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A BIFUNCTIONALIZED ALLENE ETHANOL EXTRACTS WITH ANTIFUNGAL ACTIVITY

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ABSTRACT: *Background and Purpose: Antifungal effects of a Bifunctionalized Allene with unprotected hydroxy group (3-Diphenylphosphinoyl-5-methylhepta-3,4-dien-2-ol) (BA-3) on pathogenic yeast and fungi had been established. BA-3 (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-3 on eukaryotic cells have not been studied yet. The present study was aimed to assess the antifungal activity of BA-3 on pathogenic yeast and fungi. Experimental approach: In vitro antifungal test: Aspergillus niger, Penicillium claviforme, Saccharomyces cerevisae, Candida albicans 8673 and Candida glabrata 72 were treated for 24 hours with BA-3 (50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml), Fluconazole (150 mg/ml) for yeast and Chlornitromycin (250 mg/ml) for fungi. The antifungal activity was assayed by the well diffusion method with digital caliper. Determination of minimum inhibitory concentrations(MICs): The MIC of BA-3, that shows antifungal activity, were determined by methods as described by [18] and MICs were read in µg/ml after over night incubation at 37°C. All experiments were made in replicate. Determination of Minimum fungal concentratio n(MFC):The MFC was carried out to check whether the test microbes were killed or only their growth was inhibited. Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK) was prepared and sterilized at 121°C for 15 minutes, the medium was poured into sterile petri dishes and were allowed to cool and solidify. The contents of the MIC in the serial dilution were then subcultured on to the prepared medium, incubation was made at 37°C for 24 h, after which each plate was observed for colony growth. The lowest concentration of the BA-3 without a colony growth was recorded as the MFC. BA-3 had higher antifungal activity than tested antibiotic– Fluconazole for yeast and Chlornitromycin for fungi. Key Results: The results revealed variability in the inhibitory concentrations of BA-3 for given fungi and yeast. MIC of BA-3 at concentration 50 mg/ml for 24 hours notably inhibited growth of yeast C. glabrata72 and C. albicans. In contrast, MIC of BA-3 at concentration 25 mg/ml for 24 hours notably inhibited growth only of fungi A. niger. MFC of BA-3 at concentration 25 mg/ml for 24 hours notably inhibited growth of C. glabrata and C. albicans 8673. MFC of BA-3 at concentration 12.5 mg/ml for 24 hours notably inhibited growth only of fungi A. niger. For Fungi Imperfecta from P. claviformeand years S. cerevisae MFC it was not reported. Conclusions and Implications: Based on the results obtained we can conclude that the examined BA-3 has bactericidal activity towards both pathogenic yeast and Fungi Imperfecta, but in different concentrations.The BA-3 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-cariniipneumonia (PCP) -a disease similar to AIDS[2]. In our previous studies was shown that theBifunctionalized Allenes with protected hydroxy group (Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate) (BA-1) and unprotected hydroxy group (Dimethyl 1-(1-hydroxyethyl)-3-methylpenta-1,2-dienephosphonate) (BA-2) exhibited antibacterial [8, 10] and antifungal activity [9, 11]. The*

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results obtained show for the first time the existence of antifungal activity of BA-2 towards various pathogenic yeast and fungi.

KEYWORDS: BA-3 (*3-Diphenylphosphinoyl-5-methylhepta-3,4-dien-2-ol),* antifungal activity, antibiotic.

INTRODUCTION

The lack of understanding of the unique features and risk of resistance has paved the way for the present epidemic. Moreover, few studies have been done on the magnitude of the burden to convince policy makers of the urgent need to react. Since the penicillin era, antibiotics have been viewed as wonder drugs that couldbe prescribed without fear of harm, despite early warnings of consequences such as antibiotic resistance and side effects [6,14]. Their use has spread into many nonmedical areas, and has been unregulated, both legally and illegally. Antibiotic resistance is perceived as acomplex medical problem. Antibiotics are different from all other drug groups in that the effects of their use extend far beyond individual patients. Even more worrying is the accumulating evidence that antibiotic usein seriously ill but uninfected patients can actually increase mortality [7]. Traffic and selling of antibiotics at markets, shops, and pharmacies is largely unregulated, without prescription, and even without involvement of a person with pharmaceutical training [16]. This widespread access is madeeasier by the internet and marketing stunts for free orcheap antibiotics.Use of antibiotics, which is unnecessary (eg, for growth promotion) or where alternatives exist (eg, routineprevention) should be phased out. The international organisations WHO, OIE, and FAO should provide a clear definition of "unnecessary routine prevention". Governmentsacross the globe should then revise existinglegislation or draft new legislation accordingly. Additionally, all key stakeholders should commit toprudent and rational use of antimicrobials. The environmental release of antibiotics from all sectors needsto be monitored and controlled. Strategies need to identifyand focus control on hot spots for horizontal resistancegene transfer such as wastewater treatment facilities.

