Synthesis, structural characterization, DNA-binding and antimicrobial activities of series of new macrocyclic ligands and their transition metal complexes

by

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ABSTRACT

Two series of novel mixed aza-oxo-thia macrocyclic ligands; 1,7(2,6)-ditriazina-2,6,8,12-tetraaza-3,5,9,11-tetraoxocyclododecaphan-1^4,7^4-dimethyl **(L_1)**; 1,8(2,6)-ditriazina-2,7,9,14-tetraaza-3,6,10,13-tetraoxocyclotetradecaphan-1^4,8^4- dimethyl **(L_2)**; 1,9(2,6)-di-triazina-2,8,10,16-tetra-aza-3,7,11,15-tetraoxocyclohexadecaphan-1^4,9^4-dimethyl **(L_3)**; 1,10(2,6)-ditriazina-2,9,11,18-tetraaza-3,8,12,17-tetraoxocyclooctadecaphan-1^4,10^4-dimethyl **(L_4)**; 1,9(2,6)-ditriazina-2,8,10,16-tetraaza-3,7,11,15-tetraoxo-5,13-dithiacyclo-hexadecaphan-1^4,9^4-diphenyl **(L_5)**; 1,10(2,6)-ditriazina-2,9,11,18-tetraaza-3,8,12,17-tetra-oxo-5,6,14,15-tetrathiacyclooctadecaphan-1^4,10^4-diphenyl **(L_6)**; 1,11(2,6)-ditriazina-2,10,12,20-tetraaza-3,9,13,19-tetraoxo-6,16-dithiacyclocosaphan-1^4,11^4-diphenyl **(L_7)** ; 1,12(2,6)-ditriazina-2,11,13,22-tetraaza-3,10,14,21-tetraoxo-6,7,17,18-tetrathiacyclodo-cosaphan-1^4,12^4-diphenyl **(L_8)**; 1,2-Di(*o*-aminophenylthio)ethane **(L_9)** and monometallic complexes of **(L_9)** ligand with palladium halides PdX_2 (X = Cl, Br, I) were synthesized. The structural features of the compounds have been investigated by elemental analyses, conductivity measurements, FT-Raman, FT-IR, ^1H and ^{13}C NMR spectroscopy. The antimicrobial activities of the ligands were evaluated using disk diffusion method in dimethyl sulfoxide (DMSO) as well as the minimal inhibitory concentration (MIC) dilution method, against several bacteria and yeast cultures. The obtained results from both methods were assessed in side-by-side comparison with those of Penicillin-G, Ampicilin, Cefotaxime, Vancomycin, Oflaxacin, Tetracyclin well-known antibacterial agents. The results from dilution procedure were compared with Gentamycin as antibacterial and Nystatin as antifungal. In most cases, the compounds show strong antifungal activity in the comparison tests. The binding of the ligands to genomic DNA was assessed by monitoring the change in absorption spectra at 260 nm in the presence or absence of genomic DNA. The binding ability of compounds to DNA was also determined by electrophoretic mobility

shift using polyacrylamide gel electrophoresis assay (PAGE). The effect of ligands on Taq polymerase-catalyzed gene amplification reaction was investigated by using PCR method. The cytotoxicity assay was performed using human cervical cancer HeLa cells.

Keywords: Macrocyclic Ligand; FT-Raman; Dilution; Antimicrobial; Antifungal; Cefotaxime; Gentamycin.

ÖZET

Aza-okso-tiya makrosiklik ligantlardan yeni iki seri; 1,7(2,6)-ditriazina-2,6,8,12-tetraaza-3,5,9,11-tetraoksosiklododekafan-1^4,7^4-dimetil **(L_1)**; 1,8(2,6)-ditriazina-2,7,9,14-tetraaza-3,6,10,13-tetraoksosiklotetradekafan-1^4,8^4-dimetil **(L_2)**; 1,9(2,6)-ditriazina-2,8,10,16-tetraaza-3,7,11,15-tetraoksosiklohekzadekafan-1^4,9^4-dimetil **(L_3)**; 1,10(2,6)-ditriazina-2,9,11,18-tetra aza-3,8,12,17-tetraoksosiklooktadekafan-1^4,10^4-dimetil **(L_4)**; 1,9(2,6)-ditriazina-2,8,10,16-tetraaza-3,7,11,15-tetraokso-5,13-ditiyasiklohekzadekafan-1^4,9^4-difenil **(L_5)**; 1,10(2,6)-ditriazina-2,9,11,18-tetraaza-3,8,12,17-tetraokso-5,6,14,15-tetratiyasiklo-oktadekafan-1^4,10^4-difenil **(L_6)**; 1,11(2,6)-ditriazina-2,10,12,20-tetraaza-3,9,13,19-tetra-okso-6,16-ditiyasiklo-kozafan-1^4,11^4-difenil **(L_7)**; 1,12(2,6)-ditriazina-2,11,13,22-tetraaza-3,10,14,21-tetraokso-6,7,17,18-tetratiyasiklodokozafan-1^4,12^4-difenil **(L_8)**; ve makrosiklik olmayan 1,2-Di(*o*-aminofeniltiyo)etan **(L_9)** ligandı sentezlendi**.** **(L_9)** ligandının PdX_2 (X = Cl, Br, I) ile olan monometalik kompleksleri hazırlandı. Ligantlar ve kompleksler elemental analiz, iletkenlik ölçümleri, FT-Raman, FT-IR, ^{1}H and ^{13}C NMR spektroskopi yöntemleri kullanılarak yapısal olarak karakterize edildi. Antimikrobiyal ve antifungal aktiviteleri birçok bakteri ve mantar kültürüne karşı disk difüzyon ve minimum inhibisyon konsantrasyonu (MIC) metotları kullanılarak tayin edildi. Disk difüzyon metodundan elde edilen sonuçlar bilinen antibakteriyellerden Penicilin-G, Amficilin, Cefotaxime, Vancomycin, Oflaxacin ve Tetracyclin ile tek tek karşılaştırıldı. Minumum inhibisyon konsantrasyon metodundan elde edilen sonuçlar antibakteriyel olarak Gentamycin ve antifungal olarak Nystatin ile karşılaştırıldı. Antifungal aktiviteler Candida albicans, Kluyveromyces fragilis, Rhodotorula rubra, Debaryomyces hansenii, ve Hanseniaspora guilliermondii olarak adlandırılan beş mantar kültürü üzerinde çalışılarak rapor edildi, sonuçlar ticari antifungallardan Nystatin, Ketaconazole ve Clotrimazole referans alınarak elde edildi. Ayrıca, sentezlenen ligantların DNA ile olabilecek etkileşimleri UV-görünür

spektroskopi, poliakrilamit jel elektroforesis ve polimeraz zincir reaksiyonu metotları ile incelendi. Sentezlenen ligantların sitotoksisite aktiviteleri HeLa (serviks adeno karsinoma hücresi) kanser hücrelerinde araştırıldı.

Anahtar Kelimeler: Makrosiklik Ligant; FT-Raman; Disk Difüzyon; Antimikrobiyal; Antifungal; Cefotaxime; Gentamycin.

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NOMENCLATURE

NMR	: Nuclear Magnetic Resonance
FT-IR	: Fourier transform infrared
PCR	: Polymerase chain reaction
PAGE	: Polyacrylamide gel electrophoresis
MIC	: Minimal inhibitory concentration
HeLa	: Cervical cancer cells taken from Henrietta Lacks
Nd:YAG	: Neodymium doped yttrium aluminum garnet
DMSO	: dimetyhlsulfoxide
ESI-MS	: Electrospray ionization-Mass spectrometry
Cfu	: Colony-forming unit
TBE	: Buffer containing a mixture of Tris base, boric acid and EDTA
dNTP	: Nucleotide
MTT	: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenpyltetrazolium bromide
bp	: Base pair
L_1	: 1,7(2,6)-ditriazina-2,6,8,12-tetraaza-3,5,9,11-tetraoxocyclododecaphan-1^4,7^4-dimethyl
L_2	: 1,8(2,6)-ditriazina-2,7,9,14-tetraaza-3,6,10,13-tetraoxocyclotetradecaphan-1^4,8^4-dimethyl
L_3	: 1,9(2,6)-ditriazina-2,8,10,16-tetra-aza-3,7,11,15-tetraoxocyclohexadeca-phan-1^4,9^4-dimethyl
L_4	: 1,10(2,6)-ditriazina-2,9,11,18 -tetraaza-3,8,12,17-tetraoxocyclooctadeca-phan-1^4,10^4-dimethyl
L_5	: 1,9(2,6)-ditriazina-2,8,10,16-tetraaza-3,7,11,15-tetraoxo-5,13-dithiacyclohexadecaphan-1^4,9^4-diphenyl

L_6 : 1,10(2,6)-ditriazina-2,9,11,18-tetraaza-3,8,12,17-tetraoxo-5,6,14,15-tetrathiacyclooctadecaphan-1^4,10^4-diphenyl

L_7 : 1,11(2,6)-ditriazina-2,10,12,20-tetraaza-3,9,13,19-tetraoxo-6,16-dithiacyclocosaphan-1^4,11^4-diphenyl

L_8 : 1,12(2,6)-ditriazina-2,11,13,22-tetraaza-3,10,14,21-tetraoxo-6,7,17,18-tetrathiacyclodocosaphan-1^4,12^4-diphenyl

L_9 : 1,2-Di(*o*-aminophenylthio)ethane

Chapter 1

INTRODUCTION

The design and synthesis of macrocyclic compounds have very greatly advanced over the last few decades mainly with the goal of developing complexing-agents, or molecular receptors, capable of binding very efficiently and very selectively a given substrate [1-6]. The molecular recognition has been developed by taking into account specific molecular interactions and relationship between geometrical structure and binding sites. Particular attraction in the field is its strongly interdisciplinary nature involving as it does organic, inorganic, and physical chemistry as well as the numerous analogies with biological phenomena.

Giving background information about the ring systems will be helpful to cover extensively the vast panorama of macrocyclic compounds. Based on the historical observations ring systems, cyclo $(CH_2)_n$, can be classified into four main groups: small rings (n = 3, 4), normal rings (n = 5, 6, 7), medium rings (n = 8-11), and large rings ($n \geq 12$). Normal rings date back to very beginning of organic chemistry and have been studied widely [7]. The persistent fascination in small rings should be seen in relation to the development of bonding theories. The synthesis of strained rings has been a challenge for existing theories. The chemistry of medium and large rings was well studied in the early 1900s and certain basic rules were established at that time. The names Ruggli, Ruzicka, Stoll, Lüttringhaus, Prelog and Ziegler should be cited [7]. The beginning of current macrocyclic chemistry dates back to late 1960s with the realization that macrocyclic

structures can form effective and selective complexing agents. This is the case for various natural macrocycles, e.g., a cyclic peptide type valinomycin which is extracted from the cells of several Streptomyces strains.

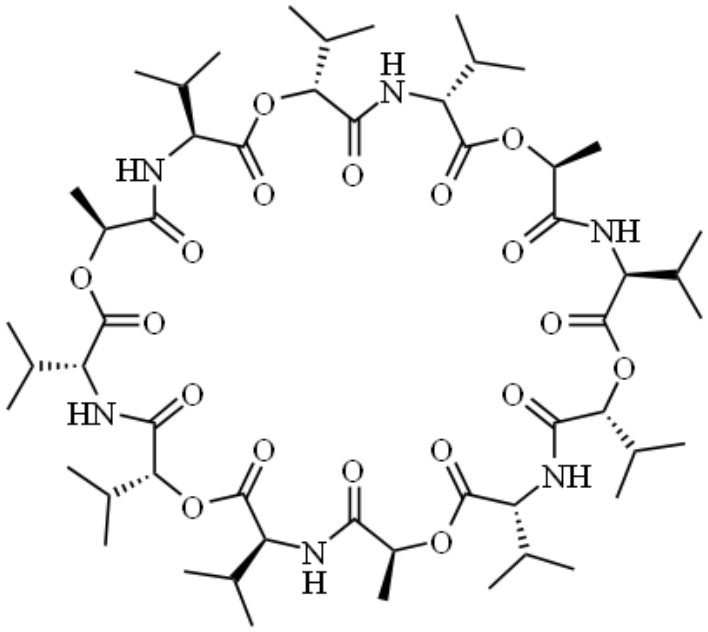


Figure 1.1. Antibiotic Valinomycin, a natural macrocycle.

On account of their multi-functional features, macrocycles have received considerable attention in research mainly as a result of the significant roles they played in inorganic chemistry as ligands, medicinal chemistry as antimicrobials, antitumors, and materials' applications such as dyes (phthalocyanines) and photography [1-10]. The fact that nature prefers certain macrocyclic ligands and their complexes for many fundamental biological functions has long been recognized. Enhanced kinetic and thermodynamic stabilities of macrocyclic structures are the good reasons for this preference. The motivation for

investigation of these systems stems from some of the biological functions such as photosynthesis (complex of magnesium in chlorophyll), formation of vitamin B_{12} from cobalt-containing corrin ring, storage (myoglobin) and transport (hemoglobin) of oxygen in mammalian and other respiratory systems. In addition to these conjugated systems, there are other different macrocyclic ligands found in nature [11-15]. For instance, antibiotic nonactin selectively binds to potassium and carries this ion across lipid barriers of cell membranes [16]. In this respect, the multi-donor macrocycles offer exciting possibilities to construct novel supramolecular assemblies that are capable of performing various specific molecular functions. These assemblies have been of remarkable versatility due to their great availability as ligands possessing several potential donor centers, binding flexibility with biomolecules, and coordination capability with various metal ions [17,18].

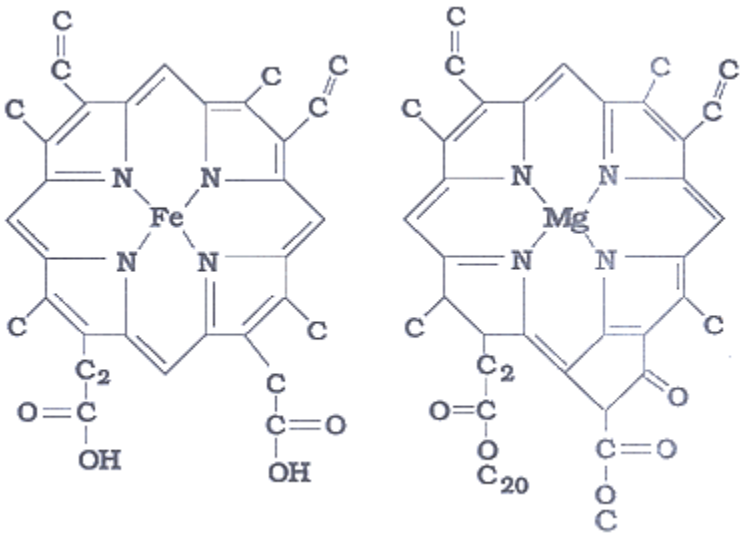


Figure 1.2. Hemoglobin (left) and Chlorophyll (right), well-known natural macrocycles [19].

Macrocycles find broad applications in medicine, cancer diagnosis and in treatment of tumors, in metal ion techniques and treatment of kidney stone [20-23]. Their successful uses in diverse processes such as separation of ions by transport through either artificial or natural membrane, liquid-liquid or solid-solid phase transfer reaction, preparation of ion-selective electrodes, isotope separation, have been known for many years [24-27].

Macrocyclic compounds containing amide linkages, namely macrocyclic peptides which show a diverse range of biological and potentially therapeutic properties are noteworthy. There are several examples of natural and synthetic macrocyclic bioactive peptides [28-32]. As a result of their structural diversity and unique properties, they provide important bio-applications including enzyme-inhibiting, antitumor, and antibiotic activities [33-35]. Macrocyclic peptides are advantageous over acyclic peptides that make them suitable for drug delivery due to their resistance to proteolytic degradation [28]. Understanding the structural, kinetic, and thermodynamic aspects of macrocyclic peptides will aid to the design and develop novel synthetic macrocycles with desired biological features.

In parallel with the discovery of the numerous natural macrocycles, the chemical synthesis of macrocycles has been developed and impressive amount of work devoted to synthetic macrocyclic ligands. Most of these macrocyclic ligands contain multi-coordination donor atoms, such as O or N and mixed O, N or N, S atoms, and exhibit remarkable properties in coordination chemistry [36-39]. Particular attraction has been directed towards nitrogen and sulfur donor ligands due to their capability of furnishing an environment of controlled geometry, their mixed hard-soft donor character, versatility in coordination behavior, and that they may have various applications e.g. antibacterial, anticancerous, antiviral, antifungal, antifertile and other biochemical properties [40-53].

Researchers in this area have synthesized novel nitrogen-sulfur donor ligands through condensation of two or four either identical or different units that has considerably

contributed to the development of synthetic macrocyclic ligands. The impressive number of members synthesized continues to increase because of intriguing observation that different ligands show different biological properties [54-66].

Ligands with a good selectivity towards particular metal ions in different chemical surroundings have found use in biological and environmental applications of coordination chemistry; also the complexes of transition metal ions with macrocyclic ligands are significant because they display the greatest resemblance to many natural systems such as porhyrins and cobalamines [67-69]. (Cobalamine is a transition metal complex of corrinoid ligands with cobalt and found in many organisms including man. Cyano derivatives of cobalamines are known as vitamin B12). Growing class of macrocyclic complexes and research corresponds to its toxicity against bacterial growth, anticancerous property and other biochemical properties [40,42,43,70,71]. The possible use of synthetic macrocycles as models for biologically important systems has initiated a vast amount of research activities, ranging from synthesis of new ring systems and studies on the properties and functions of macrocyclic complexes to their application in industry, medicine and other fields [72-78]. The substantial significance of these structures has been remarked in a review on macrocycles, emphasizing the uses in various applications i.e. molecular recognition, artificial catalyst, supramolecular structures, model mimicking naturally occurring metalloproteins, metalloenzymes, as electron carriers in redox reactions, dioxygen carriers, ionophores in a number of biochemical processes, antitumor drugs, MRI contrast agents and in radiopharmaceutical chemistry [79,80].

It is obvious that the study of metal complexes of macrocyclic ligands are of great interest in recent years in view of the possibility of obtaining coordination compound of unusual structure and stability. The selection of the metal ion is ensured by a cavity of adequate size, and the binding to the macrocycle is achieved through the presence of polar interaction sites lining the cavity. Furthermore, structural factors such as ligand rigidity, the

type of donor atoms, and their disposition have been shown to play significant roles in determining the binding features of macrocycles toward metal ion [81,82].

In this expanding field, the interest toward transition metal complexes containing multi-donor macrocyclic ligands has increased in order to generate metal-based drugs exhibiting a high biological activity together with a reduced toxicity. Almost a century, researchers have investigated these systems due to their structural similarities with biological systems that can be used as drugs and they possess a wide variety of antimicrobial activity against bacteria, fungi and certain type of tumors [83,84]. The role of metal ions in the structure and function of biomolecules is fundamental, yet often unknown at the mechanistic and molecular level. There are two main hypotheses proposing the selectivity and specificity principles of macromolecules for a certain metal. Irving-Williams series of stability [85] and hard-soft acid-base principle of Pearson-Parr [86] are the theoretical assumptions governing these principles.

The discovery of penicillin by Alexander Fleming in 1928, followed by the discovery and clinical use of sulphonamides in the 1930s, heralded the age of modern antimicrobial chemotherapy. The use of penicillin became widespread in the 1940s and the phenomenon of drug-resistance in microbial strains came out with the commercialization of penicillin. Not long after scientists began noticing the emergence of a penicillin-resistant strain of Staphylococcus aureus, a common bacterium which causes a variety of suppurative (pus-forming) infections and toxinoses in humans [87]. From that first case of resistant Staphylococcus, the problem of antimicrobial resistance has snowballed into a serious public health concern with economic, social, and political implications. Drug-resistance to antifungal agents is also of increasing concern [88]. Candida albicans is the major fungal pathogen in humans and is carried by over 50% of the population [89]. The increasing prevalence of fungal infections, especially hospital-acquired infections and infections in immuno-compromised patients has heightened the need for new anti-fungal treatments [90-

93]. Drug-resistant fungal isolates have been reported for all known cases of antifungal drugs [94-96]. Thus, there is an urgent need to develop new and more effective antifungal therapies.

In the present study, the synthesis, spectral characterizations, antimicrobial screening, DNA binding and cytotoxicity activities of two series of novel macrocyclic ligands and palladium halide complexes of the ligand 1,2-Di(o-aminophenylthio)ethane were reported. All the ligands and metal complexes were evaluated against five Gram-positive and four Gram-negative bacteria, and five pathogenic fungi. For such biological screening disc diffusion technique and minimal inhibitory concentration method (MIC) were used. The binding of the ligands to genomic DNA was assessed by UV-Vis spectroscopy, polyacrylamide gel electrophoresis (PAGE), and polymerase chain reaction (PCR) methods. The cytotoxicity assay was performed using human cervical cancer HeLa cells. The nomenclature of the compounds was done according to IUPAC Phane Nomenclature System (Fig. 2.4) [97].

Chapter 2

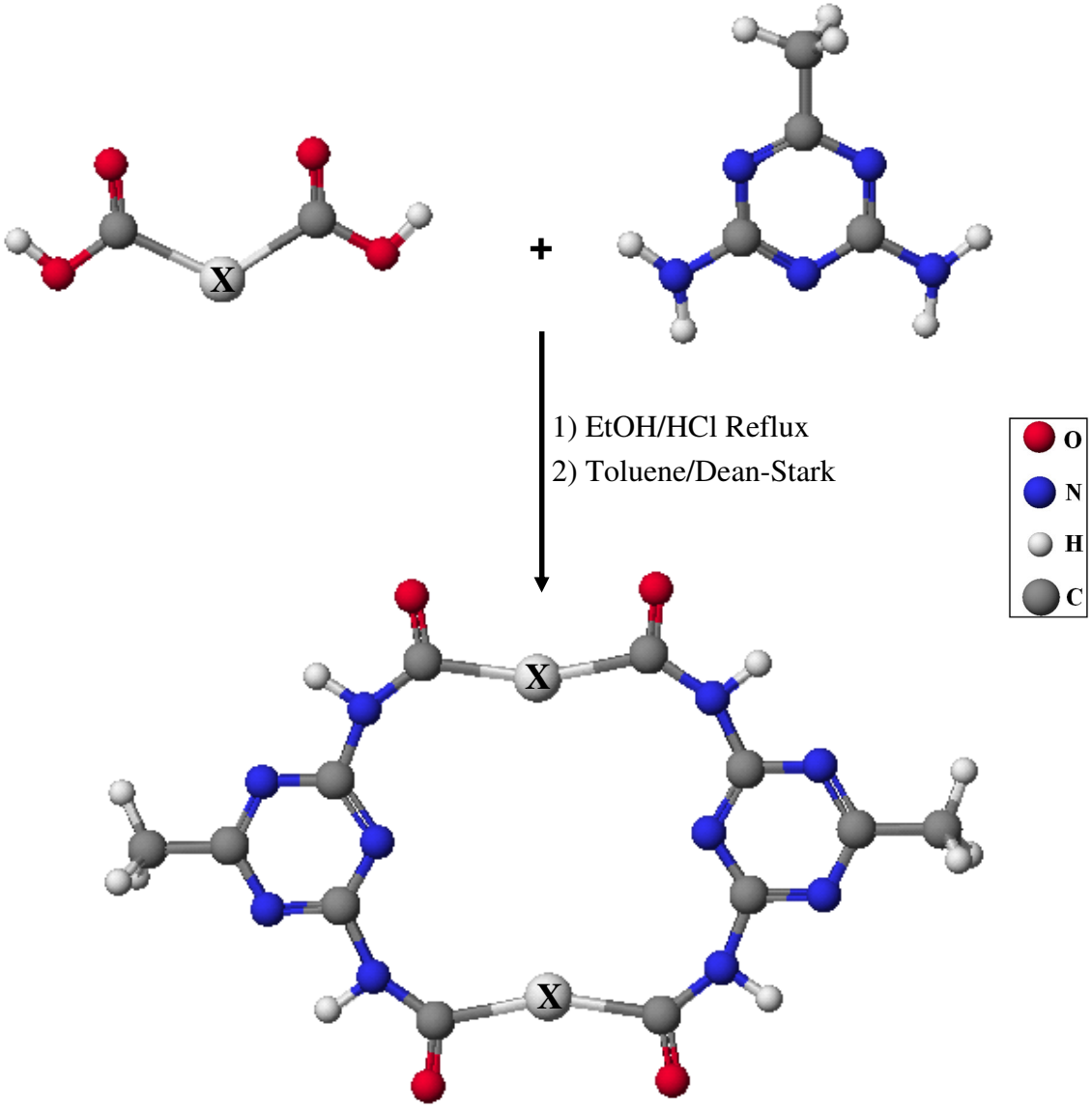
EXPERIMENTAL METHODS

2.1. Chemistry

All chemicals and solvents were reagent grade and used as purchased without further purification except toluene (Na/benzophenone under N_2). Melting points were determined using an Electro-thermal 9100 melting-point apparatus. Analytical data were obtained with a Thermo Finnigan Flash EA 1112 analyser. Mass (ESI) analyses were carried out in positive ion modes using a Thermo Finnigan LCQ Advantage MAX LC/MS/MS Spectrometer. FT-IR spectra were recorded as KBr pellets on a Jasco FT/IR-600 Plus-Spectrometer. Raman spectra were obtained from powdered samples placed in a Pyrex tube using the Bruker RFS 100/S spectrometer in the range 4000–20 cm^{-1}. The 1064-nm line, provided by a near infrared Nd:YAG air-cooled laser, was used as excitation line. The output laser power was set to 180–200 mW. Routine ^{1}H (400 MHz) and ^{13}C (100 MHz) spectra were recorded at ambient temperature in dimethyl sulfoxide (DMSO)-d_6. Chemical shifts (δ) are expressed in units of parts per million relative to TMS. Molar conductivity of the complexes was measured on a WPA CMD750 conductivity meter in DMSO at 25°C. The structure of the ligands and complexes were refined by performing a geometry optimization calculation in MOPAC using PM5 parameter available on CACHe work system pro version 6.1.10 package software. The antimicrobial activities were evaluated against Gram-positive and Gram-negative bacteria and the yeast cultures using both the disk diffusion and the dilution methods. Mueller Hinton media, Nutrient Broth and Malt

