Appl Biochem Biotechnol (2009) 159:284–293
DOI 10.1007/s12010-009-8591-2

Isolation and Difference in Anti-*Staphylococcus aureus* Bioactivity of Curvularin Derivates from Fungus *Eupenicillium* sp.

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Received: 23 August 2008 / Accepted: 20 February 2009 /
Published online: 31 March 2009

Abstract With the anti-microbial and anti-tumor composite screening model, bioassay-guided fractionation led to the isolation of two structurally related bioactive compounds, curvularin and αβ-dehydrocurvularin, from ethyl acetate extract of *Eupenicillium* sp. associated with marine sponge *Axinella* sp. Further study on the structure–activity relationship demonstrated that both compounds exhibited differences in bioactive profiles which are highly associated with their minor structural differences. Both curvularin and αβ-dehydrocurvularin have similar level of anti-fungal and anti-tumorous activity, while αβ-dehydrocurvularin is active against *Staphylococcus aureus* with a minimal inhibitory concentration of 375 μg/ml but curvularin does not. No detectable activity against Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* exists for both compounds. It is suggested that the partial planar backbone structure, due to the conjugation of π electrons in the presence of a 3,4-double bond and the carbonyl group at position C-2 in αβ-dehydrocurvularin, acts as a key factor for the inhibition of *S. aureus*, a Gram-positive low G + C bacteria that are often the hospital-acquired and/or community-acquired pathogen.

Keywords Curvularin · αβ-Dehydrocurvularin · Anti-*Staphylococcus aureus* · Bioassay-guided isolation

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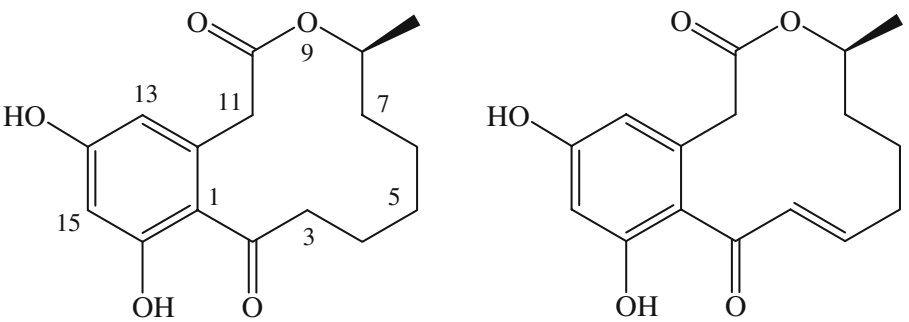
Introduction

The compound curvularin (**1**) is a macrolide, which shows antibiotic activity towards some fungi as a non-specific phytotoxin [1]. Curvularin and its derivatives could be potential nematicides against the root-lesion nematode, *Pratylenchus penetrans* [2]. More interestingly, some of these derivatives show to act as inhibitors in blocking the cell division [3] by specifically disordering the microtubule centers [4] and inducing barrel-shaped spindles [5]. It was reported that curvularin and αβ-dehydrocurvularin (**2**), as well as other derivates, are produced by some fungal species, such as *Curvularia* [6], *Aspergillus* [7], *Alternaria* [8], and *Penicillium* [9]. In this paper, we describe the bioassay-guided isolation of curvularin and αβ-dehydrocurvularin (see Scheme 1) from *Eupenicillium* sp., associated with marine sponge *Axinella* sp., and their structure elucidation. Using anti-microbial and anti-tumor composite screening models, we found that curvularin and αβ-dehydrocurvularin have slightly different bioactivity profiles.

Materials and Methods

General Experimental Procedures

Melting points were determined on a XRS-1 digital melting point apparatus. The ^{1}H and ^{13}C NMR data were collected on an Bruker AVANCE-500 (Bruker, Switzerland) spectrometer at 500 MHz, and the chemical shifts were recorded in δ (ppm) relative to Si $(Me)_4$ with coupling constants J in Hz. Electron ionization-mass spectrometry (EI-MS) was performed using a MAT95XP mass spectrometer (Thermo, USA). Silica gel (100–200 mesh) for open column chromatography and prepared silica gel (300 mesh) plates for TLC were produced by the Qingdao Marine Chemical Factory (Qingdao, China). Sephadex™ LH-20 was from GE Healthcare (GE, USA). Further purification of compounds was performed on a HITACHI L-2000 preparative high performance liquid chromatography (PHPLC) (HITACHI, Japan) with one YMC semi-preparative ODS column. All other chemicals used in this study are of analytical grade.



