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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF A BIFUNCTIONALIZED ALLENE ETHANOL EXTRACTS

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ABSTRACT

Background and Purpose: Antibacterial effects of a Bifunctionalized Allene with unprotected hydroxy group (*Dimethyl 1-(1-hydroxyethyl)-3-methylpenta-1,2-dienephosphonate)* (**BA-2**) on pathogenic Gram-positives and Gram-negatives bacteria 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 bacterial cells *in vitro*. The effects of **BA-2** on prokaryotic cells have not been studied. The present study was aimed to assess the antibacterial activity of BA-2 on pathogenic Gram-positive and Gram-negative bacteria.

Experimental approach: *In vitro* antimicrobial test: *Escherichia coli 3398, Staphylococus aureus 745, Bacillus subtilis 6633, Salmonella Typhymurium 3591, Listeria monocytogen 863 and Enterobacter aerogenes 3691*were treated for 24 hours with **BA-2** (50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml),Sefpotec (250 mg/ml).The antibacterialactivity was assayed by the well diffusion methodwith digital caliper.*Determination of minimum inhibitory concentrations(MICs)*: The MIC of **BA-2**, that shows antimicrobial activity, were determined by methods as described by [16] and MICs were read in µg/ml after over night incubation at 37°C. All experiments were made in replicate.*Determination of Minimum bacteriocidal concentration(MBC)***:** The MBC were carried out to check whetherthe test microbes were killed or only their growth was inhibited. Nutrient Agaragar 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 the serial dilution were then subcultured onto the prepared 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 MBC. **BA-2**had higher antibacterial activity than tested antibiotic– Sefpotec.

Key Results: The results revealed variability in the inhibitory concentrations of **BA-2** for given bacteria. **BA-2** at concentration 50 mg/ml for 24 hours notably inhibited growth of *E. aerogenes 3691*(25.52 mm mean zone of inhibition), ***E. coli 3398*** (30.48 mm mean zone of inhibition) and *S. Typhymurium 745* (21.22 mm mean zone of inhibition). On the contrary, **BA-2** had no activity against *L. monocytogen 863* (17.39 mm mean zone of inhibition), which are comparable to the inhibitory effectof standard drug. **BA-2** did not inhibited *S. aureus 745* and *B. subtilis 6633*.

Conclusions and Implications: Based on the results obtained we can conclude that the examined **BA-2** has bactericidal activity towards pathogenic bacteria, 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[1]. In our previous studies was shown that the 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 [10] and antifungal activity [11]. The results obtained show for the first time the existence of antibacterial activity of **BA-2** towards various pathogenic bacteria.

INTRODUCTION

The decreasing effectiveness of antibiotics in treatingcommon infections has quickened in recent years, and with the arrival of untreatable strains of carbapenemresistantEnterobacteriaceae, we are at the dawn of apostantibiotic era [7].

In high-income countries, continued high rates of antibiotic use in hospitals, the community, and agriculture have contributed to selection pressure that has sustained resistant strains [14], forcing a shift to more expensive and more broad-spectrum antibiotics. In low income and middle-income countries (LMICs), antibiotic use is increasing with rising incomes, high

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rates of hospitalisation, and high prevalence of hospital infections. Resistance arises as a consequence of mutations inmicrobes and selection pressure from antibiotic use that provides a competitive advantage for mutated strains.

Suboptimum antibiotic doses help stepwise selection ofresistance. Resistance genes are borne on chromosomal, and increasingly, on transmissible extrachromosomalelements. The resulting resistant clones—eg, meticillinresistant *Staphylococcus aureus* (MRSA) USA 300,*Escherichia coli* ST131, and *Klebsiella* ST258) are disseminatedrapidly worldwide. This spread is facilitated byinter species gene transmission, poor sanitation and hygiene in communities and hospitals, and the increasing frequency of global, travel, trade, and disease transmission [7,14,17].The lack of understanding of the unique features and risk of resistance has paved the way for the present epidemic. Countries that have implemented comprehensivenational strategies have been the most successful incontrolling resistance [6,18,19,20]. These strategies include, but are not restricted to, good health-care infrastructure and health insurance for all; limited drug advertising; surveillance of antibiotic use and to detect resistance inhuman beings and animals; policies for prudent antibiotic use in human beings and animals; standardized infection control policies and sufficient staffing; antibioticstewardship programmes in hospitals and other health-care facilities; and isolation or de con tamination ofpatients with resistant organisms [20].

In this paper, the antibacterial 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

*Escherichia coli 3398, Staphylococus aureus 745, Bacillus subtilis 6633, Salmonella Typhymurium 3591, Listeria monocytogen 863 and Enterobacter aerogenes 3691*were obtained from the Collection of the Department of General and Applied Microbiology, Sofia University. All the isolates werechecked for purity and maintained in slants of Nutrient agar.

Media used

Nutrient Agar (Bio life 272-20128, Milano, Italia) was the medium used as the growth medium for the microbes.

Compound tested

Bifunctionalized Allene with unprotected hydroxy group (*Dimethyl 1-(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) [12].

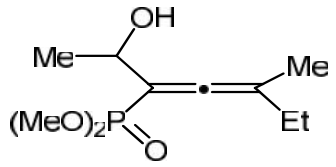

Figure 1 Structural formula of **BA-2**

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 preparedin ethanol.

