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NOTE

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Lupinacidin C, an Inhibitor of Tumor Cell Invasion from *Micromonospora lupini*

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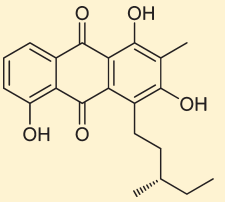
Supporting Information

ABSTRACT: A new anthraquinone derivative, lupinacidin C (**1**), was isolated from the endophytic actinomycete *Micromonospora lupini*. The structure was elucidated on the basis of spectroscopic analyses, and the absolute configuration was determined by total synthesis. Lupinacidin C (**1**) exhibited the most potent inhibitory effects among the congeners on the invasion of murine colon carcinoma cells into the reconstituted basement membrane.



Endophytic actinomycetes are known to inhabit the phyllosphere and the rhizosphere, and they are especially abundant in root tissues.[1] In particular, similar to rhizobia in legume plants, the genus *Frankia* is involved in root nodule formation and nitrogen fixation in nonlegume plants, establishing a close relationship with host plants. Different from this symbiont, biological and ecological functions of most of the endophytic actinomycetes in plants are not comprehensively understood.[1c,1d] However, these endophytes are currently of significant interest as an untapped resource of novel bioactive small molecules because their metabolites are speculated to affect the physiological conditions of host plants such as growth and disease resistance.[1d,2] *Micromonospora* is a common genus in terrestrial and aquatic environments, but our current knowledge on its inhabitation in plant tissues is limited.[3] Recently, legume root nodules have been found to commonly harbor this species, and a series of new species were recovered from this previously ignored habitat.[4] Our investigation on the secondary metabolites from legume-derived new species resulted in the discovery of new anthraquinones, lupinacidins A (**2**) and B (**3**) from *M. lupini* isolated from *Lupinus angustifolius*.[5] These compounds inhibited the tumor cell invasion into the reconstituted basement membrane in a concentration-dependent manner at noncytotoxic concentrations. Further chemical investigation of the culture extract from *M. lupini* Lupac 08 led to the identification of a new minor congener, lupinacidin C (**1**).

The 1-butanol extract of the whole culture broth of *M. lupini* Lupac 08 was subjected to normal- and reversed-phase column chromatographies and HPLC purification to yield lupinacidin C (**1**) as optically active orange needles ($[\alpha]_D$ +4.6, $CHCl_3$). The UV spectrum was closely similar to that for lupinacidins A (**2**) and B (**3**) (λ_{max} 212, 235, 253, 276, 445 nm).[5] The high-resolution FABMS gave an $[M + H]^+$ peak at *m/z* 355.1543 appropriate for $C_{21}H_{22}O_5$ (calcd for $C_{21}H_{23}O_5$ *m/z* 355.1540), corresponding to the addition of a methylene fragment (CH_2) to lupinacidin A (**2**). The ^{13}C NMR and HMQC spectra revealed the three aromatic carbons (C-6, C-7, and C-8) each bearing protons (H-6, H-7, and H-8). Contiguous couplings of these protons (H-6/H-7/H-8) were confirmed by the $^1H-^1H$ COSY spectrum. In addition, two carbonyl carbons, three hydroxylated aromatic carbons (C-1, C-3, and C-5), a singlet methyl, and six quaternary sp^2 carbons were observed. In the ^1H NMR spectrum, two hydrogen-bonded phenolic hydroxyl protons were observed at δ_H 13.0 and 14.2. All these protons and carbons attributable to the anthraquinone skeleton were almost identical with those for **2** and **3**. Comparison of the ^1H and ^{13}C NMR data for **1** with those for **2** and **3** indicated the difference in the alkyl substituent at C-4. The COSY spectrum showed three small spin systems: H_2-12/H_2-13, H-14/H_3-17, and H_2-15/H_3-16. These fragments were combined on the basis of HMBC correlations from H_3-16 to C-14 and C-15 and from H_3-17 to C-13, C-14, and C-15, thereby establishing the 3-methylpentyl group. HMBC correlations from H_2-12 to C-3 and C-4 connected this alkyl substituent to C-4.