Scheme 1 Structure of curvularin and αβ-dehydrocurvularin

Isolation and Identification of Strain *Eupenicillium* sp.

The fungi isolate was obtained by serial dilution method [10] from sponge samples collected from South China Sea (18° 13′ N; 109° 29′ E) near Sanya, Hainan, China in July 2005. The sponge sample was later identified as *Axinella* sp. by Dr. K. J. Lee (Department of Biology, Hannam University, 133 Ojungdong, Daedukgu, Daejeon, Korea) through personal communication. The bioactive fungal strain was identified to be *Eupenicillium* sp. by comparing its morphological features with the reference description [11] and by online BLAST analysis of its 18S rDNA sequence with those submitted sequences on GenBank database.

Culture Media

The strain isolation was performed on the starch casein KNO_3 agar (SCKA) medium composed of 20 g starch, 2 g KNO_3, 2 g K_2HPO_4, 2 g NaCl, 0.3 g casein, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 18 g agar in 500 ml sterile distilled water premixed with 500 ml filtered sea water.

The germination and growth of the marine fungal isolate was undertaken on the Gauze's No. 1 sea water (GSW) medium, which was composed of 20 g soluble starch, 1 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, and 30 g agar in 500 ml sterile distilled water premixed with 500 ml filtered sea water. All above inorganic chemicals were from Guangzhou Chemical Reagent Factory, and the organic reagents from Amresco Inc.

Composite Screening Model

Indicator Strains

The initial bioactivity screening procedure involved indicator organisms including two fungi, two Gram-positive (G^+), and two Gram-negative (G^-) bacteria. The two indicator G^- strains, *Escherichia coli* (GIM 1.115) and *Pseudomonas aeruginosa* (GIM 1.46), two G^+ strains, *Bacillus subtilis* (GIM 1.181) and *Staphylococcus aureus* (GIM 1.178), and a fungus, *Saccharomyces cerevisiae* Hansen (GIM 2.86), were obtained from Microbial Culture Collection Center, Guangdong Institute of Microbiology (GIMCC). Another indicator fungus *Sclerotinia sclerotiorum* (ACCC 30046), one plant pathogenic fungus, were from Agricultural Culture Collection of China (ACCC) through professor Zanmin Hu of Institute of Genetics and Developmental Biology, CAS.

Tumor Cell Lines

The further anti-tumor screening for ethyl acetate (EtOAc) extract of fermentation (see 'Separation and Purification of Metabolites') referred to four types of tumor cell including human lung adenocarcinoma epithelial cell line (A549), human Henrietta Lacks cervical cancer cell line (HeLa), mice Ehrlich ascites carcinoma cell line (Ehrlich), and human breast adenocarcinoma cell line (MCF-7) that were carried out at the Medical Science College of China Three Gorges University.

Antagonistic Test

For fungal inhibition test, an inoculum (5 mm in diameter) of each isolate, prepared by punching the growth agar gel of 72-h Petri dish culture, was transferred into the four

corners of a 9-cm-diameter Petri dish containing PDA medium. A fungal indicator organism (5 mm in diameter) was put in the middle of the same Petri dish. The inoculated Petri dish was cultured at 28 °C for 48 h. The antagonistic action [12] was evaluated by measuring the diameter of the inhibition zones with penicillin as a positive control. For bacterial inhibition test, an indicator organism was spread on top of the Petri dish containing LB agar. An inoculum (5 mm in diameter) of each isolate, prepared by punching the growth agar gel of 72-h Petri dish culture, was transferred into the four corners of the Petri dish with indicator bacteria. The inoculated Petri dish was cultured at 28 °C for 48 h. The antagonistic action was evaluated by measuring the diameter of the inhibition zones with penicillin as a positive control. Each antagonistic test was performed in triplicate.

Fermentation Condition

The fresh mycelium growing on GSW agar at 28 °C for 5 days was inoculated into 500-ml Erlenmeyer flasks containing 150 ml GSW medium. After 2 days of incubation at 28 °C on rotary shaker at 200 rpm, a 40-ml seed culture liquid was transferred into each 1,000-ml Erlenmeyer flask containing 300 ml GSW medium. The flasks were incubated for 10 days at 28 °C and 200 rpm on a rotary shaker.

