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New ethacridine derivatives as the potential antifungal and antibacterial preparations

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Key words: *ethacridine; thiazolidine; nitrofuran; antifungal activity; antibacterial activity.*

Summary. *Until the 20th century fungal infections were rather easy cured, and the need of new antifungal drugs was low. However, low choice of antifungal preparations, their toxicity, limited spectrum of action, and ability to produce resistant strains show the need of new effective medicines for systemic fungal diseases in nowadays. Our goal of research was to synthesize new antimicrobial compounds containing three or more pharmacophores in one molecule. The initial 5-substituted-2-methylmercaptothiazolidin-4-ones were subjected to S-demethylation to yield 2-amino-substituted thiazolidinones. Ethacridine, nitrofuran aldehydes and nitrobenzene aldehyde as pharmacophoric amino or aldehyde group having compounds have been used. Antimicrobial (antifungal) activity of the new compounds was screened in vitro in these bacterial cultures: Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Bacillus subtilis ATCC 6633, Klebsiella pneumoniae ATCC 33499 and fungal cultures: Candida albicans ATCC 60193, Candida glabrata, Candida krusei, Candida kefyr ATCC 8614, Candida tropicalis ATCC 8302, Candida parapsilosis. Results showed that the new compounds were significantly more effective as antimicrobial agents than initial preparation ethacridine. Ethacridine derivatives were not only effective against numerous gram-positive and some gram-negative bacteria, but the spectrum of action has been discovered against fungi. Minimal fungistatic concentration varies in the range 10.0–750 μg/mL and antibacterial concentration is in the range 62.5–1000 μg/mL. Compound 2a having nitrofuryl substituent in the fifth position of tiazolidine cycle was the most active of synthesized ethacridine compounds. The obtained results gave the opportunity to separate the perspective group of potential antiinfective compounds.*

Introduction

Until the 20th century, fungal infections were rather easy cured, and the need of new antifungal drugs was low (1). A long-term systemic fungal infections were not seriously dangerous for human health and life. Pharmaceutical manufacturers thought that there is no need for investments into investigation of new antifungal drugs (2).

The incidence of fungal infections have increased over the last two decades, and *Candida* species were the predominant mycotic pathogen (3–5). Serious invasive fungal infections represent an increasing threat to human health. *Candida* species produce broad range infections, ranging from superficial illnesses to life threatening diseases (6). In recent years, because of overuse of antibacterial antibiotics, the use of imunossupressors, cytotoxins, opportunistic mycoses become prominent (7, 8).

To decrease the increasing number of fungal pathogens and the growing problems of resistance, new antifungal compounds are required (1), and a variety of effective antifungal agents is of growing importance (9). Because of the above-stated reasons, drug chemistry specialists and pharmacologists spare no effort to discover and investigate new biologically active substances also having antimicrobial activity.

For a number of years, the Department of Pharmaceutical Chemistry of Kaunas University of Medicine has investigated new sulfanilamide compounds, which are obtained by substituting 4-amino group with heterocycles, such as thiazole derivatives, which have antimicrobial pharmacophores (10). The selection of

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such antimicrobial pharmacophores rests on our idea to investigate new compounds that have advantages vis-à-vis initial products. Nevertheless, search for a more potent antibacterial alternative is still a challenge. Higher antimicrobial activity may be also due to different mechanism of action.

Thiazole derivatives as potential drugs were taken into account in the beginning of 20th century, and nowadays this interest renewed again (11). Structure of rhodanine (2-thioxo-4-thiazolidinone) is convenient by chemical reasons: into different positions of thiazolidine cycle by introduction of various substituents it is possible to get chemical compounds having different biological activity (12, 13).

Acridine derivatives are one of the oldest classes of biologically active compounds, widely used as antibacterial agents and are potential chemotherapeutic agents (14). The use of heteroaromatic dyes as antibacterial agents was proposed by Ehrlich and Benda in 1912. Since then many compounds containing the acridine pharmacophore were synthesized and tested as antimicrobials. However, with the current massive increases in drug-resistant bacterial infection, new acridine derivatives may be of use (15).

