



Original Research Article

In Vitro Studies of Antibacterial Activity of a Bifunctionalized Allene ethanol Extracts

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A B S T R A C T

Antibacterial effects of a Bifunctionalized Allene (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate*) (BA-1) on pathogenic Gram-positives and Gram-negatives bacteria had been established. (BA-1) exerted different inhibitory effect on different bacterial cells *in vitro*. The effects of BA-1 on prokaryotic cells have not been studied yet. The present study was aimed to assess the antibacterial activity of BA-1 on pathogenic Gram-positive and Gram-negative bacteria. *In vitro* antimicrobial test: *Escherichia coli* 3398, *Staphylococcus aureus* 745, *Bacillus subtilis* 6633, *Salmonella typhimurium* 3591, *Listeria monocytogenes* 863 and *Enterobacter aerogenes* 3691 were treated for 24 hours with BA-1 (1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml), Sefpotec (250 mg/ml). The antibacterial activity was assayed by the well diffusion method. *Determination of minimum inhibitory concentrations (MICs)*: The MIC of BA-1, that shows antimicrobial activity, were determined by 2-fold dilution methods as described by Omura *et al.* (1993) and MICs were read in µg/ml after overnight incubation at 37°C. All experiments were made in replicate. *Determination of Minimum bacteriocidal concentration (MBC)*: The MBC was carried out to check whether the test microbes were killed or only their growth was inhibited. Nutrient Agar agar was prepared and sterilized at 121°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 subcultured onto 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 extracts without a colony growth was recorded as the MBC. BA-1 shows good bactericidal activity against the selected pathogens the maximum activity evinced on *Salmonella typhimurium* 3591 with zone of inhibition 20.41mm, *Listeria monocytogenes* 863 with zone of inhibition 16.68mm and *Staphylococcus aureus* 745 with zone of inhibition 15.22mm in comparison to tested antibiotic. BA-1 had higher antibacterial activity than tested antibiotic even from this fourth generation – Sefpotec. The present study indicated significant antibacterial activity of BA-1 on tested pathogenic bacteria. The inhibitory effect of Bifunctionalized Allene against several bacterial species indicates broad spectrum antimicrobial potential. This justified the use of BA-1 for the treatment of diseases of microbial origin and also makes it a potential candidate to use in drug development for treatment of infectious diseases caused by these pathogens.

Keywords

BA-1 (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate*),
Antibacterial activity,
Antibiotic

Introduction

In the past 60 years, antibiotics have been critical in the fight against infectious disease caused by bacteria and other microbes.

Antimicrobial chemotherapy has been a leading cause for the dramatic rise of average life expectancy in the Twentieth

Century. However, disease-causing microbes that have become resistant to antibiotic drug therapy are an increasing public health problem. Wound infections, gonorrhea, tuberculosis, pneumonia, septicemia and childhood ear infections are just a few of the diseases that have become hard to treat with antibiotics.

One part of the problem is that bacteria and other microbes that cause infections are remarkably resilient and have developed several ways to resist antibiotics and other antimicrobial drugs. Another part of the problem is due to increasing use, and misuse, of existing antibiotics in human and veterinary medicine and in agriculture.

Bacteria are champions of evolution, and a few microbes have adapted to a point where they pose serious clinical challenges for humans. Among the Gram-positive organisms, methicillin-resistant *Staphylococcus aureus* (NCCLS) and *E. faecium* represent the biggest therapeutic hurdles (Arias and Murray, 2009).

Nowadays, about 70% percent of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. Some organisms are resistant to all approved antibiotics and can only be treated with experimental and potentially toxic drugs.

An alarming increase in resistance of bacteria that cause community acquired infections has also been documented, especially in the staphylococci and pneumococci (*Streptococcus pneumoniae*), which are prevalent causes of disease and mortality.

In a recent study, 25% of bacterial pneumonia cases were shown to be resistant to penicillin, and an additional 25% of cases were resistant to more than one antibiotic

(Arias and Murray, 2014).

Microbial development of resistance, as well as economic incentives, has resulted in research and development in the search for new antibiotics in order to maintain a pool of effective drugs at all times.

In this paper, the antimicrobial activity of a BA-1 (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate*) has been studied as part of the exploration for new and novel bio-active compounds.

