ECOLOGICAL CHEMISTRY AND ENGINEERING A

Vol. 17, No. 10 2010

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SEARCH FOR AN EFFICIENT COMPOUND WITH ANTIFUNGAL PROPERTIES INHIBITING *Fusarium* GENUS FUNGI

POSZUKIWANIE SKUTECZNEGO ZWIĄZKU O WŁAŚCIWOŚCIACH PRZECIWGRZYBICZYCH HAMUJĄCEGO ROZWÓJ GRZYBÓW Z RODZAJU *Fusarium* NA RÓŻNYCH GENOTYPACH ZBÓŻ

Abstract: A food quality finds considerable consumer's interests. Presence of contaminants in food is one of the principle criteria of foodstuff health safety assessment. Toxins of moulds, namely of *Fusarium* genus called mycotoxins, that are characterized by acute toxic action, are one of the most dangerous biological--origin substances found in food. Farm products may be contaminated with these metabolites beginning from the crop development in a field (mainly cereals), through their harvest, to storage and transport of final products. In order to reduce the food contamination due to mycotoxins, it is necessary to apply properly selected plant protection means. Own study upon achieving the active substances allowed for selecting compounds with promising biological action, that would become potential fungicides controlling moulds.

Keywords: mycotoxins, *Fusarium* species, antifungal activity, minimal inhibitory concentration – MIC

Fusarium genus fungi are the most isolated pathogens of worldwide crops [1, 2]. They are the reason of enormous economic loss resulting from their high pathogenecity and toxin-formation abilities, thus they are counted to the most dangerous filamentous fungi.

Fusarium infects crops of the basic economical importance for human [3], they occur on all cultivated winter and spring cereal species and can infect plants at their seedling, sprouting, and flowering stages [4–6]. Head blight is the most dangerous and economically significant cereal disease caused by *Fusarium* spp. [7]. It affects the lower ear weight and grain number per ear, plant growth inhibition, lower starch content in grains, and considerable worse baking quality of a flour achieved from infected grains [8].

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Under Polish climatic conditions, head blight is most frequently caused by a complex of diverse species such as *Fusarium culmorum*, *Fusarium avanaceum*, and *Fusarium graminearum* [9]. Quite large percentage of another pathogen has been lately observed – *Fusarium poae* that infects oats and other cereals panicles in Lublin region and eastern Poland [10, 11]. In addition, weather conditions (high air moisture and high temperatures, namely during flowering), as well as improper grain storage and transport conditions, favor mould development [12]. Deoxynivalenol (DON) is the most dangerous mycotoxin produced by *Fusarium* genus fungi. It is accumulated in infected plant cells and that way it penetrates the human's and animal's nutrition chain [13, 14]. Mycotoxins are low-molecular substances, against which an organism fails to produce any antibodies. They are lipophilic and hence they are prone to be deposited in fat cell fractions at plants and animals [15]. Prolonged exposure to mycotoxin action may result in endocrinological, neurological, and tumor diseases.

The problem of raw materials and foodstuff contamination with *Fusarium* genus fungi and their toxic metabolites cannot be eliminated applying traditional agrotechnical operations. An appropriate using properly selected fungicides, both in a view of fighting against diseases, existing weather conditions, and the rate applied are needed. However, quite great resistance of fungi against nowadays used antifungal means is a serious problem at controlling head blight. Due to fast mutation of strains towards some fungal species, up-to-date applied plant protection means became ineffective, while others retained their action, although at much increased doses. Furthermore, in the case of some mould strains, the active substance should be applied only once.

Therefore, studies upon achieving modern fungicides with systemic action that would have bilateral features, are extremely important at present. The own study conducted up-to-date included synthesis of diverse 2,4-dihydroxythiobenzamide derivatives in a view of their high activity towards phytopathogenic and yeast-like fungi, as well as dermatophytes [16–18]. Achieved results are promising and allow for searching for agents efficient to a narrow selected group of fungi – those of *Fusarium* genus.

Materials and methods

The study involved 13 different thiocarbonyl derivatives (Fig. 1) that were produced by means of organic synthesis at Chemistry Department, University of Life Sciences in Lublin, in accordance with the patented reaction mechanism [19]. Applying high--performance liquid chromatography (HPLC), the purity of achieved compounds could be confirmed.

