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A New Post-PKS Modification Process in the Carbamoyltransferase Gene Inactivation Strain of *Streptomyces hygroscopicus* 17997

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Abstract Genetic manipulation of geldanamycin (GDM) producer *Streptomyces* species is a rational approach to understand biosynthesis processes and create new analogues. In this study, the carbamoyltransferase gene *gdmN* was inactivated by insertion of an apramycin-resistance gene *aac3* (IV) into the genome of the geldanamycin-producing strain *Streptomyces hygroscopicus* 17997. GDM analogues produced by this mutant strain were isolated and characterized, such as new compound 4,5-dihydro-7-*O*-descarbamoyl-7-hydroxy-19-*O*-glycylgeldanamycin. This compound could be converted to compound 4,5-dihydro-19-*O*-glycylgeldanamycin, another new GDM analogue, by a strain of *Streptomyces hygroscopicus* 17997 in which the GDM-*pks* was inactivated. These new compounds exhibited reductions of cytotoxicity against HepG2 cancer cells, but increases of aqueous solubility. These results suggest that a new post-polyketide synthase modification was involved in this process to produce new GDM analogues.

Keywords geldanamycin, carbamoyltransferase gene, gene inactivation, post-polyketide synthase modification, geldanamycin analogues

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Introduction

Geldanamycin (GDM) produced by *Streptomyces hygroscopicus* has been identified as a novel heat shock protein 90 (Hsp90) inhibitor [1, 2] and is a promising drug candidate in combating human tumor [3] and viral diseases [4～6]. Two GDM derivatives, the water-soluble 17-dimethylaminoethy-l7-demethoxygeldanamycin (17-DMAG) and 17-allylamino-17-demethoxy GDM (17-AAG), are currently in phase I and II clinical trials, respectively [7～9].

GDM is a 19-membered macrocyclic lactam that is related to the benzoquinone ansamycins, such as herbimycin and macbecin [10, 11]. Biosynthesis of this class of compounds involves the assembly of 3-amino-5-hydroxybenzoic acid as a starter unit. Following elongation with the acyl-Coenzyme A substrates malonyl-CoA, methylmalonyl-CoA, and 2-methoxymalonyl-CoA (ACP) [12～14], the presumed polyketide intermediate undergoes intramolecular lactamization by GdmF to form progeldanamycin [15], an initial polyketide synthase (PKS) product, which is, then converted to GDM by several post-PKS modification steps, such as C-17 hydroxylation, C-17 *O*-methylation, C-21 oxidation, C-7 carbamoylation, and C-4, 5 oxidation [15～17].

A set of type I PKS genes required for GDM biosynthesis have been cloned, sequenced and analyzed from several *Streptomyces* spp. [15, 19, 20]. Analysis of the GDM biosynthetic gene cluster has revealed the presence of a series of putative post-PKS modifying genes from *S. hygroscopicus* var. *geldanus* NRRL 3602 [15]. The genetic manipulation of the gene or domain of the GDM biosynthetic gene cluster is helpful for understanding the biosynthesis process, and creates new analogues of GDM

[16, 18].

Our previous studies showed that the GDM biosynthetic gene cluster from GDM-producing strain *S. hygroscopicus* 17997 (GenBank accession number: AY179507) exhibited 93% identity to *gdmN* in NRRL 3602 [19]. BLAST search analysis indicated that GdmN is highly homologous to *O*-carbamoyltransferases, NovN, Asm21 and ORF7 [21~23], which are involved in the biosynthesis of novobiocin, ansamitocin and concanamycin A, respectively.

Here we report that the *gdmN* in *S. hygroscopicus* 17997 was inactivated by insertion of an apramycin-resistance gene, *aac3* (IV) [24], and that the known GDM analogue 4,5-dihydro-7-*O*-descarbamoyl-7-hydroxygeldanamycin and new analogue 4,5-dihydro-7-*O*-descarbamoyl-7-hydroxy-19-*O*-glycylgeldanamycin were isolated from this *gdmN* inactivation strain. The C-7 *O*-carbamoylation of GDMCT-1-1 was successfully occurred by bioconversion with a *gdmN* gene-harboring strain. On the basis of the present results, we proposed that a new post-polyketide synthase modification was involved in this process to produce new GDM analogues.