To determine the absolute configuration of the stereogenic center on the side chain, the *S*-enantiomer of **1** was synthesized according to the protocol previously employed for the synthesis of lupinacidins A (**2**) and B (**3**) (Scheme 1).[6] L-Leucine (**4**) was converted into the bromo acid **5**,[7] which was further treated with $LiAlH_4$ to give alcohol **6**.[8] The optically active alcohol **6** was transformed into the corresponding iodide **7**, and an enone **8** was alkylated with **7**.[9] The resulting product **9** obtained as a ca. 1:1

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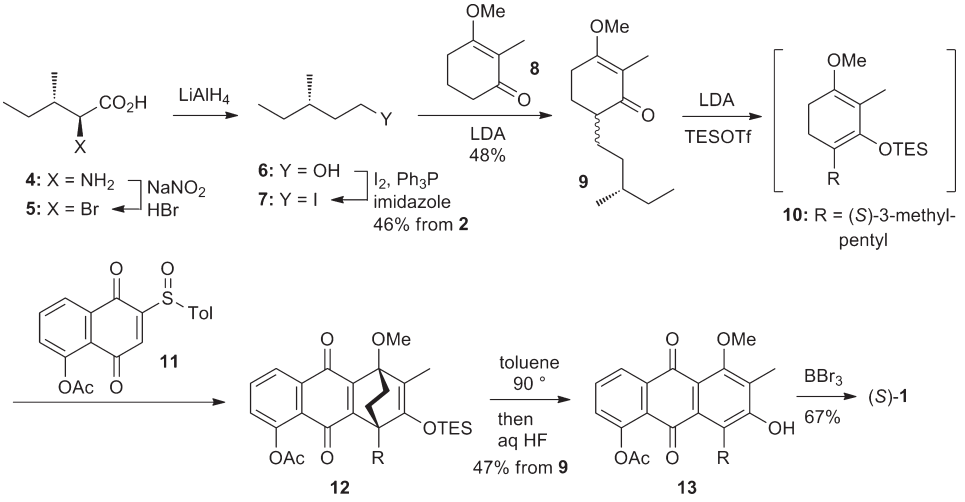
Scheme 1. Synthesis of (*S*)-1



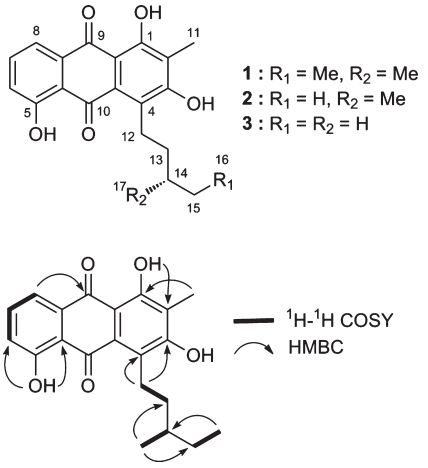


Figure 1. $^{1}H-^{1}H$ COSY and key HMBC correlations for **1**.

diastereomeric mixture was converted into silyl enol ether **10**, which was then subjected to the Diels–Alder reaction with sulfinyl quinone **11** to afford tetracyclic intermediate **12** via in situ liberation of *p*-toluenesulfenic acid from an intermediary Diels–Alder adduct. Heating of **12** in toluene brought about the elimination of ethylene to afford an anthraquinone intermediate, which was then treated with aqueous HF in one pot to give **13**. Finally, deprotection of both the methoxy and acetyl groups of **13** with BBr_3 furnished (*S*)-**1**, the ^{1}H and ^{13}C NMR spectra of which were identical with those of natural **1**. The specific rotation of (*S*)-**1**, $[\alpha]^{24}_{D}$ +5.3 (*c* 0.23, $CHCl_3$), showed a good agreement with that of the natural product, $[\alpha]^{24}_{D}$ +4.6 (*c* 0.10, $CHCl_3$). Therefore, the absolute configuration of **1** was determined to be *S*.

