to other treatments such as ionizing radiation.[10] A geldanamycin derivative (17-allylamino-17-demethoxygeldanamycin) is currently undergoing phase II clinical trials.[11]

Geldanamycin (**1**) is synthesized in *Streptomyces hygroscopicus* var. *geldanus* by a modular polyketide synthase (PKS) and several tailoring enzymes.[12] The geldanamycin PKS consists of seven polyketide chain extension modules preceded by a 3-amino-5-hydroxybenzoic acid (AHBA) loading domain. Each extension module contains an acyl transferease (AT) domain that loads a malonyl (module 6), a methylmalonyl (modules 1, 3, 4, and 7), or a methoxymalonyl (modules 2 and 5) extender unit. Replacing the AT domains of the geldanamycin PKS with a malonyl AT domain has been successful in generating several geldanamycin analogues.[13] For example, replacing AT4 by a malonyl AT domain predicts the biosynthesis of 8-demethylgeldanamycin (**2**). Here, we report the production, structure elucidation, and biological properties of the geldanamycin analogue **2** by an AT4-engineered *S. hygroscopicus* and of 4,5-epoxy-8-demethylgeldanamycin (**3**), an unexpected product.

8-Demethylgeldanamycin (**2**) was purified from a fermentation broth of *S. hygroscopicus* var. *geldanus* by reversed-phase medium-pressure column chromatography and preparative HPLC. The UV spectrum of **2** showed two strong absorptions at 256 and 305 nm consistent with dienamide and benzoquinone moieties. The infrared data of **2** displayed absorptions at 3458 and 3359 cm^{-1} characteristic of amines and hydroxyl functionalities. Carbonyl stretches were evident at 1723, 1700, 1652, and 1605 cm^{-1}, the latter corresponding to a quinoid carbonyl. 8-Demethylgeldanamycin (**2**) was assigned the molecular formula $C_{28}H_{38}N_2O_9$ on the basis of MS and NMR data.



1, R = CH_3
2, R = H



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A resonance denoting the presence of a trisubstituted benzoquinone moiety was observed at 7.23 ppm in the ^1H NMR spectrum (Table 1). Also evident were one olefinic methyl signal at 2.01 ppm, two aliphatic methyl resonances at 1.11 and 0.92 ppm, three methoxy shifts at 3.35, 3.36, and 4.11 ppm, five olefinic proton signals, and two broad NH resonances. The ^{13}C NMR spectrum displayed three methyl resonances, one of which was bonded to an olefinic carbon, and three *O*-methyl signals. The methoxyl shift at 4.11 ppm is characteristic of an aromatic *O*-methyl group.

The complete structure of **2** was elucidated by analysis of the COSY and HMBC data (Table 1). The C-2 through C-15 portion of the molecule, including the methyl substit-

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Table 1. NMR Assignments for 8-Demethylgeldanamycin (**2**) in $CDCl_3$

