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Tunicamycin A3, a new member of tunicamycins from the fermented mycelium of an actinomycete strain H83-3

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Abstract

Tunicamycin A3, a new member in family of tunicamycins, was obtained from the fermented mycelium of a marine actinomycete strains H83-3 which was isolated in the sea mud of mangrove region. The structure of the tunicamycin was elucidated on the basis of spectroscopic methods. The compounds showed potent activity against pathogenic fungus Candida albican with minimal inhibitory concentration (MIC) of 1.125 μg/mL, and weak anticancer activity against CNE cell line with the IC_{50} value of 85 μg/mL by MTT method.

Keywords

Streptomyce strain; tunicamycin A_3; antifungal activity; Candida albican.

1. Introduction

Tunicamycins are a family of nucleotide antibiotics which were produced by *Streptomyces lysosuperificus* [1], *Streptomyces clavuligerus* [2], and other *streptomyces* strains [3,4]. Structurally, they are composed of uracil, N-acetyl-glucosamine (GlcNAc), an unusual 11-carbon 2-aminodialdose (named tumicamine) and an amide-linked fatty acid [5]. The structural difference among the members of this family of compounds are only in the *N*-linked acyl chains, variant both in the number of branching methyl groups and in their locations [6]. Tunicamycins are potent inhibitors of the polyphenol-P: *N*-acetylhexosamine-1-P-translocase family and, consequently, are commonly used as tools for studying protein *N*-glycosylation and bacterial cell wall biosynthesis [7,8]. Now, tunicamycins are one of commonly used tools for studying protein *N*-glycosylation, bacterial cell wall biosynthesis, and cell biology [9]. The total synthesis of tunicamycin V further supported the structure and their biological activities [10]. Although natural tunicamycins were produced by microorganism usually in the mixture of a series of constitutional or homogenerous components with only the different *N*-linked acyl chains, these individual components can be separated and isolated by reversed phase C-18 HPLC, eluting typically with gradient acetonitrile-water or methanol-water mixture buffered with sodium acetate (pH 5.0), and detection of UV 260 nm due to common uracil moiety [11,12]. LC-ESI-MS or MABDI-TOF-MS are a very powerful tool for detecting and analyzing and characterizing tunicamycins in the mixture produced from the fermentation of some *Streptomycete* strains, even at a very low concentration (picomolar range) for the extract of fermented broth or the

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chromatographic fractions [13]. Eckardt's modified nomenclature led to a more consistency, structural logics and clear names for tunicamycins [14].

