Design and Synthesis of Aminopyrimidinimino Isatin Analogues as Novel Non-Nucleoside HIV-1 Reverse Transcriptase Inhibitors with Broad-Spectrum Antimicrobial Properties

THESIS

Submitted in partial fulfilment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

By
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Under the supervision of
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Certificate

This is to certify that the thesis entitled "**Design and Synthesis of Aminopyrimidinimino Isatin Analogues as Novel Non-Nucleoside HIV-1 Reverse Transcriptase Inhibitors with Broad-Spectrum Antimicrobial Properties**" and submitted by **Miss. Tanushree Ratan Bal, ID. No. 2002PHXF031,** for the award of Ph.D. degree of the Institute, embodies the original work done by her under my supervision.

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Date: 14.04.2005

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Date: 14/4/05 **Tanushree R. Bal**

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List of Symbols and Abbreviations

AIDS	Acquired immunodeficiency syndrome
ADAM	Alkenyl diaryl methane
α-APA	α-anilinophenylacetamide
AZT	azidothymidine
BHAP	Bis (heteroaryl) piperazine
CC_{50}	Cytotoxic concentration
$CCID_{50}$	Cell culture infective dose
ddC	dideoxycytidine
ddI	dideoxyinosine
ddN	2',3'-dideoxynucleoside
ddNDP	5'-dideoxy nucleoside diphosphate
ddNMP	5'-dideoxy nucleoside monophosphate
ddNTP	5'-dideoxy nucleoside triphosphate
d4T	didehydrodideoxythymidine
DABO	3,4-dihydro-2-alkoxy-6-benzyl-4-oxopyrimidines
DAPY	Dianilinopyrimidine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside monophosphate
EC_{50}	Effective concentration
ED_{50}	Effective dose
HIV	Human immuno deficiency virus
HCV	Hepatitis C virus
HAART	Highly active antiretroviral therapy

HEPT	1-(2-hydroxyethoxymethyl)-6-(phenylthio) thymine
IDUs	Intravenous drug users
IL-1β	Interleukin -1 beta
MABA	Microplate Alamar Blue Assay
MIC	Minimum inhibitory concentration
MTB	*Mycobacterium tuberculosis*
MTT	Methyl thiazol tetrazolium
NNRTIs	Non- nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
PBO	pyrrolobenzoxazepinone
PIs	Protease inhibitors
QXPT	quinoxalinyl-ethyl pyridyl thioureas
RMS	Root Mean Square
RNA	Ribonucleic acid
RT	Reverse transcriptase
SI	Selectivity Index
TB	Tuberculosis
3TC	3-thiadideoxycytidine
TET	Thiophene-ethyl thiourea
TIBO	4,5,6,7-tetra-hydroimidazo[4,5,1-jk] [1, 4] benzodiazepin-2 (1H)-one
TNF-α	Tumor necrosis factor - alpha
TSAO	2',5'-bis-O-(tert-butyldimethylsilyl)-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide)
WHO	World-Health Organization

Abstract

In the present thesis, three series (Series I, II, and III) of aminopyrimidinimino Isatin analogues were designed and synthesized based on 3-dimensional pharmacophore model studies using molecular mechanics (MM3) field force. The structures of the synthesized compounds were confirmed by spectral (IR, ^{1}H-NMR, Mass) and elemental analysis. The synthesized compounds were primarily evaluated for their cytopathogenicity and anti-HIV activity on replication of HIV-1 (HTLV-III$_B$ strain) on MT-4 and CEM cell line. Selected compounds were also assayed for inhibitory effects against highly purified HIV-1 reverse transcriptase enzyme by *in vitro* enzyme inhibition assay using homopolymer template primers. The synthesized compounds were also evaluated for cytotoxicity and inhibitory effects on Hepatitis C Virus viral RNA replication in Huh-7 cells, antimycobacterial activity against *Mycobacteium tuberculosis* $H_{37}Rv$ strain using microplate alamar blue asay technique (MABA) and cytotoxicity in Vero cells, *In-vitro* antibacterial activity against 24 pathogenic bacteria by conventional agar dilution procedure, and *In-vivo* antibacterial activity (some selected compounds) against experimentally induced infection of mice, to emerge as a novel non-nucleoside HIV-1 reverse transcriptase inhibitors displaying broad-spectrum antimicrobial property. The most potent compound with broad-spectrum antimicrobial activity was found to be compound M56 (1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7[[N^4-[3'-(4'-amino-5'-trimethoxybenzylpyrimidin-2'-yl)imino-1'-(5-fluoroisatinyl)]methyl]N^1-piperazinyl]-3-quinoline carboxylic acid), which exhibited anti-HIV-1 cytopathogenicity with EC_{50} value of 12.1 µM, % protection of 99.6% and also demonstrated broad-spectrum antimicrobial properties against HCV (95% inhibition of viral RNA replication), mycobacterial (MIC value 3.13 µg/ml and SI of > 3.19), and bacterial (*In vitro* MIC of 0.002 µM and *In vivo* EC_{50} of 1.87 mg/Kg body wt.) pathogenesis.

Contents

01 Introduction

INTRODUCTION

In the present era, acquired immunodeficiency syndrome (AIDS) is the most fatal and life-threatening pathogenic disorder, against which the current generation of anti-HIV drugs represents a major triumph, but no definitive cure. Despite the advances in knowledge of the pathogenesis of HIV-1 and its therapeutic intervention, the AIDS pandemic continues to be the globe's leading public health issue. which has prompted an unprecedented scientific and clinical effort to combat it.

The causative agent of AIDS has been identified as a retrovirus of the lentiviridae family (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Popovic et al., 1984) originally, referred to as HTLV-III or LAV, but now called human immunodeficiency virus (HIV) (Gallo and Montagnier 1988). HIV-1 is the predominant type of HIV and is the causative agent in the development and transmission of acquired immunodeficiency syndrome (Levy et al., 1984). By virtue of its ability to cause progressive depletion of CD4 T-lymphocytes, HIV subverts the ability of the host to mount an adequate antiviral immune response. Overtime, HIV-induced depletion of CD4 lymphocytes leads to collapse of the immune response paving the way to life-threatening immunodeficiency. This gradual deterioration of immune systems increases the vulnerability of the host to numerous viral, fungal, bacterial and protozoal organisms, otherwise held in check among individuals

with healthy immune system. The result of which is loss of ability of the body to fight infection and subsequent acquisition of infections termed 'opportunistic infections'.

1.1 HIV and Opportunistic Infections

Worldwide, tuberculosis is the leading killer and the most common opportunistic infection among HIV-seropositive individuals (Dye et al., 1999). It is one of the leading causes of illness and death among people living with AIDS (Raviglione et al., 1995). The World Health Organization (WHO) estimates that one-third of the world's population is infected with *Mycobacterium tuberculosis* (Small 1996) and approximately 10 million people are co-infected with *Mycobacterium tuberculosis* and HIV. Tuberculosis is found to kill 1 of every 3 patients infected with aquired immunodeficiency syndrome (Raviglione et al., 1995).

Tuberculosis (TB) and HIV interact in fundamentally important ways pathophysiologically, clinically and epidemiologically. CD4 cell-mediated immunity and macrophage function are critical to an effective host response against infection with *Mycobacterium tuberculosis*. But unfortunately the hallmark of HIV infection is the progressive deterioration and depletion of CD4 cells, coupled with defects in macrophage and monocytes function. Thus, the patient's defense against progression of primary infection with *Mycobacterium tuberculosis* or reactivation of latent infection is compromised in proportion to the degree of immunosuppression related to HIV infection. Thus, HIV-infected individuals are at an increased risk of developing tuberculosis in the active form (Selwyn et al., 1989; Edlin et al., 1992; Bermejo et al., 1992) as well as reactivation of latent tuberculosis (Daley et al., 1992). *Mycobacterium tuberculosis* infection of HIV-1 positive patients, occurring before the onset of AIDS, potentially reactivates HIV-1 replication resulting in increased HIV-1 viremia and hastens HIV-1 disease (Whalen et al., 1995; Goletti et al., 1996; Nakata et al., 1997; Havlir and Barnes 1999). *In vitro* studies have shown that pathogenesis of *Mycobacterium tuberculosis* induces macrophage activation and pro-inflammatory cytokine production (TNF- α, IL-1β, IL-6, IL-8) (Friedland et al., 1992; Friedland et al., 1993a; Friedland et al., 1993b; Zhang et al., 1995b; Toossi 1996) which play a key role in *Mycobacterium tuberculosis* killing, but at the same time have been implicated in enhanced HIV-1 replication (Shattock et al., 1993; Shattock et al., 1994; Zhang et al., 1995a).

Although the introduction of HAART (highly active antiretroviral therapy) have resulted in much improved prognosis (Palella, Jr. et al., 1998) for people living with HIV (declining overall mortality and increasing life-expectancy), end-stage chronic liver disease has emerged as an increasingly prevalent cause of morbidity and mortality in people with HIV (Bica et al., 2001; Cacoub et al., 2001). In a retrospective analysis of the possible causes, the main factor in the development of liver pathogenesis was associated to infection with hepatotropic viruses mainly HCV (Rodriguez-Rosado et al., 1998).

The high prevalence of co-infection with HIV and HCV has been attributed to the fact that both are transmitted through the sharing of contaminated needles, with IDUs and haemophiliacs comprising of the main risk group for coinfection (Soriano et al., 1999). Hepatitis C Virus is a serious and growing threat to human health which infects an estimated 170 million people worldwide (Choo et al., 1992; Alter 1997) and thus represents a viral pandemic, five times more widespread than infection with HIV-1 (Lauer and Walker 2001). Dual infection with HIV and HCV has emerged as an aggressive health threat affecting more than one-third of all HIV-infected subjects.

Since the outbreak of the AIDS epidemic, tremendous efforts have been directed towards the development of antiretroviral therapies that target HIV type 1 in particular. En route from their discovery as a new anti-HIV entities to their eventual use for the treatment of HIV infection, new anti-retroviral drugs have to cross many hurdles (e.g. adherence, tolerability, toxicity and virus-drug resistance at clinical level) and at each point of the developmental process, emerging pitfalls have to be balanced against potential benefits at the end. The exhaustive amount of information on HIV life cycle has led to the identification of several molecular and cellular targets for chemotherapeutic intervention of HIV replication (Lipsky 1996). A schematic representation of HIV life cycle is shown in fig. 1.1.

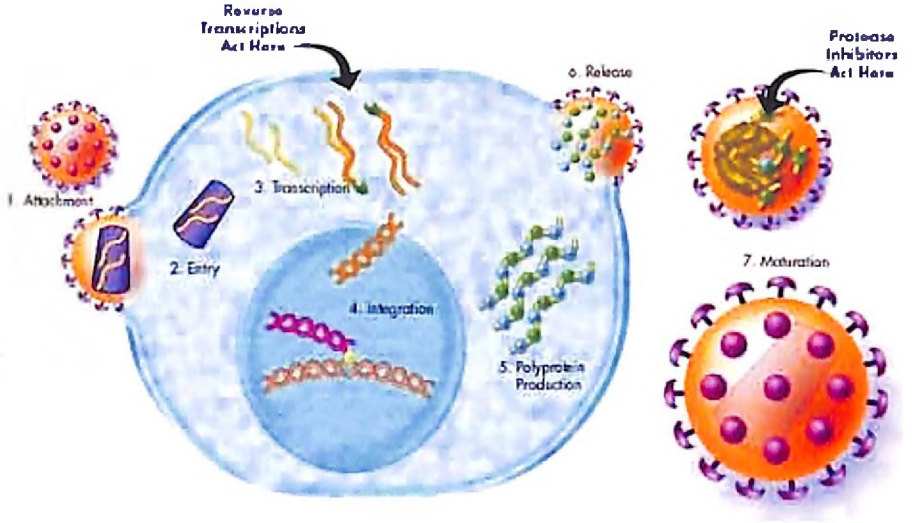


Fig. 1.1 Schematic representation of HIV life cycle

Among the several steps crucial for HIV-1 proliferation, the best known and most intensively studied current treatment strategies are mainly based on drugs that interfere with virus replication by inhibiting either HIV reverse transcriptase (RT) or HIV protease (Jonckheere et al., 1994; Katz and Skalka 1994; Jonckheere et al., 2000). The RT inhibitors can be broadly divided into two types:

(a) Nucleoside/ Nucleotide Reverse transcriptase inhibitors (NRTI's, NtRTI's).

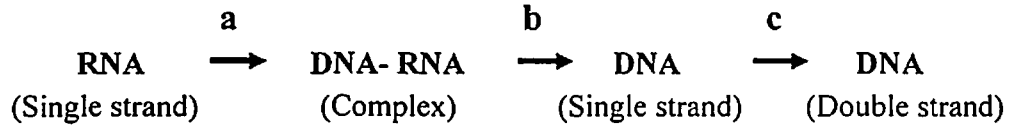
(b) Non-Nucleotide Reverse transcriptase inhibitors (NNRTI's).

The NRTI's and NtRTI's are competitive inhibitors of the 2'-deoxynucleoside triphosphate (dNTP) binding site on Reverse transcriptase and act as substrate decoys and chain terminators (Tantillo et al., 1994a), whereas the NNRTI's bind to a hydrophobic pocket close to, but distinct from the RT active site in the p66 subunit and inhibit the enzyme activity by mediating allosteric changes in the RT, thus causing a distortion of the catalytic active site aspartyl residues (Smerdon et al., 1994; Kroeger Smith et al., 1995; Esnouf et al., 1995; Das et al., 1996; Hopkins et al., 1996; Hsiou et al., 1998).

1.2 Reverse Transcriptase

Reverse transcriptase is a key enzyme, which plays an essential and multifunctional role in the replication of HIV-1 and thus constitutes an attractive target for the development of new drugs useful in AIDS therapy. It is responsible for the transcription of single-stranded viral genomic RNA into double-stranded DNA (Gilboa et al., 1979; Tanese et al., 1986; Goff 1990; Prasad and Goff 1990) which penetrates into the nucleus and is ultimately integrated to the human genome by integrase (Hao et al., 1988; Robert et al., 1990). With the completion of integration, the virus accomplishes an important requirement and is ready to produce more infectious particles and embark on its journey of immune destruction.

Reverse transcriptase is a multifunctional enzyme, which possesses RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity, and RNase activity (Fig 1.2). RT performs three important functions. First, using the RNA as a template, it catalyzes an RNA-dependent DNA synthesis to produce a minus-strand of DNA. Second, using the ribonuclease H (RNase H) section of Reverse transcriptase, the enzyme systematically degrades the genomic negative strand of RNA. Third, using a small polypurine sequence (left undigested on the portion of the digested RNA) as a specific primer, it catalyzes a DNA-dependent DNA synthesis of (+) – stranded DNA, as a complementary copy of (-) - stranded DNA. This newly formed DNA double helix is called proviral DNA, which is translocated into the nucleus and is integrated into the host genome.



a : RNA- dependent DNA polymerase ;

b: RNase H activity

c: DNA – dependent DNA polymerase

Fig 1.2 Reverse transcriptase as a multifunctional enzyme

1.3 Nucleoside Reverse Transcriptase Inhibitors (NRTI)

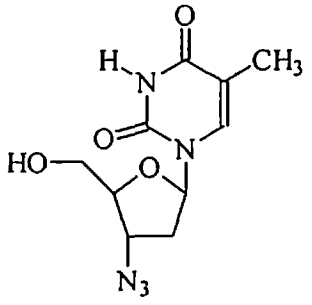
Members of NRTIs act as irreversible and competitive inhibitors for the HIV-RT. At the reverse transcriptase level, these ddN analogues (2',3'-dideoxynucleosides) interact at the substrate binding site of the enzyme, characterized by its catalytic triad constituted of three aspartic acid residues at positions 110, 185 and 186 (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993).

In order to show anti-HIV activity, by interacting with their target enzyme i.e. reverse transcriptase, the NRTIs require intracellular activation to the nucleoside triphosphate form (the nucleotide). This activation process requires three phosphorylation steps, whereby the compounds are converted successively to 5'-monophosphate (ddNMP), 5'-diphosphate (ddNDP), and 5'-triphosphate (ddNTP) form by cellular kinases (De Clercq 1995). Following intracellular phosphorylation to the 5'-triphosphate form, the nucleoside 5'-triphosphate inhibits RT by two modes; first, by acting as a competitive inhibitor of the normal nucleoside-5'-triphosphate (dNTP) for the enzyme; and second, following their incorporation into the growing DNA chain (due to the presence of 5'-hydroxy functionality), they do not permit furthur chain elongation (Huang et al., 1990) due to the lack of a necessary 3'-hydroxy moiety. This leads to the inability to form 3',5'-phosphodiester linkage thus causing DNA chain termination (Mitsuya et al., 1987; De Clercq 1987; Yarchoan et al., 1989; Herdewijn and De Clercq 1990).

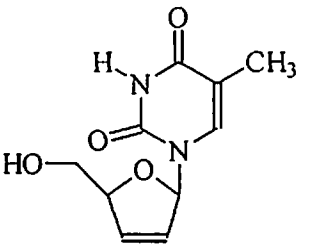
The therapy of HIV (human immunodeficiency virus) infections has, since the advent of azidothymidine (AZT) (Richman et al., 1987; Fischl et al., 1987; Chu et al., 1989), been dominated by the NRTIs (nucleoside reverse transcriptase inhibitors) or 2', 3'-dideoxynucleoside (ddN) derivatives.

The clinically licensed drugs of this group are azidothymidine (AZT, Zidovudine, Retrovir) (Fig. 1.3), which was the first member of the class to be approved (Mitsuya et al., 1985; Yarchoan et al., 1987; Herdewijn et al., 1987), dideoxyinosine (ddI, Didanosine, Videx®)(Yarchoan et al., 1989; Cooley et al., 1990; Lambert et al., 1990), dideoxycytidine (ddC, Zalcitabine, Hivid®) (Yarchoan et al., 1988), didehydrodideoxythymidine (d4T, Stavudine, Zerit®) (Baba et al., 1987; Balzarini et al., 1987; Hamamoto et al., 1987; Lin et al., 1987; Ho and Hitchcock 1989; Zhu et al., 1990; Mansuri et al., 1990; Martin et al., 1990), 3-thiadideoxycytidine (3TC, Lamivudine,

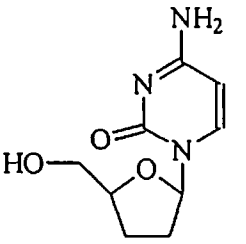
Epivir®), abacavir (ABC, Ziagen®), Emtricitabine ((-)FTC, Emtriva®) (Schinazi et al., 1992), and tenovir disoproxil fumarate (TDF, Viread®).



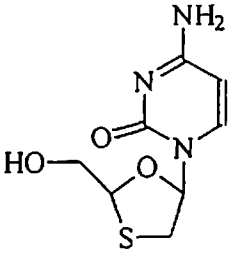
Zidovudine



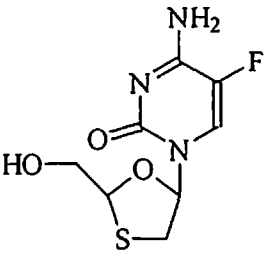
Stavudine



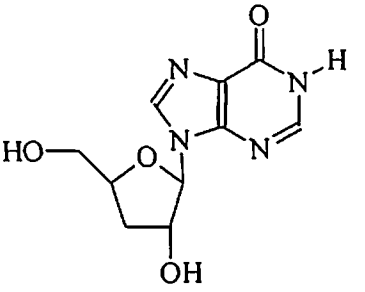
Zalcitabine



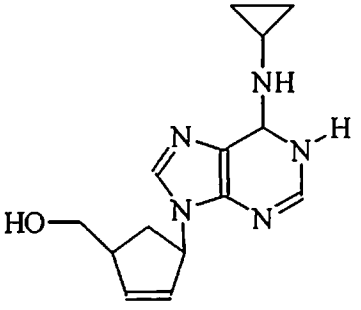
Lamivudine



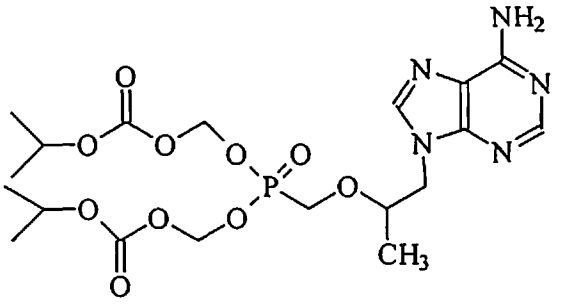
Emtricitabine



Didanosine



Abacavir



Tenofovir disproxil

Fig 1.3 Nucleoside Reverse Transcriptase Inhibitors

1.4 Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) have, in addition to the Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and protease inhibitors (PIs), gained a definitive place in the treatment of HIV-1 infections (De Clercq 1998). Non-nucleoside reverse transcriptase inhibitors during the last five years, gained an increasing momentum in the therapy of HIV infections (De Clercq 2000; Campiani et al., 2002). NNRTIs are especially attractive drug candidate because they do not function as chain terminators and do not bind at the dNTP site (Kopp et al., 1991; Romero et al., 1991a) making them less likely to interfere with the normal function of other DNA polymerases and therefore less toxic than NRTIs.

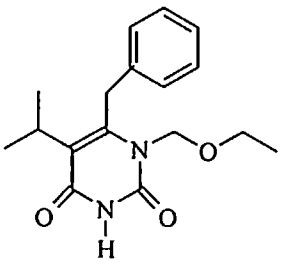
Three NNRTIs in the meantime have been licensed for clinical use: Nevirapine (Viramune®), Delavirdine (Rescriptor®), and Efavirenz (Sustiva®, Stocrin®) and several others have proceeded onto clinical development (i.e tivirapine, loviride, MKC-442, HBY 097).

The era of the NNRTIs started about a decennium ago with the discovery of 1-(2-hydroxyethoxymethyl)-6-(phenylthio) thymine (HEPT) (Miyasaka et al., 1989; Baba et al., 1989) and 4,5,6,7-tetra-hydroimidazo[4,5,1-jk] [1,4] benzodiazepin-2(1H)-one and –thione (TIBO) (Pauwels et al., 1990; Debyser et al., 1991) as specific HIV-1 inhibitors (Fig 1.4), targeted at the HIV-1 reverse transcriptase (Pauwels et al., 1990; Baba et al., 1991a; Baba et al., 1991b).

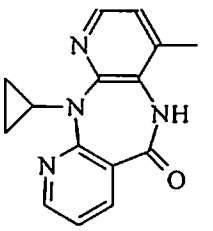
Following the HEPT and TIBO derivatives, several other compounds, viz. nevirapine (BI-RG-587) (Merluzzi et al., 1990; Koup et al., 1991), pyridinone derivatives L-696,229 and L-696,661 (Goldman et al., 1991; Goldman et al., 1992), and bis(heteroaryl)piperazine (BHAP) derivatives U-88204 and U-90152 (Romero et al., 1991a; Romero et al., 1993) were identified as specific HIV-1 inhibitors. In contrast to HEPT and TIBO which were first found to inhibit HIV-1 replication in cell culture before their action on the target HIV-1 RT was unraveled, nevirapine, pyridinone and the bis (heteroaryl)piperazine derivatives were discovered through an HIV-1 RT screening program before their antiviral activity in cell culture was established (De Clercq 1987; De Clercq 1998; De Clercq 2004).

Besides HEPT, TIBO, Nevirapine, Pyridinone and BHAP, other compounds i.e. TSAO-T and TSAO-m^3T (Balzarini et al., 1992a; Balzarini et al., 1992b; Balzarini et al., 1992c), Loviride [a-APA (R89439)] (Pauwels et al., 1993), PETT (LY 300046) (Ahgren et al., 1995), new HEPT derivatives (i.e. I-EBU (MKC-442)) (Baba et al., 1994), and TIBO (i.e. 8-chloro-TIBO, Tivirapine (R 86183)) (Pauwels et al., 1994), have been collectively referred as NNRTIs (De Clercq 1996a; De Clercq 1996b) , and to distinguish them from NRTIs (nucleoside/ nucleotide reverse transcriptase inhibitors).

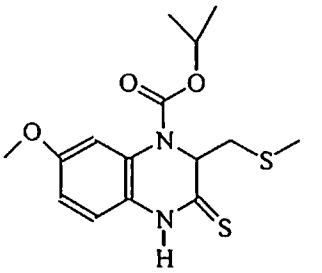
Some of the newer classes of NNRTIs, described in the last five years include the thieno [3,4-e] [1,2,4] thiadiazine derivative (QM 96521) (Witvrouw et al., 1998; Arranz et al., 1998), the imidazo derivative S-1153 (AG 1549, Capravirine) (Fujiwara et al., 1998), (-)-6-chloro-2-{[1-(furo[2,3-c]pyridin-5-yl)ethyl]thio}pyrimidin-4-amine (PNU- 142721) (Wishka et al., 1998), N-[2-(5-bromopyridyl]-N-[2-(2,5-dimethoxyphenyl)ethyl]thiourea (HI-236) (Mao et al., 1999), the pyrido [1,2-a] indole derivative BCH-1 (Taylor et al., 1999), the 4-(cyclopropylalkenyl)-3,4-dihydro-4-trifluoromethyl)quinazolin-2(1H)-ones DPC 082 and DPC 083 (Corbett et al., 2000), the thiophene-ethylthiourea (TET) derivative HI-443 (Uckun et al., 1999a), the (cyclohexenyl)ethylthiourea derivatives HI-346 and HI-445 (Uckun et al., 1999b), the cis-cyclopropylurea-PETT derivatives (Hogberg et al., 1999), the (alkenyl)(diaryl)methane (ADAM) series of compounds (Casimiro-Garcia et al., 1999; Xu et al., 2002), the pyrrolobenzoxazepinone (PBO) derivatives (Campiani et al., 1999), the (quinoxalinyl-ethyl)pyridylthioureas (QXPTs) (Campiani et al., 2001), the MKC-442 (emivirine) derivative SJ-3366 (Buckheit 2001), the 3,4-dihydro-2-alkoxy-6-benzyl-4-oxopyrimidines (DABOs) (Pani et al., 2001), the dianilinopyrimidine (DAPY) derivatives (i.e TMC 125), 4,4-disubstituted 1H, 3H-2,1,3-benzothiadiazine 2,2-dioxides (Corbett et al., 2000) N-(5-bromopyridin-2-yl)-N'-[2-(4-methylphenyl)ethyl]thiourea (Uckun et al., 2000), (quinoxalinyl ethyl) pyridyl thioureas (Campiani et al., 2000), 2,3-diaryl-1,3-thiazolidin-4-ones (Barreca et al., 2001), 2-amino-6-(arylsulfanyl)benzonitriles (Chan et al., 2001) 6-substituted 2-(aryl-sulfanyl) benzonitriles (Sharma et al., 2002), 2-(methylsulfanyl)-1H-imidazoles (Loksha et al., 2003), N-aminoimidazole derivatives structurally analogous to Capravirine (Lagoja et al., 2003), and acylthiocarbamates structurally related to (phenethyl)thiazolyl-thiourea (Ranise et al., 2003).



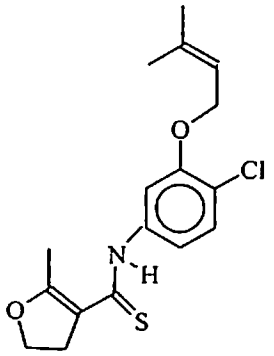
I-EBU (MKC-442)



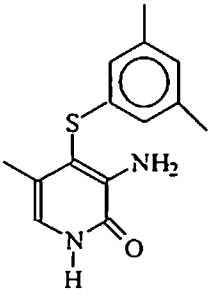
Nevirapine (BI-RG-587)



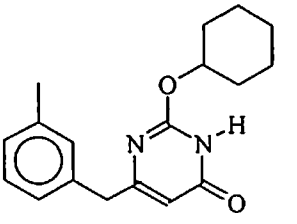
Quinoxaline HBY 097



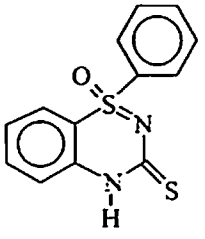
Thiocarboxanilide UC-781



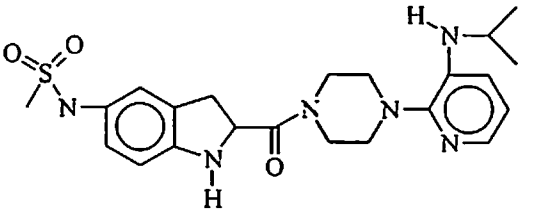
HEPT-pyridinone hybrid



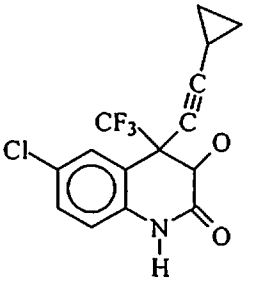
DABO



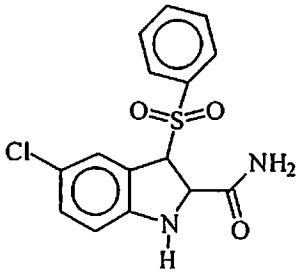
Benzothiadiazine-1-oxide



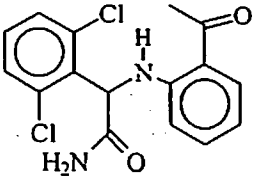
BHAP (U-90152) Delavirdine



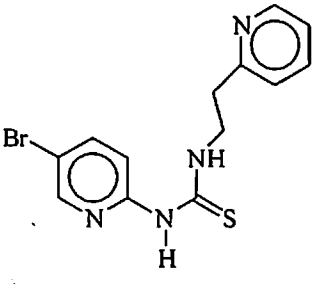
Efavirenz



Indole Carboxamide



Loviride



Trovirdine

Fig 1.4 HIV-1 Specific, Non- Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Interaction of NNRTIs at the HIV-RT

Unlike the ddN analogues (i.e. AZT, ddI etc) which following their intracellular phosphorylation to the triphosphate form, interact with the substrate-binding site of the HIV-RT, the NNRTIs block the HIV-1 RT reaction through interaction with an allosterically located, non-substrate binding site (De Clercq 1993; De Clercq 1996a; De Clercq 1996b). This NNRTI-binding site ('pocket') is located at a close distance of about 10 Å from the substrate-binding site (Tantillo et al., 1994). This binding site of NNRTI is spatially as well as functionally (Dueweke et al., 1992; Debyser et al., 1992) associated with the substrate binding site. The cooperative interaction between these two sites (Spence et al., 1995) provides a rationale to increase the effectiveness of combination therapy using NRTIs and NNRTIs.

A common mode of binding for the chemically diverse NNRTIs with their target site at the HIV-RT has been proposed after several studies (Ren et al., 1995a). It is proposed that the NNRTIs cause a repositioning of the three-stranded β-sheet in the p66 subunit which contains the catalytic trio of aspartic acid residues 110, 185, and 186 (Esnouf et al., 1995). This suggests that the NNRTIs inhibit HIV-1 RT by locking the active catalytic site in an inactive conformation, reminiscent of the conformation observed in the inactive p51 subunit (Esnouf et al., 1995). The NNRTIs appear to function as pi-electron donors to aromatic side-chain residues surrounding the pocket (Kroeger Smith et al., 1995). Side-chain residues also adapt to each bound NNRTI in a highly specific manner, closing down around the surface of the drug to make tight van der-Waals contacts (Kroeger Smith et al., 1995). In the absence of the NNRTIs, the side chains of Tyr-181 and Tyr-188 point into the hydrophobic core of the protein, as a result of which the non-nucleoside binding pocket does not exist (Rodgers et al., 1995; Ren et al., 1995b). It is only upon the contact of protein with the NNRTI that TYR-181 and TYR-188, as well as Tyr-229 move away, so as to create a hydrophobic pocket of sufficient volume to accommodate the NNRTI.

Specificity of NNRTIs for HIV-1 as compared to HIV-2

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are specifically active against HIV-1 RT and not against HIV-2 RT. It has been found that HIV-2 RT has a similar overall fold to HIV-1 RT, but has structural differences within the NNRTI pocket at both conserved and non-conserved residues. These differences can give rise to unfavorable inhibitor contacts or destabilization of part of the binding pocket at positions 101, 106, 138, 181, 188 and 190. In particular, the Y181I substitution in HIV-2 RT could be a significant contributory factor to the inherent resistance of HIV-2 to NNRTIs (Ren et al., 2002).

Although promising inhibitors of NNRTI class are being used clinically, it has become obligatory to design new and more efficacious inhibitors in light of emergence of key variants in addition to wild type to circumvent this dilemma.

02 Literature Review

2.1 Antimicrobial Properties of Pyrimidine Derivatives:-

Chan etal., synthesized conformationally restricted analogues of trimethoprim by connecting the ortho position of the benzene ring to the methylene linkage with two methylene groups, thus forming a dihydroindene derivative (Chan and Roth 1991).

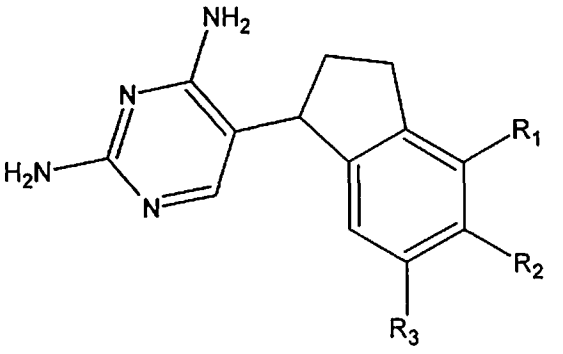


Fig:2.1 R_1= H, OCH_3 ; R_2 = OCH_3; R_3 = OCH_3

Compounds were screened for dihydrofolate reductase inhibitory activities obtained from *E. coli*, rat liver and *N. gonorrhoeae*. Compound **1** (R_1=H, R_2, R_3=OCH_3) showed 100 times less activity than trimethoprim against *E. coli* DHFR, 1.5 times less activity against rat liver DHFR (Kuyper et al., 1985) and 20 fold less activity against *N .gonorrhoeae* DHFR (Roth et al., 1988).

Tanaka et al., synthesized a seris of 5-methyl-6-(arylthio) and 5-substituted-6-(phenylthio) derivatives to investigate the effect of substitution on the pyrimidine moiety of 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) and 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)-2-thiothymine (HEPT-S), on anti-HIV activity (Tanaka et al. 1992a; Tanaka et al., 1992b).

The synthesized compounds were evaluated for anti-HIV-1 (HTLV-III_B) activity in MT-4 cell lines. Substitution at the *meta* position of the C-6-(phenylthio) ring by the methyl group, improved the original anti-HIV-1 activity of HEPT and introduction of two *m*-methyl groups to the phenylthio ring further potentiated the activity.

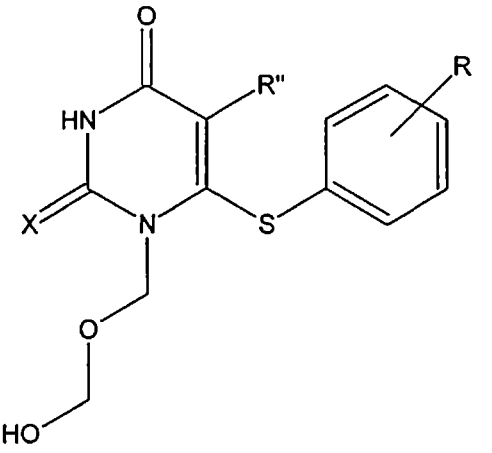


Fig.: 2.2

R = 2-CH_3, 2-Cl, 2-NO_2, 2-OCH_3, 3-CH_3, 3-C_2H_5, 3-t-butyl, 3-CH_2OH, 3-CF_3, 3-F, -Cl, 3-Br, 3-I, 3-NO_2, 3-OH, 3-OCH_3, 4-Cl, 4-F, 4-NO_2, 4-CN, 4-OH, 3, 5-$(CH_3)_2$, 3-$COOCH_3$, 3-$COCH_3$, 3-$CONH_2$, 3-CN, H, 3,5-Cl_2

R"= CH_3, $CH_2CH{=}CH_2$, $COOCH_3$, $CONHC_6H_5$, C_2H_5, propyl, *iso*-propyl

X = O, S

The analogues **2** (R=3,5-$(CH_3)_2$; R"=Me; X=O), 6-[3,5-dimethylphenyl)thio]-1-[(2-hydroxyethoxy)methyl]thymine and **3** (R=3,5-$(CH_3)_2$; R"=Me; X=S), 6-[3,5-dimethylphenyl)thio]-1-[(2-hydroxyethoxy)methyl]-2-thiothymine were found to be inhibitory with EC_{50} values of 0.26 μM and 0.22 μM respectively.

It is noteworthy that 6-[3,5-dimethylphenyl)thio]-5-ethyl-1-[(2-hydroxyethoxy)methyl]thymine derivatives and 6-[3,5-dimethylphenyl)thio]-5-isopropyl-

1-[(2-hydroxyethoxy)methyl]thymine derivatives inhibited the replication of HIV-1 in the nanomolar concentration range.

Tanaka et al., synthesized a series of deoxy analogs and related compounds to investigate the effect of substitution in the acyclic structure of 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)-thymine (HEPT), on anti HIV-1 activity (Tanaka et al., 1992b).

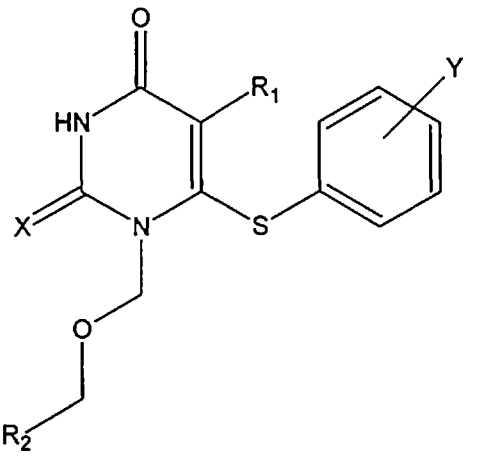


Fig.:2.3

R_2= CH_3, C_2H_5, Propyl, butyl, $CH_2C_6H_5$, $CH_2CH_2Si(CH_3)_3$, c-hexyl, $CH_2CH_2C_6H_5$, $CH_2C_6H_4(4\text{-}CH_3)$, $CH_2C_6H_4(4\text{-}Cl)$

R_1= CH_3, C_2H_5, *iso*-propyl, *c*-propyl

Y = H, 3,5-$(Me)_2$, 3,5-Cl_2

X = O, S

The synthesized compounds were evaluated for their anti-HIV-1 activity on MT-4 cell lines. Substitution with ethoxymethyl and benzyloxy methyl groups at N-1 position potentiated anti-HIV-1 activity, showing EC_{50} value of 0.33 μM and 0.088 μM for compounds **4** (R_1=CH_3; R_2=CH_2Ph; Y=H; X=O), 5-CH_3-1-(ethoxymethyl)-6-(phenylthio)uracil and **5** (R_1=CH_3; R_2=C_2H_5; Y=H; X=O), 5-CH_3-1-[(benzyloxy)methyl)-6-(phenylthio) uracil respectively.

Replacement of 5-methyl group of these two analogues by ethyl or an isopropyl group improved anti-HIV activity remarkably with EC_{50} values in the range of 0.0027-0.019 μM.

Tanaka et al., synthesized several 6-benzyl analogs of 1-[(2-hydroxyethoxy) methyl]-6-(phenylthio) thymine (HEPT) and evaluated them for anti- HIV activity. All the synthesized compounds inhibited HIV-1 replication in MT-4 cells in the submicromolar to nanomolar concentration range (Tanaka et al., 1995).

