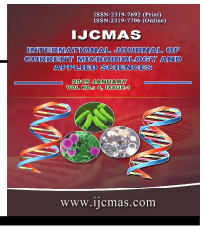
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 3 (2015) pp. 627-632**
http://www.ijcmas.com



Original Research Article

Study of antibacterial activity of br-containing oxaphosphole in vitro

Ignatova-Ivanova Ts[1]*, M. Kaschchieva[1], S. Etem[1] and Dobromir Enchev[2]

[1]Department of Biology, Faculty of Natural Sciences, Konstantin Preslavsky University of Shumen, 115, Universitetska Str., BG-9712 Shumen, Bulgaria
[2]Department of Organic Chemistry & Technology, Faculty of Natural Sciences,Konstantin Preslavsky University of Shumen, 115, Universitetska Str., BG-9712 Shumen, Bulgaria
**Corresponding author*

Keywords

Erwinia amylovora, Br-oxph (4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine2-oxide), antibacterial activity, Streptomycin

A B S T R A C T

Antibacterial effects of a Br-oxph (*4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine2-oxide)* on pathogenic Gram-negatives bacteria *Erwinia amylovora* had been established. Br-oxph exerted different inhibitory effect on two bacterial cells *in vitro*. The effects of Br-oxph on prokaryotic cells have not been studied yet. The present study was aimed to assess the antibacterial activity of Br-oxph on pathogenic Gram-negative bacteria. *In vitro* antimicrobial test: *E. amylovora NBIMCC 8518* and *E. amylovora NBIMCC 8488* were treated for 24 hours with Br-oxph (1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml), Streptomycin (250 mg/ml). The antibacterial activity was assayed by the well diffusion method with digital caliper. *Determination of minimum inhibitory concentrations(MICs):*The MIC of Br-oxph, that shows antimicrobial activity, were determined as described by [10] and MICs were read in µg/ml after over night incubation at 37^{o}C. We used four concentrations – 1mg/ml; 2mg/ml; 3mg/ml and 4mg/ml. 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^{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 four concentrations were then subcultured 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 extracts without a colony growth was recorded as the MBC. Br-oxph shows good bactericidal activity against the selected pathogens the maximum activity evinced on Br-oxph at for 24 hours inhibited growth of *E. amylovora NBIMCC 8518* (24.30 mm mean zone of inhibition) and *E. amylovora NBIMCC 8488* (23.34 mm mean zone of inhibition). For comparison, Streptomycin for 24 hours inhibited growth of *E. amylovora* (27.52 mm mean zone of inhibition). The present study indicated significant antibacterial activity of Br-oxph on tested pathogenic bacteria. The inhibitory effect of Br-oxph against several bacterial species indicates broad spectrum antimicrobial potential. This justified the use of Br-oxph 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.

Introduction

Fire blight is a dangerous, contagious and economically important bacterial disease which affects mainly the seed fruit species. The big economic importance of the disease is related with the opportunity for compromising of large areas planted with the species susceptible to that disease, and apples (*Malus domestica*) and pears (*Pyrus communis*) are especially vulnerable. Within 2-3 years, with favourable conditions for its development, the pathogen can entirely destroy the affected plants. The disease was registered in plants belonging to the family sp. Rosaceae [4,11,17], and in the recent years there is increasing isolation of new species which are hosts of the pathogen [1]. The bacterium *Erwinia amylovora* (Burr.) Winslow, belonging to the genus *Bacillus amylovorus*, is the causal pathogen of fire blight.

The fire blight disease was first discovered in 18th c. in USA. In 1957, the first mass appearance of the disease was reported in America [17]. In Europe, the appearance of this disease on the fruit trees was reported in 1950 [6,17], and after that it began to spread on the territories of the continent and in the Mediterranean countries. Currently, the disease is spread in different places of the world [3].

In Bulgaria, fire blight was first found in 1989, on a quince-tree and a pear in the region of Plovdiv. Gradually, sick trees were found in other regions of the country, and the registered hosts are permanently increasing [1]. The distribution of *E. amylovora* in Bulgaria results from the import of plants from Europe and the lack of efficient control on the disease and the reduced care in the orchards.

Preventive measures shall be undertaken against the dissemination of that dangerous disease. They include decontamination of the used trimming tools and treatment of the fruit cultures with chemical preparations (copper hydroxide, copper sulfate, and copper oxychloride) which are used with variable success.

In USA, the antibiotic streptomycin is used to destroy the causal pathogen of fire blight [13,15]. In European Union, the use of antibiotics in cultivation of fruit cultures is not allowed. Streptomycin is not registered as a plant protection preparation due to its accumulation in the produce. Residual amounts of streptomycin were found in agricultural products, in honey and in the bees [5].

For the purposes of bioproduction, the fight against *E. amylovora* is carried out also with biologically active preparations [5]. Not all registered conventional preparations for fight against *E. amylovora* are efficient in the fight against it, which imposes the development of new combinations for effect against the causal agent of fire blight.