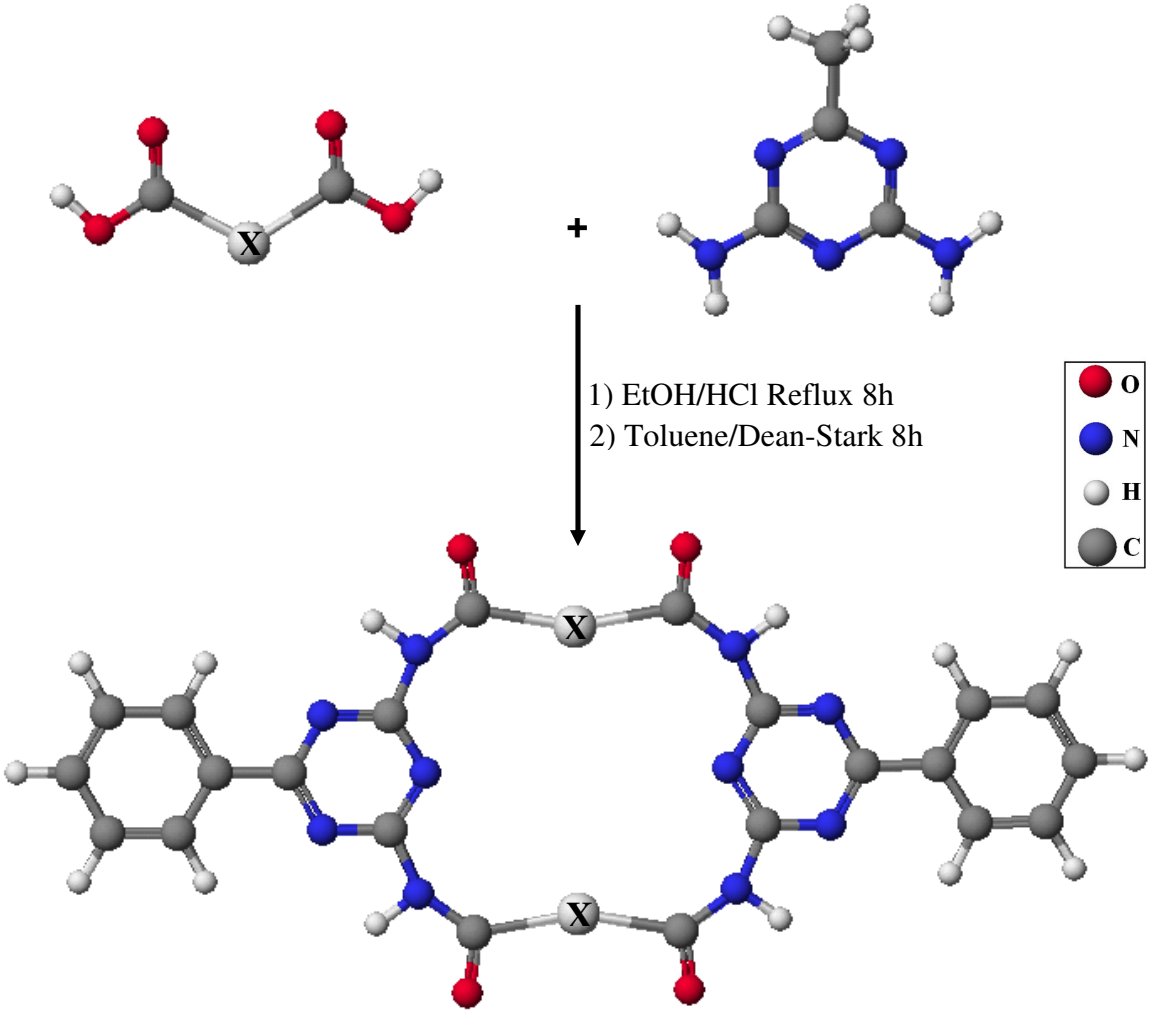
Extract Broth were purchased from Difco and yeast extracts were obtained from oxoid. The binding of the ligands to genomic DNA was assessed by monitoring the change in absorption spectra in the presence or absence of genomic DNA. The UV–Vis measurement for each ligand was performed on an ND-1000 spectrophotometer. The binding ability of compounds to DNA was also determined by electrophoretic mobility shift using polyacrylamide gel electrophoresis assay (PAGE). The effect of ligands on Taq polymerase-catalyzed gene amplification reaction was investigated by using PCR method. The cytotoxicity assay was performed using human cervical cancer HeLa cells. Synthetic routes for the ligands are given in Figures 2.1, 2.2, and 2.3.

2.1.1. Synthesis of the Ligands



X = $–CH_2–$; $–(CH_2)_2–$; $–(CH_2)_3–$; $–(CH_2)_4–$

Figure 2.1. Synthetic pathway for the macrocyclic ligands.



X = –CH_2–S–CH_2–; –CH_2–S–S–CH_2–; –$(CH_2)_2$–S–$(CH_2)_2$–; –$(CH_2)_2$–S–S–$(CH_2)_2$–

Figure 2.2. Synthetic pathway for the macrocyclic ligands.

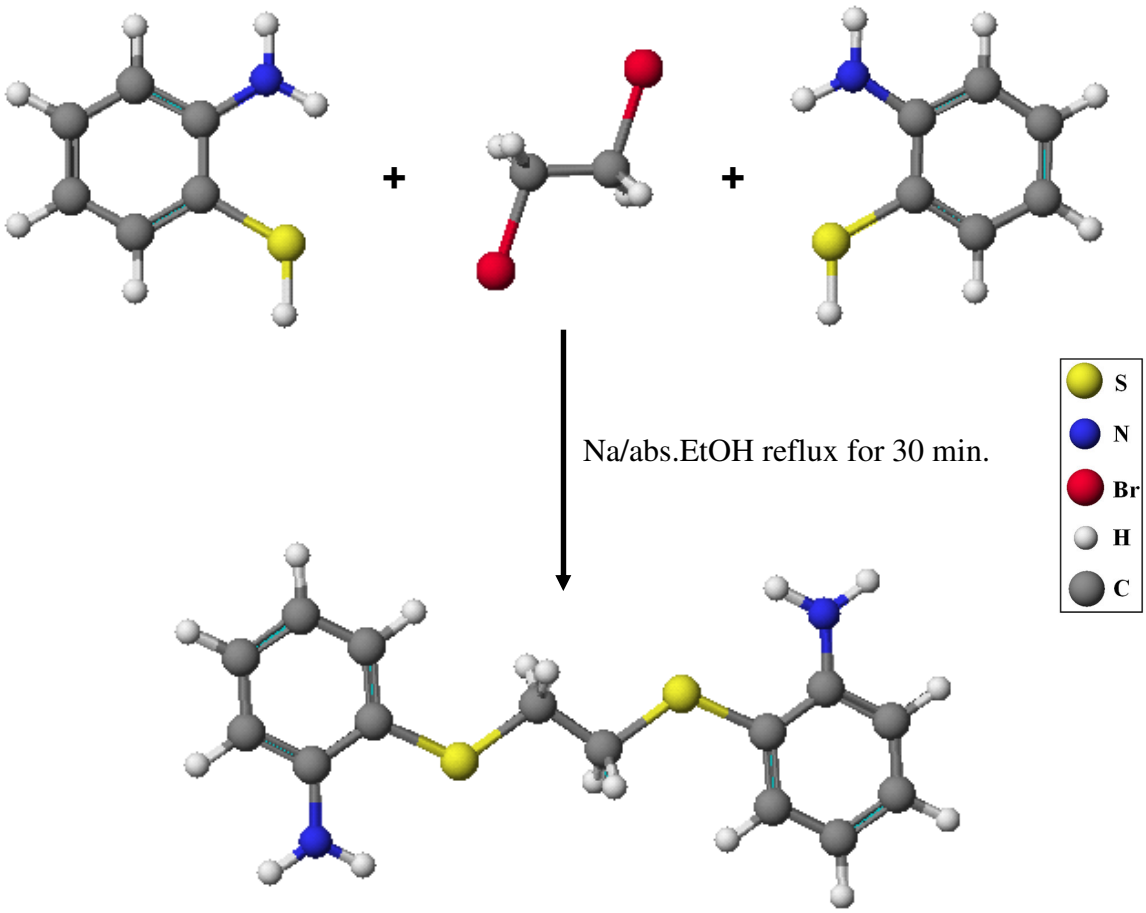


Figure 2.3. Synthetic pathway for the [1,2-Di(o-amino phenylthio)ethane] ligand.

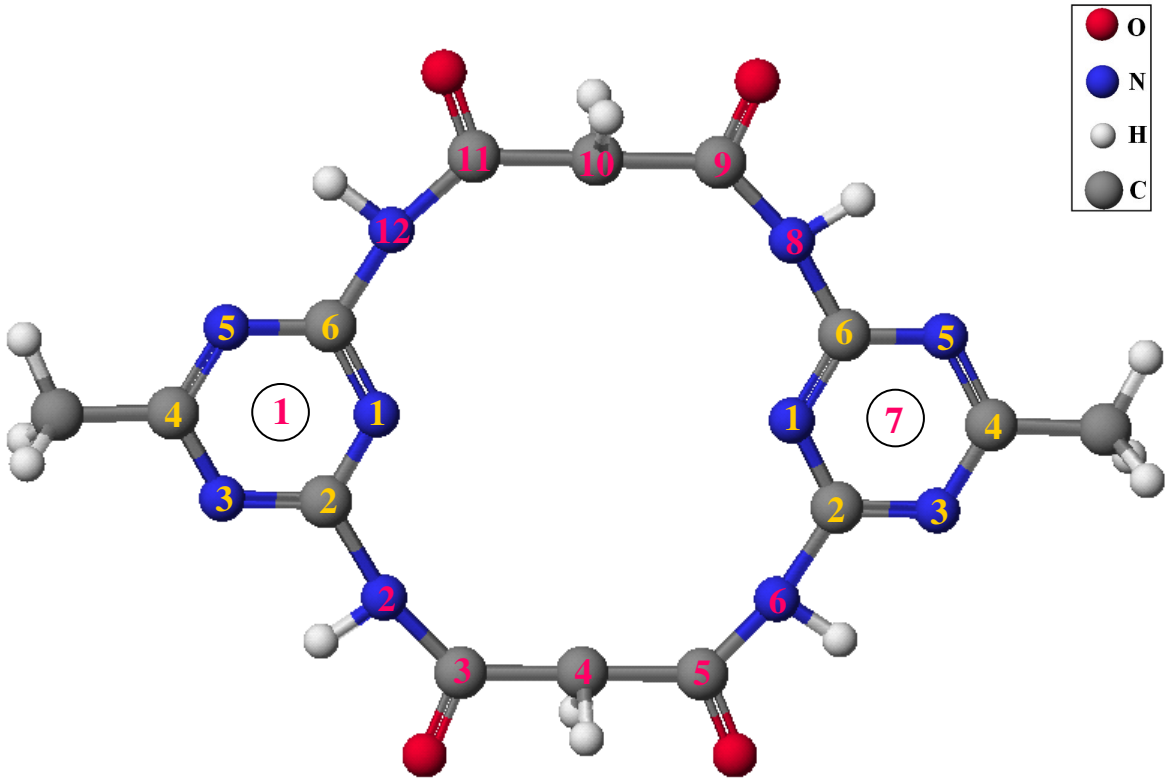


Figure 2.4. Structure and nomenclature of the (L_1) ligand using IUPAC Phane Nomenclature Sytem: **1,7**(**2,6**)-ditriazina-**2,6,8,12**-tetraaza-**3,5,9,11**-tetraoxocyclododeca-phan-$\mathbf{1}^{4}$,$\mathbf{7}^{4}$-dimethyl.

2.1.1.1. *1,7(2,6)-ditriazina-2,6,8,12-tetraaza-3,5,9,11-tetraoxocyclododecaphan-1^4,7^4-dimethyl* ***(L_1).*** Centrifuged hot ethanolic solution (30 cm^3, absolute) of 2,4-diamino-6-methyl-1,3,5-triazine (1.25 g, 10 mmol) was mixed with hot ethanolic solution of (10 cm^3) malonic acid (1.05 g, 10 mmol) in the presence of few drops of concentrated HCl. The solution mixture was refluxed for overnight at 85–90 °C. The white crystalline solid was formed, which was filtered, washed with cold EtOH and dried under vacuum. The dried solid was then suspended in dry toluene and the mixture was refluxed using a Dean-Stark trap for 8 h, before the mixture was cooled down, which was then filtered, washed with light petroleum ether and dried under vacuum (1.95 g, 85%). m.p. 260 °C. Found (calculated) (L_1), [$C_{14}H_{14}N_{10}O_4$]: C, 44.01 (43.52); H, 3.71 (3.63); N, 36.35 (36.27); O, 16.48 (16.58). Mass: 386.33. ^{1}H NMR, δ_H 2.12 (s, 6H$_e$); 3.18 (s, 4H, CH_2); 7.04 (br, 4H, NH). ^{13}C{^{1}H}NMR, δ_C 24.03 (2C$_e$, CH_3); 42.14 (2C, CH_2); 165.86 (4C$_c$, t-ring); 169.62 (2C$_d$, t-ring); 173.02 (4C, CO). Optimized structure of the (L_1) ligand is given in Figure 2.5.

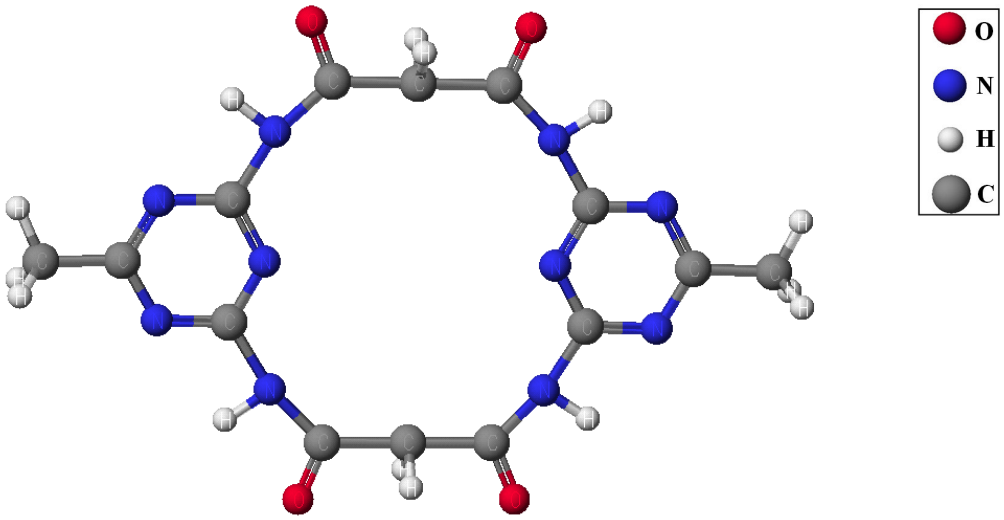


Figure 2.5. Optimized structure of the (L_1) ligand.

The other ligands were prepared in a similar manner to the ligand (L_1) and the results are presented as following:

2.1.1.2. *1,8(2,6)-ditriazina-2,7,9,14-tetraaza-3,6,10,13-tetraoxocyclotetradecaphan-1*^{4}*,8*^{4}*-dimethyl* ***(L₂).*** Hot ethanolic solution (30 cm^3, absolute) of 2,4-diamino-6-methyl-1,3,5-triazine (1.25 g, 10 mmol) was reacted with hot ethanolic solution of (10 cm^3) succinic acid (1.20 g, 10 mmol). The white crystalline solid was treated in a Dean-Stark trap (2.10 g, 87%). m.p. 238-239 °C. Found (calculated) (L_2), [$C_{16}H_{18}N_{10}O_4$]: C, 45.65 (46.38); H, 4.27 (4.35); N, 33.72 (33.81); O, 15.95 (15.46). Mass: 414.38. ^{1}H NMR, δ_H 2.07 (s, 8H, CH_2); 2.43 (s, 6H_e); 6.64 (br, 4H, NH). ^{13}C{^{1}H}NMR, δ_C 24.90 (2C_e, CH_3); 29.33 (4C, CH_2); 167.23 (4C_c, t-ring); 174.16 (2C_d, t-ring); 174.80 (4C, CO). Optimized structure of the (L_2) ligand is given in Figure 2.6.

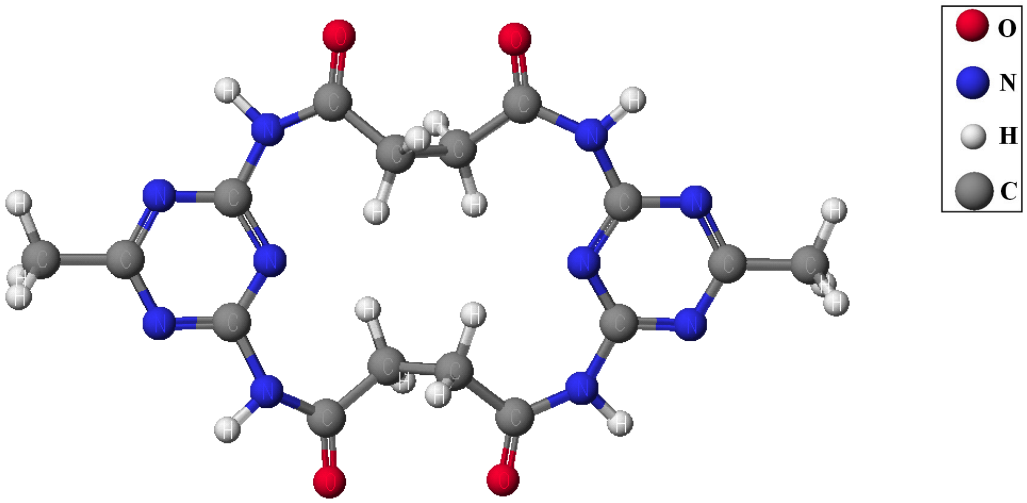


Figure 2.6. Optimized structure of the (L_2) ligand.

2.1.1.3. *1,9(2,6)-ditriazina-2,8,10,16-tetraaza-3,7,11,15-tetraoxocyclohexadecaphan-1^4,9^4-dimethyl* ***(L_3).*** Hot ethanolic solution (30 cm^3, absolute) of 2,4-diamino-6-methyl-1,3,5-triazine (1.25 g, 10 mmol) was reacted with hot ethanolic solution of (10 cm^3) glutaric acid (1.35 g, 10 mmol). The white crystalline solid was treated in a Dean-Stark trap (2.00 g, 78%). m.p. 225 °C. Found (calculated) (L_3), [$C_{18}H_{22}N_{10}O_4$]: C, 48.05 (48.87); H, 4.84 (4.98); N, 32.03 (31.67); O, 14.97 (14.48). Mass: 442.40. ^{1}H NMR, δ_H 1.72 (s, 4H$_b$); 2.07 (s, 4H$_a$); 2.07 (s, 4H$_{a'}$); 2.25 (s, 6H$_e$); 6.66 (br, 4H, NH). ^{13}C{^{1}H}NMR, δ_C 20.45 (2C$_b$) 24.85 (2C$_e$, CH$_3$); 33.27 (6C, CH$_2$); 167.21 (4C$_c$, t-ring); 174.65 (2C$_d$, t-ring), 174.80 (4C, CO). Optimized structure of the (L_3) ligand is given in Figure 2.7.

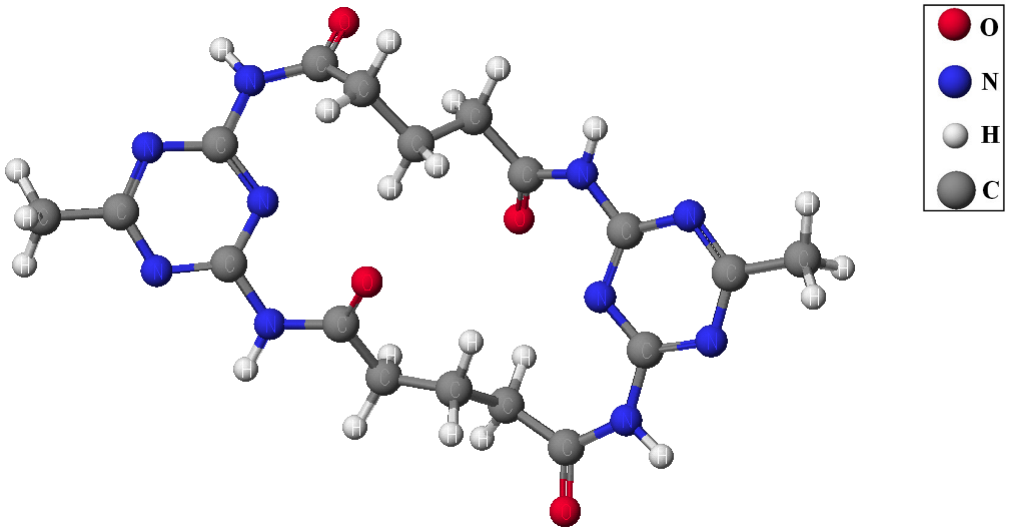


Figure 2.7. Optimized structure of the (L_3) ligand.

2.1.1.4. *1,10(2,6)-ditriazina-2,9,11,18-tetraaza-3,8,12,17-tetraoxocyclooctadecaphan-1^4,10^4-dimethyl* ***(L_4).*** Hot ethanolic solution (30 cm^3, absolute) of 2,4-diamino-6-methyl-1,3,5-triazine (1.25 g, 10 mmol) was reacted with hot ethanolic solution of (10 cm^3) adipic acid (1.50 g, 10 mmol). The white crystalline solid was treated in a Dean-Stark trap (1.85 g,

70%). m.p. 226-227 °C. Found (calculated) (L_4), [$C_{20}H_{26}N_{10}O_4$]: C, 52.08 (51.06); H, 5.45 (5.53); N, 28.78 (29.79); O, 12.99 (13.62). Mass: 470.49. ^{1}H NMR, δ_H 1.51 (t, $4H_b$); 1.51 (t, $4H_{b'}$); 2.21 (s, $4H_a$); 2.21 (s, $4H_{a'}$); 2.07 (s, $6H_e$); 6.64 (br, 4H, NH). $^{13}C\{^1H\}$NMR, δ_C 24.50 ($2C_b$) 24.91 ($2C_e$, CH_3); 33.87 (6C, CH_2); 167.27 ($4C_c$, t-ring); 174.88 ($2C_d$, t-ring), 175.05 (4C, CO). Optimized structure of the (L_4) ligand is given in Figure 2.8.

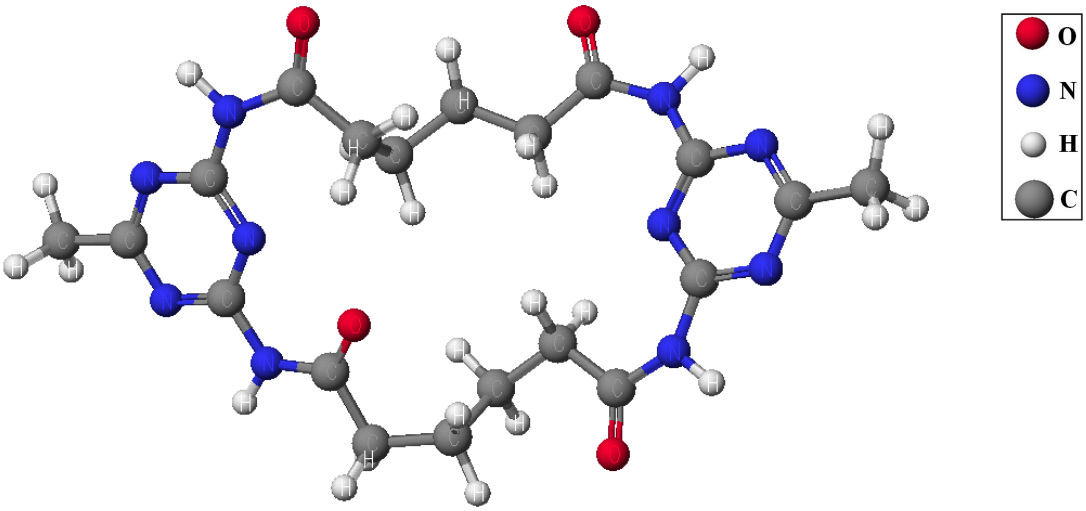


Figure 2.8. Optimized structure of the (L_4) ligand.

