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NOTE

Methanethiol as a catabolite of methionine provides methylthio- group for chemical formation of 19-*S*-methylgeldanamycin and 17,19-dimethylthioherbimycin A

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Geldanamycin (GDM) is an ansamycin produced by *Streptomyces hygroscopicus*. In our study of secondary metabolites from *S. hygroscopicus* 17997 (a GDM producing strain deposited at China Pharmaceutical Culture Collection, with an accession number CPCC 200120), we identified a natural GDM analog, 19-*S*-methylgeldanamycin (**1**, Figure 1). Compound **1** retained potent cytotoxicity against cancer cells, and meanwhile exhibited increased water solubility and photostability compared with GDM.[1]

As a methylthio-derivative of GDM, **1**'s synthetic mechanism in *S. hygroscopicus* 17997 remains unclear. The biosynthetic pathway of GDM does not contain any methylthiolation reaction.[2–4] Besides, bioinformatics analysis of GDM biosynthetic gene clusters revealed no candidate gene encoding radical-SAM enzyme for methylthiolation reaction.[5] So, 19-methylthiolation of GDM is not an essential and/or extended modification reaction in GDM biosynthesis.

An examination of natural ansamycins reveals quite a few compounds with methylthio- group (such as trierixin, quinotrierixin, awamycin and 3-methylthiorifamycin SV, Figure 1) linking to an aromatic carbon of the molecules.[6–9] Besides, urdamycin E as an angucycline antibiotic contains also a methylthio- group (Figure 1).[10]

Among these compounds, urdamycin E and 3-methylthiorifamycin SV were known to be closely related to L-methionine (Met) for their production.[8,10] Rohr[10] proposed a mechanism for chemical conversion of urdamycin A to E, which involved a Michael addition of $^{-}$SMe (derived biogenetically from Met) to urdamycin A. The mechanism may be applicable to methylthio-containing quinone antibiotics such as awamycin.[10] In fact, methanethiol can react chemically with GDM (or naphthomycin A), yielding **1** (or 30-methylthionaphthomycin A by addition-elimination).[11,12] Therefore, the methylthio- group in **1** should come from methanethiol, and methanethiol should be derived from Met as a catabolite by *S. hygroscopicus* 17997. Other natural ansamycins carrying methylthio-groups (Figure 1) must have the same biological origin and synthetic mechanism.

To demonstrate the above deduction, we supplemented Met into ISP2 medium (1.0% malt extract, 0.4% yeast extract, 0.4% glucose, 1.5% agar, 0.3% Met) to culture *S. hygroscopicus* 17997 at 28 °C for 5 days. We found that the production of **1** increased to a level of $\sim$20 mg l^{-1} (Compound **1** was only MS detectable if Met was not supplemented). Besides, a strong and repulsive smell filled the room for culturing *S. hygroscopicus* 17997. GC–MS analysis confirmed that the bad smell originated from methanethiol diffused out of the headspace gas of these Met-plus ISP2 medium plates with *Streptomyces hygroscopicus* 17997 (Figure 2). And methanethiol as a catabolite of Met by *S. hygroscopicus* 17997 reacted chemically with GDM, which yielded **1** (Figure 3 and Supplementary Figure S1).

When we supplemented L-ethionine to ISP2 medium plates inoculated with *S. hygroscopicus* 17997, we detected the production of an expected red compound with *m/z* 643, corresponding to the sodium adduct ion of 19-*S*-ethylgeldanamycin ($C_{31}H_{44}N_2O_9SNa$; see Supplementary Information for its silica gel thin layer chromatography (TLC) and MS–MS confirmation; Supplementary Figures S2 and S3). These *S. hygroscopicus* 17997 cultures also gave off a strong smell of thiols (ethanethiol). This result provided an additional evidence that the methylthio- group in **1** originated from Met, which was catabolized by *S. hygroscopicus* 17997 to methanethiol as substrate for the production of **1**.

