New Geldanamycin Analogs from *Streptomyces hygroscopicus*

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Geldanamycin (GM) and its analogs are important anticancer agents that inhibit heat shock protein (Hsp) 90, which is a major chaperone protein in cancer cells. Accordingly, based on interest in obtaining novel natural GM derivatives, the potential of *Streptomyces hygroscopicus* JCM4427, a GM producer, was explored for novel natural GM derivative(s), resulting in the discovery of new GM analogs as a biosynthetic shunt product and intermediates from its fermentation broth. In this study, the fermentation, isolation, structure determination, and biological activity of the compounds, two new tetracyclic thiazinogeldanamycin (1) and 19-hydroxy-4,5-dihydrogeldanamycin (3), together with the three known 4,5-dihydrothiazinogeldanamycin (2), reblastatin (4), and 17-demethoxy-reblastatin (5), are described.

**Keywords:** Geldanamycin, natural products, biosynthetic shunt product

Geldanamycin (GM), an anticancer antibiotic, was first isolated in 1970 from *Streptomyces hygroscopicus* var. *geldamus* var. *nova* [2]. GM is a 19-member macrocyclic lactam that binds to the N-terminal ATP binding pocket of heat shock protein 90 (Hsp90) and inhibits its ATP-dependent chaperone functions [18]. The molecular chaperone Hsp90 is responsible for maintaining correct folding and stability of many signaling proteins, and is emerging as an important target in cancer therapeutics [7, 12]. The biosynthesis of this class of compounds involves the assembly of 3-amino-5-hydroxybenzoic acid as a starter unit, followed by the addition of extender units such as acetate, propionate, and glycolate to form a polyketide backbone [14]. The nascent polyketide synthase (PKS) product is then converted to GM by the post-PKS modification steps, which include C-17 hydroxylation, C-17 O-methylation, C-21 oxidation, C-7 carbamoylation, and C-4,5 oxidation. In our previous studies, we identified the GM biosynthetic gene cluster in *S. hygroscopicus* subsp. *duamycticus* JCM4427 and characterized the GM biosynthetic pathway [4, 15, 20]. Moreover, during these studies, we reported novel GM analogs from several genetically engineered strains, and subsequently their biological potentials were evaluated [3, 8, 9, 19]. The re-engineering of pathways for such secondary metabolites to make novel molecular variants will be enabled by the understanding of the chemical logic and protein machinery in the producer microbes. In chemical diversity terms, variation of cultivation parameters also induces the production of formerly unidentified compounds such as a biosynthetic intermediate or shunt product. Moreover, an approach to the improvement of fermentation to obtain maximum production titers of desired compound brings about unexpected analogs of the main product. Accordingly, based on interest in obtaining novel natural GM derivatives, the potential of *S. hygroscopicus* JCM4427, a GM producer, was explored for novel natural GM derivative(s), resulting in the discovery of new GM analogs as a biosynthetic shunt product and intermediates from its fermentation broth. In this paper, we report the fermentation, isolation, structure determination, and biological activity of the compounds, two new tetracyclic thiazinogeldanamycin (1) and 19-hydroxy-4,5-dihydrogeldanamycin (3), together with the three known 4,5-dihydrothiazinogeldanamycin (2), reblastatin (4), and 17-demethoxy-reblastatin (5) (Fig. 1). *S. hygroscopicus* JCM4427 was obtained from the Japanese Culture Collection of Microorganisms. Fermentation was carried out in a liquid culture medium containing YEME medium (sucrose 103.0 g, yeast extract 3.0 g, peptone (Difco) 5.0 g, malt extract (Difco) 3.0 g, glucose 10.0 g, and MgCl₂·6H₂O 1.0 g in 1 L of distilled water) [4]. Spores that developed during growth on ISP4 medium were harvested and inoculated into a 1,000 ml baffled flask containing 300 ml of the YEME medium and cultured on a

