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Antifungal Activity of Protect-DRODO Ethanol Extracts.

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ABSTRACT

Antifungal effects of a bifunctionalized allenewith protected hydroxy group (*2-(2-Diphenylphosphinoyl-4-methyl-octa-2,3-dienyloxy)-tetrahydro-2H-pyran)* (Protect-DPODO) on pathogenic yeast and fungi had been established. Protect-DPODO(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 Protect-DPODO on eukaryotic cells have not been studied yet. The present study was aimed to assess the antifungal activity ofProtect-DPODO on pathogenic yeast and fungi. *In vitro* antifungal test: *Aspergillus niger, Penicillium claviforme, Saccharomyces cerevisae, Candida albicans 8673 and Candida glabrata 72* were treated for 24 hours with Protect-DPODO(50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml), Fluconazole (150 mg/ml).The antifungal activity was assayed by the well diffusion method *with* digital caliper.*Determination of minimum inhibitory concentrations(MICs):* The MIC of Protect-DPODO, that shows antifungal activity, were determined by methods as described by [1] and MICs were read in µg/ml after overnightincubation at 37°C. All experiments were made in replicate.*Determination of Minimum fungal concentration(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 theProtect-DPODOwithout a colony growth was recorded asthe MFC.Protect-DPODO had higher antifungal activity than tested antibiotic– Fluconazole for yeast and Chloronitromycin for fungi. The results revealed variability in the inhibitory concentrations of Protect-DPODO for given fungi and yeast MIC of Protect-DPODO at concentration 50 mg/ml for 24 hours notably inhibited growth of yeast *C.albicans* and fungi. In contrast, MIC of Protect-DPODO at concentration 25 mg/ml for 24 hours notably inhibited growth only of fungi *P. claviforme.* MFC of Protect-DPODO at concentration 25 mg/ml for 24 hours notably inhibited growth of *C. albicans 8673*. MFC of Protect-DPODO at concentration 12.5 mg/ml for 24 hours notably inhibited growth only of fungi *P. claviforme.* For Fungi Imperfecta from *A. niger* and years *C. glabrata 72* and *S. cerevisae*MFC it was not reported. Based on the results obtained we can conclude that the examined Protect-DPODO has bactericidal activity towards both pathogenic yeast and Fungi Imperfecta, but in different concentrations.The Protect-DPODOpossesses 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.In our previous studies was shown that another bifunctionalized allene with protected hydroxy group (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate)* (BA-1) exhibited antibacterial and antifungal activity. The results obtained show for the first time the existence of antifungal activity of Protect-DPODO towards various pathogenic yeast and fungi.

Keywords: antibiotic, antifungal activity, Protect-DPODO(*2-(2-Diphenylphosphinoyl-4-methyl-octa-2,3-dienyloxy)-tetrahydro-2H-pyran)*.

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INTRODUCTION

In 1945, Sir Alexander Fleming famously warned of the danger of over-reliance on antibiotics and the threat of bacteria developing resistance [1, 2]. 71 years later, his prediction has been realised; in The Lancet Infectious Diseases Commission on antibiotic resistance and use, Laxminarayan[3]and colleagues warn that "we are at the dawn of a postantibiotic era", with "almost all disease-causing bacteria resistant to the antibiotics commonly used to treat them".

Rarely has modern medicine faced such a grave threat. Without antibiotics, treatments from minor surgery to major transplants could become impossible, and health-care costs are likely to spiral as we resort to newer, more expensive antibiotics and sustain longer hospital admissions. Infection-related mortality rates in developed countries might return to those of the early 20th century. Microbiological diagnostic tests help to prevent unnecessary antibiotic use and narrow the spectrum of coverage needed to treat an infection, but these are often slow, and delays in treatment can be associated with increased mortality. Hence, broad-spectrum antibiotic treatment is often used. Development of rapid diagnostics could eliminate this delay, enabling targeted antibiotic or non-antibiotic treatment from the outset. Antimicrobial resistance extends far beyond human medicine: the majority of the 100 000–200 000 tonnes of antibiotics manufactured every year is used in the agricultural, piscicultural, and veterinary sectors. More research is needed on the associations between non-human use of antibiotics and the development of resistance in human beings. Reduction of antibiotic use in animal rearing must be achieved while maintaining the security of the food supply [4].