carbon #	δ C	δ H, mult., *J*	COSY	HMBC
1	168.2			
2	134.6			
3	127.6	6.94, 1H, d, *J* = 11.5 Hz	H-4, 5, 22	C-1, 19, 22
4	126.3	6.54, 1H, dd, *J* = 11.0, 11.5 Hz	H-3, 5, 6, 22	C-2, 6
5	136.8	5.86, 1H, dd, *J* = 8.0, 11.5	H-4, 6, 22	C-3
6	79.9	4.26, 1H, d, *J* = 8.0 Hz	H-4, 5, 7	C-4, 5, 8, 23
7	77.9	5.38, 1H, d, *J* = 8.0 Hz	H-8, 9	C-8, 9, 24
8	125.9	5.84, 1H, dd, *J* = 8.0, 15.5 Hz	H-6, 7, 9, 10	C-10
9	139.1	6.22, 1H, dd, *J* = 7.0, 15.5 Hz	H-8, 10	C-7, 10, 11, 26
10	36.9	2.44, 1H, m	H-8, 9, 11, 26	C-9
11	74.0	3.70, 1H, t, *J* = 5.5 Hz	H-10, 12	C-12
12	80.6	3.60, 1H, m	H-11, 13	C-27
13	35.8	1.53, 1H, m	H-12, 13b, 28	C-22
		1.83, 1H, m	H-12, 13a, 28	
14	30.1	1.56, 1H, m	H-15a/b, 28	
15	30.9	2.40, 1H, m	H-14, 15b	C-14, 16, 17, 18, 21, 28
		2.52, 1H, m	H-14, 15a, 28	C-16, 24, 28
16	127.3			
17	157.1			
18	184.1			
19	111.4	7.23, 1H, s		C-17, 20, 21
20	137.8			
21	184.4			
22	12.3	2.01, 3H, s	H-3, 4	C-1, 2, 3
23	57.8	3.35, 3H, s		C-6
24	156.0			
25				
26	13.5	1.11, 3H, d, *J* = 7.0 Hz	H-10	C-9, 10, 11
27	56.4	3.36, 3H, s		C-12
28	21.8	0.92, 3H, d, *J* = 6.0 Hz	H-14	C-13, 14
29	61.6	4.11, 3H, s		C-17
22-NH		8.8, 1H, s	H-3	C-1, 18, 19
23-NH_2		4.90, 1H, bs		
11-OH		3.60, 1H, bs		

uents, was assembled by interpretation of the COSY spectrum. HMBC correlations from H-23, H-27, and H-29 placed the methoxy groups at C-6, C-12, and C-17, respectively. The carbamate functionality was attached to C-7 on the basis of an HMBC correlation observed from H-7 to C-24. The HMBC spectrum exhibited cross-peaks between H-22 and C-1 as well as between an NH and C-1, C18, and C-19, thus linking the α,*β*,*γ*,*δ*-unsaturated amide with the benzoquinone moiety at C-20. Correlations observed from H-15 to C-16, 17, 18, and 21 allowed for the connection of the other end of the benzoquinone. The structure of **2** was thus determined as 8-demethylgeldanamycin. The configuration of the C-8,9 double bond was established as *trans* due to the large coupling constant of 15.5 Hz between H-8 and H-9.

Compound **3** was isolated as a solid, and the molecular formula, $C_{28}H_{38}N_2O_{10}$ was established from the HRMS and NMR data. Absent from the UV spectrum of **3** was the dienamide absorption at 256 nm observed in **2**, pointing to a reduction in the extent of conjugation. The IR spectrum of **3** displayed absorptions for a hydroxyl moiety (3361 cm^{-1}), NH groups (3459 cm^{-1}), and carbonyl functionalities (1715, 1605 cm^{-1}). The ^1H and ^{13}C NMR spectra of **3** were comparable to those of **2** (Tables 1 and 2); however, the olefinic proton resonances for H-4 and H-5 were absent. The H-3 and H-6 proton signals also differed by 1.0 and 1.1 ppm, respectively. Comparison of the ^{13}C NMR spectrum of **2** and **3** indicated that there were two new signals at 50.0 and 58.9 ppm in **3**, assigned to an epoxide functionality, and that the two olefinic carbon resonances for C-4 and 5 (126.3 and 136.8) of **2** were missing. Compound **3** was therefore assigned the structure of 4,5-epoxy-8-demethylgeldanamycin. This structural assignment was confirmed by detailed analyses of COSY and HMBC data (Table 2). The geometry of the epoxide was established as *cis* on the basis of a 4.0 Hz coupling constant between the H-4 and H-5 protons; this was confirmed by examination of the NOESY spectrum.

