Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 41 (2006) 101–105
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://france.elsevier.com/direct/ejmech

Short communication

Synthesis, antibacterial and antifungal activity of some new pyridazinone metal complexes

Mehmet Sönmez [a,*], İsmet Berber [b], Esvet Akbaş [a]

[a] *Department of Chemistry, Faculty of Science and Arts, Yüzüncü Yıl University, 65080 Van, Turkey*
[b] *Department of Biology, Faculty of Science and Arts, Yüzüncü Yıl University, 65080 Van, Turkey*

Received 24 March 2005; received in revised form 30 September 2005; accepted 6 October 2005
Available online 15 November 2005

Abstract

The new various metal complexes of 5-benzoyl-4-hydroxy-2-methyl-6-phenyl-2*H*-pyridazin-3-one were synthesized. All the complexes were evaluated for their antimicrobial activities against Gram-positive, Gram-negative bacteria and fungi using microdilution procedure. The Cd(II) and Ni(II) complexes exhibited selective and effective activities against one Gram-positive bacterium (*Staphylococcus aureus* ATCC 6538), one Gram-negative bacterium (*Pseudomonas putida* ATCC 12633) and against two yeast (*Candida albicans* ATCC 27541 and *Candida tropicalis* 1828) in contrast to poor activity observed other microorganisms. The new synthesized complexes were characterized using IR, ^{1}H-NMR and UV spectral data together with elemental analysis.

Keywords: Pyridazinone complexes; Antimicrobial activity

1. Introduction

In recent decades, the problems of multi-drug resistant microorganisms have reached on alarming level in many countries around the world [1–3]. A number of recent clinical reports describe the increasing occurrence of meticillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and other antibiotic-resistant human pathogenic microorganisms in the United States and European countries [4]. Infections caused by those microorganisms pose a serious challenge to the medical community and need for an effective therapy has led to a search for novel antimicrobial agents. In our previous papers [5,6] we described the synthesis and antibacterial and antifungal activities of pyrazol, pyridazinone and pyrazolo[3,4-*d*]pyridazin (Fig. 1) derivatives, respectively.

In these studies, we revealed that the pyridazin derivative compounds showed weak activity against fungi and bacteria, however, some of the prepared pyrazolo[3,4-*d*]pyridazine derivatives, containing chloro group, displayed higher MIC values than the above mentioned standard drugs.

Metal chelation is involved in many important biological processes where the coordination can occurs between a variety of metals ions and a wide range of ligands [7]. Many types of ligands are known and the properties of their derived metal chelates have been investigated [8–10]. The complex combination with copper of pyridazin derivatives and sulfamethoxypyridazine complexes of Cu(II), Ni(II), Co(II) and Ag(I) have a considerable anti-inflammatory and antimicrobial activity against Gram-positive, Gram-negative bacteria and fungi [11,12]. In this paper we report the synthesis of new Cu(II), Co(II), Cd(II), Ni(II) and Zn(II) complexes with 5-benzoyl-4-hydroxy-2-methyl-6-phenyl-2*H*-pyridazin-3-one (LH) [5]. Elemental analysis, IR, ^{1}H-NMR, UV spectral data and molar conductance were obtained to determine the structure of the complexes. Moreover, the new complexes were tested against representative Gram-negative and Gram-positive bacteria, as well as fungi.

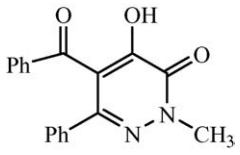

Fig. 1. Structure of the ligand (LH).

* Corresponding author.
E-mail address: vansonmez@hotmail.com (M. Sönmez).

0223-5234/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.ejmech.2005.10.003

2. Results and discussion

2.1. Chemistry

Reactions of LH with metal(II) salts yielded complexes possessing a 1:2 metal/ligand ratio. Ligand reacted with $Cu(CH_3COO)_2{\cdot}H_2O$, $Co(CH_3COO)_2{\cdot}4H_2O$, $Zn(CH_3COO)_2{\cdot}2H_2O$, $CdCl_2{\cdot}2H_2O$ $NiCl_2{\cdot}6H_2O$, and formed the complexes having only a 1:2 metal/ligand stoichiometry. The newly synthesized pyridazinon complexes are very stable at room temperature in the solid state. The metal complexes are generally soluble in DMF and DMSO. The elemental analytical data of the complexes reveal that the compounds have a metal/ligand anion stoichiometry of 1:2 corresponding to the general formulae of $[M(L)_2]{\cdot}mH_2O$ where L is the anion of LH. The analytical data are in good agreement with the proposed stoichiometry of the complexes. The colors, yields, melting points, IR and electronic absorption spectral data of all the compounds are presented in Table 1. All the complexes did not show electrolytic properties (1.35–4.50 Ω^{-1} cm^2 mol^{-1}) [7–9].

