Rev Iberoam Micol. 2012;29(1):29–33

Revista Iberoamericana de Micología

www.elsevier.es/reviberoammicol

Original article

In vitro activity of 5-(2,4-dimethylbenzyl) pyrrolidin-2-one extracted from marine *Streptomyces* VITSVK5 spp. against fungal and bacterial human pathogens

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ARTICLE INFO

Article history:
Received 26 October 2010
Accepted 8 June 2011
Available online 5 July 2011

Keywords:
Streptomyces VITSVK5 spp.
5-(2,4-Dimethylbenzyl) pyrrolidin-2-one (DMBPO)
Antibacterial activity
Antifungal activity
Marine natural product

ABSTRACT

Background: Pharmacological screening and usage of natural products for the treatment of human diseases has had a long history from traditional medicine to modern drugs. The majority of modern drugs are reported to be mostly from natural products.
Objective: The aim of the present study was to evaluate the inhibitory activity of 5-(2,4-dimethylbenzyl) pyrrolidin-2-one (DMBPO) extracted from marine *Streptomyces* VITSVK5 spp. isolated from sediment samples collected at Marakkanam coast of Bay of Bengal, India.
Methods: The lead compound was isolated by bioactive guided extraction and purified by silica gel column chromatography. Structural elucidation of the lead compound was carried out by using UV, FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT and HR-MS spectral data.
Results: Systematic screening of isolates for antimicrobial activity lead to identification of a potential strain, *Streptomyces* VITSVK5 spp. (GQ848482). Bioactivity guided extraction yielded a compound DMBPO and its inhibitory activity was tested against selected bacterial and fungal strains. DMBPO showed maximal activity against *Escherichia coli* with a MIC value of 187 μg/ml, followed by *Klebsiella pneumoniae* (MIC of 220 μg/ml and 10.3 mm zone of inhibition), *Staphylococcus aureus* (MIC of >1000 μg/ml and 4.4 mm zone of inhibition) and *Bacillus subtilis* (MIC of 850 μg/ml and 2.6 mm zone of inhibition). Furthermore, DMBPO was found to be a potent inhibitor of opportunistic fungal pathogens too. It showed a maximum activity against *Aspergillus niger* with a MIC value of 1 μg/ml and 28 mm zone of inhibition.
Conclusion: The result of this study indicates that DMBPO possess antibiotic activity to selected bacterial and fungal pathogens and exhibited better activity against fungi than bacteria.

Actividad in vitro of 5-(2,4-dimetilbencil) pirrolidin-2-uno extraído de *Streptomyces* VITSVK5 sp. marino frente a patógenos fúngicos y bacterianos humanos

Palabras clave:
Streptomyces VITSVK5 sp.
5-(2,4-Dimetilbencil) pirrolidin-2-ona (DMBPO)
Actividad antibacteriana
Actividad antifúngica
Producto natural marino

RESUMEN

Antecedentes: El cribado farmacológico y el uso de productos naturales para el tratamiento de las enfermedades humanas tiene un largo historial que comienza en la medicina tradicional y se extiende hasta los fármacos modernos. La mayoría de los fármacos modernos proceden principalmente de productos naturales.
Objetivo: El objetivo del presente estudio fue valorar la actividad inhibidora of 5-(2,4-dimetilbencil) pirrolidin-2-uno (DMBPO) extraído de *Streptomyces* VITSVK5 sp. marino aislado de muestras de sedimento recolectadas en la costa de Marakkanam de la bahía de Bengala, India.
Métodos: El compuesto principal se aisló mediante extracción bioactiva guiada y se purificó mediante cromatografía de columna de gel de sílice. La dilucidación estructural del compuesto principal se efectuó utilizando datos espectrales de las técnicas UV, FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT y HR-MS.
Resultados: El cribado sistemático de los aislamientos en busca de actividad antimicrobiana dio lugar a la identificación de una cepa potencial, *Streptomyces* VITSVK5 sp. (GQ848482). Con la extracción bioactiva guiada se obtuvo un compuesto DMBPO y su actividad inhibidora se examinó frente a cepas bacterianas y fúngicas seleccionadas. DMBPO mostró una actividad máxima frente a *Escherichia coli* con un valor de la concentración inhibitoria mínima (CIM) de 187 μg/ml, seguida de *Klebsiella pneumoniae* (CIM

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1130-1406/$ – see front matter
doi:10.1016/j.riam.2011.06.008

de 220 μg/ml y zona de inhibición de 10,3 mm), *Staphylococcus aureus* (CIM > 1.000 μg/ml y zona de inhibición de 4,4 mm) y *Bacillus subtilis* (CIM de 850 μg/ml y zona de inhibición de 2,6 mm). Además, se puso de relieve que DMBPO también fue un inhibidor potente de los patógenos fúngicos oportunistas. Se demostró una actividad máxima frente a *Aspergillus niger* con un valor de CIM de 1 μg/ml y una zona de inhibición de 28 mm.