Materials and Methods

Test organisms

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

Media used

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

Compound tested

Bifunctionalized Allene (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate*) (BA-1) was synthesised in the Laboratory of Toxicological Chemistry, Department of Organic Chemistry & Technology of the Konstantin Preslavsky University of Shumen, Bulgaria (Figure 1) (Ismailov *et al.*, 2014).

Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate

(BA-1). Orangeoil, yield: 72%. R_f 0.43; IR (neat, cm^{-1}): 1120 (C-O-C), 1254 (P=O), 1956 (C=C=C). $^1\text{H-NMR}$ (CDCl_3 , 600.1 MHz): δ 0.93 (t, $J = 7.1$ Hz, 3H, Me-(CH_2)₃), 1.43 (dd, $J = 6.3$ Hz, $J = 10.0$ Hz, 3H, Me-CHO), 1.48, 1.55, 1.64, 3.62, 4.38 (overlapping multiplets, 9H, OTHP), 1.77 (d, $J = 6.6$ Hz, 3H, Me-C=), 1.41, 1.74, 2.11 (overlapping multiplets, 6H, Me-(CH_2)₃), 3.76 (d, $J = 11.2$ Hz, 3H, MeO), 4.64(m, 1H, CHO). $^{13}\text{C-NMR}$ (CDCl_3 , 150.9 MHz) $\delta = 13.8, 18.8$ ($J = 6.5$ Hz), 19.5, 22.7, 23.5 ($J = 7.5$ Hz), 25.7, 29.6, 30.4, 32.8, 52.3 ($J = 6.2$ Hz), 62.3, 68.6 ($J = 10.2$ Hz), 91.4 ($J = 191.7$ Hz), 95.6, 103.4 ($J = 16.2$ Hz), 209.0 ($J = 5.3$ Hz). $^{31}\text{P-NMR}$ (CDCl_3 , 242.9 MHz): δ 20.5. Anal. Calcd for $\text{C}_{17}\text{H}_{31}\text{O}_5\text{P}$ (346.40): C 58.94, H 9.02. Found: C 59.01, H 8.96.

Preparing the solution of BA-1

The solutions of BA-1(1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml) were freshly prepared in ethanol.

Assay for Antimicrobial 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-1 and antibiotics tested.

After adjusting the pH at 6.5 by NaOH, the activity of the plant extracts 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 (Bertrand-Harb

et al., 2003). All experiments were performed in triplicate.

Determination of Minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations of **BA-1**, that shows antimicrobial activity, were determined by 2-fold dilution methods as described by (Omura *et al.*, 1993) and MICs were read in $\mu\text{g/ml}$ after overnight incubation at 37°C. All experiments were made in replicate.

Determination of Minimum bacteriocidal concentration (MBC)

The MBC were carried out to check whether the test microbes were killed or only their growth was inhibited. Nutrient Agar agar was prepared and sterilized at 121°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 subcultured onto 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 extracts without a colony growth was recorded as the MBC.

Results and Discussion

In the present study the effects of **BA-1** on six pathogenic bacteria (Gram-positive and Gram-negative) and 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 against both Gram-positive and Gram-negative organisms (NCCLS, 1993).

The effects of BA-1 on the microorganisms were summarized in Table 1.

The sensitivities of the test organisms to infusions were indicated by clear zone around the wells (Figure 2).

BA-1at concentration 4 mg/ml for 24 hours notably inhibited growth of *S. typhymurium* (20.41 mm mean zone of inhibition), *L. monocytogenes* (16.68 mm mean zone of inhibition) and *S. aureus* (15.22 mm mean zone of inhibition). On the contrary, Bifunctionalized Allene had no activity against *E. coli* (10.49 mm mean zone of inhibition).

Our assay for antibacterial activity of BA-1 was conducted by testing different concentrations of the compound on various pathogens to determine the MICs. We used four concentrations – 1mg/ml; 2mg/ml; 3mg/ml and 4mg/ml. The results are shown in table 2.

