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Preliminary communication

Antimicrobial activity of pyrimidinophanes with thiocytosine and uracil moieties

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ABSTRACT

Reactions of pyrimidinophanes with two 6-methylthiocytosine and one 5(6)-alkyluracil moieties bridged with each other by polymethylene spacers with methyl or nonyl *p*-toluenesulfonate, *p*-toluenesulfonic acid, methanesulfonate and trifluorosulfonate afforded amphiphilic macrocyclic bis-*p*-toluene-, methane- and trifluorosulfonates. Despite the presence of several reaction centers in the initial pyrimidinophane molecules, protonation and methylation occurred only at the N^1 atom (with quaternization) of the 6-methylthiocytosine moieties. The bacteriostatic and fungistatic activity of the products was estimated. Macrocyclic tosylates exhibit a remarkable selectivity towards *Staphylococcus aureus*, with MIC values comparable with a reference drug. Bacteriostatic activity of the amphiphilic pyrimidinophanes depends on the size of the macrocycles, and the highest activity corresponds to definite lengths of polymethylene bridges. Besides, the antimicrobial activity of the screened pyrimidine derivatives depends on their topology. While macrocyclic tosylates are more active against bacteria than against fungi, acyclic tosylate with the same structural fragments shows a dramatical decrease of MIC towards mold and yeast with respect to the corresponding macrocycle. It is found that macrocyclic and acyclic tosylates in high dilutions decrease the extracellular lipase activity.

1. Introduction

Due to the rapid growth of drug resistant strains of pathogenic bacteria, the search for new antibacterial agents is of utmost importance. At present there is no doubt that the existing arsenal of antimicrobial agents we have in hand for the treatment of infectious diseases is insufficient to protect us over a long term [1]. As a consequence, new efforts to develop new antibacterial agents are extremely necessary, and there is an upsurge of interest in developing new antibiotics based on novel chemical scaffolds.

It is generally accepted that the introduction of novel antibiotic classes would contribute to the combat of the ever lasting problem of bacterial resistance.

There is a real necessity for the discovery of new compounds endowed with antimicrobial activity, possibly acting through mechanisms of action, which are distinct from those of well-known classes of antibacterial agents to which many clinically relevant pathogens are now resistant. It should be mentioned that among the new agents under development, only a few of the compounds namely the oxazolidinone, cationic peptide and lipopeptide antibiotics can be truly regarded as novel mechanism agents [2].

In our opinion, the macrocyclic structure can be considered as a promising chemical scaffold for new antibiotics. Macrocycles are assumed to be the compounds that can possess novel mechanisms of action. In recent years there have been numerous reports describing synthesis and biological activity, in particular, antimicrobial activity of macrocycles. In most cases, synthetic azalides [3,4] and macrocyclic oligopeptides or pseudopeptides [5–7] with strong antibacterial and antifungal activity were reported. The macrocyclic scaffold of the compounds can be conjugated with specific pharmacophores, such as quinolones [3], ureas or thioureas [4], and hydroxamates [7] which emphasize their activity according to definite cultures. Other types of antimicrobial macrocycles are known as well, e.g. macrocyclis Schiff bases [8], cyclophanes [9,10], porphirines [11], macrocyclic pyridines [12] and triazines [13].

Recently, antimicrobial macrocycles with pyrimidine fragments, namely uracil rings have been reported [14,15]. These macrocycles, called pyrimidinophanes consist of two 6-methyluracil moieties [14] or 6-methyluracil and 5-decyl-6-methyluracil moieties [15] bridging each other with $-(CH_2)_nN(Et)(CH_2)_m$-chains. The nitrogen atoms in bridges were quaternized with RBr (R is benzyl or alkyl radical), and the antimicrobial activity of the obtained pyrimidinophanes was tested. As a background for the study it is assumed that pyrimidinophanes with nucleotide base units, particularly, uracil units, when attached to a fragment exhibiting

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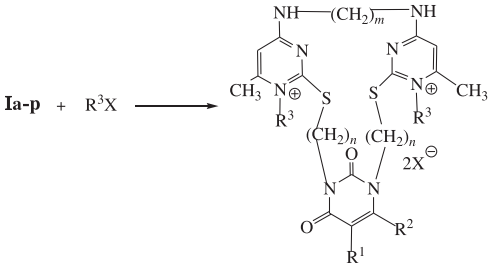
a specific activity with respect to a certain biotarget, would increase the specifity of the molecule relative to the target, provided that the macrocycle is complementary to a biotarget especially to an enzyme. Besides, the pyrimidinophane framework itself can be considered as a specific fragment capable of interfering into the principal biochemical pathways (cell wall synthesis, DNA and RNA synthesis, protein formation). The idea was to combine the pyrimidinophane framework with ammonium groups which display nonspecific antimicrobial activity through interaction with cell walls and cell membranes of microorganisms in order to obtain high antibacterial and antifungal activity. It was found that the macrocycles with two uracil units and quaternary ammonium groups with lipophilic substituents in polymethylene bridges exhibited significant bacteriostatic, fungistatic, bactericidal, and fungicidal activity comparable with standard antibacterial and antifungal drugs [14,15].

The chemical modification of nucleic acid fragments offers a continuous challenge for the organic chemists in search of compounds, in particular, macrocyclic compounds, namely pyrimidinophanes with antiviral and antibacterial activity. Herein new antimicrobial pyrimidinophanes are reported. These macrocycles differ from the previously reported pyrimidinophanes as follows: (i) instead of two 6-methyluracil or 5-decyl-6-methyluracil units in a macrocyclic framework there are three pyrimidine moieties, namely one 5(6)-alkylsubstituted uracil and two 6-methylthiocytosine (6-methyl-2-thio-4-aminopyrimidine) fragments linked to each other with polymethylene bridges, (ii) quaternized N atoms are introduced not in the bridging chains but in the pyrimidine moieties proper. As a result, pyrimidinophanes of this type are novel biologically active macrocyclic compounds and especially novel antimicrobial agents.

2. Chemistry

We previously reported on the synthesis, structure and some properties of macrocycles incorporating two thiocytosine derivatives (4-amino-6-methyl-2-thiopyrimidinyl moieties) and one 6-methyluracil fragment [16–18]. In particular, the reaction of disodium salts **2** with dibromides **3** in DMF produced pyrimidinophanes **1** (Scheme 1). Some of pyrimidinophanes **1**, namely macrocycles **1a**–**c**, **f**–**j** were described elsewhere [16,17]. In the present work new pyrimidinophanes **1d**, **e**, **k**–**p**, in which the substituents at C^5 and $C^6$of the uracil ring and the lengths of polymetylene chains $-(CH_2)_{n,m}-$ vary, were obtained in the same way



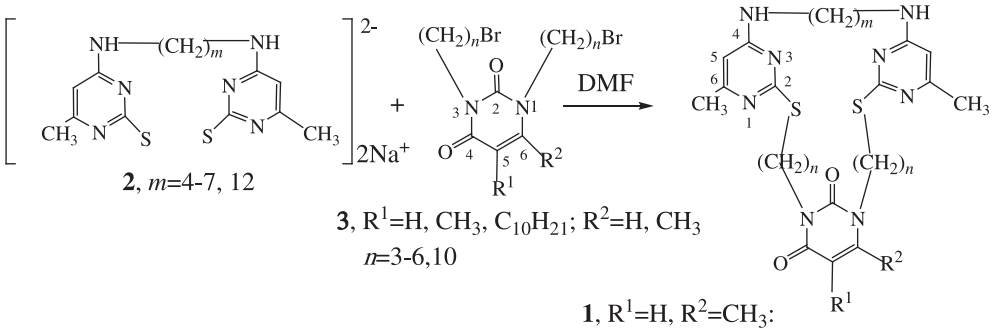
4, X=$[SO_3C_6H_4CH_3]^-$, R^1=H, R^2=CH_3, R^3=CH_3:
m=*n*=4 (**a**); *m*=5: *n*=5 (**b**) 6 (**c**), 10 (**d**);
m=6: *n*=3 (**e**), 4 (**f**), 5 (**g**), 6 (**h**), 10 (**i**);
m=7: *n*=5 (**j**), 10 (**k**); *m*=12: *n*=6 (**l**), 10 (**m**);
R^1=CH_3, R^2=H, R^3=CH_3: *m*=6, *n*=5 (**n**);
R^1=n-$C_{10}H_{21}$, R^2=CH_3, R^3=CH_3: *m*=6, *n*=5 (**o**), 6 (**p**);
R^1=H, R^2=CH_3, R^3=C_9H_{19}: *m*=6, *n*=3 (**q**); *m*=5, *n*=10 (**r**);
R^1=R^3=H, R^2=CH_3: *m*=6, *n*=5 (**s**);
X=$[SO_3CH_3]^-$, R^1=H, R^2=CH_3: *m*=5, *n*=6 (**t**);
X=$[SO_3CF_3]^-$, R^1=H, R^2=CH_3: *m*=5, *n*=6 (**u**)

Scheme 2. Synthesis of amphiphilic pyrimidinophanes **4** by alkylating and protonating of the N atom of the pyrimidine ring.

by introducing into the reaction the corresponding disodium salt **2** and dibromide **3**.

