SYNTHESIS AND APPLICATIONS OF FLAVINS AND THEIR ANALOGUES

Thesis submitted to the Delhi Technological University

for the award of the Degree of

DOCTOR OF PHILOSOPHY

by

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September 2017

Dedicated

To

My family

DECLARATION

I hereby declare that this Ph.D. thesis entitled "**Synthesis and Applications of Flavins and their analogues**" was carried out by me for the degree of Doctor of Philosophy under the joint guidance and supervision of Dr. Ram Singh, and Dr. Richa Srivastava, Department of Applied Chemistry, Delhi Technological University (DTU), Delhi, India.

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort has been made to indicate this clearly with proper citation.

For the present thesis, which I am submitting to the University, no degree or diploma has been conferred on me before, either in this or in any other University.

Place: Delhi

Date: (Deepshikha Rathore)

CERTIFICATE

This is to certify that the thesis entitled "***Synthesis and Applications of Flavins and their analogues***" submitted by **Ms. Deepshikha Rathore** to **Delhi Technological University**, for the award of the degree of "Doctor of Philosophy" is a record of bonafide work carried out by her. Ms. Deepshikha Rathore has worked under our guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to our knowledge has reached requisite standards.

The results contained in this thesis are original and have not been submitted to any other university or institute for the award of any degree or diploma.

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ABSTRACT

Flavoenzymes are colourful oxidoreductases occurring widely in living systems. The flavin cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are involved in the catalysis of a wide variety of biological redox reactions. To understand the molecular mechanism of the flavoenzymes and their applications, the quantitative amount of these cofactors and their analogues are essential. Hence, the synthesis of flavins has been the focus of research due to its paucity in the natural systems.

Chapter 1 deals with the solid and solution phase synthesis of flavin and their analogues. The traditional solution phase synthesis suffers from a number of limitations and hence a better and more environmental friendly synthesis is achieved in this chapter using the Merrifield resin as polymer support. The polymer-supported synthesis was found to be advantageous over solution phase synthesis.

Chapter 2 describes the application of flavin as catalyst for the Baeyer-Villiger (BV) Oxidation. The Baeyer Villiger (BV) oxidation is the basic reaction for the synthesis of lactones. In this chapter, different flavin derivatives have been utilized as catalyst in hydroperxy form for the transformations of cyclic ketones to corresponding lactones. The reactions have been performed to validate biomimetic reaction for BVMOs. Also, the developed catalyst served as metal free organocatalyst. Further, this chapter discussed the effect of chain length at N-10 position on BV reactions.

Chapter 3 explains about the application of flavin as catalyst for remediation of selected halogenated compounds. Halogenated organic compounds (HOCs) are considered as a major pollutant for any ecosystem due to their persistent, lipophilic and toxic nature. we have mimicked the reactions of cholorophenol monoxygenases. Under the present newly designed biomimetic experimental conditions, we confirmed the removal of chlorine in 4-halophenols using synthetic cofactor of flavoenzymes.

Chapter 4 explains the electrochemical study of flavin derivatives. In this context, we report the electrochemical oxidation-reduction process of riboflavin in buffered aqueous solution at varying pH using cyclic voltammetry (CV) techniques. The CV results indicate that the redox voltammetry behavior of riboflavin (Rf) at the sensor was reversible as well as quasi-reversible involving two anodic and cathodic peaks. Electrochemical kinetic parameters for Rf at different pH values in buffered aqueous solution have been calculated, which indicates the structural changes/ behavior of Rf at different pH conditions and provides insight into the one or two electron transfer reaction.

Chapter 5 deals with the studies of *Bombax ceiba* flower extract and their secondary metabolite conjugate with flavin. In this chapter; we have utilized the crude and semi crude extract of *B. ceiba* for identification of group of compound present in it using chemical test method. Further, the extract has been evaluated for antioxidant properties. An secondary metabolite, vanillic acid has also been conjugated with riboflavin tetracetate and evaluated for their antioxidant properties.

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List of important abbreviations

S. No.	Abbreviations	Full names
1	FMN	Flavin Mono Nucleotide
2	FAD	Flavin Adenine Dinucleotide
8	DMF	Dimethyl Formamide
4	TMS	Tetramethylsilane
5	TLC	Thin layer chromatography
6	DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
7	DMSO	Dimethyl sulfoxide
8	THF	Tetrahydrofuran
9	FTIR	Fourier Transform Infrared
10	Mp	Melting Point
11	TFA	Trifluoroacetic Acid
12	DCM	Dichloromethane
13	BVMOs	Baeyer-Villiger Monooxygenases
14	CHMO	Cyclohexanone Oxygenase
15	CPMO	Cyclopentanone Oxygenase
16	PCBs	Polychlorinated Biphenyls
17	PBDEs	Polybrominated Diphenyl Ethers
18	DDT	Dichlorodiphenyl Trichloroethane
19	EPA	Environmental Protection Agency
20	CDDs	Chlorinated Dibenzo-p-dioxins
21	CDFs	Chlorinated Dibenzofurans
22	WHO	World Health Organization
23	TDI	Tolerable Daily Intake
24	POPs	Persistent Organic Pollutants
25	HOCs	Halogenated Organic Compounds
26	RE	Reference Electrode
27	PL	Photoluminescence
28	PB	Phophate Buffer
29	DPPH	2,2-Diphenyl-1-picrylhydrazyl
30	MIC	Minimum Inhibitory Concentration

CHAPTER 1

Solid and Solution phase synthesis of Flavin and their analogues

1.1 Introduction

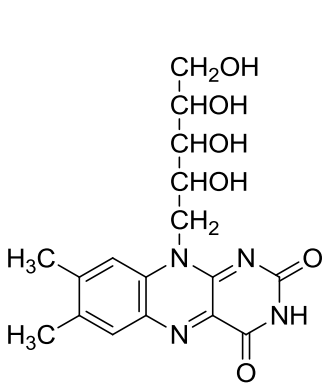
Heterocyclic compounds are the key building blocks for the development of medicinally and biologically active compounds.[1] These compounds are cyclic in nature and possess at least one atom other than the carbon (C). In most of the heterocyclic compounds, the other atoms are nitrogen (N), oxygen (O) and sulphur (S). The development of environmental friendly methods for the synthesis of these compounds is always the focus of research due to large number of applications in different fields. The increasing environmental consciousness all over the world has put a pressing need to develop an alternative synthetic approach from the traditional method of synthesis. This requires novel approaches which reduce the material and energy of chemical processes and products. The synthetic approaches must also minimize or eliminate the dispersion of harmful chemicals in the environment and meets the challenges of green chemistry.[2-5]

In general, traditional method of synthesis which is also known as conventional synthesis is used to produce existing and novel compounds. These methods suffer from one or more disadvantages. Some of the disadvantages include long reaction time, high temperature, use of corrosive acids, less atom economy and so on.[6-8] To overcome these disadvantages, the chemists are constantly looking for the alternative methods for synthesis. Solid phase synthesis is one method which overcomes some of the above mentioned disadvantages.[9,10] Polymer supported synthesis is an important class of solid phase synthesis which has been successfully applied for the synthesis of biologically active as well as non-active compounds.[11-15]

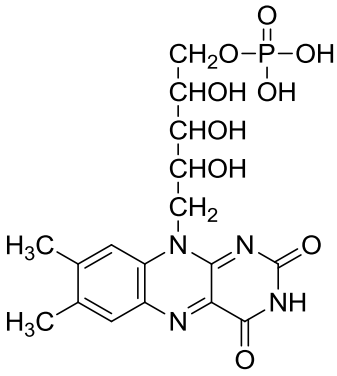
Flavin is tricyclic nitrogen containing heterocyclic molecule. The term 'flavin' refers to the yellow chromophoric and redox active prosthetic group of a class of redox enzymes occurring widely in animals and plants, called the flavoenzymes.[16-18] The term 'flavin' is considered as interchangeable with the term 'isoalloxazine'. The flavin family includes vitamin B_2 (Riboflavin, **1**), flavin mononucleotide (FMN, **2**) and flavin adenine dinucleotide (FAD, **3**) (Figure 1.1) and other synthetic derivatives of flavins.[19] The flavin cofactors FMN (**2**) and FAD (**3**) are involved in the catalysis of a wide variety of biological redox reactions, including the dehydrogenation of NAD(P)H, lipid esters and D-amino acids, the oxidation of amines to imines and N-oxides, the formation and cleavage of disulphide bonds, the hydroxylation of aromatic substrates and the activation of molecular oxygen.[20-27] The importance of compounds of flavin family has shown the need for the development of their synthetic methodology. The synthesis of flavin cofactors has been the

focus of research due to its paucity in the natural systems. To understand the molecular mechanism of the proteins containing these cofactors, the quantitative amount of these cofactors is essential.

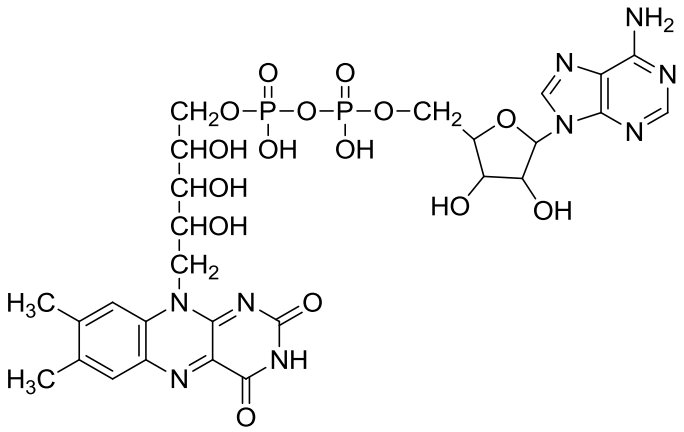
This chapter deals with synthesis of selective flavin derivatives *via* solid phase and solution phase methods of synthesis. The methodology applied is a step towards the synthesis of flavin in environmental friendly manner. In the present chapter, some modifications in the classical synthetic methods have been done for the synthesis of 10-substituted flavins (isoalloxazines) with the aim to make the synthesis environmental friendly and to increase the yields with the help of solid and solution phase methods.



Riboflavin (**1**)



FMN (**2**)



FAD (**3**)

Figure 1.1: Flavins in nature

1.2 Solid and solution phase synthesis of flavins: Literature Review

1.2.1 Solid phase synthesis of flavins

Solid phase synthesis is pioneered by Robert Bruce Merrifield.[28] The solid phase synthesis means carrying out a synthesis on solid support such as resin beads, polymer etc (Figure 1.2).[29-32] In solid phase synthesis, solid support is attached to either reactants or sometimes to a particular reagent through covalent or non-covalent interactions. This makes the reaction heterogeneous in nature. A subsequent reaction achieved the desired product, which can then again be cleaved from the solid phase-bound linker and re-used as such or after going through some minor chemical modifications. Commonly combinatorial technique uses organic synthesis on solid support for the preparation of libraries of compounds. Polymeric supports are used in organic synthesis due to their insolubilization and immobilization to the attached species. The polymer support also creates the special micro environmental, which helps the reactions to be carried out in efficient ways. This has been developed in recent years as an important tool in the synthesis of peptides, oligonucleotides, oligosaccharides and other biologically active compounds.[33,34] Further, the polymer-supported synthesis has also been applied in a variety of organic reactions including the synthesis of natural products, heterocycles, medicinal chemistry etc (Figure 1.3).[35-44] Polymer supported synthesis has also been utilized for the synthesis of flavins. Merrifield resin (chloromethylated polystyrene resin) has been used as polymer support for the synthesis of 7-carboxy-10-substituted flavins (Scheme 1.1).[44]

1.2.2 Solution phase synthesis of flavins

The solution phase synthesis is the traditional and conventional method of synthesis. These types of reactions occur in the presence of solvent and reaction takes place in solution. Usually the reactions performed in homogeneous solution are considered as solution phase synthesis. The flavin derivatives have been synthesized using solution phase method.[45-50]

The flavins are usually synthesized by the condensation of *ortho*-substituted anilines with pyrimidines. The various synthetic methodologies of 10-substituted flavins are shown in figure 1.4. The condensation of 2-arylazo compound (**5**) with barbituric acid (**6**) leads to the formation of flavins (**4**).[51-53]

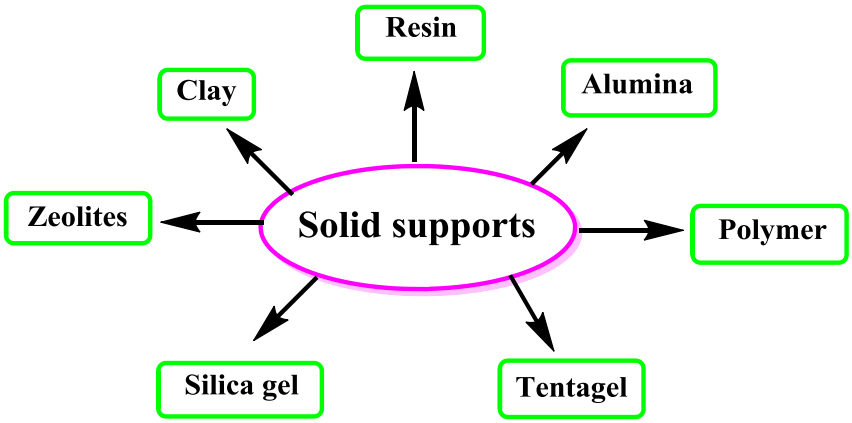


Figure 1.2: Some important solid supports for synthesis

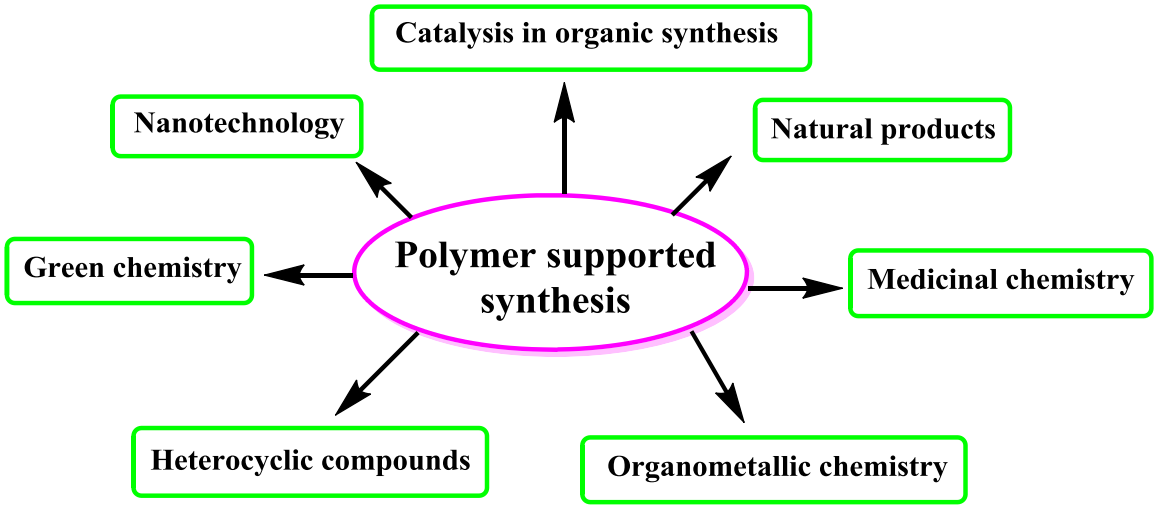
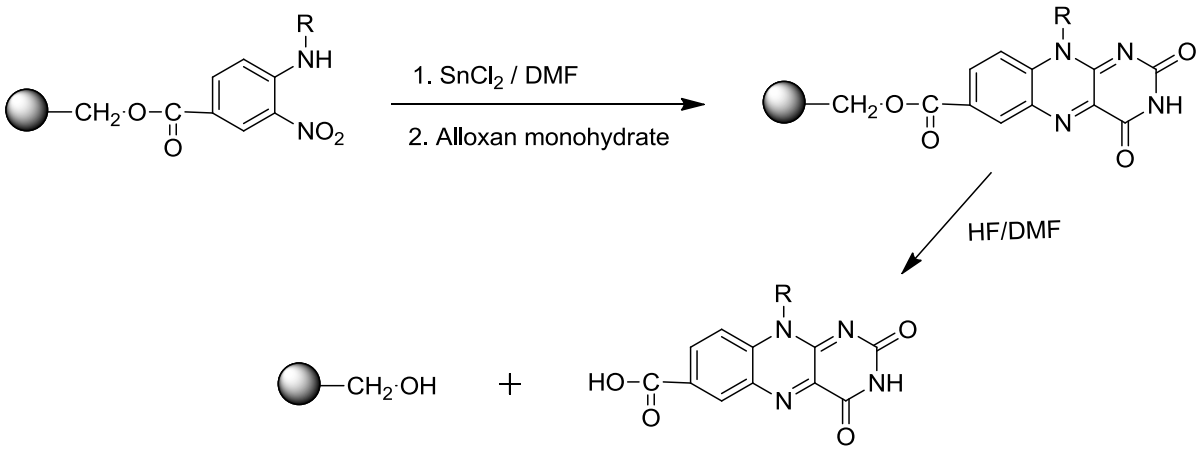


Figure 1.3: Application of polymer-supported synthesis in different fields



Scheme 1.1: Polymer supported synthesis of substituted flavins

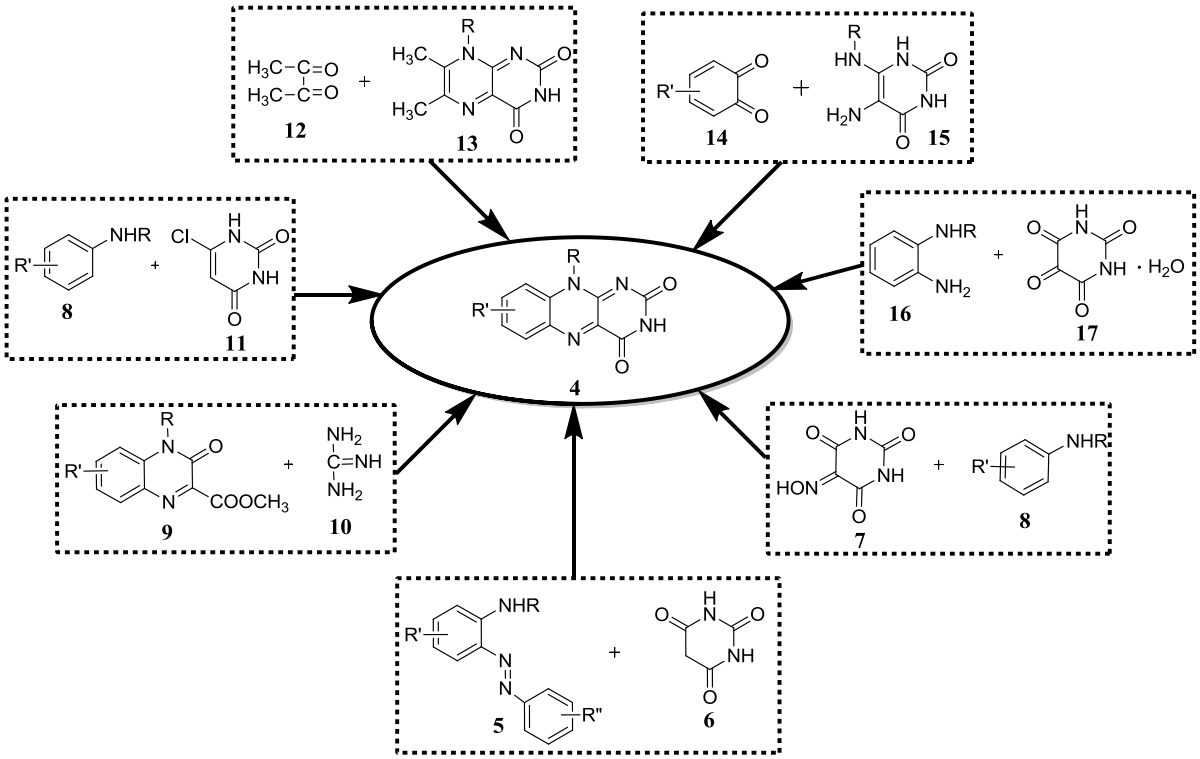


Figure 1.4: Various solution phase synthetic methods of flavins

The violuric acid (**7**) condenses with appropriate anilines (**8**) in acidic condition to give the 10-substituted flavins (**4**) (Figure 1.4). The condensation has been accomplished by heating the reactants in methanol, dilute ethanol, acetic acid, propionic acid, dilute boric acid in water and dilute sodium hydroxide solution. The flavin derivatives were also prepared by heating the quinoxaline (**9**) with guanidine (**10**) under nitrogen atmosphere and in dark in the presence of methyl, ethyl or *n*-propyl alcohol and sodium alcoholate to the refluxing temperature in 1 to 90 hours (Figure 1.4).[54,55]

The reaction of 4-chlorouracil (**11)** with appropriate aniline (**8**) gave 6-(N-substituted anilino)uracil which on nitration in acetic acid with excess of sodium nitrite produced 10-substituted flavin N^5-oxide. The reduction of this compound with sodium dithionite in water gave quantitative yields of flavin (Figure1.4).[56-59] The 10-substituted flavins were also synthesized by the reaction of monomeric biacetyl (**12**) and lumazine (**13**) at 130 ^oC for several hours (Figure 1.4).[60] This is an important reaction because it is closely related to the processes involved in the biosynthesis of riboflavin. The flavin derivatives were also synthesized from *ortho*-benzoquinone (**14**). The condensation of

ortho-benzoquinone (**14**) and diaminopyrimidine (**15**) by heating them in neutral alcohol or water solution or acetic acid solution formed 10-substituted flavins (Figure 1.4).[61,62]

The most widely used method for the synthesis of 10-substituted flavins includes the reaction between N-substituted *ortho*-phenylenediamine (**16**) and alloxan monohydrate (**17**) under acidic conditions (Figure 1.4).[63-69]

1.3 Experimental

Melting points were determined on a laboratory capillary melting apparatus and are uncorrected. The electronic spectra were recorded on a Perkin-Elmer UV-260 spectrophotometer and absorption maxima have been expressed in nanometers (nm). FTIR spectra were recorded on a Perkin Elmer 1710 FTIR spectrophotometer and the ν_{max} are expressed in cm^{-1}. ^1H NMR was recorded on a Bruker Avance-300 spectrophotometer (300 MHz) and the chemical shifts were expressed in ppm. The abbreviation s, d, t, q, m and bs stand for singlet, doublet, triplet, quartret, multiplet and broad singlet respectively. The elemental analysis was measured by Perkin Elmer 2400.

The solvents and reagents were purchased from reputed company and were used without further purifications.

Alloxan monohydrate was obtained from Acros (Belgium), substituted amines and anilines were obtained from Fluka (Switzerland). The 1-chloro-2-nitrobenzene, metal salts and other related reagents were obtained from sd Fine chemicals (India) and other reputed Indian companies.

1.3.1 Solution phase synthesis

General procedure for synthesis of 2-nitro-N-substituted anilines (20)

A mixture of 1-chloro-2-nitrobenzene (**18**) (10 mmol), aniline (**19**) (10 mmol) and salt (16.32 mmol) was taken in a round bottom flask (100 mL) and heated at ~ 50 ^oC for the time specified in the table 1.1 (results and discussion section 1.4). The reaction mixture was cooled to room temperature and partitioned between acidic water-chloroform (1:1 v/v). The chloroform layer was dried (anhydrous Na_2SO_4) and the solvent was evaporated by distillation. The residue was adsorbed on silica gel (60-120 mesh) to make slurry and subjected to column chromatography, eluting with petroleum ether (60-80 ^oC). The

unreacted 1-chloro-2-nitrobenzene (**18**) was eluted first, followed by the elution of highly orange to red coloured product **20**.

2-Nitro-N-phenylaniline (20a)

Mp.: 76 ^{o}C (lit[70] mp. 76 ^{o}C); FTIR (KBr): 3356, 2965, 2850, 1615, 1593, 1570, 1501, 1409, 1346, 1258, 1143, 1258, 1143, 1073, 1029, 741 cm^{-1}; ^{1}H NMR ($CDCl_3$): 6.74-6.80 (m, 3H, H-3', H-4' & H-5'), 7.21-7.29 (m, 2H, H-4 and H-5), 7.33-7.44 (m, 3H, H-2', H-6' and H-6), 8.20 (dd, 1H, H-3, *J* = 8.1 Hz & 2.2 Hz).

2-Nitro-N-(4'-methylphenyl)aniline (20b)

Mp.: 69 ^{o}C (lit[70] mp. 69-70 ^{o}C); FTIR (KBr): 3322, 2932, 2810, 1608, 1564, 1514, 1442, 1345, 1289, 1213, 1160, 1045, 954, 851, 773, 651 cm^{-1}; ^{1}H NMR ($CDCl_3$): 1.98 (s, 3H, CH_3), 6.49 (d, 1H, H-6, *J* = 8.1 Hz), 6.71 (t, 1H, H-5), 7.28-7.46 (m, 4H, H-2', H-3', H-5' & H-6'), 7.57 (t, 1H, H-4), 8.12 (d, 1H, H-3, *J* = 8.2 Hz).

2-Nitro-N-(2'-methylphenyl)aniline (20c)

Mp.: 76 ^{o}C (lit[70] mp. 76 ^{o}C); FTIR (KBr): 3321, 2932, 2810, 1608, 1564, 1514, 1442, 1345, 1289, 1213, 1160, 1045, 954, 851, 773, 651 cm^{-1}; ^{1}H NMR ($CDCl_3$): 1.95 (s, 3H, CH_3), 6.36 (dd, 1H, H-6, *J* = 8.0 & 2.2 Hz), 6.69 (t, 1H, H-5), 7.21-7.26 (m, 2H, H-3'& H-4'), 7.29-7.31 (m, 2H, H-6' & H-5'), 7.38 (t, 1H, H-4), 8.20 (dd, 1H, H-3, *J* = 8.0 & 2.2 Hz).

2-Nitro-N-(4'-chlorophenyl)aniline (20d)

Mp.: 146 ^{o}C (lit[70] mp. 145-146 ^{o}C); FTIR (KBr): 3314, 2930, 2860, 1570, 1532, 1440, 1355, 1258, 1147, 1042, 971, 854, 832, 650, 451 cm^{-1}; ^{1}H NMR ($CDCl_3$): 6.85-7.25 (m, 2H, H-4 & H-6), 7.45-7.60 (m, 4H, H-2', H-3', H-5' & H-6'), 7.85 (d, 1H, H-5, *J* = 8.0 Hz), 8.10 (d, 1H, H-3, *J* = 8.0 Hz).

2-Nitro-N-(2'-chlorophenyl)aniline (20e)

Mp.: 115 ^{o}C (lit[70] mp. 116-117 ^{o}C); FTIR (KBr): 3284, 2924, 2855, 1587, 1530, 1461, 1357, 1258, 1147, 1058, 953, 854, 775, 733, 649, 474, 457 cm^{-1}; ^{1}H NMR ($CDCl_3$): 6.81-7.10 (m, 2H, H-4 & H-6), 7.29-7.38 (m, 4H, H-3', H-4', H-5' & H-6'), 7.80 (d, 1H, H-5, *J* = 8.0 Hz), 8.20 (d, 1H, H-3, *J* = 8.0 Hz).

2-Nitro-N-(4'-methoxyphenyl)aniline (20f)

Mp.: 87-88 ^{o}C (lit[71] mp. 89 ^{o}C); FTIR (KBr): 3330, 2964, 2860, 1618, 1568, 1507, 1438, 1350, 1324, 1247, 1168, 1141, 1106, 1032, 848, 743 cm^{-1}; ^{1}H NMR ($CDCl_3$): 3.81 (s, 3H, OCH_3), 6.39 (1H, H-6, *J* = 8.2), 7.08-7.19 (m, 4H, H-4, H-5, H-4'& H-5'), 7.30-7.39 (m, 2H, H-3' & H-6'), 8.18 (1H, H-3, *J* = 8.3 Hz).

General procedure for synthesis of 10-substituted flavins (4)

To a solution of N-substituted-2-nitroaniline (**20**, 5 mmol) in dry ethanol (40 mL) in paar hydrogen bottle, Pd/C (20 %) (0.02 g) was added and the reaction mixture was hydrogenated at ambient pressure and temperature till the colour of the solution disappears (for 15-24 h). When the quantitative amount of hydrogen was absorbed and the reaction mixture became almost colourless, glacial acetic acid (2 mL) was added to the reaction mixture and the catalyst was filtered off. The alloxan monohydrate (**17**, 5 mmol), boric acid (5 mmol) and glacial acetic acid (2 mL) were added to the filtrate, and the reaction mixture was refluxed for 1 h. The reaction mixture was allowed to cool to room temperature and kept overnight in refrigerator. The resulting precipitate was filtered off to give 10-substituted flavins (**4**).

10-Phenylflavin (4a)

Mp. >300 ^{o}C (lit[72] mp. 335-336 ^{o}C); UV-visible (CH_3OH) λ_{max}: 262, 333, 435 nm; FTIR (Nujol): 3684, 3020, 2959, 2929, 2873, 1720, 1676, 1525, 1478, 1422, 1208, 1016, 929, 791 cm^{-1}; ^{1}H NMR (DMSO-d_6): 6.81 (d, 1H, H-9, *J* = 8.40 Hz), 7.43 (d, 2H, H-3', *J* = 6.90 Hz), 7.60-7.77 (m, 5H, Ar-H), 8.22 (d, 1H, H-6, *J* = 7.83 Hz).

10-(4'-Methylphenyl)flavin (4b)

Mp. >300 ^{o}C; UV-visible (CH_3OH) λ_{max}: 268, 338, 439 nm; FTIR (Nujol): 3520, 3320, 2922, 1714, 1664, 1547, 1460, 1272, 1196, 1090, 880, 875 cm^{-1}; ^{1}H NMR (DMSO-d_6): 1.73 (s, 3H, CH_3), 6.81 (dd, 1H, H-9, *J* = 8.0 Hz & 2.0 Hz), 7.23 (t, 1H, H-8), 7.36 (d, 2H, H-3' & H-5'), 7.63 (d, 2H, H-2' & H-6'), 7.74 (t, 1H, H-7), 8.18 (d, 1H, H-6, *J* = 8.8 Hz).

10-(2'-Methylphenyl)flavin (4c)

Mp. >300 ^{o}C (lit[72] mp. >300 ^{o}C); UV-visible (CH_3OH) λ_{max}: 265, 336, 443 nm; FTIR (KBr): 3448, 3056, 1705, 1641, 1585, 1508, 1458, 1398, 1217, 1181, 832 cm^{-1}; ^{1}H NMR

(DMSO-d_6): 2.01 (s, 3H, CH_3), 6.73 (d, 1H, H-9, J = 9.0 Hz), 7.45 (t, 1H, H-8), 7.51-7.57 (m, 4H, Ar-H), 7.80 (t, 1H, H-7), 8.32 (dd, 1H, H-6, J = 9.0 Hz & 2.2 Hz).

10-(4'-Chlorophenyl)flavin (4d)

Mp. >300^oC (lit[73] mp. >300 ^oC); UV-visible (CH_3OH) λ_{max}: 270, 333, 440 nm; FTIR (Nujol): 3322, 2923, 1718, 1663, 1547, 1460, 1273, 1196, 1090, 888 and 875; ^1H NMR (DMSO-d_6): 6.88 (dd, 1H, H-9, J = 8.1 Hz & 2.2 Hz), 7.23-7.25 (m, 1H, H-8), 7.36-7.38 (m, 2H, H-3' & H-5'), 7.63-7.64 (m, 2H, H-2' & H-6'), 7.71-7.74 (m, 1H, H-7), 8.16 (d, 1H, H-6, J = 8.4 Hz).

10-(2'-Chlorophenyl)flavin (4e)

Mp. >300 ^oC (lit[73] mp. >300 ^oC); UV-visible (CH_3OH) λ_{max}: 266, 335, 444 nm; FTIR (KBr): 3348, 3051, 2825, 1715, 1642, 1585, 1509, 1458, 1398, 1217, 1182 and 839; ^1H NMR (DMSO-d_6): 6.77 (d, 1H, H-9, J = 9.0 Hz), 7.41-7.45 (m, 1H, H-8), 7.51-7.55 (m, 4H, Ar-H), 7.79-7.81 (m, 1H, H-7) and 8.33 (dd, 1H, H-6, J = 9.0 Hz & 2.3 Hz).

10-(4'-Methoxyphenyl)flavin (4f)

Mp. >300^oC (lit[72] mp. >300 ^oC); UV-visible (CH_3OH) λ_{max}: 268, 333, 448 nm; FTIR (Nujol): 3320, 2922, 1714, 1664, 1547, 1460, 1272, 1196, 1090, 880, 875 cm^{-1}; ^1H NMR (DMSO-d_6): 3.73 (s, 3H, OCH_3), 6.81 (d, 1H, H-9, J = 8.0 Hz), 7.23 (t, 1H, H-8), 7.36 (d, 2H, H-3' & H-5'), 7.63 (d, 2H, H-2' & H-6'), 7.74 (t, 1H, H-7), 8.18 (d, 1H, H-6, J = 8.8 Hz & 3.0 Hz).

1.3.2 Solid phase synthesis

Coupling of Merrifield resin (21) with 4-chloro-3-nitrophenol (22)

Merrifield resin (**21**) was taken in dry tetrahydrofuran (THF, 100 mL). To this, 4-chloro-3-nitrophenol (**22**) (4.34 g, 25 mmol) and sodium methoxide ($NaOCH_3$, 25 mmol) were added and the reaction mixture was stirred with heating at 50 ^oC for 24 h. The solvent was removed under reduced pressure. The reaction mixture was partitioned with water and chloroform and filtered to get the polymer-supported chloronitrobenzene **23**. The 1.93 g (11.12 mmol) of the compound **22** was recovered unreacted after the reaction, which shows that approximately 13 mmol is the theoretical loading capacity of the polymer supported compound **23**. The yields of the final compounds were calculated based on the

theoretical loading.

General method for synthesis of polymer-supported N-substituted-2-nitroanilines (24)

Substituted amines/anilines (**19**) (13 mmol) and polymer-supported chloronitro-benzene **23** were taken in CH_2Cl_2-THF (50 mL, 1:1 v/v) at room temperature and the suspension was stirred for 24 h. The reaction mixture was filtered and washed subsequently with THF and CH_2Cl_2; dried in vacuo to get the polymer-supported N-substituted-2-nitroanilines (**24**). The synthesized compounds were characterized with the help of FTIR spectra (Table 1.2).

