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NOTE

7-*O*-descarbamoyl-7-hydroxygeldanamycin, a minor component from the *gdmN* disruption mutant of *Streptomyces hygroscopicus* 17997

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Geldanamycin (GDM) is a 19-membered macrocyclic lactam and is related to ansamycin antibiotics. GDM binds to heat shock protein 90, inhibiting its chaperone activity.[1] GDM and its derivatives have the potential to serve as chemotherapeutic agents in cancer and virus-infected diseases.[2] A clear understanding of GDM biosynthesis should help us to create novel GDM analogs with optimal properties by genetic manipulation of its biosynthetic pathway.

The biosynthetic gene cluster of GDM, including a set of type-I polyketide synthase (PKS) genes, a set of post-PKS tailoring genes and a separate set of genes for biosynthesis of 3-amino-5-hydroxybenzoic acid, the starter unit for GDM biosynthesis, had been cloned, sequenced and analyzed from several streptomycetes independently.[3–5] The post-PKS tailoring genes, such as *gdmN* (encoding the carbamoyltransferase for 7-*O*-carbamoylation), *gdmP* (encoding the cytochrome P450 monooxygenase for C-4,5 oxidation) and *gdmM* (encoding the FAD-binding monooxygenase for C-21 hydroxylation), had been disrupted, respectively, in *Streptomyces hygroscopicus* 17997, *S. hygroscopicus* subsp. *duamyceticus* JCM4427 or other GDM producer(s), to confirm their biological functions in GDM biosynthesis.[6–8] As a result, a brief post-PKS tailoring process of GDM biosynthesis was proposed (Figure 1, thick arrows).[6]

The *gel8* (identical to *gdmN*) disruption mutant of *S. hygroscopicus* subsp. *duamyceticus* JCM4427 produced 4,5-dihydro-7-*O*-descarbamoyl-7-hydroxygeldanamycin (CT-1-7) as major component.[6] The *gdmN* disruption mutant of *S. hygroscopicus* 17997 produced, besides CT-1-7, 4,5-dihydro-7-*O*-descarbamoyl-7-hydroxy-19-*O*-glycylgeldanamycin (CT-1-1), which is a novel GDM analog.[9]

To examine whether there are other undiscovered GDM analogs in the *gdmN* disruption mutant of *S. hygroscopicus* 17997, a time-course monitoring of its fermentation culture was conducted. The fermentation supernatants at different times (36, 60, 84, 108 h, and so on) were extracted with equal volumes of ethyl acetate (EtOAc). The organic layers were concentrated, then subjected to silica-gel TLC (developed with a mobile phase of EtOAc/CH_2Cl_2/hexane/methanol, 9:6:6:1, v/v) and then alkaline color reaction for a preliminary discrimination of GDM analogs.[10] A weak purple band appeared above the blue band of CT-1-7, which was supposed to be a novel GDM analog (at 84 h, Figure 2).

Compounds corresponding to the weak purple band of alkaline treatment were eluted out with EtOAc from silica-gel TLC. A major peak appeared by HPLC analysis, with its retention time being very close to that of CT-1-7, and its UV spectrum profile similar to that of CT-1-7 and very similar to that of GDM (see Supplementary Figure S1). The peak contained a major compound with a molecular weight of 540.5 Da ($[M+Na]^+$), which is two units less than that of CT-1-7 (542.4 Da, $[M+Na]^+$), as analyzed by liquid chromatography-ESI(+)-MS. Based on these data, we suggested the chemical structure of the major compound as 7-*O*-descarbamoyl-7-hydroxygeldanamycin (designated as CT-1-7x), with a molecular formula of $C_{28}H_{39}O_8N_1$.