Geldanamycin (**1**) and related compounds produced in *S. hygroscopicus* strains are assembled by modular polyketide synthases (PKS).[14] The intermediate produced by the PKS, pro-geldanamycin, is believed to possess the fully saturated alkane functionality at C-4, C-5, which is presumably oxidized to a double bond by a tailoring enzyme (R. Rascher, unpublished data). Thus, it is quite likely that 4,5-epoxy-8-demethylgeldanamycin (**3**) is formed from 8-demethylgeldanamycin (**2**) by the action of the same post-PKS monooxygenase responsible for the double-bond formation through a second oxidation, although another enzyme cannot be ruled out. This is the first reported geldanamycin analogue possessing an epoxide moiety produced biologically.

The cytotoxicity of both geldanamycin analogues was measured against the human breast cancer cell line SK-BR-3. The IC_{50} for compounds **2** and **3**, 481 and 280 nM, respectively, were 10−20-fold higher than 17-AAG (27 nM), the geldanamycin derivative currently in clinical trials.

In summary, genetic engineering has provided two new geldanamycin analogues, 8-demethylgeldanamycin (**2**) and a minor metabolite 4,5-epoxy-8-demethylgeldanamycin (**3**). The genetic engineering approach enables alteration of carbon substituents at remotely functionalized positions of the macrolactam that are difficult to achieve by chemical modification.

Experimental Section

General Experimental Procedures. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) were recorded in $CDCl_3$ solution with a Bruker DRX 400 spectrometer. Chemical shifts were referenced to δ 7.26 and 77.0 for ^1H and ^{13}C spectra, respectively.

Table 2. NMR Assignment for 4,5-Epoxy-8-demethylgeldanamycin (**3**) in $CDCl_3$

carbon #	δ C	δ H, mult., *J*	COSY	HMBC
1	167.2 (s)			
2	141.0 (s)			
3	128.3 (d)	5.88, 1H, dd, *J* = 1.0, 8.0 Hz	H-4, 22	C-1, 22
4	50.0 (d)	3.71, 1H, dd, *J* = 4.0, 8.0 Hz	H-3, 5	C-5
5	58.9 (d)	3.32, 1H, dd, *J* = 2.0, 4.0 Hz	H-4, 6	C-4
6	82.8 (d)	3.16, 1H, dd, *J* = 2.0, 8.0 Hz	H-5, 7	C-5, 23
7	75.3 (d)	5.33, 1H, d, *J* = 8.0 Hz	H-6, 8	C-24
8	125.4 (d)	5.75,1H, dd, *J* = 8.0, 15.5 Hz	H-7, 9	C-10
9	138.6 (d)	5.99, 1H, dd, *J* = 8.0, 15.5 Hz	H-8	C-7
10	36.9 (d)	2.43, 1H, m	H-11, 26	
11	73.9 (d)	3.54, 1H, m	H-10, 12	
12	80.5 (d)	3.47, 1H, bs	H-11, 13a, 13b, 14	C-27
13	34.1(t)	1.71, 1H, m	H-12, 13b, 28	
		1.41, 1H, m	H-12, 13a, 14	
14	29.1 (d)	1.71, 1H, m	H-12, 13b, 28	
		2.51, 1H, dd, *J* = 5.2, 12.8 Hz	H-15b	C-16, 21
15	30.6 (t)	2.31, 1H, dd, *J* = 8.0, 12.8 Hz	H-15a	C-16
16	127.7 (s)			
17	157.1 (s)			
18	184.1 (s)[a]			
19	111.9 (d)	7.22, 1H, s		C-17, 18
20	137.9 (s)			
21	183.9 (s)[a]			
22	13.0 (q)	2.11, 3H, d, *J* = 1.0 Hz	H-3	C-1, 2, 3
23	59.0 (q)	3.58, 3H, s		C-6
24	155.8 (s)			
25				
26	13.3 (q)	1.0, 3H, d, *J* = 6.8 Hz	H-10	C-9, 10, 11
27	56.5 (q)	3.33, 3H, s	H-4, 6	C-5
28	22.3 (q)	0.95, 3H, d, *J* = 6.4 Hz	H-14	C-13, 15
29	61.6 (q)	4.08, 3H, S		C-17
22-NH		8.69, 1H, bs		C-2, 18, 19
24-NH_2		4.89, 2H, bs		

[a] Signals are interchangeable.

