

## ORIGINAL ARTICLE

# Penicillenols from a deep-sea fungus *Aspergillus restrictus* inhibit *Candida albicans* biofilm formation and hyphal growth

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Penicillenols (A1, A2, B1, B2, C1 and C2) were isolated from *Aspergillus restrictus* DFFSCS006, and could differentially inhibit biofilm formation and eradicate pre-developed biofilms of *Candida albicans*. Their structure–bioactivity relationships suggested that the saturation of hydrocarbon chain at C-8, *R*-configuration of C-5 and *trans*-configuration of the double bond between C-5 and C-6 of pyrrolidine-2,4-dione unit were important for their anti-biofilm activities. Penicillenols A2 and B1 slowed the hyphal growth and suppressed the transcripts of hypha specific genes *HWP1*, *ALS1*, *ALS3*, *ECE1* and *SAP4*. Moreover, penicillenols A2 and B1 were found to act synergistically with amphotericin B against *C. albicans* biofilm formation.

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## INTRODUCTION

*Candida albicans* is a harmless commensal together with other members of the microbiota in individuals with healthy immune systems, but when the system is impaired or the microbiota is changed, *C. albicans* is capable of causing life-threatening infections, often associated with the formation of biofilms.<sup>1</sup> *C. albicans* biofilms, consisting of budding yeast, elongated hyphae and oval pseudohyphae, are inherently less sensitive to many clinical antifungal agents including fluconazole and amphotericin B and host immune defenses than planktonic cells.<sup>2,3</sup> Besides the *C. albicans* biofilm, the capacity of *C. albicans* to switch from yeast to hyphae is also associated with antifungal drug resistance, virulence, invasion and colonization, and the development of the spatially organized architecture of highly structured mature biofilms.<sup>4,5</sup> *Staphylococcus aureus* and *Pseudomonas aeruginosa* are capable of causing a series of acute and chronic infections, and biofilms protect them from antibiotics. Therefore, biofilms have shown to house a 100–1000-fold increase in tolerance towards antimicrobial agents compared with equivalent populations of their free-floating counterparts. Once biofilms colonized the indwelling medical devices, it became almost impossible to clear the infections.

In an attempt to find sources of anti-biofilm agents, many investigators have begun to extract and analyze natural products from plants and marine organisms.<sup>6</sup> For example, (-)- epigallocatechin-3-gallate from tea and 2,4-di-tert-butylphenol from marine bacterium *Vibrio alginolyticus* G16 inhibited the biofilm formation of *C. albicans*.<sup>7,8</sup> In a preliminary experiment, the ethyl acetate extract

of a culture broth of the deep-sea-derived fungus *Aspergillus restrictus* DFFSCS006 was found to exhibit significant antibacterial and anti-biofilm activity against *Staphylococcus aureus* and *C. albicans*. A further investigation on the chemical constituents of the extract led to the isolation of six penicillenols. Penicillenols A1, A2, B1, B2, C1 and C2 were first isolated from *Penicillium* sp. GQ-7 and marine-derived fungus *Xylariaceae* sp.<sup>9–10</sup> Penicillenols A1 and B1 exhibited cytotoxicity against HL-60 cell with IC<sub>50</sub> values of 0.76 and 3.2 μM, respectively.<sup>9</sup> Penicillenols B1 and B2 could inhibit the growth of *S. aureus* with MICs of 8 μM (2.2 μg ml<sup>-1</sup>) and 70 μM (19.4 μg ml<sup>-1</sup>), respectively.<sup>11</sup> Penicillenols A1 and A2 did not show obvious activity against *Bacillus subtilis* and *Escherichia coli* at 50 μg per disk (6 mm).<sup>10</sup> However, the anti-biofilm of the penicillenols discussed here has not been reported previously. In this study, penicillenols were obtained from a deep-sea-derived fungus *A. restrictus* DFFSCS006, and their anti-biofilm activities against *S. aureus* ATCC 6538, *P. aeruginosa* PAO1 and *C. albicans* SC5314, their structure–activity relationships and the mechanistic basis for penicillenols A2 and B1 mediating the inhibition of *C. albicans* biofilms were further studied.

## MATERIALS AND METHODS

### Strains

*A. restrictus* DFFSCS006 was isolated from a marine sediment sample collected from the South China Sea (17°59'742" N, 111°48'092" E, 2326 m depth)<sup>12</sup> and grown on potato dextrose agar medium supplemented with 3% sea salt. *S. aureus* ATCC 6538 and *P. aeruginosa* PAO1 (ATCC 15692) were routinely cultivated at 37 and 30 °C, respectively, in Luria Bertani medium and

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**Table 1** Sequences of the primers used for quantitative reverse transcription PCR

Gene	Name	Primer sequence (5'–3')
<i>ACT1</i>	Actin	GGTTTGAAGCTGCTGGTATTGACC/ACGTTACAGCAATACCTGGGAACATG
<i>HWP1</i>	Hyphal wall protein 1	CAAGTGGTGCTATTACTATTCCG/GCGACACTTGAGTAATTGGC
<i>ALS1</i>	Agglutinin-like sequence 1	AGCTGTTGCCAGTGCCTC/AATGTGTTGGTTGAAGGTGAG
<i>ALS3</i>	Agglutinin-like sequence 3	CAACATCAACCAACCAATCTC/TGAATAACAGAACCAGATCCG
<i>ECE1</i>	Extent of cell elongation 1	CTTCTTCAAGACTCCCAAC/TCAATACCGACAGTTTCAATG
<i>SAP4</i>	Secreted aspartyl protease 4	GGTACCGTTGATTCCAATTC/ATCTTCACTTTCACGAACACG

*C. albicans* SC5314 was cultivated in yeast peptone dextrose (YPD) medium at 37 °C.

