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Angucyclines from an insect-derived actinobacterium *Amycolatopsis* sp. HCa1 and their cytotoxic activity

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ABSTRACT

One new angucyclinone derivative, amycomycin A (**1**), and one new angucycline, amycomycin B (**2**), along with 5 known compounds (**3**–**7**), were isolated from an actinobacterium *Amycolatopsis* sp. HCa1 associated with the grasshopper, *Oxya chinensis*. Their structures were elucidated on the basis of spectroscopic methods, including extensive NMR spectra. Compounds **1**–**7** were tested in vitro for their cytotoxic effects on five cell lines including human gastric adenocarcinoma cell line (BGC823), human hepatocarcinoma cell line (HepG2), human melanoma cell line (A375), human oral squamous carcinoma cell line (KB), and ghost cell line (Ghost-R5X4). Cell viability assays showed that compound **7** was active in four cell lines with IC_{50} values less than 18.0 μM except in KB showing no activity up to 100 μM.

The angucycline antibiotics, a large group of polycyclic aromatic polyketides mainly isolated from *Streptomyces*, constitute a novel class of antitumor and antibacterial compounds.[1–6] Members of the actinobacteria have been extensively studied for their tendency to produce pharmaceutically useful compounds, and yet insect-associated actinobacteria represent unexplored sources for natural product discovery.[7–11] In our discovery for bioactive secondary metabolites from insect-associated actinobacteria, we have reported the chemical studies of a rare actinobacterium *Amycolatopsis* sp. HCa1 isolated from the gut of *Oxya chinensis*, and identified two new angucyclines[12] and eight new actinotetraoses A–H[13] from the cultivation of *Amycolatopsis* sp. HCa1 using malt extract liquid media. In order to obtain more new bioactive secondary metabolites from this strain, we vary the culture conditions, and chemical analysis of the secondary metabolites produced by the actinobacterium led to one new angucyclinone derivative, amycomycin A (**1**), and one new angucycline, amycomycin B (**2**), along with 5 known compounds (**3**–**7**) (Fig. 1), tetrangomycin (**3**),[6,14] PD116779 (**4**),[3] X-14881E (**5**),[15] sakyomicin A (**6**)[16] and sakyomicin C (**7**),[16] which were confirmed by means of spectroscopic methods. Here, we describe the isolation and structural determination of the two new compounds and the cytotoxic activity of these isolates.

The ethyl acetate extract from about 20 L liquid fermentation broth of *Amycolatopsis* sp. HCa1 was fractionated by a combination of column chromatographic methods, resulting in the isolation of amycomycins A–B (**1**–**2**), and 5 known compounds (**3**–**7**).[17] The ^1H and ^{13}C NMR spectroscopic data of **1**–**2** are listed (Table 1).

Amycomycin A (**1**) was isolated as an orange solid, with the molecular formula $C_{20}H_{18}O_8$ (12 double-bond equivalents) as derived from ESI high-resolution mass spectrometry ($[M+Na]^+$ at *m*/*z* 409.0889, calculated 409.0894) and ^1H and ^{13}C NMR spectral data (Table 1).[18] The ^1H NMR spectrum of **1** displayed two chelated phenolic hydroxyl protons (δ_H 13.01, 12.53), five aromatic protons from 7.42 to 7.85 ppm, one aliphatic methyl group (δ_H 0.99), one oxygenated methyl group (δ_H 3.67), one oxygenated methine (δ_H 3.97), two methylene protons (δ_H 3.15, 2.81) and two hydroxyl groups (δ_H 5.66, 4.70). The ^{13}C NMR spectrum showed a total of twenty resonance lines that were grouped into three carbonyl carbons (δ_C 188.5, 188.0, 173.1), twelve aromatic carbons between 162.1 and 115.5 ppm, two oxygen bearing aliphatic carbons at δ_C 77.8 and 74.5, one oxygenated methyl carbon (δ_C 51.8), one methylene carbon (δ_C 35.6) and one methyl carbon (δ_C 23.0) as edited by the DEPT135 and HSQC experiments. The complete assignment of all ^1H and ^{13}C NMR spectral data of **1** was subsequently accomplished by the correlative interpretation of its HSQC, ^1H–^1H COSY, HMBC and NOESY spectra. In this molecule, a 1,2,3-trisubstitued benzene ring and a 1,2,3,4-tetrasubstitued benzene ring were indicated by two groups of signals at δ_H 7.80 (d, J = 7.9 Hz), 7.85 (t, J = 7.9 Hz) and 7.42 (d, J = 7.9 Hz), and at δ_H 7.83 (d, J = 7.8 Hz) and 7.73 (d, J = 7.8 Hz), which were also supported by the ^1H–^1H COSY experiment. The two aromatic substructures were incorporated with two ketonic carbons to form the most probable structure of **1** according to the key HMBC correlations of the H-4 (δ_H