CT-1-7x was then purified for structural elucidation. An equal volume of EtOAc was used to extract the fermentation supernatant (about 10 l) of *gdmN* disruption mutant of *S. hygroscopicus* 17997 (culture time 84 h), then dried to a crude solid (about 3.042 g) by rotary evaporation at 37 °C. It was then fractionated by silica-gel column chromatography. The pool containing CT-1-7x (eluted by petroleum ether–EtOAc, 75:25, v/v) was dried (30.8 mg) and re-dissolved in methanol for preparative Sephadex LH-20 (φ1.8 cm×150 cm) fractionation, yielding the refined preparation

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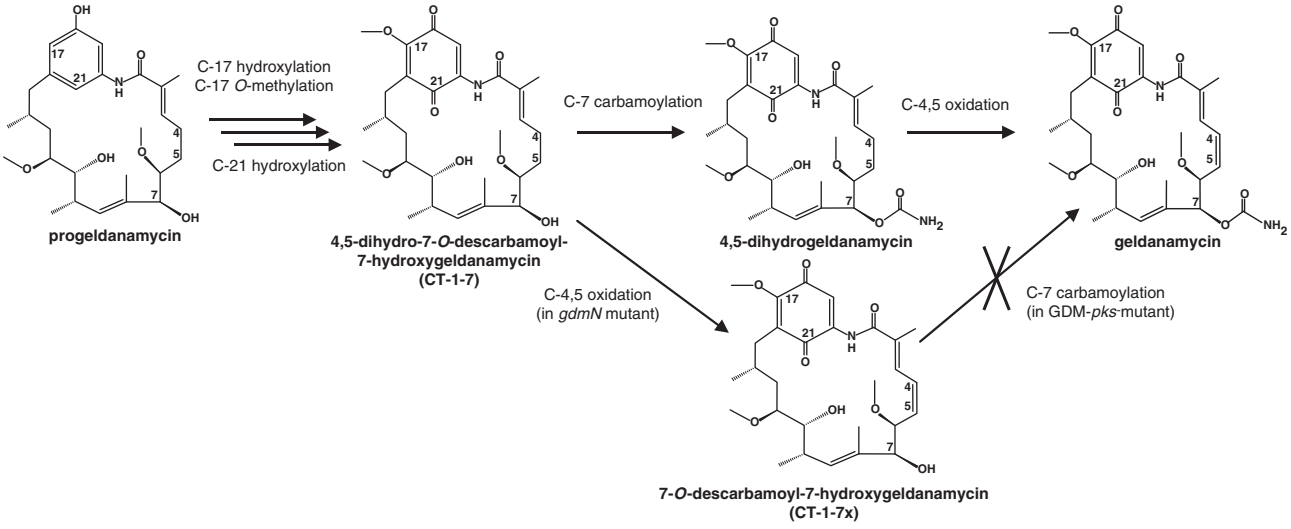

Figure 1 The post-polyketide synthase tailoring process of geldanamycin biosynthesis.

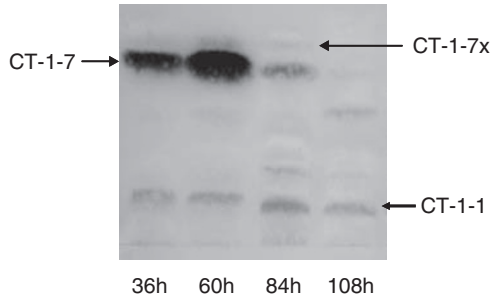

Figure 2 Silica-gel TLC (GF254) of the ethyl acetate (EtOAc) extract of *gdmN* disruption mutant of *Streptomyces hygroscopicus* 17997 at different fermentation times (after the color reaction by 2 mol per liter NaOH). The *gdmN* disruption mutant of *S. hygroscopicus* 17997 was grown at 28 °C for 7–10 days on MY agar (yeast extract 0.4%, malt extract 1.0%, glucose 0.4%, agar power 1.5%) plates (apramycin, 50 μg ml^{-1}) for sporulation, then inoculated into the fermentation medium (starch 2%, cotton seed power 0.5%, glucose 0.5%, corn steep liquor 1.0%, yeast powder 0.5%, $CaCO_3$ 0.2%) for shaking (200 r.p.m.) at 28 °C for different times, and the culture supernatants were extracted with EtOAc. CT-1-7x appeared at a culture time of 84 h with a production level of about 10 mg l^{-1}.