Most of pyrimidinophanes **1** are insoluble in water and poorly soluble in other polar solvents, such as DMSO. The quaternizing of N atoms into the pyrimidine rings or amino-substituents at the pyrimidine rings of the pyrimidinophanes **1** was aimed at screening of antimicrobial activity. On the one hand the quaternization of N atoms can provide solubility in appropriate solvents and on the other hand a nonspecific antimicrobial activity.

Quaternization of N atoms of the pyrimidinophanes with inorganic acid or alkyl halides was attempted. However, our attempts to obtain the corresponding hydrochlorides by heating pyrimidinophanes **1** in boiling hydrochloric acid or by treatment with alkyl bromides were unsuccessful: only the initial macrocycle was isolated from the reaction mixture. Amphiphilic macrocyclic bis-*p*-toluenesulfonates **4a**–**u** have been synthesized by the reaction of corresponding pyrimidinophane **1** with methyl or nonyl *p*-toluenesulfonate (tosylate) or *p*-toluenesulfonic acid (Scheme 2). The reactions were carried out in ester which served as a solvent. The yields of macrocyclic tosylates **4** are almost quantitative.



2, *m*=4-7, 12

3, R^1=H, CH_3, $C_{10}H_{21}$; R^2=H, CH_3
n=3-6,10

1, R^1=H, R^2=CH_3:
m=*n*=4 (**a**); *m*=5, *n*=5 (**b**) 6 (**c**), 10 (**d**);
m=6, *n*=3 (**e**), 4 (**f**), 5 (**g**), 6 (**h**), 10 (**i**);
m=7, *n*=5 (**j**), 10 (**k**); *m*=12, *n*=6 (**l**), 10 (**m**);
R^1=CH_3, R^2=H: *m*=6, *n*=5 (**n**);
R^1=n-$C_{10}H_{21}$, R^2=CH_3: *m*=6, *n*=5 (**o**),6 (**p**)

Scheme 1. Synthesis of pyrimidinophanes **1** with different substituents at uracil moiety and lengths of bridges.

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Theoretically, alkylation and protonation could involve both the *endo*-N^1 and N^3 atoms in the pyrimidine rings of the thiocytosine moiety and, the *exo*-N and S atoms in the bridging fragments. Earlier it was shown that thiocytosine derivatives, in particular 4-alkylamino-6-methyl-2-methylsulfanylpyrimidines, were methylated with methyl tosylate and protonated with trifluoroacetic acid exclusively at the N^1 atom of the pyrimidine ring to give the corresponding quaternary salts [19].

Unfortunately, in the case of macrocyclic salts **4** the situation is much more ambiguous. Overlapping and broadening of signals in ^{1}H NMR spectra complicate their univocal assignment and there is no straightforward identification of key proton signals that could be used to elucidate the structure of the macrocycles. However, considerable downfield shifts of the C^5H proton signals and signals from the C^4-$NHCH_2$ protons in the ^{1}H NMR spectra of **4** as compared to the corresponding signals in the spectra of the initial pyrimidinophanes **1**, and the presence of two NCH_3 singlets with the chemical shift values similar to the chemical shifts values of the $N^1_{pyr}CH_3$ groups for simple methylated thiopyrimidines [19] allow us to assume that methylation occurred at the N^1 atoms of the pyrimidine rings. This assumption was confirmed by a variety of NMR correlation experiments. In particular, by measurements of self-diffusion coefficients of pyrimidinophanes **4d**, **h** it was shown that two NCH_3 groups belong to one molecule because of the same self-diffusion coefficients. Besides, tosylate-anions are strongly complexed with the macrocycles, and in solution the tosylate-anions and the cationic pyrimidinophanes come in pairs. Finally, the binding of the methyl groups with the N^1 atoms of the pyrimidine rings was directly shown by a variety of 2D NMR correlation methods in the way as described in detail for model systems [19]. Unambiguous assignment was carried out by ^{1}H-^{13}C HMBC and, in particular ^{1}H-^{15}N HMBC experiments.

In addition to tosylates mesylate **4t** and triflate **4u** have been prepared by the reaction of pyrimidinophane **1c** with the methyl ester of methanesulfonic acid and the methyl ester of trifluoromethylsulfonic acid, respectively (Scheme 2).

For antimicrobial screening acyclic tosylates **8**–**10** (Fig. 1) were obtained by methylating compounds **5**–**7** with methyl tosylate. Pyrimidine **5**, bispyrimidine **6** and trispyrimidine **7** simulate building fragments of the pyrimidinophanes **1** and in particular pyrimidinophanes with $m = 6$ and $n = 6$. It is of interest to compare the antimicrobial properties of tosylates **9** and **10** with their macrocyclic counterparts and to elucidate the influence of compound topology on biological activity.

The physical and spectral data of new pyrimidinophanes, acyclic pyrimidines and their tosylates are presented in Supplementary material, and the selected data are in the experimental protocol.

3. Results and discussion

The in vitro antibacterial and antifungal activity of the pyrimidinophanes **1** and **4**, and acyclic pyrimidines **8**–**10** was investigated against several pathogenic representative Gram-negative bacteria (*Pseudomonas aeruginosa* 9027 and *Escherichia coli* F-50), Gram-positive bacteria (*Staphylococcus aureus* 209p, *Bacillus subtilis* 6633, *Bacillus cereus* 8035 *and Enterococcus faecalis* ATCC 8043), molds (*Aspergillus niger* BKMF-1119, *Trichophyton mentagrophytes var. gypseum* 1773) and yeast (*Candida albicans* 885-653). Pyrimidinophanes **1** are inactive against all the bacteria and fungi studied (MICs ≥500 μg/mL). However, macrocyclic salts **4** exhibit bacteriostatic and fungistatic activity, and the results are presented in Table 1. As shown in Table 1 *P. aeruginosa* and *A. niger* are in different to salts **4**, neither is the growth of *E. faecalis* and *C. albicans* (these results are not included in Table 1).

Some of macrocycles **4** exhibit significant bacteriostatic activity against gram-positive bacteria *S. aureus*, *B. subtilis* and *B. cereus* (but not against *E. faecalis*). The minimal inhibitory concentrations (MICs) of pyrimidinophanes **4a**–**u** against *S. aureus* range widely from 0.2 to 500 μg/mL, and the minimal MICs are comparable with the corresponding values for the fluoroquinolone derivative Ciprofloxacin as a standard. An interesting structure–activity profile is observed considering the following structural parameters: (1) length of polymethylene chains *m* and *n*, (2) substituent at the C^5 of uracil ring, (3) substituent at ammonium center and (4) nature of anion. The influence of the fragments on the activity of the pyrimidinophanes **4** against gram-positive bacteria is worth discussing.

The influence of the number of methylene units *m* and *n* in bridges on the activity of the macrocyclic ditosylates is evident though not simple. Fig. 2 illustrates the dependence MICs of pyrimidinophanes with the 6-methyluracil moiety, the methyl at pyrimidine ring N and tosylate as anion (R^1 = H, $R^2 = R^3 = CH_3$, X = $[SO_3C_6H_4CH_3]^-$) towards *S. aureus* on the length of three polymethylene chains. Herein the following ranges of methylene units are used: *m* vary from 4 to 12 and *n* vary from 3 to 10. In

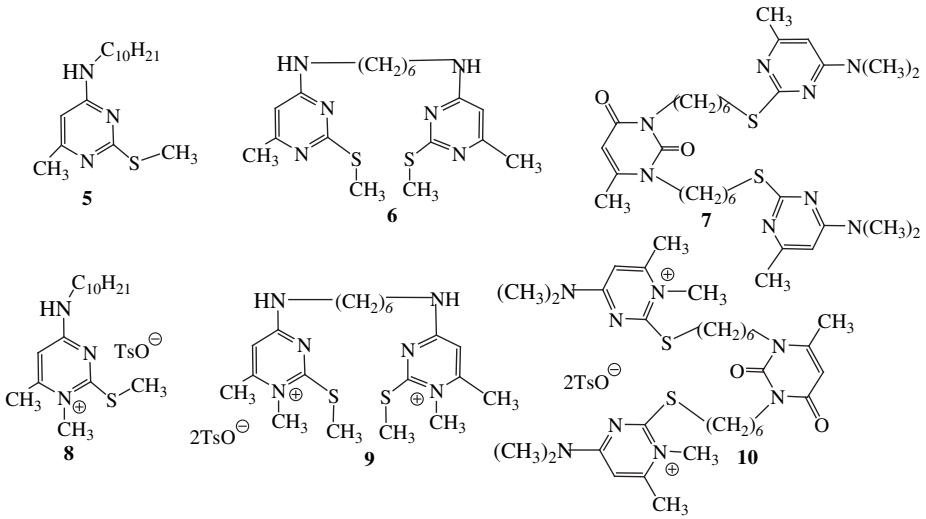

Fig. 1. Structures of the model compounds and their tosylates simulating building blocks of the pyrimidinophanes **1**.