Bidentate complexes were obtained from 1/2 molar ratio reactions with metal ions and LH ligand. The ligand LH, on reaction with metal salts yields complexes corresponding to the general formula $[Co(L)_2(H_2O)_2]{\cdot}H_2O$, $[Cu(L)_2]$, $[Zn(L)_2(H_2O)_2]$, $[Ni(L)_2(H_2O)_2]$ and $[Cd(L)_2(H_2O)_2]$. The complexes were prepared by the general reaction shown below:

$$2LH + MX_2 \cdot nH_2O \xrightarrow{\text{methanole/chloroform}} M(L)_2 \cdot mH_2O + 2HX + kH_2O$$

M =	Co(II)	Cu(II)	Zn(II)	Ni(II)	Cd(II)	LH = $C_{18}H_{14}N_2O_3$
n =	4	1	2	6	2	
m =	3	–	2	2	2	
k =	1	1	–	4		
X =	CH_3COO	CH_3COO	CH_3COO	Cl	Cl	

The metal/ligand ratio of all the complexes was found to be 1:2. They also, all the complexes except Cu(II), have two, and three additional molecule of water of coordination and water of crystallization.

2.1.1. Infrared spectral study

The most important infrared spectral bands of the investigated metal complexes are summarized in Table 1. The free ligand is characterized by strong bands at 1669, 1288 and 3117 cm^{-1} which may be ascribed to the stretching vibrations of C=O (carbonyl), C–O (phenolic) and OH (phenolic) groups, respectively [8–10]. The band at 1669 cm^{-1} due to the stretching mode of the C=O group in the spectrum of the free ligand shows a remarkable shift to lower frequency with splitting in the 1658–1648 cm^{-1} region in all the spectra of the complexes suggesting that the coordinating carbonyl oxygen atoms of the ligand are involved in complex formation [8–10,13]. In the spectra of all the complexes the phenolic C–O band at 1270–1278 cm^{-1} is shifted to lower frequency (10–18 cm^{-1}). It is suggested that the oxygen atom of this phenolic (C–O) group is involved in bridging to the metal ions. A broad band in the 3300–3490 cm^{-1} range is observed in the spectra of the Ni(II), Co(II), Cd(II) and Zn(II) complexes. An additional band at 840 cm^{-1} suggests that water molecules are coordinated to metal ions [8–10,13]. This band may be assigned to water OH stretching frequencies confirming the elemental analyses. Also, thermal studies results that indicate water molecules are involved in the chemical composition of these metal complexes. Moreover, a large number of heterocyclic ring vibrations at 1580–1200 cm^{-1} are seen during the formation of the complexes, these bands are shifted to lower wave length. Bands of M–O, appear, respectively, at 420–465 cm^{-1}.

2.1.2. Proton nuclear magnetic resonance spectra

Deuterated chloroform or deuterated DMSO were used as solvent to measure the ^{1}H-NMR spectra of the ligand [5] and its complexes Zn(II), Cd(II), respectively. The spectrum of the

Table 1
Characteristic IR and electronic absorption spectral data of the ligand and its metal complexes

Compounds	M.p. (°C)	Yield (%)	% Λ_M (Ω^{-1} cm^2 mol^{-1})	C=O	OH/H_2O	C–O	M–O	λ_{max} (ε_{max}, M^{-1} cm^{-1})
LH $C_{18}H_{14}N_2O_3$ (306.2 g mol^{-1}) (light yellow)	218	40.0	1.1	1669s	3117m, b	1288 s	–	340 (2980), 306 (6520), 281 (6800)
$[CuL_2]$ $C_{36}H_{26}CuN_4O_6$ (673.94 g mol^{-1}) (green)	> 300	80.0	1.35	1658s	–	1278m	465w 507w	524 (22), 340 (3902)
$[CoL_2(H_2O)_2]{\cdot}H_2O$ $C_{36}H_{32}CoN_4O_9$ (723.51 g mol^{-1}) (brown)	215[d]	68.0	2.70	1651m	3300–3500 w, b	1272 w	445w 510w	628 (12), 487 (816) 356 (3602)
$[NiL_2(H_2O)_2]$ $C_{36}H_{30}Ni\ N_4O_8$ (705.29 g mol^{-1}) (light green)	250[d]	78.0	3.15	1648m	3300–3490m, b	1277s	450w 511w	515 (40), 479 (115) 361 (1690)
$[ZnL_2(H_2O)_2]$ $C_{36}H_{32}N_4O_9Zn$ (711.79 g mol^{-1}) (dirty white)	183[d]	76.0	1.89	1650m	3419–3450m, b	1272m	440w 510w	343 (1434), 302 (2462), 282 (3654)
$[CdL_2(H_2O)_2]$ $C_{36}H_{30}CuN_4O_8$ (759.0 g mol^{-1}) (cream)	197[d]	69.0	4.50	1650m	3350–3500m, b	1270w	460w 510w	339 (2434), 303 (4460), 279 (6650)