(17.9 mg). The refined preparation was used for preparative HPLC (Shimadzu LC-10ATVp, Shimadzu International Trading (Shanghai) Co., Ltd, Beijing, China; Agilent ZorBax SB-C18, Agilent Technologies Co. Ltd (China), Beijing, China, 5 μm, φ9.4 mm×250 mm, methanol/water, 71:29 (v/v), 1.5 ml min^{-1}), yielding a pure preparation of CT-1-7x (10.7 mg, purity ⩾97%). The pure preparation, an amorphous yellow powder, was used for ^{1}H- and ^{13}C-NMR analyses.

The ^{13}C-NMR and DEPT spectra displayed 28 carbon signals, including 7 methyls, 2 methylenes, 11 methines and 8 quaternary carbons. The ^{13}C-NMR of CT-1-7x showed strong similarities to that of CT-1-7 except for C-4,5. The carbon signals of CH_2-4 (δ_C 23.84) and CH_2-5 (δ_C 29.19) in CT-1-7 were absent, while two additional olefinic methyl signals at δ_C 131.163 and 127.340 emerged in the ^{13}C-NMR of CT-1-7x. The hydrogen signals of CH_2-4 (δ_H 2.24/2.43) and CH_2-5 (δ_H 1.74/1.89) of CT-1-7 disappeared in CT-1-7x, while two additional hydrogen signals of CH- (δ_H 6.590, 6.075) appeared in the ^{1}H-NMR of CT-1-7x. The two newly appeared olefinic methyl signals (CH-4 and CH-5) in CT-1-7x were also confirmed by the DEPT spectra of CT-1-7x. The NMR chemical shifts were assigned completely for CT-1-7x from HSQC, COSY and HMBC (Table 1). Thus, the chemical structure of CT-1-7x was determined to be the C-4,5 olefinic form of CT-1-7, that is, 7-*O*-descarbamoyl-7-hydroxygeldanamycin.

Table 1 NMR data of CT-1-7x[a]

Carbon no.	*δ_H Mult J[b]*	*δ_C*	*COSY*	*HMBC*
1		168.268		
2		136.977		H-2-CH_3, 4, 5
3	6.929, 1H, d, 12	138.488	H-4, 5, 2-CH_3	H-2-CH_3, 4, 5
4	6.590, 1H, t, 11.4	131.163	H-3, 5	H-3, 5, 6
5	6.075, 1H, t, 10.2	127.340	H-4, 6	H-3, 4, 6
6	3.378, 1H, t, over	81.121	H-5, 7	H-4, 5, 7, 6-OCH_3
7	4.225, 1H, d, 9.6	80.629	H-6, 9	H-6-OCH_3, 8-CH_3, 9
8		134.554		H-7, 8-CH_3, 9
9	5.650, 1H, d, 10.2	125.589	H-7, 10	H-7, 8-CH_3, 10
10	2.845, 1H, m, over	32.013	H-9, 10-CH_3,11	H-9, 10-CH_3, 11
11	3.510, 1H, t	72.948	H-10, 12	H-10, 10-CH_3, 12
12	4.024, 1H, d	80.826	H-11, 13	H-11, 12-OCH_3, 13
13	1.746, 2H, m, over	34.138		H-12, 14
14	1.806, H, m, over	27.689	H-13, 15	H-13, 15
15	2.490, 2H, m	32.791		H-14, 14-CH_3,
16		127.684		H-15
17		156.945		H-15, 17-OCH_3
18		184.198		H-19
19		111.671		H-NH
20		138.144		H-19
21		184.885		H-15, 19, NH
2-CH_3	2.070, 3H, s	12.552		H-3
6-OCH_3	3.335, 3H, s	56.588		H-6
8-CH_3	0.985, 3H, d, 6.6	11.937	H-9	H-7, 9
10-CH_3	1.294, 3H, t, 7.2	14.218	H-10	H-10
12-OCH_3	3.392, 3H, s	56.801		H-12
14-CH_3	1.022, 3H, d, 6.6	23.105	H-14	H-14, 13, 15
17-OCH_3	4.161, 3H, s, over	61.709		H-19-$OCOCH_2NH_2$
NH	8.713			

[a]^{1}H- and ^{13}C-NMR spectra were obtained on Bruker Advance 600 MHz (Bruker BioSpin, AG Ltd. (PR China), Beijing, China) with tetramethylsilane (TMS) as internal standard, 2D NMR spectra were obtained on Bruker Advance 600 MHz in $CDCl_3$ at room temperature.
[b]Coupling constants are presented in Hz.