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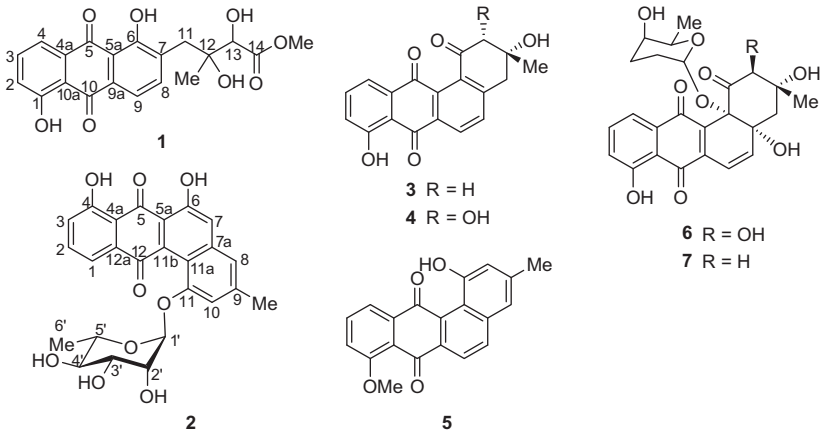

Figure 1. Structures of compounds **1**–**7**.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for **1** and **2** in DMSO-d_6

Position	1		2	
	δ_H (mult, *J* in Hz)	δ_C	δ_H (mult, *J* in Hz)	δ_C
1	–	162.1	7.56 (d, 8.0)	118.0
1-OH	12.53 (s)	–	–	–
2	7.42 (d, 7.9)	125.1	7.83 (t, 8.0)	137.8
3	7.85 (t, 7.9)	137.7	7.37 (d, 8.0)	123.2
4	7.80 (d, 7.9)	119.6	–	160.4
4-OH	–	–	11.38 (br s)	–
4a	–	133.5	–	115.8
5	–	188.5	–	191.5
5a	–	115.5	–	121.5
6	–	161.3	–	153.7
6-OH	13.01 (s)	–	11.85 (s)	–
7	–	135.8	6.61 (s)	116.7
7a	–	–	–	142.3
8	7.83 (d, 7.8)	140.3	6.59 (s)	116.7
9	7.73 (d, 7.8)	118.7	–	118.8
9-Me	–	–	2.25 (s)	22.2
9a	–	131.5	–	–
10	–	188.0	7.08 (s)	111.2
10a	–	116.4	–	–
11	2.81 (d, 13.3) 3.15 (d, 13.3)	35.6	–	154.5
11a	–	–	–	138.8
11b	–	–	–	137.9
12	–	74.5	–	185.5
12a	–	–	–	136.9
12-Me	0.99 (s)	23.0	–	–
12-OH	4.70 (br s)	–	–	–
13	3.97 (d, 6.0)	77.8	–	–
13-OH	5.66 (d, 6.0)	–	–	–
14	–	173.1	–	–
14-OMe	3.67 (s)	51.8	–	–
1′	–	–	5.47 (br s)	99.9
2′	–	–	3.89 (m)	70.1
3′	–	–	3.64(m, overlap)	70.6
4′	–	–	3.35 (m, overlap)	71.9
5′	–	–	3.64 (m, overlap)	70.2
6′	–	–	1.20 (d, 6.0)	18.1

