The Journal of Antibiotics (2012) 65, 79–82

www.nature.com/ja

NOTE

17-*O*-demethylreblastatin, a subnormal intermediate in geldanamycin biosynthesis

Changhong Jia, Linzhuan Wu, Siyang Ni, Hongyuan Wang, Xin Liu, Shufen Li, Ling Lin, Weiqing He and Yiguang Wang

The Journal of Antibiotics (2012) **65,** 79–82; doi:10.1038/ja.2011.56; published online 7 December 2011

Keywords: 17-*O*-demethylreblastatin; geldanamycin biosynthesis; *Streptomyces hygroscopicus* 17997

Geldanamycin (GDM), produced by *Streptomyces hygroscopicus*, is a 19-membered macrocyclic lactam related to benzoquinone ansamycins. Interest in GDM increased greatly upon the discovery of its remarkable antitumor properties, but severe hepatotoxicity made it unfitting for direct clinical uses.[1] At present, GDM is a promising lead compound for antitumor drug development. A clear understanding of GDM biosynthesis is of great importance in creating novel GDM analogs by genetic manipulation or combinatorial biosynthesis.

The biosynthesis of GDM involves the assembly of one starter unit (3-amino-5-hydroxybenzoic acid), with seven extender units (one acetate, four propionates and two methoxyacetates). The assembly process forms a polyketide backbone, which then undergoes a post-PKS tailoring process that includes C-7 carbamoylation, C-17 hydroxylation, C-17 methylation, C-21 hydroxylation, C-4,5 oxidation and oxidation of hydroquinone to quinone, to form GDM finally.[2,3] The genes required for GDM biosynthesis have been cloned, sequenced and analyzed from several strains of *Streptomyces*.[3–6]

Up to now, the order of post-PKS tailoring process in GDM biosynthesis is only defined partially.[3,6,7] The C-7 carbamoylation must take place before C-4,5 oxidation. The C-4,5 oxidation takes place at a later time in the post-PKS tailoring process. The order of benzoquinone modifications of the post-PKS tailoring process, in particular, the order of C-17 hydroxylation and C-21 hydroxylation remains unclear. The isolation of reblastatin from *S. hygroscopicus* subsp. *hygroscopicus* SANK61995,[8] a GDM producer, and the isolation of reblastatin-like ansamycins (17-*O*-demethylreblastatin and autolytimicin) from *Streptomyces* sp. S6699 and *S. autolyticus* JX-47,[9,10] seem to suggest that C-17 hydroxylation (and C-17 methylation) take place before C-21 hydroxylation, but biological evidences are needed to prove this point.

We are interested in understanding the biosynthetic details of GDM production. Recently, we reported the characterization of a minor component, 7-*O*-descarbamoyl-7-hydroxygeldanamycin, from the *gdmN* (encoding the enzyme for C-7 carbamoylation) disruption mutant of *S. hygroscopicus* 17997, and provided evidences that C-7 carbamoylation must take place before C-4,5 oxidation in GDM biosynthesis.[7]

Recently, in our monitoring of secondary metabolites of *S. hygroscopicus* 17997, a wild-type GDM (300–400 mg l^{-1}) producer, we found a GDM analog from the mid-stage (40–60 h) fermentation broth(s) of *S. hygroscopicus* 17997 (Supplementary Figures S1 and S2). It was found to exist also in the *gdmP* (encoding the cytochrome P450 monooxygenase for C-4,5 oxidation) disruption mutant of *S. hygroscopicus* 17997 (Figures 1 and 2). The compound disappeared from the fermentation broths in older cultures of *S. hygroscopicus* 17997 or its *gdmP* disruption mutant, coinciding with the slowdown or stop of GDM or 4,5-dihydrogeldanamycin biosynthesis. Besides, we detected the descarbamoylated form of the compound from the *gdmN* disruption mutant of *S. hygroscopicus* 17997 (Supplementary Figure S3).

The compound aroused our attention. We isolated the compound and determined its chemical structure, which is 17-*O*-demethylreblastatin (or 17-hydroxyautolytimicin, compound **3** in Figure 4), a non-benzoquinone GDM analog. In this paper, we reported the re-discovery of 17-*O*-demethylreblastatin and its verification as a subnormal intermediate of GDM biosynthesis in *S. hygroscopicus* 17997; besides, we proposed an order of benzoquinone modifications of the post-PKS tailoring process in GDM biosynthesis.