Compound **1** was tested for anti-invasive activity using murine colon 26-L5 carcinoma cells, as compounds **2** and **3** were previously tested in the same assay and showed moderately strong activities (IC_{50} values: 0.21 μM for **2**; 0.92 μM for **3**).[5] Prior to invasion assay, cytotoxicity of **1** was assessed at concentrations from 0.1 to 3 μg/mL. **1** was inactive to the cells at less than 0.3 μg/mL, and thus the inhibitory effect on invasion was assessed at concentrations less than 0.1 μg/mL. Among the congeners, **1** displayed the most potent anti-invasive activity, with an IC_{50} value of 0.019 μg/mL (0.054 μM), indicating that the hydrophobicity of the alkyl side chain is involved in potency. On the basis of these findings, chemical synthesis of additional lupinacidin analogues is in progress for evaluation as a candidate for antimetastatic agent.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined with a Yanaco MP-J3 apparatus and are uncorrected. Optical rotations were measured on a Jasco DIP-1030 or a Jasco DIP-371 polarimeter. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. IR spectra were collected on a Perkin-Elmer Spectrum 100. NMR spectra were recorded on a Jeol JMN-LA400 or a Varian MR-400 spectrometer, using the signals of the residual solvent protons and carbons as internal standard. The HRFABMS was measured using a Jeol JMS-HX110A. HREIMS were recorded on a Jeol JMS-700. Dichloromethane and THF used for chemical synthesis were distilled from CaH_2 and Na/benzophenone, respectively.

Microorganism. The actinomycete strain *Micromonospora lupini* Lupac 08 was isolated from the root nodule of *Lupinus angustifolius* collected in Saelices el Chico (Salamanca, Spain) in 2003. Isolation and characterization were described in the previous paper.[4b]

Fermentation and Isolation. Seed and production fermentations of strain Lupac 08 were carried out in V-22 and A-3 M medium, respectively, as described previously.[5] The combined 1-butanol extract from 80 × 100 mL fermentation (23 g) was subjected to silica gel column chromatography purification (Kanto Chemical Co., Inc., 63-210 mesh, 60 × 400 mm) eluting with solvent mixtures of $CHCl_3$/MeOH (1:0, 20:1, 10:1, 4:1, 2:1, and 1:1 v/v), successively. Fractions 3 and 4 (10:1 and 4:1), containing lupinacidins, were concentrated and refractionated by C-18 reversed-phase MPLC (Nacalai Tesque, Inc., silica gel 60-C18, 250–350 mesh, 55 × 250 mm) with a step gradient of MeCN/0.15% KH_2PO_4 buffer solution (pH 3.5)/MeOH as follows: fractions 1–7, MeCN/KH_2PO_4 buffer solution (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2 v/v); and fraction 8, 100% MeOH. Evaporation of fraction 8 in vacuo left a wet residue, which was taken up in EtOAc, providing 500 mg of dark brown solid. Final purification was achieved by repeated C-18 RP HPLC (Nacalai Tesque Inc., Cosmosil 5C18-AR-II, 20 × 250 mm) with 85% MeCN in 0.15% KH_2PO_4 buffer solution to afford **1** (5.8 mg).

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Table 1. ^{1}H and ^{13}C NMR Data for Lupinacidin C (1) in $CDCl_3$