Materials and Methods

Bacterial Strains, Plasmids and Culture Conditions

GDM-producing strain *S. hygroscopicus* 17997 was isolated from soil in China (Institute of Medicinal Biotechnology, Beijing). A GDM-*pks* inactivation strain was constructed by Dr. Weiqing He (Institute of Medicinal Biotechnology, Beijing) [25]. pGH112 vector was kindly provided by Prof. Keqian Yang (Institute of Microbiology, Chinese Academy of Sciences, Beijing) [26]. *Escherichia coli* ET12567/pUZ8002 was used as donor strain for conjugal transfer to *S. hygroscopicus* 17997. pUC18-Am^R (apramycin resistance) used for gene inactivation was constructed in our lab [19] and pKC1139-Km^R (kanamycin resistance) used in the complementation experiment was generated by insertion of a 1.0-kb *Bam*HI - *Eco*RI fragment from pUC119-kan^R (provided by Prof. Huarong Tan, Institute of Microbiology, Chinese Academy of Sciences, China) into the same sites of pKC1139. *E. coli* ET12567/pUZ8002 and *S. hygroscopicus* 17997 were co-cultured in Mannitol soya flour medium (MS) [27]. Media for the culture and fermentation of *S. hygroscopicus* 17997 were used as described by Gao *et al.* [28].

Construction of Plasmids, PCR Primers and Conditions

An internal 949-bp *Eco*RI - *Bam*HI fragment (fragment 1) and a 963-bp *Pst*I - *Xba*I fragment (fragment 2) in *gdmN* gene were obtained by PCR using primers N1~N4: N1 (fragment 1) (5′-CCGGAATTCACGGCCTTGGCCA-GATCC-3′ *Eco*RI), N2 (fragment 1) (5′-CGC-GGATCCATCCACCCCGCCTCGCAC-3′ *Bam*HI), N3 (fragment 2) (5′-AAAACTGCAGGCCGTTGAGGC-TGGAGTT-3′ *Pst*I), and N4 (fragment 2) (5′-CTA-GTCTAGACCGACTGGTTTGGGTGAT-3′ *Xba*I). The fragment 1 and 2 were then ligated with a 1.5-kb *Bam*HI - *Pst*I fragment carrying the apramycin-resistance gene from pUC18-Am^R and then cloned into the *EcoR*I - *Xba*I sites of vector pGH112, to generate the replacement vector pGEX-*gdmN* (Fig. 1). For gene complementation, a *Bgl*II fragment of the intact *gdmN* gene was cloned in the pKC1139-Km^R plasmid to generate the vector pKC-*gdmN*.

PCR reactions were performed with *S. hygroscopicus* 17997 and *gdmN* gene inactivation mutant genomic DNA as templates at 96°C for 4 minutes, followed by 30 cycles of 40 seconds at 94°C and 40 seconds at 59~62°C, 1.5 minutes at 72°C, and an additional 5 minutes at 72°C. The PCR products were analyzed and purified with agarose gel and PCR Clean-Up System (Promega), as described by the manufacturer.

Isolation and Identification of *gdmN* Gene Mutant Strain

Plasmid pGEX-*gdmN* was introduced into *S. hygroscopicus* 17997 by conjugation using *E. coli* ET12567/PUZ8002 as a donor. Conjugation was carried out on MS agar plates incubating at 28°C for 20 hours. Transconjugants were selected after overlaying the plates with 1.0 ml H_2O containing 200 μg/ml nalidixic acid and 50 μg/ml apramycin. Plates were incubated at 28°C for 7~10 days. Single colonies were picked and grown on MY agar plates (4.0 g yeast extract, 10 g malt extract, 4.0 g glucose, 15 g agar, per liter) for several rounds of non-selective incubation. Desired double crossover mutants (Tsr^S and Am^R) were obtained. To confirm the integration of the Am^R gene into the *gdmN* locus, genomic DNA was isolated from a number of transconjugants, and PCR was carried out using primers P1 (5′-CCCAAGCTTCTCGTGGACGGG-TTGCT-3′) and P2 (5′-CTAGTCTAGATCGAACGCC-TCCACCTC-3′).

Fermentation and Purification

The *gdmN* mutant strain and gene complementation strain were grown on MY agar plates for 7 days for sporulation. Cultures were grown in seed medium for 2 days at 28°C, and 3.0 ml was used to inoculate 50 ml of fermentation medium in 500-ml shake flasks, which were cultured at 200 rpm at 28°C for another 3 or 4 days. Fermentation broth was extracted with EtOAc, and the extract was dried to a crude solid by rotary evaporation at 37°C.