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Table 1
In vitro antibacterial and antifungal activity of pyrimidinophanes with different *n* and *m*, substituents at uracil ring and anion X.[a]

Minimal inhibitory concentration (MICs), μg/mL													
No	R^1	R^2	R^3	X	*m*	*n*	*Sa*	*Ec*	*Pa*	*Bc*	*Bs*	*An*	*Tm*
4a	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	4	4	125	500	–	250	125	–	500
4b	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	5	5	7.8	250	–	12.5	6.3	–	125
4c	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	5	6	7.8	250	–	15.6	7.8	–	125
4t	H	CH_3	CH_3	$[SO_3CH_3]^-$	5	6	15.6	–	–	125	n.d.	–	–
4u	H	CH_3	CH_3	$[SO_3CF_3]^-$	5	6	7.8	–	–	62.5	n.d.	–	–
4d	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	5	10	0.2	15.6	–	7.8	2.0	–	31.3
4r	H	CH_3	C_9H_{19}	$[SO_3C_6H_4CH_3]^-$	5	10	–	–	–	–	–	–	–
4e	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	6	3	250	–	–	–	156	–	500
4q	H	CH_3	C_9H_{19}	$[SO_3C_6H_4CH_3]^-$	6	3	7.8	250	–	15.6	7.8	–	125
4f	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	6	4	78	250	–	–	78	–	250
4g	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	6	5	6.3	250	–	125	78	–	62.3
4n	CH_3	H	CH_3	$[SO_3C_6H_4CH_3]^-$	6	5	7.8	–	–	250	n.d.	–	–
4o	$C_{10}H_{21}$	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	6	5	0.2	31.3	–	15.6	n.d.	–	31.3
4s	H	CH_3	H	$[SO_3C_6H_4CH_3]^-$	6	5	–	–	–	–	–	–	–
4p	$C_{10}H_{21}$	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	6	6	6.3	250	–	12.5	3.1	–	62.3
4h	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	6	6	3.1	250	–	6.3	3.1	–	62.3
4i	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	6	10	2.0	250	–	3.1	1.6	–	31.3
4j	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	7	5	15.6	–	–	125	78	–	62.3
4k	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	7	10	0.2	15.6	–	7.8	2.0	500	31.3
4l	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	12	6	2.0	62.3	–	12.5	3.1	–	62.3
4m	H	CH_3	CH_3	$[SO_3C_6H_4CH_3]^-$	12	10	22.5	–	–	25.0	22.5	–	–
Clotrimazole													3.1
Ciprofloxacin							0.25	0.5	0.1	0.25	0.25		
Amphotericin B												20	

n.d., Not done; -, no activity was observed (MIC ≥500 μg/mL); *Pa*, *Pseudomonas aeruginosa*; *Ec*, *Escherichia coli*; *Sa*, *Staphylococcus aureus*; *Ba*, *Bacillus subtilis*; *Bc*, *Bacillus cereus*; *An*, *Aspergillus niger*; *Tm*, *Trichophyton mentagrophytes*.

[a] The tests were performed in duplicate and repeated twice.

principle, the bacteriostatic activity of pyrimidinophanes increases with the rise in the *m* and *n* values of methylene units in the bridging fragments. The lowest activity is observed for macrocycles **4a**, **e**, **f** with minimal *m* and *n* (*m* = 4–6 and *n* = 3–6) and the highest activity (MIC against *S. aureus* is 0.2 μg/mL) is provided by pyrimidinophanes **4d** and **4k** with non-maximum *m* but medium values of *m* (*m* = 5, 7) and the maximum value of *n* (*n* = 10). It is interesting that the MIC of pyrimidinophane **4i** with *m* = 6 and *n* = 10 against *S.s aureus* is 10-fold greater than the MICs of **4d** and **4k**. In our opinion, the selectivity towards *S. aureus* and the dependence of MICs values on the size of pyrimidinopanes **4a**–**m** are an evidence of the specific mode of action of the macrocycles.

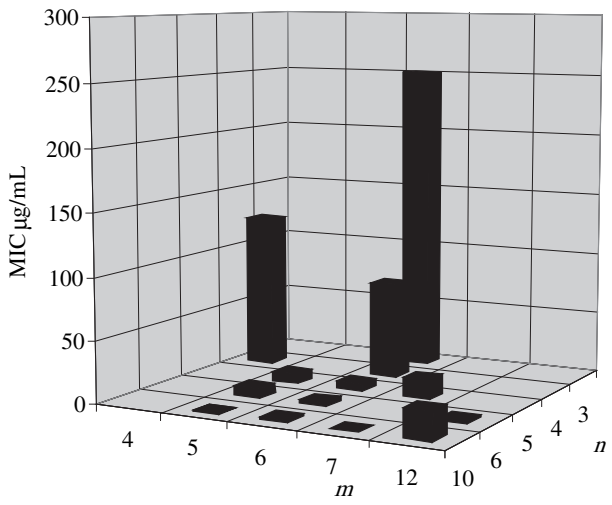

Fig. 2. Dependence of activity of pyrimidinophanes **4a**–**m** (R^1 = H, R^2 = R^3 = CH_3, X = $[SO_3C_6H_4CH_3]^-$) against *Staphylococcus aureus* on methylene units number *m* and *n* in bridging chains.

The same trend occurs towards other gram-positive bacteria *B. subtilis* and *B. cereus* and gram-negative bacteria *E. coli*. While the pyrimidinophanes **4d**, **k**, **i** with *n* = 10 have the lowest MICs values of 3.1–7.8 μg/mL against *B. subtilis* and *B. cereus*, only macrocycles **4d**, **k** exhibit an activity towards *E. coli* with MICs 15.6 μg/mL, which is 15-fold greater than that of **4i**.

Pyrimidinophanes **4a**–**m** do not exhibit any fungistatic activity towards screened fungi except the mold *T. mentagrophytes*. Among this series of macrocycles only pyrimidinophanes **4d**, **k**, **i** exhibit a modest efficacy against the fungi with MICs 31.3 μg/mL. As in the case of bacteria the minimum MICs values correspond to macrocyclic salts with the "optimal" size (*m* = 5–7, *n* = 10). However, the more expanded pyrimidinophane **4m** (*m* = 12, *n* = 10) does not inhibit the growth of fungi.

The influence of substituents at the C^5 of uracil ring and at the ammonium center of 6-methyl-2-thiocytosine rings on the activity of the pyrimidinophanes is rather ambiguous. While the lipophilic *n*-decyl substituent at the C^5 of uracil provides a dramatic decrease in the MICs values of salt **4o** (*m* = 6, *n* = 5, R^1 = $C_{10}H_{21}$, R^2 = R^3 = CH_3, X = $[SO_3C_6H_4CH_3]^-$) compared with **4g** (*m* = 6, *n* = 5, R^1 = H, R^2 = R^3 = CH_3, X = $[SO_3C_6H_4CH_3]^-$), the MICs values of **4p** (*m* = *n* = 6, R^1 = $C_{10}H_{21}$, R^2 = R^3 = CH_3, X = $[SO_3C_6H_4CH_3]^-$) and **4h** (*m* = *n* = 6, R^1 = $C_{10}H_{21}$, R^2 = R^3 = CH_3, X = $[SO_3C_6H_4CH_3]^-$) are nearly the same. The displacement of the CH_3-substituent from uracil C^6 of **4g** to uracil C^5 of **4n** (*m* = 6, *n* = 5, R^1 = R^3 = CH_3, R^2 = H, X = $[SO_3C_6H_4CH_3]^-$) leads to slight decrease in activity against bacteria and fungi (Table 1). The introduction of a lipophilic *n*-nonyl substituent at the N atom of pyrimidine rings instead of the methyl group does not affect the activity of pyrimidinophane **4r** (*m* = 5, *n* = 10, R^1 = H, R^2 = CH_3, R^3 = C_9H_{19}, X = $[SO_3C_6H_4CH_3]^-$). On the contrary the MICs of **4r** against all screened microbes dramatically increased compared with those of **4d**. On the other hand, the bacteriostatic activity of macrocycle **4q** (*m* = 6, *n* = 3, R^1 = H, R^2 = CH_3, R^3 = C_9H_{19}, X = $[SO_3C_6H_4CH_3]^-$) significantly increases as compared to macrocycle **4e** (*m* = 6, *n* = 3, R^1 = H, R^2 = CH_3, R^3 = CH_3, X = $[SO_3C_6H_4CH_3]^-$). While pyrimidinophane **4g** (*m* = 6,

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Table 2
In vitro antibacterial and antifungal activity of acyclic thiopyrimidine derivatives.[a]

Minimal inhibitory concentration (MICs) μg/mL								
No	*Sa*	*Ec*	*Pa*	*Bc*	*Bs*	*An*	*Tm*	*Ca*
8	21.9	–	–	125	31.3	–	62.5	–
9	31.3	–	–	156	62.5	–	62.5	–
10	15.6	–	–	62.3	15.6	31.3	31.3	3.9
Clotrimazole							3.1	0.4
Ciprofloxacin	0.25	0.50	0.1	0.25	0.25			
Amphotericin B						20		

-, No activity was showed; *Pa, Pseudomonas aeruginosa; Ec, Escherichia coli; Sa, Staphylococcus aureus; Ba, Bacillus subtilis; Bc, Bacillus cereus; An, Aspergillus niger; Tm, Trichophyton mentagrophytes; Ca, Candida Albicans*.

[a] The tests were performed in duplicate and repeated twice.