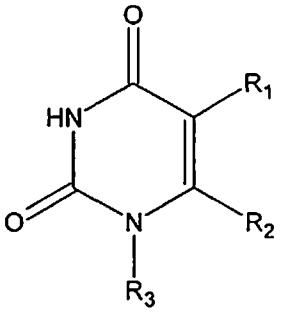


Fig.: 2.4

$R_1 = CH_3, C_2H_5$, *iso*-propyl

$R_2 = C_6H_5, CH_2CH_2C_6H_5, CH_2C_6H_5, CH_2C_6H_5$(3, 5-di-methyl)

$R_3 = HO(CH_2)_2OCH_2, C_2H_5OCH_2$, Butyl, $CH_3OCH_2CH_2$

Compound **6** (R_1= *i*-pr, $R_2 = CH_2Ph$ and $R_3 = EtOCH_2$) i.e 6-benzyl-1-(ethoxymethyl)-5-isopropyl-uracil seemed to be a highly promising candidate for the treatment of AIDS.

Mai et al., synthesized novel compounds related to 2-(cyclohexylthio)-3,4-dihydro-5-methyl-6-(3-methylbenzyl)-4-oxopyrimidine as NNRTIs with the aim to improve the anti HIV-1 activity of S-DABOs (Mai et al., 1997).

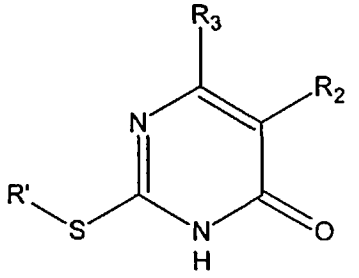


Fig.: 2.5

R_1= *sec*-butyl, *n*-undecyl, cyclopentyl, cyclohexyl, isopropyl, 2-pentyl, 3-pentyl

R_2= H, CH_3, $CH_2C_6H_5$

R_3 = C_6H_5, $(CH_2)_2C_6H_5$, $CH_2OC_6H_5$, $CH_2C_6H_5$, CH_3, $(CH_2)_2CH_3$, 1-naphthylmethyl, 2-naphthylmethyl

The synthesized compounds were evaluated for their ability to inhibit HIV-1 induced cytopathogenicity against the MT-4 cells. Most of the compounds were noncytotoxic to MT-4 cells at doses as high as 300 μM and only few compounds showed CC_{50} values at concentration around 50 μM or lower.

Compound 7 (R' = isopropyl, R_2 = H, R_3 = 1-naphthylmethyl), **8** (R' = *sec*-butyl, R_2 = H, R_3 = 1-naphthylmethyl) and **9** (R' = *sec*-butyl, R_2 = CH_3, R_3 = 1-naphthylmethyl) were endowed with the highest potency showing EC_{50} values of 1.0, 0.3 and 1.0 μM respectively and selectivity of >300, >900 and >300 respectively.

Pontikis et al., synthesized N-1 side chain-modified analogs of 1-[(2-hydroxy)methyl]-6-(phenylthio)-thymine (HEPT), and evaluated for their inhibitory effects on the replication of HIV-1 in two human T-4 lymphoblastoid cell lines, CEM-SS and MT-4 (Pontikis et al., 1997).

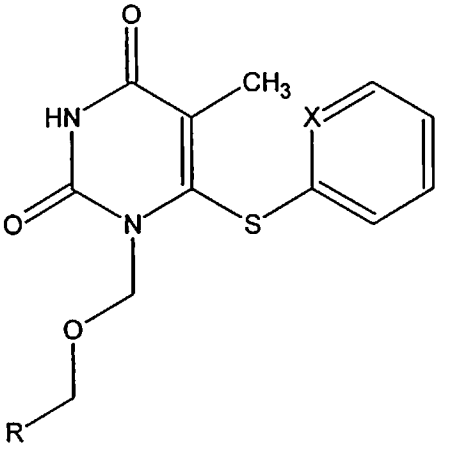


Fig.: 2.6

R = Br, I, morpholine, piperazinyl, $N(CH_2CN)_2$, NHC_6H_5, $N(C_6H_5)_2$, NH_2, $NHCOC_6H_5$, $N(COC_6H_5)_2$, $NHCO(CH_2)_2Cl$, $NHCO(CH_2)_2PO_3H$, SC_6H_5

X=CH, N

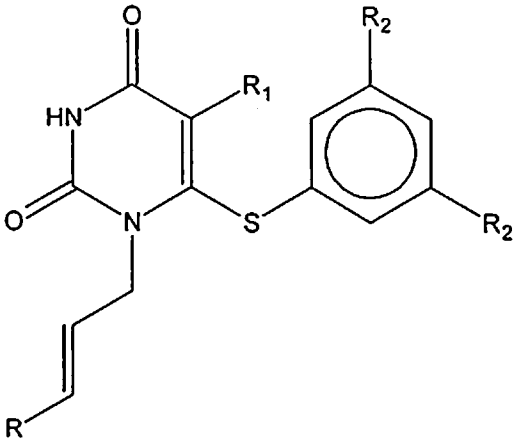


Fig.: 2.7

R=C_6H_5, cyclohexyl, 3-pyridyl, 2-furyl, 2-thienyl, 5-NO_2-2-thienyl, 2-benzofuranyl

R_1=CH_3, C_2H_5

R_2=H, CH_3

Cytotoxicity was not observed in either cell line at concentration equal to or below 1 μM. Compounds **10** (X=CH, R=NHPh), **11** (R=C_6H_5, R_1=C_2H_5, R_2=H), **12** (R=2-furyl, R_1=C_2H_5, R_2=H), **13** (R=2-thienyl, R_1=C_2H_5, R_2=H) and **14** (R=2-benzofuranyl, R_1=C_2H_5, R_2=H) bearing an aromatic ring at the end of the N-1 side chain were found to be potent than the known diphenyl-substituted HEPT analogue BPT.

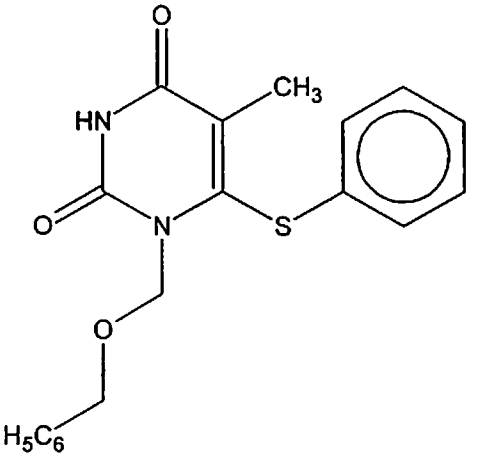


Fig.: 2.8 (BPT)

Vig et al., synthesized novel dihydroalkoxybenzyloxopyrimidine (S-DABO) derivatives targeting the non-nucleoside inhibition (NNI) binding site of the HIV reverse transcriptase (RT), using a novel computer model for the NNI binding pocket. The synthesized compounds were then tested for their RT inhibitory activity in cell-free assays (using purified recombinant HIV-RT), p-24 production in HIV-infected peripheral blood mononuclear cells and viability of HTLV-IIIB-infected peripheral blood mononuclear cells (Vig et al., 1998).

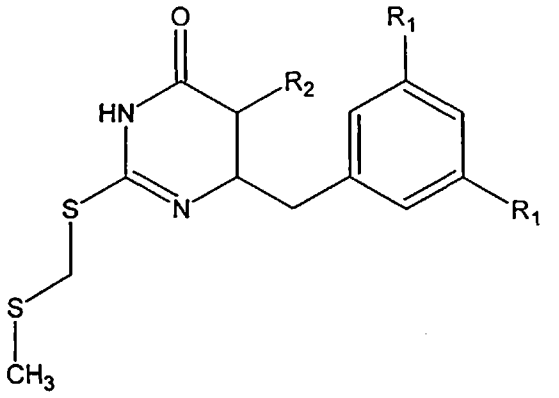


Fig.: 2.9

R_1= H, CH_3

R_2= CH_3, C_2H_5, *iso*-propyl

The lead compound in this series was identified as compound **15** (R_1=H; R_2=*iso*-propyl), 5-isopropyl-2-[(methylthiomethyl)thio]6-(benzyl)-pyrimidin-4-(1H)-one, which elicited potent anti-HIV activity with an IC_{50} value < 1nM for inhibition of HIV replication, as measured by p24 production in HIV-infected human peripheral blood mononuclear cells and showed no detectable cytotoxicity with IC_{50} [MTA] values of >100 μM for cellular proliferation.

Nugent et al., synthesized a series of pyrimidine thioethers, which were furthur evaluated for inhibitory properties against wild-type HIV-1 RT and an RT carrying resistance-conferring mutation P236L (Nugent et al., 1998).

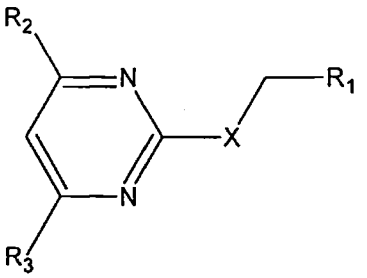


Fig.: 2.10

R_1= Ph, 2-naphthyl, 1-naphthyl, 3-CH_3Ph, cyclohexyl, COOH, $CH_2COOC_2H_5$, $CON(C_2H_5)_2$, CH=CH-Ph, CH=CH-CH_3, CH=CH-$COOCH_3$, CH=CH-$CON(CH_3)_2$, CH=CH-$CON(C_2H_5)_2$, C_6H_4(*p*-CH_3), C_6H_4(*m*-CH_3), C_6H_4(*o*-CH_3), C_6H_4(tert-butyl), C_6H_4(*p*-$COOCH_3$)

R_2 = Cl, pyrrolidino, OH, CH_3, CF_3, CN

R_3 = NH_2, Cl, PrNH, $HO(CH_2)_3NH$, cyclohexylamino, piperidino, pyrrolidino, AcNH, H

X= S, O, NH, CH_2, SO, SO_2

Modification of both the pyrimidine and the functionality attached through the thioether yielded several analogues, which demonstrated activity against both enzyme types with IC_{50} values as low as 190 nM against wild-type and 66nM against P236L RT.

Mai et al., designed and synthesized new 2,6-disubstituted benzyl-DABO derivatives as highly potent and specific inhibitors of the HIV-1 Reverse transcriptase (RT), to investigate the effect of electron-withdrawing substituents in the benzyl unit of the S-DABO skeleton versus their anti-HIV activity (Mai et al., 1999).

Synthesized compounds were evaluated for cytotoxicity and anti-HIV activity in MT-4 cells in comparison with MKC-442 and nevirapine, which were used as reference drugs.

Among the various mono- and disubstituted phenyl derivatives, the most potent were those containing a 6-(2, 6-F_2 phenyl methyl) substituent (F-DABOs), which showed EC_{50} values ranging between 40 and 90 nM and selectivity indexes upto $\geq$5000. An excellent correlation was found between EC_{50} and IC_{50} values which confirmed that these compounds act as inhibitors of the HIV-1 RT.

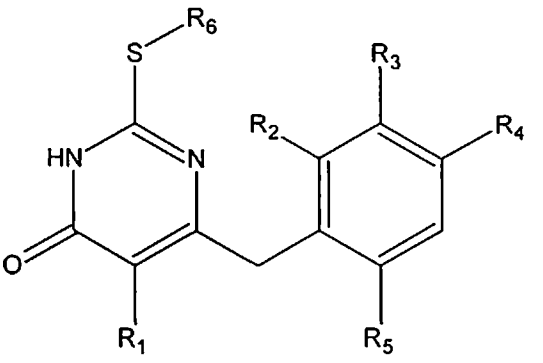


Fig.: 2.11

R_1=H, CH_3

R_2=H, Cl, F, NO_2

R_3=H, Cl, F, NO_2

R_4=H, Cl, F, NO_2

R_5=H, Cl, F

R_6= *s*-butyl, CH_3, 1-propyl, *n*-butyl, *iso*-butyl, c-pentyl, c-hexyl

Ludovici et al., synthesized a series of 2, 4-disubstituted, 2, 4, 5-trisubstituted and 2, 4, 5, 6-tetrasubstituted diarylpyrimidines (DAPYs) as potential anti-HIV drug candidates (Ludovici et al., 2001).

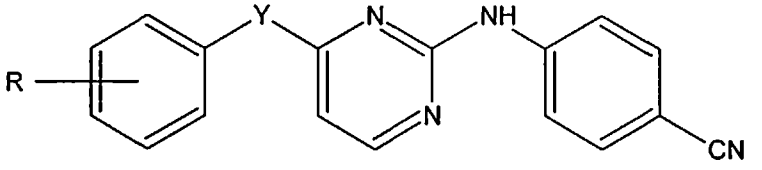


Fig.: 2.12

R= 2, 4, 6-trimethyl, 2,6-dimethyl-4-CN, 2,6-dimethyl-4-Br, 2,6-dimethyl-4-(HCC), 2,6-dibromo-6-F, 2,6-dimethyl, 2,6-dimethyl-4-Cl, 2,6-dimethyl-4-methyl, 2,6-dimethyl-4-Br

Y= N, O, S etc.

Several of the above 2, 4-disubstituted pyrimidines possessed good activity against the HIV-1 wild type and a number of clinically relevant single- and double-mutant starins. Their activity against the double mutants was only in the micromolar range.

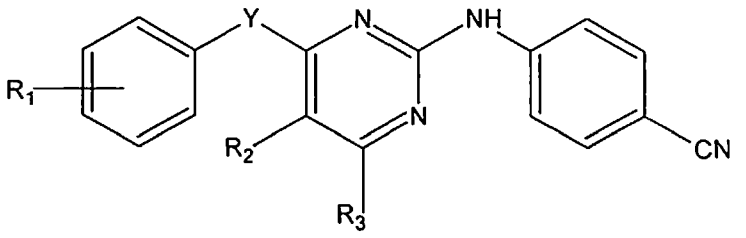


Fig.: 2.13

R_1 = 2, 4, 6-trimethyl, 2,6-dimethyl-4-CN

R_2 = Br, HCC, vinyl, phenyl, CN, Cl, Methyl, NO_2, NH_2, NHAc

R_3 = NH_2

Y = O, N

Introduction of substituent in the 5- and 6- position led to compounds that displayed excellent potency against both wild type and single- and double-mutant strains of HIV-1.

Mai et al., reported structure-based design, synthesis and biological evaluation of novel 2-Alkylthio-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkyl-pyrimidin-4(3H)-ones as non-nucleoside reverse transcriptase (RT) (Mai et al., 2001).

All the compounds were evaluated for their cytotoxicity and anti-HIV-1 activity in MT-4 cells and some of the selected compounds were assayed against highly purified recombinant wild-type HIV-1 RT using homopolymer template primers.

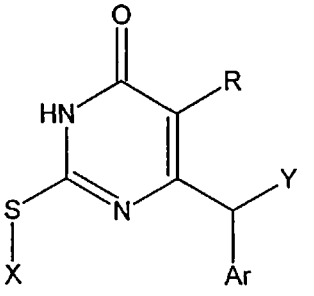


Fig : 2.14

Ar = Phenyl, 1-naphthyl, 2, 6-F_2-Ph, 2, 6-Cl_2-Ph

X = *iso*-Pentyl, *iso*-propyl, *sec*-butyl, cyclopentyl, cyclohexyl, *n*-butyl

Y = CH_3, C_2H_5

R = H, CH_3

Compound **16** (Ar = 2,6-F_2-Ph, R = Y =Me, X = *iso*-pentyl) turned out to be the most potent and selective among the S-DABOs reported till date (CC_{50} > 200 μM, EC_{50} = 6 nM, IC_{50} =5 nM and SI> 33,333) inhibiting HIV-1 replication in MT-4 cells more effectively than MKC-442 (by 5-fold) and nevirapine (by 50-fold).

El-Brollosy N R et al., (2002) reported the synthesis and antiviral activities of a series of 6-arylmethyl-1-(allyloxymethyl)-5-alkyl uracil derivatives as analogues of the anti HIV-1 drug emivirine (formerly MKC-442) (El Brollosy et al., 2002).

Synthesized compounds were evaluated for cytotoxicity and inhibitory activity against two different HIV-1 strains induced (IIIB and HxBz strains) cytopathogenicity in MT-4 cells.

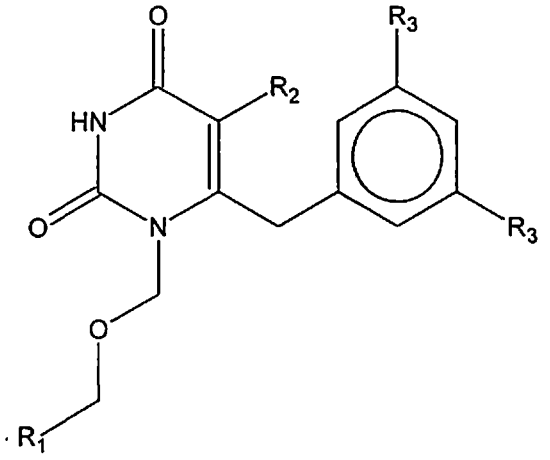


Fig.: 2.15

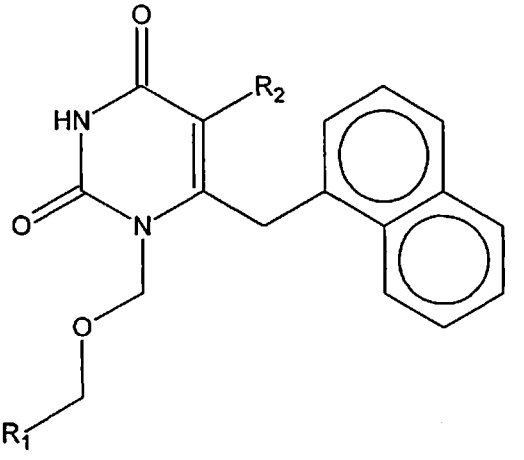


Fig.: 2.16

R_1 = CH_2=CH, $(CH_3)_2$C=CH, CH_2=C(CH_3), CH=C

R_2 = *iso*-propyl, C_2H_5

R_3 = H, CH_3

The most active compounds were found to be (N-1) allyloxymethyl- and (N-1)3-methylbut-2-enyl substituted 5-ethyl-6-(3,5-dimethylbenzyl)uracils, which showed activity against HIV-1 wild type in the picomolar range with SI greater than 5 x10^6 and activity in the submicromolar range against the clinically important Y181C and K103N mutant strains known to be resistant to emivirine.

Hockova et al., synthesized 5-substituted-2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidine, bearing various substituents at the C 5- position of the parent compound and its N 1 isomer(Hockova et al., 2003).

Three 5-substituted-6-[2-(phosphonomethoxy)ethoxy] (PMEO) pyrimidine derivative **17** (R=CH_3, R'=H), **18** (R=Br, R'=H), and **19** (R=Cl, R'=H) showed a pronounced antiviral acytivity in cell culture against retroviruses. The 5-methyl derivative was exquisitely inhibitory to HIV and Moloney murine sarcoma virus-induced cytopathicity in cell-culture (EC_{50} ~ 0.00018 μM/ml) but also cytostatic to CEM cell cultures. In contrast, 5-halogen substituted derivatives showed pronounced antiretroviral activity (EC_{50} = 0.0023-0.0110 μM/ml), comparable to that of reference drugs adefovir and tenofovir, and were devoid of measurable toxicity at 0.3 μM/ml.

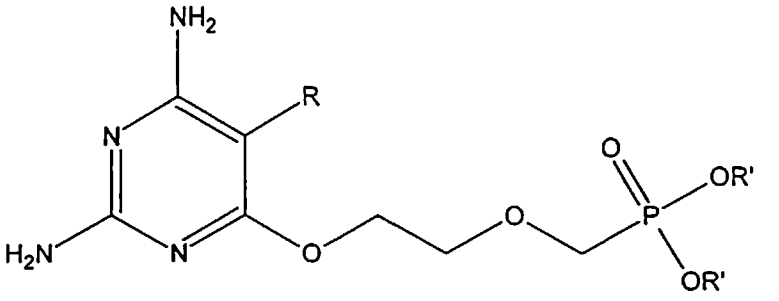


Fig.:2.17

R = allyl, benzyl, CH_2CN, $CH_2COOC_2H_5$, CH_3, phenyl, cyclopropyl, CH_2COOH, Br, Cl, I

R' = H, *iso*-propyl

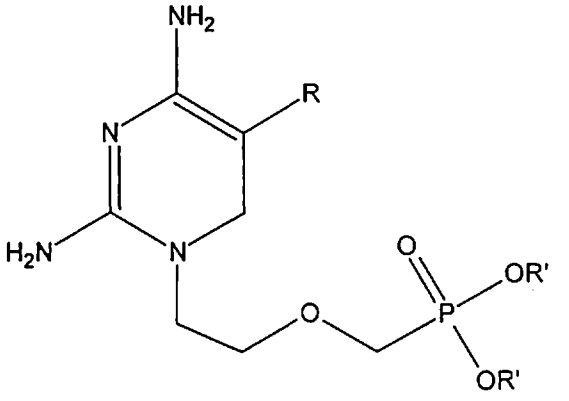


Fig.:2.18

R=R'= same as fig. 2.17

Ragno et al., synthesized 2-alkylamino-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4(3H)-ones (difluoro-amino-DABOs or F2-NH-DABOs) as new DABO prototypes belonging to NNRTI class (Ragno et al., 2004).

Various programmes (VALIDATE II and Autodock) were undertaken to predict the biological activities of the newly designed DABOs, and improved predicted biological activities prompted their synthesis and biological evaluation.

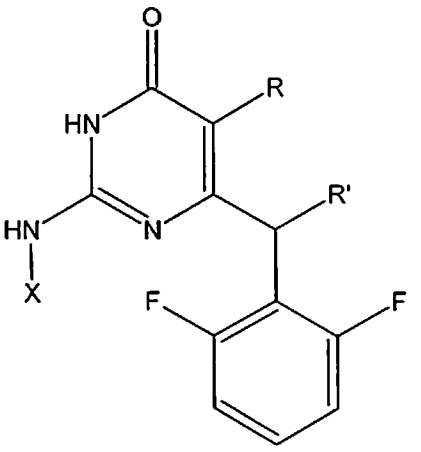


Fig.:2.19

X= NH; R= H, CH_3; R'= H, CH_3

Synthesized F_2-NH-DABOs (difluoro-amino-DABOs) were found to be highly active in both anti-RT and anti-HIV biological assays with IC_{50} and EC_{50} comparable with that of the reference compound MKC-442.

Interestingly, **20** (X=NH; R=H; R'=CH_3) (2-cyclopentylamino-6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-5-methylpyrimidin-4(3H)-one was found to be active against the Y181C HIV-1 mutant strain at submicromolar concentration, with a resistance value similar to that of efavirenz (Y181C\WTIIIB value: 5.3 of compound **20** compared to 6 of efavirenz in μM).

Rosowsky et al., synthesized 2, 4-diamino-5-(2',5'-substituted benzyl)pyrimidine with a carboxyphenyl substituent at the 5'-position of the benzyl moiety as dihyrofolate reductase inhibitors (DHFR inhibitors), in an attempt to combine the enzyme-binding selectivity of 2,4-diamino-5-(3', 4', 5'-trimethoxybenzyl) pyrimidine (i.e Trimethoprim, TMP) with the potency of 2,4-diamino-5-methyl-6-(2',5'-dimethoxybenzyl)pyrido[2,3-d]pyrimidine (i.e Piritrexim, PTX) (Rosowsky et al., 2004).

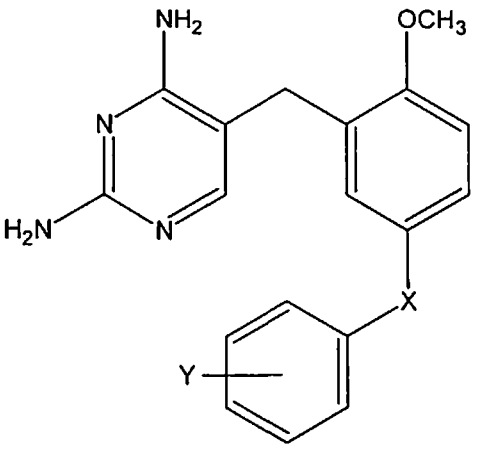


Fig.:2.20

X= OCH_2, $C{=}CCH_2O$, $CH{=}CHCH_2O$, $(CH_2)_3O$, C=C, CH_2CH_2

Y = 2-COOH, 3-COOH, 4-COOH

The synthesized analogues were tested as new lipophilic antifolate drugs against three potentially life-threatening opportunistic parasites often foun in patients with AIDS and other immune disorders, namely *Pneumocystis carinii* (Pc), *Toxoplasma gondii* (Tg) and *Mycobacterium avium* (Ma).

Among all the synthesized analogues tested for their ability to inhibit Pc, Tg and Ma DHFR, compound **21** (X= C=C; Y=3-COOH) (2,4-diamino-(2'-methoxy-5'-(3-carboxyphenyl)-ethynylbenzyl]pyrimidine), with an IC_{50} of 23 nM and a SI of 28 in the Pc DHFR assay, had about the same potency as PTX and was 520 times more potent than TMP. As an inhibitor of Tg DHFR, it had an IC_{50} of 5.5 nM (510 fold lower than TMP and similar to PTX) and a SI value of 120 (2-fold better than TMP and vastly superior to PTX). Against Ma it had an IC_{50} and SI value of 1.5 nM and 430 respectively compared with 300 nM and 610 for TMP.

Compound **22** (X= C=C; Y= 4-COOH) (2, 4-diamino-(2'-methoxy-5'-(4-carboxyphenyl)-ethynylbenzyl]pyrimidine), had 2.5-fold lower potency than 4a against Ma DHFR (IC_{50} =3.7) and was substantially weaker against Pc and Tg DHFR, but it displayed 2200-fold selectivity against the Ma enzyme and was found to be the most selective 2, 4-diamino-5-(5'-substituted benzyl) pyrimidine inhibitor of this enzyme encountered till date.

Rao et al., synthesized several 2-(2,6-dihalophenyl)-3-(pyrimidin-2-yl)-1,3-thiazolidin-4-ones derivatives and evaluated them for prevention of the cytopathic effects of HIV-1 (IIIB) and HIV-2 (ROD) in MT-4 cells (Rao et al., 2004).

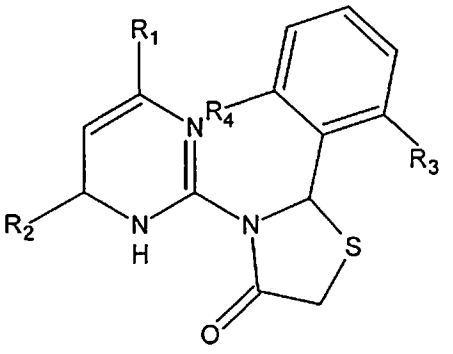


Fig.:2.21

R_1= H, CH_3

R_2= H, Cl, Me, OH, OCH_3

R_3= Cl, F

R_4=Cl, F

Compounds **23** (R_1=CH_3, R_2=H, R_3=R_4=Cl), **24** (R_1=CH_3, R_2=CH_3, R_3=R_4=Cl) and **25** (R_1=CH_3, R_2=OCH_3, R_3=R_4=Cl) emerged as the most potent inhibitors of HIV-1 in MT-4 cell cultures, showing viral replication inhibition at 10-40 nM concentration. They were found to have comparable anti-HIV-1 activity in CEM-cell cultures.

The compounds were also evaluated in CEM cell cultures against an extensive panel of mutant virus strains containing a single mutation in their RT, which is characteristic for the HIV-NNRTI resistance profile. The compounds retained activity against some mutant HIV-1 strains, whereas against other mutant viruses, their activity was reduced by 100 to 1000-fold relative to that recorded for the wild-type strain. Thus, conclusively they behaved as first-generation NNRTIs in that their activity was restricted to HIV-1, with significantly reduced potency against the characteristic NNRTI-resistant mutants K103N and Y181C.

2.2 Antimicrobial Properties of Isatin Derivatives

Sherman et al., studied the effect of N-methylisatin-β-4',4'-diethylthiosemicarbazone (M-IBDET) on the inhibition of Moloney leukaemia virus (MLV) production. The effective antiviral drug concentrations ranged between 3.4 μM and 34 μM. At viral inhibitory concentrations, the drug reduced RNA synthesis only very slightly and did not effect protein synthesis at all, although growth and DNA synthesis of host cells were suppressed (Sherman et al., 1980).

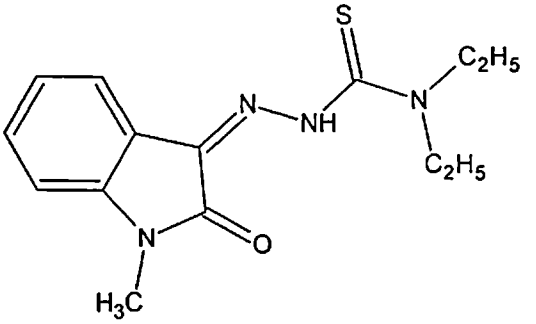


Fig.:2.22 (M-IBDET)

Comparison of M-IBDET with actinomycin D, cycloheximide and α-amanitin in terms of their inhibitory effect on the release of MLV into the culture medium showed that M-IBDET was comparable to the other antimetabolites and specifically inhibits MLV-production.

Ronen et al., studied the effect of N-methylisatin-β-4',4'-diethylthiosemicarbazone (M-IBDET) on the intracellular production of viral constituents in a mouse cell line, 3T3/MLV, chronically infected with Moloney leukaemia virus (Ronen et al., 1985).

Treatment of the cells with 17 μM M-IBDET for 6 hrs inhibited extracellular virus production by 80%, but did not affect the level of viral RNA in the cytoplasm or in the plasma membrane. Intracellular reverse transcriptase activity and levels of viral structural proteins were significantly inhibited, although the drug did not affect viral RNA.

Bankowski et al., observed about 90% inhibition of vaccinia virus in RK-13 cells by the compound N,N'-bis(methylisatin-β-thiosemicarbazone)-2-ethylpiperazine at 100 μM/L concentration. The compound (bis-IBTMP) had no influence on virus adsorption and on early stages of virus multiplication, but affect virus reproduction from 12 to 24 hr post infection (p.i) (Bankowski et al., 1986).

In the infected cells, treated with bis-IBTMP, the incorporation of 3H-thymidine into the infected cells increased during the first 10 hr post infection, but decreased gradually afterwards.

Teitz et al., studied the inhibition of production of HIV by N-methylisatin-β-4',4'-diethylthiosemicarbazone (M-IBDET) and N-allylisatin-β-4',4'-diallylthiosemicarbazone (A-IBDAT). Inhibition of HIV production was confirmed by various parameters of virus assay, employing reverse transcriptase activity, plaque forming units (PFU) and levels of viral structural proteins (Teitz et al., 1994).

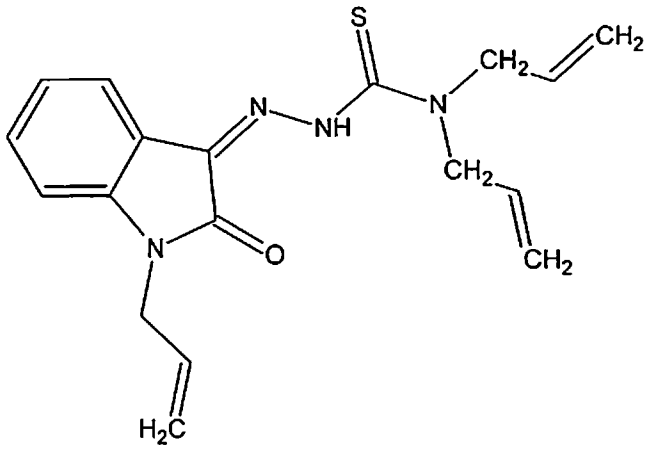


Fig.: 2.23 (A-IBDAT)

Effective antiviral thiosemicarbazone derivative's concentration ranged from 0.17 to 2.04μM for M-IBDET, and from 1.45 to 17.4 μM for A-IBDAT. Therapeutic index values of 20 and 30 were found for M-IBDET and A-IBDAT respectively. A significant inhibition of HIV structural protein synthesis was shown by both M-IBDET and A-IBDAT.

Webber et al., synthesized a novel series of 1, 5-disubstituted isatins (2, 3-dioxindoles), utilizing a combination of protein structure-based drug design, molecular modeling and structure-activity relationship (SAR), which were designed to act as reversible, non-peptidic inhibitors of human rhinovirus (HRV) 3C protease (3CP) (Webber et al., 1996).

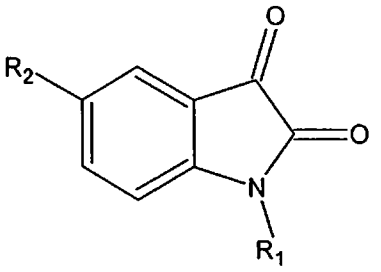


Fig.:2.24

R_1=CH_3, (E)-CH_2CH=$CHPh$, $(CH_2)_3Ph$, CH_2Ph, CH_2(4-Ph-C_6H_4), CH_2-β-naphthyl, CH_2(4-Me-C_6H_4), CH_2(3,4-diMe -C_6H_3), CH_2(3-$OMeC_6H_4$)

R_2= H, Cl, I, NO_2, COOH, CO_2CH_3, $CONH_2$, CN, $CSNH_2$, $COCH_3$, $CON(CH_3)_2$

All the synthesized compounds were tested for inhibiton of purified HRV-14 3CP. Selected compounds were assayed for antirhinoviral activity against HRV-14-infected HI-Hela cells, using a cell-protection assay.

Compounds **26** (R_1=CH_3; R_2=$CONH_2$), **27** (R_1=(E)-CH_2CH=$CHPh$; R_2=$CONH_2$), and **28** (R_1=CH_2-2-benzo[b]thiophene; R_2=$CONH_2$) were found to have excellent selectivity for HRV-14 3CP, compared to other proteolytic enzymes, including chymotrypsin and cathepsin B (Ki=0.051 ± 0.006 μM, 0.011 ± 0.002, 0.002 ± 0.002 μM respectively for **26**, **27**, and **28**).

Pandeya et al., synthesized Schiff bases by reacting isatin and its derivatives with trimethoprim. The N-mannich bases of the above Schiff bases were synthesized by condensing acidic 'NH' group of isatin with formaldehyde and secondary amines (Pandeya et al., 1998a).

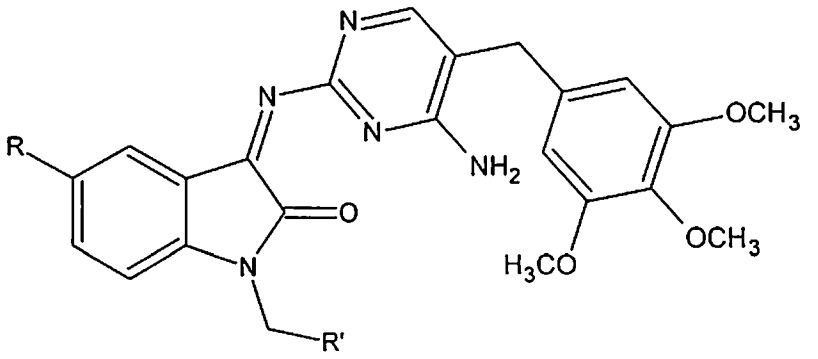


Fig.: 2.25

R = H, CH_3, Br

R' = H, -$N(CH_3)_2$, -$N(C_2H_5)_2$, -piperidine, -pyrrolidine, -morpholine,

All the synthesized compounds were screened for their antibacterial activity by agar dilution method. All the compounds showed good activity against *Vibrio cholerae non-O_1*, *Shigella boydii*, *Enterococcus faecalis* and *Edwardsiella tarda* with MIC in the range of 10-25 µg/ml.

Pandeya et al., synthesized Mannich bases of ciprofloxacin and lomefloxacin with isatin and its derivatives by condensing acidic 'NH' group of isatin with formaldehyde and secondary amino group (piperizine moiety) of ciprofloxacin and lomefloxacin (Pandeya et al., 1998b).

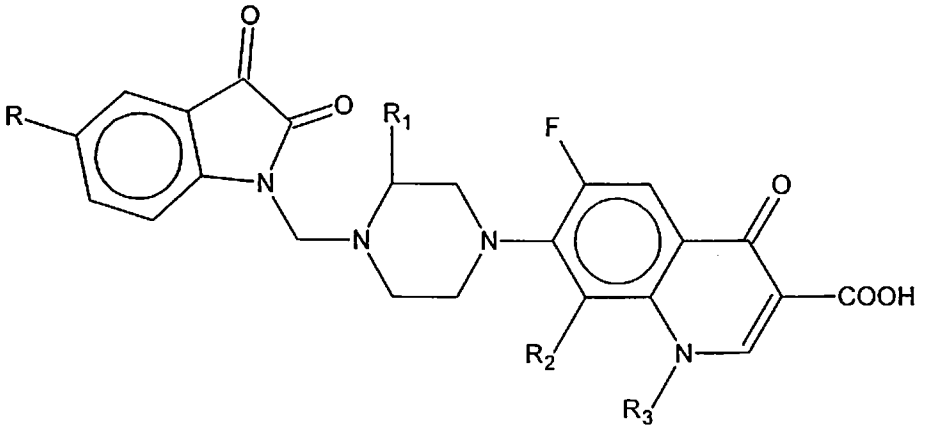


Fig.: 2.26

R=H, CH_3; R_1=CH_3, H; R_2=F, H; R_3=C_2H_5, -cyclopropyl

All the compounds were evaluated for antibacterial activity by agar dilution method against 9 pathogenic bacteria. Mannich bases of ciprofloxacin are equipotent or more potent than ciprofloxacin against certain pathogenic micro-organisms. Mannich bases of lomefloxacin are equipotent to that of lomefloxacin.

Pandeya et al., synthesized Schiff base of isatin and its derivatives with trimethoprim. N-Mannich bases were synthesized from the corresponding Schiff base by reaction with formaldehyde and various secondary amines (Pandeya and Sriram 1998).

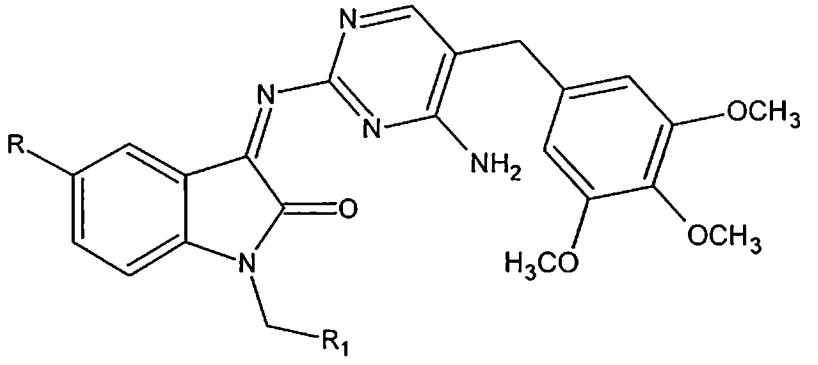


Fig.: 2.27

R = H, CH_3, Br

R'= H, -$N(CH_3)_2$, -$N(C_2H_5)_2$, -piperidine, -pyrrolidine, -morpholine

The synthesized compounds have been screened for anti-HIV activity and cytotoxicity against HIV-1 (III B) and HIV-2 (ROD) cells grown in MT-4 cell lines.The N-dimethyl and morpholino substituted mannich bases were the most active compound in the series and gave maximum protection against HIV-1 (III B) strain.