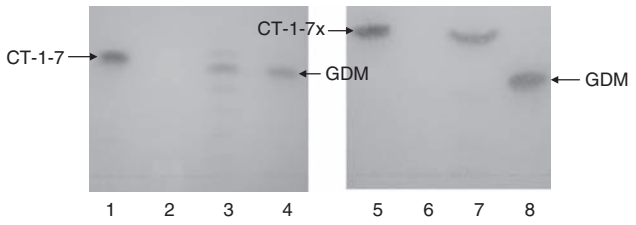

Figure 3 Bioconversion of CT-1-7 and CT-1-7x. CT-1-7 was converted to geldanamycin (GDM) without difficulty by GDM-*pks*-, while CT-1-7x failed to be converted to GDM by GDM-*pks*-. Left: 1, CT-1-7; 2, GDM-*pks*-; 3, CT-1-7+GDM-*pks*-; 4, GDM. Right: 5, CT-1-7x; 6, GDM-*pks*-; 7, CT-1-7x+GDM-*pks*-; 8, GDM. For bioconversion, flasks of 250 ml, each containing 40 ml fermentation medium (starch 2%, glucose 0.5%, cottonseed cake power 0.5%, corn steep liquor 1.0%, yeast power 0.5% and $CaCO_3$ 0.2%) were inoculated with spores of GDM-*pks*-, shaken at 28 °C with 200 r.p.m. for 60 h and then treated with 4 mg CT-1-7 or CT-1-7x (in 100 μl ethyl acetate (EtOAc)) for each flask, and shaken for another 12 h (or for an even longer time of 24–48 h). The culture broth was extracted once with 40 ml EtOAc, the organic phase was evaporated *in vacuo*, and the residue dissolved in 200 μl EtOAc. About 10 μl of each sample was used for silica-gel TLC and then alkaline color reaction to check whether GDM was produced.

CT-1-7x shared the same chemical structure as the compound designated as CP 144365,[11] whose spectroscopic data and references, if any, were not disclosed.

Shin *et al.*[7] proved that a cytochrome P450 monooxygenase encoded by *gdmP* of the GDM biosynthetic gene cluster was involved in the formation of C-4,5 double bonds of GDM in the post-PKS tailoring process of GDM biosynthesis. The discovery of CT-1-7x from the *gdmN* disruption mutant of *S. hygroscopicus* 17997 indicated that the cytochrome P450 monooxygenase encoded by *gdmP* had a relaxed substrate specificity, which is common to enzymes for microbial secondary metabolite biosynthesis.

It seems that the carbamoyltransferase in the post-PKS tailoring process of GDM biosynthesis exhibited also a relaxed substrate specificity, as several GDM biosynthetic intermediates or their analogs with 7-*O*-carbamoylation, such as KOS-1806, reblastatin and 8-demethylgeldanamycin, were discovered or isolated.[12,13] To confirm whether CT-1-7x could be further 7-*O*-carbamoylated (producing GDM), a bioconversion experiment was conducted (Figure 3). Contrary to our expectation, incubation of CT-1-7x with GDM-*pks*$^-$,[8] a GDM's PKS gene disruption mutant of *S. hygroscopicus* 17997 (it lost the ability to produce GDM, but still had a full complement of post-PKS tailoring process genes), resulted in no production of GDM, while incubation of CT-1-7 (as the positive control) with GDM-*pks*$^-$ resulted in the production of GDM as had been reported earlier by Hong *et al.*[6] This result led us to conclude that the carbamoyltransferase in the post-PKS tailoring process of GDM biosynthesis showed a special substrate specificity, that is, the C-4,5 of its substrate(s) being single-bonded. Therefore, 7-*O*-carbamoylation must take place before C-4,5 oxidation in the post-PKS tailoring process of GDM biosynthesis, but the two steps are not necessarily close to each other.

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