ANALYSIS OF SMALL MOLECULE INTERACTION WITH DNA

A DISSERTATION

Submitted in partial fulfillment of the requirements for the award of the degree

of

MASTER OF TECHNOLOGY

in

ADVANCED CHEMICAL ANALYSIS

By

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JUNE, 2010

TO

My parent and friends

Certificate

This is to certify that the thesis entitled "ANALYSIS OF SMALL MOLECULE INTERACTION WITH DNA" submitted by Hemant Kumar who got his name registered on August 2008 for the award of M.Tech. degree in Advanced Chemical Analysis of Indian Institute of Technology Roorkee, is absolutely based upon his own work under my supervision and that neither this thesis nor any part of it has been submitted for any degree/diploma or any other academic award anywhere before.

Date: 29 June 2010

Kaushik Ghosh

Dr. Kaushik Ghosh

Signature of supervisor

with official seal

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Preface

The work embodied in this thesis entitled "Analysis of small molecule interaction with DNA" was initiated August, 2009.

The thesis consists of a preamble of the present work and two chapters. DNA interaction studies on mononuclear complexes of iron and manganese derived from planar tetradentate ligand is described in chapter 1. In chapter 2, synthesis and characterization of new mononuclear Fe(II) complex and DNA interaction studies will be described.

All the ligands and complexes described in this thesis were characterized by elemental analysis followed by spectral analysis.

In keeping with general practice of reporting scientific observations, due acknowledgement has been made of the findings of other investigators. I must take the responsibility of any unintentional oversights and error which might have crept in.

ACKNOWLEDGEMENT

In the first place, I would like to express my deep and sincere gratitude to my supervisors, Dr. Kaushik Ghosh and their supervision, advice and guidance from the very early stage of this project as well as giving me extraordinary experiences throughout the work. Above all and the most needed, they provided me unflinching encouragement and support in various ways. Their scientific intuitions have made them as a constant oasis of ideas and passions in science, which inspired and enriched my growth as a student. I am indebted to them for their valuable guidance.

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I wish to thank to all my family members and friends for giving me close association as well as constant inspiration.

Financial support of the Ministry of Human Resources, Government of India is gratefully acknowledged. Lastly I am thankful to my institute (IIT Roorkee) for all the infrastructural facilities.

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Abstract

In this chapter the preamble of the work and literature review will be described. The chemical systems reported in the present study are briefly introduced. The various chemical methods and equipments used are summarized.

1. Introduction and literature review:

Cancer is generally reckoned by common people as one of the scariest diseases, but it is not a single ailment. Cancer is defined medically as a group of more than 100 life-threatening diseases which is caused by out-of-control progressive cellular growth.[1] Cancer can occur in any part of our body. The major treatment consists of radiation, surgery, chemotherapy or combination of all these three therapies.[2] The first book on chemotherapy appeared in the year of 1909, written by Nobel-prize winner Paul Ehrlich. The saga of chemotherapy started with usage of herbal extracts. The modern era of chemotherapy begins with the approval of alkylating agent, cyclophosphamide in 1959. The first metallodrug, cisplatin, is introduced in medical practice in 1978.[3,4]

1.1. Metallodrug based on platinum:

Platinum compounds in cancer chemotherapy deserve a special attention as three metallo drugs in medicinal practice are platinum drugs. Cisplatin, cis-$[PtCl_2(NH_3)_2]$ was first synthesized in 1844 by Peyrone in Turin and named as Peyrone's chloride.[5] The biological activity was discovered by serendipity in 1965 by physicist turned biophysicist Barnett Rosenberg and coworkers during investigations of the effect of electric fields on the growth of *Escherichia coli*. They were using platinum electrodes in a solution containing chloride and ammonium salts among other constituents, when they observed an unexpected event. The bacteria become long filaments, and did not replicate. Cell division in the *Escherichia coli* was thus found to be inhibited. Further extensive analysis of this unpredicted observation led them to the conclusion that the bacterial replicative cycle was inhibited by platinum ammine complexes formed by electrolysis at the platinum electrodes and to the subsequent use of cis-

$[PtCl_2(NH_3)_2]$ as an anticancer drug. Today, over 30 years after its approval as a chemotherapeutic agent by the North American Food and Drug Administration in the USA (FDA), cisplatin is still one of the world's best selling anticancer drugs, cis-platin is marketed under the names *cisplatinol*® and *platinosin*®. It is responsible for the cure of over 90% of cases of testicular cancer and it plays an important role in some cancer treatments such as ovarian, head and neck cancer, bladder cancer, cervical cancer, melanoma, and lymphomas.[6,7]

Inspired by this unexpected success thousands of platinum (similar as parent cisplatin) compounds have been synthesized and tested for antitumor efficacy. Till to date relatively few completed the clinical trials[8,9] and six of them are currently approved. The structures of these drugs are shown in Fig.1.1

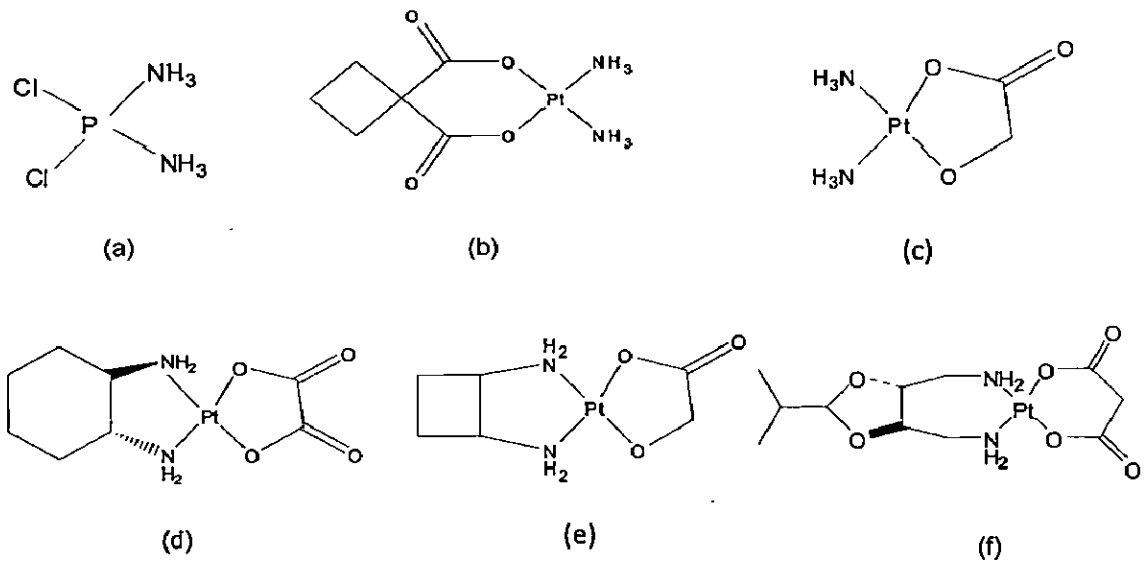


Fig. 1.1 Clinically approved platinum antitumor drugs (a) Cisplatin (b) Carboplatin (c) Nedaplatin (d) Oxaliplatin (e) Lobaplatin (f) Heptaplatin.

There are some major drawbacks in the use of cisplatin for cancer-chemotherapy. The poor solubility in saline, developments of resistance by the tumour cells and severe side-effects are the limitations. The resistance against drug may be intrinsic or acquired. Severe side effects include failure of the kidney and bone marrow (nephrotoxicity and haematological toxicity),

nausea, intractable vomiting (emesis), peripheral neuropathy, deafness and seizures[10] and myelotoxicity. In addition cisplatin is to be administered intravenously which is inconvenient to outpatient treatment.

The second generation of platinum drug from this series is carboplatin (it contains the chelating dicarboxylate cbdca: cyclobutanedicarboxylic acid), which exhibits improved therapeutic index and ameliorates some severe side effects.[11]

The third generation of platinum drug includes oxaliplatin, which also overcomes cisplatin resistance and is specific for common cancer (means testicular and ovarian because they comprise higher percentage of cancer cases). Oxaliplatin is approved for clinical use (Eloxatin™) in Europe and China for colorectal cancer[13] and it is also in medical practice in USA since 2005.

Unfortunately, none of the successors of cisplatin could be considered superior to the prototype in terms of both lower toxicity and clinical efficacy and, thus, the pursuit for novel platinum compounds with optimized pharmacological profile remains an unsolved problem.

1.2 DNA Interaction with first row transition metal:

Very few studies were undertaken to coordination with the first row transition metal ions, and much less attention was focused on the antitumor activity of these metal complexes. Among the first row transition metal ions, manganese, iron, cobalt, nickel, copper and zinc ions have come to be recognized as having important biological effects and lesser toxic. The study of the antitumor activity and DNA binding property of these metal complexes has much of our interest.[12,13]

Transition metal complexes of Fe, Cu, Zn, Mn, Ni, Co, have been reported to mediate DNA oxidation in the presence of oxidants or reductants or without any assistant agents.[14-17] These complexes attack the sugar or base moieties of DNA.[18,19] Several well known and best characterized nucleolytic agents are: $[Fe(EDTA)]^{2-}$, (EDTA=ethylene diaminetetraaceticacid),[20] $[Cu(OP)_2]^{+}$, (OP=1,10-phenanthroline),[21] Fe-BLM (BLM=bleomycin),[22] metalloporphyrins,[23] Ni-peptides,[24] and metal-salen [salen=N,N'-ethylenebis (salicylideneaminato)].[25]

1.2.1 Bleomycin and metallobleomycin:

Cu(II).BLMs (Fig. 2) and metal-free BLMs can inhibit the growth of tumor cells in tissue culture[26] and tumor growth in animals.[27,28] These results contrast dramatically with in vitro studies with Cu(II).BLM where numerous laboratories have shown its inability to degrade DNA, the molecular mechanism thought to be responsible for its cytotoxicity in vivo.[29,30] To account for this apparent inconsistency between the in vivo and in vitro data, two hypotheses were put forth (Scheme 1.1) by Umezawa and colleagues[30] and Hecht and colleagues.[31] Both models agree that free BLM rapidly forms Cu(II).BLM in the plasma and this is the species that is probably transported into the nucleus of the cell.

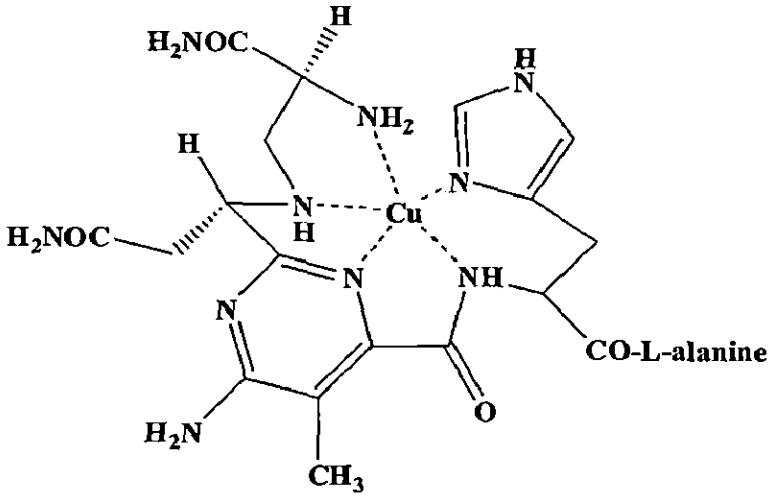
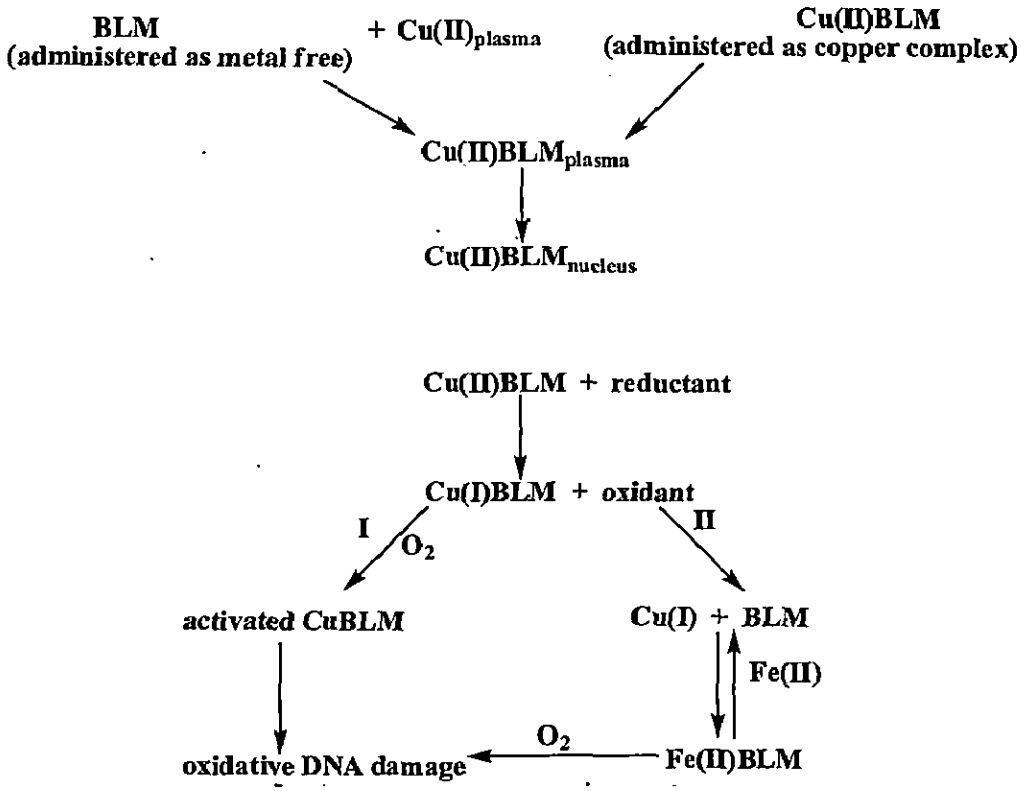


Fig. 1.2 Structure of Cu.BLM



Scheme 1.1. DNA damage studies mechanism in vivo by metallobleomycin.

The Umezawa model[30] requires that Cu(I).BLM [K_a ~4 times K_a of Fe(II).BLM] combine with "Fe(II)" in the nucleus to produce Fe(II).BLM, which is ultimately responsible for the oxidative DNA damage. While studies by both Freedman et al.[32] and Antholine et al.[26] indicate that this is a viable model in vitro, the extrapolation to in vivo conditions still warrants concern for the following reasons. First, the concentration of BLM and therefore Cu(II).BLM in the nucleus is unknown but is quite low, and therefore Cu(II).BLM should be entirely bound to the DNA. Second, the identity and concentration of the physiological reductant(s) are unknown and reduction of a Cu(II).BLM (already sterically hindered) bound to DNA would be even more difficult. Third, the concentration and source of Fe(II) in the nucleus is unknown. Future research will address these experimentally difficult questions, but at present the role of "Cu vs. Fe" BLMs as mediators of in vivo damage remains unanswered.

The coordination chemistry of Fe(II) and Fe(III).BLMs has been intensely investigated since workers in the field realized that Fe(II) was essential for BLM-mediated DNA degradation in vitro. However, the nature of these ligands[33] and their arrangement in the Fe complexes still remain controversial. (The binding of the secondary amine, pyrimidine, and imidazole is not disputed. The ligation of the P-hydroxyhistidine amide and the carbamoyl group of mannose and their role in the coordination chemistry are controversial).

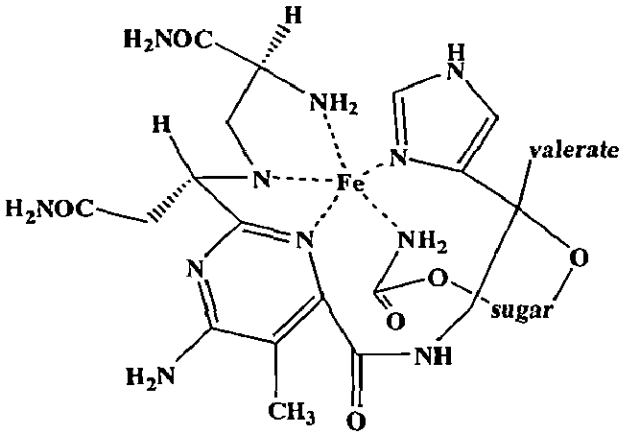
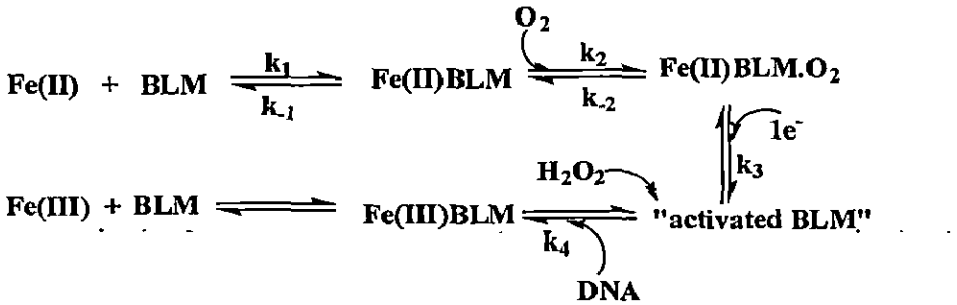


Fig. 1.3 Structure of Fe.BLM

The elegant studies of Sausville et al.[33,34] elucidated the essential requirements for the cofactors Fe(II) and O_2 in BLM-mediated degradation of DNA in vitro. These studies also showed that formation of a 1: 1 Fe(II).BLM complex was required for the production of free base and malondialdehyde like material in the presence of DNA. Haidle et al.[35] and Kuo and Haidle[36] had previously demonstrated production of free nucleic acid bases and aldehydic materials resulting from incubation of BLM with DNA. However, the work of Sausville et al.[33,34] demonstrated that Haidle's observed products, which required the presence of organic reducing agents present in their reaction mixtures. The reductants, therefore, were required to convert Fe(III) to Fe(II), the cofactor for the BLM reaction in vitro.



Scheme 1.2. DNA damage mechanism by Fe.BLM in vivo.

The established requirements for Fe(II) and O_2 triggered a large number of investigations in an attempts to unravel the chemistry of the Fe(II).O_2.BLM species (scheme 1.2) responsible for production of the products observed upon interaction of the complex with DNA or with small molecules. DNA degradation does result from oxidation of iron(II).bleomycin.DNA, the oxidized iron(III).bleomycin complex can dissociate from DNA, be reduced to iron(II)-bleomycin, bind to DNA, and again oxidize, results releasing DNA bases by successive oxidation-reduction cycles.[37]

In addition to iron and copper BLM is also capable of forming an active DNA-degrading complex with Mn(II) and Mn(III). However, the cofactors required for this activity are at present controversial.

The Hecht group[38] was the first to report studies with Mn(II).BLM and found that its incubation with DNA resulted in strand scission, producing relaxed circular and linear duplex DNA. No scission was observed under anaerobic conditions, and no detectable malondialdehyde was produced. Mn(II).BLM appeared to be about 10-fold less active than Fe(II).BLM under identical experimental conditions.

In addition, the Hecht group also examined the chemistry of Mn(III).BLM.B_2 in the presence of iodosobenzene by using the same approach as with Fe(III).BLM activation. An analysis of the reaction of Mn(III).BLM and C_6H_5IO with a number of olefins such as cis-stilbene afforded a profile of oxidized products similar to that obtained for Fe(III).BLM or (tetraphenylporphinato) manganese(III) chloride and iodosobenzene. The Mn(III).BLM system produced single and double-stranded nicks in DNA.

Shortly after the Hecht[38] Burger et al.[39] reported that Mn(II).BLM required H_2O_2 to degrade DNA, a clear contradiction of the Hecht findings. They found that malondialdehyde was formed and verified by TLC that thymine and thymine*propenal* were formed in a 5 to 1 ratio, a considerably higher ratio than that found for Fe.BLM (~1 to 1) in air. Mn(II).BLM was determined to have 1-3% of the activity of Fe-(III).BLM with H_2O_2. Furthermore, they found that aerobic solutions of 2-mercaptoethanol were inactive, and the addition of thiols rapidly terminated the $Mn^{II}.H_2O_2$ mediated degradation. Finally, Mn(II).BLM was also investigated by Suzuki et al.[40] who reported that the complex could cause DNA damage, as determined by sequencing gel analysis, in the presence of O_2 and a reductant (2-mercaptoethanol), or with H_2O_2 alone, or by irradiation with UV light. Their three induction systems afforded very similar nucleotide sequence cleavage patterns.

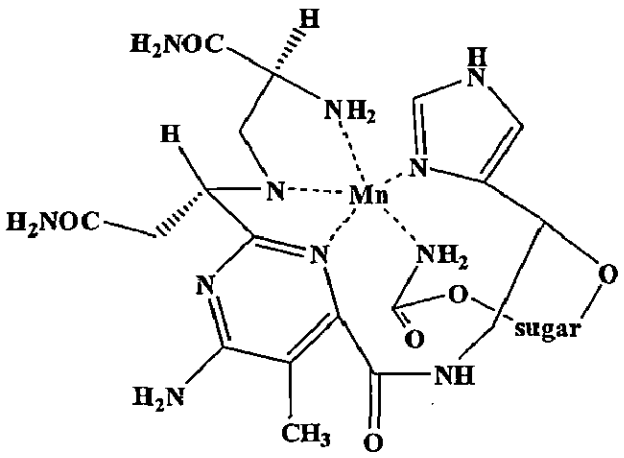


Fig. 1.4 Structure of Mn.BLM.

1.2.2 *DNA damage by $[Cu(OP)_2]^+$ complex:*

In 1979 Sigman discovered that copper ion complex to 1,10-phenothroline $[Cu(OP)_2]^+$ (Fig. 1.5)cleaves DNA.[21] Over the past two decades, copper-phenothroline complexes and their

respective conjugates have been used to footprint DNA binding molecules, map macromolecular interactions, and prepare sequence specific cleaving molecules.[41] $[Cu(OP)_2]^+$ exhibits a strong and under some conditions exclusive, preference for oxidative cleavage of B-DNA over single stranded biopolymers.[42] The general mechanism (Scheme 1.3) by which copper-phenothroline complexes damage DNA is complicated, and numbers of issues remain unresolved.

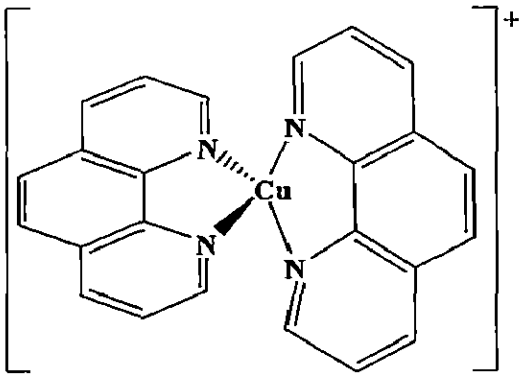
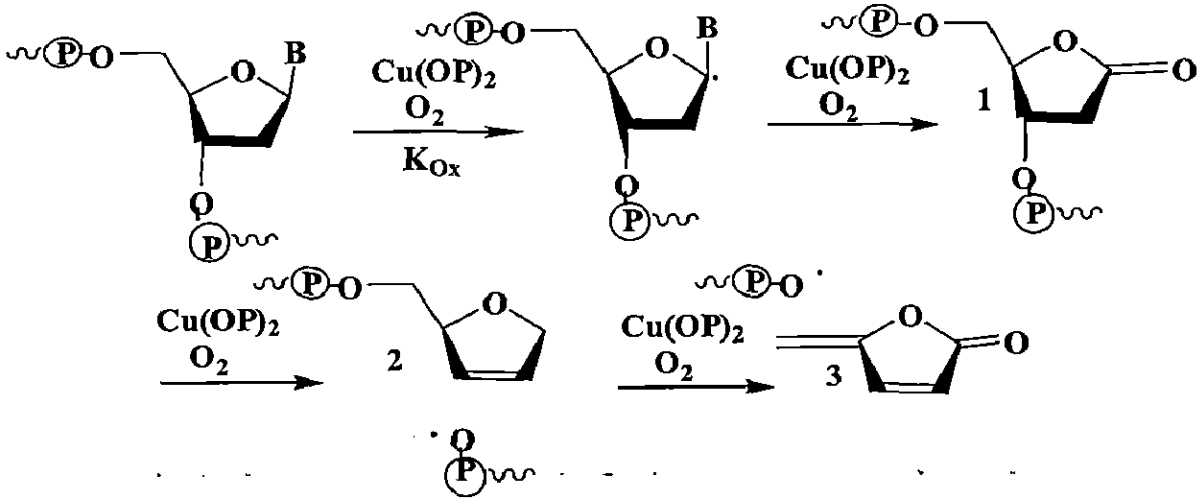


Fig. 1.5 Structure of$[Cu(OP)_2]^+$



Scheme 1.3. Mechanism of DNA scission by $[Cu(OP)_2]^+$ complex.