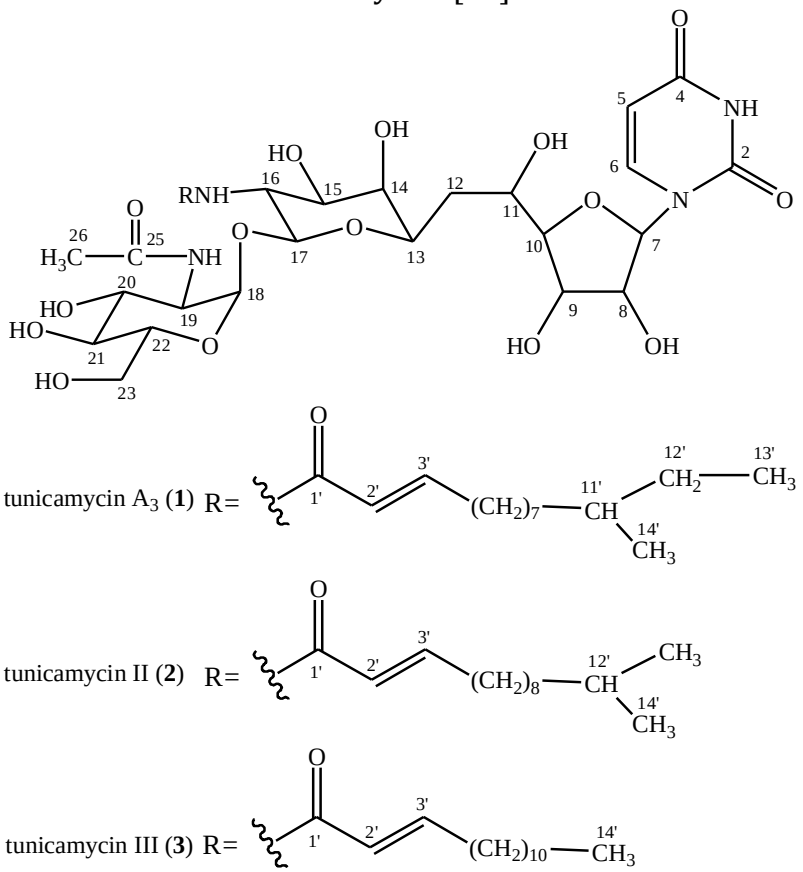

Figure 1. The structure of compounds 1, 2, and 3

2. Results and Discussion

The screen for active strains against Candida albicans from 2362 strains of actinomyces isolated from the soil and sand had targeted at 92 isolates including 43 strains of sea actinomyces and 49 strains of soil actinomyces. Amongst them, an actinomycete isolate designated as H83-3 was detected to have significant bioactivity against C. albicans. Strain H83-3 was identified to be genus Streptomyces by studying its morphology and 16S rDNA sequence analysis, most closely related to Streptomyces qiobisporus subsp. qiobisporus, sharing 99.9% of similarity. Sequence of the Streptomyce H83-3 is as follow:

AGAGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAA
GCCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGG
GACAAGCCCTGGAAACGGGGTCTAATACCGGATAATACTCCTGCCTGCATGGGTGGGGGTTGAAAG
CTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGA
CGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGA
AGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCT
GCATTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATG
CGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGC
GAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGG
TGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGG

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CCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATT
CGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCC
TTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACAGGAG
ACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGC
TGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGCGGAGCGAATCTCAAAAA
GCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAG
ATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCG
GTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTC
GGACGAAGTCGTAACAAGGTAACC.(Abbreviations: fragments shadowed were PCR amplification primers 27f and 1492r.)

According to our preliminary study on the fermentation conditions, the optimum flask-shaking conditions of H83-3 to obtain the most effective anti-fungi activity were as follow: basal culture medium for 3 days at the speed of 200 r/min under 28 °C, and the optimum expansion cultural fermentation conditions were: 10% inoculating content of 48 h-cultured mother solution with 7.0-7.2 initial pH value in basal GAUZE's Liquid Medium NO.1[15] for 3 days at 200 r/min of stirring speed under 28 °C. Total 40 L broth (2×20 L) was fermented for chemical investigation of antifungal metabolites.

Compound **1** was isolated as amorphous white solid. The molecular formula was determined as $C_{37}H_{60}N_4O_{16}$ based on its HR-ESI-MS data. UV showed the maxim absorption at 222 and 263 nm. The IR strong absorptions at 3397 and 1669 cm^{-1} indicated the presences of hydroxyl and carbonyl groups. The ^{1}H and ^{13}C NMR spectra (Table 1) exhibited the characteristic signals of an uracil moiety [δ_H 5.74 (1H, d, J = 8.0 Hz, H-5), 7.90 (1H, d, J = 8.0 Hz, H-5), and δ_C 152.70 (C-2), 166.25 (C-4), 103.41 (C-5) and 142.65 (C-6)], an acetyl group [δ_H 1.92 (3H, s), and a characteristic α,β-unsaturated carboxyl unit [δ_H 5.96 (1H, d, J=15.5 Hz, H-2'), 6.81 (1H, dt, J=15.5, 6.9 Hz, H-3'), and δ_C 169.7, 125.1, 146.6]. The uracil group was further confirmed by HMBC correlations between C-4 and H-5, and between H-4 and C-6. Successive correlations of 11 methine protons and a pair of methylene protons from H-7 to H-17 in ^{1}H-^{1}H COSY revealed the presence of an unusual 11-carbon 2-aminodialdose (that is, tunicamine) with the aids of HMBC and HSQC experiments. A *N*-acetyl glucosamine was indicated by the successive correlations of H-18~H-23 in ^{1}H –^{1}H COSY and the key HMBC correlation of H-19/C-25. The anomic carbon (C-18) from *N*-acetylglucosamine linked to the anomic carbon at the end of tunicamine. The other end of the tunicamine was connected to the *N*-atom of uracil moiety on the basis of the long rang corrections between H-1 and C-7, and between H-7 and C-2, and C-6 in HMBC. The α,β-unsaturated carboxyl unit was considered to the part of α,β-unsaturated carboxylic acyl group on the basis of the formula deduced from MS data and the signals of fatty chains from NMR spectra. The terminal of fatty chain was 2-*n*-butyl group due to partly overlap of two sets of signals from two terminal methyl groups (6H) in a triplet (3H, d, J=6.5 Hz) and a doublet (3H, t, J=6.8 Hz). Although tunicamycin with formula $C_{37}H_{60}N_4O_{16}$ and molecular weight of 816 units was isolated and determined to be tunicamycins II and III previously, compound **1** obviously had a different acyl chain. Therefore, **1** was a new tunicamycin, the constructional isomer of tunicamycins II & III [13], designated tunicamycin A_3. The compounds showed potent activity against pathogenic fungus *Candida albican* with minimal inhibitory concentration (MIC) of 1.125μg/mL, and weak anticancer activity against CNE cell line with the IC_{50} value of 85μg/mL.