$n = 5$, $R^1 = H$, $R^2 = CH_3$, $R^3 = CH_3$, $X = [SO_3C_6H_4CH_3]^-$) exhibits a modest activity towards *S. aureus* and *T. mentagrophytes*, macrocycle **4s** ($m = 6$, $n = 5$, $R^1 = R^3 = H$, $R^2 = CH_3$, $X = [SO_3C_6H_4CH_3]^-$) obtained by the tosylation of macrocycle **1g** with *p*-toluenesulfonic acid is absolutely inactive.

For varying anions the quaternization of the pyrimidine ring N atoms of macrocycle **1c** was performed with methyl ester of methanesulfonic acid and methyl ester of trifluoromethylsulfonic acid. Macrocyclic bismesylate **4t** ($m = 5$, $n = 6$, $R^1 = H$, $R^2 = CH_3$, $R^3 = CH_3$, $X = [SO_3CH_3]^-$) and bistriflate **4u** ($m = 5$, $n = 6$, $R^1 = H$, $R^2 = CH_3$, $R^3 = CH_3$, $X = [SO_3CF_3]^-$) exhibit the same bacteriostatic activity as bistosylate **4c** ($m = 5$, $n = 6$, $R^1 = H$, $R^2 = CH_3$, $R^3 = CH_3$, $X = [SO_3C_6H_4CH_3]^-$) against *S. aureus*. However, the MICs of **4t** and **4u** against other screened bacteria and fungi are significantly higher than those of **4c**.

From the structure–activity profile of pyrimidinophanes **4a**–**u** it is obvious that the size of macrocycles, in particular, the length of polymethylene chains is the crucial point providing bacteriostatic activity against gram-positive bacteria *S. aureus*, *B. subtilis*, *B. cereus* and mold *T. mentagrophytes*. To our knowledge, such "pre-organization" towards antimicrobial activity has not been described earlier. The "optimal" structure of macrocyclic salts seems to be as follows: (i) 6-methyluracil and 6-methylthiocytosine moieties bridging each other with polymethylene chains, $m = 5–7$, $n = 10$, (ii) the ring N atom of 6-methylthiocytosine is quaternized with methyl *p*-tosylate. It is assumed that the number of methylene units in bridges, the methyl substituent at the C^6 of uracil moiety, the methyl radical at N of thiocytosine rings and tosylate-anions cause the appropriate "active" conformation to interact with subcellular components. The conformational structure of pyrimidinophanes **1** is rather diverse, and is determined by the length of the spacers, intramolecular NH bonding, pH and solvent. The quaternization of pyrimidine rings and in particular their protonation leads to the disruption of intramolecular hydrogen bonds, destabilization of the folded conformation and to strong counterion assisted self-aggregation of macrocyles which can be destroyed in polar solvents [18]. The detailed study of the conformational behavior of pyrimidinophanes **4** in different solvents will be published elsewhere.

Acyclic tosylates **8**–**10** prepared from pyrimidine **5**, bispyrimidine **6** and trispyrimidine **7** can be considered to be the building units of pyrimidinophanes **4**, and especially compound **10** is the acyclic counterpart of the macrocycles. These tosylates are less active against all the bacteria screened than the pyrimidinophane **4h** with the same length of spacers ($m = n = 6$). However, the MICs of acyclic salt **10** dramatically decreases towards mold *A. niger* and yeast *C. albicans* with respect to the macrocycle (Table 2). As a matter of fact, pyrimidinophanes **4** do not exhibit any activity against those fungi at all. In our opinion, such a tuning of activity towards bacteria and fungi via the topology of the compounds can be useful in the "smart bomb" approach [1,20].

Macrocyclic and acyclic compounds with the highest bacteriostatic and fungistatic efficacy were further screened for their bactericidal and fungicidal activity. In particular, the minimal bactericidal concentrations (MBC) of the pyrimidinophanes **4d**, **4k** and **4o** against *S. aureus*, *B. subtilis*, *B. cereus*, and the minimal fungicidal concentrations (MFC) of acyclic counterpart **10** against *A. niger*, *T. mentagrophytes*, *C. albicans* were determined. Only compound **10** revealed a reasonable value of MFC against *C. albicans* (125 μg/mL). Other screened fungi and bacteria were not influenced by **4d**, **k**, **o** and **10** (their MBC and MFC are 500 μg/mL and higher). These data show that the macrocyclic and acyclic compounds studied exhibit bacteriostatic and fungistatic properties, but their bactericidal and fungicidal activity is not high.

We have evaluated the toxicity of the pyrimidinophanes **4e**, **f**, **g**, **h**, **j** and acyclic counterparts **9** and **10** in terms of lethal doses (LD_{50}) for mice. Toxicity data are presented in Table 3. According to the levels of acute toxicity for mammals [21] both the pyrimidinophanes and the acyclic compounds can be considered moderately toxic compounds. Toxicity data show that lethal doses for mammals decrease with the increase of the number of methylene units *m* and *n* in bridges of the pyrimidinophanes and consequently with the increase in their bacteriostatic and fungistatic activity. The toxicity of compound **10** is 2-fold higher than that of compound **9**, and the main contribution to the toxicity of pyrimidinophanes seems to be especially provided by the fragment in which uracil is bridged wit1h two thiocytosine moieties.

What is the mode of the bacteriostatic action of the pyrimidinophanes **4**? As a matter of fact, it is a challenging problem that such expanded molecules are capable of displaying antimicrobial activity and sufficient toxicity. In fact, pyrimidinophanes **4** can be considered amphiphilic molecules and in particular bolaamphiphiles. In spite of this, pyrimidinophanes **4** do not act as simple amphiphiles via a detergent mode of membrane disruption or cationic peptide and nonpeptidic antibacterial agents [22–26]. This is confirmed by the following observations. (1) We tried to solubilize orange OT in aqueous solutions of the pyrimidinophanes **4d**, **h**, **i**, **k** with the highest bacteriostatic activity. Orange OT is a highly hydrophobic dye that does not dissolve in water. This substance can be used to determine critical micelle concentrations (CMC) of surfactants because orange OT dissolves in the nonpolar core of surfactant aggregates [27]. However, orange OT was not solubilized by the aqueous solutions of the macrocycles. (2) The CMC value of the pyrimidinophane **4o** with a lipophilic *n*-decyl substituent at the C^5 of uracil moiety has been determined by measuring the electrical conductivity and surface tension of its aqueous solutions (aggregation data involving the macrocycles **4** will be published elsewhere). The CMC value 2300 μg/mL obtained is 11000-fold and 4.5-fold higher than MIC and MBC of **4o** against *S. aureus*, respectively. In addition the CMC of the pyrimidinophanes **4** without any lipophilic radicals at uracil or thiocytosine units significantly exceed those of **4o**. Then pyrimidinophanes **4** exhibit antibacterial activity under the CMC values, and evidently this is

Table 3
Toxicity some of pyrimidinophanes with $R^1 = H$, $R^2 = CH_3$, $R^3 = CH_3$, $X = [SO_3C_6H_4CH_3]^-$ on mice.

Compound	**4e** ($m = 6$, $n = 3$)	**4f** ($m = 6$, $n = 4$)	**4g** ($m = 6$, $n = 5$)	**4h** ($m = 6$, $n = 6$)	**4j** ($m = 7$, $n = 5$)	**9**	**10**
LD_{50}, mice, mg/kg	9.2 (6.9÷12.2)	10.6 (8.0÷14.1)	18.1 (13.4÷24.6)	21.7 (16.4÷28.7)	5.5 (4.3÷7.0)	14.7 (9.1÷18.7)	6.7 (5.0÷7.8)

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Table 4
In vitro inhibition of glucose DHO activity and extracellular lipase activity with macrocyclic tosylates and their acyclic counterparts.

Compound	Concentration, μg/mL	Inhibition of DHO activity, %		Inhibition of lipase activity, %	
		S. aureus	*C. albicans*	*S. aureus*	*C. albicans*
4d	500	100	100	95	95
	50	21	15	77	90
	5	0	0	68	28
	0.5	0	0	26	17
4k	500	100	100	100	100
	50	19	13	68	95
	5	0	0	45	48
	0.5	0	0	19	38
4h	500	89	100	68	90
	50	0	65	54	59
	5	0	0	32	38
4o	500	100	100	100	90
	50	30	27	100	90
	5	0	0	45	38
	0.5	0	0	17	28
9	500	0	100	86.3	90
	50	0	10	77	69
	5	0	0	13	28
10	500	57	100	100	100
	50	0	63	87	100
	5	0	0	23	69
	0.5	0	0	0	38

not provided by micelles. One of the proposed modes of the bactericidal effect of amphiphiles and in particular quaternary ammonium salts appears to be due to the ability of their micelles via the solubilization of bacterial cell to cause the release of their contents into the surrounding medium [28]. (3) The dependence of the pyrimidinophanes **4** selectivity against *S. aureus* on the size of the macrocycles cannot be due to the detergent-like mode of antimicrobial action. (4) Insignificant biocidal and fungicidal activity of pyrimidinophanes **4**.

