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Synthesis, characterization and microbiological activity of a Zn(II) complex of a novel benzofurazan derivative

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Dedicated to Acad. Bogdan Kurtev on the occasion of his 100th birth anniversary

Complex formation of Zn(II) and Al(III) ions with a novel 4-nitro-benzofurazan-cyclam conjugate has been investigated by ^{1}H-NMR and electronic (UV-vis and fluorescence) spectroscopy in dimethylsulfoxide. A stable Zn(II)) complex [Zn(**P1**)$(NO_3)_2$] has been isolated, characterised, and *in vitro* its antimicrobial activity was tested. Good antibacterial activity against several Gram-positive and Gram-negative bacteria and antifungal activity against two yeasts has been observed.

Key words: metal complexes, cyclam, benzofurazane, microbiological, NMR

INTRODUCTION

In recent years, much attention has focused on the synthesis of new metal complexes of biologically important metal ions such as Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+} with various organic ligands as potential antimicrobial agents which improve antifungal or antibacterial activity of existing chemotherapeutics [1–5]. On the other hand, the design and synthesis of specific ligands which can coordinate to metal ions is a major goal to many research groups because the biological activity of the metal complexes depend not only on the nature of the metal ions and the chemical structure of the ligands, but also on the type of bonding between them. Although some metal complexes with organic ligands showed very good antimicrobial activity, studies are still in search of new highly active antibacterial and antifungal compounds. This reflects the fact that many of the clinical pathogens quickly become resistant to chemotherapy applied in practice.

Cyclam (1,4,8,11-tetraazacyclotetradecane) is a cyclic polyamine most widely used as a ligand in medical chemistry research which is due to its commercial availability and the easy linking of nitrogen atoms to functional units. In this case the coordination sites of cyclam-cored derivatives are the tertiary amino groups from cyclam ring. The complexes with biologically important metal ions have been investigated with regard to their potential biomedical applications [6-10]. Recently we have investigated *in-vitro* anticancer, antibacterial and antifungal activity of a new Cu(II) complex of modified cyclam with four 7-nitro-2,1,3-benzoxadiaziles units bonded to its cyclic nitrogen atoms [11].

In this paper we present the Zn(II) complex formation of modified cyclam with four 4-amino-7-nitro-2,1,3-benzoxadiaziles units. The chemical structure, composition and association constants were confirmed and analysed by electronic (UV/Vis and fluorescence), Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR) spectroscopy, and scanning electron microscopy (SEM). The antimicrobial activity of the ligand and its Zn-complex has also been investigated against different pathogens.

EXPERIMENTAL

Materials and Methods

The synthesis of initial ligand **P1** has been described recently [11]. UV/Vis spectrophotometric investigations were performed using a Unicam Helios α spectrophotometer. The fluorescence spectra were taken on a Hitachi F-4500 spectrofluorimeter. All spectra were corrected to dilution, and the fluorescence spectra were also corrected for the inner filter effect using the

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equation $IF_{corr} = IF_{measured} \times 10^{(A_{ex}+A_{em})/2}$, where $IF_{measured}$ is the measured emitted fluorescence intensity, A_{ex} and A_{em} are the UV-Vis absorption of the solution at the wavelengths of excitation and emission, respectively [12]. All absorption and fluorescence spectra were recorded using 1 cm pathlength synthetic quartz cells at concentration of 2.2×10^{-5} mol l^{-1}. $Zn(NO_3)_2 \cdot 5H_2O$ and $Al(NO_3)_3 \cdot 9H_2O$ (Aldrich) were used as a sources of Zn(II) and Al(III) ions respectively. IR spectra were recorded on an Infrared Fourier transform spectrometer (IRAffinity-1 Shimadzu) with the diffuse-reflectance attachment (MIRacle Attenuated Total Reflectance Attachment) at a 2 cm^{-1} resolution. The ^1H NMR measurements were performed on a Bruker Avance DRX 500 spectrometer. The measurements were carried out in a DMSO-d_6 solution at ambient temperature. The chemical shifts were referenced to a tetramethylsilane (TMS) standard. Electrospray mass spectroscopic measurements were carried out using a Hewlett–Packard Series 110 0 MSD. The surface morphology of P1 and [Zn(**P1**)$(NO_3)_2$] was analyzed by scanning electron microscope (SEM) JSM-5510 (JEOL), operated at 10 kV of acceleration voltage. Before imaging, the investigated samples were coated with gold by JFC-1200 fine coater (JEOL).