In this paper, the antimicrobial activity of a Br-oxph (*4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine2-oxide)* has been studied as part of the exploration for new and novel bio-active compounds.

Materials and Methods

Test organisms

E. amylovora NBIMCC 8518 and *E. amylovora NBIMCC 8488* 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 with supplemented of 10% sucrose.

Media used

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

Compound tested

Br-oxph (*4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine2-oxide)* belongs to the family of heterocyclic organophosphorus compounds was synthesized in the Laboratory of Organic Chemistry of the University of Shumen (Bulgaria).

Preparing the solution of Br-oxph.

The solutions of Br-oxph (1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml) were freshly preparedin twice distilled water.

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 Br-oxph 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 with digital caliper [2]. All experiments were performed in triplicate.

Determination of Minimum inhibitory concentrations(MICs)

The minimum inhibitory concentrations of Br-oxph, that shows antimicrobial activity, were determined as described by [10] and MICs were read in µg/ml after over night incubation at 37^{o}C. We used four concentrations – 1mg/ml; 2mg/ml; 3mg/ml and 4mg/ml. 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^{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 four concentrations were then subcultured 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 extracts without a colony growth was recorded as the MBC.

Result and Discussion

In the present study the effects of Br-oxph on two Gram-negative pathogenic bacteria *E. amylovora* were evaluated. The effects were compared with widely used antibiotic Streptomycin. According to NCCLS, the antibiotic Streptomycin used is known to have broad spectrum antibacterial activity against *E. amylovora* [13,15]. The effects of Br-oxph 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). Br-oxph at for 24 hours inhibited growth of *E. amylovora NBIMCC 8518* (24.30 mm mean zone of inhibition) and *E. amylovora NBIMCC 8488* (23.34 mm mean zone of inhibition). For comparison, Streptomycin for 24 hours inhibited growth of *E. amylovora* (27.52 mm mean zone of inhibition).

These assay for antibacterial activity of Br-oxph 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 Br-oxph for two bacteria. MIC of Br-oxph at concentration 2 mg/ml for 24 hours notably inhibited growth of *E. amylovora NBIMCC 8488* . In contrast, MIC of Br-oxph at concentration 1 mg/ml for 24 hours inhibited growth of *E. amylovora NBIMCC 8518.* The probable reason for the higher MIC reported for Gram-negative bacteria is 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 Br-oxph. We used four concentrations – 1mg/ml; 2mg/ml; 3mg/ml and 4mg/ml. The results are shown in table 3.

MBC of Br-oxph at concentration 2 mg/ml for 24 hours inhibited growth for two bacteria.

Based on the results obtained we can conclude that the examined Br-oxph has bactericidal activity towards Gram-negative bacteria, but in different concentrations.

Oxaphospholes possess biological activity, which is not well studied. Br-oxph is a structural analogue to compounds used as antiviral drugs [7,9,12,16] and antitumor agents[8,14]. The results obtained show for the first time the existence of antibacterial activity of Br-oxph towards Gram-negative pathogenic bacteria *E. amylovora*.

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.

Table.1 Effect of Br-oxph on test organisms

Microorganisms	**Zone of inhibition (mm)**
E. amylovora NBIMCC 8518	24.30±0.05
E. amylovora NBIMCC 8488	23.34±0.03
Streptomycin 250 µg/ml	27.52±0.11

Data are presented as average values ± standard deviation in mm

Table.2 The MIC of Br-oxph

Microorganisms	**MIC (mg/ml)**			
	Br-oxph 1mg/ml	**Br-oxph 2mg/ml**	**Br-oxph 3mg/ml**	**Br-oxph 4mg/ml**
E. amylovora NBIMCC 8518	+			
E. amylovora NBIMCC 8488		+		

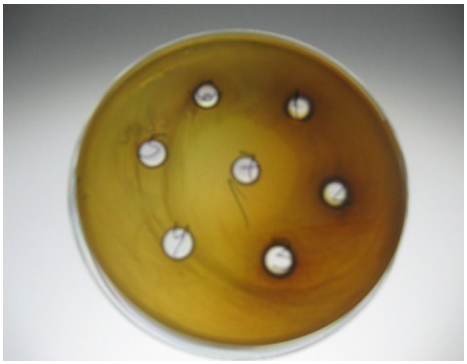
Results are mean ± SEM of three separate trails

Table.3 The MBC of Br-oxph

Microorganisms	MBC (mg/ml)			
	Br-oxph 1mg/ml	**Br-oxph 2mg/ml**	**Br-oxph 3mg/ml**	**Br-oxph 4mg/ml**
E. amylovora NBIMCC 8518		+		
E. amylovora NBIMCC 8488		+		

Results are mean ± SEM of three separate trails

Figure.2 Showing Zone of inhibition with Br-oxph along with tested antibiotic Streptomycin of 24 hous



Position 1,2 and 3) E. amylovora NBIMCC 8518; 4,5 and 6) E. amylovora NBIMCC 8488 7) Streptomycin

Nevertheless, further studies are required to explore the mechanism of biochemical active principle in the Br-oxph for the inhibitory action on Gram-negative pathogenic bacteria *E. amylovora* selected in the study.

