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Original article:

SYNTHESIS AND ANTIBACTERIAL ACTIVITY OF A NEW DERIVATIVE OF THE MELDRUN ACID: 2,2-DIMETHYL-5-(4H-1,2,4-TRIAZOL-4-YLAMINOMETHYLENE)-1,3-DIOXANE-4,6-DIONE ($C_9H_{10}N_4O_4$)

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ABSTRACT

The discovery of new substances with proven antimicrobial activity is the current study goal of various researchers. Usage of synthetic products has grown considerably in the past few years due to processing agility, and capability of going through previous chemical modifications in order to enhance its biological activity. Widespread careless use of antimicrobials has made the number of resistant microorganisms rise significantly, thus demanding more efficient drugs to fight them. One of these synthetic candidates for this purpose is the substance 2,2-Dimethyl-5-(4H-1,2,4-triazol-4-ylaminomethylene)-1,3-dioxane-4,6-dione ($C_9H_{10}N_4O_4$), aminomethylene derivative from Meldrum's acid. This substance, alone and in association with common antibiotics, were evaluated *in vitro* for antimicrobial activity, and had their minimum inhibitory concentration (MIC) towards *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 10536 and *Pseudomonas aeruginosa* ATCC 15442, as well as multiresistant strains *Escherichia coli* 27, *Staphylococcus aureus* 358 and *Pseudomonas aeruginosa* 03 determined. The antimicrobial modulation action tests of the aminoglycosides with $C_9H_{10}N_4O_4$ were performed according to the microdilution method, and resulted in observation of a positive synergic effect.

Keywords: synthetic products, antimicrobial activity, resistant microorganisms, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, aminoglycosides

INTRODUCTION

Staphylococcus aureus is a spherical bacterium from the Gram-positive cocci group, also characterized as being a facultative anaerobe pathogen. This microorganism is easily found on the skin and mucous membranes of human beings, as well as in other mammals and birds. It can cause diseases that range from simple infections (pustules, furuncles and cellulitis) to more serious ones

(pneumonia, meningitis, endocarditis, toxic shock syndrome, septicemia, etc.) (Santos et al., 2007). It is an important etiological agent associated with acquired infections in the community and in hospitals, which became a paradigm of bacterial infections. Considered as one of the main human pathogens, it is notable for its high frequency and pathogenicity, which leads to diseases both in immunocompromised individuals and in healthy ones, and also for the easy intra-hospital dissemination associated to resistance to antibiotics (Coutinho et al., 2008, 2009).

The appearance of multiresistant *S. aureus* strains in the last few years, isolated even from hospital materials and equipments, constitutes a major problem to infection control and antimicrobial therapeutics (Araújo et al., 2000). Multiresistance in *S. aureus* results from the presence of plasmids which most frequently allows for multiresistance transfer, among other factors (Freitas, 2003).

Escherichia coli takes the form of a bacillus and belongs to the Enterobacteriaceae family, being aerobe and facultative anaerobe (Murray, 2004). It normally exists in animals (including humans) intestinal tract, and exert a benefic effect on the organism, suppressing proliferation of harmful bacteria and synthesizing a considerable portion of vitamins. Among *E. coli* strains, however, there is a group which is capable of causing diseases in humans, that is collectively called enteropathogenic *E. coli* (EEC) (Silva et al., 2003).

Pathogenic lineages of *E. coli* have been evidenced as a primary cause of urinary tract infections, neonatal meningitis, hospital-acquired septicemia, and enteritis in humans. Resistance to at least two different antibiotics classes is very common, which restricts the available therapeutic options (Schneider et al., 2009).

Pseudomonas aeruginosa is characterized by a Gram-negative rod, infecting a wide variety of plant and animal hosts (Wu et al., 2005). In humans, it is responsible for chronic lung infections affecting immunocompromised patients and also patients with cystic fibrosis (Lyczak et al., 2000). *P. aeruginosa* is the third main cause of nosocomial infections (Bonomo and Szabo, 2006).

Widespread and careless use of antibiotics has led to a series of problems, among them, human ecology unbalance and microbial resistance, demanding a search for more efficient drugs to combat them. The development of any new antimicrobial drug is accompanied by microorganisms resistance, and their emergence is a threat to research advances (Lyczak et al., 2000). Due to the large raise of pathogenic microorganisms resistance to multiple drugs, there is a concern on finding new therapeutic alternatives (Oliveira et al., 2007). For patients, antimicrobial resistance augments morbidity, while for health institutions it corresponds to higher costs (Dancer, 2001).