Pandeya et al., synthesized Schiff bases of isatin and its derivatives with sulphadoxine. The corresponding N-Mannich bases were prepared by reacting the Schiff bases with formaldehyde and secondary or primary amines (Pandeya et al., 1998c) .

The synthesized compounds were evaluated for their antibacterial activity against 25 pathogenic bacteria and antifungal activity against 8 pathogenic fungi.

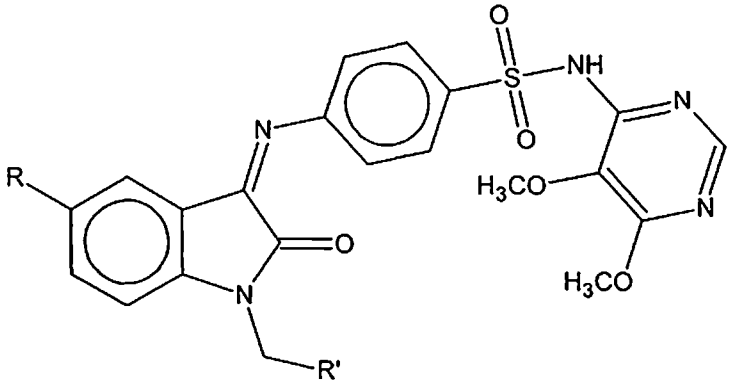


Fig.: 2.28

R = H, CH_3

R'= -$N(CH_3)_2$, -$N(C_2H_5)_2$, -piperidine, -morpholine, -pyrrolidine

All the compounds showed notable activity against the various bacterial and fungal starins. The piperidinomethyl group was found to enhance the antibacterial activity of the compounds and the morpholine derivatives decreased the activity. This observation was vice-versa in case of antifungal activity.

Pandeya et al., synthesized Schiff base of isatin and its derivatives with 3-amino-2-methylmercaptoquinazol-4(3H)-one and its corresponding N-Mannich bases by reacting it with formaldehyde and several secondary amines (Pandeya et al., 1999b).

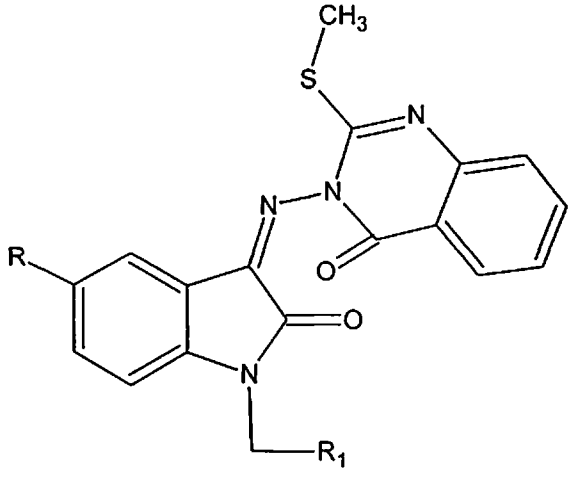


Fig.: 2.29

R = H, Cl, Br

R_1= H, -$N(CH_3)_2$, -piperidine, -morpholine

All the synthesized compounds were screened for their *in vitro* antibacterial activity against 26 pathogenic bacteria, antifungal activity against 8 pathogenic fungi and anti-HIV activity against replication of HIV-1 (IIIB) in MT-4 cells.

All the compounds exhibited mild to moderate antibacterial activity against 26 pathogenic bacteria. They also showed significant antifungal activity when compared to the reference compound clotrimazole.

Compound 5-chloro-3-(3', 4'-dihydro-2'-methylmercapto-4'-oxoquinazolin-3'-yl)-1-morpholino methyl imino isatin was found to be the most active antimicrobial compound. Among the Mannich bases, the reactivity profile was very high with morpholino derivative and substitution at the 5th position of isatin was also found to affect the activity.

Pandeya et al. synthesized Schiff bases by reacting isatin and its derivatives with N-[4-(4'-chlorophenyl)thiazol-2-yl]thiosemicarbazide. N-Mannich bases of these compounds were synthesized by reacting Schiff base with formaldehyde and three secondary amines (Pandeya et al., 1999c).

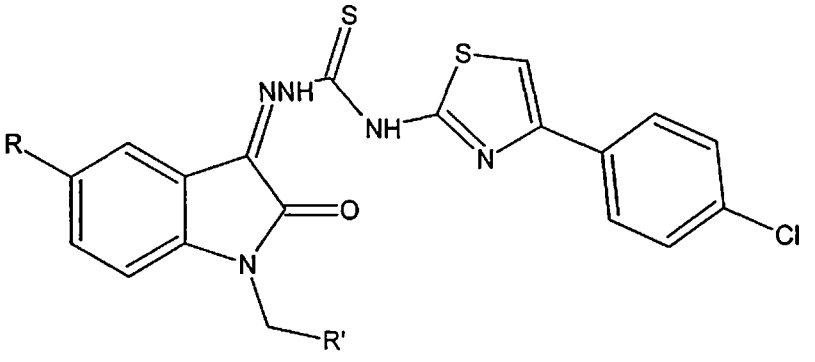


Fig.:2.30

R= H, Cl, Br

R'= -$N(CH_3)_2$, -piperidine, -morpholine

Evaluation of antimicrobial activity of compounds was done by agar dilution method against 28 pathogenic bacteria, 8 pathogenic fungi and anti-HIV activity against replication of HIV-1 (III B) in MT-4 cells.

Among the compounds tested, 1-[N,N-dimethylaminomethyl)-5-bromoisatin-3-[1'-[4''-(p-chlorophenyl)thiazol-2''-yl]thiosemicarbazone, showed the most favourable antimicrobial activity.

Pandeya et al., synthesized Schiff base by reacting isatin with N-(6-chloro-benzothiazol-2-yl)thiosemicarbazide and corresponding Mannich bases by reacting Schiff base with formaldehyde and several secondary amines (Pandeya et al., 1999a).

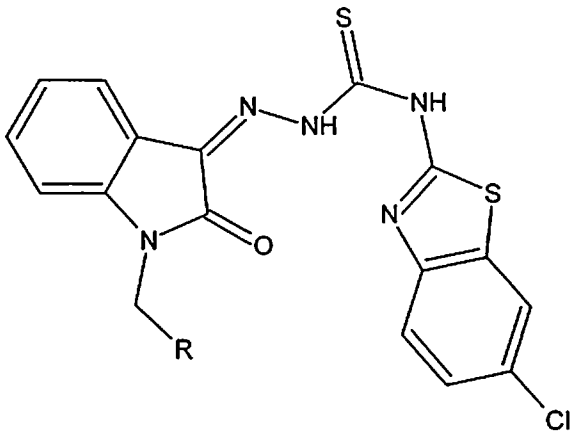


Fig.: 2.31

R = -$N(CH_3)_2$, -piperidine, -morpholine, H

The synthesized compounds were evaluated for their antibacterial activity against 25 pathogenic bacteria, antifungal activity against 8 pathogenic fungi and anti-HIV activity against replication of HIV-1 (III B) in MT-4 cells.

Among all the tested compounds, 1-[N,N-dimethylaminomethyl]isatin-3-[1'-(6''-chlorobenzothiazol-2''-yl]thiosemicarbazide showed the highest antibacterial activity.All the compounds showed significant antifungal activity when compared to clotrimazole, as three compounds were equipotent (2.44 µg/ml) against *Microsporum gypsum* and three compounds were equipotent and one more active against *Histoplasma capsulatum*. None of the compounds showed marked anti-HIV activity at a concentration significantly below their toxicity threshold.

Pandeya et al., synthesized Schiff base from reaction of isatin and its derivative with 3-(4'-pyridyl)-4-amino-5-mercapto-4-(H)-1,2,4-triazole. N-Mannich base of these compounds was synthesized by reaction with several secondary amines and formaldehyde (Pandeya et al., 2000b).

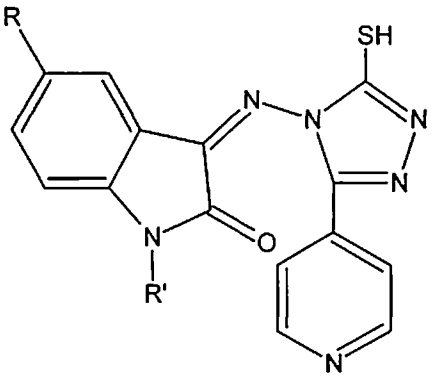


Fig.: 2.32

R = H, Cl, Br

R'= H, $-CH_2(CH_3)_2$, $-CH_2$-piperidine, $-CH_2$-morpholine

The synthesized compounds were evaluated for their *in vitro* antibacterial activity against 27 pathogenic bacteria, *in vitro* antifungal activity against 8 pathogenic fungi and anti-HIV activity against replication of HIV-1 (IIIB) in MT-4 cells.

All the compounds showed mild to moderate activity against 27 tested bacteria among which 1-(piperidinomethyl)-5-bromo-3-[3'-(4''-pyridyl)-5'-mercapto-4'-(H)-1',2',4'-triazol-4'-yl]iminoisatin showed the most favourable antibacterial activity. The most active compound showing antifungal activity was 1-(morpholinomethyl)-3-[3'-(4''-pyridyl)-5'-mercapto-4'-(H)-1',2',4'-triazol-4'-yl]iminoisatin. All the compounds were inactive against the replication of HIV-1 (III B) at subtoxic concentration in MT-4 cells.

Pandeya et al., synthesized Mannich bases of norfloxacin by reacting norfloxacin with formaldehyde and several isatin derivatives (Pandeya et al., 2000a).

The synthesized compounds were evaluated for their *in vitro* antibacterial activity against 28 pathogenic bacteria, antifungal activity against 8 pathogenic fungi and anti-HIV activity against replication of HIV-1 (III B) in MT-4 cells.

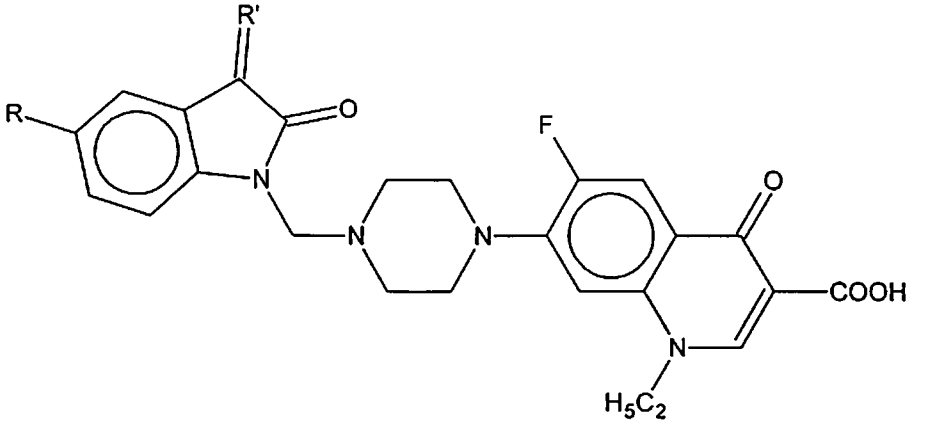


Fig.: 2.33

R = H, Cl, Br

R'= -trimethoprim, -sulphadoxine, -sulphadiazine

All the synthesized compounds were more active than norfloxacin against 13 tested bacteria. Two compounds **29** (R=Cl, R'= trimethoprim moiety) and **30** (R=Br , R'= trimethoprim moiety) showed inhibition against HIV-1 (III B) with EC_{50} values of 11.3 and 13.9 µg/ml respectively. In mouse protection test, also two compounds are more active than norfloxacin (ED_{50}: 1.25 mg/kg and 1.62 mg/kg). Among the compounds tested, 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7[[N4-[5'-bromo-3'-(4'-amino-5'-trimethoxybenzylpyrimidin-2'-yl]-imino-1'-isatinyl]methyl]N1-piperazinyl]-3-quinolinecarboxylic acid showed promising activity in all the three tests.

Sridhar et al., synthesized Schiff bases and hydrazones of substituted isatins by reacting isatin and aromatic primary amines/hydrazines respectively. A new series of the corresponding N-Mannich bases were synthesized by reacting them with formaldehyde and diphenylamine (Sridhar et al., 2001).

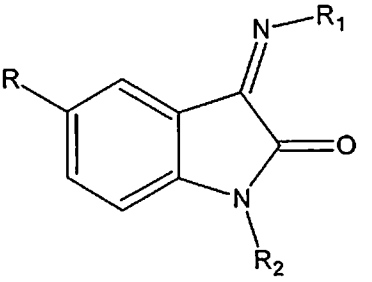


Fig.:2.34

R= H, Cl, Br, CH_3, NO_2

R_1=1-naphthyl, NH-CS-NH_2, C_6H_5 (*p*-Cl), C_6H_5 (*p*-CH_3), C_6H_5 (*p*-OCH_3), NH-C_6H_5, C_6H_5 (*p*-Br), NH-C_6H_3(2,4-dinitro)

R_2=H, CH_2N $(C_6H_5)_2$

All the synthesized compounds were screened for antibacterial activity against seven Gram positive and seven Gram negative standard and pathological bacterial strains by the paper disc diffusion technique.

Most of the compounds exhibited mild to moderate antibacterial activity. Compound **31** (R=NO_2, R_1=1-naphthyl, R_2=H) and **32** (R=H, R_1=4-bromophenyl, R_2= CH_2N $(C_6H_5)_2$) were found to be the most active compounds against the screened Gram (+) and Gram (-) standard and pathological bacterial strains. Mannich bases exhibited higher activity than the corresponding Schiff base.

Selvam et al., synthesized Schiff base by reaction of isatin and its derivatives with sulphamidine. The Schiff and corresponding Mannich bases were investigated for their anti-HIV activity against replication of HIV-1 (IIIB) and HIV-2 (ROD) strains in acutely infected MT-4 cells and were estimated by MTT assay (Selvam et al., 2001).

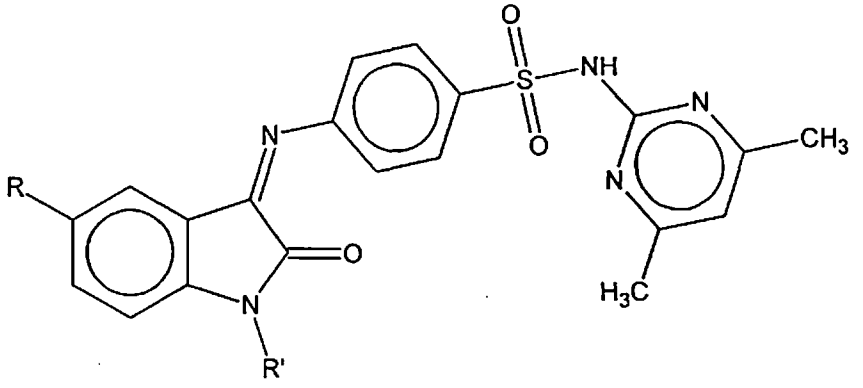


Fig.: 2.35

R=H, Cl, Br, CH_3, H

R'=H, $COCH_3$

It was found that all compounds were active against replication of HIV-1 and HIV-2 in acutely infected MT-4 cells. The effective concentration (EC_{50}) of the test compounds against the replication of HIV-1 (range: 8.0-15.3 µg/ml) was less when compared to HIV-

2 (range: 41.5- > 125 μg/ml). However, the inhibitory concentrations (IC_{50}) of compounds against the replication of HIV-1 and HIV-2 were significantly higher than the standard AZT.

Pandeya et al., synthesized Mannich bases of norfloxacin by reacting them with formaldehyde and isatin derivatives bearing isoniazid semicarbazone and thiosemicarbazone moieties, with an aim to achieve antitubercular activity (Pandeya et al., 2001).

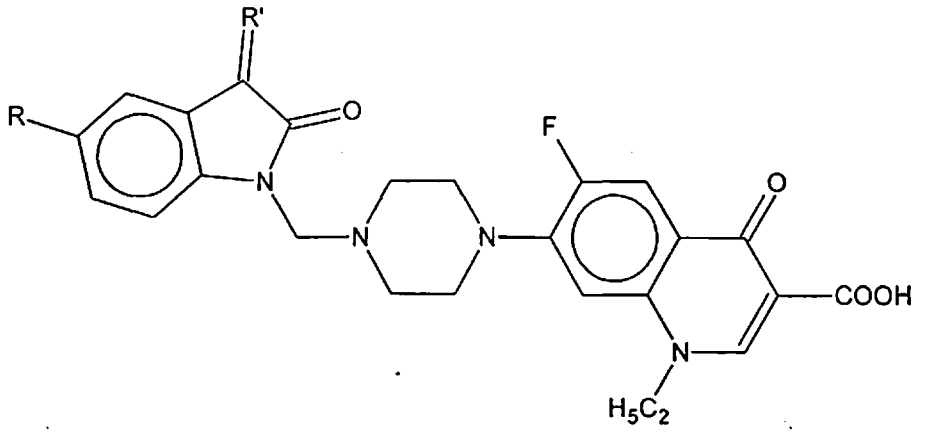


Fig.:2.36

R = H, Cl, Br

R'= O, -NNHCONH$_2$, -NNHCSNH$_2$, - NNHCO-pyridine

The synthesized compounds were evaluated *in vitro*, against *Mycobacterium tuberculosis* $H_{37}Rv$, at a concentration of 12.5 μg/ml, in BACTEC 12B medium using the BACTEC radiometric system.

The evaluated compounds showed activity against the mycobacteria with MIC values ranging from 6.25, to > 12.5 μg/ml, with a percentage inhibition ranging between 90 and 100%. The isonicotinoyl hydrazones **33** (R=H; R'=- NNHCO-pyridine) and **34** (R=Cl, R'=- NNHCO-pyridine) emerged as the most active compounds in the series.

Pandeya et al., synthesized Schiff base of isatin with pyrimethamine. The corresponding N-Mannich bases were prepared from the schiff base by condensing with formaldehyde and secondary amines (Pandeya et al., 2002).

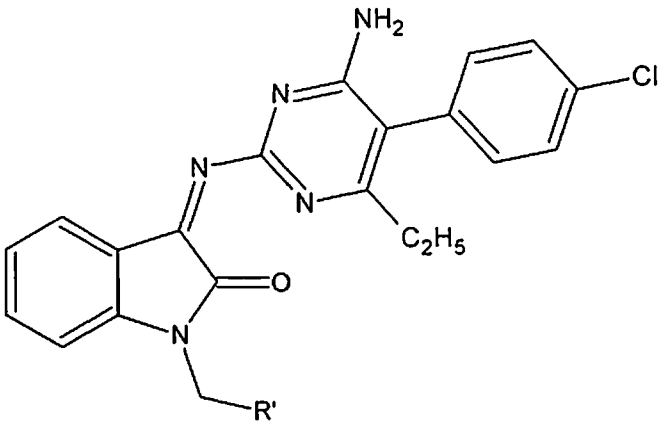


Fig.:2.37

R' = -$N(CH_3)_2$, -$N(C_2H_5)_2$, -morpholine, -piperidine, -pyrrolidine, -sulphadoxine

The synthesized compounds were screened for antibacterial activity against 25 pathogenic bacteria and antifungal activity against 6 pathogenic fungi by agar dilution method. All the compounds showed marked activity against a variety of micro-organisms. All the N-Mannich bases exhibited more potency than pyrimethamine.

Among the analogues evaluated, 2-[1''-(morpholinomethyl)-3''-isatinimino]-5-(4'-chlorophenyl)-6-ethyl-4-aminopyrimidine exhibited higher potency compared to the standard drugs against all bacteria.

Sriram et al., synthesized Schiff bases by reacting isatin and its derivatives with 4-(4-chlorophenyl)-6-(4-methylphenyl)-2-aminopyrimidine. The corresponding N-Mannich bases of these compounds were synthesized by reacting Schiff bases with formaldehyde and several secondary amines (Sriram et al., 2002).

The compounds were evaluated *in vitro* against *Mycobacterium tuberculosis* $H_{37}Rv$ at 6.25 µg/ml in BACTEC 12B medium using the BACTEC 460 radiometric system. All the synthsized compounds showed percentage inhibition ranging from 86 to 97% in preliminary screening at 6.25 µg/ml.

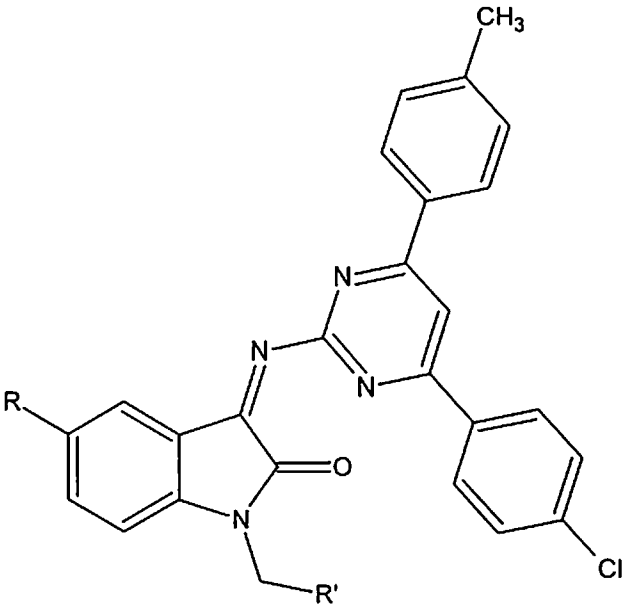


Fig.:2.38

R= H, Cl, Br

R'=H, -$N(CH_3)_2$, -piperidine, -morpholine

Among the compounds tested, 3-[4-(4-chlorophenyl)-6-(4-methylphenyl)pyrimidin-2-yl]iminoisatin showed promising activity with 97% inhibition at a concentration of 6.25 µg/ml.

03 Objective and Plan of Work

3.1 Objective

The research work was aimed to design and synthesize aminopyrimidinimino isatin analogues as novel non-nucleoside HIV-1 reverse transcriptase inhibitors with broad-spectrum antimicrobial properties.

3.2 Plan of work

The plan of work is broadly classified under three main categories:

1) Rational approach of drug design via molecular modeling strategies:

The pharmacophoric pattern was developed by deriving preferred conformations of the widely diverging class of compounds in NNRTI category and then defining and comparing the common groups in terms of specific atom types, general atom types and functional group etc. The pharmacophore model was elucidated, by joining the sites in common and calculating interatomic distances using molecular mechanics force-fields.The derived model was then matched by superimposition in least square fit calculation.

2) Synthesis:

The synthesis of various aminopyrimidinimino isatin derivatives was achieved via four steps. 5-Substituted isatins and 5-substituted benzyl-2,4-diamino pyrimidine were synthesized separately by a two step process. Various 5-substituted isatins were then condensed with 5-substituted benzyl-2, 4-diamino pyrimidine in the presence of glacial acetic acid to form Schiff bases. The N-Mannich bases of the above Schiff bases were synthesized by condensing acidic imino group of isatin with formaldehyde and various secondary amines.

3) Microbiology:

The synthesized aminopyrimidinimino isatin derivatives were evaluated primarily for their cytopathogenicity and anti-HIV activity on the replication of HIV-1 (HTLV-IIIB) in MT-4 cell line and CEM cell line. Selected compounds were assayed for inhibitory effects against highly purified HIV-1 RT using homopolymer template primers.

The synthesized compounds were also evaluated for the following antimicrobial activities to emerge as a novel non-nucleoside HIV-1 reverse transcriptase inhibitors displaying broad-spectrum antimicrobial property:

a) Cytotoxicity and inhibitory effects on the Hepatitis C Virus viral RNA replication in Huh-7 cells.
b) Antimycobacterial activity against *Mycobacteium tuberculosis* $H_{37}Rv$ strain using microplate alamar blue asay technique and cytotoxicity in Vero cells.
c) *In-vitro* antibacterial activity against 24 pathogenic bacteria by conventional agar dilution procedure.
d) *In- vivo* antibacterial activity (some selected compounds) against experimentally induced infection of mice

04 Molecular Drug Design

Molecular modeling methodology attributes to the understanding of molecular interactions, which is being regulated by subtle recognition and discrimination process, whereby the 3-dimentional features and the binding energies play an important role. It also provides the necessary tools for predicting the potential possibilities of prototype candidate molecule. This discipline has widened the horizons of pharmaceutical research by providing tools for discovering new lead structures by a rational approach. Our present study has been concerned with deriving pharmacophoric patterns in well known active molecules and designing new analogs based on the derived pharmacophore model.

The concept of a pharmacophore has been one of the central tenets of rational drug design. A pharmacophore model helps in understanding the range of biological activity observed in a series of compounds as well as to guide the design of new, potentially more potent compounds. The process of generating a pharmacophoric pattern referred as pharmacophore mapping involves three main aspects.

a) Identification of pharmacophoric elements in a set of high-affinity ligands, required for biological activity.
b) Performing an exhaustive conformational analysis for each compound in the set, in order to identify a set of low-energy conformations for each active molecule.

c) Developing molecular superimposition techniques for which the selected pharmacophoric elements superimpose.

Several studies have revealed that although the NNRTI's seemingly belong to a widely diverging classes of compounds, but on closer inspection it has been elucidated that most of them possess some features in common, that is a (thio) carboxamide, (thio) acetamide or (thio) urea entity ('*body*') which is hydrophilic in nature, surrounded by two hydrophobic, mostly aryl moieties ('*wings*'), one of which is quite often substituted by a halogen group. Thus, the overall structure may be considered reminiscent of a butterfly with hydrophilic centre ('*body*') and two hydrophobic outskirts ('*wings*'). The 'butterfly-like' conformation has been proven by crystallographic analysis for nevirapine (Mui et al., 1992) and TBZ. In the HIV-1 RT binding pocket, the NNRTI's i.e loviride (a-APA R95846) (Ding et al., 1995b), tivirapine (TIBO R86183) (Ding et al., 1995a), 9-chloro-TIBO (R 82913) (Ren et al., 1995b), and nevirapine (Kroeger Smith et al., 1995), roughly overlay each other maintaining a very similar 'butterfly-like' conformation.

A large number of structurally diverse ligands have been utilized in the derivation of the pharmacophore model. In the present study, eight well-known NNRTI's namely, Nevirapine, Delavirdine, Efavirenz, Trovirdine, Loviride, Indole carboxamide, Benzothiadiazine-1-oxide and Thiocarboxanilide, were selected to develop the pharmacophore model (Fig 4.1).

All the ligands were geometrically optimized based on the internal strain energy calculated by molecular mechanics calculations (MM3 parameterization) in Alchemy Tripos software to ensure uniform sampling of low energy conformers, based on the presumption that low energy conformations are used to generate the pharmacophore. Then the essential structural components like atoms, centroids of collection of atoms, electron lone pair positions, steric and electrostatic potentials etc were matched in the three-dimensional space of the energetically accessible conformations of the ligands, to arrive at the 3-point pharmacophore model proposed below (Fig 4.2).

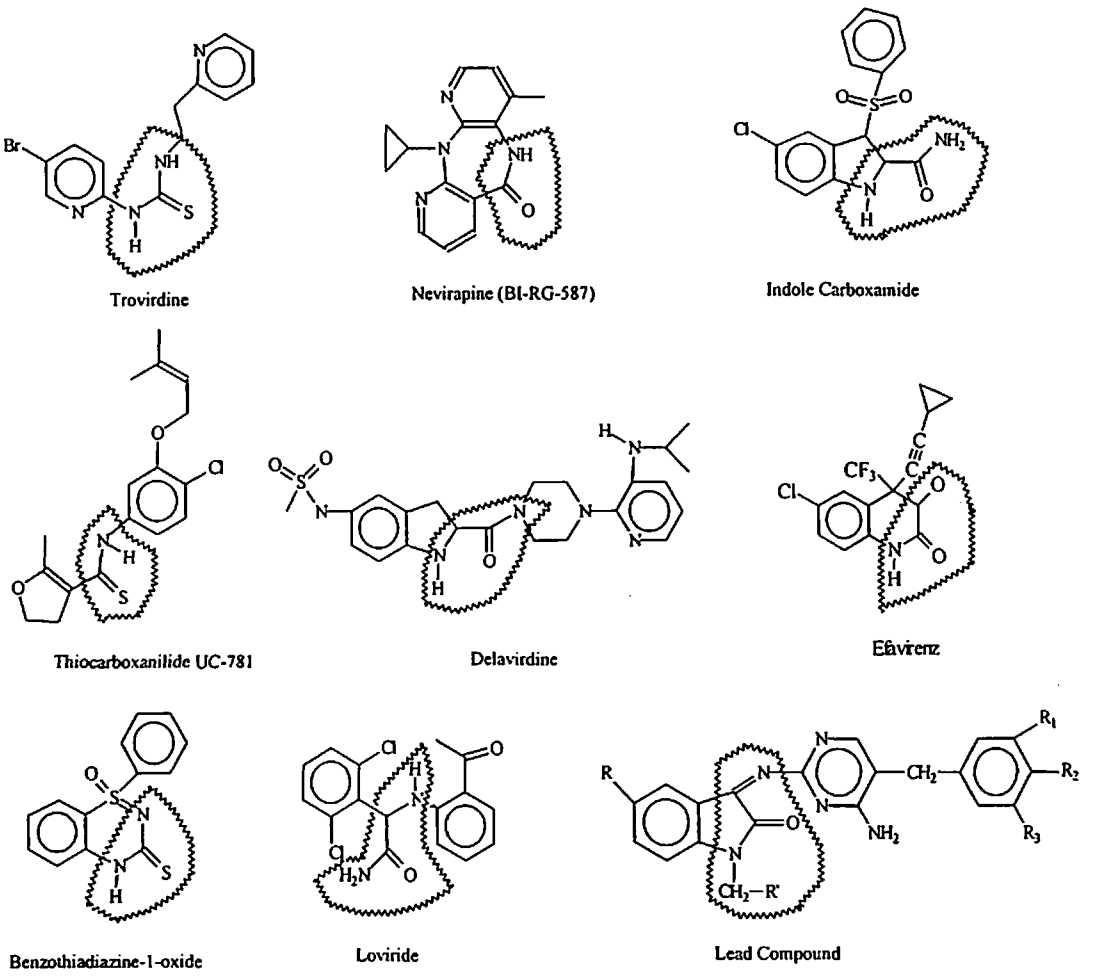


Fig.: 4.1 Schematic representation of butterfly like conformation of the existing NNRTI's and the lead compound

The crucial structural components included in the proposed model contain a hydrophilic centre (A), which is surrounded by 2 hydrophobic outskirts denoted by B and C. The distance between the 3-pharmacophoric points were calculated for minimum four different conformations and are represented as mean standard deviation (Table 4.1).

The proposed aminopyrimidinimino isatin analogue was designed based on the derived pharmacophoric model with the iminocarbamoyl moiety (-N=C-CO-N-) constituting the '*body*' and the aryl rings of pyrimidine and isatin derivative constituting the '*wings*'. This lead compound was found to fulfill the specification of the pharmacophoric distance map by complying within the defined range.

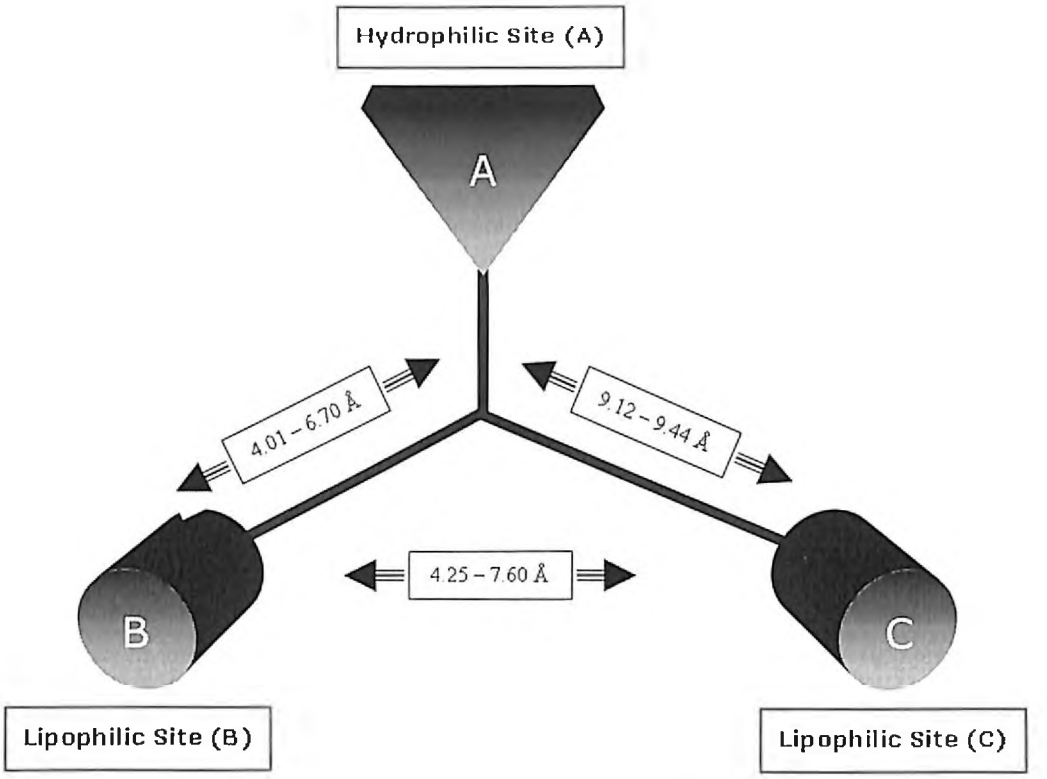


Fig 4.2: Schematic representation of a butterfly-like configuration of NNRTI'S and the pharmacophoric distance map

During the development of a 3D-pharmacophoric model, molecular superposition technique named least-squares superimposition have been used to investigate similarities and differences between the accessible conformations of different molecules with respect to the spatial positions of their pharmacophoric elements. The root mean square deviation (RMS) between the selected points in the test molecule (aminopyrimidinimino isatin analogue) and the corresponding points in the reference molecule (nevirapine, efavirenz, and delavirdine) has been calculated and are presented in Fig 4.3 - 4.5. The rms value is zero (Å) for a perfect fit and increases as the fit is decreased. It was deduced from the RMS fit value that the lead compound structure fits appreciably with delavirdine showing RMS value of 0.075.