Sources tell that nitrofurans possess a range of positive properties. They enhance phagocytosis and have little probability of developing bacterial resistance to these compounds, which is related to various and different mechanisms of action (16). Irrespective of all the facts mentioned above uses of nitrofurans in clinical practice is becoming rarer because of their relatively high toxicity and a large number of side effects. By incorporating pharmacologically active nitrofuran pharmacophores and ethacridine pharmacophores in one molecule we expected to design antimicrobial preparations.

The aim was the study of the influence of 7-ethoxyacridine-3,8-diamine (ethacridine) and nitro group having pharmacophores on the antimicrobial (antifungal) activity of thiazolidine derivatives. Therefore, it is therapeutic interest to design and to obtain the compounds containing three or more pharmacophores in one molecule.

Materials and methods

General

New ethacridine derivatives (2a, 2b, 2c) were synthesized at the Department of Pharmaceutical Chemistry of Kaunas University of Medicine.

Melting points were determined on Kofler apparatus with microscope. The IR spectra were recorded in cm^{-1} for KBr pellets on a SPECORD M80 spectrophotometer. ^{1}H-NMR spectra were recorded on a Bruker AM 300 spectrometer using DMSO-d^6 as a solvent and TMS as the internal reference standard. Chemical shifts are expressed in δ ppm. The purity of compounds was routinely checked by TLC using precoated silica gel plates (Kieselgel 0.25 mm, 60G F254, Merck, Germany). Spots were detected under UV (254 nm). Elemental analysis was performed at the Pharmaceutical Department of Jagiellonian University (Cracow, Poland).

Microbiological experiments were carried out at the Department of Microbiology in Kaunas University of Medicine.

Chemistry

General procedure A. Synthesis of 5-substituted 2-methylmercaptothiazolidin-4-ones (1a-c). To a solution of 2-methylrhodanine (1.47 g, 0.01 mol) in concentrated acetic acid (10 mL) the appropriate aldehyde (0.01 mol) was added (Fig. 1). Ammonium acetate was used as a catalyst. The reaction mixture was stirred for 1 h at 60°C. After cooling the solid obtained was filtered, washed with acetic acid, then ether and dried. The crude product was crystallized from acetic acid.

General procedure B (Fig. 2). *Synthesis of 2-(9-amino-7-ethoxyacridin-3-ylamino)-5-((5-nitrofuran-2-yl)methylen)thiazol-4(5H)-one (2a), 2-(9-amino-7-ethoxyacridin-3-ylamino)-5-(3-(5-nitrofuran-2-yl)aliliden)thiazol-4(5H)-one (2b), 2-(9-amino-7-ethoxyacridin-3-ylamino)-5-(4-nitrobenzyliden) thiazol-4(5H)-one (2c).*

A mixture of 7-ethoxyacridine-3,8-diamine (3.8 g, 0.015 mol) and solution of appropriate 5-substituted 2-methylmercaptothiazolidin-4-one in acetic acid (0.01 mol) was heated for 3 h at 90°C. The solid obtained was filtered, washed with acetic acid, dried and crystallized from a mixture of 2-butanol-dimethylformamide (1:1). All reactions proceeded smoothly a good yield.

Antimicrobial activity

Antimicrobial activity of new compounds and ethacridine was determined at the Department of Microbiology of Kaunas University of Medicine.

Antimicrobial susceptibility tests. Antimicrobial and antifungal susceptibility was tested *in vitro* using a serial broth dilution technique (in Mueller-Hinton broth II, BBL, Cockeysville, USA). Antimicrobial activity of new compounds (2a-e) and ethacridine was tested *in vitro* in these standard bacterial cultures:

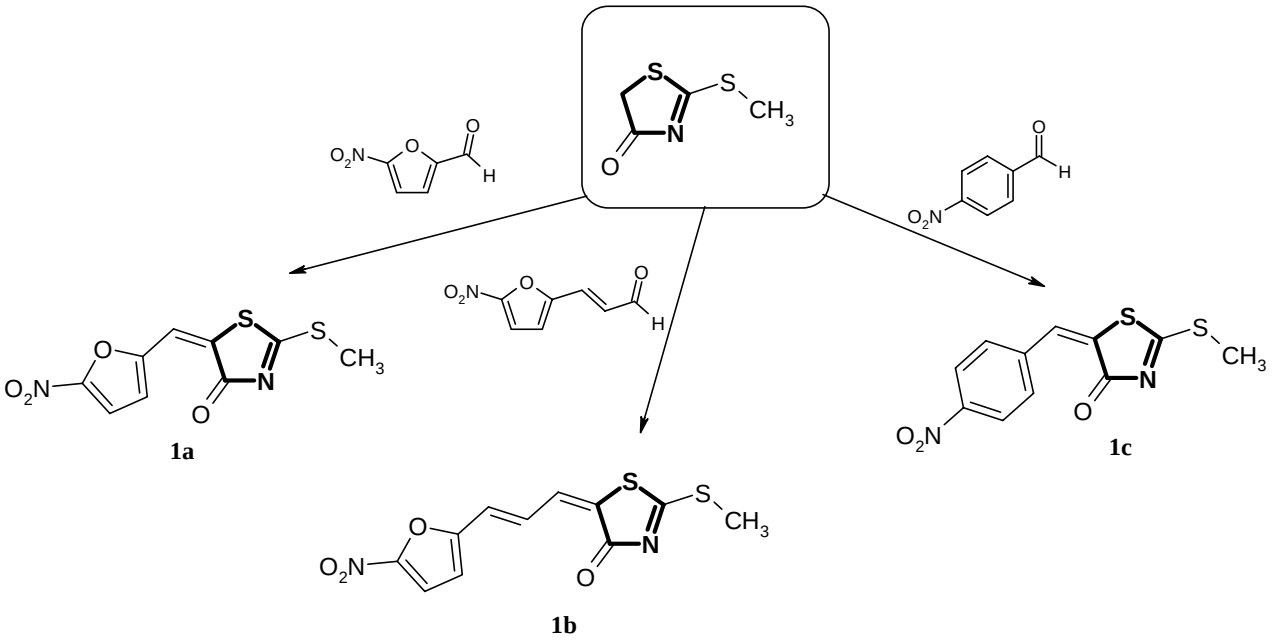


Fig. 1. **Synthesis of 5-substituted 2-methylmercaptothiazolidin-4-ones**

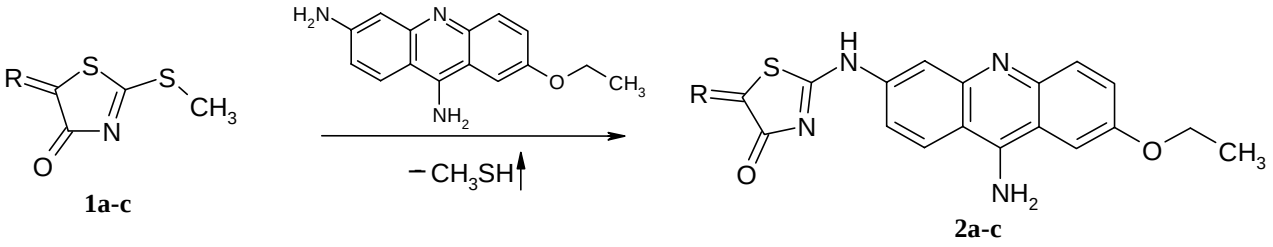


Fig. 2. **Synthesis of new compounds 2a-c**

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633*, Klebsiella pneumoniae* ATCC 33499 and fungal cultures: *Candida albicans* ATCC 60193*, C. glabrata, C. krusei, C. kefyr* ATCC 8614*, C. tropicalis* ATCC 8302*, C. parapsilosis.*

Preparation of standard microorganism cultures. Standard cultures of nonsporic bacteria *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* were cultivated for 20–24 h at the temperature of 35–37°C in Mueller-Hinton Agar (Mueller-Hinton II Agar, BBL, Cockeysville, USA). A bacterial suspension was prepared from cultivated bacterial cultures in physiological solution according to turbidity standard 0.5 McFarland.

Standard culture of sporic bacteria *Bacillus cereus* was cultivated for 7 days at the temperature of 35–37°C in Mueller-Hinton II Agar. After sporic bacteria culture had grown, it was washed away from the surface of the broth with sterile physiological solution, and the prepared suspension was being heated for 30 min at the temperature of 70°C and diluted till the concentration of spores in 1 mL ranged from 10×10^6 to 100×10^6. Sporic suspension can be kept for a long time at temperature below 4°C.

