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NOTE

Two herbimycin analogs, 4,5-dihydro-(4*S*)-4-hydroxyherbimycin B and (15*S*)-15-hydroxyherbimycin B, from *Streptomyces* sp. CPCC 200291

Wei Zhao, Bingya Jiang, Linzhuan Wu, Yanni Nan, Jing Cui, Liyan Yu, Yuzhen Wei, Jinghua Li and Guangzhi Shan

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Herbimycin A (Figure 1) is a benzoquinone ansamycin, discovered by Satoshi Ōmura in 1979, with herbicidal activity.[1] Herbimycin A possesses strong cytotoxicity against tumor cells, as it is a specific inhibitor of heat-shock protein 90, whose client proteins have important roles in cell-cycle regulation.[2,3] Chemical derivatives of herbimycin A were synthesized for improved activity against Ehrlich's carcinoma.[4,5]

Streptomyces are well known for their potential to produce secondary metabolites with antibacterial, antifungal and cytotoxic activities. *Streptomyces* sp. CPCC 200291, with strong antibacterial and antifungal activities, was a soil isolate of Hainan Province, China. In the course of studying the secondary metabolites of the isolate, which is grown by solid-state fermentation, we identified herbimycins A and C as the principal components by TLC and LC–MS, and discovered two new herbimycin analogs as minor components. Herein, we report the identification, structure determination and probable biosynthetic routes of the two new herbimycin analogs.

Spores of *Streptomyces* sp. CPCC 200291 were spread on an ISP2 medium (composition: 0.4% yeast extract; 1.0% malt extract; 0.4% glucose; and 1.5% agar powder) and the plate was incubated at 28 °C for 7 days to produce an even layer of mycelial lawn. The agar culture was cut into small pieces and extracted with ethyl acetate (EtOAc). The EtOAc extract was concentrated and then analyzed by silica gel TLC developed with EtOAc–*n*-hexane–CH_2Cl_2–CH_3OH, 6:4:4:1, v/v. The two dominant yellow bands with R_f 0.75 and 0.52 were identified as herbimycins A and C, respectively (Supplementary Figure S1). There were two minor yellow bands at the lower part of the silica gel TLC plate. Similar to herbimycins A and C, they turned purple/blue upon spraying with 2 M NaOH, a diagnostic color reaction of benzoquinone ansamycins (Supplementary Figure S1).[6] HPLC revealed peaks with UV–vis spectra similar or identical to that of herbimycins A or C, but with molecular ions $[M+Na]^+$ at *m/z* 571 and 569 (Supplementary Figures S2–S6), which differentiated these metabolites from any known natural or biotransformation herbimycin analogs.[7–12]

To purify the two new herbimycin analogs, stock spores of *Streptomyces* sp. CPCC 200291 were spread onto 8–10 ISP2-medium plates and then incubated at 28 °C for 9–10 days for spore maturation. Fresh spores were recovered from these ISP2 plates with sterile water, and these were used to inoculate ~800 ISP2 (20 l) plates. After an incubation period of 6–7 days at 28 °C, the agar cultures were pooled and extracted three times with equal volume of EtOAc. The EtOAc extract was evaporated under reduced pressure to yield a dark brown solid residue (10.98 g). This was resuspended in 200 ml EtOAc, and 200 ml aqueous $FeCl_3$ (10%) was added and the mixture was stirred for 3 h. This procedure was to facilitate the subsequent separation and purification by oxidation of hydroquinone herbimycins to the quinones. The EtOAc layer was separated and concentrated at 35 °C to afford a brown oil, which was fractionated by normal-phase flash chromatography with a petroleum ether–EtOAc step-wise gradient 25–100% on a silica gel column, 35 × 460 mm (Büchi Sepacore C-620 from BUCHI Labortechnik AG, Flawil, Switzerland). Fractions eluting at ~90% EtOAc contained the new herbimycin analogs were pooled, dried (1.7 g), and further fractionated by Sephadex LH-20 chromatography (28 × 1400 mm; CH_3OH–CH_2Cl_2–petroleum ether, 5:3:1, v/v). This resulted in a yellow fraction containing the two new herbimycin analogs (150 mg, dried). These were separated by preparative HPLC on a Shimadzu (Kyoto, Japan) Prominence System with a LC-20AP pump and an SPD-20AV UV detector; using a YMC (Kyoto, Japan) C_{18} column (21.4 × 150 mm, CH_3OH-H_2O, 58–100% in 50 min, 10 ml min^{-1}). Each of the new herbimycins was further purified by semi-preparative HPLC on a YMC C_{18} column: 10.0 × 250 mm, with isocratic ACN-H_2O elution (30:70 for **1** and 34:66 for **2**), yielding **1** (3.8 mg) and **2** (9.3 mg) as yellow amorphous powders.

