Design, Synthesis and Development of Potential anti-HIV-1 Inhibitors, Targeted To HIV-1 Associated Topoisomerase IIβ Kinase

DOCTOR OF PHILOSOPHY

By

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Enrollment No: 10LTPH03

December-2017

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A thesis Submitted to University of Hyderabad

For the award of a Ph.D. degree in Biotechnology and Bioinformatics

by

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DECLARATION

I**, Kurumurthy Kammari,** hereby declare that the work presented in this thesis, entitled as "**Design, Synthesis and Development of Potential anti-HIV-1 Inhibitors, Targeted to HIV-1 Associated Topoisomerase IIβ Kinase**" has been carried out by me under the supervision of **Prof. Anand K. Kondapi**, Department of Biotechnology and Bioinformatics. To the best of my knowledge this work has not been submitted for the award of any degree or diploma at any other university or institution. I hereby agree that my thesis can be deposited in Shodganga/INFLIBNET. A report on plagiarism statistics from the University Librarian is enclosed.

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CERTIFICATE

This is to certify that the thesis entitled "**Design, Synthesis and Development of Potential anti-HIV-1 Inhibitors, Targeted to HIV-1 Associated Topoisomerase IIβ Kinase**" is a record of bonafide work done by **Mr. Kurumurthy Kammari**, a research scholar for Ph.D. programme in department of biotechnology and bioinformatics, university of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other university or institution for the award of any degree or diploma. I recommend his thesis for submission towards the partial fulfilment of Doctor of Philosophy degree in Biotechnology and Bioinformatics.

Part of this thesis have been:

A. Published in the following publication:
1. **Kurumurthy Kammari, et. al. Future Medicinal Chemistry,11 Sep 2017; Doi.Org/10.4155/fmc-2017-0091**

B. Presented in the following conferences
1. **EMBO conference-2017, (International).**

Further, the student has passed the following courses towards fulfillment of coursework requirement for Ph.D.

S. No	Course code	Name	Credits	Pass/Fail
1	BT801	Research Methodology	4	Pass
2	BT802	Research Ethics & Management	2	Pass
3	BT803	Laboratory Work	4	Pass
4	BT804	Biostatistics	2	Pass

Supervisor **Head of Department** **Dean of School**

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Last, I thank to CSIR for giving me fellowship and DST to complete my Ph.D.

Kurumurthy Kammari

Scope of the thesis:

This thesis presents synthesis of novel molecules and methods of synthesis and characterization of synthesised compounds

Collaborations:

In silico design studies were carried out by Ms. B. Akhila

In vitro biological activity studies were carried out by Mr. D. A. Kiran Kumar

Abbreviations Table:

TOPO IIβK_{HIV}	: HIV-1 associated Topoisomerase β kinase
AIDS	: Acquired Immuno-Deficiency Syndrome
AZT	: Azidothymidine (Zidovudine)
pM	: Picomolar Concentration
nM	: Nanomolar Concentration
mmole	: Milli Mole
FDA	: Food and Drug Administration
HAART	: Highly Active Anti-Retroviral Therapy
HIV	: Human Immunodeficiency Virus
IC_{50}	: 50% Inhibitory Concentration
CC_{50}	: 50% Cytotoxic Concentration
QSAR	: Quantitative Structure Activity Relationship
3D-QSAR	: 3-Dimensional Quantitative Structure Activity Relationship
CoMFA	: Comparative Molecular Field Analysis

Contents

Chapter -1

Introduction

Human Immunodeficiency Virus-1 (HIV-1)

HIV-1 is frequently associated with the development of Acquired Immune Deficiency Syndrome (AIDS), a condition in humans with low immunity and higher susceptibility to opportunistic infections. HIV-1 belongs to the genus lenti virus and family Retroviridae. The AIDS has evolved as a major cause of T cell impairment as the virus infects $CD4^{+}$T cells of infected individuals **[1][2].**

According to The Joint United Nations Programme on HIV and AIDS (UNAIDS) estimation, so far 27 million people were died around the world with virus infection, until 2014, approximately 36.9 million people are living with HIV globally, approximately 17.2 million are men, 16.8 million are women and 3.4 million are less than 15 years old. There were about 1.8 million deaths from AIDS in 2010, down from 2.2 million in 2005 (Adopted from UNAIDS Report 2016).

The world has committed to ending the AIDS epidemic by 2030. How to reach this bold target within the Sustainable Development Goals is the central question discussed in the United Nations General Assembly high-level meeting on ending AIDS, during 8 to 10 June 2016 (Adopted from UNAIDS Report 2016). The extraordinary accomplishments of the last 15 years have inspired global confidence that this target can be achieved.

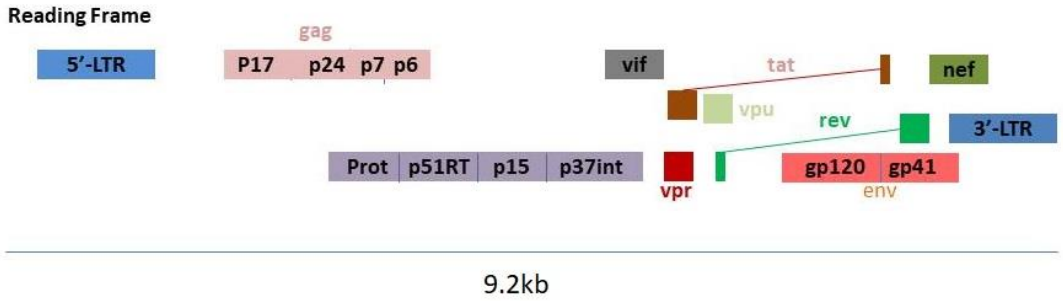
HIV-1 viral genome structure and function:

HIV-1 is a spherical virus particle and with 100 nm diameter. The HIV-1 genome size is about 9.8kb in which two structural genes (Gag and Env), three enzymes (RT, IN and PR) and six accessory genes, genome containing Long Terminal Repeats (LTR) sequence on both ends, all these components help in the viral replication **[3].**

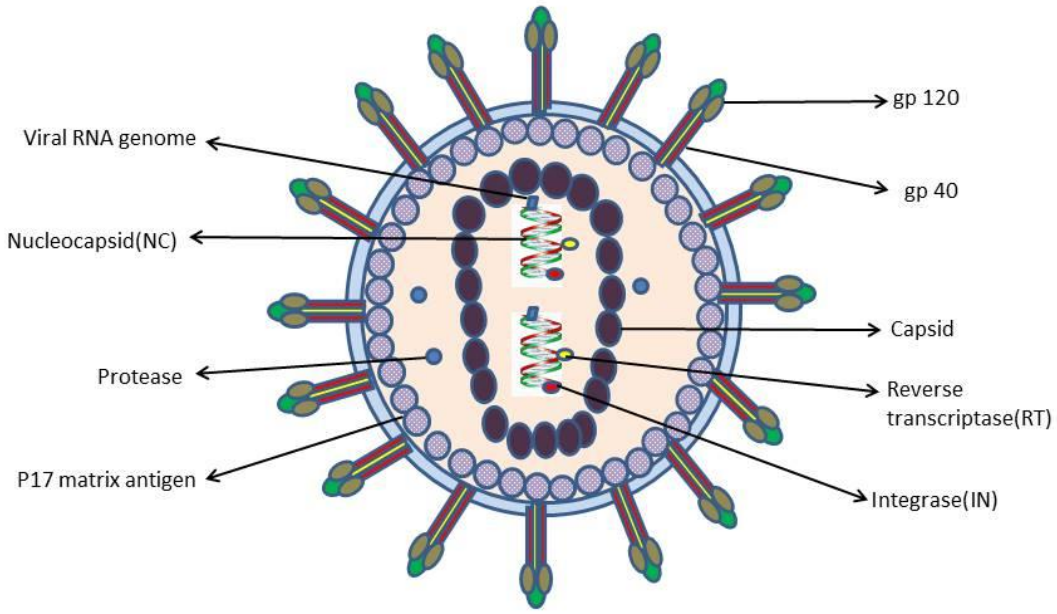
Figure 1: (A) HIV-1 genome and (B) HIV-1 virion and potential antiviral targets

Figure 1:

(A) HIV-1 genome



(B) HIV-1 virion and potential antiviral targets



Structural Genes:

Structural genes *gag*, *pol*, and *env* were synthesized as poly-protein precursors and later they were cleaved into mature proteins by viral or cellular protease.

***Gag*:** The 55-kDa gag protein codes for a precursor gag polyprotein which is processed by viral protease during maturation to (MA matrix protein), Capsid (CA), nucleocapsid (NC), spacer peptide 1 (SP1), spacer peptide 2(SP2) and p6 protein **[4] [5].**

Pol: pol gene encodes for the enzymes Reverse Transcriptase (RT), RNase H, Integrase (IN) and HIV Protease (PR).

Env: env codes for gp160, which is cleaved by host proteases in the endoplasmic reticulum of the host cell, the post-translational processing produces a surface glycoprotein, gp120 (SU) and gp41 (TM) which embedded in the viral envelope to enable the virus to attach to and fuse with target cells.

Enzymes:

Reverse Transcriptase (RT): RT is RNA dependent DNA polymerase, it is involved in viral cDNA synthesis.

Integrase (IN): IN is required for the integration of viral DNA into the host genome.

Protease (PR): PR is required for the cleaving of *gag* precursor polyprotein to structural proteins.

Regulatory proteins:

These are six regulatory proteins of HIV-1 are coded by the genes *tat*, *vif*, *vpr*, *vpu*, *nef* and *rev* which are very crucial for the viral propagation.

Viral infectivity factor (Vif): Vif inhibits the host antiviral activity of APOBEC family by directly binding to it and inducing degradation by triggering the ubiquitination pathway **[9].**

Negative regulatory factor (Nef): Nef helps in regulating the host cellular machinery by increasing the viral infectivity, it helps in down-regulating the surface molecules expressed in host immune cells such as MHC molecules present on infected APCs and T Lymphocytes. **[10] [11] [12].**

Viral protein R (Vpr): Vpr is required for efficient viral replication and immune suppression in proliferating cells by inducing G2 cell cycle arrest and apoptosis **[14] [15].** It is also important in viral replication in non-dividing cells go host such as macrophages **[16].**

Viral protein U (**Vpu):** Vpu is essential in the releasing of viral particle **[17] [18] [15].**

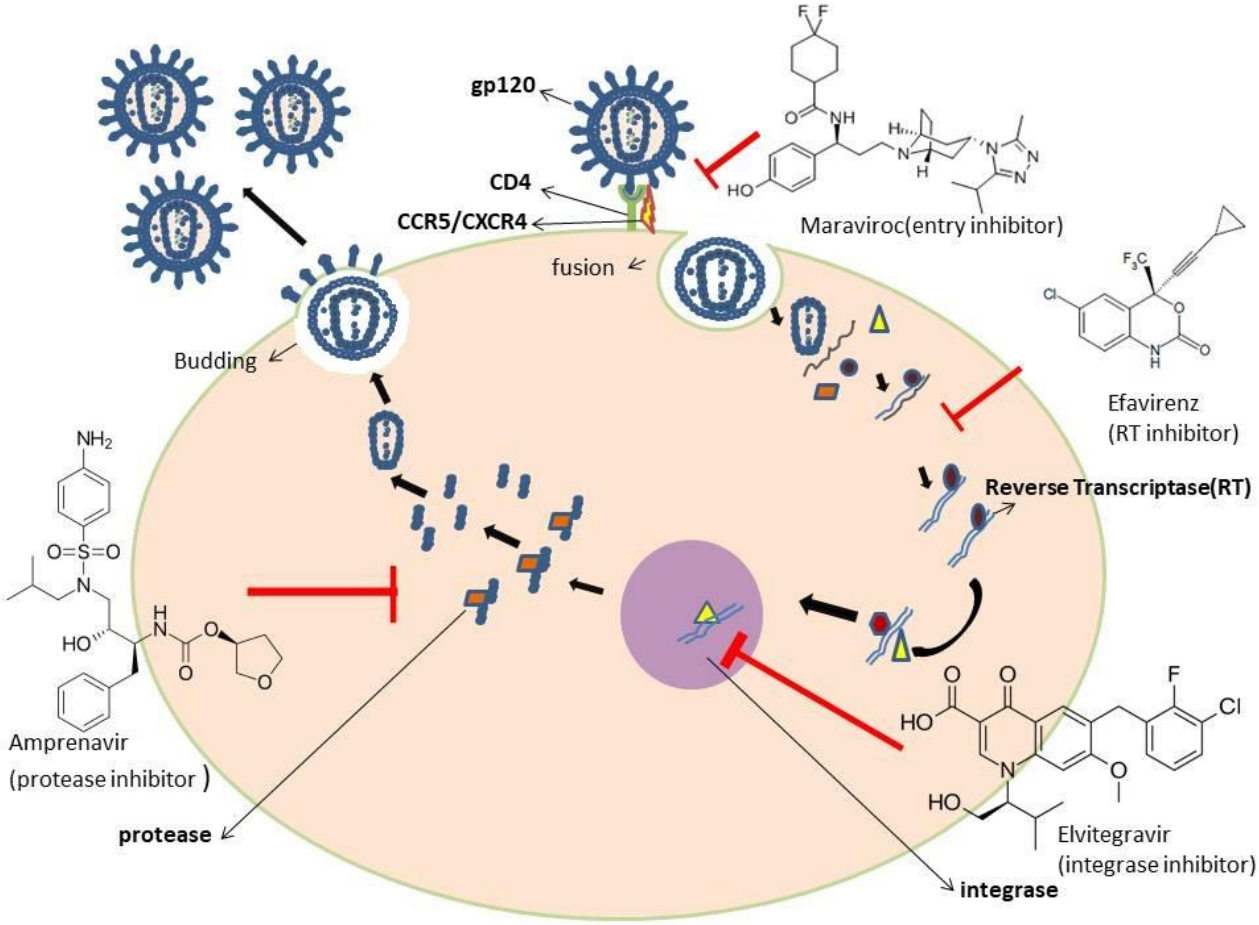
Tat: (Trans-activator of Transcription) Tat is a regulatory protein which plays crucial role in the regulation of the viral genome transcription, ensuring efficient synthesis of viral mRNAs and helps in the release of virions from infected cells. Tat is expressed as 86 to 101 amino acids depending on subtype of virus. Tat is a RNA binding protein which binds to TAR (Tans-activator Response Element) in RNA stem-loop secondary structure near the 5' LTR and stimulates the transcription of viral mRNA **[6] [7] [8].**

***Rev*:** (Regulator of Expression of Virion proteins): The Rev protein is essential for the protein expression of HIV-1. It regulates the protein expression by binding to the viral genome by specific arginine-rich RNA-binding motif which also acts as a NLS (nuclear localization signals) required for the transport of Rev to the nucleus from cytosol during viral replication. Rev also plays an important role in recognizing a complex stem-loop structure of the mRNA *env* located in the intron separating coding exon of Tat and Rev, known as the HIV Rev response element (RRE). Rev plays an important role in the synthesis of major viral proteins essential for viral replication and its propagation**[13].**

HIV-1 life cycle and possible drug targets:

Viral life cycle comprises of several steps, which can be targeted by anti-HIV-1 drugs. These steps include entry, reverse transcription, integration, transcription, assembly and budding (see in figure 2)

Figure 2: HIV-1 life cycle and drug targets



Entry (Binding and Fusion):

The life cycle of HIV-1 begins with the binding and fusion into the $CD4^{+}$T lymphocyte. Binding of the trimeric viral envelope protein gp120 with CD4 of host cell membrane along with co-receptor CXCR4 or CCR5, the gp120 and CD4 interaction induces a conformational

change in trimeric protein and forms a helix bundle that fuse in the host cell membrane leading fusion, internalization and release of viral capsid in cytosol of host cell **[22].**

Reverse Transcription:

After the entry of viral RNA genome into the helper T cell, the single stranded RNA undergoes reverse transcription to convert into double stranded DNA, this process is known as Reverse Transcription which is mediated by a viral coded enzyme Reverse Transcriptase.

Integration:

Synthesized proviral DNA in the presence of host and viral proteins forms pre-integration complexes and translocated to nucleus, where the proviral DNA integrates into host genome with the help of a viral enzyme called as integrase. The integrated DNA into the host genome is called provirus. Provirus may remain in the latent stage for several years together or it may produce new copies of HIV-1.

Transcription:

When the provirus is in active state, it uses the host RNA polymerase to synthesize the viral genetic material as short strand of RNA (mRNA) which is transcribed into new viral proteins by using host cellular machinery.

Assembly:

Assembly of virus initiated with viral capsomeres forms icosahedral particle with RNA molecules in the cytoplasm of host cell. Viral protease cleaves polyprotein gag (p55) and gag-pol (p160) to completely process as matured viral proteins. Once the viral RNA genome and all the essential proteins are synthesized, the virus assembly takes place and forms a new viral particle which is ready for maturation and release.

Maturation and Budding:

The newly assembled viral particles in the host cytoplasm of infected T cell undergo maturation and released out of the cell by a process of budding.

Antiretroviral therapy:

The first effective therapy against HIV-1 was the Nucleoside Reverse Transcriptase Inhibitor (NRTI), Zidovudine (AZT). It was approved by FDA to treat AIDS after successful clinical trials in 1987, subsequently several nucleoside inhibitors were also approved by FDA and made available to HIV-1 infected patients. Initially single drug therapy (monotherapy) was used to treat HIV infected patients and found it was not always effective in reducing viral RNA levels and this led to disease progression and emergence of drug resistant variants. Later in the late 1990s, use of combination drug therapy known as Highly Active Anti-Retroviral Therapy (HAART) was introduced for effective treatment of HIV-1 infection and AIDS progression. This therapy includes combination of three or more antiretroviral drugs which affects different sites of viral replication for neutralization of virus in HIV-1 infected patients. The Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI), Protease Inhibitor (PI) and fusion inhibitor were employed in combination therapy under HAART. HAART has been successfully altering the viral prognosis in HIV-1 infected patients from high mortality rate to chronic and manageable disease progression and this therapy has been still practicing in most of the countries in the world for the reduction of HIV-1 cases **[20] [21].** Due to several limitations of HAART for the treatment of AIDS, scientific community is still working to find the new drug targets and drugs in eradicating the HIV-1 from the infected patients. The HIV medicines are approved by USFDA is given in Table 1.

Table 1: FDA approved AIDS drugs (Adapted from www.aidsmeds.com).

Table 1: (A)

Nucleoside Reverse Transcriptase Inhibitors (NRTIs)			
S.No.	**Generic Name**	**Brand Name**	**FDA Approval Date**
1	abacavir	Ziagen	December 17, 1998
2	didanosine	Videx	October 9, 1991
3		Videx EC	October 31, 2000
4	emtricitabine	Emtriva	July 2, 2003
5	lamivudine	Epivir	November 17, 1995
6	stavudine	Zerit	June 24, 1994
7	tenofovir disoproxil fumarate	Viread	October 26, 2001
8	zidovudine	Retrovir	March 19, 1987
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)			
9	efavirenz	Sustiva	September 17, 1998
10	etravirine	Intelence	January 18, 2008
11	nevirapine	Viramune	June 21, 1996
		Viramune XR	March 25, 2011
12	rilpivirine	Edurant	May 20, 2011
Integrase Inhibitors			
13	dolutegravir	Tivicay	August 13, 2013
14	elvitegravir	Vitekta	September 24, 2014
15	raltegravir	Isentress	October 12, 2007

Table 1: (B)

Protease Inhibitors			
S.No.	**Generic Name**	**Brand Name**	**FDA Approval Date**
16	atazanavir	Reyataz	June 20, 2003
17	darunavir	Prezista	June 23, 2006
18	fosamprenavir	Lexiva	October 20, 2003
19	indinavir	Crixivan	March 13, 1996
20	nelfinavir	Viracept	March 14, 1997
21	ritonavir	Norvir	March 1, 1996
22	saquinavir	Invirase	December 6, 1995
23	tipranavir	Aptivus	June 22, 2005
Fusion Inhibitors			
24	enfuvirtide	Fuzeon	March 13, 2003
Entry Inhibitors			
25	maraviroc	Selzentry	August 6, 2007
Pharmacokinetic Enhancers			
26	cobicistat	Tybost	September 24, 2014

Table 1: (C)

Combination HIV Medicines			
S.No.	**Generic Name**	**Brand Name**	**FDA Approval Date**
27	abacavir and lamivudine	Epzicom	August 2, 2004
28	abacavir, dolutegravir, and lamivudine	Triumeq	August 22, 2014
29	abacavir, lamivudine, and zidovudine	Trizivir	November 14, 2000
30	atazanavir and cobicistat	Evotaz	January 29, 2015
31	darunavir and cobicistat	Prezcobix	January 29, 2015
32	efavirenz, emtricitabine, and tenofovir disoproxil fumarate	Atripla	July 12, 2006
33	elvitegravir, cobicistat, emtricitabine, and tenofovir alafenamide fumarate	Genvoya	November 5, 2015
34	elvitegravir, cobicistat, emtricitabine, and tenofovir disoproxil fumarate	Stribild	August 27, 2012
35	emtricitabine, rilpivirine, and tenofovir alafenamide	Odefsey	March 1, 2016
36	emtricitabine, rilpivirine, and tenofovir disoproxil fumarate	Complera	August 10, 2011
37	emtricitabine and tenofovir alafenamide	Descovy	April 4, 2016
38	emtricitabine and tenofovir disoproxil fumarate	Truvada	August 2, 2004
39	lamivudine and zidovudine	Combivir	September 27, 1997
40	lopinavir and ritonavir	Kaletra	September 15, 2000

Almost 26, anti-HIV-1 drugs have been licensed for the treatment of AIDS. Non-nucleoside reverse transcriptase inhibitors (NNRTI) - 4, Nucleoside reverse transcriptase inhibitors (NRTI) -8, Integrase inhibitor- 3, Fusion inhibitor -1, Protease inhibitor (PI)-8, Entry inhibitor-1, Pharmacokinetic-enhancer - 1. Drug resistant viral variants in HIV-1 infected patients are a failure of treatment AIDS. Highly active anti-retroviral therapy (HAART) (combinations of potent antiviral drugs) is an effective treatment than monotherapeutic drug. But prolonged HAART leads to multidrug resistance due to resistance mutations of HIV-1 [**30**]. Hence failure of HAART, emergency need of new potent drug and new viral target needed.

Even though some drug targets have been identified to neutralizing the HIV-1, emergence of resistance is continuing and search for new drug targets is highly demanding for elimination of the dreadful mutant HIV-1 strains contributing to drug resistance. Significant work is still conducting on viral and host specific interactions to understand the newer therapeutics at molecular level. As such HIV-1 associated Topoisomerase IIβ kinase is one of the new viral targets.

Recent studies on Topoisomerase IIβ phosphorylation has been identified in HIV-1viral progression. Subsequently a 72kDa protein which is Phosphorylating the Topoisomerase IIβ during HIV-1 replication was observed in a purified virus concentrate **[23]** that suggested the presence of a novel kinase. This fascinating drug target has made to explore the possibility of stymied this vital pathway in the life-cycle of HIV-1 to prevent the viral growth.

DNA Topoisomerases:

DNA Topoisomerases are the essential nuclear enzymes to all living cell, these enzymes regulate the topological conformation of DNA during the replication, transcription and segregation of chromosomes **[18].** Topoisomerases are classified into two types, Type-I and Type-II Topoisomerases. Lower eukaryotes express only one isoform of Type –II

Topoisomerase whereas; higher eukaryotes express two unique isoforms, Topoisomerases II α and β which help in cellular division and development. Both the isoforms are similar in structural and catalytical aspects, but differ in biochemical, genetical and immunological aspects. The 'α 'isoform has been shown to express in all proliferating cells and tissues, while β isoform were reported in all cell types. Topoisomerase II binding and cleaving sites have been identified in the LTR regions of HIV-1 and also in the integration site in human DNA **[24].** It has been established that the levels of Topo II α and β isoforms are not only enhanced in viral infection, but also these isoforms undergo phosphorylation during HIV-1 infection **[25] [26] [27].**

Earlier reports have shown that during the HIV-1 infection, Topoisomerase II is present as phosphoproteins in HIV-1 infected cells **[26].**

Topoisomerase II beta kinase:

Kondapi et al. (2005) characterized phophorylation of Topoisomerase II alpha and beta. These studies showed that Topo II alpha phosphorylation activity is high at 8 hour and 72 hours of post infection, while beta isoform phosphorylation is high at 4 hours and 16 hours onwards of post infection. Analysis of sensitivity of these two-kinase activities in the presence of various inhibitors showed that Topo II alpha kinase is sensitive to Map kinase inbitors, while Topo II beta exhibits very low sensitivity with the known kinase inhibitors **[28].** Topo II beta kinase was purified from the virus concentrates and enzymatic properties are analysed, results showed that beta kinase is a 72 kDa protein with serine/ threonine kinase properties. Further, pyridine derivatives showed significant inhibition of beta kinase activity along with inhibition of HIV-1 replication, thus suggesting 72 kDa HIV-1 associated Topoiosmerase II beta kinase (TopoIIβK_{HIV}) as potential target for affecting HIV-1 replication [28]. Hence, current thesis

examines interaction of TopoIIβK_{HIV} with series of organic molecular frameworks with and without pyridine.

Pyridine Derivatives as TopoIIβK_{HIV} Inhibitors:

Topo IIβK_{HIV} catalysed Topo IIβ phosphorylation experiment was carried out in the presence of diverse structural class of organic molecules with increasing concentration **[28].** Pyridine derivatives possess a significant inhibitory activity against Topo IIβK_{HIV} and have low cytotoxicity suggested by the screening results. The pyridine sub-structure has a very high prevalence among kinase inhibitors has shown by the results of a recent broad-spectrum study by Aronov *et al.*, **[29].** Because of these results we focused on the pyridine derivatives, which were chosen and analyzed for their effect on the activity of Topo IIβK_{HIV}. The experiment results have shown and expressed in terms of 50% inhibition of Topo IIβK_{HIV} activity (IC_{50}) in previously reported paper. These results indicate the significant Topo IIβK_{HIV} antagonism shown by these pyridine derivatives.

Drug Design:

Figure 3: Hypothetical pocket of Topo IIβK$_{HIV}$

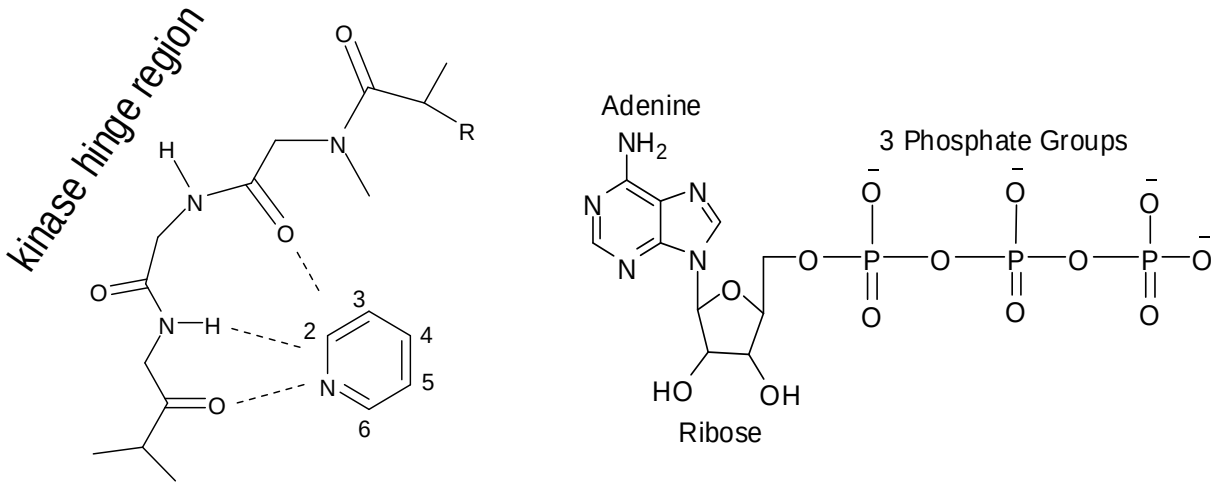


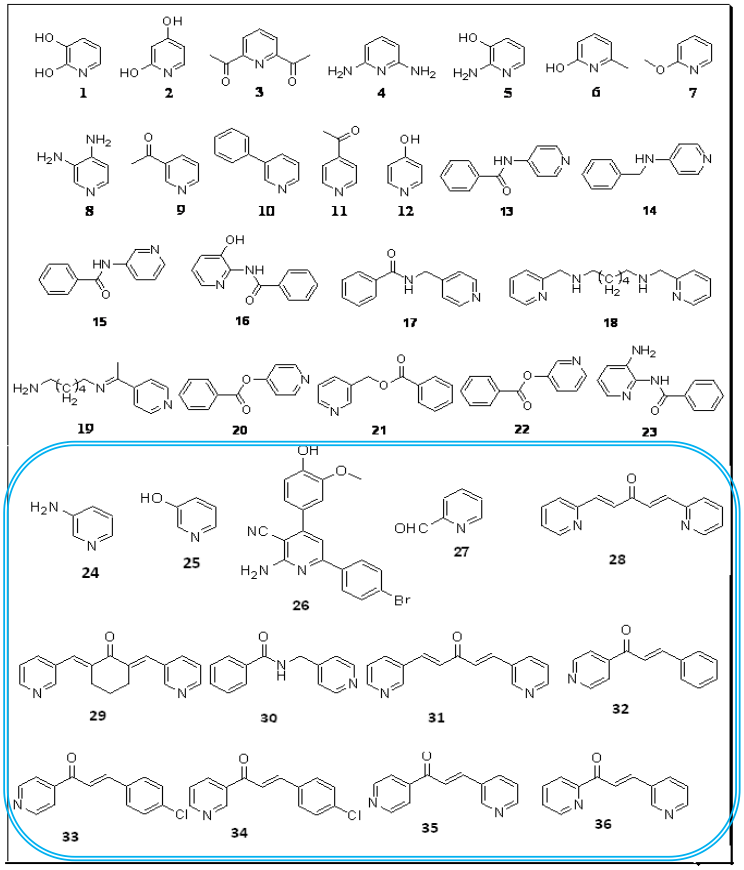
Fig.3: Hypothetical Model: A hypothetical model showing the possible interactions provided by carbonyl oxygen, amide hydrogen of hinge peptide generally present in kinase with pyridine pharmacophore at 2, 3 positions similar to the 6-membered pyrimidine ring in Adenine of ATP, while 4, 5 and 6 positions are accessible to the steric and electrostatic groups (indicated by contours) that may provide an interacting environment for the approaching substrate, Topo IIβ.

3D-QSAR (CoMFA) of kinase antagonism and antiviral activity of pyridine molecules:

The pyridine group moiety having Topo IIβK$_{HIV}$ inhibitors developed by using CoMFA contour model for better activity. The CoMFA model used for predicting the activity of the molecules in highly accurate. CoMFA involves generation of a molecular model by comparing the steric and electrostatic fields, sampled over grid points of molecular 3D space. The alignment of the molecules over one another in 3-D space forms one of the most important and basic steps of the analysis. The previous analysis, the pyridine ring was considered as a common sub-structure

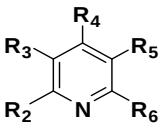
and backbone for all the molecules studied, as the core and then aligned the molecules with, **3-**phenyl pyridine as a template molecule. CoMFA model was generated using a set of pyridine molecules 1 to 20 for kinase inhibition and the same set molecules were used for antiviral activity **[28].** To strengthen the prediction statistics, molecules from 24 to 36 **(Fig 3)** have been synthesized and their inhibitory activity against Topo $II\beta K_{HIV}$ **(Table 2)** and QSAR evaluation was refined.

Figure 4: Following synthesized molecules used for the refine QSAR equation and generated new 3D-QSAR contour model for the designing new potent anti-HIV-1 molecules and Topo $II\beta K_{HIV}$ inhibitors.

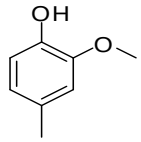
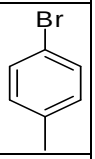
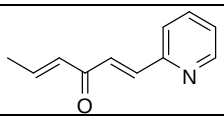
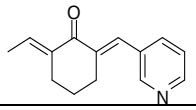
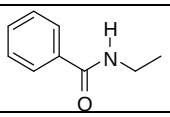
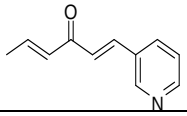
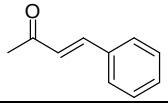
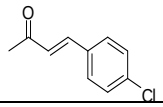
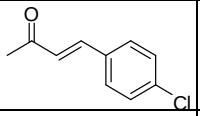
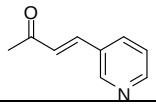
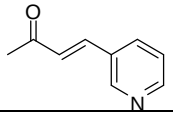


Strucure of Molecules. Molecules 1 to 20 have been studied in Kannapiran et al **[28],** molecules 24 to 36 has been sythesiszed in this thesis and used for refine 3D-QSAR model.

Table 2. Used this molecules inhibitory values for refining QSAR model



Comparative Molecular Fields of kinase inhibition and anti-HIV-1 models:

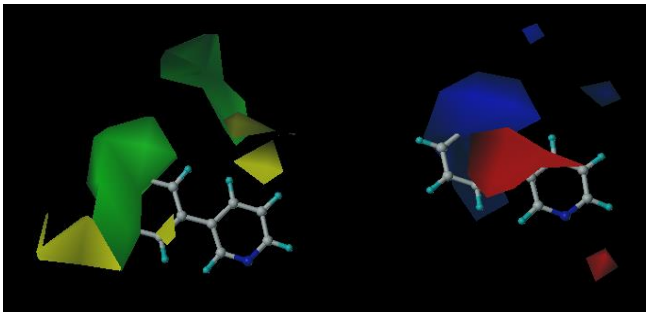
Molecule No	Kinase Inhibition(PIC_{50})	R_2	R_3	R_4	R_5	R_6
24	8.050	H	NH_2	H	H	H
25	7.610	H	OH	H	H	H
26	5.770	NH_2	CN		H	
27	6.590	CHO	H	H	H	H
28	4.045		H	H	H	H
29	3.970	H		H	H	H
30	4.031	H	H		H	H
31	4.060	H		H	H	H
32	4.045	H	H		H	H
33	4.045	H	H		H	H
34	4.031	H		H	H	H
35	4.060	H	H		H	H
36	4.060		H	H	H	H

The steric and electrostatic fields generated through CoMFA are compared to identify the common regions that correspond to both structural components associated with activity. The kinase model (**Fig.5A ii**) shows a region around 5^{th} and 6^{th} positions favoring for positive charge and at the same time a region by the side of 1^{st} position and spanning the N of the heterocyclic ring shows disfavor for positive charge. The presence of bulky group at 5^{th} position and less steric bulkiness at 2^{nd} and 6^{th} positions may be inferred to the activity of the inhibitor 3-phenyl pyridine **(Fig.5Ai)** The antiviral model **(Fig .5B)** shows a region, favoring for steric bulk at 5^{th} position and partly at 6^{th} position and regions disfavoring steric bulk mainly at positions 6^{th} and 1^{st}. A huge positive charge propensity is observed mainly at positions 5^{th}, 6^{th} and partly at 1^{st} in the plane parallel to the pyridine ring, but a small patch of negative charge is located around the N of the heterocyclic ring **(Fig .5B)**. The comparative study on both the models suggests that the native charge and the interactive environment around adjacent groups in pyridine structure affect the activity of the molecules and play important role in binding to the receptor. The region on one side of the pyridine ring structure towards 5^{th}, 6^{th} and around N has enough space to accommodate for steric modifications thereby facilitating enhancement of activity due to substitution with bulkier groups as it is evident from both the cases. The models show a distinct positive charge preference at positions on the either side of heterocyclic N but predominantly at the positions 1^{st}, 5^{th} and 6^{th} positions.

Figure 5: Contour Map for the new 3D-QSAR model generated

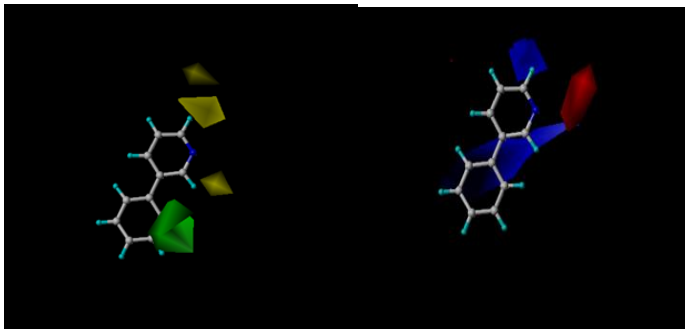
A) Kinase inhibition Activity

i) Steric ii) Electrostatic



B) Anti-Viral Activity

i) Steric ii) Electrostatic



Green : Favored Blue : Favored

Yellow : Disfavored Red : Disfavored

Fig. 5 : CoMFA model depicting the Steric and Electrostatic contour of active molecules. The 3-dimensional contour map based on (a) anti-kinase activity (IC_{50}) and (b) anti-viral activity (IC_{50}) of the molecules representing the favoured (green & blue) and disfavoured (yellow & red) regions of steric and electrostatic fields around the pyridine molecule**.**

Rationale of study:

Earlier reports have shown that the phosphorylation of Topo II α and β were observed during HIV-1 infection in the T cells, in which Topoisomerase IIβ is predominantly phosphorylated during HIV-1 infection. A 72 kDa protein has been purified from virus concentrate and reported to be associated with Topoisomerase IIβ isoform-specific phosphorylation. HIV-1 Associated Topoisomerase IIβKinase found to be expressed in gp120 expressing cell line suggesting it is originated from virus envelope.

HIV-1 Associated Topoisomerase IIβKinase has been evaluated for inhibition by a panel of serine and tyrosine kinase inhibitors and the results showed that these inhibitors do not show significant inhibitory activity against this kinase. A series of synthetic molecules from distinct structural backbone showed significant inhibitory action against HIV-1 Associated Topoisomerase IIβKinase.

These interesting findings were the inspiration to explore the possibility of blocking this crucial pathway in the life-cycle of HIV, and thereby prevent virus replication by targeting to HIV-1 associated Topo IIβ kinase (Topo $IIβK_{HIV}$). Experiments on Topo II β phosphorylation catalyzed by Topo $IIβK_{HIV}$ were carried out in the presence of increasing concentrations of diverse structural class of organic molecules in which the Pyridine derivatives were found to be active against a Topo $IIβK_{HIV}$ activity and HIV-1 replication. The kinase inhibition and anti-viral activities for various pyridine inhibitors were tested in an *in vitro* kinase and based on these results a CoMFA model was generated. On the basis of 3D-QSAR (CoMFA) study we have designed and developed potent HIV-1 associated TopoIIβ kinase inhibitors. This development of new drugs targeting HIV-1 infection is of utmost importance for the clinical use in HIV-1 patients for expansion of line of treatment of infected patients.

Based on the above rationale, the following objectives were framed.

Objectives:

1. Design, synthesis and biological evaluation of pyridine derivatives of coumarin for anti-HIV-1 and HIV-1 associated topoisomerase II β kinase inhibition.
2. Molecular derivatization studies of pyridine-coumarin analogues for improved cellular bioavailability and anti-HIV-1 activity.
3. Development of new lead molecules based on structure and biological activities of non-pyridine derivatives of coumarin.
4. Design, synthesis and evaluation of pyridine bischalcone derivatives for inhibition of HIV-1 associated topoisomerase II β kinase and anti-HIV-1 activity.

Chapter-2

Objective -1

Design, synthesis and biological evaluation of pyridine derivatives of coumarin for anti-HIV-1 and HIV-1 associated topoisomerase II β kinase.

Introduction: -

From the past two decades, HIV therapeutics research has been exponentially increased. In the present day, viral Integrase, Protease, Reverse Transcriptase, and Envelope protein, which are responsible for the entry, infectivity and establishment of the virus infection, were principally targeted by antiretroviral therapeutics (ART). Host cell CD4 receptor and CXCR4 or CCR5 co-receptors are used for HIV-1 entry and to stop the entry of HIV-1, Maraviroc [31], inhibitor was found to target CCR5 receptor, research results were identified CCR5 receptor of the host cell as potential target for this inhibitor and this inhibitor has been approved for HIV-1 treatment in 2007. However, in the wake of virus resistance to the existing drugs, there is an emergency need in identification of new targets for controlling viral infection and conquer drug resistance.

In the way of searching for the new targets in HIV life cycle, our lab focused on Topoisomerase II isoforms phosphorylation. A straight interrelationship has been noticed between the reduction in the efficiency of HIV-1 replication and the depletion of Topoisomerase II (Topo II) level by the antisense oligonucleotide [32], poisoning of Topo II and SiRNA knockdown [33]. In HIV-1 reverse transcription, SiRNA mediated knockdown of Topo II isoforms shown to play a crucial role in formation of intermediates [34] and viral mRNA synthesis mediated by HIV-1 tat [35]. Thus, in targeting HIV-1 replication, Topo II isoforms could be as potential interest. Since Topo II isoforms are cellular proteins which are essential for maintenance of

several housekeeping functions of cells, targeting these enzymes will be very harmful to cell survival.

Topo II isoforms catalytic activity has been regulated by phosphorylation, several reports on cellular kinases to phosphorylate these enzymes [36]. Our earlier studies revealed that the two fractions of purified HIV-1 lysate are differentially phosphorylated the Topo II isoforms [23]. Topo IIα isoform predominantly phosphorylated by One fraction of viral lysate and sensitive to MAP kinase inhibitor PD98059 [23]. HIV-1 Associated Topoisomerase IIβ kinase (Topo IIβK_{HIV}) is a 72 kDa novel protein has been found to be present in the purified HIV-1 virus lysate [23] [28]. Topo IIβK_{HIV} has shown to be resistant to a panel of 20 potent STK and Tyrosine kinase inhibitors including staurosporine and PD98059 and which is a Ser/Thr kinase (STK) [28], which classify that Topo IIβK_{HIV} is a novel kinase, that involved in phophorylarion of Topo IIβ enzymes during HIV-1 replication and then encapsulated into the virus. Since Topo IIβK_{HIV} does not found to be present in uninfected healthy T cells, it can form distinctive target for interfering HIV-1 replication in infected cells [28]. Based on the previous studies on Topo IIβK_{HIV} inhibitors, various pyridine derivatives were designed, synthesized and analysed for their activity.

Anti-HIV drug discovery:

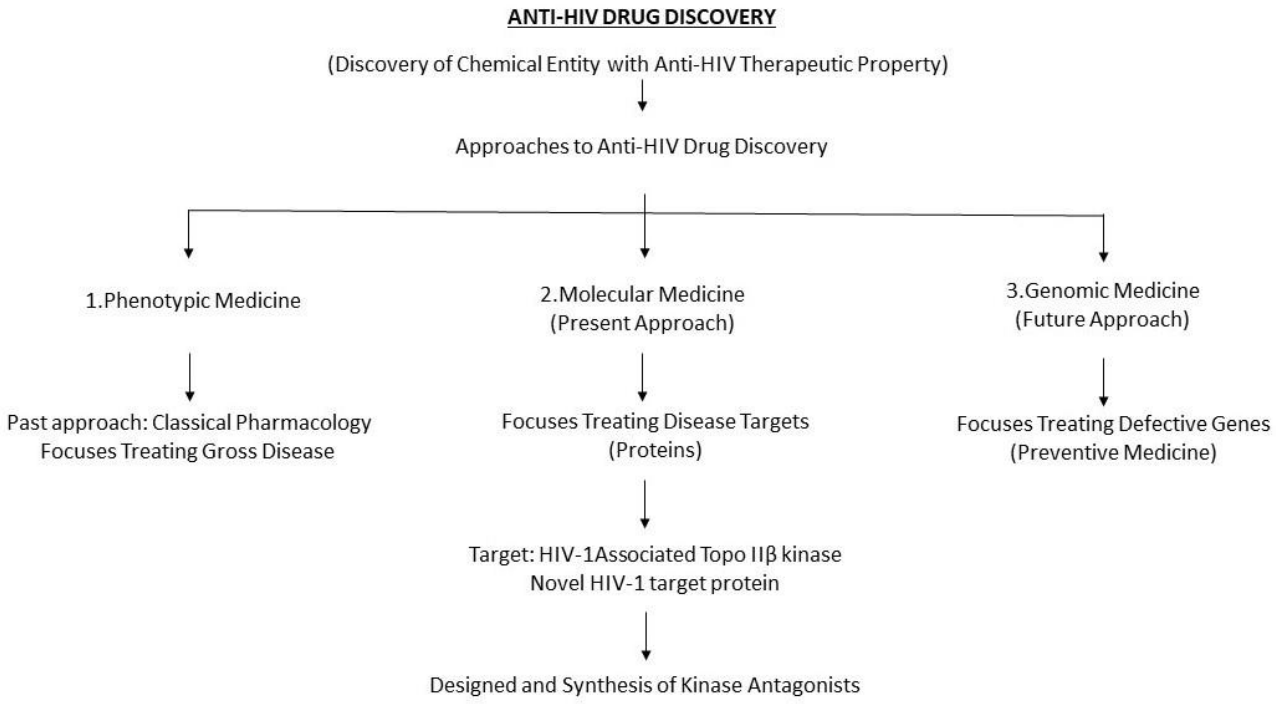
Identification of screening hits on target Topo IIβK_{HIV} protein, medicinal chemistry deals with the optimization of those hits to increase the selectivity (to lessen the potential of side effects), affinity, metabolic stability (to expand the half-life), potency/efficacy and oral bioavailability. All of these requirements have been identified and fulfilled once in a compound, the process of drug development will start prior to clinical trials.

Drug discovery approaches:

Basically, Drug discovery approaches are three types

1. Phenotypic Medicine approach
2. Molecular Medicine approach
3. Genomic Medicine approach

Figure 6: In our drug discovery studies, we followed molecular medicine approach for target in the Topo IIβK_{HIV}.



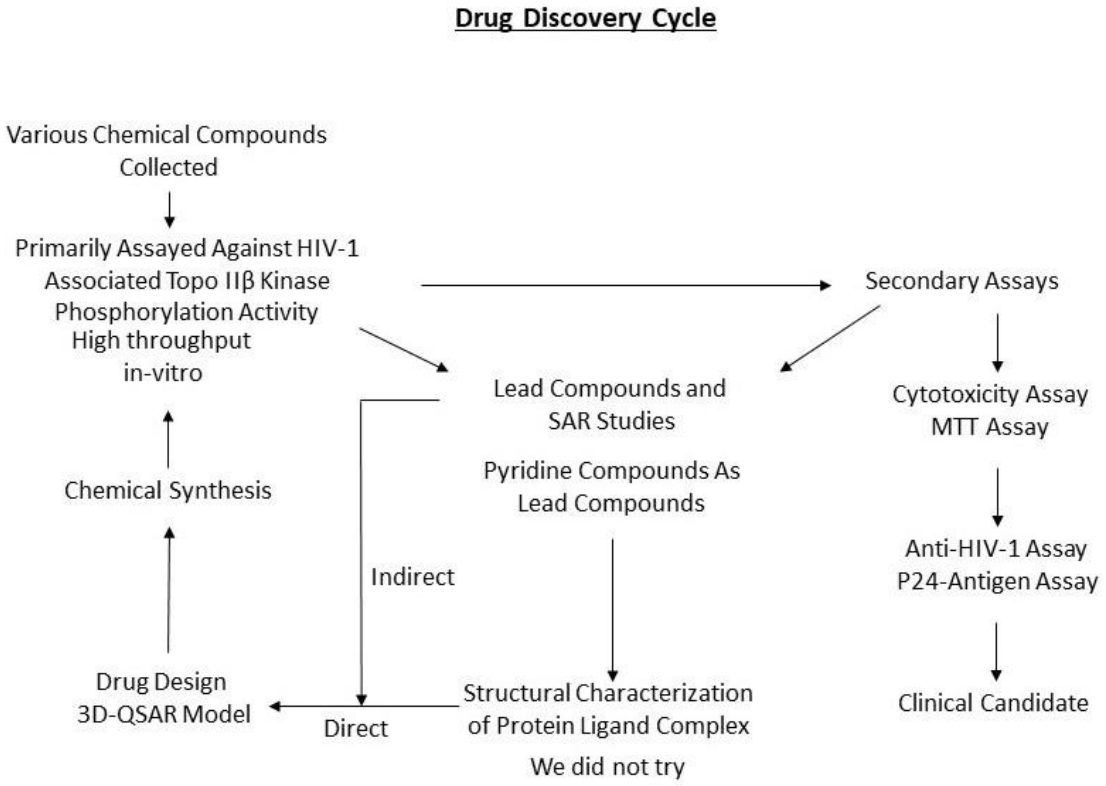
Molecular Medicine Approach Discovery Plan:

Key Steps:

1. Identified novel Topo IIβK_{HIV} protein as a target.
2. Validated a causative role for Topo IIβK_{HIV} in phosphorylating the Topo II β in HIV-1 lifecycle.
3. Discovered pyridine derivatives as lead molecules that modulate Topo IIβK_{HIV} activity.

4. Drug design, optimized pharmacophores on pyridine which have shown potential Topo IIβK_{HIV} inhibition and anti-HIV-1 activities, through 3D–QSAR and CoMFA model, synthesized and characterized.
5. Studied the taxological effects of optimized pyridine lead molecules.
6. Study the in-vitro models and the effects of drug (optimized pyridine lead molecules), But we have not done because it is time taking process and need lot of money.
7. Optimizing formulation for the lead (DPR- Drug Product Research).

Figure 7: Molecular Medicine Approach Drug Discovery Cycle:



Screening and Design:

The process of finding a drug against a chosen novel target Topo IIβK_{HIV} for AIDS disease, generally involved high throughput screening (HTS), wherein large libraries of chemicals are tested for their ability to inhibit the TopoIIβ kinase activity, which are called antagonists to the Topo IIβK_{HIV}. After HTS, we noticed selective compounds which were specifically targeting the Topo IIβK_{HIV}, as we found pyridine molecules which will interfere only with the Topo IIβK_{HIV} target, but not other related targets. To this end, other screenings assays will be made to see whether the hits against the chosen TopoIIβ kinase target will interfere with other related targets as in the process of cross screening. Cross-screening is important, because the more unrelated targets a compound hits the more likely that off-target toxicity will occur with that compound once it reaches the clinic.

From these early screening assay, one will not reach the perfect drug candidate. One of the first steps is to screen the compounds that will not to be developed into drugs, but it is considered as lead compound. For example, compounds that hits in almost every assay, as a medicinal chemist, we classified as Pan Assay Interference Compounds (PAINS), in which the compounds were separated from the chemical library. It is often observed that several PAINS are found to have some degree of activity and these compounds share common chemical features, one or more pharmacophores can then be developed.

At this point as a medicinal chemist was attempted to use structure-activity-relationships (SAR) to improve certain features of the lead compound:

1. Increase activity against the novel TopoIIβ kinase activity
2. Reduce activity of Topo IIβK_{HIV} antagonists against unrelated targets
3. Improve the drug likeness or ADME properties of the Topo IIβK_{HIV} antagonists

The process of kinase inhibition assays was carried out for the improvement of the new molecular entities and allowed the favoured compounds to go forward to *in-vitro* and *in-vivo* testing for activity in the model of choice.

Drug design:

Drug design is two types:

1. Structure- based drug design
2. Ligand- based drug design

For structure-based drug design (or direct drug design) needed the knowledge of the three-dimensional structure of the Topo IIβK_{HIV} target obtained through methods such as X-ray crystallography or NMR spectroscopy. As the experimental structure of Topo IIβK_{HIV} is not available, so we have not preferred structure based drug design in the designing of Topo IIβK_{HIV} antagonists. But in ligand–based drug design (or indirect drug design) depends only on knowledge of other molecules that bind to the Topo IIβK_{HIV} target which is very crucial in our studies. By these other molecules which were inhibited Topo IIβK_{HIV} were used to derive a pharmacophore model (3D-QSAR model) that defines the minimum necessary structural entities a molecule must possess in order to bind to this Topo IIβK_{HIV} target. Quantitative structure–activity relationship (QSAR), in which a correlation between calculated properties of molecules and their experimentally determined kinase inhibition activity values was derived. This QSAR correlation relationship was used to predict the activity of new analogues. By using this QSAR equation we have built 3D-QSAR model. By seeing this model, we realised about steric and electronic features of potent ligand that is necessary to ensure the optimal supramolecular interactions with a specific Topo IIβK_{HIV} target to trigger (or block) its phosphorylating biological response.

3D-QSAR:

According to the very recent definition by IUPAC, a pharmacophore model (3D-QSAR model) is an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target to block its biological response. In virtual screening of different chemical compounds which were collected from market and nature resource, the compounds having pyridine moiety have shown significant *in-vitro* TopoIIβK$_{HIV}$ phosphorylation inhibition, which initiated to the synthesis of various pyridine derivatives.

After that series of pyridine derivatives were evaluated and a 3D-QSAR analysis has been performed for understanding insights into the organic frame works associated in inhibition of Topo IIβK$_{HIV}$ [28]. Comparative molecular field analysis (CoMFA) generated a contour model providing predictive model of probable active site features of Topo IIβK$_{HIV}$ [28].

Figure 8:

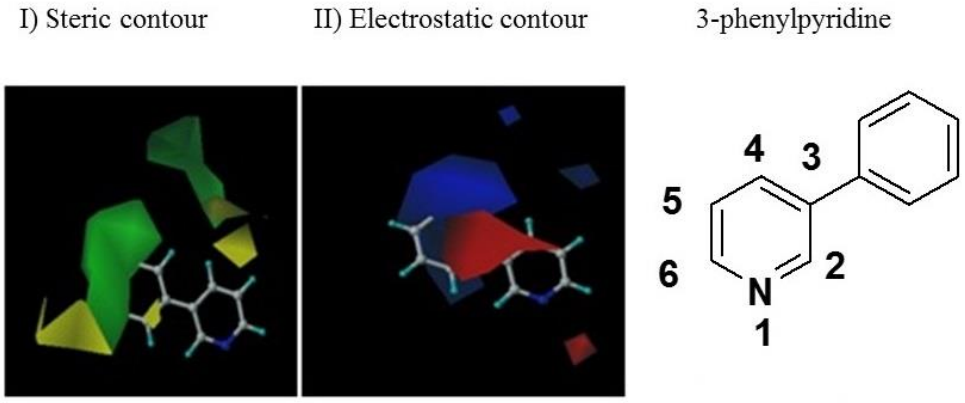


Fig. 8: 3D-QSAR model: CoMFA model illustrating the Steric (I) and Electrostatic (II) contour of active molecules. Here the standard highest activity molecule (3-Phenyl Pyridine) 3D contour maps generated by CoMFA analysis of the derivatives. (I) the binding affinity realized, through the regions, where hydrophobic substitution enhances (green) or reduces (yellow). (II) The colour coding's specifying the regions, where electronegative substituent enhances (red) or reduces (blue) the binding affinity.

Drug Discovery against HIV-1 targeting Topo IIβK$_{HIV}$:

In the present study, we have critically investigated the active site of Topo IIβK$_{HIV,}$with designed pyridine substructures having various organic structural frame works containing dicoumarol moiety, synthesized and inhibitory activity evaluated against Topo IIβK$_{HIV.}$ Actually, extensive biological activities have shown by coumarin compounds and are also used as cosmetics, optical brightening agents and supplement to food. A wide number of pharmacologically active compounds such as antibiotics and natural products have Coumarin ring system is one of the most important substructures [37] and also in antitumor drugs [38]. In the literature, few methods are reported for the synthesis of dicoumarols from 4-hydroxycoumarin and aldehydes in presence of distinct catalysts. However, exotic reaction conditions and prolonged reaction time is required for these methods and few of these methods require the use of toxic organic solvents, expensive catalysts, and tedious process. Water is an attractive medium for many organic reactions and growing recognition for water as solvent in green chemistry, resulted in less dangerous, less expensive, and environmentally friendly reactions, similar to Diels–Alder reactions [39], Claisen rearrangement reaction [40], Reformat sky reactions [41] and Pinacol-coupling reactions [42]. Recent reports suggest that in aqueous media, under various conditions, dicoumarol derivatives could be obtained through condensation of 4-hydroxycoumarin and different aldehydes [43] [44]. In this study, we have synthesized pyridine dicoumarols, under conventional reflux conditions in aqueous media (Scheme 2) and evaluated their effect on Topo IIβK$_{HIV}$. Results showed that pyridine dicoumarols and its derivatives inhibit the Topo IIβK$_{HIV}$ activity *in vitro* along with anti-HIV-1 activity.

Chemistry:

Scheme-1: New synthetic method of pyridine derivatives of 4, 4^1-epoxydicoumarin and biological evaluation against HIV-1 associated topoisomerase II β kinase.

Pyridylidene-4, 4^1-epoxy dicoumarins preparation:

4, 4^1-epoxydicoumarin derivatives (UHAKKM-1,2,3) general synthetic procedure: Dicoumarol (1mmol) and benzoyl chloride (2mmol) mixture was added in round bottom flask containing 10mL of acetonitrile, to this, 4 or 5 drops tri ethyl amine was added and stirred at room temperature or under conventional reflux conditions. The completion of reaction was monitored through Thin Layer Chromatographic analysis. After the reaction completed, the reaction mixture was cooled to room temperature. The solid products were filtered and washed with hot methanol and dried to give the desired products (UHAKKM-**1 to 3**). From the column chromatography, products were purified [45].

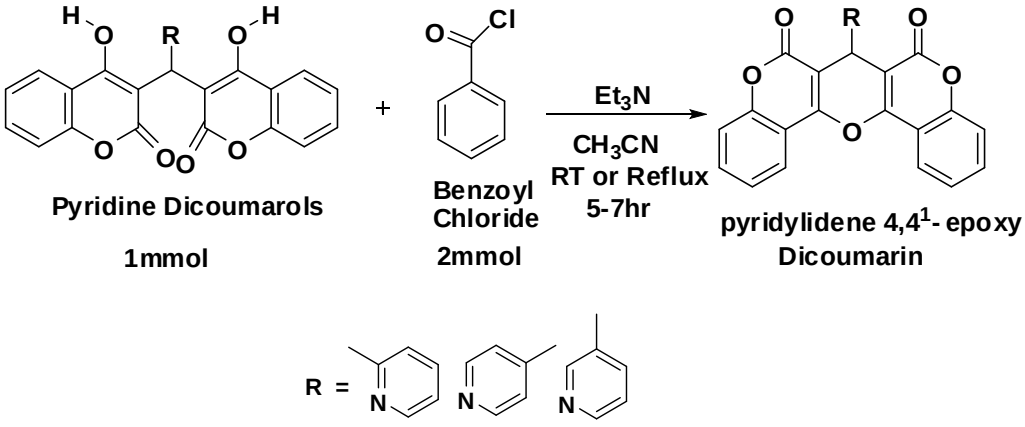


Table.3: The table specifies the reaction condition and reaction time of synthesis of Pyridylidene-4, 4^1-epoxy dicoumarin derivatives.

Entry	R	Product	Reaction condition	Reaction Time
1	2-C_5H_4N	UHAKKM-1	RT	5-6hr
2	4-C_5H_4N	UHAKKM-2	Reflux	7-8hr
3	3-C_5H_4N	UHAKKM-3	RT	4-5hr

Note: All derivatives are prepared in room temperature, except 4- pyridylidene-4,4^1-epoxy dicoumarin, which was prepared by refluxing at 82°C.

Spectral characterization (see supporting spectral information from figure-S1 to S30):

2-pyridylidene-4,4^1-epoxy dicoumarin [UHAKKM-1]**:** White solid (Yield: 90%); mp: 198-202 ^{0}C; ^{1}H-NMR (DMSO) δ: 5.28 (s, 1H, CH), 7.10–8.39 (m, 12H, Ar-H); ^{13}C-NMR ($CDCl_3$) δ: 37.41, 105.20, 113.69, 117.07, 122.52, 124.52, 125.52, 132.66, 133.92, 136.31, 149.58, 152.81, 154.42, 159.13, 160.63; IR (KBr) ν: 1719, 1671, 1609, 1388, 1048, 750 cm^{-1}; HRMS (ESI+) [M+H]: calcd. for$C_{24}H_{13}NO_5$ 396.087, found 396.100

4-pyridylidene-4,4^1-epoxy dicoumarin [UHAKKM-2**]:** White solid (Yield: 85%); mp: 213-216^{0}C; ^{1}H-NMR (DMSO) δ: 5.17 (s, 1H, CH), 7.52–8.82 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 36.01, 103.19, 113.29, 117.16, 124.11, 125.61, 128.06, 134.23, 142.61, 152.77, 154.83, 159.35, 160.20; IR (KBr) ν: 1730, 1710, 1667,1607, 1383, 757 cm^{-1}; HRMS (ESI+) [M+H]: calcd.for $C_{24}H_{13}NO_5$ 396.087, found 396.088

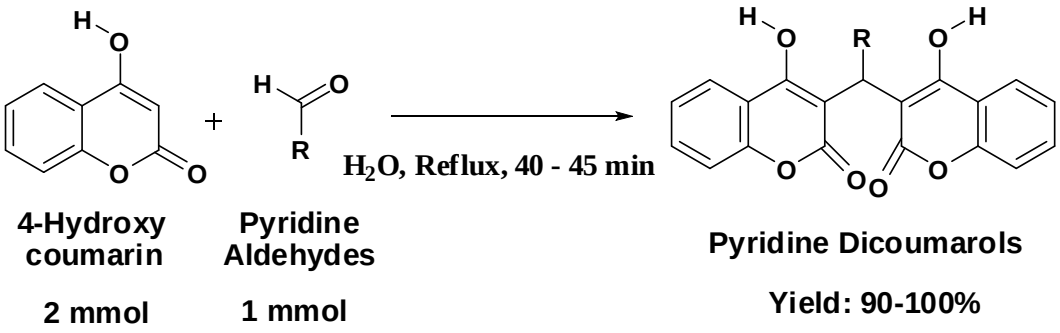
3- pyridylidene-4,4^1-epoxy dicoumarin [UHAKKM-3]**:** White solid (Yield: 94%); mp: 226-228^{0}C; ^{1}H-NMR (DMSO) δ: 5.11 (s, 1H, CH), 7.54–8.99 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 26.76, 103.84, 113.47, 117.14, 124.05, 125.53, 125.94, 133.99, 152.77, 154.60, 160.31; IR

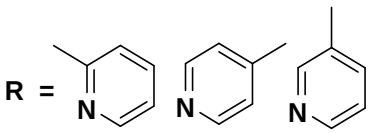
(KBr) ν: 1743, 1711, 1607, 1237, 1006, 758 cm^{-1}; HRMS (ESI+) [M+H]: calcd.for $C_{24}H_{13}NO_5$ 396.087, found 396.088.

Scheme-2: Catalyst-free and efficient eco-friendly synthesis of pyridine dicoumarol derivatives in water and their applications towards antiHIV-1 activity targeting to Topo IIβK_{HIV}.

3, 3'-(pyridine-n-yl methylene) bis(4-hydroxy-2H-chromen-2-one) derivatives preparation:

Dicoumarols general synthetic procedure: 4-hydroxycoumarin (2mmol) and aldehydes (1mmol) were taken in 25mL round bottom flask containing 10mL water and refluxed for 1h. Reaction completion was confirmed by TLC and then filtered the solid, washed with hot methanol and dried. Then we characterized the pure products which yielded about 90-100% [46].





Spectral characterization (see supporting spectral information from figure-S1 to S30):

3,3'-(pyridin-2-ylmethylene)bis(4-hydroxy-2H-chromen-2-one)[UHAKKM-4**]**: Whitesolid (Yield: 90%); mp: 224–228ºC; ^{1}H-NMR (DMSO) δ: 6.54 (s, 1H, CH), 7.26–8.65 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 37.04, 100.88, 116.27, 119.81, 123.73, 124.75, 124.88, 126.30, 132.27, 142.22, 146.82, 153.26, 157.98, 164.31, 168.97 ; IR (KBr) ν: 3124, 3068, 2879,1698, 1639, 1614, 1538, 1350, 1180, 1063, 882, 760 cm^{-1}; MS(ESI) m/z $[M+H]^+$ 414:$[M+Na]^+$ 436:HRMS (ESI+) [M+H]: calcd.for $C_{24}H_{15}NO_6$ 414.09, found 414.10

3,3'-(pyridin-4-ylmethylene)bis(4-hydroxy-2H-chromen-2-one)[UHAKKM-5**]**: White solid (Yield: 95%); mp: 248–250ºC; ^{1}H-NMR (DMSO) δ: 6.47 (s, 1H, CH), 7.25–8.69 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 38.26, 101,.90, 116.23, 119.83, 123.69, 124.69, 125.68, 132.09, 141.41, 153.16, 164.53, 165.31, 168.60; IR (KBr) ν: 3088, 3068, 2890, 2622, 1684, 1541, 1495, 1405, 1330, 1273, 1175, 1107, 855, 805, 759 cm^{-1}; MS(ESI) m/z $[M+H]^+$ 414, $[M+Na]^+$ 436:HRMS (ESI+) [M+H]: calcd.for $C_{24}H_{15}NO_6$ 414.09, found 414.10

3,3'-(pyridin-3-ylmethylene)bis(4-hydroxy-2H-chromen-2-one)[UHAKKM-6**]**: White solid (Yield: 93%); mp: 258–260ºC; ^{1}H-NMR (DMSO) δ: 6.45 (s, 1H, CH), 7.23–8.72 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 35.13, 102.10, 116.19 119.94, 123.61, 124.63, 127.11, 131.98, 139.53, 140.74, 143.27, 145.25, 153.16, 164.48, 168.46; IR (KBr) ν: 3080, 3060, 2909, 2157, 1686, 1614, 1536, 1330, 1180, 1050, 1017, 900, 759 cm^{-1}; MS(ESI) m/z $[M+H]^+$414,$[M+Na]^+$ 436:HRMS (ESI+) [M+H]: calcd.for $C_{24}H_{15}NO_6$ 414.09, found 414.10

Biological Evaluation Methods:

Kinase Phosphorylation and Inhibition Assay:

Dephosphorylated topoisomerase IIβ was treated with kinase at 100 μM cold ATP and 5 μCi of [γ-32P] hot ATP in kinase buffer in presence of test compounds at varying concentrations. 3 ng of topoisomerase II β antibodies and 6% protein A–agarose beads were added. The beads were washed with TBS and eluted with 5% trichloroacetic acid (TCA). Elute was spotted on Whattman paper discs and dried and ^{32}P was measured by liquid scintillation. Each experiment was performed in triplicate and all data points represent an average of results from the triplicate experiments. In all the 3-Phenyl Pyridine was used as a reference standard.

Cytotoxicity of compounds in SupT1 Cells:

Cytotoxicity assay was performed with MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) in SupT1 cells. Briefly, SupT1 cell were grown in RPMI 1640 media with 10% FBS, approximately 0.02 X 106cells were seeded in 100μL of complete media. After overnight incubation cell viability was determined by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reagent (5mg/mL). 10uL MTT was added to each well and incubated for 4 hours in cell culture incubator, 100uL of DMSO was added to all the wells, plate was gently swirled and kept in dark for 2 hours at RT. Absorbance was measured at 570 nm in a microtiter plate reader, each assay was repeated at least three times and in triplicates. The IC50 (Concentration of 50% Inhibition) value was calculated using Graph Pad Prism 5.0 (Graph Pad Software, Inc., San Diego, CA).

Anti-Viral Assay:

SupT1 cells with 99% confluency were seeded in 24 well plate and infected with HIV-193IN101 at a final concentration equivalent to 1ng per mL and then drug with a concentration of 100uM was added to the wells. The infected cells were incubated for 2 hours at 370C in a 5% CO_2 incubator. After 2 hours the cells were washed and pelleted at 350xg for 10 minutes, the supernatant was discarded and the Sup T1 pellet was resuspended in fresh RPMI1640 complete media containing 10% FBS. Resuspended infected SupT1 cells were further incubated for 96 hours in 5% CO2.After 96 hours the supernatant was collected and analysed by using a p24 antigen capture assay kit (Advanced Bioscience Laboratories, Kensington, MD, USA). The extent of infection in the absence of test compound was considered to be equvalent to 0% inhibition. Azidothymidine (AZT) was employed as positive control.

Results:

Design and Synthesis:

3D-QSAR studies carried out previously generated contours based on which the molecules in this study have been designed. From the **Fig 5**. (I) and (II) map the preferable and non-preferable substitutions in terms of Steric or electrostatically charged groups to the pharmacophore, can be realized based on the sub-structures. In this 3D-QSAR model the 4^{th} and 5^{th} places of the pyridine ring favour addition of steric groups, while the addition such that the conformation aligns the hydrophobic group between 2^{nd} and 3^{rd} places of pyridine ring results in reduced binding affinity. Likewise, an addition of electropositive group at 5^{th}place of pyridine ring such that the conformation aligns between structures in **Fig 9** (II) were designed to match the contours and the coumarin moiety was situated each at 4,5,6 position of the ring for compounds UHAKKM-1 to 6 respectively. From the hypothetical pocket model

recommended [28], it could be realized that the interaction of 2, 3 positions with the kinase hinge region, restricted the substitutions to only the above-mentioned positions. The coumarin moiety behaves as a hydrophobic bulky group which on substitution in the favourable region enhanced the activity of the compound. Likewise, the presence of oxygen atoms on the coumarin group resulted in fulfilling the electrostatic constraints. Due to this the synthesized compounds showed high Topo IIβK_{HIV} antagonism.

Fig.9 : a)

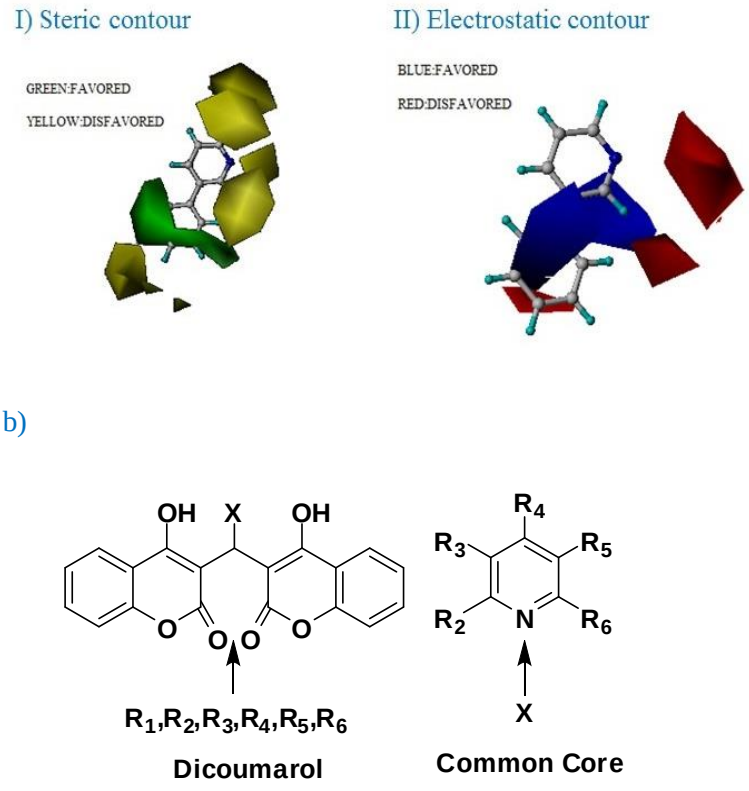


Fig.9: 3D QSAR model: CoMFA model illustrating **a)** the Steric (I) and Electrostatic (II) contour of active molecules. The 3D contour maps around the highest activity molecule (3-Phenyl Pyridine) generated by CoMFA analysis of the derivatives. (I)Regions where hydrophobic substitution enhances (green) or reduces (yellow) the binding affinity. (II) The colour coding's specify regions where electronegative substituent enhance (red) or reduce (blue) the binding affinity **b)** the structures of analogues with a coumarin moiety at different places of the core pyridine ring (X) (R1, R2, R3, R4, R5, R6 = Dicoumarol) designed based on the contours.

