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Copper Complexes with Bioactive Ligands

Part II — Antifungal Activity*

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ABSTRACT. Antifungal activity of new copper(II) complexes of 2-methylthionicotinate (2-MeSNic) of the composition $Cu(2\text{-MeSNic})_2(MeNia)_2 \cdot 4H_2O$ (where MeNia is *N*-methylnicotinamide), and $Cu(2\text{-MeSNic})_2(Nia)_2 \cdot 2H_2O$ (where Nia is nicotinamide) and $Cu(2\text{-MeSNic})_2L_2$ (where L is isonicotinamide, iNia, or ethyl nicotinate, EtNic) were tested on various strains of filamentous fungi by the macrodilution method. Most sensitive against copper(II) adducts with bioactive ligands were *Rhizopus oryzae* and *Microsporum gypseum* (IC_{50} 1.5–2.3 mmol/L). The adducts with Nia, MeNia and EtNic at 5 mmol/L induced morphological changes in growing hyphae of *Botrytis cinerea*, mainly their intensive branching attached to release of cytoplasm with partial growth inhibition. Inhibition of sporulation (>90 %) of *Alternaria alternata* by $Cu(2\text{-MeSNic})_2 \cdot H_2O$ was observed as a change in the color of the colonies. The highest resistance was marked by *B. cinerea* and *Fusarium moniliforme* (average IC_{50} values 4.25 and 3.13 mmol/L, respectively). The presence of all bioactive ligands in copper(II) complexes caused an increase in the inhibition effect against model fungi (except significant inhibition activity of EtNic on *R. oryzae*).

A number of data indicate that endogenous copper plays an important role in many biochemical processes (Gabriel *et al.* 2000). Four μg/mL $CuTAABCl_2$ caused depolymerization of filamentous structures in HepG2 cells. Urbančíková and Jantová (1997) assummed that partial restoration of actin filamentous structures in transformed cells by $CuTAABCl_2$ leads to partial restoration of the regulation mechanism of cell proliferation manifested by its inhibition.

Antimicrobial activity of selected aqua–carboxyl–cupric complexes was examined by using the 1st screening methods on anthropo- and phytopathogenic microorganisms. The effects of all aqua-complexes on bacteria and yeasts were minimal. On the other hand, the activity of these substances on the agents causing dermatomycoses (*Trichophyton terrestre*, *Microsporum gypseum*) achieved an MIC value of 500 μg/mL and lower (Sokolík *et al.* 1992). They found that the copper(II) complexes increase sporulation of *Aspergillus niger* and concurrently kills the yeast *Candida albicans*. These effects were attributed to a slow release of Cu^{2+} ions and thiophene oligomers into the culture medium (Čík *et al.* 2001). Other authors (Moncoľ *et al.* 2000) observed that in the presence of the complex $[Cu(CH_3CCl_2COO)_2Nia_2]$ (Nia = nicotinamide) *Aspergilus niger* grew as a white colony rather than a black one. *A. niger* probably lost the ability to form spores. When a white colony was moved to a cultivation medium without the compound, the colony became black again. Morphology changes were also observed with other fungi, *e.g.* hyphae of *Botrytis cinerea* were more branched and were releasing more cytoplasmic content in their tips when some quinoline derivatives were present (Strigáčová *et al.* 2000).

Results of the Ames test performed on various strains of *Salmonella typhimurium* showed that copper(II) salicylate, copper(II) niflumate and their ronicol adducts did not significantly increase the number of spontaneous revertants and showed no mutagenic activity. A lower mutagenic effect was observed in the promutagens 2-aminoanthracene and 2-aminofluorene following the treatment of all copper(II) complexes (Mikulášová *et al.* 1998). Similarly, noncoordinated 4-nitropyridine-*N*-oxide exhibited genotoxic activity, while complexation with phenoxyacetatocopper(II) complex of this chemical mutagen reduced its mutagenic effect (Bláhová *et al.* 1994).

*Part I: Copper complexes with bioactive ligands. Antimicrobial activity. *Folia Microbiol.* 46, 379–384 (2001).

In the group of mononuclear aqua–cresoxyacetato–copper^{II} complexes and binuclear phenazone–(*o*-, *m*-, *p*-cresoxyacetato)–copper^{II} complexes the antiinflammatory activity assayed on rat-paw carrageenan-induced edema, was found. In general, majority of copper(II) complexes tested were clearly more effective than the corresponding free acids or copper(II) salicylate tetrahydrate and salicylic acid (used as standard) were less active (Bláhová *et al.* 1998).