HR-ESI(+)MS (Supplementary Figure S7) established the molecular formula of **1** as $C_{28}H_{40}N_2O_9$ (measured 571.26340,

Key Laboratory of Biotechnology of Antibiotics of Ministry of Health, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
Correspondence: Dr B Jiang or Professor L Wu, Key Laboratory of Biotechnology of Antibiotics of Ministry of Health, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Tiantan Xili, Beijing 100050, China.
E-mail: jiangbingya@163.com or wulinzhuan@imb.pumc.edu.cn
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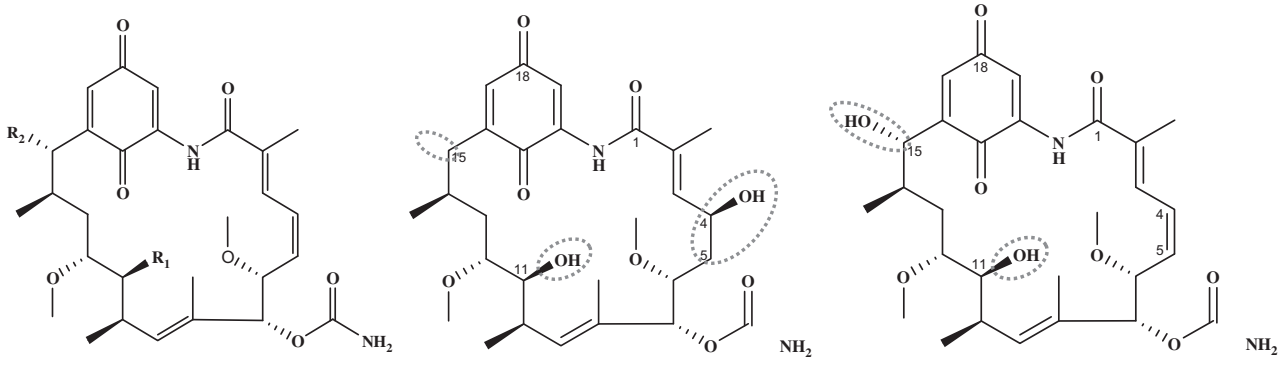
calculated 571.26260 for $C_{28}H_{40}N_2O_9Na$), which is $-C_2H_2$ less than herbimycin A ($C_{30}H_{42}N_2O_9$). The unsaturation degree of **1** is 9, whereas that of herbimycin A is 10, suggesting that **1** may contain one olefinic bond less than herbimycin A. The UV absorption profile of **1** seemed to support this prediction, as it lost the shoulder peak at 240–250 nm ascribed to the dienamide moiety of herbimycin A.[1,13] On the basis of the molecular formula and saturation degree differences, we speculated that **1** lacked two of the methyl groups in herbimycin A. The two methyl groups may be the *O*-methyl groups at C-11 and C-15, which were added in the post-PKS (polyketide synthase) tailoring process in the herbimycin A biosynthesis. The other two *O*-methyl groups (at C-6 and C-12) should be present in **1**, as they were incorporated into the molecule in the form of methoxymalonyl-CoA at the PKS stage of biosynthesis.[14]

