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Antitumor anthraquinones from an endophytic actinomycete *Micromonospora lupini* sp. *nov*.

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Abstract—Two novel anthraquinones, lupinacidins A (**1**) and B (**2**), have been isolated from the culture broth of a new endophytic actinomycete belonging to the genus *Micromonospora*. Lupinacidins were found to show significant inhibitory effects on the invasion of murine colon 26-L5 carcinoma cells without inhibiting cell growth.

Tumor metastasis is comprised of a complex cascade of sequential events including the release of tumor cells from the primary site, invasion into the connective tissue barriers, dissemination through the blood or lymphatic circulation, and invasion and proliferation at distant sites.[2] Since tumor metastasis is the leading cause of high cancer mortality, there is substantial need and interest in discovering an inhibitor for metastatic events. During the metastatic cascade, tumor cells must pass through the basement membrane consisting of various extracellular matrix proteins such as fibronectin, laminin, collagens, and proteoglycans secreted by vascular endothelial cells. The process of invasion into the basement membrane is understood to mainly involve tumor cell adhesion, enzymatic degradation of extracellular matrix proteins, and migration. Inhibition of these steps is thus expected an effective approach to control metastasis and invasion.[3] In our continuous effort to obtain an anti-invasive compound from microbial secondary metabolites, two novel anthraquinones were isolated from the culture broth of an endophytic *Micromonospora lupini* Lupac 08.[4] In this report, we have employed an experimental model of tumor cell invasion using transwell cell culture chamber and Matrigel®, the reconstituted basement membrane.[5] This assay enables to detect an inhibitor of cell adhesion, enzymatic degradation of extracellular matrix, or migration. Herein, we report the structure elucidation and anti-invasive activity of two novel anthraquinones.

Lupinacidin A (**1**) was obtained as orange needles.[6,7] The high-resolution FABMS coupled with the ^{13}C NMR indicated a molecular formula of $C_{20}H_{20}O_5$ for **1**. The ^{13}C NMR spectrum of **1** displayed carbon signals including two carbonyl, 12 carbons in the olefinic region, and six aliphatic carbons. Analysis of the combined 1D and 2D spectral data established that **1** possessed three olefinic methine, 11 quaternary sp^2 carbons, one aliphatic methine, two methylene, three methyl carbons, and two phenolic protons. The presence of another phenolic proton was suggested by the molecular formula and the presence of three oxygenated sp^2 carbons around 162 ppm. The methyl proton signal that was observed as a doublet at 0.91 ppm corresponded to six protons, suggesting the presence of an isopropyl group. The IR spectrum was consistent with the presence of hydroxyl (3450 cm^{-1}) and carbonyl functional groups (1620 and 1600 cm^{-1}). The UV–vis spectrum showed absorption at 212, 254, and 446 nm, suggesting

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an anthraquinone chromophore.[8] The 11 degrees of unsaturation inherent in the molecular formula of **1**, coupled with data showing the presence of two carbonyl and 12 olefinic carbons (8 degrees of unsaturation), indicated that **1** must possess three rings. Further analysis of ^{1}H–^{1}H COSY and HMBC NMR spectra provided three substructures including two aromatic and one aliphatic moieties. The first aromatic part, the 1,2,3-trisubstituted benzenoid substructure, was established by ^{1}H–^{1}H couplings between H-7 and H-8, and between H-8 and H-9, and the three-bond HMBC correlations from H-7 to C-5a and C-9, from H-8 to C-6 and C-9a, and from H-9 to C-5a and C-7. A three-bond HMBC correlation from H-9 to C-10 established the location of the carbonyl at C-10. The HMBC correlations from the phenolic proton at 12.72 ppm to C-5a, C-6, and C-7 showed the attachment of the hydroxyl group at C-6. Another carbonyl C-5 was suggested to connect at C-5a by the four-bond HMBC correlations from H-7 and H-9 to C-5. This connection was supported by a sharp singlet peak of 6-OH that was considered hydrogen-bonded to a carbonyl oxygen. The aliphatic substructure was elucidated starting from the two equivalent methyl proton doublets (H-15 and H-16) that showed HMBC correlations to one another, to C-13, and to C-14. The proton signal attached to C-13 showed a COSY correlation to the methylene proton signals H_2-12, which in turn showed HMBC correlations to three olefinic quaternary carbons C-3, C-4, and C-4a. The second aromatic part was assembled from the three-bond HMBC correlations from the methyl proton singlet at 2.02 ppm to C-1 (162.3 ppm) and C-3 (162.0 ppm) that indicated that the two phenolic hydroxyl groups were located at the *ortho*-positions of the methyl substituent. The aforementioned HMBC correlations from the aliphatic methylene protons H-12 to the aromatic nucleus showed the location of the aliphatic side chain at the *ortho*-position of the hydroxyl group at C-3. The phenolic hydroxyl proton 1-OH, which showed HMBC correlations to C-1, C-2, and C-10a, was observed as a sharp singlet at 14.19 ppm, indicating its hydrogen-bonding to the oxygen of a quinone carbonyl. Furthermore, the four-bond HMBC from 1-OH to C-10 established the connection between C-10 and C-10a. Finally, the connection between C-4a and C-5 was deduced from the requirement from the molecular formula to complete the structure of **1**.

