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RESEARCH ARTICLE

ANTIFUNGAL ACTIVITY OF A BIFUNCTIONALIZED ALLENE ETHANOL EXTRACTS

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ABSTRACT

Background and Purpose: Antifungal effects of a Bifunctionalized Allenewith unprotected hydroxy group (*Dimethyl 1-(1-hydroxyethyl)-3-methylpenta-1,2-dienephosphonate)* (**BA-2**) on pathogenic yeast and fungi had been established. BA-2 (50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml) exerted different inhibitory effect on different yearst and fungi cells *in vitro*. The effects of BA-2 on eukaryotic cells have not been studied yet. The present study was aimed to assess the antifungal activity of BA-2on pathogenic yeast and fungi.

Experimental approach: *In vitro* antifungal test: *Aspergillusniger, Penicilliumclaviforme, Saccharomyces cerevisae, Candida albicans 8673 and Candida glabrata 72* were treated for 24 hours with **BA-2**(50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml),Fluconazole (150 mg/ml).The antifungalactivity was assayed by the well diffusion method *with digital caliper*.*Determination of minimum inhibitory concentrations(MICs):* The MIC of **BA-2**, that shows antifungal activity, were determined by methods as described by [13] and MICs were read in µg/ml after over nightincubation at 37°C. All experiments were made in replicate.*Determination of Minimum fungalconcentration(MFC)***:**The MFC was carried out to check whetherthe test microbes were killed or only their growthwas inhibited. Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK) was preparedand sterilized at 121°C for 15 minutes, the medium waspoured into sterile petridishes and were allowed tocool and solidify. The contents of the MIC in theserial dilution were then subcultured onto theprepared medium, incubation was made at 37°C for24 h, after which each plate was observed forcolony growth. The lowest concentration of the BA-2 without a colony growth was recorded asthe MFC.BA-2had higher antifungal activity than tested antibiotic– Fluconazole.

INTRODUCTION

The present treatments of bacterial and fungal infections are a bit unsatisfactory, owing to rapidly developing drug resistance and side effects. This effect has a negative impact on the usage of most antimicrobial agents[12]. Antibiotic resistance continues to rise and the dawn of the much forewarned post-antibiotic era has arguably broken [9]. Antimicrobial resistance (AMR) is not a recent phenomenon, but it is a critical health issue today. Over several decades, to varying degrees, bacteria causing common infections have developed resistance to each new antibiotic, and AMR has evolved to become a worldwide health threat. The evolving public health threat of antimicrobial resistance (AMR) is driven by both appropriate and inappropriate use of anti-infective medicines for human and animal health and food production, together with inadequate measures to control the spread of infections. On World Health Day (WHD) 2011[13], in a six-point policy package, countries were called upon to (1) commit to a comprehensive, financed national plan with accountability and civil society engagement, (2) strengthen surveillance and laboratory capacity, (3) ensure uninterrupted access to essential medicines of assured quality, (4) regulate and promote rational use of medicines in animal husbandry and to ensure proper patient care, (5) enhance infection prevention and control, and (6) foster innovations and research and development of new tools [13].Small-molecule drugs, for the time being, remain an essential component of infection treatment and prevention. Two proven strategies within this paradigm are the development of new drugs with direct antibacterial activity (e.g. daptomycin) and adjuncts with antibiotic-enhancing activity (e.g. tazobactam) [9]. Efforts to develop synthetic antibacterial drugs have not been abandoned but are now more focused on derivatization of natural molecules and synthesis of natural-product-like compounds using well-known natural product scaffolds [4]. The current global pandemic of antibiotic resistance shows no signs of abating, although it may be changing direction. An important milestone has been the 3rd World Health Organization (WHO) Patient Safety Challenge on antibiotic resistance, subsequently upgraded to an entire WHO Concern, with last year's World Health Day on this topic. With a dearth of new antibiotics

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coming to market, the need for action to avert a developing global crisis in health care is increasingly urgent.

In this paper, the antifungal activity of a Bifunctionalized Allene with unprotected hydroxy group (*Dimethyl 1-(1-hydroxyethyl)-3-methylpenta-1,2-dienephosphonate*) (**BA-2**) has been studied as part of the exploration for new and novel bio-active compounds.

MATERIALS AND METHODS

Test organisms

Aspergillus niger, Penicillium claviforme, Saccharomyces cerevisae, Candida albicans 8673 and Candida glabrata 72 were obtained from the National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria. All the isolates were checked for purity and maintained in slants of Nutrient agar.

Media used

They were maintainedon Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK) plate sat 29^{o}C and subcultured on a monthly basis until sporulation. The spores were harvested after establishing a good growth rate of each of the fungal cultures and were filtered with sterile cotton filter, to avoid the presence of conidia and mycelia. The spore's suspensionsin PBS (pH e 7.0) were adjusted to the final concentrations in the range of 10^5-10^6 spores/mL.

