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NOTE

Identification of 6-demethoxy-6-methylgeldanamycin and its implication of geldanamycin biosynthesis

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Geldanamycin (**1**, Figure 1), the first member of benzoquinone ansamycins, was isolated from *Streptomyces hygroscopicus* in 1970.[1] Although exhibiting potent cytotoxicity against various cancer cells, **1** is not a clinical compound due to its severe hepatotoxicity and poor water solubility.[2] 17-AAG (17-allylamino-17-demethoxy-geldanamycin) as a semisynthetic derivative of **1** with much improved water solubility is currently under clinical trial for breast cancer treatment.[3] Many new analogs or derivatives of **1** have been created or discovered in the past few years.[4–8]

We are interested in natural **1** analogs and understanding their synthetic mechanisms. We identified such analogs as 4,5-dihydro-4-hydroxygeldanamycins, thiazinogeldanamycin and 19-*S*-methylgeldanamycin from *S. hygroscopicus* 17997 and characterized their synthetic mechanisms.[9–13] We also discovered a minor component 7-descarbamoyl-7-hydroxygeldanamycin from a *gdmN* disruption mutant of *S. hygroscopicus* 17997, which presented an additional proof for C-7 carbamoylation taking place before C-4,5 oxidation in **1** biosynthesis.[14]

Recently, as a result of our continued efforts for natural **1** analogs, we discovered 6-demethoxy-6-methylgeldanamycin (**2**) in **1** preparation from *S. hygroscopicus* 17997. In this paper, we reported the structure of **2** and its implication of **1** biosynthesis.

Some preparations of **1** were found to contain **1** analogs as small or trace impurities.[15,16] The HPLC of our **1** preparation (with a purity of about 90%; see Supplementary material: a brief description of **1** preparation from *S. hygroscopicus* 17997) from *S. hygroscopicus* 17997 displayed a small peak at 24.7 min (about 1.7% of the principle **1** peak at 22.3 min; Figure 2). The peak revealed a molecular ion at *m/z* 567 ($[M+Na]^+$), which exhibited a typical MS^2 fragment pattern of **1** (Supplementary Figure S1). The *m/z* 567 aroused our interests, as we could not assign a reasonable structure for it from MS data and current understanding of **1** biosynthesis.[17–19]

To elucidate the structure of the analog with *m/z* 567 (**2**) by NMR, a total amount of 1070 mg **1** preparation, dissolved in 10 ml dimethyl sulfoxide, was used to make a pure preparation of **2** by reversed-phase HPLC (Shimadzu LC-20AP, SHIMADZU, Kyoto, Japan; YMC ODS-A, 21.2 × 150 mm, mobile phase MeOH-H_2O, 62–100% in 21 min, 12.5 ml min^{-1}, wavelength 254 nm; Supplementary Figure S2). After evaporation, an amount of 5.6 mg of **2** as yellow amorphous powder was obtained. Analytical HPLC indicated that it displayed an UV absorption profile very similar to that of **1** (Supplementary Figure S3).

The molecular formula of **2** was established as $C_{29}H_{40}N_2O_8$ by HR-ESI(+)-MS (*m/z* 567.26596, calculated 567.26769 for $C_{29}H_{40}N_2O_8Na$, Supplementary Figure S4), which is one oxygen atom less than **1** ($C_{29}H_{40}N_2O_9$). The ^1H and ^{13}C NMR spectra of **2** (Supplementary Figures S5 and S6) were very similar to those of **1**.[18] Comparison of the NMR data of **2** with those of **1** revealed that the only difference between the two compounds was replacement of the 6-methoxy group in **1** by the 6-methyl group in **2**, which was confirmed by the 2D NMR data analysis of **2**. In particular, the ^1H-^1H COSY correlations of H-5/H-6/H-7, H-6/H_3-23 and HMBC correlations of H_3-23/C-4, C-6, C-5, in combination with the shifts of these proton and carbon resonances established the CH_3-6 in **2**. Therefore, the structure of **2** was determined to be 6-demethoxy-6-methylgeldanamycin (Figure 1). The NMR chemical shifts of **2** were assigned completely by HSQC, COSY and HMBC spectroscopic data (Supplementary Figures S7–S10) as indicated in Table 1.