*Synthesis of Zn(II) complex [Zn(**P1**)$(NO_3)_2$]*

The mixture of **P1** (85.2 mg 0.01 mmol) and $Zn(NO_3)_2 \cdot 5H_2O$ (27.95 mg, 0.01 mmol) in ethanol solution was refluxed for 1 hour and kept overnight at room temperature. The solid complex formed was filtered, washed with ethanol (10 ml, 3 times) and finally dried under vacuum. Yield: 90.7 mg (89.9%). FT-IR (cm^{-1}): 3097, 2948, 2860, 1613, 1540, 1492, 1428, 1276, 1235, 1101, 997, 919, 807, 738; ^1H-NMR ($CDCl_3$) ppm: 8.61-8.44 (m, 4 Ar-H), 6.94-6.547 (m, 4 Ar-H), 4.49-3.94 (m, 8H, -CH_2-), 3.02-2.89 (m, 8H, -CH_2-), 2.28-1.71 (4 H, -CH_2-). API-ES-MS (positive) m/z: calcd: 1009.73 found 1010.89 $(M+H)^+$. Elemental analysis $C_{34}H_{28}N_{18}O_{16}Zn$: Calc: C- 40.40%; H-2.77%; N-24.95%, Found: C- 40%.48; H-2.71%; N-25.06%.

Antimicrobial assay

The antimicrobial activities of the new compounds, ligand **P1** and [Zn(**P1**)$(NO_3)_2$] complex, were screened by the agar well diffusion method using DMSO as a solvent. Antibacterial activity was tested against Gram-positive bacteria *Bacillus subtilis*, *Bacillus cereus* ATCC 11778 and *Micrococcus luteus*, and Gram-negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter johnsonii*, *Xanthomonas oryzae* and *Serratia* sp. Antifungal activity was tested against the yeasts *Candida lipolytica* and *Saccharomyces cerevisiae*. The investigated compounds were dissolved in DMSO to obtain stock solution with concentration 5 mg/ml. A lawn of microbial cultures was prepared by spreading aliquots (0.1 ml) of overnight inoculums onto Müller-Hinton agar in Petri plates. Tests were performed using 0.1% and 0.5% of sample solutions, of which equal amount (40 µl) was added into wells (7 mm in diameter) bored into the agar. Simultaneously, gentamicin (G, 10 µg/disc) and nystatin (NS, 100 units/disc) were used as standard antibacterial and antifungal agents, respectively. After incubation of the plates at 25 °C for 24-48 h, the clear inhibition zones around the wells were measured (mm in diameter including well/disc).

RESULTS AND DISCUSSION

In order to study the metal ion binding properties of **P1** we applied ^1H NMR, UV-Vis and fluorimetric methods in DMSO solutions.

^1H NMR studies

The ^1H-NMR spectrum of **P1** consists of four groups of signals (Fig. 1). The aromatic protons of 7-nitrobezofurazane (NBD) moieties close to the ring nitrogen atoms and the CH_2 protons adjacent to ring nitrogens appear at 6.53 ppm and between 2.83-2.94 ppm, respectively, while the signals of C-CH_2-C protons are around 1.76 ppm.

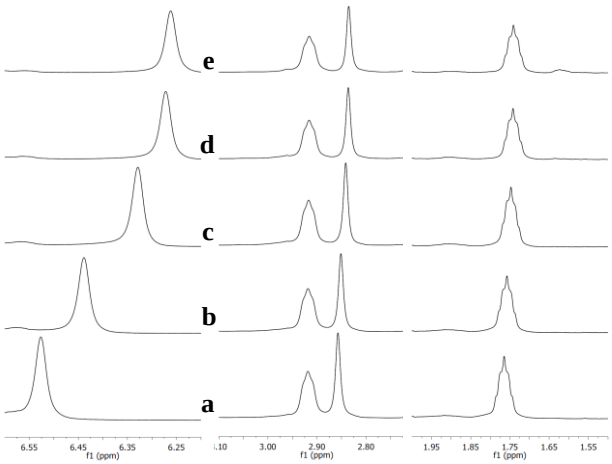


Fig. 1. ^1H NMR spectra of **P1** as a function of increasing Zn(II) concentration ([Zn(II)]/[**P1**] = 0/1 (a), 1/3 (b), 7/10 (c), 1/1 (d), 2/1 (e)), [**P1**] = 2.5 mM,).