Key: s (strong), m (medium), w (weak).

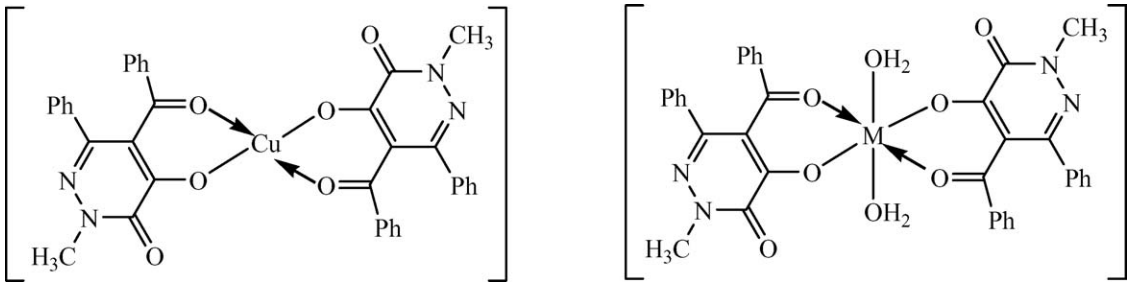

Fig. 2. Supposed structure of the M (II) complexes.

ligand shows peaks at 9.4 (s), 3.9 (s) and 7.8–7.2 (m). These peaks are attributed to –OH phenolic, $–CH_3$ and aromatic protons, respectively. In the spectrum of the Cd(II) and Zn(II) complexes $–CH_3$ and aromatic proton peaks were observed at the same position with the exception that the peak due to the phenolic group resonance is absent. This is considered as an additional evidence for the deprotonation of the phenolic OH group and shows involvement of the phenolic oxygen in bonding (Fig. 2).

2.1.3. Electronic spectra

The bands in the 340–250 nm range are attributed to the π→π* transition of the benzenoid and pyridazine rings. In the spectra of the complexes, these bands are slightly shifted to shortest wave length. On the basis of the magnetic data, the Cu(II) complex probably has a tetrahedral structure. The complex exhibits a weak transition at 524 nm corresponding to $^2B_{1g}\rightarrow{}^2A_{1g}$ which suggests the presence of four-coordinate geometry [14].

The magnetic moment value of 4.22 BM obtained for the brown Co(II) complex was in the range expected for cobalt (II) in octahedral geometry. The electronic spectra also showed a rather weak d–d transition in the visible region of the spectrum. The bands at 487 and 628 nm may be assigned to $^4T_{1g}\rightarrow{}^4T_{1g}$ (P) and $^4T_{1g}\rightarrow{}^4A_{2g}$ transitions as expected for an octahedral cobalt(II) complex [8–10,14]. The electronic spectrum of the Ni(II) complex shows two d–d transitions at 515 and 479 nm which indicate an octahedral environment around the metal ion (Fig. 2). The electronic spectrum of the Zn(II) and Cd(II) complexes, which are diamagnetic, has bands in the 302–280 nm range, and these bands may be due to the n→π* and π→π* transitions of the benzene and pyridazine rings group in a complex with octahedral geometry. These data also support the conclusion that H_2O groups are coordinated axially to all the metal complexes, except Cu(II) complex.

2.2. Biological results

Antimicrobial activities of the prepared compounds were tested against bacteria, such as *Bacillus cereus* ATCC 7064, *S. aureus* ATCC 6538, *Escherichia coli* ATCC 4230, *Pseudomonas putida* ATCC 12633 and against human pathogenic fungi (*Candida albicans* ATCC 27541, *C. albicans* 1481, *Candida tropicalis* 1828 and *Candida krusei* 24941 using broth microdilution procedure. Ampicillin trihydrate for bacteria and fluconazole for fungi were used as reference drugs. The minimal inhibitory concentrations (MICs, mg ml^{-1}) of tested compounds against bacteria and fungi are shown in Tables 2 and 3.

