

Antimicrobial Activity of 2-Nitro-6-[(4-Phenyl-Benzo[4,5]imidazo[1,2-a] Pyrimidin-2-ylimino)-Methyl]-Phenol: A Novel Schiff Base Compound

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Abstract—2-Nitro-6-[(4-phenyl-benzo[4,5]imidazo[1,2-a]pyrimidin-2-ylimino)-methyl]-phenol (LH1), a novel Schiff base compound with benzimidazole moiety was successfully synthesized and characterized using UV-VIS light, FTIR, ^1H and ^{13}C NMR. The compound was screened for its antifungal and bactericidal properties against seven fungal strains and twelve pathogenic bacteria. Disc diffusion method was used for antifungal activity and broth microdilution technique was used to determine the minimum inhibitory concentration (MIC). The compound showed strong inhibitory growth property against all fungal strains than

reference material amphotericin B. *Fusarium verticillioides* was the most sensitive fungus with 18 mm diameter of the inhibition zone. Gram-positive bacteria were more sensitive to LH1 compared to Gram-negative. LH1 showed more potent activity inducing bacteria cell death than the controls particularly with *Bacillus cereus* (MIC 7.8 $\mu\text{g/mL}$) against streptomycin and nalidixic acid (MIC 512 $\mu\text{g/mL}$). We conclude that LH1 has a low tolerance to these microbes, and promise to be a better remedy against their metabolic expressions

Keywords—Antibacterial, antifungal, antimicrobial benzimidazole, Schiff base

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I. INTRODUCTION

The screening for antimicrobial agents to target human pathogenic bacteria and the major food contaminants (fungi) remain growing challenges because of microbes resisting drugs currently in use. Microorganisms through the process of adaptation have developed some forms of resistances against antibiotics and as such turned to nullify the efficacy of reference drugs. The lost in human and animal foods due to bacterial and fungal contamination registered per annum is of worldwide concern. Details on the contamination of agricultural products by toxigenic fungi and the hazard of ingesting the contaminated food products are available [1]; [2]; [3] and the need to feed the current 7.4 billion lives demand insight into food security and food safety.

Azomethines or imines (C=N-) are carbon-nitrogen double bond compounds found in some naturally occurring molecules and can also be obtained by the condensation of a carbonyl and a primary amine as initially reported by Hugo Schiff [4]. Known as Schiff bases, imine properties have attracted scientists globally due to their multifaceted applications. Schiff bases have found interest in chemistry, biology, physics and the medical fields [5]; [6]; [7]; [8]. Schiff bases are amongst the most widely used organic molecules globally. The ease synthesis of Schiff bases by either condensation of primary amines with carbonyls in hot alcoholic solution, microwave assisted method or in water have been made known [9]; [10]. These have resulted in hundreds of thousands of compounds with diverse applications. In the search for novel Schiff base compounds with pharmacological properties, heterocycle derived Schiff bases have pointed out as better candidates and particularly those with benzimidazole moiety.

Benzimidazole is a nitrogen-based heterocyclic compound made of the fusion of a benzene ring and imidazole ring (Fig. 1). This compound is an important intermediate for the development of molecules of biological interest because of the inbuilt bioactive pharmacophore imidazole [11].

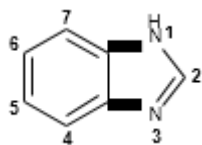


Fig. 1. Chemical structure of 1H-Benzimidazole

Incorporation of imidazole to benzene provides enough electron withdrawal to the molecule, which increases its nucleophilic property and allows substitution at different positions on the ring. Today it is a moiety of choice which possesses many biological applications and clinical properties [12]. Several derivatives of benzimidazole have been recorded together with their biological importance [13]. Benzimidazole and its derivatives have the capacity to interfere several microbial metabolic processes that in turn affect their growth and sometimes leading to cell death. They have shown good activity against bacteria [14], fungi [15], virus [16], parasites [4]. They also have good anti-inflammatory property [17] and are also good antioxidant agents [18]. Despite these remarkable inputs made globally and the continuous microbial resistance, the demand for more efforts to attend these challenges is increasing. In this study, we report the synthesis, characterization and the antimicrobial activity which include the antifungal and bactericidal activity of a novel Schiff base compound with benzimidazole moiety.