position	δ_C[a]	δ_H mult (J in Hz)[b]	HMBC[b,c]
1	162.6[d], qC		
2	117.6, qC		
3	159.7, qC		
4	128.3, qC		
4a	128.1, qC		
5	162.5[d], qC		
6	124.3, CH		
7	136.0, CH	7.27 (dd, 8.3, 1.0)	5a, 9
8	118.3, CH	7.63 (dd, 8.3, 7.6)	6, 9a
8a	133.1, qC		
9	186.9, qC	7.79 (dd, 7.6, 1.0)	5a, 7, 10
9a	110.8, qC		
10	190.4, qC		
10a	117.1, qC		
11	8.4, CH_3	2.26 (s)	1, 2, 3
12	24.5, CH_2	3.21 (dt, 11.2, 4.4)	3, 4, 13, 14
		3.16 (dt, 11.2, 4.4)	3, 4, 13, 14
13	35.4, CH_2	1.56 (m)	
		1.40 (m)	
14	35.3, CH	1.58 (m)	
15	29.3, CH_2	1.49 (m)	
		1.28 (m)	
16	11.4, CH_3	0.95 (t, 7.4)	14, 15
17	19.1, CH_3	1.05 (d, 6.3)	13, 14, 15
1-OH		14.2 (s)	1, 2
6-OH		13.0 (s)	5a, 6, 7

[a] Recorded at 100 MHz. [b] Recorded at 400 MHz. [c] HMBC correlations are from proton(s) stated to the indicated carbon. [d] Interchangeable.

*Lupinacidin C (**1**)*:. orange needles; mp 198−200 °C; $[\alpha]^{24}_D$ +4.6 (*c* 0.10, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 212 (4.41), 235 (4.17), 253 (4.21), 276 (4.21), 445 (3.97); IR (ATR) ν_{max} 3430, 1595 cm^{-1}; ^{1}H and ^{13} NMR data, see Table 1; HRFABMS $[M + H]^+$ 355.1543 (calcd for $C_{21}H_{23}O_5$, 355.1540).

*(S)-1-Iodo-3-methylpentane (**7**).* To a stirred suspension of L-isoleucine **4** (10.0 g, 76.2 mmol) in water (24 mL) was added aqueous HBr (48 wt %, 52 mL) at −10 °C, and the resulting mixture was gradually warmed to 10 °C over 1 h. To the mixture was added dropwise a solution of $NaNO_2$ (10.5 g, 152 mmol) in water (20 mL) at −10 °C over 15 min. The heavy, brown gas released from the reaction was scrubbed by 20% NaOH solution (30 mL). After being stirred at −10 °C for 30 min, the reaction mixture was gradually warmed to room temperature over 4 h and extracted with Et_2O. The extract was successively washed with saturated aqueous $Na_2S_2O_3$, water, and brine, dried ($MgSO_4$), and concentrated in vacuo to give crude **5** (14.9 g). The crude bromide (14.9 g) was dissolved in THF (20 mL) and added to a stirred suspension of lithium aluminum hydride (2.40 g 76.2 mmol) in THF (60.0 mL) at 0 °C. After being stirred for 1 day at room temperature and for an additional day at reflux, the reaction mixture was quenched with water at 0 °C, and the resultant white precipitate was dissolved with 2 N H_2SO_4 (50 mL). The mixture was extracted with Et_2O, and the extract was successively washed with water and brine, dried ($MgSO_4$), and concentrated in vacuo to give crude **6** (6.40 g), which was taken up in THF (80 mL). To the solution were successively added imidazole (6.40 g, 94.0 mmol), triphenylphosphine (18.1 g, 69.0 mmol), and iodine (19.1 g, 75.3 mmol) at 0 °C. The resulting mixture was stirred at 0 °C for 1 h and then gradually warmed to room temperature over 5 h before being quenched with saturated aqueous NH_4Cl and extracted with hexane. The extract was successively washed with saturated aqueous $NaHSO_3$, water, and brine, dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by SiO_2 column chromatography using hexane as eluent to give **7** (7.40 g, 46.0% from **4**) as a colorless oil: $[\alpha]^{27}_D$ +15.8 (*c* 0.815, $CHCl_3$); IR (ATR) ν_{max} 2959, 2925, 2873, 1460, 1178 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.25 (1H, ddd, J = 9.5, 8.6, 5.9 Hz), 3.17 (1H, ddd, J = 9.5, 8.3, 7.1 Hz), 1.88 (1H, m), 1.64 (1H, m), 1.48 (1H, m), 1.36 (1H, m), 1.18 (1H, m), 0.88 (3H, t, J = 7.6 Hz), 0.87 (3H, d, J = 6.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 40.5, 35.4, 28.7, 18.2, 11.1, 5.4; HREIMS m/z 212.0063 (calcd for $C_6H_{13}I$, 212.0062).