2.1.1.5. *1,9(2,6)-ditriazina-2,8,10,16-tetraaza-3,7,11,15-tetraoxo-5,13-dithia-cyclohexa-decaphan-1^4,9^4-diphenyl* ***(L_5).*** Centrifuged hot ethanolic solution (90 cm^3, absolute) of 2,4-diamino-6-phenyl-1,3,5-triazine (1.25 g, 10 mmol) was mixed with hot ethanolic solution of (10 cm^3) of 2,2'-thiodicetic acid (1.50 g, 10 mmol) in the presence of few drops of concentrated HCl. The solution mixture was refluxed for several hours at 85-90 ^oC. The white crystalline solid product was formed, which was filtered, washed with cold EtOH and dried under vacuum. The dried solid product was then suspended in dry toluene and the mixture was refluxed using a Dean-Stark trap for 4 h, before the mixture was cooled down,

which was then filtered, washed with light petroleum ether and dried under vacuum (1.54 g, 64%). m.p.230 ^{o}C. Found (calculated) (L_5), [$C_{26}H_{22}N_{10}O_6S_2$]: C, 52.1 (51.8); H, 3.9 (3.7); N, 22.9 (23.3); S, 11.0 (10.6). MS (ESI+) m/z (%): 602 (8). ^{1}H NMR, δ_H 3.37 (s, 8H, CH_2); 6.76 (s, 4H_g); 7.47 (m, 4H_f); 8.25 (d, 2H_h, J = 7.2 Hz); 9.0 (br, 4H, NH). ^{13}C{^{1}H}NMR, δ_C 33.99 (4C, CH_2); 128.15 (4C_f, Ph); 128.58 (4C_g, Ph), 131.51 (2C_h, Ph); 137.49 (2C_e, Ph); 167.86 (4C_c, t-ring); 170.68 (2C_d, t-ring); 171.44 (4C, CO). Optimized structure of the (L_5) ligand is given in Figure 2.9.

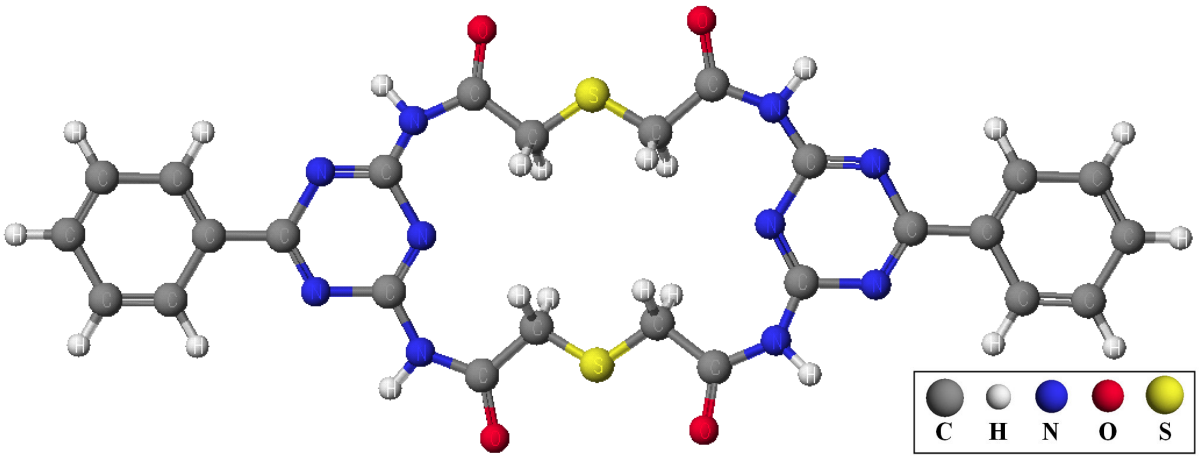


Figure 2. 9. Optimized structure of the (L_5) ligand.

The other ligands were prepared in a similar manner to the ligand (L_5) and the results are presented as following:

2.1.1.6. *1,10(2,6)-ditriazina-2,9,11,18-tetraaza-3,8,12,17-tetraoxo-5,6,14,15-tetrathia-cyclooctadecaphan-1^4,10^4-diphenyl* ***(L$_6$).*** Hot ethanolic solution (90 cm^3, absolute) of 2,4-diamino-6-phenyl-1,3,5-triazine (1.25 g, 10 mmol) was reacted with ethanolic solution of

(10 cm^3) of 2,2'-dithiodiacetic acid (1.82 g, 10 mmol) in the presence of few drops of HCl. The white crystalline solid was treated in a Dean-Stark trap (1.80 g, 67%). m.p.197-200 ^oC. Found (calculated) $(L_6)2H_2O$, $[C_{26}H_{26}N_{10}O_6S_4]$: C, 44.9 (44.4); H, 3.9 (3.7); N, 20.2 (19.9); S, 17.9 (18.2). MS (ESI) m/z (%): 666 (9), 436 (10), 241(12), 188 (100). ^1H NMR, δ_H 3.68 (s, 8H, CH_2); 6.79 (s, $4H_g$); 7.50 (m, $4H_f$); 8.26 (d, $2H_h$, J = 9.4 Hz); 9 (br, 4H, NH). $^{13}C\{^1H\}$NMR, δ_C 41.55 (4C, CH_2); 128.53 ($4C_f$, Ph); 128.99 ($4C_g$, Ph); 131.91 ($2C_h$, Ph); 137.89 ($2C_e$, Ph); 168.26 ($4C_c$, t-ring); 171.04 ($2C_d$, t-ring); 171.41 (4C, CO). Optimized structure of the (L_6) ligand is given in Figure 2.10.

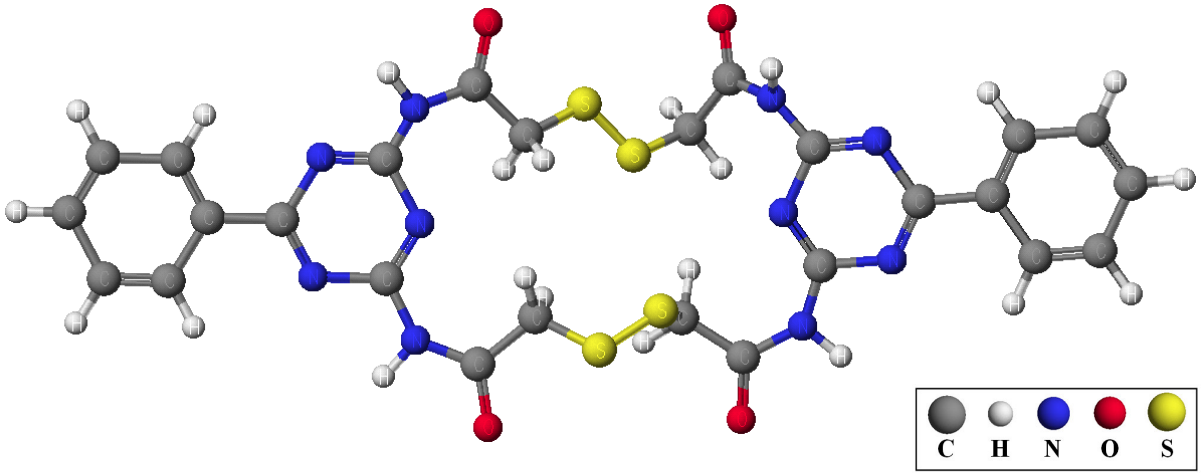


Figure 2.10. Optimized structure of the (L_6) ligand.

2.1.1.7. *1,11(2,6)-ditriazina-2,10,12,20-tetraaza-3,9,13,19-tetraoxo-6,16-dithia-cyclocosa-phan-1^4,11^4-diphenyl* ***(L_7).*** Hot ethanolic solution (90 cm^3, absolute) of 2,4-diamino-6-phenyl-1,3,5-triazine (1.25 g, 10 mmol) was reacted with ethanolic solution of (10 cm^3) of 3,3'-thiodipropionic acid (1.78 g, 10 mmol) in the presence of few drops of HCl. The white crystalline solid was treated in a Dean-Stark trap (2.34 g, 82%). m.p.210 ^oC. Found (calculated) $(L_7)(H_2O)_2$, $[C_{30}H_{34}N_{10}O_6S_2]$: C, 51.3 (51.9); H, 5.2 (4.9); N, 2.7 (20.2); S 9.5

(9.2). MS (ESI+) m/z (%): 658 (not detected), 360, 229. ^{1}H NMR, δ_H 2.51 (t, J = 6.8 Hz, 8H, CH_2─CO); 2.70 (t, J = 6.8 Hz, 8H, S─CH_2) 6.76 (s, 4H_g); 7.47 (m, 4H_f); 8.26 (d, 2H_h, J = 7.2 Hz); 10 (br, 4H, NH). ^{13}C{^{1}H}NMR, δ_C 26.41 (4C, S─CH_2); 34.55 (4C, CH_2─CO); 127.66 (4C_f, Ph); 128.08 (4C_g, Ph); 130.99 (2C_h, Ph); 137.04 (2C_e, Ph); 167.41 (4C_c, t-ring); 170.23 (2C_d, t-ring); 173.01 (4C, CO). Optimized structure of the (L_7) ligand is given in Figure 2.11.

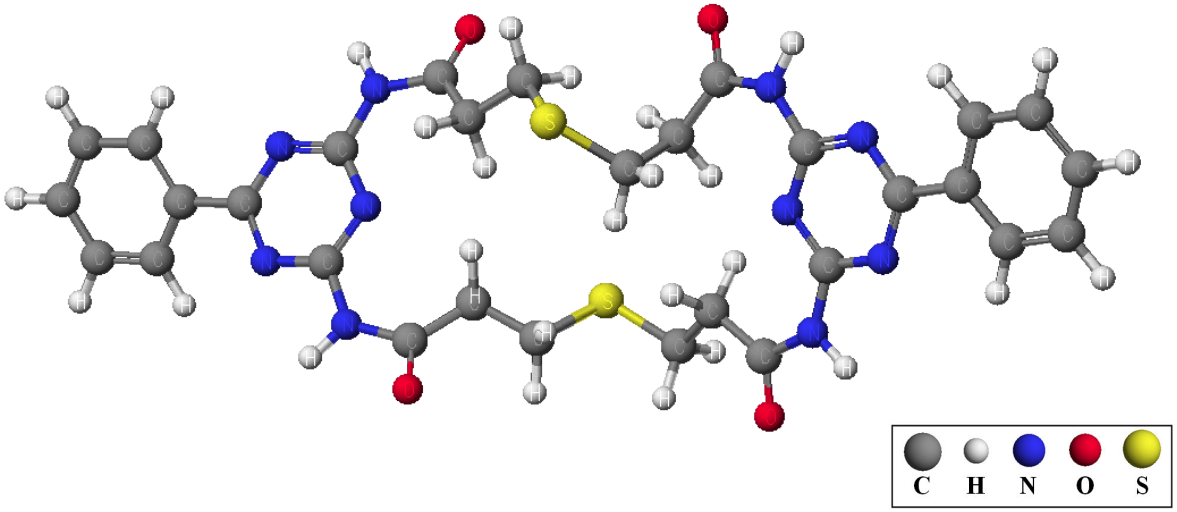


Figure 2.11. Optimized structure of the (L_7) ligand.

2.1.1.8. *1,12(2,6)-ditriazina-2,11,13,22-tetraaza-3,10,14,21-tetraoxo-6,7,17,18-tetrathia-cyclodocosaphan-1^4,12^4-diphenyl* ***(L_8).*** Hot ethanolic solution (90 cm^3, absolute) of 2,4-diamino-6-phenyl-1,3,5-triazine (1.06 g, 8.5 mmol) was reacted with ethanolic solution of (10 cm^3) of 3,3′-dithiodipropionic acid (1.78 g, 8.5 mmol) in the presence of few drops of HCl. The off white crystalline solid was treated in a Dean-Stark trap (1.77 g, 70%). m.p.165-166 ^{o}C. Found (calculated) (L_8)(H_2O), [$C_{30}H_{32}N_{10}O_5S_4$]: C, 48.7 (48.6); H, 4.7 (4.3); N, 19.3 (18.90); S, 17.5 (17.3). MS (ESI+) m/z (%): 722 (12). ^{1}H NMR, δ_H 2.64 (t, J = 6.8 Hz, 8H, CH_2─CO); 2.91 (t, J = 6.8 Hz, 8H, S─CH_2) 6.81 (s, 4H_g); 7.50 (m, 4H_f); 8.27

(d, $2H_h$, J = 9.9 Hz); 10 (br, 4H, NH). $^{13}C\{^1H\}$NMR, δ_C 33.51 (4C, S─CH_2); 34.00 (4C, CH_2─CO); 128.17 ($4C_f$, Ph); 128.64 ($4C_g$, Ph); 131.67 ($2C_h$, Ph); 137.17 ($2C_e$, Ph); 167.75 ($4C_c$, t-ring); 170.80 ($2C_d$, t-ring); 173.29 (4C, CO). Optimized structure of the (L_8) ligand is given in Figure 2.12.

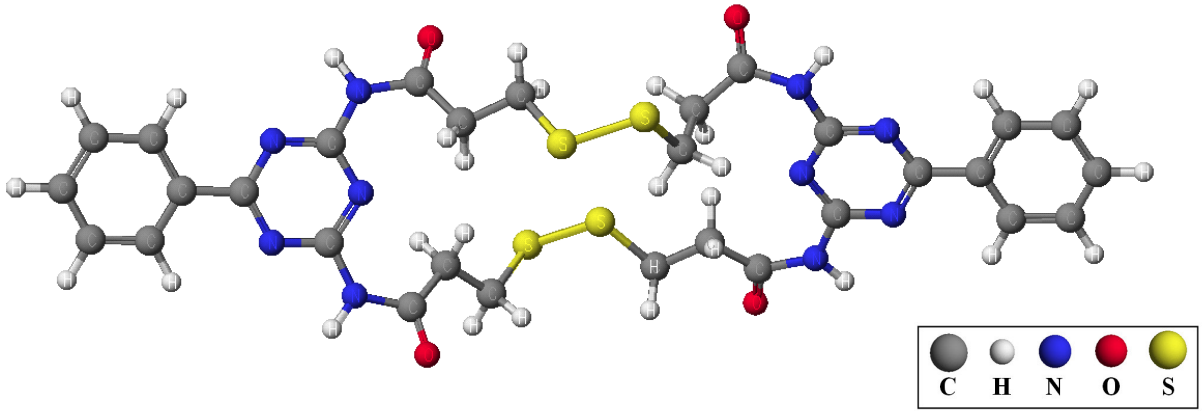
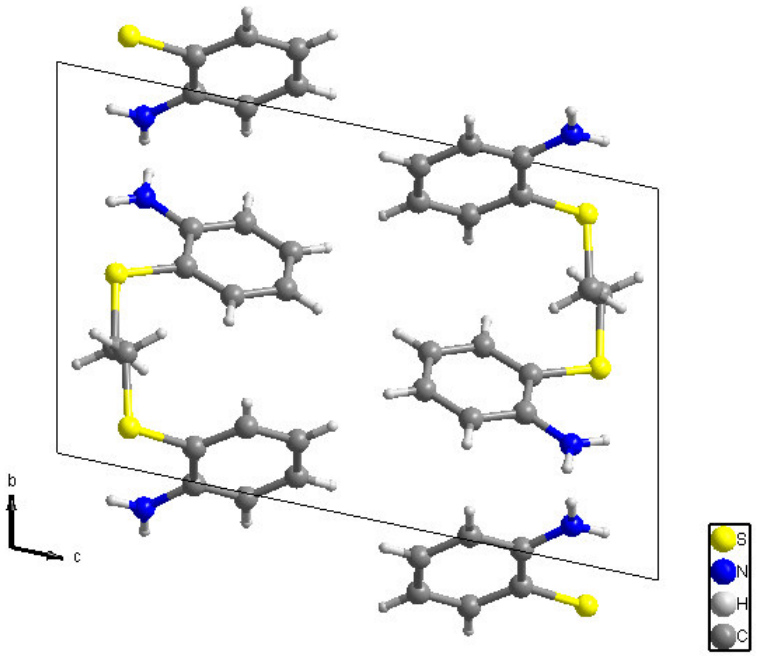


Figure 2.12. Optimized structure of the (L_8) ligand.

2.1.1.9. *1,2-Di(o-aminophenylthio)ethane* ***(L_9).*** The ligand was prepared by heating *o*-$HSC_6H_4NH_2$ (1.09 g) with absolute (99%) EtOH (3 cm^3) containing Na (0.201 g). $BrCH_2CH_2Br$ (0.372 cm^3) in EtOH (1 cm^3) was then added dropwise with constant stirring to the refluxing solution. The mixture was then cooled and poured into H_2O (300 cm^3). The solid mass so obtained, was filtered washed with H_2O and dried. The product was recrystallized from EtOH, and a yellowish residue was obtained. m.p. 75 °C. ^1H NMR: ($CDCl_3$) δ 6.3 (2H, d), δ 7.1 (2H, m), δ 7.2 (2H, d), δ 2.8 (4H, m, S–CH_2). Crystal structure of the (L_9) ligand is given in Figure 2.13.



Space Group P-1 (2)
a = 5.4477(12)
b = 9.1163(11)
c = 14.309(3)
α = 101.900(13)
β = 91.243(6)
γ = 91.027(13)
Z = 2

Figure 2.13. Crystal structure of the (L_9) ligand.

2.1.2. Synthesis of Complexes

Deoxygenated solutions of ligand 1,2-Di(o-aminophenylthio)ethane (L_9) (155 mg, 56 mmol) and Pd(II) halides (100 mg, 56 mmol) in absolute ethanol (10 mL) were refluxed under nitrogen atmosphere with constant stirring overnight. The mixture was filtered while it was hot. The filtrate was cooled to room temperature and allowed to stand to form crystals: $[Pd(L_9)]Cl_2$ pale yellow (yield 80%), $[Pd(L_9)]Br_2$ orange(yield 76%), $[Pd(L_9)]I_2$ brown (yield 70%) (decomp. 250 °C). Optimized structure of the palladium complexes is given in Figure 2.14.

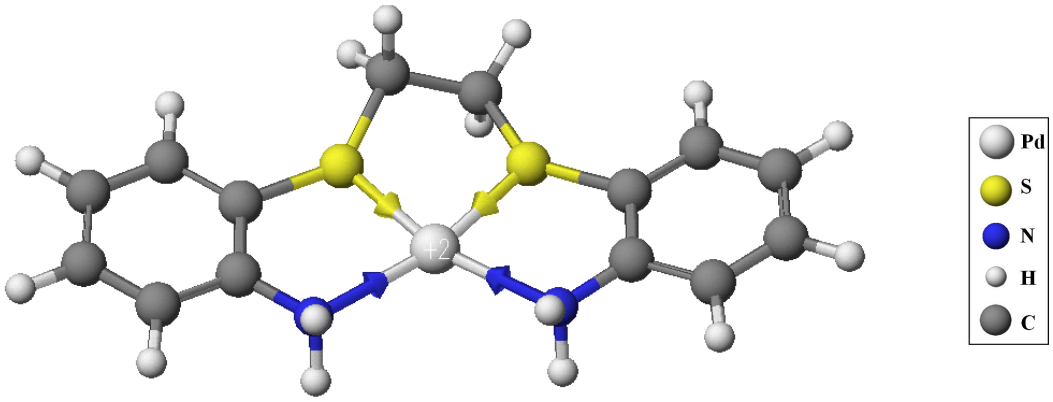


Figure 2.14. Optimized structure of the complexes.

2.2. Pharmacology

The antimicrobial activities are evaluated against Gram positive (*Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 7064, *Mycobacterium smegmatis* CCM 2067, *Listeria monocytogenes* ATCC 15313, *Micrococcus luteus* La 2971) and Gram-negative (*Escherichia coli* ATCC 11230, *Klebsiella pneumoniae* UC57, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 8427) bacteria and the yeast cultures (*Candida*

albicans ATCC 10231, *Kluyveromyces fragilis* NRRL 2415, *Rhodotorula rubra* DSM 70403, *Debaryomyces hansenii* DSM 70238 and Hanseniaspora guilliermondii DSM 3432) using both the disk diffusion and the dilution methods.

2.2.1. Biological Data

Standardized samples of Penicillin-g (blocking the formation of bacterial cell walls, rendering bacteria unable to multiply and spread; Ampicillin (penetrating and preventing the growth of Gram–negative bacteria); Cefotaxime (used against most Gram–negative enteric bacteria); Vancomycin (acting by interfering with the construction cell walls in bacteria), Ofloxacin (entering the bacterial cell and inhibiting DNA–gyrase, which is involved in the production of genetic material, preventing the bacteria from reproducing); Tetracyclines (exerting their antimicrobial effect the inhibition of protein synthesis; Nystatin (binding to sterols in the fungal cellular membrane altering the permeability to allow leakage of the cellular contents and destroying the fungus); Ketoconazole (inhibiting the growth of fungal organisms by interfering with the formation of the fungal cell wall) and Clotrimazole (interfering with their cell membranes and causing essential constituents of the fungal cells leakage). Mueller Hinton media, Nutrient Broth and Malt Extract Broth are purchased from Difco and yeast extracts is obtained from Oxoid.

2.2.2. Methods

2.2.2.1. Disk Diffusion Method

Sterilized antibiotic discs (6 mm) were used following the literature procedure [98,99]. Fresh stock solutions of the ligands were prepared in DMSO according to the needed concentrations for experiments. To ensure that the solvent had no effect on bacterial growth, a control test was performed with test medium supplemented with DMSO as the

same procedures as used in the experiments. All the bacteria were incubated at 30 ^{o}C for 24 h in Nutrient Broth. The yeasts were incubated in Malt Extract Broth for 48 h. The discs injected with solutions were placed on the inoculated agar and incubated at 35 ^{o}C (24 h) and at 25 ^{o}C (72 h) for bacteria and yeast, respectively. On each plate an appropriate reference antibiotic disc was applied depending on the test microorganisms. In each case triplicate tests were performed and the average was taken as the final reading.

2.2.2.2. Dilution Method

Screening for antibacterial and antifungal activities was carried out by preparing a broth micro dilution, following the procedure outlined in Manual of Clinical Microbial [100]. All the bacteria were incubated and activate at 30 ^{o}C for 24 h inoculation into Nutrient Broth, and the yeasts were incubated in Malt Extract Broth for 48 h. The compounds were dissolved in DMSO (2 mg mL^{-1}) and then diluted using caution adjusted Mueller Hinton Broth (Oxoid). Two-fold serial concentrations of the compounds were employed to determine the (MIC) ranging from 200 μg mL^{-1} to 1.56 μg mL^{-1}. Cultures were grown at 37 °C (20 h) and the final inoculation (inoculums) was approximately 10^{6} cfu mL^{-1}. Test cultures were incubated at 37 °C (24 h). The lowest concentrations of antimicrobial agents that result in complete inhibition of microorganisms were represented as (MIC) μg mL^{-1}. In each case triplicate tests were performed and the results are expressed as means.

2.2.2.3. UV–Vis Spectra

Ligands (L_1-L_4)

The binding of the ligands (L_1-L_4) to genomic DNA was assessed by monitoring the change in absorption of the ligands at 260 nm in the presence or absence of genomic DNA. Approximately 1 μg pACT plasmid (Promega Corporation, Madison, WI) DNA was mixed with 50 nmol chemical compound in 20 μL 1M Tris (pH 7.0) mixture and incubated for an

hour at 37 ºC prior to spectral scanning. The UV–Vis spectra measurement for each of the compounds was performed on an ND-1000 spectrophotometer (NanoDrop Technologies).

Ligands (L_5-L_8)

Calf thymus DNA was purchased from Worthington Biochemical Corp. and prepared in ddH2O at a concentration of 60 μg/μL. Purity of CT-DNA was determined by comparing UV absorbance ratios at 260 and 280 nm (1.8:1), indicating that the DNA was sufficiently free of protein. The absorption spectra of the DNA solution (250 μM) were obtained in the absence and presence of increasing amounts of the ligands [0–500 μM].

2.2.2.4. Gel Shift Assay

The binding ability of compounds to DNA was determined by electrophoretic mobility shift using polyacrylamide gel electrophoresis assay (PAGE). Purified 450bp PCR products (100 ng) were mixed with varying concentrations of the test compounds. The mixture was incubated at 37 ºC for an hour and then subjected to electrophoresis through a 10% polyacrylamide gel in 1XTBE buffer (90 mM Tris-borate and 2 mM EDTA). The gels were silver-stained and documented [101].

2.2.2.5. PCR investigation

The effect of ligands on Taq polymerase-catalyzed gene amplification reaction was investigated. A 450 bp long fragment of pACT plasmid was amplified in the presence and absence of the ligands. Briefly, PCR was performed in a total volume of 25 μL containing approximately 50 ng plasmid DNA, 2.5 μL of 10X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol forward and reverse primers, and 1 U of Taq polymerase (MBI Fermentas, Hanover, MD). The PCR program on the thermal cycler (iCyclerTM BioRad) was as follows: an initial denaturation step at 94 ºC for 5 min, followed by 15 cycles of 30 sec at 94 ºC, 30 sec at 55 ºC, 30 min at 72 ºC, and a final extension step of 10 min at 72 ºC. A

total of 10 µL from the PCR product were electrophoresed on 1% standard agarose gels at 100 Volt for 30 min. The fragments were visualized by ethidium bromide under UV transilluminator using Biodoc documentation system (Biorad).

2.2.2.6. Cytotoxicity

Ligands (L_1-L_4)

The cytotoxicity assay was performed using human cervical cancer HeLa cells. Cells were cultured at 37 °C under a humidified atmosphere of 5% CO_2 in medium supplemented with 10% fetal serum and dispersed in replicate 96- well plates with 2.5×104 cells/well. Compounds were then added. After 24-72 hours exposure to the chemical compounds, cells' viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenpyltetrazolium bromide (MTT) assay [102]. The optical density (OD) of the wells was determined using a ELISA plate reader at a test wavelength.