It is assumed that the pyrimidinophanes **4** and their acyclic counterparts **9** and **10** exhibit a specific mechanism of action towards gram-positive bacteria and fungi. To elucidate the antimicrobial targets of the macrocyles **4** a preliminary study was performed in order to establish what kind of enzyme interaction of those compounds occurs. Efficacy of glucose dehydrogenase (DHO) and extracellular triglyceride lipase from *S. aureus* and *C. albicans* was determined after treating the cells with the above compounds by decolorization time measurements of methylene blue in the absence of oxygen (Thunberg technique) [29,30] and the titrimetric method with olive oil as a substrate [31,32], respectively. Some results considering pyrimidinophanes **4d**, **k**, **h**, **o** and acyclic counterparts **9** and **10** are presented in Table 4. As is shown in the Table the significant inhibition of DHO occurs only at bactericidal and fungicidal concentrations (near 500 μg/mL), while the inhibition of lipase is observed at bacteriostatic and fungistatic concentrations (0.5–50 μg/mL). These data correlate with the MICs of the compounds against *S. aureus* and *C. albicans*, which are in the same region of concentrations. Macrocyclic and acyclic tosylates in high dilutions appear unable to inhibit bacterial enzymes involved in respiration and glycolysis. On the contrary, the compounds in the dilutions are able to inhibit lipases which hydrolyze the ester linkages of exogenous triglycerides.

4. Conclusions

We have described the antibacterial and antifungal activity of a series of pyrimidinophanes with one 6-methyluracil or 5-decyl-6-methyluraci or thymine moiety and two 6-methyl-2-thiocytosine moieties connected to each other with polymethylene spacers. Nitrogen ring atoms of 6-methyl-2-thiocytosine fragments of the pyrimidinophanes are quaternized with *p*-toluenesulfonic acid and methyl or nonyl esters of *p*-toluene-, methane- and trifluoromethylsulfonic acids. The screening data show that macrocyclic tosylates exhibit a remarkable selectivity towards gram-positive bacteria and especially *S. aureus* with the MICs values comparable with the fluoroquinolone reference drug. The bacteriostatic activity of the pyrimidinophanes unambiguously depends on the size of the macrocycles, and the highest activity corresponds to definite lengths of polymethylene bridges. Substituents at the C^5 of uracil ring and radicals at the ammonium center of thiocytosine units, as well as the nature of anion are not so important for the efficacy of the macrocycles. Besides, there exists the dependence of the antimicrobial activity of the screened pyrimidine derivatives on their topology. While macrocyclic tosylates are more active against bacteria than against fungi, acyclic tosylate with the same structural fragments shows a dramatical decrease of MIC towards mold and yeast with respect to the corresponding macrocycle. Thus, a series of macrocyclic and acyclic amphiphilic pyrimidine derivatives described herein makes it possible to tune bacteriostatic and fungistatic activity via the variation of the structural features and topology of the compounds. The mode of action of the screened compounds is unclear. However, the data obtained indicate the contribution of a specific mechanism. The study allowed us to assume that the screened compounds exhibit efficacy against bacteria and fungi inhibiting their enzymes, in particular the extracellular lipase activity.

5. Experimental

5.1. General

The NMR experiments were carried out with Bruker spectrometers AVANCE-400 (400.1 MHz (^{1}H), 100.6 MHz (^{13}C)) and AVANCE-600 (600.1 MHz (^{1}H), 150.9 MHz (^{13}C), 60.8 MHz (^{15}N)) equipped with a pulsed gradient unit capable of producing magnetic field pulse gradients in the z-direction of 53.5 Gcm^{-1}. Electron impact mass spectra were recorded on a Finnigan MAT-212 mass spectrometer (70 eV), MALDI-TOF mass spectra were recorded on a Bruker ULTRAFLEX mass spectrometer in *p*-nitroaniline matrix. The IR spectra of the compounds were recorded on a Vector 22 FTIR Spectrometer (Bruker) in the 4000–400 cm^{-1} range at a resolution of 1 cm^{-1}. Microelemental analyses data were obtained on a CHN-3 analyzer and they were within ±0.3% of the theoretical values for C, H, and N. The melting points were measured on a Boetius hot-stage apparatus. Thin layer chromatography was performed on Silufol-254 plates; visualization was carried out with UV light. For column chromatography silica gel of 60 mesh from Fluka was used. All solvents were dried according to standard protocols.

5.2. Synthesis

5.2.1. Synthesis of initial compounds

Synthesis of pyrimidinophanes **1a–c**, **f–j** was reported previously [16–18]. *N*,*N*′-(Alkane-α,ω-diyl)bis(4-amino-6-methylpyrimidine-2-thiones) and their disodium salts **2** [33], 1,3-bis(bromoalkyl)-6-methyluracils and 1,3-bis(bromoalkyl)-5-decyl-6-methyluracils **3** [14,15], pyrimidine **5** and its *p*-toluenesulfonate **8** [19], diamine **6** [17], nonyl *p*-toluenesulfonate [34] were synthesized according to known procedures. Methyl *p*-toluenesulfonate was a commercial product.

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*5.2.2. 1,3-Bis[4-(4-dimethylamino-6-methylpyrimidin-2-ylthio) hexyl]-6-methyluracil (**7**)*

1,3-Bis(6-bromohexyl)-6-methyluracil **3** (R^1 = H, n = 6) (7.23 g, 16 mmol) was added to a solution of sodium salt of 4-dimethylamino-2-mercapto-6-methylpyrimidine [35,36] (6.1 g, 32 mmol) in 400 mL of DMF. The mixture was stirred for 5 h at 80 °C and cooled, the solvent was removed under reduced pressure, and the residue was purified by column chromatography with ethyl acetate as eluent. Fractions of the eluent gave an oil (8.65 mg, 86%). ^{1}H NMR ($CDCl_3$, 600 MHz): 5.95, 5.94 (both s, 1H each, $2C^5_{pyr}H$), 5.54 (s, 1H, $C^5_{ur}H$), 3.90 (m, 2H, $N_{ur}CH_2$), 3.77 (m, 2H, $N_{ur}CH_2$), 3.02–3.06 (m, 16H, $2SCH_2$, $4NCH_3$), 2.28, 2.27 (both s, 3H each, $2C^6_{pyr}CH_3$), 2.21 (s, 3H, $C^6_{ur}CH_3$), 1.73 (m, 4H, $2CH_2$), 1.62 (m, 4H, $2CH_2$), 1.47 (m, 4H, $2CH_2$), 1.37 (m, 4H, $2CH_2$). IR (oil, cm^{-1}): ν 3090, 2929, 2865, 1736, 1701, 1662, 1588, 1509, 1432, 1403, 1369, 1306, 1242, 1202, 1171, 1092, 1034, 994, 968, 919, 811, 769. MS (EI) 628.3 (M^+). Anal Calcd for $C_{31}H_{48}N_8O_2S_2$: C: 59.20, H: 7.69, N: 17.82, S: 10.20. Found C: 59.12, H: 7.55, N: 17.91, S: 10.28.

*5.2.3. Synthesis of new pyrimidinophanes **1d**, **e**, **k**, **l**, **m**, **n**, **o**, **p***

5.2.3.1. General procedure. A mixture of corresponding diamine disodium salt **2** (10 mmol) and dibromide **3** (10 mmol) was stirred in DMF (400 mL) at room temperature for 30 h. The solvent was evaporated *in vacuo*. The residue was subjected to column chromatography using successive elution with hexane, diethyl ether, and ethyl acetate or 1:1 ethyl acetate-diethyl ether mixture. The target macrocycles **1d**, **e**, **k**, **l**, **m**, **n** were isolated from the ethyl acetate fractions, pyrimidinophanes **1o**, **p** with 5-decyl-6-methyluracil moiety were obtained from the ethyl acetate-diethyl ether mixture fractions.

*5.2.3.2. Pyrimidinophane **1d**.* Yield 16%, oil. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.83 (br.s, 2H, $2C^5_{pyr}H$), 5.55 (s, 1H, $C^5_{ur}H$), 4.84 (br.s, 2H, 2NH), 3.90 (m, 2H, $N_{yp}CH_2$), 3.79 (m, 2H, $N_{yp}CH_2$), 3.33 (m, 4H, $2NCH_2$), 3.06 (m, 4H, SCH_2), 2.25 (s, 6H, $2C^6_{pyr}CH_3$), 2.22 (s, 3H, $C^6_{ur}CH_3$), 1.62 (m, 12H, $6CH_2$), 1.42 (m, 4H, $2CH_2$), 1.28 (m, 22H, $11CH_2$). IR (oil, cm^{-1}): ν 3342, 3139, 2925, 2854, 1738, 1698, 1651, 1602, 1504, 1434, 1402, 1359, 1287, 1238, 1188, 1096, 1045, 968, 769. MS (EI) 752.5 (M^+). Anal Calcd for $C_{40}H_{64}N_8O_2S_2$: C: 63.79, H: 8.57, N: 14.88, S: 8.52. Found C: 63.92, H: 8.55, N: 14.80, S: 8.78.