The Br-oxph at 1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml concentrations showed significantanti bacterial activity on selected pathogens inclinical isolates.

Acknowledgement

Support from the Research Fund of the Konstantin Preslavsky University of Shumen (Project No. RD-08-213/10. 03. 2014, Department of Biology is acknowledged.

References

1. Atanasova I., Kabadjova P., Bogatzevska N., Moncheva P. 2005. New Host Plants of Erwinia amylovora in Bulgaria. Z Naturforsch C. Nov-Dec; 60 (11-12):893-8.
2. Bertrand-Harb C., I. Ivanova, M. Dalgalarrondo and T. Hartle. 2003. Evolutionofb-lactoglobulinanda-lactalbumincontentduring yoghurt fermentation. *Int. Dairy J. 13*, 39–45.
3. Bonn W.G., Van der Zwet T., 2000. Distribution and economic importance of fire blight. Fire Blight The Disease and its Causative Agent, Erwiniaamylovora, CABI Publiching, Wallingford, UK, pg. 37– 55)
4. Bradbury, J.F. 1986. Guide to plant pathogenic bacteria, CAB International, Wallingford, UK, 332 pp.
5. Broggini, G.A.L., Duffy, B., Holliger, E.,Scha¨rer, H.-J., Gessler C. and Patocchi, A. 2005. Detection of the fire blight biocontrol agent Bacillus subtilis BD170 (Biopro) in a Swiss apple orchard, 8 July 2004, European Journal of Plant Pathology, 111: 93–100

6. Jock S, Donat V, Lorpez MM, Bazzi C and Geider K. 2002. Following spread of fireblight in Western, Central and Southern Europe by molecular differentiation of Erwiniaamylovora strains with PFGE analysis. Environmental Microbiology 4: 106–114
7. Kamiya, N. 2003. The mechanisms of action of antivirals against hepatitis B virus infection. J Antimicrob Chemother. 51: 1085-1089.
8. Kanno, T.Y.N., L.A. Sensiate, N.A. de Paula and Salles, M.J.S. 2009. Toxic effects of different doses of cyclophosphamide on the reproductive parameters of male mice. Braz J Pharm Sci. 45: 313-319.
9. Okano, K. 2009. Synthesis and pharmaceutical application of L-ribose. Tetrahedron. 65: 1937-1949.
10. Omura, S., D.V.D. Pyl, J. Inokoshi, Y. Takahashi and H. Takeshima, 1993. Pepticinnamins, new farnesylprotein-transferase inhibitors produced by an actinomycete. I. Producing strain, fermentation, isolation andbiological activity. *Journal of Antibiotics*, 1993,46(2), 222-228.
11. Ordax M., Marco–Noales, E., Lopez M.M., and Biosca, E.G. 2006. Copper induces a viable but nonculturable (VBNC) state in Erwiniaamylovora. ActaHorticulturae 704, 205–210.
12. Pescovitz, M.D. 2008. Maribavir: a new oral anti-cytomegalovirus drug. Future Virol. 3:435-443.
13. Rezzonico, F., Stockwell, V. O., and Duffy, B. 2009. Plant Agricultural Streptomycin Formulations Do Not Carry Antibiotic Resistance Genes, Antimicrobial Agents And Chemotherapy, July 2009, p. 3173–3177 Vol. 53, No. 7 doi:10.1128/AAC.00036-09 Copyright © 2009, American Society for Microbiology.
14. Sharabi, A. and Haran-Ghera, N. 2011. Immune Recovery after Cyclophosphamide Treatment In Multiple Myeloma: Implication for Maintenance Immunotherapy. Bone Marrow. 2011: ID 269519, doi: 10.1155/2011/269519.
15. Stockwell, V.O. & Duffy B. 2012. Use of antibiotics in plant agriculture**,** Rev. sci. tech. Off. int. Epiz., 2012, 31 (1), 199-210.
16. Tam, R.C., K. Ramasamy, J. Bard, B. Pai, C. Lim and Averett, D.R. 2000. The ribavirin analog ICN 17261 demonstrates reduced toxicity and antiviral effects with retention of both immunomodulatory activity and reduction of hepatitis-induced serum alanine aminotransferase levels. Antimicrob Agents Chemother. 44:1276–1283.
17. Van der Zwet, T. and Keil, H. L. 1979. Fire Blight: A Bacterium Disease of Rosaceous Plants. Agriculture Handbook no. 510. USDA, Government Printing Of- fice, Washington, DC.