Bioassay of Fermentation Extract and Fractions

One liter culture filtrate was extracted four times with the same volume of EtOAc. After evaporation of the solvent from the combined extract *in vacuo*, 10 μl of the residue in serial concentration was used in an *in vitro* bioassay against anti-microbial screening model according to the filter paper disk diffusion methods [13] with the paper disk 5 mm in diameter. In bioassay-guided fractionation [14], the bioassay of fractions was conducted mainly against the sensitive test strains, *S. aureus* and *S. cerevisiae* Hansen, with other same parameters as above. Each fraction was tested in triplicate with dimethyl sulfoxide (DMSO) as a negative control.

Separation and Purification of Metabolites

Eupenicillium sp. was cultured in GSW medium at 28 °C and 200 rpm in a rotary shaker for 10 days. The culture broth (30 l) was filtered before being extracted with EtOAc for four times. The combined filtrates were concentrated *in vacuo*, and the obtained residue (9.8 g) was first fractionated by open column chromatography on silica gel ($CHCl_3$–MeOH gradient). The activity tracking method or the bioassay-guided fractionation was applied throughout the separation and purification procedure. One bioactive fraction (0.6 g), obtained by elution with 98% $CHCl_3$–MeOH on silica gel column, was further purified on a PHPLC equipped with a 10 mm×250 mm ODS column (MeOH–H_2O, 60:40). This fraction yielded 36 mg of compound **1** as colorless plate from MeOH. Another bioactive fraction (1.1 g), obtained by elution with 95% $CHCl_3$–MeOH on silica gel column, was further purified on a Sephadex LH-20 column ($CHCl_3$–MeOH, 1:1) and recrystallized from MeOH to yield 126 mg of compound **2** as a yellowy plate.

Bioassays and MIC Detections of the Purified Compounds

Anti-microbial activities and minimal inhibitory concentration (MIC) values of compound **1** and **2** (dissolved in DMSO) were assessed *in vitro* using anti-microbial screening strains as

above by the agar serial dilution method described by Ter Laak et al. [15], with DMSO as a negative control and penicillin as a positive control. Each isolated compound of different concentration was performed in triplicate.

Using above anti-tumor screening cells, IC_{50} value of compounds **1** and **2** (in DMSO) against the four types of tumor cell lines was tested in triplicate according to the MTT method described in the literature [16], with DMSO as a negative control and Taxol™ as a positive control.

Characterizations and Structure Elucidations of Metabolites

Curvularin (Compound **1***, See Scheme 1)*

Colorless plate (MeOH), mp. 203–207 °C. EI-MS: 293 $[M + H]^+$, MF: $C_{16}H_{20}O_5$; ^1H NMR data (acetone-D_6, 500 MHz): 9.11(1 H, s, 16-OH), 8.69(1 H, s, 14-OH), 6.39(1 H, s, H-15), 6.34(1 H, d, J=1.6 Hz, H-13), 4.91(1 H, m, H-8), 3.79(1 H, d, J=15.7 Hz, H-11a), 3.70 (1 H, d, J=15.7 Hz, H-11b), 3.08(1 H, m, H-3a), 2.74(1 H, d, m, H-3b), 1.74(1 H, m, H-4a), 1.60(1 H, m, H-7b), 1.52(1 H, m, H-4b), 1.46(1 H, m, H-6a), 1.43(1 H, m, H-7a), 1.40 (1 H, m, H-5a), 1.29(1 H, m, H-6b), 1.25(1 H, m, H-5b), 1.11(3 H, d, J=6.3 Hz, 8-CH_3); ^{13}C NMR data (acetone-D_6, 500 MHz): 207.3(C-2), 171.5(C-10), 160.6(C-16), 158.8(C-14), 137.5(C-12), 121.8(C-1), 112.8(C-13), 103.0(C-15), 73.1(C-8), 44.2(C-3), 40.2(C-11), 33.4(C-7), 28.0(C-5), 25.1(C-6), 23.9(C-4), 21.1(8-CH_3). These data are identical to those described before elsewhere by Ghisalberti et al. [3, 17].