II. MATERIAL AND METHODS

A. Material

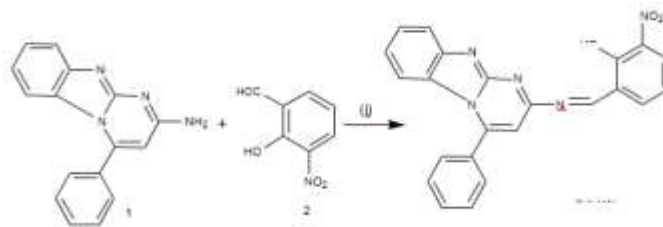
Standard grade chemicals and reagents were used throughout in this study. The starting material compound **1** was donated to us by Dr. Ndinteh Derek Tantoh from the Department of Applied Chemistry University of Johannesburg. Detailed information on compound **1** is found here [19]. 2-Hydroxy-3-nitrobenzaldehyde, ethanol, and other solvents were obtained from Sigma-Aldrich South Africa. The microorganisms used were purchased from Davies Diagnostic South Africa and glycerol stocks were prepared and stored at -8 °C.

B. Physical Measurements

The melting point of compound LHI was recorded on a hot stage electrothermal digital melting point apparatus with a maximum heating capacity of 450 °C. Fourier transform infrared (FTIR) spectrum was collected using a Spectrum 100, PerkinElmer FTIR spectrophotometer with a CsI disc in the frequency range of 4000-200 cm⁻¹. Optical measurement of the Schiff base was recorded on UV-2540 Shimadzu UV-Vis absorption spectrophotometry. The absorption was recorded between 200-800 nm. Flash 2000 organic elemental analyzer was used to determine the percentage of C, H, N contains in compound LHI. Structure elucidation by ¹H NMR and ¹³C NMR was assessed on a Bruker 400 MHz spectrometer apparatus operating at room temperature. Chemical shift values (δ) are given in ppm (parts per million) and deshield internally from tetramethylsilane (TMS).

C. Synthesis of Schiff Base Ligand LHI

Compound LHI was synthesized as described by Yousif *et al.* [20] as shown below (Scheme. 1.). Compound **1** (50 mg; 18 mmol) was mixed with a solution of compound **2** in an equimolar ratio (1:1) and refluxed in ethanol for 4 hours. The yellow solution obtained was concentrated at low pressure on a rotary pump. The resulting precipitate was washed with cold ethanol, air dried and maintained from moisture in a desiccator.



Scheme. 1. Synthesis of LHI

2-Nitro-6-[(4-phenyl-benzo[4,5]imidazo[1,2-a]pyrimidin-2-yl imino)-methyl]-phenol (LHI)

LHI, (79% yield); yellowish powder, mp = 149°C; FTIR(cm⁻¹): 3676, 2991, 1759, 1558, 1292, 1052, 921, 766; ¹H NMR (400 MHz, DMSO-d₆) δ = 10.24 (1H, s, OH), 8.16 (1H, s, CH=N-), 8.05 (1H, d, J=8.0 Hz, Ar-H), 7.78 (1H, d, J=8.0 Hz, Ar-H), 7.68-7.67 (3H, m, Ar-H), 7.56 (1H, d, J=8.0 Hz, Ar-H), 7.35 (2H, t, J=8.0 Hz, Ar-H), 6.96 (1H, t, J=8.0 Hz, Ar-H), 6.60 (1H, t, J=8.0 Hz, Ar-H), 6.38 (1H, s, Ar-H), 6.17 (1H, d, J=8.0 Hz, Ar-H). ¹³C NMR (400 MHz, DMSO-d₆) δ = 191.1, 161.8, 161.3, 151.0, 148.4, 134.9, 132.2, 131.4, 131.3, 129.4, 128.6, 127.8, 126.8, 125.5, 121.1, 114.7, 113.3, 113.0, 101.6. Anal. Calcd for C₂₃H₁₅N₅O₃ %: C,54.29; H, 3.60; N, 10.55; Found: C,54.84; H, 3.48; N,10.58.

