UNIVERSITY OF KWAZULU-NATAL

DESIGN, SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF NOVEL FUSED PYRIMIDINE ANALOGUES AS ANTICANCER AGENTS

By

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A thesis submitted to the School of Health Science, Discipline of Pharmaceutical science, Department of Pharmaceutical Chemistry, University of KwaZulu-Natal, Westville, for the degree of Doctor of Philosophy.

This thesis has been prepared according to **Format 4** (Thesis by publications) as outlined in the guidelines of College of Health Sciences, University of KwaZulu-Natal. The chapters consist of an overall introduction, chapters in discrete research papers and a final discussion. Two chapters have been published and the remaining chapters have been submitted in peer-reviewed internationally accepted journals.

As the candidate's supervisor, I have approved this thesis for examination/submission.

Supervisor: Dr. R. Karpoormath

Signed: _____   Date: 11/April/2018

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ABSTRACT

Cancer is a multifaceted disease considered as the most serious health burden all over the world. Due to existing of limited anticancer drugs and detrimental side effects, the anticancer research has been challenging. An investigation on identifying novel potential drugs is highly required to treat this serious abnormal cell growth. Advanced potential anticancer drug entrants are crucially required to combat the drawbacks linked with current drugs or line of therapies. Extensive investigations are being carried out on synthetic manipulations of heterocyclic aromatic compounds (purines) for developing efficient and potent anticancer drugs. Besides, these manipulations also offer effective leads for further optimization. Therefore, this project is an effort in detecting a novel and potent anticancer leads based on bioisostere of purines called pyrazolopyrimidines.

In this research project we have performed an comprehensive literature survey of structural isomers of pyrazolopyrimidines (pyrazolo[1,5-*a*]pyrimidine and pyrazolo[4,3-*d*]pyrimidine) for their synthetic approaches and biological activities with special emphasis on structure-activity relationship (SAR) studies. These SAR studies prompted us to implement the observed studies on one of the structural isomer of pyrazolopyrimidine called pyrazolo[3,4-*d*]pyrimidine. And further, we have synthesized some novel series of pyrazolo[3,4-*d*]pyrimidine derivatives with various substituents at C-4 and C-6 positions of the scaffold. A total 71 compounds comprising of phenethyl and pentane hybrids (**7-43**, Chapter 4), benzoyl hybrids (**5a-5h**, **6a-6d** and **7a-7c**, Chapter 5) and lastly phenylcarbamoyl acetamide hybrids (**9a-9s**, Chapter 6) have been synthesized by molecular hybridization approach as outlined in schemes of respective chapters. The completion of reaction and the purity of novel synthesized compounds were confirmed by chromatographic analysis. All the newly synthesized compounds displayed acceptable analysis for their anticipated structures, which were established based on physicochemical and spectral data (IR, ^{1}H NMR, ^{13}C NMR and HRMS).

All synthesized compounds were primarily evaluated for their *in vitro* anticancer activities at Laboratory of Growth Regulators, Centre of the Region Hana for Biotechnological and Agricultural Research, Palacky University & Institute of Experimental Botany ASCR, Slechtitelu 27, 78371 Olomouc, Czech Republic.

From the systematic analysis of anticancer activity, results obtained following key observations were made.

   i. Structural isomers of fused pyrimidines have been looked upon for molecular changes in emerging drug like candidates. Pyrazolopyrimidine is a bioisostere of purines has acquired considerable importance due to its diverse, facile and general synthetic methodologies with great medicinal importance. Several analogs of this scaffold have emerged as a promising leads in the design of some novel pharmacologically active compounds with enhanced

metabolic, pharmacokinetic and pharmacological profiles, representing that there is plenty scope for considering pyrazolopyrimidine as a structural framework for evolving effective leads.

ii. Chapter 4: From the 37 novel phenethyl and alkyl pentane pyrazolo[3,4-*d*]pyrimidine derivatives synthesized and evaluated for CDK2/Cyclin E, Abl kinase inhibitory activity and anti-proliferative activity against K-562 (chronic myelogeneous leukemia) and MCF-7 (breast adenocarcinoma) cell lines. From the tested results, compounds **11** (CDK: IC_{50} = 5.1 µM; Abl: >12.5 µM), **8** (CDK: IC_{50} = 7.8 µM; Abl: >25 µM) and **36** (CDK: IC_{50} = 8.8 µM; Abl: >25 µM) exhibited significant inhibitory activity. Further from this series, most of the synthesized compounds indicated prominent anti-proliferative effects with IC_{50} value ranging from 19.2 µM to 27.4 µM. Incorporation of monosubstituted phenyl groups at C-4 of the pyrazolo[3,4-*d*]pyrimidine nucleus had favored for most prominent anticancer activity.

iii. Chapter 5: Among the 15 novel benzoyl hybrids synthesized and evaluated, compounds **5a** and **6c** displayed (CDK2: IC_{50} = 8.8 µM, 6.8 µM) commendable inhibitory activity and notable anti-proliferative activity ranging from 18.9 µM to 89.3 µM). Presence of heteroatom containing bicyclic moieties at C-4 of the nucleus enhanced both inhibitory and anti-proliferative activity.

iv. Chapter 6: Of the 19 novel phenylcarbamoyl acetamide hybrids synthesized and tested, compounds **9a**, **9c**, **9g**, **9m** and **9p** showed moderate enzymatic inhibitory activity with an IC_{50} value >12.5 µM against both CDK2 and Abl kinases while, remaining compounds of this series could not generate IC_{50} values due to solubility limit (IC_{50} = >25 µM to >100 µM).

DECLARATION 1: PLAGIARISM

I, **Srinivasulu Cherukupalli**, declare that

i. The research reported in this dissertation, except where otherwise indicated, is my original work.

ii. This dissertation has not been submitted for any degree or examination at any other university.

iii. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

iv. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

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DECLARATION 2: PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publications

1. S. Cherukupalli, R. Karpoormath, B. Chandrasekaran, G. A. Hampannavar, N. Thapliyal, V. N. Palakollu, An insight on synthetic and medicinal aspects of pyrazolo[1,5-*a*]pyrimidine scaffold, *European Journal of Medicinal Chemistry*, **2017**, 126, 298-352, https://doi.org/10.1016/j.ejmech.2016.11.019.

   Contributions: I did the literature review and wrote the entire manuscript under the supervision of Dr. Rajshekhar Karpoormath. Rest all the co-authors assisted me in improvisation, writing up and summarizing the literature review (conclusion).

2. S. Cherukupalli, G. A. Hampannavar, C. Sampath, B. Chandrasekaran, N. Sayyad, F. Kayamba, R. R. Aleti, R. Karpoormath, An appraisal on synthetic and pharmaceutical perspectives of pyrazolo[4,3-*d*]pyrimidine scaffold. *Bioorganic and Medicinal Chemistry*, **2017**, https://doi.org/10.1016/j.bmc.2017.10.012.

   Contributions: I did the literature review and wrote the entire manuscript under the supervision of Dr. Rajshekhar Karpoormath. Rest all the co-authors assisted me in improvisation, writing up and summarizing the literature review (conclusion).

3. S. Cherukupalli, B. Chandrasekaran, N. Sayyad, R. R. Aleti, S. R. Merugu, R. Karpoormath, Synthesis, anticancer evaluation, and molecular docking studies of some novel 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines as potential cyclin dependent kinase 2 (CDK2) inhibitors. *Bioorganic Chemistry, 2018,* https://doi.org/10.1016/j.bioorg.2018.02.030.

   Contributions: I generated the rationale and did all the experimental and characterization as well as writing up of manuscript under the guidance of Dr. Rajshekhar Karpoormath (Supervisor). The co-authors assisted me in writing up of results and discussion and designing of the target molecules.

4. S. Cherukupalli, B. Chandrasekaran, N. Sayyad, R. R. Aleti, S. R. Merugu, R. Karpoormath, Synthesis of 4,6-disubstituted pyrazolo[3,4-d]pyrimidine analogues: molecular docking, anticancer evaluation as potential cyclin dependent kinase 2 (CDK2) inhibitors. *Submitted to Chemical Biology and Drug Design (CBDD).*

   Contributions: I generated the rationale and did all the experimental and characterization as well as writing up of manuscript under the guidance of Dr. Rajshekhar Karpoormath

(Supervisor). The co-authors assisted me in writing up of results and discussion and designing of the target molecules.

Conference contributions

1. **Oral presentation**: Synthesis and anticancer evaluation of some novel 4,6-disubstituted pyrazolo[3,4-*d*] pyrimidines as potential cyclin dependent kinase 2 (CDK2) inhibitors. College of Health Sciences Research Symposium, held at Nelson R Mandela School of Medicine Campus, Durban, South Africa, from 5th to 6th October **2017**.

Other publications

1. B. Chandrasekaran, R. Muthusamy, T. L. Chuin, K. P. Samukelisiwe, S. Cherukupalli, G. A. Hampannavar, N. Sayyad, E. S. Soliman, R. Karpoormath, Ligand and structure based in silico studies to identify kinesin spindle protein (KSP) inhibitors as potential anticancer agents, *Journal of Biomolecular Structure and Dynamics*, **2017,** http://dx.doi.org/10.1080/07391102.2017.1396255.

2. V. N. Palakollu, N. Thapliyal, T. Chiwunze, R. Karpoormath, S. Karunanidhi, **S. Cherukupalli**, Electrochemically reduced graphene oxide/Poly-Glycine composite modified electrode for sensitive determination of L-dopa, *Material Science and Engineering: C*, **2017**, 77, 394-404.

3. N. Thapliyal, T. Chiwunze, R. Karpoormath, **S. Cherukupalli**, Fabrication of gold nanourchins based electrochemical sensor for nanomolar determination of primaquine, *Material Science and Engineering: C*, **2017**, 74, 27-35.

4. N. Thapliyal, T. Chiwunze, R. Karpoormath, **S. Cherukupalli**, Research progress in electroanalytical techniques for determination of antimalarial drugs in pharmaceutical and biological samples, *RSC Advances*, **2016**, 6, 57580-57602.

Signed: _____ Date: 11-04-2018 _____

Dedicated

To

My Mother, a strong and gentle soul, who have raised me to be the person I am today

My Father for his unconditional care, unceasing support and Encouragement

My Sisters for their solace and love all the way of my life

My brother-in-law for his gentleness and affection without whom none of my success would be possible

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whatever I undertake well. Thank you both for pushing me to reach for the stars there by raising my spirits to achieve the same, which could never be accomplished without the support of this wonderful family. I have to specially mention my deep gratitude and love to my sisters Lakshmi and Malleswari for being with me all the times with their constant encouragement and co-operation. I have to mention special thanks and love to Supriya Reddy, whose lovable words always brightens up my day. Cheers, warm blessings and love to my nephews Sumanth Reddy, Shashank Reddy, and niece Pranavi.

Finally, I would like to take the opportunity to thank all my relatives and teachers. I ask for forgiveness for any inadvertent exclusions.

LIST OF ABBREVIATIONS

A. alternate	:	*Alternaria alternate*
A. flavus	:	*Aspergillus flavus*
A. niger	:	*Aspergillus niger*
A. terreus	:	*Aspergillus terreus*
ARB	:	Non-peptide angiotensin II receptor antagonists
B. fabae	:	*Botrytis fabae*
B. subtilis	:	*Bacillus subtilis*
B. thuringiensis	:	*Bacillus thuringiensis*
B. cereus	:	*Bacillus cereus*
BMP	:	Bone morphogenetic protein
B-Raf	:	Rapidly accelerated fibrosarcoma
BZR	:	Benzodiazepine receptor
C. albicans	:	*Candida albicans*
cAMP	:	Cyclic Adenosine Monophosphate
CB_2	:	Cannabinoid receptor type-2
CBZR	:	Central benzodiazepine receptor
CCR1	:	C-C chemokine receptor type-1
CDK	:	Cyclin-dependent kinase
CHK	:	Checkpoint kinase
CK2	:	Casein kinase-2
CNS	:	Central nervous system
COX	:	Cyclooxygenase
CRF	:	Corticotropin-releasing factor
CRK	:	Cdc2-related kinase
c-Src	:	Proto-oncogene tyrosine-protein kinase Src
CYC6	:	Cyclin 6
DNA	:	Deoxyribonucleic acid
DPP-IV	:	Dipeptidyl peptidase-4
E. coli	:	*Escherichia coli*
EAC	:	Ehrlich ascites carcinoma
EC_{50}	:	Half maximal effective concentration

ECMA	:	Endothelial cell mitogenesis assay
ED_{50}	:	Median effective dose
ER	:	Estrogen receptor
ERK	:	Extracellular signal-regulated kinase
F. oxysporum	:	*Fusarium oxysporum*
G. candidum	:	*Geotrichum candidum*
$GABA_A$	:	Gamma-aminobutyric acid
GI_{50}	:	Ealf maximal growth inhibition
GSK-3β	:	Glycogen synthase kinase 3 beta
hA	:	Human adenosine
HCV	:	Hepatitis C virus
HIV	:	Human immunodeficiency virus
HLM	:	Human liver mocrosome
HMG-CoA	:	3-hydroxy-3-methylglutaryl-coenzyme-A
HSV-1	:	Herpes simplex virus type-1
$5\text{-HT}_6\text{R}$	:	5-hydroxytryptamine subtype 6 receptor
IC_{50}	:	Half maximal inhibitory concentration
IRAK-4	:	Interleukin-1 receptor associated kinase-4
JNK-1	:	c-Jun N-terminal kinase-1
K. pneumonia	:	*Klebsiella pneumonia*
KDR	:	Kinase insert domain receptor
K_i	:	Inhibition constant
kNN-MFA	:	k-nearest neighbor molecular field analysis
Lck	:	Lymphocyte-specific protein tyrosine kinase
MAPKAP K-2	:	Mitogen-activated protein kinase-activated protein kinase-2
MIC	:	Minimum inhibitory concentration
MLC	:	Minimum lethal concentration
MMP-13	:	Matrix metalloproteinase-13
mTOR	:	Mammalian target of rapamycin
NPY1R	:	Neuropeptide Y receptor type-1
OMF	:	Mass of fabric
P. aeruginosa	:	*Pseudomonas aeruginosa*
PAH	:	Pulmonary arterial hypertension
P. chrysogenum	:	*Penicillium chrysogenum*

PBZR	:	Peripheral benzodiazepine receptor
PDE	:	Phosphodiesterase
PDK1	:	Pyruvate dehydrogenase lipoamide kinase isozyme-1
PET	:	Positron emission tomography
Pim	:	Proviral integrations of moloney
PKB	:	Protein kinase B
RNA	:	Ribonucleic acid
ROCK-II	:	Rho associated coiled-coil containing protein kinase-2
RSV	:	Human respiratory syncytial virus
S. racemosum	:	*Syncephalastrum racemosum*
S. aureus	:	*Staphylococcus aureus*
S. marcescens	:	*Serratia marcescens*
SI	:	Selectivity index
T. cruzi	:	*Trypanosama cruzi*
Thym	:	Thymus
TSPO	:	Translocator protein
TTK	:	Threonine tyrosine kinase
VEGFR-2	:	Vascular endothelial growth factor receptor-2

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Note: Referencing styles of individual chapters are as per guidelines of communicated journals.

CHAPTER 1

1 GENERAL INTRODUCTION

1.1 Background

Cancer is a generic term for a complex disease characterized by uncontrolled growth of the abnormal cells beyond their usual margins that can then enter contiguous parts of the body and/or spread to other organs. Other common terms used are malignant tumors and neoplasms. Cancer can affect almost any part of the body and has many anatomic and molecular subtypes, each one requiring particular management approaches. Cancer has an impending mortality and morbidity rate with a staggering 14.1 million people worldwide. The rise in the frequency of confirmed cancer cases remains a challenge, especially in South Africa, which ranks as one of the highest statistics in comparison to other countries that have been stricken by this devastating disease. In 2012, the World Health Organization (WHO) estimated that out of 14.1 million individuals affected with cancer, 8.8 million people (approximately 22,000 cancer deaths a day) have succumbed to this disease. By 2030, new cancer cases are expected to grow to 21.7 million with a staggering 13 million deaths as a result of the rapid growth and aging of the population unless preventative measures are put into place.[1] Some important lifestyle factors known to increase the cancer risk is the consumption of alcohol, poor diet, smoking, reproductive alterations and physical inactivity in developing nations. It is important to take note that the actual Fig.s for estimated cancer cases will be considerably larger.[2] Therefore, it is for this reason that cancer is considered as a serious public health burden worldwide as illustrated in **Fig. 1** & **2**.

1.2 Types of cancer

Cancer is a multifaceted disease, which commences when healthy cells change and grow out of control, resulting in the formation of a mass called a tumor. A tumor can be cancerous or benign. A cancerous tumor is malignant, meaning it can grow and spread to other parts of the body. A benign tumor describes a tumor that can grow but does not spread. There are almost 100 types of cancers existing in human body and these are titled according to organs or tissues in which they form. The worldwide cancer statistics from cancer research UK, the top five most commonly diagnosed cancers are the lung, breast, colorectal, prostate and stomach cancers (**Fig.3a**) accounting for more than half of total cancer deaths (**Fig. 3b**).[3] Over the last 40 years, the common cancer profiles have slightly changed with lung, liver, stomach and bowel cancers accounting for most deaths.[4]
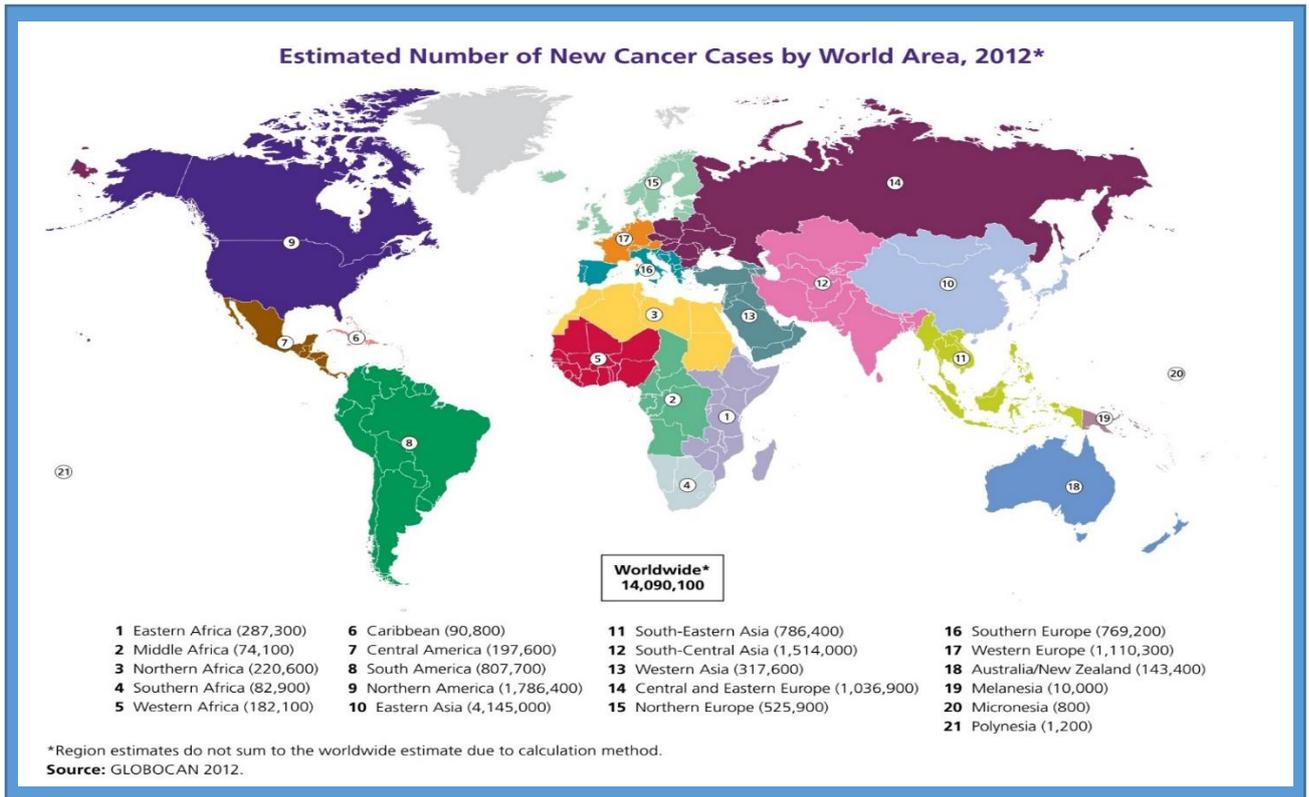
Fig. 1. Estimated number of new cancer cases by area.[1]
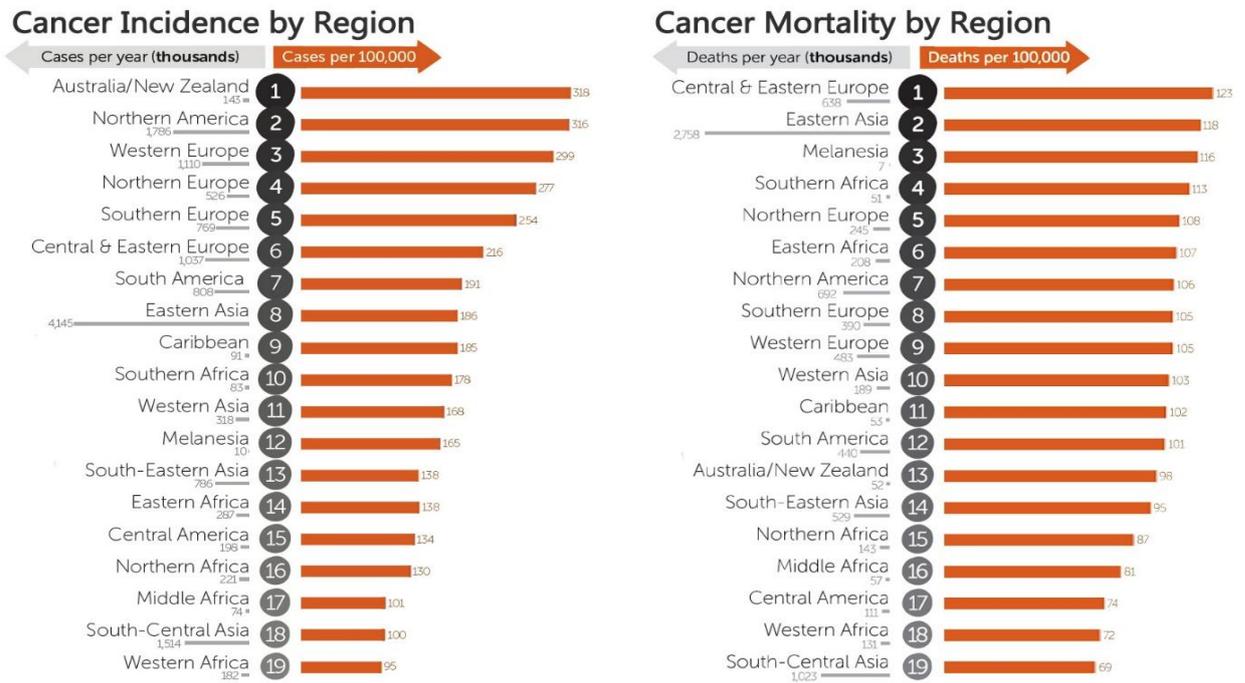
Fig. 2. Cancer Incidence and mortality by region.[3,4]
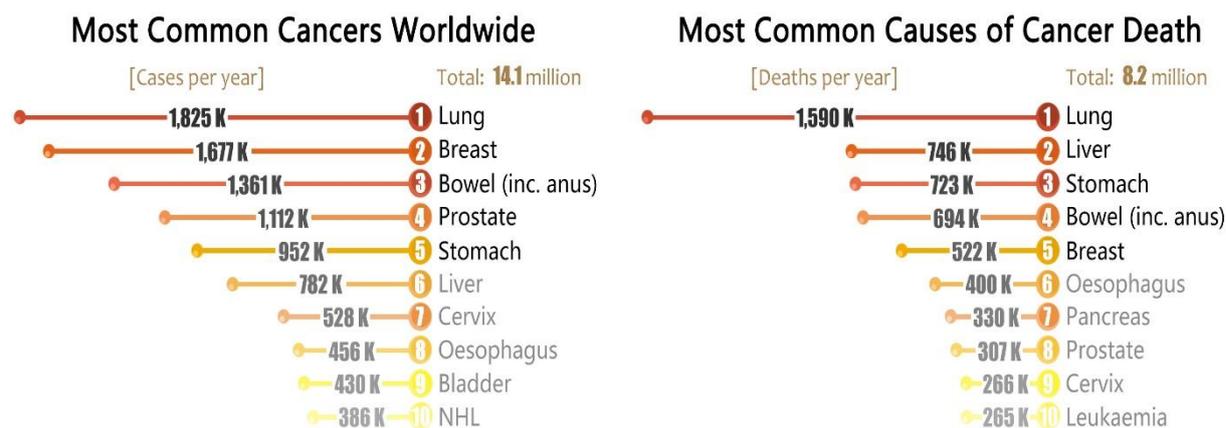
Fig. 3. **a**) top 10 most common cancers; **b**) causes of cancer death.[3,4]

1.3   Causes of cancer

Most cancers are associated with environmental, lifestyle as well as economical and behavioral factors.[5] Some of the common factors that contribute to cancer death include infections (15-20%), tobacco (25-30%), obesity/diet/physical inactivity (30-35%), radiation (up to 10%), heredity (3-10%), hormones and physical/environmental agents.[6] General signs and symptoms associated with cancer are prolonged cough, lump in the affected area, baffling weight loss, abnormal bleeding and change in bowel movements.

1.3.1   Tobacco

In 2008, WHO named *Nicotiana* as the global single greatest preventable cause of death.[7] Tobacco is a product obtained from the dried leaves of tobacco plant and mostly consumed in the form of cigarettes, cigars, pipe tobacco and other tobacco containing chewable products. Tobacco contains an alkaloid called nicotine, which is stimulant responsible for the addictive nature and affects many organs such as the heart, liver and lungs. Its use is associated with many forms of cancers resulting in 80% of lung cancer,[8] which could be attributed to approximately fifty known carcinogens such as nitrosamines and polycyclic aromatic hydrocarbons.[9]

Moreover, smoking non-tobacco containing electronic cigarettes is more harmful as it generates the chemical formaldehyde. Lung cancer is one of the most often detected cancer and the leading cause of the cancer related deaths worldwide. Globally, 1.8 million new cases have been reported in 2012, accounting for 13% total cancer detection and an estimated 1.6 million (1.1 million in men and 491,200 deaths in women).[2] In some nations, it has been shown to be the leading cause of death for women as opposed to breast cancer. **Fig. 4** unveils the prevalence of lung cancer worldwide.[1]

Males

Females

*Per 100,000, age standardized to the World Standard Population. **Source:** GLOBOCAN 2012.
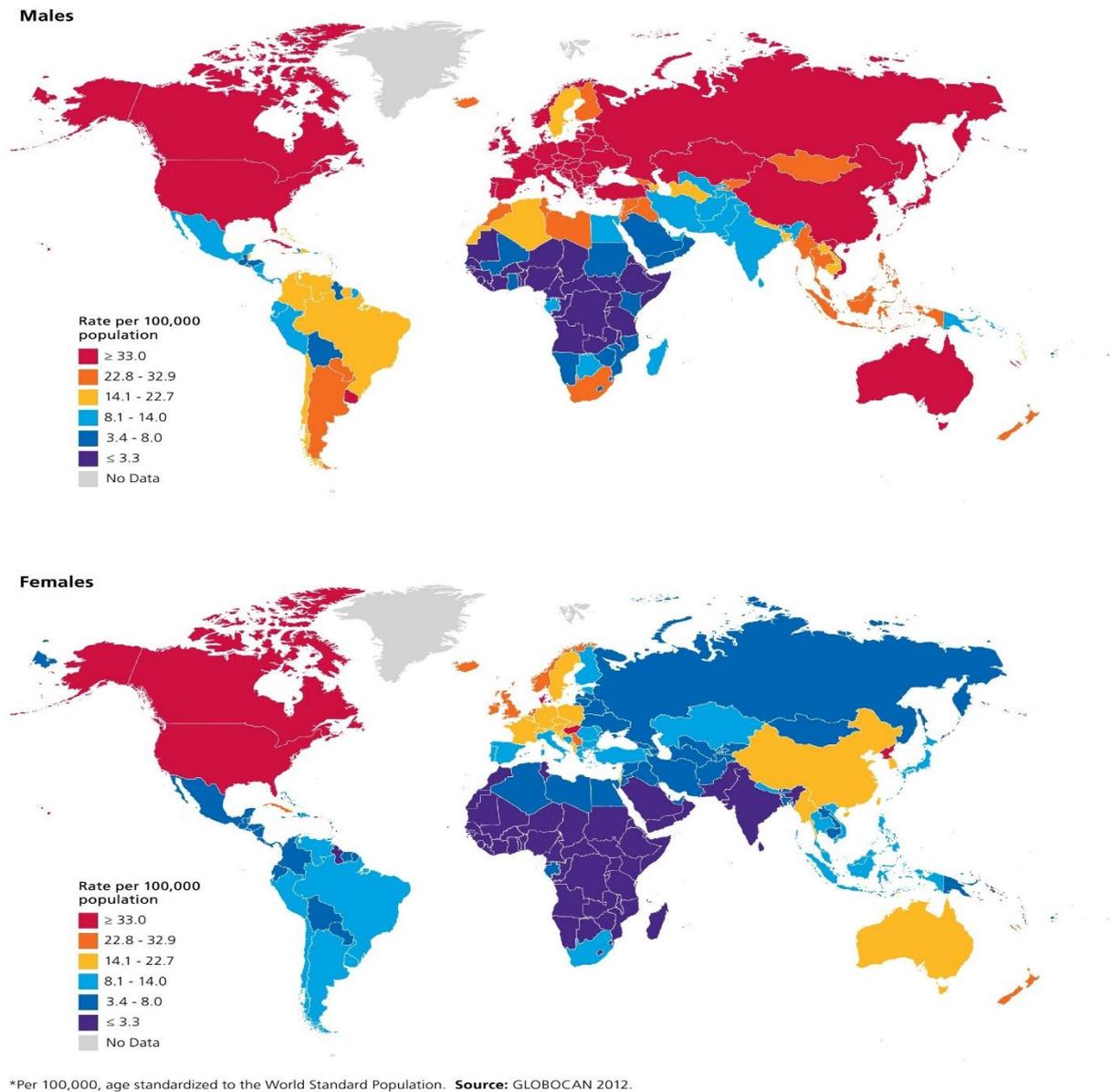
Fig. 4. International Variation in Lung Cancer Incidence Rates, 2012.[1]

1.3.2 *Physical inactivity/obesity*

Human life has been associated with several physical activities including exercising, performing household chores, working, and leisure-time activities such as hiking, walking, to name but a few. Physical activity is crucial for the human body to maintain an equilibrium between the number of calories consumed and the number of calories utilized. Using less calories than one consumes leads to obesity, in which a person has an unhealthy body fat, developed when the energy intake from food and drink exceeds energy outlay from physical activity and other metabolic processes. Obesity is a risk factor for cancer, as well as other chronic diseases such as type 2 diabetes and cardiovascular disease.[6] Obese people often have chronic low-level inflammation, which can cause DNA damage that can lead

to cancer overtime. Overweight and obese people in comparison to normal-weight people are more likely to have conditions or disorders that are connected to or that cause chronic local inflammation and those are risk factors for certain cancers.[10] Globally, 30-35% cancer deaths have been attributed to poor diet/obesity/physical inactivity. Data obtained from the National Health and Nutrition Examination Survey (NHANES) revealed that in 2011–2014, almost 70% of U.S. adults age 20 years or older were overweight and more than one-third (36.5%) were obese.[11] An increase in body weight has been linked to several different types of cancers. (**Fig. 5**) with an estimated 14-20% cancer deaths been reported.[12]
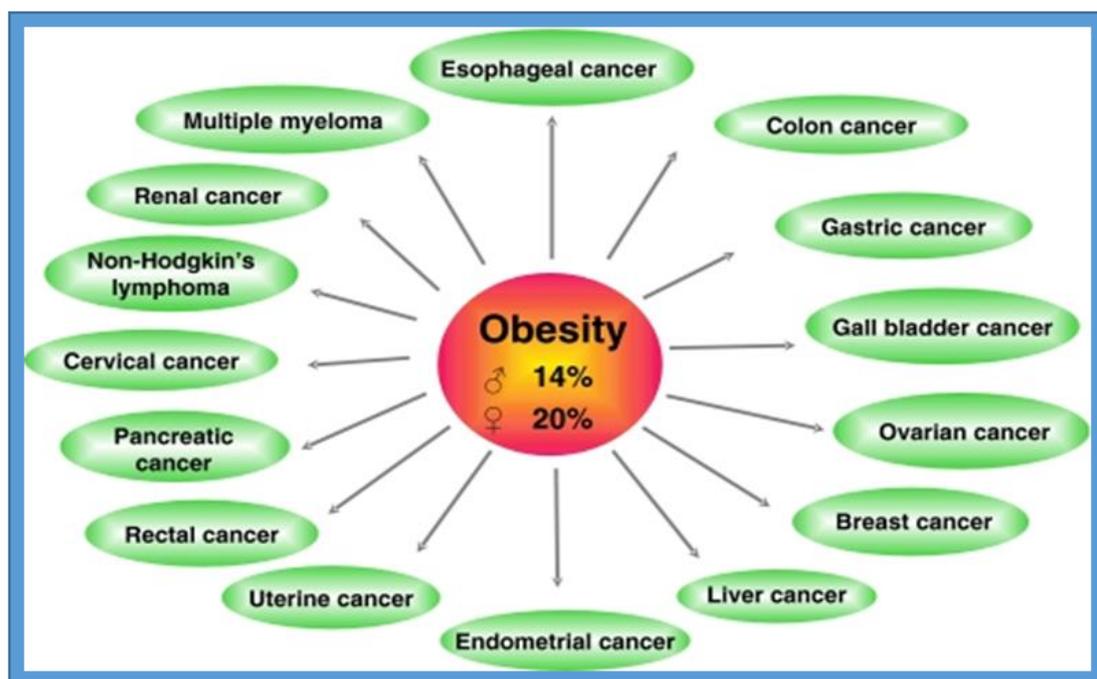
Fig. 5. Association of body fat with increased risks of a number of cancers.

1.3.3 Infectious agents

Several infectious agents such as viruses, parasites and bacteria can cause cancer or may increase the risk factor that forms cancer. In addition, some viruses can interrupt signaling that are typically responsible for cell growth and proliferation in both prokaryotes and eukaryotes. Therefore, some infections are known to weaken the immune system and the human body becomes more susceptible to these cancer causing agents. Generally, viruses that are associated with an augmented risk of cancer can be passed on from one person to another through blood and other body fluid transfusions, unprotected sex as well as the sharing of needles. Globally an estimated 16.1% of cancer cases are attributable to infectious agents,[13] especially for cervical cancers, liver cancers (80%) and other cancers (15-20%).[14] This global percentage varies from one regions to the other with as high as 32.7% in Africa to 3.3% in New Zealand and Australia.[15] A virus that could be responsible for causing cancer is called oncovirus

and is the most significant risk factor for several cancer growth in the humans.[16] The table below briefly gives the various cancers causing viruses, bacteria's and parasites.

Virus/bacteria/parasite	Name	Type of cancer
Virus	Hepatitis B	Liver cancer
	Hepatitis C	Liver cancer[15]
	Human papilloma	Cervical, nose, throat, anus, larynx and esophagus cancer[16]
	Epstein-barr	Nasopharyngeal, burkitt lymphoma and hodgkins lymphoma cancer
	Human herpesvirus 8	Kaposis sarcoma and B-cell lymphoma cancer[17]
	Human T cell lymphotropic	Adult T cell leukemia cancer[18]
	Merkel cell polyoma	Merkel cell carcinoma[19]
Bacteria	*Helicobacter pylori*	Gastric cancer[20]
	Chlamydophila pneumoniae	Lung cancer[21]
Parasite	*Schistosoma haematobium*	Bladder cancer
	Schistosoma japonicum	Colorectal cancer[22]

Table 1. Different types of known cancer causing viruses, bacteria's and parasites.

1.3.4   *Radiation*

The radiation of certain wavelengths/frequencies such as ionizing radiation has sufficient energy to damage DNA and cause cancer. Globally up to 10% of critical cancers are due to radiations, which compromises of both ionizing and non-ionizing radiation whereas most of the non-melanoma skin cancers is caused by non-ionizing ultraviolet radiation.[6] Radiation can cause cancer in any part of the human body, and at any age. Radiation influenced solid tumors generally takes 10-15 years to become clinically visible and radiation induced leukemias characteristically takes 2-10 years.[23] The sources of radiation include radon, x-rays, gamma rays, medical imaging, alpha particles, neutrons, beta particles. In addition, various medical techniques, such as positron emission tomography (PET) scans, chest x-rays, computed tomography (CT) scans and radiation therapy can also cause cell damage that can lead to cancer. However, the advantages from these medical techniques are very high while the risk posed is trivial.

1.3.5   *Heredity*

A genetic defect is usually passed from parents to their offspring, either by sexual or asexual reproduction and the progeny cells or organisms obtain the genetic information from their parents. Majority of cancers are non-hereditary whereas hereditary cancers are mainly caused by an inherited genetic defect. Globally an estimated 0.3% of people are carriers of a genetic mutation that has a great effect on cancer risk and cause around 3-10% of all cancers. In addition, a set of medical signs include inherited mutations in BRCA1 (breast cancer susceptibility gene 1) and BRCA2 (breast cancer susceptibility gene 2) which is responsible for more than 75% risk for breast cancer, ovarian cancer[24] and hereditary nonpolyposis colorectal cancer, which is existing in about 3% of persons diagnosed with colorectal cancer.[25]

1.3.6 Hormones

Hormone is a chemical messenger that is secreted into the blood to communicate between organs and tissues for physiological regulation, behavioral actions including digestion, tissue function, metabolism, sleep, respiration, lactation, sensory perception, stress, growth and development, reproduction, excretion, movement, and mood.[26] Apart from those listed, some hormones play a major role in the development of cancer by encouraging cell proliferation. Hormones are important in sex-related cancers include breast, prostate, testis, endometrium, ovary, as well as bone and thyroid cancer. One such example is estrogen a female sex hormone, known as a human carcinogen. Even though these sex hormones have crucial physiological roles in both females and males as well as linked with an increased risk of certain cancers. For example, taking combined menopausal hormone therapy (estrogen & progesterone) can increase a woman's risk of breast cancer. Menopausal hormone therapy with estrogen alone results in an increased risk of endometrial cancer. In addition, person on hormone replacement therapy would have increasingly high levels of hormones, thus a greater risk of developing cancer. On the other hand, a person who does exercise more than average will have a lower level of hormones and consequently a reduced risk of cancer.[27]

1.3.7 Physical/environmental agents

Apart from chemical agents, there are certain matter/substances which on prolonged exposure could physically induce cancer and are called physical cancer agents. For instance, continued exposure to asbestos leads to membrane cancer. Similarly, other natural and synthetic asbestos-like fibers such as glass wool, wollastonite, rock wool and attapulgite are also known to cause cancer. In addition, non-fibrous materials such as powdered metallic nickel and cobalt, crystalline silica can also induce cancer.[28]

1.4 Types of cancer treatments

Total removal of cancerous tissue without causing harm to the rest of the healthy tissue is an ideal goal of treatment. There are several types of cancer treatments and the choice of treatment depends on

location, type, grade of tumor and the phase of the disease. Most of the cancers can be treated mainly using chemotherapy, hormonal therapy, surgery, radiation therapy, immunotherapy as well as targeted therapy.

1.4.1 Surgery

Cancer surgery is used to prevent, diagnose, and treat cancer. It is the oldest type (Ancient Egypt) of cancer treatment and normally works best for solid tumors existing in specific areas. The main objective of the surgery can be removal of only the tumor or whole organ. Each cancer type has specific cancer operations such as whipple surgery for pancreatic cancer, mastectomy for breast cancer, prostatectomy for prostate cancer and lung surgery for non-small cell lung cancer.[29] In addition, surgery is often essential for cancer staging (determining the extent of the disease and whether it has metastasized to regional lymph nodes) and to control bowel obstruction or spinal cord compression.[30]

1.4.2 Chemotherapy

Chemotherapy (frequently abbreviated to CTX or chemo or CTx) is a type of cancer treatment with one or more drugs (chemotherapeutic agents) that can kill cancer cells. Combination chemotherapy is a combination of two or more cancer drugs is usually used for the efficient treatment of cancer. Chemotherapy may be specified with a curative intent (combinations of drugs), or it may aim to prolong life or to diminish symptoms (palliative chemotherapy). Traditional chemotherapeutic agents are cytotoxic as they have been seen to interfere with rapid cell division and affect the normal healthy cells as well, but cancer cells may vary broadly in their susceptibility to these agents. Chemotherapeutic drugs in a number of ways can cause interference of cell division and these include duplication of DNA and separation of just formed chromosomes. Majority of leukemia and lymphoma cancers could be treated by chemotherapy and on the other hand, chemotherapy and combination chemotherapy have the potential to damage healthy tissues or organs, particularly those tissues have a great replacement rate. However, these cells have the capability to repair themselves after chemotherapy.

1.4.3 Radiation therapy

Radiation therapy is the use of ionization radiation to kill cancer cells. This therapy can be administrated internally through brachytherapy or externally through external beam radiotherapy. Radiation therapy can kill almost every type of solid tumors, including cancers of the cervix, breast, prostate, lung, liver, brain, pancreas, uterus, larynx, skin, stomach, or soft tissue sarcomas. In addition, it is also useful in the treatment of leukemia and lymphoma. Radiation therapy harms or eliminates cells in the area being treated (target tissue) by harming their genetic material, thus making it difficult for these cells to continue to grow and divide. Although radiation harms both cancer cells and normal cells, most normal

cells can improve from the effects of radiation and function properly. The main target of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue.

1.4.4   Targeted therapy

Targeted therapy since 1990s, plays a significant role in the effective treatment of some specific cancers. In general, the target therapy utilizes small molecules as inhibitors of enzymatic domains on mutated or overexpressed target enzymes. One such example of targeted chemotherapy are the tyrosine kinase inhibitors such as imatinib and gefitinib. Monoclonal antibody therapy is another target therapy in which the therapeutic agent is antibody, which exactly binds to a protein on the surface of the cancer cells. Prominent examples are anti-HER2/neu antibody called trastuzumab used in the treatment of breast cancer, and anti-CD20 antibody called rituximab employed in the treatment of a variety of B-cell malignancies. Further, photodynamic therapy (PDT) is another strategy for treatment of basal cell carcinoma or lung cancer comprising a tissue oxygen, light and a photosensitizer. Photodynamic therapy can also be involved in eliminating traces of malignant tissue and later the surgical removal of huge tumors.[31]

1.4.5   Immunotherapy

Immunotherapy refers to a set of various therapeutic approaches aimed to induce the patient's own immunity to fight the tumor. Recent approaches for producing an immune response against tumors include intravesical Bacillus Calmette-Guerin (BCG) immunotherapy for bladder cancer, and use of interferons and other cytokines to induce an immune response in renal cell carcinoma and melanoma patients. Vaccines to produce particular immune responses are the topic of intensive research for many tumors, especially malignant melanoma and renal cell carcinoma. Sipuleucel-T is a vaccine-like strategy for prostate cancer in which dendritic cells from the patient are loaded with prostatic acid phosphatase peptides to induce a specific immune response against prostate-derived cells.

1.4.6   Hormonal therapy

The development of some cancers can be inhibited by providing or blocking some hormones. General examples of hormone-sensitive tumors include certain types of prostate and breast cancers. Eliminating or blocking testosterone or estrogen is often a significant additional treatment. In certain cancers, administration of hormone agonists, such as progestogens may be therapeutically beneficial.

1.5   Cyclin-dependent kinase (CDK) inhibitors

Cyclin-dependent kinases (CDKs) are a family of serine/threonine or mammalian heterodimeric enzymes comprising more than 13 members. These kinases are connected with regulation of cell-cycle progression by phosphorylating proteins in cell division. Dysregulation of the cell cycle control is a

common cause of various human cancers and is normally associated with aberrant activation of cyclin-dependent kinases.[32,33] CDK1 and CDK2 kinases play significant role in cell division, contribute to the phosphorylation and inactivation of the retinoblastoma (Rb) tumor suppressor protein, which is produced throughout late G1, S and G2-M phases.[34-36] Other members of this family such as CDK7, CDK8 and CDK9 etc. contribute to the regulation of RNA polymerase II and the control of cellular transcription.[37,38] Consequently, inhibition of CDKs represents an attractive therapeutic strategy in oncology. The structures of these CDK molecules are quite varied and they are generally constituted or derived from various heterocyclic families such as purines, pyrimidines, indoles, pyrazoles, thiazoles, or derived from natural products such as flavones or staurosporine. Several compounds from these families such as roscovitine, dinaciclib, palbociclib etc. are existing under clinical evaluation. **Table 2** indicates clinical drugs with their respective structures, administration mode and clinical trial stage.

Drug	Structure	Administration Mode	Clinical Trial Stage
Flavopiridol		Intravenous	II
Roscovitine		Oral	II
Dinaciclib		Intravenous	III
SNS032		Intravenous	I
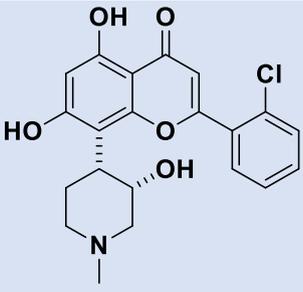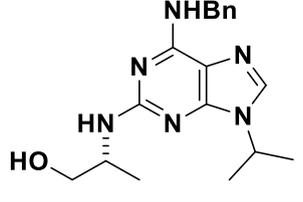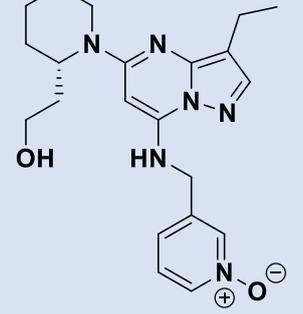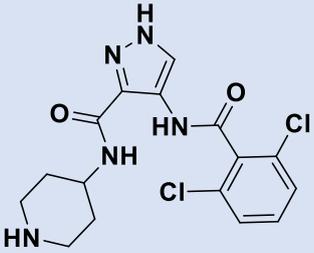
AT7519		Intravenous	I/II
Palbociclib or PD0332991		Oral	III
EM-1421		Intravenous	I/II
RGB-286638		Intravenous	I
P276-00		Intravenous	II
BAY-1000394		Oral	I
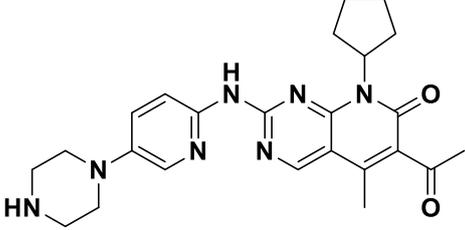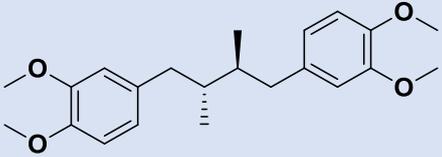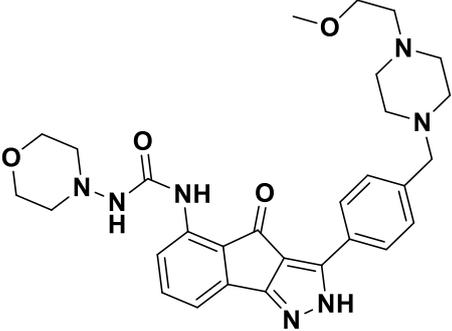
TG02/SG1317		Oral	I
PHA848125AC		Oral	II
LEE-011		Oral	III
Bemaciclib or LY2835219		Oral	I/(III)
UCN01		Intravenous	II

Table 2. Structures of CDK drugs under clinical trials

1.6 Marketed drugs containing pyrimidine/fused pyrimidine scaffold
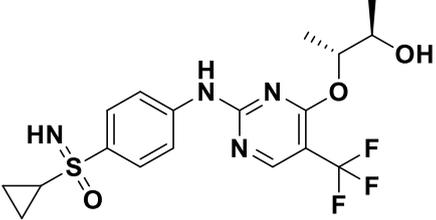
Pyrazolopyrimidines are the fused heterocyclic ring systems, which structurally resemble purines have prompted medicinal investigations to evaluate their potential therapeutic significance. Several anticancer drugs with pyrimidine/fused pyrimidine scaffold such as abemaciclib, afatinib, copanlisib, brigatinib, nelarabine, capecitabine to name but a few exist in the market and are illustrated in **Table 3**.

Drug	Structure	Target
Abemaciclib		Advanced/metastatic breast cancers.
Afatinib		Pleural mesothelioma and non-small cell lung cancer.
Copanlisib		Non-Hodgkin lymphoma and chronic lymphocytic leukemia.
Brigatinib		Anaplastic lymphoma.
Nelarabine		Acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma.
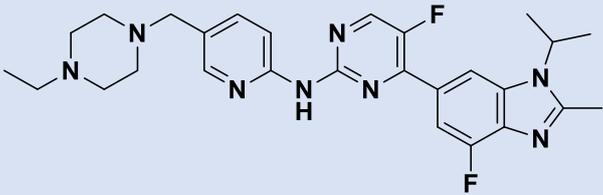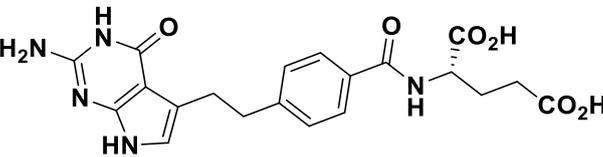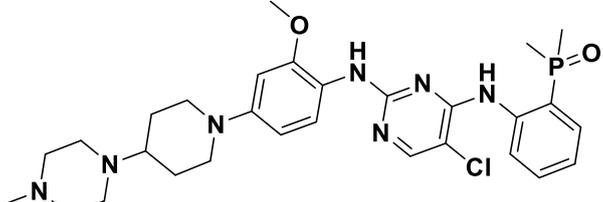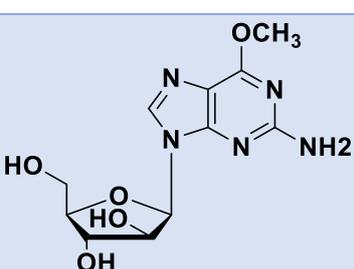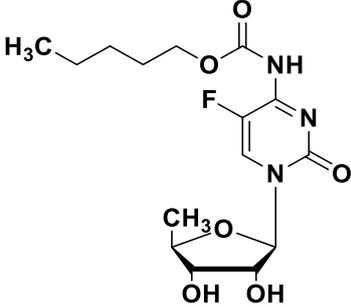
Capecitabine		Breast cancer, gastric and colorectal cancer.
Fluorouracil		Colon cancer, esophageal cancer, pancreatic cancer, breast cancer, cervical cancer.
Cladribine		Hairy cell leukemia B-cell chronic lymphocytic leukemia.
Clofarabine		Acute lymphoblastic leukemia.
Cytarabine		Acute myeloid leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, and non-Hodgkin's lymphoma.
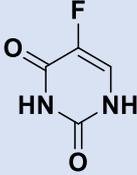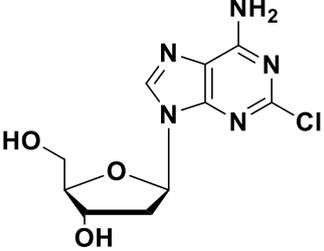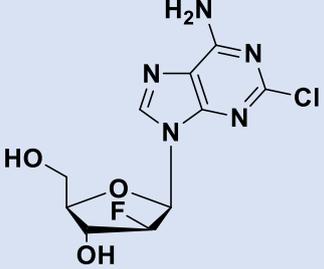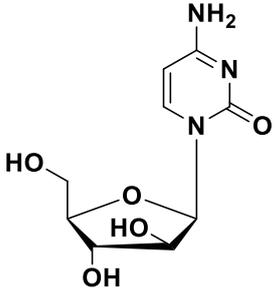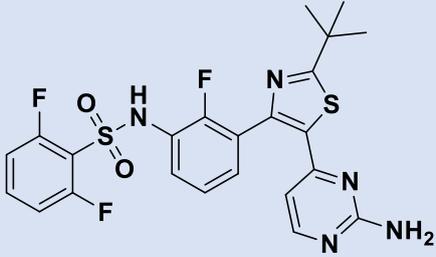
Dabrafenib		Non small-cell lung cancer.
Dasatinib		Chronic myelogenous leukemia, acute lymphoblastic leukemia.
Fludarabine		Leukemia and lymphoma.
Gemcitabine		Breast cancer, ovarian cancer, non-small cell lung cancer, pancreatic cancer.
Imatinib		Chronic myelogenous leukemia and acute lymphocytic leukemia.
Ibrutinib		Mantle cell lymphoma, chronic lymphocytic leukemia, and Waldenström's macroglobulinemia.
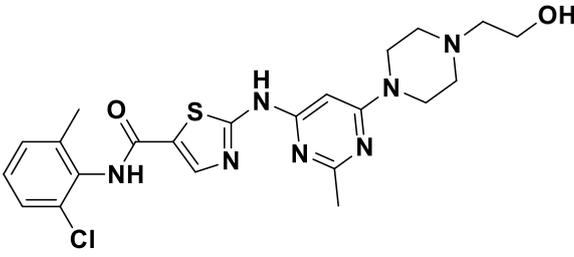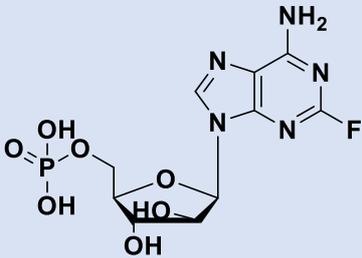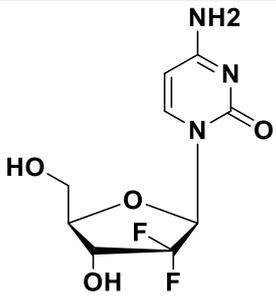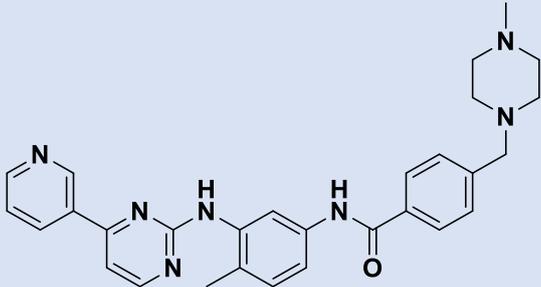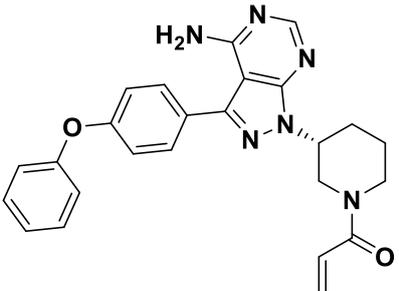
Ribociclib		Breast cancer
Mercaptopurine		Acute lymphocytic leukemia, chronic myeloid leukemia Crohn's disease.
Osimertinib		Non-small-cell lung cancer.
Pazopanib		Renal cell carcinoma and soft tissue sarcoma.
Tioguanine		Acute myeloid leukemia (AML), acute lymphocytic leukemia.
Nilotinib		Chronic myelogenous leukemia.
Trifluridine		Colorectal cancer
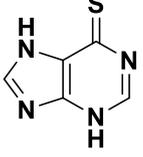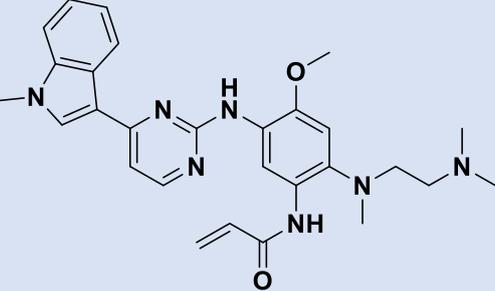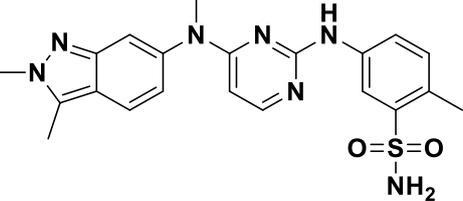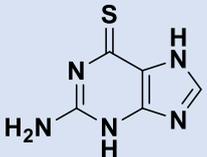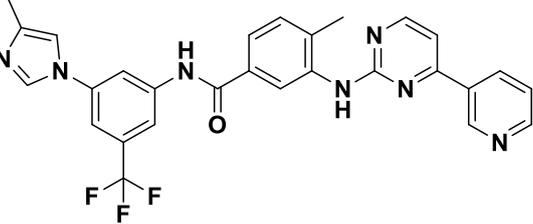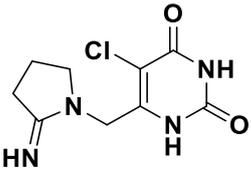
tipiracil		Colorectal cancer
Ceritinib		Metastatic non-small cell lung cancer.

Table 3. Pyrimidine/fused pyrimidine containing marketed drugs as anticancer agents

2   RATIONALE OF OUR RESEARCH

From our literature review, it was noted that countries like Africa, Northern America, Eastern Asia, Central, Eastern Europe, and Australia/New Zealand carry a massive burden of cancer making it a multifaceted disease affecting every region of the world. Clinicians handling the cancer cases, frequently encounter significant challenges such as lack of clinical experience, adverse events, lack of patients adherence, inadequate availability diagnostics or second line drugs, thus augmenting the risk of drug resistance. Therefore, after considering all the above mentioned facts, a robust and diverse drug discovery and development approach needs to be implemented to fill the pipeline with potential leads as well as developing new drug molecules against the new cancer targets. One should also look for extensive exploitation of chemical space and optimize lead hits for effective cancer drugs for future. Scaffolds such as nitrogen containing heterocyclic compounds have been widely known to display anticancer activity. One such scaffold is "Purine" which has been widely used as a building block for developing various anticancer drugs. Bioisosteric replacement of purines has resulted in several potential anticancer drugs like roscovitine, dinaciclib, nelarabine, cladribine, etc. Similarly, abemaciclib, brigatinib, capecitabine, etc, are examples of some of the significant anticancer drugs derived from pyrimidine scaffold[39]. Thus, bioisosteric replacement is an important tool/technique in developing potential anticancer drugs. Pyrazolo[3,4-*d*]pyrimidine is one such scaffold that could be used as an bioisostere in developing new potential anticancer drugs. Hence, in an effort to identify new leads, a pyrazolo[3,4-*d*] pyrimidine scaffold was exploited.
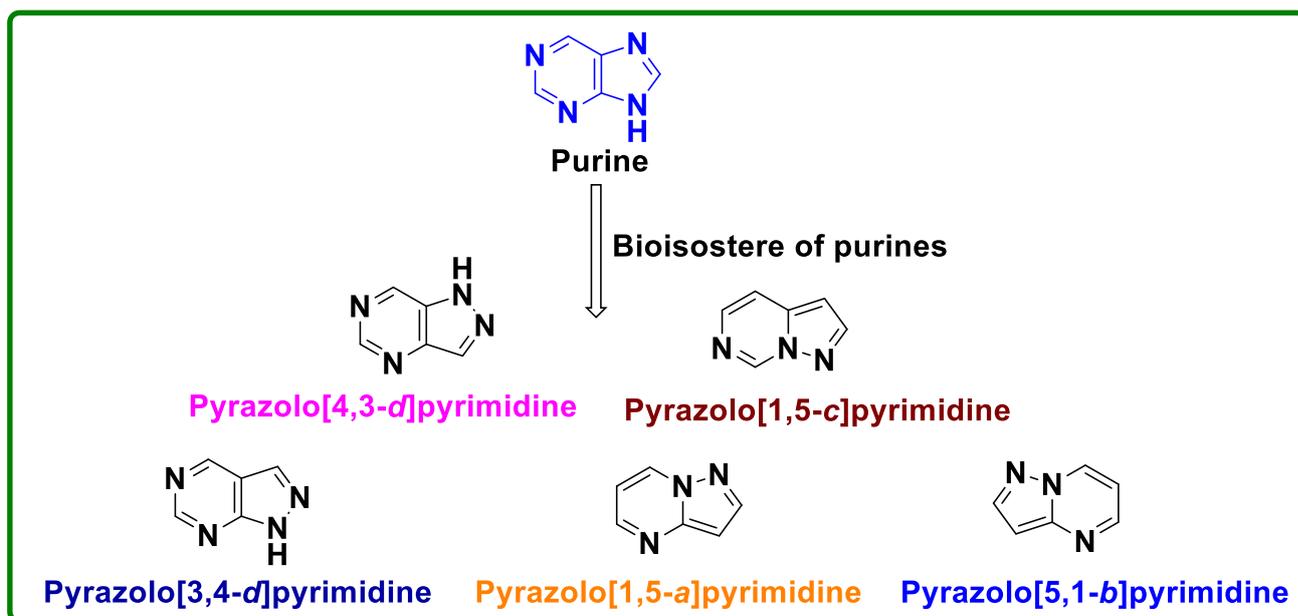
Structural isomers of pyrazolopyrimidine

Fusion of pyrazole with the pyrimidine ring results in the formation of a bicyclic system known as "pyrazolopyrimidine". Approximately five different structural isomers of this bicyclic system are known, such as pyrazolo[1,5-a]pyrimidine, pyrazolo[4,3-d]pyrimidine, pyrazolo[3,4-d]pyrimidine, pyrazolo[5,1-b]pyrimidine, and pyrazolo[1,5-c]pyrimidine. These isomers vary by the position of nitrogen, degree of saturation or unsaturation, or the number of nitrogen's in the pyrazole nucleus.

Form our extensive literature survey, it was noted that pyrazolo[3,4-d]pyrimidine is a versatile and pharmacologically significant scaffold. Pyrazolo[3,4-d]pyrimidine is also known for its diverse of biological activities namely anticancer, anti-inflammatory, antibacterial, antiviral and antifungal properties to name but a few. However, there were only few research articles on pyrazolo[3,4-d]pyrimidines as specific CDK2 inhibitors. Thus we envisaged to further exploit pyrazolo[3,4-d]pyrimidine as potential CDK2 inhibitors. We aimed to design and synthesize a library of novel pyrazolo[3,4-d]pyrimidine as potential anticancer agents keeping in mind the active site of CDK2 enzyme as well as the core structural features highly active ligands against kinases family. The paragraphs below outlines our work plan in brief.

➤ Our comprehensive literature search revealed that there were no comprehensive reviews published on structural isomers of pyrazolopyrimidines such as pyrazolo[1,5-a]pyrimidine and pyrazolo[4,3-d]pyrimidine. Hence we envisioned to write a review on synthetic and medicinal aspects of pyrazolo[1,5-a]pyrimidine and pyrazolo[4,3-d]pyrimidine with special emphasis on structure-activity relationship studies.

➢ Phenethyl and pentane groups are essential moieties in drug discovery. The derivatives from these moieties are well known to possess varied range of activities such as anti-inflammatory, anticancer, anti-HIV, antibacterial, and anti-inflammatory. However, there were no reports on phenethyl/pentane containing pyrazolo[3,4-*d*]pyrimidines as anticancer agents. Therefore, we attempted to synthesize pyrazolo[3,4-*d*]pyrimidine analogs incorporating phenethyl/pentane groups as potential anticancer agents.

➢ Ring systems such as furan, benzofuran and thiphene are pharmacologically active five and six membered heterocycles, which contain oxygen and sulphur heteroatoms. These vital core structures have been extensively investigated for numerous biological properties namely anticancer, antitubercular, anti-inflammatory, antimicrobial, antiviral and anticonvulsant. In this chapter we attempted to synthesize a number of substituted benzoate derivatives as potential CDK2 inhibitors by incorporating different acid chlorides at the C-4 position of the pyrazolo[3,4-*d*]pyrimidine scaffold while keeping phenethyl/pentane groups at C-6 of the scaffold.

➢ Phenylcarbamoyl acetamide is a well-known six membered heterocycle, which contains urea functional group. This vital core structure has been known to exhibit a significant range of biological activities namely, anticancer, anti-inflammatory, antibacterial, antifungal, antiviral, antidiabetic, anti-atherosclerosis, and antimycobacterial. Therefore, in this chapter we anticipated to synthesize phenylcarbamoyl acetamide derivatives of pyrazolo[3,4-*d*]pyrimidine as potential anticancer agents.

3 OBJECTIVES OF THE PRESENT RESEARCH WORK

Cancer is affecting humans at an alarming rate. New drugs with an ability to overcome the drawbacks of existing anticancer chemotherapy are of high priority. The field of medicinal chemistry is contributing implicitly to the process of drug discovery and development. Synthesis of novel chemical entities, modification of existing scaffolds, combining two or more bioactive molecules (hybridization), replacing groups with bioisosteres, and optimization of natural compounds to identify promising leads are some of the interesting themes in the field of medicinal chemistry. Heterocyclic scaffolds having one or more hetero atoms have become crucial in drug discovery, which is evident from the fact that more than 95% of the marketed drugs are built on heterocyclic scaffolds.

Based on the abovementioned, the aims and objectives of the present research work are:

1. To carry out a comprehensive literature review/assessment for identification of new chemical entities as anticancer activity (Identification of a research gap and defining the scope of proposed work).

2. To synthesize a novel series of pyrazolo[3,4-*d*]pyrimidine by incorporating certain structural features/groups that could lead to hit molecules as CDK2 inhibitors:
   a. Phenethyl/pentane/hexane derivatives.
   b. Furan, thiophene and benzofuran etc. derivatives
   c. Phenylcarbamoyl acetamide derivatives.

3. To purify the synthesized compounds by chromatographic techniques namely column (flash) chromatography.

4. To establish the structures of synthesized compounds by physicochemical and spectral analysis (IR, ^{1}H NMR, ^{13}C NMR and High-resolution mass spectrometry).

5. To carry out the preliminary biological evaluation of the synthesized compounds for their anticancer activity.

6. Molecular docking studies to determine the binding affinity of these molecules against CDK2 active site.

7. To generate a valid SAR based on the data obtained from the CDK2 inhibitory activity, molecular docking studies and the chemical structures of the inhibitors.

8. Based on this research to develop a future strategy to improve/optimize the lead molecules as potential CDK2 inhibitors.

The subsequent chapters unveils the extensive literature review on pyrazolo[1,5-*a*]pyrimidine, pyrazolo[4,3-*d*]pyrimidine scaffolds with emphasis on novel synthetic routes, pharmacological activity and SAR.

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CHAPTER 2

An insight on synthetic and medicinal aspects of pyrazolo[1,5-*a*]pyrimidine scaffold

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Graphical Abstract
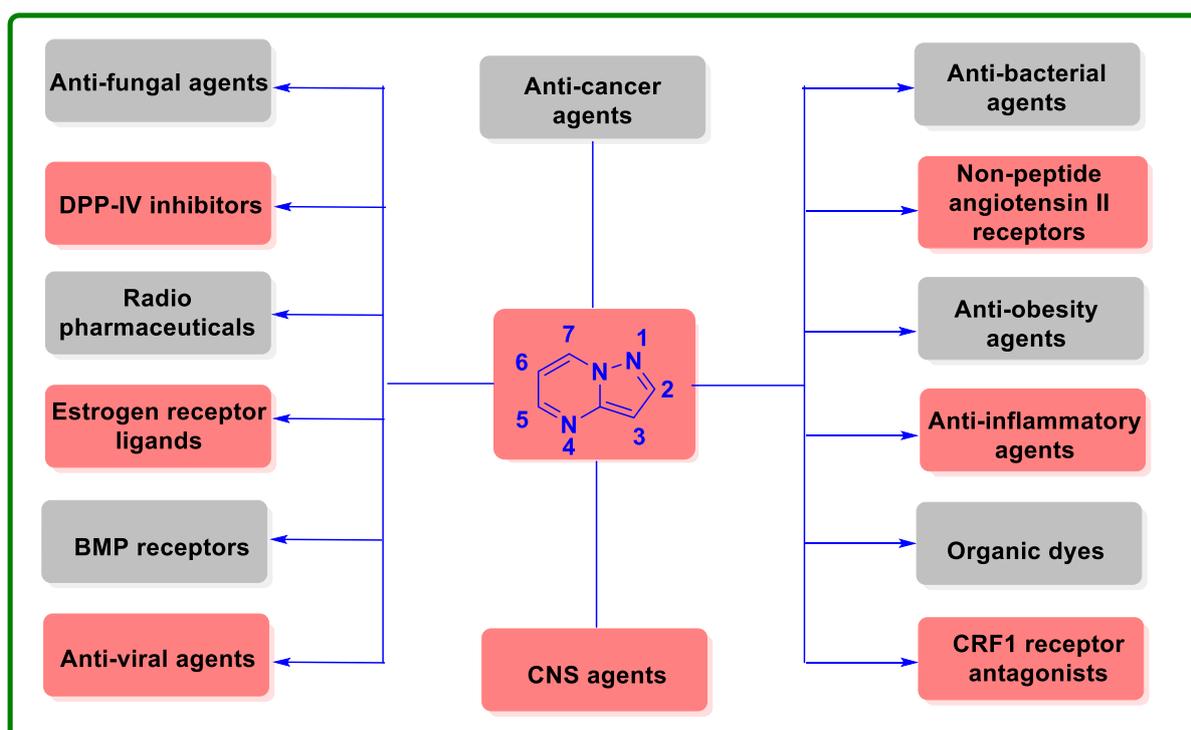
Review article

An insight on synthetic and medicinal aspects of pyrazolo[1,5-a]pyrimidine scaffold

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ARTICLE INFO

ABSTRACT

Pyrazolo[1,5-a]pyrimidine scaffold is one of the privileged hetrocycles in drug discovery. Its application as a buliding block for developing drug-like candidates has displayed broad range of medicinal properties such as anticancer, CNS agents, anti-infectious, anti-inflammary, CRF_1 antagonists and radio diagnostics. The structure-activity relationship (SAR) studies have acquired greater attention amid medicinal chemists, and many of the lead compounds were derived for various disease targets. However, there is plenty of room for the medicinal chemists to further exploit this privileged scaffold in developing potential drug candidates. The present review briefly outlines relevant synthetic strategies employed for pyrazolo[1,5-a]pyrimidine derivatives. It also extensively reveals significant biological properties along with SAR studies. To the best of our understanding current review is the first attempt made towards the compilation of significant advances made on pyrazolo[1,5-a]pyrimidines reported since 1980s.

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Abstract:

Pyrazolo[1,5-*a*]pyrimidine scaffold is one of the privileged heterocycles in drug discovery. Its application as a buliding block for developing drug-like candidates has displayed broad range of medicinal properties such as anticancer, CNS agents, anti-infectious, anti-inflammatory, CRF_1 antagonists and radio diagnostics. The structure-activity relationship (SAR) studies have acquired greater attention amid medicinal chemists, and many of the lead compounds were derived for various disease targets. However, there is plenty of room for the medicinal chemists to further exploit this privileged scaffold in devloping potential drug candidates. The present review briefly outlines relevant synthetic strategies employed for pyrazolo[1,5-*a*]pyrimidine derivatives. It also extensively reveals significant biological properties along with SAR studies. To the best of our understanding current review is the first attempt made towards the compilation of significant advances made on pyrazolo[1,5-*a*]pyrimidines reported since 1980s.

1   Introduction

Heterocycles hold a key point in organic and medicinal chemistry as they act as a bridge between life sciences and biochemical investigations. A significant amount of contemporary investigation is being currently pursued on these compounds world wide. Aza-heterocycles are essential scaffolds for generating wide range of chemical libraries/drug-like candidates for their applications to obtain desired theapeutic/pharmacological activity. Among all the aza-heterocycles, pyrazolo pyrimidine is one such essential drug-like nucleus bearing enormous biologically applicability [1]. From historic point of view, pyrazolo pyrimidines were first described as adenosine receptor antagonists [2]. Numerous isomeric forms of pyrazolo pyrimidine namely pyrazolo[5,1-*b*]pyrimidines, pyrazolo[5,1-*a*]pyrimidines, pyrazolo[4,3-*d*]pyrimidines, pyrazolo[1,5-*c*]pyrimidines and pyrazolo[3,4-*d*]pyrimidines are known [3]. Among all, pyrazolo[1,5-*a*]pyrimidine is a rigid bicyclic heterocycle core (**Fig. 1**) and has emerged a vital building block for medicinal compounds. The synthesis is accomplished by condensing 3 or 5-amino pyrazoles with sodium salts of formyl ketones [4], 1,3-diketones [5,6], enaminones [7–9], acetoacetanilides [10], β-ketoesters [11], enamino-nitriles [12], *β*-ketoaldehydes [13], 3-oxo-2-phenylpropanenitrile [14], dehydroacetic acid [15], malononitriles [16] and *β*,*γ*-unsaturated- *γ*-alkoxy-*α*-keto esters [17].
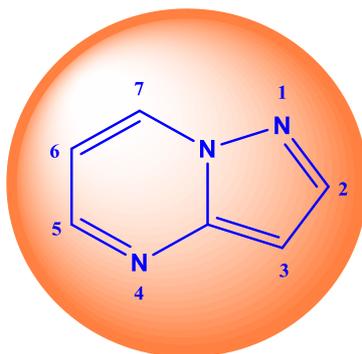
Fig. 1. The General structure of pyrazolo[1,5-*a*]pyrimidine.

These numerous synthetic pathways have prompted medicinal scientists to discover several new drugs consisting of pyrazolo[1,5-*a*]pyrimidines as a core moiety. These are also known to be purine analogues with diverse biological applications as antimetabolites in purine bio-chemical interactions, antischistosomal, antitrypanosomal and sedative [18], anxiolytic [19], AMP phosphodiesterase inhibitors [20], benzodiazepine receptor ligands [21], HMG-CoA reductase inhibitors [22], KDR kinase inhibitors [23], COX-1, COX-2 selective inhibitors [24], HCV inhibitors [25], PET tumor imaging agents [26], serotonin 5-HT$_6$ receptor antagonists [27], kinase inhibitors [28], HIV reverse transcriptase inhibitors [29], CCR1 antagonists [30], antimalarial and antifungal activities [31]. Several marketed drugs with pyrazolo[1,5-*a*]pyrimidine nucleus such as zaleplon, indiplon [32], dinaciclib [33], dorsomorphin [34], ocinaplon [1], anagliptin [35], lorediplon and pyrazophos [36] are illustrated in **Fig.**

2 with their respective structures and the approved activities. Inspired by these observations, in this review we summarize and represent the latest progress on synthetic strategies and medicinal properties of pyrazolo[1,5-*a*]pyrimidine derivatives, along with special emphasis on SAR of aforementioned derivatives.

Name of the Drug	Structure	Approved activity
Zaleplon		Insomnia
Indiplon		Hypnotic and sedative
Dinaciclib		Melanoma, chronic lymphocytic leukemia, pancreatic cancer
Dorsomorphin		Bone and cartilage
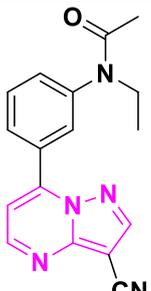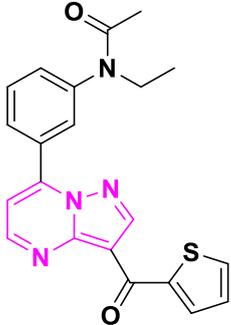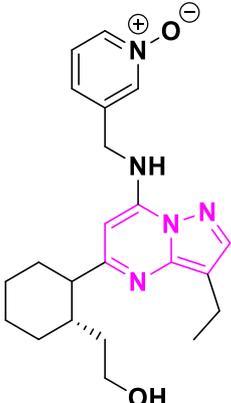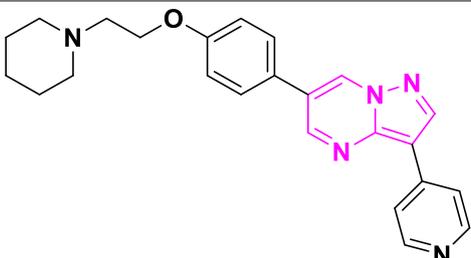
Ocinaplon		Anxiolytic, sedative and amnestic
Anagliptin		Type 2 diabetes mellitus
Lorediplon		Insomnia
Pyrazophos		Fungicide and insecticide
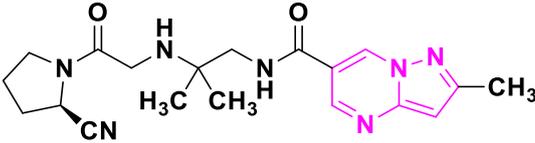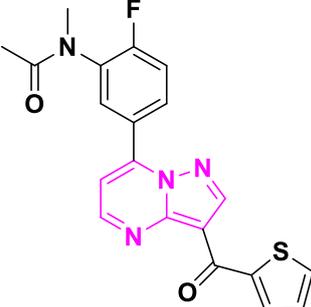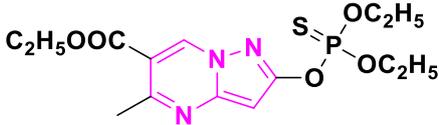
Fig. 2. Marketed drugs containing pyrazolo[1,5-*a*]pyrimidine nucleus.

2 Synthetic approaches for pyrazolo[1,5-*a*]pyrimidine scaffold

Synthesis of fused pyrimidine compounds was first reported in mid-1950's [2]. Since then thousands of derivatives have been synthesized by applying numerous synthetic strategies and analysed for their pharmacological properties. The various strategies for synthesis of pyrazolo[1,5-*a*]pyrimidines have been presented in **Fig. 3**.

One of the most common reagent, i.e. substituted 1*H*-pyrazole-5-amine (**i-xviii**), was used to obtain the target pyrazolo[1,5-*a*]pyrimidines (**P1-P18**) through numerous synthetic routes. Xu et al. prepared the desired product **P1** by cyclization of **i** with *N*-methyluracil in the presence of sodium ethoxide as a Michael acceptor in ethanol (route-a) [37]. Li and co-workers synthesized **P2** by allowing the reaction between **ii** and ethyl 4-chloroacetoacetate in acetone under refluxed conditions (route-b) [38]. Wang et al. reacted 5-amino-1*H*-pyrazol-4-yl thiophen-2-yl methanone (**iii**) with methyl (*E*)-3-(3-

(dimethylamino)acryloyl)benzoate to afford pyrazolo[1,5-*a*]pyrimidine scaffold **P3** under reflux conditions in acetone (route-c) [7]. Fraley et al. have accomplished the desired product **P4** by treating 4-phenyl-1*H*-pyrazol-5-amine (**iv**) with 3-hydroxy-2-phenylacrylaldehyde in acidic condition (route-d) [39]. Paruch et al. reported one pot synthesis of **P5** by reacting **v** with methyl-3-oxobutanoate, phosphorus oxychloride and *N*,*N*-dimethylaniline under inert conditions (route-e) [40]. Gommermann and co-workers carried out the cyclization reaction of 4-(4-(4-methylpiperazin-1-yl)phenyl)-1*H*-pyrazol-5-amine (**vi**) with 3-(dimethylamino)-2-(4-nitrophenyl)acrylonitrile to achieve **P6** under acidic conditions (1.25 M HCl in ethanol) in acetone (route-f) [41]. Frey and co-workers established cyclo-condensation reaction between 3-amino-4-bromo pyrazole (**vii**) with 3-oxo-2-phenyl propanenitrile to get target compound **P7** (route-g) [12]. Selleri et al. obtained **P8** via treating **viii** with ethyl-3-hydroxy-2-(thiophen-3-yl)acrylate (route-h) [42]. Labroli and co-workers introduced cyclization reaction of 3-amino pyrazole (**ix**) with *tert*-butyl 3-(3-methoxy-3-oxopropanoyl)piperidine-1-carboxylate to afford **P9** (route-i) [43]. Engers et al. reported condensation reaction of 1*H*-pyrazole-5-amine (**x**) with 2-(4-methoxyphenyl)malonaldehyde to achieve product **P10** under microwave irradiation conditions (route-j) [44].

Route-a:
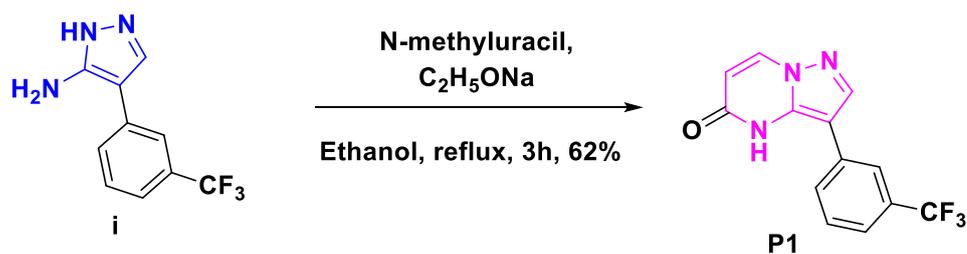
Route-b:
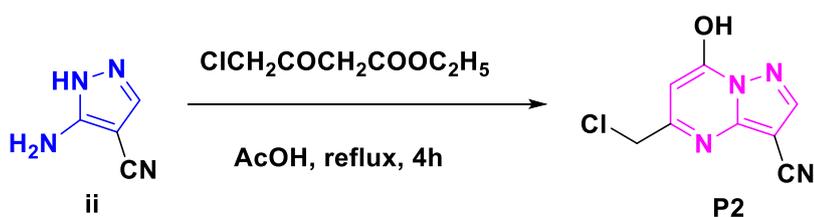
Route-c:
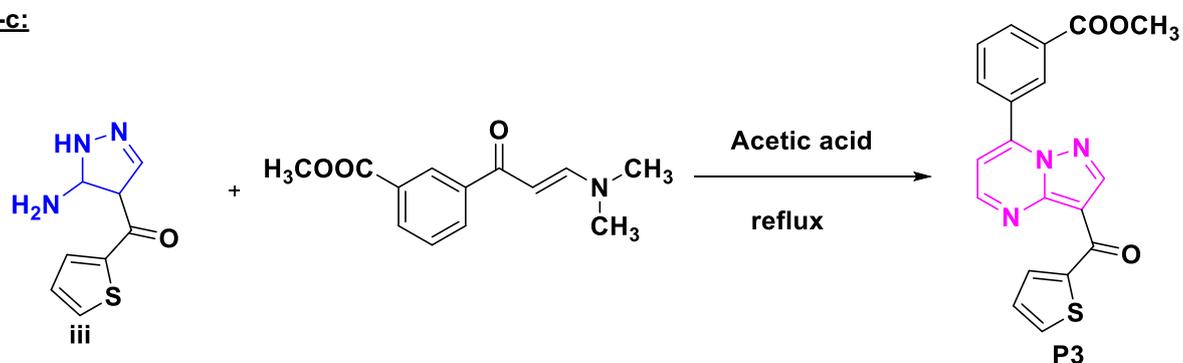
Route-d:
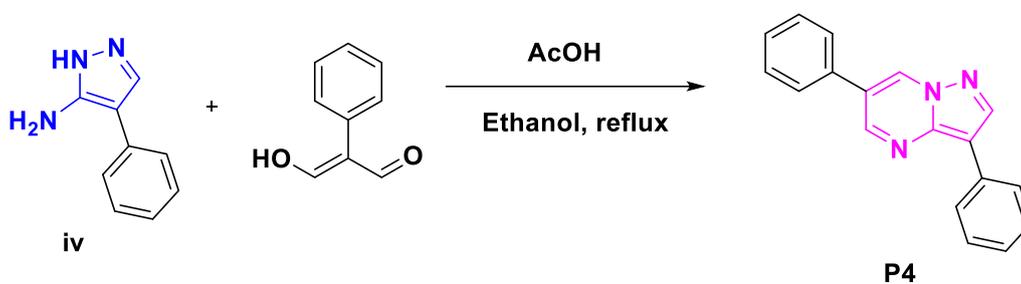
Route-e:
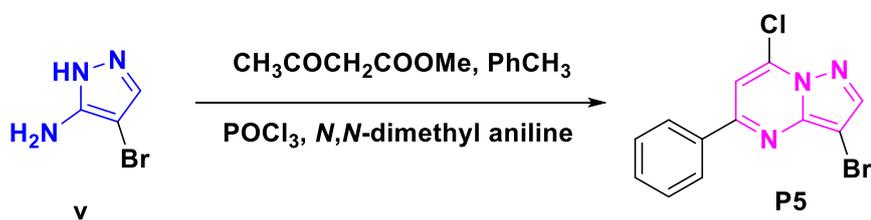
Fig. 3. Synthetic approaches for pyrazolo[1,5-*a*]pyrimidines.

Route-f:
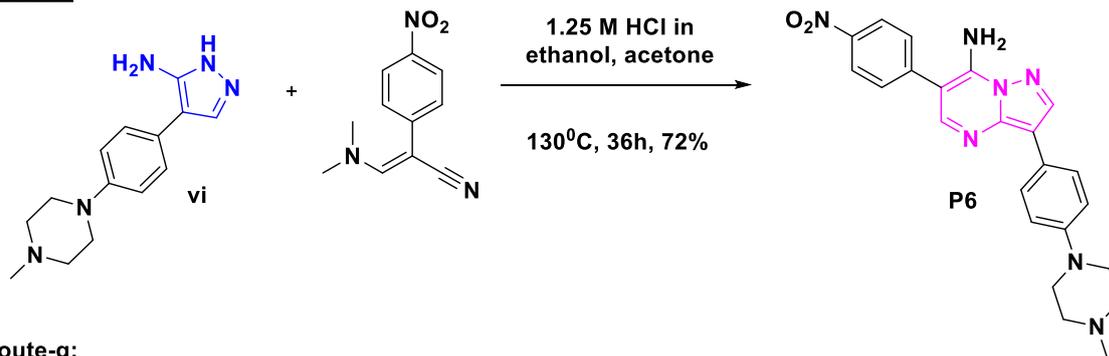
Route-g:
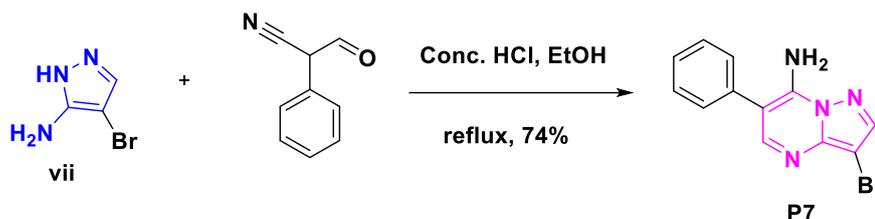
Route-h:
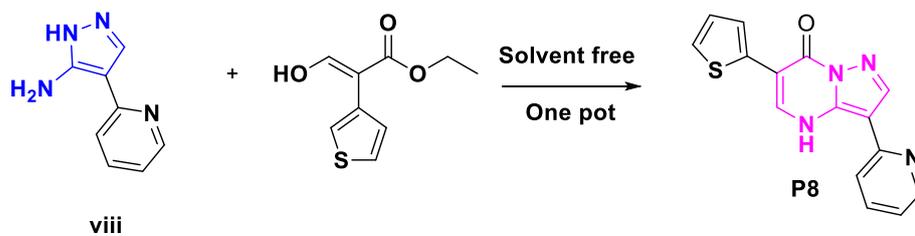
Route-i:
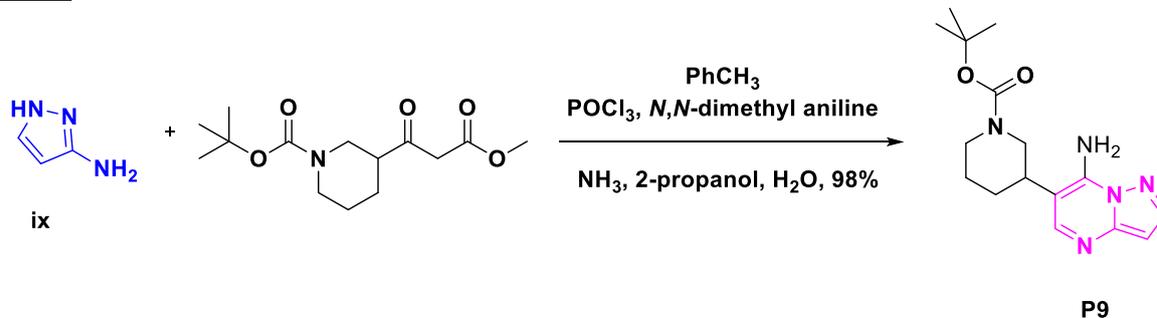
Fig. 3 (continued)**.** Synthetic approaches for pyrazolo[1,5-*a*]pyrimidines.

Dwyer et al. acquired the final compound **P11** by the condensation reaction of 3-aminopyrazole (**xi**) with 1,3-dimethyluracil (route-k) [45]. Enany et al. attempted the reaction between **xii** and malanonitrile to produce **P12** in presence of organic base like triethylamine (route-l) [46]. Kosugi and co-workers acquired **P13** by treating **xiii** with 2-substituted malonic acid diester in the presence of sodium ethoxide in ethanol under reflux conditions (route-m) [47]. Campton et al. reported the reaction of **xiv** with 1,1,3,3-tetramethoxypropane to attain the desired product **P14** (route-n) [48]. Selleri and co-workers attempted condensation reaction between 2-(5-amino-3-(*p*-tolyl)-1*H*-pyrazol-4-yl)acetic acid (**xv**) and 4,4-dimethoxy-2-butanone to attain **P15** in ethanol (route-o) [19]. Ivachtchenko et al. blended N^3–

methyl-4-(phenylsulfonyl)-4*H*-pyraole-3,5-diamine (**xvi**) and 3-aminobut-2-enenitrile in acetic acid to give **P16** (route-p) [49]. Patnaik and co-workers reported condensation of ethyl 5-amino-1*H*-pyrazole-4-carboxylate (**xvii**) with pentane-2,4-dione in the presence of aqueous NaOH and CH_3OH in acetic acid yielding **P17** (route-q) [50]. Tabrizi et al. offered synthesis of **P18** employing cyclization of **xviii** with diethyl ethoxymethylenemalonate (route-r) [51].

Route-j:
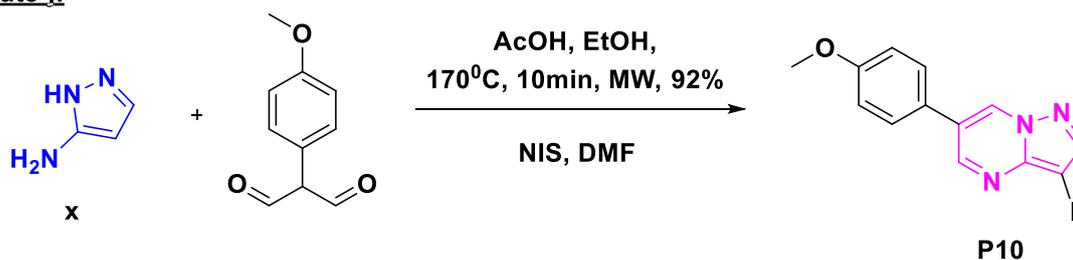
Route-k:
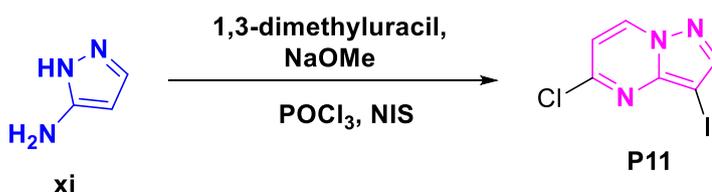
Route-l:
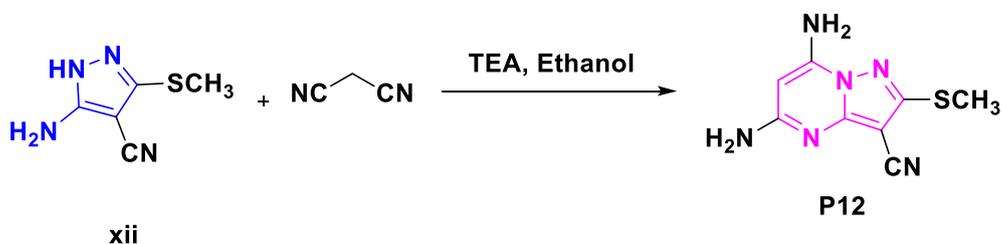
Route-m:
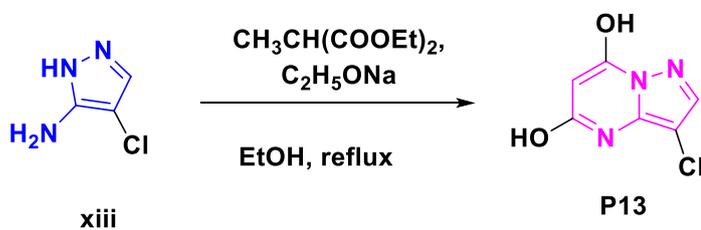
Route-n:
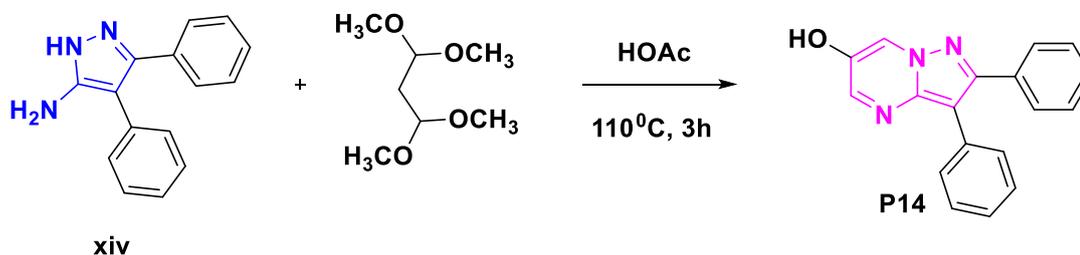
Fig. 3 (continued)**.** Synthetic approaches for pyrazolo[1,5-*a*]pyrimidines.

Route-o:
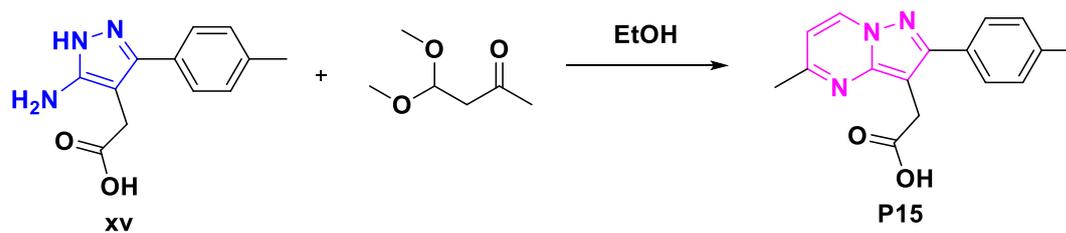
Route-p:
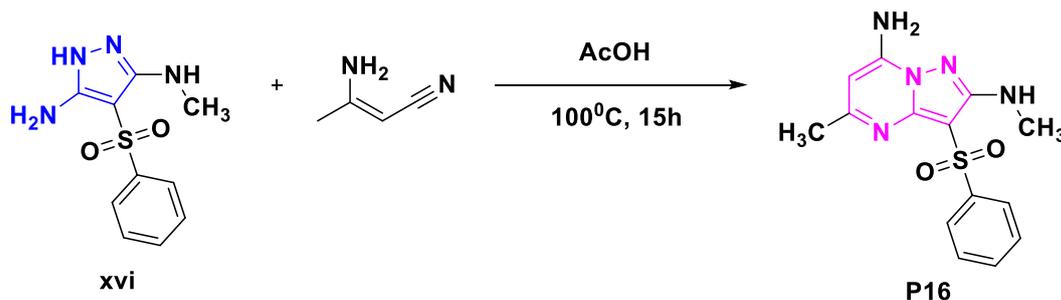
Route-q:
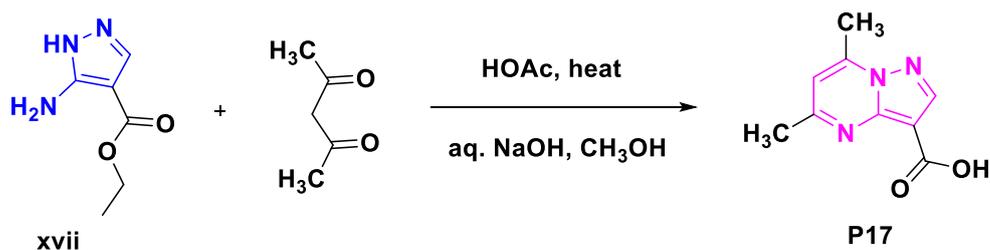
Route-r:
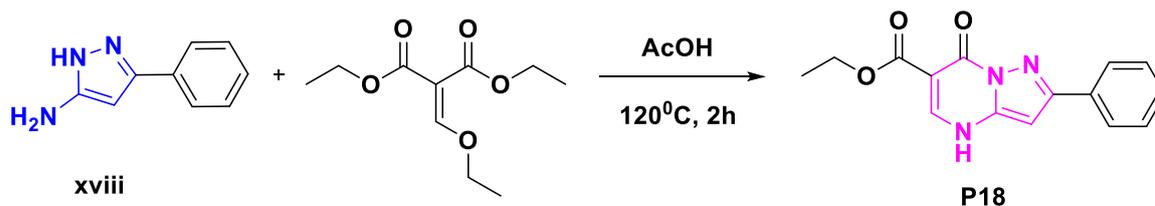
Fig. 3 (continued)**.** Synthetic approaches for pyrazolo[1,5-*a*]pyrimidines.

3 Pharmacology

During the course of literature study on pyrazolo pyrimidines such as pyrazolo[1,5-*a*]pyrimidines, pyrazolo[4,3-*d*]pyrimidines, pyrazolo[3,4-*d*]pyrimidines and pyrazolo[5,1-*b*]pyrimidines, it has been identified that pyrazolo[1,5-*a*]pyrimidines emerged as a promising lead agents against several ailments namely cancer, malaria, fungal infections, inflammation and etc. The following discussions illustrate the pharmacological applications of pyrazolo[1,5-*a*]pyrimidines against various biological properties.

It is quite evident that variations in functionalities on this scaffold brings about diverse biological activities.

3.1   Anti-cancer agents

3.1.1   Anti-proliferatives

Cell proliferation is the process resulting in a progression of cell number and is a balance between cell divisions and cell death. Deregulation in cell proliferation or suppression of cell death is a hallmark cause of varied clinical consequences including several forms of cancers. These cancer cells may abstain the normal regulatory control of cell division, thus failing to undergo appropriate cell death resulting into tumorigenic state [52]. Drugs or chemical entities that selectively kill these aberrant cells have emerged as promising antiproliferative agents in cancer drug discovery [53].

In 2005, Gopalsamy et al. reported a series of pyrazolo[1,5-*a*]yrimidin-7-yl phenyl amides as p21 chemoselective compounds for anti-proliferative activity. A p21 protein is a downstream effector of p53 gene, a major regulator of the DNA damage thus inhibiting cyclin dependent kinases (CDK) activity arresting the progression of cell cycle. The pharmacological evaluation was performed against HCT116 and 80S14 cell lines. Among the tested series, compound **1** exhibited potential activity and SAR study revealed the significance of R group as shown in **Fig. 4** [54].
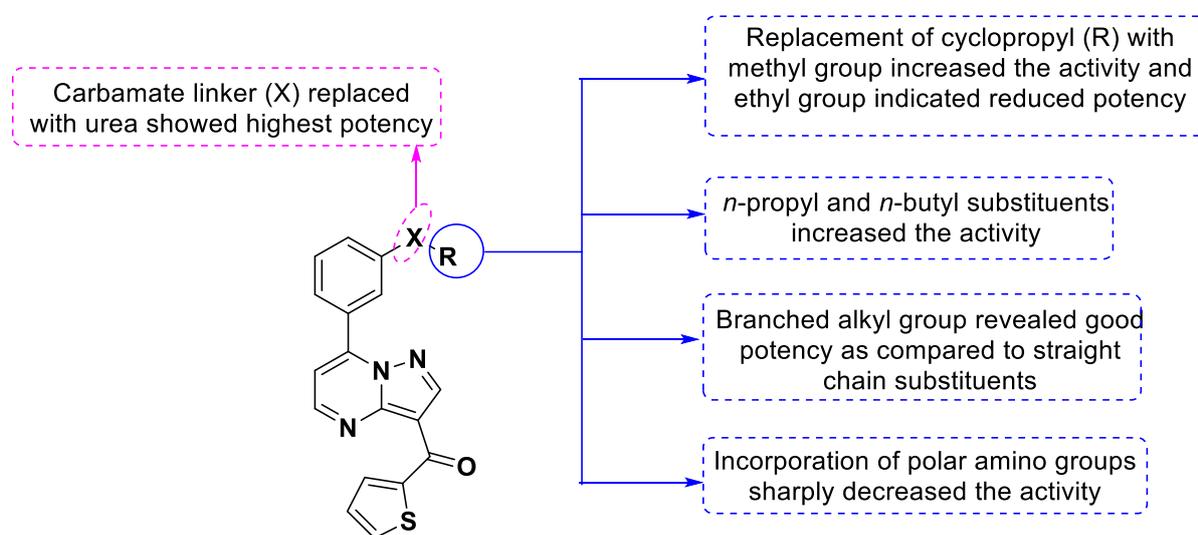
C. No	X	R^1	HCT116	80S14	Ratio
			IC$_{50}$ (µM)	IC$_{50}$ (µM)	
1	NHCONH	*i*-propyl	2.7	0.089	30

Fig. 4. SAR of p21 chemoselective pyrazolo[1,5-*a*]pyrimidin-7-yl-phenyl amides and the anti-proliferative activity of the representative compound **1**.

In 2006, Li and co-workers introduced a series of 3–cyano-5,7-disubstituted pyrazolo[1,5-*a*]pyrimidine derivatives and evaluated them for anti-tumor activity. Among the series, derivative **2** presented excellent anti-tumor activity against human liver (Bel-7402) and human fibro sarcoma (HT-1080) cancer cell lines. SAR studies revealed that compound with bis(trifluoromethyl)aniline at C-7 and piperdinyl at C-5 position enhanced anti-tumor activity as compared to other groups as displayed in **Fig. 5** [38].

C. No	R^1	NR^{2}R^3	Bel-7402	HT-1080
			IC$_{50}$ (µM)	IC$_{50}$ (µM)
2	3,5-di(trifluoromethyl)phenyl	piperdinyl	13.7	17.6
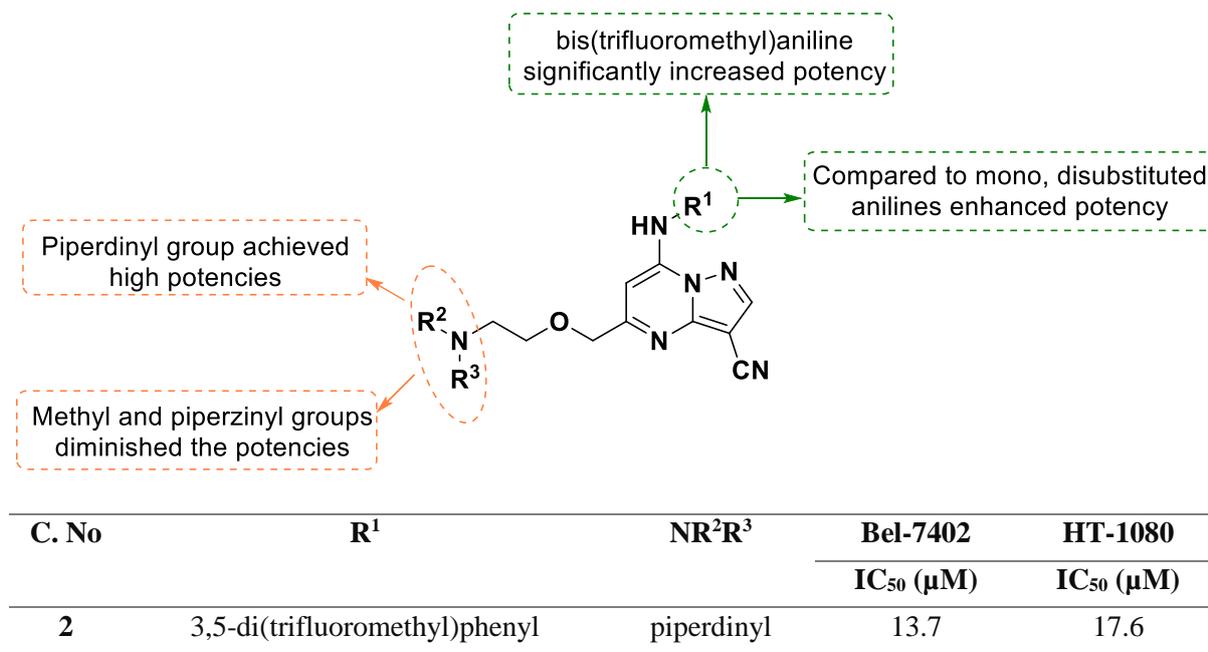
Fig. 5. SAR of amino alkoxy moiety containing pyrazolo[1,5-*a*]pyrimidines and anti-tumor activities of the representative compound **2**.

In 2007, Powell et al. discovered *N*-(3-(3-(thiophene-2-carbonyl)pyrazolo[1,5-*a*]pyrimidin-7-yl)phenyl)cyclopropane carboxamide derivatives as anti-proliferative agents. SAR studies indicated that methyl group at position 2 (R^3) and 6 (R^2) was responsible for lower activity as shown in **Fig. 6**. Among the tested series, compound **3** exhibited promising activity against HCT116 (p21+/+) and 80S14 (p21-/-) cell lines [55].

C. No	R^1	R^2	R^3	R^4	p 21+/+ IC$_{50}$ (µM)	p 21-/- IC$_{50}$ (µM)	Ratio
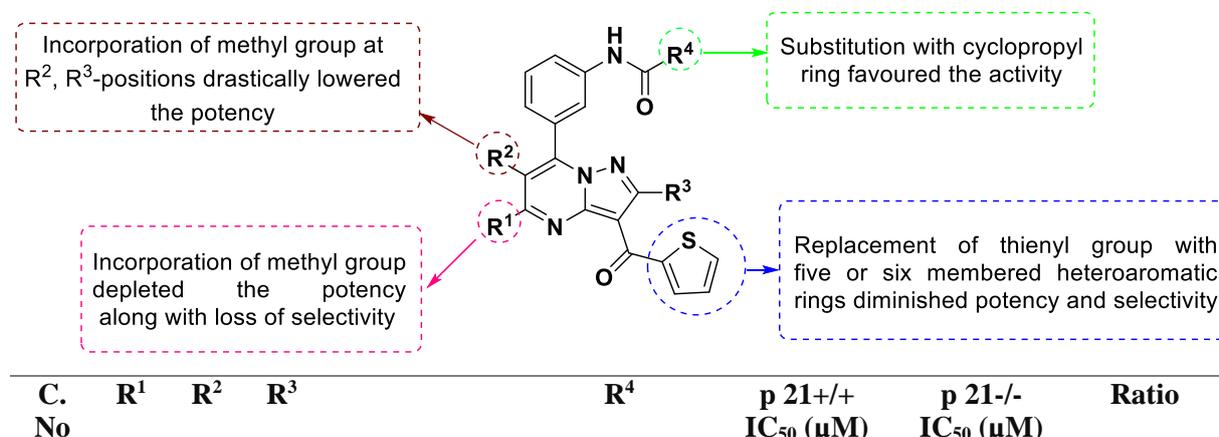
3	H	H	H	cyclopropyl	11	0.45	23

Fig. 6. SAR and anti-proliferative activity of pyrazolo[1,5-*a*]pyrimidine derivatives.

In 2009, Wang et al. derivatized a series of pyrazolo[1,5-*a*]pyrimidin-7-ylphenyl amides and evaluated them as anti-proliferative agents. SAR was carried out to correlate the importance of numerous substituents with the observed activity. From the series, compound **4** exhibited excellent potency against six colon cell lines (**Fig. 7**), namely HCT116 (p21+/+), 80S14 (p21-/-), LoVo, SW620, DLD1 and HT-29 [7] .

Potency heightened with halo groups, while dropped with alkyl/alkoxy substitutions

Fluorinated groups were more potent

Incorporation of substituents on phenyl ring demonstrated variations in activity

Urea and carbamate analogs were found essential for the activity

Small alkyl groups in the amide region were well tolerated

Carbonyl group was imperative for activity

C. No	Ar/Het	R	Y	p 21+/+	p 21-/-	LoVo	SW620	DLD1	HT-29	Ratio
				IC$_{50}$ (µM)	IC$_{50}$ (µM)	IC$_{50}$ (µM)	IC$_{50}$ (µM)	IC$_{50}$ (µM)	IC$_{50}$ (µM)	IC$_{50}$ (µM)
4	1,3-Ph-4-F	*i*-Pr	O	2.3	0.035	0.012	0015	0.015	0.015	65

Fig. 7. pyrazolo[1,5-*a*]pyrimidin-7-ylphenyl amides and their effect on colon cell lines.

In 2009, Ahmed et al. reported synthesis of novel compound **5** [*N*-(4-chlorophenyl)-2-(methylthio)-5-(naphthalene-2-yl)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide] by reacting sodium salt of 3-hydroxy-1-(2-naphthyl)prop-2-en-1-one with substituted 3-aminopyrazole as anti-tumor agent. The pharmacological evaluation was carried out at different concentrations against four cell lines, namely HCT116 (colon carcinoma), HepG2 (liver carcinoma cell line), Hela (cervix carcinoma cell line) and MCF7 (breast carcinoma). Results revealed that compound **5** exhibited effective toxicity against HCT116 and Hela cell lines as shown in **Fig. 8** [56].

	Chloro substituent on phenyl ring was crucial for activity than methyl
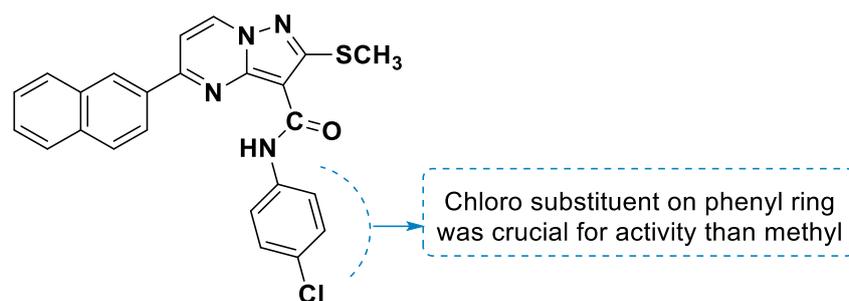
C. No	HepG2 IC$_{50}$ (µg/mL)	MCF7 IC$_{50}$ (µg/mL)	HCT116 IC$_{50}$ (µg/mL)	Hela IC$_{50}$·(µg/mL)
5	1.88	0.47	0.54	0.40

Fig. 8. Anti-tumor activity of *N*-(4-chlorophenyl)-2-(methylthio)-5-(naphthalene-2-yl) pyrazolo[1,5-*a*]pyrimidine-3-carboxamide.

In 2010, Abdel-Aziz et al. described a facile synthesis of thiazolo[3,2-*a*]benzimidazole linked pyrazolo[1,5-*a*]pyrimidines and performed *in vitro* anti-tumor activity against CaCo-2 (colon cancer cell line) and cytotoxicity against BHK (fibroblast cell line). SAR studies revealed the role of different functional groups and their effect on anti-tumor activity and cytotoxicity, which is presented in **Fig. 9**. All the compounds exhibited good activity. However, compound **6** showed potential activity for both the cell lines CaCo-2 and BHK [57].
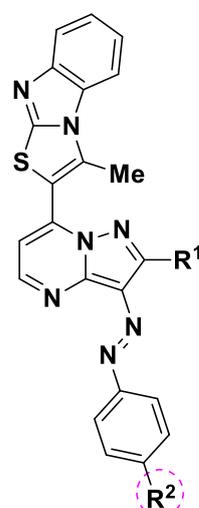
	Fluoro group favoured the anti-tumor activity compared to chloro and methyl substitutions

C. No	R^1	R^2	CaCo-2 IC$_{50}$ (µg/mL)	BHK IC$_{50}$ (µg/mL)
6	NH$_2$	F	0.5	2.3

Fig. 9. SAR and anti-tumor properties of thiazolo[3,2-*a*]benzimidazole linked pyrazolo[1,5-*a*]pyrimidines.

In 2011, El-Enany et al. described the synthesis of new pyrazolo[1,5-*a*]pyrimidine-3-carbonitriles with 7-substituted amino groups along with anti-tumor activity. Compound **7** [7-cyclohexylamino-2-methylthio-5-phenylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile] of this series displayed high potency against HCT116 cell line. SAR study revealed that the presence of functional groups at C-7 was essential for anti-tumor activity (**Fig. 10**) [46].

C. No	R	HCT116 IC$_{50}$ (µM)
7	C$_6$H$_{11}$	0.0020
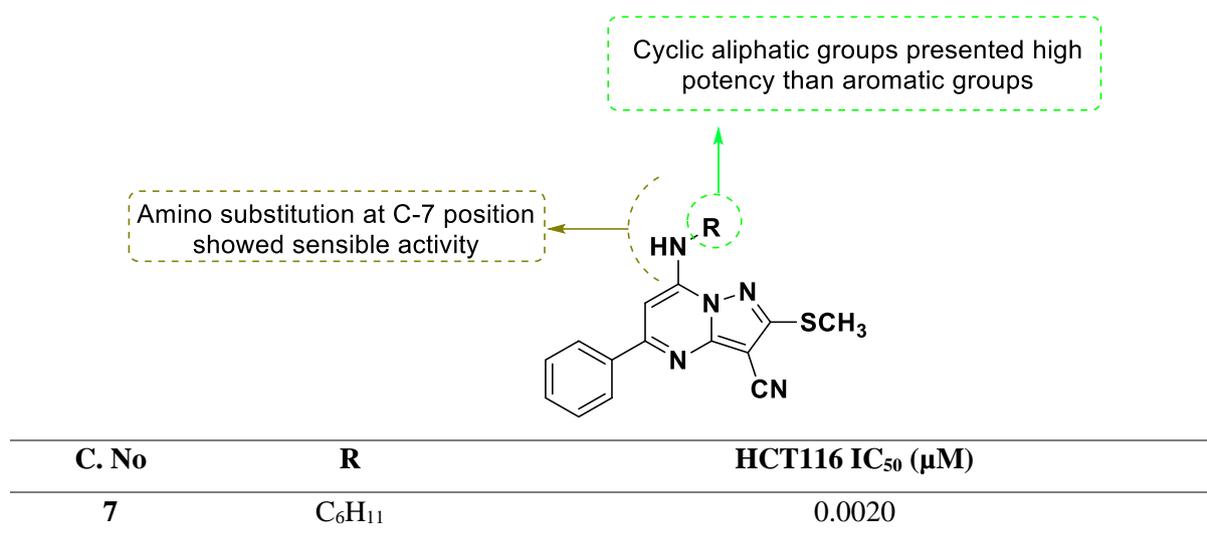
Fig. 10. SAR of pyrazolo[1,5-*a*]pyrimidine-3-carbonitriles as anti-tumor agents.

In 2012, Metwally et al. synthesized novel substituted pyrazolo[1,5-*a*]pyrimidine compounds and evaluated them for their anti-tumor and antioxidative properties. SAR study presented the significance of substituents for favourable activity. However, outcome of this work concluded that compound **8** [(*E*)-1,5-dimethyl-4-((2-methyl-7-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl)diazenyl)-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one] showed promising potency against ehrlich ascites carcinoma cells (EAC) at different effective doses (ED) (**Fig. 11**) [58].

C. No	X	EAC IC$_{50}$ (µg/mL)	ED$_{100}$ (µg/mL)	ED$_{50}$ (µg/mL)	ED$_{25}$ (µg/mL)
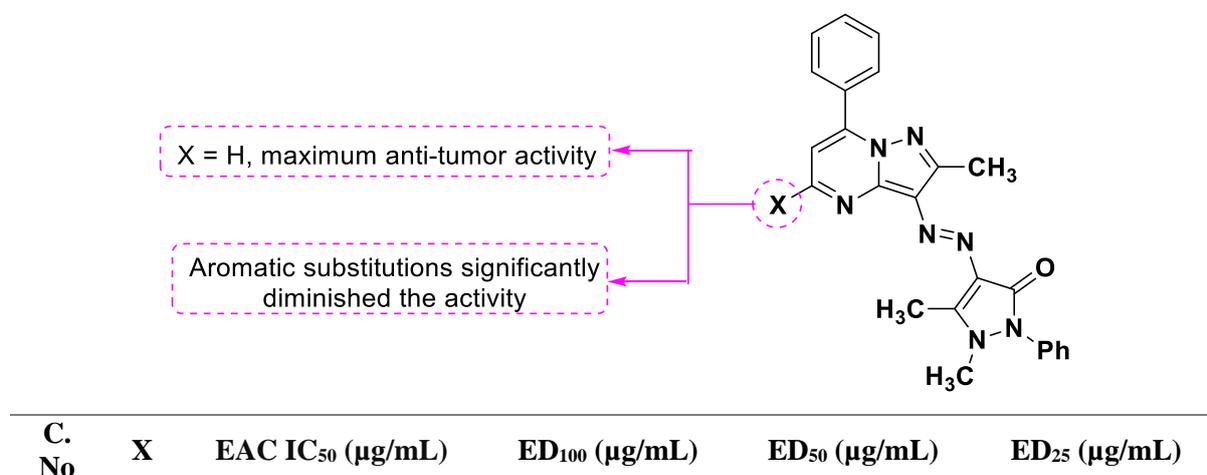
| **8** | H | 3.13 | 99.8 | 87.1 | 70.2 |

Fig. 11. SAR of pyrazolo[1,5-*a*]pyrimidine derivative as anti-tumor agent.

3.1.2 CDK Inhibitors

Cyclin-dependent kinases (CDKs) are a group of serine/threonine or mammalian heterodimeric kinase enzymes that are associated with regulation of cell-cycle progression by phosphorylating proteins involved in cell division. Regulatory subunits of these enzymes play a crucial role in controlling cell cycle, cell division and transcription mechanism in eukaryotes and hence regulate DNA replication process. Any disruptions in routine activity or deregulations result in numerous tumors, thus making the critical target for anticancer therapy. CDK inhibitors oversee the regulation of CDKs, and hence control cell cycle progression [59,60].

In 2005, Williamson et al. attempted structure-guided synthesis of pyrazolo[1,5-*a*]pyrimidines as CDK2 inhibitors. Among the synthesized series, compounds **9-11** exhibited potent activity against tested enzymes CDK2 and GSK-3β. GI$_{50}$ was also determined on a cancer cell line HCT 116. A close SAR observation (**Fig. 12**) revealed the effect of substituents [61].

C. No	X	R^1	R^2	CDK2	GSK-3β	HCT 116
				IC$_{50}$ (µM)	IC$_{50}$ (µM)	GI$_{50}$ (µM)
9	NH	Br		0.002	8.7	0.34
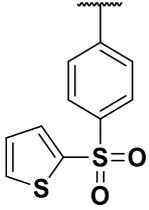
10	NH	Cl		0.011	4.0	0.12
11	NH	Br		0.016	5.3	0.08
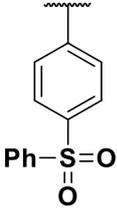
Fig. 12. SAR of pyrazolo[1,5-*a*]pyrimidines as CDK2 inhibitors.

In 2007, Paruch and co-workers prepared pyrazolo[1,5-*a*]pyrimidine derivatives as orally available CDK2 inhibitors from suitable acetonitrile and *β*-keto esters. Among the synthesised molecules, compound **12** exhibited excellent potency against CDK2/cyclin A, GSK-3*β* and Thym. Further screening against a panel of around 50 kinases (e.g. JNK 1, PKB, PDK1, ROCK-II) and 17 tumor cell lines was performed. The outcome of this work concluded that compound **12** was orally active and exhibited efficacy in A2780 mouse tumor xenograft model. SAR study depicting various substituents is elaborated in **Fig. 13** [40].

C. No	R^1	R^2	R^3	CDK2/CyclinA IC$_{50}$ (µM)	GSK-3β IC$_{50}$ (µM)	Thym IC$_{50}$ (µM)
12	Br		CH$_2$-3Pyr-O	0.013	0.13	0.21

Animal model	Dose, mpk$^\delta$ vehicle	AUC (µM h)	C_{max} (µM)	T_{max} (h)
Mouse	40 20 % HPBCD$^\phi$	17.9	6.81	2.0

mpk$^\delta$: milligrams per kilograms; HPBCD$^\phi$: Hydroxy propyl-beta-cyclodextrin

Fig. 13. SAR studies and pharmacokinetic properties of pyrazolo[1,5-*a*]pyrimidine derivatives as orally available CDK2 inhibitors.

In 2010, Heathcote et al. reported synthesis and biological evaluation of compound **13** [(2*S*,3*S*)-3-((7-(benzylzmino)-3-isopropylpyrazolo[1,5-*a*]pyrimidine-5-yl)amino)butane-1,2,4-triol] as effective inhibitor for CDK1, 2, 5 and 9 kinases. The cell line studies of this compound showed potent activity against various CDKs, namely CDK9, CDK2, CDK5, CDK1 and CDK7. This cell based study also displayed inhibition of phosphorylation of CDK substrate. The pharmacokinetic studies concluded that **13** is a potent and novel CDK inhibitor with a potential for oral delivery in cancer patients. SAR studies identified the significant role of side chain, hydroxyl group along with terminal amine group as depicted in **Fig. 14** [62].
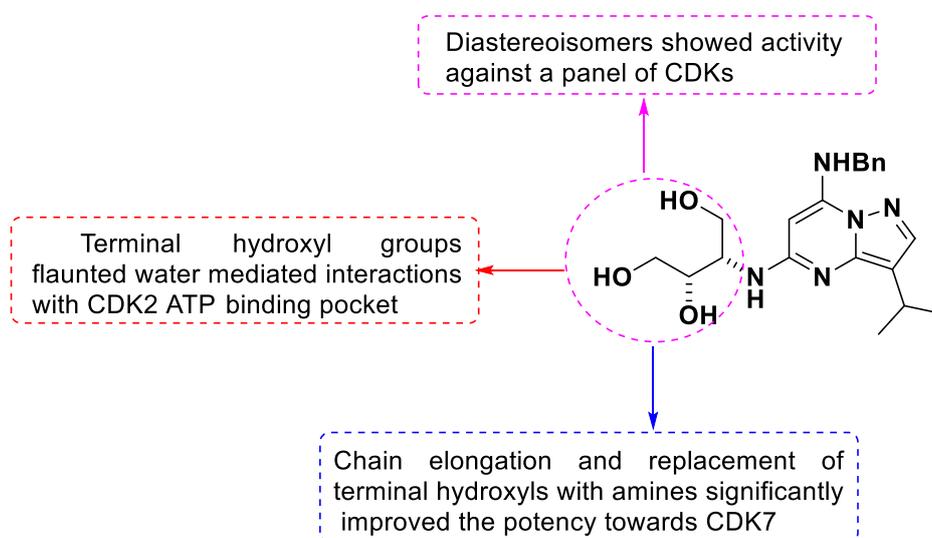
C. No	Kinase	IC$_{50}$ (µM) (SD)
	CDK1	0.033 (0.01)
	CDK2	0.003 (0.001)
	CDK4	20 (1.3)
13	CDK5	0.03 (0.006)
	CDK6	35.5 (1.3)
	CDK7	0.25 (0.04)
	CDK9	0.09 (0.01)

Fig. 14. SAR studies of (2*S*,3*S*)-3-((7-(benzylamino)-3-isopropylpyrazolo[1,5-*a*]pyrimidine-5-yl)amino)butane-1,2,4-triol as CDK inhibitors.

In 2013, Kamal et al. introduced a series of 2-aminobenzothiazole linked pyrazolo[1,5-*a*]pyrimidines and determined their anticancer activities against five cell lines A549, DU-145, ACHN, MCF-7 and Hela. From series, compounds **14** and **15** displayed promising activity with IC$_{50}$ values ranging from

2.01 to 7.07 and 1.94 to 3.46 μM respectively. Further, highly active compounds were screened on A549 cell line to know the molecular events involved in G_2/M cell cycle and the expression of CDKs. Results suggested that the most active compound arrested G_2/M cell cycle and reduced the expression level of CDKs. **Fig. 15** illustrates a brief SAR study depicting the effect of various substituents on the cancer activity [63].

C. No	R^1	R^2	R^3	A549 IC$_{50}$ (μM)	DU-145 IC$_{50}$ (μM)	MCF-7 IC$_{50}$ (μM)	ACHN IC$_{50}$ (μM)	Hela IC$_{50}$ (μM)
14	OCH$_3$	OCH$_3$	OCH$_3$	2.01	3.16	2.88	4.36	7.07
15	H	F	H	1.94	2.08	2.29	3.46	2.63

Fig. 15. SAR and effect of 2-aminobenzothiazole linked pyrimidines on human cancer cell lines.

In 2013, Li et al. selected pyrazolo[1,5-*a*]pyrimidine type cyclin A/CDK2 inhibitors and carried out an inclusive *in silico* investigation by three dimensional quantitative structure-activity relationship (3D-QSAR), MD simulations and docking experiments. The results of CoMSIA (SEE = 0.347, Q^2 = 0.516, R$_{pre}^2$ = 0.914, R$_{ncv}^2$ = 0.912, R$_m^2$ = 0.843, SEP = 0.812) with ten constituents (**16-25**) by steric, hydrophobic and H-bond donor showed internal and external predictive capacity. SAR studies were developed to determine the role of different substituents on the activity as depicted in **Fig. 16** [33].
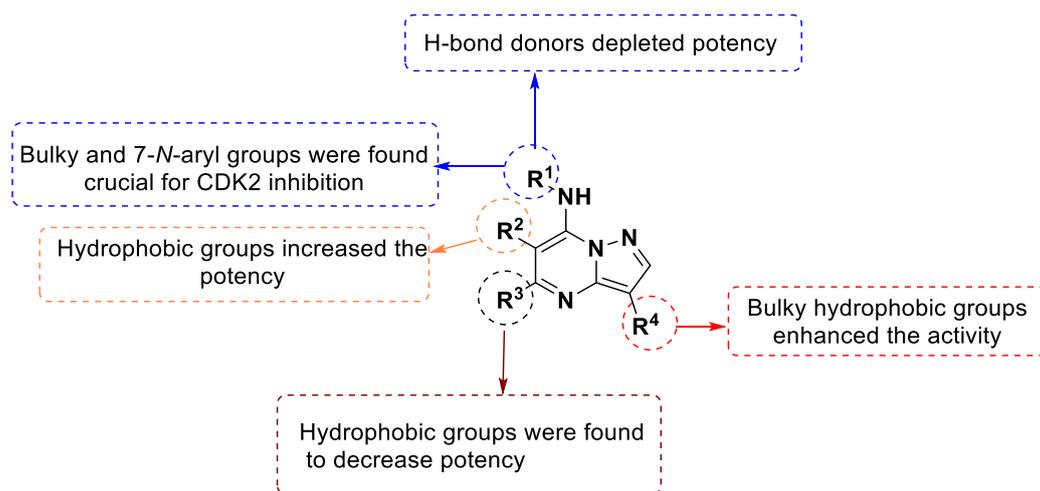
C. No	R¹	R²	R³	R⁴	pIC$_{50}$ (M)
16	H	H	HN-piperidin-3-yl	H	5.11
17	H	H	HN-piperidin-3-yl	3-hydroxyphenyl	6.19
18	benzyl	H	HN-piperidin-3-yl	N-methylpyrazol-4-yl	4.51
19	H	Et	HN-piperidin-3-yl	N-methylpyrazol-4-yl	6.48
20	H	3-pyridyl	HN-piperidin-3-yl	N-methylpyrazol-4-yl	5.82
21	H	Br	piperidin-4-ylmethyl	N-methylpyrazol-4-yl	5.55
22	pyridin-3-ylmethyl	H	Ph	c-Pr	7.15
23	pyridin-3-ylmethyl	H	Ph	S(CH$_2$)$_2$NHAc	8.70

| 24 | Pr | H | | Br | 6.79 |
| 25 | CH$_2$-3Pyr-O | H | | Br | 7.89 |
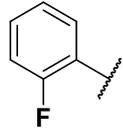
Fig. 16. SAR of pyrazolo[1,5-*a*]pyrimidine type CDK2 inhibitors.

In 2015, Phillipson et al. reported pyrazolo[1,5-*a*]pyrimidine derivatives as effective CDK9 inhibitors. Among all the synthesized molecules, compound **26** [*N*-(2-(5-chloropyrazolo[1,5-*a*]pyrimidin-3-yl)ethyl)-5-cyano-*N*,2-dimethylbenzenesulfonamide] showed significant potency against CDK9, CDK7, P13K*α* and FLT3 enzymes. SAR observations suggest the importance of functionalities as illustrated in **Fig. 17** [64]
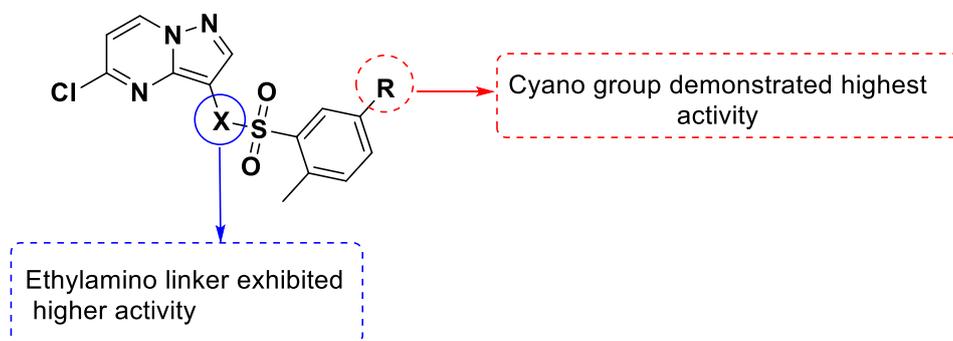
C. No	R	X	IC$_{50}$ (nM)				
			CDK9	CDK7	P13K*α*	FLT3	MV4:11
26	CN		203	>10,000	>10,000	219	0.177
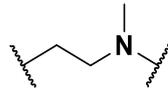
Fig. 17. SAR and pharmacological activities of compound **26** as CDK9 inhibitor.

3.1.3   c-Src, lck and chk inhibitors

Src family kinase (SFKs) is a non-receptor tyrosine kinase that plays a crucial role in tumor growth [65]. SFKs consists nine family members that share alike structural and functional features. Their over-expressed or elevated levels is known to be linked to cancer progression by mediating or promoting signal pathways of oncogenesis [66].

Lymphocyte-specific protein tyrosine kinase (Lck), a member of the Src-family is a key activator enzyme playing role in signal transductions essential in T-cell differentiation and proliferation.

Functions of T-cell are widely implicated in malignancies and autoimmune diseases. Lck inhibitors necessarily inhibit T-cell activation and are therefore widely used in T-cell mediated responses including cell proliferation and auto immune responses [67,68].

Serine/threonine checkpoint kinases (CHKs) are intracellular kinases that regulate checkpoints in cellular growth cycles [69]. CHKs specifically control both the G2/M and intra-S checkpoints and hence play a key role in cell-cycle progression [70].