Ligands (L_5-L_8)

The cytotoxic effect of the (L_5-L_8) ligands against 23132/87 (stomach adenocarcinoma, human) and A549 (non-small cell lung adenocarcinoma, human) were assayed by MTT method [102]. The cells were plated at 37 °C for 24 h on 96-well plates (Grainer) at a density of 3.10^3–5.10^3 cells per well, with RPMI 1640 medium in a humidified atmosphere (5% CO_2). The RPMI 1640 medium was supplemented with 10% (v/v) fetal bovine serum(FBS), 4mM $_L$-glutamine and antibiotics (penicillin/streptomycin). Freshly prepared ligands (DMSO) were further diluted in complete culture medium and the final volume of DMSO in the culture medium was adjusted to 0.5% (v/v). The seeded cells were exposed to 0.1, 1, 10, 100 µM concentrations of the test compounds for 24 and 48 h at 37 °C in humidified incubator with 5% CO_2 at a final volume of 150 µL. After incubation,15 µL MTT solutions was added to each well and incubated for further 4 h. The MTT solution then was carefully removed and dissolved by adding 200 µL (DMF 50%, SDS 5%)

aqueous solution and analyzed at 570 nm in a microplate reader. Cytotoxicity values were determined using $100x(OD_{570treated\ cells}/OD_{570untreated\ cell})$ equation.

2.2.2.7. DNA fragmentation

Tested cell lines were used to analyze the DNA ladder formation which is a characteristic of apoptotic cell death [103]. The cells (10×10^4) were plated on 6 well plates and exposed on the (L_5–L_8) ligands (100 μM) for 24 h. After the incubation the cell lines were collected and washed with PBS (phosphate-buffered saline). The DNAwas extracted with the Nucleospin Tissue kit. The extracts were run on 1.5% agarose gel. The products were visualized under UV-light with Bio-Rad Gel Doc gel scanner system.

2.2.2.8. Hoechst 33258 staining [104].

The cells that were cultured on 6 well plates and treated with the compound (100 μM) for 24 h. After the treatment, cells were washed with PBS (3 times) and fixed with ice cold methanol for 10 min. Following fixation, cells were incubated with Hoechst 33258 (5 mg mL^{-1}) for 5 min and then destained with double-distilled water for further 5 min and were examined by fluorescence microscopy (CarlZeis).

Chapter 3

RESULTS AND DISCUSSION

3.1. Vibrational Spectroscopy Studies

3.1.1. (L_1-L_4) Ligands

The present vibrational spectra (Fig. 3.1) can be discussed in terms of two characteristic wave regions: 3400–2900 cm^{-1} corresponding to ν(H–O–H), ν(N–H) and ν(C–H) characteristic modes, 1800–800 cm^{-1} belongs to ν(CONH–) amides, $\nu(C–N)_{tr}$, and δ(N–H). Prominent Raman and IR frequency data values and their assignments are presented in Table 3.1. The bands corresponding to free carboxylic acid (–COOH) and amine ($–NH_2$) groups are not observed in the IR spectra of the (L_1–L_4) ligands, which suggests complete condensation of the amine groups of 2,4-diamino-6-methyl-1,3,5-triazine with corresponding dicarboxylic acids. The medium to strong bands observed in the region 3380-3150 cm^{-1} in the IR spectra is assignable to amide A and amide B vibrations, respectively. The amide A is almost caused by the NH stretching vibration; this mode of vibration is not dependent on the backbone conformation but is very sensitive to the strength of a hydrogen bonding, which may caused broadening and as well as observance of multiplicity. The amide B originates from a Fermi resonance between the first overtone of amide II and the N–H stretching vibration [105,106]. Due to non-planar characteristic behavior of the compounds in the solid state, the bands could be observed as multiple signals. These lines have extremely weak Raman intensity (Figs. 3.1 and 3.2). The pure characteristic ν(CH) modes of aliphatic groups are observed in the wave region 2990–2870 cm^{-1} in both Raman and IR spectra as expected. The peptide bond formation could also be

confirmed due to the observance of two major bands in the frequency regions 1700–1640 cm^{-1} (amide I) and 1590–1540 cm^{-1} (amide II). The amide I band is the most intensive absorption band particularly in the IR spectrum and it is almost fully governed by the stretching vibration of the C=O mode with a very small contribution of the C–N groups. Amide II is more complex than amide I, which derives mainly from in-plane N–H bending vibration with certain contribution of ν(C–N) groups. Amides III and IV are very complex bands resulting from a mixture of several coordinate displacements. These modes are dominated by the out-of-plane motions particularly (N–H) wagging and (C=O) deformation vibrations as reported in Table 3.1 [105,106]. The bending vibrations for δ(N–H) in IR spectra are observed in 1640-1620 cm^{-1} frequency region(Figs. 3.1 and 3.2).

Table 3.1. Prominent IR and Raman bands for the macrocyclic (L_1-L_4) compounds.

	FT-IR (cm^{-1})	Raman (cm^{-1})
(L_1)	3388 ν(H–O–H), 3266, 3185 ν(N–H), 3103 [ν(CO)+δ(N–H)], 2966, 2904 ν(C–H), 1701 ν(C=O), 1677 δ(O–H), 1632 [δ(N–H)+ν(C–N], 1463 $\nu(C–N)_{tr}$, 1419, 1372, 1280 [ν(C–N)+δ(N–H)], 948 [ω(N–H)+ϕ(C=O)], 754, 558, 435.	3184 ν(N–H), 2965, 2939, 2907 ν(C–H), 1704, 1692 ν(C=O), 1632 δ(O–H), 1559 [δ(N–H)+ν(C–N], 1421 $\nu(C–N)_{tr}$, 1392, 1375 [ν(C–N)+δ(N–H)], 1282, 962 , 904 [ω(N–H)+ϕ(C=O)], 667, 558, 437.
(L_2)	3327 ν(H–O–H), 3210 ν(N–H), 3154 [ν(CO)+δ(N–H)], 2990 ν(C–H), 1658 ν(C=O), 1619 δ(O–H), 1547 [δ(N–H)+ν(C–N], 1534, 1460 $\nu(C–N)_{tr}$, 1399 [ν(C–N)+δ(N–H)], 1135, 917 [ω(N–H)+ϕ(C=O)], 798, 668, 653, 567.	3125 ν(N–H), 2977, 2935, 2903 ν(C–H), 1682, 1664 ν(C=O), 1622 δ(O–H) 1559 [δ(N–H)+ν(C–N], 1423 $\nu(C–N)_{tr}$, 1402, 1366 [ν(C–N)+δ(N–H)], 957 [ω(N–H)+ϕ(C=O)], 667, 575.
(L_3)	3332 ν(H–O–H), 3208 ν(N–H), 3129 [ν(CO)+δ(N–H)], 2966 ν(C–H), 1675 ν(C=O), 1623 δ(O–H), 1561 [δ(N–H)+ν(C–N], 1462 $\nu(C–N)_{tr}$, 1399, 1261 [ν(C–N)+δ(N–H)], 1115, 1056, 949 [ω(N–H)+ϕ(C=O)], 796, 754, 690, 544.	3113 ν(N–H), 2939, 2910, 2870 ν(C–H), 1673, ν(C=O), 1623 δ(O–H), 1566 [δ(N–H)+ν(C–N], 1442 $\nu(C–N)_{tr}$, 1417, 1365, 1294 [ν(C–N)+δ(N–H)], 951 [ω(N–H)+ϕ(C=O)], 883, 667, 579.
(L_4)	3411, 3327 ν(H–O–H), 3210 ν(N–H), 3123 [ν(CO)+δ(N–H)], 2950 ν(C–H), 2932, 2868 ν(C–H), 1689, 1642 ν(C=O), 1571 [δ(N–H)+ν(C–N], 1535, 1458 $\nu(C–N)_{tr}$, 1410, 1277 [ν(C–N)+δ(N–H)], 1193, 1023, 908, 812[ω(N–H)+ ϕ(C=O)], 732, 602, 574.	3137 ν(N–H), 3074, 2949, 2921, 2873 ν(C–H), 1700, ν(C=O), 1649 δ(O–H), 1600 [δ(N–H)+ν(C–N], 1499 $\nu(C–N)_{tr}$, 1414, 1298 [ν(C–N)+δ(N–H)], 1088, 977 [ω(N–H)+ϕ(C=O)], 660, 577.

ν, stretching; δ, bending; ω, out-of-plane wagging; ϕ, out-of-plane ring deformation; tr, triazine ring

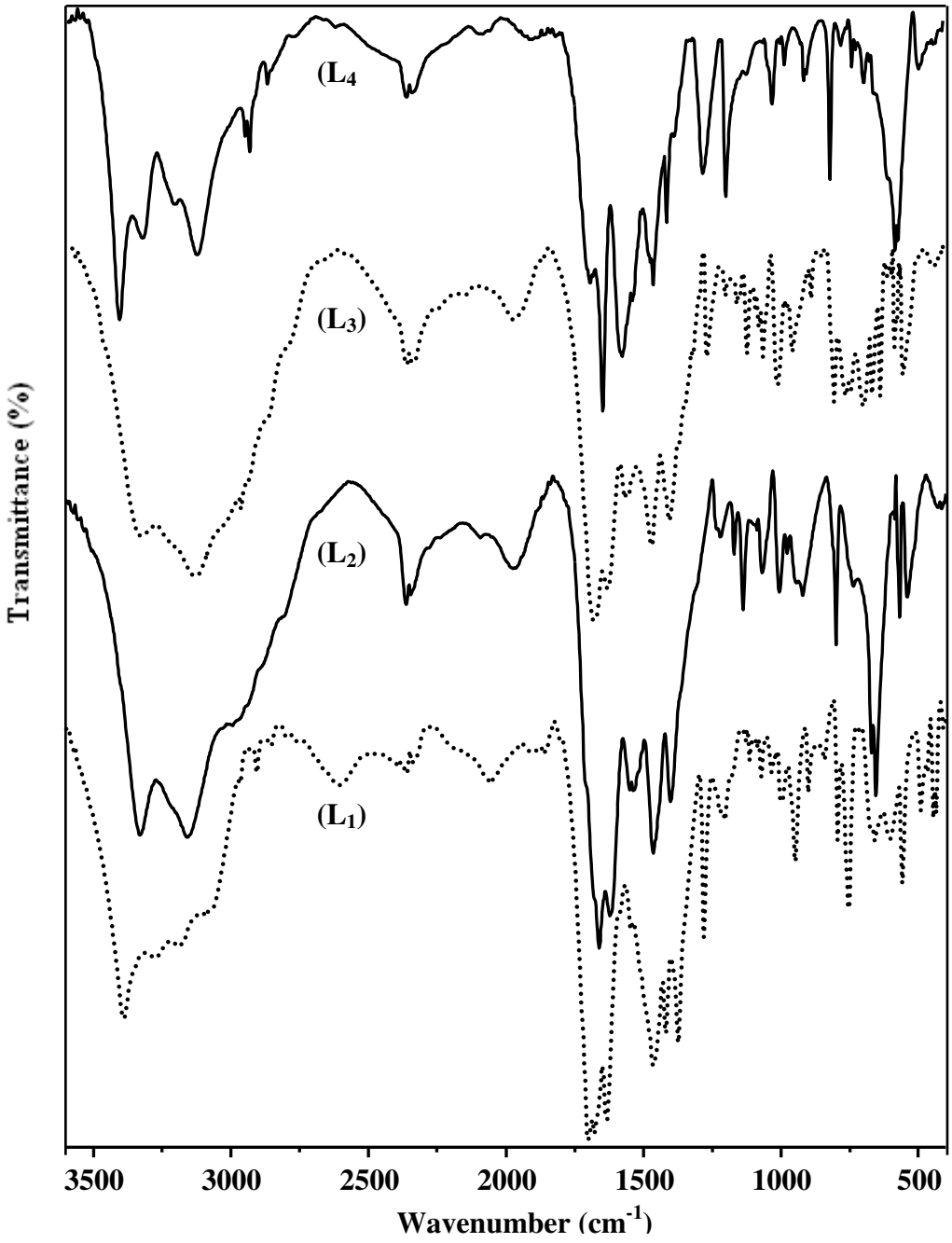


Figure 3.1. FT-IR spectrum of (L_1-L_4) compounds in the 3600– 400 cm^{-1} region.

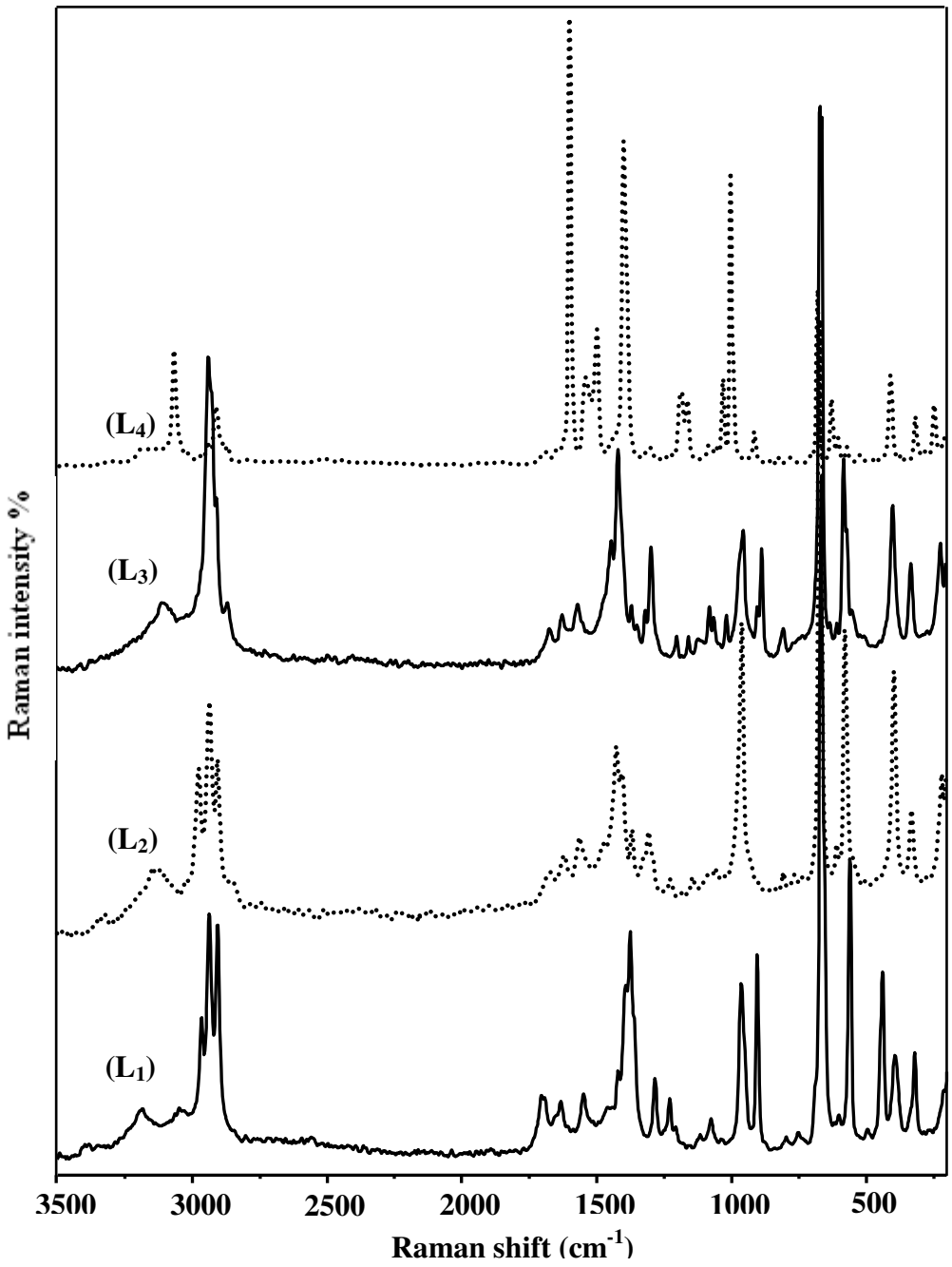


Figure 3.2. FT-Raman spectrum of (L_1-L_4) compounds in the 3500-200 cm^{-1} region.

3.1.2. (L_5-L_8) Ligands

The present vibrational spectra (Fig. 3.3.) can be discussed in terms of three characteristic wave regions: 3570-2850 cm^{-1} corresponding to ν(H–O–H) lattice water, ν(N−H) amides and ν(C−H) aliphatic and aromatic characteristic modes, 1800-800 cm^{-1} belongs to ν(CONH–) amides, $\nu(C-N)_{tr}$, δ(N−H) and 750-500 cm^{-1} frequencies regions due to the ν(C−S) and ν(S−S) characteristic stretching modes, which are clearly assignable in the Raman spectra. Prominent Raman and IR frequency data values and their some assignments are presented in Table 3.2 and Table 3.3. Appearance of a strong broad bands in the region 3490-3380 cm^{-1} in the IR spectra may be due to the presence of lattice water (H–O–H) (antisymmetric and symmetric), which was also confirmed by elemental analysis [105]. These modes are either very weak or not active in Raman spectra. The bands corresponding to free carboxylic acid (–COOH) and amine ($–NH_2$) groups are not observed in the IR spectra of the (L_5-L_8) ligands, which suggests complete condensation of the amine groups of 2,4-diamino-6-phenyl-1,3,5-triazine with corresponding dicarboxylic acids. The medium to strong bands observed in the regions 3300 and 3150 cm^{-1} in the IR spectra are assignable to amide A and amide B vibration respectively. The amide A is almost caused by the NH stretching vibration, this mode of vibration is not depend on the backbone conformation but is very sensitive to the strength of a hydrogen bonding. The amide B is originates from a Fermi resonance between the first overtone of amide II and the N-H stretching vibration [106,107]. Due to non-planar characteristic behavior of the compounds in the solid state, the bands could be observed as multiple signals. These lines have extremely weak Raman intensity, particularly in the solid form (Figs. 3.3, 3.4.). The pure characteristic ν(CH) modes of aliphatic groups are observed in the wave region 2930-2820 cm^{-1} in both Raman and IR spectra as expected. The peptide bond formation could also be confirmed due to the observance of two major bands in the frequency regions 1700-1650 cm^{-1} (amide I) and 1590-1540 cm^{-1} (amide II). The amide I band is the most intensive

absorption band particularly in the IR spectrum and it is almost fully governed by the stretching vibration of the C=O mode with a very small contribution of the C–N groups. Amide II is more complex than amide I, which derives mainly from in-plane N–H bending vibration with certain contribution of ν(C–N) groups. Amide III and IV are very complex bands resulting from a mixture of several coordinate displacements. These modes are dominated by the out-of-plane motions particularly (N–H) wagging and (C=O) deformation vibrations as reported in the Table 3.2 [106,107]. The bending vibrations for δ(H–O–H) in IR spectra are observed in the region 1640 cm^{-1} (Figs. 3.3, 3.4.).

The vibrational frequency of ν(R–S–S–R) (R = Alkyl) are observed in the region 520-480 cm^{-1} [108]. In the Raman spectra, ν(C–S) and ν(S–S) modes for the (C–S–C) and (C–S–S–C) moieties are very characteristic giving rise to a medium to strong stretching bands in the wave region 710-570 and 530-500 cm^{-1}, respectively [108,109]. The medium to strong bands at 508 of (L_6) and 505 cm^{-1} of (L_8) clearly show characteristic ν(S–S) stretching modes for these macrocycles (Fig. 3.5). The assignments are supported by the fact that the other relevant bands at this region remain almost unchanged among these compounds. No clear-cut assignments are possible for ν(C–S) modes, but bands at *ca.* 530-520 cm^{-1} could be assignable for these particular vibrations (Fig. 3.5). Because of the non-polar character of the (C–S) and (S–S) modes, their counterpart in IR are quite weak (Table 3.2, Table 3.3, and Fig. 3.3).

Table 3.2. Prominent IR bands for the (L_5-L_8) compounds.

	FT-IR (cm^{-1})
(L_5)	3371 ν(N–H), 3326 ν(N–H), 3178 [ν(CO)+δ(N–H)], 3034 $\nu(C–H)_{ar}$, 2980 ν(C–H), 1673 ν(C=O), 1592 $\nu(C–C)_{ar}$, 1535 [δ(N–H) + ν̣C–N], 1513 $\nu(C–N)_{tr}$, 1390, 1361-1273 [ν(C–N)+δ̣(N–H)], 896, 780 [ω̣(N–H)+ϕ(C=O)], 687 $\omega(C–C)_{ar}$, 621 ν(C–S), 592, 463.
(L_6)	3467 ν(O–H), 3414 ν(N–H), 3322 [ν(CO)+δ(N–H)], 3127 $\nu(C–H)_{ar}$, 2937, 2833 ν(C–H), 1686, ν(C=O), 1648 $\nu(C–C)_{ar}$, 1570 [δ(N–H)+ν̣C–N], 1530 $\nu(C–N)_{tr}$, 1396, 1296-1261 [ν(C–N)+δ̣(N–H)], 913, 817 [ω̣(N–H)+ϕ(C=O)], 780 $\omega(C–C)_{ar}$, 715, 630 ν(C–S), 586 , 470.
(L_7)	3478 ν(O–H), 3440 ν(N–H), 3332, 3215 [ν(CO)+δ(N–H)], 3073 $\nu(C–H)_{ar}$, 2975 ν(C–H), 1685 ν(C=O), 1628 $\nu(C–C)_{ar}$, 1591 [δ(N–H)+ν̣C–N], 1565 $\nu(C–N)_{tr}$, 1394-1261 [ν(C–N)+δ̣(N–H)], 1193, 908, 822 [ω̣(N–H)+ϕ(C=O)], 785 $\omega(CC)_{ar}$, 709, 621 ν(C–S), 553, 460.
(L_8)	3486 ν(O–H), ν(N–H), 3319 [ν(CO)+δ(N–H)], 3123 $\nu(C–H)_{ar}$, 2965, 2925 ν(C–H), 1703 ν(C=O), 1673 ν(C=O), 1644 $\nu(C–C)_{ar}$, 1568 [δ(N–H)+ν̣C–N], 1533 $\nu(C–N)_{tr}$, 1399-1203 [ν(C–N)+δ̣(N–H)], 1062, 820 ω̣(N-H), 773 $\omega(C–C)_{ar}$, 689, 630 ν(C–S).

ν̣, stretching; δ̣, bending; ω, out-of-plane wagging; ar, aromatic ring; tr, triazine ring.

Table 3.3. Prominent Raman bands for the (L_5-L_8) compounds.

	Raman (cm^{-1})
(L_5)	3100, 3069 ν(C–H)$_{ar}$, 2972, 2921 ν(C–H), 1681 ν(C=O), 1601 ν(C–C)$_{ar}$, 1552 ν(C–N)$_{tr}$, 1497 [ν(CO)+δ(N–H)], 1466, 1400, 1181, 1028, 1000, 776 ω(C–H)$_{ar}$, 678, 621 ν(C–S), 95.
(L_6)	3129, 3071 ν(C–H)ar, 2998, 2937 ν(C–H), 1701 ν(C=O), 1651 ν(C=O), 1601 ν(C–C)ar, 1527 [ν(CO)+δ(N–H)], 1504 ν(C–N)$_{tr}$, 1404, 1195, 1156, 1024, 1003, 913, 784 ω(C–H)$_{ar}$, 681, 631 ν(C–S), 573, 505 ν(S–S), 263, 118.
(L_7)	3198, 3081, 3069 ν(C–H)ar, 2936, 2916 ν(C–H), 1646 ν(C=O), 1602 ν(C–C)$_{tr}$, 1538 ν(C–N)$_{tr}$, 1495 [ν(CO)+δ(N–H)], 1399, 1182, 1163, 1004, 781 ω(C–H)$_{ar}$, 676, 616 ν(C–S), 395, 97, 70.
(L_8)	3064, ν(C–H)$_{ar}$, 2968, 2956, 2927, 2912 ν(C–H), 1708 ν(C=O), 1684 ν(C=O), 1622, 1605 ν(C–C)$_{tr}$, 1578 ν(C–N)$_{tr}$, 1504 [ν(CO)+δ(N–H)], 1404, 1362, 1003, 778 ω(C–H)$_{ar}$, 682, 628 ν(C–S), 612, 601, 568 , 508 ν(S–S), 487, 130, 90.

ν, stretching; δ, bending; ω, out-of-plane wagging; ar, aromatic ring; tr, triazine ring.