D. Biological Assays

Bactericidal Activity

Twelve pharmaceutical strains of bacteria were used to assess the bactericidal property of ligand LHI. The studied microbes include Gram positive (*Bacillus cereus* (ATCC10876), *Bacillus subtilis* (ATCC19659), *Enterococcus faecalis* (ATCC13047), *Mycobacterium smegmatis* (MC2155), *Staphylococcus epidermidis* (ATCC14990) and Gram-negative *Escherichia coli* (ATCC25922), *Enterobacter cloacae* (ATCC13047), *Klebsiella oxytoca* (ATCC8724), *Klebsiella pneumonia* (ATCC13882), *Proteus vulgaris* (ATCC6380), *Pseudomonas aeruginosa* (ATCC27853), *Enterobacter aerogenes* (ATCC13048). Broth microdilution method was followed as recommended here [21]. From an overnight bacterial culture prepared in nutrient broth, a suspension of 1.5 x 10⁸ cfu/mL in Muller-Hilton corresponding to the 0.5 McFarland standard was made ready. LHI was dissolved in DMSO to contain 1 mg/mL stock solution. This was then serially diluted to give 500, 250, 125, 62.5, 31.2, 15.6 and 7.8 μ g/mL of which 100 μ L of each was seeded in duplicate in 96 well plates and flooded with 100 μ L of inoculum. The mixture was then incubated at 37 °C in 5% CO₂ for 24 hours. Streptomycin and nalidixic acid were used as reference drugs for comparison. The amount of bacteria that resisted LHI exposure was determined calorimetrically in resazurin dye. The minimum inhibitory concentration was

recorded as the smallest concentration that induced bacterial cell death.

E. Antifungal Activity

The synthesized compound LH1 was evaluated through a preliminary *in vitro* antifungal screening activity against seven fungal strains (*Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus carbonarius*, *Fusarium proliferatum*, and *Fusarium verticillioides*). Disc diffusion method by CLSI [21] standard M28 was followed. Each fungal strain was allowed to produce spores after seven days incubation on potatoes dextrose agar (PDA). The spores were then harvested in Ringer solution and diluted to contain 1×10^5 spores/mL working solution. Sterile blank discs (6 mm ϕ) were impregnated with 20 μ L of 1 mg/mL of test compound LH1 and placed in triplicate on solidified fresh PDA spread with 500 μ L of inoculum. The plates were incubated for 72 hours at 37 $^{\circ}$ C. Amphotericin B and nystatin were used as positive controls. The effect of LH1 on fungal growth was observed and the diameters of the inhibition zones were measured in millimeter (mm) and compared with commercial standards.

III. RESULTS AND DISCUSSION

A. Spectroscopy Data

Schiff base compound LH1 was obtained by condensation reaction of starting materials **1** and **2** refluxed in hot ethanol for 4 hours. The yellow solution obtained was dried out and the resulting yellow precipitate was washed with cold ethanol. The compound is stable in the air and has a high melting point (149 $^{\circ}$ C). LH1 is soluble in DMSO and in hot ethanol however it precipitates in cold ethanol and is insoluble in methanol and chloroform. Electronic spectral of LH1 in DMSO showed absorption bands in the low UV range between 200–400 nm. The two strong maxima were observed at 275 nm and 316 nm. These absorption bands were assigned to the $\pi-\pi^*$ and $n-\pi^*$ respectively. The first transition ($\pi-\pi^*$) at 275 nm resulted from the phenolic ring resonance while the second ($n-\pi^*$) corresponds to the imine bond [22].

Infrared spectrum (FTIR) of LH1 showed a low or weak intensity band at 3676 cm^{-1} due to the phenolic OH group. The spectrum showed a sharp absorption signal at 1558 cm^{-1} corresponding to the C=N vibration mode. Similar results are published here [23] confirming the successful formation of the azomethine bond. The stretching vibration at 2991 cm^{-1} was conferred to the ν C-H vibration frequency of the phenyl ring [24].

The percentage composition of C, H, and N contain in LH1 was determined theoretically and compared with experimental findings. Data showed that calculated values matched experimental results with ± 0.5 units. These observations satisfied our expectations.