The results revealed variability in the inhibitory concentrations of BA-1for given bacteria. MIC of BA-1at concentration 4 mg/ml for 24 hours notably inhibited growth only of *E. coli* 3398. In contrast, MIC of bifunctionalized allene at concentration 1 mg/ml for 24 hours notably inhibited growth of *S. aureus* 745 and *B. subtilis* 6633. The probable reason for the higher MIC reported for Gram-negative bacteria are the complex structure of their cell wall.

Our next task was to determine the Minimum bactericidal concentration (MBC) in regards with determining the bactericidal or bacteriostatic activity of the examined BA-1. We used four concentrations – 1mg/ml; 2mg/ml; 3mg/ml and 4mg/ml. The results are shown in table 3.

MBC of BA-1at concentration 3 mg/ml for 24 hours notably inhibited growth only of Gram-negatives bacteria *E. aerogenes* 3691, *E. coli* 3398 and *Salmonella typhymurium*

745. For Gram-positive bacteria *S. aureus* 745, *B. subtilis* 6633 and *L. monocytogenes* 863 MBC is 1 mg/ml.

Based on the results obtained we can conclude that the examined BA-1has bactericidal activity towards both Gram-positive bacteria and Gram-negative bacteria, but in different concentrations.

The BA-1possess biological activity, which is not well studied and 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 (Beach *et al.*, 1997).

The results obtained show for the first time the existence of antibacterial activity of BA-1towards various pathogenic bacteria.

Infectious diseases have become the major cause and serious concern in public health issues. 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.

However, in the present study results also exhibited the confirmation of the antimicrobial property that showed bactericidal action on the pathogens commonly encountered in hospitalized patients. 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.

The Bifunctionalized Allenes BA-1at 1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml

concentrations showed significant anti-infective activity on selected pathogens from clinical isolates.

Table.1 Effect of BA-1 on test organisms

Microorganisms	Zone of inhibition (mm)
<i>S. aureus</i> 745 Gram- positive	15.22±0.02
<i>E. aerogenes</i> 3691 Gram-negative	11.28±0.03
<i>E. coli</i> 3398 Gram-negative	10.49±0.07
<i>B. subtilis</i> 6633 Gram- positive	13.85±0.02
<i>L. monocytogenes</i> 863 Gram- positive	16.68±0.03
<i>S. typhimurium</i> 745 Gram-negative	20.41±0.05
Ethanol(96%) (Negative control)	10.09±0.05
Sefpotec 250 µg/ml	12.52±0.19

Data are presented as average values ± standard deviation in mm

Table.2 The MIC of BA-1

Microorganisms	MIC (mg/ml)			
	BA1mg/ml	BA2mg/ml	BA3mg/ml	BA4mg/ml
<i>S. aureus</i> 745	+			
<i>E. aerogenes</i> 3691			+	
<i>E. coli</i> 3398				+
<i>B. subtilis</i> 6633	+			
<i>L. monocytogenes</i> 863		+		
<i>Salmonella</i> <i>Typhimurium</i> 745			+	

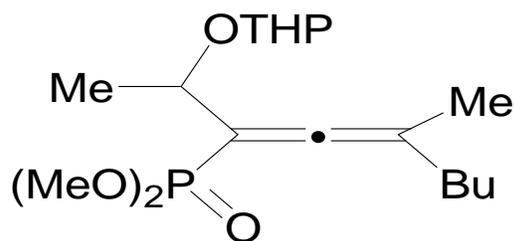
Results are mean ± SEM of three separate trails

Table.3 The MBC of BA-1

Microorganisms	MBC (mg/ml)			
	BA1mg/ml	BA2mg/ml	BA3mg/ml	BA4mg/ml
<i>S. aureus</i> 745	+			
<i>E. aerogenes</i> 3691			+	
<i>E. coli</i> 3398			+	
<i>B. subtilis</i> 6633	+			
<i>L. monocytogenes</i> 863	+			
<i>Salmonella</i> <i>Typhimurium</i> 745			+	

Results are mean ± SEM of three separate trails.

Figure.1 Structural formula of BA-1



104-c, 7e

Figure.2 Showing Zone of inhibition with BA-1 along with tested antibiotics Biseptol and Sefpotec of 24 hours *S. aureus* 745 Position 1 and 2) BA-1; 3 and 4) Sefpotec; 5 and 6) Ethanol



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