αβ-Dehydrocurvularin (Compound **2***, See Scheme 1)*

Yellowy plate (MeOH), mp. 218–223 °C. EI-MS: 291 $[M + H]^+$; MF: $C_{16}H_{18}O_5$; ^1H NMR data (acetone-D_6, 500 MHz): 10.20(1 H, s, 16-OH), 9.70(1 H, s, 14-OH), 6.77(1 H, m, H-3), 6.60(1 H, m, H-4), 6.35(1 H, s, H-13), 6.31(1 H, d, H-15), 4.74(1 H, m, H-8), 4.08(1H, d, J=17.7 Hz, H-11a), 3.62(1H, d, J=17.7 Hz, H-11b), 2.35(1 H, m, H-5b), 2.42(1 H, m, H-5a), 1.86(1 H, m, H-6a), 1.86(1 H, m, H-7a), 1.65(1 H, m, H-6b), 1.62(1 H, m, H-7b), 1.19(3H, d, J=6.4 Hz, 8-CH_3); ^{13}C NMR data (acetone-D_6, 500 MHz): 197.7(C-2), 172.3 (C-10), 166.4(C-16), 163.6(C-14), 150.0(C-4), 139.9(C-12), 133.0(C-3), 116.0(C-1), 114.2 (C-13), 103.4(C-15), 73.3(C-8), 44.1(C-11), 35.2(C-7), 33.6(C-5), 25.7(C-6), 22.0(4-CH_3). These data are identical to those described in the literature [8, 18].

Results and Discussion

With anti-microbial model, especially with G^+ indicator strain *S. aureus* and two fungal indicator strains *S. sclerotiorum* and *S. cerevisiae*, we found that one isolate from marine sponge *Axinella* sp. collected in South China Sea near Sanya, later identified as fungus *Eupenicillium* sp., had notably inhibitory activity in antagonistic test. So, further study on screening of bioactive compounds focused on *Eupenicillium* sp. Its main morphological characteristics are its tough, dense penicilli bearing long, broad columns of conidia, and its smooth-walled, unflanged ascospores which are produced within 14 days of inoculation onto GSW medium. It was reported that the oligotrophic culture media are more suitable for growth of marine-derived microbe [19, 20]. From several culture media including LB, SCKA, 2216E, PDA, YPD, PDAS, and GSW, the GSW medium with the least nutrition in 50% natural filtered sea water was selected for germination, growth, and fermentation of the

fungus isolate because *Eupenicillium* sp. grew faster and produced more active compounds in this medium.

The antagonistic actions of the fungus *Eupenicillium* sp. was shown in Table 1, although nearly no anti-bacterial activity was detected when G^- strains *E. coli* and *P. aeruginosa* were used as test strains, but its anti-*Staphylococcus aureus* and anti-fungal actions as indicated by the antagonistic tests were fairly similar to those of EtOAc extracts derived from its corresponding cultures. The results of anti-microbial activity tests showed that *Eupenicillium* sp. produced the effective anti-*Staphylococcus aureus* metabolites. Furthermore, the anti-*Staphylococcus aureus* activity of the EtOAc extract of the culture was stronger than to that of penicillin, which was used as a positive control under the same mass concentration (Table 1). As we have known, *S. aureus*, both hospital-acquired and community-acquired, which is the most common cause of staph infections, is a dangerous pathogen that involved in an increasing number of serious infections including acute bacterial meningitis with high risk for morbidity and mortality [21, 22]. Subsequently, a scaled-up culture of *Eupenicillium* sp. was prepared to purify and characterize the key anti-*Staphylococcus aureus* metabolites.

Using the anti-fungal and anti-*Staphylococcus aureus* bioassay-guided fractionation, two known metabolites (compounds **1** and **2**) were purified from the EtOAc extract of *Eupenicillium* sp. Compound **1** was identified as curvularin and compound **2** was identified as αβ-dehydrocurvularin by comparing their spectral data with those of the authentic data [3, 8, 18] after the spectral analyses including MS, ^{1}H NMR, ^{13}C NMR, and DEPT.