### Fermentation, extraction, purification and identification

*A. restrictus* DFFSCS006 was inoculated in 120 ml soluble starch medium (10 g glucose per liter, 10 g starch soluble per liter, 1 g KH<sub>2</sub>PO<sub>4</sub> per liter, 1 g MgSO<sub>4</sub> per liter, 1 g pepton per liter and 30 g sea salt per liter) in a 500 ml flask and incubated on a rotary shaker (200 r.p.m.) at 28 °C for 2 days. Then each of 5 ml cultures was added into 300 ml liquid medium (20% potato, 2% maltose, 2% mannitol, 1% glucose, 0.5% monosodium glutamate, 0.5% peptone, 0.3% yeast and 3% sea salt, w/v) in a 1 liter flask and incubated statically at 26 °C for 31 days.

After incubation, the fermentation broth was collected and filtered through cheesecloth to separate the supernatant and mycelia. The supernatant was mixed with XAD-16 resin and mycelia were extracted with 80% acetone. The resin and acetone extracts were evaporated and extracted with ethyl acetate to afford a 100 g brown crude gum.

The crude gum was chromatographed on a silica gel column eluting with various gradient concentrations of CHCl<sub>3</sub>/MeOH (100:0, 99:1, 98:2, 95:5, 90:10, 80:20 and 0:100, v/v) to obtain seven fractions. Fraction 4 (30 g) was subjected to MPLC (medium pressure preparative liquid chromatography) eluting with CHCl<sub>3</sub>/acetone (100:0, 90:10, 80:20, 70:30, 60:40 and 50:50, v/v) and CHCl<sub>3</sub>/MeOH (90:10, 80:20, 70:30 and 50:50, v/v) to give three sub-fractions (Fr.4-1 ~ Fr.4-3). Fr.4-1 was further subjected to RP-MPLC (reversed-phase-MPLC) with an ODS column eluting with MeOH/H<sub>2</sub>O (from 20:80 to 0:100, 120 min, 20 ml min<sup>-1</sup>) to give four sub-fractions (Fr.4-1-1 ~ Fr.4-4-4). Fr.4-1-1 was purified by sephedex LH-20 column eluting with CH<sub>3</sub>Cl-MeOH (1:1) and SP HPLC (semi-preparative high performance liquid chromatography; YMC-Pack, ODS-A S-5 μm × 12 mm 250 × 20 mm inner diameter, 5 ml min<sup>-1</sup>) eluting with MeOH-H<sub>2</sub>O-TFA (89:11:0.03) to get compounds penicillenol B1 (90.4 mg, t<sub>R</sub> = 49.5 min) and penicillenol B2 (30.4 mg, t<sub>R</sub> = 52.5 min). Fr.4-1-2 was purified by sephedex LH-20 column eluting with CH<sub>3</sub>Cl-MeOH (1:1) and then further purified by SP HPLC (YMC-Pack, ODS-A S-5 μm × 12 mm 250 × 20 mm inner diameter, 5 ml min<sup>-1</sup>) eluting with MeOH-H<sub>2</sub>O-TFA (74:26:0.03) to afford compounds penicillenol C2 (15.9 mg, t<sub>R</sub> = 51.0 min) and penicillenol C1 (7.8 mg, t<sub>R</sub> = 58.5 min). Fr.4-3 was also subjected to RP-MPLC with an ODS column eluting with MeOH/H<sub>2</sub>O (from 20:80 to 0:100, 120 min, 20 ml min<sup>-1</sup>) to give five sub-fractions (Fr.4-3-1 ~ Fr.4-3-5). Fr.4-3-3 was purified by sephedex LH-20 column eluting with CH<sub>3</sub>Cl-MeOH (1:1) and then further purified by SP HPLC (YMC-Pack, ODS-A S-5 μm × 12 mm 250 × 20 mm inner diameter, 5 ml min<sup>-1</sup>) eluting with MeOH-H<sub>2</sub>O-TFA (76:24:0.03) to obtain compounds penicillenol A2 (21.0 mg, t<sub>R</sub> = 51.8 min) and penicillenol A1 (22.1 mg, t<sub>R</sub> = 45.0 min).

The <sup>1</sup>H and <sup>13</sup>C NMR data of penicillenols A1, A2, B1, B2, C1 and C2 were acquired with a Bruker AV-500 MHz NMR spectrometer (Bruker, Germany) with TMS as reference.

### Determination of MICs

The MICs of penicillenols against *C. albicans* were tested by broth microdilution method in 96-well polystyrene plates (Corning, NY, USA) according to the Clinical and Laboratory Standards Institute guidelines (2008).<sup>13</sup> Wells containing 100 μl YPD with 2 × 10<sup>6</sup> cells ml<sup>-1</sup> *C. albicans* were supplemented with various concentrations (3.12 to 200 μg ml<sup>-1</sup>) of six penicillenols separately and incubated at 37 °C for 24 h. For *S. aureus* and

*P. aeruginosa*, wells with 100 μl *S. aureus*/*P. aeruginosa* dilutions (OD<sub>600</sub> = 0.01) were supplemented with different concentrations of the six penicillenols (3.12 to 200 μg ml<sup>-1</sup>) separately and incubated for 24 h. After incubation, optical densities at 600 nm (OD<sub>600</sub>) of the wells was measured using a Multiskan GO Reader (Thermo Scientific, Waltham, MA, USA).