Compound tested

Bifunctionalized Allene with unprotected hydroxy group(*Dimethyl1-(1-hydroxyethyl)-3-methylpenta-1,2 dienephosphonate)* (**BA-2**) was synthesised in the Laboratory of Toxicologycal Chemistry, Department of Organic Chemistry & Technology of the Konstantin Preslavsky University of Shumen, Bulgaria (figure 1) [7].

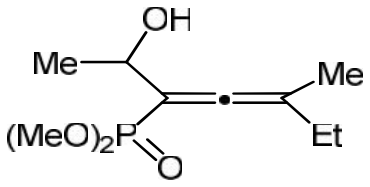

Figure 1 Structural formula of **BA-2**

Dimethyl 1-(1-hydroxyethyl)-3-methylpenta-1,2-dienephosphonate (**BA-2**). Yellow oil, yield: 80%. Rf0.58; IR (neat, cm−1): 1254 (P=O), 1956 (C=C=C), 3372 (OH). 1H-NMR (600.1 MHz): δ 0.98 (t,*J* = 7.5 Hz, 3H, Me-CH2), 1.42 (dd, *J* = 6.1 Hz, *J* = 10.2 Hz, 3H, Me-CHO), 1.78 (d, *J* = 6.6 Hz, 3H,Me-C=), 2.02–2.10 (m, 2H, Me-CH2), 2.70 (s, 1H, OH), 3.78 (d, *J* = 11.6 Hz, 3H, MeO), 4.67–4.72(m, 1H, Me-CHO). 13C-NMR (150.9 MHz) δ = 12.2 (*J* = 7.6 Hz), 18.4 (*J* = 6.4 Hz), 23.2 (*J* = 7.5 Hz),27.4 (*J* = 9.2 Hz), 52.6 (*J* = 6.2 Hz), 66.9 (*J* = 10.3 Hz), 96.3 (*J* = 192.3 Hz), 104.4 (*J* = 15.9 Hz),208.9 (*J* = 5.4 Hz). 31P-NMR (242.9 MHz): δ 21.1. Anal. Calcd forC10H19O4P (234.23): C 51.28, H8.18. Found: C 51.21, H 8.13.

Preparing the solution of BA-2

The solutions of **BA-2**(50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml)were freshly prepared in ethanol.

Assay for Antifungal Activity.

Antifungal assay was performed by the well diffusion method using soft 0.8% agar. Agar medium was added to sterile Petri dishes seeded with 100 µl of each test bacterial strains. Wells of equal distance were dug on the seeded plates. Each well was filled up with 100 µl of the BA-2 and antibiotics tested. After adjusting the pH at 6.5 by NaOH, the activity of the BA-2 was checked. The plates were incubated at 37°C for 48 hours. The antifungal activity was assayed by measuring the diameter of the inhibition zone formed around the well with digital caliper[3]. All experiments were performed in triplicate.

Determination of Minimum inhibitory concentrations(MICs)

The minimum inhibitory concentrations of **BA-2**, that shows antimicrobial activity, were determined by 2-folddilution methods as described by [11] and MICs were read in µg/ml after overnight incubation at 37^{o}C. All experiments were made in replicate.

Determination of Minimum fungal concentration(MFC)

The MFC were carried out to check whether the test microbes were killed or only their growth was inhibited. Potato Dextrose Agar was prepared and sterilized at 121^{o}C for 15 minutes, the medium was poured into sterile petridishes and were allowed to cool and solidify. The contents of the MFC in the serial dilution were then subcultured onto the prepared medium, incubation was made at 37^{o}C for24 h, after which each plate was observed for colony growth. The lowest concentration of the BA-2 without a colony growth was recorded as the MFC.

RESULTS

In the present study the effects of **BA-2**on five pathogenic fungiand were evaluated. The effects were compared with widely used antibioticFluconazole. According to NCCLS, the antibiotic Fluconazole used is known to have broad spectrum antifungal activity [10].The effects of **BA-2**on the microorganisms were summarized in Table 1.

Table 1 Effect of **BA-2**on test organisms.

Microorganisms	Zone of inhibition (mm)[a]
A. niger	15.53±0.19
P. claviforme	0
S. cerevisae	0
C. albicans 8673	13.84±0.18
C. glabrata 72	16.25±0.02
Ethanol(96%) (Negative control)	13.22±0.01
Fluconazole150µg/ml	13.49±0.02

[a]Data are presented as average values ± standard deviation in mm.

