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Antimicrobial and Biological Effects of Bomphos and Phomphos on Bacterial and Yeast Cells

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In this study, the antimicrobial effects of monophosphazenes such as SM, BOMPHOS, and PHOMPHOS were examined on bacterial and yeast strains. In addition, the biological effects of these compounds were tested on the *Saccharomyces cerevisiae* and *Candida albicans* cells. The SM has an antimicrobial effect on the bacterial and yeast strains within the range of 100 and 1500 μg. When the concentration was increased, the inhibition zone expanded on the growth media ($P < 0.01$; $P < 0.001$). Like SM, BOMPHOS molecule has antimicrobial activity on the bacterial and yeast cells. The most effective concentrations of BOMPHOS on the microorganisms were observed by 1500 μg ($P < 0.001$). The PHOMPHOS did not effect on the bacterial and yeast cells between 100 and 1000 range, but it has an antimicrobial effect in 1500 μg. *In vitro* media, the biological effects of these molecules were compared with vitamin E, melatonin, and fish oil on the yeast cells. In *S. cerevisiae* growth media, the cell densities were increased SM, BOMPHOS, and PHOMPHOS after 20, 30, and 45 h. The highest increase in the cell density were observed in media of BOMPHOS. In *C. albicans* growth media, the cell density was increased by melatonin after 20, 30, and 45 h, but were decreased by other supplemental groups. Lipid level of *S. cerevisiae* was reduced by administered 300 and 1000 μg vitamin E and fish oil ($P < 0.01$). In addition, the lipid level of the same yeast cell were diminished by the 1000 μg melatonin and 300 μg PHOMPHOS ($P < 0.05$, $P < 0.01$). The lipid level of *C. albicans* were increased by vitamin E and BOMPHOS and fish oil, but was decreased with PHOMPHOS ($P < 0.01$). In conclusion, while high concentration of PHOMPHOS has antimicrobial effects on the bacterial and yeast cells, the SM and BOMPHOS have antimicrobial effects in all the concentrations. PHOMPHOS decreased the lipid level of *C. albicans*, but BOMPHOS increased in the the same yeast cell. In addition, the antioxidants such as vitamin E, melatonin, and fish oils have affected on the lipid synthesis of yeast cells.

Key Words: monophosphazenes; antimicrobial and biological effects; vitamin E; melatonin; fish oil; bacterial and yeast cells.

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INTRODUCTION

Phosphazenes are compounds that contain alternating phosphorus and nitrogen atoms in their skeleton. These compounds fall into three categories: the cyclo, the monophosphazenes, and phosphazenes. The cyclo or polyphosphazenes are probably the best known and most intensively studied phosphorus-nitrogen derivatives. Monophosphazenes have the structure RN=PR3. They show many fundamental similarities to the cyclic and polymeric phosphazenes. They are particularly interesting from the view point of their bonding structure and much of the physical-inorganic work carried out on these compounds has an important bearing on the skeletal bonding found in cyclic and polymeric phosphazenes (*1,2*).

Phosphazenes possess a number of characteristics such as use as drug components for chemotheropeutic applications, and antibacterial activity. These compound are used in the structure of medical implants and drug delivery systems. In such applications where the polymer serves a temporary function, the ultimate degradability of the material to harmless fragments is a prerequisite for successful medical use. Phosphazene compounds and derivatives are widely used against bacterial agents in medicine on the surface cover in cardiovascular devices, knee and hip joints and inocular lens. These compounds covalently bind to the surface or with their side groups bind strongly to surface though ionic and hydrogen binding or van der Waals forces. Maximum interactions with microoganisms would be expected for polymer chain sites of a soluble macromolecule that is anchored to a surface (*2–5*).