*(R/S)-3-Methoxy-2-methyl-6-[(S)-3-methylpentyl]-2-cyclohexen-1-one (**9**).* To a stirred solution of LDA [prepared by treating a solution of diisopropylamine (0.42 mL, 3.0 mmol) in THF (1.5 mL) with butyllithium (1.6 M in hexane, 1.9 mL, 3.0 mmol) at −10 °C for 20 min] was added dropwise a solution of **8** (280 mg, 2.00 mmol) in THF (3.5 mL) at −78 °C. After 2 h, HMPA (1.0 mL, 5.7 mmol) and a solution of **7** (212 mg, 1.00 mmol) in THF (4 mL) were successively added at the same temperature, and the resulting mixture was gradually warmed to room temperature over 4 h. The reaction mixture was quenched with saturated aqueous NH_4Cl and extracted with Et_2O. The extract was successively washed with water and brine, dried ($MgSO_4$), and concentrated in vacuo. The residue was chromatographed over SiO_2 (hexane/EtOAc, 5:1) to give 107 mg (47.7 mmol, 48%) of **9** as a white solid: mp 55.5−56.5 °C; IR (ATR) ν_{max} 1619, 1602, 1378 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.80 (3H, s), 2.63 (1H, m), 2.48 (1H, m), 2.04−2.16 (2H, m), 1.69−1.89 (2H, m), 1.68 (3H, t, J = 1.6 Hz), 1.24−1.41 (4H, m), 1.09−1.19 (2H, m), 0.83−0.88 (6H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.9, 170.3, 114.1, 54.9, 44.8/44.7, 34.61/34.56, 34.1/33.9, 29.5/29.1, 27.2/27.1, 25.7/25.5, 23.5/23.4, 19.2/19.1, 11.4/11.3, 7.56; HREIMS m/z 224.1779 (calcd for $C_{14}H_{24}O_2$, 224.1776).

*5-Acetoxy-3-hydroxy-1-methoxy-2-methyl-4-[(S)-3-methylpentyl]-9,10-anthracenedione (**13**).* To a stirred solution of LDA [prepared by treating a solution of diisopropylamine (0.18 mL, 1.3 mmol) in THF (0.84 mL) with a solution of butyllithium (1.57 M in hexane, 0.74 mL, 1.2 mmol) at −10 °C for 20 min] was added a solution of **9** (95 mg, 0.42 mmol) in THF (0.84 mL) at −78 °C. After 2 h, TESOTf (0.25 mL, 1.1 mmol) was added, and the resulting mixture was stirred at −78 °C for 2 h. To the mixture containing **10** was added dropwise a solution of **11** (177 mg, 0.5 mmol) in CH_2Cl_2 (5 mL), and the mixture was stirred at −78 °C for 12 h. The mixture was quenched with MeOH/AcOH (3:1) at −78 °C and then gradually warmed to 0 °C before being diluted with saturated aqueous NH_4Cl and extracted with Et_2O. The extract was successively washed with saturated aqueous $NaHCO_3$ and brine, dried ($MgSO_4$), and concentrated in vacuo. The residue was partially purified by SiO_2 column chromatographed (hexane/EtOAc, 10:1) to give a mixture (120 mg) containing **12** as the major component. The product was dissolved in toluene (2 mL) and stirred at 95 °C for 9 h. To the resulting solution were successively added MeCN (3 mL) and several drops of hydrofluoric acid (46 wt % in water) at 0 °C. The mixture was stirred overnight at room temperature, diluted with water (10 mL), and extracted with Et_2O. The extract was washed with brine, dried ($MgSO_4$), and concentrated in vacuo. The residue was chromatographed over SiO_2 (hexane/EtOAc, 10.1−5:1) to give **13** (81 mg, 47% from **9**) as a yellow solid: mp 116.0−117.0 °C; $[\alpha]^{27}_D$ +6.61 (*c* 0.880, $CHCl_3$); IR (ATR) ν_{max} 3451, 1772, 1670, 1596, 1552, 1190 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.07 (1H, dd, J = 7.9, 1.2 Hz), 7.67 (1H, t, J = 7.9 Hz), 7.29 (1H, dd, J = 7.9, 1.2 Hz), 5.73 (1H, br s, O*H*), 3.88 (3H, s), 2.96 (1H, m), 2.85 (1H, m), 2.42 (3H, s), 2.25 (3H, s), 1.64 (1H, m), 1.44−1.58 (3H, m), 1.28 (1H. m), 1.04 (3H, d, J = 6.2 Hz), 0.95 (3H, t, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 185.7, 181.7, 169.8, 158.4, 158.3, 148.3, 136.3, 134.0, 133.8, 127.8, 127.1, 126.6, 124.5, 124.0, 120.0, 61.6, 35.8,

