https://doi.org/10.31925/farmacia.2018.4.15 **ORIGINAL ARTICLE**

SCREENING OF HETEROCYCLIC SUBSTITUTED SYDNONES FOR POTENTIAL BIOLOGICAL ACTIVITY

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Abstract

Nowadays, the emergence of bacterial resistance to antibiotics has become a major public health concern; therefore the development of new compounds overcoming the bacterial resistance is essential. Thus, this study evaluates the *in vitro* antimicrobial activity of some recently synthesized sydnone derivatives, on Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*), Gram-negative (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*) bacteria and fungi (*Candida albicans*, *Aspergillus niger*) and estimates the influence of sub-inhibitory concentrations of these newly synthesized compounds on the expression of some virulence factors by bacterial strains and on adhesion ability to inert substrates.

Rezumat

În prezent, apariția rezistenței bacteriene la antibiotice a devenit o problemă majoră de sănătate publică, prin urmare dezvoltarea de antibiotice noi este esențială. Acest studiu evaluează activitatea antimicrobiană *in vitro* a unor sydnone sintetizate recent, folosind bacterii Gram-pozitive (*Staphylococcus aureus*, *Bacillus subtilis*) şi Gram-negative (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*), dar şi fungi (*Candida albicans*, *Aspergillus niger*) şi determină influenţa concentraţiilor subinhibitoare a acestor compuşi nou sintetizaţi asupra exprimării unor factori de virulenţă la tulpinile bacteriene şi asupra aderenţei bacteriene.

Keywords: sydnone derivatives, antimicrobial activity, virulence factors expression, inhibition of microbial adherence

Introduction

Sydnones, heterocyclic compounds with mesoionic structure, have been synthesized the first time in 1935 [12]. Compounds belonging to this class have been studied in the laboratories of the Centre of Organic Chemistry since 1965 [17], their structure and properties being of continuous interest, fact reflected in a number of published papers, some of them being mentioned here [9-11]. The sydnones are interesting because these compounds revealed a variety of biological activities like: antimicrobial, antifungal, antihelmintic, antiinflamatory or analgesic [1]. Recently a number of heterocyclic substituted sydnones (**1-6**) (as presented in Table I) have been prepared and their structure was confirmed by spectral analyses (IR, UV-Vis, NMR and X-ray) [2, 3]. In the view of finding new compounds having anti-bacterial or antifungic activity, this paper presents the evaluation of the effects of these recently synthesized sydnones on bacterial and fungal strains.

Materials and Methods

The structures of the sydnone derivatives **1-6** [2] are presented in Table I. Stock solutions of 1 mg/mL, prepared by dissolving the test compounds **1-6** in sterile dimethyl sulphoxide (DMSO), were stored at 4°C.

The antimicrobial properties of these compounds were tested against reference strains from the "Cantacuzino" Institute Collection (IC), Bucharest, Romania: *Staphylococcus aureus* (*S. aureus*) IC 13204, *Bacillus subtilis* (*B. subtilis*) IC 12488, *Escherichia coli* (*E. coli*) IC 13529, *Klebsiella pneumoniae* (*K. pneumoniae*) IC 13420, *Pseudomonas aeruginosa* (*P. aeruginosa*) IC 13202, *Candida albicans* (*C. albicans*) IC 249, *Aspergillus niger* (*A. niger*) IC 13534 and, also, against bacteria strains recently isolated from clinical infections: *P. aeruginosa* 211349, *P. aeruginosa*, *E. coli* 211602, *E. coli* 211850, *K. pneumoniae* 211790, *K. pneumoniae* 210703, *K. pneumoniae* 63. The bacterial strains were identified with VITEK 2 System Version 07.01. The VITEK cards for identification

were inoculated and incubated as previously described [15].

The microbial inocula were prepared in sterile saline solution from 18 - 24 h microbial cultures (4 - 5 isolated colonies) developed on solid media and adjusted by nephelometry to a standard density 0.5 McFarland (corresponding to 1.5 x 10^8 CFU/mL for bacteria and 5 x 10^6 CFU/mL for fungi). For *A. niger* a sporal suspension with 0.5 McFarland density was used [5, 6].

The *qualitative screening* of the antimicrobial properties for compounds **1-6** was performed by a procedure adapted from Kirby-Bauer diffusion method. Petri dishes with nutrient media: Mueller Hinton agar, for bacterial strains and Yeast Extract–Peptone–Glycerol (YPG) for fungal strains, were seeded with bacterial/fungal inocula of 0.5 McFarland optical densities, then 10 µL of the compounds **1-6** solutions were added in spots. The Petri dishes were left at room temperature to allow a drop of solution to be adsorbed in the medium and afterwards they were incubated at 37ºC during 24 h for bacteria and 48 h for fungi. The DMSO solvent was also tested to evaluate its potential antimicrobial activity. The bactericidal/fungicidal effect was observed by the occurrence of a growth inhibition zone around the spots. The results were expressed as diameters of the inhibition zones generated by the tested compounds [5].