In 2008, Makaiyama et al. reported synthesis of pyrazolo[1,5-*a*]pyrimidines as c-Src kinase inhibitors to decrease I_{Kr} channel blockade. Among all, compound **27** [7-((*S*)-1-benzyl-2-hydroxyethylamino)-5-cyclopropyl-2-(3,5-dimethoxyphenylaminopyrazolo[1,5-*a*]pyrimidine-3-carboxamine] surfaced as the most potent structure having good activity with less I_{Kr} channel blockade and excellent *in vivo* efficacy in middle cerebral artery (MCA) in rat. SAR study (**Fig. 18**) revealed the effect of incorporating different substituents on pyrazolo[1,5-*a*]pyrimidine scaffold for c-Src kinase activity [71].
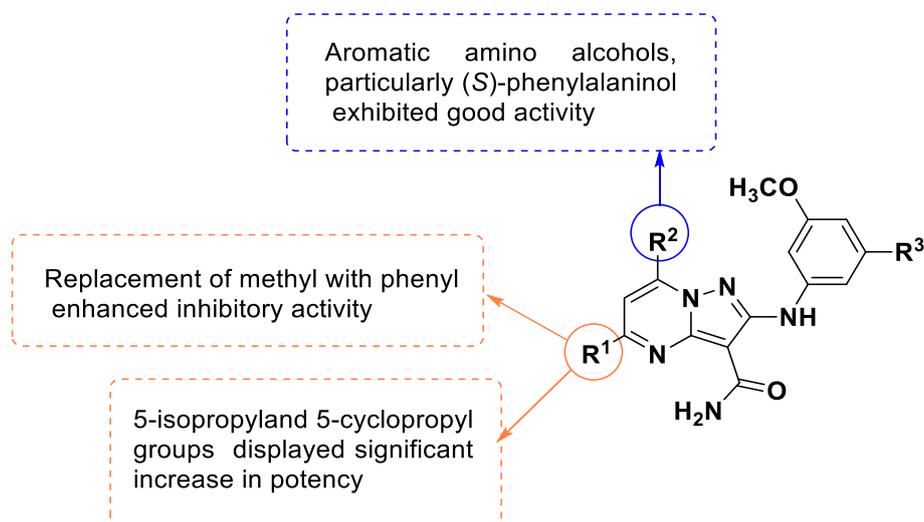
C. No	R^1	R^2	R^3	c-Src		ELISA	I_{Kr}
				IC$_{50}$ (μM)	% inhibition at 1 μM	IC$_{50}$ (μM)	% inhibition
27			OCH$_3$	0.003	100	0.1	23.4

Animal Model	AUC (μg h/mL)	CI (mL/min/kg)	$t_{1/2}$ (min)	V_{ss} (L/kg)

Rat	1.99	25	22	0.53

Fig. 18. SAR and pharmacokinetic properties of (7-((*S*)-1-benzyl-2-hydroxyethylamino)-5-cyclopropyl-2-(3,5-dimethoxyphenylamino)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide.

In 2010, Gommermann and co-workers reported the design and synthesis of pyrazolo[1,5-*a*]pyrimidine compounds as orally active inhibitors of Lck. A SAR study towards an effective Lck activity and also for selectivity against Hck, cSrc and KDR is shown in **Fig. 19**. Among the series of compounds, **28** [Isobutyl (4-(7-amino-3-(3-(piperzin-1-yl)pyrazolo[1,5-*a*]pyrimidin-6-yl)phenyl)carbamate] was optimized as active Lck inhibitor which also indicated excellent selectivity against Hck, cSrc and KDR [41].
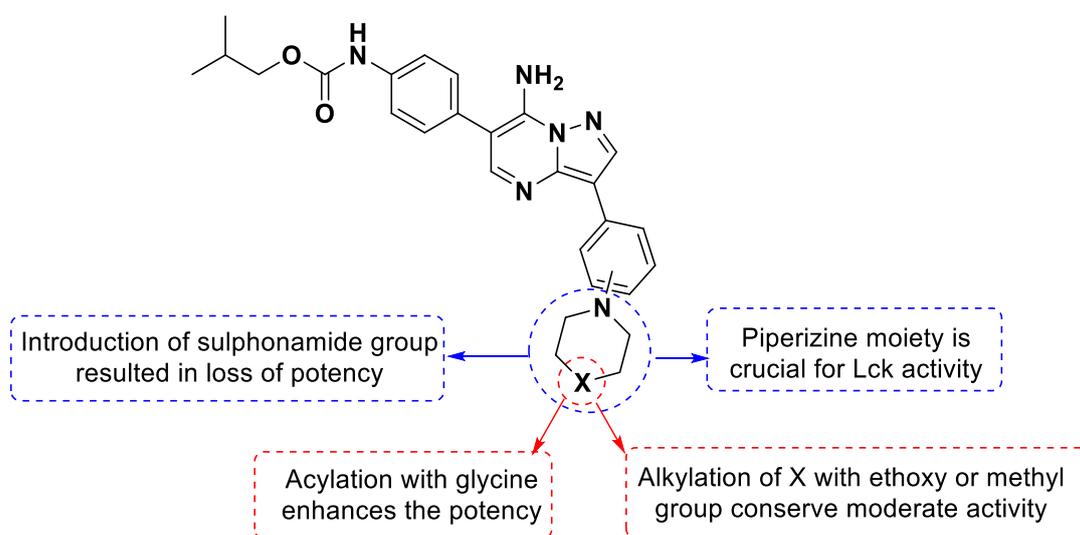
C. No	X	Lck lance* IC$_{50}$ (nM)	cSrc lance IC$_{50}$ (nM)	Hck lance IC$_{50}$ (nM)	KDR IC$_{50}$ (nM)
28	NH, *meta*	7	642	310	2100

*Lance is a trademark for assay kits by PerkinElmer, Inc.

Fig. 19. SAR and selectivity values of Isobutyl(4-(7-amino-3-(3-(piperzin-1-yl)pyrazolo[1,5-*a*]pyrimidin-6-yl)phenylcarbamate.

In 2011, Dwyer et al. reported the synthesis of a series of pyrazolo[1,5-*a*]pyrimidine compounds as CHK1 and CDK2 inhibitors. Compound **29** [3-methyl-*N*-(3-(1-methyl-1*H*-pyrazol-4-yl)-5-(piperidin-3-yl)pyrazolo[1,5-*a*]pyrimidin-7-yl)isothiazol-5-amine] emerged as the most active against CHK1 enzyme and also displayed selectivity against CDK2 enzyme. Single X-ray crystal structure of compound **29** bound to CHK1 protein was determined. Molecular docking studies of **29** presented significant interactions between pyrazolo[1,5-*a*]pyrimidine scaffold and CHK1 active site. Further, from SAR studies it was observed that substitution at C-7 was crucial for activity as shown in **Fig. 20** [11].

Aryl and heteroaryl analogues potentiated the activity

Aminoisothiazole substitution was essential for activity

Alkyl groups (Simple/branched/cyclo) reduced the potency

Interactions with carboxylate of Glu 91 and carbonyl of Glu 134 were observed

C. No	R	CHK1	CDK2/Cyclin A
		IC$_{50}$ (μM)	IC$_{50}$ (μM)
29		0.009	40
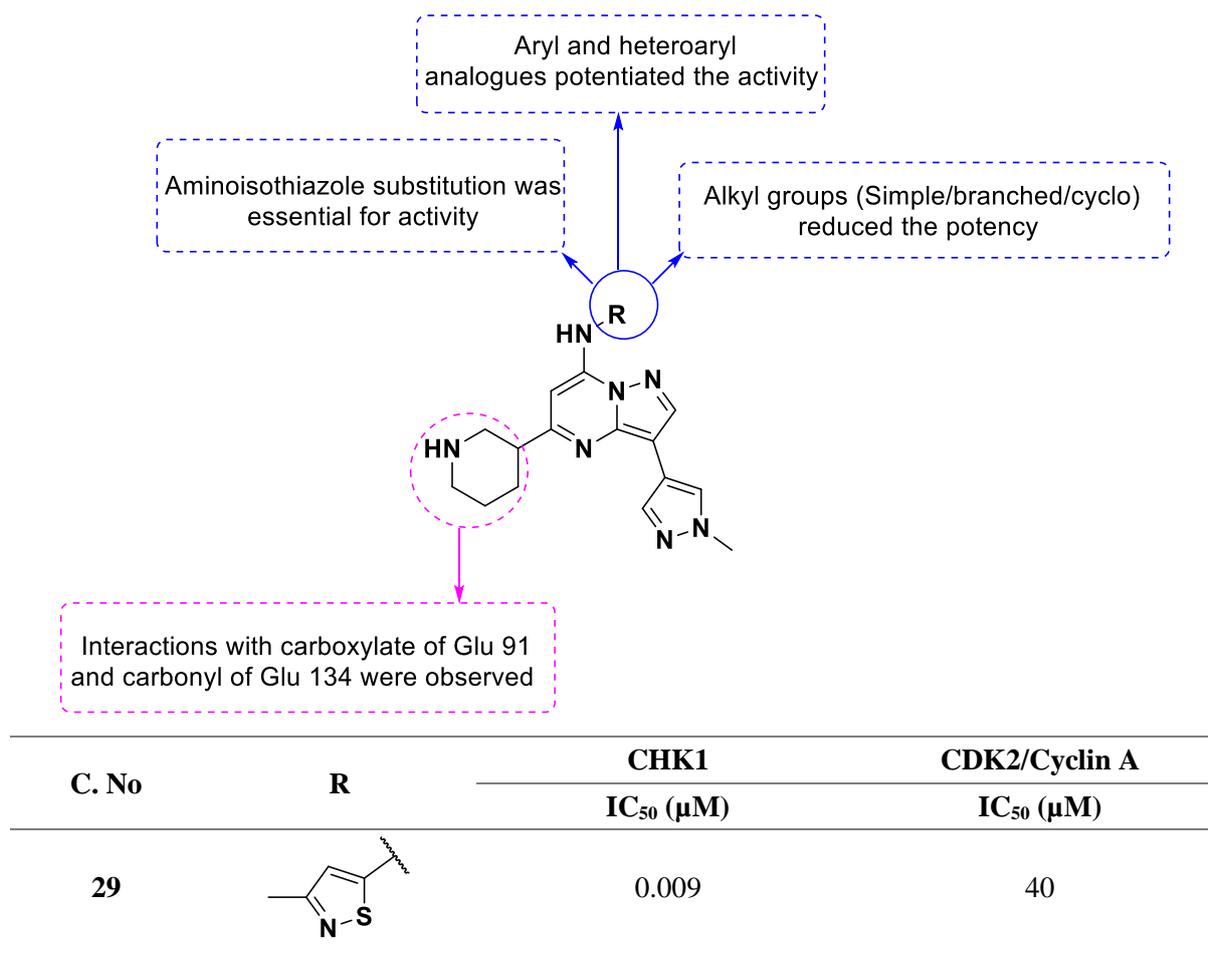
Fig. 20. SAR and molecular interactions of 3-methyl-*N*-(3-(1-methyl-1*H*-pyrazol-4-yl)-5-(piperidin-3-yl)pyrazolo[1,5-*a*]pyrimidin-7-yl)isothiazol-5-amine.

In 2011, Labroli et al. reported the preparation and pharmacological properties of novel pyrazolo[1,5-*a*]pyrimidines as CHK1 inhibitors. Compounds **30**-**32** from this series were highly potent against CHK1 and CDK2. X-ray crystallographic studies of potent compound **31**, revealed that the -NH$_2$ group at C-7 displayed strong hydrogen bonding interactions with the water molecule at the CHK1 enzyme active site. The SAR study is elaborated in **Fig. 21** [43].

Homologated and exocyclic analogues exhibited good activity than acyclic amines

NH of the C5 3-piperidinyl group was observed to form several key hydrogen bond interactions

Hetero atoms were found to be well tolerated
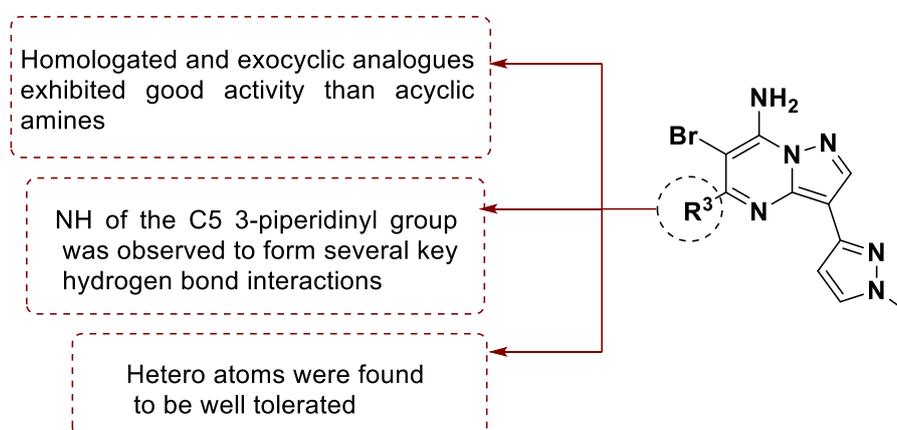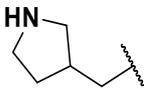
C. No	R³	CHK1 IC₅₀ (µM)	CDK2/Cyclin A IC₅₀ (µM)
		IC₅₀ (µM)	**IC₅₀ (µM)**
30		0.007	0.84
31		0.007	2.4
32		0.005	0.44
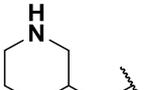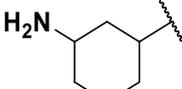
Fig. 21. SAR studies of 5-(3-aminocyclohexyl)-6-bromo-3-(1-methyl-1*H*-pyrazol-4-yl)pyrazolo[1,5-*a*]pyrimidin-7-amines derivatives.

3.1.4   B-Raf kinase inhibitors

Rapidly accelerated fibrosarcoma (Raf) kinase is a class of serine/threonine protein kinases and a key component in growth and survival of the cell. It is also known to be an important constituent of Raf-MEK-ERK signalling pathway. B-Raf kinase is active in several human cancers and has therefore been a potential target for inhibition in cancer therapeutics [72-74].

In 2009, Gopalsamy and co-workers reported the synthesis of a series of novel pyrazolo[1,5-*a*]pyrimidine-3-carboxylates and evaluated these compounds as B-Raf kinase inhibitors. In this series, compounds **33**-**35** exhibited potent activity. A brief SAR study of this series is presented in **Fig. 22** [75].
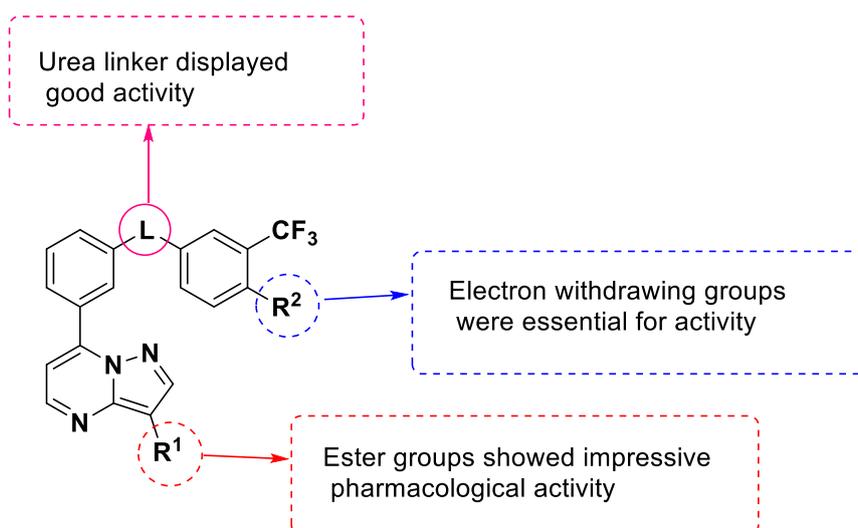
C. No	R¹	R²	Linker (L)	B-Raf kinase IC₅₀ (µM)

33	$CONHCH_2CH_2N(C_2H_5)_2$	Cl	NH-CO-NH	0.17
34	$CONH(CH_2)_3OCH_3$	Cl	NH-CO-NH	0.27
35	$CONHCH_2CH_2$-*N*-morpholinyl	Cl	NH-CO-NH	0.16

Fig. 22. SAR study of pyrazolo[1,5-*a*]pyrimidine-3-carboxylates as potent B-Raf kinase inhibitors.

In 2009, Berger et al. reported synthesis of novel 3-substituted pyrazolo[1,5-*a*]pyrimidin-7-yl)phenyl)-3-(trifluoromethyl)benzamides as active B-Raf kinase inhibitors. Potent B-Raf kinase activity was observed for the compounds **36-38** from this series. Compounds were also tested against WM 266-4 and HT29 cell lines and the results are shown in Fig. 23. From SAR studies, it was noted that higher potency was exhibited for compounds with basic amine residue at C-3 position (**Fig. 23**) [76].
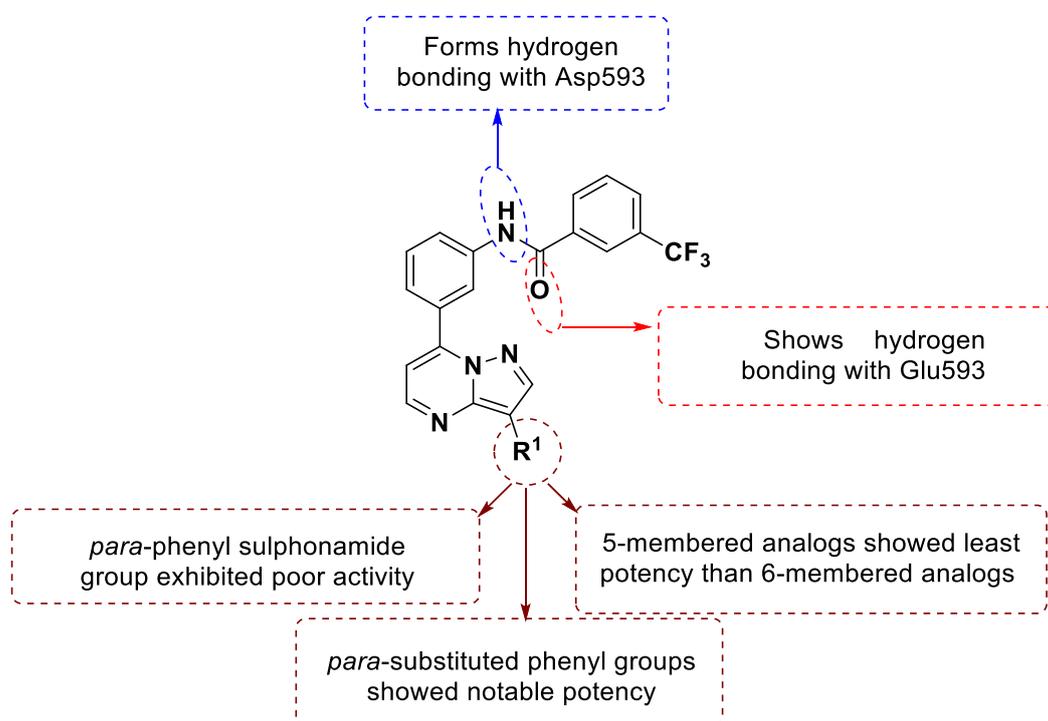
C. No	R^1	B-Raf	HT29	WM 266-4
		IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
36	4-Ph-CH$_2$-N(CH$_3$)$_2$	0.024	0.78	0.92
37	3-Pyridinyl-6-NHCH$_2$CH$_2$N(CH$_3$)$_2$	0.030	0.46	0.92
38	3-Pyridinyl-6-N-methylpiperazine	0.044	0.31	0.74

Fig. 23. SAR and B-Raf kinase activity of 3-substituted *N*-(3-(pyrazolo[1,5-*a*]pyrimidin-7-yl)phenyl)-3-(trifluoromethyl)benzamides.

In 2009, Grandi and co-workers reported the synthesis of 3,7-disubstituted pyrazolo[1,5-*a*]pyrimidines and evaluated them as B-Raf kinase inhibitors. SAR study revealed that the compounds with tropanes

at C-7, indazole at C-3 positions were well tolerated as depicted in **Fig. 24**. From the synthesized compounds, **39**-**41** exhibited good activity against B-Raf, A375 and WM266-4 cell lines [77].
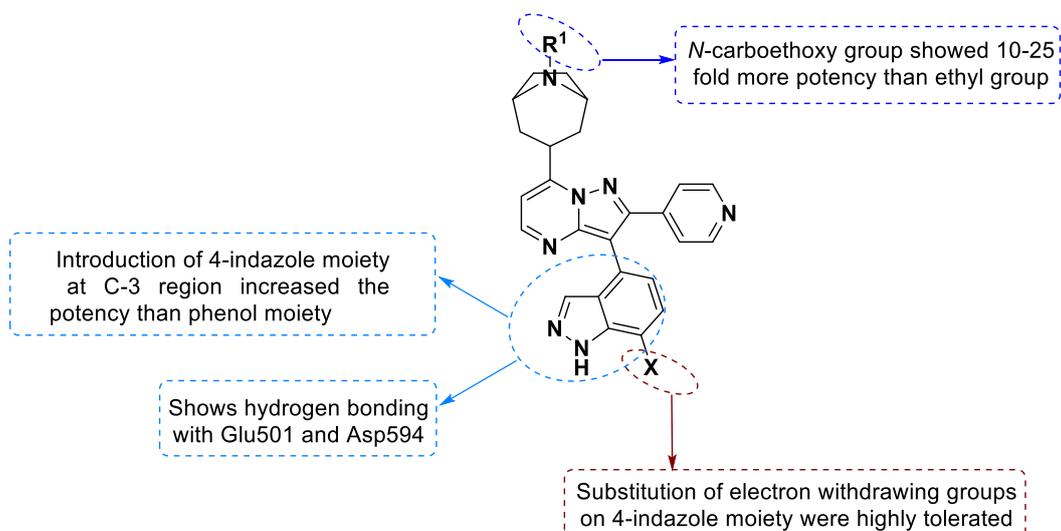
C. No	X	R¹	B-Raf	A375	WM266-4
			IC$_{50}$ (µM)	IC$_{50}$ (µM)	IC$_{50}$ (µM)
39	H	COOC$_2$H$_5$	0.002	0.38	0.30
40	Cl	COOC$_2$H$_5$	0.0004	0.58	0.28
41	F	COOC$_2$H$_5$	0.0004	0.62	0.30

Fig. 24. B-Raf kinase activity and SAR studies of disubstituted pyrazolo[1,5-*a*]pyrimidines.

Wang et al. in 2009 reported a series of novel pyrazolo[1,5-*a*]pyrimidines and as B-Raf type-1 kinase inhibitors. Compound **42** [3-(7-(2-chloro-4-((1*S*,4*S*)-5-methyl-2,5-diazabicyclo[2.2.1]heptan-2-yl)phenyl)-2-(pyridin-4-yl)pyrazolo[1,5-*a*]pyrimidin-3-yl)phenol] was highly selective and potent against B-Raf kinase as well as significantly effective against A 375 cell line. **Fig. 25** briefly describes the SAR studies for this series [78].

Methyl substitution significantly increased the activity

Introduction of diazabicyclo[2.2.1]heptanyl group enhanced both enzymatic and cellular activity

Pyridinyl nitrogen formed hydrogen bonding with Cys532

Chloro group showed better potency than H, methyl and F groups

Showed hydrogen bonding with Glu501 and Asp594 residues

C. No	R^1	R^2	B-Raf	A375
			IC$_{50}$ (nM)	IC$_{50}$ (μM)
42	CH$_3$	Cl	<0.3	<0.010

Fig. 25. SAR and B-Raf kinase activity of lead compound consisting pyrazolo[1,5-*a*]pyrimidine.

3.1.5 *Pim kinase inhibitors*

The PIM family of serine/threonine kinases are highly homologous (60-70%) in their kinase domains. They are mainly comprised of three members, Pim-1, Pim-2, and Pim-3 that regulate numerous signalling pathways essential in tumor development and progression. Overexpression of these kinases results in several forms of cancers namely pancreatic, prostate, bladder, haematological and many others. PIM inhibitors reduced the survival, progression and migration of these tumor cells thus proving to be effective in cancer treatment [79].

In 2013, Wang and co-workers reported structure and property based pyrazolo[1,5-*a*]pyrimidines as pan-pim inhibitors. For this series, SAR studies and lead optimization techniques identified a potent compound **43** [*N*-(5-(2-fluorophenyl)-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)-5-(((3-fluoropiperidin-4-yl)methyl)amino)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide] (**Fig. 26**). Along with pan-pim activity, **43** also exhibited good potency (IC$_{50}$ of 1.9 μM) against multiple myeloma cell line (MM1s) [80].

Fig. 26. Pin-pam inhibitory activities and SAR of *N*-(5-(2-fluorophenyl)-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)-5-(((3-fluoropiperidin-4-yl)methyl)amino)pyrazolo[1,5-*a*]pyrimidine-3-carboxamide.
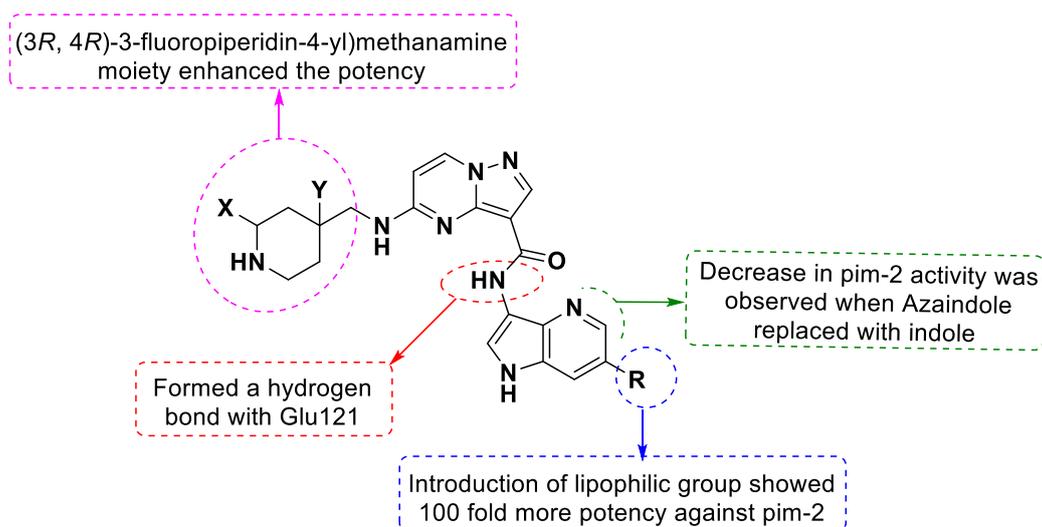
C. No	X	Y	R	$^{\Psi}$SC	K_i (nM)			$^{¥}$c.pKa
					Pim-1	**Pim-2**	**Pim-3**	
43	F	H	2-F-Ph	Trans	0.003	0.032	0.009	8.3

$^{\Psi}$SC: relative stereochemistry amid X- and -NHCH$_2$- functional groups.
$^{¥}$c.pKa: Calculated pKa of piperidine nitrogen using MoKa 1.1.0 through the proprietary roche model.

Dwyer et al. in 2013 reported the synthesis of C-3, C-5 di-substituted pyrazolo[1,5-*a*]pyrimidines as pim inhibitors. Among this series, compound **44** [*N*1-(3-(3-(5-methyl-1,3,4-oxadiazol-2-yl)phenyl)pyrazolo[1,5-*a*]pyrimidin-5-yl)ethane-1,2-diamine] exhibited potent inhibitory activity against pim-1 and pim-2 as well as indicated moderate selectivity against CDK2, pim-3, CK2, CHK1, P13K and mTOR kinases. It was observed from SAR studies that appropriate substitution at C-3 position influenced the activity. **Fig. 27** concisely presents the SAR studies and various kinase enzyme activities for the potent compund **44** [45].
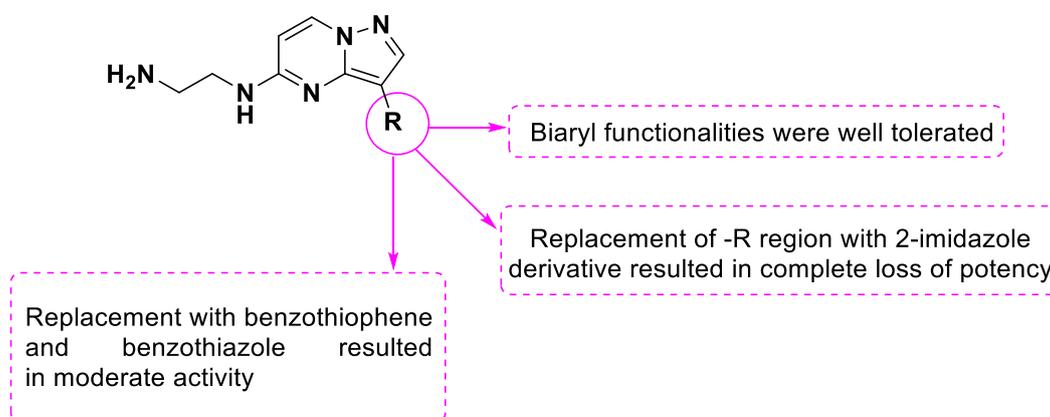
C. No	R	Pim-1	Pim-2
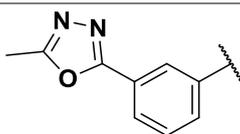
		IC$_{50}$ (nM)	IC$_{50}$ (nM)
44		1.3	7.3

Kinase	IC$_{50}$ (nM)
CDK2	580
Pim-3	1.8
CK2	950
CHK1	7100
mTor	>3000
P1K	>3000

Fig. 27. SAR, Pim and kinase activity of 3,5-disubstituted pyrazolo[1,5-*a*]pyrimidine.

Xu and co-workers in 2015 reported synthesis of disubstituted pyrazolo[1,5-*a*]pyrimidine derivatives and evaluated their pharmacological properties as potent pim-1 and Flt-3 kinase inhibitors. Compounds **45**-**49** in this series unveiled good inhibition. Further, it was revealed that compound **49** also exibited strong inhibition of BAD phosphorylation at 1 µM concentration. A brief SAR study has been represented in **Fig. 28** [37].

Introduction of *trans*-4-aminocyclohexanol enhanced potency by 100 folds

Substitutions with -OH group exhibited greater potency than ketone, ether and sulfone functionalities

Amino substituent displayed most potent pim-1 inhibition
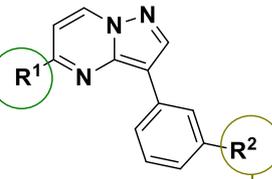
Electron withdrawing groups namely -Cl, trifluoro indicated a major role in pim-kinase activity

C. No	R^1	R^2	Pim-1 IC$_{50}$ (nM)	Pim-2 IC$_{50}$ (nM)	Flt-3 IC$_{50}$ (nM)
45		OCF$_3$	45	1491	ND
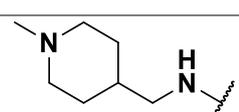
No.	Structure	R	pim-1	pim-2	pim-3
46		CF$_3$	25	282	157
47		OCF$_3$	27	269	53
48		CF$_3$	17	525	271
49		Cl	23	228	125
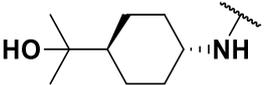
Fig. 28. SAR and pim activities of disubstituted pyrazolo[1,5-*a*]pyrimidine derivatives.

3.1.6   KDR kinase inhibitors

The kinase insert domain-containing receptor (KDR), also referred to as VEGFR-2, is one of the vital mediators of vascular endothelial growth factor (VEGF) functions in endothelial cells, and a key regulator of angiogenesis and subsequent progression of the tumor. Thus making it a potential and valid target for anticancer drug discovery [81].

In 2002, Fraley et al. synthesized 3,6-diaryl pyrazolo[1,5-*a*]pyrimidines by the condensation reaction between 2-arylmalondialdehydes and 3-amino-4-aryl pyrazoles and evaluated their activity as KDR kinase inhibitors. SAR studies suggested that substituents at C-6 and C-3 positions are essential for potency and are presented in **Fig. 29**. Among the series, compounds **50**-**53** showed moderate to good activity against KDR kinase and these compounds were also further screened against highly homologous receptor tyrosine kinases (PDGFR*β*, FLT-4, FGFR-1 and FLT-1) and SRC kinase [39].

C. No	R^1	R^2	KDR IC$_{50}$ (nM)
50	(4-pyridyl)	(4-pyridyl)	224
51	(4-methoxyphenyl)	(4-pyridyl)	37
52	(4-pyridyl)	(3-thienyl)	24
53	(4-methoxyphenyl)	(3-thienyl)	19

C. No	PDGFRβ	FGFR-1	FLT-1	FLT-4	SRC
50	0.4	59	4.6	2.7	20
51	0.5	54	5.7	2.2	27
52	11.4	104	7.1	9.2	146
53	1.8	>100	10.0	10.0	>100
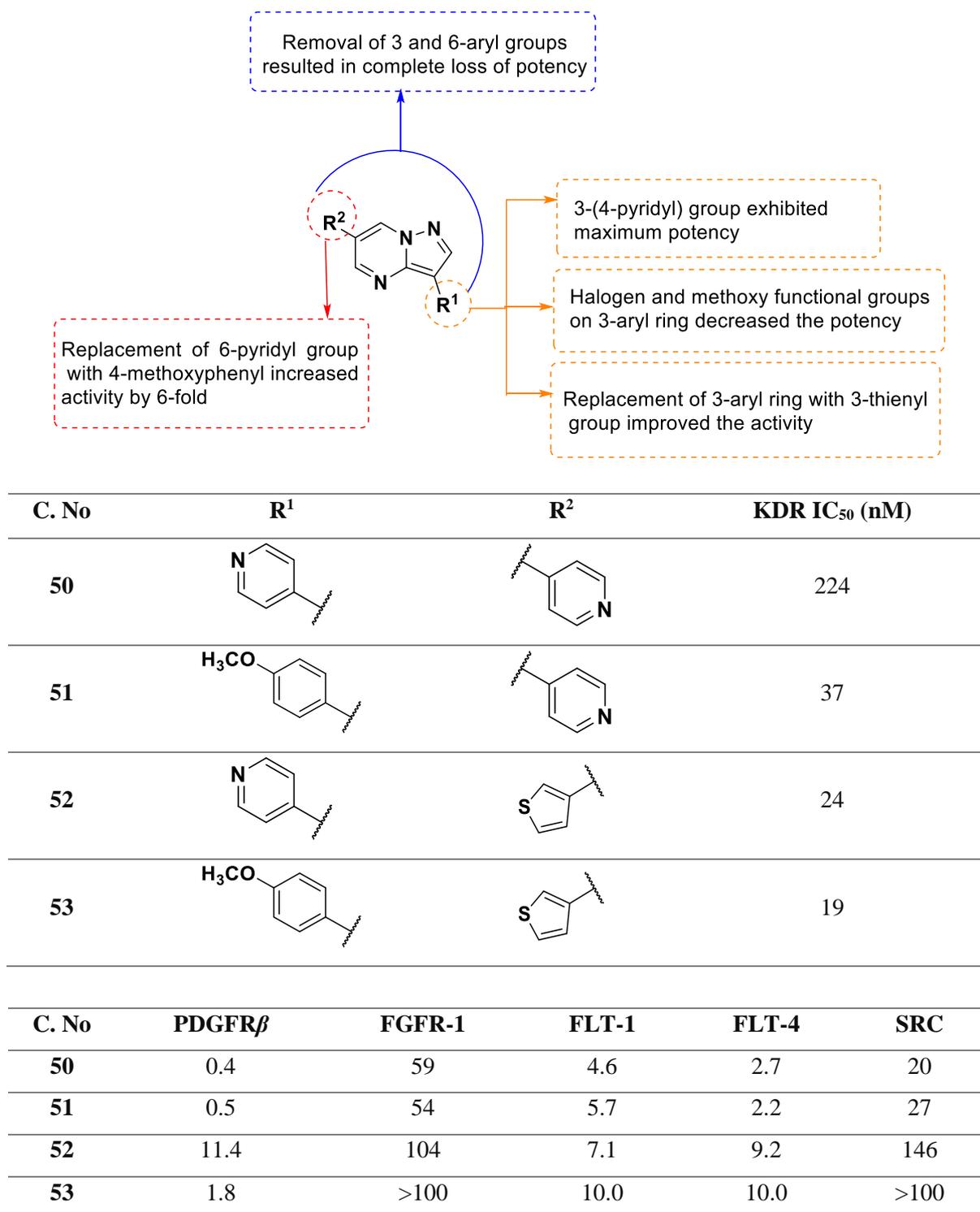
Fig. 29. SAR and KDR kinase and KDR selectivity values of 3,6-diaryl pyrazolo[1,5-*a*]pyrimidines.

The same research group in order to improve the solubility and biological property, reported a novel series of pyrazolo[1,5-*a*]pyrimidines as KDR kinase inhibitors. From the reported series, compounds **54-56** exhibited promising potency against KDR kinase, thus inferring the importance of physical

properties in improving the activity. SAR study reveals the importance of structural modifications at C-6 of pyrazolo[1,5-*a*]pyrimidine core to afford greater kinase activity as depicted in **Fig. 30** [20].

C. No	Side chain position	R¹	R²	KDR IC$_{50}$ (nM)	ECMA IC$_{50}$ (nM)
54	*para*			4	22
55	*para*			9	32
56	*para*			3	18
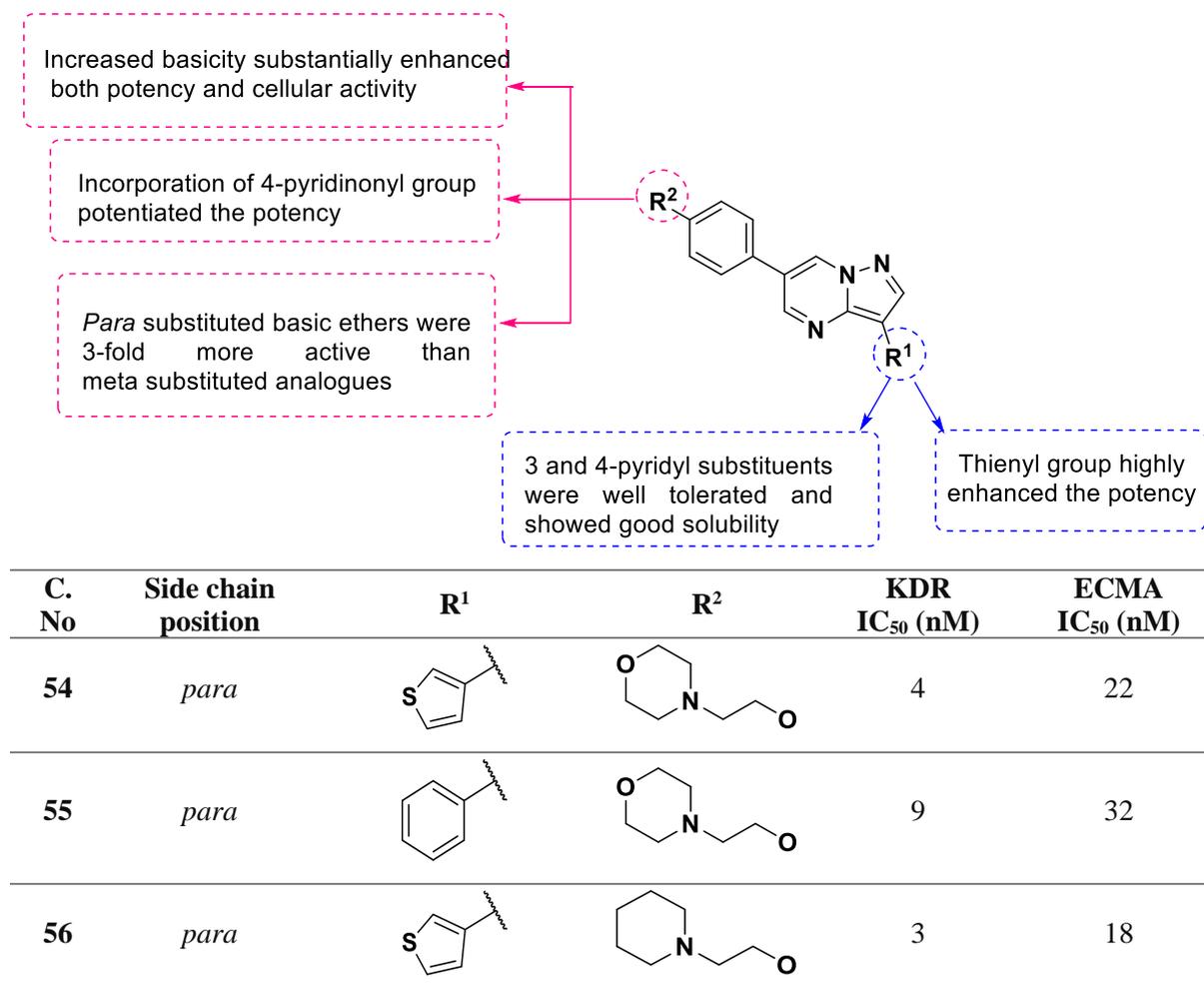
Fig. 30. KDR kinase and ECMA activities.

In 2008, Frey et al. reported the synthesis of 7-aminopyrazolo[1,5-*a*]pyrimidines as KDR inhibitors. SAR study revealed that incorporation of *N*,*N*-diaryl urea moiety at C-6 region and *N*-methyl pyrazole at C-3 region enhanced the kinase and cellular activity as shown in **Fig. 31**. It was noted that compound **57** exhibited greater KDR kinase and cellular activity. The compounds were also found to have good pharmacokinetic profiles and efficacy in estradiol-induced murine uterine edema (UE) assay [12].

The following SAR annotations appear in the figure:

- Incorporation of *N,N*-diaryl urea moieties with *para* substituted 6-phenyl ring enhanced the potency
- Methyl and trifluoromethyl groups exhibited good potency than fluoro or chloro
- Introduction of nitrile, acidic or amide groups at C-3 resulted in loss of activity
- Incorporation of 3-pyridine and *N*-methylpyrazole at 3-position showed good cellular activity

C. No	R	KDR IC$_{50}$ (nM)	KDR (cell) IC$_{50}$ (nM)	UE ED$_{50}$ (mg/kg)
57	3-CF$_3$	3	0.7	1.4

Fig. 31. Structural activity studies, KDR and UE results of 7-aminopyrazolo[1,5-*a*]pyrimidines.

3.2 Central nervous system (CNS) agents

3.2.1 *Benzodiazepine receptor modulators*

Benzodiazepines are a class of drugs affecting the central nervous system. They have been commonly prescribed as medications mainly as anxiolytics, hypnotics, sedatives and as anticonvulsants. The pharmacological response is believed to be mediated by the benzodiazepine receptors (BZR) in the brain [82]. This receptor is known to be a supramolecular complex consisting of the binding site for benzodiazepine, the recognition sites for GABA, and the GABA-dependent chloride channel, thus bringing about two types of pharmacological profile namely benzodiazepine agonists (anxiolytic, anticonvulsant and sedative), and inverse agonists (cause anxiety and convulsions) [83].

In 1999, Selleri et al. synthesized thienyl and methoxyphenyl substituted pyrazolo[1,5-*a*]pyrimidines and evaluated their biological activity against BZR. Within the series, compounds **58** [3-(4-methoxyphenyl)-6-(thiophen-3-yl)pyrazolo[1,5-*a*]pyrimidin-7(4*H*)-one and **59** [3,6-di(thiophen-3-yl)pyrazolo[1,5-*a*]pyrimidin-7(4*H*)-one] were reported as highly active compounds. SAR study is briefed in **Fig. 32** [84].

Substitution of 3-thienyl group displayed potent activity

Incorporation of methyl group on nitrogen resulted in reduction of potency

Introduction of 3-thienyl and 4-methoxy phenyl groups displayed significant potency

C. No	R	R^1	ϕInhibition (%)	K_i (nM)	ψGABA ratio
58			92 ± 0.8	42.2 ± 1.7	1.25
59			100 ± 6.7	16.3 ± 1.3	1.30

ϕInhibition (%): percent of inhibition of [3H]RO15-1788 at 10 µM concentrations are means $\pm$ 5 SEM of five determinations; ψGABA ratio: IC_{50} compound/IC_{50} compound + 10 µM GABA.

Fig. 32. SAR and activity values of thienyl and methoxyphenyl substituted pyrazolo[1,5-*a*]pyrimidines.

The same research group in 2001, reported the synthesis of 2-aryl pyrazolo[1,5-*a*]pyrimidine-3-yl acetamides as potent peripheral and central BZR ligands (PBZR & CBZR). Binding assays were performed for PBZR ([3H]PK 11195 and [3H]Ro 5-4864) and CBZR ([3H]Ro 15-1788) using radio ligands. SAR studies concluded with a key factor that was anticipated to enhance the cellular activity for both CBZR and PBZR ligands as shown in **Fig. 33**. Among the series, compounds **60**-**64** were found to be more selective and potent inhibitors [21].

Inclusion of phenyl ring resulted in loss of activity

Alkyl substitutions showed good potency

Introduction of methyl group at C-5 and C-7 positions showed highest activity
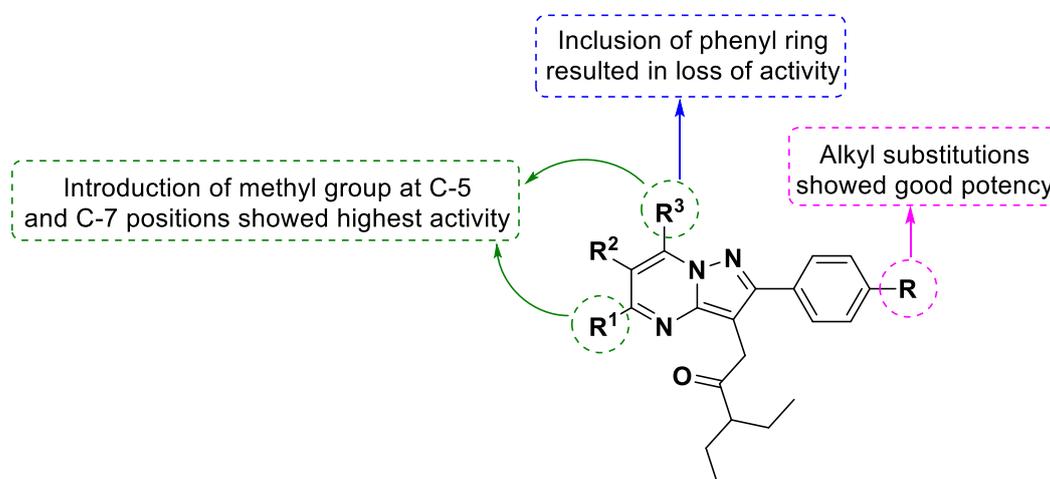
C. No	R^1	R^2	R^3	R	K_i (nM) PBZR		K_i (nM) CBZR
					[^{3}H]PK 11195	[^{3}H]Ro 5-4864	
60	CH$_3$	H	CH$_3$	Cl	2.4 ± 0.2	1.4 ± 0.2	>10.00
61	CH$_3$	H	CH$_3$	CH$_3$	0.8 ± 0.1	1.7 ± 0.2	>10.00
62	CH$_3$	H	CH$_3$	OCH$_3$	4.7 ± 0.4	3.1 ± 0.2	>10.00
63	CH$_3$	H	Ph	Cl	2.4 ± 0.2	2.5 ± 0.2	>10.00
64	Ph	H	CH$_3$	Cl	3.4 ± 0.2	2.7 ± 0.2	>10.00

Fig. 33. SAR and PBBR, CBZR studies of *N,N*-diethyl-(2-arylpyrazolo[1,5-*a*]pyrimidin-3-yl acetamides.

In 2003, Selleri et al. reported the synthesis of 3-aryl-6-(3-thienyl)pyrazolo[1,5-*a*]pyrimidin-7-ones and *in vitro* biological evaluation on Bz/GABA$_A$ and recombinant BZRs (α1235β2/3γ2). Compound **65** [3-(pyridin-2-yl)-6-(thiophen-3-yl)pyrazolo[1,5-*a*]pyrimidin-7(4*H*)-one] was found to be the most active in comparison to the standard drugs (diazepam, zolpidem) and a brief SAR study has been depicted in **Fig. 34**. [42].
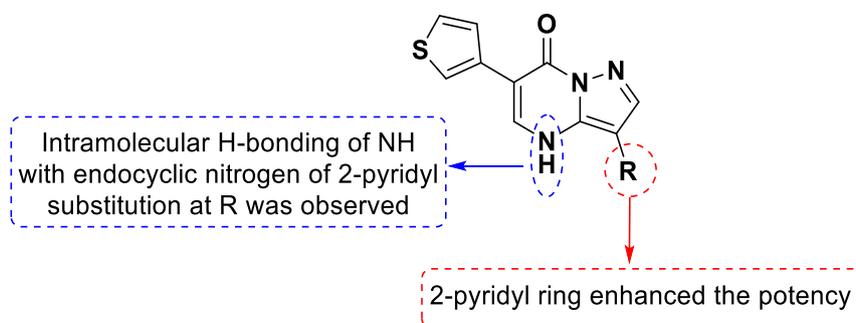
C. No	R	Inhibition (%)	K_i (nM)	*GR	α 1	α 2	α 3	α 5
65	2-pyridyl	98 ± 1	3.9 ± 0.5	1.10	7.0 ± 0.8	927 ± 83	ND	740 ± 51
Diazepam		NR	10	1.5	14	20	15	11
Zolpidem		NR	NR	NR	26.7	156	383	>10000

ND: Not determined; NR: Not reported; *GR: GABA ratio.

Fig. 34. SAR and activity data of 3-aryl-6-(3-thienyl)pyrazolo[1,5-*a*]pyrimidin-7-ones against recombinant BZRs.

In 2005, the same research group reported the synthesis and biological properties of 2-phenyl pyrazolo[1,5-*a*]pyrimidin-3-yl acetamides as peripheral benzodiazepine receptor (PBZR) ligands. SAR study is briefed in **Fig. 35**. Among the discovered series, ligands **66-68** showed prominent affinity for both PBZR and central benzodiazepine receptors (CBZR) [85].

Chain length of amide nitrogen
is crucial for activity
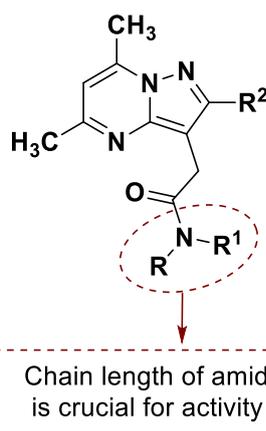
C. No	R	R¹	R²	PBR K_i (nM)	CBR K_i (nM)
66	C_2H_5	C_2H_5	4-CH₃-Ph	0.8	>10.00
67	*n-but*	*n-but*	Ph	4.5	10.00
68	C_2H_5	Ph	Ph	0.8	10.00

Fig. 35. SAR and activity studies of 2-phenyl pyrazolo[1,5-*a*]pyrimidin-3-yl acetamides.

The same research group (in 2005) reported the synthesis of compound **69** [*N,N*-dimethyl-2-(5-methyl-2-(*p*-tolyl)pyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide] as selective GABA$_A$ α1 receptor antagonist. *In vitro* studies were performed in comparison with the standard drugs, namely zolpidem and diazepam, on bovine brain homogenate along with recombinant BZRs (*αxβ2/3γ2*, x = 123&5). The results emphasise the binding affinities only for α1 as illustrated in **Fig. 36** [19].

Dimethylamino group is crucial for activity
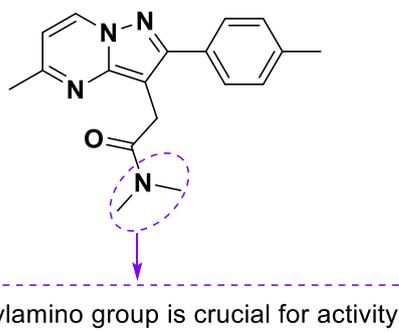
C. No	K_i (nM)			
	α 1	*α 2*	*α 3*	*α 5*
69	31 ± 4	>10000	>10000	>10000
Zolpidem	26.7	156	383	>10000
Diazepam	14	20	15	11

Fig. 36. Recombinant BZRs affinity values of *N,N*-dimethyl-2-(5-methyl-2-(*p*-tolyl)pyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide.

In 2010, Reynolds et al. discovered the synthesis of pyrazolo[1,5-*a*]pyrimidine acetamides bearing phenyl alkyl ether functional groups and evaluated them as translocator protein (TSPO) and CBZR ligands. All the synthesized compounds **70-75** exhibited good affinity for TSPO with broad selectivity over CBZR as well as CNS transporters and receptors, as illustrated in **Fig. 37** [86].

C. No	R	TSPO K_i (nM)	CBR K_i (nM)
70	CH_3	4.7 ± 0.2	>10000
71	CH_2CH_3	5.7 ± 0.5	>10000
72	CH_2CH_2F	7.0 ± 0.4	>10000
73	$(CH_2)_2CH_3$	1.4 ± 0.2	>10000
74	$(CH_2)_3CH_3$	1.1 ± 0.1	>10000
75	H_2C—CH	4.8 ± 0.5	>10000
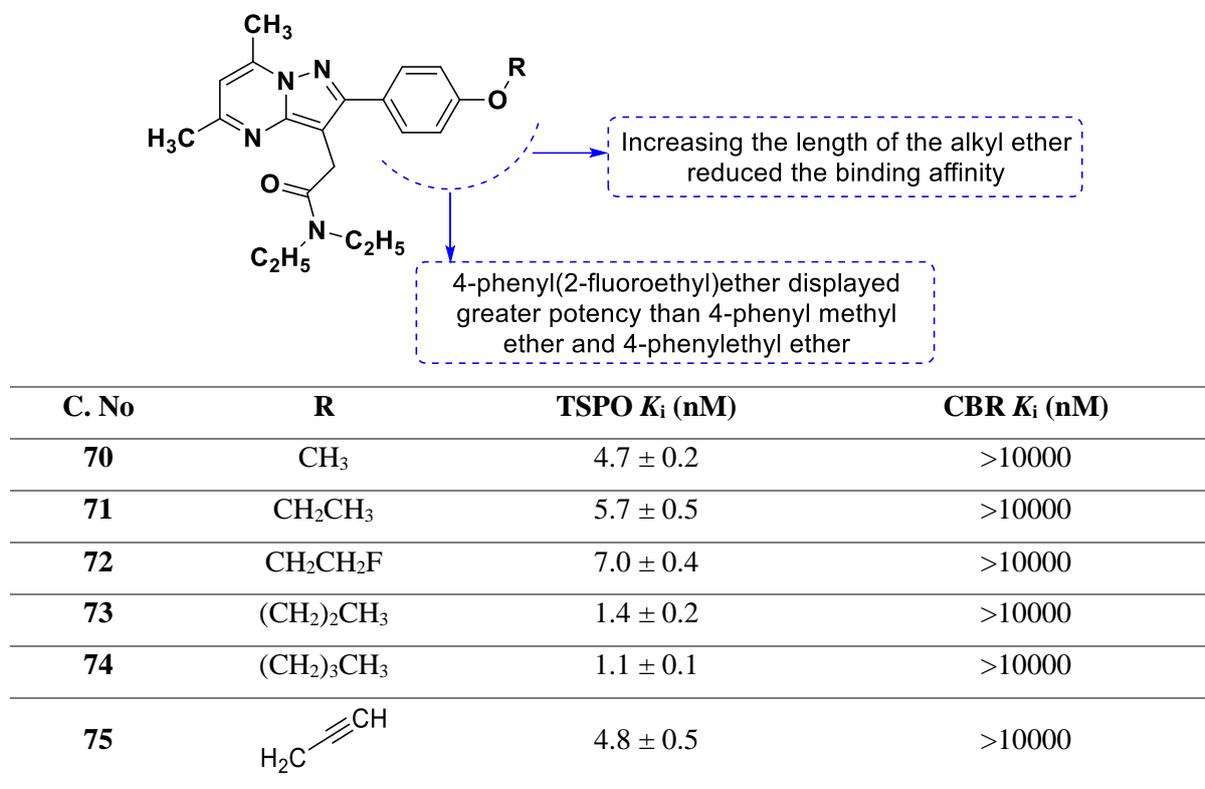
Fig. 37. TSPO and CBZR binding and selectivity studies of pyrazolo[1,5-*a*]pyrimidines.

3.2.2　5-HT₆ receptor antagonists

5-Hydroxytryptamine subtype 6 receptor (5-HT$_6$R) is a recently discovered serotonin receptor (a typical G protein-coupled receptor) and a promising target for cognitive disorders like Alzheimer's, schizophrenia, anxiety and obesity [87,88].

In 2010, Ivachtchenko et al. reported the synthesis of (3-phenylsulfonylcycloalkano[*e* and *d*]pyrazolo[1,5-*a*]pyrimidin-2-yl)amines as serotonin 5-HT$_6$ receptors. Among the series, compounds **76** and **77** exhibited the maximum affinity against 5-HT$_6$ receptor. A brief SAR study has been depicted in **Fig. 38** [27].
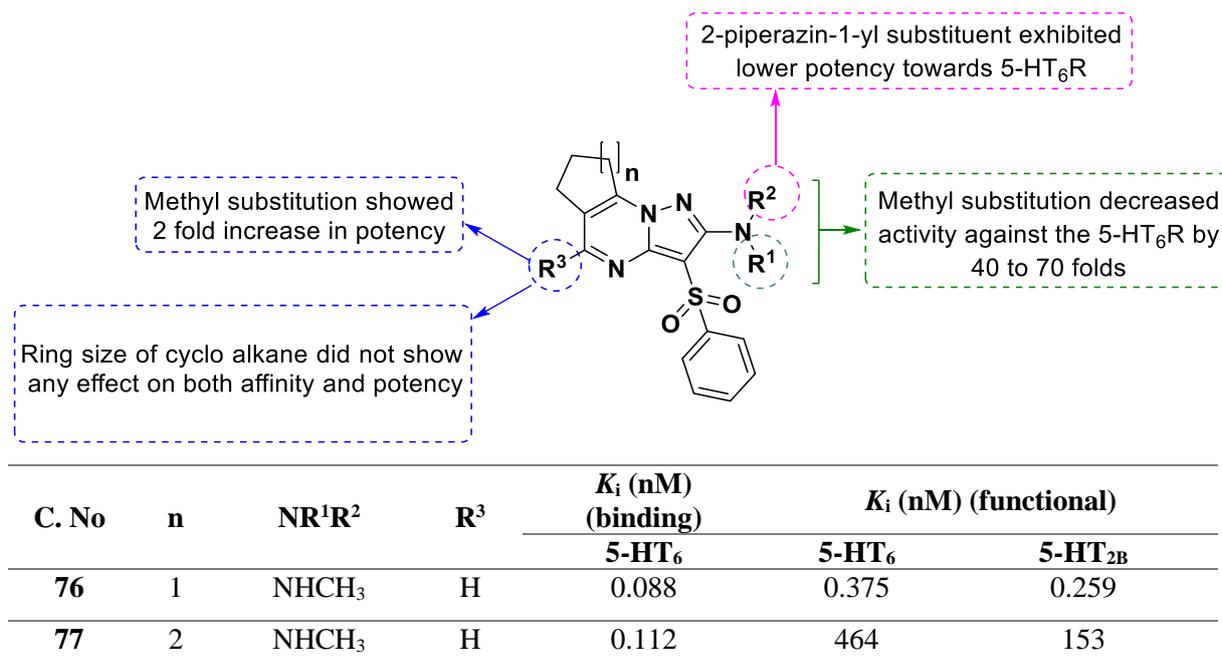
Fig. 38. SAR and 5-HT$_6$ receptor studies of pyrazolo[1,5-*a*]pyrimidines.

The same research group (in 2011) reported the synthesis and SAR studies of substituted 5,N^2-dimethyl-3-phenylsulfonyl-pyrazolo[1,5-*a*]pyrimidine-2-amines and evaluated them as 5-HT$_6$ receptor ligands. SAR studies reveal that the formation of intermolecular hydrogen bond between 2-methylamino and 3-sulfo group highly enhanced the potency and selectivity to block serotonin responses in HEK-293 cells. Among the series, compounds **78-81** were found to be more potent inhibitors of 5-HT$_6$ receptors and the activity values are represented in **Fig. 39** [49].

C. No	n	NR^{1}R^2	R^3	K_i (nM) (binding)	K_i (nM) (functional)	
				5-HT$_6$	**5-HT$_6$**	**5-HT$_{2B}$**
76	1	NHCH$_3$	H	0.088	0.375	0.259
77	2	NHCH$_3$	H	0.112	464	153

C. No	R^1	R^2	R^3	R^4	PK_i (nM)	pA$_2$	ALogP98
78	H	H	H	H	9.52	9.3	1.89
79	H	H	H	Cl	9.66	ND	2.55
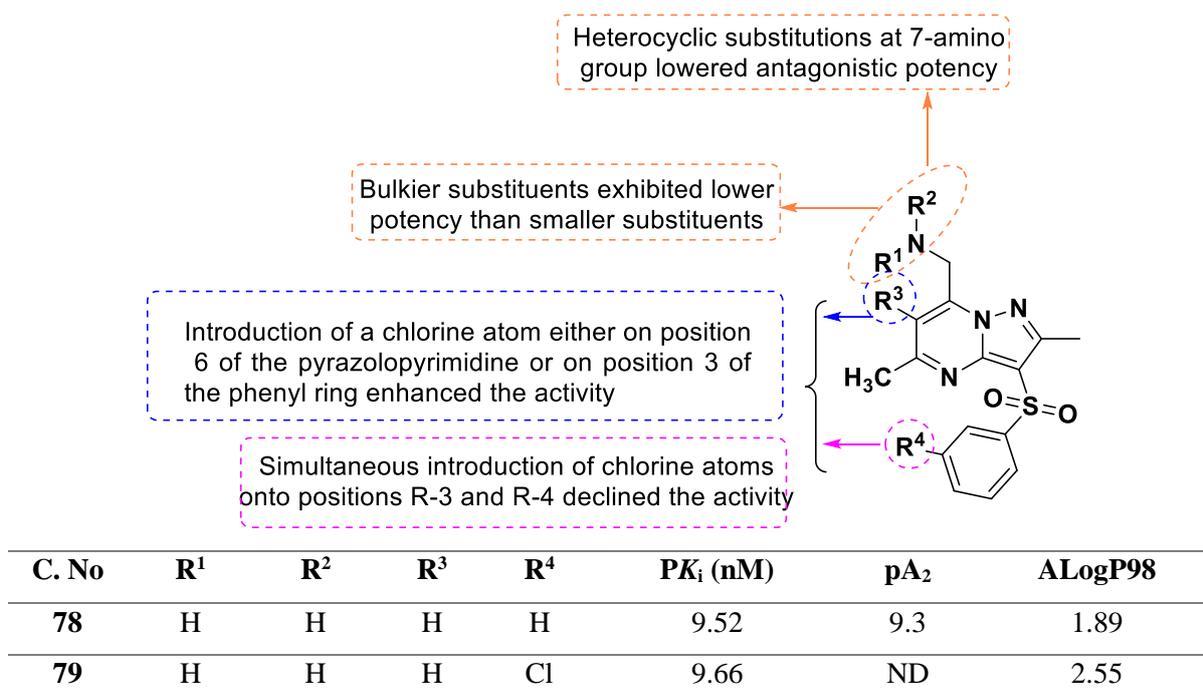
80	H	H	Cl	H	9.85	ND	2.55
81	CH₃	CH₃	H	H	9.46	ND	2.80

ND: Not determined.

Fig. 39. SAR, 5-HT$_6$R activity profiles of substituted 5,N^2-dimethyl-3-phenylsulfonyl-pyrazolo[1,5-a]pyrimidine-2-amines.

3.3 Anti-infectious agents

Infectious diseases are major threat to human kind from pre-historic era. In recent decades, the emergence of drug-resistance microbial infections has generated serious life-threatening health issues and are resposible for highest mortality [89]. Various analogs of pyrazolo[1,5-a]pyrimidines scafold have been reported possess effective antimicrobial activity as mentioned in the succeeding sections.

In 1975, Senga et al. discovered the synthesis of pyrazolo[1,5-a]pyrimidines as antitrichomonal agents. In this series, compound **82** [6-carbethoxy-4-ethyl-3-nitropyrazolo[l,5-a]pyrimidin-7-one] demonstrated potent antitrichomonal activity than the standard drug metronidazole. From the SAR, it was deduced that 3-nitro, 6-carbethoxy and 4-ethyl groups are essential for activity. The structure of potent compound has been described in **Fig. 40** including its inhibitory potency [90].

C. No	R	R^1	R^2	Conc (μg/mL)	
				MIC^a	**MLC^b**
82	NO₂	C₂H₅	COOC₂H₅	1.0	3.2
Metronidazole				3.2	10
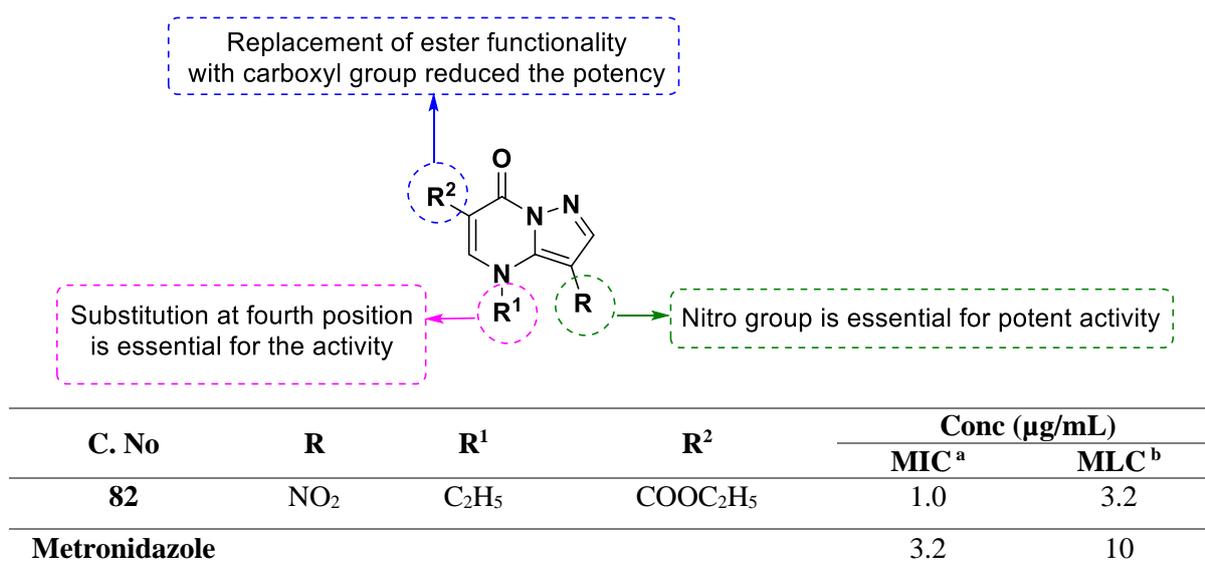
MIC^a: Minimum inhibitory concentration; MLC^b: Minimum lethal concentration.

Fig. 40. SAR of multi substituted pyrazolo[1,5-a]pyrimidines as antitrichomonal agents.

In 1976, Novinson and co-workers reported the synthesis of hydrazine containing pyrazolo[1,5-a]pyrimidine derivatives and evaluated them for *in vitro* antitrypanosomal activity. Further, the authors have screened the title compounds for *in vitro* and *in vivo* activities against *Trypanosama cruzi*. Among all the synthesized molecules, compound **83** showed significant activity. A brief SAR study on the scaffold is presented in **Fig. 41** [91].

C. No	R^1	R^2	R^3	*In vitro* *MIC/MLC**	*In vivo* $^\Delta$IMST
83	H	H	CH$_3$	10/10	63.4
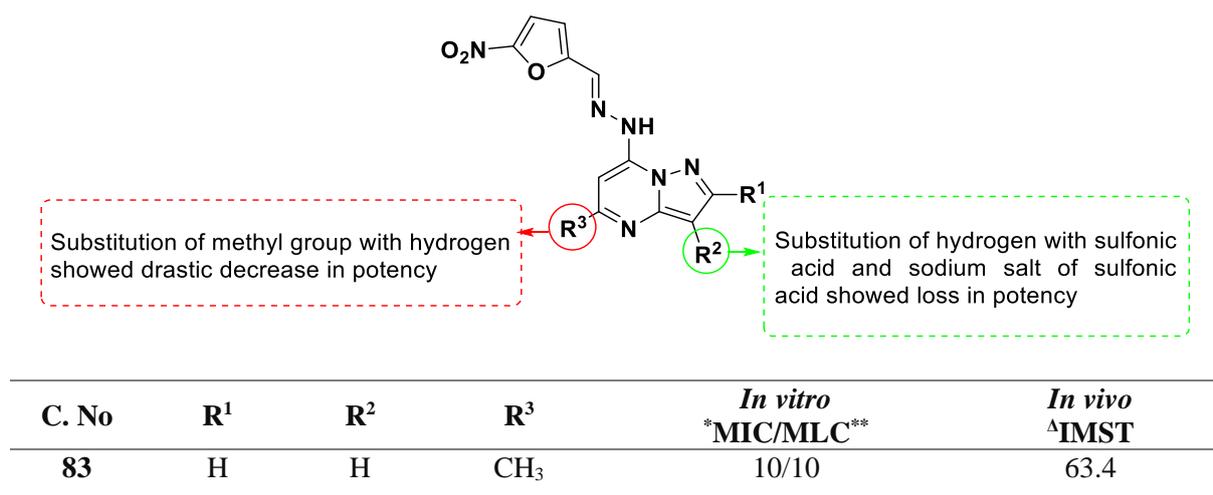
*MIC: Minimum inhibitory concentration; MLC**: Minimum lethal concentration expressed in µg/mL; $^\Delta$IMST: Increase in mean survival time was expressed as present increase beyond survival time of control mice 100 mg/kg twice daily by gavage.

Fig. 41. SAR and antitrypanosomal activity studies of compound **83**.

In 1977, Novinson et al. synthesized 7-alkylamino substituted pyrazolo[l,5-*a*]pyrimidines and *in vitro* antifungal activity against *Trichophyton mentagrophytes* was performed. Out of 22 tested derivatives, compounds **84** and **85** revealed potent fungicidal activity (*in vitro*). It was also observed that compound **85** was one fold more potent than **84**. However, their topical applications on unabraded and abraded guinea pig skin (*in vivo*) generated skin irritation. Preliminary SAR and structures of active compounds are presented in **Fig. 42** [92].

C. No	R	R^1	R^2	R^3	Conc (in µmol/mL)	
					MIC	MLC
84	H	CH$_3$	H	NH(CH$_2$)$_7$CH$_3$	0.02	0.16
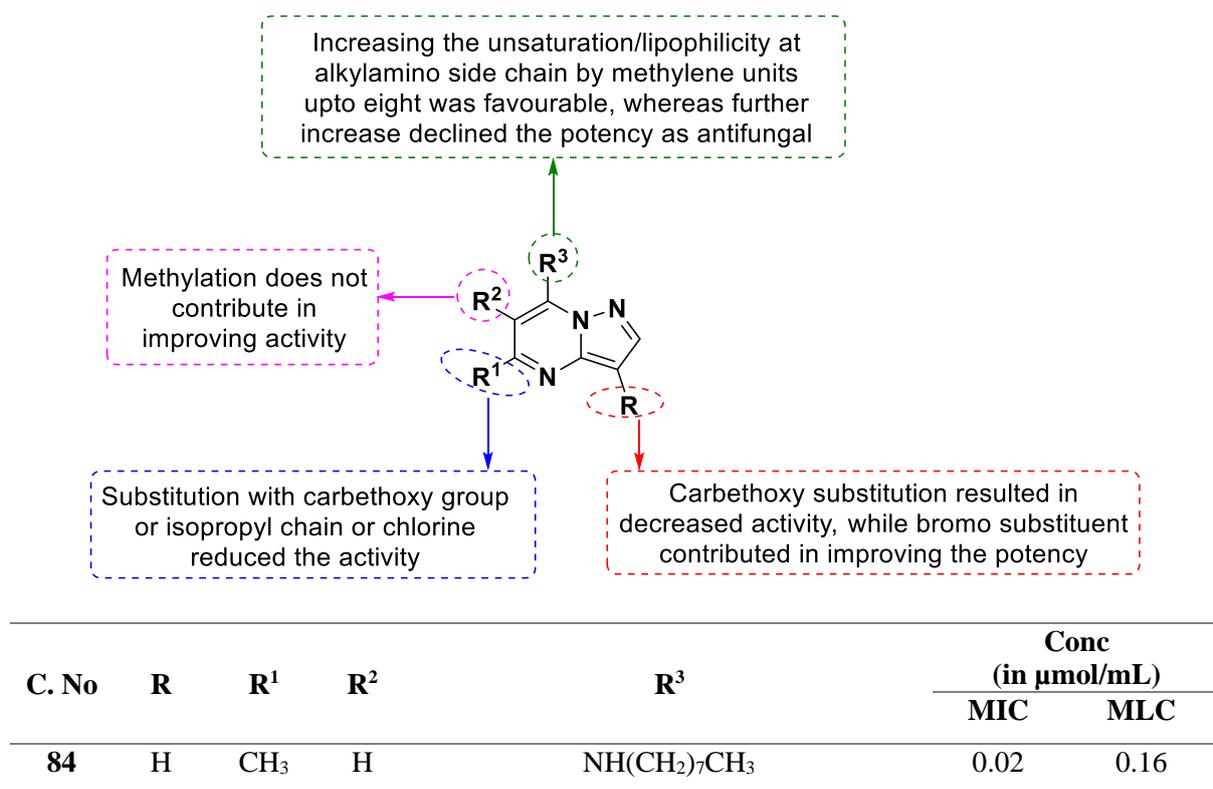
85	Br	CH$_3$	H	NH(CH$_2$)$_8$CH=CH(CH$_2$)$_7$CH$_3$	0.01	0.02

Fig. 42. SAR of 7-alkylamino substituted pyrazolo[l,5-*a*]pyrimidines as antifungal compounds.

El-Gaby and co-workers in 2000 carried out the synthesis and antibacterial evaluation of pyrazolo[1,5-*a*]pyrimidines bearing sulfonamido moieties. Of the screened compounds, four derivatives (**86-89**) exhibited potent activity against gram negative than gram positive bacterial strains in comparison to the standard drug streptomycin. The preliminary SAR study suggested arylamino substitutions on basic nucleus contributed maximum inhibition against gram negative bacterial strains, whereas unsubstitution on sulphonamido moiety generated weak inhibition. The antibacterial screening results for the active compounds are presented in **Fig. 43** [93].
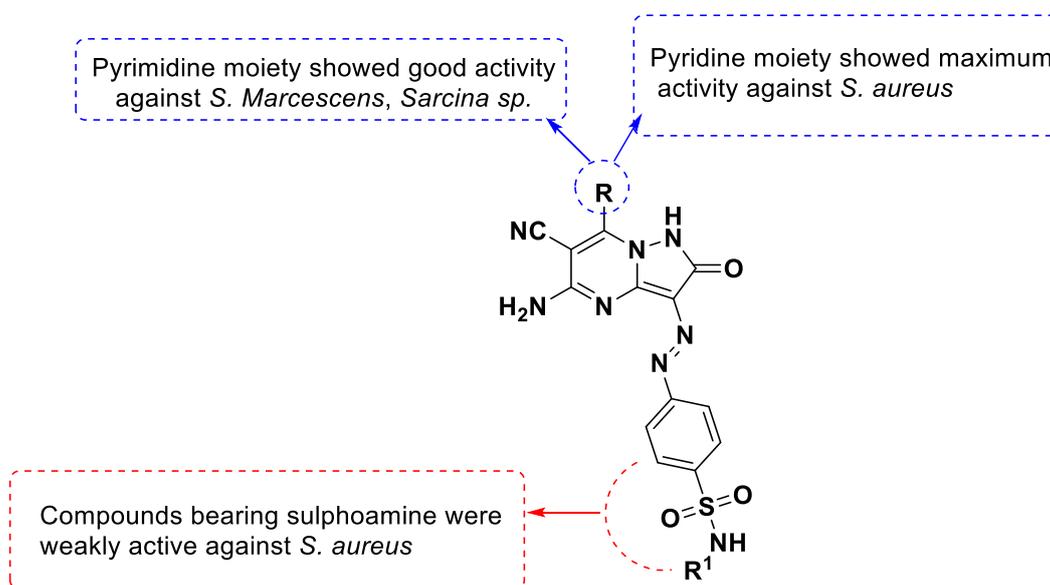
C. No	R	R^1	Gram positive			Gram negative	
			Staphylococcus aureus	*Bacillus subtilis*	*Sarcina sp.*	*Escherichia coli*	*Serratia marcescens*
86	2-Pyrimidinyl	NH-C$_6$H$_4$-O-CH$_3$-*p*	++	+	+++	+++	+++
87	2-(4-Methyl pyrimidinyl)	NH-C$_6$H$_4$-O-CH$_3$-*p*	++	+	+++	++	+++
88	H	C$_6$H$_4$-O-CH$_3$-*o*	+	+++	++	++	++
89	2-Pyridinyl	C$_6$H$_4$-O-	+++	+++	++	++	++

CH₃-*o*					
Streptomycin	++++	++++	++++	++++	++++

+: moderately sensitive giving a zone of inhibition 9–11 mm; ++: sensitive giving a zone of inhibition 12–14 mm; +++: very sensitive giving a zone of inhibition 15–18 mm.

Fig. 43. SAR and active antibacterial pyrazolo[1,5-*a*]pyrimidines.

In 2008, Bondock et al. synthesized new heterocyclic analogs including pyrazolo[1,5-*a*]pyrimidines containing antipyrine moiety and evaluated for antimicrobial potency against bacterial and fungal strains along with reference drugs like ampicillin, chloramphenicol and fluconazole. Compounds **90** and **91** exhibit higher antimicrobial properties than the evaluated reference drugs. The SAR of the same is shown in **Fig. 44** [94].

Incorporation of antipyrine at position 3 via a carboxamide linker demonstrated high antimicrobial activity
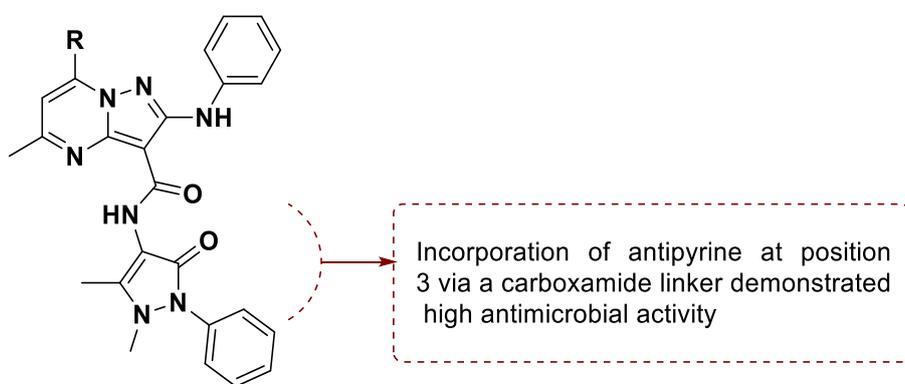
C. No	R	Zone inhibition (in mm)			
		Gram positive bacteria	Gram negative bacteria	Fungi	
		B. thuringiensis	*K. pneumoniae*	*B. fabae*	*F. oxysporum*
90	CH₃	21	28	24	25
91	OH	20	24	27	25
Ampicillin		18	19	17	15
Chloramphenicol		23	20	16	15
Fluconazole		NA	NA	22	16

NA: No activity.

Fig. 44. Structures and results of the antimicrobial (inhibition zone) potency of pyrazolo[1,5-*a*]pyrimidines.

Popovici-Muller et al. (2009) reported a novel series of pyrazolo[1,5-*a*]pyrimidines as Hepatitis-C Virus (HCV) RNA polymerase inhibitors. Authors synthesized three different analogues by modifying different functional groups/moieties at C-3 (carboxylic acid group), C-7 (cyclohexyl group) and C-6 (aromatic substituents) positions. Authors evaluated all the synthesized compounds for HCV RNA

polymerase inhibition. Of the evaluated compounds (**92-95**), **95** was discovered as potent HCV RNA polymerase inhibitor. **Fig. 45** illustrates a brief SAR study and active compounds of the series [95].

C. No	R^1	R^2	HCV NS5B IC$_{50}$ (µM)
92			0.09
93			0.042
94			0.02
95			0.011
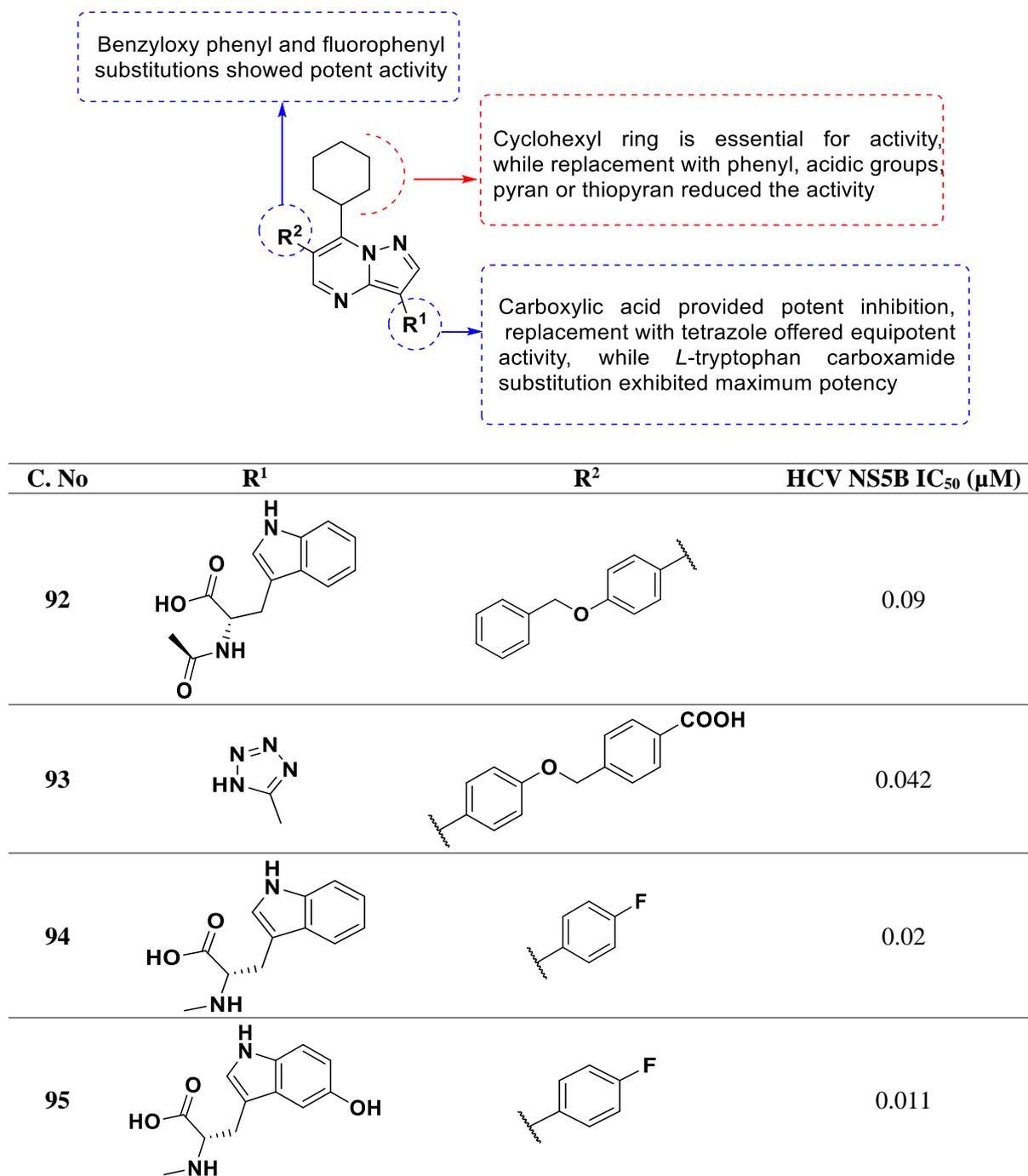
Fig. 45. SAR and antiviral properties of active compounds.

In 2010, Abdelhamid et al. carried out the synthesis and antimicrobial activity of some fused pyrimidine compounds including pyrazolo[1,5-*a*]pyrimidines bearing thiazole moiety. From the eight synthesized pyrazolo[1,5-*a*]pyrimidines, only four compounds (**96-99**) were evaluated for their biological activity. Moderate activity was observed as compared to the standard drugs (**Fig. 46**) [96].

Methyl group showed significant activity than phenyl, hydrogen and cyano groups

Phenyl group is crucial for activity

C. No	X	Y	*S. aureus*	*B. subtilis*	*E. coli*	*P. aeruginosa*	*C. albiacns*
96	Ph	H	≥800	≥800	≥800	≥800	≥800
97	H	Ph	≥800	≥400	≥800	≥400	≥400
98	4-CH$_3$	Ph	≥800	≥400	≥400	≥800	≥400
99	CN	H	≥800	≥800	≥800	≥800	≥400
Ciprofloxacin			≤25	≤25	≤25	400	≥800
Triflucan			≥800	≥800	≥800	≥800	≤25
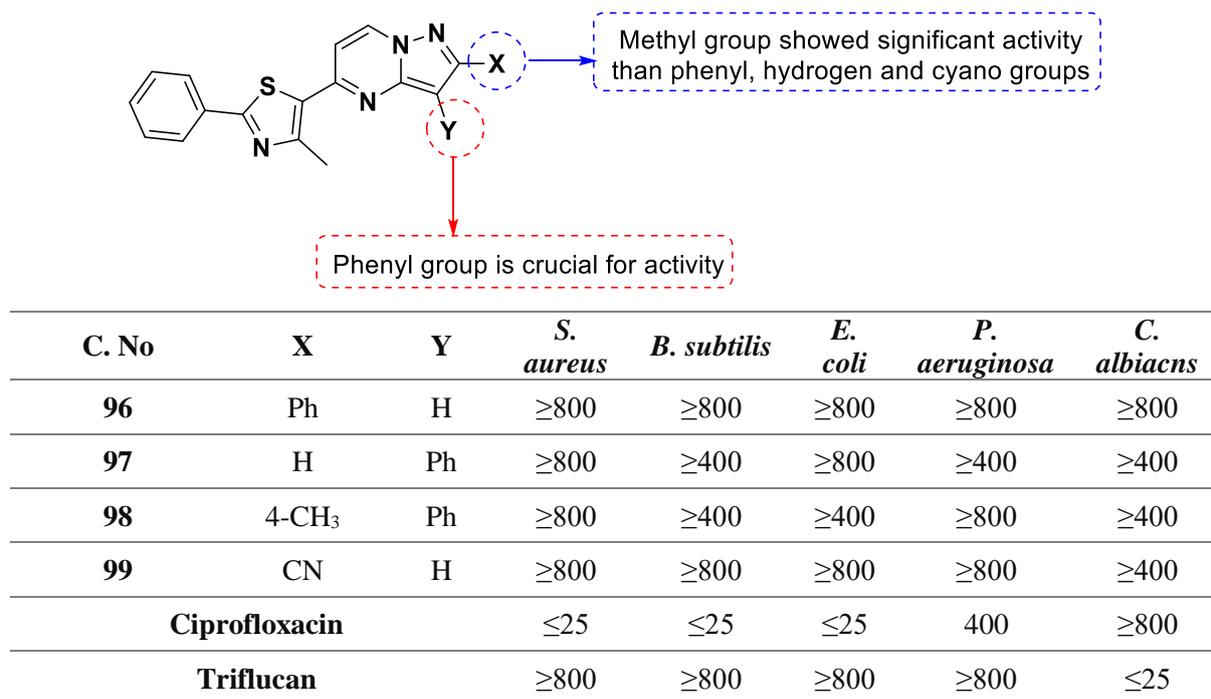
Fig. 46. Structures and MIC values of pyrazolo[1,5-*a*]pyrimidines against different bacterial and fungal strains.

In 2011, Aggarwal et al. synthesized pyrazol-1-ylpyrazolo[1,5-*a*]pyrimidines regioselectively. All the synthesized molecules were tested for antimicrobial properties against two gram positive and gram negative bacterial strains along with four phytopathogenic fungi. Among the screened molecules, compound **100** and **101** exhibited maximum antibacterial potency on par with standard dugs, gentamycin and linezolid. Compound **102** displayed potent antifungal activity (200 mg/mL) than the standard drugs gentamycin, linezolid and mancozeb. SAR study on this series has been presented in **Fig. 47** [97].

para-substituted phenyl ring with methyl or chloro groups increased antibacterial activities, whereas methoxy, bromine groups on phenyl and 2-thienyl group diminished the activities. Also, alkyl groups reduced the potency

Replacement with ethylene moiety reduced the antimicrobial potency

Ethylene group decreased the antimicrobial property

R^2	R^3	Zone of inhibition (in mm)	% Inhibition at 200 μg/mL

C. No	R1		R4	Gram negative bacteria		Gram positive bacteria		*A. terreus*	*A. alternata*	*F. oxysporum*	*Helmin-Thosporo-rium sp.*
				E. coli	*P. aeruginosa*	*S. aureus*	*B. cereus*				
100	H	4-CH3-C6H4-	H	10.5	8	8	8.5	ND	ND	ND	ND
101	H	4-Cl-C6H4-	H	13	10	9	13	12.5	0	16.3	36.6
102	H	CH3 CH3	H	7	7	7	7	73.2	73.1	71.9	79.2
	Gentamycin			24	12	14	14	ND	ND	ND	ND
	Linezolid			12	8.5	18	10	ND	ND	ND	ND
	Mancozeb			ND	ND	ND	ND	76.40	65.90	81.11	70.00

ND: Not determined.

Fig. 47. SAR and antimicrobial results of pyrazolo[1,5-*a*]pyrimidines.

In the same year, Shaikh et al. reported an eco-friendly green synthesis and *in vitro* antimicrobial screening studies for pyrazolo[1,5-*a*]pyrimidine compounds. Of the evaluated compounds, **103**-**105** exhibited maximum inhibition against *E. coli*, while **106** and its analog **107** showed good activity against *A. niger*. The structures of the active compounds with screening results and SAR studies are disclosed in **Fig. 48** [98].

Methyl group was found less active than chloro for both antibacterial and antifungal

$R^1 = R^2 = H$ is crucial for both antibacterial and antifungal activity

C. No	R1	R2	R3	Zone of inhibition (in mm)							
				Bacterial strains				Fungal strains			
				E. coli	*B. subtilis*	*S. aureus*	*S. typhi*	*A. niger*	*A. flavus*	*C. albicans*	*P. chrysogenum*
103	H	H	Cl	18	12	15	15	20	NA	11	13
104	I	H	Cl	17	15	18	16	26	21	19	17

105	Br	CH$_3$	Cl	19	NA	10	30	14	17	15	16
106	Cl	H	Cl	26	16	NA	24	18	21	17	24
107	H	CH$_3$	Cl	16	18	14	NA	16	10	20	19

NA: No activity.

Fig. 48. Structures and antimicrobial results of pyrazolo[1,5-*a*]pyrimidines.

In 2013, Al-Adiwish et al. synthesized derivatives of pyrazolo[1,5-*a*]pyrimidine, pyrazolo[5,1-*c*][1,2,4]triazine and evaluated for their antibacterial and cytotoxicity properties. Further, these compounds were screened against a panel of two gram positive and three gram negative bacterial strains. The prepared compounds exhibited moderate to low antibacterial activity than the standard drugs (chloramphenicol and streptomycin). All the compounds were demonstrated to be non-cytotoxic to Vero cells. **Fig. 49** illustrates the SAR, highly active structures (**108**-**110**) and MIC values of evaluated compounds [99].

Presence of piperidine group increased antibacterial activity than pyrrolidine and morpholine groups

C. No	R	R^1	R^2	R^3	Gram positive bacteria Zone of inhibition (in mm)		Gram negative bacteria Zone of inhibition (in mm)		
					B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. marcescens*
108	NH$_2$	CN	SCH$_3$	piperidine	10 ± 0.57	11 ± 0.57	10 ± 0.55	7 ± 1.15	7 ± 1.00
109	CH$_3$	H	OH	piperidine	10 ± 0.58	12 ± 0.58	11 ± 0.58	7 ± 0.00	7 ± 1.15
110	CH$_3$	H	CH$_3$	morpholine	9 ± 0.58	9 ± 0.55	10 ± 0.57	7 ± 0.57	6 ± 0.00
Chloramphenicol					25 ± 0.28	25 ± 0.28	NT	NT	NT
Streptomycin					NT	NT	13 ± 0.58	14 ± 0.57	20 ± 0.57

NT: Not tested.

Fig. 49. SAR, antibacterial activity of pyrazolo[1,5-*a*]pyrimidines.

In the same year, Ishak et al. reported the synthesis and antimicrobial screening of pyrazolo[1,5-a]pyrimidine derivatives. Of the thirteen derivatives synthesized, six compounds were evaluated for both antibacterial and antifungal activities. Among the screened derivatives, compounds **111-113** showed potent activity comparable to that of standard drugs. **Fig. 50** represents the structures, zone of inhibition (values) of active compounds and reference drugs along with SAR studies [31].
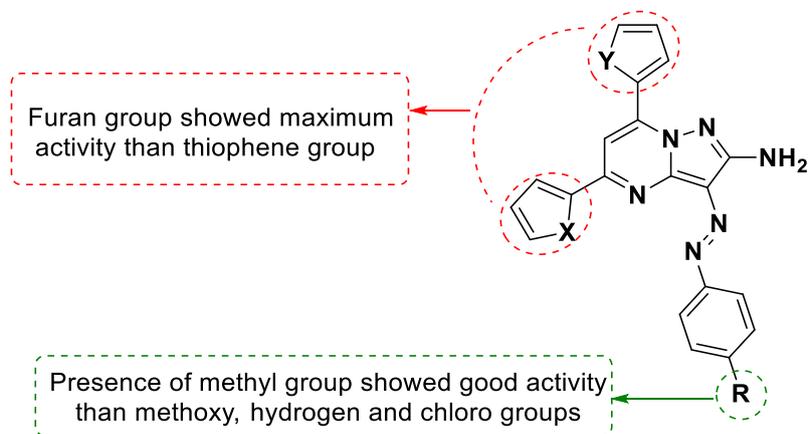
C. No	R	X	Y	Zone of inhibition (in mm)							
				Bacterial strains				Fungal strains			
				S. aureus	B. subtilis	P. aeruginosa	E. coli	A. fumigates	G. candidum	C. albicans	S. racemosum
111	H	S	S	15.3 ± 0.03	17.9 ± 0.09	9.3 ± 0.05	14.9 ± 0.07	15.2 ± 0.08	11.3 ± 0.05	10 ± 0.04	8.2 ± 0.06
112	H	O	O	16.7 ± 0.05	15.7 ± 0.3	NA	15.2 ± 0.2	14.2 ± 0.09	15.3 ± 0.3	NA	10.2 ± 0.06
113	CH₃	O	O	22.2 ± 0.09	23.3 ± 0.2	18.5 ± 0.08	21.9 ± 0.1	21.8 ± 0.2	19.5 ± 0.08	18.1 ± 0.3	14.8 ± 0.09
Pencillin G				30.1 ± 0.06	31.6 ± 0.05	28.3 ± 0.08	33.1 ± 0.09	ND	ND	ND	ND
Streptomycin				28.1 ± 0.07	29.7 ± 0.06	25.2 ± 0.09	29.7 ± 0.09	ND	ND	ND	ND
Itraconazole				ND	ND	ND	ND	27.4 ± 0.05	24.2 ± 0.09	25.2 ± 0.07	23.9 ± 0.04
Clotrimazole				ND	ND	ND	ND	26.3 ± 0.08	23.2 ± 0.03	20.8 ± 0.02	21.4 ± 0.05

NA: No activity; ND: Not determined.

Fig. 50. SAR and antimicrobial studies of pyrazolo[1,5-*a*]pyrimidines.

Recently in 2014, Tian et al. reported the synthesis and medicinal properties of new pyrazolo[1,5-*a*]pyrimidines as HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTIs). Within the evaluated derivatives, compound **114** was found to be the most potent inhibitor with an EC_{50} of 0.07 µM against wild-type HIV-1 with high selectivity index (SI, 3999). Moreover, **114** was determined as an effective NNRTI than the standard drugs (nevirapine and delavirdine). Authors also performed HIV-1 RT inhibitory assay for compound **114**, which showed an IC_{50} of 2.26 µM. The SAR and anti-HIV evaluation results of active compound are given in **Fig. 51** [29].
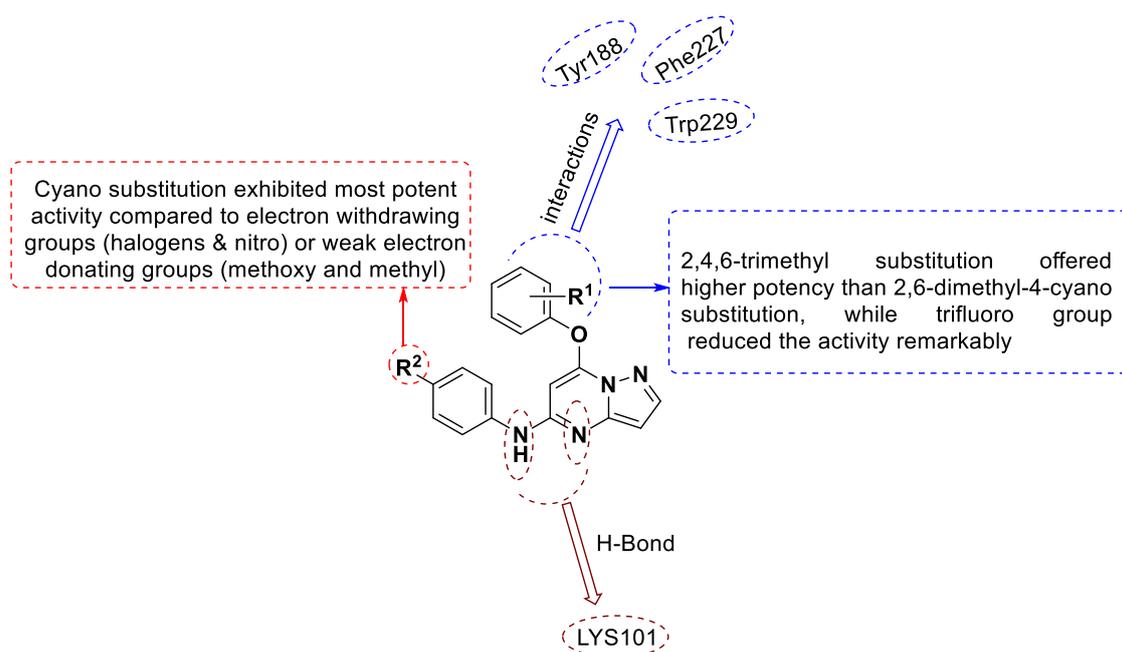
| C. No | R^1 | R^2 | EC$_{50}$ (µM) | | CC$_{50}$ (µM) | SI | | RT inhibition (µM) |
			HIV-1 IIIB	HIV-2 ROD		HIV-1 IIIB	HIV-2 ROD	
114	2,4,6-Trimethyl	4-CN	0.07 ± 0.01	>276	276 ± 53.7	3999	<1	2.26
	Nevirapine		0.07 ± 0.06	NA	>14.99	>89	NA	ND
	Delavirdine		0.16 ± 0.15	NA	>43.81	>277	NA	ND

ND: Not determined; NA: No activity.

Fig. 51. SAR and activity data of active compounds.

Recently (2015) Mackman et al. discovered new pyrazolo[1,5-*a*]pyrimidine derivatives as orally bio-available respiratory syncytial virus (RSV) fusion inhibitor through hit-to-lead optimization campaign. Authors conducted phenotypic screen using HEp-2 cells infected with RSV A2 virus on a library of ∼4,00,000 molecules and identified compound **115** as a lead antiviral molecule (EC_{50} = 65 nM) for

optimization. Further, X-ray crystallography of **115** confirmed the *S*-configuration in which 2-heteroaryl functional group on piperidine ring positioned into an axial alignment with a dihedral angle of 95°. The pharmacokinetic study of **115** indicated the poor passive permeability and oral bioavailability as depicted in **Fig. 52a**. Hence, pyrazolo[1,5-*a*]pyrimidin-2-yl C-5 *N*-linked pyrrolidine and azetidine analogues were developed to obtain a potent compound with enhanced permeability or efflux properties. Among the evaluated derivatives, compound **116** displayed higher potency with favourable properties as illustrated in **Fig. 52b**. Apart from pharmacokinetic studies, authors also investigated *in vivo* preclinical (cotton rat model) and clinical evaluation of **116** that demonstrated antiviral efficacy in a dose-dependent manner [100].

Modification to bridged bicyclics and acyclic *N*-methyl alanine moiety reduced the antiviral potency

S-configuration and optimal dihedral angle favoured the activity

115
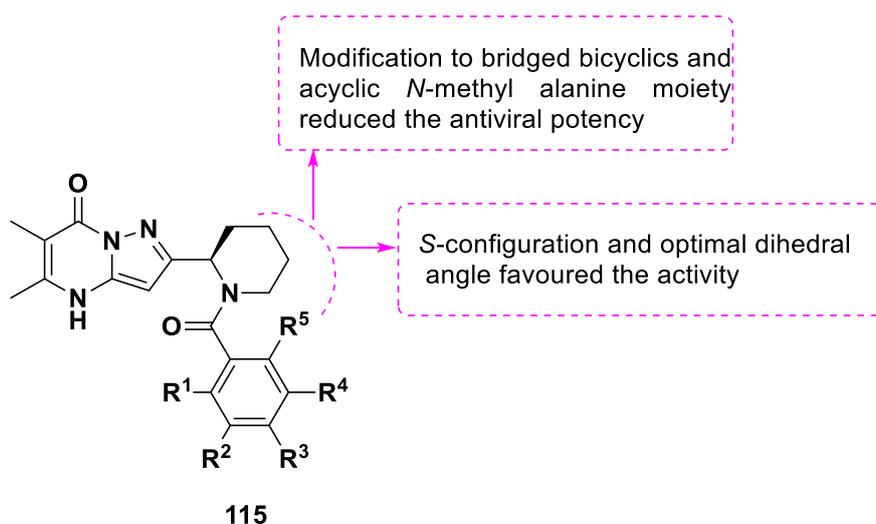
C. No	R^1	R^2	R^3	R^4	R^5	RSV EC$_{50}$ (nM)	log D	Caco2 AB/BA (× 10^{-6} cm/s)	% Free human plasma	Fold plasma shift
115	H	NHSO$_2$CH$_3$	H	H	H	65	1.3	0.33/2.2	6.5	13

Fig. 52a. SAR and biological studies of a lead compound.

116
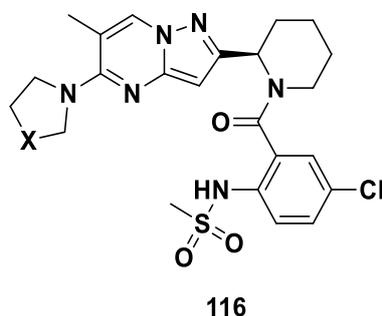
C. No	X	RSV EC$_{50}$ (nM)	log D	Caco2 AB/BA (× 10^{-6} cm/s)	% Free human plasma	MS pred CL human/rat/dog (L/h/kg)	Fold plasma shift	SD rat F %
116	CH-NH$_2$(S)	0.37	2.0	6.4/19	4.4	<0.16/0.26/0.41	22	46

Fig. 52b. Pharmacokinetic properties of potent derivative **116**.

3.4 Anti-inflammatory agents

Inflammation is a beneficial and defensive biological response to tissue damage, infections, toxins or autoimmune injury in order to abolish or limit the blow-out of an injurious agent. The process is a complex phenomenon involving the role of several cellular mediators [101]. Management of inflammatory disorders encompasses usage of drugs or anti-inflammatory agents.

In 1974, Novinson and co-workers reported the synthesis of 3-substituted 5,7-dimethyl pyrazolo[1,5-*a*]pyrimidines and evaluated them against 3,5-cyclic-AMP phosphodiesterase enzyme. Out of the 15 synthesized molecules, compounds **117-120** having halogen (-Br, Cl, I) and acetyl groups at C-3 position were found to be more potent in inhibiting phosphodiesterase enzyme as illustrated in **Fig. 53** [23].
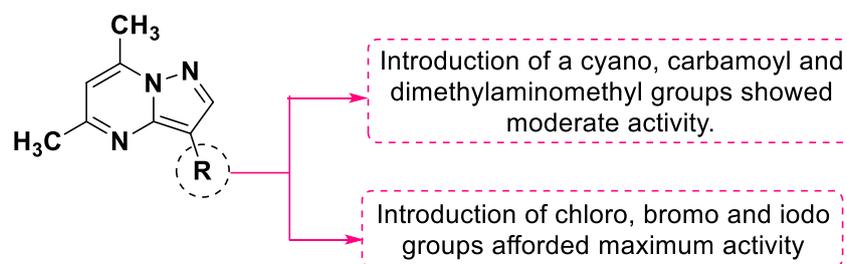
C. No	R	High K_m PDE IC$_{50}$ (nM)		Low K_m PDE IC$_{50}$ (nM)	
		Rabbit kidney	**Rabbit lung**	**Beef heart**	**Rabbit lung**
117	Br	2.20	2.40	1.7	0.7
118	Cl	1.33	3.11	1.7	2.2
119	I	1.23	3.55	1.5	3.5
120	COCH$_3$	0.57	1.5	0.4	1.0

Fig. 53. 3,5-cyclic-AMP phosphodiesterase inhibitory abilities of active compounds.

In 1982, Springer et al. synthesized pyrazolo[1,5-*a*]pyrimidines and their related analogs for adenosine cyclic 3',5'-phosphate phosphodiesterase (PDE) inhibition. Of the tested derivatives, **121** and **122** were determined to be potent inhibitors. In the case of ADP-induced platelet aggregation inhibitory studies, **123** was identified as a potent compound. A brief SAR of this series and the activity data of most active compounds is illustrated in **Fig. 54** [102].

Alkylamino and alkoxy or alkylthio groups provided better PDE inhibition than 7-hydroxy, -amino or -chloro substituents

Ethoxy substitution promoted inhibition

Methyl group increased the potency

Ethyl or bromo substitution enhanced the PDE Inhibition
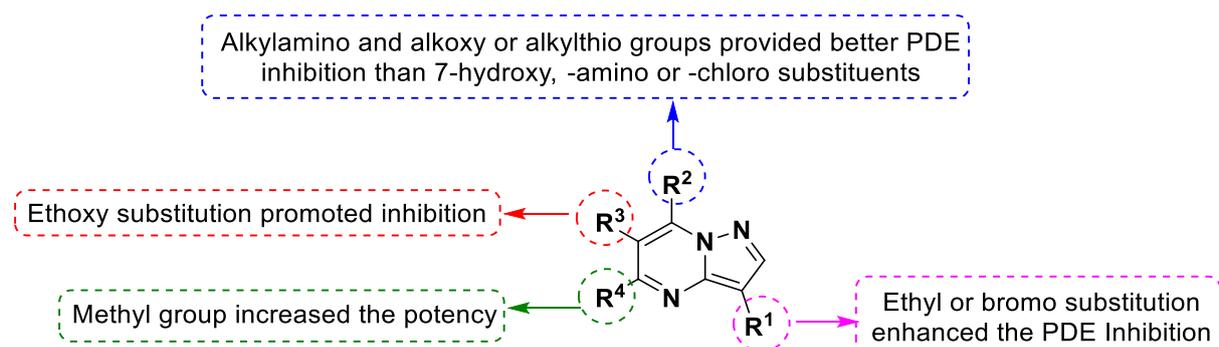
C. No	R^1	R^2	R^3	R^4	PDE Inhibition			Inhibition of ADP-induced platelet Aggregation	
					Lung	Kidney	Heart	Conc.	Inhibition
121	Br	OH	COOC$_2$H$_5$	H	0.5	1.0	NA	50	34
122	H	NHCH$_2$CH$_2$OH	H	CH$_3$	0.5	NA	3.7	NA	NA
123	Br	NH$_2$	COOC$_2$H$_5$	H	NA	NA	NA	25	67

NA: No activity.

Fig. 54. SAR and 3',5'-phosphate phosphodiesterase inhibitor studies of substituted pyrazolo[1,5-a]pyrimidines.

In 1983, Auzzi et al. synthesized 2-phenylpyrazolo[1,5-a]pyrimidin-7-ones and explored their antipyretic and anti-inflammatory properties. While deriving a relationship between chemical structure and anti-inflammatory potential, authors further synthesized some more derivatives with modifications of an elongated side chain at the C-4 position. Among the tested derivatives, compound **124** [4-ethyl-5,6-dihydro-2-phenylpyrazolo[1,5-a]pyrimidine-7-one] exhibited potent anti-inflammatory and moderate antiulcer activity. **Fig. 55** illustrates a brief SAR and activity profile of compound **124** [103].

Phenyl substitution retains the activity

Alkylation or bromination reduced the activity

Functionalization with long alkyl chains decreased the activity

Substitutions other than hydrogen reduce the potency
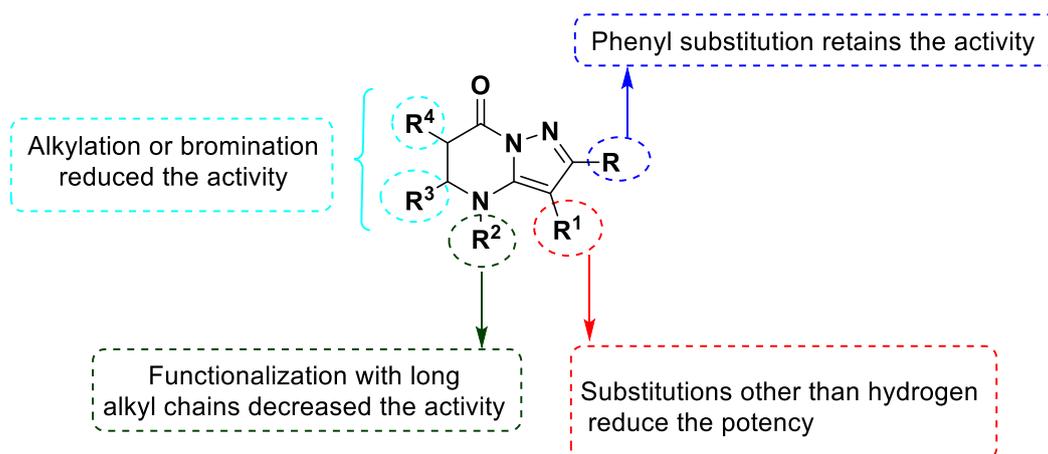
R	R^1	R^2	R^3	R^4	Anti-inflammatory activity

C. No						Dose (mg)	% reduction of edema, 3 h	ED$_{50}$ mg/kg
						10	54.1 ± 4.9	
124	Ph	H	C$_2$H$_5$	H	H	25	69.8 ± 4.1	7.3 (2.4-22.1)
						100	90.1 ± 2.6	

Fig. 55. SAR and anti-inflammatory properties of 2-phenylpyrazolo[1,5-*a*]pyrimidin-7-ones.

In 2001, Almansa et al. reported the synthesis and anti-inflammatory evaluation of a series of bicyclic pyrazolo[1,5-*a*]pyrimidines. Authors performed *in vitro* COX-1 and COX-2 enzyme inhibition assay and *in vivo* (carrageenan-induced paw edema, air-pouch model) studies. From this series, compound **125** exhibited potent and selective COX-2 inhibition. The SAR and pharmacological data of the potent molecule **125** are presented in **Fig. 56** [24].
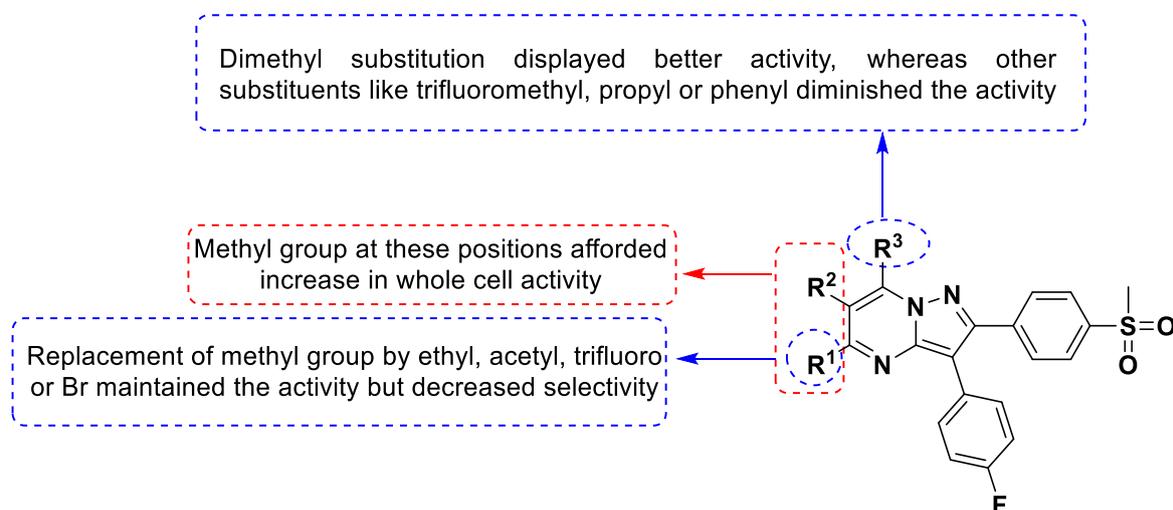
C. No	R^1	R^2	R^3	% Inh HWB		IC$_{50}$ Whole Cell	
				COX-1	COX-2	COX-1	COX-2
				10 (μM)	10 (μM)	(μM)	(μM)
125	H	CH$_3$	CH$_3$	47.5	96.4	>10	0.012

Fig. 56. SAR and the data of potent molecule.

In 2008, Shaaban et al. synthesized pyrazolo[1,5-*a*]pyrimidines, pyrimido[1,2-*a*]benzimidazole and triazolo[1,5-*a*]pyrimidine ring systems and evaluated all the synthesized compounds for the analgesic and anti-inflammatory properties. Compounds **126** [2-(4-bromophenyl)-6-(phenylsulphonyl)-7-(4-methylphenyl)-pyrazolo[1,5-*a*]pyrimidine] and **127** [3-bromo-2-phenyl-6-(phenylsulphonyl)-7-(4-methylphenyl)-pyrazolo[1,5-*a*]pyrimidine] were reported as potent anti-inflammatory as well as analgesic agents. SAR study demonstrated that the other scaffolds exhibited lower activity as compared to pyrazolo[1,5-*a*]pyrimidine system. Among the series, bromine substituent on pyrazole moiety **127**

enhanced the analgesic effect. In compound **126**, the electronic influence of bromine on aryl ring over pyrazole exhibited higher anti-inflammatory activity. The pharmacological evaluation results and SAR of active compounds are given in **Fig. 57** [104].
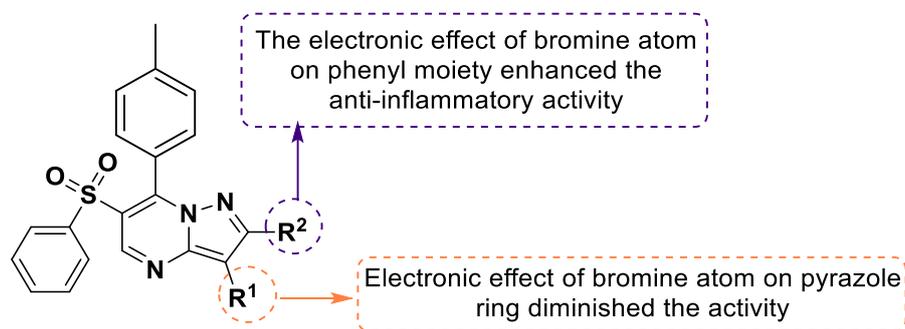
C. No	R^1	R^2	Anti-inflammatory profile						Analgesic profile		
			% edema inhibition						Number of writhing		
			1h	2h	3h	4h	5h	6h	30 min	% Change	Potency
126	H	4-Br-C_6H_4	74.1	72.6	69.2	26.6	31.7	54.3	29.5 ± 1.4	65.2	0.76
127	Br	Ph	65.5	61.0	54.8	12.5	0	6.6	12.2 ± 1.1	85.6	0.99

Fig. 57. Pharmacological evaluation results of active compounds.

In 2012, Gege et al. discovered non-Zn chelating, selective matrix metalloproteinase 13 (MMP-13) inhibitors for the cure of osteoarthritis. Of the synthesized molecules, compound **128** was considered as intra-articular disease modifying osteoarthritic drug (IA-DMOAD). Further, it had a favourable pharmacokinetic profile which minimized the total exposure. Insignificant levels of the constituent were noticed in the plasma resulting 1 mg/kg (IV route) or 0.5 mg/kg (IA route) administration to rats. Moreover, derivative **128** exhibited good *ex vivo* and *in vitro* efficacy, effectively blocking collagen degradation in a dose-dependent manner with an IC_{50} of 20 nM as depicted in **Fig. 58**. This study concluded that **128** possess long permanency in joints, penetrates cartilage efficiently with no measurable systemic exposure and has significant efficacy [105].
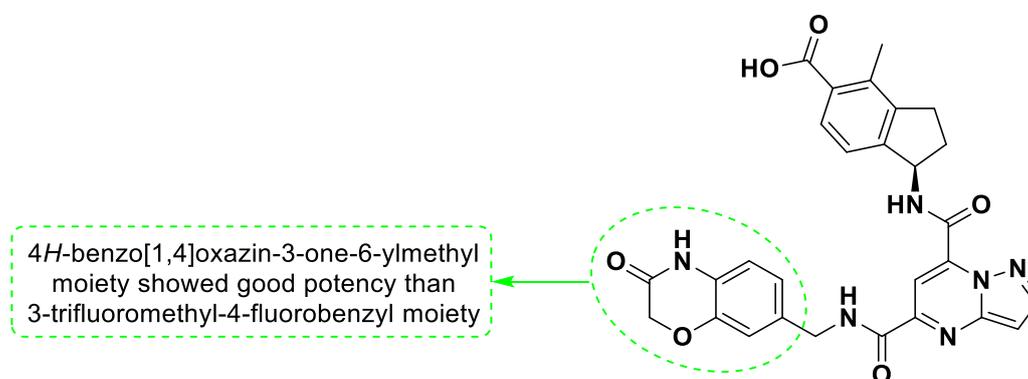
C. No	IC$_{50}$ (nM) vs. catalytic domain										
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-12	MMP-13	MMP-14	TACE	Agg1
128	>20000	>20000	8200	>20000	3200	>20000	655	0.03	>20000	>20000	2400

Fig. 58. Selectivity and potency of compound **128** towards MMP-13.

In 2012, Kosugi et al. reported the synthesis of 5,6,7-trisubstituted pyrazolo[1,5-*a*]pyrimidines and evaluated their biological properties as mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP K-2) inhibitors. SAR studies were performed to optimize highly potent and selective analogues and revealed that substitution at C-6 position was necessary to enhance the activity as depicted in **Fig. 59**. All the synthesized derivatives exhibited good activity and compounds **129**-**131** were most potent [47].
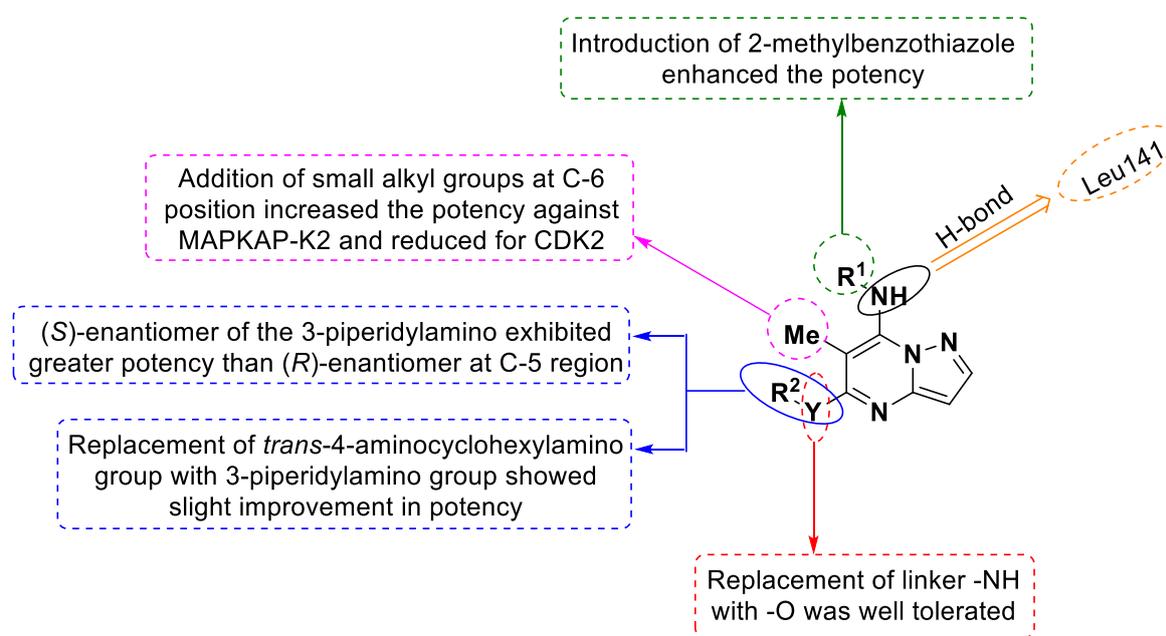
C. No	R^1	Y-R^2	MAPKAP-K2	CDK2	Selectivity CDK2/ MAPKAP-K2
			IC$_{50}$ (µM)	IC$_{50}$ (µM)	
129			0.054	25	463
130			0.057	21	368

| 131 | | | 0.076 | 27 | 355 |

Fig. 59. SAR, MAPKAP-K2 kinase and CDK2 selectivity values of 5,6,7-trisubstituted pyrimidines.

Recently (2015), Lim et al. developed a methodology for the synthesis of 5-amino-*N*-(1*H*-pyrazol-4-yl)pyrazolo[1,5-*a*]pyrimidine-3-carboxamides as Interleukin-1 receptor associated kinase 4 (IRAK4) inhibitors. Initially, authors identified compound **132** as a lead molecule through high-throughput screening (HTS) campaign. Encouraging results of compound **132** with an IC_{50} value of 110 nM and high ligand binding efficiency (LBE) of 0.44 encouraged the authors to further explore the pyrazolo[1,5-*a*]pyrimidine scaffold as inhibitors to IRAK4, which is a remarkable target for many inflammatory diseases. A library of pyrazolopyrimidines bearing amine substitutions at the 5-position of the ring followed by carboxamide replacement and finally diamino substitutions at the 5th position to obtain potent inhibitor **133** was generated. Robust PK/PD response in the R848-induced rat model was reported for compound **134**. Molecular modelling guided SAR studies and pharmacological evaluation including cell potencies, kinase selectivities, and pharmacokinetic profiles are given in **Fig. 60** [106].
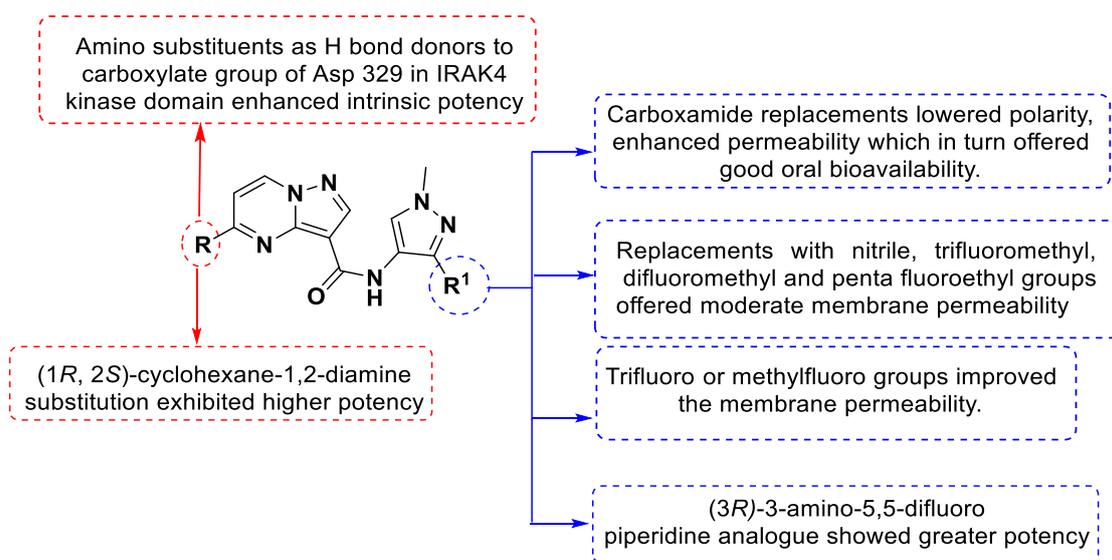
C. No	R	R^1	IRAK4 IC$_{50}$ (nM)	cLogD	Polar surface area (A^2)	Cell permeability (Papp)
132	H	CONH$_2$	110	1.7	127	17
133		-CHF$_2$	< 0.5	-2.8	ND	30
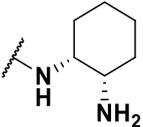
| 134 | | -CONH$_2$ | 0.3 | -4.3 | 152 | 2 |

ND: Not determined.

C. No	hPBMC IC$_{50}$ (nM)	rWB IC$_{50}$ (nM)	No. of kinases tested	% of kinases	rat Cl$_p$ (mL min^{-1} kg^{-1})	rat %F
133	12	81	101	> 95	46	42
134	31	300	264	99	56	0

Fig. 60. SAR and IRAK4 inhibitory, pharmacokinetic activities of pyrazolo[1,5-*a*]pyrimidine-3-carboxamide derivatives.

In 2016, Roux and co-workers reported novel substituted pyrazolo[1,5-*a*]pyrimidines and evaluated them as potent phosphodiesterase-4 (PDE4) inhibitors. These tested molecules showed moderate to high activity against PDE4. Among all, compounds **135** and **136** revealed 200 fold improvement in both activity and cellular potency. SAR was performed to optimize the potent molecules as PDE-4 inhibitors and is illustrated in **Fig. 61** [107].
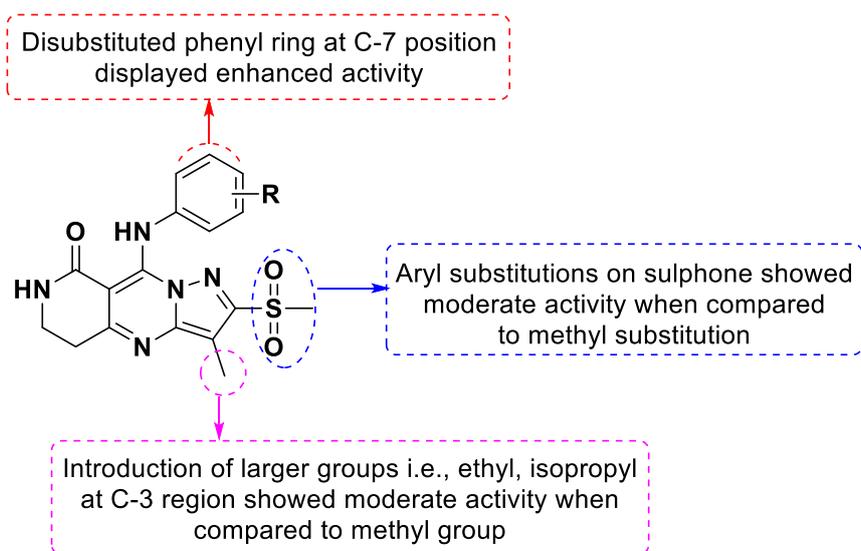
C. No	R	PDE4B1 IC$_{50}$ (nM)
135	3-OCH$_3$	0.1
136	3,5-Cl	0.03

Fig. 61. SAR and PDE4 inhibitory studies.

3.5 CRF-1 receptor antagonists

Corticotropin-releasing factor (CRF) is a 41-amino-acid neuropeptide produced in the paraventricular nucleus of the hypothalamus and released in response to stress and pain [108]. CRF binds to CRF receptors in the anterior pituitary, resulting in release of adrenocorticotropic hormone (ACTH) [109,110].

In 1998, Wustrow et al. reported the synthesis, X-ray crystallography studies and evaluation of pyrazolo[1,5-*a*]pyrimidines for their binding affinity towards human CRF-1 receptor. SAR and X-ray crystallographic studies revealed the extension of the 3-phenyl ring and alkyl group on 7-position of the amino substituent are essential for CRF binding affinity as illustrated in **Fig. 62**. Among the series, compound **137** was identified as essential CRF inhibitor with high affinity [111].
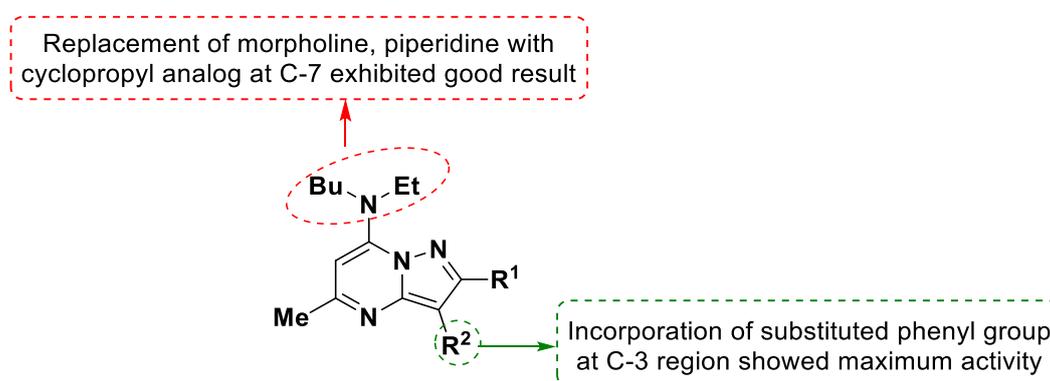
C. No	R	R^1	R^2	CRF$_1$ Binding (K_i nM)
137	Ethyl	Butyl	2,4-Dichlorophenyl	5

Fig. 62. CRF binding affinity studies of pyrazolo[1,5-*a*]pyrimidine derivative.

In 2000, Gilligan et al. discovered a series of novel pyrazolo[1,5-*a*]pyrimidines as corticotropin-releasing factor (hCRF$_1$) antagonists. In this series, compounds **138**-**140** demonstrated good hCRF$_1$ activity. Results of their work concluded that compound **140** showed good anxiety efficacy in the dog (0.3 mg/kg). The results of key intravenous (iv) pharmacokinetic parameters ($t_{1/2}$, *CL* and $V_{d, ss}$) and a brief SAR study are depicted in **Fig. 63** [112].

Compounds containing diethylamino, 3-pentyl amino and 2,4-disubstituted patterns exhibited good receptor binding affinities

Acylamino analogue acted as poor ligand for receptor binding affinity

Replacement of -Ar group with 2,4-dimethyl phenyl, 4-Cl-2-Me phenyl, 2-Cl-4-Me phenyl and 2,4-dichlorophenyl groups enhanced the binding affinities

Introduction of alkoxy substituents (bromine) at 5-position highly reduced the potency

C. No	R	Ar	Mean $hCRF_1$ K_i (nM)
138	NH-3-pentyl	2-CH$_3$-4-ClPh	1.7 ± 0.9
139	NH-3-pentyl	2-Cl-4-CH$_3$Ph	1.5 ± 1.0
140	NH-3-pentyl	2-Me-4-CH$_3$OPh	1.0 ± 0.2

Parameter	iv (intravenous)	po (peroral)
$t_{1/2}$ **(h)**	46.4 ± 7.6	45.1 ± 10.2
CL **(L/h/kg)**	049 ± 0.08	NA
$V_{d, ss}$ **(L/kg)**	23.0 ± 4.2	NA

NA: No activity

Fig. 63. SAR and single dose pharmacokinetic data of pyrazolo[1,5-*a*]pyrimidines.