Lupinacidin B (**2**) was isolated as orange needles.[9] Its molecular formula was deduced as $C_{19}H_{18}O_5$ from high-resolution FABMS and ^{13}C NMR data (Table 1). All of the resonances attributable to the anthraquinone skeleton (C-1 to C-11) were present in the ^{1}H and ^{13}C NMR spectra of **2** (Table 1). The differences observed in the NMR spectra corresponded to differences in the aliphatic side chain (C-12 to C-15). The most significant change was the loss of the resonance observed for the doublet methyl groups and the addition of resonance

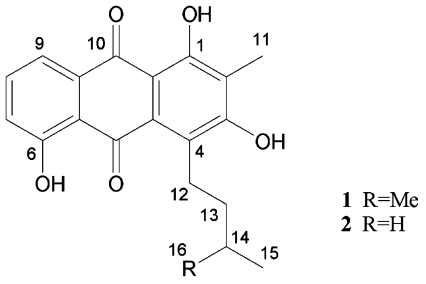

Figure 1. Structures of lupinacidins A (**1**) and B (**2**).

Table 1. ^{1}H and ^{13}C NMR data for lupinacidins A (**1**) and B (**2**) in DMSO-d_6[a]

Position	Lupinacidin A (**1**)			Lupinacidin B (**2**)	
	δ_H mult (*J* in Hz)	δ_C	HMBC	δ_H mult (*J* in Hz)	δ_C
1		162.3			162.4
2		117.8			117.8
3		162.0			162.3
4		130.6			130.3
4a		127.3			127.6
5		189.9			190.1
5a		116.5			116.7
6		161.6			161.5
7	7.18 (1H, d, 8.3)	123.8	5, 5a, 9	7.28 (1H, dd, 8.3, 1.0)	123.9
8	7.62 (1H, d, 8.3, 7.6)	136.4	6, 9a	7.72 (1H, t, 7.8)	136.5
9	7.51 (1H, d, 7.6)	117.7	5a, 7, 10	7.64 (1H, dd, 7.6, 1.0)	117.9
9a		132.6			132.8
10		185.4			185.5
10a		108.9			108.9
11	2.02 (3H, s)	9.1	1, 2, 3	2.12 (3H, s)	9.1
12	2.94 (2H, t, 7.9)	24.6	3, 4, 4a	3.10 (2H, m)	26.2
13	1.24 (2H, m)	37.3		1.43 (2H, m)	30.8
14	1.62 (1H, m)	28.4		1.43 (2H, m)	22.8
15	0.91 (3H, d, 5.9)	22.5	13, 14, 16	0.94 (3H, t, 6.8)	13.9
16	0.91 (3H, d, 5.9)	22.5	13, 14, 15		
1-OH	14.19 (1H, s)		1, 2, 10, 10a	14.20 (1H, s)	
6-OH	12.72 (1H, s)		5a, 6, 7	12.77 (1H, s)	

[a] ^{1}H spectrum was recorded at 400 MHz referenced to residual (CHD_2)$_2$SO (2.50 ppm). ^{13}C spectrum was recorded at 100 MHz referenced to (CD_3)$_2$SO (39.5 ppm).