The structure of the title compound was confirmed using ^1H and ^{13}C NMR analysis. In the ^1H NMR spectrum showed a strong signal at $\delta = 8.16$ ppm appearing as a singlet which was

assigned to the imine C=N-H proton. Phenolic proton (Ar-OH) appeared at $\delta = 10.24$ ppm with reference to the degree of the downfield medium. Aromatic protons are peaked as doublet (d), doublet the double (dd), triplet (t), and multiplet (m) signals at $\delta = 6-8.03$ ppm with a coupling constant $J = 8$ Hz. No undesired proton was recorded on the spectrum. ^{13}C NMR spectrum of the prepared Schiff base LH1 showed characteristic signals of imine and aromatic carbon atoms in the expected ranges of chemical shift values. At $\delta = 161.85$ and $\delta = 161.35$ ppm, the imine groups (C=N-C) and (C=N-H) are observed. All carbons of the aromatic rings were observed between $\delta = 101-155$ ppm [25]. The signal seen at $\delta = 191.12$ ppm assigned to the (N=C-N=C-H) appeared downfield as a result of the electronegativity of the N atom together with a free electron density of the aromatic rings.

B. Biological Data

Bactericidal Activity

Antimicrobial activity by broth microdilution method of compound LH1 against Gram-positive and Gram-negative bacteria was studied. The tested organisms were all sensitive to LH1 and the minimum inhibitory concentrations are summarized in Table. 1. We clearly see that the ligand has a low tolerance for Gram-positive bacteria (MIC 7.8 $\mu\text{g/mL}$) compared to Gram-negative (15.6 $\mu\text{g/mL}$) affecting more the former. The compound exhibited strong activity against some pathogenic strains with greater potency than the reference drugs particularly *Bacillus cereus* (MIC 7.8 $\mu\text{g/mL}$) against standards (MIC 32 $\mu\text{g/mL}$). This makes LH1 compound four times more potent than streptomycin and nalidixic acid. Moreover, *Mycobacterium smegmatis* MIC 31.2 $\mu\text{g/mL}$ against nalidixic acid MIC 512 $\mu\text{g/mL}$ proved LH1 to be sixteen times strongly active than the control against the bacterium. The potency of LH1 to induce cell death in Gram-negative bacteria was observed against *Escherichia coli* and *Enterobacter cloacae* with MIC 250 $\mu\text{g/mL}$ and 15.6 $\mu\text{g/mL}$ respectively against commercial drugs (MIC 512 $\mu\text{g/mL}$). These results, therefore, suggest that the compound can be considered as a candidate drug for the treatment of infections caused by these pathogens.

The cell walls of bacteria are made of lipids that regulate and control the flow of molecules in or outside the cells. The passage of molecules/ions across the cell membrane is polarity dependent. Lipids based membranes will interact better with lipid soluble compounds, therefore, favor their penetration into the cytoplasm. Lipophilicity (cLogP) is an important factor for controlling the antimicrobial activity of target drugs was determined theoretically to be cLogP = 5.37. This high value could also be the reason behind the strength of compound LH1. Because high lipophilicity generally leads to compounds with high rapid turnover [26], this increases the chemical interaction between the compound and bilayer membrane. As lipophilicity increases so are the cells membrane permeability which might result in an alteration in cellular metabolic activity leading to cell death. The hydrophobic core of the LH1 could also be associated with its aromatic rings which allow lipid solubility

and consequently facilitates its passage across the cell membrane. [27].

TABLE I: DIAMETER (MM) OF ZONE OF INHIBITION OF LIGAND LH1, AMPHOTERICIN B AND NYSTATIN ($\mu\text{g}/\text{mL}$)

Test compounds	Bacteria											
	Gram-positive					Gram-negative						
	BS	EF	SE	BC	MS	EC	PV	KO	EA	PA	KP	EC
LH1	31.2	250	125	7.8	31.2	250	250	125	125	125	250	15.6
STM	16	128	8	32	4	64	0.032	16	16	8	64	512
NLD	16	512	64	32	512	512	0.008	8	256	64	64	16

STM: Streptomycin; NLD: Nalidixic acid; BC: Bacillus cereus, BS: Bacillus subtilis, EF: Enterococcus faecalis, MS: Mycobacterium smegmatis, SE: Staphylococcus epidermidis, ECL: Enterobacter cloacae, EC: Escherichia coli, EA: Enterobacter aerogenes, PV: Proteus vulgaris, KO: Klebsiella oxytoca, KP: Klebsiella pneumonia, and PA: Pseudomonas aeruginosa