Phosphazene polymers with an amino acid ester as cosubstituents are potentially biodegradable *in vivo*. Polyphosphazenes with amino acid groups are flexible, generally biodegradable, and erode hydrolytically to the amino acid, phosphate, and ammonia. Also, their derivatives have been stated to be biodegradable and biocompatible molecules. Consequently these compounds are of potential interest as biomaterials (*2–4*).

Vitamin E is the major lipid soluble antioxidant present in blood and cell membranes of higher organisms. It acts synergistically with other antioxidants in cells, to protect them from damage and lysis induced by oxidative stress (*6*). Tocopherols can undergo oxidation–reduction reactions on the aromatic ring (*7,8*). The vitamin activity of tocopherols likely results from their ability to prevent oxidative damage to the lipids of cellular membranes (*9–12*).

It is known that the pineal hormone melatonin is a powerful free radical scavenger and likely to be a general promoter of anti-oxidative mechanisms. Both *in vitro* and *in vivo* studies have shown that the pineal hormone melatonin is a potent antioxidant (*13*). Fish oils contain highly unsaturated fatty acids such as eicosapentaenoic (20:5), docosapentaenoic (22:5), and docosahexaenoic acid (22:6). In addition, the precursors of essential fatty acids such as linoleic (18:2), especially linolenic (18:3), acids are present in fish oil content. These unsaturated fatty acids depress lipid synthesis in liver and help maintain the normal level of serum fatty acids (*14–16*).

In this paper, we determined the antimicrobial effects of monophosphazenes such as the SM, BOMPHOS, and PHOMPHOS. In addition, the biological effects of these molecules were compared with potential antioxidants vitamin E, melatonin, and fish oil on the lipid levels of yeast cells such as *S. cerevisae* and *C. albicans*.

MATERIAL AND METHOD

Material

Benzene, benzyl alcohol, phurphuryl alcohol, ammonium sulfate, and chlorobenzene were supplied by Merck. Solvents and other liquids used in experimental works was dried by conventional methods. All reactions were monitored using Kieselgel 60F254 (silica gel) precoated TLC plates, and the optimal separating conditions were determined. Separation of products was carried out by flash column chromatography using Kieselgel 60.

The purity of each monophosphazene compound was established by column chromatography, NMR, mass spectroscopy, and elemental analysis. IR spectra were recorded with a Midiac 1700 M FTIR spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded using a Geminine Varian 200 spectrometer. ^{31}P-NMR spectra were recorded. Fisions EA 1108 and Leco 932 CHNS-O elemental analysis apparatus was used for out microanalysis.

Synthesis of Monophosphazenes

The SM ($Cl_3P{=}N\text{-}P\ (O)\ Cl_2$) was prepared by the method of Emsly *et al.* (*17*) and purified by vacuum distillation. Phosphorus pentachloride (93.5 g) and ammonium sulfate (13.2 g) were mixed with 200 ml chlorobenzene and refluxed under back cooler at 130–135°C for 2 h. Then, the solvent mixture was derived by evaporation and the product was obtained (mp, 32°C; bp, 110–115°C) The alcohol derivatives were synthesized as follows:

In order to synthesize BOMPHOS, 50 ml benzyl alcohol was mixed with metallic sodium metal particles to form alcoholate, which was then cooled to 10°C. Then 2.2 g of SM was added and refluxed under back cooling for 3 h. At the end of reaction, the products formed were extracted with 3 × 50-ml benzene. After the water had been removed from the benzene phase, the solvent mixture was rotary evaporated. Then the products of reaction were separated by Thin Layer Chromatography (TLC) by using a mixture of benzene:methanol:acetone:chloroform (10:1:3:2, v/v/v/v). After the value of R_f of product had been determined as (0.64), it was purified in a silica-gel column (n^{20} D = 1.443; d^{20} = 1.065 g/cm^3). The reaction product was then identified by IR, mass, and NMR spectroscopy.