Successful treatment of infectious diseases requires choice of the most suitable antimicrobial agent, comprising consideration of drug pharmacokinetics (PK), including penetration into infection site, pathogen susceptibility, optimal route of drug administration, drug dose, frequency of administration, duration of therapy, and drug toxicity. Antimicrobial pharmacokinetic/pharmacodynamic (PK/PD) studies consider these variables and have been useful in drug development, optimizing dosing regimens, determining susceptibility breakpoints, and limiting toxicity of antifungal therapy [1,2].

The rapid pandemic spread of multiresistant bacteria and the paucity of new effective antibiotics is placing patients' safety at risk worldwide. The infrastructure of antibiotic discovery both in academia and in industry is at a dangerously low level and needs to be rebuilt. A new sustainable global model for the discovery, development, and distribution of antibiotics needs be developed in which the private and public sectors work in partnership and the large scientific bottlenecks for discovery of antibiotics with new mechanisms of action are solved in collaboration between academia, SMEs, and major pharmaceutical companies [15].

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In this paper, the antifungal activity of a Bifunctionalized Allenewith unprotected hydroxy group(*3-Diphenylphosphinoyl-5-methylhepta-3,4-dien-2-ol*) (**BA-3**) 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 Bankfor Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria. All the isolates were checked for purity and maintained in slants of Nutrient agar.

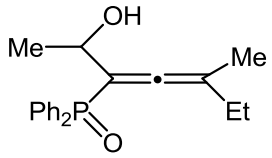
Media used

They were maintainedon Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK) platesat 29ºC and subcultured on a monthly basis until sporulation. Thespores were harvested afterestablishing a good growth rate of eachof the fungal cultures and were filtered with sterile cotton filter, toavoid the presence of conidia and mycelia. The spore's suspensionsin PBS (pH 7.0) were adjusted to the final concentrations in therange of 10^5-10^6 spores/mL.

Compound tested

Bifunctionalized Allenewith unprotected hydroxy group(*3-Diphenylphosphinoyl-5-methylhepta-3,4-dien-2-ol)* (**BA-3**) was synthesised in the Laboratory of Toxicologycal Chemistry, Department of Organic Chemistry & Technology of the Konstantin Preslavsky University of Shumen, Bulgaria (figure 1) [12].

Figure 1.Structural formula of BA-3



3-Diphenylphosphinoyl-5-methylhepta-3,4-dien-2-ol (**BA-3**). Light orange oil, yield: 87%. R_f 0.59; IR(neat, cm^{-1}): 1174 (P=O), 1441, 1490 (Ph), 1951 (C=C=C), 3369 (OH). ^{1}H-NMR (600.1MHz): δ 0.86(t, *J* = 7.4 Hz, 3H, Me-CH_2), 1.35 (dd, *J* = 6.2 Hz, *J* = 9.4 Hz, 3H, Me-CHO), 1.58 (d, *J* = 6.3 Hz, 3H,Me-C=), 1.78–1.90 (m, 2H, Me-CH_2), 2.70 (s, 1H, OH), 4.59–4.63 (m, 1H, Me-CHO), 7.35–7.90(m, 10H, 2Ph). ^{13}C-NMR (150.9 MHz) δ = 12.4, 18.5 (*J* = 5.4 Hz), 22.4 (*J* = 7.6 Hz), 26.7, 64.2(*J* = 7.4 Hz), 96.5 (*J* = 104.2 Hz), 105.1 (*J* = 13.4 Hz), 129.1–132.4 (2Ph), 204.1 (*J* = 7.1 Hz). ^{31}P-NMR(242.9 MHz): δ 34.2. Anal. Calcd for $C_{20}H_{23}O_2P$ (326.37): C 73.60, H 7.10. Found: C 73.67, H 7.05.

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Preparing the solution of BA-3

The solutions of **BA-3**(50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml)werefreshly 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-3** and antibiotics tested. After adjusting the pH at 6.5 by NaOH, the activity of the BA-3 was checked. The plates were incubated at 37°C for 48 hours. The antifungalactivity was assayed by measuring the diameter of the inhibition zoneformed around the wellwith digital caliper[4]. All experiments were performed in triplicate.