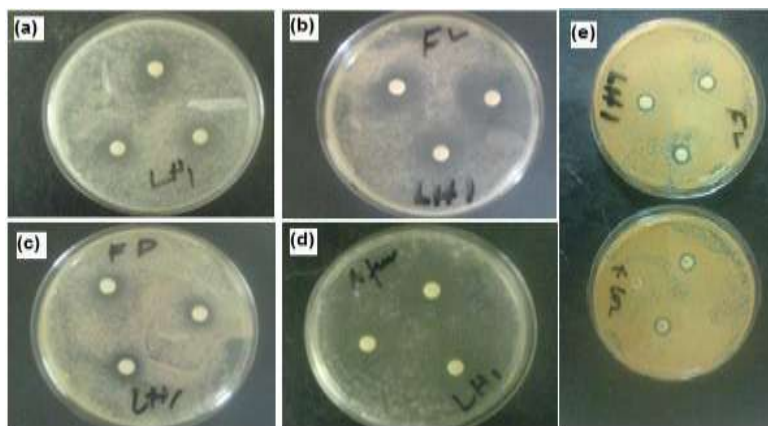


Fig. 1. Photography images showing the area of inhibition exhibited by ligand LH1 against (a) *Aspergillus flavus*, (b) *Fusarium verticillioides*, (c) *Fusarium proliferatum*, (d) *Aspergillus fumigatus*, and (e) *Fusarium verticillioides* against amphotericin B and nystatin standards (20 μg). (Here 20 μL of LH1 corresponding to 20 μg of LH1 was impregnated on each disc in triplicate)

Antifungal Activity

Disc diffusion assay was used to assess the antifungal activity of ligand LH1 against seven fungal strains. Sterile blank discs were soaked with 20 μL of test compound containing 20 μg of the analyte and placed on solidified PDA conditioned with 1×10^5 spores/mL of each fungus. Compound 1 affects the ease propagation of all fungi, inhibiting their growth as seen in Fig. 2. The compound has greater activity against the references materials used. The clear zones (no growth) around the discs were measured diagonally and the average values are summarized in Table. II. together with commercial drugs as diameters of inhibitory zones.

TABLE II: DIAMETER (MM) OF ZONE OF INHIBITION OF LIGAND LH1, AMPHOTERICIN B (AMB) AND NYSTATIN (NYT)

Fungal strains	Standards		
	LH1	AMB	Nystatin
<i>Aspergillus carbonarius</i>	13	~	6
<i>Aspergillus flavus</i>	14	10	12
<i>Aspergillus fumigatus</i>	16	9	14
<i>Aspergillus niger</i>	13	10	17
<i>Aspergillus parasiticus</i>	15	~	12
<i>Fusarium proliferatum</i>	16	7	7
<i>Fusarium verticillioides</i>	18	7	14

Compound LH1 has strong antifungal activity against all the tested fungal strains and in some cases greater than the positive controls studied. The compound showed greater activity than

amphotericin B. The compound LH1 displayed the highest activity against *Fusarium verticillioides* (Fig. 1 (b)) followed by *Fusarium proliferatum* (Fig. 1 (c)) and *Aspergillus fumigatus* (Fig. 1 (d)). The lowest activity of LH1 was noted against *Aspergillus niger* (Fig. not shown) yet with a diameter (ϕ 10 mm) higher than that of standard amphotericin B (ϕ 8 mm). Compound LH1 has a broader spectrum of activity exhibiting more strength against amphotericin B resistant strains like *Aspergillus carbonarius* and *Aspergillus parasiticus* with ϕ 13 mm against 0 mm activity for AMB image not shown.

The antifungal activity of LH1 was similar to that of nystatin with slight differences. The potency of the compound can be explained by the similar reasons mentioned above. Though prokaryotic mechanisms of defense differ from fungi's and other large molecular genomes (eukaryotes), the same factors that control the movement of molecules in and outside the cells are maintained. The composition of bacteria cell membrane might differ from that of fungi but the major components are lipids and therefore the overturn theory [26] still applies.

IV. CONCLUSION

A novel Schiff base compound LH1 with benzimidazole moiety was successfully synthesized, characterized, and studied for its antibacterial and antifungal properties. Spectroscopy data confirmed the structure of the ligand synthesized. The compound (LH1) showed strong antibacterial and antifungal activities against all the microorganisms tested. LH1 was cytotoxic to all bacterial strains analyzed in this study showing

greater bactericidal potency than some reference materials. In addition, LH1 inhibits fungal growth far better than amphotericin B against all the fungi used, suggesting consideration of the compound as candidate remedy for these toxigenic fungi and pathogenic strains.

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