Table 4: Theoretical and experimental activities of the designed and synthesized compounds:

S.No	R	Molecules	Theoretical pIC_{50} Values	Experimental pIC_{50} values	IC_{50}concentration (pM)
1	2-C_5H_4N	UHAKKM-4	8.89	9.56	$2.7\pm 0.4 \times 10^2$
2	2-C_5H_4N	UHAKKM-1	9.09	9.52	$3.0\pm 0.21 \times 10^2$
3	4-C_5H_4N	UHAKKM-5	9.49	9.56	$2.8\pm0.14 \times 10^2$
4	4-C_5H_4N	UHAKKM-2	9.09	9.04	$2.3\pm0.17 \times 10^2$
5	3-C_5H_4N	UHAKKM-6	9.77	9.51	$3.1\pm0.21 \times 10^2$
6	3-C_5H_4N	UHAKKM-3	9.84	9.38	$4.2\pm0.34 \times 10^2$

All the molecules of the pyridyl- dicoumarol derivatives (1-6) in table-2 were designed based on the contours generated from the 3D-QSAR studies, synthesized and the activities were predicted using the generated equation. The high predicted values were found to be similar to the experimental value; all molecules were showing IC_{50} at picomolar concentration. As indicated in the Table 4, the IC_{50} concentration of Topo IIβK_{HIV} inhibition and respective pIC_{50} values.

Fig.10: Cytotoxicity assay (MTT):

The cytotoxicity of molecules in SupT1 cells was examined and all the compounds showed CC_{50} (50% cytotoxic concentration) above 1mM concentration.

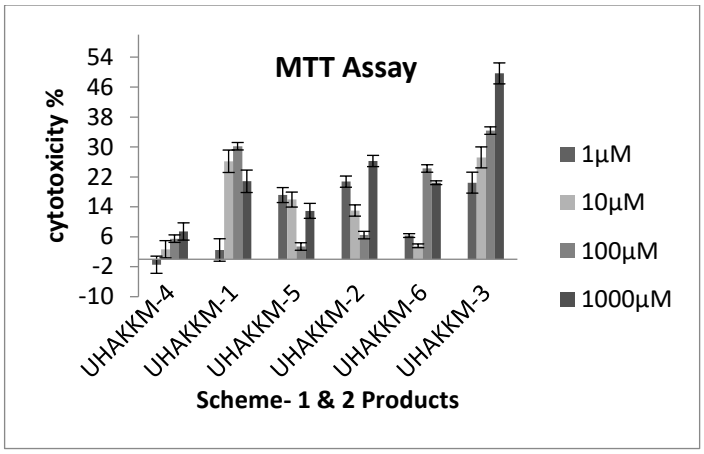


Fig.10: The graph indicates UHAKKM-1 to 6 have shown less cytotoxicity.

Figure 11: HIV-$I_{93IN101}$ Viral Inhibition Assay:

The anti-HIV-1 activity (inhibiting the viral replication) of the molecules was tested using the p24 antigen capture sandwich ELISA method (p24 assay) for UHAKKM-1 to 6.

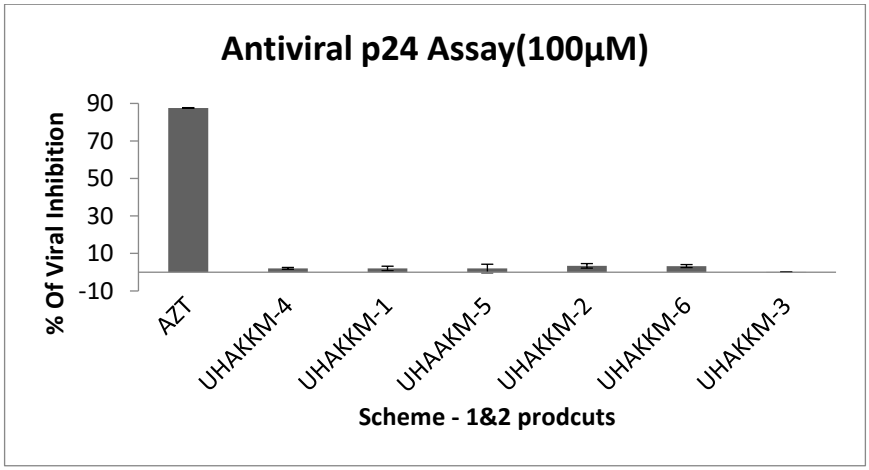


Fig. 11: Above antiviral p24 assay graph showed, antiviral activity of UHAKKM-1 to 6 is shown less even at 100µM concentration.

Table 5: Anti-viral assay results

Compound	Anti-viral activity	Kinase inhibitionIC_{50}(pM)
UHAKKM-1	Very low	$2.7\pm 0.4 \times 10^2$
UHAKKM-2	Very low	$3.0\pm 0.21 \times 10^2$
UHAKKM-3	Very low	$2.8\pm0.14\times 10^2$
UHAKKM-4	Low	$2.3\pm0.17\times10^2$
UHAKKM-5	Low	$3.1\pm0.21\times10^2$
UHAKKM-6	Low	$4.2\pm0.34\times10^2$

Table.5 and Anti-viral assay graph: Table.5 indicates the activity of the molecules in inhibiting the viral replication was evaluated by p24 assay using a previously established protocol. The molecules UHAKKM-1 to 6 have shown only 20% inhibition at 100µM concentration.

Discussion:

We have taken previous results from a comparative molecular field analysis (CoMFA) of a set of pyridine analogues that inhibit the Topo IIβK_{HIV} *invitro* phosphorylation assay. The results indicated a strong correlation between the inhibitory activity of these pyridine analogs and the steric and electrostatic fields around them. CoMFA quantitative structure-activity relationship models (3D-QSAR model) with considerable predictive ability were obtained.

Based on 3D-QSAR, process of rational drug design, synthesis and development of potential HIV-1 inhibitors, from CoMFA contour model, we noticed steric factor, electrostatic factor and pyridine moiety should be needed for drug with good activity against HIV-1 and Topo IIβK_{HIV} inhibition. Before selecting pharmacophore group on pyridine, we consider all the knowledge of active site of Topo II$\beta K_{HIV,}$ including this knowledge, we also kept an eye on below features as a medicinal chemist and consider easily public availability of product.

Medicinal chemist must and should be consider these features before drug synthesis:

1. Rationale for pharmacophore selection, for this we read previous details of anti-HIV drug discovery.

2. Ease of synthesis, for this in drug synthetic process should have less steps, followed green chemistry rules for reduce environment pollution.

3. We considered also patentable drug synthesis.

4. We also focused on competitive with market available drugs anti-HIV-1 activity.

Pharmacophore selection:

To identify pharmacophore moiety that has desired therapeutic property when kept on pyridine common core, we searched whatever sources are available like drugs from microbial sources, drugs from animal sources, drugs from plant sources and different drug features which are market available. Finally, we found very interesting pharmacophore that is coumarin. Based on QSAR analysis dicoumarol placed on pyridine have shown TopoIIβ kinase inhibition predicted pIC_{50} values are high. Hence, we have chosen dicoumarol as pharmacophore on pyridine base molecule.

Rationale for coumarin pharmacophore selection:

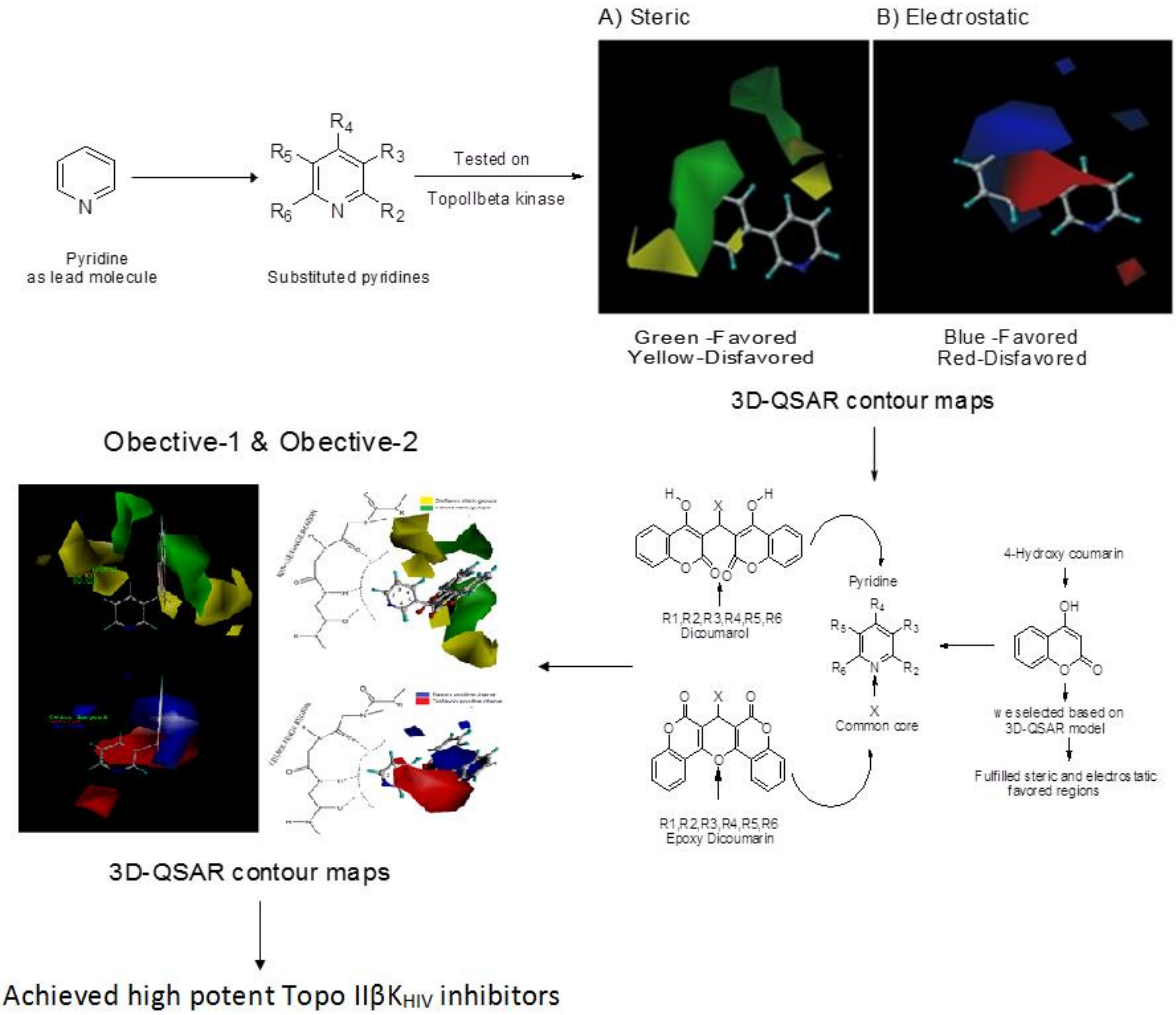
Coumarin is a phytochemical found in a large number of plants like Tonka beans, lavender, licorice etc that has been shown to have anti-tumour, anti-fungal and anti-coagulant activities. Number of drugs with coumarin moiety against chronic infections, inflammation, blood anti-coagulation, and high protein edema [47] have been studied and reported. A previous study of this structural frame works (coumarin moiety having compounds) reported a considerable activity against the HIV-1 integrase [48]. We noticed pyridine-dicoumarol compounds have fulfilled complementary binding at steric and electronic regions of TopoIIbetakinase in

CoMFA model. Hence, we have been selected dicoumarol moiety as pharmacophore for Topo IIβK_{HIV} inhibition.

Ease of pyridine-coumarin derivative synthesis and economic favourable:

In among coumarin, we have chosen 4-hydroxy coumarin, reason for that the 4-hydroxycoumarin condensation with pyridine aldehydes gives product is a concerted reaction. In our drug development process, we achieved catalyst-free and efficient eco-friendly synthesis of pyridine dicoumarol Derivatives in Water with excellent yields (see scheme-2). An efficient, a simple green protocol for their synthesis that gives excellent yields in water medium under conventional reflux in a short time. This aqueous mediated reaction of 4-hydroxycoumarin and three pyridine-aldehydes avoids the use of corrosive, toxic solvents and provides several advantages such as short reaction time and scalable green synthesis. 4-hydroxycoumarin is economically very inexpensive compound.

Fig.12: Over all brief discussion about objective -1 cycle map:



Scheme-2 products (UHAKKM-4 to 6) Topo IIβK$_{HIV}$ inhibition and antiHIV-1 activity:

Succeeding the synthesis of three pyridine dicoumarols UHAKKM-4 to 6 (scheme-2 products), we evaluated pyridine dicoumarols against Topo IIβK$_{HIV}$ phosphorylation *invitro* assay, resulted Topo IIβK$_{HIV}$ inhibition IC$_{50}$ values in picomolar concentration. In that UHAKKM-4 has shown highest inhibition activity. But surprisingly this UHAKKM-4 to 6 compounds has not shown anti-HIV-1 activity (less antiHIV-1 activity). But we had confident on some

structural studies leads to modification of UHAKKM-4 to 6 compounds will have shown result as potential antiHIV-1 activity. As predicted before by 3D-QSAR models 4^{th},5^{th},6^{th}position steric group contained pyridine derivatives shows high activity against kinase, experimentally also 3,3'-(pyridin-2-ylmethylene) bis(4-hydroxy-2H-chromen-2-one) (UHAKKM-4) showed highest activity among scheme-2 pyridine-dicoumarols. Because in UHAKKM-4 dicoumarol moiety situated on 6^{th}position on pyridine but it is expanded major part of dicoumarol until 5^{th} position, because of that UHAKKM-4 has shown little bit highest kinase inhibition activity among scheme-2 products.

Synthetic method of pyridine dicoumarol derivatives:

Scheme-2: Catalyst-free, fast and efficient eco-friendly synthesis of pyridine - dicoumarols in water (UHAKKM-4 to 6)

Table 6: Three pyridine dicoumarols preparation reaction condition

Entry	R	Product	Time (min)	Yield (%)
1	2-C_5H_4N	1	45	90
2	4-C_5H_4N	**2**	40	95
3	3-C_5H_4N	**3**	35	93

Table 6: Indicate pyridine-dicoumarols synthesized in short time with high yields without column chromatography.

Table 7: Evaluation of catalytic activity of different catalysts for the synthesis of dicoumarol **UHAKKM-6** in water.[a]

Entry	Catalyst	Mol (%)	Time	Yield (%)
1	AcOH	10	5 h	50
2	I_2	10	4 h	55
3	Ptsa	10	10 h	45
4	NH_4Cl	10	10 h	58
5	LiBr	10	3 h	64
6	NH_4OAc	10	1 h	58
7	$Na_2.EDTA.H_2O$	10	50 min	75
8	Catalyst-free	—	35 min	93

[a]Reaction conditions: 4-hydroxycoumarin (2.0 mmol); pyridine-3-aldehyde (1.0 mmol); Catalyst (10 mol%); water (15 mL) under reflux conditions.

Table 7: Here we wish to report a catalyst-free green approach for the synthesis of dicoumarol derivatives under conventional reflux conditions in water. In our initial study, 4-hydroxycoumarin was reacted with pyridine-3-aldehyde in the presence of NH_4OAc in water under refluxing temperature for 1 h and the expected product was obtained in 58 % yield (Table 4, entry 6). The use of other catalysts (Table, entries 1-5) does not improved the yields even after several hours in water under reflux conditions, and where the reaction was carried out in presence of disodium EDTA hydrate salt in water under the same conditions the reaction was completed within 50 min affording the desired product in 75% yield.

However, excellent yield was achieved when the reaction was carried out in absence of catalyst under conventional reflux temperature in water and the reaction was completed within 35 min yielded 93% (Table.7, entry 8). In order to optimize the catalyst-free reactions, we have evaluated the reaction of 4-hydroxycoumarin and pyridine-3-aldehyde to afford in various other organic solvents such as CH_3OH, dichloromethane and CH_3CN under reflux conditions for 24

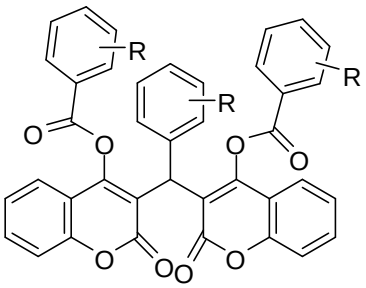
h, and percentage of yields are very poor (40, 25 and 30) with compare to the water-mediated green protocol (Table.7, entry 8).

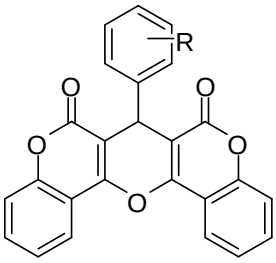
These observations encouraged us to expand the scope and generality of this standardized (catalyst-free, water-mediated) reaction methodology, three possible pyridine-dicoumarol derivatives were synthesized starting from 4-hydroxycoumarin and various pyridine aldehydes under conventional reflux conditions. The yields were obtained excellent for pyridine-dicoumarol derivatives and were in the range of the 90-98%. we used this method for the synthesis of 3,3'-(pyridin-n-yl methylene) bis(4-hydroxy-2H-chromen-2-one) (pyridine-dicoumarol derivatives) which have shown high inhibition of Topo IIβ Kinase activity at picomolar concentration.

Scheme-1 products (UHAKKM-1 to 3) TopoIIβK$_{HIV}$ inhibition and antiHIV-1 activity:

An efficient new synthetic industrial procedure for the preparation of 3,3^{1}-arylidene-4,4^{1}-epoxy dicoumarin via the elimination of the water molecule from the reaction of dicoumarol and benzoyl chloride in the presence of tri ethylamine in acetonitrile at room temperature reported. The advantages of this method are high yields, no need column chromatography but for biological activity studies purpose we did small pack column chromatography to get pure compounds. These compounds have shown high inhibition of HIV-1 associated Topoisomerase IIβ kinase activity at very low concentration(picomolar). But surprisingly this UHAKKM-1 to 3 compounds has not shown anti-HIV-1 activity (very less antiHIV-1 activity). In this new method, we expected dibenzoyl derivative of dicoumarol derivative, but obtained 3,3^{1}-arylidene- 4,4^{1}-epoxy dicoumarin.

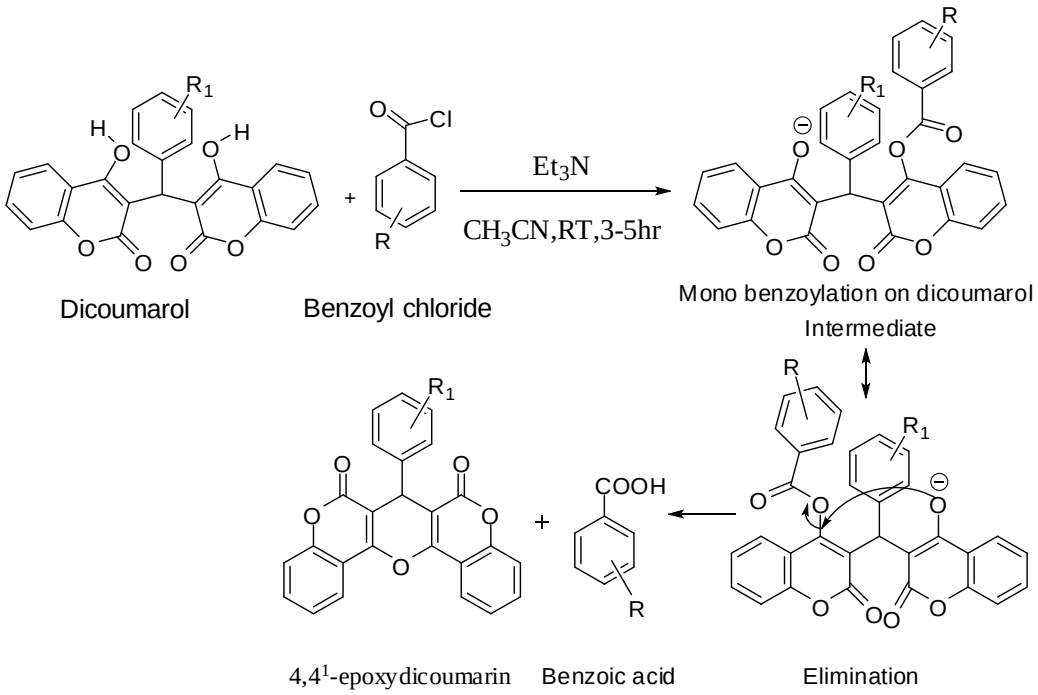
Expected product **Final product**





Dibenzoyl derivative of dicoumarol **4,4[1]-epoxydicoumarin**

Scheme-1 proposed mechanism:



By using scheme-2 products we were synthesized scheme-1 products. This is novel method. We expected dibenzoyl derivative of scheme-1 product but we achieved finally 4,4[1]-epoxy dicoumarin products.

We tried to synthesis, benzoylation of scheme-2 products because reduce the hydroxyl group interference at the time of cleavage the dicoumarols (scheme-1 products) lactone ring. For

that first to protect the hydroxyl group of scheme-2 products needed. If lactone ring cleavage was happened scheme-2 products can expand structure and conformational flexibility enhancement happened, which leads to optimum comparative binding at the TopoIIβK_{HIV} target, which can show potential anti-HIV-1 activity. Scheme-1 reaction very specific. We tried 11th combinations of reactants, solvent, catalyst. But we achieved 10th combination showed good yield see table 6. I observed low solubility in DMSO also; hence we cannot get ^{13}C NMR spectra clearly. Because^{13}C NMR spectra required more solubility of compound in deuterated solvent.

Table 8: Scheme-2 method different trial combinations for the synthesis of UHAKKM-1 to 3

Entry	Reactant	Reactant	Catalyst	Solvent	Product
1	Dicoumarol	Acetyl Chloride	K_2CO_3	CH_3OH	No
2	Dicoumarol	Acetyl Chloride	K_2CO_3	Acetone	No
3	Dicoumarol	Acetyl Chloride	NaOH	CH_3OH	No
4	Dicoumarol	Acetyl Chloride	NaOH	Acetone	No
5	Dicoumarol	BenzoylChloride	K_2CO_3	CH_3OH	No
6	Dicoumarol	BenzoylChloride	NaOH	CH_3OH	No
7	Dicoumarol	BenzoylChloride	NaOH	Acetone	No
8	Dicoumarol	BenzoylChloride	K_2CO_3	Acetone	Yes
9	Dicoumarol	BenzoylChloride	Et3N	Acetone	Yes
10	Dicoumarol	BenzoylChloride	Et3N	Acetonitrile	Yes
11	Dicoumarol	BenzoylChloride	K_2CO_3	Acetonitrile	Yes

Table 8: Scheme-1 method: For standardizing scheme-1 method, we tried to synthesis of these compounds (UHAKKM-1 to 3) in 11 combinations i.e. different reactant and solvents and catalysts, resulted 10th combination good method for the synthesis of scheme-1 compounds.

Table. 9: scheme-1 method reaction conditions and yields:

Product Name	Yield	Temperature	Reactant	Catalyst	Solvent	Time
UHAKKM-1	90%	RT	Benzoyl Chloride	Et_3N	CH_3CN	6hr
UHAKKM-2	95%	RT	Benzoyl Chloride	Et_3N	CH_3CN	5hr
UHAKKM-3	84%	Reflux	Benzoyl Chloride	Et_3N	CH_3CN	12hr

Table. 9: Except UHAKKM-3, remain UHAKKM-1 and 2 can synthesize in room temperature. For the synthesis of UHAKKM-3 reflux condition is required.

Table 10: various benzoyl chlorides effect on scheme-2 method:

Entry	Reactant	Temperature	Yield	Time
1	Benzoyl Chloride	RT	95%	5-7hr
2	3-fluoro benzoyl chloride	RT	82%	5-7hr
3	4-Methyl Benzoyl Chloride	RT	70%	5-7hr
4	3-methoxy benzoyl chloride	RT	80%	5-7hr

Table 10: has shown scheme-1 product yield affected by various substituted benzoyl chlorides. Above the table have shown unsubstituted benzoyl chloride involved scheme-1 reaction method product yields are more than substituted benzoylcholride. Here we used UHAKKM-2 for standardization.

These scheme-1 products showed Topo-IIβ kinase inhibition, but less many activities than scheme-2 products activity. In these scheme-1 products also 6thposition substituted pyridine more activity against Topo IIβ Kinase (UHAKKM-1) as we explained previous scheme2

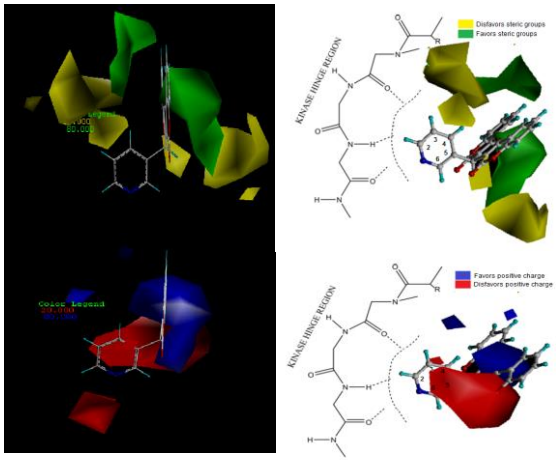
discussion. These experimental Topo IIβ Kinase inhibition values almost correlated with 3D-QSAR predicted kinase inhibition activity values.

In this study, we have designed and developed a series of dicoumarol derivatives with potential TopoIIβkinase inhibition. These compounds specifically targeted the novel Topo $IIβK_{HIV}$ that was shown to be responsible for phosphorylation of Topoisomerase IIβ [3] necessary for HIV-1 replication.

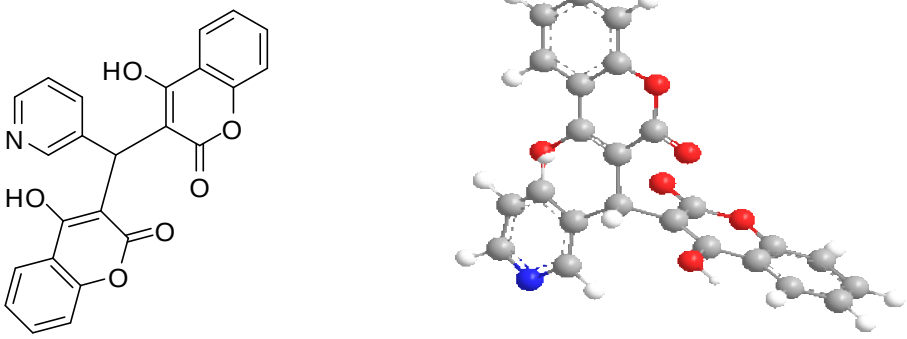
The designed structures satisfied the contour constraints as shown in **Fig.13** and thus showed good Topo $IIβK_{HIV}$ antagonism.

Fig.13: 3D-QSAR model of 3,3'-(pyridin-3-ylmethylene)bis(4-hydroxy-2H-chromen-2-one)

a)



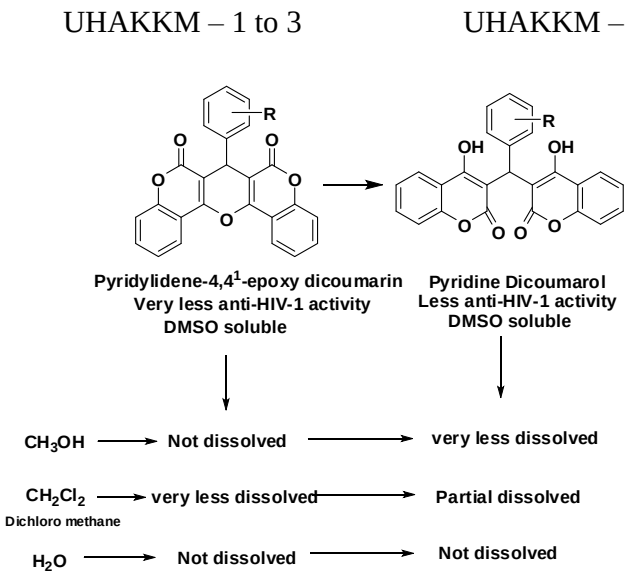
b)



3,3'-(pyridin-3-ylmethylene)bis(4-hydroxy-2H-chromen-2-one)

Fig.13: 3D-QSAR Contours overlaid on the structure Pyridine Dicoumarol derivative UHAKKM-6 a) I) steric II) electrostatic contours obtained from 3D QSAR equation. b) Structure of 3,3'-(pyridin-3-ylmethylene) bis(4-hydroxy-2H-chromen-2-one). The bulk dicoumarol ring of the compound lies overlapped with the green region of the contour, suggesting the role of the bulk group in the activity of the compound. Similarly, the overlap of red region with the enolic Oxygen group substitution suggests the role of electronegative groups in the enhanced TopoIIβK_{HIV} antagonism.

Observation: Reason for low antiviral activity of UHAKKM-1 to 6



Note: During synthesis, we noticed solubility problem of UHAKKM-1 to 6 compounds in organic solvents. From the result of solubility problems, we noticed pharmacodynamics and pharmacokinetic problems can affect to reach the target in live cell leads to null antiviral activity.

Pyridine epoxy dicoumarin derivatives (UHAKKM-1 to 3) have shown high inhibition of Topo IIβK_{HIV} activity at low concentration but surprisingly have not shown anti-HIV-1 activity even at high concentration (100µM). To increase the small extent of the conformational flexibility of UHAKKM-1 to 3, another set of compounds, Pyridine dicoumarol derivatives (UHAKKM-4 to 6) were synthesized, these are de-cyclized structures with open ring system that have hydroxyl groups and thus reducing the rigidity. Interestingly, these compounds also showed similar anti-HIV-1 activity (little bit more) as UHAKKM-1 to 3. In the second set of compounds (UHAKKM-4 to 6), the presence of hydroxyl groups leads to the formation of intra-molecular hydrogen bonds further making the compounds rigid [49]. From the above results, it was suspected that these molecules showed low activity because of lack the conformational flexibility to reach the intracellular target, as seen in p24 assay.

Thus, to increase the flexibility of pyridine dicoumarols (UHAKKM-4 to 6), the intramolecular hydrogen bonding removal needed. It was also observed that the absence of pyridine moiety showed TopoIIβK_{HIV} inhibition activity decreased. Hence it was concluded that the pyridine ring, enolic oxygen and flexibility plays a crucial role in inhibition of TopoIIβK_{HIV} bioactivity of dicoumarol derivatives

Conclusion:

In conclusion, a structure-based evaluation of substructures needed to reach target in live cell during the process of drug development. We noticed scheme-1 and scheme-2 products (UHAKKM-1 to 6) have faced pharmacokinetics and pharmacodynamic problems to reach the target in live cell. We have been strongly believed, structure modification of pyridine dicoumarol derivatives leads to get optimal partition coefficient, can able to an increase in inhibition of the Topo IIβK_{HIV} activity and antiviral activity, reason for that we have already known TopoIIβK_{HIV} inhibition IC_{50} values in picomolar concentration.

Summary:

We present a study of Pyridine-dicoumarol derivatives for action against a novel Topoisomerase II Beta Kinase, identified in the HIV-1 viral lysate. These derivatives were designed based on previous 3D-QSAR studies. An efficient and simple green protocol for their synthesis that gives excellent yields in water medium under conventional reflux in short time is also reported. An efficient novel industrial procedure for the preparation of 3, 3^1-arylidene-4,4^1-epoxy dicoumarin via the elimination of the water molecule from the reaction of dicoumarol and benzoyl chloride in the presence of tri-ethylamine in acetonitrile at room temperature. These compounds have shown inhibition of HIV-1 associated Topoisomerase IIβ kinase activity at picomolar concentration.

Future Perspective:

This study demonstrates, TopoIIβK_{HIV} as a potential target to control HIV-1 infection. We demonstrated the achievement of new method (Scheme-1) found during the process of drug development. Scheme-1 and 2 compounds have shown high potential inhibition at picomolar concentration. We noticed that pharmacokinetics and pharmacodynamics problems of UHAKKM-1 to 6 leads to absence of anti-HIV-1 activity.

Conclusions:

1. 3D-QSAR was employed to design potent compounds to inhibit Topo IIβ$K_{HIV.}$
2. 4-Hydroxy Coumarin was selected and used as the starting material and 6 compounds were designed and synthesized successively.
3. UHAKKM-1 to 3 compounds synthesized by novel method.
4. Synthesized compounds showed high TopoIIβK_{HIV} inhibition.

In conclusion, based on 3D-QSAR we selected dicoumarin steric group as appropriate pharmacophore placed on pyridine lead to the development of potent anti-HIV-1 compounds targeted to Topo IIβK_{HIV}.

Chapter-3

Objective-2

Molecular derivatization studies of pyridine-coumarin analogues for improved cellular bioavailability and anti-HIV-1 activity.

In objective-1, we designed several pyridine derivatives of coumarin, based on the 3D QSAR analysis model, these studies yielded novel coumarin derivatives with significant inhibitory activity against TopoIIβk_{HIV} in vitro. When anti-HIV-1 activity of these molecules analysed in T cells, the results showed that UHAKKM-1 to UHAKKM-3 possess very low anti-HIV-1 activity and UHAKKM-4 to UHAKKM-6 showed slightly increased anti-HIV-1 activity, but anti-HIV-1 activity is insignificant. A molecule that is active against target in vitro and fail to confer similar activity could be due to (a) its inability to enter cells, (b) loss of stability under cellular environment, (c) intracellular modification of the target.

Rationale:

Compounds UHAKKM-1 to UHAKKM-6 are soluble in DMSO and were not soluble in water. This made to hypothesize whether the low activity could be due to its low aquatic solubility. This problem is solved by some structural medications to compounds UHAKKM-4 to UHAKKM-6 to enhance their solubility in water.

To enhance solubility, sodium salt of UHAKKM-4 to 6 was prepared as per Scheme-3 and analyzed the results significantly enhanced solubility and anti-HIV-1 activity.

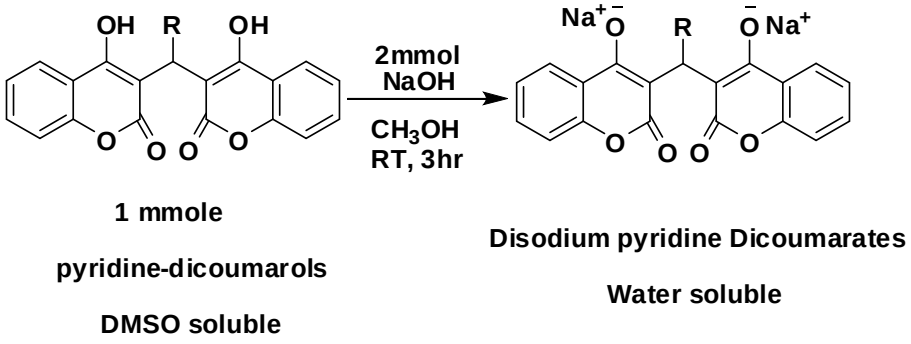
Methods:

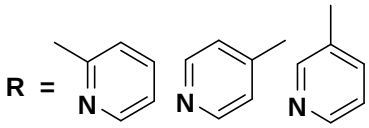
Chemistry:

Scheme-3:

Disodium pyridine dicoumarate derivatives:

Pyridine dicoumarols (1mmol) **(UHAKKM-4 to 6)** were added to 10 ml methanol in round bottom flask (25ml) and 2 mmol NaOH was added, then the reaction mixture was stirred for 3 hrs. After obtaining the clear solution, under reduced pressure, the solvent was evaporated and recovered as solid products. The obtained products are thoroughly soluble in water [19].





Results:

Characterization of compounds:

Disodium pyridine dicoumarates IR-spectral analysis: The binding mode of the pyridine dicoumarols to sodium was explained by IR spectra of the disodium pyridine dicoumarates as compared with the free pyridine dicoumarols.

IR-spectra of the compounds were analysed by JASCO IR-A-302 Spectrometer and FTIR in the range of 4000-400 cm^{-1} as shown in the table 11.

Table 11: Specific IR frequencies of the pyridine dicoumarols and disodium pyridine dicoumarates. **(see supporting spectral information from figure-S1 to S30):**

Compound	ν OH/H_2O	ν (C=O)	ν (C=C)
UHAKKM-4	3124m 3068m	1698s 1639s	1614s 1538s
UHAKKM-5	3088m 3068m	1684s 1625s	1598s 1541s
UHAKKM-6	3135m 3060m	1686s 1614s	1536s 1462s
UHAKKM-7	3211br	1630s 1595s	1496s
UHAKKM-8	3216br	1629s 1597s	1476s
UHAKKM-9	3212br	1630s 1598s	1511s

IR-spectrum of disodium pyridine-2-dicoumarate (UHAKKM-7):

Bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-pyridin-2-yl-methane (UHAKKM-4) bands appeared in the IR spectrum at 3124,3068,1698,1639,1614,1538 cm^{-1}. 1698 and 1639 cm^{-1} bands can be attributed to the carbonyl groups of the lactone rings stretching vibrations. 1614 and 1538 cm^{-1} Bands can be related to the conjugated olefinic system stretching vibrations. A characteristic broad band of νOH with coordinated water was observed in the range 3100-3500 cm^{-1} in the IR spectrum of the UHAKKM-7 (disodium pyridine-2-dicoumarate). We observed at 3124 and 3068 cm^{-1} band in the spectrum of the UHAKKM-4 was disappeared in the spectrum of the UHAKKM-7. A comparative study of the IR – spectrum of the UHAKKM-4

and UHAKKM-7 disclose the disappearance of absorption bands observed in the UHAKKM-4 at 3124 and 3068 cm^{-1} directing the disappear of enolic protons on UHAKKM-7 compound. The ν (C=O) bands at 1698 and 1639 cm^{-1} exhibits a shift of 40-60 cm^{-1} to lower wavenumber values on UHAKKM-7, which may be taken as confirmation for the involvement of the C =O groups in coordination with sodium ion.

Remaining compounds (UHAKKM-8, UHAKKM-9) also show similar IR spectral properties as UHAKKM-7.

HRMS: (see supporting spectral information from figure-S1 to S30):

UHAKKM-7[Disodium-3,3'-(pyridin-2-ylmethylene)bis(2-oxo-2H-chromen-4-olate)]: HRMS (ESI+) [M+H]: calcd. for $C_{24}H_{13}NO_6^{2-}.2Na^{\oplus}$ 458.067478, found 458.0617

UHAKKM-8[Disodium-3,3'-(pyridin-4-ylmethylene)bis(2-oxo-2H-chromen-4-olate)]: HRMS (ESI+) [M+H]: calcd. for $C_{24}H_{13}NO_6^{2-}.2Na^{\oplus}$ 458.067478, found 458.0611

UHAKKM-9[Disodium-3,3'-(pyridin-3-ylmethylene)bis(2-oxo-2H-chromen-4-olate)]: HRMS (ESI+) [M+H]: calcd. for $C_{24}H_{13}NO_6^{2-}.2Na^{\oplus}$ 458.067478, found 458.0618.

Invitro - TopoIIβK_{HIV} inhibition assay results:

Table 12: Theoretical and experimental activities of the designed compounds

S.No	R	Molecules	Theoretical pIC_{50} Values	Experimental pIC_{50} values	IC_{50}concentration (pM)
1	2-C_5H_4N	UHAKKM-4	8.89	9.56	2.7± 0.4 x 10^2
2	2-C_5H_4N	UHAKKM-1	9.09	9.52	3.0± 0.21 x 10^2
3	4-C_5H_4N	UHAKKM-5	9.49	9.56	2.8±0.14x 10^2
4	4-C_5H_4N	UHAKKM-2	9.09	9.04	2.3±0.17x10^2
5	3-C_5H_4N	UHAKKM-6	9.77	9.51	3.1±0.21x10^2
6	3-C_5H_4N	UHAKKM-3	9.84	9.38	4.2±0.34x10^2

7	2-C_5H_4N	UHAKKM-7	11.89	12.04	0.9±0.03
8	4-C_5H_4N	UHAKKM-8	11.72	12.05	0.8±0.06
9	3-C_5H_4N	UHAKKM-9	11.94	12.06	0.8±0.05

All the molecules of the pyridine-dicoumarol derivatives and disodium pyridine dicoumarate derivatives (1-9) in table-12 were designed based on the contours generated from the 3D-QSAR studies and the activities were predicted using the generated equation. The high predicted values were found to be similar to the experimental value; all molecules were showing IC_{50} at picomolar concentration. As indicated in the Table 12, the IC_{50} concentration of Topo IIβK_{HIV} inhibition and respective pIC_{50} values

MTT assay results:

The toxicity of molecules in SupT1 cells was tested and found CC_{50} concentration. All the compounds showed CC_{50} (50% cytotoxic concentration) above 1mM concentration have shown below.

Table 13: CC_{50} concentration of the UHAKKM-1 to UHAKKM-9.

S. No	R	Molecules	CC_{50} concentration (mM)
1	2-C_5H_4N	UHAKKM-1	2.1±0.21
2	4-C_5H_4N	UHAKKM-2	3.7±0.16
3	3-C_5H_4N	UHAKKM-3	1.3±0.04
4	2-C_5H_4N	UHAKKM-4	4.3±0.31
5	4-C_5H_4N	UHAKKM-5	2.4±0.19
6	3-C_5H_4N	UHAKKM-6	3.1±0.16
7	2-C_5H_4N	UHAKKM-7	1.8±0.05
8	4-C_5H_4N	UHAKKM-8	0.8±0.03
9	3-C_5H_4N	UHAKKM-9	1.3±0.17

The above table indicates the CC_{50} (50% Cytotoxic Concentration) of the compounds, the compounds have 50% cell cytotoxicity even at high concentration (>1mM), 3-phenyl pyridine used as a positive control.

Action of compound on the replication of HIV-$I_{93IN101}$:

The activity of the molecules in inhibiting the replication HIV-$I_{93IN101}$ in Sup T1 cells was estimated using the p24 antigen capture sandwich ELISA method (p24 assay) on the day 4 in the infection conducted in the presence of UHAKKM-1 to 9.

Results in Table No.6, the molecules UHAKKM-1 to 6 have shown low viral inhibition (10-20%) at more than 100 µM and the molecules UHAKKM-7 to 9 have shown significantly high anti-viral activity (13.5 to 21.4 nM) and higher inhibition of Topo IIβK_{HIV} (0.8 to 0.9 pM).

Table.14: Anti-HIV-1 assay results of UHAKKM-1 to 9

Molecule	**Anti-viral IC_{50}(nM)**	**Kinase inhibitionIC_{50}(pM)**
UHAKKM-1	Below10% at 100µM	3.0± 0.21 x 10^2
UHAKKM-2	Below10% at 100µM	2.3±0.17x10^2
UHAKKM-3	Below10% at 100µM	4.2±0.34x10^2
UHAKKM-4	Below10% at 100µM	2.7± 0.4 x 10^2
UHAKKM-5	Below10% at 100µM	2.8±0.14x 10^2
UHAKKM-6	Below10% at 100µM	3.1±0.21x10^2
UHAKKM-7	13.5+1.3	0.9±0.03
UHAKKM-8	16.4+1.7	0.8±0.06
UHAKKM-9	21.4+1.9	0.8±0.05

Table.14: Anti-viral assay. The activity of the molecules in inhibiting the viral replication was estimated by p24 assay using a previously established protocol. The molecules UHAKKM-1 to 6 have shown only 10% inhibition at 100µM concentration. But water-soluble disodium pyridine dicoumarate molecules UHAKKM-7 to 9 have shown high inhibition at low concentration, AZT and 3-Phenylpyridine were used as positive controls. Data were represented as mean ± standard deviation. The value of significance, **P<0.005, *P<0.05.

Discussion:

Many efforts have been made to synthesize large number of compounds in several steps, in those studies some compounds may not exhibit expected biological activity, and thus they lose further investigation. In view of this several important compounds did not reach the therapeutic evaluation. Similarly, UHAKKM-1 to 6 showed significant inhibition of Topo IIβK_{HIV} *in-vitro*, when we evaluated in virus reutilization studies, they did not exhibit significant activity. When we enumerated in cells treated with these compounds, it is found that they are in very low concentrations in cells. Hence, modifications of the compounds have been taken-up, various structural modification of pyridine dicoumarols have been evaluated for enhancing solubility and bioavailability.

Quantitative structure activity relationship study on pyridine dicoumarols to achieve potential anti-HIV-1 activity compounds:

Now we can discuss systematically, how achieved our goal, that potential anti-HIV-1 compounds.

Hansch and co-workers in the early 1960's proposed that the biological activity of a compound is a function of its ability to reach and bind to its target site. Hansch proposed that drug actions could be divided into two stages.

1) The transport of the drug to its site of action - pharmacokinetics
2) The binding of the drug to the target site - pharmacodynamics

During the transport of the drug from the point of administration to its site of action, they pass through numerous membranes and aquatic compartments. So, the ability of the drug to reach its target is dependent on its optimum lipophilicity (amphiphilicity). Therefore, the transport characteristics of different lead analogues are expected to be different.

Any ideal drug candidate depends on below physico-chemical parameters

1. Lipophilicity of the drug
2. Steric and Electronic factors

Lipophilicity is the main factor governing absorption, distribution, metabolism, and elimination (ADME) of drugs in biological systems, this lipophilicity of drug play key role in pharmacokinetic studies. Similarly, electronic and steric features are vital in drug and optimal supramolecular binding at receptor site, means electronic and steric features play key role in pharmacodynamic process of a drug.

1. Lipophilicity of drug:

Following two parameters are commonly used to relate distribution of drug with biological activity.

A) Partition coefficient (P)
B) Lipophilicity substituent constant (π)

Partition coefficient refers to whole molecule; lipophilicity substituent constant is related to substituent groups present on the main compound.

A) Partition coefficient:

A drug has to pass through a number of biological interface compartments in order to reach its site of action. Each biological interface is surrounded by lipid phase and aqueous phase (water). Partition coefficient is an important parameter where it gives the solubility of the drug in lipid

phase and water phase. To determine the partition coefficient n-octanol/H_2O system is frequently chosen, because it has the most extensive database.

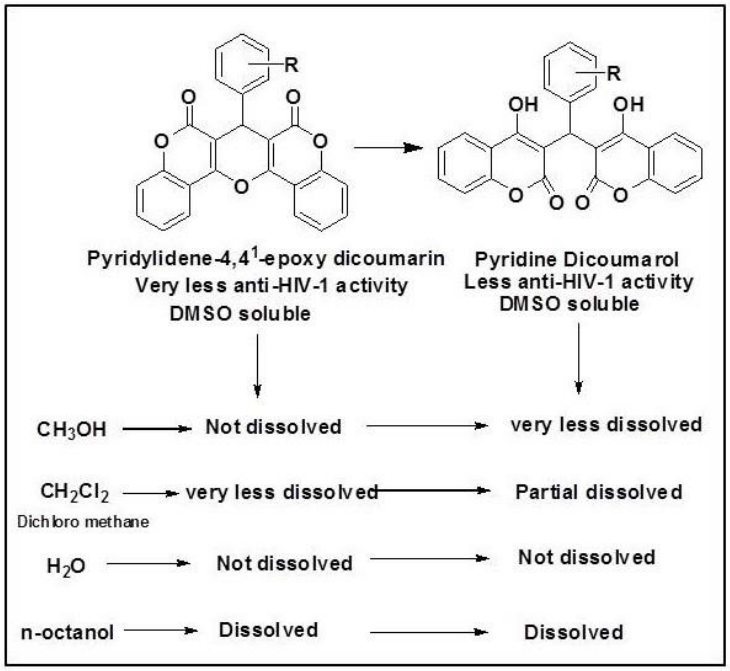
P = n-octanol/water if p >1 = lipophilic, p < 1 = hydrophilic

If 'p' is more than 1, then the drug molecule is more lipophilic, if it is less than 1, the drug molecule is less lipophilic more hydrophilic. If 'p' is more than 2, then the drug can pass through blood brain barrier.

Partition coefficient application to our scheme-1 and scheme-2 products:

Herein we tested solubility in n – octanol and water and also in different solvents. For partition coefficient calculation of these compounds we consider only two solvents i.e. n–octanol and water.

UHAKKM-1 to 3 UHAKKM-4 to 6



In 'n-octanol' both scheme-1 and scheme-2 products are dissolved and in water these compounds not dissolved. From this we can say partition coefficient (p) value more than 1 or

infinitive. Hence, partition coefficient value determines the ability of these compounds to cross lipid bilayer of the cell, but not aquatic layer, in aqua phase layer these compounds glued. That's why we observed *in vitro* kinase phosphorylation assay, we saw kinase inhibition IC_{50} in picomolar concentration and in anti-HIV-1 p24-ELISA assay very less activity, is almost nil.

B) Lipophilicity substituent constant (π):

Lipophilic substituent constant (**π**) is also known as hydrophobic substituent constant. According to Hansch and Co-workers it can be calculated as follows

$$\pi = \log p_x - \log p_H$$

Where $\log p_x$ = Partition coefficient of substituted compound

$\log p_H$ = Partition coefficient of unsubstituted compound (standard compound)

A positive '**π**' value indicates that a substituent has a higher lipophilicity than' H', and so will increase the concentration of the compound in the n–octanol layer and by increase its concentration in the lipid material of biological system. Conversely a negative '**π**' value shows that the substituent has a lower lipophilicity than 'H' and so probably increases the concentration of the compound in the aqua media of biological system.

Linear relationship between log p and biological activity:

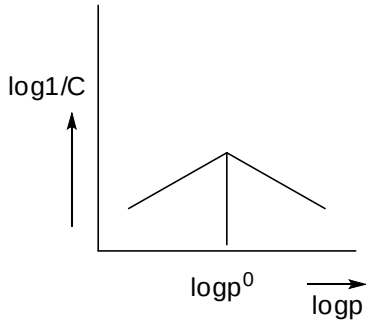
The nature of the relationship obtained depends on the range of 'p' values for the compounds used. If this range is small, the result may be expressed as a straight-line equation, having the general formula.

$$\log (1/C) = K_1 \log p + K_2$$

Where K_1 and K_2 are constants, this equation indicates a linear relationship between the activity of the drug and its partition coefficient. In such a linear relationship, the biological activity increases as the lipophilicity increases.

Non-linear relationship between log p and biological activity:

Linear relationship between lipophilicity and biological activity apply to only certain range of lipophilicity values. If lipophilicity exceeds a definite limit a more/less sharp decrease of biological activity. In linear equation the lipophilicity limits are still beyond the range of optimum lipophilicity. In series of compounds biological activity increases to a maximum (log P) and then decreases. If a plot is drawn between biological activity (B.A) (log1/C) and partition coefficient (logp) a parabola is obtained.

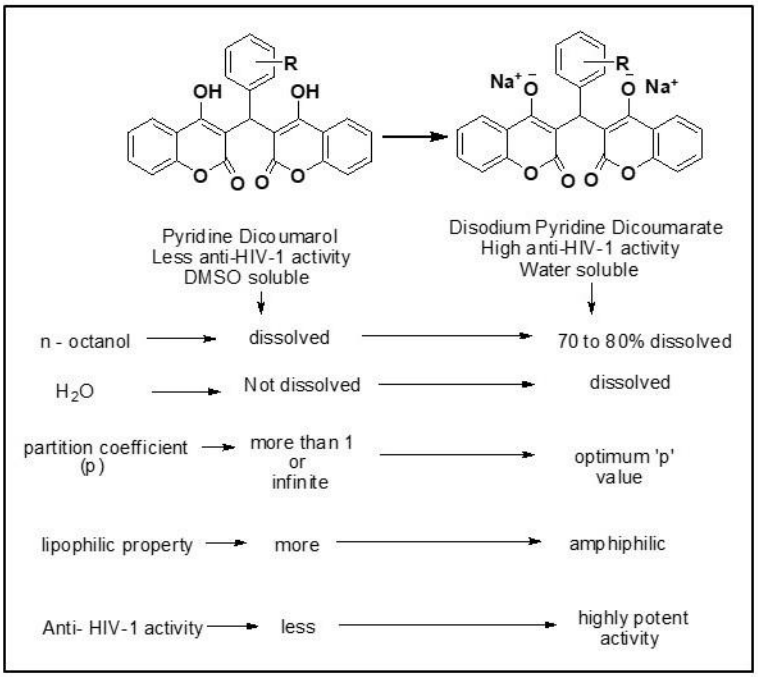


P^0 = the optimum value for the partition coefficient where the biological activity is maximum, but after that optimum partition coefficient, biological activity decreases. The main reason for the decrease in the biological activity is due to increase in lipophilicity above optimum. Because of the high lipophilicity of the drug molecule the compound becomes so lipid soluble that it no longer can circulate in the blood stream but merely becomes glued or buried in lipid membrane. Highly polar compounds are so insoluble in organic phases, that they cannot cross lipid membranes and will remain in the aqueous phase. Hence only compounds of intermediate

lipophilicity will be able to pass through or cross lipophilic as well as hydrophilic barrier to reach their intracellular target.

Lipophilicity substituent constant (π) application to our UHAKKM-4 to 6 and UHAKKM -7 to 9:

UHAKKM-4 to 6 **UHAKKM -7 to 9**



We were interested to calculate lipophilic substituent constant (**π**) formula; it can be calculated as follows for estimating lipophilicity

$$\pi = \log p_x - \log p_H$$

Where '**π**' is lipophilic substituent constant of Disodium pyridine dicoumarate compounds (UHAKKM – 7 to 9)

Where log p_x = Partition coefficient of disodium pyridine dicoumarate compounds

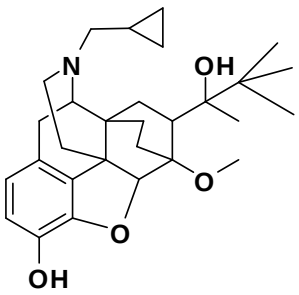
Log p_H = Partition coefficient of pyridine dicoumarol compounds (standard compounds)

For disodium pyridine dicoumarate compounds have shown a negative '**π**' values, that the removal of hydrogen substituent from enolic hydroxyl group of pyridine dicoumarol has a

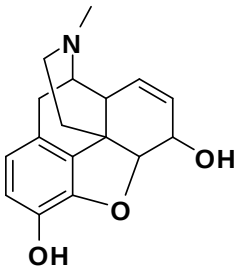
lower lipophilicity than pyridine dicoumarols and so probably increases the concentration of the compound in the aqua media of biological system. We also noticed pyridine dicoumarols (UHAKKM -4 to 6) are not dissolved in water and dissolved in n – octanol, so partition coefficient value(P) is high, this leads to perform high lipophilicity and glued in lipid phase of cell membrane. Finally resulted less anti-HIV-1 activity by pyridine dicoumarols. When hydrogen was removed from enolic hydrogen of hydroxyl group of pyridine dicoumarols (i.e. disodium dicoumarates UHAKKM – 7 to 9) only 70-80% dissolved in n – octanol and dissolved total in water. Resulted disodium dicoumarate compounds reached optimum partition coefficient, gained amphiphilic behaviour, which can easily pass through blood brain barrier and aqua phase in biological system. Because of optimum partition coefficient and disodium pyridine dicoumarates have shown high potential anti-HIV-1 activity through reaching the target in HIV-1 infected cell.

2. Steric and Electronic factors of drug:

In order for a drug to bind effectively to its target site, the dimensions of the pharmacophore positions of the drug must be complementary to those of the target site. The Taft steric substituent constant **(E_s)** was the first attempt to show the dimensions of the target site and drug's activity. Steric parameters of the drug marked by affect the drug- receptor interactions reflecting the change in the onset and duration of biological action.



Bupre norphine



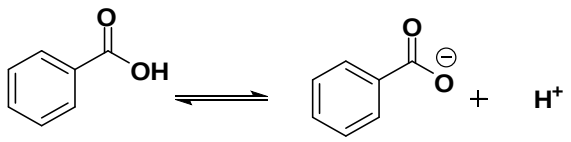
Morphine

For example, Bupre norphine, a more lipophilic drug than morphine and is expected to enter the CNS (central nervous system) rapidly. However, because of bulky substituent at nitrogen, it needs time to get oriented in a favourable conformation. The bulky substituent also delays the detachment of drug from the receptor. This leads to late onset and long duration of action.

Substituent constant or Electronic parameter also known as Hammet constant is important physicochemical parameter played an important role in the design of drugs, which is based on the metabolism and elimination pattern of the drug and drug receptor interactions.

The distribution of the electron in a drug molecule will have a considerable influence in the distribution and activity of a drug, which in turn effects the biological activity. The first attempt to quantify the electron effects of groups on the physicochemical properties of compound was made by Hammet in 1940. He studied the rate of dissociation of benzoic acid (unsubstituted acid) and substituted benzoic acid (substituents may be electron releasing or withdrawing group).

Dissociation of unsubstituted benzoic acid can be represented on



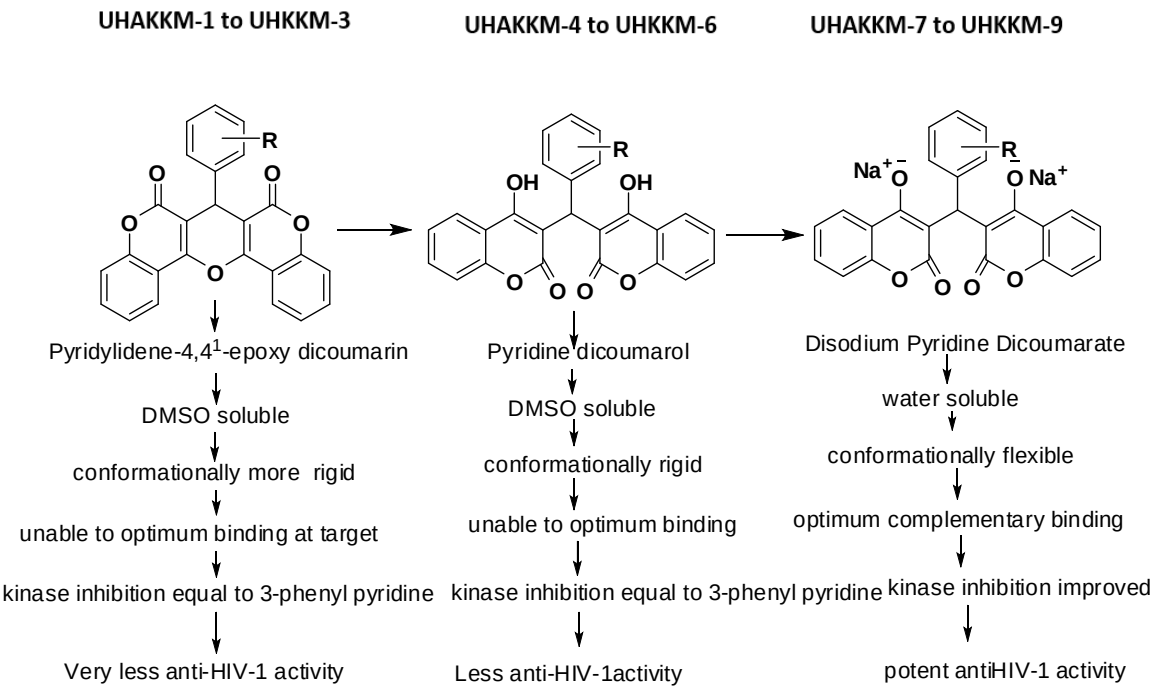
$$\text{Dissociation Constant (k)} = \frac{[\text{phcoo}^-][\text{H}^+]}{[\text{phcooH}]}$$

If the dissociation constant is more, acidic strength is more. The value of **'k'** depends on substituting different substitutions (electron releasing and withdrawing). If the electron withdrawing group is present on benzoic acid, acidic strength of benzoic acid increases. In the presence of electron donating group on benzoic acid, acidic strength of benzoic acid decrease. Hence, different electronic effects needed for the enzyme inhibition.

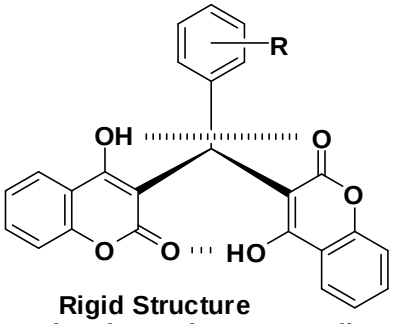
Steric and Electronic parameters favours in enhancement of TopoIIβK$_{HIV}$ inhibition and improved potential anti-HIV-1 activity:

Before synthesis of 7 to 9, we have designed Scheme-1 and 2 products (i.e. UHAKKM-1 to 6) on the basis of 3D-QSAR CoMFA contour model. Their theoretical predicted Topo IIβK$_{HIV}$ inhibition pIC$_{50}$ values lies within 9 to 10. Those designed scheme-1 and 2 products were synthesized and characterized. After confirmed structures of designed scheme-1 and 2 compounds by spectral characterization, evaluated against Topo IIβK$_{HIV}$ by an *in vitro* phosphorylation assay. These scheme-1 and 2 compounds have shown experimental Topo IIβK$_{HIV}$ inhibition pIC$_{50}$ values approximately equal to theoretical predicted pIC$_{50}$ values. We noticed that the standard compound, 3-phenyl pyridine also have shown kinase inhibition pIC$_{50}$ value also lies between 9 to 10 [**28**]. Structurally 3 – phenyl pyridine has one phenyl group as steric group, but comparative scheme-1 and 2 products have larger dicoumarol moiety. So, we expected kinase inhibition pIC$_{50}$ values are more than 3-phenyl pyridine. We thought that if we can answer for this solution, we can achieve enhancement of anti-HIV-1 activity of scheme-1 and 2 compounds. We started investigation on scheme-1 and 2 structural conformation studies. We found reason for this equal pIC$_{50}$ values of scheme-1 and 2 compounds could be due to rigid conformational structure. Therefore scheme-1 and 2 compounds have not shown conformational flexibility to move and optimum complementary binding at its Topo IIβK$_{HIV}$ target.

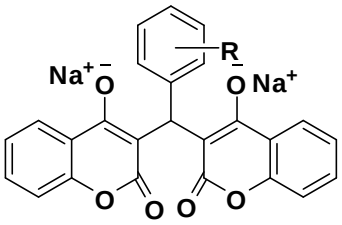
Relaxation of conformational rigidity of scheme-1 and 2 products subsequently leads to enhancement of Topo IIβK_{HIV} inhibition and anti-HIV-1 activity:



The activity of the molecules in inhibiting viral replication was tested using the p24-ELISA assay. It was observed thatUHAKKM-1 to 6 showed low anti-HIV-1 activity even at concentrations higher than 100µM. However, water soluble disodium pyridine dicoumarate molecules UHAKKM-7 to 9 have shown high viral inhibition at low concentration (IC_{50}<38nM). We also tested other disodium aromatic dicoumarate compounds but surprisingly they did not show any viral inhibition and very low TopoIIβK_{HIV} inhibition.



Rigid Structure
Intramolecular Hydrogen Bonding



Water Soluble
Flexibility Increased
No Intramolecular Hydrogen Bonding

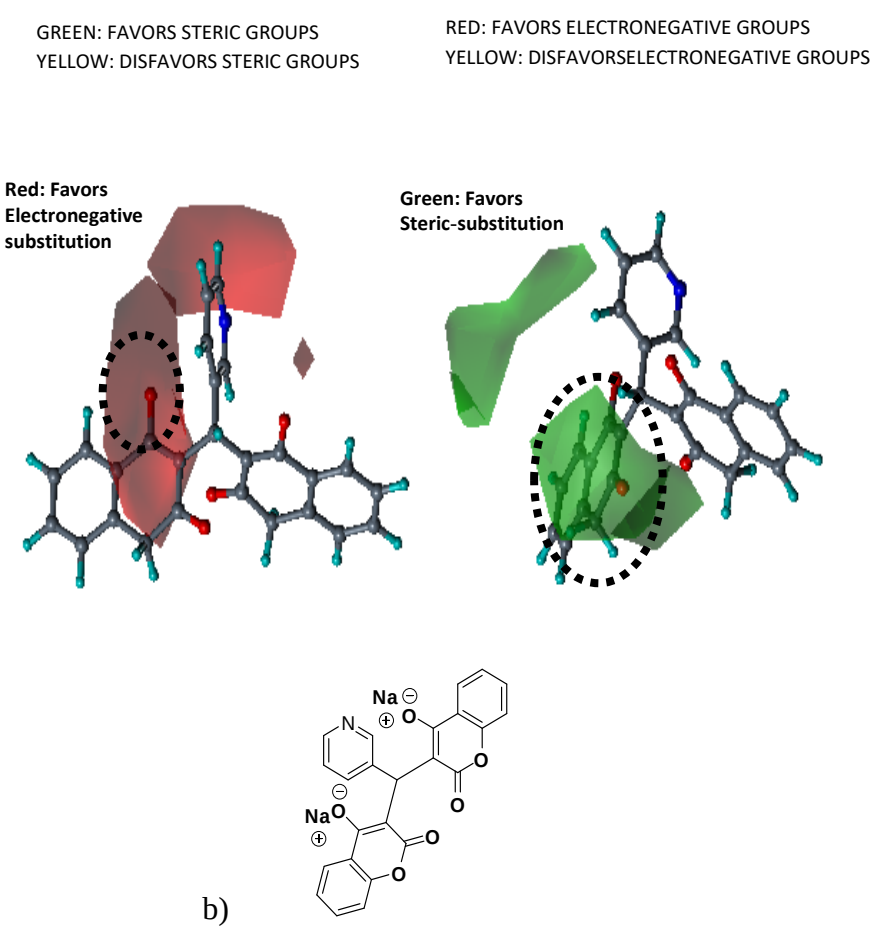
Pyridine epoxy dicoumarin derivatives (UHAKKM-1 to 3) and 3-phenyl pyridine have shown equal inhibition of Topo IIβK_{HIV} activity at low concentration but surprisingly scheme-1 products have not shown anti-HIV-1 activity even at high concentration (µM). To increase the conformational flexibility of UHAKKM-1 to 3, another set of compounds, Pyridine dicoumarol derivatives (UHAKKM-4 to 6) were synthesized, these are de-cyclized structures with open ring system that have two hydroxyl groups and thus reducing the rigidity compared to scheme-1 products. Interestingly, these compounds also showed similar activity as UHAKKM-1 to 3. In the second set of compounds (UHAKKM-4 to 6), the presence of hydroxyl groups leads to the formation of intra- molecular hydrogen bonds further making the compounds rigid [22]. From the above results, it was suspected that these molecules showed low activity because of lack of the conformational flexibility to reach the intracellular target and proper optimum complementary binding, as seen in p24 assay results.

Thus, to increase the conformational flexibility of pyridine dicoumarols (UHAKKM-4 to 6), the intra-molecular hydrogen bonding was removed by using alcoholic NaOH. Because of increased conformational flexibility and formed disodium pyridine dicoumarate salts were soluble in water. Hence the compounds (UHAKKM-7 to 9) acquire the required properties appropriate to reach the target and the placement of negative charges on enolic Oxygens resulting from formation of salts enhances the activity permits the molecule interact at the

phosphate interacting domain of the kinase. It was also observed that the absence of pyridine moiety showed decreased antiviral and TopoIIβK_{HIV} inhibition, as seen in case of other disodium aromatic dicoumarate compounds. Hence it was concluded that the pyridine ring, negatively charged enolic oxygen and conformational flexibility plays a crucial role in bioactivity of dicoumarol derivatives.

Fig.14: Sodium3,3'-(pyridin-3-ylmethylene)bis(2-oxo-2H-chromen-4-olate) UHAKKM-9 molecule 3D-QSAR contour map

a) i) ii)



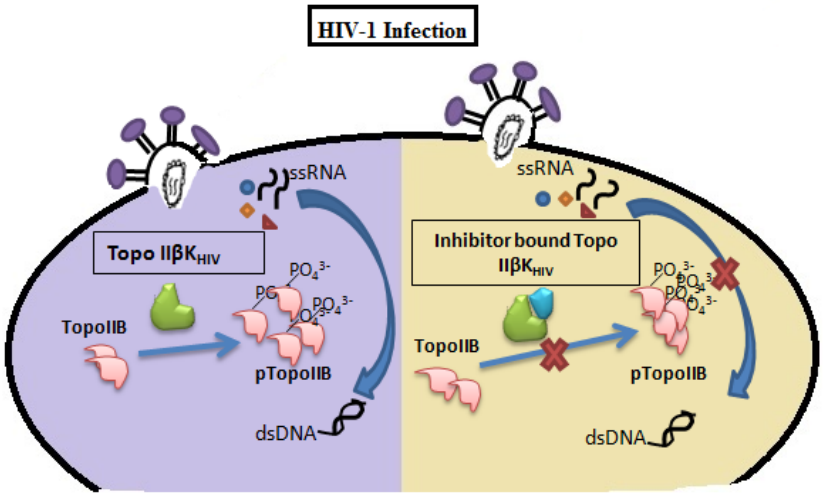
b)

Fig.14: 3D-QSAR Contours overlaid on the structure Pyridine Dicoumarol derivative UHAKKM-9 a) i) steric ii) electrostatic contours obtained from 3D QSAR equation. b) Structure of Disodium-3,3'-(pyridin-3-ylmethylene) bis(2-oxo-2H-chromen-4-olate). The bulk dicoumarol ring of the compound lies overlapped with the green region of the contour, suggesting the role of the bulk group (dicoumarol moiety) in the activity of the

compound. Similarly, the overlap of red region with the negatively charged enolic Oxygen group substitution suggests the role of electronegative groups in the enhanced Topo$II\beta K_{HIV}$ antagonism.

In this study, we have designed and developed a series of dicoumarol derivatives with potential anti-HIV-1 activity. These compounds specifically target the novel Topo $II\beta K_{HIV}$ that was shown to be responsible for phosphorylation of Topoisomerase IIβ [3] necessary for HIV-1 replication. The compound UHAKKM-7 showed anti-viral activity with an IC_{50} of 13 nM, which was seen to be on par with the widely used and clinically used, Azidothymidine (AZT) that has an IC_{50} of 25nM. The potency of this compound is also better than many of the FDA approved anti-HIV-1 drugs. The design of compounds UHAKKM-7, 8 and 9 was done based on 3D-QSAR contours obtained in a previous study and the structures were then optimized to obtain better activity. The designed structures satisfied the contour constraints as shown in **Fig.14** and thus showed good Topo $II\beta K_{HIV}$ antagonism. A model depicting action of inhibitors during HIV-1 replication is presented in Fig.9.

Fig.15: TopoIIβK$_{HIV}$ role in HIV-1 life cycle



Summary:

A structural study of Pyridine Dicoumarol derivatives with potential activity against a novel Topoisomerase II β Kinase (TopoIIβk$_{HIV}$), identified in the HIV-1 viral lysate and anti-HIV-1 activity. These derivatives are designed based on a 3D-QSAR study of a set of Pyridine derivatives. Herein we demonstrate the achievement of water soluble, high antiHIV-1 activity (IC_{50} <38nM) and less cytotoxicity disodium pyridine dicoumarate derivatives from DMSO soluble, less anti-HIV-1 activity pyridine dicoumarol derivatives. The study is expected to provide a crucial point for further development of pyridine dicoumarol series as HIV-1 associated TopoIIβk$_{HIV}$ inhibitors for clinical application against AIDS.

Conclusion:

In conclusion, a structure based evaluation of substructures would lead to drug development. Structure modification of pyridine dicoumarol derivatives has shown an increase in inhibition of the Topo IIβK_{HIV} activity and antiviral activity.

Future Perspective:

This study demonstrates, HIV-1 Associated Topo IIβK_{HIV} as a potential target to control HIV-1 infection. We demonstrated the achievement of water soluble disodium pyridine dicoumarate derivatives showing high anti-HIV-1 activity (IC_{50}<38nM) which provides a platform for development of new class of inhibitors for clinical application against AIDS. Further studies on preclinical and clinical evaluation is required.

Conclusions:

1.3D QSAR was employed to design potent compounds to inhibit Topo IIβK_{HIV}

2.4-Hydroxy Coumarin was used as the starting material and 9 compounds were designed and synthesized successively.

3. Synthesized compounds have shown high TopoIIβK_{HIV} inhibition.

4. Water soluble compounds UHAKKM-7 to 9 showed high anti-HIV-1 activity.

5. In conclusion, structural studies on Dicoumarols lead to the development of potent anti-HIV-1 compounds targeted to Topo IIβK_{HIV}.