According to Montanari (1995), molecular modification is the most used method to obtain new drugs. It consists, basically, in starting with a well characterized molecule, with known biological action, as a model or prototype, from which new compounds will be synthesized. The final product will be structurally similar, homolog, or analog, to the starting substance. Thus, the pharmaceutical industry is constantly developing research on the correlation of molecular structure and pharmacological activity of new synthetic products (Cechinel Filho and Yunes, 1998).

The synthesis of isolated pure substances has become a tool of great importance to the development of drugs with enhanced pharmacological properties, and nowadays the use of molecular modeling contributes markedly to the discovery of new drugs, which are safer and more efficient. Many heterocyclic derivatives of Meldrum acid (2,2-dimethyl-1,3-dioxane-4,6-dione) have been prepared and studied from the synthetic and structural point of view (Chen, 1991), but, however, its biological properties have not received the same attention.

In order to fill this gap, in this study we evaluate the antibacterial and the enhancement of antibiotic effect of 2,2-dimethyl-5-

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(4H-1,2,4-triazol-4-ylaminomethylene)-1,3-dioxane-4,6-dione ($C_9H_{10}N_4O_4$) against standard and multiresistant (clinical origin) strains of *E. coli, S. aureus* and *P. aeruginosa.*

MATERIAL AND METHODS

Synthesis of compound

The search for a study to demonstrate the biological activity with 5-aminomethylene Meldrum´s acid derivatives led to a patent in 1965 (Sterling Drug, 1966). In addition, different heterocyclic rings were condensed to the 5-methoxymethylene Meldrum's acid in order to evaluate their potential biological activity as antileishmanicidal, antitrypanosomal and antiviral (Silva, 2006). In this paper, we choose to synthesize the of compound 2,2-Dimethyl-5-(4H-1,2,4-triazol-4-ylaminomethylene)-1,3-dioxane-4,6-dione, $C_9H_{10}N_4O_4$, an aminomethylene derivative from Meldrum's acid in order to explore it biological application. The aminomethylene derivative can be obtained by condensation of an arylamine with 5-(metoximethylene)-2,2-dimethyl-1,3-dioxane-4,6-dione generated *in situ* from a reaction with trimethyl orthoformate (Cassis et al., 1985). A solution of Meldrum acid (36 mmol) in thimethyl orthoformate (50 mL) was heated under reflux for 2 h, then the corresponding arylamine (30 mmol) was added. Reflux was kept for additional 30 minutes. The material thus formed was filtered and washed with methanol. The synthesis can be described by the scheme shown in Figure 1. The product of this synthesis was the compound 2,2-dimethyl-5-(4H-1,2,4-triazol-4-ylaminomethylene)-1,3-dioxane-4,6-dione, $C_9H_{10}N_4O_4$, a crystalline solid with melting point between 170 °C and 172 °C (Joussef et al., 2005).

Bacterial material

Standard and multiresistant lineages of *E. coli* (*E. coli* ATCC 10536 and *E. coli* 27), *S. aureus* (*S. aureus* ATCC 25923 and *S. aureus* 358) and (*P. aeruginosa* ATCC 15442 and *P. aeruginosa* 03) were assayed; the resistance profile is shown in Table 1. All lineages were kept in Hear Infusion Agar (HIA, Difco Laboratories Inc.). Before the essays were carried out, lineages were cultivated at 37 °C during 18 h in Brain Hear Infusion broth (BHI, DIfco Laboratories Inc.).

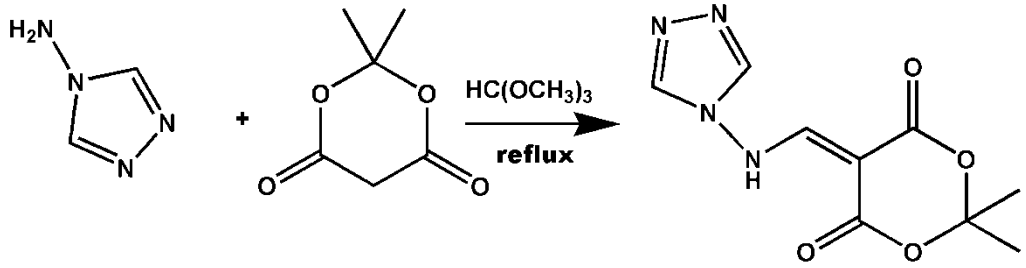

Figure 1: Synthesis of the compound 2,2-Dimethyl-5-(4H-1,2,4-triazol-4-ylaminomethylene)-1,3-dioxane-4,6-dione, $C_9H_{10}N_4O_4$