Frozen stock spores of the *gdmP* disruption mutant of *S. hygroscopicus* 17997 were thawed and spread onto ISPII (0.4% yeast extract, 1.0% malt extract, 0.4% glucose and 1.5% agar power) plates, incubated at 28 °C for 8–10 days, for mycelium growth and sporulation, then slices of the plate culture were picked up as seed and inoculated into the fermentation medium (starch 2 °C, 0.5% cottonseed meal, 0.5% glucose, 1.0% corn steep liquor, 0.5% yeast

Key Laboratory of Biotechnology of Antibiotics of Ministry of Health, Institute of Medicinal Biotechnology, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing, China
Correspondence: Professor L Wu, Key Laboratory of Biotechnology of Antibiotics of Ministry of Health, Institute of Medicinal Biotechnology, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing 100050, China.
E-mail: wulinzhuan@yahoo.com.cn
Received 13 December 2010; revised 7 May 2011; accepted 3 June 2011; published online 7 December 2011

powder, 0.2% $CaCO_3$) for shaking (200 r.p.m.) at 28 °C for 48 h. The fermentation supernatant was extracted with an equal volume of EtOAc. The organic layer was concentrated, then subjected to silica gel TLC for chromatography (developed with a mobile phase of EtOAc/CH_2Cl_2/hexane/MeOH, 9:6:6:1.5, v/v). Upon spraying of 2.0 mol l^{-1} NaOH onto the TLC plate, a method for preliminary discrimination of GDM and its analogs,[11] a red band of R_f=0.16 appeared (for 4,5-dihydrogeldanamycin, R_f=0.66; Figure 1). Upon spraying of $FeCl_3$ (1% $FeCl_3$ and 1% $K_3Fe(CN)_6$, mix before use, 1:1, v/v) onto the TLC plate for detecting compound(s) with phenol moiety, a blue band of the same position appeared. Compound(s) within this band (before color reaction), which aroused our interest, was eluted out by EtOAc extraction, and then dried and re-dissolved in MeOH for LC-ESI(+)-MS analysis (Figure 2). In LC, a major elution peak with a retention time of 16.83 min appeared. In MS^1, the major elution peak contained a principal molecular ion with *m/z* 557 ($[M+Na]^+$), whose fragment ions displayed a MS^2 pattern typical of GDM analogs.[12] On the basis of these results, we believed that the compound of *m/z* 557 was an analog of GDM.

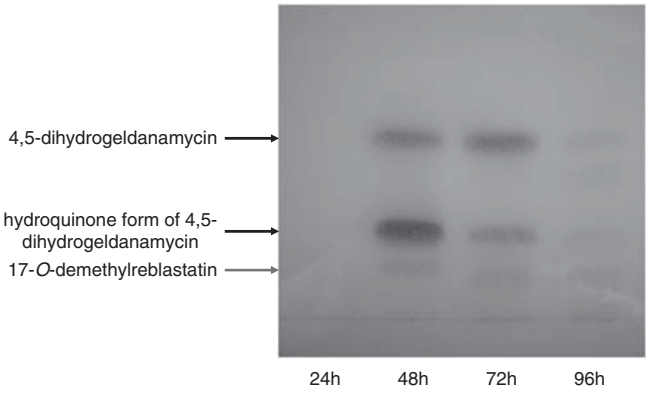

Figure 1 Silica gel TLC of EtOAc extract of fermentation cultures at 24, 48, 72 and 96 h of the *gdmP* disruption mutant of *S. hygroscopicus* 17997 (after color reaction by 2.0 mol l^{-1} NaOH). A red band, which was characterized later as 17-*O*-demethylreblastatin, appeared from the EtOAc extract of fermentation cultures at 48 h. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

The molecular formula of the compound was deduced to be $C_{28}H_{42}N_2O_8$ (exact mass, $[M+Na]^+$, 557.28388) from its accurate mass of 557.28155 ($[M+Na]^+$) by HR-ESI(+)-MS. On the basis of its molecular formula and the possible structures that could possibly appear in the post-PKS tailoring process of GDM biosynthesis, we assumed the compound was either 17-*O*-demethylreblastatin, a known compound that possessed potent inhibitory activity in a cell-based oncostatin M signaling assay and was isolated from *Streptomyces* sp. S6699,[9] or 17-demethoxy-21-hydroxyreblastatin, a putative intermediate of GDM biosynthesis.