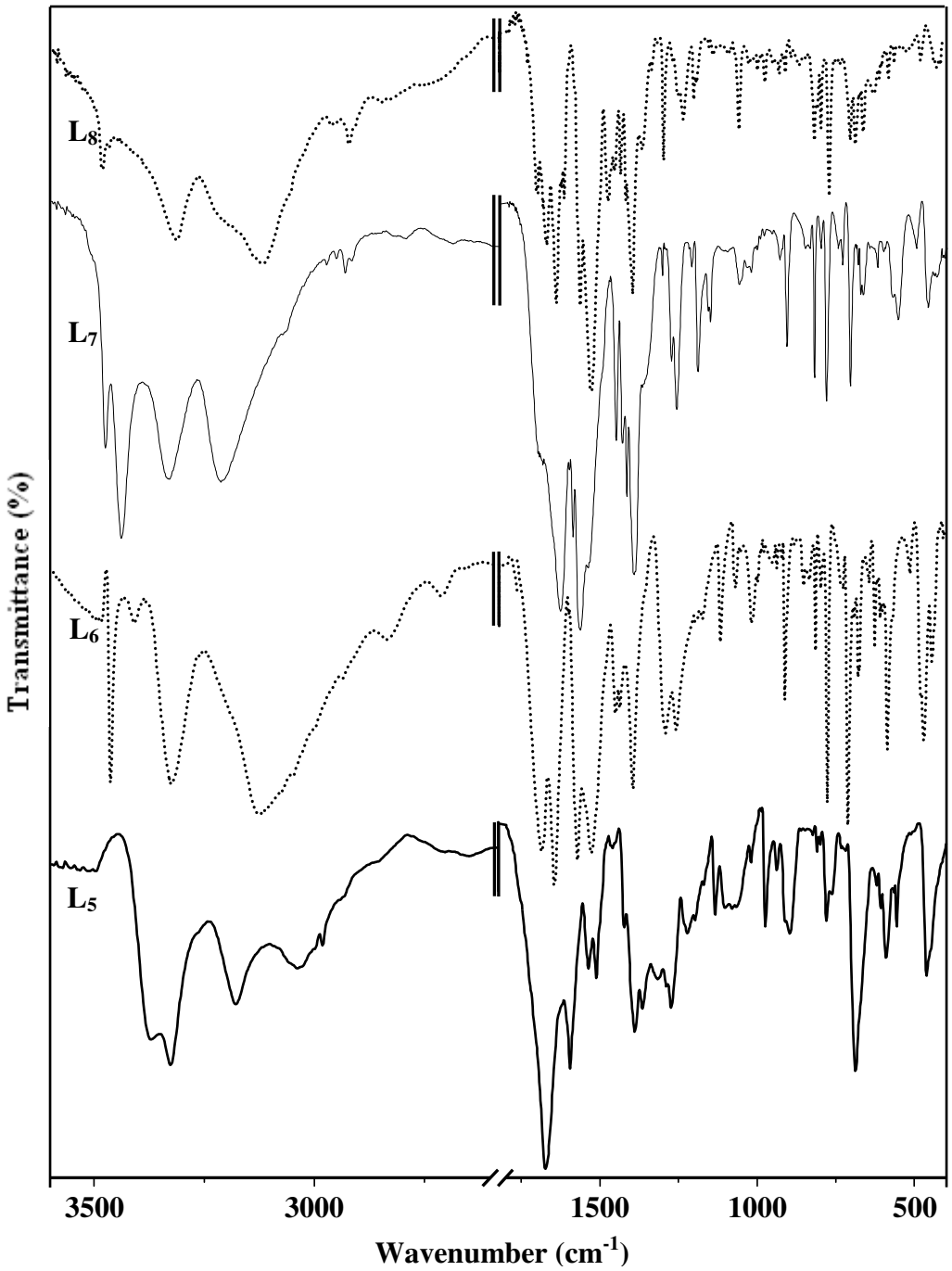


Figure 3.3. FT-IR spectra of (L_5-L_8) compounds in the 3600-2600//1800-400 cm^{-1} regions.

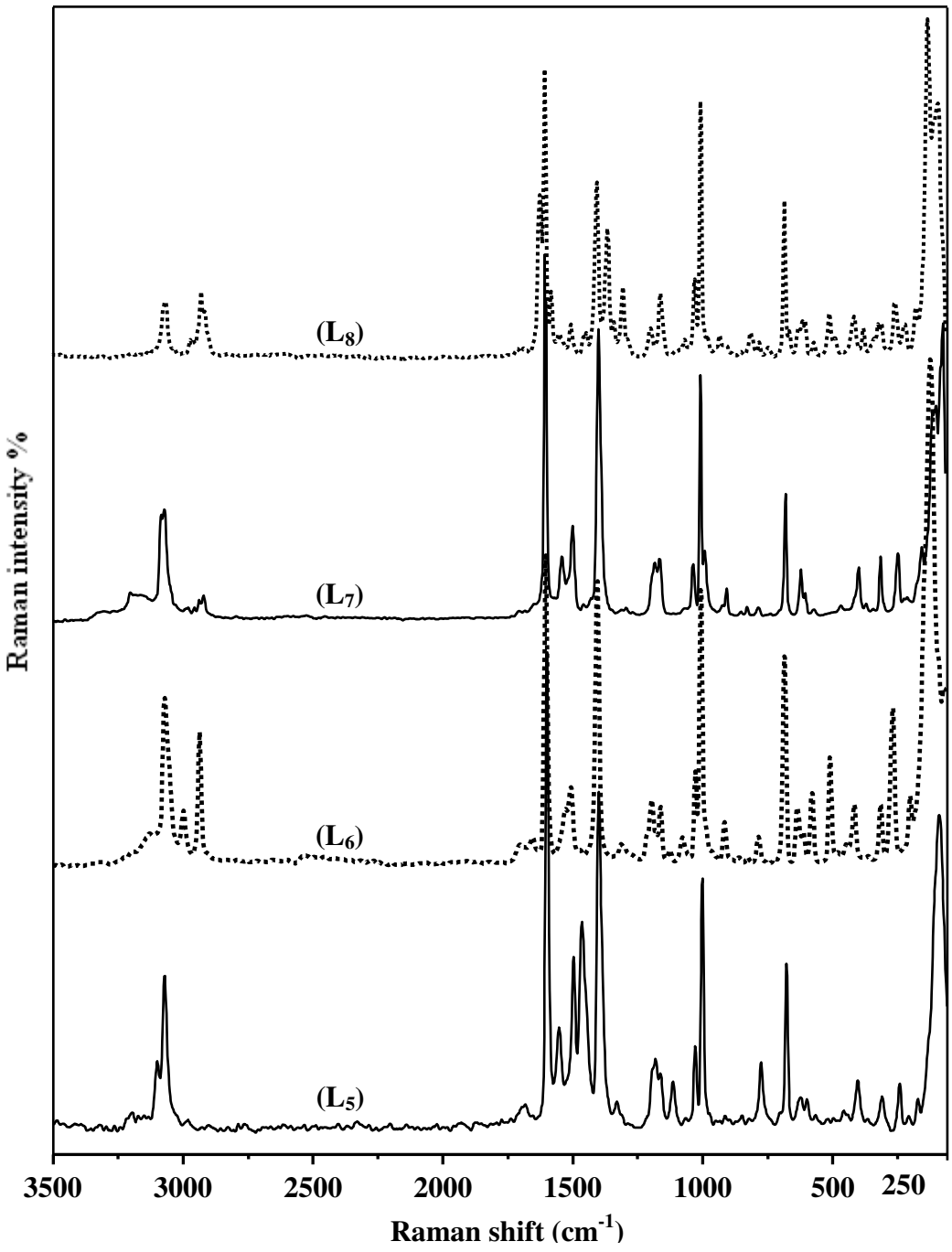


Figure 3.4. FT-Raman spectra of (L_5-L_8) compounds in the 3500-60 cm^{-1} region.

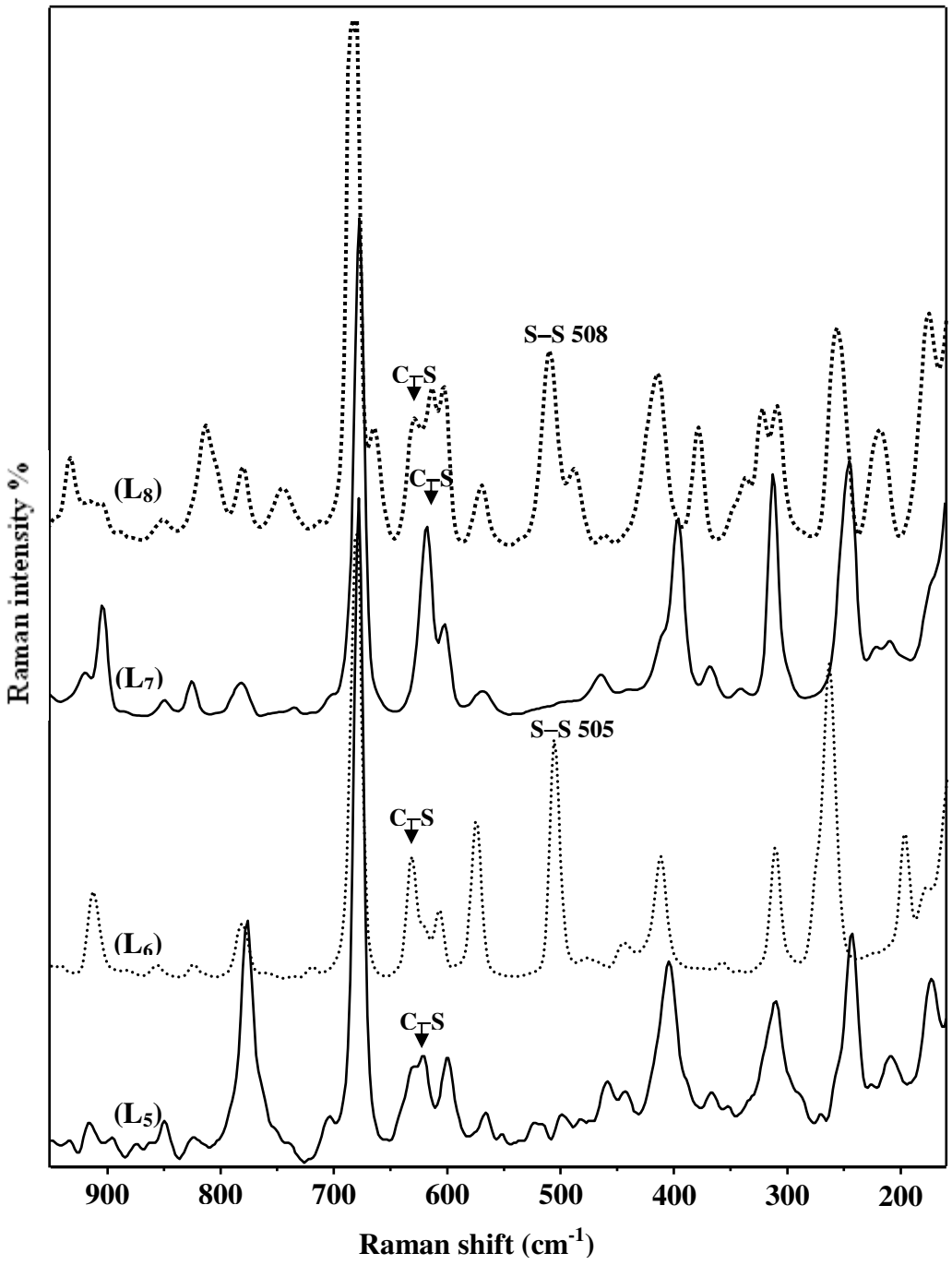


Figure 3.5. FT-Raman spectra of (L_5-L_8) compounds in the 950-160 cm^{-1} region.

3.1.3. Pd(II) halide complexes

The present vibrational spectra can be discussed in terms of three characteristic wave regions: 3500-2800 cm^{-1} corresponding to υ(N–H), aromatic and aliphatic υ(C–H) modes, 2000-600 cm^{-1} frequencies due to υ(C=C), υ(C–N), υ(C–C), and (CH) deformations, and 600-200 cm^{-1} frequency regions due to the υ(Pd–S) and υ(Pd–N) characteristic stretching modes. The complexed υ(N–H) modes could not be observed clearly due to the coordination of nitrogen atom to Pd(II) , appears as a double peak of medium intensity at 3340-3300 cm^{-1} in the free ligand (Fig.3.6).

υ(Pd–S) stretching modes were observed in the 450-300 cm^{-1} frequency region as expected. In the IR spectrum of the complex $[Pd(L_9)]X_2$, the υ(M-X) and υ(S–M–S) modes were registered at 365-325 cm^{-1} and 422-400 cm^{-1}, respectively. Hereby, the anti-symmetric υ_{asym}(S–Pd–S) stretch should be seen clearly in the infrared spectra. Accordingly, the medium to intense bands at 422 cm^{-1} are assigned to the υ_{asym}(S–M–S) vibrations.

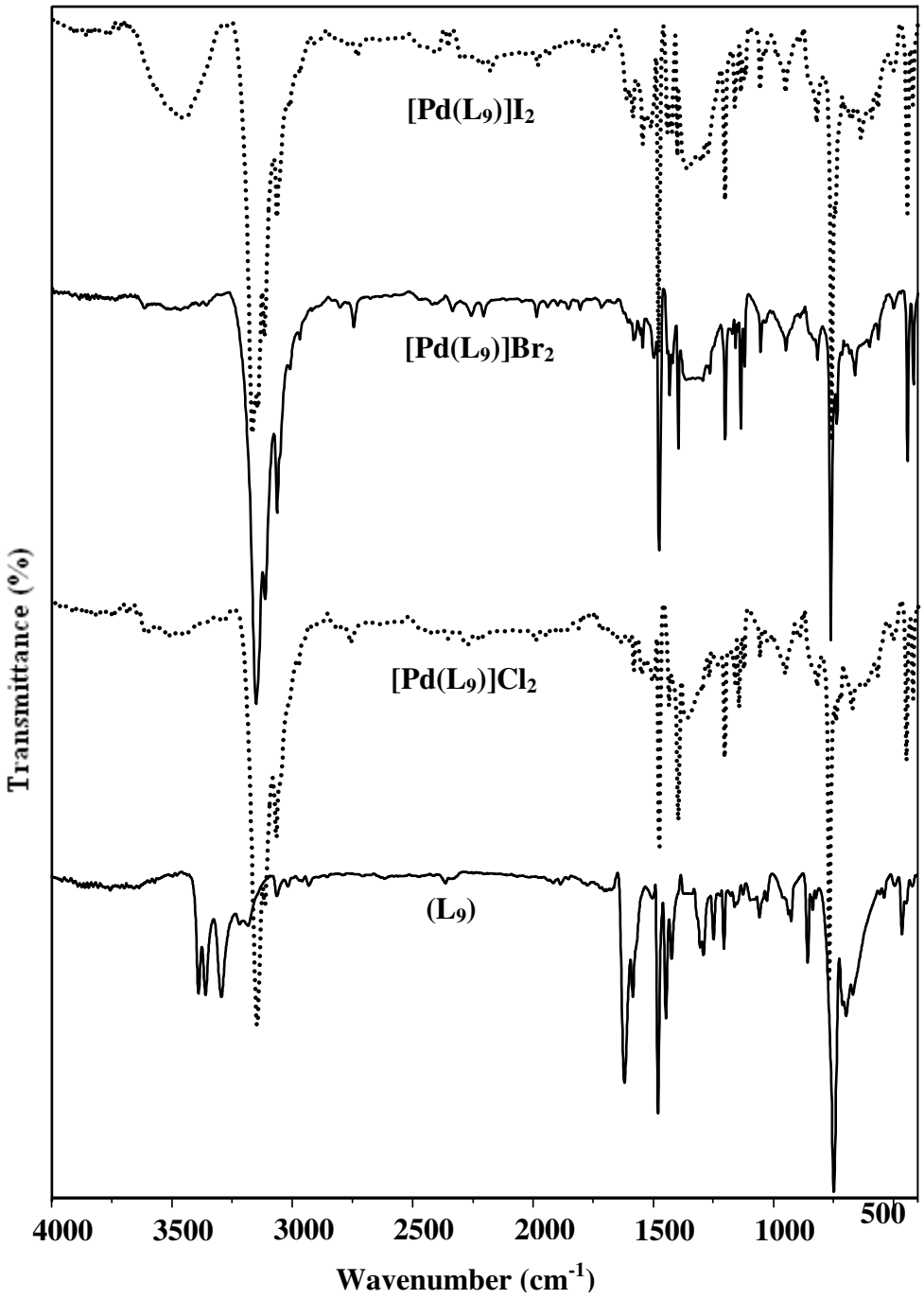


Figure 3.6. FT-IR spectrum of (L_9), [Pd(L_9)]Cl_2, [Pd(L_9)]Br_2 and [Pd(L_9)]I_2 in the 3600-400 cm^{-1} region.

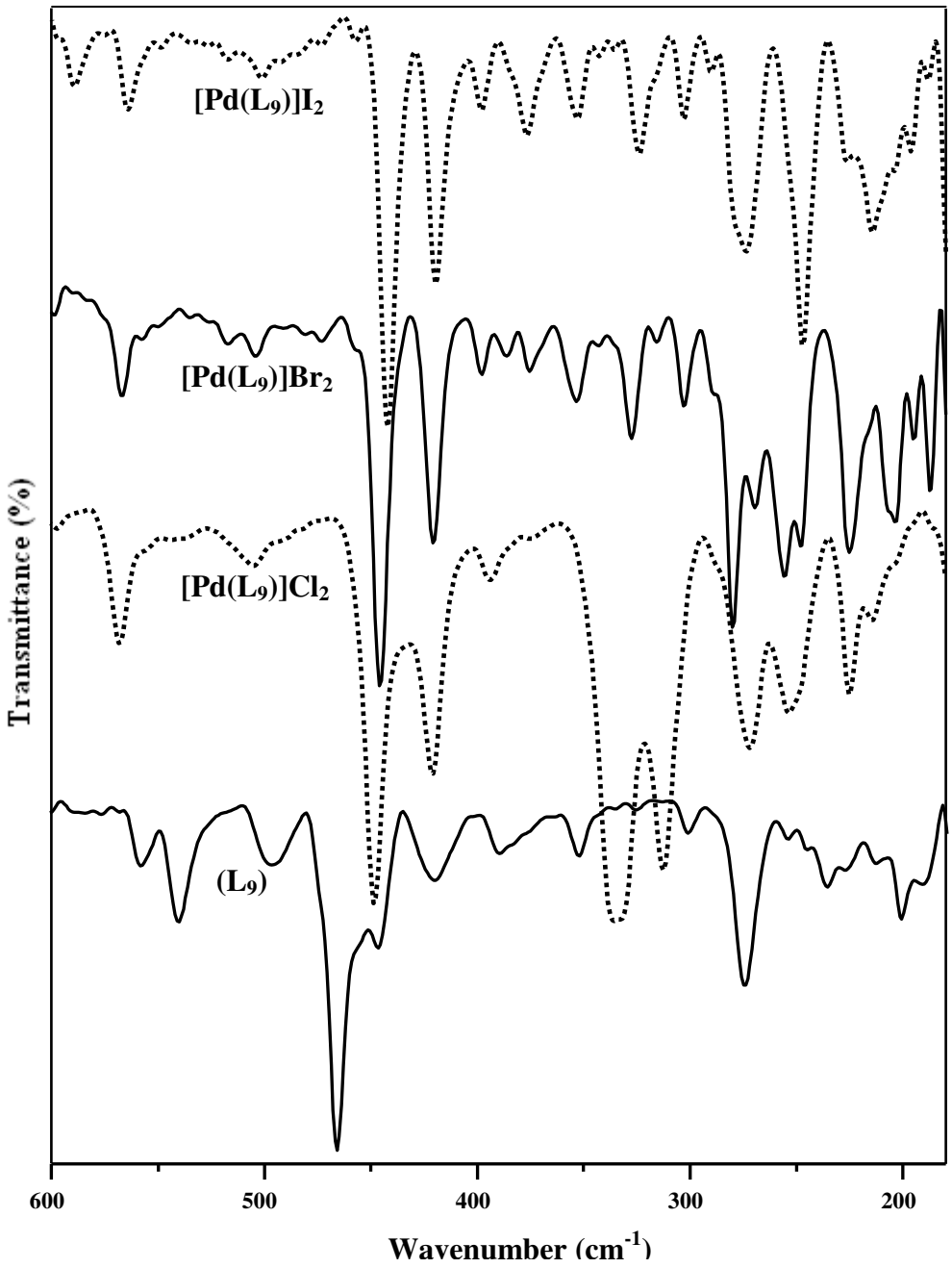


Figure 3.7. FT-IR spectrum of (L_9), [Pd(L_9)]Cl_2, [Pd(L_9)]Br_2 and [Pd(L_9)]I_2 in the 600-175 cm^{-1} region.

3.2. Conductivity Measurements

Conductivity measurements are strongly temperature dependant. After assessing the thermal stabilities of $[Pd(L_9)]Cl_2$, $[Pd(L_9)]Br_2$ and $[Pd(L_9)]I_2$ complexes by TGA Mettler TG500, conductivity measurements were done by using solid-state conductometer at temperature range 20-250 ˚C. The results (Figs. 3.8.a,b,c,d) suggest that halide ions are located outside the coordination sphere since as ionic radius of anions increase, the conductivity decreases. This trend may be interpreted by taking into account the fact that larger ions should be moving smaller in the matrix of a substance, thus causing overall decrease in conductivity. It has been long known that ionic size of counter ions of large molecules is strictly related to the conductivity and dielectric properties of a substance [34].

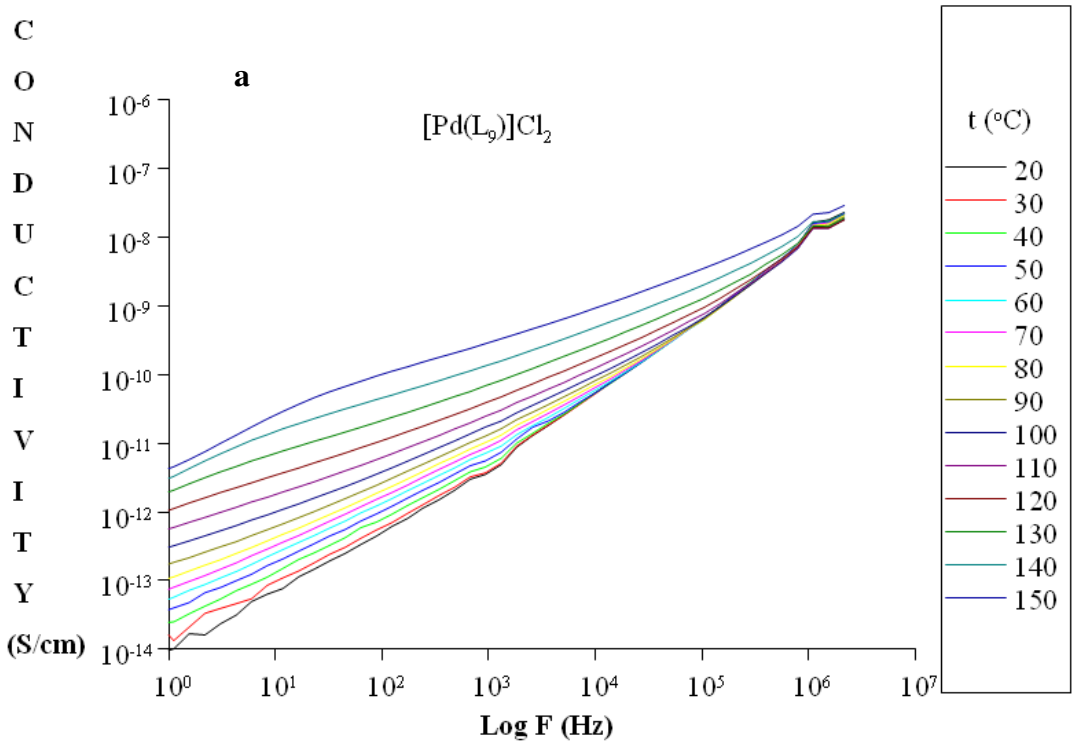
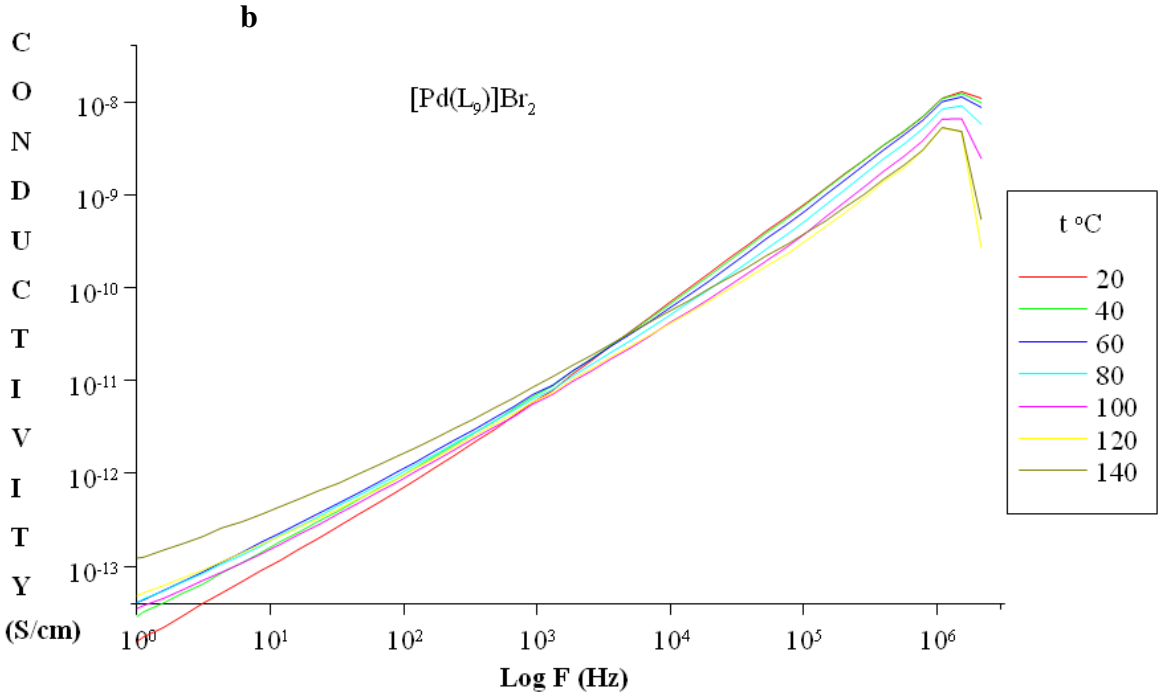
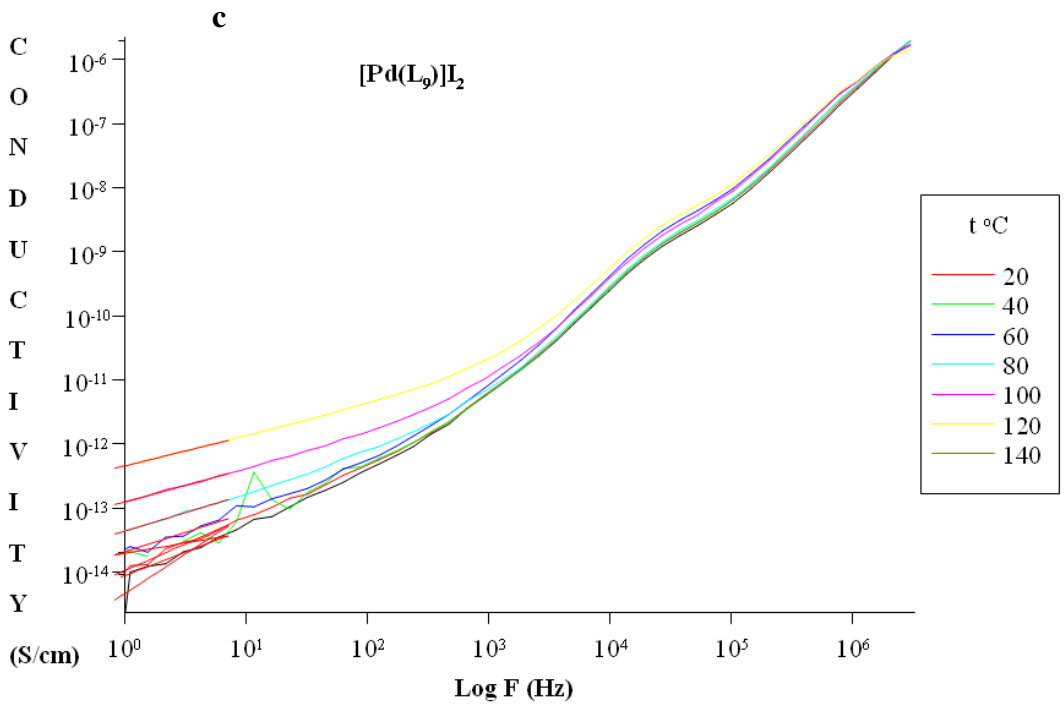
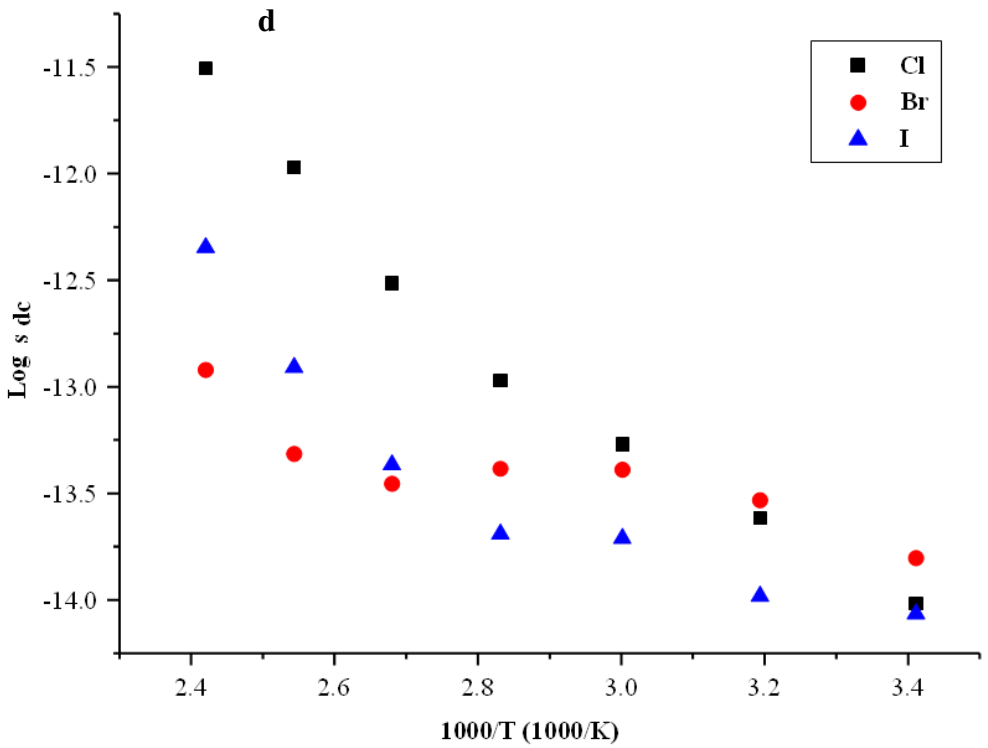