Increasing concentration of the diamagnetic Zn(II) ions results upfield shift of the aromatic signals (Fig. 1). This may indicate the involvement of these units in the metal ion binding. The shift of aromatic protons as a function of Zn(II)-to-ligand P1 ratio is depicted in Fig. 1. Since at $[Zn^{2+}]/[\mathbf{P1}] = 1$ a sharp breakpoint can be observed (Fig. 2), the stoichiometry of the formed species is clearly 1:1, however based on these data only a lower limit of the stability constant of $[Zn(\mathbf{P1})]^{2+}$ can be estimated ($\log K \geq 5.5$).

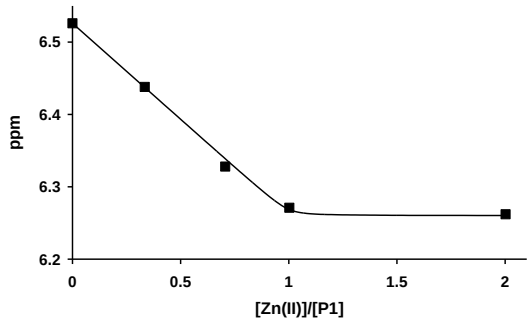


Fig. 2. Shift of the aromatic protons of **P1** as a function of zinc(II)/[**P1**] ratio (see also Fig. 1). The black line is the calculated curve with log K = 5,5.

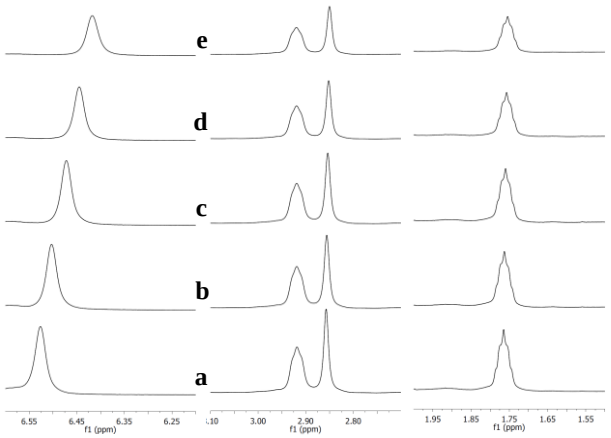


Fig. 3. ^{1}H NMR spectra of **P1** as a function of increasing Al(III) concentration ([Al(III)]/[**P1**] = 0/1 (a), 1/3 (b), 1/1 (c), 2/1 (d), 5/1 (e), [**P1**] = 2.5 mM).

Similarly to Zn(II), the interaction between ligand **P1** and Al(III) ions results in up field shift of the proton signals (Fig. 3). The effect of increasing Al(III) concentration on the chemical shift of aromatic protons are plotted in Fig. 4. Assuming the formation of a single $[Al(P1)]^{3+}$ species the experimental data can be well reproduced using log $K_{Al(P1)} = 2.49$.

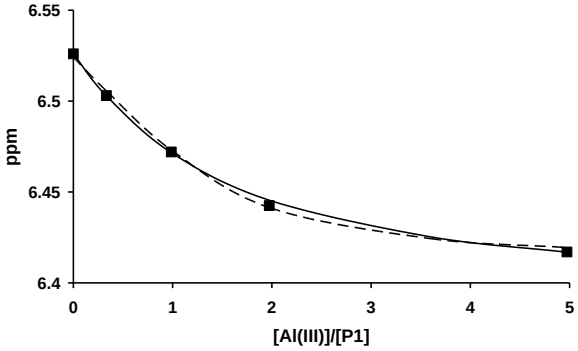


Fig. 4. Shift of the aromatic protons of **P1** as a function of Al(III)/[**P1**] ratio. The solid line is fitted curve with log K = 2.49, for the dashed line see the text.