In 2011, Saito and co-workers discovered 6,7-dihydro-5*H*-cyclopenta[*d*]pyrazolo[1,5-*a*]pyrimidines as novel CRF$_1$ antagonists. SAR studies revealed the role of different functional groups on the moiety, which is presented in **Fig. 64**. Among the synthesized compounds, **141**-**143** exhibited both binding affinities and potent antagonist activity. Later, pharmacokinetic studies were carried out in a rat model and it was concluded that compound **141** was orally effective CRF$_1$ antagonist [30].

Introduction of ether oxygen into fused five member ring afforded potent activity

Removal of methyl group from pyrazol moiety showed 4 fold reduction in binding affinity

Replacement of methyl group with ethyl, cyclobutyl, trifluoromethyl and methylthiomethyl groups showed reduction in affinity values

2,4-disubstituted phenyl group on pyrazole moiety displayed potential activity compared to 2,5-disubstitution

C. No	X	Y	Binding affinity IC$_{50}$ (nM) Human	Antagonist activity EC$_{50}$ (nM) Human
141	H	H	4	40
142	O	H	7	4
143	H	O	84	8

Fig. 64. SAR and CRF$_1$ affinity studies of pyrazolo[1,5-*a*]pyrimidines.

In the same year, the same research group reported the synthesis of pyrazolo[1,5-*a*]pyrimidines and triazolo[1,5-*a*]pyrimidines as novel CRF$_1$ receptor antagonists. SAR studies revealed that the significant activity was afforded by changing substitutions at C-5 and C-7 positions as depicted in **Fig. 65**. Among the series, compounds **144-147** showed potent results for binding affinity and antagonist activity [113].

Incorporation of methyl and ethyl groups at C-6 position increased the affinity and antagonist values when compared to bulkier groups

Replacement of -NH moiety with *N*-allyl, *N*-methoxymethyl at 5-position of pyrazolopyrimidine afforded good activity
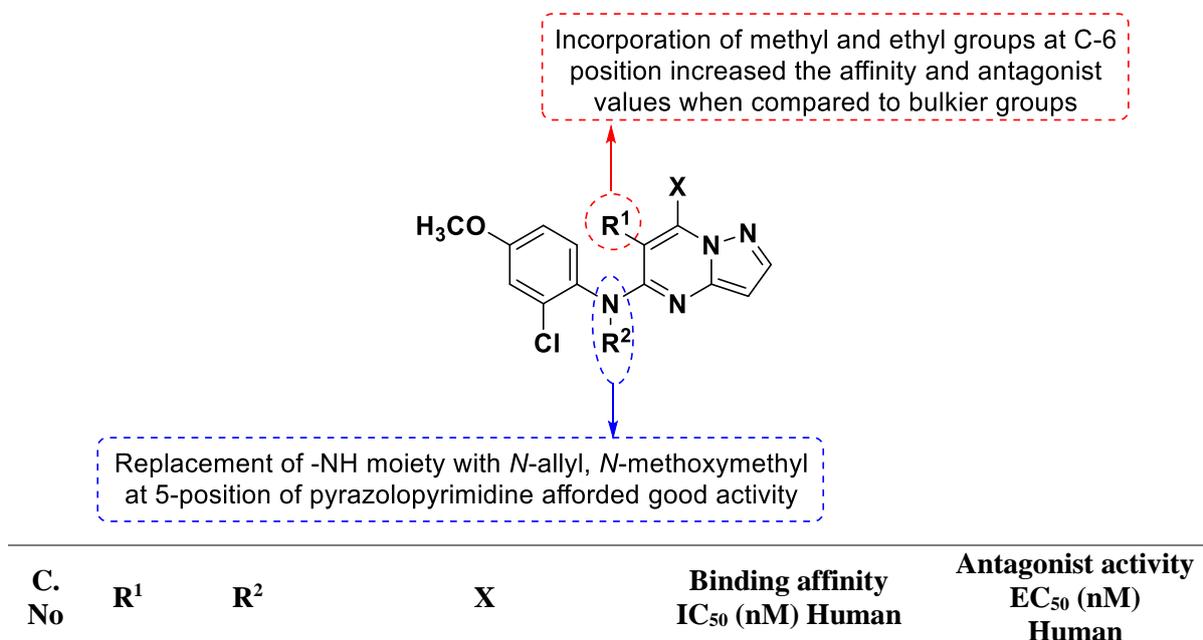
C. No	R^1	R^2	X	Binding affinity IC$_{50}$ (nM) Human	Antagonist activity EC$_{50}$ (nM) Human

144	CH$_3$	H	![structure]	7.4	7.4
145	CH$_3$	H	![structure]	8	4
146	C$_2$H$_5$	H	![structure]	4.2	8.6
147	H	CH$_2$OCH$_3$	![structure]	15	4.4

Fig. 65. CRF$_1$ binding affinity and antagonist studies of pyrazolo[1,5-a]pyrimidines.

3.6   Radiopharmaceuticals

In 2010, Ding et al. reported the synthesis and medicinal valuation of pyrazolo[1,5-a] pyrimidines as imaging agents for tumors. Initially, authors synthesized 5-((2-aminoethylamino)methyl)-7-(4-bromoanilino)-3-cyanopyrazolo[1,5-a]pyrimidine (ABCPP) and conjugated the compound with *N*-mercaptoacetylglycine (MAG), MAF (*N*-mercaptoacetyl-phenylalanine) and *N*-mercaptoacetylvaline (MAA). All the conjugates were radiolabelled with isotope [^{99m}TcN]$^{2+}$ and studied for their bio-distribution towards tumor-bearing mice. Of the evaluated complexes, *N*-mercaptoacetylglycine tagged compound exhibited maximum favorable tumor/muscle ratios, tumor/blood ratios reaching 2.97 and 1.51 at thirty minute post-injection. Structure and bio-distribution results are presented in **Fig. 66** for the favourable complex ^{99m}TcN-MAG-ABCPP (**148**) [114].

148
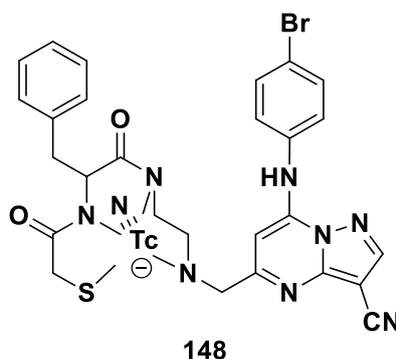
C. No	Tissue	5 min	30 min	60 min	120 min
	Heart	0.93 ± 0.11	0.32 ± 0.06	0.35 ± 0.02	0.37 ± 0.08
	Blood	2.38 ± 0.17	0.77 ± 0.01	0.46 ± 0.04	0.38 ± 0.01
148	Spleen	19.90 ± 1.70	14.38 ± 1.64	8.16 ± 0.10	8.32 ± 1.05
	Stomach	0.69 ± 0.12	0.54 ± 0.11	0.32 ± 0.04	0.09 ± 0.02
	Kidney	11.08 ± 1.13	5.06 ± 1.16	3.58 ± 0.21	3.35 ± 0.29

Liver	23.32 ± 1.32	23.88 ± 2.83	14.67 ± 0.87	12.84 ± 0.91
Muscle	0.72 ± 0.05	0.39 ± 0.08	0.34 ± 0.07	0.22 ± 0.03
Small Intestine	8.82 ± 1.29	3.42 ± 0.42	0.64 ± 0.08	0.62 ± 0.15
Large Intestine	1.62 ± 0.20	0.62 ± 0.08	0.68 ± 0.02	0.67 ± 0.06
Tumor	1.23 ± 0.13	1.16 ± 0.14	0.86 ± 0.14	0.45 ± 0.07
Bone	1.25 ± 0.74	0.95 ± 0.27	0.73 ± 0.28	0.86 ± 0.09
Brain	0.13 ± 0.01	0.04 ± 0.004	0.08 ± 0.01	0.07 ± 0.01
Lung	4.53 ± 0.99	2.30 ± 0.60	0.86 ± 0.07	0.73 ± 0.41
*T/B ratio	0.52	1.51	1.84	1.17
§T/M ratio	1.71	2.97	2.49	2.01

§T/M: tumor-to-muscle; *T/B: tumor-to-blood; All data are the mean percentage (n = 3) of the injected dose per gram of tissue, ±: the standard deviation of the mean.

Fig. 66. Structure and bio-distribution of ^{99m}TcN-MAG-ABCPP in mice bearing S 180 tumor (% ID/g).

In 2011, Xu et al. synthesized 7-(2-[^{18}F]fluoroethylamino)-5-methylpyrazolo[1,5-*a*] pyrimidine-3-carbonitrile ([^{18}F]FEMPPC, [^{18}F]1) and evaluated as positron emission tomography (PET) imaging agents for tumor detection. Initially, authors conducted uptake characteristics of [^{18}F]1 against S180 tumor cells and mice bearing S180 tumor followed by *ex vivo* bio distribution studies and concluded higher features for [^{18}F]1 with respect to the total tracer accumulation and preservation in the tumor. *In vitro* and *in vivo* results suggested that [^{18}F]1 (**149**) could be a favorable PET tracer for tumor detection. The structure and bio-distribution results are presented in **Fig. 67** for [^{18}F]1 (**149**) [115].

149
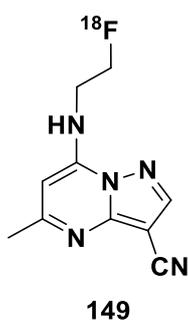
C. No	Tissue	5 min	15 min	30 min	60 min	120 min
149	Lung	6.61 ± 0.54	3.79 ± 0.93	3.77 ± 0.31	2.78 ± 0.19	2.54 ± 0.96
	Heart	5.53 ± 0.61	3.57 ± 0.65	4.06 ± 0.09	3.46 ± 0.78	2.59 ± 0.18
	Spleen	4.66 ± 0.26	3.42 ± 0.52	3.43 ± 0.39	2.82 ± 0.31	2.15 ± 0.94
	Brain	3.17 ± 0.48	2.70 ± 0.07	2.47 ± 0.24	1.93 ± 0.25	1.79 ± 0.27
	Blood	4.83 ± 0.45	4.65 ± 0.05	4.49 ± 0.31	3.63 ± 0.33	2.95 ± 0.30

Kidney	7.93 ± 1.27	5.04 ± 0.48	3.28 ± 0.20	2.58 ± 0.16	1.92 ± 0.40
Tumor	1.88 ± 0.63	4.37 ± 0.30	5.51 ± 0.31	2.95 ± 0.36	2.88 ± 0.34
Muscle	3.99 ± 1.05	3.84 ± 0.45	3.05 ± 0.22	2.31 ± 0.48	2.64 ± 0.60
Liver	6.99 ± 1.00	4.02 ± 0.20	3.17 ± 0.16	2.45 ± 0.30	1.84 ± 0.22
T/muscle ratio	0.47	1.14	1.81	1.28	1.09
T/Blood ratio	0.39	0.94	1.23	0.81	0.98
T/brain ratio	0.59	1.62	2.23	1.53	1.61

Fig. 67. Structure and bio-distribution in mice bearing S180 for [^{18}F]1 expressed as % injected dose per gram.

3.7   Organic dyes

Dyes are coloured substances having affinity to the substrate being applied on. They usually possess chromophoric groups and have a conjugated system [116].

Al-Etaibi et al. in 2011 synthesized dyes consisting of pyrazolo[1,5-*a*]pyrimidine moiety and applied those dyes to polyester fibers employing high temperature method using microwave as heating source. Results of the study indicated that the dyed fabrics demonstrated reasonable light fastness and good washing fastness characteristics. Structures of dyes (**150**-**153**) and the results are given in **Fig. 68** [117].
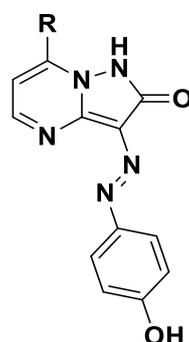
C. No.	R	Wash Fastness			Light fastness
		(4 % o.m.f. dyeing)			
		$^{\Psi}$**Alt**	***SC**	$^{\Delta}$**SW**	
150	Ph	5	5	5	2
151	4-Cl-Ph	5	5	5	2
152	-Fur-2-yl	4	5	4	2
153	-Thien-2-yl	4-5	4-5	4-5	2

ISO CO2/CO41; $^{\Psi}$Alt: alteration; *SC: staining on cotton; $^{\Delta}$SW: staining on wool; o.m.f: mass of fabric.

Fig. 68. Organic dye properties of synthesized compounds.

In 2014, Kamel et al. prepared bifunctional bis-sulphatoethylsulphone (**154**) and bis-monochlorotriazine (**155**) reactive dyes bearing pyrazolo[1,5-*a*]pyrimidine as a basic nucleus. Authors tested the synthesized dyes for their behavior on cotton, wool and silk fabrics. The results of this study indicated maximum fixation values and level of exhaustion for the dyes and the dyed fabrics exhibited nice light fastness, good rubbing, washing and perspiration fastness. The chemical structure of dyes and the study results at 4% shade are depicted in **Fig. 69** [118].

154   **155**
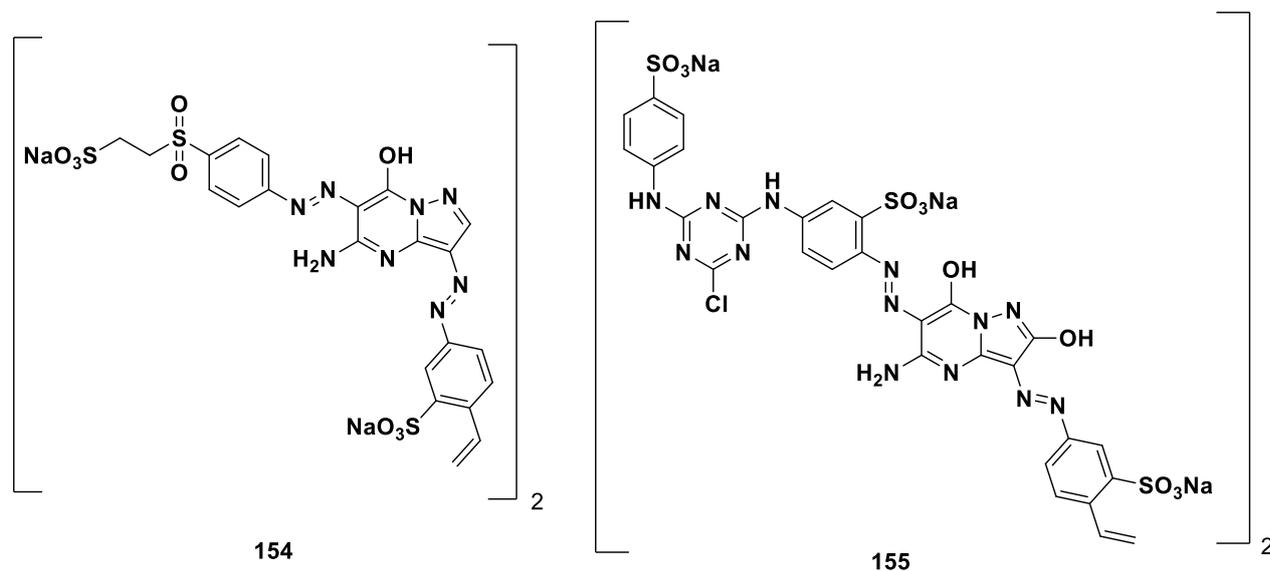
C. No	Dyed samples	K/S	Fastness to rubbing		Wash Fastness			Fastness to perspiration						Light
			Wet	Dry				Alkaline			Acidic			
					ΨAlt	*SC	ΛSW	Alt	SC	SW	Alt	SC	SW	
154	C	12.51	4-5	5	5	4-5	4-5	5	4-5	4-5	5	4-5	5	4-5
	W	20.45	5	4-5	4-5	5	4-5	5	4-5	5	5	5	5	5-6
	S	15.95	5	5	5	5	5	4-5	5	5	4-5	5	4-5	4-5
155	C	14.71	5	5	5	5	5	4-5	5	4-5	4-5	5	4-5	6
	W	26.85	4-5	4-5	4-5	4-5	4-5	4-5	4-5	4-5	5	4-5	4-5	5-6
	S	16.35	4-5	4-5	4-5	4-5	4-5	5	4-5	5	5	4-5	5	6

ΨAlt: alteration; *SC: staining on cotton; ΛSW: staining on wool; K/S: color strength.

Fig. 69. Dye properties of bis-sulphatoethylsulphone and bis-monochlorotriazine containing pyrazolo[1,5-*a*]pyrimidines.

Moreover, the same research group reported the synthesis of heterocyclic reactive dyes based on disazo pyrazolopyrimidine analogs possessing a sulfatoethylsulfone reactive group and applied to cotton, wool

and silk fabrics. Their research findings evidenced that the dyed fabrics manifested notable light fastness, good rubbing, washing and perspiration fastness. The chemical structure of representative dye **156** and its fastness properties as well as colour yields on the test fabrics are given in **Fig. 70** [119]
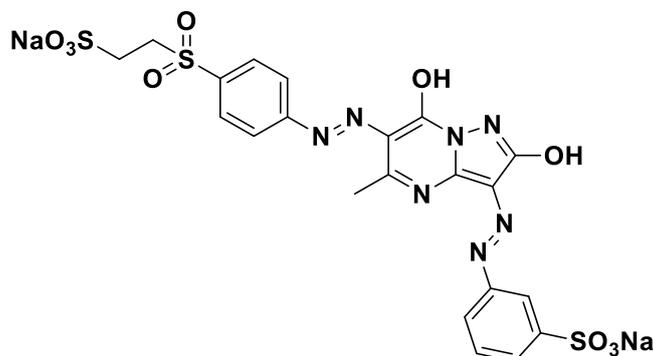
C. No	Shade (%)	Sample dyed	K/S	Fastness to rubbing		Wash fastness		Fastness to perspiration						Light
				Wet	Dry			Alkaline			Acidic			
						Acidic	Alkaline	Alt	SC	SW	Alt	SC	SW	
156	2	C	1.93	4-5	4-5	4-5	5	5	5	4-5	5	4-5	4-5	3-4
		W	13.19	4-5	4-5	5	4-5	4-5	4-5	5	4-5	4-5	5	6
		S	4.44	4-5	4-5	5	4-5	5	5	5	5	4-5	5	5-6
	4	C	4.12	4-5	4-5	5	4-5	5	5	4-5	4-5	4-5	5	2-3
		W	18.99	5	5	4-5	5	5	5	4-5	5	4-5	5	5-6
		S	6.35	5	5	5	5	4-5	4-5	5	5	5	4-5	2-3

Fig. 70. Dye properties of the representative compound.

3.8 Miscellaneous agents

In 1995, Kiyama et al. reported the synthesis and evaluation of pyrazolo[1,5-*a*]pyrimidine derivatives as non-peptide angiotensin II receptor antagonists. Among the evaluated compounds, derivative **157** decreased the mean blood pressure by more than 30 mmHg from the normal at a dose of 1 mg/kg by intravenous administration spontaneously to hypertensive rats. **Fig. 71** illustrates the SAR studies and the structures of active compounds (**158** and **159**) in the angiotensin II receptor binding assay [120].

Fig. 71. SAR and the binding affinity of active compounds towards angiotensin II receptors.
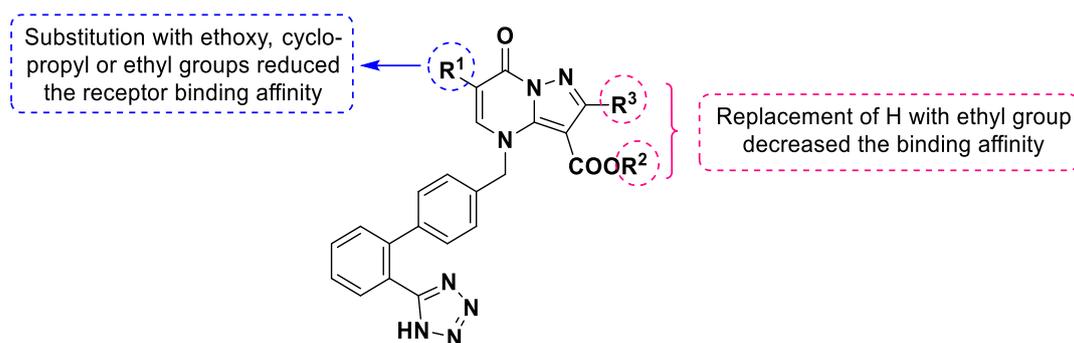
C. No	R^1	R^2	R^3	Receptor Assay (K_i nM) Human	Receptor Assay (K_i nM) Rat
157	*n*-Propyl	H	H	0.68	2.5
158	*n*-Pentyl	H	H	0.36	3.4
159	*n*-Butyl	H	H	0.85	2.5

In 2011, Kato et al. discovered a series of pyrazolo[1,5-*a*]pyrimidine derivatives as novel Dipeptidyl peptidase-IV (DPP-IV) inhibitors. Among the series, compounds **159**-**161** were discovered as potent DPP-IV inhibitors. Pharmacokinetics studies revealed compound **159** exhibited metabolic stability and well balanced elimination. SAR, pharmacological and selected pharmacokinetic data are presented in **Fig. 72** [35].
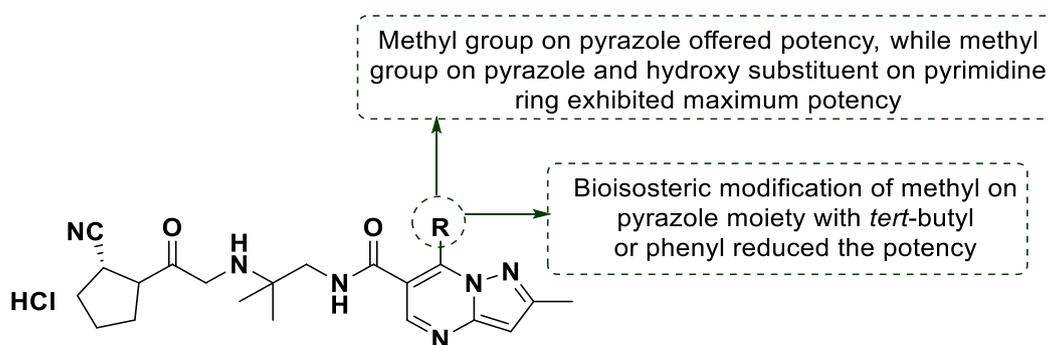
C. No	R	IC$_{50}$ (nM) DPP-IV	IC$_{50}$ (nM) DPP-8	IC$_{50}$ (nM) DPP-9	Serum Protein binding (%) 20 hg/mL Rat	Serum Protein binding (%) 20 hg/mL Dog	Serum Protein binding (%) 20 hg/mL Human	Metabolic activity [CL'int (l/h/kg)] Rat	Metabolic activity [CL'int (l/h/kg)] Dog	Metabolic activity [CL'int (l/h/kg)] Human
159	H	3.8	68	60	77.3	64.8	29.2	1.4	0.1	0.3
160	CH$_3$	13	76	64	22.1	22.5	18.9	NA	NA	NA
161	OH	1.8	58	45	NA	NA	NA	NA	NA	NA

NA: No activity.

Fig. 72. SAR, pharmacological and selected pharmacokinetic data of active compounds.

In the same year (2011), Griffith et al. discovered and synthesized pyrazolo[1,5-*a*]pyrimidine derivatives as neuropeptide Y1 receptor (an anti-obesity drug target) antagonists. Authors prepared thirteen new final compounds and evaluated (*in vitro*) using human Y1R radioligand binding assay. Within the series, compound **162** was discovered as a potent antagonist with the binding affinity value of 1 nM with comparatively active compound **163**. Further, compound **162** inhibited NPY-induced rises in blood pressure (BP) and food intake after intravenous and intracerebroventricular administration in an animal model. In feeding behavioural studies of **162**, modest inhibitions of food intake were reported in several lean rodent models. SAR, binding affinity and selected pharmacokinetic data are demonstrated in **Fig. 73** [121].
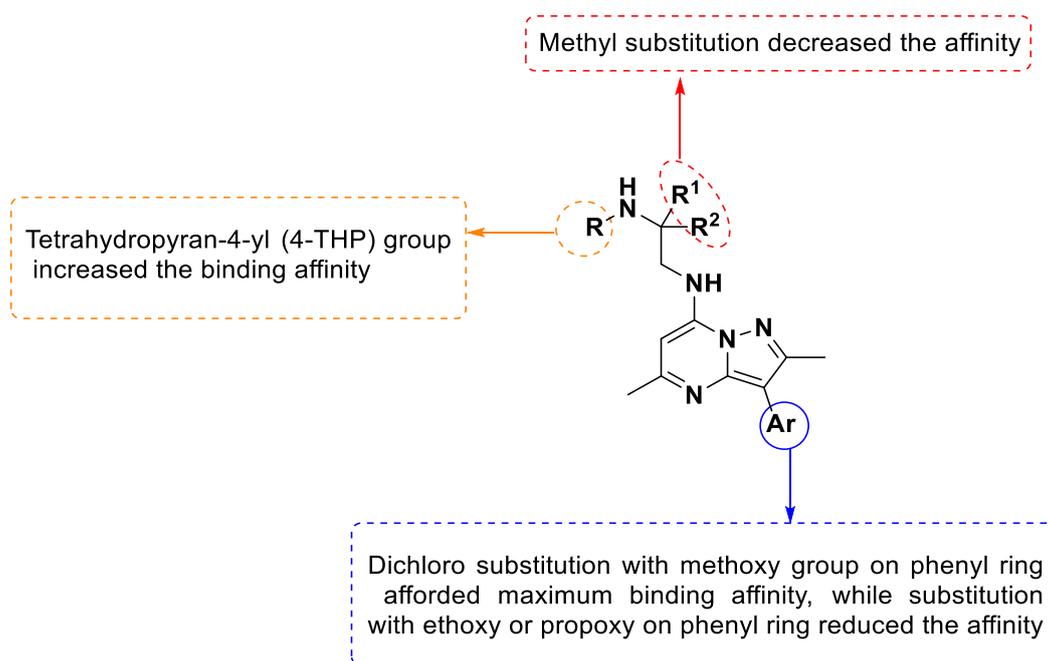
C. No	Ar	R^1, R^2	R	*cE* log *D*	$^\Psi K_i$ (nM)	Rat Clp (mL/min/kg)	Rat fu,p	Rat Clp, u (mL/min/kg)	NPY pressor response (% inh at 3 mg/kg iv)
162	2,6-Cl-4-CH$_3$O-Ph	H,H	tetrahydro-pyran-4-yl	2.6	1	45	0.059	763	92
163	2,6-Cl-4-C$_2$H$_5$O-Ph	H,H	tetrahydro-pyran-4-yl	2.8	3	28	0.029	996	47

$^\Psi K_i$: Human Y1R radioligand binding assay; Rat Clp: Rat plasma clearance; Rat fu,p: Rat functional potency; Rat Clp, u: Rat plasma clearance, unbound clearance; iv: intra venous.

Fig. 73. SAR, binding affinity and selected pharmacokinetic data of potent inhibitors.

In 2004, Campton and co-workers discovered and optimized the pyrazolo[1,5-*a*]pyrimidines as estrogen receptor (ER) ligands. Among those, compounds **164-166** exhibited promising binding affinities for ER*α* and ER*β*. The optimized SAR studies revealed that incorporation of –OH group on the structure resulted in low affinity values at C-6 as shown in **Fig. 74**, which indicated that ligand core was attached to the ER in a reverse mode and served as isostere of the A-ring of estradiol [48].

C. No	R	R^1	R^2	ERα IC$_{50}$ (nM)	ERβ IC$_{50}$ (nM)
164	H	OH	H	0.010	0.050
165	OH	H	H	0.007	0.023
166	OH	OH	H	0.004	0.100
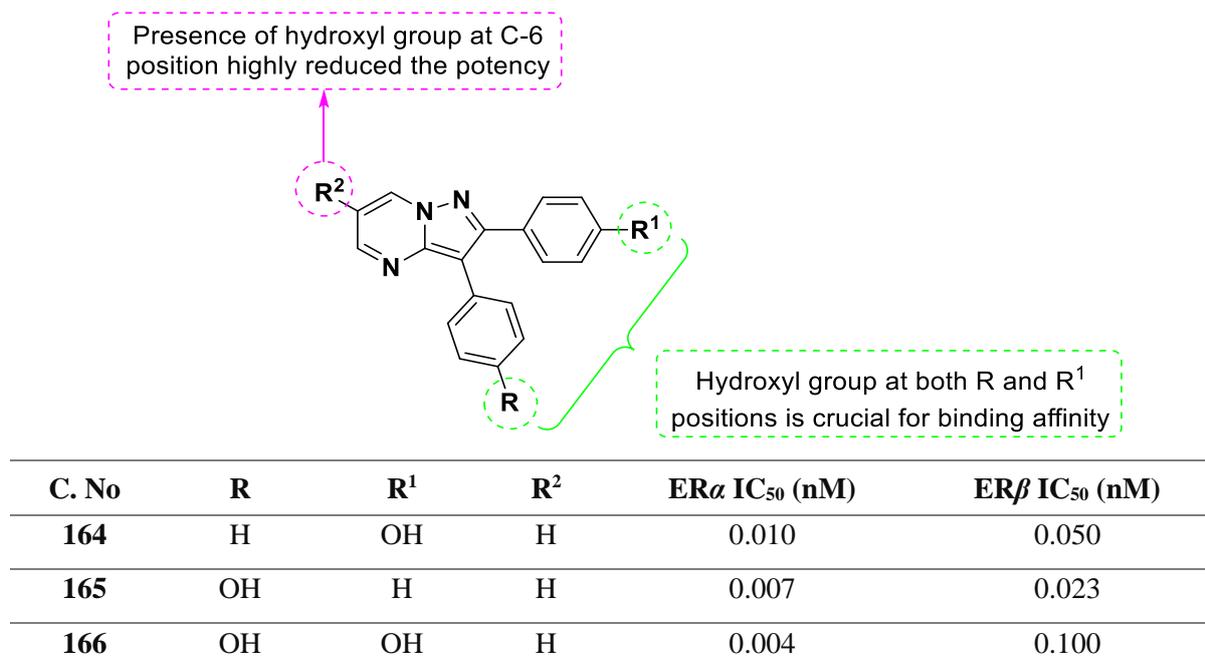
Fig. 74. Estrogen receptor ligands bearing pyrazolo[1,5-*a*]pyrimidine scaffold.

In 2015, Hassan and co-workers synthesized pyrazolo[1,5-*a*]pyrimidine derivatives by reacting 5-amino-*N*-aryl-1*H*-pyrazoles with acetylacetone and 2-(4-methoxybenzylidene)- malononitrile. All the synthesized compounds were tested against Ehrlich ascites carcinoma (EAC) cell line for their *in vitro* cytotoxic activity. Among those, compounds **167** and **168** displayed potential activity when compared to doxorubicin as a reference drug. A brief SAR study has been presented in **Fig. 75** [32].

Phenyl group showed maximum activity than 4-methylphenyl and 4-chloro phenyl

167

4-chlorophenyl group showed maximum activity than 4-methylphenyl and phenyl groups

168

C. No	R	EAC IC$_{50}$ (μg/mL)
167	Ph	10
168	4-Cl-Ph	25
Doxorubicin		37.4
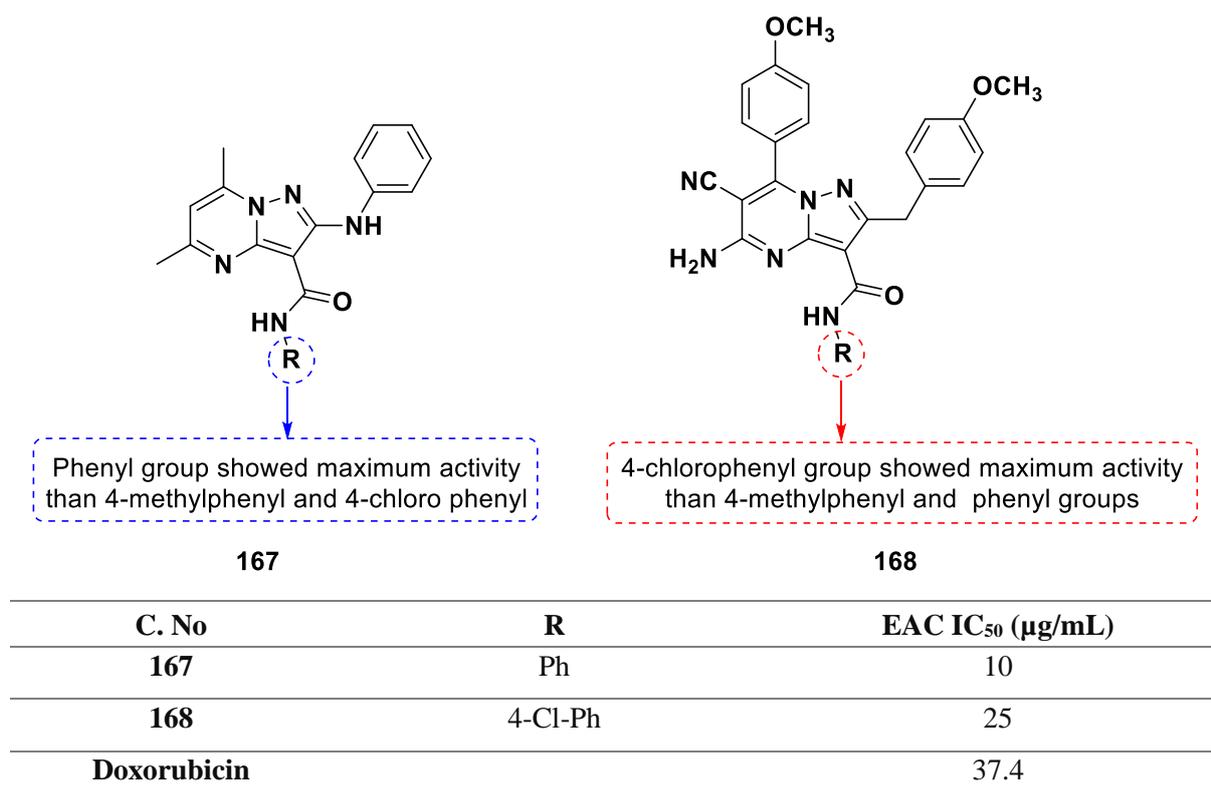
Fig. 75. Cytotoxicity studies of pyrazolo[1,5-*a*]pyrimidines with reference drug doxorubicin.

In 2015, the same above mentioned authors (Hassan and co-workers) reported the synthesis and *in vitro* cytotoxic activities of 7-hydroxy-5-methyl-*N*-(aryl)pyrazolo[1,5-*a*] pyrimidines. All the synthesized compounds were tested against four human cancer cell lines (lung A549, liver HepG, colon HCT116 and breast MCF-7). Within the series, compounds **169** and **170** showed significant cytotoxic activities when compared to reference drug doxorubicin. SAR studies have been represented in **Fig. 76** [122].

Phenyl group showed excellent potency when compared to substituted phenyl group at R-position

C. No	R	Human cancer cell lines			
		Colon HCT116 IC$_{50}$ (μg/mL)	Lung A549 IC$_{50}$ (μg/mL)	Liver HepG2	Breast MCF-7 IC$_{50}$ (μg/mL)
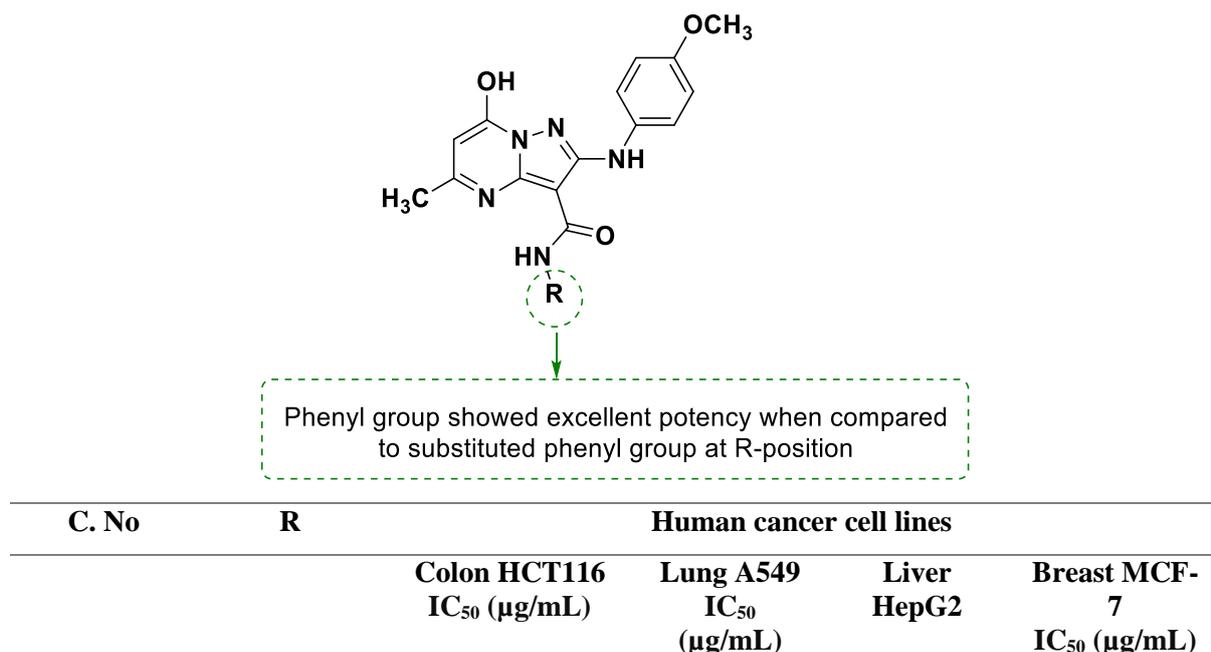
				IC$_{50}$ (µg/mL)	
169	Ph	NA	5.00 ± 0.50	4.00 ± 0.44	4.60 ± 0.55
170	4-Cl-C$_6$H$_4$	NA	5.45 ± 0.62	6.10 ± 0.62	4.20 ± 0.60
Doxorubicin		6.30 ± 0.60	5.10 ± 0.50	4.20 ± 0.46	4.70 ± 0.55

NA: No activity.

Fig. 76. SAR and cytotoxic properties of highly active compounds consisting pyrazolo[1,5-*a*]pyrimidine scaffold.

Engers and co-workers reported the synthesis and pharmacological evaluation of 3,6-disubstituted pyrazolo[1,5-*a*]pyrimidine derivatives as bone morphogenetic protein (BMP) receptors. SAR studies were carried out to determine the active functional groups at C-6, C-3 positions in order to enhance the potency and also carried out comparative studies with known drugs such as dorsomorphin, LDN-193189 and DMH-1 as represented in **Fig. 77**. Among the series, compounds **171**-**173** exhibited equipotent activity against BMP receptors (BMP4 cell IC$_{50}$ = <1 nM) [44].

C. No	R^1	R^2	BMP4 cell IC$_{50}$ (nM)
171		-OCH$_3$	<1
172			<1
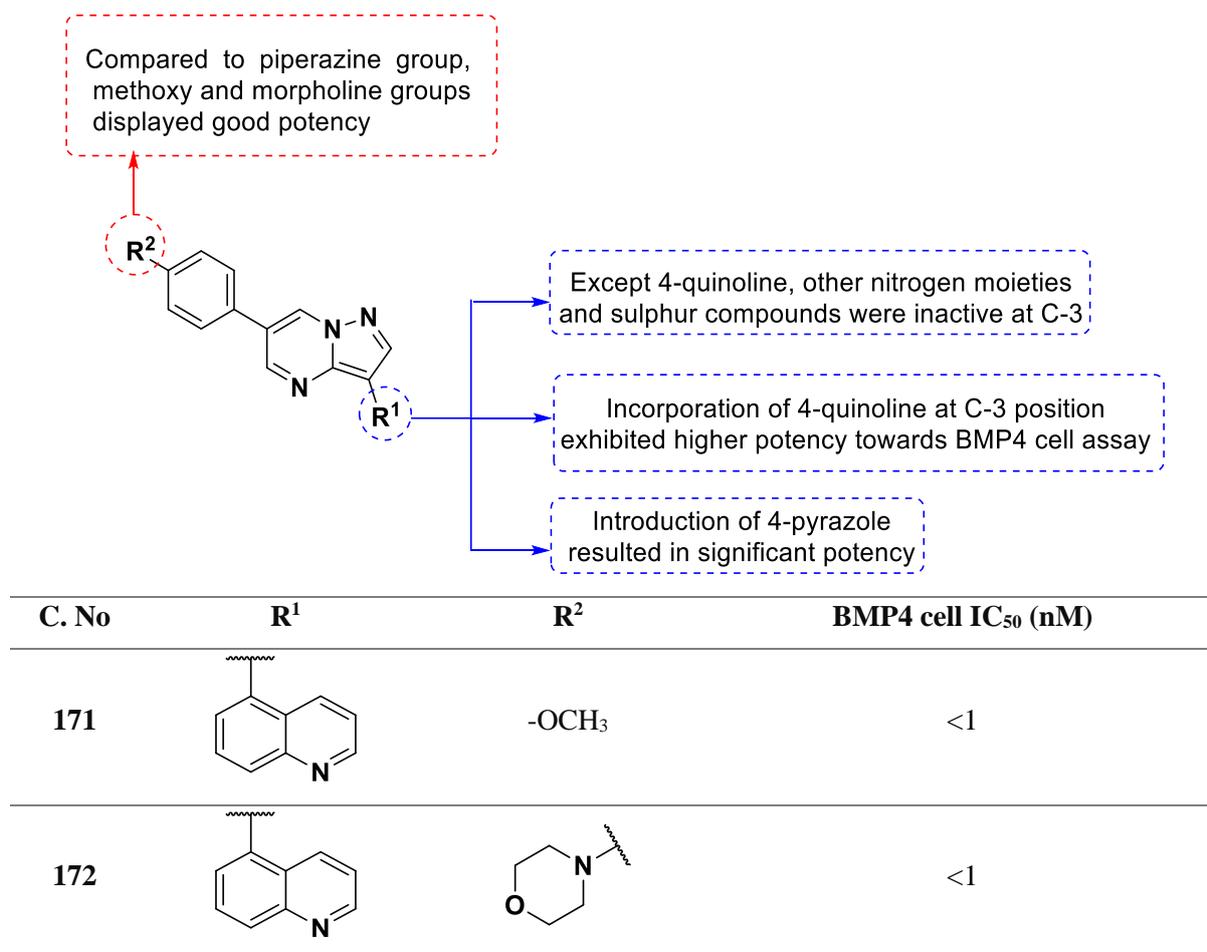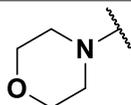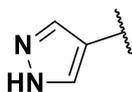
173			<1

Fig. 77. SAR and BMP4 cell line properties of 3,6-difunctionalized pyrazolo[1,5-*a*]pyrimidines.

In 2013, Tabrizi and co-workers reported the synthesis of 7-oxopyrazolo[1,5-*a*]pyrimidine-6-carboxamides and evaluated their pharmacological activity as effective and selective cannabinoid receptor inverse agonists. SAR studies were performed to find out the effective functional groups at C-6 and C-2 positions to achieve potent cannabinoid receptor type-2 (CB$_2$) receptors. Among the series, compounds **174**-**177** displayed potent and selective CB$_2$ receptor affinities as depicted in **Fig. 78** [51].

Admant-1-yl group is essential for activity

Unsubstituted phenyl ring showed good activity when compared to substituted phenyl
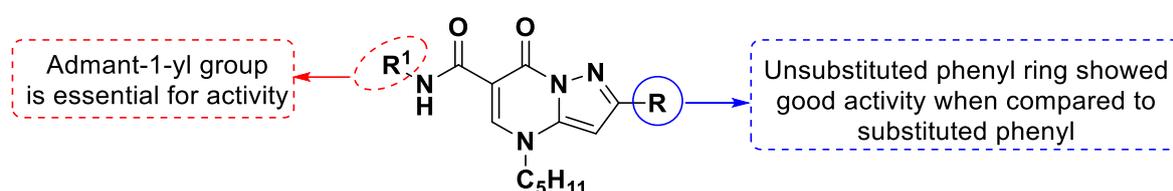
C. No	R	R^1	K_i (nM)				
			^4rCB$_1$	$^*r$CB$_2$	$^\psi$hCB$_1$	§hCB$_2$	$^\phi$SI
174	Ph	Adamant-1-yl	> 10000 (40%)	2.74 ± 0.28	>10000 (40%)	2.56 ± 0.22	>3906
175	2-CH$_3$-Ph	Adamant-1-yl	> 10000 (30%)	3.21 ± 0.30	>10000 (22%)	2.86 ± 0.25	>3496
176	4-Cl-Ph	Adamant-1-yl	> 10000 (10%)	4.21 ± 0.42	>10000 (13%)	3.88 ± 0.31	>2577
177	Furan-2-yl	Adamant-1-yl	> 10000 (40%)	5.14 ± 0.42	>10000 (34%)	4.92 ± 0.43	>2032

Here ^4rCB$_1$: rat brain for CB$_1$ receptors; $^*r$CB$_2$: rat spleen for CB$_2$ receptors; $^\psi$hCB$_1$: human CB$_1$ CHO membrane; §hCB$_2$: human CB$_2$ CHO membrane; $^\phi$SI: selectivity.

Fig. 78. SAR and cannabinoid receptor studies of 7-oxopyrazolo[1,5-*a*]pyrimidine-6-carboxamides.

In 2016, Liu et al. reported the discovery of pyrazolo[1,5-*a*]pyrimidines as threonine tyrosine kinase (TTK) inhibitors. All synthesized molecules were screened against various cancer cell lines. Compound **178** indicated potent activities against all tested cancer cell lines. SAR studies concluded that assimilation of polar, basic and solubilizing groups in the hydrophobic and solvent accessible areas modulated physiochemical assets while maintaining potency, as illustrated in **Fig. 79** [123].

Basic morpholine and pyran functionl groups showed less potency when compared to hydroxyl cyclobutanes at C-7 position

Addition of hydroxyl functionalities to solvent accessible region showed potent activity

Incorporation of pyridyl group exhibited significant potency

Cyclopropyl benzamide carbonyl and -NH were coordinated with side chain of Pro673 and Ile663

C. No	R	R^1	TTK IC$_{50}$ (nM)	Cancer cell GI$_{50}$ (µM)		
				MDA-MB-468	HCT116	OVCAR-3
178			1.7	0.002	0.002	0.004

Fig. 79. SAR and TTK activity studies.

3.9 Patents covering pyrazolo[1,5-*a*]pyrimidine nucleus and their target activity

Medicinal importance of pyrazolo[1,5-*a*]pyrimidine scaffold is further evidenced based on numerous patents registered from 1980 to recent past. Various research groups that patented this scaffold for different therapeutic segments are concisely presented in **Table-1**.

Research group	Patent number	Target activity	Year
Dusza	US4626538	Anxiolytic, antiepileptic, sedative-hypnotic agents [9]	1986
Nugent	US005397774A	Anti-inflammatory[124]	1995
Inoue	US005688949A	Anti-inflammatory [125]	1997
Inoue	US005843951A	Analgesic [126]	1998
Levin	US20070219183A1	Cancer [127]	2007
Chen	WO2008036579A1	CRF$_1$ receptor antagonists [128]	2008
Andrews	WO2011029027A1	mTOR inhibitors [129]	2011

Zhao	WO2012027239A1	mTOR inhibitors [130]	2012
Marugan	WO2012078855A1	Glucocerebrosidase activators [131]	2012
Bearss	US8710057B2	Protein kinase inhibitors [132]	2014
Ahmad	WO2014089379A9	ATR kinase inhibitors [133]	2014

Table-1. Pyrazolo[1,5-*a*]pyrimidine nucleus containing patents having numerous biological activities.
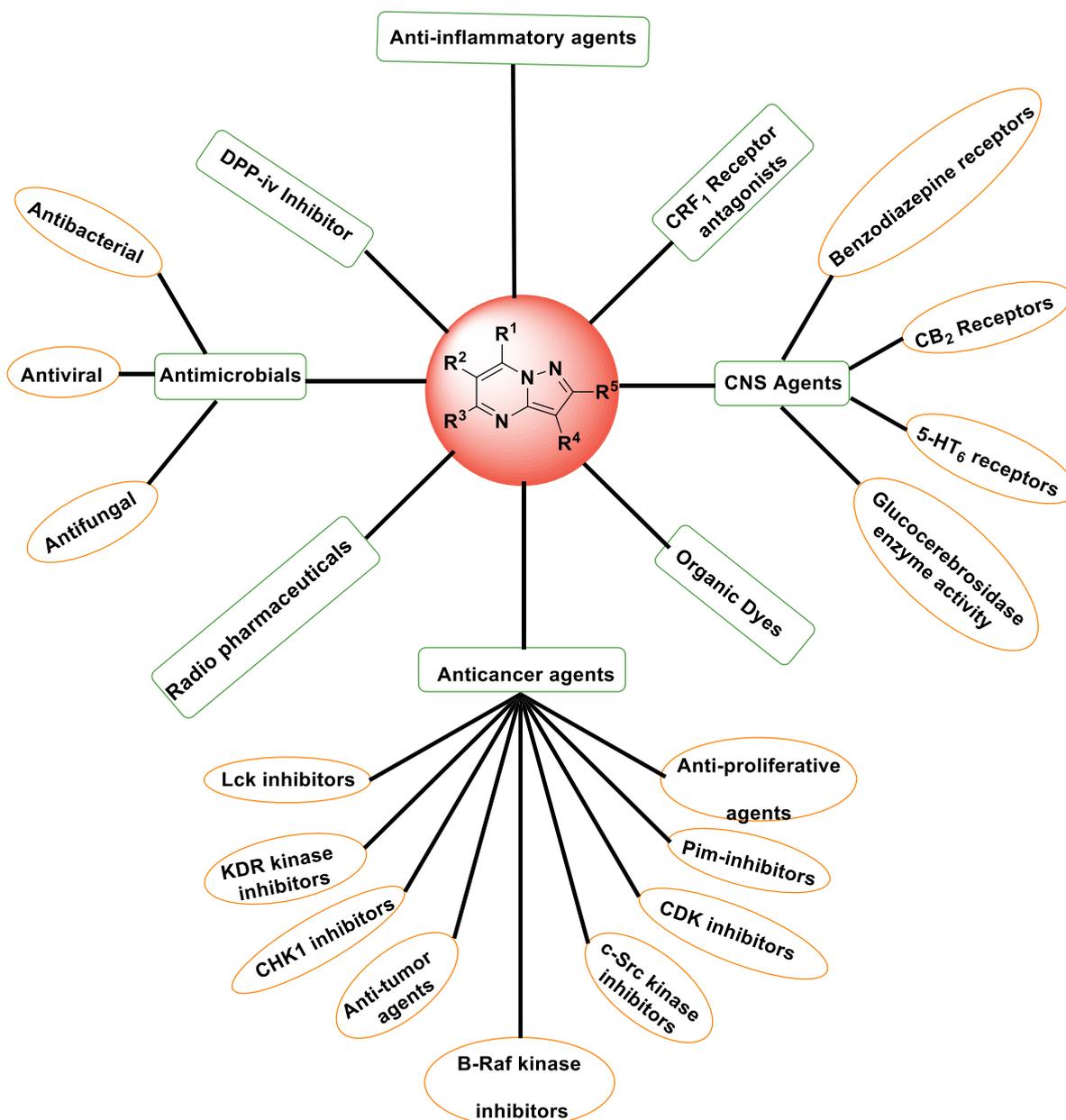
Fig. 80. Biological activities of pyrazolo[1,5-*a*]pyrimidines.
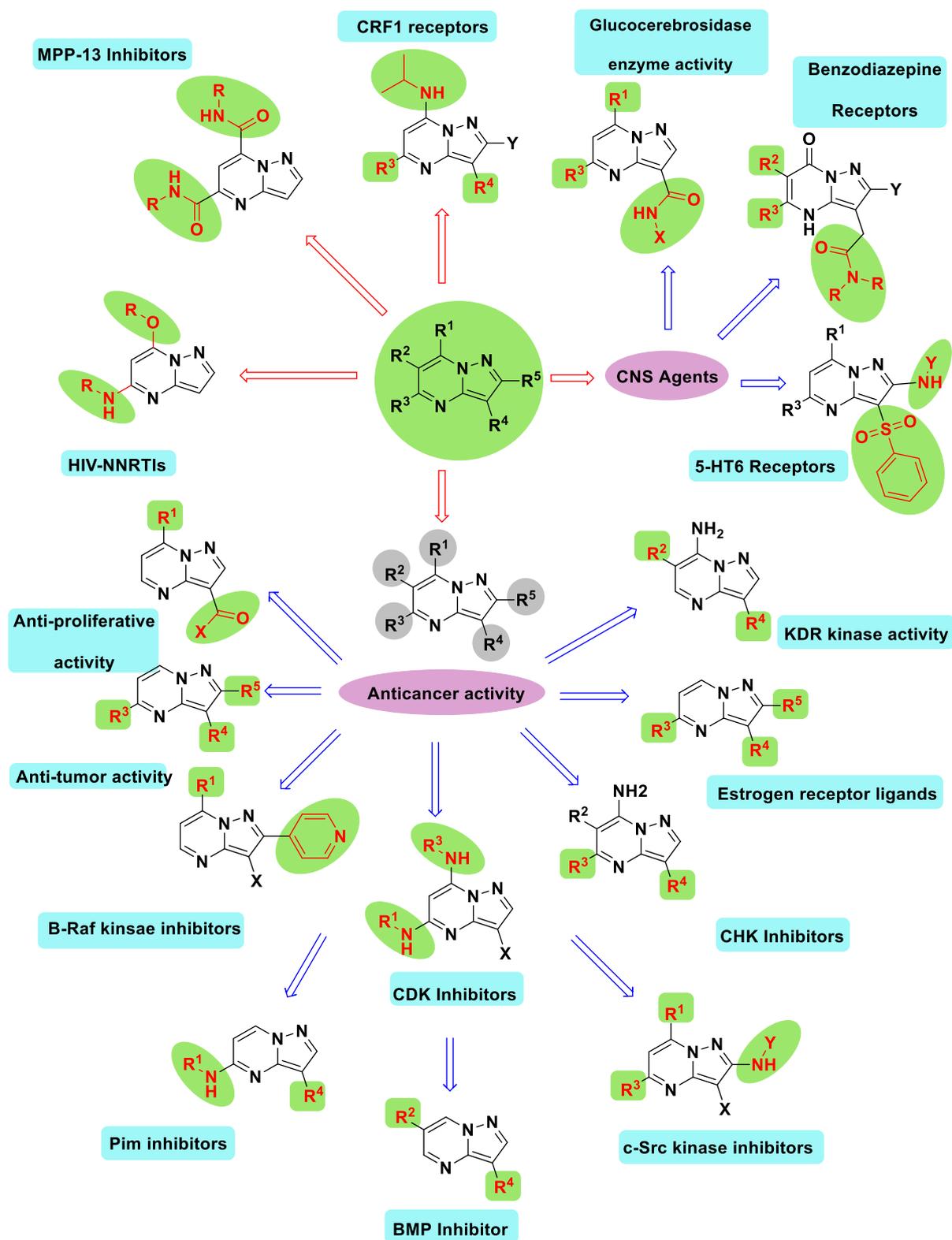
Fig. 81. Summary of structural modifications to influence the activity.

4 Conclusion

Literature survey indicated pyrazolo[1,5-*a*]pyrimidine as a privileged scaffold in medicinal chemistry with diverse pharmacological activities. In this review, we initially discussed various strategies employed for the syntheses of substituted pyrazolo[1,5-*a*]- pyrimidines through multi-component reactions (MCRs), conventional heating and/or microwave-assisted organic reactions (MAORs). Moreover, these synthetic protocols offer pharmaceutical chemists to generate a library of pyrazolo[1,5-*a*]pyrimidines for high throughput screening (HTS) analysis. Clinically administered drugs such as zaleplon, indiplon, ocinaplon, dinaciclib, dosomorphin, anagliptin, pyrazophos and lorediplon bearing pyrazolo[1,5-*a*]pyrimidine nucleus have been effectively used till date for therapy of numerous ailments. Medicinal attributes of pyrazolo[1,5-*a*]pyrimidines have been extensively studied for different biological activities such as anticancer, antimicrobials, CNS depressant and other applications such as organic dyes, and in radiopharmaceuticals, as illustrated in **Fig. 80**. Recently, several therapeutic applications and patents on this scaffold have been emerged and much more is yet to be explored. A number of researchers and scientists analyzed SAR of pyrazolo[1,5-*a*]pyrimidines and deduced the bioactive structures in a quantitative manner (QSAR). The C-3 position of pyrazolo[1,5-*a*]pyrimidines accomplished favourable CNS activity, whereas modifications at C-2, C-3, C-5, C-6 and C-7 positions attributed towards different anticancer targets. Further, change in substitution patterns on C-5 and C-7 positions imparted higher affinity towards HIV-NNRTIs and CRF_1 receptors. Overall structural diversifications and their effects on biological activities are presented in **Fig. 81**. A wide range of pharmaceutical properties displayed by this privileged scaffold will definitely serve the purpose for developing effective potent chemotherapeutics. This review aims to provide an extensive information to the scientific community to design novel, target selective, optimized and varied pyrazolo[1,5-*a*]pyrimidine analogs for the treatment of multifactorial diseases.

5 Conflicts of Interest

Authors hereby declare that there are no financial/commercial conflicts of interest.

6 Acknowledgement

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CHAPTER 3

An appraisal on synthetic and pharmaceutical perspectives of pyrazolo[4,3-*d*]pyrimidine scaffold

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Graphical Abstract
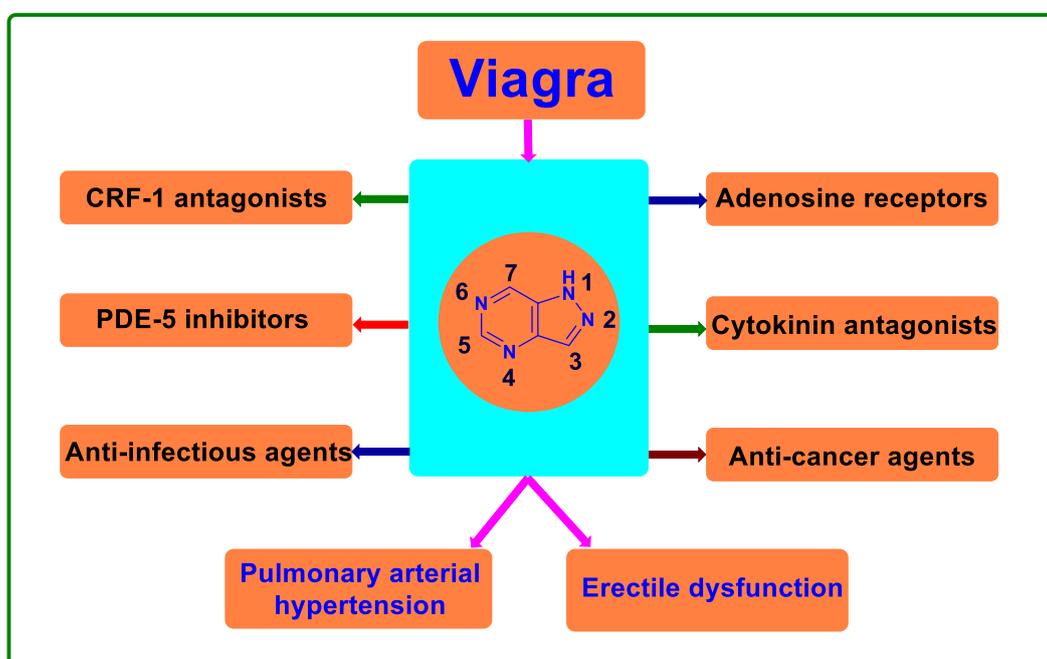
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Highlights

- Pyrazolo[4,3-*d*]pyrimidine: Nitrogen containing bicyclic heterocyclic ring with diverse biological activities.

- Synthetic strategies for variously substituted pyrazolo[4,3-*d*]pyrimidine.

- The numerous pharmacological activities of pyrazolo[4,3-*d*]pyrimidine has been discussed.

- SAR of pyrazolo[4,3-*d*]pyrimidine scaffold for miscellaneous activities has been explained.

- This review will be helpful for medicinal chemists in designing future drugs.

Abstract:

Pyrazolo[4,3-*d*]pyrimidine, a fused heterocycle bearing pyrazole and pyrimidine portions has gained a significant attention in the field of bioorganic and medicinal chemistry. Pyrazolo[4,3-*d*]pyrimidine derivatives have demonstrated numerous pharmacological activities particularly, anti-cancer, anti-infectious, phosphodiesterase inhibitors, adenosine antagonists and cytokinin antagonists etc. This review extensively unveils the synthetic and pharmacological diversity with special emphasis on structural variations around pyrazolo[4,3-*d*]pyrimidine scaffold. This endeavour has thus uncovered the medicinal worthiness of pyrazolo[4,3-*d*]pyrimidine framework. To the best of our knowledge this review is the first compilation on synthetic, medicinal and structure activity relationship (SAR) aspects of pyrazolo[4,3-*d*]pyrimidines since 1956.

1   Introduction

Heterocyclic compounds have been significant part of both organic and medicinal chemistry research. Majority of the commercially available drugs are built on heterocyclic scaffolds and these scaffolds are core part of the drugs responsible for desired pharmacological activity. Over the years, these heterocycles have been synthesized with numerous improved synthetic methods.[1] Further these compounds have also been a valuable source of intermediates in the synthesis of several fused heterocyclic compounds of biological importance.[2] These fused heterocyclic compounds have major roles in biological processes, and they are present in a wide variety of drugs, antibiotics, vitamins, natural products and many other biomolecules. In addition, nitrogen bearing fused heterocycles have gained a substantial attention and occur in a variety of bioactive natural products, pharmaceuticals, organic materials, dyes and agrochemicals.[3] Among these *N*-fused heterocycles, pyrazolopyrimidine is one of the attractive fused heterocyclic moiety owing to its synthesis and immense pharmacological importance. Some of the marketed drugs containing pyrazolopyrimidine core structure are allopurinol,[4] zaleplon, indiplon,[5] dinaciclib,[6] dorsomorphin,[7] ocinaplon,[8] anagliptin,[9] lorediplon and pyrazophos,[10] sildenafil,[11] tisopurine.[12] Pyrazolopyrimidines show pharmacological properties such as cyclin-dependent kinase (CDK) inhibitors,[13,14] anti-proliferative,[15] anti-bacterial,[16] anti-fungal,[17,18] anti-viral agents,[19] anti-leishmanial.[20] Furthermore, pyrazolopyrimidines acts as central nervous system depressants,[21] COX-1, COX-2 selective inhibitors,[22] antitrypanosomal and sedative,[23] serotonin 5-HT$_6$ receptor antagonists,[24,25] corticotropin-releasing factor (CRF) 1 receptor antagonists,[26] tuberculostatic[27] and PET tumor imaging agents.[28]

Several isomeric forms of pyrazolopyrimidines are well-known such as pyrazolo[1,5-*c*]pyrimidines, pyrazolo[5,1-*b*]pyrimidines, pyrazolo[5,1-*a*]pyrimidines, pyrazolo[4,3-*d*]pyrimidines and pyrazolo[3,4-*d*]pyrimidines.[29] Of them, pyrazolo[4,3-*d*]pyrimidine (**Fig. 1**), which is an isostere of purines has acquired considerable importance due to its diverse, facile and general synthetic methodologies with great medicinal importance. In 1958, Robins reported the synthesis of pyrazolo pyrimidines as potential purine antagonists.[30] Substituted 1*H*-pyrazole and 1,3-dimethylpyrimidine-2,4-(1*H*,3*H*)-dione are the most common substrates for synthesizing pyrazolo[4,3-*d*]pyrimidine derivatives.

a   b
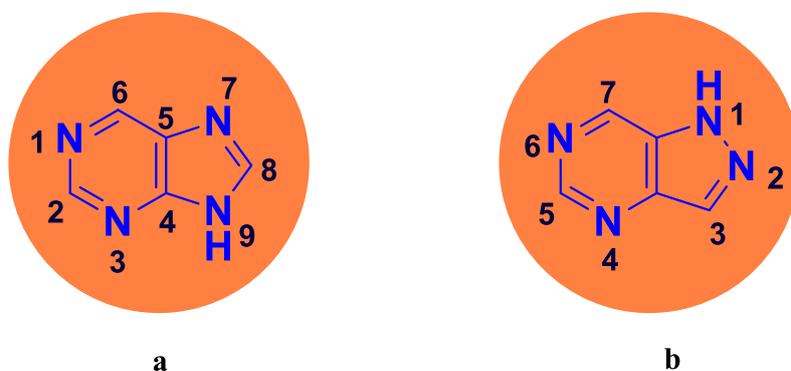
Fig. 1. General structures of purine (**a**) and pyrazolo[4,3-*d*]pyrimidine (**b**).

The versatile synthetic approaches for pyrazolo[4,3-*d*]pyrimidine derivatives have gained significant interest of the medicinal chemists due to its wide range of pharmacological applications namely, adenosine receptor antagonists,[31] cytokinin antagonists,[32] corticotrophin-releasing factor receptor antagonists,[33] anti-leishmanial,[19] phosphodiesterase 5 (PDE5) inhibitors,[34] anti-viral, anti-fungal,[35] diagnostic agents,[36] anti-inflammatory,[37] agents in male and female sexual dysfunctions[38] etc. Marketed drug, Sildenafil is a well-known example of a drug containing pyrazolo[4,3-*d*]pyrimidine scaffold. This drug is marketed with brand names as Viagra® and Revatio®, is the first drug of its kind to be used for the treatment of male erectile dysfunction and pulmonary arterial hypertension (PAH)[39] (**Fig. 2**). Pyrazolo[4,3-*d*]pyrimidine scaffold also forms a vital component of naturally occurring nucleoside antibiotics such as formycin A and B.[40]

Recently, our research group reported a review on pyrazolo[1,5-*a*]pyrimidine framework emphasising on methods of synthesis, structure activity correlations with their reported pharmacological activities.[41] In continuation of our interest on pyrazolopyrimidines, the current review reveals various synthetic approaches and medicinal properties of pyrazolo[4,3-*d*]pyrimidine derivatives. To the best of our understanding, this review is the first of its kind with extensive compilation on synthesis, structure-activity relationship (SAR) and medicinal properties of pyrazolo[4,3-*d*]pyrimidine derivatives.
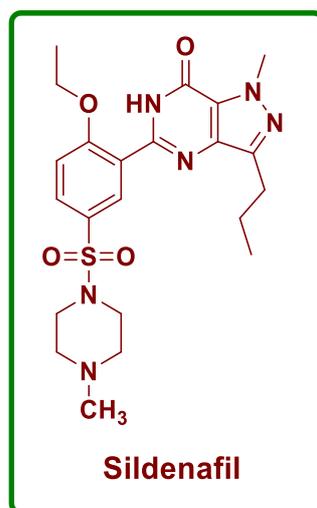
Fig. 2. Marketed drug Sildenafil containing pyrazolo[4,3-*d*]pyrimidine scaffold.

2   Synthetic methodologies for pyrazolo[4,3-*d*]pyrimidine scaffold

Pyrimidine is a six membered 1,3-diazine heterocycle containing two imine nitrogen atoms. These two nitrogens exhibit electron withdrawing tendency on the surrounding atoms thus making pyrimidine ring more resistant towards electrophilic substitution while facilitating nucleophilic attack.[42] On the other hand, pyrazole a five membered 1,2-diazine heterocycle bearing two nitrogen atoms adjacent to each other and is a structural isomer of imidazole having nitrogen atoms at 1- and 3-position.[43] Fusion of pyrazole with the

pyrimidine ring results in the formation of new bicyclic system known as "pyrazolopyrimidine". There are almost five different structural isomers of this bicyclic system, which exists due to the varying position of nitrogen, degree of saturation or unsaturation, or the number of nitrogens in the pyrazole nucleus.[44] Among those, pyrazolo[4,3-*d*]pyrimidine scaffold is privileged and medicinally significant as it has multiple reaction centres (Nitrogen at 1, 2, 4 and 6 positions and Carbon at 3, 5 and 7 positions). Their manifestation contributes to the expression of dual or multiple reactive abilities in terms of electrophilic as well as nucleophilic substitution reactions (**Fig. 3**). These properties outlined above, prompted chemists to synthesize pyrazolopyrimidine derivatives for their potential application in medicinal chemistry. From the historic point of view, synthesis of pyrazolo[4,3-*d*]pyrimidines was first reported in 1956,[30] since then numerous derivatives have been synthesized by applying various synthetic methodologies and were evaluated for their biological properties. The numerous synthetic strategies of pyrazolo[4,3-*d*]pyrimidines are represented in **Fig. 4**.
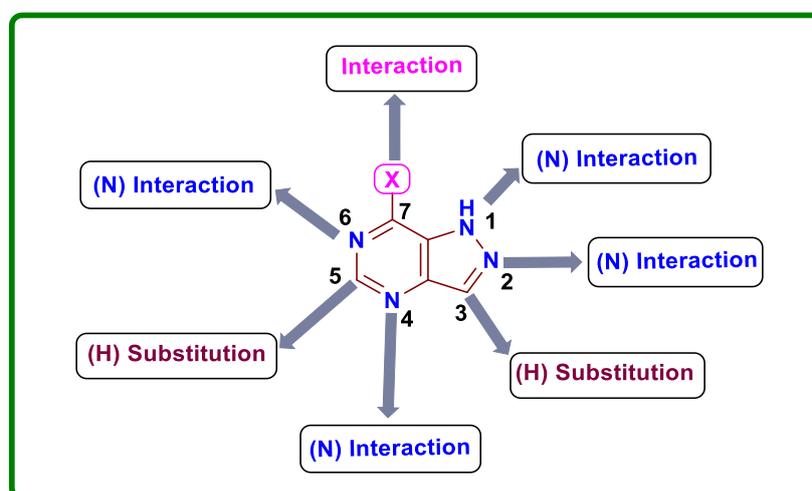
Fig. 3. Possible reaction centres of pyrazolo[4,3-*d*]pyrimidine scaffold.

The synthesis of pyrazolo[4,3-*d*]pyrimidine derivatives (**P1**-**P39**) have been achieved by utilizing several simple substituted 1*H*-pyrazoles (**i-xxxvi**) and substituted 1,3-dimethylpyrimidine-2,4(1*H*,3*H*)-diones (**xxxvii-xxxix**) by employing different synthetic routes. The following discussion divulges in detail about the synthesis of pyrazolo[4,3-*d*]pyrimidines derivatives.

Robins *et al*. accomplished the desired product **P1** by the cyclization of **i** (4-amino-3-methyl-1*H*-pyrazole-5-carboxamide) with thiourea under reflux conditions (scheme-1).[45] Robins *et al*. treated 4-amino-1*H*-pyrazole-5-carboxamide (**ii**) with formamide under boiling conditions to afford 7-hydroxypyrazolo[4,3-*d*]pyrimidine (**P2**) as a target molecule (scheme-2).[46] Long and co-workers introduced a fusion reaction by ring annulation of **iii** (4-amino-3-methyl-1*H*-pyrazole-5-carbothioamide) with urea to achieve **P3** (scheme-3).[47] Acton and co-workers carried out the Curtius rearrangement of **iv** in boiling toluene to achieve pyrazolo[4,3-*d*]pyrimidinedione (**P4**) as a final compound (scheme-4).[48] Takei *et al*. attempted the reaction

between **v** (ethyl 4-amino-5-oxo-4,5-dihydro-1*H*-pyrazole-3-carboxylate hydrochloride) and formamidine acetate to attain **P5** in presence of triethylamine as a base in 2-ethoxy ethanol (scheme-5).[49] Wierzchowski and co-workers reported the cyclization reaction of ethyl 4-amino-1-ethyl-3-propyl-1*H*-pyrazole-5-carboxylate (**vi**) with formamide to afford **P6** under reflux conditions (scheme-6).[50] Lewis *et al*. obtained **P7** via treating methyl (*Z*)-*N*-benzoyl-*N*'-(5-carbamoyl-1*H*-pyrazol-4-yl)carbamimidothioate (**vii**) with a saturated solution of ammonia in dimethylformamide and dilute NaOH solution under reflux conditions (scheme-7).[51] Ochi and co-workers have reported hydrogenation and subsequent cyclization of **viii** (4-nitroso-5-hydroxy-1-phenyl-1*H*-pyrazole-3-carboxylate) with formamide to obtain **P8** under inert conditions at 180-190 ^{0}C (scheme-8).[52]
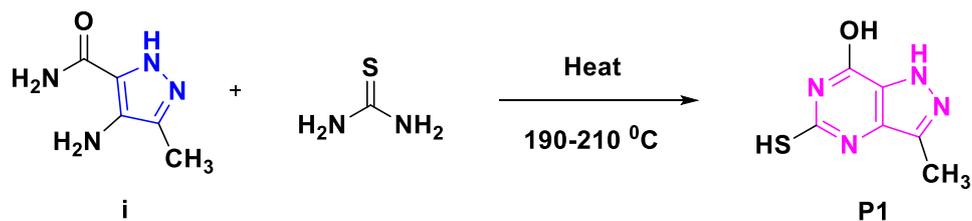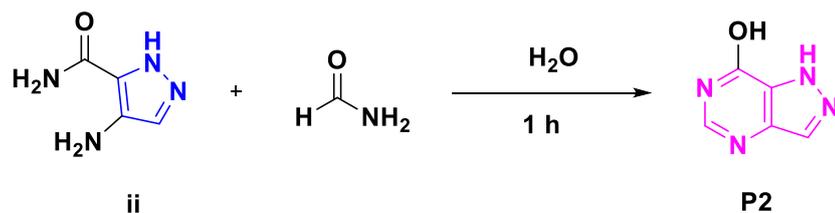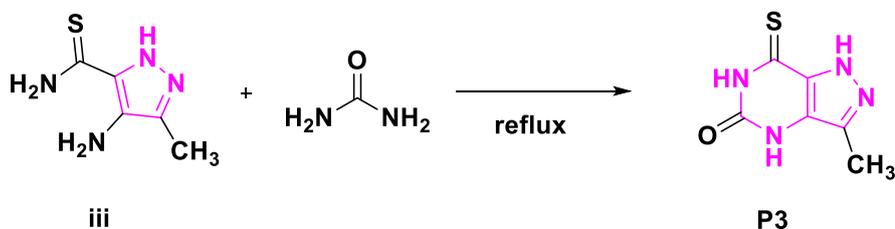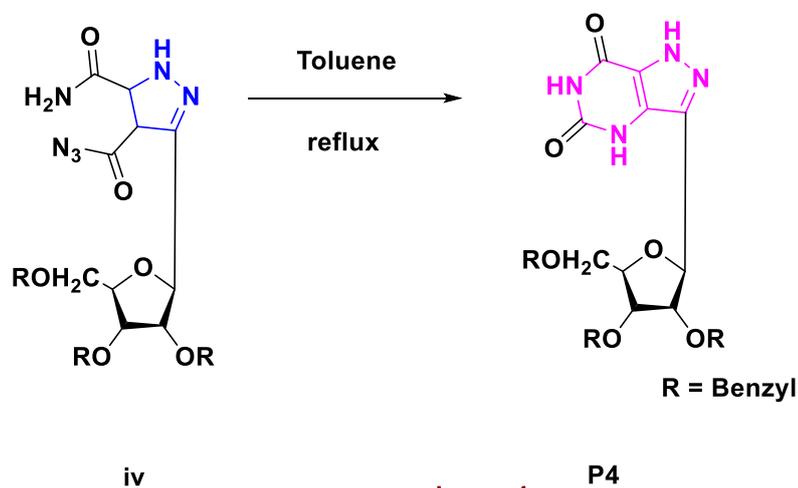
Fig. 4. Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.
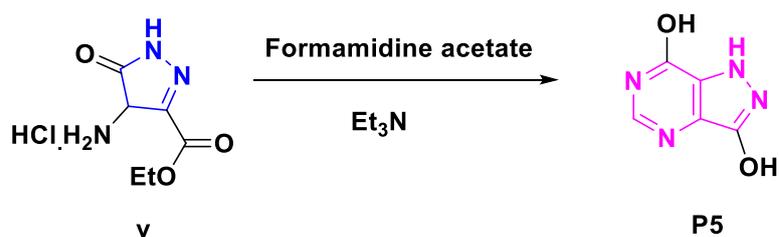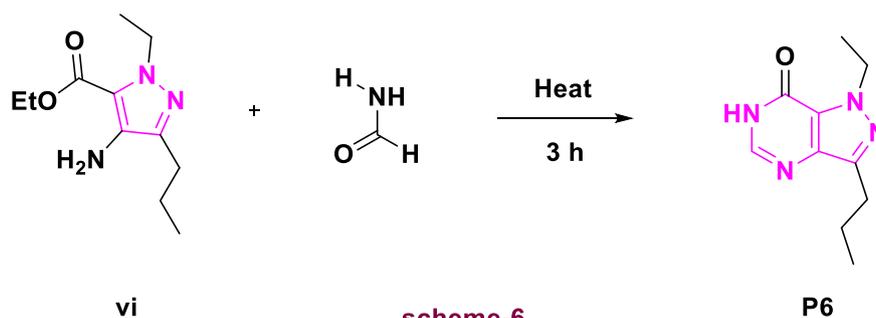
scheme-5

scheme-6
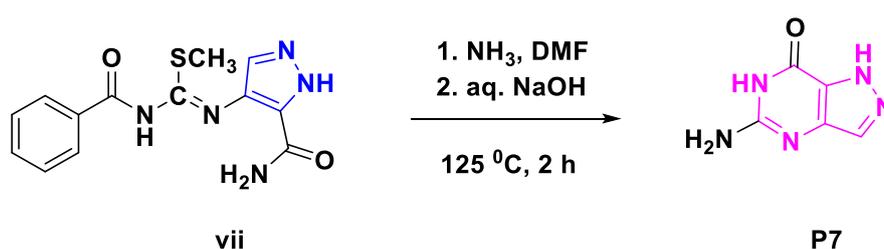
scheme-7
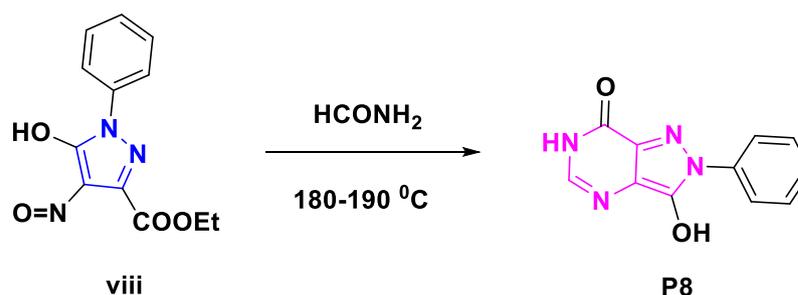
scheme-8

Fig. 4. (*Continued*). Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.

Acton *et al*. have obtained the desired product **P9**, by cyclization reaction of **ix** with ammonia (scheme-9).[53] Baraldi and co-workers synthesized **P10** by allowing the cyclization reaction between 4-amino-*N*-(4-chlorophenyl)-3-methyl-1*H*-pyrazole-5-carboxamide (**x**) and formamide (scheme-10).[17] Hamilton *et al*. have performed cyclization reaction between 4-amino-1,3-dimethyl-1*H*-pyrazole-5-carboxamide (**xi**) and

4-methylbenzoic acid to obtain **P11** in polyphosphoric acid (scheme-11).[31] Buchanan and co-workers have attained the desired product **P12** by cyclization of **xii** with ammonia in dimethylformamide (scheme-12).[54] Haddad *et al*. have succefully carried out the cyclization reaction of methyl 1,3-diphenyl-4-(3-phenylureido)-1*H*-pyrazole-5-carboxylate (**xiii**) with sodium ethoxide in ethanol under reflux conditions to achieve **P13** (scheme-13).[55] Dale and co-workers have performed cyclization reaction of 4-(2-ethoxybenzamido)-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide (**xiv**) in presence of sodium hydroxide as a base to afford **P14** (scheme-14).[56] El-abadelah *et al*. have reported cyclo-condensation reaction between 4-amino-1-methyl-5-propyl-1*H*-pyrazole-3-carboxamide (**xv**) and 2-ethoxybenzoic acid in polyphosphoric acid at 130-140 ^{0}C to obtain **P15** (scheme-15).[57] Yuan and co-workers attempted and achived the reaction between ethyl 4-amino-5-(2,4-dichlorophenyl)-1-methyl-1*H*-pyrazole-3-carboxylate (**xvi**) and benzyl thioacetimidate hydrobromide in pyridine to afford **P16** (scheme-16).[33]
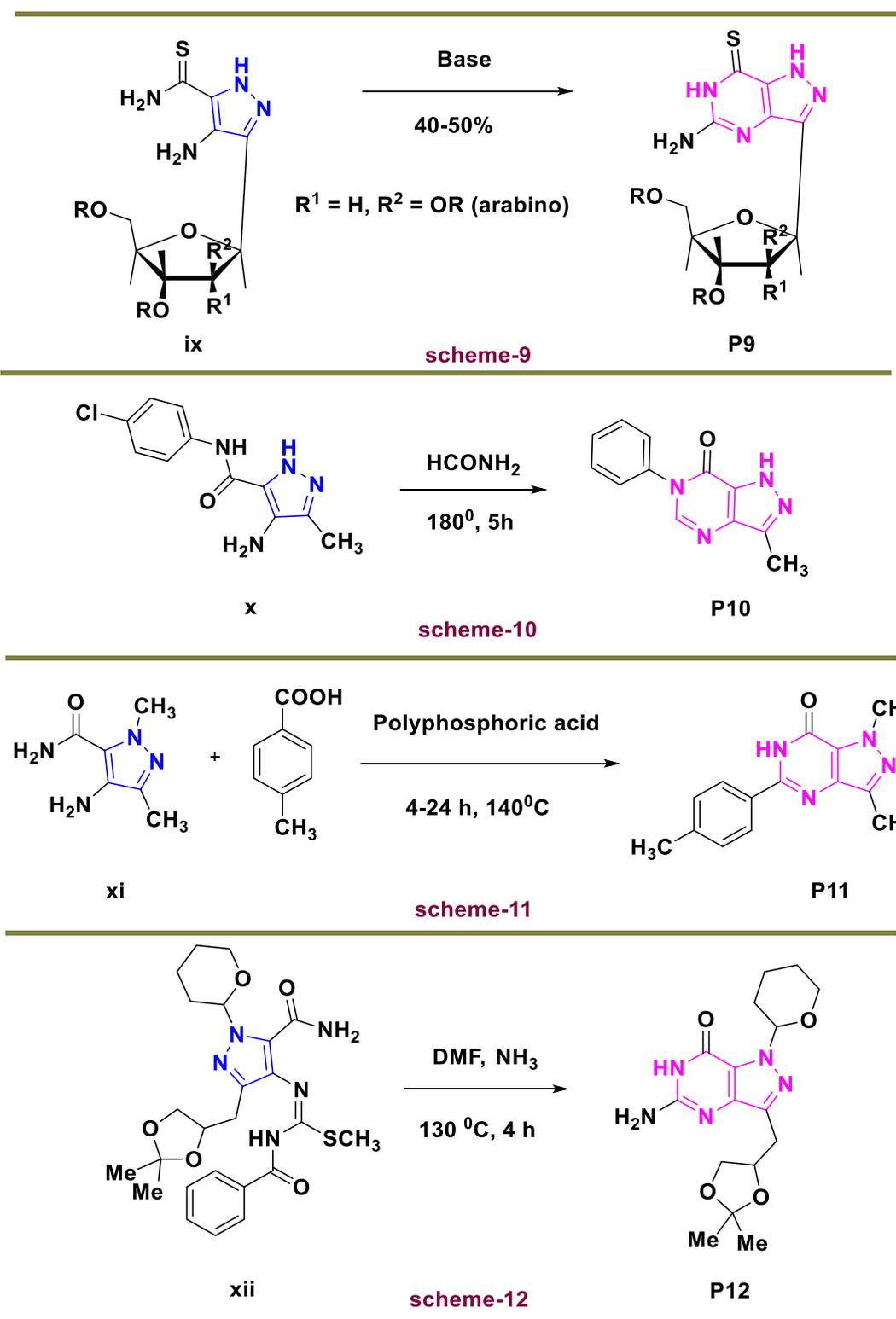
scheme-9

scheme-10

scheme-11

scheme-12

Fig. 4. (*Continued*). Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.

xiii　scheme-13　**P13**

xiv　scheme-14　**P14**

xv　scheme-15　**P15**

xvi　scheme-16
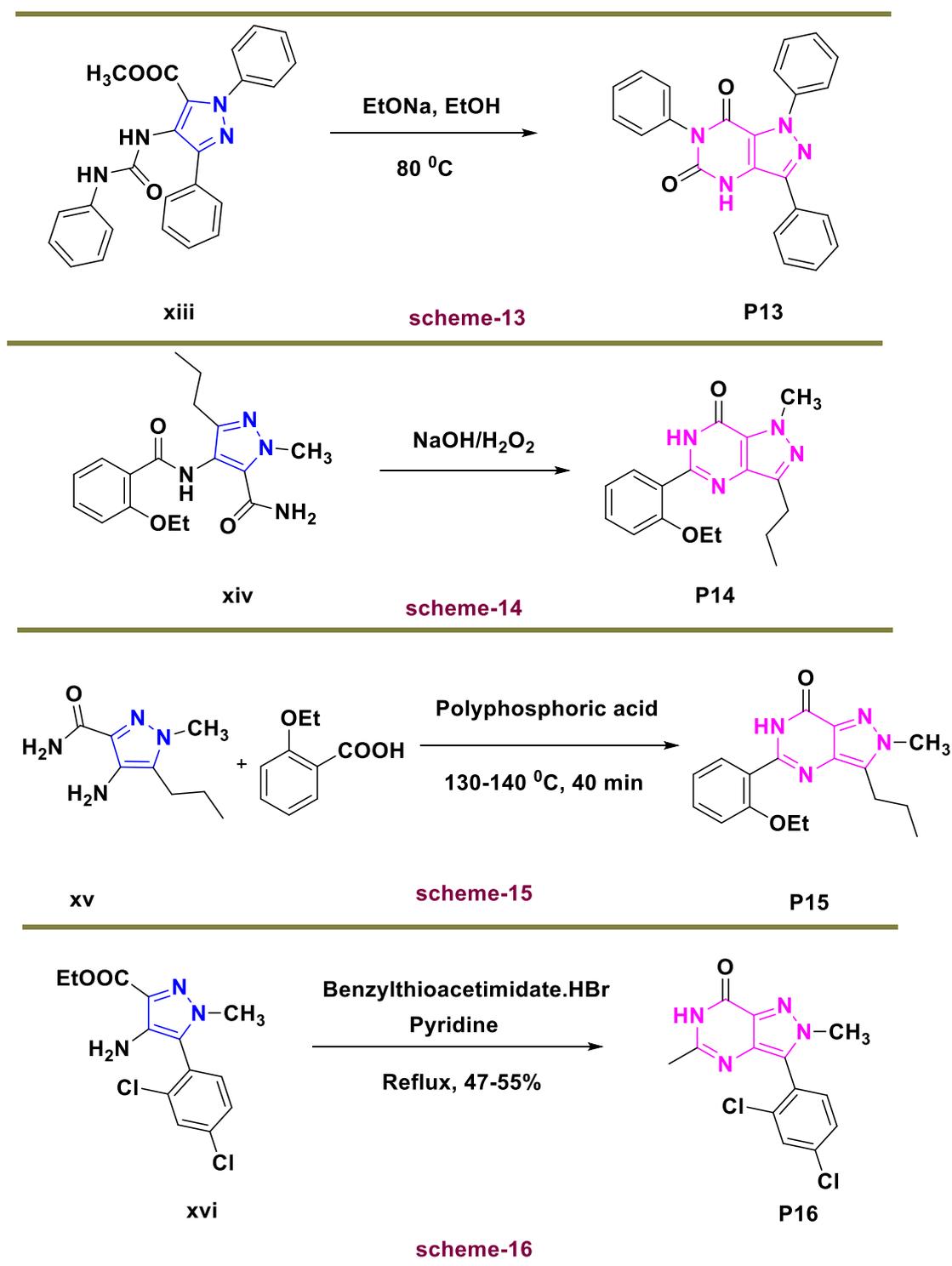
Fig. 4. (*Continued*). Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.

Moravcova and co-workers acquired the desired compound **P17** by the cyclization reaction of methyl 4-amino-3-isopropyl-1*H*-pyrazole-5-carboxylate (**xvii**) with formamidine acetate in presence of triethylamine (scheme-17).[14] Reddy *et al*. attempted the reaction between 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-

carboxamide (**xviii**) and benzaldehyde to attain **P18** in acetic acid containing catalytic amount of *p*-toluene sulfonic acid (scheme-18).[58] Khan and co-workers obtained **P19** via treating 4-(3-bromobenzamido)-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide (**xviv**) with basic alumina under microwave irradiation conditions (scheme-19).[59] Krystof *et al*. reported fusion reaction of 4-amino-3-isopropyl-1*H*-pyrazole-5-carboxamide (**xx**) with urea to achieve compound **P20** (scheme-20).[60] Brady and co-workers acquired **P21** by treating 4-amino-*N*-ethyl-1-methyl-3-phenyl-1*H*-pyrazole-5-carboxamide (**xxi**) with carbonyldiimidazole or other phosgene equivalent reagents (scheme-21).[61] Lenzi *et al*. attempted the cyclization reaction of ethyl 4-amino-1-(4-methoxyphenyl)-1*H*-pyrazole-3-carboxylate (**xxii**) with 1,1,1-triethoxyethane in presence of ammonium acetate to afford **P22** under microwave irradiation conditions (scheme-22).[62] Tollefson and co-workers carried out the cyclization reaction of 4-amino-3-ethyl-1-(2-(2,2,2-trifluoroethoxy)ethyl)-1*H*-pyrazole-5-carboxamide (**xxiii**) with carbonyldiimidazole to get target molecule **P23** (scheme-23).[34] Reddy *et al*. obtained the final compound **P24** by condensation reaction of methyl 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxylate (**xxiv**) with triethyl orthoformate in presence of aromatic amines (scheme-24).[63]
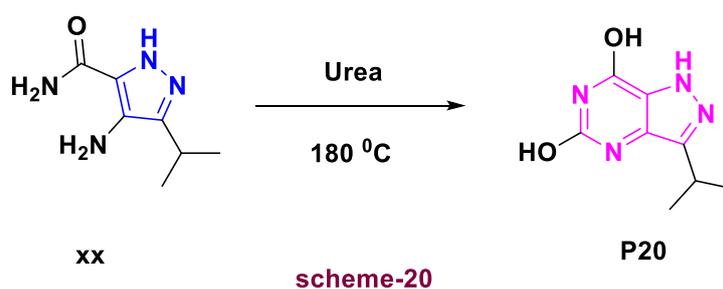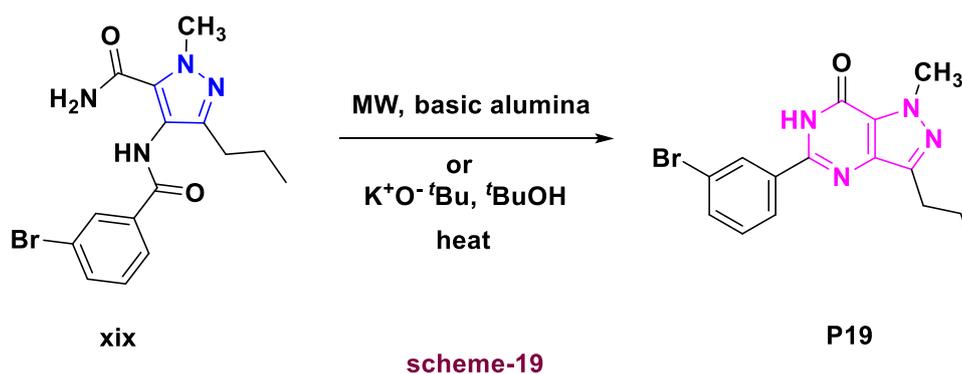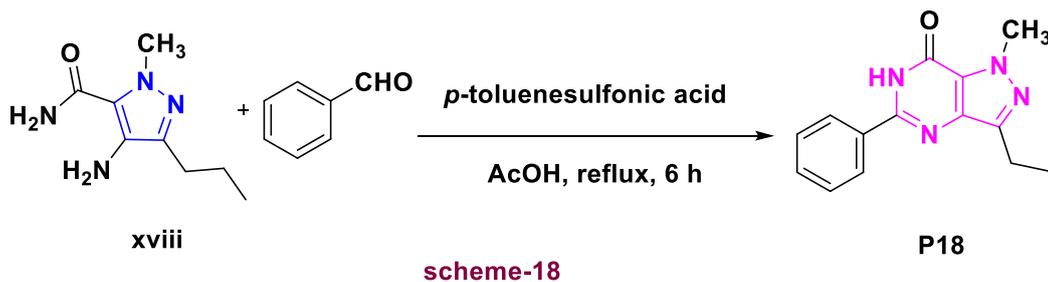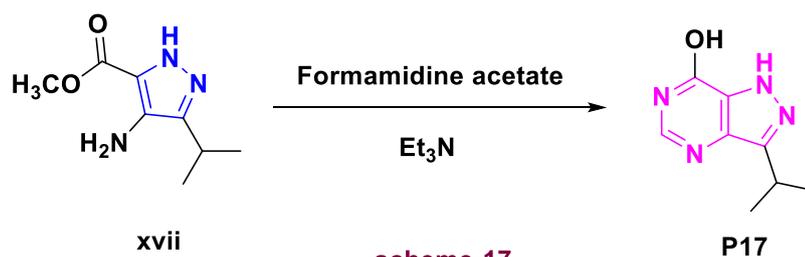
Fig. 4. (*Continued*). Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.

scheme-21

scheme-22

scheme-23

scheme-24
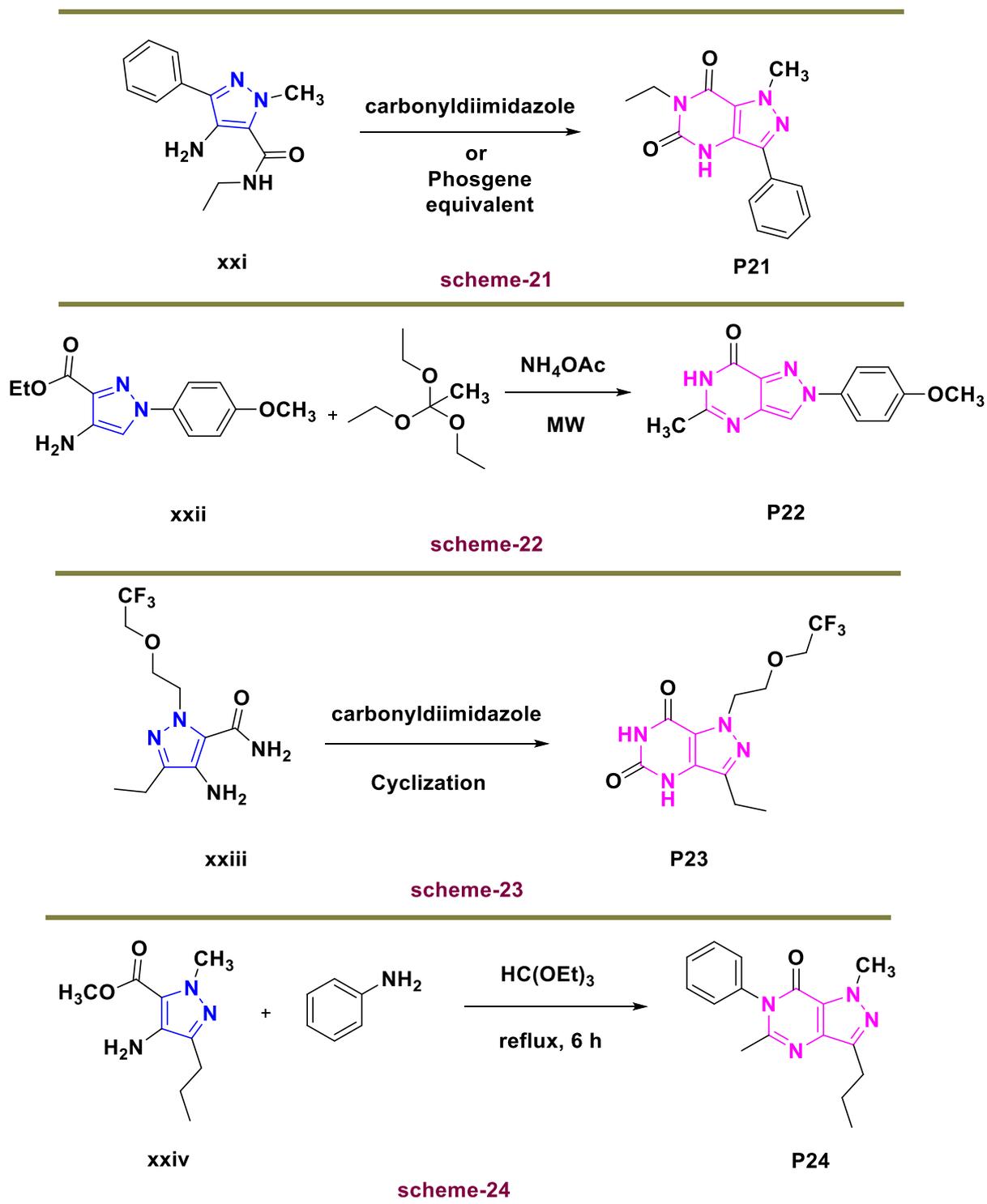
Fig. 4. (*Continued*). Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.

Jorda and co-workers established fusion reaction between 4-amino-3-isopropyl-1*H*-pyrazole-5-carboxamide (**xxv**) with thiourea under inert conditions to attain the desired product **P25** (scheme-25).[13] Geffken *et al*. offered synthesis of desired compound **P26** employing cyclization of ethyl 4-amino-3-phenyl-1*H*-pyrazole-5-carboxylate (**xxvi**) with formamidine acetate under reflux conditions (scheme-26).[64] Nayak

and co-workers blended 4-iodo-1,5-diphenyl-1*H*-pyrazole-3-carbaldehyde (**xxvii**) and acetamidine hydrochloride in presence of catalytic amount of copper iodide and cesium carbonate to achieve **P27** (scheme-27).[65] Bratenko and co-workers attempted intramolecular cyclization reaction of **xxviii** by treating with potassium hydroxide or potassium *tert*-butoxide in ethanol to afford **P28** (scheme-28).[66] Squarcialupi *et al*. established one pot synthesis of **P29** by reacting **xxix** (4-amino-1-(4-methoxyphenyl)-1*H*-pyrazole-3-carbonitrile) with triethyl orthobenzoate and ammonium acetate under microwave conditions (scheme-29).[67] Reddy and co-workers reported the cyclization reaction of 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide (**xxx**) with 2-ethoxybenzaldehyde to afford **P30** in presence of catalytic amount of potassium persulfate in H_2O:DMSO (1:1) under microwave irradiation conditions (scheme-30).[68] Squarcialupi *et al*. reacted 4-amino-1-phenyl-1*H*-pyrazole-3-carbonitrile (**xxxi**) with ethyl (*E*)-3-(4-methoxyphenyl)acrylimidate hydrochloride to attain **P31** in presence of ammonium acetate under microwave conditions (scheme-31).[69] Rote and co-workers have carried out the regioselective condensation of 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide (**xxxii**) with benzaldehyde in presence of acetonitrile and slight excess of molecular iodine to afford **P32** (scheme-32).[70]
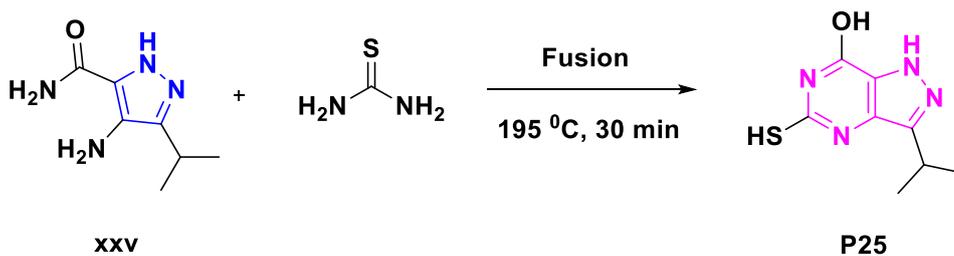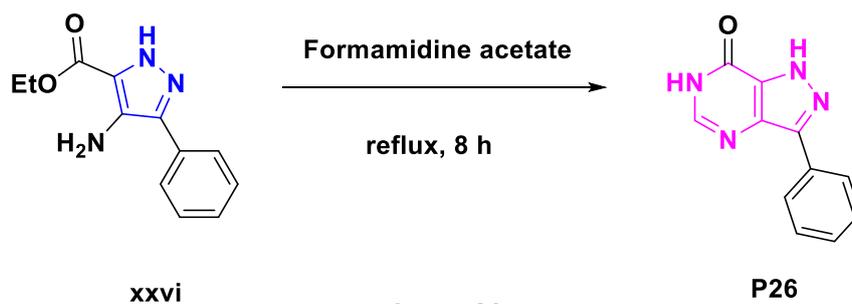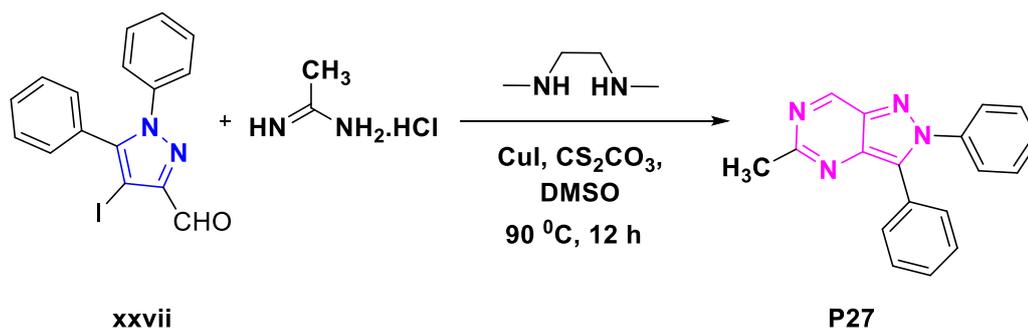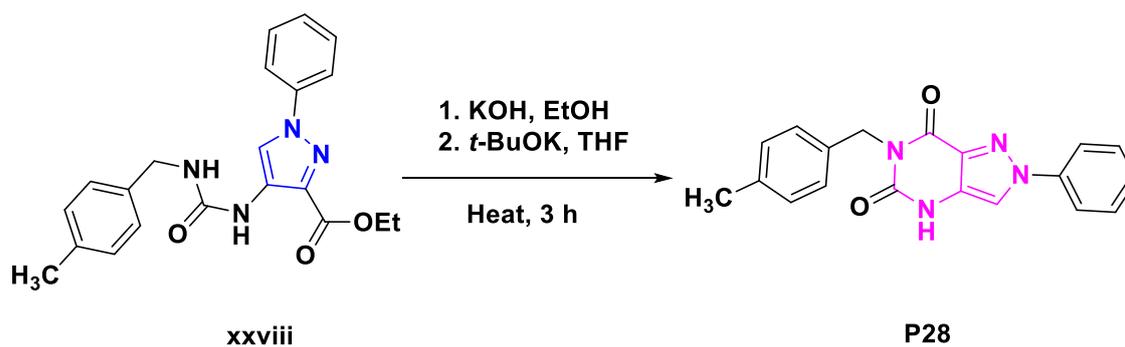
Fig. 4. (*Continued*). Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.
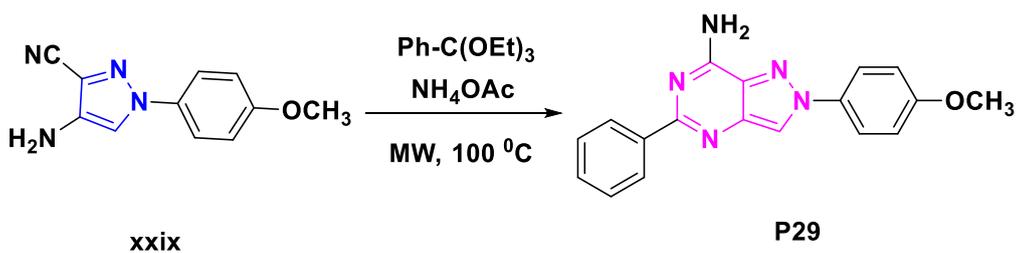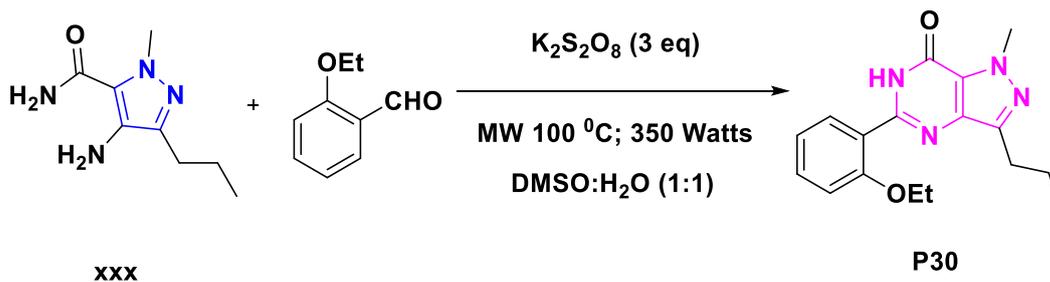
scheme-29

scheme-30
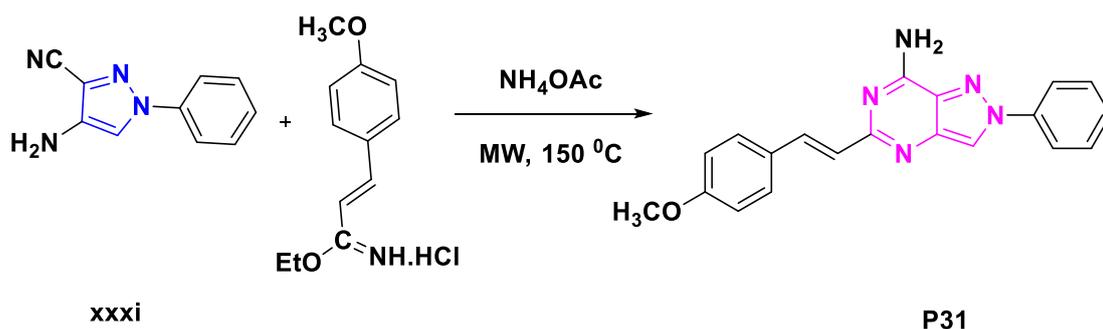
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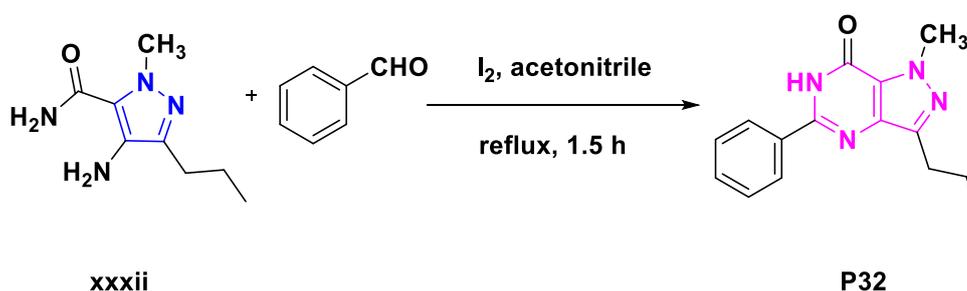
scheme-32

Fig. 4. (*Continued*). Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.

Mohammed and co-workers have treated 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide (**xxxiii**) and 1-(4-nitrophenyl)ethan-1-one to achieve **P33** in presence of catalytic amount of molecular iodine (scheme-33).[71] Hafez *et al*. have obtained **P34** by treating (*E*)-*N*-(5-(2-benzylidenehydrazine-1-carbonyl)-3-(4-chlorophenyl)-1*H*-pyrazol-4-yl)acetamide (**xxxiv**) with sodium ethoxide under reflux conditions (scheme-34).[35] Squarcialupi and co-workers have accomplished the target product **P35** by reacting 4-amino-1-(2-hydroxyphenyl)-1*H*-pyrazole-3-carbonitrile (**xxxv**) with ethyl iminoester hydrochloride (scheme-35).[72] Squarcialupi *et al*. treated methyl 4-amino-1-methyl-1*H*-pyrazole-3-carboxylate (**xxxvi**) with (triethoxymethyl)benzene to get target compound **P36** under microwave irradiation conditions (scheme-36).[73] Pepesch and co-workers have treated **xxxvii** with strong alkali (NaOH) to afford the desired molecule **P37** (scheme-37).[74] Senda *et al*. have accomplished the desired molecule **P38** by reacting 6-(bromomethyl)-1,3-dimethyl-5-nitropyrimidine-2,4(1*H*,3*H*)-dione (**xxxviii**) with methylamine in ethanol under reflux conditions (scheme-38).[75] Hirota and co-workers introduced cyclization of 1,3-dimethyl-5-nitro-6-((phenylamino)methyl)pyrimidine-2,4(1*H*,3*H*)-dione (**xxxix**) to achieve the desired molecule **P39** (scheme-39).[76]
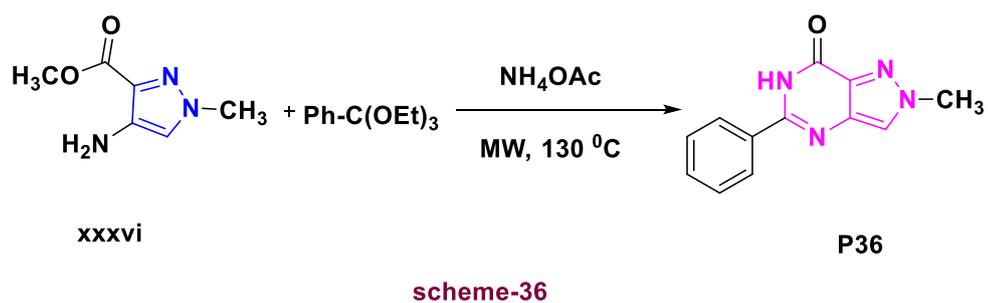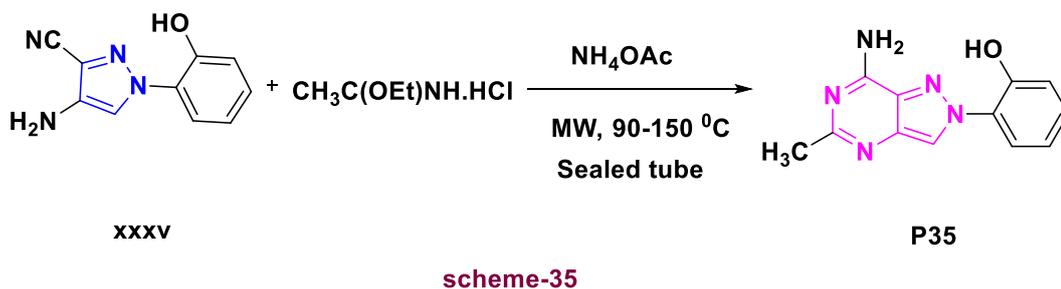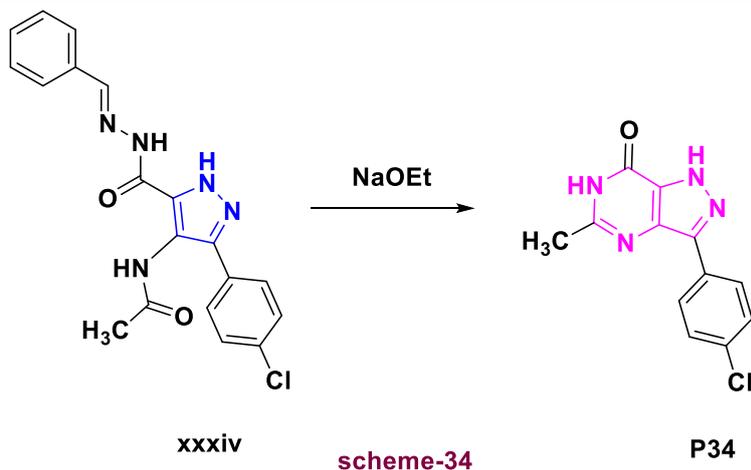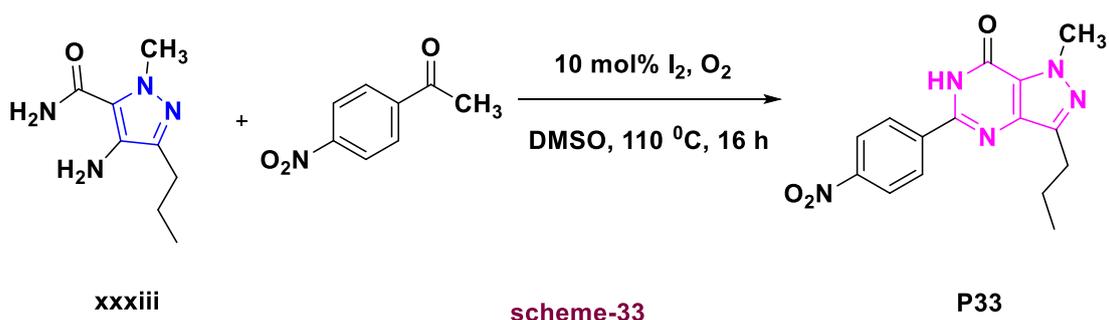
xxxiii

scheme-33

P33

xxxiv

scheme-34

P34

xxxv

P35

scheme-35

xxxvi

P36

scheme-36

Fig. 4. (*Continued*). Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.

scheme-37

scheme-38

scheme-39
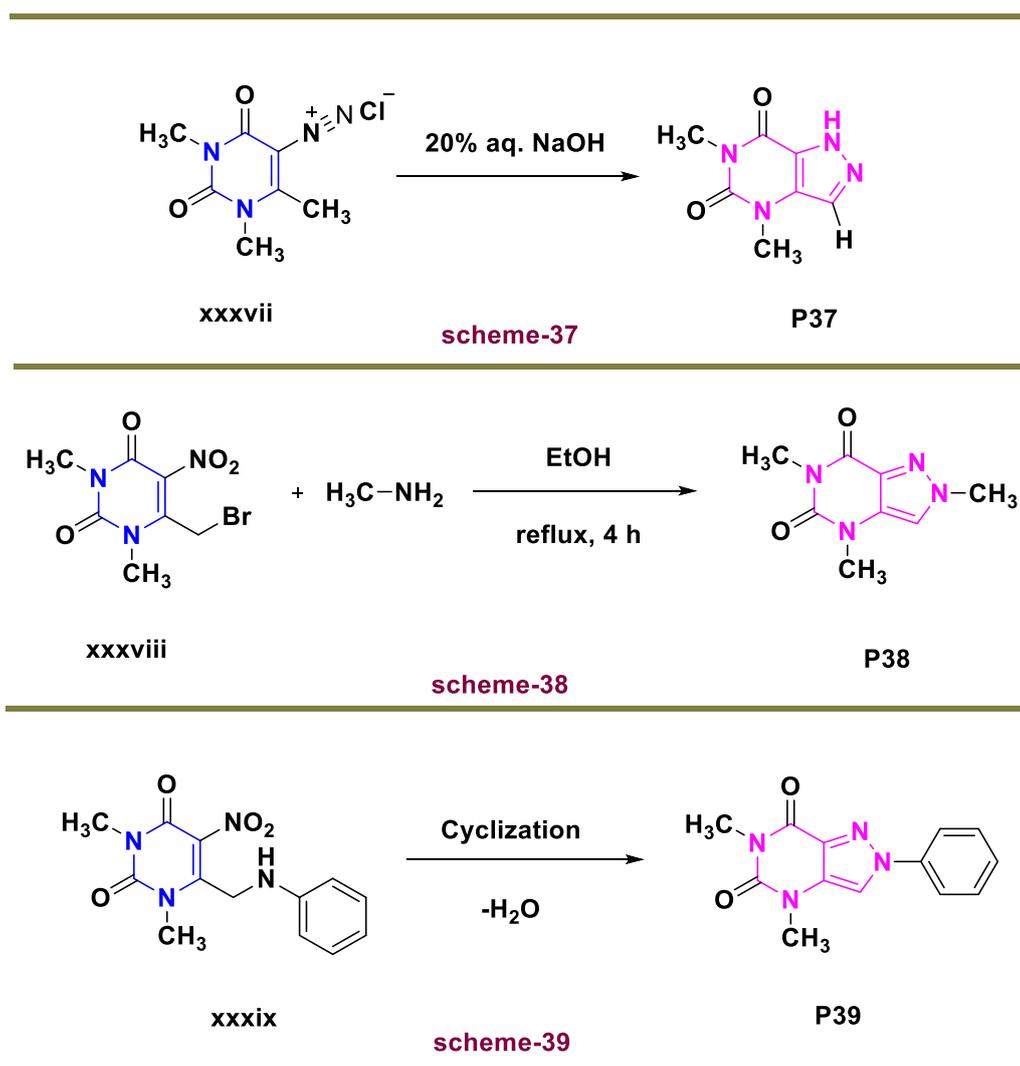
Fig. 4. (*Continued*). Synthetic strategies for pyrazolo[4,3-*d*]pyrimidines.

3 Biological activities

3.1 Anti-cancer agents

In 2003, Moravcova *et al*. attempted novel synthesis of 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines and evaluated their anticancer activity against CDK1/Cyclin B kinase and anti-proliferative activity against myeloid leukemia cell line (K-562). All the molecules exhibited good potency against both CDK1/Cyclin B kinase and K-562 cell line. From the series, compound **1** (2-(((3-isopropyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-7-yl)amino)methyl)phenol) showed maximum CDK1 inhibitory activity ($IC_{50} = 0.44 \, \mu moL/dm^3$) as well as K-562 cell line activity ($IC_{50} = 54 \, \mu moL/dm^3$) when compared to reference drug olomoucine (CDK1: $IC_{50} = 7 \, \mu moL/dm^3$; K-562: $IC_{50} = 163 \, \mu moL/dm^3$). SAR studies revealed that the presence of hydroxy benzyl functional group at C-7 was essential for both anticancer and anti-proliferative activity **Fig. 5**.[14]

Inclusion of 2-bromobenzyl group resulted in complete loss of activity

Introduction of hydroxy benzyl analogues significantly increased potency

Certain loss in activity was observed after substitution of the C-7 with effective cytokinin or anticytokinin side chains like furfuryl, isopent-2-en-1-yl and pentyl

R = 2-hydroxy benzyl, maximum CDK activity

C. No	R	CDK1/Cyclin B IC$_{50}$ (µM)	K-562 IC$_{50}$ (µM)
1	HO	0.44	54
Olomoucine		7	163

Fig. 5. SAR and anticancer properties of 2-(((3-isopropyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-7-yl)amino)methyl)phenol.

In 2006, Krystof and co-workers synthesized 3-isopropyl-N^5,N^7-bis(4-methoxybenzyl)-1*H*-pyrazolo[4,3-*d*]pyrimidine-5,7-diamine (**2**) as CDK1 inhibitor. Compound **2** was also tested against a panel of human cancer cell lines (A431, A549, BT474, CEM, G361, HBL100, HeLa, K562, MCF7 and many others) for their anti-proliferative activity. Of the tested cell lines, compound **2** showed significant potency against HeLa cell line. From SAR studies, it was revealed that amino linkage at C-5 and C-7 positions have greatly influenced the activity as depicted in **Fig. 6**.[60]

Amino linkage is crucial for activity
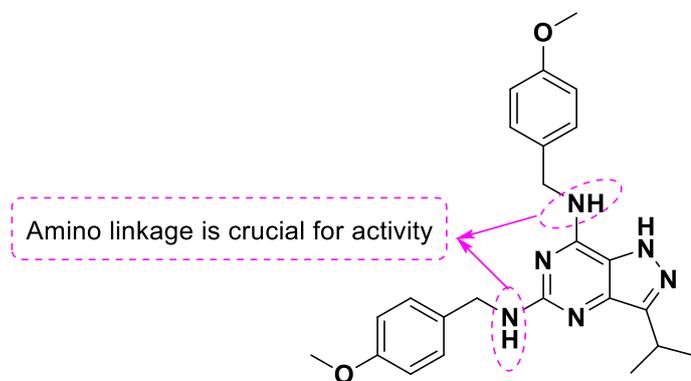
C. No	CDK1 IC$_{50}$ (μM)
2	4.1 ± 1.3

C. No	Cell lines	IC$_{50}$ (μM)
2	A431	6.9 ± 0.2
	A549	6.8 ± 0.4
	BT474	6.8 ± 0.2
	CEM	6.3 ± 0.6
	G361	6.7 ± 0.2
	HBL100	6.4 ± 0.1
	HeLa	5.2 ± 0.4
	K562	6.2 ± 0.2
	MCF7	6.4 ± 0.5

Fig. 6. SAR and antiproliferative activity of compound **2** on various cancer cell lines.

In 2011, Jorda and co-workers reported synthesis of (*R*)-2-((7-(benzylamino)-3-isopropyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl)amino)butan-1-ol (**3**) and determined its anti-proliferative activity against a panel of human cancer cell lines (MCF-7, HCT-116, RPMI-8226, CEM, G-361, A-549, A-431 and others). Further, compound **3** was found to have potent inhibitory activity against several CDKs, namely CDK2, CDK5, CDK7 and CDK9. **Fig. 7** briefly displays the anti-proliferative and CDK activity of compound **3**.[13]
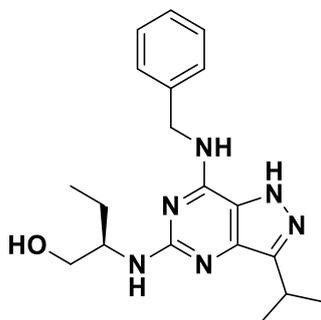
| C. No	Human tumor Cell lines	IC$_{50}$ (μM)
3	MCF-7	7.5 ± 2.3
	HCT-116	11.0 ± 1.8
	RPMI-8226	3.6 ± 0.3
	CEM	3.8 ± 0.8
	G-361	4.8 ± 1.6
	A-549	7.1 ± 2.1
	A-431	7.7 ± 0.3

C. No	IC$_{50}$ (µM)			
	CDK2/Cyclin E	CDK5/P35	CDK7/Cyclin H/MAT1	CDK9/Cyclin T1
3	0.04	0.20	0.16	1.00

Fig. 7. Anticancer activity on various cancer cell lines and kinase selectivity profile for compound **3**.

In 2013, Weitensteiner *et al*. observed potential anti-angiogenic activity of the newly synthesized 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidine derivatives by employing three *in vitro* assays (proliferation, cell migration and tube formation) and substantiated based on *n vivo* chorioallantoic membrane assay. The most active compounds were examined further for their kinase selectivity profile against a panel of 24 different kinases including other isoforms of CDK (**Fig. 8**). From the experimental data, compound **4** and **5** displayed high selectivity towards Cdk2 and Cdk5. Authors also investigated to understand the relationship between the CDK-5 inhibition and anti-angiogenic properties of **4** and **5** by quantifying lamellipodia formation followed by immunocytochemistry analysis. Thus, concluding that these two compounds (**4** and **5**) inhibited angiogenesis (*in vitro*) *via* CDK5 inhibitory mechanism.[77]
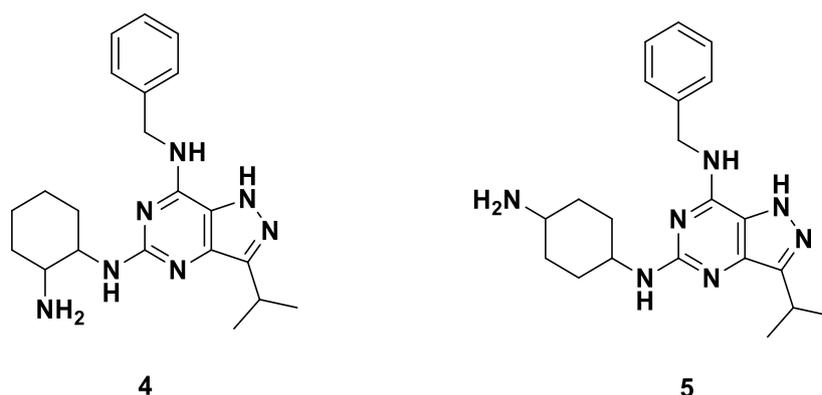
C. No	CDK1 IC$_{50}$	CDK2 IC$_{50}$	CDK4 IC$_{50}$	CDK5 IC$_{50}$	CDK6 IC$_{50}$	CDK7 IC$_{50}$	CDK9 IC$_{50}$
4	3.2×10^{-6}	9.9×10^{-7}	1.5×10^{-5}	4.4×10^{-7}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-6}$
5	5.8×10^{-6}	1.5×10^{-6}	6.6×10^{-6}	1.6×10^{-6}	9.1×10^{-5}	$> 10^{-4}$	1.9×10^{-6}

IC$_{50}$ values are given in molar.

Fig. 8. CDK kinase activity of lead compounds consisting trisubstituted pyrazolo[4,3-*d*]pyrimidine scaffold.

In 2014, Reddy and co-workers reported a microwave assisted synthesis of 5-substituted-1*H*-pyrazolo[4,3-*d*]pyrimidine-7(6*H*)-one analogs and evaluated their pharmacological activity against various human cancer

cell lines (HeLa, CAKI-1, PC-3, MiaPaca-2 and A-549). From this series, compound **6**, (5-(3,5-dimethoxyphenyl)-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one displayed potent activity against all cancer cell lines. SAR studies revealed that appropriate substitutions at C-5 influenced the potency as shown in **Fig. 9**.[68]
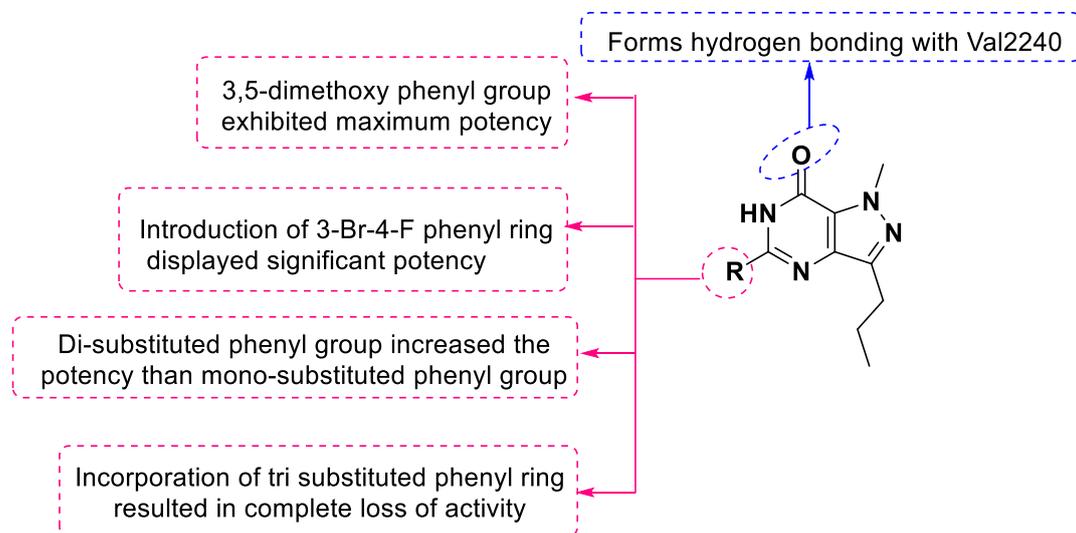
C. No	R	IC$_{50}$ (μM)				
		Hela	CAKI-1	PC-3	Miapaca-2	A-549
6		19	17	37	24	14

Fig. 9. SAR and anti-proliferative activity of compound **6** on various human cancer cell lines.

In 2015, Reznickova *et al*. reported the synthesis of 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidines and evaluated their CDK inhibitor activity. All the synthesized molecules having isopropyl group at C-3 position showed significant activity. From the series, compounds **7**, **8** and **9** exhibited potent CDK2 inhibitory activity and excellent cytotoxicity against MCF-7 and K-562 human cancer cell lines as represented in **Fig. 10**. From SAR studies, it was concluded that substitutions at C-5 and C-7 positions were necessary for both CDK2 and cancer activities.[78]
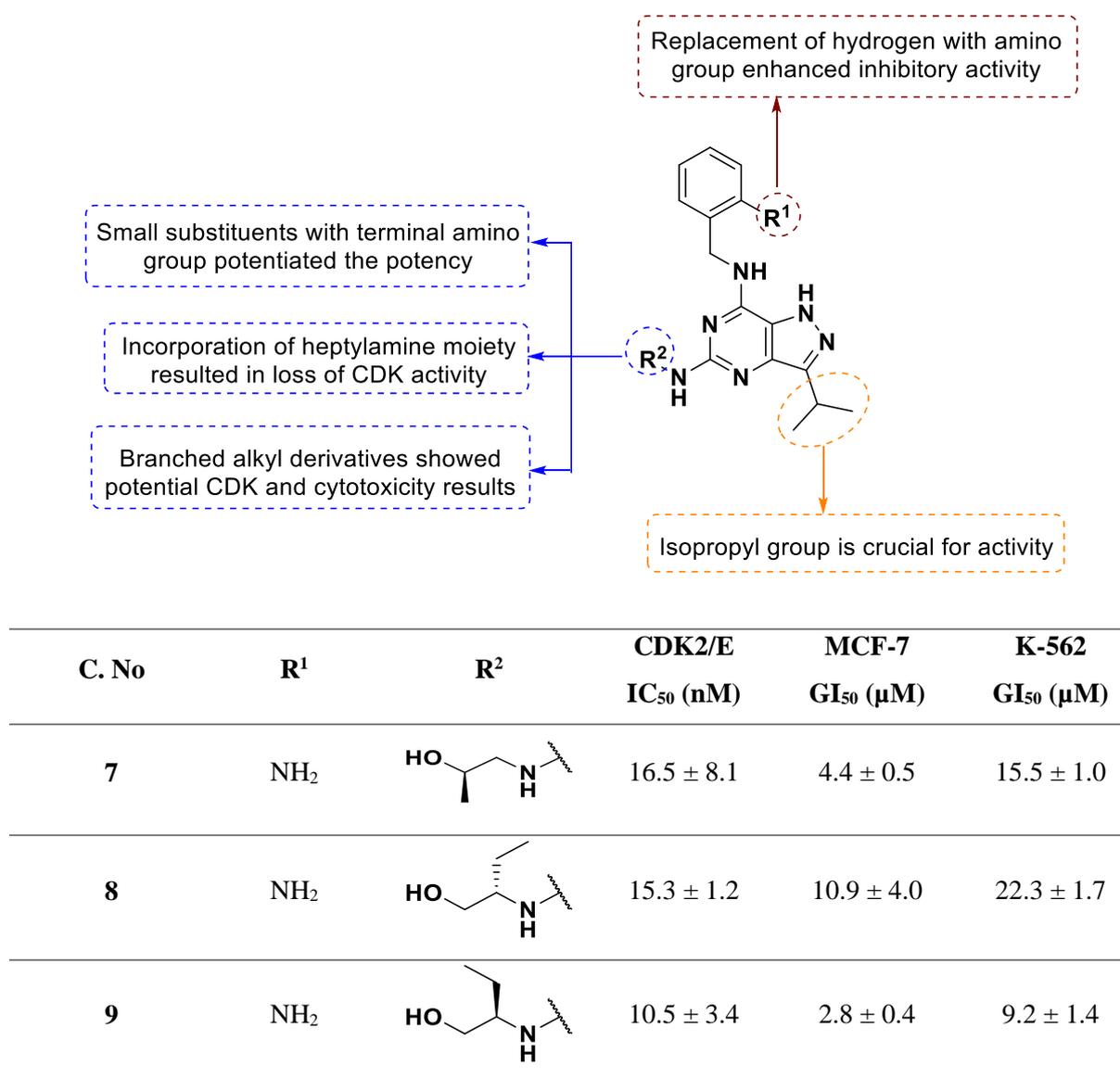
Fig. 10. Anticancer activity and SAR studies of 3,5,7-trisubstituted pyrazolo[4,3-*d*]pyrimidines.