1.2.3 Fe-EDTA complex (DNA Foot printing agent):

Iron atom has very good redox-active properties that can catalyze the reduction of hydrogen peroxide, generating hydroxide anion and the highly reactive hydroxyl radical.

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + {}^{\bullet}OH + OH^{-} \qquad 1$$

If the reaction is to cycle, the $M^{(n+1)+}$ produced must be reduced again by a one-electron donor. A particularly efficient reductant is superoxide anion (O_2^{-}):

$$M^{(n+1)+} + O^{2-} \longrightarrow M^{n+} + O_2 \qquad 2$$

The overall reaction summarized in eq 3 has been termed Fenton Chemistry and may play a central role in oxygen toxicity.[43] Iron and, to a lesser extent, copper, are primarily responsible for carrying out the reaction *in vivo*.[44]

$$O_2^{-} + H_2O_2 \longrightarrow O_2 + OH^{-} + {}^{\bullet}OH \qquad 3$$

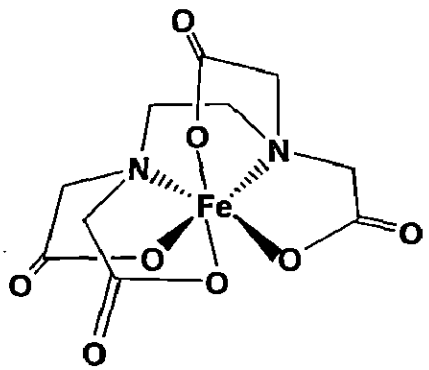


Fig. 1.6 Structre of Fe.EDTA

Ferrous-EDTA (Fig. 1.6) is an efficient mediator of Fenton chemistry. It is readily reduced by superoxide and ascorbic acid and efficiently oxidized by hydrogen peroxide. Moreover, its compact structure restricts its reactivity with the hydroxyl radicals that it

generates. Hydroxyl radicals generated by γ irradiation or chemically by ferrous-EDTA cleave DNA efficiently by reaction mechanism(s) which have not yet been fully elucidated.[45,46] With either radiatively or chemically generated hydroxyl radicals, the 5'-termini are phosphomonoesters and the 3'-termini are 3'-phosphomonoesters or 5'-phosphomonoesters. The former products presumably are generated by C-1 hydrogen abstraction while the latter are generated by 4-H hydrogen abstraction from the deoxyribose. Although free bases can be detected, the bases themselves can also react.[47]

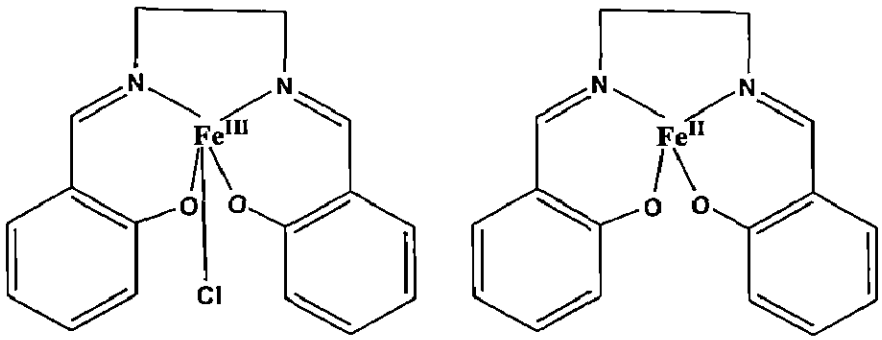
Reaction with the bases does not in itself lead to cleavage of the DNA, but can result in cleavage after subsequent chemical treatment. Aruoma et al.[48] observed two major base damage products, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 8-hydroxyguanine, when DNA was treated with hypoxanthine/xanthine oxidase/Fe^{3+}-EDTA. Hydroxyl radical was confirmed as the causative agent because ·OH radical scavengers such as dimethylsulfoxide inhibited base damage. Furthermore, superoxide dismutase, catalase, and desferrioxamine also inhibited base damage.

1.2.4 Porphyrin and salen complexes:

The scission chemistry of metalloporphyrins coordinated to metal ions (e.g. Co^{3+}, Fe^{3+} and Mn^{3+}) which retain their axial ligands has been studied. For the redox-active metal ion, Fe^{3+} scission is observed in the presence of either ascorbic acid, superoxide, or iodosobenzene in A-T, but not G-C rich regions. A possible explanation for this observation is that the porphyrin is a minor-groove ligand whose interaction with DNA is inhibited by the 2-NH_2 of guanine. Analysis of the reaction products indicates the formation of phosphomonoester

termini but no thiobarbituric acid positive base-propenals. As would be expected for a ribose-directed oxidative activity, scission is observed at all four nucleotides.[49]

Due to their diverse redox and structural properties, metal complexes of salen (bis(salicylidene)ethylenediamine) have been used extensively as catalyst for epoxidation of olefins.[50]



A B

Fig. 1.7 Structure of Fe.salen complex

The oxidative nature of metallosalens attracted many biological chemists to develop novel class of DNA binding and modifying agents.[51] Various types of metallosalens have been developed over the period of last two decades and their DNA binding and damaging properties have been studied in detail. For example, Routier et al.[51] have studied Fe(II)-salen (Fig.1.7) and its various hydroxyl derivatives that bind and cleave DNA in a sequence neutral fashion.[52] Burrows and Rokita synthesized cationic Ni(II) salen derivatives that couple accessible guanine residues and cross-link DNA strands.[53] Mandal et al.[54] have developed a set of water soluble Mn(III)-salen and Ni(II)-salen derivatives which cleave DNA in the presence of co-oxidant magnesium monoperoxyphthalate with A-T and G-C rich sequence preferences

respectively. To enhance their targeting property or sequence specificity, aromatic ring of metallosalens have been modified by attaching various sequence specific DNA binders such as distamycin and ellipticine.[55,56]

Although, many metallo salen derivatives have been developed and their DNA damaging properties have been studied, most of these investigations are limited to only in vitro experiments and very little is known about the function of these metallosalens in live cells and in vivo. Fe(III).salen produces free radicals under reducing environment and cleaves DNA in vitro. Most importantly, we demonstrated that Fe(III).salen induces apoptosis in human cell via mitochondrial pathway.

The ever-increasing numbers of biomolecules found to contain manganese include manganese containing superoxide dismutase (Mn-SOD), catalase, Mn-ribonucleotide reductase, Mn-peroxidase, the oxygen evolving center (OEC) of photosystem-II (PS-II), and Mn-thiosulfate oxidase. The mechanisms of action of these enzymes are very diverse and include oxo-atom transfer (WOC; the four-electron oxidation of water to dioxygen in PS-II, extradiol dioxygenase), electron transfer (SOD, catalase), the reduction of ribonucleotides to water and deoxyribonucleotides, and the oxidation of thiosulfate to sulfate.[57]

In 1990, Jacobsen and Katsuki independently reported highly selective (> 99% ee) asymmetric epoxidation (Fig. 8) of alkenes using Mn(salen) catalysts.[58] Since then, the area of Mn(salen) mediated epoxidation has expanded greatly. This interest in the Mn(salen) systems is due to its utility as well as the intriguing mechanism in terms of catalytic cycle and mode of reaction of active species.

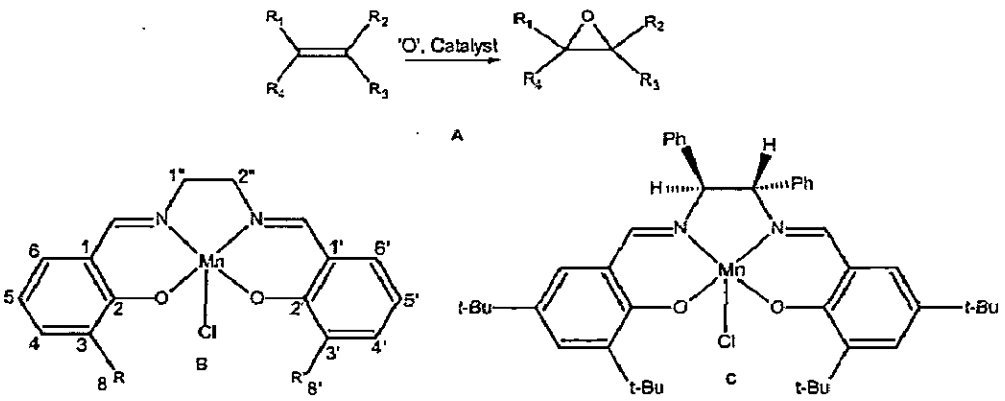


Fig. 1.8 A: The epoxidation reaction; B: Structure of Mn(salen) complex; C: jacobsen's Mn(salen) catalysts.

Products of DNA cleavage were separated by electrophoresis on a 1% agarose gel, visualized by ethidium bromide staining/fluorescence. At micromolar concentration in the presence of oxidant, single-strand DNA cleavage, as evidenced by the conversion of pBR322 from Form I (supercoiled) to Form II (nicked). At higher concentrations of complex, the production of Form III (linear) DNA was observed, which is consistent with independent.[49]

Manganese-porphyrin complexes are mainly studied for oxidation of alkenes and alkanes as structural and functional models of the P-450 enzyme. Investigations initially were directed at regioselective epoxidations with simple porphyrins, such as tetraphenylporphyrin (TPP) and tetramesitylporphyrin (TMP).[59] A large volume of data is now available on the binding specificity and on the mechanism of DNA cleavage by MnTMPyP activated by chemical oxidants. Analysis of MnTMPyP-mediated strand scissions on a 139 base-pair fragment of pBR322 DNA reveals that the minimum metalloporphyrin cleavage site is one A-T triplet (three consecutive A-T base pairs).[50] This DNA binding specificity was confirmed

by the DNase I footprinting method.[60] MnTMPyP suggest that high-valent manganese-oxo species might be responsible for DNA cleavage. Hydrogen peroxide is at least 3 orders of magnitude less efficient than potassium monopersulfate.[61] and breaks are observed in A-T regions, at the preferred binding sites of MnTMPyP, suggesting that diffusible hydroxyl radicals are not responsible for the single-strand breaks.[50] These strand scissions are observed on double-stranded DNA at manganese porphyrin concentrations as low as 0.5 nM with short incubation times of 1 min. At low-complex concentration, up to five single-strand breaks per MnTMPyP are observed.[61]

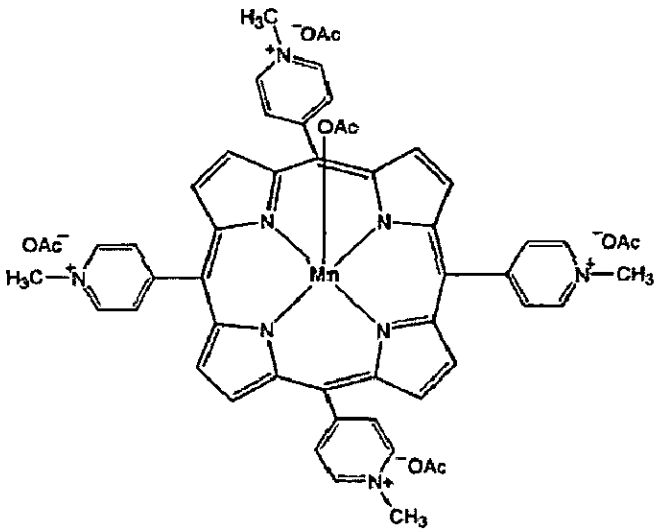


Fig. 1.9 Structure of MnTMPyP.

2. Mechanism of DNA interaction:

With nucleic acids being polyanions at physiological pH and beyond (one negative charge per phosphate diester entity), they require cations for charge neutralization. These can be metal ions, protonated amines (spermine, spermidine) or protonated amino acid side chains (lysine, arginine). Non coordinating alkali ions lead to partial charge neutralization in the case of double stranded DNA by condensing around DNA in a cylindrical fashion. Despite this partial charge neutralization (76% for +1 cations according to Manning's theory), DNA still has a pronounced affinity for cationic metal entities. Metal species can interact with DNA or nucleic acids in either of the two following ways: First, directly via coordination to phosphate oxygen atoms, sugar oxygen atoms, atoms of the heterocyclic bases (N, C, O), or combinations thereof. Second, indirectly via its other ligands. This latter possibility includes hydrogen bond formation, e.g. between aqua or amine ligands and suitable acceptors of the nucleic acid, which can be considerable if multiple hydrogen bonds are possible, or p-p interactions between the nucleic acid and the metal entity (intercalation; groovebinding), if the metal carries hetero aromatic auxiliary ligands. Again, combinations of all possibilities are feasible. Durgs bind to DNA by covalently or noncovalently.[62].

2.1 Covalent binding:

Covalent binding in DNA is irreversible and invariably leads to complete inhibition of DNA processes and subsequent cell death. Cis-platin (cis-diamminedichloroplatinum) is a famous covalent binder used as an anticancer drug, and makes an intra/inter strand cross-link via the chloro groups with the nitrogens on the DNA bases.

Fig. 1.10 Cis-platin binds to guanine in the major groove of DNA by covalently.

The binding of cisplatin to DNA is kinetically rather than thermodynamically controlled and the hydrolysis reaction of chloride ions is the rate-determining step for DNA binding. The N7 atoms of the imidazole rings of guanine and adenine located in the major groove of the double helix are the most accessible and reactive nucleophilic sites for platinum binding. The reaction of cisplatin with DNA may lead to various structurally different adducts. The binding sites on the nucleobases and different probable crosslinks in presence of cisplatin are shown in (Fig. 1.11). Initially, monofunctional DNA adducts are formed, but most of them react further to produce interstrand or intrastrand crosslinks, which block replication and/or prevent transcription.[63]

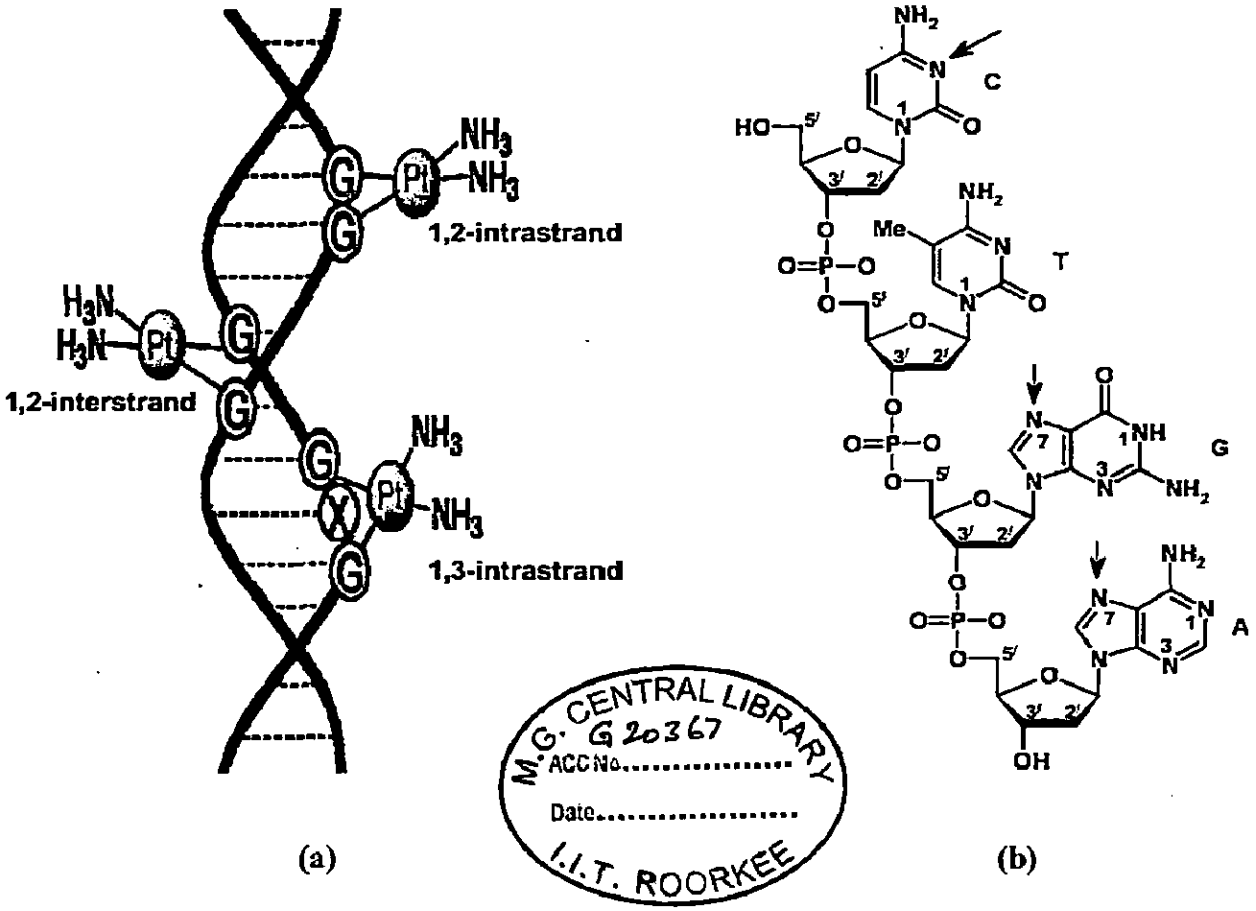
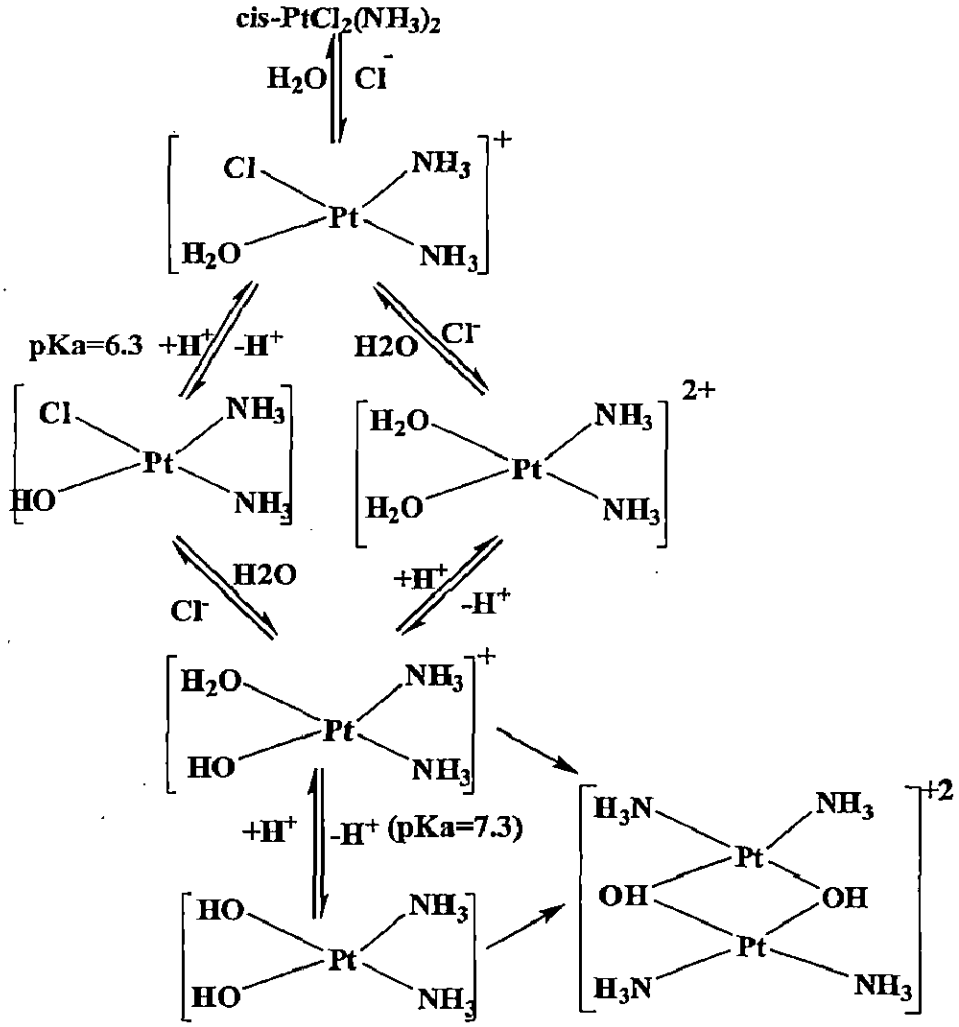


Fig. 1.11(a) Possible platination sites on DNA nucleobases (indicated with arrows) and (b) various possible crosslinks on DNA.

The interactions that may occur in living tissue and during the transport of drugs through the body are of great importance to understand the mechanism of action. Most of the present knowledge is based on the behaviour of the classical compound cisplatin in aqueous solution, which may be hydrolysed depending upon the Cl^- ion concentration and pH in the medium. The hydrolysis reaction mechanism of Martin is shown in scheme 4.[64]



Scheme 1.4. A hydrolytic mechanism of cis-platin under physiological conditions.

Under conditions with a high Pt-concentration and high pH values, formation of dinuclear and trinuclear hydroxo-bridged Pt-species does occur. However, these species are unlikely to be formed under biological conditions. In aqueous solution cisplatin will lose Cl^-, and aqua or/and hydroxo (at high pH) species are formed. In the body, the Cl^- concentration outside cells is rather high, (about 100 mM), and therefore hydrolysis is largely prevented there, but inside cells the Cl^- concentration is about 4mM, allowing the hydrolysis process. [64,65]

2.2 Non covalent binding:

Non covalent interaction basically is due to interaction of the compound with DNA by hydrogen bonding, electrostatic forces, Van der waals, groove binding, intercalation etc. It is mainly three types of interaction.

(i) Groove binding (ii) External binding (iii) Intercalation

2.2.1 *Groove binding:*

Twin helical strands form the DNA backbone. Another double helix may be found by tracing the spaces, or grooves, between the strands. These voids are adjacent to the base pairs and may provide a binding site. As the strands are not directly opposite each other, the grooves are unequally sized. One groove, the major groove, is 22 Å wide and the other, the minor groove, is 12 Å wide[66], (Fig. 1.12) the narrowness of the minor groove means that the edges of the bases are more accessible in the major groove. As a result, proteins like transcription factors that can bind to specific sequences in double-stranded DNA usually make contacts to the sides of the bases exposed in the major groove. This situation varies in unusual conformations of DNA within the cell but the major and minor grooves are always named to reflect the differences in size that would be seen if the DNA is twisted back into the ordinary B form.

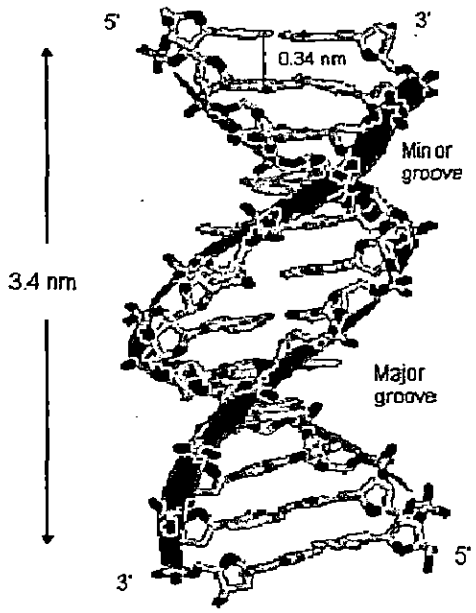


Fig. 1.12A Groove in helical form of B-DNA

The molecules approaches within van der Waals contact and resides in the DNA groove. Hydrophobic and/or hydrogen-bonding are usually important components of this binding process, and provide stabilization. The antibiotic netropsin is a model groove-binder (Fig. 1.14). Geometric and steric factors also play a role as shown with $[Ru(TMP)_3]^{2+}$ (TMP = 3,4,7,8-tetramethyl phenanthroline) (Fig. 1.13)where the methyl groups prevent intercalation.[67]

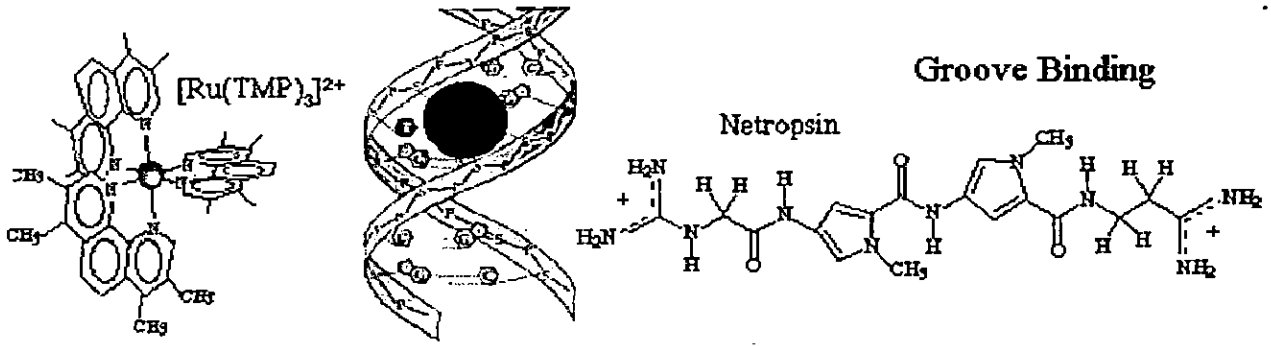


Fig. 1.13 Groove binding by metal complex and netropsin.