Conclusión: El resultado del presente estudio indica que DMBPO posee actividad antibiótica frente a patógenos bacterianos y fúngicos seleccionados y exhibió una mejor actividad frente a hongos que bacterias.

Natural products are chemical compounds derived from living organisms e.g., plants, animals and microorganisms. They can be defined as chemical compounds isolated or derived from primary or rather secondary metabolism of organisms concerned naturally. Nature acts as a prominent reservoir for new and novel therapeutics. By employing sophisticated techniques under various screening programs, the rate of discovery of natural compounds exceeded 1 million so far, out of which 22,500 biologically active compounds that have been extracted are from microbes; 45% are produced by actinobacteria, 38% by fungi and 17% by unicellular bacteria[5]. Over the past 75 years, natural product derived-compounds have led to the discovery of many drugs to treat numerous human diseases[6]. The oceans cover more than 70% of earth surface and little is known about the microbial diversity of marine sediments, which is an inexhaustible resource that has not been fully exploited. Marine extremophiles serves as valuable natural resource for novel products such as antibiotics, antitumor agents, and other therapeutic substances[1]. Microbial secondary metabolites have been known as one of the immense reservoir of natural chemical diversity with potent biological activity[4,5]. Most bacterial secondary metabolites are generated through a unique, multi-step biosynthetic process with specific enzymes for each complex structure formation. Their encoding genes are normally clustered within the genome of the organism and the precursors for the biosynthesis are derived from primary metabolites. Marine actinomycetes are potential provider of novel bioactive metabolites and have currently emerged as an important source for natural products with unique chemical diversity. Members of the class actinobacteria especially *Streptomyces* spp. have long been recognized as prolific sources of useful bioactive metabolites, providing more than 85% of the naturally occurring antibiotics discovered to date and continuing as a rich source of new bioactive metabolites[3]. Actinomycetes are the most abundant group in antibiotic production, and a good number of antibiotics available in the market are extracted from marine actinomycetes[17].

Actinobacteria or actinomycetes are a group of Gram-positive bacteria with high G + C ratio. Actinobacteria are widely distributed in terrestrial and aquatic ecosystems, especially in soil, where they play a crucial role in the recycling of refractory biomaterials by decomposing complex mixtures of polymers in dead plant, animal and fungal materials. Marine actinomycetes exhibit very different 16S rRNA sequences compared to their terrestrial counterparts. Marine and terrestrial actinomycetes produce many different metabolites due to their taxonomic distance and difference between them. Marine actinomycetes produced many secondary metabolites and served as a potential source for new anti-infective drugs[13]. These represent one of the most studied and exploited classes of bacteria for their ability to make a wide range of biologically active metabolites[8]. The actinobacteria play an important role among the marine bacterial communities, because of its diversity and ability to produce novel chemical compounds of high commercial value[1,7]. Studies on marine actinomycetes are very limited in the Indian sub-continent and most of the actinomycetes isolated are yet to be screened for bioactive secondary metabolites.

In this study we have reported the antibiotic activity of the compound 5-(2,4-dimethylbenzyl) pyrrolidin-2-one extracted from marine *Streptomyces* VITSVK5 spp. against selected bacterial and fungal pathogens.

Materials and methods

Isolation of actinomycetes

The strain *Streptomyces* VITSVK5 spp. was isolated from the salt marsh of Marakkanam coast of Bay of Bengal, southern India. The strain was selectively isolated on starch casein agar, ISP No.1 medium and the culturing conditions were optimized.

Extraction and purification of compound

Well grown slant culture of the *Streptomyces* VITSVK5 spp. was used for preparation of seed culture. The seed culture was inoculated in 50 ml medium containing the optimized production medium prepared with sea water 50%, distilled water 50%, pH 8.2 and incubated for 2 days in rotary shaker (200 rpm) at 30 °C. The inocula (10%) were then transferred into 200 ml production medium in 1 l Erlenmeyer flasks and kept for shake flask growth for a week. After fermentation, the broth was centrifuged at 4000 rpm for 10 min at 10 °C and the supernatant was separated by using 0.2 μm membrane filter. The supernatant was extracted twice with n-butanol (400 ml) and washed with 500 ml water. Then the culture was harvested by centrifugation for 10 min at 4000 rpm at 10 °C and the filtrate was separated by centrifugation (Remi High speed centrifuge). After separation, the organic phase was dried over Na_2SO_4 (anhydrous). The extract was then concentrated in rotary vacuum and lyophilized using a freeze drier (Thermo, USA) at 5 °C for 5 h. The crude extracts were stored at −20 °C. The butanol layer was concentrated and the residual suspension (750 mg) was chromatographed over silica gel column and eluted with chloroform:methanol (10:0, 9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5, 7:3). The active fractions were collected, concentrated and further separated by preparative thin layer chromatography (TLC) on silica gel with chloroform:methanol (8:2) and the purity of the compound was analyzed.

