

NOTE

Xiakemycin A, a novel pyranonaphthoquinone antibiotic, produced by the *Streptomyces* sp. CC8-201 from the soil of a karst cave

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Pyranonaphthoquinone (PNQ) antibiotics represent an interesting family of biologically active secondary metabolites. The basic skeleton of PNQ antibiotics is the naphtho[2,3-*c*]pyran-5,10-dione ring system. PNQ lactones contain an additional γ -lactone ring fused to the dihydropyran moiety¹ such as lactoquinomycin A (**2**)² and its related aglycones, kalafungin (**3**),³ as shown in Figure 1. Many members of PNQ lactones were produced by actinomycetes, especially, *Streptomyces* spp., and found to be selective inhibitors of the serine-threonine kinase AKT, also known as protein kinase B.⁴

In the course of our screening for new antibiotics produced by actinomycetes from poorly explored habitats, a series of PNQ antibiotics with activity against *Staphylococcus aureus* ATCC 25923, were discovered from the secondary metabolites of a cave-derived actinomycete, CC8-201. The strain was isolated from the soil of a remote karst cave located at a suburb of Chongqing city, China, and was identified as a *Streptomyces* sp. based on a 16S rRNA gene analysis (GenBank accession number: KJ480796). Their UV and visible (UV/Vis) spectra were similar to that of PNQ antibiotics such as **2** and **3**. Under guidance of bioassay and UV/Vis spectra, fractionation and purification were conducted. As a result, a novel PNQ antibiotic was purified and identified. To the best of our knowledge, the compound is undoubtedly the first new antibiotic discovered from Chinese karst cave, hence, named as xiakemycin A (**1**) (Figure 1), in honor of Xia-ke Xu, the first explorer of karst caves in China.⁵ In this paper, we report the fermentation, isolation, physico-chemical properties, structural elucidation and bioactivities of **1**.

A stock culture of the strain *Streptomyces* sp. CC8-201 was maintained at 4 °C on YIM 38 agar slant consisting of yeast extract (Beijing Aoboxing Biotechnology, Beijing, China) 5.0 g, malt extract (Beijing Aoboxing Biotechnology) 4.0 g, glucose 4.0 g, phenylalanine 0.001 g, alanine 0.0003 g, thiamine 0.001 g, pyridoxine 0.001 g, riboflavin 0.001 g, niacin 0.001 g, biotin 0.001 g, FeSO₄·7H₂O 0.002 g, MnCl₂·4H₂O 0.001 g, ZnSO₄·7H₂O 0.001 g and agar 20.0 g

in 1.0 l distilled water (pH 8.0).⁶ The stock culture was inoculated into 500 ml Erlenmeyer flasks containing 100 ml of seed medium, which was the same as above, but without agar. The flask culture was incubated on a rotary shaker (180 r.p.m.) at 28 °C for 48 h. One hundred milliliters of the flask culture was transferred to a 5000-ml Erlenmeyer flask containing 1000 ml of the producing medium, which contained the same ingredients as seed medium and was supplemented with 2% soluble starch (Beijing Qi Te Xin Chemical Co. Ltd., Beijing, China). The fermentation was carried out at 28 °C for 168 h on a rotary shaker (180 r.p.m.). One hundred liters of supernatant from the fermentation broth was absorbed on a column containing 10 l Diaion HP-20 (Mitsubishi Chemical Holdings Corp., Tokyo, Japan) and the column was eluted successively with 30 l each of distilled water, 30, 50 and 80% acetone-distilled water to yield four fractions (Fr. A–D). Fr. D (2.2 g) showed bioactivity and was subjected to gel chromatography on Sephadex LH-20 (2.5 × 100 cm², Pharmacia Company, Uppsala, Sweden) using methanol as eluant to further purify. The first brown bioactive fraction (150 ml) was concentrated to give a brown material (160 mg). After being dissolved in 1.6 ml of methanol, the sample was filtered through a syringe filter unit with 0.22 μ m polytetrafluoroethylene (PTFE) membranes (Tianjin Jinteng Experiment Equipment Co., Ltd., Tianjin, China) and further purified by HPLC (Agilent 1200, Agilent Technologies Inc., Santa Clara, CA, USA) on a Zorbax SB-C18 column (9.4 × 250 mm², 5 μ m, Agilent Technologies Inc.) with methanol/water, 65:35 (v/v) at 1 ml min⁻¹. The peak at R_t = 35 min, with UV/Vis absorption maxima at 212, 263, 355 and 444 nm, was collected and pooled to yield 3 mg of **1** as an orange powder.