The available evidence supports a dimeric structure for $Cu(2\text{-MeSNic})_2 \cdot H_2O$. The remaining complex was also studied by X-ray analysis (Mikloš *et al.* 2001). Some of the biological activities of these copper(II) complexes were described by Dudová *et al.* (2001).

The aim of this paper was to determine the antifungal activity of 10 newly synthesized copper(II) complexes with bioactive ligands using various filamentous representatives, *e.g.* phytopathogenic and dermatophytic fungi. We also describe the observed morphology changes of two model representatives.

MATERIALS AND METHODS

Materials. Filamentous fungi *Rhizopus oryzae*, *Alternaria alternata*, *Fusarium moniliforme* (*Collection of Microorganisms of Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology*, Bratislava, Slovakia), *Botrytis cinerea* CCM F-16 and *Microsporum gypseum* (*Laboratory of Medical Mycology, Postgraduate Medical Institute*, Bratislava, Slovakia) were used.

The derivatives of chromatographic-grade purity were dissolved in Me_2SO, the final concentration of which never exceeded 1 % (*V*/*V*).

Antifungal assay. The effects of Cu^{II} complexes on filamentous fungi were tested during static cultivation. Fifty μL of the tested compound in Me_2SO were added to Petri dishes (diameter 50 mm, the final concentrations of Cu^{II} complexes ranged from 1 to 10 mmol/L) just before pouring 5 mL Sabouraud's (dermatophytes) or malt extract (other tested fungi) agar (*Imuna*, Šarišské Michaľany, Slovakia). The solidified plates were then inoculated in the center with 5 μL of the spore suspension (approximate spore concentration 100/μL in 0.1 % aqueous Tween 80) of filamentous fungi from 21-d-old strains. Triplicate sets of agar plates were incubated at 25 °C and the diameter of growing colonies was measured (Hudecová *et al.* 1996).

The antimicrobial effect was characterized by IC_{50} and MIC values, which were read from the toxicity curves.

Effect on the morphology of Botrytis cinerea. Microphotographs of hyphae growing into the cultivation medium with the compounds were taken *in situ* after staining with 0.5 % (*W*/*V*) methyl blue in lactophenol (Hudecová *et al.* 1994).

Inhibition of the sporulation of Alternaria alternata. The suspension of spores was prepared from a 7-d-old colony of the fungus (obtained in the same way as in the antifungal assay) by scraping it into 5 mL 0.1 % aqueous Tween 80 and after the removal of mycelia by filtration through a nylon mesh. The number of spores was determined using a hæmocytometer.

RESULTS AND DISCUSSION

Antifungal activity of Cu^{II} complexes is summarized in Table I. The tested complexes vary greatly in their antifungal activity. The highest inhibition effect on *R. oryzae* was observed with $Cu(2\text{-MeSNic})_2(EtNic)_2$ (IC_{50} 1.5 mmol/L). The decrease in growth, caused by the presence of EtNic adduct in the growth medium, is demonstrated in Fig. 1. A significant inhibition effect of EtNic (IC_{50} 2.1 mmol/L) was manifested in the above case, probably because the free bioactive ligand alone represented an inhibition force similar to some other adducts, even if it has not influenced the viability of *R. oryzae* in any concentration tested (MIC > 10 mmol/L). The other adducts with bioactive ligands influenced *R. oryzae* at approximately same level (IC_{50} 2.0–2.3 mmol/L) and they stopped growth completely at 5 mmol/L, with a static effect on spores.

Only two compounds (*viz.* $Cu(2\text{-MeSNic})_2 \cdot H_2O$ and $Cu(2\text{-MeSNic})_2(iNia)_2$; both with IC_{50} 1.8 mmol/L) were significantly active against *A. alternata*. The rest of the complexes showed weak effects (IC_{50} 2.3–3.0 mmol/L). At the same time, $Cu(2\text{-MeSNic})_2 \cdot H_2O$ inhibited the sporulation of this fungus by more than 90 % at 5 mmol/L. The influence on sporulation was observed as changes of

colony color from black to white caused by the decrease of the spore concentration. The counts of spores after action of this derivative are shown in Table II.