C. No	R^1	R^2	CDK2/E IC$_{50}$ (nM)	MCF-7 GI$_{50}$ (µM)	K-562 GI$_{50}$ (µM)
7	NH$_2$		16.5 ± 8.1	4.4 ± 0.5	15.5 ± 1.0
8	NH$_2$		15.3 ± 1.2	10.9 ± 4.0	22.3 ± 1.7
9	NH$_2$		10.5 ± 3.4	2.8 ± 0.4	9.2 ± 1.4

In 2016, Vymetalova and co-workers reported the synthesis of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)*H*-pyrazolo[4,3-*d*]pyrimidines and evaluated for their CDK inhibition activity. SAR study revealed that compounds contained hydroxyalkylamines at C-5 exhibited maximum potency for both CDK2 and CDK5. Compounds were also tested against K-562, MCF-7, G-361 and HCT-116 cell lines and the activity results are shown in **Fig. 11**. From the synthesized series, compounds **10**, **11, 12** and **13** exhibited significant potency against CDK2 and CDK5 kinases, K-562, MCF-7, G-361 and HCT-116 cell lines. Further highly active compound **10** was screened against a panel of 50 protein kinases revealed its selectivity for CDKs.[79]
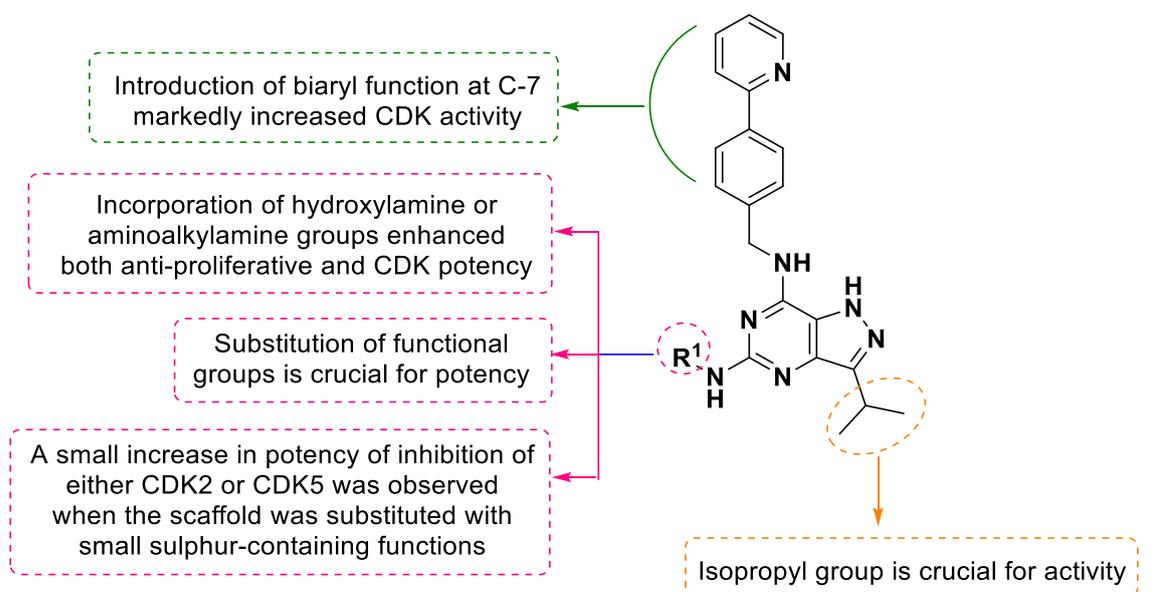
C. No	R^1	IC$_{50}$ (µM)					
		K-562	**MCF-7**	**G-361**	**HCT-116**	**CDK2**	**CDK5**
10	HO—C—CH₂—NH (structure)	0.029	0.024	0.048	0.085	0.009	0.001
11	HO—CH₂—CH(OH)—CH₂—NH (structure)	0.047	0.059	0.072	0.087	0.012	0.021
12	morpholine-N (structure)	0.110	0.163	0.197	0.270	0.018	0.005
13	H₂N—CH₂—CH₂—NH (structure)	0.063	0.062	0.230	0.227	0.018	0.008

Fig. 11. SAR study of 3-isopropyl-7-[4-(2-pyridyl)benzyl]amino-1(2)*H*-pyrazolo[4,3-*d*]pyrimidines as potent CDK inhibitors.

3.2   Anti-infectious agents

In 1984, Baraldi and co-workers reported the synthesis and antiviral activity of 1-*β*-D-ribofuranosyl-3-methyl-6-substituted-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-ones. From SAR studies, it was noted that significant activity was displayed for compounds with 4-methoxybenzyl and 4-chlorophenyl moieties at C-6 as shown in **Fig. 12**. From the series, compounds **14**, **15** and **16** exhibited moderate activity against herpes simplex virus type-1 (HSV-1) when compared to 5-iodo-2-deoxyuridine (IDU) as reference drug.[17]
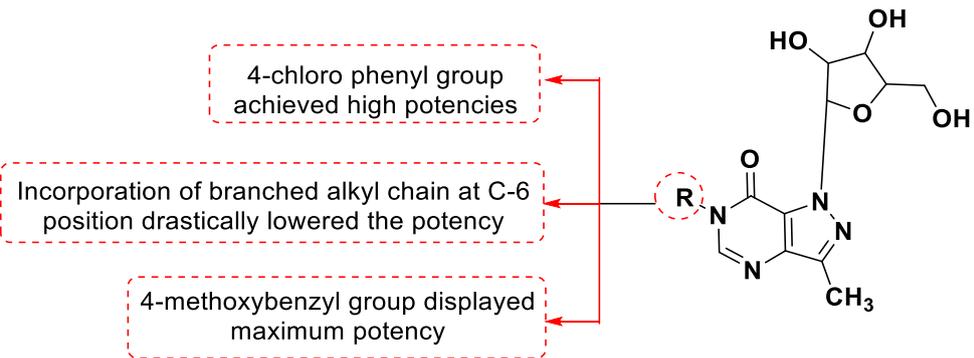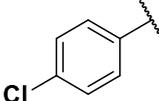
C. No	R	HSV-1 ED$_{50}$ (µM)
14		50
15		152
16		49.5
IDU		1.2
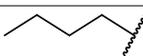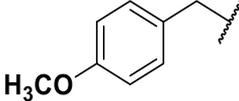
Fig. 12. Structures and anti-viral activity of 1-β-D-ribofuranosyl-3-methyl-6-substituted-7H-pyrazolo[4,3-d]pyrimidin-7-ones against HSV-1.

In 1991, Sanghvi *et al*. reported *in vivo* anti-viral activity of pyrazolo[4,3-d]pyrimidines and related guanosine analogues prepared from formycin. Three derivatives were prepared and tested for their ability to inhibit certain RNA and DNA viral replication by *in vitro* and semliki forest virus infection by *in vivo* methods. From the synthesized compounds, 5-amino-1-methyl-3-β-D-ribofuranosyl-pyrazolo[4,3-d]pyrimidin-7(6H)-one (**17**) exhibited favourable protection survivor against a lethal dose of semliki forest virus infection in mice model as compared to the standard drug. A brief SAR study and activity data have been represented in **Fig. 13**.[80]

Incorporation of methyl group on
nitrogen exhibited maximum activity

R = H, resulted in loss of activity
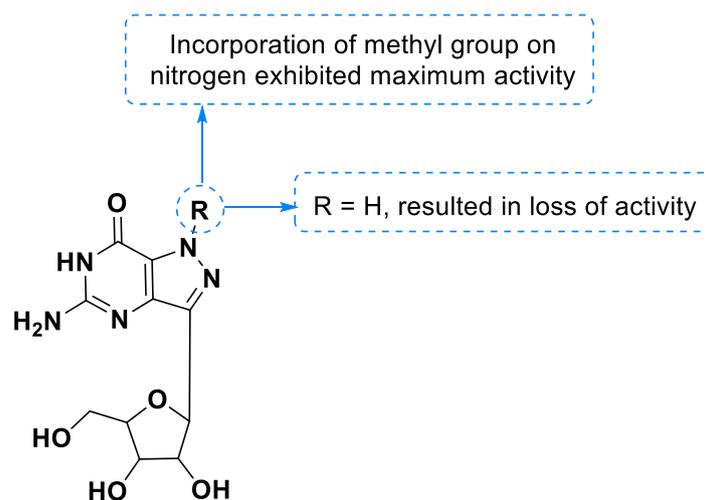
C. No	R	Dose[a] (mg/kg)	Survivors/total (%)[b]	Mean survival time[c]
		200	7/12 (58)	7 ± 0.8
		100	8/12 (67)	8 ± 0.8
17	CH₃	50	8/12 (67)	6 ± 0.8
		30	6/12 (50)	9.2 ± 2.9
		10	1/12 (8)	9.6 ± 2.4
7-thia-8-oxo-guanosine		100	11/12 (92)	8 ± 0.0

a: Half daily doses were administered intraperitoneally at 24 and 18 hours relative to virus inoculation; b: Statistically significant ($p < 0.025$) determined by the two-tailed fisher exact test; c: Dead mice.

Fig. 13. Anti-viral activity of 5-amino-1-methyl-3-*β*-D-ribofuranosyl-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one.

In 2011, Geffken and co-workers reported design and synthesis of pyrazolo[4,3-*d*]pyrimidin-4-ones and evaluated their pharmacological activity against bacterial and fungal strains. Of the screened compounds, 3-(4-bromophenyl)-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (**18**) exhibited moderate activity against *C. albicans* and inactive against other bacterial strains as compared to the standard drugs ampicillin and clotrimazole. SAR and antibacterial and fungal screening results are presented in **Fig. 14**.[64]
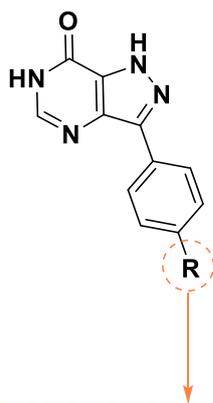
Bromo substitution exhibited favorable activity than chloro, fluoro groups

C. No	R	S. aureus (μg mL⁻¹)		E. Coli (μg mL⁻¹)		P. aeruginosa (μg mL⁻¹)		C. albicans (μg mL⁻¹)	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
18	**Br**	NA	NA	NA	NA	NA	NA	250	250
Clotrimazole		NA	NA	NA	NA	NA	NA	7.81	15.62
Ampicillin		3.9	15.62	7.81	31.25	250	500	NA	NA

NA: No activity.

Fig. 14. Anti-microbial values of 3-(4-bromophenyl)-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one against different bacterial and fungal strains.

In 2016, Hafez and co-workers synthesized a series of novel 6-amino-3-(4-chlorophenyl)-5-methyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]-pyrimidin-7-one derivatives and evaluated them for their antimicrobial properties. All the synthesized molecules exhibited potent activity against gram positive and gram negative bacterial and fungal strains, comparable to that of standard drug. Compounds were also tested against HT29, HePG2 and MCF-7 cancer cell lines. Compounds **19** and **20** showed prominent results for both antimicrobial and anti-cancer activity. **Fig. 15** represents the structures, SAR studies and biological properties of active compounds along with reference drugs.[35]

Incorporation of propyl-2-methoxyacetate resulted in reduction of activity

Introduction of 3,6-dihydropyran enhanced both microbial and anticancer activity

C. No	R	Bacterial Strains				Fungi MIC (μg mL^{-1})	
		Gram -ve ¥ZI(mm)/MIC (μg mL^{-1})		Gram +ve ZI(mm)/MIC (μg mL^{-1})			
		E. Coli	P. aeruginosa	S. lactis	S. aureus	C. albicans	A. flavus
19		12/50	10/35	15/35	18/40	22/-	25/-
20		14/40	14/55	20/40	24/40	18/-	24/-
Cefatoxime		26/13	18/10	30/12	31/15	-/-	-/-
Nystatin		-/-	-/-	-/-	-/-	25/20	30/25

-/-: No activity; ¥Results expressed as inhibition zone (ZI) diameter in mm./Minimal inhibitory concentration (MIC)

C. No	R	HT-29 IC$_{50}$ (μg mL^{-1})	HePG-2 IC$_{50}$ (μg mL^{-1})	MCF-7 IC$_{50}$ (μg mL^{-1})
19		1.52 ± 0.09	1.62 ± 0.11	1.77 ± 0.15
20		0.88 ± 0.04	0.42 ± 0.12	0.62 ± 0.03

**Fig. 15**. Antimicrobial and anti-cancer properties of 6-amino-3-(4-chlorophenyl)-5-methyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]-pyrimidin-7-one derivatives.

3.3. CNS Agents

3.3.1. *Phosphodiesterase-5 inhibitor activity*

In 2010, Tollefson et al. introduced a series of 1-(2-ethoxyethyl)-1*H*-pyrazolo[4,3-*d*]pyrimidines and determined their biological activity as potent phosphodiesterase-5 inhibitors (PDE5). From SAR study, it was revealed that the substitution of functional groups at C-3, C-5 and C-7 positions are necessary to achieve significant potency. From this series, compounds **21**-**24** exhibited significant potency, selectivity and efficacy against PDE5 as shown in **Fig. 16**. Further, the most active compound **24** of the series was incubated with human liver microsomes and observed that 87% of compound remained after 30 minutes time.[81]
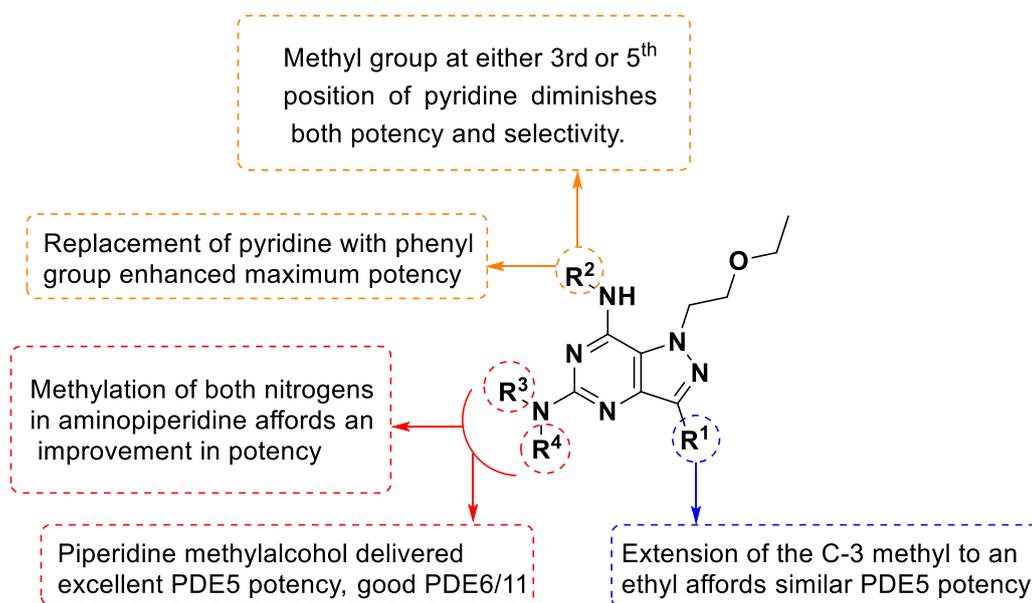
C. No	R^1	R^2	NR^{3}R^4	PDE5 IC$_{50}$ (nM)	PDE6/PDE5^a ratio	PDE11/PDE5^a ratio	HLM^b
21	Et	phenyl	piperazine	0.07	140×	160×	81%

22	Et		0.087	110×	540×	36%
23	Et		0.07	100×	120×	28%
24	Et		0.04	100×	530×	87%

a: Ratios of IC$_{50}$'s.; b: Human liver microsome stability, % compound remaining after 30 minutes.

Fig. 16. SAR and phosphodiesterase inhibitory abilities of active compounds.

In 2010, Tollefson *et al*. reported the synthesis of a series of 1-(2-(2,2,2-trifluoroethoxy)ethyl-1*H*-pyrazolo[4,3-*d*]pyrimidines as potent phosphodiesterase (PDE5/6/11) inhibitors. Among the series, compounds **25**-**28** displayed potent activity against PDE5. From SAR studies, it was revealed that the presence of trifluoroethoxyethyl group at N-1 position was necessary for significant PDE5 activity. The pharmacokinetic data of active compounds were examined by using dogs. Apart from these studies, authors also investigated *in vivo* model for efficacy in spontaneously hypertensive rats (SHR) to investigate the compound levels and blood pressure. Results of the active compounds and pharmacokinetic data are represented in **Fig. 17**.[34]
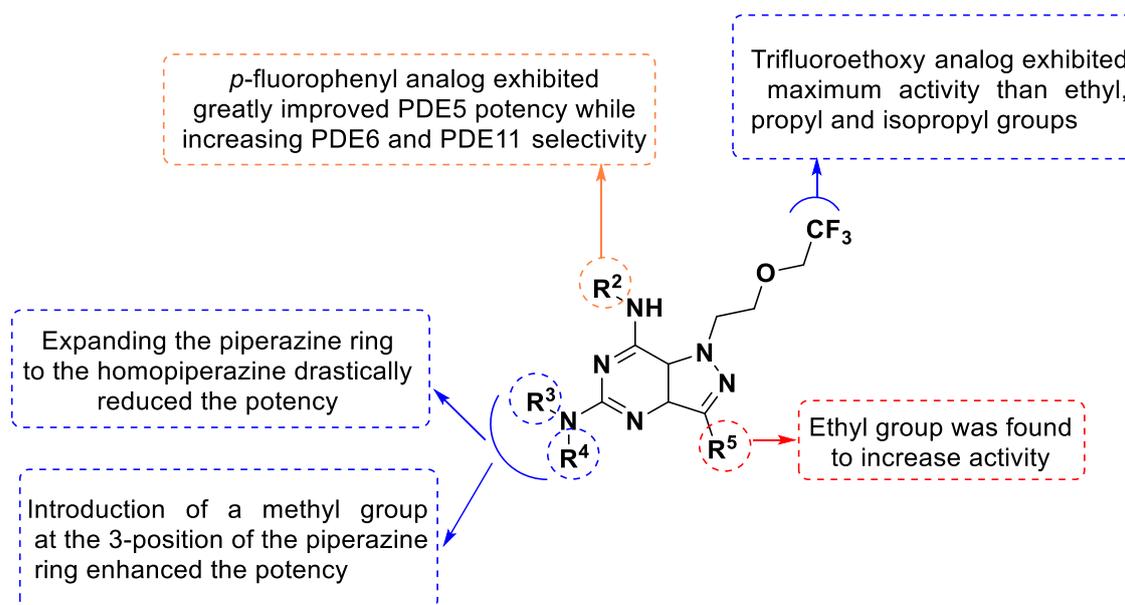
C. No	R²	NR³R⁴	R⁵	PDE5 IC$_{50}$ (nM)	PDE6/PDE5[a] ratio	PDE11/PDE5[a] ratio	HLM[b]
25			Me	0.78	420×	440×	83
26			Et	0.15	120×	2800×	96
27			Me	0.14	312×	14,700×	76 min[c]
28			Et	0.07	125×	27,000×	42 min[c]

a: Ratios of IC$_{50}$'s.; b: Human liver microsome stability, % compound remaining after 30 minutes; c: HLM half life.

C. No	Dofetilide[a] (%)	hERG[b] IC$_{50}$ (μM)	IV Dog PK[c]			SHR[d]
			$t_{1/2}$	Cl	V$_{dss}$	
25	40.0	5.6	ND	ND	ND	ND

| 26 | 10.7 | 5.1 | 6.7 | 20.2 | 8.0 | + |
| 27 | 24.7 | 9.0 | 4.9 | 22 | 9.4 | ++ |
| 28 | 18.8 | 1.4 | 5.1 | 18.5 | 7.8 | ++ |

a: Percent inhibition of [3H]-dofetilide binding to the hERG protein stably expressed on HEK-293 cells following a 10 lM dose of test compound; b: hERG patch clamp electrophysiology assay, IC_{50}; c: Compound dosed at 0.2f–0.5e mpk in 10 kg beagles. Halflife ($t_{1/2}$) in h, clearance (Cl) in mL/min/kg, volume of distribution (V_{dss}) in L/kg; d: Compound dosed orally in spontaneously hypertensive rats (SHR) while monitoring MAP, + = decrease of 10–15 mmHg, ++ decrease of >15 mmHg.

Fig. 17. SAR and pharmacokinetic properties of piperazine linked pyrazolo[4,3-*d*]pyrimidines as potent phosphodiesterase inhibitors.

In 2015, Choudhari *et al*. reported the three dimensional quantitative structure-activity relationship (3D-QSAR), pharmacophore identification studies on a series of 1-(2-ethoxyethyl)-1*H*-pyrazolo[4,3-*d*]pyrimidines as PDE5 inhibitors. Two different QSAR models (multiple linear regression analysis and kNN-MFA analysis) were used to carry out identification studies on 32 molecules and observed that both techniques showed similar results. From the obtained results, they concluded kNN-MFA technique can be utilized for cross validation of the results of multiple linear regression studies. A brief SAR and QSAR results of compounds **29**-**35** are presented in **Fig. 18**.[82]
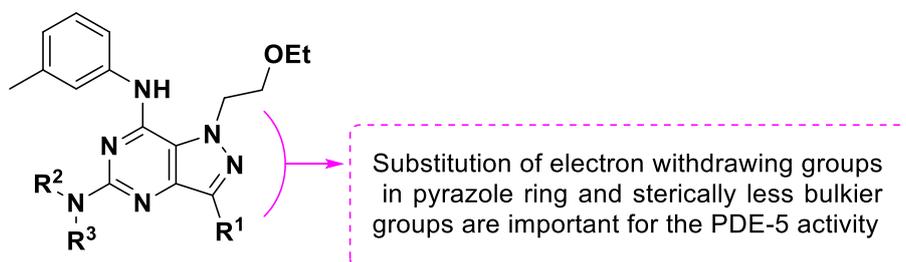
C. No	R¹	NR²R³	3D QSAR by MLRᵃ			D QSAR by kNN-MFAᵇ		
			Obs. Act§	Pre. Act€	Res.	Obs. Act	Pre. Act	Res.
29	Me	*(piperazine structure)*	-0.41497	-0.50069	0.085718	-0.41497	-0.34802	-0.06696
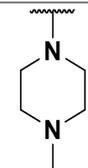
|								
30	Et		0.05061	0.043787	0.006823	0.05061	-0.07732	0.127927
31	Et		-0.39794	-0.12422	-0.27373	-0.39794	-0.35668	-0.04126
32	Et		-0.07918	-0.03801	-0.04117	-0.07918	-0.2384	0.159214
33	Et		0.119186	0.098904	0.020282	0.119186	-0.08439	0.203571
34	Me		-0.36173	-0.27329	-0.08844	-0.36173	0.03908	0.40075
35	Et		0.69897	0.87657	-0.1776	0.69897	0.47816	0.220824
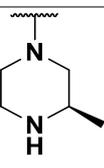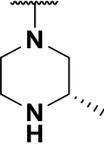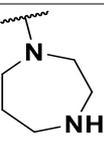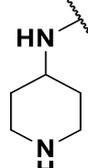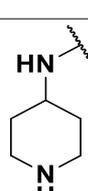
a: Multiple linear regression; b: k-nearest neighbour molecular field analysis; §: Observed activity; €: previous activity.

Fig. 18. 3D-QSAR studies of 1-(2-ethoxyethyl)-1*H*-pyrazolo[4,3-*d*]pyrimidines as PDE5 inhibitors.

In 2016, Rabal and co-workers described the synthesis and biological evaluation of pyrazolo[4,3-*d*]pyrimidines as first-in-class dual acting histone deacetylases (HDACs) and PDE5 inhibitors for the treatment of Alzheimer's disease. Role of functional groups at C-5 position in achieving potent inhibitory activity against PDE-5, HDAC-2 and HDAC-6 has been elaborated in **Fig. 19**. From the tested series, compounds **36-39** showed promising activity with IC_{50} values ranging from 0.5 to 60 nM against PDE-5, while the hit compound **37** exhibited PDE-5 inhibition at 3 nM. The identified lead compound **39** demonstrated higher selectivity for HDAC-6 (IC_{50} = 91 nM) over other isoforms of HDAC.[83]

Incorporation of *N*-hydroxy-3-cyclo butane-1-carboxamide moiety through alkyl linker resulted in good activity and selectivity against both PDE-5, HDAC-6

Observed more flexibility between sildenafil core and hydrophobic linker by introducing secondary amine as an attachment point

Pyrimidyl groups presented high potency than phenyl groups

Replacement of the sulfonamide group tended to result in a slight decrease in the potency for PDE-5
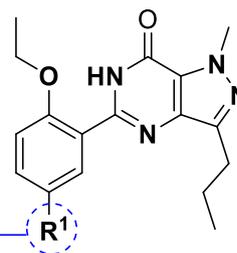
| C. No	R^1	IC$_{50}$ (nM)				
		PDE-5	HDAC-1	HDAC-2	HDAC-3-NCOR-2	HDAC-6
36		3	8	117	36	268
37		3	10500	>20000	NA	2360
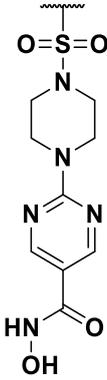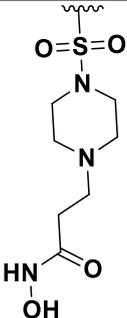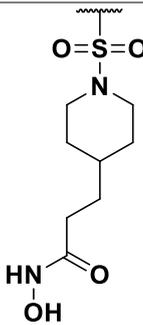
38		0.5	406	1940	NA	87
39		60	310	490	322	91
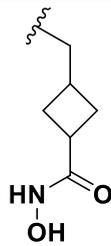
NA: No activity; HDACs: dual acting histone deacetylases.

Fig. 19. Structures, biological results of active compounds as potent PDE5 inhibitors.

### 3.3.2. Adenosine receptor antagonist's activity

In 1987, Hamilton and co-workers reported synthesis of 1,3-dialkylpyrazolo[4,3-*d*]pyrimidin-7-ones and evaluated their pharmacological properties for adenosine receptor antagonists. From the synthesized series, compounds **40**-**42** exhibited excellent potency towards adenosine A_1 receptors. SAR studies indicated that *N*-(2 (dimethylamino)ethyl) benzenesulfonamide group at C-5 and methyl group at C-3 position was responsible for higher activity as depicted **Fig.20**.[31]
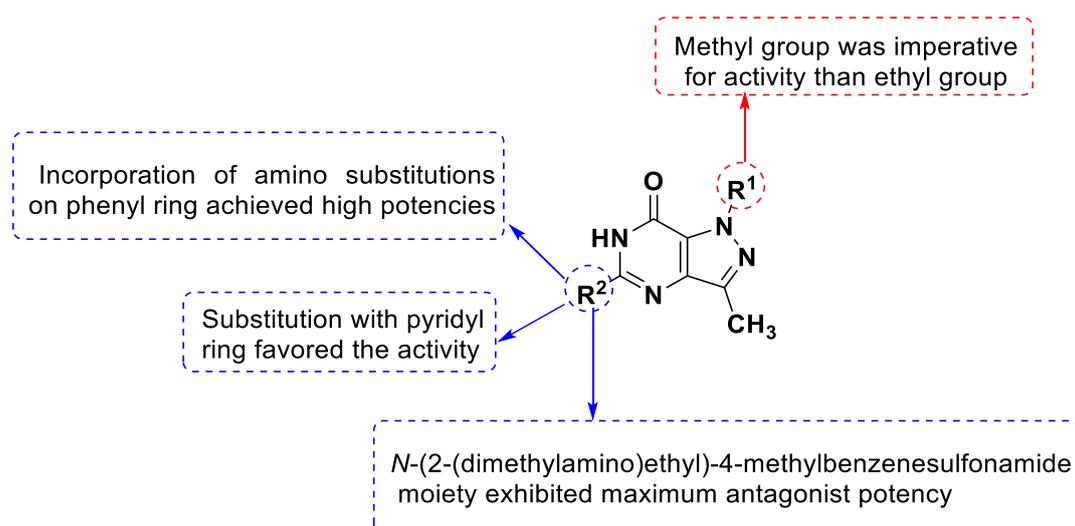
C. No	R^1	R^2	Antagonist activity IC$_{50}$ (nM)
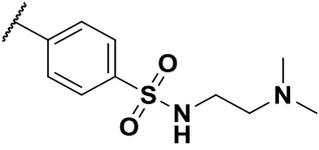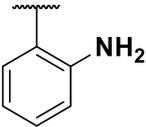
| 40 | CH$_3$ | | 68 |
| 41 | CH$_3$ | | 230 |
| 42 | C$_2$H$_5$ | 4-pyridyl | 250 |

Fig. 20. SAR and adenosine receptor antagonist activity of 1,3-dialkyl pyrazolo[4,3-*d*]pyrimidin-7-ones.

In 2009, Lenzi and co-workers reported synthesis and biological evaluation of 2-phenyl pyrazolo[4,3-*d*]pyrimidines as potent and selective human A$_3$ adenosine receptor antagonists. SAR studies indicated that presence of small lipophilic substituent at C-5 position was important for significant activity. From series, compound **43** [2-(4-methoxyphenyl)-5-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one] exhibited potent activity as represented in **Fig. 21**.[62]
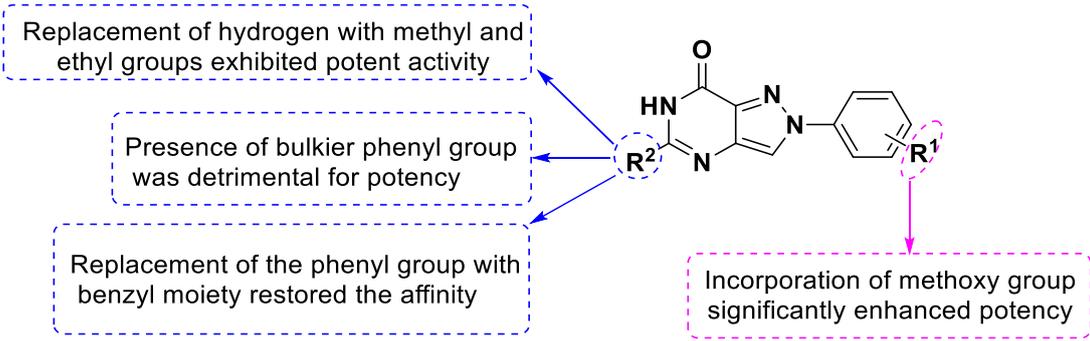
| C. No | R^1 | R^2 | Binding experiments K_i (nM) | | | cAMP assays IC$_{50}$ (nM) | |
			hA$_3$[a]	hA$_1$[b]	hA$_{2A}$[c]	hA$_{2B}$[d]	hA$_3$[e]
43	4-OCH$_3$	CH$_3$	1.2 ± 0.1	5%	1%	2%	5.2 ± 0.5

a: Displacement of specific [^{125}I]AB-MECA binding to hA$_3$ CHO cells, where K_i values are mean values (SEM of four separate assays each performed in duplicate. Percentage of inhibition in [^{125}I]AB-MECA competition binding assays to hA$_3$CHO cells at 1μM tested compounds; b: Percentage of inhibition in [^{3}H]DPCPX competition binding assays to hA$_1$CHO cells at 1 μM tested compounds; c: Percentage of inhibition in [^{3}H]ZM241385 competition binding assays to hA$_{2A}$CHO cells at 1μM tested compounds; d: Percentage of inhibition on cAMP experiments in hA$_{2B}$CHO cells, stimulated by 200 nM NECA, at 1 μM examined compounds; e: IC$_{50}$ values are expressed as mean values (SEM of four separate cAMP experiments in hA$_3$CHO cells, inhibited by 100 nM Cl-IB-MECA.

Fig. 21. SAR and human A$_3$ adenosine receptor antagonist activity of 2-(4-methoxyphenyl)-5-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one.

In 2013, Squarcialupi *et al.* reported synthesis, molecular modelling and pharmacological evaluation of 2-arylpyrazolo[4,3-*d*]pyrimidin-7-amino derivatives as human A_3 adenosine receptor antagonists. SAR studies suggested that *para*-methoxy group on 2-phenyl ring at C-2 position, lipophilic groups at C-5 and acyl groups at C-7 position are essential for significant potency. From the set of series synthesized, compounds **44-46** exhibited good activity. A brief SAR along with active most compounds are presented in **Fig. 22**.[84]
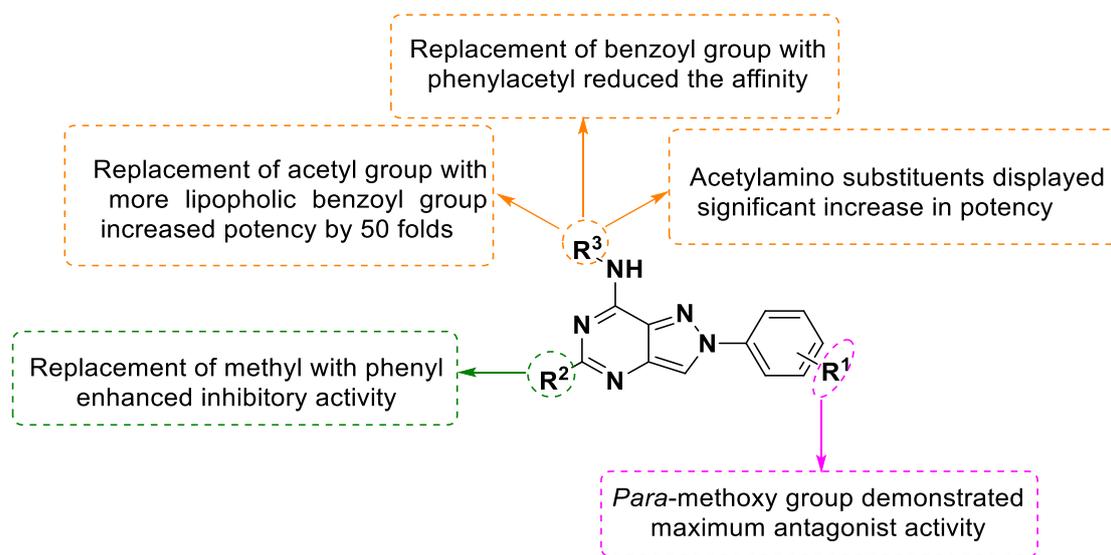
| C. No | R^1 | R^2 | R^3 | Binding experiments[a] | | | | cAMP assays | |
| | | | | K_i (nM) or I% | | | | IC_{50} (nM) | |
				hA_1[b]	hA_{2A}[c]	hA_3[d]	rA_3[e]	hA_{2B}[f]	hA_3[g]
44	H	CH_3	$CO-C_6H_4-OCH_3$	4%	1%	2.4 ± 0.2	NIL	1%	NIL
45	H	CH_3	CO-Ph	30%	1%	5.6 ± 0.5	6%	2%	2.4 ± 0.2
46	OCH_3	Ph	CO-Ph	3%	1%	18 ± 2	12%	2%	63 ± 5

a: K_i values are means $\pm$ SEM of four separate assays each performed in duplicate. Percentage of inhibition (*I*%) is determined at 1 µM concentration of the tested compounds; b: Displacement of specific [^{3}H]DPCPX competition binding assays to hA_1CHO cells; c: Displacement of specific [^{3}H]ZM241385 competition binding to hA_{2A}CHO cells; d: Displacement of specific [^{125}I]AB-MECA competition binding to hA3CHO cells; e: Percentage of inhibition (I%) in [^{125}I]AB-MECA competition binding assays to rA3HEK cells. fcAMP experiments in hA_{2B}CHO cells, stimulated by 200 nM NECA. IC_{50} values are expressed as means $\pm$ SEM of four separate cAMP experiments. Percentage of inhibition (*I*%) is determined at 1 µM concentration of the tested compounds; g: IC_{50} values are expressed as means $\pm$ SEM of four separate cAMP experiments in hA3CHO cells, in the presence of 100 nM Cl-IB-MECA.

Fig. 22. Structures, binding results of 7-amino-2-phenylpyrazolo[4,3-*d*]pyrimidines as A_3 adenosine receptor antagonists.

In 2014, Squarcialupi *et al*. reported molecular modeling and biological properties of 7-amino-2-phenylpyrazolo[4,3-*d*]pyrimidines as A_1 and A_{2A} adenosine receptors. SAR studies revealed that affinity and selectivity were based on the nature of the functional groups at C-5 position as depicted in **Fig. 23**. In this series, compounds **47**-**52** exhibited potent activity against both human A_1 and A_{2A} adenosine receptors.[69]


| C. No | R[1] | Binding experiments[a] | | | cAMP assays |
		K_i (nM) or I%			IC_{50} (nM) or I%
		hA_1^b	hA_{2A}^c	hA_3^d	hA_{2B}^e
47	2-F-C_6H_4-CH_2-	35 ± 4	154 ± 13	37%	123 ± 11
48	Ph-$(CH_2)_3$	5.31 ± 0.42	55 ± 5	12%	42%
49	2-OMe-C_6H_4-$(CH_2)_3$	1.31 ± 0.15	1.53 ± 12	47%	387 ± 36
50	3-OMe-C_6H_4-$(CH_2)_3$	0.15 ± 0.02	147 ± 13	33%	334 ± 27
51	2-OH-C_6H_4-$(CH_2)_3$	0.54 ± 0.05	102 ± 9	36%	247 ± 18
52	3-OH-C_6H_4-$(CH_2)_3$	0.22 ± 0.03	146 ± 15	46%	314 ± 26

a: K_i values are means ± SEM of four separate assays each performed in duplicate. Percentage of inhibition (I%) are determined at 1 µM concentration of the tested compounds; b: Displacement of specific [³H]DPCPX competition binding assays to hA_1CHO cells; c: Displacement of specific [³H]ZM241385 competition binding to hA_{2A}CHO cells; d: Displacement of specific [¹²⁵I]AB-MECA competition binding to hA_3CHO cells; e: cAMP experiments in hA_{2B}CHO cells, stimulated by 200 nM NECA. IC_{50} values are expressed as means ± SEM of four separate cAMP experiments. Percentage of inhibition (I%) are determined at 1 µM concentration of the tested compounds.

Fig. 23. SAR and A_1 and A_{2A} adenosine receptor activity of pyrazolo[4,3-*d*]pyrimidines.

In 2016, Squarcialupi *et al*. reported structural refinement of pyrazolo[4,3-*d*]pyrimidine derivatives to obtain potent and selective antagonists for the human A_3 adenosine receptors. SAR studies presented the significance of phenyl group at C-2, 2-thienyl moiety at C-5 and acyl groups at C-7 positions for favorable activity as illustrated in **Fig. 24**. However, outcome of this work concluded that compounds **53**-**55** exhibited potent activity against A_3 adenosine receptors.[73]

Incorporation of benzyl, phenyl and 2-furoyl groups depleted the potency

4-methoxyphenyl and pyridyl groups exhibited significant potency

Phenyl group is crucial for activity

Introduction of methyl and benzyl groups depleted the potency

Thienyl group achieved high potencies than phenyl, methyl and benzyl groups
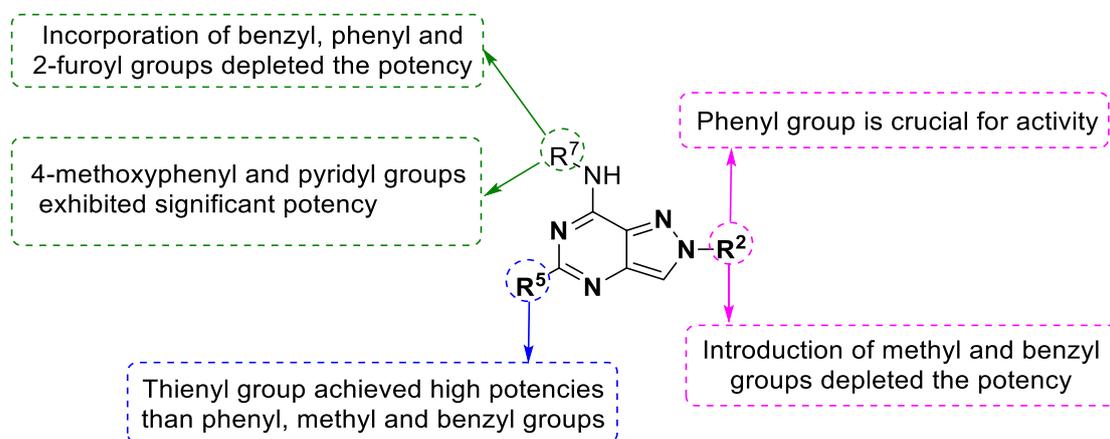
C. No	R²	R⁵	R⁷	Binding experiments[a] K_i (nM) or I%			cAMP assays IC_{50} (nM) or I%	
				hA₁[b]	hA₂ₐ[c]	hA₃[d]	hA₂ᵦ[e]	hA₃[f]
53	Ph	2-thienyl	C₆H₄-OMe	1%	1%	0.027 ± 0.003	1%	0.11 ± 0.01
54	Ph	2-thienyl	3-pyridyl	764 ± 68	3%	0.41 ± 0.04	1%	0.01 ± 0.09
55	Ph	C₆H₄-OMe	C₆H₄-OMe	3%	1%	1.31 ± 0.12	1%	4.27 ± 0.03

a: K_i values are means ± SEM of four separate assays each performed in duplicate. Percentage of inhibition (I%) are determined at 1 µM concentration of the tested compounds; b: Displacement of specific [³H]DPCPX competition binding assays to hA₁CHO cells; c: Displacement of specific [³H]ZM241385 competition binding to hA₂ₐCHO cells; d: Displacement of specific [¹²⁵I]AB-MECA competition binding to hA₃CHO cells; e: cAMP experiments in hA₂ᵦCHO cells, stimulated by 200 nM NECA. IC_{50} values are expressed as means ± SEM of four separate cAMP experiments. Percentage of inhibition (I%) are determined at 1 µM concentration of the tested compounds.

Fig. 24. SAR and human A_3 adenosine receptor activity of lead compounds consisting pyrazolo[4,3-*d*]pyrimidine.

In 2016, Squarcialupi *et al*. synthesized 7-aminopyrazolo[4,3-*d*]pyrimidines to evaluate structural modification at C-2 and C-5 positions to afford significant activity against human A_1 and A_{2A} adenosine receptors. SAR study revealed that incorporation of 2-methoxybenzyl group at C-2 region and 5-

methylfuran-2-yl at C-5 region enhanced the adenosine activity as shown in **Fig. 25**. From the series, compounds **56-59** displayed promising activity against both A_1 and A_{2A} receptors.[72]
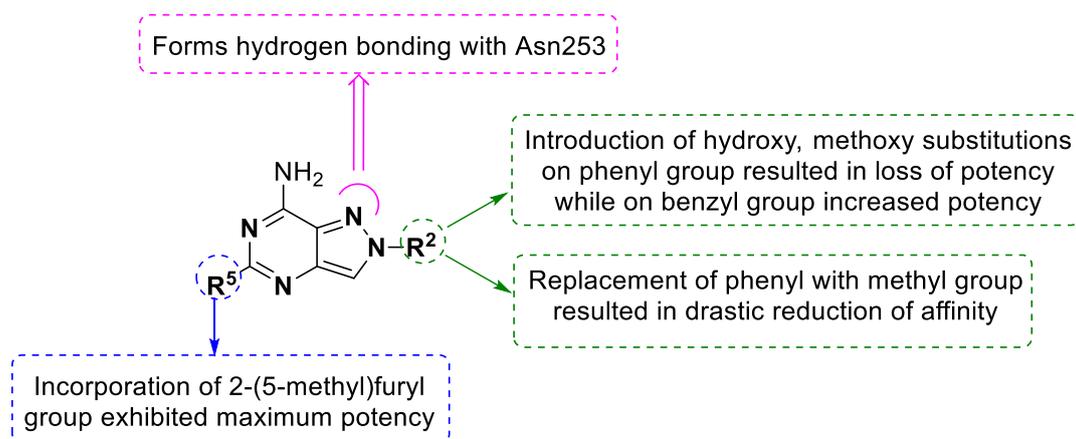
C. No	R[2]	R[5]	^aBinding experiments			cAMP assays
			K_i (nM) or I%			IC$_{50}$ (nM) or I%
			hA$_1$	hA$_{2A}$	hA$_3$	hA$_{2B}$
56	(2-OCH₃ benzyl)	(5-methylfuran-2-yl)	18 ± 2	3.62 ± 0.34	82 ± 7	30%
57	(2-OH benzyl)	(5-methylfuran-2-yl)	126 ± 11	5.26 ± 0.47	88 ± 6	293 ± 26
58	(3-OCH₃ benzyl)	(5-methylfuran-2-yl)	308 ± 8	57 ± 6	498 ± 47	354 ± 32
59	(3-OH benzyl)	(5-methylfuran-2-yl)	252 ± 21	51 ± 5	427 ± 41	421 ± 39

a: K_i values are means $\pm$ SEM of four separate assays each performed in duplicate. Percentage of inhibition (I%) are determined at 1 µM concentration of the tested compounds; b: Displacement of specific [³H]DPCPX competition binding assays to hA₁CHO cells; c: Displacement of specific [³H]ZM241385 competition binding to hA₂ACHO cells; d: Displacement of specific [¹²⁵I]AB-MECA competition binding to hA₃CHO cells; e: cAMP experiments in hA₂BCHO

cells, stimulated by 200 nM NECA. Percentage of inhibition (I%) are determined at 1 μM concentration of the tested compounds.

Fig. 25. Structures and biological activity of 7-aminopyrazolo[4,3-*d*]pyrimidines as human A$_1$ and A$_{2A}$ adenosine receptors.

3.4.   Cytokinin activity

In 1971, Hecht and co-workers synthesized 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines and evaluated their cytokinin activity in tobacco bioassay. The anti-cytokinin activity was determined by the growth of tobacco callus on a standard medium to which 6-aminobenzylpurine and other prepared compounds were added at various concentrations. From the series, compounds **60** and **61** exhibited potent cytokinin activity of 0.081 and 0.24 μM respectively. SAR studies indicated that isopentenyl substitution at C-7 position was responsible for higher activity as depicted in **Fig. 26**.[85]

Fig. 26. Structures of lead compounds with potent cytokinin activity.

In 1972, Skoog *et al*. reported the synthesis of 7-substituted 3-methylpyrazolo[4,3-*d*]pyrimidines and evaluated their biological properties as cytokinin antagonists in the tobacco bioassay. Potent cytokinin antagonist activity was determined for the compounds **62**-**64** from this synthesized series. SAR study revealed that compounds with linear groups such as *n*-pentyl and *n*-butyl at C-7 position were well favored for activity as depicted in **Fig. 27**.[86]

Linear substitutions (via. *n*-pentyl, *n*-butyl etc.) exhibited fair potency than branched (i.e., *i*-pentyl, *i*-butyl) substitutions

Introduction of dibutyl group resulted in complete loss of antagonist activity

Hydroxy and methylthio moieties were found inactive

Incorporation of cyclopentyl and cyclohexyl groups were found to decrease potency

C. No	R	Range tested	Cytokinin activity IC$_{50}$ (µM)		Antagonist activity IC$_{50}$ (µM)	
			Detection	Maximum growth	Detection	Lethal dosage
62		0.009-20	NA	NG	0.03	0.2
63		0.009-20	NA	NG	0.03	0.5
64		0.009-20	NA	NG	0.1	0.73

NA: No activity; NG: No growth.

Fig. 27. SAR of 7-substituted 3-methylpyrazolo[4,3-*d*]pyrimidines as cytokinin antagonists in tobacco bioassay.

In 1980, Gregorini and co-workers reported synthesis of 7-(pentylamino), 7-(benzylamino)-3-methylpyrazolo[4,3-*d*]pyrimidines and evaluated them as cytokinin antagonists on suspension-cultured tobacco cells. All molecules were found highly inhibitory to cytokinin-autonomous and cytokinin-requiring tobacco cells. The structures of active compounds (**65**, **66**) and cytokinin activity results are represented in **Fig. 28**.[32]

65   **66**
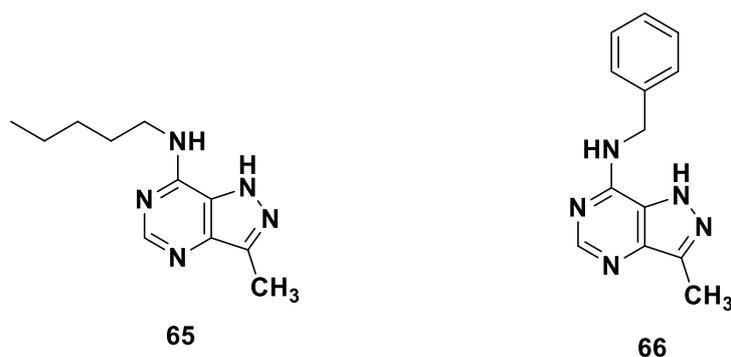
C. No	cytokinin-autonomous tobacco cells		cytokinin-requiring tobacco cells	
	IC$_{50}$ (µM)		IC$_{50}$ (µM)	
	I$_{50}$*	I$_{100}$*	I$_{50}$	I$_{100}$
65	0.025	0.05	0.02	0.05
66	0.03	0.1	0.04	0.15

*I$_{50}$ and I$_{100}$ correspond to concentrations of analogs which produce 50% and 100% of growth inhibition, respectively.

Fig. 28. Cytokinin activity of pyrazolo[4,3-*d*]pyrimidines on various tobacco cells.

3.5. Miscellaneous agents

In 2002, Yuan and co-workers reported the synthesis and pharmacological evaluation of 3-aryl pyrazolo[4,3-*d*]pyrimidine derivatives as potential carticotropin releasing factor-1 (CRF-1) antagonists. SAR studies reveal the effect of substitutions on nitrogen at C-7 and at C-3 positions as depicted in **Fig. 29**. From the reported series, compounds **67-70** exhibited significant antagonist activity against nonpeptide CRF-1.[33]
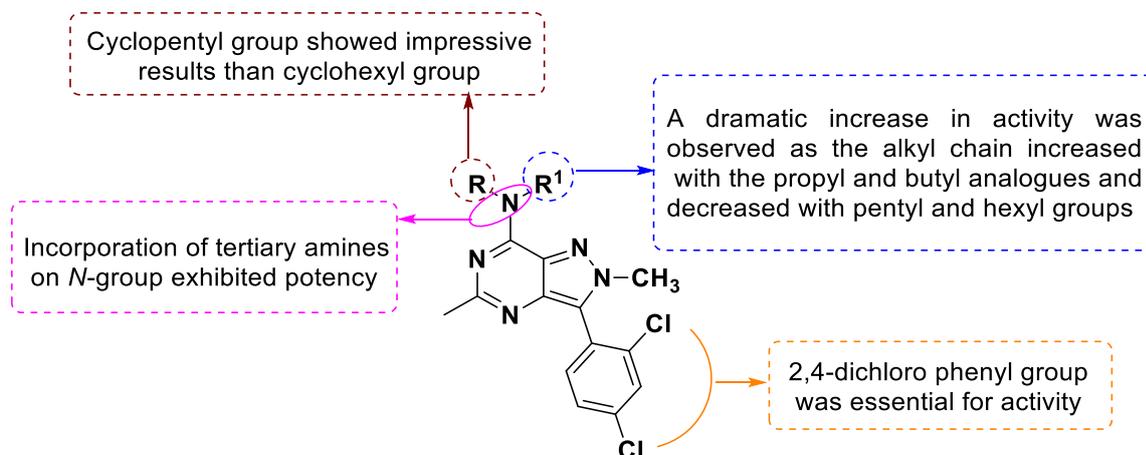
C. No	R	R[1]	CRF$_1$ Binding K_i (nM)
67	*n*-propyl	*n*-propyl	2 ± 1
68	(CH$_2$)$_2$OCH$_3$	(CH$_2$)$_2$OCH$_3$	3 ± 2
69	Ethyl	*n*-butyl	2 ± 1
70	Ethyl	*n*-propyl	3 ± 2

Fig. 29. SAR, structures and CRF-1 binding affinity studies of lead compounds.

In 2011, Jorda *et al*. reported a series of 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidine derivatives and evaluated them for anti-leishmanial and CRK/CYC6 (cdc2-related kinase/ Cyclin 6) kinase activity. Among the tested series, compounds **71-74** exhibited potential activity whose SAR studies revealed that the lipophilic groups (namely adamantyl, halo substituted phenyl and 2-hydroxybenzyl) at C-7 position were responsible for increased activity whereas, non-lipophilic (furyl) and lipophilic (pentyl) groups lowered the activity as discussed in **Fig. 30**.[19]
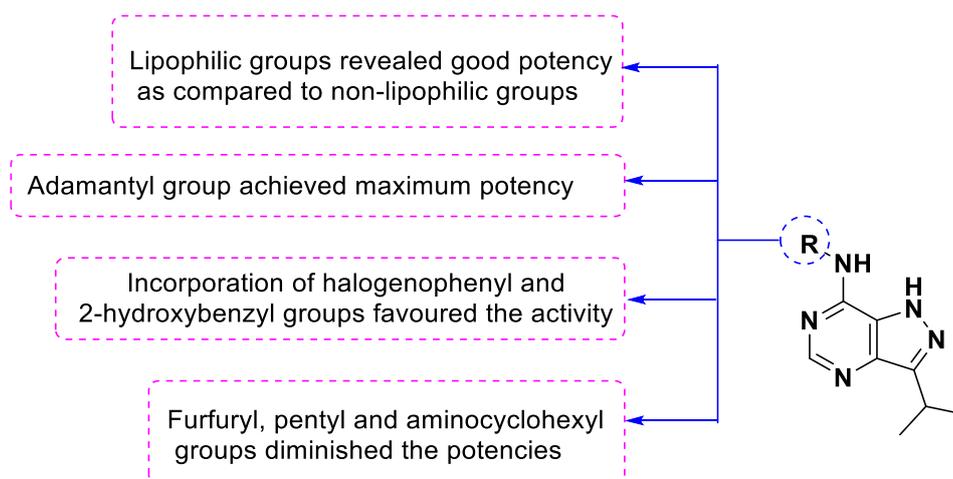
C. No	R	Leishmanial donovani axeaxenicaxenic amastigotes inhibition		CRK/CYC6 kinase inhibiton	
		(%)[a]	EC$_{50}$ (μM)	(%)[b]	IC$_{50}$ (μM)[c]
71	2-hydroxybenzyl	87.4 ± 0.4	35.7	68.0 ± 2.1	11.91
72	Adamantan-1-yl	73.2 ± 0.0	1.22	93.8 ± 0.3	1.82
73	4-F-Ph	75.8 ± 1.7	11.6	78.8 ± 0.4	6.8

| 74 | 3-Cl-Ph | 73.3 ± 1.1 | 12.4 | 81.3 ± 2.3 | 9.86 |

a: In the presence of 30 µM compound; b: In the presence of 15 ATP with 30 µM compound; c: In the presence of 15 µM ATP.

Fig. 30. Anti-leishmanial activity and SAR studies of 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines.

3.6. Patents covering pyrazolo[4,3-d]pyrimidine scaffold with diverse biological activities

Pharmacological significance of pyrazolo[4,3-d]pyrimidine scaffold is further proved based on several patents registered from 1976 to 2012. Different research groups patented this nucleus for numerous therapeutic sections are briefly presented in **Table-1**.

Research group	Patent number	Target activity	Year
Ratajczyk	US3939161	Anti-convulsant, sedative, anti-inflammatory and gastric anti-secretory agents.[87]	1976
Hecht	US4282361	Various schemes for synthesis.[88]	1981
Fujikawa	US4654348	Anti-hyperlipidemic agents.[89]	1987
Hamilton	US4663326	A_1 adenosine and cardiovascular.[90]	1987
Hamilton	US4666908	PDE5 inhibitors.[91]	1987
Hamilton	WO8800192	Anti-anxiety, asthma and allergic agents.[92]	1988
Yuan	US5723608	CRF_1 receptor antagonists.[93]	1998
Zhihua	WO0014088	Male erectile dysfunction.[94]	2000
Lu	US6204383 B1	Synthetic process for preparing sildenafil.[95]	2001
Yuan	US6211187 B1	CRF_1 receptor ligands.[96]	2001
Bunnage	WO0127113 A2	Male erectile dysfunction and female sexual dysfunction.[38]	2001
Daniela	EP1348707 A1	Diagnostic agents.[36]	2003
Jonas	US6777419 B1	PDE5 inhibitors.[97]	2004
Eggenweiler	US20040023990	Asthma, allergic and female sexual disorder.[98]	2004
Eggenweiler	US20040063730 A1	Hypertension, angina and pulmonary hypertension.[99]	2004
Eggenweiler	US20040077664 A1	Hypertension, angina and bronchitis.[100]	2004

Hodgetts	CA2537916 A1	CRF$_1$ receptor modulators.[101]	2005
Hodgetts	WO2005028480 A2	Selective modulators of CRF$_1$ receptors.[102]	2005
Bell	WO2005097799 A1	PDE5 inhibitors.[103]	2005
Pal	US20060128729 A1	Anti-inflammatory.[37]	2006
Acker	WO2006046135 A2	PDE5 inhibitors.[104]	2006
Bell	US7262192 B2	PDE5 inhibitors.[105]	2007
Butora	US7534767 B2	RNA dependent RNA viral polymerase.[106]	2009
Bell	US7569572 B2	PDE5 inhibitors.[107]	2009
Bell	US7572799 B2	PDE5 inhibitors.[108]	2009
Moravcova	US7745450 B2	Cancer.[109]	2010
Bell	US8097621 B2	PDE5 inhibitors.[110]	2012

Table 1. Pyrazolo[4,3-*d*]pyrimidine nucleus containing patents having numerous biological Activities.
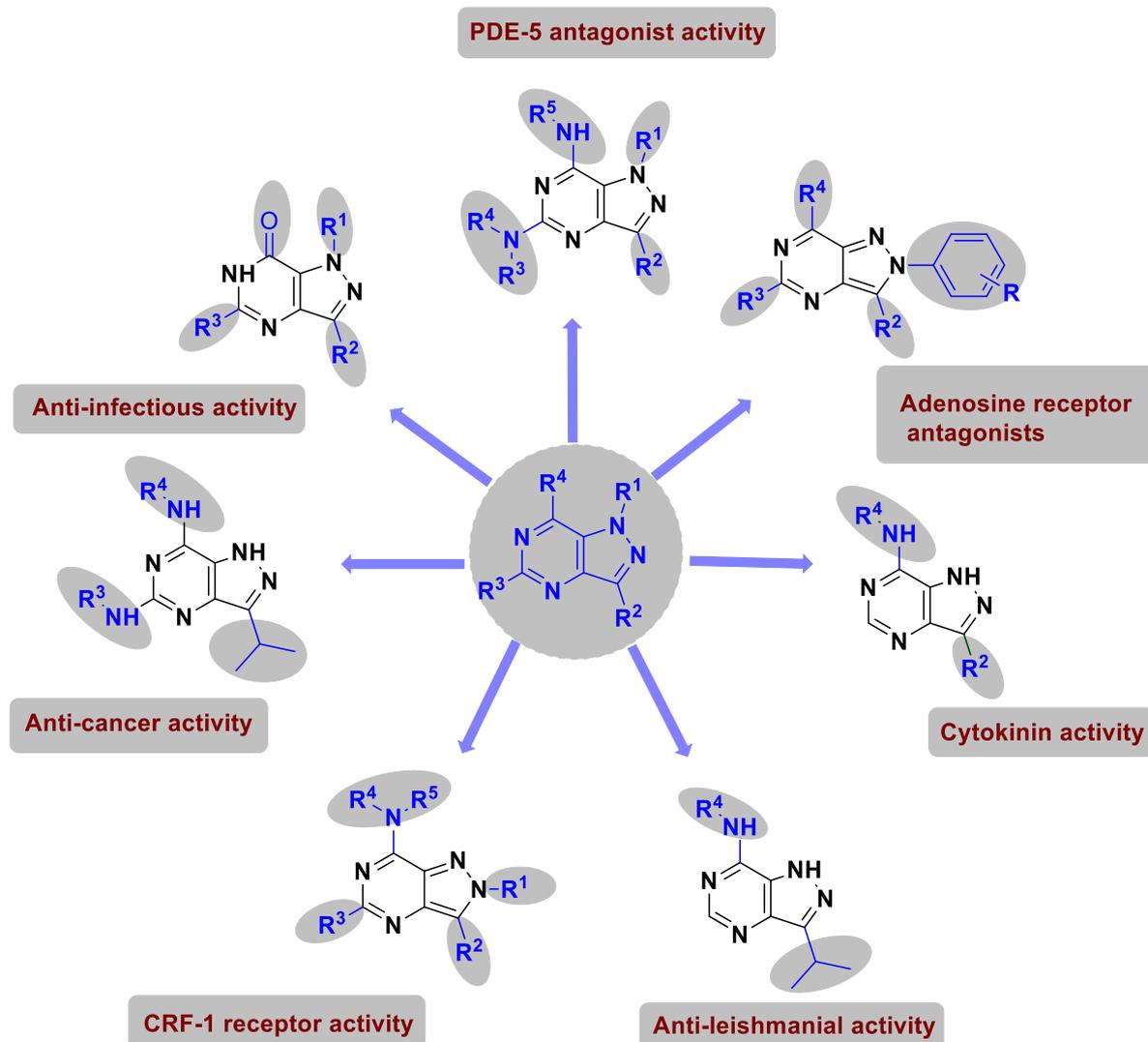
Fig. 31. Summary of structural amendments to influence the biological activity.

4. Conclusion

Nitrogen containing heterocycles have been a versatile class of compounds displaying array of biological properties. Pyrazolo[4,3-*d*]pyrimidine is a significant framework that is being explored exponentially for its multifarious medicinal properties. The present manuscript at the beginning has emphasized numerous methods for synthesizing variously substituted pyrazolo[4,3-*d*]pyrimidines through conventional, multi-component and microwave-assisted reaction methods. Apart from remarkable synthetic evolutions, pyrazolo[4,3-*d*]pyrimidine derivatives have been found to unveil a diverse set of pharmaceutical properties. Numerous applications along with patents (**Table 1**) have been explored based on their biochemical, biophysical and medicinal aspects and much more is yet to be explored. Medicinal properties of pyrazolo[4,3-*d*]pyrimidines have been broadly examined for diverse biological activities such as

antimicrobial, human adenosine antagonists, anticancer, CNS depressant, phosphodiesterase 5 inhibitors and other applications such as anti-leishmanial and cytokinin inhibitors. Many of the researchers have successfully achieved the modifications over the pyrazolo[4,3-*d*]pyrimidine scaffold and have investigated them on diverse biological activities. These activity profiles could be regulated by suitable selection of the fused heterocycles and the substitutions on the pyrazolo[4,3-*d*]pyrimidine scaffold. Substitutions on C-3, C-5 and C-7 positions of pyrazolo[4,3-*d*]pyrimidine showed distinct anti-cancer activity, whereas substitutions at N-1, C-3 and C-5 positions showed antibacterial activity. Further, the sites of modifications on pyrazolo[4,3-*d*]pyrimidine scaffold and variation of biological activity has been represented in **Fig. 31**. A wide range of synthetic pathways and varied biological properties have opened the door for the discovery of novel drug candidates and other target activities. In addition, the current manuscript can be valuable to researchers to find out novel, optimized and target selective pyrazolo[4,3-*d*]pyrimidine compounds and its use in the treatment of some more incurable diseases.

5 Conflicts of Interest

Authors hereby declare that there are no financial/commercial conflicts of interest.

6 Acknowledgement

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CHAPTER 4

Synthesis, anticancer evaluation, and molecular docking studies of some novel 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines as cyclin dependent kinase 2 (CDK2) inhibitors

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Graphical Abstract

Abstract:

A novel series of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines (**7-43**) bearing various anilines at C-4 position and thiophenethyl or thiopentane moieties at C-6 position have been designed and synthesized by molecular hybridization approach. All the synthesized compounds were evaluated for *in vitro* CDK2/cyclin E and Abl kinase inhibitory activity as well as anti-proliferative activity against K-562 (chronic myelogeneous leukemia), and MCF-7 (breast adenocarcinoma) cell lines. The structure-activity relationship (SAR) studies revealed that compounds with thiophenethyl group at C-6 with mono-substituted anilines at C-4 exhibited better CDK2 inhibitory activity compared to alkyl group (thiopentane) at C-6 and di-substituted anilines at C-4 of the scaffold. In particular, compounds having 2-chloro, 3-nitro and 4-methylthio aniline groups at C-4 displayed significant enzymatic inhibitory activity against CDK2 with single digit micro molar IC_{50} values. The *in silico* molecular docking studies suggested possible binding orientation and the binding energies were in agreement with the observed SAR as well as experimental results. In addition, some of the synthesized compounds indicated anti-proliferative effects against K-562 and MCF-7 cancer cell lines with IC_{50} values in a micro molar range. Thus, the synthesized compounds could be considered as new anticancer hits for further lead optimization.

Key words: Pyrazolo[3,4-*d*]pyrimidine; cyclin dependent kinase inhibitor; Anti-proliferative activity; Molecular docking; GLIDE.

1 Introduction

Cancer is an enormous global health burden, affecting almost every region and socio-economic level. It is the second leading cause of death worldwide, accounted for 8.8 million deaths in 2015 and nearly 1 in 6 of all global deaths.[1] The new cancer cases are expected to increase to 15 million per year by 2020, according to the World Health Organization (WHO), unless further precautionary measures are followed.[2] Wide efforts are being carried out in order to discover new treatment approaches as well as to improve prevention and molecular diagnostic systems.[3,4] It is becoming noteworthy to investigate new druggable molecular targets, identify and develop their modulators as novel drugs for the treatment of cancer. Amongst others, protein kinases have become an important group of drug targets and number of kinase inhibitors in clinical development is rapidly increasing.[5]

Cyclin-dependent kinases (CDKs) are a group of serine/threonine kinases comprising 20 members, of which some are linked with regulation of cell-cycle progression by phosphorylating proteins involved in cell division. For example, formation of active complex composed of CDK2 and cyclin E enables pRb phosphorylation, activation of transcription factor E2F which initiates S phase of the cell cycle.[6] CDK2 then also associates with cyclin A, governing continuous DNA replication and properly programed deactivation of E2F. Deregulations of CDKs or cyclins, as well as the loss of endogenous inhibitory proteins, result in abrogation of cell cycle control, which is connected with development of tumors. Thus CDKs are considered important targets for anticancer drugs.[7,8] The lack of clarity as to which CDK is the most suitable drug target followed by poor selectivity hindered the clinical development of specific CDK inhibitors.[9]

Initially, the importance of CDK2 as a drug target for cancer therapy was in question since CDK2 knockdown investigations failed to block cell proliferation in a number of tumour cell lines,[10] and by mouse knockout investigations where the animals were viable.[11,12] Current investigations employing a chemical genetic method in which CDK2 countenance was maintained, but enzymatic activity was inhibited, affords interesting sign that CDK2 is a valid anticancer drug target.[6] Up to date many CDK2 inhibitors have been developed and some of them (including roscovitine, CYC065, dinaciclib, AT7519, milciclib) undergo clinical evaluation.[13,14] CDK2 inhibitors are also anticipated to have efficacy in combinations with other drugs or where synthetic lethality can be recognized.[15] Recent study proved that a combination of phosphatidylinositol-3-kinase and CDK2 inhibitors induced apoptosis in malignant glioma xenografts via synthetic-lethal interaction.[16] CDK2 was proved as a therapeutic target in BRCA-deficient cancers,[17] ovarian cancer.[18]

Fused pyrimidines have received a great deal of attention due to their immense role as active pharmacophores.[19] The pyrazole annulated on the pyrimidine scaffold leads to pyrazolopyrimidine, which can be looked upon as the bio-isostere of purine.[20] The pharmacological significance of purine

nucleus is well established. Oxypurinol and its congeners allopurinol and thiopurinol (Tisopurine) which contains pyrazolo[3,4-*d*]pyrimidine scaffold inhibit xanthine oxidase enzyme and interfere with the biosynthesis of uric acid, further allopurinol acting as a causative agent for gout.[21] Ibrutinib/PCI-32765 was recently approved by the US FDA for the treatment of mantle cell lymphoma, chronic lymphocytic leukemia and Waldenstrom's macroglobulinemia diseases (**Figure 1**).[22]
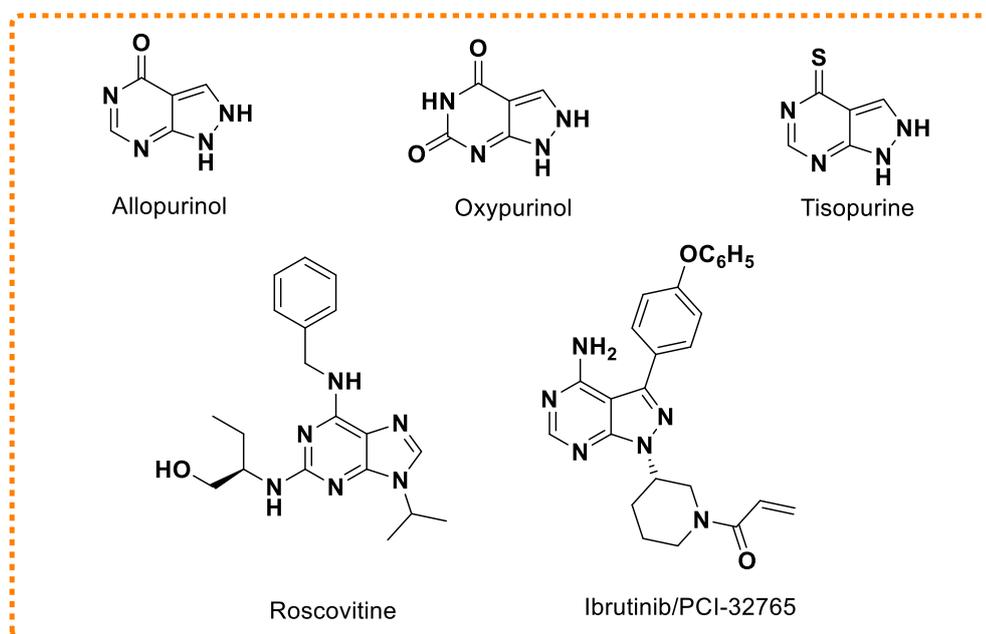
Figure 1. Structures of active drugs containing fused pyrimidine.

Pyrazolo[3,4-*d*]pyrimidine is also reported to encompass biological potential as anticancer, antiviral, antimicrobial, herbicidal, CNS agents (phosphodiesterase 9 and benzodiazepine receptors), anti-inflammatory and cardiovascular activities.[23] **Figure 2** depicts several reported analogs of pyrazolo[3,4-*d*]pyrimidine with anti-cancer activities related to inhibition of various protein kinases.[24-29]

Based on the above mentioned facts and in continuation of our research work on anticancer drug discovery, we envisaged to further exploit the pyrazolo[3,4-*d*]pyrimidine scaffold to synthesize novel CDK2 inhibitors.[30,31] Thus a series of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine was synthesized with a design strategy: a) bioisosteric replacement of purine nucleus, b) incorporation of more lipophilic aromatic amines (C-4) and aliphatic or aromatic groups (C-6). These derivatives were also evaluated against Abl kinase and two cancer cell lines K-562 (chronic myelogeneous leukemia) and MCF-7 (breast adenocarcinoma). Further, *in silico* molecular docking studies were performed to calculate the binding energies and orientations of these compounds with respect to the active site of CDK-2 protein. The computational results were in agreement with our experimental observations.
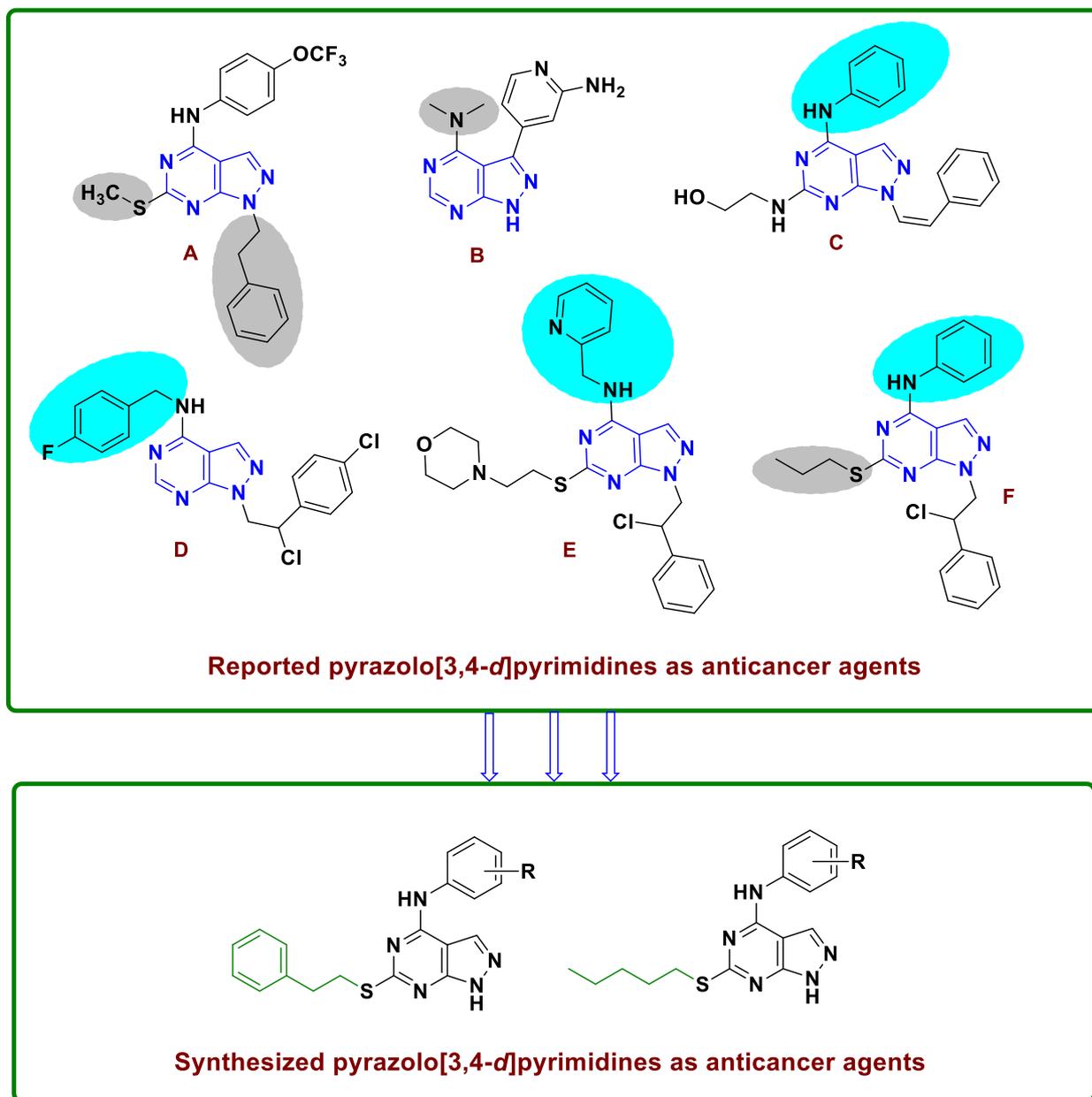
Figure 2. Known derivatives of pyrazolo[3,4-*d*]pyrimidine and their anticancer activities along with the designed molecules. **A**: (K_{i50} against Src, AblT315I = 0.056, 0.01 μM);[24] **B**: (IC$_{50}$ against CDK9 = 17 nM);[25] **C**: (IC$_{50}$ against CDK2 = 0.5μM);[26] **D**: (K_{i50} against Abl = 80 nM);[27] **E**: (K_{i50} against cSrc, Abl = 0.21± 0.02, 0.15±0.02 μM);[28] **F**: (IC$_{50}$ against Src = 1.2 ± 0.4 μM).[29]

2 Results and discussion

2.1 Chemistry

The synthesis of novel series of pyrazolo[3,4-*d*]pyrimidine hybrids was achieved through an efficient and versatile synthetic route as depicted **Scheme 1**. On the pyrazolopyrimidine scaffold, chloro group was introduced at C-4 as a good leaving group, which acted as a most reactive site for different nucleophiles, while thiophenethyl or thiopentane groups were introduced at C-6 position. With a view

to prepare the target hybrid molecules (**7-43**), the key intermediates 4-chloro-6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6a**), 4-chloro-6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6b**) were synthesized from ethoxymethylene malononitrile (**1**) as illustrated in **Scheme 1**.

From the earlier reported literature methods compound **4** was successfully synthesized.[32] Further under microwave irradiation alkylation of compound **4** was achieved by reacting with 2-chloroethyl benzene in presence of anhydrous potassium carbonate in *N,N*-dimethylformamide (DMF) to obtain 6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**5a**). Similarly reacting **4** with 1-bromopentane in 1M NaOH solution at 70 °C resulted in 6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**5b**) in moderate yield. Chlorination of **5a** and **5b** was easily achieved with Vilsmeier complex (POCl₃:DMF) to obtain halogenated key intermediates 4-chloro-6-(phenethyl/pentylthio)-1*H*-pyrazolo[3,4-*d*]-pyrimidine that is **6a** and **6b**. As displayed in **Scheme 1**, the synthesis of the final hybrid compounds (**7-43**) in good yield (75-95%) was achieved by nucleophilic substitution with various primary amines at C-4. The IR, ¹H and ¹³C NMR spectroscopic data of all the novel compounds were in agreement with the predicted structures, which was further validated by the HR-MS data (Supporting information).
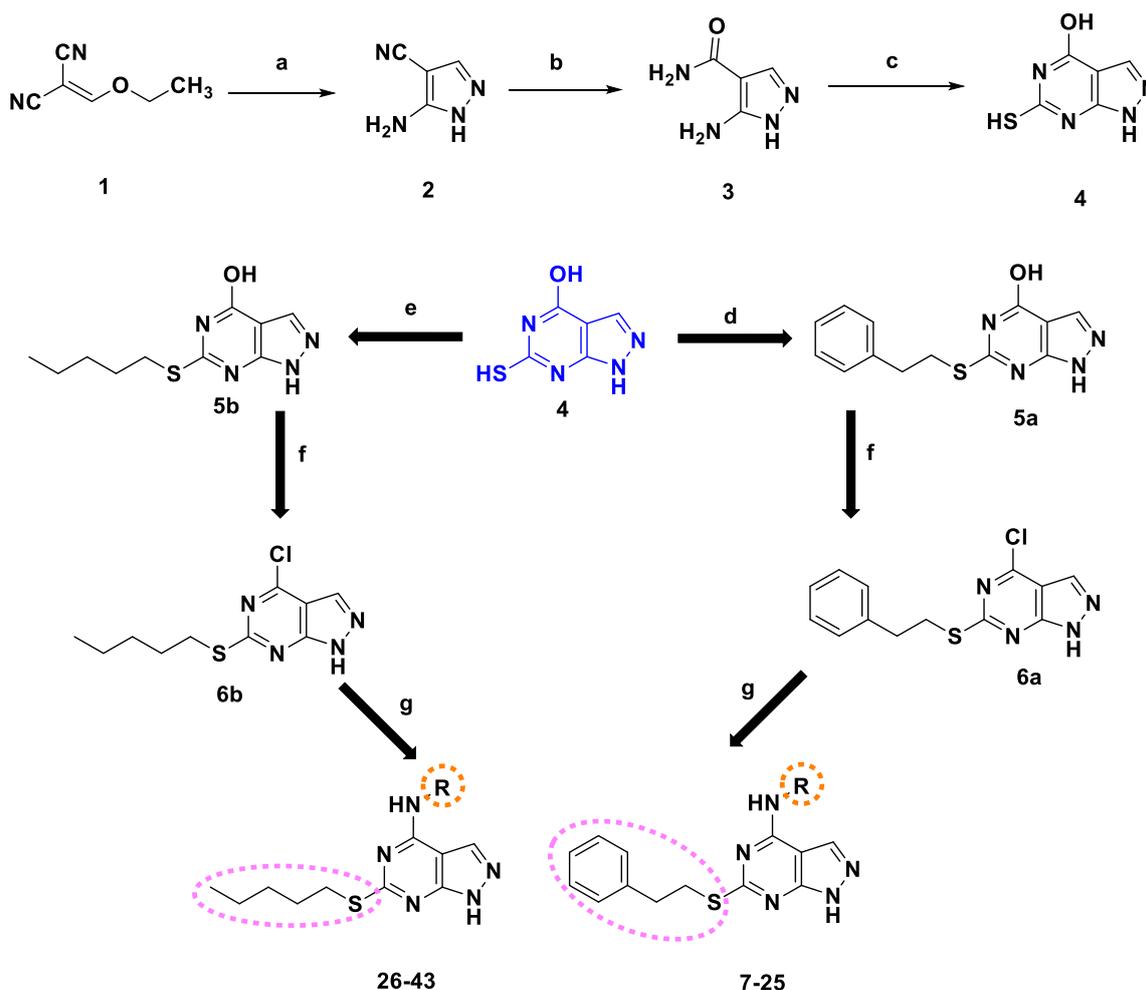
Scheme 1: Synthetic exploration for the preparation of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine analogues (**7-43**).

Reagents and conditions: (a) hydrazine hydrate, ethanol, 80 °C, 3h, 92%; (b) Conc. H_2SO_4, NH_4OH, H_2O, 50 °C, 5h, 90%; (c) potassium ethyl xanthogenate, DMF, 120 °C, 6h, 82%; (d) 2-chloroethyl benzene, K_2CO_3, DMF, 70 °C, microwave, 20 min, 76%; (e) 1-bromopentane, NaOH, H_2O, glacial acetic acid, 50 °C, 5h, RT, overnight, 80%; (f) $POCl_3$, DMF, 80 °C, 2h, 85%; (g) $R-NH_2$, EtOH, 80 °C, 2h, 75-95%.

The ^{1}H NMR of compound **4** exhibited very distinct singlet signals resonating at around δ 13.61, 13.03, 11.86 and 8.42 ppm, was attributed to the N-H, S-H, O-H and Ar-H protons of pyrazole ring. Thus, indicating the formation of the fused pyrazolopyrimidine by ring annulation of 5-amino-1*H*-pyrazole-4-carboxamide (**3**) with potassium ethyl xanthogenate. For compounds **5a** and **5b**, the distinctive methylene signals (Ph-C$\underline{H_2}$-C$\underline{H_2}$-S- and -S-C$\underline{H_2}$-C$\underline{H_2}$-C$\underline{H_2}$-C$\underline{H_2}$-) appeared around δ 3.43-3.39, 3.01-2.97 ppm and δ 3.17-2.87, 1.73-1.43, 1.41-1.14 respectively, while the methyl peak for **5b** was observed at δ 0.86-0.80 ppm. In particular, the disappearance of a distinct singlet signal at around δ 13.03 ppm for mercapto (-S$\underline{H}$) group evidently indicated the successful alkylation of pyrimidine scaffold. Whereas the most characteristic singlet, doublet and triplet signals at around δ 7.32-7.20 ppm was attributed to the aromatic protons (C$_6\underline{H_5}$-CH$_2$-CH$_2$-S-) at C-6 of the pyrimidine ring. Further, the absence of characteristic singlet signal at around δ 12.22-12.28 ppm for (-O$\underline{H}$) at C-4 of pyrimidine ring confirmed the formation of halogenated (-**Cl**) key intermediates 4-chloro-6-(phenethyl/pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6a** and **6b**). These findings corroborated with their respective ^{13}C NMR, where the methylene carbon peaks at δ 34.61, 31.08 ppm (C$_6$H$_5$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-) and δ 30.61-30.37, 30.16-29.69, 29.22-28.11, 21.78-21.73 ppm (-S-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-) were assigned to **5a** and **5b**. The single methyl peak (**5b)** appeared around 13.90-13.83 ppm while the prominent aromatic signals (**5a** and **5b**) resonated around δ 167.89-109.79 ppm.

The IR spectra of the title compounds **7-43** displayed a reasonably sturdy and characteristic bands around 2921-3033 cm^{-1}, 1270-1300 cm^{-1} accounting for N-H and C-S stretching respectively, while the most prominent band of C-N appearing around 1180-1184 cm^{-1} indicated the formation of final hybrid molecules. Further, the prominent ^{1}H-NMR signals of the title compounds **7-43** resonated around δ 13.39-10.28 ppm (exocyclic-N-H), δ 9.94-7.75 ppm (ring-N-H) and 10.02-8.19 ppm (C-3 aromatic proton) while, the singlet or multiplet aromatic proton peaks appeared around δ 8.47-6.84 ppm. For compounds **7-25** the methylene protons (-S-C$\underline{H_2}$-C$\underline{H_2}$-Ph) were observed around δ 3.43-2.81 ppm while for compounds **26-43** the signals (-S-C$\underline{H_2}$-C$\underline{H_2}$-C$\underline{H_2}$-C$\underline{H_2}$-) appeared around δ 3.10-1.20 ppm. The methyl protons for **26-43** resonated around δ 0.87-0.80 ppm. Further the methylthio (-Ph-SC$\underline{H_3}$), methyl (Br-Ph-C$\underline{H_3}$), methoxy (Ph-OC$\underline{H_3}$) and ethynyl-$\underline{H}$ protons resonated around δ 2.47-2.44 ppm, δ 2.30-

2.18 ppm, δ 3.77-3.71 ppm and 4.18 ppm respectively. The ^{13}C NMR spectra further confirmed the structures of the title compounds. The characteristic carbon signals C-6, C-4 and C-3 of the pyrimidine ring were observed around δ 167.41-166.40, 155.73-153.11 and 132.88-130.56 ppm, while the various aromatic/heteroaromatic carbons resonated between δ 153.62-98.12 ppm. Further, the prominent carbon signals observed around δ 55.66-55.61 ppm δ 21.78-17.74 ppm and 30.17-15.31 ppm were attributed to the methoxy (-O<u>C</u>H₃), methyl and thiomethyl carbons respectively. Further, for compounds **7-25** the methylene carbon peaks (-S-<u>C</u>H₂-<u>C</u>H₂-Ph) were observed around δ 35.51-31.08 ppm while for compounds **26-43** the signals (-S-<u>C</u>H₂-<u>C</u>H₂-<u>C</u>H₂-<u>C</u>H₂-) appeared around δ 30.62-21.73 ppm and the methyl peak resonated around δ 13.89-13.83 ppm. In addition, the HR-MS spectrums of the title compounds (**7-43**) displayed accurate molecular ion peaks, which were in agreement with their expected molecular weights (supporting information).

2.2 *In vitro* evaluation for CDK2 and Abl kinase inhibitors

All the final compounds were evaluated for CDK2/cyclin E kinase inhibition and the IC$_{50}$ values of various *in vitro* anticancer profiles of the final compounds are summarized in **Table 1**. Abl kinase inhibition was evaluated as a counter screen, to get a preliminary information about selectivity.

Code	R	IC$_{50}$ (µM)[a]			
		CDK2/Cyclin E	**Abl**	**K-562**	**MCF-7**
7	Ph	>12.5	>12.5	19.9	19.2
8	2-ClPh	7.8	>12.5	>6.25	>6.25
9	3-ClPh	13.3	>25	>25	24.0
10	4-ClPh	>25	>25	>25	>25
11	3-NO₂Ph	5.1	>25	24.6	24.3
12	4-NO₂Ph	>12.5	>12.5	>6.25	>6.25
13	2-BrPh	>12.5	>12.5	>6.25	>6.25
14	3-BrPh	>25	>25	27.4	23.9
15	4-BrPh	>25	>25	>50	>50
16	2,4-(CH₃)₂Ph	>12.5	>12.5	>6.25	>6.25
17	SCH₃Ph	>25	>25	>6.25	>6.25
18	4-Cl-3-CF₃Ph	13.4	>25	>6.25	>6.25
19	4-Br-3-CF₃Ph	>25	>25	>6.25	>6.25
20	4-Cl-3-NO₂Ph	>25	>25	>6.25	>6.25
21	4-F-2-CH₃Ph	>12.5	>12.5	>6.25	>6.25
22	3-Br-4-CH₃Ph	>25	>25	>6.25	>6.25

23	2-Cl-4-FPh	>12.5	>12.5	>12.5	>12.5
24	3,4-(OCH$_3$)Ph	15.9	>12.5	>6.25	>6.25
25	4-(ethynyl)Ph	>25	>25	>12.5	>12.5
26	Ph	>50	>50	>100	>100
27	2-ClPh	8.7	>12.5	>25	>25
28	3-ClPh	>25	>25	>12.5	>12.5
29	4-ClPh	>12.5	>12.5	>6.25	>6.25
30	3-NO$_2$Ph	17.7	>25	>12.5	>12.5
31	4-NO$_2$Ph	>25	>25	>6.25	>6.25
32	2-BrPh	>25	>25	>12.5	>12.5
33	3-BrPh	>25	>25	>6.25	>6.25
34	4-BrPh	>25	>25	>6.25	>6.25
35	2,4-(CH$_3$)$_2$Ph	>25	>25	>12.5	>12.5
36	SCH$_3$Ph	8.8	>25	>6.25	>6.25
37	4-Cl-3-CF$_3$Ph	>25	>25	>12.5	>12.5
38	4-Br-3-CF$_3$Ph	>25	>25	>6.25	>6.25
39	4-Cl-3-NO$_2$Ph	>25	>25	>12.5	>12.5
40	4-F-2-CH$_3$Ph	>12.5	>12.5	>12.5	>12.5
41	3-Br-4-CH$_3$Ph	>25	>25	>6.25	>6.25
42	2-Cl-4-FPh	>12.5	>12.5	>6.25	>6.25
43	3,4-(OCH$_3$)Ph	>12.5	>12.5	>6.25	>6.25
	Roscovitine	0.1	>100	42	11
	Imatinib	>100	0.2	0.5	>10

[a] IC$_{50}$ values were determined in triplicate in the range of 0.05 to 100 μM. IC$_{50}$ value indicates concentration (μM) that inhibits activity of the tested enzyme to 50%. For cytotoxic assays, IC$_{50}$ means the concentration (μM) that inhibits the growth of 50% of cells during a three-day cultivation

Table 1. Anticancer evaluation of novel 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine derivatives.

Some synthesized compounds displayed an activity in the single digit micro molar range against CDK2/cyclin E. Fascinatingly, it was observed that some compounds containing thiophenethyl group at C-6 displayed prominent anticancer activity (**7**, **9**, **11**, **14**) as compared to compounds **26-43** having thiopentane group. It was also observed that incorporation of phenyl/substituted phenyl groups at C-4 of the pyrazolo[3,4-*d*]pyrimidine nucleus was essential for CDK2 activity. From the tested thiophenethyl series the highest CDK2 inhibitory activity was recorded for compounds **11** (IC$_{50}$ = 5.1 μM) and **8** (IC$_{50}$ = 7.8 μM) with 3-nitroaniline and 2-chloroaniline group at C-4 respectively. Notable significant inhibition was also observed for compounds bearing 3-chloroaniline (**9**; IC$_{50}$ = 13.3 μM), 4-

chloro-3-trifluoromethylaniline (**18**; IC_{50} = 13.4 µM) and 3,4-dimethoxyaniline (**24**; IC_{50} = 15.9 µM). For the remaining compounds of this thiophenethyl series, IC_{50} values could not be measured due to a solubility limit (IC_{50} value >12.5 or >25 µM).

Similarly, some thiopentane derivatives (series **26-43**) also exhibited a reasonable activity profile with IC_{50} starting from 8.7 µM. From this series, compounds with 2-chloroaniline (**27**) and 4-methylthioaniline (**36**) showed the best activity with IC_{50} 8.7 µM and 8.8 µM, respectively; this was followed by 3-nitroaniline derivative (**30**) with IC_{50} 17.7 µM. The remaining compounds in this series were less soluble and their IC_{50} were not achieved. In addition, all compounds (**7-43**) were also evaluated against Abl kinase but none of the compounds showed any inhibition in the assayed concentration range, confirming reasonable selectivity towards CDK2 over unrelated Abl.

2.3 Anti-proliferative activity against K-562 and MCF-7 cell lines

All the newly synthesized 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine analogues **7-43** were further evaluated for their *in vitro* anti-proliferative activity against K-562 (chronic myelogeneous leukemia) and MCF-7 (breast adenocarcinoma) cell lines. Several compounds are displaying appreciable activity with measurable IC_{50} values against the two cell lines, such as compounds **7** (IC_{50} = 19.9, 19.2 µM), **9** (IC_{50} = >25, 24 µM), **11** (IC_{50} = 24.6, 24.3 µM), and **14** (IC_{50} = 27.4, 23.9 µM). The remaining compounds were not active in the tested concentration range.

2.4 Structure-activity relationship (SAR) studies

In general, a careful observation of the structure-activity relationship (SAR) indicated that the CDK inhibitory activity was considerably affected by the nature of various substituents present at C-4 (aromatic ring) and C-6 positions on pyrazolo[3,4-*d*]pyrimidine scaffold. From the two series of compounds (phenethyl and pentane at C6), the phenethyl series gave most number of active compounds (8, 9, 11, 18 and 24), while in pentane series only three molecules (**27**, **30** and **36**) were active. Further analysis of both the series revealed that compounds **8**, **9**, **11**, **27**, **30** and **36** with mono substituted phenyl ring (2-Cl-Ph, 3-Cl-Ph, 3-NO$_2$-Ph, and 4-SCH$_3$-Ph groups) at C-4 showed better activity than compounds with disubstituted phenyl groups except for compounds **18** and **24** (4Cl-3CF$_3$-Ph and 3,4-OCH$_3$-Ph). Compound **11** followed by **8**, **9**, **27**, **30** and **36** presented highest inhibitory activity.

Further, no specific activity was observed for all the compounds against Abl kinase (IC_{50} = >12.5 to >50.0). However, compounds 7, 11 and 14, displayed specific anti-proliferative activity against K-562 (IC50 = 19.9, 24.6 and 27.4) and MCF-7 (IC50 = 19.2, 24.3 and 23.9), indicating that mono-substitutions at C4 with phenethyl at C6 was favorable. In summary, compound 11 was most active,

however due to low solubility profile for most of the compounds a conclusive SAR could not be derived. **Figure 3** provides a brief overview of the SAR.
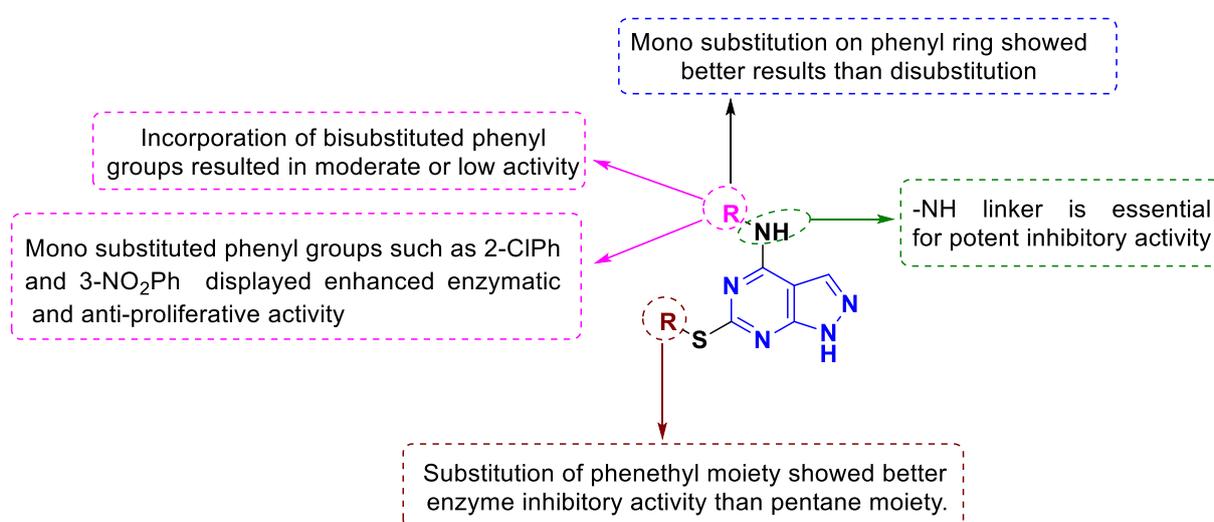
Fig. 3. SAR study of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines as potent anticancer agents.

2.5 Molecular docking study

Computational and bioinformatics tools have become essential part for the design and development of therapeutically effective novel chemical entity.[33] Dinaciclib, a pyrazolo[1,5-*a*]pyrimidine compound, displayed higher binding affinity towards CDKs.[34] Hence, to further understand and substantiate our observed experimental data, molecular docking simulation was performed for the synthesized compounds with the target CDK2 protein.

To validate the docking protocols and to reproduce the reported orientation of R-roscovitine in the predefined binding site of CDK2 (PDB ID: 2A4L), docking studies were performed using Glide program of Schrodinger-Maestro 11.2. From the docking results, the pose of *R*-roscovitine obtained revealed similar molecular interactions as reported.[35] The docked complex presented characteristic hydrogen bonding (H-bond) interactions with crucial residues of the active-site, such as Leu83 interacted with roscovitine by forming a strong [C=O with benzylamino NH (1.91 Å)] and a weak [(C=O with ring nitrogen (2.39 Å)] H-bond. Similarly, the residue Asp86 interacted with OH group of roscovitine via a strong H-bond (1.67 Å), whereas Lys89 exhibited π–cation interaction with phenyl ring of the ligand. **Figure 4** represents the reproduced 3D molecular interactions of the docked pose, validating the docking protocols.
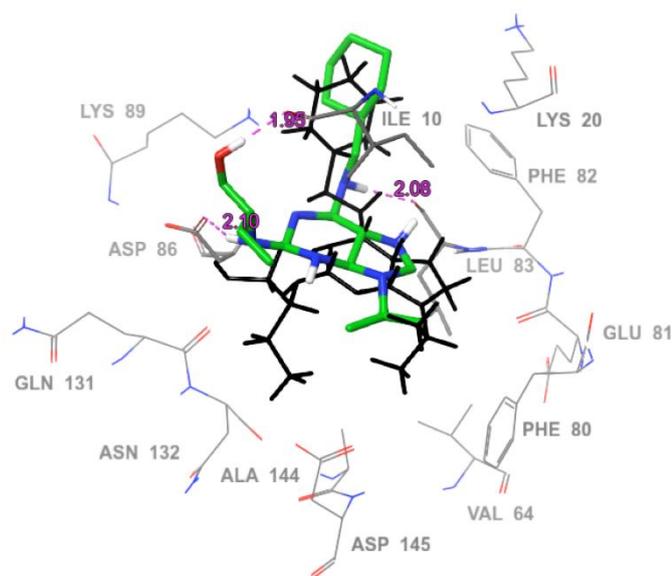
Figure 4: Reported pose of Roscovitine (black thin tube model) and docked pose (thick tube model) into the active-site showing similar interactions (docking validation). Magenta coloured dashed lines indicate hydrogen bonding, while grey-coloured thin tube amino acids are considered as crucial residues.

The docking experimental data with our synthesized compounds revealed that they fit well into the binding-site and display favourable interactions with the crucial amino acid residues. Interestingly, two nitrogen atoms of pyrazole ring of the best active compound **11** (IC$_{50}$ = 5.1 µM) displayed strong H bond interactions with NH of Leu83 (2.37 Å) and C=O of Glu81 (2.02 Å) respectively, signifying these H bond interactions were crucial for the CDK-2 inhibition. The nitro group of the ligand was in close proximity to Lys89 presenting weak vdW interaction, whereas the acidic and basic amino acid residues (Lys129, Glu131, Asn132 and Asp145) surrounded thiophenethyl group. The exocyclic NH and the side chain sulphur displayed no characteristic interactions with the protein (**Figure 5a**). In the case of moderately active compound **27**, the ring NH displayed H bond interaction with Leu83 (1.69 Å), while the exocyclic NH presented weak H bond interaction (2.59 Å) with Asp86. The side chain was anchored within the catalytic residues Phe80 and Asp145, whereas the pocket consisting of Val18, Lys33, and Asp145 residues surrounded chlorophenyl ring. While in the case of the least active compound **30**, the orientation of the docked pose was observed to be different with respect to best and the moderately active compounds (**11** and **27**). The nitro group showed weak H bond interactions with the basic residues (Lys33 and Asn 132), which could have contributed to the altered pose of the ligand. Furthermore the aliphatic side chain occupied the hydrophobic region (Val64, Phe80, Leu134, and Ala144) resulting in H-bond interactions of the ring and exocyclic nitrogen with different residues of the active site (Leu83 and Ile10). **Figure 5** presents the different molecular interactions of **11**, **27** and **30** with the active site residues of CDK-2 protein (PDB ID: 2A4L).
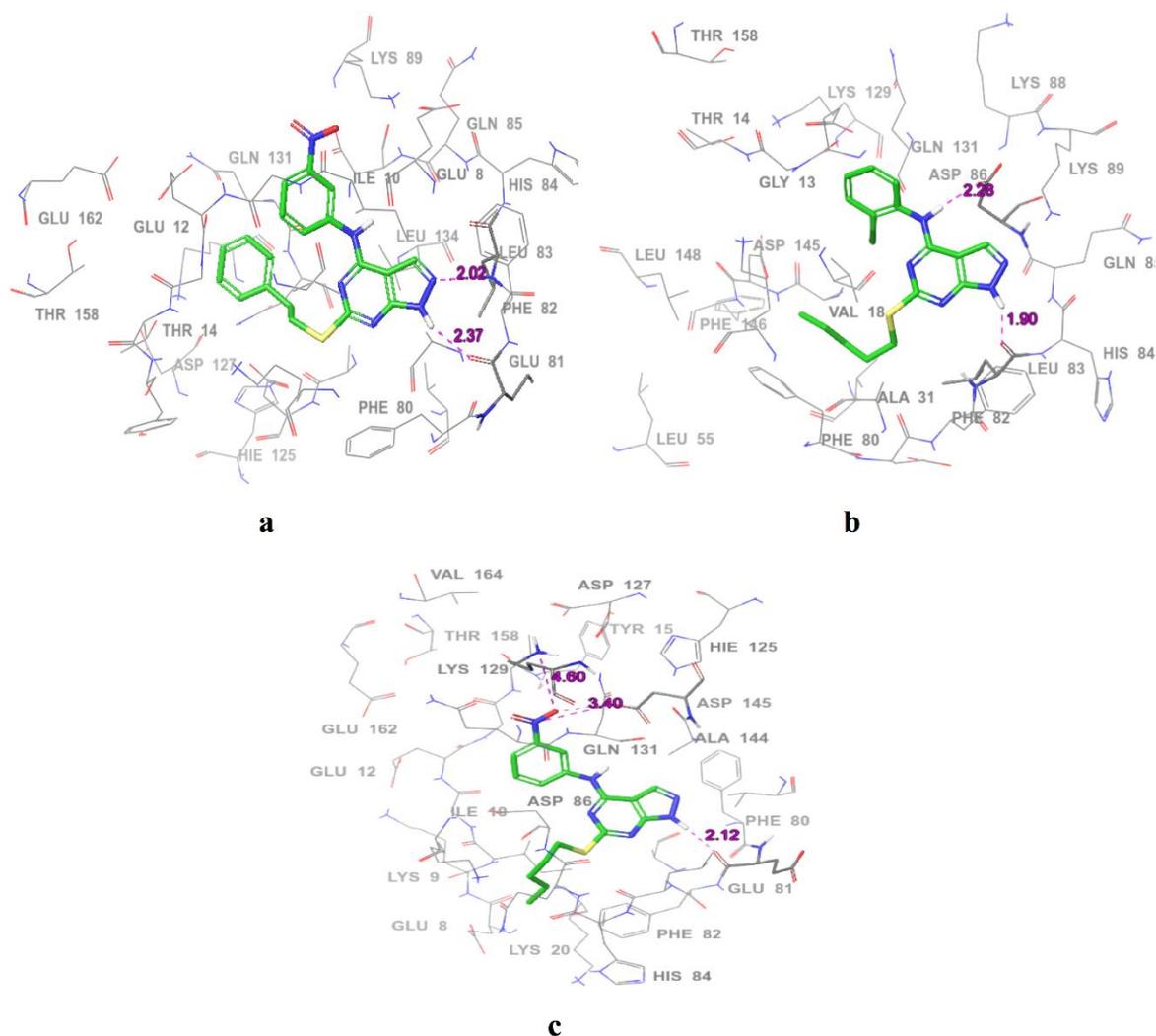
Figure 5. Molecular interactions of a) best active compound **11** b) moderately active compound **27** c) less active compound **30** into the binding site of CDK2/cyclin E protein. Nonpolar hydrogens were hidden for clarity and pink dashed lines indicate H bond.

3 Conclusion

In summary, we have successfully synthesized and characterized a new series of pyrazolo[3,4-*d*]pyrimidine derivatives **7**-**43** in good yields. The key intermediates 4-chloro-6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6a**), 4-chloro-6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6b**) allowed us to generate a library of condensed pyrimidines. All synthesized compounds were evaluated for their *in vitro* enzymatic inhibitory activity against CDK2/cyclin E and four compounds (**8**, **11**, **27**, and **36**) were significantly active with IC$_{50}$ values ranging from 5.1 μM to 8.8 μM. From the SAR, it was clear that the presence of thiophenethyl group at C-6 with mono-substituted anilines at C-4 of the pyrazolo[3,4-*d*]pyrimidine nucleus was essential for anticancer activity. In addition, the binding

energies of the best active compounds were in agreement with the experimental data and supported the SAR studies. However, the moderate to poor activity observed for the majority of the compounds could be attributed to the solubility limit and this is currently being addressed in our laboratory. Thus, these preliminary research findings can further guide the researchers at large in developing novel pyrazolo[3,4-*d*]pyrimidine based CDK2 inhibitors as potential anticancer agents.

4 Experimental Section

All the chemicals used in this research work were purchased from Sigma-Aldrich and Merck Millipore, South Africa. All the solvents, except those of laboratory-reagent grade, were dried and purified when necessary according to previously published methods. The progress of the reactions was monitored by thin-layer chromatography (TLC) on pre-coated silica gel plates procured from E. Merck and Co. (Darmstadt, Germany) using 36% ethyl acetate in *n*-hexane as the mobile phase and iodine vapor as the visualizing agent. Purification of crude compounds were performed by crystallization using appropriate solvent and by flash column chromatography using 100-200 mesh silica gel with Methanol (MeOH) and DCM as solvents. The melting points of the synthesized compounds were determined using a Thermo Fisher Scientific (IA9000, UK) digital melting point apparatus and are uncorrected. The IR spectra were recorded on a Bruker Alpha FT-IR spectrometer (Billerica, MA, USA) using the ATR technique. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 and 600 MHz (Bruker, Rheinstetten/Karlsruhe, Germany) spectrometers using $CDCl_3$ and DMSO-d_6. The chemical shifts are reported in δ ppm units with respect to TMS as an internal standard. HR-MS was recorded on an Autospec mass spectrometer with electron impact at 70 eV.

4.1 Synthesis of 6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (5a)

To a stirred solution of 6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (compound **4**, 0.3g, 0.00179 mol) in *N,N*-dimethyl formamide (2 mL), K_2CO_3 (0.247 g, 0.0017 9 mol) was added and stirred at room temperature for 10 min. To this constantly stirred reaction mass, 2-chloroethyl benzene (0.28 mL, 0.002 mol) was slowly added dropwise and heated at 80 °C for 20 min. in a microwave reactor at 150 psi. After completion of reaction (monitored on TLC), the reaction mixture was poured in ice cold water and extracted with dichloromethane (DCM). The extracted organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure to obtain crude product (dark brown viscous liquid) which was further purified by flash silica column [MeOH/ DCM, 10:90] to afford the desired compound **5a** as light brown solid. Yield: 76%; mp 210-212 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3022 (NH Str.), 2920 (Ar-H Str. of Pyr.), 1671 (C=O Str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.59 (s, 1H, NH), 12.22 (s, 1H, OH), 8.03 (s, 1H, ArH), 7.31 (t, *J* = 2.52 Hz, 4H, ArH), 7.25-7.20 (m, 1H, ArH), 3.41 (t, *J* = 5.04 Hz, 2H, C$\underline{H}_2$), 2.99 (t, *J* = 7.56 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 158.20, 139.93, 128.73, 128.62, 128.41, 126.41, 102.89, 34.61 ($\underline{C}H_2$), 31.08 ($\underline{C}H_2$) ppm.

4.2 Synthesis of 6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (5b)

To a stirred solution of 6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (compound **4**, 1g, 0.005 mol) in 1M NaOH solution (12 mL), 1-bromopentane (1.48 mL, 0.011 mol) was added dropwise and heated at 70 °C for 6h and later slowly brought to RT and continued stirring for overnight. After completion of reaction (monitored on TLC), glacial acetic acid was added dropwise to yield the crude solid, which further washed with petroleum ether and purified by flash silica column [MeOH/DCM, 05:95] to afford the desired compound **5b** as yellow solid. Yield: 80%; mp 201-203 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3180 (NH Str.), 2953 (Ar-H Str.), 1678 (C=O Str.); ^{1}H-NMR (400 MHz, DMSO-*d*$_6$) δ: 13.54 (s, 1H, NH), 12.28 (s, 1H, OH), 7.93 (s, 1H, ArH), 3.15 (t, *J* = 7.26 Hz, 2H, C$\underline{H}_2$), 1.70-1.63 (m, 2H, C$\underline{H}_2$), 1.39-1.26 (m, 4H, (C$\underline{H}_2$)$_2$), 0.86 (t, *J* = 7.08 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ: 159.49, 157.75, 135.24, 30.37 ($\underline{C}H_2$), 29.69 ($\underline{C}H_2$), 28.24 ($\underline{C}H_2$), 21.66 ($\underline{C}H_2$), 13.84 ($\underline{C}H_3$) ppm.

4.3 Synthesis of 4-chloro-6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine(6a)

Vilsmeier-Haack reagent was freshly prepared by the careful addition of POCl$_3$ (3.75 mL, 0.041 mol) in DMF (0.85 mL, 0.011 mol) at 0 °C with constant stirring. To this reaction mixture (maintained at 0 °C), added 6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (5a, 1g, 0.003 mol) and stirred initially for 30 min and later slowly brought to RT and continued stirring for another 30 min. Finally, the reaction mixture was allowed to reflux at 80 °C for 2 h until the TLC showed full consumption of starting material. The reaction mixture was then poured on ice cold water and neutralized with 10% NaOH solution. Thus, the obtained precipitate was filtered under suction and further purified by flash silica column [MeOH/DCM, 05:95] to afford the desired compound **6a**, as light yellow solid. Yield: 85%; mp 218-220 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3208 (NH Str.), 2927 (Ar-H Str.); ^{1}H-NMR (400 MHz, DMSO-*d*$_6$) δ: 14.24 (s, 1H, NH), 8.30 (s, 1H, ArH), 7.31 (t, *J* = 2.48 Hz, 4H, ArH), 7.25-7.19 (m, 1H, ArH), 3.41 (t, *J* = 7.66 Hz, 2H, C$\underline{H}_2$), 3.01 (t, *J* = 7.62 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ: 167.89, 140.07, 128.73, 128.63, 128.39, 126.36, 109.79, 34.58 ($\underline{C}H_2$), 31.93 ($\underline{C}H_2$) ppm.

4.4 Synthesis of 4-chloro-6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (6b)

Vilsmeier-Haack reagent was freshly prepared by the careful addition of POCl$_3$ (4.29 mL, 0.047 mol) in DMF (0.97 mL, 0.012 mol) at 0 °C with constant stirring. To this reaction mixture (maintained at 0 °C) was added 6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (5b, 1g, 0.004 mol) and stirred initially for 30 min and later slowly brought to RT and continued stirring for another 30 min. Finally, the reaction mixture was allowed to reflux at 80 °C for 2 h until the TLC showed full consumption of starting material. The reaction mixture was then poured in ice cold water and neutralized with 10% NaOH solution. Thus, the generated precipitate was filtered under suction and further purified by flash silica column [MeOH/DCM, 05:95] to afford the desired compound **6b** as light yellow solid. Yield: 85%; mp 194-196 °C; FTIR (ATR, cm^{-1})ν_{max}: 3095 (NH Str.), 2956 (Ar-H Str.), 1602 , 1556, 1465; ^{1}H-NMR

(400 MHz, DMSO-d_6) δ: 14.19 (s, 1H, NH), 8.28 (s, 1H, ArH), 3.16 (t, J = 7.32 Hz, 2H, C$\underline{H}_2$), 1.73-1.66 (m, 2H, C$\underline{H}_2$), 1.42-1.27 (m, 4H, (C$\underline{H}_2$)$_2$), 0.87 (t, J = 7.16 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 168.23, 109.70, 30.42 ($\underline{C}H_2$), 30.38 ($\underline{C}H_2$), 28.11 ($\underline{C}H_2$), 21.66 ($\underline{C}H_2$), 13.84 ($\underline{C}H_3$) ppm.

4.5 General procedure for the synthesis of 6-(phenethylthio/pentylthio)-*N*-phenyl-1*H* pyrazolo[3,4-*d*]pyrimidin-4-amines (7-43)

To a well stirred solution of compound **6a** or **6b** (0.2g, 1Eq.) in 10 mL absolute ethanol, was added an appropriately substituted anilines (1.1Eq.) and the reaction mixture was refluxed for 2-3 h until TLC showed full consumption of starting materials. The excess of solvent was evaporated under reduced pressure to yield the crude solids, which were further purified by recrystallization with methanol to afford the desired title compounds (**7-43**).

4.5.1. 6-(phenethylthio)-N-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (7)

White solid; yield: 90%; mp 226-228 °C; FTIR (ATR, cm^{-1})ν$_{max}$: 3363, 3119, 2918, 1621, 1567, 1491, 1470, 1301, 1183; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.44 (s, 1H, NH), 10.05 (s, 1H, ArH), 8.17 (s, 1H, NH), 7.77 (d, J = 7.92 Hz, 2H, ArH), 7.35-7.19 (m, 7H, ArH), 7.10 (t, J = 7.34 Hz, 1H, ArH), 3.36 (t, J = 5.14 Hz, 2H, C$\underline{H}_2$), 2.97 (t, J = 5.14, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.40, 155.51, 153.48, 140.38, 138.90, 128.69, 128.52, 128.31, 126.20, 123.59, 121.34, 98.41, 35.30 ($\underline{C}H_2$), 31.31 ($\underline{C}H_2$) ppm; HRMS (ESI) for C$_{19}$H$_{16}$N$_5$S, [M+H]$^+$ calcd: 346.1126, found: 346.1126.

4.5.2. N-(2-chlorophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (8)

White solid; yield: 92%; mp 268-270 °C; FTIR (ATR, cm^{-1})ν$_{max}$: 3055, 2918, 2718, 1626, 1560, 1379, 1269, 1184; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.47 (s, 1H, NH), 7.98 (s, 1H, NH), 7.62-7.58 (m, 2H, ArH), 7.41-7.08 (m, 8H, ArH), 3.20 (t, J = 7.68 Hz, 2H, C$\underline{H}_2$), 2.83 (t, J = 7.56 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.95, 155.03, 140.21, 134.86, 130.46, 129.95, 129.39, 128.49, 128.29, 127.81, 126.20, 98.12, 35.33 ($\underline{C}H_2$), 31.37 ($\underline{C}H_2$) ppm.

4.5.3. N-(3-chlorophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (9)

White solid; yield: 85%; mp 216-218 °C; FTIR (ATR, cm^{-1})ν$_{max}$: 3311, 3210, 3132, 3023, 2917, 1635, 1558, 1473, 1388, 1309, 1176; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.46 (s, 1H, NH), 8.35 (s, 1H, NH), 8.08 (s, 1H, ArH), 7.73 (dd, J = 8.08, 1.04 Hz, 1H, ArH), 7.36-7.12 (m, 8H, ArH), 3.39 (t, J = 7.62 Hz, 2H, C$\underline{H}_2$), 2.98 (t, J = 7.62 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.27, 155.19, 153.30, 140.47, 140.24, 132.99, 132.48, 130.28, 128.57, 128.32, 126.25, 123.05, 120.55, 119.31, 35.06 ($\underline{C}H_2$), 31.52 ($\underline{C}H_2$) ppm; HRMS (ESI) for C$_{19}$H$_{15}$N$_5$SCl, [M+H]$^+$ calcd: 380.0733, found: 380.0737.

4.5.4. N-(4-chlorophenyl)-6-(3-phenylpropyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (10)

White solid; yield: 85%; mp 286-288 °C; FTIR (ATR, cm^{-1})ν_{max}: 3052, 2911, 2725, 1627, 1566, 1487, 1383, 1276, 1184, 1080, 1015; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.81 (s, 1H, NH), 8.49 (s, 1H, NH), 7.84 (d, J = 8.80 Hz, 2H, ArH), 7.35-7.18 (m, 8H, ArH), 3.37 (t, J = 7.74 Hz, 2H, CH$_2$), 2.95 (t, J = 7.74 Hz, 2H, CH$_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6):δ 166.99, 154.47, 153.53, 140.20, 137.55, 132.23, 128.56, 128.51, 128.31, 127.63, 126.26, 123.16, 98.76, 35.21 (CH$_2$), 31.38 (CH$_2$) ppm.

4.5.5. N-(3-nitrophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (11)

Yellow solid; yield: 81%; mp 249-251°C; FTIR (ATR, cm^{-1})ν_{max}: 3378, 3100, 3030, 2828, 1629, 1566, 1525, 1478, 1429, 1349, 1250, 1109; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.63 (s, 1H, NH), 8.91 (t, J = 2.12 Hz, 1H, ArH), 8.34 (s, 1H, NH), 8.22 (dd, J = 8.06, 1.62 Hz, 1H, ArH), 7.91 (dd, J = 8.14, 1.98 Hz, 1H, ArH), 7.61 (t, J = 8.18 Hz, 1H, ArH), 7.28-7.17 (m, 6H, ArH), 3.41 (t, J = 7.62 Hz, 2H, CH$_2$), 2.97 (t, J = 7.60 Hz, 2H, CH$_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.38, 155.45, 153.12, 147.90, 140.35, 140.23, 132.53, 130.00, 128.47, 128.29, 126.46, 126.22, 117.51, 114.85, 98.72, 35.03 (CH$_2$), 31.44 (CH$_2$) ppm.

4.5.6. N-(4-nitrophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (12)

White solid; yield: 88%; mp 265-267 °C; FTIR (ATR, cm^{-1})ν_{max}: 3033, 2902, 2820, 2752, 1627, 1570, 1515, 1338, 1274, 1183, 1110; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.06 (s, 1H, NH), 9.94 (s, 1H, NH), 8.54 (s, 1H, ArH), 8.17 (s, 4H, ArH), 7.29-7.19 (m, 5H, ArH), 3.41 (t, J = 7.70 Hz, 2H, CH$_2$), 3.00 (t, J = 7.68 Hz, 2H, CH$_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.34, 155.39, 153.01, 145.64, 141.83, 140.27, 132.88, 128.51, 128.32, 126.29, 124.72, 120.14, 99.19, 35.08 (CH$_2$), 31.39 (CH$_2$) ppm; HRMS (ESI) for C$_{19}$H$_{15}$N$_6$O$_2$S, [M+H]$^+$ calcd: 391.0974, found: 391.0977.

4.5.7. N-(2-bromophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (13)

White solid; yield: 85%; mp 266-268 °C; FTIR (ATR, cm^{-1})ν_{max}: 3052, 2921, 2709, 1625, 1585, 1564, 1547, 1507, 1415, 1380, 1268, 1185; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.57 (s, 1H, NH), 7.96 (s, 1H, NH), 7.76 (dd, J = 8.0, 1.0 Hz, 1H, ArH), 7.57 (dd, J = 7.80, 1.52 Hz, 1H, ArH), 7.44 (dd, J = 7.62, 1.16 Hz, 1H, ArH), 7.32-7.24 (m, 4H, ArH), 7.18 (t, J = 7.28 Hz, 1H, ArH), 7.08 (d, J = 7.28 Hz, 2H, ArH), 3.19 (t, J = 7.72 Hz, 2H, CH$_2$), 2.82 (t, J = 7.68 Hz, 2H, CH$_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.85, 155.15, 140.18, 136.35, 133.12, 131.87, 129.79, 128.88, 128.50, 128.29, 126.20, 35.32 (CH$_2$), 31.39 (CH$_2$) ppm.

4.5.8. N-(3-bromophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (14)

White solid; yield: 81%; mp 265-267 °C; FTIR (ATR, cm^{-1})ν_{max}: 3053, 2914, 2724, 1625, 1558, 1507, 1472, 1379, 1273, 1184; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.61 (s, 1H, NH), 8.42 (s, 1H, NH), 8.23 (s, 1H, ArH), 7.80 (t, J = 2.66 Hz, 1H, ArH), 7.29-7.20 (m, 8H, ArH), 3.39 (t, J = 7.58 Hz, 2H, CH$_2$), 2.98 (t, J = 7.56 Hz, 2H, CH$_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.16, 153.39, 140.52, 140.17,

132.46, 130.56, 128.59, 128.33, 126.26, 126.09, 123.55, 121.41, 119.85, 98.75, 35.02 ($\underline{C}H_2$), 31.56 ($\underline{C}H_2$) ppm; HRMS (ESI) for $C_{19}H_{15}N_5SBr$, $[M+H]^+$ calcd: 424.0224, found: 424.0232.

4.5.9. N-(4-bromophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (15)

White solid; yield: 78%; mp 284-286 °C; FTIR (ATR, cm^{-1})ν$_{max}$: 3100, 3025, 2917, 1613, 1579, 1495, 1473, 1304, 1225, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.84 (s, 1H, NH), 8.51 (s, 1H, NH), 7.79 (d, J = 8.76 Hz, 2H, ArH), 7.46 (d, J = 8.80 Hz, 2H, ArH), 7.30-7.18 (m, 6H, ArH), 3.37 (t, J = 5.16 Hz, 2H, CH$_2$), 2.95 (t, J = 7.74 Hz, 2H, CH$_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.98, 153.50, 140.20, 137.97, 132.22, 131.47, 128.51, 128.33, 126.27, 123.52, 115.74, 98.81, 35.21 ($\underline{C}H_2$), 31.38 ($\underline{C}H_2$) ppm.

4.6.0. N-(2,4-dimethylphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (16)

White solid; yield: 76%; mp 238-240 °C; FTIR (ATR, cm^{-1})ν$_{max}$: 3100, 3025, 2917, 1613, 1579, 1495, 1473, 1335, 1304, 1225, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ:13.29 (s, 1H, NH), 9.66 (s, 1H, NH), 7.29-7.04 (m, 9H, ArH), 3.22 (s, 2H, C$\underline{H}_2$), 2.88 (s, 2H, C$\underline{H}_2$), 2.31 (s, 3H, C$\underline{H}_3$), 2.14 (s, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.40, 155.73, 140.50, 133.89, 132.55, 131.23, 128.51, 128.25, 127.04, 126.13, 35.51 ($\underline{C}H_2$), 31.28 ($\underline{C}H_2$), 20.64 ($\underline{C}H_3$), 17.74 ($\underline{C}H_3$) ppm; HRMS (ESI) for $C_{21}H_{20}N_5S$, $[M+H]^+$ calcd: 374.1447, found: 374.1439.

4.6.1. N-(4-(methylthio)phenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (17)

White solid; yield: 90%; mp 262-264 °C; FTIR (ATR, cm^{-1})ν$_{max}$: 3054, 2921, 2809, 2752, 1625, 1583, 1586, 1488, 1417, 1385, 1274, 1243, 1184; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.50 (s, 1H, NH), 8.36 (s, 1H, NH), 7.74 (d, J = 8.52 Hz, 2H, ArH), 7.31-7.19 (m, 8H, ArH), 3.37 (t, J = 7.80 Hz, 2H, C$\underline{H}_2$), 2.96 (t, J = 7.76 Hz, 2H, C$\underline{H}_2$), 2.44 (s, 3H, SC$\underline{H}_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.07, 154.78, 153.49, 140.28, 135.91, 132.20, 128.51, 128.31, 126.70, 126.24, 122.23, 98.58, 35.31 ($\underline{C}H_2$), 31.31 ($\underline{C}H_2$), 15.31 ($\underline{C}H_3$) ppm; HRMS (ESI) for $C_{20}H_{18}N_5S_2$, $[M+H]^+$ calcd: 392.1000, found: 392.1004.

4.6.2. N-(4-chloro-3-(trifluoromethyl)phenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d] pyrimidin-4-amine (18)

White solid; yield: 89%; mp 273-275 °C; FTIR (ATR, cm^{-1})ν$_{max}$: 3033, 2920, 2808, 2752, 1628, 1566, 1507, 1498, 1399, 1315, 1274, 1171, 1153, 1107, 1037; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.93 (s, 1H, NH), 8.49 (d, J = 2.52 Hz, 1H, ArH), 8.47 (s, 1H, NH), 8.18 (dd, J = 8.82, 2.50 Hz, 1H, ArH), 7.61 (d, J = 8.80 Hz, 1H, ArH), 7.29-7.20 (m, 6H, ArH), 3.37 (t, J = 7.68 Hz, 2H, C$\underline{H}_2$), 2.96 (t, J = 7.66 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.21, 155.12, 153.17, 140.21, 138.63, 132.61, 131.89, 126.76, 126.46, 125.52, 124.15, 123.86, 121.44, 119.62, 119.57, 98.83, 35.03 ($\underline{C}H_2$), 31.40 ($\underline{C}H_2$) ppm; HRMS (ESI) for $C_{20}H_{15}N_5F_3SCl$, $[M+H]^+$ calcd: 449.0689, found: 448.0611.

4.6.3. N-(4-bromo-3-(trifluoromethyl)phenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (19)

White solid; yield: 86%; mp 273-275 °C; FTIR (ATR, cm^{-1})ν_{max}: 3033, 2905, 2809, 2752, 1628, 1562, 1507, 1473, 1381, 1315, 1273, 1182, 1131, 1097; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.71 (s, 1H, NH), 8.48 (s, 1H, NH), 8.37 (s, 1H, ArH), 8.08 (t, J =6.48 Hz, 1H, ArH), 7.76 (t, J = 7.44 Hz, 1H, ArH), 7.29-7.20 (m, 6H, ArH), 3.37 (t, J =7.66 Hz, 2H, C$\underline{H}_2$), 2.96 (t, J =7.54 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.27, 153.11, 140.23, 139.12, 135.27, 132.61, 128.50, 128.32, 128.26, 126.26, 125.49, 124.25, 121.53, 119.82, 35.04 (C$\underline{H}_2$), 31.38 (C$\underline{H}_2$) ppm; HRMS (ESI) for C$_{20}$H$_{15}$N$_5$F$_3$SBr, [M+H]$^+$ calcd: 493.0184, found: 492.0105.

4.6.4. N-(4-chloro-3-nitrophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (20)

White solid; yield: 75%; mp 265-267 °C; FTIR (ATR, cm^{-1})ν_{max}: 3023, 2899, 2827, 2759, 1628, 1557, 1531, 1494, 1432, 1376, 1338, 1275, 1182, 1048; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.95 (s, 1H, NH), 8.75 (s, 1H, ArH), 8.43 (s, 1H, NH), 8.16 (d, J = 8.84 Hz, 1H, ArH), 7.68 (dd, J = 8.82, 1.66 Hz, 1H, ArH), 7.29-7.18 (m, 6H, ArH), 3.40 (t, J = 7.58 Hz, 2H, C$\underline{H}_2$), 2.97 (t, J = 7.56 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.28, 155.29, 153.02, 147.02, 140.21, 139.17, 132.71, 131.82, 128.50, 128.30, 126.25, 125.57, 118.35, 117.18, 98.82, 34.98 (C$\underline{H}_2$), 31.45 (C$\underline{H}_2$) ppm.

4.6.5. N-(4-fluoro-2-methylphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (21)

White solid; yield: 80 %; mp 268-270 °C; FTIR (ATR, cm^{-1})ν_{max}: 3103, 3026, 3000, 2934, 1593, 1580, 1492, 1313, 1303, 1210, 1180, 1144; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.34 (s, 1H, NH), 9.72 (s, 1H, NH), 7.39 (q, J = 4.76 Hz, 1H, ArH), 7.29-7.04 (m, 8H, ArH), 3.20 (s, 2H, C$\underline{H}_2$), 2.87 (d, J = 6.92 Hz, 2H, C$\underline{H}_2$), 2.19 (s, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.41, 161.66, 155.71, 140.44, 132.79, 132.48, 128.47, 128.24, 126.15, 117.12, 116.89, 113.21, 113.00, 35.50 (C$\underline{H}_2$), 31.28 (C$\underline{H}_2$), 17.90 (C$\underline{H}_3$) ppm; HRMS (ESI) for C$_{20}$H$_{17}$N$_5$FS, [M+H]$^+$ calcd: 378.1191, found: 378.1189.

4.6.6. N-(3-bromo-4-methylphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (22)

White solid; yield: 85%; mp 279-281 °C; FTIR (ATR, cm^{-1})ν_{max}: 3056, 2923, 2745, 1626, 1583, 1550, 1487, 1383, 1270, 1184, 1145, 1038; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.67 (s, 1H, NH), 8.43 (s, 1H, NH), 8.19 (d, J = 1.84 Hz, 1H, ArH), 7.68 (dd, J = 8.24, 2.00 Hz, 1H, ArH), 7.28-7.18 (m, 7H, ArH), 3.38 (t, J = 7.66 Hz, 2H, C$\underline{H}_2$), 2.96 (t, J = 7.62 Hz, 2H, C$\underline{H}_2$), 2.30 (s, 3H, CH$_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.06, 154.62, 153.45, 140.16, 137.83, 130.85, 128.58, 128.30, 126.25, 124.55, 123.64, 120.54, 119.29, 35.07 (C$\underline{H}_2$), 31.56 (C$\underline{H}_2$), 21.78 (C$\underline{H}_3$) ppm; HRMS (ESI) for C$_{20}$H$_{18}$N$_5$SBr, [M+H]$^+$ calcd: 439.0466, found: 438.0388.

4.6.7. N-(2-chloro-4-fluorophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (23)

White solid; yield: 83 %; mp 282-284 °C; FTIR (ATR, cm^{-1})ν_{max}: 3037, 2907, 2724, 1629, 1569, 1488, 1380, 1269, 1256, 1196, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.28 (s, 1H, NH), 8.08 (s, 1H, NH), 7.65-7.57 (m, 2H, ArH), 7.28-7.17 (m, 5H, ArH), 7.09 (d, J = 10.24 Hz, 2H, ArH), 3.18 (t, J = 7.76 Hz, 2H, C$\underline{H}_2$), 2.82 (d, J = 5.80 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 155.10, 140.17, 131.55, 130.92, 130.82, 128.41, 128.26, 126.21, 117.24, 116.98, 115.09, 114.87, 35.36 ($\underline{C}H_2$), 31.32 ($\underline{C}H_2$) ppm.