Table 1: Resistance profile of microorganisms

Microorganism	Origin	Resistance Profile
Escherichia coli 27	Surgical wound	Ast; Ax; Amp; Ami; Amox; Ca; Can; Caz; Cip; Cfc; Cf; Clo; Im; Szt; Tet; Tob
Staphylococcus aureus 358	Surgical wound	Ami; But; Can; Gen; Neo; Net Oxa; Para; Sis; Tob;
Pseudomonas aeruginosa 03	Urine culture	Com; Ctz; Im; Cip; Ptz; Lev; Mer; Ami

Ast – Aztreonam; Ax – Amoxicillin; Amp – Ampicillin; Ami – Amikacin; Amox – Amoxicillin; But – Butirosina; Ca – Cefadroxil; Can – Kanamycin; Caz – Ceftazidime; Cip – Ciprofloxacin; Cfc – Cefaclor; Cf – Cephalothin; Clo – Chloramphenicol; Gen – Gentamicin; Im – Imipenem; Neo – Neomycin; Oxa – Oxacillin; Para – Paramomicina; Sis – Sisomicin; Szt – Sulfamethoxazole and Trimethoprim; Tet – Trimethoprim; Tob – Tobramycin; Com: Cefepime; Ctz: ceftazidima; Ptz: piperacilina-tazobactam; Lev: levofloxacin; Mer: meropenen

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Antimicrobial activity assay

The initial solution was prepared by dissolving 0,01 g of the synthetic compound $C_9H_{10}N_4O_4$ in 1000 mL of DMSO (Merck), to result in a stock solution with concentration 10 mg/mL. This was diluted to 1024 µg/mL, and successive 1:2 dilutions with distilled water yielded concentrations from 512 to 0.5 µg/mL. The compound $C_9H_{10}N_4O_4$ was dissolved using dymethilsulfoxide (DMSO - Merck, Darmstadt, Alemanha) using the following proportions: 0.01 g of compound dissolved in 1 mL of DMSO, obtaining a solution with 10 mg/mL. This solution was diluted to 1024 µg/mL and sequentially diluted with sterile water in a range varying from 512 to 0.5 µg/mL. The microorganisms employed in this study were provided by Instituto Nacional de Controle de Qualidade em Saúde (INCQS) from Fundação Oswaldo Cruz, Ministério da Saúde, Brasil. The MIC of the synthetic compound $C_9H_{10}N_4O_4$ was determined by the microdilution in broth method, using concentrations ranging from 512 to 8 µg/mL. Previously standardized bacterial suspensions were diluted in 1:10 proportion with BHI broth to yield a final concentration of 10^5 cells/mL (NCCLS, 2003). This method uses small volumes of medium (BHI 10 %) and sample ($C_9H_{10}N_4O_4$) distributed in cavities of sterile microplates. Sample was prepared in doubled concentration (1024 µg/mL) in relation to the defined initial concentration (512 µg/mL) with 100 µL volume, and then diluted 1:1 serially in BHI 10 %. Using 100 µL of culture medium per cavity, bacterial suspension was diluted at 1:10 proportion. Negative, positive (medium + microorganism), and inhibition controls were used with the culture medium, in concentrations ranging from 512 to 8 µg/mL. The filled plates were incubated at 35 °C (±2 °C) for 24 h (Javadpour et al., 1996). A sodium resazurine indicator solution was prepared with sterile distilled water (0.01 % w/v) in order to determine the MIC of solutions toward standard bacterial lineages. After incubation, 20 µL of the indicator solution was added to each cavity, and 1 hour later, under room temperature, results were read. A color change from blue to pink is due to resazurine reduction and indicates that bacterial growth has occurred (Palomino et al., 2002). The MIC can thus be verified, which is defined as the smallest drug concentration capable of inhibiting bacterial growth, evidenced by the unaltered blue color. The assessment of $C_9H_{10}N_4O_4$ as a possible modulator of antibiotic action was done by determining the MIC of antibiotics from the aminoglycoside group (amikacin, gentamicin e neomycin), with and without that substance, in sterile microplates. Aminoglycosides concentrations ranged from 2500 to 2.5 µg/ml. The $C_9H_{10}N_4O_4$ solution was added to BHI 10 % broth with subinhibitory concentrations, obtained after the MIC assessment, whereas for the modulation test, an 8 fold concentration reduction (MIC/8) was employed. Antibiotics solutions were prepared in doubled concentration (5000 µg/ml), relative to the initial one, with sterile distilled water. In each cavity of the sterile microplate there were 100 µl of the culture medium and the diluted (1:10) bacterial suspension. The controls used for MIC assessment of $C_9H_{10}N_4O_4$ were the same as the ones used for modulation tests (Sato et al., 2004). The filled microplates were incubated at 35 °C (±2 °C) for 24 h, then reading was performed using the resazurine indicator solution, as previously described for the MIC assessment.