*5.2.3.3. Pyrimidinophane **1e**.* Yield 15%, m.p. 224–226 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.84, 5.82 (both s, 1H each, $2C^5_{pyr}H$), 5.56 (s, 1H, $C^5_{ur}H$), 4.84 (br. S, 2H, 2NH), 3.93, 3.82 (both m, 2H each, $2N_{ur}CH_2$), 3.37 (m, 4H, $2NCH_2$), 3.09, 3.07 (both m, 2H each, $2SCH_2$), 2.24, 2.23 (both s, 3H each, $2C^6_{pyr}CH_3$), 2.22 (s, 3H, $C^6_{ur}CH_3$), 1.99 (m, 4H, $2CH_2$), 1.62 (m, 4H, $2CH_2$), 1.62 (m, 4H, $2CH_2$). IR (KBr pellet, cm^{-1}): ν 3326, 3270, 3139, 2957, 2925, 2855, 1703, 1660, 1603, 1509, 1458, 1401, 1355, 1290, 1231, 1186, 1093, 1034, 969, 767. MS (EI) 570.3 (M^+). Anal Calcd for $C_{27}H_{38}N_8O_2S_2$: C: 56.82, H: 6.71, N: 19.63, S: 11.24. Found C: 56.90, H: 6.75, N: 19.50, S: 11.38.

*5.2.3.4. Pyrimidinophane **1k**.* Yield 15%, oil. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.83 (br.s, 2H, $2C^5_{pyr}H$), 5.55 (s, 1H, $C^5_{ur}H$), 4.77 (br.s, 2H, 2NH), 3.90 (m, 2H, $N_{ur}CH_2$), 3.78 (m, 2H, $N_{ur}CH_2$), 3.31 (m, 4H, $2NCH_2$), 3.01–3.08 (m, 4H, $2SCH_2$), 2.24 (s, 6H, $2C^6_{pyr}CH_3$), 2.22 (s, 3H, $C^6_{ur}CH_3$), 1.72–1.60 (m, 14H, $7CH_2$), 1.37–1.25 (m, 28H, $14CH_2$). IR (oil, cm^{-1}): ν 3333, 3141, 2927, 2853, 1737, 1699, 1662, 1592, 1505, 1432, 1402, 1359, 1285, 1240, 1187, 1093, 1044, 968, 819, 768. MS (EI) 780.5 (M^+). Anal Calcd for $C_{42}H_{68}N_8O_2S_2$: C: 64.58, H: 8.77, N: 14.34, S: 8.21. Found C: 64.65, H: 8.75, N: 14.40, S: 8.28.

*5.2.3.5. Pyrimidinophane **1l**.* Yield 16%, m.p. 134–136 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.83 (s, 2H, $2C^5_{pyr}H$), 5.55 (s, 1H, $C^5_{ur}H$), 4.77 (br.s, 2H, 2NH), 3.90 (m, 2H, $N_{ur}CH_2$), 3.78 (m, 2H, $N_{ur}CH_2$), 3.31 (m, 4H, $2NCH_2$), 3.08–3.01 (m, 4H, $2SCH_2$), 2.24 (s, 6H, $2C^6_{pyr}CH_3$), 2.22 (s, 3H, $C^6_{ur}CH_3$), 1.72–1.60 (m, 8H, $4CH_2$), 1.37–1.25 (m, 28H, $14CH_2$). IR (KBr pellet, cm^{-1}): ν 3296, 3136, 2934, 2925, 2854, 1702, 1657, 1595, 1507, 1463, 1401, 1359, 1287, 1236, 1187, 1093, 1034, 970, 816, 767. MS (EI) 738.4 (M^+). Anal Calcd for $C_{39}H_{62}N_8O_2S_2$: C: 63.38, H: 8.46, N: 15.16, S: 8.68. Found C: 63.50, H: 8.39, N: 15.08, S: 8.58.

*5.2.3.6. Pyrimidinophane **1m**.* Yield 16%, oil. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.82 (s, 2H, $2C^5_{pyr}H$), 5.54 (s, 1H, $C^5_{ur}H$), 4.79 (br.s, 2H, 2NH), 3.89 (m, 2H, $N_{ur}CH_2$), 3.77 (m, 2H, $N_{ur}CH_2$), 3.30 (m, 4H, $2NCH_2$), 3.06–3.02 (m, 4H, $2SCH_2$), 2.23 (s, 6H, $2C^6_{pyr}CH_3$), 2.21 (s, 3H, $C^6_{ur}CH_3$), 1.69 (m, 4H, $2CH_2$), 1.58 (m, 8H, $4CH_2$), 1.41–1.26 (m, 40H, $20CH_2$). IR (oil, cm^{-1}): ν 3343, 3149, 2920, 2850, 1699, 1655, 1598, 1507, 1467, 1433, 1401, 1357, 1287, 1218, 1184, 1096, 970, 914, 814, 768. MS (EI) 850.6 (M^+). Anal Calcd for $C_{47}H_{78}N_8O_2S_2$: C: 66.31, H: 9.24, N: 13.16, S: 7.53. Found C: 66.39, H: 9.33, N: 13.26, S: 7.68.

*5.2.3.7. Pyrimidinophane **1n**.* Yield 9%, m.p. 187–190 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 6.95 (s, 1H, $C^6_{ur}H$), 5.82, 5.81 (both s, 1H each, $2C^5_{pyr}H$), 3.96 (m, 2H, $N_{ur}CH_2$), 3.72 (m, 2H, $N_{ur}CH_2$), 3.30 (m, 4H, $2NCH_2$), 3.07–3.01 (m, 4H, $2SCH_2$), 2.24 (s, 6H, $2C^6_{pyr}CH_3$), 1.92 (s, 3H, $C^5_{ur}CH_3$), 1.79–1.53 (m, 12H, $6CH_2$), 1.45 (m, 8H, $4CH_2$). IR (oil, cm^{-1}): ν 3375, 3136, 2925, 2856, 1698, 1655, 1596, 1506, 1464, 1430, 1401, 1354, 1293, 1212, 1183, 1090, 970, 912, 823, 766. MS (EI) 626.3 (M^+). Anal Calcd for $C_{31}H_{46}N_8O_2S_2$: C: 59.39, H: 7.40, N: 17.87, S: 10.23. Found C: 59.22, H: 7.53, N: 18.00, S: 10.08.

*5.2.3.8. Pyrimidinophane **1o**.* Yield 10%, m.p. 115–117 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.83, 5.81 (both s, 1H each, $2C^5_{pyr}H$), 5.15 (br.s, 2H, 2NH), 3.94 (m, 2H, $N_{ur}CH_2$), 3.83 (m, 2H, $N_{ur}CH_2$), 3.30 (m, 4H, $2NCH_2$), 3.09–3.01 (m, 4H, $2SCH_2$), 2.41 (t, 2H, $C^5_{ur}CH_2$, J = 6.8 Hz), 2.25, 2.24, 2.23 (all s, 3H each, $C^6_{ur}CH_3$, $2C^6_{pyr}CH_3$), 1.79–1.61 (m, 16H, $8CH_2$), 1.49 (m, 4H, $2CH_2$), 1.41 (m, 4H, $2CH_2$), 1.26 (m, 12H, $6CH_2$), 0.88 (t, 3H, CH_3, J = 7.2 Hz). IR (KBr pellet, cm^{-1}): ν 3370, 3133, 2923, 2858, 1699, 1655, 86, 1503, 1464, 1432, 1403, 1356, 1290, 1210, 1180, 1092, 971, 915, 818, 761. MS (EI) 766.5 (M^+). Anal Calcd for $C_{41}H_{66}N_8O_2S_2$: C: 64.19, H: 8.67, N: 14.61, S: 8.36. Found C: 64.31, H: 8.59, N: 14.68, S: 8.18.

*5.2.3.9. Pyrimidinophane **1p**.* Yield 8%, m.p. 99–101 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.83, 5.82 (both s, 1H each, $2C^5_{pyr}H$), 4.89 (br.s, 2H, 2NH), 3.92 (m, 2H, $N_{ur}CH_2$), 3.82 (m, 2H, $N_{ur}CH_2$), 3.32 (m, 4H, $2NCH_2$), 3.09–3.01 (m, 4H, $2SCH_2$), 2.40 (t, 2H, $C^5_{ur}CH_2$, J = 7.6 Hz), 2.25, 2.24, 2.22 (all s, 3H each, $C^6_{ur}CH_3$, $2C^6_{pyr}CH_3$), 1.72–1.64 (m, 16H, $8CH_2$), 1.47–1.25 (m, 24H, $12CH_2$), 0.88 (t, H, CH_3, J = 6.8 Hz). IR (KBr pellet, cm^{-1}): ν 3370, 3133, 2923, 2858, 1699, 1655, 86, 1503, 1464, 1432, 1403, 1356, 1290, 1210, 1180, 1092, 971, 915, 818, 761. MS (EI) 794.5 (M^+). Anal Calcd for $C_{43}H_{70}N_8O_2S_2$: C: 64.95, H: 8.87, N: 14.09, S: 8.06. Found C: 65.04, H: 8.79, N: 13.98, S: 8.21.

5.2.4. Synthesis of macrocyclic and acyclic bis(p-toluene-, methane- and trifluoromethylsulfonates)

5.2.4.1. General procedure. A mixture of 150 mg of pyrimidinophane **1a**–**p** or diamine **6** or compound **7** and 0.9 g of methyl *p*-toluenesulfonate, methyl methanesulfonate or methyl trifluoromethylsulfonate was stirred for 7 h at 90–100 °C. The mixture was cooled to room temperature, 40 mL of diethyl ether was added, and the liquid phase was separated from the precipitate by decanting. This procedure was repeated 3–6 times, and the crystalline or oily product was dried under reduced pressure.