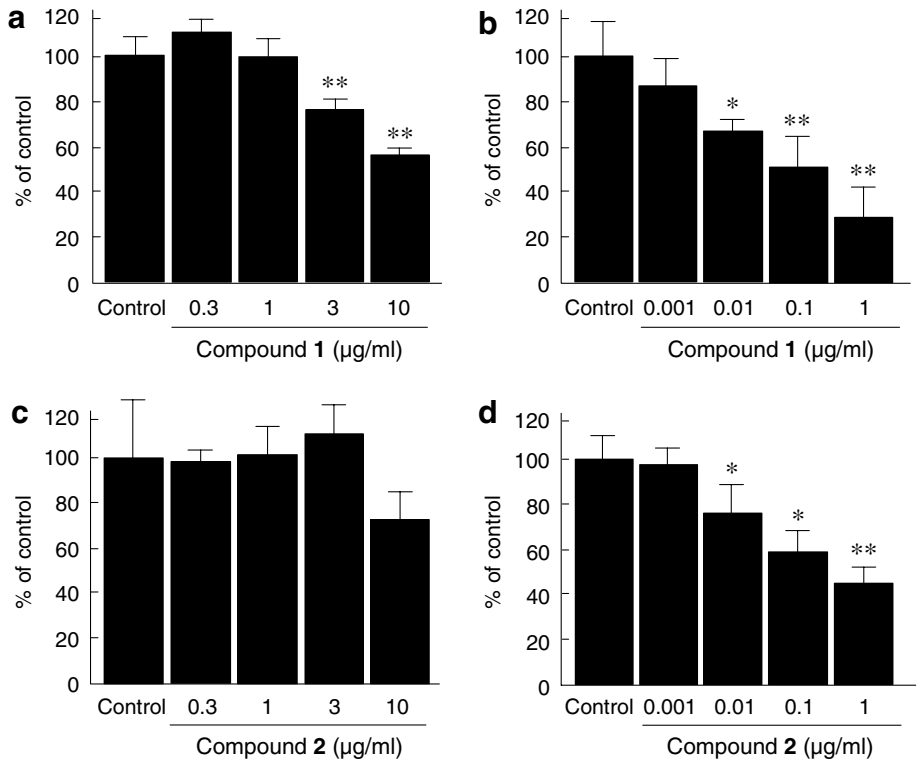

Figure 2. Effects of compounds **1** and **2** on proliferation and invasion of murine colon 26-L5 cells. The cytotoxicity was determined by incubation of the cells for 24 h with various concentrations of **1** and **2**, using WST-1 staining method (a and c). In the invasion assay, colon 26-L5 cells were seeded into Transwell chambers and cultured for 8 h in the presence of compound **1** or **2**. Invading cells were detected by the crystal violet staining method (b and d). $^{*}P < 0.05$, $^{**}P < 0.01$.

for a triplet methyl group. COSY correlations from H_3-15 to H_2-12 and the HMBC correlations from H_3-15 to C-13 and C-14 allowed the assignment of a butyl group in the aliphatic side chain (Fig. 1).

The effects of novel anthraquinones on the proliferation of murine colon 26-L5 carcinoma cells were initially assessed at concentrations from 0.3 to 10 μg/ml (Figs. 2a and c). Lupinacidin A (**1**) showed inhibitory effects on cell growth at 3 μg/ml and more, while lupinacidin B (**2**) was less toxic than compound **1** and not cytotoxic even at 3 μg/ml. Anti-invasive effects of lupinacidins were next examined at non-cytotoxic concentrations.[1] Compounds **1** and **2** exhibited dose-dependent inhibition of in vitro invasion of colon 26-L5 cells with IC_{50} values of 0.07 μg/ml (=0.21 μM) and 0.3 μg/ml (=0.92 μM), respectively (Figs. 2b and d). Compound **1** was more potent both in cytotoxic and anti-invasive activities than compound **2**, suggesting that the alkyl substituent is involved in these activities. Among a number of naturally occurring anthraquinones, R1128 B (1,3,6-trihydroxy-8-*n*-butylanthraquinone) is structurally similar to lupinacidins.[10] R1128 B is the estrogen receptor (ER) antagonist and has been shown to exhibit antitumor activity in mouse model experiments. Recently, inhibition of tumor cell invasion by ER antagonist, tamoxifen, has been demonstrated, although detailed mechanisms remain to be solved.[11] In addition, two classes of microbial aromatic polyketides, anthracyclines[12] and tetracycline derivatives[13], have been reported to inhibit tumor cell invasion through inhibition of collagenase gene expression and matrix metalloproteases, respectively. Lupinacidins described herein represent an additional candidate for the development of anti-invasive drugs. Further investigations on their biological activities are in progress.

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omy of the strain Lupac 08 will be reported in a separate paper. The strain was subcultured on Bennet's agar for 7 days at 32 °C. It was used to inoculate 500 ml K-1 flasks each containing 100 ml of V-22 medium.[14] A volume of 3 ml of seed culture was used to inoculate 100 ml of A-3M medium in 500 ml K-1 flasks.[14] At the end of the fermentation period, 50 ml of 1-butanol was added to each flask, and they were allowed to shake for 1 additional hour. The mixture was centrifuged at 2000 rpm for 15 min and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the extraction solvent in vacuo provided approximately 2.88 g of extract per 1 L of culture.

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7. Lupinacidin A (**1**): orange needles; mp 234–238 °C; UV (MeOH) λ_{max} (log ε) 212 (4.32), 236 (4.08), 254 (4.13), 276 (4.12), 294 (sh, 3.92), 339 (3.36), 422 (sh, 3.81), 446 (3.88), 466 (sh, 3.78) nm; (0.01 N HCl–MeOH) 212 (4.36), 236 (4.15), 252 (4.20), 276 (4.18), 296 (sh, 4.00), 423 (sh, 3.88), 446 (3.93), 469 (sh, 3.82); (0.01 N NaOH–MeOH) 206 (4.97), 258 (4.14), 275 (4.11), 333 (4.18), 457 (3.82), 517 (3.81); IR (KBr) ν_{max} 3450, 2950, 2920, 1620, 1600, 1400, 1380, 1250, 1200, 1160, 1085 cm^{-1}; ^1H and ^{13}C NMR (Table 1); HRFABMS $[M+H]^+$ *m/z* obsd 341.1385 (calcd for $C_{20}H_{21}O_5$ 341.1388, −0.3 mmu).
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