### Biofilm formation and destruction assay

The effects of penicillenols on the formation of *C. albicans* biofilms were assayed in the pre-sterilized polystyrene flat-bottomed 96-well plates (Corning, NY, USA).<sup>8,14</sup> Briefly, wells containing 150 μl mineral medium (0.7 g KH<sub>2</sub>PO<sub>4</sub> per liter, 0.3 g K<sub>2</sub>HPO<sub>4</sub> per liter, 0.5 g MgSO<sub>4</sub> per liter, 2 g NaNO<sub>3</sub> per liter, 0.5 g KCl per liter, 0.01 g FeSO<sub>4</sub> per liter, pH 7) with 10<sup>6</sup> cells were subjected to different concentrations of penicillenols (6.25 to 200 μg ml<sup>-1</sup>) and then incubated at 37 °C for 48 h. After incubation, planktonic cells were discarded, and the wells were washed three times with sterile distilled water, dried, stained with 0.1% crystal violet (Shanghai Chemical Reagents Co. Ltd, China) for 30 min and dissolved with 30% acetic acid. OD<sub>570</sub> was determined using a Multiskan GO Reader (Thermo Scientific). Biofilm inhibition was calculated using the following formula: Percentage biofilm inhibition = [(Control OD<sub>570</sub> - Treated OD<sub>570</sub>) / Control OD<sub>570</sub>] × 100. The biofilms of *S. aureus* and *P. aeruginosa* were also measured according to the above procedures with the initial OD<sub>600</sub> of 0.01 and 24 h incubation. All the experiments were performed in eight replicates.

Moreover, the effects on biofilm disruption were detected with the biofilms grown in 96-well plates for 48 h as described above. The biofilms were treated with 12.5, 25, 50 and 100 μg ml<sup>-1</sup> penicillenols, separately, for 48 h, and then stained with crystal violet.

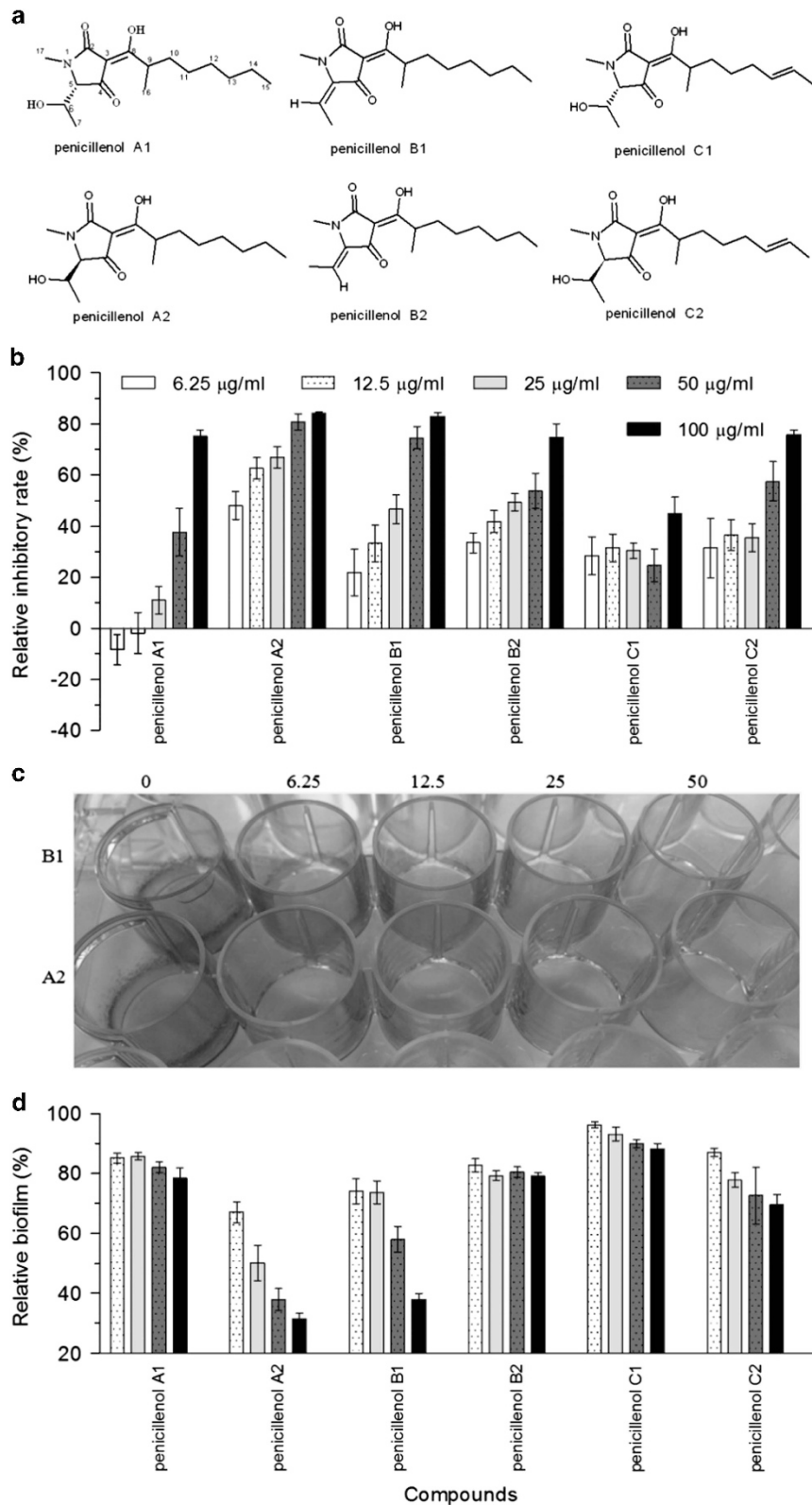
### Effects of penicillenols on hyphal growth

Hyphal growth assay was performed in 5 ml of YPD medium and YPD medium with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). A total 2 × 10<sup>7</sup> cells ml<sup>-1</sup> *C. albicans* in medium were incubated with different concentrations of penicillenols (25 and 50 μg ml<sup>-1</sup>) at 37 °C with agitation (200 r.p.m.). After 5 h, aliquots of samples were observed under bright field using × 40 objective lens by optical microscope and photographed.