ISSN: 1813-4890

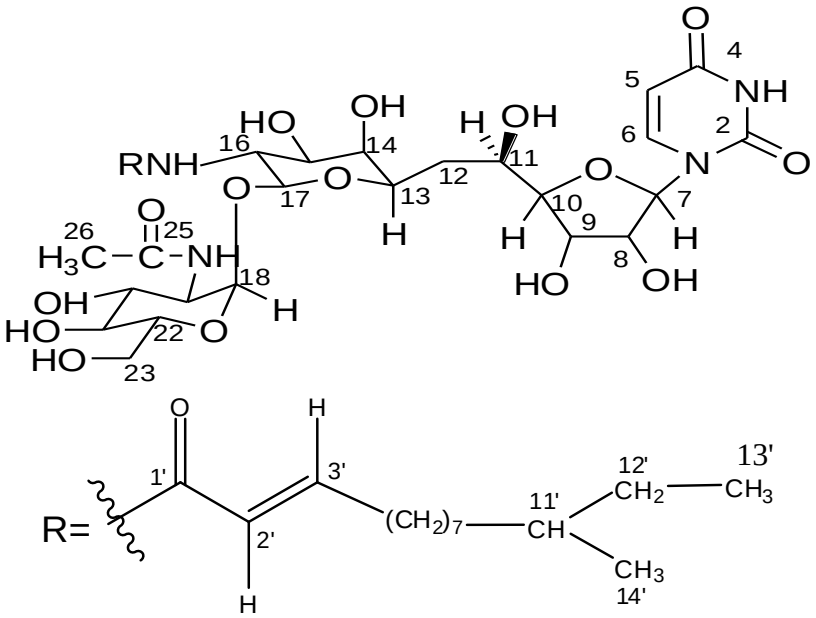
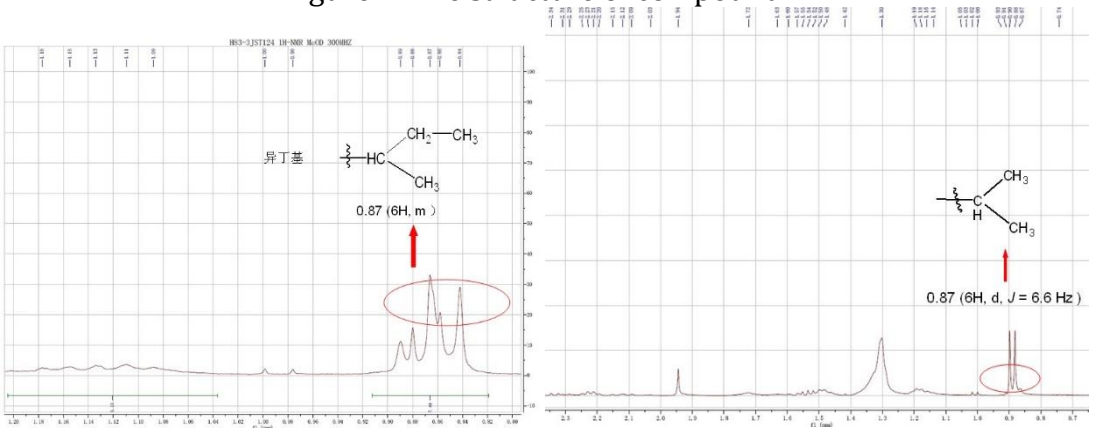
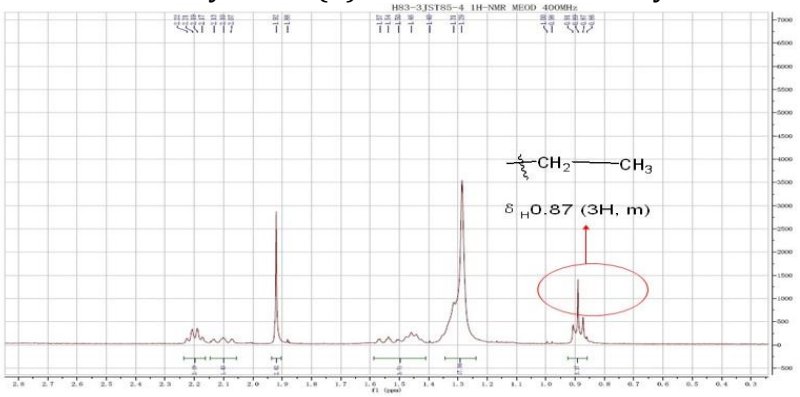