Table 4.1.The pharmacophoric distance model of bioactive NNRTI's and lead compound by molecular mechanics (MM3) force fields

COMPOUND	AB (IN Å)		BC (IN Å)		CA (IN Å)	
	LOWER LIMIT	UPPER LIMIT	LOWER LIMIT	UPPER LIMIT	LOWER LIMIT	UPPER LIMIT
DELAVIRDINE	4.328 ± 0.04	6.705 ± 0.15	4.256 ± 0.19	7.542 ± 0.35	9.156 ± 0.04	9.382 ± 0.04
TROVIRDINE	4.235 ± 0.01	6.635 ± 0.02	4.289 ± 0.08	7.269 ± 0.16	9.168 ± 0.04	9.426 ± 0.04
LOVIRIDE	4.356 ± 0.03	6.709 ± 0.12	4.254 ± 0.06	7.129 ± 0.14	9.132 ± 0.04	9.368 ± 0.04
INDOLE CARBOXAMIDE	4.359 ± 0.01	6.705 ± 0.20	4.562 ± 0.14	7.478 ± 0.07	9.125 ± 0.04	9.434 ± 0.04
EFAVIRENZ	4.425 ± 0.05	6.689 ± 0.16	4.257 ± 0.23	7.211 ± 0.21	9.145 ± 0.04	9.440 ± 0.04
NEVIRAPINE	4.014 ± 0.02	6.538 ± 0.04	4.268 ± 0.31	7.603 ± 0.02	9.215 ± 0.04	9.421 ± 0.04
BENZOTHIADIAZINE-1-OXIDE	4.512 ± 0.02	6.459 ± 0.03	4.269 ± 0.14	7.545 ± 0.01	9.129 ± 0.04	9.406 ± 0.04
THIOCARBOXANILIDE	4.229 ± 0.04	6.523 ± 0.11	4.263 ± 0.07	7.147 ± 0.39	9.169 ± 0.04	9.398 ± 0.04
LEAD COMPOUND	4.235 ± 0.18	6.459 ± 0.12	4.258 ± 0.09	7.547 ± 0.15	9.159 ± 0.01	9.431 ± 0.02

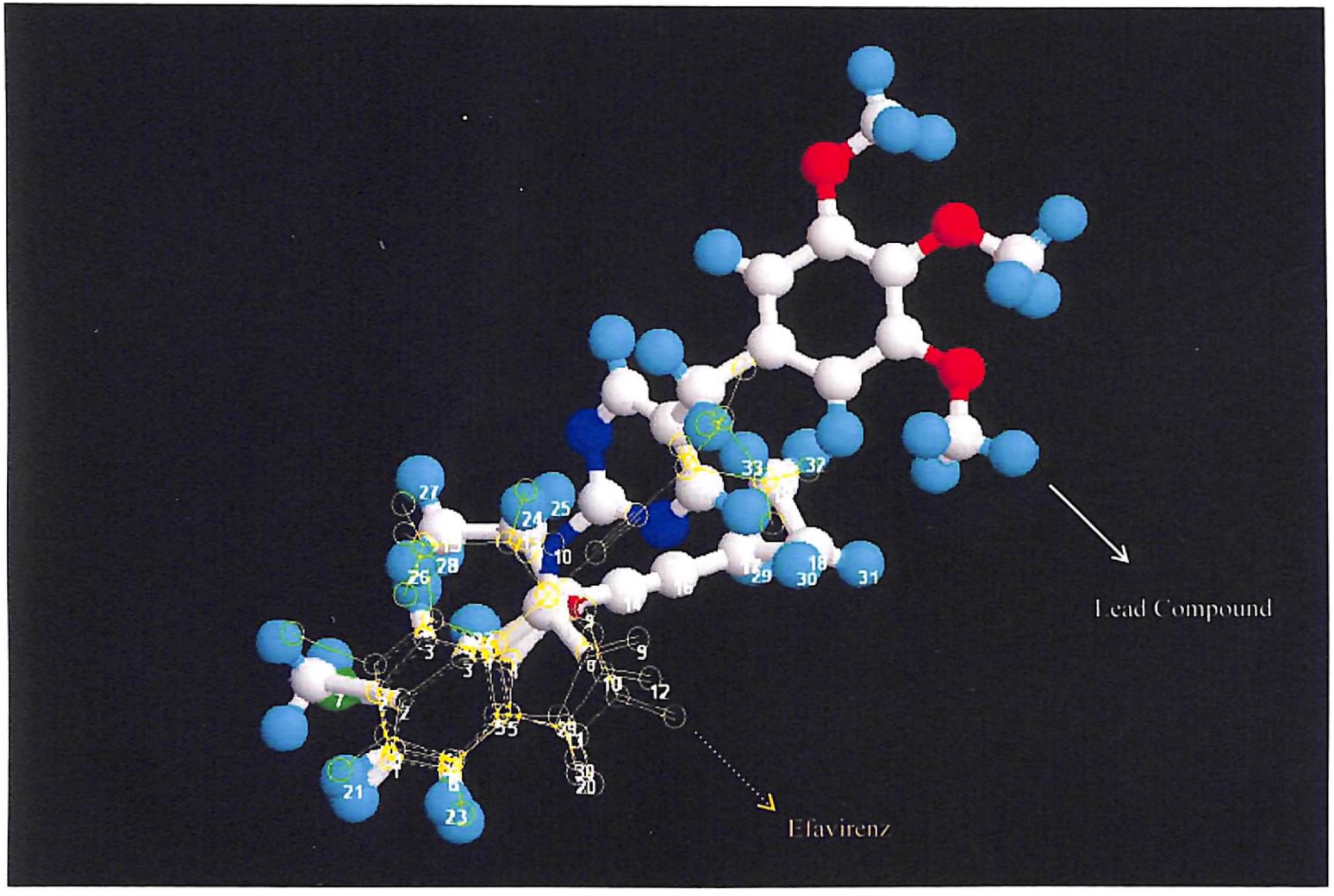


Fig.4.3 Superimposition and RMS fit of the proposed lead compound and Efavirenz (RMS value = 0.282)

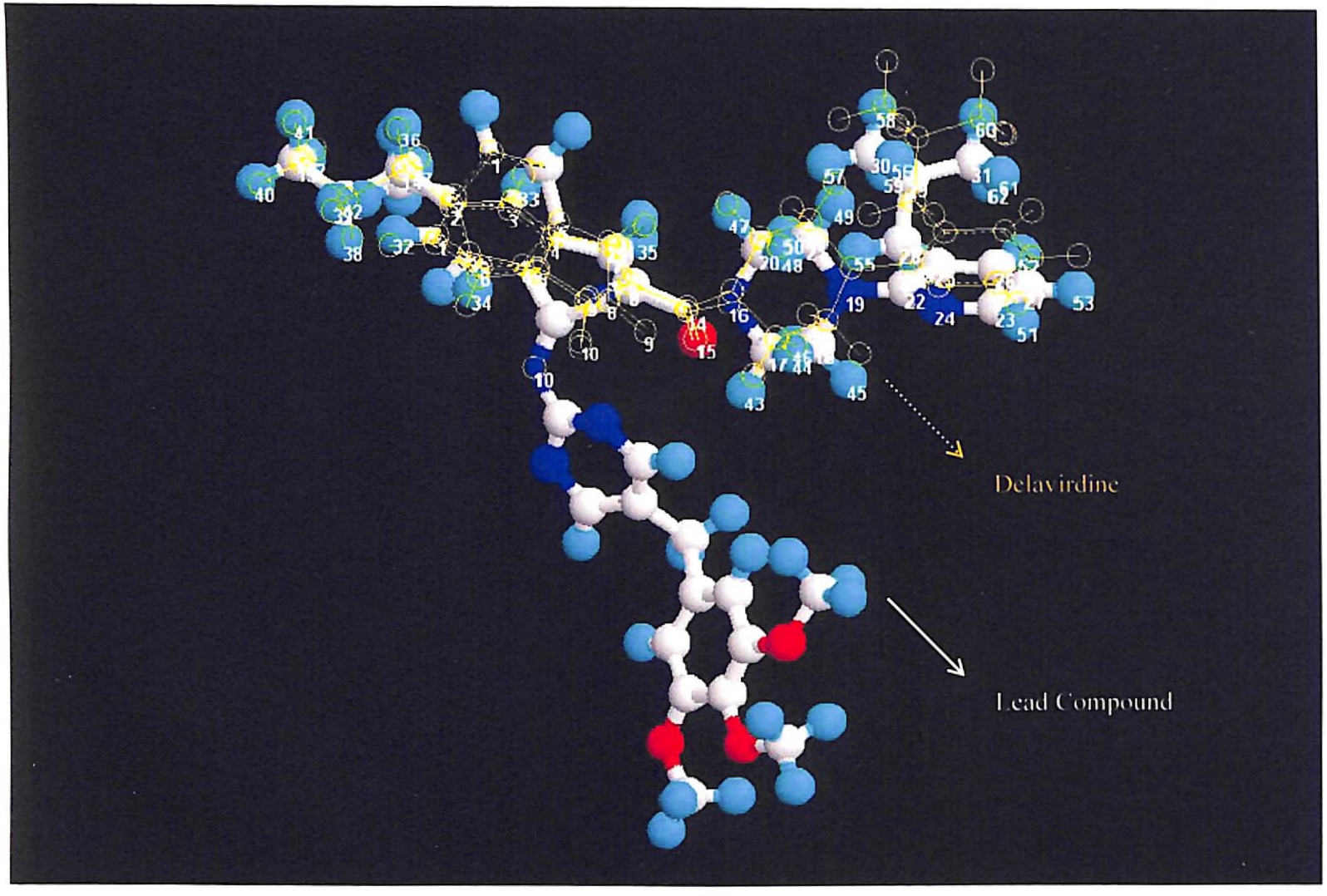


Fig.: 4.4 Superimposition and RMS fit of the proposed lead compound and Delavirdine (RMS value = 0.075)

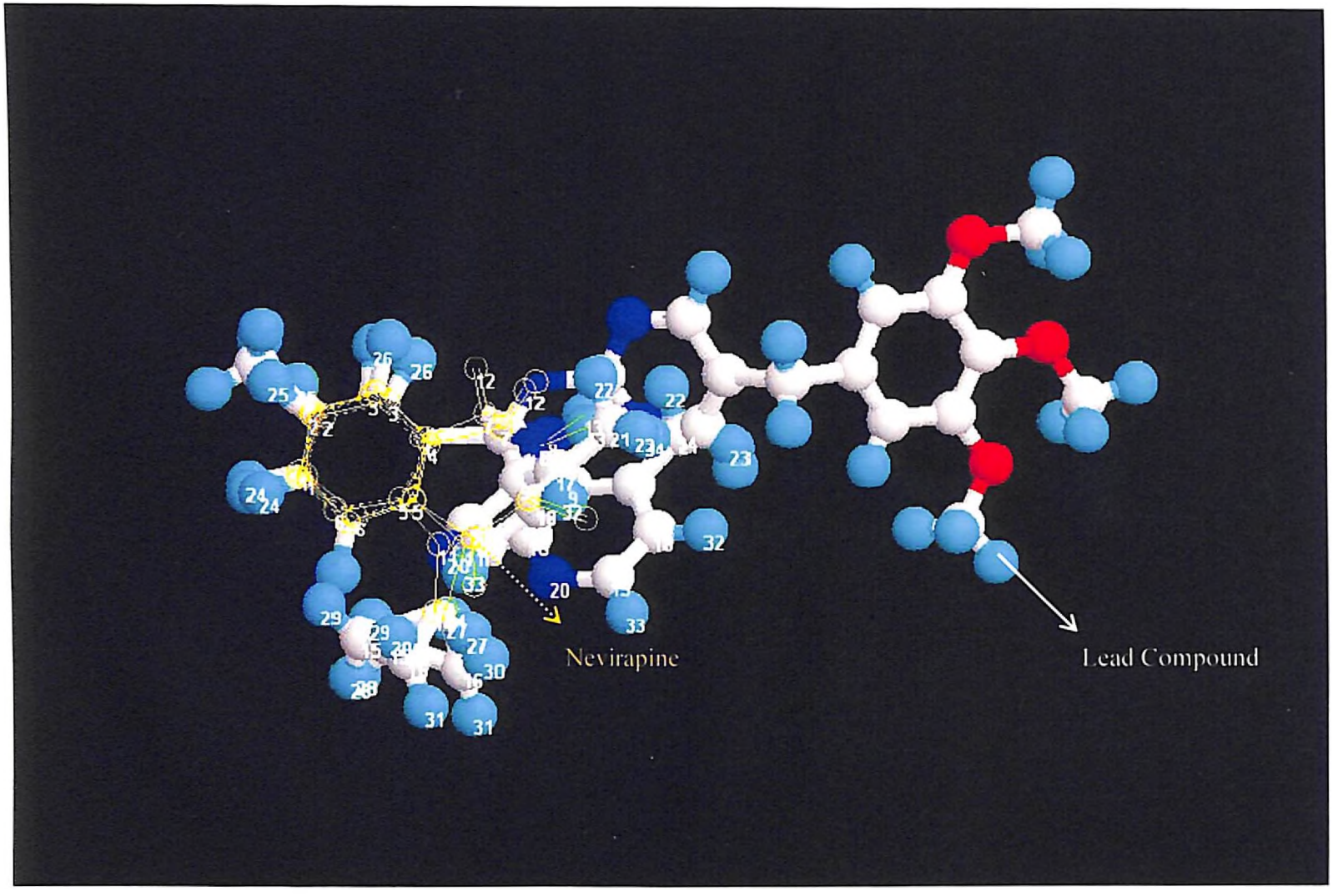


Fig.: 4.5 Superimposition and RMS fit of the proposed lead compound and Nevirapine (RMS value = 0.224)

05 Synthesis and Characterization

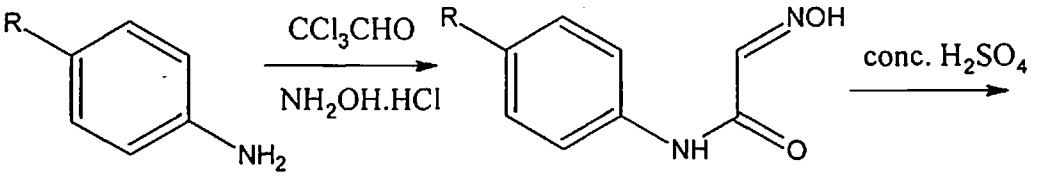
5.1 Materials and Methods:

Melting points were determined in one end open capillary tubes on a Büchi 530 melting point apparatus and are uncorrected. Infra-red spectra (IR) were recorded (in KBr) on Jasco IR Report 100 and are reported in reciprocal centimeters (cm^{-1}). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on Bruker Avance (300 MHz) spectrophotometer in the indicated solvent. Chemical shifts are expressed in δ units (ppm) using tetramethylsilane (TMS) as an internal reference standard, and signals are expressed as a s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or coupling constants (J) in Hz. Mass spectra of the compounds were measured with Shimadzu GC-MS-QP5000 spectrophotometer. Elemental analyses (C, H and N) were undertaken with Perkin-Elmer model 240C analyzer. The progress of reactions was monitered by ascending thin-layer chromatography (TLC) on silica gel (precoated silica gel plate 60 F_{254}, Merck) and visualized by using iodine vapour. Developing solvents were toluene-methanol (8:2). A domestic microwave oven with the following specifications had been used: Make LG; Input 220V~50 Hz, 980 W, 4.7 A; Frequency 2450 MHz. The log P values were determined using Scilog P software.

5.2 Synthesis:

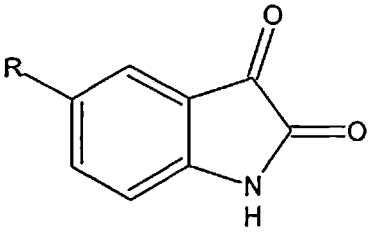
The synthesis of aminopyrimidinimino isatin derivatives is carried out in four steps.The first step involved the synthesis of 5-substituted isatin starting with *p*-substituted aniline along with chloral hydrate and hydroxylamine hydrochloride to yield the intermediate named isonitrosoacetanilide, which on treatment with conc. sulphuric acid gave 5-substituted isatin, as depicted in the synthetic scheme below.

Step I: Synthesis of 5-substituted isatin



p - Substituted aniline

Isonitrosoacetanilide

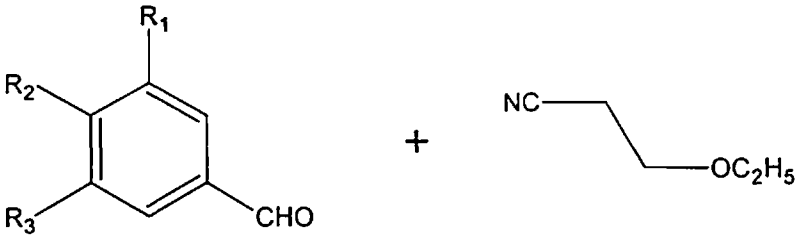


R = H, Cl, Br, CH_3, F

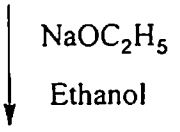
5-Substituted isatin

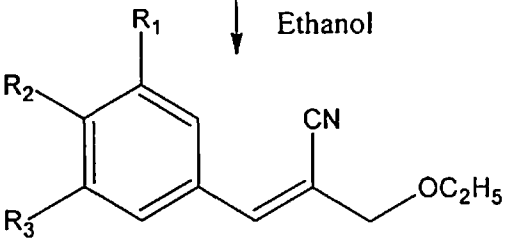
Step II: Synthesis of 2, 4-diamino-5-(substituted benzyl) pyrimidine

In the second step, substituted benzaldehydes were condensed with β-ethoxy propionitrile in the presence of alkoxide ion (as a base), to yield the intermediate product which on cyclization with excess of guanidine hydrochloride resulted in the final product.

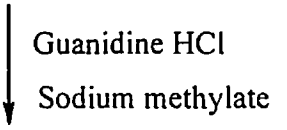


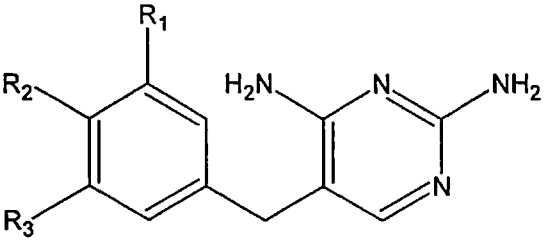
Substituted benzaldehyde ß-Ethoxy propionitrile





2-(substituted benzyl)-3-ethoxy-2-propionitrile





2, 4-diamino-5-(substituted benzyl) pyrimidine

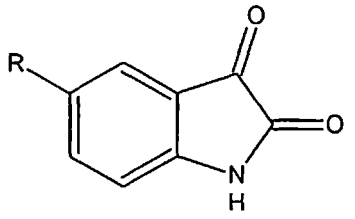
R_1=H, R_2=Cl, R_3=H

R_1=H, R_2=$N(CH_3)_2$, R_3=H

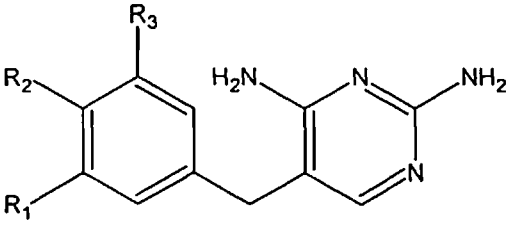
R_1=R_2=R_3=OCH_3

Step III: Synthesis of 3-{[4-amino-5-(substituted benzyl)pyrimidin-2-yl]imino}-5-substituted-1,3-dihydro-2H-indol-2-one (SCHIFF BASE)

5-substituted isatins were treated with 2,4-diamino-5-(substituted) pyrimidine in the presence of an acid catalyst to form the product (Schiff base).



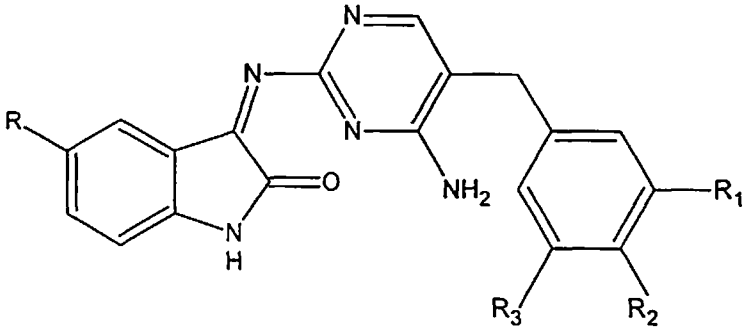
5-Substituted isatin



2, 4-diamino -5 -(substituted benzyl) pyrimidin

Glacial acetic acid/Ethanol

SCHIFF's REACTION



3-{[4-amino-5-(substituted benzyl) pyrimidin2-yl] imino}-

5-substituted-1, 3-dihydro-2H-indol-2-one

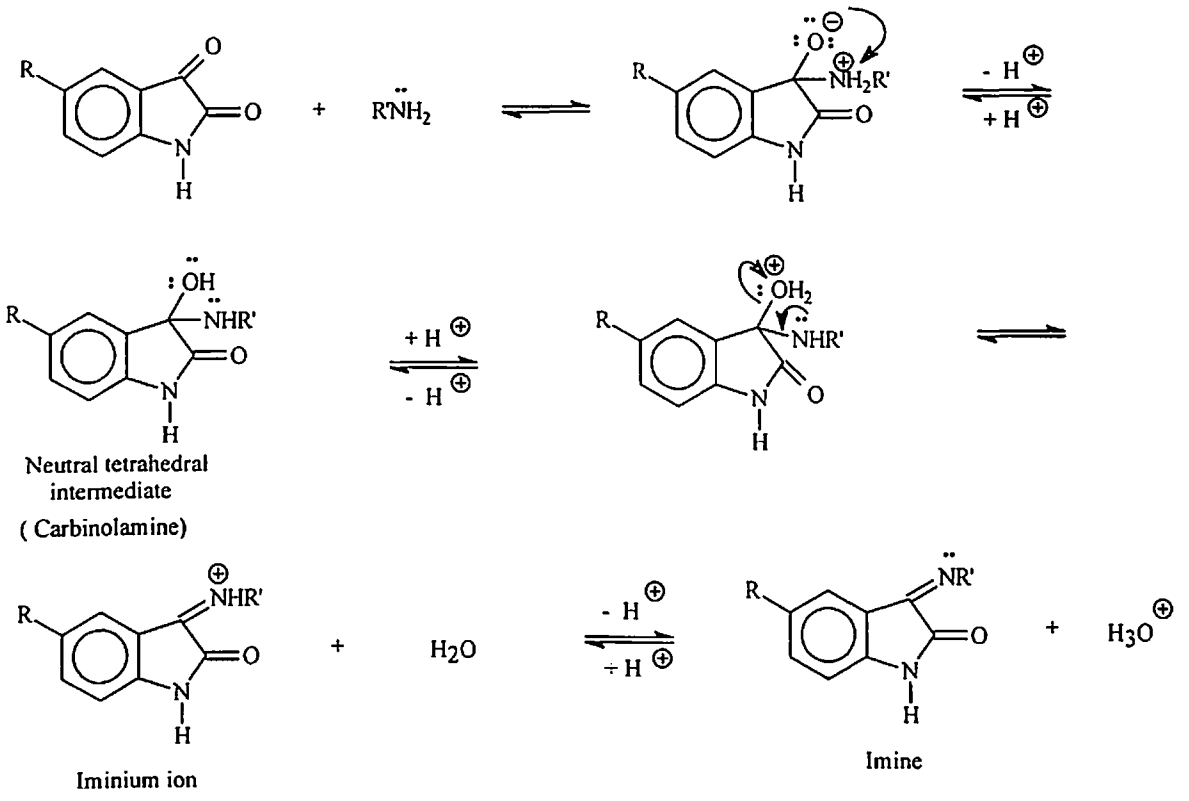
R = H, Cl, Br, CH_3, F

R_1= R_3= H, R_2=Cl

R_1= R_3= H, R_2=$N(CH_3)_2$

R_1=R_2=R_3=OCH_3

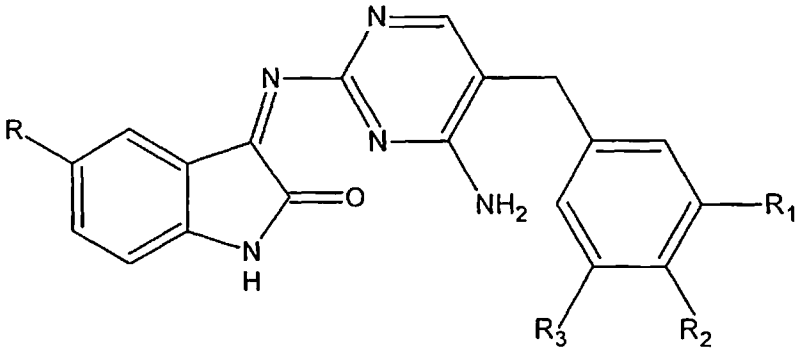
Mechanism of Schiff's Reaction



This is nucleophilic addition-elimination reaction, wherby nucleophilic addition of an amine leads to the formation of unstable tetrahedral compound followed by elimination of water. In the first step of the mechanism of imine formation, the amine, which acts as a nucleophile, attacks the carbonyl carbon. Proton transfer from ammonium ion to alkoxide ion leads to neutral tetrahedral intermediate called carbinolamine. The pH at which imine formation is carried out should be controlled. There must be sufficient acid present to protonate the tetrahedral intermediate, so that the better leaving group i.e water rather than the more basic hydroxyl group is liberated. The lone pair on the amine comes down to push out water, giving rise to a protonated imine. Finally, water accepts the proton from the iminium ion, regenerating the acid catalysts.

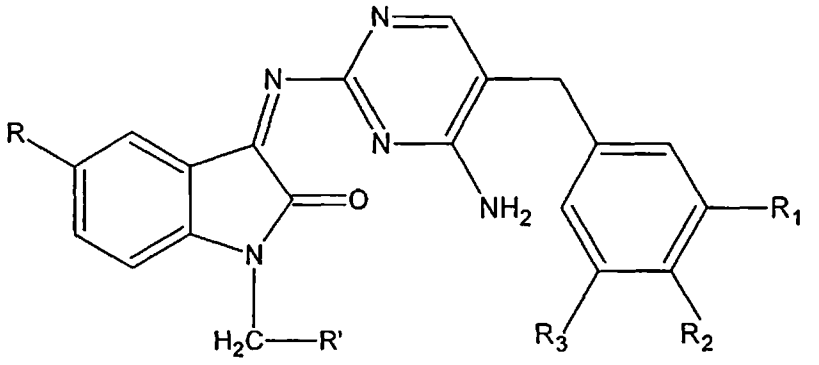
Step IV: Synthesis of 3-{[4-amino-5-(substituted benzyl)pyrimidin-2-yl]imino}-5-substituted-1-[(substitutedamino)methyl]-1,3-dihydro-2H-indol-2-one (MANNICH BASE)

The Schiff base when treated with various secondary amines, in the presence of formaldehyde undergoes Mannich's reaction to give the final product.



3-{[4-amino-5-(substituted benzyl) pyrimidin-2 -yl] imino}-5-substituted- 1, 3 - dihydro-2H-indol-2-one

MANNICH's REACTION ↓ HCHO , Secondary amines



Mannich Base

where, R = H, Cl, Br, CH_3, F

R_1= R_3= H, R_2=Cl

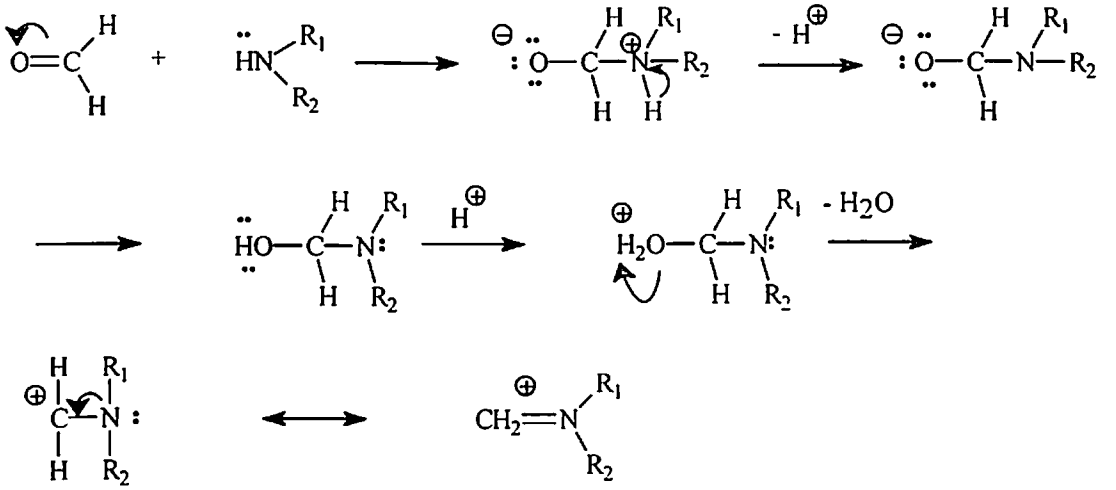
R_1= R_3= H, R_2=$N(CH_3)_2$

R_1=R_2=R_3=OCH_3

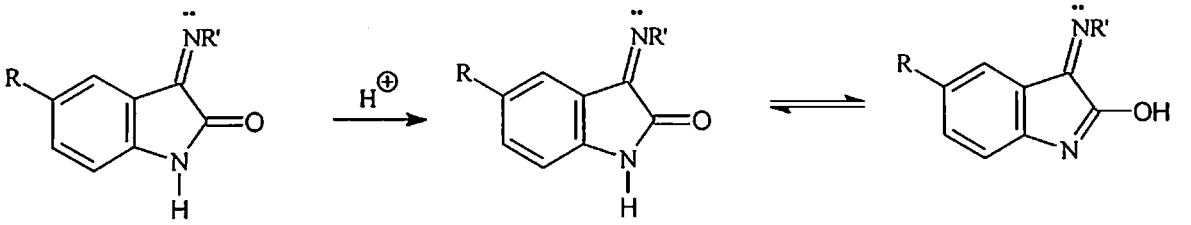
R' = secondary amines

Mechanism of Mannich Reaction

Step I: Iminium ion formation



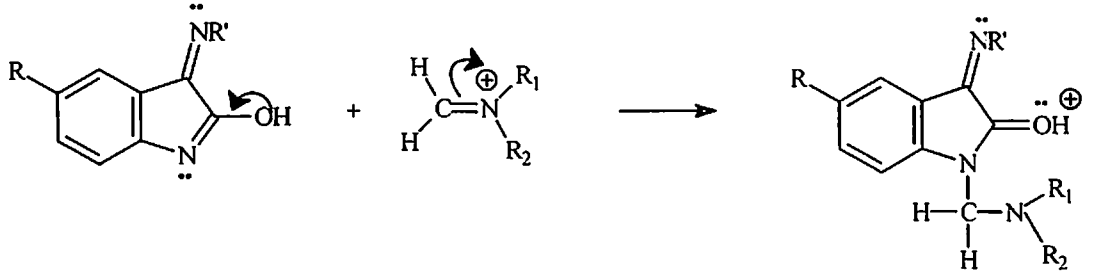
Step II: Tautomerism (Lactam-Lactim)



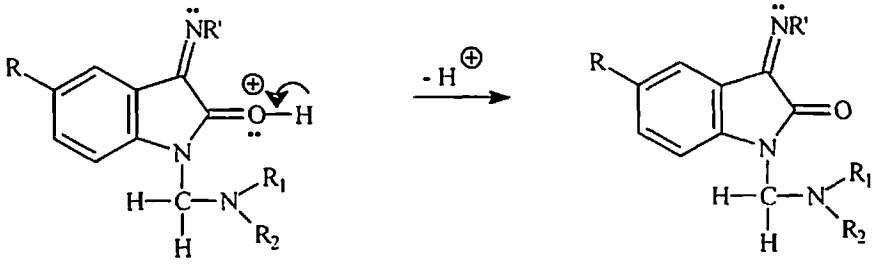
Lactam

Lactim

Step III: Carbon-Nitrogen bond formation



Step IV: Proton transfer



In the frist step, the lone pair of electrons present on the nitrogen atom of the secondary amine attacks the carbonyl carbon of formaldehyde. This leads to the formation of protonated ion i.e hydroxymethyldialkyl amine which on elimination of water molecule forms a reactive species known as iminium ion. In the second step, the lactam undergoes tautomerism to form lactim which acts as a nucleophile. The lactim form of Schiff base then attacks the electrophilic carbon of iminium ion to form carbon-nitrogen bond in the third step. In the last step, loss of proton from the oxonium ion intermediate occurs, leading to the formation of final product.

5.3 Procedure

Step I: Synthesis of Isatin

a) Synthesis of isonitrosoacetanilide from aniline (Marvel and Hiers 1943)

In a round bottom flask, 18 g (0.108 moles) of chloral hydrate in 240 ml of water was placed. To this solution, are then added, in order, 26 g of sodium sulphate, a solution of 9.3 g (0.1 mol) of aniline in 60 ml of water containing 10.24 gm (8.6 ml, 0.104 mol) of concentrated hydrochloric acid (sp. gr. 1.19) to dissolve the amine, and finally, a solution of 22 g (0.316 moles) of hydroxylamine hydrochloride in 100 ml of water. The flask was then heated, so that vigorous boiling begins in about forty to forty-five minutes. After one to two minutes of vigorous boiling, the reaction was completed. During the heating period, some crystals of isonitrosoacetanilide separated. On cooling the solution in running water, the remainder crystallized. It was filtered with suction and air-dried. The yield was 13.40 g (86% of the theoretical yield) and melting point of the product was found to be 176°C.

b) Synthesis of isatin from isonitrosoacetanilide

120 g (65 ml) of concentrated sulphuric acid (sp.gr. 1.84) was warmed to 50°C and to this, 15 g (0.0914 moles) of dry isonitrosoacetanilide was added at such a rate so as to keep the temperature between 60°C and 70°C but not higher. External cooling was applied at this stage, so that the reaction could be carried out more rapidly. After the addition of the isonitrosoacetanilide was finished, the solution was heated to 80°C and was kept at this temperature for about 10 minutes to complete the reaction. Then the reaction mixture was cooled to room temperature and poured upon ten to twelve times its volume of cracked ice. After standing for about 90 minutes, the isatin was filtered with suction, washed several times with cold water to remove sulphuric acid, and then dried in the air. The yield of crude isatin was 10 g (73% of the theoretical yield) and the melting point was found to be 192°C.

Purification of isatin

10 g of crude isatin was suspended in 50 ml of hot water and treated with a solution of 4.4 gm of sodium hydroxide in 10 ml of water. The solution was stirred and isatin passed into the solution. Dilute hydrochloric acid was then added, with stirring until a

slight precipitate appears. This required about 14.5 ml to 15.0 ml of an acid made by diluting one volume of conc. hydrochloric acid (sp. gr. 1.19) with two volumes of water. The mixture was filtered at once, the ppt was rejected and the filtrate was made acidic to congo red paper with HCl. The solution is then cooled rapidly and the isatin, which separated, was filtered with suction and dried in the air. Isatin was crystallized from glacial acetic acid. Yield: 62%, m.p: 197°C.

Synthesis of 5-substituted isatins

5-chloro, 5-bromo, 5-methyl and 5-fluoro isatin were prepared in the same manner as given above using appropriate moles of 4-chloroaniline, 4-bromoaniline, 4-methylaniline, and 4-fluoroaniline respectively.

Step II: Synthesis of 2, 4-diamino-5-(substituted benzyl) pyrimidine

a) Synthesis of 2-(4-chlorobenzyl)-3-ethoxy-2-propionitrile (Stenbuck et al., 1963)

To a solution of 2.04 g (0.03 moles) of sodium ethoxide in 14.0 ml of absolute ethanol were added 2.81 g (0.02 moles) of p-chloro benzaldehyde and 2 g (2.195 ml, 0.02 moles) of β-ethoxypropionitrile. The reaction mixture was heated under a fractionating column for around 4 hrs. The solution was then concentrated in vacuo to thick syrup and partitioned between ether and cold water. Much of the colour went into the aqueous layer. The ethereal layer was washed with water until the washings were neutral and then dried over sodium sulphate. The ethereal solution was filtered, the solvent was evaporated and the residue was distilled at 0.45 mm. From a portion of this distillate, a solid portion was obtained which was recrystallized from ethanol. The yield obtained was 1.8 g (68% of the theoretical yield) and melting point was 58°C.

b) Synthesis of 2, 4-diamino-5-(4-chlorobenzyl) pyrimidine

A guanidine solution was prepared from 3 g (0.03 moles) of guanidine hydrochloride and sodium methylate (0.72 g of sodium). The sodium chloride was filtered off and the solution was concentrated in vacuo. To it was added 1.023 g (0.0055 moles) of 2-(4-chlorobenzyl)-3-ethoxy-2-propionitrile. The solution was stirred and refluxed for 20 hrs. Stirring was continued, while the solution was allowed to cool and 50 ml of water was added. A solid substance separated out that was filtered off, washed with ether and dried.

The yield obtained was 0.716 g (66.0 % of the theoretical yield) and melting point was 165-166°C.

Step III: Synthesis of Schiff base (Pandeya et al., 1999c)

Equimolar quantities (0.01 mol) of 5-substituted isatin and 5-(substituted benzyl)-2,4-diamino pyrimidine were dissolved in warm ethanol, containing 1 ml of glacial acetic acid as acid catalyst. The reaction mixture was irradiated in an unmodified domestic microwave oven at 80% intensity with 30 second/cycle for 4 minutes, set aside and kept in cold condition for crystallization. The resultant crystals were washed with dilute ethanol, dried and recrystallized from ethanol-chloroform mixture.

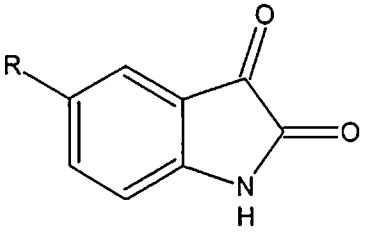
Step IV: Synthesis of Mannich base (Pandeya et al., 1999c)

To a solution of 37% formaldehyde (0.5 ml) and appropriate secondary amines (0.02 moles) in ethanol, was added 3-{[4-amino-5- (substituted benzyl) pyrimidin-2-yl] imino}-5-substituted-1, 3-dihydro-2H-indol-2-one (SCHIFF BASE) (0.02 mol) and the above mixture was irradiated in a microwave oven at an intensity of 60% with 30 sec/cycle turn. The number of cycles in turn depended on the completion of the reaction, which was checked regularly by TLC. The reaction timing varied from 2.5-5 min. The clear solution obtained after the completion of the reaction was kept for crystallization at 0°C for 30 min and the resulting precipitate was recrystallized from chloroform.

5.4 Results and Discussion

Step I:

Table 5.1: Physical data of 5-substituted isatin



Compound	R	Yield (%)	m.p. (°c)	Molecular Formula	Molecular weight
I-1	H	62	197	$C_8H_5NO_2$	147.13
I-2	Cl	61	246	$C_8H_4ClNO_2$	181.58
I-3	Br	60	252	$C_8H_4BrNO_2$	226.03
I-4	CH_3	67	180	$C_9H_7NO_2$	161.16
I-5	F	62	225	$C_8H_4FNO_2$	165.12

Table 5.2: Spectral data of the synthesized 5-substituted isatin

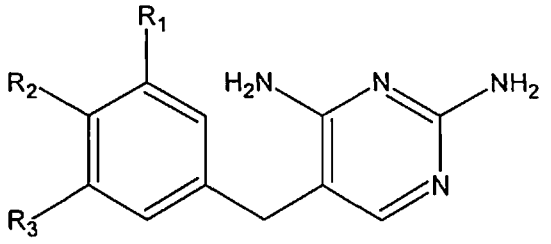
Compound	IR spectroscopy (cm^{-1}; KBr)	^1H- NMR (δ ppm, DMSO-d_6)
I-1	1720 (α-diketone), 3200 (amide N-H), 1610 (ring C=C stretch)	6.832-7.36 (m, 5H, Ar-H), 10.6 (s, 1H, N-H D_2O exchangeable)
I-4	1720 (α-diketone), 3200 (amide N-H), 1600-1590 (ring C=C stretch)	2.34 (s, 3H, CH3), 6.912-7.30 (m, 5H, Ar-H), 10.64 (s, 1H, N-H D_2O exchangeable)

Of the many methods developed for the synthesis of isatin and its derivatives, the two-step synthetic procedure developed by Sandmeyer was employed. The first step involved the synthesis of isonitrosoacetanilide intermediate from aniline, chloral hydrate and hydroxylamine hydrochloride. In the second step, the isonitrosoacetanilide was converted into isatin via cyclization in the presence of conc. sulphuric acid.

The % yield of isatin and its 5-substituted derivatives were found in the range of 60-67% after purification step. The structures of the compound (**I-1** to **I-5**) were elucidated by spectral analysis (IR and ^{1}H-NMR). In the IR spectra, all the compounds showed bands in the region of 1720 cm^{-1} (characteristic of α-diketone) and 3200 cm^{-1} (amide N-H stretching). The structure was further confirmed by using ^{1}H-NMR spectra. In the NMR spectra, the compounds showed a sharp singlet at δ 10.6 ppm (s, 1H, NH of isatin, D_2O exchangeable).

Step II:

Table 5.3: Physical data of 2, 4-diamino-5-(substituted benzyl) pyrimidine



Comp. No.	R_1	R_2	R_3	Yield (%)	m.p. (°C)	Molecular formula	Molecular Weight
P1	$-OCH_3$	$-OCH_3$	$-OCH_3$	64.5	199	$C_{14}H_{18}N_4O_3$	290.318
P2	-H	-Cl	-H	59	166	$C_{11}H_{11}ClN_4$	234.685
P3	-H	$-N(CH_3)_2$	-H	55	176	$C_{13}H_{17}N_5$	243.308

Table 5.4: Spectral data of the synthesized 2, 4-diamino-5-(substituted benzyl) Pyrimidine

Compd No.	IR spectroscopy (cm^{-1}; KBr)	^1H- NMR (δ ppm, DMSO-d_6)
P1	3516 (Asymmetric NH_2 Stretch), 3414 (symmetric NH_2 Stretch), 3010 (Aromatic C-H stretch), 2966, 2940, 2840 (Aliphatic C-H stretch), 1616, 1593 (NH_2 deform. overlap with aromatic ring), 1506 (Aromatic ring), 1452 (CH deformation), 1236, 1129 (Aromatic –OCH_3)	3.54 (s, 2H, -CH_2), 3.63 (s, 3H, -OCH_3) 3.94 (s, 6H, -OCH_3), 5.80 (s, 2H, NH_2), 6.16 (s, 2H, -NH_2), 6.58-7.36 (m, 3H, Ar-H)
P3	3506 (Asymmetric NH_2 Stretch, 3405 (symmetric NH_2 Stretch), 1620 (N-H bend), 1596 (N-H bend)	1.82 (s, 6H, -N(CH_3)$_2$), 3.50 (s, 2H, -CH_2), 5.80 (s, 2H, NH_2) 6.12 (s, 2H, -NH_2)

The 2,4-diamino-5-(substituted benzyl)pyrimidine was synthesized via two step procedure. Variously substitued benzaldehyde undergo base-catalyzed condensation with β–ethoxypropinitrile to form β-ethoxy-α-(substituted benzylidene) propionitrile, which on reaction with considerable excess of guanidine (2-3 equivalents) undergo cyclization to form the required pyrimidine moiety.