Compound **1** was soluble in dimethyl sulfoxide, MeOH, CHCl₃ and CH₃CN. Its m.p. was 143–144 °C (dec). The molecular formula of **1** was determined to be C₂₂H₂₀O₈ by HR-ESI-MS (m/z , found = 413.1231 [M+H]⁺, calcd = 413.1231), which has thirteen degrees of unsaturation.

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The IR spectrum (film) of **1** exhibited absorption bands at 3266, 3212, 2977, 2934, 2956, 1784, 1656, 1622, 1585, 1467, 1366, 1230, 1066, 792, 764 cm^{-1} , which indicated the presence of a lactone (1784 cm^{-1}), a non-chelated quinone carbonyl group (1656 cm^{-1}) and a chelated quinone carbonyl group (1622 cm^{-1}). The UV/Vis absorption spectra in methanol showed maxima at 212 ($\epsilon = 44\,646$), 263 (16\,405), 355 (3558), 444 (4270). Comprehensive analysis

of $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, $^{13}\text{C-}^1\text{H}$ COSY and DEPT spectra of **1** indicated 22 carbon signals could be attributed to 5 oxygenated methine groups, 2 methylene groups, 2 methyl groups, 10 olefinic carbons, 2 carbonyl carbons and 1 ester carbonyl carbon. NMR data of **1** in CDCl_3 (I) and CD_3OD (II) were listed in Table 1.

The UV/Vis spectrum of **1** is similar to that of PNQ lactones, such as **2** and **3**. NMR data comparison between **1** and **2** as shown in

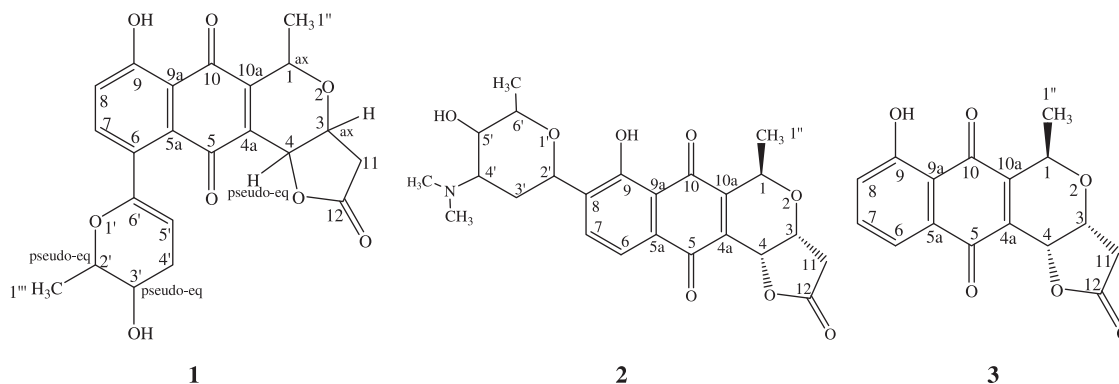


Figure 1 Structures of **1** (xiakemycin A), **2** (lactoquinomycin A) and **3** (kalafungin).

