The Annals of the University Dunarea de Jos of Galati
Fascicle VI – Food Technology 35(2) 66-74

ORIGINAL RESEARCH PAPER

STUDIES ON ANTIMICROBIAL ACTIVITIES OF SOME NEWBIS-PYRIDINIUM DERIVATIVES

BIANCA FURDUI[1*], OANA CONSTANTIN[2], RODICA DINICĂ[1], GABRIELA BAHRIM[2]
[1] *Dunărea de Jos University of Galaţi, Faculty of Science, 47, Domnească Street, 800008, Galaţi, Romania*
[2]*Dunărea de Jos University of Galaţi, Faculty of Food and Science Engeneering, 47, Domnească Street, 800008, Galaţi, Romania*
**Corresponding author: bfurdui@ugal.ro*

Received 17 August 2011
Revised 13 September 2011

The antimicrobial activity against food spoilage microorganisms of some new symmetrical diquaternary salts of 1, 2-bis(4-pyridinium)-ethane obtained from the reaction of 1,2-bis-(4-pyridil)-ethane with reactive halides, was comparatively investigated. Some of these compounds were found to exhibit inhibitory and antimicrobial activity against several bacteria, yeasts and fungus, in function of structure and doses. The inhibitory effect against yeasts and moulds was very low, comparatively with the antibacterial activity against both Gram-positive and Gram-negative bacteria. Very interesting are the spectacular bactericide effects (D_{IZ}>64mm) against *Bacillus* and *Pseudomonas* tested strains, of N,N' – diphenacyl - 1,2 – bis - (4 - pyridinium) ethane dibromide and N,N'-di (4,5 – dibromo – 2 – hydroxyl -propiophenacyl) - 1,2 – bis - (4 - pyridinium) - ethane dibromide.

Keywords: quaternary ammonium salts, pyridinium quaternary salts, antimicrobial activity

Introduction

The alarming increase of bacterial resistance to many antibacterial agents has limited the use of commercial disinfectants and imposed the necessity of a permanent identification of new compounds with microbiostatic and microbicide effect.

Quaternary ammonium salts (QAC) are one of the most used classes of disinfectants with a large applicability in hospital environments, water treatment, textile, paint and food industries due to their relatively low toxicity to human and animals and to their broad specificity of antimicrobial activity (Block, 2001). Among them, pyridinium and bispyridinium quaternary salts represent an important group of chemicals widely used as biocides (Chanawanno *et al.*, 2010;

Kourai. *et al.*, 2006), drugs (Hu *et al.*, 2009; Fujimoto *et al.*, 2006) and herbicides (Summers, 1980; Ukai *et al.*, 1989) due to their strong antimicrobial effect even at very small concentrations, on a broad range of gram-positive and gram-negative bacteria (i.e.. *Bacillus subtilis*, *Sarcina lutea*, *Streptococcus pneumoniae, Staphylococcus aureus, Micrococcus luteus, Escherichia coli* etc.) and on some moulds species (*Aspergillus niger*, *Aspergillus glaucus*, *Geotrichum candidum*, *Fusarium graminearum* etc.) and yeasts (*Saccharomyces cerevisiae*, *Rhodoturula glutinis* etc.) (Perles *et al*., 2008; Ng *et al*., 2007; Zetterberg *et al*., 1970). In regard to recent results on the low cytotoxic effect of some antimicrobial agents from bis-quaternary pyridinium salts group on human cells (Nagamune *et al.*, 2000) we may expect that pyridinium and bispyridinium salts are potential agents recommended for use in composition of hygiene products, in food industry (technological places, equipments, surfaces), and in catering units, hospitals, etc.

Past studies include the development of nitrogen quaternary salts in particular derived from 4,4'-bipyridil with antimicrobial properties (Dinica *et al*., 2008; Furdui *et al.*, 2007).

In order to find new and more efficient antimicrobial agents with possible applicability in food industry disinfection processes, we have investigated the antimicrobial potential of some new symmetrical diquaternary salts derived from 1, 2-bis-(4-pyridinium)-ethane against food spoilage microorganisms.