In order to synthesize PHOMPHOS, 60 ml phurphuryl alcohol was mixed with 1.5 g metallic sodium metal particles and cooled to 10°C. Then 2.7 g of SM was added and refluxed under back cooling for 3 h. The product formed was extracted with 3 × 50-ml benzene. After the water had been removed, the solvent was evaporated. The obtained product was separated on TLC by using a mixture of benzene: ethyl alcohol: acetone (10: 1: 7; v/v/v/) and its R_f value was determined (0.59). The product was then refined by column chromatography using a mixture of benzene:ethyl alcohol:acetone (10: 1: 7; v/v/v/) and was identified with IR, mass, and NMR spectroscopy.

Bacterial and Yeast Strains

Bacterial cells. Listeria monocytogenes SCOTT A; Bacillus megaterum DSM 32; Escherichia coli ATCC 25922; Enterobacter aeroginase CCM 2531; Bacillus brevis

FMC 3; Micrococcus luteus, Psedomonos vulgaris and *Staphylococcus aureus CO-VAN 1*.

Yeast cells. Saccharomyces cerevisiae FMC 16; Candida albicans FMC 17.

Preparation of Microbial Culture Media

Bacterial strains were inoculated to Nutrient Buyyon culture (Difco) and incubated at 30 ± 0.1°C for 24 h. Yeast cells were inoculated into Malt Extract Buyyon (Difco) and incubated at 30°C for 48 h. In order to test the antimicrobial effects, the SM, BOMPHOS, and PHOMPHOS 15-ml Hilton agar were placed in petri dishes, which were then inoculated with yeast by taking 100 μl into culture media. After agar had hardened, discs of 6-mm diameter and to which monophosphazene compounds had been absorbed were placed on agar. Petri dishes were left at 4°C for 2 h. Then bacterial cultures were incubated at 35 ± 0.1°C for 18–24 h, and yeast cultures were incubated at 30 ± 0.1°C for 70 h. At the end of incubation time, the inhibition zones on the bacterial and yeast nutrient media were measured in millimeters.

Preparation of Disks

In order to prepare the discs, the SM, BOMPHOS, and PHOMPHOS compounds were dissolved in toluene and absorbed onto disks, which are made from filter paper, by using a microinjector. In addition, purified toluene was absorbed onto control disks.

Preparation of Yeast Strains Growth Media

The strains of *S. cerevisiae* and *C. albicans* were grown in YDP medium. One percent (w/v) yeast extract, 2% (w/v) bactopeptone, 2% glucose (w/v) from Difco. After the growth media had been prepared, their turbidites were measured 650 nm by using an Ultra spectrophotometer, and then 100 μl of cultured yeast cells were inoculated into culture. After the inoculation, the turbidity of culture media at 30°C was monitored for 12–20 h until absorbance values were in the range 0.150–200 absorbency. Then the monophosphazene compounds, vitamin E, melatonin, and fish oil were added to the culture media, i.e., both *S. cerevisiae* and *C. albicans*. Yeast cells turbidites in the growth media were measured at 650 nm for 4, 20, 30, and 45 h after the compounds had been added. Cells were then collected by centrifugation at 3000*g* for 10 min and cell pellets were washed three times with 0.05 M potassium phosphate buffer (pH = 6.8). Cell pellets were maintained at −10°C until the lipid analysis.

Lipid Extraction

The total lipids were extracted with chloroform–methanol (2:1, v/v) by the method of Folch *et al.* (*18*) as previously described (*19*). The cell pellets of *S. cerevisiae* and *C. albicans* were taken and homogenized with the mixture of chloroform–methanol (2:1, v/v) in a test tube with Teflon flask. Nonlipid contaminants were removed by washing with 0.88% KCl solution. The solvents were evaporated from the extracts in a rotary evaporator at 45°C under vacuum and lipid residues were dissolved in purified hexane and stored at −10°C.