UV-Vis and fluorescence study

Due to the intense UV-Vis and fluorescence spectra of **P1** these techniques allow to study the metal-ligand interaction at considerably lower concentration range (0.01-0.02 mM) and therefore at much higher metal/ligand ratios than in the case of NMR spectroscopy. Parallel absorption and fluorimetric measurements have been performed in the case of Zn(II). The both methods showed similar change upon increasing concentration of Zn(II): a smaller but well detectable change of intensities up to 1/1 metal to ligand ratio, and a more important change at much higher metal-to-ligand ratio (Fig. 5 and Fig. 6).

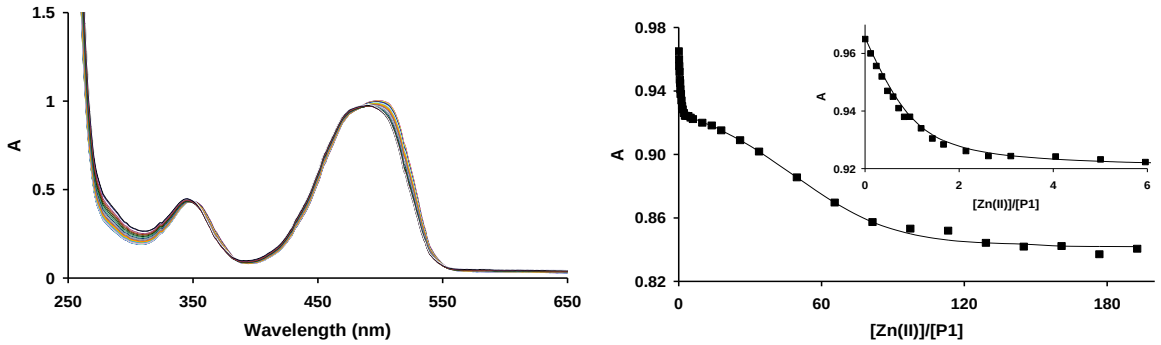


Fig. 5. Absorption spectra (left) and their absorption change at λ_A = 510 nm (right) of **P1** (c = 0,022 mM) at different concentration of Zn (II) ions (c = 0 – 4.2 mM); The solid line is fitted curve with log $K_{Zn(\mathbf{P1})} = 5.3$ and log $K_{Zn_2(\mathbf{P1})} = 2.6$.

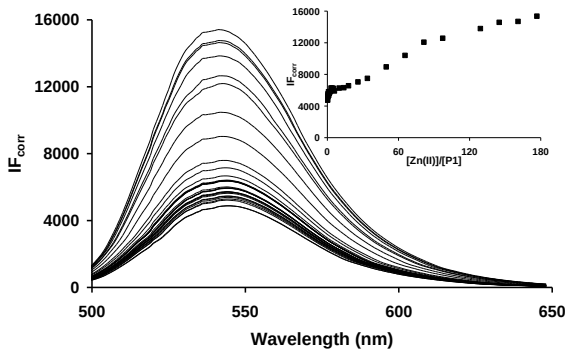


Fig. 6. Fluorescence spectra of the **P1** (c = 0.022 mM), as a function of Zn(II) ions concentration (c = 0–3.9 mM), λ_{ext} =480 nm. The insert shows the intensity change at λ_F = 540 nm.

These facts indicate the successive formation of two species. The first complex $[Zn(\mathbf{P1})]^{2+}$ has rather high thermodynamic stability, while the second one has a lower association constant ($[Zn(\mathbf{P1})]^{2+} + Zn^{2+} = [Zn_2(\mathbf{P1})]^{4+}$, log $K_{Zn_2(\mathbf{P1})}$). Since the precision of the absorption spectra is much higher than that of the fluorescence spectra, the former was used to derive the stability constants of the Zn(II) complexes (log $K_{Zn(\mathbf{P1})}$ = 5.3 ± 0.2 and log $K_{Zn_2(\mathbf{P1})}$ = 2.6 ± 0.3, see Fig. 5 right). The stability constant obtained for the species $[Zn(\mathbf{P1})]^{2+}$ from the UV-Vis data agree well with that calculated based on the NMR measurements (the formation of the dinuclear $[Zn_2(\mathbf{P1})]^{4+}$, complex was negligible under the conditions used for the NMR study). On the other hand, the formation of dinuclear complex indicates the participation of NBD moieties, too, in the metal ion coordination at higher concentrations of Zn(II) ions.