The standard fungal cultures: *Candida albicans*, *C. glabrata*, *C. krusei*, *C. kefyr*, *C. tropicalis* and *C. parapsilosis* were cultivated for 20–24 h at 30°C in Mueller-Hinton Agar (Mueller-Hinton II Agar, BBL, Cockeysville, USA). A fungal suspension was prepared from cultivated fungal cultures in physiological solution according to the turbidity standard 0.5 McFarland.

Preparation of investigative compounds for microbiological analysis. The main solution of new compounds (VIL-1, VIL-2, VIL-3) and comparison compound (ethacridine) (20 000 μg/mL) was prepared in dimethylsulfoxide because of its slight solubility in other solvents. Then dilutions of 0.6, 1.25, 2.5, 5, 10, 15.6, 31.25, 62.5, 125, 250, 500, 750, 1000 μg/mL were carried out under aseptic conditions by transferring the necessary amount of the analyzed solution

analyzed using a sterile pipette to other tubes filled with 2 ml of Mueller-Hinton broth.

The minimal dilution, *i.e.* the lowest concentration in μg/mL of the investigative and comparison compound that inhibit the growth of bacteria, was determined by the first tube in the series which inhibited visible growth – it was the minimal inhibitory concentration (MIC).

The minimal bactericidal (fungicidal) concentration, defined as the minimal concentration of antimicrobial (antifungal) compound that prevents any growth of the tested microorganisms (fungi), was determined by subculturing MIC broth tube without visible growth to a Mueller-Hinton agar and incubating for 20–24 h at 35–37°C temperature (for bacteria) and at 30°C (for fungi).

Results and discussion

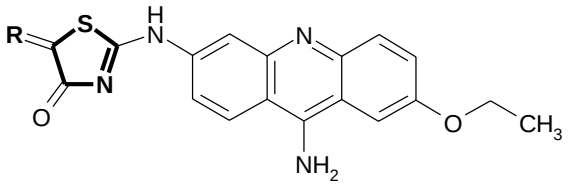
All new compounds were successfully synthesized. The structures of new compounds (2a-c) were confirmed by elemental analysis and spectral data (IR, NMR). All characterization data of new compounds are shown in Table 1.

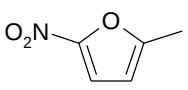
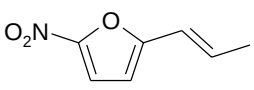
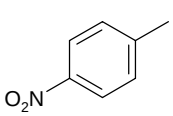
Aldehydes react with 2-methylrhodanine upon heating in acetic acid at 60°C. Boiling made reactions go faster but yields of products were lower, most likely because 2-methylrhodanine decomposed. As catalyst ammonium acetate was used because higher yields are obtained in this case.

Duration of reaction depends on nature of aldehyde. Reactions with nitrofuran derivatives are going faster (by forming compounds 1a and 1b), it is enough to heat these compounds with 2-methylrhodanine for 0.5 h. Reactions with 4-nitrobenzaldehyde are going slower (by forming 1c), in this case reaction mix has to be heated for 1 h. Compound 1c is better soluble in acetic acid compared to compounds having nitrofuran cycle in their structure so reaction mixture of compounds 1a is cooled in ice bath for longer time (for 4 h). Reaction yields also differs (82–91%), it depends on the nature of aldehyde. Yield of reaction between 4-nitrobenzaldehyde and 2-methylrhodanine is the lowest (82%).

Reactions of ethacridine derivatives were going in acetic acid. Solvent was chosen according to solubility of initial compounds 1a-c. Reaction mixture was heated at constant 90°C temperature because reaction products decomposed at higher temperature. Reaction process was monitored using lead acetate indicator. Reaction yields of all ethacridine derivatives were rather high, especially of those having 4-nitrobenzaldehyde moiety in fifth position of thiazolidine cycle (68–90%). Upon taking double excess of ethacridine reaction is going faster but excretion of reaction product is more complicated so it was taken only 1.5 times bigger amount of ethacridine.