4.6.8. N-(3,4-dimethoxyphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (24)

Light yellow solid; yield: 78%; mp 265-267 °C; FTIR (ATR, cm^{-1})ν_{max}: 3063, 2930, 2716, 1629, 1584, 1505, 1457, 1388, 1259, 1232, 1204, 1184, 1129; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.97 (s, 1H, NH), 8.63 (s, 1H, NH), 7.39-7.14 (m, 8H, ArH), 6.85 (d, J = 8.40 Hz, 1H, ArH), 3.73 (s, 3H, OCH$_3$), 3.71 (s, 3H, OC$\underline{H}_3$), 3.38 (t, J = 7.80 Hz, 2H, C$\underline{H}_2$), 2.93 (t, J = 7.74 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.40, 148.58, 140.05, 131.11, 128.52, 128.30, 126.29, 111.69, 55.66 (O-$\underline{C}H_3$), 55.51 (O-$\underline{C}H_3$), 35.19 ($\underline{C}H_2$), 31.46 ($\underline{C}H_2$) ppm.

4.6.9. N-(3-ethynylphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (25)

Light yellow solid; yield: 78%; mp 254-256 °C; FTIR (ATR, cm^{-1})ν_{max}: 3230, 3065, 2914, 2750, 1627, 1571, 1543, 1509, 1381, 1257, 1167, 1051; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.82 (s, 1H, NH), 8.51 (s, 1H, NH), 8.03 (s, 1H, Ar$\underline{H}$), 7.86 (d, J = 4.54 Hz, 1H, ArH), 7.35-7.18 (m, 8H, ArH), 4.18 (s, 1H, Ethynyl-$\underline{H}$), 3.39 (t, J = 7.54 Hz, 2H, C$\underline{H}_2$), 2.97 (t, J = 7.52 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.99, 153.62, 140.13, 138.92, 129.13, 127.04, 126.27, 124.41, 122.09, 122.04, 98.80, 83.30, 80.75, 35.04 ($\underline{C}H_2$), 31.56 ($\underline{C}H_2$) ppm.

4.7.0.   6-(pentylthio)-N-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (26)

White solid; yield: 86%; mp 202-204 °C; FTIR (ATR, cm^{-1})ν_{max}: 3079, 2924, 1624, 1568, 1540, 1507, 1496, 1309, 1185; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.39 (s, 1H, NH), 10.02 (s, 1H, ArH), 8.15 (s, 1H, NH), 7.78 (d, J=7.88 Hz, 2H, ArH), 7.38 (t, J = 7.92 Hz, 2H, ArH), 7.12 (t, J = 7.32 Hz, 1H, ArH), 3.09 (t, J = 7.38 Hz, 2H, C$\underline{H}_2$), 1.66 (m, 2H, C$\underline{H}_2$), 1.25-1.38 (m, 4H, (C$\underline{H}_2$)$_2$), 0.85 (t, J = 7.16 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.73, 155.51, 153.39, 138.96, 128.64, 123.53, 121.22, 98.34, 30.57 ($\underline{C}H_2$), 29.97 ($\underline{C}H_2$), 28.97 ($\underline{C}H_2$), 21.75 ($\underline{C}H_2$), 13.85 ($\underline{C}H_3$) ppm; HRMS (ESI) for C$_{16}$H$_{18}$N$_5$S, [M+H]$^+$ calcd: 312.1284, found: 312.1283.

4.7.1.   N-(2-chlorophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (27)

White solid; yield: 75%; mp 231-233 °C; FTIR (ATR, cm^{-1})ν_{max}: 3054, 2924, 2719, 1624, 1571, 1383, 1273, 1185, 1057; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.48 (s, 1H, NH), 7.99 (S, 1H, NH), 7.57-7.61 (m, 2H, ArH), 7.35-7.44 (m, 2H, ArH), 2.90 (t, J = 7.44 Hz, 2H, C$\underline{H}_2$), 1.46-1.53 (m, 2H, C$\underline{H}_2$), 1.14-1.22 (m, 4H, (C$\underline{H}_2$)$_2$), 0.82 (t, J = 6.86 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.35,

154.87, 134.96, 132.05, 129.84, 129.43, 128.19, 127.70, 97.95, 30.50 (C$\underline{H}_2$), 29.99 (C$\underline{H}_2$), 29.11 (C$\underline{H}_2$), 21.74(C$\underline{H}_2$), 13.87 (C$\underline{H}_3$) ppm.

4.7.2. N-(3-chlorophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (28)

White solid; yield: 92%; mp 224-226 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2928, 1623, 1558, 1541, 1473, 1386, 1304, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.35 (s, 1H, NH), 8.30 (s, 1H, NH), 8.12 (s, 1H, ArH), 7.70 (d, *J* = 8.28 Hz, 1H, ArH), 7.39 (t, *J* = 8.10 Hz, 1H, ArH), 7.15 (dd, *J* = 7.90, 1.70 Hz, 1H, ArH), 3.12 (t, *J* = 7.32 Hz, 2H, C$\underline{H}_2$), 1.63-1.70 (m, 2H, C$\underline{H}_2$), 1.21-1.41 (m, 4H (C$\underline{H}_2$)$_2$), 0.84 (t, *J* = 7.16 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.64, 155.27, 153.21, 140.53, 132.96, 132.43, 130.28, 123.00, 120.47, 119.19, 98.62, 30.51 (C$\underline{H}_2$), 30.14 (C$\underline{H}_2$), 28.75 (C$\underline{H}_2$), 21.76 (C$\underline{H}_2$), 13.87 (C$\underline{H}_3$) ppm.

4.7.3. N-(4-chlorophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (29)

White solid; yield: 88%; mp 253-255 °C; FTIR (ATR, cm^{-1})ν_{max}: 3056, 2923, 2717, 1625, 1567, 1541, 1384, 1267, 1183, 1080, 1013; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.58 (s, 1H, NH), 8.39 (s, 1H, NH), 7.85 (d, *J* = 8.88 Hz, 2H, ArH), 7.43 (d, *J* = 8.88 Hz, 2H, ArH), 3.09 (t, *J* = 7.38 Hz, 2H, C$\underline{H}_2$), 1.61-1.68 (m, 2H, C$\underline{H}_2$), 1.22-1.37 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, *J* = 7.16 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.45, 153.38, 137.76, 132.27, 128.51, 127.40, 122.95, 98.60, 30.58 (C$\underline{H}_2$), 30.12 (C$\underline{H}_2$), 28.88 (C$\underline{H}_2$), 21.76 (C$\underline{H}_2$), 13.87 (C$\underline{H}_3$) ppm.

4.7.4. N-(3-nitrophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (30)

Brown solid; yield: 84%; mp 210-212 °C; FTIR (ATR, cm^{-1})ν_{max}: 3080, 2928, 2757, 1625, 1558, 1522, 1348, 1299, 1175, 1072; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.70 (s, 1H, NH), 8.96 (t, *J* = 2.14 Hz, 1H, ArH), 8.37 (s, 1H, ArH), 8.20-8.23 (m, 1H, ArH), 7.92-7.94 (m, 1H, ArH), 7.66 (t, *J* = 8.22 Hz, 1H, ArH), 3.15 (t, *J* = 7.30 Hz, 2H, C$\underline{H}_2$), 1.62-1.69 (m, 2H, C$\underline{H}_2$), 1.21-1.38 (m, 4H, (C$\underline{H}_2$)$_2$), 0.82 (t, *J* = 7.20 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.72, 155.34, 153.14, 147.93, 140.40, 132.55, 130.00, 126.49, 117.56, 114.85, 98.72, 30.49 (C$\underline{H}_2$), 30.16 (C$\underline{H}_2$), 28.57 (C$\underline{H}_2$), 21.74 (C$\underline{H}_2$), 13.86 (C$\underline{H}_3$) ppm.

4.7.5. N-(4-nitrophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (31)

Yellow solid; yield: 93%; mp 268-270 °C; FTIR (ATR, cm^{-1})ν_{max}: 3108, 2923, 1626, 1568, 1508, 1336, 1297, 1250, 1182, 1111; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.66 (s, 1H, NH), 8.33 (s, 1H, NH), 8.27 (d, *J* = 9.24 Hz, 2H, ArH), 8.13 (d, *J* = 9.24 Hz, 2H, ArH), 3.14 (t, *J* = 7.32 Hz, 2H, C$\underline{H}_2$), 1.66-1.73 (m, 2H, C$\underline{H}_2$), 1.25-1.43 (m, 4H, (C$\underline{H}_2$)$_2$), 0.85 (t, *J* = 7.18 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.77, 155.64, 152.77, 145.70, 141.75, 132.57, 124.79, 119.87, 99.00, 30.60 (C$\underline{H}_2$), 30.12 (C$\underline{H}_2$), 28.73 (C$\underline{H}_2$), 21.77 (C$\underline{H}_2$), 13.86 (C$\underline{H}_3$) ppm.

4.7.6.   *N-(2-bromophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (32)*

White solid; yield: 81%; mp 255-257 °C; FTIR (ATR, cm^{-1})ν_{max}: 3052, 2924, 2725, 1626, 1570, 1542, 1507, 1379, 1275, 1184, 1044; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.53 (s, 1H, NH), 7.96 (s, 1H, NH), 7.75-7.78 (m, 1H, ArH), 7.53-7.56 (m, 1H, ArH), 7.45-7.49 (m, 1H, ArH), 7.29-7.33 (m, 1H, ArH), 2.90 (t, *J* = 7.42 Hz, 2H, C$\underline{H}_2$), 1.45-1.53 (m, 2H, C$\underline{H}_2$), 1.14-1.22 (m, 4H, (C$\underline{H}_2$)$_2$, 0.82 (t, *J* = 6.98 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.15, 155.06, 136.37, 133.01, 131.81, 129.82, 128.80, 128.40, 121.69, 97.97, 30.49 (C$\underline{H}_2$), 30.06 (C$\underline{H}_2$), 29.15 (C$\underline{H}_2$), 21.75 (C$\underline{H}_2$), 13.88 (C$\underline{H}_3$) ppm.

4.7.7.   *N-(3-bromophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (33)*

Brown solid; yield: 76%; mp 232-234 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2927, 1622, 1557, 1472, 1298, 1178, 1063; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.41 (s, 1H, NH), 8.32 (s, 1H, NH), 8.26 (t, *J* = 1.78 Hz, 1H, ArH), 7.75 (d, *J* = 8.04 Hz, 1H, ArH), 7.27-7.35 (m, 2H, ArH), 3.12 (t, *J* = 7.28 Hz, 2H, C$\underline{H}_2$), 1.63-1.71 (m, 2H, C$\underline{H}_2$), 1.23-1.41 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, *J* = 7.22 Hz, 3H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.59, 155.19, 153.18, 140.63, 132.41, 130.56, 125.88, 123.32, 121.39, 119.57, 98.61, 30.49 (C$\underline{H}_2$), 30.13 (C$\underline{H}_2$), 28.70 (C$\underline{H}_2$), 21.75 (C$\underline{H}_2$), 13.86 (C$\underline{H}_3$) ppm.

4.7.8.   *N-(4-bromophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (34)*

White solid; yield: 90%; mp 256-258 °C; FTIR (ATR, cm^{-1})ν_{max}: 3056, 2924, 2718, 1626, 1559, 1474, 1383, 1269, 1183, 1065; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.53 (s, 1H, NH), 8.36 (s, 1H, NH), 7.80 (d, *J* = 8.64 Hz, 2H, ArH), 7.55 (d, *J* = 8.80 Hz, 2H, ArH), 3.09 (t, *J* = 7.36 Hz, 2H, C$\underline{H}_2$), 1.61-1.68 (m, 2H, C$\underline{H}_2$), 1.22-1.37 (m, 4H, (C$\underline{H}_2$)$_2$), 0.85 (t, *J* = 7.08 Hz, 3H, C$\underline{H}_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.48, 153.34, 138.22, 132.27, 131.41, 123.25, 98.61, 30.59 (C$\underline{H}_2$), 30.12 (C$\underline{H}_2$), 28.89 (C$\underline{H}_2$), 21.76 (C$\underline{H}_2$), 13.89 (C$\underline{H}_3$) ppm; HRMS (ESI) for C$_{16}$H$_{17}$N$_5$SBr, [M+H]$^+$ calcd: 390.0383, found: 390.0388.

4.7.9. *N-(2,4-dimethylphenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (35)*

Brown solid; yield: 90%; mp 256-258 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2922, 1614, 1578, 1473, 1315, 1223, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.24 (s, 1H, NH), 9.62 (s, 1H, ArH), 7.17 (t, *J* = 9.64 Hz, 2H, ArH), 7.06 (d, *J* = 7.68 Hz, 1H, ArH), 2.95 (s, 2H, C$\underline{H}_2$), 2.31 (s, 3H, C$\underline{H}_3$), 2.13 (s, 3H, C$\underline{H}_3$), 1.56 (s, 2H, C$\underline{H}_2$), 1.25 (s, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, *J* = 6.68 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.72, 155.74, 133.93, 132.51, 131.16, 127.51, 127.00, 30.58 (C$\underline{H}_2$), 29.80 (C$\underline{H}_2$), 29.10 (C$\underline{H}_3$), 21.76 (C$\underline{H}_2$), 20.62 (C$\underline{H}_3$), 17.72 (C$\underline{H}_2$), 13.90 (C$\underline{H}_3$) ppm; HRMS (ESI) for C$_{18}$H$_{22}$N$_5$S, [M+H]$^+$ calcd: 340.1601, found: 340.1596.

4.8.0.   *N-(4-(methylthio)phenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (36)*

Brown solid; yield: 77%; mp 242-244 °C; FTIR (ATR, cm^{-1})ν_{max}: 3064, 2923, 2724, 1624, 1558, 1520, 1507, 1386, 1185, 1083; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.65 (s, 1H, NH), 8.42 (d, J = 9.84, 1H, NH), 7.75 (d, J = 8.60 Hz, 2H, ArH), 7.29 (d, J = 8.64 Hz, 2H, ArH), 3.09 (t, J = 7.42 Hz, 2H, C$\underline{H}_2$), 2.47 (s, 3H, SC$\underline{H}_3$), 1.60-1.68 (m, 2H, C$\underline{H}_2$), 1.21-1.36 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, J = 7.12 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.25, 153.57, 135.82, 131.96, 126.74, 122.41, 98.61, 30.61 ($\underline{C}H_2$), 30.17 ($\underline{C}H_2$), 28.98 ($\underline{C}H_2$), 21.77 ($\underline{C}H_2$), 15.38 ($\underline{C}H_3$), 13.90 ($\underline{C}H_3$) ppm.

4.8.1. N-(4-chloro-3-(trifluoromethyl)phenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (37)

White solid; yield: 83%; mp 258-260 °C; FTIR (ATR, cm^{-1})ν_{max}: 3101, 2927, 1628, 1567, 1478, 1437, 1385, 1321, 1256, 1176, 1132; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.60 (s, 1H, NH), 8.51 (d, J = 2.52 Hz, 1H, ArH), 8.32 (s, 1H, ArH), 8.10 (dd, J = 8.80, 2.52 Hz, 1H, ArH), 7.71 (d, J = 8.80 Hz, 1H, ArH), 3.10 (t, J = 7.34 Hz, 2H, C$\underline{H}_2$), 1.61-1.68 (m, 2H, C$\underline{H}_2$), 1.21-1.38 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, J = 7.18 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.67, 155.41, 152.99, 138.72, 132.49, 131.94, 126.45, 125.33, 123.73, 119.47, 98.66, 30.48 ($\underline{C}H_2$), 30.01 ($\underline{C}H_2$), 28.57 ($\underline{C}H_2$), 21.73 ($\underline{C}H_2$), 13.83 ($\underline{C}H_3$) ppm.

4.8.2. N-(4-bromo-3-(trifluoromethyl)phenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (38)

Brown solid; yield: 79%; mp 257-259 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2926, 1633, 1559, 1541, 1474, 1436, 1257, 1145, 1097; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.62 (s, 1H, NH), 8.51 (d, J = 2.52 Hz, 1H, ArH), 8.34 (s, 1H, NH), 8.03 (dd, J = 8.74, 2.50 Hz, 1H, ArH), 7.86 (d, J = 8.72 Hz, 1H, ArH), 3.10 (t, J = 7.32 Hz, 2H, C$\underline{H}_2$), 1.61-1.68 (m, 2H, C$\underline{H}_2$), 1.22-1.38 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, J = 7.14 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.64, 155.37, 152.98, 139.16, 135.28, 132.50, 128.54, 125.39, 124.29, 119.73, 111.31, 98.70, 30.48 ($\underline{C}H_2$), 30.00 ($\underline{C}H_2$), 28.56 ($\underline{C}H_2$), 21.73 ($\underline{C}H_2$), 13.83 ($\underline{C}H_3$) ppm; HRMS (ESI) for C$_{17}$H$_{16}$N$_5$F$_3$SBr, [M+H]$^+$ calcd: 458.0258, found: 458.0262.

4.8.3. N-(4-chloro-3-nitrophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (39)

Yellow solid; yield: 91%; mp 251-253 °C; FTIR (ATR, cm^{-1})ν_{max}: 3098, 2925, 1626, 1558, 1540, 1473, 1339, 1297, 1265, 1182; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.73 (s, 1H, NH), 8.78 (d, J = 2.52 Hz, 1H, ArH), 8.33 (s, 1H, ArH), 8.08 (dd, J = 8.92, 2.56 Hz, 1H, ArH), 7.76 (d, J = 8.88 Hz, 1H, ArH), 3.12 (t, J = 7.26 Hz, 2H, C$\underline{H}_2$), 1.61-1.68 (m, 2H, C$\underline{H}_2$), 1.20-1.39 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, J = 7.14 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.70, 155.45, 152.90, 147.12, 139.23, 132.54, 131.83, 125.33, 118.20, 116.94, 98.71, 30.48 ($\underline{C}H_2$), 30.14 ($\underline{C}H_2$), 28.50 ($\underline{C}H_2$), 21.76 ($\underline{C}H_2$), 13.86 ($\underline{C}H_3$) ppm.

4.8.4. N-(4-fluoro-2-methylphenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (40)

White solid; yield: 90%; mp 261-263 °C; FTIR (ATR, cm^{-1})ν_{max}: 3100, 2953, 1577, 1490, 1206, 1181; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.29 (s, 1H, NH), 9.69 (s, 1H, ArH), 7.33-7.36 (m, 1H, ArH), 7.20 (t, J = 4.76 Hz, 1H, ArH), 7.01-7.11 (m, 1H, ArH), 2.93 (s, 2H, C$\underline{H}_2$), 2.18 (s, 3H, C$\underline{H}_3$), 1.54 (s, 2H, C$\underline{H}_2$), 1.22 (s, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, J = 6.96 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.72, 155.80, 132.83, 132.39, 129.41, 117.01, 116.79, 113.15, 112.90, 30.59 ($\underline{C}H_2$), 29.84($\underline{C}H_2$), 29.13 ($\underline{C}H_3$), 21.76 ($\underline{C}H_2$), 17.87 ($\underline{C}H_2$), 13.87 ($\underline{C}H_3$) ppm.

4.8.5. N-(3-bromo-4-methylphenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (41)

Brown solid; yield: 86%; mp 237-239 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2923, 1623, 1556, 1474, 1395, 1301, 1178; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.63 (s, 1H, NH), 8.30 (s, 1H, NH), 8.23 (d, J = 1.84 Hz, 1H, ArH), 7.65 (dd, J = 8.28, 2.08 Hz, 1H, ArH), 7.34 (d, J = 8.32 Hz, 1H, ArH), 3.12 (t, J = 7.28 Hz, 2H, C$\underline{H}_2$), 1.62-1.70 (m, 2H, C$\underline{H}_2$), 1.22-1.40 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, J = 7.22 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.54, 153.23, 138.05, 132.30, 131.98, 130.82, 124.29, 123.61, 120.19, 98.52, 30.50 ($\underline{C}H_2$), 30.13 ($\underline{C}H_2$), 28.72 ($\underline{C}H_2$), 21.75($\underline{C}H_2$), 21.73($\underline{C}H_3$), 13.86 ($\underline{C}H_3$) ppm; HRMS (ESI) for C$_{17}$H$_{19}$N$_5$FS, [M+H]$^+$ calcd: 344.1348, found: 344.1345.

4.8.6. N-(2-chloro-4-fluorophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (42)

White solid; yield: 80%; mp 247-249 °C; FTIR (ATR, cm^{-1})ν_{max}: 3054, 2925, 2716, 1625, 1573, 1488, 1382, 1257, 1181; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.44 (s, 1H, NH), 8.04 (s, 1H, ArH), 7.59-7.62 (m, 2H, ArH), 7.29-7.34 (m, 1H, ArH), 2.89 (t, J = 7.42 Hz, 2H, C$\underline{H}_2$), 1.45-1.52 (m, 2H, C$\underline{H}_2$), 1.16-1.22 (m, 4H, (C$\underline{H}_2$)$_2$), 0.82 (t, J = 6.88 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.20, 161.54, 159.08, 155.10, 131.86, 131.01, 117.13, 116.87, 115.01, 114.78, 98.01, 30.59 ($\underline{C}H_2$), 30.09 ($\underline{C}H_2$), 29.19 ($\underline{C}H_2$), 21.76 ($\underline{C}H_2$), 13.86 ($\underline{C}H_3$) ppm.

4.8.7. N-(3,4-dimethoxyphenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (43)

Brown solid; yield: 78%; mp 247-249 °C; FTIR (ATR, cm^{-1})ν_{max}: 3055, 2951, 2725, 1625, 1507, 1260, 1233, 1026; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.52 (s, 1H, NH), 8.43 (s, 1H, NH), 7.40 (s, 1H, ArH), 7.28 (s, 1H, ArH), 6.97 (d, J = 8.72 Hz, 1H, ArH), 3.76 (d, J = 2.12 Hz, 6H, (OC$\underline{H}_3$)$_2$), 3.10 (t, J = 7.38 Hz, 2H, C$\underline{H}_2$), 1.59-1.64 (m, 2H, C$\underline{H}_2$), 1.20-1.34 (m, 4H, (C$\underline{H}_2$)$_2$), 0.83 (t, J = 7.08 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.72, 153.97, 148.59, 131.38, 114.58, 111.74, 107.58, 98.57, 55.73 (O$\underline{C}H_3$), 55.59 (O$\underline{C}H_3$), 30.50 ($\underline{C}H_2$), 30.28 ($\underline{C}H_2$), 28.81 ($\underline{C}H_2$), 21.72 ($\underline{C}H_2$), 13.83 $\underline{C}H_3$) ppm; HRMS (ESI) for C$_{18}$H$_{22}$N$_5$O$_2$S, [M+H]$^+$ calcd: 372.1496, found: 372.1494.

5   Biological activity

5.1   CDK2 and Abl kinase inhibition assays

CDK2/cyclin E and Abl kinases were produced in Sf9 insect cells via baculoviral infection and purified on a NiNTA column. The kinase reactions were assayed with suitable substrates (1 mg/mL histone H1 for CDK2 and 500 μM peptide GGEAIYAAPFKK for Abl) in the presence of 15 or 10 μM ATP for CDK2 and Abl, respectively, 0.05 μCi [γ-^{33}P]ATP, and the test compound in a final volume of 10 μL, all in a reaction buffer (60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM Na-orthovanadate, 1.2 mM DTT, 2.5 μg / 50 μl $PEG_{20.000}$). The reactions were stopped by adding 5 μL of 3% aq. H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose (Whatman), washed 3× with 0.5% aq. H_3PO_4 and finally air-dried. Kinase inhibition was quantified using a FLA-7000 digital image analyzer. The concentration of the test compounds required to reduce the kinase activity by 50 % was determined from dose-response curves and recorded as their IC_{50}.

5.2 Anti-proliferative evaluation for K-562 and MCF-7 cell lines

The tumor cells (purchased from the American Type Culture Collection) were grown in DMEM medium (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum and L-glutamine (0.3 g/L) and were maintained at 37°C in a humidified atmosphere with 5% CO_2. For anticancer cytotoxicity estimations, 10^4 cells were seeded into each well of a 96-well plate, allowed to stabilize for 20 h, and the test inhibitors were then added at different concentrations (ranging from 0.1 to 100 μM or to a solubility limit) in triplicate. Three days after addition of the inhibitors, calcein AM solution (Molecular Probes) was added. One hour later, fluorescence of cells was quantified using a Fluoroskan Ascent (Labsystems) reader and cytotoxic effective concentrations were calculated and expressed as IC_{50} values from dose-response curves. Roscovitine and imatinib were used as reference drugs.

6 Molecular docking simulation

Molecular docking experiments were performed using Glide software package[36] implemented in Schrodinger Suite (2017-2) (Schrödinger, Inc., USA)[37] running on Intel CORE i7 based hpZ230 workstation with the Microsoft Windows 10 OS. In this protocol, the protein was kept rigid, while the ligands were allowed to be flexible throughout the docking simulation.

6.1 Protein preparation

The starting X-ray solved protein crystal structure of cyclin dependent kinase-2 bound with *R*-roscovitine was retrieved from protein data bank (PDB) bearing ID 2A4L.[35] The protein was prepared by automatic preparation by Protein Preparation Wizard of Glide employing the Optimized Potentials for Liquid Simulations 3 (OPLS3) forcefield. During the pre-processing stage, crystallographic water molecules were removed and added missing hydrogens to the protein structure corresponding to pH 7.0 was achieved. The protein metal ions and cofactors were viewed and removed from the protein structure. The tool neutralized the side chains that are not close to the binding cavity and do not

participate in salt bridges. The pre-processed protein structure was refined initially by optimizing the sample-water orientation followed by restrained minimization of co-crystallized complex using OPLS3, which reorients side chain hydroxyl groups and alleviates potential steric clashes. Thus, the complex obtained was minimized until it reaches the convergent of heavy atom to RMSD 0.3 Å and taken finally in .mae format.

6.2 Grid file generation

Receptor grid generation protocol of Maestro 11.2 was used to define the binding-site of the protein (2A4L) for docking simulation by excluding any co-crystallized metals, co-factors, water molecules all of which may have crystallized during experimental crystallization of the CDK-2 protein. A grid box was generated around the centroid of the cognate ligand (*R*-roscovitine) specifying the size for the docking ligands (20 Å) with default settings.

6.3 Ligand preparation

The structures of the synthesized ligands and standard *R*-roscovitine were sketched using built panel of Maestro and taken in .mae format. LigPrep is a utility of Schrodinger software suit that combines tools for generating 3D structures from 1D (Smiles) and 2D (SDF) representation, searching for tautomers, steric isomers and perform a geometry minimization of the ligands. By employing Ligprep protocol, all the ligands were prepared using OPLS3 with default settings and the output file was saved in maegz format automatically.

6.4 Docking simulation

For precision and accuracy of the docking protocols, the co-crystallized ligand was extracted from the crystal structure of 2A4L and re-docked using Glide docking algorithm (Schrodinger Inc) in its extra precision (XP) mode with default settings without applying any constraints. A good agreement of the obtained pose of docked *R*-roscovitine with cognate ligand indicated the reliability of the selected docking parameters for docking of the synthesized ligands. Hence, by specifying the ligands against the receptor grid, molecular docking was performed using default settings in Glide XP mode.

6.5 Binding mode analysis

The protein-ligand complexes were analysed to investigate various types of interactions by utilizing XP visualizer protocol. For the best-scored ligands, the 2D and 3D plots of molecular ligand-receptor interactions were analysed for hydrogen bond, halogen bond, salt bridges, π-π stacking, and π-cation interactions.

Conflict of Interest

Authors hereby declare that there are no financial/commercial conflicts of interest.

Acknowledgment

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CHAPTER 5

Synthesis of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine analogues: molecular docking, anticancer evaluation as cyclin dependent kinase 2 (CDK2) inhibitors

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Graphical Abstract

Most active compound

Molecular interactions of most active compound in the binding site of CDK2 protein
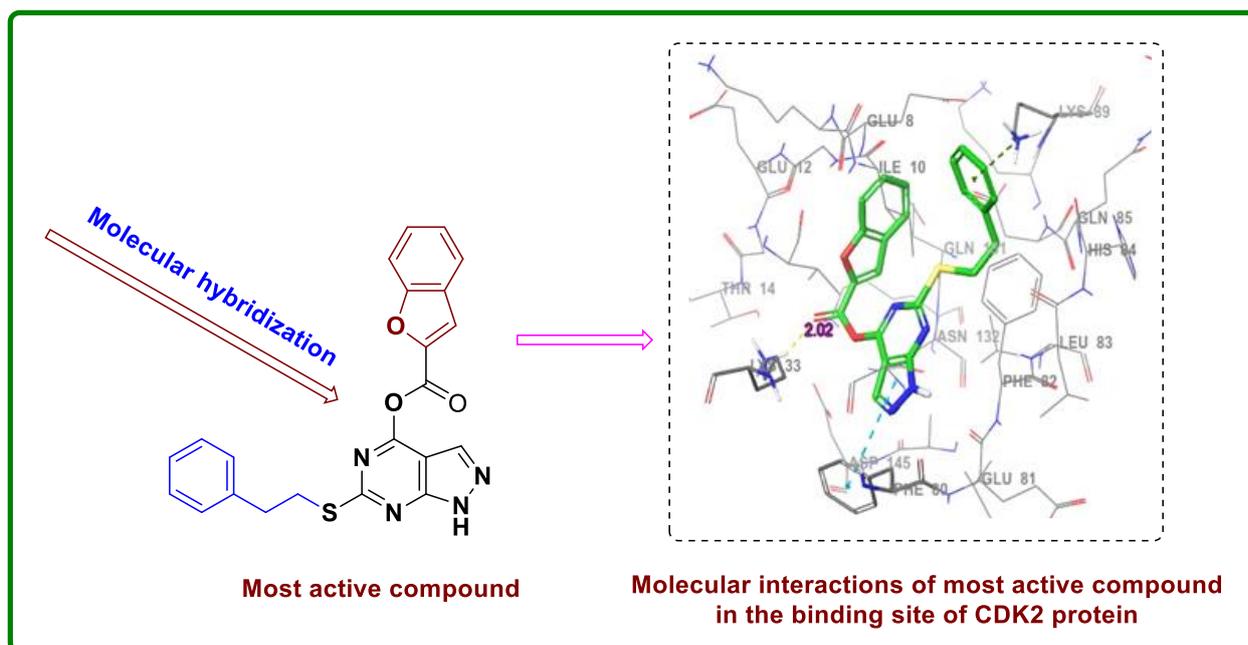
Abstract:

A series of novel 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines (**5a-5h, 6a-6d, 7a-7c**) bearing different mono & bicyclic heterocycles at C-4 position in a combination of pentane/phenethyl/hexane substituents at C-6 position has been designed and synthesized. All the novel compounds were evaluated for *in vitro* CDK2/cyclin E and Abl kinase inhibitory activity as well as anti-proliferative activity against K-562 (chronic myelogeneous leukemia), MCF-7 (breast adenocarcinoma) cell lines. The structure-activity relationship studies (SAR) revealed that the compounds with pentane/phenethyl group at C-6 with heteroatom containing bicyclic moiety at C-4 exhibited commendable CDK2 inhibitory compared to hexane group at C-6 with monocyclic groups at C-4 of the scaffold. From the tested results, compounds having benzofuran moiety at C-4 showed single digit micro molar IC_{50} values. Further from *in silico* molecular docking studies, it was suggested that possible binding orientations and binding energies were in agreement with the observed SAR as well as experimental results. In addition, some of the synthesized compounds indicated anti-proliferative effects against K-562 and MCF-7 cancer cell lines with IC_{50} values in a micromolar range. Thus, the research findings on the pyrazolo[3,4-*d*]pyrimidine hybrids specified the prospective greatness of molecular hybridization and strongly encouraged us for further lead optimization with an aim to develop potential anticancer agents.

Key words: Pyrazolo[3,4-*d*]pyrimidine; Cyclin dependent kinase inhibitor; Anti-proliferative activity; Molecular docking; GLIDE.

1   Introduction

Cancer is one of the most serious health burden touching all over the world. After the cardiovascular, cancer is the second leading cause of death.[1] The understanding of the molecular mechanism of cancer improved strongly in recent years and has deeply impacted on experimental, slowly also on clinical tumor therapy.[2] Predominant efforts are being carried out in order to identify advanced treatments and developments in prevention and chemotherapeutic organization.[3,4] Apart from surgical treatment and irradiation methods, chemotherapy remains a significant route for cancer therapy by the progress of molecular targets that exactly interfere with the vital mechanisms involved in expansion and progression of various types of cancer.[5] Among others, protein kinases have become a significant group of drug targets and number of kinase inhibitors in clinical development is rapidly increasing.[6]

Cyclin-dependent kinases (CDKs) are a family of serine/threonine kinase comprising 20 members, which participates in regulation of cell-cycle progression by phosphorylating proteins in cell division. These enzymes play a crucial role in cell division, transcription, post-transcriptional modification and controlling cell cycle.[7] Among the 20 existing CDK proteins, CDK2 actively participates in the G1/S checkpoint and initiates the cell cycle through S-phase results in apoptosis.[8] For example, formation of active complex composed of CDK2 and cyclin E enables pRb phosphorylation, activation of transcription factor E2F which and initiation of S phase of the cell cycle. CDK2 then also associates with cyclin A, governing continuous DNA replication and properly programed deactivation of E2F. Thus, CDK2 became as prospective target for the treatment of tumors by initiation of apoptotic pathways as contrasting to cell cycle arrest.[9] Up to date many CDK2 have been developed and some of them (including roscovitine, CYC065 , dinaciclib, AT7519, milciclib) undergo clinical evaluation.[10,11] To support these findings, recent research study evidenced that both phosphatidylinositol-3-kinase and CDK2 inhibitors together induced apoptosis in malignant glioma xenografts via a synthetic-lethal interaction.[12] In addition, CDK2 inhibitor also proved as a therapeutic target in ovarian cancer,[13] neuroblastoma[14] and BRCA-deficient cancers.[15] Positively these results, taken together with clinical data, have led to a revival of interest in CDK2 inhibitors as anti-cancer agents.

It is a well-known fact that, broad range of investigations have been conducted on pyrazolo[3,4-*d*]pyrimidines due to their enormous role as potent pharmacophore. Fusion of pyrazole on pyrimidine leads to bicyclic system known as pyrazolopyrimidine, which can be looked upon as the bioisostere of purine, thus exhibits promising antitumor activity by acting as ATP competitive inhibitor for several kinase enzymes.[16,17] Several compounds of this family were found to induce apoptosis and/or reduce cell proliferation in various solid tumor and leukemia cell lines.[18-21] Their cytotoxic activities might be accredited to inhibition of various enzymes such as mammalian target of rapamycin (mTOR),[22] Src/Abl kinase,[23] glycogen synthase kinase (GSK),[24] tyrosine kinase,[25] cyclin dependent kinase (CDK)[26] and

xanthine oxidase inhibitors.[27] Also reported to encompass biological potential as CNS agents, antiviral, anti-inflammatory, herbicidal, anticancer, antimicrobial and cardiovascular activities.[28] **Fig. 2** depicts several reported derivatives of pyrazolo[3,4-*d*]pyrimidine with anticancer activities related to inhibition of different protein kinases.[25, 29-34]

Based on the above mentioned facts and in continuation of our research work on anticancer drug discovery,[35,36] we envisaged to further exploit the pyrazolo[3,4-*d*]pyrimidine scaffold to synthesize novel CDK2 inhibitors. In the present study a novel series of pyrazolo[3,4-*d*]pyrimidine derivatives (**scheme-1**) has been synthesized by substituting various chemical entities at C-4 and C-6 positions through efficient synthetic method. Further, these synthesized molecules were also evaluated against Abl kinase as well as K562 and MCF7 cancer cell lines. In addition, the *in silico* molecular docking studies were performed to analyze the binding energies and orientations of these compounds with respect to the active site of CDK-2 protein. The computational results were in agreement with our experimental observations.

Fig. 1. Structures of the CDK2 inhibitors in clinical trials.
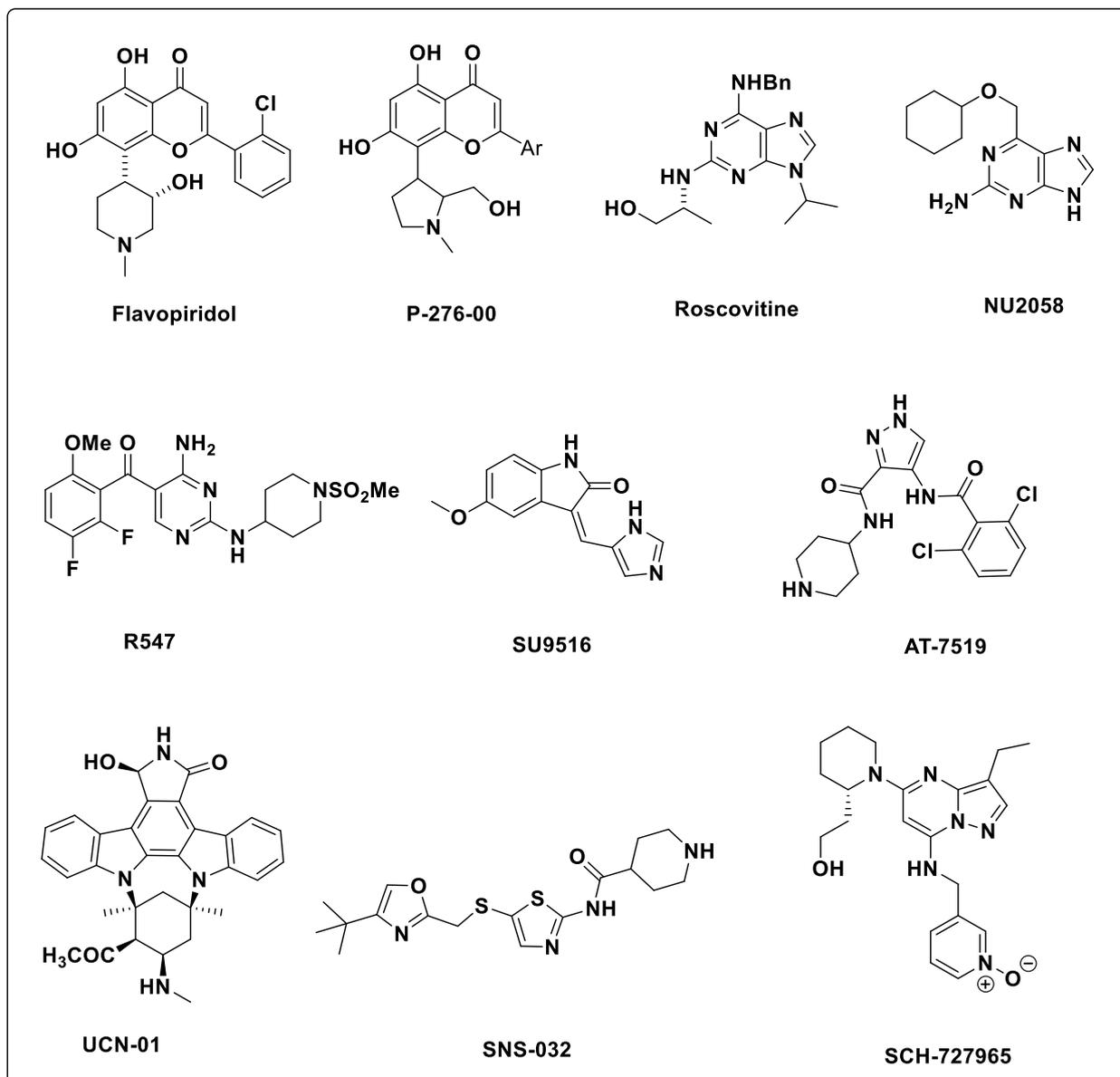
Reported pyrazolo[3,4-d]pyrimidines as anticancer agents
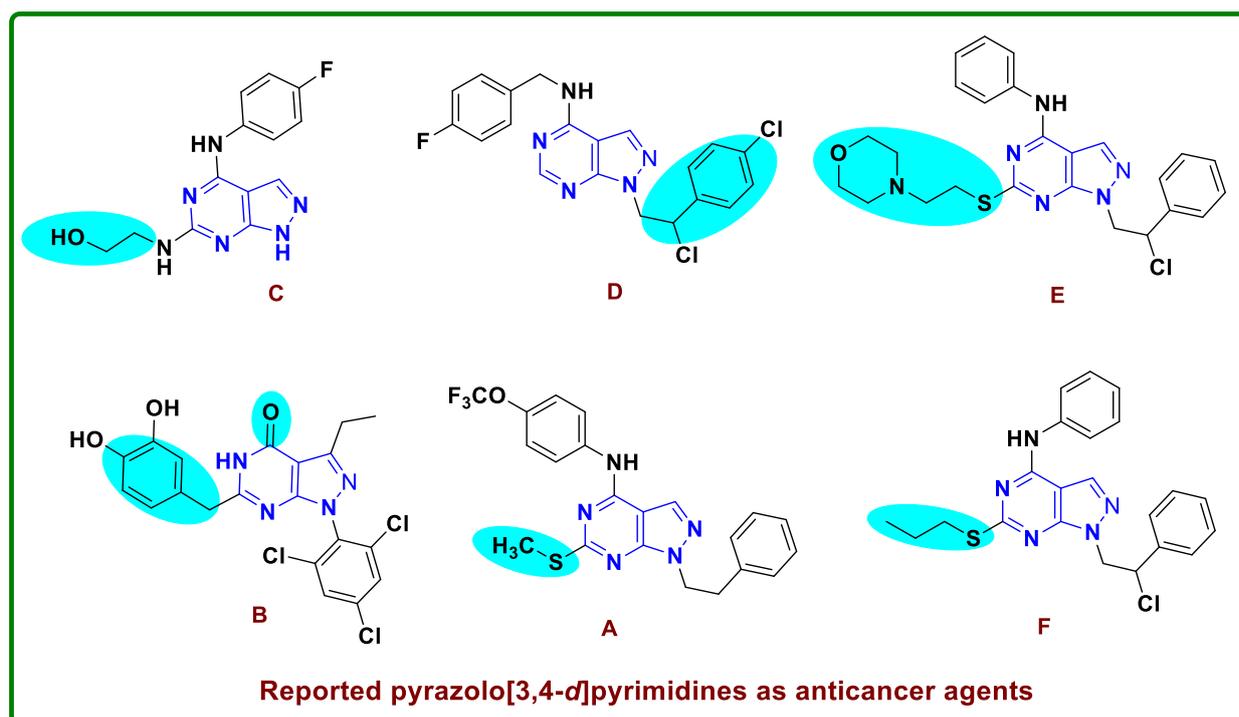
Fig. 2. Known derivatives of pyrazolo[3,4-d]pyrimidine and their anticancer activities. Compound **A**: (K_{i50} against Src, AblT315I = 0.056, 0.01 μM)[29]; **B**: (IC_{50} against CDK2 =0.020 μM)[30]; **C**: (IC_{50} against CDK2 = 0.5 μM)[31]; **D**: (K_{i50} against Abl = 80 nM)[32]; **E**: (K_{i50} against cSrc, Abl = 0.21± 0.02, 0.15±0.02 μM)[33]; **F**: (IC_{50} against Src = 1.2 ± 0.4 μM)[34].

2   Results and discussion

2.1   Chemistry

6-mercapto-1H-pyrazolo[3,4-d]pyrimidin-4-ol (**1**), is a key intermediate for the synthesis of desired hybrid molecules as shown in **Scheme 1**. On the pyrazolopyrimidine scaffold, the hydroxy group was introduced at C-4 as a nucleophilic substituent, which acted as a most reactive site for different nucleophiles, while mercapto group was introduced at C-6 as a precursor to perform the alkylation with different aliphatic and aromatic substituents.

From the previous reported literature methods compound **1** was successfully synthesized.[37] Further, to increase the structural exploration around the nucleus, alkylation of compound **1** was done with 1-bromopentane to acheive 6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-ol (**2**) and also with 2-chloroethyl benzene, 1-bromohexane to obtain 6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-ol (**3**) and 6-(hexylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-ol (**4**) respectively. As displayed in **scheme 1** the nucleophilic substitution of compounds **2**, **3** and **4** at C-4 with acid chlorides was successfully accomplished the final compounds (**5a-5h**, **6a-6d** and **7a-7c**) in THF containing catalytic amount of

pyridine in good yield (70-85%). The IR, ^{1}H and ^{13}C NMR spectroscopic data of all the novel compounds were in agreement with the predicted structures and were further corroborated by HR-MS information, which is precised in supporting information.
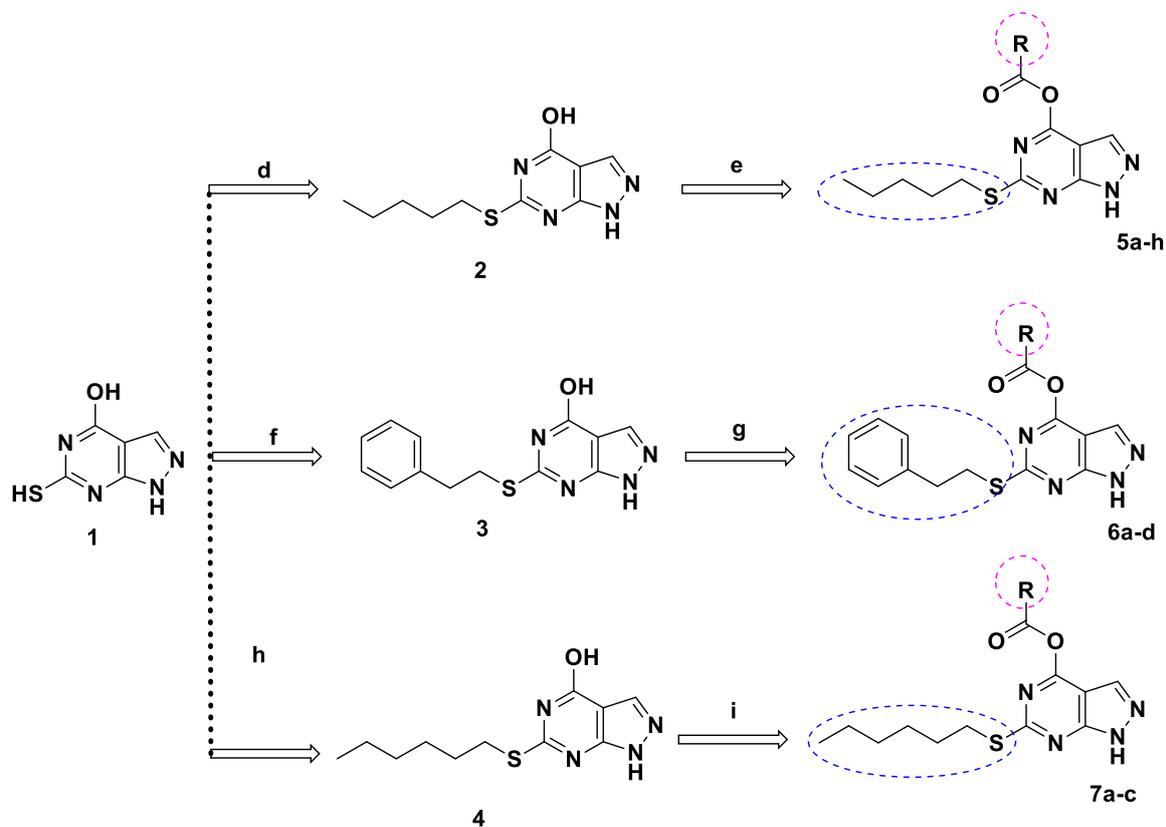
Scheme 1: Synthesis of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine derivatives.

Reagents and conditions: (a) hydrazine hydrate, ethanol, 80 °C, 3h, 92%; (b) Conc. H_2SO_4, NH_4OH, H_2O, 50 °C, 5h, 90%; (c) potassium ethyl xanthogenate, DMF, 120 °C, 6h, 82%; (d) 1-bromopentane, NaOH, H_2O, glacial acetic acid, 50 °C, 5h, RT, overnight, 80%; (e) acid chlorides, pyridine, RT, 2h, 75-85%; (f) 2-chloroethyl benzene, K_2CO_3, DMF, 70 °C, microwave, 20 min, 76%; (g) acid chlorides, pyridine, THF, RT, 2h, 70-85%; (h) 1-bromohexane, NaOH, H_2O, glacial acetic acid, 50 °C, 5h, RT, overnight, 70%; (i) acid chlorides, pyridine, RT, 2h, 65-85%.

The ^{1}H NMR of compound **1** exhibited the presence of a very distinct singlet signals resonating at around δ 13.61, 13.03, 11.86 and 8.42 ppm for N-H proton, S-H proton, O-H proton and Ar-H proton of pyrazole ring. Thus, indicated the formation of bicyclic moiety by fusion of 5-amino-1*H*-pyrazole-4-carboxamide with potassium ethyl xanthogenate. ^{1}H NMR of compounds **2**, **3** and **4** displayed the characteristic methylene signals (-S-C**H₂**-C**H₂**-C**H₂**-C**H₂**-, Ph-C**H₂**-C**H₂**-S- and -S-C**H₂**-C**H₂**-C**H₂**-

C$\underline{H}_2$-C$\underline{H}_2$-) around δ 3.17-2.87, 1.73-1.43, 1.41-1.14 ppm; δ 3.43-3.39, 3.01-2.97 ppm; δ 3.17-3.13, 1.69-1.62, 1.41-1.36, 1.34-1.26 ppm respectively, while the methyl signals for **2** and **4** were observed at around δ 0.86-0.80 ppm. In particular, the disappearance of a distinct singlet signal at around δ 13.60-13.54 ppm for mercapto (-S$\underline{H}$) group evidently indicated the successful alkylation of pyrimidine scaffold. Whereas most distinctive singlet, doublet and triplet signals at around δ 7.32-7.20 ppm was attributed to the aromatic protons of **11** (C$_6\underline{H}_5$-CH$_2$-CH$_2$-) at C-6 of pyrazolopyrimidine ring. These findings were further validated with their respective ^{13}C NMR, where the most prominent methylene carbon signal resonated at around δ 30.37, 29.69, 28.24, 21.66 ppm (-S-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-), δ 34.61 and 31.08 ppm (-C$_6$H$_5$-$\underline{C}$H$_2$-$\underline{C}$H$_2$) and δ 30.74, 29.75, 28.50, 27.85, 22.00 ppm (-S-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-) were assigned for compounds **2**, **3** and **4**. The distinctive methyl peaks (**2** and **4)** appeared around δ 13.84 and 13.88 ppm while the prominent aromatic signals (**2**, **3** and **4**) resonated around δ 173.28-130.19 ppm. Further, the characteristic singlet signal disappeared at around δ 12.28-12.31 ppm, which was accounted for (-O$\underline{H}$) at C-4 evidently indicated the formation of title compounds (**5a**-**5h**, **6a**-**6d** and **7a**-**7c**) with ester linkage as displayed in **scheme 2**.

The IR spectra of the title compounds (**5a**-**5h**, **6a**-**6d** and **7a**-**7c**) showed a reasonably sturdy and distinctive bands around 2904-3290 cm^{-1}, 1232-1292 cm^{-1} accounting for N-H and C-S stretching respectively, while the most distinctive bands of both ester carbonyl group (-**O**-**C**=**O**) and ketone carbonyl (-**C**=**O**) were appearing around 1667-1715 cm^{-1} indicated the formation of title molecules. Further, the ^{1}H NMR signals of the title compounds displayed distinctive singlet signal around δ 12.36-12.17 ppm for cyclic –NH proton, while the hydroxyl proton (-OH) resonated around at δ 11.47-10.65 ppm. For the title compounds the singlet signal resonated around δ 10.78-8.96 ppm for C-3 proton while, the doublet or multiplet aromatic peaks existed around δ 8.61-6.87 ppm. Further, for compounds **5a**-**5h**, **6a**-**6d** and **7a**-**7c** the methylene protons were appeared around δ 3.52-1.23 ppm while, the methyl protons for **5a**-**5h** and **7a**-**7c** were resonated around δ 0.90-0.83 ppm. Further, the most characteristic singlet signals resonated at δ 4.27 ppm, δ 2.57 ppm and δ 1.05 ppm for cyclobutane C-$\underline{H}$ (**5d**), methylthio and -C$\underline{H_3}$)$_3$ (**5c**) protons respectively. The ^{13}C NMR spectra was further confirmed the structures of the final compounds. The characteristic carbon peaks for C-3, C-4 and C-6 of pyrazolopyrimidine ring were appeared at around δ 131.96-130.19, 158.87-158.19 and 173.28-161.87 ppm while, different aromatic carbons were observed between δ 159.78-107.87 ppm. Further, the methylene peaks observed for compounds **5a**-**5h** (-S-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$: δ 30.37-21.66 ppm), **6a**-**6d** (-S-$\underline{C}$H$_2$-$\underline{C}$H$_2$-Ph: δ 34.24-31.20 ppm) and for **7a**-**7c** (-S-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$-$\underline{C}$H$_2$: δ 30.74-22.00 ppm) respectively. The methyl peak for **5a**-**5h** and **7a**-**7c** appeared around δ 13.84-13.36 ppm while, the prominent carbon peaks resonated around δ 13.88 ppm (–S-$\underline{C}$H$_3$), δ 44.80 and 31.04-29.86 ppm (**13c**: -$\underline{C}$H$_2$-C($\underline{C}$H$_3$)$_3$). In addition, the HR-MS spectrums of the final compounds (**5a-5h, 6a-6d** and **7a-7c**)

showed accurate molecular ion peaks, which were in agreement with their expected molecular weights (supporting information).

2.2 *In vitro* evaluation for CDK2 and Abl kinase inhibitors

All the final compounds were evaluated for CDK2/cyclin E kinase inhibition and the IC_{50} values of various *in vitro* anticancer profiles are summarized in **Table 1**. Abl kinase inhibition was evaluated as a counter screen, to get a preliminary information about selectivity. We carried a considerable effort in optimizing the tail groups at both C-4 and C-6 of the pyrazolo[3,4-*d*]pyrimidine scaffold. Thus, substituted various bioactive groups such as pentane, phenethyl, hexane groups at C-6 as well as mono and bicyclic aromatic/heteroaromatic groups at C-4 through ester linkage (**Scheme 1**). The tested compounds from **scheme 1** belongs to three series; pentane (**5a-5h**), phenethyl (**6a-6d**) and hexane series (**7a-7c**). Interestingly, it was observed that the compounds from phenethyl series (**6a-6d**) showed prominent anticancer activity as compared to pentane (**5a-5h**) and hexane (**7a-7c**) series, also observed that the incorporation of aromatics/heteroaromatics at C-4 of the nucleus is necessary for potent activity. From the tested pentane/phenethyl/hexane series, compounds **5a** and **6c** bearing benzofuran moiety at C-4 indicated the best CDK2 activity profile with IC_{50} = 8.8 μM and 6.8 μM respectively. Further, notable activity profile was also observed for compounds bearing 3,3-dimethylbutane (**5c:** IC_{50} = 16.9 μM), phenyl (**7a:** IC_{50} = 14.8 μM) and 2-furan (**7b:** IC_{50} = 21.2 μM) groups. In addition, these pentane/phenethyl/hexane series of compounds where evaluated against Abl kinase, but none of the compounds showed any inhibition in the assayed concentration range, confirming reasonable selectivity towards CDK2 over unrelated Abl.

C. No	R	IC_{50} (μM)[a]			
		CDK2	**Abl**	**K-562**	**MCF-7**
5a		8.8	>12.5	>50	>50
5b		>12.5	>12.5	>50	>50
5c		16.9	>50	73.4	89.3
5d		>25	>25	25.0	23.0
5e		>12.5	>12.5	>25	>25
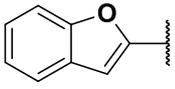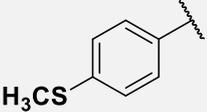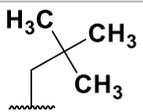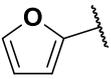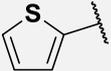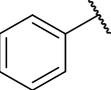
5f		>12.5	>12.5	>25	>25
5g		>12.5	>12.5	>12.5	>12.5
5h		>12.5	>12.5	>50	>50
6a		>12.5	>12.5	>12.5	>12.5
6b		>12.5	>12.5	20.4	25
6c		6.8	>12.5	19.8	18.9
6d		>12.5	>12.5	23.2	18.9
7a		14.8	>12.5	>50	>50
7b		21.2	>25	>12.5	>12.5
7c		>25	>25	>6.25	>6.25
	Roscovitine	0.1	>100	42	11
	Imatinib	>100	0.2	0.5	>10
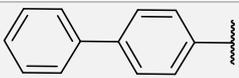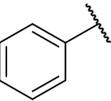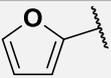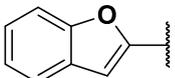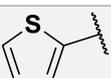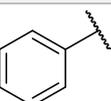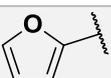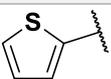
[a] IC_{50} values were determined in triplicate in the range of 0.05 to 100 μM. IC_{50} value indicates concentration (μM) that inhibits activity of tested enzyme to 50% or for cytotoxic assays, concentration (μM) that reduces 50% of cells during a three-day cultivation

Table 1. Anticancer evaluation of novel 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine derivatives.

2.3 Anti-proliferative activity against K-562 and MCF-7 cell line

All the novel mono and disubstituted pyrazolo[3,4-*d*]pyrimidine analogues (**5a-5h**, **6a-6d** and **7a-7c**) were evaluated for their *in vitro* anti-proliferative activity against K-562 (chronic myelogeneous leukemia) and MCF-7 (breast adenocarcinoma) cell lines. Several compounds displaying appreciable activity with measurable IC_{50} values against the two cell lines, such as compounds **6c** (IC_{50} = 19.8, 18.9 μM), **6d** (IC_{50} = 23.2, 18.9 μM) and **6b** (IC_{50} = 20.4, 25 μM). Further, notable anti-proliferative profile

was observed for compounds **5d** (IC$_{50}$ = 25.0, 23.0 µM), and **5c** (IC$_{50}$ = 73.4, 89.3 µM) respectively. The remaining compounds were not active in the tested concentration range.

2.4 Structure-activity relationship (SAR) Studies

In general, careful observation of the structure-activity relationship (SAR) indicated that the anticancer activity was considerably affected by the nature of different substituents present at C-4 and C-6 positions on pyrazolo[3,4-*d*]pyrimidine scaffold. Initially it was observed that the compounds only substituted at C-6 showed least activity whereas compounds substituted at both C-4 and C-6 positions exhibited potent activity, indicated that the incorporation of heterocyclic moieties at C-4 resulted in significant activity. Further analyses of the data revealed that benzofuran group (**5a**, **6c**) at C-4 was more favorable for anticancer and anti-proliferative activity than furan (**5e**) and thiophene (**6d**) indicated that the presence of bi-heterocyclic moiety at C-4 was resulted in best activity. Surprisingly from **scheme 1**, it was observed that phenethyl pentane groups at C-6 showed better activity results than hexane group as illustrated in **Fig. 3**.
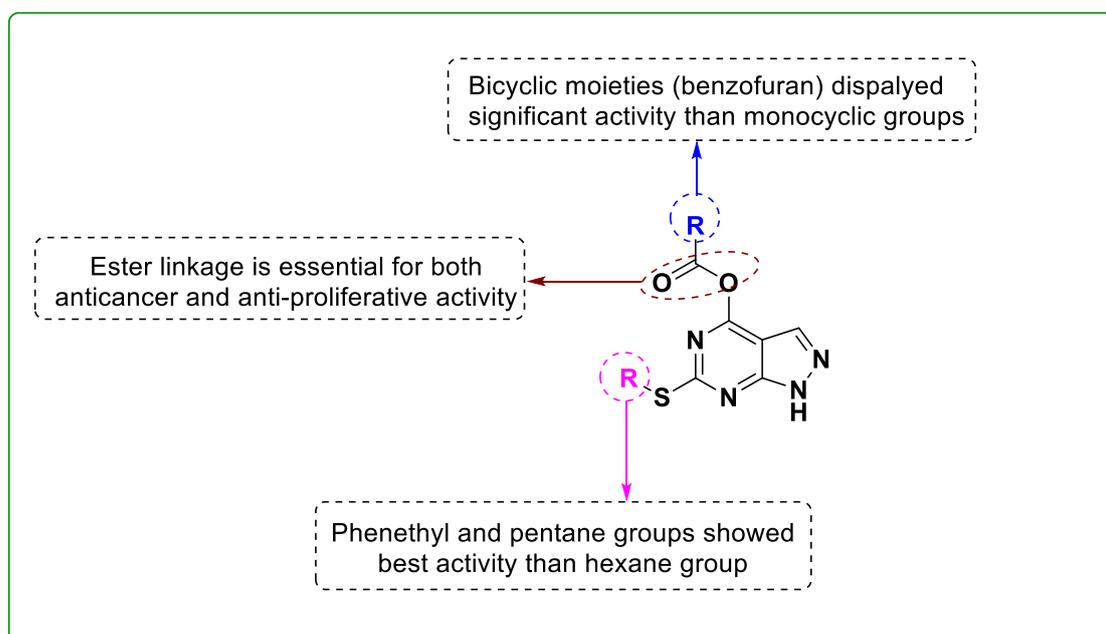
Fig. 3. SAR study around pyrazolo[3,4-*d*]pyrimidine scaffold towards potent activity.

2.5 Molecular docking study

Bioinformatics has become an essential part in the design of therapeutically active novel chemical entity (NCE).[38] From the literature, higher binding affinity reported for a pyrazolo[1,5-*a*]pyrimidine-based experimental drug (dinaciclib) towards CDKs motivated us to conduct molecular docking of our synthesized compounds to further understand and substantiate our observed *in vitro* experimental data.[39]

To validate the docking protocols and to reproduce the protein data bank (PDB) reported orientation of *R*-roscovitine (PDB ID: 2A4L),[40] docking studies were performed using Glide program of Schrodinger-Maestro 11.2. From the docking experiments, the obtained pose of *R*-roscovitine revealed similar molecular interactions as reported in the PDB. The docked complex presented characteristic hydrogen bonding (H-bond) interactions with crucial residues of the active-site, such as Leu83 with roscovitine by forming a strong [C=O with benzylamino NH (1.91 Å)] and a weak [(C=O with ring nitrogen (2.39 Å)] H-bonds. Similarly, the residue Asp86 interacted with OH group of roscovitine *via* a strong H-bond (1.67 Å), whereas Lys89 exhibited π–cation interaction with phenyl ring of *R*-roscovitine. **Fig. 4** represents the reproduced 3D molecular interactions of the docked pose together with the reported pose of *R*-roscovitine.
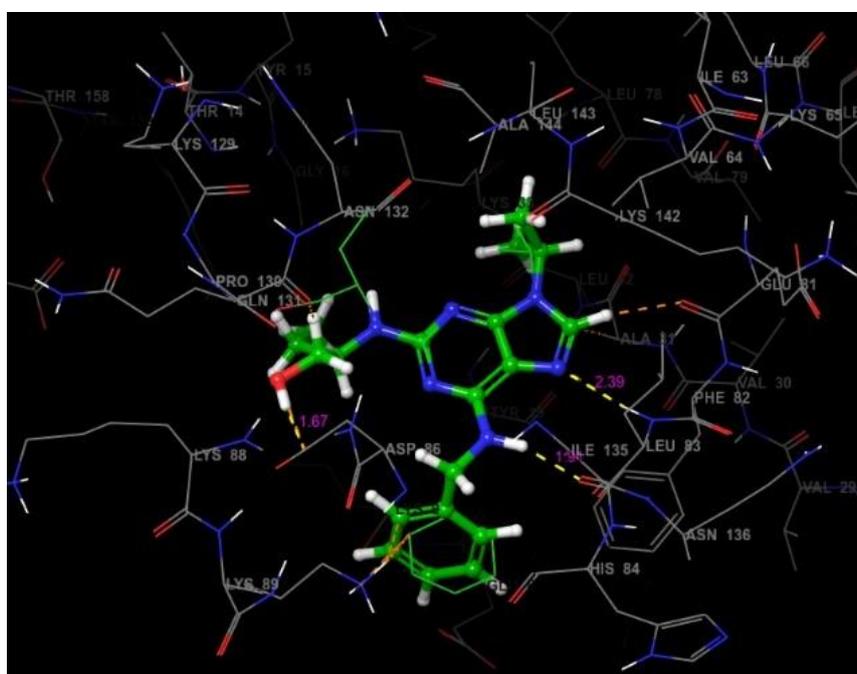
Fig. 4. Reported pose (wire-frame model) and Docked pose (thick tube model) into the active-site showing similar interactions (docking validation).

The docking experimental data of our synthesized compounds revealed that they docked well into the binding-site and displayed favourable interactions with the crucial amino acid residues. Interestingly, the most active compound **6c** showed three significant molecular interactions with crucial amino acid residues of CDK2. The carbonyl oxygen (C=O) of **6c** exhibited a strong H-bond interaction with NH of the basic residue Lys33 (2.02 Å). Further, the pyrazole ring and phenyl ring of thiophenethyl substitution were presented π-π (Phe80) and π-cation (Lys89) interactions, respectively (**Fig. 5a**). Hence, these three molecular interactions were considered as crucial which might have contributed significantly to the potent *in vitro* CDK-2 inhibition.

In the case of less active compound **7c**, the orientation of the docked pose was observed to be different wherein the pyrazole NH presented a strong H-bond interaction with Leu83 (1.68 Å). There is no other characteristic molecular interactions observed for **7c** with active-site residues of CDK2. Hence, lacking of two of the crucial interactions may have contributed to the less *in vitro* CDK-2 inhibition potential of **7c**, while comparing with the most active **6c**. **Fig. 5** presents different molecular interactions of **6c** and **7c** with the active site residues of CDK-2 (PDB ID: 2A4L).
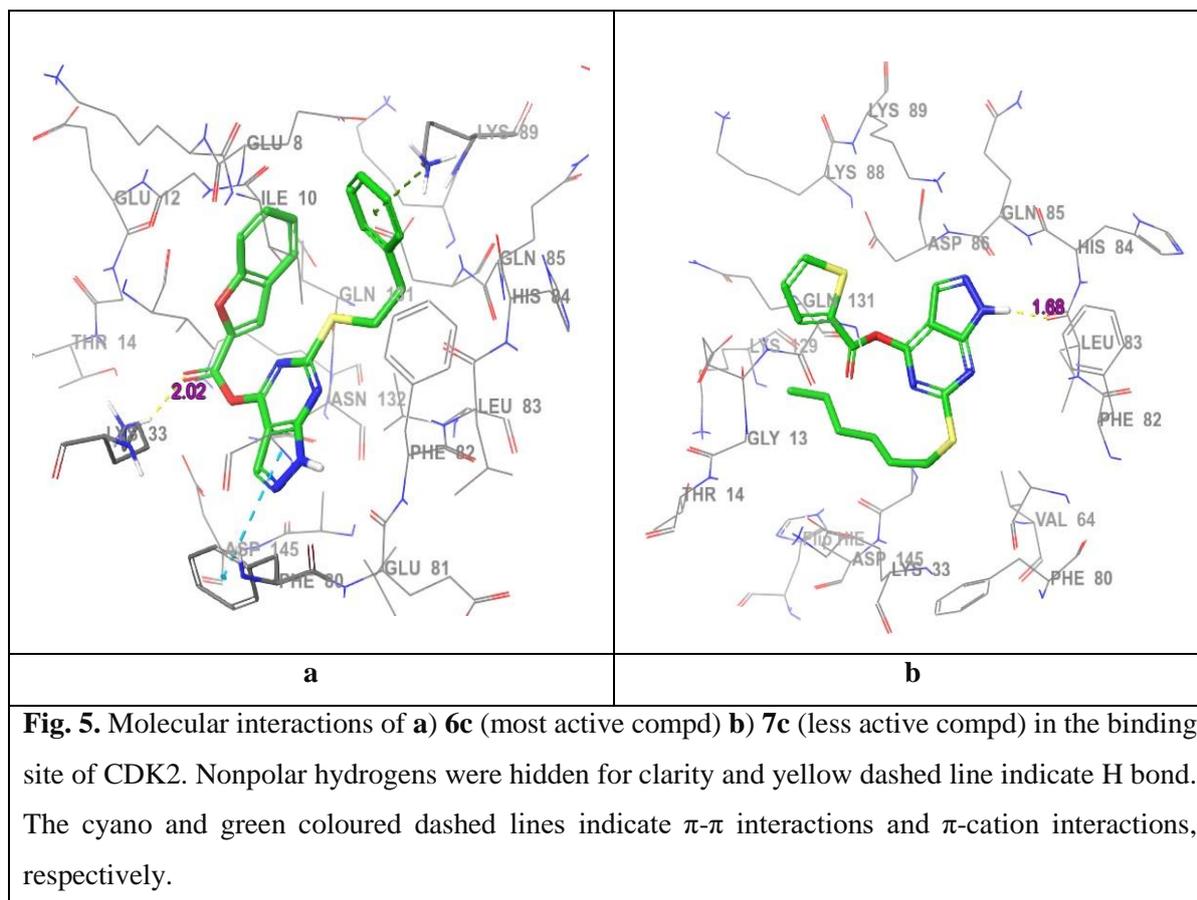
a	b

Fig. 5. Molecular interactions of **a**) **6c** (most active compd) **b**) **7c** (less active compd) in the binding site of CDK2. Nonpolar hydrogens were hidden for clarity and yellow dashed line indicate H bond. The cyano and green coloured dashed lines indicate π-π interactions and π-cation interactions, respectively.

3   Conclusion

In summary, we have successfully synthesized and characterized a new series of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine derivatives with good yields. The key intermediates 6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**1**), 6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**2**), 6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**3**) and 6-(hexylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**4**) allowed us to increase a library of total 34 fused pyrimidine derivatives (**5a-5h**, **6a-6d**, **7a-7c**). All synthesized compounds were evaluated for *in vitro* enzymatic activity against CDK2/cyclin E, Abl kinases as well as anti-proliferative activity against K-562 and MCF-7 cancer cell lines. Interestingly, it was observed that compounds **6c**, **5a**, **7a**, **5c** and **7b** showed most potent CDK2/cyclin E activity with IC$_{50}$ values ranging from 6.8 to 21.2 μM. Further, compounds **6b** (IC$_{50}$=

20.4, 25 µM), **6c** (IC$_{50}$= 19.8, 18.9 µM) and **6d** (IC$_{50}$= 23.2, 18.9 µM) displayed appreciable anti-proliferative activity at specific IC$_{50}$ values. From the SAR study, it was clear that the presence of bi heterocyclic group (benzofuran) at C-4 of the scaffold led to prominent activity. In addition, the *in silico* binding interaction and energies of the best active compound (**6c**) were in agreement with the experimental data and supported the SAR studies. Thus, these research outcomes can further guide the researchers in emerging novel pyrazolo[3,4-*d*]pyrimidine based CDK-2 inhibitors as potential anticancer agents.

4 Experimental Section

4.1 Chemistry protocol

All the chemicals used in this research work were purchased from Sigma-Aldrich and Merck Millipore, South Africa. All the solvents, except those of laboratory-reagent grade, were dried and purified when necessary according to previously published methods. The progress of the reactions and the purity of the compounds were monitored by thin-layer chromatography (TLC) on pre-coated silica gel plates procured from E. Merck and Co. (Darmstadt, Germany) using 36% ethyl acetate in n-hexane as the mobile phase and iodine vapor as the visualizing agent. The melting points of the synthesized compounds were determined using a Thermo Fisher Scientific (IA9000, UK) digital melting point apparatus and are uncorrected. The IR spectra were recorded on a Bruker Alpha FT-IR spectrometer (Billerica, MA, USA) using the ATR technique. The 1H NMR and 13C NMR spectra were recorded on a Bruker AVANCE 400 and 600 MHz (Bruker, Rheinstetten/Karlsruhe, Germany) spectrometers using CDCl3 and DMSO-*d6*. The chemical shifts are reported in δ ppm units with respect to TMS as an internal standard. HRMS spectra was recorded on an Autospec mass spectrometer with electron impact at 70 eV.

4.2 Synthesis of 6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (2)

To a stirred solution of 6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (compound **1**, 1g, 0.00595mol) in 1M NaOH solution (12 mL), 1-bromopentane (1.48 mL, 0.01190mol) was added dropwise and heated at 70^0 C for 6h and later slowly brought to RT and continued stirring for overnight. After completion of reaction (monitored on TLC), glacial acetic acid was added dropwise to yield the crude solid, which further washed with petroleum ether and purified by flash silica column [MeOH/DCM, 05:95] to afford the desired compound (**2**), as yellow solid. Yield: 80 %; mp 201-203 °C; FTIR (ATR, cm^{-1}) ν$_{max}$: 3180.27 (NH Str.), 2953 (Ar C-H Str.), 2925, 1678 (C=O Str.), 1556, 1390, 1240, 1154, 1123, 961, 874, 773, 665, 588, 534; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.54 (s, 1H, NH), 12.28 (s, 1H, OH), 7.93 (s, 1H, ArH), 3.15 (t, *J* = 7.26 Hz, 2H, CH$_2$), 1.70-1.63 (m, 2H, CH$_2$), 1.39-1.26 (m, 4H, (CH$_2$)$_2$), 0.86 (t,

$J = 7.08$ Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 159.49, 157.75, 135.24, 30.37 (C$\underline{H}_2$), 29.69 (C$\underline{H}_2$), 28.24 (C$\underline{H}_2$), 21.66 (C$\underline{H}_2$), 13.84 (C$\underline{H}_3$).

4.3   Synthesis of 6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (3)

To a stirred solution of 6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (compound **1**, 0.3g, 0.00179mol) in *N,N*-dimethyl formamide (2mL), K$_2$CO$_3$ (0.247g, 0.00179mol) was added and stirred at room temperature for 10 min. To this constantly stirred reaction mass, 2-chloroethyl benzene (0.28 mL, 0.00214mol) was slowly added dropwise and heated at 80 °C for 20 minutes in microwave reactor at 150 psi. After completion of reaction (monitored on TLC), the reaction mixture was poured on ice cold water and extracted with dichloromethane (DCM). The extracted organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure to obtain dark brown gel liquid, was further purified by flash silica column [MeOH/DCM, 10:90] to afford the desired compound (**3**), as light brown solid. Yield: 72 %; mp 210-212 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3022 (NH Str.), 2920 (Ar C-H Str. of Pyr.), 1671 (C=O Str.), 1571, 1239, 1144, 963, 952, 774, 757, 701, 617; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.59 (s, 1H, NH), 12.22 (s, 1H, OH), 8.03 (s, 1H, ArH), 7.31 (t, $J = 2.52$ Hz, 4H, ArH), 7.25-7.20 (m, 1H, ArH), 3.41 (t, $J = 5.04$ Hz, 2H, C$\underline{H}_2$), 2.99 (t, $J = 7.56$ Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 158.20, 139.93, 128.73, 128.62, 128.41, 126.41, 102.89, 34.61 (C$\underline{H}_2$), 31.08 (C$\underline{H}_2$) ppm.

4.4   6-(hexylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (4)

To a stirred solution of 6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (compound **1**, 1g, 0.00595mol) in 1M NaOH solution (12 mL), 1-bromohexane (1.65 mL, 0.01190mol) was added dropwise and heated at 70^0 C for 6h and later slowly brought to RT and continued stirring for overnight. After completion of reaction (monitored on TLC), glacial acetic acid was added dropwise to yield the crude solid, which further washed with petroleum ether and purified by flash silica column [MeOH/DCM, 05:95] to afford the desired compound (**4**), as yellow solid. Yield: 75 %; mp 211-213 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3174 (NH Str.), 2925 (Ar C-H Str.), 2851, 1670 (C=O Str.), 1595, 1395, 1240, 1151, 1053, 961, 876, 780, 538; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.54 (s, 1H, NH), 12.28 (s, 1H, OH), 7.93 (s, 1H, ArH), 3.15 (t, $J = 7.22$ Hz, 2H, C$\underline{H}_2$), 1.69-1.62 (m, 2H, C$\underline{H}_2$), 1.41-1.34 (m, 2H, C$\underline{H}_2$), 1.27 (t, $J = 3.62$ Hz, 4H, (C$\underline{H}_2$)$_2$), 0.85 (t, $J = 6.96$ Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 159.48, 157.72, 153.37, 135.23, 102.54, 30.74 (C$\underline{H}_2$), 29.75 (C$\underline{H}_2$), 28.50 (C$\underline{H}_2$), 27.85 (C$\underline{H}_2$), 22.00 (C$\underline{H}_2$), 13.88 (C$\underline{H}_3$) ppm.

4.5   General procedure for synthesis of final compounds (5a-5h, 6a-6d and 7a-7c)

To a constantly stirred solution of compounds 6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**5**), 6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**7**) 6-(hexylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**9**) (0.2g, 0.00074mol) in THF was added pyridine (0.060ml, 0.00047mol) and stirred for 10 min. To this reaction mixture were added different acid chlorides (0.00074mol), continued stirring for 60 min. Progress of the reaction was monitored on TLC. On completion, the reaction mixture was poured into ice cold water to yield the crude solids, which were further purified by recrystallization with ethanol to afford the desired title compounds (**5a-5h, 6a-6d and 7a-7c**).

4.5.1   6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl benzofuran-2-carboxylate (5a)

White solid; yield: 80 %; mp 205-207 °C; FTIR (ATR, cm^{-1}) ν_{max}: 2954 (NH Str.), 2927 (Ar C-H Str.), 2865 (alkane C-H Str.), 1714 (ester C=O Str.), 1442, 1371, 1291, 1177, 1129, 946, 877, 735; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.36 (s, 1H, NH), 9.18 (s, 1H, ArH), 8.58 (s, 1H, ArH), 7.99 (d, *J* = 7.80 Hz, 1H, ArH), 7.79 (d, *J* = 8.52 Hz, 1H, ArH), 7.61 (t, *J* = 7.80 Hz, 1H, ArH), 7.41 (t, *J* = 7.50 Hz, 1H, ArH), 3.19 (t, *J* = 7.24 Hz, 2H, C$\underline{H}_2$), 1.73-1.66 (m, 2H, C$\underline{H}_2$), 1.41-1.28 (m, 4H, (C$\underline{H}_2$)$_2$), 0.88 (t, *J* = 7.08 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.14 (C=O), 159.78, 158.33, 155.54, 155.32, 144.07, 130.46, 129.52, 126.81, 124.62, 124.39, 121.83, 112.17, 108.27, 30.38 (CH$_2$), 29.93 (CH$_2$), 27.99 (CH$_2$), 21.69 (CH$_2$), 13.83 (CH$_3$) ppm; HRMS (ESI, m/z) [M+H]$^+$; calculated for C$_{19}$H$_{18}$N$_4$O$_3$S , 381.1027; found 381.1021.

4.5.2   6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl 4-(methylthio)benzoate (5b)

White solid; yield: 78 %; mp 187-189 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3056 (NH Str.), 2923 (Ar C-H Str.), 2854 (alkane C-H Str.), 1686 (ester C=O Str.), 1586, 1453, 1355, 1239, 1093, 966, 896, 774, 741; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.31 (s, 1H, NH), 9.10 (s, 1H, ArH), 8.06 (d, *J* = 8.56 Hz, 2H, ArH), 7.45 (d, *J* = 8.56 Hz, 2H, ArH), 3.16 (t, *J* = 7.24 Hz, 2H, C$\underline{H}_2$), 2.57 (s, 3H, S-C$\underline{H}_3$), 1.70-1.63 (m, *J* = 7.26 Hz, 2H, C$\underline{H}_2$), 1.40-1.25 (m, 4H, (C$\underline{H}_2$)$_2$), 0.86 (t, *J* = 7.08 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 165.32, 161.52 (C=O), 159.28, 158.54, 146.95, 132.19, 130.61, 125.76, 124.46, 107.90, 30.36 (C$\underline{H}_2$), 29.86 (C$\underline{H}_2$), 28.13 (C$\underline{H}_2$), 21.67 (C$\underline{H}_2$), 13.88 (S-C$\underline{H}_3$), 13.81 (C$\underline{H}_3$) ppm; HRMS (ESI, m/z) [M+H]$^+$; calculated for C$_{18}$H$_{20}$N$_4$O$_2$S$_2$, 387.0944; found 387.0949.

4.5.3   6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl 3,3-dimethylbutanoate (5c)

Light brown solid; yield: 75 %; mp 174-176 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3191 (C-H Str. of CH$_3$), 3090 (N-H Str.), 2929 (Ar C-H Str.), 2856 (alkane C-H Str.), 1667 (ester C=O Str.), 1575, 1388, 1245, 1221, 1155, 964, 938, 750, 664; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.28 (s, 1H, NH), 9.00 (s, 1H, ArH), 3.16 (t, *J* = 7.26 Hz, 2H, C$\underline{H}_2$), 3.12 (s, 2H, (C$\underline{H}_2$), 1.71-1.63 (m, 2H, C$\underline{H}_2$), 1.41-1.26 (m, 4H, (C$\underline{H}_2$)$_2$), 1.05 (s, 9H, (C$\underline{H}_3$)$_3$), 0.87 (t, *J* = 7.12 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 170.74,

161.41 (C=O), 158.87, 158.62, 127.94, 108.30, 44.80, 31.04, 30.38 (C$\underline{\text{H}}_2$), 29.86 (C$\underline{\text{H}}_2$), 29.23, 28.20 (C$\underline{\text{H}}_2$), 21.70 (C$\underline{\text{H}}_2$), 13.84 (C$\underline{\text{H}}_3$) ppm.

4.5.4   6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl cyclobutanecarboxylate (5d)

White solid; yield: 75 %; mp 165-167 °C; FTIR (ATR, cm^{-1}) ν$_{max}$: 3057 (Ar C-H Str.), 2917 (N-H Str.), 2855 (alkane C-H Str.), 1698 (ester C=O Str.), 1599, 1452, 1367, 1249, 1136, 965, 779; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.27 (s, 1H, NH), 8.95 (s, 1H, ArH), 4.29-4.20 (m, 1H, alicyclic C$\underline{\text{H}}$), 3.16 (t, J = 7.24 Hz, 2H, C$\underline{\text{H}}_2$), 2.35-2.29 (m, 4H, alicyclic (C$\underline{\text{H}}_2$)$_2$), 2.11-2.00 (m, 1H, alicyclic C$\underline{\text{H}}$), 1.91-1.82 (m, 1H), 1.71-1.63 (m, 2H, C$\underline{\text{H}}_2$), 1.41-1.28 (m, 4H, (C$\underline{\text{H}}_2$)$_2$), 0.87 (t, J = 7.04 Hz, 3H, C$\underline{\text{H}}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 173.28, 161.32 (C=O), 159.13, 158.57, 128.25, 108.12, 37.41, 30.38 (CH$_2$), 29.84 (CH$_2$), 28.18 (C$\underline{\text{H}}_2$), 24.70, 21.69 (C$\underline{\text{H}}_2$), 17.82, 13.84 (CH$_3$) ppm.

4.5.5   6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl furan-2-carboxylate (5e)

White solid; yield: 80 %; mp 197-199 °C; FTIR (ATR, cm-1) ν$_{max}$: 3058 (Ar C-H Str.), 2974 (N-H Str.), 2887 (alkane C-H Str.), 1688 (ester C=O Str.), 1603, 1459, 1373, 1285, 1084, 936, 863, 763; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.33 (s, 1H, NH), 9.12 (s, 1H, ArH), 8.25 (d, J = 0.80 Hz, 1H, ArH), 8.11 (d, J = 3.60 Hz, 1H, ArH), 6.88 (dd, J = 3.68, 1.64 Hz, 1H, ArH), 3.19 (t, J = 7.24 Hz, 2H, C$\underline{\text{H}}_2$), 1.73-1.65 (m, 2H, C$\underline{\text{H}}_2$), 1.40-1.29 (m, 4H, (C$\underline{\text{H}}_2$)$_2$), 0.88 (t, 7.08 Hz, 3H, C$\underline{\text{H}}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 161.91 (C=O), 159.68, 158.43, 154.15, 150.64, 143.50, 130.19, 126.39, 113.50, 108.03, 30.37 (C$\underline{\text{H}}_2$), 29.91(C$\underline{\text{H}}_2$), 28.09 (C$\underline{\text{H}}_2$), 21.66 (C$\underline{\text{H}}_2$), 13.83 (C$\underline{\text{H}}_3$) ppm; HRMS (ESI, m/z) [M+H]$^+$; calculated for C$_{15}$H$_{16}$N$_4$O$_3$S, 331.0854; found 331.0865.

4.5.6   6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl thiophene-2-carboxylate (5f)

White solid; yield: 85 %; mp 205-207 °C; FTIR (ATR, cm-1) ν$_{max}$: 3040 (Ar C-H Str.), 2927 (N-H Str.), 2854 (alkane C-H Str.), 1691 (ester C=O Str.), 1608, 1452, 1352, 1242, 1088, 887, 773, 426; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 12.34 (s, 1H, NH), 9.12 (s, 1H, ArH), 8.44 (d, J = 3.24 Hz, 1H, ArH), 8.26 (d, J = 4.32 Hz, 1H, ArH), 7.34 (t, J = 4.42 Hz, 1H, ArH), 3.20 (t, J = 7.22 Hz, 2H, C$\underline{\text{H}}_2$), 1.73-1.66 (m, 2H, C$\underline{\text{H}}_2$), 1.42-1.28 (m, 4H, (C$\underline{\text{H}}_2$)$_2$), 0.88 (t, J = 7.02 Hz, 3H, C$\underline{\text{H}}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 161.97 (C=O), 159.33, 158.39, 158.35, 140.13, 139.59, 130.95, 129.92, 128.25, 108.37, 30.33 (C$\underline{\text{H}}_2$), 29.89 (C$\underline{\text{H}}_2$), 28.00 (C$\underline{\text{H}}_2$), 21.62 (C$\underline{\text{H}}_2$), 13.80 (C$\underline{\text{H}}_3$) ppm; HRMS (ESI, m/z) [M+H]$^+$; calculated for C$_{15}$H$_{16}$N$_4$O$_2$S$_2$, 347.0634; found 347.0636.

4.5.7   6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl benzoate (5g)

White solid; yield: 76 %; mp 168-170 °C; FTIR (ATR, cm-1) ν$_{max}$: 3040 (Ar C-H Str.), 2927 (N-H Str.), 2854 (alkane C-H Str.), 1687 (ester C=O Str.), 1597, 1452, 1363, 1238, 1090, 898, 773, 706; ^{1}H-NMR

(400 MHz, DMSO-d_6) δ: 12.32 (s, 1H, NH), 9.13 (s, 1H, ArH), 8.06 (d, J = 7.40 Hz, 2H, ArH), 7.74 (t, J =7.42 Hz, 1H, ArH), 7.60 (t, J = 7.70 Hz, 2H, ArH), 3.16 (t, J = 7.22 Hz, 2H, C$\underline{H}_2$), 1.70-1.63 (m, 2H, C$\underline{H}_2$), 1.39-1.23 (m, 4H, (CH$_2$)$_2$), 0.85 (t, J =7.04 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.24, 161.61 (C=O), 159.33, 158.53, 133.65, 131.44, 130.64, 130.45, 128.29, 108.11, 30.33 ($\underline{C}H_2$), 29.84 ($\underline{C}H_2$), 28.11 ($\underline{C}H_2$), 21.64 ($\underline{C}H_2$), 13.79 ($\underline{C}H_3$) ppm; HRMS (ESI, m/z) [M+H]$^+$; calculated for C$_{17}$H$_{18}$N$_4$O$_2$S , 341.1071; found 341.1072.

4.5.8   6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl [1,1'-biphenyl]-4-carboxylate (5h)

White solid; yield: 84 %; mp 173-175 °C; FTIR (ATR, cm-1) ν$_{max}$: 3036 (Ar C-H Str.), 2904 (N-H Str.), 2873 (alkane C-H Str.), 1691 (ester C=O Str.), 1604, 1448, 1367, 1283, 1253, 1137, 903, 735, 692; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.17 (s, 1H, NH), 9.09 (s, 1H, ArH), 8.21 (d, J = 8.34 Hz, 2H, ArH), 7.90 (d, J = 8.34 Hz, 2H, ArH), 7.79 (d, J = 7.56 Hz, 2H, ArH), 7.53 (t, J = 7.59 Hz, 2H, ArH), 7.45 (t, J = 7.47 Hz, 1H, ArH), 3.20 (t, J = 7.26 Hz, 2H, C$\underline{H}_2$), 1.73-1.68 (m, 2H, C$\underline{H}_2$), 1.42-1.30 (m, 4H, (C$\underline{H}_2$)$_2$), 0.87 (t, J = 7.20 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 165.51, 161.43 (C=O), 159.17, 158.19, 144.98, 138.50, 131.96, 128.92, 128.82, 128.29, 126.79, 126.23, 107.87, 30.04 ($\underline{C}H_2$), 29.75 ($\underline{C}H_2$), 27.84 ($\underline{C}H_2$), 21.27 ($\underline{C}H_2$), 13.36 ($\underline{C}H_3$) ppm.

4.5.9   6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl benzoate (6a)

White solid; yield: 80 %; mp 198-200 °C; FTIR (ATR, cm-1) ν$_{max}$: 3061 (Ar C-H Str.), 2982 (N-H Str.), 2880 (alkane C-H Str.), 1704 (ester C=O Str.), 1684, 1597, 1455, 1362, 1232, 1135, 898, 698; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.34 (s, 1H, NH), 9.15 (s, 1H, ArH), 8.08 (d, J = 7.32 Hz, 1H, ArH), 7.74 (t, J =7.46 Hz, 1H, ArH), 7.61 (t, J =7.74 Hz, 2H, ArH), 7.32-7.26 (m, 4H, ArH), 7.23-7.19 (m, 1H, ArH), 3.45 (t, J = 7.34 Hz, 2H, C$\underline{H}_2$), 3.0 (t, J =7.34 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.26, 161.41 (C=O), 159.35, 158.55, 139.77, 133.72, 131.51, 130.75, 130.45, 128.60, 128.40, 128.33, 126.42, 108.15, 34.23 ($\underline{C}H_2$), 31.20 ($\underline{C}H_2$) ppm; HRMS (ESI, m/z) [M+H]$^+$; calculated for C$_{20}$H$_{16}$N$_4$O$_2$S , 375.0920; found 375.0916.

4.6.0   6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-ylfuran-2-carboxylate (6b)

White solid; yield: 78 %; mp 230-232 °C; FTIR (ATR, cm-1) ν$_{max}$: 3072 (Ar C-H Str.), 2927 (N-H Str.), 2855 (alkane C-H Str.), 1688 (ester C=O Str.), 1608, 1453, 1419, 1368, 1271, 1243, 1139, 1090, 865, 773, 693; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.34 (s, 1H, NH), 9.14 (s, 1H, ArH), 8.27 (d, J = 0.96 Hz, 1H, ArH), 8.14 (d, J = 3.44 Hz, 1H, ArH), 7.32 (d, J = 4.48Hz, 4H, ArH), 7.23 (m, 1H, ArH), 6.89 (dd, J = 3.7, 1.6 Hz, 1H, ArH), 3.47 (t, J = 7.42 Hz, 2H, C$\underline{H}_2$), 3.03 (t, J = 7.44 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.65 (C=O), 159.64, 158.38, 154.11, 150.63, 143.49, 139.85, 130.22, 128.63, 128.43, 126.42, 113.52, 108.05, 34.27 ($\underline{C}H_2$), 31.28 ($\underline{C}H_2$) ppm.

4.6.1 6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl benzofuran-2-carboxylate (6c)

White solid; yield: 75 %; mp 230-232 °C; FTIR (ATR, cm-1) ν_{max}: 3120 (Ar C-H Str.), 3028 (N-H Str.), 2876 (alkane C-H Str.), 1706 (ester C=O Str.), 1604, 1542, 1542, 1454, 1373, 1292, 1141, 946, 866, 742, 694; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.37 (s, 1H, NH), 9.21 (s, 1H, ArH), 8.61 (s, 1H, ArH), 8.01 (d, J =7.84 Hz, 1H, ArH), 7.80 (d, J = 8.36 Hz, 1H, ArH), 7.61 (t, J = 7.78 Hz, 1H, ArH), 7.42 (t, J =7.54 Hz, 1H, ArH), 7.33 (d, J = 4.36 Hz, 4H, ArH), 7.24 (m, 1H, ArH), 3.50 (t, J = 7.36 Hz, 2H, C$\underline{H}_2$), 3.04 (t, J = 7.40 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 161.92 (C=O), 159.78, 158.32, 155.55, 155.33, 144.09, 139.81, 130.53, 129.53, 128.64, 128.44, 126.83, 126.46, 124.68, 124.39, 121.87, 112.17, 108.30, 34.13 (C$\underline{H}_2$), 31.30 (C$\underline{H}_2$) ppm.

4.6.2 6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl thiophene-2-carboxylate (6d)

White solid; yield: 80 %; mp 252-254 °C; FTIR (ATR, cm-1) ν_{max}: 3124 (Ar C-H Str.), 3027 (N-H Str.), 2879 (alkane C-H Str.), 1693 (ester C=O Str.), 1606, 1454, 1374, 1350, 1244, 1093, 887, 742, 725, 693; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.35 (s, 1H, NH), 9.14 (s, 1H, ArH), 8.45 (d, J = 2.80 Hz, 1H, ArH), 8.26 (d, J = 4.40 Hz, 1H, ArH), 7.32 (d, J = 3.92 Hz, 5H, ArH), 7.23 (d, J = 3.84 Hz, 1H, ArH), 3.48 (t, J = 7.18 Hz, 2H, C$\underline{H}_2$), 3.03 (t, J = 7.14 Hz, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 161.76 (C=O), 159.30, 158.37, 140.21, 139.86, 139.62, 130.95, 129.93, 128.62, 128.43, 128.25, 126.43, 108.40, 34.24 (C$\underline{H}_2$), 31.34 (C$\underline{H}_2$) ppm; HRMS (ESI, m/z) [M+H]$^+$; calculated for $C_{18}H_{14}N_4O_2S_2$, 381.0481; found 381.0480.

4.6.3 6-(hexylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl benzoate (7a)

White solid; yield: 76 %; mp 167-169 °C; FTIR (ATR, cm-1) ν_{max}: 3140 (Ar C-H Str.), 2924 (N-H Str.), 2847 (alkane C-H Str.), 1713 (ester C=O Str.), 1610, 1449, 1368, 1285, 1203, 1090, 900, 705; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.33 (s, 1H, NH), 9.14 (s, 1H, ArH), 8.06 (d, J = 7.32 Hz, 2H, ArH), 7.74 (t, J = 7.40 Hz, 1H, ArH), 7.60 (t, J = 7.76 Hz, 2H, ArH), 3.16 (t, J = 7.24 Hz, 2H, C$\underline{H}_2$), 1.69-1.62 (m, 2H, C$\underline{H}_2$), 1.41-1.34 (m, 2H, C$\underline{H}_2$), 1.28-1.24 (m, 4H, (CH$_2$)$_2$), 0.84 (t, J = 6.74 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.27, 161.63 (C=O), 159.34, 158.55, 133.67, 131.46, 130.67, 130.46, 128.30, 108.12, 30.71 (C$\underline{H}_2$), 29.87 (C$\underline{H}_2$), 28.40 (C$\underline{H}_2$), 27.83 (C$\underline{H}_2$), 21.96 (C$\underline{H}_2$), 13.87 (C$\underline{H}_3$) ppm.

4.6.4 6-(hexylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl thiophene-2-carboxylate (7b)

White solid; yield: 78 %; mp 205-207 °C; FTIR (ATR, cm-1) ν_{max}: 3143 (Ar C-H Str.), 2915 (N-H Str.), 2850 (alkane C-H Str.), 1692 (ester C=O Str.), 1606, 1453, 1372, 1350, 1243, 1140, 1088, 887, 853,724; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.35 (s, 1H, NH), 9.14 (s, 1H, ArH), 8.45 (d, J = 3.28 Hz, 1H, ArH), 8.26 (d, J = 4.68 Hz, 1H, ArH), 7.35 (t, J = 4.34 Hz, 1H, ArH), 3.21 (t, J = 7.14 Hz, 2H, C$\underline{H}_2$),

1.73-1.66 (m, 2H, C$\underline{H}_2$), 1.40 (d, J = 6.00 Hz, 2H, C$\underline{H}_2$), 1.29 (d, J = 3.36 Hz, 4H, (C$\underline{H}_2$)$_2$), 0.87 (t, J = 6.42 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 161.99 (C=O), 159.35, 158.41, 158.37, 140.15, 139.61, 130.97, 129.98, 128.29, 108.38, 30.70 (C$\underline{H}_2$), 29.93 (C$\underline{H}_2$), 28.30 (C$\underline{H}_2$), 27.82 (C$\underline{H}_2$), 21.97 (C$\underline{H}_2$), 13.90 (C$\underline{H}_3$) ppm; HRMS (ESI, m/z) [M+H]$^+$; calculated for C$_{16}$H$_{18}$N$_4$O$_2$S$_2$, 361.0793; found 361.0793.

4.6.5   6-(hexylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl furan-2-carboxylate (7c)

White solid; yield: 78 %; mp 152-154 °C; FTIR (ATR, cm-1) ν$_{max}$: 3092 (Ar C-H Str.), 2924 (N-H Str.), 2849 (alkane C-H Str.), 1712 (ester C=O Str.), 1604, 1462, 1376, 1288, 1263, 1146, 936, 865, 772; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.34 (s, 1H, NH), 9.14 (s, 1H, ArH), 8.27 (s, 1H, ArH), 8.13 (d, J = 3.60 Hz, 1H, ArH), 6.89 (s, 1H, ArH), 3.22-3.13 (m, 2H, C$\underline{H}_2$), 1.73-1.62 (m, 2H, CH$_2$), 1.40 (s, 2H, C$\underline{H}_2$), 1.29 (d, J = 3.60 Hz, 4H, (CH$_2$)$_2$), 0.86 (d, J = 5.60 Hz, 3H, CH$_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 161.87 (C=O), 159.65, 158.41, 158.38, 154.12, 143.48, 130.21, 129.98, 126.34, 113.46, 108.02, 30.69 (C$\underline{H}_2$), 29.89 (C$\underline{H}_2$), 28.35 (C$\underline{H}_2$), 27.82 (C$\underline{H}_2$), 21.95 (C$\underline{H}_2$), 13.88 (C$\underline{H}_3$) ppm; HRMS (ESI, m/z) [M+H]$^+$; calculated for C$_{16}$H$_{18}$N$_4$O$_3$S, 345.1017; found 345.1021.

5   Biological activity protocol

5.1   CDK2 and Abl kinase inhibition assays

CDK2/cyclin E and Abl kinases were produced in Sf9 insect cells via baculoviral infection and purified on a NiNTA column. The kinase reactions were assayed with suitable substrates (1 mg/mL histone H1 for CDK2 and 500 μM peptide GGEAIYAAPFKK for Abl) in the presence of 15 or 10 μM ATP for CDK2 and Abl, respectively, 0.05 μCi [γ-^{33}P]ATP, and the test compound in a final volume of 10 μL, all in a reaction buffer (60 mM HEPES-NaOH, pH 7.5, 3 mM MgCl$_2$, 3 mM MnCl$_2$, 3 μM Na-orthovanadate, 1.2 mM DTT, 2.5 μg / 50 μl PEG$_{20.000}$). The reactions were stopped by adding 5 μL of 3% aq. H$_3$PO$_4$. Aliquots were spotted onto P-81 phosphocellulose (Whatman), washed 3× with 0.5% aq. H$_3$PO$_4$ and finally air-dried. Kinase inhibition was quantified using a FLA-7000 digital image analyzer. The concentration of the test compounds required to reduce the kinase activity by 50 % was determined from dose-response curves and recorded as their IC$_{50}$.

5.2   Anti-proliferative (K-562 and MCF-7) activity assays

The tumor cells (purchased from the American Type Culture Collection) were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum and L-glutamine (0.3 g/L) and were maintained at 37 °C in a humidified atmosphere with 5% CO$_2$. For anticancer cytotoxicity estimations, 10^4 cells were seeded into each well of a 96-well plate, allowed to stabilize for 20 h, and the test

inhibitors were then added at different concentrations (ranging from 0.1 to 100 μM or to a solubility limit) in triplicate. Three days after addition of the inhibitors, calcein AM solution (Molecular Probes) was added. One hour later, fluorescence of cells was quantified using a Fluoroskan Ascent (Labsystems) reader and cytotoxic effective concentrations were calculated and expressed as IC_{50} values from dose-response curves. Roscovitine and imatinib were used as reference drugs.

6   Molecular docking simulation

Molecular docking experiments were performed using Glide software package[41] implemented in Schrodinger Suite (2017-2) (Schrödinger, Inc., USA)[42] running on Intel CORE i7 based hpZ230 workstation with the Microsoft Windows 10 OS. In this protocol, the protein was kept rigid, while the ligands were allowed to be flexible throughout the docking simulation.

6.1   Protein preparation

The starting X-ray solved protein crystal structure of cyclin dependent kinase-2 bound with *R*-roscovitine was retrieved from protein data bank (PDB) bearing ID 2A4L.[43] The protein was prepared by automatic preparation by Protein Preparation Wizard of Glide employing the Optimized Potentials for Liquid Simulations 3 (OPLS3) forcefield. During the pre-processing stage, crystallographic water molecules were removed and added missing hydrogens to the protein structures corresponding to pH 7.0 was achieved. The protein metal ions and cofactors were viewed and removed from the protein structure. The tool neutralized the side chains that are not close to the binding cavity and do not participate in salt bridges. The pre-processed protein structure was refined initially by optimizing the sample-water orientation followed by restrained minimization of co-crystallized complex using OPLS3, which reorients side chain hydroxyl groups and alleviates potential steric clashes. Thus, the complex obtained was minimized until it reaches the convergent of heavy atom to RMSD 0.3 Å and taken finally in .mae format.

6.2   Grid file generation

Receptor grid generation protocol of Maestro 11.2 was used to define the binding-site of the protein (2A4L) for docking simulation by excluding any co-crystallized metals, co-factors, water molecules all of which may have crystallized during experimental crystallization of the CDK-2 protein. A grid box was generated around the centroid of the cognate ligand (*R*-roscovitine) specifying the size for the docking ligands (20 Å) with default settings.

6.3   Ligand preparation

The Structures of the synthesized ligands and standard *R*-roscovitine were sketched using built panel of Maestro and taken in .mae format. LigPrep is a utility of Schrodinger software suit that combines tools for generating 3D structures from 1D (Smiles) and 2D (SDF) representation, searching for tautomers, steric isomers and perform a geometry minimization of the ligands. By employing Ligprep protocol, all the ligands were prepared using OPLS3 with default settings and the output file was saved in .maegz format automatically.

6.4 Docking simulation

For precision and accuracy of the docking protocols, the co-crystallized ligand was extracted from the crystal structure of 2A4L and re-docked using Glide docking algorithm (Schrodinger Inc) in its extra precision (XP) mode with default settings without applying any constraints. A good agreement of the obtained pose of docked *R*-roscovitine with cognate ligand indicated the reliability of the selected docking parameters for docking of the synthesized ligands. Hence, by specifying the ligands against the receptor grid, molecular docking was performed using default settings in Glide XP mode.

6.5 Binding mode analysis

The protein-ligand complexes were analysed to investigate various types of interactions by utilizing XP visualizer protocol. For the best-scored ligands, the 2D and 3D plots of molecular ligand-receptor interactions were analysed for hydrogen bond, halogen bond, salt bridges, π-π stacking, and π-cation interactions. G-score and relevant docking descriptors were computed for each of the best docked pose.

Conflict of Interest

Authors hereby declare that there are no financial/commercial conflicts of interest.

Acknowledgment

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CHAPTER 6

Design, synthesis and biological evaluation of novel pyrazolo[3,4-*d*]pyrimidine analogues as anticancer agents

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Graphical Abstract
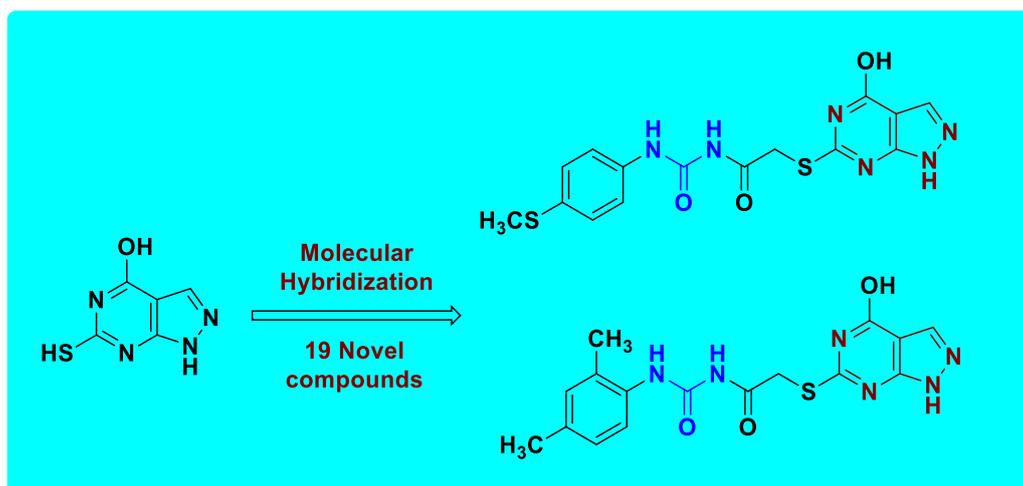
Abstract:

A new series of pyrazolo[3,4-*d*]pyrimidine (**9a-9s**) possessing phenylcarbamoyl acetamide at C-6 position were designed and synthesized. The synthesized compounds were evaluated for anticancer activity against CDK2/Cyclin E and Abl kinase enzymes and further evaluated for anti-proliferative activity against K-562 (chronic myelogeneous leukemia) andMCF-7 (breast adenocarcinoma) cell lines. The structure-activity relationship studies (SAR) revealed that the compounds with mono substitution on phenylcarbamoyl acetamide moiety exhibited commendable activity compared to disubstitution. For all the synthesized molecules from this series, IC_{50} values could not be measured due to solubility limit (IC_{50} >12.5 µM or >25 µM). Therefore, the observed findings on the pyrazolo[3,4-*d*]pyrimidine scaffold with phenylcarbamoyl acetamide group seems to suggest need for further lead optimization with an aim to improve solubility and anticancer activity.

1 Introduction

Cancer is a multifaceted disease characterized by uncontrolled growth of the malignant cell population. Cancer is considered as the most serious health burden touching every region of the world.[1] It is a second leading cause of the death worldwide, accounted for 8.8 million death in 2015. According to the World Health Organization (WHO), the new cancer cases are anticipated to rise by as much as 15 million per year by 2020 unless actions that are more preventive.[2] Although, chemotherapy is the key remedy for cancer treatment, but the use of existing chemotherapeutics is often limited due to existing of limited anticancer drugs and detrimental side effects.[3] It is thus momentous to identify new targets and agents for the cure of cancer. Thus, a substantial research for innovative anticancer agents has been fueled by many academics and industries to unveil novel targets and mechanisms based on the lead candidates of various classes of compounds.[4]

Cyclin-dependent kinases (CDKs) are a group of serine/threonine kinase comprising more than 20 members that are associated with regulation of cell-cycle progression by phosphorylating proteins involved in cell division. Controlling subunits of these enzymes play a key role in regulatory cell cycle, cell division and transcription mechanism in both eukaryotes and prokaryotes.[5] From historic point of view, the first CDK2 inhibitor to be known as 6-dimethylaminopurine (IC$_{50}$: 120 μM).[6,7] From the existing CDK2 inhibitors only few molecules are in different levels of clinical trials. The molecules AT7519, R547 and SNS-032 have reached to phase-I; flavopiridol, roscovitine, P-276-00 and CSH727965 have touched phase-II, while PHA-793887 and AG-024322 have been dismissed in phase-I clinical trials for either the insufficiency of perspicacity from other treatment modalities[8] or the causing of simple hepatic toxicity.[9] **Fig. 1** represents the structures of CDK inhibitors under clinical trials. To support these findings, a study proved that both phosphatidylinositol-3-kinase and CDK2 inhibitors together induced apoptosis in malignant glioma xenografts via a synthetic-lethal interaction.[10] Further, CDK2 inhibitors also evidenced as therapeutic target in neuroblastoma[11] BRCA-deficient cancers[12] and ovarian cancer.[13]
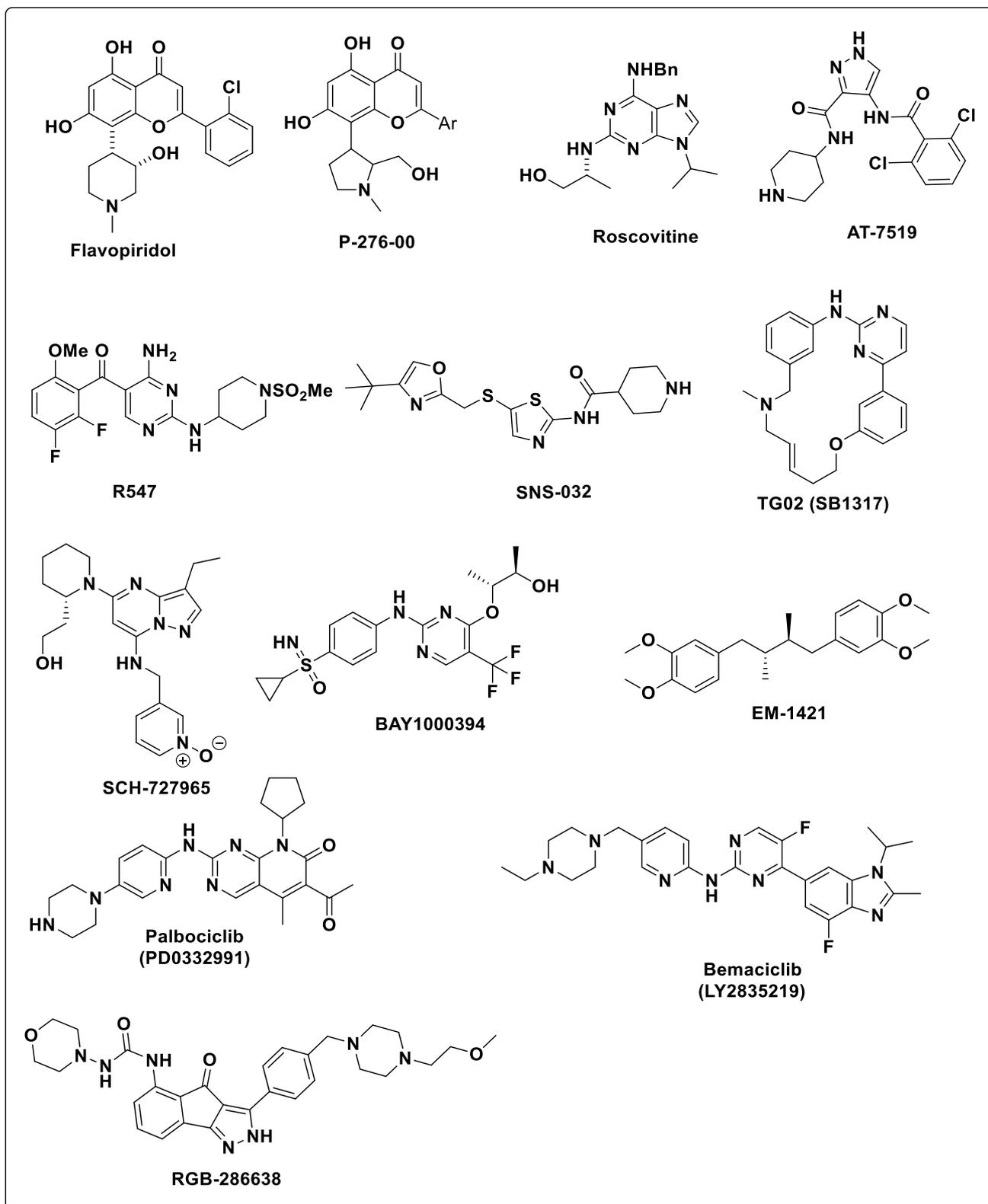
Fig. 1. Structures of the CDK2 inhibitors in clinical trials.

In the past few years, significant amount of contemporary investigations have been conducted on aza-heterocycles for producing wide range of chemical libraries/drug-like candidates. Among all,

pyrazolopyrimidine is one such important drug-like nucleus synthesized by fusion of pyrazole with pyrimidine.[14] Several isomeric forms of pyrazolo pyrimidine namely pyrazolo[3,4-*d*]pyrimidines, pyrazolo[5,1-*b*]pyrimidines, pyrazolo[5,1-*a*]pyrimidines, pyrazolo[1,5-*c*]pyrimidines, pyrazolo[4,3-*d*]pyrimidines are known.[15] From the existing isomers, pyrazolo[3,4-*d*]pyrimidine is an bioisostere of purines with many pharmacological applications as Src/Abl kinase,[16] glycogen synthase kinase (GSK),[17] mammalian target of rapamycin (mTOR),[18] xanthine oxidase inhibitors ,[19] tyrosine kinase[20] and cyclin dependent kinase (CDK).[21] Many pyrazolo[3,4-*d*]pyrimidine derivatives bearing different substitutions on nucleus are stated for diverse cancer targets as illustrated in **Fig. 2**.[22-27]

Inspired by the important findings of pyrazolo[3,4-*d*]pyrimidines as anticancer agents and in continuation of our research work involving the identification of novel anticancer analogues,[28,29] in the current study a novel series of pyrazolo[3,4-*d*]pyrimidine derivatives have been synthesized and evaluated for *in vitro* anticancer activity towards CDK2, Abl kinase inhibitors and K-562, MCF-7 cell lines. In this work we carried out chemical modifications at C-6 of the pyrazolo[3,4-*d*]pyrimidine nucleus (**scheme-1**) by efficient synthetic method.
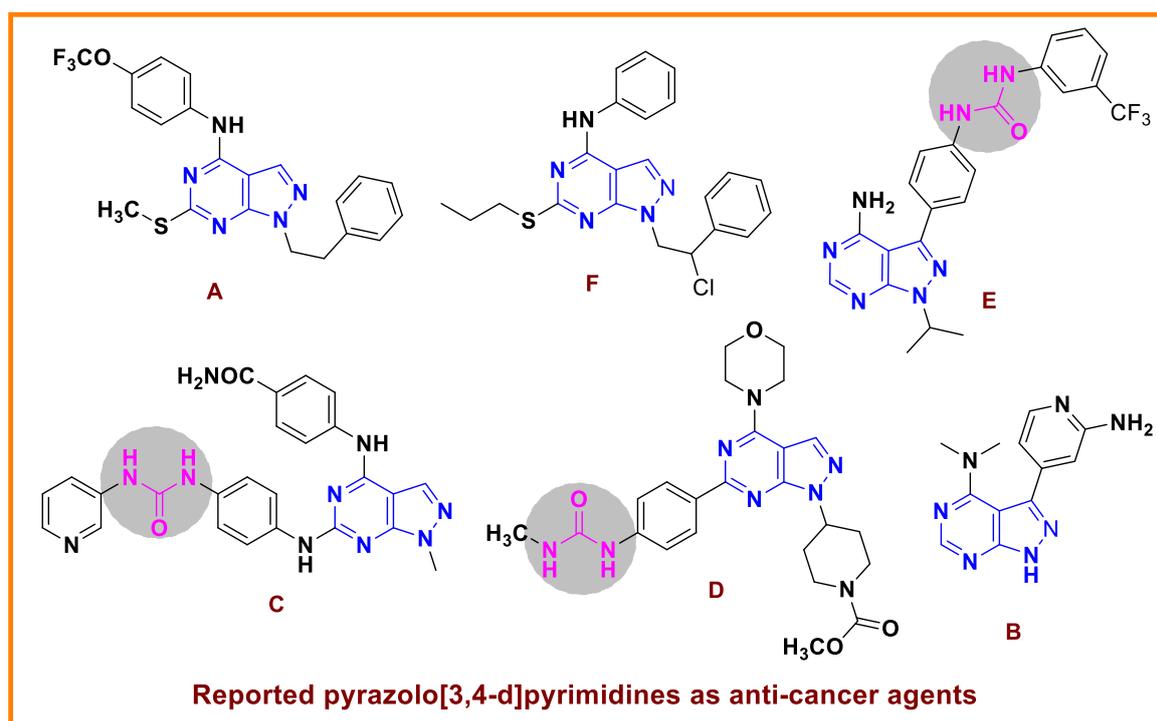
Fig. 2. Known derivatives of pyrazolo[3,4-*d*]pyrimidine analogues and their anticancer activities. **A**: (K_{i50} against Src, AblT315I = 0.056, 0.01 μM);[22] **B**: (IC_{50} against CDK9 = 17 nM);[23] **C**: (IC_{50} against mTOR = 13 nM);[24] **D**: (IC_{50} against mTOR = 9 nM);[25] **E**: (K_{i50} against cSrc, Abl = 25, 41 nM);[26] **F**: (IC_{50} against Src = 1.2 ± 0.4 μM).[27]

2   Results and discussion

2.1 Chemistry

6-mercapto-1H-pyrazolo[3,4-d]pyrimidin-4-ol (**4**), as a key intermediate for the synthesis of desired hybrid molecules was accomplished from 2-(ethoxymethylene)malononitrile (**1**) by the sequence of reactions shown in **Fig. 1**. The synthesis was performed in a process that the five membered pyrazole ring was first accomplished and the pyrimidine ring formation was followed. In pyrazolopyrimidine scaffold, the hydroxy group as nucleophilic substituent was introduced at C-4 and the methylmercapto group was employed at C-6 as a precursor to perform the nucleophilic substitution with different phenylcarbamoyl acetamides.
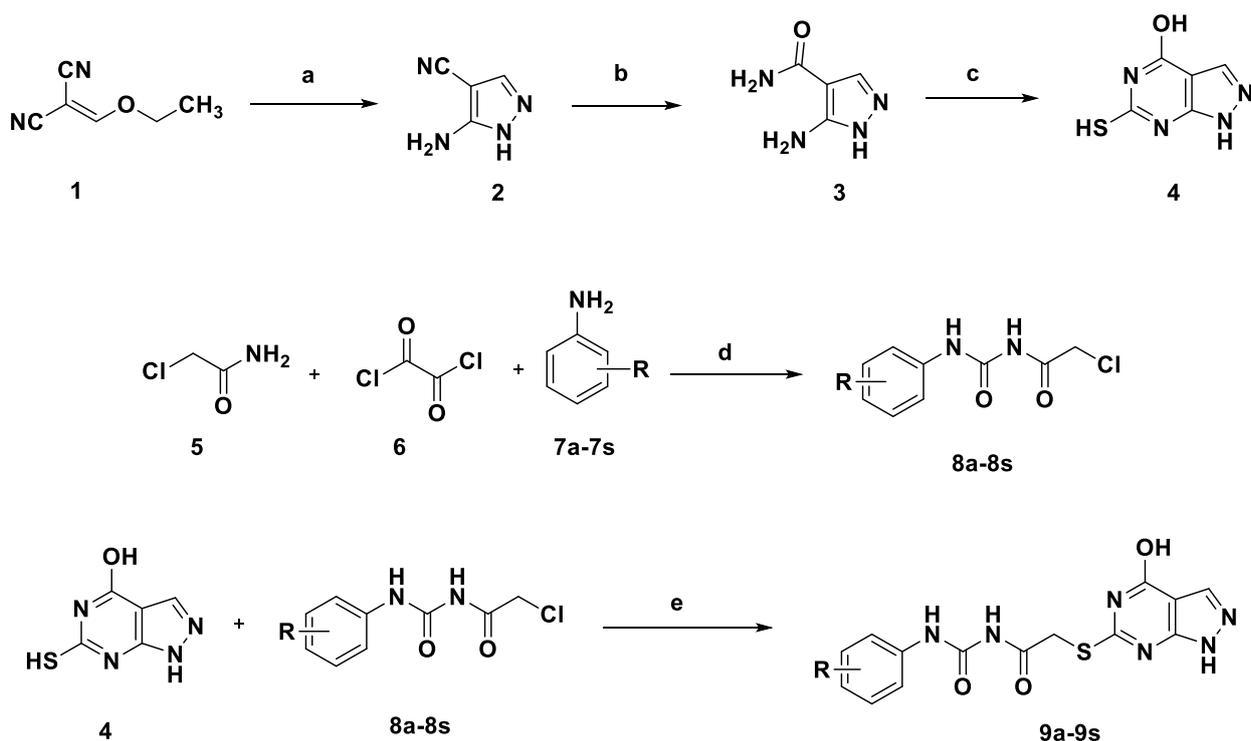
Scheme 1. Synthesis of 4-substituted pyrazolo[3,4-d]pyrimidine hybrid molecules.

Reagents and conditions: (a) hydrazine hydrate, ethanol, 80^0 C, 3h, 92%; (b) Conc. H_2SO_4, NH_4OH, H_2O, 50^0 C, 5h, 90%; (c) potassium ethyl xanthogenate, DMF, 120^0 C, 6h, 82%; (d) 1,2-dichloroethane, 90^0 C, 6h, 90%; (e) 1M KOH, acetone, 60^0 C, 2-3h, 80-96%.

Nucleophilic substitution of commercially available 2-(ethoxymethylene) malononitrile (**1**) with hydrazine hydrate in ethanol under reflux conditions afforded 5-amino-1H-pyrazole-4-carbonitrile (**2**) which underwent partial hydrolysis of nitrile group with 98% sulfuric acid to attain 5-amino-1H-pyrazole-4-carboxamide (**3**) in excellent yield (90%). Subsequent fusion of carboxamide (**2**) with potassium ethyl xanthogenate in N,N-dimethylformamide accomplished the cyclic product 6-mercapto-1H-pyrazolo[3,4-d]pyrimidin-4-ol (**4**) in good yield (82%).[30] Further, various substituted

phenylcarbamoyl acetamides (**8a-8s**) were prepared by allowing the reaction of 2-chloroacetamide (**5**) and oxalyl chloride (**6**) with different amines (**7a-7s**) in anhydrous 1,2-dichloroethane under reflux conditions as reported in literature.[31] The nucleophilic substitution of compound **4** at C-6 with substituted phenylcarbamoyl acetamides (**8a-8s**) was carried out in presence of 1M KOH solution under heat conditions to attain the desired final products (**9a-9s**) as presented in **scheme 1**. The anticipated structures of newly prepared final compounds were in agreement with physicochemical and spectral (IR, ^{1}H NMR and ^{13}C NMR) data attained and were further sub-stantiated by HR-MS information, which is precised in supporting information.

The ^{1}H NMR of compound **4** exhibited the presence of a very distinct singlet signals resonating at around δ 13.61, 13.03, 11.86 and 8.42 ppm attributed for N-H proton, S-H proton, O-H proton and C-3 Ar-H protons respectively, indicating its formation of bicyclic system (pyrazolopyrimidine) by a process of simple condensation reaction by ring annulation of 5-amino-1*H*-pyrazole-4-carboxamide with potassium ethyl xanthogenate. Further, from ^{1}H NMR of compounds **9a-9s**, we observed most informative singlet signals at around δ 13.57-13.07, 12.51-12.16, 8.00-7.94 ppm accounted for three -NH protons, while the characteristic singlet signal appearing at δ 4.28-4.20 ppm attributing to methylene group (-S-C**H₂**-NH-NH-). Thus confirmed the alkylation of mercapto group (-**SH**-) with substituted phenylcarbamoyl acetamides (**8a-8s**) to achieve the desired final products (**9a-9s**) as represented in **scheme 1**.

Further, from the IR spectra of the title compounds (**9a-9s**) we observed a reasonably sturdy and characteristic bands around 2904.93-3290.96cm⁻¹, 1232.49-1292.89cm⁻¹ accounting for N-H and C-S groups respectively, while most characteristic band of -**C=O** appearing around 1667.34-1715.29 cm⁻¹ indicated the formation of final hybrid molecules. Further, the ^{1}H NMR spectrum of the final compounds displayed some characteristic singlet signals at around δ 12.36-12.17 ppm for ring N-H, δ 13.57-13.07, 8.00-7.94 ppm for two exocyclic –NH protons (-N**H**-CO-N**H**-Ph), while the hydroxyl proton (-OH) on aromatic ring resonated as singlet signal around at δ 11.47-10.65 ppm. Further, the most informative singlet signals resonated around δ 10.78-8.96 ppm (C-3 aromatic proton), δ 4.28-4.20 ppm for methylene protons of carbamoyl acetamide chain (-S-C**H₂**-NH-CO-NH-). Further, various signals appeared as doublet or multiplets at around δ 8.61-6.87 ppm accounted for other aromatic protons. The ^{13}C NMR spectra further confirmed the structures of the title compounds. The characteristic carbon signals resonated at around δ 173.28-161.87, 158.87-158.19 and 131.96-130.19 ppm were assigned to C-6, C-4 and C-3 carbons of pyrazolopyrimidine ring. Further, other aromatic/heteroaromatic carbons resonated between δ 159.78-107.87 ppm while, the prominent carbon peaks observed at around δ 34.43-34.29 ppm for methylene (-S-**C**H₂-NH-CO-NH-), δ 55.76 ppm for methoxy (-O**C**H₃) and δ 20.03-17.05 ppm for methyl groups respectively. In addition, the formation of

the final pyrazolo[3,4-*d*]pyrimidine derivatives (**9a-9s**) was confirmed by calculating their individual mass spectrums (HR-MS), which displayed accurate molecular ion peaks that were in agreement with their expected molecular weights (supporting information).

2.2 *In vitro* evaluation for anti-cancer (CDK2 & Abl) and anti-proliferative (K-562 & MCF-7) activity

All the final compounds were evaluated for CDK2/cyclin E kinase inhibition and the IC_{50} values of various *in vitro* anticancer profiles are summarized in **Table 1**. Abl kinase inhibition was evaluated as a counter screen, to get a preliminary information about selectivity. In addition, to explore the biological significance, these compounds were further evaluated for their *in vitro* anti-proliferative activity against K-562 (chronic myelogeneous leukemia) and MCF-7 (breast adenocarcinoma) cell lines. We studied the effect of various substitutions on phenyl ring of the phenylcarbamoyl acetamide moiety at C-6, which was in turn connected to pyrazolopyrimidine nucleus through a sulphur bridge. From the obtained results, it was observed that for all the synthesized molecules from this series, IC_{50} values could not be measured due to solubility limit (IC_{50} >12.5 µM or >25 µM).

C. No	R	IC_{50} (µM)			
		CDK2	Abl	K-562	MCF-7
9a	2,4-CH$_3$	>12.5	>12.5	>25	>25
9b	2-Cl	>25	>25	>25	>25
9c	3-Cl	>12.5	>12.5	>25	>25
9d	4-Cl	>25	>25	>25	>25
9e	2-Br	>25	>25	>25	>25
9f	3-Br	>25	>25	>12.5	>12.5
9g	4-Br	>12.5	>12.5	>100	>100
9h	2-NO$_2$	>25	>25	>25	>25
9i	3-NO$_2$	>25	>25	>25	>25
9j	4-NO$_2$	>50	>50	>100	>100

9k	4-Cl-3-NO$_2$	>25	>25	>50	>50
9l	4-Br-3-CF$_3$	>25	>25	>50	>50
9m	4-SCH$_3$	>12.5	>12.5	>12.5	>12.5
9n	4-F-2-CH$_3$	>25	>25	>12.5	>12.5
9o	2-Cl-4-F	>25	>25	>50	>50
9p	4-OCH$_3$-2-NO$_2$	>12.5	>12.5	>50	>50
9q	2-Cl-5-NO$_2$	>25	>25	>25	>25
9r	H	>25	>25	>50	>50
9s	N	>25	>25	49.9	>50
	Roscovitine	0.1	>100	42	11
	Imatinib	>100	0.2	0.5	>10

[a] IC$_{50}$ values were determined in triplicate in the range of 0.05 to 100 μM. IC$_{50}$ value indicates concentration (μM) that inhibits activity of tested enzyme to 50% or for cytotoxic assays, concentration (μM) that reduces 50% of cells during a three-day cultivation

Table 1. Anticancer evaluation of novel mono substituted pyrazolo[3,4-*d*]pyrimidines against CDK2/Abl kinases and K-562/MCF-7 cell lines.

3 Conclusion

In summary, a series of new mono substituted pyrazolo[3,4-*d*]pyrimidines with substituted phenylcarbamoyl acetamide side chain at C-4 positon has been designed and synthesized. The key intermediate 6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol allowed us to increase our library of compounds. All synthesized compounds were evaluated for *in vitro* enzymatic activity against CDK2/cyclin E, Abl kinases as well as anti-proliferative activity against K-562 (chronic myelogeneous leukemia) and MCF-7 (breast adenocarcinoma) cell lines. From the obtained results, it was observed that for all the synthesized molecules from this series, IC$_{50}$ values could not be measured due to solubility limit (IC$_{50}$ >12.5 μM or >25 μM). This research outcome promotes the advantage of interaction of phenycarbomoyl acetamides to pyrazolopyrimidine scaffold through sulphur linkage, thus

offers an idea for further compound optimization and functionalization to enhance the solubility and anticancer activity, which deserves further investigation.

4   Experimental Section

All the chemicals used in this research work were purchased from Sigma-Aldrich and Merck Millipore, South Africa. All the solvents, except those of laboratory-reagent grade, were dried and purified when necessary according to previously published methods. The progress of the reactions and the purity of the compounds were monitored by thin-layer chromatography (TLC) on pre-coated silica gel plates procured from E. Merck and Co. (Darmstadt, Germany) using 36% ethyl acetate in n-hexane as the mobile phase and iodine vapor as the visualizing agent. The melting points of the synthesized compounds were determined using a Thermo Fisher Scientific (IA9000, UK) digital melting point apparatus and are uncorrected. The IR spectra were recorded on a Bruker Alpha FT-IR spectrometer (Billerica, MA, USA) using the ATR technique. The 1H NMR and 13C NMR spectra were recorded on a Bruker AVANCE 600 and 600 MHz (Bruker, Rheinstetten/Karlsruhe, Germany) spectrometers using CDCl3 and DMSO-$d6$. The chemical shifts are reported in δ ppm units with respect to TMS as an internal standard. HRMS spectra were recorded on an Autospec mass spectrometer with electron impact at 70 eV.

4.1   General procedure for the synthesis of compounds (9a-9s)

To a well-stirred solution of compound **4** (0.5g, 0.00298mol) in 1M KOH solution, added equimolar amount of a solution of compounds **8s-8s** in acetone (10mL) was added. The reaction mixture was stirred for 1h at room temperature then heated at 50 °C for 2h. Upon completion, the precipitated product was filtered off to afford the crude product. The crude product was recrystallized from THF to yield the appropriate pure product.

4.1.1   N-((2,4-dimethylphenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)acetamide (9a)

Brown solid; yield: 84 %; mp 212-214°C; FTIR (ATR, cm-1) ν_{max}: 3487.05, 3101.10, 2930.55, 1687.76, 1585.78, 1521.91, 1444.10, 1244.37, 1155.79, 966.32, 840.46, 748.54, 676.59, 553.68; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.46 (s, 1H, NH), 12.27 (s, 1H, NH), 10.80 (s, 1H, OH), 9.99 (s, 1H, ArH), 7.99 (s, 1H, NH), 7.72 (d, *J* = 8.22 Hz, 1H, ArH), 7.02 (s, 1H, ArH), 6.97 (d, *J* = 8.28 Hz, 1H, ArH), 4.24 (s, 2H, C$\underline{H}_2$), 2.23 (s, 3H, C$\underline{H}_3$), 2.16 (s, 3H, C$\underline{H}_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.85 (C=O), 150.20, 133.00, 132.87, 130.52, 127.72, 126.48, 121.22, 34.37 (C$\underline{H}_2$), 20.03 (C$\underline{H}_3$), 17.05 (C$\underline{H}_3$) ppm.