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rotary shaker operating at 160 rpm for 3 days at 28°C. Equal volumes of the seed culture [final 5% (w/w)] were inoculated into 50 baffled flasks containing 300 ml of YEME medium and cultured on a rotary shaker at 160 rpm for 7 days at 28°C. The resulting culture (approximately 15 L) was extracted twice with an equal volume of ethyl acetate (EtOAc). The EtOAc-soluble material (15.0 g) was subjected to silica gel chromatography using a stepwise gradient elution of mixtures of CH₂Cl₂ and methanol (MeOH). The fraction eluted with CH₂Cl₂:MeOH (85:15) was further purified by an octadecyl silica (ODS)-A gel (YMC, Kyoto, Japan) open column to yield 10 fractions using a stepwise gradient elution with MeOH (50–100%). The second fraction eluted was further purified by reverse-phase high-performance liquid chromatography (HPLC) using YMC-J’sphere ODS-H80 (10 × 250 mm, 3 ml/min) with a linear gradient from 25% to 100% acetonitrile (MeCN) containing 0.05% trifluoroacetic acid (TFA) to yield the compounds 1 and 2 mixture (36 mg). Compounds 1 (12 mg) and 2 (18 mg) were further purified by reverse-phase HPLC using YMC-J’sphere ODS-H80 (10 × 250 mm, 3 ml/min; YMC, Kyoto, Japan) with 30% MeCN containing 0.05% TFA. The fourth fraction eluted was further purified by reverse-phase HPLC using YMC-J’sphere ODS-H80 (10 × 250 mm, 3 ml/min) with a linear gradient from 40% MeCN containing 0.05% TFA to yield compound 3 (16 mg). Similarly, the third fraction was also purified by HPLC (YMC, Kyoto, Japan) with 30% MeCN containing 0.05% TFA to yield compounds 4 (15.0 mg) and 5 (6.5 mg), respectively. The structures of isolated compounds were elucidated by analysis of NMR and MS data, together with comparison of their spectral data with those in the literature [11, 12, 16, 17].

Tetracyclic thiazinogeldanamycin (1): white powder; [α]D 25 +17.48 (c, 0.05, MeOH); UV (MeOH) λmax (log ε) 202 (4.25), 234 (4.04), 280 (4.08); 1H-NMR: (400 MHz, DMSO-d6) δ 9.91 (1H, s, 18-NH), 6.68 (1H, dd, J = 4.8, 9.3 Hz, H-3), 5.54 (1H, d, J = 9.2 Hz, H-7), 4.93 (1H, d, J = 9.2 Hz, H-7), 3.82 (1H, m, H-11), 3.70 (3H, s, 17-OCH3), 3.52 (2H, m, H-26), 3.36 (3H, s, 6-OCH3), 3.30 (3H, s, 12-OCH3), 3.26 (1H, m, H-12), 3.16 (1H, m, H-6), 2.99 (1H, d, J = 11.6 Hz, H-15a), 2.61 (1H, overlap, H-10), 2.61 (1H, overlap, H-15b), 2.42 (2H, m, H-4), 2.32 (1H, m, H-14), 2.09 (3H, s, H-22), 1.72 (1H, overlap, H-5a), 1.72 (2H, overlap, H-13), 1.49 (3H, s, H-23), 1.43 (1H, m, H-5b), 0.90 (3H, d, J = 6.8 Hz, H-24), 0.76 (3H, d, J = 6.4 Hz, H-25); 13C NMR: (100 MHz, DMSO-d6) δ 165.4 (C-27), 164.0 (C-1), 156.1 (7-OCONH2), 144.9 (C-17), 144.2 (C-21), 140.0 (C-3), 135.4 (C-9), 134.7 (C-18), 130.1 (C-8), 128.2 (C-20), 122.9 (C-2), 115.7 (C-16), 108.9 (C-19), 82.2 (C-12), 81.2 (C-7), 79.2 (C-11), 78.8 (C-6), 61.6 (17-OCH3), 58.6 (6-OCH3), 56.3 (12-OCH3), 34.6 (C-13), 32.8 (C-10), 32.4 (C-15), 29.6 (C-5), 29.0 (C-14), 28.8 (C-26), 23.0 (C-4), 20.7 (C-25), 14.0 (C-24), 12.4 (C-22), 11.3 (C-23); HRESIMS m/z 616.2697 [M-H] (calcd for C31H27N2O8S, 616.2698)