Quantitative assays, for establishing the minimal inhibitory concentration (MIC), were performed by the binary microdilution method, in 96-well plates. Binary serial dilutions of the stock solutions were prepared in 150 µL medium, after which the wells were seeded with 10 µL of microbial suspension of 0.5 McFarland density values, 1:10 diluted. The plates were incubated at 37ºC for 24 h for bacteria and 48 h for fungi. For each test, a microbial culture control and a sterility control were performed. DMSO was also tested by binary serial dilution to evaluate its potential antimicrobial activity [8]. The reference substances used were: ciprofloxacin (CIP) a fluoro-quinolone antibiotic for bacterial strains and fluconazole (FLU) a triazole derivative for fungal strains, each as a 128 µg/mL solution. MICs were read by the wells observation. The MICs values were confirmed by absorbance measurement at 620 nm [5, 6].

For *investigating the influence of the tested compounds on the expression of different virulence factors*, bacterial strains were cultivated in the presence of sub-inhibitory concentrations of compounds **1-6**. Then, 10 µL of each bacterial culture were inoculated in spots on specific media, simultaneously with control samples, to determine the expression of the virulence factors [7]. *β-Haemolysin* was evidenced by spotting strains on blood agar plates containing 5% (v/v) sheep blood. After incubation at 37°C for 24 h, the clear areas (total lysis of the red blood cells) around the colonies point out a positive reaction. For the *caseinase* activity, the strains were spotted on 10% soluble casein agar as substrate. After incubation at 37°C for 24 h, a precipitation zone surrounding the bacteria growth indicated the casein production. For the *lecithinase* production, the cultures were spotted into 8% eggs yolk agar and were incubated at 37°C for 7 days. An opaque (precipitation) zone around the spot indicates the *lecithinase* production. For the investigation of *lipase* production, the strains were spotted on Tween 80 (oleic acid ester) with agar as a substrate, at a final concentration of 0.5% and the plates were incubated at 37°C for 7 days. An opaque (precipitation) zone around the spot was registered as a positive reaction [4, 13].

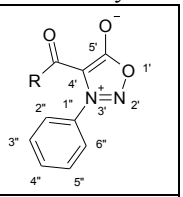
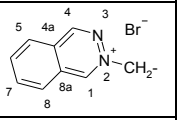
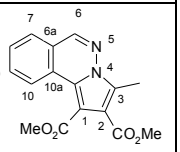
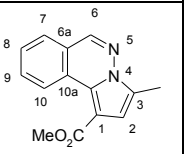
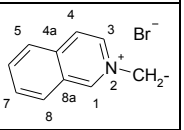
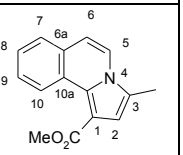
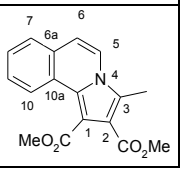
To study *the susceptibility of compounds* **1-6** *to inhibit biofilm formation*, an experimental model was developed. Thus, after the MIC assay, the liquid cultures from the 96-well plates were removed and the wells were washed three times with phosphate buffer saline (PBS), fixed with methanol for 5 minutes, stained with 1% violet crystal solution for 30 minutes, washed with distilled water and dissolved in 33% acetic acid, and thereafter, the adhesion ability to the abiotic surface was determined by reading the coloured suspension density to an Elisa reader at 490 nm [16].

Results and Discussion

Six recently synthesized compounds with a sydnone moiety [2] have been tested, *in vitro*, against pathogenic bacteria and fungi. Table I contains the structures and some spectral data of these compounds.

Table I

The chemical structure of the tested sydnones

General formula	
Compound	***R***
2-[2-(3-Phenylsydnon-4-yl)-2-oxoethyl] phthalazinium bromide (**1**), brown solid, m. p. 231 - 234°C.	
Dimethyl 3-[(3-phenyl sydnon-4-yl)-oxomethyl]-pyrrolo[2,1-a]phthlazine-1,2 dicarboxylate (**2**), yellow solid, m. p. 214 - 215ºC.	
Methyl 3-[(3-phenyl sydnon-4-yl)-oxomethyl] -pyrrolo[2,1-a]phthlazin-1-carboxylate (**3**), yellow solid, m. p. 244 - 246ºC.	
2-[2-(3-Phenylsydnon-4-yl)-2-oxoethyl] isoquinolinium bromide (**4**), yellow solid, m. p. 245 - 246ºC.	
Methyl 3-[(3-phenylsydnon-4-yl)-oxomethyl]-pyrrolo[2,1-a]isoquinoline-1-carboxylate (**5**), yellow solid, m. p. 227 - 228ºC.	
Dimethyl 3-[(3-phenylsydnon-4-yl)-oxomethyl]-pyrrolo[2,1-a]isoquinoline-1,2-dicarboxylate (**6**), yellow solid, m. p. 171 - 173ºC.	