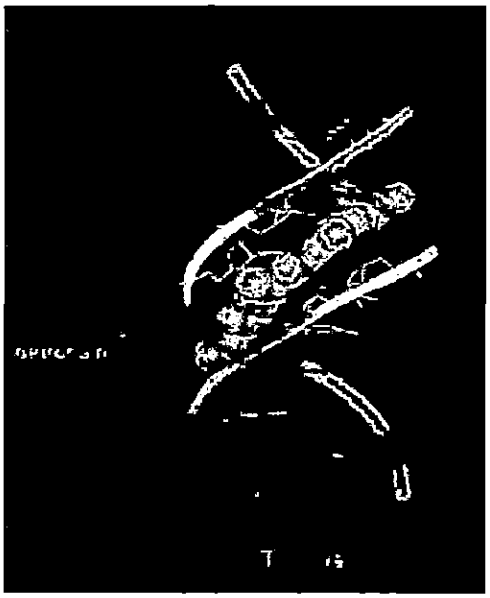


Fig. 1.14 A groove binding by netropsin in DNA

The netropsin molecule displaces the spine of hydration and fits snugly within the minor groove in the A-A-T-T centre. It widens the groove and bends the helix axis back by 8 degrees but neither unwinds or elongates the double helix. The drug molecule held in place by amide NH hydrogen bond that bridge adinine N-3 and thymine O-2 atoms, exactly as with the spine of hydration.[68]

2.2.2 *External binding*:

Of all direct coordination modes, binding to the phosphate group is probably most important in the case of alkali and alkaline earth metal ions, which represent 'natural' counter ions of nucleic acids in cells.

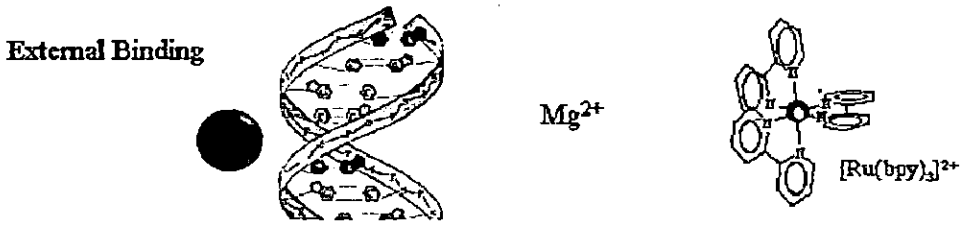


Fig. 1.15 Interaction of metal positive charge centre to DNA.

This association is electrostatic in nature (Ru(II) complexes are 2+ positively charged and the DNA phosphate sugar backbone is negatively charged. This association mode was proposed for $[Ru(bpy)_3]^{2+}$ as the luminescence enhancement of this complex upon binding to DNA is strongly dependent on the ionic strenght. Cations as Mg^{2+}, Ca^{2+} (Fig. 1.15) usually interact also in this way.[69]

2.2.3 *Intercalation*:

Intercalation occurs when ligands of an appropriate size and chemical nature fit themselves in between base pairs of DNA. These ligands are mostly polycyclic, fused aromatic and planar and therefore often make good nucleic acid stains. Intensively studied DNA intercalators include berberine, ethidium bromide, proflavine, daunomycin, doxorubicin, and thalidomide.

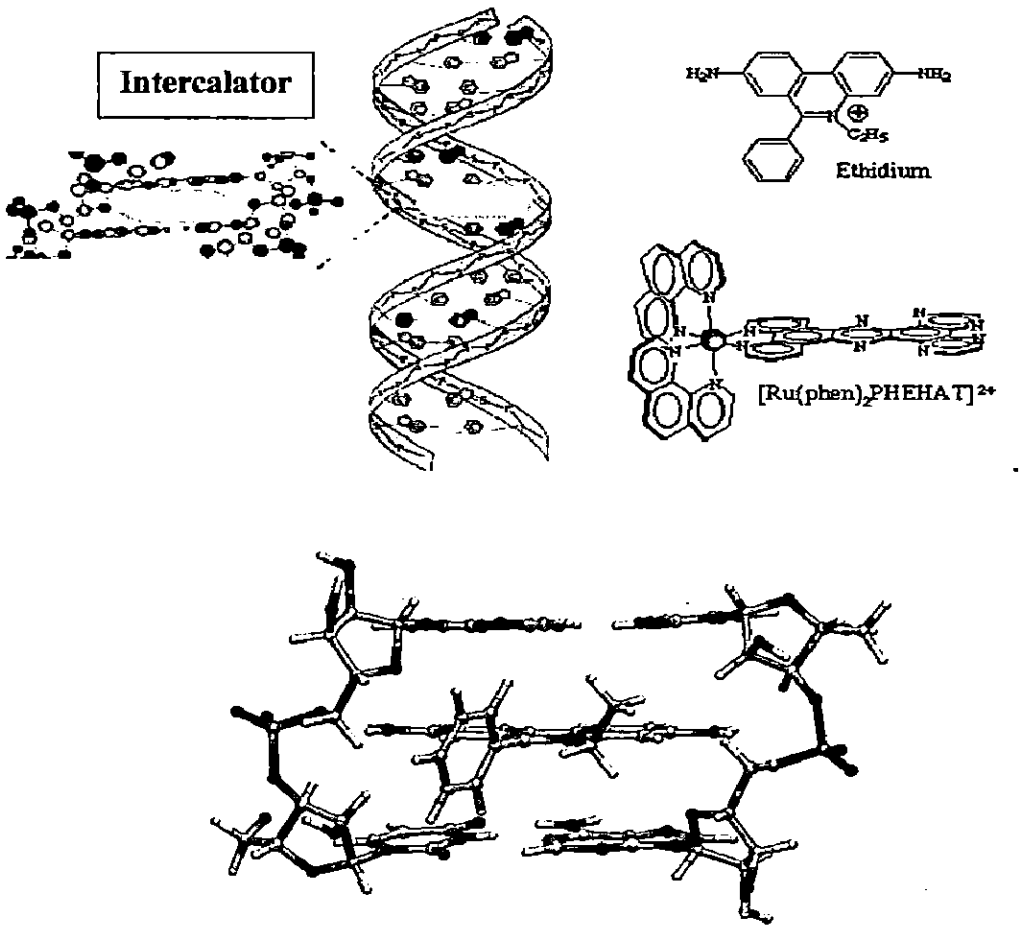


Fig. 1.16 Intercalation by metal complex and Ethidium cation.

Some metal complexes of Ru atom with ligands PHEHAT[70] are used as DNA intercalators (Fig. 1.16) in chemotherapeutic treatment to inhibit DNA replication in rapidly growing cancer cells. $[Ru(TAP)2PHEHAT]^{2+}$ is a photo-oxidizing complex and present a high affinity for DNA (afforded by the intercalating ligand PHEHAT). The luminescence of this complex is quenched very efficiently by DNA. $[Ru(phen)_2PHEHAT]^{2+}$ present a singular photo physical behaviour. In aqueous solution, this complex does not emit light at room temperature. Indeed this is related to the fact that the luminophore in this case is the Ru-PHEHAT transition which is very efficiently quenched by non-radiative deactivation through

OH vibrators. In the presence of DNA, the luminophore is protected from aqueous deactivation as it is tightly bound to DNA and the complex exhibit intense luminescence. This "light switch" ON effect is promising in order to detect efficient photoprobes of the genetic material.

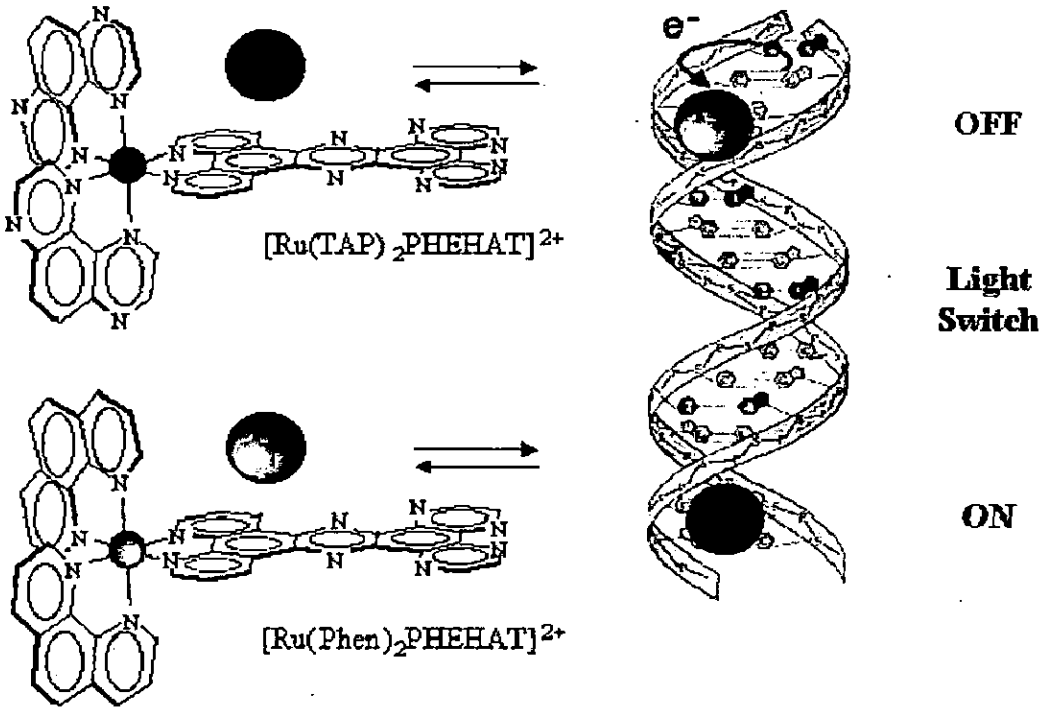


Fig. 1.17 Light switch behaviour of $[Ru(TAP)_2PHEHAT]^{2+}$ and $[Ru(phen)_2PHEHAT]^{2+}$ upon interaction with DNA.

It illustrates also how to tune the properties of Ru(II) complexes simply by changing the ligands. Compared to the first complex, the 2 TAP ligands have been replaced by 2 phen ligands which differ only by 2N.[70]

3. Mechanism of nuclease activity:

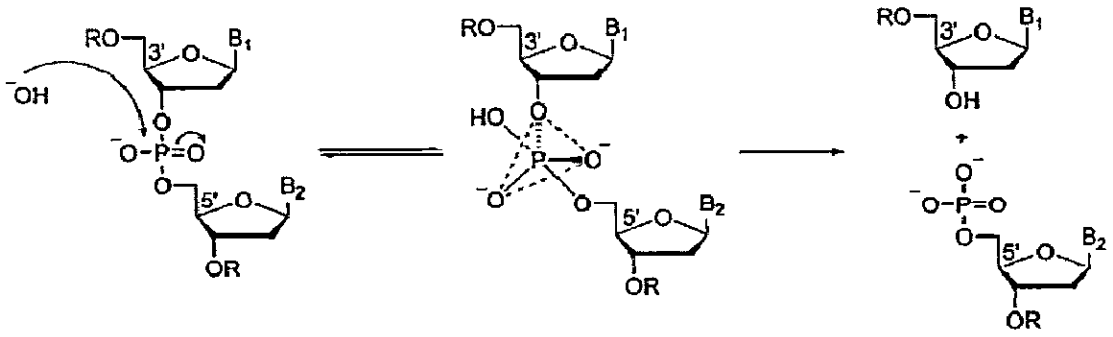
Cleavage of a DNA or RNA strand as a result of chemical reactions initially occurring on the nucleobase usually requires a second chemical step such as heat, alkali, or enzymatic treatment to affect strand scission. In general, three different types of DNA cleavage can be distinguished, namely:

(i) Hydrolytic cleavage (ii) Photolytic cleavage (iii) Oxidative cleavage

On above given methods first two categories are quite closely related.

3.1 Hydrolytic cleavage:

The half life of a typical phosphate diester bond of DNA in neutral water under ambient conditions (25°C) is estimated to be in the order of tens to hundred billions of years[71]. This means that a catalyst has to accelerate this reaction 10^{17} fold to achieve an effective hydrolysis of the phosphate backbone of DNA within an acceptable timeframe (i.e. a couple of min). The hydrolysis reaction is facilitated by the presence of metal ions, acting as Lewis acids. These Lewis acids can activate the phosphate group towards nucleophilic attack, activate water or hydroxide as nucleophile or increase the leaving group ability of the departing alcohol. The general accepted mechanism of the DNA hydrolysis reaction is a nucleophilic attack at the DNA phosphate backbone, to form a five coordinate intermediate, which can be stabilized by the catalyst.[72] Subsequent cleavage of either the 3'-PO (as seen is most often in enzymatic systems) or the 5'-PO results in a strand scission.



Scheme 1.5. Proposed reaction mechanism for the hydrolysis of DNA.

The enzymatically promoted 3'-PO scission is shown. The Lewis acids can be involved in activating water or hydroxide as nucleophile or activating the phosphate group as towards nucleophilic attack (left). Alternatively, the leaving group ability of the departing alcohol can be increased.

3.2 Oxidative cleavage:

Oxidative damage to nucleobases is a major pathway for DNA and RNA cleavage. Much attention has focused on oxidative events in the deoxyribose sugar moiety because this can lead to direct strand scission.[73] Mechanism of bleomycin complexes were described as strand scission via the oxidation of deoxyribose sugar moiety. Indeed, many of the common DNA targeted chemotherapeutics, which are natural products, utilize this mechanism.[74] It is interesting that these natural products as well as bleomycin bind DNA very specifically by positioning their reactive group in a precise location of DNA for the abstraction of a sugar hydrogen atom. In the absence of such specific-binding interactions, one would expect oxidants and free radical species generated by oxidants to react with the electron rich

nucleobases. However, the free radicals (mainly hydroxyl radicals) not only participate in the oxidation of the nucleobases but also could oxidize deoxyribose sugar moiety.[75]

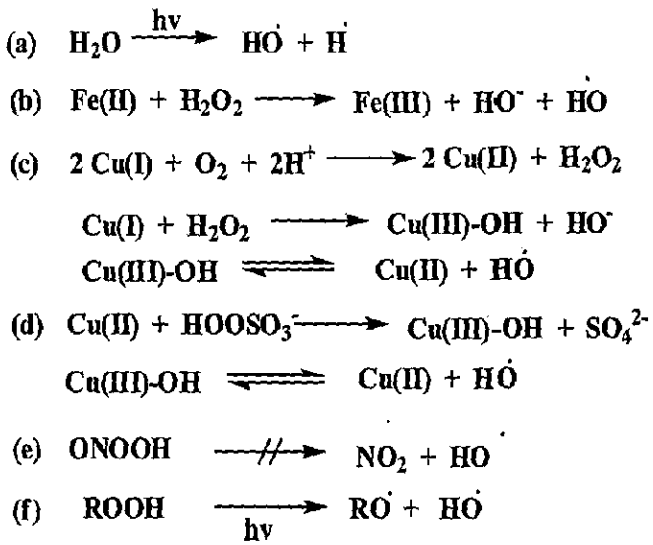
3.2.1 *Reactive oxygen species:*

The sequential reduction of molecular oxygen can generate a group of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH). The •OH radicals an extremely strong and highly diffusible oxidant, which can mediate DNA damage by adding to double bonds of DNA bases or abstracting hydrogen atoms from sugar moiety.[76] Singlet oxygen ($^1O_2$) is another ROS derived from oxygen, which is often involved in the oxidative cleavage of DNA with energy transfer. Transition metal ions in a variety of ligand environments play a key role in the redox cycle to form primary and secondary ROS as well as the reactive metal oxygen species (RMOS). The typical Fenton type reaction produces hydroxyl radicals which are responsible for the nonselective DNA scission induced by $[Fe(EDTA)]^{2-}$ [20,77] (as shown in following equation below). RMOS is more diverse than ROS, which includes end-on and side-on peroxo/superoxo metal species (M–OH/M–OOH) and even high valent metal oxo species (M=O).[17] These radical intermediates may result in DNA-cleavage at certain domains or sites by binding DNA in a selective way.

$$[Fe(EDTA)]^{-2} + H_2O_2 \longrightarrow [Fe(EDTA)]^{-1} + \dot{O}H + OH^-$$

Other transition metals may produce hydroxyl radicals by mechanisms similar to the above reaction. It is still unknown whether the active oxidant is actually free hydroxyl radical or a metal bound species, such as a highvalent M^{n+1}-OH or deprotonated M^{n+2}=O (Fig. 7c). This question is still unresolved for $Cu(I)/H_2O_2$.[78] The metal catalyzed decomposition of

peracids can also lead to HO· like species (Scheme 1.6d). The decomposition of peroxynitrous acid does not require transition metal catalysis, but it is pH dependent. It has been used as a hydroxyl radical DNA footprinting agent, but recent studies argue strongly that HO· is not formed in its decomposition (Scheme 1.6e).[19]

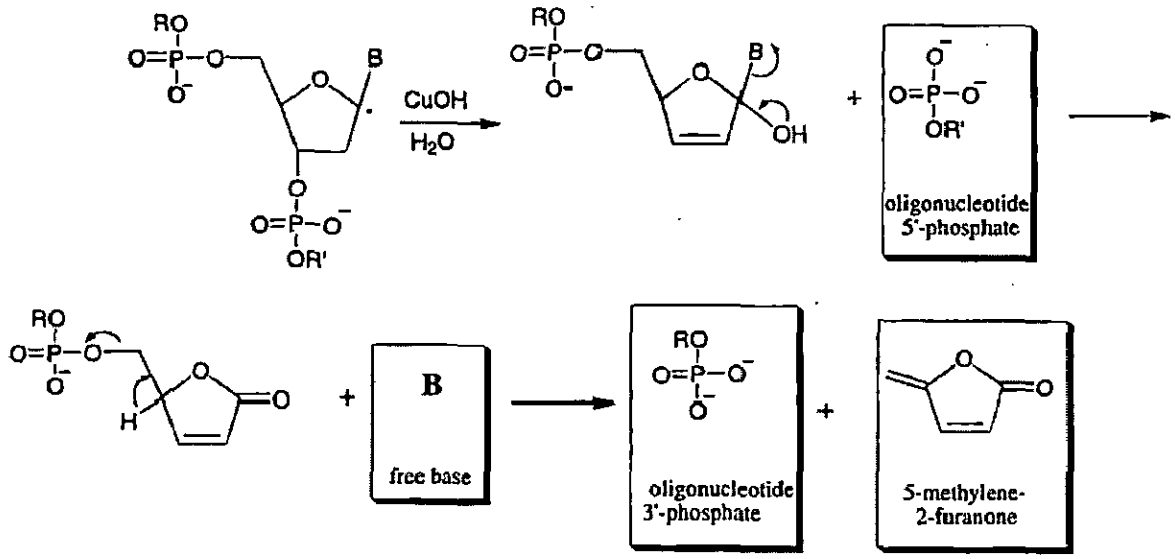


Scheme 1.6. Mechanism of ROS species generation by metal centre.

3.2.2 *Abstraction of H-1':*

In a B-DNA helix, H-1′ is one of the hydrogens in the ribose sugar moiety which is buried in the minor groove and is inaccessible to solvent compared to H-4′ and H-5′. Likewise, deuterium kinetic isotope effect experiments for the reaction of the hydroxyl radical (generated by Fenton chemistry) with duplex DNA indicated low reactivity at this position.[79] Hence, the importance of H-1′ as a reactive site appears to be limited primarily to minor groove binding molecules in which the oxidant is generated within the groove and oriented toward this hydrogen. One of the best characterized DNA cleavage reactions resulting from H-1′

abstraction is nuclease activity by bis(1,10-phenanthroline) copper(I) complex $[Cu(OP)_2]^+$. This artificial nuclease, discovered by Sigman and co-workers[21] and binds to DNA in the minor groove in a sequence dependent manner. It degrades double stranded DNA in the presence of hydrogen peroxide. Investigation of the mechanism predicted that HO· radical is not involved in this process rather the role of MROS species like $[CuO]^+$, $[CuOH]^{2+}$ or CuO_2H are involved in nuclease activity.[18,80]



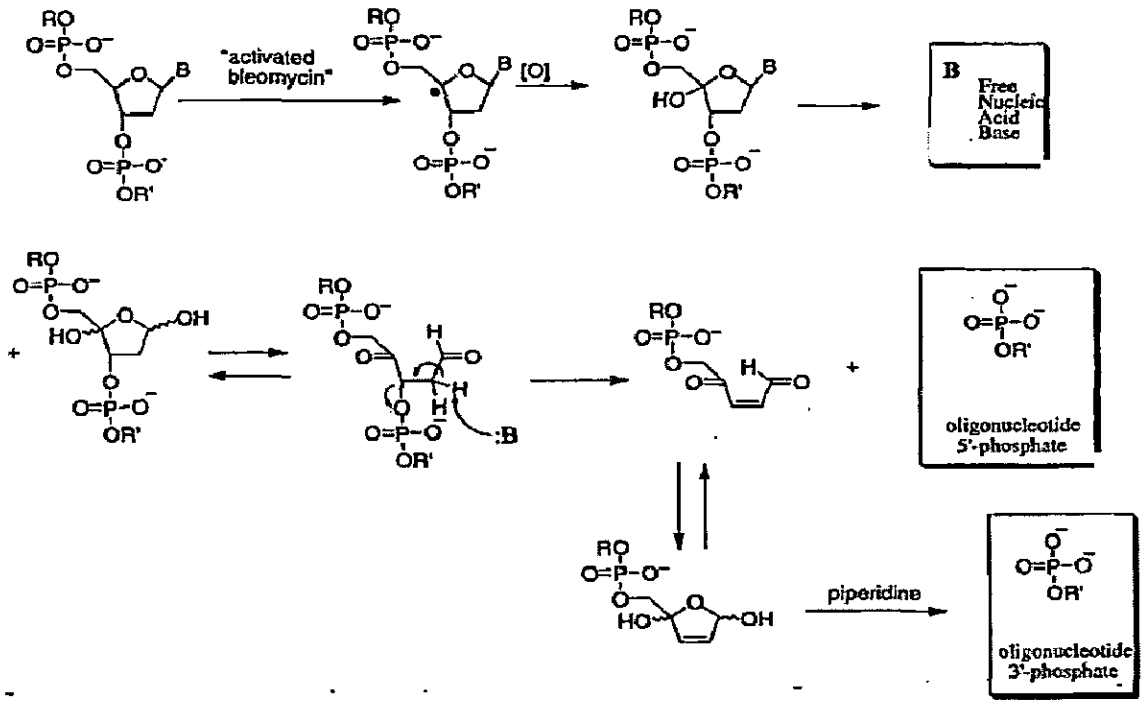
Scheme 1.7. Proposed H-1′-Abstraction Pathway for Oxidative DNA Strand Scission by Bis(1,10-phenanthroline)copper(I).[80]

3.2.3 *Abstraction of* H-4':

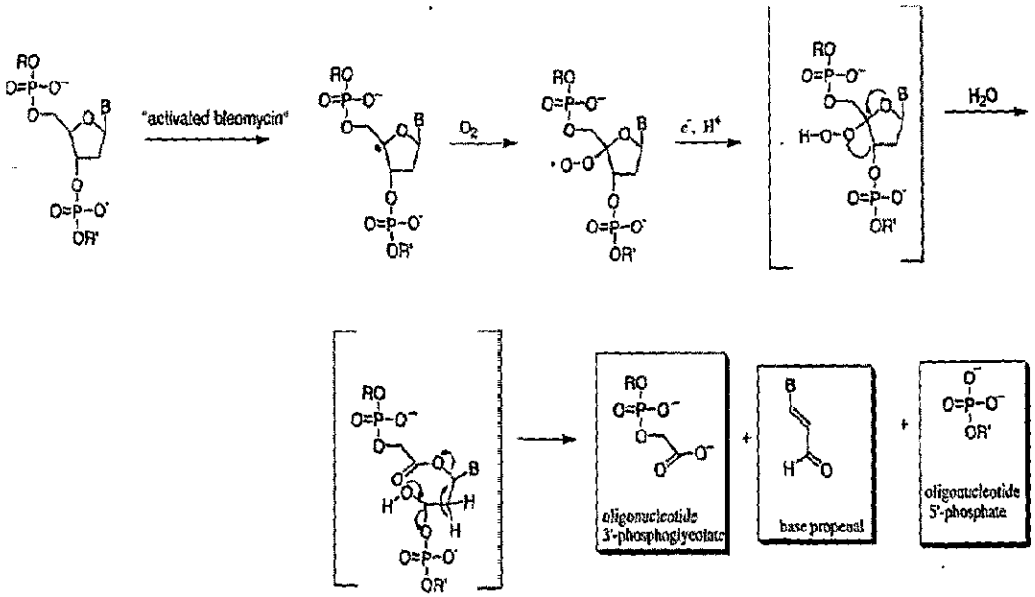
Strand scission resulting from abstraction of H-4′ has been observed in many systems. This deoxyribose position was found to be more accessible than H-1′ but lesser than H-5′ in B-form DNA; [81,82] therefore it is not surprising that many DNA cleavage molecules will prefer to react at this site. DNA damage initiated from 4′-hydrogen abstraction has been proposed for by

ionizing radiation,[83] and methidiumpropyl(EDTA)Fe(II),[47] Fenton-generated hydroxyl radicals,[77] and the drugs bleomycin.[74] A pathway involving H-4′ may also be of minor importance in DNA scission mediated by $Cu(OP)_2$.[21]

One of the best characterized path ways of DNA cleavage from H-4′ abstraction is that mediated by the anti tumor antibiotic bleomycin Fe(II) (Fig.1.3). Bleomycin-mediated cleavage mechanisms are discussed in detail by Burger.[22] The drug binds to DNA in the minor groove and is activated either by molecular oxygen and two electrons, or by H_2O_2.[84] The actual identity of the oxidizing species inactivated bleomycin has been an issue of debate. Electro spray mass spectrometry has provided evidence of a ferric hydroxide or a high valent iron oxo species such as $Fe^{V}=O \longleftrightarrow Fe^{IV}\text{-}\dot{O}$ capable of abstracting a hydrogen atom from deoxyribose.



