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

C. PAZ*, D. CAJAS-MADRIGA, C. TORRES, Y. MORENO, M. J. FERNÁNDEZ, J. BECERRA* AND M. SILVA

1Laboratorio de Química de Productos Naturales, Facultad de Ciencias Naturales, Universidad de Concepción, Chile
2Fac. Cs. Químicas-Centro de Biotecnología, Universidad de Concepción, Chile
3Laboratorio de Genómica y Biodiversidad (LGB), Departamento de Ciencias Naturales, Universidad del Bío-Bío, Chillán, Chile

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

ABSTRACT

The ligand (z)-2-(3-methoxyphenylamino)-4-oxo-4-phenyl-2-enolic 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 forest1, 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 mining2. For this reason, the damages caused by the fungi stainers of processed wood become one of the risk factors most relevant to the forestry sector3. 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 contamination4. It is common to find stacks of sawn timber with serious damage caused by fungi of the Ceratocystis genera5, however, in recent years new strains have been detected, among them we find representatives of Mucor genera, of which Mucor plumbeus Bon. is one of the most common6. 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 timber7. Contamination affects not only the processed wood, can also affect humans, putting at risk the health of the population1. The most used products for the pathogenic control of wood are chemical synthetic-based copper salts or chlorinated phenolic compounds8. Searching for new compounds with antifungal activity we studied the acid 4-phenyl-2,4-dioxobutanoic acid, which reported important biological activities like enzyme inhibitor of HIV-1 integrase9, in bacteria inhibits KDPG aldolase10 and derivates have shown analgesic activity11. From the 2,4-dioxobutanoic acid, we synthesized the ligand keto-enamine (z)-2-(3-methoxyphenylamino)-4-oxo-4-phenyl-2-enolic acid (L) and their Ni (II) and Cu (II) complexes and studied their fungicide activity in the wood stain fungi Mucor plumbeus.

EXPERIMENTAL

The 1H-NMR spectra were determined using a Bruker ARX 300 instrument, operating at 300.1 MHz (1H) and 75.5 MHz (13C). 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.

Keywords: wood stain fungi, Mucor plumbeus, Ni-complex.
**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, NiCl₂·6H₂O (119 mg, 0.5 mmol) or CuSO₄·5H₂O (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⁻¹): 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 sterile water, and dilutions of this suspension were streaked out onto YMG agar augmented with 200 mg l-1 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. This strain has been deposited at the collection of the Laboratory of Natural Products Chemistry of University of Concepción, Chile (accession number LQM-P-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. DNA was extracted from fruit bodies using the E.Z.N.A. fungal DNA MiniKit (Omegabiotek). PCR reactions were performed using Lr0R (5’-gtacccgctgaacttaagc-3’) as forward primer and LR06 (5’-gcggaccctggcttacc-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 MgCl₂, 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 s, followed by a final step of 5 min at 72°C. After PCR purification and sequencing (both directions) by Macrogen sequencing software, the 28S nuclear ribosomal large subunit rRNA (LSU) gene, D2, and D3 regions were analyzed in triplicate.

**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 10⁶ 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. The activity was evaluated by spectroscopic methods at 450 nm concentrations from 400 μg/mL to 3,2 μg/mL. Plates were 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. This strain has been deposited at the collection of the Laboratory of Natural Products Chemistry of University of Concepción, Chile (accession number LQM-P-001).

**Microtiter plate test**

The minimal inhibitory concentration (MIC) was determined following the method described by Sarker et al 2007. Microplates were cultivated in liquid media YMG, pH 5.5 and inoculated with 25 μL of solution 10⁻¹ 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-enolic acid (L), displays a conjugate system, enaminoketone, give rise to intramolecular hydrogen bonding. The IR spectrum of L exhibits bands at 3320 and 1604 cm⁻¹ 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, showed in the scheme 3 and 4.

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. In spite of this, an assignation for the bands is proposed, Table 1.

**Scheme 3**

**Scheme 4**

**Scheme 5**

**Scheme 6**
**Table 1: Infra-red analysis for Ni(II) complex.**

<table>
<thead>
<tr>
<th>Molecular vibration</th>
<th>Ligand (cm⁻¹)</th>
<th>Complex (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H</td>
<td>3219</td>
<td>-</td>
</tr>
<tr>
<td>C=O</td>
<td>1604</td>
<td>1591</td>
</tr>
<tr>
<td>C=N</td>
<td>1575</td>
<td>1575</td>
</tr>
</tbody>
</table>

**Fungal strain**

The microscopic analysis of the strain is coincident with the description for Mucor species presented by Schipper in 1976; colony varying from 2-20 mm in height, Mouse Gray, Deep Mouse Gray or Light Olive Gray colour; sporangiohores branching in a sympodial and in a monopodial fashion (Fig. 1A), up to 21 μm in diam., constricted and infrequently recurved below sporangium, with slightly incrusted walls. Columellae pyriform, obovoid on a truncate base, ellipsoidal to cylindrical-ellipsoidal, with incrusted 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)²⁷.

**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. 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 unknown²⁷. 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 equivalents²⁷, ²⁸.

**CONCLUSIONS**

We have synthesized and evaluated in vitro the antifungal activity of the ligand (z)-2-(3-methoxyphenylamino)-4-oxo-4-phenylbut-2-enolic acid and its metal complex’s of nickel and cupper against the wood stain fungus M. 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.

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**

2. Instituto Forestal (INFOR). El sector forestal chileno en una mirada. Santiago, Chile, 2005; pp. 68