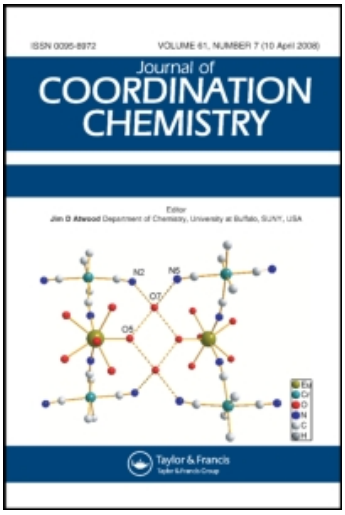

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Synthesis in solvent-free conditions, characterization and biological activity of a new Schiff-base ligand and its Cu(II) complex

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Synthesis in solvent-free conditions, characterization and biological activity of a new Schiff-base ligand and its Cu(II) complex

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A new *bis*-imine Schiff base (N_8O_2 donor, LH_2) has been synthesized by melt condensation of 3-amino-1,2,4-triazole with 1,5-*bis*(2′-formylphenyl)-1,5-dioksapentane in 2 : 1 molar ratio, in solvent and solvent-free conditions. The ligand has been characterized using FTIR, UV–Vis, ^{1}H NMR, ^{13}C NMR, mass spectra, elemental analysis, and thermal (TGA-DSC) techniques. A new complex of LH_2 with Cu(II) has been synthesized in solvent and solvent-free conditions. FTIR, UV–Vis mass spectra, and thermal analysis (TGA-DSC) have been used to characterize the Cu(II) complex. Thermal degradation showed that the final products were black carbon and metallic copper. Distorted octahedral geometry has been observed around $CuLH_2Cl_2 \cdot LH_2$ and its Cu(II) complex have been screened for *in vitro* antifungal and antioxidant activities. Antioxidation was determined for their superoxide scavenging and reducing activities. The ligand and its Cu(II) complex have strong antifungal activity against *Aspergillus niger*. The compounds have significant superoxide anion radical scavenging activity and reducing power against various antioxidant systems *in vitro*.

Keywords: Synthesis; Schiff base; Solvent-free reaction; Cu(II) complex; Antifungal activity; Antioxidant activity

1. Introduction

Schiff bases show biological applications including antibacterial, antifungal, and antitumor activity [1–5]. Triazoles and their heterocyclic derivatives also possess a wide spectrum of biological activities, such as analgesic, antiviral, antifungal, and anticancer activities [6–9]; 1,2,4-triazole and its derivatives constitute an interesting class of heterocycles, which gained considerable attention as a hybrid of pyrazole and imidazole with regard to arrangement of ring nitrogens [10]. Synthetic chemistry is developing approaches to profitable products with less environmental impact. One approach is solvent-free organic synthesis [11].

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In the present study, we report synthesis of a new triazole derivative *bis*-imine Schiff base and its Cu(II) complex using solvent-free conditions with solid–solid melt condensation reactions.

Schiff bases and complexes can be pro- or anti-oxidants. Structure and antioxidant activity relationships are inconsistent, but the quality of antioxidant action depends on the type of ligands forming the bioactive complexes [12]. Widespread use of antifungal agents has resulted in resistance to these drugs by pathogenic microorganisms [13]. Antioxidants are inhibitors against oxidative damage, helping to reduce oxidative damage from free radicals and reactive oxygen species [14]. The aim of this study is to investigate the antifungal and antioxidant activity of the ligand and its Cu(II) complex and to elucidate their antioxidative mechanism. Antifungal activity was determined against *Aspergillus niger* compared with Fluconazole as a standard antifungal drug. The ligand (LH_2) and its Cu(II) complex are also screened for superoxide radical scavenging and reducing activities, compared with synthetic antioxidants, e.g. butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), trolox or α-tocopherol.