Meanwhile, the effects of penicillenols on colony morphology were measured on YPD agar plates and YPD agar plates with 0.1 and 0.2 g Congo red per liter (Sigma, USA), respectively. One microliter cell suspension (10<sup>6</sup> cells ml<sup>-1</sup>) was spotted at the center of the agar plates supplemented with/without 30 μg ml<sup>-1</sup> penicillenols, incubated at 37 °C for 48 and 96 h and then photographed.

### RNA isolation and quantitative reverse transcription PCR

The cells grown in YPD supplemented with/without 25 μg ml<sup>-1</sup> penicillenols with agitation for 5 h were collected, and the total RNAs were extracted using RNAiso Plus Reagent (TaKaRa, Dalian, China). The complementary DNA was synthesized with PrimeScript RT reagent kit (TaKaRa) and 10-fold dilutions were used as templates to analyze the transcriptional levels of five hypha specific genes via quantitative reverse transcription PCR with paired primers and housekeeping internal control (*ACT1*; Table 1) as an internal standard with SYBR Premix Ex Taq (TaKaRa). The relative transcription level of each gene was evaluated as the ratio of its transcript in YPD with penicillenols over that in YPD medium with DMSO using the 2<sup>-ΔΔCt</sup> method.<sup>15</sup>



**Figure 1** Penicillensols inhibited the *C. albicans* biofilm formation and eradicated the developed biofilms. (a) The chemical structures of six penicillensols. (b) The relative inhibitory rate of biofilm formation after treatment with penicillensols was quantified compared with the treatment without any penicillensol after 48 h in 96-well plates. (c) Crystal violet staining of biofilms treated with penicillensols A2 and B1 (0–50 µg ml<sup>-1</sup>). (d) The effects of six penicillensols on the 48 h grown biofilms were assayed in 96-well plates. Error bar: s.d. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

**Table 2** List of respective MIC and BIC<sub>80</sub> of penicillens against *S. aureus* ATCC 6538, *P. aeruginosa* PAO1 (ATCC 15692) and *C. albicans* SC5314

Compound	MIC ( $\mu\text{g ml}^{-1}$ )			BIC <sub>80</sub> ( $\mu\text{g ml}^{-1}$ )		
	<i>S. aureus</i>	<i>C. albicans</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>P. aeruginosa</i>
Penicillol A1	100	>200	>200	100	200	>200
Penicillol A2	12.5	>200	>200	25	50	>200
Penicillol B1	12.5	>200	>200	50	100	>200
Penicillol B2	200	>200	>200	200	200	>200
Penicillol C1	>200	>200	>200	>200	>200	>200
Penicillol C2	>200	>200	>200	>200	200	>200
Amphotericin B	—*	1.6	—	—	0.4	—
Gentamycin sulfate	—	—	4	—	—	4
Chloramphenicol	12.5	—	—	10	—	—

\*Not tested.

### Extracellular polymeric substance inhibition and phospholipase production

Extracellular polymeric substance (EPS) inhibition was evaluated by the carbohydrate estimation method.<sup>8</sup> Biofilms grown on glass slide (1×1) in mineral medium with/without penicillens (25 and 50  $\mu\text{g ml}^{-1}$ ) were transferred into a glass tube separately, and 500  $\mu\text{l}$  of 0.9% NaCl, 500  $\mu\text{l}$  of 5% phenol and 2.5 ml of 0.2% (w/v) hydrazine sulfate in concentrated H<sub>2</sub>SO<sub>4</sub> were added, and the tubes were incubated in dark for 1 h. Absorbance at OD<sub>490</sub> was recorded using a Multiskan GO Reader (Thermo Scientific). EPS inhibition was calculated using the following formula: % EPS inhibition = [(Control OD<sub>490</sub> - Treated OD<sub>490</sub>)/Control OD<sub>490</sub>] × 100.

For phospholipase production, 1  $\mu\text{l}$  cell suspension (10<sup>6</sup> cells ml<sup>-1</sup>) was placed at the middle of the phospholipase agar medium (1% peptone, 3% glucose, 5.73% NaCl, 0.055% CaCl<sub>2</sub>, 10% egg yolk emulsion and 1.8% agar, w/v) supplemented with/without 25  $\mu\text{g ml}^{-1}$  penicillens and incubated at 37 °C for 96 h.<sup>8</sup> Diameters of the colonies and the opaque precipitation zones around it were read and the formula: Phospholipase activity = Diameter of the colony/(Diameter of the colony+the precipitation zone) was used to analyze the phospholipase activity (Pz). Pz=1 means negative phospholipase activity, Pz=0.64–0.99 shows positive phospholipase activity and Pz≤0.63 means very strong activity.<sup>16</sup>

### Combination testing of penicillens with amphotericin B on *C. albicans* biofilm formation

The synergistic effects of penicillens with amphotericin B (AmB) were studied at their sub-BIC<sub>80</sub> concentrations. Biofilm formation was initiated in the presence of the combination of each penicillol (1.5 and 3.0  $\mu\text{g ml}^{-1}$ ) and AmB (0.05 and 0.1  $\mu\text{g ml}^{-1}$ ) or single compound in 96-well plates and incubated at 37 °C for 48 h as described above. The absorbance at 570 nm was determined using a Multiskan GO Reader (Thermo Scientific). All the experiments were performed in six replicates.

