

# SYNTHESIS OF THE LIGAND (Z)-2-(3-METHOXYPHENYLAMINO)-4-OXO-4-PHENYLBUT-2-ENOIC ACID AND ITS ANTIFUNGAL ACTIVITY AGAINST THE WOOD STAIN FUNGI *MUCOR PLUMBEUS*

C. PAZ<sup>1</sup>\*, D. CAJAS-MADRIAGA<sup>1</sup>, C. TORRES<sup>3</sup>, Y. MORENO<sup>2</sup>, M. J. FERNÁNDEZ<sup>1</sup>,  
J. BECERRA<sup>1</sup> AND M. SILVA<sup>1</sup>

<sup>1</sup>Laboratorio de Química de Productos Naturales, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Chile

<sup>2</sup>Fac. Cs. Químicas-Centro de Biotecnología, Universidad de Concepción, Chile

<sup>3</sup>Laboratorio de Genómica y Biodiversidad (LGB), Departamento de Ciencias Naturales, Universidad del Biobío, Chillán, Chile

(Received: August 6, 2012 - Accepted: January 30, 2013)

## ABSTRACT

The ligand (z)-2-(3-methoxyphenylamino)-4-oxo-4-phenylbut-2-enoic acid and its Ni (II) complex were synthesized and their antifungal activity against the fungi wood stain *Mucor plumbeus* was evaluated. The ligand displayed fungostatic activity while the Ni (II) complex exhibited antifungal activity with a MIC of 50 µg/mL, moreover the copper complex did not showed biocide activity.

**Keywords:** wood stain fungi, *Mucor plumbeus*, Ni-complex.

## INTRODUCTION

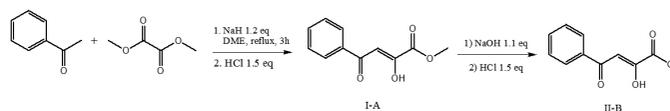
In the last three decades there has been an increase in sustainable forest plantations in the central-southern zone of Chile. More than 40% of the arable land is apt for forest soils, this has allowed the development of a dynamic forestry sector and industrial basis through monospecific plantations of forest<sup>1</sup>, which corresponds to 2.4 million hectares of plantations of exotic species of fast-growing *eucalyptus* and *Pinus* genera. The forest industry consequently represents 3.1% of the gross domestic product and is the second most important economic activity after mining<sup>2</sup>. For this reason, the damages caused by the fungi stainers of processed wood become one of the risk factors most relevant to the forestry sector<sup>3</sup>. In this regard, the fungi stainers have been a problem without a solution, by considerably diminishing the commercial value of the sawn timber due to the appearance of unusual colours and the high risk of contamination<sup>4</sup>. It is common to find stacks of sawn timber with serious damage caused by fungi of the *Ceratocystis* genera<sup>5</sup>, however, in recent years new stainers have been detected, among them we find representatives of *Mucor* genera, of which *Mucor plumbeus* Bon. is one of the most common<sup>6</sup>. This fungus is characterized by forming clear light to dark olive grey colonies sized from 2 to 20 mm occurring on the surface of sawn timber<sup>7</sup>. Contamination affects not only the processed wood, can also affect humans, putting at risk the health of the population<sup>8</sup>. The most used products for the pathogenic control of wood are chemical synthetic-based copper salts or chlorinated phenolic compounds<sup>9</sup>.

Searching for new compounds with antifungic activity we studied the acid 4-phenyl-2,4-dioxobutanoic acid, which reported important biological activities like enzyme inhibitor of HIV-1 integrase<sup>10</sup>, in bacteria inhibits KDPG aldolase<sup>11</sup> and derivatives have shown analgesic activity<sup>12</sup>. From the 2,4-dioxobutanoic acid, we synthesized the ligand keto-enamine (z)-2-(3-methoxyphenylamino)-4-oxo-4-phenylbut-2-enoic acid (L) and their Ni (II) and Cu (II) complexes and studied their fungicide activity in the wood stain fungi *Mucor plumbeus*.