Table 1.2: FTIR spectral values of compounds **24**

Comp. No.	Compound name	FTIR (cm^{-1})
24a	Polymer supported N-propyl-2-nitroaniline	3320, 2928, 2919, 1550, 1543, 1418, 1333, 1237, 867, 735
24b	Polymer supported N-butyl-2-nitroaniline	3331, 2931, 2893, 1545, 1432, 1335, 1242, 888, 812, 742
24c	Polymer supported N-cyclohexyl-2-nitroaniline	3318, 2922, 2899, 1515, 1438, 1336, 1246, 1150, 826, 665
24d	Polymer supported N-phenyl-2-nitroaniline	3328, 2929, 2901, 1570, 1520, 1440, 1338, 1322, 1246, 848, 821
24e	Polymer supported N-(4'-chlorophenyl)-2-nitroaniline	3320, 2931, 2900, 1565, 1516, 1430, 1348, 1262, 1250, 856, 751
24f	Polymer supported N-(4'-methoxyphenyl)-2-nitroaniline	3309, 2922, 2880, 1540, 1435, 1343, 1280, 1287, 1243, 1141, 850, 759

General method for synthesis of polymer-supported N-substituted-2-aminoanilines (25)

To the polymer-supported nitro compounds (**24**) were added $SnCl_2.H_2O$ (14 mmol) and dimethylformamide (DMF, 50 mL) at room temperature. The suspensions was stirred at room temperature for 24 h, filtered and washed subsequently with H_2O, CH_2Cl_2 and CH_3OH. The residue was dried in vacuo to get polymer-supported diamino compounds (**25**). The synthesized compounds were characterized with the help of FTIR spectra (Table 1.3).

Table 1.3: FTIR spectral values of compounds **25**

Comp. No.	Compound name	FTIR (cm^{-1})
25a	Polymer supported N-propyl-2-aminoaniline	3434, 3325, 2932, 2829, 1365, 1245, 1103, 918, 846, 765
25b	Polymer supported N-butyl-2-aminoaniline	3430, 3342, 2992, 2833, 1375, 1251, 1100, 979, 856, 727
25c	Polymer supported N-cyclohexyl-2-aminoaniline	3438, 3322, 2915, 2860, 1448, 1243, 1145, 1111, 1051, 856
25d	Polymer supported N-phenyl-2-aminoaniline	3455, 3341, 3320, 2965, 1433, 1247, 1118, 849, 780, 765
25e	Polymer supported N-(4'-chlorophenyl)-2-aminoaniline	3444, 3390, 3311, 2930, 1500, 1490, 1268, 1251, 881
25f	Polymer supported N-(4'-methoxyphenyl)-2-aminoaniline	3440, 3329, 3310, 2990, 2828, 1438, 1411, 1290, 1236, 1131, 929, 856

General method for the synthesis of 7-polymer-supported 10-substituted flavin (26)

To the polymer-supported diamine **25** taken in ethanol (50 mL), alloxan monohydrate (**17**) (13 mmol) and dilute hydrochloric acid (2 mL) were added. The suspension was stirred at room temperature for 12 h, filtered and washed with H_2O, CH_2Cl_2 and CH_3OH. The residue was dried in vacuo to get the polymer-supported flavins (**26**). The synthesized compounds were characterized with the help of FTIR spectra (Table 1.4).

General method for cleavage of flavin (27) from 7-polymer-supported 10-substituted flavins (26)

To the polymer-supported flavins **26**, trifluoroacetic acid (TFA) and CH_2Cl_2 (1:1 v/v) (5 mL) were added and the reaction mixture was stirred for 3 h at room temperature. The suspension was filtered and washed with mixture of CH_3CH_2OH-CH_3OH (1:1 v/v 30 mL). The filtrate was concentrated under reduced pressure to get the desired products (**27**), which was further recrystallized from CH_3CH_2OH. The products were characterized by spectroscopic data.

Table 1.4: FTIR spectral values of compounds **26**

Comp. No.	Compound name	FTIR (cm^{-1})
26a	Polymer supported 10-propylflavin	3433, 3312, 3155, 1729, 1680, 1660, 1573, 1519, 1445, 1334, 1243, 1236, 1131, 834, 776
26b	Polymer supported 10-butylflavin	3442, 3308, 2969, 1722, 1675, 1663, 1522, 1519, 1458, 1399, 1303, 1241, 1236, 1115, 990, 842, 777
26c	Polymer supported 10-cyclohexylflavin	3450, 3332, 2926, 2833, 1720, 1680, 1635, 1575, 1560, 1433, 1356, 1243, 1178, 1111, 981, 879, 794
26d	Polymer supported 10-phenylflavin	3445, 3300, 3159, 3030, 2928, 2854, 1716, 1653, 1625, 1571, 1591, 1555, 1457, 1405, 1305, 1240, 1120, 975, 878, 753
26e	Polymer supported 10-(4'-chlorophenyl)flavin	3442, 3310, 3016, 2950, 2866, 1722, 1658, 1622, 1581, 1466, 1375, 1278, 1143, 988, 856, 781
26f	Polymer supported 10-(4'-methoxyphenyl)flavin	3429, 3313, 2926, 2855, 1721, 1672, 1623, 1540, 1500, 1400, 1368, 1290, 985, 867

7-Hydroxy-10-propylflavin (**27a**)

Yield: 77%; mp.: >300 ^{o}C; UV-Vis (DMSO) λ_{max}: 280, 340, 429 nm; FTIR (KBr): 3460, 3027, 2842, 1714, 1659, 1580, 1551, 1429, 1256, 1098, 884, 836, 773 cm^{-1}; ^{1}H NMR (DMSO-d_6): 1.23 (t, 3H, CH_3), 1.80-1.86 (m, 2H, CH_2), 4.64 (t, 2H, $N_{10}CH_2$), 7.78 (d, 1H, H-9, J = 8.7 Hz), 8.03 (bs, 1H, H-3), 8.46 (d, 1H, H-8, J = 8.8 Hz), 8.80 (s, 1H, H-6); Elemental analysis for $C_{13}H_{12}N_4O_3$ (%): Found C 57.25; H 4.49; N 20.69, Calculated C 57.35; H 4.44; N 20.58.

7-Hydroxy-10-butylflavin (**27b**)

Yield: 77%; mp.: >300 ^{o}C; UV-Vis (DMSO) λ_{max}: 287, 330, 435 nm; FTIR (KBr): 3470, 3169, 2952, 1720, 1660, 1555, 1519, 1343, 1250, 1236, 1099, 843, 769 cm^{-1}; ^{1}H NMR (DMSO-d_6): 0.91 (t, 3H, CH_3), 0.98-1.92 (m, 4H, $(CH_2)_2$), 4.65 (t, 2H, $N_{10}CH_2$), 7.55 (bs, 1H, NH), 7.93 (d, 1H, H-9, J = 9.0 Hz), 8.40 (d, H-8, J = 8.7 Hz), 9.03 (s, 1H, H-6);

Elemental analysis for $C_{14}H_{14}N_4O_3$ (%): Found C 58.69; H 4.99; N 19.63, Calculated C 58.73; H 4.93; N 19.57.

7-Hydroxy-10-cyclohexylflavin (**27c**)

Yield: 80%; mp.: >300 ^oC; UV-Vis (DMSO) λ_{max}: 280, 333, 431 nm; FTIR (KBr): 3424, 3027, 2843, 1713, 1659, 1581, 1531, 1429, 1256, 1098, 889, 849 cm^{-1}; ^1H NMR (DMSO-d_6): 1.15-2.08 (m, 10H, cyclohexyl-H), 4.17-4.19 (m, 1H, $N_{10}CH$), 7.81 (d, 1H, H-9, *J* = 8.8 Hz), 8.22 (bs, 1H, H-3), 8.57 (d, 1H, H-8, *J* =8.9 Hz), 8.88 (s, 1H, H-6); Elemental analysis for $C_{16}H_{16}N_4O_3$ (%): Found C 61.61; H 5.20; N 17.82 Calculated C 61.53; H 5.16; N 17.94.

7-Hydroxy-10-phenylflavin (**27d**)

Yield: 81%; mp.: >300 ^oC; UV-Vis (DMSO) λ_{max}: 283, 334, 445 nm; FTIR (KBr): 3452, 3223, 2926, 1718, 1654, 1588, 1420, 1380, 1279, 1189, 907, 816 cm^{-1}; ^1H NMR (DMSO-d_6): 6.97 (d, 1H, H-9, *J* =8.2 Hz), 7.35-7.79 (m, 5H, 10-phenyl H), 7.84 (d, 1H, H-8, *J* = 8.1 Hz), 8.23 (s, 1H, H-6); Elemental analysis for $C_{16}H_{10}N_4O_3$ (%): Found C 62.64; H 3.39; N 18.37 Calculated C 62.74; H 3.29; N 18.29.

7-Hydroxy-10-(4'-chlorophenyl)flavin (**27e**)

Yield: 74%; mp.: >300 ^oC; UV-Vis (DMSO) λ_{max}: 288, 337, 432 nm; FTIR (KBr): 3420, 3399, 3065, 2933, 1725, 1660, 1575, 1399, 1289, 1199, 846 cm^{-1}; ^1H NMR (DMSO-d_6): 7.66 (d, 1H, H-9, *J* = 9.0 Hz), 7.74-7.76 (m, 2H, H-2', H-6'), 7.91-7.93 (m, 2H, H-3', H-5'), 8.21 (d, 1H, H-8, *J* =8.9 Hz), 8.60 (bs, 1H, H-3), 8.82 (s, 1H, H-6); Elemental analysis for $C_{16}H_9N_4O_3Cl$(%): Found C 56.39; H 2.55; N 16.52 Calculated C 56.40; H 2.66; N 16.44.

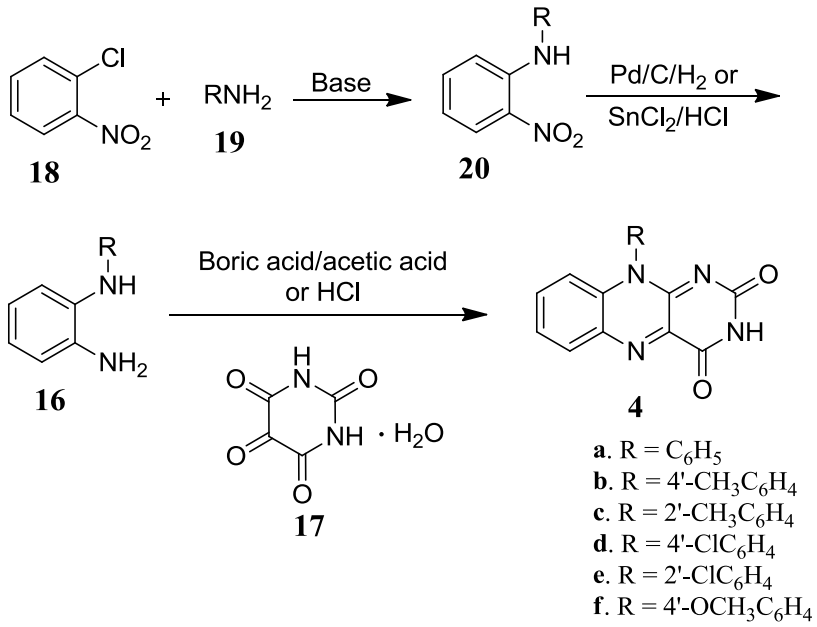
7-Hydroxy-10-(4'-methoxyphenyl)flavin (**27f**)

Yield: (77%); mp.: >300 ^oC; UV-Vis (DMSO) λ_{max}: 281, 341, 432 nm; FTIR (KBr): 3424, 3326, 2921, 1725, 1660, 1607, 1499, 1469, 1219, 1177, 1040, 833 cm^{-1}; ^1H NMR (DMSO-d_6): 4.16 (s, 3H, OCH_3), 7.61 (d, 1H, H-9, *J* = 9.0 Hz), 8.11-8.13 (m, 2H, H-3', H-5'), 8.20-8.23 (m, 2H, H-2', H-6'), 8.31 (d, 1H, H-8, *J* = 9.0 Hz), 8.70 (s, 1H, H-6); Elemental analysis for $C_{17}H_{12}N_4O_4$ (%): Found C 60.77; H 3.65; N 16.51 Calculated C 60.71; H 3.60; N 16.66.

1.4 Results and Discussion

1.4.1 Solution phase synthesis of 10-substituted flavin

The reaction of 1-chloro-2-nitrobenzene (**18**) with substituted amines/anilines (**19**) in the presence of base gives 2-nitro-N-substituted aniline (**20**) which on reduction gives the required diamino compound (**16**). The cyclocondensation of diamine (**16**) with alloxan monohydrate (**17**) have been achieved in acidic condition. A combination of alloxan monohydrate, boric acid, dilute acetic acid and alloxan monohydrate, hydrochloric acid have been very successful.[63-69,74-76] Even in 30% sulphuric acid solution the condensation has been successful.[77] The condensation may be accomplished by mixing the components, shaking them or sometime refluxing them for one hour and leaving them at room temperature for one or two days. Sometimes the flavins precipitate from the reaction mixture in crystalline form and require very little purification, but on other occasions they can be extremely difficult to isolate and purified by column chromatography.



Scheme 1.2: Solution phase synthesis of 10-substituted flavins (**4**)

The reduction of **20** with Pd/C/H_2 gives the required diamino compound (**16**), which was not isolated, that on subsequent cyclocondensation with alloxan monohydrate (**17**) afforded 10-substituted flavins (**4**) in 40-55% yields with respect to reactant **18**. The cyclo-condensation was performed in boric acid, dilute acetic acid. The condensation was accomplished by mixing the components and refluxing them for one hour. The reaction

mixture was cooled to room temperature and kept overnight in refrigerator. The required products **4** were precipitated out and filtered.

The formation of flavin (**4**) has been confirmed by various spectroscopic data including UV-visible, Fourier transform infrared (FTIR) and ^{1}H NMR spectroscopy. The absorption maxima in the UV-visible spectrum of 10-phenylflavin (**4a**) has been observed in methanol at 435, 333 and 262 nm (Figure 1.5), which are characteristic peaks for the flavin moiety.[78] In the FTIR spectrum of **4a**, appearance of peaks at 1676 and 1720 cm^{-1} indicate the presence of two carbonyl groups at 2- and 4-positions respectively (Figure 1.6), which are also characteristics of the flavin moiety.[79] The structure has been further confirmed by ^{1}H NMR spectroscopy (Figure 1.7). In the ^{1}H NMR spectrum of **4a**, a doublet at 6.81 ppm for one proton has been assigned to the H-9 aromatic proton. Doublets at 7.43 and 8.22 ppm for two and one protons respectively have been assigned to the 2H-3' and one H-6 protons respectively. The rest aromatic protons have been observed in the region 7.60-7.77 ppm (Figure 1.7). These ^{1}H NMR spectral data are also in comparison with the literature of flavin nucleus.[78,79]

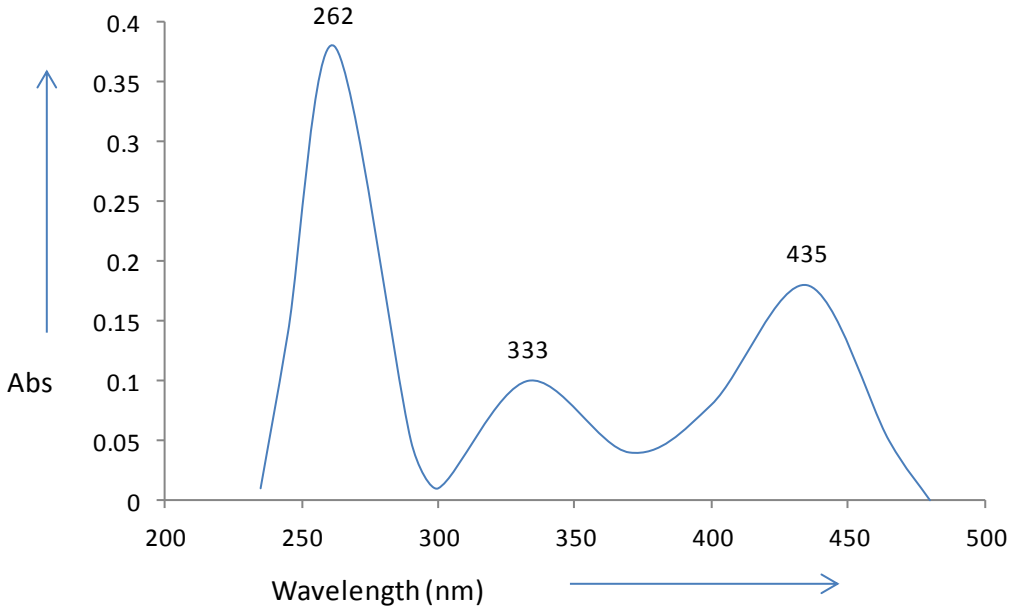


Figure 1.5: UV-visible spectrum of 10-phenyl flavin (**4a**) in methanol

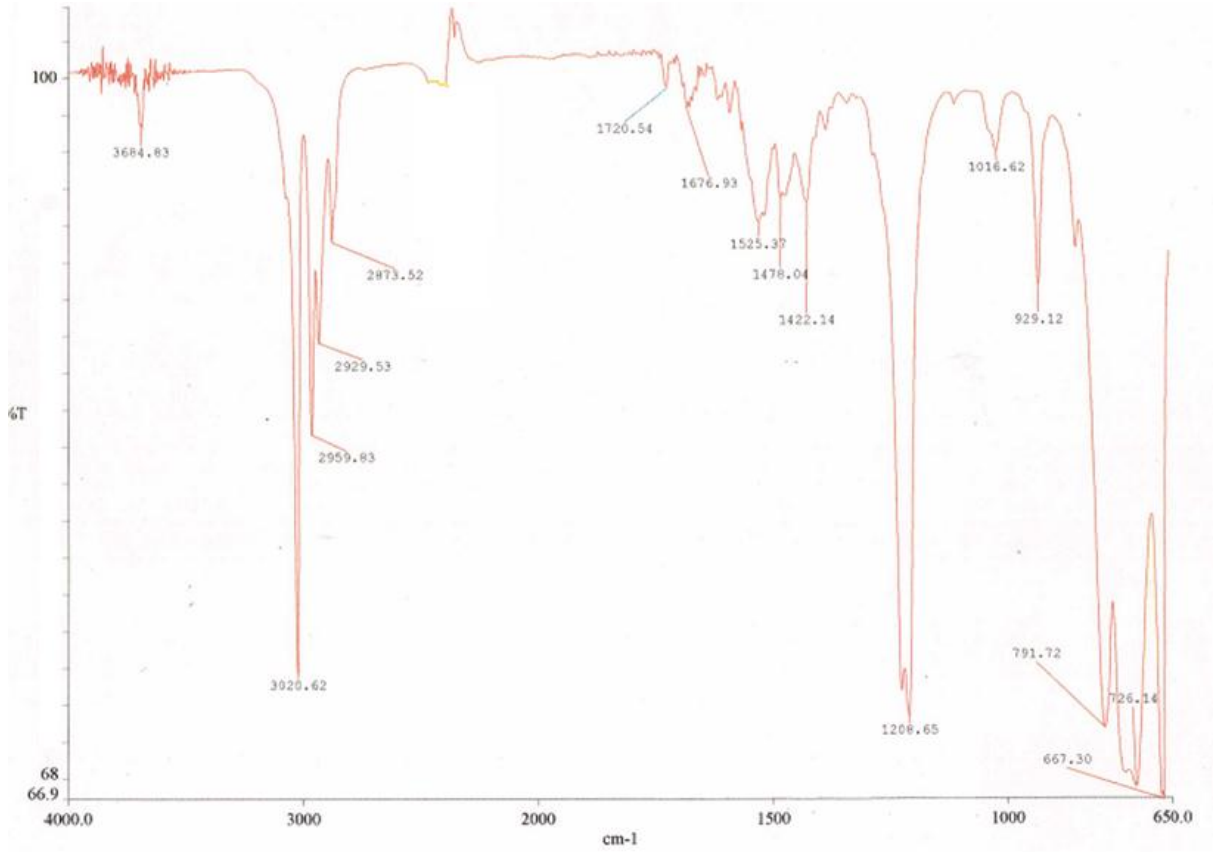


Figure 1.6: FTIR spectrum of 10-phenylflavin (**4a**)

The first step in the synthesis of 10-substituted flavins plays crucial role in the synthesis and also responsible for low yield. The reaction seems to be very simple, but in practical, this requires high temperature and tedious work up. This step was modified to improve the overall yield of the flavin to perform the reaction under solvent free condition at comparable temperature.

Synthesis of 2-nitro-N-substituted anilines

The 2-nitro-N-substituted anilines are important intermediates for the synthesis of widely used biologically active molecules like, flavins, benzimidazoles, benzotrizoles & other biologically active benzo-fused hetrocycles.[80,81] These group of compounds find their applications in pharmaceuticals,[82] agrochemicals,[83] and natural as well synthetic products[84]. The halogen atom in aryl halides is relatively inert and requires activation from electron withdrawing groups attached at 2- and/or 4-position while undergoing nucleophilic aromatic substitution.[85,86]

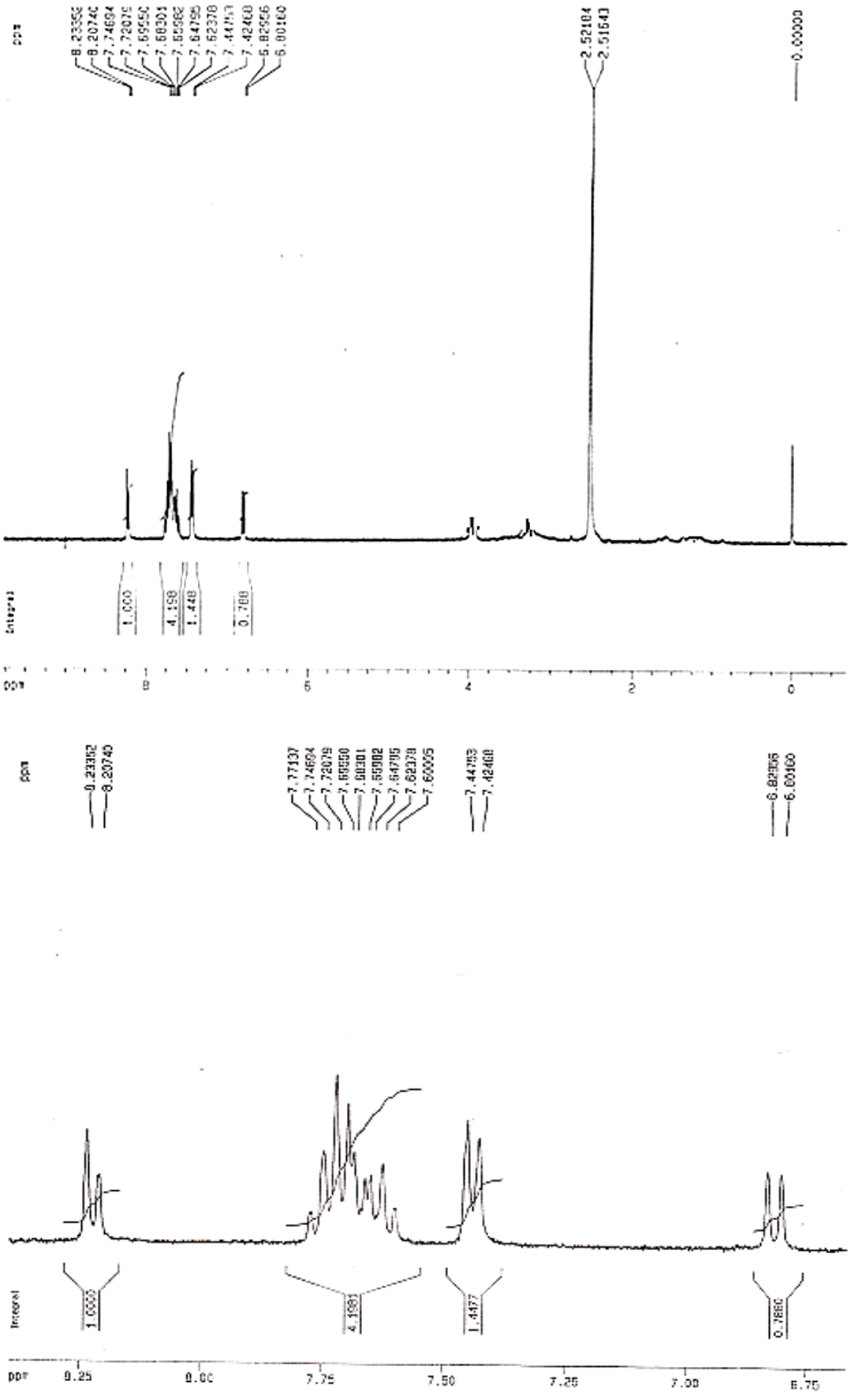


Figure 1.7: ^{1}H NMR spectrum of 10-phenylflavin (**4a**)

The traditional method involves long reaction time, high temperature, use of high boiling solvents like DMF, DMSO, ionic liquids etc, expensive bases or combination of above factors.[87,88] Solvent free organic synthesis is a versatile technique in promoting variety of chemical reactions and a good approach towards green chemistry.[89,90] The present section discusses a mild and efficient procedure for the synthesis of N-substituted 2-nitroanilines under solvent free condition.

In a typical case, the solvent free reaction of 1-chloro-2-nitrobenzene (**18**) with aniline (**19a**) in the presence of sodium carbonate for 5 hours at ~ 50^{o}C gave 2-nitro-N-phenylaniline (**20a**) in 88% yield (Table 1.1, entry 1). No further increase in product formation achieved even after heating the reaction mixture for 24 hours (Table 1.1, entry 1). Similarly, **18** was reacted with other anilines (**19b-f**) to give appreciable yield of **20b-f** (Table 1.1, entry 2-6). The reaction was extended to 24 hours duration to check the optimization of the reaction. It was observed that the extension of reaction time at same temperature (~ 50 ^{o}C) have very little effect on the isolated yield.

To explore the scope of this reaction, substituent having electron withdrawing (-Cl) and releasing (-CH_3) groups have been chosen and synthesized. In all the cases, the nucleophilic substitution reaction was successful in good to excellent yield with sodium carbonate. The electron withdrawing nature of the chloro group is responsible for the lower yield of products in case of **20d** and **20e**. The products were separated by simple extraction with chloroform-water mixture, followed by column chromatography (Figure 1.8).

To check the effect of salt on product formation, four different types of salts were tried for the reaction. The maximum yield was obtained with sodium carbonate. We tried this reaction with simple zinc dust also, but failed to get any desired product. The basic purpose of using these salts is to scavenge the released hydrochloric acid (HCl) as their chloride salts so that the reaction can proceed in forward direction. If the hydrochloric acid is not removed, this reacts with the reactant aniline to for anilinium salt which reduces the nucleophilicity of aniline. This does not allow the reaction to proceed further. The amount of sodium carbonate salt was also optimized by taking it from smaller quantity to higher quantity (Figure 1.9). All the products were purified by column chromatography over silica gel and characterized by melting point, mixed melting point, FTIR and ^{1}H NMR spectroscopic data (Experimental section).

In the ^{1}H NMR spectrum of 2-nitro N-phenylaniline (**20a**), three multiplets in the region 6.74-6.80; 7.21-7.29 and 7.33-7.44 ppm for three, two and three protons respectively has been assigned to the protons of aromatic region. The double doublet peak

at 8.20 for one proton has been assigned to H-3 proton. This confirmed the formation of **20a**. The ^{1}H NMR data of all other compounds are comparable with the structural values.

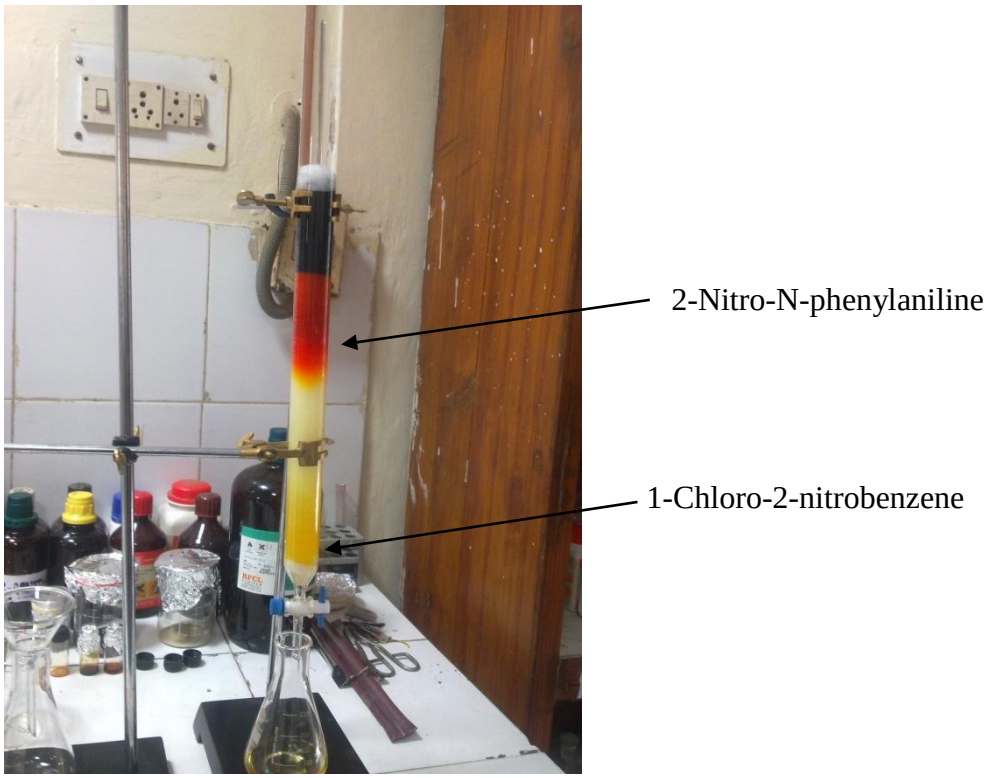


Figure 1.8: Column chromatographic purification of 2-Nitro-N-phenylanilines (**20a**)

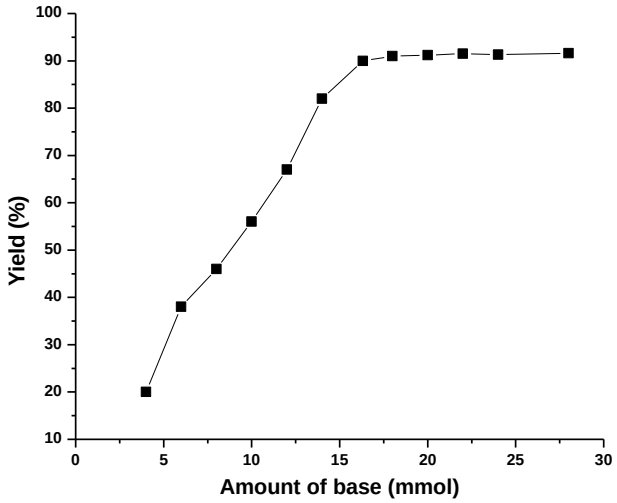
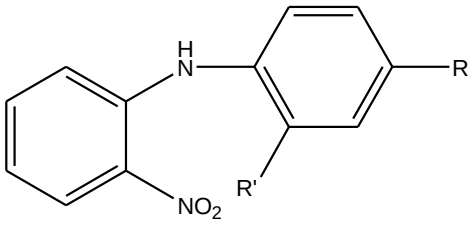


Figure 1.9: Optimization of sodium carbonate salt

Table 1.1: Synthesis of N-Substituted 2-nitroanilines (**20**)



S. No.	Salts used	Compounds prepared		Isolated Yield (%)	
		R	**R'**	**5 h**	**24 h**
1	Sodium carbonate	H	H	88	88
2	Sodium carbonate	CH_3	H	90	90
3	Sodium carbonate	H	CH_3	79	80
4	Sodium carbonate	Cl	H	75	75
5	Sodium carbonate	H	Cl	70	70
6	Sodium carbonate	OCH_3	H	81	82
7	Potassium acetate	H	H	55	58
8	Potassium acetate	CH_3	H	59	63
9	Potassium acetate	H	CH_3	55	60
10	Potassium acetate	Cl	H	42	49
11	Potassium acetate	H	Cl	41	45
12	Copper acetate monohydrate	H	H	48	48
13	Copper acetate monohydrate	CH_3	H	31	33
14	Copper acetate monohydrate	H	CH_3	28	30
15	Copper acetate monohydrate	Cl	H	40	41
16	Copper acetate monohydrate	H	Cl	28	28
17	Lead acetate	H	H	43	45
18	Lead acetate	CH_3	H	30	33
19	Lead acetate	H	CH_3	23	25
20	Lead acetate	Cl	H	28	31
21	Lead acetate	H	Cl	23	28

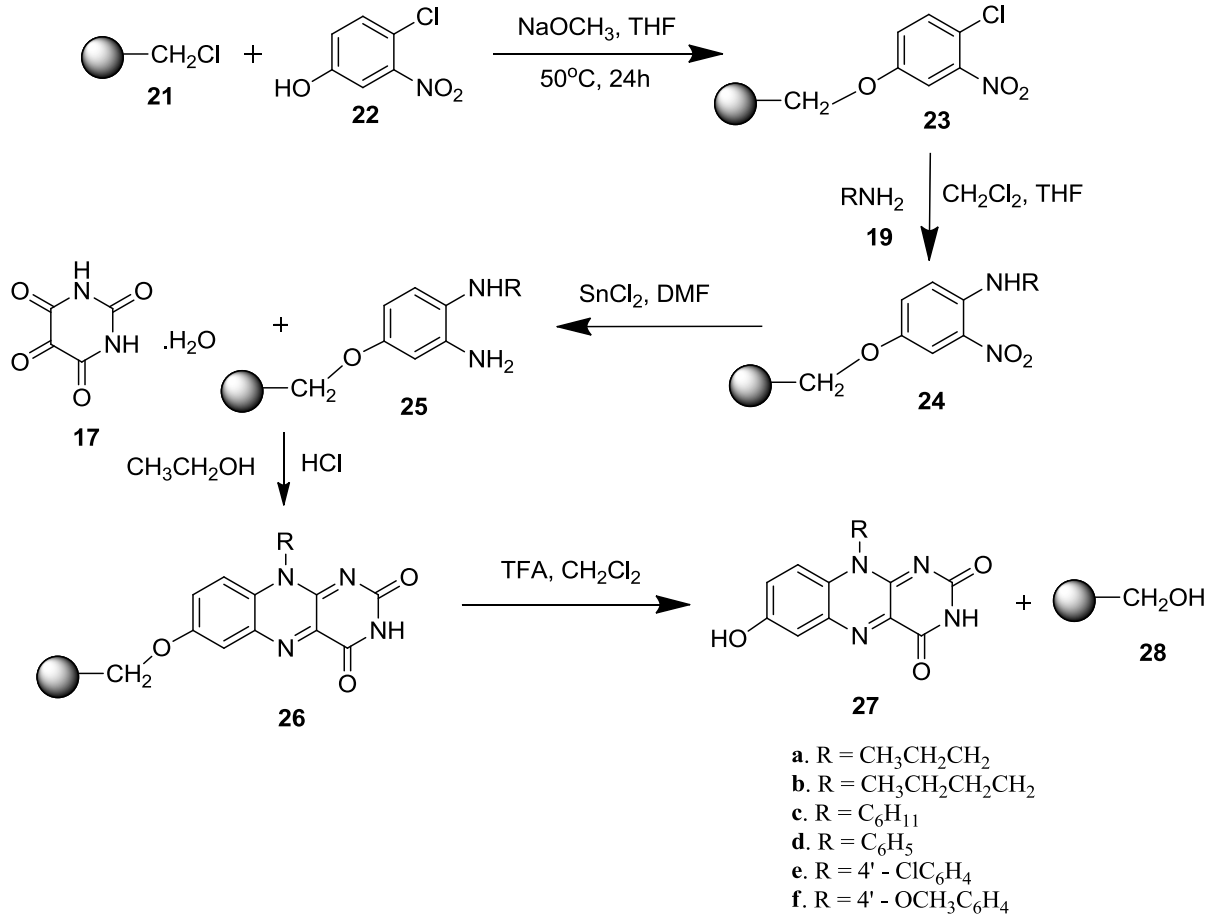
1.4.2 Solid phase synthesis of 10-substituted flavins

The polymer supported organic synthesis is a powerful method for the synthesis of heterocyclic molecules.[91-93] The concept of polymer-supported synthesis was first realized when Merrifield published his synthesis of L-leucyl-L-alanylglycyl-L-valine *via* attachment of the intermediate to a polymer backbone.[28] Soon this concept was utilized by organic chemists for the synthesis of diverse group of compounds having diverse applications.[91-93]

In this method of synthesis, an inert insoluble polymer is coupled to one of the substrate usually through a reaction dependent stable covalent bond. The synthesis has also been carried out using polymer-support reagents and/or catalysts and soluble polymer supports.[94]

The Merrifield resin (chloromethylated styrene-divinylbenzene copolymer, 2% cross-linked, 2.50 mequiv of Cl/g, **21**) was reacted with 4-chloro-3-nitrophenol (**22**) in the presence of sodium methoxide in dry tetrahydrofuran (THF) to give polymer supported chloronitrobenzene **23**. The reaction of **23** with substituted amines/anilines (**19**) in the solvent DCM:THF (1:1 v/v) gave polymer-supported nitroanilines **24**. The reduction of **24** with $SnCl_2$ gave polymer-supported diamines **25**. The appearance of absorptions between 3400-3500 cm^{-1} for primary amine in FTIR spectroscopy and absence of absorptions in the range 1300-1400 & 1500-1600 cm^{-1} for nitro group also confirmed the formation of resin bound diamines **25**. The spectrum of nitro group usually shows two very intense peaks in the range 1300-1400 (sym) and 1500-1600 (asym). The cyclocondensation of diamines **25** with alloxan monohydrate **7** in the presence of dilute HCl in ethanol gave the polymer-supported flavins **26** (Scheme 1.3). The formation of the polymer-supported flavins was characterized by FTIR spectroscopy.