For each compound, ^1H, ^{13}C, COSY, constant time HMBC, and multiplicity-edited HSQC experiments were carried out. HRESIMS were obtained by manual peak matching versus internal standards by high-resolution mass spectrometry using an Applied Biosystems Mariner TOF spectrometer configured with a Turbo-Ionspray source in positive ion mode. Infrared spectra were measured on a Perkin-Elmer 1600 series FTIR spectrophotometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Preparative HPLC was carried out on a Polaris C_{18} column (MetaChem, 10 μm, 21.2 × 250 mm).

Culture Conditions. Spores from *S. hygroscopicus* K309-27-1 were used to inoculate 50 mL of YPD medium in a 250 mL flask. The culture was grown for 1 day at 30 °C, and then 25 mL was transferred into 500 mL of YPD medium in a 2.8 L Fernbach flask. The culture was incubated at 30 °C for 1 day and then used to inoculate 20 L bioreactors at 4% (v/v). Two 20 L bioreactors (New Brunswick Scientific BioFlo IV) with a 12 L working volume were used to produce material. The temperature was maintained at 30 °C. The pH was initially controlled at 6.5 with 2.5 N H_2SO_4 or 2.5 N NaOH. Two days after inoculation, the pH was reduced to 6.0 for the rest of the process. Dissolved O_2 (DO) was controlled above 30% air saturation with agitation (200−400 rpm). The overall pressure in the bioreactors was manually set at 6 psi, and airflow was controlled at 1 vessel volume per minute (vvm). Foaming was controlled by automatic addition of 50% (v/v) Antifoam B (JT Baker). The bioreactors were harvested 4 days after inoculation.

Media. YPD seed medium was prepared according to the manufacturer's instructions (Sigma-Aldrich). The 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 oatmeal (Gerber), and 10 g of beet molasses (Minn-dak). The medium was sterilized in place in the bioreactors for 30 min at 121 °C. Glucose was added to the bioreactors to a final concentration of 36.4 g/L using a sterile 650 g/L stock solution.

Extraction and Isolation of 2 and 3. Celite (50 g/L) and Cycep 349 (2.5 mL/L) were added to the fermentation broth of two bioreactors (26 L). The broth was filtered, and the clarified solution (23 L) was loaded onto an equilibrated Diaion HP20 column (10 × 13 cm, 1 L). The column was eluted with MeOH, and the resulting mixture (4 L) was concentrated in vacuo to 250 mL. The mixture was diluted to 25% (v/v) MeOH and loaded onto a preconditioned C-18 column (5 × 20 cm, 400 mL). The column was washed with 50% (v/v) MeOH (800 mL) and eluted with 60% (v/v) MeOH. Fractions 11−20 contained **2**, and 8−10 contained **3** by HPLC. Fractions 11−20 were combined and concentrated to afford a dark brown solid. This material was further purified by preparative HPLC on a Polaris C18 column using 60% (v/v) MeOH as the mobile phase. After evaporation to dryness and crystallization from MeOH, **2** (470 mg) was obtained as a brown amorphous solid. Fractions containing **3** were concentrated in vacuo and subjected to purification by preparative HPLC on a Polaris C18 column using 57% (v/v) MeOH−H_2O as the eluant. Fractions containing **3** were pooled and concentrated to afford a tan solid (6 mg).

8-Demethylgeldanamycin (2): brown amorphous solid; $[\alpha]^{25}_D$ +3.8 (*c* 0.009, MeOH); UV (MeOH) λ_{max} (log ϵ) 256 (1.41), 305 (1.44) nm; IR (NaCl disk) ν_{max} 3458, 3359, 1723, 1700, 1651, 1605, 1594, 1367, 1329, 1195, 1136, 1090, 1056 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESITOFMS *m/z* 569.2470 $[M + Na]^+$ (calcd for $C_{28}H_{38}N_2O_9Na$, 569.2466).