7.80) with C-5 (δ_C 188.5) and C-10a (δ_C 116.4), and of the H-9 (δ_H 7.73) with C-5a (δ_C 115.5) and C-10 (δ_C 188.0). Meanwhile, two phenolic singlets, observed at lower field at δ_H 13.01 and 12.53 owing to hydrogen bonds with the neighboring ketonic groups, showed HMBC correlations to C-5a, C-6 and C-7, and to C-1, C-2 (δ_C 125.1) and C-10a, which positioned the hydroxyl group at the deshielded C-6 (δ_C 161.3) and C-1 (δ_C 162.1), respectively. Another spin system from C-11 to C-14 was established by the key HMBC correlations from 12-Me protons (δ_H 0.99) to C-11 (δ_C 35.6), C-12 (δ_C 74.5), and C-13 (δ_C 77.8), and from H-13 (δ_H 3.97) to C-12 and C-14 (δ_C 173.1). HMBC correlations from hydroxyl proton (12-OH, δ_H 4.70) to C-11, C-12 and 12-Me, and from hydroxyl proton (13-OH, δ_H 5.66) to C-12 also supported this spin system. A $^{3}J_{C-H}$ diagnostic correlation from the oxygenated methyl protons (δ_H 3.67) to C-14 was also observed. The connectivity between C-11 and C-7 was deduced by the HMBC correlations of the H-11 (δ_H 3.15, 2.81) with C-6, C-7 (δ_C 135.8) and C-8 (δ_C 140.3); and H-8 (δ_H 7.83) with C-11, thus establishing the planar structure of **1** as shown in Figure 1. The relative stereochemistry of **1** was confirmed by a NOESY experiment, which showed the NOESY correlations between 12-Me and H-13; H-11α (δ_H 2.81) and 12-OH, 13-OH. However, due to its limited amount, the absolute configuration was not assigned.

Amycomycin B (**2**) was isolated as a brown solid.[18] Its molecular formula was determined to be $C_{25}H_{22}O_9$ with fifteen unsaturation degrees determined by HRESIMS and from ^{13}C NMR spectra. The ^{1}H NMR spectrum of compound **2** clearly showed two methyls (δ_H 1.20, 2.25), one anomeric proton of alpha-glycosidically linked sugar at δ_H 5.47 (br s), six aromatic protons (δ_H 7.83, 7.56, 7.37, 7.08, 6.61, 6.59), and two phenolic hydroxyl protons (δ_H 11.85, 11.38). The ^{13}C NMR (DEPT) spectroscopic data (Table 1) revealed the presence of two methyl groups, five oxygenated methines, sixteen olefinic carbons and two ketone carbonyls. The IR spectrum of **2** exhibited the absorption bands at 3649.0 cm^{-1} (hydroxyl group) and 1736.3 cm^{-1} (carbonyl group). The gross structure of the aglycone of **2** was determined by analysis of the NMR data and by comparison with the NMR data of dehydrorabelomycin.[19] Comparing the NMR data of **2** with those of dehydrorabelomycin indicated that **2** was similar to dehydrorabelomycin except for one rhamnose unit in **2**. The linkage of the rhamnose to the aglycone was established by the key HMBC correlations of H-1′ (δ_H 5.47) with C-11 (δ_C 154.5). Moreover, the presence of L-rhamnose was further evidenced by the acid hydrolysis.[20] Based on the above evidences, compound **2** was identified as dehydrorabelomycin-11-*O*-α-L-rhamnopyranoside, named amycomycin B, the structure of which was further confirmed by HSQC, HMBC, ^{1}H–^{1}H COSY and NOESY experiments (Fig. 2).

In this study, the cytotoxic activities of compounds **1**–**2** against A375 and KB, and the cytotoxic activities of compounds **3**–**7** against BGC823, HepG2, A375, KB, and Ghost-R5X4 cells, were examined (Table 2).[21] As a result, compound **1** and **2** didn't exhibit cytotoxic activities against the tested A375 and KB cells at a concentration of 100 μM; compounds **3**–**5**, and **7** displayed different cytotoxic activity against Ghost-R5X4 cells with the IC_{50} values in a range from 14.0 to 89.04 μM. Both of compounds **6** and **7**

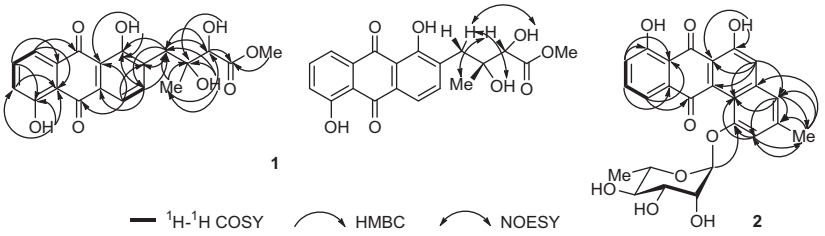

Figure 2. Selected key 2D correlations of compounds **1** and **2**.