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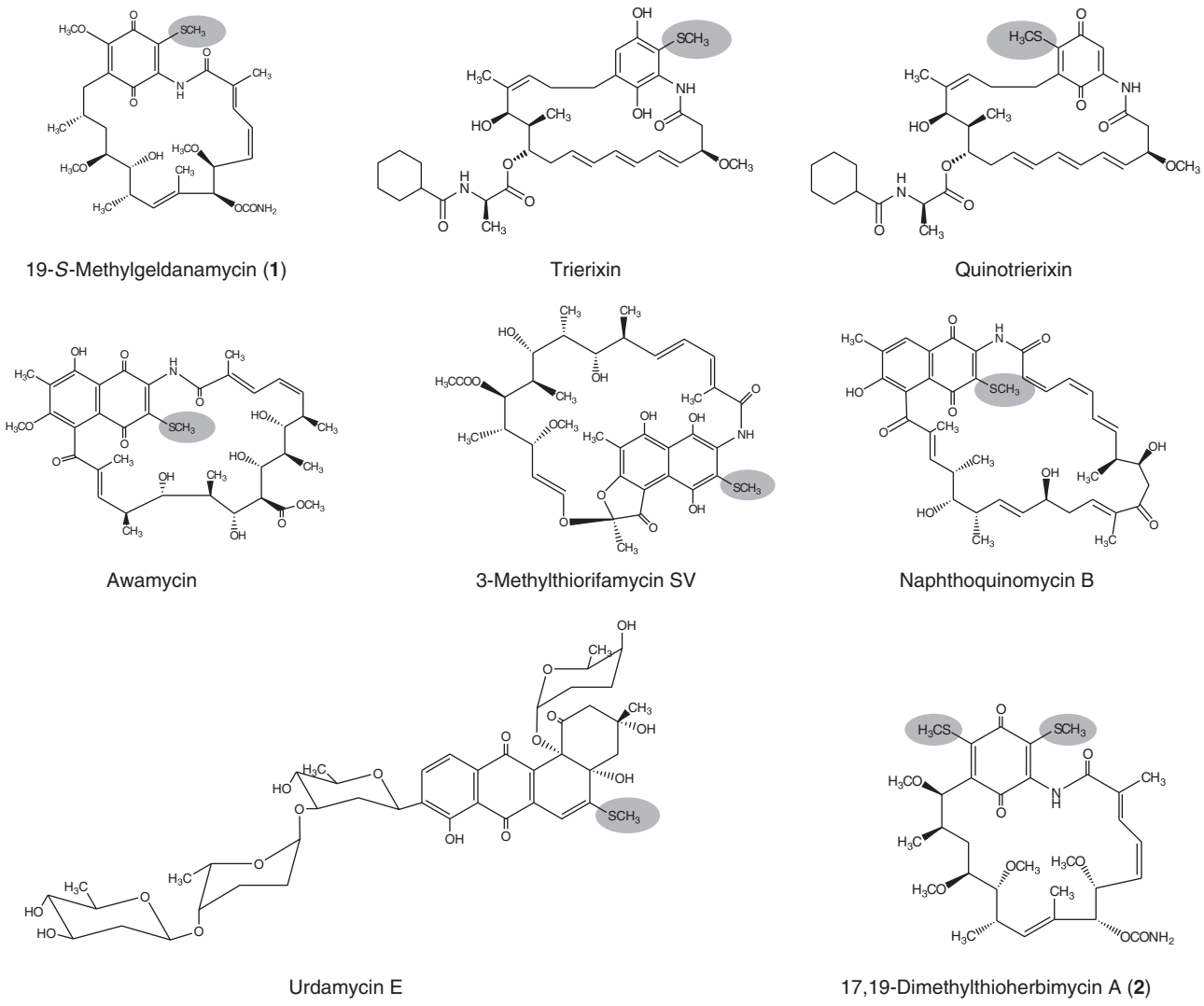

Figure 1 Chemical structures of some natural products carrying methylthio-group. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