The increasing concentration of Al(III) also resulted in well-defined changes on the spectral properties. The effect on the UV-Vis spectra was similar to that of Zn(II) (Fig. 7). However, considerable increase on the emission spectra was observed (Fig. 8), even at low Al(III) concentrations. The concentration dependence of the emission intensity at λ_F = 544 nm is depicted in the insert of Fig. 8. Again, these data can be fitted only by the assumption that two successively formed complexes are present, like in the case of Zn(II). The best fit of the experimental data was obtained by log $K_{Al(\mathbf{P1})}$ = 4.5±0.2 and log $K_{Al_2(\mathbf{P1})}$ = 2.8±0.3 (Fig. 8). This observation is, however, virtually very different from that obtained by NMR. However, the fluorescence study allowed to collect considerably higher number of experimental points in a wider range of [Al(III)]/[**P1**] ratios, it provides more complete and therefore more correct description of complex formation equilibria. Indeed, using the latter model, the NMR data can be reproduced very well (within 0.003 ppm, see dashed line in Fig. 4).

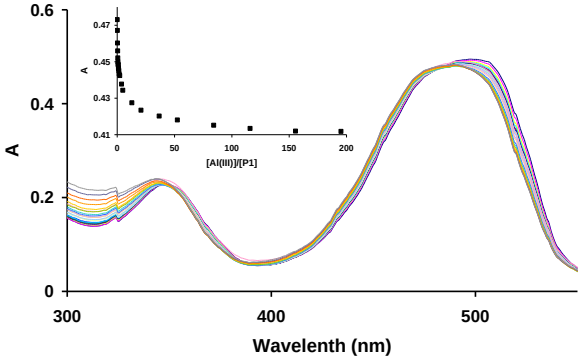


Fig. 7. Absorption spectra of **P1** (c=0,0104 mM) in the presence of different concentrations of Al(III) (c = 0–2.0 mM). The insert shows the changes of absorbance at 510 nm.

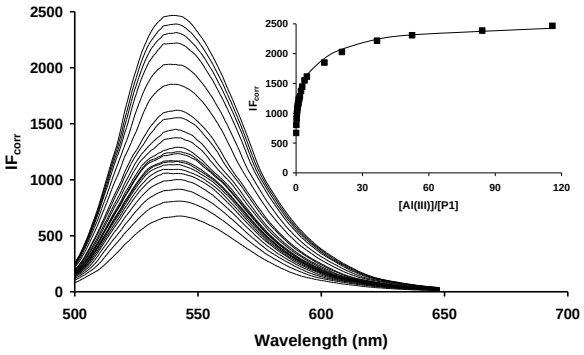


Fig. 8. Fluorescence spectra of **P1** (c = 0,0104 mM) in the presence of different concentrations of Al(III) (c = 0–1.3 mM), λ_{ex} = 480 nm); The insert shows the intensity change at λ_F = 540 nm. The solid line is fitted curve with log $K_{Al(P1)}$ = 4.5 and log $K_{Al_2(\mathbf{P1})}$ = 2.8.

FT-IR spectral characteristics of P1 and [Zn(P1)(NO$_3$)$_2$]

In Fig. 9 the IR spectra of ligand **P1** and its Zn(II) complex are plotted. Their comparison shows a significant difference in the spectra of the metal complex where the characteristic peaks for nitrate group (-NO_3) were registered in the 1100-1600 cm^{-1} region. Valence asymmetric vibrations were occurs at 1530-1580 cm^{-1} and the respective symmetrical vibrations are at 1230-1290 cm^{-1}. In this spectral region the characteristic bands assigned to benzofurazan structure are superimposed with those of the nitrate ions, bonded

to the Zn(II) ions. The only differences in the spectrum of the complex are the intensity of the bands. Furthermore, there is no difference in the position of the respective peaks in the spectral region where other functional groups from benzofurazane moiety absorb, what indicates that they do not participate in the formation of coordination bonds with Zn(II). That allows the assumption that, the coordination is realized via the tertiary amine groups in the cyclam ring, as shown in Formula 1.