## EXPERIMENTAL

The <sup>1</sup>H-NMR spectra were determined using a Bruker ARX 300 instrument, operating at 300.1 MHz (<sup>1</sup>H) and 75.5 MHz (<sup>13</sup>C). EI MS spectra were measured on Trace DSQII GC/MS-system (Axel Semrau GmbH & Co). FT-IR-spectrometer Nicolet 6700 from Thermo Electron Corporation with the ATR-unit Smart Performer. Melting points were determined on a Melting Point SMP10 (Stuart) uncorrected. Column Chromatography was performed using Merck silica gel 60 (0.063–0.200 mm). TLC was carried out on a Merck silica gel 60 PF254. Solvents used in this study were distilled prior to use and dried over appropriate drying agents.

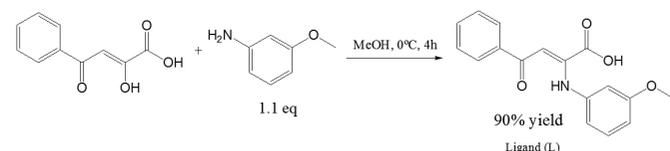
## General synthesis



Scheme 1

(Z)-Methyl 2-hydroxy-4-oxo-4-phenylbut-2-enoate (I-A): A mixture of acetophenone (1.0 g, 8.3 mmol) and dimethyl oxalate (1.2 g, 10 mmol) were stirred in 150 mL of anhydrous dimethoxyethane, then sodium hydride (240 mg, 10 mmol) was added. The mixture was stirred under reflux for 4 h. The reaction was cooled and quenched with water 100mL and HCl until pH 2. The mixture was extracted 3 times with EtOAc, and the organic layer was evaporated in vacuo. The compound precipitated as a white solid (1.49 g, 84%). <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 300 MHz) ppm: 3.94 (3H, s); 7.10 (1H, s); 7.50 (2H, m); 7.61 (1H, m); 8.00 (2H, m), 15.83 (1H, s, interchangeable). <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 75 MHz) ppm: 55.4; 99.4; 127.5; 129.2; 130.2; 135.2; 162.6; 169.1; 190.8. EI-MS: 206 g/mol

(Z)-2-Hydroxy-4-oxo-4-phenylbut-2-enoic acid (II-B): Hydrolysis of I-A (1.0 g, 4.9 mmol) was carried out with NaOH (194 mg, 4.9 mmol) in 20mL of water and stirred at 50°C for 15 min. The reaction was filtered and the reaction mixture was extracted three times with 10 mL of ethyl acetate. The water layer was acidified with HCl until pH 2. The product precipitated as a white solid, then filtered off and dried (752 mg, 80%), mp= 160°C. <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 300 MHz) ppm: 6.75 (1H, br); 7.52 (2H, m); 7.53 (1H, d, J= 4.2 Hz); 7.87 (2H, d, J= 4.5 Hz). <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 75 MHz) ppm: 98.0; 127.5; 128.0; 128.4; 128.9; 133.0; 135.4; 163.8. EI-MS: 192 g/mol



Scheme 2

(z)-2-(3-methoxyphenylamino)-4-oxo-4-phenylbut-2-enoic acid (L): Condensation of II-B (700 mg, 3.6 mmol) with 3-methoxy aniline (493 mg, 4.0 mmol) was carried out in methanol 50 mL at 0 °C by 4 h. The product precipitated as a yellow solid, 962 mg, 90% yield, mp = 168-169°C. <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) ppm: 3.73 (3H, s); 6.12 (1H, s); 6.70 (2H, m); 6.79 (1H, d, J= 2Hz); 7.22 (1H, t, J= 8.1 Hz); 7.42 (1H, m); 7.48 (2H, m), 7.55 (1H, d, J= 7.2 Hz); 7.92 (2H, d, J= 7.2 Hz); 12.22 (1H, s). <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 75 MHz) ppm: 55.1; 9.44; 106.3; 110.2; 112.9; 127.1; 128.7; 129.9; 131.8; 138.7; 140.5; 154.9; 159.9; 165.7; 1889.5. IR-KBr (cm<sup>-1</sup>): 3219 NH; 1604 C=O; 1575 N=O.

### Complex synthesis

The complexes of Ni(II) and Cu(II) were obtained by reflux of 300 mg (1.0 mmol) of the ligand, dissolved in 30 ml of methanol, with a solution of 0.5 equivalents of the corresponding metal salt, NiCl<sub>2</sub>·6H<sub>2</sub>O (119 mg, 0.5 mmol) or CuSO<sub>4</sub>·5H<sub>2</sub>O (125 mg, 0.5 mmol) in 15 ml water was added dropwise with continuous stirring. The mixture was stirred under reflux for 6 h. The solvent was removed in vacuum obtaining a green solid that was washed with ethyl acetate three times, followed by cold methanol-water ratio 1:1. The complex was then dried in vacuum desiccators.