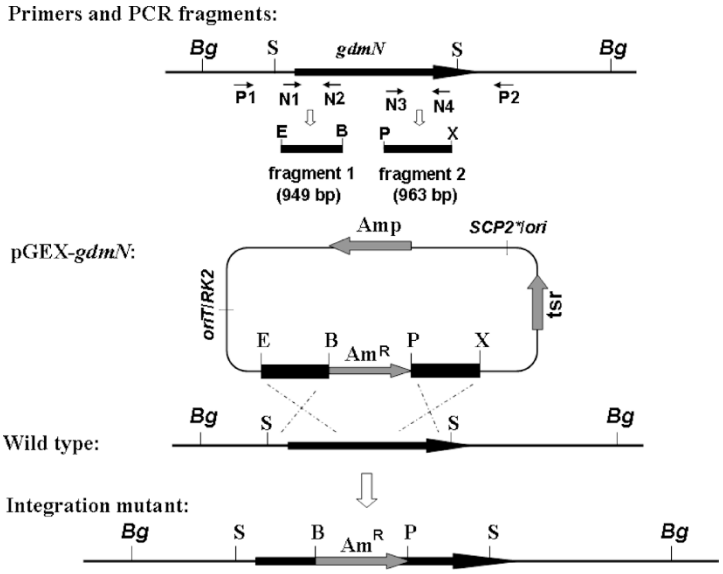

Fig. 1 Inactivation of *gdmN* of the GDM biosynthetic gene cluster.

Am^R, apramycin resistance gene; tsr, thiostrepton resistance gene; B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; P, *Pst*I; S, *Sac*I; X, *Xba*I.

Fractionation was initiated by silica gel chromatography using CH_2Cl_2/MeOH [19 : 1 (v/v, volume in volume)] as the mobile phase, and fractions obtained were pooled based on TLC and HPLC analysis. Pools containing compounds GDMCT-1-1 were further purified by preparative HPLC [Shimadzu ODS-C18; 150×20 mm, MeOH/H_2O, 14 : 11 (v/v), 5.0 ml/minute], to yield GDMCT-1-1 [retention time (Rt) 34.6, 33 mg]. A similar procedure was applied to prepare the standard samples of GDM and 4,5-dihydro-7-*O*-descarbamoyl-7-hydroxygeldanamycin (GDMCT-1-7)

Complementation of *gdmN* Gene

Plasmid pKC-*gdmN* was introduced to the *gdmN* inactivation strain by conjugation. *gdmN* gene complementation strains were grown in fermentation medium containing apramycin (50 μg/ml) and kanamycin (50 μg/ml). Culture broth was extracted with ethyl acetate and analyzed by HPLC.

HPLC was carried out by a Shimadzu LC-6A with a diode array UV detector set at 304 nm, fitted with a Diamonsil^{TM} RP-C18 column (5.0 μm; 4.6×150 mm). Samples were eluted with a gradient (1.0 ml/minute) of MeOH/H_2O (40 : 60 to 100 : 0) over 30 minutes.

Non-enzymatic Conversion Experiment of GDMCT-1-7

10 μl of purified GDMCT-1-7 (80 mg/ml in DMSO) was incubated with 50 ml fermentation medium in 500-ml shake flasks at 200 rpm at 28°C for 3 days. Samples were monitored daily by HPLC and TLC analyzes.

Bioconversion and Purification

The GDM-*pks* inactivation strain was grown on MY agar plates for 7 days for sporulation. Culture was grown in seed medium for 2 days at 28°C, then 5.0 ml seed culture with addition of 10 μl GDMCT-1-1 (80 mg/ml in DMSO) was transferred into 50 ml fermentation medium in 500-ml shake flasks and incubated at 200 rpm at 28°C for 4 days. 4.0 liters fermentation broth was extracted twice with EtOAc and the extract was dried to a crude solid by rotary evaporation at 37°C. Fractionation was initiated by silica gel chromatography using CH_2Cl_2/MeOH [16 : 1 (v/v)] as mobile phase, and fractions obtained were pooled based on TLC and HPLC analysis. Pools containing compounds GDMCT-1-2 were further purified by preparative HPLC [Waters 2996 ODS-C18; 150×20 mm, MeOH/H_2O, 14 : 11 (v/v), 5.0 ml/minute], to yield GDMCT-1-2 (Rt 23.1, 10 mg).