To further confirm the above synthetic mechanism, we supplemented Met ($3.0\,g\,l^{-1}$) in culturing *S. hygroscopicus* N02Z-0421 (a herbimycin producer isolated by China NCPC New Drug Research and Development Co. Ltd.) to obtain methylthio- derivative of herbimycin, which is a close analog of GDM.[13] An expected red compound was detected in the secondary metabolites of *S. hygroscopicus* N02Z-0421 by silica gel TLC (Figure 4). The red compound was then purified by a procedure of ethyl acetate extraction, silica gel chromatography and reversed-phase HPLC, and 10 mg pure preparation of the red compound (purity >98%, calculated by area% of HPLC at 250 nm; Supplementary Figure S4) were obtained from 1.2 l fermentation supernatant of *S. hygroscopicus* N02Z-0421. (See Supplementary Information for detailed descriptions of fermentation of *S. hygroscopicus* N02Z-0421 and purification of the red compound.)

HR-ESI(+)-MS of the red compound revealed a principal peak at *m/z* 689.25554 ($[M+Na]^+$) and a minor (^{34}S isotope) peak at *m/z* 691.24797 (Supplementary Information, Supplementary Figure S5). The molecular formula of the red compound was thus established as $C_{32}H_{46}N_2O_9S_2$ (exact mass 689.25369 for $[M+Na]^+$), which is $S_2C_2H_4$ (or two SCH_2) more than herbimycin A ($C_{30}H_{42}N_2O_9$).

The NMR spectra of the red compound (Supplementary Figures S6–S11) were highly similar to those of herbimycin A except a few differences.[14,15] In the ^{13}C-NMR, two additional carbon signals of -SCH_3 at δ_C 15.1 and δ_C 16.3 showed up; in the ^1H-NMR, two additional hydrogen signals (δ_H 2.52, δ_H 2.60) from two -SCH_3 appeared, whereas the hydrogen signals of C-17 and C-19 in herbimycin A disappeared. Long-range correlations from methyl proton at δ_H 4.28 (H-15) and -SCH_3 proton at δ 2.52 to aromatic carbon C-17 suggested that this -SCH_3 (δ_H 2.52) group was connected to C-17. The other -SCH_3 group was connected to C-19, as indicated by its methyl proton at δ_H 2.60 exhibiting long-range correlation to the aromatic carbon C-19. Therefore, the chemical structure of the red compound was 17,19-dimethylthioherbimycin A (**2**). Its NMR chemical shifts were assigned completely by HSQC, COSY and HMBC (Table 1 and Figure 5).

A comparison of the benzoquinone moiety of GDM and herbimycin A shows that C-17 of GDM links to a methoxyl group, while