The appearance of strong peaks in the regions 1640-1660 and 1710-1730 cm^{-1} confirms the presence of two carbonyl groups (C=O) in the molecule which is attributed to the carbonyls at positions 2 and 4 respectively of the flavin ring.[79] Another broad peak in the region 3460-3420 cm^{-1} is due to the presence of -NH group at position 3 of the flavin ring. The polymer-support from compound **26** was cleaved using TFA in dichloromethane to give 7-hydroxy-10-substituted flavins (**27**) in 74-81% yields. The formation of **27** was confirmed by different spectroscopic data such as UV-Visible, FTIR, ^1H NMR spectroscopy and elemental analysis (experimental section).



Scheme 1.3: Polymer supported synthesis of 7-hydroxy-10-substituted flavins

In the ^{1}H NMR spectrum of **27b**, a triplet at 0.91 ppm for three protons has been assigned to CH_3 group of the butyl chain. A multiplet in the region 0.98-1.92 ppm and triplet at 4.65 ppm have been assigned to two CH_2 groups and $N^{10}CH_2$ group respectively (Figure 1.10). The other peaks have been observed in the aromatic region confirmed the formation of flavin **27**. [78,79]

This method is advantageous over the conventional solution-phase method due to the ease of product isolation through only filtration and further gets cleaved from the polymer support. A hindrance to polymer-supported reactions has been the lack of analytical methods to establish the degree to which the expected product has formed.

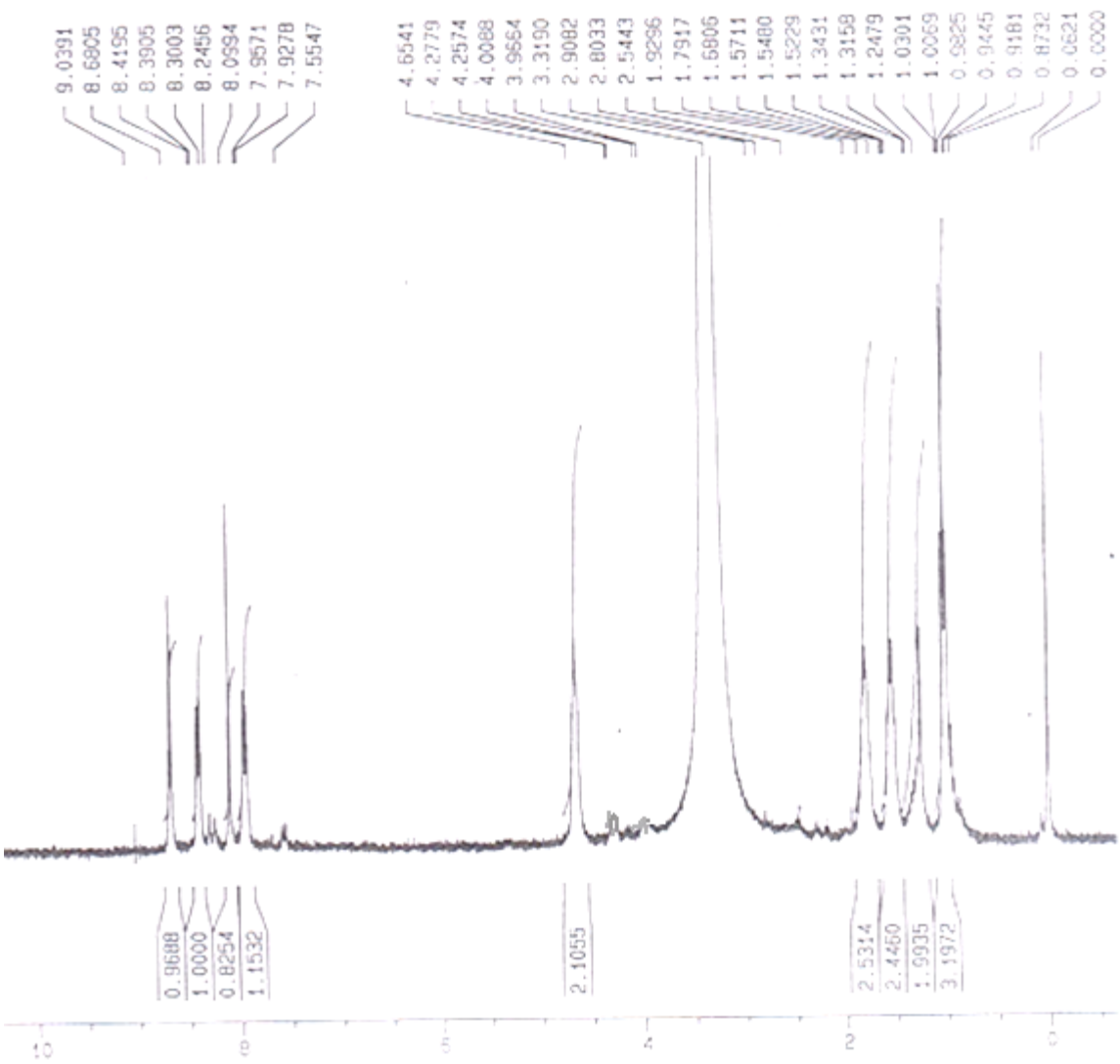


Figure 1.10: ^{1}H NMR Spectrum of 7-hydroxy-10-butylflavin (**27b**)

1.5 Conclusions

Flavoenzymes are redox enzymes which are involved in various biological processes. They contain FAD or FMN as their cofactors to carry out their reactions. Flavin (or isoaloxazine) is a core heterocyclic moiety present in FAD or FMN and hence their synthesis is always a focus of research. This chapter described the solid and solution phase synthesis of 10-substituted flavins.

The synthesis of 10-substituted flavins was achieved from 1-chloro-2-nitrobenzene which on reaction with anilines/amines gave N-substituted nitro anilines. This on reduction and further cyclocondensation with alloxan monohydrate gave the desired flavins. We have developed a rapid, efficient and solvent free procedure for the synthesis of 2-nitro-N-

arylanilines by aromatic nucleophilic substitution of 1-chloro-2-nitrobenzene with various anilines in the presence of sodium carbonate. The formation of product in the presence of other salts like potassium acetate, lead acetate and copper acetate has also been studied. Good to excellent yields were obtained in very short reaction time with sodium carbonate.

The solid phase synthesis was achieved through polymer supported reaction using Merrifield resin. The reaction was performed by simply stirring the reaction at room temperature.

The polymer-supported synthetic method is an efficient and free from tedious workup process for the synthesis of 7-hydroxy-10-substituted flavin in good yields. This is more attractive and advantageous in comparison to their solution phase synthesis where each synthetic step requires tedious purification process including column chromatography. This is an environmental friendly method of synthesis.

1.6 References

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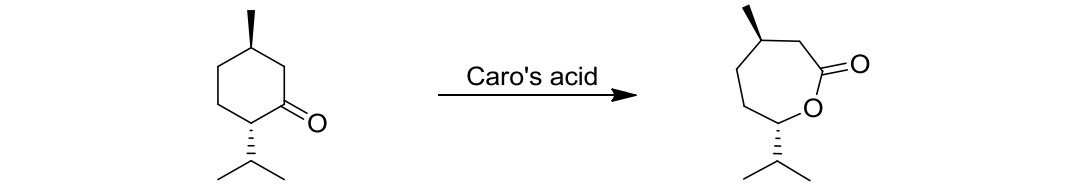
CHAPTER 2

Application of Flavins as catalyst for Baeyer-Villiger oxidation

2.1 Introduction

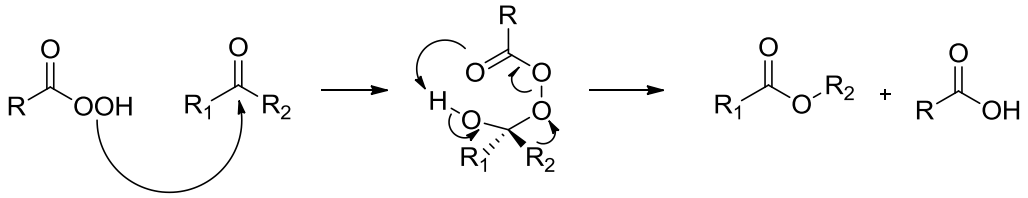
Esters are an important class of compounds having wide range of applications. Those esters which are cyclic in nature are called lactones. They possess two or more carbon atoms and single endocyclic oxygen coupled with an adjacent ketone. The two great scientists of late 1800 AD, Adolf von Baeyer and Victor Villiger discovered the organic transformation of ketones into esters and cyclic ketones into lactones.[1] After their name, this transformation was named as Baeyer-Villiger oxidation. They had used Caro's acid for the oxidation of menthone (Scheme 2.1).[1] Caro's acid is a mixture of concentrated sulphuric acid and sodium persulphate. Since then chemists tried various enzymatic and non-enzymatic methods for the oxidation of ketones or cyclic ketones to esters or lactones respectively.[2-5]



Scheme 2.1: BV oxidation of menthone

During the reaction of Baeyer-Villiger (BV) oxidation by peracids, the carbonyl carbon of ketone acts as electrophilic centre and is attacked by oxygen nucleophile of the peracids.[6] This forms a tetrahedral intermediate which is unstable and hence rearranges itself to ester and carboxylic acid (Scheme 2.2).[6] This rearrangement takes place with the help of carbon-carbon bond migration where the most substituted carbon centre moves with retention of configuration. The migration is also influenced by other factors like electronic, conformational, steric and peracid used.[3]

The peracids like percarboxylic acid, 3-5-dinitroperbenzoic acid, *meta*-chloroperbenzoic acid etc act as oxygen donor.[7] Even after so much of work done, this reaction suffers from several limitations especially with the use of peracids and halogenated solvents. Environmental concern is one of the important limitations where the by-products of peracids, carboxylic acid and their salts provide problem with its disposal.[7] Also, the organic peracids have limitations in their commercial use due to their hazardous expensive and explosive nature.[6,8] This led to the utilization of various other oxidants and catalysts which have their own advantages and disadvantages.[9-15]



Scheme 2.2: Mechanism of BV oxidation catalyzed by per-acids

Some of the heterogenous catalysts (Figure 2.1) utilized along with suitable oxygen donor for the BV oxidation includes solid acid[9], zeolites[10], titanium silicate[11], selenium[12] and arsenic (As)[13]. The homogeneous catalysts based on platinum (Pt)[14], zirconium (Zr)[15], rhenium (Re)[16], selenium (Se)[17] and molybdinum (Mo)[18] have also been used for BV oxidation. Some of the other systems utilized for these oxidations are molecular iodine with H_2O_2[19], copper with a combination of molecular oxygen and aldehyde[20], platinum with H_2O_2[21], and almunium with cumene hydroperoxide.[22] Despite these effects, the design and development of metal-free organocatalysts based on the biomimetic concepts would be an important alternative for BV oxidation. This chapter describes the biomimetic concepts and effects of chain length at N^{10}-position of flavin on BV oxidation of cycloketones.

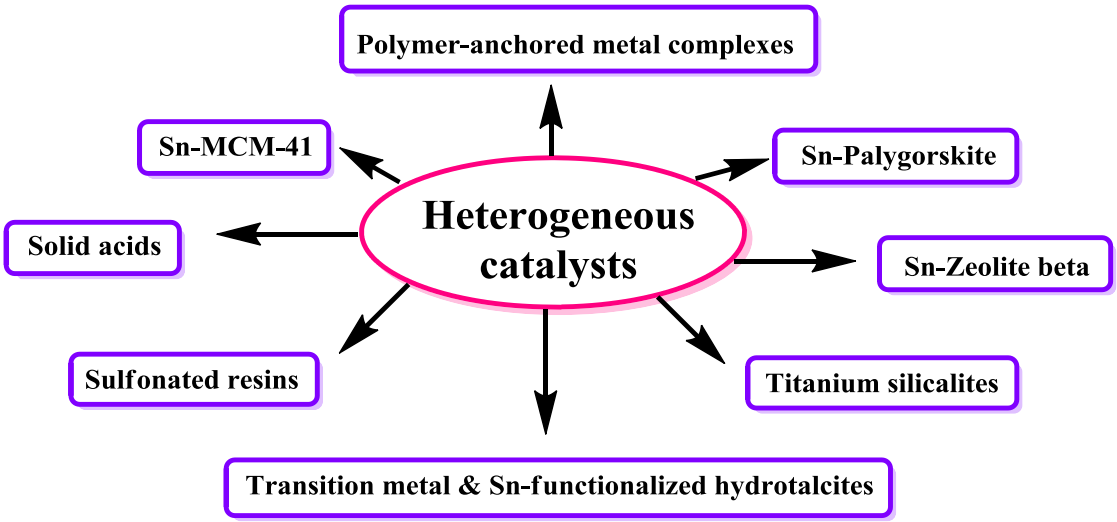
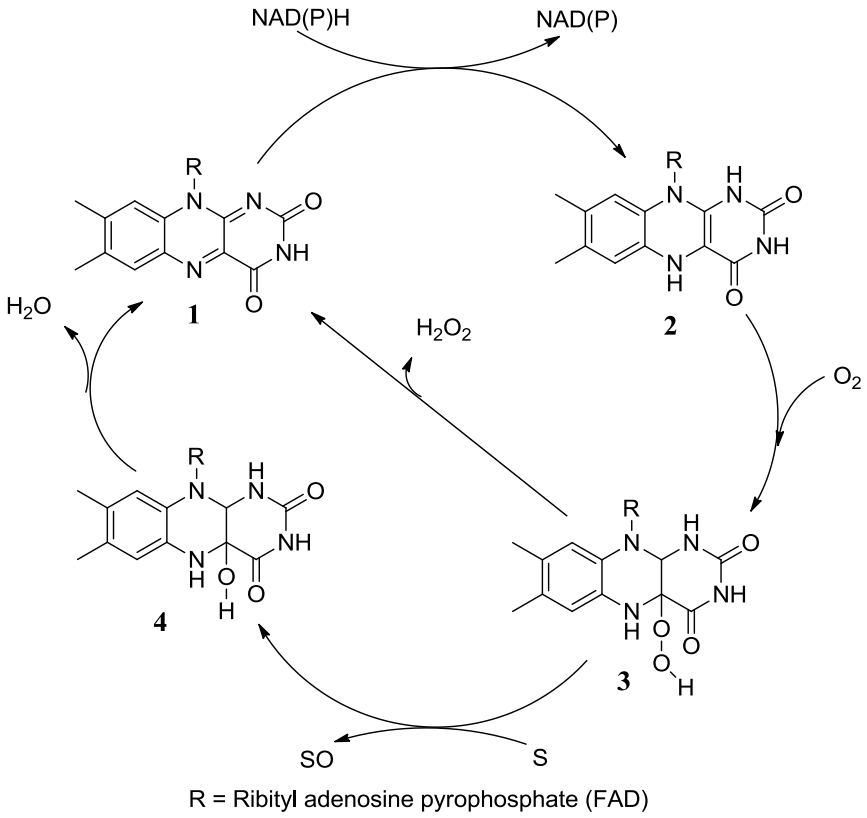


Figure 2.1: Important heterogeneous catalyst utilized for BV oxidation

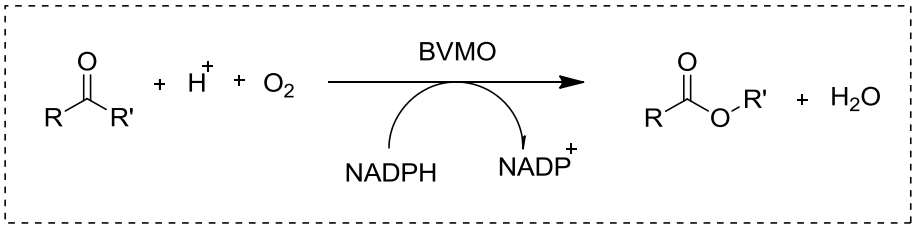
2.2 Flavin-catalyzed BV oxidation: Literature review

The cofactor engineering is emerging as an important process in industrial biotechnology based on biomimetic concept. This process utilizes the idea of cofactor function to carry out reactions. This helps in the development of artificial biocatalysts.[23-28] This concept has also been utilized for the development of artificial biocatalysts for BV oxidation.[29] The enzyme responsible for carrying out the BV oxidation type reaction in living organism is known as Baeyer-Villiger monooxygenases (BVMOs)[6] which are NAD(P)H-dependent flavoproteins. The cofactor of BVMOs, FAD in their catalytic cycle get reduced to dihydroflavin (**2**) with simultaneous oxidation of NAD(P)H to NAD(P) (Scheme 2.3).[30] The reduced flavin is oxidized by dioxygen (O_2) to give the 4a-hydroperoxyflavin (**3**) which transfer oxygen to the substrate to give the oxygenated product.[30] During this process 4a-hydroxyperoxyflavin (**3**) gets converted to hydroxyflavin (**4**) that releases water molecule to regenerate FAD again and completes the catalytic cycle of BVMO.[30]



Scheme 2.3: Catalytic cycle of BVMOs

If substrates is not present, the **3** gets directly converted to FAD by eliminating hydrogen peroxide (H_2O_2).[31,32] When the substrate is ketone or cyclic ketone, the oxygenated product is either ester or lactone respectively (Scheme 2.4).[33,34]



Scheme 2.4: BV oxidation catalyzed by BVMOs

The discovery of BVMOs opened the pathways for many biocatalytic processes for BV oxidation using either whole cells, isolated enzymes or through synthetic flavins.[35-40] The isolation of cyclohexanone oxygenase (CHMO) from *Acinetobacter* was reported by Trudgill's group in 1970s which converted a large number of cyclic ketones to their respective lactones.[41] This CHMO became a representative BV enzyme which acted as enantioselective biocatalysts for a number of reactions.[42]

In 1976, Griffin et al purified cyclopentanone monooxygenases (CPMO) from *Comamonas* sp.[43] The ketones having C_4 to C_8 carbon atoms along with norbornanone have been the preferred substrate for CPMO. In many cases, the substrate overlap of CPMO with CHMO has been observed however their enantioselectivity differs.[6] For example, Iwaki et al has shown that the substrate 4-methylcyclohexanone get converted to the corresponding (S)-lactone by CHMO whereas the CPMO convert it to (R)-lactone.[44] The whole cell of CPMO have been employed for most of the biocatalytic processes. The CPMOs have actively being used as biocatalyst for the formulation of δ-valerolactones which has their further applications in the synthesis of biologically active compounds.[6,45]

Some other monooxygenases like cyclododecanone monoxygenase[46], 4-hydroxyacetophenone monooxygenase[47] have also been utilized as biocatalysts for BV oxidation. Some of the human flavin containing monooxygenases have also been used as biocatalysts for BV oxidations.[48,49]

Studies have also been done by using the synthetic flavin as BV metal-free organocatalysts to mimic the reactions of BVMOs. Furstoss et al[50] have reported in 1996 that flavin can activate the hydrogen peroxide[51] for the oxidation of cyclobutanones. A planar-chiral bisflavin has also been used to catalyze the BV oxidation of

cyclobutanones.[52] Imada et al have also reported BV oxidation of cyclic ketones to lactones with the synthetic flavin catalysts under the O_2 atmosphere (1 atm).[53]

2.3 Experimental

Melting points were determined on a laboratory capilary melting apparatus and are uncorrected. Electronic spectra were recorded on a Perkin-Elmer UV-260 spectrophotometer and absorption maxima have been expressed in nanometers (nm). FTIR spectra were recorded on a Perkin Elmer 1710 FTIR spectrophotometer and the ν_{max} are expressed in cm^{-1}. ^1H NMR was recorded on a Bruker Avance-300 spectrophotometer (300 MHz) and the chemical shifts were expressed in ppm. The abbreviation s, d, t, q, m and bs stand for singlet, doublet, triplet, quartret, multiplet and broad singlet respectively.

The solvents and reagents were purchased from reputed company and were used without further purifications. Alloxan monohydrate was obtained from Acros (Belgium), substituted amines were obtained from Fluka (Switzerland). The 1-chloro-2-nitrobenzene, metal salts and other related reagents were obtained from sd Fine chemicals (India) and other reputed Indian companies.

General procedure for synthesis of 2-nitro-N-substituted anilines (7)

A mixture of amine (**6**) (12 mmol), 1-chloro-2-nitrobenzene (**5**) (10 mmol) and fused potassium acetate (10 mmol) was heated at 150 ^oC for 24 hours. The reaction mixture was allowed to cool to room temperature and partitioned between water-chloroform (1:1) (2×100 mL). The chloroform layer was further washed with 20% HCl (2×50 mL) and water (2×100 mL). The resulting chloroform layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was subjected to column chromatography over silica gel (60-120 mesh) using petroleum ether (60-80 ^oC) as eluent to get the desired product **7**.

2-Nitro-N-ethylaniline (7a)

Orange liquid; FTIR (KBr): 3479, 3355, 3174, 3100, 1630, 1591, 1572, 1469, 1349, 1282, 1172, 1169, 1006, 873, 849 cm^{-1}; ^1H NMR ($CDCl_3$): 1.03 (t, 3H, CH_3), 3.40-3.45 (m, 2H, CH_2), 6.69 (d, 1H, H-6, J = 7.81 Hz), 6.77-6.79 (m, 1H, H-4), 7.21-7.23 (m, 1H, H-5), 8.01 (d, 1H, H-3, J = 8.00 Hz).

2-Nitro-N-propylaniline (7b)

Orange liquid; FTIR (KBr): 3477, 3345, 3164, 3090, 1632, 1581, 1562, 1469, 1352, 1281, 1172, 1166, 1000, 877, 849 cm^{-1}; ^1H NMR ($CDCl_3$): 1.00 (t, 3H, CH_3), 1.58-160 (m, 2H, CH_2), 3.12-3.15 (m, 2H, CH_2), 6.66 (d, 1H, H-6, *J* = 7.88 Hz), 6.75-6.77 (m, 1H, H-4), 7.29-7.31 (m, 1H, H-5), 8.01 (d, 1H, H-3, *J* = 8.01 Hz).

2-Nitro-N-butylaniline (7c)

Orange liquid; FTIR (Nujol): 3440, 3389, 3092, 2955, 2877, 1601, 1570, 1531, 1459, 1428, 1419, 1350, 1278, 1239, 1164, 1056, 977, 860, 756, 652 cm^{-1}; ^1H NMR ($CDCl_3$): 1.00 (t, 3H, CH_3), 1.25-1.55 (m, 4H, 2×CH_2), 2.85-3.00 (m, 2H, CH_2), 6.68 (dd, 1H, H-6, *J* = 7.81 & 1.88 Hz), 6.72-6.74 (m, 1H, H-4), 7.11-7.13 (m, 1H, H-5), 8.11 (d, 1H, H-3, *J* = 8.0 Hz).

2-Nitro-N-pentylaniline (7d)

Orange liquid; FTIR (KBr): 3470, 3325, 3104, 3098, 1642, 1571, 1562, 1429, 1349, 1281, 1172, 1166, 1000, 877, 849 cm^{-1}; ^1H NMR ($CDCl_3$): 0.96 (t, 3H, CH_3), 1.36-1.88 (m, 6H, 3×CH_2), 3.18-3.20 (m, 2H, CH_2), 6.63 (d, 1H, H-6, *J* = 7.98 Hz), 6.77-6.79 (m, 1H, H-4), 7.29-7.31 (m, 1H, H-5), 8.01 (d, 1H, H-3, *J* = 8.11 Hz).

2-Nitro- N-Hexylaniline (7e)

Orange liquid; FTIR (Nujol): 3394, 3098, 2935, 2867, 1600, 1575, 1521, 1479, 1428, 1419, 1358, 1278, 1239, 1164, 1056, 978, 860, 757, 650 cm^{-1}; ^1H NMR ($CDCl_3$): 0.99 (t, 3H, CH_3), 1.15-1.65 (m, 8H, 4×CH_2), 2.80-3.01 (m, 2H, CH_2), 6.58 (dd, 1H, H-6, *J* = 7.71 & 1.80 Hz), 6.82-6.85 (m, 1H, H-4), 7.14-7.17 (m, 1H, H-5), 8.13 (dd, 1H, H-3, *J* = 8.0 & 2.0 Hz).

General procedure for synthesis of 10-substituted flavins (8)

To a solution of N-substituted-2-nitroaniline (**7**, 5 mmol) in dry ethanol (40 mL) in paar hydrogen bottle, Pd/C (20 %) (0.02 g) was added and the reaction mixture was hydrogenated at ambient pressure and temperature till the colour of the solution disappears (for 15-24 h). When the quantitative amount of hydrogen was absorbed and the reaction mixture became almost colourless, glacial acetic acid (2 mL) was added to the reaction mixture and the catalyst was filtered off. The alloxan monohydrate (5 mmol), conc. HCl

(2 mL) were added to the filtrate, and the reaction mixture was refluxed for 1 h. The reaction mixture was allowed to cool to room temperature and kept overnight in refrigerator. The resulting precipitate was filtered off to give 10-substituted flavins (**8**).

10-Ethylflavin (8a)

Mp. 299 ^{o}C; UV-visible (CH_3OH) λ_{max}: 266, 335, 440 nm; FTIR (KBr): 3400, 3100, 1715, 1660, 1572, 1423, 1281, 1266, 1180, 1093, 881, 860 cm^{-1}; ^{1}H NMR ($CDCl_3$): 1.27 (t, 3H, CH_3), 4.13 (q, 2H, N^{10}-CH_2), 7.64-7.69 (m, 2H, H-8 & H-9), 7.92-7.97 (m, 1H, H-7), 8.33-8.36 (m, 1H, H-6), 8.58 (s, 1H, NH).

10-Propylflavin (8b)

Mp. 293-294 ^{o}C; UV-visible (CH_3OH) λ_{max}: 268, 338, 449 nm; FTIR (Nujol): 3399, 3320, 2922, 1714, 1664, 1547, 1460, 1272, 1196, 1090, 880, 875 cm^{-1}; ^{1}H NMR (DMSO-d_6): 1.10 (t, 3H, CH_3), 1.81-1.89 (m, 2H, CH_2), 4.62 (t, 2H, N^{10}-CH_2), 7.96-8.05 (m, 3H, H-9, H-7 & H-8), 8.40 (d, 1H, H-6, J = 9.12 Hz), 8.71 (s, 1H, NH).

10-Butylflavin (8c)

Mp.: 294-296 ^{o}C (dec.); UV-visible (CH_3OH) λ_{max}: 269, 333, 441 nm; FTIR (KBr): 3469, 3225, 2955, 1720, 1664, 1549, 1503, 1400, 990, 773 cm^{-1}; ^{1}H NMR ($CDCl_3$): 0.93 (t, 3H, CH_3), 1.33-1.78 (m, 4H, 2×CH_2), 4.69 (t, 2H, $N^{10}CH_2$), 6.99 (d, 1H, H-9, J = 7.90 Hz), 7.64-7.79 (m, 2H, H-7 & H-8), 7.99 (d, 1H, H-6, J = 8.01 Hz), 8.40 (bs, 1H, N^{3}H).

10-Pentylflavin (8d)

Mp.: 274-275 ^{o}C; UV-visible (CH_3OH) λ_{max}: 273, 338, 447 nm; FTIR (KBr): 3459, 3222, 2956, 1721, 1663, 1541, 1500, 1401, 993, 773 cm^{-1}; ^{1}H NMR ($CDCl_3$): 0.91 (t, 3H, CH_3), 1.13-1.79 (m, 6H, 2×CH_2), 4.59 (t, 2H, $N^{10}CH_2$), 7.01 (d, 1H, H-9, J = 7.93 Hz), 7.66-7.81 (m, 2H, H-7 & H-8), 8.11 (d, 1H, H-6, J = 8.11 Hz), 8.44 (bs, 1H, N^{3}H).

10-Hexylflavin (8e)

Mp.: 254-256 ^{o}C (lit.[54] mp. 254-256 ^{o}C); UV-visible (CH_3OH) λ_{max}: 266, 335, 440 nm; FTIR (KBr): 3466, 3220, 2925, 1723, 1674, 1549, 1503, 1404, 990 and 771 cm^{-1}; ^{1}H NMR ($CDCl_3$): 0.92 (t, 3H, CH_3), 1.36-1.49 & 1.85-2.18 (m, 8H, 4×CH_2), 4.71 (t, 2H,

$N^{10}CH_2$), 7.64-7.69 (m, 2H, H-7 & H-9), 7.97 (dd, 1H, H-8, *J* = 7.66 & 1.40 Hz), 8.36 (d, 1H, H-6, *J* = 7.88 Hz), 8.50 (bs, 1H, N^3H).

General procedure for the N-3 alkylation of 10-substituted isoalloxazines

DBU (0.5 mmol) was added to a solution of alkyl halide (0.6 mmol) and flavin (**8**) (0.5 mmol) in dry benzene (50 mL). The reaction mixture was stirred at room temperature for 2 hours and washed with water. The organic layer was dried over anhydrous sodium sulphate and the solvent was removed under reduced pressure. The residue was further washed with petroleum ether to remove the alkylating agent, if present and recrystallized from petroleum ether-benzene. In a few cases where starting materials were present, the residue was chromatographed over silica gel (60-120 mesh) using chloroform as an eluent.

10-Ethyl-3-methylflavin (9a)

Mp. 296-297 ^oC; FTIR (KBr): 3090, 1713, 1661, 1573, 1423, 1283, 1266, 1182, 1093, 880, 760 cm^{-1}; ^1H NMR (DMSO-d_6): 1.24 (t, 3H, CH_3), 3.33 (s, 3H, N^3-CH_3), 4.11 (q, 2H, N^{10}-CH_2), 7.61-7.67 (m, 1H, H-9), 7.93-7.99 (m, 2H, H-7 & H-8), 8.33 (d, 1H, H-6, *J* = 9.12 Hz).

3-Methyl-10-propylflavin (9b)

Mp. 290-291 ^oC; FTIR (Nujol): 2922, 2870, 1719, 1663, 1545, 1277, 1200, 1196, 1093, 883, 775 cm^{-1}; ^1H NMR (DMSO-d_6): 1.07 (t, 3H, CH_3), 1.89-1.93 (m, 2H, CH_2), 3.37 (s, 3H, N^3-CH_3), 4.51 (t, 2H, N^{10}-CH_2), 7.93-8.15 (m, 3H, H-9, H-7 & H-8), 8.41 (d, 1H, H-6, *J* = 9.12 Hz).

10-Butyl-3-methylflavin (9c)

Mp.: 301-303 ^oC (lit.[55] mp. 307-308 ^oC); FTIR (KBr): 2925, 2856, 2362, 1710, 1650, 1610, 1580, 1540, 1500, 1461, 1363, 1270, 1190, 1170, 1100, 1040, 960, 865, 767, 720 cm^{-1}; ^1H NMR ($CDCl_3$): 0.90 (t, 3H, CH_3), 1.35-1.75 (m, 4H, 2×CH_2), 3.55 (s, 3H, N^3-CH_3), 4.70 (t, 3H, N^{10}-CH_2), 6.89 (d, 1H, H-9, *J* = 8.1 Hz), 7.55-7.66 (m, 2H, H-7 & H-8), 8.12 (dd, 1H, H-6, *J* = 8.2 & 1.8 Hz).

3-Methyl-10-pentylflavin (9d)

Mp.: 281-283 ^{o}C; FTIR (KBr): 2956, 2871, 1722, 1663, 1540, 1500, 1455, 1400, 983, 773 cm^{-1}; ^{1}H NMR ($CDCl_3$): 0.93 (t, 3H, CH_3), 1.29-1.39 (m, 6H, 2×CH_2), 3.56 (s, 3H, N^3-CH_3), 4.55-4.57 (t, 2H, $N^{10}CH_2$), 7.31 (d, 1H, H-9, *J* = 8.03 Hz), 7.69-7.83 (m, 2H, H-7 & H-8), 8.07 (d, 1H, H-6, *J* = 8.17 Hz).

10-Hexyl-3-methylisoalloxazine (9e)

Mp.: 180-181 ^{o}C (lit.[55] mp. 180 ^{o}C); FTIR (KBr): 2925, 2856, 2362, 1710, 1650, 1610, 1580, 1540, 1500, 1461, 1363, 1270, 1190, 1170, 1100, 1040, 960, 865, 767 and 720 cm^{-1}; ^{1}H NMR ($CDCl_3$): 0.90 (t, 3H, CH_3), 1.35-1.70 (m, 8H, 4×CH_2), 3.45 (s, 3H, N^3-CH_3), 4.70 (t, 3H, N^{10}-CH_2), 6.89 (d, 1H, H-9, *J* = 8.1 Hz), 7.53-7.55 (m, 1H, H-7), 7.90-7.92 (m, 1H, H-8), 8.28 (dd, 1H, H-6, *J* = 7.8 & 1.2 Hz).