Cytotoxicity Assay of GDM Analogues

The compounds were dissolved in DMSO as 100 mg/ml stock solutions before use and stored at −20°C. Human liver cancer HepG2 cells (the American Type Culture Collection, Rockville, MD) were cultured in MEM medium supplemented with 10% Fetal Bovine Serum. HepG2 cells were maintained in a 37°C, 5.0% CO_2 humidified incubator. For cytotoxicity assays, 5000 of HepG2 cells were plated in 100 μl per well into 96-well micro titer plates. Cells were allowed to adhere for 24 hours. Each compound ranging from 0.1 to 100 μg/ml in 100 μl was added to cells in duplicate wells. After 24 hours incubation,

cell viability was determined by sulforhodamine B (SRB) assay [29]. IC_{50} is defined as the concentration of compounds required for inhibiting cell growth by 50%.

Water Solubility Test of GDM Analogues

Standard curves of relationship between OD251 and OD304 values and concentrations of GDM analogues in MeOH were established. Saturated aqueous solution of GDM analogues was made in 50 mM PBS at pH 7.0 with magnetic stir at room temperature for 12 hours in dark, and then centrifuged at 100,000 *g* for 10 minutes at 4°C. Supernatant were measured by BECKMAN DU® 800 nucleic acid/protein analyzer at OD251 (GDMCT-1-1 and GDMCT-1-2) and OD304 (GDMCT-1-7 and GDM). The concentration of the saturated aqueous solutions was determined according to standard curves.

Results

Inactivation of *gdmN* Gene

Inactivation of *gdmN* in *S. hygroscopicus* 17997 was performed by insertion of the Am^R gene into the *gdmN* locus as shown in Fig. 1, and confirmed by PCR analysis using P1 and P2 primers. Predicted sizes (3.1-kb and 4.5-kb) of PCR products were obtained using the genome DNA of *S. hygroscopicus* 17997 and *gdmN* inactivation strain as templates, respectively. Complementation analysis was performed to ensure the phenotypic change of the *gdmN* inactivation strain was solely caused by the inactivation of *gdmN*.

HPLC analysis showed that the ability of GDM production was completely lost by *gdmN* inactivation (Fig. 2B) and restored by its complementation (Fig. 2C). In contrast, two major components (GDMCT-1-1 and GDMCT-1-7) with Rt 12.9 and 25.8 minutes were observed in the *gdmN* inactivation strain (Fig. 2B). It is interesting to note that the ratio of GDMCT-1-1 and GDMCT-1-7 produced by the *gdmN* inactivation strain was changed in a time dependent manner. As shown in Fig. 3, GDMCT-1-7 was produced early and then gradually declined after 3 days fermentation, meanwhile GDMCT-1-1 had significantly accumulated during day 4 and day 5.

Structural Elucidation of GDMCT-1-7 and GDMCT-1-1

Compound GDMCT-1-7 displayed ESI-MS patterns resembling those of GDM. The ^{1}H-NMR, ^{13}C-NMR and MS data were identical with those of compound **3** [20] and were consistent with the molecular formula $C_{28}H_{41}N_1O_8$ obtained by negative HRFAB-MS ($[M-H]^-$, *m/z* 518.2740). Accordingly, this compound is the 4,5-dihydro-7-*O*-descarbamoyl-7-hydroxygeldanamycin (GDMCT-1-7) being the main product of the carbamoyltransferase gene inactivation strain of *S. hygroscopicus* JCM4427 as

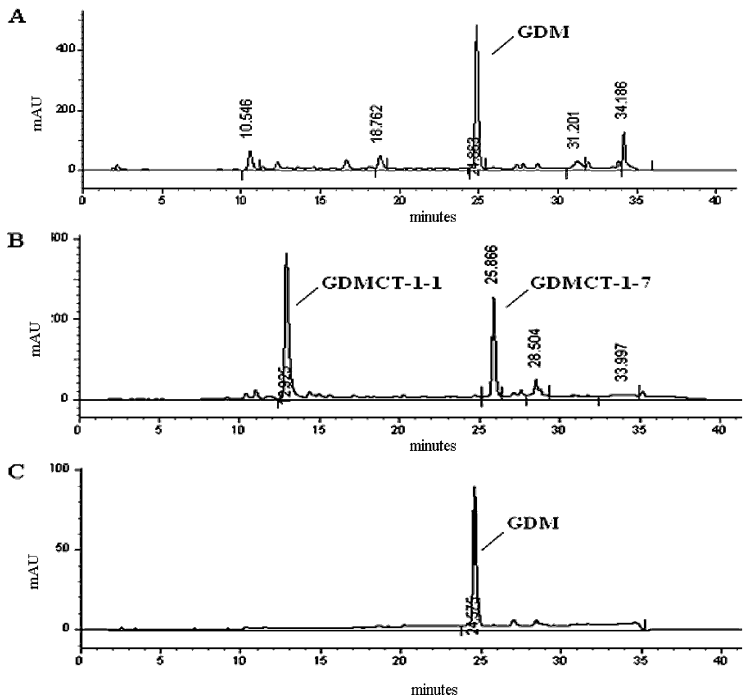

Fig. 2 HPLC analysis of GDM analogues produced by wild-type and *gdmN* inactivation strains of *S. hygroscopicus* 17997. A, wild-type; B, *gdmN* inactivation strain; C, *gdmN* complement.

reported by Hong *et al.* [20].