Table I. Antifungal activity of Cu^{II} complexes characterized by IC_{50} (mmol/L) and MIC (mmol/L)†

Compound	*Rhizopus oryzae*		*Alternaria alternata*		*Botrytis cinerea*		*Fusarium moniliforme*		*Microsporum gypseum*	
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
2-MeSNic	inact		9.0	>10	inact		9.7	10	6.4	>10
$Cu(2\text{-MeSNic})_2 \cdot H_2O$	7.0	10^S	1.8	10^C	8.0	>10	7.0	10^S	2.1	5^S
$Cu(2\text{-MeSNic})_2(Nia)_2 \cdot 2H_2O$	2.0	5^S	3.0	10^S	3.9	10^S	2.1	5^S	1.5	5^C
$Cu(2\text{-MeSNic})_2(iNia)_2$	2.3	5^S	1.8	10^S	3.5	>10	3.1	10^S	2.3	5^C
$Cu(2\text{-MeSNic})_2(MeNia)_2 \cdot 4H_2O$	2.0	5^S	2.3	>10	7.0	>10	5.2	>10	1.7	5^C
$Cu(2\text{-MeSNic})_2(EtNic)_2$	1.5	5^S	3.0	10^S	2.6	10^S	2.1	5^S	1.8	5^C
Nia	inact		inact		inact		inact		8.2	>10
iNia	inact		inact		inact		inact		8.3	>10
MeNia	inact		6.5	>10	6.3	>10	inact		inact	
EtNic	2.1	>10	8.5	>10	9.8	>10	inact		7.8	>10

^{S}Concentration inducing a fungistatical effect. ^{C}Concentration inducing a fungicidal effect (MMC).
†inact — inactive compound (IC_{50} > 10 mmol/L).

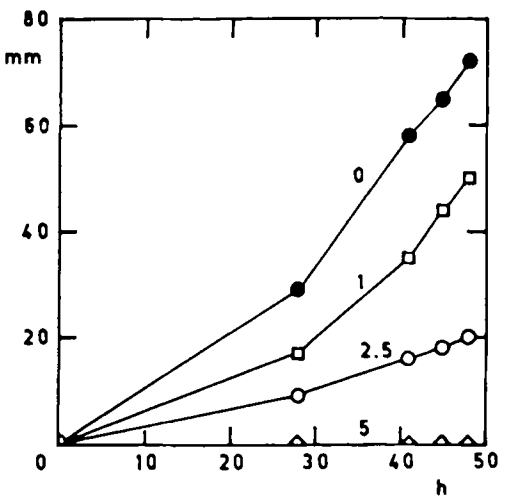

Fig. 1. Growth inhibition (colony diameter, mm) of *R. oryzae* by $Cu(2\text{-MeSNic})_2(EtNic)_2$; *numbers at curves* — final concentration (mmol/L).

The lowest antifungal effect was found with *B. cinerea* and *F. moniliforme*. The majority of the complexes influenced growth weakly. Only the effects of $Cu(2\text{-MeSNic})_2(Nia)_2 \cdot 2H_2O$ and $Cu(2\text{-MeSNic})_2(EtNic)_2$ against *F. moniliforme* (with IC_{50} 2.1 mmol/L and MIC 5 mmol/L) and the effect of $Cu(2\text{-MeSNic})_2(EtNic)_2$ against *B. cinerea* could be noticed. Significant morphological changes of *B. cinerea* accompanied by partial growth inhibition were also observed. $Cu(2\text{-MeSNic})_2(Nia)_2 \cdot 2H_2O$ at 5 mmol/L and the derivative $Cu(2\text{-MeSNic})_2(MeNia)_2 \cdot 4H_2O$ at 10 mmol/L had a ramification effect on growing hyphal tips (Fig. 2B,C). These changes were accompanied by the release of cytoplasm as a result of the presence of $Cu(2\text{-MeSNic})_2(EtNic)_2$ at 5 mmol/L (Fig. 2A). *M. gypseum* was the fungus most sensitive to the tested compounds. Even $Cu(2\text{-MeSNic})_2 \cdot H_2O$ alone showed a strong antimicrobial activity against *M. gypseum* (IC_{50} 2.1 mmol/L) and this efficiency depended on the genesis of adducts.