The NMR spectra (Supplementary Figures S8–S13) of **1** were very similar to herbimycin A.[4,15] They showed signals characteristic of benzoquinone ansamycins, such as the 2,5-disubstituted-*p*-benzoquinone ring. Compound **1** revealed resonances for two tri-substituted double bonds at δ_H 6.03 (dd, $J=9.0$, 1.2 Hz, H-3) and 5.53 (d, $J=9.6$ Hz, H-9), whereas herbimycin A displayed resonances for one tri-substituted conjugated double bond (that is, two double bonds) and one tri-substituted double bond. On the basis of these differences, together with the aid of ^{1}H-^{1}H COSY correlations of H-3/H-4/H_2-5/H-6/H-7 (Figure 2) and their chemical shifts (δ_H 6.03 (1H, dd, $J=9.0$, 1.2 Hz, H-3), 4.74 (1H, m, H-4), 1.93 (2H, m, H-5), δ_C 136.6 (C-3), 65.2 (C-4), 37.1 (C-5)), we concluded that **1** had a C-4,5 single bond and a hydroxyl group at C-4, rendering C-4 chiral.

The ^{1}H-^{1}H COSY correlations of H-10/H-11/H-12, H_2-13/H-14/H_2-15 and HMBC correlations from H-9, CH_3-10 and H_2-13 to C-11, and from H-21 to C-15 (Figure 2) established that **1** had a hydroxyl group bonded to C-11 (δ_C 73.7) and a methylene at C-15 (δ_C 38.7), which implied that **1** had lost the C-11 *O*-methyl group and the C-15 methoxyl group in herbimycin A. The NMR spectra of **1** indicated only two methoxy singlet peaks (δ_H 3.45 and 3.33) assigned to C-6 and

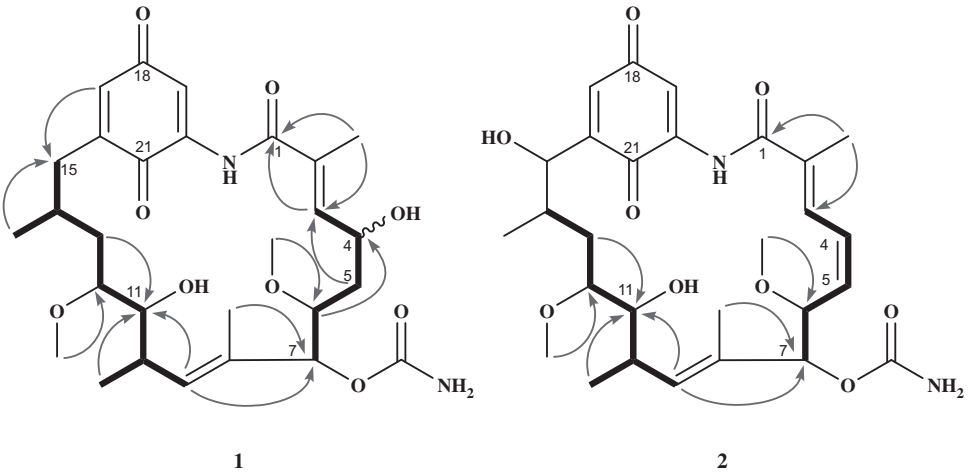


herbimycin A R_1=OCH_3 R_2=OCH_3

herbimycin B R_1=OH R_2=H **1** **2**

herbimycin C R_1=OH R_2=OCH_3

Figure 1 The chemical structures of herbimycins A/B/C, 4,5-dihydro-(4*S*)-4-hydroxyherbimycin B (**1**) and (15*S*)-15-hydroxyherbimycin B (**2**). A full color version of this figure is available at *The Journal of Antibiotics* journal online.



1 **2**

Figure 2 ^{1}H-^{1}H COSY (thick lines) and main HMBC (blue arrows) correlations of **1** and **2.** A full color version of this figure is available at *The Journal of Antibiotics* journal online.

C-12, respectively, by HMBC correlations from 6-OCH_3 to C-6, 12-OCH_3 to C-12, and COSY correlations of H-4/H_2-5/H-6/H-7 and H-9/H-10/H-11/H-12/H_2-13 (Figure 2).