Materials and methods

Chemical compounds tested

The antimicrobial potential of ten new symmetrical diquaternary salts derived from 1,2-bis-(4-pyridinium)-ethane (**1-10)**, obtained from the reaction of 1,2-bis-(4-pyridil)-ethane with reactive halides (Furdui B. et al., 2011) was investigated. The structures of investigated compounds are presented in Figure 1 and their names are:

1. *N,N'-diphenacyl-1,2-bis-(4-pyridinium)ethane dibromide*
2. *N,N'-di(p-bromophenacyl)-1,2-bis-(4-pyridinium)ethane dibromide*
3. *N,N'-di(m-metoxyphenacyl)-1,2-bis-(4-pyridinium)ethane dibromide*
4. *N,N'-di(p-nitrophenacyl)-1,2-bis-(4-pyridinium)ethane dibromide*
5. *N,N'-di(3,4-dihydroxyphenacyl)-1,2-bis-(4-pyridinium)ethane dichloride*
6. *N,N'-di(5-bromo-2-hydroxy-propiophenacyl)-1,2-bis-(4-pyridinium)ethane dibromide*
7. *N,N'-di(4,5-dibromo-2-hydroxy-propiophenacyl)-1,2-bis-(4-pyridinium) ethane dibromide*
8. *N,N'-di(carbomethoxy-methyl)-1,2-bis-(4-pyridinium)ethane dibromide*
9. *N,N'-di(carboethoxy-methyl)-1,2-bis-(4-pyridinium)ethane dibromide*
10. *N,N'- di(4'-nitrofuran)-1,2-bis-(4-pyridinium)ethane dibromide*

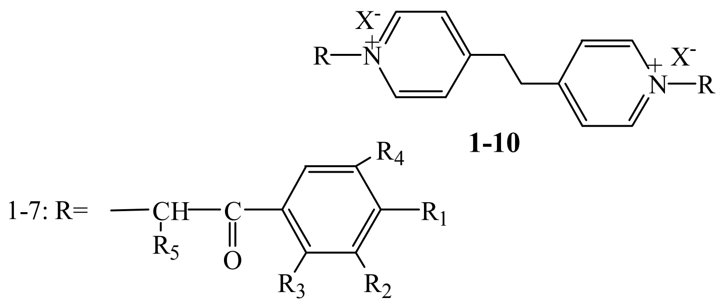
Strains and media

The evaluation of the inhibitory potential of the investigated compounds was made on ten microorganism strains:

- bacteria: Gram-positive bacteria (*Bacillus subtilis* ATCC 19659, *Bacillus cereus* ATCC 10876 and *Sarcina lutea*), Gram-negative bacterium (*Pseudomonas fluorescens* ATCC 13525);
- yeasts (*Rhodotorula glutinis*, *Candida mycoderma* and *Saccharomyces cerevisiae*);
- moulds (*Aspergillus niger*, *Geotrichum candidum* and *Fusarium graminearum*).

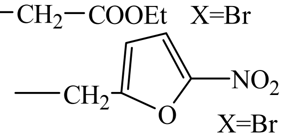
The ATCC bacterial strains were purchased from the American Type Culture Collection. All wild bacterial and fungal strains were isolated from food spoilage microbiota.

The cultures of test microorganisms were maintained in medium agar slants at 4°C and used as stock cultures. Bacterial strains were grown at 37°C in PCA medium (casein peptone 5.0 g/l, yeast extract 2.5 g/l, dextrose 1.0 g/l, agar 15.0 g/l, pH 7.2) and the final concentration used for antimicrobial activity was 106 CFU/ml. Yeasts were grown at X°C in MEA medium (maltose 12.75 g/l, peptone 0.78 g/l, dextrin 2.75 g/l, agar 15.00 g/l, glycerol 2.35 g/l, pH 4.7) and the final concentration of 10^6 CFU/mL was obtained by viable counts serial dilutions. Spore suspension was obtained from the stock culture in a final concentration of 10^5-10^6 spores/ml).



1: R_1=H, R_2=H, R_3= H, R_4 =H, R_5=H, X=Br
2: R_1=Br, R_2=H, R_3=H, R_4 =H, R_5=H, X=Br
3: R_1=H, R_2=OCH_3, R_3=H, R_4 =H, R_5=H, X=Br
4: R_1=NO_2, R_2=H, R_3=H, R_4 =H, R_5=H, X=Br
5: R_1=OH, R_2=OH, R_4 =H, R_5=H, X=Cl
6: R_1=H, R_2=H, R_3=OH, R_4 =Br, R_5=CH_3, X=Br
7: R_1=Br, R_2=H, R_3=OH, R_4 =Br, R_5=CH_3, X=Br