Structure elucidation

The UV spectra of the compound were measured using UV–visible spectrophotometer (Techcomp, Hong Kong). The sample was lyophilized and mixed with KBr (1:20; 0.02 g of sample with KBr at a final weight of 0.4 g) and the pure compound was then grounded, desorbed at 60 °C for 24 h and pressed to obtain IR-transparent KBr-pellets. Infrared spectra of the compound were obtained using a Fourier Transform Infrared Spectrometer (FT-IR-AVATAR 330). The spectra were collected within a scanning range of 400–4000 cm^{-1}. The FT-IR was first calibrated for background signal scanning with a control sample of pure KBr, and then the

experimental sample was scanned. The spectrum obtained was analyzed for various functional groups.

The proton NMR (^{1}H NMR) and carbon NMR (^{13}C NMR, V Bruker Avance III 500 MHz–AV 500) spectra of the compound were obtained by using a dimethyl sulfoxide d6 (DMSO-d6) as solvent. It was further evaluated with DEPT-135, and further confirmed by mass spectroscopy (HRMS, Jeol GCMATE II). The structure of the compound was established with the help of spectral data obtained from various spectroscopic techniques. The 3D structure of the compound was obtained by using Chemdraw software (Ultra 8.0).

Bacterial and fungal pathogens

The following bacterial strains *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10273), *Bacillus cereus* (ATCC 14579), *Streptococcus pneumoniae* (ATCC 6301), *Proteus mirabilis* (ATCC 8259) and fungal strains *Candida albicans* (ATCC 10231) *Aspergillus fumigatus* (ATCC 6645), *Aspergillus niger* (ATCC 6404), *Cryptococcus albidus* (ATCC 60109) and *Trichophyton rubrum* (ATCC 14001) were used in this study.

Assay of antibacterial activity

The antibacterial activity of lead compound was tested by agar diffusion assay[2]. The plates were incubated at 37 °C for 24 h during which activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated three times and the antibacterial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the compound when compared to positive controls (chloramphenicol). Agar well diffusion method was further evaluated by determining the minimum inhibitory concentration (MIC) by the broth two-fold macro dilution method. The compound was serially diluted at varying concentration of 1500–1 μg/ml in Mueller Hinton broth. Culture broth (0.1 ml) was added to the each tube containing the compound. The tubes were incubated aerobically at 37.8 °C for 24 h. Positive controls were prepared separately with respective organisms in the same culture media without the compound. After incubation, the tube with the least concentration of the compound showing no growth was taken as the MIC value for the respective organism.

Assay of antifungal activity

The antifungal activity of lead compound was tested by agar diffusion assay. *C. albicans* inocula was prepared by picking five distinct colonies of approximately 1 mm from 48 h old culture grown on Sabouraud dextrose agar and incubated at 35 ± 2 °C. Colonies were suspended in 5 ml of sterile 0.85% saline. Muller Hinton agar plates were prepared with 2% glucose and 0.5 μg/ml methylene blue dye medium and further used for carrying out antifungal activity by agar diffusion method. The antifungal activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the lead compound when compared to the control (amphotericin B). Agar well diffusion method was further evaluated by broth microdilution method using the standard protocol (CLSI M38-A). Fungal conidia were taken from well grown slant culture, adjusting a suspension to a concentration of 1×10^6 conidia/ml using RPMI medium. It was further diluted with PBS/Tween-20 to have a suspension of 2×10^3 conidia/ml. Dilutions were plated and spread over Sabouraud's dextrose agar plates. These plates are then incubated at 37 °C for 48 h. For each isolates add 100 μl of inocula into all the wells in the appropriate row, which makes the final inoculum to 5×10^5 conidia/ml. Microtitre plates were then incubated for 48 h at 37 °C in a moist chamber and the MIC was read visually.

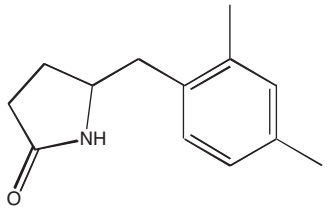

Figure 1. Structure of 5-(2,4-dimethylbenzyl) pyrrolidin-2-one ($C_{13}H_{17}NO$).