19-Hydroxy-4,5-dihyrogeldanamycin (3): red solid; [α]D 25 +208.0 (c, 0.10, MeOH); UV (MeOH) λmax (log ε) 230 (4.09), 307 (3.95); 1H-NMR: (400 MHz, DMSO-d6) δ 8.65 (1H, s, NH), 5.55 (1H, d, J = 8.4 Hz, H-3), 5.10 (1H, d, J = 10.0 Hz, H-9), 4.90 (1H, d, J = 6.4 Hz, H-7), 3.88 (3H, s, 17-OCH3), 3.47 (1H, dd, J = 2.8, 8.8 Hz, H-11), 3.32 (3H, s, 6-OCH3), 3.20 (3H, s, 12-OCH3), 3.16 (1H, m, H-6), 2.96 (1H, dt, J = 3.2, 10.4 Hz, H-12), 2.60 (1H, dd, J = 5.2, 12.0 Hz, H-15a), 2.28 (1H, dd, J = 4.0, 12.0 Hz, H-15b), 2.16 (1H, overlap, H-10), 2.16 (1H, overlap, H-4), 1.95 (1H, m, H-14), 1.64 (3H, s, H-22), 1.59 (1H, m, H-13a), 1.32 (3H, s, H-23), 1.18 (2H, m, H-5), 0.96 (3H, d, J = 6.4 Hz, H-24), 0.74 (1H, m, H-1b), 0.49 (3H, d, J = 6.8 Hz); 13C NMR: (100 MHz, DMSO-d6) δ 184.0 (C-21), 179.0 (C-18), 173.1 (C-1), 155.8 (7-OCONH2), 154.4 (C-17), 145.1 (C-20), 132.6 (C-9), 132.5 (C-2), 131.4 (C-3), 129.1 (C-16), 128.9 (C-8), 120.6 (C-9), 79.6 (C-6), 79.2 (C-12), 79.0 (C-7), 71.4 (C-11), 60.7 (17-OCH3), 58.0 (6-OCH3), 55.9 (12-OCH3), 34.4 (C-10), 31.4 (C-13), 29.5 (C-5), 29.2 (C-15), 28.7 (C-14), 22.8 (C-4), 17.8 (C-24), 17.5 (C-25), 13.1 (C-22), 11.7 (C-23); HRESIMS m/z 577.2768 [M-H] (calcd for C32H31N2O8S, 577.2767)

The molecular formula of 1 was established as C31H27N2O8S on the basis of the HR-ESIMS and NMR data. This
Fig. 2. Key $^1$H-$^1$H COSY and HMBC correlations of 1 and 3.

The compound had very similar $^1$H and $^{13}$C NMR spectra to those of known 4,5-dihydrothiazinogeldanamycins [11, 13]. The $^{13}$C NMR, DEPT, and HMQC spectra revealed the presence of 31 carbon signals comprising three carbonyl, six aromatic quaternary, two olefinic quaternary, two olefinic methine, four oxymethine, two aliphatic methine, five aliphatic methylene, three methoxy, and four methyl carbons. In the $^{13}$C NMR spectrum of 1, the absence of signals corresponding to the benzoquinone at C-18 and C-21, and the presence of two additional oxygenated signals at δ $^{13}$C 134.7 and 144.2, respectively, suggested that the benzoquinone moiety was replaced to a hydroquinone or phenolic form. Interpretation of the 2D-NMR data (Fig. 2) including COSY, HMQC, and HMBC spectra enabled the construction of compound 1. In particular, the $^{13}$C and HMQC spectra showed an absence of the C-19 methine carbon and a newly appearing methylene carbon (C-26, δ 28.8) and two quaternary carbon (C-19, δ 108.9; C-27, δ 164.5) signals, and the position of the newly formed thiazino ring was confirmed by the observation of key HMBC correlations as follows: H-26/C-19 and C-27, 18-NH/C-17, C-19, C-26, and C-27. The positions of the functional groups were determined with HMBC correlations (6-OCH$_3$/C-6, 12-OCH$_3$/C-12, 17-OCH$_3$/C-17, and H-7/7-OCONH$_2$). A complete analysis of MS and NMR data confirmed that 1 is a non-quinone (hydroquinone or phenolic form) derivative of 4,5-dihydrothiazinogeldanamycin [11], with the difference of 18 mass units. The overall analysis of the NMR spectra revealed that this compound must be a tetracyclic skeleton compound based on unsaturation equivalents calculated from the molecular formula. Consequently, the compound 1 was suggested to form an ether linkage between oxygenated C-21 and C-11 to satisfy the molecular formula. The presence of an ether linkage between C-11 and C-21 was confirmed by a deuterium-induced differential isotope shift $^{13}$C NMR experiment [6]. The Δδ values [δc (DMSO- $d_6$/H$_2$O) − δc (DMSO-$d_6$/D$_2$O)] were 0.041 (C-11) and -0.035 (C-21), respectively, indicating the absence of the hydroxyl groups at these two carbons. From these analyses, the structure of the new tetracyclic thiazinogeldanamycin (1) was assigned as shown in Fig. 1.