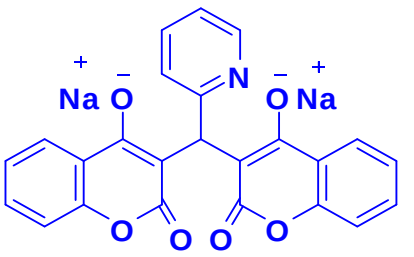
Chapter-4

Objective-3

Development of new lead molecules based on structure and biological activities of non-pyridine derivatives of coumarin.

Abstract: We have synthesized sixty-nine non-pyridine dicoumarol derivatives following scheme-3 and biologically evaluated against TopoIIβK_{HIV} activity and HIV-1 replication. We have noticed methoxy groups placed on benzene ring (which was replaced pyridine ring) in non-pyridine dicoumarol derivatives enhances TopoIIβK_{HIV} inhibition and anti-HIV-1 activities over the methoxy unsubstituted. Furthermore, disodium pyridine dicoumarate derivative form showed significant enhancement of biological activity of methoxy groups substituted benzene ring in dicoumarol derivatives. Same as disodium pyridine dicoumarate salts, disodium methoxy group's substituted benzene dicoumarate salts have shown anti-HIV-1 activity, but less than those pyridine-dicoumarate salts. Thus, the methoxy substituted disodium pyridine dicoumarate compounds exhibits higher TopoIIβK_{HIV} inhibition and anti-HIV-1 activity than methoxy unsubstituted disodium pyridine dicoumarate compounds (scheme-3-products).

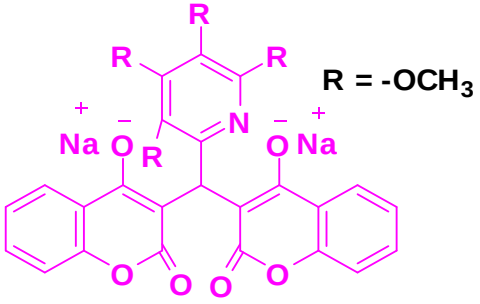
Methoxy Unsubstituted pyridine dicoumarate



TopoIIbeta kinase inhibition

Anti-HIV-1 activity

Methoxy substituted pyridine dicoumarate



TopoIIbeta kinase inhibition

Anti-HIV-1 activity

Introduction:

Previous objectives 1 & 2 reveals pyridine having dicoumarol derivatives have shown significant inhibition of TopoIIβK_{HIV} and HIV-1 activities. In objective-2, scheme-3 water soluble disodium pyridine dicoumarates (UHAKKM-7 to 9) have shown potent TopoIIβK_{HIV} inhibition and anti-HIV-1 activity (IC_{50}<25nM). In this study, distinct structural class of molecules having dicoumarol moiety without pyridine moiety was analyzed for potential activity against TopoIIβK_{HIV}. The dicoumarol moiety having derivatives which were designed based on a 3D-QSAR study of diverse structural class of molecules, which has pyridine as base. From 3D-QSAR model we observed that dicoumarol moiety is acceptable on pyridine, which is favorable for potential activity for TopoIIβK_{HIV} inhibition. In this study we observed pyridine which has steric group (dicoumarol moiety) has shown high inhibition on TopoIIβK_{HIV} than non-pyridine derivatives.TopoIIβK_{HIV} Inhibition activity is attributed to the dicoumarol group in the hinge region of the kinase which reveals the crucial pharmacophore for the development of HIV-1 inhibitors. For the further development of disodium pyridine dicoumarate compounds (UHAKKM-7 to 9) needed rational clue, reason for that in the present study pharmacophore group dicoumarol moiety placed on substituted aromatic ring instead of pyridine and tested these non-pyridine-dicoumarol derivatives against TopoIIβkinase and HIV-1. Results of these non-pyridine-dicoumarol derivatives bio-activity, we can develop bioactivity of disodium pyridine dicoumarate compounds (UHAKKM-7 to 9).

Rationale:

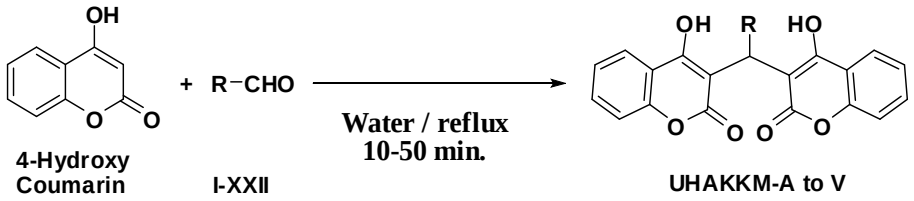
From Chapter-2 and Chapter-3, we have concluded that the pyridine ring, negatively charged enolic oxygen and conformational flexibility plays a crucial role in bioactivity of dicoumarol derivatives. To further verify the role of pyridine ring (heteroatom nitrogen substituted six membered ring) substitutions on the bioactivity of coumarin derivatives, we replaced substituted pyridine ring with substituted benzene and verified activity. From these studies at least, we can get clue for further development of disodium pyridine dicoumarate activity against HIV-1 targeting TopoIIβK_{HIV}.

Chemistry:

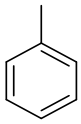
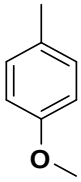
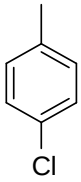
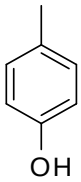
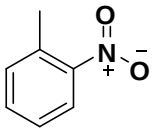
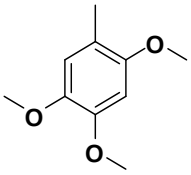
Synthesis of 3, 3'-(aryl-methylene) bis (4-hydroxy-2H-chromen-2-one) derivatives:

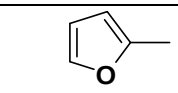
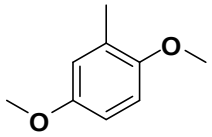
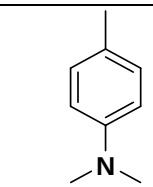
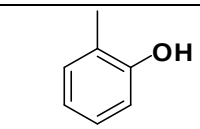
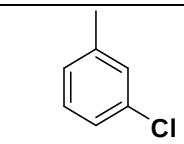
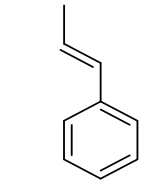
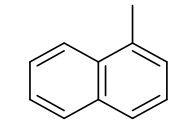
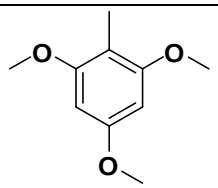
General synthetic procedure for the dicoumarols (UHAKKM-A to V): 4-hydroxycoumarin (**1**) (2 mmol) and aromatic/hetero-aromatic aldehydes (**I to XXII**) (1 mmol) mixture was taken in 15 mL of water and stirred under conventional reflux conditions for the appropriate time (10 to 50 min) in Table 1. The reaction completion was monitored by TLC (Thin Layer Chromatography) system. Once the reaction completion is confirmed, then the reaction mixture was allowed to obtain room temperature. The products, were collected by filtration methods, ultimately washed with hot methanol and dried to give the desired products (**UHAKKM-A to V**).

Table-15: synthesis of dicoumarols UHAKKM-A to V in water.[a]



[a]Reaction conditions: 4-hydroxycoumarin (2.0 mmol); aldehyde (1.0 mmol); 15 mL water under reflux condition.

Entry	R	Product	Yield (%)
I		UHAKKM-A	96
II		UHAKKM-B	93
III		UHAKKM-C	95
IV		UHAKKM-D	94
V		UHAKKM-E	80
VI		UHAKKM-F	85

VII		UHAKKM-G	94
VIII		UHAKKM-H	70
IX		UHAKKM-I	87
X	H	UHAKKM-J	85
XI	CH_3	UHAKKM-K	90
XII		UHAKKM-L	82
XIII		UHAKKM-M	94
XIV		UHAKKM-N	80
XV		UHAKKM-O	76
XVI		UHAKKM-P	78

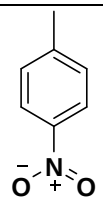
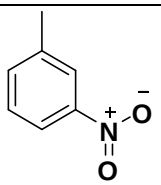
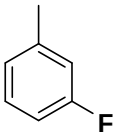
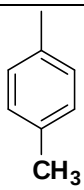
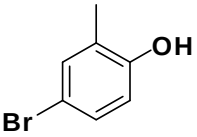
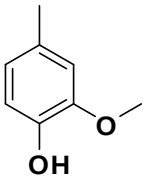
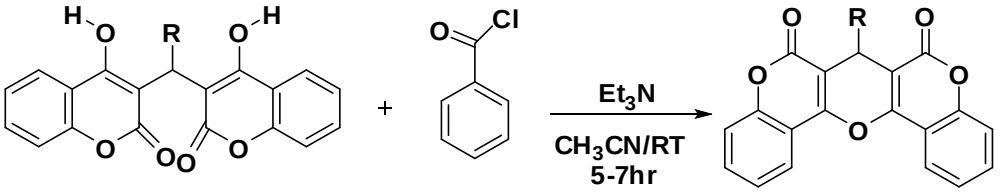
XVII		UHAKKM-Q	97
XVIII		UHAKKM-R	90
XIX		UHAKKM-S	95
XX		UHAKKM-T	88
XXI		UHAKKM-U	86
XXII		UHAKKM-V	92
XXIII	ORTHO TOLYL	UHAKKM-W	84

Table-16: Arylidine-4, 4[1]-epoxy dicoumarin derivatives synthesis:

General synthetic procedure for the 4, 4[1]-epoxydicoumarin derivatives (UHAKKM-A to V): Dicoumarols (1 mmol) and benzoyl chloride (2 mmol) mixture was added to 10mL of acetonitrile in the round bottom flask(25mL) and in the presence of 4 or 5 drops tri ethyl amine, reaction mixture was stirred at room temperature. The reaction completion was monitored by

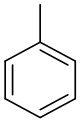
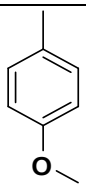
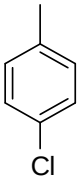
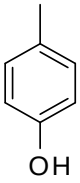
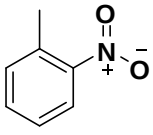
Thin Layer Chromatographic analysis. After the reaction completion, the solid products were collected by filtration methods, finally washed with acetone, water and dried to give the desired products (UHAKKM-**a-to-v**). Pure products were obtained from column chromatography [17].

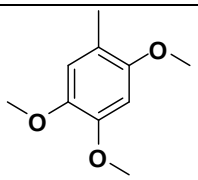
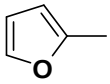
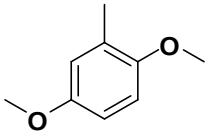
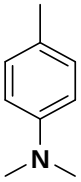
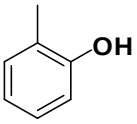
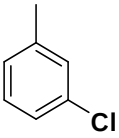
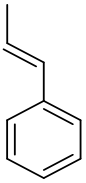
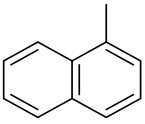


Dicoumarol Derivatives
UHAKKM-A to V
1mmole

Benzoyl Chloride
2mmole

Arylidine-4,4[1]- epoxy Dicoumarin
UHAKKM-A1 to V1

Entry	R	CHEMICAL FORMULA	Product	Yield (%)
I		C6H5	UHAKKM-A1	98
II		4-CH_3O-C_6H_4	UHAKKM-B1	96
III		4-Cl-C_6H_4	UHAKKM-C1	92
IV		4-HO-C_6H_4	UHAKKM-D1	98
V		2-O_2N-C_6H_4	UHAKKM-E1	97

VI		2,4,5-Tri-CH_3O-C_6H_2	UHAKKM-F1	90
VII		2-C_4H_3O	UHAKKM-G1	95
VIII		2,5-Di-CH_3O-C_6H_3	UHAKKM-H1	86
IX		4-$(CH3)_2N$-C_6H_4	UHAKKM-I1	92
X	H	H	UHAKKM-J1	88
XI	CH_3	CH_3	UHAKKM-K1	94
XII		2-HO-C_6H_4	UHAKKM-L1	96
XIII		3-Cl-C_6H_4	UHAKKM-M1	95
XIV		-CH=CH-C_6H_5	UHAKKM-N1	93
XV		1-Naphthyl	UHAKKM-O1	86

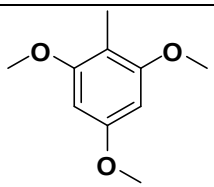
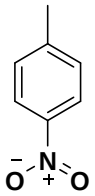
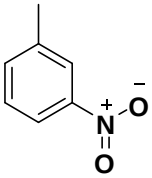
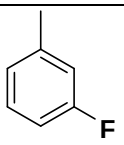
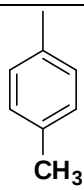
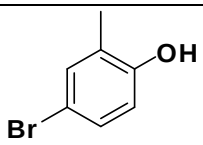
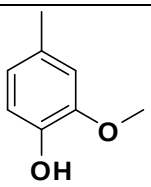
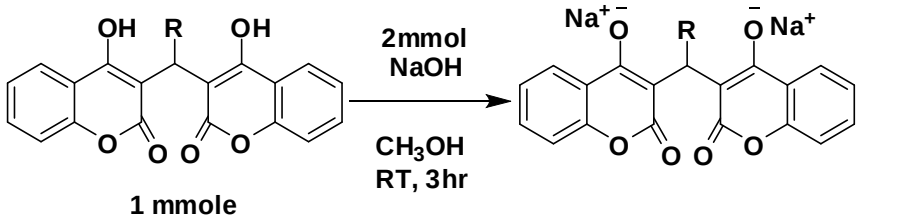
XVI		2,4,6-Tri-CH_3O-C_6H_2	UHAKKM-P1	93
XVII		4-O_2N-C_6H_4	UHAKKM-Q1	98
XVIII		3-O_2N-C_6H_4	UHAKKM-R1	92
XIX		3-F-C_6H_4	UHAKKM-S1	88
XX		4-H_3C-C_6H_4	UHAKKM-T1	90
XXI		2-Br-5-OH-C_6H_3	UHAKKM-U1	89
XXII		4-OH-3-$OCH_3C_6H_3$	UHAKKM-V1	96
XXIII	ORTHO TOLYL	2-CH3-C6H4	UHAKKM-W1	87

Table.17: Synthesis of Disodium Arylidene dicoumarates:

General synthetic procedure of disodium arylidene dicoumarates: Dicoumarols (1mmol) **(UHAKKM-A to V)** were taken in 25 ml round bottom flask, to this 10mLmethanol and 2 mmol NaOH were added, the reaction mixture was stirred for 3 hrs. After obtaining clear solution, the solvent was removed by evaporation, under reduced pressure and solid products obtained. The dicoumarate salts can soluble (UHAKKM-A2 to V2) in water absolutely [19].



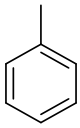
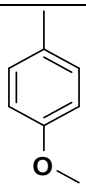
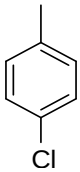
1 mmole

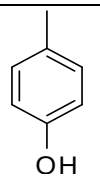
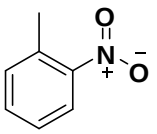
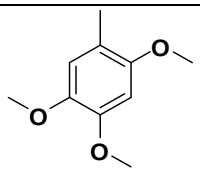
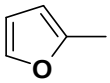
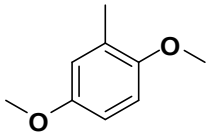
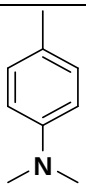
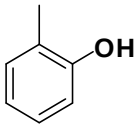
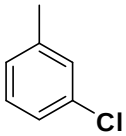
Dicoumarols (UHAKKM-A to V)

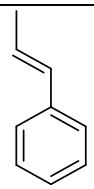
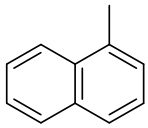
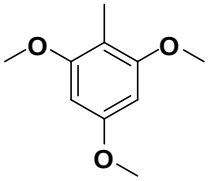
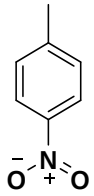
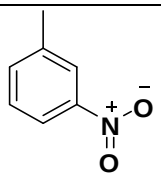
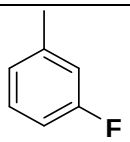
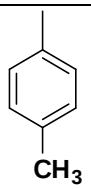
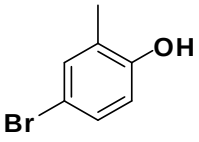
DMSO soluble

Disodium Arylidine Dicoumarates

Water soluble

Entry	R	Name	Product
I		Phenyl-	UHAKKM-A2
II		4-methoxy phenyl	UHAKKM-B2
III		4-chloro-phenyl	UHAKKM-C2

IV		4-hydroxy phenyl	UHAKKM-D2
V		2-nitro-phenyl-	UHAKKM-E2
VI		2,4,5-Tri-methoxy-phenyl-	UHAKKM-F2
VII		2-furyl	UHAKKM-G2
VIII		2,5-Di-methoxy-phenyl-	UHAKKM-H2
IX		4-N,N-Di-methyl-phenyl	UHAKKM-I2
X	H	H	UHAKKM-J2
XI	CH_3	Methyl-	UHAKKM-K2
XII		2-hydroxy- phenyl-	UHAKKM-L2
XIII		3-Chloro-phenyl-	UHAKKM-M2

XIV		cinnamyl-	UHAKKM-N2
XV		1-Naphthyl	UHAKKM-O2
XVI		2,4,6-Tri-methoxy-phenyl-	UHAKKM-P2
XVII		4-nitro-phenyl-	UHAKKM-Q2
XVIII		3-nitro-phenyl-	UHAKKM-R2
XIX		3-Fluoro-phenyl-	UHAKKM-S2
XX		4-tolyl-	UHAKKM-T2
XXI		5-bromo-2-hydroxy-phenyl-	UHAKKM-U2

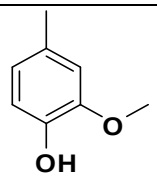
XXII		4-hydroxy-3-methoxy-phenyl-	UHAKKM-V2
XXIII	ORTHO-TOLYL	2-methyl-phenyl-	UHAKKM-W2

Table.18: TopoIIβkinase inhibitory activity values of non-pyridine dicoumarol derivatives:

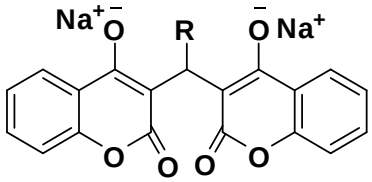
Entry	R	Product Code	pIC $_{50}$	IC$_{50}$(pM)
1	2,5-Di-OCH_3-C_6H_3	UHAKKM-H	11.154	7.012
2	2,4,5-Tri-OCH_3 C_6H_2	UHAKKM-F	11.354	4.417
3	2,4,6-Tri-$OCH_3C_6H_2$	UHAKKM-P	11.358	4.380
4	4-$(CH_3)_2N$-C_6H_4	UHAKKM-I	Not Done	Not Done
5	5-Br-2-OH-C_6H_3	UHAKKM-U	8.99	$1.0x10^3$
6	3-F-C_6H_4	UHAKKM-S	8.32	$4.760x10^3$
7	4-OH-3-$OCH_3C_6H_3$	UHAKKM-V	8.75	$1.770x10^3$
8	2,5-Di-OCH_3-C_6H_3	UHAKKM-H1	11.355	4.410
9	2,4,5-Tri-OCH_3 C_6H_2	UHAKKM-F1	11.133	7.352
10	2,4,6-Tri-$OCH_3C_6H_2$	UHAKKM-P1	11.258	5.510
11	4-$(CH_3)_2N$-C_6H_4	UHAKKM-I1	8.47	$3.340x10^3$
12	5-Br-2-OH-C_6H_3	UHAKKM-U1	8.77	$1.660x10^3$
13	3-F-C_6H_4	UHAKKM-S1	8.69	$2.030x10^3$
14	4-OH-3-$OCH_3C_6H_3$	UHAKKM-V1	Not Done	Not Done
15	4-NO2-C_6H_4	UHAKKM-Q	9.12	$7.57x10^2$
16	3-NO2-C_6H_4	UHAKKM-R	9.657	$2.20x10^2$
17	2-NO2-C_6H_4	UHAKKM-E	9.012	$9.71x10^2$
18	4-CH_3-C_6H_4	UHAKKM-T	9.09	$8.10x10^2$
19	4-OCH_3-C_6H_4	UHAKKM-B	8.97	$1.05x10^3$
20	3-Cl-C_6H_4	UHAKKM-M	9.003	$9.93x10^2$

21	2-C_4H_3O	UHAKKM-G	8.76	$1.720x10^3$
22	4-$NO_2C_6H_4$	UHAKKM-Q1	8.394	$4.030x10^3$
23	3-NO2C6H4	UHAKKM-R1	8.804	$1.570x10^3$
24	2-$NO_2C_6H_4$	UHAKKM-E1	9.182	$6.57x10^2$
25	4-$CH_3C_6H_4$	UHAKKM-T1	9.105	$7.85x10^2$
26	4-CH_3O-C_6H_4	UHAKKM-B1	11.066	8.58
27	3-Cl-C_6H_4	UHAKKM-M1	8.66	$2.15x10^3$
28	2-C_4H_3O	UHAKKM-G1	7.82	$14.930x10^3$

Table.18: We have screened all non-pyridine dicoumarol, 3, 3'-arylidene-4, 4'-epoxy dicoumarins and disodium arylidine dicoumarate derivatives (69 compounds) against TopoIIβK_{HIV}. In that 28 compounds have shown better TopoIIβK_{HIV} inhibition activity, shown in Table-4. Among all kinase inhibitory values UHAKKM- F, H, P, B1 compounds have shown better activity. We noticed that these compouds have methoxy groups on benzene ring, which cause to better activity than others. But these compounds have shown kinase inhibition less than disodium pyridine dicoumarate compounds (UHAKKM-7 to 9).

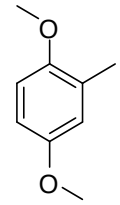
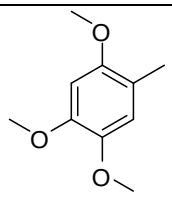
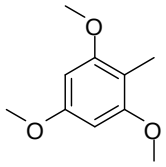
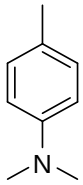
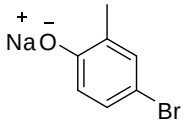
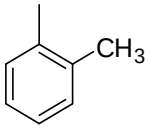
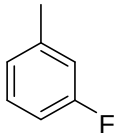
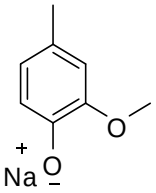
Compounds synthesized by Scheme -3

Disodium Arylidine Dicoumarates



water soluble

Table. 19: Synthesized disodium di-coumarate derivates (replaced by pyridine ring):

Code No	Code Name	R	Group Name
26	UHAKKM-H2		2,5-Di-Methoxy-Phenyl-
27	UHAKKM-F2		2,4,5-Tri-Methoxy-Phenyl-
28	UHAKKM-P2		2,4,6-Tri-Methoxy-Phenyl-
29	UHAKKM-I2		4-N,N-Di-Methyl-Phenyl-
30	UHAKKM-U2		5-Bromo-2-phenoxide-nyl-
31	UHAKKM-T2		2-Tolyl-
32	UHAKKM-S2		3-Fluoro-phenyl-
33	UHAKKM-V2		3-Methoxy-4-phenoxide-nyl-

Based on in-vitro kinase phosphorylation inhibition assay results, among sixty-nine molecules above substituted benzene sodium dicoumarate molecules, we have selected for further MTT and anti-viral studies

MTT assay results of various substituted benzene having disodium dicoumarates:

Fig.16:

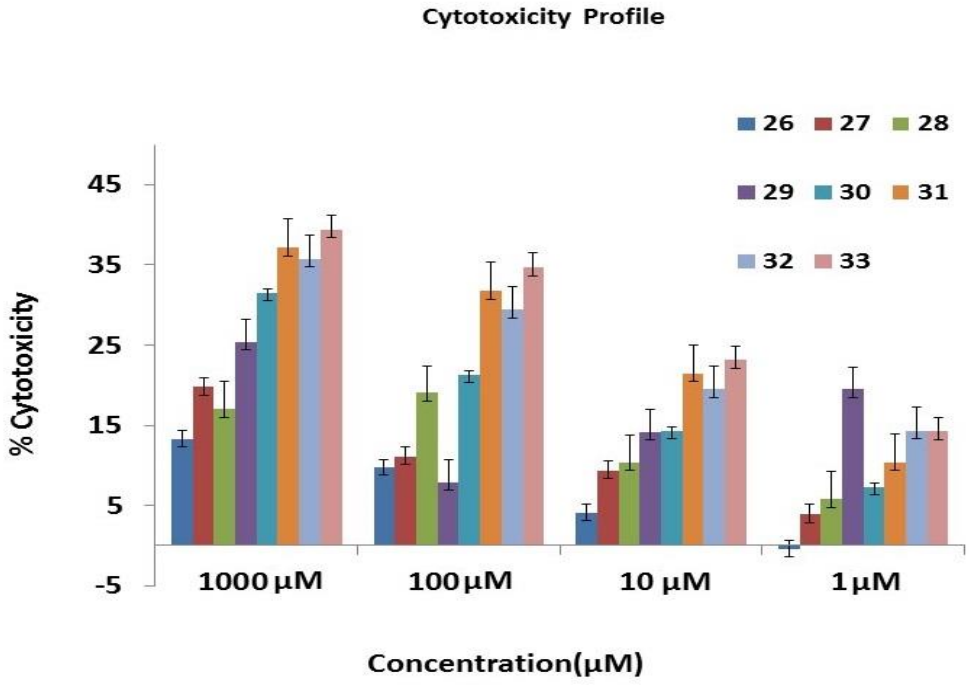
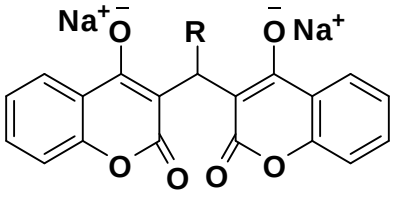


Fig.16: Above figure depicts that the molecules various substituted benzene having disodium dicoumarates (without pyridine) have shown below 45%cytotoxicity even at 1000µM concentration.

Anti-HIV-1 assay results of various substituted benzene having disodium dicoumarates:

Disodium Arylidine Dicoumarates



water soluble

Fig.17:

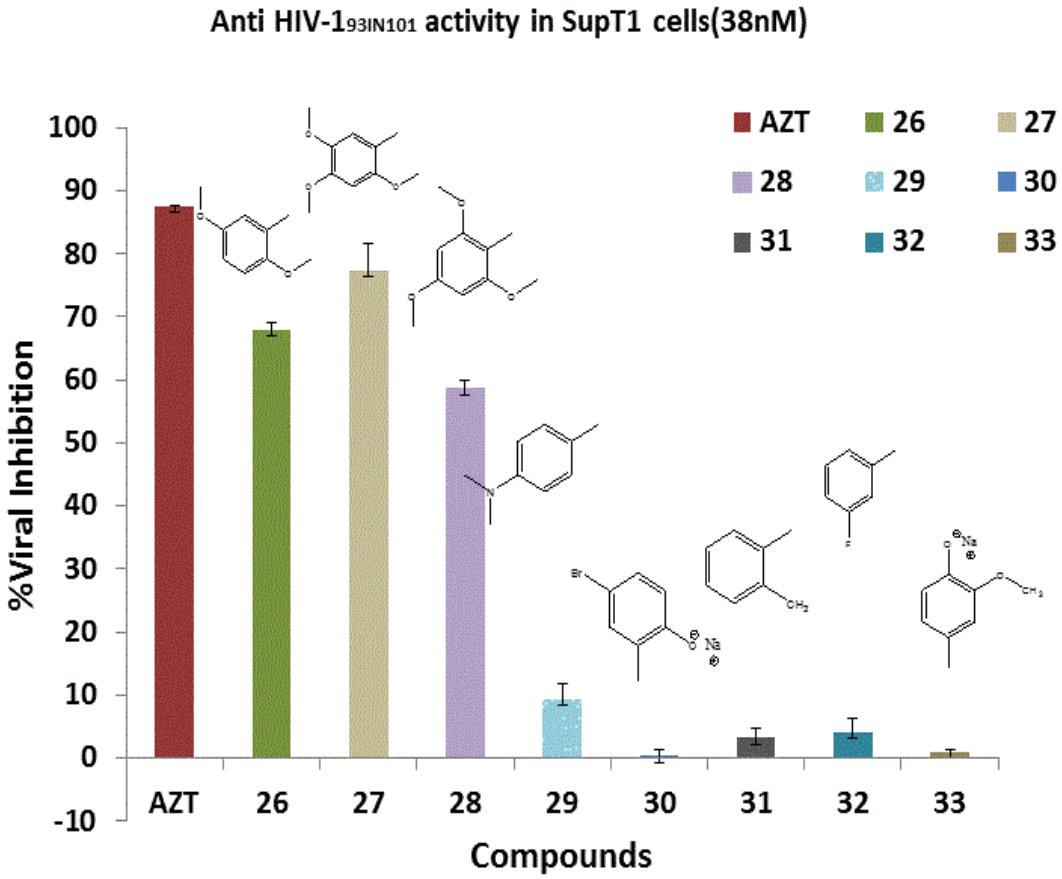
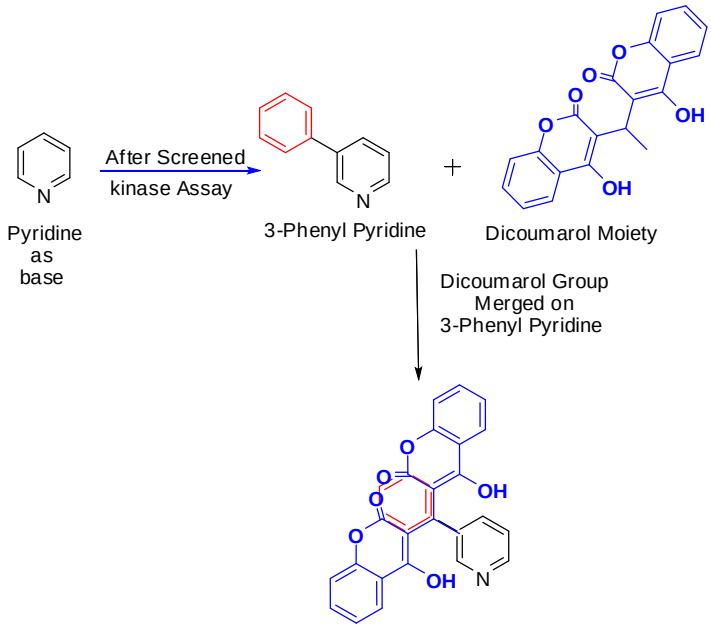


Fig.17: Above figure 11 indicates methoxy substituted benzene disodium dicoumarates shown improved anti-viral activity than methoxy unsubstituted derivatives.

Discussion:

In obective-1 we have chosen dicoumarol group as pharmacophore moiety on pyridine and evaluated against HIV-1 targeted TopoIIβkinase.

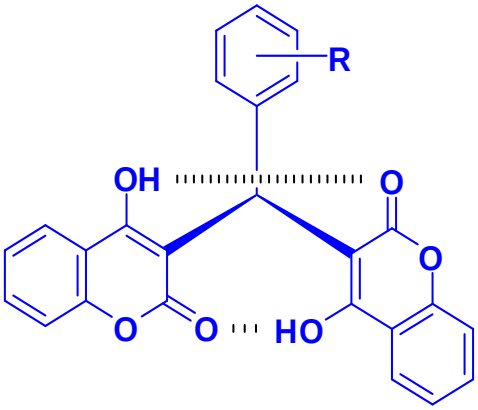


Observation: These pyridine dicoumarols have shown potential inhibition on TopoIIβK_{HIV} and less anti-HIV-1 activity.

In objective-2, we have optimized for the anti-HIV-1 activity, pharmacokinetic and pharmacodynamics activity of pyridine dicoumarols. The intra-molecular hydrogen bonding in these molecules conferred conformational rigidity, which limited them in intracellular localization and reaching the proximity of target, TopoIIβK_{HIV} in live cell. This conformational rigidity due to hydroxyl hydrogens was eliminated by converting them into sodium salt using alcoholic sodium hydroxide. Conversion of sodium salt prevented intramolecular Hydrogen bond and introduced conformational flexibility in these pyridine dicoumarols. Due to the effect of conformational flexibility, solubility of sodium pyridine dicoumarates in 1-octanol significantly along with higher solubility in water. These soluble species of that sodium

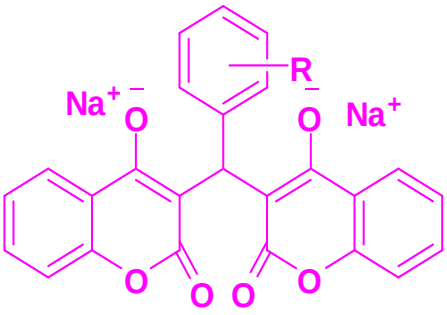
pyridine dicoumarates slowed cellular permeability of molecules exhibited significant anti-HIV-1activity along with further enhancement of TopoIIβK_{HIV} inhibition.

Pyridine-Dicoumarol



Conformational Rigid Structure
Intramolecular Hydrogen Bonding

Sodium pyridine dicoumarate



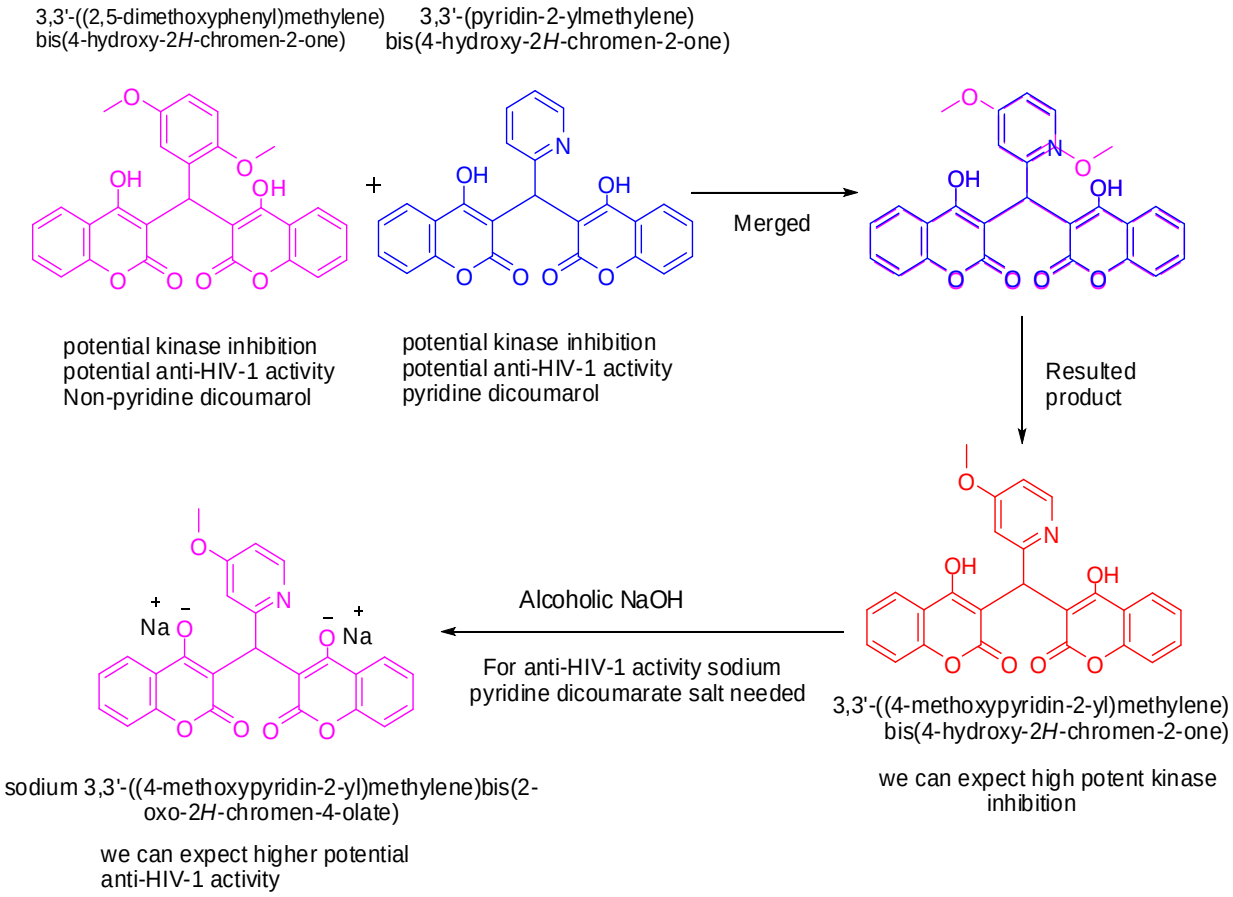
Water Soluble
Conformational Flexibility Enhanced
No Intramolecular Hydrogen Bonding

In this objective, we have analysed the activity of substitution groups on pyridine ring on TopoIIβK_{HIV} and HIV-1 activities. Hence, we have synthesized sixty-nine non-pyridine dicoumarols and tested against HIV-1 and TopoIIβK_{HIV}. Among these non-pyridine-dicoumarols, methoxy substituted benzene ring (in place of pyridine) having arylidene dicoumarols have shown potential TopoIIβK_{HIV} inhibition and anti-HIV-1 activity.

We have also noticed in sixty-nine non-pyridine-dicoumarols, only methoxy substituted benzene ring having sodium arylidene dicoumarates have shown anti-HIV-1 activity, but less than sodium pyridine dicoumarates. Result of that leads to development of sodium pyridine dicoumarates anti-HIV-1 activity and TopoIIβK_{HIV} inhibition, only when methoxy groups placed on pyridine ring. The comparison of structure of active molecules provided an indication that methoxy substituted benzene ring having dicoumarols merged on pyridine dicoumarols may have active environment for TopoIIβK_{HIV} interaction. This lead to development of merged

derivatives for the development of pyridine dicoumarols with improved inhibition of TopoIIβK_{HIV} and anti-HIV-1 activity.

Design and Drug Development process from objective-3:



From above results, merged 4-methoxy-pyridine-2-dicoumarol molecule would provide further inhibition of TopoIIβK_{HIV} along with anti-HIV-1 activity from its salt.

Conclusion:

In conclusion, a structural based evaluation of non-pyridine-dicoumarols leads to understand TopoIIβK_{HIV} binding cavity and further development of sodium pyridine dicoumarates (UHAKKM-7 to 9) during the process of drug design and development. Results showed methoxy substituted benzene ring having non-pyridine dicoumarol products possess higher

TopoIIβK_{HIV} inhibition and its sodium dicoumarate salts have shown highest anti-HIV-1 activity. Among methoxy substituted benzene ring having non-pyridine dicoumarols, 2,5-di-methoxy substituted sodium arylidene dicoumarate compound have shown highest anti-HIV-1 activity. Even though 2, 5-di-methoxy substituted sodium arylidene dicoumarate (UHAKKM-H2) having highest anti-HIV-1 activity among methoxy substituted non-pyridine-sodium dicoumarates, with lower anti-HIV activity than sodium pyridine dicoumarates. From this study, it is understood that the kinase binding site is located in a cavity for supramolecular binding of drug. The merged 2,5-di-methoxy substituted sodium arylidene dicoumarate (UHAKKM-H2) and sodium pyridine-2-dicoumarate (UHAKKM-7), resulted molecule is sodium 4-methoxy-pyridine-2-dicoumarate possess higher TopoIIβK_{HIV} inhibition and anti-HIV-1 activity.

Future Perspective:

This study demonstrates that dicoumarol moiety a potential pharmacophore which is placed on pyridine ring to control HIV-1 infection through targeting TopoIIβK_{HIV}. We demonstrated that the achievement of TopoIIβK_{HIV} binding site located cavity and its physical features using diverse structural series of non-pyridine dicoumarols. Pyridine-dicoumarols and methoxy substituted non-pyridine dicoumarols merged; resulted compounds will have shown high potential TopoIIβK_{HIV} inhibition and anti-HIV-1 activity. In this objective we have also observed that sodium methoxy substituted dicoumarate salts have shown highest TopoIIβK_{HIV} inhibition and anti-HIV-1 activity.

Executive summary:

1.Sixty-six non-pyridine dicoumarols employed for understanding kinase binding cavity to design potent anti-HIV-1 compounds to inhibit Topo II$\beta K_{HIV.}$

2.2, 5-di-methoxy-arylidene-dicoumarol (UHAKKM-H2) was selected and used as the merged or molecule structure, reason of that which has shown highest TopoIIβK_{HIV} inhibition and anti-HIV-1 activity among all non-pyridine dicoumarols.

3. UHAKKM-H2 dimethoxy compound merged with UHAKKM-4, pyridine-2-dicoumarol, resulted 4-methoxypyridine-2-dicoumarol structure have shown close to binding site cavity of TopoIIβK_{HIV} physical features.

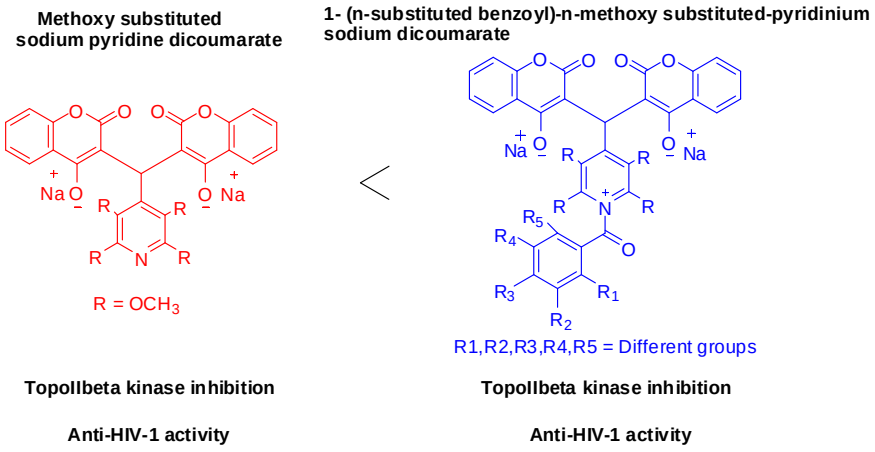
In conclusion, based on non-pyridine-dicoumarols and activity relationship, we have selected methoxy group placed on 4^{th} place of pyridine-2-dicoumarol. Methoxy group also one of an appropriate pharmacophore placed on pyridine lead to the development of potent anti-HIV-1 compounds targeted to Topo IIβK_{HIV}.

Chapter - 5

Objective-4

Design, synthesis and evaluation of pyridine bis-chalcone derivatives for inhibition of HIV-1 associated Topoisomerase II β kinase and anti-HIV-1 activity.

Abstract: In Chapter IV under OBJECTIVE-3, our analysis of molecule structure (methoxy substituted sodium pyridine-dicoumarate) promoted higher biological activity conferred importance of Kinase binding cavity in potent interaction of inhibitor. In this study, we have synthesized four molecules which have positive charge on pyridine ring nitrogen and length of the molecule more than sodium pyridine dicoumarate and these molecules were tested against TopoIIβ$K_{HIV-1}$and HIV-1. Results showed that pyridinium bischalcone derivatives have shown activity against TopoIIβK_{HIV} and HIV-1, but less than 3-phenyl pyridinum. Thus, suggesting a presence of positive charged environment at the surface of the cavity that provide an interaction with these molecules and stabilizes the binding. Furthermore, 3-phenyl pyridinium molecule have shown less inhibition than sodium pyridine dicoumarates against TopoIIβK_{HIV} and HIV-1 suggesting presence of free hetero nitrogen is important for conferring kinase interaction through electronegative hetero nitrogen for interaction with electropositive Hydrogen through hydrogen bonding.



Rationale:

Based on 3D-QSAR contour maps in Chapter-2 (Objective-1), we have chosen dicoumarol moiety on pyridine, synthesized, characterized, tested against TopoIIβK_{HIV} and HIV-1, resulted potent in-vitro inhibition of TopoIIβK_{HIV}, but they have not shown anti-HIV-1 activity. Then in Chapter-3 (objective-2), we have carried out physico-organic structural studies on pyridine dicoumarol leads to enhancement of inhibition against TopoIIβK_{HIV} and HIV-1. Subsequently, we have studied in Chapter-4 (objective-3) on sodium pyridine-dicoumarates, which groups on pyridine ring cause to enhance kinase inhibitory activity and pyridine ring importance on TopoIIβK_{HIV}. We have studied activities of non-pyridine-dicoumarols and observed that methoxy groups substituted aromatic ring having non-pyridine dicoumarols have shown potent inhibitory activity against TopoIIβK_{HIV} and HIV-1. These studies given understanding that pyridine ring plays important role in interaction with TopoIIβK_{HIV} and methoxy substituted pyridine ring dicoumarols can show enhanced inhibition against TopoIIβK_{HIV} and HIV-1. From those studies we infer that environment in TopoIIβK_{HIV} binding site cavity is an important feature for enhancing selectivity and activity of molecules against TopoIIβK_{HIV} and anti-HIV-1 activity. In this Chapter-5, we have studied the physico-chemical properties of the interacting environment in the kinase with aid of charged molecules.

Introduction:

Much efforts have been spent on searching for better pyridine dicoumarol derivatives to achieve most potential TopoIIβK_{HIV} inhibition and anti-HIV-1 activity on drug resistant virus. During the process of development pyridine dicoumarol, previous chapter (cf Chapter-4), we have understood methoxy substituted pyridine ring in pyridine dicoumarol derivatives can show potential TopoIIβK_{HIV} inhibition than unsubstituted. In this chapter our approach was to target the length of end to end binding site, hydrogen bond acceptors and charge centers in

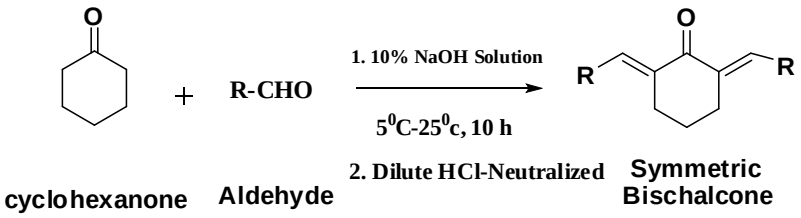
interaction with TopoIIβK_{HIV} activity. Symmetric methyl pyridinium bischalcones and 3-phenyl methyl pyridinum compounds were selected for analysis of TopoIIβK_{HIV}inhibition to understand interacting environment at TopoIIβK_{HIV}binding site for optimal binding interaction. Symmetric pyridinium bis-chalcones having cyclohexanone as linker, 3-phenylpyridinium molecules have to synthesize and characterize to test against the Topo IIβkinase TopoIIβK_{HIV} and HIV-1.

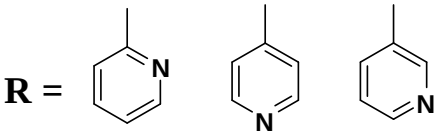
Chemistry:

Scheme-4:

Preparation of 2, 6-bis (pyridyl-methylene) cyclohexanone derivatives:

pyridine aldehyde (4mmol) and cyclohexanone (2mmol) mixture in water (10mL) was stirred with cooling to 5^0C, and added dropwise 1mL of 10% sodium hydroxide solution. Then the mixture was stirred at 25^0C for 10 h and then neutralized with a dilute hydrochloric acid solution, when confirmed reaction completion by thin layer chromatography. The precipitate was filtered off and recrystallized from ethanol. Purified product achieved by column chromatography (ethyl acetate/hexane) [**50**] [**51**].

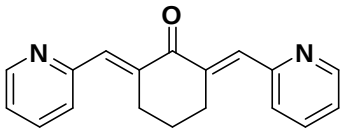




We have synthesized three different symmetric pyridine bischalcones:

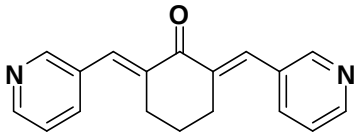
70

(2*E*,6*E*)-2,6-bis(pyridin-2-ylmethylene)cyclohexanone



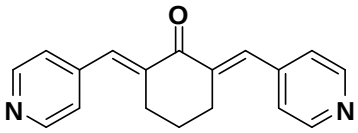
71

(2*E*,6*E*)-2,6-bis(pyridin-3-ylmethylene)cyclohexanone



72

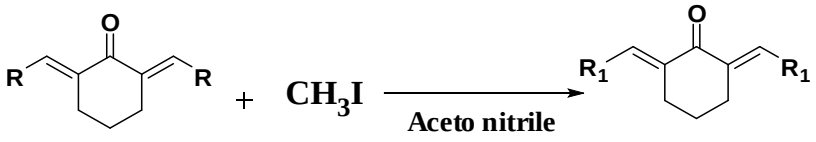
(2*E*,6*E*)-2,6-bis(pyridin-4-ylmethylene)cyclohexanone



Scheme-5:

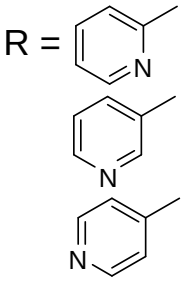
2, 6-Bis [(*E*)-(pyridyl) methylene] cyclohexanone methylation:

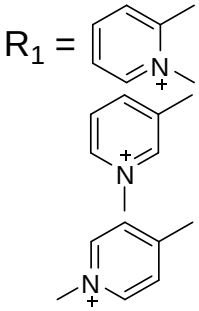
Preparation-procedure:2,6-Bis[(*E*)-(pyridyl)methylene]cyclohexanone(2mmol) derivatives(scheme-4 products) in acetonitrile (10ml) was stirred with cooling to 5^{0}C in25mL round bottom flask with tightly closed rubber septum , and 5mL of methyl iodide solution was injected. The mixture was stirred at 5^{0}C for 12 h and then at room temperature was stirred for 72 hrs. After that formed precipitate was filtered, washed with little amount of acetonitrile and dried [**52**].



Methyl iodide 5°C- 12h

2,6 - Bis[(E) - (pyridyl)methylene] cyclohexanone RT-72h **Pyridine-N-Methylation symmetric bischalconeproduct**

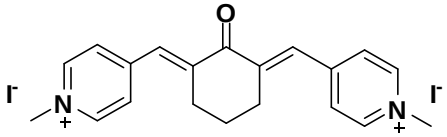




We have synthesized three different symmetric pyridinium bischalcones:

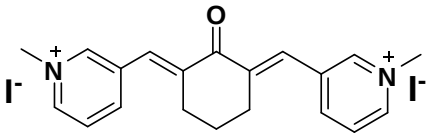
UHAKKM-X: 73

4,4'-(1*E*,1'*E*)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1-methylpyridinium) iodide



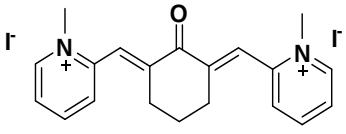
UHAKKM-Y :74

3,3'-(1*E*,1'*E*)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1-methylpyridinium) iodide



UHAKKM-Z:75

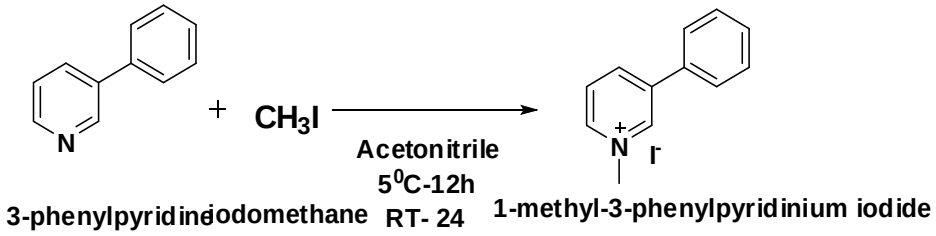
2,2'-(1*E*,1'*E*)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1-methylpyridinium) iodide



Scheme-6:

1-methyl - 3- phenyl pyridinium preparation:

3-phenyl pyridine (2mmol) in acetonitrile (10 ml) was stirred while cooling to 5^0C in 25ml round bottom flask with tightly closed rubber septum, and 5mL of methyl iodide solution was injected. The mixture was stirred at 5^0C for 12h and then at room temperature was stirred for 24h.The precipitate was filtered and washed with little amount of acetonitrile [**53**].



(UHAKKM-ZA:76)

Scheme-4 compounds have confirmed molecular structure by LC-MS spectrum only.

(2E,6E)-2, 6-bis(pyridin-n-ylmethylene) cyclohexanone.

Chemical Formula: C18H16N2O

Exact Mass: 276.13

Molecular Weight: 276.33

m/z: 276.13 (100.0%), 277.13 (19.7%), 278.13 (2.1%)

Found ESI-MS (M+H): 277; Found: 277

Scheme-5 compounds spectral characterization:

4,4'-(1E,1'E)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1-methylpyridinium).(UHAKKM-W: 73)

Yield: 610 mg (99%), m.p: 196-198 °C; AT-FTIR: ν (cm-1) 3455.97, 3334.04, 3014.65, 1672.74,1637.77; $_1$H NMR (400 MHz,d6-DMSO), δ (ppm): 9.03 (4H), 8.26 (4H), 7.71 (2H), 4.37 (6H), 3.01 (4H), 1.80 (2H); $_{13}$C NMR (100MHz, d6-DMSO), δ (ppm): 188.72, 151.01, 145.65, 144.26, 131.09, 128.23, 48.14,28.02,21.63; HRMS(ESI+): m/z: calculated for $C_{20}H_{22}N_2O^{2+}$(M^{2+}): 153.0850; found: 153.0861.

3,3'-(1E,1'E)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1-methylpyridinium). (UHAKKM-X: 74)

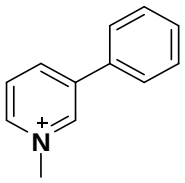
Yield: 602 mg (98%), m.p: 230-232 °C; AT-FTIR : ν (cm^{-1}) 3040.16, 2980.92, 2871.51, 1671.92,1617.45; ^{1}H NMR (400 MHz,d6-DMSO), δ (ppm): 9.54 (2H), 9.00 (2H), 8.734 (2H), 8.230 (2H), 7.68 (2H); 4.41 (6H); 3.01 (4H); 1.78 (2H); ^{13}C NMR (100MHz, d6-DMSO), δ (ppm): 188.44, 146.63, 145.50, 144.98, 141.48, 135.06, 129.64, 127.92, 48.80, 27.86,22.00; HRMS(ESI+): m/z: calculated for $C_{20}H_{22}N_2O^{2+}$(M^{2+}): 153.0850; found: 153.0877.

2,2'-(1E,1'E)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1-methylpyridinium) . (UHAKKM-Y: 75)

Yield: 479 mg (78%), m.p: 187-189 °C; AT-FT IR : ν (cm^{-1})3600.41,3434.18, 3019.39, 2997.49,1682.61, 1621.76; ^{1}H NMR (400 MHz,d6-DMSO), δ (ppm): 9.19 (2H), 8.64 (2H), 8.15 (4H), 7.66 (2H), 4.29 (6H); 2.85 (4H); 1.77 (2H); ^{13}C NMR (100MHz, d6-DMSO), δ (ppm): 187.78 ,150.65 ,147.55 ,145.57 ,144.90, 129.55, 127.51, 126.43, 46.75,28.06,21.82; HRMS(ESI+): m/z: calculated for $C_{20}H_{22}N_2O^{2+}$(M^{2+}): 153.0850; found: 153.0892.

Scheme-6 compound has confirmed molecular structure by LC-MS.

1-methyl-3-phenyl-pyridinium iodide (UHAKKM-Z:76)



1-methyl-3-phenylpyridinium
Chemical Formula: C12H12N+
Exact Mass: 170.10
Molecular Weight: 170.23
m/z: 170.10 (100.0%), 171.10 (13.1%)

Found ESI-MS (M^{+}): 170; Found: 170

Kinase Inhibitory Activity:

Analysis of kinase inhibitory activity of compounds showed that all four compounds are highly active, wherein 1-methyl-3-phenyl pyridinium showed highest activity of 1 pM, while methyl pyridinium bischalcones showed 5 pM.

Symmetrical methyl pyridinium bischalcones:

Table 20 : Methyl pyridinium bischalcones kinase inhibition activity values:

S.No	Molecule code	pIC_{50} value	IC_{50} concentration(M)
73	UHAKKM-W	11.239	$5.8X10^{-12}$
74	UHAKKM-X	11.265	$5.4X10^{-12}$
75	UHAKKM-Y	11.334	$4.6X10^{-12}$

Above table have shown potent kinase inhibition by methyl pyridinium bischalcones, but lower than UHAKKM-7 to 9.

1-methyl-3-phenyl pyridinium:

Table 21: I-Methyl-3-phenyl pyridinium molecule kinase inhibition activity:

S.No	Molecule code	pIC_{50} value	IC_{50} concentration(M)
76	UHAKKM-Z	12.049	$0.9X10^{-12}$

Above table have shown potent kinase inhibition by I-Methyl-3-phenyl pyridinium molecule, but less than UHAKKM-7 to 9.

MTT-assay results:

Molecules are non-toxic to Sup T1 cells

Fig.18: Symmetrical Methyl-Pyridinium-Bischalcones cytotoxicity profile

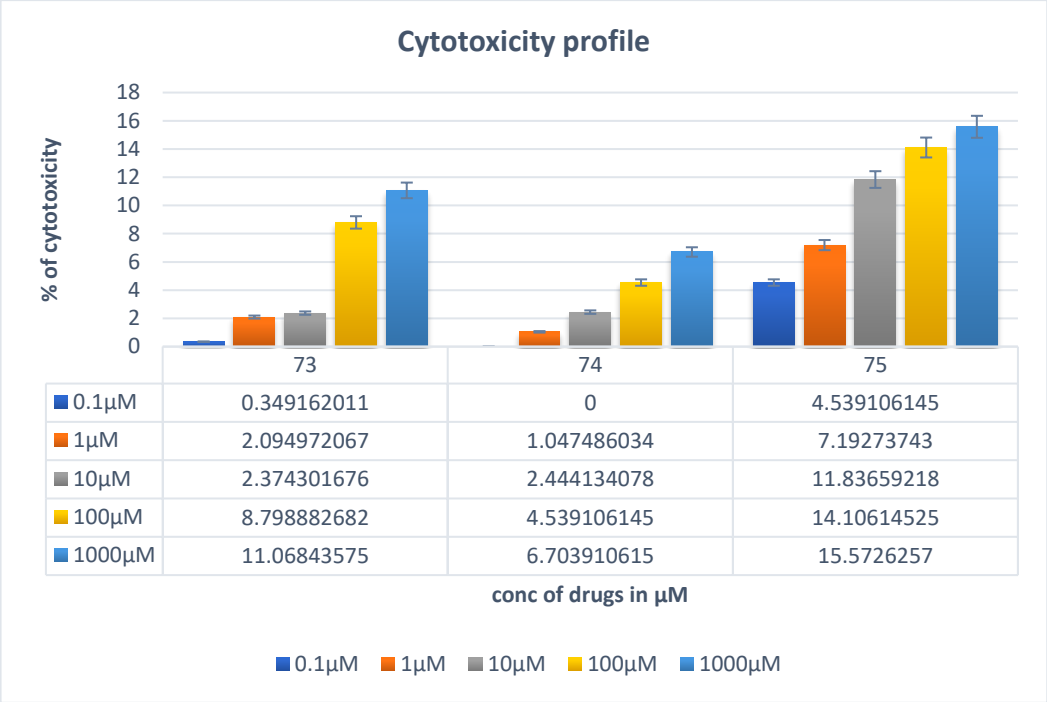


Fig.18: Symmetrical Methyl-Pyridinium-Bischalcones have shown less cytotoxicity (below 20%)even at 1000µM.

Fig.19: 1-Methyl-3-phenyl-pyridinium cytotoxicity profile:

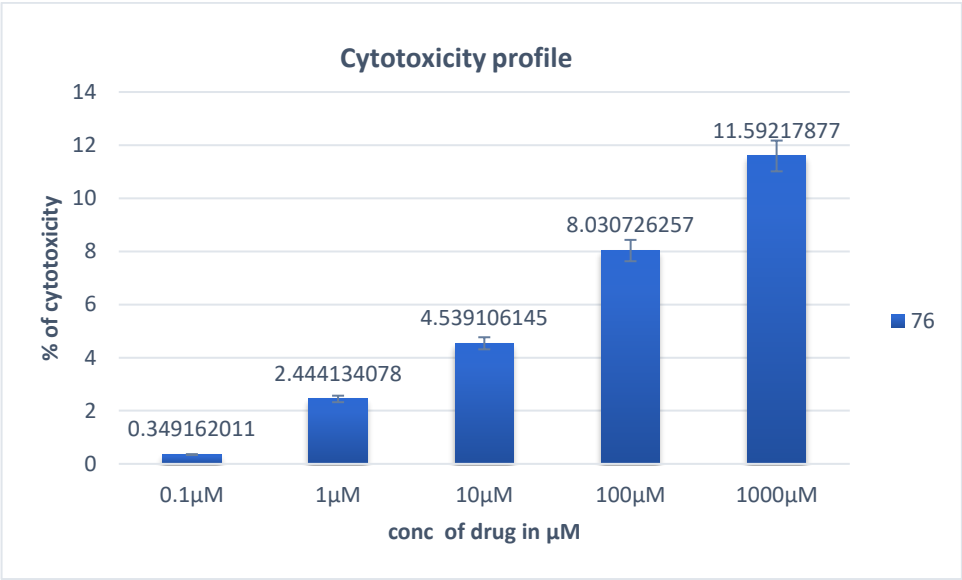


Fig.19: 1-Methyl-3-phenyl-pyridinium molecule have shown less cytotoxicity (below 15%)even at 1000µM.

Anti-HIV-$1_{93IN101}$ activity in SupT1 cells (38nM):

Anti-HIV-1 activity is analysed at 38nM, results showed that 1-methyl-3-phenyl-pyridinium possess highest anti-HIV-1 activity followed by methyl-pyridinium-bischalcones, suggesting 1-methyl-3-phenyl-pyridinium possess highest activity due to specific interactions conferred by this compound to kinase active site.

Fig.20: Symmetric methyl-pyridinium-bischalcones anti-HIV-$1_{93IN101}$ activity(38nM):

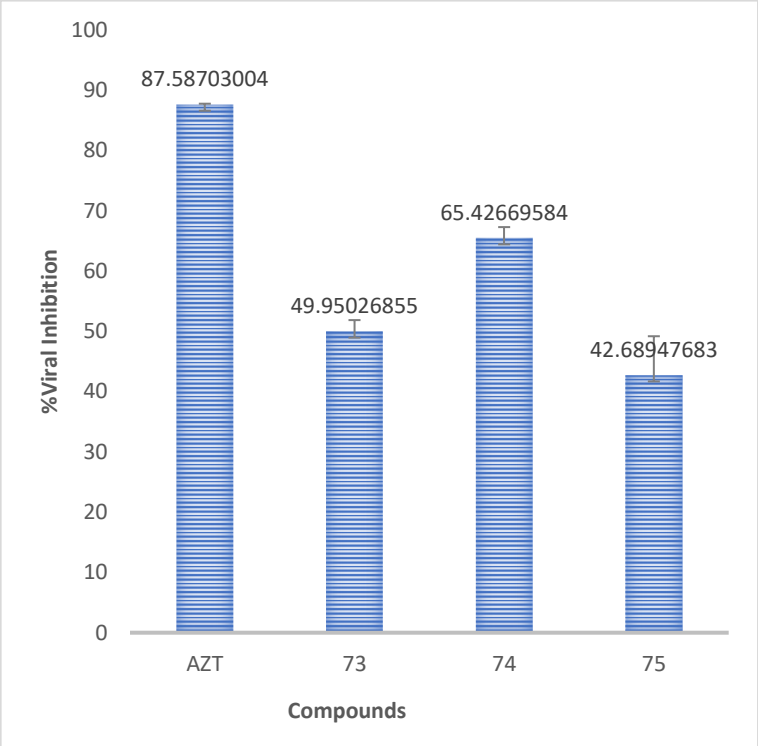


Fig.20: Symmetric methyl-pyridinium-bischalcones have shown approximately 50% anti-HIV-$1_{93IN101}$ activity(IC_{50}) at 38 nM analysed by p24-assay. 3,3'-(1E,1'E)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1- methylpyridinium)(UHAKKM-X: 74) showed better activity than others.

Fig.21: 1-methyl-3-phenyl-pyridinium anti-HIV-$1_{93IN101}$ activity(38nM):

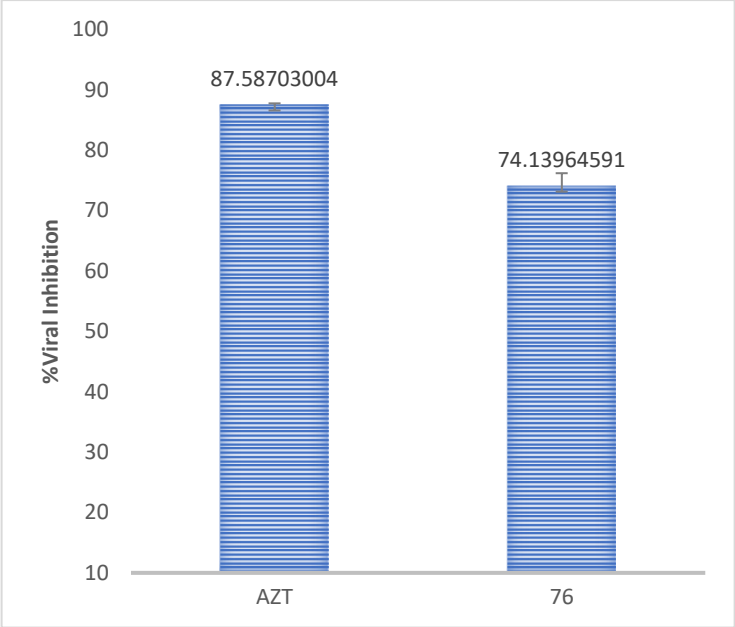


Fig.21: 1-methyl-3-phenyl-pyridinium (UHAKKM-Z:76) has shown 74% anti-HIV-$1_{93IN101}$ activity at 38nM.This molecule exhibits IC_{50} below 30nM against HIV-1.

We have compared results of UHAKKM-7 to 9 against TopoIIβK_{HIV} and HIV-1 with 73, 74, 75 and 76. UHAKKM-7 to 9 compounds have shown IC_{50} less than 30nM against HIV-1 replication, which was the objective of this study.

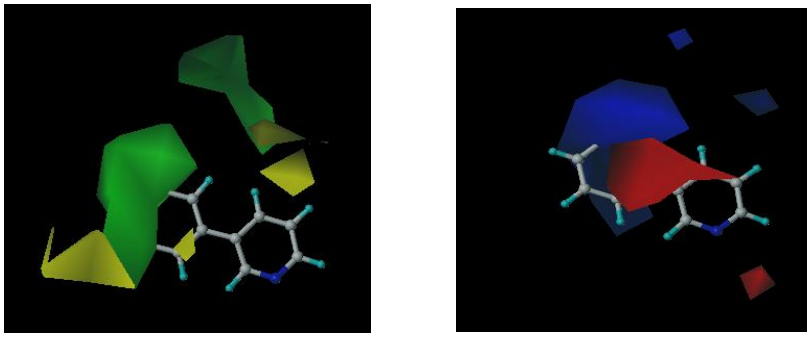
Discussion:

In the objective-1 we have selected dicoumarol group as a steric group on pyridine. 3D-QSAR model have also shown green and blue regions fulfilled by the dicoumarol group as steric group and enolic oxygens as electronegative group (see objective-2). Pyridine dicoumarols have shown experimentally significant kinase inhibition, it is approximately equal to predicted kinase inhibitory values through 3D-QSAR analysis. Higher activity 1-Methyl-3-phenyl-pyridinium could be due to charge nitrogen center with hydrophobic phenyl located in the cavity located in kinase active site, N-methyl may be helping in blocking non-specific

interaction of electronegative nitrogen. A series of compounds with and without pyridine have been designed based on the following QSAR model

3D-QSAR model:

Contour Map For the new model generated for kinase inhibition:

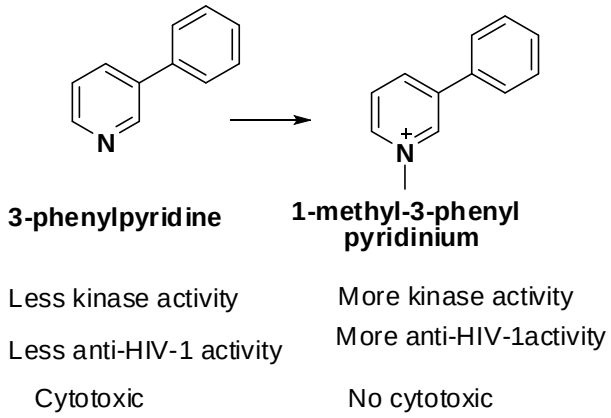


Green: Favor steric groups, Yellow: Disfavor Steric group
Blue: Favor Electronegative group, Red: Favor Electropositive

These molecules were synthesized, characterised and molecular activity against TopoIIβK_{HIV} and HIV-1 replication.

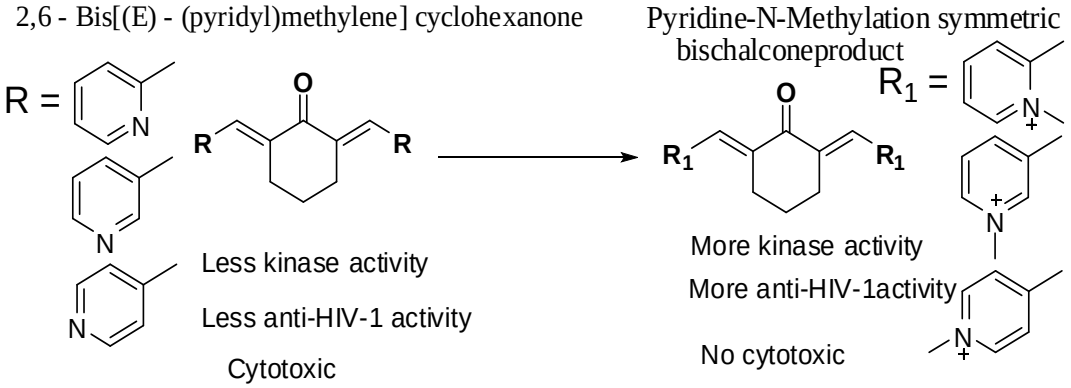
In the objective-2, we applied physico-organic studies on pyridine dicoumarols to overcome pharmacokinetic, pharmacodynamic problems and achieve anti-HIV-1 activity. Result of that sodium pyridine dicoumarates have shown good anti-HIV-1 activity and TopoIIβK_{HIV-1} inhibition. In the objective-3, we have noticed methoxy substituted pyridine ring having sodium pyridine dicoumarates have shown better inhibition than methoxy unsubstituted derivative.

In objective-4, we obtained higher active molecules than methoxy substituted sodium pyridine dicoumarate. Indeed 3D-QSAR contour maps showed the presence of electropositive center at hetero-N atom of pyridine ring required to confer enhanced inhibition of kinase which has shown red region. Hence, we have synthesized 1-methyl-3-phenyl pyridinium molecule from methylation of 3-phenyl pyridine.



We have tested 3-phenyl pyridine and 1-methyl-3-phenyl pyridinium against TopoIIβKinase and HIV-1; results have shown 1-methyl -3-phenyl pyridinium possesses higher activity than 3-phenyl pyridine. This data reveals that positive charge needed on pyridine ring nitrogen, means that near pyridine ring proximity negative charge environment at binding site.

In this objective, we have also studied end to end length of the binding sites at receptor site, for that pyridine bischalcones as cyclohexanone linker. We have synthesized pyridine symmetric bischalcones, cyclohexanone as linker and from these compounds we have also synthesized pyridinium symmetric bischalcones as cyclohexanone linker and tested against TopoIIβK_{HIV} and HIV-1.