Determination of Minimum inhibitory concentrations(MICs)

The minimum inhibitory concentrations of **BA-3**, that shows antimicrobial activity, were determined by 2-folddilution methods as described by [18] and MICs were read in µg/ml after over nightincubation at 37^{o}C. All experiments were made in replicate.

Determination of Minimum fungal concentration (MFC)

The MFC were carried out to check whetherthe test microbes were killed or only their growthwas inhibited. Potato Dextrose Agar agar was preparedand sterilized at 121^{o}C for 15 minutes, the medium waspoured into sterile petridishes and were allowed tocool and solidify. The contents of the MFC in theserial dilution were then subcultured onto theprepared medium, incubation was made at 37^{o}C for 24 h, after which each plate was observed forcolony growth. The lowest concentration of the BA-3 without a colony growth was recorded asthe MFC.

Results

In the present study the effects of **BA-3** on five pathogenic fungi and were evaluated. The effects were compared with widely used antibiotics Fluconazole for yeast and Chlornitromycin for fungi. According toNCCLS, the antibiotic Fluconazole used is known to have broad spectrum antiyeast activity and Chlornitromycin used is known to have broad spectrum antifungal activity [17]. The effects of **BA-3** on the microorganisms were summarized in Table 1.

Table 1. Effect of BA-3on test organisms.

Microorganisms	**Zone of inhibition (mm)**[a]
A. niger	**15.42±0.18**
P. claviforme	**0**
S. cerevisae	**0**
C. albicans 8673	**13.60±0.02**
C. glabrata 72	**15,52±0.02**
Ethanol(96%) (Negative control)	**12.65±0.05**
Fluconazole (150 mg/ml)	**13.52±0.02**
Chlornitromycin (250 mg/ml)	**14.06±0.19**

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

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BA-3 at concentration 50 mg/ml for 24 hours notably inhibited growth of *C. glabrata*72 (15.52 mm mean zone of inhibition) and ***A. niger*** (15.42mm mean zone of inhibition). On the contrary, **BA-3** had no activity against *C. albicans*(13.60mm mean zone of inhibition), which are comparable to the inhibitory effectof standard drug Fluconazole. **BA-3** did not inhibited *P. claviforme* and *S. cerevisae*.

Our assay for antifungal activity of **BA-3** 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 ofBA-3

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-3** for given fungi. MIC of **BA-3**at concentration 50 mg/ml for 24 hours notably inhibited growth of yeast *C. glabrata*72 and *C. albicans*. In contrast, MIC of **BA-3** at concentration 25 mg/ml for 24 hours notably inhibited growth only of fungi *A. niger*. 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-3**. 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.

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Table 3. The MFC of BA-3

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-3** at concentration 25 mg/ml for 24 hours notably inhibited growth of *C. glabrata* and *C. albicans 8673*. MFC of **BA-3** at concentration 12.5 mg/ml for 24 hours notably inhibited growth only of fungi *A. niger*. 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-3** has bactericidal activity towards both pathogenic yeast and Fungi Imperfecta, but in different concentrations.

The **BA-3** 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[13].In our previous studies was shown that theBifunctionalized Alleneswith protected hydroxy group (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate)* (**BA-1**) and unprotected hydroxy group (*Dimethyl 1-(1-hydroxyethyl)-3-methylpenta-1,2-dienephosphonate*) (**BA-2**) exhibited antibacterial [8, 10] and antifungal activity [9, 11]. The results obtained show for the first time the existence of antifungal activity of **BA-3** towards various pathogenic yeast and fungi.

In Europe and the United States of America (USA), *Candida glabrata* has emerged as the second most common cause of invasive candidiasis and an increasing number of reports show its importance in mucosal or bloodstream infections [20]. Systemic infections due to *C. glabrata* are characterized by a high mortality rate and they are difficult to treat due to their intrinsically low susceptibility to azoles, particularly fluconazole [19]. Numerous *C. glabrata* isolates have shown primary resistance to fluconazole, while others easily develop fluconazole resistance after exposure to the treatment [3].Chapeland-Leclerc et al. [5]reported a case of IC due to *C. glabrata* in which the infecting strain acquired resistance to flucytosine, fluconazole, voriconazole, and caspofungin through successive independent events following prolonged exposure to each class of antifungal agent.

Consequently, the continued application of antifungal susceptibility testing for the conventional and new antifungal agents is critical to detect the emergence of resistance in this important opportunistic fungal pathogen. Future studies should include testing

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Bifunctionalized Allenes and as a component of combined antifungal therapy for invasive and refractory mould infections.

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 hydroxyl group **BA-3** at 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml concentrations showed significant antifungial activity on selected pathogens inclinical isolates.

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