### Data analysis

The results from eight replicates were expressed as mean ± standard deviation (s.d.) and statistical analysis was subjected to one-factor analysis of variance performed with the Data Processing System software.<sup>17</sup> It is considered to be statistically significant when \*P<0.05 in all the experiments.

## RESULTS AND DISCUSSION

### Penicillens showed antimicrobial and anti-biofilm activity against *S. aureus* and *C. albicans*

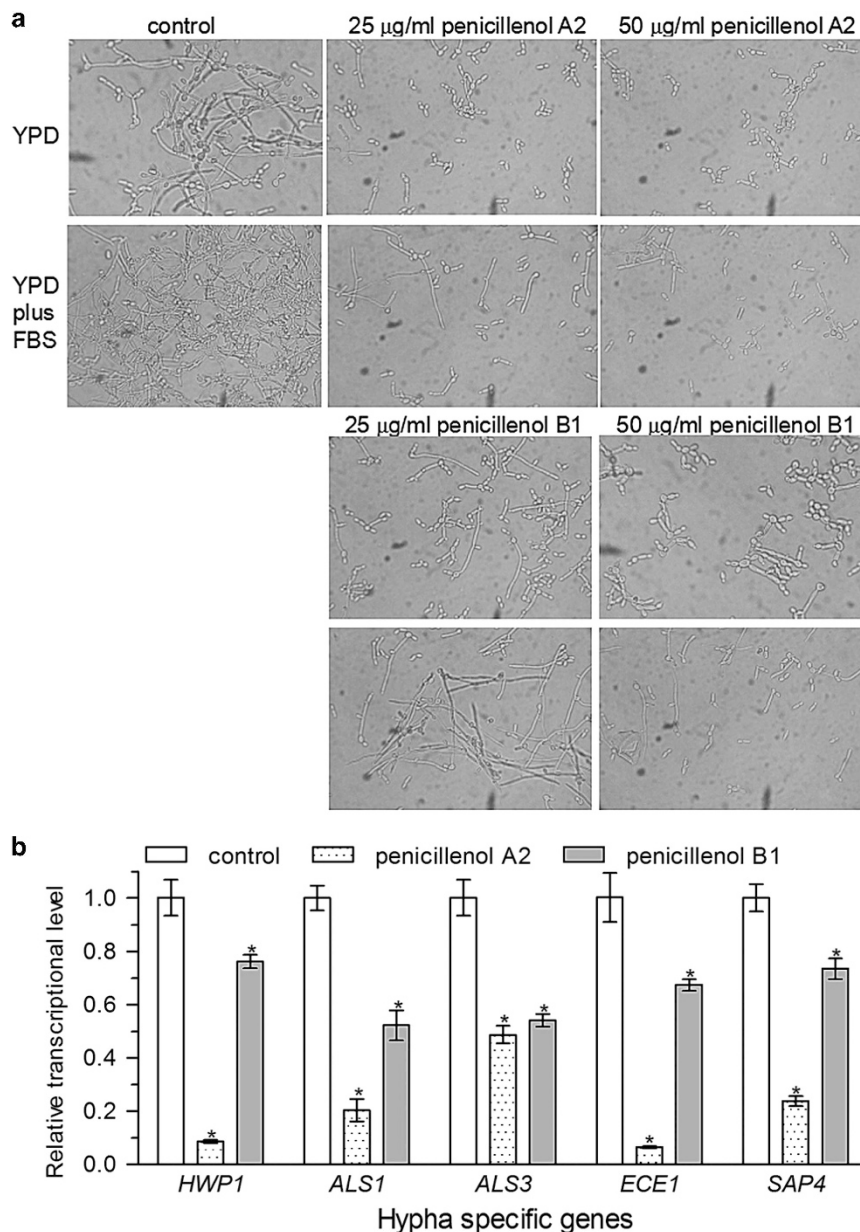
Penicillens (A1, A2, B1, B2, C1 and C2) were extracted and purified from the deep-sea-derived fungus *A. restrictus* DFFSCS006, and their structures (Figure 1a) were identified by comparison of their NMR data with reported data.<sup>9,10</sup> Previous studies reported that the six penicillens showed cytotoxic activities against several cell lines, and