Figure 1. The structure of compound 1



A: Tunicamycin A3 (1) B: Tunicamycin II



C: Tunicamycin III

Figure 2. The comparison of the terminal methyl ^{1}H NMR signals of acyl fatty chains between tunicamycin A_3 (**1**) and tunicamycins II & III (=streptovirudin C_2)

ISSN: 1813-4890

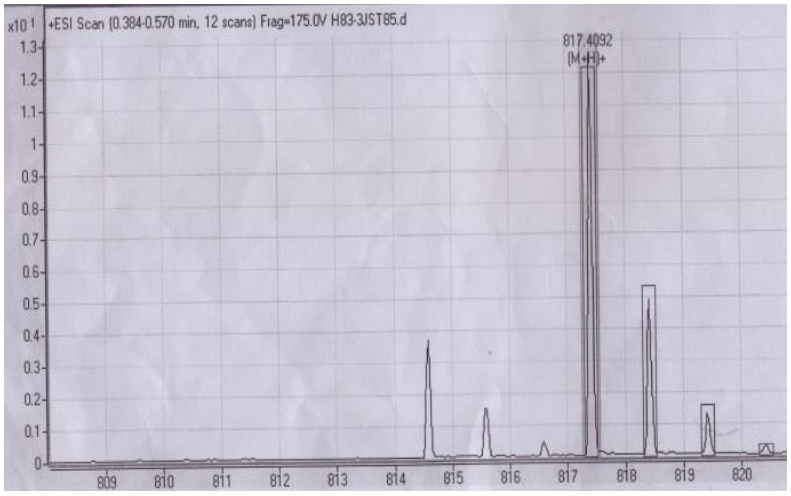


Figure 3. The high resolution ESI mass spectrum of **1**

Table 1. The ^{1}H and ^{13}C NMR (in MeOH-d_4) data of compound **1**

No.	δH (number of H, multiplet, J in Hz)	δC
2		152.70
4		166.25
5	5.74(1H, d, J = 8.0 Hz)	103.41
6	7.90 (1H, d, J = 8.1Hz)	142.65
7	5.90 (1H, d, J = 5.8 Hz)	90.42
8	4.20 (2H, m)	75.44
9	4.20 (2H, m)	70.89
10	3.84 (2H, m)	89.63
11	4.01 (3H, m)	68.45
12	2.09 (1H, t, J = 11.1 Hz)	35.98
13	3.77 (2H, m)	72.56
14	4.01 (3H, m)	74.31
15	3.64 (3H, m)	72.89
16	3.84 (2H, m)	54.95
17	4.92 (1H, d, J = 3.4 Hz)	100.18
18	4.57 (1H, d, J = 8.5 Hz)	102.04
19	4.01 (3H, m)	54.53
20	4.01 (3H, m)	73.29
21	3.64 (3H, m)	72.07
22	3.34 (lH, t, J = 9.1 Hz)	72.64
23	3.77 (2H, m)	63.19
25		173.45
26	1.92 (3H, s)	23.24
1′		169.71
2′	5.96 (1H, d, J = 15.5 Hz)	125.13
3′	6.81 (1H, dt, J = 15.5, 6.9 Hz)	146.61
4′~12′,	1.16 ~1.57 (2H, m) 1.28 (br s) 1.57-1.40 (4H, m)	40.27 33.00 31.01 30.74 30.67 30.55

ISSN: 1813-4890

		30.36 29.46 29.44 28.14
13′	0.87 (3H, t, J = 6.6 Hz)	19.62
14′	0.87 (3H, d, J = 6.8 Hz)	11.72