Table 1 NMR data of **1** (xiakemycin A) in CDCl_3 (I) and CD_3OD (II) and NMR data comparison of common structural portions between xiakemycin A (**1**) and lactoquinomycin A (**2**) in CDCl_3 *

Position	Lactoquinomycin A ²		Xiakemycin A			
	CDCl_3		CDCl_3 (I)		CD_3OD (II)	
	$\delta_{\text{C}a}$	$\delta_{\text{H}b}$ (mult, J Hz)	$\delta_{\text{C}c}$	$\delta_{\text{H}d}$ (mult, J Hz)	$\delta_{\text{C}c}$	$\delta_{\text{H}d}$ (mult, J Hz)
1	66.3	5.08 (q, 7.0)	66.2	5.04 (q, 7.2)	67.7	4.95 (q, 7.2)
1''	18.8	1.57 (d, 7.0)	18.6	1.56 (d, 7.2)	18.5	1.51 (d, 6.6)
3	66.5	4.69 (dd, 5.1, 2.9)	66.5	4.66 (dd, 4.8, 3.6)	68.1	4.71 (dd, 4.8, 3.0)
4	68.7	5.25 (d, 2.9)	68.3	5.28 (d, 3.6)	70.6	5.33 (d, 3.0)
4a	134.9	—	136.1	—	137.4	—
5	180.8	—	181.5	—	183.1	—
5a	129.7	—	129.0	—	131.3	—
6	119.6	7.71 (d, 7.8)	130.9	—	131.4	—
7	133.5	7.91 (d, 7.8)	139.9	7.56(d, 9.0)	140.8	7.55(d, 8.4)
8	138.6	—	124.4	7.25(d, 9.0)	124.6	7.19(d, 9.0)
9	157.7	—	162.5	—	163.3	—
9-OH	—	12.20 (br s)	—	12.32 (s)	—	—
9a	114.0	—	115.2	—	116.8	—
10	187.8	—	188.4	—	190.1	—
10a	149.2	—	148.2	—	149.3	—
11a	37.0	2.69 (d, 17.6)	36.9	2.69 (d, 18.0)	37.7	2.49(d, 18.0)
11b	—	2.97 (dd, 17.6, 5.1)	—	2.95 (dd, 18.0, 4.8)	—	3.09(dd, 18.0, 5.4)
12	173.5	—	174.0	—	177.2	—
2'	—	—	75.9	4.20 (quint, 6.6)	78.0	3.89 (m)
1'''	—	—	17.2	1.28 (d, 6.6)	17.5	1.24 (d, 6.6)
3'	—	—	67.1	3.80 (m)	69.6	3.57 (m)
3'-OH	—	—	—	3.31 (d, 9.0)	—	—
4'a	—	—	27.5	2.22 (m)	31.8	2.12 (ddd, 17.4, 9.0, 3.0)
4'b	—	—	—	2.50 (m)	—	2.46 (ddd, 17.4, 6.0, 4.8)
5'	—	—	95.9	4.83 (dd, 3.6, 4.2)	99.2	4.86 (dd, 4.8, 3.0)
6'	—	—	151.1	—	154.1	—

a. 100 MHz; b. 400 MHz; c. 125 MHz; d. 600 MHz

*NMR data of the substitution moiety at the C-8 position in lactoquinomycin A (**2**) did not shown in Table 1.