The relationship between chemical structure of the medicinal agents and their biological activity i.e. structure–activity relationship (SAR) is one of the long standing problems in medicinal and structural chemistry. Based on the assumption that the similar compounds have similar physical and biological properties, the biological effects of a new chemical compound can be predicted without the cost of performing the traditional toxicology studies and/or in vitro/in vivo assays [5].

Over several decades, to varying degrees, bacteriacausing common infections have developed resistance to eachnew antibiotic. Antibiotic resistance continues to rise and the dawn of the much forewarned post- antibiotic era has arguably broken [3]. The present treatments of bacterial and fungal infections are abit unsatisfactory, owing to rapidly developing drug resistanceand side effects. This effect has a negative impact on the usage of most antimicrobial agents [6].

In the design of new drugs, the development of hybrid molecules may lead to compounds with interesting biological profiles.

In this paper, the antifungal activity of a bifunctionalized allenewith protected hydroxy group (*2-(2-Diphenylphosphinoyl-4-methyl-octa-2,3-dienyloxy)-tetrahydro-2H-pyran*) (Protect-DPODO)has been studied as part of the exploration for new and novel bio-active compounds.

MATERIALS AND METHODS

Test organisms

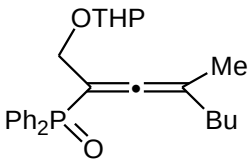
Aspergillus niger, Penicillium claviforme, Saccharomyces cerevisae, Candida albicans 8673 and Candida glabrata 72 were obtained from the National Bankfor Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria. All the isolates werechecked for purity and maintained in slants ofNutrient agar.

Media used

They were maintainedon Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK) platesat 29°C 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 e 7.0) were adjusted to the final concentrations in therange of 10^5-10^6 spores/mL.

Compound tested

Protect-DPODOwas synthesised in the Laboratory of Toxicologycal Chemistry, Department of Organic Chemistry & Technology of the Konstantin Preslavsky University of Shumen, Bulgaria (Figure 1)[7].



Protect-DPODO

Figure 1: Structural formula of Protect-DPODO

2-(2-Diphenylphosphinoyl-4-methyl-octa-2,3-dienyloxy)-tetrahydro-2H-pyran(**Protect-DPODO)**. Yellow oil, yield:84%. R_f 0.57; IR (neat, cm^{-1}): 1120 (C-O-C), 1155 (P=O), 1438, 1482 (Ph), 1954 (C=C=C). ^{1}H-NMR(600.1 MHz): δ 0.81 (t, *J* = 7.3 Hz, 3H, Me-CH_2), 1.07–1.18, 3.41–3.45 (mm, 6H, $(CH_2)_3$-Me),1.34–1.74, 3.71–3.77, 4.58–4.61 (overlapping multiplets, 9H, OTHP), 1.51 (d, *J* = 6.6 Hz, 3H, Me-C=),4.25–4.52 (m, 2H, CH_2O), 7.30–7.82 (m, 10H, 2Ph). ^{13}C-NMR (150.9 MHz) δ = 13.9, 17.7 (*J* = 5.6 Hz),18.9, 22.2, 25.4, 30.1, 29.2, 32.8, 61.7, 64.3 (*J* = 9.6 Hz), 95.2 (*J* = 104.5 Hz), 97.8, 103.0 (*J* = 13.3 Hz),131.5–133.4 (2Ph), 208.5 (*J* = 6.4 Hz). ^{31}P-NMR (242.9 MHz): δ 29.8. Anal. Calcd for $C_{26}H_{33}O_3P$(424.51): C 73.56, H 7.84. Found: C 73.64, H 7.91.

Preparing the solution of Protect-DPODO

The solutions of Protect-DPODO(50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml)werefreshlypreparedin 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 Protect-DPODO and antibiotics tested. After adjusting the pH at 6.5 by NaOH, the activity of the Protect-DPODO was checked. The plates were incubated at 37°C for 48 hours. The antifungal activity was assayed by measuring the diameter of the inhibition zoneformed around the wellwith digital caliper[8]. All experiments were performed in triplicate.