*5.2.4.2. Macrocyclic bistosylate **4d**.* Yield 215 mg (96%), oil. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 9.42, 9.37 (both s, 1H each, 2NH), 7.75, 7.14 (both d, 4H each, $8C_{Ar}H$, both J = 7.8 Hz), 6.78 (s, 2H, $2C^5_{pyr}H$), 5.57 (s, 1H, $C^5_{ur}H$), 3.91 (m, 2H, $N_{ur}CH_2$), 3.81 (m, 2H, $N_{ur}CH_2$), 3.60 (m,

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10H, $2NCH_2$, $2N_{pyr}CH_3$), 3.15 (m, 4H, $2SCH_2$), 2.35, 2.33 (both s, 6H each, $2C^6_{pyr}CH_3$, $2C_{Ar}CH_3$), 1.72–1.62 (m, 14H, $7CH_2$), 1.30 (m, 24H, $12CH_2$). ^{13}C NMR ($CDCl_3$, 151 MHz): δ 182.1, 177.5, 167.5, 166.1, 161.7, 160.8, 160.3, 160.2, 158.6, 154.8, 141.0, 130.4, 129.4, 128.7, 126.8, 126.1, 109.6, 109.1, 105.1, 105.0, 102.0, 66.4, 64.1, 63.8, 62.1, 59.8, 56.7, 51.0, 45.6, 44.1, 42.0, 41.5, 36.7, 36.4, 34.8, 33.1, 31.5, 30.3, 29.8, 29.6, 29.2, 28.8, 27.8, 26.9, 26.7, 23.3, 22.8, 22.3, 21.9, 20.9, 20.4, 15.8, 14.7. IR (oil, cm^{-1}): ν 3252, 3129, 3080, 2974, 2927, 2856, 1698, 1642, 1516, 1497, 1452, 1400, 1358, 1213, 1189, 1123, 1034, 1012, 817, 740, 682, 567. MS MALDI-TOF ($[M\text{-}OTs]^+$) 954. Anal Calcd for $C_{56}H_{84}N_8O_8S_4$: C: 59.76, H: 7.52, N: 9.96, S: 11.40. Found C: 59.93, H: 7.72, N: 10.14, S: 11.26.

5.2.4.3. Macrocyclic bistosylate ***4f***. Yield 209 mg (85%), m.p. 64–66 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 9.51, 9.34 (both s, 1H each, 2NH), 7.76, 7.15 (both d, 4H each, $8C_{Ar}H$, both $J = 7.9$ Hz), 6.64, 6.52 (both s, 1H each, $2C^5_{pyr}H$), 5.54 (s, 1H, $C^5_{ur}H$), 3.87 (m, 2H, $N_{ur}CH_2$), 3.77 (m, 2H, $N_{ur}CH_2$), 3.70 (br.s, 6H, $2N_{pyr}CH_3$), 3.64 (m, 4H, $2NCH_2$), 3.22 (m, 2H, SCH_2), 3.17 (m, 2H, SCH_2), 2.33 (br.s, 12H, $2C_{Ar}CH_3$, $2C^6_{pyr}CH_3$), 2.25 (s, 3H, $C^6_{ur}CH_3$), 1.94–1.70 (m, 16H, $8CH_2$). ^{13}C NMR ($CDCl_3$, 151 MHz): δ 159.0, 158.4, 151.8, 146.4, 141.7, 140.3, 129.9, 128.8, 128.1, 126.2, 118.3, 106.9, 104.2, 103.1, 101.3, 100.8, 98.6, 97.5, 96.2, 62.1, 56.1, 44.9, 41.3, 40.7, 35.9, 32.4, 32.2, 28.6, 27.5, 27.3, 27.0, 26.7, 25.6, 21.4, 20.3. IR (KBr pellet, cm^{-1}): ν 3245, 3155, 3077, 2921, 2858, 1699, 1642, 14945, 1456, 1403, 1378, 1314, 1223, 1172, 1120, 1099, 1031, 1007, 818, 680. MS MALDI-TOF ($[M\text{-}OTs]^+$) 799. Anal Calcd for $C_{45}H_{62}N_8O_8S_4$: C: 55.65, H: 6.43, N: 11.54, S: 13.21. Found C: 55.51, H: 6.52, N: 11.40, S: 13.36.

5.2.4.4. Macrocyclic bistosylate ***4g***. Yield 203 mg (85%), m.p. 44–46 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 9.23, 9.15 (both s, 1H each, 2NH), 7.72, 7.14 (both d, 4H each, $8C_{Ar}H$, both $J = 7.6$ Hz), 6.75, 6.68 (both s, 1H each, $2C^5_{pyr}H$), 5.56 (s, 1H, $C^5_{ur}H$), 3.87 (m, 2H, $N_{ur}CH_2$), 3.74 (m, 2H, $N_{ur}CH_2$), 3.60 (m, 10H, $2NCH_2$, $2N_{pyr}CH_3$), 3.15 (m, 4H, $2SCH_2$), 2.33 (br.s, 12H, $2C_{Ar}CH_3$, $2C^6_{pyr}CH_3$), 2.20 (s, 3H, $C^6_{ur}CH_3$), 1.80 (m, 4H, $2CH_2$), 1.67 (m, 8H, $4CH_2$), 1.45 (m, 8H, $4CH_2$). ^{13}C NMR ($CDCl_3$, 151 MHz): δ 180.9, 178.7, 177.5, 173.4, 171.2, 167.4, 165.0, 158.4, 139.7, 131.7, 129.2, 128.1, 127.4, 125.1, 115.3, 103.7, 102.4, 65.2, 55.5, 53.9, 44.3, 40.8, 40.3, 31.9, 30.3, 29.1, 28.6, 27.9, 27.5, 26.6, 25.7, 25.5, 21.0, 20.7, 19.7, 19.1, 14.6, 13.5. IR (KBr pellet, cm^{-1}): ν 3247, 3158, 3073, 2924, 2855, 1698, 1640, 1495, 1459, 1402, 1377, 1316, 1225, 1174, 1121, 1097, 1033, 1009, 817, 681. MS MALDI-TOF ($[M\text{-}OTs]^+$) 827. Anal Calcd for $C_{47}H_{66}N_8O_8S_4$: C: 56.49, H: 6.66, N: 11.21, S: 12.83. Found C: 56.57, H: 6.74, N: 11.31, S: 12.96.

5.2.4.5. Macrocyclic bistosylate ***4h***. Yield 205 mg (87%), m.p. 51–53 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 9.38, 9.35 (both s, 1H each, 2NH), 7.73, 7.13 (both d, 4H each, $8C_{Ar}H$, both $J = 7.8$ Hz), 6.80, 6.79 (both s, 1H each, $2C^5_{pyr}H$), 5.55 (s, 1H, $C^5_{ur}H$), 3.87 (m, 2H, $N_{ur}CH_2$), 3.78 (m, 2H, $N_{ur}CH_2$), 3.74 (s, 6H, $2N_{pyr}CH_3$), 3.60–3.46 (m, 4H, $2NCH_2$), 3.19–3.14 (m, 4H, $2SCH_2$), 2.38, 2.37 (both s, 3H each, $2C^6_{pyr}CH_3$), 2.34 (s, 6H, $2C_{Ar}CH_3$), 2.22 (s, 3H, $C^6_{ur}CH_3$), 1.74–1.60 (m, 12H, $6CH_2$), 1.46–1.37 (m, 12H, $6CH_2$). ^{13}C NMR ($CDCl_3$, 151 MHz): δ 167.7, 162.9, 159.6, 154.7, 152.5, 152.0, 142.8, 140.7, 132.9, 130.4, 129.4, 128.7, 126.6, 104.9, 102.1, 66.4, 56.7, 45.5, 42.1, 41.6, 36.5, 36.4, 33.3, 33.2, 29.6, 29.4, 29.2, 28.8, 27.9, 26.9, 21.9, 20.8, 20.3, 15.8. IR (KBr pellet, cm^{-1}): ν 3240, 3161, 3068, 2927, 2849, 1701, 1645, 1500, 1463, 1400, 1372, 1314, 1222, 1182, 1123, 1100, 1035, 1000, 1097, 685. MS MALDI-TOF ($[M\text{-}OTs]^+$) 855. Anal Calcd for $C_{49}H_{70}N_8O_8S_4$: C: 57.28, H: 6.87, N: 10.91, S: 12.48. Found C: 57.37, H: 6.85, N: 10.80, S: 12.62.