Table II. Inhibition of sporulation (%) of *A. alternata* by $Cu(2\text{-MeSNic})_2 \cdot H_2O$ (*c*, mmol/L)

c	Colony	Colony, cm^2
0	100	100
1	2	9

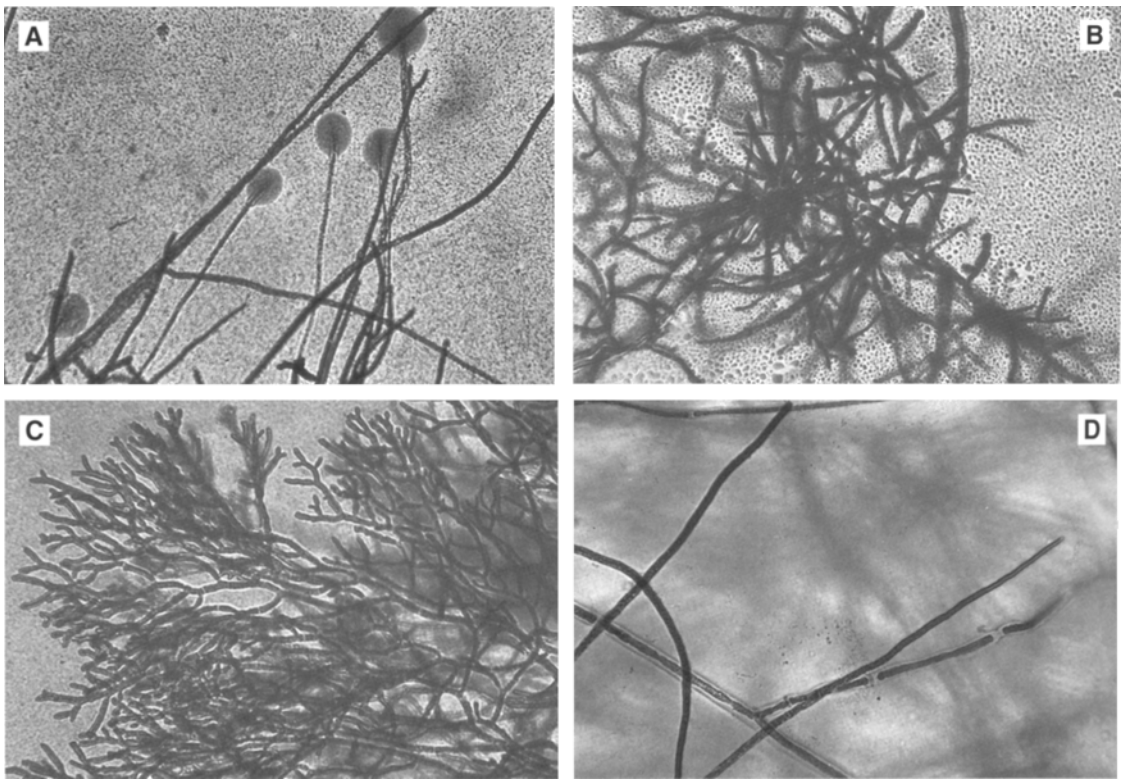

Fig. 2. Changes in the morphology of *B. cinerea* hyphal tips induced by $Cu(2\text{-MeSNic})_2(EtNic)_2$ (5 mmol/L; **A**), $Cu(2\text{-MeSNic})_2(MeNia)_2{\cdot}4H_2O$ (10 mmol/L; **B**), $Cu(2\text{-MeSNic})_2(Nia)_2{\cdot}2H_2O$ (5 mmol/L; **C**), and control (**D**); magnification 140×.

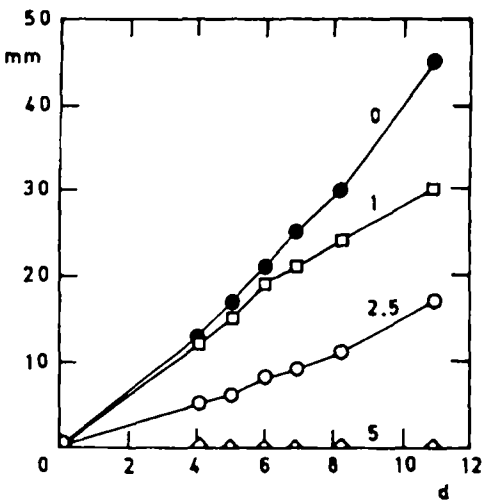

Fig. 3. Growth inhibition (colony diameter, mm) of *M. gypseum* by $Cu(2\text{-MeSNic})_2(Nia)_2{\cdot}2H_2O$; *numbers at curves* — final concentration (mmol/L).