penicillens B1 and B2 also showed dose-dependent antimicrobial activity against *S. aureus*.<sup>9,11</sup> Their anti-biofilm activities have not been reported. Here, initial studies were conducted to determine the antimicrobial activities of penicillens *in vitro*. The results (Table 2) showed that the penicillens inhibited differentially the growth of *S. aureus*, and the MICs of penicillens A2 and B1 are close to that of the antibiotic chloramphenicol, whereas all the penicillens exhibited weak inhibition of *C. albicans* and *P. aeruginosa* with MICs >200  $\mu\text{g ml}^{-1}$ . Meanwhile, the anti-biofilm activities of penicillens were investigated in 96-well microtiter plates. The BIC<sub>80</sub> was defined to be the lowest concentration where the biofilm formation was inhibited by 80% as compared with control. We found that six penicillens could inhibit the biofilm formation of *C. albicans* and *S. aureus* in varying degrees, where penicillol A2 has the lowest BIC<sub>80</sub> estimate, followed by penicillol B1, penicillol A1, penicillol B2, penicillol C2 and penicillol C1 in turn (Table 2). For *S. aureus*, BIC<sub>80</sub> estimates were greater than or equal to their MICs, which indicated that the anti-biofilm activity were due to the inhibition of *S. aureus* growth. For *C. albicans*, BIC<sub>80</sub> estimate was 50  $\mu\text{g ml}^{-1}$  for penicillol A2, 100  $\mu\text{g ml}^{-1}$  for penicillol B1, 200  $\mu\text{g ml}^{-1}$  for penicillol A1, B2 and C2, and >200  $\mu\text{g ml}^{-1}$  for penicillol C1, respectively, which were lower than their MICs. However, all the penicillens showed a BIC<sub>80</sub> >200  $\mu\text{g ml}^{-1}$  against *P. aeruginosa* (Table 2). These data indicated that penicillens could inhibit the growth of *S. aureus* and the biofilm formation of *C. albicans* at the sub-MIC concentrations, but did not significantly affect the cell growth and biofilm formation of *P. aeruginosa*, which promoted us to choose *C. albicans* as the tested strain in the following experiments.

### Penicillens inhibit biofilm formation and eradicate the preformed biofilm in *C. albicans*

Penicillens inhibited the *C. albicans* biofilm formation in a dose-dependent manner (Figures 1b and c). At 100  $\mu\text{g ml}^{-1}$ , their inhibitory rates were up to 50% except penicillol C1, and at 50  $\mu\text{g ml}^{-1}$ , the inhibitory rate was 81% for penicillol A2, 75% for penicillol B1, 58% for penicillol C2, 54% for penicillol B1 and 38% for penicillol A1, respectively (Figure 1b). Furthermore, the susceptibilities of penicillens against preformed biofilms were tested in 96-well microtiter plates with biofilms grown for 48 h. Figure 1d showed that penicillens A2 and B1 could accelerate the eradication by up to 50% (62–68%) at 100  $\mu\text{g ml}^{-1}$ , whereas other penicillens showed no obvious effect on the eradication of the developed biofilms. Meanwhile, the inhibitory rate of penicillol





**Figure 2** Effects of penicilllenols A2 and B1 on *C. albicans* hyphal growth. (a) *C. albicans* were grown in YPD medium (upper) and YPD with 10% FBS (lower) at the indicated concentration of penicilllenols A2 and B1 for 5 h with agitation. Each sample was photographed at  $\times 40$  magnification. (b) The relative transcriptional levels of hyphal specific genes in the presence of  $25 \mu\text{g ml}^{-1}$  penicilllenols A2 and B1 with respect to those in the absence of penicilllenols A2 and B1 were displayed after normalization with internal control housekeeping gene *ACT1*. Error bar: s.d. FBS, fetal bovine serum; YPD, yeast peptone dextrose. The asterisked bar (\*) in each three-bar group differs significantly from those unmarked ( $P < 0.05$ ).

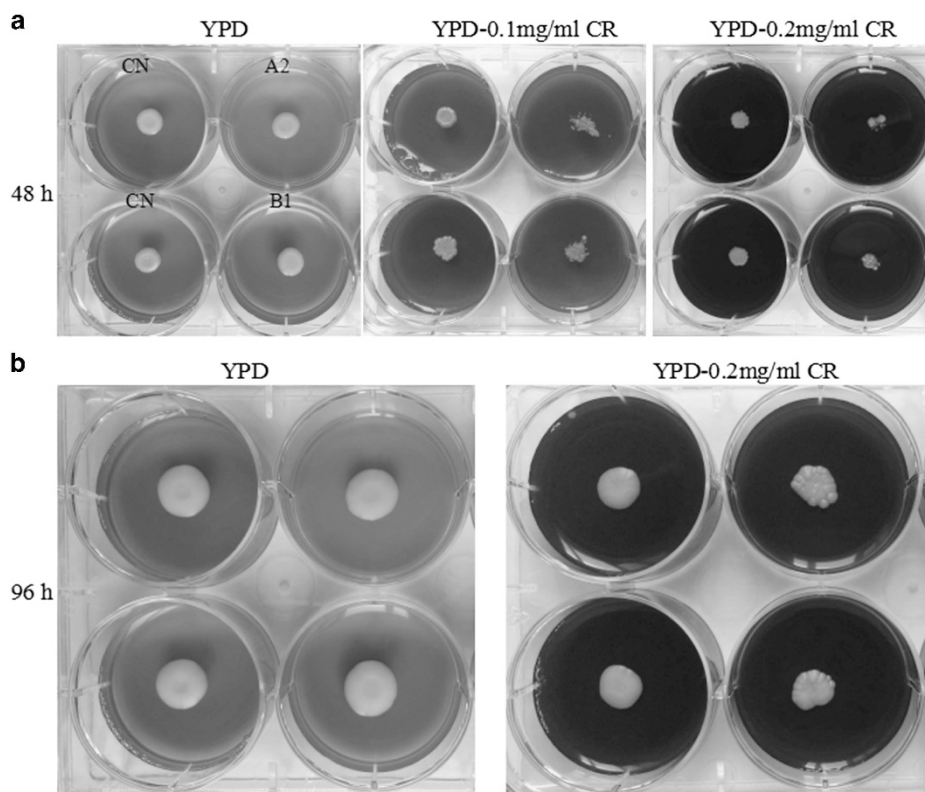
A2 was a little higher than that of penicilllenol B1. These data demonstrated that penicilllenols A2 and B1 were more active than other four penicilllenols.