Scheme 1.8(a). Proposed H-4′-Abstraction Pathway for DNA Strand Scission Mediated by Activated Bleomycin Fe(II) under Anaerobic Conditions.[85,86]

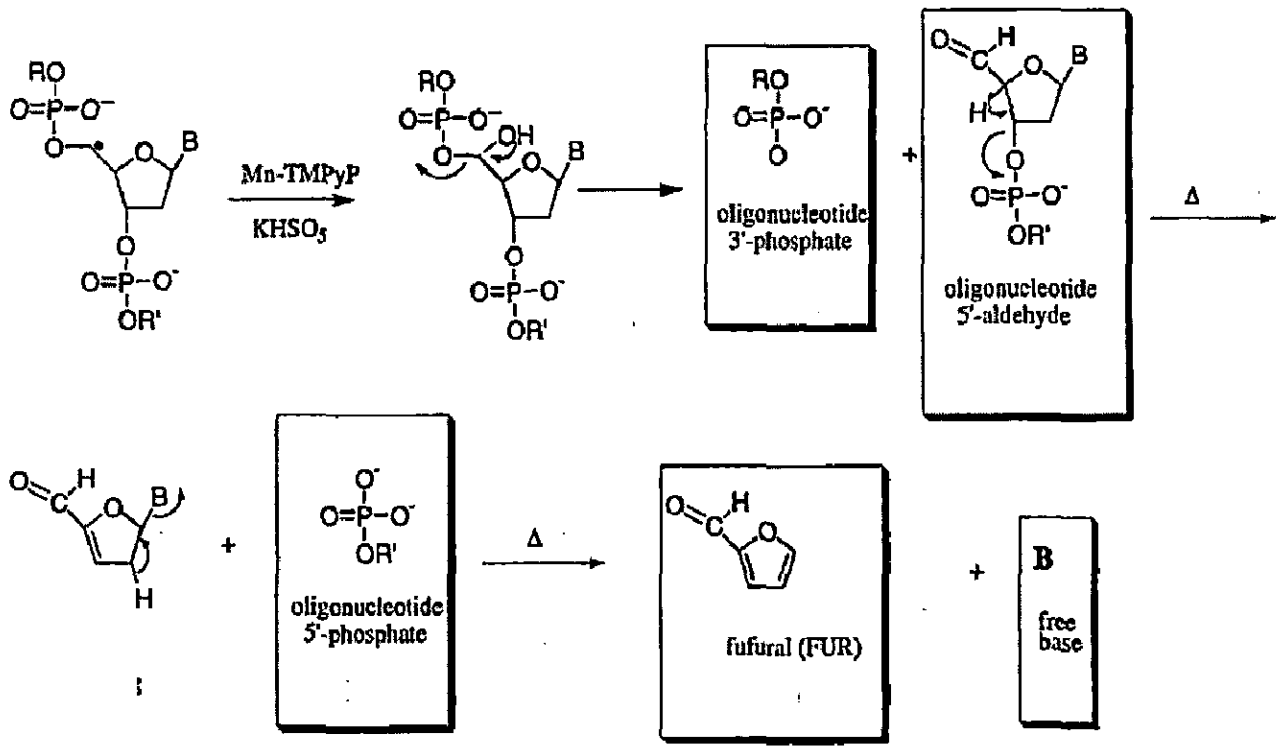


Scheme 1.8(b) Proposed H-4'-Abstraction Pathway for DNA Strand Scission Mediated by Activated Bleomycin Fe(II) under Aerobic Conditions.[85,86]

3.2.4 *Abstraction of H-5':*

The two hydrogen atoms attached to the 5'-carbon also are most access among the H-1' H-2' H-3' and H-4' in a B-DNA helix.[82,87] When the sum of the accessible surface areas of both 5'-hydrogens is considered, solvent accessibility is even greater. While both atoms are accessible from the minor groove, it should be noted that one atom points away from the groove toward solvent. Pathways involving abstraction of hydrogen from the 5'-position have been proposed for DNA scission mediated by the enediyne antibiotics,[88] Fenton generated hydroxyl radicals[79], gamma radiolysis[83], cationic metal porphyrins[47]. A different type of

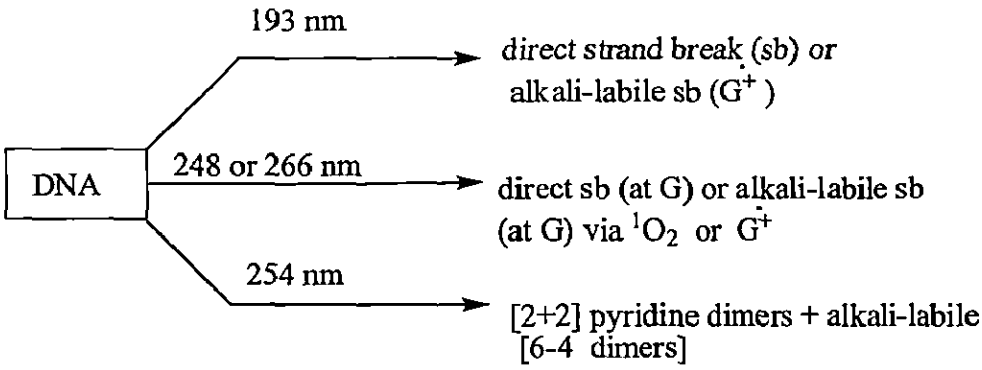
degraded sugar is observed as a product of 5′ chemistry mediated by cationic metal porphyrins. meso-Tetrakis(N-methylpyridinium-4-yl)porphyrinato manganese(III)pentaacetate [Mn-TMPyP] (Fig. 1.9), in the presence of an oxygen atom donor such as $KHSO_5$, creates strand breaks in DNA.[89] The complex can abstract either a1′-H or a 5′-H atom, depending on sequence. The 5′-mechanism appears predominant in poly (dA) poly (dT) sequences, but less important in mixed sequence DNA. The proposed pathway of oxidation is shown in Scheme 1.9. A unique product produced by this pathway is an oxidized sugar bearing an aldehyde, known as furfural (FUR).



Scheme 1.9. Proposed H-5′ Abstraction Pathway for DNA Strand Scission Mediated by the Cationic Manganese Porphyrin meso-tetrakis(N-methylpyridinium-4-yl) porphyrinato manganese(III)Pentaacetate [Mn-TMPyP].[89]

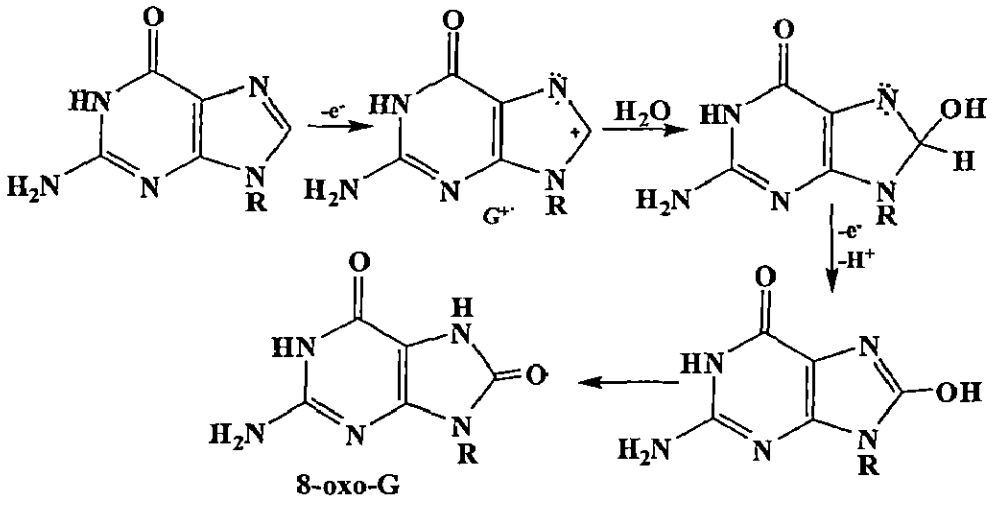
3.3 Photolytic cleavage

The direct irradiation of DNA with ultraviolet light leads to a variety of lesions, many of which are centered on the nucleobase and are alkali labile (Scheme 1.10).[90] The wavelength and intensity of light determine to some extent the type of lesion obtained.



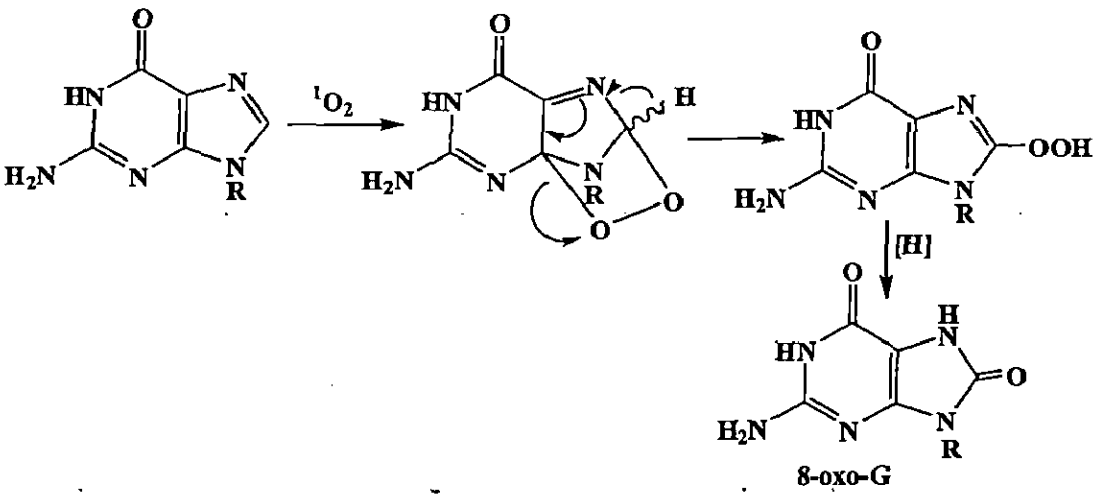
Scheme 1.10. Strand break (*sb*) of DNA by direct irradiation of radiation.

By using a 193nm laser source, a monophotonic process is observed that gives principally alkali labile guanine lesions through one electron oxidation of guanine (scheme 1.11) and direct strand breaks are also observed.[91] Since guanine is the most easily oxidized base, much of the initial photo ionization is thought to occur at Gs.[92] The guanine radical cation produced, then yield alkali-labile product including 8-oxo-G and others. The source of the direct strand breaks on photo ionization is not well understood, but may result either from C4′-hydrogen atom abstraction by nucleobase radicals or from formation of phosphate radicals. The involvement of guanine radical cation is implied from the fact that direct strand breaks occur preferentially at guanines.[93]



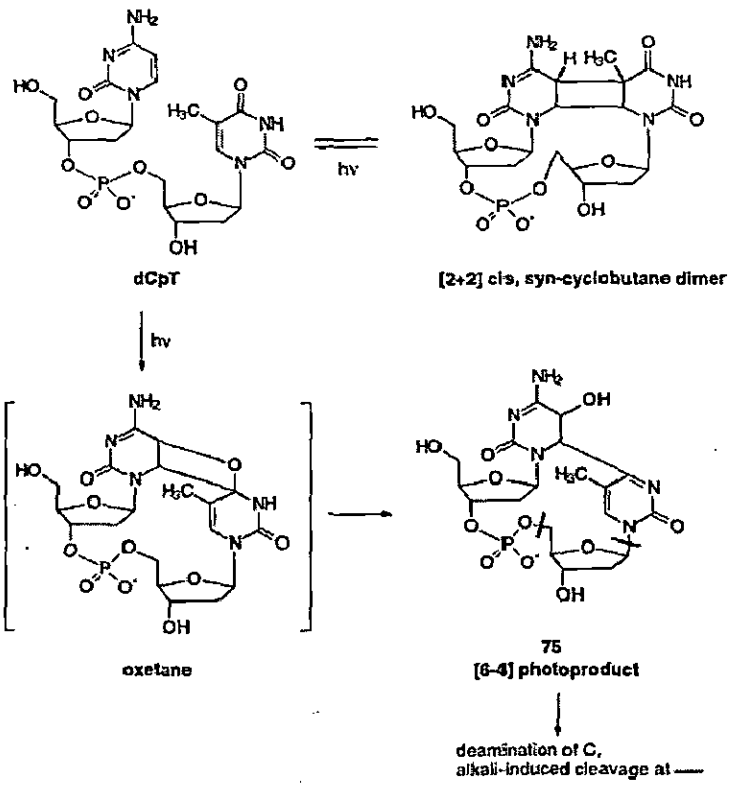
Scheme 1.11. Mechanism of sb of DNA via $G^{+\cdot}$ radical pathway.

A two photon photo oxidation of DNA using either 248 or 266 nm light sources also leads to alkali labile guanine oxidation, but this process may be due to formation of singlet oxygen ($^1O_2$). Curiously, the two photon process, like the monophotonic, 193nm irradiation, also leads to direct strand breaks that occur on the 3′ side of a guanine.



Scheme 1.12. Mechanism of sb of DNA via singlet oxygen pathway.

When 254-nm light is used to mimic photo lesions formed from sunlight, the principle products are pyrimidine photo adducts rather than photo-oxidation.[90] (Scheme 1.13)

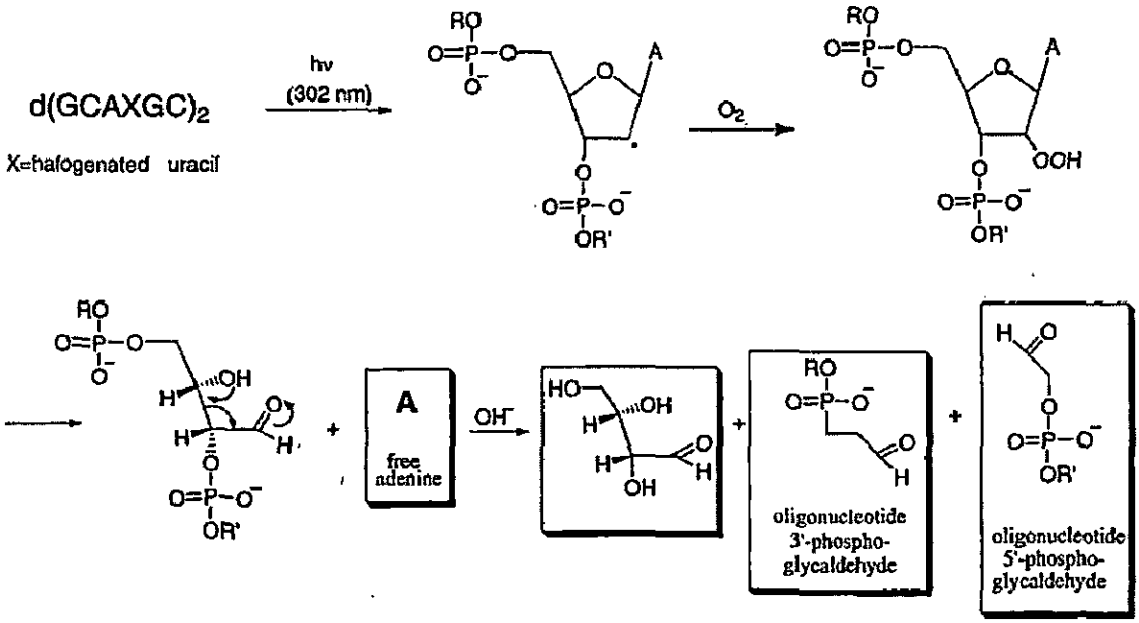


Scheme 1.13. An adduct formation by irradiating of 254 nm of radiation.

3.3.1 *Abstraction of ribose sugar* H-2′ and H-3′ *hydrogens:*

To date, the 2′-position of deoxyribose is not a commonly invoked abstraction site in DNA, presumably because of the low accessibility or low reactivity of these hydrogens. An aldehyde sugar thought to a rise from H-2′ abstraction was identified from gamma ray treated poly(U) by GC/MS.[94] Quantum mechanical calculations suggest that in short single-stranded oligonucleotides, ionizing radiation can induce a conformational change that shortens the distance between the base and the sugar, enabling a base radical to abstract a 1′-or 2′-hydrogen

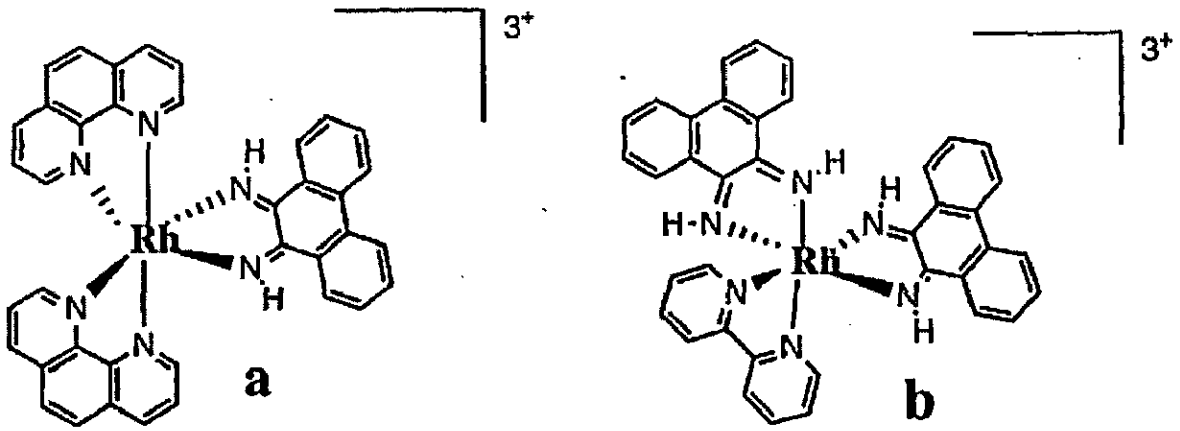
from the sugar.[97] Uracil, furthermore, is known to form radicals that are powerful hydrogen abstractors and may be able to abstract hydrogen atoms from adjacent sugars as well. An erythrose containing site thought to arise from H-2′ abstraction also was reported as a product of gamma radiolysis of CT-DNA,[96,97] and the hydroxyl radicals generated by the Fenton reaction showed a small kinetic isotope effect on the production of phosphate terminated DNA strands when the 2′-position of double stranded DNA was dideuterated[79]. These results suggest that abstraction of a 2′-hydrogen atom may contribute to strand scission even in B-form DNA. However, the contribution of this cleavage pathway appears to be minor.