8: R = $-CH_2-COOMe$ X=Br
9: R = $-CH_2-COOEt$ X=Br
10: R =



X=Br

Figure 1. Structure of tested diquaternary salts

Antimicrobial activity assay

In this study, two methods were used to determine the antimicrobial activity, as following:

a. Agar diffusion test

The qualitative evaluation of the inhibitory potential of the investigated compounds was tested by the agar diffusion test. The diffusion method involves inoculation of high cells concentrations of the test microorganisms on specific agar culture medium, at 42°C, in sterile Petri's plates. After homogenization and solidification of the medium, on the surface plate, sterile filter paper discs (Φ= 19 mm) were placed, coated (equal time, 10 minutes) with aqueous solutions (5 mg/mL) of the tested chemicals. The blank was a disc soaked in distilled water. The plates were incubated at optimal conditions for test culture growth (at 37°C, 48 hour for bacteria, and at 25°C, for 3-5 days, for yeasts and moulds). The evaluation of microbiostatic or microbicide effects of the studied chemical compounds was made by measuring at every 24 hours, the inhibition zone diameter (D_{IZ}, mm) and by checking some morphological characteristics of colonies, i.e. pigmentation, sporulation intensity.

A product is considered active if the difference between the inhibition zones of this product and that of the blank is at least of 2 mm.

Considering the diameter of the tests paper discs (19 mm), the following classification of the chemical compounds activity was proposed, depending on the dimensions of the inhibition zones:

- with low inhibitory effect, $D_{iz} \leq 20$ mm;
- with medium inhibitory effect, D_{iz}= 20-50 mm;
- with strong inhibitory effect, $D_{iz} \geq 50$ mm.

b. Minimum inhibitory concentration

The quantitative effect of the substances with the higher inhibitory potential of cells growth was determined *in vitro,* by bacterial cultivation in stationary conditions, in liquid nutrient broth medium, on the basis of the minimum inhibitory concentration (MIC) values. The most sensible microorganisms were used for quantitative evaluation. Serial dilutions (5, 2.5, 1, 0.5 and 0.25 mg chemical compound /ml medium) were prepared from stock solutions (5 mg/ml); 0.5 ml of each standardized bacterial suspension was added to an equal volume of each chemical compound dilution (excluding the sterility control). After incubation for 24 h ± 1 h at 37°C, the turbidity of the cultures associated with the veil or derma formation at the medium's surface was visually assessed. The microorganism's growth was monitored by optical density determination (OD_{600}). The tests were performed simultaneously on negative controls (only medium), growth controls (medium + test microorganism) and sterility controls (medium + chemical compounds). The lowest concentration of antimicrobial agent, that inhibits the development of visible growth after 24 h of incubation at 37°C, was taken as the minimum inhibitory concentration.

All the experiments were performed in triplicate.

Table 1. The qualitative antibacterial and antifungal effect of ten diquaternary salts derived from 1,2-bis(4-pyridil)-ethane (Sorces: 1 - American Type Culture Collection; 2 - Food Spoilage Microbiota

Microorganisms	Source	Tested compounds/D_{IZ}, mm										
		H_2O	1	2	3	4	5	6	7	8	9	10
Bacillus subtilis	1	0	24.33 ±0.57	0	33.66 ±0.57	34.66 ±1.52	22.33 ±0.57	38±1	30±1	0	25.33 ±0.57	34.66 ±1.52
Bacillus cereus	1	0	0`	25.66 ±0.57	31.33 ±1.15	0	30.33 ±0.57	41.33 ±0.57	64.66 ±0.57	0	0	45.33 ±0.57
Sarcina lutea	2	0	0	34.66 ±0.57	0	0	29.33 ±0.57	0	0	31.66±0 .57	0	25.66 ±0.57
Pseudomonas fluorescens	1	0	69.66 ±0.57	27.33 ±0.57	0	0	0	38.66 ±0.57	69.66 ±1.52	0	0	25.33 ±0.57
Rhodotorula glutinis	2	0	0	0	24.66 ±0.47	0	0	0	0	0	0	0
Candida mycoderma	2	0	0	0	0	0	0	0	0	0	0	0
Saccharomyces cerevisiae	2	0	0	0	0	0	0	0	0	0	0	0
Aspergillus niger	2	0	0	0	0	0	0	0	0	0	0	0
Geotrichum candidum	2	0	20.66 ±0.57	0	0	0	0	0	0	0	0	0
Fusarium graminearum	2	0	0	0	0	0	0	0	0	0	0	0

Results and discussions

The results obtained through the diffusion method, after 24 h of cultivation, are presented in Table 1.