Synthesis of catalyst (5-ethyl-3-methylflavinium perchlorate salt) (10)

Synthesis of **2** was achieved using the reported procedure with 10-substituted flavin.[56,57] 10-Substituted-3-methylflavin (1 mmol) and Pd/C (10%) were suspended in ethanol (15 mL). The mixture was purged with nitrogen. Concentrated HCl (1.5 mL) and freshly distilled acetaldehyde (1.5 mL) were added to the mixture and was hydrogenated at room temperature. The mixture was filtered to give yellow colour solution. Since this compound as reported is very sensitive towards air, it was not isolated. This was further dissolved in 10 mL of dithionite containing ammonia and filtered. The filtrate was added to 70% $HClO_4$ solution (15 mL). Further, $NaNO_2$ (7 mmol) and $NaClO_4$ (4 mmol) were added to the reaction mixture. The reaction mixture was stirred at 10°C for 2 h. The resulting light purple precipitate was filtered, washed with water, CH_3OH, ether and $CHCl_3$ to yield flavinium perchlorate salt (**10**).

5,10-Diethyl-3-methylflavinium perchlorate salt (10a)

^{1}H NMR (CD_3CN): 0.96 (t, 3H, CH_3), 1.11 (t, 3H, CH_3), 1.81 (m, 2H, CH_2), 3.21 (s, 3H, N^3CH_3), 3.92 (t, 2H, $N^{10}CH_2$), 7.34 (d, 1H, H-9, *J* = 7.91), 7.67-7.68 (m, 2H, H-7 & H-8), 7.96 (d, 1H, H-6, *J* = 7.88 Hz).

5-Ethyl-3-methyl-10-propylflavinium perchlorate salt (10b)

^{1}H NMR (CD_3CN): 0.98 (t, 3H, CH_3), 1.17 (t, 3H, CH_3), 1.71 (m, 2H, CH_2), 1.91 (m, 2H, CH_2), 3.16 (s, 3H, N^3CH_3), 3.93 (t, 2H, $N^{10}CH_2$), 7.33 (d, 1H, H-9, J = 7.71), 7.77-7.88 (m, 2H, H-7 & H-8), 7.91 (d, 1H, H-6, J = 7.78 Hz).

10-Butyl-5-ethyl-3-methylflavinium perchlorate salt (10c)

^{1}H NMR (CD_3CN): 0.91 (t, 3H, CH_3), 1.09 (t, 3H, CH_3), 1.22–1.92 (m, 6H, 3×CH_2), 3.25 (s, 3H, N^3CH_3), 4.02 (t, 2H, $N^{10}CH_2$), 7.31–7.41 (m, 2H, H-7 & H-9), 7.77-7.79 (m, 1H, H-8), 8.06 (d, 1H, H-6, J = 7.99 Hz).

5-Ethyl-3-methyl-10-pentylflavinium perchlorate salt (10d)

^{1}H NMR (CD_3CN): 1.01 (t, 3H, CH_3), 1.16 (t, 3H, CH_3), 1.19–1.99 (m, 8H, 4×CH_2), 3.22 (s, 3H, N^3CH_3), 3.86 (t, 2H, $N^{10}CH_2$), 7.39 (d, 1H, H-9, J = 8.11 Hz), 7.55–7.68 (m, 2H, H-7 & H-8), 7.86 (d, 1H, H-6, J = 7.79 Hz).

5-Ethyl-10-hexyl-3-methylflavinium perchlorate salt (10e)

^{1}H NMR (CD_3CN): 0.96 (t, 3H, CH_3), 1.11 (t, 3H, CH_3), 1.20–2.01 (m, 10H, 5×CH_2), 3.21 (s, 3H, N-CH_3), 3.92 (t, 2H, $N^{10}CH_2$), 7.34–7.41 (m, 2H, H-7 & H-9), 7.67-7.68 (m, 1H, H-8) & 7.86 (d, 1H, H-6, J=7.88 Hz).

BV oxidation with the catalyst prepared

Ketone (**11**, 5 mmol) and the catalyst **10** (100 mg) were dissolved in *t*-BuOH (10 mL), and 1 mL of 35% H_2O_2/water solution was added *via* syringe in a 50 mL round bottom flask. The mixture was stirred at nitrogen atmosphere at room temperature for 24 h. The progress of the reaction was monitored with TLC using authentic sample. The evaporation of the solvent under reduced pressure afforded the lactones (**12**).

δ-Valerolactone

Viscous liquid; FTIR (nujol): 2960, 2903, 1745, 1475, 1460, 1440, 1430, 1339, 1235, 1210, 1190, 1070, 930 cm^{-1}; ^{13}C NMR ($CDCl_3$, 300 MHz): 174, 66, 31, 30, 27 ppm.

ε-Caprolactone

Viscous liquid; FTIR (nujol): 2985, 2898, 1750, 1491, 1440, 1423, 1403, 1350, 1246, 1188, 1165, 1083, 941 cm^{-1}; ^{13}C NMR ($CDCl_3$, 300 MHz): 177, 69, 34, 32, 29, 23 ppm.

2.4 Results and Discussion

This study is based on the reactions carried out by BVMOs.[35-40] This work mimics the oxidation work performed by BVMOs using the synthetic analogue of their cofactor, 10-substituted flavins. Further, we tried to establish the relationship between N10-chain length and the percent of transformation from cyclic ketone to lactone.

2.4.1 Synthesis of 3,10-disubstituted flavins (9)

The flavin (isoalloxazine) derivatives were synthesized by using the published procedure and as given in experimental section (Chapter 1) (Scheme 2.5).[58-60] In this chapter, 10-substituted flavins have been synthesized with aliphatic amines. The aliphatic chain was chosen as the N^{10}-substitution in natural flavin starts with aliphatic chain.

The reaction of 1-chloro-2-nitrobenzene (**5**) with aliphatic amines (**6**) in the presence of fused potassium acetate gave N-substituted-2-nitroaniline (**7**) in 40-45% yield. In a typical case, the formation of N-hexyl-2-nitroaniline (**7e**) has been confirmed by various spectroscopic data. In the ^{1}H NMR spectra of **7e**, a triplet at 0.99 ppm for three protons has been assigned to the terminal CH_3 group. Two multiplets in the region 1.15-1.65 and 2.80-3.01 ppm for ten protons is due to the aliphatic side chain. Two double doublets at 6.58 and 8.13 ppm for one proton each has been observed due to the aromatic protons H-6 and H-3 respectively. The presence of two multiplets in the region 6.82-6.85 and 7.14-7.17 ppm has been assigned to the H-4 and H-5 protons respectively. These ^{1}H NMR values confirmed the formation of **7e** (experimental section).

The reduction of **7** with $Pd/C/H_2$ gives the required diamino compound which on subsequent cyclocondensation with alloxan monohydrate affords 10-substituted flavins (**8**) in 33-54% yields. The formation of flavins (**8**) has been confirmed by various spectroscopic data including UV-visible, infrared (FTIR) and ^{1}H NMR spectroscopy. In a typical case, the absorption maxima in the UV-visible spectrum of 10-ethylflavin (**8a**) has been observed in methanol at 266, 335 and 440 nm, which are characteristic of the flavin moiety (Figure 2.2).[61] In the IR spectra of **8a**, the appearance of peaks at 1660 and 1715 cm^{-1} indicate the presence of two carbonyl groups at 2- and 4-positions respectively, which are also characteristics of the flavin moiety.[61-63] The structure has further been confirmed by ^{1}H NMR spectroscopy.

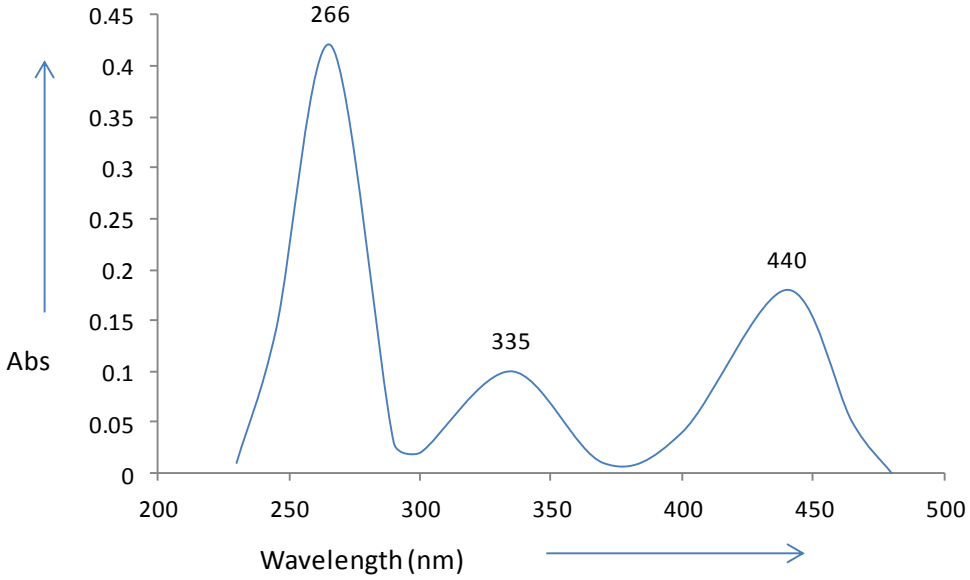


Figure 2.2: UV-visible spectrum of 10-ethylflavin (**8a**)

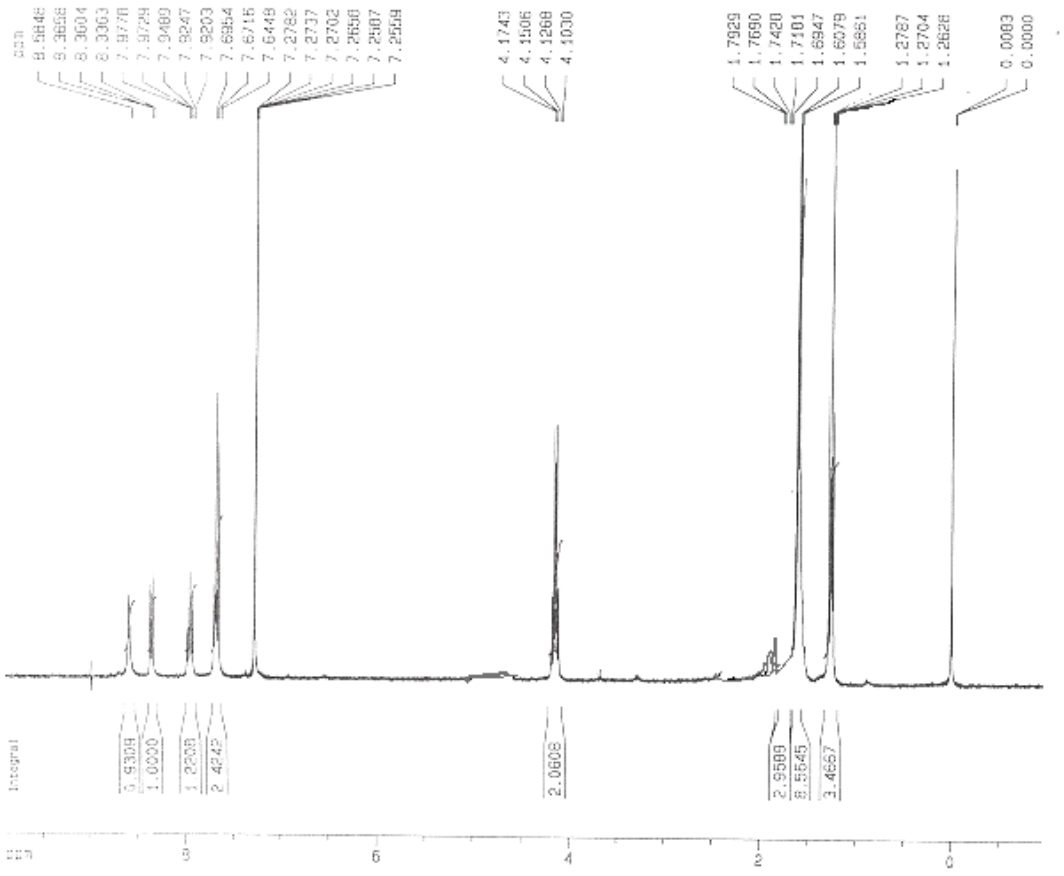


Figure 2.3: ^{1}H NMR spectrum of 10-ethylflavin (**8a**)

In the ^{1}H NMR spectrum of **8a** recorded in $CDCl_3$, a triplet at 1.27 for three protons and a quartet at 4.13 ppm have been assigned to the methyl group and $N^{10}CH_2$ protons. Other multiplets in the aromatic regions have been consistent with the ^{1}H NMR spectrum of flavin moiety (Figure 2.3).[61,62] In the ^{1}H NMR spectrum of 10-propylflavin (**8b**), a triplet at 1.10 ppm and multiplet in the region 1.81-1.89 ppm for two protons have been assigned to the CH_3 and CH_2 groups respectively. Another triplet at 4.62 ppm is for N^{10}-CH_2 group. Other peaks in the aromatic regions further confirmed the formation of **8b** (Figure 2.4).

The reaction of 10-substituted flavin (**8**) with methyl iodide using DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) as a base in dry benzene at room temperature gave 3,10-disubstituted flavins (**9**) in 87-97% yields (Scheme 2.5).[55] The formation of **9** has been confirmed by various spectroscopic data including infrared (FTIR) and ^{1}H NMR spectroscopy. The alkylation at N-3 position in **9a** has been confirmed by ^{1}H NMR where an additional singlet appeared at 3.33 ppm. The other peaks have been observed in agreement with the flavin moiety.

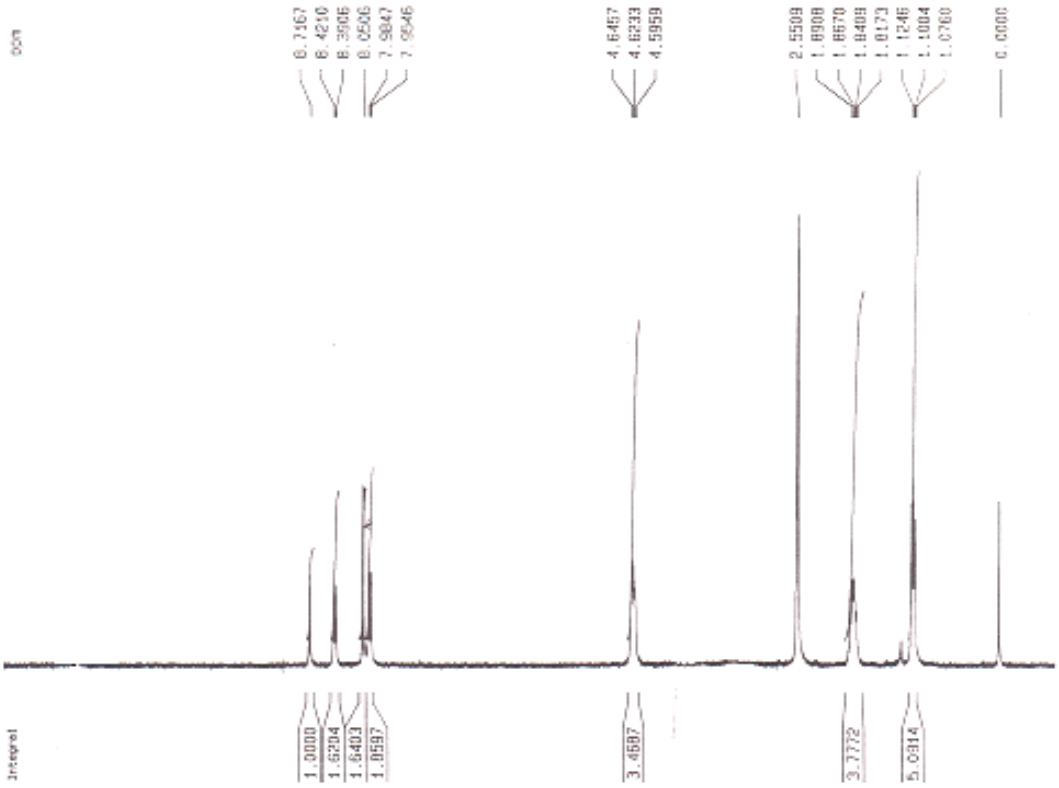
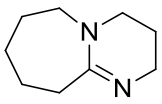


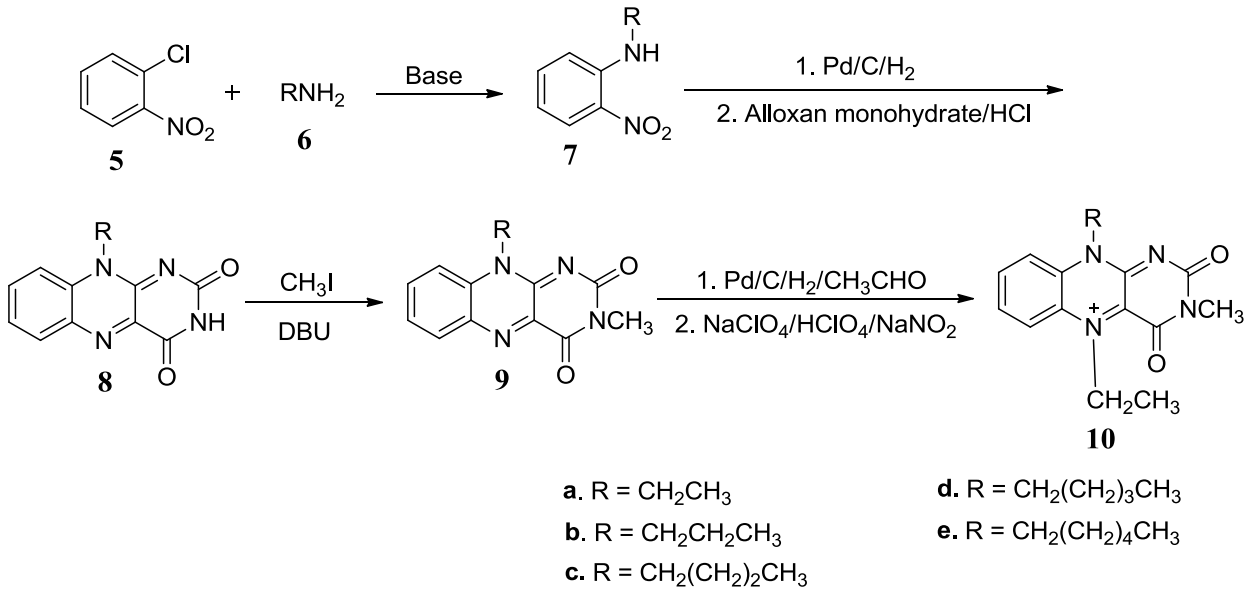
Figure 2.4: ^{1}H NMR spectrum of 10-propyflavin (**8b**)



1,8-Diazabicyclo[5.4.0]undec-7-ene

2.4.2 Synthesis of 5-ethyl-3-methylflavinium perchlorate salt (10)

The synthesis of the catalyst **10** was achieved using the reported method (Scheme 2.5).[56,57] The flavin **9** on reductive alkylation with Pd/C (10%) and acetaldehyde and further treatment without isolation with $HClO_4$/ $NaClO_4$ in the presence of $NaNO_2$ gave a purple colour compound **10**. This catalyst was used for the oxidation of lactones. These cationic flavins are known to give an intermediate flavin hydroperoxide on reaction with hydrogen peroxide (H_2O_2) which is an active oxygen donor.



Scheme 2.5: Synthesis of 10-substituted 5-ethyl-3-methylflavinium perchlorate (**10**)

2.4.3 Baeyer-Villiger oxidation of lactones with synthesized catalyst

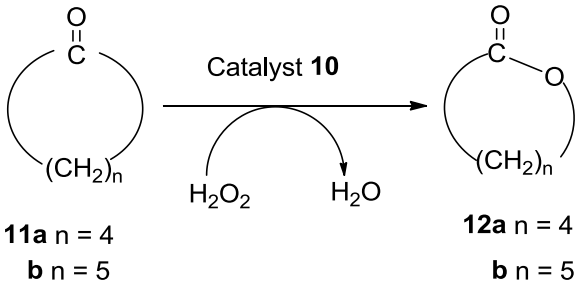
The reaction of cyclopentanone (**11a**) with hydrogen peroxide solution (35% w/w) in the nitrogen atmosphere and the flavin catalyst **10** in *t*-butanol resulted in the conversion of cyclopentanone (**11a**) to δ-valerolactone (**12a**) in 89% yields after 24 hours of the reaction at room temperature (Scheme 2.6). The progress of reaction was monitored with thin layer chromatography (TLC) using authentic sample of **12a**. The formation of the product was confirmed by FTIR and ^{13}C NMR spectroscopy. The decrease in the

frequency has been observed for carbonyl group in lactones. The FTIR spectra of **12a** is given in figure 2.5 and ^{13}C NMR of **12b** is given in figure 2.6.

To check the use of catalyst, the reaction was performed under same condition without the use of catalyst **10**. In the first 24 hours, no product was obtained as observed from TLC. The reaction was allowed to run for further 48 hours. No product was obtained even after 48 hours. The TLC of the reaction mixture showed the presence of unreacted **11a**. The reaction was also repeated with cyclohexanone (**11b**) and found the similar results.

The reaction with respect to the use of solvent was also optimized for the biomimetic reaction leading to BV oxidation (Table 2.1). Four solvents were tried, water, methanol, acetonitrile and *t*-butanol. The reaction in *t*-butanol gave the best yield and hence was chosen as the appropriate solvent for the reaction.

The role of hydrogen peroxide (H_2O_2) was also confirmed as oxygen donor as no reaction was observed without its presence (Table 2.1, entry 5). However, when reaction was performed with H_2O_2 but in the presence of molecular oxygen, about 30% conversion observed.



Scheme 2.6: BV oxidation of cyclic ketones

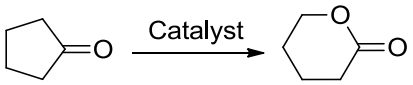
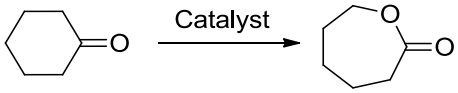
To check the biomimetic prospects of the reaction, catalyst with different chain length at N^{10}-position which were prepared have been applied for the BV oxidation with the substrates, **11a** and **11b** (Table 2.2). The reaction with the catalyst 10-hexyl-5-ethyl-3-methylflavinium perchlorate salt) (**10e**) gave better yield in both the cases. Also, the difference in yield between the catalysts **10e** and **10d** was found to be very low (Table 2.2). This suggests that the chain length of 5 to 6 carbon atoms at the N^{10}-position is ideal to carry out this BV oxidation using metal-free organocatalyst based on the reaction pathways of BVMOs.

Table 2.1: Optimization of reaction conditions for BV oxidation of **11a**

S. No.	Reaction conditions used[#]	Isolated % Yield of 12a
1.	**11a/10a/** H_2O_2/water	30
2.	**11a/10a** /H_2O_2/methanol	40
3.	**11a/10a** /H_2O_2/acetonitrile	60
4.	**11a/10a** /H_2O_2/*t*-butanol	89
5.	**11a**/ H_2O_2/ *t*-butanol	No reaction
6.	**11a/10a** /*t*-butanol	No reaction
7.	**11a/10a**/mol O_2 /*t*-butanol	30

#stirred for 24 h in nitrogen atmosphere at room temperature

Table 2.2: Comparative yield of cyclic ketones with catalysts **10**

S. No.	Catalyst	Yield (%, Isolated)
 **11a** **12a**		
1	**10a,** N[10]-ethyl	78
2	**10b,** N[10]-propyl	78
3	**10c,** N[10]-butyl	80
4	**10d**, N[10]-pentyl	87
5	**10e,** N[10]-hexyl	89
 **11b** **12b**		
6	**10a,** N[10]-ethyl	80
7	**10b,** N[10]-propyl	80
8	**10c,** N[10]-butyl	82
9	**10d**, N[10]-pentyl	89
10	**10e,** N[10]-hexyl	91

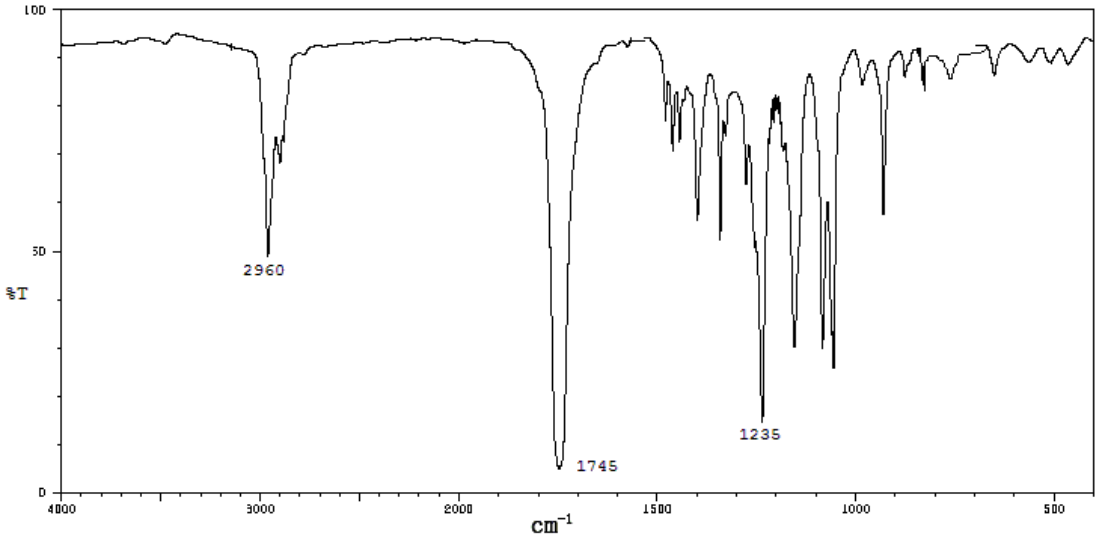


Figure 2.5: FTIR spectrum of δ-valerolactone

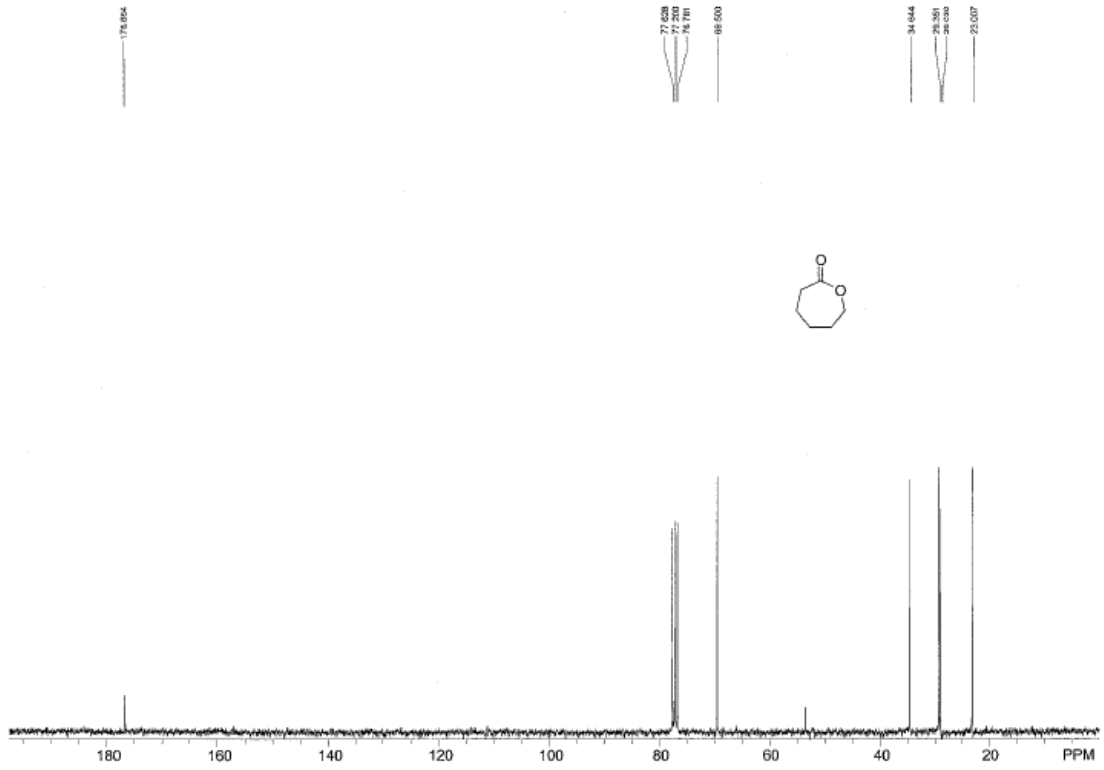


Figure 2.6: ^{13}C NMR spectrum of ε-caprolactone in $CDCl_3$

2.5 Conclusions

Esters and cyclic esters (lactones) are an important class of compounds having wide range of applications. One of the versatile methods for their synthesis is their transformation from corresponding ketones or cyclic ketones using BV oxidation. The BV oxidations have been achieved by enzymatic and chemical methods.

The chapter deals with the synthesis of lactones using chemical methods based on biomimetic approach. The results support the existing thought that the flavin derivatives can be used as enzyme models for catalyzing the BV oxidation of ketones. The reaction can be run in a very simple fashion at room temperature using hydrogen peroxide as a cheap oxidant using metal-free organocatalysts.

In the present study, for the first time we have initiated the effect of chain length at N-10 position of the catalyst to understand the molecular mechanism of BVMOs o n BV oxidation reactions. The study suggested that the chain length of 5 to 6 carbon atoms at the N^{10}-position is ideal to carry out this BV oxidation based on the reaction pathways of BVMOs.

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CHAPTER 3

Application of Flavins as catalyst for Remediation of selected Halogenated compounds

3.1 Introduction

The compounds possessing one or more, same or different halogen atoms are known as halogenated compounds. The annual global production and consumption of halogenated compounds is very high.[1-4] These compounds are regarded as persistent pollutants and unfortunately accurate data is not available. The use and misuse of halogenated organic compounds in industrial and agricultural sectors allows entry of huge amount of these chemicals into the environment leading to environmental contamination.[1-4] Some of the other applications of these compounds include solvents, antifouling, flame retardants and so on.[5] As per a report of Meyer et al. about 30% of agrochemicals and 20% of small molecule drugs are halogenated compounds.[5] The percentage distribution of the emission of halogenated compounds with same or different halogen atoms from different sources is given in figure 3.1.[5] Apart from this, about 10% of halogenated compounds possess two or even more different types of halogens in the same molecules.[5] The environment contaminated with halogenated compound pose various types of risks to the ecosystems (Figure 3.2). The amounts of chlorinated compounds are higher in composition to other halogenated compounds. The volatile chlorinated compounds have usually been found in industrial areas.[6,7] The high molecular weight chlorine containing organic compounds such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), perfluorooctane sulfonate (PFOS), hexachlorobenzene (HCB), dichlorodiphenyl trichloroethane (DDT) etc are commonly known toxic pollutants which causes adverse effects to the health of humans and wildlife.[8] These compounds have very long persistent time and are highly toxic in nature. The presence of strong carbon-chlorine bond is responsible for their stability and persistent in the environment.

There are many examples where particular halogenated compounds have been very useful but later withdrawn due to its toxic nature. One of the best examples is DDT. The compound DDT had find applications in the past but later this had been banned by United States Environmental Protection Agency (EPA) due to its toxic and adverse effect to the health of wildlife and humans.[9] It causes very harmful disease such as cancer, tumor and birth defect. Other halogenated compounds like PCBs[10] and PBDEs[11] are other two important and extensively used industrialized chemicals that have been produced in wide range over the past three decades. Their use and misuse represents a huge amount of pollution in the environment.[12] The above few examples are sufficient to understand the need for their remediation leading to healthy life.

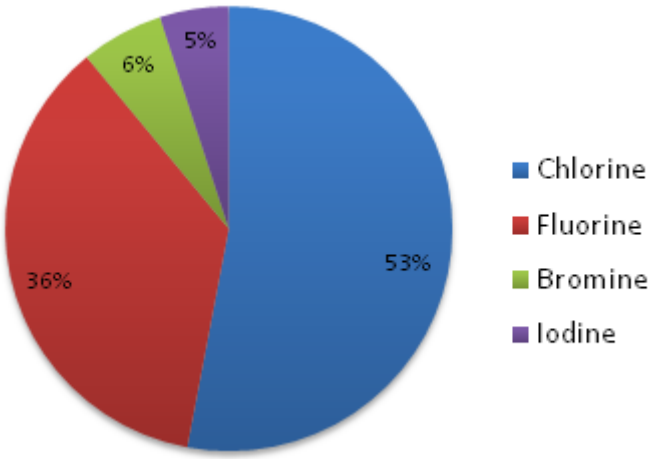


Figure 3.1: Emission of halogenated compounds

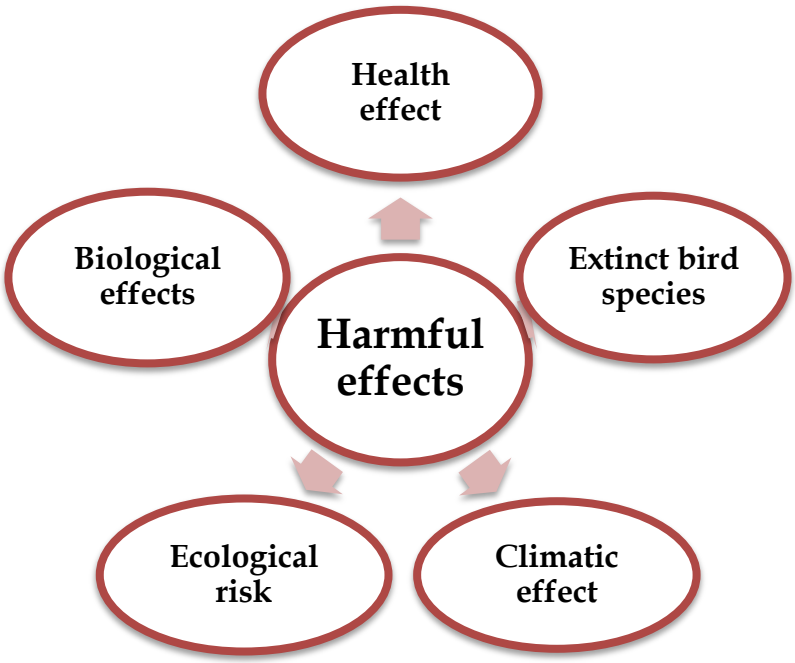


Figure 3.2: Harmful effects of halogenated compounds

The quality of our life is directly proportional to the quality of the environment. A better environment always gives a healthy life. Unfortunately, we failed to balance between the progress in science, technology, and industry and their impact on our environment and hence life. Despite the enormous progress made from traditional disposal method of digging a hole and filling it with waste material to new technologies that use high-temperature incineration and chemical decomposition, a lot of involvement is

required in remediation process to get away from associated deficiencies.[13,14] The remediation process helps in the removal of pollutants or contaminants from environmental media like soil, water, or air.[15,16] Therefore, there is urgent need to develop an efficient method to minimize the presence of toxic chemicals into the environment. This chapter is a step towards the chemical catalytic remediation of halophenols based on biomimetic reaction.