Table 2
In vitro cytotoxicity of compounds **1**-**7** against BGC823, HepG2, A375, KB, and Ghost-R5X4 cell lines (IC_{50} μM)

	BGC823	HepG2	A375	KB	Ghost-R5X4
1	ND	ND	NA	NA	ND
2	ND	ND	NA	NA	ND
3	36.07	NA	NA	23.7	17.74
4	NA	NA	NA	NA	37.64
5	NA	NA	NA	NA	89.04
6	15.06	34.87	NA	NA	NA
7	11.03	17.36	17.5	NA	14.0
Doxorubicin·HCl[a]	0.01	1.11	0.73	0.01	5.73

ND = no data. NA = not active up to 100 μM.
[a] Used as a positive control

showed cytotoxicity against BGC823 cells with IC_{50} values of 15.06 and 11.03 μM, respectively, and against HepG2 cells with IC_{50} values of 34.87 and 17.36 μM, respectively. While in KB cells, only compound **3** showed cytotoxicity with IC_{50} value of 23.7 μM, and in A375 cells, only compound **7** exhibited cytotoxicity with IC_{50} value of 17.5 μM. It was noteworthy to mention that compounds **3**–**4** and **6**–**7** also showed cytotoxic against human cervical cancer HeLa cells, human gastric adenocarcinoma SGC-7901 cells, human lung adenocarcinoma SPC-A-1 cells and mouse macrophage RAW264.7 cells in a previous study.[12]

In conclusion, one new oxidatively opened angucyclinone derivative (**1**), one new angucycline (**2**), and five known compounds (**3**–**7**), were isolated from the broth of the rare actinobacterium *Amycolatopsis* sp. HCa1, which was isolated from the gut of *Oxya chinensis*. Their structures were established by spectroscopic analysis. Their biological activity was evaluated in a cytotoxic assay. Compound **7** showed moderate cytotoxic activity against humuan tumor cell lines BGC823, HepG2, and A375 with IC_{50} values of 11.03, 17.36, 17.5 μM, respectively. These findings indicated that insect-associated bacterial communities are a promising source of new valuable bioactive natural products and are worthy of further investigation.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.048.

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17. *Strain, fermentation, extraction and isolation*: *Amycolatopsis* sp. HCa1, previously isolated from the gut of healthy grasshopper (*Oxya chinensis*) collected from the suburb of Nanchang, Jiangxi Province, PR China, was grown in inorganic salts-starch liquid media (consisting of 1.0 g/L KNO_3, 0.5 g/L $MgSO_4$, 0.5 g/L K_2HPO_4, 0.5 g/L NaCl, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 20 g/L soluble starch, and deionized water) at 28 °C and 140 rpm for 72 h. 30 mL of the seed culture was inoculated into each 1000 mL Erlenmeyer flask of production medium composed of (per litre): 10 g glucose, 10 g beef extract, 1 g peptone, and 5 g NaCl; the pH was adjusted to 7.0. They were incubated at 28 °C for 13 days on a rotary shaker at 140 rpm. The liquid filtrate from about 20 L of fermentation broth was collected and extracted four times with ethyl acetate (20 L × 4 times) at room temperature. The EtOAc solutions were combined and the solvents were evaporated under reduced pressure to afford a crude yellow oil extract (3.1 g), which was fractionated by silica gel (31 g, 200–300 mesh) column chromatography (CC) (4 × 60 cm) eluted with a gradient of $CHCl_3$/MeOH (v/v 100:0→0:100, each 500 mL) to give eight fractions (Fr.1–Fr.8). Fr. 1 ($CHCl_3$/MeOH, 100:0) (203.2 mg) was subsequently separated by Sephadex LH-20 CC (2 × 30 cm) eluted with $CDCl_3$ (400 mL) to give subfraction 1.4, which was recrystallized from the $CDCl_3$/MeOH mixture (v/v 1:1) at room temperature to yield **5** (4.2 mg). Fr. 2 ($CHCl_3$/MeOH, 100:1) (332.6 mg) was orange solid, which was then recrystallized from the $CDCl_3$/MeOH mixture (v/v 1:1) to yield **4** (12.6 mg), was removed by filtration. The filtrate (105.6 mg) of Fr. 2 was refractionated by ODS column (2.5 × 40 cm) with a gradient of MeOH/H_2O (v/v 40:60, 50:50, 60:40, 70:30, 100:0, each 500 mL) to obtain five subfractions. Subfraction 2.3 (MeOH/H_2O, 60:40) was chromatographed on Sephadex LH-20 column (1.5 × 40 cm) with MeOH (500 mL) to give **3** (3.6 mg). Subfraction 2.4 (MeOH/H_2O, 70:30) was purified by semipreparative reversed-phase HPLC (MeOH/H_2O, 65:35; 2 mL/min) to yield **1** (1.5 mg, t_R = 11.72 min). Fr. 4 ($CHCl_3$/MeOH, 100:4) (411.6 mg) was separated on ODS CC (2.5 × 40 cm) with a gradient of MeOH/H_2O (v/v 40:60, 50:50, 60:40, 70:30, 100:0, each 500 mL) to give five subfractions. Purification of subfraction 4.1 (MeOH/H_2O, 40:60) and 4.2 (MeOH/H_2O, 50:50) were conducted on Sephadex LH-20 column (2 × 45 cm) with MeOH (each 500 mL) to yield **6** (5.5 mg) and **7** (6.2 mg), respectively. Subfraction 4.3 (MeOH/H_2O, 60:40) was further fractioned again by Sephadex LH-20 CC (2 × 45 cm) using MeOH (500 mL) as eluents to give **2** (4.1 mg).
18. Physical and spectroscopic data of compounds **1** and **2**:
Amycomycin A (**1**), orange solid; $[\alpha]_D^{25}$ −29.1 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 206 (4.34), 227 (4.23), 280 (4.42) nm; IR (KBr) ν_{max}: 3589.9, 2921.7, 1738.0, 1683.1, 1650.9, 1539.8, 1457.2, 1421.0 cm^{-1}; ^{1}H and ^{13}C NMR spectroscopic data: see Table 1; HRESIMS: *m*/*z* 409.0889 [M+Na]$^+$ (calcd. for