The compound 2 was obtained as a white powder. Unexpectedly, the satisfactory NMR data of 2 were not obtained because of its instability. During the chemical study, compound 2 easily converted to 1 in the methanol solvent system, as monitored by HPLC (data not shown). The compound 2 was predicted to be the reported 4,5-dihydrothiazinogeldanamycin by the LC/MS data (m/z 634 [M-H$^-$] and 1269 [2M-H$^-$]), the UV absorbance spectrum (250nm), and its $^1$H NMR data [11].

The molecular formula of 3 was determined to be C$_{29}$H$_{30}$O$_{18}$ on the basis of the HR-ESIMS data (found, m/z 577.2768 [M-H$^-$]; calculated 577.2767) and NMR data. This compound had very similar $^1$H and $^{13}$C NMR spectra to those of known 4,5-dihydrogeldanamycins [10], except for the absence of an aromatic proton signal at position C-19 (δ 120.6, quaternary) and 16 mass units more than that of 4,5-dihydrogeldanamycin. These data suggested the substitution of a hydroxyl group at C-19. The positions of other functional groups were also determined with HMBC correlations (6-OCH$_3$/C-6, 12-OCH$_3$/C-12, 17-OCH$_3$/C-17, and H-7/7-OCONH$_2$; Fig. 2). Thus, the structure of 3 was determined to be 19-hydroxy-4,5-dihydrogeldanamycin, as shown in Fig. 1. Compounds 4 and 5 were identified as reblastatin and 17-demethoxy-reblastatin by the comparison of NMR and ESI-MS data with those in the literature, respectively [16, 17]. Reblastatin inhibited ongostatin M signaling and Rb protein phosphorylation in cell-based assay [16]. The presence of reblastatin, which contains a 17-methoxy group on the benzene ring, meant that the methylation reaction followed to 17-hydroxylation without the 18,21-oxidation reaction. It could be a shunt process on the main GM biosynthetic pathway. 17-Demethoxy-reblastatin

<p>| Table 1. Biological activities of geldanamycin analogs from Streptomyces hygroscopicus JCM4427. |
|-------------------------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Yeast Hsp90 ATPase inhibition assay (IC$_{50}$)</th>
<th>Cell viability assay (SK-Br3, IC$_{so}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geldanamycin</td>
<td>3.19 µM</td>
<td>7.0 nm</td>
</tr>
<tr>
<td>1</td>
<td>&gt;100 µM</td>
<td>32.4 µM</td>
</tr>
<tr>
<td>19-Hydroxy-4,5-dihydrogeldanamycin (3)</td>
<td>&gt;100 µM</td>
<td>11.0 µM</td>
</tr>
<tr>
<td>Reblastatin (4)</td>
<td>0.32 µM</td>
<td>0.69 µM</td>
</tr>
<tr>
<td>17-Demethoxy-reblastatin (5)</td>
<td>1.82 µM</td>
<td>0.66 µM</td>
</tr>
</tbody>
</table>
(5), a non-quinoid geldanamycin analog with a monophenolic structure, is a major intermediate immediately after the amide ring formation [15].

All isolated GM analogs were evaluated for their biological activities using in vitro assay (Table 1). Reblastatin (4) and 17-demethoxy-reblastatin (5) showed potent activity of Hsp90 ATPase inhibition with IC₅₀ values of 0.32 and 1.82 µM, respectively. Compounds 1 and 3 showed no activities of Hsp90 ATPase inhibition at our experimental range (below 100 µM). The functionality of the C-19 position of benzoquinone-type GM derivatives showed the non-enzymatic conjugation with glutathione, leading to cellular depletion, which is a major factor of the hepatotoxicity of benzoquinone compounds. Therefore, the C-19 modification of compounds 1 and 3 would be originated from the chemical reactivity of the benzoquinone ring. In particular, the probable biosynthesis of the benzoquinone-type GM derivatives showed the amide ring formation [15].

The structure, is a major intermediate immediately after the condensation of benzoquinone with cysteine [1, 5].

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