Ni-Complex: green solid, decomposition over 210°C. IR-KBr (cm<sup>-1</sup>): 1591 C=O; 1575 N=O.

### Fungus isolation

*Mucor plumbeus* was isolated from wood stain collected in a sawmill near Concepción, VIII Region, Chile (36°48'S - 72°56'O). Infected wood samples were suspended in steril water, and dilutions of this suspension were streaked out onto YMG agar augmented with 200 mg l<sup>-1</sup> of streptomycin, at pH 5.5 and incubated at 22° C by 7 days, procedure repeated 3 times till pure colonies grow up in the plate as gray mycelium which was verified by microscopic examination<sup>13</sup>. This strain has been deposited at the collection of the Laboratory of Natural Products Chemistry of University of Concepción, Chile (accession number LQMP-001).

### Fungus Identification

In order to validate the morphological identification of *Mucor plumbeus* strain, it was amplified the 28S nuclear ribosomal large subunit rRNA (LSU) using Lr0R/Lr6 primer combination that covers D1, 28S Ribosomal RNA gene, D2, and D3 regions<sup>14</sup>. DNA was extracted from fruit bodies using the E.Z.N.A. fungal DNA MiniKit (Omega-Biotek). PCR reactions were performed using LROR (5'-gtaccgcgtgaacttaagc-3') as forward primer and LR06 (5'-cgccagttctgctacc-3') as reverse primer. Each reaction was conducted in a 15 µL volume containing 30-50 ng of DNA, 1X of PCR buffer, 2 mM MgCl<sub>2</sub>, 0.1 µM of each dNTP, 0.5 µM of forward and reverse primers, and 1 U of Taq DNA polymerase. PCR amplification was carried out with an initial denaturation of 4 min at 94°C, and then 35 cycles of 30 s at 94°C, 60 s at 50°C and 60 s at 72°C, followed by a final step of 5 min at 72°C. After PCR purification and sequencing (both directions) by MacroGen sequencing service (Seoul, Korea), analyses of sequences were edited using Genious v5.4 software<sup>15</sup>. The sequence of *M. plumbeus* 28S Ribosomal RNA gene was analyzed with BLAST (Basic Local Alignment Search Tool) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the percentage of maximal identity with the sequences of the global database. Finally, the sequence obtained from the present study was deposited in GenBank with the code *BankIt1532924 Seq1 JX123134*.

### Antifungal activity determination

#### Agar diffusion test

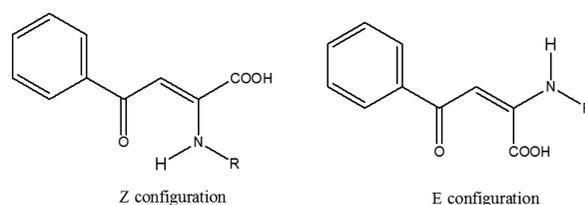
Antifungal activity of L and their Ni(II) and Cu(II) complexes was qualitatively evaluated by diffusion test in agar YMG. Plates were inoculated with a spore concentration of 10<sup>6</sup> spores/mL of *M. plumbeus*. Paper disks (6 mm) were impregnated with 200 µg of ligand L, Ni(II) complex and Cu(II) complex. As positive control was used antiblue375 and DMSO as negative control (solvent). Plates were incubated at 22°C by 21 days<sup>16</sup>.

#### Microtitre plate test

The minimal inhibitory concentration (MIC) was determined following the method described by Sarker et al 2007<sup>17,18</sup>. Microplates were cultivated in liquid media YMG, pH 5.5 and inoculated with 25 µL of solution 10<sup>5</sup> FCU/mL per well. Solutions of L and Ni(II) complex were added in decreasing concentrations from 400 µg/mL to 3,2 µg/mL. Plates were incubated at 22°C by 21 days. The activity was evaluated by spectroscopic methods at 450 nm in Epoch™ (BIOTEK®) spectrophotometer recording the absorbance of each well after incubation and correcting the blank control. Each sample was analyzed in triplicate.