4,5-Epoxy-8-demethylgeldanamycin (3): tan solid; UV (MeOH) λ_{max} (log ϵ) 303 (2.21) nm; IR (NaCl disk) ν_{max} 3459, 3361, 1715, 1605, 1594, 1455, 1361, 1324, 1195, 1120, 1088, 1047 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESITOFMS *m/z* 585.24187 $[M + Na]^+$ (calcd for $C_{28}H_{38}N_2O_{10}Na$, 585.2439).

Biological Assay. The human breast cancer cell line SK-BR-3 was obtained from the American Type Culture Collection (Manassas, VA) and maintained in McCoy's 5A modified medium (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone; Logan, UT) and 2 mM glutamine, in humidified air with 5% CO_2 at 37 °C. Cells were seeded in

duplicate in 96-well black tissue culture microtiter plates at ~4000 cells per well and allowed to attach for 15 h. Serial 10-fold dilutions of compounds were added to the wells, and the cells were incubated for 72 h. Cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega; Madison, WI). IC_{50} is defined as the concentration of the compound required for inhibiting cell growth by 50%.

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References and Notes

(1) Deboer, C.; Meulman, P. A.; Wnuk, R. J.; Peterson, D. H. *J. Antibiot*. **1970**, *23*, 442−447.
(2) Johnson, R. D.; Haber, A.; Rinehart, K. L., Jr. *J. Am. Chem. Soc.* **1974**, *96*, 3316−3317.
(3) Take, Y.; Inouye, Y.; Nakamura, S.; Allaudeen, H. S.; Kubo, A. *J. Antibiot.* **1989**, *42*, 107−110.
(4) Heisey, R. M.; Putman, A. R. *J. Nat. Prod.* **1986**, *49*, 859−63.
(5) Andrus, M. B.; Meridith, E. L.; Sekar, B. B. V. *Org. Lett.* **2001**, *3*, 259−262.
(6) Li, Y.; Tao, P.; Liu, Y.; Jiang, J. *Antimicrob. Agents Chemother.* **2004**, 867−872.
(7) Bedin, M.; Gaben, A.; Saucier, C.; Mester, J. *Int. J. Cancer* **2004**, *109*, 643−652.
(8) Roe, S. M.; Prodromou, C.; O'Brien, R.; Ladbury, J. E.; Piper, P. W.; Pearl, L. H. *J. Med. Chem.* **1999**, *42*, 260−266.
(9) Berghe, T. V.; Kalai, M.; Loo, G. V.; Declercq, W.; Vandenabeele, P. *J. Biol. Chem.* **2003**, *278*, 5622−5629.
(10) Bisht, K. S.; Bradbury, C. M.; Mattson, D.; Kaushal, A.; Sowers, A.; Markovina, S.; Ortiz, K. L.; Sieck, L. K.; Isaacs, J. S.; Brechbiel, M. W.; Mitchell, J. B.; Neckers, L. M.; Gius, D. *Cancer Res.* **2003**, *63*, 8984−8995.
(11) Sausville, E. A.; Tomaszewski, J. E.; Ivy, P. *Curr. Cancer Drug Targets* **2003**, *3*, 377−383.
(12) Staunton, J.; Weissman, K. *J. Nat. Prod. Rep.* **2001**, *18*, 380−416.
(13) Patel, K.; Piagentini, M.; Rascher, A.; Tian, Z.; Buchanan, G. O.; Regentin, R.; Hu, Z.; Hutchinson, C. R.; McDaniel, R. *Chem. Biol.* **2004**, *11*, 1625−1633.
(14) Rascher, A.; Hu, Z.; Viswanathan, N.; Schirmer, A.; Reid, R.; Nierman, W. C.; Lewis, M.; Hutchinson, C. R. *FEMS Microbiol. Lett.* **2003**, *218*, 223−230.

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