Determination of Total Lipids

Total lipids were determined according to the method of Frings *et al.* (*20*). Portions (200 μl) of each solution from extracted lipids were treated with 500 μl of concentrated sulfuric acid and left in boiling water for 10 min. Then 5 ml of phosphovanilin reagent (*20*) was added and the mixture was incubated at 25°C for 20 min. After incubation the absorbency of samples was read at 540 nm against a blank. To calculate the level of lipids of cell pellets, a standard curve was prepared under the same conditions using U.S.A. grade of olive oil (Sigma, St. Louis, MO).

RESULTS

The Antimicrobial Effects of Monophosphazenes

It has been observed that the starting material containing chloride atoms was effective on the bacterial and yeast cells at all the administered concentrations. When the concentration in disc was elevated, the diameter of inhibition zone was larger than with the initial concentration ($P < 0.01$; $P < 0.001$) (Table 1). In addition, we have observed that BOMPHOS has synthesized from SM is effective bacterial and yeast cells at all the administered concentration. However, the most effective concentrations of BOMPHOS was 1000 and 1500 μg ($P < 0.01$, $P < 0.001$; Table 2). PHOMPHOS only showed antimicrobial activity on the bacterial and yeast cells in 1500 μg, but this activity lowed according to SM and BOMPHOS molecules ($P < 0.05$) (Tables 1–3).

Variation of Cells Densities in the Growth Media

In *S. cerevisiae* growth media the cell densities in culture with vitamin E, melatonin, and fish oil were lower than in control group cells after 4 h, but the BOMPHOS

TABLE 1

The Antimicrobial Effect *N*-Dichlorophosphonil-*P*-trichlora Monophosphazene Compound on the Bacterial and Yeast Cells

	100 μg	300 μg	600 μg	1000 μg	1500 μg
Bacterial strains					
Listeria monocytogenes	0.00	15.00 ± 0.57^a	18.00 ± 0.58^b	20.00 ± 0.57^b	25.00 ± 0.57^c
Bacillus megaterum	10.00 ± 0.57	12.67 ± 0.88^a	18.00 ± 1.15^b	20.00 ± 0.55^b	21.00 ± 0.56^b
Escherichia coli	0.00	10.00 ± 0.57^a	11.00 ± 0.57^a	13.00 ± 0.57^b	15.00 ± 0.55^b
Enterobacter aeroginase	8.00 ± 0.58	17.00 ± 0.58^b	18.00 ± 0.56^b	19.00 ± 0.54^b	20.00 ± 0.57^b
Bacillus brevis	9.00 ± 0.54	21.00 ± 0.57	21.67 ± 0.88^b	23.00 ± 0.59^b	23.00 ± 0.57^b
Micrococcus luteus	0.00	12.33 ± 1.20^a	15.00 ± 0.88^b	16.00 ± 0.564^b	17.00 ± 0.57^b
Psedomonos vulgaris	8.00 ± 0.57	11.33 ± 0.88^a	17.00 ± 0.56^b	18.00 ± 0.57^b	19.00 ± 0.56^b
Staphylococcus aureus	0.00	18.00 ± 0.57^b	20.00 ± 0.58^c	20.00 ± 0.58^c	21.00 ± 0.58^c
Yeast strains					
Saccharomyces cerevisiae	9.00 ± 0.58	15.00 ± 0.58^a	17.00 ± 0.57^b	19.00 ± 0.55^c	20.00 ± 0.57^c
Candida albicans	7.00 ± 0.60	10.00 ± 0.58^a	14.00 ± 0.57^b	15.33 ± 0.33^b	15.00 ± 0.58^b

^{a} $P < 0.05$.
^{b} $P < 0.01$.
^{c} $P < 0.001$.