3.2 Remediation of halogenated compounds: Literature review

Due to the existence of halogenated pollutants like PCBs, PBDEs, perfluorooctane sulfonate (PFOS) etc in the environment several remediation methodology including dehalogenation have been developed and adopted.[17-20] The dehalogenation of halogenated compounds has been achieved by several methods like electrochemical, photochemical, ultrasonic technique etc. Considering the methods of remediation process, they are grouped into two important categories, one is chemical dehalogenation (chemremediation) and other is bioremediation methods.[21,22]

3.2.1 Chemremediation

The chemremediation or chemical degradation method uses catalysts, metals, non-metals, their salts to convert the halogenated compounds to non-toxic compounds.[23-25] Many researchers have used catalytic dehydrohalogenation for the remediation of halogenated compounds.[24,26] This is the potential methodology developed by the scientists. This method involves hydrogenolysis of the carbon-halogen bond to remove halogen atoms from halogenated molecules.

Reports are there for the application of zeolite based catalysts also for dehalogenation reactions.[27-31] Creyghton et al. have used the platinum loaded HBEA zeolite for the gas phase hydrogenation of chlorobenzene.[27] Shin and Keane also contributed towards similar activity using nickel-silica and nickel-Y zeolite catalysts for gas phase hydrodechlorination of pentachlorophenol.[28,29]

The study has also shown that the 4-chlorophenol was converted into cyclohexanone with the help of molecular hydrogen under the solvent free condition.[19] The chemical reductive methods generally adopt transition metals as catalysts, based on nickel[32,33], palladium[34,35], rhodium[36,37] and platinum[38,39]. Palladium (Pd) chemistry has

been the most widely used catalyst among all the other catalysts and studied intensively by the researchers.[40,41] The studies have shown that the catalytic activity of Raney Ni was comparable to that of Pd/C for the dehalogenation of compounds like 4-bromobiphenyl and 2-chlorophenol under mild reaction conditions.[42,43]

The studies have also shown that the rhodium complexes bearing bis (imino)pyridine ligands generate rhodium nanoparticles, which catalyze the dehalogenation of aromatic compounds, including PCBs and the hydrogenation of benzene and functionalized aromatic compounds. This study revealed that the complex of rhodium possesses powerful activity towards dehalogenation under mild reaction conditions.[44]

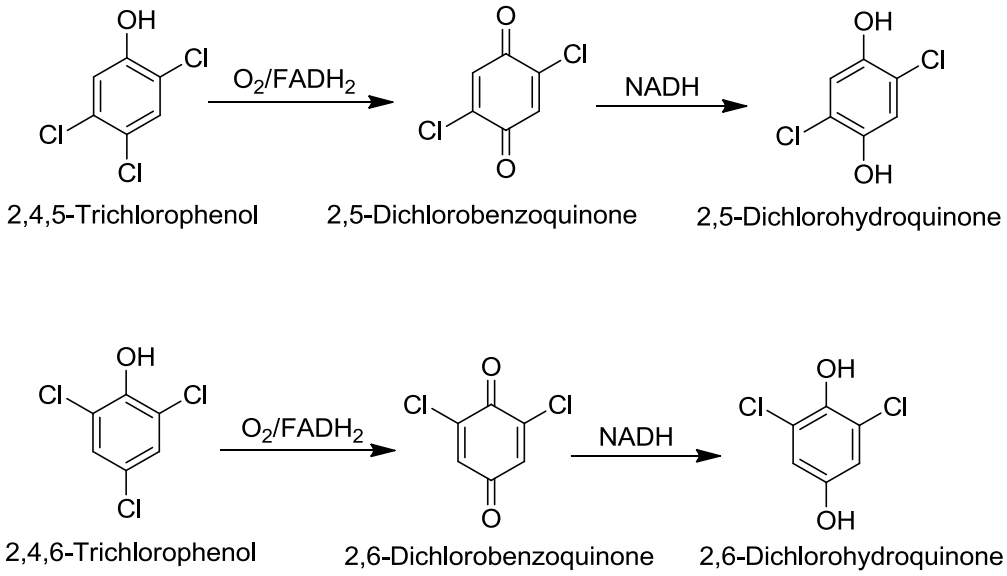
In the field of chemical dehalogenation, a new methodology was developed by the researchers with help of indium chemistry for degradation of halogenated environmental. The indium chemistry has also been used for the remediation process.[23,45] To make the reaction process more environmental friendly, green solvents[46,47] like, ionic liquids (ILs) have also been used as an alternative solvent for the dehalogenation reactions.[48]

The solvent free dehalogenation of aromatic halides by using molecular hydrogen has also been achieved which has advantages of non-toxicity and green environment efficiency.[49] Another study demonstrated the PANI/Fe^0 catalyzed dehalogenation process. This is an excellent example of nanotechnology application in dehalogenation. This showed the remediation of variety of aryl halides with Grignard reagent as a reductant under optimized condition reaction.[50]

3.2.1 Bioremediation

The bioremediation process utilizes natural systems like enzymes, microorganisms etc for the dehalogenation process.[51] The bioremediation process is safe but very slow in execution. Most of this remediation system works under aerobic conditions. Enzymes being a well known biocatalysts are an unopposed choice for bioremediation and hence many oxygenases mediate the dehalogenation process.[51] Flavin dependent monooxygenases or flavoenzymes are an ubiquitous and diverse class of biological redox catalysts involved in dehalogenation reactions.[51-53] The active site responsible for the activation of molecular oxygen has been established as the enzyme-bound 4a-hydroperoxyflavin (Chapter 2, Scheme 2.3).[54,55] These 4a-hydroperoxyflavin have been utilized by enzymes for the removal of halogens during the bioremediation of chlorinated

phenols. Flavoenzymes like pentachlorophenol 4-monooxygenase (PcpB), chlorophenol 4-monooxygenase catalyzes hydroxylation at *para*-position with removal of the chloride ion in the degradation of chlorophenols using NADPH or NADH as a co-substrate (Scheme 3.1), although both the enzymes possess similarities and differences.[56]



Scheme 3.1: Bioremediation of chlorophenols with flavoenzymes

In this chapter, we have developed a new facile and efficient biomimetic methodology for the dehalogenation of halogenated pollutants as environment friendly method. The biomimetic pathways always attract interest because of the limited availability of enzymes or due to difficulties of enzyme expression or isolation.

3.3 Experimental

Melting points were determined on a laboratory capillary melting apparatus and are uncorrected. FTIR spectra were recorded on a Perkin Elmer 1710 FTIR spectrophotometer and the ν_{max} are expressed in cm^{-1}. ^1H NMR was recorded on a Bruker Avance-300 spectrophotometer (300 MHz) and the chemical shifts were expressed in ppm.

The solvents and reagents were purchased from reputed company and were used without further purification. Thin-layer chromatography was performed on aluminium coated silica plates.

Synthesis of catalyst (10-substituted-5-ethyl-3-methylflavinium perchlorate salt) (4):

The synthesis of the catalysts **4** was done in chapter 2, experimental section and utilized for the dehalogenation reaction in this chapter.

Procedure for the dehalogenation reaction of 4-chlorophenol with flavin catalyst 4:

Hydrogen peroxide solution (35% w/w) was added to a mixture of 4-chlorophenol (0.75 mmol) and flavin catalyst (0.019 mmol) in acetonitrile (1 mL). The mixture was stirred for 18 h in nitrogen atmosphere at 80 ^{o}C. Upon cooling, the organics were then extracted three times with ethyl acetate (3×5 mL). The combined organic extracts were dried over $NaSO_4$ and solvent removed in vacuo to get the benzoquinone. The progress of reaction was monitored with TLC using authentic sample of benzoquinone and chlorophenol.

Similarly, this reaction was repeated with 4-bromophenol.

The formation of benzoquinone was characterized by melting point (115 °C) and ^{1}H NMR ($CDCl_3$): 6.90 ppm.

3.4 Results and Discussion

Halogenated organic compounds (HOCs) are one of the most versatile and widely used classes of compounds in the industrial world.[57] Unfortunately they are considered as a major pollutant for any ecosystem due to their persistent, lipophilic and toxic nature.[58,59] Compounds like pentachlorophenol (PCP) which has been widely used to preserve lumber, is currently listed as one of the major environmental pollutants in North America.[60,61] The exposure to penta is known to cause cancer and birth defects in laboratory animals, and chromosome abnormalities, blood disorders, and nerve damage in humans.[62] Due to its toxic nature, this compound was banned in ten countries and severely restricted in nine.[62,63] Chlorinated phenols and their derivatives have also been widely used as pesticides and herbicides, increasing the list of environmental pollutant. The complete or partial removal of halogens from these compounds is an important method of remediation. This study is based on the bioremediation study of halogenated phenols by chlorophenol monooxygenases. This work mimics the remediation work performed by chlorophenol monooxygenases using the synthetic analogue of their flavin cofactor.

3.4.1 Synthesis of 3,10-disubstituted flavins (1)

The flavin (isoalloxazine) derivatives were synthesized by published procedure and as given in (Chapter 2, Experimental section). To a solution of N-substituted-2-nitroanilines in absolute ethanol in paar hydrogen bottle, Pd/C was added and the reaction mixture was hydrogenated at ambient pressure and temperature for 24 hours. When the quantitative amount of hydrogen was absorbed and the reaction mixture became almost colourless, the hydrogenation apparatus was stopped and hydrogen gas was released. Glacial acetic acid was added to the reaction mixture and the catalyst was filtered off. The alloxan monohydrate under the acidic condition was added and the reaction mixture was refluxed for one hour. The reaction mixture was allowed to cool to room temperature and kept overnight in refrigerator to get the desired product 10-substituted flavins in 40-45% yields. This was further methylated at N-3 position with CH_3I using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base.

3.4.2 Synthesis of catalyst (5-ethyl-3-methylflavinium perchlorate salt) (4)

The synthesis of the catalyst **4** was achieved using the published procedure[64,65] and as given in experimental section (Scheme 3.2). The flavin **1** on reductive alkylation with Pd/C (10%) and acetaldehyde gave the compound **3**. This was further treated without isolation with $HClO_4$/ $NaClO_4$ in the presence of $NaNO_2$ to get a purple colour compound **4**. This catalyst was used for the oxidation of halogenated organic compounds. These cationic flavins are known to give an intermediate flavin hydroperoxide (**5**) on reaction with hydrogen peroxide (H_2O_2) which is an active oxygen donor (Scheme 3.2).

3.4.3 Biomimetic reaction of halogenated compound with catalyst 4

The reaction of 4-halophenol (**7**) with hydrogen peroxide solution (35% w/w) in the nitrogen atmosphere and the flavin catalyst **4** in acetonitrile resulted in substitution of the chloro group with hydroxyl group (**7**-**9**, Scheme 3.2). The progress of reaction was monitored with thin layer chromatography (TLC) using authentic sample of benzoquinone and 4-halophenols. To check the use of catalyst, the reaction was performed under same condition without the use of catalyst **4**. The reaction was allowed to run for 24 hours. No product was obtained even after 24 hours. The TLC of the reaction mixture showed the presence of unreacted 4-halophenols.

The reaction with respect to the use of solvent was also optimized for the biomimetic reaction leading to chemical remediation of halogenated compounds (Table 3.1). Three solvents were tried, methanol, acetonitrile and *t*-butanol. The reaction in acetonitrile gave the best yield and hence was chosen as the appropriate solvent for the reaction.

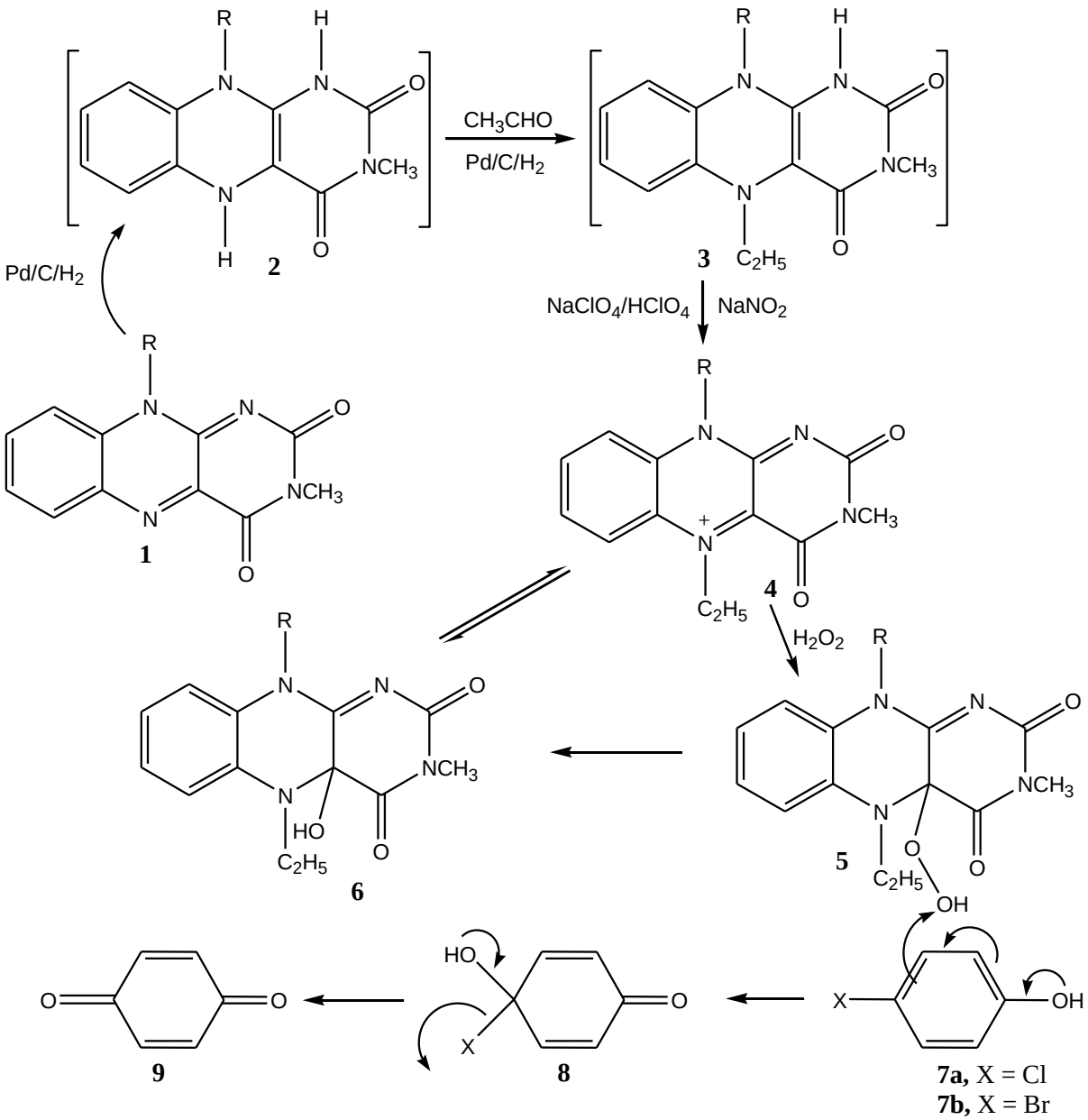
The chemremediation reaction was also tried without the use of catalyst **4** under similar reaction condition. No product was obtained after 18 hours of reaction (Table 3.1, entry 4). The reaction was continued for another 6 hours but still no product was obtained. The role of hydrogen peroxide (H_2O_2) was also confirmed as oxygen donor as no reaction was observed without its presence (Table 3.1, entry 5).

Table 3.1: Optimization of reaction conditions for remediation of 4-chlorophenol (**7a**)

S. No.	**Reaction conditions used**[#]	**% Yield**
1.	**7a**/**4e**/H_2O_2/methanol	40
2.	**7a**/**4e**/H_2O_2/acetonitrile	62
3.	**7a**/**4e**/H_2O_2/*t*-butanol	45
4.	**7a**/ H_2O_2/Acetonitrile	No reaction
5.	**7a**/**4e**/Acetonitrile	No reaction

#stirred for 18 h in nitrogen atmosphere at 80 ^oC.

To check the biomimetic prospects of the reaction, catalyst with different chain length at N^{10}-position which were prepared in chapter 2 were tried with the substrates, 4-chlorophenol and 4-bromophenol. The 4-chlorophenol was found to be better substrate for remediation in comparison to the 4-bromophenol. The maximum remediation was observed 62% with the substrate **7a** whereas with **7b** 58% benzoquinone was formed (Table 3.2). The reaction with the catalyst 10-hexyl-5-ethyl-3-methylflavinium perchlorate salt) (**4e**) gave better yield in both the cases. Also, the difference in yield between the catalysts **4e** and **4d** was found to be very low (Table 3.2).



a. R = CH_2CH_3 **b.** R = $CH_2CH_2CH_3$ **c.** R = $CH_2(CH_2)_2CH_3$
d. R = $CH_2(CH_2)_3CH_3$ **e.** R = $CH_2(CH_2)_4CH_3$

Scheme 3.2: Catalytic chemremediation of 4-halophenols (**7**)

This suggests that the chain length of 5 to 6 carbon atoms at the N^{10}-position is ideal to carry out this chemremediation using metal-free organocatalyst based on the reaction pathways of chlorophenol monooxygenases. The above results also confirmed that the remediation of halogens from halophenols is a step towards biomimetic reactions. These results are also in agreement with the results obtained in chapter 2.

Table 3.2: Chemremediation reaction of 4-halophenols with catalysts **4**.

S. No.	**Reaction conditions used**[#]	**% Yield***	**S. No.**	**Reaction conditions used**[#]	**% Yield***
*Chlorophenol(**7a**)*			*Bromophenol (**7b**)*		
1	**4a**/H_2O_2/acetonitrile	40	6	**4a**/H_2O_2/acetonitrile	33
2	**4b**/H_2O_2/acetonitrile	40	7	**4b**/H_2O_2/acetonitrile	38
3	**4c**/H_2O_2/acetonitrile	45	8	**4c**/H_2O_2/acetonitrile	45
4	**4d**/H_2O_2/acetonitrile	60	9	**4d**/H_2O_2/acetonitrile	55
5	**4e**/H_2O_2/acetonitrile	62	10	**4e**/H_2O_2/acetonitrile	58

#stirred for 18 h in nitrogen atmosphere at 80 ^oC.
*isolated yield

3.5 Conclusions

Halogenated organic compounds are well known environmental pollutants due to their stable and persistent nature. The presence of strong carbon-halogen bond is responsible for their stability in the environment. The remediation of halogen from these molecules always remains a challenge. Chemical and biological remediation processes are two most widely used methods to convert them into less toxic molecules. Enzymatic remediation is very important class of biological remediation.

Flavoenzymes are involved in the dehalogenation of various halogenated organic compounds. This enzyme works with the help of its cofactor flavin. The chapter deals with the dehalogenation of halophenols using chemical methods based on biomimetic approach. The results support the existing thought that the flavin derivatives can be used as enzyme models for catalyzing the dehalogenation reaction. The reaction can be run in a very simple fashion at room temperature using hydrogen peroxide as a cheap oxidant

using metal-free organocatalysts. The effect of chain length at N^{10} position of the catalyst was also studied to understand the molecular mechanism of halophenol monooxygenases. The study suggested that the chain length of 5 to 6 carbon atoms at the N^{10}-position is ideal to carry out this remediation based on the reaction pathways of flavoenzymes.

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CHAPTER 4

Electrochemical studies of Flavin derivative

4.1 Introduction

Electrochemical studies or electrochemistry is a part of chemical science but in totality, it is a subject in itself. This discipline deals with the dependence of chemical and electrical properties of any substances on each other. Starting from chemical sciences to biology to engineering, the field of electrochemistry has wide range of applications.[1-4] This discipline uses a measurement of potential and current to determine the concentration or chemical reactivity and sometimes vice-versa also. In most of the electrochemical studies, the study has been done with the help of three electrodes – the working electrode (WE), the reference electrode (RE) and the counter (or auxiliary) electrode (CE). These all three electrodes are connected to a potentiogalvanostat, a device that delivers a constant electric current at a constant voltage. This device controls the potential of the working electrode to estimate the resulting current.[5] In one typical electrochemical experiment, a potential is applied to the working electrode and the resulting current is measured. This further plotted versus time as shown in figure 4.1. In another experiment, the potential is varied and the resulting current is plotted versus the applied potential. In a solution, the equilibrium concentrations of the reduced and oxidized forms of a redox couple are linked to the potential (E) *via* Nernst's equation[6]:

$$E = Eo + \frac{RT}{nF} \ln C\ (oxi)/C(red)$$

Where, E_o is equilibrium, F is Faraday's constant, T is absolute temperature,
$C_{(oxi)}$ and $C_{(red)}$ are concentrations of oxidation and reduction points.

When the potential is applied to WE, the redox couples present at electrode adjust their concentration ratios according to the Nernst's equation. The signal transduction and the electrochemical (CV) performance of sensors have been studied using potentiostat/galvanostat. An accurately determined and maintained potential is produced by a potentiostat through the employment of DC power source and drawing small currents at constant voltage into the system.

In cyclic voltammeter, the working electrode potential is swept at a specific sweep rate (in volts/second) and the resulting current *vs* time curve is recorded. Generally, the sweep is reversed at a specific switching potential, and this process is known as cyclic voltammetry.[7] Since the sweep rate is constant and the initial and switching potentials are known, the time can be easily converted to potential and current *vs* applied potential can

be recorded. Cyclic voltammetry is applied to gain information on electrochemical reactions behavior of electroactive species consisting of known redox potential. The current is observed at the WE (as the CE conducts electricity from the signal source to the WE) during the potential scans against a constant RE potential. The electrolytic solution is used in order to provide ions to the electrodes during redox process. The resulting current *vs* applied potential curve (a cyclic voltammogram) is predicted for an ideal, reversible system to have the shape as given in the figure 4.1.

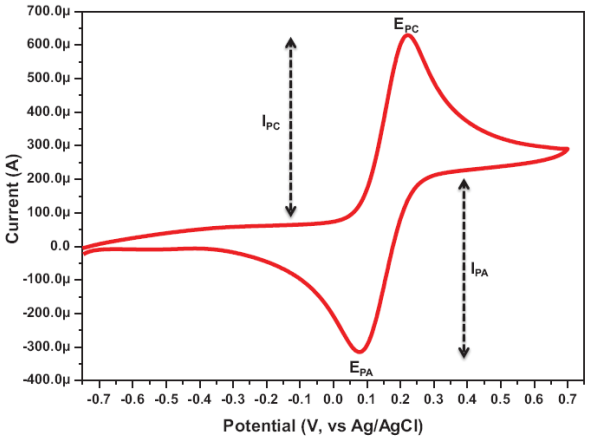
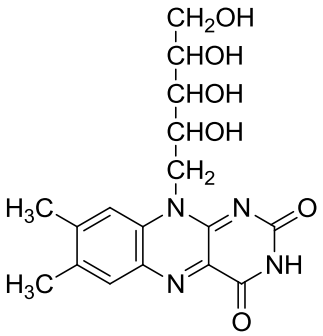


Figure 4.1: Cyclic voltammogram for an ideal, reversible system

Electrochemistry plays vital role in living organisms. The redox processes are involved in the substrate metabolism and energy conversion.[8] The processes like photosynthesis and respiratory electron transport along with others are only possible with a series of oxidation-reduction reactions.[8,9] The process like oxidative phosphorylation involves the oxidation of $FADH_2$ to FAD and NADH to NAD^+.[10,11] The redox character study of biomolecules with the help of electrochemical techniques is very helpful in understanding their mechanistic, kinetic and thermodynamic pathways.[8-12]

Flavoenzymes are important and most studied oxidoreductase enzymes. These enzymes possess flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) as their cofactors which are involved in various metabolic pathways through both $1e^-$ and $2e^-$ transfer reactions.[13,14] The electron transfer reactions are facilitated through the core flavin moiety which is a tricyclic molecule having fused pyrazine-pyrimidine rings.[15] This chapter deals with the electrochemical studies of riboflavin (**1**) under different environment to understand its molecular mechanism in living system.



Riboflavin (**1**)

4.2 Electrochemical studies of riboflavin: Literature review

Riboflavin (Rf, **1**) was formerly known as vitamin G.[16] This is a water soluble vitamin known as Vitamin B_2 which is discovered in 1920 AD.[17] First time, this was isolated in 1933 and synthesized in 1935 AD.[17] The molecule got its name from its sugar, ribitol. Its IUPAC name is 7,8-dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxypentyl) isoalloxazine. This is yellow-orange solid substance with poor solubility in water as compared to other B vitamins. The World Health Organization (WHO) lists this compound as essential medicine.[18] The natural sources of this vitamin includes foods such as almonds, eggs, whole grains, dairy products, organ meats, and dark green vegetables.

Rf is an electroactive components of flavoenzymes which consists of a planar isoalloxazine ring and are involved in many redox reactions.[19] This is amphiphillic in nature, due to the presence of hydrophilic ribityl side chain as well as hydrophobic part present within the structure.[20] In the mitochondria, riboflavin is phosphorylated to form FMN by riboflavin kinase, which is further converted into FAD. Both FMN and FAD (Figure 1.1, Chapter 1) act as an important cofactors that forms an integral part of the redox active sites of many different enzymes involved in a range of biological systems such as dehydrogenation reactions, dioxide activation, electron transfer reaction, etc.[21-23] These cofactors are unique due to their ability to undergo one and two electron redox processes.[24] The covalent attachment of redox cofactor onto a variety of flavin has sparked considerable interest in electrochemistry.[25] When the covalent attachment is through the secondary amino group in the isoalloxazine ring, then the redox behavior of aromatic ring would be considerably changed.[26] On the other hand, the linkage through the hydroxyl

group of ribose sugar unit from the ribityl side chain leaves the redox characteristics of riboflavin unchanged, as it does not affect the electron density of the ring.[25] These reversible redox reactions are necessary for cellular respiration and energy supplementation.[20,27]

Several electrochemical investigations on the Rf and their derivatives have been conducted to elucidate the mechanism of the redox reactions using cyclic voltammetry (CV),[28-31] UV-visible spectrophotometer (UV-Vis)[32-34] and photoluminescence (PL) spectra[35-39]. In this context, Bailey et al examined the reduction and oxidation of riboflavin by performing surface-enhanced raman scattering with simultaneous electrochemical detection of riboflavin at different pH values.[40] They have obtained the combination of results and illustrate the presence of three ionic forms, oxidized, semi-quinone, and reduced form of Rf.[41,42] Similarly, Tan et al explained the electrochemical reduction mechanisms of flavin in buffered and unbuffered aqueous solution at pH 3.0 – 11.0 using variable scan-rate (0.1-20 Vs^{-1}).[28] Further, Bagoji et al. have improved the electrochemical oxidative determination of Rf at a thin graphene film modified glassy carbon electrode and demonstrated the redox voltammetric behavior of Rf at the sensor was quasi-reversible involving two electrons-two protons.[38] It was also observed that the redox reaction was adsorption-controlled at the surface of modified electrode.

Another investigation have been done by Stankovic et al. to determine the behavior of riboflavin in pharmaceutical tablets by square wave voltammetry using boron-doped diamond electrode.[43] They showed riboflavin undergoes a diffusion-controlled quasi-reversible electron transfer reaction at pH 2 (buffer solution) *via* electrochemical analysis that is cyclic voltammetry. Berchmans and Vijayavalli have used covalent linkage to attached riboflavin part of FAD and FMN using carbodiimide reagent to the surface of glassy carbon.[44] The flavins have been used as good electron mediator because they easily forms thermodynamically reversible systems. The mediators are attached with the electrode surface by covalent linkage[44,45], adsorption[46,47] or entrapment[48].

In this chapter, efforts have been made to investigate the unique redox behavior of riboflavin by various electrochemical methods and under different pH conditions to understand the pH dependence of the biological and non-biological reaction mechanisms carried out by flavoenzymes. The studies have been performed using CV, UV-Vis and PL spectra. The electrochemical studies have been used to determine the electron transfer through the semi-quinone radical in one electron or two-electron process or two-proton

reduction. Further, different electrochemical kinetics parameters were calculated[49] for Rf at different pH values in buffered aqueous solution.

4.3 Experimental Section

4.3.1 Chemicals

Riboflavin (Rf; ≥ 98%) and sodium hydroxide (NaOH; 99.99%) were purchased from Sigma-Aldrich, India. All the other chemicals were of analytical grade and used without any further purification.Buffers were made using citric acid, sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$; >99.99%) and sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$). The pH of the solution was adjusted by adding NaOH and for all the experiments deionized water (18 MΩ cm) was used.

4.3.2 Preparation of Phosphate buffer and Citrate-phosphate buffer

Phosphate buffered solution was prepared by mixing the 0.2 M (15.6 g in 500 mL) solution of $NaH_2PO_4.2H_2O$ and 0.2 M (17.8 g in 500 mL) of $Na_2HPO_4.2H_2O$ in deionized water. The pH was adjusted using dilute solution of NaOH. Citrate-phosphate buffer solution was prepared by mixing the 0.1 M (21 g in 1L) solution of citric acid and 0.2 M (35.59 g in 1L) solution of $Na_2HPO_4.2H_2O$ in deionized water. This solution was then added to 100 μM of Rf. Before the voltammetry analysis, solution of Rf in phosphate buffer and citrate-phosphate buffer were deoxygenated by purging with high purity of inert gas (argon gas) at room temperature.

4.3.3 Characterization

UV-Visible absorption spectra were obtained using a thin layer quartz cell at room temperature and recorded on a Perkin-Elmer model UV-260 spectrophotometer, with absorption maxima in nm. Fluorescence spectra were recorded at the excitation value of 420 nm on a photoluminescence spectrophotometer (PL), and emission maxima were expressed in nm. A solution of Rf in phosphate buffer and citrate-phosphate buffer at different pH values are used for UV-Visible spectrophotometer and fluorescence spectra analysis at room temperature. The CV experiment was conducted on an Autolab potentiostat/galvanostat (Metrohm Autolab) using a three-electrode system with Au as the

working electrode, Ag/AgCl as a reference electrode, and a platinum wire as a counter electrode, in phosphate-buffer. The working electrode (Au disk) has a diameter of 1mm. A phosphate buffer and citrate-phosphate buffer with varying pH values were used as the supporting electrolytes. All CV experiments were conducted under an inert atmosphere to avoid sample reaction with dissolved molecular oxygen.

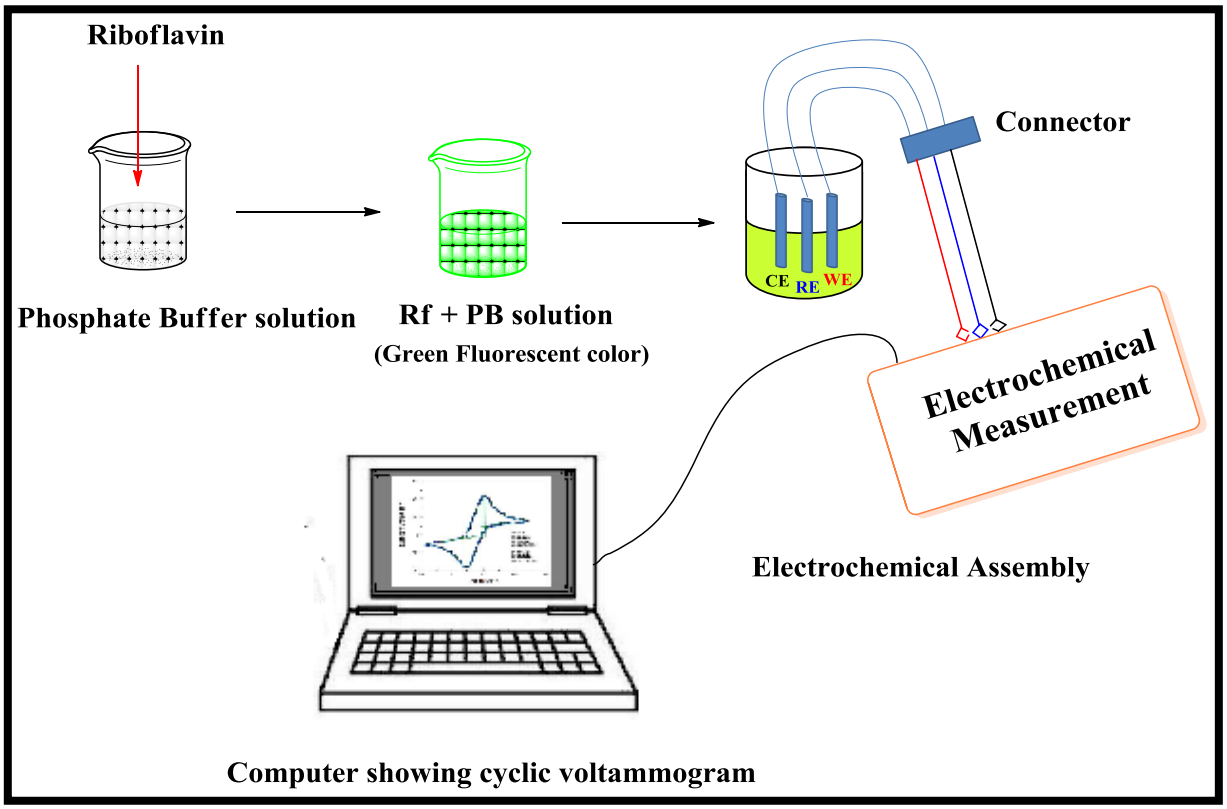
4.4 Results and Discussion

4.4.1 Electrochemical studies

Electrochemical analysis has been conducted using CV to study the redox behavior of riboflavin under different pH conditions (Scheme 4.1). Figure 4.2a shows the CV of 100 μM Rf in 0.1 M citrate-phosphate buffer at pH 3.0 with varying scan rate from 0.1 to 1.0 V s^{-1}. The voltammograms shows a well-defined cathodic and anodic peak, which correlate to the reversible reduction and oxidation of Rf. Further, CV studies were also performed at different pH (5.0, 7.0, 9.0, and 12.0) where the scan rate was increased from 0.1 to 1.0 V s^{-1} and the results are shown in figure 4.2(b, c) and figure 4.3(a, b). The appearance of two anodic and cathodic peaks (i & ii) have been observed. The Ip (anodic) and Ip (cathodic) peak value of Rf at various pH (3-12) has been summarized in table 1 and the peak current increases with increase in the scan rate.

Figure 4.4 (a & b) shows the CV of Rf at various pH in 0.1 M phosphate buffer solution where the scan rate was (0.7 Vs^{-1}). The voltammograms showed well-defined cathodic and anodic peaks, which correlate to reversible reduction and oxidation of riboflavin at different pH (Table 4.1). It was observed that on increasing the pH of Rf, there was an increase in the peak current. Similarly, the peak separation increases with increasing in the pH values. The change in peak current is consistent with the shift expected from altering the pH values during redox process of Rf.[50] As the scan rate increases, the reduction wave split into two parts, that may be possibly due to the presence of hydrogen bonded dianion RF^{2-} and RFH^{-} (Figures 4.3a & b and 4.5).

The peak current (I_p) for both oxidation and reduction follow a linear relation with respect to the square root of scan rate that summarized diffusion-controlled electrochemical reaction rates for the Rf at the electrode shown in figure 4.6.