In order to evaluate the biological activity of newly synthesized derivatives, *Fusarium* genus fungal species were tested; they were selected due to their presence in cereals and cereal-origin products.

Material for study comprised moulds. The material for inoculation was transferred onto Sabouraud substrate. Inoculum was made up of suspension containing 10^5 cfu per 1 cm^3 (milliliter). Aliquots of 20 mm^3 (μl) suspension were transferred onto Petri dishes with studied mediums containing corresponded concentrations of tested compounds.

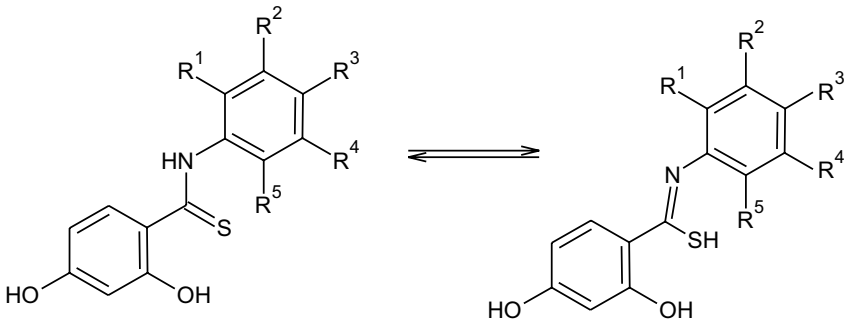

Fig. 1. Chemical structures of substituted 2,4-dihydroxythioamides

The control was made up of above inoculum on studied mediums that lack tested compounds or with 1 % DMSO. The incubation was performed at 27 ^{o}C, and *minimal inhibitory concentration* (MIC) reading was made after 5 days. Tests were conducted at Department of Pharmaceutical Microbiology, University of Medicine in Lublin.

Subsequent part of biological examinations was performed at Sub-department of Pesticide Applying and Formulation, Institute of Organic Industry in Warsaw (GLP certificate, Statement of GLP Compliance No. G013). Following standard fungicides were applied: carbendazyme (commercial name – Sarfun 500 S.C.) and precymidone (commercial name – Sumilex 500 S.C.). Assays were made in accordance with laboratory method for antifungal activity assessment (procedure SPR/FA_2/11) by estimating the influence of particular concentrations of tested compound present in a medium on growth of fungal colony that causes crop diseases.

Solution or suspension of tested compound was added to sterile and cooled PDA medium (3.9 g of DDA medium (Difco) + 0.2 g of agar in 100 cm^3 of distilled water) to achieve needed concentration in substrate. Tested compounds were dissolved in acetone and water mixture (1.5:1), while fungicides were dissolved in water.

Inoculum with mycelium of tested strains was put into solidified medium on 5 cm diameter Petri dishes. Dishes were incubated at 21 ^{o}C for 5 days. Then, the diameter of fungal colonies was measured. Based on Abbot's formula, it served for calculating the inhibition efficiency of tested compound or standard at particular concentration, the inhibition of mycelium linear growth was measured.

Following criteria of action efficiency were used:

Efficiency of linear growth [%]	Ranking of fungicidal control	
	score	action
Above 80 %	3	good
50–80 %	2	moderate
20–50 %	1	poor
20 % and less	0	no action

The assessment was made at 200 mg/dm^3 and 20 mg/dm^3 concentrations. The experiment was carried out in two replications for each combination.

Table 1

The MIC (*minimal inhibitory concentration*) values mg/dm^3 of itraconazole and fluconazole and thioamide compounds against *Fusarium* genus fungi, n = 11, on the Sabouraud's medium after 5 days of incubations