As **1** was co-produced with herbimycin A and shared the PKSs in herbimycin A biosynthesis, the absolute configurations (except at C-4) of **1** and herbimycin A are presumed to be the same. The absolute configuration of C-4 in **1** was determined to be *S* by Mosher's method (Figure 3; Supplementary Figures S14–S19).[6,16] Therefore, the structure of **1** was determined to be 4,5-dihydro-(4*S*)-4-hydroxy-11-*O*-demethyl-15-demethoxyherbimycin A, or 4,5-dihydro-(4*S*)-4-hydroxyherbimycin B (Figure 1). The NMR spectra of **1** are assigned completely in Table 1.

HR-ESI(+)MS (Supplementary Figure S20) established the molecular formula of **2** as $C_{28}H_{38}N_2O_9$ (measured 569.24777 and calculated 569.24695 for $C_{28}H_{38}N_2O_9Na$), which was -C_2H_4 less than herbimycin A ($C_{30}H_{42}N_2O_9$). Compound **2** had the same unsaturation degree as herbimycin A. In addition, **2** exhibited nearly identical UV–vis absorption profile to that of herbimycin A. Therefore, **2** should be a close analog of herbimycin A, probably lacking two methyl groups.

A comparison of the NMR data of **2** (Supplementary Figures S21–S26) with that of herbimycin A confirmed the speculation on the structure of **2**. The ^{1}H-NMR spectrum indicated the presence of only two methoxyl groups in **2** rather than four in herbimycin A. These were assigned as attached to C-6 and C-12 by ^{1}H-^{1}H COSY correlations of H-5/H-6/H-7, H-9/H-10/H-11/H-12 and by HMBC correlations of 6-OCH_3/C-6, 12-OCH_3/C-12 (Figure 2). Thus, **2** is assigned the structure without the two *O*-methyl groups linked to C-11 and C-15, which were added in the post-PKS tailoring in the biosynthesis of herbimycin A. The ^{1}H-^{1}H COSY correlations of H-9/H-10/H-11/H-12/H_2-13/H-14, together with their chemical shifts, indicated that C-11 of **2** carried a hydroxyl group (Figure 2). The C-15 of **2** is also associated with a hydroxyl group from its δ_C 71.7 (78.7 in herbimycin A), although the proton signal of C-15 was not observed. Other sections of the ^{1}H-NMR of **2** were very similar or identical to that of herbimycin A. As **2** was co-produced with herbimycin A and shared the same PKS for biosynthesis, the absolute configurations of chiral carbon atoms (including C-11 and C-15) of **2** are presumed to be the same as in herbimycin A. Thus, the structure of **2** was assigned as 11,15-di-*O*-demethylherbimycin A, or (15*S*)-15-hydroxyherbimycin B (Figure 1). The NMR spectra of **2** are assigned completely as Table 1.

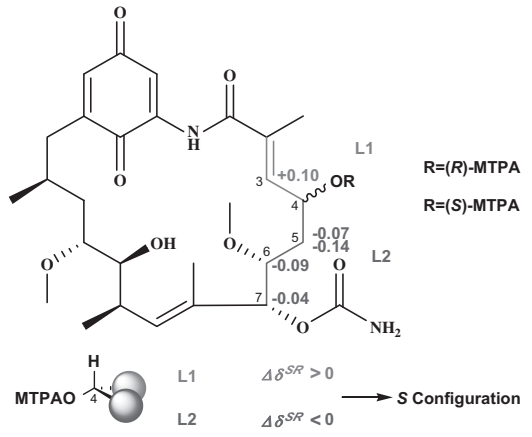


Figure 3 The $\Delta\delta^{SR}$ values of **1*R*** and **1*S*** from Mosher's reactions and the absolute configuration of *Sec*-OH at C-4 in **1**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Nine natural herbimycin analogs (herbimycins A–F, 17,19-dimethylthioherbimycin A, **1** and **2**) have been identified.[9,11] Of them, herbimycin A is the primary major component. Herbimycins B and C could arise from incomplete post-PKS tailoring modifications in the biosynthesis of herbimycin A. Herbimycin D is the result of the addition of cysteine to herbimycin A,[17] and 17,19-dimethylthioherbimycin A is the result of the addition of two methanethiols to herbimycin A.[10] Herbimycins E and F may be shunt products in the biosynthesis of herbimycin A, with some new modifications (Figure 4).