The % yield of 2,4-diamino-5-(substituted benzyl) pyrimidine derivatives were found in the range of 55-65% after purification step. The structures of the compound (**P-1** to **P-3**) were elucidated by spectral analysis (IR and ^1H-NMR). In the IR spectra, the compounds showed bands in the region 3516 cm^{-1} (Asymmetric NH_2 stretching), 1616, 1593 (NH_2 deformation overlap with aromatic ring). In the ^1H-NMR spectra, a singlet was obtained at δ 3.54 ppm (s, 2H, -CH_2), δ 5.80 ppm (s, 2H, 4-NH_2), and δ 6.16 ppm (s, 2H, 2-NH_2),

Step III:

Table 5.5: Physical data of synthesized Schiff base

#	R	R_1	R_2	R_3	Yield (%)	m.p. (°C)	Molecular formula (Mol.Wt.)
S1	-Cl	$-OCH_3$	$-OCH_3$	$-OCH_3$	71	188	$C_{22}H_{20}ClN_5O_4$ (453.878)
S2	-Br	$-OCH_3$	$-OCH_3$	$-OCH_3$	63	210	$C_{22}H_{20}BrN_5O_4$ (498.329)
S3	$-CH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	60	179	$C_{23}H_{23}N_5O_4$ (433.460)
S4	-F	$-OCH_3$	$-OCH_3$	$-OCH_3$	67	182	$C_{22}H_{20}FN_5O_4$ (437.424)
S5	-Cl	-H	-Cl	-H	76	195	$C_{19}H_{13}Cl_2N_5O$ (398.245)
S6	$-CH_3$	-H	-Cl	-H	69	166	$C_{20}H_{16}ClN_5O$ (377.827)
S7	-F	-H	-Cl	-H	63	174	$C_{19}H_{13}ClFN_5O$ (381.791)
S8	-H	-H	$-N(CH_3)_2$	-H	65	162	$C_{21}H_{20}N_6O$ (372.423)
S9	$-CH_3$	-H	$-N(CH_3)_2$	-H	61	168	$C_{22}H_{22}N_6O$ (386.450)
S10	-F	-H	$-N(CH_3)_2$	-H	64	185	$C_{21}H_{19}FN_6O$ (390.414)

Table 5.6: Spectral data of the synthesized Schiff base

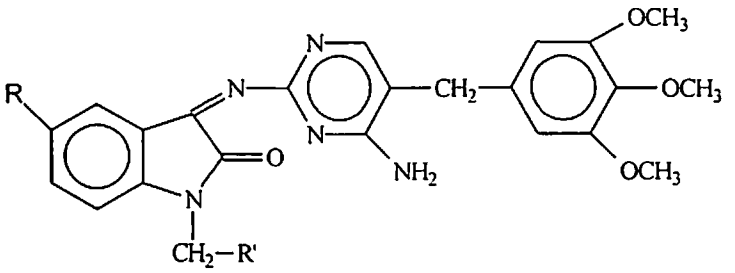
Compound	IR spectroscopy (cm^{-1}; KBr)	^{1}H- NMR (δ ppm, DMSO-d_6)
S1	3200 (N-H Stretch), 1740 (amide Stretch), 1625 (C=N stretch), 3400 (N-H stretch), 3010 (Aromatic C-H stretch), 1660, 1616, 1586 (Aromatic C-C ring stretch)	3.18 (s, 2H, $-CH_2$), 3.7 (s, 9H, $-OCH_3$) 5.80 (s, 2H, NH_2) 6.7-7.2 (m, 6H, Ar-H), 10.7 (s, 1H, -NH)
S5	3300 (N-H Stretch), 1740 (amide Stretch), 1660 (C=N stretch), 3400 (N-H stretch), 3010 (Aromatic C-H stretch), 1660, 1620, 1580 (Aromatic C-C ring stretch)	3.18 (s, 2H, $-CH_2$), 3.7 (s, 9H, $-OCH_3$) 5.6 (s, 2H, NH_2) 6.7-7.2 (m, 6H, Ar-H), 10.7 (s, 1H, -NH)

5-substituted isatins were condensed with 2,4-diamino-5-(4'chlorobenzyl)-pyrimidine in the presence of glacial acetic acid to form Schiff bases. The % yield of the synthesized Schiff base was found in the range of 60-76% after recrystallization with ethanol-chloroform mixture. The structures of the compound (**S-1** to **S-10**) were elucidated by spectral analysis (IR and ^{1}H-NMR). In the IR spectra, the compounds showed peak in the region 1640-1625 cm^{-1} which was due to the presence of azomethine (C=N). There was a peak at 3200 cm^{-1}, which was due to the amidic N-H group. In the Schiff base, there was no peak in the region 1725 cm^{-1}, which was characteristic for α-diketones, and the presence of azomethine group indicated that the primary amino group of pyrimidine reacted with the keto group at C-3 position of isatin.

In the ^{1}H-NMR spectra, the 2, 4-diamino-5-(substituted benzyl) pyrimidine showed a singlet at δ 6.16 due to 2-NH_2 proton, which was absent in the Schiff base.

Step IV:

Table 5.7: Physical data of synthesized Mannich bases (M1-M5)



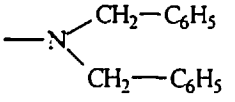
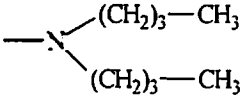
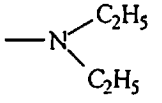
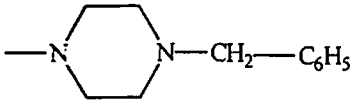
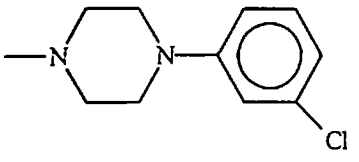
#	R	R'	Molecular Formula (Mol. Wt)	Yield (%)	m.p (°c)	Log P
M1	-Cl		$C_{37}H_{35}N_6O_4Cl$ (663.16)	72.0	122	9.28
M2	-Cl		$C_{31}H_{39}N_6O_4Cl$ (595.13)	68.0	108	7.98
M3	-Cl		$C_{27}H_{31}N_6O_4Cl$ (539.02)	66.0	100	4.38
M4	-Cl		$C_{34}H_{36}N_7O_4Cl$ (642.14)	73.5	109	8.45
M5	-Cl		$C_{33}H_{33}N_7O_4Cl_2$ (662.56)	71.5	81	8.55

Table 5.7: Physical data of synthesized Mannich bases (M6-M12)

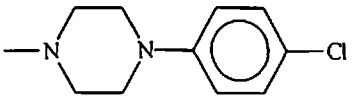
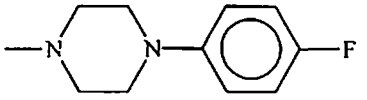
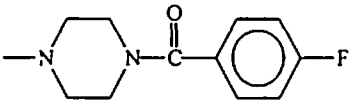
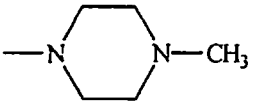
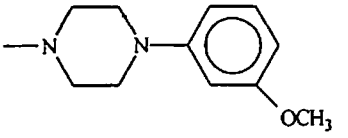
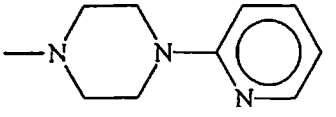
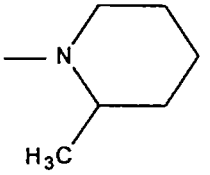
#	R	R'	Molecular Formula (Mol. Wt)	Yield (%)	m.p (°c)	Log P
M6	-Cl		$C_{33}H_{33}N_7O_4Cl_2$ (662.56)	71.0	77	8.55
M7	-Cl		$C_{33}H_{33}N_7O_4ClF$ (646.11)	69.0	86	8.23
M8	-Cl		$C_{34}H_{33}N_7O_5ClF$ (674.12)	66.0	72	8.03
M9	-Cl		$C_{28}H_{32}N_7O_4Cl$ (566.05)	66.50	94	3.45
M10	-Cl		$C_{34}H_{36}N_7O_5Cl$ (658.14)	67.0	92	8.25
M11	-Cl		$C_{32}H_{33}N_8O_4Cl$ (629.10)	74.0	84	8.02
M12	-Cl		$C_{29}H_{33}N_6O_4Cl$ 565.06	71.50	102	4.70

Table 5.7: Physical data of synthesized Mannich bases (M13-M19)

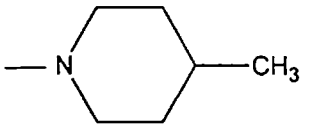
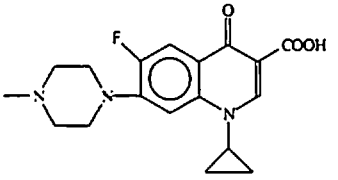
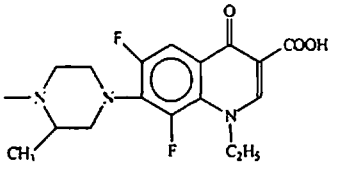
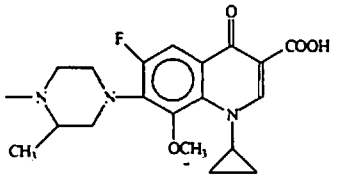
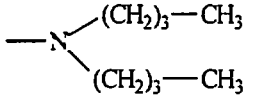
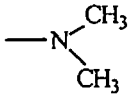
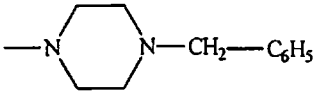
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log P
M13	-Cl		$C_{29}H_{33}N_6O_4Cl$ (565.06)	71.0	98	4.97
M14	-Cl		$C_{40}H_{38}N_8O_7FCl$ (797.23)	67.00	247	5.06
M15	-Cl		$C_{40}H_{39}N_8O_7F_2Cl$ (817.23)	64.0	250	5.66
M16	-Cl		$C_{42}H_{42}N_8O_8FCl$ (841.28)	68.0	140	5.72
M17	-Br		$C_{31}H_{39}N_6O_4Br$ (639.58)	69.0	104	7.89
M18	-Br		$C_{27}H_{31}N_6O_4Br$ (583.49)	70.0	68	4.82
M19	-Br		$C_{34}H_{36}N_7O_4Br$ (686.60)	75.0	87	7.75

Table 5.7: Physical data of synthesized Mannich bases (M20-M26)

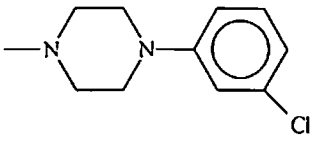
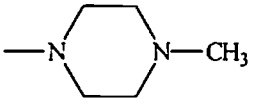
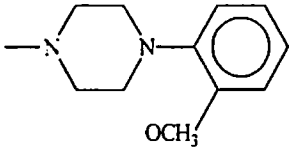
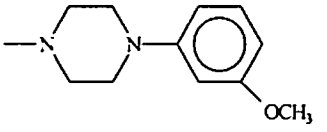
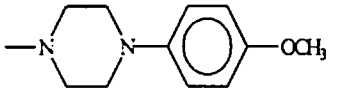
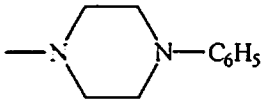
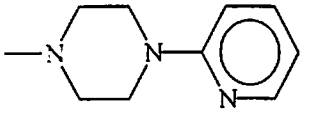
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log P
M20	-Br		$C_{33}H_{33}N_7O_4ClBr$ (707.01)	63.0	84	7.65
M21	-Br		$C_{28}H_{32}N_7O_4Br$ (610.50)	65.0	85	4.06
M22	-Br		$C_{34}H_{36}N_7O_5Br$ (702.59)	69.50	117	7.48
M23	-Br		$C_{34}H_{36}N_7O_5Br$ (702.59)	72.0	82	7.49
M24	-Br		$C_{34}H_{36}N_7O_5Br$ (702.59)	71.0	83	7.51
M25	-Br		$C_{33}H_{34}N_7O_4Br$ (672.57)	71.0	96	7.42
M26	-Br		$C_{32}H_{33}N_8O_4Br$ (673.56)	72.0	76	7.12

Table 5.7: Physical data of synthesized Mannich bases (M27-M33)

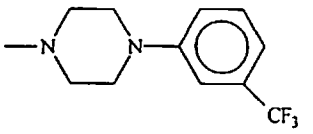
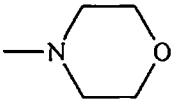
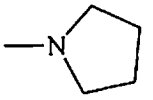
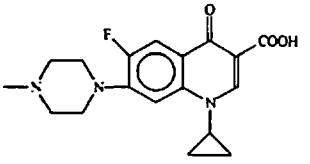
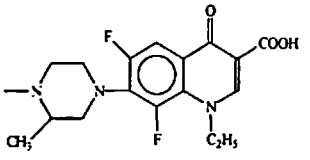
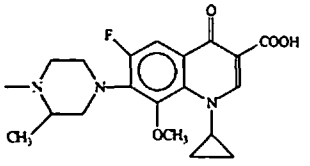
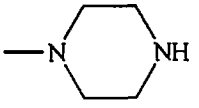
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log P
M27	-Br		$C_{34}H_{33}N_7O_4F_3Br$ (740.57)	66.0	146	8.62
M28	-Br		$C_{27}H_{29}N_6O_5Br$ (597.46)	65.50	88	3.81
M29	-Br		$C_{27}H_{29}N_6O_4Br$ (581.46)	65.0	94	4.49
M30	-Br		$C_{40}H_{38}N_8O_7FBr$ (841.68)	76.0	222 -	5.30
M31	-Br		$C_{40}H_{39}N_8O_7F_2Br$ (861.68)	76.50	257	5.88
M32	-Br		$C_{42}H_{42}N_8O_8FBr$ (885.73)	76.0	137	5.77
M33	$-CH_3$		$C_{28}H_{33}N_7O_4$ (531.60)	65.50	162	6.12

Table 5.7: Physical data of synthesized Mannich bases (M34-M40)

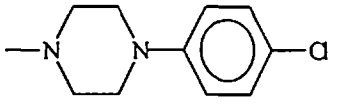
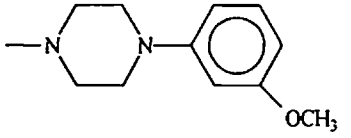
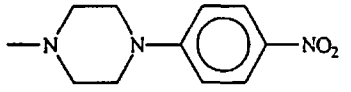
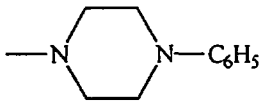
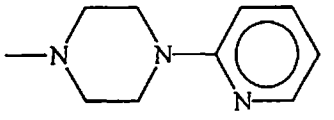
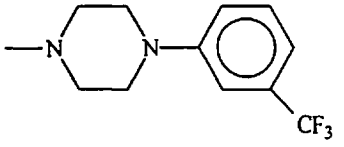
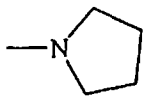
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log p
M34	$-CH_3$		$C_{34}H_{36}N_7O_4Cl$ (642.14)	60.0	79	8.04
M35	$-CH_3$		$C_{35}H_{39}N_7O_5$ (637.72)	58.0	132	7.99
M36	$-CH_3$		$C_{34}H_{36}N_8O_6$ (652.7)	67.0	80	7.01
M37	$-CH_3$		$C_{34}H_{37}N_7O_4$ (607.70)	64.50	97	7.84
M38	$-CH_3$		$C_{33}H_{36}N_8O_4$ (608.69)	68.00	87	7.54
M39	$-CH_3$		$C_{35}H_{36}N_7O_4F_3$ (675.70)	65.0	64	9.29
M40	$-CH_3$		$C_{28}H_{32}N_6O_4$ (516.59)	60.50	117	4.01

Table 5.7: Physical data of synthesized Mannich bases (M41-M47)

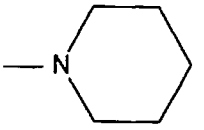
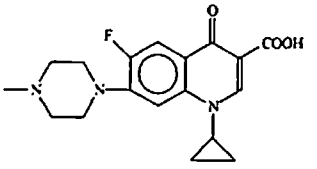
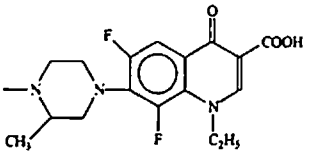
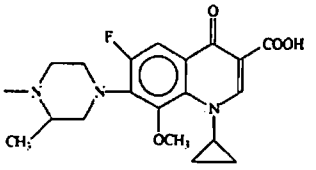
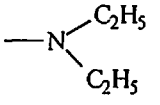
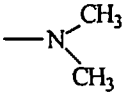
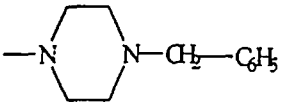
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log p
M41	$-CH_3$		$C_{29}H_{34}N_6O_4$ (530.61)	61.50	60	4.52
M42	$-CH_3$		$C_{41}H_{41}N_8O_7F$ (776.81)	70.50	164	5.69
M43	$-CH_3$		$C_{41}H_{42}N_8O_7F_2$ (796.819)	69.00	147	6.29
M44	$-CH_3$		$C_{43}H_{45}N_8O_8F$ (820.86)	72.50	141	6.20
M45	-F		$C_{27}H_{31}N_6O_4F$ (522.571)	62.0	123	4.00
M46	- F		$C_{25}H_{27}N_6O_4F$ (494.518)	60.0	77	3.23
M47	- F		$C_{34}H_{36}N_7O_4F$ (625.693)	60.5	79	8.30

Table 5.7: Physical data of synthesized Mannich bases (M48-M54)

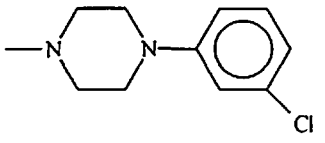
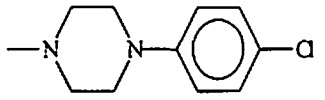
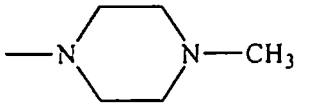
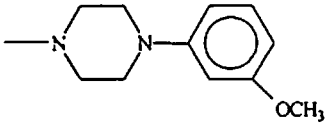
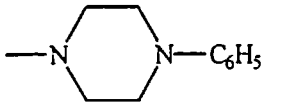
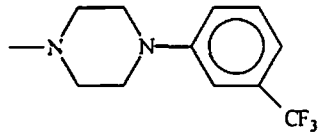
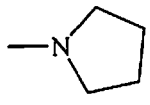
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log p
M48	F		$C_{33}H_{33}N_7O_4ClF$ (646.111)	66.0	112	8.32
M49	- F		$C_{33}H_{33}N_7O_4ClF$ (646.111)	66.0	114	8.31
M50	- F		$C_{28}H_{32}N_7O_4F$ (549.597)	69.0	111	6.28
M51	- F		$C_{34}H_{36}N_7O_5F$ (641.692)	60.50	132	8.09
M52	- F		$C_{33}H_{34}N_7O_4F$ (611.666)	62.50	60	8.01
M53	- F		$C_{34}H_{33}N_7O_4F_4$ (679.664)	65.0	76	8.89
M54	- F		$C_{27}H_{29}N_6O_4F$ (520.556)	68.0	137	3.58

Table 5.7: Physical data of synthesized Mannich bases (M55-M59)

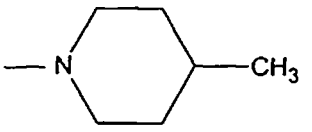
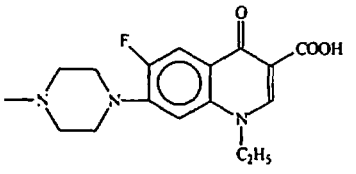
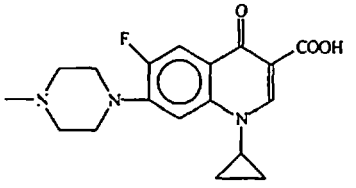
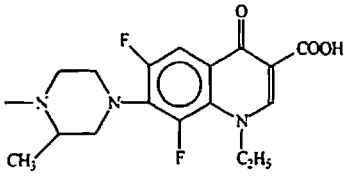
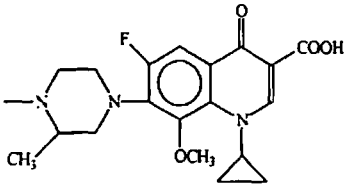
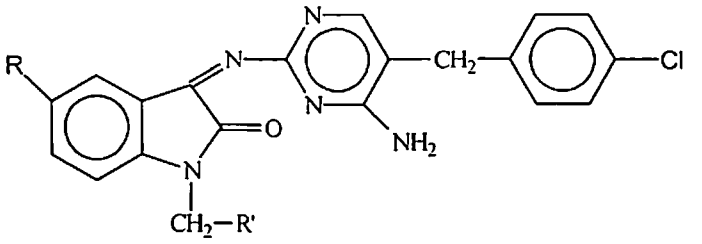
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log p
M55	F		$C_{29}H_{33}N_6O_4F$ (548.609)	65.0	99	4.59
M56	- F		$C_{39}H_{38}N_8O_7F_2$ (768.766)	63.0	264	5.33
M57	- F		$C_{40}H_{38}N_8O_7F_2$ (780.776)	64.50	230	5.29
M58	-F		$C_{40}H_{39}N_8O_7F_3$ (800.783)	68.0	212	5.71
M59	- F		$C_{42}H_{42}N_8O_8F_2$ (824.829)	69.0	144	5.86

Table 5.8: Physical data of synthesized Mannich bases (C1-C5)



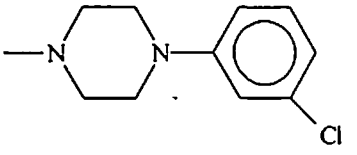
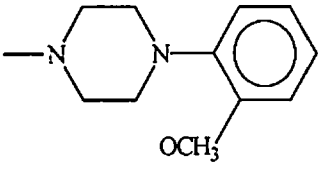
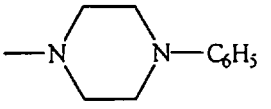
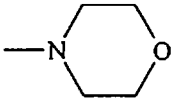
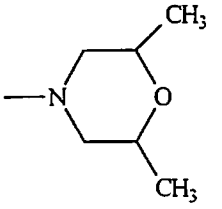
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log p
C1	-Cl		$C_{30}H_{26}N_7OCl_3$ (606.932)	69.5	162	8.78
C2	-Cl		$C_{31}H_{29}N_7O_2Cl_2$ (602.513)	70.0	144	8.49
C3	-Cl		$C_{30}H_{27}N_7OCl_2$ (572.487)	72.5	102	8.50
C4	-Cl		$C_{24}H_{22}N_6O_2Cl_2$ (497.376)	71.0	172	7.22
C5	-Cl		$C_{26}H_{26}N_6O_2Cl_2$ (525.429)	69.0	138	7.51

Table 5.8: Physical data of synthesized Mannich bases (C6-C10)

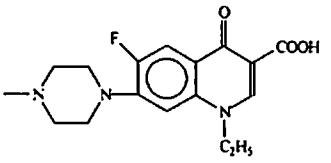
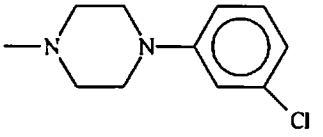
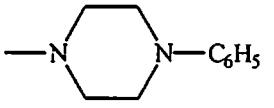
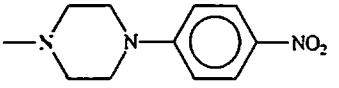
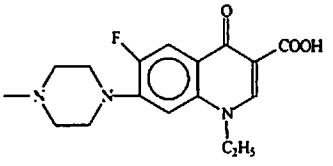
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log p
C6	-Cl		$C_{36}H_{31}N_8O_4Cl_2F$ (729.587	66.0	177	4.10
C7	$-CH_3$		$C_{31}H_{29}N_7OCl_2$ (586.514)	66.0	121	8.19
C8	$-CH_3$		$C_{31}H_{30}N_7OCl$ (552.069)	68.50	150	7.89
C9	$-CH_3$		$C_{31}H_{29}N_8O_3Cl$ (597.067)	70.0	170	6.95
C10	$-CH_3$		$C_{37}H_{34}N_8O_4ClF$ (709.169)	72.5	184	4.77

Table 5.8: Physical data of synthesized Mannich bases (C11-C15)

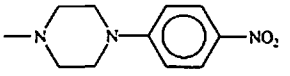
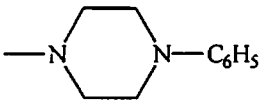
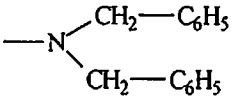
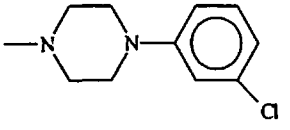
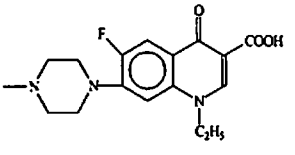
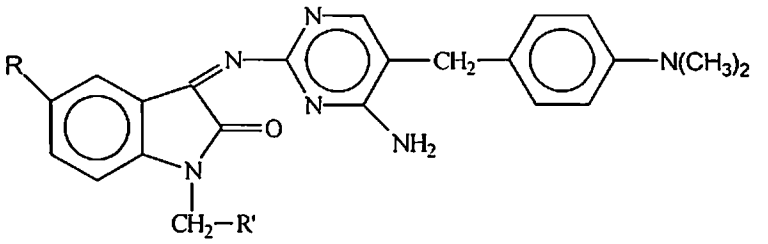
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log p
C11	-F		$C_{30}H_{26}N_8O_3ClF$ (601.031)	63.0	167	7.32
C12	- F		$C_{30}H_{27}N_7OClF$ (556.033)	68.50	110	8.36
C13	- F		$C_{34}H_{28}N_6OClF$ (591.077)	68.0	81	9.44
C14	- F		$C_{30}H_{26}N_7OCl_2F$ (590.478)	74.00	141	8.64
C15	- F		$C_{36}H_{31}N_8O_4ClF_2$ (713.132)	76.0	211	3.95

Table 5.9: Physical data of synthesized Mannich bases (D1-D6)



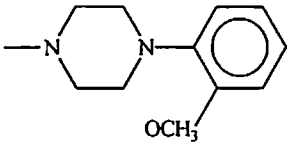
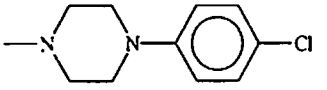
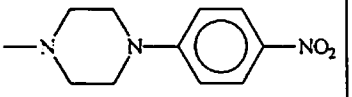
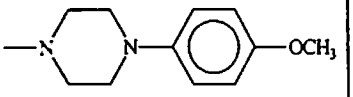
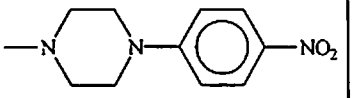
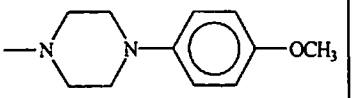
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log p
D1	- H		$C_{33}H_{36}N_8O_2$ (576.692)	69.50	150	7.09
D2	- H		$C_{32}H_{33}N_8OCl$ (581.110)	72.00	160	7.30
D3	- H		$C_{32}H_{33}N_9O_3$ (591.663)	68.00	156	6.13
D4	- H		$C_{33}H_{36}N_8O_2$ (576.692)	65.50	132	7.14
D5	$-CH_3$		$C_{33}H_{35}N_9O_3$ (605.690)	73.50	166	6.63
D6	$-CH_3$		$C_{34}H_{38}N_8O_2$ (590.718)	71.50	142	7.62

Table 5.9: Physical data of synthesized Mannich bases (D7-D13)

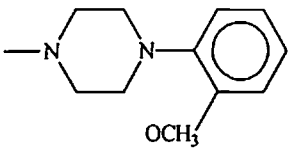
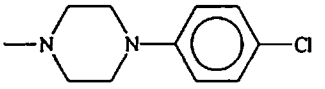
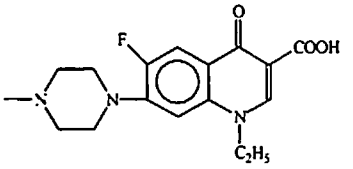
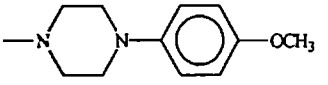
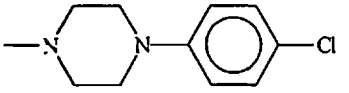
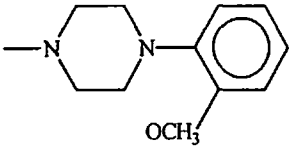
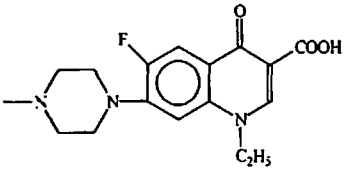
#	R	R'	Molecular Formula (Mol Wt)	Yield (%)	m.p (°c)	Log p
D7	$-CH_3$		$C_{34}H_{38}N_8O_2$ (590.718)	66.5	149	7.58
D8	$-CH_3$		$C_{33}H_{35}N_8OCl$ (595.137)	62.5	136	7.66
D9	$-CH_3$		$C_{39}H_{40}N_9O4F$ (717.792)	68.0	225	5.23
D10	- F		$C_{33}H_{351}N_8O_2F$ (594.682)	67.5	128	7.84
D11	-F		$C_{32}H_{32}N_8OClF$ (599.101)	73.5	140	8.04
D12	- F		$C_{33}H_{35}N_8O_2F$ (594.682)	71.0	146	7.80
D13	- F		$C_{38}H_{37}N_9O_4F_2$ (721.755)	72.5	217	5.28

Table 5.10: Spectral and Elemental analysis data of synthesized compounds

Comp. No	IR spectroscopy (cm^{-1}; KBr)	^1H- NMR (δ ppm, DMSO-d_6)	Elemental Analysis (Calculated/ Found)		
			C	H	N
M3	3010, 2850, 2840, 1730, 1616, 1506, 1236, 1129	1.86 (t, 6H, CH_3 of $-C_2H_5$), 3.2 (s, 2H, CH_2 of benzyl), 3.7(s, 9H, $-OCH_3$), 4.2 (q, 4H, CH_2 of $-C_2H_5$), 5.2 (s, 2H, $-NCH_2N-$), 5.6 (s, 2H, NH_2), 6.8-7.28 (m, 6H, Ar-H)	60.16 60.18	5.8 5.69	15.59 15.60
M5	3010, 2856, 2830, 1720, 1620, 1500, 1240	3.17 (s, 2H, CH_2 of benzyl), 3.65(s, 9H, $-OCH_3$), 3.9-4.1 (m, 8H, piperazine-H), 5.2 (s, 2H, $-NCH_2N-$), 5.65 (s, 2H, NH_2), 6.67-7.82 (m, 10H, Ar-H))	59.82 60.04	5.02 5.11	14.8 14.72
M14	3010, 2850, 2840, 1736, 1620, 1506, 1236, 1125	0.88-1.12 (m, 4H, cyclopropyl-H), 3.2 (s, 2H, CH_2 of benzyl), 3.5 (m, 1H, cyclopropyl-H), 3.60 (s, 9H, $-OCH_3$), 3.7-4.1 (m, 8H, piperazine-H), 5.1 (s, 2H, $-NCH_2N-$), 5.8 (s, 2H, NH_2), 6.58-8.66 (m, 9H, Ar-H), 8.6 (s, 1H,)	60.26 60.19	4.8 4.71	14.06 14.13
M18	3010, 2850, 2840, 1730, 1616, 1506, 1236,1129	1.86 (t, 6H, CH_3 of $-C_2H_5$), 3.16 (s, 2H, CH_2 of benzyl), 3.7(s, 9H, $-OCH_3$), 4.2 (q, 4H, CH_2 of $-C_2H_5$), 5.1 (s, 2H, $-NCH_2N-$), 5.6 (s, 2H, NH_2), 6.8-7.26 (m, 6H, Ar-H)	55.58 55.48	5.36 5.39	14.4 14.60
M19	3010, 2850, 2840, 1730, 1616, 1500, 1240	3.17 (s, 2H, CH_2 of benzyl), 3.65 (s, 9H, $-OCH_3$), 3.9-4.1 (m, 8H, piperazine-H), 4.36 (s, 2H, CH_2 of benzyl piperazine), 5.2 (s, 2H, $-NCH_2N-$), 5.65 (s, 2H, NH_2), 6.67-7.82 (m, 11H, Ar-H)	59.48 59.60	5.28 5.20	14.28 14.32
M30	3010, 2850, 2840, 1736, 1620, 1506, 1236, 1125	0.88-1.1 (m, 4H, cyclopropyl-H), 3.3 (s, 2H, CH_2 of benzyl), 3.5 (m, 1H, cyclopropyl-H), 3.62 (s, 9H, $-OCH_3$), 3.7-4.1 (m, 8H, piperazine-H), 5.1 (s, 2H, $-NCH_2N-$), 5.8 (s, 2H, NH_2), 6.58-8.60 (m, 9H, Ar-H), 8.6 (s, 1H, C_2-H)	57.08 57.12	4.55 4.61	13.31 13.30

Table 5.11: Spectral and Elemental analysis data of synthesized compounds

Comp. No	IR spectroscopy (cm^{-1}; KBr)	^1H- NMR (δ ppm, DMSO-d_6)	Elemental Analysis (Calculated/ Found)		
			C	H	N
M34	3010, 2850, 2830, 1730, 1620, 1500, 1240	3.18 (s, 2H, CH_2 of benzyl), 3.6(s, 9H, $-OCH_3$), 3.9-4.2 (m, 8H, piperazine-H), 5.2 (s, 2H, $-NCH_2N-$), 5.65 (s, 2H, NH_2), 6.7-7.82 (m, 10H, Ar-H)	63.59 63.60	5.65 5.60	15.27 15.12
M37	3010, 2850, 2830, 1730, 1616, 1500, 1240	1.8 (t, 6H, CH_3 of $-C_2H_5$), 3.17 (s, 2H, CH_2 of benzyl), 3.65 (s, 9H, $-OCH_3$), 3.9-4.1 (m, 8H, piperazine-H), 5.2 (s, 2H, $-NCH_2N-$), 5.65 (s, 2H, NH_2), 6.67-7.82 (m, 11H, Ar-H)	67.2 67.02	6.14 6.20	16.13 16.32
M42	3010, 2850, 2840, 1736, 1620, 1506, 1236, 1125	0.88-1.12 (m, 4H, cyclopropyl-H), 1.84 (s, 3H, CH_3), 3.3 (s, 2H, CH_2 of benzyl), 3.5 (m, 1H, cyclopropyl-H), 3.62 (s, 9H, $-OCH_3$), 3.7-4.1 (m, 8H, piperazine-H), 5.16 (s, 2H, $-NCH_2N-$), 5.8 (s, 2H, NH_2), 6.58-8.40 (m, 9H, Ar-H), 8.6 (s, 1H, C_2-H)	63.39 63.28	5.32 5.36	14.42 14.40
M46	3010, 2850, 2840, 1730, 1616, 1506, 1236, 1129	2.5 (s, 6H, $-N(CH_3)_2$), 3.16 (s, 2H, CH_2 of benzyl), 3.7 (s, 9H, $-OCH_3$), 5.1 (s, 2H, $-NCH_2N-$), 5.6 (s, 2H, NH_2), 6.8-7.26 (m, 6H, Ar-H)	60.72 60.13	5.55 5.42	16.99 17.09
M49	3010, 2850, 2830, 1730, 1620, 1500, 1240	3.17 (s, 2H, CH_2 of benzyl), 3.65 (s, 9H, $-OCH_3$), 3.9-4.1 (m, 8H, piperazine-H), 4.36 (s, 2H, CH_2 of benzyl piperazine), 5.2 (s, 2H, $-NCH_2N-$), 5.65 (s, 2H, NH_2), 6.67-7.82 (m, 10H, Ar-H)	61.35 61.6	5.15 5.2	15.17 15.22
M56	3010, 2850, 2840, 1736, 1620, 1596, 1506, 1236, 1125	1.28 (t, 3H, CH_3 of $-C_2H_5$), 3.3 (s, 2H, CH_2 of benzyl), 3.62 (s, 9H, $-OCH_3$), 3.7-4.1 (m, 8H, piperazine-H), 4.25 (q, 2H, CH_2 of $-C_2H_5$), 5.1 (s, 2H, $-NCH_2N-$), 5.8 (s, 2H, NH_2), 6.58-8.60 (m, 9H, Ar-H), 8.6 (s, 1H, C_2-H)	60.93 60.82	4.98 4.91	14.58 14.30

Table 5.12: Spectral and Elemental analysis data of synthesized compound

Comp. No.	IR spectroscopy (cm^{-1}; KBr)	^{1}H- NMR (δ ppm, DMSO-d_6)	Elemental Analysis (Calculated/ Found)		
			C	H	N
C1	3010, 2850, 2830, 1730, 1620, 1500, 1240	3.18(s, 2H, CH_2 of -chlorobenzyl), 3.9-4.26 (m, 8H, piperazine-H), 5.2 (s, 2H, -NCH_2N-), 5.65 (s, 2H, NH_2), 6.67-7.80 (m, 12H, Ar-H)	59.37 59.17	4.32 4.19	16.15 16.22
C10	3016, 2856, 2830, 1736, 1620, 1596, 1506, 1236, 1125	1.28 (t, 3H, CH_3 of -C_2H_5), 1.85 (s, 3H, CH3), 3.3 (s, 2H, CH_2 of benzyl), 3.7-4.1 (s, 9H, -OCH_3), 3.7-4.1 (m, 8H, piperazine-H), 4.25 (q, 2H, CH2 of –C_2H_5), 5.1 (s, 2H, -NCH_2N-), 5.8 (s, 2H, NH_2), 6.58-8.60 (m, 11H, Ar-H), 8.6 (s, 1H, C_2-H)	62.67 62.82	4.83 4.91	15.8 15.70
C12	3010, 2850, 2830, 1730, 1620, 1500, 1240	3.17 (s, 2H, CH_2 of -chlorobenzyl), 3.9-4.1 (m, 8H, piperazine-H), 5.2 (s, 2H, -NCH_2N-), 5.65 (s, 2H, NH_2), 6.67-8.02 (m, 13H, Ar-H))	64.8 64.60	4.89 4.82	17.63 17.70
D2	3010, 2850, 2840, 1730, 1616, 1506, 1236, 1129	1.82 (s, 6H, -N $(CH_3)_2$), 3.18 (s, 2H, CH_2 of benzyl), 3.9-4.2 (m, 8H, piperazine-H), 5.2 (s, 2H, -NCH_2N-), 5.65 (s, 2H, NH_2), 6.7-7.82 (m, 12H, Ar-H)	55.58 55.48	5.36 5.39	14.4 14.60
D13	3010, 2850, 2840, 1730, 1616, 1500, 1240	1.82 (s, 6H, -N $(CH_3)_2$),1.28 (t, 3H, CH_3 of -C_2H_5), 3.3 (s, 2H, CH_2 of benzyl), 3.7-4.1 (m, 8H, piperazine-H), 4.25 (q, 2H, CH_2 of –C_2H_5), 5.1 (s, 2H, -NCH_2N-), 5.8 (s, 2H, NH_2), 6.58-8.60 (m, 9H, Ar-H), 8.6 (s, 1H, C_2-H)	59.48 59.60	5.28 5.20	14.28 14.32

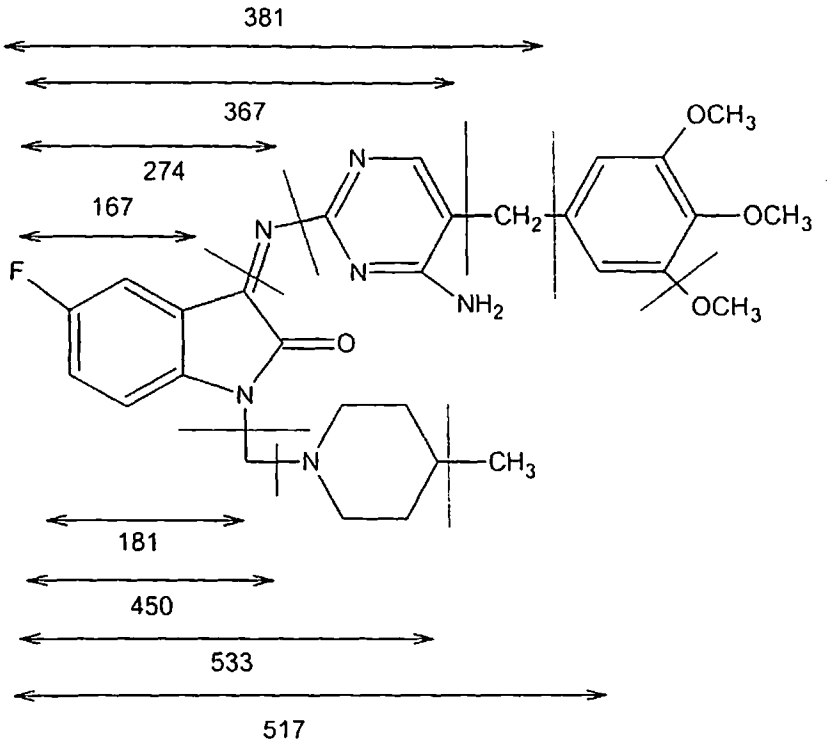
The N-Mannich bases of the Schiff base (**S1-S10**) were synthesized by condensing acidic imino group of isatin with formaldehyde and various secondary amines.