The UV profile of the compound exhibited a maximal absorption at 290 nm (Figure 2), at which 17-*O*-demethylreblastatin showed also maximal absorption.[9] As the UV absorption data of 17-demethoxy-21-hydroxyreblastatin is not available for comparison, and its UV profile is supposed to be very similar to that of 17-*O*-demethylreblastatin, the identity of the compound can only be determined by NMR analysis.

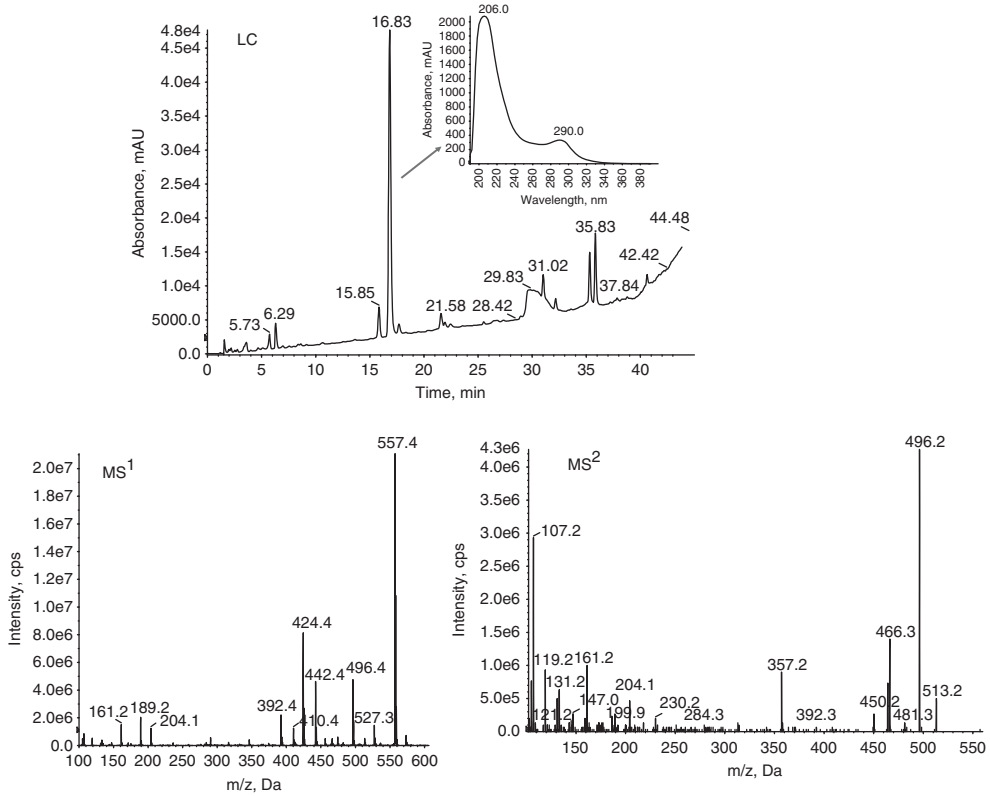

Figure 2 LC-MS analysis of the compound from the *gdmP* disruption mutant of *S. hygroscopicus* 17997. LC: a major elution peak at 16.83 min (Dikma Diamonsil C_{18} column, 5 μm, 150×4.6 mm; gradient elution, 30% MeOH-water to 100% MeOH in 30 min; flow rate, 1 ml min^{-1}; detection wave, 252 nm) had a UV maximal absorption at 290 nm. MS^1 of the elution peak displayed a compound of *m/z* 557 $[M+Na]^+$. MS^2 of *m/z* 557: 557 $[M+Na]^+$, 513 $[M+Na–CO_2]^+$, 496 [base peak, $M+Na–HOCONH_2]^+$, 466 $[M+Na–HOCONH_2–OCH_2]^+$, 161 [C_{2-10}, $^+C(CH_3)$=CH-CH_2-CH_2-CC-C(CH_3)=CH-CH_2CH_3].