The results obtained from the *qualitative* tests showed an antimicrobial activity of compound **4** on both Gram-positive bacteria species (d = 8 - 10 mm), and Gram-negative bacteria species (d = 5 - 6 mm). For the other compounds there is no evidence of inhibition zones around the spots (d ~ 0 mm). The DMSO has no effect on the checked microorganisms.

The results of the *quantitative* assay of the antimicrobial activity against reference strains showed that compound **4** exhibited the highest activity against *E. coli* (MIC of 62.5 µg/mL), similar action against *P. aeruginosa*, *K. pneumonia*, *S. aureus* and *A. niger* (MIC of 125 µg/mL) and lower inhibitory effect on *B. subtilis* and *C. albicans* (MIC of 250 - 500 µg/mL) (Table II). In the case of clinically isolated Gram-negative strains, an antimicrobial activity was observed with MIC = 250 µg/mL, only on the *E. coli* strains (*E. coli* 211602 and *E. coli* 211850).

Table II

The results of the antimicrobial activity (MIC values in µg/mL)

	MIC (µg/mL)							
Microbial strains	**1**	**2**	**3**	**4**	**5**	**6**	CIP	FLU
P. aeruginosa IC 13202	500	500	500	125	> 500	> 500	0.25	-
E. coli IC 13529	500	500	> 500	62,5	> 500	> 500	< 0.125	-
K. pneumoniae IC 13420	500	500	> 500	125	> 500	> 500	< 0.125	-
S. aureus IC 13204	500	500	> 500	125	> 500	> 500	0.25	-
B. subtilis IC 12488	> 500	> 500	> 500	500	> 500	> 500	0.25	-
C. albicans IC 249	> 500	> 500	> 500	250	> 500	> 500	-	15.62
A. niger IC 13534	500	125	500	125	500	500	-	15.62

According to the experimental results, compounds **1-6** have no influence on the secretion of soluble bacterial virulence factors (*β-haemolysin*, *caseinase*, *lecithinase*, *lipase*) implicated in the infectious process progression.

The notion of adherence in biology is an important concept, because fixation mechanisms represent the stage preceding the onset of an infectious process. The bacterial adhesion at the host tissues or the prosthetic medical devices, determinate morphological and behavioural changes in bacterial cells, leading to the development of biofilms conferring high resistance to the antibiotic therapy [14].

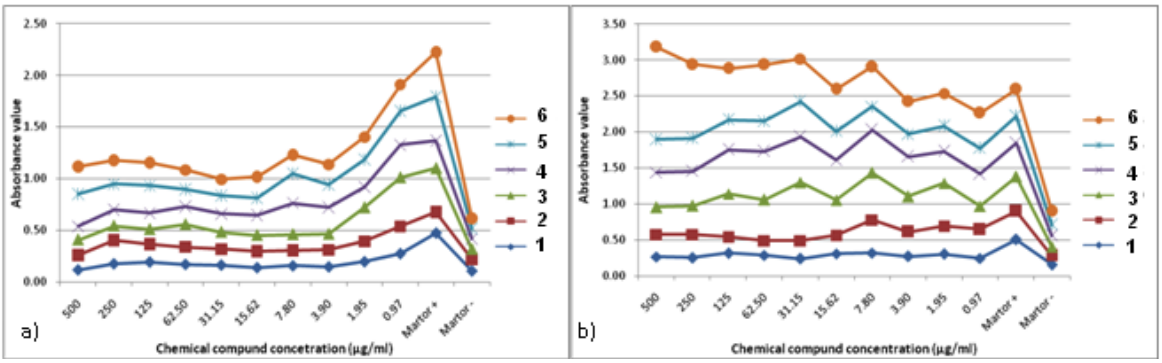


Figure 1.

The influence of sub-inhibitory concentrations of compounds **1-6** upon bacterial adherence on an inert substrate: a) *P. aeruginosa* IC 13202; b) *B. subtilis* IC 12488

It was observed that the sub-inhibitory concentration of almost all compounds tested caused an inhibition of adhesion ability to inert substrates of the Gram-negative reference bacterial strains. Exceptions are compounds **5** and **6**, which caused a slight stimulation of adherence to the inert substrate for *E. coli* IC 13529. It could be hypothetically explained by the stimulation of the bacterial *adhesins* expression, causing colonization and biofilm formation. In the case of Gram-positive bacteria significant changes were not observed in the adherence ability to the inert substrate, except for the compound **6** which determined stimulation of the adherence to the inert substrate of *B. subtilis* IC 12488. For clinically isolated strains it was noticed that compound **4** caused an inhibition of adhesion ability to the inert substrate (Figure 1).

Conclusions

According to the experimental results, the isoquinoline sydnone derivative **4** shows a potential antimicrobial activity. Starting from this compound, further structure-activity relationship and molecular docking studies have to be performed for designing and synthesizing new molecules with better antimicrobial activity and higher efficiency in bacterial biofilm destruction.

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