The antimicrobial activity results showed that these new bis-pyridinium derivates exhibited interesting antibacterial activities; all compounds being active against at least one of the tested strains, with medium or strong inhibitory effect. The compounds **1** and **3** present the largest antimicrobial inhibition spectrum.

The tested compounds are more effective against bacteria than against yeasts or moulds. They proved to be active both on Gram-positive and Gram-negative bacteria, very interesting being the spectacular bactericide effect against *Bacillus* and *Pseudomonas* tested strains, of some compounds (**1** and **7**). The compound **10** is the only compound with a medium inhibitory effect against all tested bacteria strains.

The inhibitory effect on the yeasts and moulds is very low, the salt **3** being the only compound that partially inhibits the growth of *Rhodotorula glutinis* (Figure 2), while the salt **1** is the only one that inhibit one mould strain *Geotrichum candidum*. All the tested compounds are completely inactive against *Candida mycoderma*, *Saccharomyces cerevisiae*, *Aspergillus niger* and *Fusarium graminearum* strains.

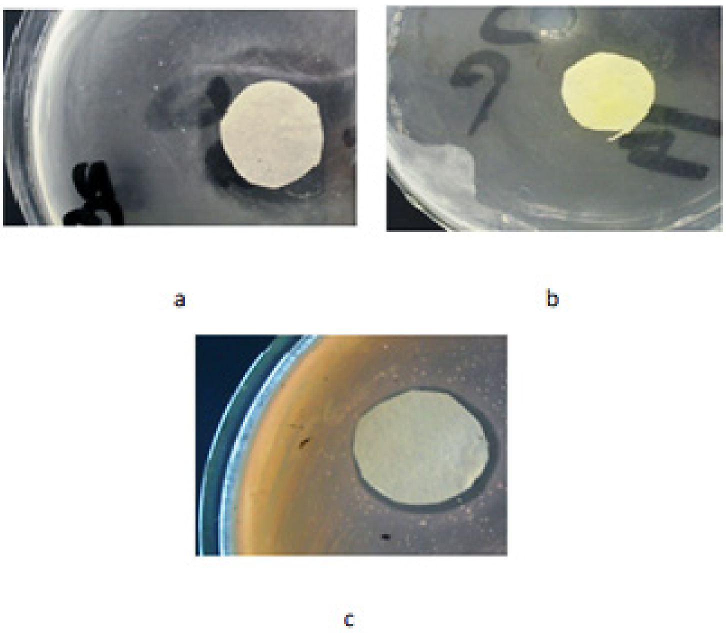


Figure 2. Antimicrobial effect of some bis-pyridinium derivatives, after 48 h of cultivation on solid agar media; a) compound coded **3** against *Bacillus subtilis*; b) compound coded **7** against *Bacillus cereus*; c) compound coded **3** against *Rhodotorula glutinis*

Regarding the results obtained by diffusion method, the quantitative evaluation tests were performed only on the bacterial strains and using the most active

compounds for each individual strain. The cultures' growth was evaluated for the bacterial strains by the increasing of the turbidity associated with a veil formation at the culture surface for the *Bacillus* and *Pseudomonas* strains and respectively with a sediment formation for *Sarcina lutea*. The turbidity was spectrophotometrically evaluated by measuring the optical density of the cultures comparatively with a sterility sample (medium+chemical compound).
The obtained results show that the highest activity of the tested diquaternary salts is against *Bacillus subtilis* strain. Its growth is completely inhibited by the diquaternary salts coded **3** and **4**, at all the tested doses, while the other tested salts are less active, with MIC of 0.5 mg/ml (salt **10**) or 1 mg/ml (salts **6** and **7**). Against the other bacterial strain, the activity of the tested compounds is lower (MIC≥2.5mg/ml) (Table 2).

Table 2. MIC values for of some 1,2-bis(4-pyridinium)-ethane diquaternary salts

Bacterial strains Microorganisms	Tested compound	MIC, mg/ml
Bacillus subtilis	3	0.25
	4	0.25
	6	1.00
	7	1.00
	10	0.50
Bacillus cereus	3	5.00
	5	5.00
	6	2.50
	7	2.50
	10	5.00
Pseudomonas fluorescens	1	2.50
	6	2.50
	7	2.50
Sarcina lutea	2	2.50
	8	2.50

* MIC – the lowest concentration of compound at which the microorganism tested does not show visible growth

Conclusions

The present study shows that the newly synthesized symmetrical diquaternary salts of 1,2-bis-(4-pyridinium)-ethane present interest as antimicrobial agents in order to produce desifectants or detergents. The inhibitory effect against yeasts and moulds is very low, comparatively with the antibacterial activity against both Gram-positive and Gram-negative bacteria.
Regarding the relations structure-activity, we can observe that the antimicrobial activity is quasi-general and not at all influenced by the nature of the R substitutes. The substitute's nature and the position in the phenyl ring do not have a decisive

influence on the biological activity, influencing mostly the selectivity. The biological activity of the tested compounds could be explained by their ionic structure, being influenced by their water solubility too. The difference between the observed antimicrobial activities is probably due to their molecular hydrophobicity, absorbability, and the electron density of the ammonium nitrogen atom.