As shown in Tables 2 and 3, the ligand showed weak activity, whereas the complex compounds had the highest antimicrobial activities against Gram-positive, Gram-negative bacteria and fungi with minimum inhibitory concentrations in the range of 0.16–0.005 mg ml^{-1}. The results of antibacterial activities indicated that slight differences between the activities of all the complex compounds against tested bacteria, except Cd (II) complex. The Cd(II) complex showed selective and effective antibacterial activity against one of the Gram-positive bacteria (*S. aureus* ATCC 6538) and one of the Gram-negative bacteria (*P. putida* ATCC 12633), also poor activity against other tested microorganisms (*E. coli* ATCC 4230 and *B. cereus* ATCC 7064). *S. aureus* ATCC 6538 strain is highly pathogenic to humans. The Cd(II) complex showed highest antibacterial activity against this bacterium compared to other tested bacterial strains (Table 2).

Table 3 summarized antifungal activities of the tested complexes against human pathogenic yeast species. According to these results revealed that the newly synthesized Cd(II) and Ni (II) complexes had promising antifungal activities against two yeast strains (*C. albicans* ATCC 27541 and *C. tropicalis* 1828) and poor activities against other yeast. These results suggested that the Cd(II) and Ni(II) complexes had effective and selective antimicrobial activities against both bacteria and yeast.

Cu, Co, Ni and Zn are essential for microorganisms as trace nutrients. In contrast, Cd has not been identified as a trace nutrient and is (in fact) thought to have no beneficial roles in bacteria and fungi. Therefore, cadmium, and its derivatives are more toxic than other trace elements against microorganisms at micro or millimolar levels. Although microbial resistance to antibiotics and the divalent cations carried on plas-

Table 2
MICs* of the compounds LH, Co(II), Cu(II), Cd(II), Ni(II) and Zn(II) complexes against Gram-positive and Gram-negative bacteria

Compounds	*P. putida* ATCC 12633	*E. coli* ATCC 4230	*B. cereus* ATCC 7064	*S. aureus* ATCC 6538
LH	1.28	0.64	1.28	0.64
$[CoL_2(H_2O)_2]\cdot H_2O$	0.04	0.08	0.08	0.16
$[CuL_2]$	0.16	0.16	0.16	0.16
$[CdL_2(H_2O)_2]$	0.01	0.04	0.04	0.005
$[NiL_2(H_2O)_2]$	0.04	0.08	0.08	0.08
$[ZnL_2(H_2O)_2]$	0.04	0.08	0.04	0.04
Ampicilline	0.02	0.005	0.005	0.01

* MICs values were determined as mg ml^{-1} active compounds in medium.

Table 3
MICs* of the compounds LH, Co(II), Cu(II), Cd(II), Ni(II) and Zn(II) complexes against fungi

Compounds	*C. albicans* ATCC 27541	*C. albicans* 1481	*C. tropicalis* 1828	*C. krusei* 24941
LH	0.64	1.28	0.64	1.28
$[CoL_2(H_2O)_2]\cdot H_2O$	0.02	0.02	0.04	0.04
$[CuL_2]$	0.01	0.02	0.02	0.04
$[CdL_2(H_2O)_2]$	0.005	0.04	0.005	0.04
$[NiL_2(H_2O)_2]$	0.005	0.02	0.02	0.04
$[ZnL_2(H_2O)_2]$	0.01	0.04	0.01	0.04
Fluconazole	0.005	0.01	0.005	0.01

* MICs values were determined as mg ml^{-1} active compounds in medium.

mids, a mechanism resistance to organometallic compounds of these metals is still unknown [15]. Moreover, several studies reported that the organometallic compounds of the divalent cations are more toxic than their metallic forms, particularly when compared to their own inorganic equivalents [16,17]. Here, we proposed that the reason for this higher antimicrobial activity might be related to the structure of the Cd(II) complex rather than the presence of the metal form of cadmium. Also, the other cause for the effect could be bounded to the damaged of the membrane permeabilization and the membrane lipid composition.