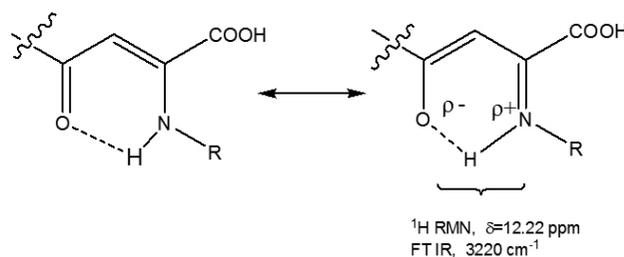
## RESULTS AND DISCUSSION

The synthesized compound (z)-2-(3-methoxyphenylamino)-4-oxo-4-phenylbut-2-enoic acid (L), displays a conjugate system, enaminoketone, give rise to intramolecular hydrogen bonding. The IR spectrum of L exhibits bands at 3220 and 1604 cm<sup>-1</sup> for the NH and CO stretching modes, respectively. The ligand conjugate system undergoes a tautomeric equilibrium (keto-enol/imine-enamine) as is reported in other similar systems<sup>19-22</sup>, showed in the scheme 3 and 4.



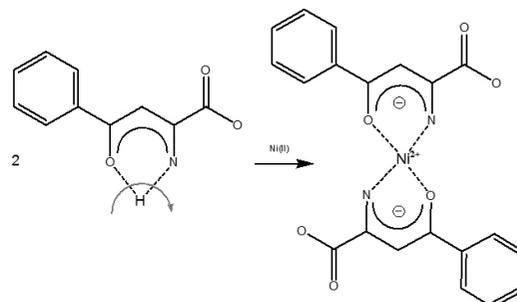
Scheme 3

The <sup>1</sup>H NMR spectrum of this compound in DMSO-d<sub>6</sub> at 20°C exhibits sharp signals characteristic of a single conformer (Z). Therefore L presents an intramolecularly H-bonded Z configuration<sup>23</sup>.



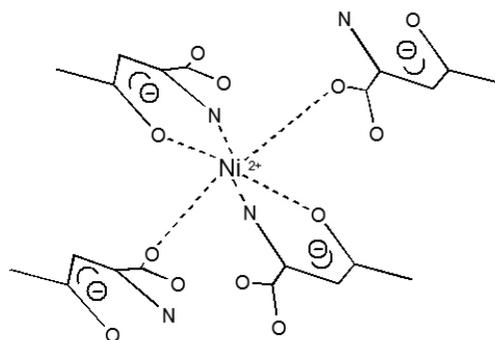
Scheme 4

With this in mind, for the reaction between the ligand and nickel (II), it has been postulated the following complex (Scheme 5).



Scheme 5

The infrared spectrum does not have a normal N-H band, because the system has been deprotonated. Instead the CO stretching mode has been shifted to 1591 cm<sup>-1</sup> due to the Ni-O=C coordination. The apical position could be occupied by a molecule of oxygen from the carboxylate group of a neighboring ligand; if possible then it will generate an infinite chain (Scheme 6).



Scheme 6

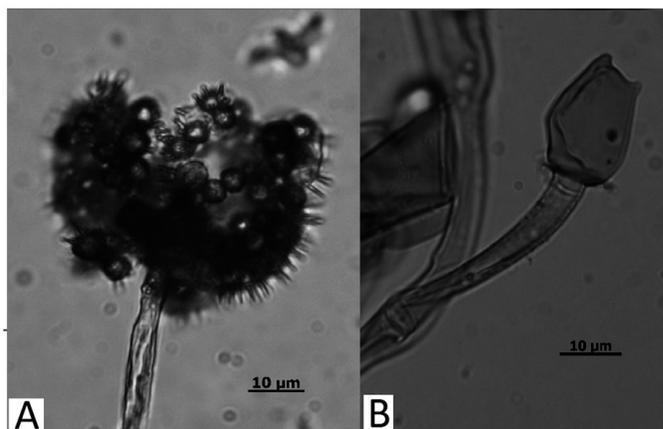
The interpretation of infrared to the complex is difficult because the metal ion coordination causes displacement of the bands; and it is possible that one of the tautomeric forms predominates over the other<sup>24</sup>. In spite of this, an assignation for the bands is proposed, Table 1.