Scheme 1.14. Proposed H-2′ Abstraction Pathway for Photolysis induced DNA Strand Scission at a Nucleotide Containing a Halogenated Uracil[98]

One class of metal complexes has been established to cleave DNA through abstraction of the 3′-hydrogen. Rhodium (III) coordinated by well known intercalating ligand is able to bind to duplex DNA and generate strand breaks in the presence of light. It is interesting to note

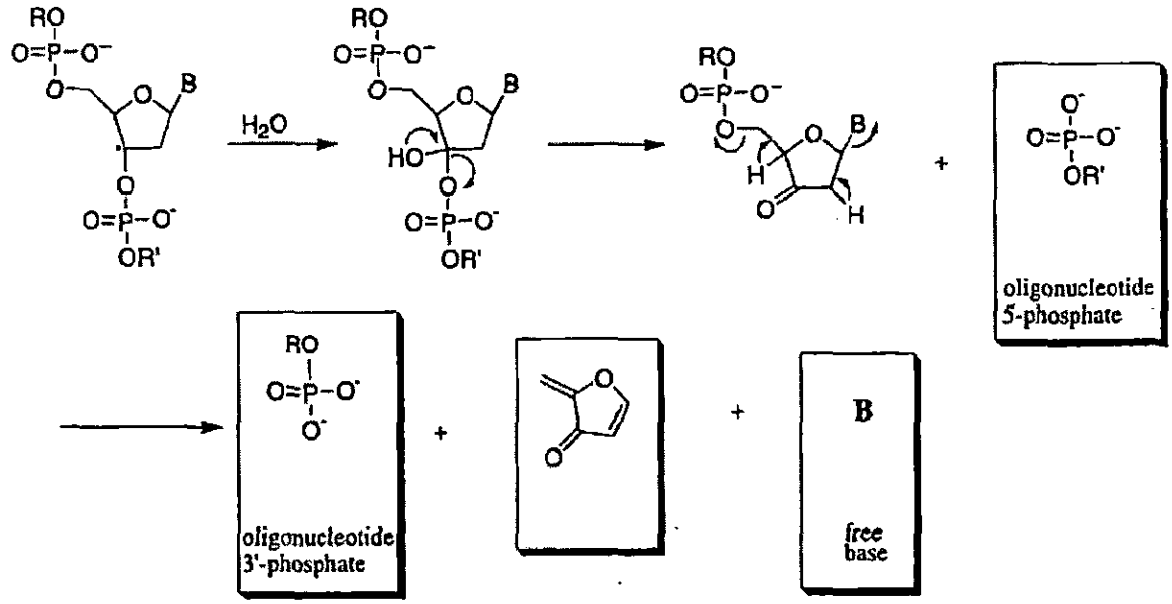
that these complexes also slightly unwind DNA. This group of photo reactive agents includes $[Rh(phen)_2phi]^{3+}$, $[Rh(phi)_2bpy]^{3+}$ (Fig. 1.18), and both enantiomers of $[Rh(en)_2\text{-}phi]^{3+}$ (where en=ethylenediamine, phen=1,-10-phenanthroline, bpy=2,2′-bipyridyl, and phi=9,10-phenanthrenequinone diimine).[99]



$[Rh(phen)_2(phi)]^{3+}$ (a) and $[Rh(phi)_2(bpy)]^{3+}$ (b)

Fig. 1.18

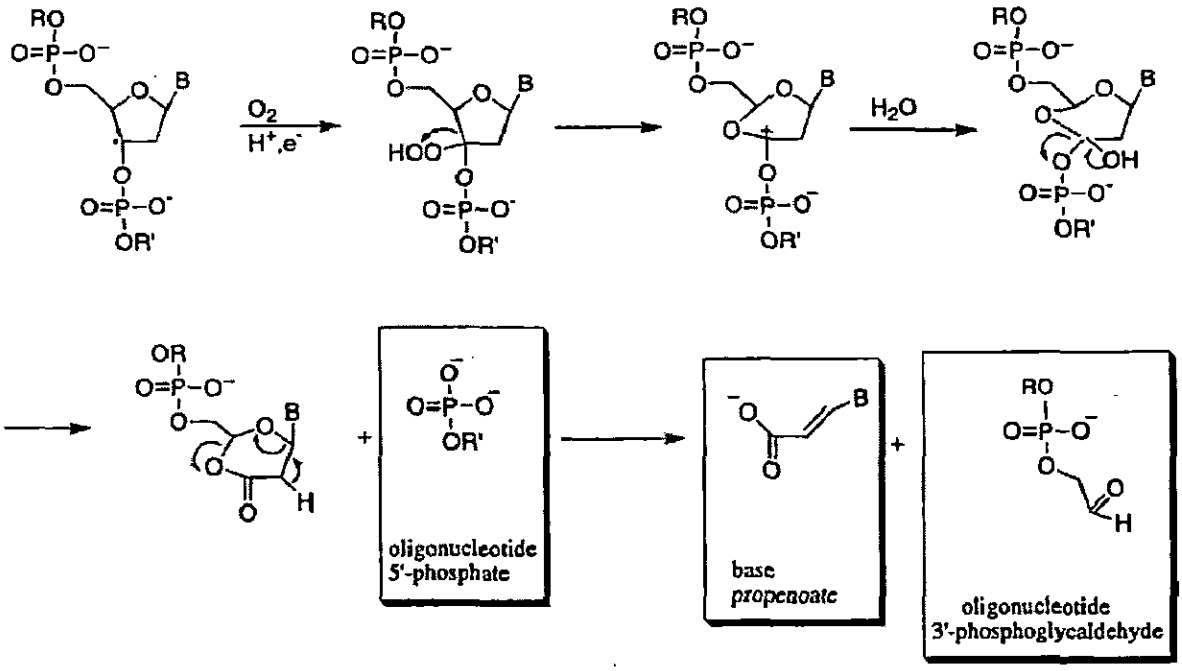
The rhodium complex is believed to form a phi cation radical via photoinduced ligand to metal charge transfer. In the metal DNA complex, the cation radical is proposed to be pointed toward H-3′. Chemical modification, HPLC analysis, and gel electrophoresis studies revealed the products of this reaction to be, as shown in Scheme 1.15 and 1.16. Oxygen was found to be necessary for formation of the 3′-phosphoglycaldehyde terminated fragment and base propenoic acid, but not for formation of free base or the 3′-phosphate terminated product. The partitioning between anaerobic and aerobic pathways best correlated with how the shape of the rhodium complex was expected to limit access of oxygen to the cleavage site.[100]



Scheme 1.15. Proposed H-3'Abstraction Pathway for DNA Strand Scission Mediated by $[Rh(phen)_2(phi)]^{3+}$ or $[Rh(phi)_2bpy]^{3+}$ under Anaerobic Conditions.[100]

The anaerobic pathway (Scheme 1.14) involves oxidation of the radical, followed by solvolysis to yield an alcohol that then undergoes elimination of the phosphate and release of free base. This mechanism predicts the production of an unopened sugar lactone derivative.

In the oxygen dependent mechanism (Scheme 1.16), molecular oxygen reacts with the 3'-radical to give the hydroperoxide radical. Subsequent rearrangement, results in insertion of an oxygen atom into the deoxyribose ring, forming intermediate. Decomposition yields base propenoic acid and DNA fragments with 5'-phosphate or 3'-phosphoglycaldehyde termini.



Scheme 1.16. Proposed H-3′-Abstraction Pathway for DNA Strand Scission Mediated by $[Rh(phen)_2(phi)]^{3+}$ or $[Rh(phi)2bpy]^{3+}$ under Aerobic Conditions.[100]

Work of present study we have synthesized following ligand H_2bpb [*1,2-bis(pyridine-2-carboxamido)benzene*] characterized manganese and iron complexes of this ligand and studied DNA interaction and nuclease activity.

4. References:

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Chapter-1

DNA interaction studies on mononuclear complexes of iron and manganese derived from planar tetradentate ligand

Abstract

A tetradentate binegative planar ligand H_2bpb was synthesized and characterized. Manganese and iron complexes of H_2bpb were synthesized and characterized. DNA interactions of these complexes were studies. Nuclease activity was examined and mechanism was investigated.

1. Introduction:

In the recent years studies on interaction of redox active transition metal complexes with DNA received considerable interest due to their plural applications in metallopharmaceutical research for the development of novel drugs[1] as well as biomolecular probes[2]. In this regard, first row transition metals namely manganese, iron and copper complexes are preferred because they are biologically relevant and lesser toxic.[1,3] Our quest originated from our interest to study the interaction of DNA with metal complexes derived from the ligand containing carboxamido nitrogen donor(s).

Amides are biologically ubiquitous functional groups. The chemistry of metal peptide complexes has attracted the unabated interest of inorganic and biological chemists because of their biomimetric relevance[4a,b] in prion protein,[5,6] photo labile nitrosyl complex synthesis[7,8] and research in green chemistry[9] as well as for their pharmaceutical importance.[10,11] Deprotonated amides are efficient ligands for stabilizing high oxidation states[12,13] of metals and the resultant complexes often act as efficient oxidation catalysts.[14,15] Pyridine-2-carboxamido ligand could be relatively inexpensive and easy to prepare and modify. These results prompted us to examine the DNA interaction studies of manganese and iron complexes of a tetradentate ligand, H_2bpb which possesses two N_{am} donors (shown in Fig. 2.1).

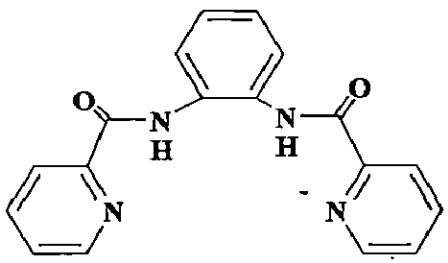


Fig. 2.1 H_2bpb [1,2-bis(pyridine-2-carboxamido)benzene]

It is well known that redox active manganese and iron posses similar chemistry[16] and their metal complexes could participate in Fenton type reactions. Manganese and iron complexes derived from H_2bpb ligand were used for several purposes. Firstly, complexes of iron were used for synthesis of photolabile nitrosyl complex synthesis[17]. Secondly, oxo-transfer reaction was studies for both iron and manganese bpb complexes.[18,19] Thirdly SOD activity studies,[20] however this was never used for DNA interaction studies. On the other hand it is reported in the literature that this planar tetradentate ligand got certain similarities with ligands like salen and prophyrin.[2,17] However, DNA interaction studies with salen complexes of manganese and iron was studied.[21,22] However, to the best of our knowledge DNA interaction studies with bpb complexes with manganese and iron were not reported. Herein we report the DNA interaction and nuclease activity of manganese and iron complexes derived from H_2bpb.

2. Experimental:

2.1 Materials and method:

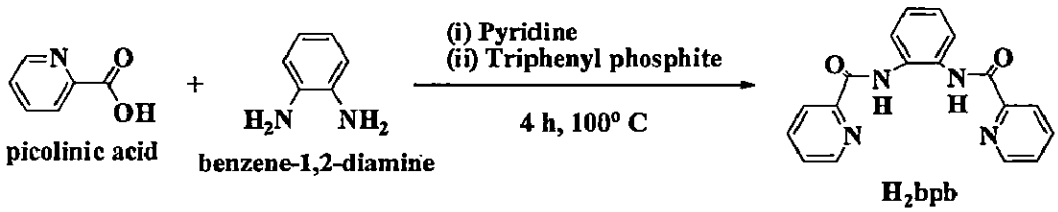
All the solvents used were reagent grade. Removal of all solvents was carried out under reduced pressure. Toluene, diethyl ether, dimethylsulfoxide (DMSO), dimethylformamide (DMF), chloroform and dichloromethane were purified by distillation over 4Å molecular sieves and stored over sieves. Methanol was purified by distillation over 3Å molecular sieves and stored over sieves. Acetonitrile, acetone were dried by refluxing and distillation over P_2O_5 (0.5%,w/v). Analytical grade reagents triethylamine (ThomasBaker, India), $Mn(CH_3COO)_3.2H_2O$ (Sigma Aldrich, Steinheim, Germany) were used as obtained, $FeCl_3$ (anhydrous, Rankem, RFCL limited). Picolinic acid (Wilson laboratory Bombay) and tripheny phosphate (Acros organic). Xanthine, nitro blue tetrazolium (NBT) and catalase were obtained

from Himedia and xantine oxidase (XO) from bovine milk, agarose (molecular biology grade, Himedia) and ethidiumbromide (EB) were purchased from Sigma. The supercoiled pBR322 DNA and calf thymus DNA (CT-DNA) were purchased from Bangalore Ge-Nei (India).Tris buffer was prepared in deionised water.

2.2 Synthesis:

2.2.1 *H_2bpb [1,2-bis(2-pyridinecarboxamido)benzene]:*

To a solution of 2-pyridinecarboxylic acid (2mmol) in pyridine (0.5ml) was added into 1,2-diaminebenzene (1mmol). After triphenyl phosphite (1mmol) addition the reaction solution was heated (90°C) for 4 h on oil bath and allowed to stand at room temp overnight. A brown oily viscous liquid resulted. Treated this brown oily viscous liquid by ethanol and filtered off, results a white color solid compound (96%) obtained.[23] Yield 2.11 g (72 %). IR (KBr, υ_{max}/cm^{-1}): 1672, $\upsilon_{N\text{-}H}$ 3314, GC-MS (CH_2Cl_2, m/z): 318 M^+. All the spectra are shown in appendice A_1, A_2, A_3.



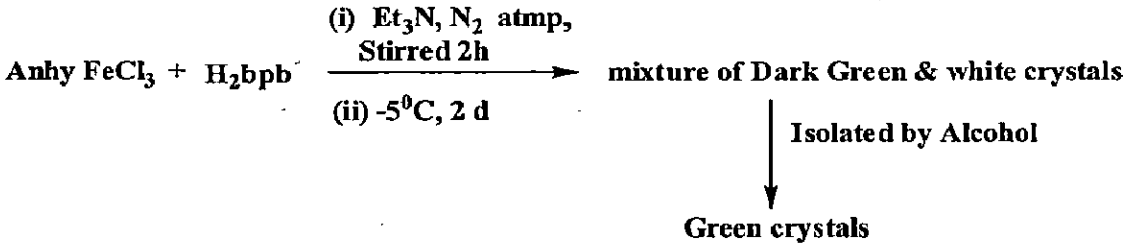
2.2.2 *[Mn(bpb)Cl(H_2O)]:*

The compounds were prepared by using similar procedures as outlined in the literature.[24]

To a mixture of H_2bpb (160mg, 1 mmol) and $[Mn(OAc)_3.2H_20]$ (130 mg, 1 mmol) in methanol (15 ml) was added solid LiCl (300 mg, 10 mmol). The mixture was stirred at 298 K for 15 min and then heated to reflux for 15 min. It was then cooled and the precipitated brown microcrystalline complex was filtered off, washed with a small amount of methanol and dried in vacuo (yield ca. 70%). This simple procedure afforded the complex in good yield and purity (Found: C, 53.50; H, 3.15; N, 13.70. Calc. for $C_{18}H_{12}C_lMnN,O_2$: C, 53.15;H,2.95;N, 13.80%). (70 %). IR (KBr, υ_{max}/cm^{-1}): 1645, $\upsilon_{C=O}$, no N-H stretch, UV-visible [DMF, λ_{max}/nm]: 273 nm. $\Lambda_M/\Omega^{-1}cm^2\ mol^{-1}$ (in DMF), 12. IR spectra shown in appendix A_4.

2.2.3 *[Fe(bpb)Cl$_2$]Et$_3$NH:*

The compounds were prepared by using similar procedures as outlined in the literature.[25]The ligand 1,2-bis(2-pyridinecarboxamide)-benzene (H_2bpb) (1mmol, 318mg) solution in acetonitrile was added to anhydrous $FeCl_3$ (1mmol, 162mg) and triethylamine (2mmol, 210mg) in a nitrogen atmosphere. The solution turned dark green immediately. The solution was stirred for approximately 2 h and a small amount of brown precipitate was removed by filtration. After reducing the volume the solution, it was cooled at -5°C for 2 days. A mixture of dark green crystal of $(Et_3NH)[Fe(bpb)Cl_2]$and white crystals of Et_3NHCl formed and isolated these crystal by the help of ethyl alcohol because solubility differences in ethanol. Yield 50 %, IR (KBr, υ_{max}/cm^{-1}): 1620, $\nu_{C=O}$, no N-H stretch, UV-visible [DMF, λ_{max}/nm ($\varepsilon/M^{-1}cm^{-1}$)]: 276 nm; $\Lambda_M/\Omega^{-1}cm^2mol^{-1}$ (in DMF) 60. Analytical calculation for $[Fe(bpb)Cl_2]Et_3NH$ ($FeC_{24}N_5O_2Cl_2H_{30}$):Fe, 9.25; C, 53.25; H, 5.34; N, 13.37; Cl, 10.98. IR Spectra are shown in appendix A_5.



2.3 Physical Measurements:

Elemental analyses were carried out microanalytically at Elemenlar Vario ELIII. Melting point were obtned using Perfit (India) melting point apparatus. IR spectra were obtained as KBr pellets with Thermo Nikolet Nexus FT-IR spectrometer, using 50 scans and were reported in cm^{-1}.GC-MS data were obtained on a quadrupole Perkin Elemer Clarus 500MS coupled to a Perkin Elemer Clarus 500GC fitted with an Elite1 column and mass detector was operated at 70eV. Electronic absorption spectra were recorded in dichloromethane, acetonitrile, DMF or DMSO solvents with an Evolution600, Thermo Scientific UV-visible spectrophotometer. Emission quenching titrations were carried out on RF-5301pc fluorescence spectrophotometer. Magnetic susceptibilities were determined at 297K with Vibrating Sample Magnetometer model155, using nickel as a standard. Diamagnetic corrections were carried out with Pascal's increments. Molar conductivities were determined in DMF at 10^{-3} Mat 25°C with a Systronics304 conductometer. All experiments were performed at room temperature, and solutions were thoroughly degassed with nitrogen prior to beginning the experiments, and during the measurements nitrogen atmosphere was maintained.

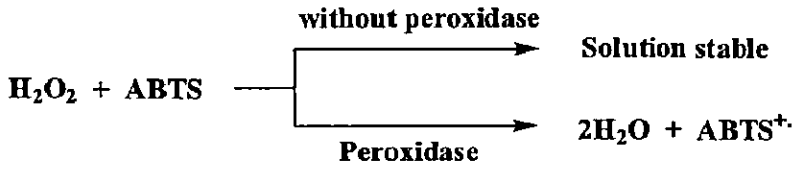
2.3.1 *Superoxide Dismutase activity:*

The SOD activity of the Mn-bpb complex was assayed previously using the standard NBT assayed[20] however SOD activity of Fe-bpb was not reported yet. The SOD activity of complexe **2** was tested by indirect method using xanthine/xanthine oxidase nitro blue-tetrazolium (NBT) assay.[26a-c] In this method superoxide anion is generated insitu enzymetically by xanthine/xantine oxidase system and detected spectrophotometrically by reduction of NBT which produced a formazan dye of blue color. Absorbance at 560 nm got increased because of production of formazan dye.[27] However, the increase rate of the absorbance was reduced with the increase of complexes concentration. The assay was performed in 50 mM potassium phosphate buffer at pH 7.2 using 0.6 mM xanthine, 0.3 mM NBT, 0.07 U/mL xanthine oxidase and catalase 1000 U/mL (final volume = 750 µL). The reaction was started after adding 0.07 U/mL xanthine oxidase and measurement was started after 3 min for each experiment.

2.3.2 *Peroxidase probe:*

The peroxidase-like activity of the complexes was determined by the oxidation of the ABTS [2,2'-azinobis(3-ethylbenzothiazoline)sulfonic] to $ABTS^{+\cdot}$ at pH 6.8. ABTS is a colourless water-soluble compound used for clinical peroxidase assays because of its excellent characteristics.[28,29] ABTS on oxidation forms a stable green cation radical ($ABTS^{+\cdot}$) with several strong absorptions in the visible. The formation of $ABTS^{+\cdot}$ is monitored conveniently at its wavelengths of 415, 650, 735 and 815 nm[30] and measured quantitatively at λ=650 nm, since ε=12000 $M^{-1}cm^{-1}$ has been determined. The oxidation potentials of ABTS and the fully reduced species to provide $ABTS^{+\cdot}$ are invariable over a wide range of pH. In the absence of

the complex (peroxidase), a solution of ABTS and H_2O_2 is stable for several hours without showing any formation of $ABTS^{+\cdot}$. The reaction of ABTS with H_2O_2 in the presence of complex (peroxidase) generates $ABTS^{+\cdot}$ and the characteristic absorption bands of this species could be established. Further oxidation to the corresponding dication[31] was not observed.



2.3.3 *DNA binding:*

The experiments were carried out in 0.1M phosphate buffer at pH7.2 using a solution of calf thymus DNA (CT-DNA) which gave a ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) of ca.1.8, indicating that the CT-DNA was sufficiently protein free.[32] The concentration of DNA solution was determined by UV–visible absorbance at 260 nm. The extinction coefficient ε_{260} was taken as $6600M^{-1}cm^{-1}$ as reported in literature.[33]

2.3.3.1 *Fluorescence spectroscopic studies:*

Fluorescence quenching experiments were carried out by the successive addition of 0-12 μM of the complexes to the DNA (25 μM) solutions containing 0.5 μM EthBr in 0.1 M phosphate buffer (pH 7.2) in 5% dimethylformamide (DMF). These samples were excited at 250 nm and emission was observed at 602 nm. EthBr emits intense fluorescence in presence of DNA due to its strong intercalation between the adjacent DNA base pairs. If the complexes can

intercalate into DNA, the binding sites of DNA accessible for EthBr will be reduced and therefore the fluorescence intensity of EthBr will be quenched. The emission spectra of EthBr bound to DNA in the absence and the presence of the metal complexes are given according to Stern-Volmer equation.[34]

$$F_o/F = 1 + K_{SV}[R] \text{ .....(i)}$$

Where F_o and F are the fluorescence intensities in the absence and the presence of complexes, respectively, K_{SV} is a linear Stern-Volmer quenching constant, [R] is the concentration of quencher. In the quenching plot of F_o/F vs [R], Ksv is given by the ratio of the slope to intercept.

2.3.3.2 ***Circular dichroism:***

Circular dichroic spectra of DNA were obtained in the region between 220 and 320 nm was scanned in 1 mm path length cuvette with 10 min incubation time for each sample. Each spectrum was averaged from three successive accumulations and smoothed within permissible limits using the software of the unit. DNA concentration was used 50 μM for 200 μM concentration of each complex in 10% methanol in phosphate buffer.

2.3.4 ***DNA cleavage*:**

DNA cleavage was measured by the conversion of supercoiled pBR322 plasmid DNA to nicked circular and linear DNA forms. Supercoiled pBR32 DNA (200 ng) in (TBE) Tris-boric acid-EDTA buffer (pH 8.2) was treated with complexes **1** and **2** taken dimethylformamide (10%) in the presence or absence of additives. The oxidative DNA

cleavages by the complexes are studies in the presence of H_2O_2 (1.6 mM, oxidizing agent). The samples were incubated at 37 °C, added loading buffer (25% bromophenol blue and 30% glycerol). The agarose gel (0.8%) containing 0.4 µg/mL of ethidium bromide (EB) was prepared and the electrophoresis of the DNA cleavage products was performed on it. The gel was run at 60 V for 2 h in TBE buffer and the bands were identified by placing the stained gel under an illuminated UV lamp.

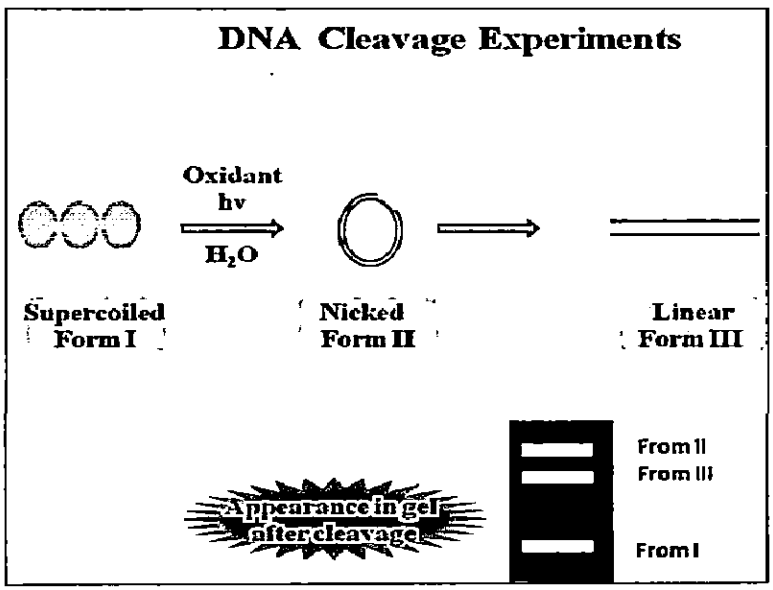


Fig. 2.2 DNA cleavage appeared after gel electrophoresis in gel photo.

3. Results and Discussion:

3.1 Superoxide Dismutase activity:

The SOD activity of complexe 2 was tested by indirect method using xanthine-xanthine oxidase-nitro blue tetrazolium (NBT) assay.[26] In this assay, while $O_2^{\cdot-}$ is generated, NBT is reduced, producing blue (formazan dye) colour which increases absorbance at 560 nm. The

more effective the compound for SOD activity, lower will be the compound concentration which inhibits the NBT reduction in 50% (IC_{50}). The IC_{50} value is the concentration of the complex which exerts activity equal to one unit of that of native SOD. The complexes described in this work show a moderate SOD-like activity comparable to that of a large number of published SOD-mimic complexes.[35,36] The complex **2** shows an IC_{50} value of 4.1 μM which indicates that this compound can be used as small molecule SOD mimic. The inhibition curve of the SOD activity for complex **2** is shown in Fig. 2.3.

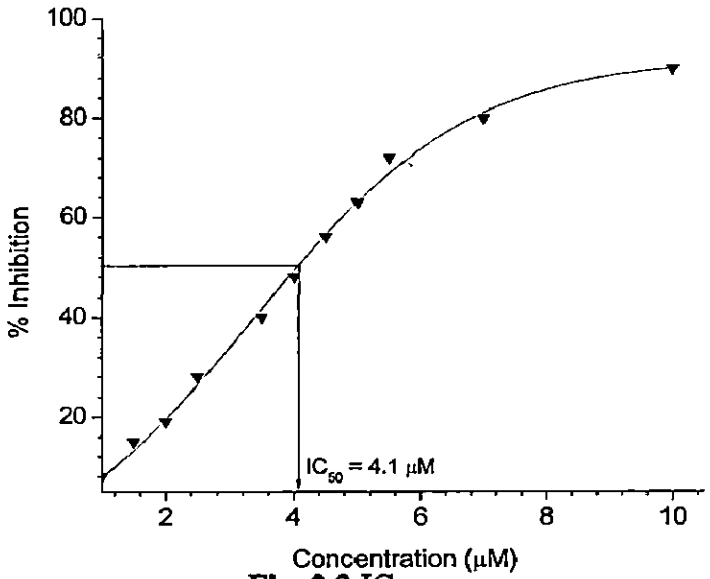


Fig. 2.3 IC_{50} curve

3.2 Peroxidase probes:

The peroxidase-like activity of the complexes was determined by the oxidation of the ABTS to $ABTS^{+\cdot}$ at pH 6.8. ABTS is particularly appropriate as substrate and it is generally used for the quantification of the enzymatic activity of peroxidises[37], ABTS is colourless and reacts readily with H_2O_2 in presence of peroxidase catalyst to yield a stable green coloured

radical cation which shows characteristic absorption at 415, 650, 735 and 815 nm, in general, wavelength at 415 nm (strongest absorption) is used for detection purposes.[30,38] In absence of peroxidase catalyst ABTS and H_2O_2 solution remain stable without the formation of $ABTS^{+}$ Fig. 2.4 shows the UV spectrum of the ABTS radical cation acquired after hydrogen peroxide decomposition by complexes **1** and **2**. The reaction of ABTS with H_2O_2 in the presence of **1, 2** (complex concentration: 3.3 x 10^{-6}M) generates green color $ABTS^{+}$. The oxidised form, $ABTS^{+}$, can be clearly identified by its characteristic absorption bands as shown in Table1.