Figure 3.8. Solid-state conductivity measurements for the complexes.


b
[Pd(L9)]Br2
CONDUCTIVITY (S/cm)
10-8
10-9
10-10
10-11
10-12
10-13
100
101
102
103
104
105
106
Log F (Hz)
t °C
20
40
60
80
100
120
140


c
[Pd(L9)]I2
CONDUCTIVITY (S/cm)
10-6
10-7
10-8
10-9
10-10
10-11
10-12
10-13
10-14
100
101
102
103
104
105
106
Log F (Hz)
t °C
20
40
60
80
100
120
140



3.3. Nuclear Magnetic Resonance Studies

3.2.1. (L_1-L_4) Ligands

The ^{1}H NMR spectra of the (L_1-L_4) ligands in DMSO-d_6 do not give any signal corresponding to primary amine –NH_2 (*ca.* 6 ppm) and carboxylic acid –COOH (*ca.* 12 ppm), instead they show broad bands at *ca.* 7 ppm corresponding to amide (4H) protons. The low field position as well as broadness of NH protons could be attributed to the combination of deshielding via weak hydrogen bonding due to amide atoms. As expected no significant changes is observed for the methyl protons chemical shifts compared with starting material (*ca.* 2.0 ppm). Singlets at 3.18 and 2.07 ppm are attributed to the methylene protons of the (L_1) and (L_2) ligands, respectively. Ligand (3) shows a triplet at

2.07 ppm and quintet at 1.72 ppm which are assignable to methylene protons of CO–CH_2 and –CH_2– units, respectively. Two triplets are observed for the ligand (L_4) at 2.21, 1.51 ppm representing the methylene protons of α and β positions to the peptide bond.

As expected no significant changes are observed for the methyl and triazine carbon chemical shift values due to the cyclic formation. The methyl-triazine carbon atom chemical shifts are observed approximately at: 174-175 (C_d), 166-167 (C_c), 24-25 (C_e) ppm. The signals in the 173-175 ppm regions are assignable to CO of the CO–NH amide group. Similarly a singlet at 42.14 ppm is attributed to methylene carbons of ligand (L_1) and a singlet at 29.33 ppm is for the methylene carbons of the ligand (L_2) (Fig. 3.10.). Two singlets are appeared at 33.27 and 20.45 ppm for the methylene carbons of CO–CH_2 and CH_2 groups of the (L_3) ligand (Fig. 3.11.), respectively. In the same manner two singlets are appeared for the (L_4) ligand at 33.87 and 24.50 ppm for methylene carbons of CO–CH_2 and CH_2–CH_2 groups, respectively. In conclusion, presented generic structure in Figure 3.9 is in best accord with the experimental data obtained from the analytical, vibrational and nuclear magnetic resonance spectra.

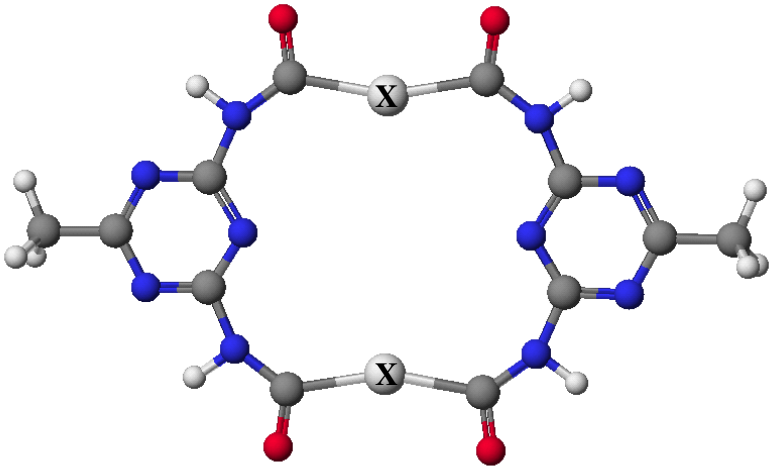


Figure 3.9. General proposed structure of the ligands: X = –CH_2–; –$(CH_2)_2$–; –$(CH_2)_3$–; –$(CH_2)_4$–.

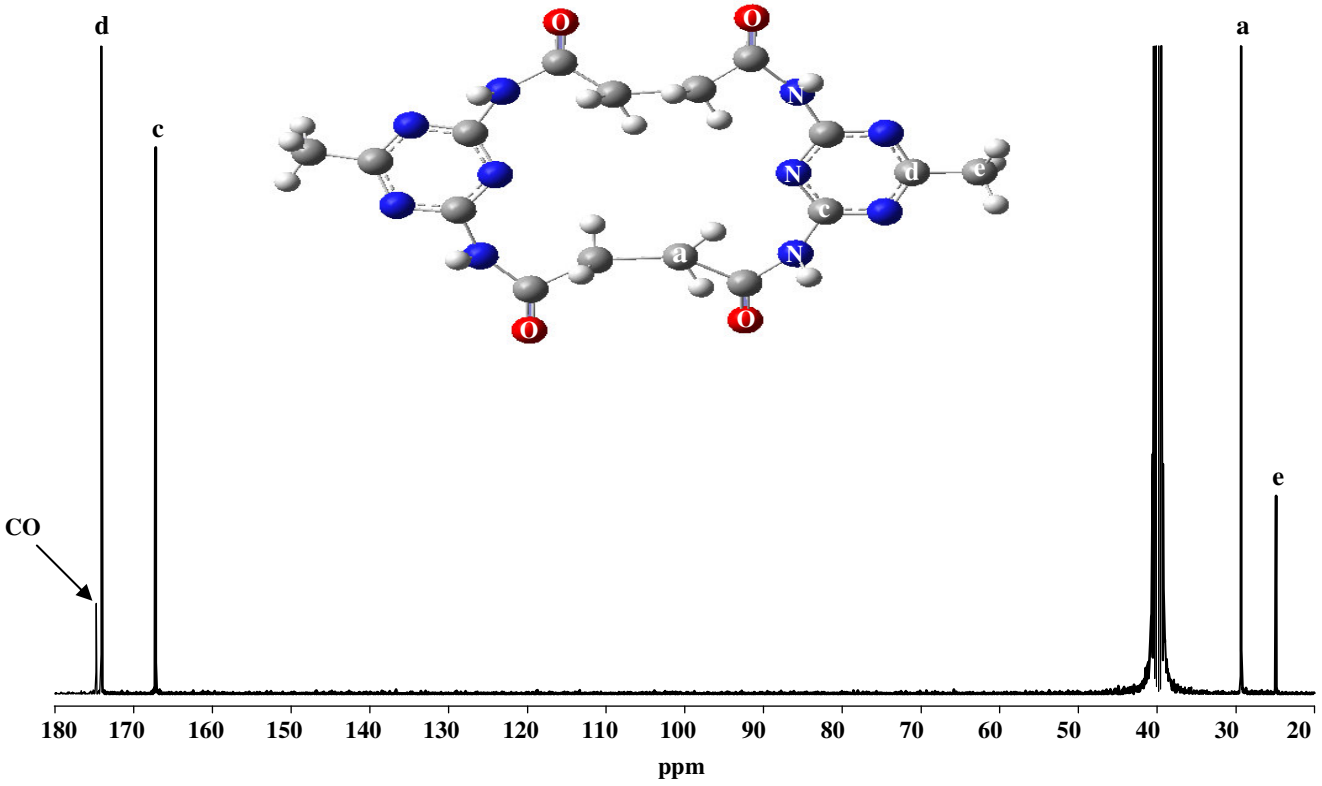


Figure 3.10. ^{13}C {H} NMR spectrum of (L_2) ligand.

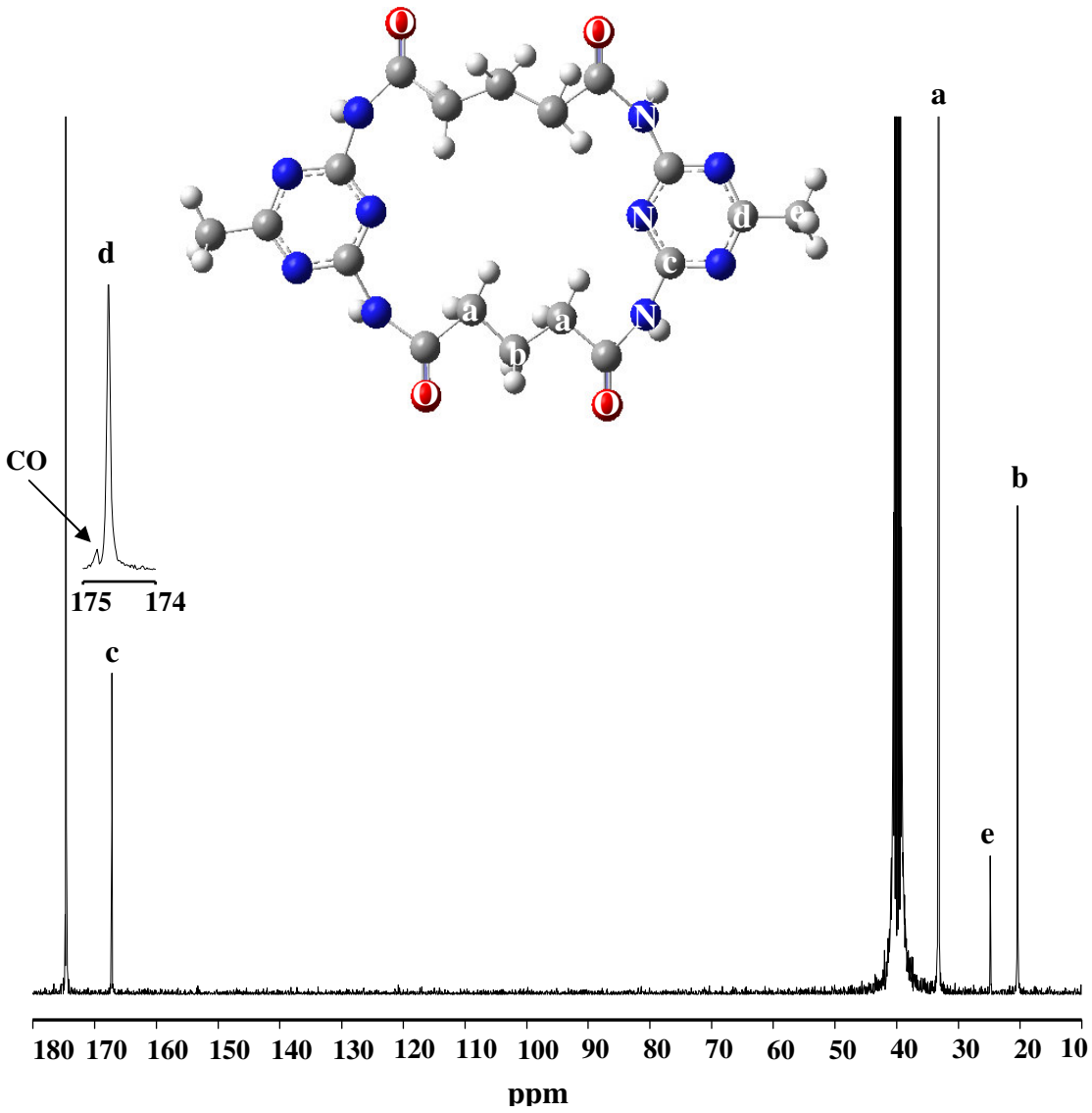


Figure 3.11. ^{13}C {H} NMR spectrum of (L_3) ligand.

3.2.2. (L_5-L_8) Ligands

The ^1H NMR spectra of the (L_5-L_8) ligands in DMSO-d_6 do not give any signal corresponding to primary amine $–NH_2$ (at 6.8 ppm) and carboxylic acid –COOH (*ca.* 11 ppm), instead they show broad bands in the region 10-9 ppm corresponding to amide (4H) protons. The low field position as well as broadness of NH protons could be attributed to the combination of deshielding via weak hydrogen bonding due to amide and sulphur atoms. As expected no significant changes is observed for the phenyl protons chemical shifts compared with starting material. Two singlets at 3.37 and 3.68 ppm are attributed to the methylene protons of $S–CH_2–CO$ groups for the (L_5) and (L_6) ligands, respectively. Two triplets are observed for the (L_7) and (L_8) ligands at 2.70, 2.51 and 2.91, 2.64 ppm for the methylene protons of $S–CH_2–CH_2–CO$ (8H) and $S–S–CH_2–CH_2–CO$ (8H) groups, respectively. Considering the slight stronger deshielding characteristic of sulphur atoms compared with amide groups, the slight higher chemical shift values 2.70 (L_7) and 2.91 (L_8), may be assignable to the methylene protons that are adjacent to the sulphur atoms [110].

No significant changes are expected for the phenyl and triazine carbon chemical shift values due to the cyclic formation. The phenyl-triazine carbon atom chemical shifts are observed approximately at: 170-171 (C_d), 67-168 (C_c), 137-138 (C_e), 131-132 (C_h), 128-129 (C_g), 128 (C_f) [109]. The signals in the 171-173 ppm regions are assignable to CO of the CO–NH amide group. Similarly two singlets at 33.75 and 40.97 ppm are attributed to methylene carbons of $S–CH_2–CO$ group for (L_5) and (L_6) ligands, respectively.

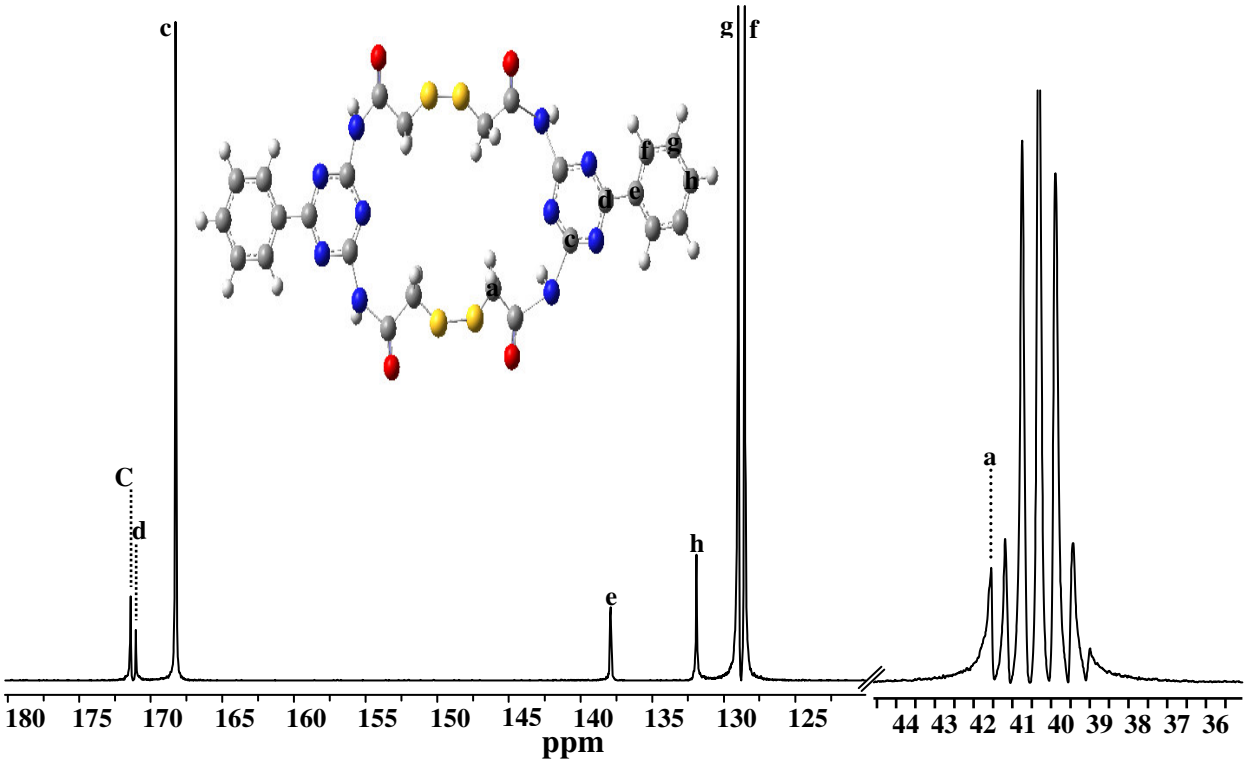


Figure 3.12. $^{13}C\{H\}$ NMR spectrum of (L_6) ligand.

Two singlets are appeared for each of the (L_7) and (L_8) ligands at 26.41, 34.55 and 33.51, 34.0 ppm for methylene carbons of S–CH_2–CH_2–CO and S–S–CH_2–CH_2–CO residues, respectively. Considering the weaker deshielding characteristic of sulphur atoms in comparison with amide group on the carbon chemical shift values, the lower ppm values 26.41 (L_7) and 33.51 (L_8), may be assignable to the methylene carbons that are close to the sulphur atoms (Figs. 3.12, 3.13) [110].

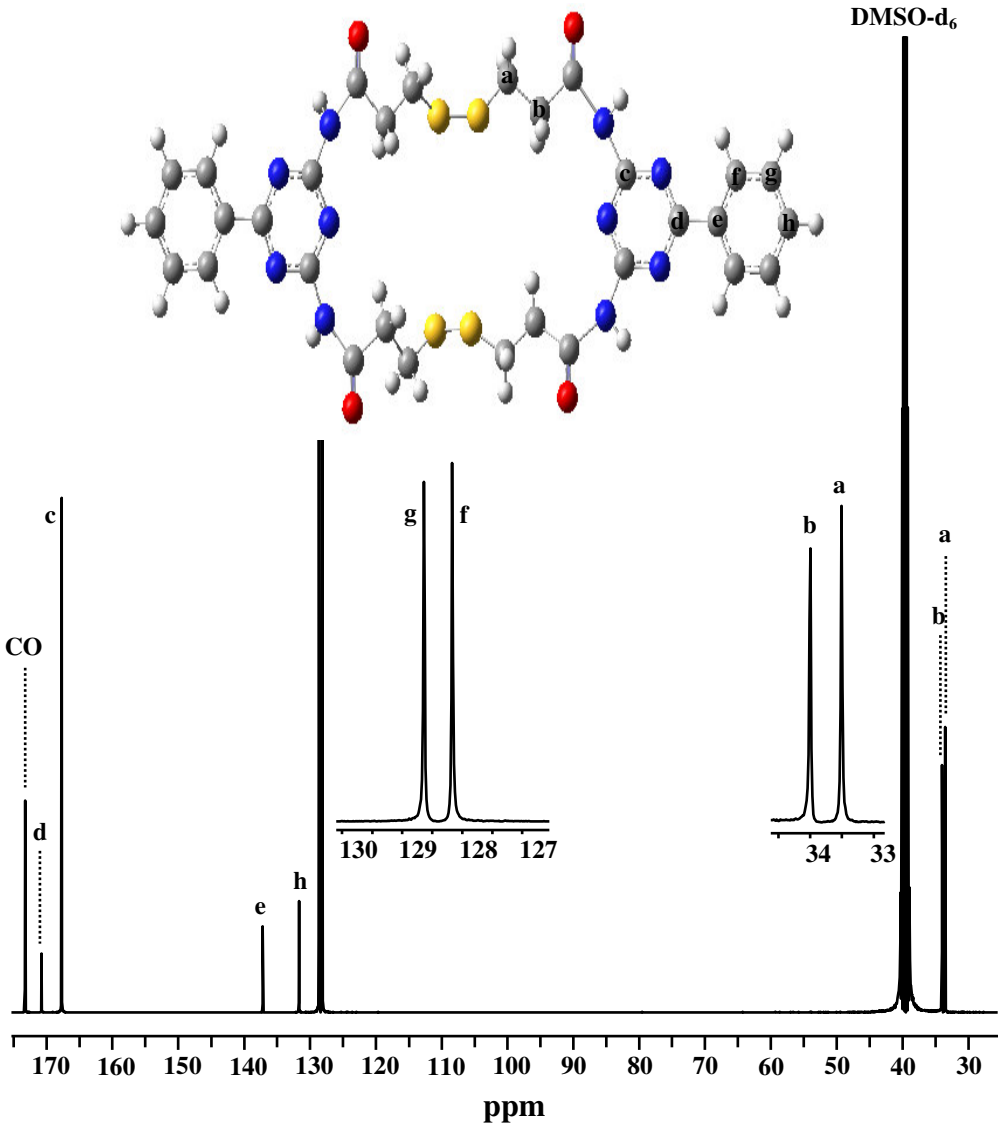


Figure 3.13. $^{13}C\{H\}$NMR spectrum of (L_8) ligand.