Compound **1** is probably a shunt metabolite in the biosynthesis pathway to herbimycin A without the benefit of the normal post-PKS tailoring enzymes, which would result in C-15 hydroxylation, and C-11 and C-15 *O*-methylations. The mechanism of the apparent C-4,5 stereospecific hydration is more difficult to speculate, as the position of the hydroxyl is such that it cannot have arisen in the normal functioning of a PKS. However, in our previous study of the biosynthesis of geldanamycin (a close analog of herbimycin A), we identified 4,5-dihydro-(4*R*/*S*)-4-hydroxygeldanamycins as a pair of minor products from the geldanamycin producer, *Streptomyces hygroscopicus* 17997.[6]

Table 1 The NMR spectra data of 1 and 2

	1		*2*	
Position	δ_C, *type*	δ_H (J *in Hz*)	δ_C, *type*	δ_H (J *in Hz*)
1	169.6, C	—	168.0, C	—
2	134.4, C	—	135.0, C	—
3	136.6, CH	6.03 dd (9.0, 1.2)	127.2, CH	6.91 br d (11.4)
4	65.2, CH	4.74 m	126.3, CH	6.54 t (11.4)
5	37.1, CH_2	1.93 (2H, m)	136.4, CH	5.87 t (10.2)
6	77.6, CH	3.80 m	80.7, CH	4.32 d (9.0)
7	80.0, CH	5.22 d (3.6)	81.5, CH	5.18 s
8	130.6, C	—	133.6, C	—
9	131.0, CH	5.53 d (9.6)	132.5, CH	5.70 br d (9.6)
10	33.1, CH	2.68 m	32.4, CH	2.79 m
11	73.7, CH	3.53 dd (6.0, 5.4)	72.9, CH	3.55 m
12	81.3, CH	3.42 m	80.7, CH	3.42 m
13	34.6, CH_2	1.74 m, 1.50 m	33.1, CH_2	1.88 (2H, m)
14	29.2, CH	1.67 m	29.7, CH	1.85 m
15	38.7, CH_2	2.66 m, 2.05 m	71.7, CH	—[a]
16	145.2, C	—	146.6, C	—
17	134.8, CH	6.47 d (2.4)	133.2, CH	6.74 s
18	184.0, C	—	184.6, C	—
19	113.5, CH	7.34 d (2.4)	113.5, CH	7.44 d (3.0)
20	139.0, C	—	138.2, C	—
21	188.1, C	—	187.8, C	—
2-CH_3	13.0, CH_3	1.97 s	12.5, CH_3	2.02 s
6-OCH_3	58.5, CH_3	3.45 s	56.8, CH_3	3.37 s
7-$OCONH_2$	156.3, C	—	156.0, C	—
8-CH_3	13.4, CH_3	1.64 s	13.0, CH_3	1.78 s
10-CH_3	14.1, CH_3	0.99 d (7.2)	12.8, CH_3	1.01 d (6.6)
12-OCH_3	56.4, CH_3	3.33 s	57.3, CH_3	3.29 s
14-CH_3	21.8, CH_3	0.95 d (6.6)	14.3, CH_3	0.93 d (6)

^{1}H and ^{13}C NMR spectra data (δ) were obtained at 600 and 150 MHz, respectively, with a Varian (Palo Alto, California, CA, USA) VNS-600 spectrometer, and measured in $CDCl_3$ at room temperature.
[a]Signal not found.