The % yield of synthesized Mannich base was found in the range of 60-76.5% after recrystallization from chloroform. The purity of the compounds was checked by TLC. The elemental analysis of the compounds (Table 5.10-5.12) for C, H, and N were within ± 0.04% of the theoretical values. IR and ^{1}H-NMR spectra were also consistent with the assigned structures.

In the IR spectra, the mannich bases showed new absorption band in the region 2850 cm^{-1} due to methylene group ($-CH_2$). Absence of peak at 3200 cm^{-1}, due to amide (N-H group) group indicated the replacement of active hydrogen atom of isatin with aminomethyl group.

The structures were further confirmed by using ^{1}H-NMR spectra. In the NMR spectra, Schiff base showed a sharp singlet at δ 10.4 ppm due to –NH group of isatin. In the case of Mannich base, the singlet at δ 10.4 ppm disappeared and a new singlet appeared at δ 5.1-5.2 ppm, which accounted for the $-NCH_2N-$ protons of the Mannich base.

Mass Spectrum of compound **M55** showed a molecular ion peak at m/z 548.2, base peak at m/z of 381. The other major fragments were at m/z 167, 181, 274, 367, 450, 517 and 533.



M55

06 Anti-HIV Activity

6.1 Test Protocol:

a) In MT-4 cells

Cell cultures

The compounds were tested for anti-HIV activity against replication of HIV-1 (III_B) in MT-4 cells. The MT-4 cells were grown in RPMI-1640 DM (Dutch modification) medium (Flow lab, Irvine Scotland), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and 20 µg/mL gentamicin (E. Merck, Darmstadt, Germany) (Pauwels et al., 1988; Balzarini et al., 1993). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Every 3-4 days, cells were spun down and seeded at 3 x 10^5 cells/ml in new cell culture flasks. At regular time intervals, the MT-4 cells were analyzed for the presence of mycoplsma and consistently found to be mycoplasma-free.

Virus

HIV-1 (strain HTLV- III_B) (Popovic et al., 1984) were obtained from the culture supernatant of HIV-1 infected MT-4 cell lines (Pauwels et al., 1987). The virus titer of the supernatant was determined in MT-4 cells. The virus stocks were stored at –70°C until used.

Anti-HIV assay

Flat bottom, 96-well plastic microtiter trays (Falcon, Becton Dickinson, Mountain View, CA) were filled with 100 µL of complete medium using a Titertek Multidrop dispenser (Flow Laboratories). This eight-chanel dispenser could fill a microtiter tray in less than 10s. Subsequently, stock solutions (10 x final test concentration) of compounds were added in 25 µl volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on HIV and mock-infected cells. Serial five-fold dilutions were made directly in the microtiter trays using a Biomek 1000 robot (Beckman). Untreated control HIV- and mock-infected cell samples were included for each compound.

50 µl of HIV at 100 $CCID_{50}$ or medium was added to either infected or mock-infected part of a microtiter tray. Exponentially growing MT-4 cells were centrifuged for 5 min at 140 x g and the supernatants were discarded. The MT-4 cells were resuspended at 6 x 10^5 cells/ml in a flask which was connected with an autoclavable dispensing cassette of a Titertek Multidrop dispenser. Under slight magnetic stirring 50 µl volumes were then transferred to the microtiter tray wells. The outer row wells were filled with 200 µl of medium. The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells remained in contact with the test compounds during the whole incubation period. Five days after infection the viability of mock- and HIV-infected cells was examined spectrophotometrically by the MTT method.

MTT assay

The MTT assay is based on the reduction of the yellow coloured 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan which can be measured spectrophotometrically. Therefore, to each well of the microtiter trays, 20 µl of a solution of MTT (7.5 mg/ml) in a phosphate-buffered saline was added using a Titertek Multidrop. The trays were furthur incubated at 37°C in a CO_2 incubator for 1 h. A fixed volume of medium (150 µl) was then removed from each cup using a M96 Washer (ICN flow) without disturbing the MT-4 cell clusters containing the formazan crystals.

Solubilization of the formazan crystals was achieved by adding 100 µl 10 % (v/v) Triton X-100 in acidified isopropanol (2 ml concentrated HCl per 500 ml solvent) using

the M96 Washer (ICN flow). Complete dissolution of the formazan crystals could be obtained after the trays had been placed on a plate shaker for 10 min (ICN flow). Finally, the absorbances were read in an eight-channel computer-controlled photometer (Multiskan MCC, ICN Flow) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically substracted from the absorbance at 540 nm, so as to eliminate the effects of non-specific absorption. Blanking was carried out directly on the microtiter trays with the first column wells which contained all reagents except for the MT-cells. All data represent the average values for a minimum of three wells. The 50% cytotoxic dose (CD_{50}) was defined as the concentration of compound that reduced the absorbance (OD_{540}) of the mock-infected control sample by 50%. The percent protection achieved by the compounds in HIV-infected cells was calculated by the following formula:

$$\text{Expressed in \%} \quad \frac{(OD_T)_{HIV}-(OD_C)_{HIV}}{(OD_C)_{mock}-(OD_C)_{HIV}}$$

Whereby $(OD_T)_{HIV}$ is the optical density measured with a given concentration of the test compound in HIV-infected cells; $(OD_C)_{HIV}$ is the optical density measured for the control untreated HIV-infected cells; $(OD_C)_{mock}$ is the optical density measured for the control untreated mock-infected cells; all OD values determined at 540 nm. The dose achieving 50% protection according to the above formula was defined as the 50% effective dose (ED_{50}).

B) In CEM cells

Candidate agents were dissolved in dimethylsulfoxide, and then diluted 1:100 in cell culture medium before preparing serial half- $\log_{10}$ dilutions. T4 lymphocytes (CEM cell-line) were added and after a brief interval HIV-1 was added, resulting in a 1:200 final dilution of the compound. Uninfected cells with the compound served as a toxicity control, and infected and uninfected cells without the compound served as basic controls. Cultures were incubated at 37°C in a 5% carbon dioxide atmosphere for 6 days. The tetrazolium salt, XTT was added to all the wells, and cultures were incubated to allow formazan color development by viable cells. Individual wells were analyzed spectrophotometrically to quantitate formazan production, and in addition were viewed microscopically for detection of viable cells and confirmation of protective activity.

Drug-treated virus-infected cells were compared with drug-treated non-infected cells and with other appropriate controls (untreated infected, untreated noninfected, drug-containing wells without cells etc) on the same plate. All tests were compared with atleast one positive control (e.g. AZT-treated) done at the same time under identical conditions.

C) HIV-1 RT assay

The reaction mixture (50µl) contained 50 mM Tris–HCl (pH 7.8), 5 mM dithiothreitol, 30 mM glutathione, 50 µM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 µg of bovine serum albumine, an appropriate concentration of the radiolabelled substrate [^{3}H] dGTP, 0.1 mM poly(vC)•oligo(dG) as the template/primer, 0.06% Triton X-100, 10 µl of inhibitor solution (containing various concentrations of compounds), and 1 µl of RT preparation.The reaction mixtures were incubated at 37°C for 15 min, at which time 100 µl of calf thymus DNA (150 µg/ml), 2 ml of $Na_4P_2O_7$ (0.1 M in 1 M HCl), and 2 ml of trichloroacetic acid (10% v/v) were added. The solutions were kept on ice for 30 min, after which the acid-insoluble material was washed and analysed for radioactivity. For the experiments in which 50% inhibitory concentration (IC_{50}) of the test compounds was determined, fixed concentration of 2.5 µM [^{3}H] dGTP was used.

6.2 Results

Anti HIV-1 activity: (Series I)

The synthesized compounds were screened for anti-HIV activity on the replication of HIV-1 (III_B) in MT-4 cell line and CEM cell line. The EC_{50}, CC_{50} and % protection values are reported in table 6.1-6.6.

Table 6.1: Cytotoxicity and biological activity of compounds (M1- M16) against HIV-1 (HTLV-III_B) in MT-4 and CEM cell line.

Comp. No.	Anti-HIV activity (μM)					
	MT-4 cell line			CEM cell line		
	EC_{50}[a]	CC_{50}[b]	% Protection	EC_{50}[a]	CC_{50}[b]	% Protection
M1	10.4	44.1	95.1	> 37.1	37.1	20.9
M2	16.1	62.6	73.2	NT	NT	NT
M3	23.6	79.1	64.1	> 98.4	98.4	20.6
M4	> 36.1	36.1	41.8	> 94.7	94.7	22.3
M5	> 39.2	39.2	20.9	> 34.4	34.4	23.00
M6	>38.6	38.6	32.6	> 40.1	40.1	22.6
M7	56.1	65.6	59.6	> 71.0	71.0	22.9
M8	> 60.6	60.6	30.2	> 51.0	51.0	23.3
M9	> 80.7	80.7	48.6	> 88.0	88.0	21.0
M10	> 32.6	32.6	10.7	> 23.3	23.3	21.9
M11	> 39.2	39.2	16.6	> 29.4	29.4	23.5
M12	32.6	97.3	62.6	> 83.8	> 83.8	20.7
M13	> 67.4	67.4	41.7	> 46.3	46.3	22.1
M14	9.4	186.6	101.0	> 200	> 200	33.3
M15	17.2	36.9	89.0	> 18.7	18.7	48.3
M16	NT	NT	NT	> 67.0	67.0	20.0

*NT indicates not tested

[a] 50% Effective concentration, or concentration required to inhibit HIV-1 induced cytopathicity in cell lines by 50%.

[b] 50% Cytotoxic concentration, or concentration required to reduce the viability of mock-infected cell lines by 50%.

Table 6.2: Cytotoxicity and biological activity of compounds (M17- M32) against HIV-1 (HTLV-IIIB) in MT-4 and CEM cell line

Comp. No.	Anti-HIV activity (μM)					
	MT-4 cell line			CEM cell line		
	EC_{50}	CC_{50}	% Protection	EC_{50}	CC_{50}	% Protection
M17	> 36.1	36.1	29.6	NT	NT	NT
M18	19.2	62.6	72.6	> 42.8	42.8	39.9
M19	7.8	79.1	88.6	> 74.0	74.0	42.4
M20	> 41.6	41.0	29.1	> 37.2	37.2	29.7
M 21	> 49.6	49.6	38.1	> 45.3	45.3	38.3
M 22	> 47.1	47.1	26.2	> 45.3	45.3	38.3
M 23	22.6	46.4	62.6	> 42.2	42.2	38.3
M 24	> 59.1	59.1	36.1	> 54.4	54.4	22.2
M 25	> 46.2	46.2	31.6	> 36.3	36.2	20.3
M 26	> 69.7	69.7	26.6	> 55.9	55.9	21.3
M 27	> 61.6	61.6	20.1	NT	NT	NT
M 28	5.6	72.6	126	> 55.1	55.1	48.4
M 29	> 96.2	96.2	39.6	> 123	123.0	22.2
M 30	12.3	64.6	68.4	NT	NT	NT
M 31	7.6	92.1	93.6	> 81.9	81.9	35.7
M 32	19.2	34.6	56.6	NT	NT	NT

Table 6.3: Cytotoxicity and biological activity of compounds (M33- M48) against HIV-1 (HTLV-IIIB) in MT-4 and CEM cell line

Comp. No.	Anti-HIV activity (μM)					
	MT-4 cell line			CEM cell line		
	EC_{50}	CC_{50}	% Protection	EC_{50}	CC_{50}	% Protection
M 33	>94.2	94.2	19.2	> 89.2	89.2	13.0
M 34	17.2	49.2	72.6	> 36.1	36.1	28.2
M 35	>32.1	32.1	26.94	> 11.9	11.9	13.2
M 36	> 47.96	47.96	16.4	> 37.4	37.4	17.6
M 37	> 116.1	116.1	26.2	> 121	121	11.2
M 38	>119.6	119.6	14.20	> 121	121	11.2
M 39	> 106.2	106.2	26.4	> 118	118	10.0
M 40	> 124	124	34.6	> 122	122	11.8
M 41	> 124.6	124.6	24.6	> 105	105	10.8
M 42	11.6	86.1	84.9	NT	NT	NT
M 43	28.4	92.6	64.0	NT	NT	NT
M 44	21.2	81.4	66.8	NT	NT	NT
M 45	> 141.01	141.0	30.2	> 116.0	116.0	11.2
M 46	> 62.7	62.7	12.1	NT	NT	NT
M47	> 139.3	139.3	34.6	> 112.0	112.0	11.5
M48	> 121.6	121.6	46.1	> 104.0	104.0	15.5

Table 6.4: Cytotoxicity and biological activity of compounds (M49- M59) against HIV-1 (HTLV-IIIB) in MT-4 and CEM cell line

Comp No.	Anti-HIV activity (μM)					
	MT-4 cell line			CEM cell line		
	EC_{50}	CC_{50}	% Protection	EC_{50}	CC_{50}	% Protection
M49	36.2	90.1	59.4	> 95.6	95.6	47.4
M50	> 106.7	106.7	42.6	> 115.0	115.0	13.9
M51	> 81.6	81.6	22.1	NT	NT	NT
M52	> 84.7	84.7	21.6	NT	NT	NT
M53	62.1	136.6	54.2	> 127.0	127	10.9
M54	> 129.6	129.6	40.1	> 121	121	14.2
M55	> 69.6	69.6	23.6	NT	NT	NT
M56	12.1	160.2	99.6	50.8	139	63.8
M57	17.9	141.6	86.2	NT	NT	NT
M58	57.1	130.9	61.6	NT	NT	NT
M59	25.2	91.6	56.8	NT	NT	NT
Nevirapine	0.35	> 200	NT	0.12	> 200	NT

Series II

Table 6.5: Cytotoxicity and biological activity of compounds (C1- C15) against HIV-1 (HTLV-IIIB) in MT-4 and CEM cell line

Comp No.	Anti-HIV activity (μM)					
	MT-4 cell line			CEM cell line		
	EC_{50}	CC_{50}	% Protection	EC_{50}	CC_{50}	% Protection
C1	20.0	39.4	62.6	> 25.8	> 25.8	13.8
C2	> 21.6	21.6	29.1	> 18.7	18.7	22.1
C3	> 42.1	42.1	36.8	NT	NT	NT
C4	> 42.8	42.8	18.0	> 34.9	34.9	12.7
C5	> 46.9	46.9	21.1	> 36.6	36.6	11.8
C6	11.2	69.8	88.4	> 26.2	26.2	26.6
C7	26.3	109.2	72.1	> 108.0	108.0	25.4
C8	> 143.9	143.9	27.6	NT	NT	NT
C9	> 113.1	113.1	42.1	> 104.0	104.0	42.7
C10	9.4	143	102.6	> 130.0	130.0	11.4
C11	> 69.1	69.1	32.1	NT	NT	NT
C12	> 72.7	72.7	8.6	NT	NT	NT
C13	> 120.9	120.9	21.1	> 116.0	116	12.2
C14	21.6	73.1	69.9	> 65.8	65.8	29.3
C15	14.6	131.6	90.1	> 123.0	123.0	19.9
Nevirapine	0.35	> 200	NT	0.12	> 200	NT

Series III

Table 6.6: Cytotoxicity and biological activity of compounds (D1- D13) against HIV-1 (HTLV-IIIB) in MT-4 and CEM cell line

Comp No.	Anti-HIV activity (μM)					
	MT-4 cell line			CEM cell line		
	EC_{50}	CC_{50}	% Protection	EC_{50}	CC_{50}	% Protection
D1	> 96.5	96.5	29.8	>92.4	92.4	12.3
D2	24.2	46.2	65.2	> 39.2	39.2	27.8
D3	> 32.2	32.2	31.6	NT	NT	NT
D4	> 40.1	40.1	30.6	NT	NT	NT
D5	> 33.6	33.6	39.8	> 85.4	85.4	17.6
D6	>38.2	38.2	36	> 90.6	90.6	11.6
D7	> 112.6	112.6	10.1	> 109	109	21.1
D8	23.6	49.5	68	> 32.5	32.5	30.6
D9	8.6	126.5	95	> 28.2	28.2	42.2
D10	> 35.2	35.2	32.6	> 71.0	71.0	18.0
D11	19.2	60.6	75.2	> 31.0	31.0	26.8
D12	> 102.2	102.2	12.2	> 52	52	14.1
D13	8.2	134.6	110	62.6	137	52.3
Nevirapine	0.35	> 200	NT	0.12	> 200	NT

Table 6.7: Inhibitory activity of selected compounds against HIV-1 RT

Selected compounds were tested for their ability to inhibit HIV-1 RT in an *in vitro* enzyme inhibition assay (IC_{50}) and the results are summarized below.

Compound No.	IC_{50} (μM) against HIV-1 RT
M14	18.4 ± 2.4
M28	12.2 ± 2.4
M30	28.4 ± 4.4
M31	20.2 ± 8.6
M42	28.4 ± 3.4
M56	27.2 ± 3.4
M57	38.2 ± 4.2
C10	21.4 ± 3.12
C15	34.5 ± 5.2
D9	20.8 ± 5.2
D13	20.4 ± 6.0
Nevirapine	4.8 ± 1.7

6.3 Discussion:

Anti-HIV activity:

The synthesized compounds in series I (**M1-M59**), series II (**C1-C15**), and series III (**D1-D13**) were evaluated for their cytotoxicity and anti-HIV activity on the replication of HIV-1 (HTLV-III_B) in MT-4 cell line and CEM cell line, and nevirapine was used as a reference drug for comparison. The results are summarized in Table 6.1-6.6. Some of the selected compounds were assayed for inhibitory effects against highly purified HIV-1 RT using homopolymeric template primers (Table 6.7). The results of the biological evaluations are expressed as CC_{50} (Cytotoxicity), EC_{50} (anti HIV-1 activity), % protection, and IC_{50} (RT inhibitory activity) values.

MT-4 cell line:

Series I

The anti-HIV-1 activity data revealed that the test compounds **M1-3, M14-15, M18-19, M23, M28, M30-32, M34, M42, M44,** and **M56-57** exhibited significant biological activity ranging (EC_{50}) from 5.6 μM to 23.6 μM with maximum protection range from 62.6% to 126%. Compounds **M14, M28, M31** and **M56** exhibited best combination of high potency and low toxicity in this series.

Compound **M28** and **M31** showed the highest activity against HIV-1 infected MT-4 cell lines with EC_{50} value of 5.6 μM and 7.6 μM respectively and their selectivity index ($SI=CC_{50}/EC_{50}$) was found to be more than 12 with maximum % protection range of 93.6-126%.

However in terms of toxicity data, compound **M14** and **M56** showed better selectivity index of > 19 and >13 respectively. They were not cytotoxic to MT-4 cells at CC_{50} value of 186.6 and 160.2 μM respectively. When compared to Nevirapine, compound **M14** was considerably less active (EC_{50} value of 9.4 μM compared to 0.35 μM of Nevirapine), more toxic (CC_{50} of 186.8 μM compared to > 200 μM of Nevirapine) and exhibited lower selectivity index (> 19 compared to > 571 of Nevirapine).

Series II

In series II, compounds **C6**, **C10**, **C14** and **C15** exhibited marked anti-HIV activity with EC_{50} values ranging from 9.4 to 21.6 μM, with maximum protection range of 69.92-102.6% (table 6.5).Compound **C10** exhibited maximum activity as well as selectivity in this series with EC_{50} value of 9.4 μM, SI of > 15 and maximum protection of 102.6%.The remaining compounds didn't show any marked anti-HIV activity below their toxicity threshold.

Series III

In series III, compounds **D2**, **D8**, **D9**, **D11** and **D13** inhibited the cytopathic effect of HIV-1 (HTLV-III_B) with EC_{50} values ranging from 8.2 to 24.2 μM, with maximum protection range of 65.2-110% (table 6.6).

Compound **D9** and **D13** were found to be the most potent compound in this series with EC_{50} value of 8.6 and 8.2 μM respectively, SI of >15 and maximum protection range of 95-110%. They were not cytotoxic to MT-4 cells at CC_{50} value of 126.5 and 134.6 μM respectively.

CEM cell line:

Series I

The anti-HIV activity of synthesized compounds, measured in HIV-1 III_B-infected CEM cells revealed that all the compounds exhibited biological activity at EC_{50} values ranging from > 18.7 to > 200 μM, with maximum protection range of 10.01-63.84%, and a maximum selectivity index of > 2. The loss of activity might be due to degeneration / rapid metabolism in the culture conditions used in the screening procedure.

The most active compound in this series was found to be compound **M56**, which showed EC_{50} value of 50.8 μM and maximum protection of 63.84%, while not being cytotoxic to the mock infected CEM cells at CC_{50} value of 139 μM.

Series II

The compounds in this series exhibited anti-HIV activity in CEM cells with EC_{50} values ranging from > 18.7 to >130 μM with maximum protection range between 11.37 to

42.71%. None of the compounds revealed any marked anti-HIV activity below their toxicity threshold.

Series III

The most active compound in this series was found to be compound **D13**, which showed EC_{50} value of 62.5 μM and maximum protection of 52.25%, while not being cytotoxic to the mock infected CEM cells at CC_{50} value of 137 μM. Other tested compounds showed marked anti-HIV activity (12.29-42.24%) at a concentration below their toxicity threshold.

HIV-1 reverse transcription (HIV-1 RT) inhibition:

Series I

Selected compounds were evaluated for inhibition of HIV-1 RT, and the resulting *in vitro* IC_{50} values with poly(vC)•oligo(dG) as the template/primer were found to be ranging from 12.2 ± 2.4 to 38.2 ± 4.2 μM. Compound **M28** was found to be the most active inhibitor of HIV-1 RT with IC_{50} value of 12.2 ± 2.4 μM, followed by compound M14 with IC_{50} value of 18.4 ± 2.4 μM.

Series II

Compounds **C10** and **C15** exhibited *in vitro* IC_{50} values of 21.4 ± 3.12 μM and 34.5 ± 5.2 μM respectively against inhibition of HIV-1 RT.

Series III

Compound **D9** inhibited HIV-1 RT with an IC_{50} value of 20.8 ± 5.2 μM followed by compound **D13** with an IC_{50} value of 20.4 ± 6.0 μM. In comparison to Nevirapine, the compounds exhibited low degree of inhibition of HIV-1 RT.

The *in vitro* IC_{50} values for the compounds against HIV-1 RT with poly(vC)•oligo(dG) as the template/primer was found to be significantly higher than its corresponding EC_{50} values for inhibition of the cytopathic effect of HIV-1 III_B in MT-4 cells. This discrepancy is not unusual for the NNRTI's, as it may reflect the differences between the *in vitro* assay in which a synthetic template/primer has been added and the cellular system.

6.4 Structure-activity relationship study (SAR):

MT-4 cell line:

Series I:

From the structure-activity relationship (SAR) point of view, the following salient features have been observed regarding the nature of the substituents at N-1 and C-5 position of aminopyrimidinimino isatin analogues in series I (59 compounds).

At N-1 position, phenyl piperazine moiety with electron withdrawing groups like chloro and fluoro at para position (compound **M6**, **M7**, **M34**, **M49**) indicated an improvement in activity (EC_{50} range: 17.2 - >38.6 μM), in comparison to groups like methoxy and nitro (compound **M24** and **M36**), which didn't show any marked anti-HIV activity (EC_{50} range: >47.96 - >59.1 μM).

At N-1 position, substitution with fluoroquinolone derivatives has shown consistently higher activity, lower toxicity profile and higher % protection with chloro, bromo, methyl and Fluoro substitution at C-5 position (compound **M14**, **M15**, **M30**, **M31**, **M32**, **M42**, **M43**, **M44**, **M56**, **M57**, **M58**, and **M59**).

Compound **M28**, which possessed a morpholine moiety at N-1 position and bromo at C-5 position of isatin, exhibited the highest activity (EC_{50} value: 5.6 μM and max. protection of 126%) in series I against MT-4 cell line.

Among the 5-substituted isatins, the anti-HIV activity was found to follow the trend: $Br > Cl > F > CH_3$

To confirm the above mentioned findings and to acquire more insight into the SAR of this class of compounds, we extended our study to the synthesis and anti-HIV evaluation of a new series (series II and III) of aminopyrimidinimino isatin analogues, where the trimethoxy groups of benzyl pyrimidine was replaced with functional groups like *para* chloro (series II) and *para* dimethylamine (series III) substitution.

Series II:

At N-1 position, phenyl piperazine with electron-withdrawing group like chlorine at *meta* position (compound **C1,** and **C7**) exhibited better anti-HIV activity (EC_{50} range: 20.02 – 26.3 μM), in comparison to unsubstituted phenyl piperazine (compound **C3, C8,** and **C12**) (EC_{50} range: > 42.1 - > 143.92 μM). *Para* substitution of phenyl piperazine moiety with nitro group, at N-1 position, (compound **C9** and **C11**) is not showing any marked improvement in activity (EC_{50} range: > 69.12 - > 113.12 μM), compared to unsubstituted phenyl piperazine (compound **C3, C8,** and **C12**) (EC_{50} range: >42.1 - > 43.92 μM). At N-1 position, substitution with fluoroquinolone derivative (compound **C6, C10** and **C15**) has indicated significant improvement in selectivity as well as activity, in comparison with any other N-1 substitution (EC_{50} range: 9.4 - 14.9 μM).

When comparing C-5 substitution, it is found that methyl group at C-5 position of isatin is showing marginally higher activity as well as better toxicity profile compared to fluoro substitution. The activity trend shows the following pattern: - CH_3> F> H

Series III:

Para chloro substitution of phenyl piperazine at N-1 position (compound **D2, D8** and **D11**) is showing improved activity (EC_{50} range: 19.2 - 24.2 μM) in comparison with groups like nitro and methoxy (compound **D3, D4, D5, D6, D10**) (EC_{50} range: >32.2->40.1 μM). Substitution of ortho-methoxy group on phenyl piperazine moiety at N-1 position (compound **D1, D7** and **D12**) is showing less activity, higher toxicity data as well as low percentage protection (EC_{50} range: >96.5-112.6 μM).

At N-1 position, fluoroquinolone derivative (compound **D9** and **D13**) is showing significant anti-HIV activity (EC_{50} range: 8.2 - 8.6 μM) with various 5-substituted as well as unsubstituted isatin derivatives, when compared to any other substitution at this position.

In general, the order of activity of the substituents at the 5th position was F> CH_3> H.

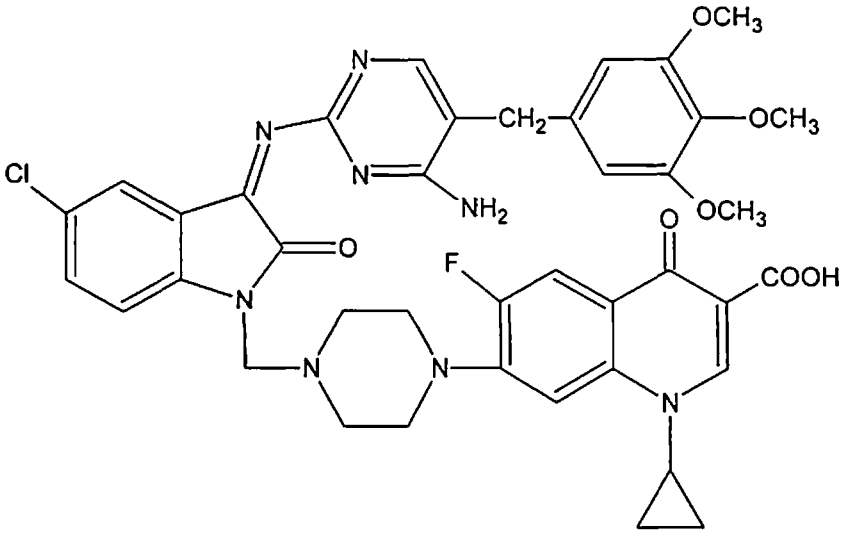
CEM cell line:

In CEM cell line, the structure-activity relationship is not correlating well in view of the nature of substituents, to provide a proper inference regarding all the key structural requirements for a potent anti-HIV compound.

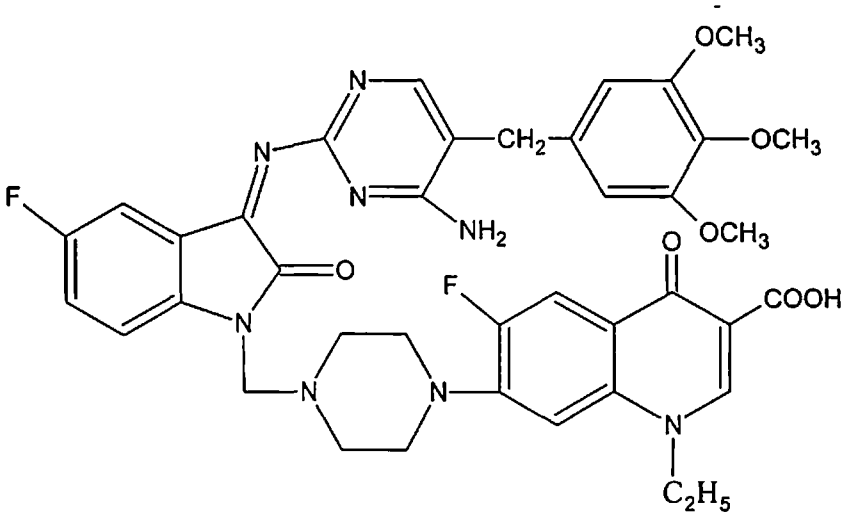
In series I, the fluoroquinolone derivative at N-1 position (compound **M56**) is the only substituent which is providing marked anti-HIV activity with EC_{50} value of 50.8 μM and maximum protection of 63.84%, compared to any other substitution. With respect to substitution at the 5th position of isatin, fluorine is the only atom to exhibit activity. In series II and III, no specific structure-activity relationship can be established with respect to the variously substituted compounds.

6.5 Conclusion

Among the synthesized compounds, compound **M14** was found to exhibit maximum inhibition of the cytopathic effect of HIV-1(III_B), in MT-4 cell line, with selectivity index of 19.85, EC_{50} value of 9.4 μM and percentage protection of 101%, followed by compound **M56** which exhibited selectivity index of 13.23, EC_{50} value of 12.1 μM and percentage protection of 99.6 %.



M14



M56

07 Anti-HCV Activity

7.1 Antiviral and Cytotoxicity Assay for HCV:

Cell culture

Huh-7 cells harbouring the subgenomic HCV replicon BM4-5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% L-pyruvate, 1% penicillin and 1% streptomycin supplemented with 500 µg/mL G418 (Geneticin, Invitrogen) (Saito et al., 2003). Cells were passaged every 4 days.

Cytotoxicity asay

Huh-7 cells were seeded at a density of 3 $x10^4$ cells/well in 96-well plates for the cell-viability assay. Sixteen hours post seeding, cells were treated with the compounds at 50 µg/mL for 3 days. The administration of each drug was renewed each day. Ribavirin (ICN Pharmaceuticals, USA), mycophenolic acid (Sigma, USA), and interferon alpha- 2b (IntronA) were used in the same conditions as positive controls. At the end of treatment, cell viability assays were performed with the 96-well plates using Neutral Red assay (Sigma).

Neutral Red (NR) cytotoxicity assay

The NR cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay, based on the ability of viable cells to incorporate and bind neutral red, a supravital dye. Neutral red is a weak cationic dye that readily penetrates the cell membranes by non-ionic diffusion, accumulating intracellularly in lysosomes, where it binds with anionic sites in the lysosomal matrix. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics, result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged or dead cells, which are the basis of this assay.

Antiviral assay

Huh-7 cells were seeded at a density of 6 x 10^5 cells/well in six - well plates for the antiviral assay. Total RNA (tRNA) was extracted from six-well plates with the 'Extract All' reagent (Eurobio), which is a mixture of guanidinium thiocyanate–phenol–chloroform. Northern Blot analysis was then performed using the Northern MaxTM-Gly (Ambion) kit. Ten micrograms of tRNA was denatured in glyoxal buffer at 50°C for 30 min and separated by agarose gel electrophoresis. It was then transferred for 12 h onto a charged nylon membrane (Biodyne B, Merck Eurolab). Hybridisation was carried out with three different [^{32}P] CTP-labelled riboprobes obtained by in-vitro transcription (Promega). These probes were complementary to the NS5A region of the HCV genome, and to the cellular gene GAPDH, respectively. First, the blot was hybridized with two riboprobes directed against the negative strand of HCV RNA and the GAPDH mRNA, respectively. After one night of hybridization at 68°C, the membrane was washed, then exposed to X-ray film and a phosphor screen for quantitative analysis. The amount of GAPDH mRNA was used as an internal loading control to standardize the amount of HCV RNA detected. The same membrane was subsequently hybridized with a negative-sense riboprobe to determine the level of HCV-positive strand RNA using the same approach.

7.2 Results

Anti HCV Activity

The synthesized comounds in series I were evaluated for their inhibitory effects on HCV viral RNA replication in Huh-7 cells and the results are summarized in Table 7.1-7.3.

Table 7.1: Anti- HCV activity of compounds (M1-M20) in Huh-7 cells

Compound no.	Anti - HCV activity at 50 μg/ml	
	Cell growth (%)	Inhibition of viral RNA replication (%)
M1	65	81
M2	48	84
M3	72	67
M4	79	66
M5	67	96
M6	56	99
M7	64	80
M8	71	91
M9	83	71
M10	71	100
M11	101	89
M12	112	89
M13	108	81
M14	109	86
M15	85	60
M16	NT	NT
M17	11	100
M18	84	76
M19	62	74
M20	70	80

Table 7.2: Anti- HCV activity of compounds (M21-M40) in Huh-7 cells

Compound no.	Anti - HCV activity at 50 µg/ml	
	Cell growth (%)	Inhibition of viral RNA replication (%)
M 21	83	76
M 22	83	45
M 23	54	94
M 24	60	83
M 25	62	96
M 26	88	84
M 27	51	98
M 28	62	80
M 29	59	100
M 30	88	81
M 31	80	80
M 32	86	80
M 33	57	96
M 34	67	90
M 35	15	100
M 36	74	93
M 37	78	91
M 38	78	72
M 39	81	87
M 40	93	90

Table 7.3: Anti- HCV activity of compounds (M41-M59) in Huh-7 cells

Compound no.	Anti - HCV activity at 50 μg/ml	
	Cell growth (%)	Inhibition of viral RNA replication (%)
M 41	74	99
M 42	81	97
M 43	68	92
M 44	76	65
M 45	90	82
M 46	55	100
M47	87	97
M48	63	92
M49	66	90
M50	68	96
M51	10	100
M52	91	98
M53	71	88
M54	90	88
M55	97	100
M56	48	95
M57	38	100
M58	73	89
M59	89	92
Interferon α-2B (100 IU/ml)	98	88
Ribavirin (0.12 μg/ml)	72	42

7.3 DISCUSSION:

All the synthesized compounds in series I were evaluated for their inhibitory effects on the HCV viral RNA replication in Huh-7 cells at 50 μg/ml and the results are summarized in Table 7.1-7.3.

Compounds **M11-14, M26, M30-32, M39-40, M42, M45, M47, M52, M54-55,** and **M59** were found to be more potent against HCV replication and less toxic to Huh-7 cells, as they exhibited cell growth of > 80% and inhibited HCV viral RNA replication at about 80-100%.

Some compounds (**M10, M17, M29, M35, M46, M51** and **M57**) exhibited 100% inhibition of viral RNA replication, but they were found to be toxic to Huh-7 cells, as they showed cell viability of ≤ 71%, with compound **M17, M35** and **M51** demonstrating cell growth between 10-15%.

Compound **M11-M14** exhibited >100% cell growth and inhibited replication of HCV viral RNA at about 81-89%, indicating no toxicity at the tested concentration i.e. 50 μg/ml. Most active compound in this series was found to be compound **M55** which showed 100% inhibition of viral RNA replication and at the same time offered least toxicity to Huh-7 cells with cell growth of 97%.

All the compounds are less active when compared to reference drug i.e. Ribavirin and Interferon α-2B at the specified concentration.

7.4 Structure-activity relationship study (SAR):

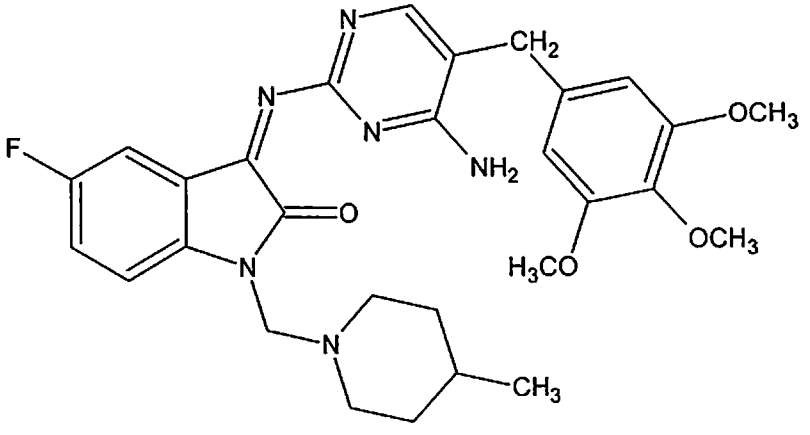
A preliminary structure-activity relationship study is shown below to asses the effect of variety of substituents on anti-HCV activity at N-1 and C-5 position of the synthesized lead moiety in series I (**M1-M59**). 2- pyridinyl piperazine moiety at N-1 position (Compound **M11**) has exhibited significant antiviral activity with 101% cell growth and 89% inhibition of viral RNA replication.

2 or 4-methyl piperidine moiety at N-1 position showed most potent anti-HCV activity with 100% inhibition of viral RNA replication (compound **M55**) as well as low toxicity to cells by facilitating 97% of cell growth (maximum), in comparison to any other substitution at that position.

Fluoroquinolone derivatives (compound **M14**) demonstrated 86% of viral RNA replication inhibition and cell growth of 109%. Order of activity at C-5 position has been found to follow the below mentioned trend: F> Cl> Br> CH_3

7.5 Conclusion

The anti-HCV activity against the viral RNA replication in Huh-7 cells harbouring the subgenomic HCV replicon BM4-5 cells showed that, compound **M55** was the most potent compound which exhibited 100% inhibition of viral RNA replication and showed less toxicity to the Huh-7 subgenomic cells with 97% cell growth.