An analysis of the 1D and 2D NMR spectra of GDMCT-1-1 suggested that it is a derivative of GDM. From the ^{1}H- and ^{13}C-NMR spectra of GDMCT-1-1, the upfield shift of C-7 signals at δ_H 3.87 and δ_C 79.69 indicated that GDMCT-1-1 has a free hydroxyl group at C-7 rather than a carbamoyl group, as expected. Furthermore, two olefinic methine signals (C-4 and C-5) of GDMCT-1-1 were not detected, suggesting that its *cis* double bond had been hydrogenated. The signal of C-19 hydrogen was not detected, but an additional carbonyl signal (δ_C 165.52) and a secondary carbon (δ_C 29.21) hydrogen (δ_H 3.55) signal which has correlation with the C-19 carbon signal (δ_C 117.90) (Fig. 4) of the benzene ring as supported by HMQC (heteronuclear multiple quantum coherence) and HMBC (heteronuclear multiple bond coherence) spectra appeared, this suggesting that there is a *O*-glycyl group at C-19. A combination of COSY (combination of correlation spectroscopy), NOESY (nuclear overhauser effect spectroscopy), NMR and IR (infrared spectroscopy) data were used to assign the ^{1}H- and ^{13}C-NMR data unambiguously (Table 1). Accordingly, the structure of this new metabolite was elucidated as 4,5-dihydro-7-*O*-descarbamoyl-7-hydroxy-19-*O*-glycylgeldanamycin (GDMCT-1-1) with molecular formula $C_{30}H_{44}N_2O_{10}Na$ which was consistent with results obtained by positive HRFAB-MS (*m/z* 615.2996).

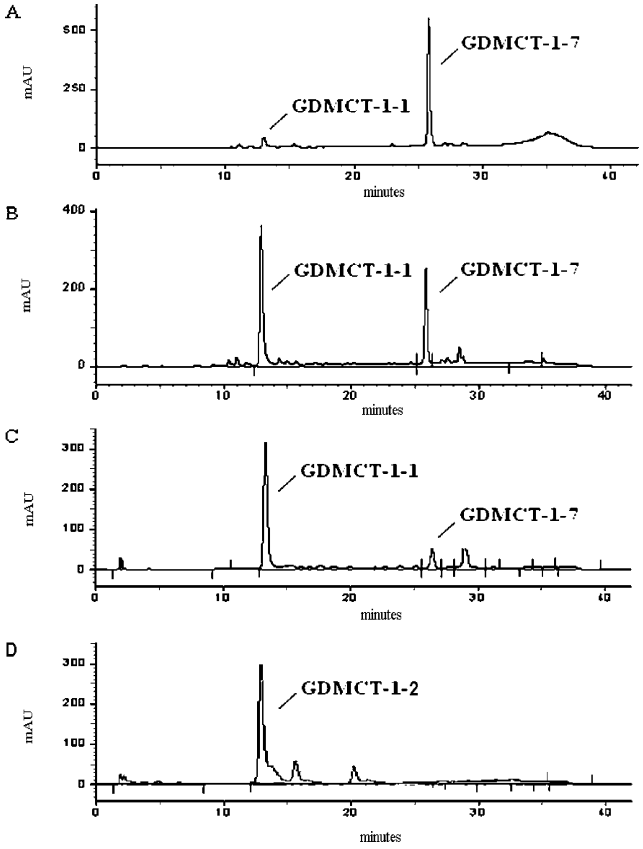

Fig. 3 HPLC analysis of GDM analogues produced by *gdmN* inactivation strain of *S. hygroscopicus* 17997 at the fermentation times of 3 (A), 4 (B) and 5 (C) days, and bioconversion of GDMCT-1-1 for 4 days (D).