These in vitro results pyridine symmetric bischalcones have shown less TopoIIβK_{HIV-1} and anti-HIV-1activity than pyridinium symmetric bischalcones. Hence kinase inhibition and anti-HIV-

1 activity of a molecule is contributed by the positive charge on pyridine ring nitrogen. Another important feature we noticed was pyridinium symmetric bischalcones possess less kinase inhibition and anti-HIV-1 activity than 1-methyl-3-phenyl pyridinium molecule. From this result, it reveals that one benzene ring length enough than two rings having linker cyclohexanone for activity, indeed molecular volume plays a role in interaction. We have noticed 1-methyl-3-phenyl pyridinium has also shown lesser kinase inhibition and anti-HIV-1 activity than sodium pyridine dicoumarates, reason of that sodium pyridine dicoumarate length is optimal length for better activity against kinase.

Conclusion:

In conclusion, ligand based evaluation of 1-methyl-3-phenyl pyridinium and three N-methyl pyridinium bischalcones having cyclohexanone linker molecules provide interactive environment with kinase interaction and further development of sodium methoxy substituted pyridine dicoumarates enhanced anti-HIV-1 activity of drug. Molecules having electron withdrawing groups substituted benzoyl moiety having on pyridine ring nitrogen, sodium 1-(n-benzoyl)-n-metoxy-substituted pyridinium sodium dicoumarate products showed highest kinase inhibition and anti- HIV-1 activity.

Future Perspective:

This study demonstrates 3D-QSAR contour maps features approximately almost achieved by this proposed molecule structure. Steric groups regions satisfied by that dicoumarol moiety which indicates green colour in contour maps, electronegative regions fulfilled by enolic oxygens and potential pharmacophore which indicates blue region in contour maps, electropositive region satisfied by benzoyl substituted pyridinium ring, which indicates red

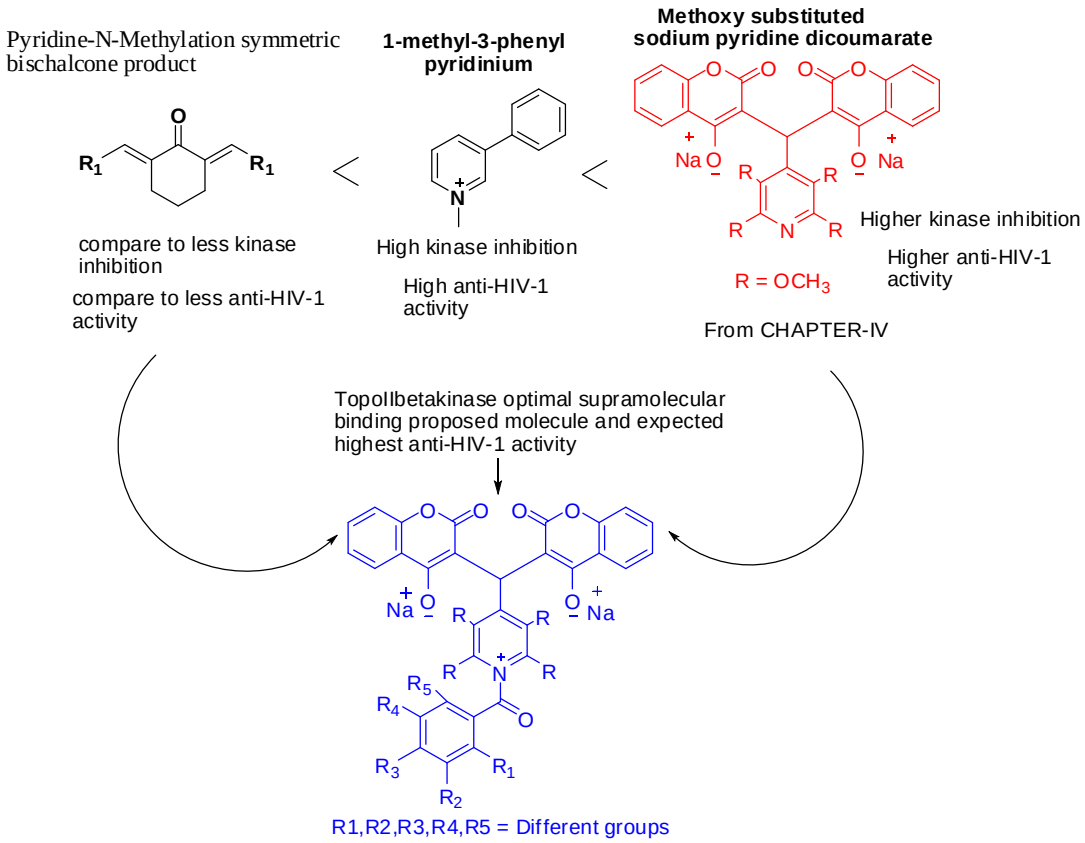
region in contour maps. Father studies required for analysis of molecular action of inhibitors on HIV-1 replication intermediates.

Executive summary:

1-methyl-3-phenyl-pyridinium and three methyl pyridinium symmetric bischalcones which have cyclohexanone as linker were employed for understanding kinase binding cavity to design potent anti-HIV-1 compounds to inhibit Topo IIβ$K_{HIV.}$

Overall Summary of the thesis:

Summary of the thesis results are given in the following schematic representation:

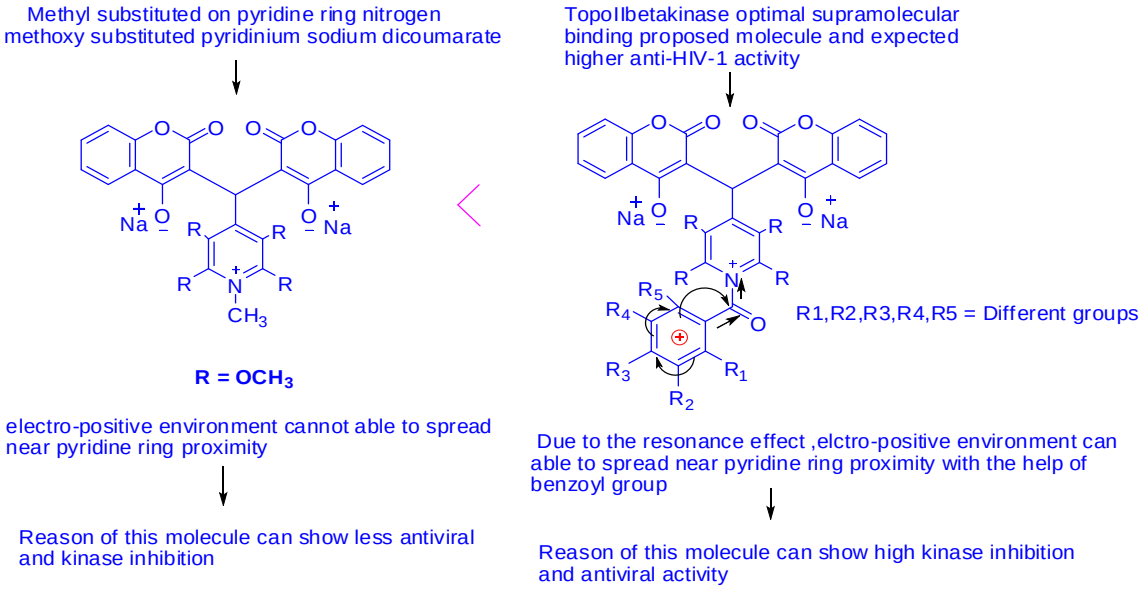


Finally, we have constructed one predictive structure for best kinase inhibition and anti-HIV-1 activity which has N-substituted benzoyl ring on pyridine ring nitrogen, result of that positive charge having 1-(N-substituted benzoyl)-n-methoxy substituted pyridinium sodium

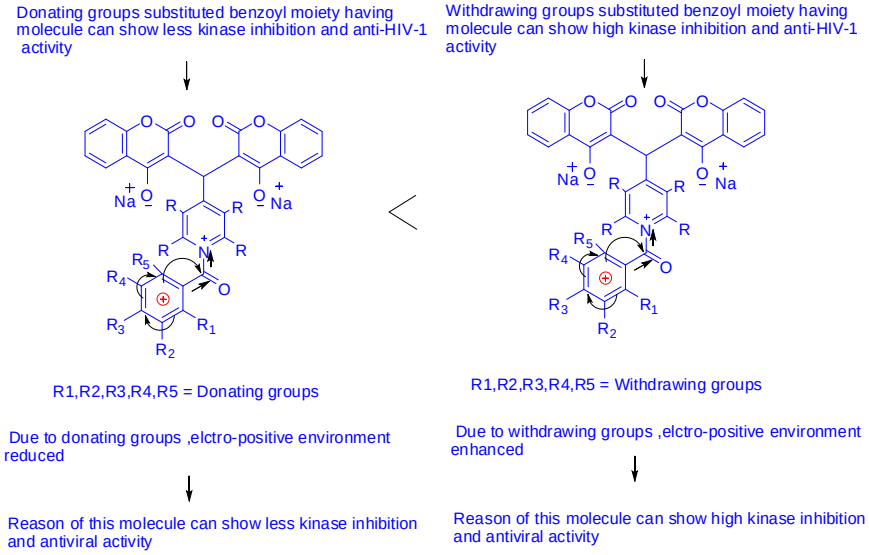
dicoumarate, which exhibits highest kinase and anti-HIV-1 activities for further drug development.

In objective 4, results show pyridine ring proximity electro-positive environment is required.

These observations are depicted in the following diagram:



Further, electro-positivity in pyridine structure having substituted benzoyl derivative show highest activity against kinase and HIV-1 than pyridine N-methyl substitution substituted.



Supporting Spectral characterization information

OBJECTIVE-1 SPECTRAL DATA

Figure-S1:

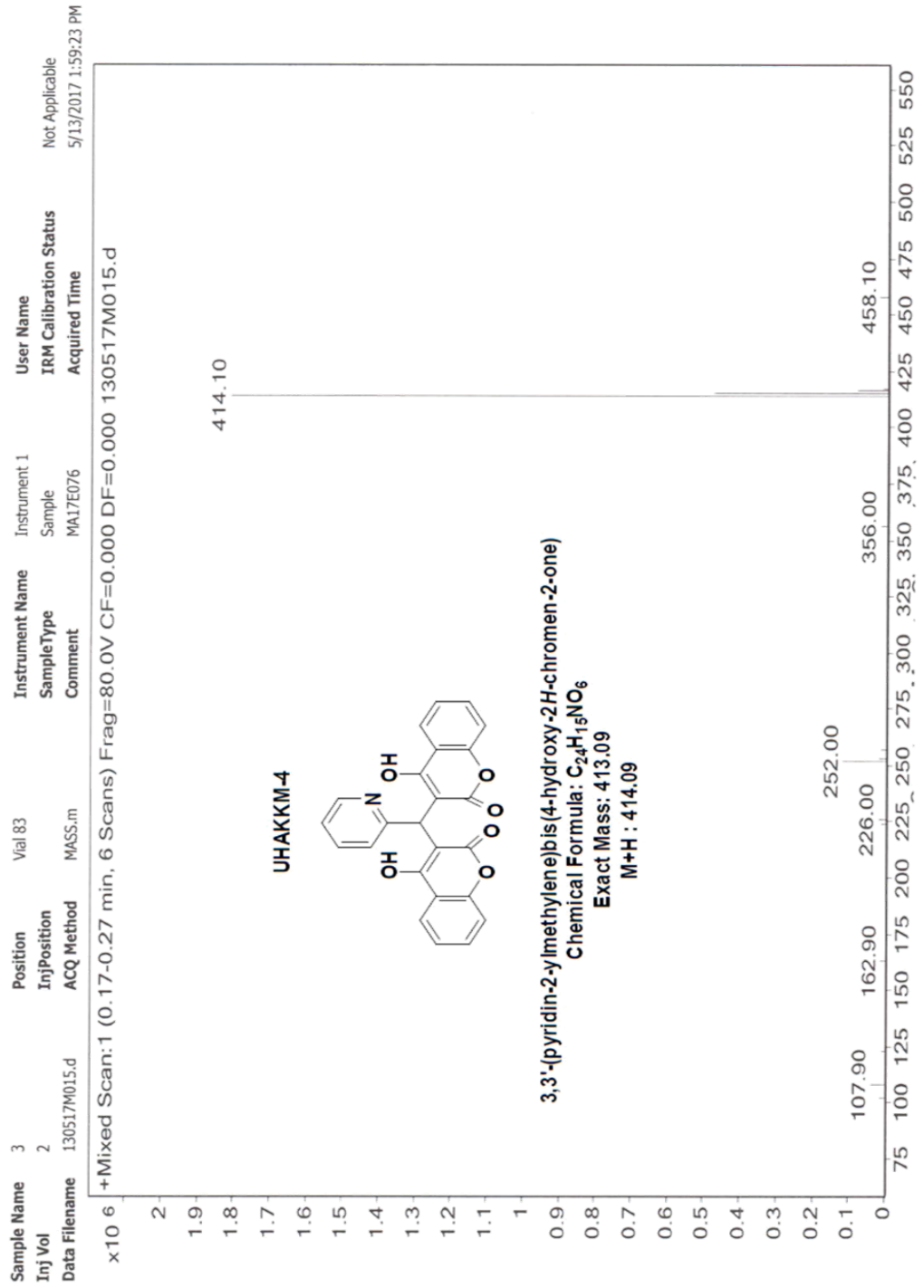


Figure-S1: The ESI-HRMS spectrum of UHAKKM-4

Figure-S2:

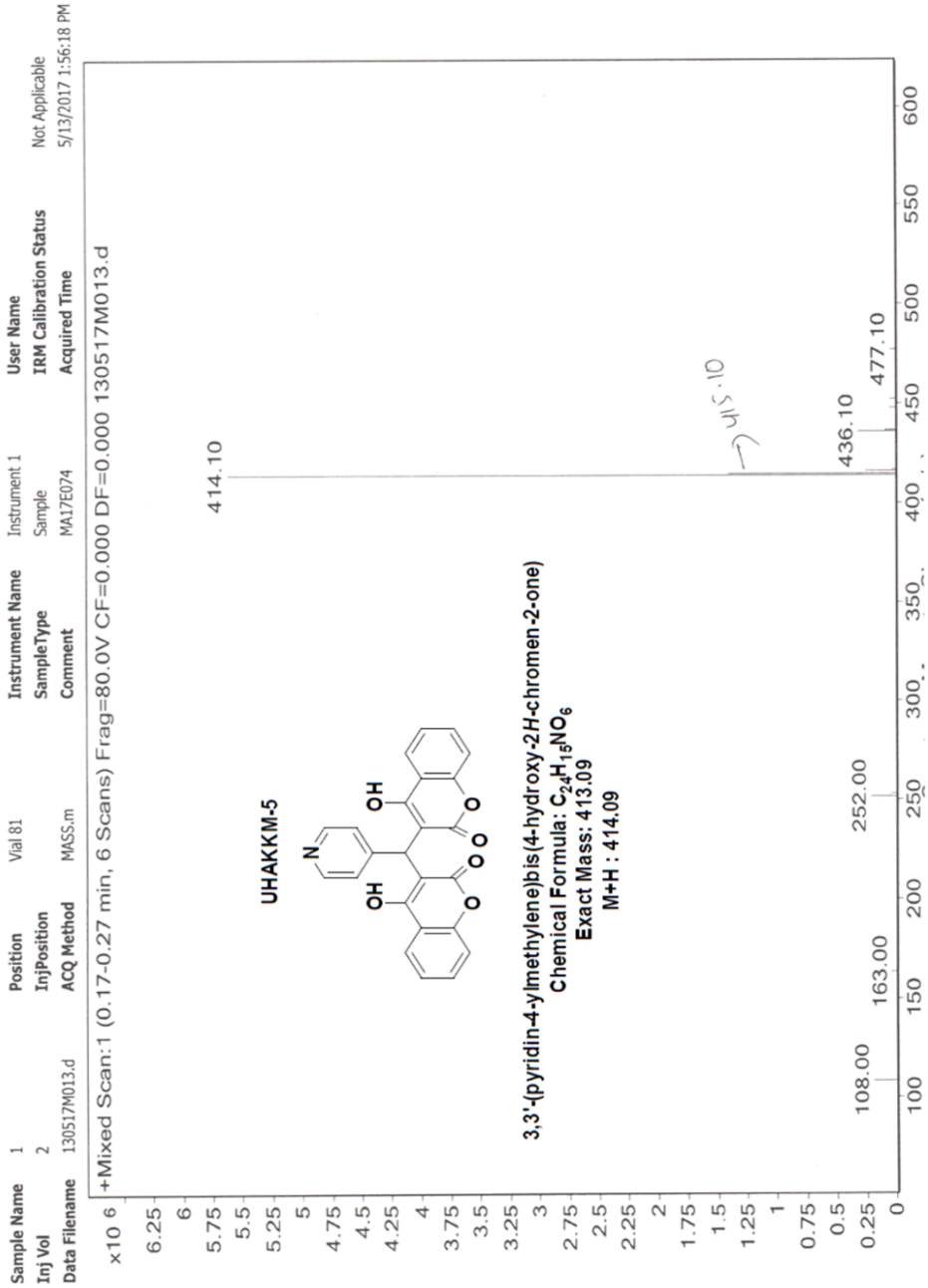


Figure-S2: The ESI-HRMS spectrum of UHAKKM-5

Figure-S3:

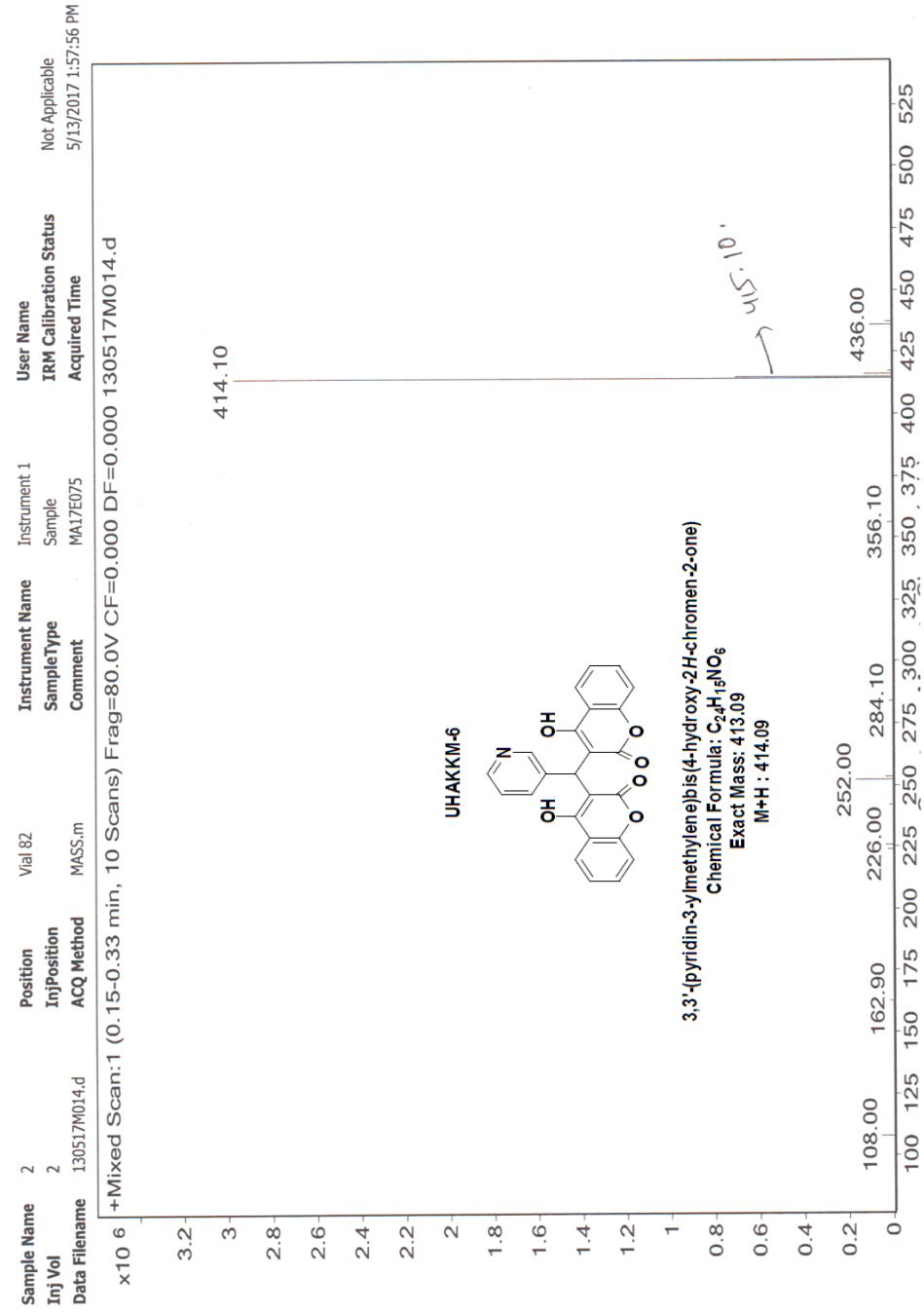


Figure-S3: The ESI-HRMS spectrum of UHAKKM-6

Figure-S4:

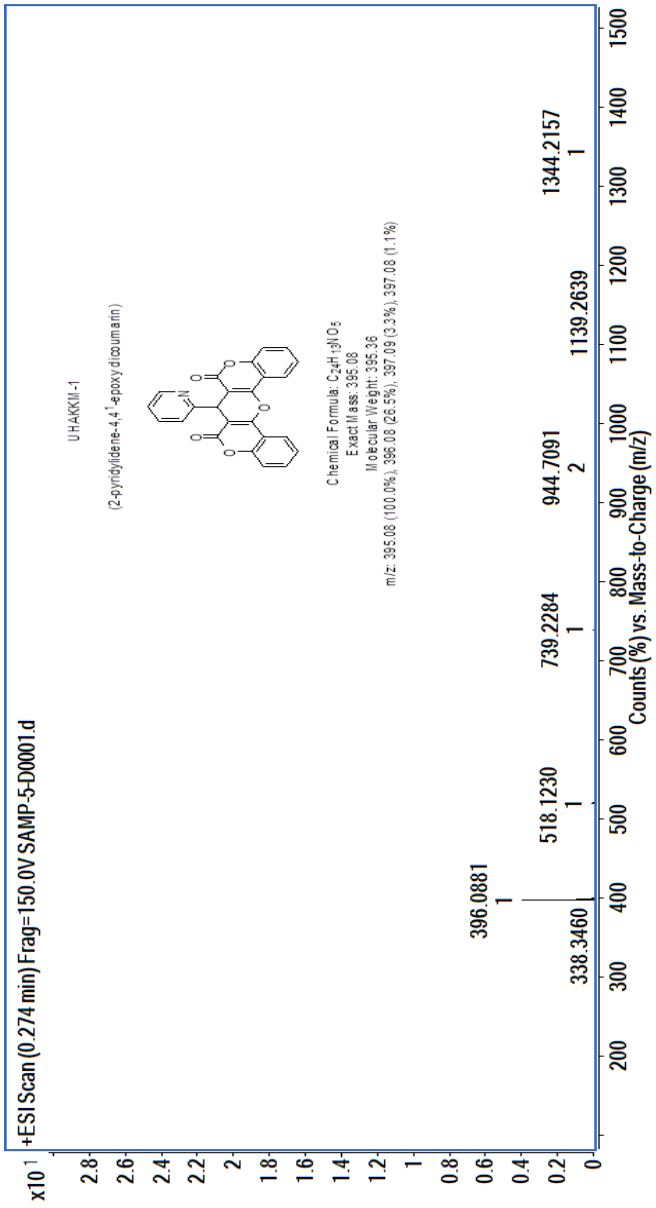


Figure-S4: The ESI-HRMS spectrum of UHAKKM-1

Figure-S5:

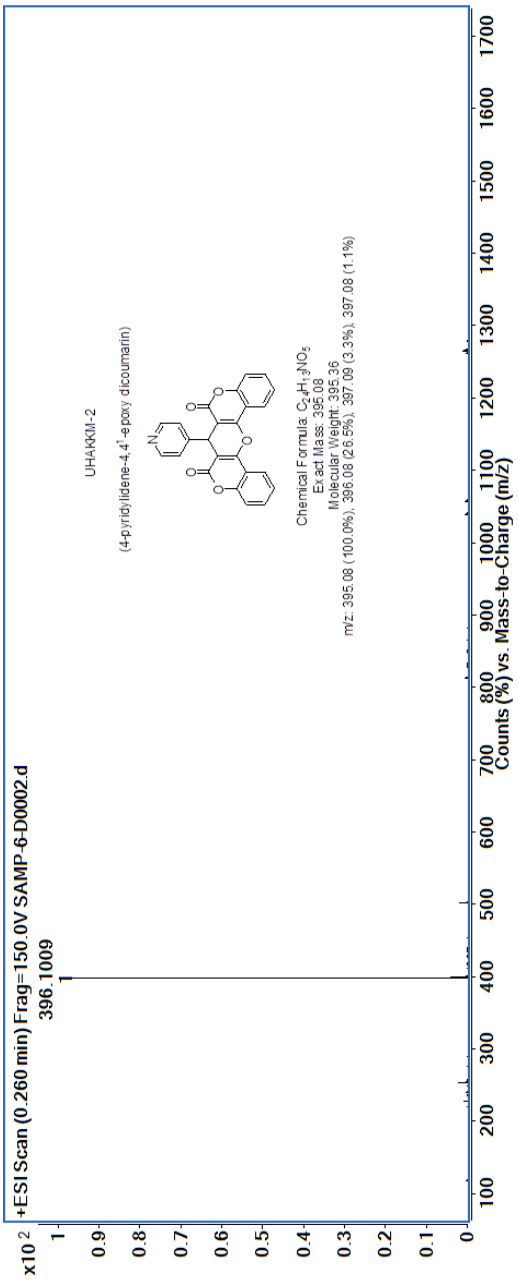


Figure-S5: The ESI-HRMS spectrum of UHAKKM-2

Figure-S6:

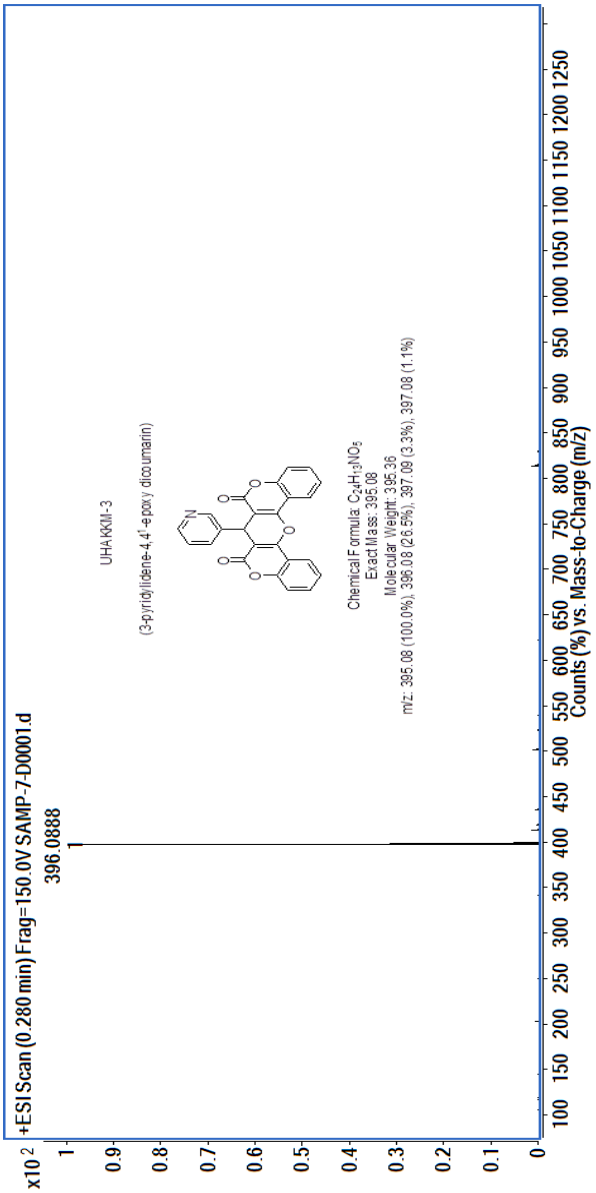


Figure-S6: The ESI-HRMS spectrum of UHAKKM-3

Figure-S7:

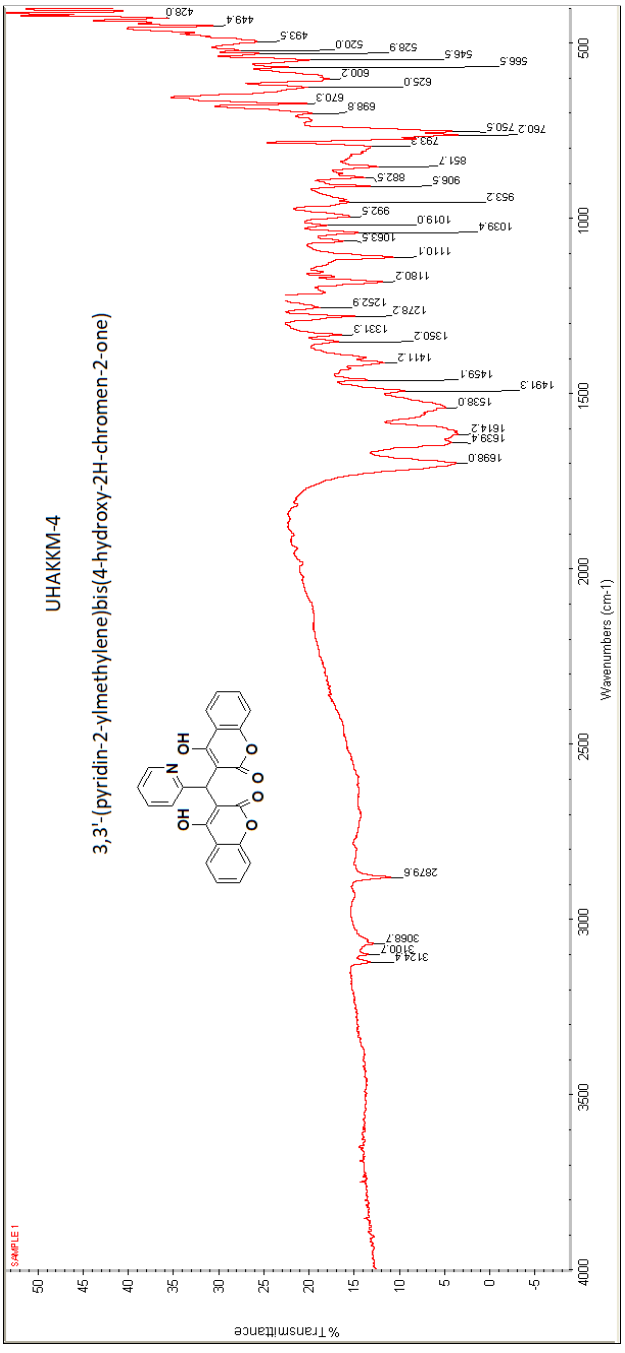


Figure-S7: The FTIR spectrum of UHAKKM-4

Figure-S8:

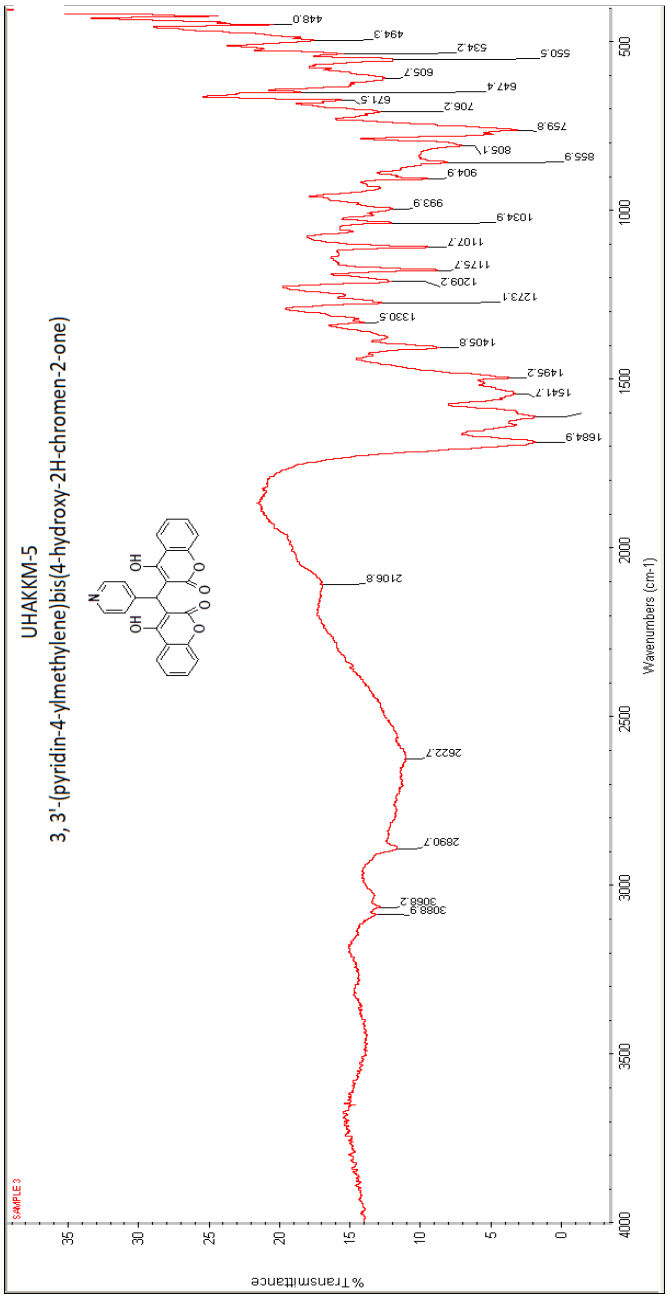


Figure-S8: The FTIR spectrum of UHAKKM-5

Figure-S9:

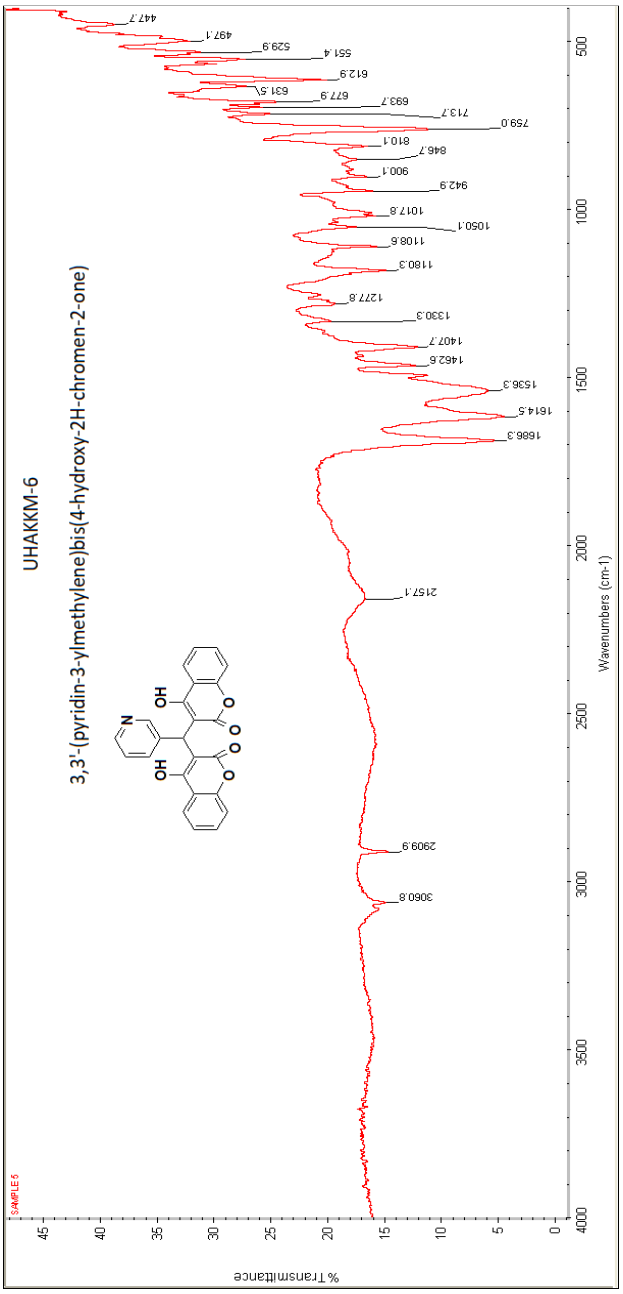


Figure-S9: The FTIR spectrum of UHAKKM-6

Figure-S10:

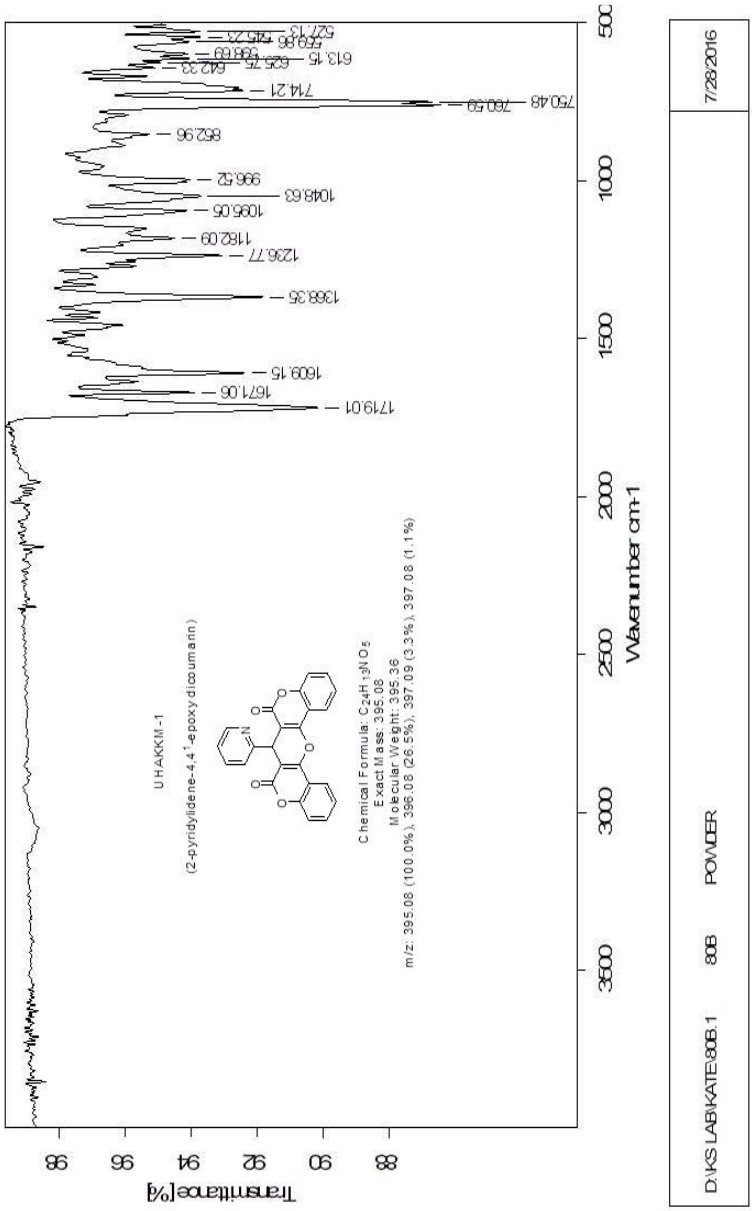


Figure-S10: The FTIR spectrum of UHAKKM-1

Figure-S11:

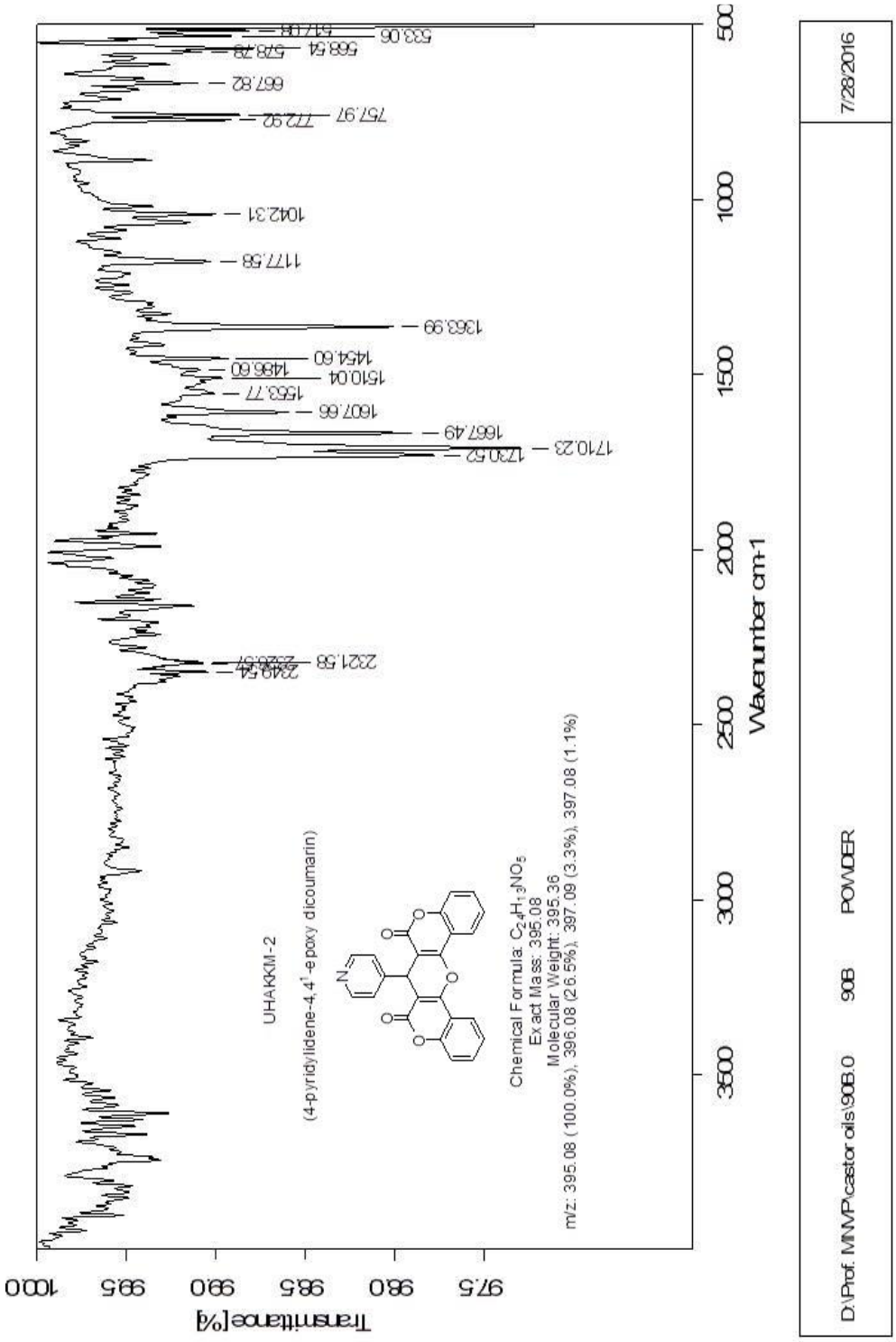


Figure-S11: The FTIR spectrum of UHAKKM-2

Figure-S12:

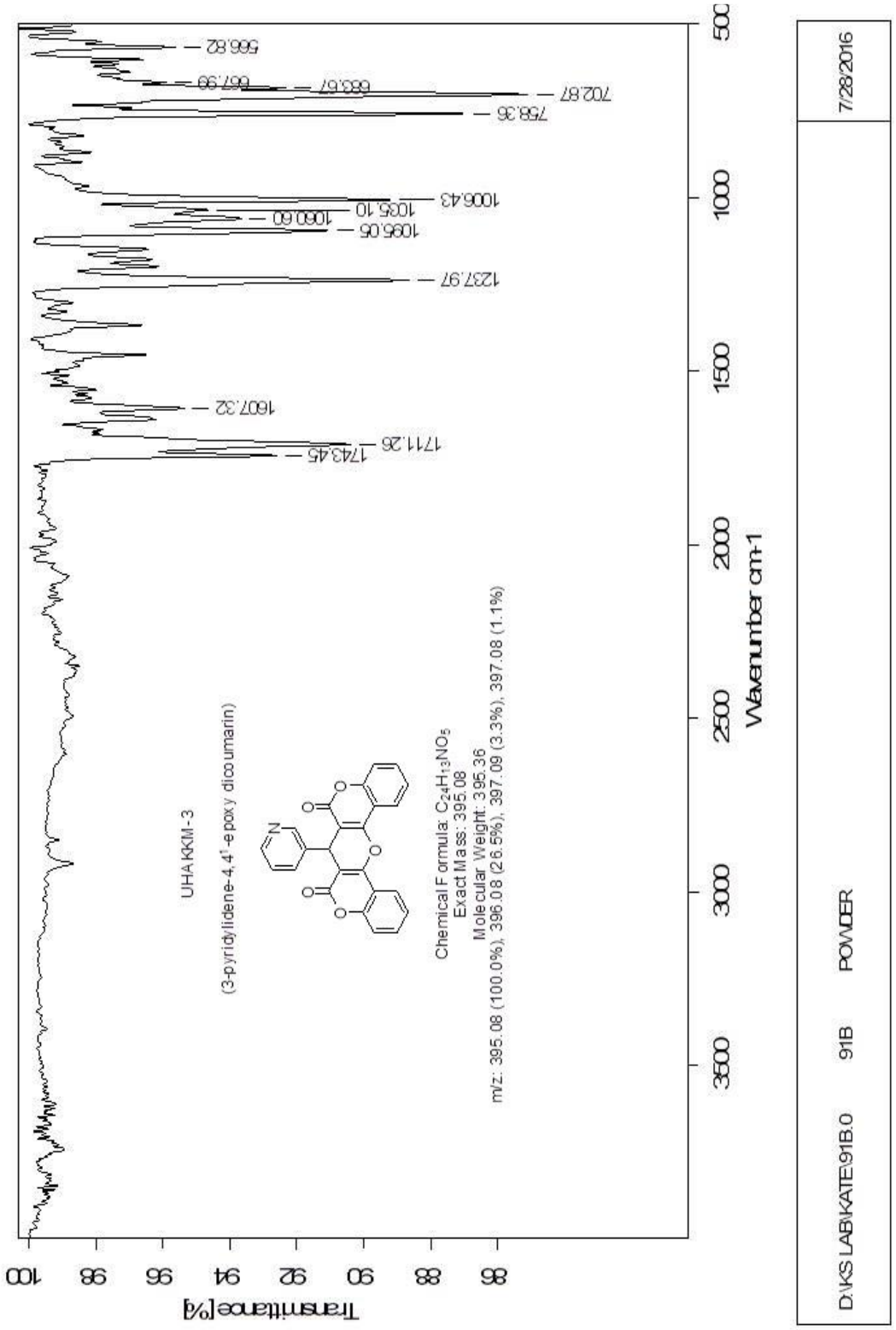


Figure-S12: The FTIR spectrum of UHAKKM-3

Figure-S13:

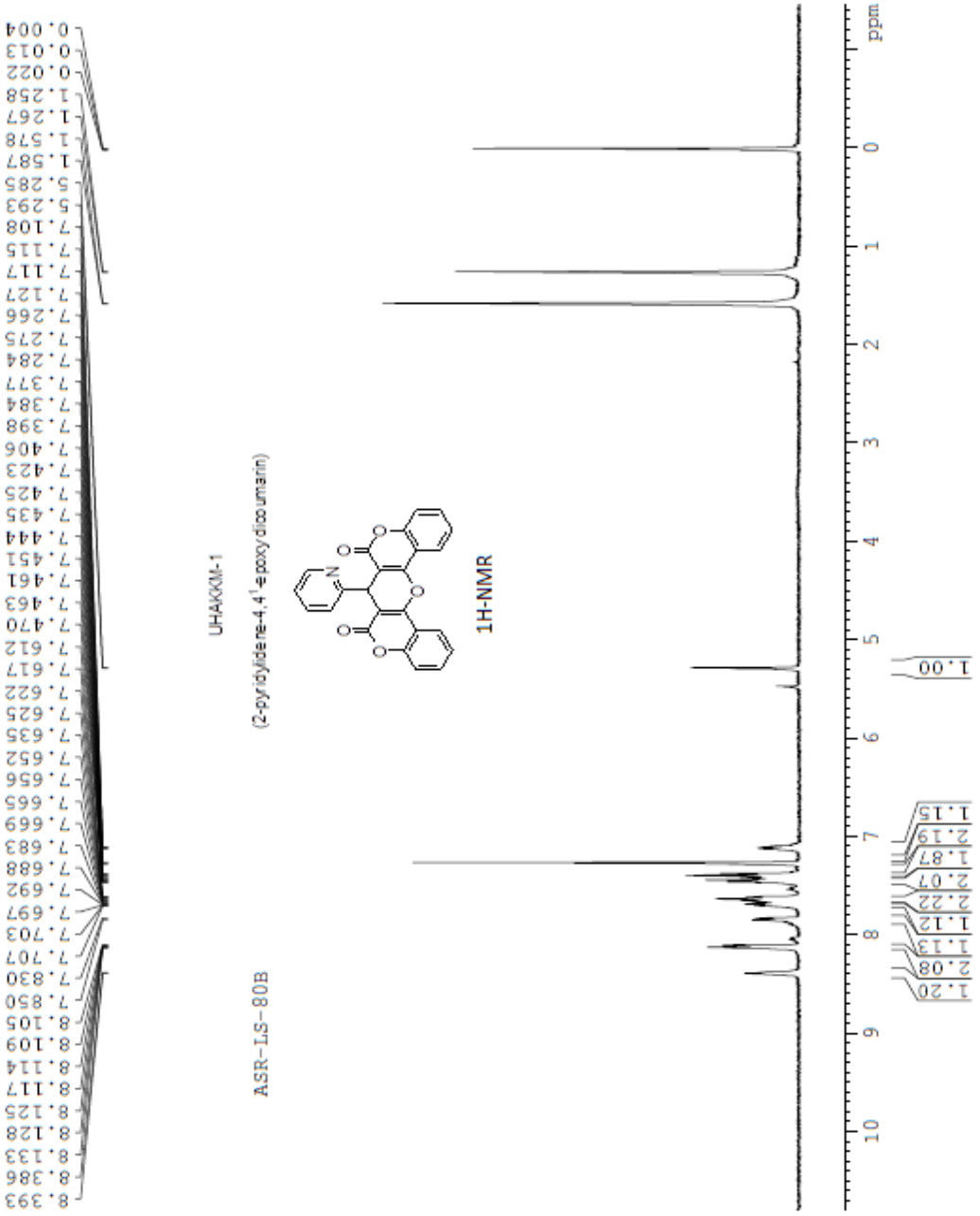


Figure-S13: The ^{1}H-NMR spectrum of UHAKKM-1

Figure-S14:

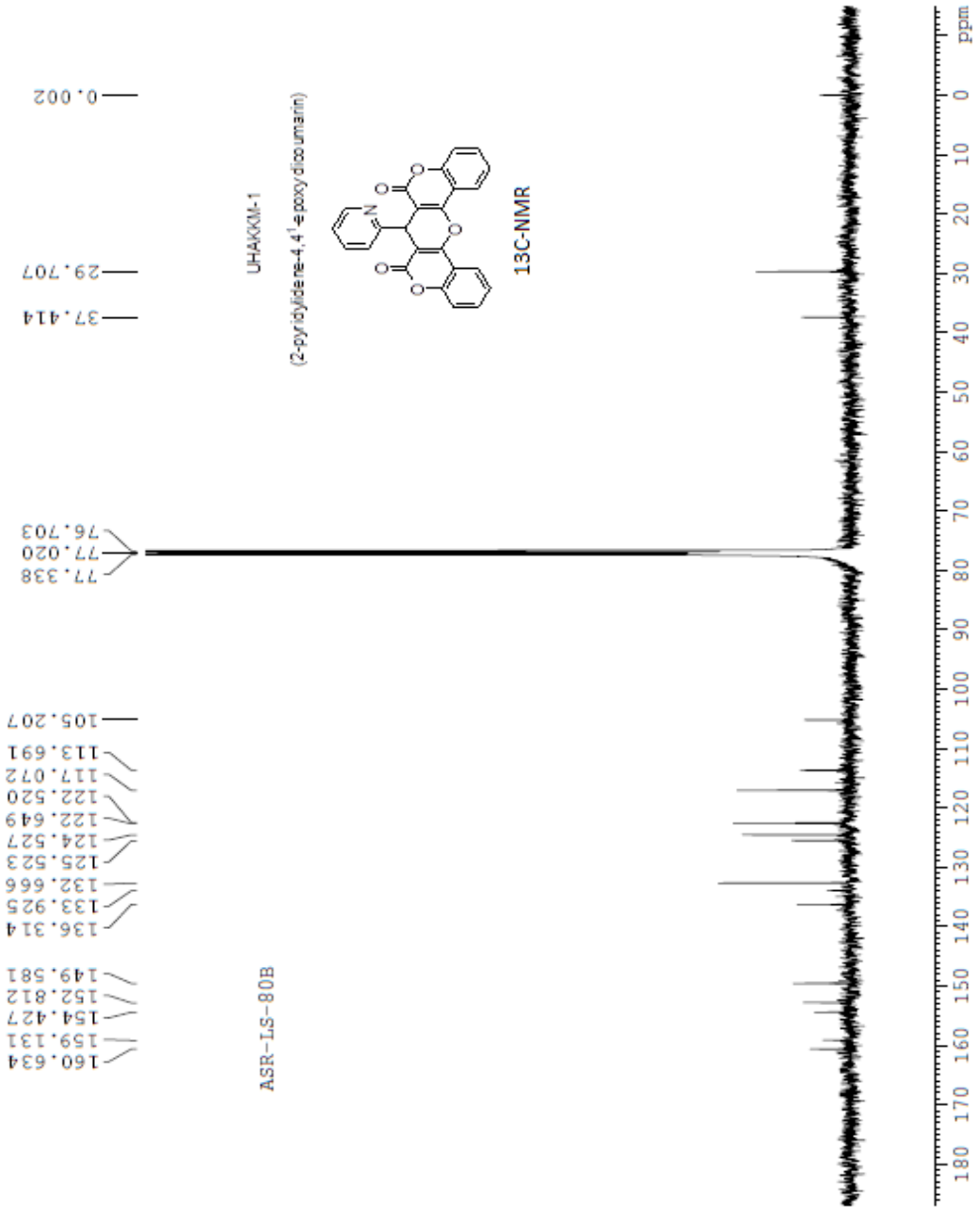


Figure-S14: The ^{13}C-NMR spectrum of UHAKKM-1

Figure-S15:

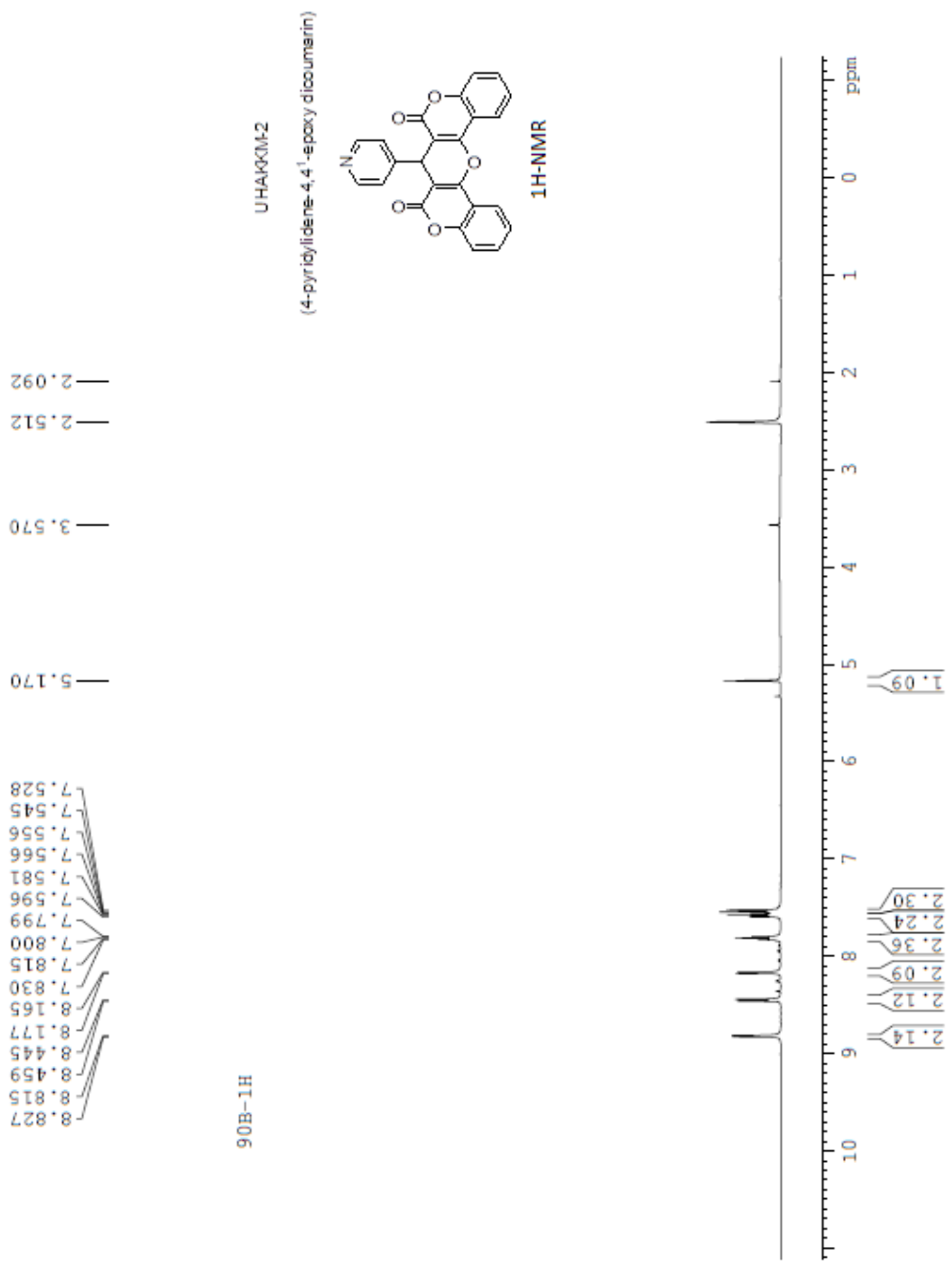


Figure-S15: The ^{1}H-NMR spectrum of UHAKKM-2

Figure-S16:

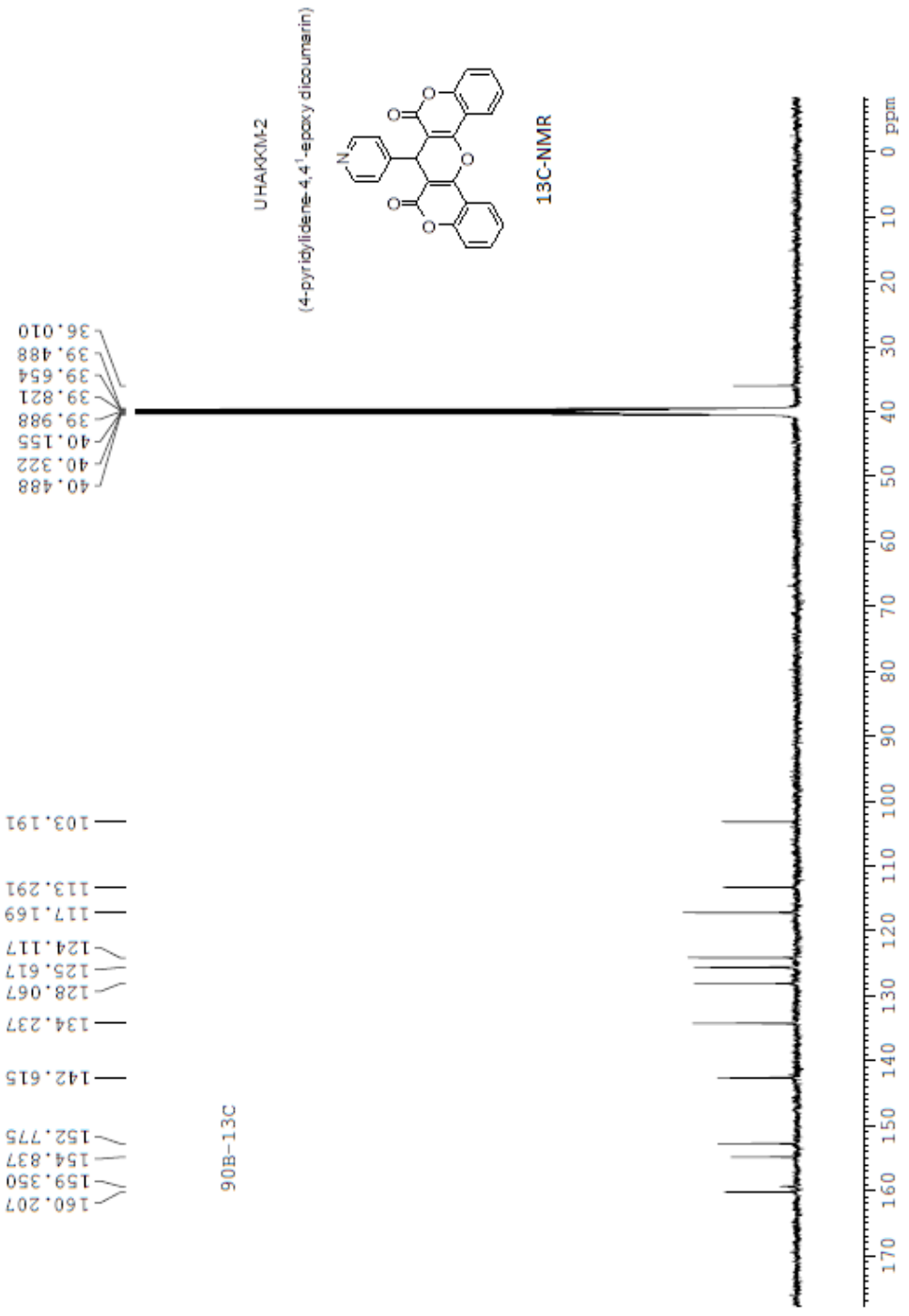


Figure-S16: The ^{13}C-NMR spectrum of UHAKKM-2

Figure-S17:

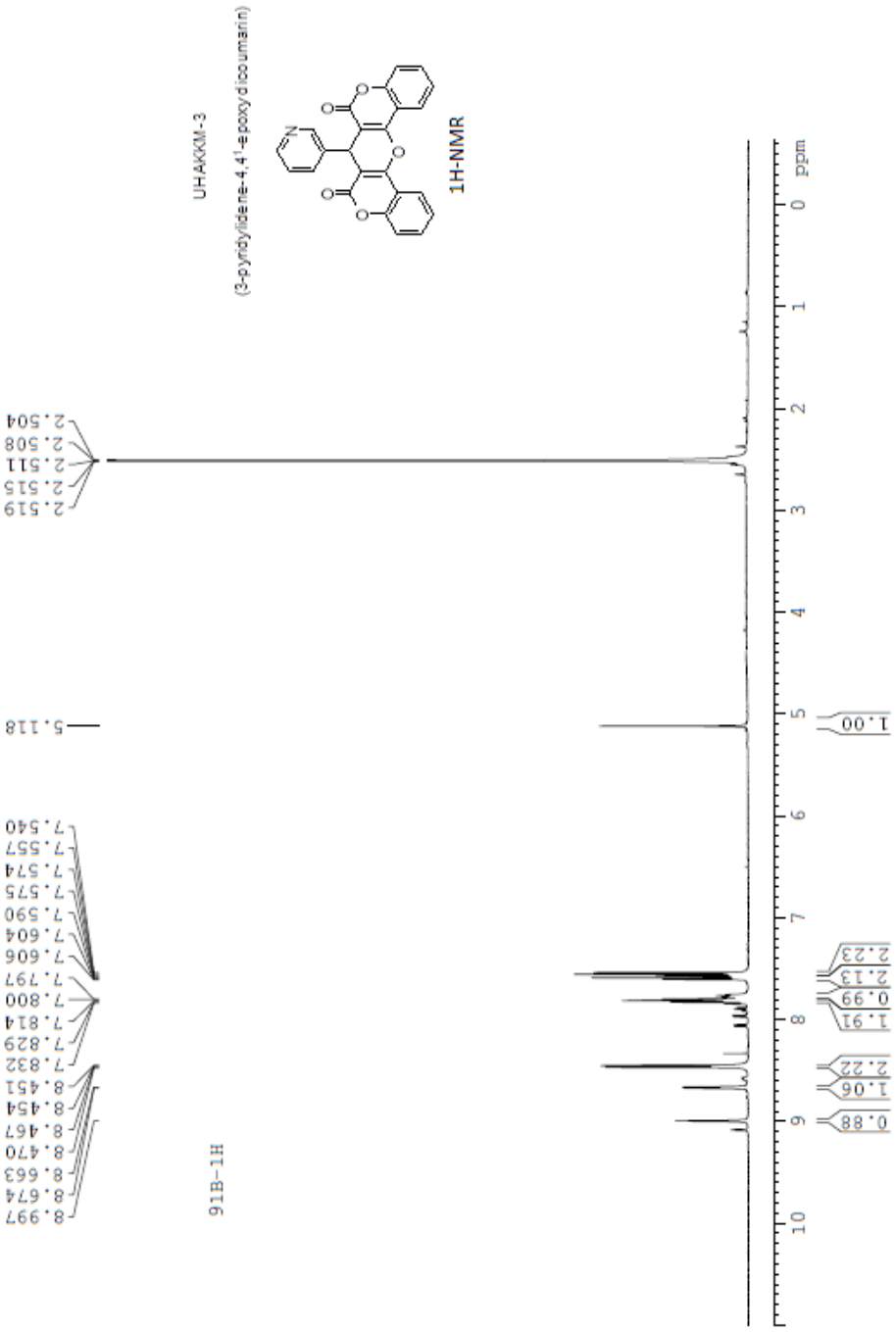


Figure-S17: The ^{1}H-NMR spectrum of UHAKKM-3

Figure-S18:

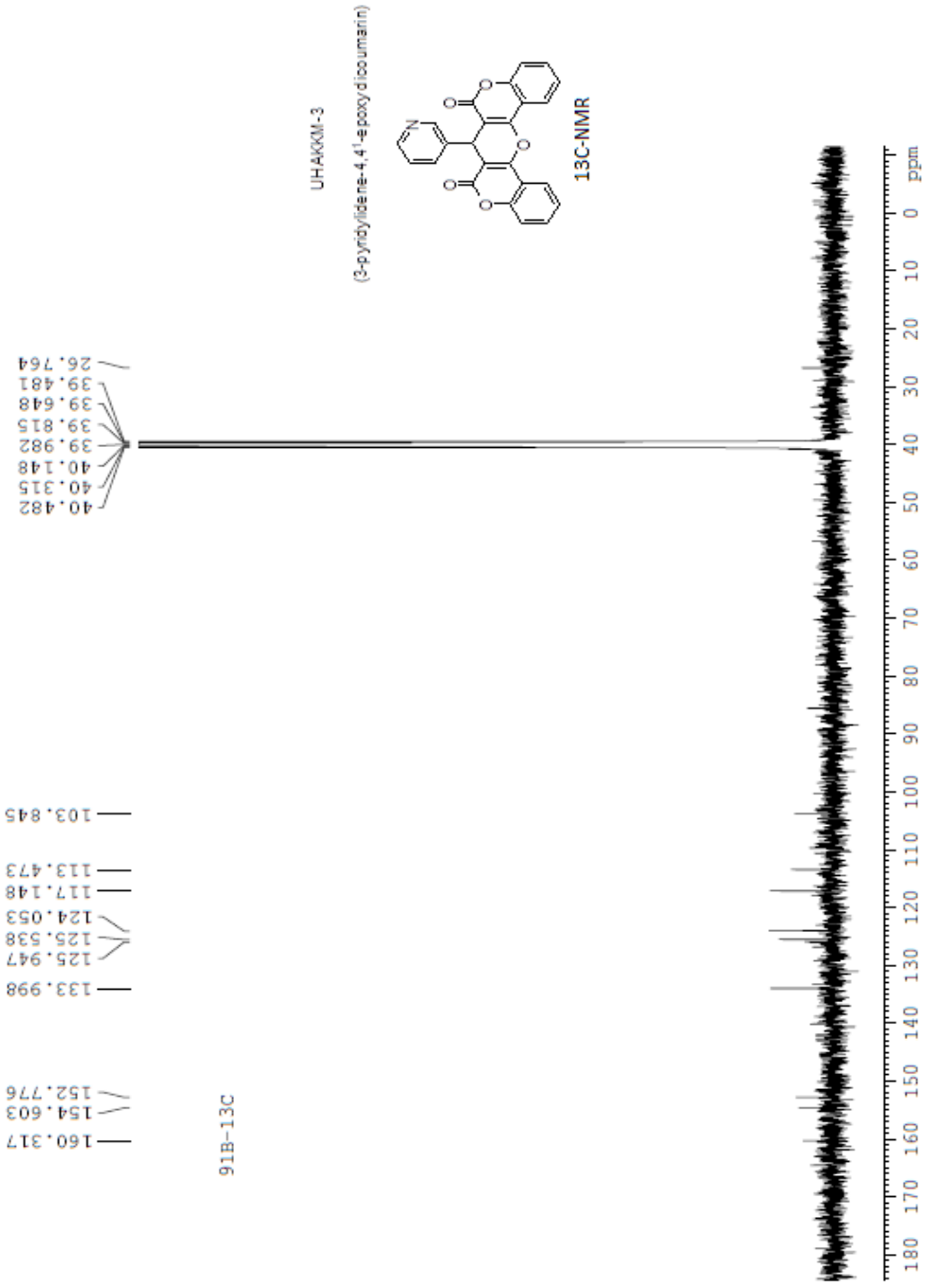


Figure-S18: The ^{13}C-NMR spectrum of UHAKKM-3

Figure-S19:

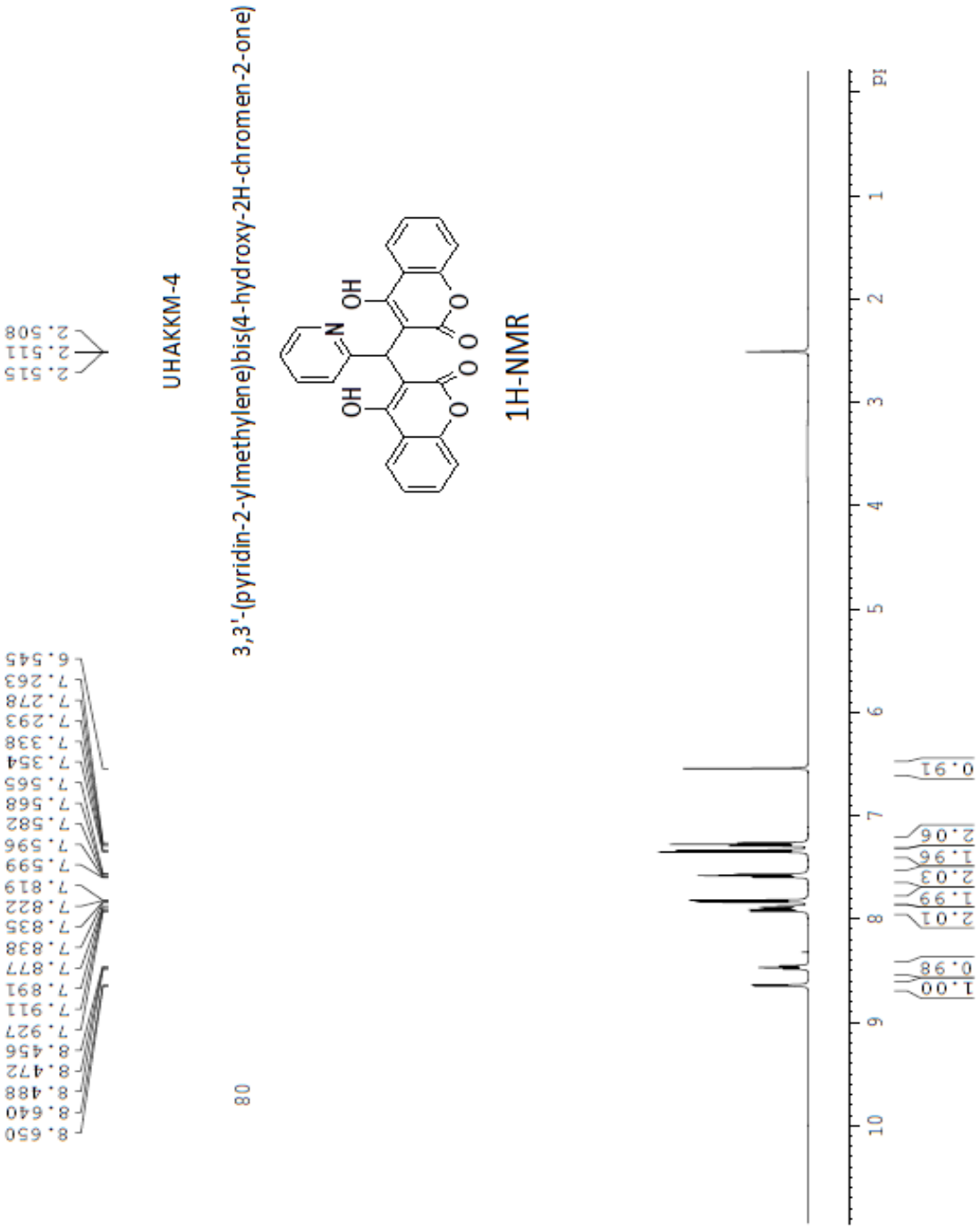


Figure-S19: The ^{1}H-NMR spectrum of UHAKKM-4

Figure-S20:

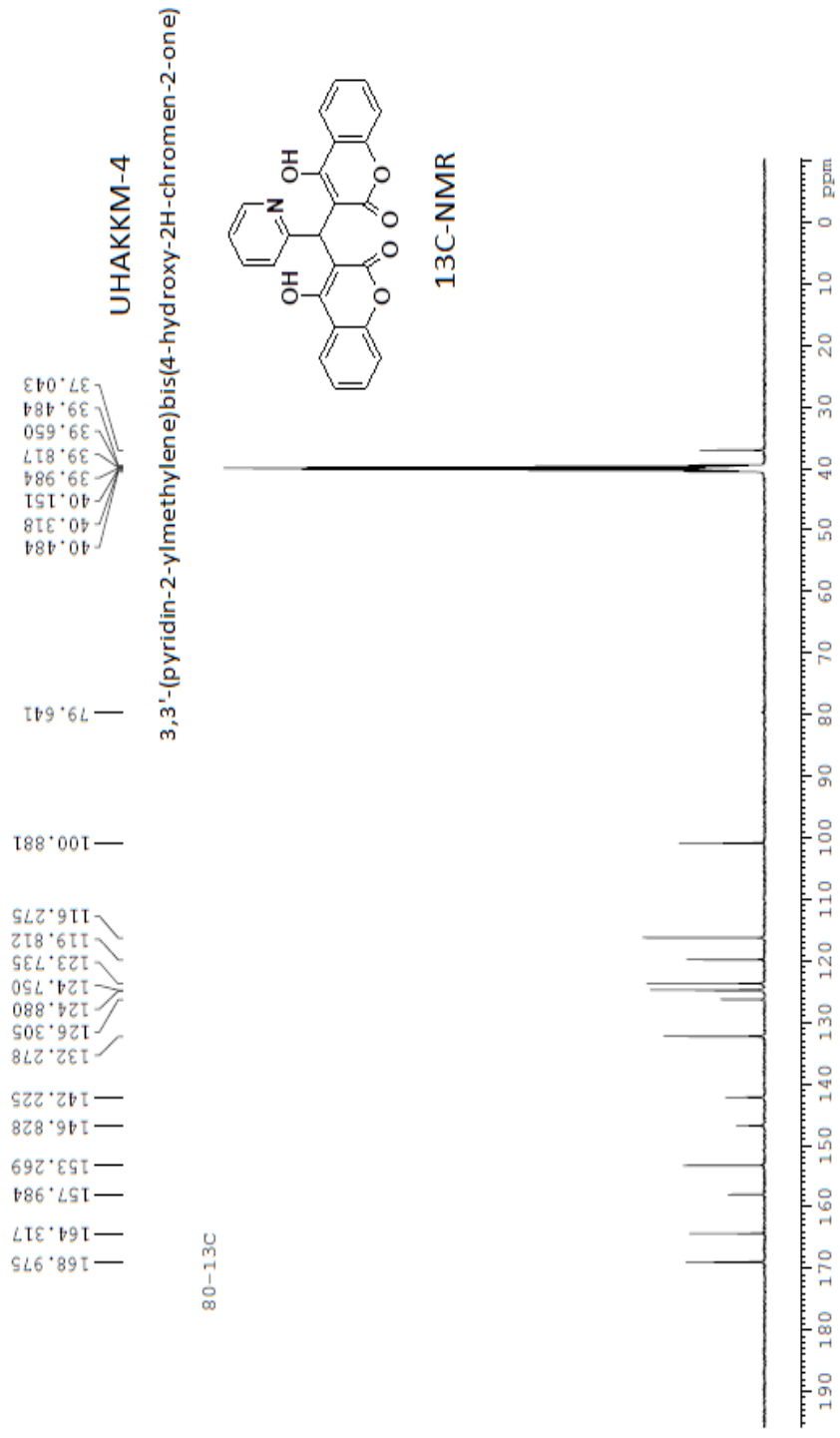


Figure-S20: The ^{13}C-NMR spectrum of UHAKKM-4

Figure-S21:

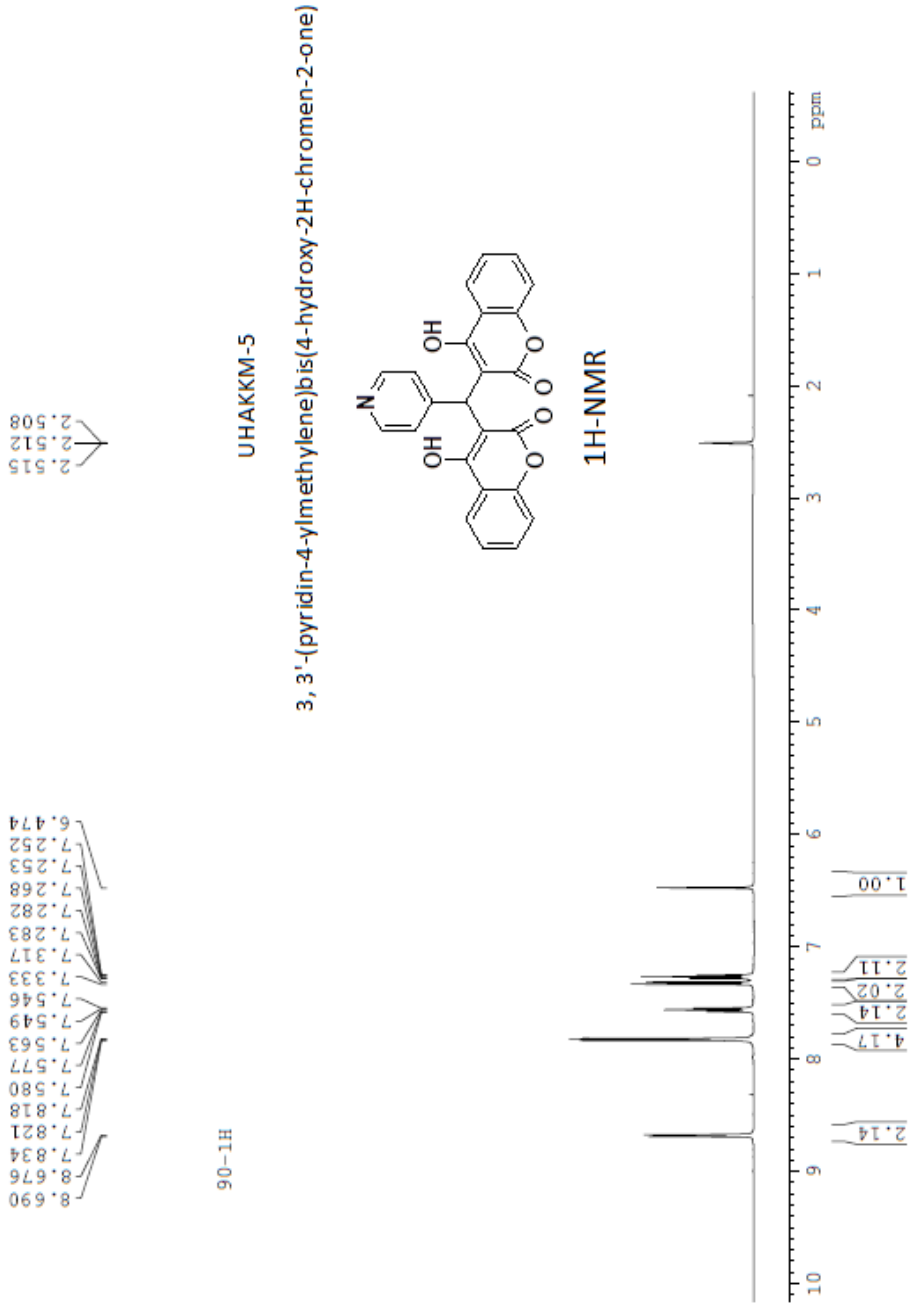


Figure-S21: The ^{1}H-NMR spectrum of UHAKKM-5

Figure-S22:

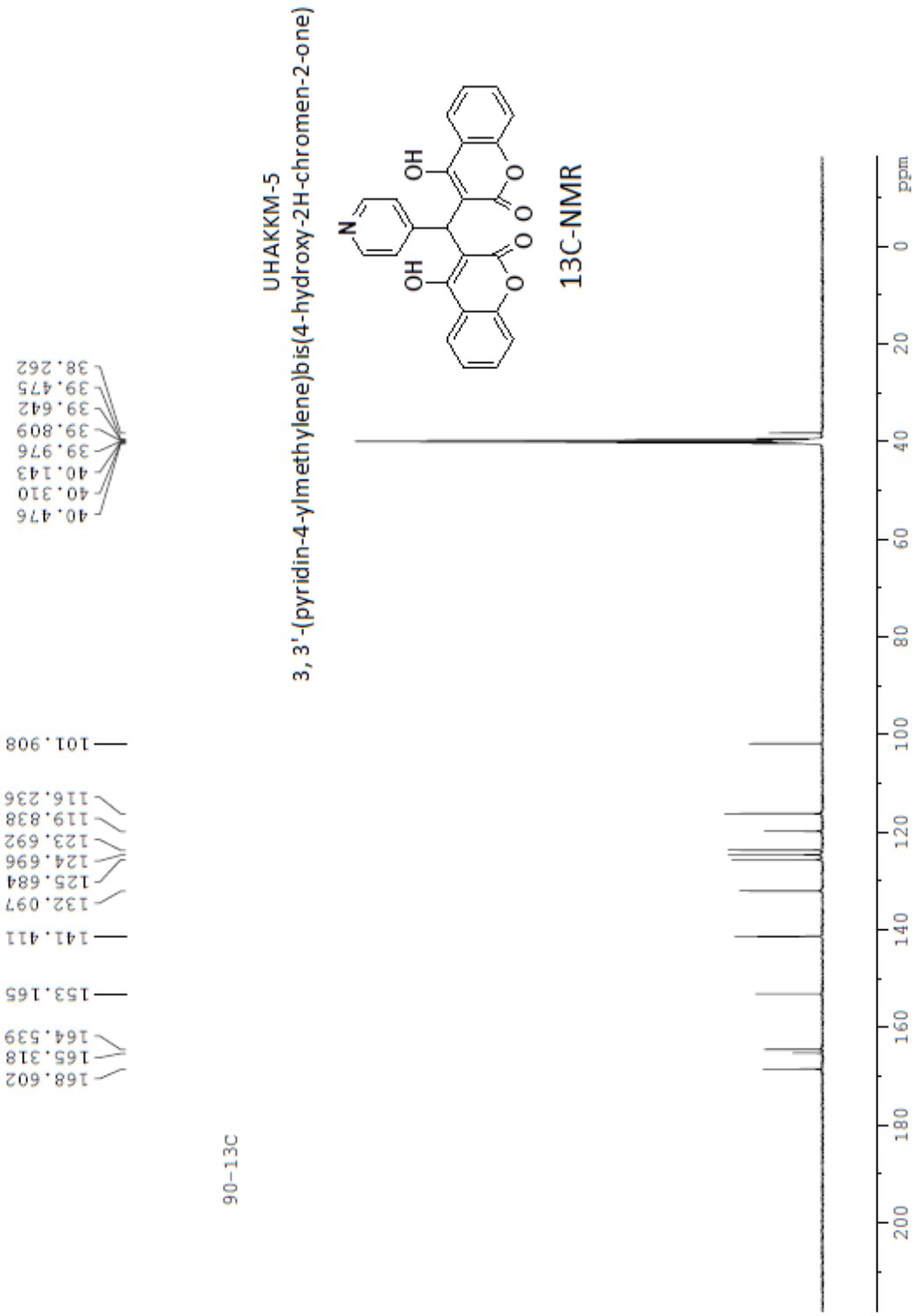


Figure-S22: The ^{13}C-NMR spectrum of UHAKKM-5

Figure-S23:

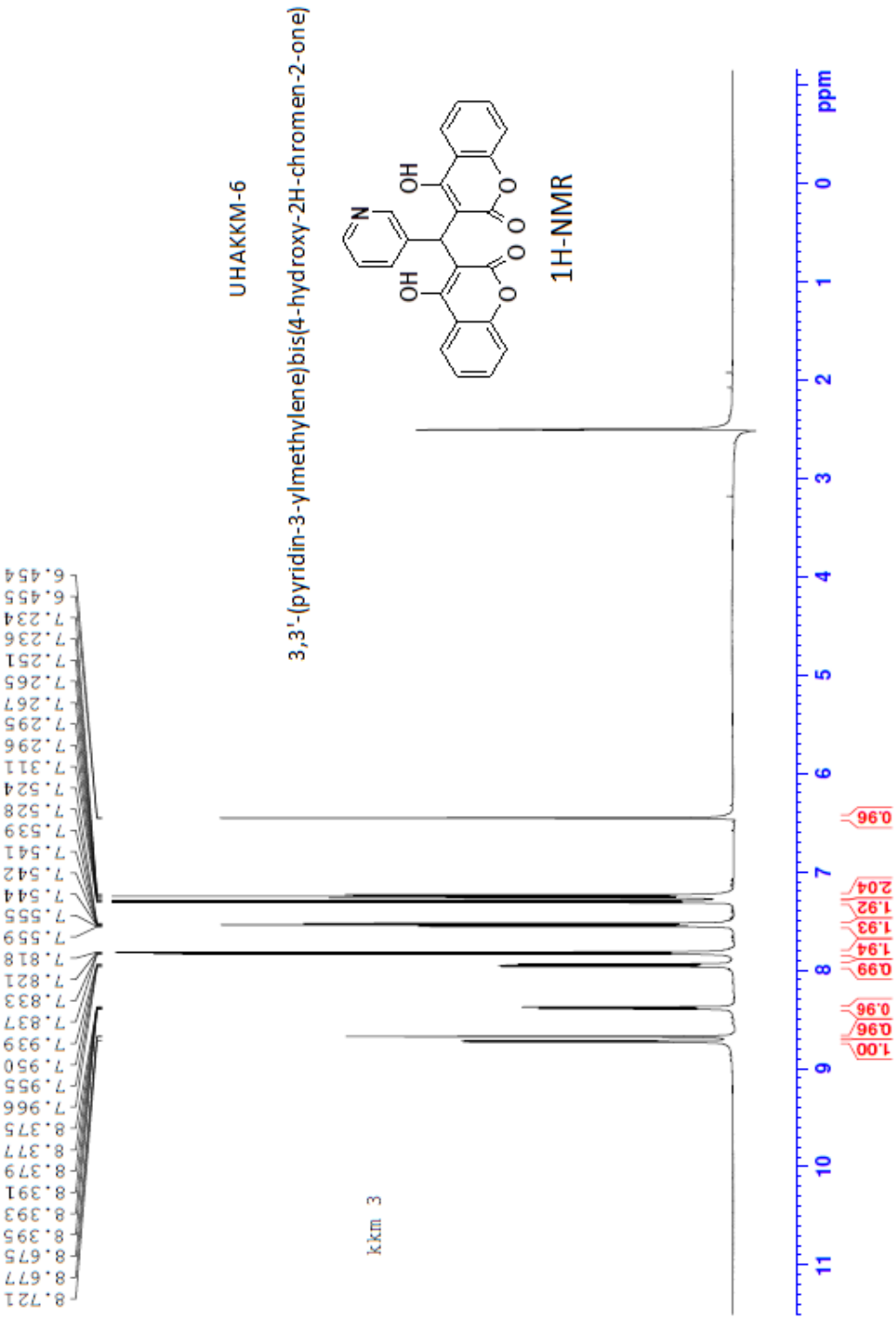


Figure-S23: The ^{1}H-NMR spectrum of UHAKKM-6

Figure-S24:

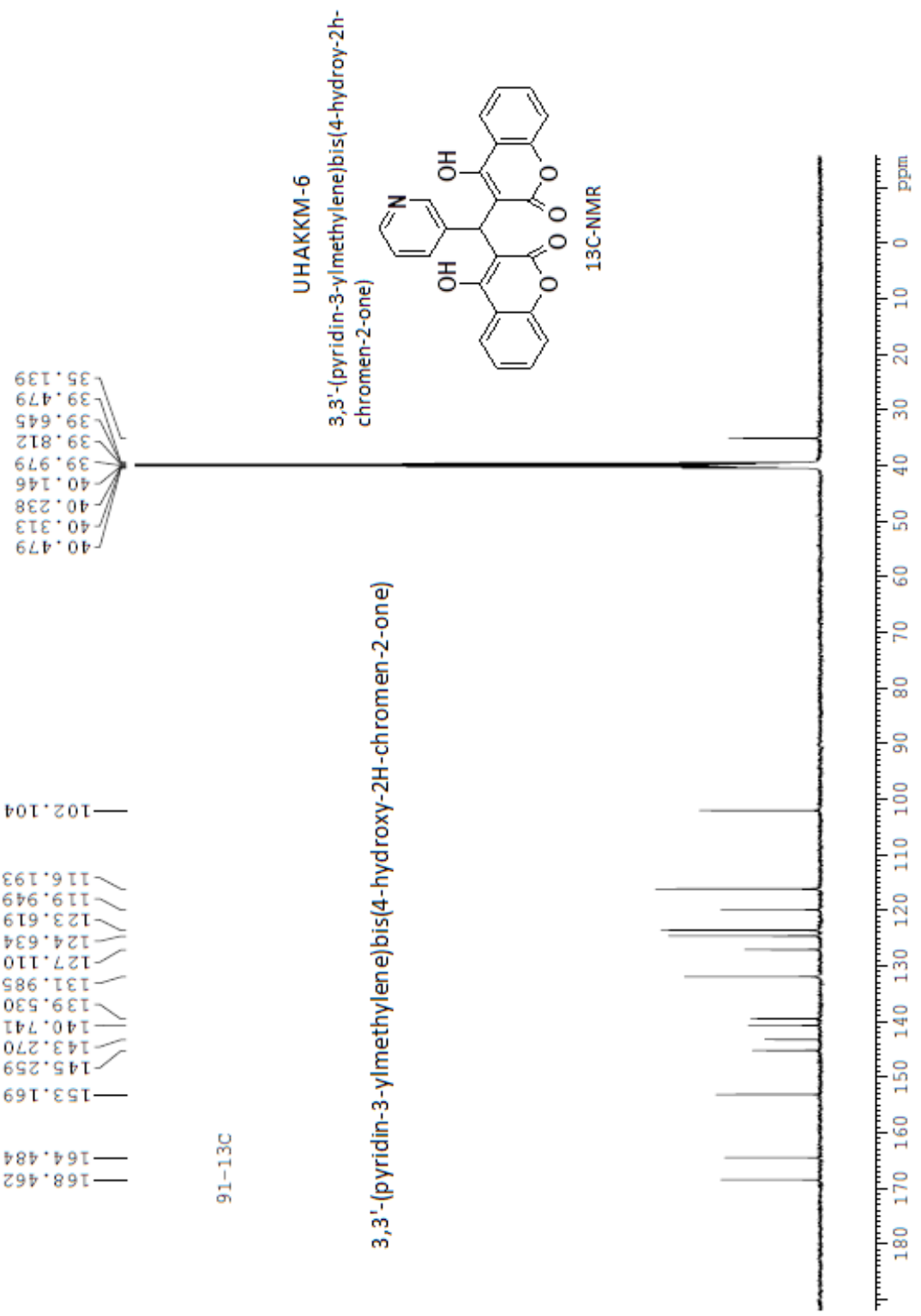


Figure-S24: The ^{13}C-NMR spectrum of UHAKKM-6

OBJECTIVE-2 SPECTRAL DATA

Figure-S25:

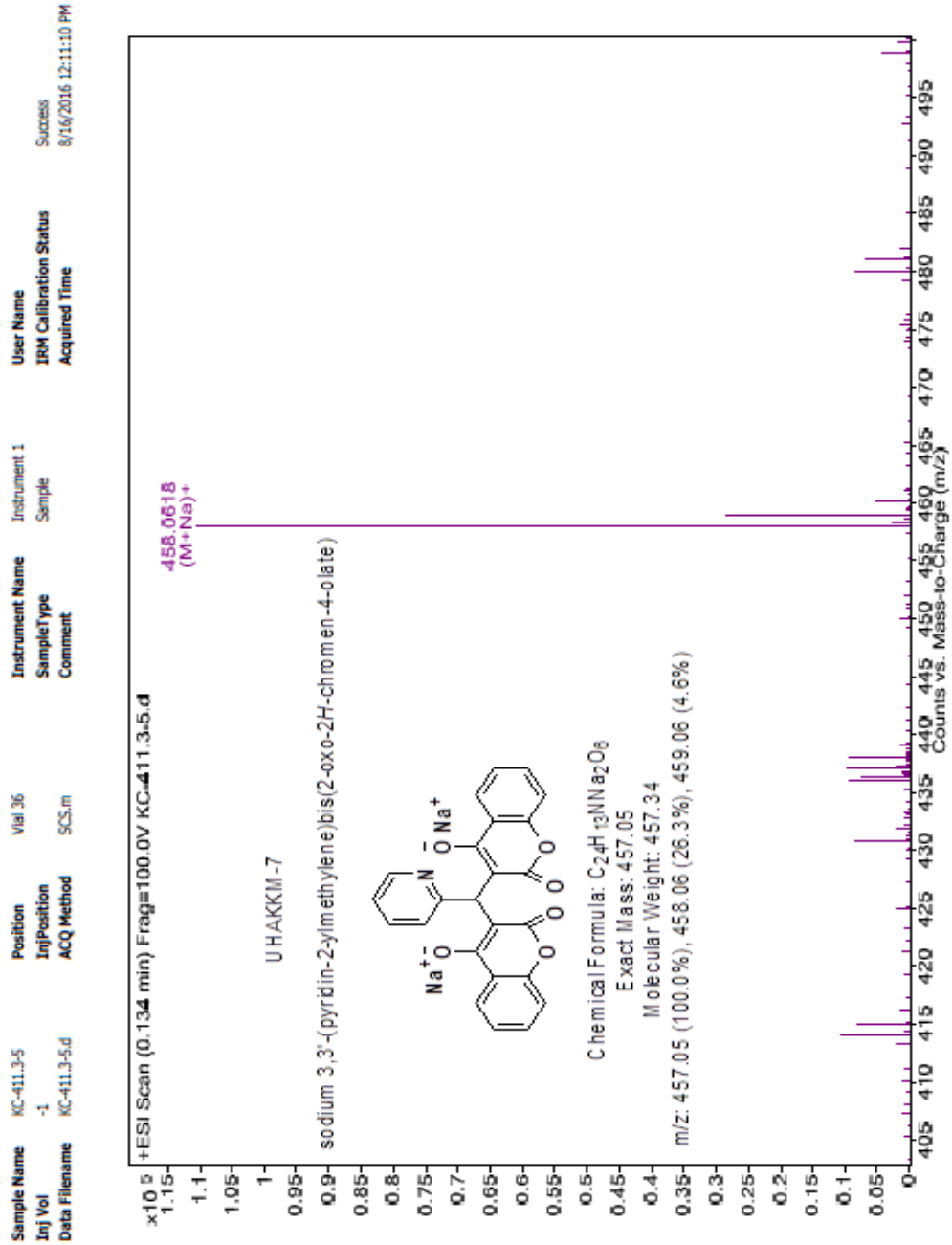


Figure-S25: The ESI-HRMS spectrum of UHAKKM-7

Figure-S26:

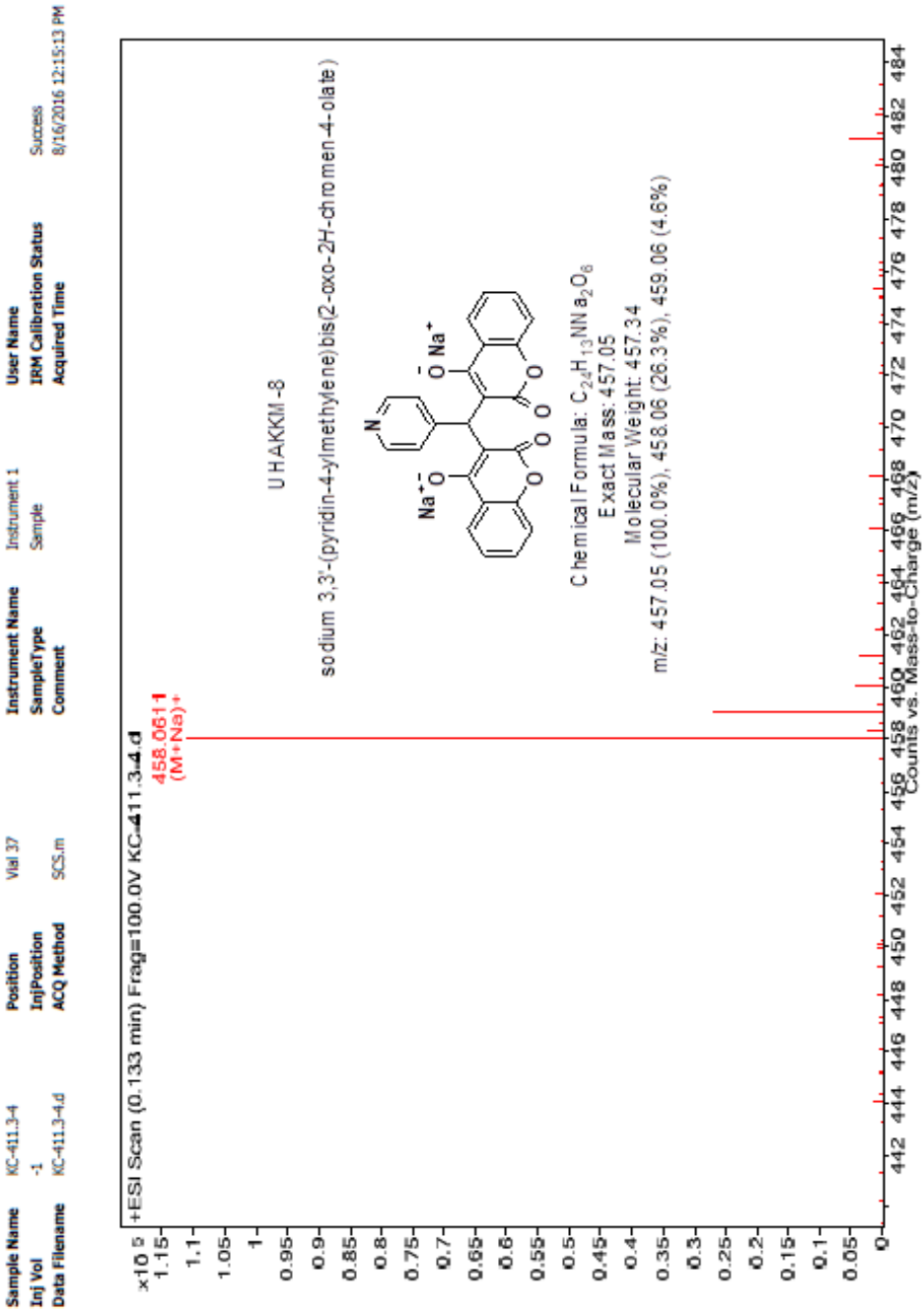


Figure-S26: The ESI-HRMS spectrum of UHAKKM-8

Figure-S27:

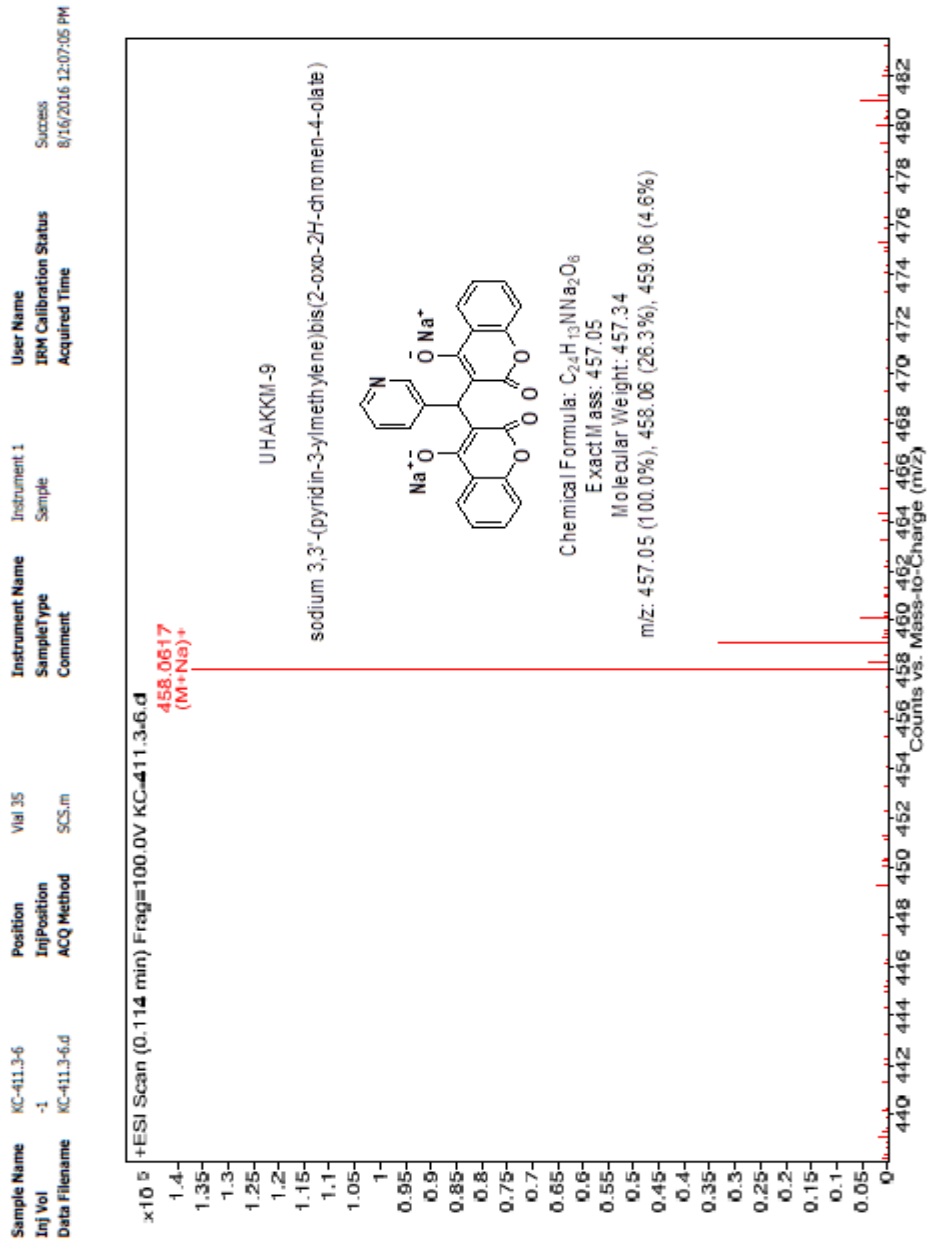


Figure-S27: The ESI-HRMS spectrum of UHAKKM-9

Figure-S28:

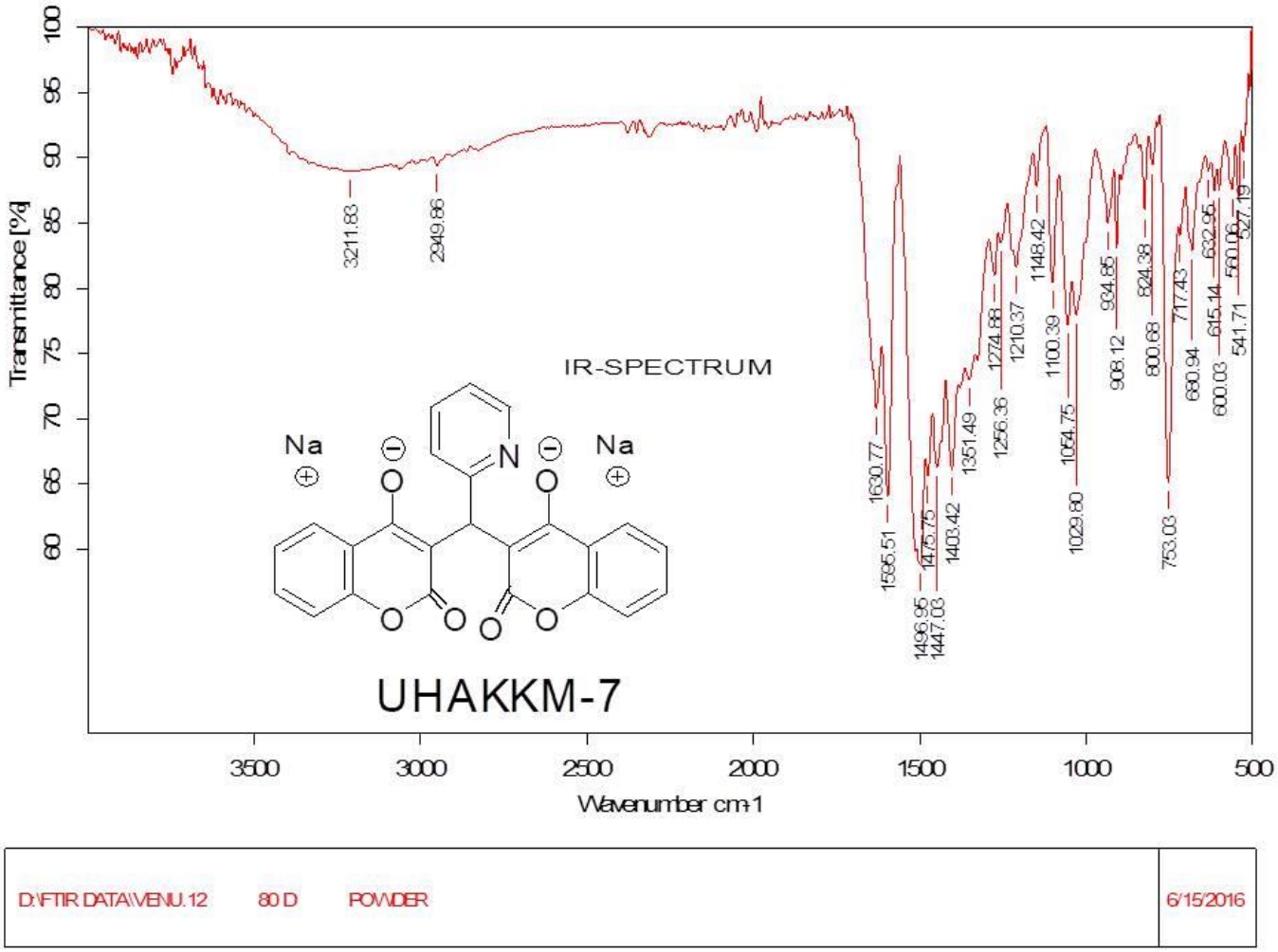


Figure-S28: The FTIR spectrum of UHAKKM-7

Figure-S29:

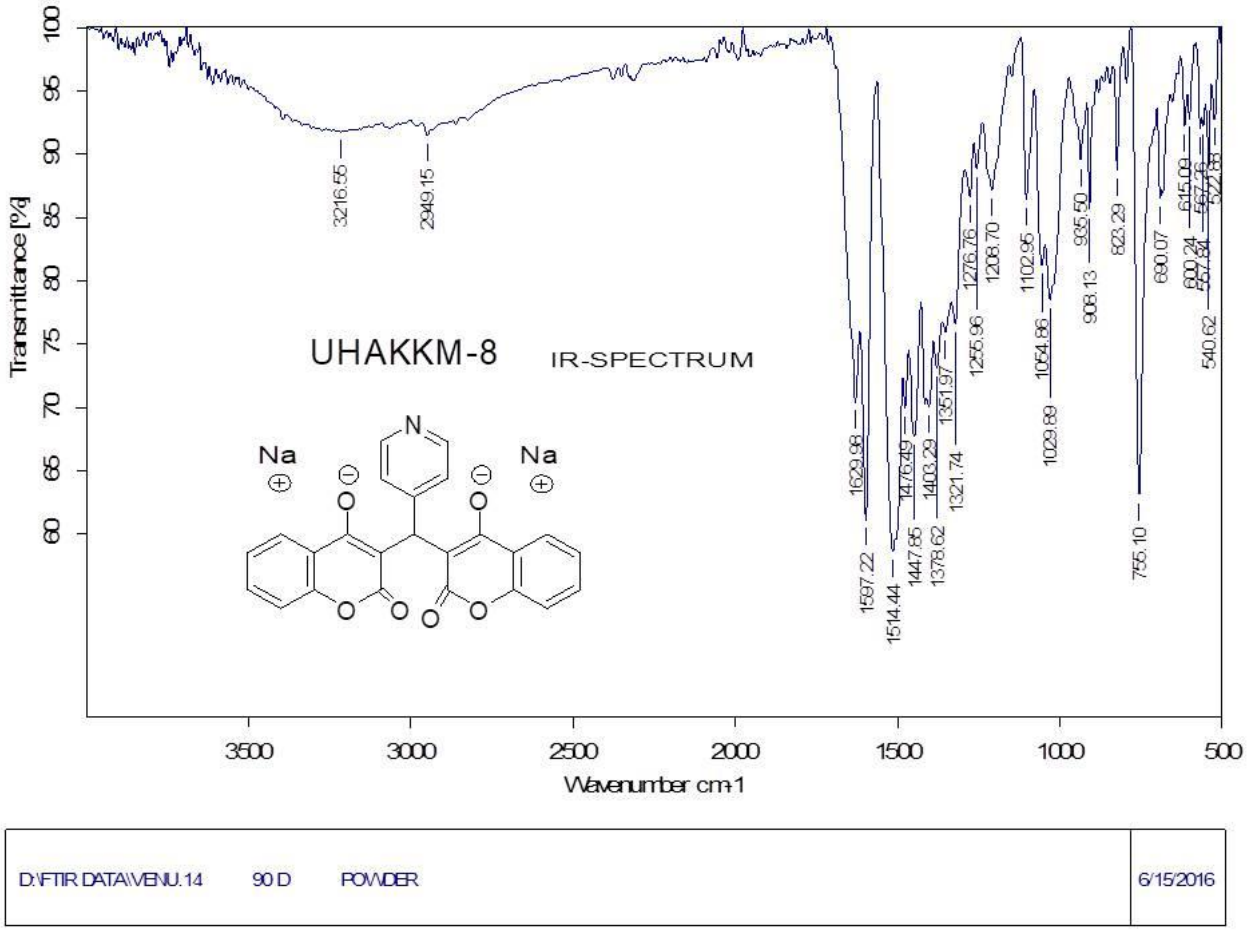


Figure-S29: The FTIR spectrum of UHAKKM-8

Figure-S30:

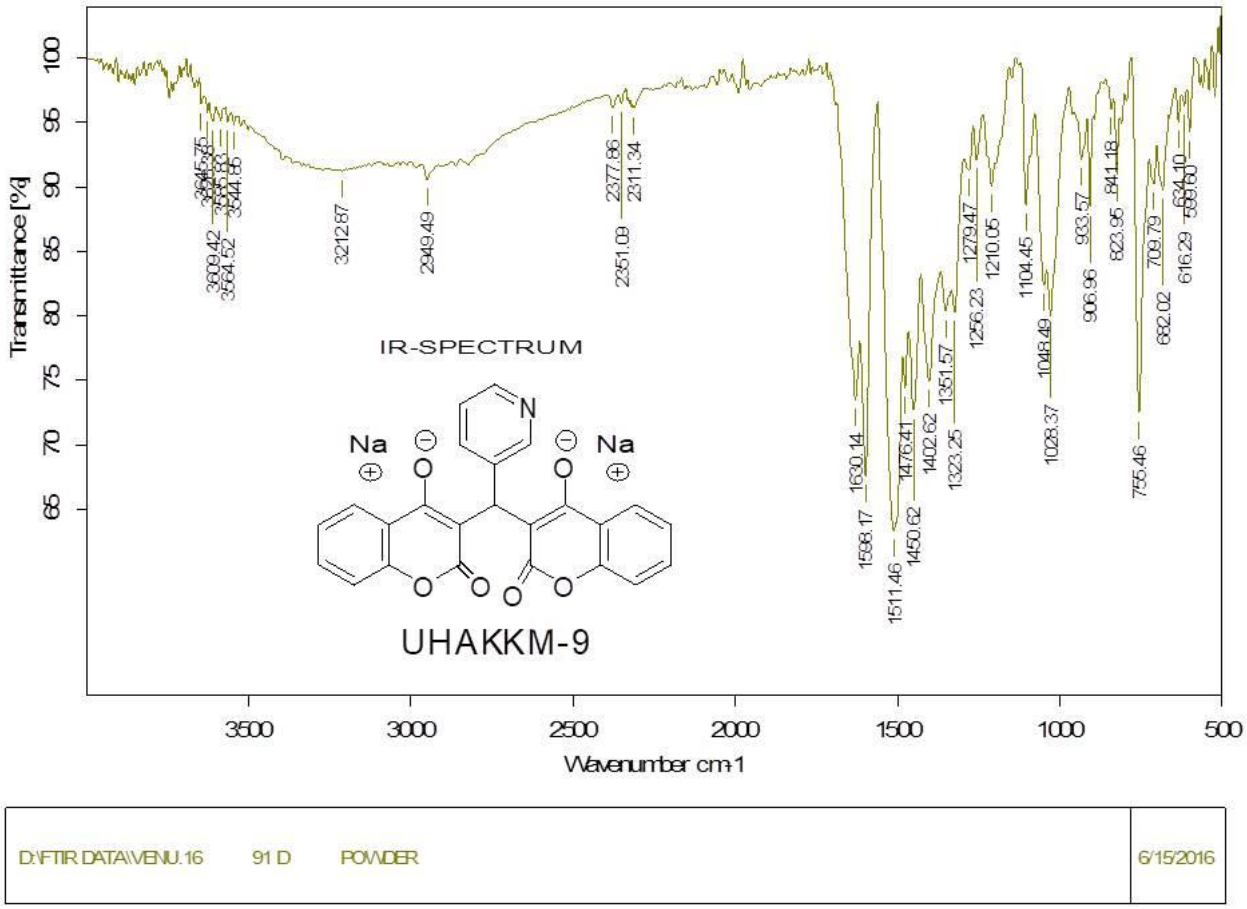


Figure-S30: The FTIR spectrum of UHAKKM-9

OBJECTIVE-3 SPECTRAL DATA

Figure-S31:

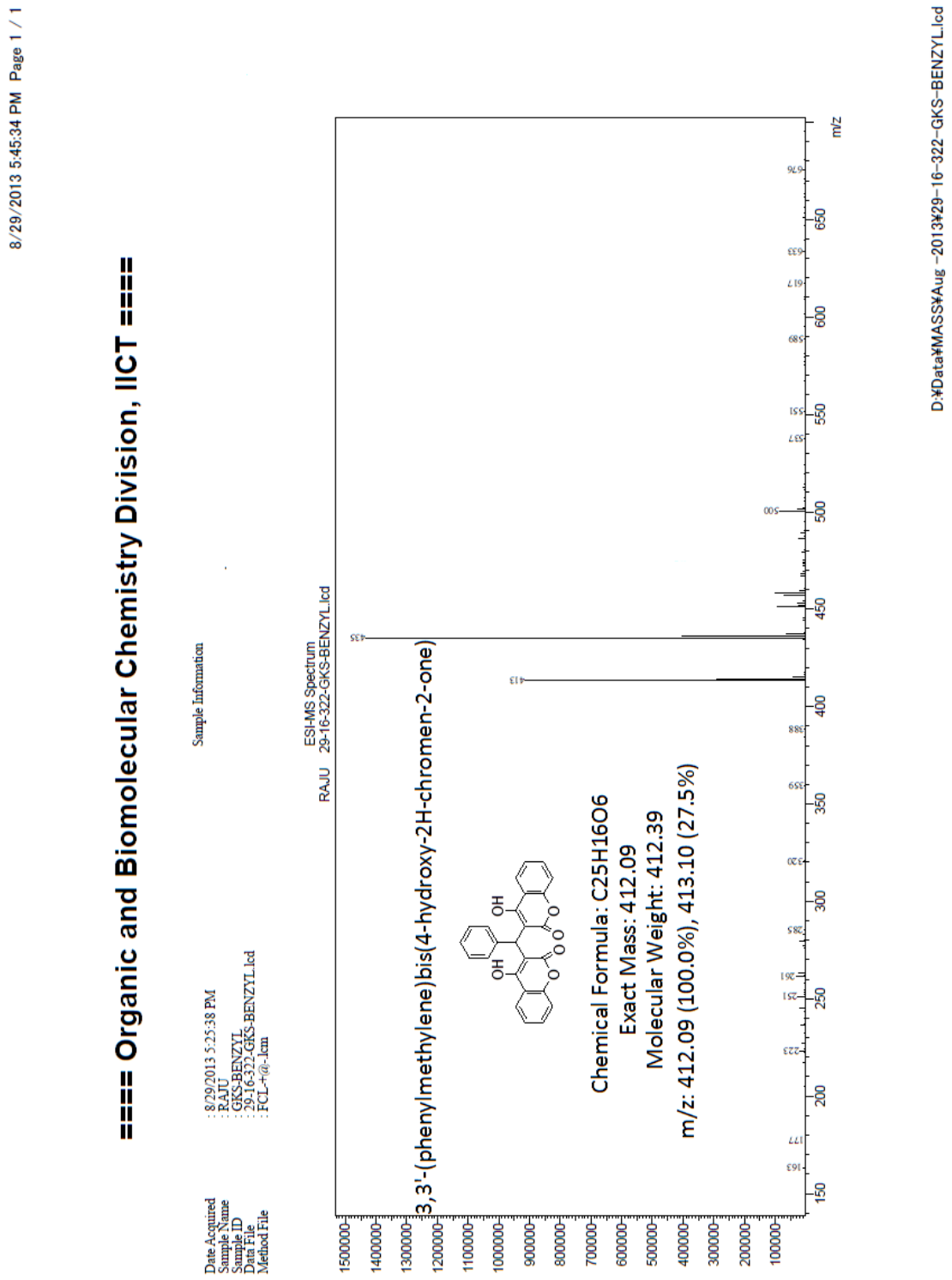


Figure-S31: The ESI- LCMS spectrum of UHAKKM-A

Figure-S32:

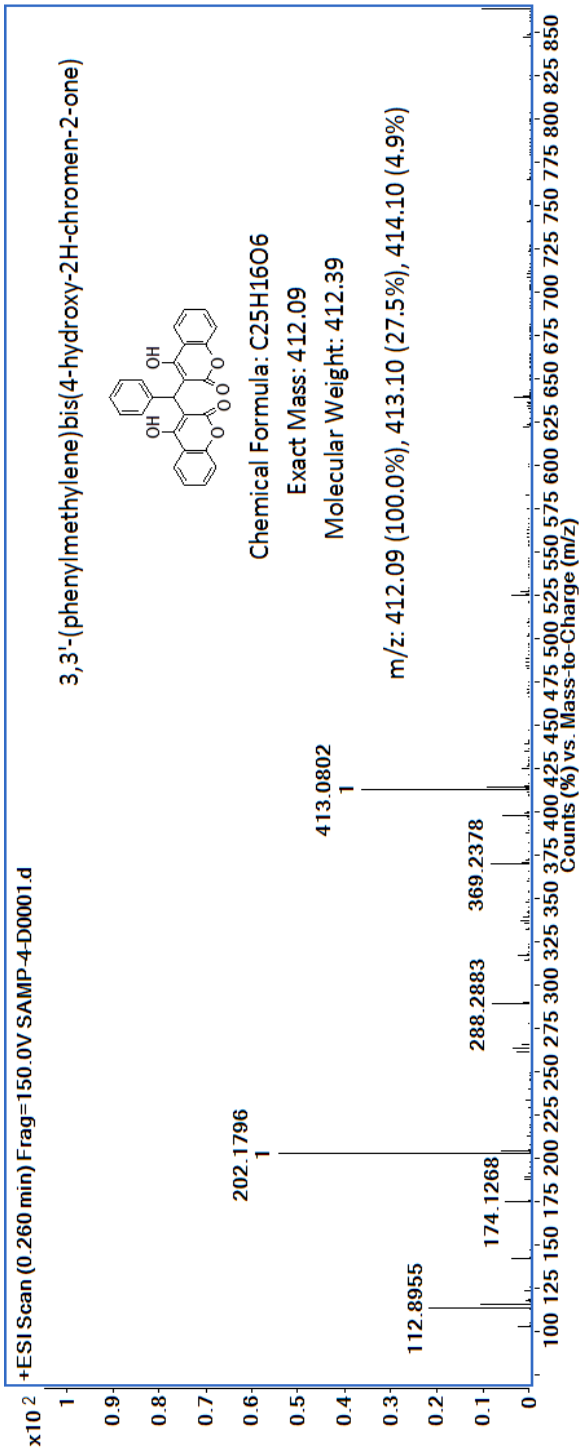


Figure-S32: The ESI- HRMS spectrum of UHAKKM-A

Figure-S33:

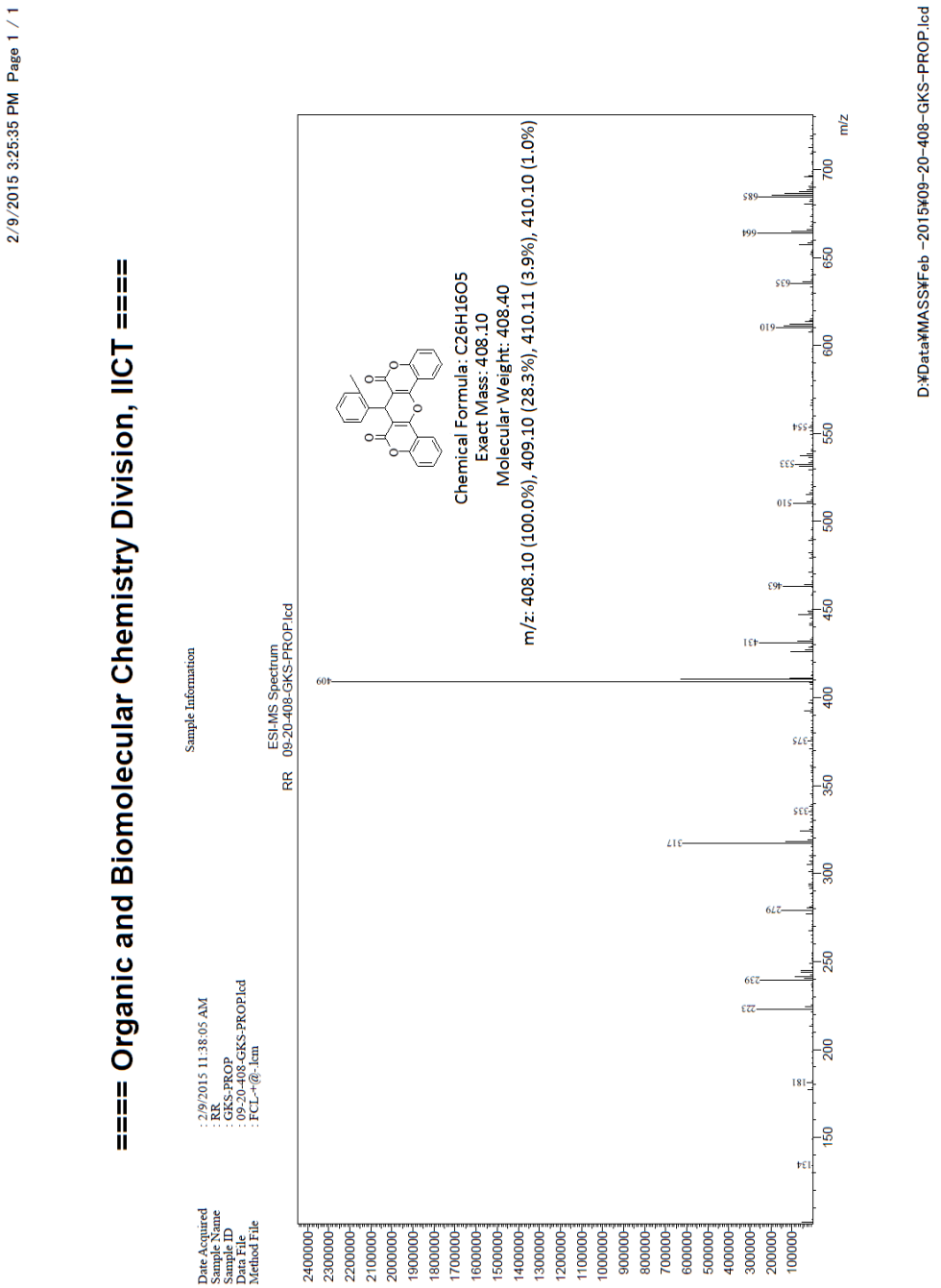


Figure-S33: The ESI- LCMS spectrum of UHAKKM-W1

Figure-S34:

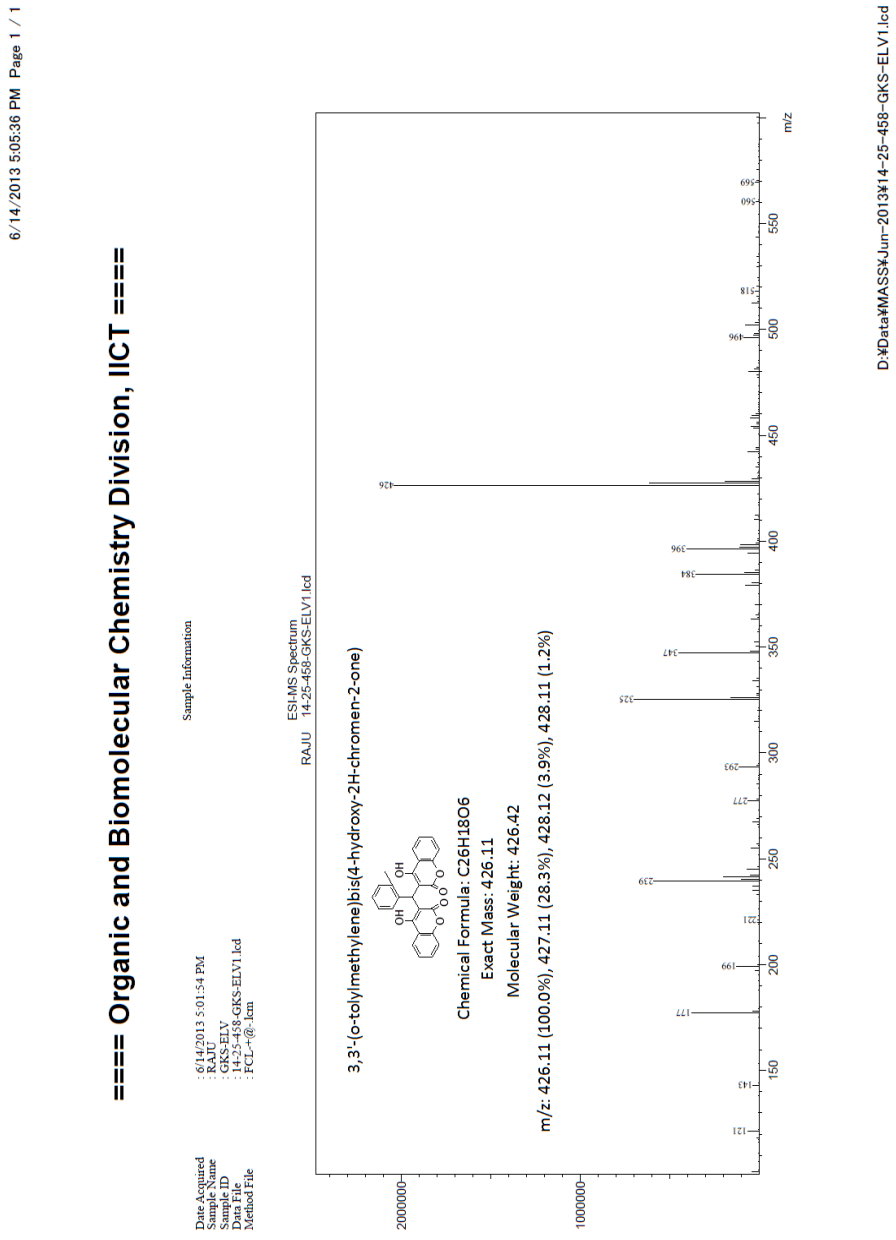


Figure-S34: The ESI- LCMS spectrum of UHAKKM-W

Figure-S35:

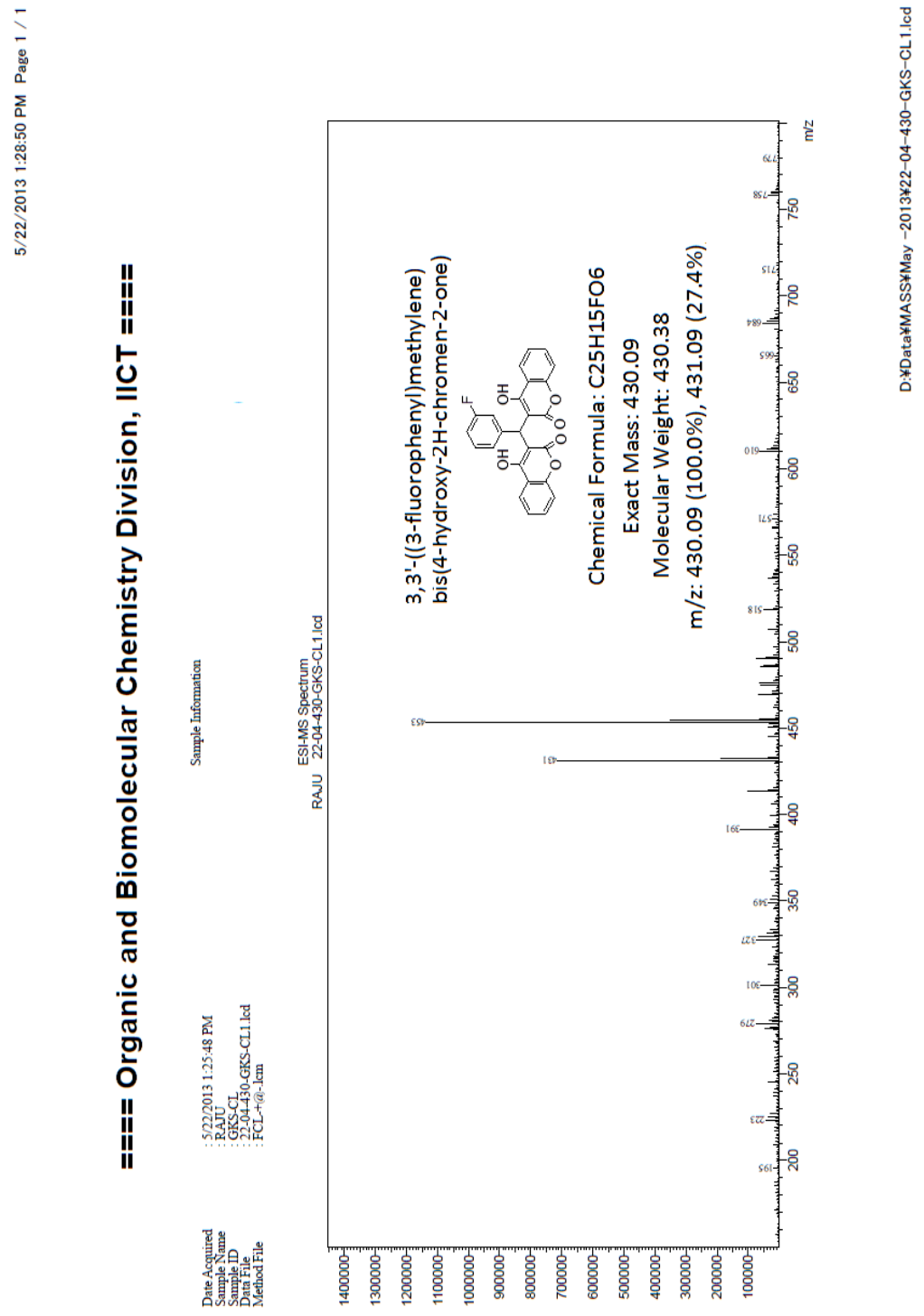


Figure-S35: The ESI- LCMS spectrum of UHAKKM-S

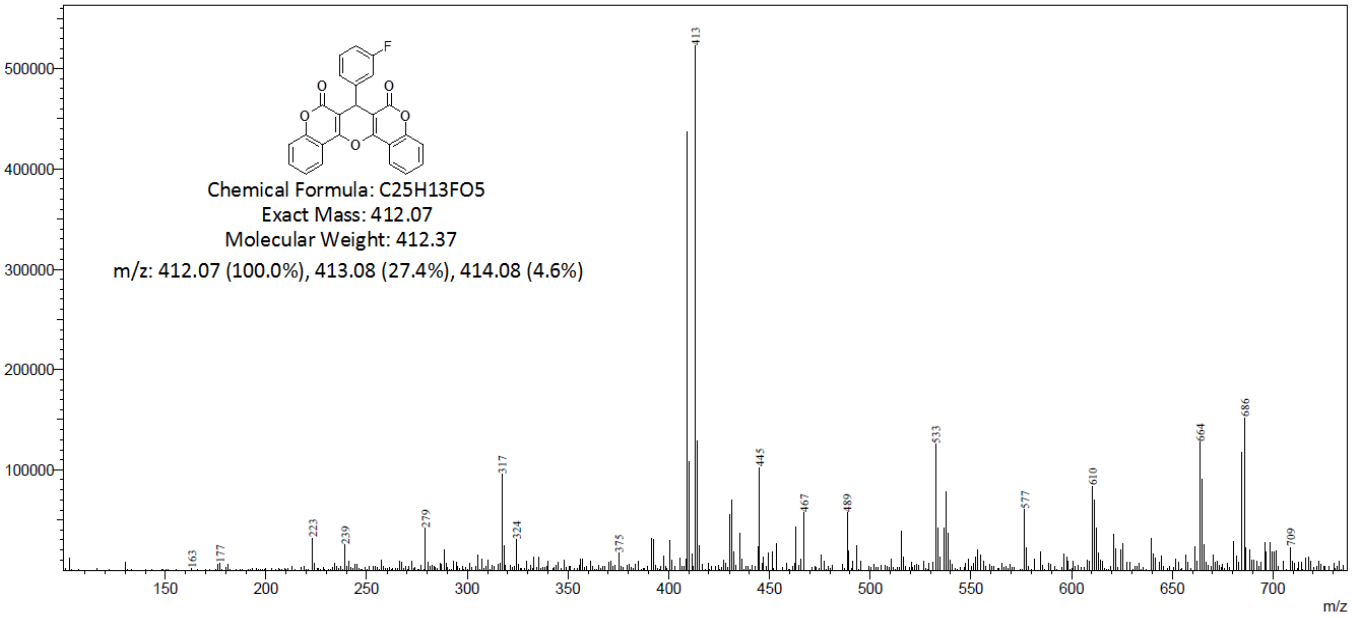
Figure-S36:

==== Organic and Biomolecular Chemistry Division, IICT ====

Sample Information

Date Acquired : 2/9/2015 11:40:52 AM
Sample Name : RR
Sample ID : GKS-BUT
Data File : 09-21-412-GKS-BUT.lcd
Method File : FCL-+@-.lcm

ESI-MS Spectrum
RR 09-21-412-GKS-BUT.lcd



D:¥Data¥MASS¥Feb -2015¥09-21-412-GKS-BUT.lcd

Figure-S36: The ESI- LCMS spectrum of UHAKKM-S1

Figure-S37:

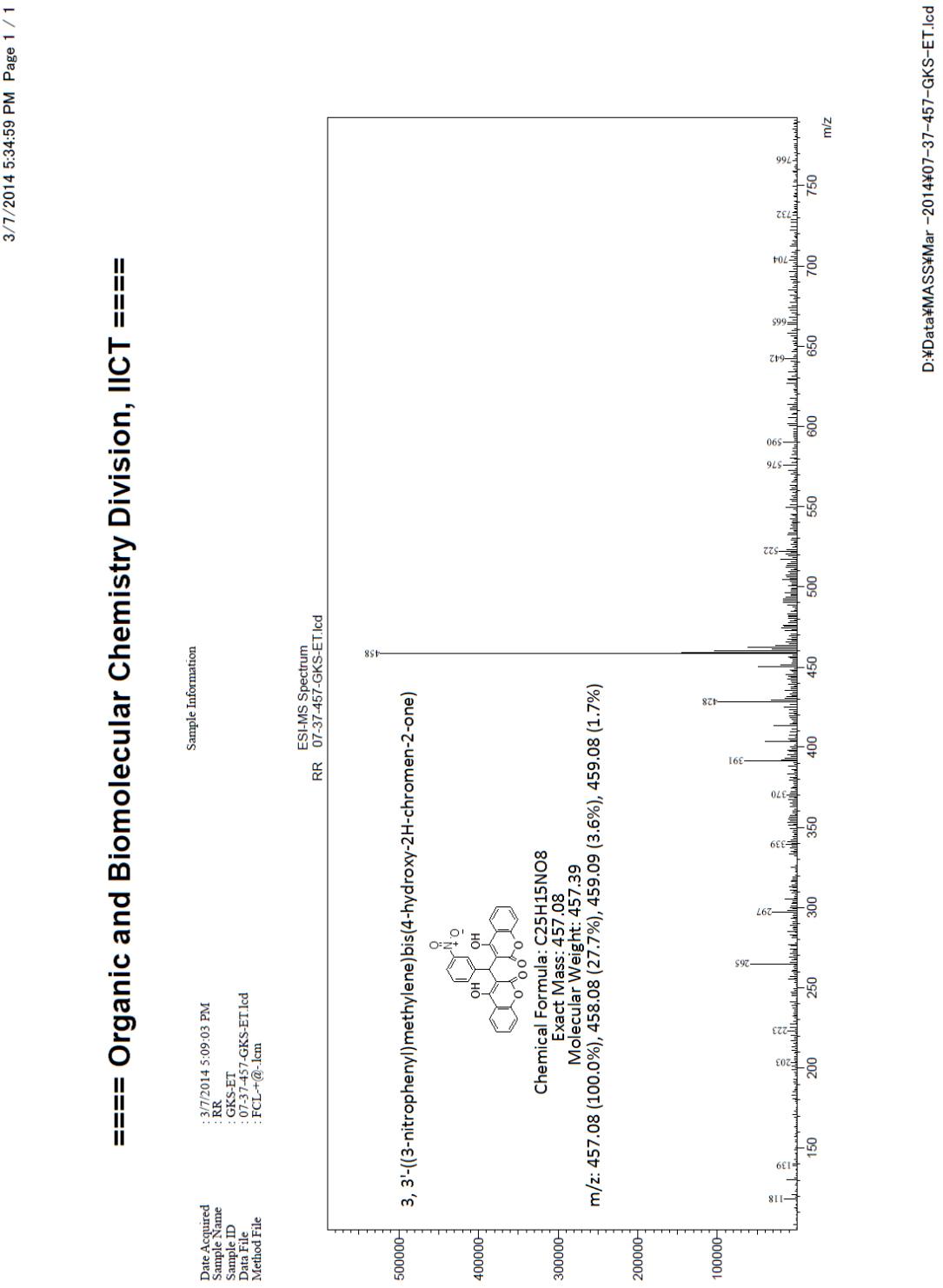


Figure-S37: The ESI- LCMS spectrum of UHAKKM-R

Figure-S38:

Figure-S38: The ESI- LCMS spectrum of UHAKKM-Q

Figure-S39:

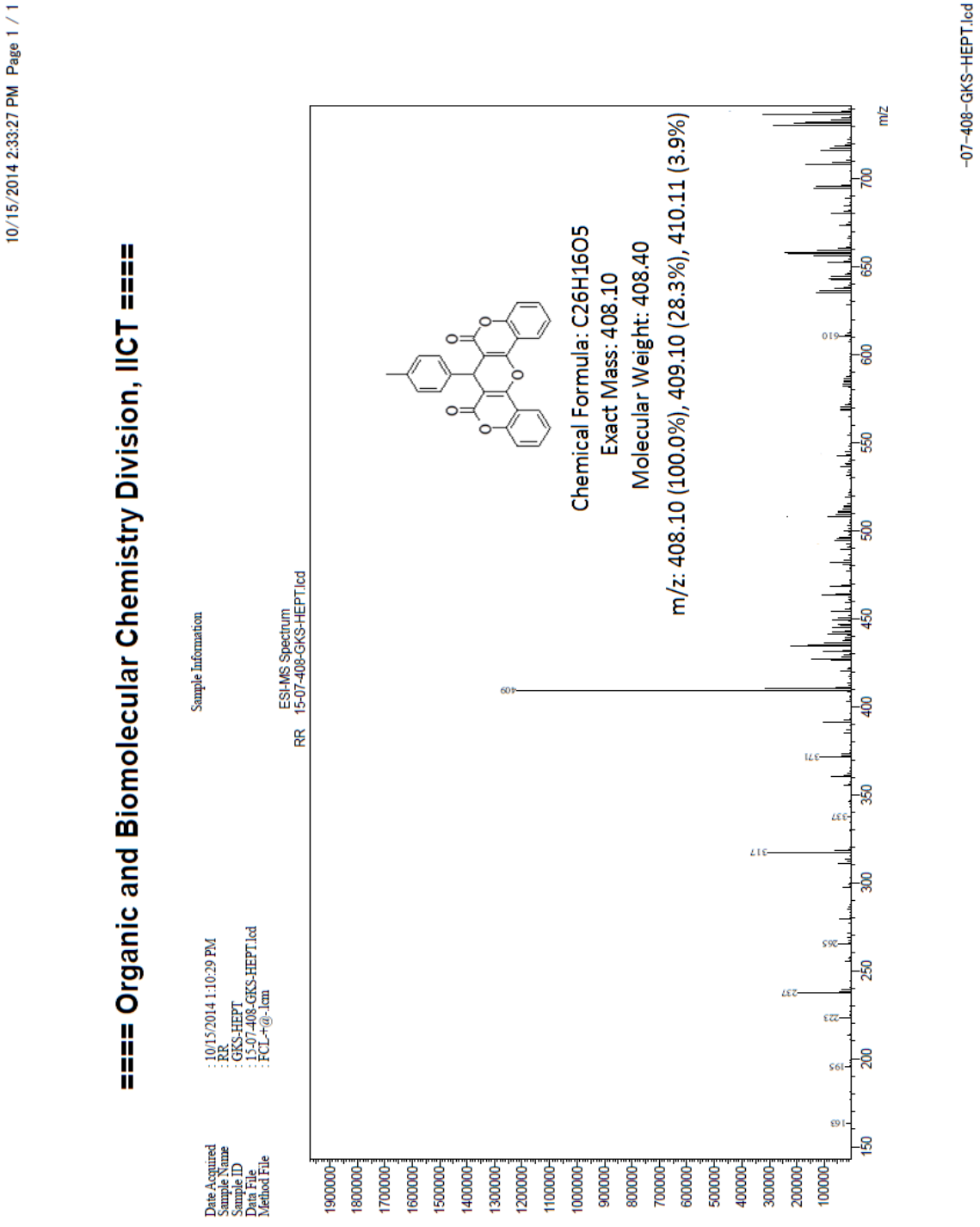


Figure-S39: The ESI- LCMS spectrum of UHAKKM-T1

Figure-S40:

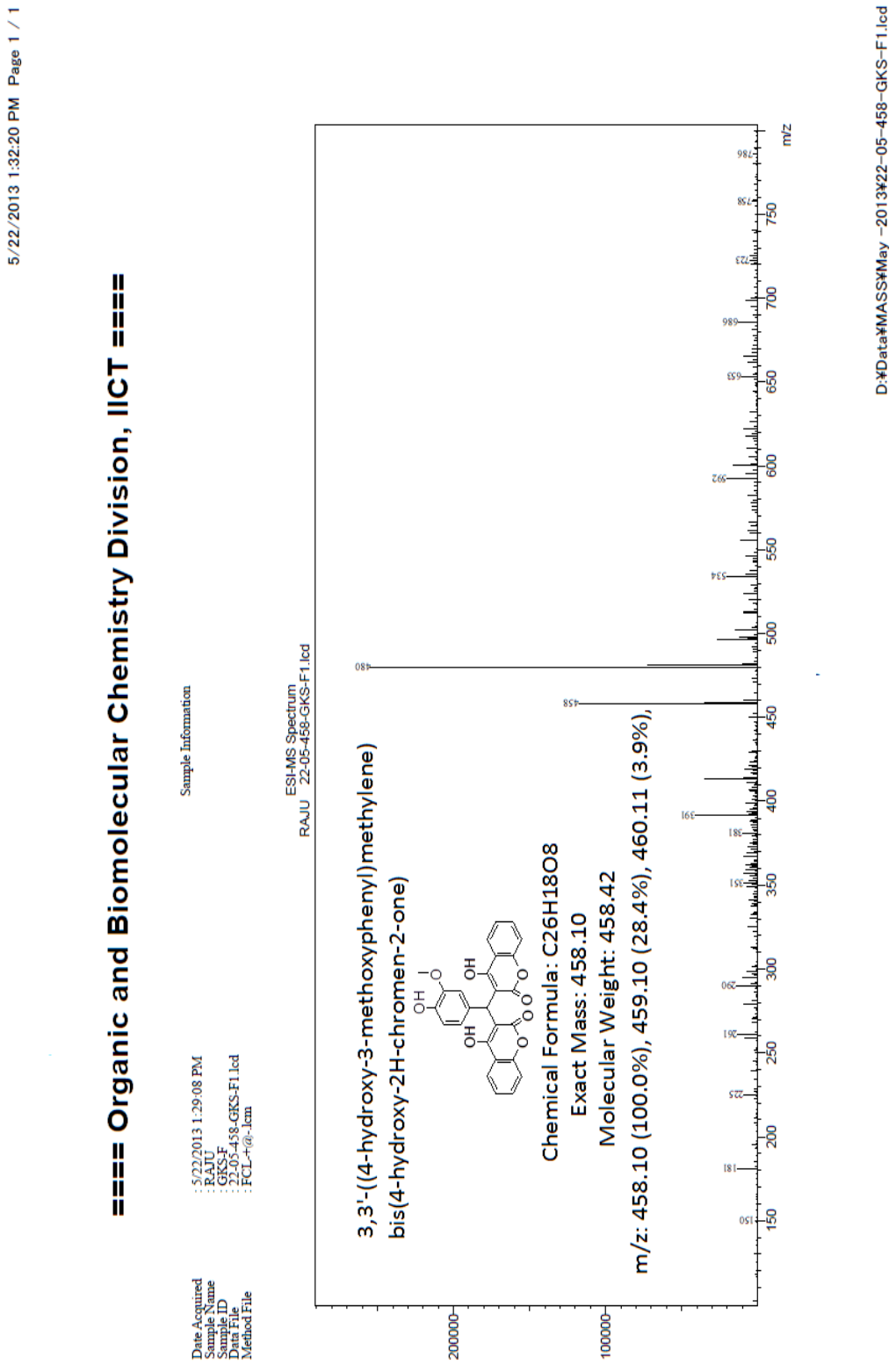


Figure-S40: The ESI- LCMS spectrum of UHAKKM-V

Figure-S41:

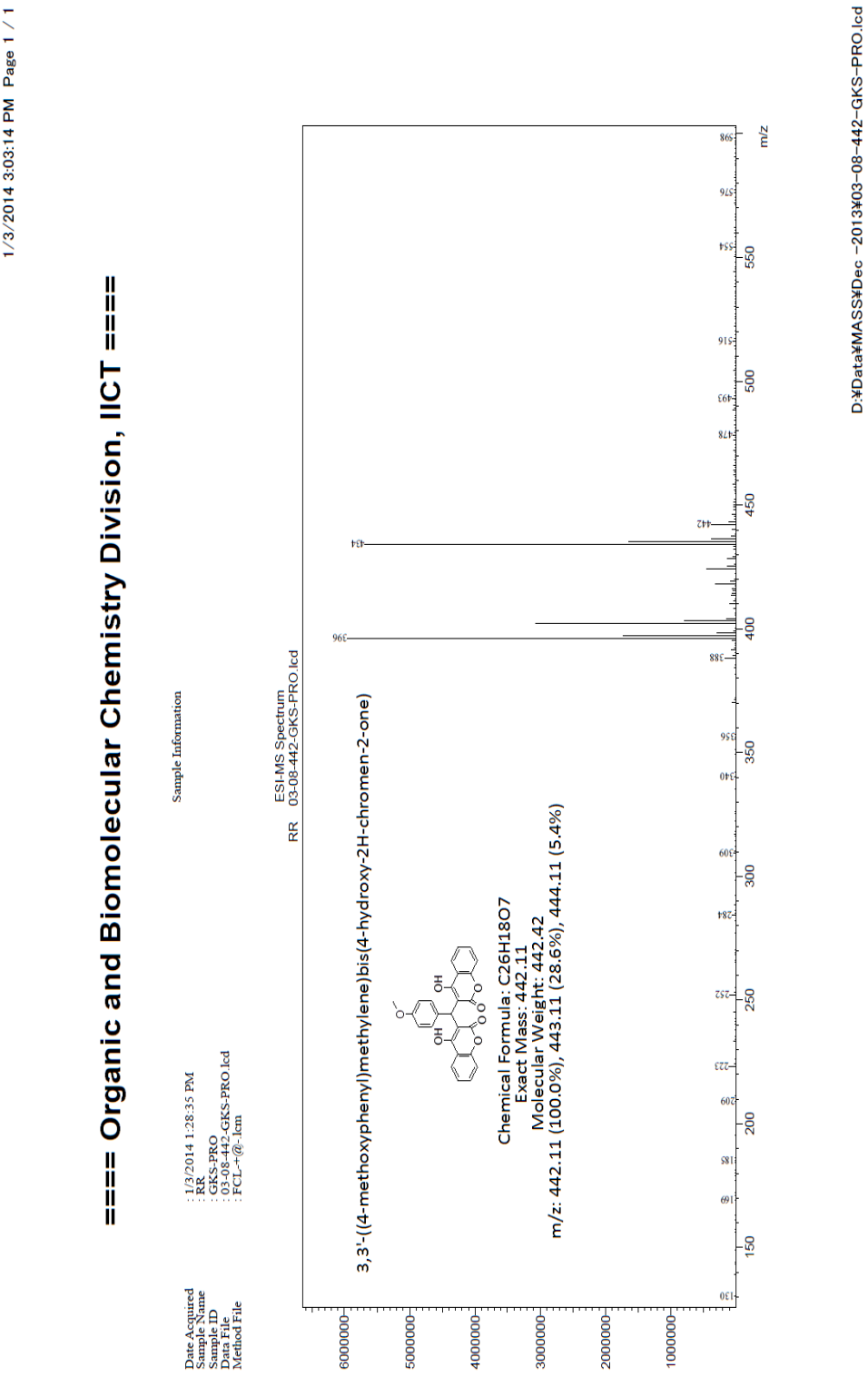


Figure-S41: The ESI- LCMS spectrum of UHAKKM-B

Figure-S42:

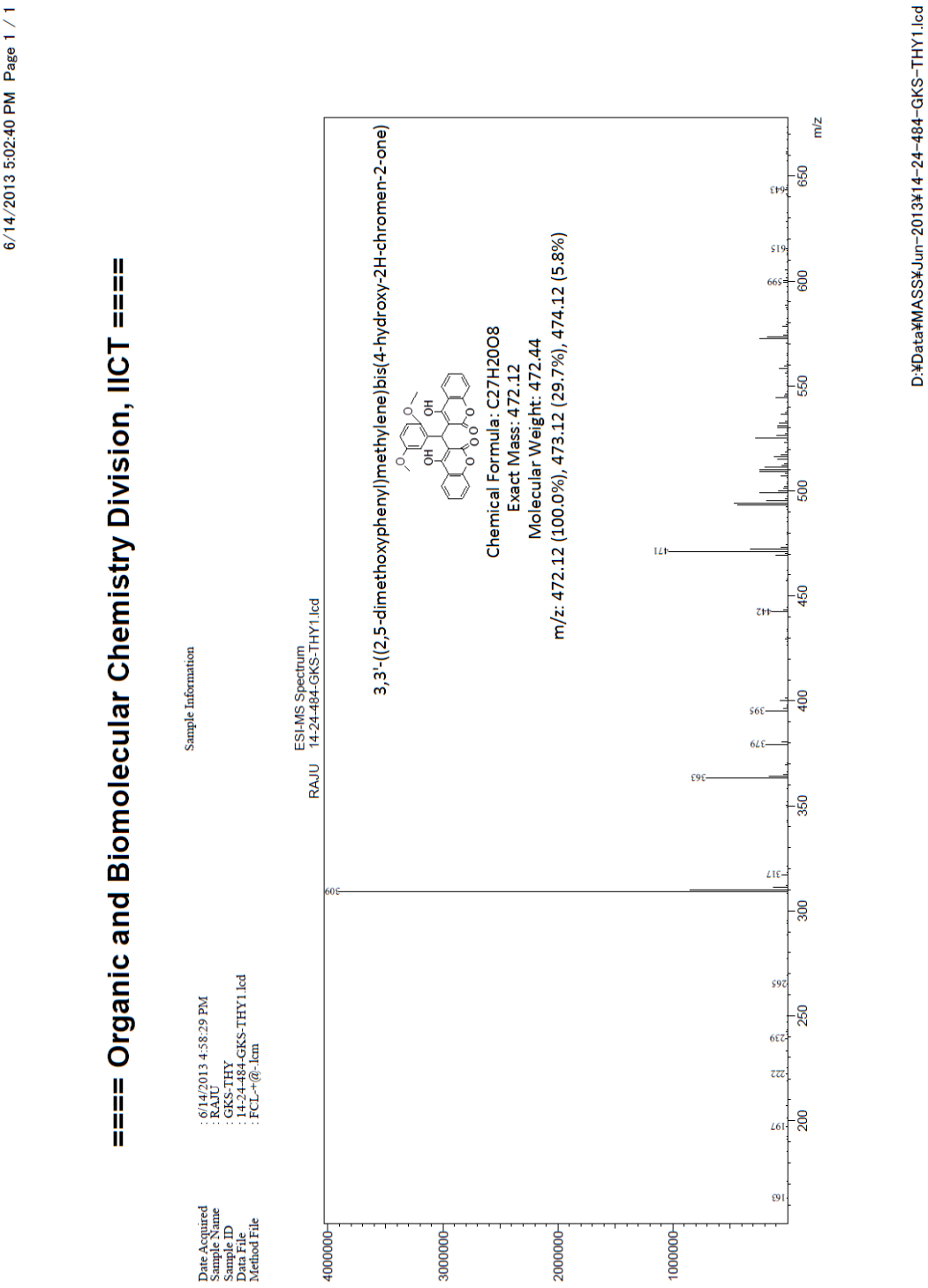


Figure-S42: The ESI- LCMS spectrum of UHAKKM-H

Figure-S43:

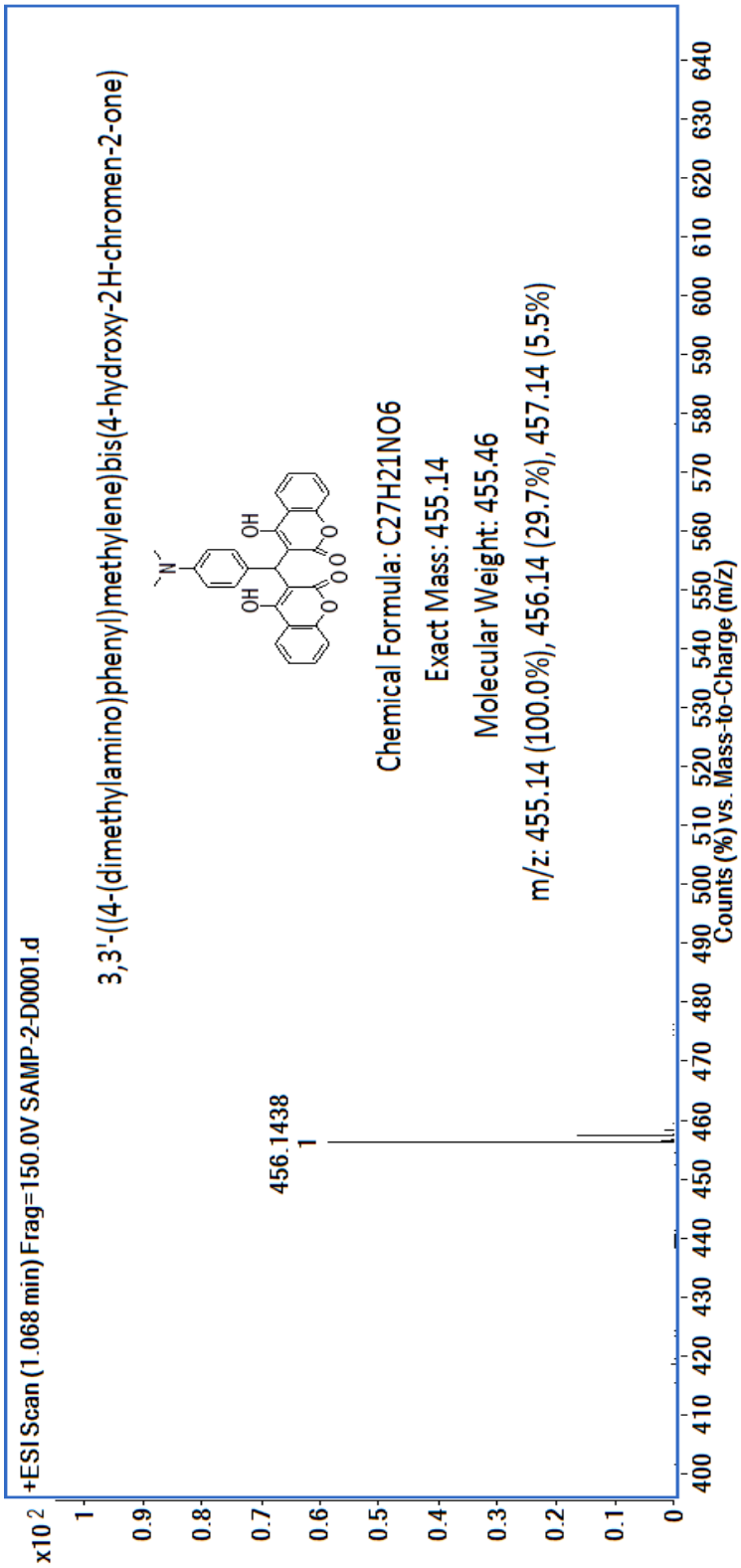


Figure-S43: The ESI- HRMS spectrum of UHAKKM-I

Figure-S44:

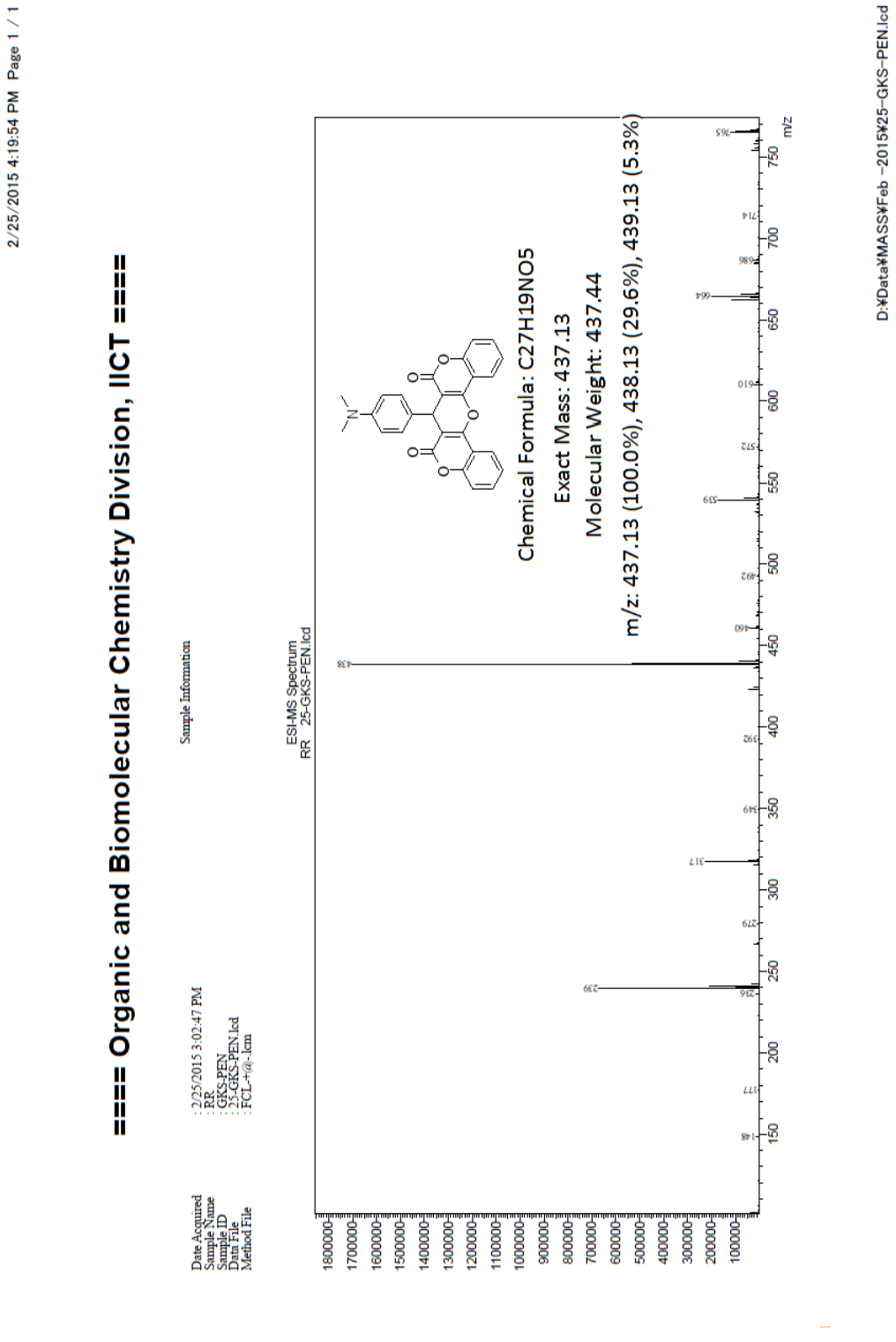


Figure-S44: The ESI- LCMS spectrum of UHAKKM-I1

Figure-S45:

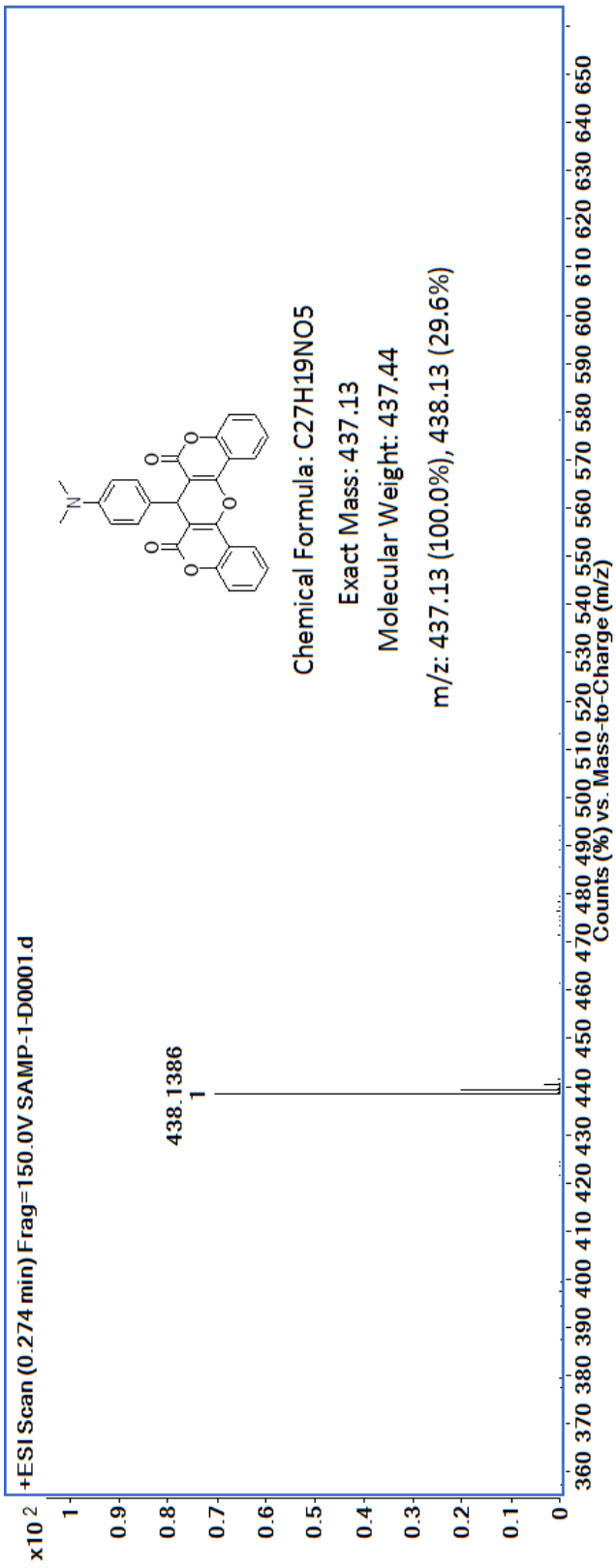


Figure-S45: The ESI- HRMS spectrum of UHAKKM-I1

Figure-S46:

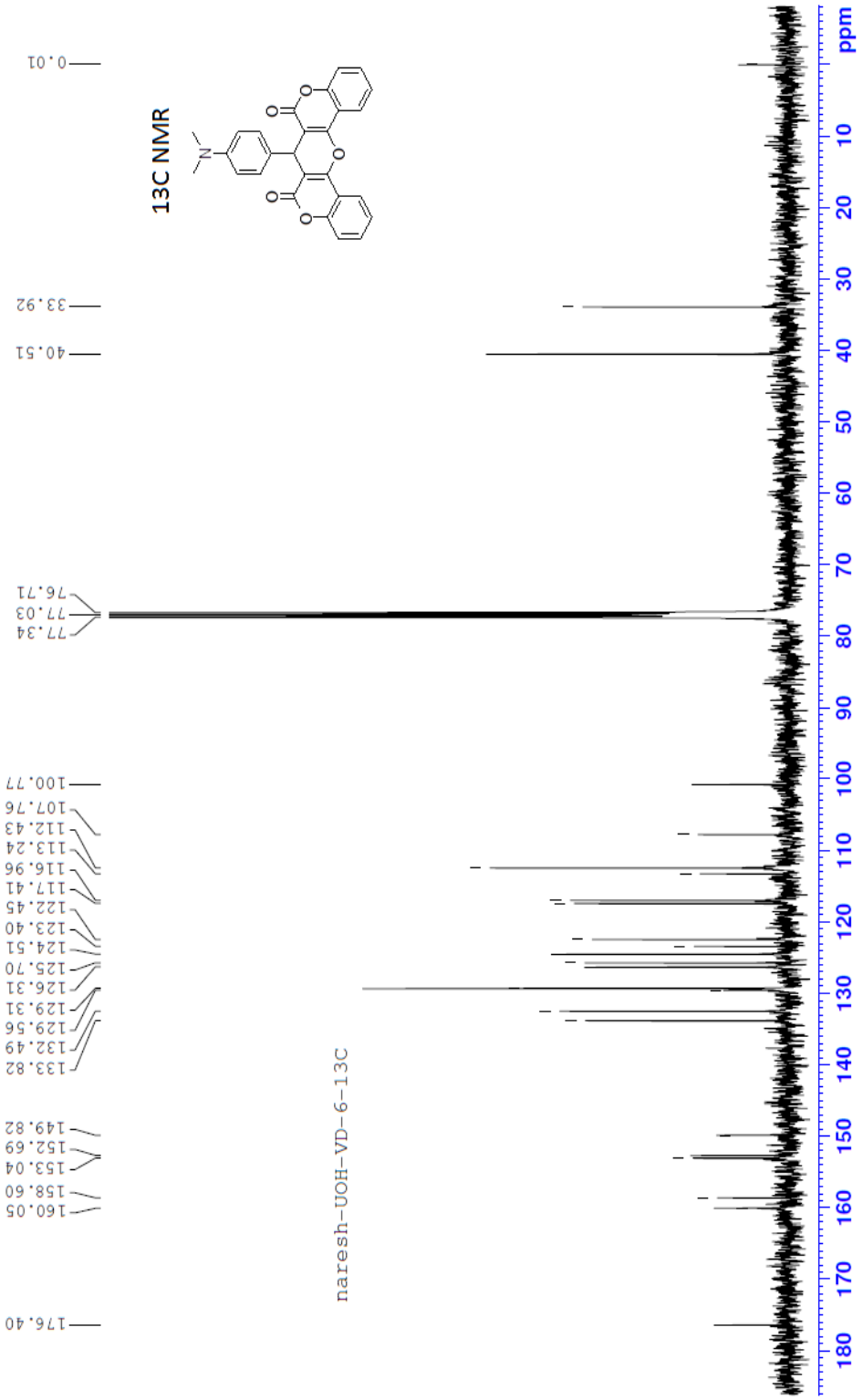


Figure-S46: The [13] C-NMR spectrum of UHAKKM-I1

Figure-S47:

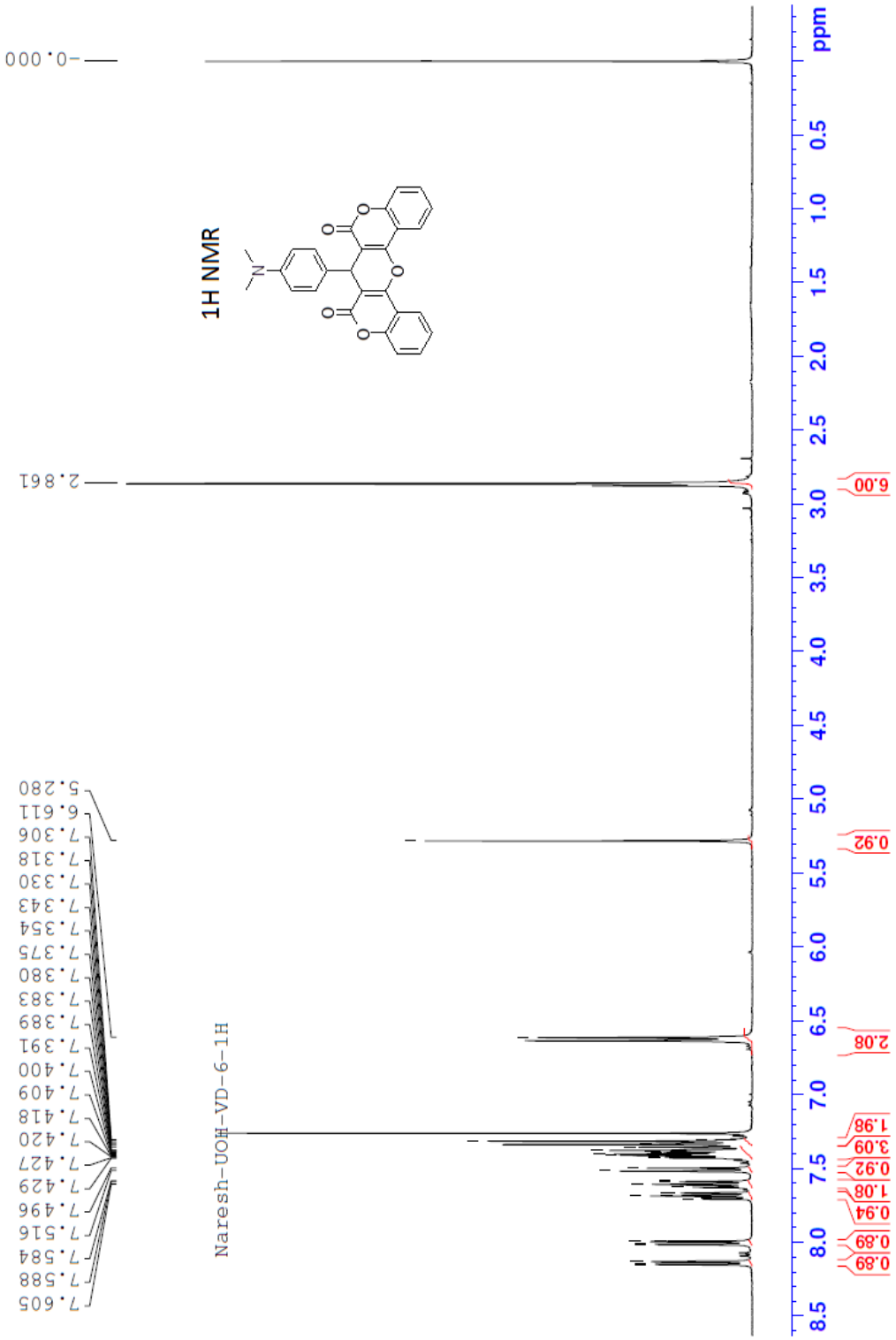


Figure-S47: The ^{1}H-NMR spectrum of UHAKKM-I1

Figure-S48:

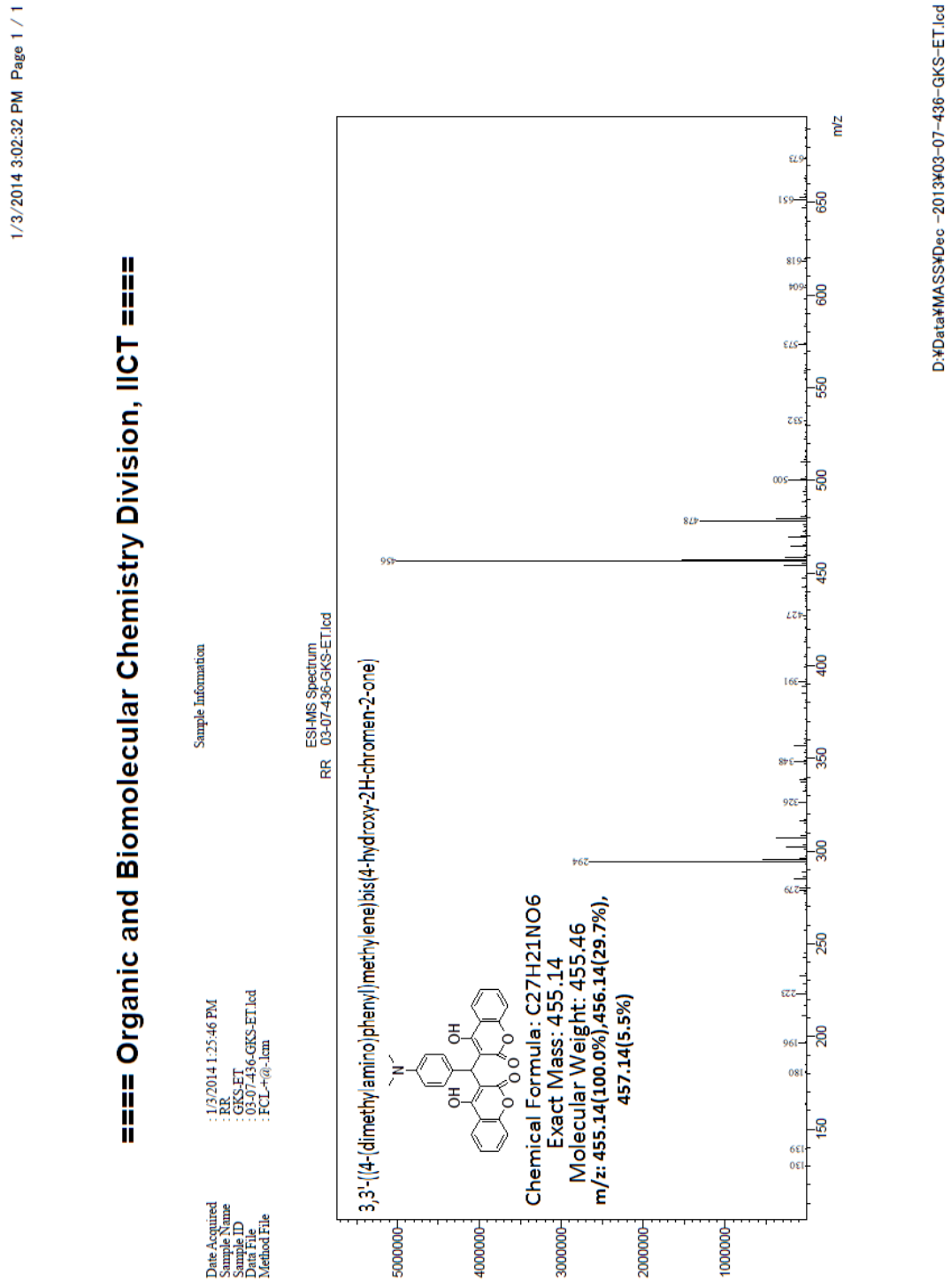


Figure-S48: The ESI- LCMS spectrum of UHAKKM-I

Figure-S49:

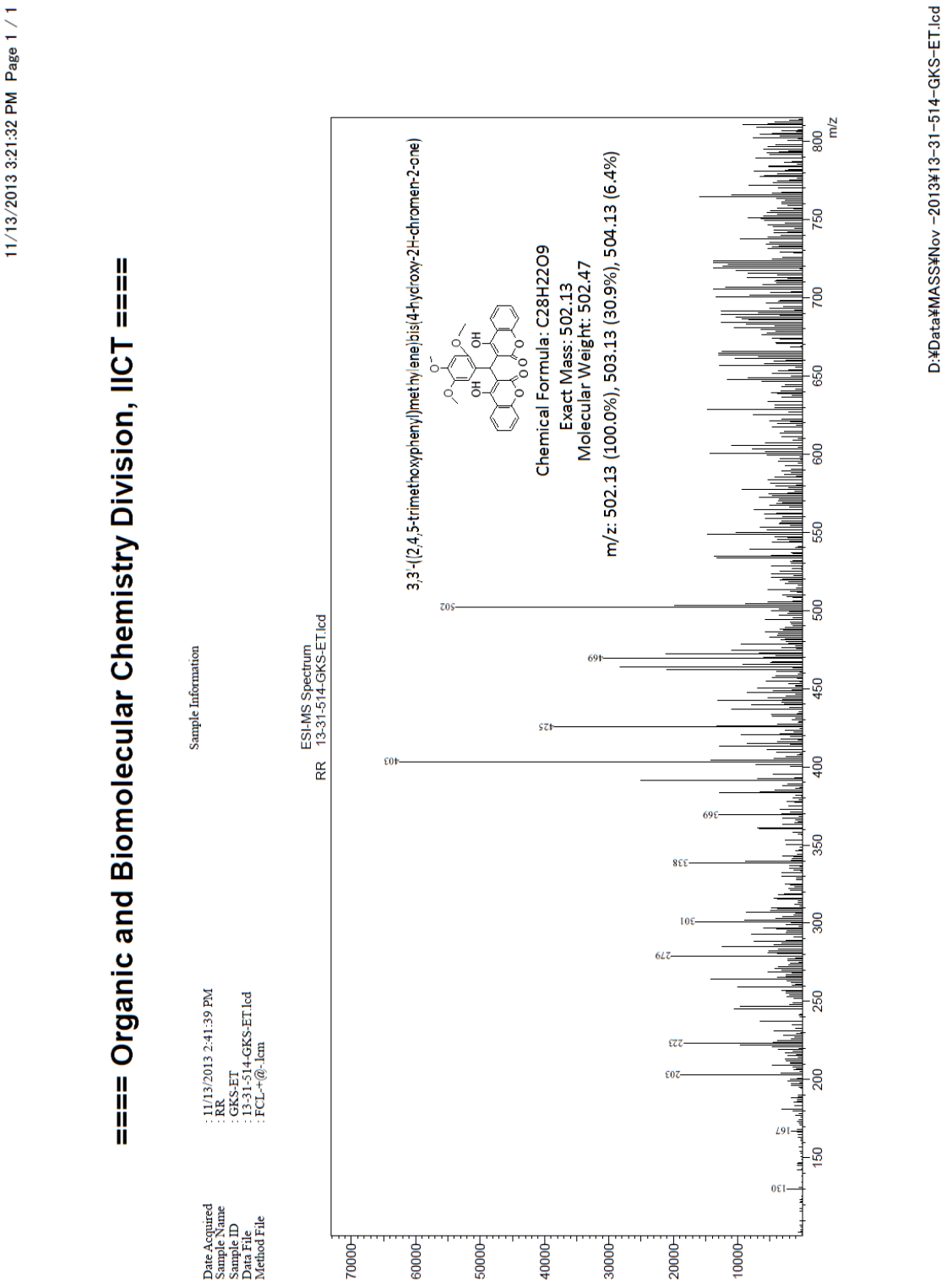


Figure-S49: The ESI- LCMS spectrum of UHAKKM-F

Figure-S50:

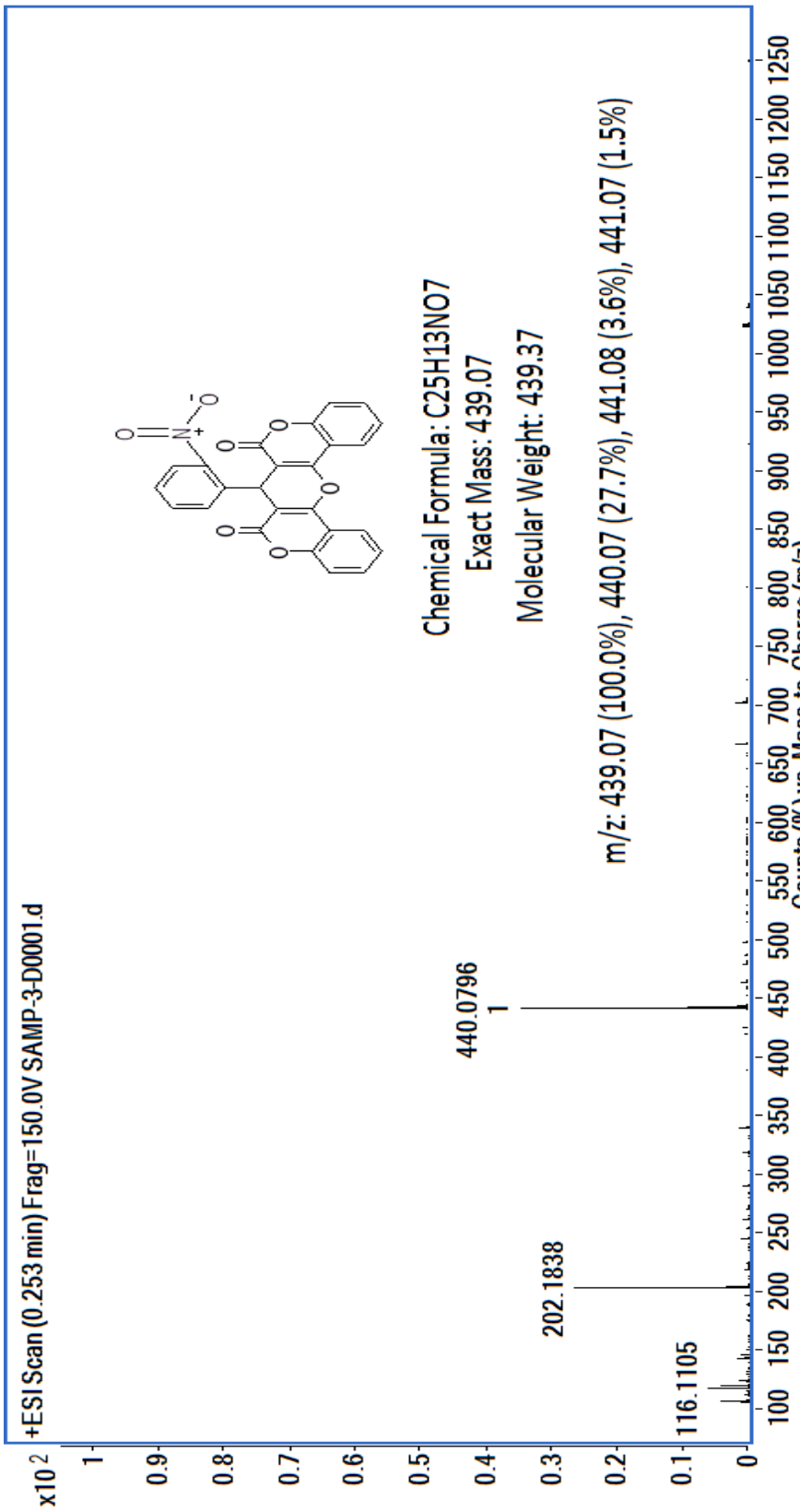


Figure-S50: The ESI-HRMS spectrum of UHAKKM-E1

Figure-S51:

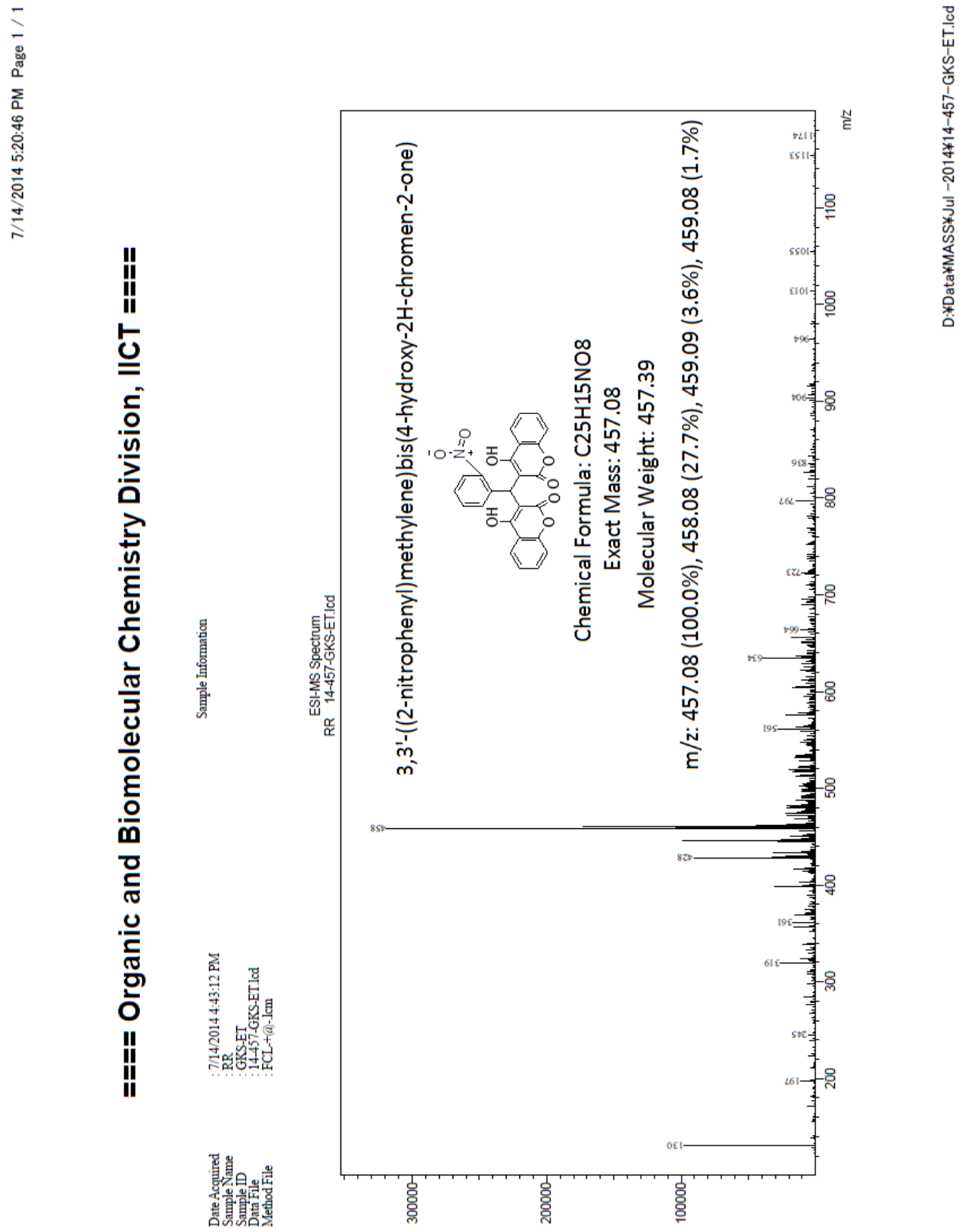


Figure-S51: The ESI- LCMS spectrum of UHAKKM-E

Figure-S52:

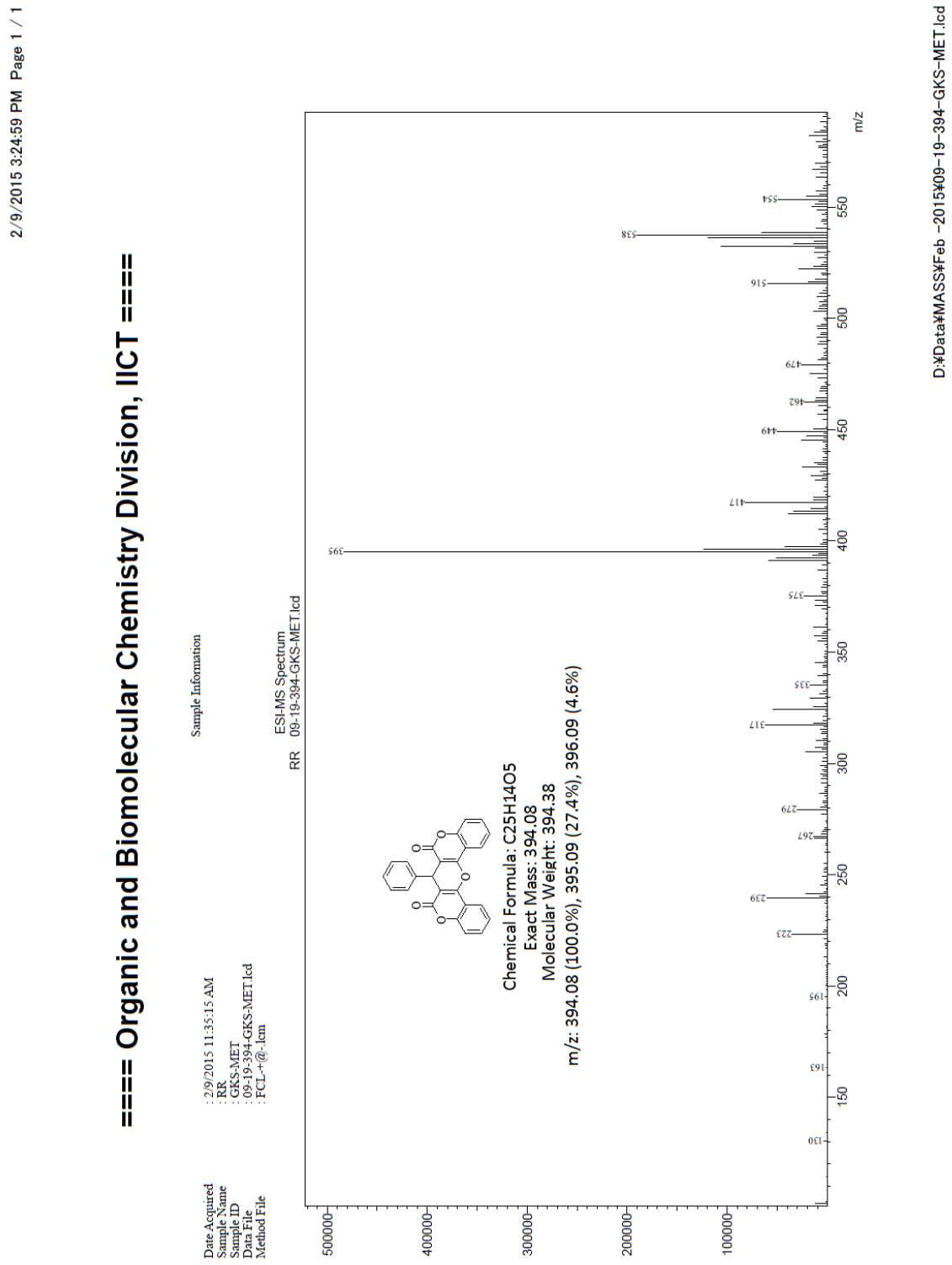


Figure-S52: The ESI- LCMS spectrum of UHAKKM-A1

Figure-S53:

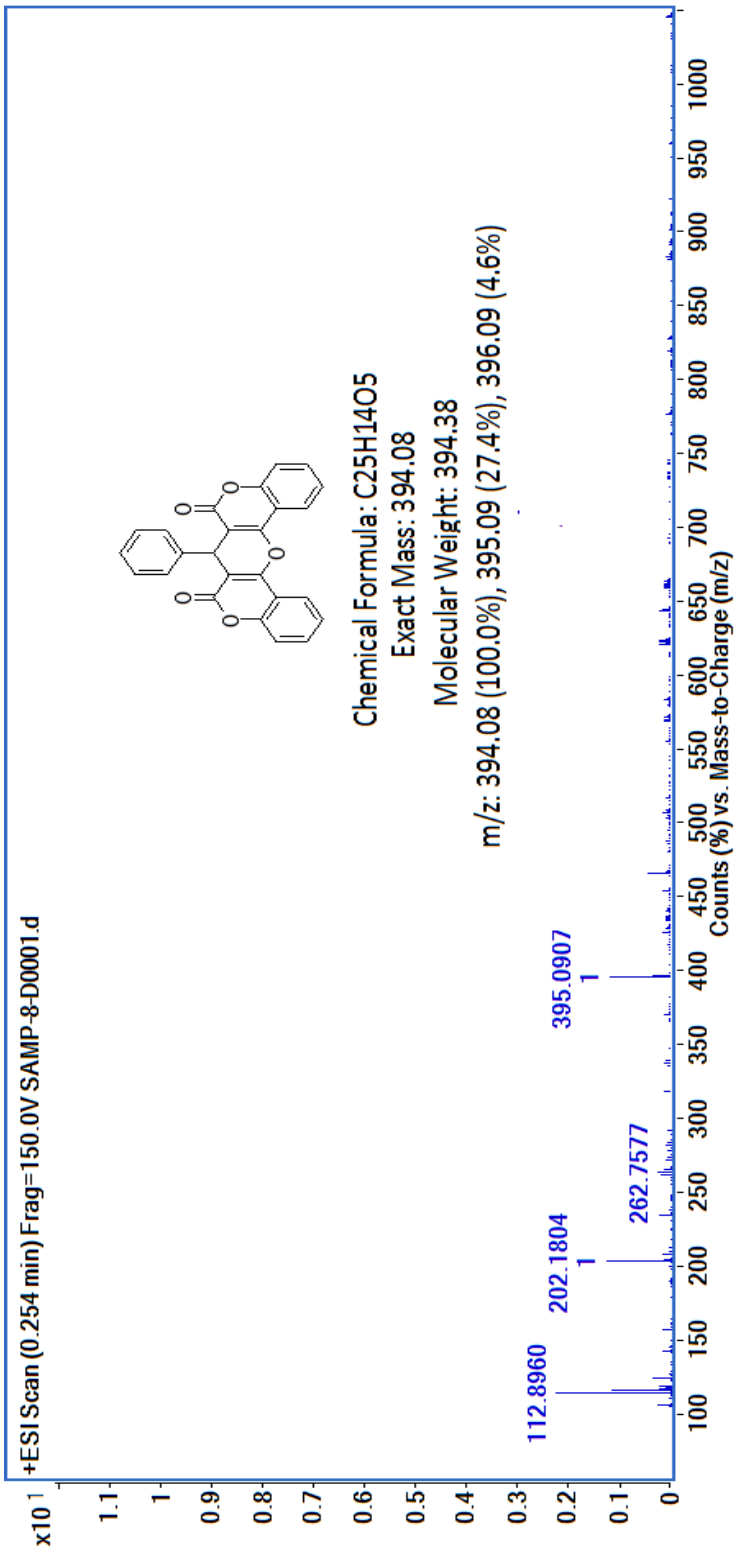


Figure-S53: The ESI-HRMS spectrum of UHAKKM-A1

Figure-S54:

Figure-S54: The ESI-HRMS spectrum of UHAKKM-A

OBJECTIVE-4 SPECTRAL DATA

Figure-S55:

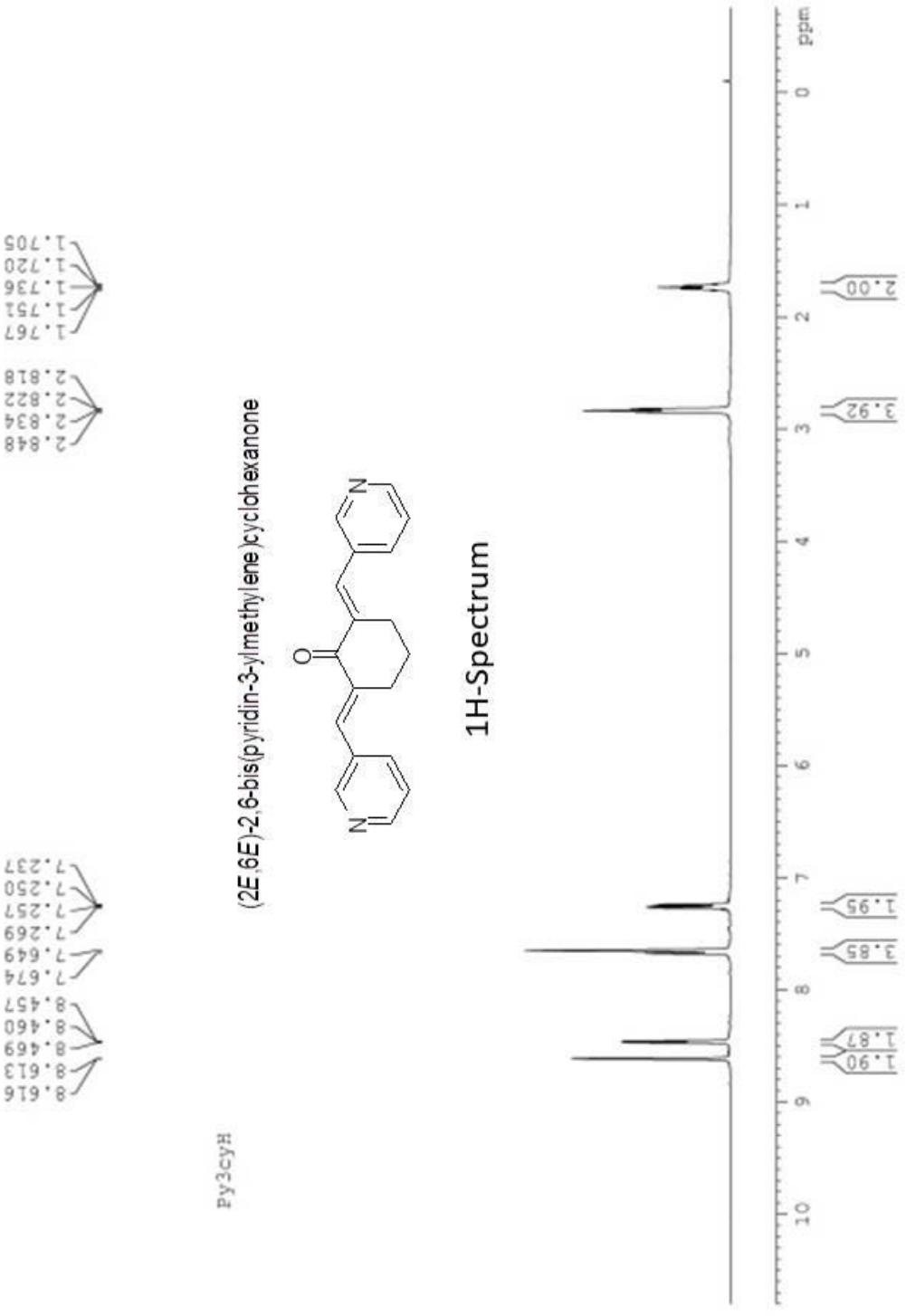


Figure-S55: The ^{1}H-NMR spectrum of MOLECULE-71

Figure-S56:

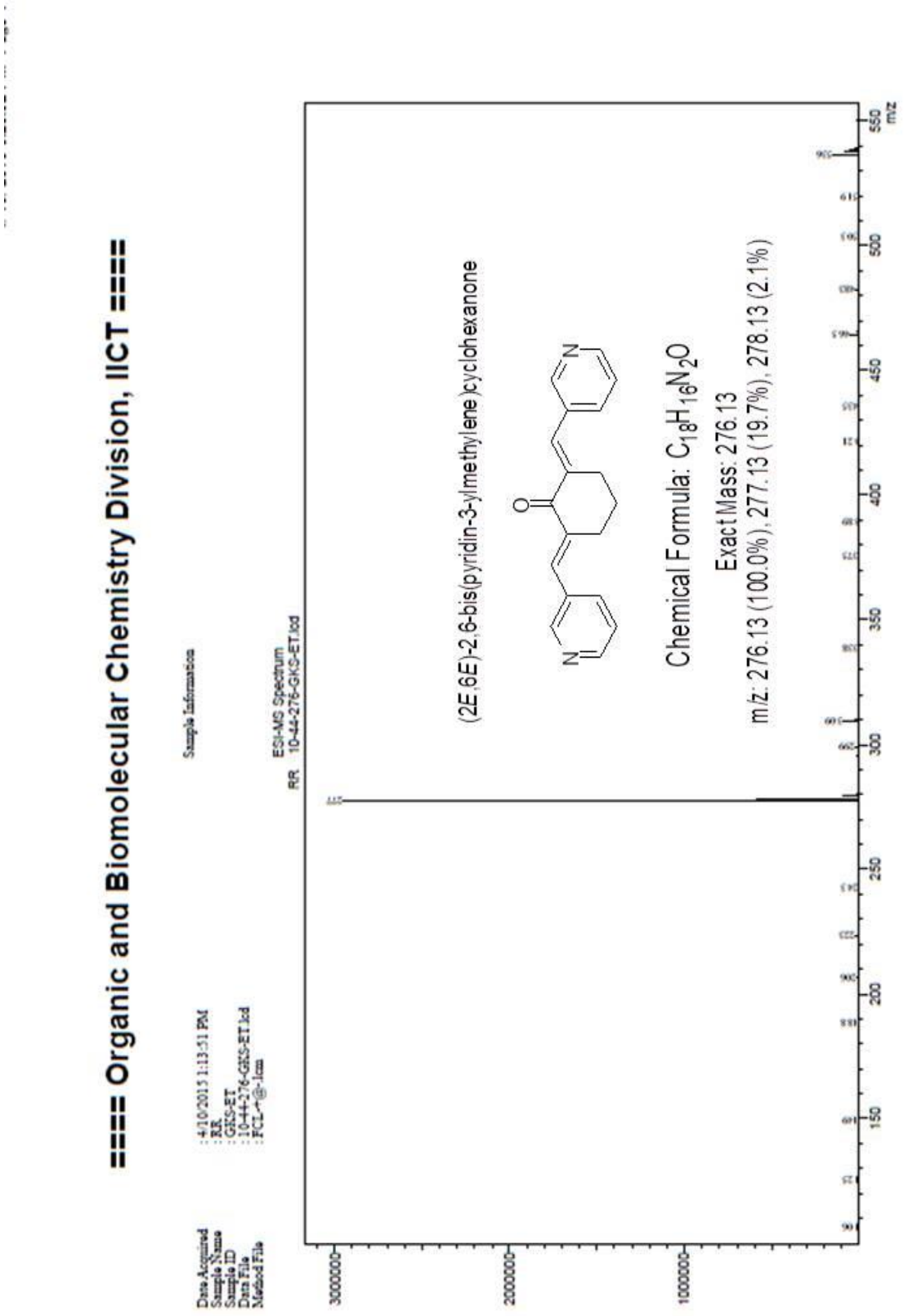


Figure-S56: The ESI-LCMS spectrum of MOLECULE-71

Figure-S57:

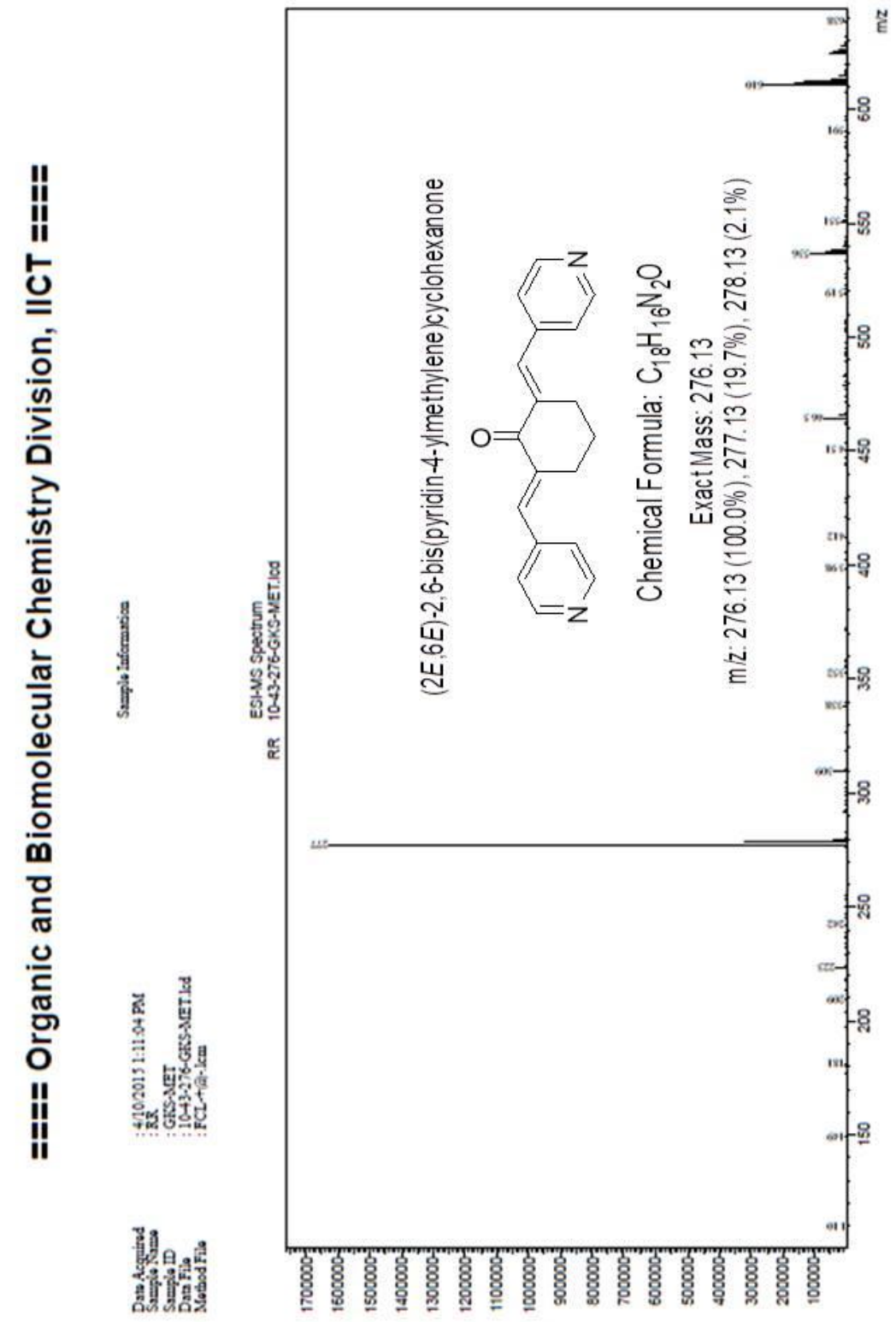


Figure-S57: The ESI-LCMS spectrum of MOLECULE-72

Figure-S58:

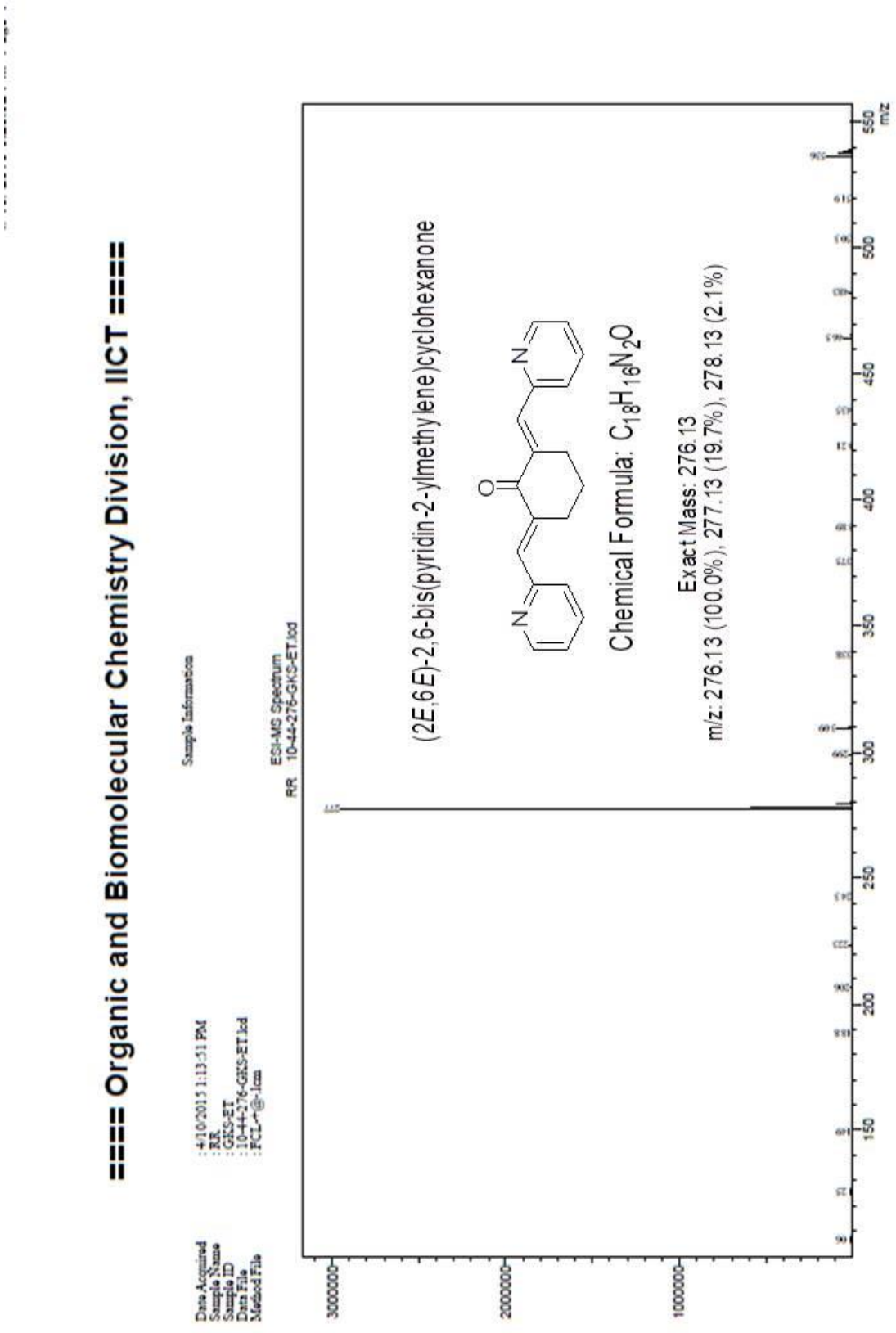


Figure-S58: The ESI-LCMS spectrum of MOLECULE-70

Figure-S59:

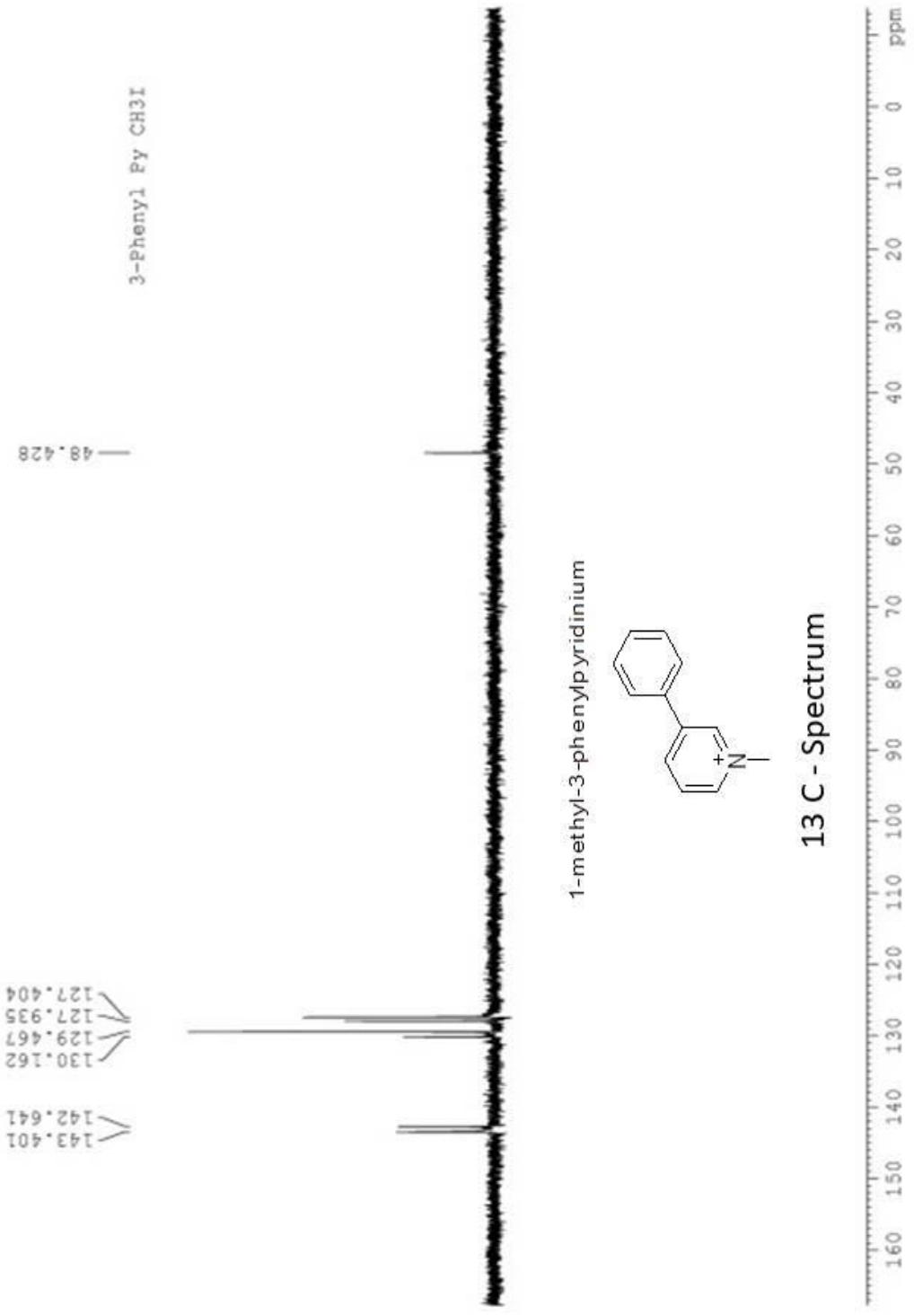


Figure-S59: The ^{13}C-NMR spectrum of UHAKKM-ZA(MOLECULE-76)