RESULTS AND DISCUSSION

Antimicrobials association is still studied due to the possibility of suppressing the appearance of resistant mutants, and producing an *in vivo* synergic effect. An attempt to maintain current antimicrobials active could be successful by combining them with other substances, presenting a therapeutic option to treat infections by *S. aureus* and other pathogens, regarding the growing cases of multiple resistance (Maia et al., 2009).

The MIC assessment test using the substance $C_9H_{10}N_4O_4$ indicated that the MIC is higher than 1024 µg/ml. According with

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Houghton et al. (2007), this result indicates that the compound does not present a clinically relevant antibiotic activity.

In the modulation of antibiotic-activity test with aminoglycosides it was observed that, in relation to *Escherichia coli* 27, $C_9H_{10}N_4O_4$ presented a synergism with amikacin, reducing the inhibitory concentration from 19.5 to 4.9 µg/mL; whereas no noticeable effect was observed with gentamicin and neomycin. In relation to *Staphylococcus aureus* 358, $C_9H_{10}N_4O_4$ presented synergism with amikacin, gentamicin and neomycin, reducing the inhibitory concentration from 19.5 to 2.4 µg/mL, 9.8 to 2.4 µg/mL and 312.5 to 78.125 µg/ml, respectively. With respect to *Pseudomonas aeruginosa* 03, $C_9H_{10}N_4O_4$ showed synergy only with gentamicin, decreasing the inhibitory concentration from 156.2 to 39.1 µg/mL (Table 2). These results express the same mechanisms of action described by Bolla et al. (2011), that reports that these synergism is due the interaction with the bacterial cell membrane or with the lipopolysacharide barrier (LPS), enhancing the antibiotic influx, increasing by this way the intracellular concentration, or inhibiting the antibiotic efflux due the activity as a blocker of efflux pump (BEP).

Table 2: MIC values (µg/mL) of aminoglycosides in the absence and presence of the compound $C_9H_{10}N_4O_4$ in multiresistant strains

	$C_9H_{10}N_4O_4$ + antibiotic	**antibiotic alone**
***Escherichia coli* 27**		
Amikacin	4.9 (S)	19.5 (I)
Gentamicin	4.9 (R)	4.9 (R)
Neomycin	1250 (R)	1250 (R)
***Staphylococcus aureus* 358**		
Amikacin	2.4 (S)	19.5 (I)
Gentamicin	2.4 (S)	9.8 (R)
Neomycin	78.1 (R)	312.5 (R)
Pseudomonas aeruginosa 03		
Amikacin	78.1 (R)	78.1 (R)
Gentamicin	39.1 (R)	156.2 (R)
Neomycin	1250 (R)	1250 (R)

MIC – Minimum Inhibitory Concentration; S – Sensitivity; I – intermediary resistance; R – Resistance.

Combination of antibiotics with laboratory synthesized isolated substances could serve as a therapeutic option for the treatment of infections caused by *S. aureus*, *E. coli* , *P. aeruginosa* and other pathogens, as well as an important approach to diminish multiple resistance.

Several compounds, as natural or synthetic, had demonstrated the capacity to modulate the antibiotic activity. Phenotiazines, in a general form, are capable to inhibit the bacterial efflux systems, enhancing the antibiotic activity. This activity was demonstrated by Chlorpromazine and thioridazine against several multiresistant bacteria (Amaral et al., 2010). As the phenotiazines, natural products can enhance the antibiotic activity inhibiting the bacterial efflux system, but also affecting the fluidity of the plasmatic membrane. Natural products as flavonoids (quercetin, isoquercetin, Rutin) (Veras et al., 2011; Araruna et al., 2012), alkaloids (Pilocarpine) (Araruna et al., 2012), terpenes (α–bisabolol) (Santos et al., 2011) had demonstrated this activity due the cited mechanisms. Both natural or synthetic compounds, modifying the antibiotic activity, can modify also the phenotype of the bacterial strains, reversing the resistant and intermediary phenotype to the sensitivity phenotype, as demonstrated by the compound $C_9H_{10}N_4O_4$ against the *E. coli, S. aureus* and *P. aeruginosa* strains.

CONCLUSIONS

According to the results obtained, it can be seen that the organic compound studied in this paper presents a modulator effect on antimicrobial activity, fulfilling expectations and providing a possible alternative in future therapeutics. In particular, it was shown that $C_9H_{10}N_4O_4$ presents antibiotic activity against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

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