From bioassay-guided fractionation procedure, it was ascertained that both purified compounds inhibited against two fungal indicator strains at a similar concentration. And more interestingly, αβ-dehydrocurvularin inhibited strongly against *S. aureus*, a Gram-positive low G + C bacteria that are often hospital-acquired and/or community-acquired pathogen, whereas curvularin has no activity at all (Fig. 1). Looking through their chemical structure (see Scheme 1), there is only one functional group difference between the two compounds; αβ-dehydrocurvularin has a double bond between C-3 and C-4 position but curvularin has none in the corresponding position. Zhang et al. [23] demonstrated that a double bond, the only structural difference between two flavonoids (as one counterpart), is one of the important structural properties essential for potent interaction between flavonoid and the breast cancer resistance protein, and the flavonoid compound with double bond is more active than its counterpart. It was reported that the

Table 1 The anti-microbial activity of *Eupenicillium* sp. from marine sponge *Axinella* sp.

Strain	Antagonistic action						Inhibition by EtOAc extract					
	G^+ bacteria		G^- bacteria		Fungi		G^+ bacteria		G^- bacteria		Fungi	
	Bs	Sa	Ec	Pa	Sc	Ss	Bs	Sa	Ec	Pa	Sc	Ss
Eupenicillium sp.	+	++	−	−	++	+	+	+++	−	−	++	+
Penicillin	++	+	+++	++	++	++	++	+	+++	++	++	++

Bs, *Bacillus subtilis*; *Sa*, *Staphylococcus aureus*; *Ec*, *Escherichia coli*; *Pa*, *Pseudomonas aeruginosa*; *Sc*, *Saccharomyces cerevisiae* Hansen; *Ss*, *Sclerotinia sclerotiorum*

'−', no inhibition; '+', '+ +', and '+ + +', the diameter of observed inhibition zones is in the ranges of 1–10, 11–20, and 21–30 mm, respectively

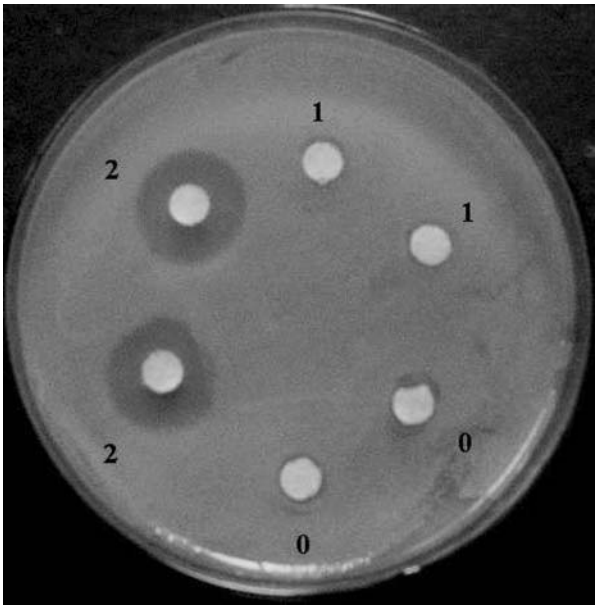


Fig. 1 The anti-*Staphylococcus aureus* activity difference between curvularin and αβ-dehydrocurvularin. *0*: DMSO; *1*: 500 μg/ml curvularin in DMSO; *2*: 500 μg/ml αβ-dehydrocurvularin in DMSO assayed with the paper disk 5 mm in diameter on GSW medium using filter paper disk diffusing method described by de Beer and Sherwood [13]

bioactivity of some other types of chemical compounds with double bond is notably stronger than those of their corresponding hydrogenated derivates [24–26]. The presence of a 3,4-double bond in αβ-dehydrocurvularin exhibited the inhibitory activity against *S. aureus* whereas curvularin did not, indicating that the presence of the 3,4-double bond is critical for inhibiting *S. aureus*. Because αβ-dehydrocurvularin has a partial planar backbone structure due to the conjugation of π electrons in the presence of a 3,4-double bond and the carbonyl group at position C-2, in contrast to a non-planar conformation of curvularin lacking this double bond, it is very likely that this partial planar conformation may be beneficial for the binding of αβ-dehydrocurvularin to the binding site(s) of *S. aureus*. Respecting no anti-bacterial activity against G^- strains for *Eupenicillium* sp., whether the mechanism of the activity against G^+ bacteria is related to the bacterial cell wall or not needs further investigation.