The compound was purified for NMR analysis (for the isolation of the compound and its NMR data, see supplementary information). The NMR signals of the compound were in nearly complete accordance with those of 17-*O*-demethylreblastatin;[9] and like 17-*O*-demethylreblastatin, the compound had a low solubility in DMSO-d^6 and its NMR peaks broadened when acquired at room temperature. Besides, the compound is much more stable than hydroquinone 4,5-dihydrogeldanamycin and hydroquinone GDM (with two Ar-OH in *para*-positions, change to the corresponding quinone forms upon exposure to air (O_2) for a few hours at room temperature). Therefore, the compound is determined to be 17-*O*-demethylreblastatin (compound **3** in Figure 4).

The appearance of 17-*O*-demethylreblastatin during GDM production in *S. hygroscopicus* 17997 or its *gdmP* disruption mutant, and its disappearance with the slowdown of GDM or 4,5-dihydrogeldanamycin biosynthesis, made us believe that 17-*O*-demethylreblastatin is an intermediate in GDM biosynthesis. To confirm this speculation, bioconversion of 17-*O*-demethylreblastatin to GDM was conducted in GDM-*pks*$^-$, a GDM polyketide synthase gene disruption mutant of *S. hygroscopicus* 17997, which had a full complement of post-PKS tailoring process genes for GDM biosynthesis but lost the ability to produce GDM. To our expectation, 17-*O*-demethylreblastatin could be bioconversed to GDM in GDM-*pks*$^-$ (Figure 3). GDM appeared evidently after 3-days incubation of 17-*O*-demethylreblastatin in GDM-*pks*$^-$, whereas GDM appeared evidently after 1-day incubation of 4,5-dihydro-7-descarbamoyl-7-hydroxygeldanamycin (a normal intermediate of GDM biosynthesis) in GDM-*pks*$^-$. Besides, the efficiency of 17-*O*-demethylreblastatin to GDM was about 1/2 by visual estimation of silica gel TLC plate after color reaction with 2.0 mol l^{-1} NaOH (half of the 17-*O*-demethylreblastatin added remained unchanged), whereas the efficiency of 4,5-dihydro-7-descarbamoyl-7-hydroxygeldanamycin to GDM was about 100%. Therefore, 17-*O*-demethylreblastatin is not a normal intermediate as we expected originally, but a subnormal (slow and incomplete) intermediate in GDM biosynthesis.

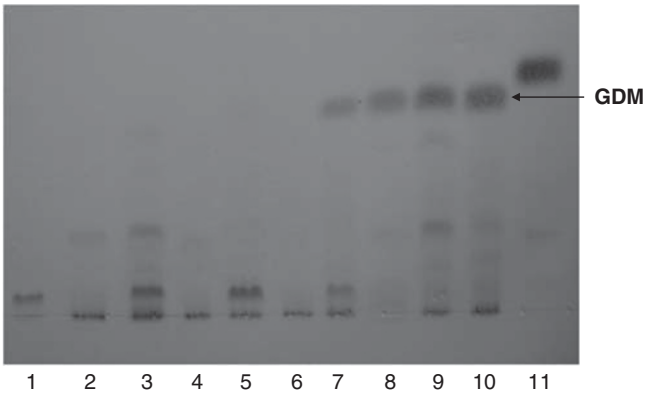

Figure 3 Silica gel TLC of bioconversion product(s) of 17-*O*-demethylreblastatin. Three ISPII plates (each with a diameter of 8.5 cm, containing 25 ml medium) were inoculated with spores of GDM-*pks*$^-$, incubated at 28 °C for 4 days with white aerial mycelia visible evidently. One plate was then overlayed with about 1.0 mg 17-*O*-demethylreblastatin (dissolved in 100 μl dimethylformamide+200 μl H_2O), another with about 3.0 mg 4,5-dihydro-7-descarbamoyl-7-hydroxygeldanamycin (dissolved also in 100 μl dimethylformamide+200 μl H_2O; as positive control), the third without anything as a blank. The plates were incubated at 28° C for another 3 days; and for each day, 1/3 cultures of each plate were cut out for extraction (24 h) with EtOAc. Each EtOAc extraction solution was poured out, air-concentrated to a final volume of about 200 μl. For silica gel TLC detection, 15 μl of each concentrated extraction solution was used. 1, 17-*O*-demethylreblastatin; 2, GDM-*pks*$^-$ (1 day); 3, 17-*O*-demethylreblastatin+GDM-*pks*$^-$ (1 day); 4, GDM-*pks*$^-$ (2 days); 5, 17-*O*-demethylreblastatin+GDM-*pks*$^-$ (2 days); 6, GDM-*pks*$^-$ (3 days); 7, 17-*O*-demethylreblastatin+GDM-*pks*$^-$ (3 days); 8, GDM; 9, 4,5-dihydro-7-descarbamoyl-7-hydroxygeldanamycin+GDM-*pks*$^-$ (1 day); 10, 4,5-dihydro-7-descarbamoyl-7-hydroxygeldanamycin+GDM-*pks*$^-$ (2 days); 11, 4,5-dihydro-7-descarbamoyl-7-hydroxygeldanamycin. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