Comparison of the structure–bioactivity of the penicilllenols evaluated suggested that the saturated hydrocarbon chain at C-8 and *trans*-configuration of the double bond between C-5 and C-6 might significantly affect their anti-biofilm activities against *C. albicans*, which is consistent with the previous results that the presence of a saturated fatty chain at C-8 might be essential for the pharmacophore and penicilllenol B1 (*trans*-configuration) was more active than penicilllenol B2 in inhibiting the growth of *S. aureus*.<sup>11,12</sup> Meanwhile, the *R*-configuration of C-5 of pyrrolidine-2,4-dione unit was also

implicated to be important for the anti-biofilm activity, which is different from the study where the *S*-configuration might be essential for anti-cancer activity.<sup>9</sup>

#### Penicilllenols inhibit *C. albicans* hyphal growth

Hyphae participate in the biofilm formation and mediate the dissemination of *C. albicans* to the host tissues by invasion.<sup>18</sup> Figure 2a showed that after 5 h, massive hyphae were observed in control samples without any penicilllenol, however, hyphal growth was modest in  $25 \mu\text{g ml}^{-1}$  penicilllenol A2 and  $50 \mu\text{g ml}^{-1}$  penicilllenol B1, while hyphae were shortest in  $50 \mu\text{g ml}^{-1}$  penicilllenol A2 in both media, indicating the dose-dependent inhibition of hyphal growth



**Figure 3** Effects of  $30 \mu\text{g ml}^{-1}$  penicillensols A2 and B1 on the *C. albicans* colonies growth in the YPD agar medium and YPD with Congo red (CR) agar medium after being grown for 48 h (a) and 96 h (b). A2, medium with  $30 \mu\text{g ml}^{-1}$  penicillensols A2; B1., medium with  $30 \mu\text{g ml}^{-1}$  penicillensols B1; CN, control treatment without penicillensols A2 and B1; YPD, yeast peptone dextrose. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

by penicillenol A2 and penicillenol B1. Nevertheless, the growth of hyphae in YPD medium with FBS was slightly better than those in YPD medium (Figure 2a). These results indicated two penicillensols inhibited the hyphal growth, which might be a potential explanation for their inhibition of the biofilm formation in *C. albicans*.

To further investigate the potential mechanism of penicillensols A2 and B1 on the inhibition of *C. albicans* hyphal growth, we analyzed the transcripts of important genes associated with hyphal growth, including hyphal wall protein 1 (*HWPI*), agglutinin-like sequence 1 and 3 (*ALS1* and *ALS3*), extent of cell elongation 1 (*ECE1*) and secreted aspartyl protease 4 (*SAP4*), in YPD medium and YPD medium supplemented with  $25 \mu\text{g ml}^{-1}$  penicillenol A2 or B1, respectively. Compared with the transcripts in untreated cells, transcripts of *HWPI*, *ALS1*, *ALS3*, *ECE1* and *SAP4* were suppressed by 92%, 80%, 51%, 94% and 76% in penicillenol A2-treated cells, respectively, and 24%, 48%, 46%, 33% and 27% in penicillenol B1-treated cells, respectively (Figure 2b). It is reported that mutants of *HWPI*, *ALS1* and/or *ALS3* showed a defect in biofilm formation of *C. albicans*.<sup>19–21</sup> Thus, the downregulation of these genes and the inhibition of hyphal growth mediated by penicillensols A2 and B1 could be a reason for the inhibition of *C. albicans* biofilm formation.

Meanwhile, penicillensols A2 and B1 reduced the growth of *C. albicans* colonies on the plates containing Congo red, a cell wall-disrupting agent interfering with the construction and stress response of the cell wall.<sup>22</sup> As shown in Figure 3a, there was no obvious difference among all the colonies in YPD agar plates, whereas both penicillenol A2 and penicillenol B1 weakened the growth of *C. albicans* colonies in YPD agar plates with 0.1 and 0.2  $\text{mg ml}^{-1}$  Congo red after