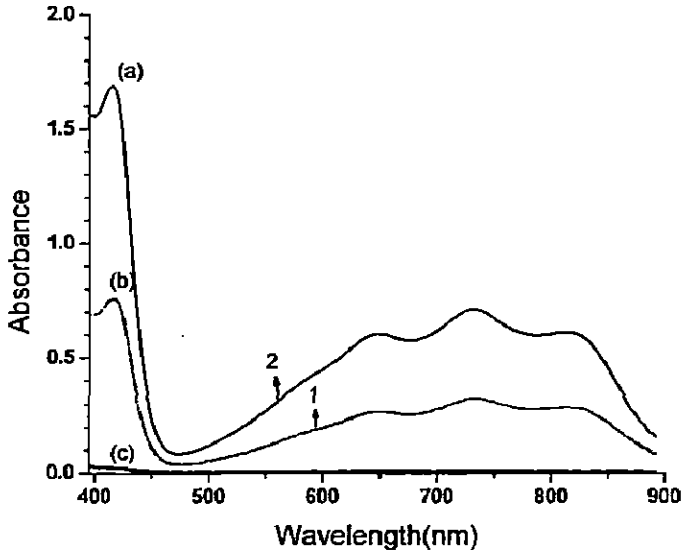


Fig. 2.4 UV absorption spectra of (a) ABTS + H_2O_2 + **2**, (b) ABTS + H_2O_2 + **1**, (c) ABTS + H_2O_2 (blank), each recorded 10 min after mixing the solutions at pH = 7.

$$\mathbf{H_2O_2 + ABTS \xrightarrow{Complex\ 1,\ 2} 2H_2O + ABTS^{+\cdot}}$$

The high rate of formation of $ABTS^{+}$ indicates a significant ability of disproportionation of hydrogen peroxide by both complexes, behaving as efficient peroxidase mimics. *Explaination for better catalytic activity is that complex is able to easily coordinate*

the substrate molecule. This is preferred if the catalyst has a vacancy in the coordination sphere or have a labile ligand. Since both complexes **1**, **2** have labile ligands, which may generate a vacant site in coordination sphere to accommodate the substrate molecule, as the good peroxidase activity of few mangase-Schiff base systems is explained on this hypothesis.[39-41]

Table: 1 Values of the intensity of the wavelengths of the UV spectra corresponding to the $ABTS^{+}$ formed in the peroxidase-like experiment.

Complex	Absorbance of $ABTS^{+}$ UV signals			
	λ = 415	**λ = 650**	**λ = 735**	**λ = 815**
1	0.765	0.281	0.333	0.300
2	1.695	0.615	0.719	0.621

3.3 DNA binding studies:

We followed two techniques namely EB fluorescence quenching studies and circular dichroic spectral studies.

3.3.1 *Fluorescence spectroscopic studies:*

In binding of both the metal complexes to DNA were examined by EthBr fluorescence displacement experiments. EthBr can be used as a probe to determine the interaction of complexes with DNA as EthBr emits intense fluorescence in presence of DNA. If the complexes can intercalate into DNA, the binding sites of DNA accessible for EthBr will be

reduced and therefore the fluorescence intensity of EthBr will be quenched. In our experiments, as described in Fig. 2.5 the fluorescence intensity of EthBr shows a remarkable decreasing trend with increasing concentration of the metal complexes. This indicates that some EthBr molecules are free from EthBr-DNA after a swapping with the complex resulting the fluorescence reducing of EthBr. The emission spectra of EthBr bound to DNA in absence and presence of complexes are shown in Fig. 2.5. The fluorescence quenching curve of DNA-bound EthBr by complexes (inset of Fig. 2.5 (a) and (b)) illustrate that the quenching of EthBr bound to DNA by complexes is in good agreement with the linear Stren-Volmer equation. The K_{SV} values (using equation (i)) for complexes **1** and **2** are 4.88 x 10^4 and 6.64 x 10^4. These results also prove that the partial replacement of EthBr bound to DNA by the complexes results in a decrease of the fluorescence intensity. The K_{sv} values indicate weak interaction of manganese complex than iron complex. From K_{sv} data external binding with moderate intercalation was speculated. Same types of observation were found with Mn-salen complexes.[42]

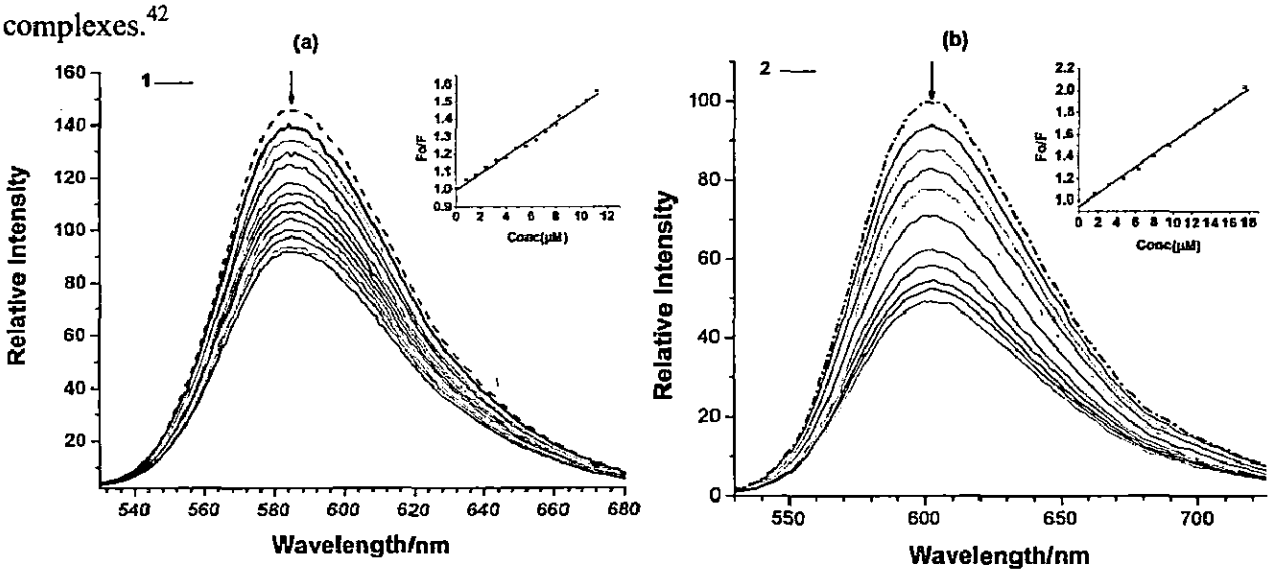


Fig. 2.5 The fluorescence quenching of EthBr–DNA systems by titrations of complexes **1** and **2**.

Fluorescence emission spectra of the EB-CT-DNA ([DNA] = 25 μM) system in absence (.....) and in presence of the complexes (0-32 μM). Inset of both figures show Stern–Volmer plots of F_0/F *vs* [R] for complexes. Tests were performed in the conditions of 50 mM phosphate buffer (pH 7.2) at 298 K, C_{EtBr} = 0.5 μM; λ_{ex} = 250 nm. (a) Complex **1** (b) Complex **2**.

3.3.2 *Circular dichroic spectral studies:*

Circular dichroic spectrum of CT-DNA exhibits a positive band at 275 nm due to base stacking, and a negative band at 245 nm due to the right handed helicity (Fig. 2.6).[43] Interaction of complex with DNA may cause significant changes in DNA structure in wide-ranging simple groove binders and electrostatic interaction of complexes show less or no perturbation on the base-stacking and helicity bands whereas intercalators increase the intensities of both the bands stabilizing the right-handed B conformation of CT-DNA. On addition of complexes **1** and **2**, the CD spectrum of DNA undergoes changes in both the positive and negative bands but no shift in the band positions. The intensities of the negative bands decrease, on addition of the both the complexes **1** and **2**. In the presence of complex **1**, the positive band showed remarkable decrease; however there is no change in positive band in case of complex **2**. Decrease in the 275 nm band on addition of complex **1** and no change in case of complex **2** exclude the possibility of intercalation mode. Decrease in the intensity of DNA helicity (245 nm) band indicates the hydrophobic interaction of –NH– groups with DNA, which is characteristic of B to A conformational change. These results may be suggested that these complexes interact with CT-DNA, showing some conformational changes.[44]

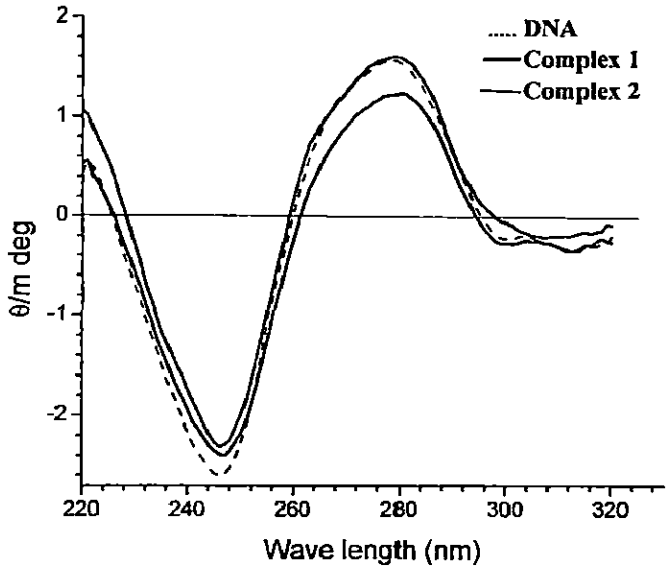


Fig. 2.6 Induced CD spectra of CT DNA (25 μM) in absence (dashed line) and in presence (solid line) of complexes **1** and **2** in phosphate buffer at pH 7.2 (10% methanol).

3.4 Nuclease activity:

The DNA cleavage activity of **1** and **2** has been studies under physiological pH and temperature by gel electrophoresis using supercoiled pBR322 plasmid as the substrate. Both complexes **1** and **2** are cleavage inactive in the presence of reducing agents (BME). The oxidative DNA cleavage activity of the complex **2** was studied in the presence of H_2O_2. Control experiments show that H_2O_2 or **2** alone, under aerobic conditions, is cleavage inactive. A 50 μM solution of **2** significantly produce double strand cleavage on treatment with 75 μM or 300 μM H_2O_2 in 2h (incubation time). Mechanism for nuclease activity (Fig. 2.9c) was examined by using different radical scavengers such as urea, DMSO, EtOH (•OH scavengers), L-histidine and NaN_3 ($^1O_2$ scavenger)[45] during DNA cleavage activity. Nuclease activity was inhibited to small extent in presence of EtOH, suggesting the possibility of the formation of

•OH radicals as the reactive species from probable hydroperoxo intermediate: $[Fe^{III}–OOH^{-}]^{2+}$ → $[Fe^{IV}=O]^{2+}$ + •OH.[46] Hence, possible participation of reactive oxygen species (ROS) was involved for DNA cleavage.

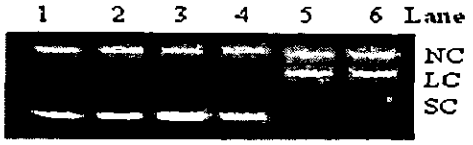


Fig. 2.7a Gel electrophoresis separations showing the oxidative cleavage of super-coiled pBR322 DNA (200 ng) by complexes **1** and **2** in10% DMF incubated at 37°C for 3h. DNA (lane 1); DNA+ H_2O_2 (300 μM) (lane 2); DNA+ **2** (50 μM) (lane 3); DNA+ **2** (100 μM) (lane 4); DNA+ **2** (50 μM) + H_2O_2 (300μM) (lane 5); DNA+ **2** (60 μM) + H_2O_2 (300 μM) (lane 6).

Fig. 2.8b Gel electrophoresis separations showing the oxidative cleavage of super-coiled pBR322 DNA (200 ng) by complex **2** in10% DMF incubated at 37°C for 2h using H_2O_2 (75 μM) and BME (75 μM). DNA (lane 1); DNA + H_2O_2 (lane 2); DNA + BME (lane 3); DNA+ **2** (100 μM) (lane 4); DNA + **2** (5 μM) + H_2O_2 (lane 5); DNA + **2** (10 μM) + H_2O_2 (lane 6); DNA+ **2** (20 μM) + H_2O_2 (lane 7) ; DNA + **2** (50 μM) + H_2O_2 (lane 8) ; DNA + **2** (75 μM) + H_2O_2 (lane 9); DNA + **2** (100 μM) + H_2O_2 (lane 10); DNA + **2** (5 μM) + BME (lane 11); DNA + **2** (10 μ M) + BME (lane 12); DNA+ **2** (20 μM) + BME (lane 13); DNA+ **2** (50μ M) + BME (lane 14); DNA+ **2** (75 μM) + BME (lane 15).

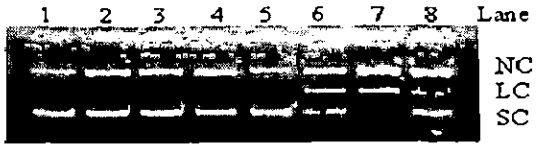


Fig. 2.9c DNA (Lane 1); DNA + H_2O_2 (200 μM) + **2** (50 μM) (Lane 2); DNA + H_2O_2(200 μM) (Lane 3); DNA + H_2O_2 (200 μM) + **2** (50 μM) + Urea (200 μM) (Lane 4); DNA + H_2O_2 (200 μM) + **2** (50 μM) + EtOH (200 μM) + (Lane 5); DNA + H_2O_2 (200 μM) + **2** (50 μM) + DMSO (200 μM) (Lane 6); DNA + H_2O_2 (200 μM) + **2** (50 μM) + L-His (200 μM) (Lane7); DNA+ H_2O_2 (200 μM) + **2** (50 μM) + NaN_3 (200 μM) (Lane 8).

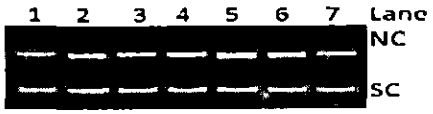


Fig. 2.10d DNA(Lane 1) ; DNA + H_2O_2(300μM) (Lane 2); DNA + H_2O_2(300μM) + **1** (25 μM) (Lane 3); DNA + H_2O_2(300μM) + **1** (50μM) (Lane 4); DNA + H_2O_2(300μM) + **1** (75μM) (Lane 5); DNA + H_2O_2(300μM) + **1** (100μM) (Lane 6); DNA + **1** (100μM) (Lane7).

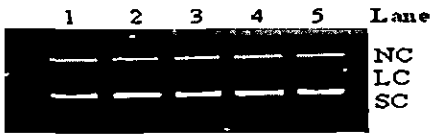


Fig. 2.11e DNA (Lane 1); DNA + BME (300 μM) (Lane 2); DNA + BME (300 μM) + 1 (80 μM) (Lane 3); DNA + BME (300μM) + **1** (100μM) (Lane 4); DNA + BME (300μM) + **1** (150μM) (Lane 5).

4. Conclusion:

In this study we prepared tetradentate [N_4] H_2bpb ligand and their complexes with iron and manganese as reported in literature. The complexe **2** described in this work shows a moderate SOD-like activity comparable to that of a large number of published SOD-mimic complexes in the literature. The complex **2** shows an IC_{50} value of 4.1 μM which indicated that this compound can be used as small molecule SOD mimic. Complexes **1**, **2** showed peroxidase activity, which have labile ligands, that may generate a vacant site in coordination sphere to accommodate the substrate molecule. DNA binding studies of complexes **1** and **2** shows that intercalation or external binding with CT-DNA has been suggested. Complex **1** did not exhibit the nuclease activity either BME or H_2O_2 but complex **2** shows the nuclease only with H_2O_2, not with BME. Partial inhibition of nuclease activity in the presence of EtOH indicated the probable participation of reactive oxygen species in DNA cleavage.

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7th July 2010

Dr. Vikas Pruthi
Associate Professor
Dept. of Biotechnology, IITR

Subject: M.Tech Dissertation evaluation – External Examinar

Dear Dr. Vikas Pruthi,

Thank you very much for kindly accepting to act as an external examiner as recommended by our Department Academic Committee to evaluate M. Tech dissertation entitled: "ANALYSIS OF SMALL MOLECULE INTERACTION WITH DNA". As per your consent viva-voce will be held on 13th July at 10.30 AM at the Seminar Hall (C-103), Chemistry Department, IITR. Kindly make it convenient to attend the same.

A copy of the dissertation is enclosed herewith for your kind reference and evaluation.

Thanking you,

Yours sincerely

(KAMALUDDIN)
Prof. and Head

Enclosed: A copy of dissertation

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Chapter-2

Synthesis and characterization of new mononuclear Fe(II) complex and DNA interaction studies.

Abstract

A tridentate neutral ligand, Simpy was synthesized and characterized. Mononuclear iron (II) complex was synthesized and characterized. We investigated DNA interaction studies by spectrophotofluorometer and circular dichroism studies and nuclease activity was examined.

1. Introduction:

Iron bleomycin is already FDA approved drug.[1-6] Some iron complexes are used for DNA footprinting also.[7,8] There are several reports in the literature where iron complexes and the DNA interaction were examined.[1,2,9-15] Hence iron is an important metal for DNA interaction studies because iron variable oxidation states, spin states and coordination numbers and redox properties in different iron complexes.[16] In the present study we have designed the following ligand shown in Fig. 3.1 and synthesized iron(II) complex of the ligand. The resultant complex was characterized by elemental analysis, UV-Vis, IR and NMR spectral studies. DNA interaction was investigated by fluoroscence spectral and CD studies.

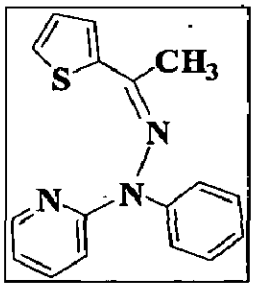


Fig. 3.1 Structure of ligand Simpy

2. Experimental:

2.1 Materials and methods:

All the solvents used were reagent grade. Purification of solvents was done as mentioned in the ***chapter 1***. $Fe(ClO_4)_2$ (Sigma Aldrich, Steinheim, Germany) were used as obtained. Other reagents, that was used same as in ***chapter 1*** also.

2.2 Synthesis:

2.2.1 *Synthesis of 2-(1-phenyl-2-(1-(thiophen-2-yl)ethylene)hydrazinyl)pyridine [Simpy]*

2-(1-phenylhydrazinyl)pyridine and 2-acetyl thiophene were prepared by reported procedure.[17] The reaction mixture of 2-acetyl thiophene (10 mmol, 1.26 g) and 2-(1-phenylhydrazinyl)pyridine (10 mmol, 1.85 g) in presence of concentrated HCl (2-3 drops) was refluxed for 4 h in 20 mL methanol. This reaction mixture was kept for slow evaporation at room temperature. After 2-3 days a yellow needle shaped crystal were appeared. Crystals were filtered, washed with small amount of methanol and dried in vacuum. Yield 2.11 g (72 %). IR (KBr, ν_{max}/cm^{-1}): 1585, $\nu_{C=N}$, UV-visible [DMF, λ_{max}/nm ($\varepsilon/M^{-1}cm^{-1}$)]: 361 (75040), GC-MS ($CH_2Cl_2$, m/z): 293 M^+ (2.13%), 169 (100%), 278 (26%); ^1H NMR (500MHz, $CDCl_3$) 2.08 (s, 3H), 6.83 (t, 1H, J= 6), 7.03 (d, 1H, J= 8.5) 7.07 (t, 1H, J= 4), 7.17 (dt, 1H, J= 7, 1), (7.40-7.33 m, 6H), 7.57 (t, 1H, J= 8) 8.23 (d 1H, J= 4.5). All the spectra are shown in appendice A_1-A_6.

2.2.2 *Synthesis of complex:*

Caution! Perchlorate salts of metal complexes with organic ligands are potentially explosive. Only small quantities of these compounds should be prepared and handled with proper protection.

Synthesis of [Fe(Simpy)$_2$](ClO$_4$)$_2$

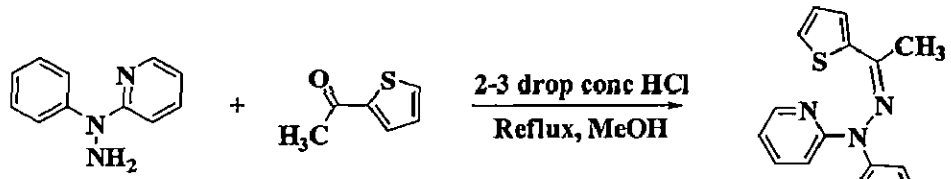
A solution of $Fe(ClO_4)_2 \cdot xH_2O$ (1 mmol, 256 mg) and ligand Simpy (1 mmol, 293 mg) in 15 ml methanol was stirred at room temperature for 48 h. Initially no change in color but after 12 h dirty purple solution was obtained which become darker with stirring time. This solution was further stirred for 36 hr and no color change is observed now. After that the solution was filtered and filtrate rum through alumina column and red purple solution come in dichloromethane and hexane (50:50). This solution was concentrate and kept for crystallization

in hexane diffusion in dichloromethane:methanol solution. Dark red purple colors of crystals were obtained after 4 days. IR data (KBr, ν_{max}/cm^{-1}): 1625, $\nu_{C=N}$, 1120, 1104, ν_{ClO4^-}, UV-visible [CH_2Cl_2, λ_{max}/nm ($\varepsilon/M^{-1}cm^{-1}$)]: 518 (2620), 349 (8480), 311 (13240). $\Lambda_M/\Omega^{-1}cm^2\ mol^{-1}$ (in acetone): 154.

3. *Results and discussion:*

3.1 Synthesis and characterization of ligand:

Tridentate ligand Simpy (N_2S) was prepared by refluxing of 2-acetyl thiophene and 2-(1-phenylhydrazinyl)pyridine in methanol, with few drops of concentrated HCl and their synthetic procedure is shown in scheme 3.1. This ligand was soluble in several organic solvent like dichloromethane, methanol, acetonitrile, dimethylformamide and acetone.



2-(1-phenylhydrazinyl)pyridine **2-acetyl thiophene** **Simpy**

Scheme 3.1. Reaction pathway of the ligamd (Simpy) synthesis.

A characterization of this ligand was performed by elemental analysis, UV–visible, FT-IR, GC-MS spectrometry and NMR (^1H and ^{13}C) spectroscopic studies. All the spectral data were tabulated in Table 1. The IR-spectra of Schiff base ligand, Simpy show $\nu_{C=N}$ band at 1583 cm^{-1} which is consistent with reported literature values.[18] The stretching band for C-H in CH_3 was observed at 3005 cm^{-1} and C-S at 774 cm^{-1}. UV-visible spectra of free ligand, Simpy show λ_{max} at around 281 nm, 360 nm, (75040). All these bands were assigned as

intramolecular π-π*, n-π* transitions.[19] The characterization of ligand Simpy was also established unambiguously by GC-MS spectrometry (Fig. 3.2), ^{1}H and ^{13}C NMR spectroscopic studies (Fig. 3.3). The MS spectra of ligand Simpy gives molecular ion (M^{+}) peak at 293 (2.13 %), M^{+}-CH_3 peak at 278 (26 %) and shows base peak at 169 (100%) for N-phenylpyridylamine ion (probable fragmentation patterns of ligand Simpy are shown in Scheme 3.2).