3.3. Biological Activity

3.3.1. (L_1-L_4) Ligands

3.3.1.1. Antimicrobial activity

The results concerning in vitro antimicrobial activities of the macrocycles together with the inhibition zone (mm) and (MIC) values of compared antibiotic and antifungal reagents are listed in Tables 3.4 and 3.5. All the compounds tested exhibit moderate antimicrobial activity. Of all the test compounds at tempted, (L_2) and (L_4) showed slightly higher activities. The MIC values in Table 3.5 indicate that all the compounds tested exhibit moderate to strong antimicrobial activity on the tested microorganisms. Once again the data indicate that the (L_2) and (L_4) macrocycles have slightly stronger activity against both Gram-positive and Gram-negative bacteria such as *Micrococcus luteus* (L_2 = 6.25 and L_4 = 6.25 μg mL^{-1}) and *Proteus vulgaris* (L_2 = 6.25, L_4 = 3.125 μg mL^{-1}) compared with Gentamycin on these microorganisms 25 and 6.25 μg mL^{-1}, respectively (Table 3.5). All the tested compounds show quite strong activity against the yeast cultures. For instance, all the compounds showed superior activity (MIC = 1.56 μg mL^{-1}) against *Debaryonmyces hansenii* culture compared with Nystatin antifungal agent. Once more the data indicate that the (L_2) and (L_4) macrocycles have slightly stronger activity against yeast culture *candida albicans* (1.56 μg mL^{-1}) in comparison with Nystatin antifungal agent. No clear-cut correlation could be made between the inhibition activity of the compounds and their for example molecular weight.

Table 3.4. *In vitro* antimicrobial activity of the (L_1-L_4) compounds and the standard reagents (inhibition zone mm).

Microorganisms/Compounds	**(L_1)**	**(L_2)**	**(L_3)**	**(L_4)**	P10	AMP	CTX	VA	OFX	TE	NY	KET	CLT
Escherichia coli(-)	11.0	13.0	10.0	14.0	18	12	10	22	30	28	-	-	-
Staphylococcus aureus(+)	11.0	12.0	12.0	13.0	13	16	12	13	24	26	-	-	-
Klebsiella pneumoniae(-)	14.0	16.0	13.0	17.0	18	14	13	22	28	30	-	-	-
Bacillus cereus(+)	10.0	10.0	9.0	10.0	8	10	54	10	44	34	-	-	-
Micrococcus luteus(+)	14.0	15.0	13.0	15.0	10	16	18	20	28	26	-	-	-
Proteus vulgaris(-)	10.0	12.0	14.0	17.0	14	12	14	18	30	25	-	-	-
Mycobacterium smegmatis(+)	10.0	10.0	9.0	11.0	15	21	11	20	32	24	-	-	-
Listeria monocytogenes(+)	12.0	14.0	11.0	15.0	10	12	16	26	30	28	-	-	-
Pseudomonas aeruginosa(-)	14.0	16.0	12.0	15.0	36	32	32	34	28	22	-	-	-
Kluyveromyces fragilis	16.0	20.0	15.0	17.0	-	-	-	-	-	-	20	21	15
Rhodotorula rubra	15.0	17.0	16.0	16.0	-	-	-	-	-	-	18	16	18
Candida albicans	18.0	22.0	20.0	24.0	-	-	-	-	-	-	18	22	16
Hanseniaspora guilliermondii	17.0	21.0	17.0	19.0	-	-	-	-	-	-	21	24	22
Debaryomyces hansenii	20.0	22.0	18.0	24.0	-	-	-	-	-	-	16	14	18

P10, Penicillin G (10 Units); AMP, Ampicillin 10 µg; CTX, Cefotaxime 30 µg; VA, Vancomycin 30 µg; OFX, Ofloxacin 5 µg; TE, Tetracycline 30 µg; NY, Nystatin 100 µg; KET, Ketaconazole 20 µg; CLT, Clotrimazole 10 µg.

Furthermore, in classifying the antibacterial activity as Gram-positive or Gram-negative, it would generally be expected that a much greater number would be active against Gram-positive than Gram-negative bacteria. In this study, the compounds are relatively active against on both types of the bacteria and as well as strongly active against yeasts, which may indicate a broad-spectrum affect. The results of our study indicate that the compounds have the potential to generate novel antimicrobial properties by displaying moderate to high affinities for most of the receptors particularly against yeast cultures.

Table 3.5. *In vitro* antimicrobial activity (MIC, µg mL^{-1}) of the (L_1-L_4) compounds.

Microorganisms/Compounds	**(L_1)**	**(L_2)**	**(L_3)**	**(L_4)**	GEN	NYS
Escherichia coli(-)	25	12.5	25	12.5	6.25	-
Staphylococcus aureus(+)	25	12.5	12.5	12.5	25	-
Klebsiella pneumoniae(-)	12.5	6.25	12.5	6.25	6.25	-
Bacillus cereus(+)	25	25	25	25	6.25	-
Micrococcus luteus(+)	12.5	6.25	12.5	6.25	25	-
Proteus vulgaris(-)	25	12.5	12.5	3.125	6.25	-
Mycobacterium smegmatis(+)	25	25	50	25	12.5	-
Listeria monocytogenes(+)	12.5	12.5	25	6.25	12.5	-
Pseudomonas aeruginose(-)	12.5	6.25	12.5	6.25	6.25	-
Kluyveromyces fragilis	6.25	3.125	6.25	3.125	-	6.25
Rhodotorula rubra	6.25	6.25	6.25	6.25	-	6.25
Candida albicans	3.125	1.56	3.125	1.56	-	3.125
Hanseniaspora guilliermondii	3.125	1.56	3.125	3.125	-	3.125
Debaryomyces hansenii	1.56	1.56	3.125	1.56	-	12.5

GEN, Gentamycin; NYS, Nystatin

DNA binding

UV–Vis spectra

The spectrophotometric analyses of the ligands (L_1-L_4) were shown in Figure 3.14. The UV spectra of the compounds showed two distinct absorbance peaks at 220-230 nm and 250-260 nm. The concentration of the compounds was 1 nmol/µL. Interestingly, although the absorption values were in order with the molecular weight of the ligands, the absorption value for the (L_2) ligand was higher than the others (Fig. 3.14.a).

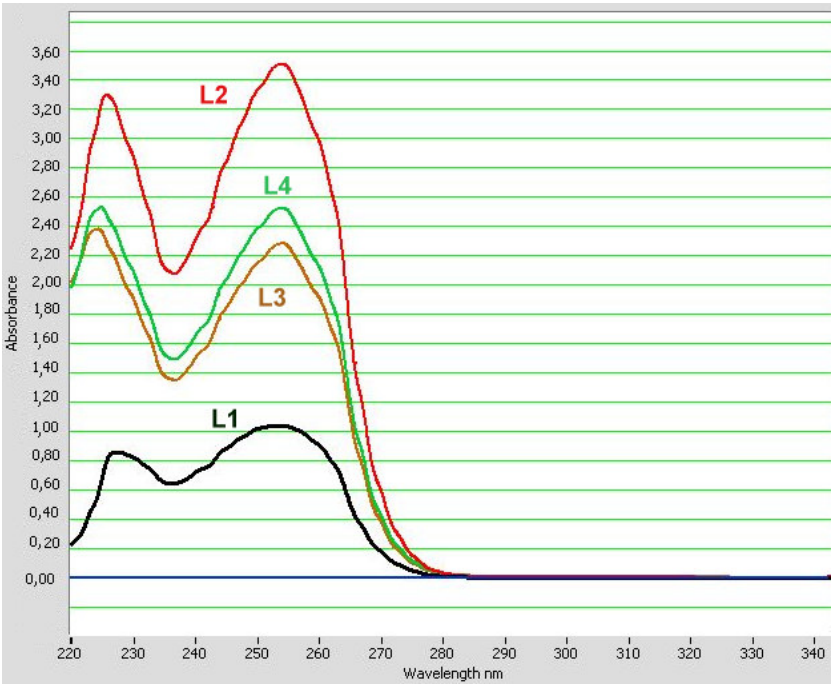


Figure 3.14.a. UV-visible absorption spectrum of (L_1-L_4) compounds.

The change in the absorption spectrum of the ligands upon addition of plasmid DNA was also tested. It was observed that the addition of plasmid DNA to the solution of (L_1) induced remarkable hyperchromism (60%) and bathochromic shift (5 nm, from 228 to 223

nm) (Fig. 3.14.b). Other compounds showed very little hyperchromicities and bathochromic shifts (data not shown).

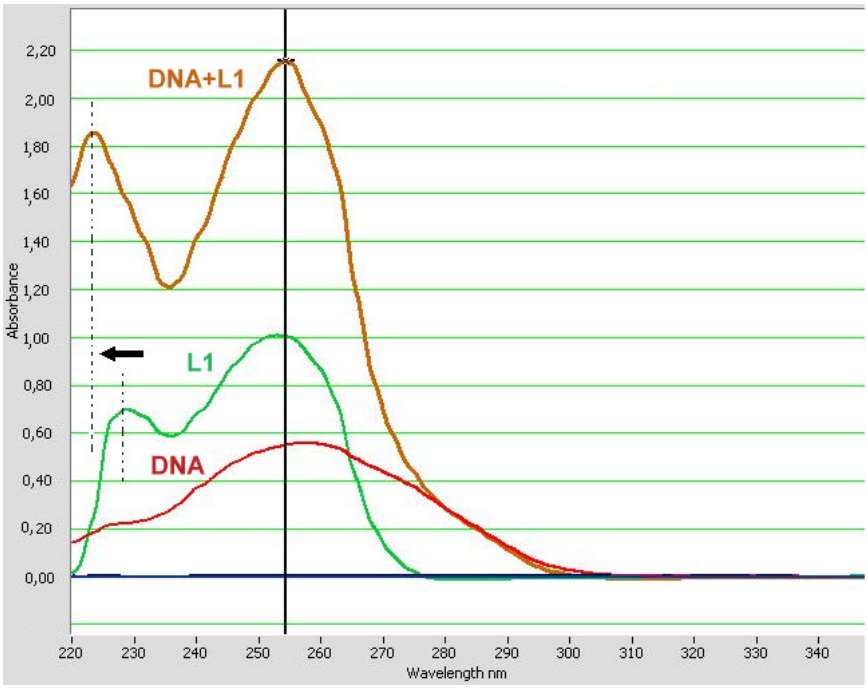


Figure 3.14.b. UV-visible spectrum changes of (L_1) aqueous solution (2.5 mmol/µl) upon addition of pACT plasmid DNA (50 ng/µl). Arrow shows the bathochromic shift.

PAGE analysis

PAGE analysis was performed to determine the effect of compounds on the electrophoretic mobility shift of the DNA. The 450 bp purified PCR products were mixed with different amount of compounds. The incubation with compounds resulted in the retardation of electrophoretic mobility when compared with naked DNA (Fig. 3.15).

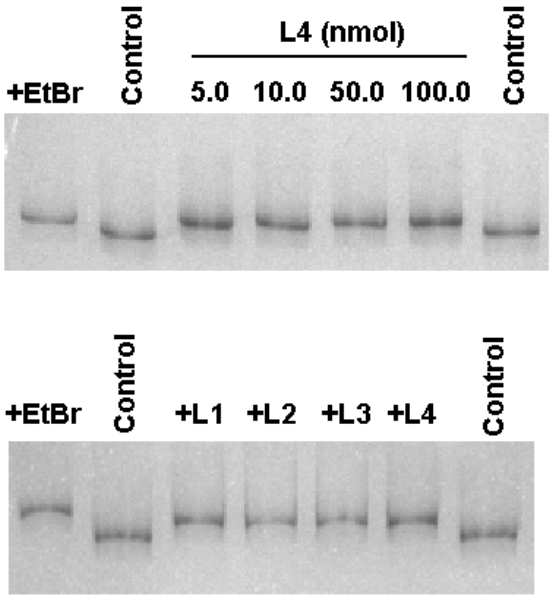


Figure 3.15. Gel electrophoresis lines for (L_1-L_4) treated DNA fragments.

A well known DNA intercalating agent, Ethidium Bromide (EtBr), was used as a control. Each compound resulted in similar retardation in the mobility. Although different concentrations of compounds were tested, the mobility shifts were similar (Fig. 3.15). These results indicated that compounds bind to the DNA fragments and caused mobility shift which is consistent with UV-vis spectrophotometer analysis.

PCR Investigation

The effect of the (L_1-L_4) ligands on the replication was assessed indirectly via its effect on amplification by Taq polymerase-mediated reactions in vitro to see whether compounds can intercalate to DNA and prevent DNA amplification. The amplification of 450 bp long fragment of pACT plasmid was examined with different amount of compounds. The compounds (1 or 5 nmol) were added to the PCR reaction. A concentration dependant

decrease in the intensity of the amplified bands was observed with all compounds when compared with controls (Fig. 3.16.a). Similar results were obtained when the amount of Taq polymerase was increased (Fig. 3.16.b). To eliminate possibility of the compounds that bind Taq polymerase and prevent activity of enzyme and in turn aberrant DNA amplification we have used excess amount Taq polymerase. All these results confirm that the (L_1-L_4) ligands in fact bind DNA selectively and prevent DNA amplification.

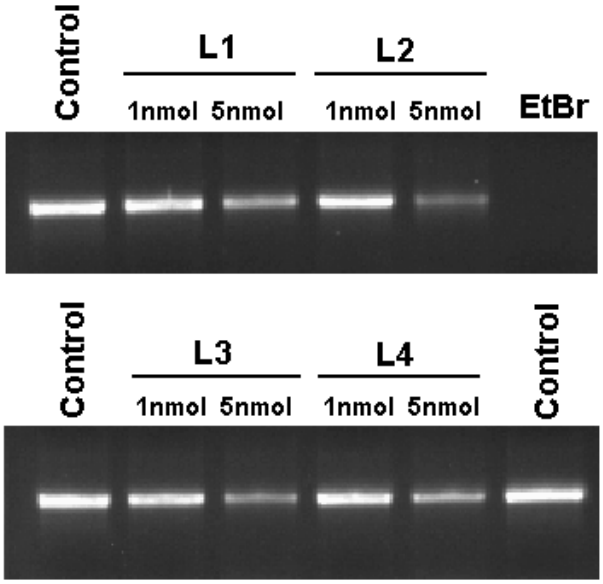


Figure 3.16.a. Agarose gel electrophoresis showing the effects of (L_1-L_4) compounds on the inhibition of Taq polymerase mediated PCR.

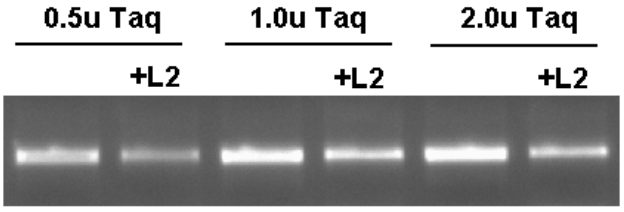


Figure 3.16.b. The effect of the (L_2) ligand on the replication (PCR).

3.3.1.2. Cytotoxicity

The cytotoxic effect of the ligands was tested against human cervical cancer HeLa cells after incubation periods of 24-72 h. The cytotoxicity results are shown in Figure 3.17 and indicate that the compounds showed mild cytotoxic effects with an IC50 ≥2mM after 72 h incubation.

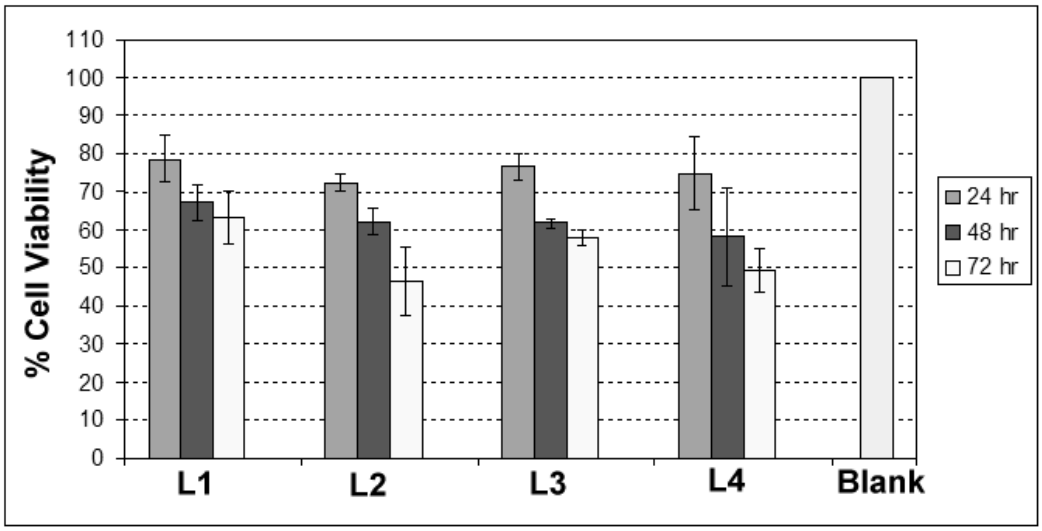


Figure 3.17. The cytotoxic effect of the (L_1-L_4) compounds (2mM) against human cervical cancer HeLa cells after incubation periods of 24-72 h.

In the present study we have tested DNA binding properties of the ligands (L_1), (L_2), (L_3), and (L_4). UV-Vis spectra and PAGE analyses results suggest the DNA binding properties of the macrocycles. The effect of the (L_1-L_4) compounds on the replication was assessed indirectly via its effect on amplification by Taq polymerase-mediated PCR reactions. A concentration dependent inhibition of amplification has been observed. On the other hand, compounds showed mild cytotoxic effect on the human cell line, HeLa. This unexpected result might be due to poor absorption of compounds by cell membrane. For

evaluation of the therapeutic efficacy of a candidate drug, absorption, distribution, metabolism, and excretion (ADME) processes play a pivotal role. Optimizing the chemical structure of candidates with respect to the ADME processes has become an integral part of the drug discovery paradigm. In pharmacokinetics, the distribution coefficient (LogP) has a strong effect on the ADME properties of a drug molecule. Since the drug must pass through lipid bilayers during cellular transportation, for an efficient transport, the compound must be hydrophobic enough to partition in the bilayer membrane [112]. To evaluate drug likeness several methodologies were developed and but in each method, hydrophobicity (LogP) is an important parameter. Based on the Lipinski's Rule of Five, LogP should be less than 5, where as Ghose et al. gave a range from -0.4 to +5.6. Interestingly, compounds (L_1-L_4) have the LogP values ≤ 0.4. This low LogP values might partially explain the mild cytotoxic effect of compounds [113,114].

3.3.2. (L_5-L_8) Ligands

3.3.2.1. Antimicrobial activity

The results concerning in vitro antimicrobial activities of the macrocycles together with the inhibition zone (mm) and (MIC) values of compared antibiotic and antifungal reagents are listed in Tables 3.6 and 3.7. All the compounds tested exhibit strong or moderate antimicrobial activity. Of all the test compounds at tempted, (L_6) and (L_8) showed slightly higher activities against most Gram-positive than Gram-negative bacteria, but all compounds show strong activity on the yeast cultures. The MIC values in Table 3.7 indicate that all the compounds tested exhibit moderate to strong antimicrobial activity on the tested microorganisms.

Once again the data indicate that the (L_6) and (L_8) macrocycles have slightly stronger activity against some Gram-positive bacteria such as *Bacillus cereus* (L_6 = 3.125 and L_8 = 6.25 µg mL^{-1}) and *Micrococcus luteus* (L_6 = 3.125 and L_8 = 6.25 µg mL^{-1}) compared with

Gentamycin on these microorganisms 25 and 6.26 µg mL^{-1}, respectively (Table 3.7). Similarly these two compounds show same activity with Gentamycin against Gram-negative *Klebsiella* bacteria (6.25 µg mL^{-1}). All the tested compounds show very strong activity against the yeast cultures. For instance, all the compounds tested showed superior activity (MIC = 1.56 µg mL^{-1}) against *Hanseniaspora guilliermondii* culture compared with Nystatin antifungal agent. Once more the data indicate that the (L_6) and (L_8) macrocycles have stronger activity against yeast cultures *Kluyveromyces fragilis* (1.56 µg mL^{-1}) and *Debaryomyces hansenii* (1.56 µg mL^{-1}) in comparison with Nystatin antifungal agent, showing MIC values 6.25 and 12.50 µg mL^{-1} on the same micro-organisms, respectively.

Table 3.6. *In vitro* antimicrobial activity of the (L_5-L_8) compounds and the standard reagents (inhibition zone mm).

Microorganisms/Compounds	**(L_5)**	**(L_6)**	**(L_7)**	**(L_8)**	P10	AMP	CTX	VA	OFX	TE	NY	KET	CLT
Escherichia coli(-)	10.0	10.0	10.0	10.0	18	12	10	22	30	28	-	-	-
Staphylococcus aureus(+)	12.0	14.0	10.0	12.0	13	16	12	13	24	26	-	-	-
Klebsiella pneumoniae(-)	13.0	15.0	13.0	16.0	18	14	13	22	28	30	-	-	-
Bacillus cereus(+)	14.0	17.0	13.0	15.0	8	10	54	10	44	34	-	-	-
Micrococcus luteus(+)	14.0	18.0	12.0	17.0	10	16	18	20	28	26	-	-	-
Proteus vulgaris(-)	12.0	11.0	11.0	13.0	14	12	14	18	30	25	-	-	-
Mycobacterium smegmatis(+)	10.0	10.0	9.0	11.0	15	21	11	20	32	24	-	-	-
Listeria monocytogenes(+)	10.0	10.0	10.0	10.0	10	12	16	26	30	28	-	-	-
Pseudomonas aeruginose(-)	9.0	11.0	11.0	11.0	36	32	32	34	28	22	-	-	-
Kluyveromyces fragilis	15.0	19.0	16.0	21.0	-	-	-	-	-	-	20	21	15
Rhodotorula rubra	17.0	18.0	16.0	21.0	-	-	-	-	-	-	18	16	18
Candida albicans	16.0	19.0	19.0	19.0	-	-	-	-	-	-	18	22	16
Hanseniaspora guilliermondii	20.0	20.0	19.0	22.0	-	-	-	-	-	-	21	24	22
Debaryomyces hansenii	18.0	21.0	18.0	22.0	-	-	-	-	-	-	16	14	18

P10, Penicillin G (10 Units); AMP, Ampicillin 10 µg; CTX, Cefotaxime 30 µg; VA, Vancomycin 30 µg; OFX , Ofloxacin 5 µg; TE, Tetracycline 30µg; NY, Nystatin 100 µg; KET, Ketaconazole 20 µg; CLT, Clotrimazole 10 µg

Table 3.7. *In vitro* antimicrobial activity (MIC, μg mL^{-1}) of the (L_5-L_8) compounds

Microorganisms/Compounds	**(L_5)**	**(L_6)**	**(L_7)**	**(L_8)**	GEN	NYS
Escherichia coli(-)	25	25	25	25	6.25	-
Staphylococcus aureus(+)	12.5	12.5	25	12.5	25	-
Klebsiella pneumoniae(-)	12.5	6.25	12.5	6.25	6.25	-
Bacillus cereus(+)	6.25	3.125	12.5	6.25	6.25	-
Micrococcus luteus(+)	12.5	3.125	12.5	6.25	25	-
Proteus vulgaris(-)	12.5	25	25	12.5	6.25	-
Mycobacterium smegmatis(+)	25	25	50	25	12,5	-
Listeria monocytogenes(+)	25	25	25	25	12,5	-
Pseudomonas aeruginose(-)	50	25	25	25	6.25	-
Kluyveromyces fragilis	6.25	1.56	6.25	1.56	-	6.25
Rhodotorula rubra	6.25	3125	6.25	3.125	-	6.25
Candida albicans	6.25	3.125	3.125	3.125	-	3.125
Hanseniaspora guilliermondii	1.56	1.56	1.56	1.56	-	3.125
Debaryomyces hansenii	3.125	1.56	3125	1.56	-	12.5

GEN, Gentamycin; NYS, Nystatin.

The inhibition activity of the compounds seems to be governed in certain degree by the percentage amount of the sulphur presence in the compounds, because the dithio (L_6) and (L_8) macrocycles seems indicate more activity against the most tested micro-organisms, compared to the monothio macrocycles. Furthermore, in classifying the antibacterial activity as Gram-positive or Gram-negative, it would generally be expected that a much greater number would be active against Gram-positive than Gram-negative bacteria. This conception seems partially true, but in this study, the compounds are relatively active against on both types of the bacteria and as well as strongly active against yeasts, which may indicate a broad-spectrum affect. The results of our study indicate that

the compounds have the potential to generate novel antimicrobial properties by displaying moderate to high affinities for most of the receptors particularly against yeast cultures.

3.3.2.2. Cytotoxicity

The pattern of cytotoxic activity of the ligands is similar to each other and indicates that none of the ligand show significant cytotoxic effect on the tested cell lines. Cytotoxicity ranges from 0-14% for both of the cell lines, indicating a high percentage of the cell lines capability to survive (86-100%). The cytotoxic effects on both cell lines seem not dependence on the concentration and time. There was a fluctuation in the survival of the cells lines (86-100%) in a dose-independent manner (Fig. 3.18). In certain cases the survival rates seems increased suggesting an attempt of the cell to develop a defense mechanism against the ligands. In overall, these compounds seem to have a very low toxic effect against tested cell lines.

3.3.2.3. Detection of apoptosis

To analyze the effects of the compounds on DNA of the cell lines, we performed nuclear Hoechst staining and DNA fragmentation assays that give clue about the apoptotic cell death. Following Hoechst staining under inverted fluorescence microscope, the nucleus of apoptotic cells can be differentiated from the nucleus of non-apoptotic cells due to DNA damage. We observed that the control and the treated cells had the same nuclear appearance following Hoechst staining (Figs. 3.19, 3.20).