Acknowledgments

This work has benefited from financial support through the 2010 POSDRU/89/1.5/S/52432 project, ORGANIZING THE NATIONAL INTEREST POSTDOCTORAL SCHOOL OF APPLIED BIOTECHNOLOGIES WITH IMPACT ON ROMANIAN BIOECONOMY, project co-financed by the European Social Fund through the Sectorial Operational Programme Human Resources Development 2007-2013.

References

Block, S.S. 2001. *Disinfection, Sterilization, and Preservation*, fifth ed. Lippincott, Williams & Wilkins, Philadelphia, U.S.A.

Chanawanno, K., Chantrapromma, S., Anantapong, T., Kanjana-Opas, A., Fun, H.-K. 2010. Synthesis, structure and in vitro antibacterial activities of new hybrid disinfectants quaternary ammonium compounds: Pyridinium and quinolinium stilbene benzenesulfonates. *European Journal of Medicinal Chemistry*, 45, 4199-4208.

Dinica, R., Furdui, B., Bahrim, G., Demeunynck, M. 2008. Precursseurs de Nouveaux Heterocycles avec interet biologiques. *Revue Roumaine de Chimie*; 53(1), 21-24.

Furdui, B., Bahrim, G., Dinica, R., Druta, I., Demeunynck, M. 2007. In Vitro Antimicrobial Activity of New Nitrogen Heterocycles, *Roumanian Biotechnological Letters*, 12 (1), 3073-3078.

Kourai, H., Yabuhara, T., Shirai, A., Maeda, T., Nagamune, H. 2006. Syntheses and antimicrobial activities of a series of new bis-quaternary ammonium compounds. *European Journal of Medicinal Chemistry,* 41 (4), 437-444.

Hu, D., Li, X., Sreenivasan, P. K., DeVizio W. 2009. A randomized, double-blind clinical study to asses the antimicrobial effects of a cetylpyridinium chloride mouth rinse on dental plaque bacteria. *Clinical Therapeutics*, 31 (11), 2540-2548.

Fujimoto, K., Morisaki, D., Yoshida, M., Namba, T., Hye-Sook, K., Wataya, Y., Kurai, H., Kakuta, H., Sasaki, K. 2006. Antimalarial effect of bis-pyridinium salts, N,N'-hexamethylenbis(4-carbamoyl-1-alkylpyridinium bromide). *Bioorganic & Medicinal Chemistry Letters*, 16 (10), 2758-2760.

Nagamune, H., Maeda, T., Ohkura, K., Yamamoto, K., Nakajima, M., Kourai, H. 2000. Evaluation of cytotoxic effects of bis-quaternary ammonium antimicrobial reagents on human cells. *Toxicology in Vitro*, 14 (2), 139-147.

Ng, C. L.L., Singhal, V., Widmer F., Wright, L. C., Sorell, T. C., Jolliffe, K. A. 2007. Synthesis, antifungal and haemolytic activity of a series of bis(pyridinium)alkanes. *Bioorganic & Medicinal Chemistry*, 15 (10), 3422-3429.

Summers, L.A. 1980. *The bipyridinium herbicides*, Academic Press, London, 189.

Ukai, S., Nagai, K., Tsuchyia, T., Kikata, T. 1989. Safenend Herbicides containing bipyridinium salts and saccaride sulfates. Jpn. Kokai Tokkyo Koho JP 01228904.12.09.1989. *Chem. Abstr*., 112, 134522.

Perles, C.E., Matheus, A., Volpe, P.L.O. 2008. The effect of quaternary ammonium surfactants on the inhibition of the aerobic metabolism of Sacharomyce cerevisiae – A calorimetric study. *Thermochimica Acta*, 479 (1-2), 28-31.

Zetterberg, G., Person, T. 1970. Effects of paraquat on *Escherichia* coli and yeast. *Estrato Bolletino Chim. Farmac*., 109 (12), 728-732.