Microbial Conversion of GDMCT-1-1

In order to clarify the possible C-7 carbamoylation of GDMCT-1-1, a GDM-*pks* inactivation strain of *S. hygroscopicus* 17997, which did not produce GDM but retained the *gdmN* encoding enzyme activity, was used in the microbial conversion of GDMCT-1-1. The results indicated that a compound GDMCT-1-2 was detected with Rt 12.3 minutes by HPLC analysis (Fig. 3D).

Compound GDMCT-1-2 displayed ESI-MS patterns that

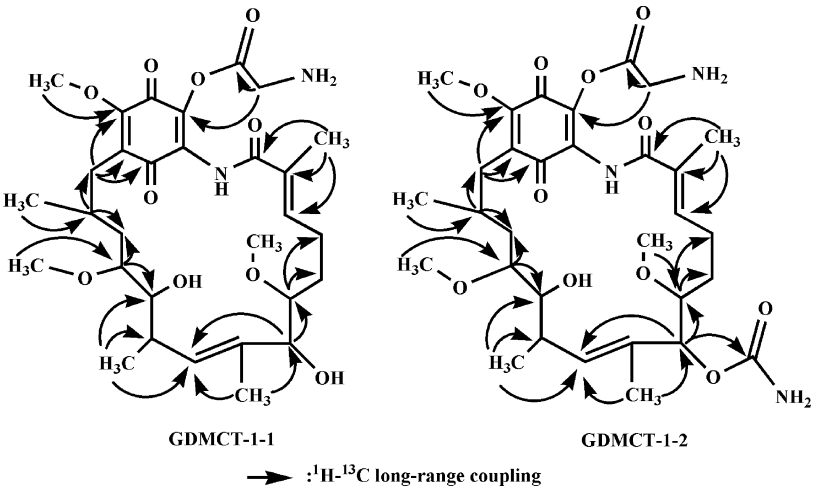

Fig. 4 ^{1}H-^{13}C HMBC correlations analysis of GDMCT-1-1 and GDMCT-1-2.

Table 1 ^{1}H- and ^{13}C-NMR data of compound GDMCT-1-1 (500 MHz) and GDMCT-1-2 (600 MHz)

Position	δ_H (CD_3OD)		δ_C (CD_3OD)	
	GDMCT-1-1	GDMCT-1-2	GDMCT-1-1	GDMCT-1-2
1	—	—	176.23	177.61
2	—	—	129.88	131.31
2-CH_3	1.80	1.73	12.21	13.57
3	5.62	5.51	133.44	134.52
4	2.05/1.99	1.89/2.02	23.40	24.56
5	1.08/0.98	0.88/0.98	29.90	31.09
6	3.06	2.98	81.92	82.50
6-OCH_3	3.44	3.36	57.58	59.39
7	3.87	4.86	79.69	81.92
8	—	—	133.44	131.12
8-CH_3	1.45	1.38	10.41	12.37
9	5.04	5.05	130.70	134.01
10	2.45	2.35	34.74	36.23
10-CH_3	1.08	1.04	17.33	18.73
11	3.65	3.50	72.94	74.05
12	3.14	2.47	81.24	82.06
12-OCH_3	3.33	3.24	55.41	56.77
13	1.76/0.98	1.70/0.88	32.15	33.23
14	2.05	1.92	31.33	32.37
14-CH_3	0.7	0.61	16.39	17.20
15	3.06/2.58	2.97/2.50	29.90	31.45
16	—	—	120.04	121.23
17	—	—	146.70	148.10
17-OCH_3	3.69	3.57	60.01	61.40
18	—	—	120.03	121.67
19	—	—	117.90	119.10
20	—	—	123.20	124.66
21	—	—	148.62	150.08
7-O$\underline{C}ONH_2$	—	—	—	159.02
19-O$\underline{C}OCH_2NH_2$	—	—	165.52	166.85
19-OCO$\underline{CH_2}NH_2$	3.55/3.33	3.47/3.21	29.21	30.50

resembled those of GDMCT-1-1. An analysis of the 1D and 2D NMR spectra of GDMCT-1-2 suggested that it was a derivative of GDMCT-1-1. From the ^{1}H- and ^{13}C-NMR spectra of GDMCT-1-2, the upfield shift of C-7 signals at δ_H 4.86 and δ_C 81.92 indicated that GDMCT-1-2 had a carbamoyl group at C-7 rather than a free hydroxyl group (Fig. 4), as expected; this was coincident with the new carbonyl C signal at δ_C 159.02. These results were consistent with the molecular formula $C_{31}H_{45}N_3O_{11}Na$ obtained by positive HRFAB-MS (*m/z* 658.2972). A combination of COSY, HMQC, HMBC and NMR data were used to assign the ^{1}H- and ^{13}C-NMR data unambiguously (Table 1). Compared to GDMCT-1-1, the structure of this new metabolite was elucidated as 4,5-dihydro-19-*O*-glycylgeldanamycin (GDMCT-1-2).