Scheme 4.1: Schematic illustration for the electrochemical analysis of Rf

It has been observed that the oxidation peak shifts to a more positive value and the reduction peak to more negative values with an increase of the scan rate and both the peak potentials are linearly dependent on the log of scan rate, and may be given as:

$$E_{pa} = E^{o} + X \ln [(1-\alpha)\, Fv/RTK_s] \ldots\ldots\ldots\ldots..(1)$$

$$E_{pc} = E^{o} + Y \ln [(\alpha)\, Fv/RTK_s] \ldots\ldots\ldots\ldots\ldots (2)$$

$$\ln K_s = \alpha \ln (1-\alpha) + (1-\alpha) \ln \alpha - \ln (RT\ln Fv - \alpha(1-\alpha)n\, F\, \Delta E_p/RT) \ldots\ldots\ldots.. (3)$$

where, E_{pa} and E_{pc} are anodic and cathodic peak potentials, α is electron transfer coefficient, K_s is the charge transfer rate constant, F is Faraday constant (96500); R is gas constant (8.314 $J \cdot mol^{-1} \cdot K^{-1}$).

The plot of ln v *versus* E_{pa} and E_{pc} yields two straight lines having the slope; X = RT/(1- α)nF and Y = RT/αnF, respectively. Based on the equations 1 and 3, the values of α and K_s have been determined and is shown in table 4.2.

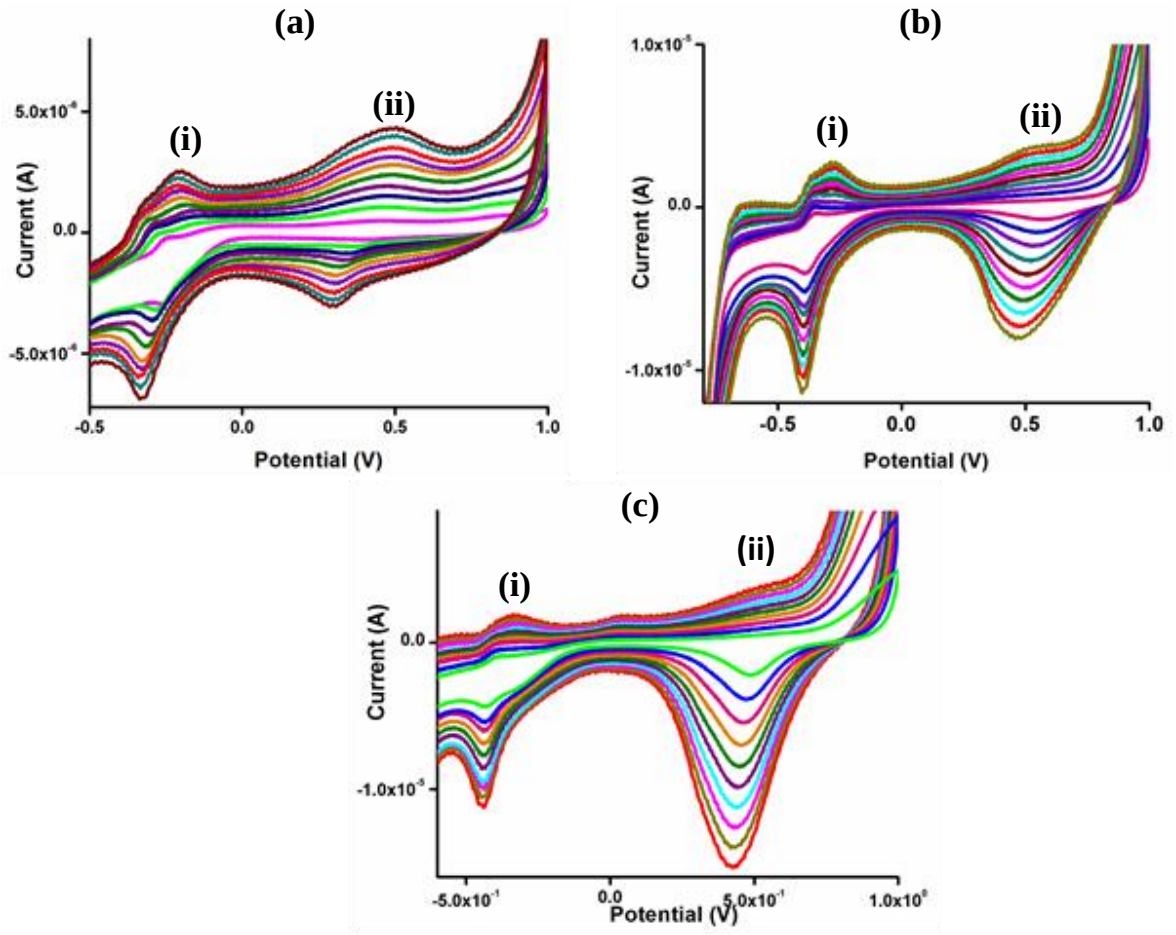


Figure 4.2: Cyclic voltammogram of Rf at (a) pH 3.0, (b) pH 5.0 and (c) pH 7.0 with scan rate from 0.1 to 1.0 Vs^{-1}

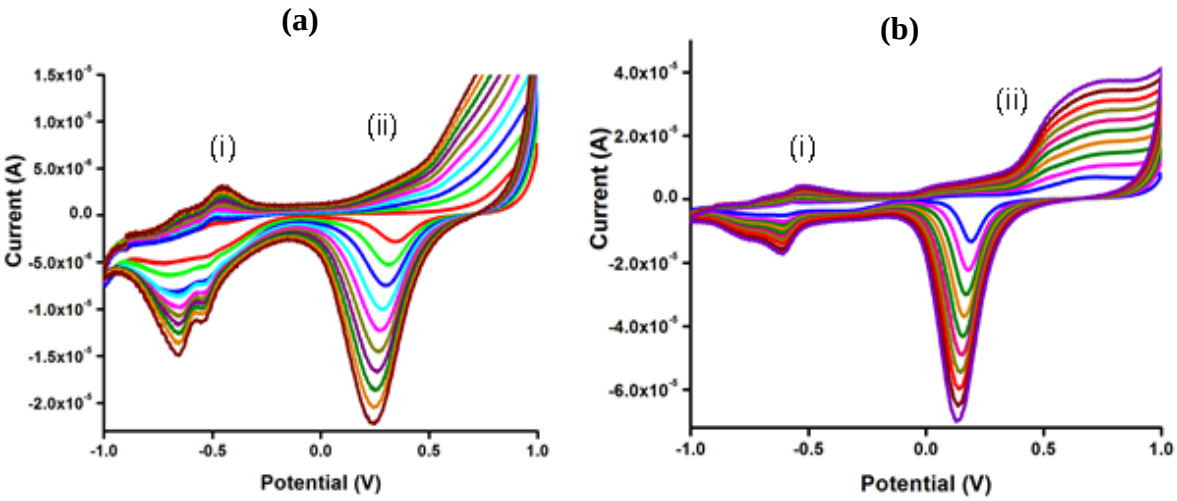


Figure 4.3: Cyclic voltammogram of Rf at (a) pH 9.0, (b) pH 12.0 with scan rate from 0.1 to 1.0 Vs^{-1}

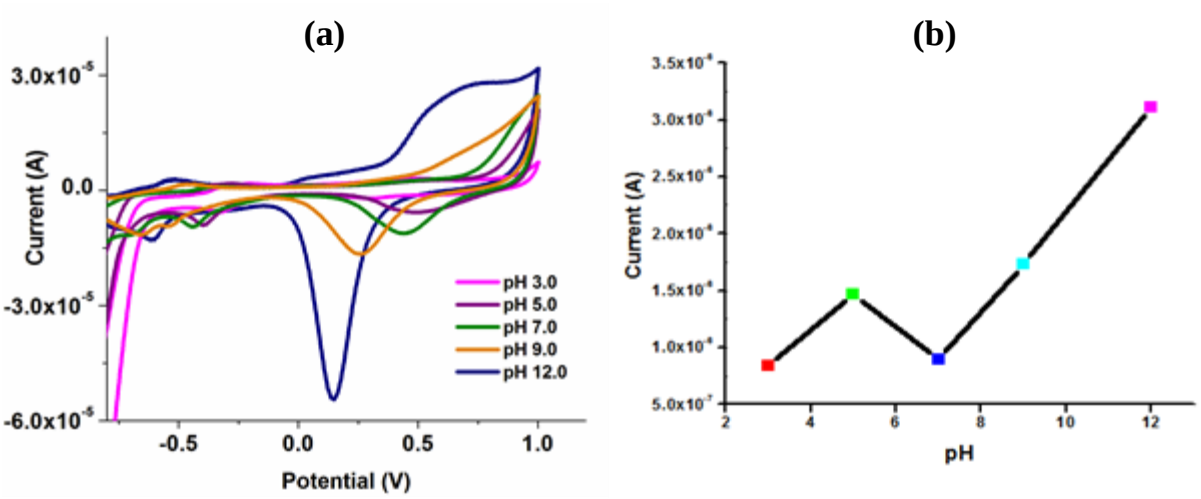


Figure 4.4: (a) Cyclic voltammetry study of Rf at different pH and (b) Plot showing the change in current with respect to change in the pH of Rf

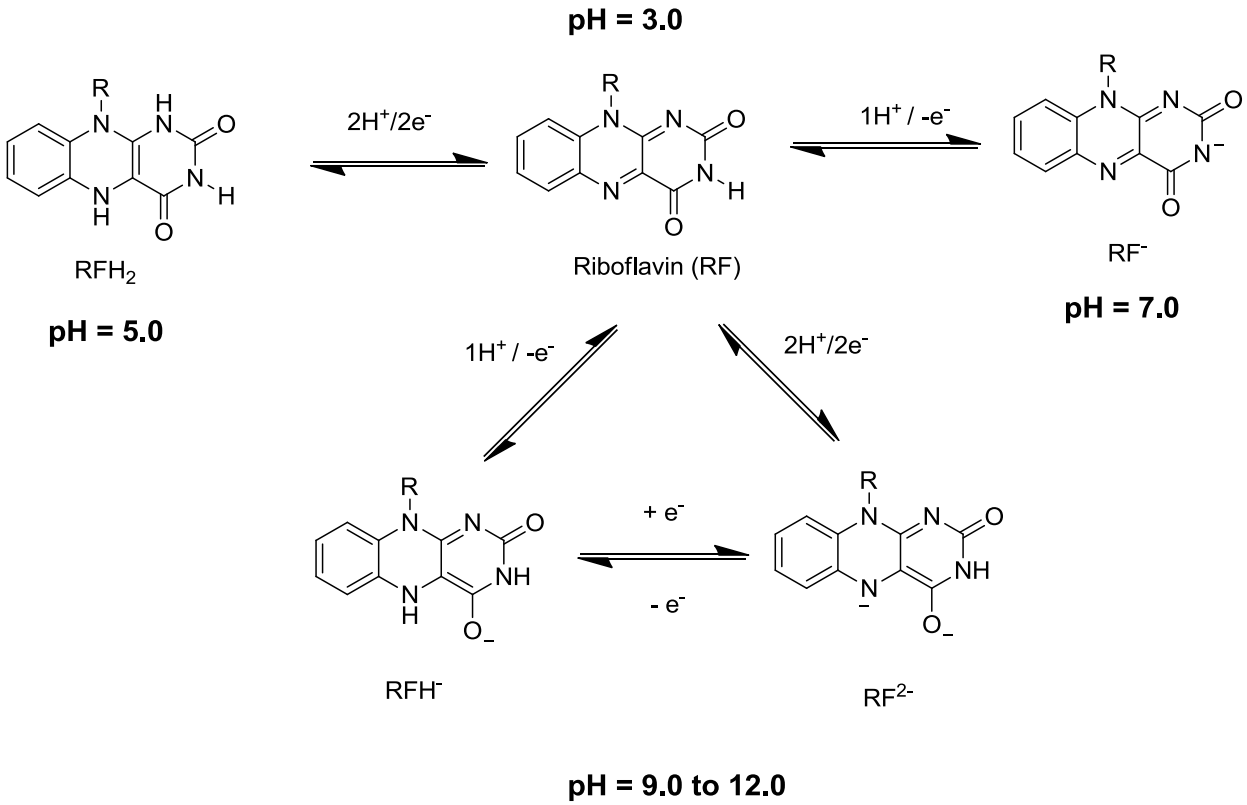


Figure 4.5: Structural changes in flavin with different pH

Table 4.1: Peak current (Anodic & Cathodic) at different pH values in buffered aqueous solution with scan-rate was 0.7 Vs^{-1}

S. No.	pH	(Ip) peak current (Anodic)	(Ip) peak current (Cathodic)	(Ip) peak current (Anodic)	(Ip) peak current (Cathodic)
1	3.0	1.4241×10^{-6}	-5.4832×10^{-6}	2.9846×10^{-6}	-1.0062×10^{-6}
2	5.0	1.7566×10^{-6}	-9.1159×10^{-6}	1.0915×10^{-6}	-5.7134×10^{-6}
3	7.0	1.0910×10^{-6}	-9.5764×10^{-6}	1.4496×10^{-6}	-1.1341×10^{-5}
4	9.0	1.4241×10^{-6}	-1.1571×10^{-5}	1.4496×10^{-6}	-1.6611×10^{-5}
5	12.0	2.7544×10^{-6}	-1.2978×10^{-5}	4.2637×10^{-6}	-5.4422×10^{-5}

Table 4.2: Kinetic parameters calculated for riboflavin at different pH values*

S. No.	pH	Electron transfer coefficient (α)	Charge transfer rate constant (K_s/S^{-1})	Effective surface area (A_{eff}/mm^2)	Average Surface coverage (Γ/mol cm^{-2})	Diffusion coefficient ($D/cm^2\ s^{-1}$)
1	3.0	0.75	2.6384×10^{-9}	0.2779	3.5572×10^{-12}	4.10×10^{-14}
2	5.0	0.85	1.8262×10^{-5}	0.2805	4.0386×10^{-12}	4.34×10^{-14}
3	7.0	0.82	2.8509×10^{-4}	0.2818	3.4364×10^{-12}	3.75×10^{-14}
4	9.0	0.76	0.6177	0.2798	5.0022×10^{-12}	5.60×10^{-14}
5	12.0	0.76	0.7929	0.2775	6.5207×10^{-12}	7.49×10^{-14}

* For pH 3 electrode used is citrate-phosphate buffer and for other pH values electrode used is phosphate buffer.

According to Laviron's equation,[51] the relationship between peak current (I_p) and surface coverage can be described as:

$$I_p = n^2F^2vA\Gamma(4RT)^{-1} \text{ ..... (4)}$$

where, n is the number of electrons transferred, A is the electrode area (0.28 cm^2), and Γ is the average surface coverage of the electrode redox substance (mol cm^{-2}).

Interestingly at basic pH (9.0 and 12.0) the Γ value obtained was higher compared to the acidic (3.0 and 5.0) and neutral solutions (7.0). Further, taking the average of both the cathodic and anodic results, Γ has been determined and is shown in table 4.2. On the basis of the linear slope of the anodic peak currents on the square root of the potential sweep rates, and the Randles-Sevcik equation,[49,52]

$$(I_p = (2.99 \times 10^5)\alpha^{1/2}n^{3/2}ACD^{1/2}v^{1/2})$$

The diffusion coefficient (D) is calculated, for Rf at different pH and the data has been shown in table 4.2. Here, n is the number of electrons transferred for the redox reaction (n = 2), C is the molar concentration of riboflavin in buffered aqueous solution (0.1 mM), and v is the scan rate (0.7 V s^{-1}).

It was observed that the D value is higher at the basic pH (9.0 and 12.0) while at neutral pH (7.0) lower D was obtained suggesting that at the basic pH there is more diffusion of the species towards the electrode surface. By performing a linear regression for I_p versus $v^{1/2}$, the slope S can be obtained, and the effective surface area of the electrode (A) is calculated for all pH values by using equation (5):

$$A = S/(2.99 \times 10^5)n^{3/2}\alpha^{1/2}CD^{1/2} \text{.....(5)}$$

For comparison, experiments have been carried out using different pH values in the same electrode (buffered aqueous solution), and the values of all kinetic parameters are given in table 4.2.

4.4.2 UV-Visible studies of Rf in buffered aqueous solution at different pH values

UV-Visible spectra were recorded at varying pH and are shown in figure 4.7. The spectral behavior of riboflavin at pH 3.0, 5.0, 7.0, 9.0, and 12.0 shows two identical peaks at 370 nm (S_2) and 443 nm (S_3) which are the characteristic of the isoalloxazine ring.[53] The absorption maxima (λ_{max}) of riboflavin at different pH values are summarized in table 4.3. The results indicate that the spectra of absorption maxima at pH values 3.0, 5.0, and 7.0 have no structural changes within the chromophore of isoalloxazine ring and shows an

absorption maxima at 263 (S_1), 370 (S_2) and 443 nm (S_3). Interestingly, at pH 9.0 the absorption maxima were obtained at 267 (S_1), 358 (S_2), and 445 (S_3). Similarly, at pH 12.0, the absorption maxima were obtained at 261 (S_1), 352 (S_2), and 443 (S_3). This change in the absorption maxima as compared to the original peaks suggest the structural changes occurred within the chromophore of isoalloxazine ring at basic pH.

4.4.3 Fluorescence studies in buffered aqueous solution at different pH values

The fluorescence spectra were recorded at different pH values and are shown in figure 4.8. The peak at 350 nm and 440 nm shows the excited PL spectra of Rf at different pH values, normalized with respect to their maximum intensity at 521 nm. At pH 9.0 and 12.0 the PL spectra excited at 350 nm shows two emission spectra (447 nm and 521 nm). Whereas, at 440 nm excitation the PL spectra of Rf at same pH value gives only one emission maxima at 521 nm, which is the characteristic emission of the isoalloxazine (flavin) moiety.[54] Moreover, at pH 3.0, 5.0, and 7.0, the PL spectra excited at 350 nm and 440 nm, shows identical emission maxima at 521 nm (Table 4.4). These results indicate that at basiccondition (9.0 and 12.0), riboflavin shows two emission maxima, which is probably due to the structural changes within the core of isoalloxazine ring as compared to acidic and neutral pH condition of Rf buffered aqueous solution. This result is in accordance with the results obtained by UV-visible spectrophotometer.

4.4.4 Chemistry of redox behavior of riboflavin in buffered solution

The Rf is an important redox moiety for one and two electron transfer cycles in cell membranes[54,55] as well as used as biocatalysis.[56,57] It has been explained that the redox behavior arises from the basic core or aromatic ring of isoalloxazine in Rf.[1,7] Riboflavin can exist in three different forms[15] in the appropriate positions based on the pH values and the applied potential. These three different forms are fully oxidized, semi-quinone radical, and fully reduced flavin (Figure 4.5). Generally, flavin is brightly fluorescent in the oxidized state and dark in the reduced state, while the semi-quinone is some-where in between in nature.[3,13,19] It also consists of different protonation forms depending upon the pH of the solution.[9] The positions N-1 and N-5 (Rf, **1**) of the isoalloxazine ring are the typical imides that are protonated upon reduction process.[3] The semi-quinone radical is a short-lived species and is the intermediate of one or two electron transfer process at a time. The results obtained by the CV illustrates the reversible oxidation and reduction of

riboflavin, correlating the spectral changes to the peak potentials with changes in pH values from acidic to basic conditions. These three ionic forms illustrate with fluorescence and absorbance experiments of Rf at different pH values demonstrate the neutral form is present from pH 3.0 to 7.0 and anionic at a pH greater than 8.0 (9.0 and 12.0). The oxidized and reduced forms of Rf (figure 4.5) have their first absorption maxima at 443 and 370 nm, indicating the presence in isoalloxazine ring. PL spectra (350 nm excited) have characteristic emission at 520 nm (figure 4.8) shows the redox properties of Rf. This provides an additional indication for the presence of redox properties of Rf. These results agree with previous studies of fluorescence and absorbance experiments[3,9, 13-15]. Moreover, the pH dependence of peak potential by CV suggests that Rf does not become deprotonated up to pH values 3.0 to 7.0 (acidic to neutral conditions).

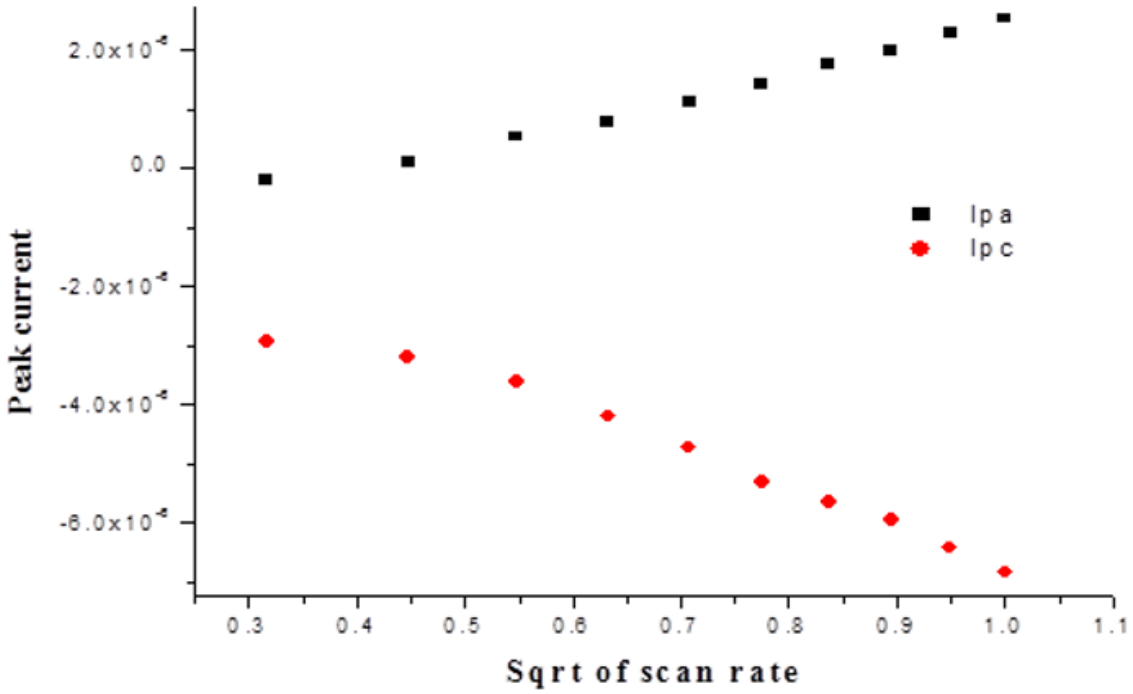


Figure 4.6: The plot showing linear relationship of peak current with respect to square root of scan rate

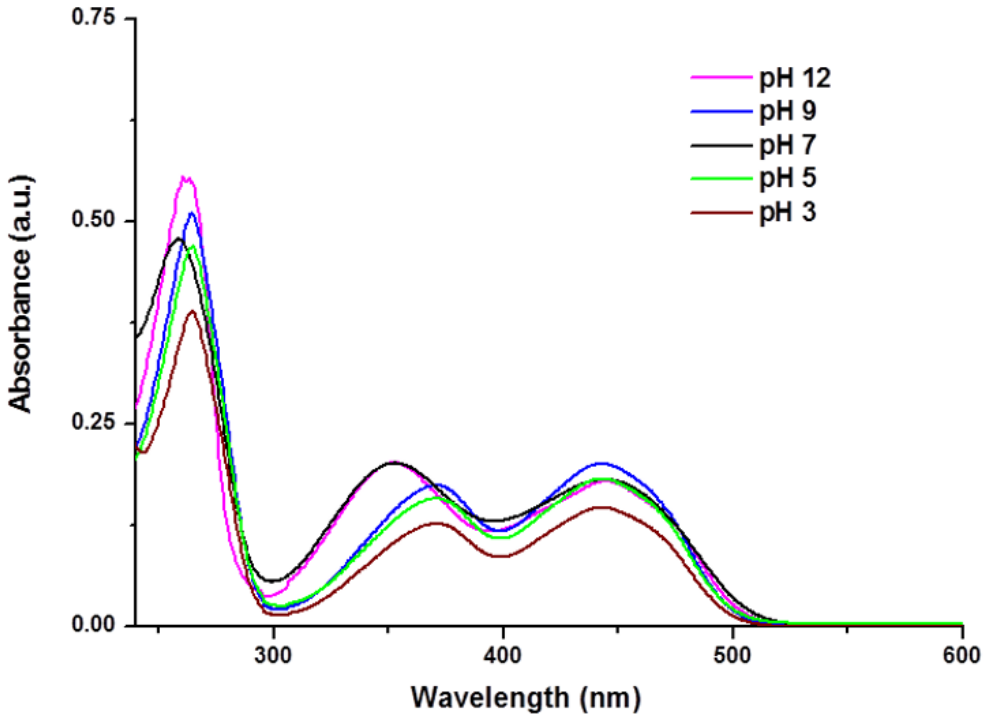


Figure 4.7: UV-Visible spectra of Rf at different pH values

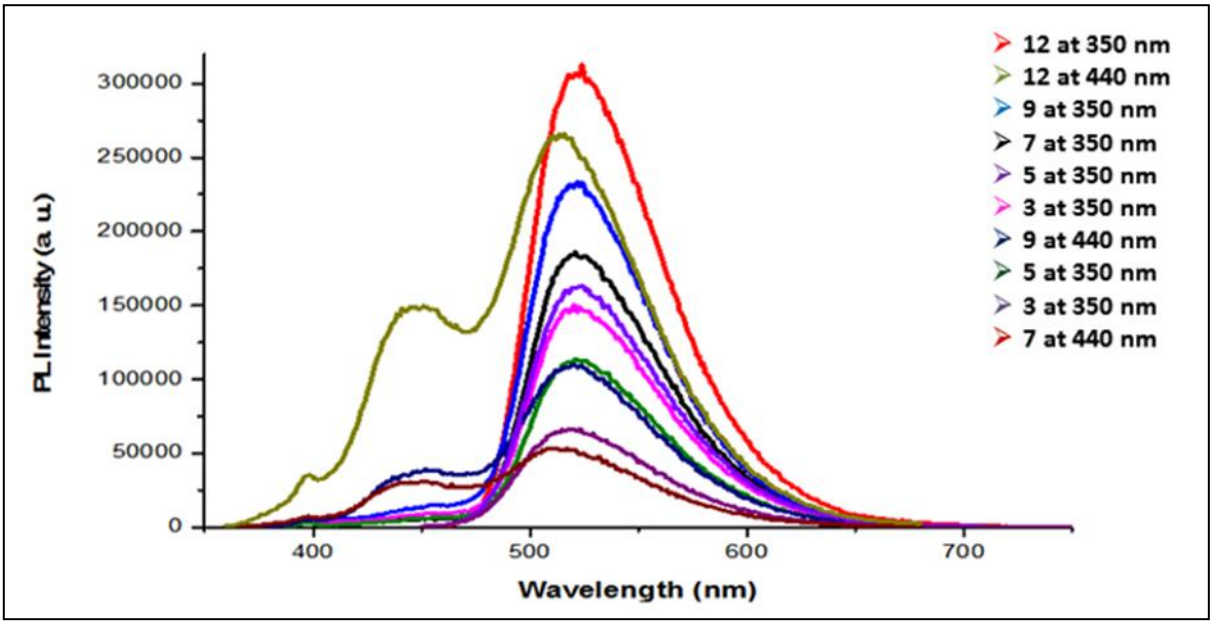


Figure 4.8: PL spectra of Rf at different pH values excited at 350 nm and 440 nm

Table 4.3: Absorption maxima (nm) of riboflavin in buffered aqueous solution at different pH values at room temperature*

S. No.	pH values	S_1 (nm)	S_2 (nm)	S_3 (nm)
1.	3	263	370	443
2.	5	263	371	442
3.	7	264	370	443
4.	9	267	358	445
5.	12	261	352	443

*S = Shoulder

Table 4.4: Emission maxima (nm) of riboflavin in buffered aqueous solution at different pH values at room temperature

S. No.	pH values	350 nm excitation		440 nm excitation	
		S-1 (nm)	S-2 (nm)	S-1 (nm)	S-2 (nm)
1.	3	520	-	522	-
2.	5	520	-	522	-
3.	7	521	-	522	-
4.	9	521	447	521	-
5.	12	520	446	521	-

4.5 Conclusions

In this study we have reported the behavior and structural changes in the aspect of electron transfer in redox process of riboflavin at different pH values in buffered aqueous solution. The electrochemical analysis confirmed that the oxidation and reduction occurred in buffered aqueous solution of riboflavin at different pH values. By increasing pH values from acidic to basic condition (pH 3.0 to 12.0), structural changes occurred in the riboflavin. At higher pH and low scan rates showed a significantly broadened

oxidation peak that separates into two oxidation peak when the scan rate was increased. Further, results of these studies have demonstrated all the kinetic parameters of riboflavin in aqueous solution at varying pH values, which helps to know about the behavior of riboflavin at the electrode. Moreover, UV-Vis spectroscopic experiment confirms the neutral form of riboflavin at pH 3.0 to 7.0, but at pH 9.0 & 12.0 Rf shows the anionic structure. PL studies conducted at 350 nm excitation & 440 nm excitation shows identical emission at 520 nm and by increasing pH value after 7.0 or basic condition split peak into two emission spectra indicates change in the structure of riboflavin.

4.6 Referencess

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CHAPTER 5

Studies of *Bombax ceiba* flower extracts and their Secondary metabolite conjugate with Flavin

5.1 Introduction

The genus *Bombax* belongs to tropical trees family and generally found in Indian subcontinents, part of Australia and Western Africa.[1] This genus belongs to the subfamily Bombacoideae and family Malvaceae. According to the plant list (www.theplantlist.org retrieved on 8-04-17), there are 171 species, out of which 7 are the accepted names which accounts for 4.1% of total known 171 species. The rest 77.8% are synonym, 5.8% unplaced and 12.3% are unassessed. The seven accepted names of the species are: *B. albidum*; *B. anceps*; *B. blancoanum*; *B. buonopozense*; *B. ceiba*; *B. costatum* and *B. insigne*.

Bombax is an age old ethnic medicinal plant. The various parts of these plants have wide range of traditional medicinal applications. They have been used for the treatment of various diseases for both human and animals.[2-4] The plant have been reported to be useful in cancer[5,6], HIV[7], diarrhoea[7], dysentery[7], urinary troubles[7], gynaecological problems,[7] bladder disorders[7], heart diseases[8], debility[7], diabetes[9,10] and impotence[3,8]. Besides having immense medicinal potential, the plants have also been used for commercial and industrial purposes.[1]

Out of the seven species, *Bombax ceiba* is highly explored plant. This is commonly known as a silk cotton tree or kapok tree (Figure 5.1). In Hindi, it is called Semal or Shalmali.[2] *B. ceiba* is growing most often in tropical and subtropical India and also occurs in Sri Lanka, Pakistan, Bangladesh, Myanmar, Malaysia, Java, Sumatra and Northern Australia.[2] This tree bears beautiful red coloured flowers during February to April (Figure 5.2). The various parts of this plant like roots, stem, bark, gum, leaves, flowers, fruits have been used in traditional system of medicines to treat different diseases of both human and animals.[1,3] The plant either whole or their parts have been reported to be useful in cancer[4,5], HIV, diarrhoea, dysentery, urinary troubles, bladder disorders[6], gynaecological problems[7], heart diseases[8], diabetes[9], impotence[1], analgesic[10], hepatoprotective[11], antiulcer[12], antiangiogenic[13], antioxidant[14], antimicrobial[15], hypotensive[16] and hypoglycaemic activities[17].

The flowers of *B. ceiba* also possess medicinal properties[3,18] apart from its beautiful red colour which may be useful in value added products. To utilize this flower as biocolours in food, pharmaceuticals & cosmetics a qualitative phytochemical screening along with antioxidant and antimicrobial studies has been performed in this chapter. This chapter also deals with the synthesis of flavin conjugate with vanillic acid.

Figure 5.1: *Bombax ceiba* tree in Delhi Technological University campus

Figure 5.2: Flowers of *Bombax ceiba*

5.2 Phytochemistry and pharmacology of *Bombax ceiba*: Literature review

5.2.1 Phytochemistry of *B. ceiba*

Various compounds isolated from different part of *B. ceiba* have been classified and discussed under different categories, like flavonoids, xanthones, coumarins, anthocyanins, terpenoids, steroids, quinines and their glycosides. Some of the miscellaneous compounds have also been isolated and characterized by different group of phytochemists. The lists of compounds from different parts of the plant are given in tables 5.1-5.15, followed by their structures.