4.1.2 N-((2-chlorophenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)acetamide (9b)

White solid; yield: 90%; mp 216-218°C; FTIR (ATR, cm-1) ν_{max}: 3487.05, 3101.10, 2952.37, 1687.76, 1585.78, 1521.91, 1444.10, 1296.62, 1244.37, 1155.79, 966.32, 863.26, 748.54, 676.59; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.41 (s, 1H, NH), 12.20 (s, 1H, NH), 10.95 (s, 1H, OH), 10.65 (s, 1H, ArH), 8.21 (dd, J = 8.28, 1.32 Hz, 1H, ArH), 7.96 (s, 1H, NH), 7.47 (dd, J = 8.22, 1.32 Hz, 1H, ArH), 7.34-7.31 (m, J = 4.27 Hz, 1H, ArH), 7.12-7.10 (m, J = 3.43 Hz, 1H, ArH), 4.25 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.92 (C=O), 149.99, 134.30, 128.91, 127.39, 124.46, 122.43, 121.43, 34.35 ($\underline{C}H_2$) ppm.

4.1.3 N-((3-chlorophenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)acetamide (9c)

White solid; yield: 87 %; mp 211-213°C; FTIR (ATR, cm-1) ν_{max}: 3290.67, 3055.43, 2976.09, 1672.71, 1487.68, 14783.48, 1245.54, 1148.70, 966.87, 772.62, 657.05, 532.95; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.41 (s, 1H, NH), 12.16 (s, 1H, NH), 10.75 (s, 1H, OH), 10.19 (s, 1H, ArH), 8.00 (s, 1H, NH), 7.72 (s, 1H, ArH), 7.37 (d, J = 7.74 Hz, 1H, ArH), 7.32 (t, J = 8.01 Hz, 1H, ArH), 7.11 (d, J = 8.52 Hz, 1H, ArH), 4.25 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.51 (C=O), 150.08, 138.78, 133.00, 123.14, 119.14, 118.05, 34.40 ($\underline{C}H_2$) ppm.

4.1.4 N-((4-chlorophenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)acetamide (9d)

White solid; yield: 85%; mp 244-246°C; FTIR (ATR, cm-1) ν_{max}: 3242.17, 3137.95, 2975.08, 1704.19, 1675.55, 1599.49, 1556.21, 1493.47, 1231.28, 1152.00, 949.78, 776.09, 708.23, 509.89; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.57 (s, 1H, NH), 12.48 (s, 1H, NH), 11.00 (s, 1H, OH), 10.29 (s, 1H, ArH), 7.95 (s, 1H, NH), 7.56 (d, J = 8.88 Hz, 2H, ArH), 7.36 (d, J = 8.88 Hz, 2H, ArH), 4.23 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.43 (C=O), 157.57, 150.04, 136.24, 128.35, 128.26, 127.23, 121.90, 121.15, 102.74, 34.39 ($\underline{C}H_2$) ppm.

4.1.5 N-((2-bromophenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)acetamide (9e)

Brown solid; yield: 84%; mp 240-242°C; FTIR (ATR, cm-1) ν_{max}: 3488.26, 3117.93, 2916.20, 1692.18, 1580.58, 1511.78, 1295.08, 1228.77, 1156.20, 966.88, 739.17, 616.56, 546.38; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.07 (s, 1H, NH), 11.67 (s, 1H, OH), 10.78 (s, 1H, ArH), 8.19 (dd, J = 8.28, 1.28 Hz,

1H, ArH), 7.83 (s, 1H, NH), 7.64 (dd, J = 8.00, 1.12 Hz, 1H, ArH), 7.39-7.35 (m, J = 4.21 Hz, 1H, ArH), 7.06-7.02 (m, J = 3.35 Hz, 1H, ArH), 4.05 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 150.09, 135.69, 132.15, 131.69, 127.85, 127.82, 124.91, 122.01, 117.20, 115.25, 113.07, 107.33, 34.36 ($\underline{C}H_2$) ppm.

4.1.6   N-((3-bromophenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)acetamide (9f)

White solid; yield: 87 %; mp 226-228°C; FTIR (ATR, cm-1) ν$_{max}$: 3251.64, 2874.39, 2824.05, 1686.84, 1595.07, 1551.77, 1491.77, 1259.54, 1218.24, 1158.08, 769.81, 534.27; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.41 (s, 1H, NH), 12.18 (s, 1H, NH), 10.74 (s, 1H, OH), 10.18 (s, 1H, ArH), 8.01 (s, 1H, NH), 7.87 (s, 1H, ArH), 7.41 (m, J = 2.19 Hz, 1H, ArH), 7.26 (t, J = 2.91 Hz, 2H, ArH), 4.25 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.48 (C=O), 150.05, 138.89, 130.34, 126.4, 121.97, 121.28, 118.44, 34.38 ($\underline{C}H_2$) ppm.

4.1.7   N-((4-bromophenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)acetamide (9g)

Brown solid; yield: 81%; mp 232-234°C; FTIR (ATR, cm-1) ν$_{max}$: 3236.82, 3133.87, 2922.02, 1702.62, 1672.40, 1547.00, 1489.02, 1392.06, 1311.37, 1227.97, 1118.73, 775.66, 665.01, 506.53; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.41 (s, 1H, NH), 12.19 (s, 1H, NH), 10.72 (s, 1H, OH), 10.15 (s, 1H, ArH), 7.98 (s, 1H, NH), 7.48 (s, 4H, ArH), 4.25 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.44 (C=O), 150.01, 136.67, 131.28, 121.52, 115.13, 34.38 ($\underline{C}H_2$) ppm.

4.1.8   2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)-N-((2-nitrophenyl)carbamoyl) acetamide (9h)

White solid; yield: 82%; mp 227-229°C; FTIR (ATR, cm-1) ν$_{max}$: 3216.47, 2872.46, 1680.99, 1581.77, 1483.18, 1436.94, 1339.98, 1243.75, 1157.59, 857.52, 743.68, 529.26; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.42 (s, 1H, NH), 12.17 (s, 1H, NH), 11.59 (s, 1H, OH), 11.01 (s, 1H, ArH), 8.39 (d, J = 8.22 Hz, 1H, ArH), 8.10 (dd, J = 8.25, 1.05 Hz, 1H, ArH), 8.03 (s, 1H, NH), 7.74-7.72 (m, J = 4.26 Hz, 1H, ArH), 7.32-7.30 (m, J = 4.19 Hz, 1H, ArH), 4.25 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.33 (C=O), 15138.55, 134.52, 132.41, 129.77, 125.00, 123.67, 123.17, 117.85, 34.36 ($\underline{C}H_2$) ppm.

4.1.9   2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)-N-((3-nitrophenyl)carbamoyl) acetamide (9i)

Brown solid; yield: 90%; mp 210-212°C; FTIR (ATR, cm-1) ν_{max}: 3514.66, 3244.67,3128.20, 2885.63, 1694.75, 1659.36, 1598.09, 1524.26, 1346.53, 1234.93, 1156.82, 735.81, 671.97; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.42 (s, 1H, NH), 12.19 (s, 1H, NH), 10.84 (s, 1H, OH), 10.41 (s, 1H, ArH), 8.56 (s, 1H, ArH), 7.99 (s, 1H, NH), 7.91 (dd, J = 8.25, 1.95 Hz, 1H, ArH), 7.82 (d, J = 8.28 Hz, 1H, ArH), 7.58 (t, , J = 8.19 Hz, 1H, ArH), 4.27 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.50 (C=O), 150.29, 147.97, 138.61, 129.78, 125.73, 117.86, 113.82, 34.43 ($\underline{C}H_2$) ppm.

4.2.0   *2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)-N-((4-nitrophenyl) carbamoyl) acetamide (9j)*

White solid; yield: 95%; mp 232-234°C; FTIR (ATR, cm-1) ν_{max}: 3226.58, 2874.63, 1685.85, 1595.53, 1487.60, 1336.76, 1244.88, 1215.59, 1161.94, 1111.08, 853.82, 770.56, 715.98, 534.24; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.41 (s, 1H, NH), 12.17 (s, 1H, NH), 10.90 (s, 1H, OH), 10.53 (s, 1H, ArH), 8.18 (d, J = 9.06 Hz, 2H, ArH), 7.98 (s, 1H, NH), 7.78 (d, J = 9.12 Hz, 2H, ArH), 4.27 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.64 (C=O), 150.01, 143.58, 142.59, 124.44, 124.24, 120.38, 119.27, 34.47 ($\underline{C}H_2$) ppm.

4.2.1   *N-((4-chloro-3-nitrophenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d] pyrimidin-6-yl)thio)acetamide (9k)*

Brown solid; yield: 92%; mp 214-216°C; FTIR (ATR, cm-1) ν_{max}: 3523.06, 3231.76, 2920.69, 1693.28, 1656.76, 1595.61, 1527.54, 1303.35, 1246.28, 1155.31, 969.66, 824.35, 764.03, 699.69; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.41 (s, 1H, NH), 12.20 (s, 1H, NH), 10.90 (s, 1H, OH), 10.42 (s, 1H, ArH), 8.35 (d, J = 2.46 Hz, 1H, ArH), 7.96 (s, 1H, NH), 7.79 (dd, J = 8.79, 2.37 Hz, 1H, ArH), 7.67 (d, J = 8.88 Hz, 1H, ArH), 4.26 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.39 (C=O), 150.20, 147.22, 137.44, 131.68, 131.46, 125.22, 124.53, 118.56, 115.90, 34.37 ($\underline{C}H_2$) ppm.

4.2.2   *N-((4-bromo-3-(trifluoromethyl)phenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo [3,4-d]pyrimidin-6-yl)thio)acetamide (9l)*

Brown solid; yield: 83%; mp 225-227°C; FTIR (ATR, cm-1) ν_{max}: 3488.74, 3100.86, 2930.06, 1691.15, 1597.49, 1521.09, 1419.88, 1305.27, 1227.40, 1134.94, 827.90, 775.05, 730.96; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.39 (s, 1H, NH), 12.16 (s, 1H, NH), 10.81 (s, 1H, OH), 10.34 (s, 1H, ArH), 8.11 (d, J = 2.58 Hz, 1H, ArH), 7.96 (s, 1H, NH), 7.78 (d, J = 8.70 Hz, 1H, ArH), 7.70 (dd, 8.73, 2.55 Hz, 1H, ArH), 4.26 (s, 2H, C$\underline{H}_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.36 (C=O), 150.18, 137.39, 135.06, 128.56, 128.36, 124.67, 123.30, 121.49, 118.89, 118.85, 111.71, 34.35 ($\underline{C}H_2$) ppm.

4.2.3 2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)-N-((4-(methylthio)phenyl) carbamoyl)acetamide (9m)

Yellow solid; yield: 88%; mp 236-238°C; FTIR (ATR, cm-1) ν_{max}: 3251.97, 3152.40, 2916.97, 1673.16, 1578.09, 1541.67, 1492.36, 1313.17, 1222.84, 1152.68, 966.09, 801.32, 687.63, 665.54, 507.81; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.41 (s, 1H, NH), 12.21 (s, 1H, NH), 10.66 (s, 1H, OH), 10.08 (s, 1H, ArH), 7.94 (s, 1H, NH), 7.46 (d, J = 8.76 Hz, 2H, ArH), 7.24 (d, J = 8.52 Hz, 2H, ArH), 4.24 (s, 2H, CH$_2$), 2.44 (s, 3H, CH$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.39 (C=O), 149.96, 134.83, 132.29, 127.34, 120.24, 34.34 (CH$_2$), 15.49 (CH$_3$) ppm.

4.2.4 N-((4-fluoro-2-methylphenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d] pyrimidin-6-yl)thio)acetamide (9n)

Yellow solid; yield: 81%; mp 237-239°C; FTIR (ATR, cm-1) ν_{max}: 3230.66, 2822.22, 1684.11, 1598.02, 1554.55, 1491.53, 1267.43, 1162.90, 1267.43, 1162.90, 867.32, 772.26, 675.14, 536.08; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.42 (s, 1H, NH), 12.24 (s, 1H, NH), 10.77 (s, 1H, OH), 9.97 (s, 1H, ArH), 7.93 (s, 1H, NH), 7.80 (q, J = 4.82 Hz, 1h, ArH), 7.06 (dd, J = 9.48, 2.94Hz, 1H, ArH), 7.00-6.97 (m, J = 4.06 Hz, 1H, ArH), 4.25 (s, 2H, CH$_2$), 2.21 (s, 3H, CH$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.79 (C=O), 159.17, 157.57, 150.26, 131.77, 131.11, 131.06, 123.26, 116.30, 112.15, 34.31 (CH$_2$), 16.98 (CH$_3$) ppm.

4.2.5 N-((2-chloro-4-fluorophenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d] pyrimidin-6-yl)thio)acetamide (9o)

White solid; yield: 92%; mp 236-238°C; FTIR (ATR, cm-1) ν_{max}: 3231.76, 2871.85, 1681.96, 1595.75, 1545.77, 1483.91, 1383.91, 1246.39, 1158.54, 1050.89, 862.59, 770.91, 697.93; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.40 (s, 1H, NH), 12.25 (s, 1H, NH), 10.97 (s, 1H, OH), 10.56 (s, 1H, ArH), 8.18 (q, J = 5.00 Hz, 1H, ArH), 7.93 (s, 1H, NH), 7.44 (dd, J = 8.34, 2.94 Hz, 1H, ArH), 7.23-7.19 (m, J = 4.06 Hz, 1H, ArH), 4.25 (s, 2H, CH$_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.86 (C=O), 158.34, 156.71, 150.06, 131.01, 130.99, 123.56, 122.96, 116.10, 115.92, 114.31, 114.17, 34.29 (CH$_2$) ppm.

4.2.6 2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)-N-((4-methoxy-2-nitrophenyl) carbamoyl)acetamide (9p)

Yellow solid; yield: 96%; mp 213-215°C; FTIR (ATR, cm-1) ν_{max}: 3433.23, 3132.91, 2945.11, 1693.03, 1506.83, 1441.97, 1267.52, 1150.08, 1035.73, 831.13, 774.25, 527.88; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.57 (s, 1H, NH), 12.51 (s, 1H, NH), 11.47 (s, 1H, OH), 11.18 (s, 1H, ArH), 8.24 (d, J = 9.20

Hz, 1H, ArH), 7.94 (s, 1H, NH), 7.58 (d, J = 3.00 Hz, 1H, ArH), 7.36 (dd, J = 9.28, 3.00 Hz, 1H, ArH), 4.22 (s, 2H, CH$_2$), 3.82 (s, 3H, CH$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.27 (C=O), 154.97, 150.28, 139.68, 125.21, 121.27, 108.86, 55.76 (CH$_3$), 34.34 (CH$_2$) ppm.

4.2.7   *N-((2-chloro-5-nitrophenyl)carbamoyl)-2-((4-hydroxy-1H-pyrazolo[3,4-d] pyrimidin-6-yl)thio)acetamide (9q)*

Brown solid; yield: 81%; mp 214-216°C; FTIR (ATR, cm-1) ν$_{max}$: 3251.94, 3129.53, 2962.12, 1691.89, 1659.73, 1565.17, 1344.81, 1227.89, 1150.27, 1060.79, 830.04, 738.55; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.39 (s, 1H, NH), 12.18 (s, 1H, NH), 11.23 (s, 1H, OH), 11.01 (s, 1H, ArH), 9.13 (d, J = 2.52 Hz, 1H, ArH), 8.00 (s, 1H, NH), 7.94 (m, J = 3.84 Hz, 1H, ArH), 7.79 (d, J =8.88 Hz, 1H, ArH), 4.28 (s, 2H, CH$_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.30, 150.11, 146.44, 135.41, 130.01, 128.47, 118.54, 115.01, 34.34 (CH$_2$) ppm.

4.2.8   *2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)-N-(phenylcarbamoyl) acetamide (9r)*

Brown solid; yield: 80%; mp 222-224°C; FTIR (ATR, cm-1) ν$_{max}$: 3244.88, 3140.28, 2976.12, 1700.27, 1672.42, 1548.77, 1490.83, 1449.90, 1223.68, 1152.02, 948.72, 857.35, 760.69, 700.47, 532.97; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.41 (s, 1H, NH), 12.19 (s, 1H, NH), 10.65 (s, 1H, OH), 10.10 (s, 1H, ArH), 7.99 (s, 1H, NH), 7.49 (d, J = 8.04 Hz, 2H, ArH), 7.31 (t, J = 7.92 Hz, 2H, ArH), 7.08 (t, J = 7.44 Hz, 1H, ArH), 4.25 (s, 2H, CH$_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.49 (C=O), 150.03, 137.23, 128.52, 123.41, 119.53, 34.39 (CH$_2$) ppm.

4.2.9   *2-((4-hydroxy-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)-N-(pyridin-2-ylcarbamoyl   acetamide (9s)*

Brown solid; yield: 89%; mp 215-217°C; FTIR (ATR, cm-1) ν$_{max}$: 3160.39, 2981.81, 2865.05, 1729.62, 1688.34, 1575.47, 1504.97, 1434.23, 1299.93, 1162.51, 778.15; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 13.40 (s, 1H, NH), 12.20 (s, 1H, NH), 10.92 (s, 1H, OH), 10.46 (s, 1H, ArH), 8.27 (t, J = 2.94 Hz, 1H, ArH), 7.91 (d, J = 8.34 Hz, 2H, ArH + NH), 7.80-7.77 (m, J = 4.33 Hz, 1H, ArH), 7.11-7.09 (m, J = 3.92 Hz, 4.27 (s, 2H, CH$_2$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.54 (C=O), 150.65, 149.82, 147.70, 138.01, 119.17, 112.82, 34.60 (CH$_2$) ppm.

5   Biological activity protocol

5.1   CDK and Abl kinase inhibition assays

CDK2/cyclin E and Abl kinases were produced in Sf9 insect cells via baculoviral infection and purified on a NiNTA column. The kinase reactions were assayed with suitable substrates (1 mg/mL histone H1 for CDK2 and 500 μM peptide GGEAIYAAPFKK for Abl) in the presence of 15 or 10 μM ATP for CDK2 and Abl, respectively, 0.05 μCi [γ-^{33}P]ATP, and the test compound in a final volume of 10 μL, all in a reaction buffer (60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM Na-orthovanadate, 1.2 mM DTT, 2.5 μg / 50 μl $PEG_{20.000}$). The reactions were stopped by adding 5 μL of 3% aq. H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose (Whatman), washed 3× with 0.5% aq. H_3PO_4 and finally air-dried. Kinase inhibition was quantified using a FLA-7000 digital image analyzer. The concentration of the test compounds required to reduce the kinase activity by 50 % was determined from dose-response curves and recorded as their IC_{50}.

5.2 Anti-proliferative (K-562 and MCF-7) activity assays

The tumor cells (purchased from the American Type Culture Collection) were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum and L-glutamine (0.3 g/L) and were maintained at 37 °C in a humidified atmosphere with 5% CO_2. For anticancer cytotoxicity estimations, 10^4 cells were seeded into each well of a 96-well plate, allowed to stabilize for 20 h, and the test inhibitors were then added at different concentrations (ranging from 0.1 to 100 μM or to a solubility limit) in triplicate. Three days after addition of the inhibitors, calcein AM solution (Molecular Probes) was added. One hour later, fluorescence of cells was quantified using a Fluoroskan Ascent (Labsystems) reader and cytotoxic effective concentrations were calculated and expressed as IC_{50} values from dose-response curves. Roscovitine and imatinib were used as reference drugs.

Conflict of Interest

Authors hereby declare that there are no financial/commercial conflicts of interest.

Acknowledgment

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CHAPTER 7

1 Summary and conclusion

Cancer is a disease caused by an uncontrolled growth of abnormal cells. Recent advancement in understanding the molecular mechanism of cancer and the factors causing it has deeply impacted in the innovation of cancer chemotharpy. Predominant efforts are being carried out in order to identify advanced treatments through enhanced imaging, molecular diagnostic approaches and improvements in prevention and chemotherapeutic organization. Every year the number people with cancer is drastically increasing world over. Thus, it is becoming imperative to investigate and discover new agents and targets for the treatment of cancer.

Cyclin-dependent kinases (CDK/Cyclins) constitute a family of serine/threonine kinases that involve in the regulation of cell cycle progression, transcription by phosphorylating proteins involved in cell division. Twenty different CDKs have been reported in mammalian cells till date. Among them, CDK1, CDK2 and their associated Cyclins A, B, D, E are considered as *bona fide* cell cycle regulators. For example, formation of active complex composed of CDK2 and cyclin E enables pRb phosphorylation, activation of transcription factor E2F which and initiation of S phase of the cell cycle. CDK2 then also associates with cyclin A, governing continuous DNA replication and properly programed deactivation of E2F. Deregulations of CDKs or cyclins, as well as the loss of endogeneous inhibitory proteins, result in abrogation of cell cycle control, which is connected with development of tumors, thus CDKs are considered important targets for anticancer drugs.

The aim of the present study was to design and identify newer potential anticancer leads by bioisosteric replacement of purine by pyrazolopyrimidines. There are almost five different structural isomers of this bicyclic system (pyrazolo[5,1-*b*]pyrimidines, pyrazolo[5,1-*a*]pyrimidines, pyrazolo[4,3-*d*]pyrimidines, pyrazolo[1,5-*c*]pyrimidines and pyrazolo[3,4-*d*]pyrimidines), which exists due to the varying position of nitrogen, degree of saturation or unsaturation, or the number of nitrogen's on the pyrazole nucleus. Pyrazolo[3,4-*d*]pyrimidine scaffold was substituted with various five/six membered heterocycles to yield novel multifarious pyrazolo[3,4-*d*]pyrimidine derivatives. This research work resulted in 71 novel derivatives as CDK2 inhibitors and the novel desired structures were confirmed by thin layer chromatography (TLC), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (^{1}H, ^{13}C NMR), and high resolution mass spectrometry (HRMS). These compounds were evaluvated for their inhibitory activity against CDK2 and Abl kinase enzymes as well as for their anti-proliferative activity (K-562 and MCF-7 cell lines). The pharmacological activity data indicated that the designed some

compounds were explicably active against CDK2 enzyme. Thus indicating that the pyrazolo[3,4-*d*]pyrimidine neuclus as potential building block in further desigining newer anticancer agents.

In chapter 2, we have extensively performed literature assessment on pyrazolo[1,5-*a*]pyrimidine for its various reported pharmacological activities. From our literature search, it was quite evident that pyrazolo[1,5-*a*]pyrimidine is a privileged scaffold, which was evident from the its marketed drugs (zeleplon, indiplon, dinaciclib, dorsomorphin, etc.) that have this scaffold. In addition, several research groups worldwide have exploited this scaffold as a building block for developing drug-like candidates with broad range of medicinal properties such as anticancer, CNS agents, anti-infectious, anti-inflammatory, CRF_1 antagonists and radio diagnostics etc. Apparently, there were no concised review article on pyrazolo[1,5-*a*]pyrimidine and its application in medicinal chemistry. Thus we envisaged in writing a review on pyrazolo[1,5-*a*]pyrimidine and its derivatives describing its biological properties with special emphasis on structure-activity relationship (SAR) studies. This work was published as a review article in *European Journal of Medicinal Chemistry* journal published in **2017**, volume **126** and page number **298** to **352**. https://doi.org/10.1016/j.ejmech.2016.11.019.

Similarly in chapter 3, we have performed comprehensive literature survey on another exploited scaffold that is pyrazolo[4,3-*d*]pyrimidine which has demonstrated numerous pharmacological activities particularly, anti-cancer, anti-infectious, phosphodiesterase inhibitors, adenosine antagonists and cytokinin antagonists etc. This extensive review unveils the synthetic and pharmacological diversity with special emphasis on structural variations around pyrazolo[4,3-*d*]pyrimidine scaffold indicating the medicinal worthiness of pyrazolo[4,3-*d*]pyrimidine framework. This review was published in *Bioorganic and medicinal chemistry* journal, **2017**, https://doi.org/10.1016/j.bmc.2017.10.012.

In chapter 4, inspired by the biological significance of pyrazolopyrimidine isomers, we synthesized novel derivatives of pyrazolo[3,4-*d*]pyrimidine by attaching aromatics at C-4 position through -NH linkage and phenethyl & pentane functional groups at C-6 via sulphur linkage by effectual synthetic route. This strategic synthetic scheme was more pragmatic in producing decent yields (75-95%) of final compounds. Synthesis of novel derivatives was achieved by nucleophilic substitution reaction of compound 4-chloro-6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6a**), 4-chloro-6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6b**) with various appropriately substituted anilines at C-4 position as depicted in scheme 1 of chapter 4. Structures of synthesized compounds were characterized by spectral data (IR, ^{1}H NMR, ^{13}C NMR and HRMS). Laboratory of Growth Regulators, Palacky University, Slechtitelu 27, 78371, Olomouc, Czech Republic, performed anticancer screening against CDK2 & Abl kinase inhibitors and K-562 & MCF-7 human cancer cell lines. Compounds **8**, **11** and **36** having 2-chloro, 3-nitro and 4-methylthio aniline groups at C-4 respectively displayed significant enzymatic

inhibitory activity against CDK2 (IC$_{50}$ = 5.1 µM, 7.8 µM, 8.8 µM) and Abl (IC$_{50}$ = >25 µM, >12.5 µM, >25 µM) as well as prominent anti-proliferative effects against K-562 and MCF-7 cancer cell lines with IC$_{50}$ value ranging from 19.2 to 27.4 µM. Further, the *in silico* molecular docking studies displayed good binding interactions and the binding energies were in agreement with the observed SAR as well as experimental results as discussed in chapter 4. This work This review was published in ***Bioorganic Chemistry*** journal, **2018**, https://doi.org/10.1016/j.bioorg.2018.02.030.

In chapter 5, as a continued effort in synthesizing pyrazolo[3,4-*d*]pyrimidine inspired anticancer compounds (CDK2 and Abl kinase inhibitors), pyrazolo[3,4-*d*]pyrimidine with different substituted heterocyclic moieties at C-4 position through ester linkage and phenethyl, pentane and hexane functional groups at C-6 via sulphur linkage for their anticancer activity. Diverse novel compounds of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine derivatives (**5a-5h**, **6a-6d** and **7a-7c**) were synthesized by efficient and adaptable synthetic route (scheme 1) as described in chapter 5. These compounds were well characterized by IR, ^{1}H, ^{13}C NMR, and HRMS. Laboratory of Growth Regulators, Palacký University, Slechtitelu 27, 78371, Olomouc, Czech Republic, evaluated obtained compounds for their in vitro anticancer activity against CDK2 & Abl kinase inhibitors and K-562 & MCF-7 human cancer cell lines. From the tested series, compounds **5a** (CDK2: IC$_{50}$ = 8.8 µM) and **6c** (CDK2: IC$_{50}$ = 6.8 µM) displayed significant enzymatic inhibitory activity and prominent anti-proliferative effects against K-562 and MCF-7 cancer cell lines with IC$_{50}$ value ranging from 18.9 to 89.3 µM. In addition, the binding energies of the best active compounds were in agreement with the experimental data and supported the SAR studies as discussed in chapter 5. This work has been communicated for publication in ***Chemical Biology and Drug Design***.

Finally, in chapter 6, as an ongoing endeavour in synthesizing pyrazolo[3,4-*d*]pyrimidine inspired anticancer compounds, we have synthesized phenylcarbamoyl acetamide fused pyrazolo[3,4-d]pyrimidines. Compounds **9a**-**9s** were synthesized with diverse structural variations by simple and effective synthetic route as described in chapter 6. Several phenylcarbamoyl acetamides (**8a-8s**) were efficiently synthesized and used for synthesis of desired final derivatives. All the final compounds were well characterized by IR, ^{1}H, ^{13}C NMR and HRMS, thus conforming their formation. *In vitro* anticancer screening was performed at Laboratory of Growth Regulators, Palacký University, Slechtitelu 27, 78371, Olomouc, Czech Republic. For all the synthesized molecules from this series, IC$_{50}$ values could not be measured due to solubility limit (IC$_{50}$ >12.5 µM or >25 µM) as described in chapter 6. The overall finds suggest the significance of hybridisation to achieve fused pyrazolopyrimidines as anticancer agents. The manuscript for this work has been drafted and is ready for communication to an appropriate peer reviewed international journal.

2   Future work

A comprehensive literature review and the anticancer activity data of the synthesized molecules displayed the significance of the pyrazolo[3,4-*d*]pyrimidine scaffold. However, one of the drawbacks of these molecules was the solubilty, which could have affected their activity profile. For better pharmacological activity, bioavailability, and efficacy profiles further design and optimization of pyrazolo[3,4-*d*]pyrimidine based molecules is needed. This could be achieved by carefully substitution of hydrophilic and charged bioactive moieties at various positions of the pyrazolo[3,4-*d*]pyrimidine scaffold through efficient synthetic methods. In parallel, 3D-QSAR-based pharmacophore models could be generated form the available dataset molecules and validated using the test set ligands. Thus, ligand-based virtual screening of the best pharmacophore model against drug-like database (ZINC, Maybridge, Chembridge and NCI) can be performed to identify new set of anticancer hits which can also be considered for strategic chemical synthesis. Further, molecular docking studies (structure-based drug design) can also offer comparatively enhanced solutions for optimization of anticancer leads. Thus, the presented work will contribute existing literature on pyrazolo[3,4-*d*]pyrimidine scaffold and assist in developing novel class of pyrazolo[3,4-*d*]pyrimidine based anticancer compounds as potential drug candidates.

APPENDIX – I

SUPPLIMENTARY INFORMATION

CHAPTER 4

Synthesis, anticancer evaluation, and molecular docking studies of some novel 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines as cyclin dependent kinase 2 (CDK2) inhibitors

Department of Pharmaceutical Chemistry, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa.
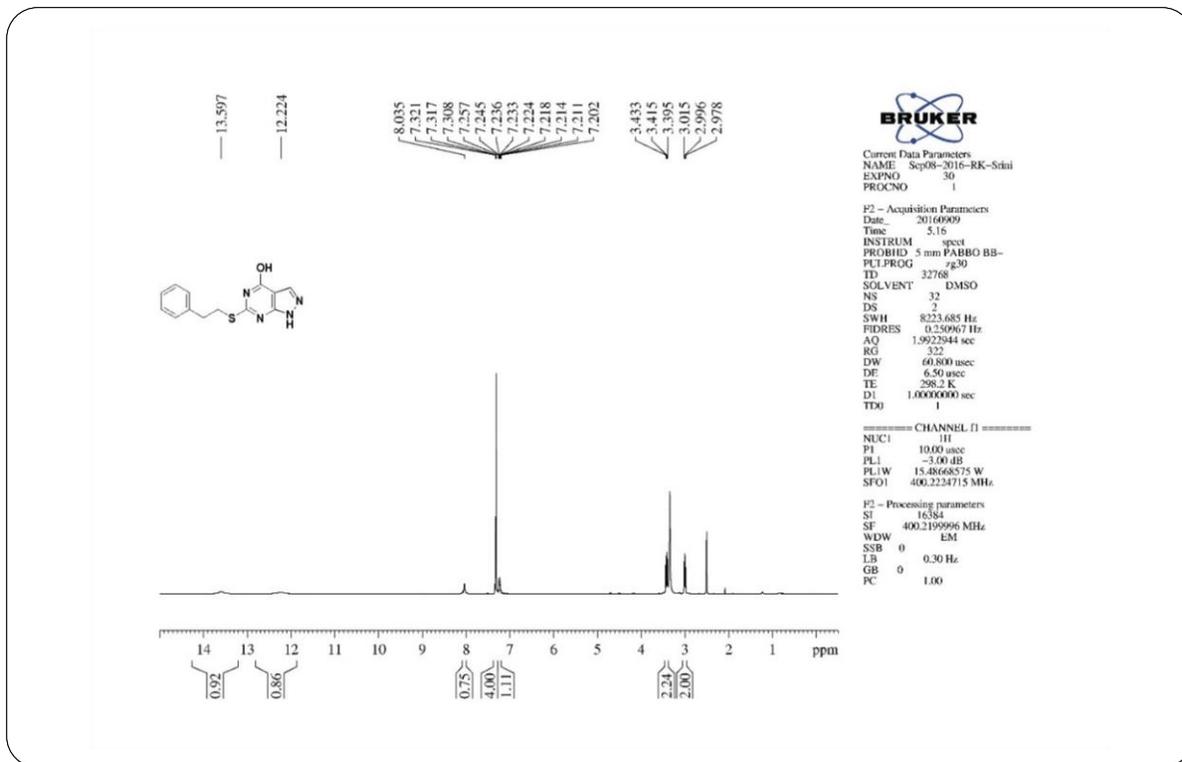
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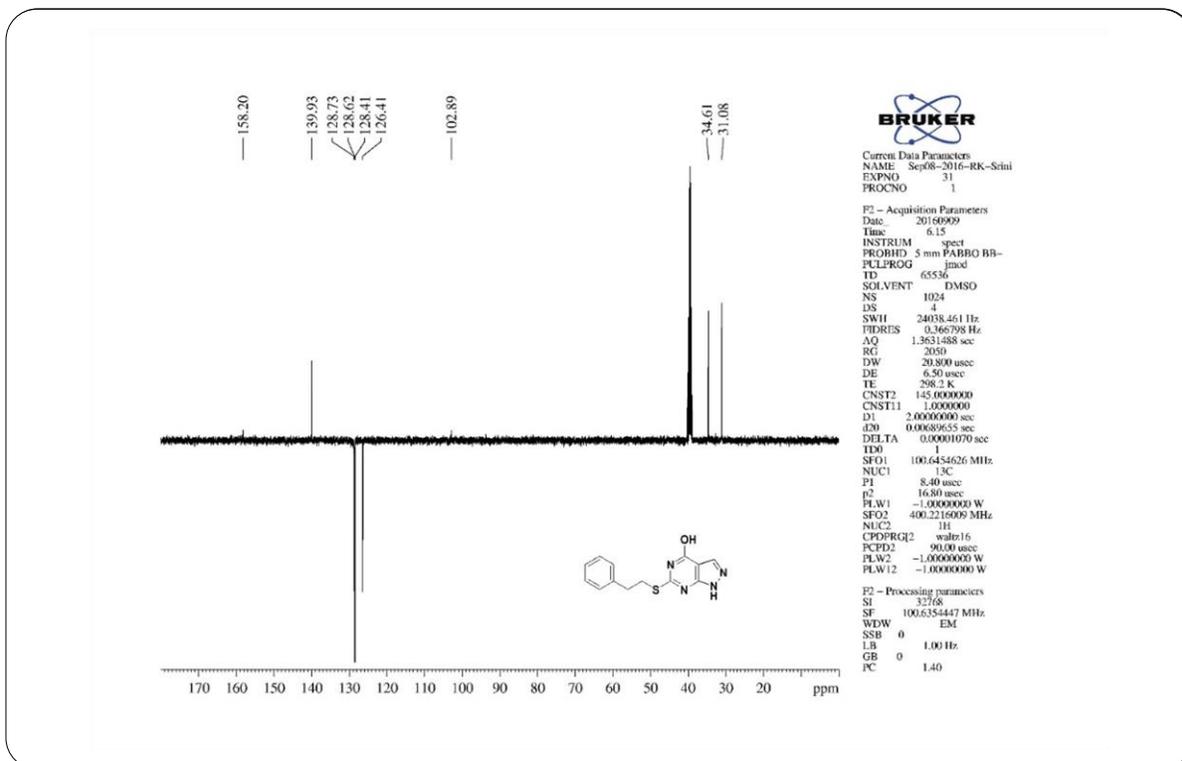
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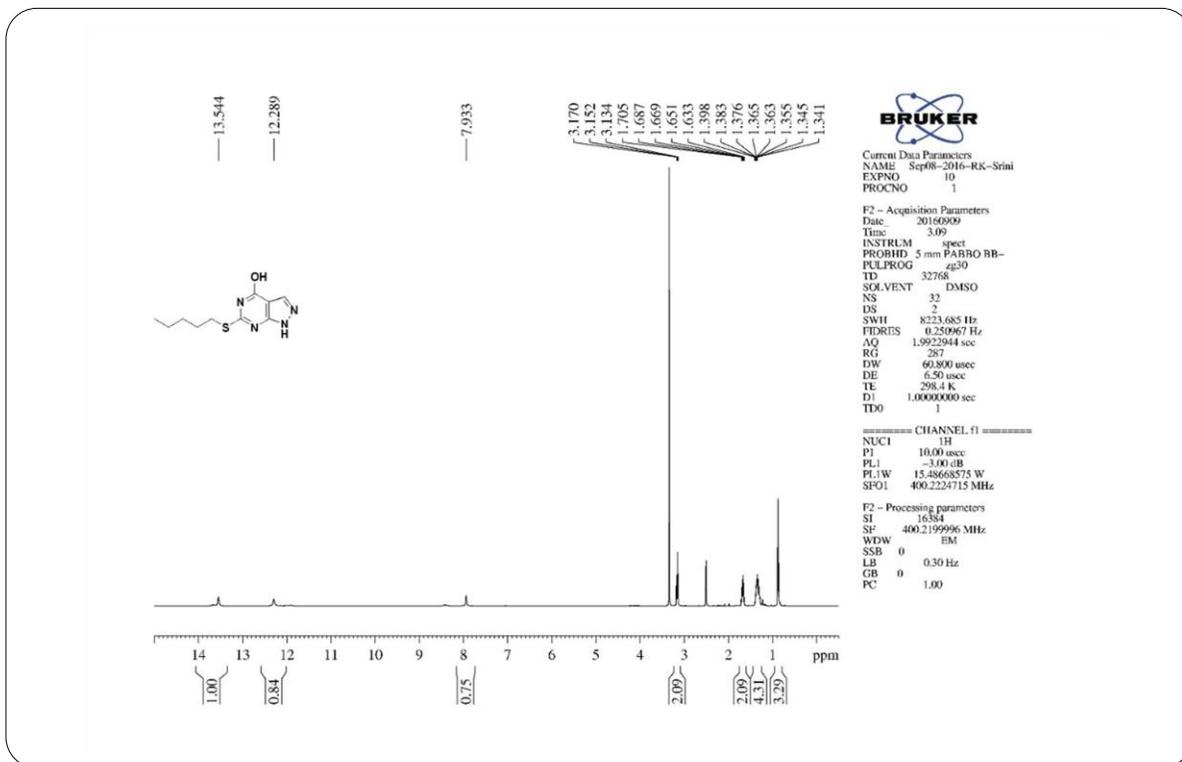
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¹³C NMR Spectrum of Compound 5b

¹H NMR Spectrum of Compound 6a

¹³C MNR Spectrum of Compound 6a
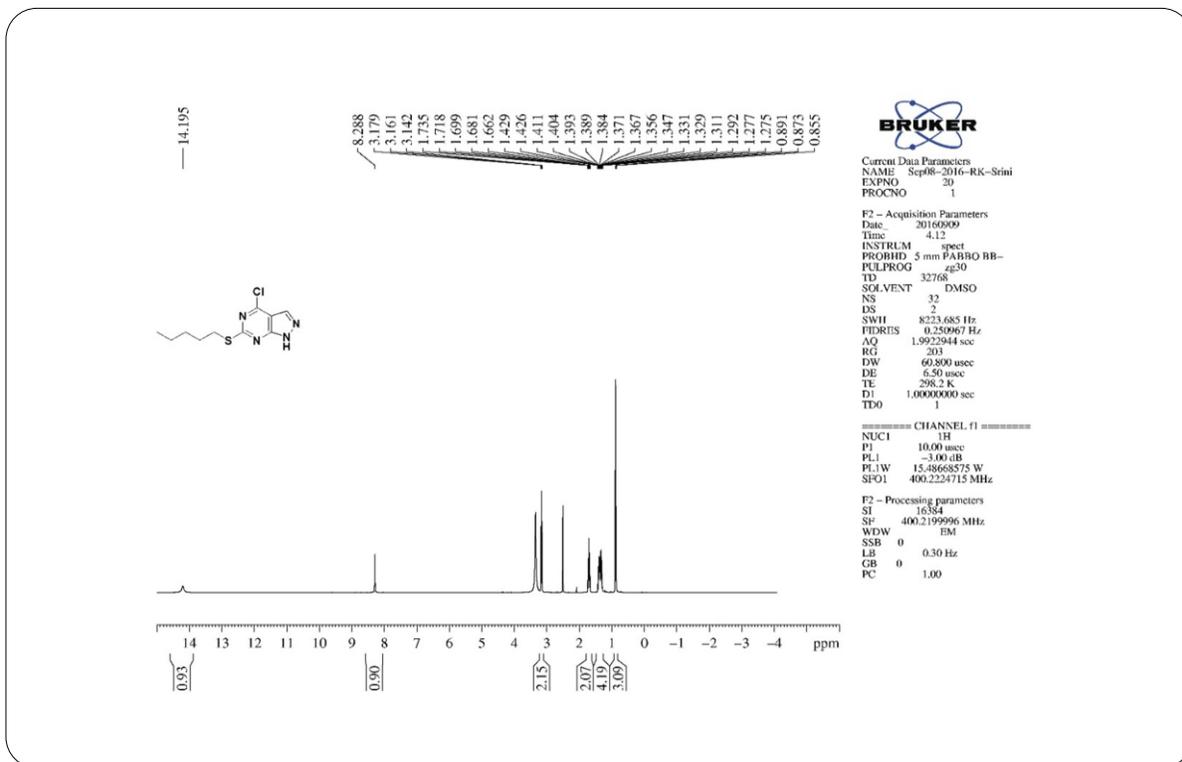
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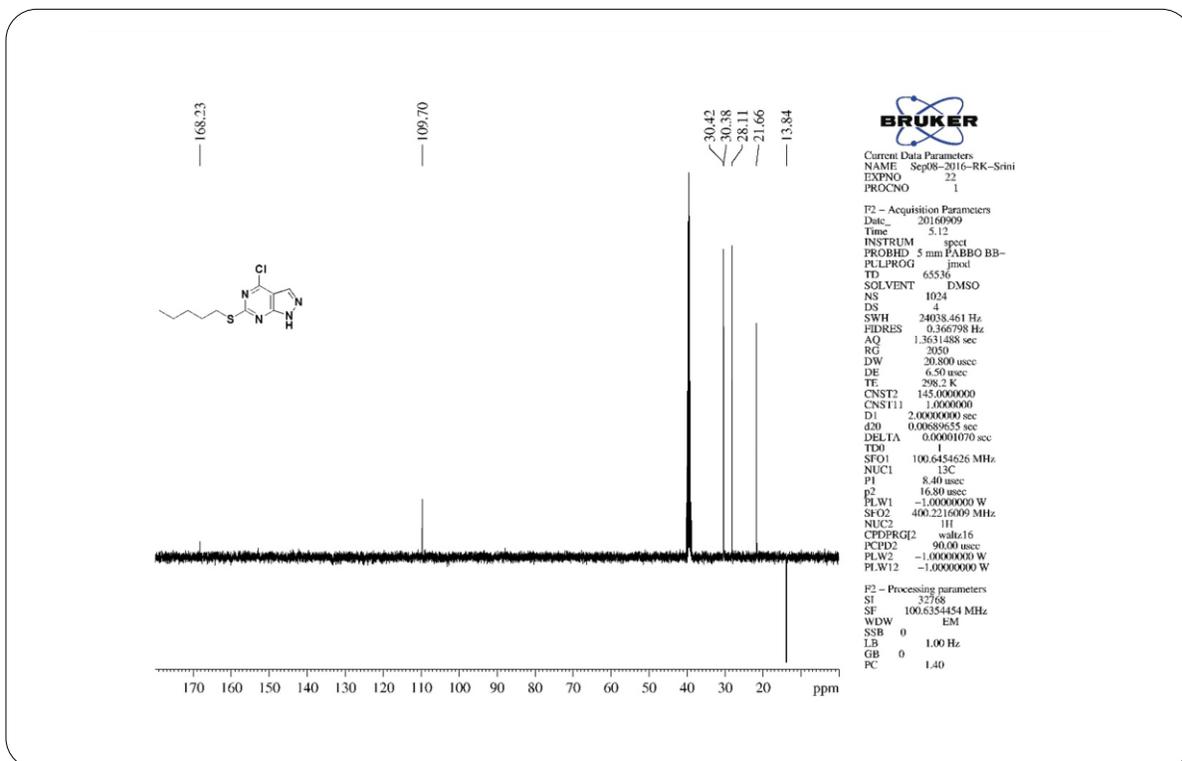

^{13}C NMR Spectrum of Compound 6b

IR Spectrum of Compound 7

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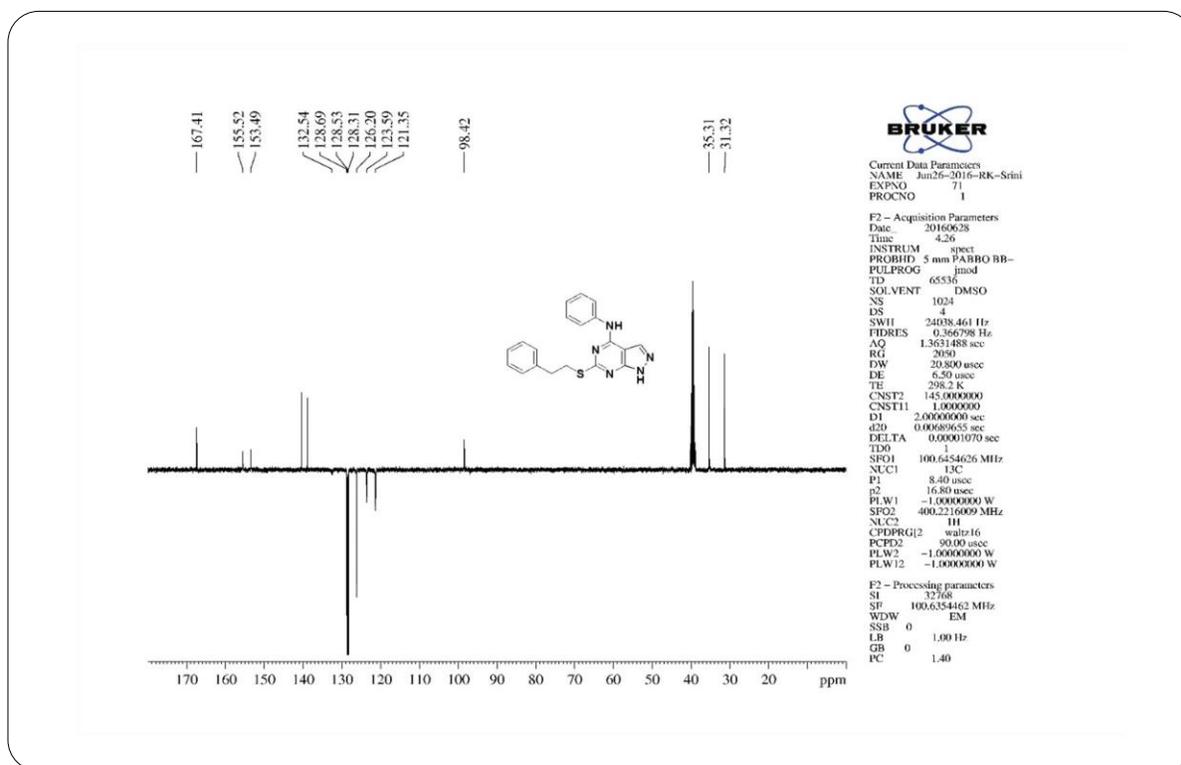
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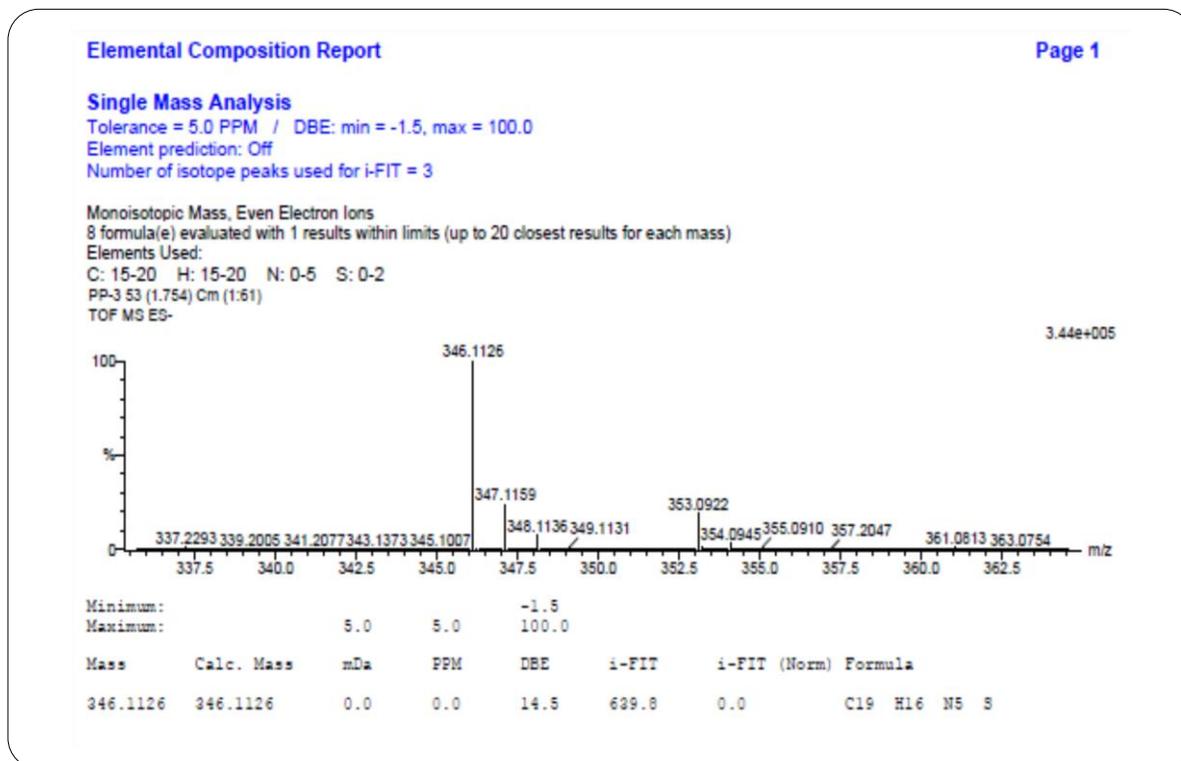
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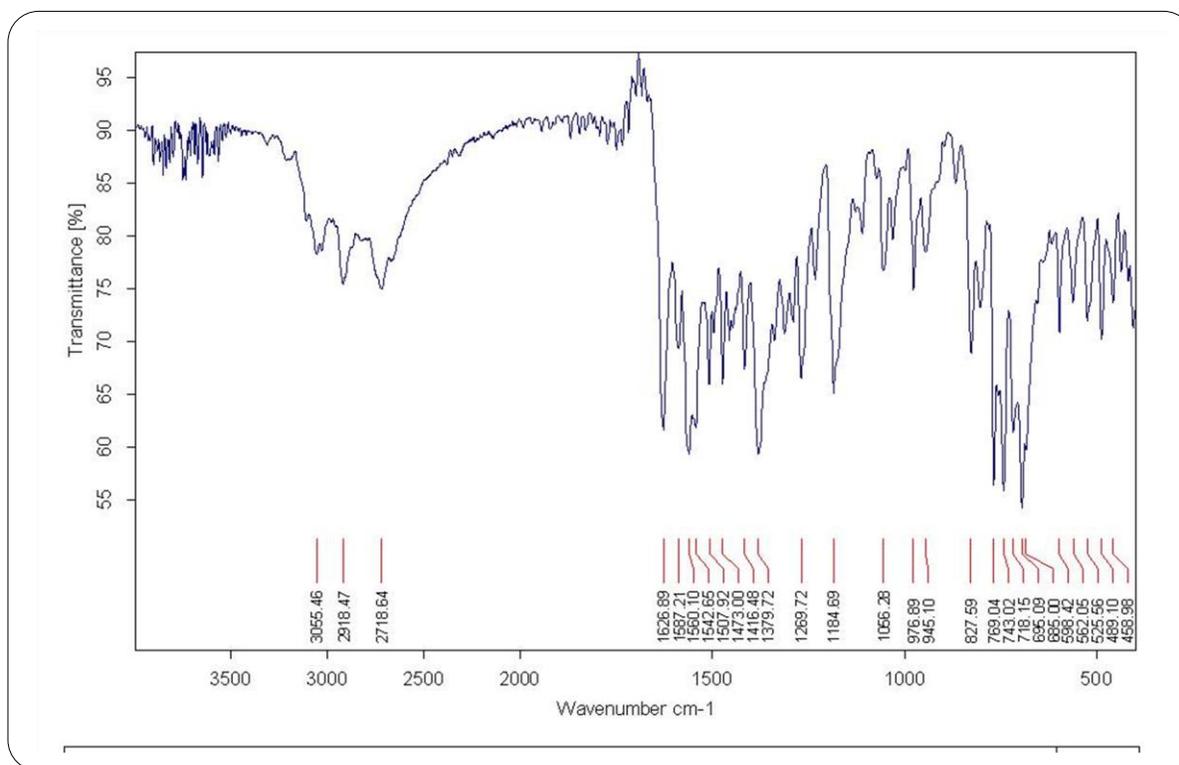
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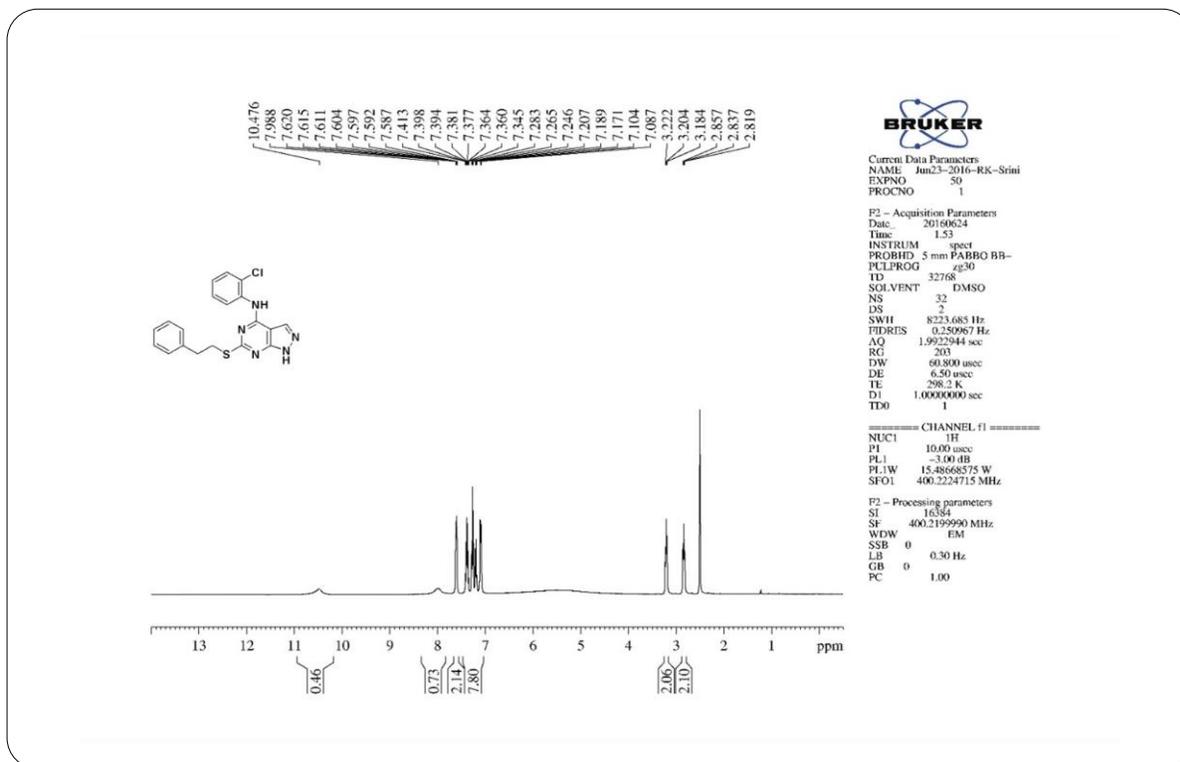
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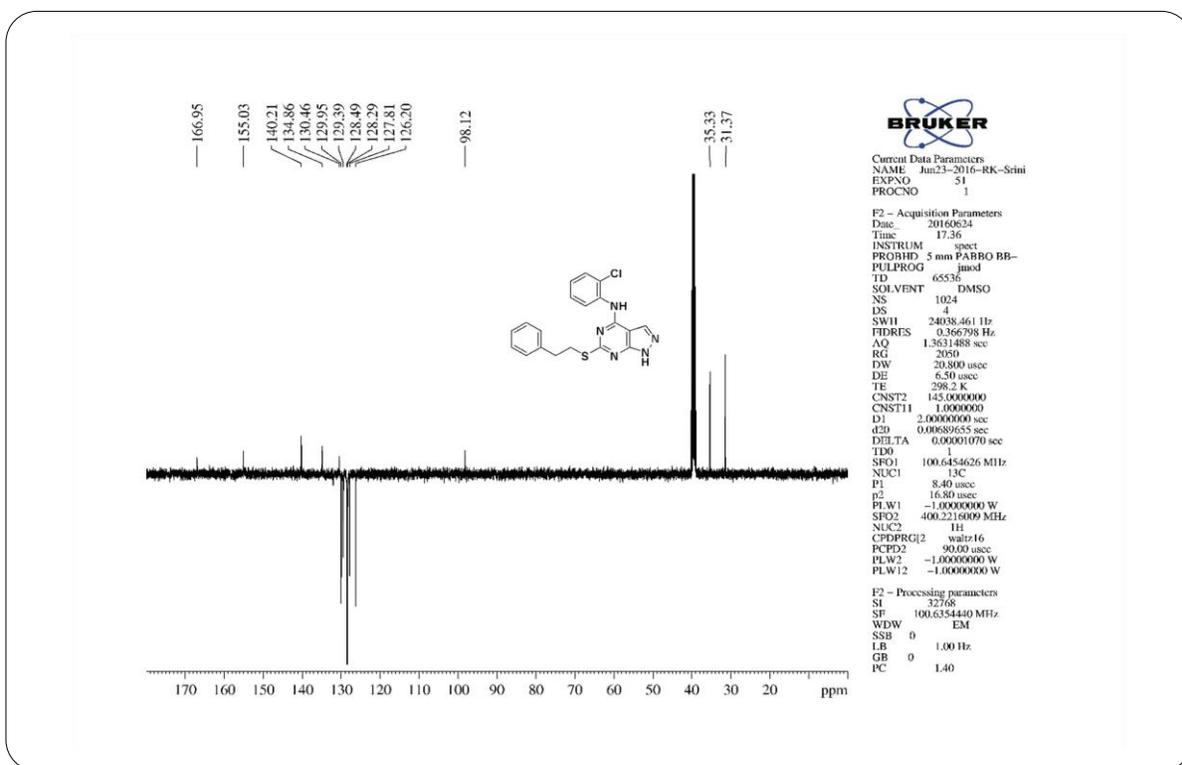
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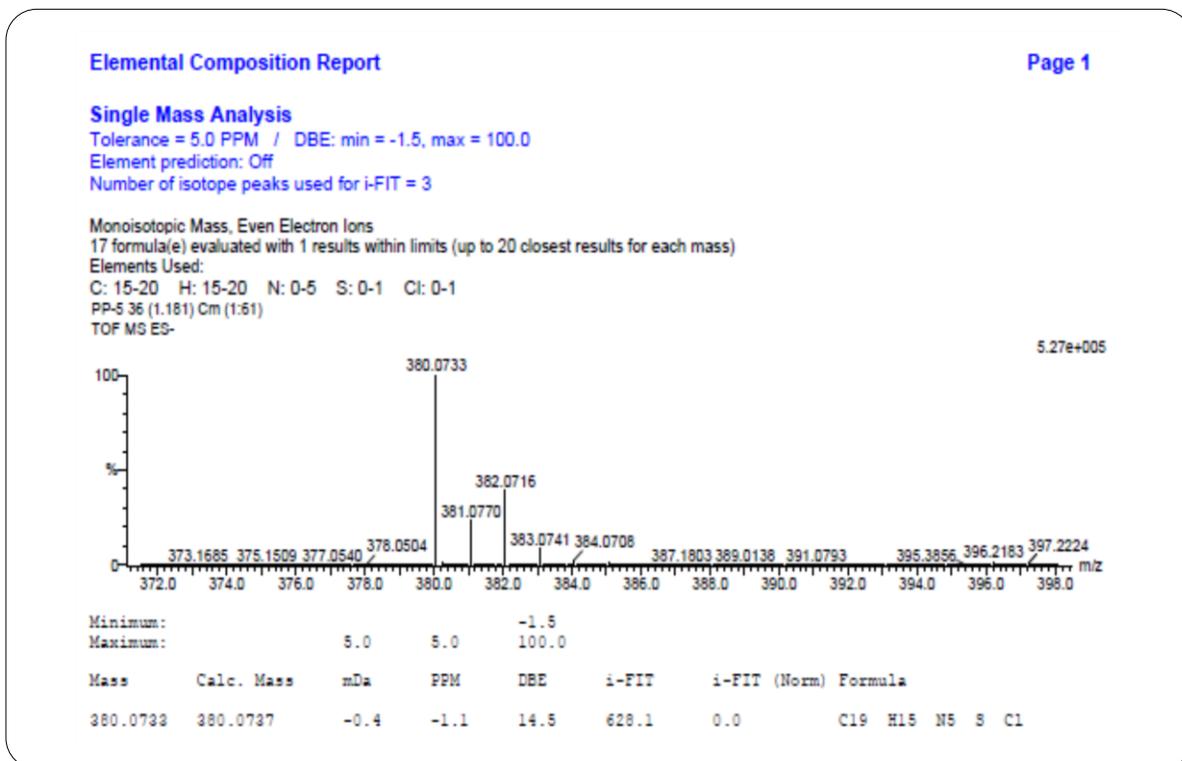
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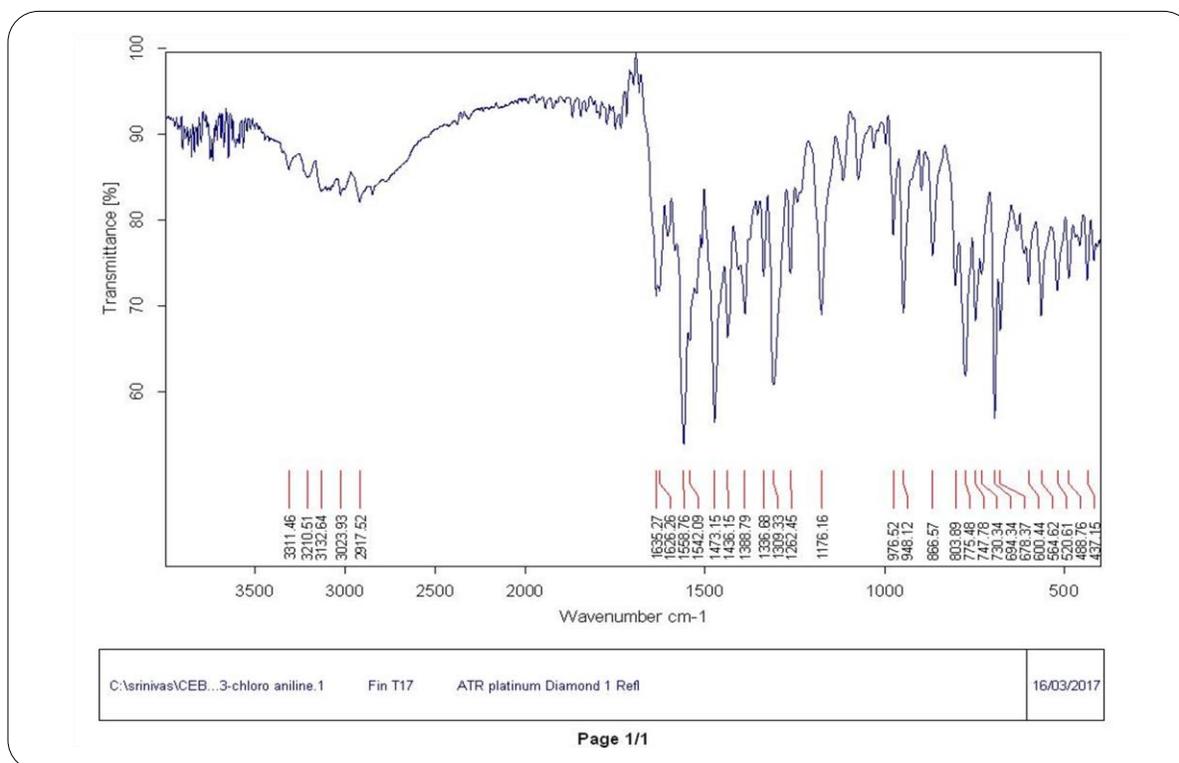
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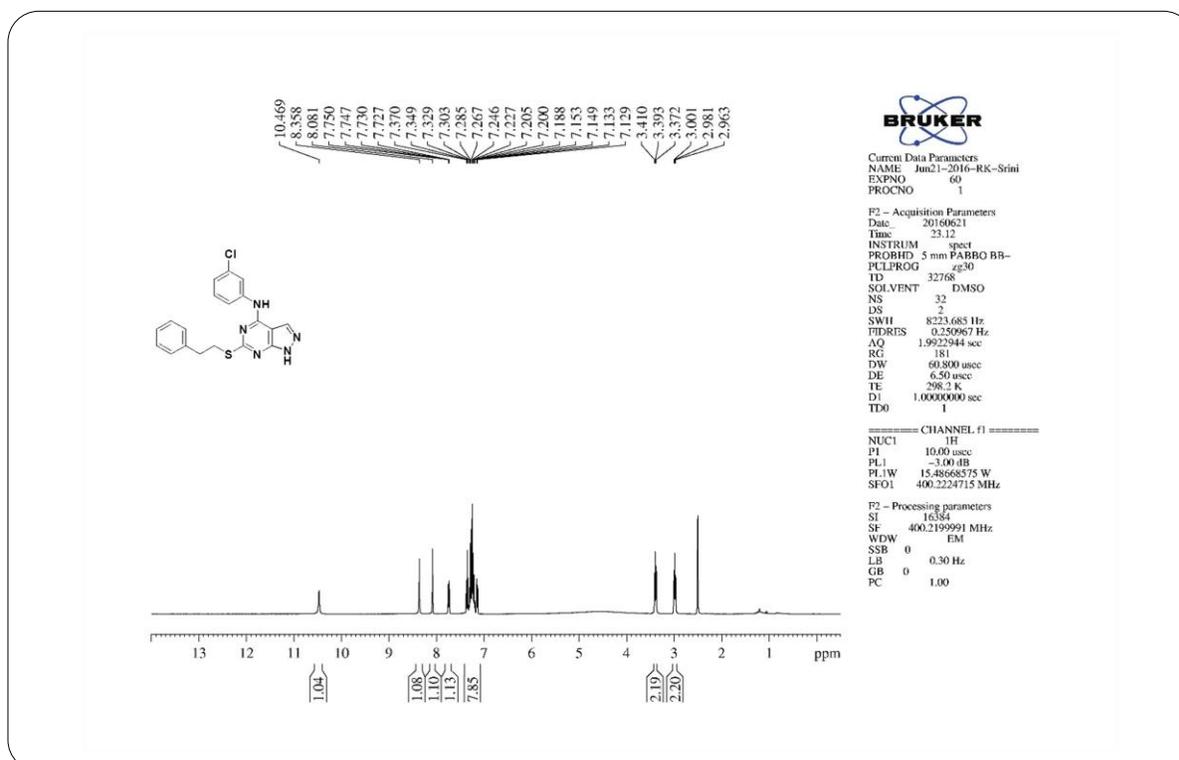
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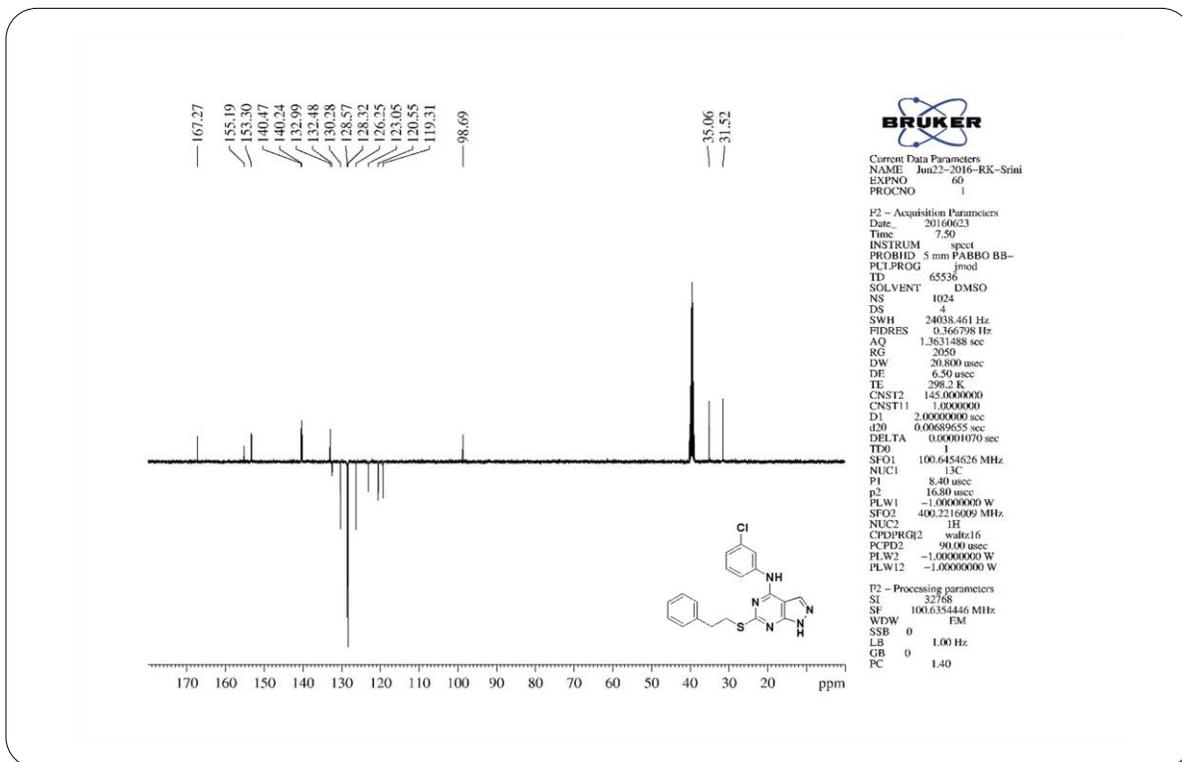
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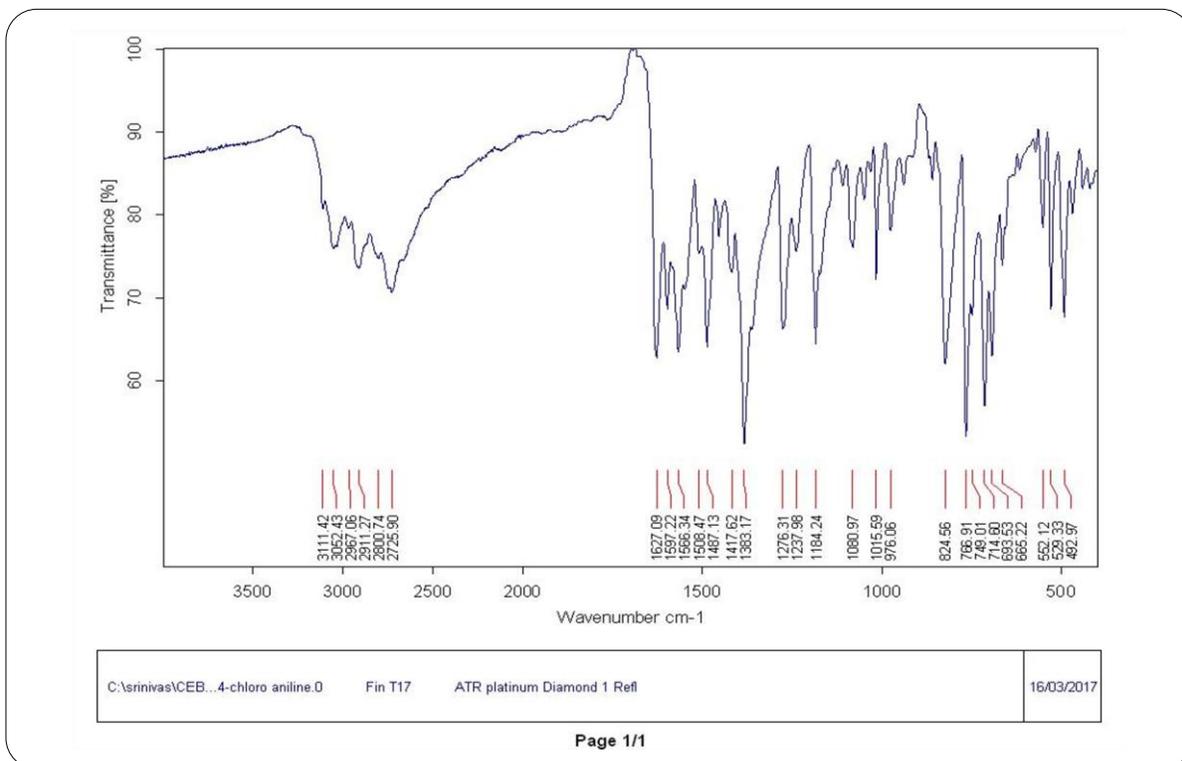
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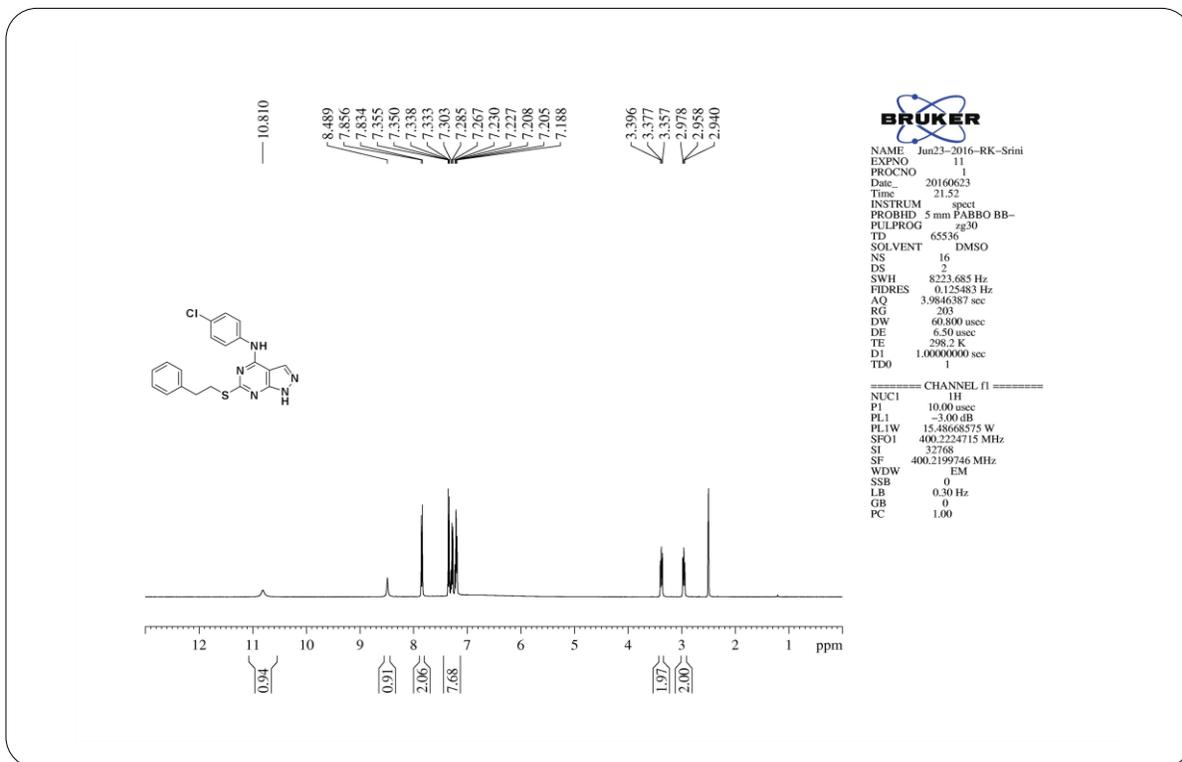
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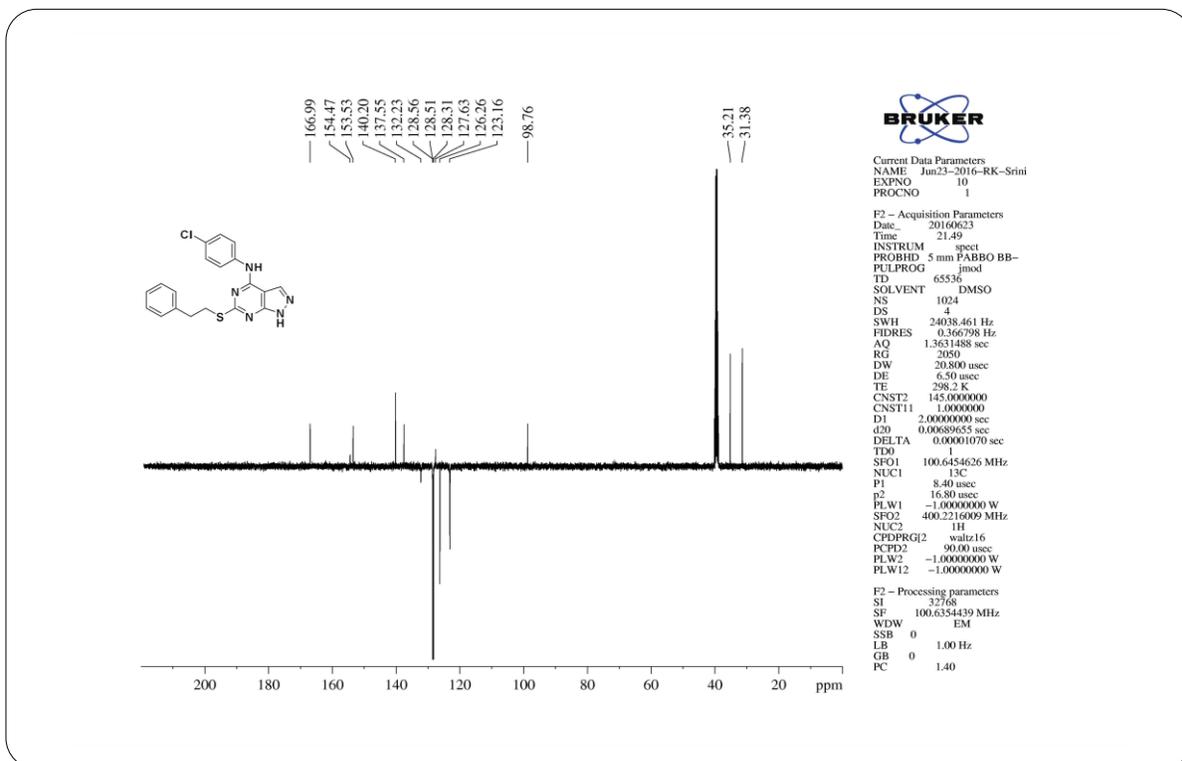
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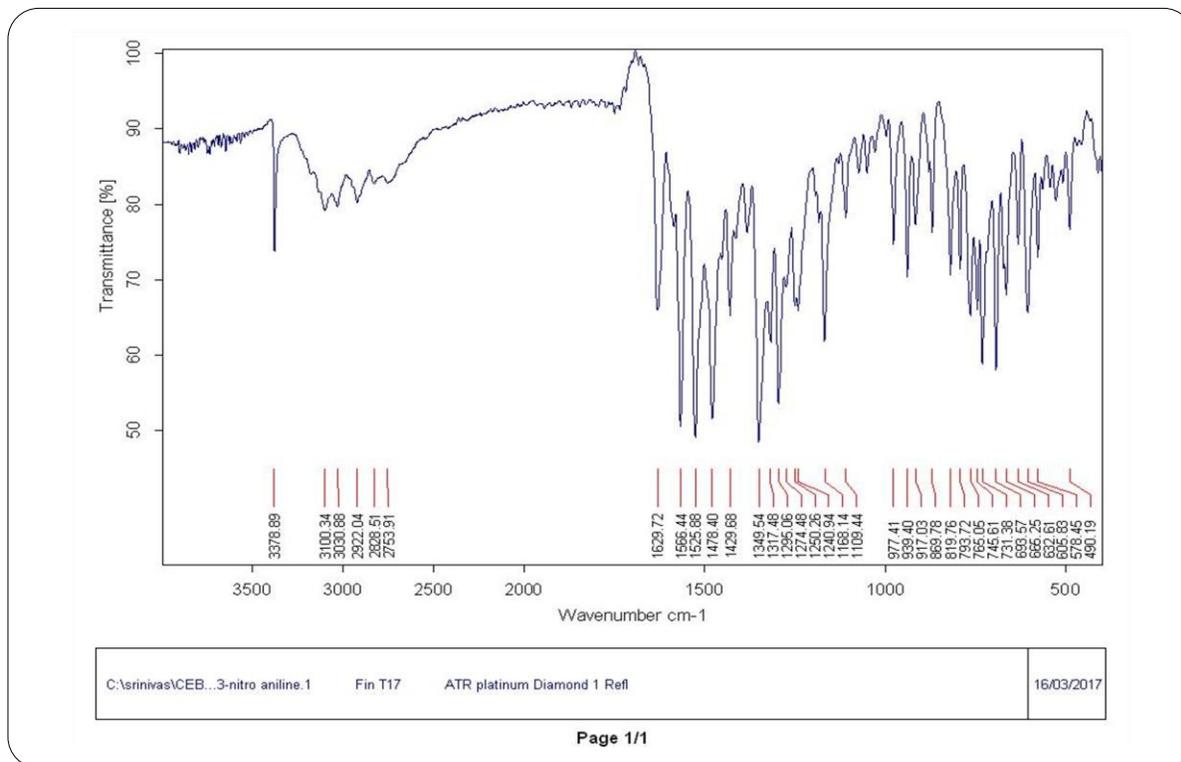
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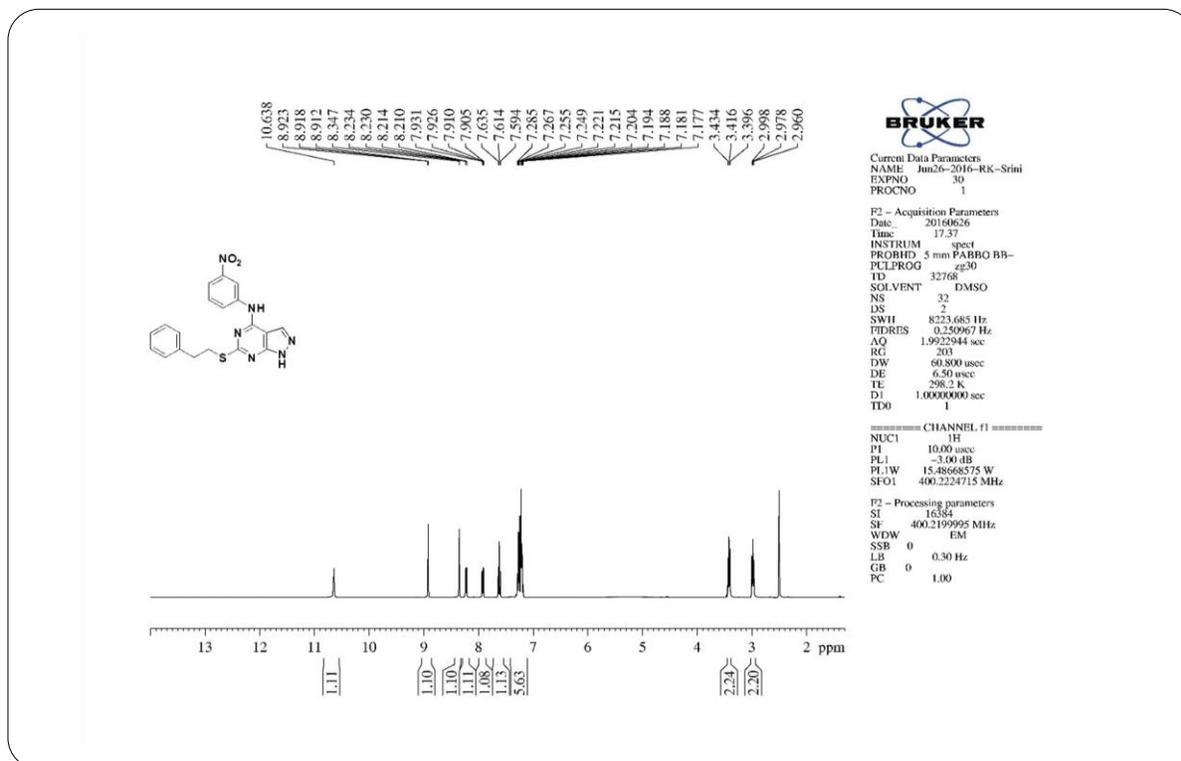

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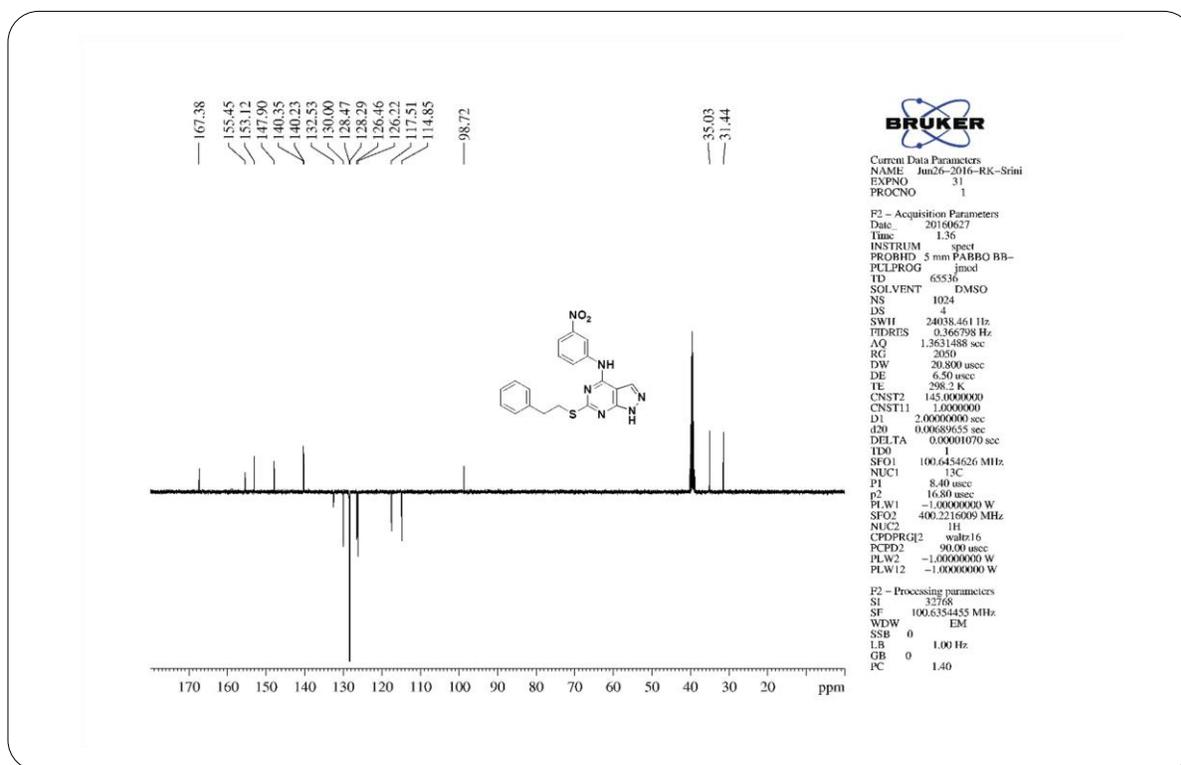
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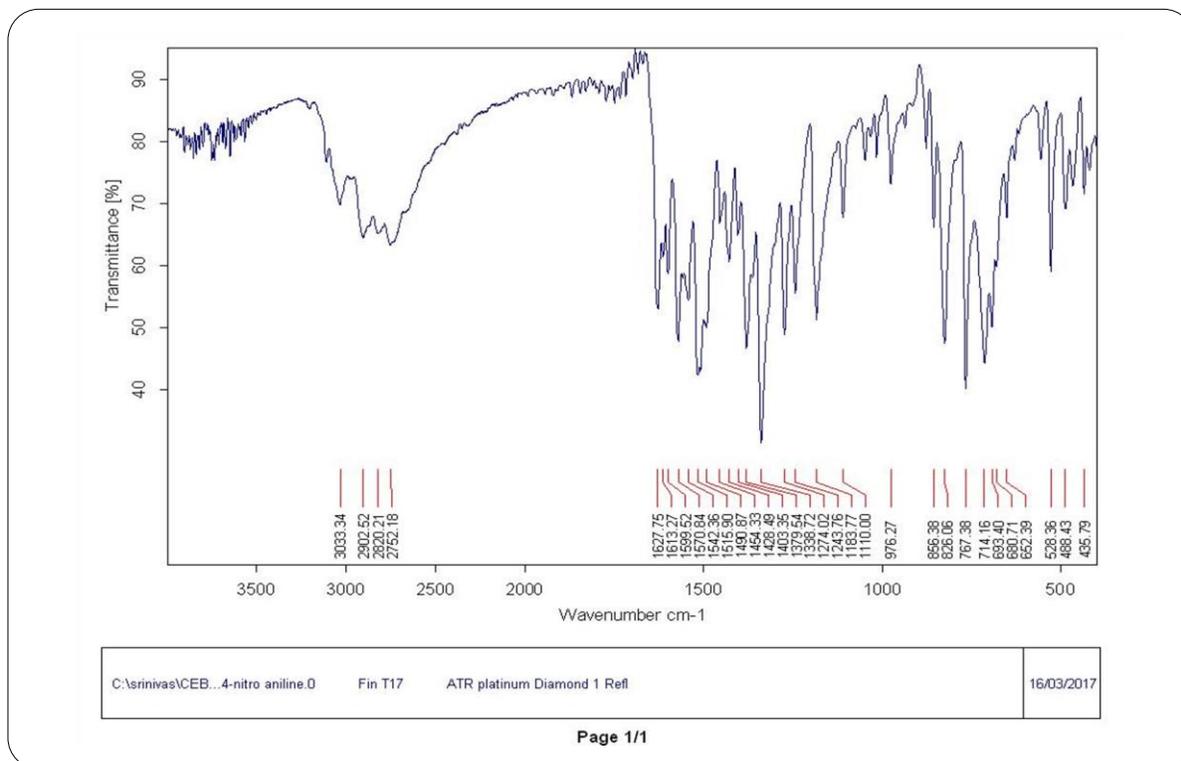
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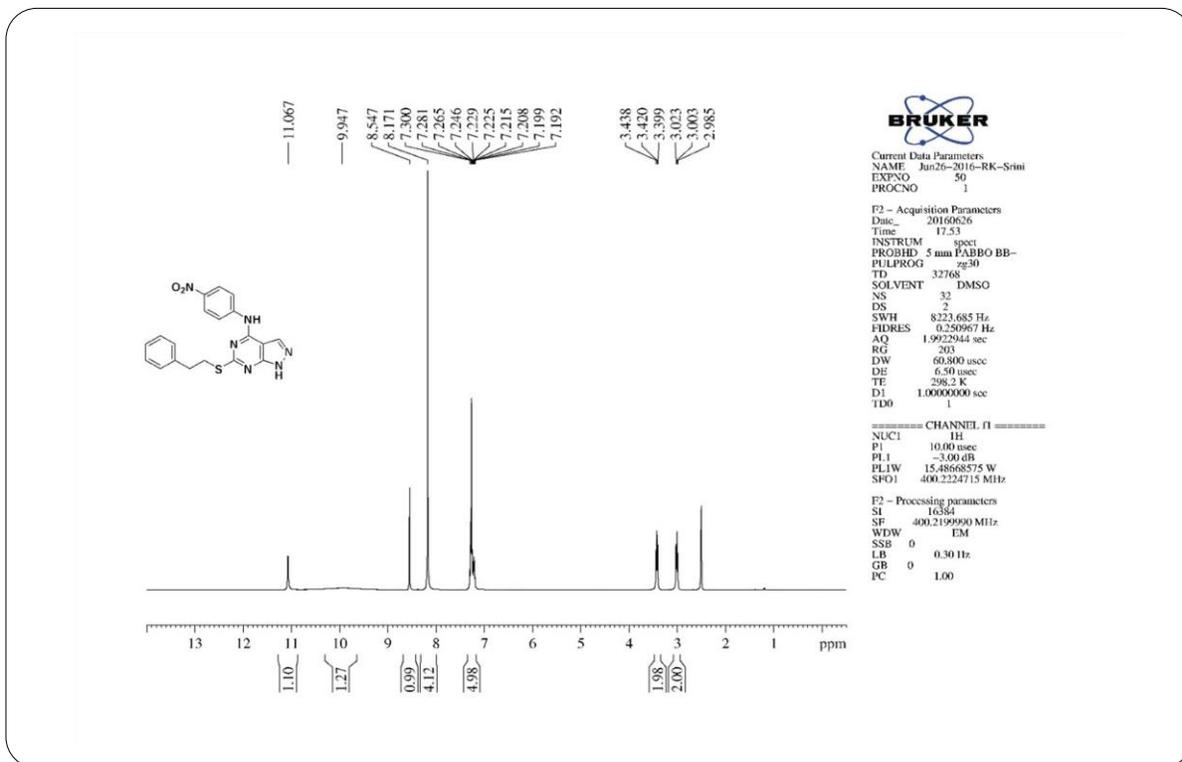
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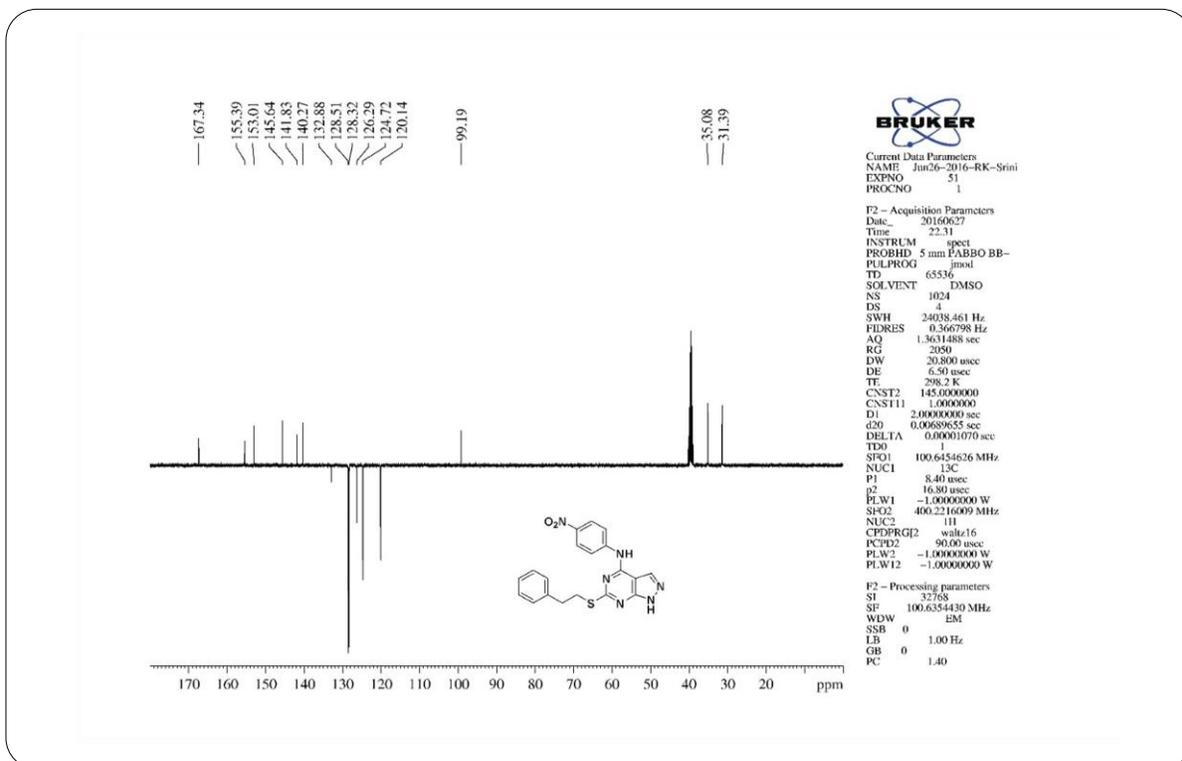
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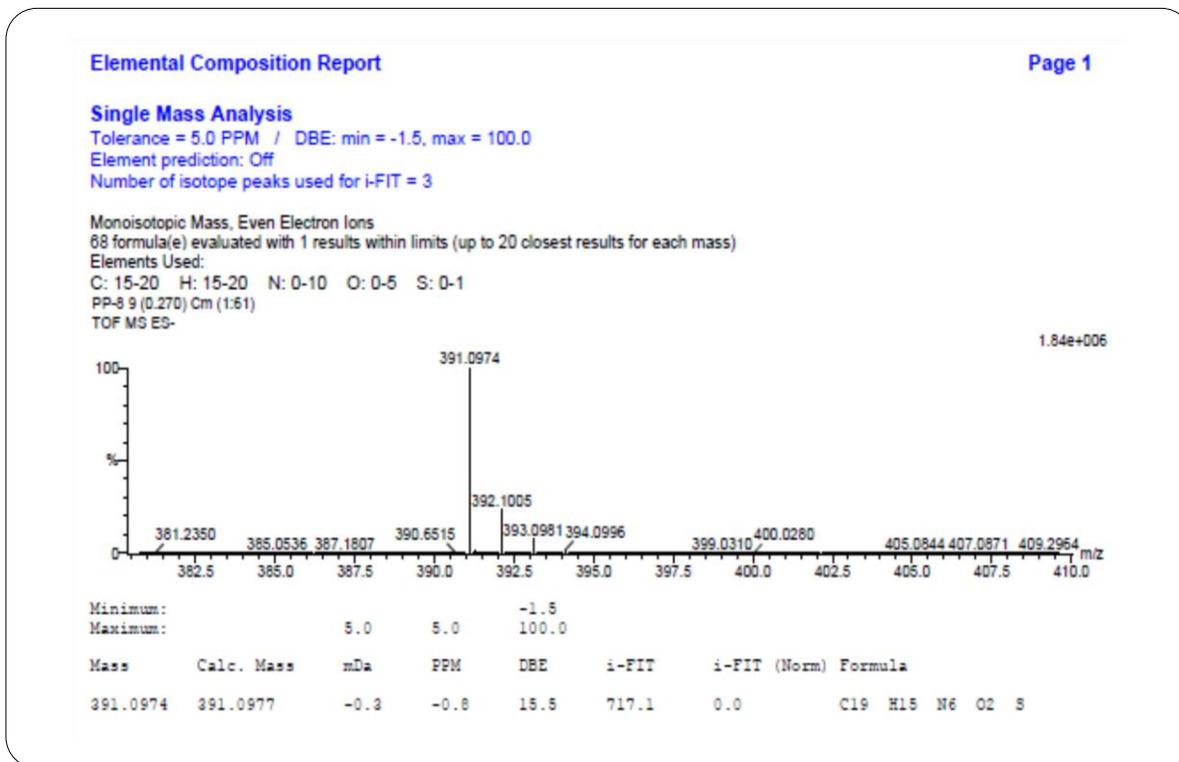
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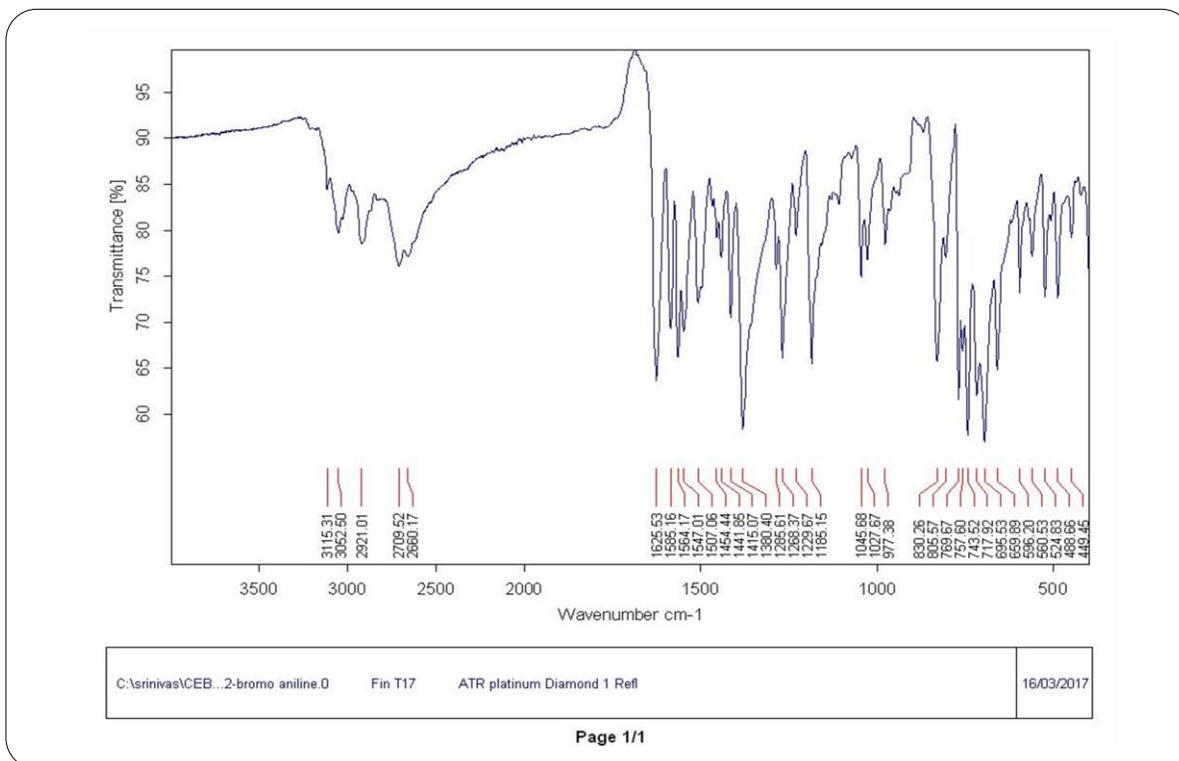
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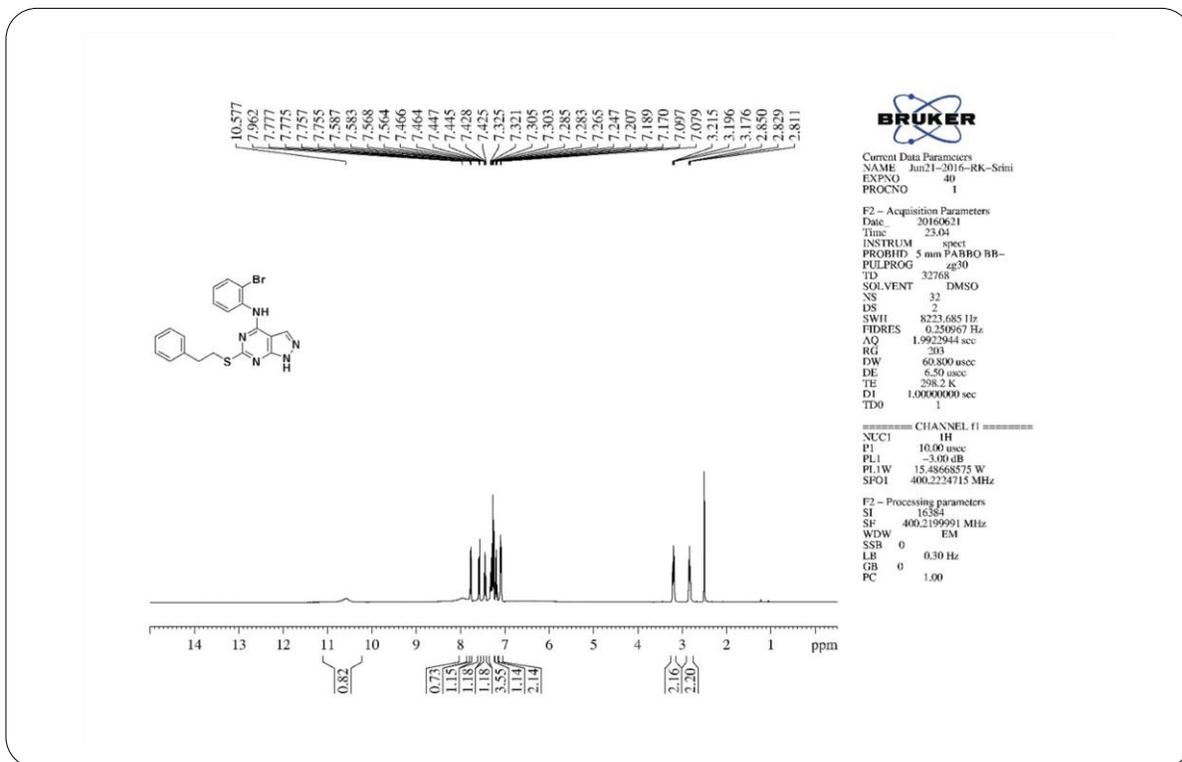
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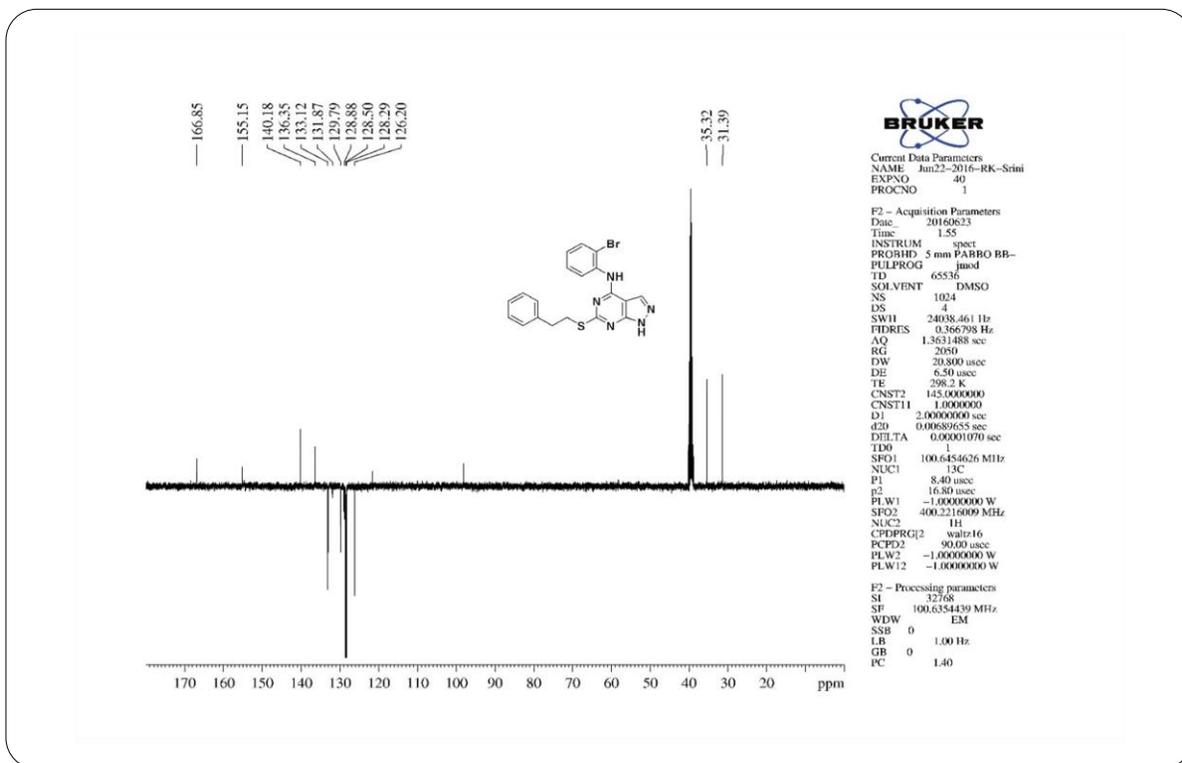
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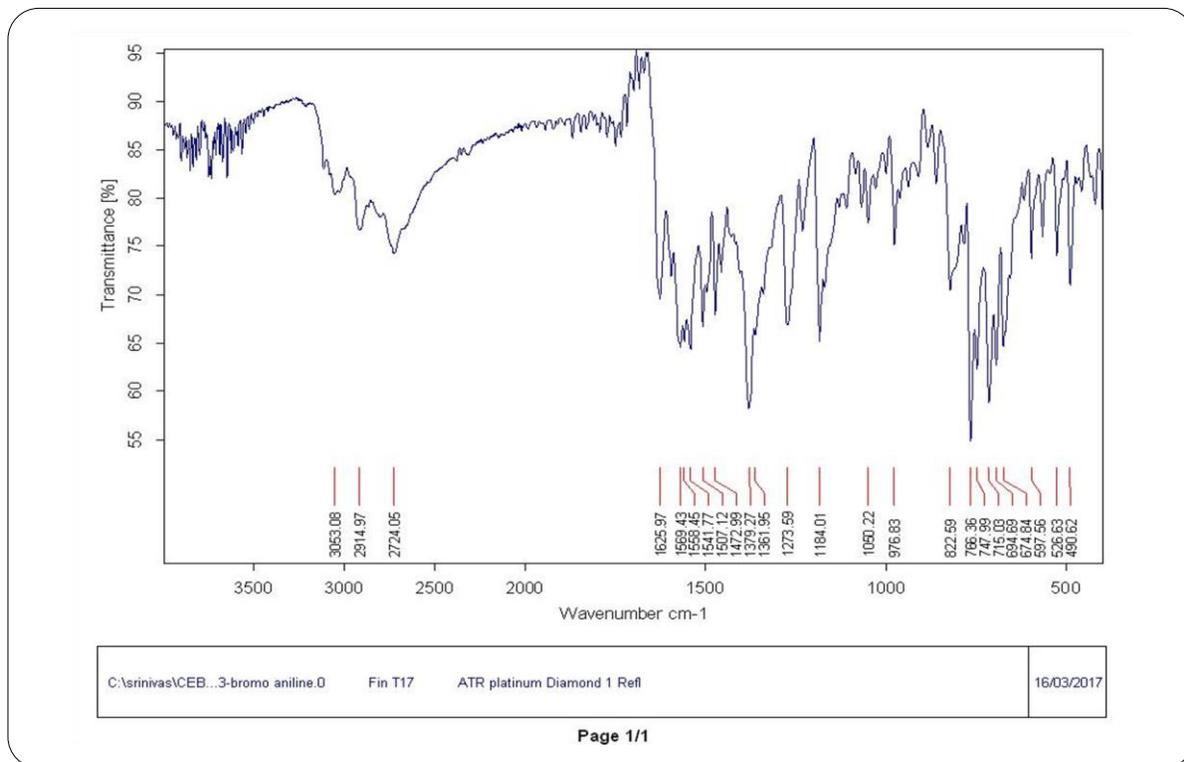
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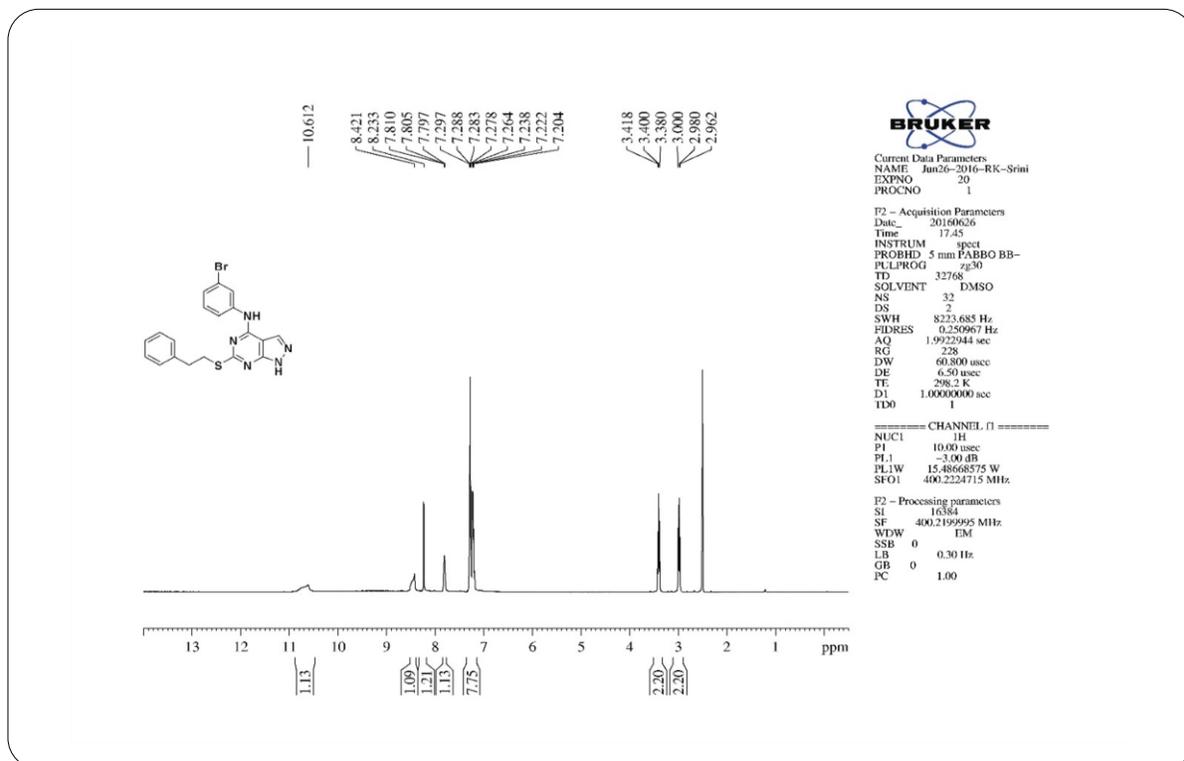
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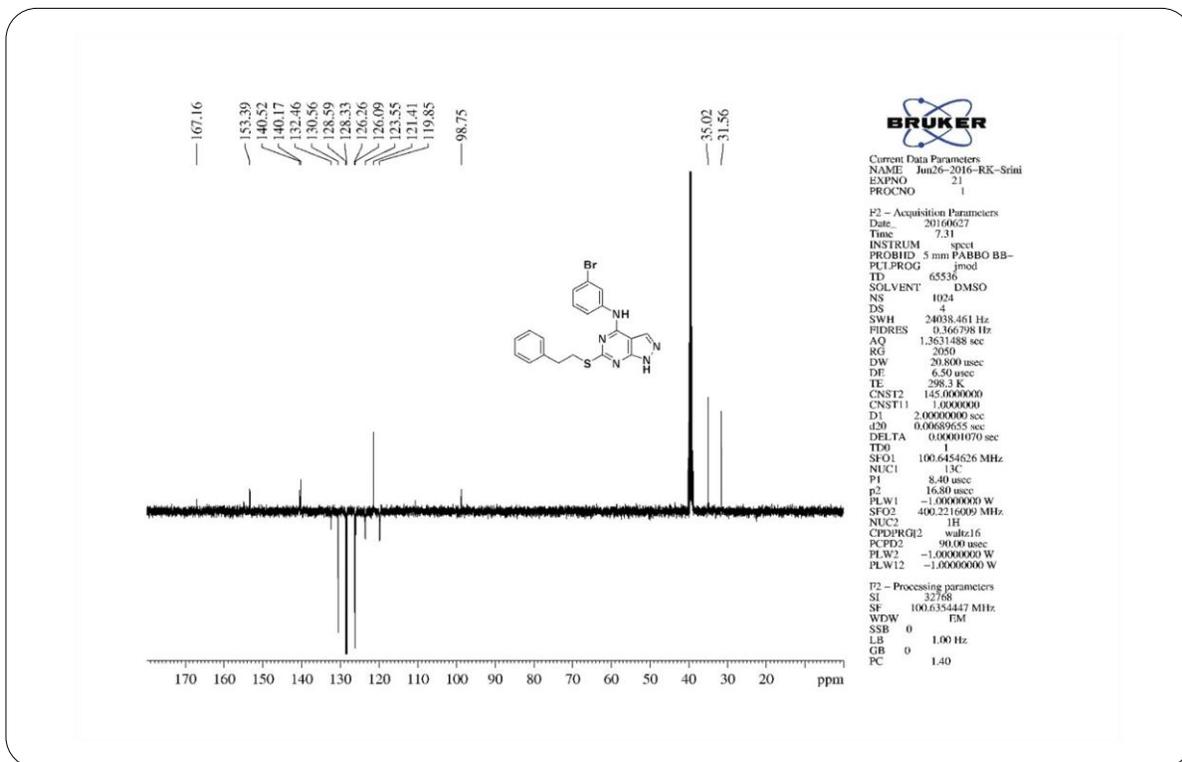
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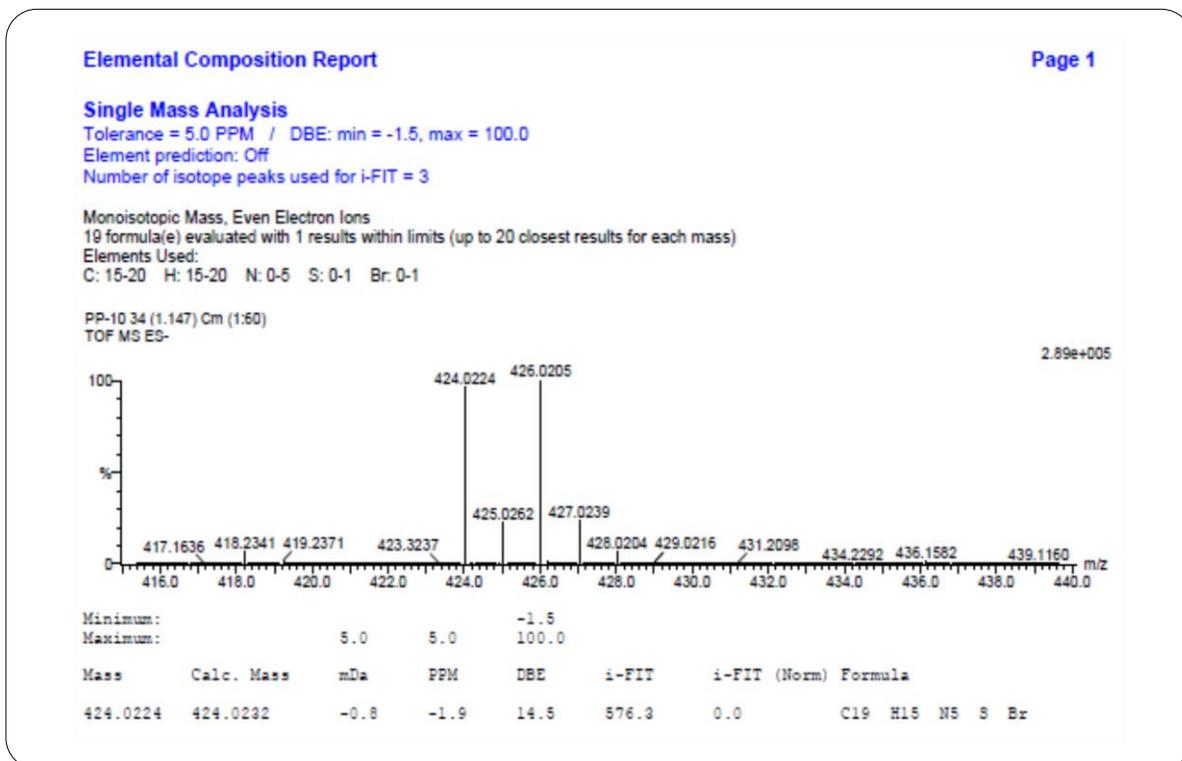
HRMS Spectrum of Compound 14
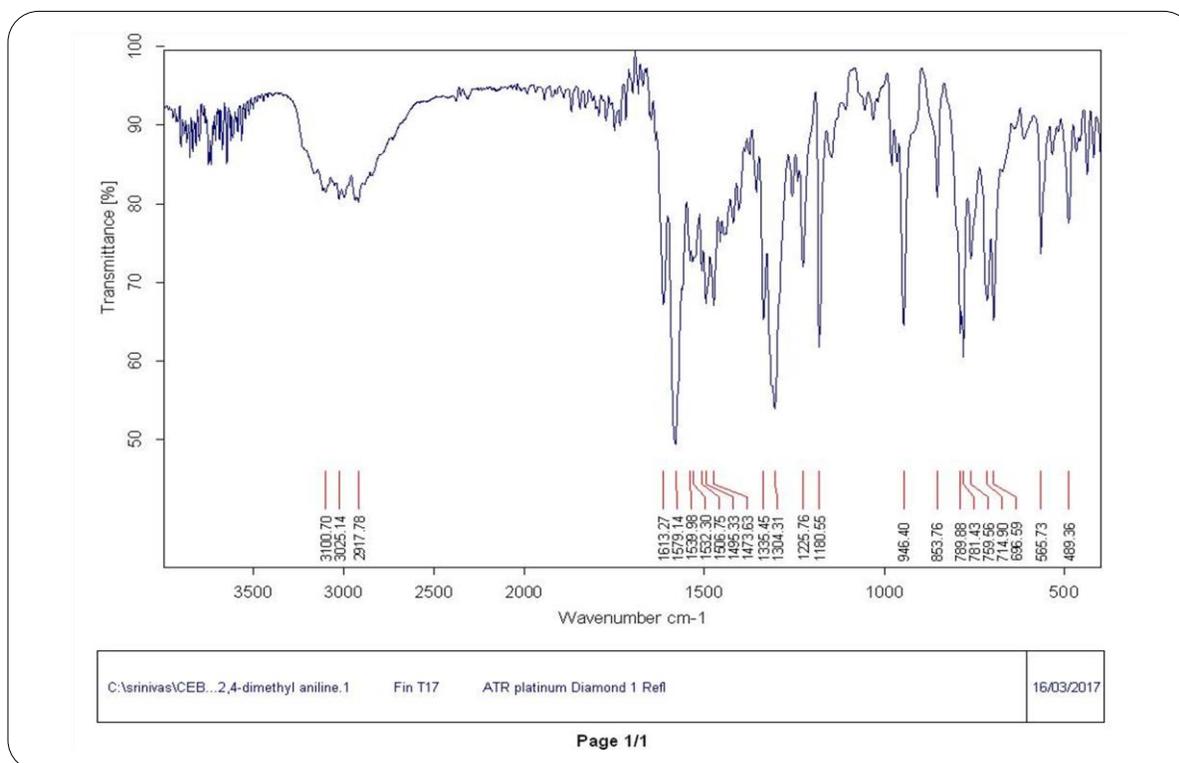
**IR Spectrum of Compound 15**
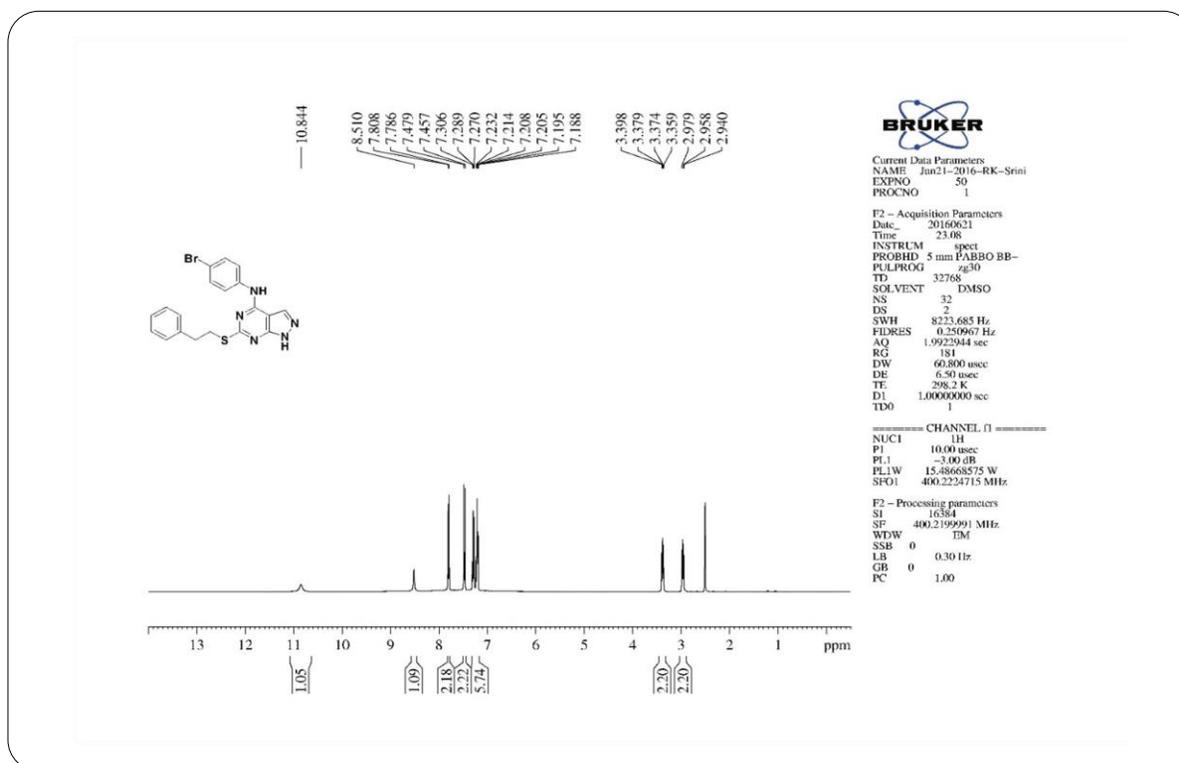
^{1}H NMR Spectrum of Compound 15
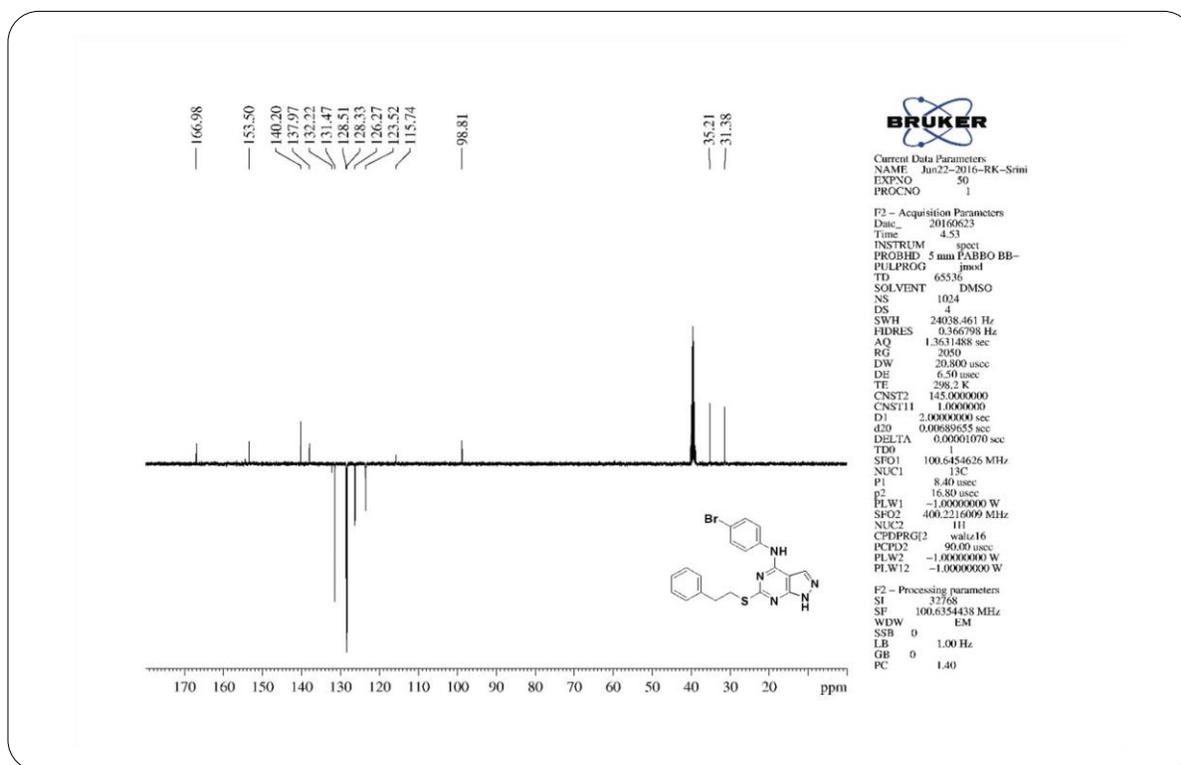
¹³C NMR Spectrum of Compound 15
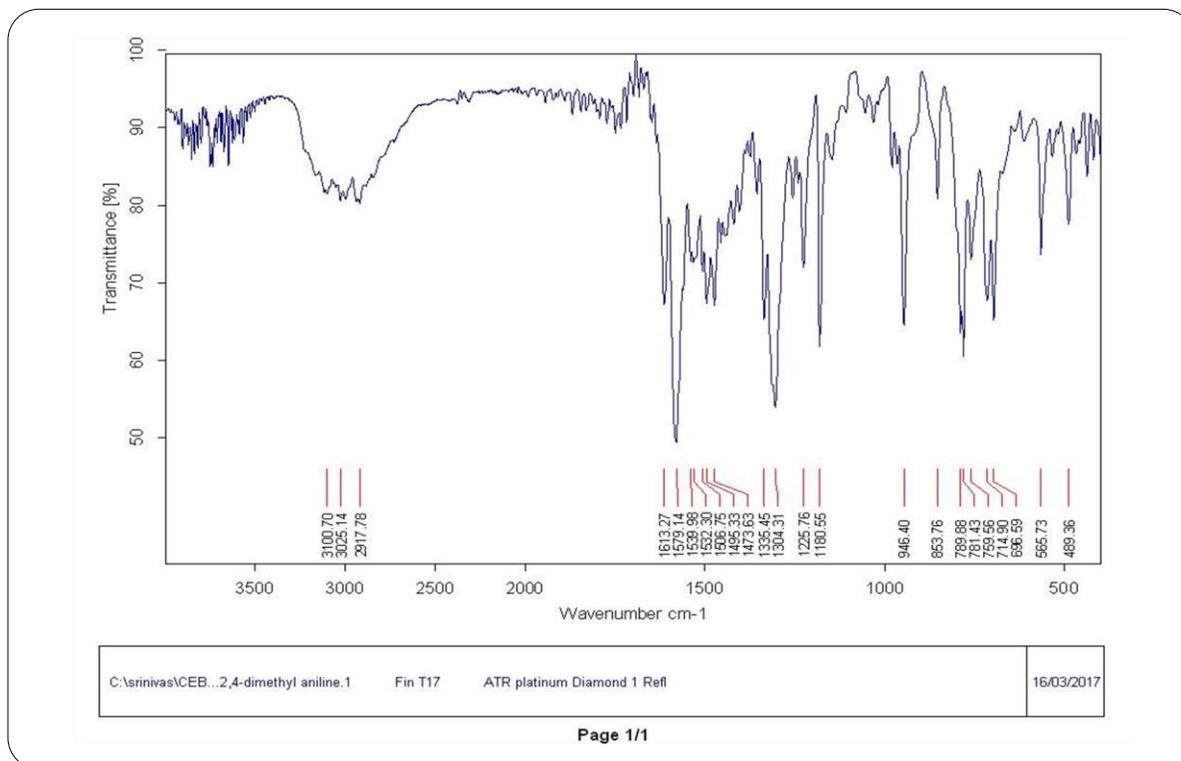
IR Spectrum of Compound 16
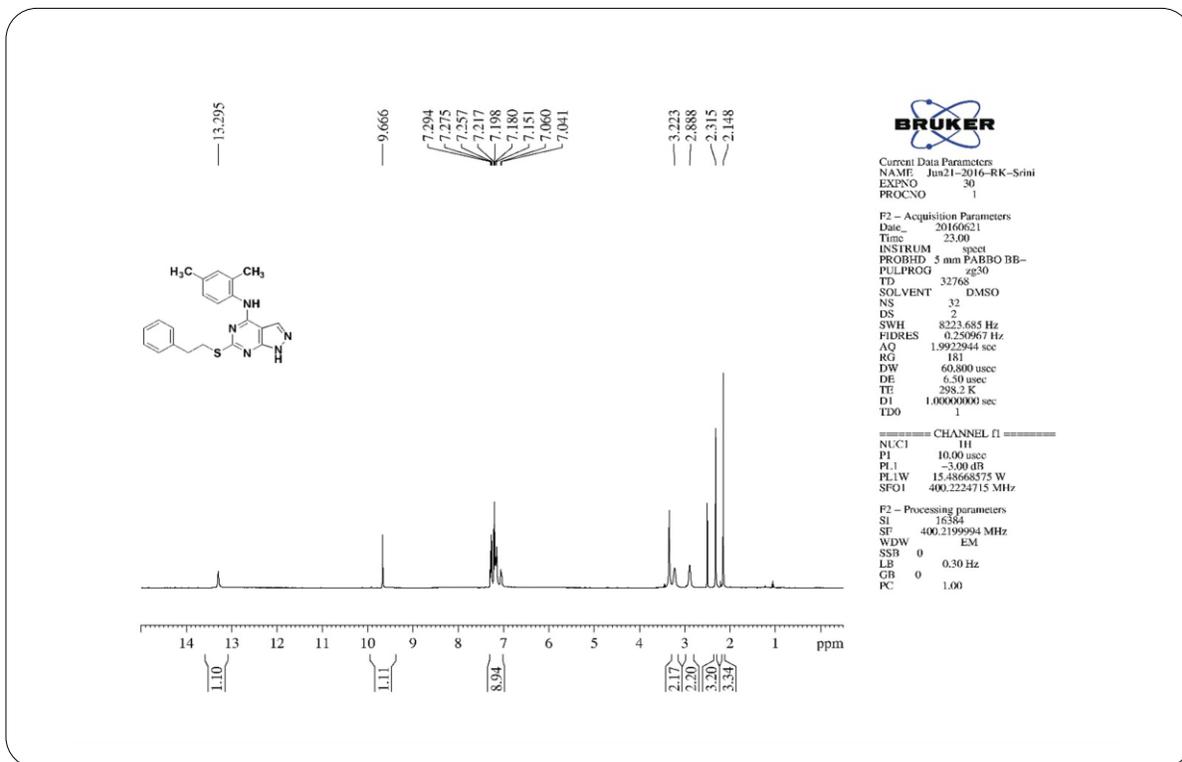
**¹H NMR Spectrum of Compound 16**
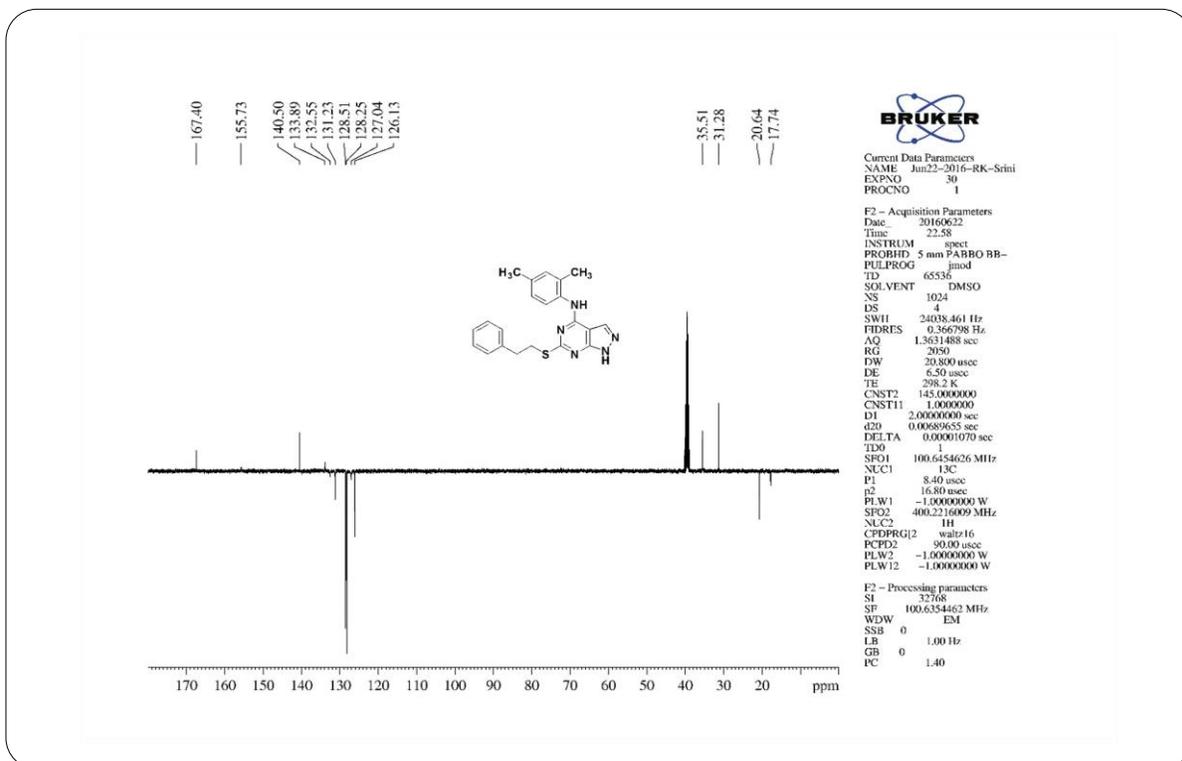