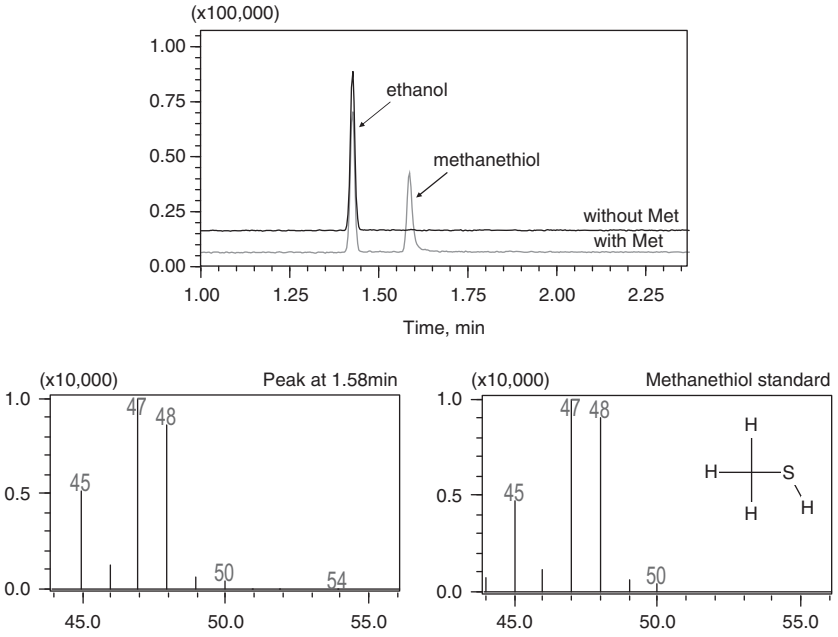

Figure 2 GC–MS of headspace gas of ISP2 medium (with and without supplementing Met) plates cultured with *Streptomyces hygroscopicus* 17997 at 28 °C for 5 days. The GC–MS was performed on an Agilent QP2010 with a column Rxi-5MS (30 m × 0.25 mm) under the following parameters. For GC, column oven temperature 35 °C, injection temperature 200 °C, flow control mode linear velocity (36.0 cm s^{-1}), helium as carrier gas at flow rate 1.0 ml min^{-1} and pressure 47.6 kPa, split ratio 100. For MS, ion source temperature 200 °C, interface temperature 250 °C, acquisition mode scan (285 per second), time 1.0–5.0 min, *m/z* 45–100. Samples (10 μl) were injected manually in split mode at 100:1, with injector temperature 200 °C. GC (upper): a methanethiol peak at 1.58 min appeared only from the headspace gas of culture medium with Met (3.0 g l^{-1}). The peak was proved to be methanethiol by MS. MS (lower): left, MS spectrum of the peak at 1.58 min; right, standard MS spectrum of methanethiol from US National Institute of Standards and Technology. The two MS spectra were identical (*m/z* 48, M^+, molecular ion; *m/z* 47, M + -H; *m/z* 45, M^+-H-2H). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

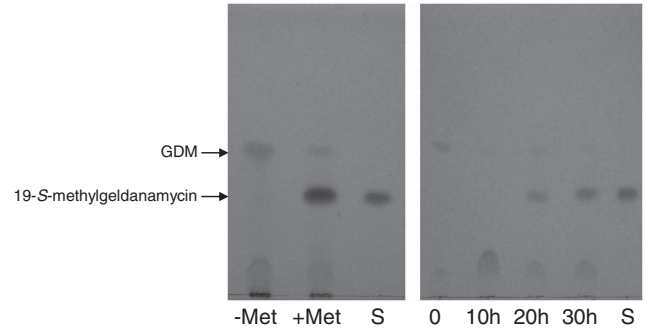

Figure 3 Silical gel TLC for 19-*S*-methylgeldanamycin production. Two ISP2 plates (one with 3.0 g l^{-1} L-Met, the other without L-Met as control) inoculated with *Streptomyces hygroscopicus* 17997 were incubated at 28 °C for 3 days for mycelia growth. The third ISP2 plate was prepared without inoculating *Streptomyces hygroscopicus* 17997, but added Geldanamycin (GDM) (purity ⩾95%, prepared by the author's laboratory) to a final concentration of 50 mg l^{-1}. Upon removing the lids, the third ISP2 plate was placed upside down over the former one ISP2 plate with L-Met, and then incubated at 28 °C for 0, 10, 20 and 30 h to detect 19-*S*-methylgeldanamycin production. At each time, 1/4 agar culture of each plate was cut off for ethyl acetate extraction. The organic extract mixed with a small volume of 10% FeCl3 for 30 min at room temperature, to oxidize hydroquinones to quinones, before silica gel TLC with a mobile phase of EtOAc/CH2Cl2/hexane/methanol (9:6:6:1, *v/v*), in which GDM had a *R*f of 0.55 and 19-*S*-methylgeldanamycin 0.38. The significant increase of 19-*S*-methylgeldanamycin production indicated that 19-*S*-methylgeldanamycin came from GDM reacting chemically with methanethiol. Left: 19-*S*-methylgeldanamycin production in the two ISP2 plates with *Streptomyces hygroscopicus* 17997. Right: 19-*S*-methylgeldanamycin production in the ISP2 plate without *Streptomyces hygroscopicus* 17997 (cell-free plate). S stands for 19-*S*-methylgeldanamycin standard (purity ⩾93%, prepared by the author's laboratory). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