2. Experimental

2.1. *Materials and methods*

FTIR spectra were recorded as KBr disks on a Perkin–Elmer Spectrum One Bv 5.0 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian UNITY INOVA 500 MHz (DMSO-d_6) spectrometer. TMS was used as internal standard in ^{1}H NMR. Mass spectra (LC-MS/MS) were determined on a Finnigan^{TM} LCQ^{TM} Advantage MAX spectrometer. Absorption spectra were recorded on an Agilent 8453 UV–Vis spectrophotometer. Elemental analyses were determined on a Thermo Finnigan Flash EA 112. TGA-DSC curves were obtained with a TA SDT Q 600 thermal analyzer using flowing nitrogen 100 mL min^{-1} from temperature range 50–1200°C, at heating rate of 10°C min^{-1}. Zinc was a reference standard. Melting points were obtained with a Büchi Melting point B-540 apparatus in open capillaries. 3-Amino-1,2,4-triazole is obtained from Merck company. Nicotinamide adenine dinucleotide (NADH), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH$^{\bullet}$), and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich GmbH, Steinheim, Germany. BHA and BHT were provided from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade from Sigma-Aldrich, Fluka or Merck. 1,5-*Bis*(2′-formylphenyl)-1,5-dioksapentane was prepared according to the published method [15].

2.2. *Syntheses*

2.2.1. Preparation of the Schiff base in solvent-free conditions (LH_2). 1,5-*Bis*(2′-formylphenyl)-1,5-dioksapentane (1.42 g, 5 mmol) was added to 3-amino-1,2,4-triazole (0.84 g, 10 mmol), stirred, and heated to 150°C for 4 h. The product was cooled giving a pale yellow solid which was washed with ethanol and water. It is soluble in DMSO and

DMF and slightly soluble in methanol. Yield 86%; m.p. 116°C; FTIR (KBr): 3225–3123 (NH), 3069 (Ar–CH), 2936–2882 (CH_2), 1598–1555 (C=N), 1244 (Ar–O–C) cm^{-1}. ^1H NMR: δ, 9.50 (N–CH=N), 7.77 (N=C–H), 7.06 −8.17 (Ar–H), 5.80 (N–H) (disappeared with D_2O exc.), 4.35 (Ar–O–CH_2) and 1.05 (CH_2) ppm. ^{13}C NMR: 159 (N–C=N), 135 (C=N), 115–125 (Ar–C), 62 (O–C) and 30 ppm (C–C). Anal. Calcd for $C_{21}H_{20}N_8O_2$ (416 g mol^{-1}): C, 60.57; H, 4.84; N, 26.91. Found: C, 60.52; H, 4.50; N, 26.75%. LC-MS: m/z: 415 [M−1].

2.2.2. Preparation of the LH_2 in solvent. A solution of 1,5-*bis*(2′-formylphenyl)-1, 5-dioksapentane (1.42 g, 5 mmol) in 50 mL absolute ethanol was added to 3-amino-1,2,4-triazole (0.84 g, 10 mmol) dissolved in 50 mL absolute ethanol. After refluxing the mixture for 6 h, a pale yellow precipitate was obtained, filtered off, and washed with ethanol and water. Yield 60%. All spectroscopic results are similar to the product from solvent-free conditions.

2.2.3. Preparation of the Cu(II) complex in solvent-free condition. LH_2 (0.2 mmol, 0.0836 g) was added to $CuCl_2 \cdot 2H_2O$ (0.2 mmol, 0.0340 g), stirred, and heated to 150°C for 3 h. The product was cooled and washed with ethanol, methanol, and water. The Cu(II) complex was green and slightly soluble in DMSO and DMF. Yield 92%; m.p. >360°C; FTIR (KBr): 3225–3124 (NH), 3070 (Ar), 1642 (C=N), 1239 (Ar–O–C) cm^{-1}. LC-MS: m/z: 553 [M + 2].

2.2.4. Preparation of the Cu(II) complex in solvent. A solution of LH_2 (2 mmol, 0.836 g) in 50 mL of absolute ethanol was added to $CuCl_2 \cdot 2H_2O$ (2 mmol, 0.340 g) in 50 mL of absolute ethanol. After the addition of 0.05 M NaOH solution in ethanol to raise the pH to 7.0–7.5, the mixture was stirred on a water bath at 60–65°C for 1 h. The green product precipitated, was filtered off, washed with ethanol, methanol, and water, and then dried. Yield: 52%. All spectroscopic results are similar to that of solvent-free condition.