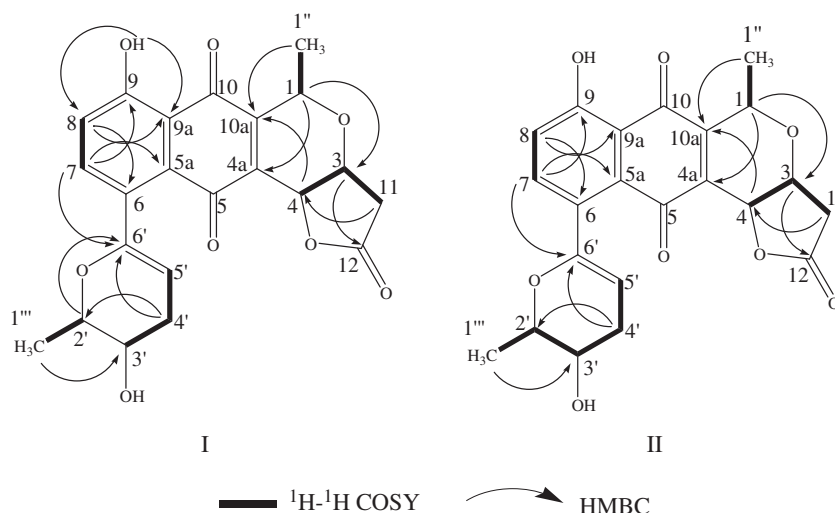


Figure 2 Summary of ^1H - ^1H COSY and key HMBC correlations for **1** in CDCl_3 (I) and in CD_3OD (II).

Table 1 revealed the similarities of chemical structure between **1** and **2**, and they are different in the substituted group and substituted position in juglone moiety. By careful analysis of 1D and 2D-NMR in both CDCl_3 and CD_3OD as shown in Figure 2, planar structure of **1** was elucidated as 6-substituted kalafungin.

The identification of Juglone moiety presented in **1** was started from two aromatic protons in ^1H -NMR in CDCl_3 : 7-H at δ 7.56 and 8-H at δ 7.25, which were readily observed. C-7 at δ 139.9 and C-8 at δ 124.4 were assigned by ^{13}C - ^1H COSY in CDCl_3 . The two aromatic protons showed cross peak in ^1H - ^1H COSY in CDCl_3 and formed AB spin system with coupling constant as 9.0 Hz, which demonstrated the existence of 1, 2, 3, 4-tetrasubstituted benzenoid ring. The other four aromatic carbons: C-9 at δ 162.5, C-9a at δ 115.2, C-5a at δ 129.0 and C-6 at δ 130.9 were observed in DEPT and ^{13}C -NMR spectra in CDCl_3 and were assigned by tracing the cross peaks in HMBC in CDCl_3 from 7-H to C-5a and C-9, from 8-H to C-6 and C-9a, from 9-OH proton at 12.32, which disappeared in ^1H -NMR in CD_3OD , to C-8 and C-9a. Chemical shift of 9-OH proton at low field indicated the phenol hydroxyl proton formed intra-molecular hydrogen bonded with carbonyl group in *peri*-position, thus, C-10 at 188.4, chemical shift at relatively low field was assigned and formed juglone moiety of **1** with another carbonyl group, C-5 at 181.5, chemical shift at relatively high field.

Juglone moiety was fused with dihydropyran moiety of **1** by two olefinic carbons: C-4a at δ 136.1 and C-10a at δ 148.2. The identification of the dihydropyran moiety was started from protons of $1''$ - CH_3 observed as a doublet signal at δ 1.56 in ^1H -NMR in CDCl_3 . By tracing the cross peaks from $1''$ -H to 1-H at δ 5.04, from 3-H at δ 4.66 to 4-H at δ 5.28 in ^1H - ^1H COSY in CDCl_3 , and the cross peaks between $1''$ -H and C-10a, between 1-H and C-4a, between 4-H and C-10a in HMBC in CDCl_3 , structural moiety as $\text{CH}_3(1'')\text{-CH}(1)\text{-C}(10\text{a})\text{-C}(4\text{a})\text{-CH}(4)\text{-CH}(3)$ was identified. The dihydropyran ring was closed by a oxygen atom between C-1 at δ 66.2 and C-3 at δ 66.5, which was further confirmed by a cross peak between 1-H and C-3 observed in HMBC in CDCl_3 .

Dihydropyran ring was fused with γ -lactone moiety of **1** by C-3 and C-4 at δ 68.3, which were identified by tracing cross peaks from 3-H to 11-H_a at δ 2.69 and 11-H_b at δ 2.95 in ^1H - ^1H COSY in CDCl_3 . By tracing the cross peaks between 3-H and C-12, the ester carbonyl carbon at δ 174.0, between 11-H_a and C-4 in HMBC in CDCl_3 ,

structural moiety as $\text{CH}(4)\text{-CH}(3)\text{-CH}_2(11)\text{-C}(12)$ was identified and further formed a γ -lactone ring closed by ester oxygen atom with C-4. Thus, planar structure of **1** was elucidated as 6-substituted kalafungin.