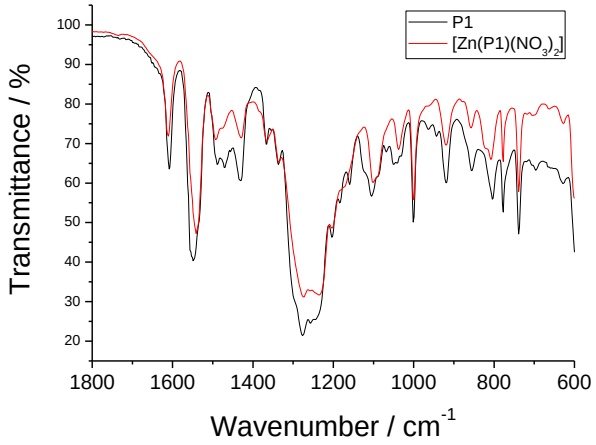
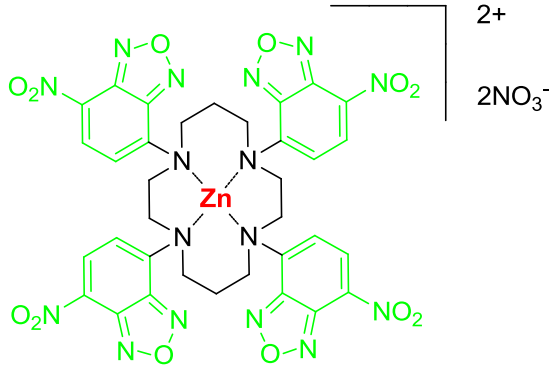


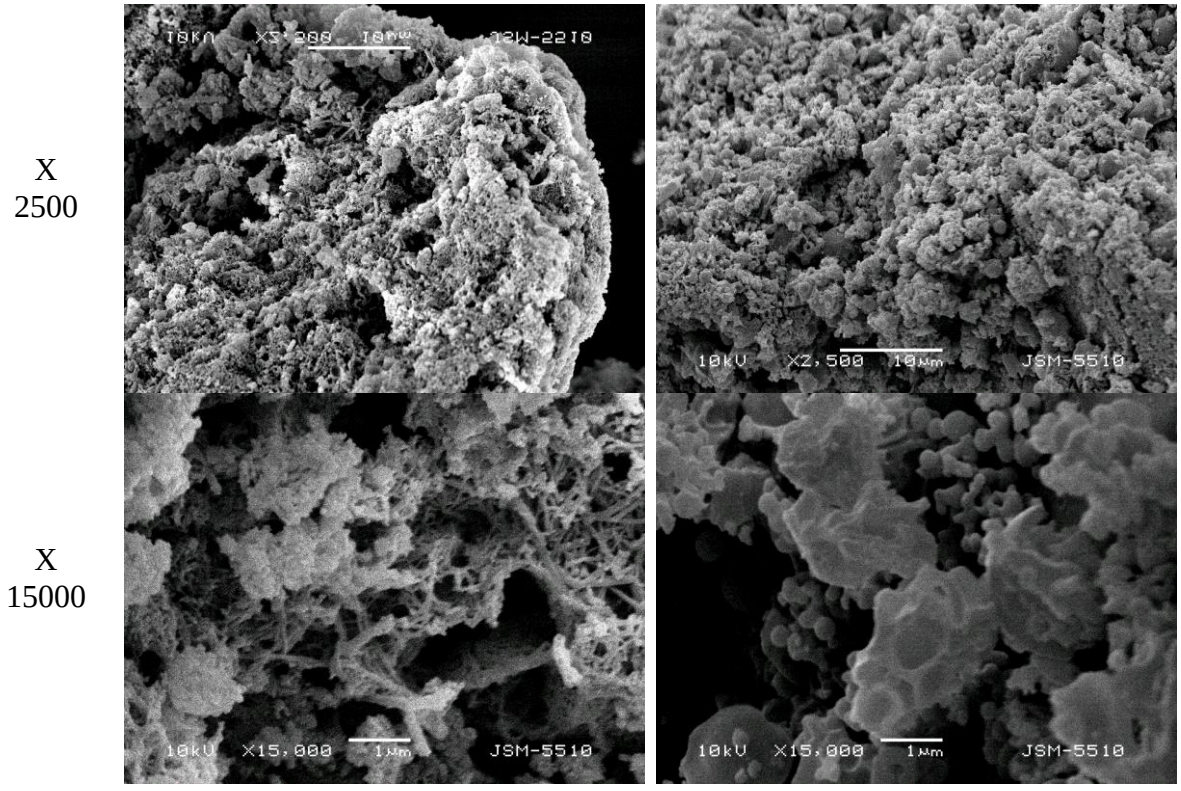
Fig. 9. IR spectra of ligand **P1** and its Zn(II) complex.