$C_{20}H_{18}O_8Na$, 409.0894).
Amycomycin B (**2**), brown solid; $[\alpha]_D^{25}$ −90.5 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 249 (4.38), 427 (3.93) nm; IR (KBr) ν_{max}: 3649.0, 1736.3, 1698.9, 1683.1, 1558.4, 1509.3 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data: see Table 1; HRESIMS: *m*/*z* 489.1154 $[M+Na]^+$ (calcd for $C_{25}H_{22}O_9Na$, 489.1156).

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20. *Acid hydrolysis of compound **2***: compound **2** (3.0 mg) in MeOH (2 mL) containing 5% H_2SO_4 solution (2 mL) was heated in a boiling water-bath for 6 h, then cooled for 10 min. The mixture was diluted with distilled water (8 mL), and partitioned between $CHCl_3$ and H_2O. The aqueous layer was neutralized with aqueous $NaHCO_3$ solution (1 M) and evaporated to give a residue of white solid. The residue and authentic sugar were analyzed by TLC ($CHCl_3$/MeOH 8:2, v/v, R_f = 0.41) with phenylene diamine-aniline-phosphoric acid used as spray reagent, suggesting the presence of L-rhamnose in **2**, which was also confirmed by measurement of its optical rotation ($[\alpha]_D^{25}$ +10.2, (*c* 0.2, H_2O)). Meanwhile, some solutions of residue and L-rhamnose were mixed and then analyzed by TLC with phenylene diamine-aniline-phosphoric acid used as spray reagent, which showed the same R_f value, also suggesting the residue is L-rhamnose.
21. *In vitro cytotoxicity test*: the cytotoxic activities for compounds **1**–**7** were tested in vitro against five cell lines including BGC823 (human gastric adenocarcinoma cell line), HepG2 (human hepatocarcinoma cell line), A375 (human melanoma cell line), KB (human oral squamous carcinoma cell line), and Ghost-R5X4 (ghost cell line). These cell lines were purchased from the Jiangsu Provincial Center for Disease Prevention and Control. The purity of the tested compounds and doxorubicin·HCl was determined to be over 95% by using the HPLC-DAD method. The cytotoxic effects on these tested cell were assessed by the IC_{50} values, and determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colometric method as described in reference [Ge, H.M.; Yu, Z.G.; Zhang, J.; Wu, J.H.; Tan, R.X. *J. Nat. Prod.* **2009**, *72*, 753.]. Each set of test was conducted three times to confirm reproducibility of the results. These compounds were dissolved in DMSO (dimethyl sulfoxide), and doxorubicin·HCl was used as a positive control, and the medium without test compound as a negative control in the bioassay.