2.3. *Biological activity*

2.3.1. Antifungal activity against *A. niger*. The antifungal activities of LH_2 and its Cu(II) complex were determined on *A. niger* using agar well-diffusion method as performed by Schillinger and Lücke [16]. Stock solution (1 mg mL^{-1}) of the test chemical was prepared by dissolving the test compound in DMF or DMSO. The stock solution was suitably diluted with sterilized distilled water to get 25, 50, and 100 μg mL^{-1}. Control for each dilution was prepared by diluting the solvent instead of stock solution with sterilized distilled water. The fungus was sub-cultured in potato dextrose agar medium (PDA). PDA plates were inoculated with freshly grown culture by spreading method. Wells (6 mm diameter) were punched in the agar and loaded with 150 μL samples of the complexes. Standard antifungal drug (Fluconazole) was used for comparison. The plates were incubated at 37°C for 48 h. Activity was determined by measuring the diameter of the zone showing complete inhibition (cm). In order to clarify any effect of DMF or DMSO on the biological screening, separate studies were

carried out with solutions alone of DMF or DMSO, and they showed no activity against the fungal strain. Antifungal activity was calculated as a mean of three replicates.

2.3.2. Antioxidant activity evaluation

2.3.2.1. *Superoxide radical scavenging activity*. Measurement of superoxide scavenging activity of LH_2 and its Cu(II) complex were based on the method described by Liu *et al*. [17]. Superoxide anions were generated in a non-enzymatic phenazine methosulfate–nicotinamide adenine dinucleotide (PMS–NADH) system by oxidation of NADH and assayed by reduction of NBT. In this experiment, the superoxide anion was generated in 3 mL of *Tris*-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 μM) solution, 1 mL of NADH (78 μM) solution, and 100 μg mL^{-1} concentration of sample solution. The reaction was started by adding 1 mL of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25°C for 5 min and absorbance at 560 nm was recorded against blank samples in a spectrophotometer. BHA, BHT, and trolox were used as standard samples (100 μg mL^{-1}). The inhibition of superoxide radical generation (%) was calculated by the equation:

$$\text{Inhibition (\%)} = [(A_0 - A_1)/A_0 \times 100]$$

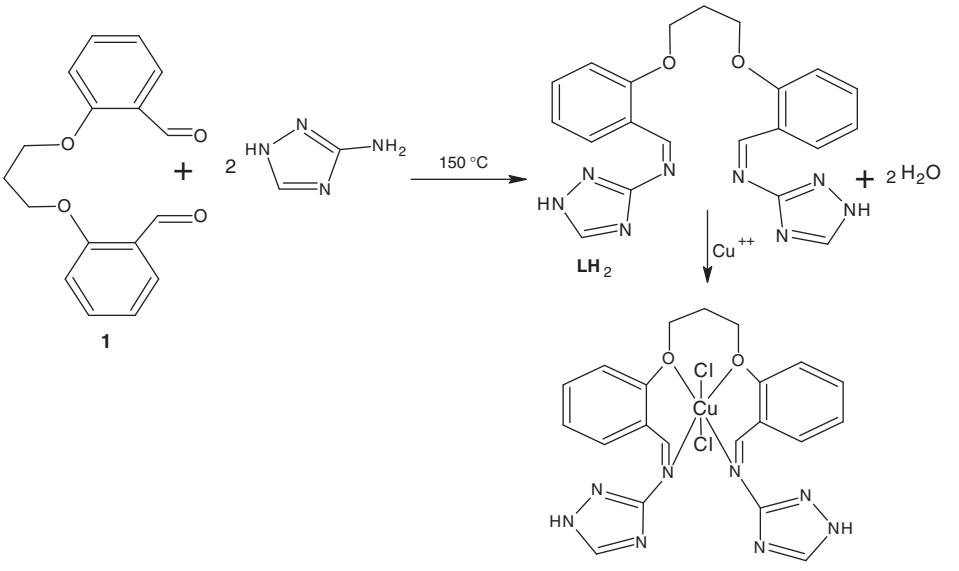
where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample or standards.

2.3.2.2. *Reducing power*. The reducing power of LH_2 and its Cu(II) complex were measured according to the method of Oyaizu [18]. Various concentrations of the samples (10–50 μg) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v), and the mixture was incubated at 50°C for 30 min. Afterwards, 2.5 mL of TCA (10%, w/v) was added to the mixture and centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of upper-layer solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%, w/v), and the absorbance was measured at 700 nm. α-Tocopherol, BHA and BHT were used as standard antioxidants. Higher absorbance of the reaction mixture indicated greater reducing power.

3. Results and discussion

3.1. *Preparation of LH_2 and the Cu(II) complex*

1,5-*Bis*(2′-formylphenyl)-1,5-dioksapentane (**I**) has been synthesized by Williamson condensation of salicylaldehyde and 1,3-dibromopropane [15]. Pure LH_2 was obtained as pale yellow microcrystalline solid by condensation of 1,5-*bis*(2′-formylphenyl)-1,5-dioksapentane with 3-amino-1,2,4-triazole. Condensation was carried out under solvent-free conditions and in solvent, yields 86 and 60%, respectively. The Cu(II) complex was prepared by treating LH_2 with $CuCl_2$ in 1 : 1 ratio, under solvent-free condition and in solvent with yields of 92 and 52%, respectively. These reactions are shown in scheme 1.