**Table 1:** Infra-red analysis for Ni(II) complex.

Molecular vibration	Ligand (cm <sup>-1</sup> )	Complex (cm <sup>-1</sup> )
N-H	3219	-
C=O	1604	1591
C=N	1575	1575

**Fungal strain**

The microscopic analysis of the strain is coincident with the description for *Mucor* species presented by Schipper<sup>7</sup> in 1976; colony varying from 2-20 mm in height, Mouse Gray, Deep Mouse Gray or Light Olive Gray colour; sporangiophores branching in a sympodial and in a monopodial fashion (Fig. 1A), up to 21 µm in diam., constricted and infrequently recurved below sporangia, with slightly incrustated walls. Columellae pyriform, obovoid on a truncate base, ellipsoidal to cylindrical-ellipsoidal, with incrustated walls that rupture at maturity (Fig. 1B). The analysis of the RNA sequence fragment 972 pb, performed in BLAST, confirmed the identity of the fungus as *M. plumbeus* (*BankIt1532924 Seq1 JX123134*) with a one hundred percent match to *M. plumbeus* strain available from Genbank database (JN938896)<sup>25</sup>.

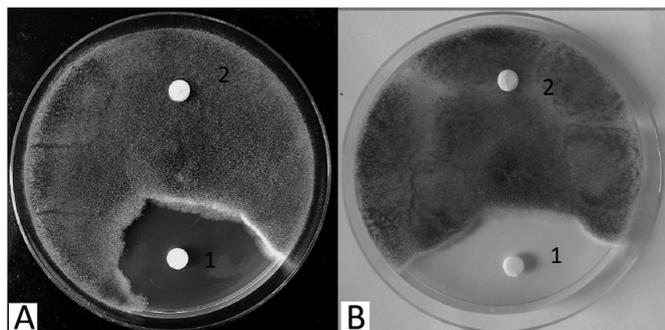


**Figure 1.** Microscopic features of *Mucor plumbeus*. A) Sporangiophore and spores growing on YMG agar. B) Pyriform columella without spores.

**Antifungal activity**

Qualitative agar diffusion test of Cu-Complex, Ni-Complex, Ligand and control were carried out against the native fungus *M. plumbeus* LQMP-001. The Ni complex displayed strong antifungal activity at concentration of 200 µg/disk, evidenced with a halo of 31 mm around disk after 21 days, figure 2A (1). The Cu complex did not show inhibitory halo at concentration of 200 µg/disk, figure 2A (2).

The ligand (L) did not show antifungal activity at concentration of 200 µg/disk, but it delayed the mycelial growth in the first 72 h, suggesting that it has fungostatic activity over *M. plumbeus* LQMP-001, figure 2B (2). The positive control, antiblue 375, displayed an inhibitory halo of 36 mm around disk at concentration of 200 µg/disk, figure 2B (1).



**Figure 2.** Antifungal activity against *Mucor plumbeus* at 200 µg/disk after 21 days. A) Complex LNi (1) and complex LCu (2). B) Positive Control, Antiblue 375 (1) and ligand (2).

Further Quantitative assays against the native fungus *M. plumbeus* LQMP-001 by microtitre plate test, n=3, determinate that the Ni complex has a minimal inhibitory concentration MIC, of 50 µg/mL. The Cu-complex has a MIC higher than 200 µg/mL. The control antiblue375, showed a MIC of 10 µg/mL, n=3.

**CONCLUSIONS**

We have synthesized and evaluated *in vitro* the antifungal activity of the ligand (z)-2-(3-methoxyphenylamino)-4-oxo-4-phenylbut-2-enoic acid and its metal complex's of nickel and copper against the wood stain fungus *Mucor plumbeus* LQMP-001. According to the *in vitro* results, the ligand displays fungostatic activity. The incorporation of Ni to the ligand molecule, formed a Ni(II) complex with antifungal activity, MIC of 50 µg/mL. Furthermore, the Cu (II) complex did not have bioactivity at concentration of 200 µg/mL or lower. The Ni complex could be a promising candidate as new antifungal agent for control of wood stain fungi.

The mechanism resistance to organometallic compounds of these metals is still unknown<sup>26</sup>. Furthermore, 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<sup>27,28</sup>.

**ACKNOWLEDGEMENTS**

Authors would like to thank the financial support from the Project Fondecyt 3130378, Project Basal PFB-27 (PCS-009), Universidad de Concepción and CONICYT for of its Doctoral Scholarship Program.