BA-2 at concentration 50 mg/ml for 24 hours notably inhibited growth of *A. niger* (15.53 mm mean zone of inhibition) and *C. glabrata*72 (16.25 mm mean zone of inhibition). On the contrary, **BA-2** had no activity against *C. albicans* (13.84 mm

mean zone of inhibition), which are comparable to the inhibitory effect of standard drug. **BA-2** did not inhibited *P. claviforme* and *S. cerevisae*.

Our assay for antifungal activity of **BA-2**was conducted by testing different concentrations of the compound on various pathogens to determine the MICs. We used five concentrations – 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml. The results are shown in Table 2.

Table 2 The MIC of **BA-2**

Microorganisms	MIC (mg/ml)				
	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.125mg/ml
A. niger	+				
P. claviforme	-	-	-	-	-
S. cerevisae	-	-	-	-	-
C. albicans 8673		+			
C. glabrata 72		+			

[a]Results are mean ± SEM of three separate trails.

The results revealed variability in the inhibitory concentrations of **BA-2** for given fungi. MIC of **BA-2** at concentration 50 mg/ml for 24 hours notably inhibited growth only of *A. niger*. In contrast, MIC of **BA-2** at concentration 25 mg/ml for 24 hours notably inhibited growth of yeast *C. glabrata* 72 and *C. albicans.* The probable reason for the higher MIC reported for eukaryotic microorganisms is the complex structure of their cell.

Our next task was to determine the Minimum fungal concentration(MFC) in regards with determining the bactericidal or bacteriostatic activity of the examined BA-2. We used five concentrations – 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml. The results are shown in Table 3.

Table 3 The MFC of **BA-2**

Microorganisms	MFC (mg/ml)[a]				
	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.125mg/ml
A. niger	+				
P. claviforme	-	-	-	-	-
S. cerevisae	-	-	-	-	-
C. albicans 8673	+				
C. glabrata 72	+				

[a]Results are mean ± SEM of three separate trails.

MFC of **BA-2**at concentration 50 mg/ml for 24 hours notably inhibited growth of *A. niger, C. glabrata* and *C. albicans 8673*. For Fungi Imperfecta from *P. claviforme* and years *S. cerevisae* MFC it was not reported.

Based on the results obtained we can conclude that the examined BA-2has bactericidal activity towards both pathogenic yeast and Fungi Imperfecta, but in different concentrations.

The **BA-2**possesses biological activity, which is not well studied. We know only from literary data that they are used for inhibiting the biosynthesis of sterol from the pathogen responsible for *Pneumocystis-carinii*pneumonia (PCP) -a disease similar to AIDS[12]. In our previous studies was shown that theBifunctionalized Allenewith protected hydroxy group (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate)* (**BA-1**) exhibited antibacterial [5] and antifungal activity [6].The results obtained show for the first time the existence of antifungal activity of **BA-2** towards various pathogenic yeast and fungi.

Invasive aspergillosis in immunosuppressed patients is difficult to diagnose, is problematic to treat, and results in a high mortality rate. Resistance in *Aspergillus* and *Candida* has been increasingly investigated and reported because standards for susceptibility testing and associated breakpoints became available and as a consequence of the increased use of antifungal compounds. From an epidemiological point of view, less is known about the true prevalence of resistant *Aspergillus* infections than about resistant infections for most other organisms. This is due to the fact that most routine laboratories do not susceptibility test their *Aspergillus*isolates and many laboratories find species (or even genus) identification of aspergilli difficult. Additionally, national surveillance programmes are lacking [1].

Antibiotics represent one of the most successful forms of therapy in medicine. But the efficiency of antibiotics is compromised by a growing number of antibiotic-resistant pathogens. Antibiotic resistance, which is implicated in elevated morbidity and mortality rates as well as in the increased treatment costs, is considered to be one of the major global public health threats (www.who.int/drugresistance/en/) and the magnitude of the problem recently prompted a number of international and national bodies to take actions to protect the public [8]:

(http://ec.europa.eu/dgs/health_consumer/docs/road-map-amr_en.pdf;
http://www.who.int/drugresistance/amr_global_action_plan/en/ ;
http://www.whitehouse.gov/sites/default/files/docs/carb_national_strategy.pdf).

The occurrence of drug resistant strains with less susceptibility to antibiotics due to mutation challenges the researchers to invent newer drugs. At this scenario, evaluation of antimicrobial substances from various sources is considered to be a pivotal role. Nevertheless, further studies are required to explore the mechanism of biochemical active principle in the Bifunctionalized Allenes for the inhibitory action on various pathogens selected in the study.

CONCLUSION

The Bifunctionalized Allenewith unprotected hydroxy group **BA-2** at 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/mlconcentrations showed significant antifungial activity on selected pathogens inclinical isolates.

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