Table 5.1: Flavonoids from *B. ceiba*

Comp. No.	Name of the compounds	Plant part(s) used[ref]
1	Quercetin	Flower[7,12,15]
2	Quercetin-3-O-β-D-glucopyranoside	Flower[15]
3	Quercetin-3-O-β-D-glucoronopyranoside	Flower[12,15]; Leaves[19]
4	Vitexin	Flower[12]; Leaves[19]
5	Isovitexin	Flower[7,12]; Leaves[19]
6	Vicenin	Flower[12]
7	Rutin	Flower[12]
8	Kaempferol	Flower[7,12,15]
9	Kaempferol-3-O-rutinoside	Flower[12]
10	Kaempferol-3-O-β-D-glucoronopyranoside	Flower[12]
11	Sexangularetin-3-O-sophoroside	Flower[12]
12	Apigenin	Flower[7]
13	Isoorientin	Leaves[19]
15	Apigenin-7-O- β-neohesperidoside	Flower[20]
16	Vicenin-2	Flower[20]
17	Naringenin	Flower[20]
18	Linarin	Flower[7]
19	Cosmetin	Flower[7]

Table 5.2: Xanthones from *B. ceiba*

Comp. No	Name of the compound	Plant part(s) used[ref]
14	Nigricanside	Leaves[19]
20	Isomangiferin	Flower[12], Leaves[19]
21	Mangiferin	Flower[12,15], Leaves[19],
22	7-O-Methylmangiferin	Flower[12]
23	2-*C*-β-d-Glucopyranosyl-1,6,7- trihydroxy-3-*O*-(*p*-hydroxybenzoyl)-9*H*-xanthen-9-one	Leaves[21]
24	4-*C*-β-d-Glucopyranosyl-1,6,8- trihydroxy-3,7-di-*O*- (*p*-hydroxybenzoyl)-9*H*-xanthen-9-one	Leaves [21]
25	4-*C*-β-d-Glucopyranosyl-1,3,6, 8-tetra hydroxy-7-*O*-(*p*-hydroxybenzoyl)- 9*H*-xanthen-9-one	Leaves [21]

Table 5.3: Coumarins from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
26	Esculetin	Flower[12]
27	Scopoletin	Flower[12]
28	Fraxetin	Flower[12]
29	Scopolin	Flower[12]

Table 5.4: Anthocyanins from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
30	Cyanidin-3,5-diglucoside	Flower[21]
31	Cyanidin-7-methyl	Flower[21]
32	Pelargonidin-5-β-d-glucoside	Flower [21]
33	Pelargonidin-3,5-diglucoside	Flower [21]
34	Pelargonidin	Flower[22]
35	Cyanidin	Flower [21]

Table 5.5: Aromatic-Glycosides from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
36	Shamimicin	Leaves[15]
37	Epicatechin-7-O- β-xylopyranoside	Stem bark[13]
38	Epicatechin-5-O- β-xylopyranoside	Stem bark[13]
39	Opuntiol	Stem bark[7,13]
40	Shamiminol	Stem bark[7,13]
41	Shamimin	Leaves[7]
42	Simalin A (6-O-(4-Hydroxy-3-methoxy benzoyl)-α,α-trehalose)	Stem bark[13]
43	Simalin B (3,4,5-Trimethoxyphenol-1-O (2-1) xylopyranosyl(6-1)α-rhamnopyranosyl-β-glucopyranoside)	Stem bark[13]

Table 5.6: Phenolic compounds from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
44	α,β,γ-tocophenol	Seeds[7]
45	Vanilic acid	Flower[12,20,21]
46	Syringic acid	Flower[20]
47	Ferulic acid	Flower[20]
48	Caffeic acid	Flower[20]
49	Syringin	Flower[20]
50	Bombalin	Flower [20], Root[9]
51	4-Hydroxy-5-(2-oxo-1-pyrrolidinyl)-benzoic acid	Flower [20]
52	Eugenyl-β-rutinoside	Flower[20]
53	3-Hydroxy-1-(4-hydroxy-3,5-dimethoxy phenyl)-1-propanone	Flower [20]
54	Protocatechulic acid	Flower[12, 20]

Table 5.7: Sesquiterpenoids from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
55	Isohemigosspol-1-2-dimethyl ether	Root[7]
56	Isohemigossypol-2-methyl ether	Root, Root bark [21]
57	2-*O*-Methylisohemigossylic acid lac tone	Root[23,24]
58	Lacinilene C	Root[9, 10, 24]
59	Bombamalone A	Root [24]
60	Bombamalone B	Root [24]
61	Bombamalone C	Root [24]
62	Bombamalone D (5,8-Dihydro-2-hydroxy-4-isopropyl-7-methoxy-6-methyl-5,8-dioxonaphthalene-1-carboxylic acid)	Root [24]
63	Bombamaloside (4-*O*-Glucopyronosyl-6,7-dihydro-2,2,8-trimethyl-6,7-dioxo-2*H*-naphtho[1,8-*bc*]furan-5-carboxylic acid)	Root[24]
64	Blumenol C-glucopyranoside	Flower[12]
65	7-Hydroxycadalene	Heartwood[25]
66	Gossypol	Root bark[21]
67	Hemigossypol	Root bark[21]
68	Hemigossypol 6-methyl ether	Root bark[21]
69	Hemigossypol 1,6,7-trimethyl ether	Root bark [21]
70	Isohemigossypol	Root bark [21]
71	Isohemigossypol-2,7-dimethyl ether	Root[21]
72	Hemigossylic acid lactone-2-hydroxy- 7-methyl ether	Root[23], Root bark[21]
73	6-Hydroxy-5-isopropyl-3-methyl-7- methoxy-8,1-naphthalene carbolactone	Root [21]
74	5-Isopropyl-3-methyl-2,7-dimethoxy- 8,1-naphthalene carbolactone	Root bark[26], Root [21]
75	5-Isopropyl-3-methyl-2,4,7-trimethoxy- 8,1-naphthalene carbolactone	Root bark[26], Root [21]
76	2,7-Dihydroxy-5-isopropyl-3-methyl -8,1-naphthalene carbolactone	Root[21,23]

Table 5.8: Triterpenoids from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
77	Lupenone	Stem bark[13,15]
78	Lupeol	Stem bark[7, 13, 16, 17], Leaves[15], Root[15]
79	Squalene	Leaves[12]
80	Taraxerone	Leaves[12]
81	Taraxerol	Leaves[12]

Table 5.9: Steroids from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
82	β-Sitosterol	Stem bark[7,16], Root[15], Flower[21]
83	Stigmasta-3,5-diene	Stem bark[13]
84	4-Methyl stigmast 7-en-3-ol	Leaves[12]
85	β-Sitosterolpalmitate	Leaves[12]
86	β-Sitosterol-3-*O*-*β*-d-glucopyranoside (Daucosterol)	Flower[21]
87	Stigmasterol (24-ethyl-5,22-cholestadien-3*β*-ol)	Flower[21]
88	Cholesterol	Flower[21]
89	Campesterol (24-Methyl-cholesterol)	Flower[21]
90	24β-Ethylcholest-5-en-3β- yl-O-α-l-arabinosyl-(1→ 6)-β-d-glucopyranoside	Flower[21]

Table 5.10: Quinones from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
91	Bombaxquinone B	Root[2,24]
92	Isohemigossypolone (2,7-dihydroxy-8-formyl-5-isopropyl- 3-methyl-1,4-naphthoquinone)	Root[21]
93	11-Nor-isohemigossypolone-2-methyl ether (7-hydroxy-5-isopropyl-2-me- thoxy-3-methyl-1,4-naphthoquinone	Heart wood[21]
94	Hemigossypolone	Root bark[21]
95	Hemigossypolon-6-methyl ether	Root bark[21]
96	8-Formyl-7-hydroxy-5-isopropyl-2-methoxy-3-methyl-1,4-naphthoquinone	Heartwood[25], Root[7,25]
97	7-Hydroxy-5-isopropyl- 2-methoxy-3-methyl-1,4-naphthoquinone	Heartwood[25]

Table 5.11: Lignans from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
98	Bombasin	Flower[21]
99	Bombasin-4-*O*-*β*-glucoside	Flower[21]
100	Dihydro-dehydro-diconiferyl alcohol- 4-*O*-*β*-d-glucopyranoside	Flower[21]

Table 5.12: Tannins from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
101	Tannic acid	Gum[7], Seeds[7]
102	Gallic acid	Gum[7], Seeds[7]
103	1-Gallayl- β-glucose	Seeds[7]
104	Ethyl gallate	Seeds[7]

Table 5.13: Fatty acids from *B. ceiba*

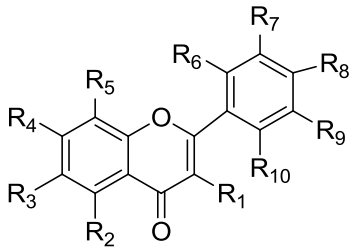
Comp. No.	Name of the compound	Plant part(s) used[ref]
105	Palmitic acid	Seed[7], Flower[21]
106	Stearic acid	Seed [21]
107	Linoleic acid	Seed[21]
108	Vernolic acid	Seed [21]
109	Myristic acid	Seed[21], Flower[21]
110	Palmitoleic acid	Seed[21]
111	Heptadecanoic acid	Seed[21]
112	Behenic acid	Seed[21]
113	Octylpalmitate	Seed[21]
114	Octadecylpalmitate	Seeds[7, 21]

Table 5.14: Amino acids from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
115	Arginine	Flower[21]
116	Alanine	Flower[21]
117	Glutamic acid	Flower[21]
118	Glycolol	Flower[21]
119	Lysine	Flower[21]

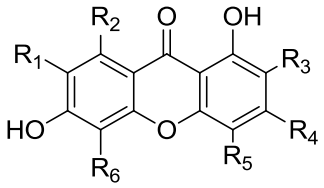
Table 5.15: Miscellaneous compounds from *B. ceiba*

Comp. No.	Name of the compound	Plant part(s) used[ref]
120	D-Glacturonic acid	Gum[7]
121	N-Triacontanol	Root[27]
122	N-Hexacosanol	Seeds[7]
123	Hentriacontane	Flower[7,12,15]
124	Hentriacontanol	Flower[7,12,15]
125	Chlorogenic acid	Flower[12]
126	Benzyl-β-D-glucopyranoside	Flower[12]
127	Methyl Chlorogenate	Flower[12]
128	Saponarin	Flower[7]
129	Xanthomicrol	Flower[7]
130	Phenylethylrutinoside	Flower[12]
131	5-(hydroxymethyl) furfural	Leaves[12]
132	1H-Indole-3-carboxylic acid	Leaves[12]
133	7-dihydroxy-5-0-xylopyranosyloxy-2H-1-benzopyran	Stem bark[7]
134	Trans-3-(p-coumaroyl)-quinic acid	Flower[28]
135	3-Methyl-2(3*H*)-benzofuranone	Flower[21]
136	*α*-Amyrin	Flower[21]
137	Oleanolic acid	Root[21]
138	α-Cedrol	Flower[21]
139	β-Cedrol	Flower[21]

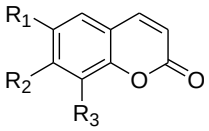


Comp. No.	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}
1	OH	OH	H	OH	H	H	OH	OH	H	H
2	O-β-D-Gluco pyranoside	OH	H	OH	H	H	OH	OH	H	H
3	O-β-D-glucorono pyranoside	OH	H	OH	H	H	OH	OH	H	H
4	H	OH	H	OH	β-D-glucopyranoside	H	H	OH	H	H
5	H	OH	-β-D-glucopyranoside	OH	H	H	H	OH	H	H
6	H	OH	-β-D-glucopyranoside	OH	-β-D-glucopyranoside	H	H	OH	H	H
7	O-Glc. (6→1) Rha	OH	H	OH	H	H	H	OH	OH	H

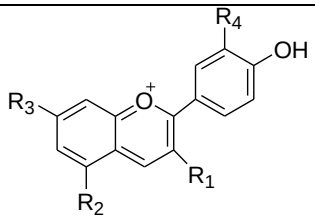
8	OH	OH	H	OH	H	H	H	OH	H	H
9	O-Rutinoside	OH	H	OH	H	H	H	OH	H	H
10	O-C-D-Glucoronopyranoside	OH	H	OH	H	H	H	OH	H	H
11	O-(β-D-Glc)$_2$	OH	H	OH	OCH_3	H	H	OH	H	H
12	H	OH	H	OH	H	H	H	OH	H	H
13	H	OH	-β-D-glucopyranoside	OH	H	H	H	OH	OH	H
15	H	OH	H	α-(1→2)-L-rhamnopyranosyl)-β-D-glucopyranosyl	H	H	H	OH	H	H
16	H	OH	β-D-Glc.	OH	β-D-Glc.	H	H	OH	H	H
17	H	OH	H	OH	H	H	H	OH	H	H
18	H	OH	H	O-Glc. (6→1) Rha	H	H	H	OCH_3	H	H
19	H	OH	H	β-O-Glc.	H	H	H	OH	H	H



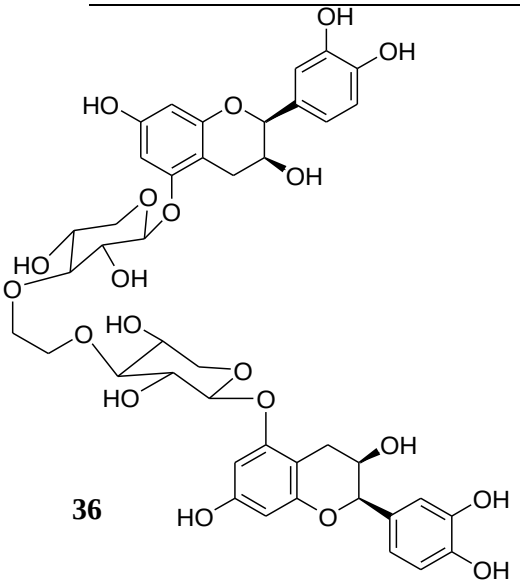
Compound No.	R_1	R_2	R_3	R_4	R_5	R_6
14	β-D-Glcoupyranoside	OH	H	H	OH	H
20	OH	H	H	OH	β-D-Glcoupyranoside	H
21	OH	H	β-C-Glc.	OH	H	H
22	OCH_3	H	β-D-Glcoupyranoside	OH	H	H
23	OH	H	β-C-Glc.	O-p-hydroxybenzoyl	H	H
24	O-p-hydroxybenzoyl	OH	H	O-p-hydroxybenzoyl	β-C-Glc.	H
25	OH	O-*p*-hydroxybenzoyl	H	OH	β-C-Glc.	H



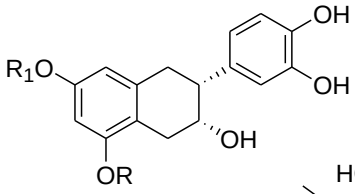
Comp. No.	R_1	R_2	R_3
26	OH	OH	H
27	OCH_3	OH	H
28	OCH_3	OH	OH
29	OCH_3	O-β-D-Glc	H



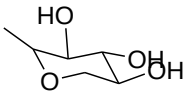
Comp. No.	R_1	R_2	R_3	R_4
30	β-O-Glc.	β-O-Glc.	OH	OH
31	β-O-Glc.	OH	OCH_3	OH
32	OH	β-O-Glc.	OH	H
33	β-O-Glc.	β-O-Glc.	OH	H
34	OH	OH	OH	H
35	OH	OH	OH	OH



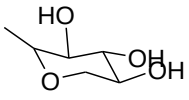
36

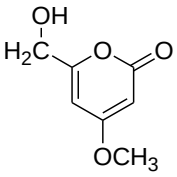


37. R = H; R1 =

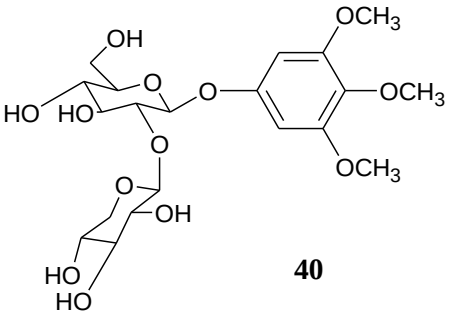


38. R1 = H; R =

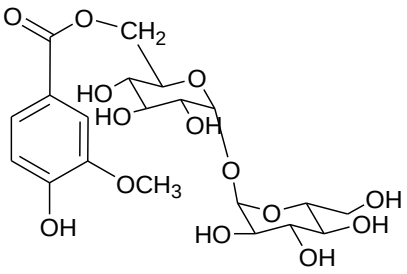




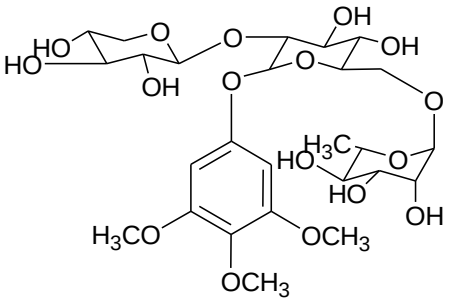
39



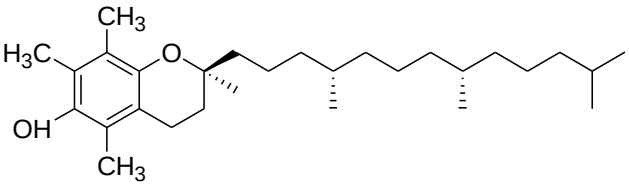
40



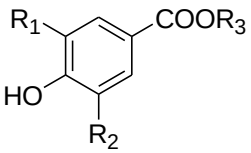
42



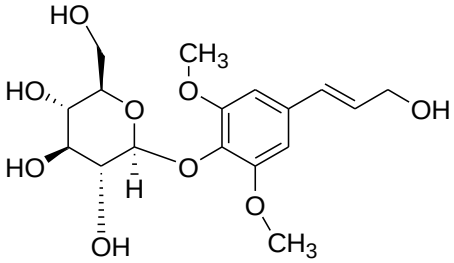
43



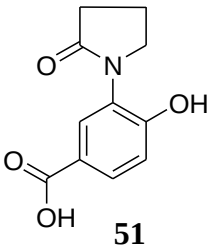
44



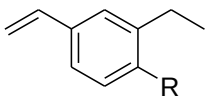
Comp. No.	R_1	R_2	R_3
45	OCH_3	H	H
46	OCH_3	OCH_3	H
102	OH	OH	H
103	OH	OH	Glc
104	OH	OH	C_2H_5



49

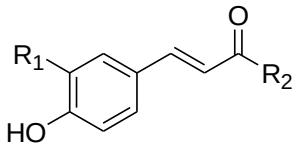


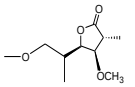
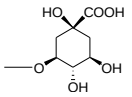
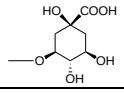
51

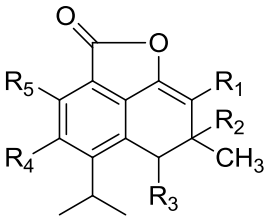


R = O-β-rutinoside

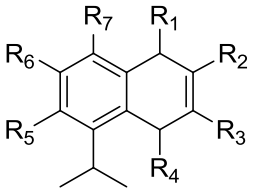
52



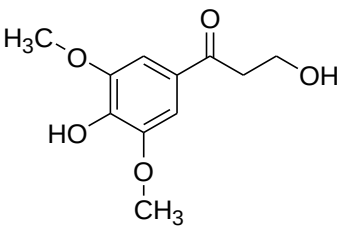
Comp. No.	R_1	R_2
47	OCH_3	OH
48	OH	OH
50	H	
125	OH	
134	H	



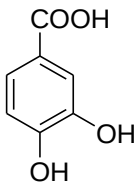
Comp. No.	R_1	R_2	R_3	R_4	R_5
57	OCH_3	H	H	H	OH
59	OCH_3	OH	O	H	OH
72	OH	H	H	H	OCH_3
73	H	H	H	OH	OCH_3
74	OCH_3	H	H	H	OCH_3
75	OCH_3	H	OCH_3	H	OCH_3
76	OH	H	H	H	OH



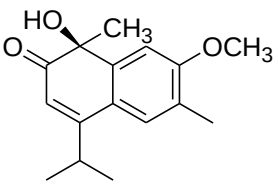
Comp. No.	R_1	R_2	R_3	R_4	R_5	R_6	R_7
55	OCH_3	OCH_3	CH_3	H	H	OH	CHO
56	OH	OCH_3	CH_3	H	H	OH	CHO
62	=O	OCH_3	CH_3	=O	H	OH	COOH
65	H	OH	CH_3	H	H	H	CH_3
67	OH	H	CH_3	H	OH	OH	CHO
68	OH	H	CH_3	H	OCH_3	OH	CHO
69	OCH_3	H	CH_3	H	OCH_3	OCH_3	CHO
70	OH	OH	CH_3	H	H	OH	CHO
71	OH	OCH_3	CH_3	H	H	OCH_3	CHO
91	=O	OCH_3	CH_3	=O	H	OH	CHO
92	=O	OH	CH_3	=O	H	OH	CHO
93	=O	OCH_3	CH_3	=O	H	OH	H
94	=O	H	CH_3	=O	OH	OH	CHO
95	=O	H	CH_3	=O	OCH_3	OH	CHO
96	=O	OCH_3	OCH_3	=O	H	OH	CHO
97	=O	OCH_3	OCH_3	=O	H	OH	H



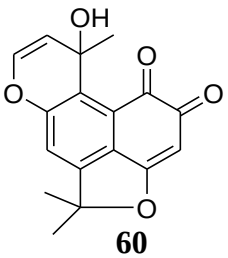
53



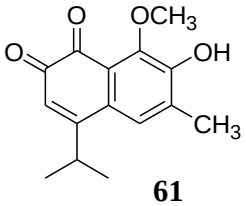
54



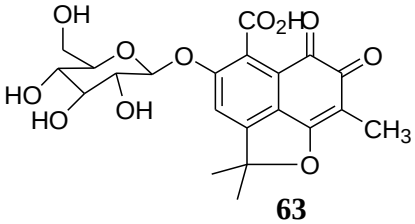
58



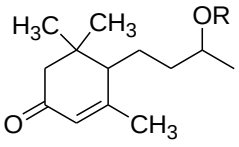
60



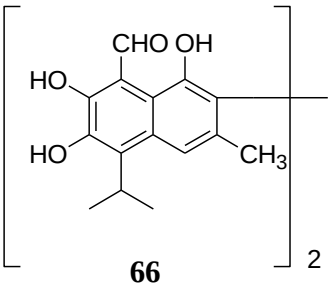
61



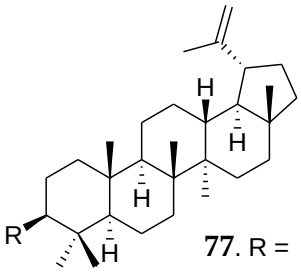
63



64 R = β-D-Glc

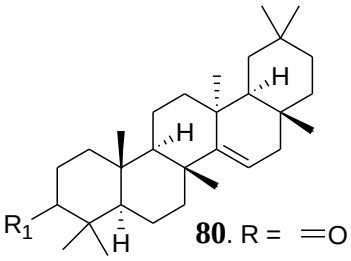


66



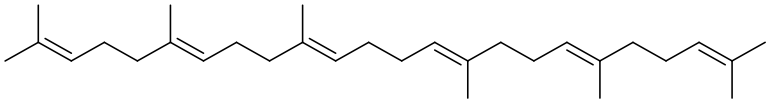
77. R = =O

78. R = β-OH

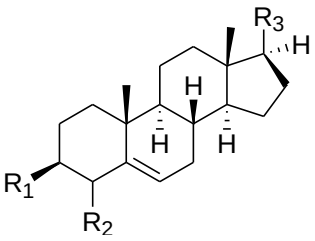


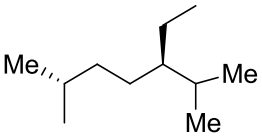
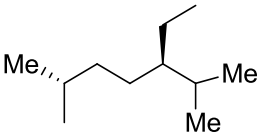
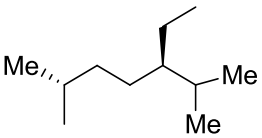
80. R = =O

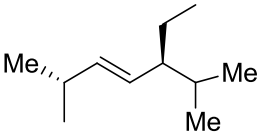
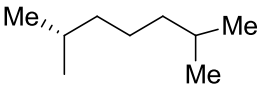
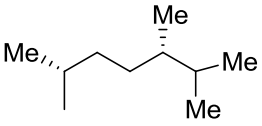
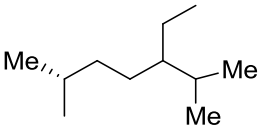
81. R = OH

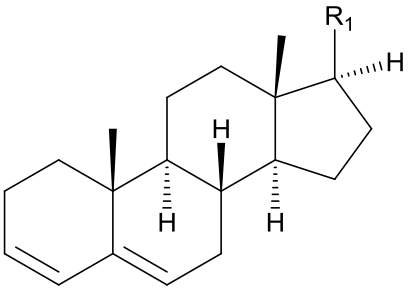


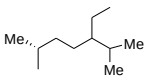
79



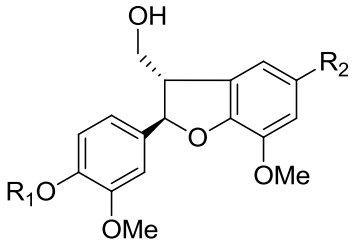
Comp. No.	R_1	R_2	R_3
82	OH	H	
84	OH	CH_3	
86	β-O-Glc	H	

87	OH	H	
88	OH	H	
89	OH	H	
90	O-Glc (6→1)Ara	H	

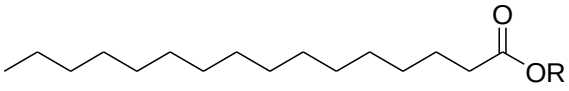


R₁ = 

83

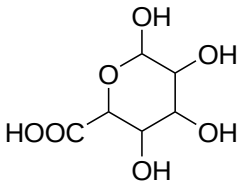


Comp. No.	R_1	R_2
98	H	-CO-CH_3
99	Glc	-CO-CH_3
100	Glc	-$(CH_2)_3$-OH

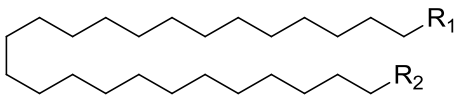


105. R = H

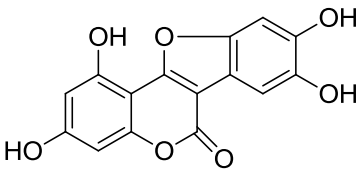
114. R = $C_{18}H_{37}$



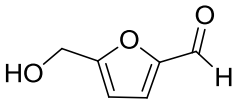
120



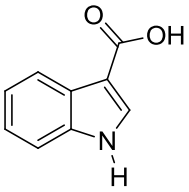
Comp. No.	R_1	R_2
121	$(CH_2)_2$-OH	$(CH_2)_2$-CH_3
122	OH	CH_3
123	$(CH_2)_2$-H	$(CH_2)_2$-CH_3



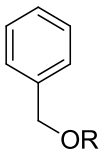
124



131

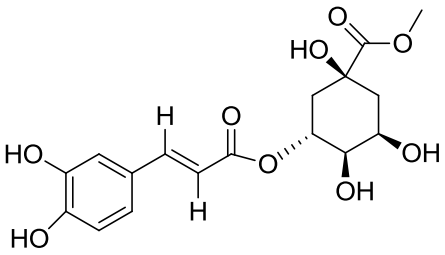


132

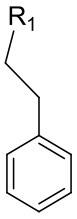


(R = β-D-Glc)

126

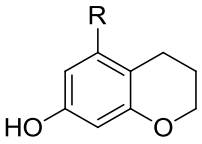


127



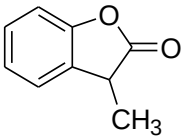
(R = O-rutinoside)

130

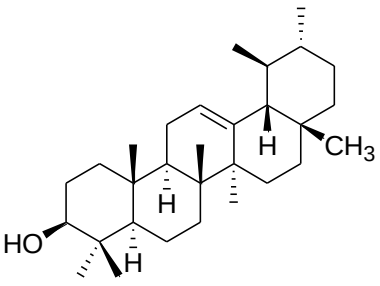


(R = O-β-D-xyalopyranose)

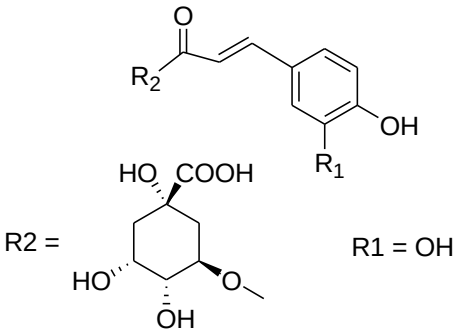
133



135



136



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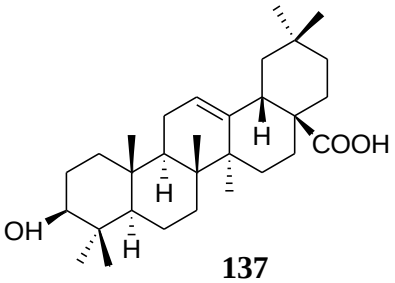
R1 = OH



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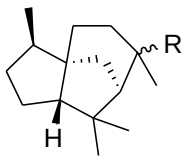


138 β-OH

139 α-OH

5.2.2 Pharmacological applications of *B. ceiba*

In Ancient time, *B. ceiba* has been widely used in varied fields. This has a place among the five scared plants of "panchwati" and utilized for providing food, fodder, fuel, and fibre along with medicinal applications. Almost all parts of the plant are reported to possess medicinal properties in various ethnobotanical studies.

The roots, stem-bark and flowers are employed to treat various ailments.[18] The whole plant is very effective in treating diseases like gastrointestinal[8], skin diseases[30], gynecological, urino-genital disorder[7], general debility[7], diabetes[8], impotence[8], hypotensive[17], hypoglycaemic[31], antidiarrhoeal[7], moderate oxytocic, musculotropic, cardiac stimulant[7] and so on. The various parts of *B. ceiba* have been reported for analgesic[32], hepatoprotective[33], antiulcer[34], antiangiogenic[35], antioxidant[35], antimicrobial[6,16], hypotensive[17] and hypoglycaemic activities[31]. Also it was used for the treatment of sexual debility[36], bleeding wounds[37] and vaginal infections[6]. The following sections discuss the traditional and modern pharmacological applications of *B. ceiba*.

Traditional pharmacological uses

B. cieba as whole plant is an important medicinal plant of tropical and subtropical India. Its medicinal usage has been reported in the traditional systems of medicine such as Ayurveda, Sidha and Unani.[11] In Ayurveda system of medicine, *B. ceiba* are most popular and considered as good remedy for tvakdosa (skin disease), Asmari (Urolithiasis), Raktarsa (Bleeding piles), Daha (Burning sensation), Vranasotha (wound), AtisaraArsa, Dantavikara, Mukhapaka-vrana.[38-41] This is also used in many polyherbal formulations for various ailments and cosmetic preparations because of its varied medicinal properties. The

whole plant or plant parts either used as decoction or powder or in formulations. The traditional applications are listed in table 5.16[6].

Table 5.16: Traditional pharmacological uses of *B. ceiba*

S. No.	Part of the plants	Traditional pharmacological applications
1.	Roots	Diabetes, sexual weakness, diarrhoea, dysantry, boils of burns, diabetes, snake bite, urinary troubles, leucorrhoea, syphilis[8,14]
2.	Stem barks	Acrid, demulcent, diuretic, inflammation, slightly astringent and heart tonic, kidney stone, headache, snake bite, tonic in boils & acne / pimples.[14]
3.	Flowers	Astringent, good for skin troubles, splenomegaly, and haemorrhoids, anemia, leucorrhoea, gonorrioea, splenomegaly, cancer, diuretic and laxative.[7]
4.	Gum	Asthma, bleedius piles, diarrhoea, dental caries, uterine disorder, menorrhagia and leucorrhoea[14]
5.	Seeds	Chicken pox and small pox[14]
6.	Leaves	Strangury and skin eruption, antidysentric, leucorrhoea, anemia, infertility, rheumatism laxative, haematinic[14]
7.	Fruits	Calculous affection, chronic inflammation, ulceration of the bladder and kidneys including strungury, dysuria, weakness of genital organ, antifertility, uterus protrusion[38-41]

Modern pharmacological uses

Antiangiogenic Activity

Angiogenesis means the growth of new blood vessels. The inhibition helps by cutting the blood supply for tumors to grow and hence antiangiogenic activity is important. Methanolic extract of the stem barks exhibited antiangiogenic activity.[43] The active compound was lupeol (**78**) which at 50 and 30 μg/mL showed 80% inhibition on *in-vitro* umbilical venous endothelial cells (HUVEC) tube formation.[43] In further studies done by Nam et al, and Rani et al found that **78** only stop the growth of HUVEC tube

while this does not affect the tumor cell lines such as SK-MEL-2, A549 and B16-F10 melanoma growth.[5,44]

Antidiabetic Activity/ Hypoglycemic effects

Methanolic extract of the bark of *B. ceiba* were evaluated for its antidiabetic activities.[27] This extracts exhibited reducing power activity in dose dependent manner. Continuous administration of methanol extracts in alloxan induced diabetic rats at a dose of 200 mg/kg body weight significantly decreased blood glucose level. The IC_{50} value of the extract was found to be 32.1 μg/ml. The extract showed significant decrease of blood sugar after glucose loading points of 30, 60 and 120 min. In addition, the bark of *B. ceiba* possesses high phenol content (74.38±7.42 mg/g of gallic acid). This finding suggested that the potential antidiabetic effect could be due to the presence of phenolic compounds.[27]

B. ceiba bark extract was evaluated for its hypoglycemic and hypolipidemic effect. The study was done through normal and streptozotocin-induced diabetic rats with oral doses of 200, 400, 600 mg/kg/day for 21 days. The results showed that a dose of 600 mg/kg of *B. ceiba* extract is the most effective to cause significant ($p<0.001$) hypoglycemic and/or hypolipidemic effects. This dose also reduced the total cholesterol and triglyceride level in severely diabetic rats. The extract was found to be rich in the triterpenoid compounds that may account for its activity.[10]

Anti-inflammatory Activity

An ethanol extract showed significant anti-inflammatory activity. The presence of phytochemicals like tannins[45], flavanoids[46], triterpenoids[47] and phenolic compounds[48] are responsible for the anti-inflammatory activity of *B. ceiba* extracts. The *in-vitro* preliminary evaluations have demonstrated that folk medicine of *B. ceiba* can be used to cure the inflammation.[49]

The effects of methanolic flower extract on NO induced by lipopolysaccharide (LPS) in mouse macrophages RAW 264.7 cell line was used to demonstrate the beneficial effect of *B. ceiba* extracts in treating inflammatory diseases. The extract could inhibit NO generation in a dose dependent manner. The inhibitory effect was found with IC_{50} value of 512.53 μg/ml. In mouse macrophage model stimulated by LPS, *B. ceiba* exhibited a significant anti-inflammatory effect. These effects were mainly due to high content of total phenol and flavonoids, which could serve as a natural resource of food and medicine.[50]

Antimicrobial Activity

The methanolic extracts of leaves of *B. ceiba* showed antimicrobial activity against acne-inducing bacteria[51] having minimal inhibitory concentration (MIC) values of 0.0050 mg/ml.[52]

Nagamani et al also evaluate the aqueous, methanol, acetone, diethyl ether, chloroform and hexane extracts of seeds for the antibacterial property. The study was done against five bacterial species namely *Escherichia coli, Bacillus Subtilis, Staphyolococcus aureus, Enterococcus faecalis,* and *Alcaligenes faecalis.*[44, 53]

Antiobesity Activity

The methanolic extract of stem barks has significant effect against High Fat Diet (HFD). The study was performed with HFD induced obesity in rats.[54] The dose for the study was chosen as 200 mg & 400 mg/Kg. The possible mechanism is through modulation of FAS (fatty acid synthase) and PTP-1B (protein tyrosine phosphate-1B) signalling due to the presence of flavonoids and lupeol in the extract.[54] This makes it as potential component in any herbal formulation to prevent obesity.

The antiobesity activity of *B. ceiba* stem bark was also studied by Patra et al.[55] They studied on model such as male, wistar albino rats. They found that active constituent gemfibrozil reverses the effects of HFD treatment on serum parameters. Antiobesity activity may be due to the inactivation of acetyl-coA carboxylase, as a result of AMPK activation that mediates thermogenesis and FAS inhibition.[55]

Antioxidant Activity

The extracts of *B. ceiba* was evaluated for its antioxidant activity.[56-58] A methanol extracts exhibited DPPH free radical scavenging activity and reducing power activity in dose dependent manner.[56] This finding suggested that the potential antioxidant activity of bark extract could be due to the presence of phenolic compounds.[57] The extract have shown DPPH radical scavenging activity with an IC_{50} of 32.1 µg/ml where standard ascorbic acid has shown the IC_{50} of 16.5 µg/ml. DPPH is commonly used as a tool to identify antioxidant molecules present in the plant extracts.[27,56] The extracts rich in the total content of phenol and flavonoids shows better antioxidant activity. The studies have shown that the high total phenolic (> 85 mg/g) and flavonoids contents (> 30 mg/g) are present in the extract of *B.* ceiba.[50] The variations are possible with change in

environmental condition. This is the reason for variation in antioxidant properties observed for the plants collected from different ecological area.[59]

Hepatoprotective Activity

The ability to protect damage to the liver is known as Hepatoprotection. The extracts of flowers have shown hepatoprotective activity.[33,60] The methanolic extract was found to be active in doses of 150, 300 and 450 mg/kg to anti-tubercular challenge after taking for 10 and 21 days.[33] The aqueous extract of flower also showed hepatoprotective activity in CCl_4 based hepatotoxicity induced rats.[60]

Hypotensive Activity

Saleem et al studied the hypotensive activity of *B. ceiba* leaves. The extract found to be useful from moderate to high dose of the extract. The toxicological studies revealed that 500 mg/kg is a lethal dose but it did not cause any mortality in mice at the dose of 1 g/kg.[31] A novel constituent, shamimicin (1,1-bis-2-(3,4-dihydroxyphenyl)-3,4-dihydro-3,7 dihydroxy-5-*O*-xylopyranosyloxy-2*H*-1-benzopyran, **36**) along with lupeol (**78**), which possesses powerful hypotensive activity has been isolated from *B. ceiba* stem bark. However, the active hypotensive fractions showed its adverse effects on heart, liver and kidneys of mice at the dose of 1000 mg/kg/d as lethal dose (LD100).[17]

5.3 Experimental Section

Melting points were determined on a laboratory capillary melting apparatus and are uncorrected. The electronic spectra were recorded on a Perkin-Elmer UV-260 spectrophotometer and absorption maxima have been expressed in nanometers (nm). FTIR spectra were recorded on a Perkin Elmer 1710 FTIR spectrophotometer and the ν_{max} are expressed in cm^{-1}. ^1H NMR was recorded on a Bruker Avance-300 spectrophotometer (300 MHz) and the chemical shifts were expressed in ppm. The elemental analysis was measured by Perkin Elmer 2400. The solvents and reagents were purchased from reputed company and were used without further purifications.