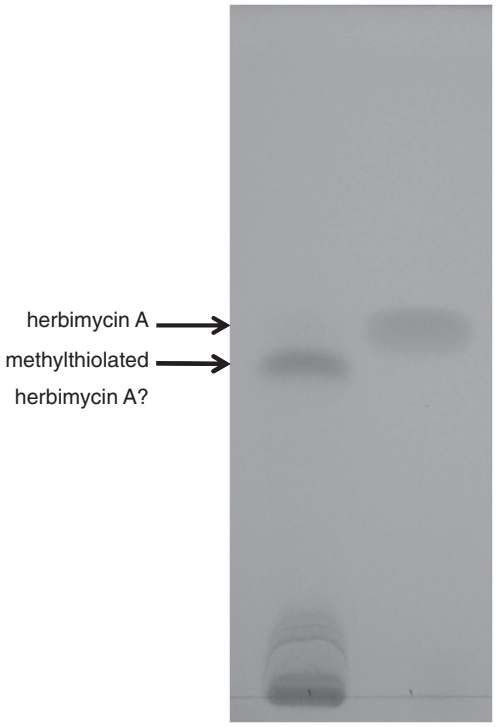

Figure 4 Silica gel TLC (developed with the same mobile phase as that of Figure 3) of ethyl acetate extract of fermentation supernatant of *Streptomyces hygroscopicus* N02Z-0421. Herbimycin A showed a R_f value of 0.60, and its methylthiolated derivative 0.55. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 1 NMR data of 17,19-dimethylthioherbimycin A

Position	δ_C	δ_H (J *in Hz)*
1	174.5	—
2	138.2	—
3	123.8	6.38, overlap
4	127.5	6.34, overlap
5	131.7	5.28, t (10.2)
6	75.9	4.06, dd (10.2, 9.0)
7	81.0	4.90, d (9.0)
8	128.7	—
9	134.3	5.18, d (10.8)
10	35.5	2.34, brs
11	86.9	3.13, overlap
12	82.2	3.13, overlap
13	30.0	0.93, overlap
		0.91, brs
14	32.7	2.94, brs
15	82.5	4.28, d (9.0)
16	139.7	—
17	150.4	—
18	178.8	—
19	135.2	—
20	138.4	—
21	176.9	—
2-CH_3	12.6	1.91, s
6-OCH_3	55.8	3.12, s
7-$OCONH_2$	157.7	—
8-CH_3	11.9	1.48, s
10-CH_3	16.4	0.93, d (6.6)
11-OCH_3	60.8	3.44, s
12-OCH_3	55.6	3.27, s
14-CH_3	17.0	1.07, d (6.6)
15-OCH_3	56.1	3.07, s
17-SCH_3	15.1	2.52
19-SCH_3	16.3	2.60

^{1}H- and ^{13}C-NMR spectra were obtained on a Varian VNS-600 (^{1}H-NMR at 600 MHz, ^{13}C-NMR at 150 MHz) with TMS as internal standard, and measured in CD_3OD at room temperature.

C-17 of herbimycin A links to a hydrogen atom that can be easily substituted by other groups. Accordingly, GDM can be mono-methylthiolated at C-19, while herbimycin A di-methylthiolated at C-17,19. But it is interesting to note that both trierixin and quinotrierixin (Figure 1) are mono-methylthiolated derivatives of ansatrienin/mycotrienin,[16,17] although ansatrienin contains two aromatic carbons for methylthiolation like herbimycin A.

Ansamitocin is a benzenic ansamycin produced by *Actinosynnema pretiosum* ATCC 31565.[18] When supplementing Met to culture medium, we detected no methylthio-derivative of ansamitocin from *A. pretiosum* ATCC 31565. This result suggests that only antibiotics with quinone moiety (such as benzoquinone and naphthoquinone ansamycins) are able to react chemically with methanethiol to form methylthio-derivatives.