Scheme 1. Synthesis of the Schiff base and the Cu(II) complex.

3.2. *Characterization of LH_2 and the Cu(II) complex*

FTIR spectra confirmed synthesis of LH_2 by the presence of strong C=N at 1598 cm^{-1} and the aromatic ether (Ar–O–C) at 1244 cm^{-1}. These bands shifted to 1642 and 1239 cm^{-1} in the complex. The shifting of C=N to higher frequency and Ar–O–C to lower frequency suggest that coordination of the Schiff-bases occurred through Ar–O–C oxygen and nitrogen of azomethine. Coordination of the ligand with the metal was confirmed by appearance of a weak frequency at 497 cm^{-1} due to metal-nitrogen stretch [19]. All results are in agreement with the molecular formula of the ligand and complex.

Absorption spectra of the ligand in DMF consist of bands centered at 279 and 331 nm assigned to π–π^* and n–π^* of C=C, C=N, and NH groups. For the complex, bands at 268 and 318 nm are weaker than for the ligand, with a small blue shift indicating coordination *via* the azomethine nitrogen atom. A small band at 480 nm is the d–d transition for Cu(II) ion in a tetragonally elongated octahedral geometry around Cu^{2+} in $CuLH_2Cl_2$.

The NMR spectra and mass spectra (as given in section 2) are consistent with the structure.

These conclusions were also supported by thermal analyses. The TGA-DSC curves of LH_2 and its Cu(II) complex are provided in "Supplementary material". Samples were heated from 50 to 1200°C. Decomposition of LH_2 showed two steps and is thermally stable to 200°C. The endothermic peak at 116°C is the melting point. The largest weight loss rate was at 320°C (exothermic) (42.53%). Total weight loss is 80% from the beginning, with only black carbon at the end. Thermal analysis of the Cu(II) complex shows three stages of decomposition from 193–989°C. TGA-DSC curve of the complex shows endothermic peaks at 241 and 279°C corresponding to loss of 2Cl and $(CH_2)_3$ (Found 6.89 and 13.42%. Calcd 7.62 and 12.8%). Reduction of Cu(II) is shown as endothermic peak at 1070–1078°C. The final products, at ca 1200°C, are black carbon (20%) and metallic copper (Found: 12.5, Calcd 12.3%).

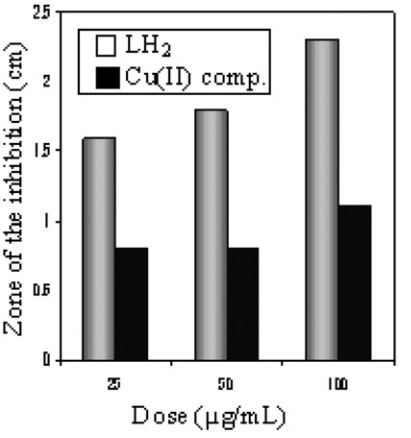


Figure 1. Antifungal activity of LH_2 and its Cu(II) complex.

In conclusion, a new *bis*-imine Schiff base and its complex have been synthesized at higher yield in solvent-free conditions. The higher concentration of reactants in the absence of solvents leads to more favorable kinetics than in solution. Here, low-melting reactants have been used.

3.3. *Biological activity*

3.3.1. Antifungal activity against *A. niger*. The compounds showed remarkable antifungal activity against *A. niger* (figure 1). LH_2 showed enhanced activity compared to its Cu(II) complex. Standard antifungal drug Fluconazole exhibited 2.5 cm of inhibition zone at a 100 µg mL^{-1} concentration.