We analyzed also the metabolites of *S. hygroscopicus* 17997 on ISPII plate with a culture time of 3–4 days at 28 °C. 17-*O*-demethylreblastatin, reblastatin, and 4,5-dihydrogeldanamycin were detected by LC-ESI(+)-MS, in addition to a large amounts of GDM. This may be regarded as another proof that 17-*O*-demethylreblastatin was an intermediate in GDM biosynthesis.

The appearance of 17-*O*-demethylreblastatin as an intermediate in GDM biosynthesis demonstrates that C-17 hydroxylation is able to take place before C-21 hydroxylation in the post-PKS tailoring process of GDM biosynthesis.

We noticed the reports about the characterization of KOS-1806, that is, 17-demethoxy-reblastatin, from the *gel7* (encoding a cytochrome P450 monooxygenase) disruption mutant of *S. hygroscopicus* subsp. *duamyceticus* JCM4427 (a GDM producer), and the *gdmM* (identical to *gel7*) disruption mutant of *S. hygroscopicus* NRRL3602 (another GDM producer). Both of them demonstrated that the enzyme encoded by *gdmM* was involved in the modification(s) of benzoquinone moiety in GDM biosynthesis, but its precise biological function(s) as catalyzing C-17 hydroxylation or C-21 hydroxylation, or even both, remained obscure.[3,6] And no definite conclusion can be drawn regarding the order of C-17 and C-21 hydroxylation in GDM biosynthesis just from the characterization of KOS-1806, which lacks both C-17 and C-21 hydroxyl groups.

The isolation of 17-*O*-demethylreblastatin and its verification as a subnormal intermediate in GDM biosynthesis suggest that C-17 hydroxylation occur after C-21 hydroxylation in the major pathway of GDM biosynthesis, although it may also be able to take place before C-21 hydroxylation to a small degree. On the basis of this understanding, and our unpublished findings that hydroquinone GDM and hydroquinone 4,5-dihydrogeldanamycin appeared as the major/predominant metabolites in mid-stage fermentation cultures of *S. hygroscopicus* 17997 and its *gdmP* disruption mutant, respectively, (Our laboratory's unpublished results: hydroquinone geldanamycin and hydroquinone 4,5-dihydrogeldanamycin were the major/predominant products of *S. hygroscopicus* 17997 and its *gdmP* disruption mutant, respectively, in mid-stage fermentation broths (see Figure 1 and Supplementary Figure S1). The two compounds can be oxidized spontaneously to GDM and 4,5-dihydrogeldanamycin upon exposure to air (O_2). This result is somewhat different to that of Lee *et al.*,[13] who suggested that hydroquinone GDM could not be oxidized spontaneously to GDM.) together with Hong YS's and Shin JC's published results on GDM biosynthesis,[1,2] we proposed a possible and more detailed order of post-PKS tailoring process of GDM biosynthesis, as illustrated in Figure 4, in which C-17 hydroxylation taking place before C-21 hydroxylation was a branched, minor pathway (dashed line), whereas C-17 hydroxylation taking place after C-21 hydroxylation was the major/normal pathway, in GDM biosynthesis.

In the LC-ESI(+)-MS analysis of GDM analogs/derivatives from *S. hygroscopicus* 17997, a putative GDM analog with *m/z* 571 in trace amount was detected (Supplementary Figure S4). It was regarded as the *para*-quinone form of compound **9** in Figure 4 (**9** should be quite unstable and oxidized rapidly before analysis), which may be regarded as another hint that C-17 hydroxylation take place after C-21 hydroxylation.