5.3.1 Plant material collection

The flowers of *B. ceiba* were collected from the campus of Delhi Technological University (DTU), Bawana Road, Delhi – 110042, India during the period of February to April. The flowers were air dried in shade at room temperature and powdered.

5.3.2 Preparation of sample

The *B. ceiba* flowers in powdered form (1.5 kg) were extracted with methanol (3 L) in soxhlet extractor (Figure 5.3) using round bottom flask (5 L) for 24 hours. The methanolic extract was filtered and the solvent was removed under reduced pressure to get the crude residue (73 g). The above prepared methanol extracts were used for preliminary phytochemical screening, antioxidant and antimicrobial properties using the standard methods.

Figure 5.3: Soxhlet extractor with flower of *B. ceiba*

5.3.3 Phytochemical screening

The presence of different group of compounds was screened using the standard protocol: carbohydrates (Molisch's test); phenols (ferric chloride test); saponins (foam test); sterols (Liebermann-Burchard test); tannins (Braymer's test); amino acid & proteins (ninhydrin test) and alkaloids (Mayers test).[61]

5.3.4 Antioxidant study

The antioxidant activities were performed for three different fractionation set of methanol extract. The extracts from different set of solvent system were studied for their free radical scavenging assay using DPPH (2,2 diphenyl-1-picryl hydrazyl) method. The solution of DPPH was prepared by dissolving 1.2 mg in 50 mL of ethanol.

Set I

The methanolic flower extract of *B. ceiba* (100 mg) was mixed with in 25 mL of different solvents and stirred at 25 ^{o}C for 1½ h on water bath (50 ^{o}C). The filtrates were used for different type of studies. The solvents used include hexane, benzene, chloroform, ethyl acetate, acetone, methanol and ethanol. Each extract (1 mL) was mixed with DPPH solution (3 mL) and allowed to react for 45 min at room temperature. This solution was analysed for antioxidant activity using UV-visible spectroscopy.

Set II

The 5 mL of each solution from set I was subjected on water-bath to evaporate the solvent. To this extract, 1 mL of ethanol was added and shaked to dissolve the same. To this ethanol solution, 3 mL of DPPH solution was added and allowed to react for 45 min at room temperature. This solution was analysed for antioxidant activity using UV-visible spectroscopy.

Set III

The methanolic flower extract of *B. ceiba* (100 mg) was mixed with in 25 mL of hexane and stirred at 25 ^{o}C for 1½ h on water bath (50 ^{o}C). The solvent was filtered and the residue was again mixed with benzene (25 mL), stirred at 25 ^{o}C for 1½ h on water bath (50 ^{o}C). This process was repeated for chloroform, ethyl acetate, acetone, methanol and ethanol respectively. Each extract (1 mL) was mixed with DPPH solution (3 mL) and allowed to react for 45 min at room temperature. This solution was analysed for antioxidant activity using UV-visible spectroscopy.

5.3.5 Antimicrobial susceptibility screening

The different groups of microorganisms were used for antimicrobial activity; *Klebsiellap neumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* (gram-negative) as

well as *Staphylococcus aureus* I, *Staphylococcus aureus* II and *Bacillus subtilis* (gram-positive). The different media required for the preparation of lawn culture of microorganisms were prepared. Mueller-Hinton (MH) medium was used as the growth media for these microorganisms.

Sterilization procedure

In order to avoid any type of contamination and cross contamination by the test organisms, the antimicrobial screening was done in Laminar Hood with all the precautions, UV light was switched on one hour before working in the Laminar Hood. Petri dishes and other glass wares, micro tips, loop, spreader, disks were sterilized by autoclaving at a temperature of 121 ^{o}C and a pressure of 15lbs./sq. inch for 20 minutes.

Mueller-Hinton Agar (9.00 g and 13.75 g) were dissolved in distilled water (250 mL) in Erlenmeyer flasks (500 mL). The pH of the medium was adjusted to 7.3. The flasks were plugged with non-absorbent cotton and sterilized in autoclave at 15 psi for 20 minutes. The media (15 mL) was poured in petri plates. The respective seed media were inoculated with cells from the surface growth of 1 day old slant of different bacteria with the help of a sterile inoculating loop wire aseptically. Then, the petri plates were placed in an incubator at 37 ^{o}C for 24 h.

Preparation of the test organism suspension

The lawn cultures for different bacteria were prepared as mentioned above and incubated at 37 ^{o}C for 24 h. The suspensions of test organism were prepared by the following steps:

a) One loop full of culture was taken from the lawn culture and then smeared on the wall of test tube of normal saline (0.85% NaCl) and mixed it.
b) The opacity of the above solution was matched with the standard cell suspension (10^{6} cell/mL). After that, the swab was taken and dipped into the test tube of normal saline and remove excess saline by pressing the swab on the wall of the test tube.

Determination of zone of inhibition by Kirby-Baur's method

The antibacterial susceptibility test was done by determining zone of inhibition by Kirby-Baur's method. The stock solution was prepared in water/methanol and was serially doubly diluted (200 µg/mL: 100, 50 and 25 µg/mL). These solutions were poured over sterilized filter disks and disks were subsequently dried to remove excess solvent.

Different bacterial strains (200 μL) selected along with culture broth were added on the plates and spread with the help of sterile spreader. The soaked filter paper disks were placed aseptically over the inoculated plates using sterile forceps. The plates were incubated at 37 ^{o}C for 24 h, in upright position. The zone of inhibition was measured using scale.

Determination of MIC by the micro dilution broth susceptibility test method

The extract was analyzed by the broth dilution assay to determine its minimum inhibitory concentration (MIC) values as per the standard protocol. The stock solution of 200 μg/mL was prepared in water and was serially diluted as 100, 50, 25, 12.5, 6.25, 3.125 μg/mL. Different concentrations were prepared in sterile dry test tubes to determine MIC. Nutrient broth was prepared and 2.9 mL of it was taken in each test tube and were sterilized after plugging. After cooling, 0.1 mL of each dilution was added to the test tubes and the final volume was made up to 3.0 mL. To each test tube 0.1 mL of bacterial culture broth was added to bring the turbidity level to 0.5 McFarlands. The test tubes were shaken to mix the inoculums with the broth uniformly. The tubes were incubated at 37 ^{o}C for 24 h and observed for any visible turbidity. The lowest concentration, at which no growth of microorganism observed, was termed as MIC.

5.3.6 Synthesis of flavin-vanillic acid conjugate (142)

Thionyl chloride (300 mg) was added to vanillic acid (186 mg) and the reaction mixture was refluxed for 1 hour. The reaction was allowed to cool to room temperature and poured into the ice cold water. The vanillic acid chloride was separated out as thick solid mass. This was decanted and further added to dry benzene. The solvent benzene was evaporated under reduced pressure to get the highly viscous jelly type product. This was further subjected to dry DMF. To this solution, riboflavin tetracetate (270 mg) and potassium carbonate (100 mg) was added. The reaction mixture was refluxed for 1 hour. The progress of the reaction was monitored with TLC. On completion of the reaction, the solvent was removed under reduced pressure. The solid product was washed with water and methanol and dried. This was further recrystallized from hot ethanol. The formation of the conjugate 142 was confirmed by FTIR, ^{1}H NMR and elemental analysis.

Yield: 39%; mp.: >300 ^{o}C; FTIR (KBr): 3433, 3023, 2955, 2843, 1713, 1678, 1659, 1583, 1533, 1429, 1256, 1099, 889, 849 cm^{-1}; ^{1}H NMR (DMSO-d_6): 2.20 (s, 12H, 4×CH_3), 2.48 (s, 6H, 2×CH_3), 3.73 (s, 3H, OCH_3), 3.91 (d, 2H, CH_2), 4.22-4.34 (m, 5H, 3×CH, 1CH_2), 6.58 (s, 1H, H-9), 6.80 (d, 1H, *J* = 7.92 Hz), 7.20 (s, 1H, H-6), 7.68 (s, 1H, Ar-H), 7.72 (d, 1H, *J* = 8.12 Hz); Elemental analysis for $C_{33}H_{34}N_4O_{13}$ (%): Found C 57.06; H 4.93; N 8.07; Calculated C 57.09; H 4.86; N 8.10.

5.4 Results and Discussion

5.4.1 Phytochemical screening of *B. ceiba* flowers methanol extract

Phytochemicals are bioactive chemicals of plants and regarded as secondary metabolites. The screening for the presence of a phytochemical group helps in its further isolation, purification and characterization. The phytochemical screening of the methanolic extract of *B. ceiba* flowers has been done according to standard literature procedure.[61] The results obtained for qualitative screening are presented in table 5.17. Of the seven phytochemical group screened for, four were present in methanol extracts of flower of *B. ceiba*. They are alkaloid, phenolics, tannins, amino acids and proteins. This suggests that the flower of *B. ceiba* offers a wider array of phytochemicals and could serve as a source of useful value added products including drugs and neutraceuticals.

5.4.2 Antioxidant study

The antioxidant activity (AA) usually determined by using methods like, 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay[62,63], Nitric Oxide radical scavenging assay[64], Hydroxyl radical scavenging assay[65], Superoxide radical scavenging assay[66] and Trolox equivalent anti-capacity (TEAC) assay[67]. In this paper, DPPH method has been used (Figure 5.4 & 5.5).

The flower extract of *B. ceiba* obtained from seven different solvents were studied for their free radical scavenging assay using DPPH (2,2-diphenyl-1-picryl hydrazyl) method. Pure DPPH solution gives absorbance at 517 nm in UV-visible spectrophotometer. The solution of DPPH in ethanol was used as blank, whereas 0.6 mg of flower extract in 25 ml ethanol was used as reference.

Percentage of antioxidant activity was calculated using the formula:

$$AA(\%) = [(A_b - A_s) / A_b] \times 100$$

Where AA = Antioxidant activity; A_b = Absorbance of blank; A_s = Absorbance of sample

The results of the AA have been summarized in table 5.18. It was found that the AA was comparable in all the three sets of experiments performed for individual solvents. The solutions of extract from polar solvents gave better AA in comparison to non-polar solvents. This may be due to the presence of large number of compounds in polar solvents. The AA for polar solvent was found to be closure to the AA of Gallic acid.

Table 5.17: Phytochemical screening of methanol extract of *B. ceiba* flower

S. No.	Test	Chemicals used	Observation	Inferences
1.	Molisch test	1-Naphthol in ethanol, H_2SO_4	No red to violet color ring appear at the junction	Test for carbohydrate is negative
2.	Ferric chloride test	Aqueous ferric chloride	Green color observed	Test for phenolics is positive
3.	Foam test	Water	No foam is observed	Test for saponins is negative
4.	Libermann-Buchard test	$CHCl_3$, acetic anhydride, conc. H_2SO_4	No pink or red colour appeared	Test for sterols is negative
5.	Braymer test	Alcoholic ferric chloride	Greenish colour appeared	Test for tannins is positive
6.	Ninhydrin test	1% Ninhydrin in acetone	Purple color observed	Test for amino acids & proteins is positive
7.	Mayer's Test	Mayer's reagent	creamy ppt. observed	Test for alkaloid is positive

5.4.3 Antimicrobial susceptibility screening

The different groups of microorganisms were used for antimicrobial activity; *Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa* (gram-negative) and *Staphylococcus aureus* I, *Staphylococcus aureus* II and *Bacillus subtilis* (gram-positive).

Table 5.18: Antioxidant activity (AA) of methanol extract of *B. ceiba* flower

Exp	Hexane	Benzene	Chloroform	Ethyl acetate	Acetone	Methanol	Ethanol	A_b
Set I	84.09	89.77	92.04	89.77	94.31	95.45	94.31	0.88
Set II	80.27	88.65	89.73	89.38	89.49	91.79	94.16	0.91
Set III	82.90	85.19	83.90	86.76	84.34	93.66	94.70	0.98

Gallic acid: Set I: 95.56; Set II: 95.71; Set III: 98.21
Range of UV-visible spectrophotometer: 400 to 650 nm

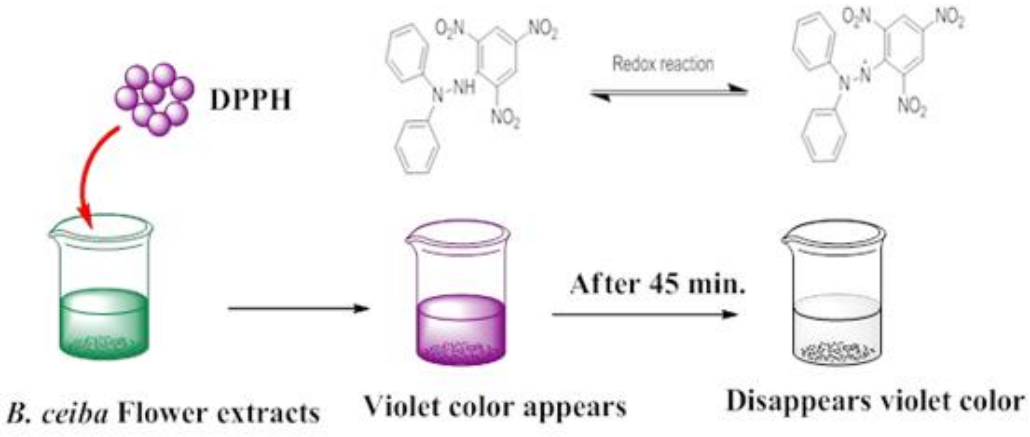


Figure 5.4: Antioxidant evaluation with DPPH

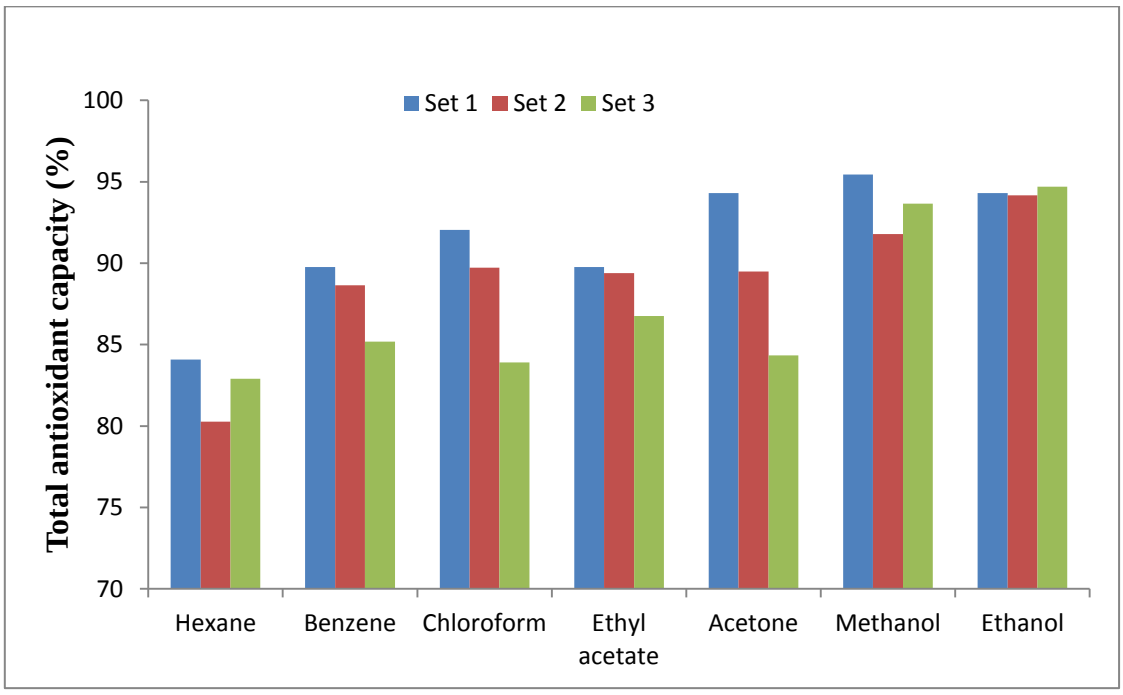


Figure 5.5: Antioxidant activity

The different media required for the preparation of lawn culture of microorganisms were prepared. The Mueller-Hinton (MH) medium for was used as the growth media for these microorganisms. In order to avoid any type of contamination and cross contamination by the test organisms, the antimicrobial screening was done in Laminar Hood with all the precautions. 9.00 and 13.75 g of Mueller- Hinton Agar were dissolved in 250 mL of distilled water in Erlenmeyer flasks of volume 500 mL. The pH of the medium was adjusted to 7.3. The flasks were plugged with non-absorbent cotton and sterilized in autoclave at 15 psi for 20 minutes. The media (15 mL) was poured in petri plates. The respective seed media were inoculated with cells from the surface growth of 1 day old slant of different bacteria with the help of a sterile inoculating loop wire aseptically. Then, the petri plates were placed in an incubator at 37 ^{o}C for 24 h (Figure 5.6).

Table 5.19: Antibacterial activity of methanolic extract of flower of *B. ceiba*

Sample	Conc (μg/mL)	Zone of Inhibition (mm)					
		Gram-negative			Gram-positive		
		K. pneumoniae	*E. coli*	*P. aeruginosa*	*S. aureus* I	*S. aureus* II	*B. subtilis*
Flower extract	25	8	7	3	7	-	8
	50	11	8	5	10	-	10
	100	15	14	7	14	2	13
	200	20	16	8	16	5	18
Vanco-mycin	25	10	8	5	10	4	10
	50	12	11	8	13	5	13
	100	15	13	9	15	8	15
	200	24	22	15	22	10	22

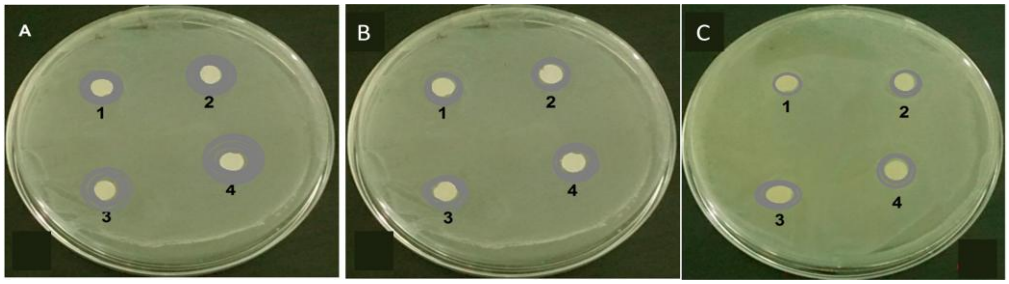
The antibacterial susceptibility test was done by determining zone of inhibition by Kirby- Baur's method. The zone of inhibition was measured using scale (Table 5.19). The size of zone of inhibition indicates the level of sensitivity. The extract was further analyzed by the broth dilution assay to determine its minimum inhibitory concentration

(MIC) values as per standard protocol. The lowest concentration, at which no growth of microorganism observed, was termed as MIC. The results are presented in table 5.20.

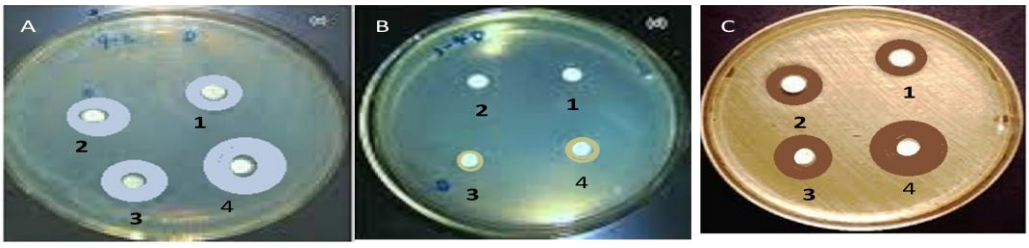
Table 5.20: The MIC determination of flower extract for different organisms

Sample (µg/mL) →	200	100	50	25	12.5	6.25	3.125
Bacteria↓							
K. pneuminoae	X	X	X	X	X	X	√
E .coli	X	X	X	X	X	X	√
P. aeruginosa	X	X	X	X	√	√	√
S. aureus I	X	X	X	√	√	√	√
S. aureus II	X	X	X	X	√	√	√
B. subtilis	X	X	X	X	X	X	√

X: No growth; √: Growth



A. *K. pneumoniae:* 1) 25, 2) 50, 3) 100 and 4) 200 (µg/mL)
B. *E. coli:* 1) 25 , 2) 50 , 3) 100 , and 4) 200 (µg/mL)
C. *P. aeruginosa:* 1) 25 2) 50 , 3) 100 , and 4) 200 (µg/mL)



A. *S. aureus* I: 1) 25, 2) 50, 3) 100, and 4) 200 (µg/mL)
B. *S. aureus* II*:* 1) 25, 2) 50, 3) 100, and 4) 200 (µg/mL)
C. *B. subtilis :* 1) 25, 2) 50, 3) 100, and 4) 200 (µg/mL)

Figure 5.6: Petri plates showing antibacterial activity

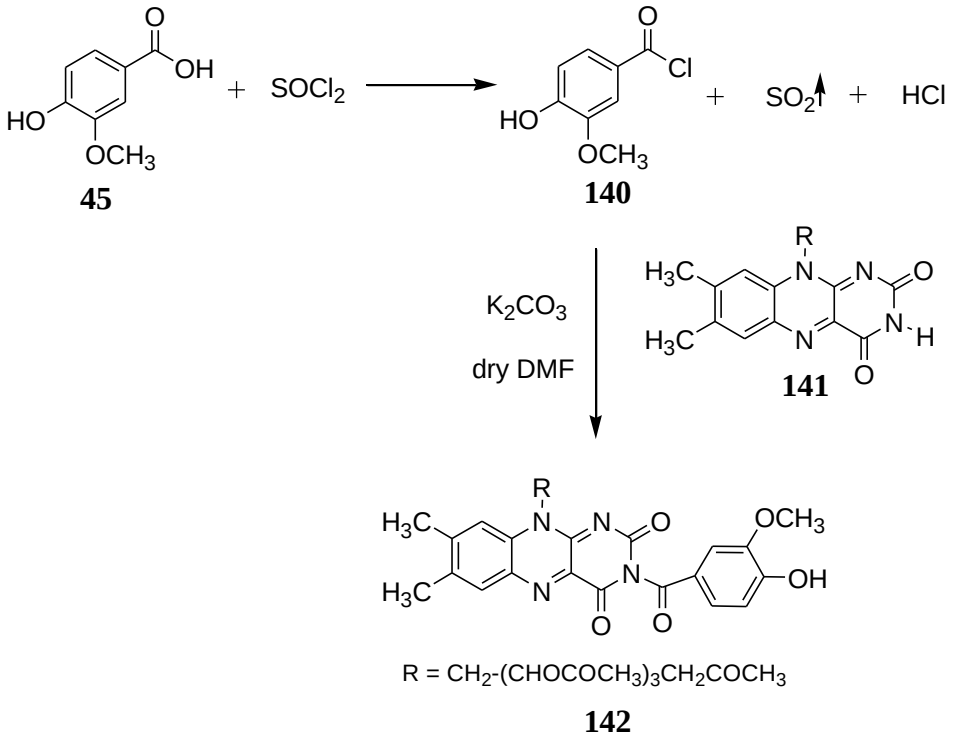
The extract exhibited potent activity against both gram-negative and gram-positive bacteria including *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The activity against *Staphylococcus aureus II* is inferior in comparison to the gram-negative bacteria. The possible reason for activity against gram-positive can be due to the fact that gram-positive bacteria are surrounded by a thick peptidoglycan layer or cell wall. It has little resistance to the diffusion of small molecules. The molecules reported here might diffuse easily through the loose outer wall of the gram-positive bacteria. Whereas, the pathogenic ability of gram-negative bacteria is usually associated with certain components of gram-negative cell walls, in particular the lipopolysaccharide (LPS) layer. They have narrow porins or channels through which the newly synthesized semi-synthetic molecules might diffuse easily and can show antibacterial activity. Enhancement in activity of all the molecules can also be due to the higher cellular uptake and thus enhanced bioavailability of the drug molecules. The extract is showing antibacterial activity of MIC ranging between 3.125-12.5 μg/ml.

5.3.6 Synthesis of flavin-vanillic acid conjugate (142)

The vanillic acid (4-hydroxy-3-methoxybenzoic acid, **45)** is a secondary metabolite present in the flower of *B. ceiba*.[20,21,68,69] This is used as a flavoring agent. Hence, this was chosen as the choice of compound for linking with another bioactive compound, riboflavin tetraacetate through its N^3-position (Scheme 5.1). The carboxylic acid group of **45** was converted into its corresponding acid chloride by using thionyl chloride. The formation of acid chloride was confirmed by FTIR spectroscopy where the peak for carnonyl group of acid chloride was found to be at higher frequency than the corresponding acid group. This acid chloride was further subjected to dry DMF. To this solution, riboflavin tetracetate and potassium carbonate were added. The reaction mixture was refluxed for 1 hour and the progress of the reaction was monitored with TLC. The formation of the conjugate **142** was confirmed by FTIR, ^{1}H NMR and elemental analysis. In ^{1}H NMR spectrum, the appearance of singlets in the aliphatic region at 2.20, 2.48 and 3.73 ppm have been assigned to the seven methyl groups present in the molecule. The other peaks in the aromatic region were in accordance to the structure.

The synthesized compound was evaluated for antioxidant properties using DPPH method. The results are summarized in table 5.21. The result did not show any improvement in antioxidant activity with respect to secondary metabolite **45** and hence the further study

was not performed. The antioxidant activity in vanilic acid (**45**) have been found to be higher in comparision to riboflavin tetraacetate and riboflavin-vanilic acid conjugate.



45 **140** **141** **142**

Scheme 5.1: Synthesis of flavin-vanillic acid conjugate

Table 5.21: Antioxidant activity determination by DPPH assay method

S. No.	Samples	1 h	24 h	48 h
1	Vanilic acid	×	√	√
2	Riboflavin tetracetate (RFT)	×	×	×
3	Vanilic acid-RFT conjugate	×	×	×

5.5 Conclusion

The genus *Bombax* belongs to tropical trees family, generally found in Indian subcontinents, part of Australia and Western Africa and possesses different medicinal values. This genus contains 7 species out of which *B. ceiba* is widely explored.

The secondary metabolites present in the *B. ceiba* belongs to the different categories, like flavonoids, xanthones, coumarins, anthocyanins, terpenoids, steroids, quinines and their glycosides. Due to its richness in different groups of compounds, this plant has been utilized for traditional and modern pharmaceutical applications.

The methanolic extract of *B. ceiba* flowers, obtained from the University campus was found to be rich in alkaloid, phenolics, tannins, amino acids and proteins. The extracts showed antibacterial and antioxidant properties. The flowers can be used as natural antioxidants and preservatives in food and non-food systems and hence utilized in value addition to the products. However, further phytochemical studies are required to authenticate the presence of bioactive molecules. The antioxidant activity of secondary metabolite vanilic acid was found to possess better antioxidant property in comparison to its conjugate with riboflavin tetraacetate.

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Summary of the Thesis

The term 'flavin' refers to the yellow chromophoric and redox active prosthetic group of a class of respiratory enzymes occurring widely in animals and plants, namely the flavoproteins. The flavin cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are involved in the catalysis of a wide variety of biological redox reactions, including the dehydrogenation of NAD(P)H, lipid esters and D-amino acids, the oxidation of amines to imines and N-oxides, the formation and cleavage of disulphide bonds, the hydroxylation of aromatic substrates and the activation of molecular oxygen. A large number of synthetic flavoenzyme models designed to simulate a particular feature of the protein in a minimised system, have been studied. The synthesis of flavin cofactors has been the focus of research due to its paucity in the natural systems. To understand the molecular mechanism of the proteins containing these cofactors, the quantitative amount of these cofactors is essential. The work embodied in this thesis involves the synthesis and applications of flavins.

Chapter 1 deals with the solid and solution phase synthesis of flavin and their analogues. The concept of polymer-supported synthesis was first realized when Merrifield published his synthesis of L-leucyl-L-alanylglycyl-L-valine via attachment of the intermediate to a polymer backbone. This was soon to revolutionize the synthesis of biologically active compounds including peptides, oligonucleotides and oligosaccharides. The present chapter deals with the polymer-supported and solution-phase synthesis of flavin derivatives. A comparative study of polymer-supported and solution phase synthesis of flavin derivatives has also been carried out in this chapter. The polymer-supported synthesis was found to be advantageous over solution phase synthesis.

Chapter 2 describes the application of flavin as catalyst for the Baeyer-Villiger (BV) Oxidation. The synthesis of lactones is a central theme of organic synthesis and the area of metal free organocatalysis is continue to progress from both the chemical and biological perspective. The Baeyer Villiger (BV) oxidation is the basic reaction for the synthesis of lactones. The metal-free organocatalytic reactions have attracted increasing attention as a complement to metal-catalyzed and enzyme-catalyzed reactions. In this chapter, different flavin derivatives have been utilized as catalyst in hydroperxy form for the transformations of cyclic ketones to corresponding lactones. The reactions have been performed to validate biomimetic reaction for BVMOs. Also, the developed catalyst served as metal free

organocatalyst. Further, this chapter discussed the effect of chain length at N-10 position on BV reactions. It was observed that the carbon chain with 5 or 6 carbon gave better result.

Chapter 3 explains about the application of flavin as catalyst for remediation of selected halogenated compounds. Halogenated organic compounds (HOCs) are one of the most versatile and widely used classes of compounds in the industrial world. Unfortunately, they are considered as a major pollutant for any ecosystem due to their persistent, lipophilic and toxic nature. The bioremediation of HOCs have been achieved using flavoenzymes. In this chapter we have mimicked the reactions of cholorophenol monoxygenases. Under the present newly designed biomimetic experimental conditions, we confirmed the removal of chlorine and bromine in 4-halophenol using synthetic cofactor of flavoenzymes. Further, this chapter discussed the effect of chain length at N-10 position on remediation reactions. It was observed that the carbon chain with 5 or 6 carbon gave better result.

Chapter 4 explains the electrochemical study of flavin derivatives. The flavin derivatives are highly versatile electroactive molecules, which catalyze a large number of redox reactions in biological systems. In this context, we hereby report the electrochemical oxidation-reduction process of riboflavin in buffered aqueous solution at varying pH (3.0, 7.0, 9.0 and 12.0) using cyclic voltammetry (CV) techniques. The CV results indicate that the redox voltammetry behavior of riboflavin (Rf) at the sensor was reversible as well as quasi-reversible involving two anodic and cathodic peaks. Electrochemical kinetic parameters for Rf at different pH values in buffered aqueous solution have been calculated, which indicates the structural changes/ behavior of Rf at different pH conditions and provides insight into the one or two electron transfer reaction.

Chapter 5 deals with the studies of *Bombax ceiba* flower extract and their secondary metabolite conjugate with flavin. *B. ceiba* which belongs to a family bombacaceae is commonly known as a silk cotton tree. ***B. c****eiba* is growing most often tropical and subtropical India. Semal trees bear beautiful red coloured flowers during January to March every year. *B. ceiba* is an age old ethnic medicinal plant in India. In this chapter; we have utilized the crude and semi crude extract of *B. ceiba* for identification of group of compound present in it using chemical test method. Further, the extract has been evaluated for antioxidant properties. A secondary metabolite vanillic acid has also been conjugated with riboflavin tetracetate and evaluated for antioxidant properties.

Publications

Papers in peer-reviewed journals

1. Ram Singh, Deepshikha Rathore, Geetanjali and Richa Srivastava; Solvent free reaction of 1-chloro-2-nitrobenzene with anilines; *Der Chemica Sinica*, 4(5), 102-105, **2013**.
2. Deepshikha Rathore, Ram Singh, Geetanjali and Richa Srivastava, Use of Flavins as Catalyst for the Remediation of Halogenated Compounds, *Applied Biochemistry and Biotechnology*, 174(3), 1151-1156, **2014**.
3. Deepshikha Rathore, Richa Srivastava and Ram Singh; Polymer-supported synthesis of 7-hydroxy-10-substituted isoalloxazines; *Indian Journal of Heterocyclic* Chemistry, 27, 13-16, **2017**.
4. Deepshikha Rathore, Richa Srivastava and Ram Singh; Effect of chain length at N-10 position of isoalloxazine on BV oxidation; *Heterocyclic Communications*, Submitted, **2017**.
5. Deepshikha Rathore, Richa Srivastava and Ram Singh; Phytochemical screening, antioxidant and antimicrobial activities of *Bombax ceiba* flower; *Morracan Journal of Chemistry*, Submitted, **2017**.
6. Deepshikha Rathore, Chandra Mouli Pandey, Richa Srivastava and Ram Singh; Electrochemical Analysis of Riboflavin under Different pH Conditions; *Chemistry - A European Journal***,** Submitted, **2017.**

General Article

1. Deepshikha Rathore, Geetanjali and R. Srivastava, Polymer Templet: A boon to organic synthesis, *Rasayan*, 6, 7-11, **2013**.
2. Deepshikha Rathore, R. Srivastava and R. Singh; Migraine: A Neurovascular Disease, *Rasayan*, 7, 28-30, **2014**.

Poster/Oral Presentation in Symposia/Conferences

1. Deepshikha Rathore, R. Srivastava, Geetanjali, and R. Singh; Flavins as metal free Organocatalysts for Baeyer–Villiger Reactions; *National Conference on Advances in Chemical Sciences (ACS-2013)*, March 1-2, **2013**, organized by Department of Chemistry, Maharshi Dayanand University, Rohtak, Haryana, India.

2. Deepshikha Rathore, R. Srivastava, Geetanjali, and R. Singh; Flavins as catalyst for remediation of halogenated compounds; *India-Japan workshop on 'Biomolecular Electronics & Organic Nanotechnology for Environment Preservation* (IJWBME-2013), December 13-15, **2013**, organized by Department of Biotechnology, Delhi Technological University, Delhi, India.
3. Deepshikha Rathore, Geetanjali, R. Singh, and R. Srivastava, Synthesis of novel Flavin Derivatives as Anti-Migraine Drugs, *National Symposium on "Chemistry at the interface of Innovative Researchers in Science and Technology*" held on 27-28 February **2014**, organised by Department of Chemistry, University of Allahabad.
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6. Ram Singh, Deepshikha Rathore, Geetanjali and Richa Srivastava; Flavins as catalyst for Bayer-Villiger Oxidation: Effect of N^{10} chain length; 10th National conference on Solid State Chemistry and Allied areas (ISCAS-2017); Delhi Technological University, (pp: 55), 1-3 July **2017**.