In conclusion, we have synthesized and evaluated in vitro the antimicrobial activity of the new various metal complexes of 5-benzoyl-4-hydroxy-2-methyl-6-phenyl-2*H*-pyridazin-3-one. According to the in vitro results indicated that the new various metal complexes of pyridazin-3-one had commonly of greater toxicological significance than the ligand. Especially we suggested that the Cd(II) and Ni(II) complexes might be a promising candidate of new antimicrobial agents.

3. Experimental protocols

MeOH, EtOH, $CHCl_3$, DMF, n-butanol, toluene, diethylether, n-heptane and were obtained from E. Merck and Aldrich. 5-Benzoyl-4-hydroxy-2-methyl-6-phenyl-2*H*-pyridazin-3-one was synthesized according to the literature [5]. The metal salts $Cu(CH_3COO)_2\cdot H_2O$, $Co(CH_3COO)_2\cdot 4H_2O$, $Zn(CH_3COO)_2\cdot 2H_2O$, $CdCl_2\cdot 2H_2O$ and $NiCl_2\cdot 6H_2O$ were obtained from E. Merck. All solvents were dried and purified before use. Elemental analyses (C, H, N) were performed by using a Carlo Erba 1106 elemental analyzer. The IR spectra were obtained using KBr discs (4000–400) cm^{-1} on a Bio-Rad-Win-IR spectrophotometer. The electronic spectra in the 200–900 nm range were obtained in DMF on a Unicam UV2-100 UV/visible spectrophotometer. Molar conductance of the ligand and their transition metal complexes were determined in DMF at room temperature by using a Jenway model 4070 conductivitymeter. The ^{1}H-NMR spectra of the some complexes were recorded with a Varian XL-200 NMR instrument. All experiments were followed by TLC using DC Alufolien Kieselgel 60 F 254 Merck and Camag TLC Lamp (254/366 nm).

3.1. Synthesis

3.1.1. Synthesis of the ligand (LH)

The LH was synthesized according to the literature [5]. An equimolar mixture of furandione (0.278 g, 1 mmol) and methylhydrazine (3.1 ml, 1 mmol) was mixture in room temperature in dry benzene (30 ml) for approximately 60 min. After the precipitate was filtered off solvent was removed by evaporation, the oily residue treated with ether and the formed crude product was recrystallized from acetic acid to give 0.122 g (40%); ^{1}H-NMR ($CDCl_3$): $\delta = 9.41$ (b, H, OH), $\delta = 7.8$–7.2 (m, 10H, H_{arom}), 3.9 ppm (s, 3H, CH_3); ^{13}C-NMR ($CDCl_3$): $\delta = 193.56$ (C=O, benzoyl), 159.21 (C=O), 152.34 (C_4), 148.88 (C_5), 138.20, 137.11, 136.15, 131.52, 131.22, 130.81, 130.46, 130.27, 121.99, 42.56 ppm (N–CH_3) [5].

3.1.2. Synthesis of the complexes

0.61 g (2.00 mmol) of the ligand was dissolved in 30 ml of chloroform, and a solution of 1.00 mmol of the metal salt [$Cu(CH_3COO)_2\cdot H_2O$ (0.20 g), $Co(CH_3COO)_2\cdot 4H_2O$ (0.25 g), $Zn(CH_3COO)_2\cdot 2H_2O$ (0.22 g), $CdCl_2\cdot 2H_2O$ (0.22 g) and $NiCl_2\cdot 6H_2O$ (0.24 g)] in 15 ml methanol was added dropwise with continuous stirring. The mixture was stirred further for 1.5–2.5 h at 80 °C. The precipitated solid was then filtered off, washed with diethyl ether, followed by cold methanol/chloroform (1:1 ratio) and dried in vacuum desiccators.

3.2. Biological assays

3.2.1. Compounds

Test compounds were dissolved in DMSO (12.5%) at an initial concentration of 2.5 mg ml^{-1} and then were serially diluted in culture medium.

3.2.2. Cells

Bacterial strains and *C. albicans* ATCC 27541 were supplied from American Type Culture Collection (ATCC). Other human pathogenic fungal isolates (*C. albicans* 1481, *C. tropicalis* 1828 and *C. krusei* 24941) were obtained from Department of Infectious Diseases, Faculty of Medicine, Yüzüncü Yıl University, Van, Turkey.