M55

08 Antimycobacterial Activity

8.1 Test Protocol:

Antimycobacterial drug screening:

Cell culture medium and growth conditions

Mycobacterium tuberculosis $H_{37}Rv$ ATCC 27294 were obtained from the American Type Culture Collection (Rockville, Md) (Collins and Franzblau 1997). For the first three (of four) replicate experiments, $H_{37}Rv$ inocula were passaged in radiometric BACTEC 12B (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) medium, until the growth index (GI) reached 800 to 999. For the fourth replicate experiment, $H_{37}Rv$ were grown in 100 ml of 7H9GC-Tween medium. Cultures were incubated in 500 ml nephelometer flask on a rotary shaker (New Brunswick Scientific, Edison, NJ) at 150 rpm and 37°C, until they reached an optical density of 0.4 to 0.5 at 550 nm. Bacteria were washed and suspended in 20 ml of phosphate-buffered saline and passed through an 8 µm-pore size filter to eliminate clumps. The filtrates were aliquoted, stored at –80°C and used within 30 days.

Microplate Alamar Blue Assay (MABA) test-

Antimicrobial susceptibility test was performed in black, clear-bottomed, 96-well microplates (Packard Instrument Company, Meriden, Conn.) in order to minimize backgroun fluorescence. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either DMSO or distilled deionized water, and subsequent two-fold dilutions were performed in 0.1 ml of 7H9GC (no Tween 80) in the microplates. Frozen bacterial inocula were initially diluted 1:20 in BACTEC 12B medium, followed by 1:50 dilution in 7H9GC. Addition of 1/10 ml to wells resulted in final bacterial titers of 2.0 $x10^5$ CFU/ml of $H_{37}Rv$. Well containing only drug was kept to detect autofluorescence of compounds. Additional control wells consisting of only bacteria (B) and only medium (M) were also kept. Plates were kept for incubation at 37°C. On day fourth of incubation, 20 µl of 10X alamar Blue solution (Alamar Biosciences/ Accumed, Westlake, Ohio) and 12.5 µl of 20% Tween 80 were added to one B well and one M well, and the plates were reincubated at 37°C. Wells were observed at 12 hr and 24 hr for a colour change from blue to pink and for a reading of ≥ 50,000 fluorescence units (FU). Fluorescence was measured in a cytofluor II microplate fluorometer (Perspective Biosystems, Framingham, Mass) in bottom reading mode with excitation at 530 nm and emmision at 590 nm. If the B wells became pink by 24 hr, then reagents were added to the remaining plates. If the B wells remained blue or demonstrated ≤ 50,000 FU, then additional M and B wells were tested daily until a colour change occurred, at which time reagents were added to all the remaining wells. Plates were then incubated at 37°C, and results were recorded at 24 hr post-reagent adition. For fluorometric MIC's, a background subtraction was performed on all wells with a mean of triplicate M wells. Percent inhibition was defined as

1 – (test well FU)/(mean FU of triplicate B wells) X 100

The lowest drug concentration effecting an inhibition of ≥ 90% was considered the MIC. Visual MIC'S were defined as the lowest concentration of drug that prevented a colour change.

DNA gyrase inhibition:

Enzymes and substrate preparation

M. Smegmatis SN2 cells were used for purification of DNA gyrase (Chatterji et al., 2001a). *M. Smegmatis* SN2 cells were grown in modified Youman and Karlson's medium (Nagaraja and Gopinathan 1980) to mid-log phase (12-14h of growth) and harvested. The pellet was subsequently resuspended in TGEM 150 mM Tris-HCl pH 7.5, 5%(v/v) glycerol, 1mM EDTA and 2mM b-mercaptoethanol), sonicated and centrifuged at 100,000g for 90 min. The supernatant (S100) was subjected to an ammonium sulphate fractionation (70% saturation). The pellet was dissolved in and dialysed against TGEM, and loaded on to a novobiocin-sepharose column. The column was washed with TGEM, and the holoenzyme was eluted with 5M urea. The proteins were renatured by step dialysis against TGEM containing 4, 3, 2, 1 and 0 M urea. The proteins were stored in TGEM containing 100 mM potassium glutamate. Specific activity of purified DNA gyrases was calculated with 1U defined as the amount of enzyme required to completely supercoil 500 ng of relaxed pUC18 DNA at 37°C in 30 min.

Enzyme assays

Supercoiling assays were carried out by incubating 500 ng of relaxed pUC18 at 37°C in supercoiling buffer [35 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 25 mM potassium glutamate, 2mM spermidine, 2 mM ATP, 50 mg/L bovine serum albumin (BSA) and 90 mg/L yeast RNA in 5% (v/v) glycerol]. After 30 min, the reaction was stopped with 0.6% SDS. Drug-induced cleavage was performed in supercoiling buffer with supercoiled pBR322 as substrate. The reactions were carried out at 30°C for 60 min in the presence of varied amounts of inhibitors and the gyrase-DNA complex was trapped by adding 0.2% SDS followed by proteinase K digestion (final concentration of 0.8 g/L) for 30 min. The reaction mixtures were resolved on a 0.8% agarose gel in 40 mM Tris-acetate buffer containing 1mM EDTA. One or 10 U of enzyme was used for supercoiling and cleavage reactions, respectively. All experiments were performed in triplicate.

8.2 Results

Anti mycobacterial activity:

The synthesized compounds were evaluated for antimycobacterial activity at 6.25 μg/ml against *Mycobacterium tuberculosis* $H_{37}Rv$ strain in BACTEC 12B medium using the microplate alamar blue assay as summarized in table 8.1-8.3.

Series I

Table 8.1: Antimycobacterial activity of compounds (M1-M32) by Microplate Alamar Blue Assay test (MABA)

Compound no.	Antimycobacterial activity at 6.25 μg/ml	Compound no.	Antimycobacterial activity at 6.25 μg/ml
	% Inhibition		% Inhibition
M1	97	M17	68
M2	67	M18	46
M3	53	M19	NT
M4	50	M20	11
M5	44	M 21	62
M6	40	M 22	60
M7	35	M 23	57
M8	38	M 24	NT
M9	35	M 25	49
M10	33	M 26	38
M11	23	M 27	69
M12	10	M 28	6
M13	15	M 29	10
M14	100	M 30	100
M15	100	M 31	100
M16	100	M 32	100

Table 8.2: Antimycobacterial activity of compounds (M33-M59) by Microplate Alamar Blue Assay test (MABA)

Compound	Antimycobacterial activity at 6.25 µg/ml	Compound	Antimycobacterial activity at 6.25 µg/ml
	% Inhibition		% Inhibition
M 33	24	M50	24
M 34	28	M51	63
M 35	15	M52	33
M 36	30	M53	40
M37	18	M54	36
M38	25	M55	24
M39	21	M56	100
M40	19	M57	100
M 41	22	M58	100
M 42	100	M59	100
M 43	100	Isoniazid	100
M 44	100	Ethionamide	90
M 45	19	PAS	92
M 46	19	Ethambutol	98
M47	37	Ciprofloxacin	95
M48	20	Kanamycin	90
M49	45	Rifampicin	95

Series II and Series III:

Table 8.3: Antimycobacterial activity of compounds (C1-C15 and D1-D13) by Microplate Alamar Blue Assay test (MABA)

Compound	Antimycobacterial activity at 6.25 µg/ml	Compound	Antimycobacterial activity at 6.25 µg/ml
	% Inhibition		% Inhibition
C1	63	D4	31
C2	52	D5	43
C3	28	D6	58
C4	34	D7	50
C5	81	D8	86
C6	100	D9	100
C7	67	D10	60
C8	43	D11	88
C9	62	D12	62
C10	100	D13	100
C11	28	Isoniazid	100
C12	24	Ethionamide	90
C13	79	PAS	92
C14	83	Ethambutol	98
C15	100	Ciprofloxacin	95
D1	46	Kanamycin	90
D2	74	Rifampicin	95
D3	35		

Table 8.4: Secondary level antimycobacterial screening of selected test compounds

Compound No.	MIC (µg/ml)	IC_{50} (µg/ml)	SI
M1	> 6.25	NC	NC
M14	3.13	> 10	> 3.19
M15	1.56	> 10	> 6.41
M16	3.13	> 10	> 3.19
M30	3.13	> 10	> 3.19
M31	3.13	> 10	> 3.19
M32	3.13	> 10	> 3.19
M42	3.13	> 10	> 3.19
M43	3.13	> 10	> 3.19
M44	3.13	> 10	> 3.19
M56	3.13	> 10	> 3.19
M57	0.78	> 62.5	> 80.13
M58	6.25	> 10	> 1.6
M59	3.13	> 10	> 3.19
C6	3.13	> 10	> 3.19
C10	3.13	> 10	> 3.19
C15	1.56	> 10	> 3.19
D9	3.13	> 10	> 3.19
D13	3.13	> 10	> 3.19
Isoniazid	0.05	-	-
Ethionamide	2.5	-	-
PAS	8	-	-
Ethambutol	1.88	-	-
Ciprofloxacin	2	-	-
Kanamycin	5	-	-
Rifampicin	0.125	-	-

NC - Not Clear

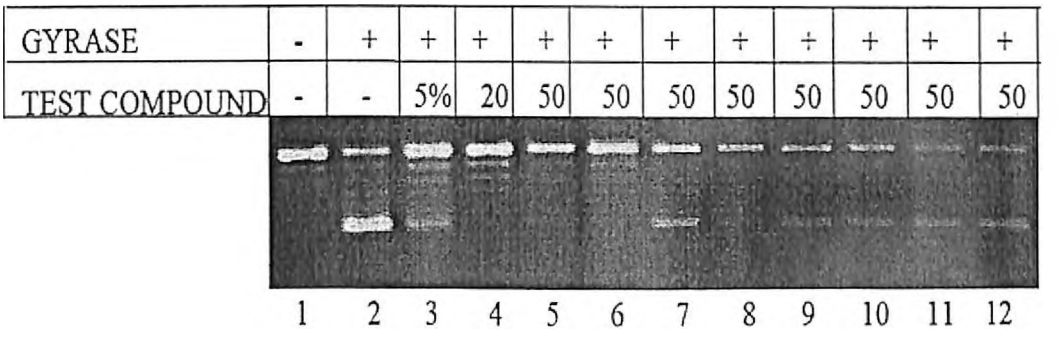
DNA gyrase Inhibition:

Compounds which exhibited significant activity in the secondary level antimycobacterial screening against *Mycobacterium tuberculosis* $H_{37}Rv$, were tested for inhibition of DNA gyrase obtained from *M. smegmatis* as reported in Table 8.5. Fig 8.1 to 8.3 represents the supercoiling assays.

Table 8.5: Summary of DNA gyrase assay with tested compounds

Compound No.	Solubility	Inhibition (µg/ml)			
		10	20	40	50
M14	DMSO	No	No	No	Yes
M16	DMSO	No	No	Yes	Yes
M30	DMSO	No	No	No	NC
M32	DMSO	No	No	Yes	Yes
M42	DMSO	-	-	-	No
M44	DMSO	-	-	-	No
M56	DMSO	-	-	-	No
M57	DMSO	-	-	-	No

NC – Not clear



Lane 1. Relaxed substrate, Lane2. Supercoiling reaction in absence of the drug,
Lane3. DMSO control, Lane 4. Drug control (MFX), Lane5-12. Compounds 1-8, 50µg/ml.

Fig 8.1: Representative Data of Supercoiling Assays

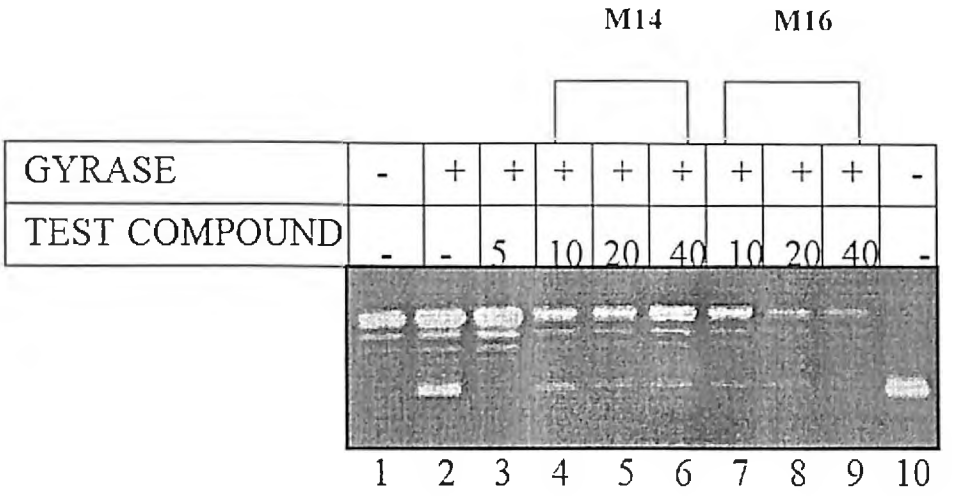


Fig 8.2: Assay with lower concentration of the compounds (Compound M14 and M16)

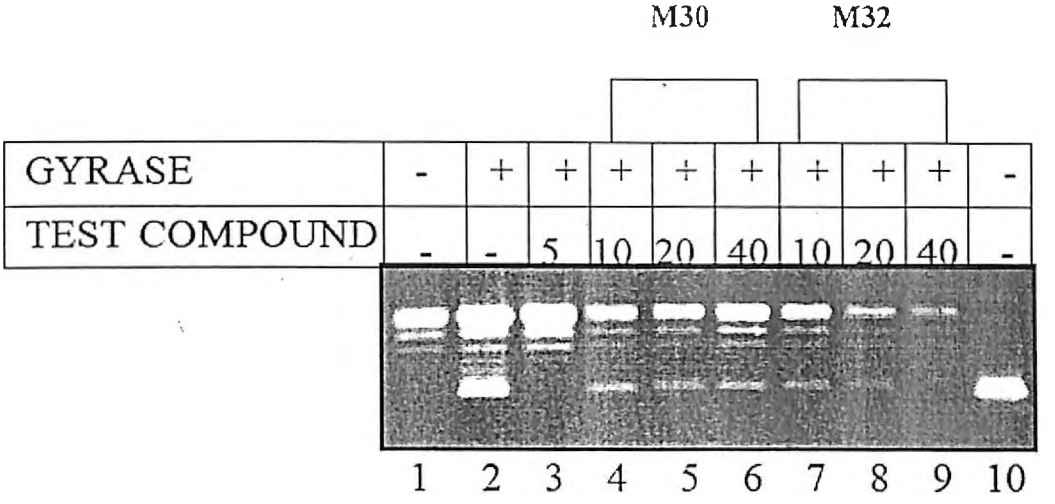


Fig 8.3: Assay with lower concentration of the compounds (Compound M30 and M32)

8.3 Discussion

Anti mycobacterial activity:

All the synthesized compounds in series I, II and III were initially screened for their antimycobacterial activity at 6.25 μg/ml against *Mycobacterium tuberculosis* $H_{37}Rv$ strain in BACTEC 12B medium using the microplate alamar blue assay (Table 8.1-8.3). Reference drugs have been included for comparison.

Compounds exhibiting percentage inhibition of ≥ 90% were considered active in this primary assay data, and were retested by serial dilutions, (beginning at 6.25 μg/ml) to determine the MIC at secondary level assay. The compounds were also screened by serial dilution to assess toxicity (IC_{50}) to a Vero cell line.

Series I

Compounds **M1, M14** to **M16, M30-32, M42-M44** and **M56-M59** exhibited greater than 90% inhibition against *M. tuberculosis* $H_{37}Rv$ strain at 6.25 μg/ml in the primary level screening. The above selected compounds were screened at secondary level for the determination of MIC value and cytotoxicity to Vero cells, where the compounds exhibited MIC values ranging from 0.78 μg/ml to > 6.25 μg/ml and selectivity index value between > 3.19 to > 80.13.

Compound **M1** showed MIC value of > 6.25 μg/ml and hence no further screening was anticipated. Compound **M58** showed MIC value of 6.25 μg/ml, while not being cytotoxic upto > 10 μg/ml to Vero cells.

Compound **M14, M16, M30-32, M42-44, M56** and **M59** exhibited MIC values of 3.13 μg/ml and IC_{50} values of >10 μg/ml. Compound **M15** exhibited MIC value of 1.56 μg/ml and IC_{50} values of >10 μg/ml.

Compound **M57** was found to be the most potent compound in this series with lowest MIC value of 0.78 μg/ml, and was not cytotoxic upto > 62.5 μg/ml to the Vero cells, resulting in a selectivity index (SI= IC_{50}/MIC) of greater than 80.13.

Compared to standard drugs, 18 drugs were found to be equipotent in the primary level screening. In the secondary level screening, compound **M57** was more potent in

terms of MIC value when compared to Ethionamide, PAS, Ethambutol, Ciprofloxacin and Kanamycin and less potent than Isoniazid and Rifampicin.

Series II

In the primary level screening, compound **C6**, **C10** and **C15** exhibited > 90% inhibition of *Mycobacterium tuberculosis* $H_{37}Rv$ strain at 6.25 μg/ml. These three compounds were selected for secondary level screening, where they showed MIC values of 3.13 μg/ml while not being cytotoxic to the Vero cells at a concentration greater than 10 μg/ml.

Series III

In the primary level screening, compound **D9** and **D13** exhibited > 90% inhibition of *Mycobacterium tuberculosis* $H_{37}Rv$ strain at 6.25 μg/ml. These compounds exhibited MIC values of 3.13 μg/ml and were not cytotoxic upto >10 μg/ml in secondary level screening,

DNA gyrase Inhibition

Some of the selected compounds, which exhibited significant activity in the secondary level antimycobacterial screening against *Mycobacterium tuberculosis* $H_{37}Rv$, were tested for inhibition of DNA gyrase obtained from *M. smegmatis*. The gyrase subunits from *M. smegmatis* are > 90% similar (Gyr A, 93.7%; Gyr B, 92%) to those present in *M. tuberculosis* at the amino acid level (Chatterji et al. 2001b).

Compound **M14**, **M16** and **M32** showed inhibition of the supercoiling reaction catalysed by DNA gyrase at 50 μg/ml concentration. Compound **M42**, **M44**, **M56** and **M57** did not interfere with supercoiling reaction at the same concentration, but showed some degree of resistance to this process when compared to control i.e. supercoiling in the absence of drug.

8.4 Structure-activity relationship study (SAR):

Anti mycobacterial activity:

Series I

Influence of various substituents at N-1, C-5 and benzyl ring of pyrimidine moiety were investigated to draw the following structure-activity relationships:

At N-1 position, dibenzylamine substitution (Compound **M1**) exhibited significant antimycobacterial activity, in preliminary screening (97% antimycobacterial activity at 6.25 µg/ml), but failed at later stage i.e. secondary level screening (MIC: - >6.25 µg/ml).

Fluoroquinolone derivative at N-1 position (Compound **M14** to **M16**, **M30-32**, **M42-44** and **M56-59**) showed promising antimycobacterial activity in primary (100% activity at 6.25 µg/ml) as well as secondary level screening (MIC : 0.78 to 3.13 µg/ml and SI between >3.19 to >80.13).

At C-5 position, substitution with the most electronegative atom i.e. fluorine is exhibiting the most potent antimycobacterial activity. In general, the order of activity with respect to 5-substituted isatin is: F> Cl> Br> CH_3

Series II

Fluoroquinolone derivative at N-1 position (Compound **C6**, **C10** and **C15**) showed promising antimycobacterial activity in primary (100% activity at 6.25 µg/ml) as well as secondary level screening (MIC: 1.56 to 3.13 µg/ml and SI between >3.19 to >6.41).

At C-5 position, substitution with the most electronegative atom i.e. fluorine is exhibiting the most potent antimycobacterial activity. In general, the order of activity with respect to 5-substituted isatin is: F> Cl> CH_3

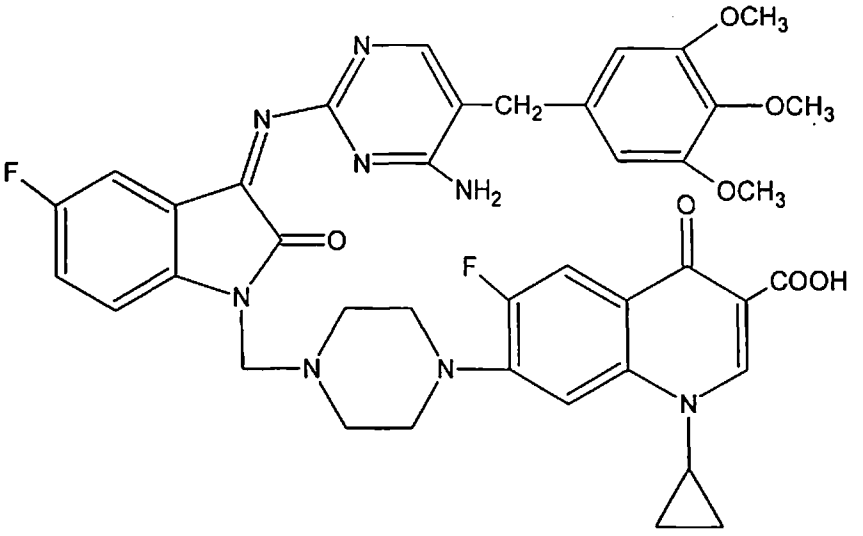
Series III

The antimycobacterial activity results emphasized, that fluoroquinolone derivative at N-1 position (Compound **D9** and **D13**) showed promising antimycobacterial activity in primary (100% activity at 6.25 µg/ml) as well as secondary level screening (MIC:- 3.13 µg/ml and SI greater than 3.19).

Fluorine substitution at C-5 position is exhibiting the most potent antimycobacterial activity, followed by $-CH_3$ and then unsubstituted isatin.

8.5 Conclusion

At N-1 position, presence of fluoroquinolone moiety is exhibiting most potent activity with compound **M57** showing lowest MIC value of 0.78 µg/ml, and was not cytotoxic upto >62.5 µg/ml to the Vero cells, resulting in a selectivity index (SI= IC_{50}/MIC) of greater than 80.13.



M57

09 Anti-bacterial Activity

9.1 Test Protocol:

***In vitro* antibacterial activity:**

Compounds were evaluated for their *in-vitro* antibacterial activity against twenty four pathogenic bacterial strains procured from the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, India. The antibacterial activity was established by agar dilution method, using Mueller-Hinton (MH) agar (Hi-Media) as the medium. Nutrient agar medium was prepared as recommended by the manufacturer.

A dilution series of test compound was prepared in separate test-tubes starting from 50 mg/ml concentration of the stock solution in DMSO. 1 ml of test compound from each of the diluted series of test tubes were taken and added to separate petri plate. To this, 19.0 ml of the prepared molten nutrient agar medium was added and mixed thoroughly. A drug-free control was also kept. All the petri-plates were then allowed to set at room temperature. Suspensions of each microorganism were prepared to contain approximately 10^6 colony forming units (cfu/ml) and were inoculated to the agar plates containing serially diluted compounds in DMSO to be tested, including the control, via standard loop. The plates were incubated at 37°C overnight (approx. 18-20 hr). The minimum inhibitory concentration (MIC) was considered to be the lowest concentration that

completely inhibited visible growth on agar plates as judged by the naked eye, disregarding a single colony or a faint haze caused by the inoculated spots.

***In vivo* antibacterial activity: (mouse protection test)**

The *in-vivo* antibacterial activity of the test compounds was determined in CF-strain male mice (20–25 g body weight, six per group). The animals used for experimental purpose has been approved by Institutional Animal Ethics Committee (IAEC) (Protocol no. IAEC/RES/11 on 21/04/2003). The mice were infected intraperitoneally with a suspension containing an amount of the indicated organism slightly greater than its lethal dose 100 (LD_{100}). The mice were then treated orally (p.o.) with a specific amount of the test compound (diluted in phosphate buffer saline and suspended with carboxy methyl cellulose) administered at 1 and 4 h after infection. ED_{50} values were calculated by interpolation among survival rates in each group after a week. It signifies the total dose of compound (mg/kg) required to protect 50% of the mice from an experimentally induced lethal systemic infection of the indicated organism.

9.2 RESULTS:

***In vitro* antibacterial activity: (Series I)**

The synthesized compounds in series I, II and III were evaluated for *in-vitro* antibacterial activity against twenty four pathogenic bacteria and the results are reported in Table 9.1-9.17.

Table 9.1 *In vitro* antibacterial activity (MIC's in μM) of compounds (M1-M6)

M. o*	M1	M2	M3	M4	M5	M6
a	37.7000	42.0000	23.1750	38.9000	37.7000	37.7000
b	37.7000	42.0000	46.3500	19.4500	37.7000	37.7000
c	37.7000	42.0000	46.3500	38.9000	37.7000	37.7000
d	1.1800	1.3125	0.7242	1.2156	0.5890	0.5890
e	0.0368	1.3125	1.4484	1.2156	0.5890	1.1781
f	18.8500	21.0000	23.1750	19.4500	37.7000	37.7000
g	18.8500	42.0000	23.1750	19.4500	37.7000	37.7000
h	37.7000	84.0000	46.3500	38.9000	4.7125	75.4000
i	0.1500	0.1640	0.3621	0.0759	0.2945	0.2954
j	37.7000	42.0000	23.1750	19.4500	18.8500	37.7000
k	9.4300	21.0000	11.5875	19.4500	18.8500	37.7000
l	0.0368	0.0205	0.0453	0.1520	0.0184	Q.1473
m	0.5900	0.6563	1.4484	0.3039	0.0368	0.0736
n	18.8500	42.0000	46.3500	19.4500	37.7000	37.7000
o	18.8500	42.0000	46.3500	19.4500	37.7000	18.8500
p	18.8500	42.0000	23.1750	19.4500	37.7000	37.7000
q	18.8500	42.0000	11.5875	19.4500	37.7000	37.7000
r	37.7000	42.0000	11.5875	19.4500	18.8500	37.7000
s	18.8500	21.0000	2.8969	19.4500	37.7000	18.8500
t	18.8500	42.0000	46.3500	19.4500	37.7000	37.7000
u	18.8500	21.0000	23.1750	19.4500	37.7000	37.7000
v	37.7000	42.0000	11.5875	19.4500	37.7000	37.7000
w	18.8500	42.0000	11.5875	19.4500	18.8500	37.7000
x	37.7000	42.0000	46.3500	19.4500	37.7000	37.7000

*a. *K. ozaenae*; b. *K. pneumoniae*; c. *S. sonnei*; d. *Plesiomonas*; e. *S. boydii*; f. *M. morganii*; g. *S. aureus*; h. *P. aeroginosa*; i. *V. mimicus*; j. *V. fluvialis*; k. *V. cholerae 0139*; l. *V. cholerae 01*; m. *V. parahaemolyticus*; n. *E. Coli NCTC10418*; o. *E. tarda*; p. *P. vulgaris*; q. *P. mirabilis*; r. *S. typhimurium*; s. *S. paratyphi A*; t. *S. typhi*; u. *S. enteritidis*; v. *C. ferundii*; w. *Enterobacter*; x. *B. megatherius*

Table 9.2: *In vitro* antibacterial activity (MIC's in μM) of compounds (M7-M12)

M. o*	M7	M8	M9	M10	M11	M12
a	19.3500	74.2000	44.1500	37.9500	19.8750	44.2000
b	38.7000	74.2000	44.1500	18.9750	39.7500	44.2000
c	38.7000	74.2000	44.1500	18.9750	39.7500	44.2000
d	0.3023	0.2898	0.1725	0.0185	0.3105	0.6906
e	0.6047	1.1593	0.6898	0.0185	0.0776	0.1727
f	19.3500	74.2000	44.1500	75.9000	9.9375	44.2000
g	19.3500	74.2000	44.1500	4.7438	39.7500	11.0500
h	38.7000	74.2000	44.1500	75.9000	39.7500	44.2000
i	0.1512	0.0724	0.0431	0.0185	0.0388	0.0216
j	38.7000	74.2000	44.1500	37.9500	39.7500	44.2000
k	19.3500	37.1000	11.0375	9.4875	9.9375	22.1000
l	0.1512	1.1594	0.0431	0.0371	0.3105	0.1727
m	1.2093	0.1449	0.0216	0.0741	0.0388	0.0432
n	19.3500	74.2000	44.1500	75.9000	39.7500	22.1000
o	19.3500	74.2000	22.0750	37.9500	39.7500	44.2000
p	19.3500	74.2000	44.1500	37.9500	19.8750	22.1000
q	19.3500	74.2000	44.1500	75.9000	39.7500	22.1000
r	19.3500	74.2000	44.1500	75.9000	39.7500	44.2000
s	9.6750	18.5500	5.5188	4.7438	9.9375	22.1000
t	19.3500	74.2000	44.1500	37.9500	39.7500	44.2000
u	19.3500	74.2000	44.1500	37.9500	39.7500	22.1000
v	19.3500	74.2000	44.1500	37.9500	19.8750	22.1000
w	19.3500	18.5500	11.0375	37.9500	39.7500	22.1000
x	19.3500	74.2000	44.1500	37.9500	39.7500	22.1000

Table 9.3: *In vitro* antibacterial activity (MIC's in μM) of compounds (M13-M18)

M. o*	M13	M14	M15	M16	M17	M18
a	22.1000	0.1225	0.4773	0.0010	39.0000	42.9000
b	44.2000	0.0077	0.2387	0.0580	39.0000	42.9000
c	44.2000	0.0077	0.1193	0.0036	39.0000	42.9000
d	1.3183	0.0306	0.0298	0.0010	39.0000	42.9000
e	0.1727	0.0612	0.0075	0.0010	39.0000	42.9000
f	44.2000	0.0306	0.0037	0.0010	39.0000	42.9000
g	44.2000	0.1225	0.0019	0.0005	39.0000	42.9000
h	44.2000	0.0306	0.0037	0.0005	39.0000	42.9000
i	0.0432	0.0002	0.0010	0.0005	2.4400	1.3400
j	44.2000	0.0002	0.0005	0.0005	2.4400	2.6800
k	44.2000	0.0005	0.0010	0.0036	2.4400	0.0210
l	0.1727	0.1225	0.0002	0.0145	1.2200	0.0048
m	0.0863	0.0019	0.0001	0.0018	2.4400	2.6800
n	44.2000	0.0010	0.0037	0.0018	39.0000	42.9000
o	44.2000	0.0005	0.0019	0.0018	39.0000	42.9000
p	44.2000	0.0002	0.0037	0.0018	39.0000	42.9000
q	44.2000	0.0005	0.0019	0.0018	39.0000	42.9000
r	44.2000	0.0001	0.0010	0.0010	39.0000	42.9000
s	11.0500	0.0038	0.0005	0.0036	39.0000	10.7000
t	44.2000	0.0077	0.0005	0.0005	39.0000	42.9000
u	44.2000	0.0153	0.0002	0.0005	19.5000	42.9000
v	11.0500	0.0306	0.0001	0.0095	0.0190	0.6100
w	44.2000	0.0010	0.0002	0.0005	0.0381	42.9000
x	22.1000	0.0005	0.0005	0.0005	19.5000	42.9000

Table 9.4: *In vitro* antibacterial activity (MIC's in μM) of compounds (M19-M24)

M. o*	M19	M20	M21	M22	M23	M24
a	18.2000	17.7000	0.6400	17.8000	0.0090	0.5550
b	18.2000	17.7000	5.1200	17.8000	0.5550	17.8000
c	18.2000	17.7000	0.6400	1.1100	0.2780	0.2780
d	0.5690	2.2100	0.6400	17.8000	0.5550	0.2780
e	18.2000	17.7000	10.2000	17.8000	1.1100	17.8000
f	18.2000	17.7000	20.5000	17.8000	1.1100	35.6000
g	18.2000	17.7000	20.5000	17.8000	1.1100	0.5550
h	36.4000	17.7000	20.5000	35.6000	0.2780	35.6000
i	0.5690	0.0086	0.3200	0.0086	0.1390	0.0086
j	0.0090	0.0086	0.3200	0.0086	0.0086	0.0086
k	0.0090	0.0173	0.3200	0.0086	0.0086	0.0086
l	2.2800	1.1000	1.2800	2.2200	0.0086	0.2780
m	1.1400	2.2100	2.5600	1.1100	0.0086	0.0086
n	18.2000	17.7000	20.5000	17.8000	0.0086	35.6000
o	36.4000	17.7000	20.5000	8.8900	0.1390	0.1390
p	18.2000	17.7000	20.5000	8.8900	0.2780	2.2200
q	18.2000	17.7000	20.5000	17.8000	0.2780	71.1000
r	18.2000	8.8400	20.5000	35.6000	8.8900	71.1000
s	18.2000	17.7000	2.5600	2.2200	0.1390	2.2200
t	18.2000	17.7000	20.5000	17.8000	0.0090	71.1000
u	18.2000	17.7000	20.5000	0.0086	0.1390	2.2200
v	2.2800	2.2100	0.6400	1.1100	0.2780	2.2200
w	0.5690	8.8400	10.2000	1.1100	0.0086	8.8900
x	18.2000	17.7000	10.2000	0.0086	0.0086	0.2780

Table 9.5: *In vitro* antibacterial activity (MIC's in μM) of compounds (M25-M30)

M. o*	M25	M26	M27	M28	M29	M30
a	4.6400	1.1600	0.5270	20.9000	21.5000	0.0290
b	18.6000	18.6000	16.9000	20.9000	21.5000	0.0002
c	0.0091	0.0091	0.0082	20.9000	21.5000	0.0000
d	18.6000	18.6000	16.9000	20.9000	21.5000	0.0036
e	37.2000	18.6000	33.8000	20.9000	43.0000	0.0002
f	37.2000	18.6000	33.8000	20.9000	43.0000	0.0002
g	2.3200	1.1600	1.0500	20.9000	43.0000	0.0004
h	37.2000	74.2000	67.5000	2.6200	86.0000	0.0004
i	0.0091	0.0091	0.0082	0.0102	0.0105	0.0290
j	0.0091	0.0091	0.0082	1.3100	1.3100	0.0004
k	0.0091	0.0091	0.0082	0.0102	0.0105	0.0290
l	1.1600	0.1450	0.0082	0.0102	0.0105	0.0290
m	0.5800	1.1600	0.5270	0.3270	0.6720	0.0004
n	37.2000	18.6000	33.8000	20.9000	86.0000	0.0004
o	37.2000	18.6000	16.9000	20.9000	43.0000	0.0004
p	37.2000	18.6000	16.9000	20.9000	43.0000	0.0004
q	37.2000	18.6000	16.9000	20.9000	43.0000	0.0004
r	37.2000	18.6000	16.9000	20.9000	43.0000	0.0002
s	0.2900	0.5800	4.2200	20.9000	21.5000	0.0036
t	37.2000	18.6000	16.9000	20.9000	21.5000	0.0002
u	9.2900	4.6400	8.4400	0.6540	21.5000	0.0002
v	0.2900	0.5800	0.5270	2.6200	1.3400	0.0290
w	4.6400	4.6400	0.2640	20.9000	21.5000	0.0002
x	18.6000	9.2800	16.9000	20.9000	21.5000	0.0002

Table 9.6 *In vitro* antibacterial activity (MIC's in μM) of compounds (M31-M36)

M. o*	M31	M32	M33	M34	M35	M36
a	0.0002	0.0017	0.0459	0.0095	0.0096	0.0094
b	0.0035	0.0138	11.7562	38.9500	19.6000	9.5750
c	0.0035	0.0017	0.0115	0.0095	0.0096	0.0748
d	0.0002	0.0035	0.0115	0.1521	0.0766	0.0374
e	0.0002	0.0008	11.7562	38.9500	19.6000	19.1500
f	0.0071	0.0008	11.7562	38.9500	19.6000	19.1500
g	0.0071	0.0008	0.0229	0.0380	0.0096	0.0094
h	0.0071	0.0278	47.0250	77.9000	78.4000	38.3000
i	0.0002	0.0017	0.0115	0.0190	0.0096	0.0374
j	0.0566	0.0008	23.5125	77.9000	19.6000	19.1500
k	0.0002	0.0017	23.5125	38.9500	19.6000	38.3000
l	0.0566	0.0069	0.0115	0.0095	0.1531	0.1496
m	0.0018	0.0035	0.1837	0.0380	0.1531	0.0374
n	0.0035	0.0017	11.7562	38.9500	19.6000	19.1500
o	0.0071	0.0035	11.7562	1.2172	19.6000	19.1500
p	0.0002	0.0008	11.7562	19.4750	19.6000	19.1500
q	0.0002	0.0035	0.0459	0.0761	0.0383	0.0094
r	0.0566	0.0035	11.7562	38.9500	19.6000	19.1500
s	0.0142	0.0069	2.9390	0.0095	9.8000	4.7875
t	0.0000	0.0004	11.7562	38.9500	19.6000	19.1500
u	0.0000	0.0008	2.9390	19.4750	9.8000	19.1500
v	0.0071	0.0002	2.9390	2.4343	4.9000	4.7875
w	0.0035	0.0004	2.9390	2.4343	1.2250	19.1500
x	0.0000	0.0004	11.7562	38.9500	0.6125	4.7875

Table 9.7: *In vitro* antibacterial activity (MIC's in μM) of compounds (M37-M42)

M. o*	M37	M38	M39	M40	M41	M42
a	0.0100	0.0090	0.0118	0.0236	0.1840	0.0002
b	20.5675	20.5350	18.5000	0.0118	1.4723	0.0002
c	0.0803	0.1604	0.0301	0.0945	0.0115	0.0079
d	0.0201	0.1604	0.0301	0.0945	0.0115	0.0079
e	20.5675	20.5350	18.5000	48.3900	1.4723	0.0079
f	20.5675	20.5350	18.5000	48.3900	23.5575	0.0079
g	0.0100	0.0090	0.0118	0.1890	0.0920	0.0002
h	41.1350	41.0700	37.0000	48.3900	23.5575	0.0002
i	0.0402	0.0802	0.0722	0.0473	0.0460	0.0157
j	20.5675	41.0700	37.0000	48.3900	1.4723	0.0157
k	10.2838	41.0700	37.0000	0.0118	1.4723	0.0157
l	0.1607	0.1604	0.0722	0.0236	0.0460	0.0005
m	0.0803	0.0401	0.0181	0.0945	0.0460	0.0002
n	20.5675	41.0700	18.5000	48.3900	23.5575	0.0002
o	20.5675	20.5350	18.5000	24.1950	23.5575	0.0002
p	20.5675	20.5350	18.5000	48.3900	0.0115	0.0002
q	0.0100	0.0090	0.1445	0.1890	0.0115	0.0157
r	20.5675	20.5350	37.0000	48.3900	0.0115	0.0002
s	20.5675	10.2675	9.2500	1.5122	5.8894	0.0002
t	20.5675	20.5350	18.5000	48.3900	0.0115	0.0002
u	20.5675	20.5350	18.5000	24.1950	23.5575	0.0002
v	5.1418	20.5350	18.5000	24.1950	23.5575	0.0002
w	20.5675	20.5350	18.5000	1.5122	11.7788	0.0005
x	10.2838	10.2675	9.2500	24.1950	11.7788	0.0157

Table 9.8 *In vitro* antibacterial activity (MIC's in μM) of compounds (M43-M48)