Comp. no.	Biological activity compounds	*Fusarium moniliforme* n = 2	*Fusarium graminearum* n = 1	*Fusarium poae* n = 1	*Fusarium culmorum* n = 2	*Fusarium oxysporum* n = 3	*Fusarium avanaceum* n = 2
1	*N*-5(2'methoxy,phenyl)-2,4-dihydroxythiobenzamid	100	100	100	100	100	100
2	*N*-1-(4'-benztrifluoromethyl)-2,4-dihydroxythiobenzamid	50	100	100	200	50	172.4
3	*N*-1-(3',5'-ditrifluoro methylbenz)-2,4-dihydroxythiobenzamid	50	50	50	100	50	50
4	*N*-1-(2'-izopropylo-4'-chlorobenz)-2,4-dihydroxythiobenzamid	50	25	50	100	50	100
5	*N*-1-(4'-fluoro,3'nitrobenz)-2,4-dihydroxythiobenzamid	25	50	100	200	100	100
6	*N*-2-(1'-phenylpirolo)2,4-dihydroxythiobenzamid	12.5	100	25	100	100	100
7	*N*-1-(5,6,7,8-tetrahydronaphthyl)-2,4-dihy droxythiobenzamid	100	100	200	200	200	100
8	*N*-1-(3',4'-benzmethyldiokso)-2,4-dihyroxythiobenzamid	200	200	200	200	200	200
9	*N*-1-(3,4-benzyldiokso)-2,4-dihydroxythiobenzamid	50	100	200	200	100	100
10	*N*-3(1,2,4-dithiazolo-5-thiono)-2,4-dihydroxythiobenzamid	25	78.5	25	200	46.8	62.4
11	*N*-(phenyl)-5-chloro-2,4-dihydroxythiobenzamid	25	100	100	200	180	187.5
12	*N,N*-[2-ethyl-3-(4'-chlorobenz)-crotoniano]-2,4-dihydroxythiobenzamid	25	50	25	100	50	25
13	*N,N*-[5-(1,2,3,4-tiatriazolo)-phenyl]-2,4-dihydroxythiobenzamid	25	62	25	50	23.5	29.2
14	Itraconazole	12.5	50	100	200	150	183.3
15	Fluconazole	100	100	200	200	200	100

Results and discusion

Evaluating the inhibition action of tested derivatives was expressed by means of determining the lowest inhibitive concentration MIC, ie the lowest concentration of subsequent dilutions of biologically active substance at which no microorganism growth can be observed. The MIC values are considered as a measure of compound effectiveness. MIC values were achieved for all tested preparations and for two reference substances towards *Fusarium* genus fungi (Table 1).

Fusarium culmorum appeared to be the most resistant – mean MIC value for applied derivatives amounted to 150 mg/dm^3, while *Fusarium moniliforme* was the least resistant – mean MIC was –56.73 mg/dm^3. Action of itraconasole and fluconasole also confirmed results achieved for tested compounds (*F. culmorum* – average MIC – 200 mg/dm^3, *F. moniliforme* – average MIC – 56.25 mg/dm^3).

The result analysis revealed that majority of tested compounds showed quite good biological activity, and their action was comparable with that of standard preparations. Derivatives No. 7, 8, and 9 were characterized by moderate activity – MIC values for them were within range of 125–200 mg/dm^3. Results for compounds No. 3, 4, 12, and 13 were much more promising – mean activities were 3-5-fold higher than for itraconasole and fluconasole. Particularly high action was recorded for *N,N*-[5-(1,2,3,4--thiatriasol)-phenyl]-2,4-dihydroxythiobenzamide (compound No. 13) with mean MIC – 35.78 mg/dm^3. It manifested 8-fold higher activity in relation to the most resistant *F. culmorum* than reference preparations and other compounds used in tests. Similar high action was observed towards other *Fusarium* genus fungi species.

Activity and functioning of tested compounds towards phytopathogenic fungi

During the laboratory tests (Table 2) at 200 mg/dm^3 concentration, all studied compounds revealed significant fungistatic activity (inhibition level remained at the level about 61–100 %). Compounds No. 3, 4, 5, 6, 10, 12 and 13 showed extremely strong inhibition action by complete inhibition of all three pathogens. Other derivatives also acted towards all three *Fusarium* genus fungi species although with 61–80 % efficiency. When compounds were applied at lower concentration (20 mg/dm^3), for majority of them, the inhibition activity decreased, or even complete lack of biological action was observed in some cases. However, derivatives No. 4, 6, 10, 12 and 13 showed quite good inhibition activity – depending on the pathogen type, their action was within 41–100 % range. On a base of earlier own studies [17, 20] and along with analysis of here achieved results, it can be confirmed that chemical structure of a molecule determines the biological action of these compounds. At the most active derivatives No. 6, 10 and 13, presence of heterocyclic substituents (pyrole, dithiasole, and tiatriasole) apparently affected the elevated activity. Presence of sulfur and nitrogen atoms having free electron pairs favors the compound's conformation during penetrating the barrier of biological membrane, thus has positive effects of derivative's biological action.