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35.3, 29.2, 24.8, 21.1, 19.0, 11.5, 9.1; HREIMS m/z 411.2174 (calcd for $C_{25}H_{31}O_5$, 411.2171).

*1,3,5-Trihydroxy-2-methyl-4-[(S)-3-methylpentyl]-9,10-anthracenedione [(S)-**1**].* To a stirred solution of **13** (30.7 mg, 74.8 mmol) in CH_2Cl_2 (3 mL) was added BBr_3 (1.0 M in CH_2Cl_2, 0.40 mL, 0.40 mmol) at −78 °C. The mixture was gradually warmed to 0 °C over 4 h and then quenched with saturated aqueous $NaHCO_3$. The resulting mixture was stirred overnight at room temperature before being extracted with CH_2Cl_2. The extract was successively washed with saturated aqueous NH_4Cl, water, and brine, dried ($MgSO_4$), and concentrated in vacuo. The residue was chromatographed over SiO_2 (hexane/EtOAc, 10:1) to give (*S*)-**1** (17.6 mg, 67%) as an orange solid: mp 198.0−198.5 °C; $[\alpha]^{24}_D$ +5.3 (*c* 0.23, $CHCl_3$); IR (ATR) ν_{max} 3458, 1618, 1605, 1240, 1192 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 14.2 (1H. s, O*H*), 13.0 (1H, s, O*H*), 7.79 (1H, dd, J = 7.5, 1.1 Hz), 7.62 (1H, dd, J = 8.4, 7.5 Hz), 7.26 (1H, dd, J = 8.4, 1.1 Hz), 5.67 (1H, s, O*H*), 3.12−3.27 (2H, m), 2.26 (3H. s), 1.53−1.62 (2H, m), 1.47 (1H, m), 1.40 (1H, m), 1.28 (1H, m), 1.05 (3H, d, J = 6.4 Hz), 0.95 (3H, t, J = 7.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 190.3, 186.9, 162.6, 162.4, 159.6, 136.0, 133.0, 128.2, 128.0, 124.3, 118.3, 117.5, 117.0, 110.7, 35.4, 35.2, 29.3, 24.5, 19.1, 11.4, 8.4; HREIMS m/z 355.1550 (calcd for $C_{21}H_{23}O_5$, 355.1545).

Biological Assays. Cytotoxic assay and invasion assay were carried out according to the procedures previously described.[10]

■ ASSOCIATED CONTENT

Supporting Information. ^1H and ^{13}C NMR spectra of lupinacidin (**1**) and synthetic (*S*)-**1**, **7**, **9**, and **13**. This material available is free of charge via the Internet at http://pubs.acs.org.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on Jan 12, 2011, with an error in the fourth paragraph. The value of the anti-invasive activity for compound **1** was corrected to 0.54 μM. The corrected version was reposted on Feb 10, 2011.