In addition, the two purified fungal metabolites were bioassayed against test G^+ bacteria *B. subtilis* and *S. aureus*, and test fungi *S. sclerotiorum* and *S. cerevisiae* by the two-fold serial dilution method (DMSO as solvent) starting from initial concentration 1.2×10^4 μg/ml. The MIC values are shown in Table 2. The results based on bioassay-guided fractionation and MICs test suggested that curvularin and αβ-dehydrocurvularin are the two major anti-fungal components, and αβ-dehydrocurvularin is the only anti-*Staphylococcus aureus* component in the fungal culture of *Eupenicillium* sp. Both compounds were relative weakly bioactive against the two test fungi, but especially αβ-dehydrocurvularin was more strongly active against *S. aureus* with the MIC just one order of magnitude higher than that of penicillin used as a positive control.

Curvularin and its derivates, as cell division inhibitors [3, 27] and unique spindle poisons [5], can be used as mold compounds for research on molecular biology. Furthermore, it was reported that curvularin, an inhibitor against A549 cell, is a new transcriptionally based inhibitors of iNOS (inducible nitric-oxide synthase) acting on the

Table 2 The minimal inhibitory concentration (MIC) values of curvularin and αβ-dehydrocurvularin (in μg/ml).

	Bs	Sa	Sc	Ss
Curvularin (1)	>3,000	–	750	>3,000
αβ-Dehydrocurvularin (2)	1,500	375	375	>3,000
Penicillin[a]	93.8	46.9	46.9	187.5
DMSO (solvent)	–	–	–	–

Bs, *Bacillus subtilis*; *Sa*, *Staphylococcus aureus*; *Sc*, *Saccharomyces cerevisiae* Hansen; *Ss*, *Sclerotinia sclerotiorum*

'–' means no inhibition

[a] Penicillin was co-assayed as positive control

Janus tyrosine kinase-STAT (the signal transducer and activator of transcription) pathway [28]. So it may represent lead structures for the development of drugs inhibiting iNOS-dependent overproduction of NO in pathophysiological situations. To fully understand the anti-tumor activity of curvularin and αβ-dehydrocurvularin, the IC_{50} values of the two compounds were assayed according to the MTT method. The results showed that both curvularin and αβ-dehydrocurvularin are actively against the four tumor cell lines (Table 3). The IC_{50} values of the two compounds against the four types of tumor cell lines, A549, HeLa, Ehrlich, and MCF-7, are listed in Table 3. The IC_{50} values of curvularin are almost always one order of magnitude higher than those of αβ-dehydrocurvularin against corresponding test cell lines, which might be due to the lack of the 3,4-double bond in the former compound.

In conclusion, this study demonstrated that curvularin and αβ-dehydrocurvularin isolated from *Eupenicillium* sp., a symbiotic fungus in marine sponge *Axinella* sp., were the two main structurally related secondary metabolites with slightly different antibiotic profiles. Both compounds are active against fungi and numerical cancer cell lines. But αβ-dehydrocurvularin showed bioactivity against *S. aureus* while curvularin did not, which indicates that the partial planar backbone structure in the presence of a 3,4-double bond plays an important role in the inhibition of *S. aureus*, a Gram-positive low G + C bacteria that are often the hospital-acquired and/or community-acquired pathogen.

Table 3 The IC_{50} values of compounds **1** and **2** against tumor cell lines (in μg/ml).

	A549	HeLa	Ehrlich	MCF-7
Curvularin (1)	142.6	92.5	47.8	113.6
αβ-Dehydrocurvularin (2)	15.3	10.3	20.4	19.8
Taxol[a]	4.5	7.4	8.6	3.2
DMSO (solvent)	–	–	–	–

A549, human lung adenocarcinoma epithelial cell line; *HeLa*, human Henrietta Lacks cervical cancer cell line; *Ehrlich*, mice Ehrlich ascites carcinoma cell line; *MCF-7*, human breast adenocarcinoma cell line

'–' means no inhibition

[a] Taxol was co-assayed as positive control

Acknowledgements This research was partially funded by the Knowledge Innovation Program of the Chinese Academy of Sciences (KZCX2-YW-216, KZCX2-YW-211) and National Supportive Plan Project of Science and Technology (2006BAB19B02). Parts of this study were carried out at the Key Laboratory of Microbial Application and Innovative Technology of Guangdong Province as an open funding project. We also thank the financial support of the Hundred Talents Program of Chinese Academy of Sciences. Many thanks to Dr. K. J. Lee for identification of the marine sponge, to Prof. Zanmin Hu and Prof. Honghui Zhu for providing some of the indicator organisms, and to Dr. Donna Smith for critical reading of this manuscript.

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