5.2.4.6. Macrocyclic bistosylate ***4j***. Yield 230 mg (97%), oil. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 9.40 (br.s, 2H, 2NH), 7.75, 7.14 (both d, 4H each, $8C_{Ar}H$, both $J = 7.9$ Hz), 6.87, 6.85 (both s, 1H each, $2C^5_{pyr}H$), 5.57 (s, 1H, $C^5_{ur}H$), 3.89 (m, 2H, $N_{ur}CH_2$), 3.79 (m, 2H, $N_{ur}CH_2$), 3.63, 3.62 (both s, 3H each, $2N_{pyr}CH_3$), 3.52 (m, 4H, $2NCH_2$), 3.16 (m, 4H, $2SCH_2$), 2.34 (br.s, 12H, $2C^6_{pyr}CH_3$, $2C_{Ar}CH_3$), 2.23 (s, 3H, $C^6_{ur}CH_3$), 1.72–1.29 (m, 22H, $11CH_2$). ^{13}C NMR ($CDCl_3$, 151 MHz): δ 166.9, 162.2, 159.0, 154.2, 151.6, 141.8, 140.3, 129.8, 128.8, 128.1, 126.1, 104.4, 101.4, 96.1, 45.0, 41.6, 41.0, 35.9, 32.6, 28.8, 28.4, 27.1, 26.5, 21.4, 20.3, 19.8. IR (oil, cm^{-1}): ν 3246, 3156, 3078, 2930, 2852, 1699, 1639, 1460, 1402, 1377, 1315, 1223, 1171, 1120, 1095, 1032, 1009, 846, 816, 769, 740, 680. MS MALDI-TOF ($[M\text{-}OTs]^+$) 841. Anal Calcd for $C_{49}H_{70}N_8O_8S_4$: C: 56.89, H: 6.76, N: 11.06, S: 12.66. Found C: 57.02, H: 6.65, N: 10.91, S: 12.80.

5.2.4.7. Acyclic bistosylate ***9***. Yield 258 mg (92%), m.p. 164–166 °C. ^{1}H NMR ($CDCl_3$, 600 MHz): 9.61 (br.s, 2H, 2NH), 7.76, 7.13 (both d, 4H each, $8C_{Ar}H$, both $J = 7.8$ Hz), 6.93 (s, 2H, $2C^5_{pyr}H$), 3.64 (s, 6H, $2N_{pyr}CH_3$), 3.48 (m, 4H, $2CH_2$), 2.59 (s, 6H, $2SCH_3$), 2.40, 2.33 (both s, 6H each, $2C^6_{pyr}CH_3$, $2C_{Ar}CH_3$), 1.66 (m, 4H, $2CH_2$), 1.42 (m, 4H, $2CH_2$). IR (KBr pellet, cm^{-1}): ν 3255, 3084, 2925, 2855, 1645, 1520, 1497, 1459, 1377, 1317, 1225, 1181, 1122, 1102, 1034, 1011, 846, 815, 682. MS MALDI-TOF ($[M\text{-}OTs]^+$) 563. Anal Calcd for $C_{32}H_{42}N_6O_6S_4$: C: 52.29, H: 5.76, N: 11.43, S: 17.45. Found C: 52.42, H: 5.60, N: 11.61, S: 17.32.

5.2.4.8. Acyclic bistosylate ***10***. Yield 232 mg (97%), oil. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.68, 7.09 (both d, 4H each, $8C_{Ar}H$, both $J = 7.7$ Hz), 6.84, 6.77 (both s, 1H each, $2C^5_{pyr}H$), 5.55 (s, 1H, $C^5_{ur}H$), 3.90 (m, 2H, $N_{ur}CH_2$), 3.78 (m, 2H, $N_{ur}CH_2$), 3.67 (s, 3H, $N_{pyr}CH_3$), 3.65 (s, 3H, $N_{pyr}CH_3$), 3.28 (s, 3H, NCH_3), 3.27 (s, 3H, NCH_3), 3.25 (s, 3H, NCH_3), 3.22 (s, 3H, NCH_3), 3.19–3.15 (m, 4H, $2SCH_2$), 2.45 (s, 3H, $C^6_{pyr}CH_3$), 2.32 (br.s, 9H, $C^6_{pyr}CH_3$, $2C_{Ar}CH_3$), 2.21 (s, 3H, $C^6_{ur}CH_3$), 1.76 (m, 4H, $2CH_2$), 1.63 (m, 4H, $2CH_2$), 1.48 (m, 4H, $2CH_2$), 1.37 (m, 4H, $2CH_2$). MS MALDI-TOF ($[M\text{-}OTs]^+$) 829. Anal Calcd for $C_{47}H_{68}N_8O_8S_4$: C: 56.37, H: 6.84, N: 11.19, S: 12.81. Found C: 56.50, H: 6.99, N: 11.11, S: 12.72.

5.3. Culture conditions, antibacterial and antifungal activity

The in vitro antibacterial and antifungal activity of the macrocyclic and acyclic synthesized compounds was investigated against several pathogenic representative Gram-negative bacteria (*P. aeruginosa* 9027, *E. coli* F-50), Gram-positive bacteria (*S. aureus* 209p, *B. subtilis* 6633, *B. cereus* 8035, *E. faecalis* ATCC 8043), molds (*A. niger* BKMF-1119, *T. mentagrophytes var. gypseum* 1773) and yeast (*C. albicans* 885-653). Minimal inhibitory concentrations (MICs) were estimated by conventional dilution methods for bacteria [37] and fungi [38]. The antibacterial and antifungal assays were performed in Hottinger broth (bacteria 3×10^5 cfu/mL) and Sabouraud dextrose broth (fungi $2 \times 10^{3-4}$ CFU/mL). The compounds were dissolved in water or DMSO and then diluted using cautiously adjusted appropriate broth.

Commercial antibiotics Ciprofloxacin, Amphotericin B and Clotrimazole were used as standard drugs. Positive growth control and standard drug controls were also run simultaneously. In the case of solutions of investigated compounds in DMSO the solvent was used as a negative control. The MICs were defined as the lowest concentrations that showed no growth and recorded by visual observation every 24 h during 5 days for bacteria and after incubation during 14 days for fungi.

The bactericidal and fungicidal activity was determined as follows: assay tubes were filled with 1 mL of test compound solution in nutrient agar. Concentrations of test compounds were varied from 12.5 to 10^4 μg/mL. Normal saline broth (bacteria 3×10^5 CFU/mL), 1 mL was added to the tubes and for 4 h the inocula were prepared by transferring the broth onto petri plates containing meal-peptone agar. Petri plates were incubated at 37 °C

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and the MBC recorded as the test compound dilution affecting the total cell death. For fungicidal activity determination the tubes with the test compounds and fungi were incubated at 26 °C. For 4 h the inocula were prepared in Sabouraud dextrose broth and incubated at 26 °C.

Two replicas were done for each compound and the experiment was repeated twice.

5.4. Enzyme assay

In measuring the dehydrogenase activity [29,30], 0.5 mL of *S. aureus* or *C. albicans* suspension and 0.5 mL of the solution of the compound investigated with desired concentration were introduced into the Thunberg tube. Instead of the compound the solution 0.5 mL of saline was added into the control tube. The Thunberg tubes were incubated at 37.5 °C for 2 h, and the following reagents were introduced into each tube: 0.8 mL of phosphate buffer, pH 7.4, and 0.5 mL of 1% glucose solution. Methylene blue solutions (0.008%–0.0001%, 0.2 mL) were placed in the stopper of the Thunberg tubes, and their evacuation was followed for 2 min at about 1 Torr, after which the contents were placed into incubator for an equilibrium period of 2 min. Then methylene blue and the buffers were mixed, and this was considered the beginning of the reaction. The decolorization time of the resulting mixtures was determined visually, and the control time was compared with the time of the compound investigated. The mixtures in the Thunberg tubes were observed for 4 h in a water incubator. If the mixture was not decolorized, it was placed into an air incubator at 37 °C. If the color was retained for a day, this meant a complete (100%) inhibition of DHO. The DHO activity was expressed as the ratio decolorization time of the control tube/decolorization time of the tube with the compound ×100%, and consequently the inhibition of the DHO activity was expressed as 100% minus the calculated DHO activity.

In measuring the lipase activity [31,32], a mixture of 1.5 mL of *S. aureus* or *C. albicans* suspension with 1.5 mL of solution of the investigated compound with the desired concentration was added to the assay substrate containing 1.3 mL of olive oil, 2 mL of Twin-80 and 1 mL of phosphate buffer, pH 7.4. The resulting mixture was shaken and incubated at 37 °C for 24 h. To stop the reaction, 15 mL ethanol was added to the reaction mixture. The liberated fatty acids were titrated with 0.1 mol/L NaOH. In the control experiment the procedure was the same except that 1.5 mL of saline was added instead of the compound solution. The absence of liberated fatty acids in the broth meant complete (100%) inhibition of lipase. Extracellular lipase activity was expressed as the difference between the mL of NaOH in control and the mL of NaOH in the presence of the compound multiplied by 0.31. This value is the ratio of the volume of the resulting mixture (22.3 mL) and the product of incubation time (24 h) with the volume of the mixture of microbial culture and the investigated compound (3 mL). The inhibition of the lipase activity was expressed as 100% minus the calculated lipase activity.

5.5. Toxicity of the compounds studied

Toxicity tests were carried out via single peroral (per os) introductions of pyrimidinophane aqueous solutions in acute tests on white outbreed mice of both sexes with the mass of 17–21 g. The observation period was 72 h. Average lethal doses were used – LD_{50} as the criteria of toxicity. To measure these values each compound was introduced to 5 groups of mice (10 mice per dose; $n = 50$). The results were processed using the program ToxCalc™ v.5.0.23E (Tidepool Scientific Software; USA).

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.05.034.

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