3. Experimental

3.1. General Procedure

Optical rotations were measured by a Jasco P-1020 digital polarmeter. Ultraviolet absorption spectra were recorded using a Jasco V-550 UV/VIS spectrophotometer. IR spectrum was scanned by a Jasco FT/IR-480 Plus spectrophotometer with KBr pellets. The 1D and 2D NMR spectra were recorded on a Bruker AV-600 spectrophotometer. Chemical shifts (δ) were expressed in ppm with reference to the residual solvent signals. ESI-Mass spectrum was obtained with a Finigan LCQ Advantage MAX mass spectrometer. HR-ESI-MS was determined on a Micromass Q-TOF mass spectrometer. Column chromatography was performed with silica gel (200-300 mesh, Qingdao Marine Chemical Plant, Qingdao, China), reversed phase C-18 (50 μm, YMC, Japan) and Sephadex LH-20 (25-100 μm, Fluka, Switzerland). Preparative HPLC was performed on a Varian Prostar system equipped with UV detectors (λ = 220 nm) and a preparative Cosmosil C18 column (20×250 mm). Strain *Candida albican* in paraffinic oil suspension solution of the spore was purchased from Guangdong Institute for Drug Control. Human nasopharyngeal carcinoma epithelial cell (CNE) was purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All the reagents were purchased from Tianjin Damao Chemical Company (Tianjin, China).

3.2. Isolation of strain H83-3

Actinomycetes were isolated from the sea soil and sand collected from all over the world and purely cultured on GAUZE's Medium NO.1. Purified isolates were preserved in sterilized sand tubes at 4 °C respectively.

For preliminary screening, isolates were grown in sterilized rice, 1 g of each was collected in a tube, and 2 mL of 70% ethanol was added. After immersing overnight, 2 mL of 95% ethanol was added, mixed, and then ultrasonicly vibrated for 30 min under 50°C. The mixture was then filtered and the filtrate was kept in the tube for dry preservation. Kirby Bauer Method was performed to test the antifugal activities of the actinomycetes. In detail, 0.1 mL of the *Candida albicans* culture was inoculated and laid evenly on Martin Agar Medium. Filter wafer (d=6 mm) imersed in the above filtrate was laid on the medium and inoculated at 37 °C. The diameters of the inhibition zones were measured after one day. Each treatment was repeated three times with triplicates.

Dual culture assay was performed for re-screening with *C. albicans* as the test microorganism. Briefly, actinomycete isolates with 20 mm of inhibition zone diameter or bigger were selected and revived on GAUZE's Medium NO. 1 for two generations. Films with 6 mm of diameter were punched and placed on Martin Agar Medium laid with *C. albicans* and inoculated at 37°C. The diameters of the inhibition zones were measured after one day. Each treatment was repeated three times with triplicates.

3.3. Taxonomic identification of strains H83-3 by genetic DNA sequencing

Actinomycete genomic DNA was isolated from the hypae cultured on GAUZE's Medium NO. 1 for 2 days by means of CTAB Method for 16S rDNA fragment gene amplification [15,16], PCR primers 27f (5'-AGAGTTTGATCATGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTTC-3')

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were used. PCR was performed in 50 μL of a reaction mixture containing 1×*TransStart FastPfu* Buffer, 250 *μ*M dNTPs, 0.4 μM of each primer, 2.5 U *TransStart FastPfu* DNA Polymerase (TransGen Biotech) and 2 *μ*L of template DNA. The amplification programm consisted of an initial denaturation (95°C, 2 min) followed by 30 cycles of denaturation (94°C, 30 sec), annealing (51°C, 30 sec), and extension (72°C, 2 min) with a single final extension step (72°C, 7 min). Then 1× PCR buffer, 200 *μ*M dNTPs, and 2.5 U *FastTaq* DNA Polymerase (TransGen Biotech) were added to the PCR product for an extension step (72°C, 30 min).

PCR product was analysed on 1% agarose gel containing with ethidium bromide and purified with PCR purification kit (TransGen Biotech) according to the manufacturer's instructions. After purification, the amplicicon was cloned in the pEASY-T vector and transformed into *Escherichia coli* DH5*α* competent cells, and the transformants were selected on LB agar supplemented with 100 *μ*g/ml Ap, which were then sent to Shanghai Invitrogen Biotechnology Co. for DNA sequencing. A BLAST search using the sequence of 16S rDNA was conducted in NCBI database.