We conducted a preliminary assay (using sulforhodamine B) of the cytotoxicity of 17-*O*-demethylreblastatin against HepG2 cells.[14]

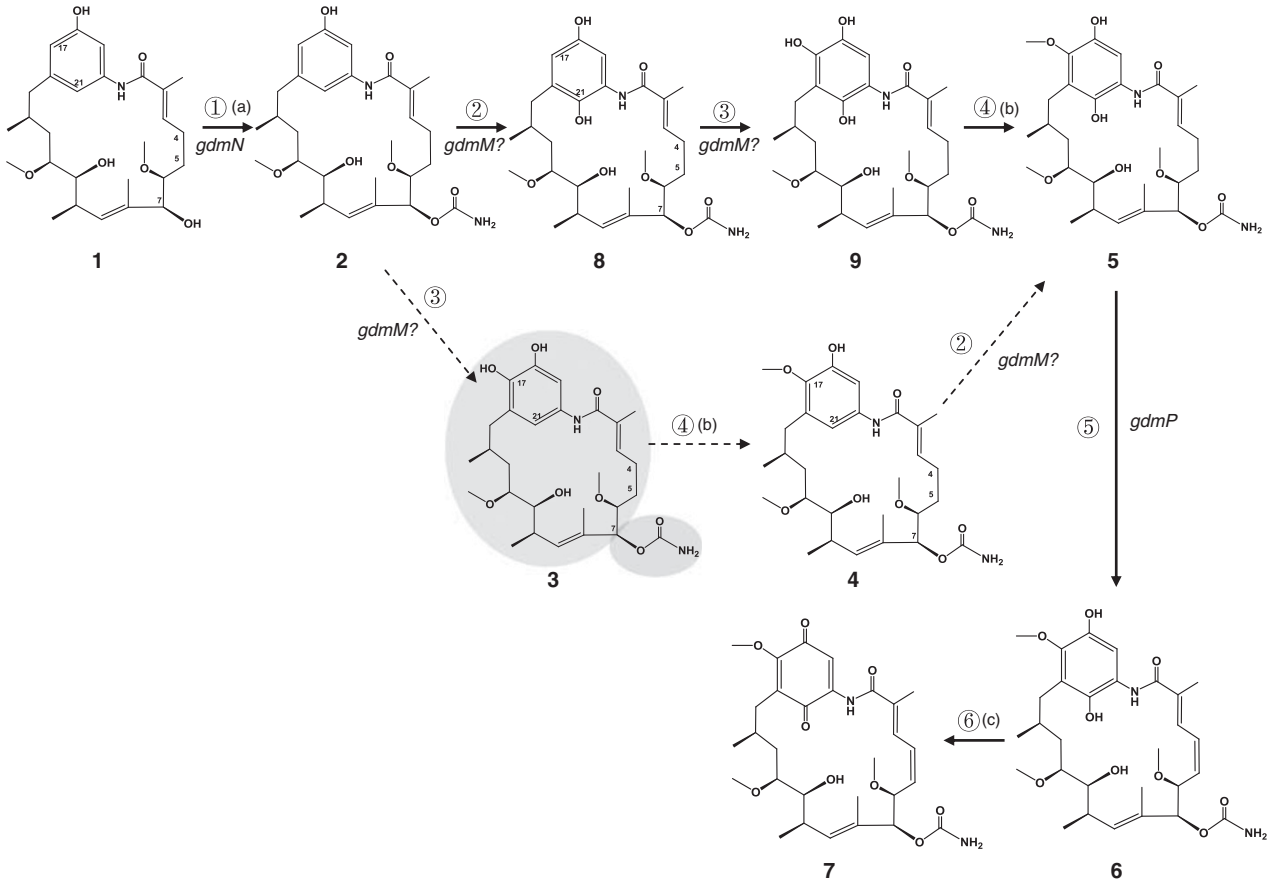

Figure 4 A possible, more detailed order of post-PKS tailoring process in GDM biosynthesis. ① C-7 carbamoylation; ② C-21 hydroxylation; ③ C-17 hydroxylation; ④ 17-*O*-methylation; ⑤ C-4,5 oxidation; ⑥ oxidation of hydroquinone to quinone. **1,** progeldanamycin; **2**, 17-demethoxyreblastatin; **3**, 17-*O*-demethylreblastatin; **4**, reblastatin; **5**, hydroquinone 4,5-dihydrogeldanamycin; **6**, hydroquinone GDM; **7**, GDM; **8**, 17-demethoxy-21-hydroxyreblastatin; **9**, 17-*O*-demethyl-21-hydroxyreblastatin. (a) It may happen at any step before C-4,5 oxidation, (b) occurring position remains uncertain and (c) spontaneous reaction that could happen at any step after C-17 hydroxylation. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