Table 2 Solubility and cytotoxicity of GDM analogues

Compound	Solubility (mg/ml)	IC_{50} (μM)
GDM	0.017	0.59
GDMCT-1-1	0.402	277.89
GDMCT-1-2	0.573	302.30
GDMCT-1-7	0.039	24.55

Water Solubility and Cytotoxicity of GDM Analogues

As shown in Table 2, the results of the solubility assay showed that the solubility of GDMCT-1-1 and GDMCT-1-2 was approximately 23-fold higher than that of GDM in aqueous solution.

The cytotoxicity of the compounds against HepG2 cells was investigated. As shown in Table 2, the results indicated that the cytotoxicity of GDMCT-1-1 and GDMCT-1-7 was almost 41 to 471-fold lower than that of GDM.

Discussion

To our best knowledge, this is the first report that GDMCT-1-1 and GDMCT-1-2 were produced by engineered biosynthesis. The new compound GDMCT-1-1 was purified from the fermentation broth of the *gdmN* inactivation strain, which was not detected in wild-type *S. hygroscopicus* 17997. It has been reported that this compound is not produced in the same gene (*gel*8) inactivation strain of *S. hygroscopicus* JCM4427 [20].

It is notable that inactivation of *gdmN* gene in *S. hygroscopicus* 17997 resulted in production of GDMCT-1-1 (Fig. 3A∼C). The accumulation of descarbamoylated compounds GDMCT-1-7 and GDMCT-1-1 in the *gdmN* inactivation strain of *S. hygroscopicus* 17997 confirmed that the *gdmN* encodes a carbamoyltransferase. We observed that GDMCT-1-7 was produced at an early stage, and then declined simultaneously with an increase of GDMCT-1-1 (Fig. 3A∼C), suggesting that GDMCT-1-7 might serve as a precursor for the biosynthesis of GDMCT-1-1 at least in the *gdmN* inactivation strain of *S. hygroscopicus* 17997. GDMCT-1-1 could be converted to GDMCT-1-2 by the *gdmN* containing strain of *S. hygroscopicus* 17997.

In our study, GDMCT-1-1 was not monitored in the nonenzymatic conversion experiment of GDMCT-1-7 (data not shown), indicating that GDM C-19 modification enzymes (glycyltransferase or a bifunctional C-19 hydroxy/glycyltransferase) were possibly involved in GDMCT-1-1 biosynthesis in the *gdmN* inactivation strain of *S. hygroscopicus* 17997. It has been reported that an *N*-glycyltransferase is discovered in *S. sannanensis* IFO 14239 (sannamycin-producer), and the gene sequences of similar glycyltransferase have been detected in other aminoglycoside antibiotic producers by DNA hybridization [30]. Up to now, there has been no report that the biological modification of GDM analogues at C-19, although this