Results

Our systematic screening of marine actinomycetes isolates for antimicrobial activity resulted in the selection of the strain *Streptomyces* VITSVK5 spp. The strain was identified by molecular taxonomic characterization using 16S rRNA partial gene sequence analysis and 16S rDNA sequence analysis. The 16S rDNA sequence (1424 base pairs) of *Streptomyces* VITSVK5 spp. has been submitted to NCBI under the accession no. GQ848482. The crude extract obtained from the isolate showed significant antifungal activity against drug resistant strains of *Aspergillus* clinical isolates. Since the butanolic extract of *Streptomyces* VITSVK5 spp. showed the maximum inhibitory activity against drug resistant *Aspergillus* clinical isolates, it was subjected to bioactive guided extraction and purification of active principle. Extraction and purification of 10 l of the culture broth yielded 112.3 mg of pure compound.

The purity of compound was checked by thin layer chromatography with R_f value of 0.43 (chloroform–methanol, 8:2) and single band obtained was visualized by iodine reagent and sulphuric acid. The spectral data (UV, FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT, and HR-MS) obtained for the compound were used to establish the structure of the compound. UV/vis (MeOH) λ_{max} 290; FT-IR cm^{-1} 3436 (–NH), 2928, 1729 (C=O); ^{1}H NMR (DMSO-d6 500 MHz): 0.871 (s, J = 64 Hz, $-CH_3$), 1.23–1.39 (m, J = 44 Hz, $2 \times CH_2$), 3.37 (t, 1H), 4.29 (s, 2H), 7.25 (s, 1H), 7.65–7.69 (t, 2H), 8.17 (s, 1H); ^{13}C NMR (DMSO-d6 500 MHz): 13.80 (CH_3), 18.60 (CH_3), 34.65($2 \times CH_2$), 60.34(CH_2), 64.98 (CH); 128.62, 131.4, 133.6, 134.3, 136.7, 144.7, 166.9. DEPT, HR-MS and m/z (found/cal.): 203.1325/203.1310.

Based on the spectral data the structure of the compound extracted from *Streptomyces* VITSVK5 spp. was identified as 5-(2,4-dimethylbenzyl) pyrrolidin-2-one (DMBPO) and the molecular formula was determined as $C_{13}H_{17}NO$. The structure of the compound is illustrated in Fig. 1.

Antibacterial and antifungal activity

Among the microorganisms tested, Gram-negative bacteria were found to be more susceptible to DMBPO (Table 1). DMBPO showed maximal activity against *E. coli* with the MIC value of 187 μg/ml when compared to chloramphenicol (3.16 μg/ml). It also showed a significant activity against *K. pneumoniae* (MIC of 220 μg/ml and 10.3 mm zone of inhibition), *S. aureus* (MIC of >1000 μg/ml and 4.4 mm) and *B. subtilis* (MIC of 850 μg/ml and 2.6 mm) (Table 1). DMBPO was found to be a potent inhibitor for opportunistic fungal pathogens too. It showed significant zone of inhibition of 18 mm (MIC – 150 μg/ml), 24 mm (MIC – 1 μg/ml) and 28 mm (MIC – 1 μg/ml) against *C. albicans*, *A. fumigatus* and *A. niger* respectively. No zone of inhibition was observed against *B. cereus, S. pneumoniae*, *P. mirabilis*, *C. albidus* and *T. rubrum*.

Discussion

The DMBPO extracted from *Streptomyces* VITSVK5 spp. exhibited antimicrobial activity against all the bacterial and fungal pathogens with the MIC value ranging from 187 to 1000 μg/ml for bacteria and

Table 1
Effect of DMBPO against tested bacterial and fungal pathogens.

Microorganism	Zone of inhibition (mm)	MIC (μg/ml)
Bacteria		
Escherichia coli (ATCC 25922)	12.1 (20.5)	187 (3.16)
Klebsiella pneumoniae (ATCC 10273)	10.3 (18.5)	220 (26.0)
Staphylococcus aureus (ATCC 25923)	4.4 (19.8)	>1000 (11.9)
Bacillus subtilis (ATCC 6633)	2.6 (22.1)	850 (24.8)
Bacillus cereus (ATCC 14579)	NZ (19.8)	ND
Streptococcus pneumoniae (ATCC 6301)	NZ (21.5)	ND
Proteus mirabilis (ATCC 8259)	NZ (17.8)	ND
Fungi		
Candida albicans (ATCC 10231)	18 (11.6)	150 (8.0)
Aspergillus fumigatus (ATCC 6645)	24 (11.6)	1 (1.0)
Aspergillus niger (ATCC 6404)	28 (11.6)	1 (2.0)
Cryptococcus albidus (ATCC 60109)	NZ (8.0)	ND
Trichophyton rubrum (ATCC 14001)	NZ (5.5)	ND

The zones of inhibition and the MIC value for the standard antibiotic (μg/ml) are given within parenthesis. Chloramphenicol and amphotericin B were used as a positive control.
NZ – No zone of inhibition; ND – Not done.