Table 1. **Characterization data of compounds 2a-c**



Compound No.	R	Yield %	Melting point °C	Mol. formula M_r	Elemental analysis Calcd./found (%)			
					C	H	N	S
2a		78	>280	$C_{23}H_{17}N_5O_5S$ 475.49	58.10 58.73	3.60 3.89	14.73 14.23	6.74 6.78
2b		66	>280	$C_{25}H_{19}N_5O_5S$ 501.52	59.87 59.73	3.82 4.15	13.96 13.99	6.39 7.95
2c		71	>280	$C_{25}H_{19}N_5O_4S$ 485.53	61.85 61.55	3.94 3.75	14.42 14.53	6.60 6.80

For microbiological analysis of synthesized compounds bacteria *Staphylococcus aureus* (gram-positive bacterium), *Escherichia coli* (gram-negative bacillus), *Bacillus subtilis* (sporic bacterium) and *Kl. pneumoniae* (capsule forming bacterium) were chosen considering their different structural peculiarities.

As it is announced in literature *Candida* species are predominant mycotic pathogens (5, 8) so activity of synthesized compounds was investigated against several *Candida* species: *Candida albicans, C. glabrata, C. kefyr, C. krusei, C. parapsilosis,* and *C. tropicalis.*

The results which are obtained from antifungal and antimicrobial activities of new compounds (2a-c) are presented in Table 2.

Microbiological analysis showed that all new ethacridine derivatives posses antimicrobial and antifungal properties. New compounds 2a-c are characterized by higher antifungal and antimicrobial activity *in vitro* than ethacridine (MIC of ethacridine is >1000 μg/mL against all tested microorganisms). It is important to note that the activities of new ethacridine derivatives are different. The pattern of a compound that contains a nitrofuryl group in its structure (2a) is more active than others against tested bacteria and fungi. The lowest antibacterial activity of all new compounds is against capsule forming bacteria *K. pneumoniae*; compounds 2b and 2c are not very active against *E. coli* (MIC is in a range of 666–1000 μg/mL). Similar principles of structure – activity relationship were observed in our previous research (17). However, compound 2b having additional double bond in nitrofuran substituent is less active compared to compound 2a. The compound having nitrobenzene substituent in its structure (2c) possesses similar activity against bacteria and fungi as compared to nitrofuran component having ethacridine derivatives (2a-b). Our presumption regarding introduction of nitrofuran component especially having additional double bound in side chain of molecule for higher antimicrobial activity has not proven in this time. Nevertheless, activity of compound 2a (having nitrofuryl group) on tested bacteria is 2 to 8 times higher in comparison to the compound 2c (it has nitrobenzene group).

Though nitrofurilalilidene and nitrobenzene substituents having compounds possess similar activity

Table 2. **Antimicrobial and antifungal activity of new compounds**

			Compound		
			2a	2b	2c
MIC, μg/mL	Antibacterial data	*Staphylococcus aureus* ATCC 25923	62.5±4.6	500.0±46.6	250.0±18.9
		Escherichia coli ATCC 25922	83±6.3	666.0±49.2	1000.0±90.4
		Bacillus subtilis ATCC 6633	62.5±4.3	62.5±5.1	125.0±12.3
		Klebsiella pneumoniae ATCC 33499	500.0±44.3	500.0±41.7	>1000.0
	Antifungal data	*Candida albicans*	62.5±4.8	500.0±42.3	500.0±48.3
		Candida glabrata	16.5±0.8	333.0±22.9	500.0±49.7
		Candida krusei	62.5±4.5	500.0±43.3	250.0±19.7
		Candida kefyr ATCC 8614	16.5±0.7	666.0±54.1	250.0±21.1
		Candida tropicalis ATCC 8302	31.3±2.1	500.0±40.7	500.0±49.1
		Candida parapsilosis	10.0±0.6	750.0±59.4	750.0±58.5
MBC, μg/mL	Antibacterial data	*Staphylococcus aureus* ATCC 25923	250.0±18.0	1000.0±87.0	750.0±50.0
		Escherichia coli ATCC 25922	666.0±51.3	>1000.0	>1000.0
		Bacillus subtilis ATCC 6633	500.0±49.0	1000.0±86.9	1000.0±91.4
		Klebsiella pneumoniae ATCC 33499	>1000.0	>1000.0	>1000.0
	Antifungal data	*Candida albicans*	250.0±15.8	>1000.0	1000.0±86.7
		Candida glabrata	208.0±17.3	1000.0±79.8	>1000.0
		Candida krusei	250.0±19.0	>1000.0	750.0±49.8
		Candida kefyr ATCC 8614	125.0±12.1	1000.0±91.8	500.0±47.5
		Candida tropicalis ATCC 8302	333.0±20.5	1000.0±88.4	1000.0±95.6
		Candida parapsilosis	125.0±11.6	>1000.0	>1000.0

MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration.

against bacteria *in vitro*, nitrofuryl group enhance activity of such compounds.