Microorganisms such as *Streptomyces* can catabolize Met to methanethiol. Ashraf[19] reported that L-methioninases, ubiquitous in all organisms except mammals, catalyzed the α, γ-elimination of L-Met to α-ketobutyrate, methanethiol and ammonia. A blast search of genome sequences of *Streptomyces* in NCBI indicated that L-methioninases existed in various species of *Streptomyces* including *Streptomyces hygroscopicus* (most of these L-methioninases were annotated as either methionine gamma-lyase or cystathionine gamma-synthases). When we added some Met to ISP2 medium to

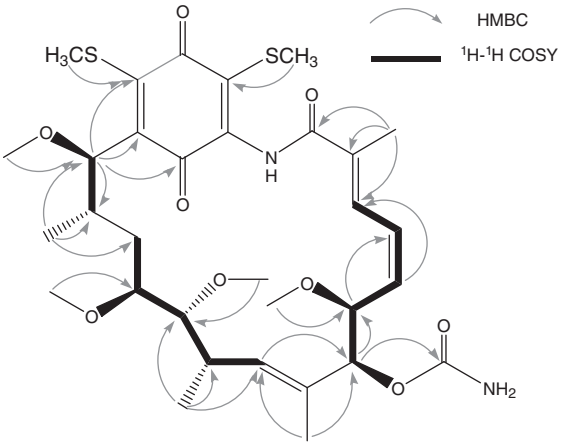

Figure 5 NMR correlations for 17,19-dimethylthioherbimycin A. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

culture *Streptomyces lividans* TK24, a model streptomycete strain, we could smell the appearance of methanethiol (if GDM was also added, **1** could be detected by silica gel TLC).

S. hygroscopicus 17997 produced low level (about 3 mg l^{-1}) of **1** in a culture medium consisted of 2% starch, 0.5% glucose, 0.5% cottonseed meal, 1% cornsteep liquor, 0.5% yeast powder and 0.2% $CaCO_3$. One possible explanation for low level production of **1** may be that this culture medium with rich organic nitrogen resources, could provide more sulfur-containing amino acids (Met and cysteine) than needed for normal growth of *S. hygroscopicus* 17997. Therefore, a part of the surplus Met was catabolized to methanethiol, which then reacted chemically with GDM to form **1**.

Thiol-containing compounds such as ethanethiol, glutathione (GSH), *N*-acetyl-L-cysteine, and so on, can also react chemically with benzoquinone or naphthoquinone ansamycins, producing many semisynthetic or natural thioansamycins.[12,20,21] Recently, Yang *et al.*[22] reported two thionaphthomycins (naphthomycins M and N). Naphthomycin M contains a thioglycolic acid group connected to C-30 of the naphthomycin skeleton via sulfur, and naphthomycin N is a dimer-like molecule, with a 2-aminoethanethiol group as bridge to connect two naphthomycin monomers. But the (bio)synthetic mechanism(s) of naphthomycins M and N remains unclear.[22]

Ōmura[23] and Shibata[24] reported chemical modification of herbimycin A, and obtained more than a dozen herbimycin A derivatives with various modifications at C-17 or C-19 of the benzoquinone moiety of herbimycin A. Among them, derivatives with a methylpiperazino group or bromine substituent at C-19 showed high antitumor activity.[23,24] Our herbimycin A derivative **2** contains two identical substituents (methylthio-) at the benzoquinone moiety of herbimycin A, one at C-17 and the other at C-19 of the molecule. A preliminary cytotoxicity assay of **2** against HepG2 cancer cells was conducted by us using MTT assay.[25] Compound **2** showed potent cytotoxic activity against HepG2 cancer cells, with an IC_{50} of 18.7 μM (for herbimycin A, 14.0 μM), almost the same to **1** with an IC_{50} of 19.0 μM.[1]

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