1–150 μg/ml for fungi. For antibacterial activity the concentration of DMBPO required to exhibit minimal inhibitory activity was comparatively higher than chloramphenicol MIC value. DMBPO exhibit better antifungal activity against *A. niger* and *A. fumigatus* with the MIC value equivalent or less than the MIC value of amphotericin B (Table 1). However, DMBPO exhibits comparatively higher MIC value against *C. albicans* when compared to amphotericin B. The results of our study show that DMBPO is very selective to inhibit the growth of *A. fumigatus* and in the case of *A. niger* DMBPO exhibits better antifungal activity with lesser MIC value when compared to amphotericin B. Our findings demonstrated the broad spectrum of antimicrobial activity of the compound DMBPO against selected human pathogens and have shown better antifungal activity than antibacterial activity. It was found to have potent inhibitory activity against opportunistic fungi, which usually infect pre-infected patients with less immunity. Invasive aspergillosis occurs predominantly in highly immunocompromised patients and it can cause up to 100% mortality if untreated. *A. fumigatus* is the most common causative species when compared to other *Aspergillus* species.

Microorganisms from extreme environments have gained considerable attention in recent years because of its diversity and biological activities, mainly due to its ability to produce novel chemical compounds of high commercial value. Recent investigations using enrichment techniques, new selection methods and media have led to the isolation of novel actinomycetes from sediment samples. Similarly, *Streptomyces* VITSVK5 spp. growth has been optimized with different process parameters[10–12]. Several earlier studies reported on the antagonistic activity of several novel marine natural products isolated from marine algae, fungi, bacteria, sponges and sea stars[14–17]. One important reason for discovering novel secondary metabolites is to circumvent the problem of resistant pathogens, which are no longer susceptible to the currently used drugs. The number of deaths due to these pathogenic organisms is on the rise, which needs to be controlled. Immuno-compromised patients are in higher risk of infection and they require a new class of antibacterial and antifungal drugs with less toxicity. In vitro antimicrobial activity of different secondary metabolites extracted from *Streptomyces* species isolated from soil samples have been reported earlier[18]. Kokare et al.[9] reported the isolation of halophilic *Actinopolyspora* species AHI from the sediments of Alibag coast of Maharashtra India. The cell free supernatant extracted from the strain exhibited good antagonistic activity under in vitro conditions against Gram-positive bacteria viz. *S. aureus*, *Staphylococcus epidermidis*, *B. subtilis* and fungi such as *A. niger*, *A. fumigatus*, *Aspergillus flavus*, *Fusarium oxysporum*, *Penicillum* sp. and *Trichoderma* sp. It did not show any antibacterial activity against Gram-negative bacteria such as *E. coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Enterobacter aerogenes* and against the fungi, *C. albicans* and *Cryptococcus* sp. Although the exploitation of marine actinomycetes as a source for discovery of novel secondary metabolites is at an early stage, numerous novel metabolites have been isolated in the past.

Streptopyrrolidine, a benzyl pyrrolidine derivative isolated from the fermentation broth of a marine *Streptomyces* sp. KORDI-3973 has been shown to exhibit significant anti-angiogenesis activity toward capillary tube formation of human umbilical vein endothelial cells.[20] Romanenko et al.[19] have isolated *Pseudomonas* (strain KMM 3042), a Gram-negative Gammaproteobacteria dwelling on lithosphere as well as in a marine environment capable of producing diverse secondary metabolites including pyrrolidinedione capable of acting as antimicrobial agent. Ours is the first report on isolation DMBPO from marine *Streptomyces* VITSVK5 spp. In the present study, the efficacy of the bioactive compound DMBPO against *A. fumigatus* and *A. niger* clinical isolates was found to be equivalent to that of amphotericin B. It is apparent that the bioactive compound DMBPO from *Streptomyces* VITSVK5 spp. has antimicrobial potential against selected bacterial and fungal pathogens. However, in vivo animal model studies are required to elucidate bio-distribution, toxicity and serum levels of DMBPO to authenticate its antimicrobial potential.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgements

Authors thank the management of VIT University for providing facilities to carry out this research.

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