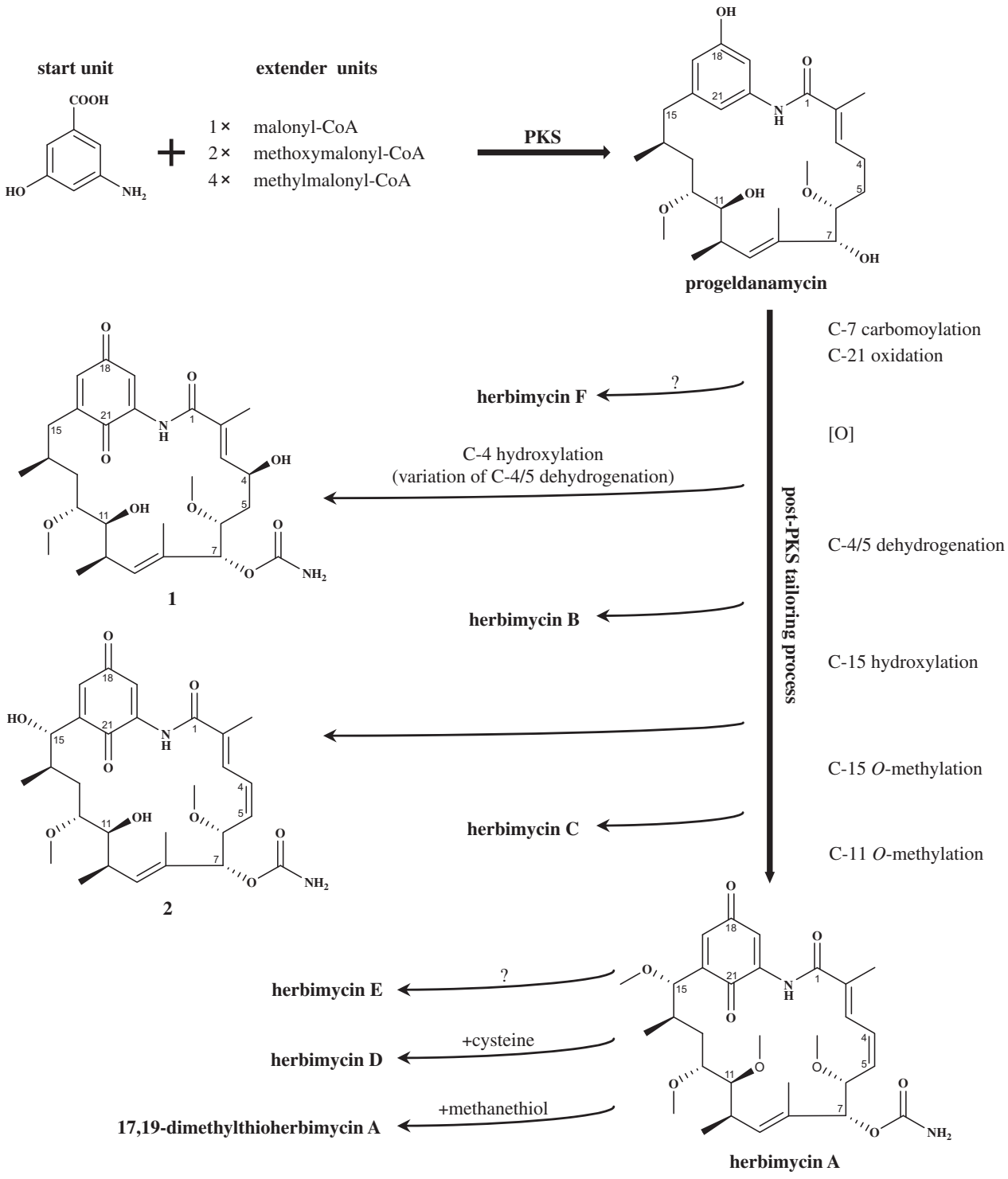


Figure 4 Compounds **1**, **2** and other natural herbimycin analogs (B–F) as shunt or as intermediate products of herbimycin A biosynthesis.

Compound **2** is presumably an intermediate of herbimycin A biosynthesis, lacking both C-11 and C-15 *O*-methylations (Figure 4).

The relative polarity of **1** and **2** compared with other herbimycins suggests greater water solubility. We assayed their water solubility, and found that **1** and **2** had slightly higher water solubility than herbimycin C (0.9422, 0.9418 and 0.8943 mg ml^{-1}, respectively), and >17-fold higher than herbimycin A (0.0512 mg ml^{-1}). This suggests better bioavailability of **1** and **2** than the water-insoluble herbimycin A.

A preliminary cytotoxicity comparison of **1**, **2** and herbimycin A against HCT116 (colon carcinoma cell line), Hela (cervical cancer cell line), A549 (lung cancer cell line) and HepG2 (liver hepatocellular carcinoma cell line) was conducted. Except for **1** showing slightly higher cytotoxicity against HCT116 than herbimycin A (IC_{50}: 24.1 compared with 36.6 μM), **1** and **2** displayed less cytotoxicity than

herbimycin A against all the cancer cell lines tested (Supplementary Table S1).