Assay for Antifungal Activity

Antimicrobial 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 antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well [4]. 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 [16] 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 (MBC)

The MBC were carried out to check whether the test microbes were killed or only their growth was inhibited. Nutrient Agaragar 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 MIC in the serial dilution were then sub cultured onto the prepared medium, incubation was made at 37^{o}C for 24 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 MBC.

RESULTS

In the present study the effects of **BA-2** on six pathogenic Gram-positive and Gram-negative bacteria were evaluated. The effects were compared with widely used antibiotic Sefpotec. According to NCCLS, the antibiotic Sefpotec used is known to have broad spectrum antibacterial activity [15]. 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]
S. aureus 745 Gram- positive	-
*E. aerogenes 3691*Gram-negative	25.52±0.12
E. coli 3398 Gram-negative	30.48±0.02
*B. subtilis 6633*Gram- positive	-
L. monocytogen 863 Gram- positive	17.39±0.10
S. Typhymurium 745 Gram-negative	21.22±0.17
Ethanol(96%) (Negative control)	11.20±0.19
Sefpotec250µg/ml	21.10±0.05

[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 ***E. aerogenes 3691*** (25.52mm mean zone of inhibition), ***E. coli 3398*** (30.48mm mean zone of inhibition) and ***S. Typhymurium 745*** (21.22 mm mean zone of inhibition). On the contrary, **BA-2** had no activity against ***L. monocytogen 863*** (17.39mm mean zone of inhibition), which are comparable to the inhibitory effectof standard drug. **BA-2** did not inhibited ***S. aureus 745*** and ***B. subtilis 6633***.

Our assay for antibacterial 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.

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.

MBC of **BA-2**at concentration 50 mg/ml for 24 hours notably inhibited growth only Gram-negative bacteria of *Salmonella Typhynurium 745*, *E. aerogenes 3691*and*E. coli 3398*For *S. aureus 745*and *B. subtilis 6633*MC it was not reported. MBC of **BA-2** at concentration 25 mg/ml for 24 hours notably inhibited growth only Gram-positive bacteria of *L. monocytogen 863*.

Based on the results obtained we can conclude that the examined **BA-2** has bactericidal activity towards both pathogenic bacteria, 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[16].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 [10] and antifungal activity [11]. The results obtained show for the first time the existence of antifungal activity of **BA-2** towards various pathogenic yeast and fungi. Even before penicillin was introduced, resistant strains ofbacteria had been detected [1-3]. The selection pressure causedby the use of millions of tonnes of antibiotics over the past75 years since antibiotics were introduced has made almostall disease-causing bacteria resistant to antibioticscommonly used to treat them.

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

Microorganisms	MIC (mg/ml)[a]				
	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.125mg/ml
S. aureus 745	-	-	-	-	-
E. aerogenes 3691			+		
E. coli 3398			+		
B. subtilis 6633	-	-	-	-	-
L. monocytogen 863		+			
Salmonella Typhynurium 745		+			

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

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

Microorganisms	MBC (mg/ml)[a]				
	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.125mg/ml
S. aureus 745	-	-	-	-	-
E. aerogenes 3691	+				
E. coli 3398	+				
B. subtilis 6633	-	-	-	-	-
L. monocytogen 863		+			
Salmonella Typhynurium 745	+				

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

The results revealed variability in the inhibitory concentrations of **BA-2** for given bacteria. MIC of **BA-2** at concentration 25 mg/ml for 24 hours notably inhibited growth of ***L. monocytogen 863*** **and** ***Salmonella Typhynurium 745***. In contrast, MIC of **BA-2** at concentration 12.5 mg/ml for 24 hours notably inhibited growth only of Gram-negative bacteria ***E. aerogenes 3691*** and ***E. coli 3398***. Our next task was to determine the Minimum bactericidal concentration(MBC) in regards with determining the bactericidal or bacteriostatic

The rapid evolution of bacterial resistance is clear in the case of β-lactamases class of antibiotics. Nearly 1000 resistance-related β-lactamases that inactivate these antibiotics have been identified, a tentimesincrease since before 1990 [8]. Antibiotic use is a main driver of selection pressure that contributes to resistance, and because consumers do notunderst and this problem, the drugs are among the world's most commonly purchased. In high-incomecountries, patients with resistant infections can turn to more expensive, newer-generation

antibiotics, but in developing countries, where infectious diseases are common and the burden is high, patients might be unable to obtain or to afford second-line treatments. Large diff erences in the frequency of resistant infections have been noted, both across European countries [9] and among regions of the USA [5,13].

Bacteria which cause disease react to the antibiotics used as treatment by becoming resistant to them, sooner or later. This natural process of adaptation, antimicrobial resistance, means that the effective lifespan of antibiotics is limited. Unnecessary use and inappropriate use of antibiotics favours the emergence and spread of resistant bacteria. A crisis has been building up over decades, so that today many common and life-threatening infections are becoming difficult or even impossible to treat, sometimes turning a common infection into a life-threatening one. It is time to take much stronger action worldwide to avert a situation that entails an ever increasing health and economic burden [20]. Considering antibiotics resistance of most Gram-positive and Gram-negative bacteria, these Bifunctionalized Allene offer good and promising prospects for the treatment of diseases caused by these. 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 unprotected hydroxy group **BA-2** at 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125 mg/ml concentrations showed significantanti bacterial activity on selected pathogens inclinical isolates.

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