Formula 1. Chemical structure of [Zn(**P1**)$(NO_3)_2$].

SEM-analysis

Scanning electron microscopy technique has been used to study the morphological change in **P1** before and after the complex formation with Zn(II). At low amplification an agglomerated morphology has been observed for **P1**. At higher amplification microstructure of **P1** showed compact structure with smaller flakes, however after complex formation the micro structure changes and convert to homogeneous solid with grains as shown in Fig. 10.



P1 [Zn(**P1**)$(NO_3)_2$]

Fig. 10. SEM micrographs of **P1** and [Zn(**P1**)$(NO_3)_2$] at different amplifications.

The difference is best pronounced at amplification of 15000 where **P1** ligand shows a lattice and reticulation structure while the complex [Zn(**P1**)$(NO_3)_2$] retains the structure of microspheres. Most probably the interaction of Zn(II) with the cyclam ring causes this change in the morphology.

Antimicrobial activity

The antimicrobial activities of the new compounds were tested *in vitro* against eight bacterial and two yeasts cultures and expressed by the zones of inhibition. Free ligand and its zinc complex showed variable inhibition activity against different test cultures except *P. aeruginosa*, and this activity was differently enhanced on coordination (Fig. 11). The inhibition effect of the complex [Zn(**P1**)$(NO_3)_2$] was better or comparable to that expressed by the control **P1** against the eight bacterial test cultures, and about 2.5-fold higher than P1 against the yeasts *C. lipolytica* (Fig. 11 and Fig. 12). The antimicrobial activity of the zinc complex ranged from good against the yeasts and the test bacteria *B. cereus*, *X. oryzae* and *Serratia* sp. (zones of inhibition in the range 16-20 mm), to moderate against *B. subtilis* and *E. coli* (zones of inhibition 12-13 mm) and weak (zones of inhibition 11 mm) against *M. luteus* and *A. johnonii*. The obtained results of antimicrobial activity of the zinc complex are similar to those reported for the copper complex [11].

Coordination to biologically active molecules to metal ions can be used as a strategy to enhance their biological activity [2, 3]. The enhancement in biological activity of metal complexes upon coordination can be explained on the basis of Overtone's concept and chelation theory [13].