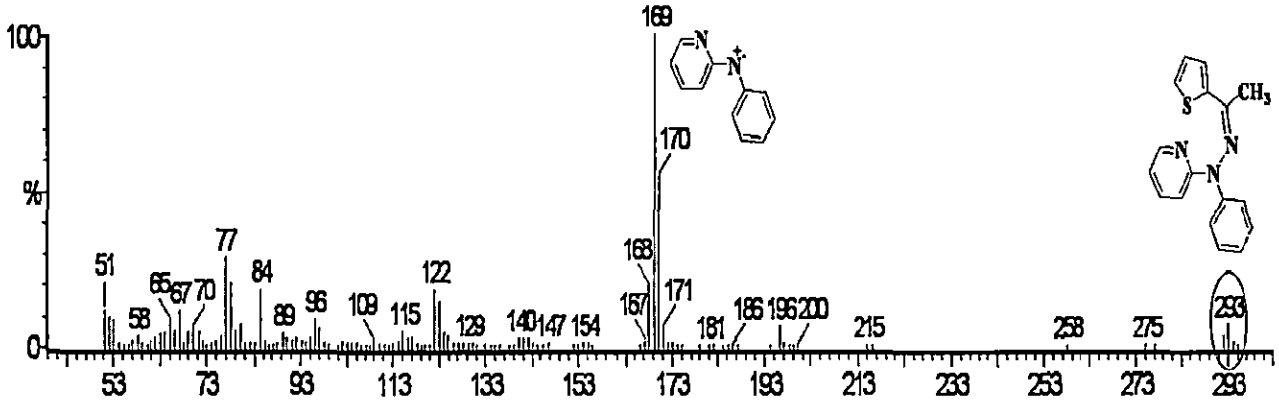
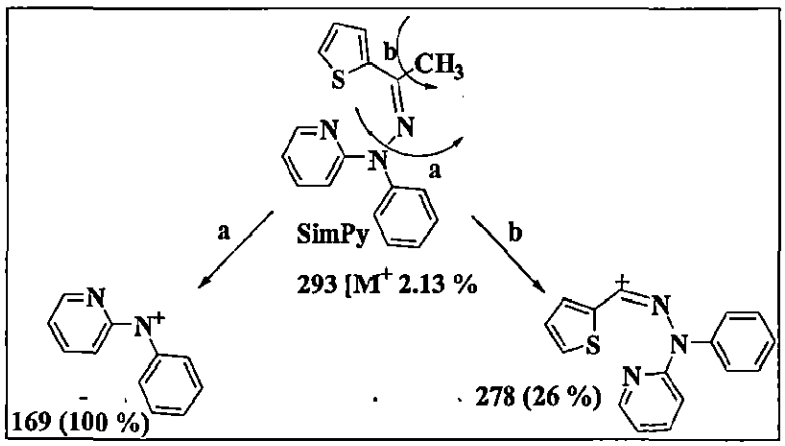


Fig. 3.2 Mass spectra of ligand (Simpy)



Scheme 3.2. Mass fragmentation pattern of ligand Simpy.

^{1}H NMR of Simpy

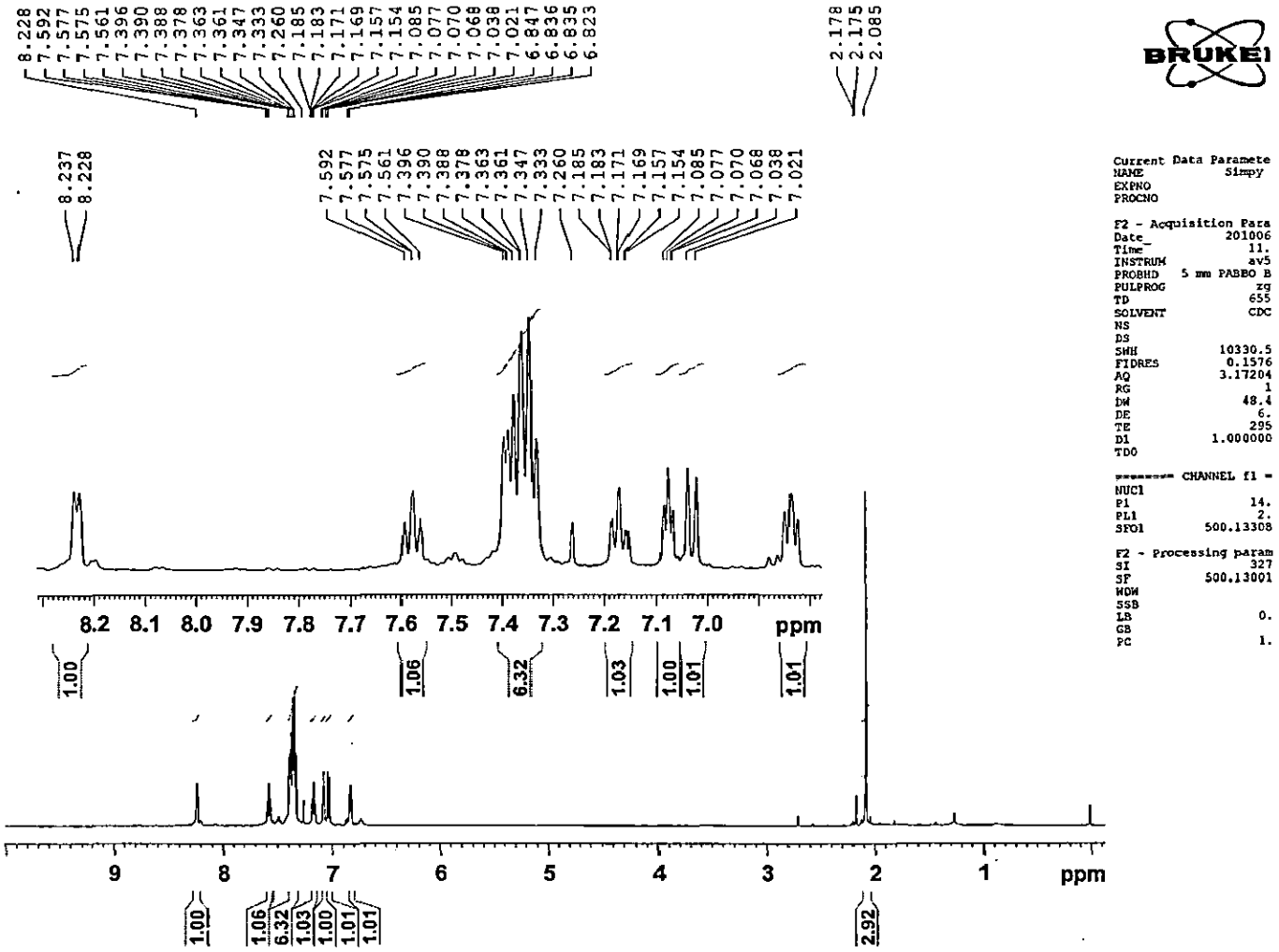


Fig. 3.3 ^{1}H NMR spectra of ligand, Simpy in $CDCl_3$ solvent.

*Table 2.1: selected spectral data of ligand [Simpy]**

Ligand	IR ν/cm^{-1}	UV-visible λ_{max}/nm ($\varepsilon/M^{-1}cm^{-1}$)	GC-MS m/z
Simpy	1583, $\nu_{-C=N}$ 1120, 1084, υ_{ClO4^-} 774, ν_{C-S}	360 nm, (75040)	293 (2.13%) 278 (26 %) 169 (100%)

**Spectra's are shown in Appendice A_6- A_8.*

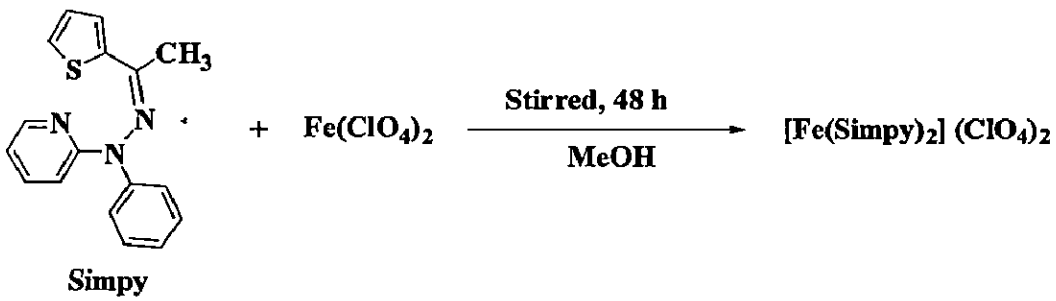
*Table 2.2: ^1H NMR and ^{13}C NMR spectral data of ligand [Simpy]**

Ligand	^1H NMR, $CDCl_3$ δ (ppm from TMS), J/Hz	^{13}C NMR δ (ppm from TMS)
Simpy	2.08 (s, 3H), 6.83 (t, 1H, J= 6), 7.03 (d, 1H, J= 8.5) 7.07 (t, 1H, J= 4), 7.17 (dt, 1H, J= 7, 1), (7.40-7.33 m, 6H), 7.57 (t, 1H, J= 8) 8.23 (d 1H, J= 4.5).	17.77, 111.62, 116.51, 125.01, 125.34, 127.33, 127.52, 128.75, 128.99, 137.48, 143.57, 145.60, 147.70, 159.07, 161.40.

**Solvents was used $CDCl_3$; ^{s}singlet, ^{d}doublet, ^{t}triplet, ^{m}multiplet; spectra of ^{13}C is shown in*

3.2 Syntheses and characterization of metal complex:

Complexes was synthesized by the stirring of ligand, Simpy with iron(II) salts in methanol for 48 h. (scheme 3.3). In both complexes there was no any change in oxidation state of metal centre. Bis complexes were obtained even though reaction started by 1:1 ratio of ligand and salts or 2:1. This was concluded by the conductivity measurement shown in table 2. By the magnetic moment, and NMR data was established the low spin and diamagnetic nature of complex.



Scheme 3.3. Synthesis of complex $[Fe(Simpy)_2](ClO_4)_2$

3.2.1 *IR spectral studies:*

The azomethine (-C=N-) characteristic band in IR for the free ligand was observed at ≈1530-1600 cm^{-1}. Coordination of the nitrogen to the metal centre, generally reduced the electron density in the azomethine moiety and thus lowered the (-C=N-) frequency[20], but here the (-C=N-) frequency (from 1583 cm^{-1} to ~1625). This is due to the back bonding by metal atom towards ligand.[21] Increase in stretching frequencies of $\nu_{C=N}$ in $[Fe(Simpy)_2](ClO_4)_2$ clearly indicates the ligation of azomethine nitrogen (-C=N-) to metal centre. (Fig. 4) The $\nu_{C=N}$

for $[Fe(Simpy)_2](ClO_4)_2$, 1625 respectively. IR bands ν_{ClO4^-} 1116, 1084 cm^{-1} together with a band at 621 cm^{-1} were found. All values are tabulated in table 2.

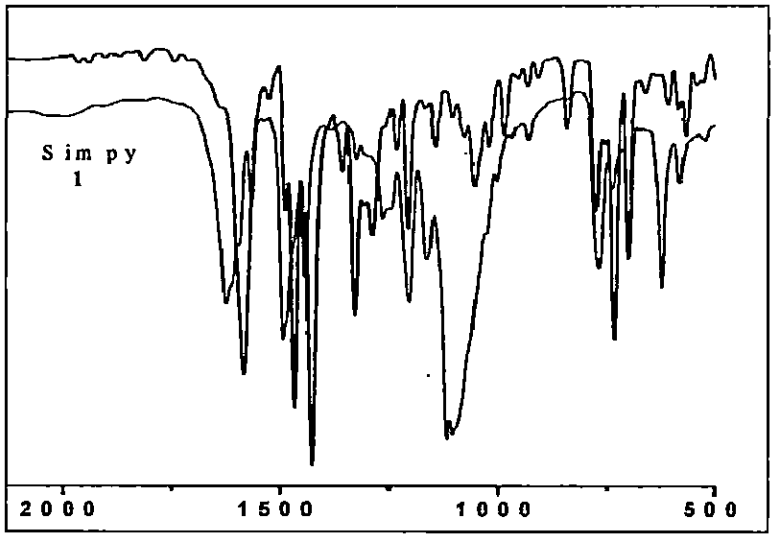


Fig. 3.4 IR spectra of ligand(-----) and complex(-----)

3.2.2 *Conductivity studies:*

Experimental data for molar conductivity are tabulated in Table 2. The molar conductivity measurements in acetone at *ca.* 10^{-3} M determined at 298 K for complex was found to be 154 $\Omega^{-1}cm^2mol^{-1}$. This value confirmed that uni-bivalent (1:2) electrolyte behaviour in solution.[21]

3.2.3 *Magnetic moment study:*

For complex magnetic moments were measured at room temperature (298 K). Complex $[Fe(Simpy)_2](ClO_4)_2$ was diamagnetic in nature and stabilized the low spin d^6 system.[18]

3.2.4 *Electronic spectral studies:*

The absorption spectra of complex $[Fe(Simpy)_2](ClO_4)_2$ were recorded in dimethylformamide at room temperature. The electronic spectral data are depicted in Table 2.3

and spectra are shown in Fig. 3.5. The transition around 275 nm is designated as π-π* transition which is observed in ligand also. Two shoulder are also appeared near 311 nm and 349 nm with extinction coefficient values of 13,240 and 8480 $M^{-1}cm^{-1}$ respectively. The absorption maxima at 518 nm of complex was assigned to metal-ligands charge transfer transitions.[19]

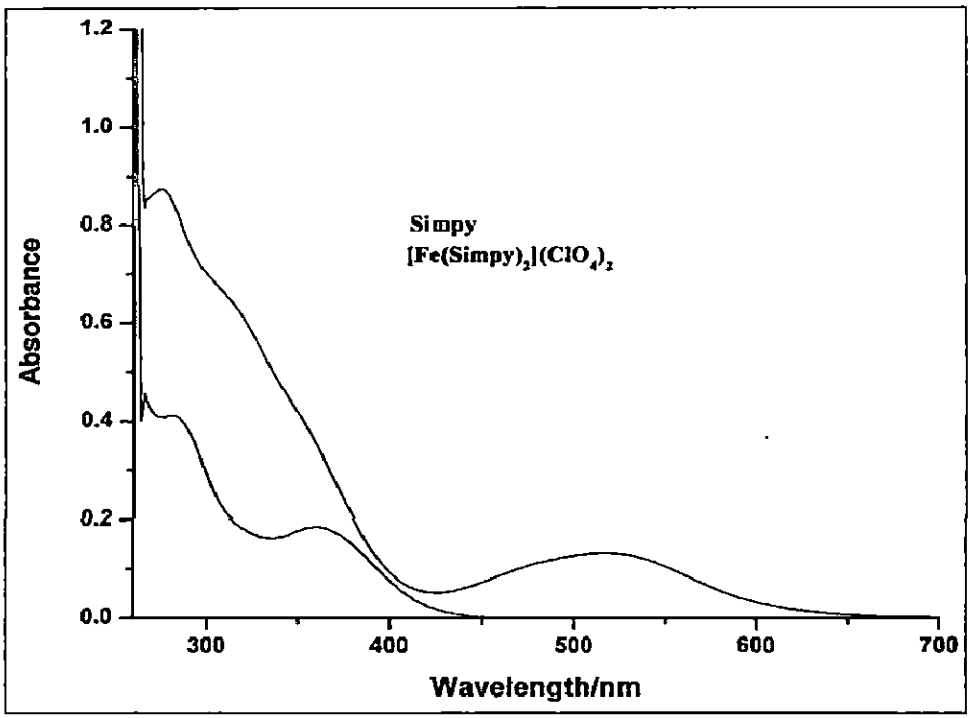


Fig. 3.5 UV-visible spectra of ligand Simpy and complex $[Fe(Simpy)_2](ClO_4)_2$

Table 2.3: Selected spectral data of metal complex *[Appendice A_{10}- A_{12}]*

Complex	λ_{max}/nm (ε/$M^{-1}cm^{-1}$)	Conductivity ($\Omega^{-1}cm^2mol^{-1}$)	IR ν/cm^{-1}
$[Fe(Simpy)_2](ClO_4)_2$	518 (2620), 349 (8480), 311 (13240), 275 (17750)	154	$\nu_{C=N}$ 1625, ν_{ClO4^-} 1116, 1084, 621

3.3 DNA interaction studies

3.3.1 *Fluorescence spectroscopic studies:*

In binding of the metal complex to DNA was examined by EthBr fluorescence displacement experiments. Principle and methods of the experiment is same as given in ***chapter 1***. In my experiment, as described in Fig. 6 the fluorescence intensity of EthBr shows a remarkable decreasing trend with increasing concentration of the metal complexes. This indicates that some EthBr molecules are free from EthBr-DNA after a swapping with the complex; results the fluorescence reducing of EthBr. The emission spectra of EthBr bound to DNA in absence and presence of complexes are shown in Fig. 6.

The fluorescence quenching curve of DNA-bound EthBr by complexes (Fig. 3.6) illustrate that the quenching of EthBr bound to DNA by complex is in good agreement with the linear Stren-Volmer equation.[22] The K_{SV} values (using equation (i)) for complex $[Fe(Simpy)_2](ClO_4)_2$ was 6.785×10^4. This result also proves that the partial replacement of EthBr bound to DNA by the complex results in a decrease of the fluorescence intensity. From K_{SV} data external binding with partial intercalation was speculated.

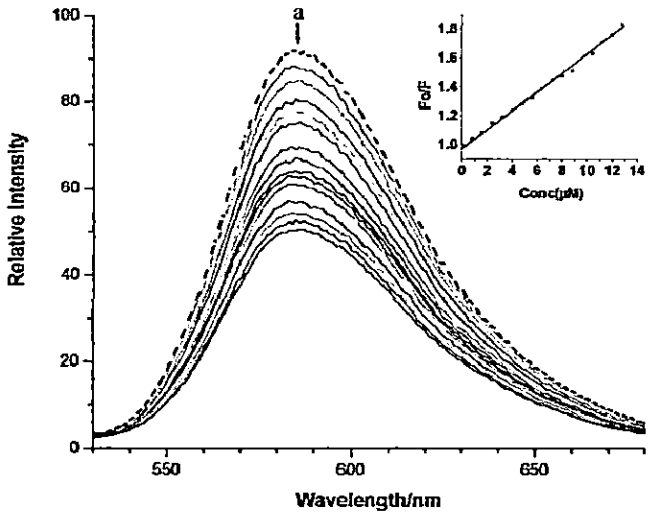


Fig. 3.6 The fluorescence quenching of EthBr–DNA systems by titrations of complex $[Fe(Simpy)_2](ClO_4)_2$.

3.3.2 *Circular dichroism:*

Addition of complex in CT-DNA solution gave rise to decrease in intensity in both negative and positive peaks. This data indicate the complex is interacting through external or surface binding.[23]

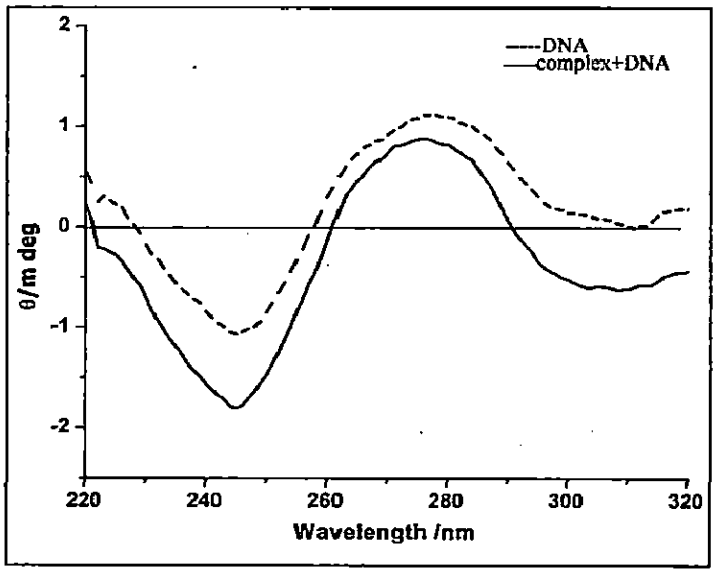


Fig. 3.7 Induced CD spectra of CT DNA (25 μM) in absence (dashed line) and in presence (solid line) of complex $[Fe(Simpy)_2](ClO_4)_2$ in phosphate buffer at pH 7.2 (10% methanol).

3.3.3 *DNA cleavage:*

The DNA cleavage activity of complex has been studies under physiological pH and temperature by gel electrophoresis using super coiled pBR322 plasmid as the substrate. Complex did not show any nuclease activity in the presence of oxidizing agent (H_2O_2).

Fig. 3.8 Gel electrophoresis separations showing the oxidative cleavage of super-coiled pBR322 DNA (200 ng) by complex in10% DMF incubated at 37°C for 3h using H_2O_2 (300 μM).

DNA (lane 1); DNA + H_2O_2 (lane 2); DNA + **1** (100 μM) (lane 3); DNA+ **1** (50 μM) + H_2O_2 (lane 4); DNA + **1** (75 μM) + H_2O_2 (lane 5); DNA + **1** (100 μM) + H_2O_2 (lane 6).

* *'1' is denoted here complex* $[Fe(Simpy)_2](ClO_4)_2$.

4 Conclusion:

In this study we prepared tridentate (N_2S) Simpy ligand and reaction Simpy with iron(II) salt gave rise to complex $[Fe(Simpy)_2](ClO_4)_2$. The new synthesized ligand Simpy stabilized the complex $[Fe(Simpy)_2](ClO_4)_2$ with iron in low spin, diamagnetic state. It was proved by magnetic moment and ^1H NMR spectral studies. DNA binding studies of complex showed that a partial intercalation or external binding with CT-DNA. These were supported by Ksv and circular dichroism spectral investigations. The complex was not efficient in nuclease activity.