The Cu^{II} complexes with bioactive ligands not only stopped the growth by 100 % at 5 mmol/L, but they a had lethal effect on spores. The IC_{50} values for this dermatophyte ranged from 1.5 to 2.3 mmol/L. All free bioactive ligands were inactive except for the mentioned EtNic effect against *R. oryzae* (IC_{50} 2.1 mmol/L). The Cu^{II} compounds inhibited the used biological objects more than free 2-MeSNic (a compound without copper at $IC_{50} \geq 6.4$ mmol/L) did. This inhibition activity was more pronounced when bioactive ligands were present in molecules, while on their own they showed only a weak activity ($IC_{50} \geq 6.3$ mmol/L).

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REFERENCES

BLÁHOVÁ M., SOKOLÍK J., LAHITOVÁ N., MURÍN A.: Mutagenic activity of phenoxyacetatocopper(II) complexes. *Čes.Slov.Farm.* **43**, 240–242 (1994).

BLÁHOVÁ M., TŮMOVÁ I., SOKOLÍK J., OROSOVÁ I., ŠVEC P.: Antiinflammatory activity of (cresoxyacetato)copper(II) complexes. *Čes.Slov.Farm.* **47**, 166–169 (1998).

ČÍK G., BUJDANOVÁ H., SERSEN F.: Study of fungicidal and antibacterial effect of the Cu(II)-complexes of thiophene oligomers synthesized in ZSM-5 zeolite channels. *Chemosphere* **44**, 313–319 (2001).

DUDOVÁ B., HUDECOVÁ D., POKORNÝ R., MIKULÁŠOVÁ M., PALICOVÁ M., SEGĽA P., MELNÍK M.: Copper complexes with bioactive ligands. Part I. Antimicrobial activity. *Folia Microbiol.* **46**, 379 384 (2001).

GABRIEL J., SHAH V., NESMĚRÁK K., BALDRIAN P., NERUD F.: Degradation of polycyclic aromatic hydrocarbons by the copper(II)–hydrogen peroxide system. *Folia Microbiol.* **45**, 573–575 (2000).

HUDECOVÁ D., JANTOVÁ S., MELNÍK M., UHER M.: New azidometalkojates and their biological activity. *Folia Microbiol.* **41**, 473–476 (1996).

HUDECOVÁ D., VAREČKA Ľ., VOLLEK V., BETINA V.: Growth and morphogenesis of *Botrytis cinerea*. Effects of exogenous calcium ions, calcium channel blockers and cyclosporin A. *Folia Microbiol.* **39**, 269–275 (1994).

MIKLOŠ D., SEGĽA P., PALICOVÁ M., KOPCOVÁ M., MELNÍK M., VALKO M., GLOWIAK T., KORAIBIK M., MROZINSKI J.: Synthesis, spectral and magnetic properties, and crystal structures of copper(II) 2-methylthionicotinate adducts with chelating ligands. *Polyhedron* **20**, 1867–1874 (2001).

MIKULÁŠOVÁ M., SZABOVÁ E., MELNÍK M.: Antimutagenic effect of copper(II) complexes. *Biologia* **53**, 719–726 (1998).

MONCOĽ J., KOMAN M., MELNÍK M., ČERNÁKOVÁ M., GLOWIAK T.: Study of copper(II) chloro- and dichloropropionates, crystal and molecular structure of *trans*-bis(methyl-3-pyridylcarbamate)bis(α,α-dichloropropionato)copper(II). *Polyhedron* **19**, 2573–2577 (2000).

SOKOLÍK J., BLÁHOVÁ M., LUČANSKÁ B., MLYNARČÍK D., VALENT A., ANDRIEZÁLOVÁ L., ŠVAJLENOVÁ O., ŽEMLIČKA M.: Antimicrobial activity of selected aqua–carboxyl–cupric complexes. *Čes.Slov.Farm.* **41**, 40–46 (1992).

STRIGÁČOVÁ J., HUDECOVÁ D., VAREČKA Ľ., LÁSIKOVÁ A., VEGH D.: Some biological properties of new quinoline-4-carboxylic acid and quinoline-4-carboxamide derivatives. *Folia Microbiol.* **45**, 305–309 (2000).

URBANČÍKOVÁ M., JANTOVÁ S.: The effect of Cu^{2+} complex induces partial restoration of actin filamentous structures in transformed cells. (In Slovak) *Vojenské zdravotnícke listy, Suppl.* **66**, 16 17 (1997).