Identification of 6-substituted group in **1** was started from $1'''$ -H of a methyl group observed in ^1H -NMR in CDCl_3 , as a doublet signal at δ 1.28. By tracing the cross peaks from $1'''$ -H to 2'-H at δ 4.20, from 2'-H to 3'-H (in CD_3OD), from 3'-H at δ 3.80 to 4'-H_a at δ 2.22 and 4'-H_b at δ 2.50, from 4'-H to 5'-H at δ 4.83 in ^1H - ^1H COSY in CDCl_3 , structural moiety as $\text{CH}_3(1''')\text{-CH}(2')\text{-CH}(3')\text{-CH}_2(4')\text{-CH}(5')$ was identified and further confirmed by the cross peaks observed between $1'''$ -H and C-3' at δ 67.1, between 4'-H and C-2' at δ 75.9 in HMBC in CDCl_3 .

A hydroxyl proton at δ 3.31 as a doublet signal with coupling constant as 9.0 Hz in ^1H -NMR in CDCl_3 , but disappeared in CD_3OD , indicated the hydroxyl group must attached to C-3', a oxygenated methine carbon in DEPT. Chemical shift of 2'-H and C-2' were at lower field than that of 3'-H and C-3', which indicated C-2' must attach to an oxygen atom and the oxygen atom must bind with C-6', a quaternary carbon, as a cross peak between 2'-H and C-6' was observed in HMBC in CDCl_3 . Chemical shift of C-5' at 95.9 and C-6' at 151.1, together with a cross peak observed between 4'-H and C-6' in HMBC in CDCl_3 revealed both C-6' and C-5' were olefinic carbon and bind each other to form a dihydropyran ring. Thus, the substitution moiety at the C-6 position was elucidated as 3-hydroxyl-2-methyl-2, 3-dihydropyran. The cross peak observed in HMBC between 7-H and C-6' linked the substituted group with juglone moiety of **1** through C-6' and C-6. Finally, the planar structure of **1** was elucidated as shown in Figure 1, and the chemical name for **1** is 9-hydroxyl-6-(3-hydroxyl-2-methyl-2,3-dihydropyran) 1-methyl-1,3,4,11-tetrahydro-2*H*-furo[3,2-*b*]naphtho[2,3-*d*]pyran-5,10,12-trione.

The relative stereochemistry of **1** was determined by analysis of NOESY in CD_3OD (Figure 3). The cross peaks observed between $1''$ -H and 3-H, between 3-H and 4-H demonstrated that $1''$ - CH_3 and 3-H were in axial orientation and 4-H was in pseudo-equatorial orientation, the coupling constants between 3-H and 4-H as 3.0 Hz in CD_3OD further confirmed the judgment. In addition, the cross peaks observed between $1'''$ -H and 3'-H, between 3'-H and 4'-H_b, between 4'-H_a and 2'-H revealed that $1'''$ - CH_3 and 3'-OH were in pseudo-equatorial orientation, 2'-H, 3'-H were in axial orientation, the

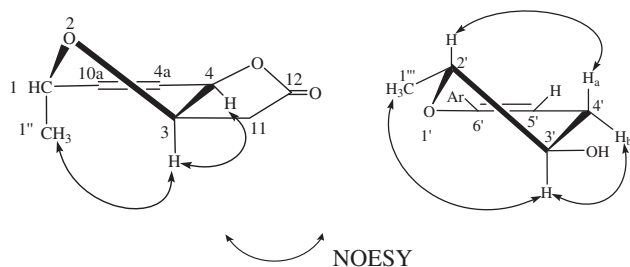


Figure 3 Key NOESY correlations of **1** in CD₃OD.