3.3.2. Antioxidant activity evaluation

3.3.2.1. *Superoxide radical scavenging activity*. Superoxide radical is harmful to cellular components as a precursor of the more reactive oxygen species, contributing to tissue damage and various diseases. In biological systems, its toxic role can be eliminated by superoxide dismutase [20]. Radicals also play an important role during peroxidation of unsaturated fatty acids. In the PMS–NADH–NBT system, superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction reduces NBT. Decrease of absorbance at 560 nm with antioxidants indicates consumption of the superoxide anion. Figure 2 shows the superoxide radical scavenging activity by 100 µg mL^{-1} of LH_2 and its Cu(II) complex in comparison to same amount of BHA, BHT, and trolox. LH_2 showed higher superoxide radical scavenging activity than BHT while Cu(II) complex showed the highest activity when compared with BHA, BHT, and trolox. The superoxide radical scavenging activity of those samples were in the order: Cu(II) complex > trolox > BHA > LH_2 > BHT. IC_{50} values for scavenging abilities on superoxide radicals were 9.8 and 3.5 µg mL^{-1} for LH_2 and Cu(II) complex, respectively.

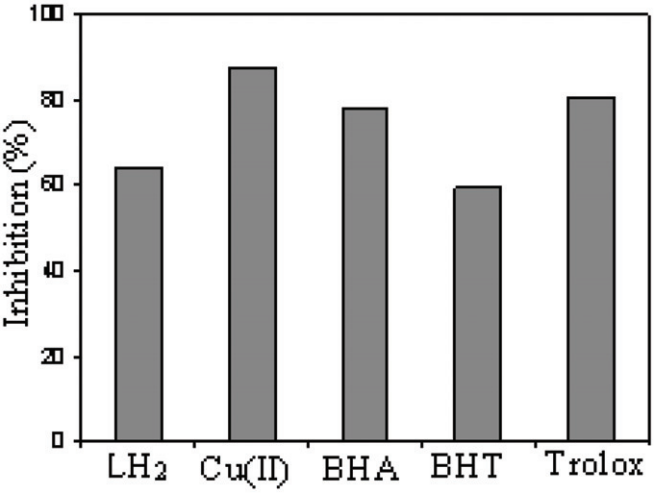


Figure 2. Superoxide radical scavenging activity of LH_2, its Cu(II) complex, BHA, BHT and, trolox at $100\,\mu g\,mL^{-1}$ concentration.

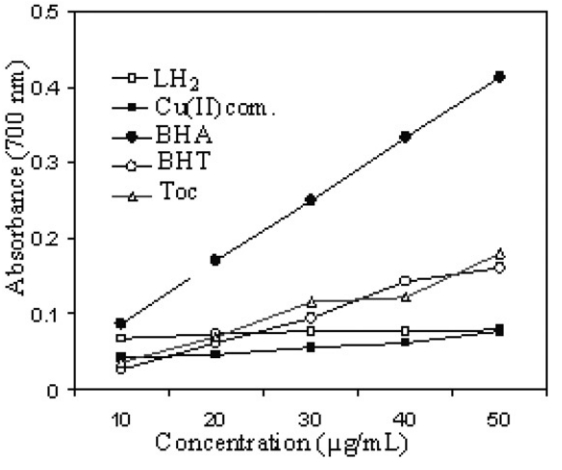


Figure 3. Reducing power of LH_2, its Cu(II) complex, BHA, BHT, and α-tocopherol on different concentrations.

3.3.2.2. *Reducing power*. The reducing power is one antioxidant capability indicator [21]. In the reducing power assay, the presence of reductants (antioxidants) in the tested samples results in reduction of Fe^{3+}/ferricyanide complex to Fe^{2+}. The amount of Fe^{2+} complex can be monitored by formation of Perl's Prussian Blue at 700 nm [20].

Figure 3 shows the reducing power of LH_2 and its Cu(II) complex. The reducing power of the samples was not concentration dependent. Based on comparison of the absorbance at 700 nm, the reducing power of LH_2 and its Cu(II) complex was higher than that of BHT and α-Tocopherol at $10\,\mu g\,mL^{-1}$. The synthesized compounds were good electron and hydrogen donors and could terminate the radical chain reaction, converting free radicals to more stable products.

4. Conclusions

We report synthesis and characterization of a new triazole *bis*-imine Schiff base and its Cu(II) complex using solvent-free conditions with solid–solid melt condensation. LH_2 and its Cu(II) complex have been characterized by elemental analyses, UV–Vis, FTIR, ^{1}H NMR, ^{13}C NMR, LC-MS spectra, and TGA-DSC techniques.

LH_2 and its Cu(II) complex have strong antifungal activity against *A. niger* and also exhibit antioxidant activity in all tests. The compounds have significant superoxide anion radical scavenging activity and reducing power against various antioxidant systems *in vitro*. Cu(II) complex showed excellent scavenging for superoxide radical. Therefore, this complex might be a new kind of scavenger of ROS.

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