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1 Ōmura, S. *et al.* Herbimycin, a new antibiotic produced by a strain of *Streptomyces*. *J. Antibiot.* **32**, 255–261 (1979).
2 Supino-Rosin, L., Yoshimura, A., Yarden, Y., Elazar, Z. & Neumann, D. Intracellular retention and degradation of the epidermal growth factor receptor, two distinct processes mediated by benzoquinone ansamycins. *J. Biol. Chem.* **275**, 21850–21855 (2000).
3 Pratt, W. B. The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. *Proc. Soc. Exp. Biol. Med.* **217**, 420–434 (1998).
4 Shibata, K. *et al.* Chemical modification of herbimycin A. Synthesis and in vivo antitumor activities of halogenated and other related derivatives of herbimycin A. *J. Antibiot.* **39**, 415–423 (1986).
5 Ōmura, S. *et al.* Chemical modification and antitumor activity of herbimycin A. 8,9-epoxide, 7,9-cyclic carbamate, and 17 or 19-amino derivatives. *J. Antibiot.* **37**, 1264–1267 (1984).
6 Li, T. *et al.* Identification of 4,5-dihydro-4-hydroxygeldanamycins as shunt products of geldanamycin biosynthesis. *J. Nat. Prod.* **75**, 1480–1484 (2012).
7 Iwai, Y. *et al.* Herbimycin B, a new benzoquinonoid ansamycin with anti-TMV and herbicidal activities. *J. Antibiot.* **33**, 1114–1119 (1980).
8 Shibata, K., Satsumabayashi, S., Nakagawa, A. & Ōmura, S. The structure and cytocidal activity of herbimycin C. *J. Antibiot.* **39**, 1630–1633 (1986).
9 Shaaban, K. A. *et al.* Herbimycins D-F, ansamycin analogues from *Streptomyces* sp. RM-7-15. *J. Nat. Prod.* **76**, 1619–1626 (2013).
10 Raju, R., Piggott, A. M., Khalil, Z., Bernhardt, P. V. & Capon, R. J. Heronamycin A: a new benzothiazine ansamycin from an Australian marine-derived *Streptomyces* sp. *Tetrahedron. Lett.* **53**, 1063–1065 (2012).
11 Li, S. F. *et al.* Methanethiol as a catabolite of methionine provides methylthio- group for chemical formation of 19-S-methylgeldanamycin and 17,19-dimethylthioherbimycin A. *J. Antibiot.* **66**, 499–503 (2013).
12 Xie, L. W. *et al.* Microbial biotransformation of water-insoluble herbimycin A to 11-hydroxy-(11-demethoxy)-herbimycin C by *Eupenicillium* sp. SD017. *J. Mol. Catal. Ser. B* **62**, 76–80 (2010).
13 Buchanan, G. O. *et al.* Production of 8-demethylgeldanamycin and 4,5-epoxy-8-demethylgeldanamycin from a recombinant strain of *Streptomyces hygroscopicus*. *J. Nat. Prod.* **68**, 607–610 (2005).
14 Rascher, A., Hu, Z. H., Buchanan, G. O., Reid, R. & Hutchinson, C. R. Insights into the biosynthesis of the benzoquinone ansamycins geldanamycin and herbimycin, obtained by gene sequencing and disruption. *Appl. Environ. Microbiol.* **71**, 4862–4871 (2005).
15 Lin, L. Z., Blaskó, G. & Cordell, G. A. ^{1}H-NMR analysis of herbimycins and dihydro-herbimycins. *J. Nat. Prod.* **51**, 1161–1165 (1988).
16 Seco, J. M., Quiñoá, E. & Riguera, R. The assignment of absolute configuration by NMR. *Chem. Rev.* **104**, 17–117 (2004).
17 Ni, S. Y. *et al.* Thiazinogeldanamycin, a new geldanamycin derivative produced by *Streptomyces hygroscopicus* 17997. *J. Microbiol. Biotechnol.* **21**, 599–603 (2011).

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