Figure-S60:

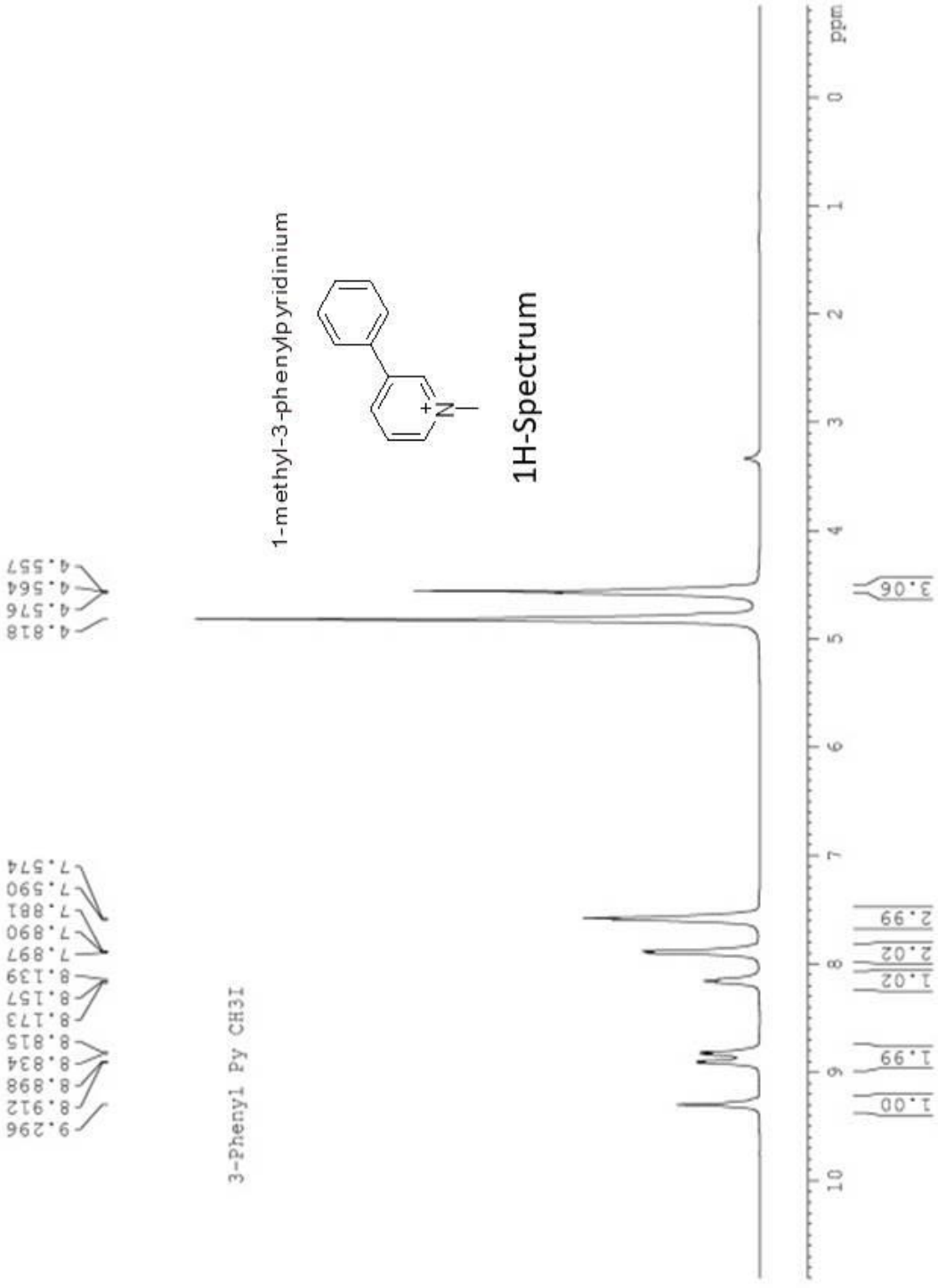


Figure-S60: The ^{1}H-NMR spectrum of UHAKKM-ZA(MOLECULE-76)

Figure-S61:

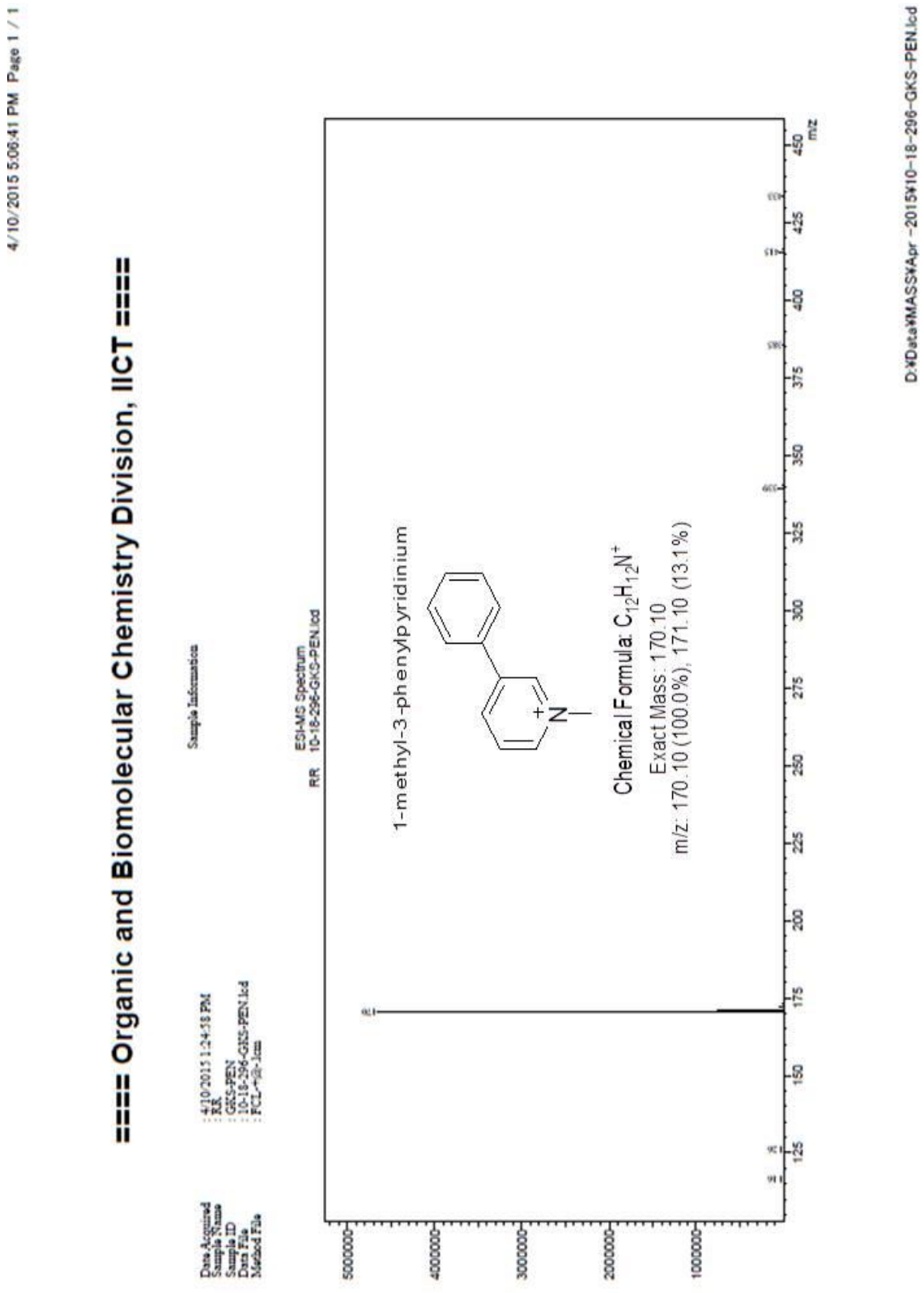


Figure-S61: The ESI-LCMS spectrum of UHAKKM-ZA(MOLECULE-76)

Figure-S62:

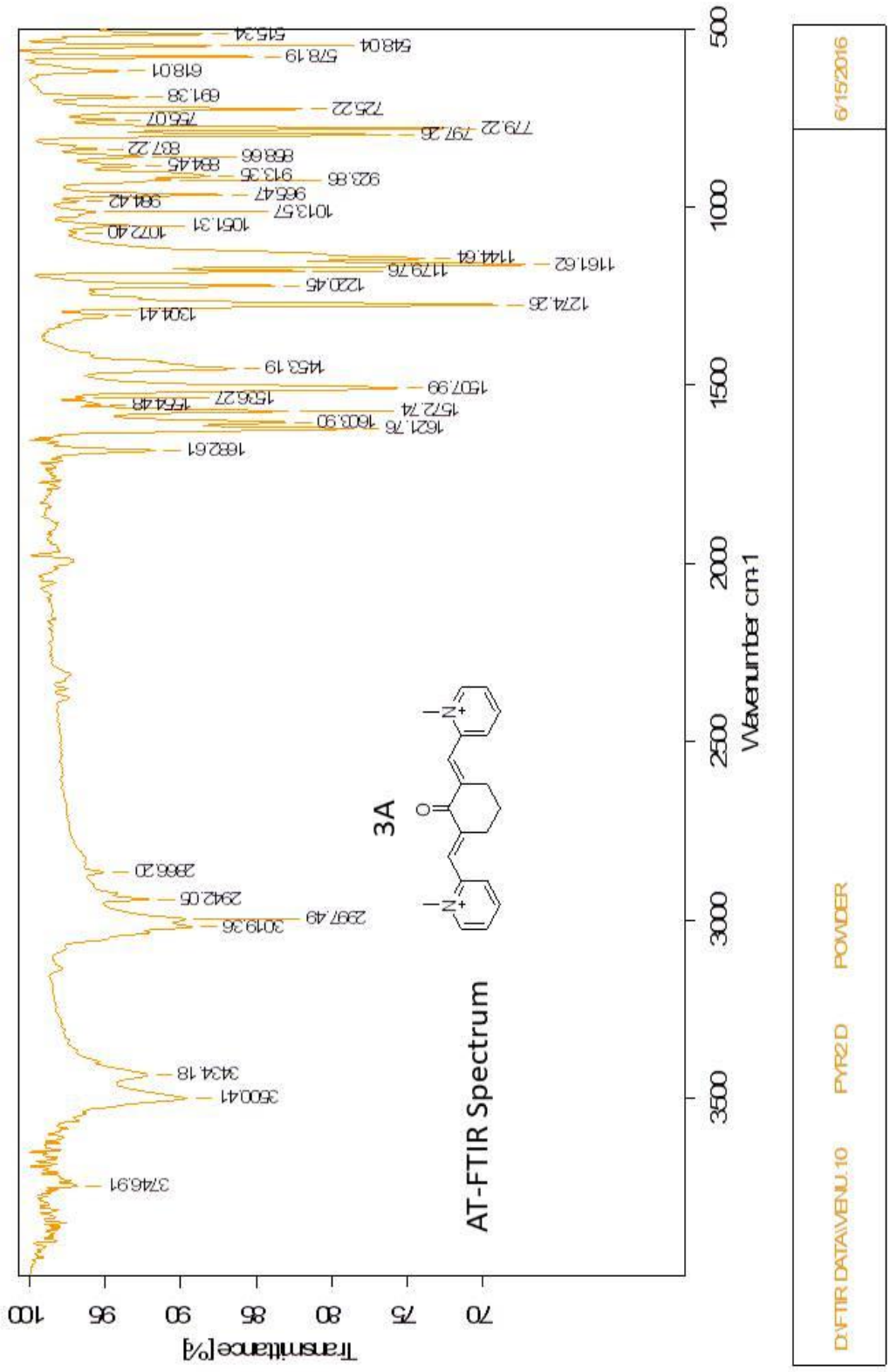


Figure-S62: The FTIR spectrum of UHAKKM-Z(MOLECULE-75)

Figure-S63:

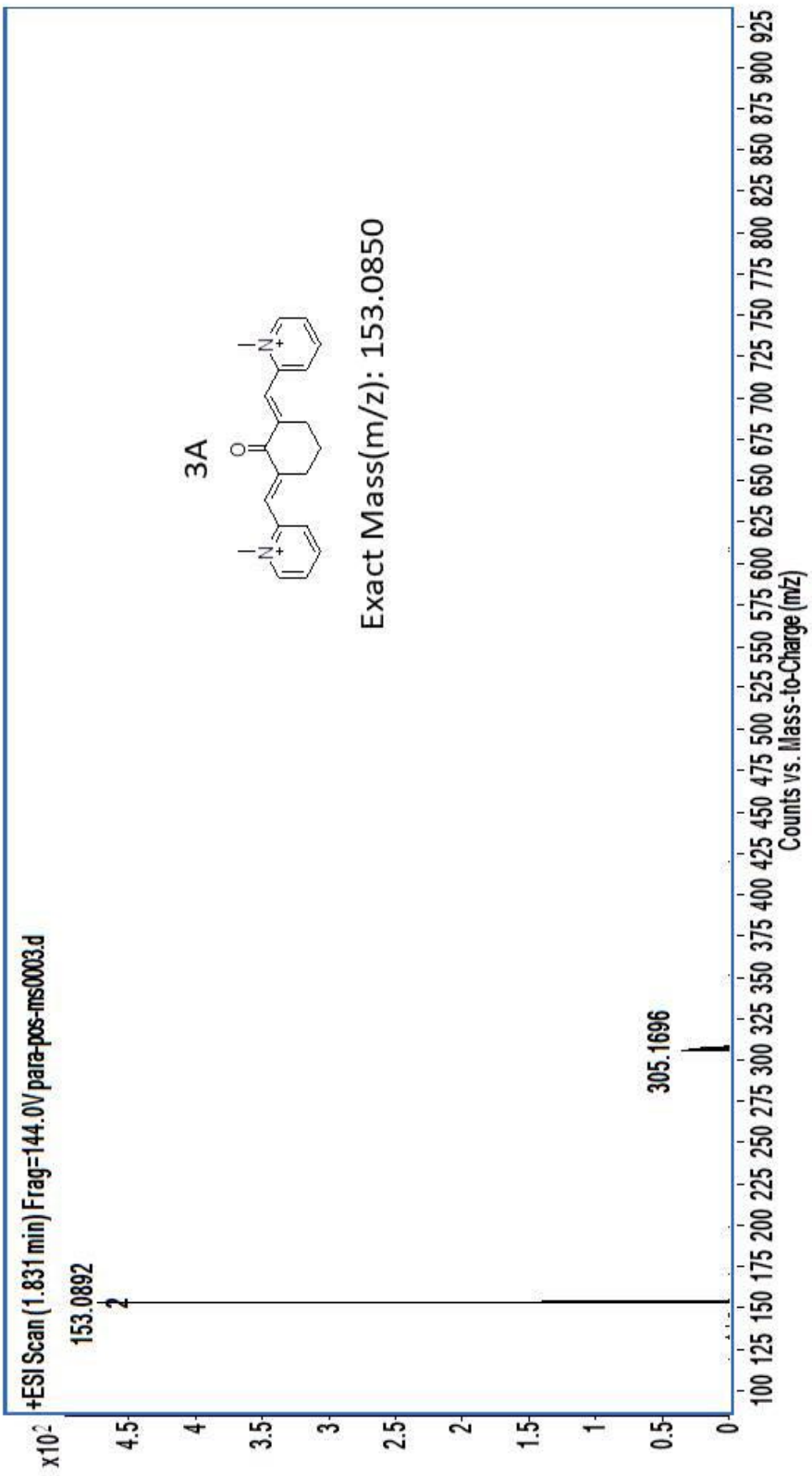


Figure-S63: The ESI-HRMS spectrum of UHAKKM-Z(MOLECULE-75)

Figure-S64:

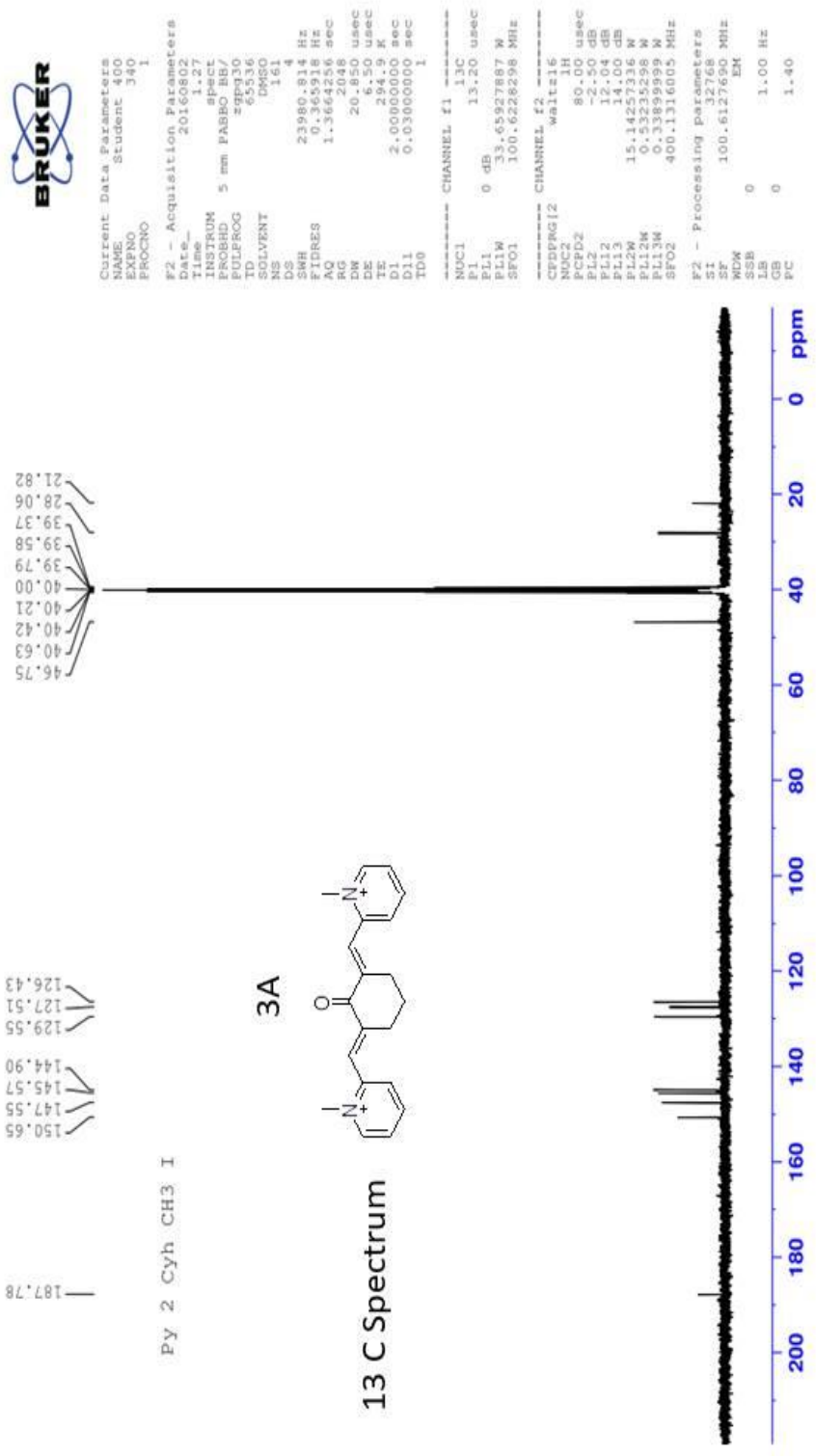


Figure-S64: The ^{13}C-NMR spectrum of UHAKKM-Z(MOLECULE-75)

Figure-S65:

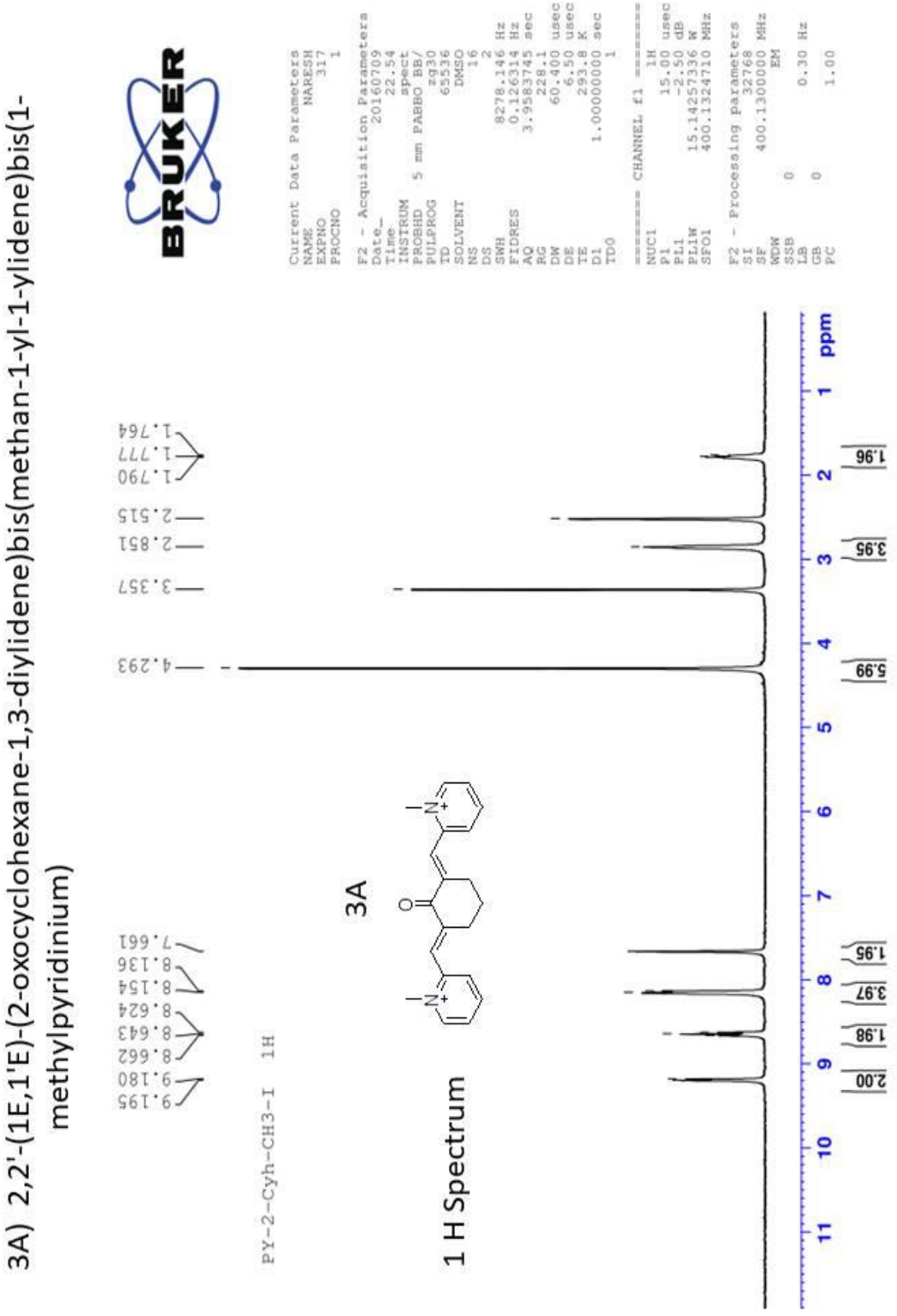


Figure-S65: The ^{1}H-NMR spectrum of UHAKKM-Z(MOLECULE-75)

Figure-S66:

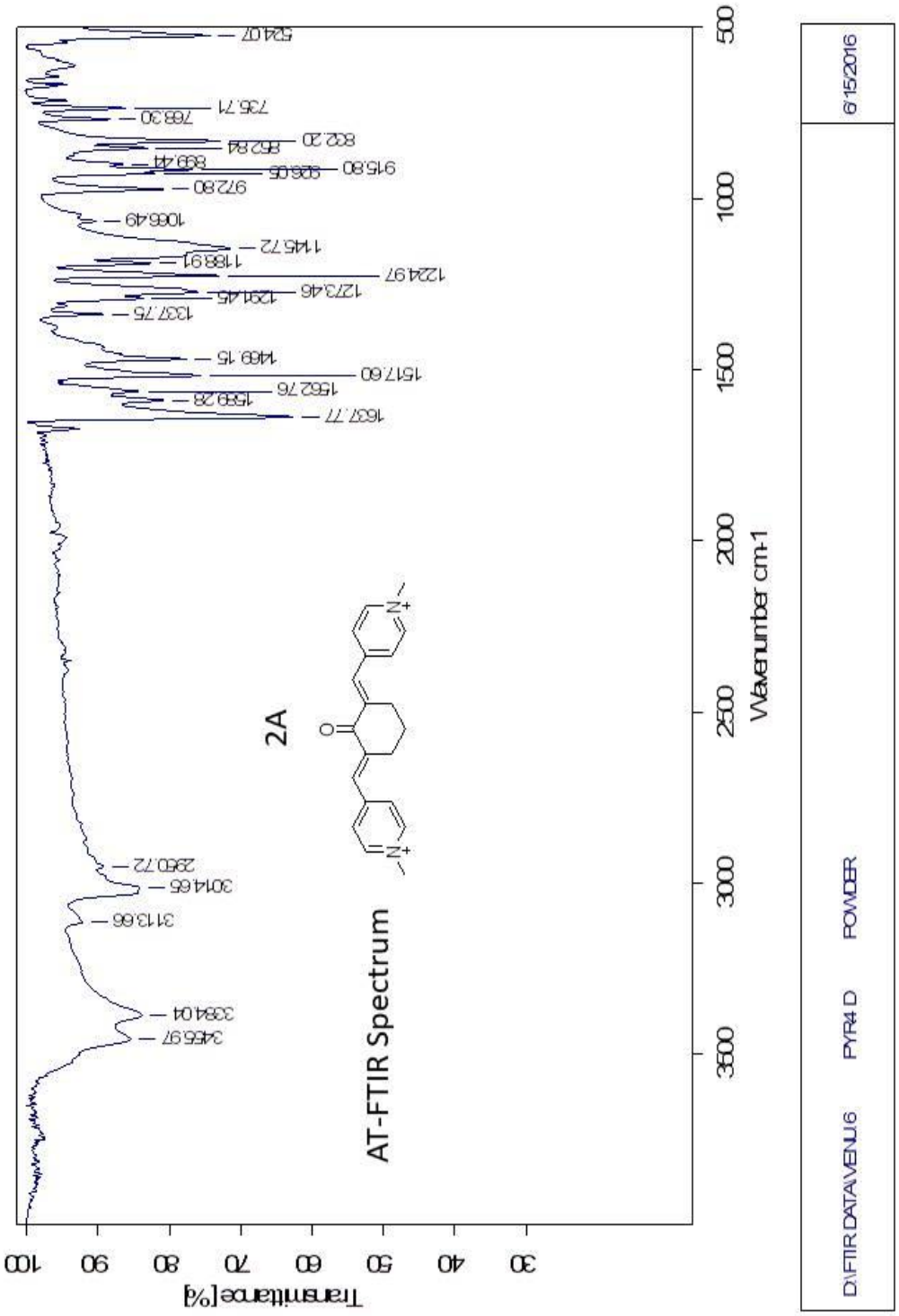


Figure-S66: The FTIR spectrum of UHAKKM-X(MOLECULE-73)

Figure-S67:

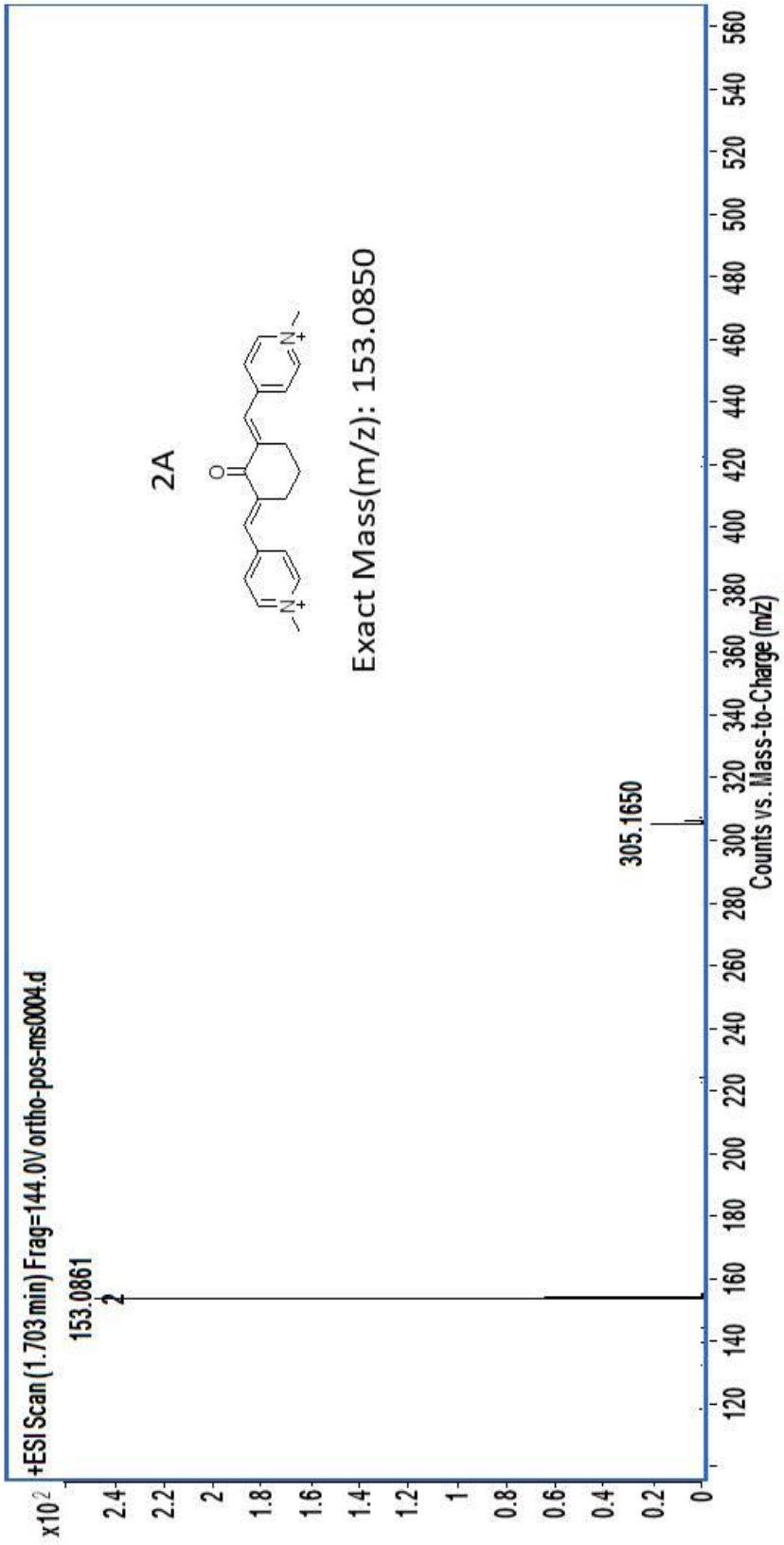


Figure-S67: The ESI-HRMS spectrum of UHAKKM-X(MOLECULE-73)

Figure-S68:

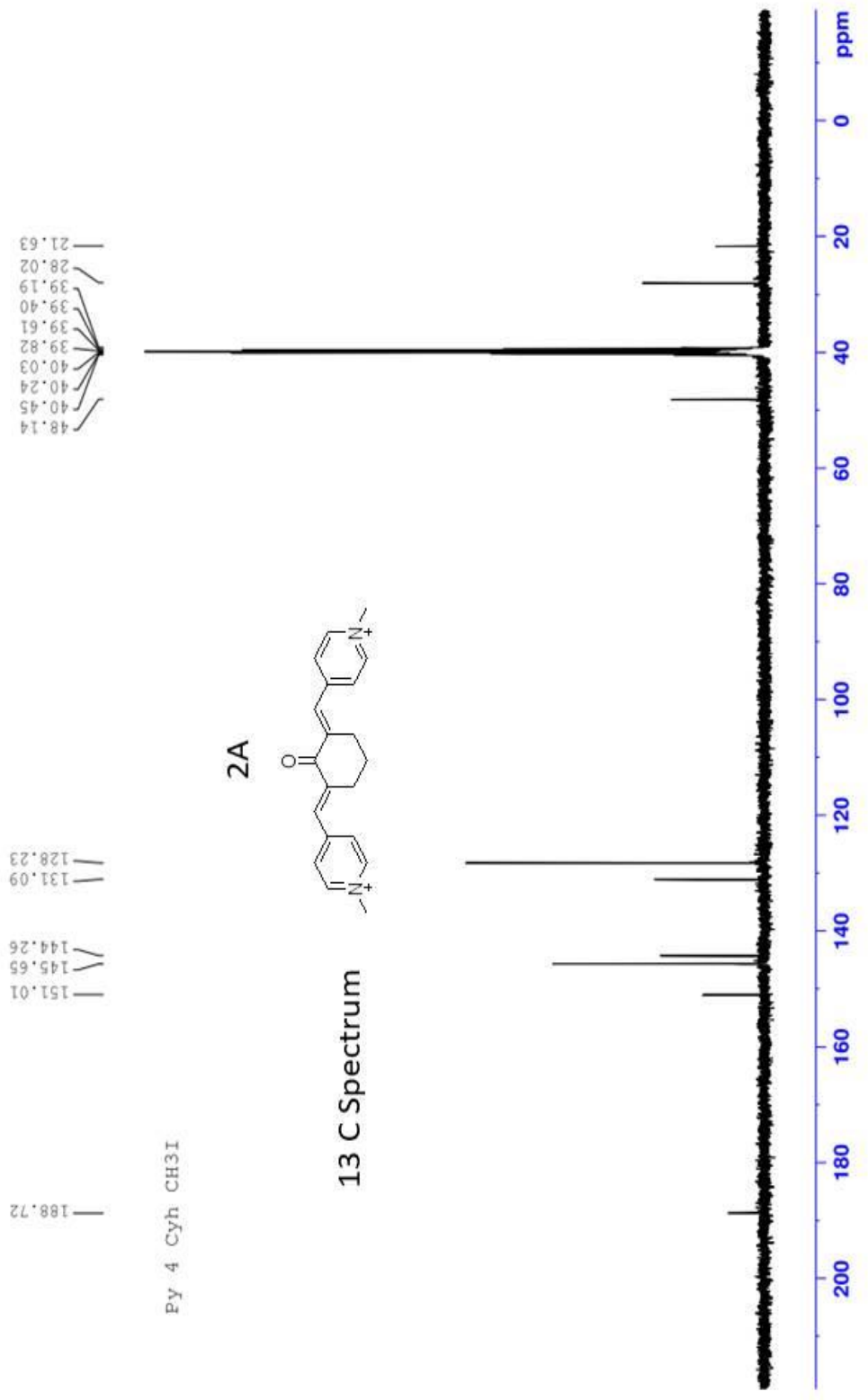


Figure-S68: The ^{13}C-NMR spectrum of UHAKKM-X(MOLECULE-73)

Figure-S69:

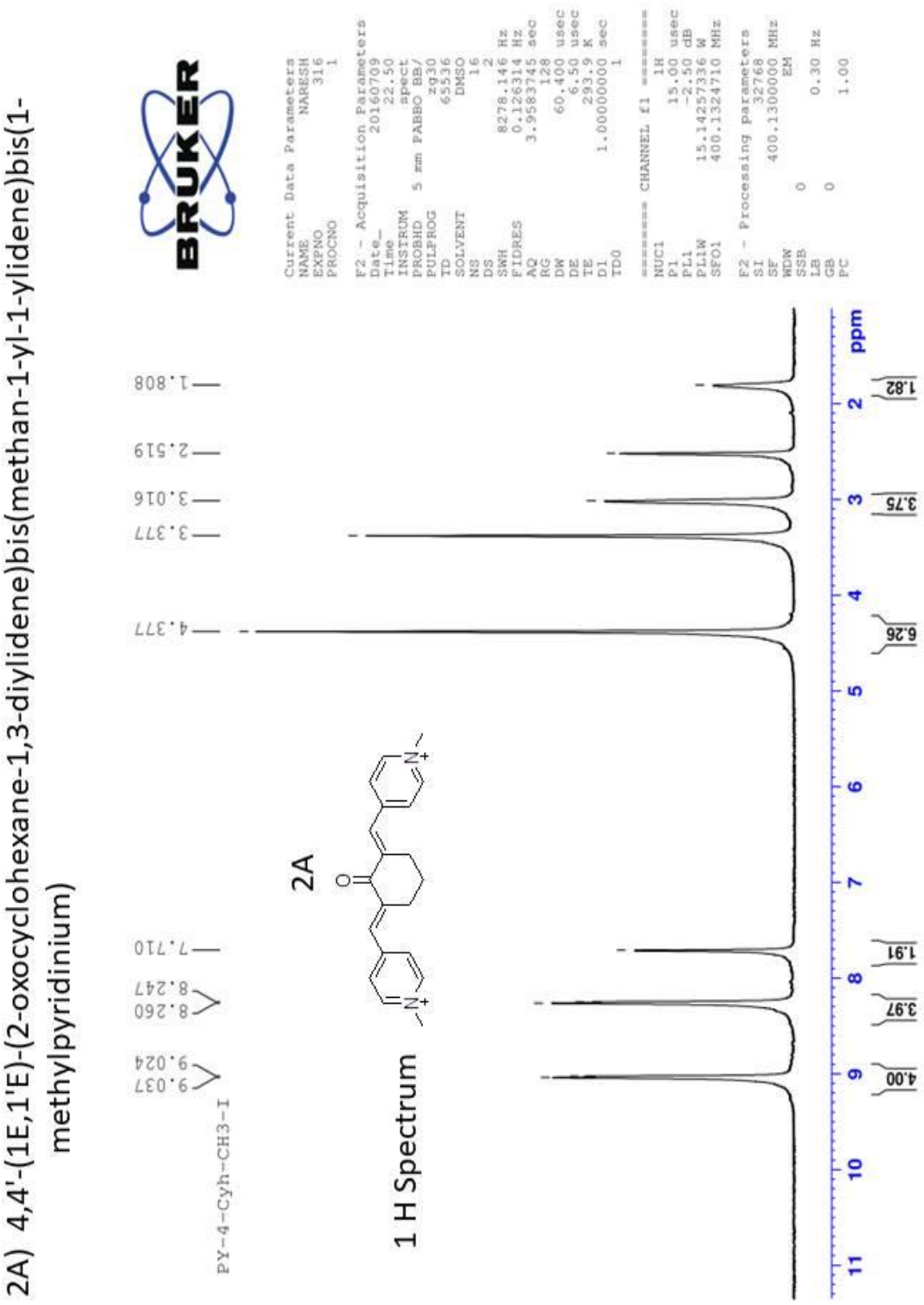


Figure-S69: The ^{1}H-NMR spectrum of UHAKKM-X(MOLECULE-73)

Figure-S70:

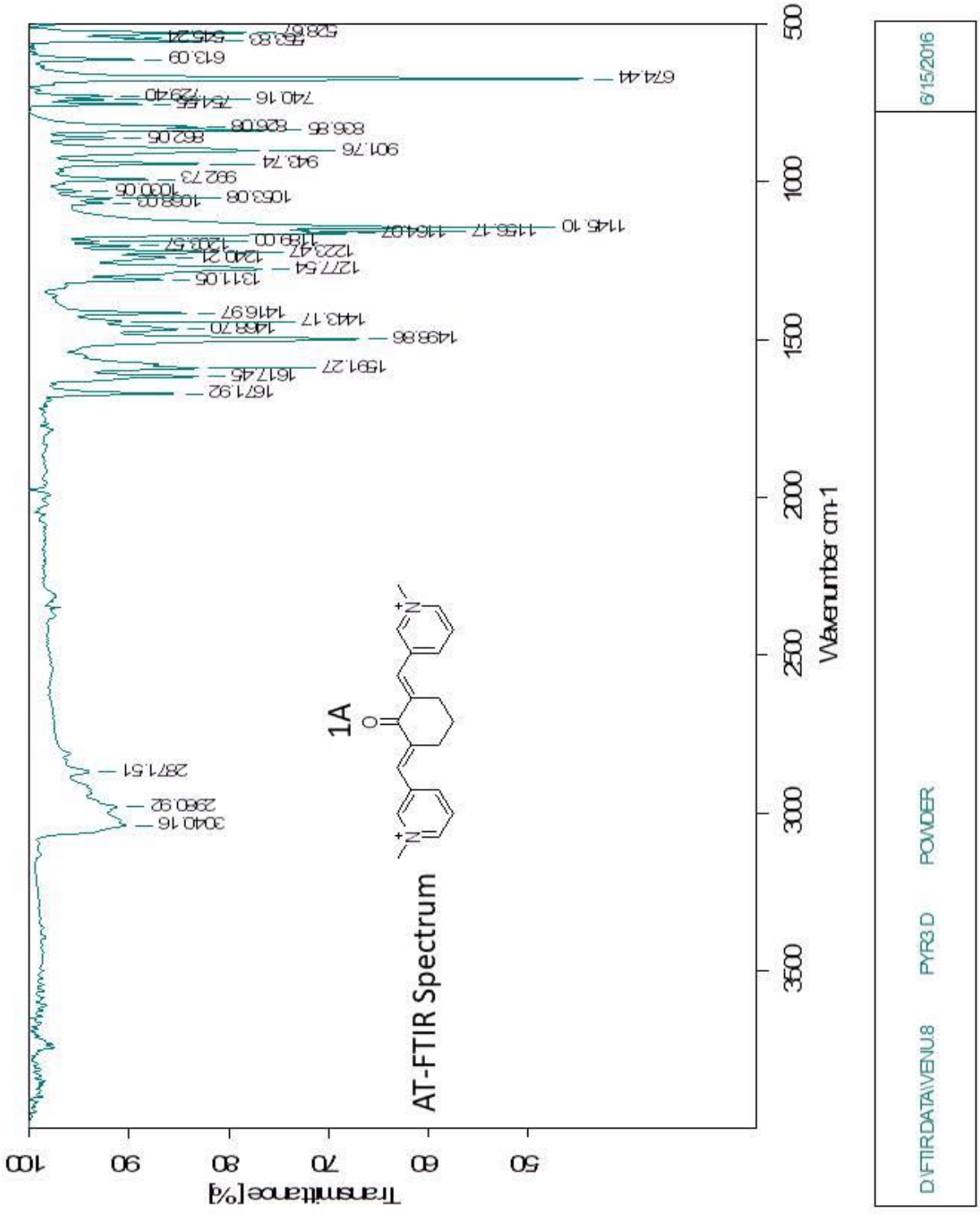


Figure-S70: The FTIR spectrum of UHAKKM-Y(MOLECULE-74)

Figure-S71:

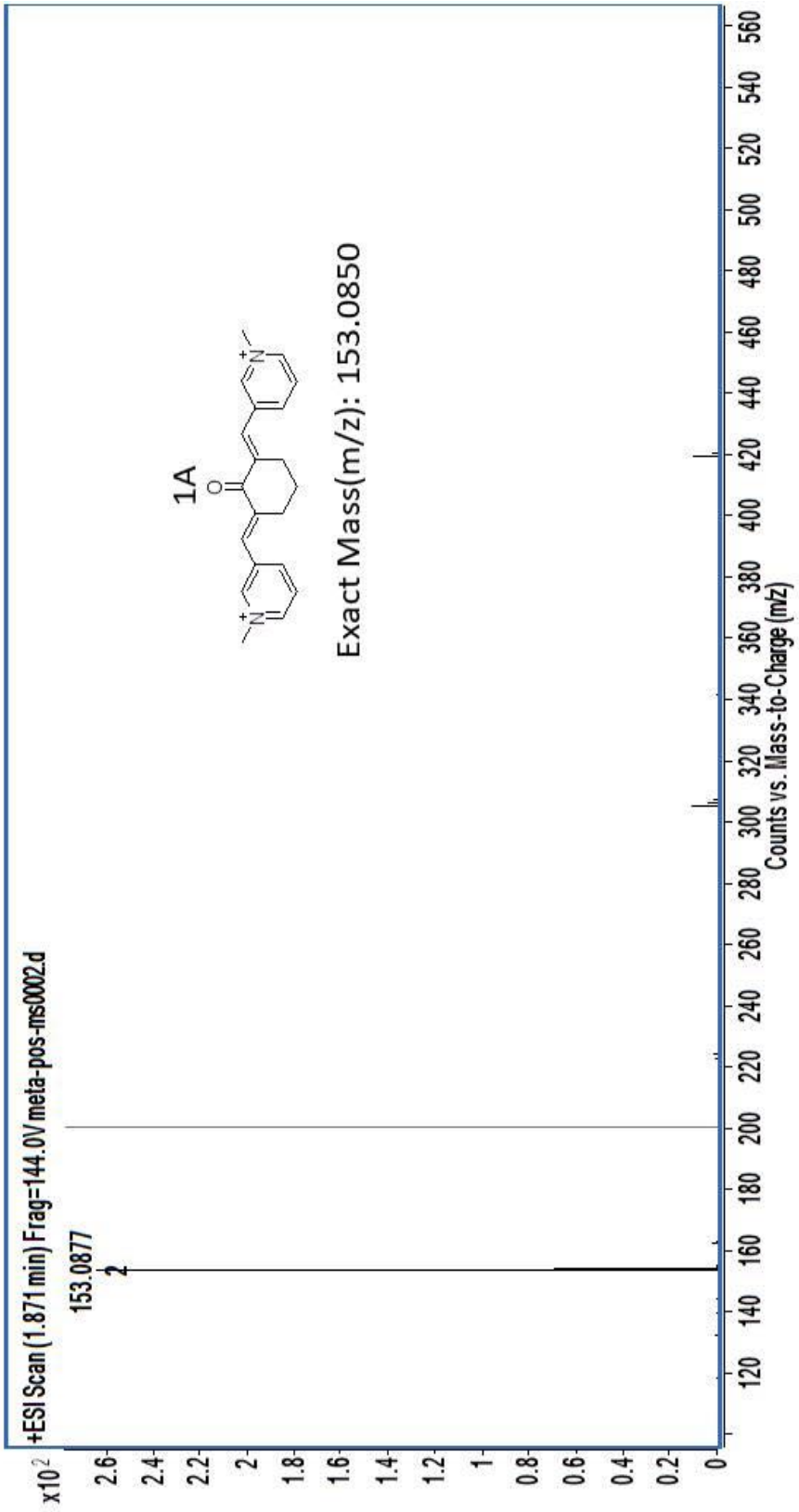


Figure-S71: The ESI-HRMS spectrum of UHAKKM-Y(MOLECULE-74)

Figure-S72:

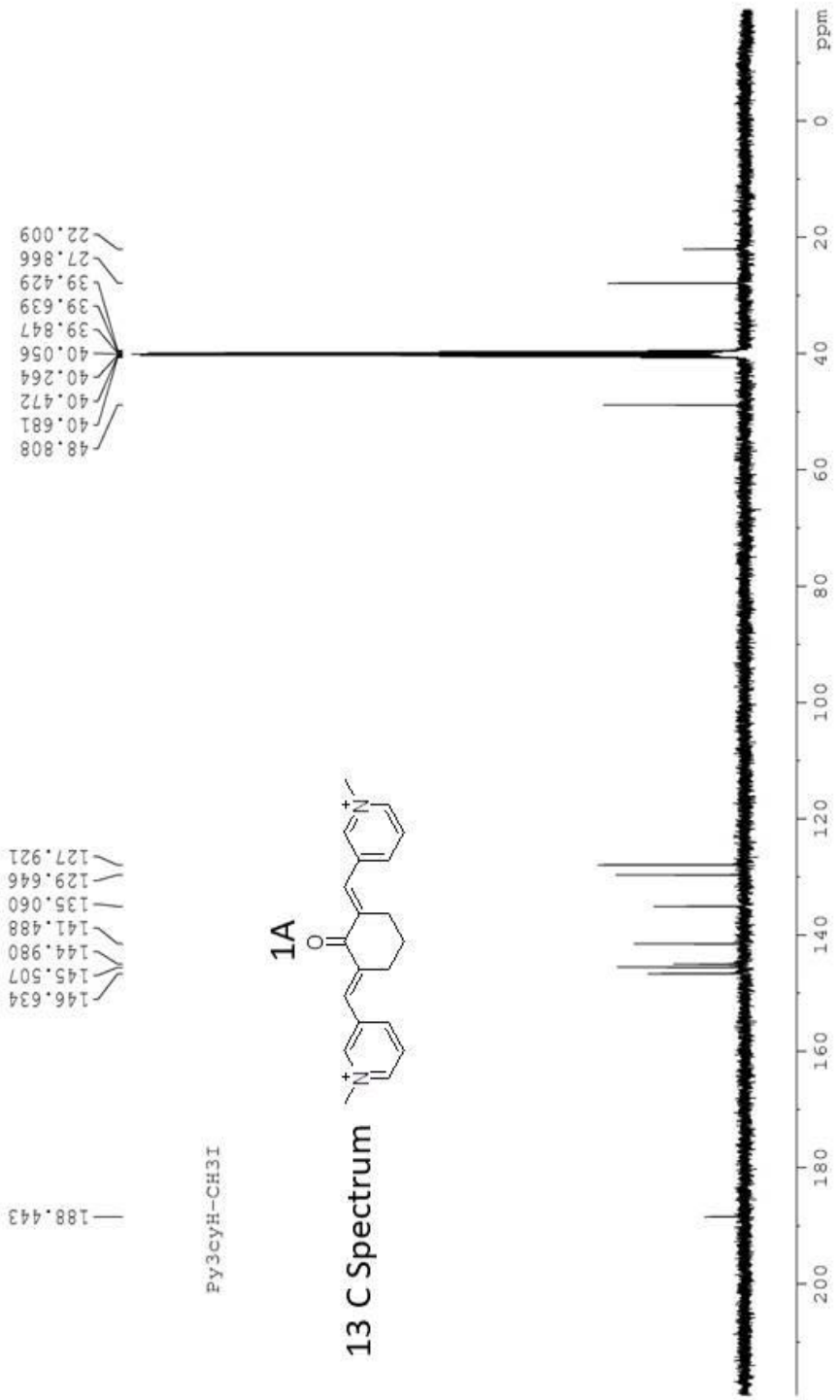


Figure-S72: The [13] C-NMR spectrum of UHAKKM-Y(MOLECULE-74)

Figure-S73:

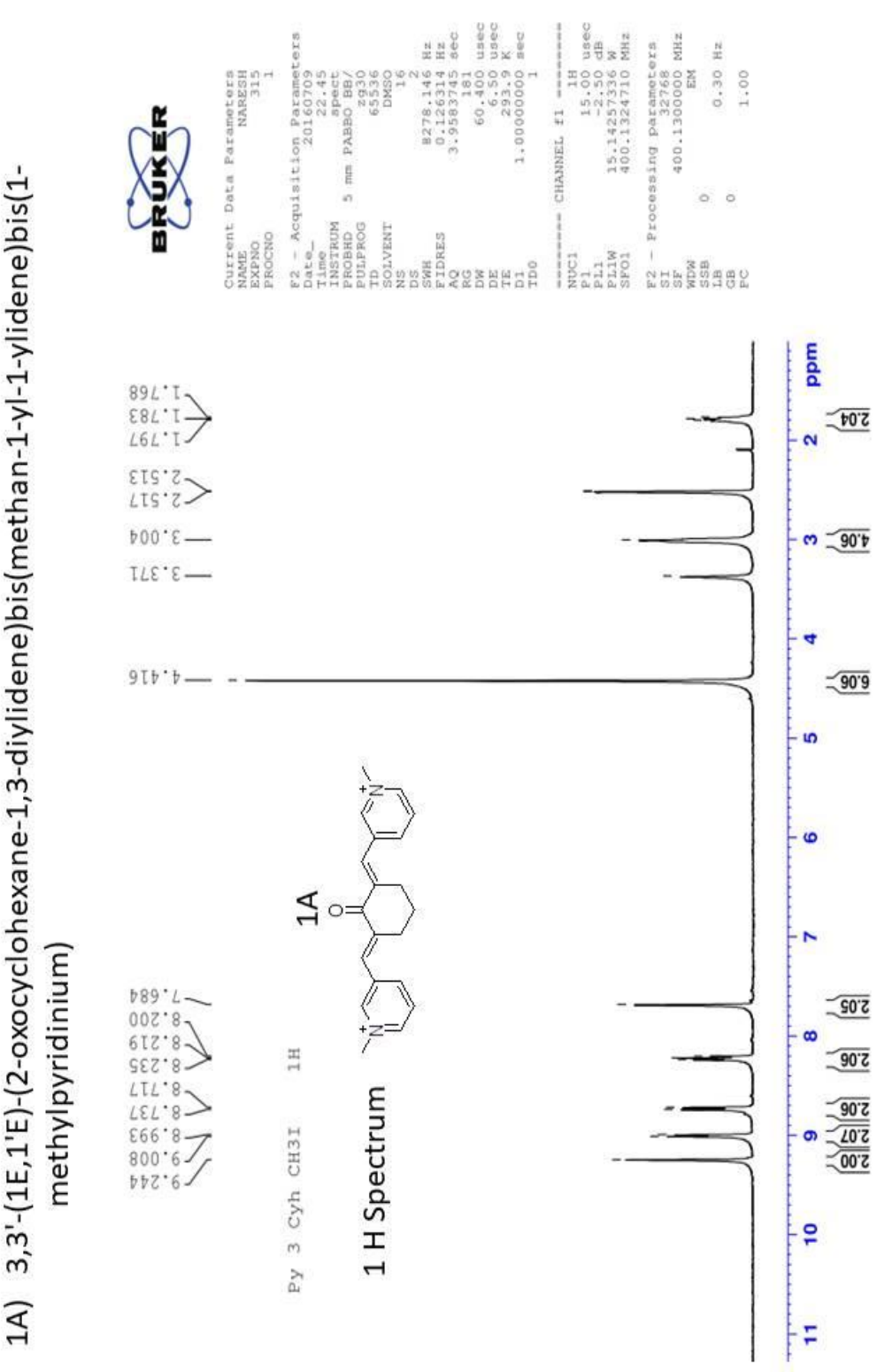


Figure-S73: The ^{1}H-NMR spectrum of UHAKKM-Y(MOLECULE-74)

Figure-S74:

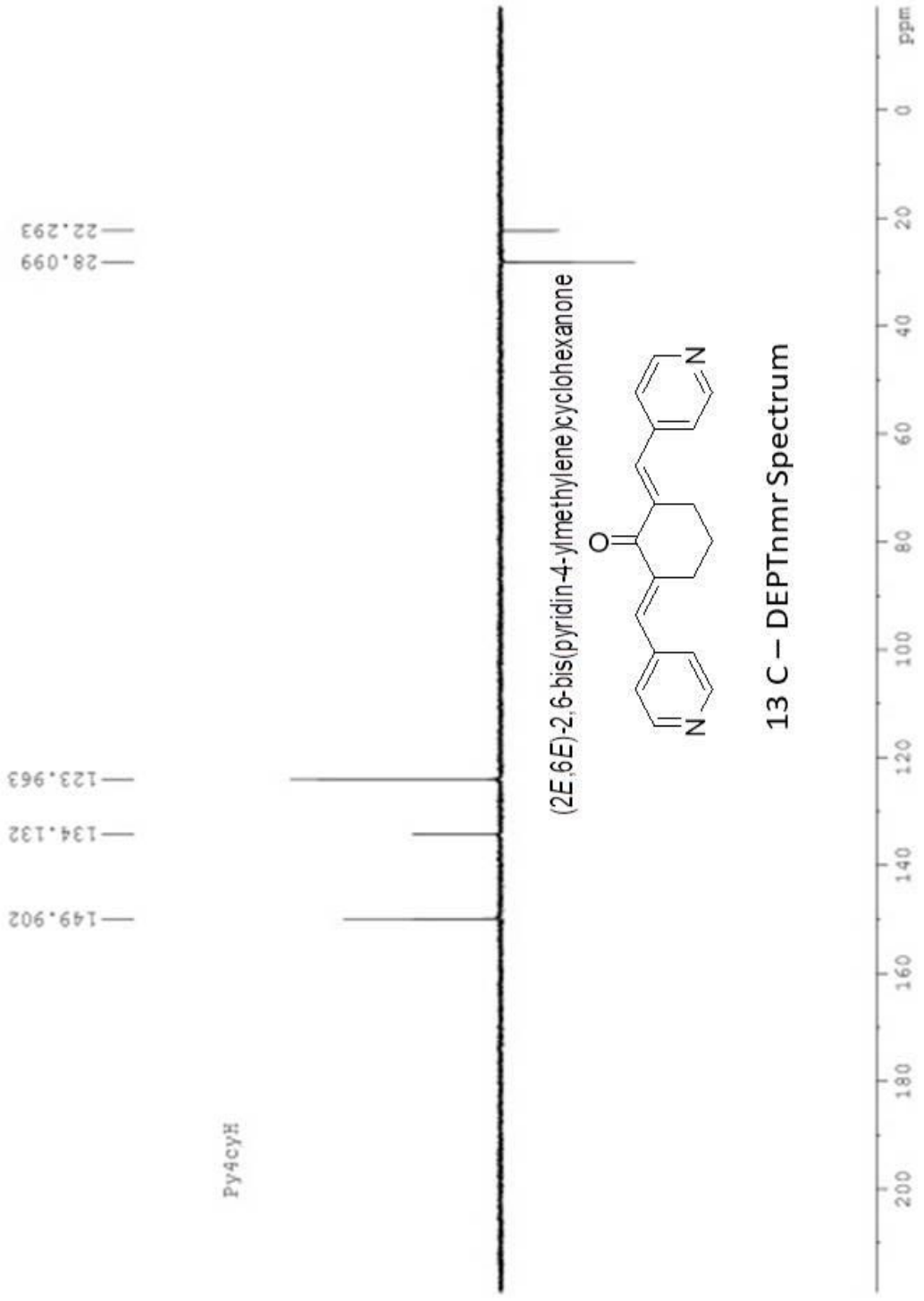


Figure-S74: The ^{13}C-DEPT NMR spectrum of MOLECULE-72

Figure-S75:

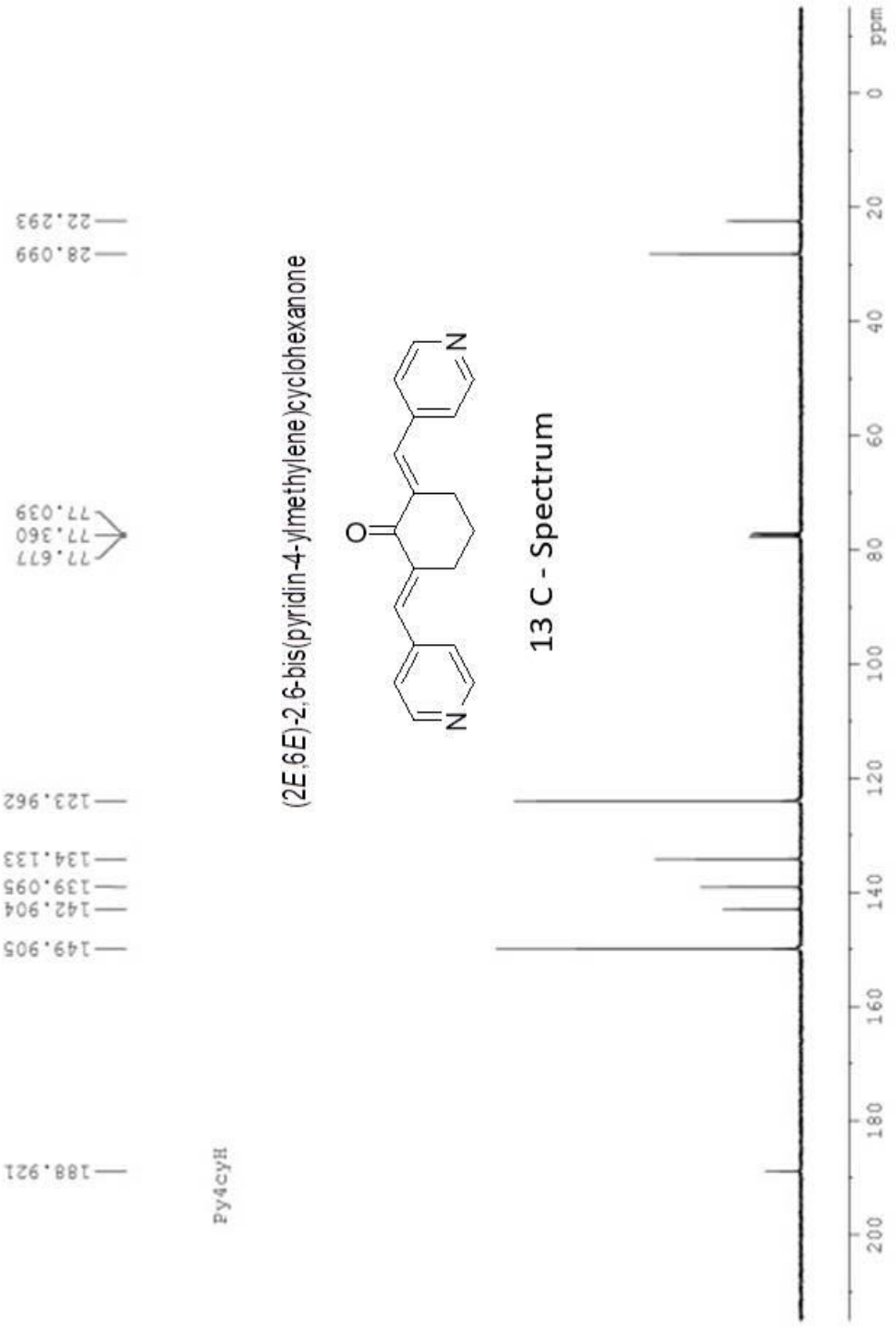


Figure-S75: The ^{13}C- NMR spectrum of MOLECULE-72

Figure-S76:

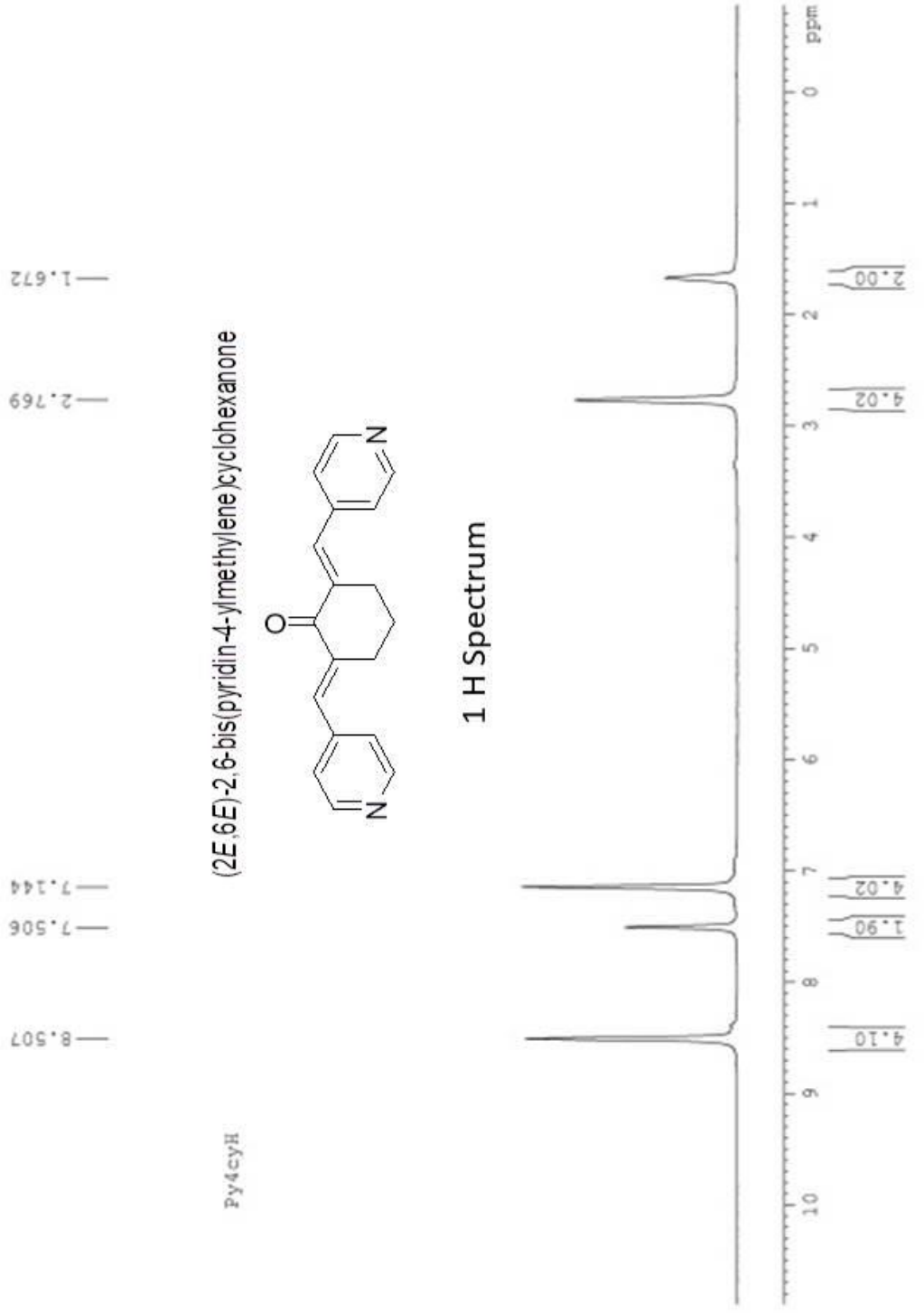


Figure-S76: The ^{1}H - NMR spectrum of MOLECULE-72

Figure-S77:

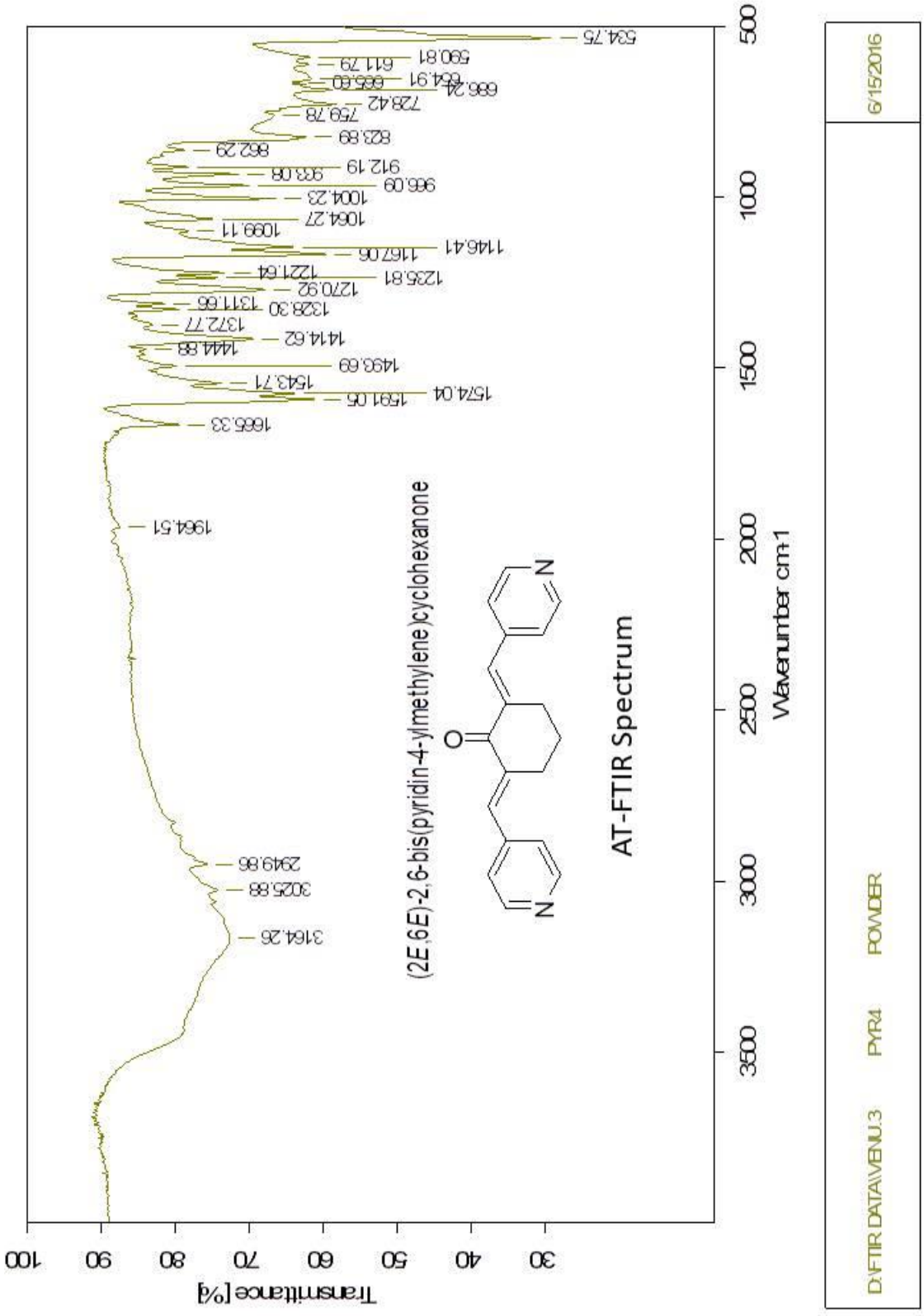


Figure-S77: The FTIR spectrum of MOLECULE-72

Figure-S78:

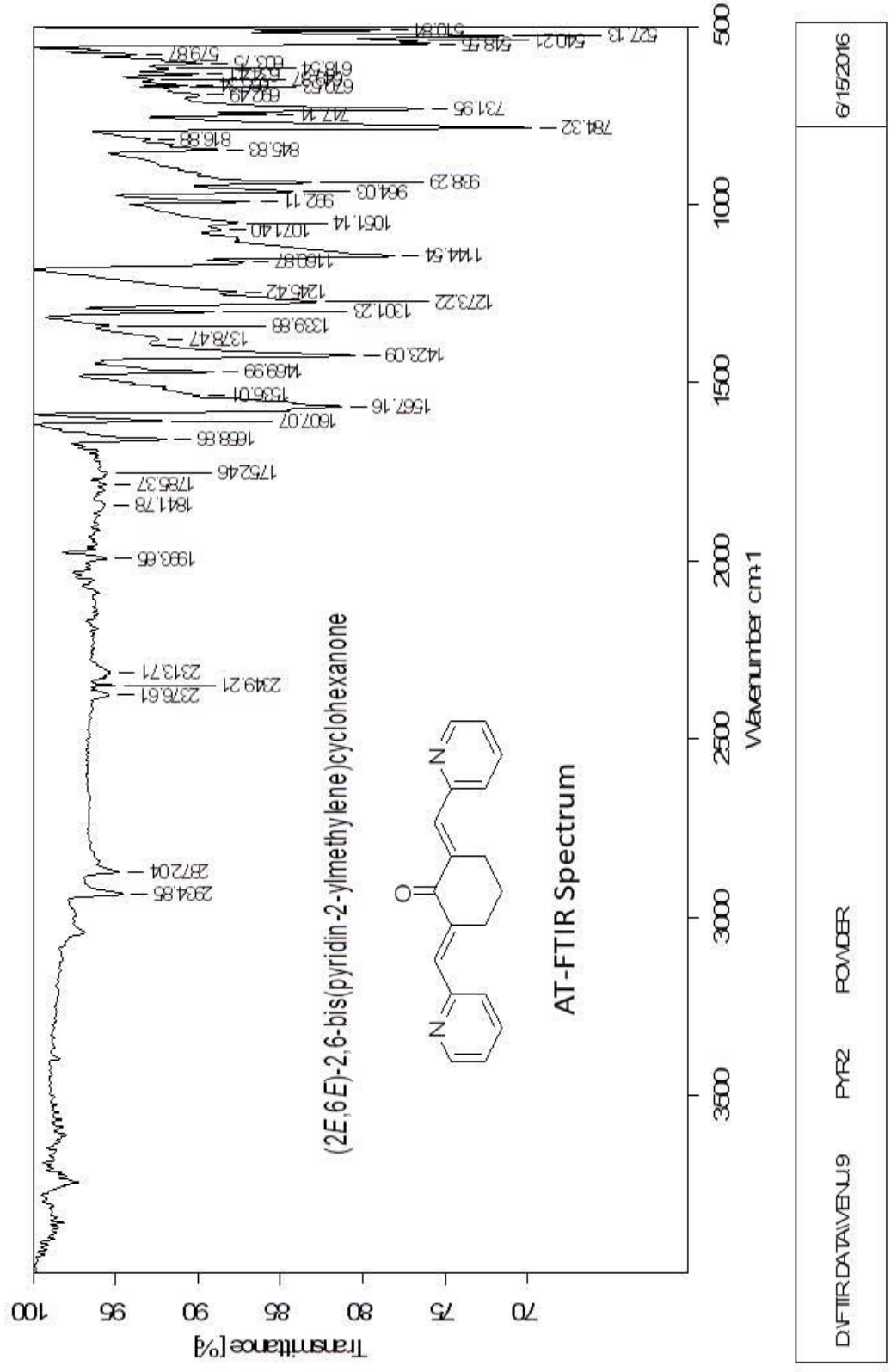


Figure-S78: The FTIR spectrum of MOLECULE-72

Figure-S79:

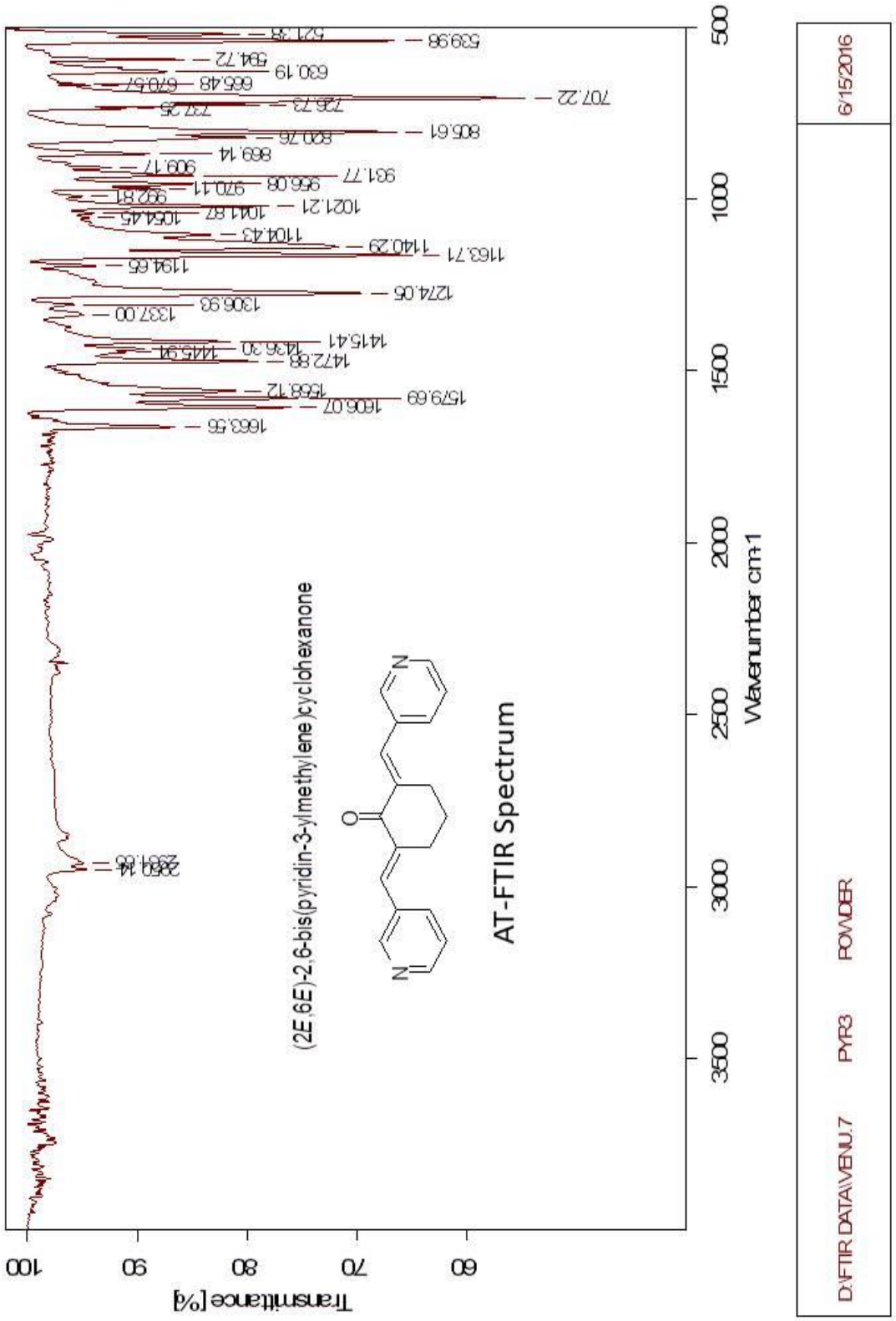


Figure-S79: The FTIR spectrum of MOLECULE-71

Figure-S80:

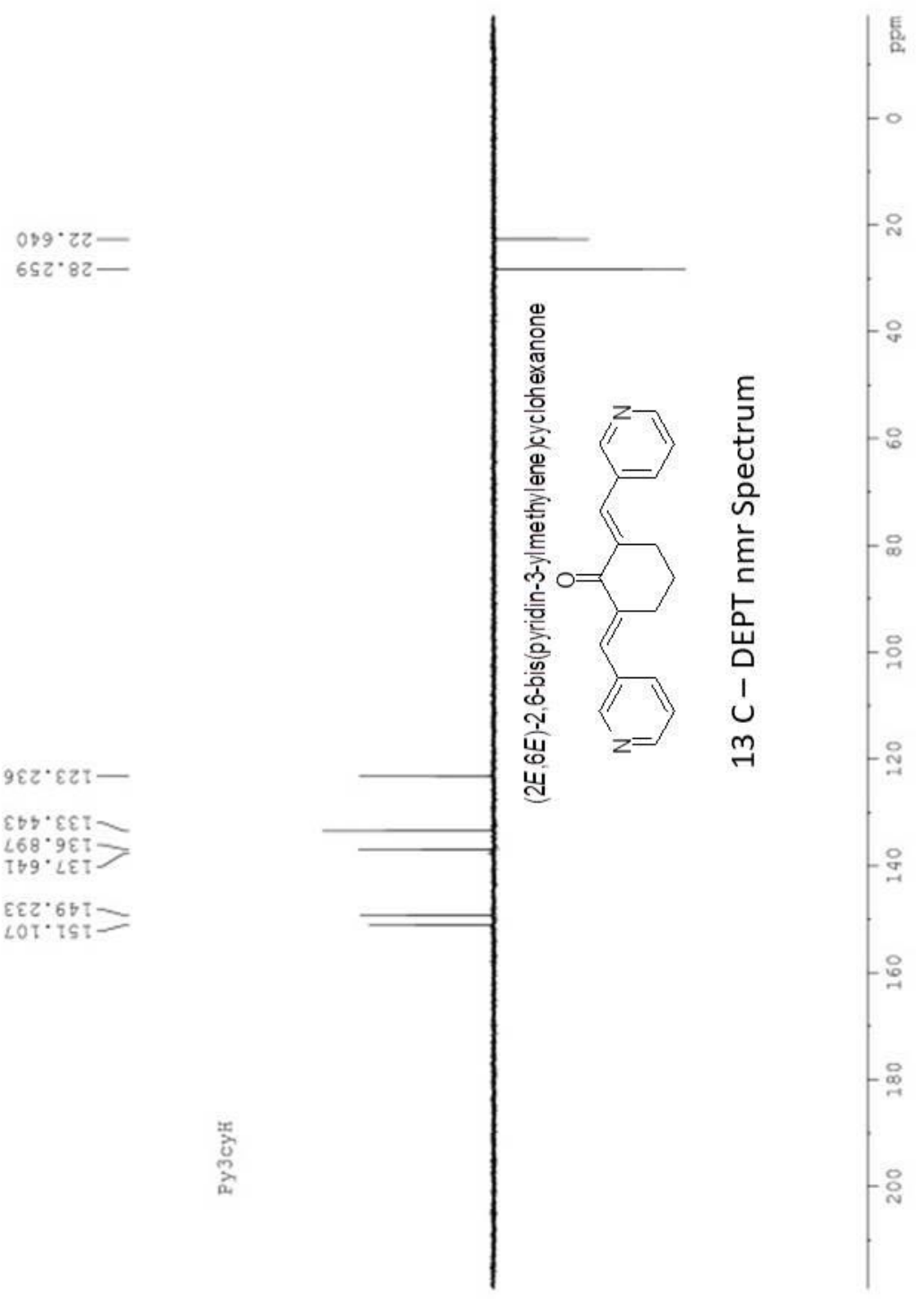


Figure-S80: The DEPT-^{13}C-NMRspectrum of MOLECULE-71

Figure-S81:

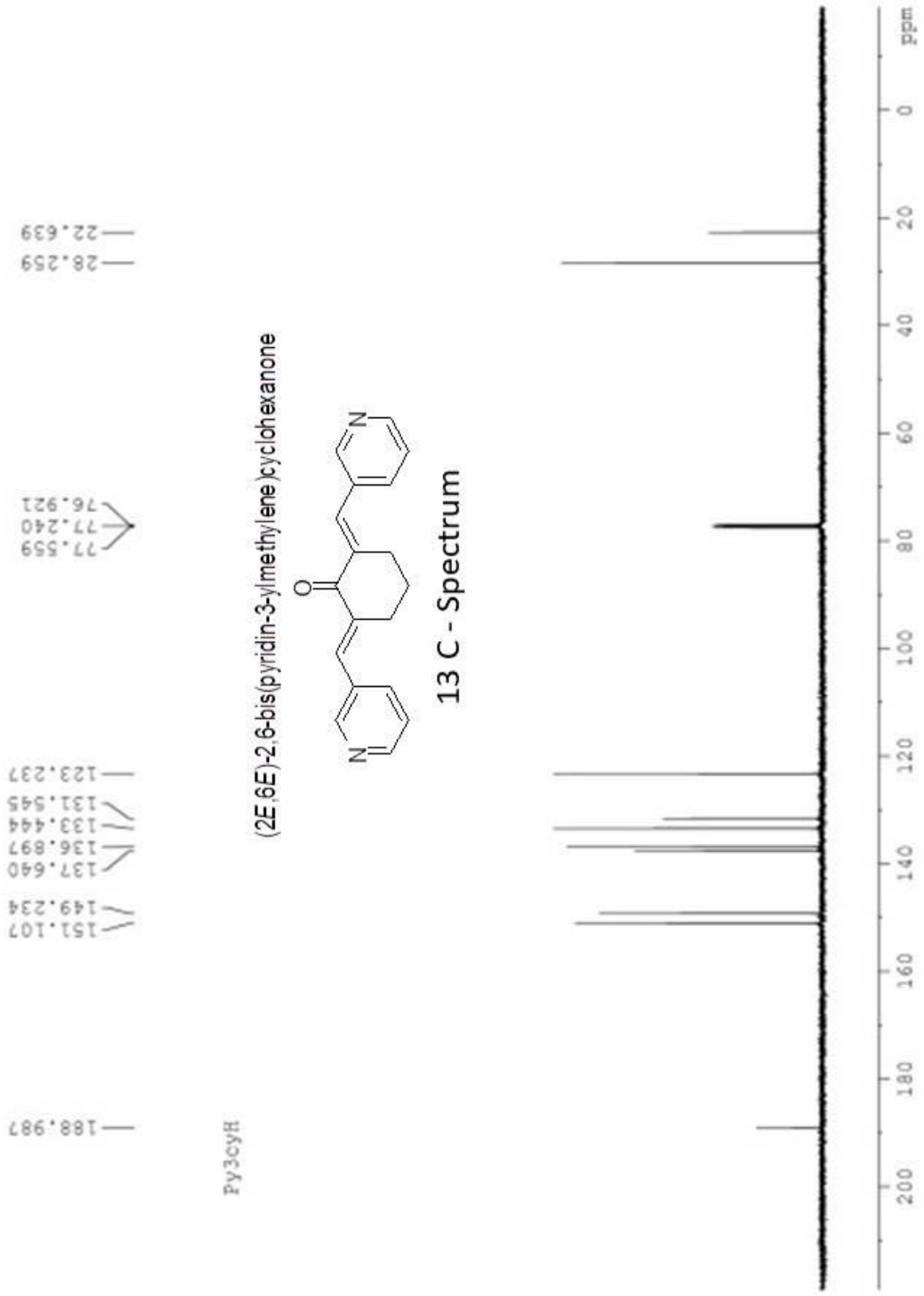


Figure-S81: The ^{13}C-NMR spectrum of MOLECULE-71

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Research Article

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Future Medicinal Chemistry

Development of pyridine dicoumarols as potent anti HIV-1 leads, targeting HIV-1 associated topoisomeraseIIβ kinase

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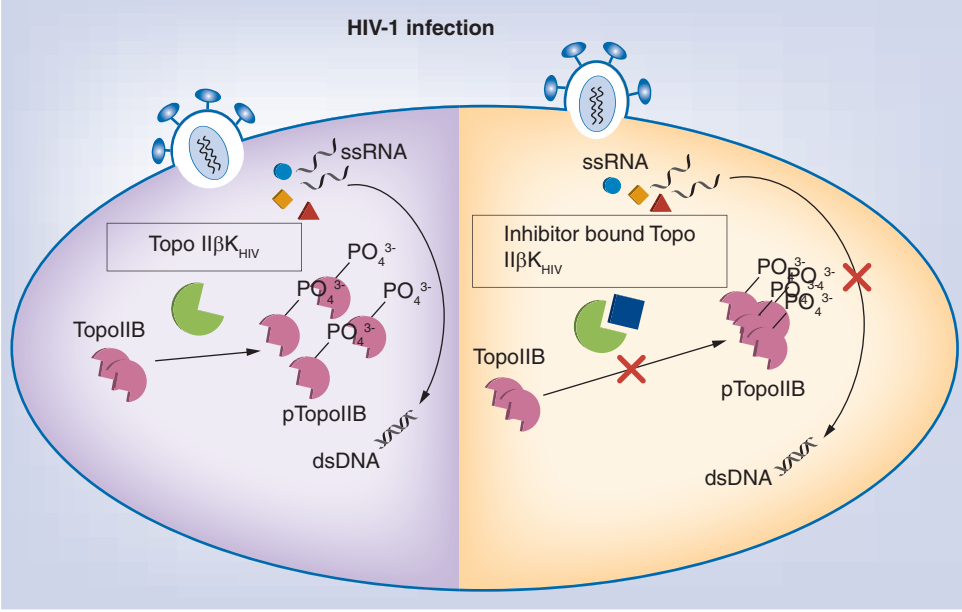
Aim: A structural study of a series of pyridine dicoumarol derivatives with potential activity against a novel Topoisomerase IIβ kinase which was identified in the HIV-1 viral lysate, compounds were designed and synthesized based on a 3D-QSAR study. **Materials & methods:** Based on QSAR model we have designed and synthesized a series of pyridine dicoumarol derivatives and characterized by spectral studies, all the molecules are biologically evaluated by kinase assay, cytotoxicity assay, ELISA and PCR method. **Result:** We demonstrated the achievement of water soluble disodium pyridine dicoumarate derivatives showing high anti-HIV-1 activity (IC_{50} <25 nM) which provides a crucial point for further development of pyridine dicoumarol series as HIV-1-associated topoisomerase IIβ kinase inhibitors for clinical application against AIDS. **Conclusion:** A new class of anti-HIV-1 lead compounds have been designed and tested. Further studies would result in development of novel and potential drugs.



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Keywords: 3D-QSAR • 3-phenyl pyridine • 4-hydroxycoumarin • CoMFA • drug design • HIV-1 • p24 • pyridine aldehydes • pyridine dicoumarols • topoisomerase II β kinase

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Research on HIV therapeutics has exponentially increased in the past two decades. The current antiretroviral therapeutics principally target viral integrase, reverse transcriptase, protease and envelope protein, that are responsible for the entry, infectivity and establishment of the virus infection. HIV-1 entry is mediated in CD4 receptor and CXCR4 or CCR5 co-receptors; their inhibitors were analyzed for targeting HIV-1 infection, these studies identified CCR5 receptor of the host cell as potential target. Maraviroc [1], an inhibitor of CCR5, has been approved in 2007 for HIV-1 treatment experienced patients with prior knowledge in safety of this drug in these patients. However, in the wake of emergence of virus resistance to the existing drugs, there is an urgent need in identification of new targets for controlling virus infection and overcome drug resistance.

A direct correlation has been observed between the decrease in the efficiency of HIV-1 replication and the depletion of Topoisomerase II (Topo II) level by the antisense oligonucleotide [2], SiRNA knockdown and poisoning of Topo II [3]. SiRNA-mediated knockdown of Topo II isoforms shown to play a critical role in formation of intermediates in HIV-1 reverse transcription [4] and HIV-1 tat mediated viral mRNA synthesis [5]. Thus, Topo II isoforms could be of potential interest in targeting HIV-1 replication. Since Topo II isoforms are cellular proteins and required for maintenance of several housekeeping functions of cells, targeting these enzyme will be lethal to cell survival.

Catalytic activity of Topo II isoforms has been regulated by phosphorylation, several cellular kinases reported to phosphorylate these enzymes [6]. Our earlier studies showed that Topo II isoforms are differentially phosphorylated by two fractions of purified HIV-1 lysate [7]. One fraction that predominantly phosphorylated Topo IIα isoform is sensitive to MAP kinase inhibitor PD98059 [7]. A novel, 72 kDa protein, HIV-1-associated Topoisomerase IIβ kinase (Topo $IIβK_{HIV}$), has been found to be present in second fraction of purified HIV-1 virus lysate [7,8]. Topo $IIβK_{HIV}$ is a Ser/Thr kinase (STK) and is shown to be resistant to a panel of 20 potent STK and tyrosine kinase inhibitors including staurosporine and PD98059 [8], thus establishing Topo $IIβK_{HIV}$ is a novel kinase that is expressed during HIV-1 infection and encapsulated into the virus. Since Topo $IIβK_{HIV}$ is not found to be present in healthy uninfected T cells, it can form unique target for interfering HIV-1 replication in infected cells [8].

A series of pyridine derivatives were evaluated and a 3D-QSAR analysis has been performed for understanding insights into the organic frameworks associated in inhibition of Topo $IIβK_{HIV}$ [8]. Comparative molecular field analysis (CoMFA) generated a contour model providing predictive model of probable active site features of Topo $IIβK_{HIV}$ [8]. In the current study, we have critically examined this active site of Topo $IIβK_{HIV}$, designed various organic structural frameworks containing pyridine substructures with a dicoumarol moiety, synthesized and evaluated for inhibitory activity against Topo $IIβK_{HIV}$. Indeed, coumarin compounds are known to possess extensive biological activities and are also used as supplement to food, cosmetics and optical brightening agents. Coumarin ring system is one of the most important substructures found in a wide number of natural products and pharmacologically active compounds such as antibiotics [9] and antitumor drugs [10]. Several methods are reported in the literature for the synthesis of dicoumarols from 4-hydroxycoumarin and aldehydes in the presence of different catalysts. However, these methods require prolonged reaction

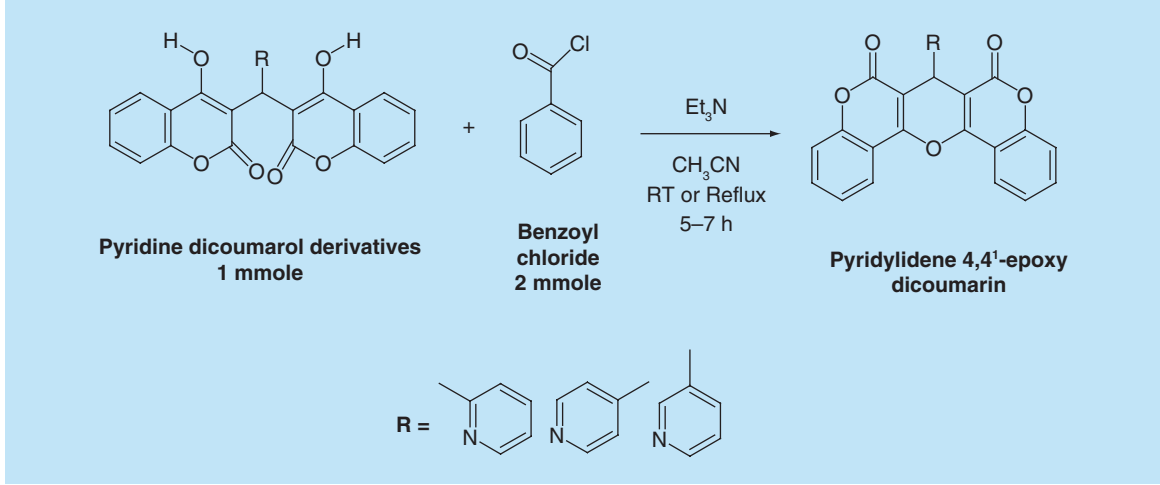

Figure 1. Synthesis of pyridylidene 4,4¹-epoxy dicoumarin derivatives.

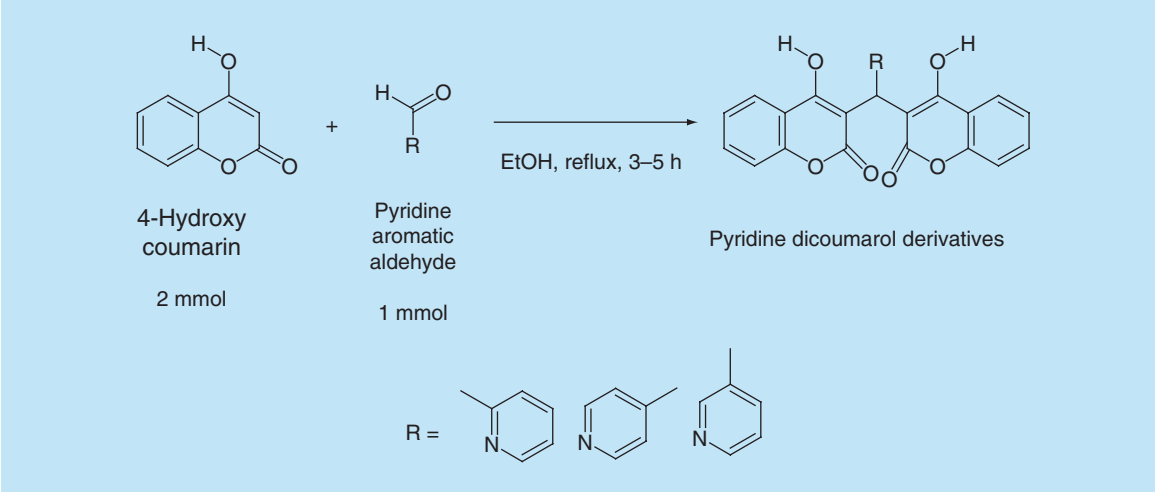

Figure 2. Synthesis of pyridine dicoumarol derivatives.

time and exotic reaction conditions and some of these procedures require the use of toxic organic solvents, expensive catalysts and tedious process. There has been growing recognition for water as an attractive medium for many organic reactions, resulting in less expensive, less dangerous and environmentally friendly reactions, such as Diels–Alder reactions [11], Claisen rearrangement reaction [12,14], Reformatsky reactions [13,16] and Pinacol-coupling reactions [14]. Recent reports suggest that dicoumarol derivatives could be obtained through condensation of 4-hydroxycoumarin and different aldehydes under various conditions in aqueous media [15,16]. In this study we synthesized pyridine dicoumarol derivatives under conventional reflux conditions in ethanol and analyzed their effect on Topo IIβK_{HIV}. Results showed that pyridine dicoumarol derivatives inhibit TopoIIβ kinase activity *in vitro* along with anti-HIV-1 activity.

Chemistry

Pyridylidene-4, 4¹-epoxy dicoumarin derivatives

General procedure for the synthesis of 4,4¹-epoxydicoumarin derivatives (UHAKKM-**1,2,3**): a mixture of dicoumarol derivative (1 mmol) and benzoyl chloride (2 mmol) in 10 ml of acetonitrile in the presence of 4 or 5 drops triethylamine were stirred at room temperature or under conventional reflux conditions (Figure 1). The completion of reaction was monitored by thin layer chromatographic analysis. After completion of the reaction, the reaction mixture was cooled to room temperature. The solid products were collected by filtration methods, finally washed with hot methanol and dried to give the desired products (UHAKKM-**1, 2, 3**). Pure products were obtained from column chromatography [17].

Note: Except 4- pyridylidene-4,4¹-epoxy dicoumarin, all derivatives were prepared at room temperature.

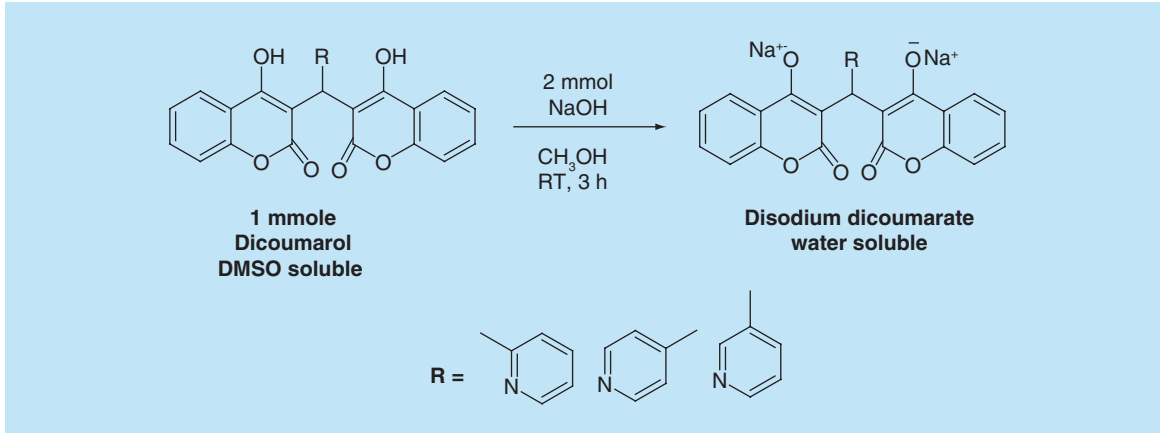

Figure 3. Synthesis of disodium dicoumarate derivatives.

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Table 1. Reaction condition and reaction time of synthesis of pyridylidene-4, 4[1]-epoxy dicoumarin derivatives.

Entry	R	Product	Reaction condition	Reaction time
1	2-C_5H_4N	UHAKKM-1	RT	5–6 h
2	4-C_5H_4N	UHAKKM-2	REFLUX	7–8 h
3	3-C_5H_4N	UHAKKM-3	RT	4–5 h

Spectral characterization

UHAKKM-1: (2-pyridylidene-4,4[1]-epoxy dicoumarin)

White solid (yield: 90%); mp: 198–202°C; ^{1}H-NMR (DMSO) δ: 5.28 (s, 1H, CH), 7.10–8.39 (m, 12H, Ar-H),; ^{13}C-NMR ($CDCl_3$) δ: 37.41, 105.20, 113.69, 117.07, 122.52, 124.52, 125.52, 132.66, 133.92, 136.31, 149.58, 152.81, 154.42, 159.13, 160.63,; IR (KBr) ν: 1719, 1671, 1609, 1388, 1048, 750 cm^{-1}; HRMS (ESI+) [M+H]: calcd. for$C_{24}H_{13}NO_5$ 396.087, found 396.100.

UHAKKM-2: (4- pyridylidene-4,4[1]-epoxy dicoumarin)

White solid (yield: 85%); mp: 213–216°C; ^{1}H-NMR (DMSO) δ: 5.17 (s, 1H, CH), 7.52–8.82 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 36.01, 103.19, 113.29, 117.16, 124.11, 125.61, 128.06, 134.23, 142.61, 152.77, 154.83, 159.35, 160.20; IR (KBr) ν: 1730, 1710, 1667,1607, 1383, 757 cm^{-1}; HRMS (ESI+) [M+H]: calcd. for $C_{24}H_{13}NO_5$ 396.087, found 396.088.

UHAKKM-3: (3- pyridylidene-4,4[1]-epoxy dicoumarin)

White solid (yield: 94%); mp: 226–228°C; ^{1}H-NMR (DMSO) δ: 5.11 (s, 1H, CH), 7.54–8.99 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 26.76, 103.84, 113.47, 117.14, 124.05, 125.53, 125.94, 133.99, 152.77, 154.60, 160.31; IR (KBr) ν: 1743, 1711, 1607, 1237, 1006, 758 cm^{-1}; HRMS (ESI+) [M+H]: calcd. for $C_{24}H_{13}NO_5$ 396.087, found 396.088.

Table 2. Selected experimental IR frequencies of the pyridine dicoumarols and disodium pyridine dicoumarates.

Compound	ν OH/H_2O	ν (C=O)	ν (C=C)
UHAKKM-4	3124 m 3068 m	1698 s 1639 s	1614 s 1538 s
UHAKKM-7	3211 br	1630 s 1595 s	1496 s
UHAKKM-5	3088 m 3068 m	1684 s 1625 s	1598 s 1541 s
UHAKKM-8	3216 br	1629 s 1597 s	1476 s
UHAKKM-6	3135 m 3060 m	1686 s 1614 s	1536 s 1462 s
UHAKKM-9	3212 br	1630 s 1598 s	1511 s

3, 3′-(Pyridine-n-ylmethylene)bis(4-hydroxy-2H-chromen-2-one) derivatives

4-hydroxycoumarin (2 mmol) was reacted with pyridine-aldehydes (1 mmol) in ethanol solution under refluxing temperature for 3–5 h and the solid white crystals were obtained and filtered off and recrystallized from dioxane (Figure 2). After that we characterized the pure products. We synthesized three pyridine possible dicoumarol derivatives [18].

Spectral characterization

UHAKKM-4

3,3'-(Pyridin-2-ylmethylene)bis(4-hydroxy-2H-chromen-2-one): whitesolid (yield: 50%); mp: 224–228°C; ^{1}H-NMR (DMSO) δ: 6.54 (s, 1H, CH), 7.26–8.65 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 37.04, 100.88, 116.27, 119.81, 123.73, 124.75, 124.88, 126.30, 132.27, 142.22, 146.82, 153.26, 157.98, 164.31, 168.97; IR (KBr) ν: 3124, 3068, 2879,1698, 1639, 1614, 1538, 1350, 1180, 1063, 882, 760 cm^{-1}; MS(ESI) m/z [M+H]$^+$ 414: [M+Na]$^+$ 436:HRMS (ESI+) [M+H]: calcd. for $C_{24}H_{15}NO_6$ 414.09, found 414.10.

UHAKKM-5

3,3'-(Pyridin-4-ylmethylene)bis(4-hydroxy-2H-chromen-2-one): white solid (yield: 63%); mp: 248–250°C; ^{1}H-NMR (DMSO) δ: 6.47 (s, 1H, CH), 7.25–8.69 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 38.26, 101, .90, 116.23, 119.83, 123.69, 124.69, 125.68, 132.09, 141.41, 153.16, 164.53, 165.31, 168.60; IR (KBr) ν: 3088, 3068, 2890, 2622, 1684, 1541, 1495, 1405, 1330, 1273, 1175, 1107, 855, 805, 759 cm^{-1}; MS (ESI) m/z [M+H]$^+$ 414, [M+Na]$^+$ 436:HRMS (ESI+) [M+H]: calcd. for $C_{24}H_{15}NO_6$ 414.09, found 414.10.

UHAKKM-6

3,3'-(Pyridin-3-ylmethylene)bis(4-hydroxy-2H-chromen-2-one): white solid (yield: 75%); mp: 258–260°C; ^{1}H-NMR (DMSO) δ: 6.45 (s, 1H, CH), 7.23–8.72 (m, 12H, Ar-H); ^{13}C-NMR (DMSO) δ: 35.13, 102.10, 116.19 119.94, 123.61, 124.63, 127.11, 131.98, 139.53, 140.74, 143.27, 145.25, 153.16, 164.48, 168.46; IR (KBr) ν: 3080, 3060, 2909, 2157, 1686, 1614, 1536, 1330, 1180, 1050, 1017, 900, 759 cm^{-1}; MS (ESI) m/z [M+H]$^+$414, [M+Na]$^+$ 436:HRMS (ESI+) [M+H]: calcd. for $C_{24}H_{15}NO_6$ 414.09, found 414.10.

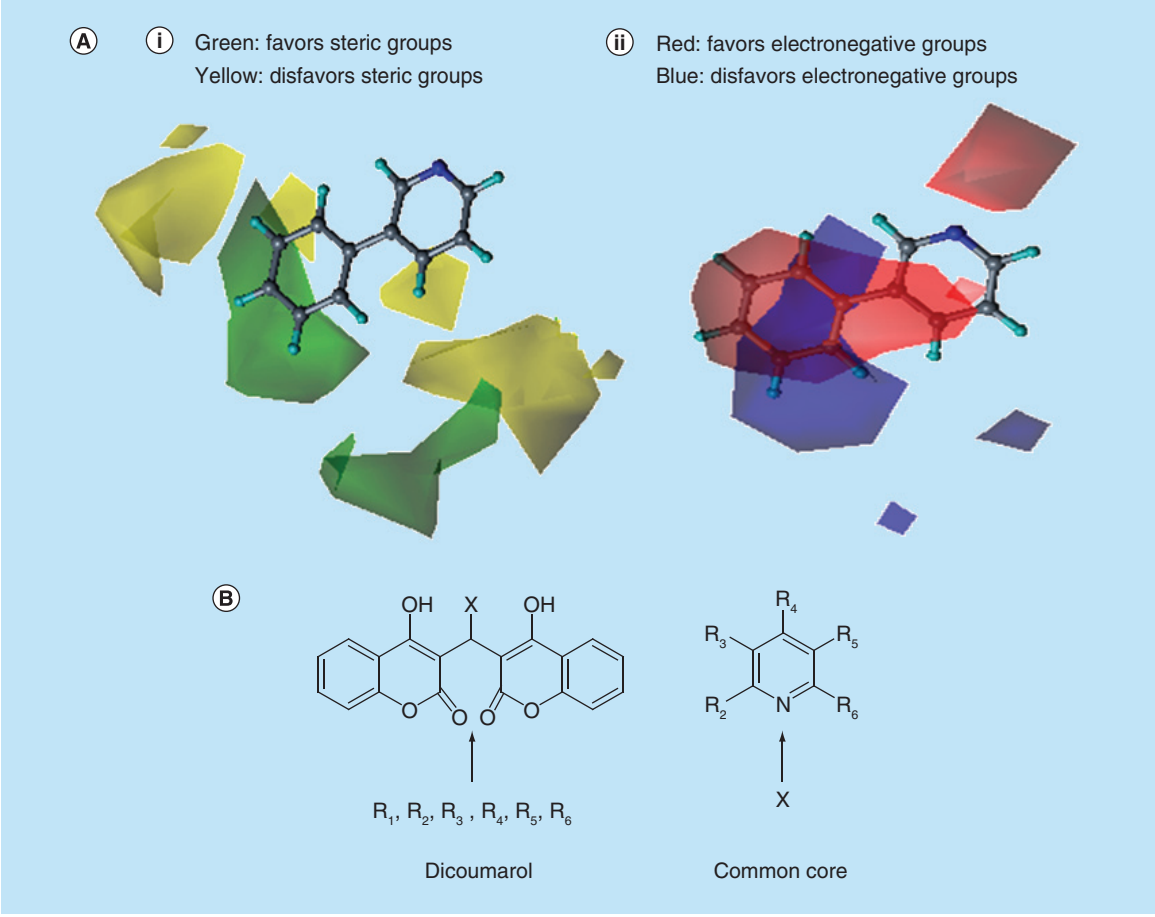

Figure 4. 3D-QSAR model. (A) CoMFA model depicting the steric electrostatic contour of active molecules. The 3D contour maps around the highest activity molecule (3-phenyl pyridine) generated by CoMFA analysis of the derivatives. **(i)** Regions where hydrophobic substitution enhances (green) or reduces (yellow) the binding affinity. **(ii)** The color codings indicate regions where electronegative substituent enhance (red) or reduce (blue) the binding affinity. **(B)** The structures of derivatives with a coumarin moiety at different positions of the core pyridine ring (X) (R1, R2, R3, R4, R5, R6 = dicoumarol) designed based on the contours.
CoMFA: Comparative molecular field analysis.
For color figures please see www.future-science.com/doi/full/10.4155/fmc-2017-0091

Disodium pyridine dicoumarate derivatives

Pyridine dicoumarol derivatives (1 mmol) were taken in 25-ml round bottom flask, to this 10 ml methanol and 2 mmol NaOH were added, the reaction mixture was stirred for 3 h. After obtaining clear solution, the solvent was evaporated under reduced pressure and solid products were recovered (Figure 3). The products soluble in water completely [19].

Spectral characterization

IR-spectral analysis of disodium pyridine dicoumarate compounds

The mode of binding of the pyridine dicoumarol derivatives to sodium was elucidated by recording the IR spectra of the disoidium pyridine dicoumarate derivatives as compared with this of the free pyridine dicoumarol derivatives.

IR-spectra of the compounds were recorded on solid state in JASCO IR-A-302 Spectrometer and Fourier transform IR in the range 4000–400 cm^{-1}. The data of the IR spectra of pyridine dicoumarol derivatives and disodium pyridine dicoumarol derivatives are presented in Table 2.

IR-spectrum of disodium pyridine-2-dicoumarate (UHAKKM-7)

The bands appear in the IR spectrum of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-pyridin-2-yl-methane(UHAKKM-4) at 3124, 3068, 1698, 1639, 1614, 1538 cm^{-1}. The bands at 1698 and 1639 cm^{-1}can be attributed to the stretching vibrations of the carbonyl groups of the lactone rings. Bands at 1614 and 1538 cm^{-1} can be related to the stretching vibrations of conjugated olefinic system. A broad band, characteristic of

$_V$OH of coordinated water was observed in the range 3100–3500 cm^{-1} in the spectrum of the disodium pyridine-2-dicoumarate (UHAKKM-7). The weak bands observed at 3124 and 3068 cm^{-1}in the spectrum of the UHAKKM-4 is missing in the spectrum of the UHAKKM-7. A comparison of the IR – spectrum of the UHAKKM-4 and UHAKKM-7 reveals the disappearance of absorption bands observed in the UHAKKM-4 at 3124 and 3068 cm^{-1} indicating the loss of enolic protons on UHAKKM-7 compound. The $_V$ (C=O) bands at 1698 and 1639 cm^{-1} exhibit a shift of 40–60 cm^{-1} to lower wave number values on UHAKKM-7, which may be taken as evidence for the participation of the C =O groups in coordination with sodium ion.

Remaining compounds (UHAKKM-8, UHAKKM-9) also show similar IR spectral properties as UHAKKM-7.

HRMS

UHAKKM-7(disodium-3,3'-[pyridin-2-ylmethylene] bis[2-oxo-2H-chromen-4-olate]): HRMS (ESI+) (M+H): calcd. for

$C_{24}H_{13}NO_6^{2-}.2Na^{\oplus}$ 458.067478, found 458.0617.

UHAKKM-8 (disodium-3,3'-[pyridin-4-ylmethylene]bis[2-oxo-2H-chromen-4-olate]): HRMS (ESI+) (M+H): calcd. for

$C_{24}H_{13}NO_6^{2-}.2Na^{\oplus}$ 458.067478, found 458.0611.

UHAKKM-9 (disodium-3,3'-[pyridin-3-ylmethylene]bis[2-oxo-2H-chromen-4-olate]): HRMS (ESI+) (M+H): calcd. for

$C_{24}H_{13}NO_6^{2-}.2Na^{\oplus}$ 458.067478, found 458.0618.

Biological evaluation

Kinase phosphorylation and inhibition assay: 72kDa Topo IIβK_{HIV} was purified and its activity tested as per the protocol mentioned in the supplementary section (Supplementary Figure 1). The Topo IIβK_{HIV} activity in phosphorylating Topoisomerase II β was measured at increasing concentrations. Caesin Kinase II was used as a standard (Supplementary Figure 2). Purified Topoisomerase II β was obtained and dephosphorylated as mentioned in the supplementary section. Dephosphorylated topoisomerase II β was treated with Topo IIβK_{HIV} at 100 µM cold ATP and 5 µCi of [γ -^{32}P] hot ATP in 1× kinase buffer in the presence of test compounds at varying concentrations, 3 ng of topoisomerase II β antibodies and 6% protein A–agarose beads were added, the beads were washed with TBS and eluted with 5% trichloroacetic acid (TCA). Elute was spotted on Whattman paper discs and dried and ^{32}P was measured by liquid scintillation. Each experiment was performed in triplicate and all data points represent an average of results from the triplicate experiments. In all the assays, 3-phenyl pyridine was used as a reference standard [8].

Assay to measure inhibition on HIV-I infectivity in SupT1 cells

Cytotoxicity of compounds in SupT1 Cells

Cytotoxicity assay was performed with MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) in SupT1 cells. Briefly, SupT1 cells were grown in RPMI 1640 media with 10% FBS (fetal bovine serum), approximately 0.02 × 10^6 cells were seeded in 100 µl of complete media. After overnight incubation with compounds, cell viability was determined by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reagent (5 mg/ml). Ten microliter MTT was added to each well and incubated for 4 h in CO_2 incubator, 100 µl of DMSO was added to all the wells, plate was gently swirled and kept in dark for 2 h at RT. Absorbance was measured at 570 nm in a microtiter plate reader, each assay was

Table 3. Theoretical and experimental activities of the designed compounds.

S. No	R	Molecules	Theoretical pIC_{50} values	Experimental pIC_{50} values	IC_{50}concentration (pM)
1	2-C_5H_4N	UHAKKM-4	8.89	9.56	2.7 ± 0.4 × 10^2
2	2-C_5H_4N	UHAKKM-1	9.09	9.52	3.0 ± 0.21 × 10^2
3	4-C_5H_4N	UHAKKM-5	9.49	9.56	2.8 ± 0.14 × 10^2
4	4-C_5H_4N	UHAKKM-2	9.09	9.04	2.3 ± 0.17 × 10^2
5	3-C_5H_4N	UHAKKM-6	9.77	9.51	3.1 ± 0.21 × 10^2
6	3-C_5H_4N	UHAKKM-3	9.84	9.38	4.2 ± 0.34 × 10^2
7	2-C_5H_4N	UHAKKM-7	11.89	12.04	0.9 ± 0.03
8	4-C_5H_4N	UHAKKM-8	11.72	12.05	0.8 ± 0.06
9	3-C_5H_4N	UHAKKM-9	11.94	12.06	0.8 ± 0.05

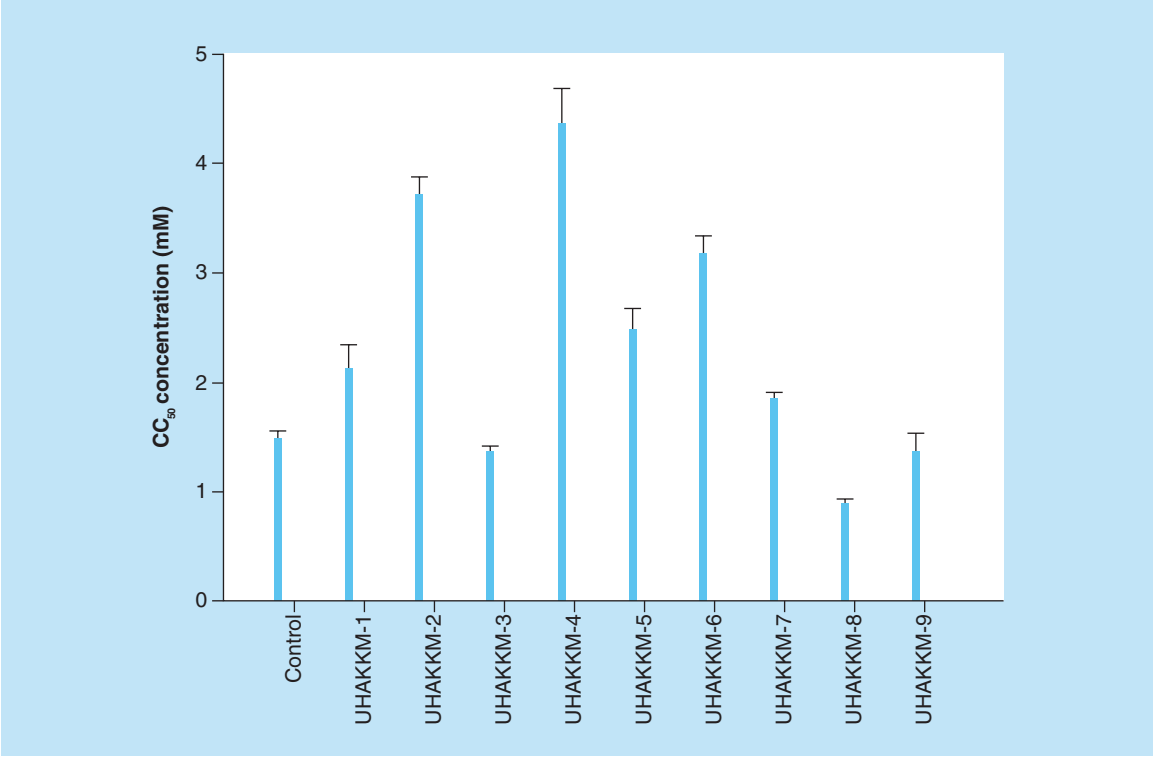

Figure 5. MTT cytotoxicity assay. The graph depicting the CC_{50} (50% cytotoxic concentration) of the compounds, the compounds have 50% cell cytotoxicity even at high concentration (>1 mM), 3-phenyl pyridine used as a positive control.

repeated at least three times and in triplicates. The IC_{50} (concentration of 50% inhibition) value was calculated using SigmaPlot v11.0 (Systat Software, CA, USA).

Antiviral assay

SupT1 cells with 99% confluence were seeded in 24-well plates and infected with HIV-$1_{93IN101}$ at a final concentration equivalent to 1 ng^{-1} ml and then compounds were added to the wells. The infected cells were incubated for 5 h at 37°C in a 5% CO_2 incubator. After 5 h the cells were washed and pelleted at 350× *g* for 10 min, the supernatant was discarded and the infected Sup T1 pellet was resuspended in fresh RPMI1640 complete media containing 10% FBS and further incubated for 96 h in 5% CO_2.

After 96 h the supernatants were collected and analyzed by using a p24 antigen capture ELISA method (Advanced Bioscience Laboratories, MD, USA). The extent of infection in the absence of test compound was considered to be equivalent to 0% inhibition. Azidothymidine (AZT) and 3-phenyl pyridine were employed as positive controls.

Analysis of proviral DNA synthesis in the presence of drug

SupT1 cells with 99% confluency were seeded in 24-well plate and infected with HIV-$1_{93IN101}$ at a final concentration equivalent to 1 ng per ml and then drug with a varying concentrations was added to the wells. After 5 h of time point the infected cells were harvested and genomic DNA was isolated by conventional phenol/chloroform method and analyzed for the amplification of proviral DNA by specific primers SK 38/39 codes for the *gag* region. Here in this experiment the AZT was used as positive control and the data were analyzed by image J software (US NIH, MA, USA)

Results & discussion

Design & synthesis

3D-QSAR studies carried out previously [8] generated contours which were further refined. Based on the refined contours the molecules in this study have been designed (Figure 4A). From the map (Figure 4A) the desirable and undesirable substitutions in terms of Steric or electrostatically charged groups to the core molecule can be understood. In this model the 4th and 5th positions of the pyridine ring favor addition of steric groups, while the addition such that the conformation aligns the hydrophobic group between 2nd and 3rd positions results in reduced binding affinity. Similarly, an addition of electropositive group at 5th position such that the conformation aligns between structures in Figure 1B were designed to match the

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contours and the coumarin moiety was placed each at 4th, 5th and 6th positions of the ring for compounds UHAKKM-1 to 9, respectively. From the hypothetical pocket model proposed [8], it could be understood that the interaction of 2nd, 3rd positions with the kinase hinge region, restricted the substitutions to only the above-mentioned positions. The coumarin moiety acts as a hydrophobic bulky group which, on substitution in the favorable region, increased the activity of the compound. Similarly, the presence of oxygen atoms on the coumarin group resulted in satisfying the electrostatic constraints. Owing to this the synthesized compounds showed high Topo IIβK_{HIV} antagonism.

Assay to measure the inhibition of kinase activity in presence of synthesized compounds

The inhibition of phosphorylating activity was tested by the kinase assay as per the method specified. The compounds showed inhibition at picomolar concentration. IC_{50} of the compounds was calculated and reported in the Table 3. All the compounds synthesized and tested showed nearly same IC_{50} concentration. The high activity could be explained by the alignment of the bulky groups as specified by the contour map. But disodium pyridine dicoumarate salts show high Topo IIβK_{HIV} inhibition than pyridine dicoumarol derivatives.

All the molecules of the pyridyl- dicoumarol derivatives and disodium pyridine dicoumarate derivatives (1–9) in Table 1 were designed based on the contours generated from the 3D-QSAR studies and the activities were predicted using the generated equation. The high predicted values were found to be similar to the experimental value, all molecules were showing IC_{50} at picomolar concentration. As indicated in the Table 3, the IC_{50} concentration of Topo IIβK_{HIV} inhibition and respective pIC_{50} values.

Compound	Anti-viral) IC_{50} (nm)	Kinase inhibition IC_{50} (pm)
UHAKKM-7	13.5 + 1.3	0.9 ± 0.03
UHAKKM-8	16.4 + 1.7	0.8 ± 0.06
UHAKKM-9	21.4 + 1.9	0.8 ± 0.05

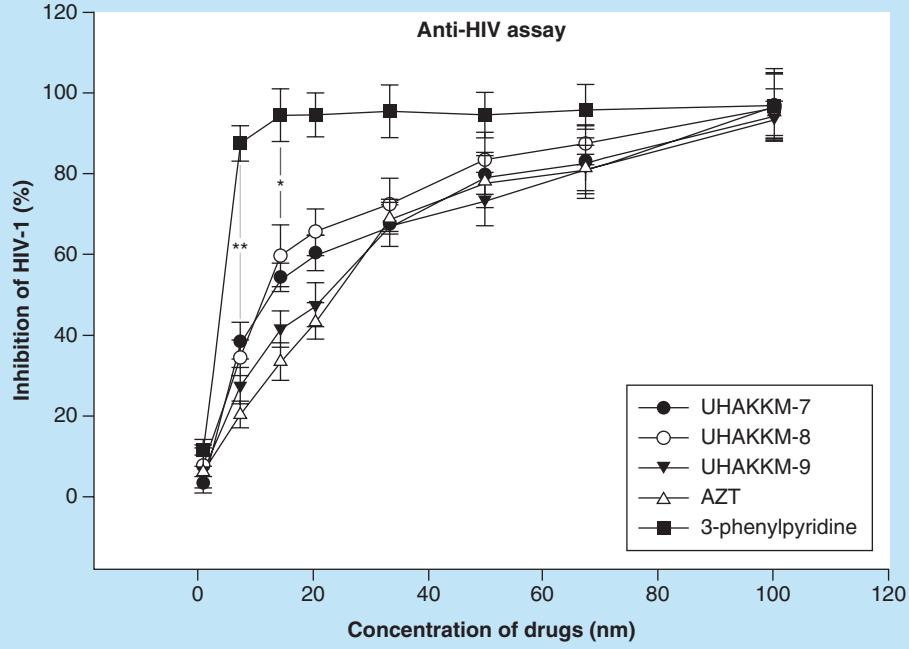

Figure 6. Antiviral assay. The activity of the molecules in inhibiting the viral replication was estimated by p24 assay using a previously established protocol. The molecules UHAKKM-1 to 6 have shown only 20% inhibition at 100 μM concentration. But water soluble disodium pyridine dicoumarate molecules UHAKKM-7 to 9 have shown high inhibition at low concentration, AZT and 3-phenylpyridine were used as positive controls. Data were represented as mean ± standard deviation.
*$p < 0.05$.
**$p < 0.005$.

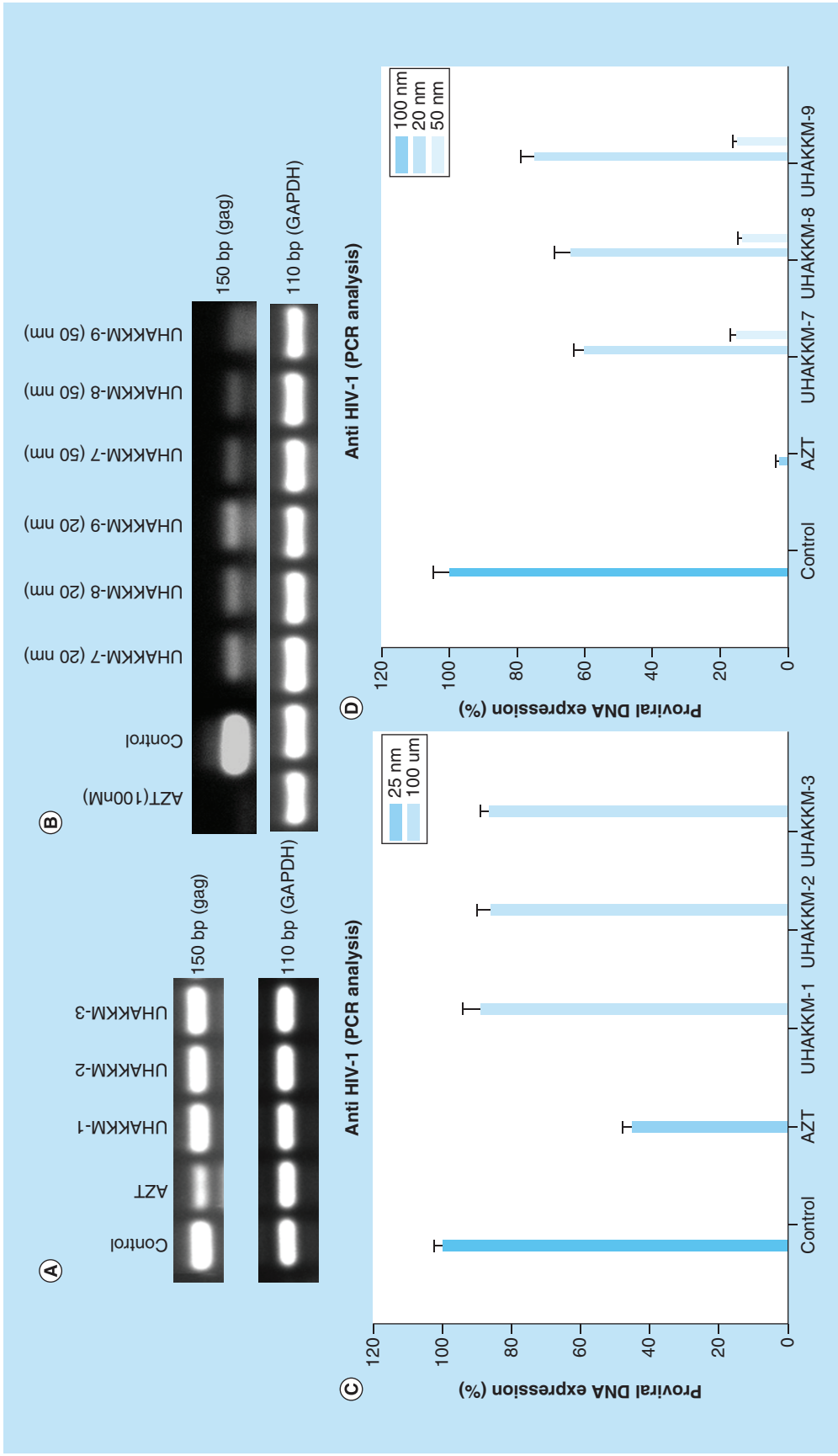

Figure 7. Proviral DNA analysis (PCR analysis). (A) Anti-HIV-1 activity of drug compounds UHAKKM-1, UHAKKM-2 and UHAKKM-3 as depicted by the amplification of *gag* region using sk 38/39 primers. Anti-HIV-1 activity of the drug compounds deduced by image J software. **(B)** Anti-HIV-1 activity of drug compounds UHAKKM-7, UHAKKM-8 and UHAKKM-9 at concentrations 20 and 50 nM as depicted by the amplification of *gag* region using sk38/39 **(C)** Anti-HIV-1 activity of the drug compounds UHAKKM-1,UHAKKM-2 and UHAKKM-3 deduced by image J software. **(D)** Anti-HIV-1 activity of the drug compounds UHAKKM-7,UHAKKM-8 and UHAKKM-9 deduced by image J software.

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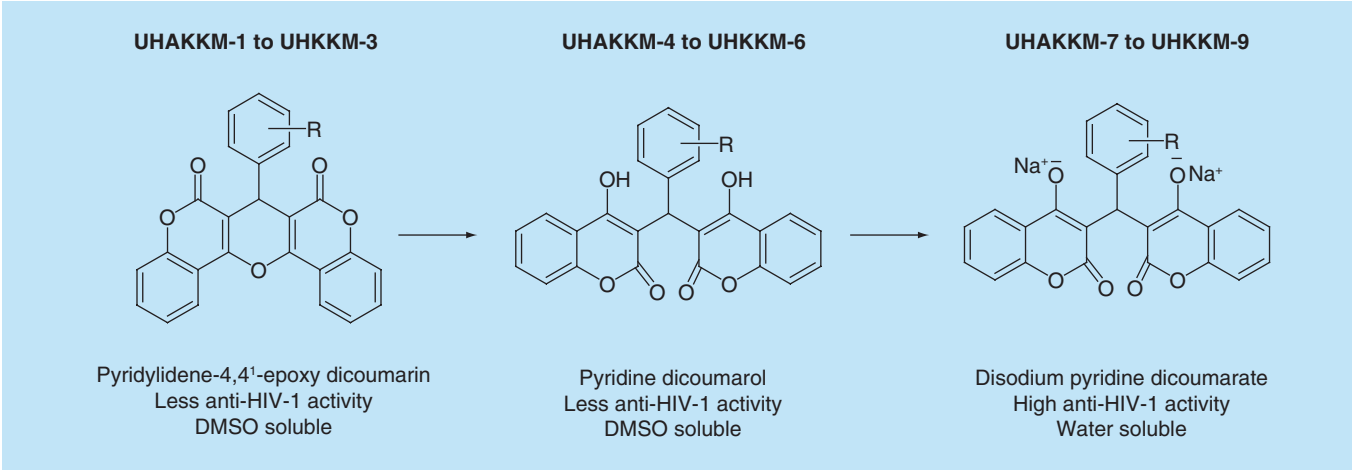

Figure 8. Sequential development of water soluble disodium pyridine dicoumarate derivatives.

MTT assay

The toxicity of molecules in SupT1 cells was tested and plotted (Figure 5). All the compounds showed CC_{50} (50% cytotoxic concentration) above 1 mM concentration.

HIV-$I_{93IN101}$ viral inhibition assay

The activity of the molecules in inhibiting the viral replication was tested using the p24 antigen capture sandwich ELISA method (p24 assay) for UHAKKM-1 to 9 and the proviral DNA was analyzed by PCR method for UHAKKM-1 to 3 and UHAKKM-7 to 9 by using the primers sk38 and sk39 specific to *gag* region of HIV-1.

The molecules UHAKKM-1 to 6 have shown low viral inhibition (20–30%) at more than 100 µM and the molecules UHAKKM-7 to 9 have shown high inhibition at low concentration (Figure 6).

Proviral DNA analysis (PCR analysis)

SupT1 cells were infected with HIV-$1_{93IN101}$ for 5 h in the presence of below the CC_{50} concentration, after 5 h DNA was isolated and amplification was quantified by using sk38/39 specific primers to *gag* region, AZT was used as positive control at (25 and 100 nM) and GAPDH was used as PCR internal control as shown, and the drug compounds UHAKKM-7, UHAKKM-8, UHAKKM-9 are showing viral inhibition at 20 and 50 nM concentration, as shown in Figure 7C & D.

Discussion

Coumarin is a phytochemical found in a large number of plants like Tonka beans, lavender, licorice, among others, that has been shown to have antitumor, antifungal and anticoagulant activities. Number of drugs with coumarin moiety against chronic infections, inflammation, blood anticoagulation and high protein edema [20] have been studied and reported. A previous study of this structural framework reported a considerable activity against the HIV-1 integrase [21].

In this study, we have designed and sequentially developed a series of pyridine dicoumarol derivatives with potential anti-HIV-1 activity (Figure 8). These compounds specifically target the novel Topo IIβK_{HIV} that was shown to be responsible for phosphorylation of topoisomerase IIβ [3] necessary for HIV-1 replication. The compound UHAKKM-7 showed antiviral activity with an IC_{50} of 13 nM, which was seen to be on par with the widely used and clinically used, AZT that has an IC_{50} of 25 nM. The potency of this compound is also better than many of the US FDA-approved anti-HIV-1 drugs. The design of compounds UHAKKM-7, 8 and 9 was done based on 3D-QSAR contours obtained in a previous study and the structures were then optimized to obtain better activity. The designed structures satisfied the contour constraints as shown in Figure 9 and thus showed good Topo IIβK_{HIV} antagonism.

The activity of the molecules in inhibiting viral replication was tested using the p24-ELISA as described earlier. It was observed that UHAKKM-1 to 6 showed low anti-HIV-1 activity even at concentrations higher than 100 µM. However, water soluble disodium pyridine dicoumarate molecules UHAKKM-7 to 9 have shown high inhibition at low concentration (IC_{50} < 30 nM). We also tested other disodium aromatic dicoumarate compounds but surprisingly they did not show any viral inhibition and very low TopoIIβK_{HIV} inhibition.

Pyridine epoxy dicoumarin derivatives (UHAKKM-1 to 3) showed high inhibition of Topo IIβK_{HIV} activity at low concentration but surprisingly have not shown anti-HIV-1 activity even at high concentration (µM). To increase the flexibility of UHAKKM-1 to 3, another set of compounds,

pyridine dicoumarol derivatives (UHAKKM-4 to 6) were synthesized, these are decyclized structures with open ring system that have hydroxyl groups and thus reducing the rigidity. Interestingly, these compounds also showed similar activity as UHAKKM-1 to 3. In the second set of compounds (UHAKKM-4 to 6), the presence of hydroxyl groups leads to the formation of intramolecular hydrogen bonds further making the compounds rigid [22]. From the above results it was suspected that these molecules showed low activity because of the lack of flexibility to reach the intracellular target, as seen in p24 assay and also the PCR results.

Thus to increase the flexibility of pyridine dicoumarols (UHAKKM-4 to 6), the intramolecular hydrogen bonding was removed by using alcoholic NaOH. The resulting compounds solubilized in water because of increased flexibility and formed disodium pyridine dicoumarate salts (Figure 10). Hence, the compounds (UHAKKM-7 to 9) acquire the required properties appropriate to reach the target and the placement of negative charges on enolic oxygen resulting from formation of salts enhances the activity. It was also observed that the absence of pyridine moiety showed decreased antiviral and TopoIIβK$_{HIV}$ inhibition, as seen in case of other disodium aromatic dicoumarate compounds. Hence it was concluded that the pyridine ring, negatively charged enolic oxygen and flexibility plays a crucial role in bioactivity of dicoumarol derivatives.

Conclusion

In conclusion, a structure-based evaluation of substructures would lead to drug development. Structure modification of pyridine dicoumarol derivatives has shown an increase in inhibition of the Topo IIβK$_{HIV}$ activity and antiviral activity.

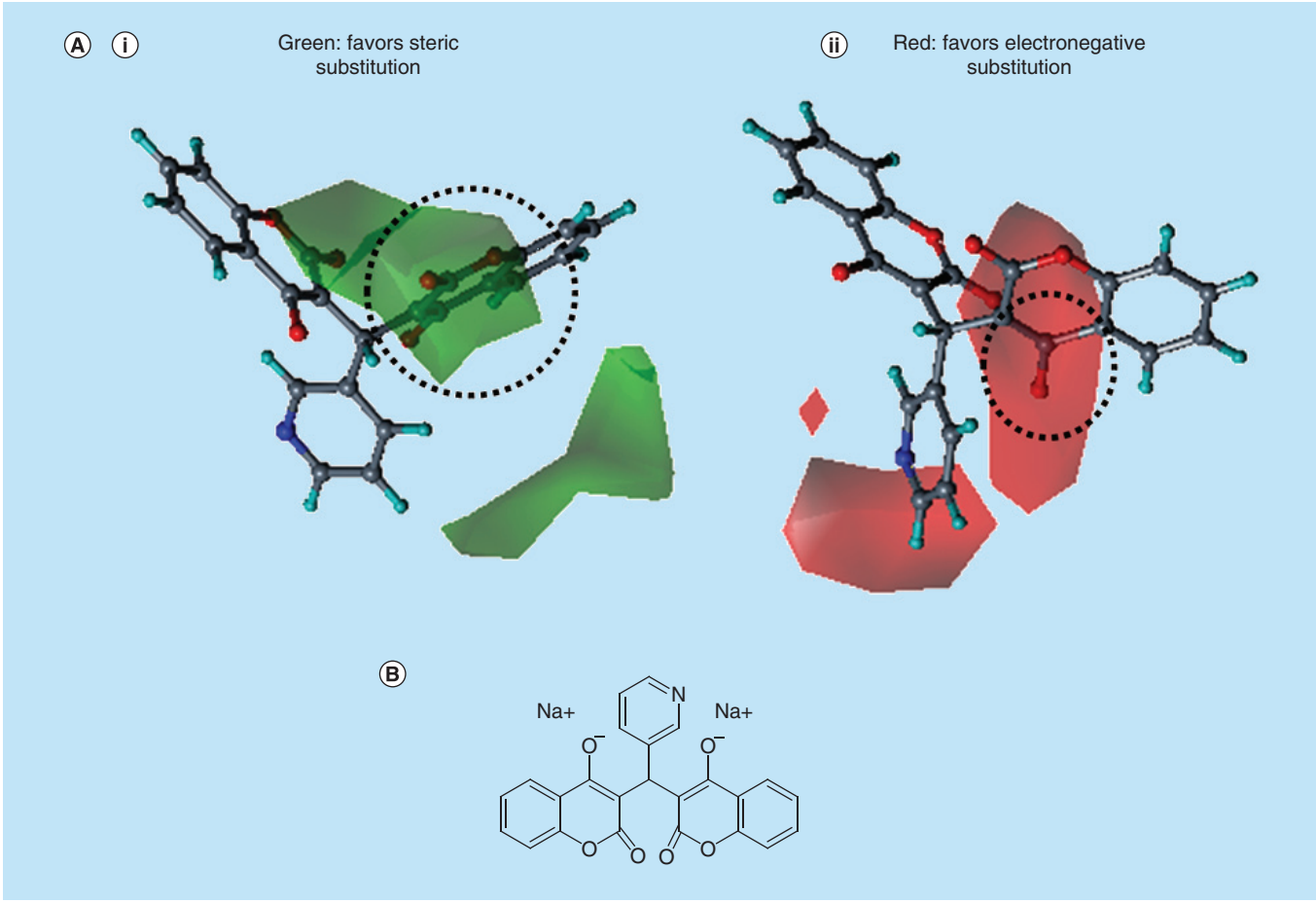

Figure 9. 3D-QSAR contours overlaid on the structure pyridine dicoumarol derivative UHAKKM-9. (A) (i) steric, (ii) electrostatic contours obtained from 3D-QSAR equation. **(B)** Structure of disodium-3,3′-(pyridin-3-ylmethylene)bis(2-oxo-2H-chromen-4-olate). The bulk dicoumarol ring of the compound lies overlapped with the green region of the contour, suggesting the role of the bulk group in the activity of the compound. Similarly, the overlap of red region with the negatively charged enolic oxygen group substitution suggests the role of electronegative groups in the enhanced TopoIIβK$_{HIV-1}$ antagonism.

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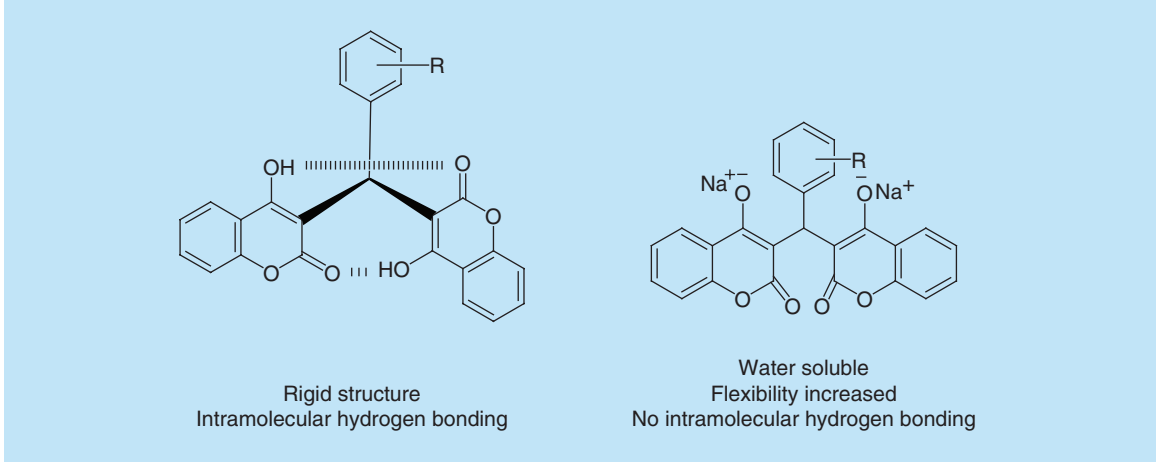

Figure 10. Comparison of structural flexibility of disodium dicoumarate derivatives with pyridine dicoumarol derivates.

Future perspective

This study demonstrates, HIV-1-associated Topo IIβK_{HIV} as a potential target to control HIV-1 infection. We demonstrated the achievement of water soluble disodium pyridine dicoumarate derivatives showing high anti-HIV-1 activity (IC_{50} <30 nM) which provides a platform for development of new class of inhibitors for clinical application against AIDS.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Summary points

- 3D-QSAR was employed to design potent compounds to inhibit Topo IIβK_{HIV}.
- 4-Hydroxy coumarin was used as the starting material and nine compounds were designed and synthesized successively.
- UHAKKM-1 to 3 compounds were synthesized by novel method.
- Synthesized compounds showed high TopoIIβK_{HIV} inhibition.
- Water soluble compounds UHAKKM-7 to 9 showed high anti-HIV-1 activity.
- In conclusion, structural studies on dicoumarols lead to the development of potent anti-HIV-1 compounds targeted to Topo IIβK_{HIV}.

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Design, Synthesis and Development of Potential anti-HIV-1 Inhibitors, Targeted To HIV-1 Associated Topoisomerase IIβ Kinase

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