¹³C NMR Spectrum of Compound 16
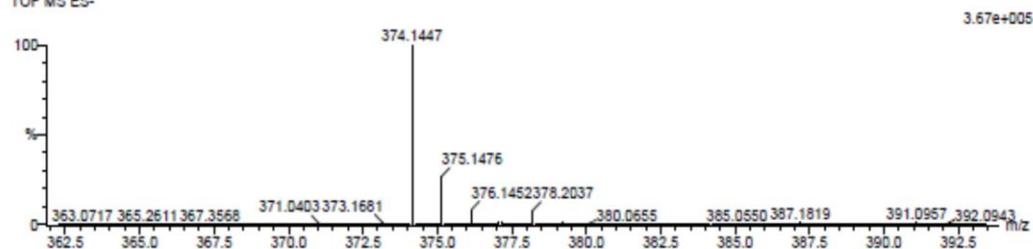
HRMS Spectrum of Compound 16

IR Spectrum of Compound 17

^{1}H NMR Spectrum of Compound 17
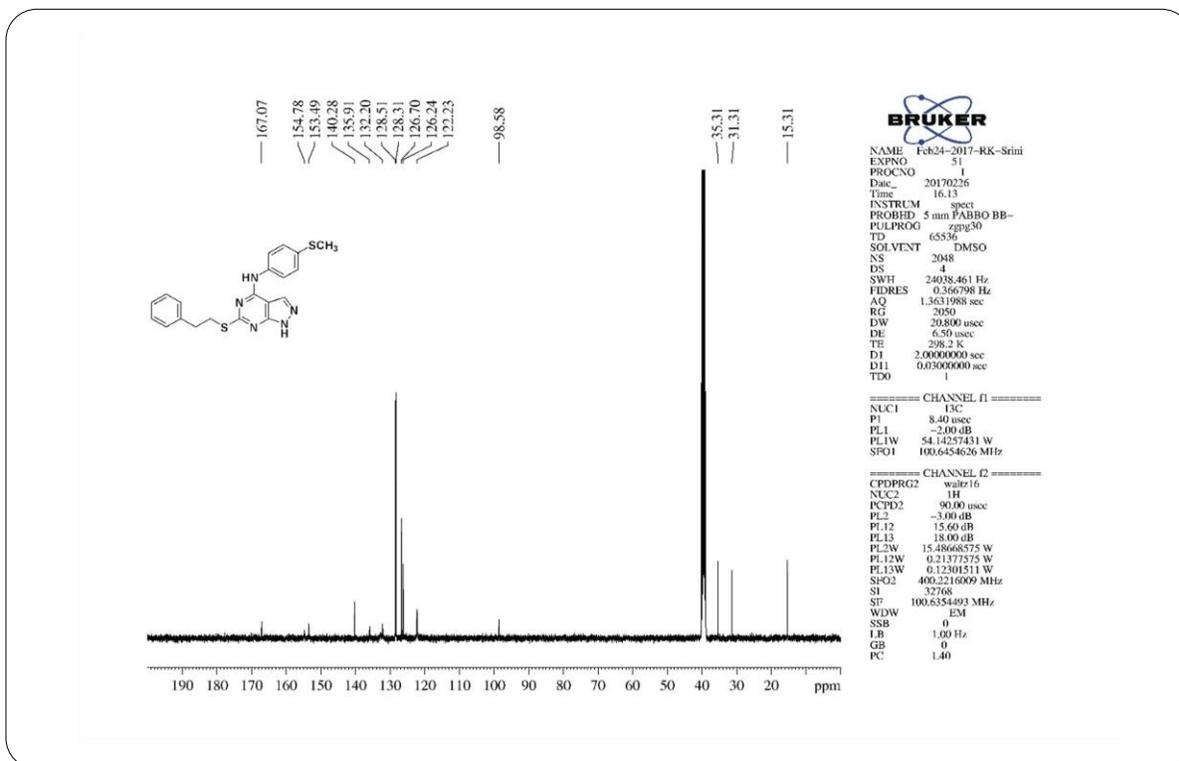
^{13}C NMR Spectrum of Compound 17

Elemental Composition Report   **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
14 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 15-20   H: 15-20   N: 0-5   S: 0-2
PP-13 2 (0.034) Cm (1:61)
TOF MS ES-
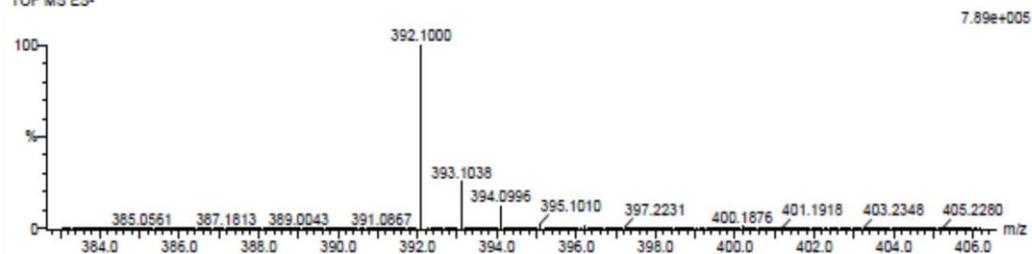
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
		Minimum:		-1.5			
		Maximum: 5.0	5.0	100.0			
392.1000	392.1004	-0.4	-1.0	14.5	675.3	0.0	C20 H18 N5 S2

HRMS Spectrum of Compound 17
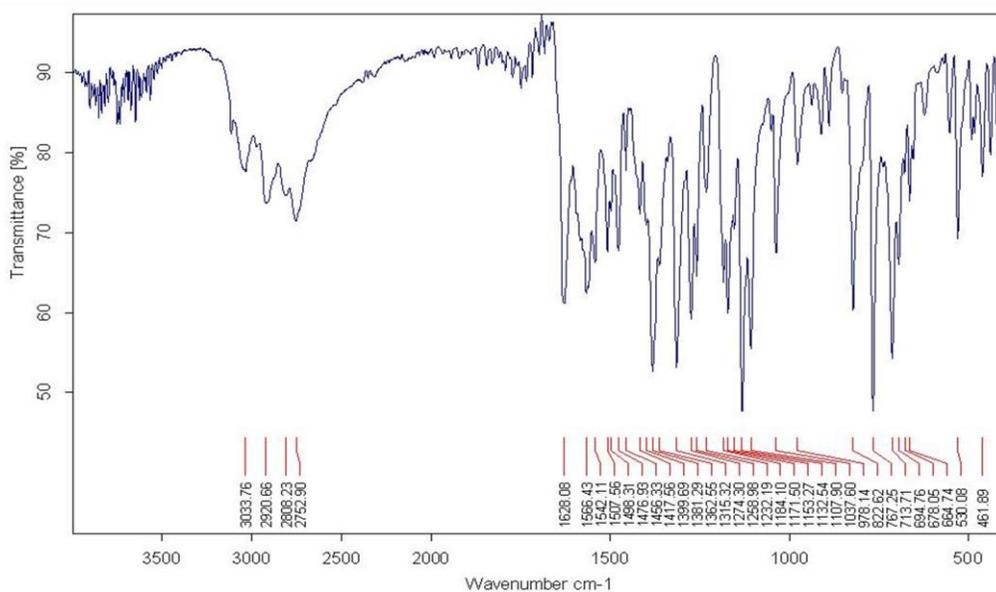
IR Spectrum of Compound 18
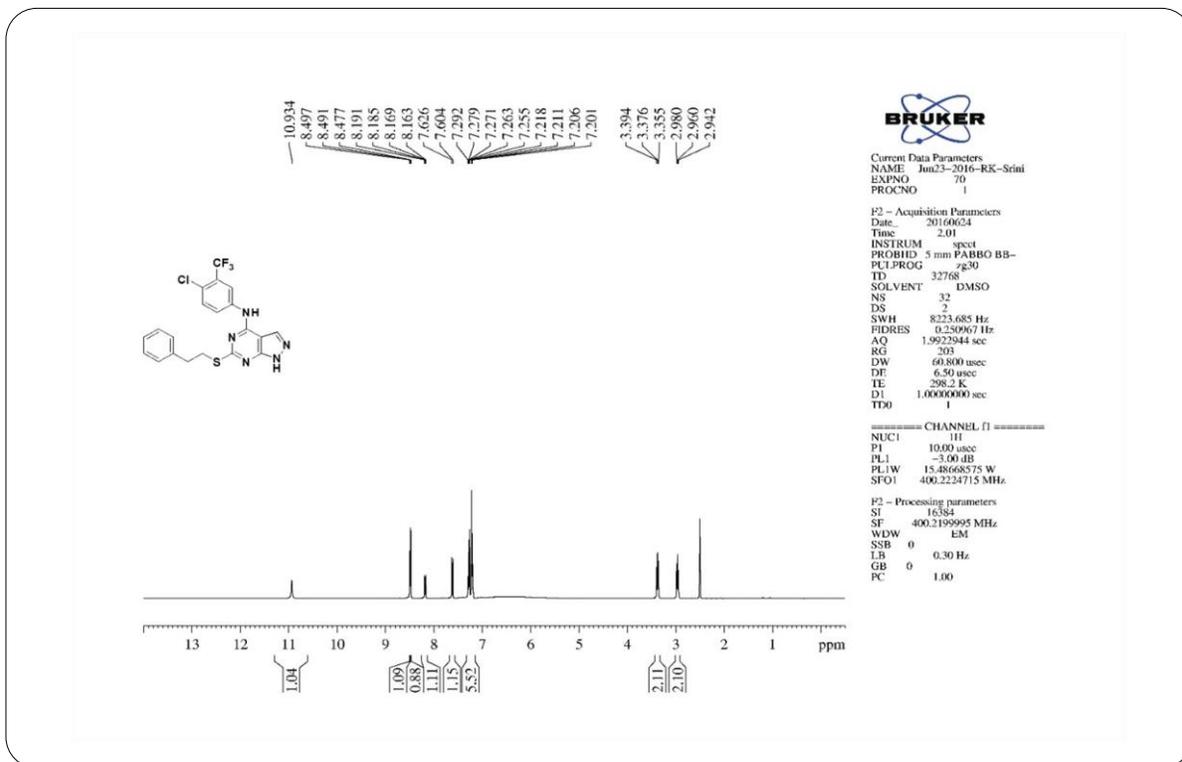
^{1}H NMR Spectrum of Compound 18

^{13}C NMR Spectrum of Compound 18

Elemental Composition Report   Page 1

Single Mass Analysis
Tolerance = 5.0 PPM   /   DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
88 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 15-20   H: 10-20   N: 0-5   F: 0-3   S: 0-1   Cl: 0-1
PP-14 2 (0.034) Cm (1:61)
TOF MS ES-
                                                                                        1.55e+006

Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
448.0605	448.0611	-0.6	-1.3	14.5	649.9	0.0	C20 H14 N5 F3 S Cl

HRMS Spectrum of Compound 18

IR Spectrum of Compound 19
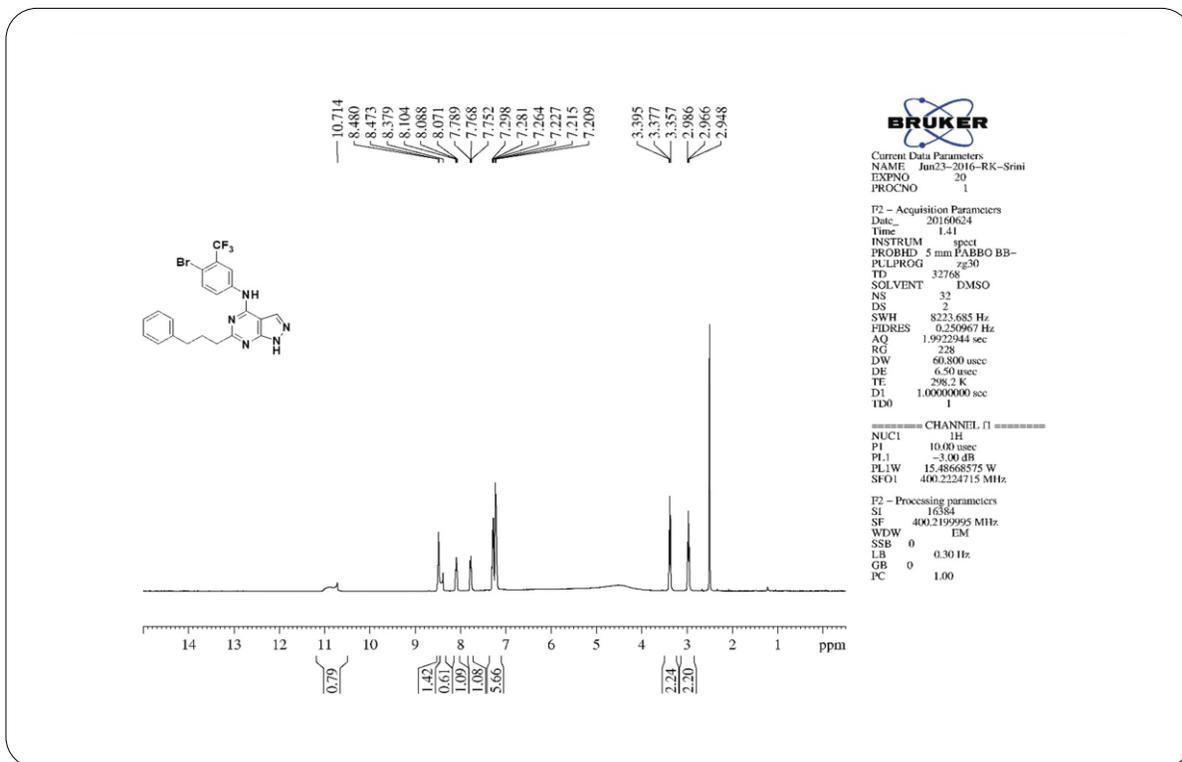
^{1}H NMR Spectrum of Compound 19

^{13}C NMR Spectrum of Compound 19

Elemental Composition Report   Page 1

Single Mass Analysis
Tolerance = 5.0 PPM   /   DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
89 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 15-20   H: 10-20   N: 0-5   F: 0-3   S: 0-1   Br: 0-1

PP-15 2 (0.034) Cm (1:61)
TOF MS ES-
                                                                                                        7.13e+005

Minimum:			-1.5			
Maximum:	5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm) Formula
492.0104	492.0105	-0.1	-0.2	14.5	575.1	0.0 C20 H14 N5 F3 S Br

HRMS Spectrum of Compound 19

IR Spectrum of Compound 20
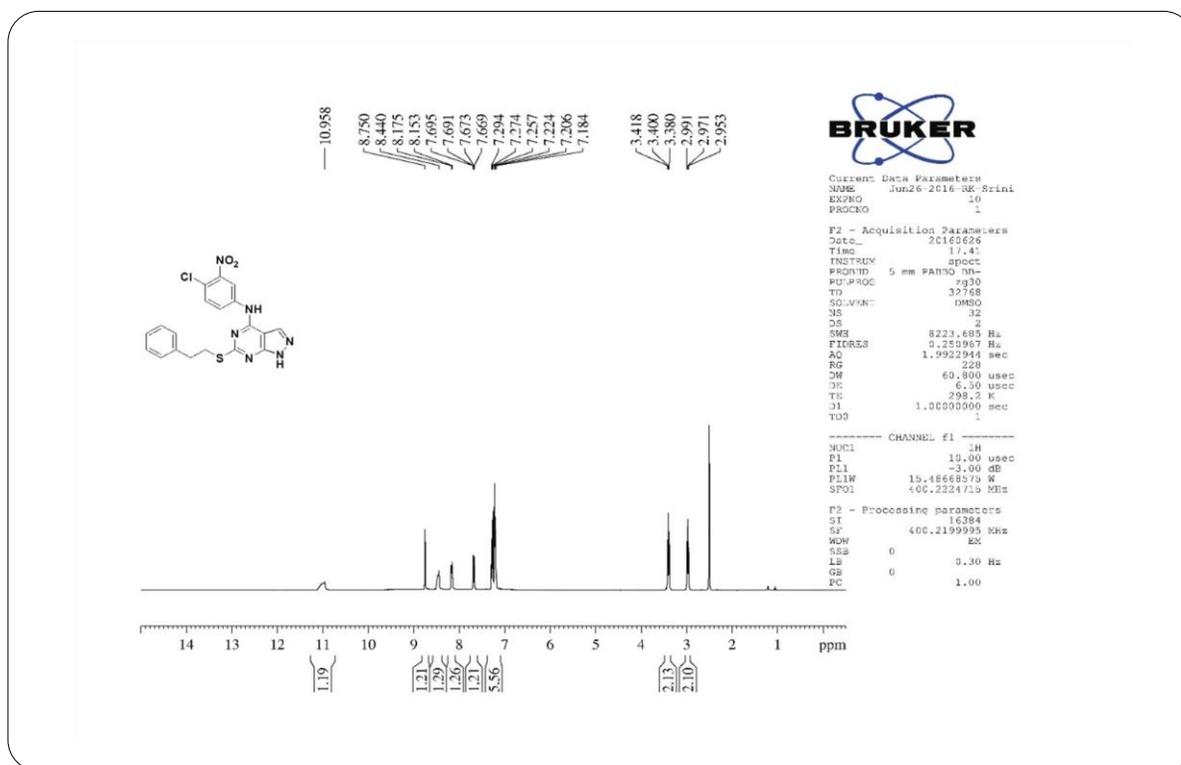
**¹H NMR Spectrum of Compound 20**
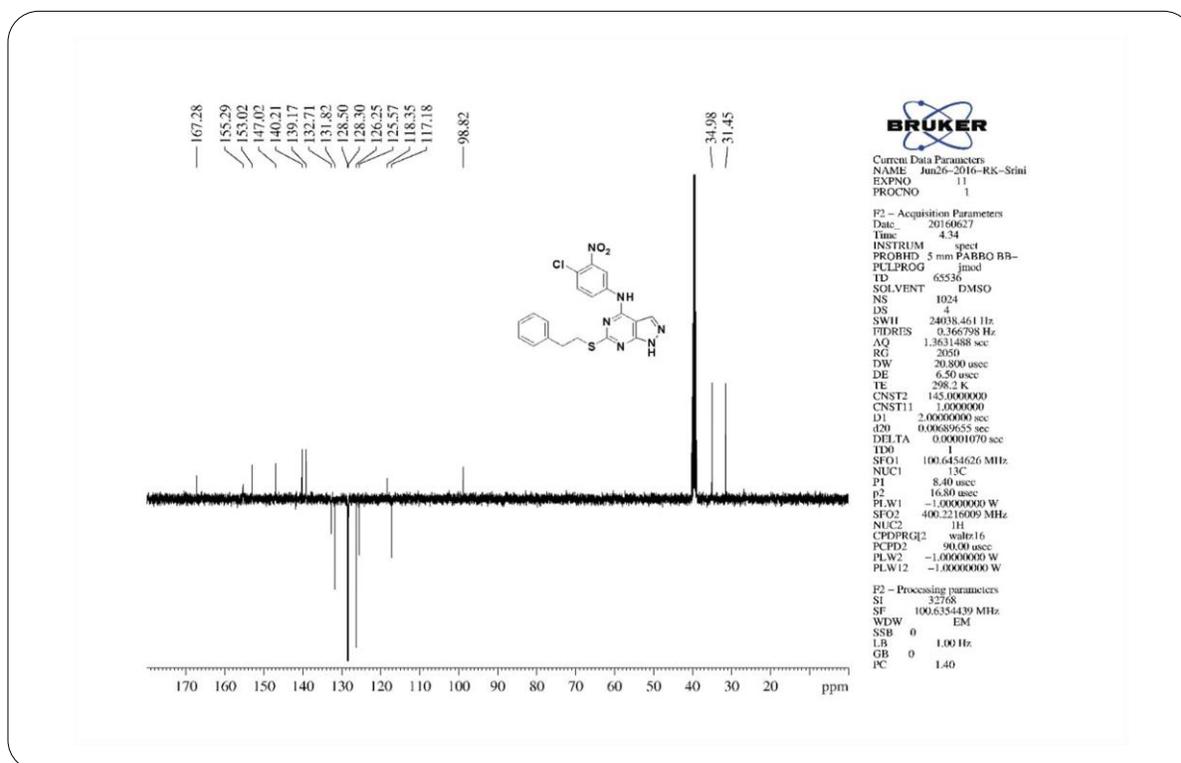
**¹³C NMR Spectrum of Compound 20**
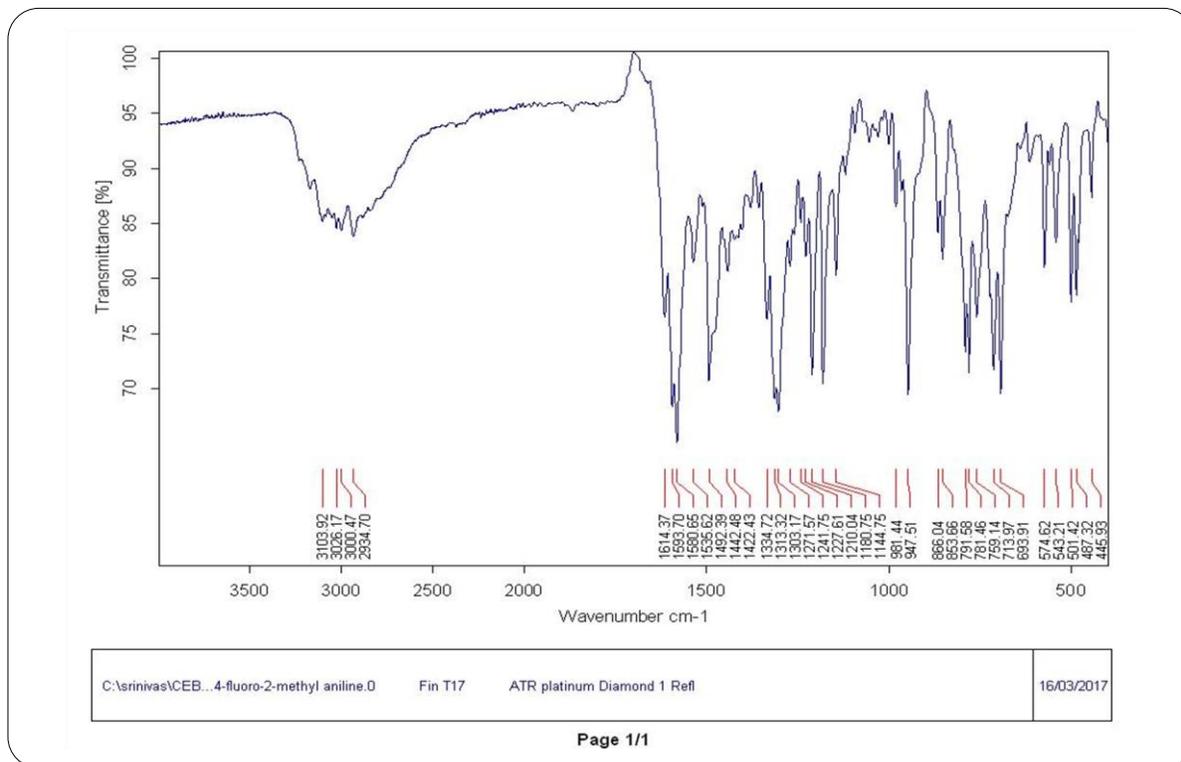
IR Spectrum of Compound 21
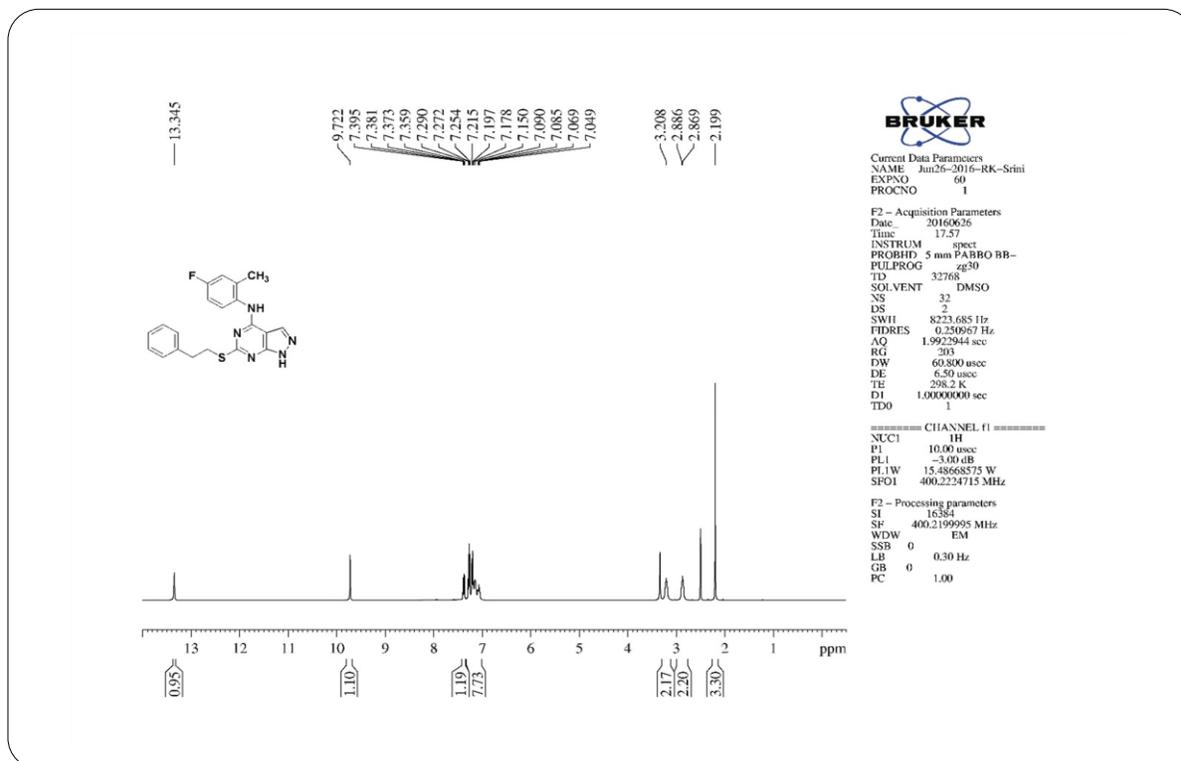
¹H NMR Spectrum of Compound 21

¹³C NMR Spectrum of Compound 21

HRMS Spectrum of Compound 21

IR Spectrum of Compound 22


^{1}H NMR Spectrum of Compound 22
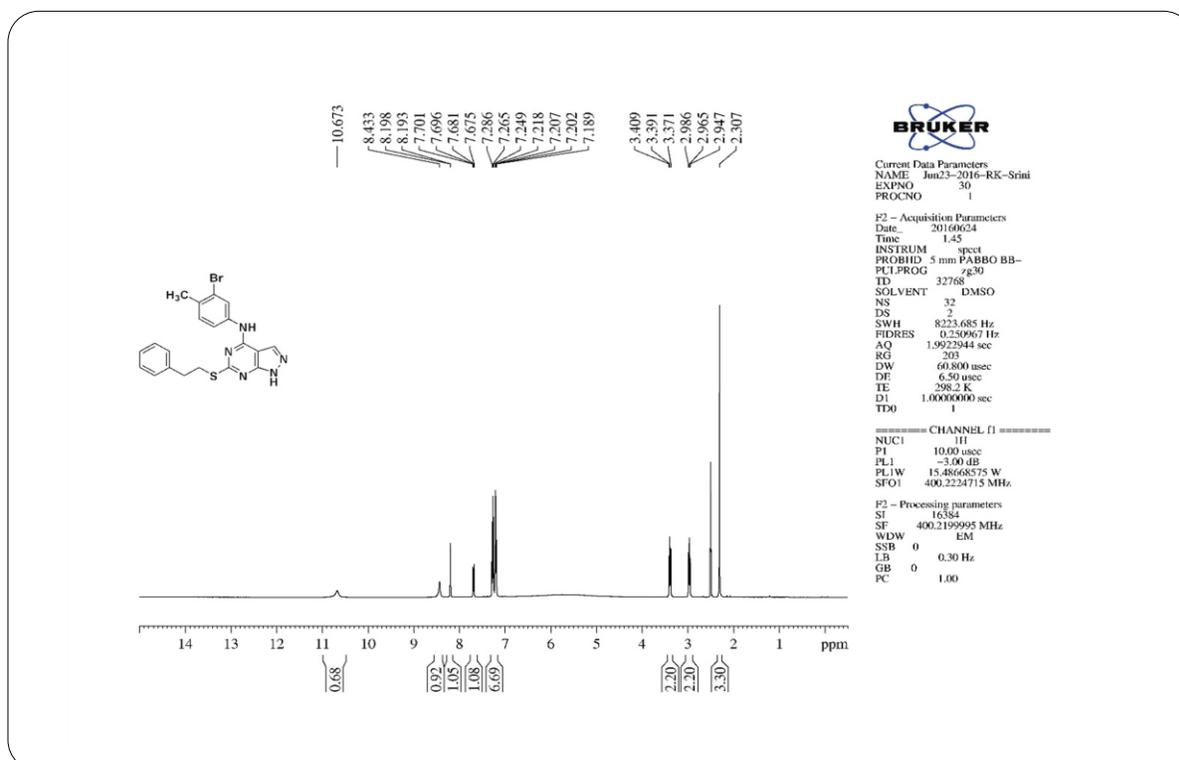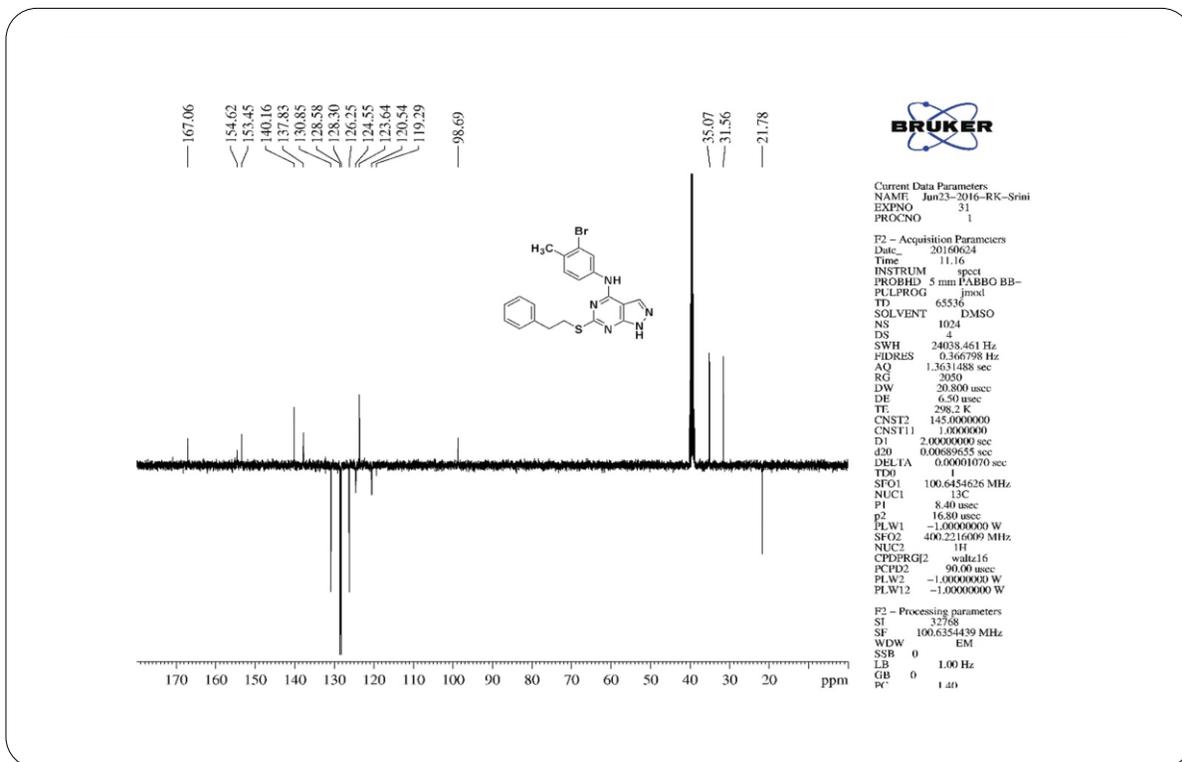
¹³C NMR Spectrum of Compound 22
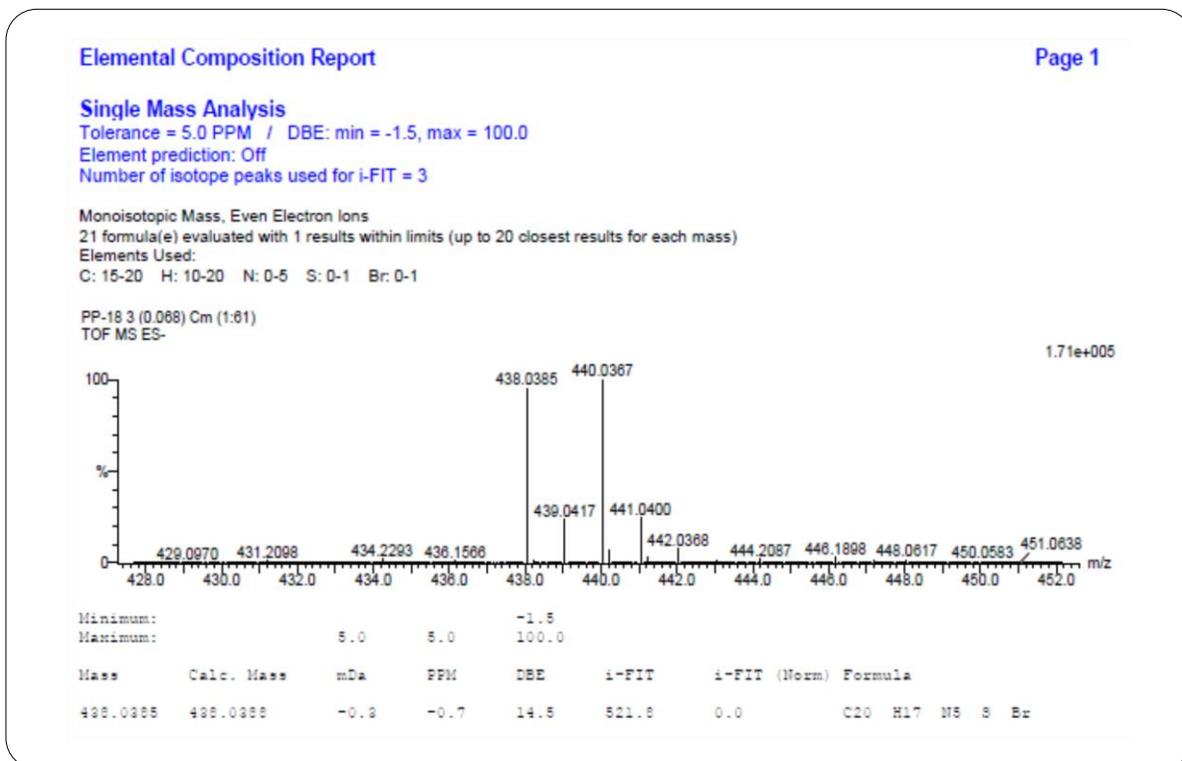
HRMS Spectrum of Compound 22
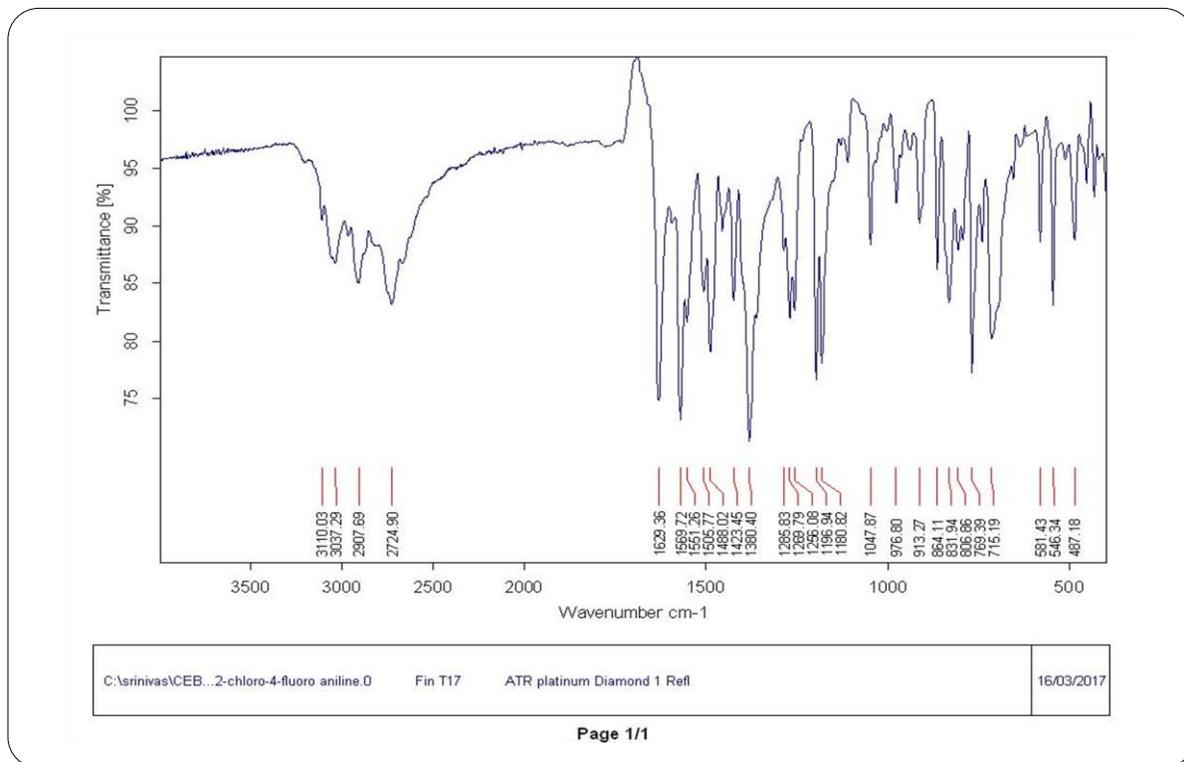
**IR Spectrum of Compound 23**
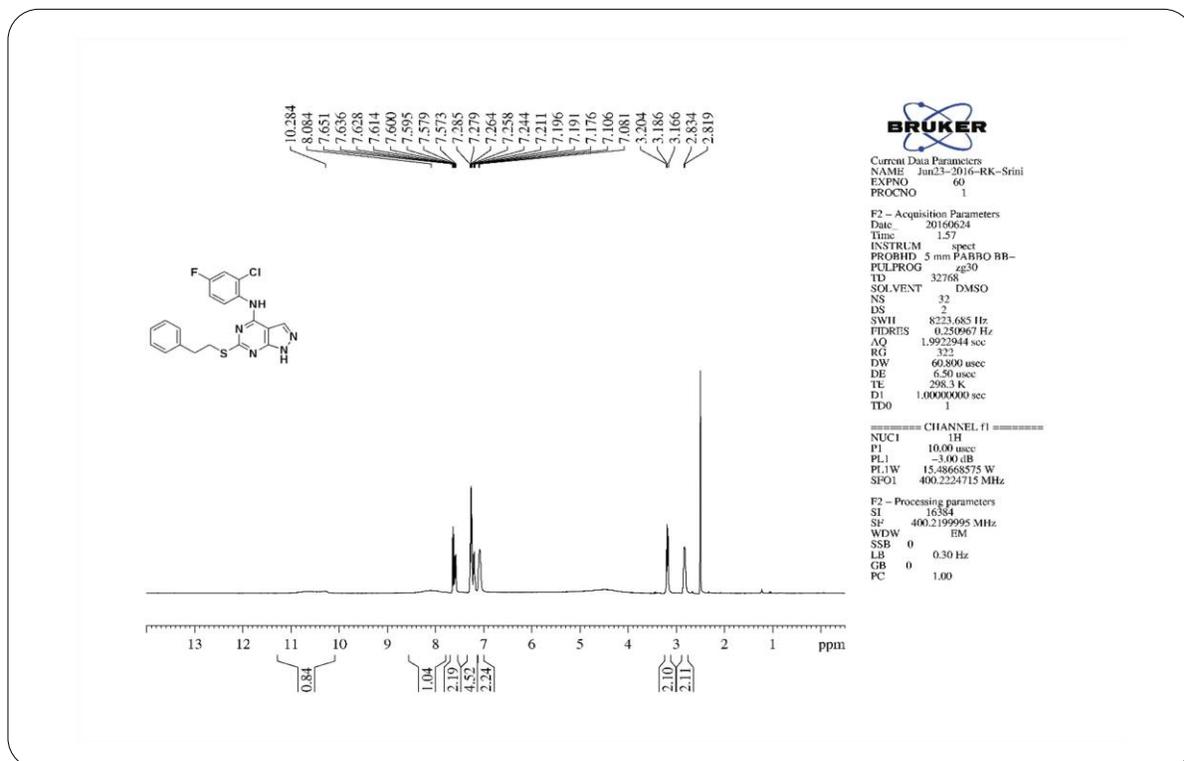
¹H NMR Spectrum of Compound 23
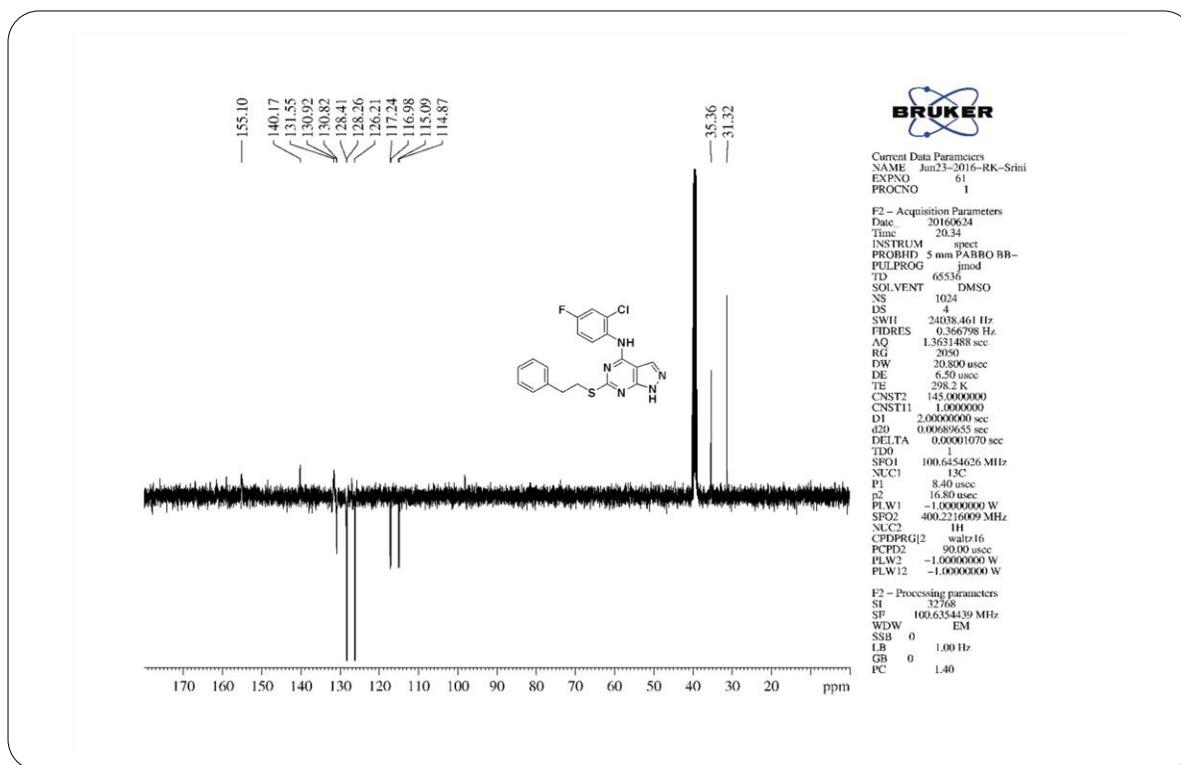
¹³C NMR Spectrum of Compound 23
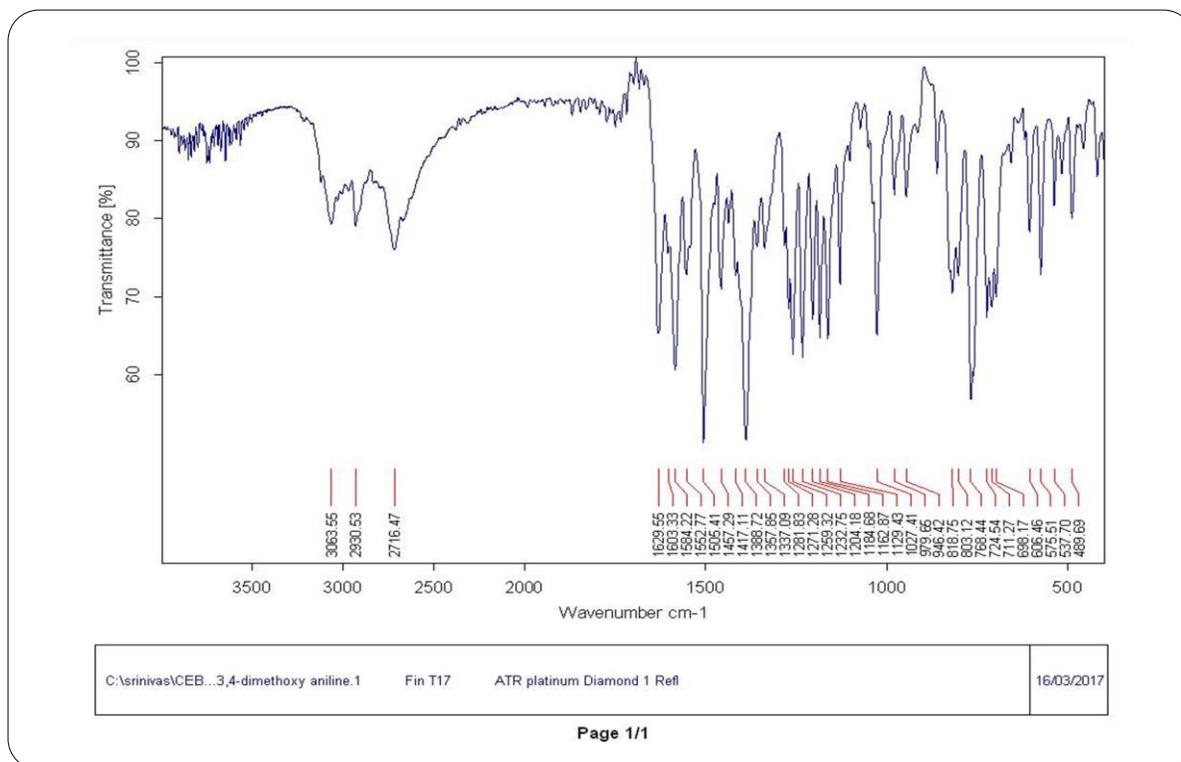
IR Spectrum of Compound 24
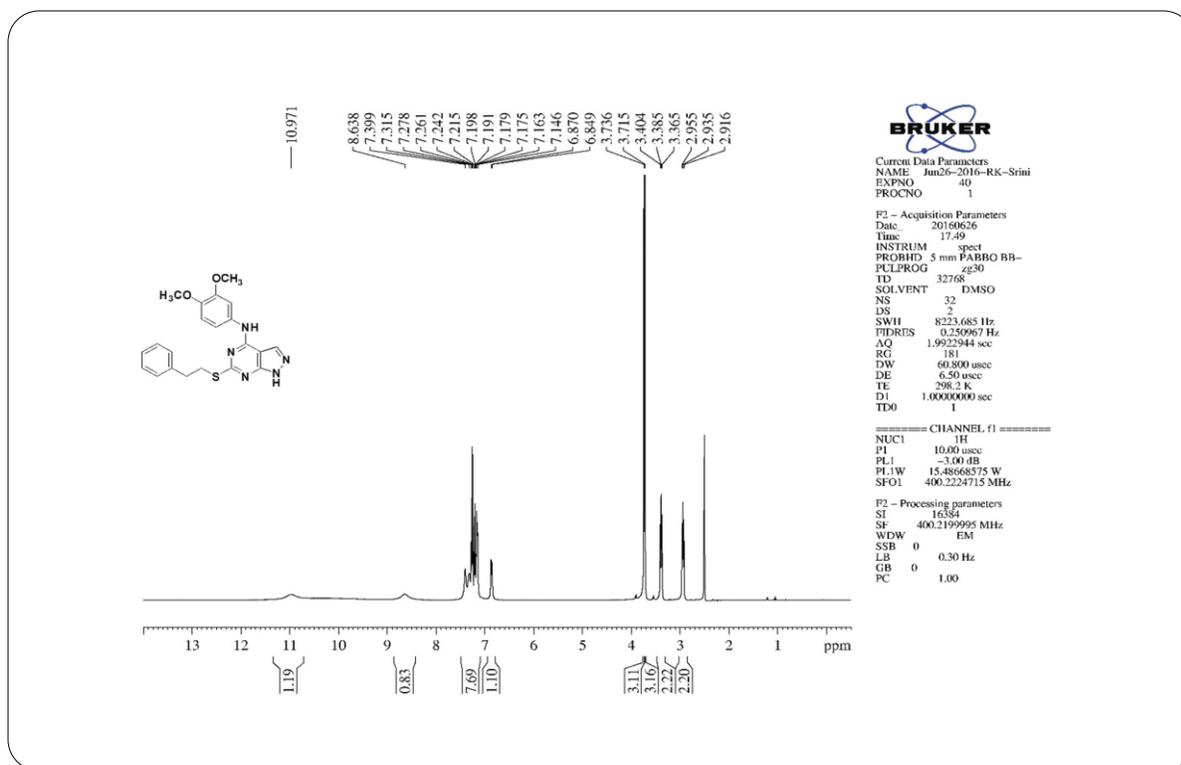
¹H NMR Spectrum of Compound 24
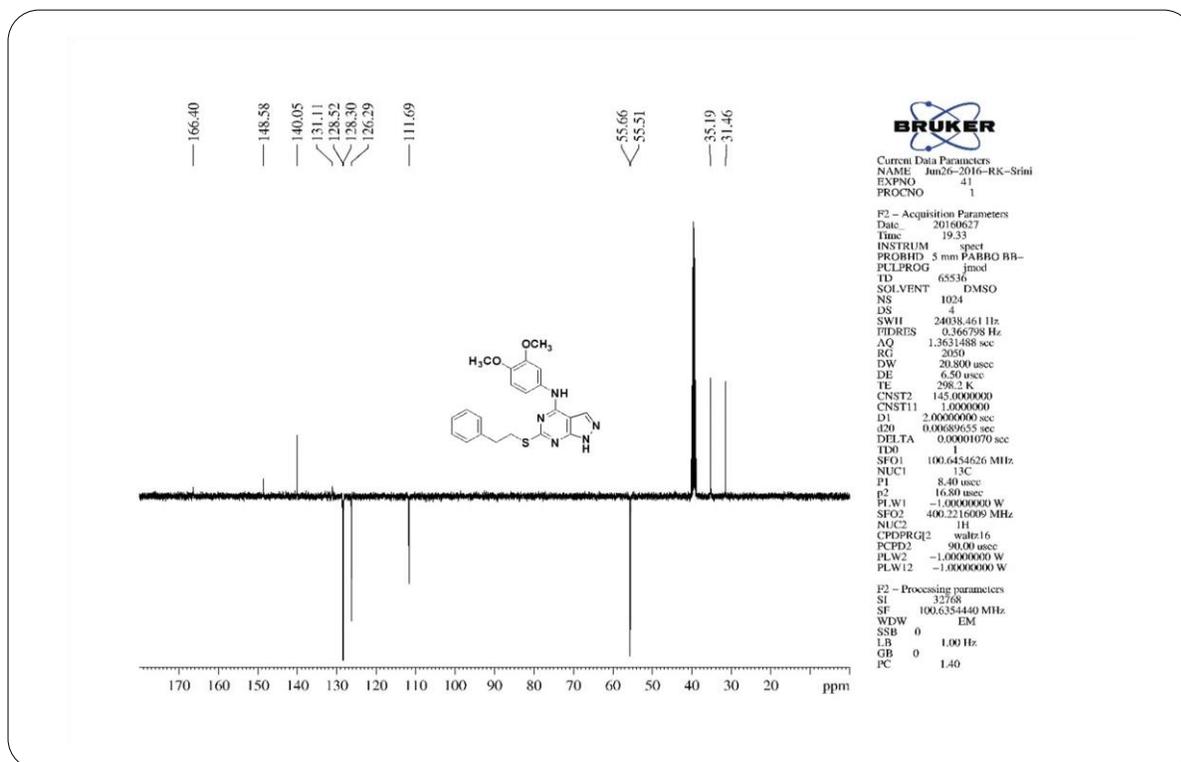
¹³C NMR Spectrum of Compound 24
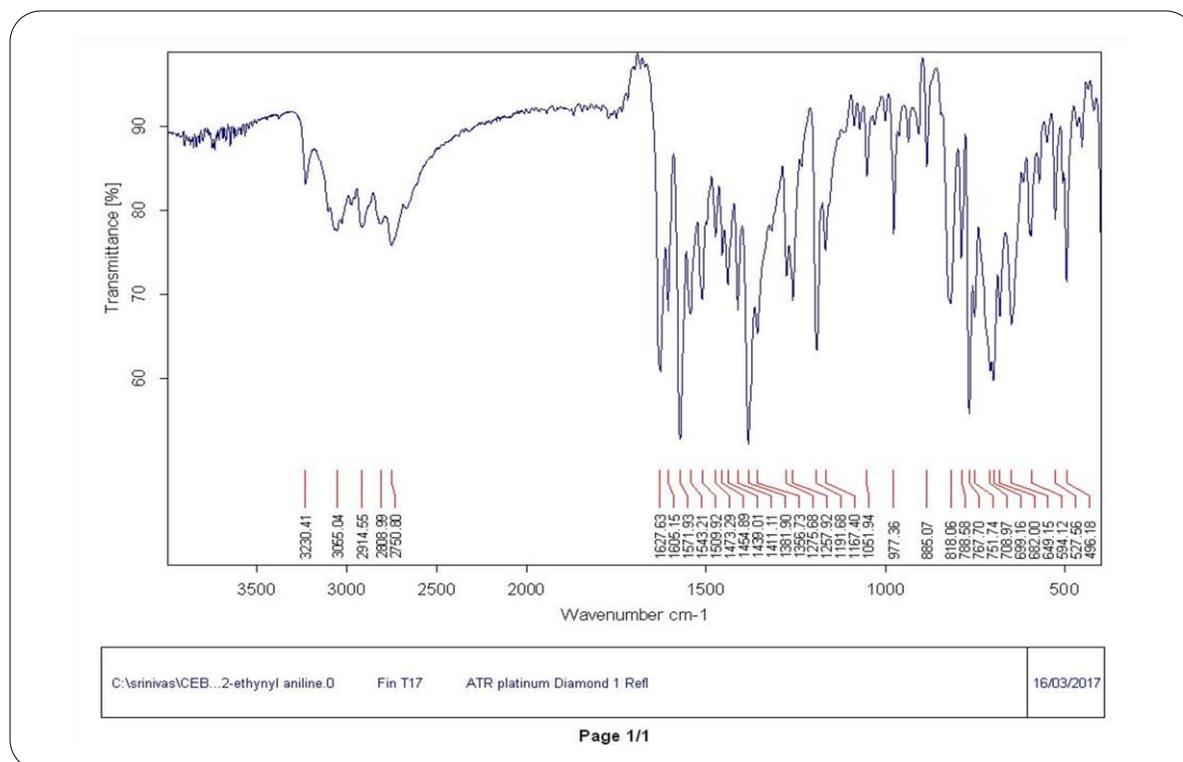
**IR Spectrum of Compound 25**
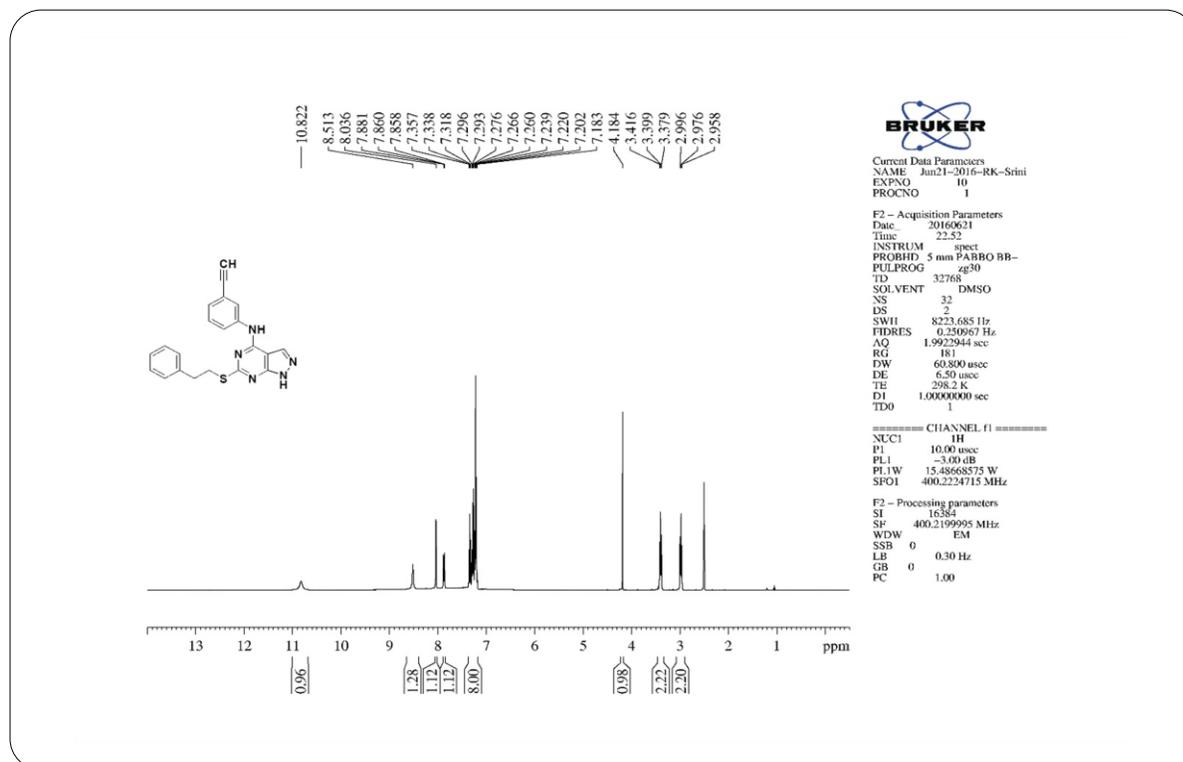
¹H NMR Spectrum of Compound 25
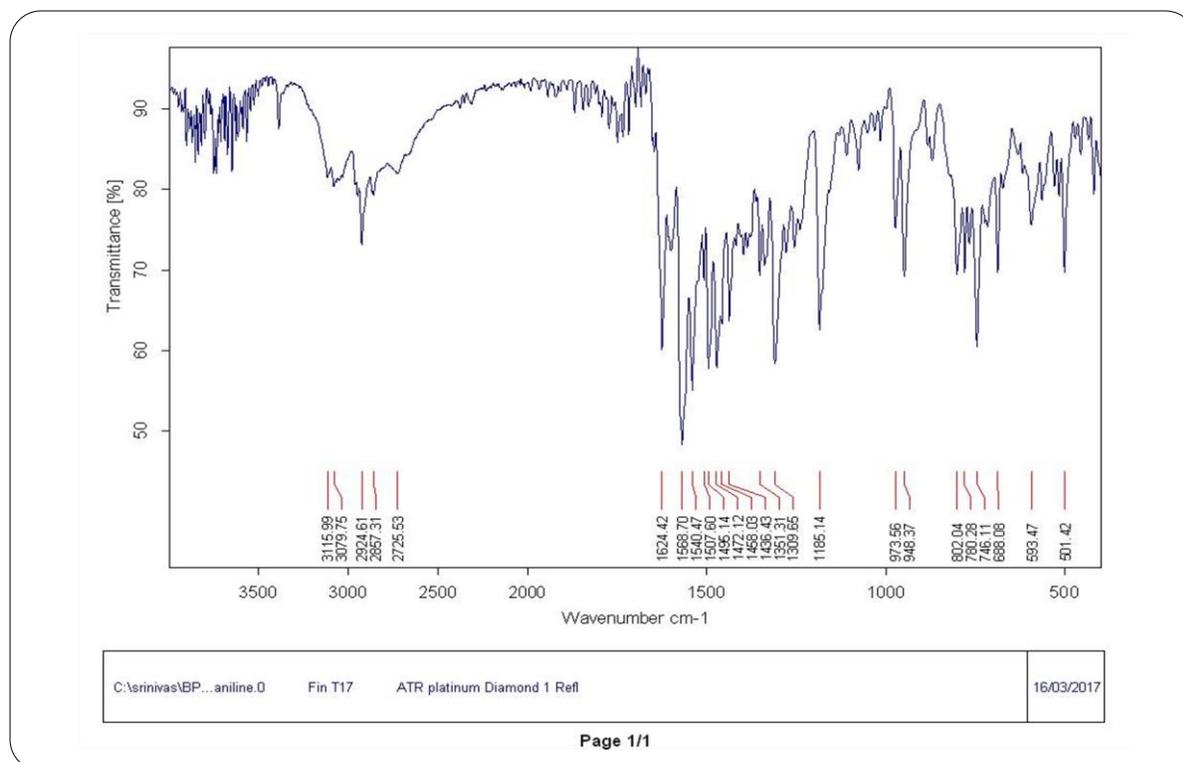
IR Spectrum of Compound 26
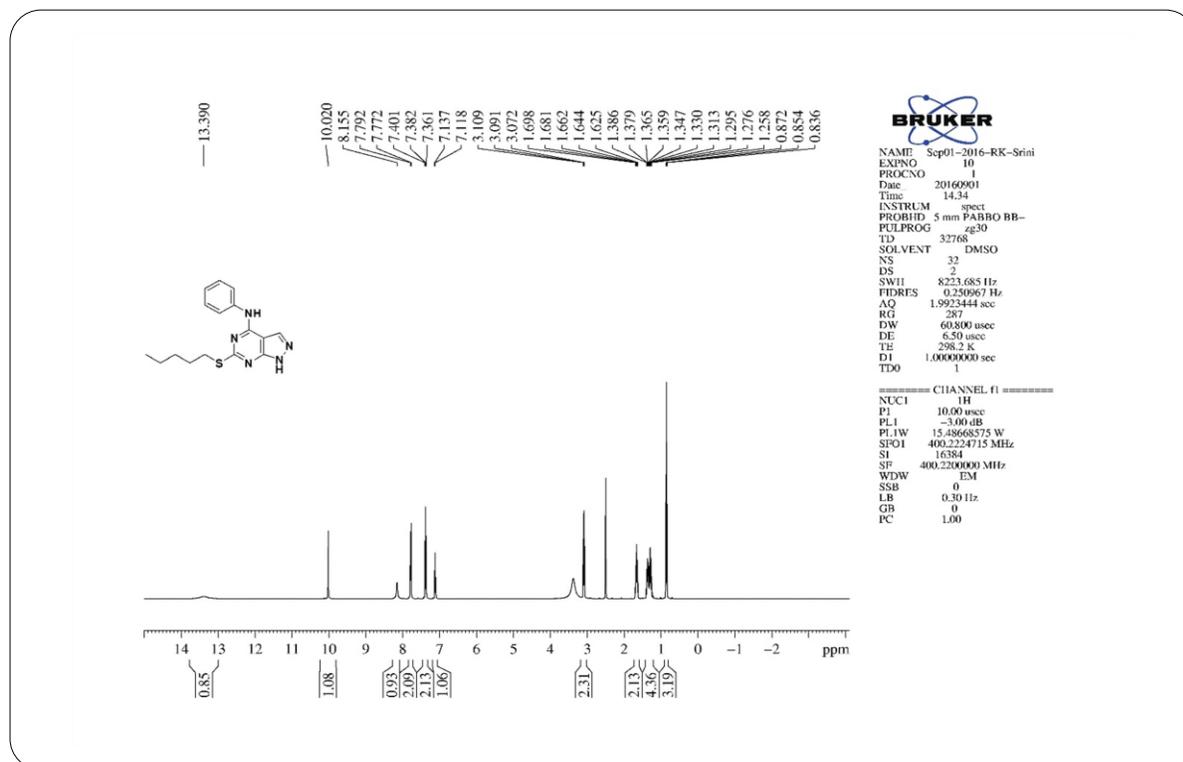
¹H NMR Spectrum of Compound 26
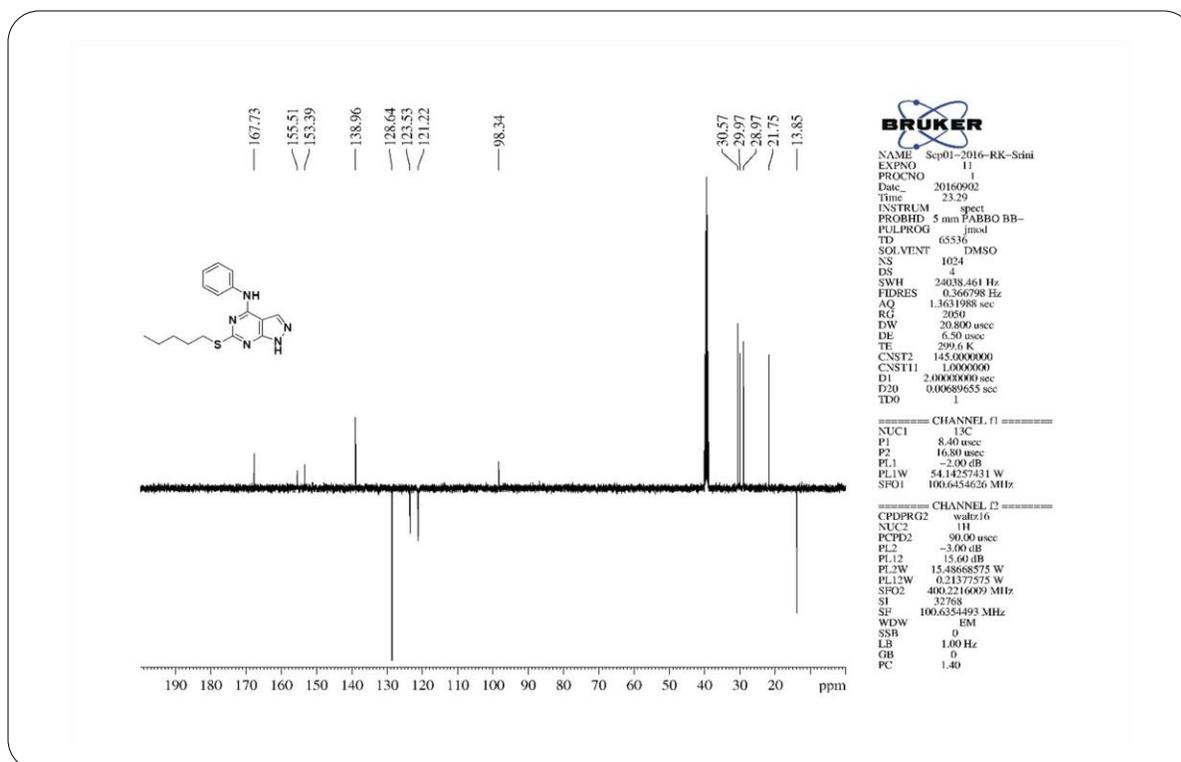
^{13}C NMR Spectrum of Compound 26
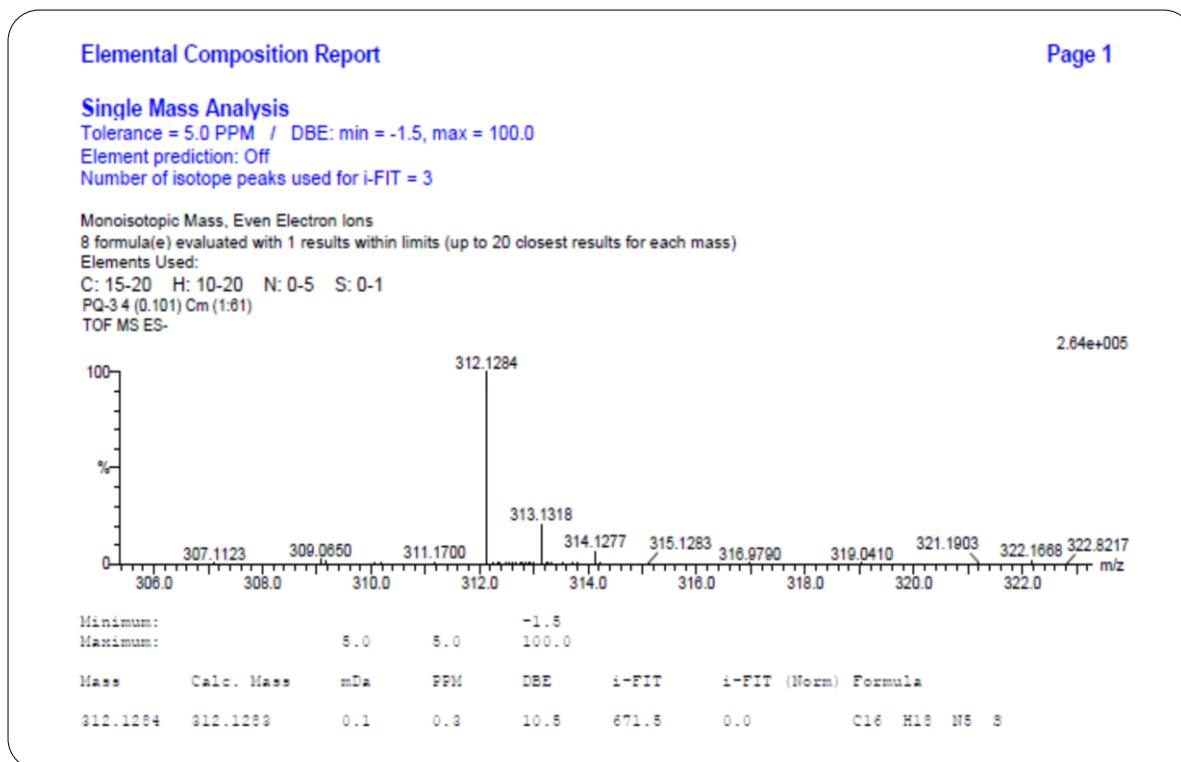
HRMS Spectrum of Compound 26
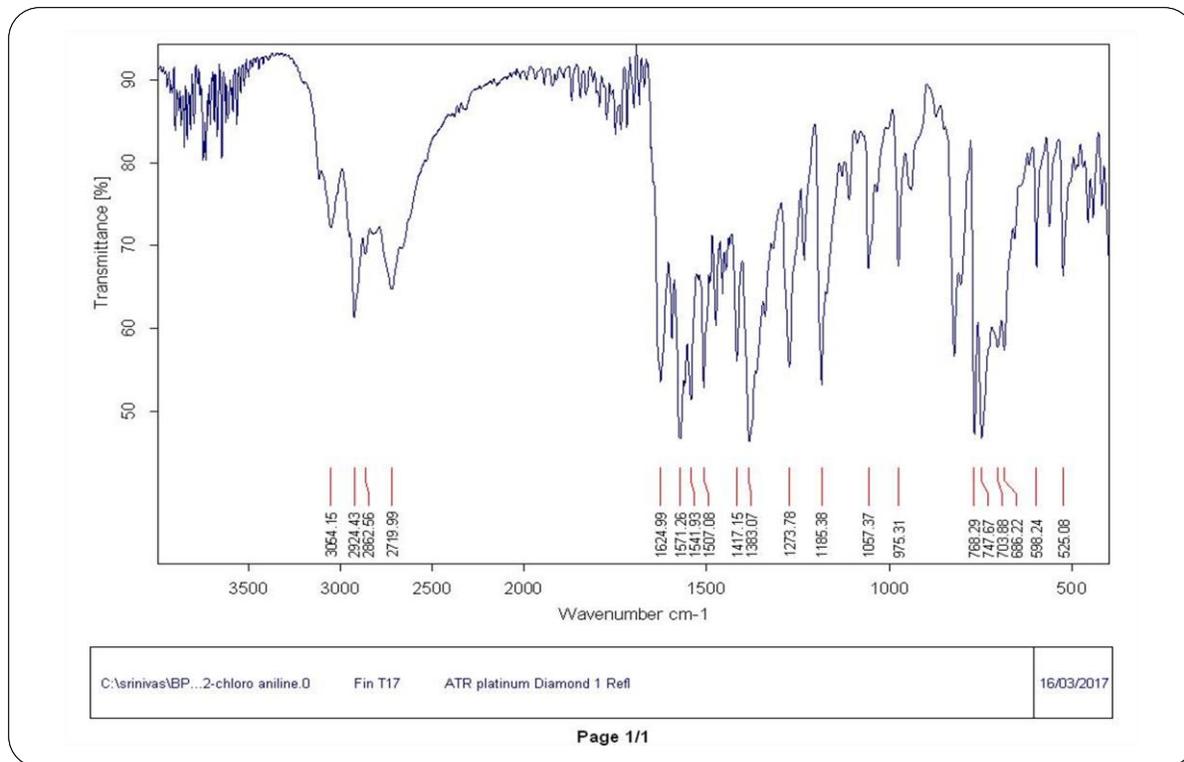
IR Spectrum of Compound 27
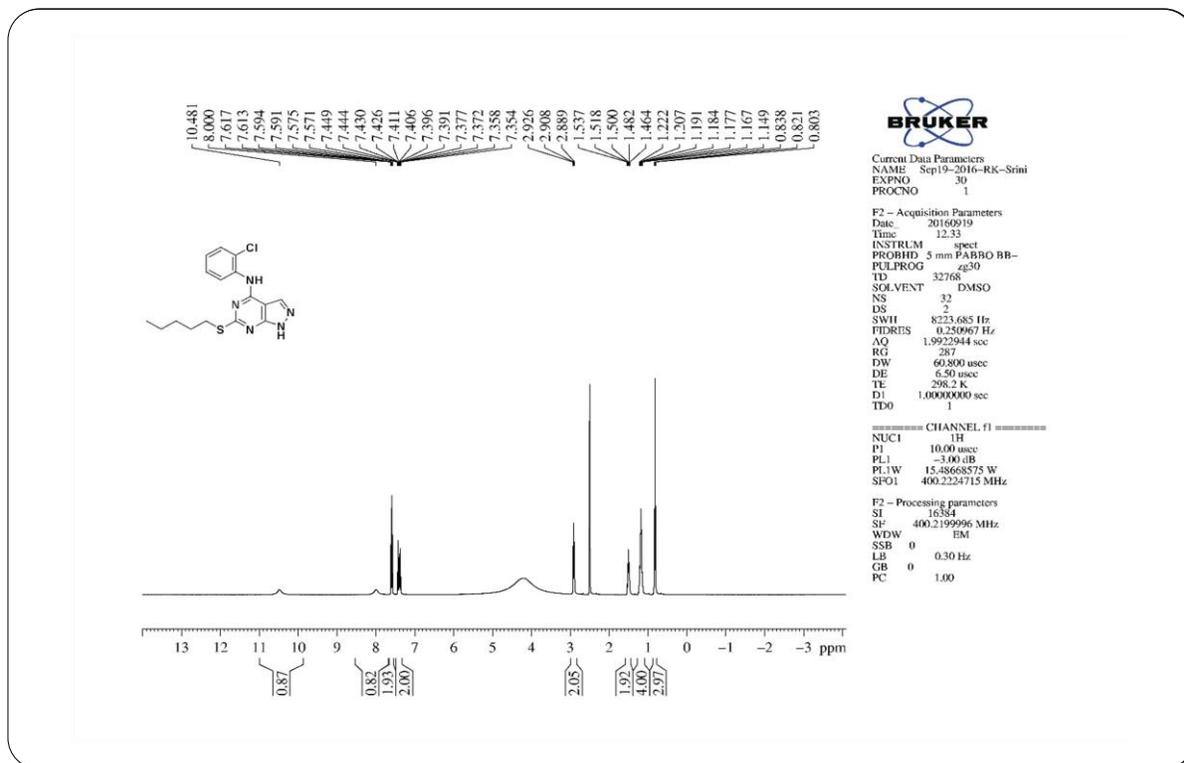
**¹H NMR Spectrum of Compound 27**
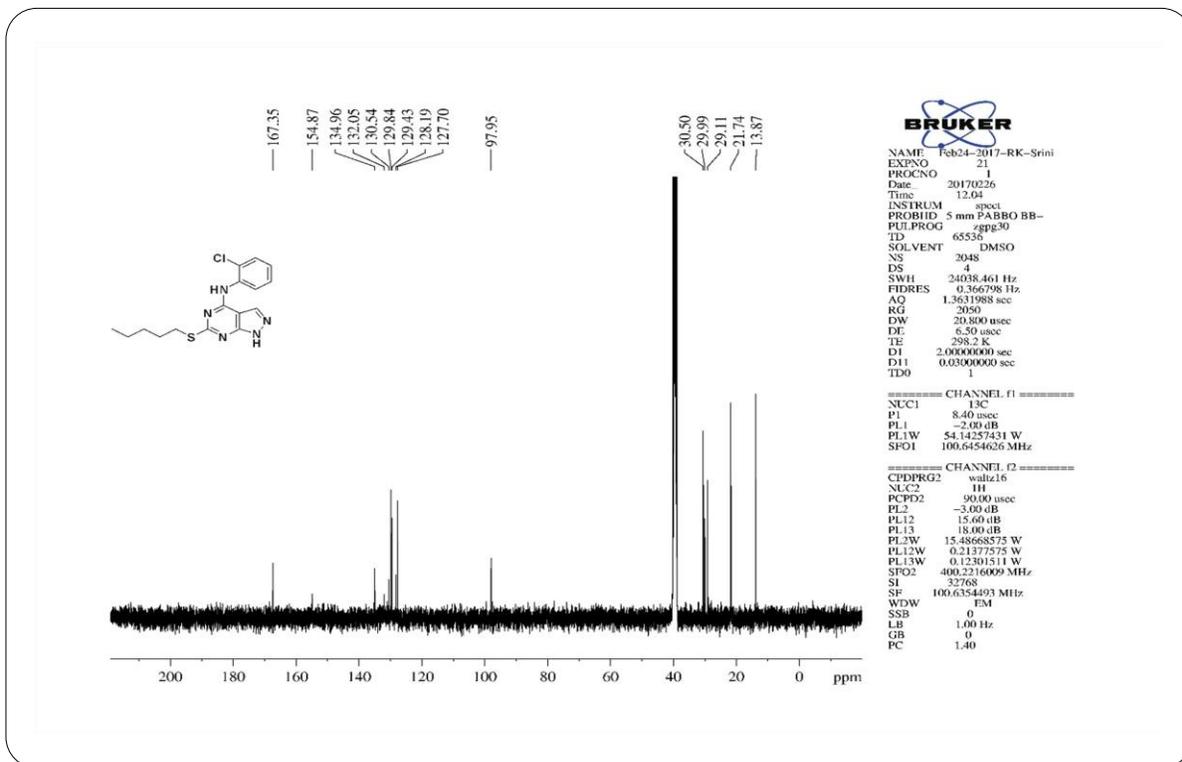
¹³C NMR Spectrum of Compound 27
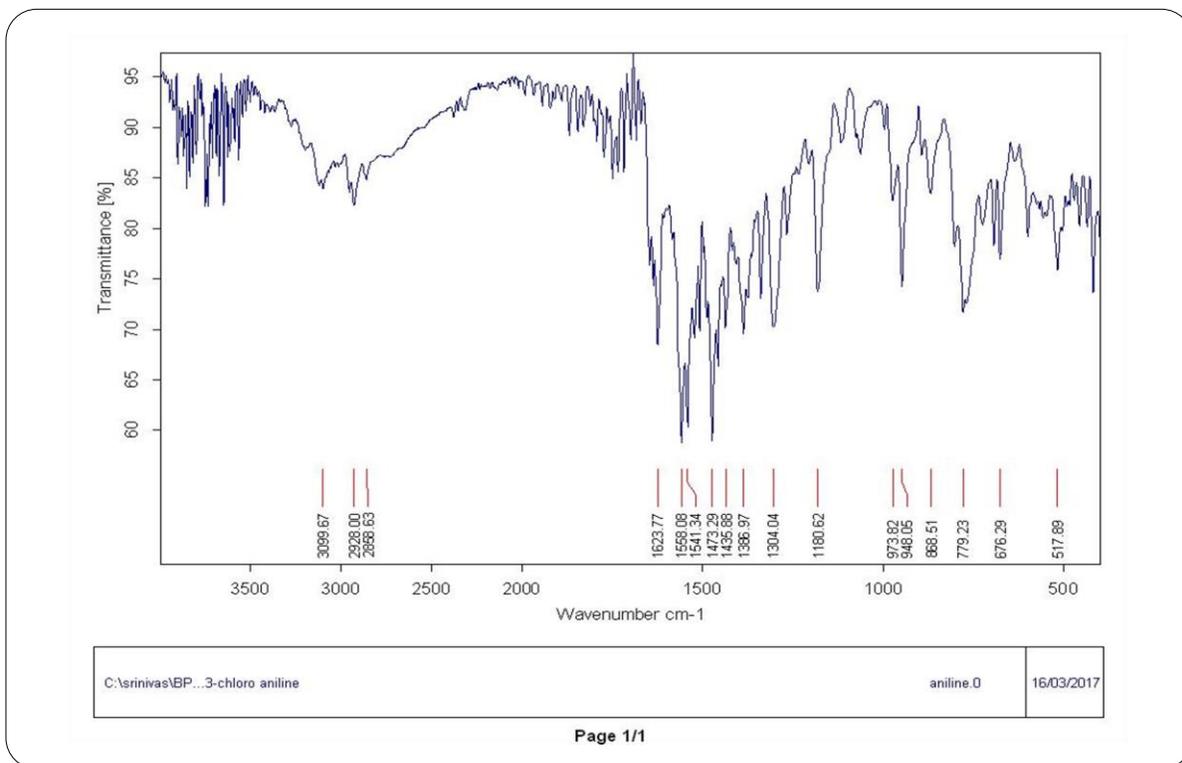
IR Spectrum of Compound 28
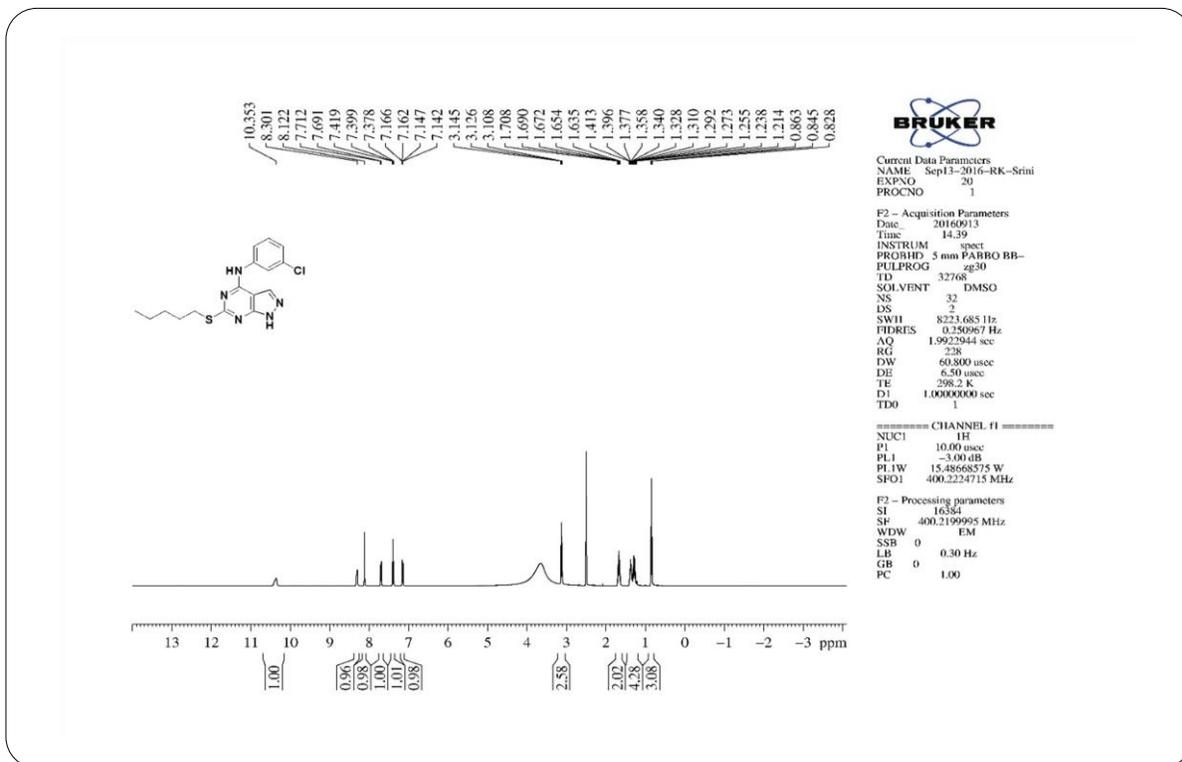
¹H NMR Spectrum of Compound 28
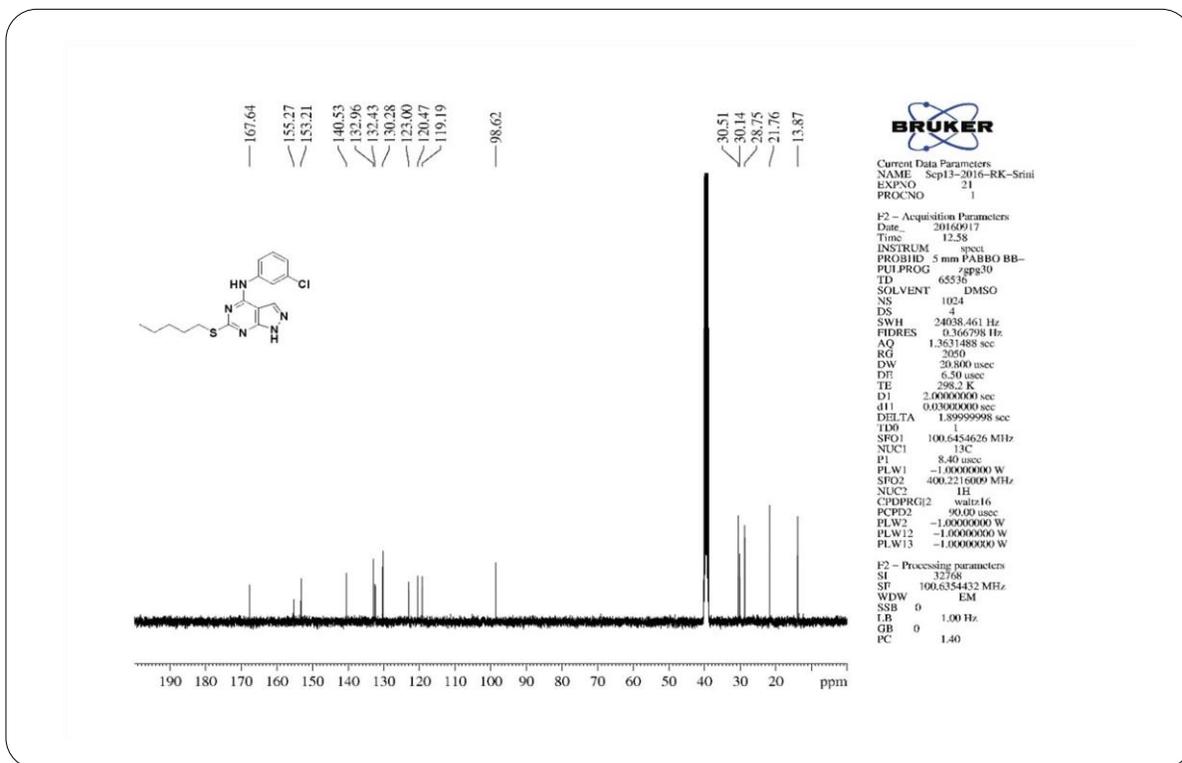
¹³C MNR Spectrum of Compound 28
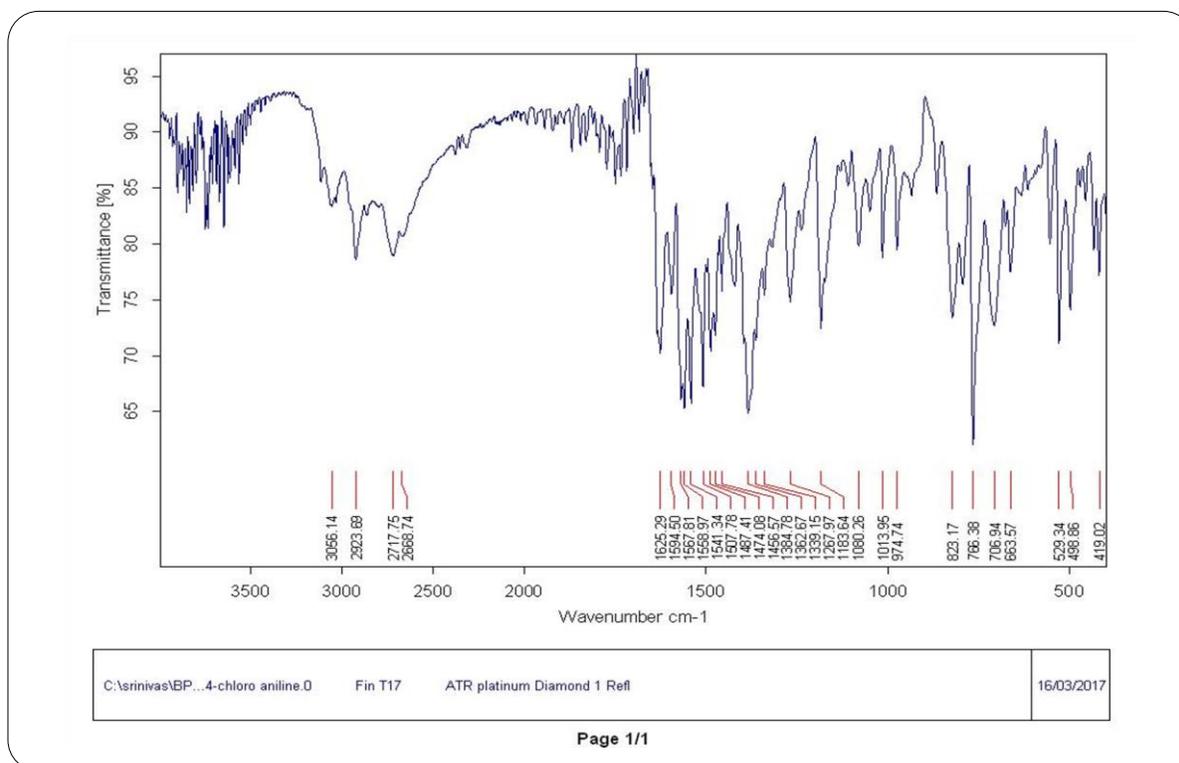
IR Spectrum of Compound 29
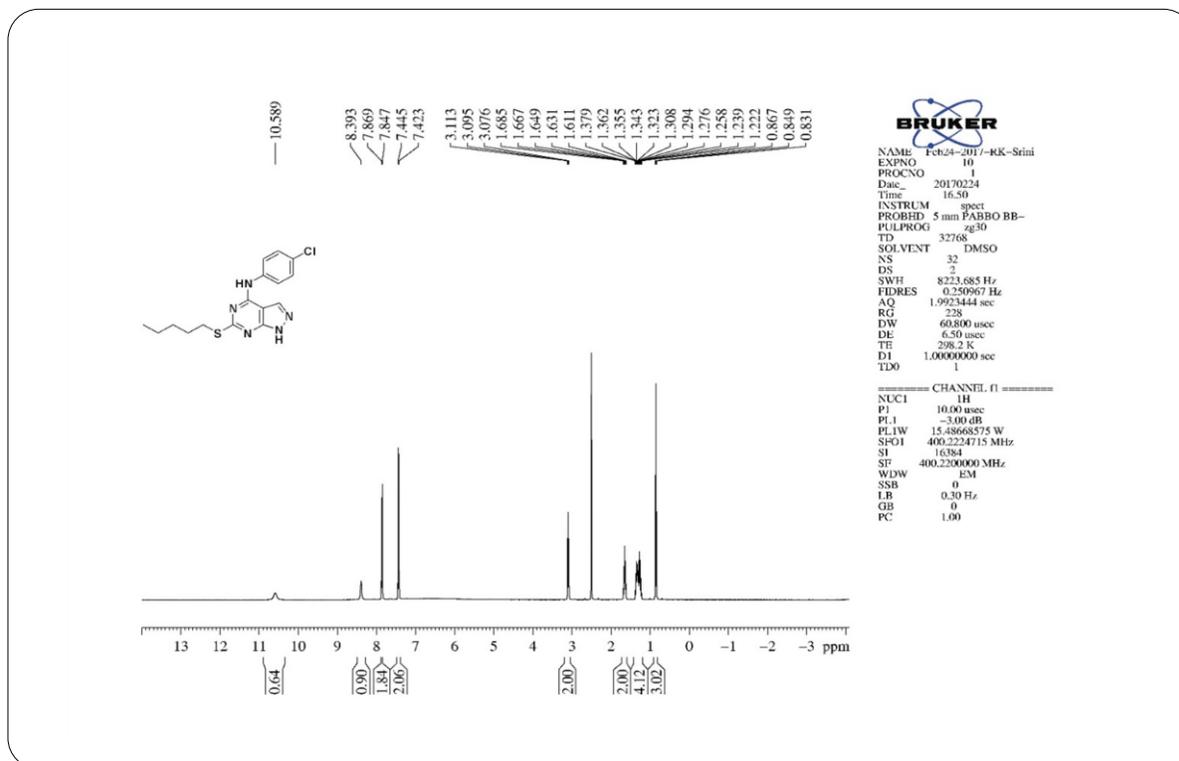
¹H NMR Spectrum of Compound 29
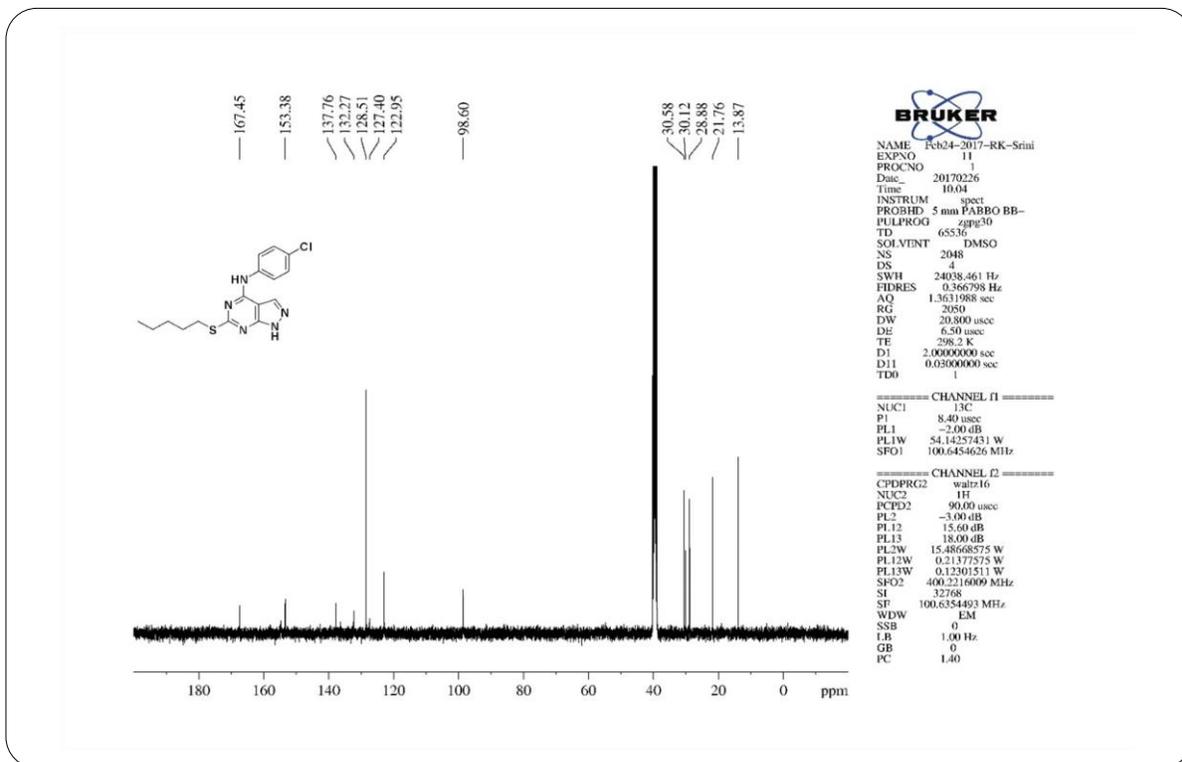
¹³C NMR Spectrum of Compound 29
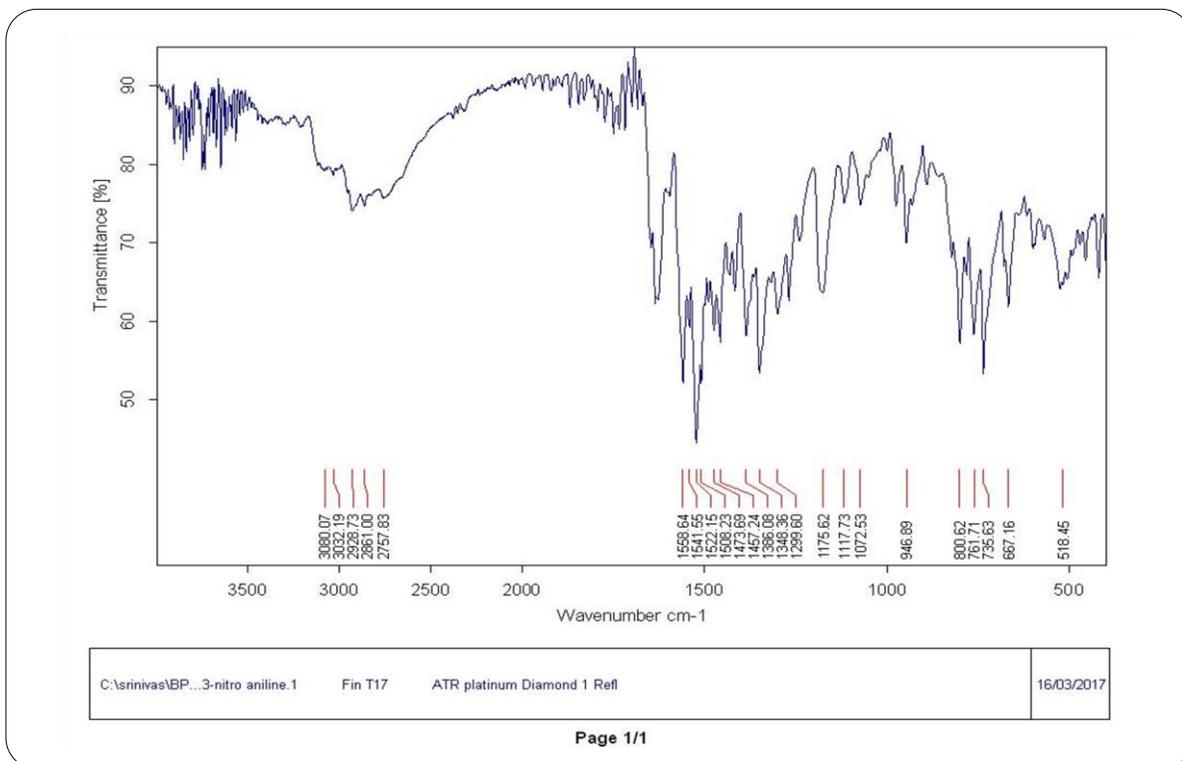
IR Spectrum of Compound 30
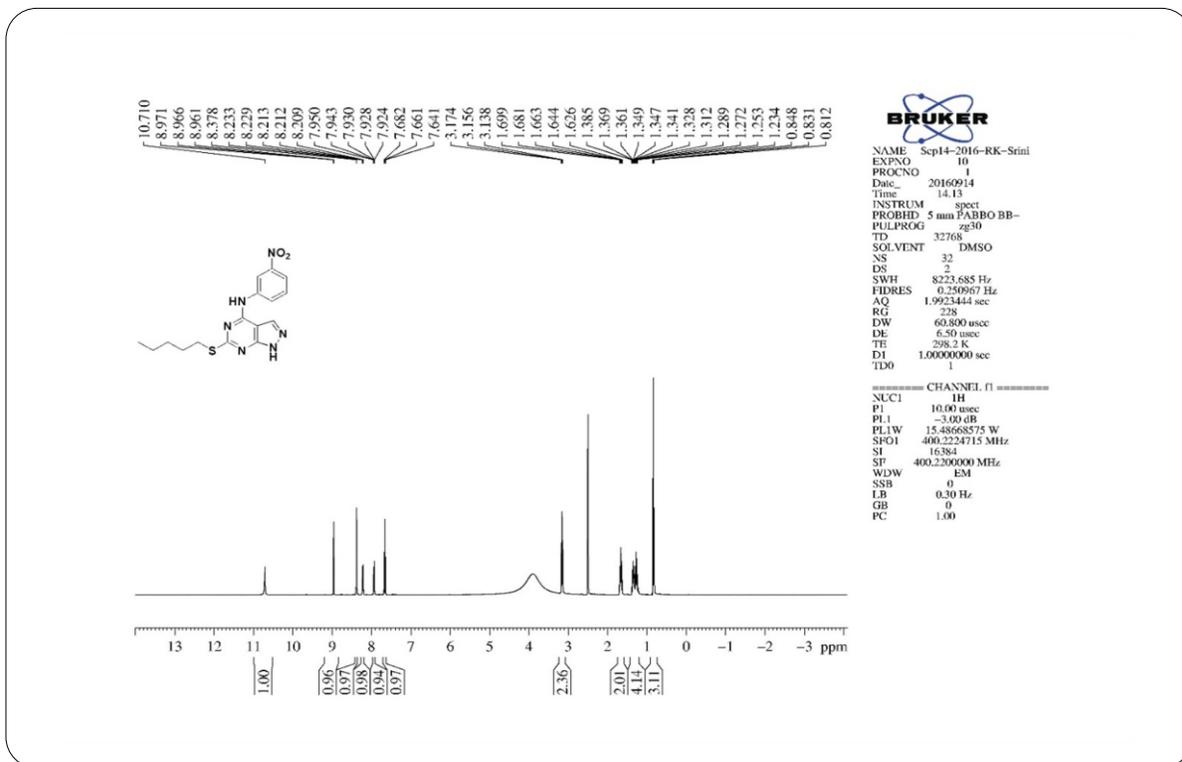
^{1}H NMR Spectrum of Compound 30
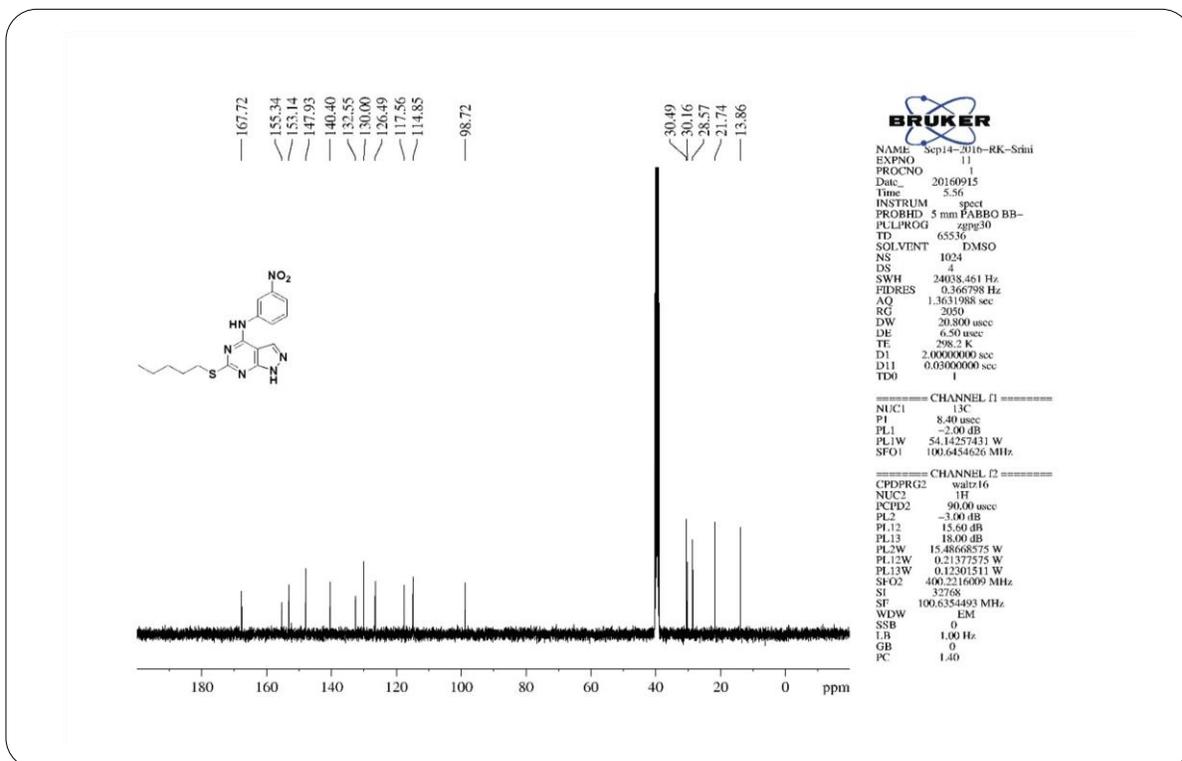
^{13}C NMR Spectrum of Compound 30
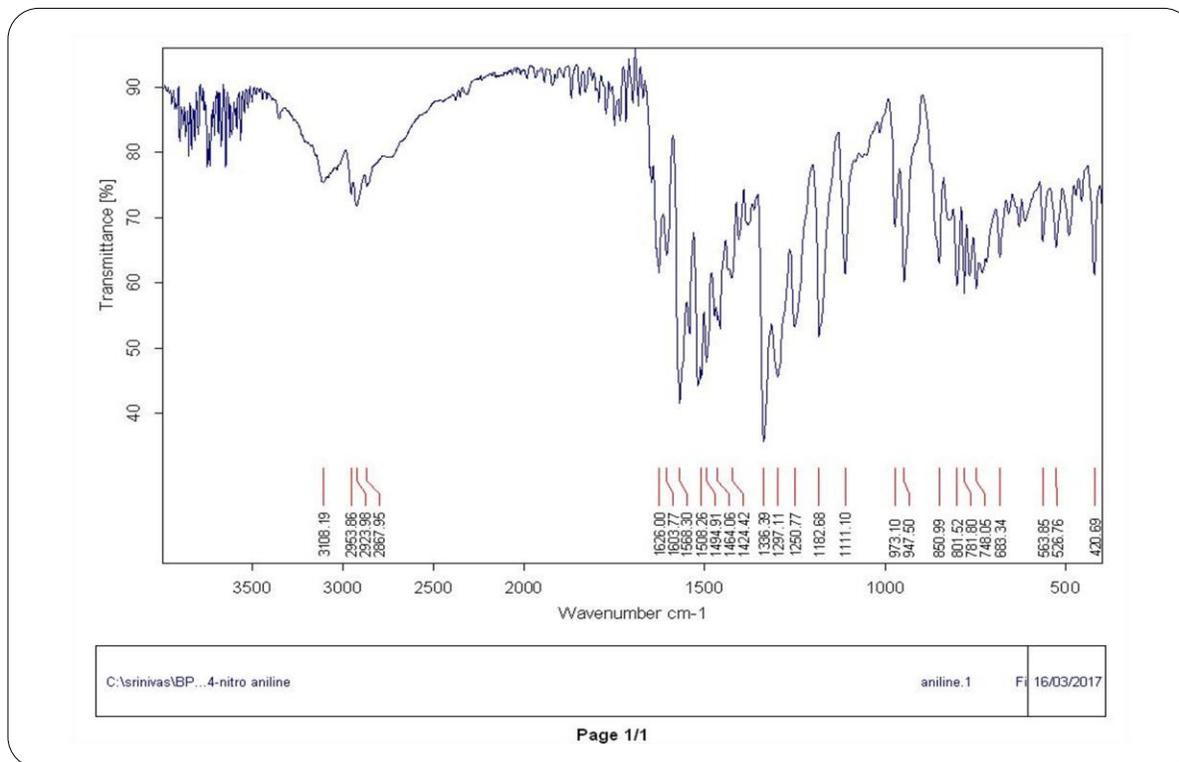
IR Spectrum of Compound 31
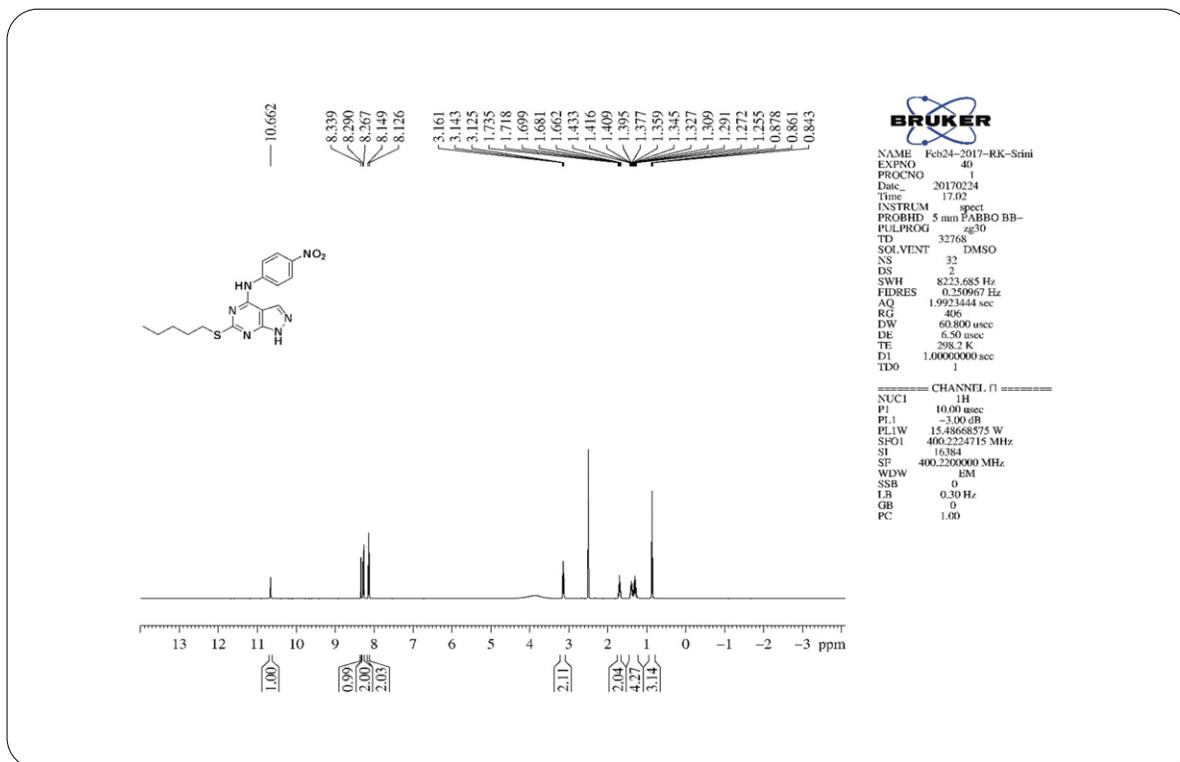
¹H NMR Spectrum of Compound 31
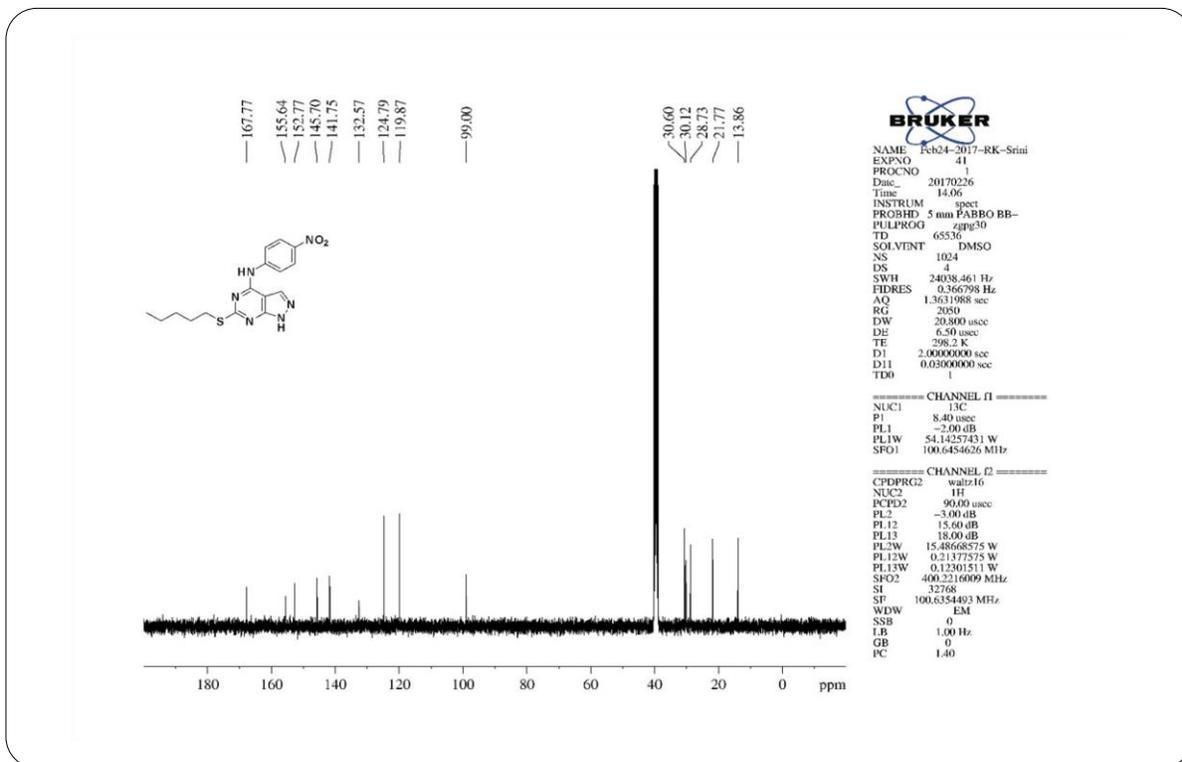
^{13}C NMR Spectrum of Compound 31
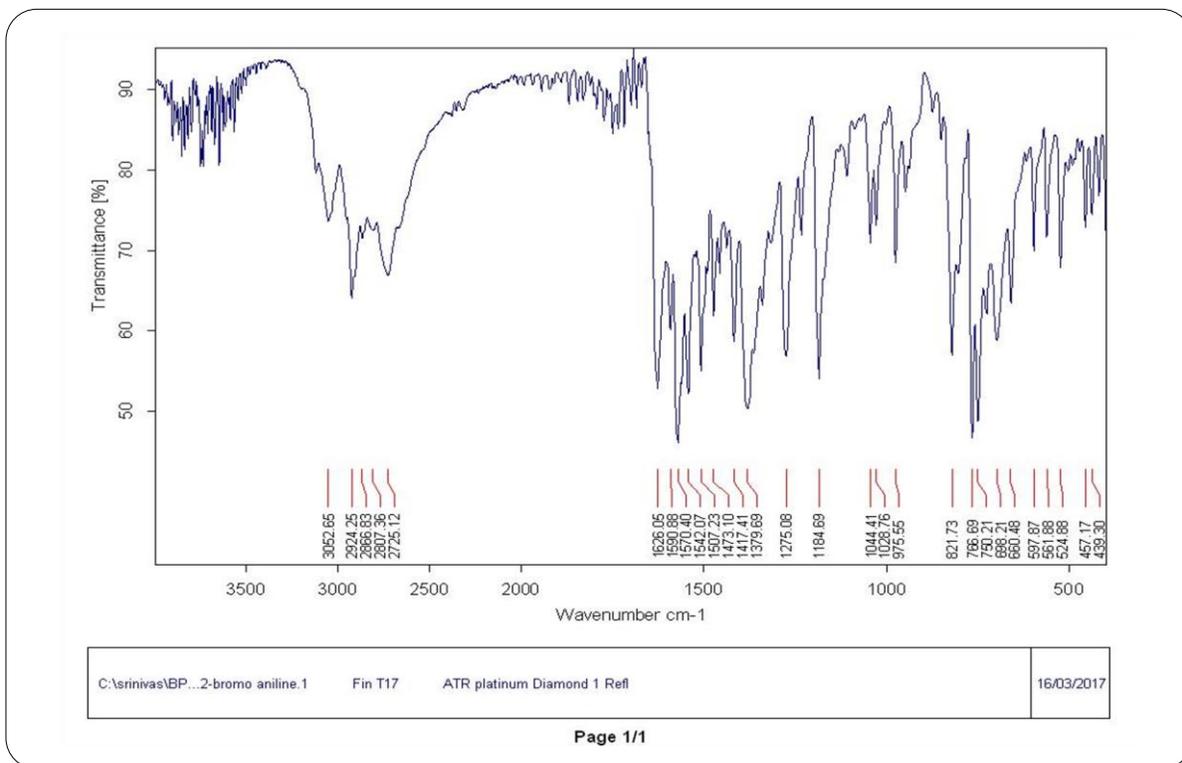
IR Spectrum of Compound 32
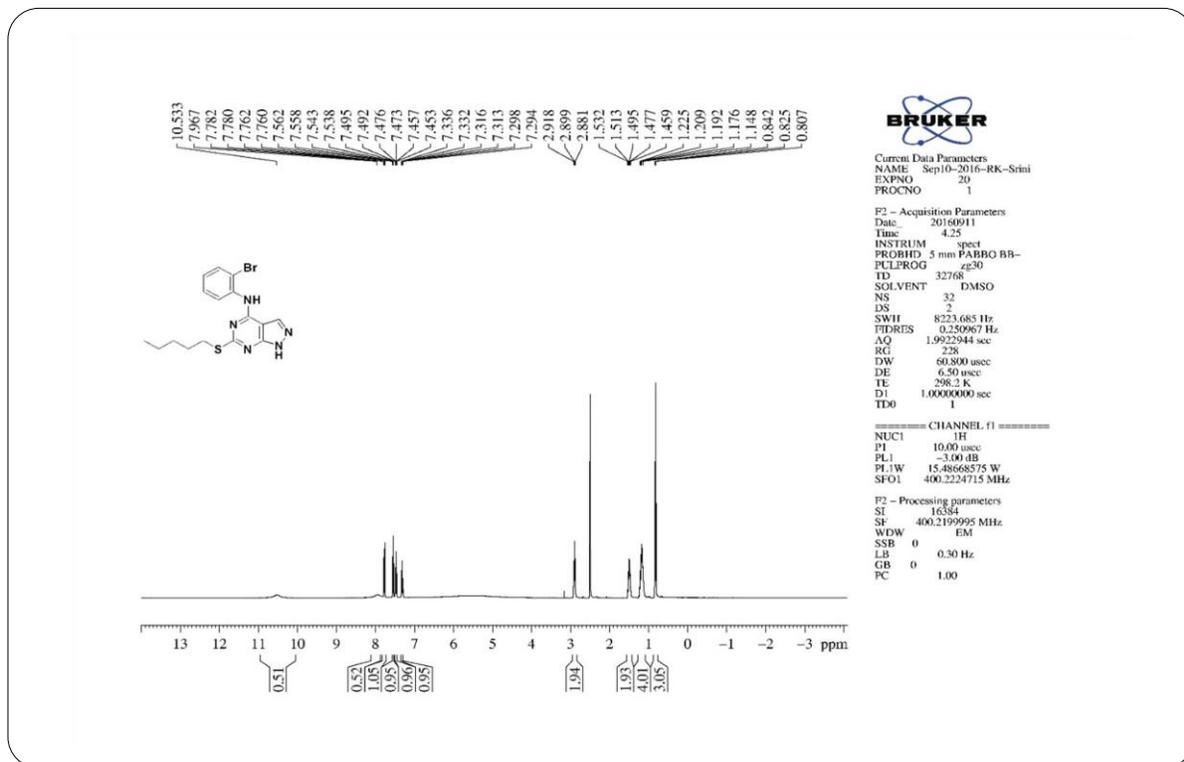
¹H NMR Spectrum of Compound 32
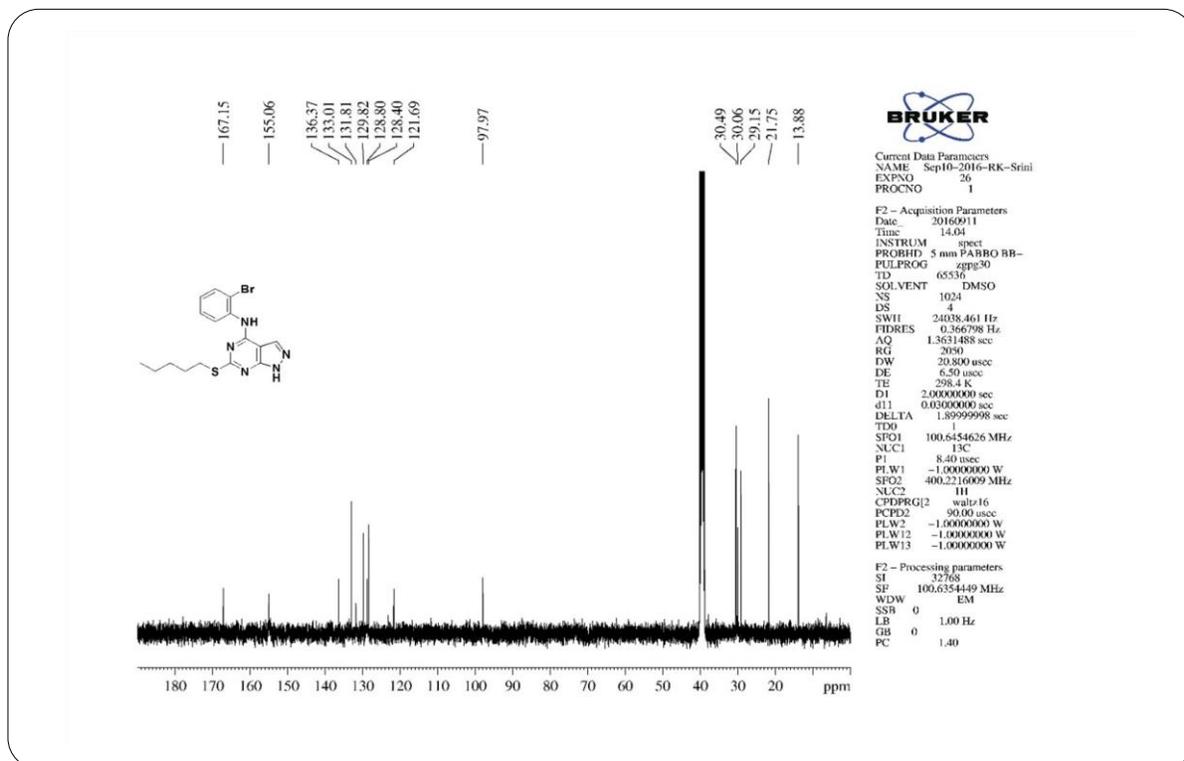
¹³C NMR Spectrum of Compound 32
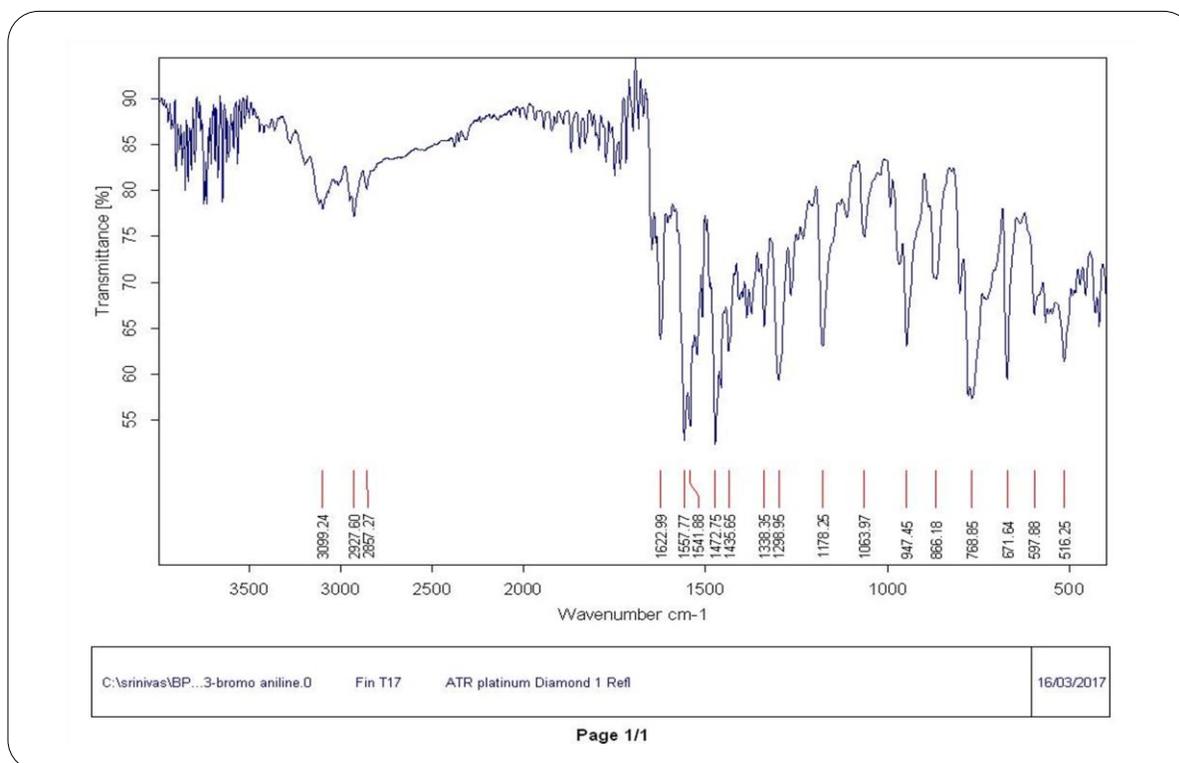
IR Spectrum of Compound 33
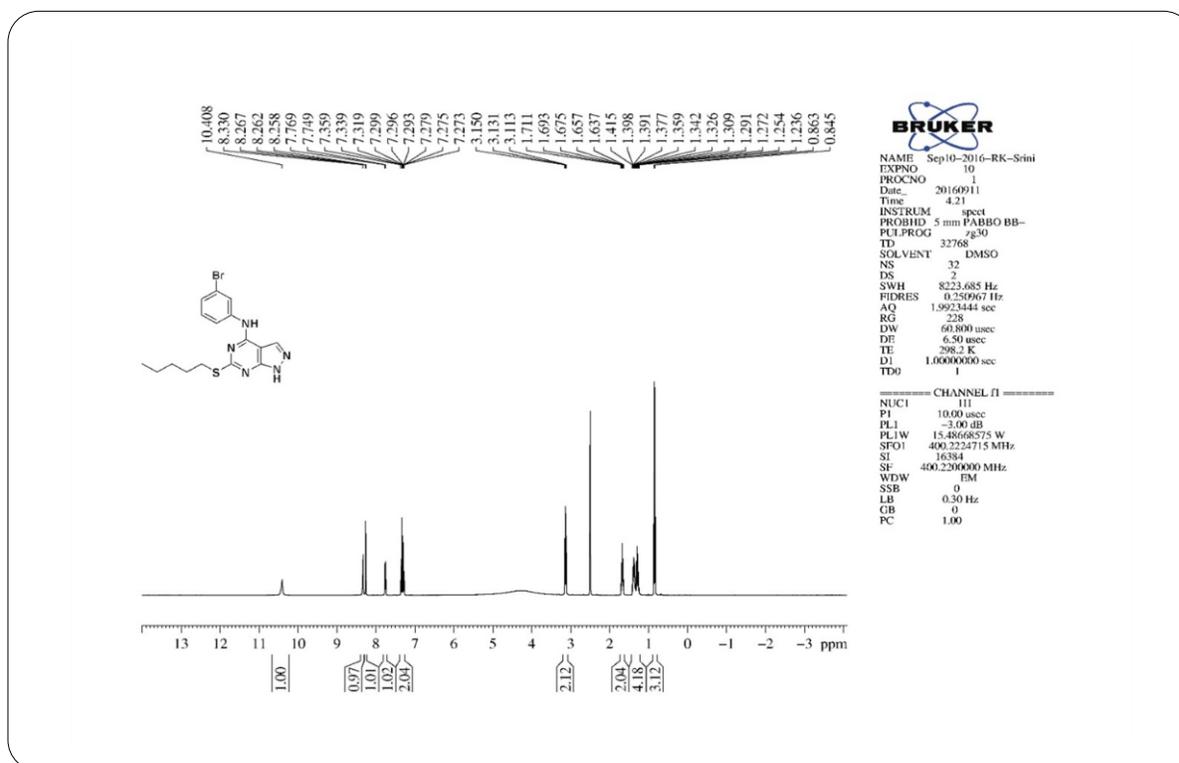
¹H NMR Spectrum of Compound 33
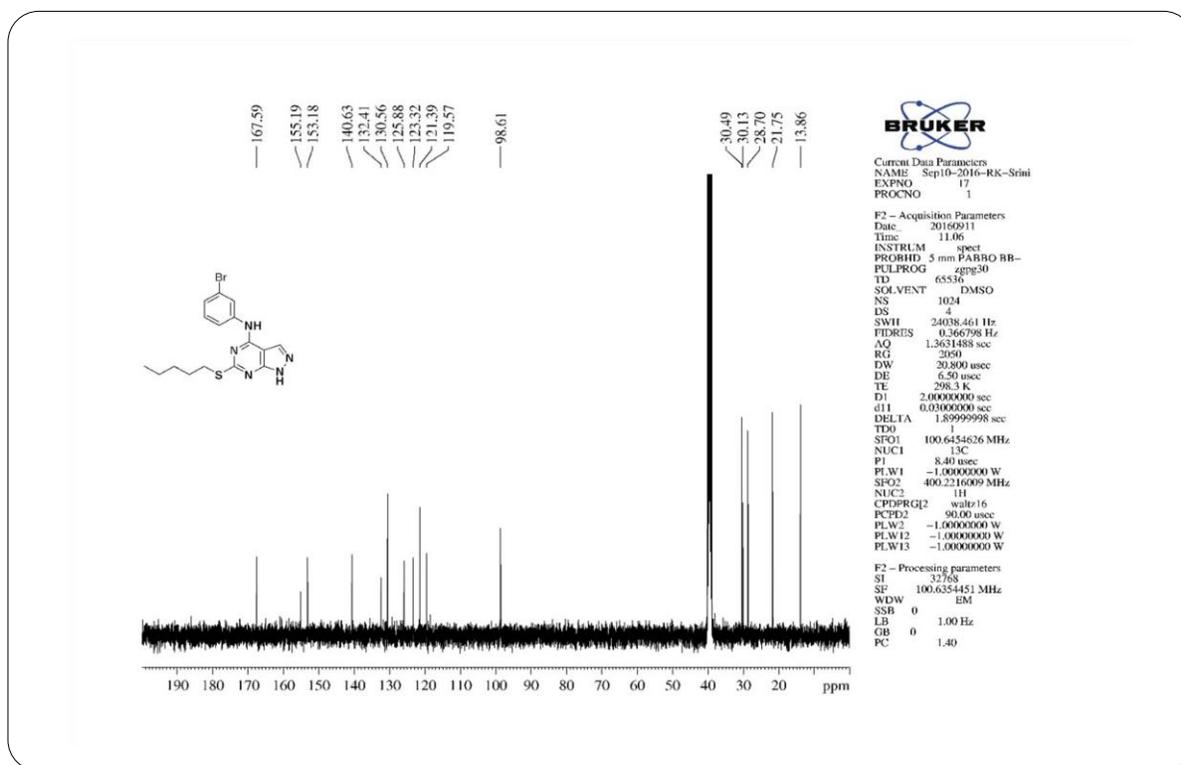
¹³C NMR Spectrum of Compound 33
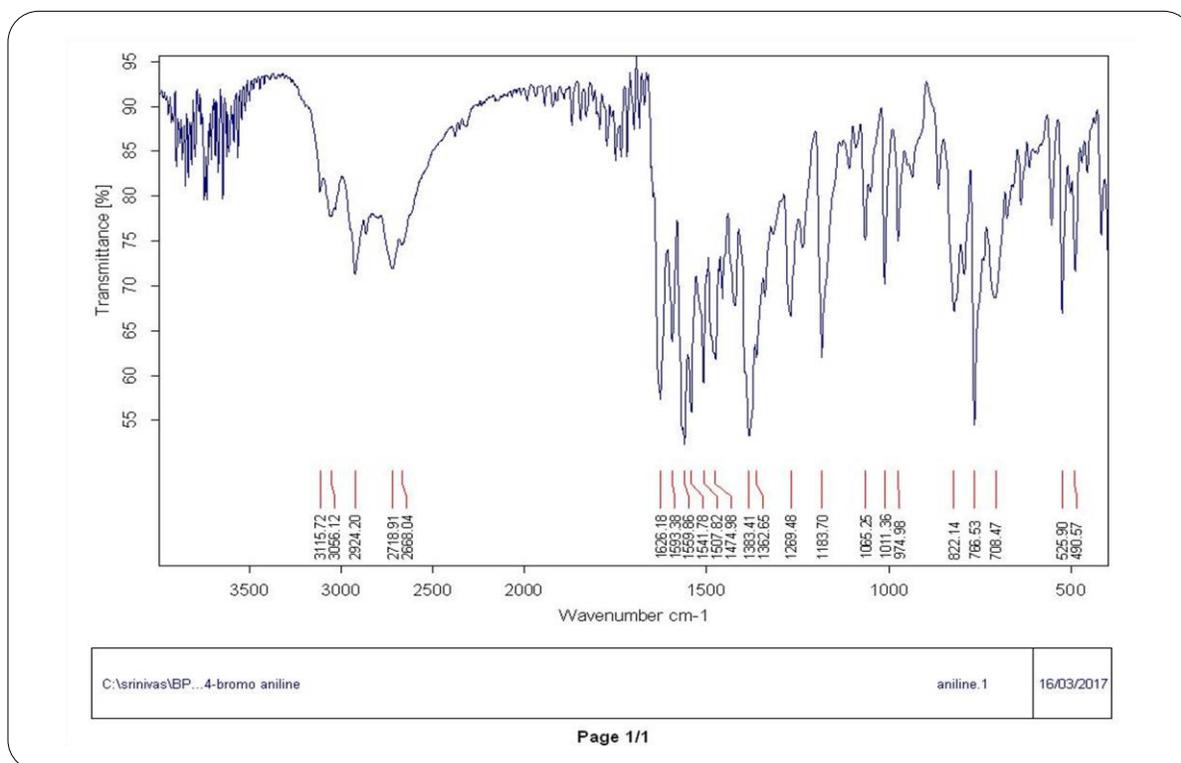
IR Spectrum of Compound 34
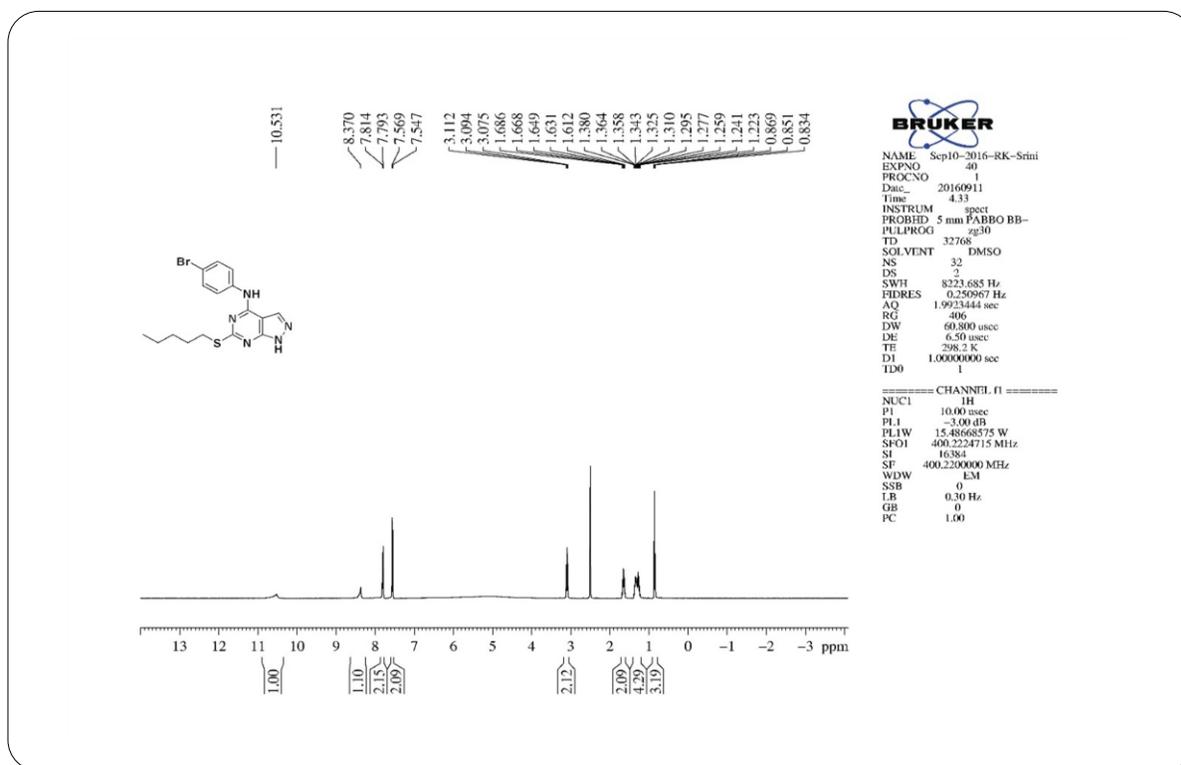
^{1}H NMR Spectrum of Compound 34
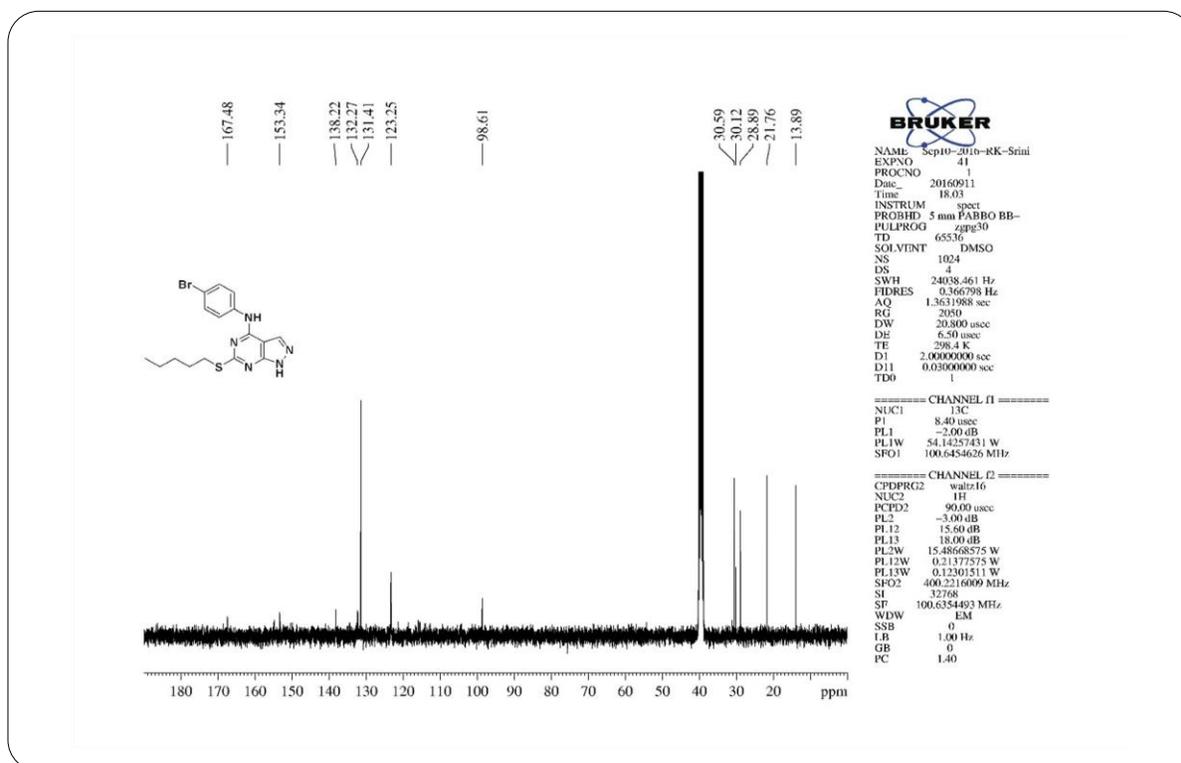
^{13}C NMR Spectrum of Compound 34
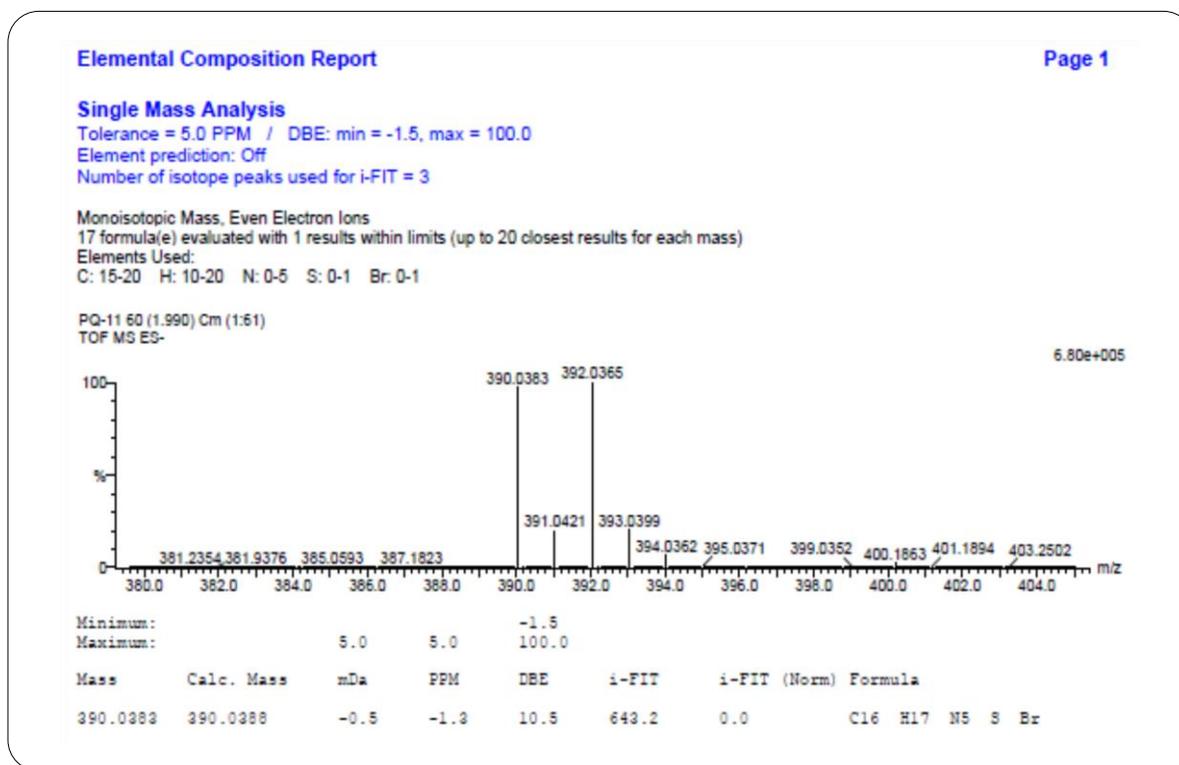
HRMS Spectrum of Compound 34
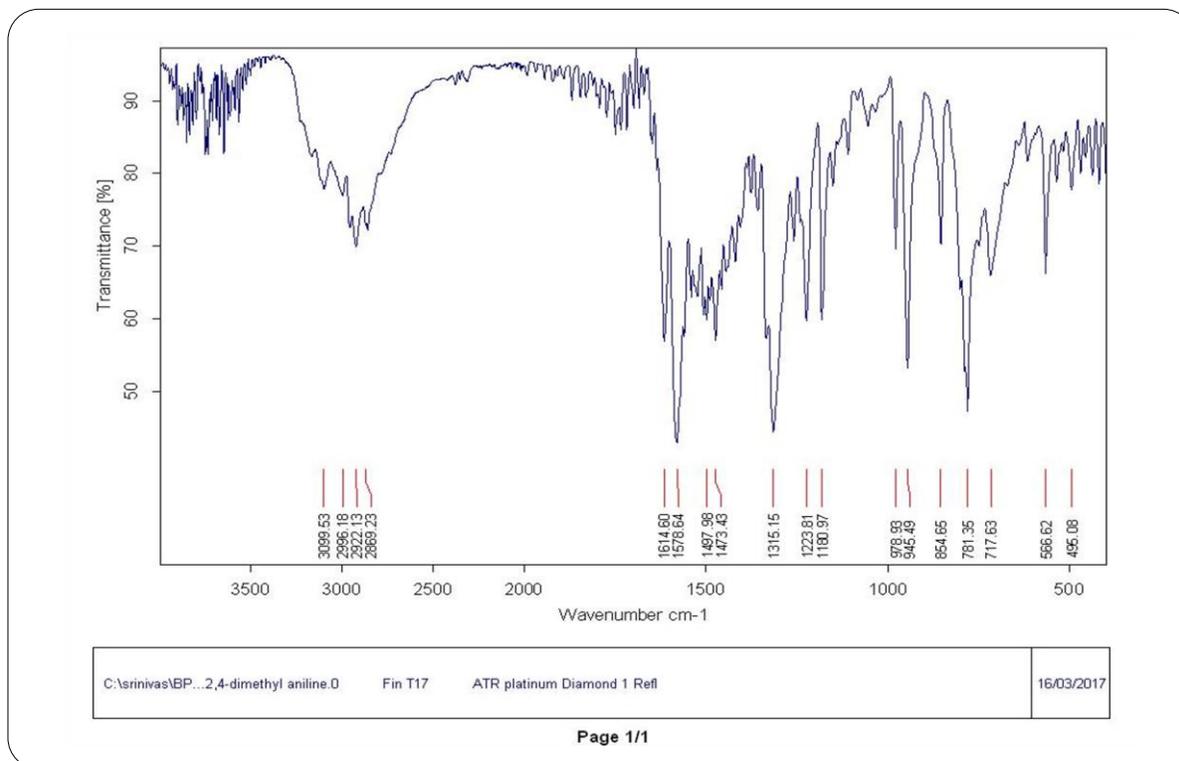
IR Spectrum of Compound 35
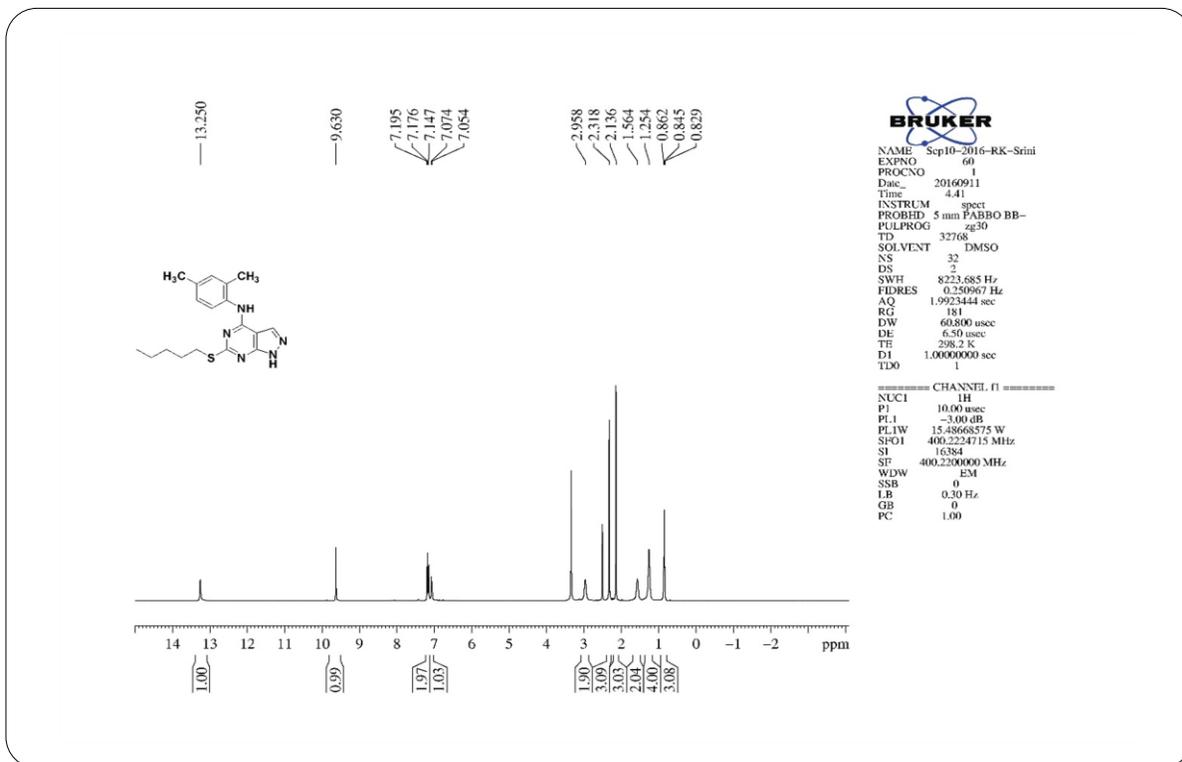
¹H NMR Spectrum of Compound 35
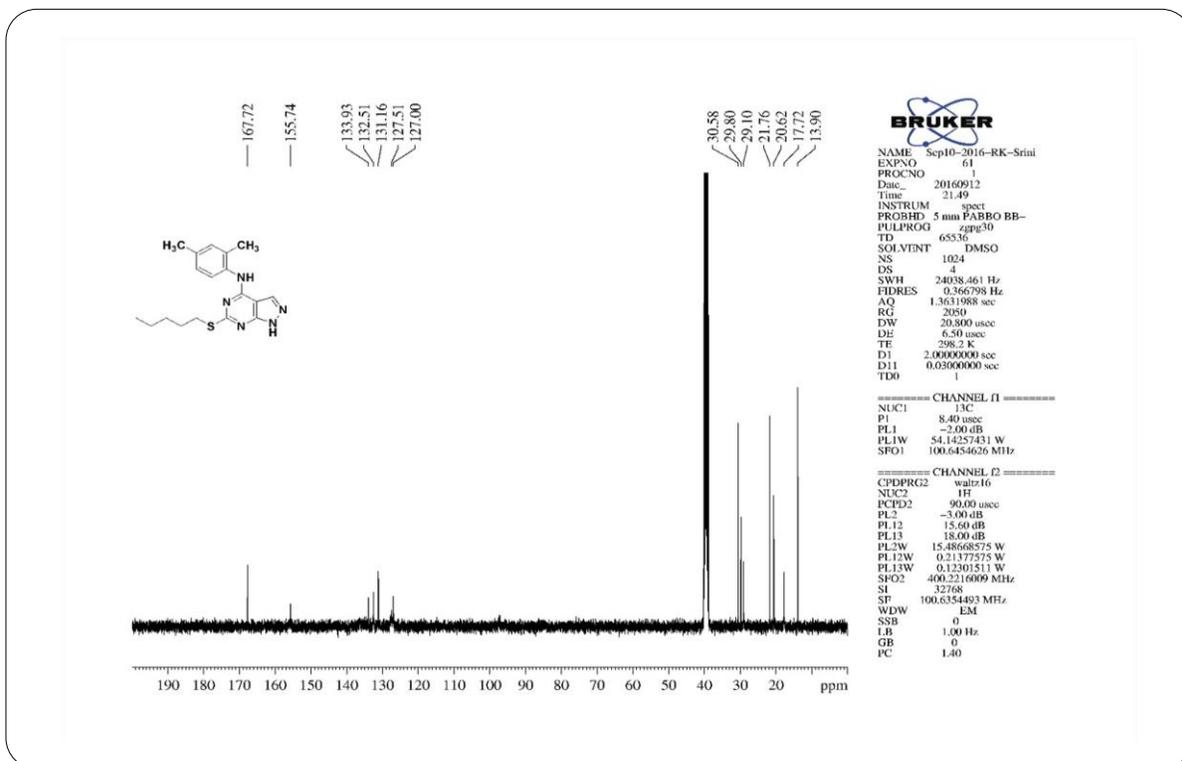
¹³C NMR Spectrum of Compound 35
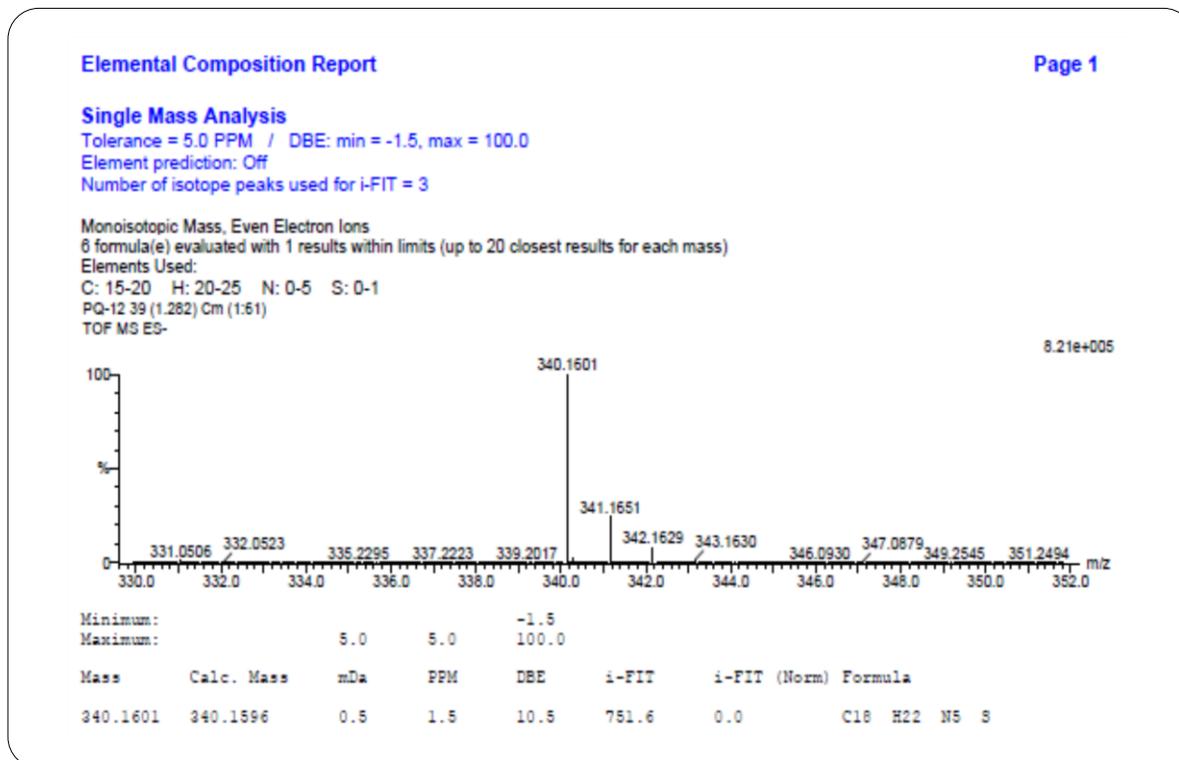
**HRMS Spectrum of Compound 35**
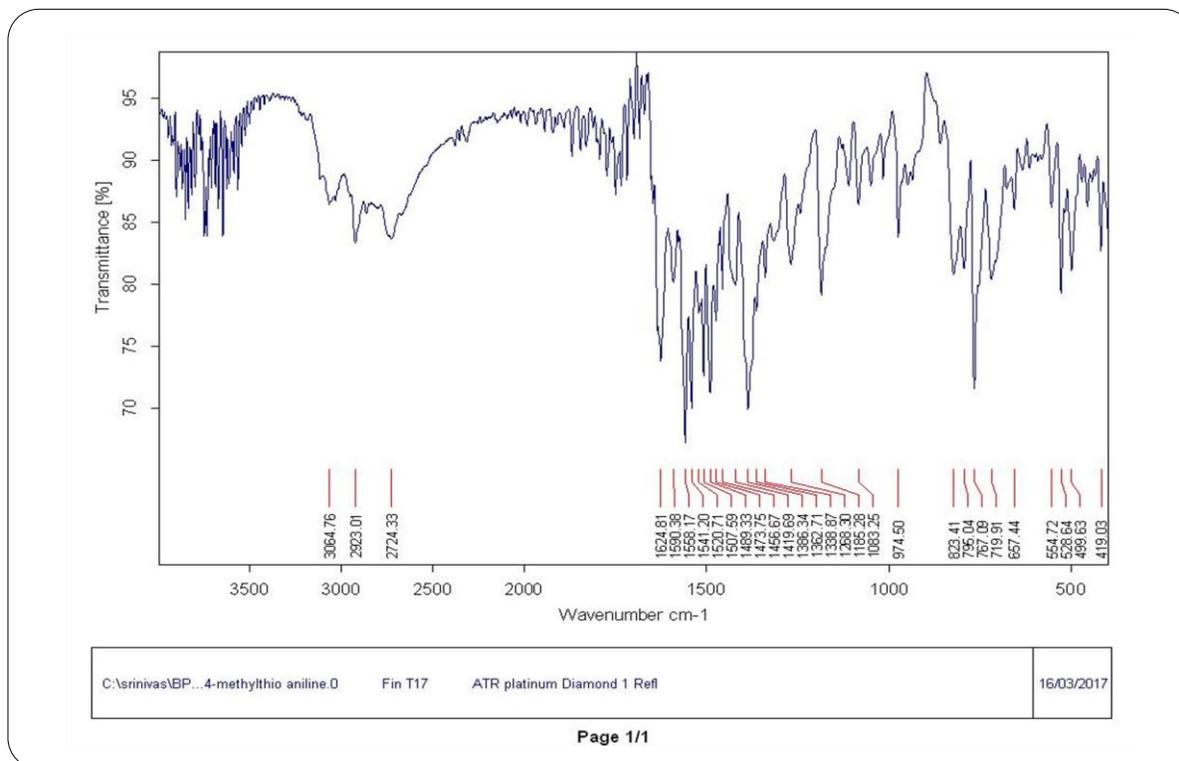
IR Spectrum of Compound 36
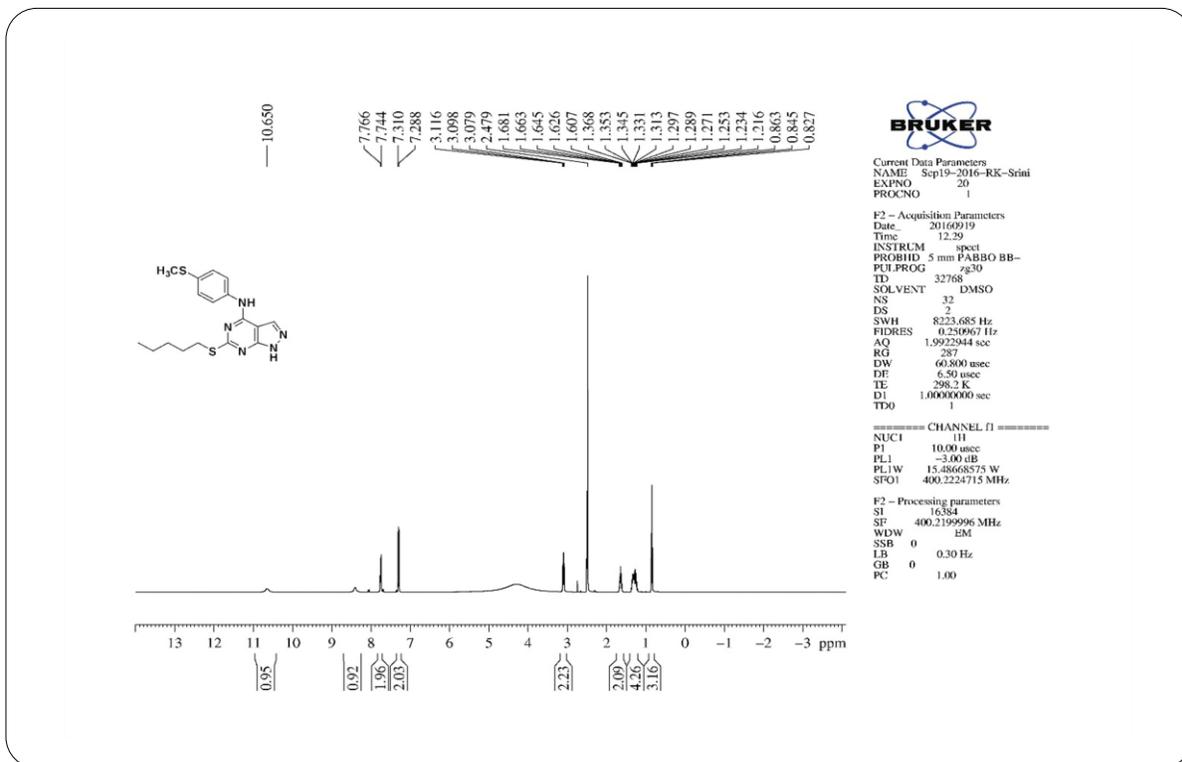
^{1}**H NMR Spectrum of Compound 36**
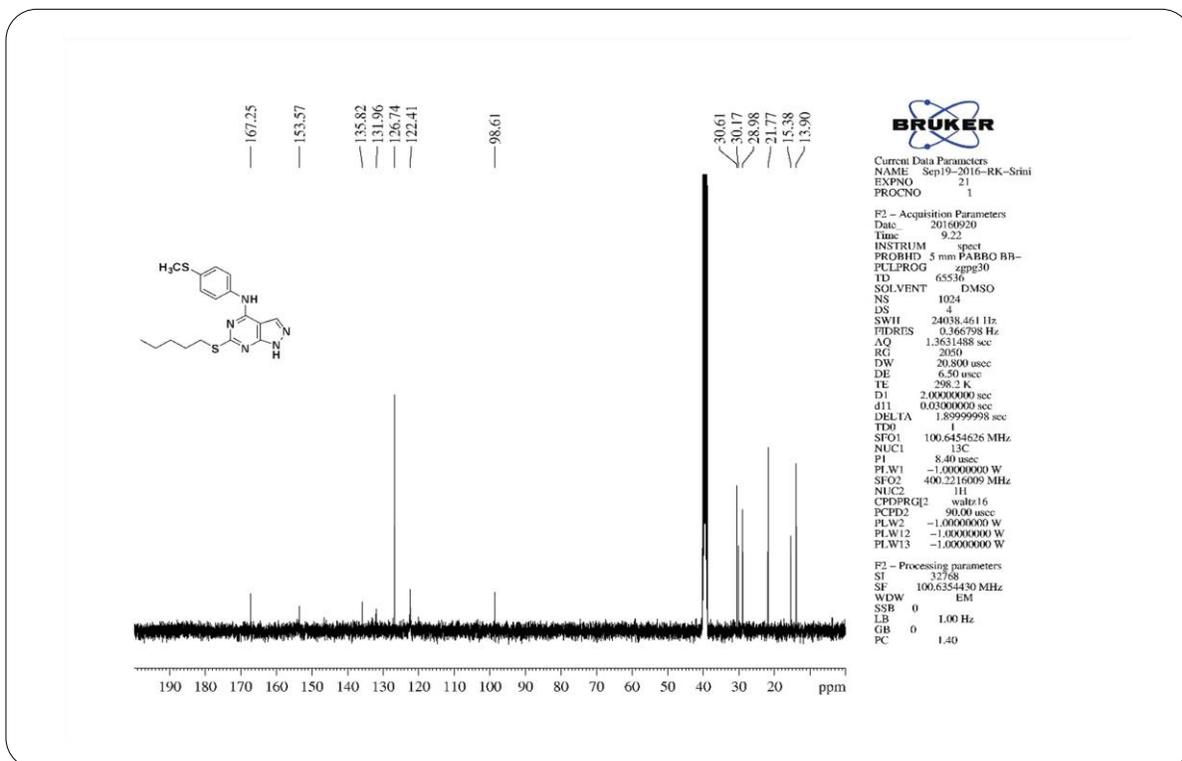
^{13}**C NMR Spectrum of Compound 36**
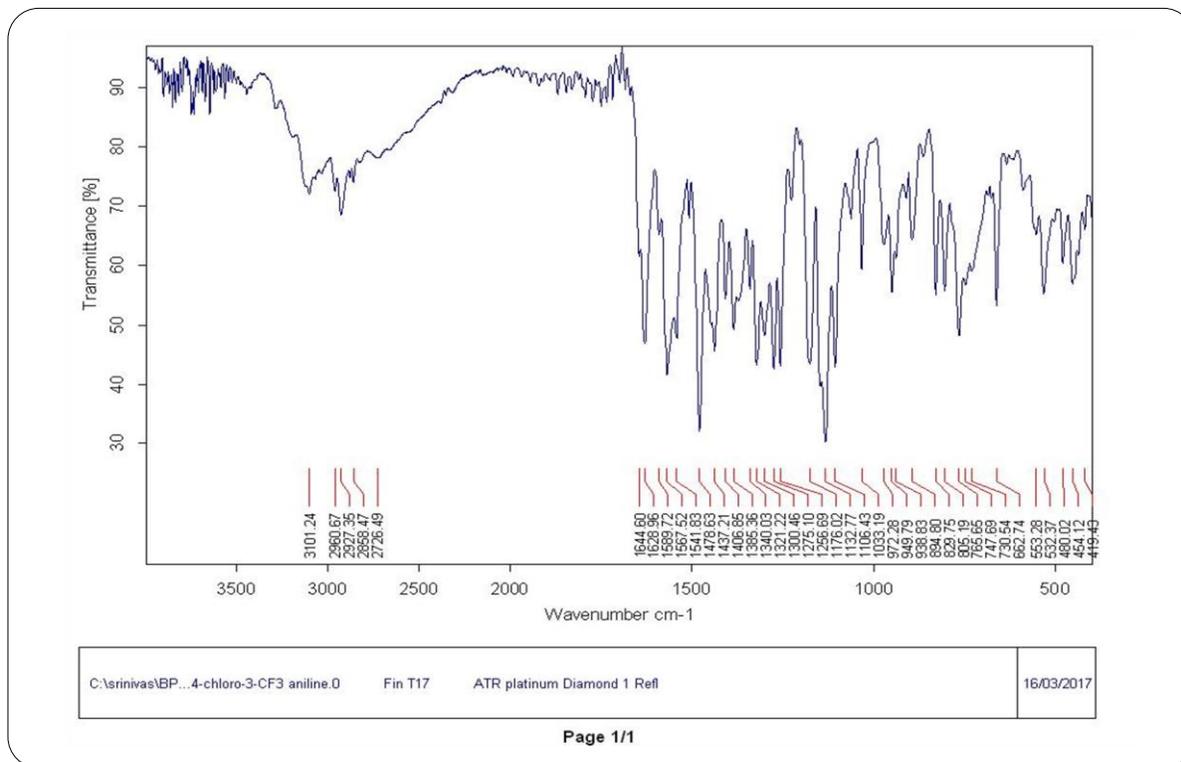
IR Spectrum of Compound 37
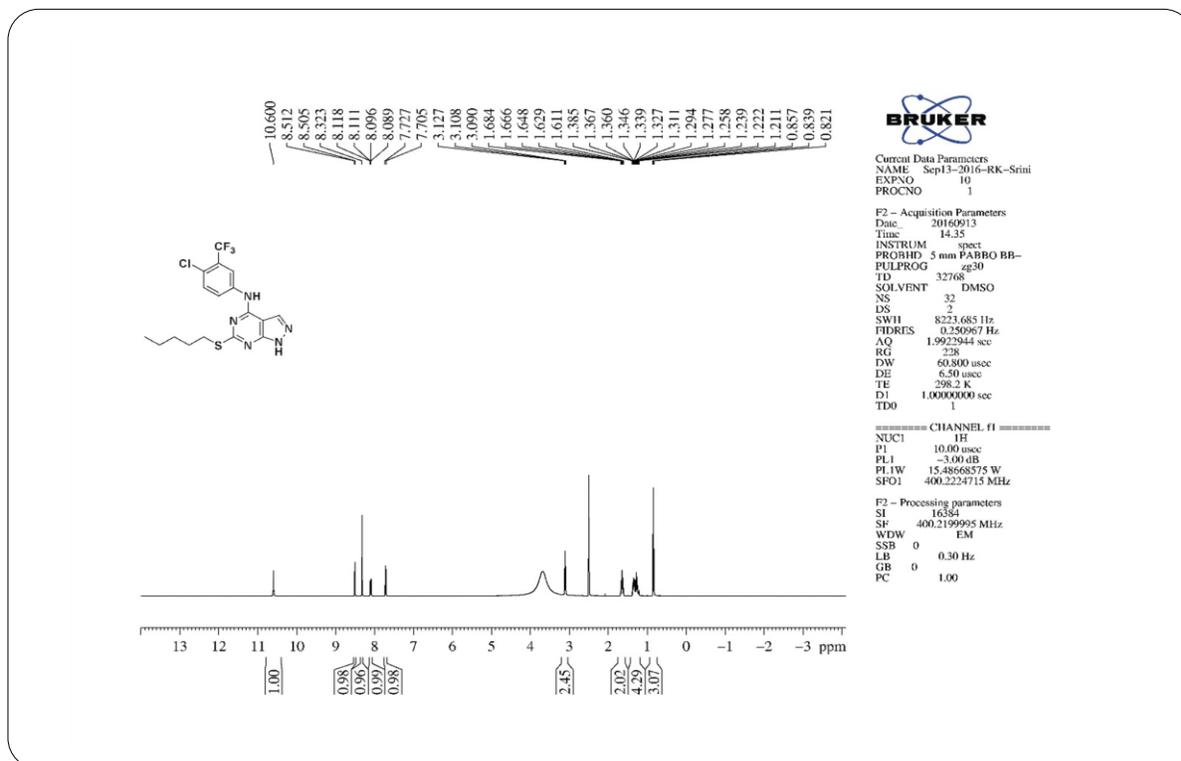
¹H NMR Spectrum of Compound 37
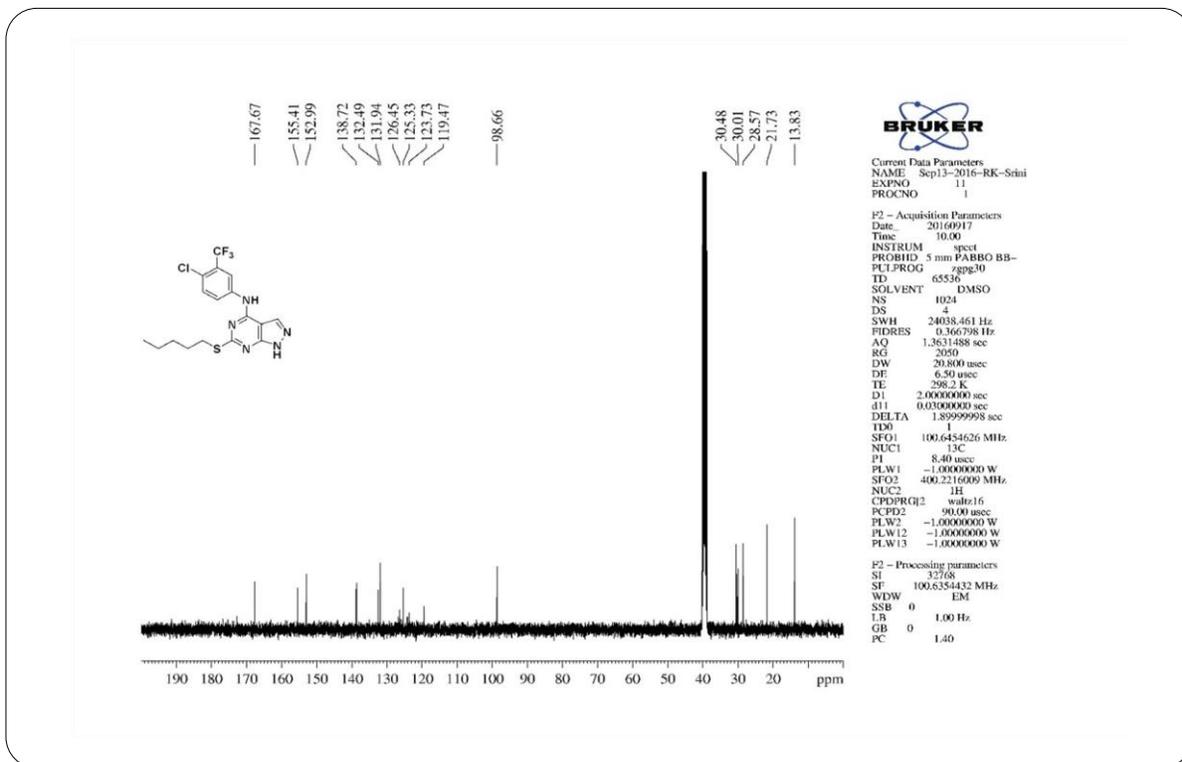
**¹³C NMR Spectrum of Compound 37**
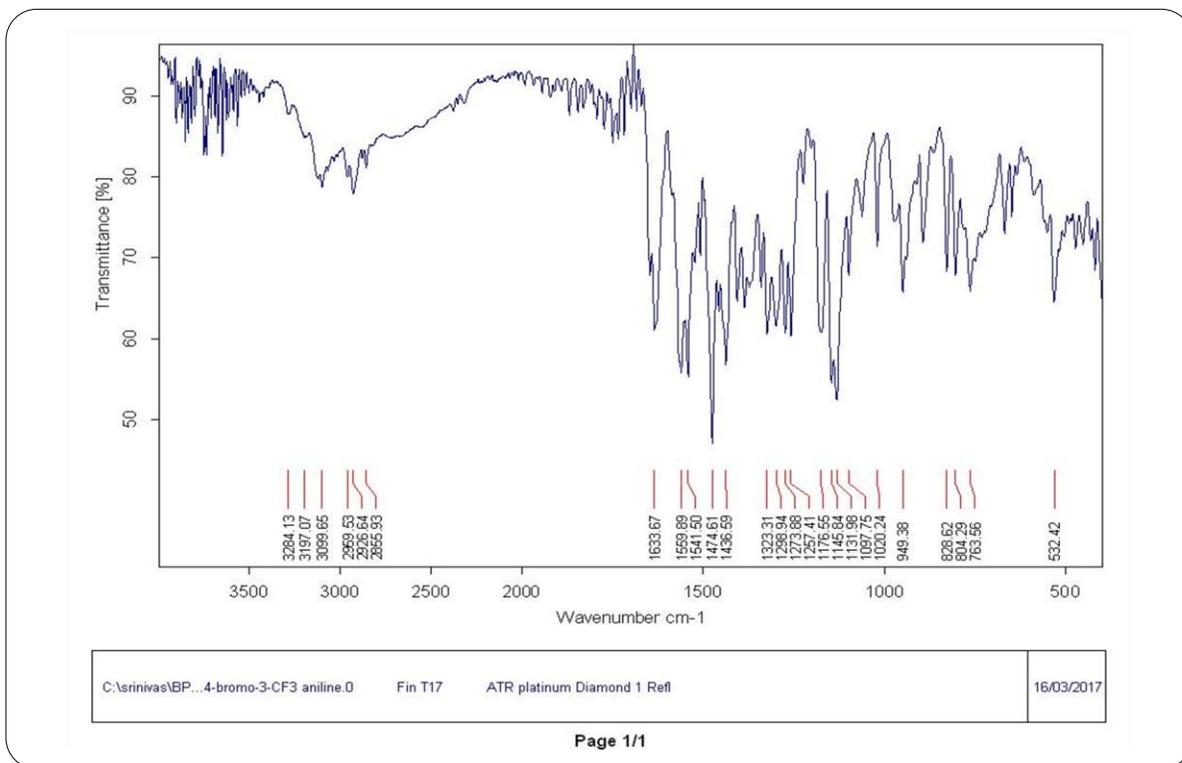
**IR Spectrum of Compound 38**
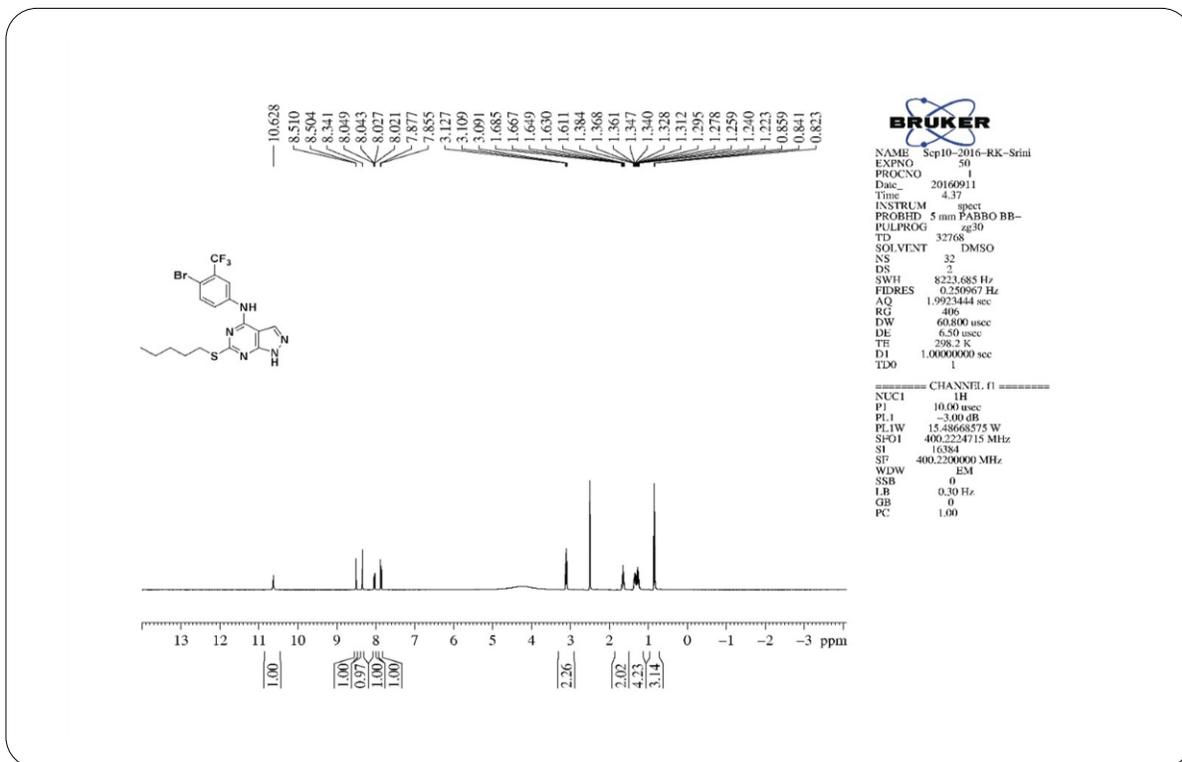
¹H NMR Spectrum of Compound 38
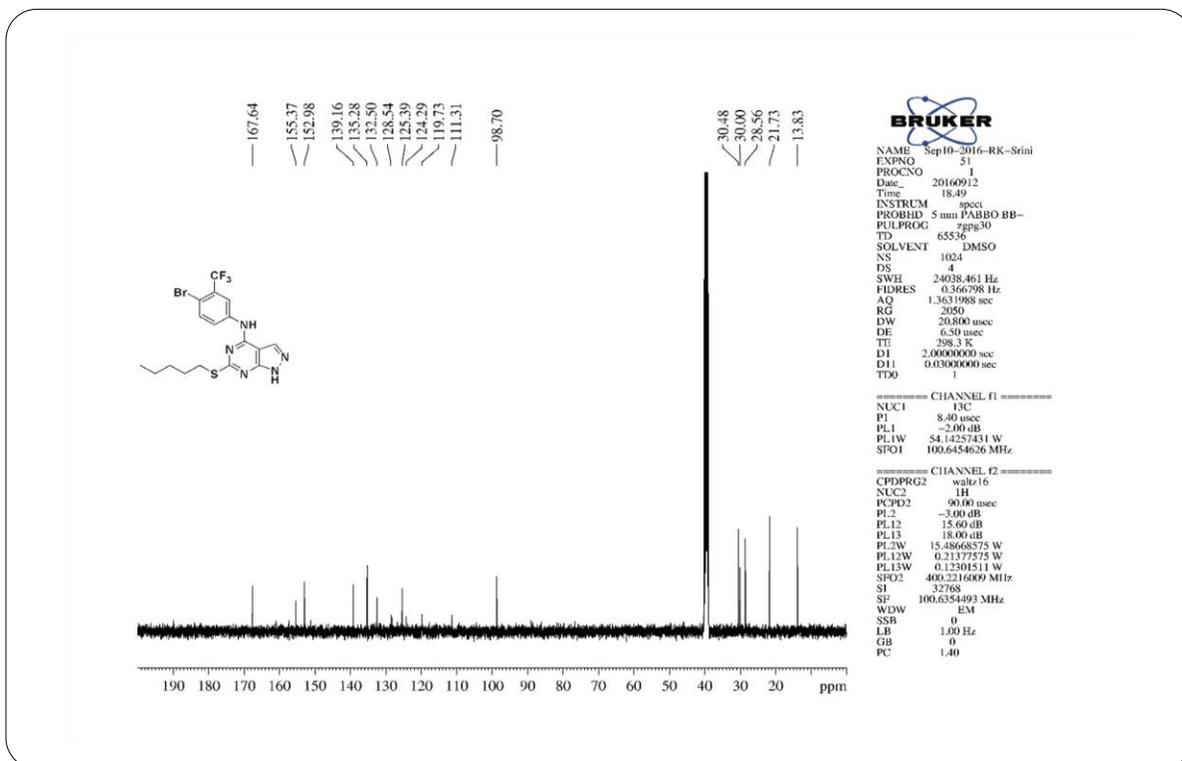