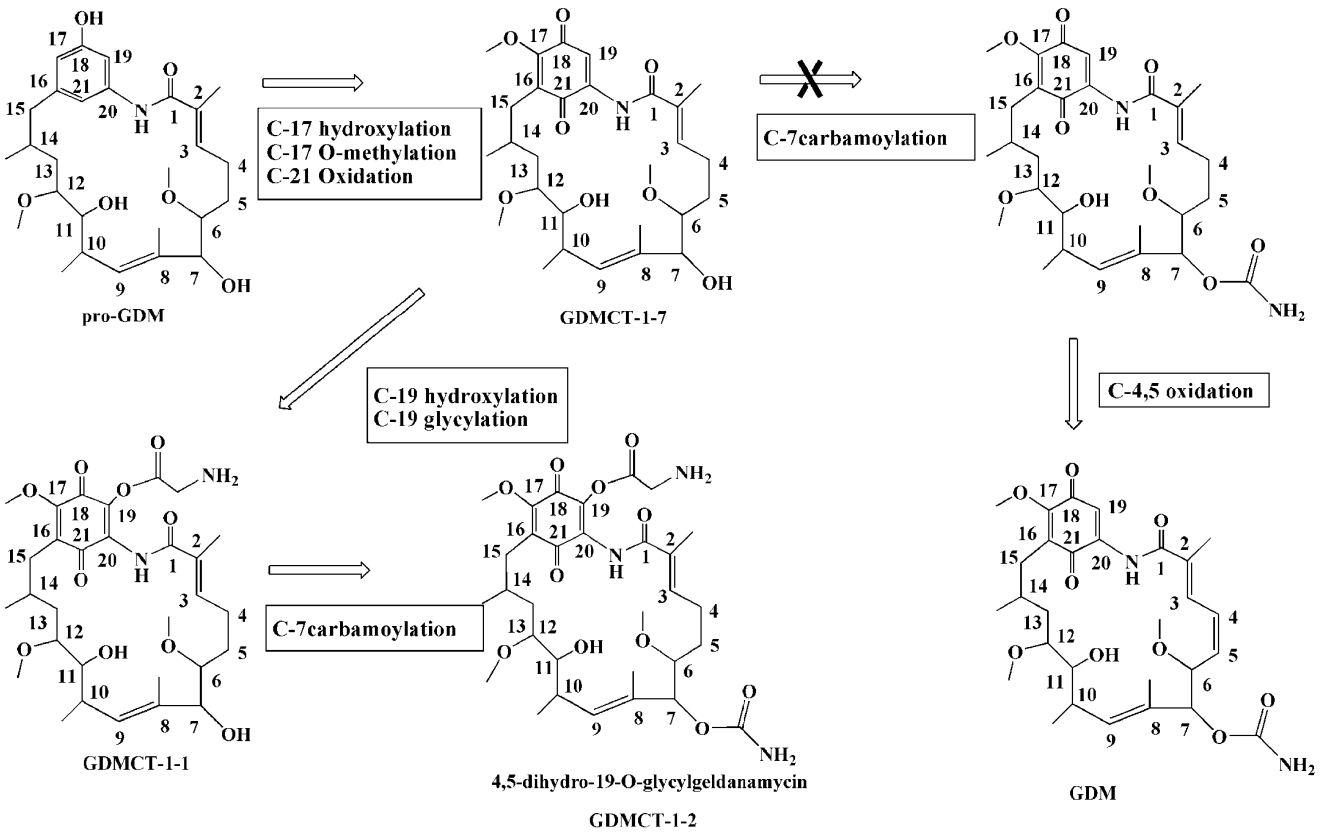

Fig. 5 The proposed post-PKS modification process of GDM analogues biosynthesis.

position can be nonenzymatically modified by glutathione [31]. GDMCT-1-1 was not observed in the wild-type *S. hygroscopicus* 17997 strain, because its intermediate product (GDMCT-1-7, a possible substrate of the putative glycyltransferase) is rapidly carbamoylated by GdmN, and therefore is not available for latter glycylation reaction. The conversion of GDMCT-1-7 to GDMCT-1-1 in the *gdmN* inactivation strain fermentation process may suggest that this is likely a spurious cellular event caused by a series of enzymes that are not directly involved in the biosynthesis of GDM. The mechanism of the *O*-glycylation of GDMCT-1-7 C-19 will be investigated in further experiments of gene expression and purified enzymes.

It is interesting that the GDM analogues GDMCT-1-7, GDMCT-1-1 and some other precursors [32] can be recognized by C-7 carbamoylase, which indicates that the C-7 carbamoylation enzyme possesses a rather broad substrate specificity. GDMCT-1-1 and GDMCT-1-2 do not carry a double bond at the C-4 and C-5 positions, the possible reason for the inhibition of C-4, 5 oxidation of GDMCT-1-1 and GDMCT-1-2 is currently undergoing investigation. Taken together, we speculate a new post-PKS modification process of GDM analogue biosynthesis in *S. hygroscopicus* 17997 as described in Fig. 5.

So far, the effective chemical modification of GDM is mostly restricted to one region of the compound, the C-17 position of the benzoquinone moiety [33～35]. However, due to the occurrence of resistance and the hepatotoxicity of the current analogues, new GDM analogues with improved efficacy, reduced toxicity and favorable pharmacological profiles are in great demand [33, 36]. Consequently, clear understand of GDM biosynthesis and the discovery of new post-tailoring enzymes should be useful for the development of new analogues.

Although the two new compounds GDMCT-1-1 and GDMCT-1-2 showed reductions of cytotoxicity against HepG2 cells (Table 2), they exhibited a notable increase of water-solubility relative to GDM and GDMCT-1-7 (Table 2), which is an important characteristic for the innovation of GDM-derived compounds. Our results provide a new modification position of GDM and contribute to better understanding of GDM biosynthesis, and this would provide valuable information for the design of new GDM analogues with enhanced biological activity.

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