Table 2 Antibacterial activity of **1**

Test organisms	MIC ($\mu\text{g ml}^{-1}$)
<i>Staphylococcus aureus</i> ATCC 29213 (MSSA)	4
<i>Staphylococcus aureus</i> ATCC 33591 (MRSA)	4
<i>Staphylococcus aureus</i> 15 (MSSA)	4
<i>Staphylococcus aureus</i> 12-28 (MSSA)	8
<i>Staphylococcus aureus</i> 12-33 (MRSA)	8
<i>Staphylococcus epidermidis</i> ATCC 12228 (MSSE)	2
<i>Staphylococcus epidermidis</i> 12-6 (MSSE)	2
<i>Staphylococcus epidermidis</i> 12-8 (MRSE)	2
<i>Enterococcus faecalis</i> ATCC 29212 (VSE)	4
<i>Enterococcus faecalis</i> ATCC 51299 (VRE)	16
<i>Enterococcus faecalis</i> 12-5 (VSE)	16
<i>Enterococcus faecalis</i> 09-9 (VRE)	8
<i>Enterococcus faecium</i> ATCC 700221 (VRE)	16
<i>Enterococcus faecium</i> 12-1 (VRE)	8
<i>Enterococcus faecium</i> 12-3 (VSE)	16

coupling constants between 4'-H_a and 3'-H as 9.0 Hz in CD₃OD further confirmed the judgment. Thus, the relative stereochemistry of **1** was determined as Figure 1.

The MIC values against Gram-positive bacteria of **1** were listed in Table 2, compound **1** showed strong inhibitory activities against Gram-positive bacteria, but exhibited weak activities against *Candida parapsilosis* ATCC22019, *Candida albicans* CCTCCAY93025,

Cryptococcus laurentii CCTCCAY91013 and *Candida tropicalis* CCTCCAY91009, with MIC values $\geq 64 \mu\text{g ml}^{-1}$. MICs were determined by agar disk dilution method according to Clinical and Laboratory Standards Institute.^{7,8} The cytotoxicities of **1** *in vitro* against human lung cancer A549 cells, breast cancer MCF-7 cells, hepatoma HepG-2 cells, cervical cancer HeLa cells, colon carcinoma HCT-116 cell p53 wt cells, neuroblastoma SH-SY5Y cells and human prostate cancer PC-3 cells were measured by the methylthiazolyldiphenyl-tetrazolium bromide assay.⁹ Compound **1** exhibited *in vitro* cytotoxicity with IC₅₀ values of 2.77, 1.82, 1.54, 0.98, 0.59, 0.55 and 0.43 μM , separately.

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- Brimble, M. A., Duncalf, L. J. & Nairn, M. R. Pyranonaphthoquinone antibiotics— isolation, structure and biological activity. *Nat. Prod. Rep.* **16**, 267–281 (1999).
- Okabe, T. *et al.* Lactoquinomycin, a novel anticancer antibiotic II. Physico-chemical properties and structure assignment. *J. Antibiotics* **38**, 1333–1336 (1985).
- Johnson, L. E. & Dietz, A. Kalafungin, a new antibiotic produced by *Streptomyces tanashiensis* strain Kala. *Appl. Environ. Microb.* **16**, 1815–1821 (1968).
- Salaski, E. J. *et al.* Pyranonaphthoquinone lactones: a new class of AKT selective kinase inhibitors alkylate a regulatory loop cysteine. *J. Med. Chem.* **52**, 2181–2184 (2009).
- Ravbar, N. The earliest Chinese karstologist xu xiake. *Acta. Carsologica.* **32**, 243–254 (2003).
- Jiang, Y. *et al.* *Streptomyces hainanensis* sp. nov., a novel member of the genus *Streptomyces*. *Int. J. Syst. Evol. Microbiol.* **57**, 2694–2698 (2007).
- Clinical and Laboratory Standards Institute. Performance Standard for Antimicrobial Disk Susceptibility Tests; Approved Standard-11th edn. CLSI document M02-A11 (Clinical and Laboratory Standards Institute, 2012).
- Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. CLSI document M100-S23 (Clinical and Laboratory Standards Institute, 2014).
- Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **65**, 55–63 (1983).

Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)