**REFERENCES**

- R. Juacida, S. Benedetti. *BOSQUE*. **18**, 87, (1997)
- Instituto Forestal (INFOR). El sector forestal chileno en una mirada. Santiago, Chile, 2005; pp. 68
- I. Vives, S. Ide, H. Peredo. *BOSQUE*. **25**, 79, (2004)
- C. Behrendt, R. Blanchette, R. Farrel. *Phytopathology*. **85**, 92, (1995)
- H. Butin and H. Peredo in Hongos parásitos en coníferas del sur con especial referencia a Chile. Biblioteca Mycologica. Band 101. J. Cramer. pp 100, 1986
- L. Giordano, P. Gonthier, G.C. Varese, L. Miserere, G. Nicolotti. *Fungal Diversity*. **38**, 69, (2009)
- M. Schipper. Studies in Micology N° 12. Institute of the Royal Netherlands, Academy of Arts and Sciences. Netherland. pp 40. 1976
- J. Webster and R. Weber. Introduction to fungi. Cambridge, 3<sup>rd</sup> edition. pp 841. 2007
- P. Montes, H. Peredo, D. Lanfranco, S. Ide and H. Dolz. *BOSQUE*. **22**, 85, (2001)
- M. Sechi, F. Carta, L. Sanna, R. Dallochio, A. Dessi, R. Al-Safi, N. Neamati. *Antiviral Research.*, **81**, 267, (2009)
- R. Braga, L. Hecquet, C. Blonski. *Bioorganic and Medicinal Chemistry*. **12**, 2965, (2004)
- E. Koz'minykh, A. belyaev, E. Berezina, V. Koz'minykh, R. Makhmudov, T. Odegova, *Pharmaceutical Chemistry Journal*. **36**, 643, (2002)
- M. Carvalho, S. Tavares, J. Medeiros, O. Núñez, H. Gallart-Ayala, M. Leitão, M. Galceran, A. Hursthouse, C. Silva. *Journal of Hazardous Materials*. **198**, 133, (2011)
- L. Tedersoo, T. Suvi, T. Jairus, S. Kõljalg. *Environmental microbiology*. **10**, 1189, (2009)
- A.J. Drummond, B. Ashton, S. Burton, M. Cheung, A. Cooper, J. Heled, R. Moir, S. Stones-Havas, S. Sturrock, T. Thierer, *Geneious 5.4*, Available from: <http://www.geneious.com/>. (2011)
- S. Suzuki, T. Murayama, Y. Shiono. *Phytochemistry*. **66**, 2329, (2005)
- D. Sarker, L. Nahar, Y. Kumarasamy. *Methods*. **42**, 321, (2007)
- Y. Morera, J. Torres-Rodríguez, T. Jiménez. *Rev Iberoam Micol*. **22**, 105, (2005)
- J. Lee, T. Lee, L. Chang, C. Lin, H. Lee, L. Hung, A. Datta, J. Huang, *Journal of Molecular Structure*, **929**, 207, (2009)
- V. Rybalkin, L. Popova, A. Dubonosov, E. Shepelenko, Y. Revinskii, V. Bren, V. Minkin, *Russian Journal of Organic Chemistry*, **37**, (2001)
- X. Yang, J. Liu, F. Zhao. *Journal of Fluorine Chemistry*. **125**, 415, (2004)
- M. Habibi, H. Loghmani-Khouzani, M. Mirmohammad Sadeghi, R. Harrington, W. Clegg, M. Ghorbani. *Analytical Sciences*, **22**, (2006)
- A. Dixit, K. Reddy, A. Deshmukh, S. Rajappa, B. Ganguly, J. Chandrasekhar. *Tetrahedron*. **51**, 1437, (1995)

24. J. Ledbetter. *The Journal of Physical Chemistry*. **81**, (1977)
25. C.L. Storch, K.A. Seifert, S. Huhndorf, V. Robert, J.L. Spouge, C.A. Levesque, W. Chen, Fungal Barcoding Consortium. *Proceedings of the National Academy Science, USA*. **109**, 6241, (2012)
26. H.N. Dietric. *Plasmid*. **27**, 17, (1992)
27. K. Fent, *Crit. Rev. Toxicol.* **26**, 1, (1996)
28. M. Anderiana, S.V. Avery, M.A. Zoroddu, G.M. Gadd, *FEMS Microbiol. Lett.* **167**, 321, (1998)