Very similar principles could be applied for structure – antifungal activity of tested compounds 2a-c. Compound 2a was found the most active against all tested *Candida* spp. It is 8 to 75 times more active than other ethacridine derivatives against tested fungi. It can be presumed that introduction of nitrofuryl group into fifth position of thiazolidine ring having 2-ethacridine substituent has the biggest influence on antifungal activity. Nevertheless, more detailed experiments are required in this case. Compound 2a possesses similar anticandidal activity as fluconazole – the most popular antifungal preparation in nowadays (18).

It should be noted that nitrofuryl group in fifth position of thiazolidine cycle remarkably enhances activity of ethacridine derivatives against all tested bacteria and fungi *in vitro*.

New compounds are bacteriostatic and fungistatic in lower concentrations, but bactericidal and fungicidal in higher concentrations.

Obtained results could further help to design more active antimicrobial compounds having thiazole cycle in their structure.

Conclusions

- New compounds 2a-c are characterised by higher antifungal and antimicrobial activity *in vitro* than ethacridine;
- All new ethacridine compounds 2a-c posses antibacterial and antifungal activity *in vitro*;
- New ethacridine derivative 2a is 2 to 8 times more active compared to others and 8 to 75 times more active than others against tested *Candida* strains *in vitro*.

Nauji etakridino dariniai – potencialūs priešgrybeliniai ir antibakteriniai vaistai

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Raktažodžiai: etakridinas, tiazolidinas, nitrofuranas, priešgrybelinis aktyvumas, antibakterinis aktyvumas.

Santrauka. Iki 20 a. 8-ojo dešimtmečio grybelinės infekcijos buvo gana lengvai išgydomos, todėl nebuvo naujų vaistų poreikio. Vis dėlto menkas priešgrybelinių preparatų pasirinkimas ir esamų vaistų toksiškumas, ribotas jų veikimas, polinkis sukelti atsparių padermių radimąsi rodo naujų veiksmingų vaistų, kuriais būtų galima išgydyti sistemines grybelines infekcijas, kūrimo poreikį šiandien.

Darbo tikslas – sintezuoti antimikrobinius junginius, savo struktūroje turinčius tris farmakoforus. Pradiniai junginiai – 5-pakeisti 2-metilmerkaptotiazolidin-4-onai buvo S-demetilinti ir sintezuoti 2-aminopakeisti tiazolidinonai. Kaip farmakoforai, turintys amino ar aldehido grupę, vartoti etakridinas, nitrofurano ir nitrobenzeno aldehidai. Antimikrobinis (priešgrybelinis) naujų junginių aktyvumas nustatytas *in vitro* su šiomis mikroorganizmų kultūromis: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 33499 ir grybelinėmis kultūromis: *Candida albicans* ATCC 60193, *Candida glabrata, Candida krusei, Candida kefyr* ATCC 8614, *Candida tropicalis* ATCC 8302, *Candida parapsilosis*. Nustatyta, kad naujų junginių antimikrobinis aktyvumas yra didesnis palyginti su pradiniu junginiu etakridinu. Etakridino dariniai aktyvūs ne tik prieš gramteigiamas ir gramneigiamas bakterijas, bet ir prieš tirtus grybelius. Mažiausia junginių fungistatinė koncentracija yra 10,0–750 μg/ml, o antibakterinė koncentracija – 62,5–1000 μg/ml. 2a junginys, kurio tiazolidino cikle penktoji padėtis papildyta nitrofurilo pakaitu, yra aktyviausias iš sintezuotų etakridino darinių. Remiantis šio tyrimo duomenimis, būtų galima išskirti perspektyvią potencialių antiinfekcinių junginių grupę.

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