Table 2

Antifungal efficiency of 13 compounds and 2 standard fungicides against *Fusarium* genus fungi

Compound	*Fusarium culmorum*		*Fusarium graminearum*		*Fusarium poae*	
	200 mg/dm^3	20 mg/dm^3	200 mg/dm^3	20 mg/dm^3	200 mg/dm^3	20 mg/dm^3
1	3	1	3	1	3	0
2	3	1	3	1	3	1
3	4	2	4	1	4	1
4	4	3	4	4	4	2
5	4	2	4	3	4	3
6	4	3	4	4	4	3
7	3	2	3	2	3	1
8	3	1	3	2	3	1
9	3	0	3	1	3	0
10	4	2	4	4	4	4
11	3	1	3	3	3	2
12	4	3	4	4	4	2
13	4	2	4	4	4	4
Carbendazyme	4	4	4	4	4	4
Precymidone	—	—	4	4	4	4

Efficiency of linear growth in %:
[0] – 0–20 %; [1] – 21–40 %; [2] – 41–60 %; [3] – 61–80 %; [4] – 81–100 %.

Among tested fungi, *Fusarium culmorum* was the most resistant, while *Fusarium graminearum* the least resistant; for the latter, values of mycelium growth inhibition were the highest, both at 200 mg/dm^3 and 20 mg/dm^3 concentrations of tested compounds.

Having taken into account the results from studies involving standards being biologically active substances (procymidone and karbendazyme), achieved derivatives may be interesting research objects. It refers in particular to compounds, the inhibition action of which is at the same level as that of applied standard fungicides. They can be considered as new leading systems and use them in further *in vitro* tests towards other types of pathogens as well as at *in vivo* studies.

Conclusions

Conducting more detailed and wider *in vitro* and *in vivo* experiments seems to be purposeful, because it would allow for more accurate learning the antifungal action scope of high-activity compounds.

Modifying the molecule structure, mainly by introducing the heterocyclic substituents containing nitrogen and sulfur atoms might be also efficient, which seems to have positive influence of increased biological activity of synthesized derivatives.

Performed experiments and tests give the hope for isolating the compound that would be highly effective in controlling the moulds responsible for introducing mycotoxins into the food and cereal products.

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POSZUKIWANIE SKUTECZNEGO ZWIĄZKU O WŁAŚCIWOŚCIACH PRZECIWGRZYBICZYCH HAMUJĄCEGO ROZWÓJ GRZYBÓW Z RODZAJU *Fusarium* NA RÓŻNYCH GENOTYPACH ZBÓŻ

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Abstrakt: Jakość żywności wzbudza szczególne zainteresowanie konsumentów. Obecność zanieczyszczeń w żywności jest jednym z podstawowych kryteriów oceny bezpieczeństwa produktów żywnościowych. Do niebezpiecznych substancji pochodzenia biologicznego coraz częściej spotykanych w żywności należą toksyny grzybów pleśniowych, szczególnie z rodzaju *Fusarium* zwane mikotoksynami, charakteryzujące się ostrym działaniem toksycznym. Produkty rolne mogą ulec zanieczyszczeniu tymi metabolitami począwszy od rozwoju roślin na polu (głównie zboża), poprzez zbiór, po przechowywanie i transport gotowych produktów. W celu ograniczenia skażenia żywności mikotoksynami konieczne jest stosowanie odpowiednio dobranych środków ochrony roślin. Badania własne nad otrzymywaniem substancji aktywnych pozwoliły wybrać związki o obiecującym działaniu biologicznym, które mogą stać się potencjalnymi fungicydami, zwalczającymi grzyby pleśniowe.

Słowa kluczowe: mikotoksyny, grzyby z rodzaju *Fusarium*, aktywność przeciwgrzybicza, MIC, 2,4-dihydroxytiobenzamidy