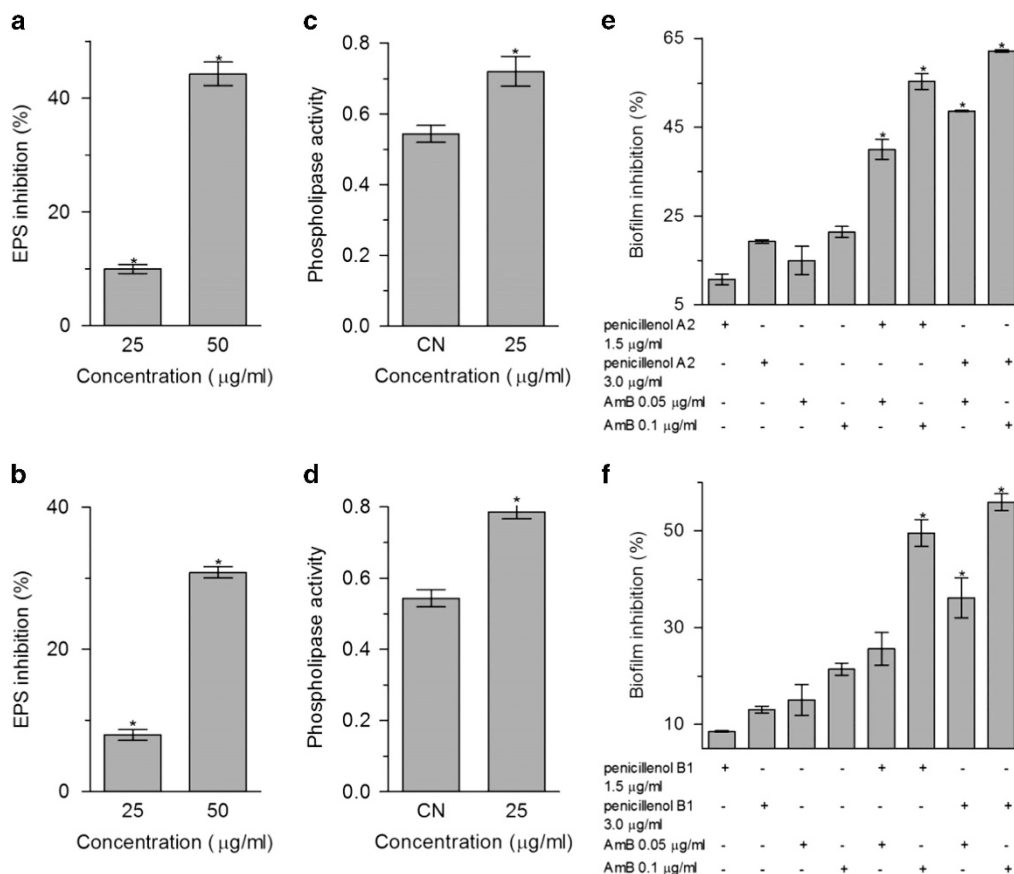
incubation for 48 h (Figure 3a) and 96 h (Figure 3b), which suggested that the two penicillensols might cause a defective cell wall, which is important for fitness and virulence.<sup>20</sup>

#### Effects of penicillensols A2 and B1 on EPS and phospholipase production and their synergistic effects with AmB on *C. albicans* biofilm formation

The EPS molecules, a major factor influencing the microbial biofilm formation process, were inhibited by 44% and 31% at  $50 \mu\text{g ml}^{-1}$  penicillensols A2 and B1, respectively, and less than 10% by both  $25 \mu\text{g ml}^{-1}$  penicillensols A2 and B1 (Figures 4a and b).

Phospholipases, hydrolyzing enzymes vital for virulence, could cleave phospholipids in cell membranes, helping *C. albicans* invade host tissues. Phospholipase activity (Pz) testing showed that control *C. albicans* exhibited strong phospholipase activity, with a Pz of 0.54, which was reduced to 0.72 and 0.79 on treatment with penicillensols A2 and B1, respectively. The results demonstrated that penicillensols A2 and B1 reduced the phospholipase activity of *C. albicans*.

The activities of penicillensols A2 and B1 together with AmB against *C. albicans* biofilm formation were assayed in 96-well plates. The results showed that 1.5 and  $3 \mu\text{g ml}^{-1}$  penicillenol A2, 1.5 and  $3 \mu\text{g ml}^{-1}$  penicillenol B1, and 0.05 and  $0.1 \mu\text{g ml}^{-1}$  AmB, inhibited the biofilm formation only by 11%, 19%, 9%, 13%, 15% and 21% respectively, but when 1.5 and  $3 \mu\text{g ml}^{-1}$  penicillenol A2 were combined with 0.05 and  $0.1 \mu\text{g ml}^{-1}$  AmB, respectively, the biofilm inhibitory rates were enhanced to 40%, 55%, 49% and 62%, respectively, which were higher than the sums of those of corresponding penicillenol A2 and AmB (Figure 4e). And the inhibitory rates



**Figure 4** Effects of penicilllenol A2 (a, c, e) and penicilllenol B1 (b, d, f) on the EPS inhibition (a, b), phospholipase activity (c, d) and anti-biofilm activity after combined with AmB (e, f). CN, control treatment without penicilllenols A2 and B1; EPS, extracellular polymeric substance. Error bar: s.d. The asterisked bar (\*) in each three-bar group differs significantly from those unmarked ( $P < 0.05$ ).

were 50% for 1.5 µg ml<sup>-1</sup> penicilllenol B1 plus 0.1 µg ml<sup>-1</sup> AmB, 36% for 3 µg ml<sup>-1</sup> penicilllenol B1 plus 0.05 µg ml<sup>-1</sup> AmB and 56% for 3 µg ml<sup>-1</sup> penicilllenol B1 plus 0.1 µg ml<sup>-1</sup> AmB, respectively, which were also higher than the sums of those of corresponding penicilllenol B1 and AmB (Figure 4f). These data demonstrated that both penicilllenols A2 and B1 might synergistically act with the antifungal agent AmB on *C. albicans* biofilm formation in varying degrees.

## CONCLUSION

Penicilllenols A2 and B1, isolated from the deep-sea-derived fungus *A. restrictus* DFFSCS006, inhibited the *C. albicans* biofilm formation by mediating the hyphal growth and the transcripts of related important genes including *HWP1*, *ALS1*, *ALS3*, *ECE1* and *SAP4*. They also caused the inhibition of EPS and the decline of the phospholipase activity and could improve the anti-biofilm activity of AmB, implying a promising synergistic combination for the treatment of candidiasis. And the structure–activity relationship of penicilllenols A1, A2, B1, B2, C1 and C2 revealed the saturated hydrocarbon chain at C-8, R-configuration of C-5 and trans-configuration of the double bond between C-5 and C-6 of pyrrolidine-2,4-dione unit might be important for their anti-biofilm activities against *C. albicans*.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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