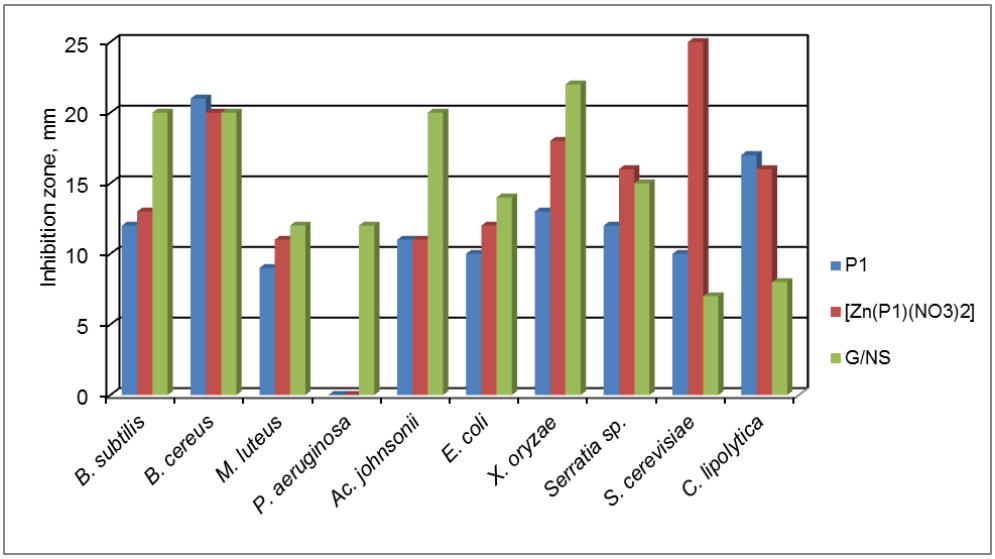


Fig. 11. Inhibition of the growth of test bacteria and yeasts by 0.5% solutions of ligand **P1** and complex [Zn(**P1**)$(NO_3)_2$]. G/NS, Gentamicin/Nystatin was used as a standard antibacterial/antifungal agent.

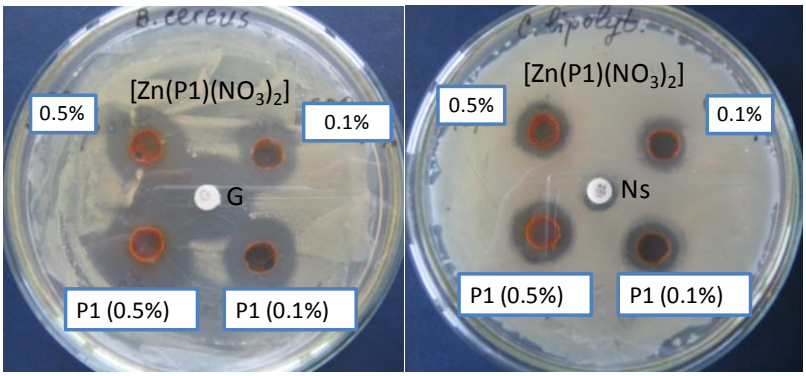


Fig. 12. Zones of inhibition of the growth of *B. cereus* and *C. lipolytica* by 0.1% and 0.5% **P1** and [Zn(**P1**)$(NO_3)_2$] compounds. Gentamicine (G) and Ns, (Nystatin) were used as a standard antibacterial and antifungal agent, respectively.

CONCLUSION

The effect of Zn(II) and Al(III) ions on the UV-Vis, fluorescence and ^{1}H NMR spectra of **P1** was studied in DMSO solution and it was shown that cyclam-4-nitro-benzofurazan conjugate may form both mono- and dinuclear complexes. The formation of dinuclear complexes at higher metal-to-ligand ratios indicates the participation of NBD moieties, too, in the metal ion coordination. On the other hand, the relatively strong binding of **P1** to Al(III) may suggest the participation of oxygen atoms of NBD residues. The antimicrobial studies suggested that the ligand is biologically active, and its zinc complex showed enhanced antibacterial and antifungal activity against microbial strains in comparison to the free ligand. Significant activity of the new compounds observed against the test pathogenic *B. cereus* and *C. lipolytica* makes them promising candidates in designing new antimicrobial preparations. Besides, significant activity of the zinc complex against phytopathogenic bacteria *X. oryzae* suggests its potential suitability for use for plant protection.

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СИНТЕЗ, ОХАРАКТЕРИЗИРАНЕ И МИКРОБИОЛОГИЧНА АКТИВНОСТ НА НОВ Zn(II) КОМПЛЕКС С ПРОИЗВОДНО НА БЕНЗОФУРАЗАНА

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(Резюме)

Чрез ^{1}H ЯМР, UV-Vis и флуоресцентна спектроскопия е проследено образуването на Zn(II) и Al(III) комплекси с използване на модифициран с 4-нитро-бензофуразан циклам като лиганд. Стабилен Zn(II)) комплекс [Zn(**P1**)$(NO_3)_2$] е изолиран, охарактеризиран и неговата антимикробна активност е изследвана *in vitro* спрямо различни грам положителни и грам отрицателни бактерии и два вида дрожди. Установена е добра микробиологична активност спрямо тестваните култури.