3.4. Fermentation of strains H83-3

Mycelial pellets were added into 50 mL of flask shaking fermentation medium in a 250 mL flask at 30°C, 200 r/min for 2 days as mother solution, for exploring optimum fermentation condition, same volume of which was added to the basal flask shaking fermentation medium under different temperature conditions of 26, 28, 30 or 32°C for 2, 3, 4 or 5 days at speeds of 100 r/min, 150 r/min, 200 r/min or 250 r/min respectively to measure the anti-fungal activities.

Based on the flask shaking fermentation condition, expansion cultural fermentation was conduced in 20 L of shaking fermentation medium with a BIOSTATC 30 L fermentation tank under the optimum fermentation conditions as 10% of inoculating content at 300 r/min of stirring speed with 15 L/min of fermentation clean air at 28 °C for 3 days.

3.5. Extraction and isolation

Fermented broth was centrifugalized to divided two parts: mycelium and solution. The mycelium was overnight soaked with 95% ethanol in 1:5 ratio (*w*/*v*) each time for 4 times. The filtered solutions were concentrated *in vacuum* to obtain 74 g residue. Then, the residue was dissolved in 300 mL 90% methanol and partitioned with petrol ether in same volume for 4 times. The aqueous methanol layer was concentrated in vacuum to get the polar residue. The residue was suspended with 200 mL distilled water, then, successively partitioned with chloroform ($CHCl_3$) and ethyl acetate (EtOAc) to yield $CHCl_3$ fraction (Fr.c 22 g), EtOAc fraction (Fr.e 18 g), and H_2O layer residue (15 g). The Fr.e with potential antifungal activity was subjected to open silica gel column chromatography, eluting with gradient EtOAc-MeOH (*v*/*v* 50:1, 20:1, 10:1, 5:1, 1:1). The fractions from EtOAc-MeOH (*v*/*v* 1:1) were further separated by open ODS column chromatography eluting with gradient aqueous methanol (40%-100%) to afford 21 subfractions (Fr. 1~21). Some fractions eluted from 70% methanol were purified by preparative reversed phase C-18 HPLC with 68% aqueous methanol as moving phase to obtain **1** as well as tunicamycins II, III, V, VII, X, U18i (**2**-**7**).

3.6. Spectroscopic data

Compound **1** White amorphous solid, mp 242~244 °C; $[\alpha]_D^{25}$ +1.85°; UV_{max}^{MeOH} (nm): 221, 264; IR (KBr): 3390, 3015, 2936, 2853, 1665, 1558, 1098, 1023 cm^{-1}; ESI-MS *m/z* 839.5 $[M+Na]^+$, 815.7 $[M-H]^-$; HR-ESI-MS: *m/z* 817.4092 $[M+H]^+$, calcd. for $C_{37}H_{61}N_4O_{16}$, 817.4084, diff. 0.0008); ^1H NMR (400 MHz, MeOH-d_4) and ^{13}C NMR (125 MHz, MeOH-d_4) data, see Table 1.

3.7. Measure of MIC

Minimum inhibitory concentration (MIC) against *Candida albicans* was determined in 96 well plates with DMSO solution of compounds. *C. albicans* were grown in modified Martin medium

(composed of glucose 20g, peptone 5g, Yeast extract 4g, $K_2HPO_4 \cdot 7H_2O$ 0.63g, $MgSO_4 \cdot 7H_2O$ 1.8g in 1000 mL distilled water) for 12 hours and dispensed into 96 well plates, 200 μL each well and quadru-repeats. The sample solution containing compounds was added into the plate wells at final concentrations of 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 μg/mL. Plates were incubated for 20 h, with DMSO as negative control. MIC was determined as the concentration of compound that totally inhibited growth of *C. albicans*.

3.8. Cytotoxicity assay against CNE

The IC_{50} of compound **1** against CNE was assayed by MTT method. Briefly, freshly trypsinized CNE cell suspensions were seeded in 96-well microtiter plates at densities of 8000 cells per well in the absence or presence of tested samples (compound **1**), which were dissolved with DMSO. Doxorubicin was used as a positive control. After 72h of cultivation, attached cells were incubated with MTT (5 mg/mL) for 1 h. The absorbance at 570 nm was measured using a microplate reader. These results represented the average from at least three independent experiments.

Acknowledgements

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