M. o*	M43	M44	M45	M46	M47	M48
a	0.0613	0.0149	0.0117	0.0123	0.0098	0.0098
b	0.0002	0.0074	95.6800	50.5000	39.9550	19.9775
c	0.0002	0.0149	23.9200	25.2500	19.9775	0.3121
d	0.0002	0.0019	95.6800	25.2500	19.9775	19.9775
e	0.0005	0.0019	95.6800	25.2500	19.9775	19.9775
f	0.0002	0.0019	95.6800	25.2500	19.9775	19.9775
g	0.0002	0.0297	23.9200	12.6250	2.4972	9.9888
h	0.0613	0.0074	95.6800	50.5000	39.9550	19.9775
i	0.0306	0.0074	0.0117	0.0123	0.0098	0.0049
j	0.0613	0.0037	95.6800	50.5000	19.9775	19.9775
k	0.0153	0.0074	47.8400	50.5000	19.9775	19.9775
l	0.0153	0.0297	0.0117	0.0123	0.0098	0.0098
m	0.0002	0.0005	0.7475	3.1563	2.4972	0.6243
n	0.0010	0.0005	0.0117	0.0123	0.0098	0.0390
o	0.0005	0.0009	23.9200	25.2500	0.0098	19.9775
p	0.0002	0.0009	23.9200	25.2500	0.0098	19.9775
q	0.0002	0.0019	0.0117	0.0123	0.0098	0.0098
r	0.0077	0.0037	0.0117	0.0123	0.0049	0.0098
s	0.0002	0.0074	95.6800	50.5000	39.9550	19.9775
t	0.0002	0.0149	95.6800	25.2500	0.0195	19.9775
u	0.0002	0.0074	0.0117	0.0123	0.0098	0.0098
v	0.0002	0.0037	23.9200	25.2500	0.0195	9.9888
w	0.0002	0.0018	0.3738	6.3125	2.4972	9.9888
x	0.0002	0.0009	23.9200	25.2500	0.0390	9.9888

Table 9.9: *In vitro* antibacterial activity (MIC's in μM) of compounds (M49-M53)

M. o*	M49	M50	M51	M52	M53
a	0.0047	0.0444	0.0761	0.0399	0.0719
b	0.0378	90.9800	77.9200	81.7400	36.7850
c	0.0094	22.7450	19.4800	10.2175	18.3925
d	0.0189	22.7450	38.9600	20.4350	18.3925
e	0.0189	22.7450	77.9200	20.4350	18.3925
f	0.0189	11.3725	77.9200	0.6386	18.3925
g	0.0189	45.4900	77.9200	2.5544	18.3925
h	0.0378	0.0444	38.9600	40.8700	36.7850
i	0.0094	0.0056	0.0048	0.0399	0.0359
j	0.0378	11.3725	77.9200	0.0120	36.7850
k	0.0094	0.0222	77.9200	40.8700	36.7850
l	0.0094	1.4216	77.9200	40.8700	0.1437
m	0.0756	1.4216	2.4350	0.0120	1.1495
n	0.0094	0.0444	0.0761	0.0050	0.0359
o	0.0094	11.3725	0.1522	10.2175	1.1495
p	0.1512	11.3725	0.0761	10.2175	4.5981
q	0.0094	0.0222	0.0761	0.0120	0.0180
r	0.0094	0.0222	0.0380	0.0120	0.0180
s	0.0094	22.7450	77.9200	0.0399	36.7850
t	0.0047	22.7450	77.9200	10.2175	36.7850
u	0.0094	0.0444	0.0761	0.0399	0.0719
v	0.0094	0.0444	38.9600	0.0399	36.7850
w	0.1512	11.3725	38.9600	10.2175	9.1963
x	0.0094	22.7450	38.9600	20.4350	73.5700

Series II

Table 9.10 *In vitro* antibacterial activity (MIC's in μM) of compounds (M54-M58)

M. o*	M54	M55	M56	M57	M58
a	0.0938	0.0445	0.0318	0.0078	0.0002
b	96.0500	91.1400	0.0079	0.0005	0.0152
c	96.0500	45.5700	0.0040	0.0039	0.0152
d	48.0250	91.1400	0.0040	0.0039	0.0152
e	48.0250	91.1400	0.0318	0.0010	0.0002
f	24.0125	91.1400	0.0040	0.0039	0.0002
g	48.0250	91.1400	0.3051	0.0020	0.0002
h	96.0500	45.5700	0.0318	0.0005	0.0010
i	0.0938	0.0890	0.0318	0.0039	0.0002
j	48.0250	45.5700	0.0640	0.0039	0.0002
k	48.0250	91.1400	0.0318	0.0156	0.0305
l	48.0250	91.1400	0.0318	0.0039	0.1220
m	1.5007	2.8481	0.0020	0.0002	0.0152
n	0.0469	0.0445	0.0020	0.0002	0.0076
o	0.1876	11.3925	0.0040	0.0078	0.0076
p	24.0125	22.7850	0.0005	0.0002	0.0002
q	0.0117	0.0445	0.0318	0.0002	0.0002
r	0.0235	0.0445	0.0318	0.0002	0.0002
s	3.0016	91.1400	0.0040	0.0005	0.0076
t	24.0125	91.1400	0.0079	0.0005	0.0002
u	0.0469	0.0890	0.0318	0.0002	0.0002
v	12.0063	45.5700	0.0318	0.0002	0.0076
w	48.0250	45.5700	0.0159	0.0002	0.0002
x	48.0250	45.5700	0.0040	0.0005	0.0038

Table 9.11: *In vitro* antibacterial activity (MIC's in μM) of compounds (M59 and reference compounds)

M. o*	M59	Cipro	Lome	Gati	Nor
a	0.0074	0.0092	0.0629	0.0037	0.0686
b	0.0370	0.0023	0.1259	0.0037	0.1372
c	0.0018	0.0023	2.0156	0.0037	0.5488
d	0.0009	0.0023	0.0629	0.1182	0.1372
e	0.0002	0.0023	0.5039	0.0590	0.0686
f	0.0018	0.0023	0.0629	0.0009	0.5488
g	0.0037	0.0023	0.0314	0.0009	2.1953
h	0.0074	0.0092	0.2519	0.0074	1.0976
i	0.0009	0.0023	0.0629	0.0074	0.0686
j	0.0018	0.0023	0.0629	0.0009	0.0686
k	0.0037	0.0023	0.1259	0.0009	0.1372
l	0.0296	0.0023	0.0009	0.0009	0.1372
m	0.0592	0.0023	2.0156	0.4727	0.1372
n	0.0037	0.0011	0.0314	0.0009	0.0171
o	0.0018	0.0023	0.2519	0.0009	0.5488
p	0.0004	0.0023	0.0314	0.0009	0.0343
q	0.0002	0.0023	0.1259	0.0009	0.0686
r	0.0010	0.0023	0.2519	0.0009	0.0171
s	0.0018	0.0023	0.0314	0.0009	0.0343
t	0.0010	0.0023	0.5039	0.0009	0.0686
u	0.0005	0.0023	1.0078	0.0009	0.5488
v	0.0018	0.0023	1.0078	0.0037	0.5488
w	0.0037	0.0023	1.0078	0.0009	0.1372
x	0.0005	0.0023	1.0078	0.0037	0.2744

Series III

Table 9.12: *In vitro* antibacterial activity (MIC's in μM) of compounds (C1-C5)

M. o*	C1	C2	C3	C4	C5
a	20.5950	20.7475	21.8350	3.1563	0.7434
b	0.0402	0.0405	0.0213	0.0247	0.0058
c	0.1609	0.1621	0.0853	0.0986	0.0232
d	41.1900	41.4950	0.6823	3.1563	0.7434
e	0.0201	0.0203	0.0107	0.0247	0.0116
f	41.1900	41.4950	21.8350	25.2500	23.7900
g	41.1900	41.4950	21.8350	25.2500	23.7900
h	41.1900	41.4950	43.6700	25.2500	23.7900
i	2.0574	1.2967	0.6823	0.0123	0.3717
j	2.0574	2.5934	0.0107	0.0123	0.3717
k	0.0050	0.0051	0.0053	0.0062	0.0015
l	1.2872	0.0405	2.7294	1.5781	1.4869
m	2.0574	2.5934	1.3647	3.1563	2.9738
n	0.0050	0.0051	0.0053	0.0062	0.0058
o	0.1609	0.1621	0.0426	0.0493	0.0929
p	41.1900	41.4950	21.8350	25.2500	23.7900
q	41.1900	41.4950	21.8350	25.2500	23.7900
r	41.1900	41.4950	21.8350	12.6250	23.7900
s	41.1900	10.3738	21.8350	25.2500	2.9738
t	0.0201	0.0203	0.0107	0.0247	0.0232
u	0.1609	0.1621	0.0853	0.0986	0.0929
v	0.0201	0.0203	2.7294	3.1563	0.7434
w	0.0402	41.4950	0.6823	12.6250	11.8950
x	20.5950	41.4950	21.8350	25.2500	11.8950

Table 9.13 *In vitro* antibacterial activity (MIC's in μM) of compounds (C6-C10)

M. o*	C6	C7	C8	C9	C10
a	0.0084	0.6660	0.7076	5.2338	0.0043
b	0.0005	0.0013	0.0221	0.0204	0.0011
c	0.0021	0.0416	0.0442	0.0818	0.0003
d	0.0669	0.6660	0.3538	20.9350	0.0689
e	0.0021	0.0052	0.0111	0.0204	0.0005
f	0.1338	1.3320	45.2850	41.8700	0.0344
g	0.0669	1.3320	0.7076	2.6169	0.0344
h	0.1338	0.3330	45.2850	41.8700	0.1377
i	0.0026	0.1665	0.0111	0.0102	0.0086
j	0.0026	0.0104	0.0111	0.0102	0.0086
k	0.0010	0.0026	0.0028	0.0051	0.0005
l	0.0167	0.0104	0.3538	1.3084	0.0172
m	0.0167	0.0104	0.0111	0.6542	0.0043
n	0.0010	0.0052	0.0028	0.0051	0.0005
o	0.0167	0.1665	0.1769	0.0818	0.0172
p	0.0021	0.3330	2.8303	41.8700	0.0011
q	0.0167	0.3330	90.5700	41.8700	0.0172
r	0.0042	10.6563	90.5700	41.8700	0.0043
s	0.0084	0.1665	2.8303	0.3271	0.0043
t	0.0042	0.0104	0.0221	0.0204	0.0022
u	0.0084	0.1665	0.0442	0.0051	0.0022
v	0.0335	0.3330	2.8303	0.3271	0.0086
w	0.0084	0.0104	11.3213	5.2338	0.0344
x	0.0084	0.0104	0.3538	20.9350	0.0172

Table 9.14: *In vitro* antibacterial activity (MIC's in μM) of compounds (C11-C15)

M. o*	C11	C12	C13	C14	C15
a	0.6499	22.4800	21.1475	0.0413	0.7076
b	0.0203	0.0220	0.0207	0.0026	0.0221
c	0.0101	0.1756	0.1652	0.0413	0.0442
d	20.7975	22.4800	21.1475	0.0052	0.3538
e	0.0203	0.0220	0.0207	0.0052	0.0111
f	41.5950	22.4800	21.1475	0.3308	45.2850
g	1.2998	22.4800	21.1475	1.3231	0.7076
h	83.1900	2.8100	84.5900	1.3231	45.2850
i	0.0102	0.0110	0.0103	0.0413	0.0111
j	0.0102	1.4050	2.6434	0.0413	0.0111
k	0.0406	0.0870	0.0413	0.0207	0.0028
l	0.0102	0.0110	0.0103	0.0413	0.3538
m	0.6499	0.3513	0.6609	0.0052	0.0111
n	0.0051	0.0055	0.0052	0.0026	0.0028
o	0.1625	0.1756	0.1652	0.0413	0.1769
p	20.7975	22.4800	42.2950	21.1700	2.8303
q	20.7975	22.4800	42.2950	10.5850	90.5700
r	20.7975	22.4800	42.2950	10.5850	90.5700
s	5.1994	22.4800	21.1475	5.2925	2.8303
t	0.0203	0.0110	0.0103	0.0207	0.0221
u	0.1625	0.0878	0.0826	0.0413	0.0442
v	0.6499	2.8100	1.3217	0.0413	2.8303
w	0.3250	22.4800	21.1475	0.3308	11.3213
x	20.7975	22.4800	21.1475	5.2925	0.3538

Table 9.15 *In vitro* antibacterial activity (MIC's in µM) of compounds (D1-D6)

M. o*	D1	D2	D3	D4	D5	D6
a	21.6750	10.7550	5.2818	1.3546	0.6449	0.0103
b	0.0846	0.0210	0.0103	0.0106	0.0050	0.0003
c	0.1693	0.0840	0.0825	0.0423	0.0202	0.0013
d	0.0212	0.0210	0.6602	1.3200	0.0101	0.0413
e	0.0423	0.0210	0.0053	0.0106	0.0202	0.0012
f	86.7000	21.5100	10.5640	5.4187	20.6370	0.0826
g	43.3500	43.0200	5.2819	10.8375	20.6370	0.0413
h	86.7000	21.5100	21.1275	10.8375	0.3224	0.0413
i	2.7093	0.6721	0.3301	0.0053	0.6449	0.0052
j	1.3543	1.3443	0.0052	0.0106	0.0013	0.0052
k	0.0052	0.0026	0.0052	0.0053	0.0101	0.0013
l	0.0212	0.0210	0.0026	0.6773	0.0101	0.0013
m	0.0423	0.0105	0.0825	0.0106	0.0202	0.0103
n	0.0053	0.0026	0.0026	0.0053	0.0050	0.0103
o	0.0846	0.1680	0.0413	0.0423	0.0202	0.0052
p	86.7000	43.0200	10.5638	21.6750	20.6375	0.0103
q	43.3500	43.0200	21.1275	21.6750	20.6375	0.0013
r	43.3500	43.0200	10.5638	10.8375	1.2898	0.0103
s	86.7000	10.7550	10.5638	10.8375	0.0101	0.0026
t	0.0212	0.0105	0.0052	0.0106	0.0202	0.0052
u	0.1693	0.1680	0.0825	0.0847	0.3224	0.0026
v	0.0211	0.0105	0.0026	0.0423	0.1612	0.0207
w	0.0423	10.7550	0.3301	10.8375	10.3188	0.0051
x	21.6750	21.5100	10.5638	21.6750	5.1593	0.1033

Table 9.16: *In vitro* antibacterial activity (MIC's in μM) of compounds (D7-D12)

M. o*	D7	D8	D9	D10	D11	D12
a	0.3306	0.3282	0.0680	0.6569	0.0051	21.0200
b	0.0006	0.0205	0.0085	0.0205	0.0013	0.0205
c	0.0207	0.0410	0.0340	0.0103	0.0003	0.0821
d	0.3306	0.0103	0.0021	0.0411	0.0815	0.1642
e	0.0051	0.0205	0.0085	0.0205	0.0006	0.0103
f	0.3306	0.0103	0.0085	42.0400	0.0408	21.0200
g	0.6613	0.6503	1.0880	1.3138	0.0408	21.0200
h	0.3306	42.0050	0.0021	84.0800	0.1630	2.6275
i	0.1653	0.0103	0.0085	0.0103	0.0102	0.0103
j	0.0827	0.0103	0.0680	0.0103	0.0102	1.3138
k	0.0052	0.0026	0.0043	0.0411	0.0006	0.0821
l	0.0013	0.0410	0.0043	0.0103	0.0203	0.0205
m	0.0103	0.0103	0.0021	0.0205	0.0051	0.0051
n	0.0103	0.0103	0.0085	0.0051	0.0006	0.0103
o	0.0026	0.0820	0.0021	0.1642	0.0204	21.0200
p	0.0827	1.3126	0.0680	21.0200	0.0013	21.0200
q	0.1653	42.0050	0.0085	21.0200	0.0204	21.0200
r	0.1653	84.0100	0.0043	21.0200	0.0051	21.0200
s	5.2900	2.8303	0.0021	5.2550	0.0051	0.0103
t	0.0207	0.0103	0.0680	0.0205	0.0003	0.0205
u	0.0052	0.0103	0.0085	0.1642	0.0101	0.1642
v	0.1653	0.0205	0.0043	0.3284	0.0408	0.0821
w	0.3306	1.3126	0.0021	0.3284	0.0204	2.6275
x	0.1653	0.3281	0.0680	21.0200	0.0051	10.0821

Table 9.17: *In vitro* antibacterial activity (MIC's in μM) of compounds (D13 and reference compounds)

M. o*	D13	Cipro	Lome	Gati	Nor
a	0.0002	0.0092	0.0629	0.0037	0.0686
b	0.0005	0.0023	0.1259	0.0037	0.1372
c	0.0011	0.0023	2.0156	0.0037	0.5488
d	0.0109	0.0023	0.0629	0.1182	0.1372
e	0.0005	0.0023	0.5039	0.0590	0.0686
f	0.0085	0.0023	0.0629	0.0009	0.5488
g	0.0338	0.0023	0.0314	0.0009	2.1953
h	0.2707	0.0092	0.2519	0.0074	1.0976
i	0.0005	0.0023	0.0629	0.0074	0.0686
j	0.0042	0.0023	0.0629	0.0009	0.0686
k	0.0005	0.0023	0.1259	0.0009	0.1372
l	0.0021	0.0023	0.0009	0.0009	0.1372
m	0.0021	0.0023	2.0156	0.4727	0.1372
n	0.0011	0.0011	0.0314	0.0009	0.0171
o	0.0085	0.0023	0.2519	0.0009	0.5488
p	0.0005	0.0023	0.0314	0.0009	0.0343
q	0.0005	0.0023	0.1259	0.0009	0.0686
r	0.0042	0.0023	0.2519	0.0009	0.0171
s	0.0085	0.0023	0.0314	0.0009	0.0343
t	0.0021	0.0023	0.5039	0.0009	0.0686
u	0.0005	0.0023	1.0078	0.0009	0.5488
v	0.0085	0.0023	1.0078	0.0037	0.5488
w	0.0042	0.0023	1.0078	0.0009	0.1372
x	0.0169	0.0023	1.0078	0.0037	0.2744

***In* vivo antibacterial activity:**

In-vivo antibacterial activity of some selected compounds against an experimentally induced infection of mice after oral administration is presented in Table 9.18.

Table 9.18 *In vivo* antibacterial study on *E.coli* NCTC 10419 strain

Compounds	*In vitro* MIC (in μM)	*In Vivo* ED_{50} (in mg/Kg body wt.)
M14	0.0010	0.62
M15	0.0037	1.25
M30	0.0005	0.46
M31	0.0035	1.87
M42	0.0002	0.62
M43	0.0010	1.87
M56	0.0020	1.87
M57	0.0002	0.62
M58	0.0076	1.25
Norfloxacin	0.0171	6.0
Ciprofloxacin	0.0011	1.25
Lomefloxacin	0.0314	1.87

9.3 DISCUSSION:

***In vitro* antibacterial activity**

All the synthesized compounds were evaluated for *in-vitro* antibacterial activity against twenty four pathogenic bacteria by conventional agar dilution method and results of these assays are summarized in Table 9.1-9.17. The data for norfloxacin, ciprofloxacin, lomefloxacin and gatifloxacin are included for comparison.

The antibacterial data revealed that all the test compounds showed mild to moderate activity against tested bacteria. The most sensitive organisms for the tested compounds were *S. sonnei*, *V. mimicus*, *V. cholerae 0139*, *V. parahaemolyticus*, *Prot. mirabilis*, *S. typhimurium*, *S. enteritidis*, *C. ferundii*, as the test compounds inhibited them at a concentration less than 2 μM.

Series I:

Eleven compounds (**M33** to **M40**, **M42** to **M44**) were found to be more active (MIC: 0.0002-0.0613 μM) than lomefloxacin (MIC: 0.0629 μM) against *K. ozaenae*. Twenty eight compounds (**M21** to **M27**, **M30** to **M32**, **M33** to **M44**, **M48** to **M49**, and **M56** to **M59**) were more active (MIC: 0.0002-1.11 μM) when compared to lomefloxacin (MIC: 2.0156 μM) against *S. sonnei*. Eight compounds (**M1**, **M10** to **M16**) were more active (MIC: 0.0010 - 0.1727 μM) than lomefloxacin (MIC: 0.5039 μM) against *S. boydii*.

When compared to lomefloxacin (MIC: 0.0314 μM), nine compounds (**M33**, **M35** to **M39**, **M41** to **M44**) were more active (MIC: 0.0002-0.0297 μM) against *S. aureus*. Twenty nine compounds (**M9** to **M16**, **M20**, **M22**, **M24** to **M37**, **M40** to **M44**) were more potent (MIC: 0.0473 -0.0002 μM) than lomefloxacin (MIC: 0.0629 μM) against *V. mimicus*. Fourteen compounds (**M18** to **M20**, **M22** to **M32**, **M40**, **M42** and **M44**) were more active (MIC: 0.0002-0.0210 μM) than lomefloxacin (MIC: 0.1259μM) against *V. cholerae 0139*. Fifty one compounds (**M1** to **M2**, **M4** to **M6**, **M8** to **M16**, **M19**, **M22** to **M44**, **M45**, **M48** to **M50**, **M52** to **M54**, **M56** to **M59**) were more active (MIC: 1.4484-0.0002 μM) than lomefloxacin (MIC: 2.0156 μM) against *V. parahaemolyticus*. Twelve compounds (**M33** to **M44**) were more active (MIC: 0.1837-0.0002 μM) than gatifloxacin (MIC: 0.4727 μM) against *V. parahaemolyticus*. Against *E. coli NCTC 10439*, eight compounds (**M45** to **M47**, **M49**, **M52**, **M56** to **M59**) were more active (MIC: 0.0123-

0.0002 μM) when compared to lomefloxacin (MIC: 0.0314 μM). Twenty five compounds (**M33** to **M38**, **M41** to **M59**) were more active (MIC: 0.0761-0.0002 μM) than lomefloxacin (MIC: 0.1259 μM) against *P. mirabilis*. Fifteen compounds (**M45** to **M59**) were more active (MIC: 0.0445-0.0002 μM) than lomefloxacin (MIC: 0.2519 μM) against *S. typhimurium*.Fifteen compounds (**M45** to **M59**) were more active (MIC: 0.0890-0.0002 μM) than lomefloxacin (MIC: 1.0078 μM) against *S. enteritidis*. Seventeen compounds (**M17** to **M18**, **M25** to **M27**, **M30** to **M32**, **M47**, **M49** to **M50**, **M52**, **M56**, **M59**) were found to be more active (MIC: 0.6100-0.0002 μM) than lomefloxacin (MIC: 1.0078 μM) against *C. ferundii*. Seven compounds (**M17**, **M19**, **M23**, **M27**, **M30** to **M32**) were more active (MIC: 0.5690-0.0002 μM) than lomefloxacin (MIC: 1.0078 μM) against *Enterobacter*.

Compound **M56**, which contained norfloxacin at N-1 position was found to be more active, in comparison to norfloxacin, against twenty two tested bacteria. Compound **M30**, which contains ciprofloxacin at N-1 position, was found to be more active than ciprofloxacin against seventeen tested bacteria. Other compound like **M14**, **M42** and **M57**, which also contains ciprofloxacin at N-1 position, was found to be active against eleven, fifteen and sixteen tested bacteria respectively.

Compound **M32**, bearing gatifloxacin at N-1 position was found to be more potent than ciprofloxacin against sixteen tested bacteria. Other compound like **M16**, **M59** and **M44**, which also contains gatifloxacin at N-1 position, was found to be active against thirteen, ten and five tested bacteria respectively.

Compound **M49** was found to be more potent, than lomefloxacin, against twenty two tested bacteria. When compared to lomefloxacin, compound **M31**, **M43** and **M58** (lomefloxacin derivative) were found to be more active than lomefloxacin against twenty three tested bacteria, whereas compound **M15** (lomefloxacin derivative) was found to be active against twenty two tested bacteria.

Series II:

All fifteen compounds (**C1** to **C15**) were found to be more active against *S. typhi* (MIC: 0.0021-0.0247 μM) and *S. boydii* (MIC: 0.0005-0.0247 μM), in comparison to norfloxacin (MIC: 0.0686 μM). When compared to norfloxacin (MIC: 0.5488 μM), compounds **C1** to **C15** were found to be more potent against *enteritidis* (MIC: 0.0005-0.1665 μM), *S. sonnei* (MIC: 0.0003-0.1756 μM), and *E. tarda* (MIC: 0.0086-0.1769 μM). Against *K. pneumonia* (MIC: 0.0005-0.0405 μM), and *V. cholerae 0139* (MIC: 0.0005-0.087 μM), all the fifteen compounds are more potent, when compared to norfloxacin (MIC: 0.1372 μM).

All fifteen compounds (**C1** to **C15**) were found to be more active against *E. coli* (MIC: 0.0005-0.0062 μM) when compared to norfloxacin (MIC: 0.0171 μM). Ten compounds (**C4**, **C6**, **C8-C15**) were found to be more potent (MIC: 0.0005-0.0413 μM), compared to norfloxacin (MIC: 0.0686 μM) against *V. mimicus*. Against *Plesiomonas*, four compounds (**C6**, **C10**, **C14**, and **C15**) were found to be more active (MIC: 0.0052-0.0689 μM), when compared to norfloxacin (MIC: 0.1372 μM). Nine compounds (**C2**, **C6**, **C7**, **C10**, **C11-C15**) were found to be potent (MIC: 0.0021-0.0413 μM) against *V. cholerae 01*, in comparison to norfloxacin (MIC: 0.1372 μM).When compared to norfloxacin (MIC: 0.0021-0.0167 μM), five compounds (**C6**, **C7**, **C8**, **C10**, **C14**, and **C15**) were found to be active (MIC: 0.0021-0.0167 μM) against *V. parahaemolyticus*. Against *C. ferundii*, eight compounds (**C1-C2**, **C6**, **C7**, **C9-C10**, **C14**, and **C15**) were found to be more active (MIC: 0.0086-0.3330 μM), when compared to norfloxacin (MIC: 0.5488 μM).

Compound **C6**, **C10** and **C15**, bearing norfloxacin at N-1 position, was found to be more potent than norfloxacin against all the twenty four tested bacteria. Compound **C14** was found to be active against seventeen tested bacteria when compared to norfloxacin.

Series III:

Against *K. pneumonia* (MIC: 0.0003-0.0846 μM), *V. cholerae 0139* (MIC: 0.0005-0.0401 μM), and *V. parahaemolyticus* (MIC: 0.0021-0.0825 μM), all the thirteen compounds are more potent, when compared to norfloxacin (MIC: 0.1372 μM). All thirteen compounds (**D1** to **D13**) were found to be more active against S. typhi (MIC:

0.0003-0.0680 μM) and S.boydii (MIC: 0.0006-0.0423 μM), in comparison to norfloxacin (MIC: 0.0686 μM).

When compared to norfloxacin (MIC: 0.5488 μM), compounds **D1** to **D13** were found to be more potent against *S. enteritidis* (MIC: 0.0005-0.3224 μM), *S. sonnei* (MIC: 0.0003-0.1693 μM), and *E. tarda* (MIC: 0.0026-0.1769 μM) and *C. ferudii* (MIC: 0.0085-0.3284 μM).

All thirteen compounds (**D1** to **D13**) were found to be more active against *E. coli* (MIC: 0.0006-0.0103 μM) when compared to norfloxacin (MIC: 0.0171 μM).

Eight compounds (**D4**, **D6**, **D8** to **D13**) were found to be more potent (MIC: 0.0005-0.0103 μM), compared to norfloxacin (MIC: 0.0686 μM) against *V. mimicus*. Against *Plesiomonas*, nine compounds (**D1** to **D2**, **D5** to **D6**, **D8** to **D13**) were found to be more active (MIC: 0.0101-0.0815 μM), when compared to norfloxacin (MIC: 0.1372 μM). Twelve compounds (**D1** to **D3**, **D5** to **D13**) were found to be potent (MIC: 0.0021-0.0410 μM) against *V. cholerae 01*, in comparison to norfloxacin (MIC: 0.1372 μM).

Compound **D11** is active in comparison to norfloxacin against all the twenty four pathogenic micro-organism.

When compared to norfloxacin, compound **D9** and **D13** (norfloxacin derivative) were found to be more active than lomefloxacin against twenty four tested bacteria

In-vivo antibacterial activity

In-vivo antibacterial activity of some selected compounds against an experimentally induced infection of mice after oral administration are presented in Table 9.18, along with the *in-vitro* activity against the infecting organism *E. coli NCTC 10418*. Norfloxacin, ciprofloxacin and lomefloxacin were used as reference compounds. Compound **M14** was found to be 2 times more active (ED_{50}: 0.62 mg/kg body weight) than ciprofloxacin (ED_{50}: 1.25 mg/kg) while compound **M15** was slightly more active (ED_{50}: 1.25 mg/kg) than lomefloxacin (ED_{50}: 1.87 mg/kg) against the tested bacteria. Compound **M30** was found to be 3 times more active (ED_{50}: 0.46 mg/kg body weight) than ciprofloxacin (ED_{50}: 1.25 mg/kg) while compound **M31** was equally active as lomefloxacin with ED_{50} of 1.87 mg/kg against the tested bacteria. Compound **M42** was twice more active than

ciprofloxacin with ED_{50} of 0.62 mg/kg and compound **M43** was equally active as lomefloxacin (ED_{50}: 1.87 mg/kg) against the tested bacteria. Compound **M56** was found to be 3 times more active (ED_{50}: 1.87 mg/kg body weight) than norfloxacin (ED_{50}: 6.0 mg/kg) while compound **M57** was twice more active than ciprofloxacin with ED_{50} of 0.62 mg/kg and compound **M58** was slightly more active (ED_{50}: 1.25 mg/kg) than lomefloxacin (ED_{50}: 1.87 mg/kg) against the tested bacteria. The better antibacterial activity of these compounds might be due to the inhibitory effect of both bacterial dihydrofolate reductase and DNA gyrase enzymes.

9.4 Structure-activity Relationship Study (SAR):

***In-vitro* antibacterial activity**

Series I

The antibacterial activity of the substituents at the 5th position was found to be in the following order: F> Br> CH_3> Cl

In case of substituents at N-1 position in mannich bases, it was found that unsubstituted piperazine (**M33**) exhibited marked activity against seven tested bacteria when compared to lomefloxacin. Substituted piperazine at N-1 position showed activity, with compound **M27, M52, M50** and **M23** exhibiting more activity, in comparison to lomefloxacin against seven, eight, ten and twelve tested bacteria respectively. N-1 substitution with piperidine derivative also demonstrated activity against eight tested bacteria (**M41**), in comparison to lomefloxacin.

Compound bearing fluoroquinolone derivatives at N-1 position were found to exhibit the most promising activity (**M31, M43, M58**) as they inhibited twenty three tested bacteria with lower MIC value in comparison to lomefloxacin, followed by 4- chloro phenyl piperazine substituent at N-1 position (**M49**), which exhibited higher potency against twenty two tested bacteria against the same. Other compounds bearing fluoroquinolone derivatives **M14, M16, M30, M32, M42, M44, M56, M57**, and **M59** also exhibited significant activities compared to their reference compounds.

Series II

Substituted piperazine (3-chloro phenyl piperazine) at N-1 position of compound **C14** showed significant activity against all the twenty four tested bacteria. Compounds bearing fluoroquinolone derivative at N-1 position were found to exhibit promising activity, with compound **C6, C10** and **C15**, inhibiting twenty four tested bacteria with MIC value lower than norfloxacin.

The antibacterial activity of the substituents at the 5th position was found to be in the following order: F> Cl> CH_3.

Series III

Compound **D9** and **D13** (norfloxacin derivative) exhibited more potency against all the twenty four tested bacteria when compared to norfloxacin. Compound **D11** bearing para-chloro phenyl piperazine moety at N-1 position demonstrated more activity, when compared to norfloxacin, against all the twenty four tested bacteria.

The order of activity of the substituents at C-5 position was the following: F> CH_3> H

***In-vivo* antibacterial activity**

Compound with fluoroquinolone derivatives at N-1 position demonstrated equal or more potency, in terms of activity, when compared to the corresponding reference drug. The in vivo antibacterial activity of the substituents at the 5th position was found to be in the following order: Br> Cl> F> CH_3

9.5 CONCLUSION

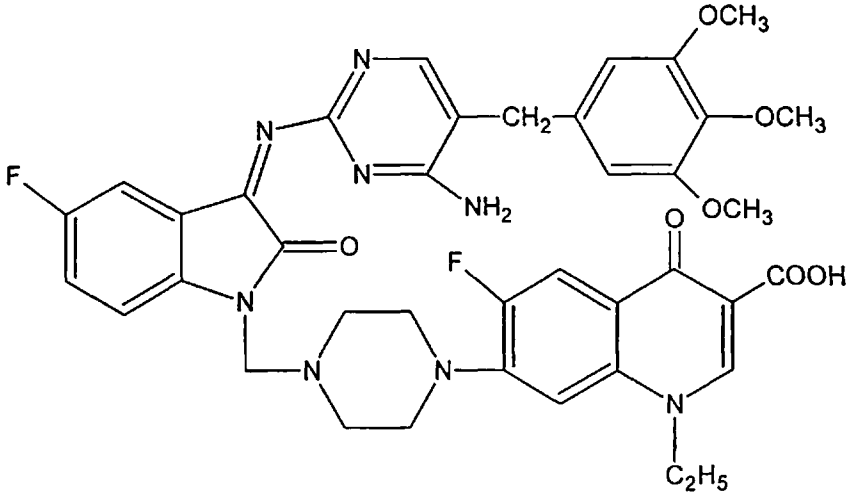
***In-vitro* antibacterial activity**

Compound **C6, C10, C15, D9** and **D13**, bearing norfloxacin at N-1 position was found to be more potent than norfloxacin against all the twenty four tested bacteria. Compound **M31, M43** and **M58** (lomefloxacin derivative) were found to be more active than lomefloxacin against twenty three tested bacteria.

Compound **D11** and **M49**, bearing 4-chloro phenyl piperazine moiety at N-1 position, was found to be active in comparison to norfloxacin against twenty four and twenty two pathogenic micro-organism respectively. Compound **C14**, containing 3-chloro phenyl piperazine at N-1 position, was found to be active against seventeen tested bacteria when compared to norfloxacin.

***In-vivo* antibacterial activity**

Compound **M30** and **M56**, containing fluoroquinolone moiety at N-1 position and electron withdrawing group (Br, F) at C-5 position were found to be the most active compound which exhibited 3 times more activity (ED_{50}: 0.46 mg/kg and 1.87 mg/kg body weight respectively) when compared to ciprofloxacin (ED_{50}: 1.25 mg/kg) and norfloxacin (ED_{50}: 6.0 mg/kg) respectively.



M56

10 Summary and Conclusion

SUMMARY AND CONCLUSION

Schiff bases of isatin and its various 5-substituted derivatives (Cl, Br, CH_3, and F) with 2,4-diamino-5-(substituted benzyl)pyrimidine were synthesized by Schiff reaction. Mannich bases of the corresponding Schiff base were prepared with different secondary amines (Total 87 compounds).

Purity of the compounds was ascertained by TLC and their structures were elucidated by spectral (IR, ^{1}H-NMR, Mass) and elemental analysis.

The synthesized compounds were evaluated for anti-HIV activity as well as anti-HCV, antimycobacterial, and antibacterial (both *In vitro* and *in vivo*) activity for broad-spectrum antimicrobial properties.

Mannich bases with fluoroquinolone moieties in series I, II and III demonstrated maximum inhibition of the cytopathic effect of HIV-1(IIIB), in MT-4 cell line, with selectivity index up to 19.85.

The anti-HIV activity of synthesized mannich bases, measured in HIV-1 III_B-infected CEM cells, revealed that fluoroquinolone derivative at N-1 position is the only substituent in all the 3 series, which provided marked anti-HIV activity with selectivity index of > 2.

Mannich base with morpholine substituent, followed by fluoroquinolone moiety, at N-1 position, exhibited considerable inhibition of HIV-1 RT.

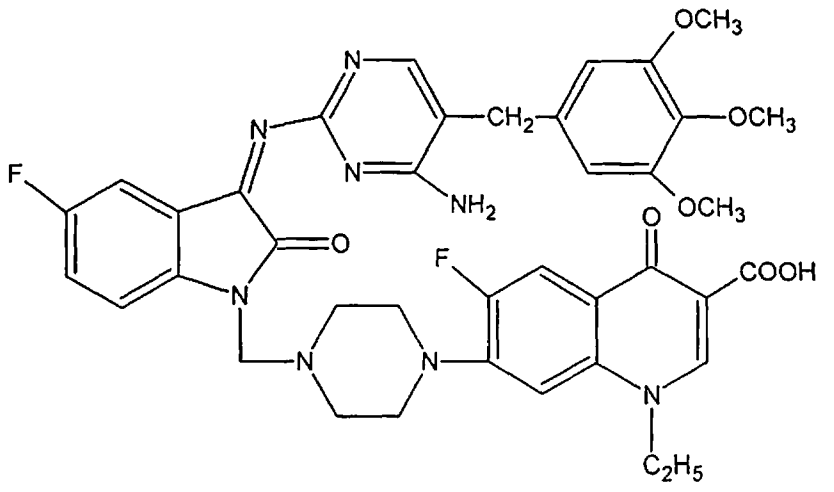
The anti-HCV activity against the viral RNA replication in Huh-7 cells showed that, ortho or para substituted methyl group on piperidine moiety demonstrated the most potent activity with 100% inhibition of viral RNA replication and facilitating 97% cell growth.

Antimycobacterial activity against *Mycobacterium tuberculosis* $H_{37}Rv$ strain showed that fluoroquinolone derivatives were the most active, with MIC value of 0.78 µg/ml and selectivity index (SI= IC_{50}/MIC) of greater than 80.13 in secondary level screening. Mannich bases containing fluoroquinolone moieties showed inhibition of the supercoiling reaction catalysed by DNA gyrase.

In vitro antibacterial studies have shown that compound with fluoroquinolone derivatives and substituted piperazine moiety exhibited potent activity compared to reference drugs. *In vivo* antibacterial studies have shown that compound with fluoroquinolone derivatives demonstrated potent activity compared to reference drugs.

The most potent compound was found to be compound **M56**, which exhibited anti-HIV-1 cytopathogenicity with EC_{50}=12.1 µM. SI=13.23, and %protection of 99.6% and also demonstrated broad-spectrum antimicrobial properties against HCV (95% inhibition of viral RNA replication), mycobacterial (MIC value 3.13 µg/ml and SI of > 3.19), and bacterial (*In vitro* MIC of 0.002 µM and *in vivo* EC_{50} of 1.87 mg/Kg body wt.) pathogenesis.

1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7[[N^4-[3'-(4'-amino-5'-trimethoxybenzylpyrimidin-2'-yl)imino-1'-(5-fluoroisatinyl)] methyl]N^1-piperazinyl]-3-quinoline carboxylic acid



Future perspectives

Although thousand of compounds have been identified as inhibitors of HIV-1 RT *In vitro* but further studies can only confirm whether they possess the appropriate pharmacological properties, such as deliverable formulation, bioavailability and attainment of effective plasma concentration and low-toxicity, which render them useful as anti-HIV therapeutics.

Reverse transcriptase has been and will continue to be a major target for anti-HIV drug development. Current NNRTI's in preclinical evaluation are exceptionally potent but drug resistance is an issue, which will continue to plague HIV therapy. Rigorous investigations of the mechanisms of NNRTI inhibition and resistance will assist in defining possible new therapeutic combination. However, the numerous advances that are being made in our understanding of RT structure and function will certainly figure prominently in future RT inhibitor design and may finally yield a drug with long-term benefit in infected patients.

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Appendix

Published

1. D. Sriram, **T. R. Bal**, P. Yogeeswari; "Design, synthesis and biological evaluation of novel non- nucleoside HIV-1 reverse transcriptase inhibitors with broad-spectrum chemotherapeutic properties", *Bioorg. Med. Chem.*, 12, 5865-5873, 2004.
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