Compound **2** is a shunt product in **1** biosynthesis. Compound **1**'s biosynthesis consists of a starter unit (3-amino-5-hydroxybenzoic acid) assembly, extender units (one malonyl, two 2-methoxymalonyl and four 2-methylmalonyl units for polyketide chain building) condensation and tailoring modifications.[17–19] Obviously, **2** is derived from mis-incorporation of a 2-methylmalonyl unit in place

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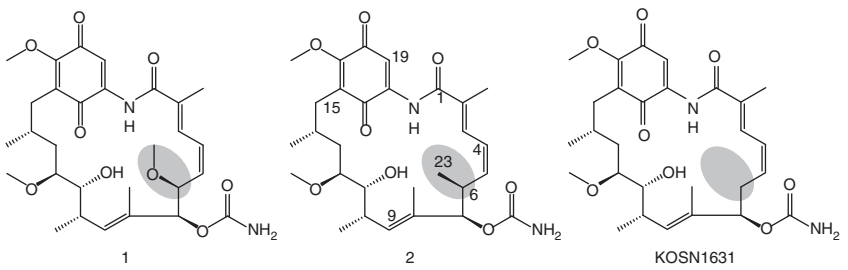

Figure 1 Chemical structure of **1**, **2** and KOSN1631. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

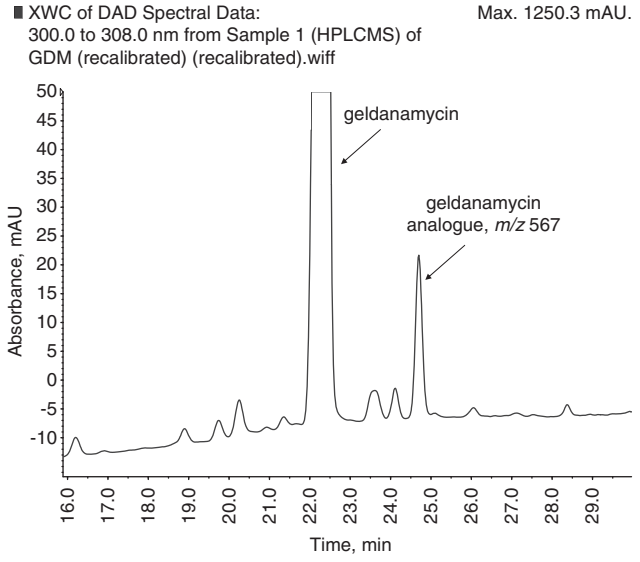

Figure 2 HPLC of geldanamycin (**1**) preparation from *S. hygroscopicus* 17997. HPLC parameters: Agilent 1200 RRLC system (Agilent, Waldbronn, Germany); Dikma Diamonsil C_{18} column (4.6 × 150 mm, 5 μm, DIKMA, Beijing, China), mobile phase MeOH-H_2O, 40–100% in 30 min, 1.0 ml min^{-1}, wavelength 304 nm. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 1 The NMR spectra data of 2

	2	
Position	δ_C	δ_H(*J in Hz*)
1	168.7	–
2	132.9	–
3	128.6	7.02, d (11.4)
4	122.4	6.30, dd (11.4, 10.8)
5	142.4	5.83, dd (10.8, 8.4)
6	39.3	2.96, dq (8.4, 6.6)
7	81.2	5.29, s
8	133.3	–
9	131.1	5.62, d (9.6)
10	32.5	2.70, m
11	73.1	3.49, m
12	81.0	3.40, m
13	34.6	1.77, m 1.65, overlap
14	28.6	1.66, overlap
15	32.2	2.45, overlap 2.43, overlap
16	127.7	–
17	156.9	–
18	184.2	–
19	111.3	7.25, s
20	138.3	–
21	184.9	–
22	12.4	1.99, s
23	14.2	1.09, d (6.6)
24	156.1	–
25	13.3	1.68, s
26	12.9	0.98, d (6.6)
27	56.5	3.34, s
28	22.8	0.96, d (7.2)
29	61.6	4.11, s
1-*NH*-	–	8.80, s
7-OCO*NH*$_2$	–	4.70, brs
11-*OH*	–	2.84, d (5.4)