17-*O*-demethylreblastatin exhibited decreased but still very strong cytotoxicity against the hepatoma cells, with an IC_{50} of 5.0 μmol l^{-1} (for GDM, IC_{50}=0.096 μmol l^{-1}). PS: Yin *et al.* reported recently that 17-*O*-methylation was the last step in post-PKS tailoring process of GDM biosynthesis.[15]

ACKNOWLEDGEMENTS

This work was supported by the National S&T Major Special Project on Major New Drug Innovation (Item Number: 2009ZX09501-008 and 2009ZX09301-003).

1 Supko, J. G., Hickman, R. L., Grever, M. R. & Malspeis, L. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother. Pharmacol.* **36,** 305–315 (1995).
2 Hong, Y. S. *et al.* Inactivation of the carbamoyltransferase gene refines post-polyketide synthase modification steps in the biosynthesis of the antitumor agent geldanamycin. *J. Am. Chem. Soc.* **126,** 11142–11143 (2004).
3 Shin, J. C. *et al.* Characterization of tailoring genes involved in the modification of geldanamycin polyketide in *Streptomyces hygroscopicus* JCM4427. *J. Microbiol. Biotechnol.* **18,** 1101–1108 (2008).
4 Rascher, A. *et al.* Cloning and characterization of a gene cluster for geldanamycin production in *Streptomyces hygroscopicus* NRRL 3602. *FEMS Microbiol. Lett.* **218,** 223–230 (2003).
5 He, W. Q. & Wang, Y. G. Cloning and analysis of geldanamycin partial biosynthetic gene cluster of *Streptomyces hygroscopicus* 17997. *Chin. J. Biotech.* **22,** 902–906 (2006).
6 Rascher, A., Hu, Z., 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).
7 Ni, S. *et al.* 7-*O*-descarbamoyl-7-hydroxygeldanamycin, a minor component from the *gdmN* disruption mutant of *Streptomyces hygroscopicus* 17997. *J. Antibiot (Tokyo).* **63,** 623–625 (2010).
8 Takatsu, T., Ohtsuki, M., Muramatsu, A., Enokita, R. & Kurakata, S. I. Reblastatin, a novel benzenoid ansamycin-type cell cycle inhibitor. *J. Antibiot. (Tokyo).* **53,** 1310–1312 (2000).
9 Stead, P. *et al.* Discovery of novel ansamycins possessing potent inhibitory activity in a cell-based oncostatin M signalling assay. *J. Antibiot. (Tokyo)* **53,** 657–663 (2000).
10 Li, M. G. *et al.* Isolation and structure elucidation of autolytimycin, a new compound produced by *Streptomyces autolyticus* JX-47. *Chin. Chem. Lett* **12,** 903–906 (2001).
11 Liu, A. M. *et al.* A color reaction method for early preliminary discrimination of benzenic ansamycins (in Chinese). *Chin. J. Antibiot.* **33,** 403–406 (2008).
12 Lang, W. *et al.* Biotransformation of geldanamycin and 17-allylamino-17-demethoxygeldanamycin by human liver microsomes: reductive versus oxidative metabolism and implications. *Drug. Metab. Dispos.* **35,** 21–29 (2007).
13 Lee, D. *et al.* Biosynthesis of the heat-shock protein 90 inhibitor geldanamycin: new insight into the formation of the benzoquinone moiety. *Chembiochem.* **7,** 246–248 (2006).
14 Li, Y. Y., He, W. Q., Wang, Y. G. & Shao, R. G. A new post-PKS modification process in the carbamoyltransferase gene inactivation strain of *Streptomyces hygroscopicus* 17997. *J. Antibiot (Tokyo).* **61,** 347–355 (2008).
15 Yin, M. *et al.* The missing C-17 *O*-methyltransferase in geldanamycin biosynthesis. *Org. Lett.* (doi:10.1021/ol201383w).

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