TABLE 2

The Antimicrobial Effect 1, 1- Bis (benzyloksi) phosphoril - 2, 2, 2- tris (2 - benzyloxy) Phosphazene Compound on the Bacterial and Yeast Cells (Inhibition Zone = mm)

	100 μg	300 μg	600 μg	1000 μg	1500 μg
Bacterial strains					
Listeria monocytogenes	0.00	11.66 ± 0.33^{c}	12.66 ± 0.88^{c}	18.00 ± 0.58^{d}	19.33 ± 0.33^{d}
Bacillus megaterum	9.00 ± 0.58	12.00 ± 0.58^{b}	13.00 ± 0.57^{c}	14.00 ± 0.57^{c}	18.00 ± 0.57^{d}
Escherichia coli	11.00 ± 0.57^{a}	11.00 ± 1.15^{a}	12.33 ± 0.66^{a}	11.00 ± 0.57^{a}	10.33 ± 0.33^{a}
Enterobacter aeroginase	9.00 ± 1.15^{a}	11.00 ± 0.58^{a}	12.00 ± 0.58^{a}	12.33 ± 0.33^{a}	11.67 ± 0.38^{a}
Bacillus brevis	9.00 ± 1.73^{a}	11.00 ± 0.58^{a}	12.33 ± 0.67^{b}	14.00 ± 0.57^{c}	16.67 ± 0.83^{d}
Micrococcus luteus	0.00	11.00 ± 1.15^{b}	13.00 ± 0.00^{c}	13.67 ± 0.33^{c}	14.00 ± 0.58^{c}
Psedomonos vulgaris	9.00 ± 0.88^{a}	10.00 ± 0.58^{a}	11.33 ± 0.33^{b}	13.67 ± 0.33^{c}	16.00 ± 0.58^{d}
Staphylococcus aureus	0.00	10.33 ± 0.88^{b}	10.67 ± 0.33^{b}	11.00 ± 0.00^{c}	11.00 ± 0.58^{c}
Yeast strains					
Saccharomyces cerevisiae	10.00 ± 0.58	13.00 ± 0.58^{b}	13.00 ± 0.58^{b}	14.17 ± 0.33^{c}	18.33 ± 0.33^{d}
Candida albicans	10.00 ± 1.15^{a}	11.67 ± 0.33^{a}	12.00 ± 0.58^{b}	14.67 ± 0.33^{c}	17.67 ± 0.33^{d}

[a] $P > 0.05$.
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] $P < 0.001$.

group was higher than in control group. At the end of 30, 45 h, we found that the highest increase in culture occurred with vitamin E and BOMPHOS (Fig. 2)

In the growth media of *C. albicans* examined after 4 h, the densities of cells in

TABLE 3

The Antimicrobial Effect 1, 1 - Bis (phurphuryloxy) fosforil - 2, 2, 2 - tris (phurphuryl) Phosphazene Compound on the Bacterial and Yeast Cells (Inhibition Zone = mm)

	100 μg	300 μg	600 μg	1000 μg	1500 μg
Bacterial strains					
Listeria monocytogenes	0.00	0.00	0.00	0.00	9.00 ± 0.57^{a}
Bacillus megaterum	0.00	0.00	0.00	0.00	9.67 ± 0.33^{a}
Escherichia coli	0.00	0.00	0.00	0.00	9.67 ± 0.33^{a}
Enterobacter aeroginase	0.00	0.00	0.00	10.67 ± 0.33^{a}	12.00 ± 0.58^{b}
Bacillus brevis	0.00	0.00	0.00	0.00	0.00
Micrococcus luteus	0.00	0.00	0.00	0.00	9.67 ± 0.33^{a}
Psedomonos vulgaris	0.00	0.00	0.00	0.00	9.00 ± 0.33^{a}
Staphylococcus aureus	0.00	0.00	80.00	0.00	0.00
Yeast strains					
Saccharomyces cerevisiae	0.00	0.00	0.00	0.00	12.00 ± 0.58^{b}
Candida albicans	0.00	0.00	0.00	9.33 ± 0.33^{a}	10.33 ± 0.33^{b}

[a] $P < 0.01$.
[b] $P < 0.001$.