^{1}H and ^{13}C NMR spectra data (δ) were obtained at 600 and 125 MHz, respectively, on INOVA-501 with tetramethylsilane as internal standard, and measured in $CDCl_3$ at room temperature.

of normal incorporation of a 2-methoxymalonyl unit into the polyketide chain in **1** biosynthesis. It is interesting to note that macbecin (or ansamitocin) as a close **1** analog from *Actinosynnema pretiosum* ATCC 31280 (or 31565) also contains a methyl (not a methoxyl) side group at C-6 of its polyketide chain, indicating that a 2-methylmalonyl unit incorporates into the polyketide chain in the corresponding condensation reaction of macbecin (or ansamitocin) biosynthesis.[20,21]

Compound **2** must share the same absolute configurations with **1**, as **2** is co-produced with **1** and must be biosynthesized by the same set of enzymes as **1**.[22–24] In particular, the incorporation of 2-methylmalonyl (not 2-methoxymalonyl) unit into the polyketide chain for **2** biosynthesis should not change the configuration of C-6, which is presumably determined by the enoylreductase domain of module 5 of polyketide synthases (PKS) for **1** biosynthesis.[22]

The identification of **2** in **1** preparation indicates that the acyltransferase domain of module 5 (AT5) of PKS for **1** biosynthesis shows a promiscuous substrate specificity for 2-methoxymalonyl CoA and, to a small degree, 2-methylmalonyl CoA. This phenomenon is observed occasionally in the biosynthesis of some microbial polyketides. For examples, the two components (A and B) of galbonolide are derived from substrate tolerance (for 2-methoxymalonyl and 2-methylmalonyl CoA) of AT5 of PKS in galbonolide biosynthesis.[25]

The two components (A and B) of epothilone are also derived from relaxed substrate specificity (for malonyl and 2-methylmalonyl CoA) of acyltransferase domain of module 3 of PKS in epothilone biosynthesis.[26] Recently, three rapamycin analogs (as impurities) were reported, with each one resulted from mis-incorporation of a 2-ethylmalonyl unit (in place of normal incorporation of a 2-methylmalonyl unit) into the polyketide chain at corresponding positions by the acyltransferase domain of module 3, 7 or 13 of PKS in rapamycin biosynthesis.[27]

To date, it is still difficult to foretell whether an acyltransferase domain in modular PKS possesses promiscuous substrate specificity or not, or even to predict its substrate specificity for 2-methoxymalonyl CoA, because only a few such acyltransferase domains are reported. We believe that the AT5 of PKS for **1** biosynthesis may be useful in establishing an *in silico* method to predict substrate promiscuity of acyltransferase domains of modular PKS in the future.

Patel *et al.*[28] reported an **1** analog 6-desmethoxygeldanamycin (KOSN1631, Figure 1) produced by an AT5-engineered strain of *S. hygroscopicus*, in which the AT5 of **1** PKS was replaced with the acyltransferase domain (with a substrate specificity for malonyl CoA) of module 2 of rapamycin PKS by genetic recombination. Compared with **1**, KOSN1631 showed a significant decrease in cytotoxicity against human breast adenocarcinoma cell line SKBr3, with an IC_{50} value of 3.2 μM (for **1**, 0.041 μM). We conducted a preliminary cytotoxicity assay of **2** against human liver hepatocellular carcinoma cell line HepG2 by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).[29] Compound **2** also exhibited lower cytotoxicity than **1** against HepG2, with an IC_{50} value of 10.5 μM (for **1**, 0.37 μM), suggesting that the 6-methoxy group may have an important role in **1**'s cytotoxicity against cancer cells.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)