5 References:

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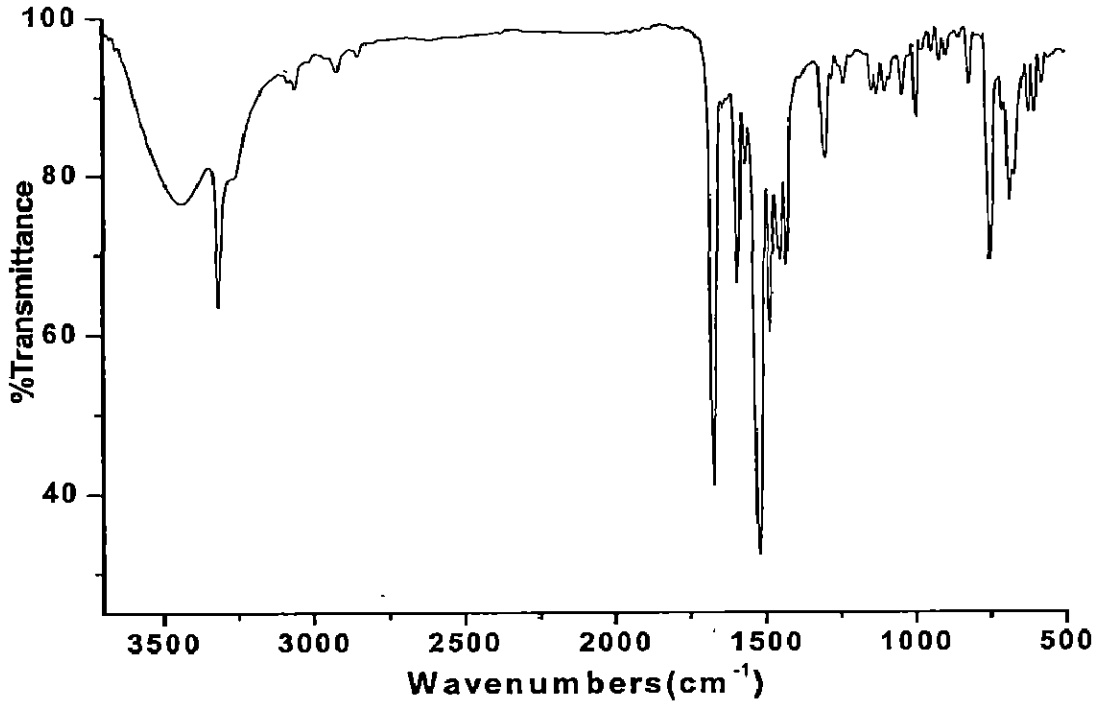
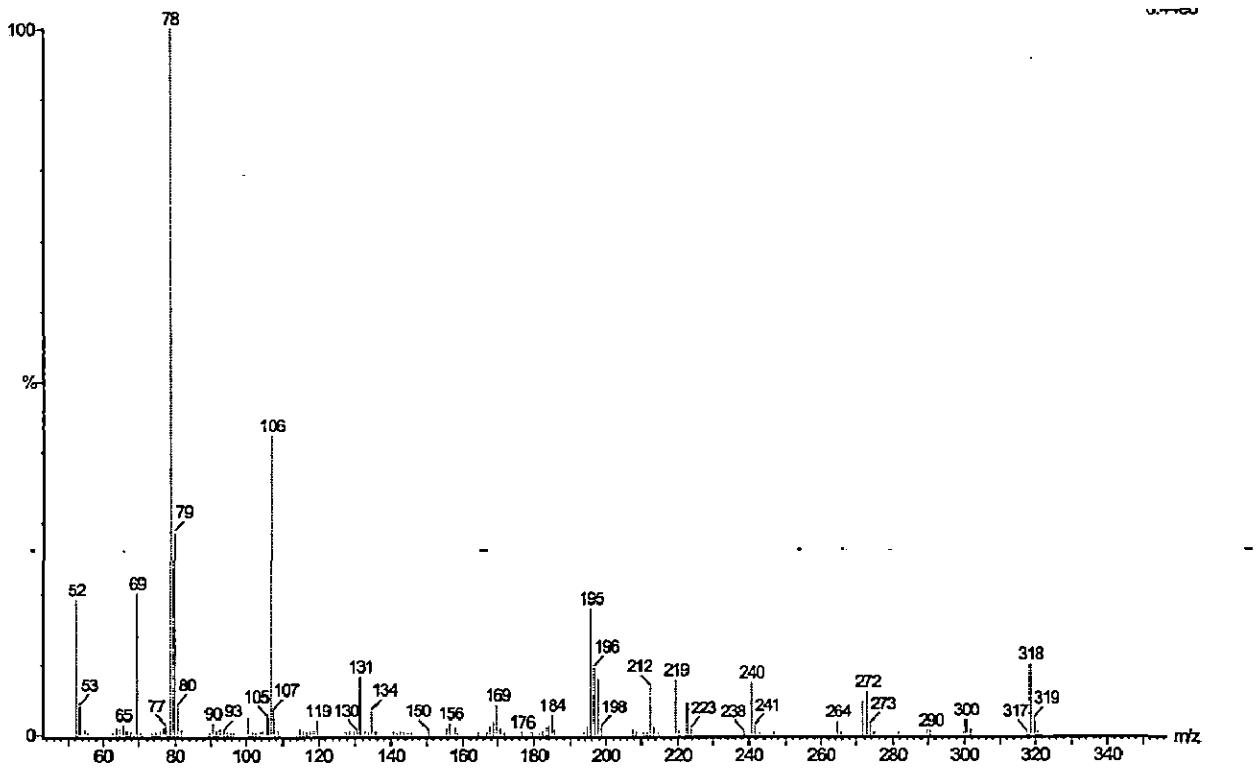
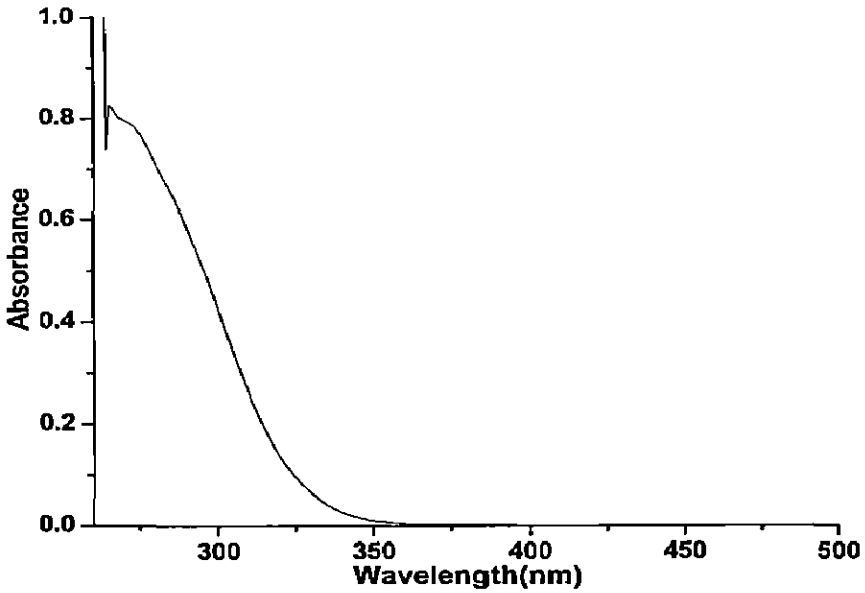
Appendix A_1: IR spectra of ligand H_2bpb



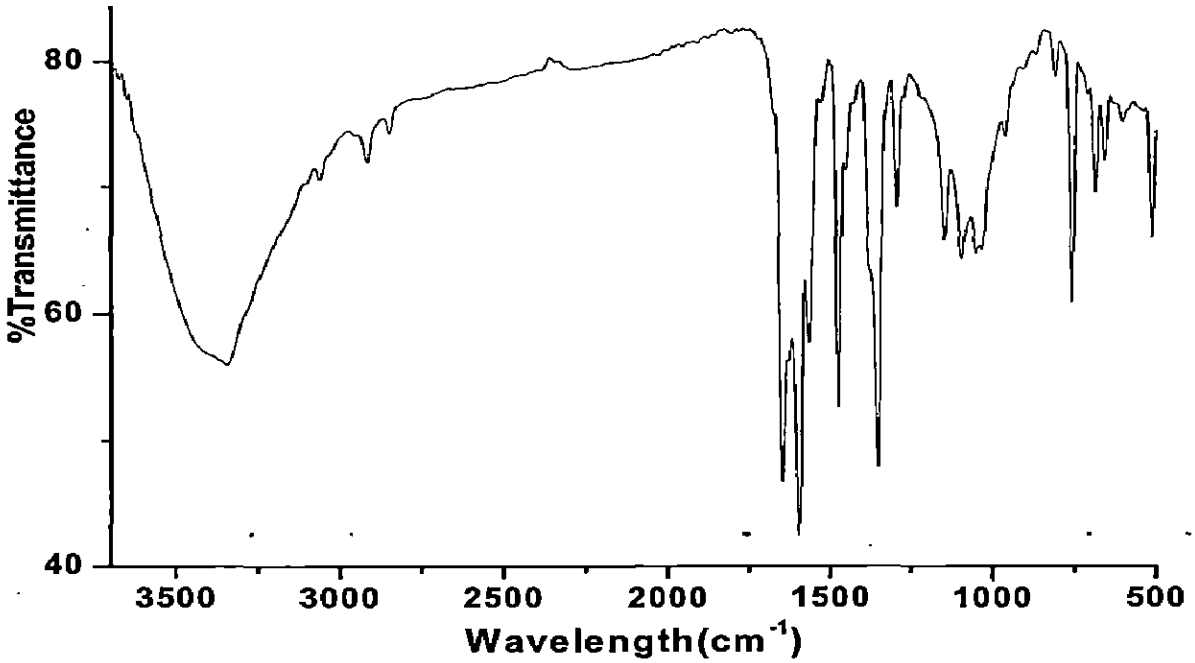
Appendix A_2: Mass spectrometry spectra of ligand H_2bpb



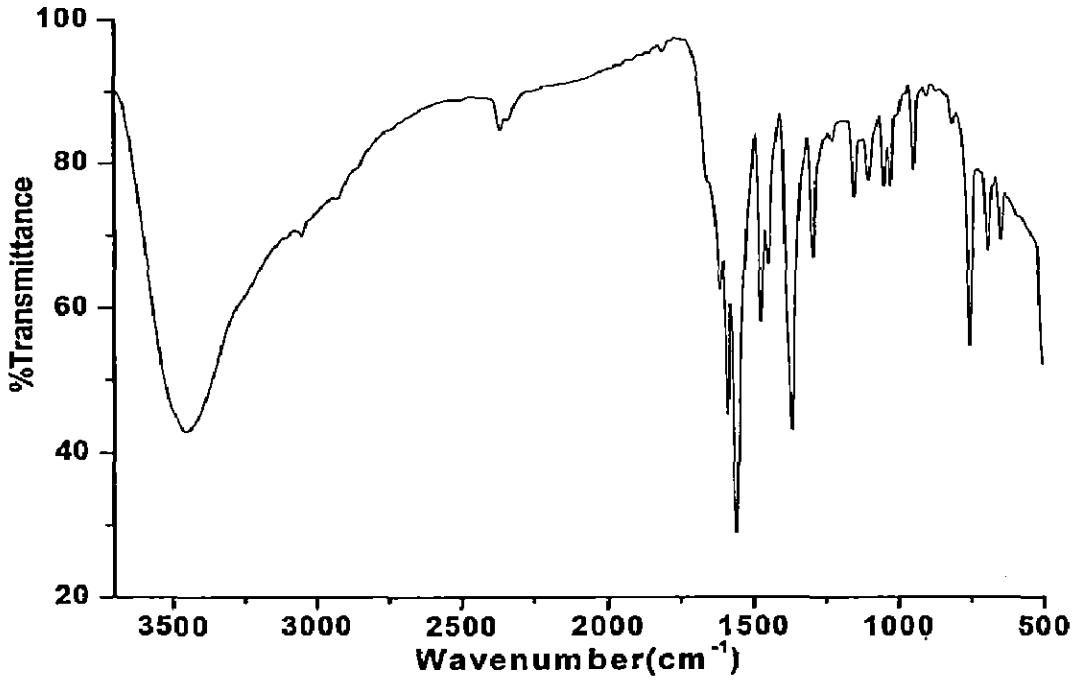
Appendix A_3: UV-vis spectra of ligand H_2bpb



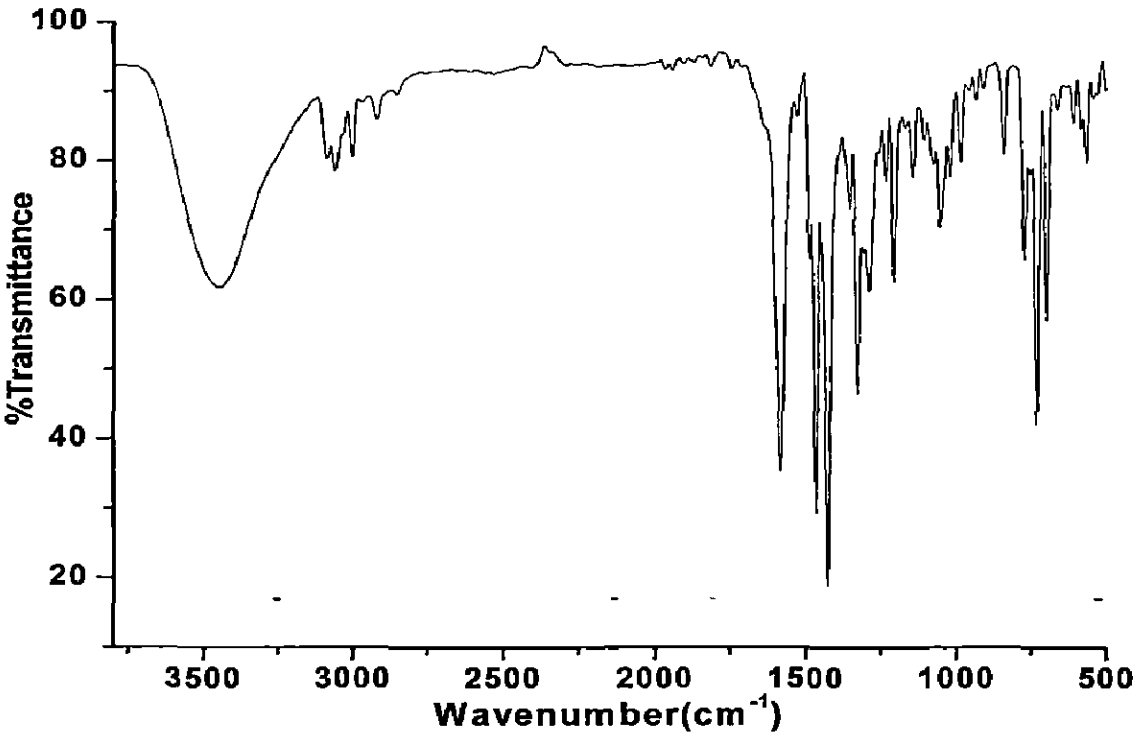
Appendix A_4: IR spectra of complex **1** [Mn(bpb)(Cl)(H_2O)]



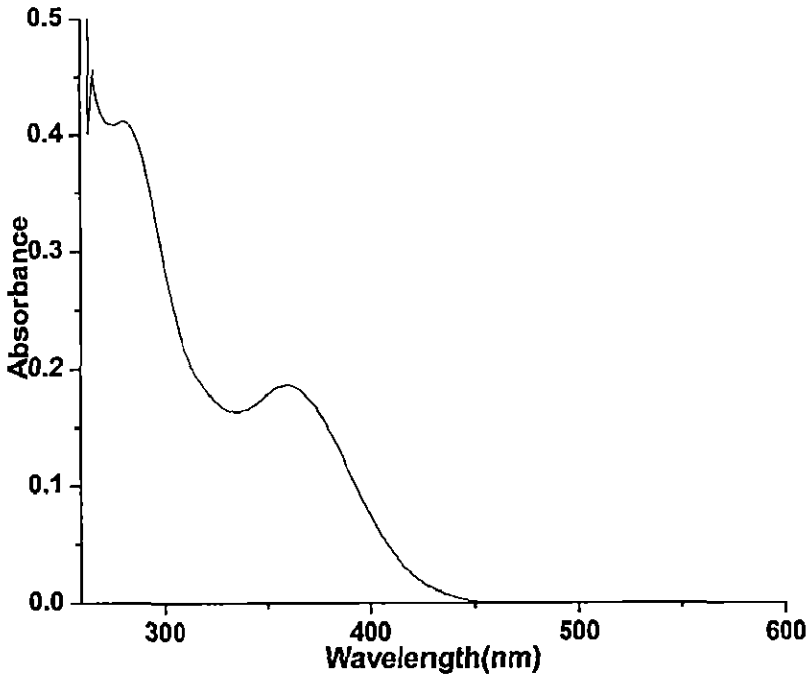
Appendix A_5: IR spectra of complex 2 $[Fe(bpb)(Cl_2)]Et_3NH$



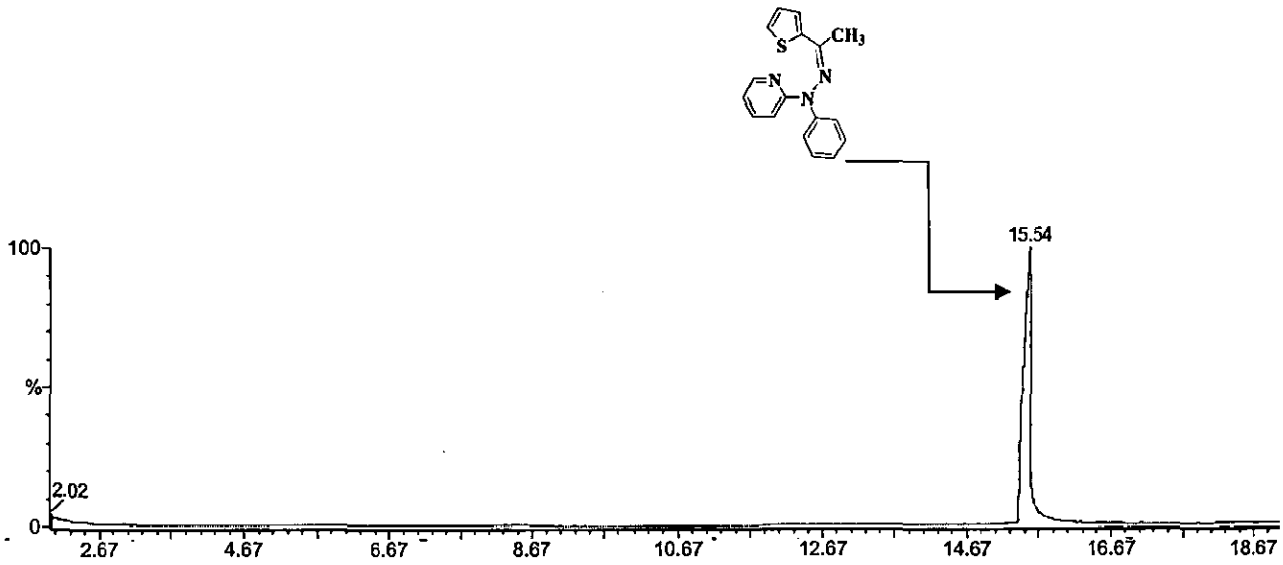
Appendix A_6: IR spectra of ligand Simpy



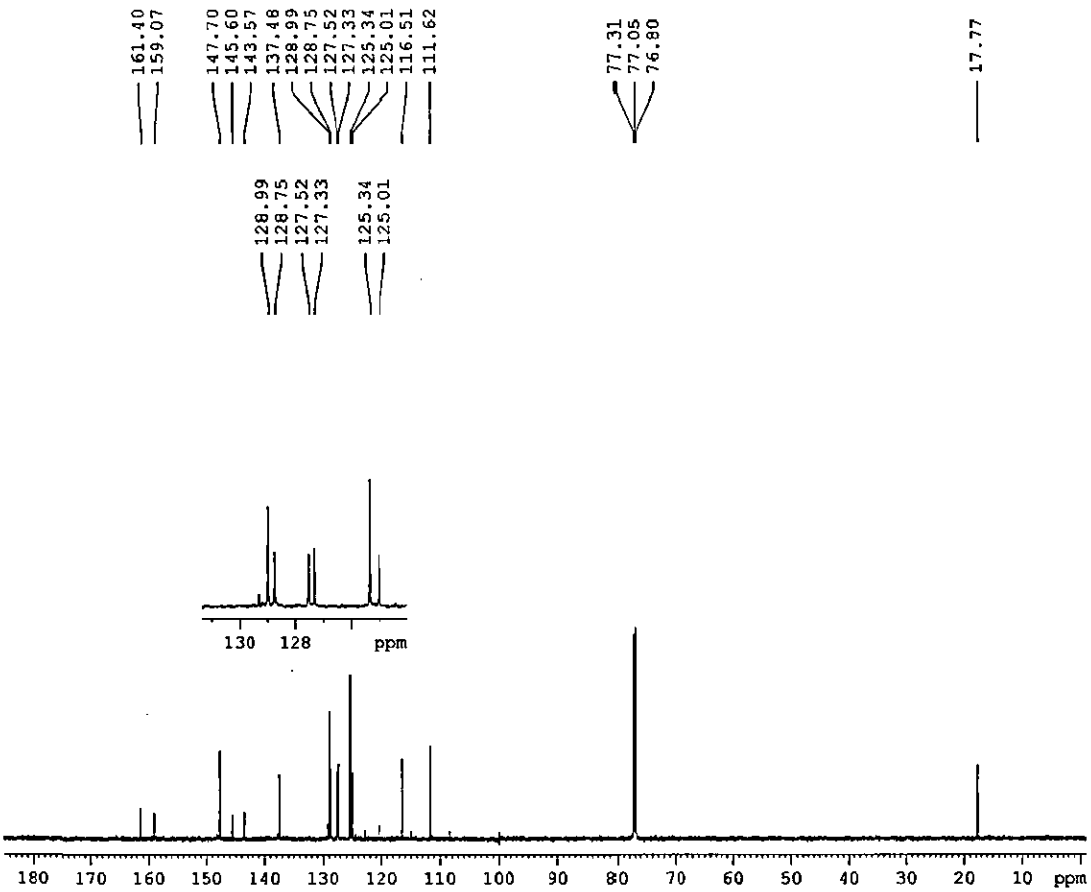
Appendix A_7: UV-vis spectra of ligand Simpy



Appendix A_8: Gas chromatogram of ligand Simpy



Appendix A_9: ^{13}C NMR of Simpy



BRUKER

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PROCNO 1

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RG 1030
DW 16.650 usec
DE 6.00 usec
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TD0 1

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PL1 1.00 dB
SFO1 125.7703643 MHz

======== CHANNEL f2 ========
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PL2 2.00 dB
PL12 16.60 dB
PL13 20.60 dB
SFO2 500.1320005 MHz

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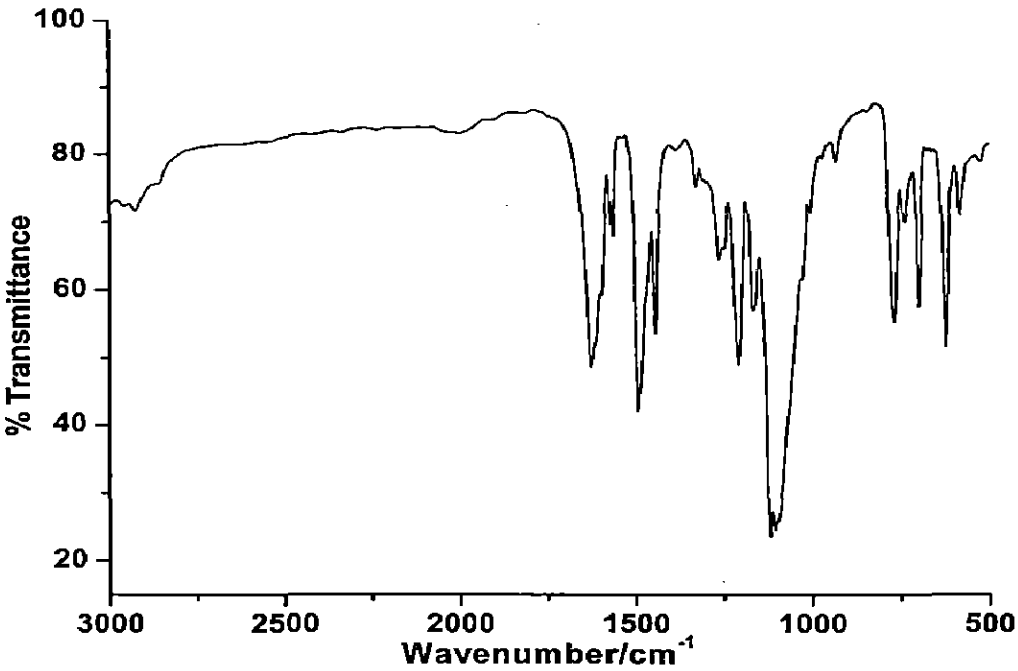
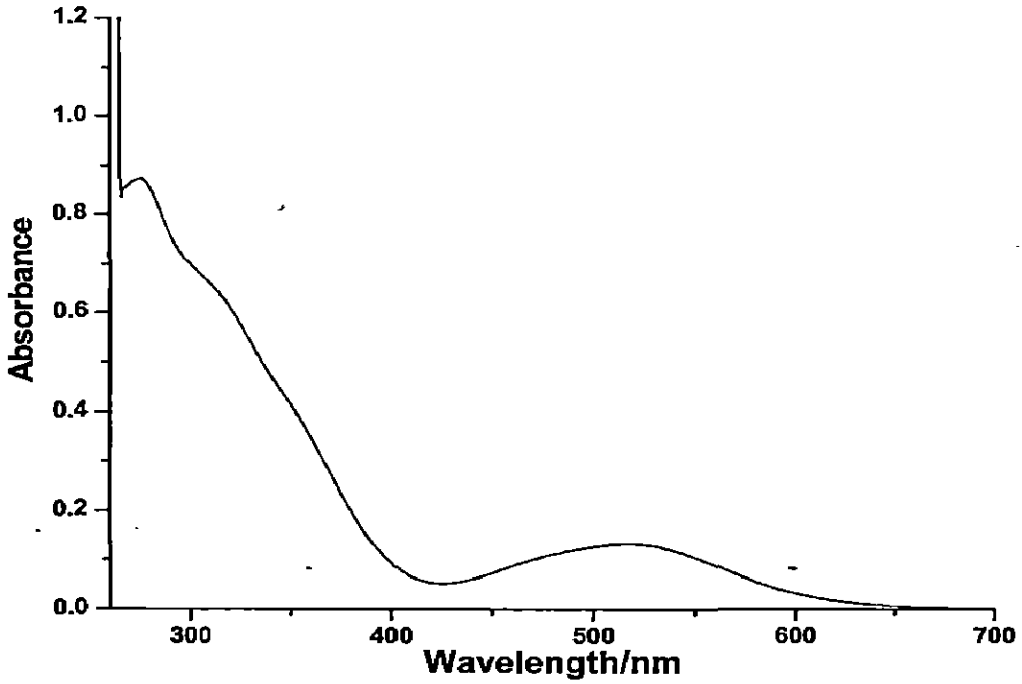
Appendix A_{10}: IR spectra of complex $[Fe(Simpy)_2](ClO_4)_2$



Appendix A_{11}: UV-vis spectra of complex $[Fe(Simpy)_2](ClO_4)_2$



pendix A_{12}: ^{1}HNMR of complex $[Fe(Simpy)_2](ClO_4)_2$