3.2.3. Antibacterial assays

The MICs of the chemical compounds assays were carried out as described by Clause [18] with minor modifications. Ampicillin trihydrate was used as reference antibacterial agent. Solutions of the test compounds and reference drug were dissolved in DMSO at a concentration of 2.56 mg ml^{-1}. The twofold dilution of the compounds and reference drug were prepared (1.28, 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01, 0.005 >) mg ml^{-1}. Antibacterial activities of the bacterial strains were carried out in Muller–Hinton broth (Difco) med-

ium, at pH 7.2, with an inoculum of (1–2) × 10^3 cells ml^{-1} by the spectrophotometric method and an aliquot of 100 μl was added to each tube of the serial dilution. The chemical compounds-broth medium serial tube dilutions inoculated with each bacterium were incubated on a rotary shaker at 37 °C for 18 h at 150 rpm. The minimum inhibitory concentrations of the chemical compounds were recorded as the lowest concentration of each chemical compounds in the tubes with no growth (i.e. no turbidity) of inoculated bacteria.

3.2.4. Antifungal assays

Antifungal activities of the yeast were performed by following the guidelines in NCCLS document M27-A using the microdilution broth method [19]. Fluconazole was used as reference antifungal agent. Solutions of the test compounds and reference drug were dissolved in DMSO at a concentration of 2.56 mg ml^{-1}. The twofold dilution of the compounds and reference drug were prepared (1.28, 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01, 0.005 >) mg ml^{-1}. Antifungal activities of the yeast were performed in RPMI 1640 medium (Sigma) which had been buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (Sigma), as outlined in document M27-A. The stock yeast inoculum suspensions were adjusted to concentration of (0.5–2.5) × 10^3 cells ml^{-1} by the spectrophotometric method and an aliquot of 100 μl was added to each tube of the serial dilution. The chemical compounds-broth medium serial tube dilutions inoculated with each yeast were incubated on a rotary shaker at 37 °C for 18 h at 150 rpm. The minimum inhibitory concentrations of the chemical compounds were recorded as the lowest concentration of each chemical compounds in the tubes with no growth (i.e. no turbidity) of inoculated yeast.

References

[1] S. Harbart, W. Albrich, D.A. Goldman, J. Huebner, Lancet Infect. Dis. 1 (2001) 251–261.
[2] L.A. Mitscher, S.P. Pillai, E.J. Gentry, D.M. Shankel, Med. Res. Rev. 19 (1999) 477.
[3] I. Berber, C. Cokmus, E. Atalan, Microbiol. 72 (2003) 54–59.
[4] P. Viksveen, Homeopathy 92 (2003) 99–107.
[5] E. Akbaş, I. Berber, A. Şener, B. Hasanov, Farmaco 60 (2005) 23–26.
[6] E. Akbaş, I. Berber, Eur. J. Med. Chem. 40 (2005) 401–405.
[7] D. Gamovskii, A.L. Nivorozhkin, V.I. Mimkin, Coord. Chem. Rev. 126 (1993) 1.
[8] M. Sönmez, J. Polish, Chem. 77 (2003) 397.
[9] M. Sönmez, M. Şekerci, Synth. React. Inorg. Met. Org. Chem. 33 (10) (2003) 1747.
[10] M. Sönmez, M. Şekerci, Synth. React. Inorg. Met. Org. Chem. 34 (3) (2004) 485.
[11] M. Dorneanu, E. Stefanescu, O. Paduraru, M. Pavelescu, A. Hriscu, Rev. Med. Chir. Soc. Med. Nat. Iasi. 100 (1–2) (1996) 167–171.
[12] S.C. Chaturvedi, S.H. Mishra, K.L. Bhargava, Y. Avadhoot, Indian Drugs 16 (8) (1979) 178–181.
[13] L.J. Bellamy, The Infrared Spectra of Complex Molecules, Chapman and Hall, London, 1978.
[14] A.B.P. Lever, Inorganic Electronic Spectroscopy, Elsevier, Amsterdam, 1984.
[15] H.N. Dietric, Plasmid 27 (1992) 17–28.
[16] K. Fent, Crit. Rev. Toxicol. 26 (1996) 1–117.
[17] M. Anderiana, S.V. Avery, M.A. Zoroddu, G.M. Gadd, FEMS Microbiol. Lett. 167 (1998) 321–326.
[18] G.W. Clause, Understanding Microbes: A Laboratory Textbook for Microbiology, W.H. Freeman and Company, New York, USA, 1989.
[19] National Committee for Clinical Laboratory Standards, Reference method for broth dilution antifungal susceptibility testing for yeasts; approved standard Document M27-A, Villanova, National Committee for Clinical Laboratory Standards 17 (1997) 1–29.