Determination of Minimum inhibitory concentrations(MICs)

The minimum inhibitory concentrations of Protect-DPODO, that shows antimicrobial activity, were determined by 2-folddilution methods as described by[1] and MICs were read in µg/ml after over nightincubation at 37°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°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°C for24 h, after which each plate was observed forcolony growth. The lowest concentration of theProtect-DPODOwithout a colony growth was recorded asthe MFC.

RESULTS

In the present study the effects of Protect-DPODO on five pathogenic fungi and were evaluated. The effects were compared with widely used antibiotics Fluconazole for yeast and Chloronitromycin for fungi. According to NCCLS, the antibiotic Fluconazole used is known to have broad spectrum antiyeast activity and

Chloronitromycin used is known to have broad spectrum antifungal activity[9].The effects of **Protect-DPODO**on the microorganisms were summarized in Table 1.

Protect-DPODO at concentration 25 mg/ml for 24 hours notably inhibited growth of *C. albicans 8673* (12.50 mm mean zone of inhibition) and *P. claviforme*(17.32 mm mean zone of inhibition). **Protect-DPODO** did not inhibited *A. niger, C.glabrata 72,* and *S. cerevisae*.

Table 1: Effect of Protect-DPODOon test organisms.

Microorganisms	Zone of inhibition (mm)[a]
A. niger	0
P. claviforme	17.32±0.18
S. cerevisae	0
C. albicans 8673	12.50±0.18
C. glabrata 72	0
Ethanol(96%) (Negative control)	10.60±0.05
Fluconazole150µg/ml	12.00±0.02
Chloronitromycin (250 µg/ml)	13.51±0.19

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

Our assay for antifungal activity of **Protect-DPODO** 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 ofProtect-DPODO

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 Protect-DPODO for given fungi. MIC of Protect-DPODO at concentration 50 mg/ml for 24 hours notably inhibited growth of yeast *C. albicans* and fungi. In contrast, MIC of Protect-DPODO at concentration 25 mg/ml for 24 hours notably inhibited growth only of fungi *P. claviforme.* 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 **Protect-DPODO**.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 ofProtect-DPODO

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 Protect-DPODO at concentration 25 mg/ml for 24 hours notably inhibited growth of *C. albicans 8673*. MFC of Protect-DPODO at concentration 12.5 mg/ml for 24 hours notably inhibited growth only

of fungi *P. claviforme.* For Fungi Imperfecta from *A. niger*and years *C. glabrata 72* and *S. cerevisae* MFC it was not reported.

Invasive infection due to *Candida* species is largely a conditionassociated with medical progress, and is widely recognized as amajor cause of morbidity and mortality in the healthcare environment.There are at least 15 distinct *Candida* species that causehuman disease, but >90% of invasive disease is caused by the5 most common pathogens, *C. albicans, C. glabrata, C. tropicalis,C. parapsilosis*, and *C. krusei*. Each of these organisms has uniquevirulence potential, antifungal susceptibility, and epidemiology,but taken as a whole, significant infections due to theseorganisms are generally referred to as invasive candidiasis[10].

Candida albicans is an opportunistic fungal pathogen that can be found in the gut and urinary tracts of around 70% of healthy adults[11]. Though this organism exists in the gut without causing disease under typical immune conditions, immunosuppression can cause this typically commensal microbe to act as a dangerous pathogen. *C. albicans* can be isolated from approximately 60% of all infections with *Candida* species, making it a very important subject of study for researchers attempting to develop new treatment options[12]. Crucial to this organism's ability to cause disease is its ability to spontaneously and reversibly change phenotype[13,14]. Depending on a number of external factors*, C. albicans* can shift from a yeast-like form, which is normally associated with a commensal relationship, to a hyphal form, a filamentous shape more commonly associated with invasive infections. These factors include temperature, pH, available nutrition, and cell density.

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

The Protect-DPODO 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[6].In our previous studies was shown that another bifunctionalized allenewith protected hydroxy group (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate)* (BA-1) exhibited antibacterial[15] and antifungal activity[16].The results obtained show for the first time the existence of antifungal activity of Protect-DPODO towards various pathogenic yeast and fungi.

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 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 allene with protected hydroxy group (*2-(2-Diphenylphosphinoyl-4-methyl-octa-2,3-dienyloxy)-tetrahydro-2H-pyran)* (**Protect-DPODO**) at 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml concentrations showed significantantifungial activity on selected pathogens inclinical isolates.

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