DNA fragmentation which is another indication for apoptosis was tested following treatment of the cell lines with compounds. DNA of the cell lines was intact suggesting that these ligands do not induce DNA fragmentation. The experiment did not indicate any apoptotic cell death and therefore no further experiments were performed (Fig. 3.21).

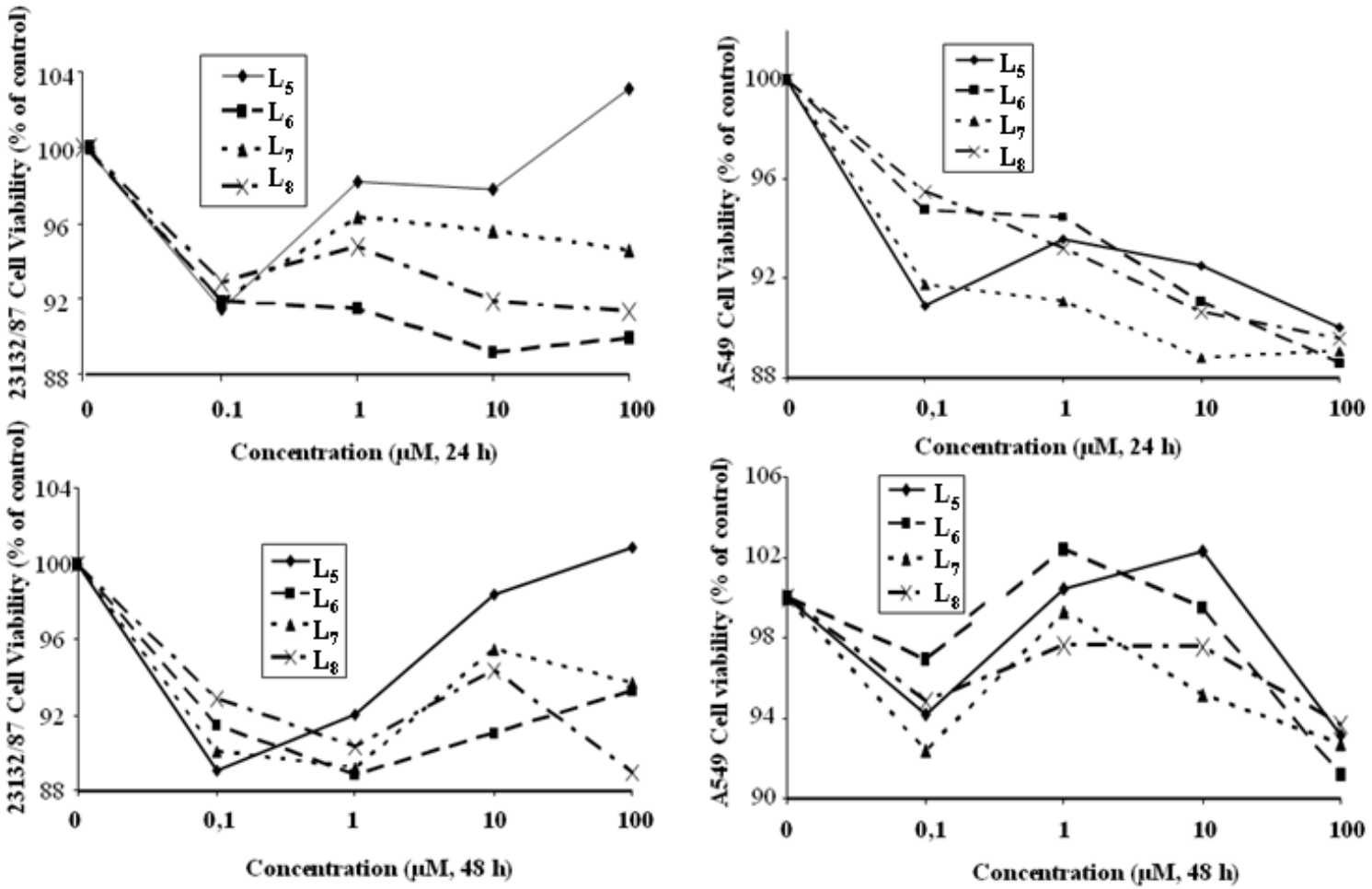


Figure 3.18. Effect of (L_5-L_8) compounds on the viability of 23132/87 and A549 cell lines (0–100 μM, 24, 48 h).

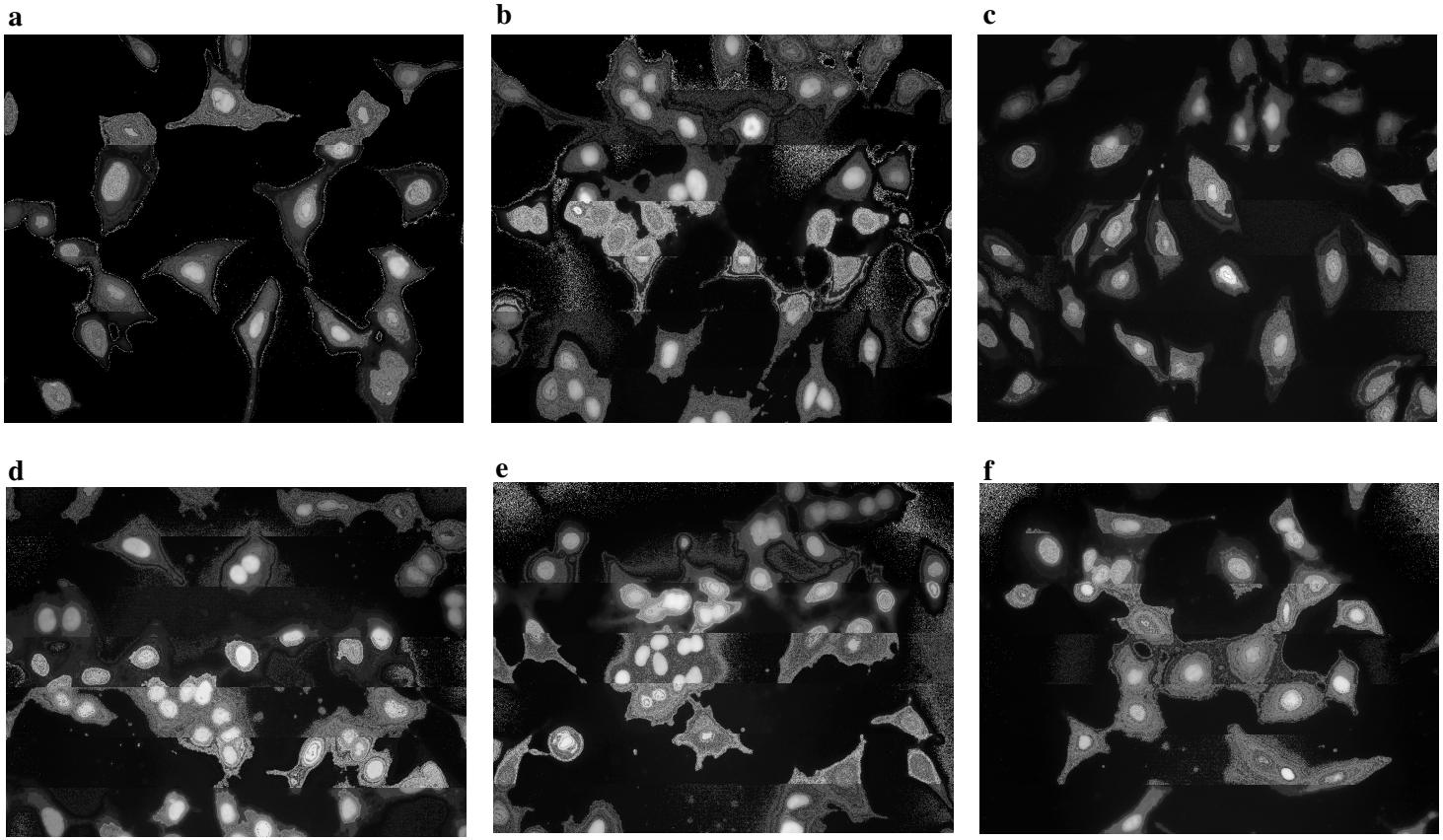


Figure 3.19. Hoechst nuclear staining of A549 cells with (100 μM, 24 h) a, untreated; (b-e) with (L_5-L_8), respectively; f, treated DMSO.

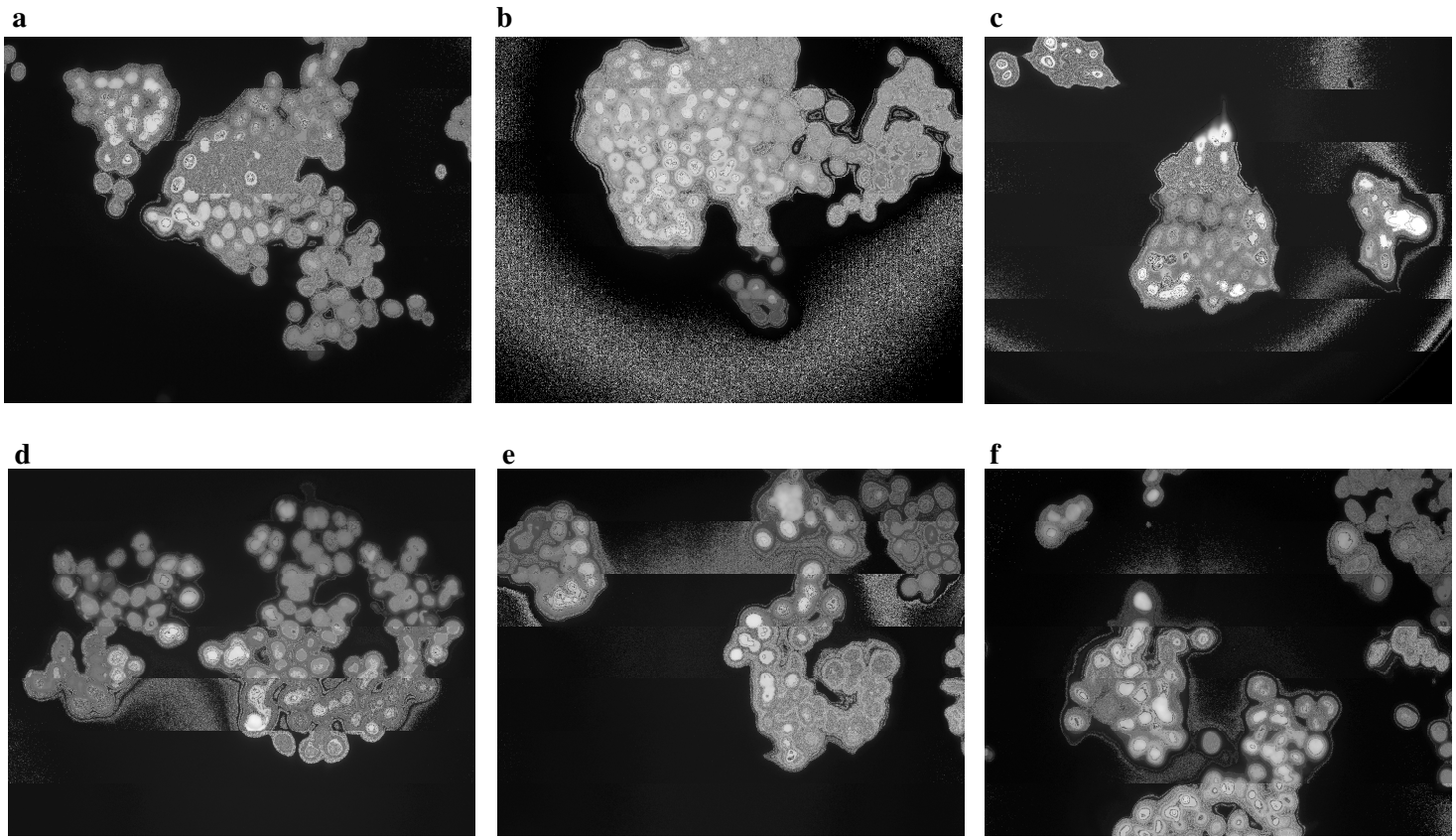


Figure 3.20. Hoechst nuclear staining of 23132/87 cells with (100 μM, 24 h) a, untreated; (b-e) with (L_5-L_8), respectively; f, treated DMSO.

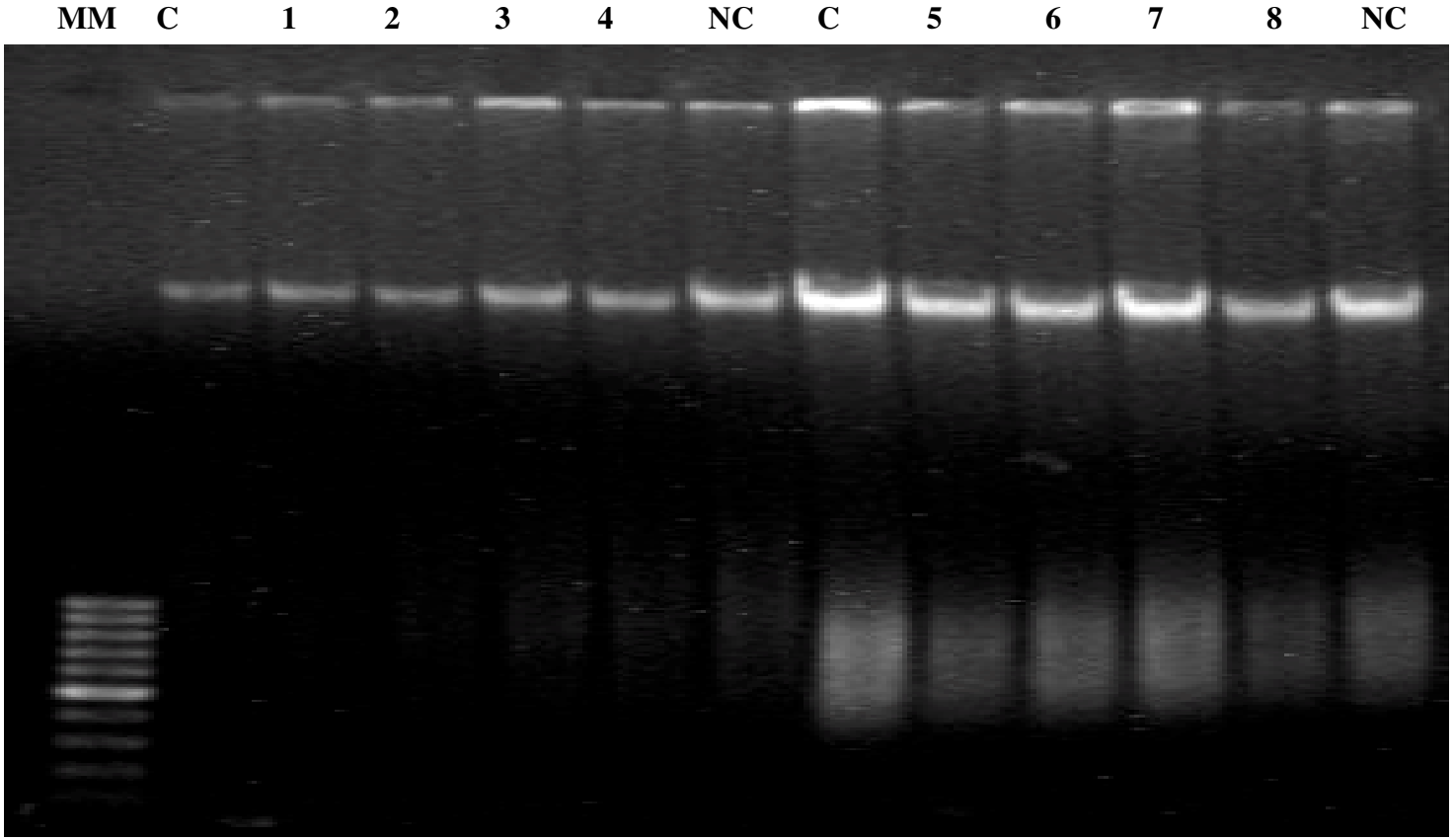


Figure 3.21. Gel electrophoresis Lines (1-4) 23132/87 cells treated (L_5-L_8) (100 µM, 24 h); Lines (5-8) A549 cells treated (L_5-L_8) (100 µM, 24 h); MM, 100 bp molecular marker; NC, DNA extracted from cells treated with DMSO (24 h); C, untreated cells.

3.3.2.4. DNA binding

Electrostatic or direct hydrogen bonding of the macrocyclic ligands with DNA is expected to bring about marked changes in its electronic spectrum. However, no marked changes have been observed in the electronic spectrum of the DNA with the addition of the ligands, ruling out the possibility of their direct binding to DNA. With the addition of ligands, the DNA systems exhibit only small changes in their electronic spectrum. Such a small change may arise with groove binding, leading to small perturbations. This hyperchromism in the absorption intensity may probably be due to the dissociation of ligand aggregates [115] or due to its external contact (surface binding) with the ligands [116]. Rather small changes in UV-vis spectra didn't allow calculation of binding constants (log Ks) for any of the ligands.

3.3.3. Pd(II) complexes

3.3.3.1. Antimicrobial activity

The results concerning in vitro antimicrobial activities are presented in Tables 3.8 and 3.9. The compounds exhibit moderate to strong antimicrobial activity on the most tested organisms. The palladium complex showed the highest activities against most bacteria as well as against yeast cultures. Apart from few exceptions particularly $[Pd(L_9)]I_2$, the MIC values in Table 3.8 indicate that all the compounds exhibit moderate antimicrobial activity on the tested microorganisms. But the $[Pd(L_9)]I_2$ showed high activity against *Staphylococcus aureus* (3.125 μg mL^{-1}), *Klebsiella pneumoniae* (3.μg mL^{-1}), and M. smegmatis (3.125 μg mL^{-1}) organisms than did the reference Gentamycin with values 25, 6.25 μg mL^{-1} , respectively, on the same organisms. The complexes show a superior antifungal activity against C. albicans (1.56 μg mL^{-1}) and *Rhodotorula rubra* (1.56 μg mL^{-1}) than occurred with the reference nystatin (Table 3.8).

Table 3.8. *In vitro* antimicrobial activity (MIC, µg mL^{-1}) of the complexes.

Microorganisms/Compounds	**L_9**	**$[Pd(L_9)]Cl_2$**	**$[Pd(L_9)]Br_2$**	**$[Pd(L_9)]I_2$**	GEN	NYS
Escherichia coli(-)	25	6.25	12.5	12.5	6.25	-
Staphylococcus aureus(+)	12.5	12.5	12.5	3.125	25	-
Klebsiella pneumoniae(-)	12.5	6.25	6.25	3.125	6.25	-
Bacillus cereus(+)	12.5	12.5	12.5	12.5	6.25	-
Micrococcus luteus(+)	25	12.5	12.5	25	25	-
Proteus vulgaris(-)	12.5	12.5	12.5	6.25	6.25	-
Mycobacterium smegmatis(+)	12.5	12.5	12.5	25	12.5	-
Listeria monocytogenes(+)	25	25	25	25	12.5	-
Pseudomonas aeruginosa(-)	12.5	12.5	12.5	12.5	6.25	-
Kluyveromyces fragilis	12.5	6.25	3.125	6.25	-	6.25
Rhodotorula rubra	6.25	6.25	1.56	3.125	-	6.25
Candida albicans	6.25	3.125	1.56	6.25	-	3.125
Hanseniaspora guilliermondii	12.5	3.125	6.25	6.25	-	3.125
Debaryomyces hansenii	12.5	6.25	6.25	6.25	-	12.5

GEN, Gentamycin; NYS, Nystatin.

Table 3.9. *In vitro* antimicrobial activity of the complexes and the standard reagents (inhibition zone mm).

Microorganisms/Comp.	**L_9**	**$[Pd(L_9)]Cl_2$**	**$[Pd(L_9)]Br_2$**	**$[Pd(L_9)]I_2$**	P10	AMP	CTX	VA	OFX	TE	NY	KETO	CLT
Escherichia coli(-)	11	16	15	14	18	12	10	22	30	28	-	-	-
Staphylococcus aureus(+)	14	15	15	21	13	16	12	13	24	26	-	-	-
Klebsiella pneumoniae(-)	14	17	18	20	18	14	13	22	28	30	-	-	-
Pseudomonas aeruginosa(-)	12	13	14	14	8	10	54	10	44	34	-	-	-
Proteus vulgaris(-)	13	15	15	18	10	16	18	20	28	26	-	-	-
Bacillus cereus(+)	12	14	12	13	14	12	14	18	30	25	-	-	-
Mycobacterium smegmatis(+)	12	12	14	11	15	21	11	20	32	24	-	-	-
Listeria monocytogenes(+)	10	11	11	9	10	12	16	26	30	28	-	-	-
Micrococcus luteus(+)	11	12	12	10	36	32	32	34	28	22	-	-	-
Candida albicans	16	18	22	16	-	-	-	-	-	-	20	21	15
Kluyveromyces fragilis	13	16	20	15	-	-	-	-	-	-	18	16	18
Rhodotorula rubra	15	17	21	19	-	-	-	-	-	-	18	22	16
H. guilliermondii	14	18	18	16	-	-	-	-	-	-	21	24	22
Debaryomyces hansenii	14	15	17	15	-	-	-	-	-	-	16	14	18

P10, Penicillin G (10 Units); AMP, Ampicillin 10 µg; CTX, Cefotaxime 30 µg; VA, Vancomycin 30 µg; OFX, Oflaxacin 5 µg; TE, Tetracyclin 30 µg; NY, Nystatin 100 µg; KETO, Ketaconazole 20 µg; CLT, Clotrimazole 10 µg.

The inhibition zone value of the palladium complexes also indicates moderate activity towards most tested organisms. The cellular target of metallic complexes is generally considered to be DNA, although the reaction mechanism, which causes the cell death are not very clear. The strong microbial activity of the palladium complexes in our case, particularly compared with the palladium chloride salt and the free ligand, may indicate that the inhibition activity of the organisms could be governed to a certain degree by the facility of binding at the metal center of the complex. The reference tetracycline type antibiotics are also thought to exert their antimicrobial effect by the inhibition of protein synthesis through binding to DNA and ofloxacin works by entering the bacterial cell and inhibiting DNA-gyrase. The results of our study indicate that the palladium complex have the potential to generate novel metabolites, by displaying moderate to high affinities for most of the receptors. The aforementioned compound could be evaluated as potential drug against certain infections by being subjected to further pharmacological tests.

Chapter 4

CONCLUSIONS

In this thesis; the facile synthesis and characterization of two series of novel macrocyclic ligands of varying ring size via condensation of diamines and various dicarboxylic acids in 1:1 molar ratios, and palladium halide compexes of 1,2-Di(o-aminophenylthio)ethane (L_9) ligand were described.

These macrocyclic ligands have been fully characterized using FT-IR, Raman, ^{1}H-NMR, ^{13}C-NMR and elemental analysis. The spectroscopic techniques with this observation provides a deeper look on the stability and characterizibility of the compounds with donor atoms in a cyclic environment. Raman spectroscopy was particularly important for the characterization of –S–S– and –C–S– bands, which is not active or very weak in the infrared spectrum. The strong peaks in the Raman spectra at 505 and 508 cm^{-1} clearly show characteristic ν(S–S) stretching modes for (L_6) and (L_8) macrocycles, respectively (Figure 3.5).

To contribute to the field of bioinorganic and organic chemistry, the synthesized compounds have been evaluated for their antibacterial and antifungal actions. Both antimicrobial and antifungal tests were carried out by using disc diffusion and minimum inhibition concentration methods. All the macrocyclic ligands and complexes in this thesis have strong activity against yeast cultures. The MIC values in Tables 3.5, 3.7 and 3.8 indicate that all the compounds tested exhibit moderate to strong antimicrobial activity on the tested microorganisms. The data indicate that (L_2), (L_4), (L_6) and (L_8) macrocycles have slightly stronger activity against most Gram-positive and Gram-negative bacteria compared

with (L_1), (L_3), (L_5) and (L_7) ligands. The inhibition activity seems to be governed in certain degree by the percentage amount of the sulfur presence in these compounds. Also all the macrocycles are active against both types of the bacteria and as well as active against yeast, which may indicate a broad-spectrum effect.

Moreover, DNA-binding properties were examined with diverse biochemical techniques, such as absorption spectra and PAGE analysis. The study of the interaction of the compounds with DNA has revealed that the ligands can be bound to DNA, but the exact mode of binding cannot be safely concluded. The effect of the compounds on the replication was assessed indirectly via its effect on amplification by Taq polymerase-mediated PCR reactions and a concentration dependent inhibition of amplification was observed. Cell viability measurements were done to assess healthy cells in the ligand samples. The macrocycles showed mild cytotoxic effect on the human cell line, HeLa. An increase in cell viability indicates cell growth, while a decrease in viability can be interpreted as the result of either toxic effects of the compounds.

The palladium complexes of (L_9) ligand exhibit moderate to strong antimicrobial activity on the most tested organisms. The minimum inhibitory concentration values in Table 3.8 indicate that all the complexes exhibit moderate antimicrobial activity on the tested microorganisms. However, $[Pd(L_9)]I_2$ complex possesses a higher antimicrobial activity against Staphylococcus aureus, Klebsiella pneumoniae, and M. smegmatis organisms than did the commercial antibiotic Gentamycin on the same organisms. The complexes show a better antifungal activity than Nystatin against C. albicans and Rhodotorula rubra (Table 3.8).

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