¹³C NMR Spectrum of Compound 38

Elemental Composition Report   **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM   /   DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
74 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 15-20   H: 15-20   N: 0-5   F: 0-3   S: 0-1   Br: 0-1

PQ-15 2 (0.034) Cm (1:61)
TOF MS ES-   1.44e+006
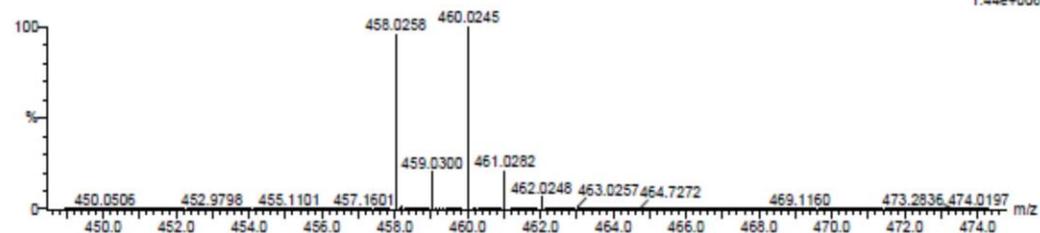
Minimum: -1.5
Maximum: 5.0   5.0   100.0

Mass   Calc. Mass   mDa   PPM   DBE   i-FIT   i-FIT (Norm) Formula

458.0258   458.0262   -0.4   -0.9   10.5   673.0   0.0   C17 H16 N5 F3 S Br

HRMS Spectrum of Compound 38
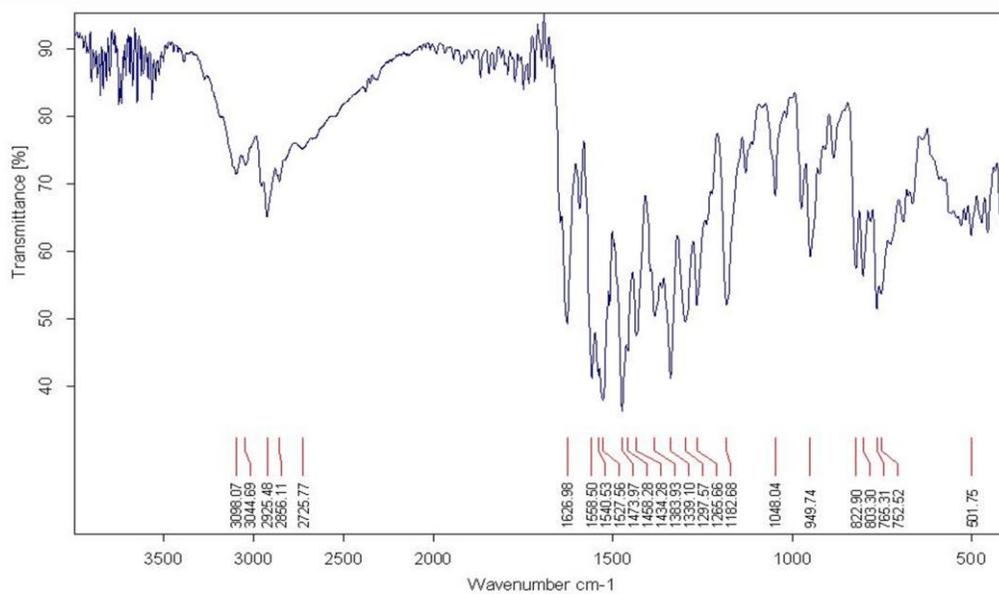
C:\srinivas\BP…4-chloro-3-nitro aniline.0   Fin T17   ATR platinum Diamond 1 Refl   16/03/2017

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IR Spectrum of Compound 39
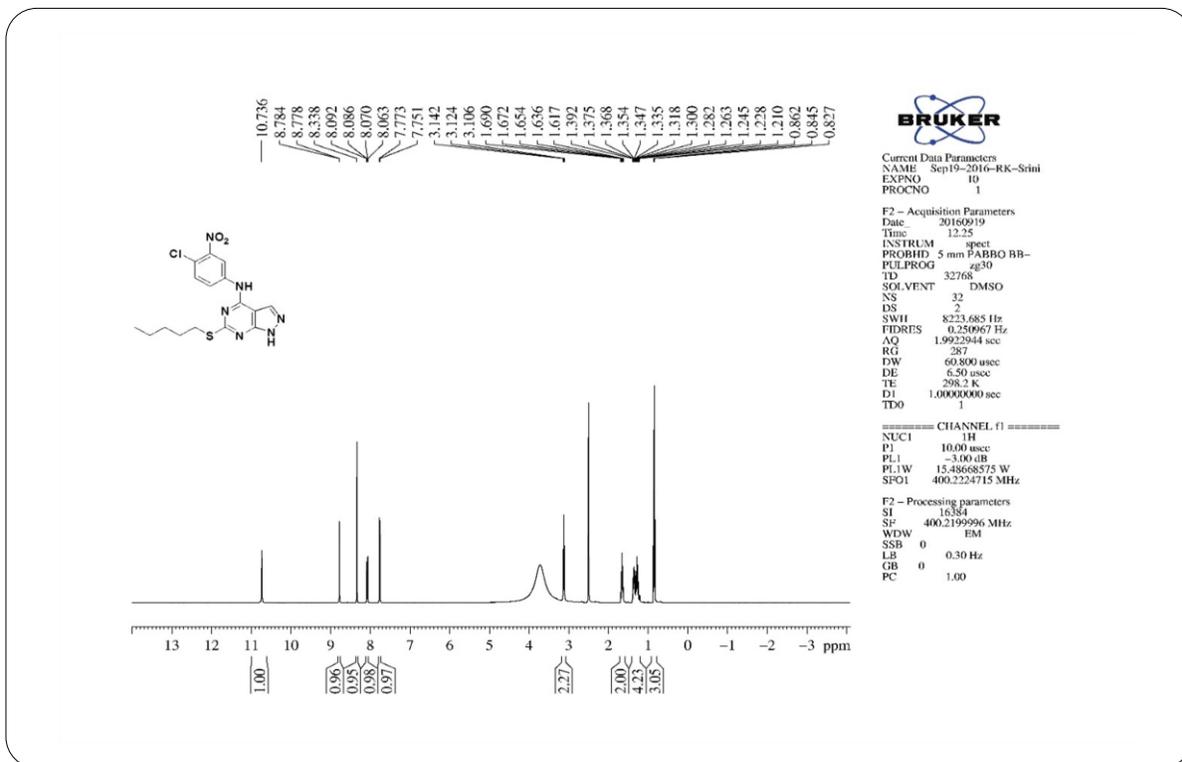
¹H NMR Spectrum of Compound 39
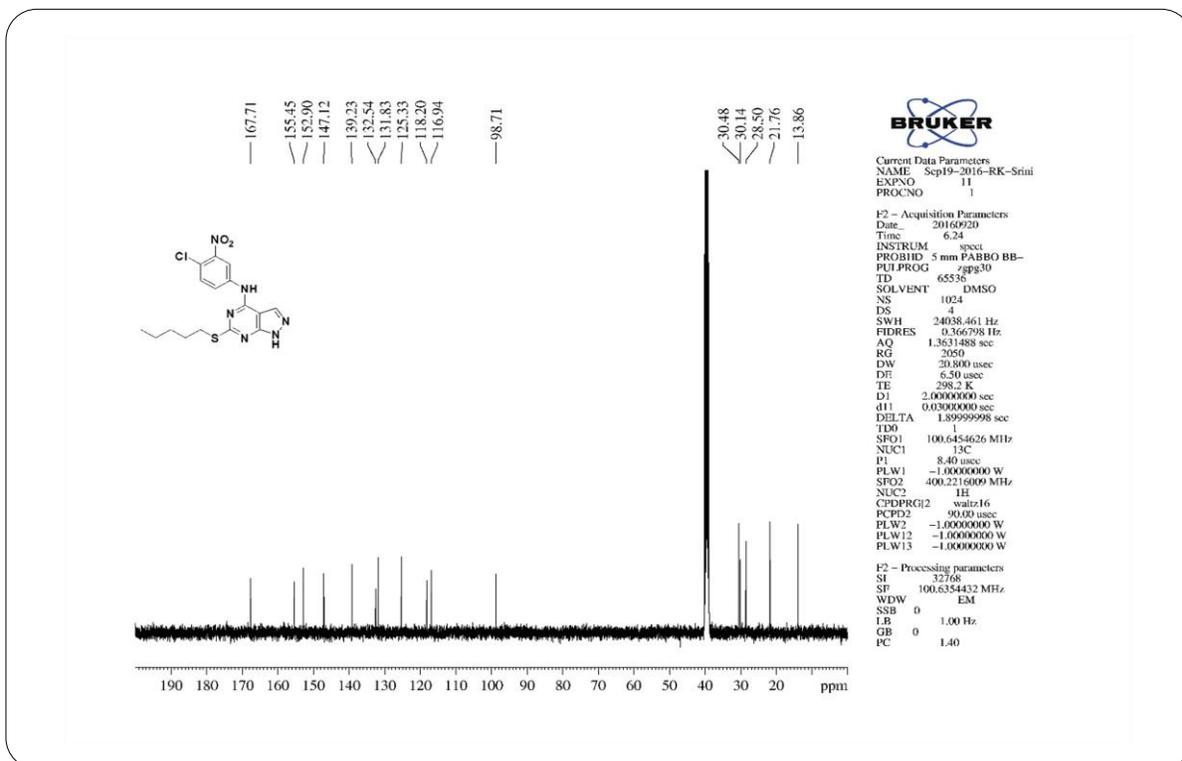
¹³C NMR Spectrum of Compound 39
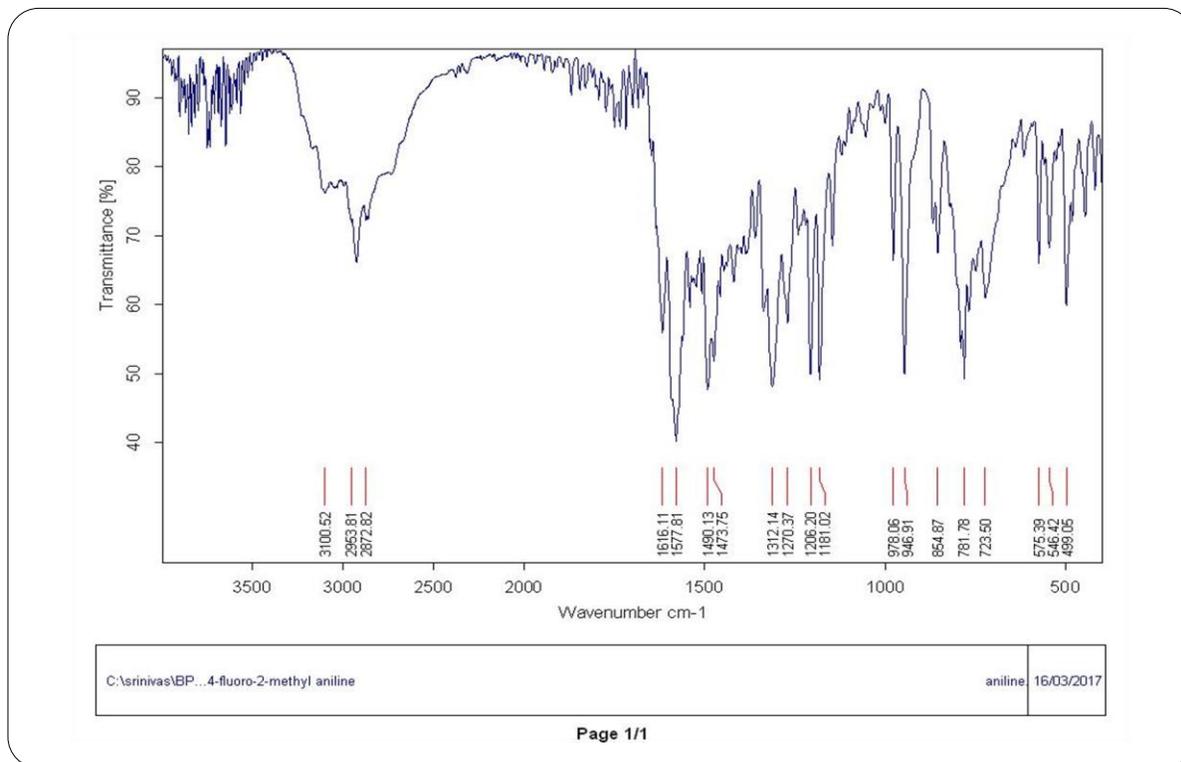
IR Spectrum of Compound 40
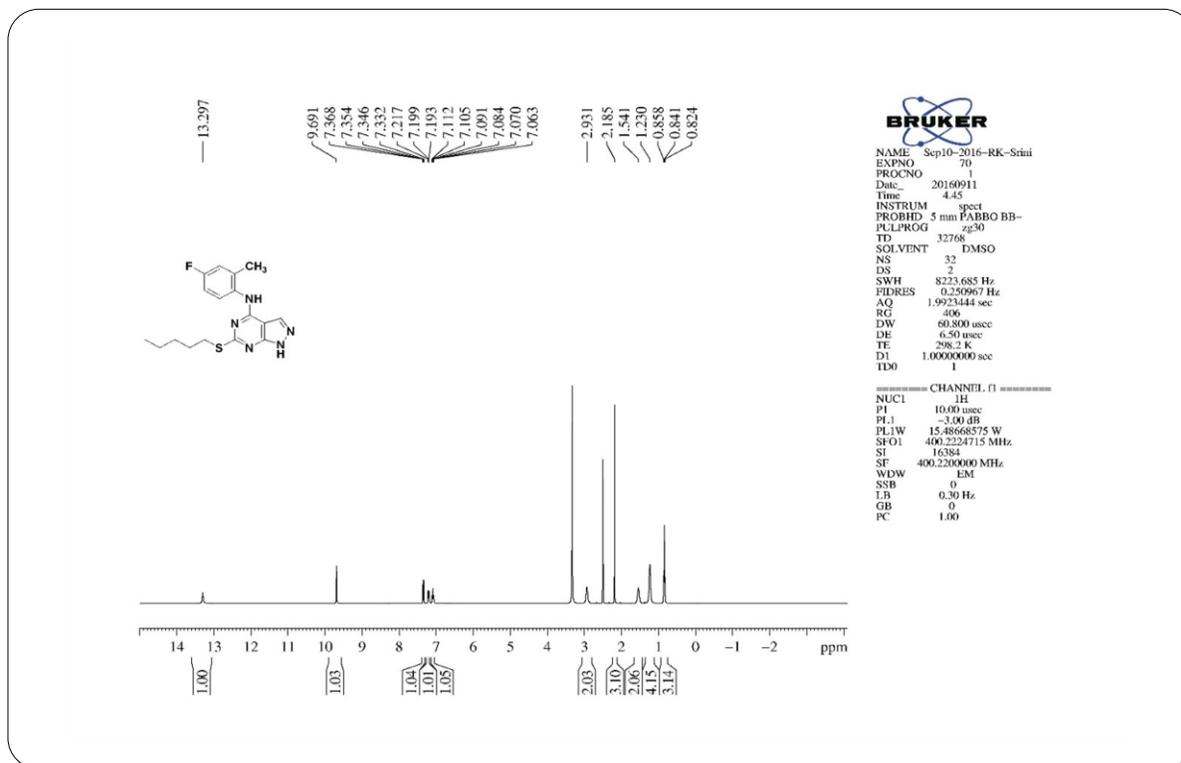
¹H NMR Spectrum of Compound 40
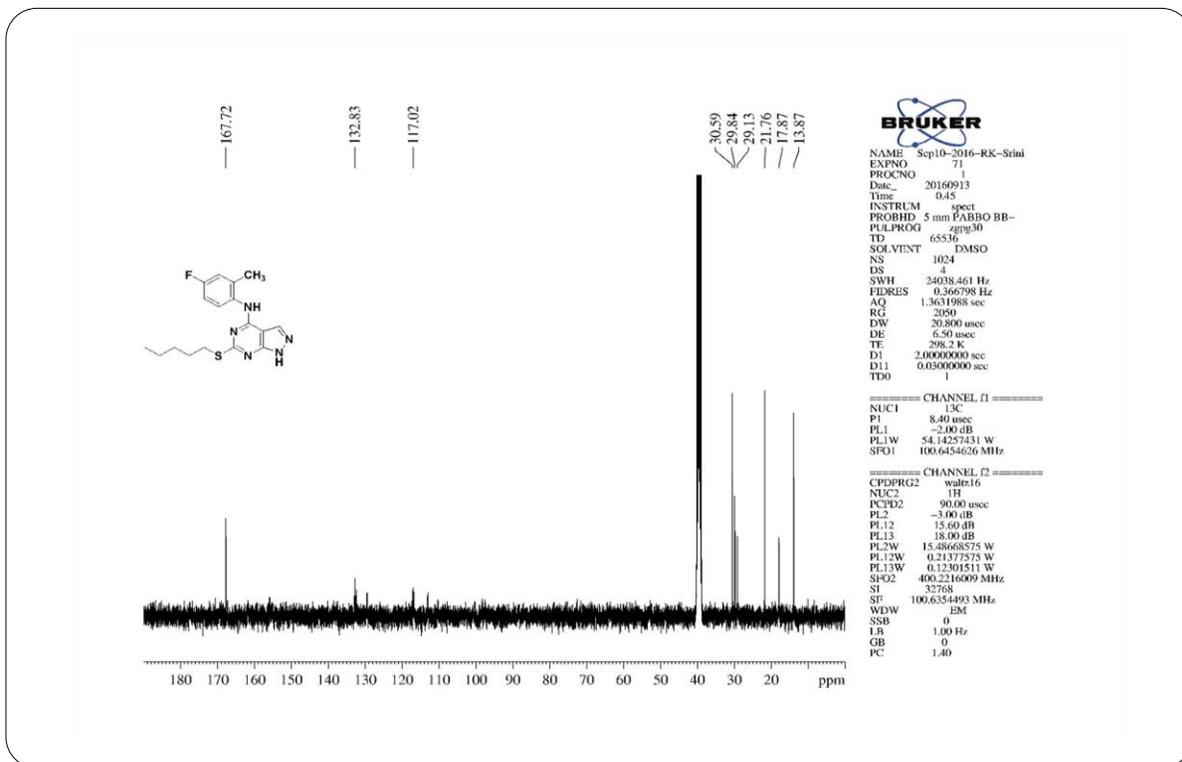
¹³C NMR Spectrum of Compound 40
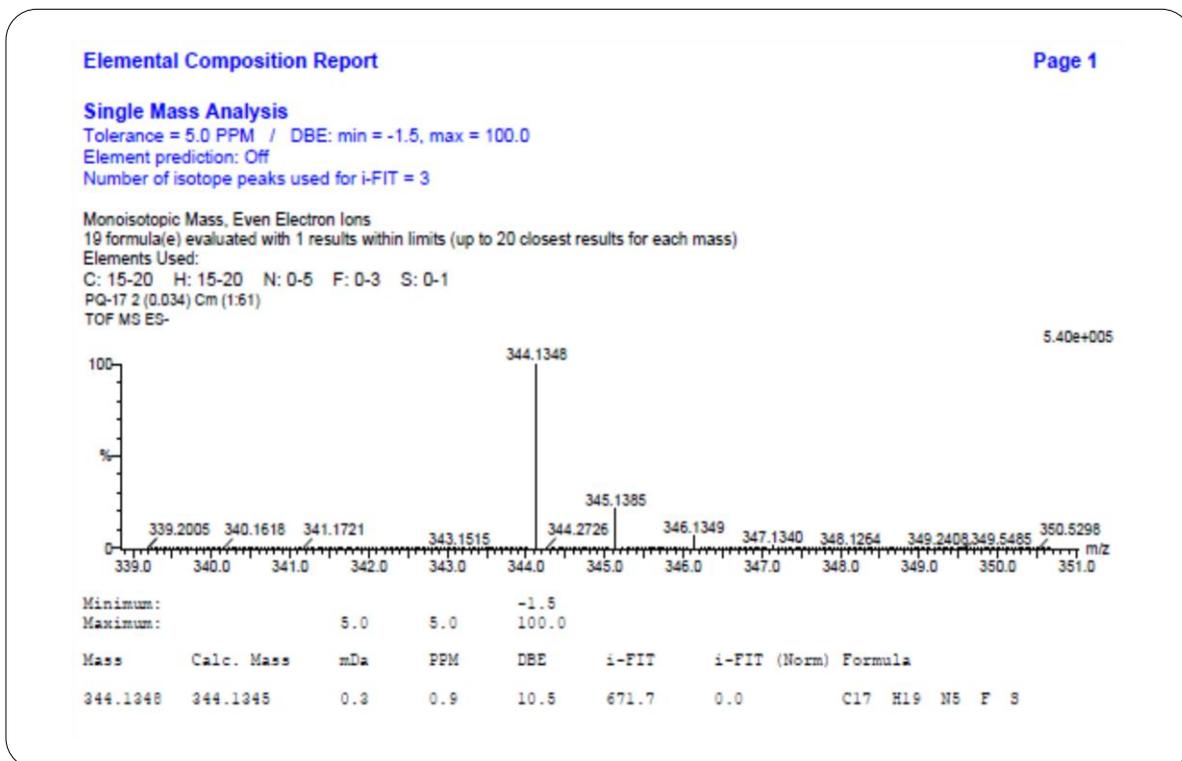
HRMS Spectrum of Compound 40
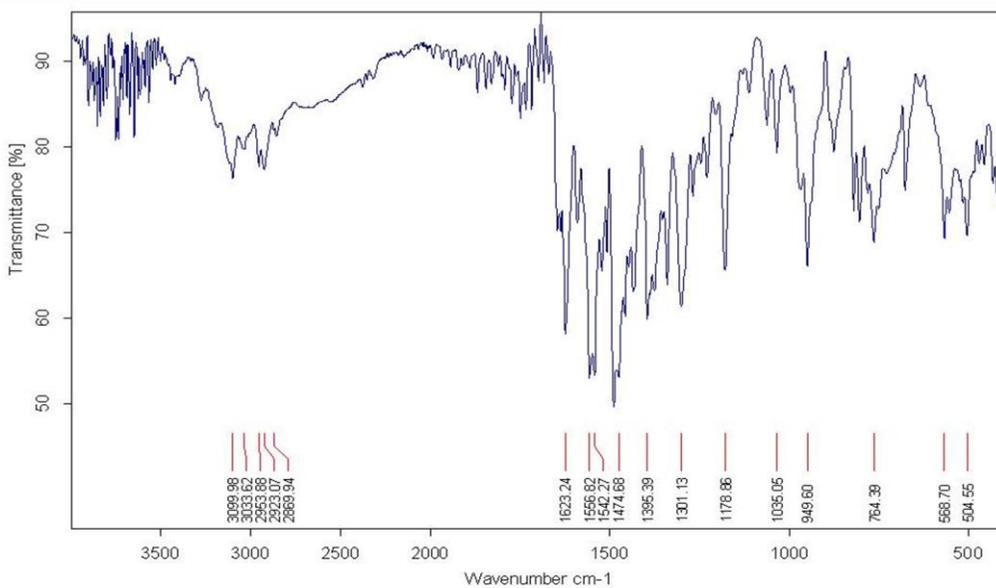
IR Spectrum of Compound 41
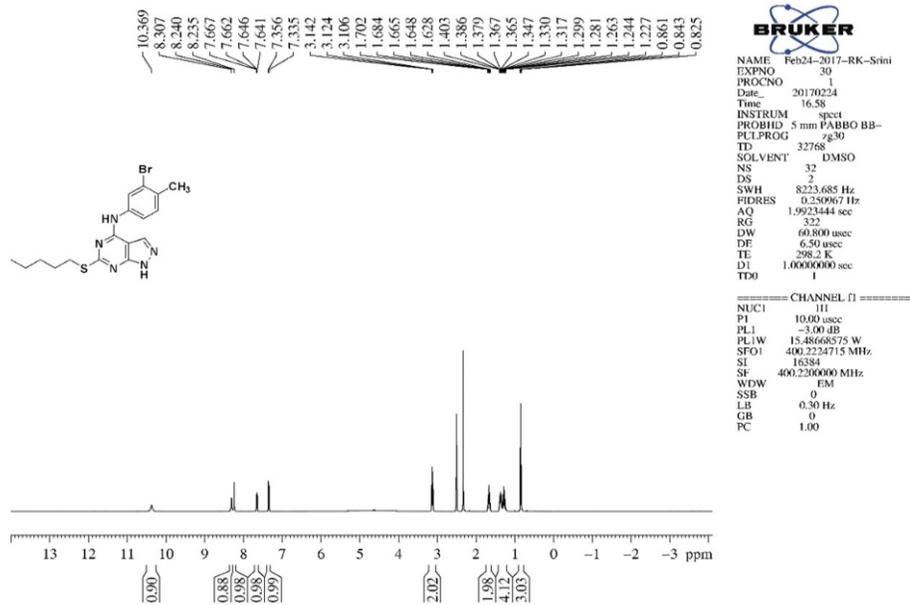
1H NMR Spectrum of Compound 41
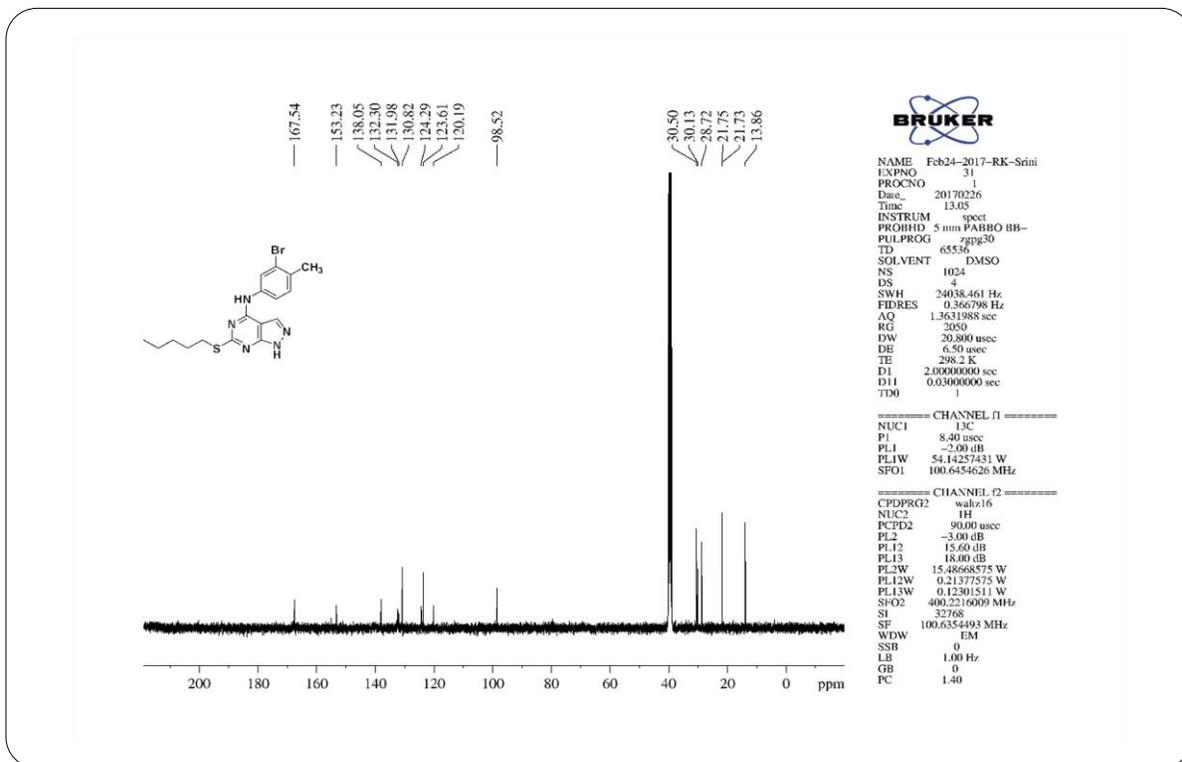
¹³C NMR Spectrum of Compound 41
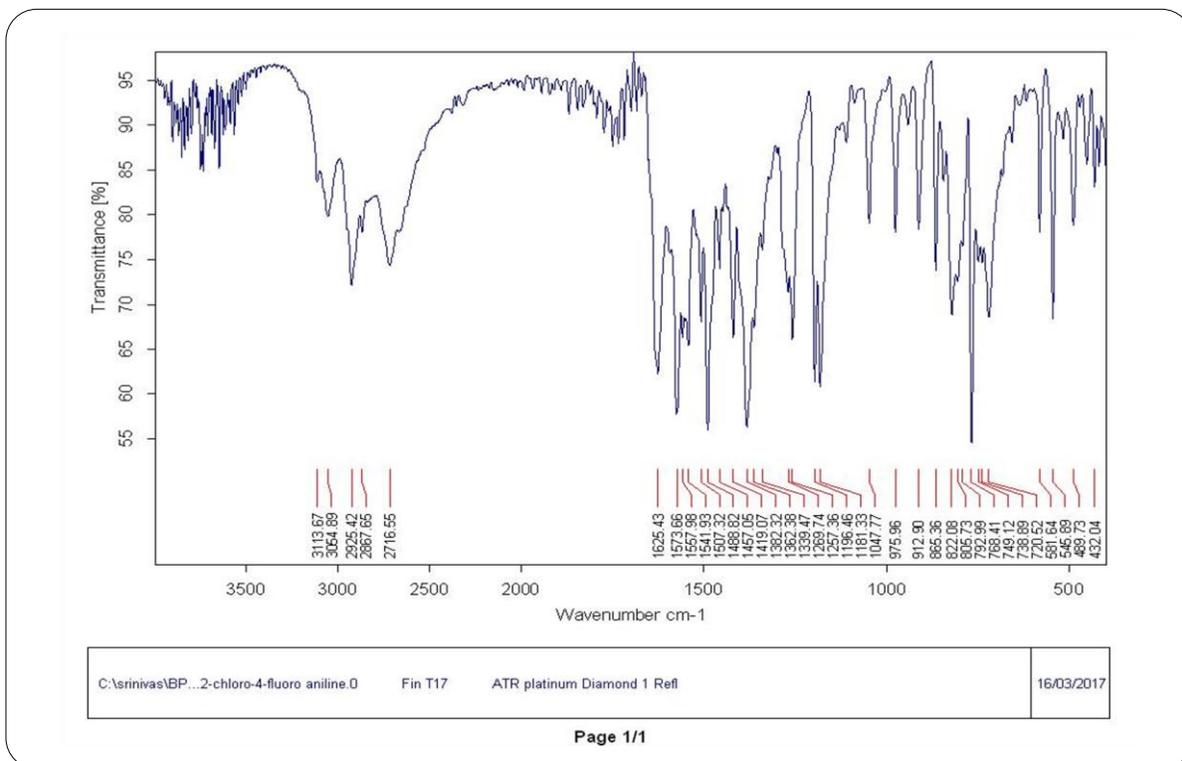
IR Spectrum of Compound 42
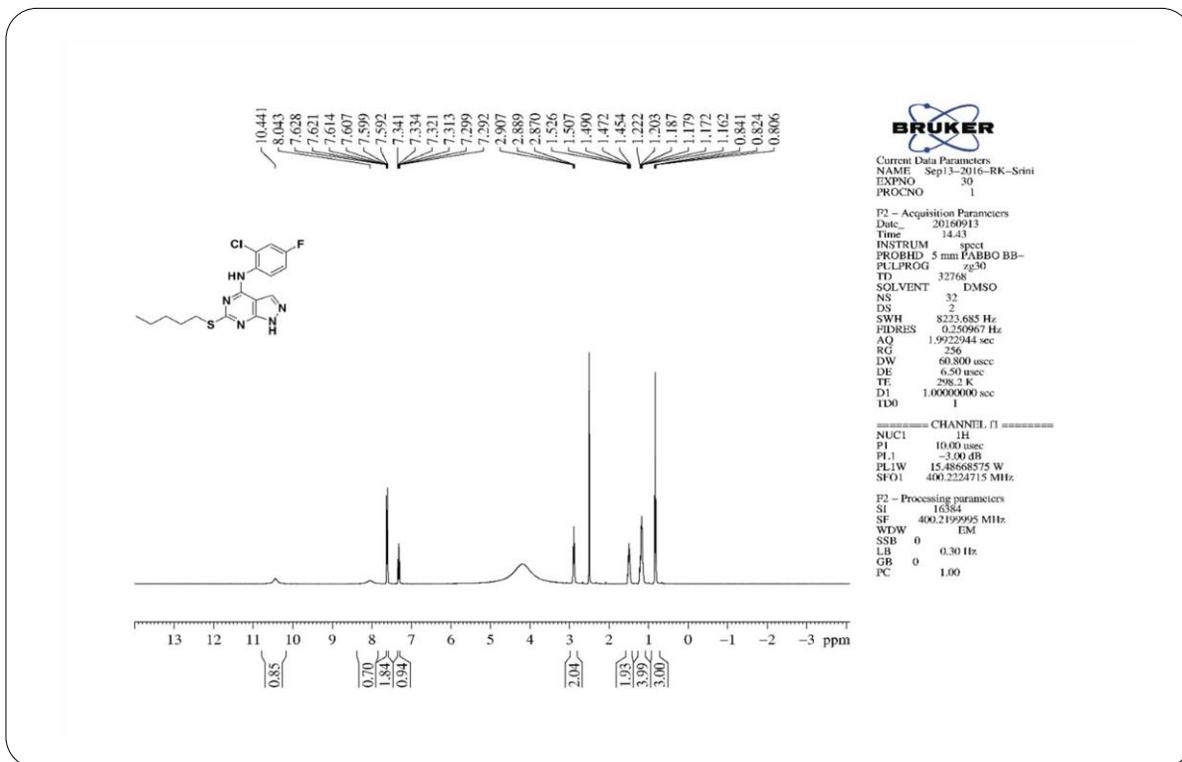
¹H NMR Spectrum of Compound 42
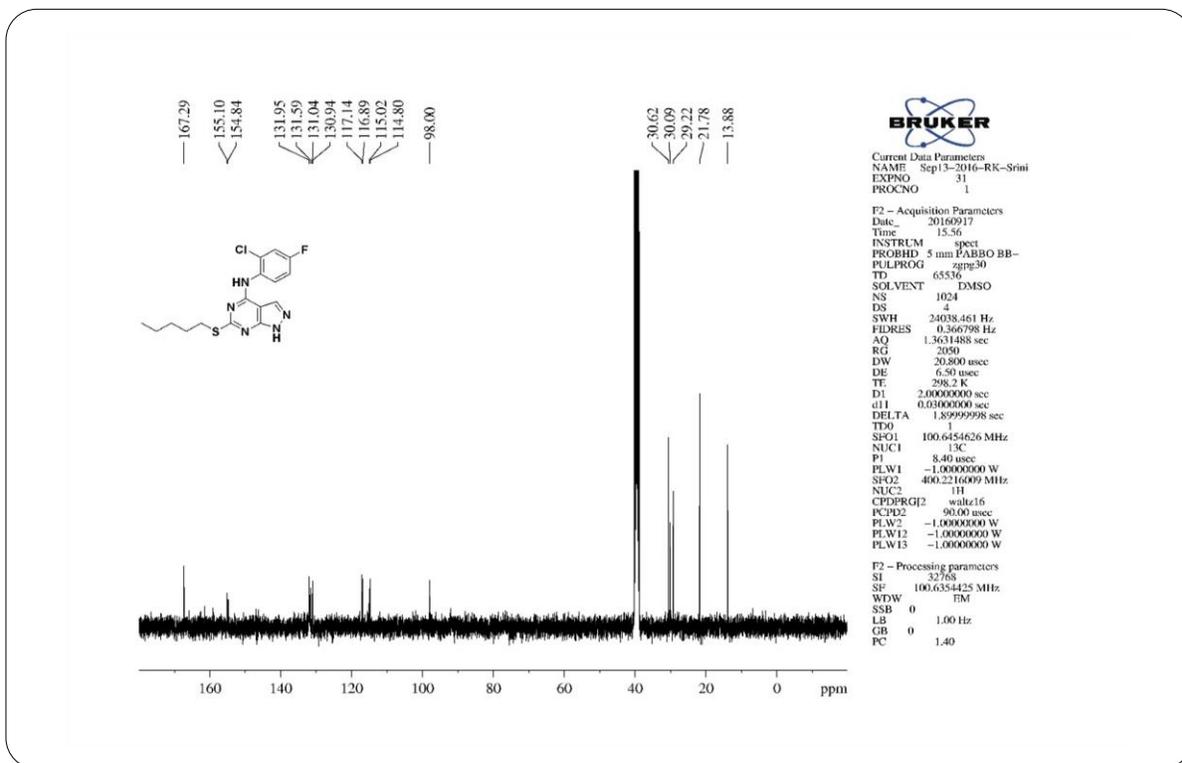
¹³C NMR Spectrum of Compound 42
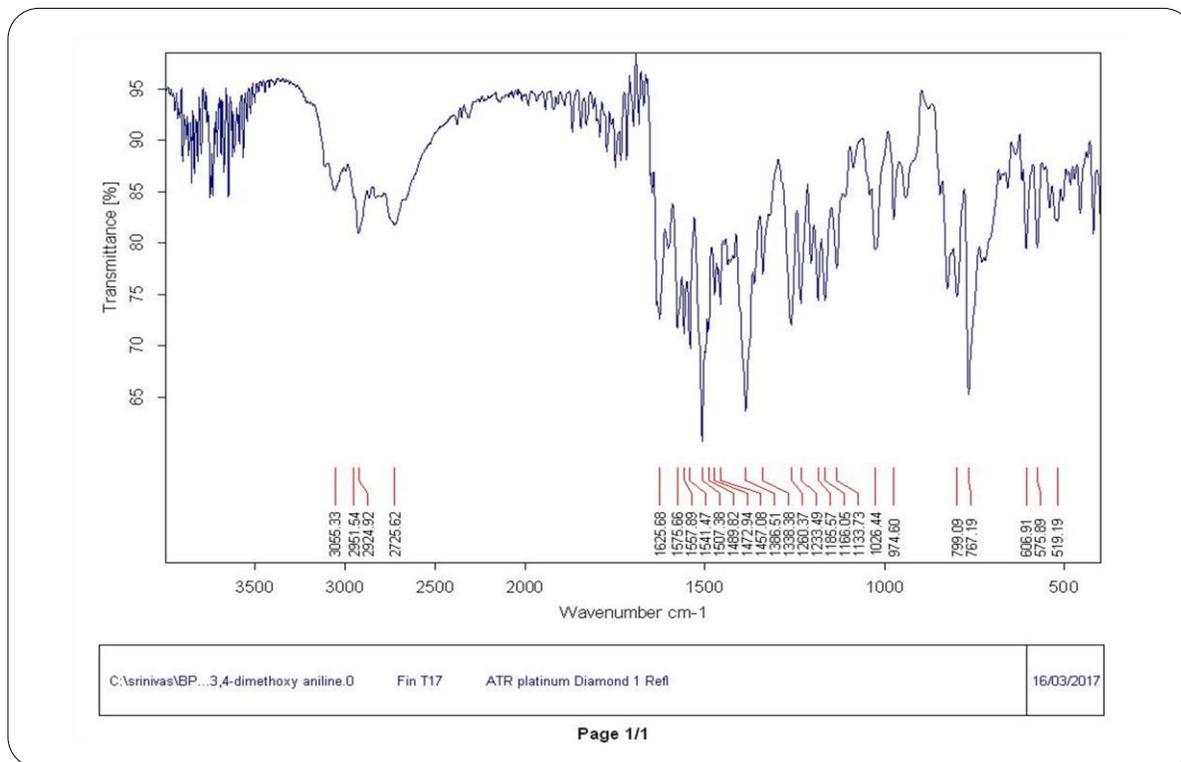
IR Spectrum of Compound 43
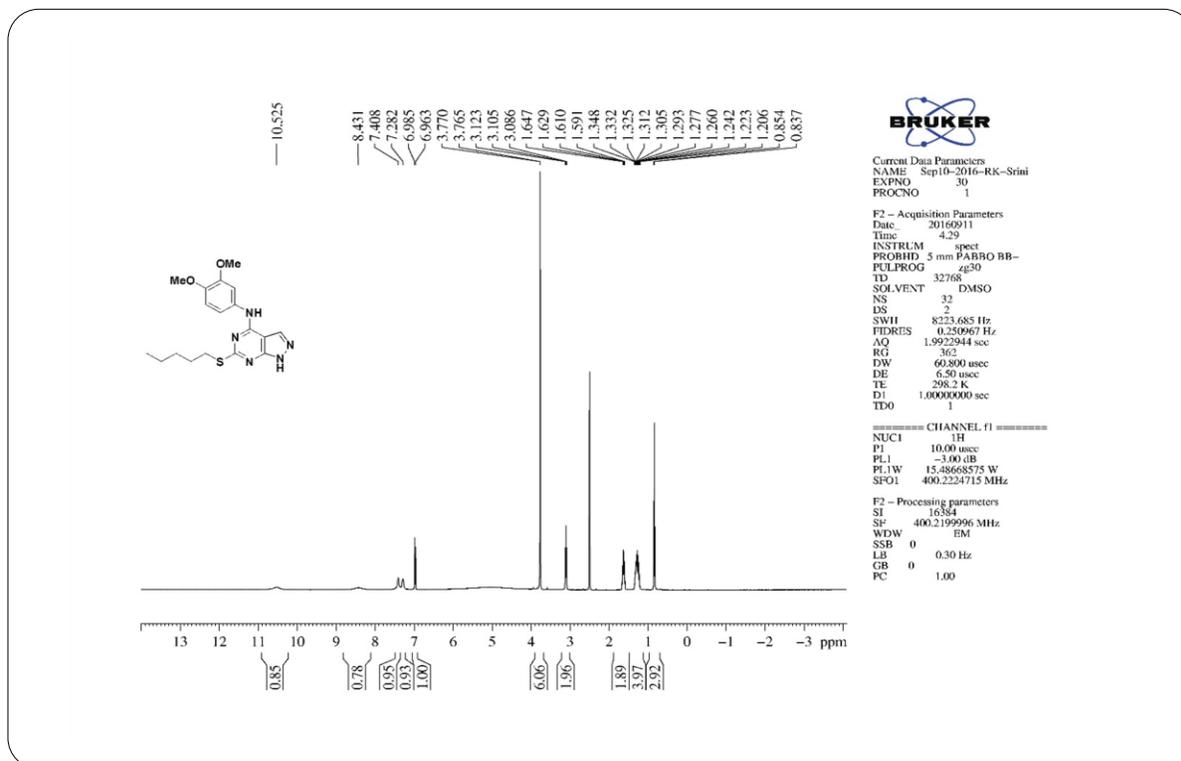
¹H NMR Spectrum of Compound 43
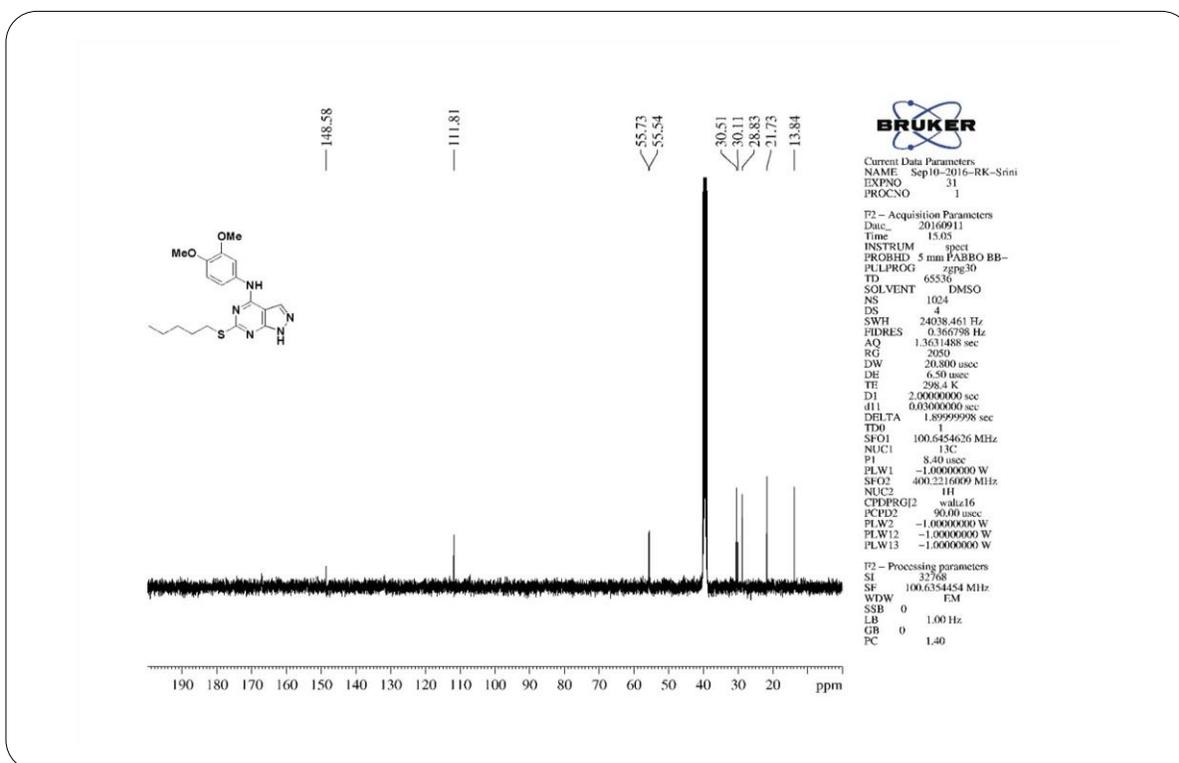
¹³C NMR Spectrum of Compound 43

HRMS Spectrum of Compound 43

APPENDIX – II

SUPPLIMENTARY INFORMATION

CHAPTER 5

Synthesis of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine analogues: molecular docking, anticancer evaluation as cyclin dependent kinase 2 (CDK2) inhibitors

Department of Pharmaceutical Chemistry, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa.
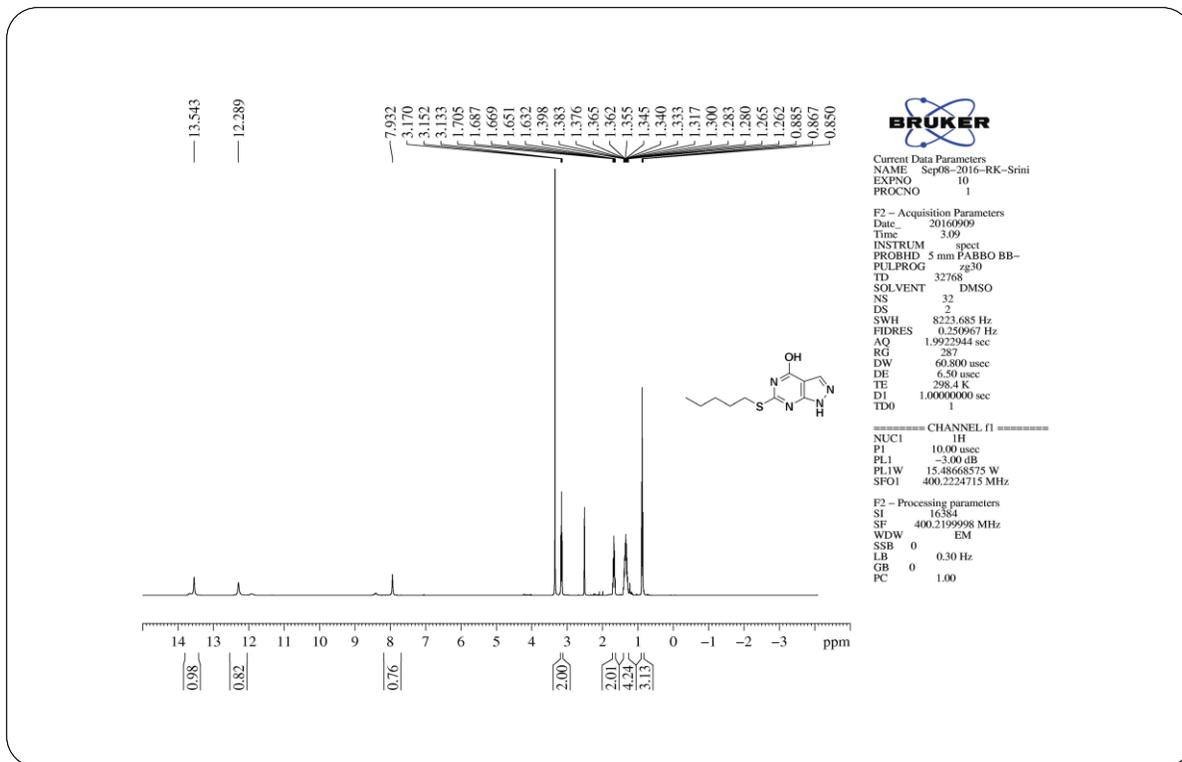
^{1}H NMR Spectrum of Compound 2
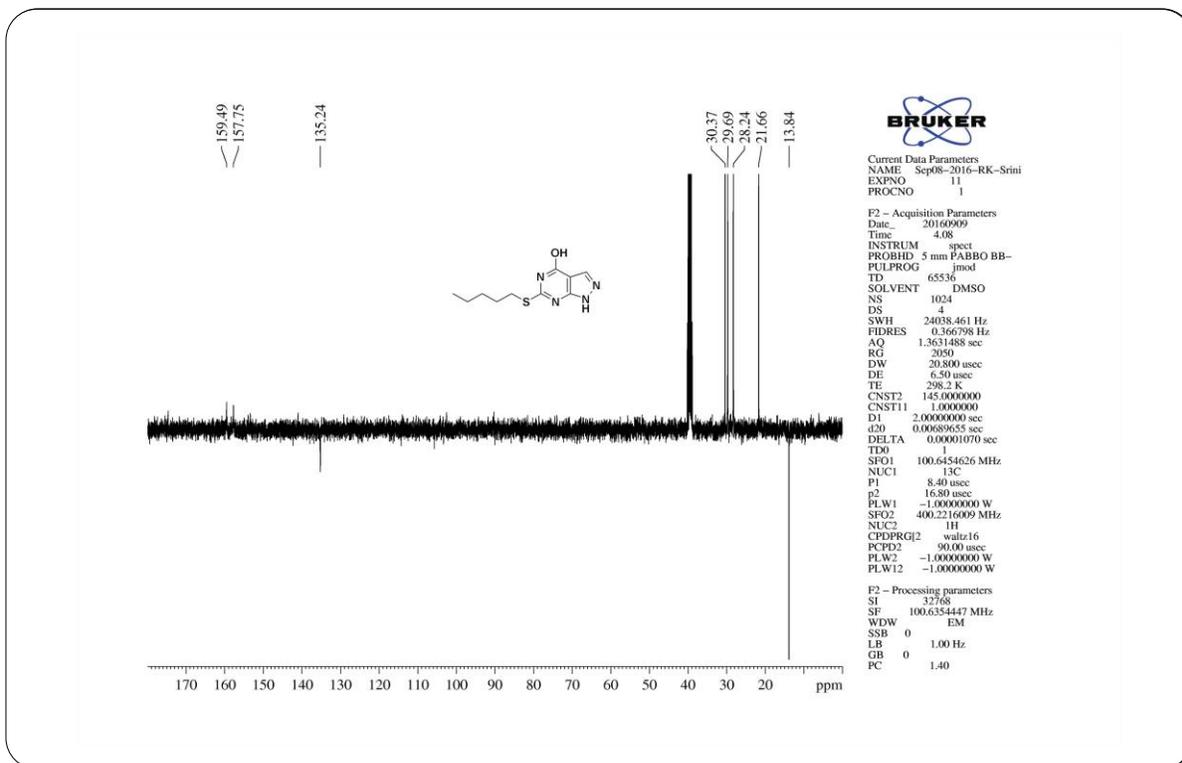
**^{13}C NMR Spectrum of Compound 2**
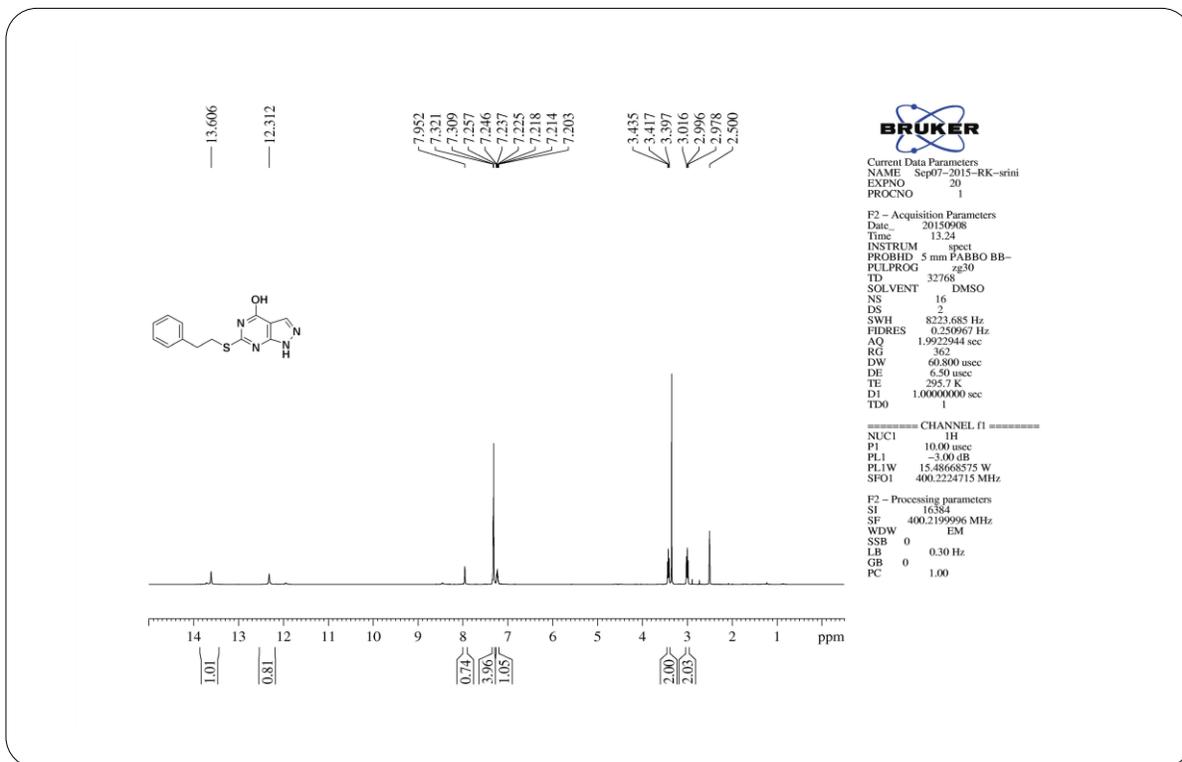
¹H NMR Spectrum of Compound 3
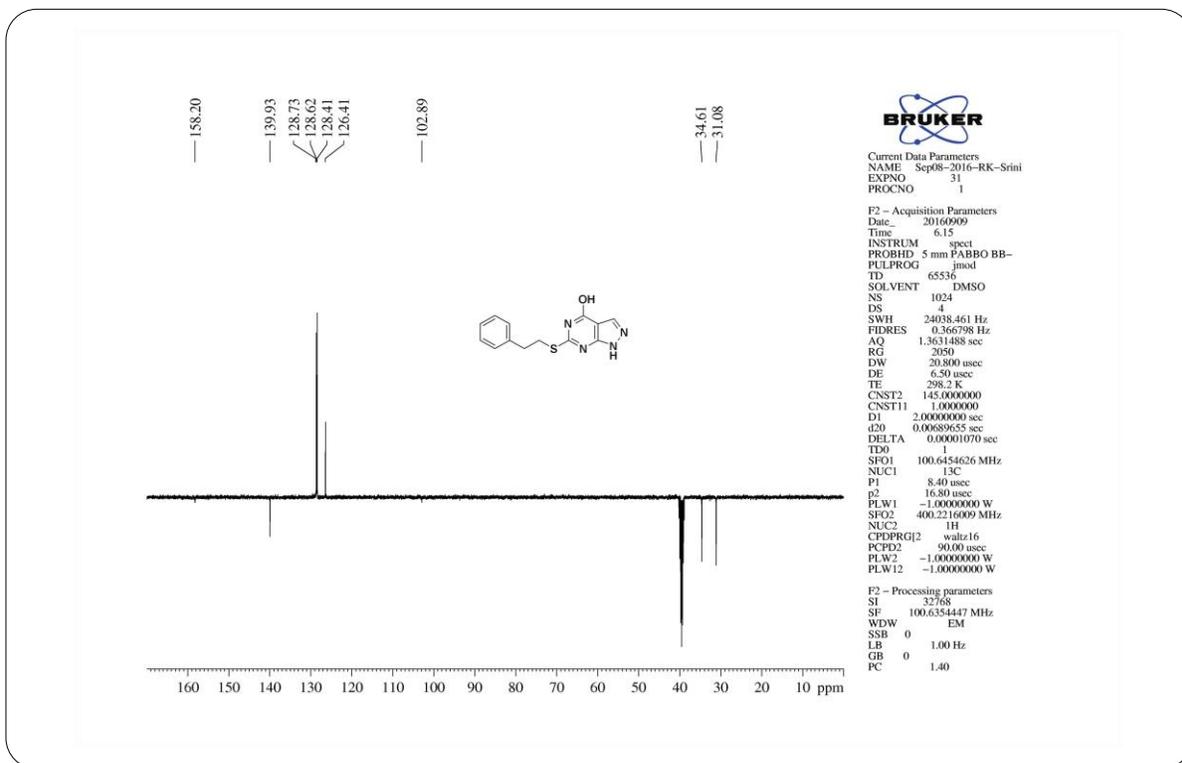
¹³C NMR Spectrum of Compound 3
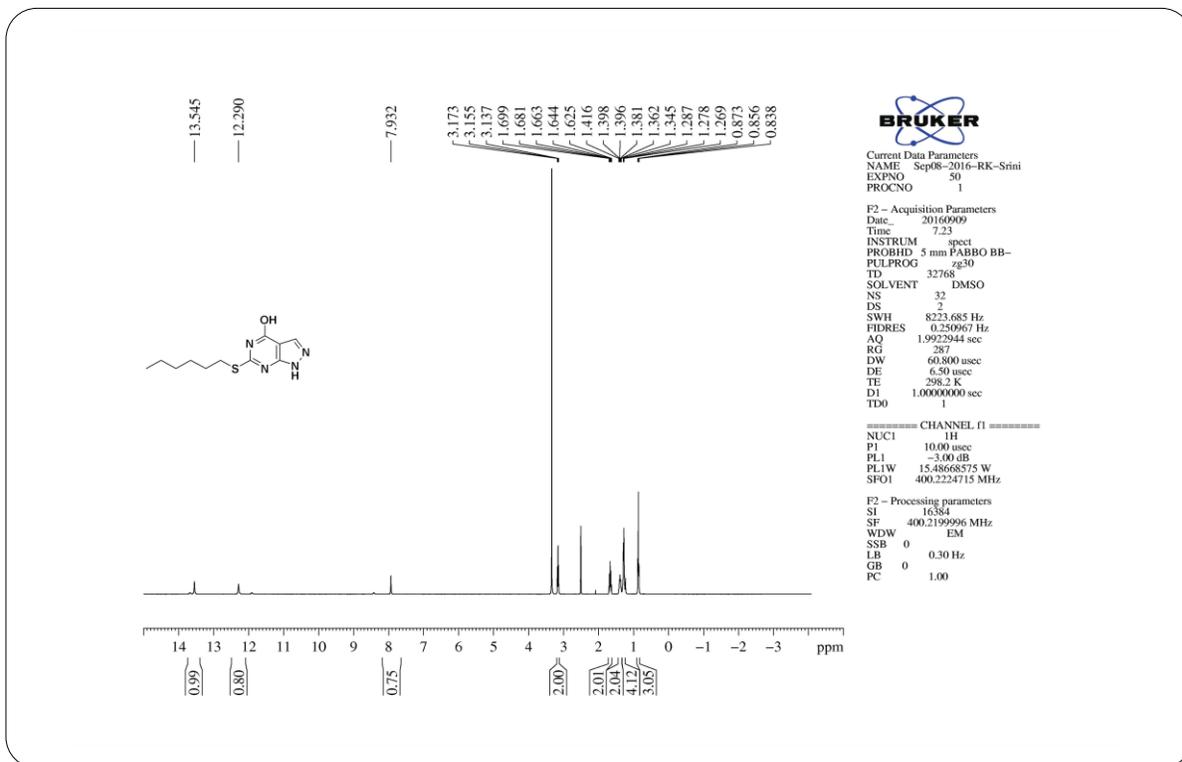
¹H NMR Spectrum of Compound 4
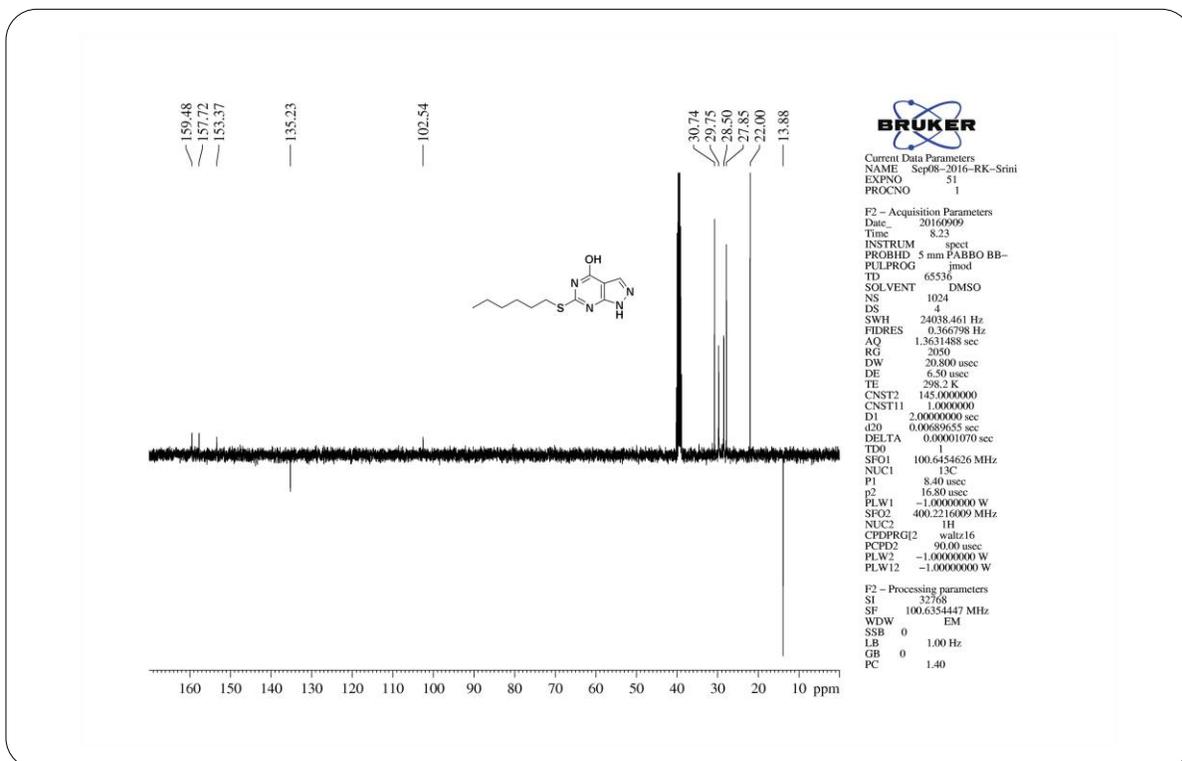
¹³C MNR Spectrum of Compound 4
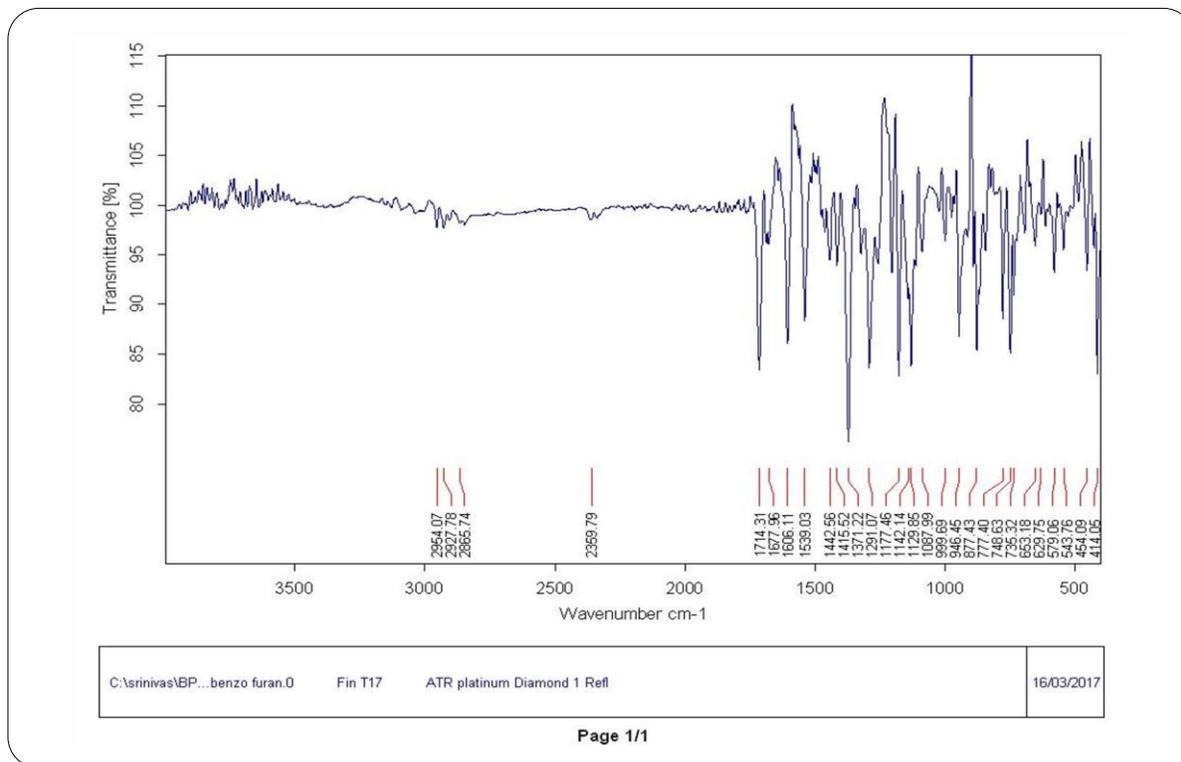
IR Spectrum of Compound 5a

¹H NMR Spectrum of Compound 5a

¹³C NMR Spectrum of Compound 5a

Elemental Composition Report   **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM   /   DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
41 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 15-20   H: 15-20   N: 0-5   O: 0-5   S: 0-1
BP-5 53 (1.754) Cm (1:61)
TOF MS ES-

Minimum:			-1.5			
Maximum:	5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm) Formula
381.1027	381.1021	0.6	1.6	13.5	620.9	0.0 C19 H17 N4 O3 S

HRMS Spectrum of Compound 5a
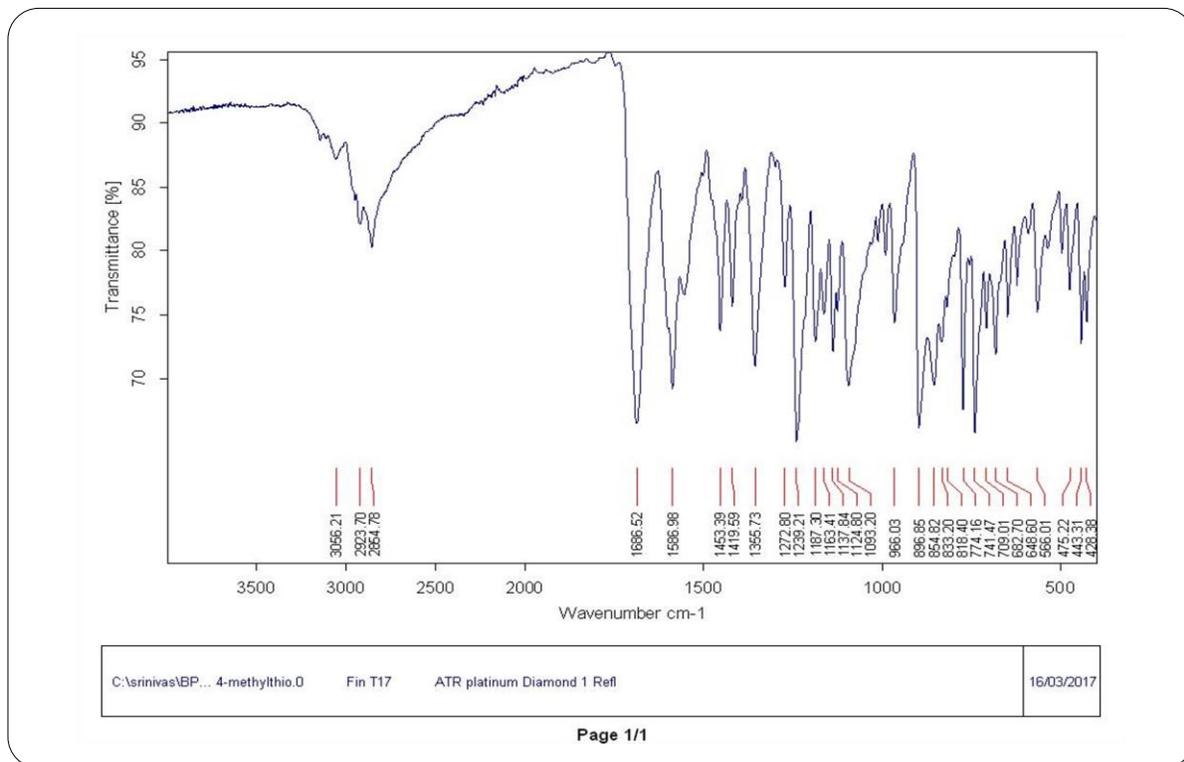
IR Spectrum of Compound 5b
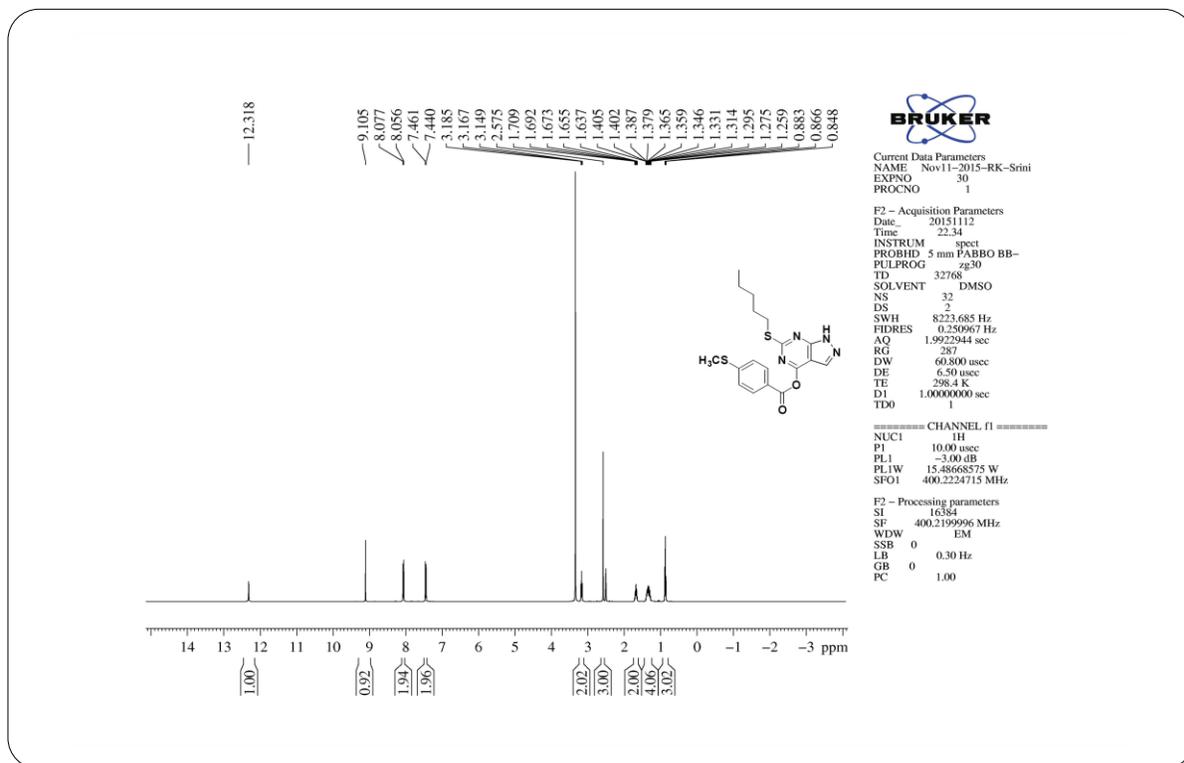
1H NMR Spectrum of Compound 5b
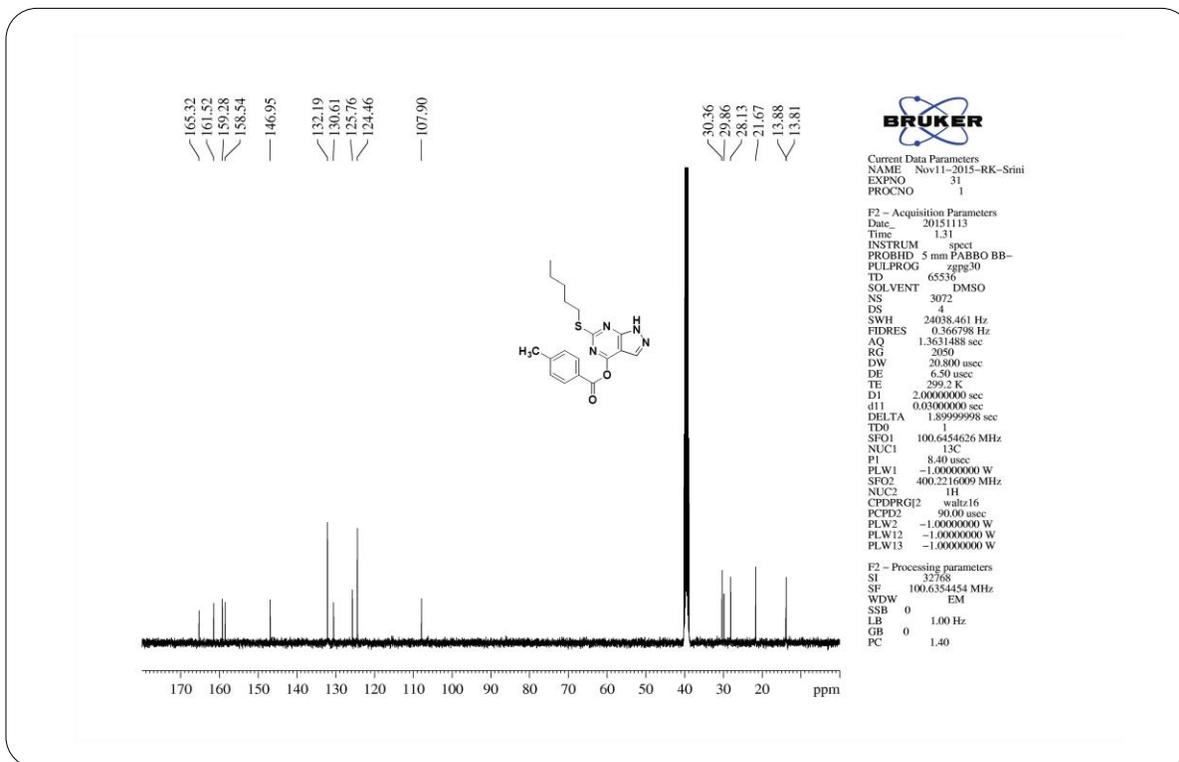
^{13}C NMR Spectrum of Compound 5b
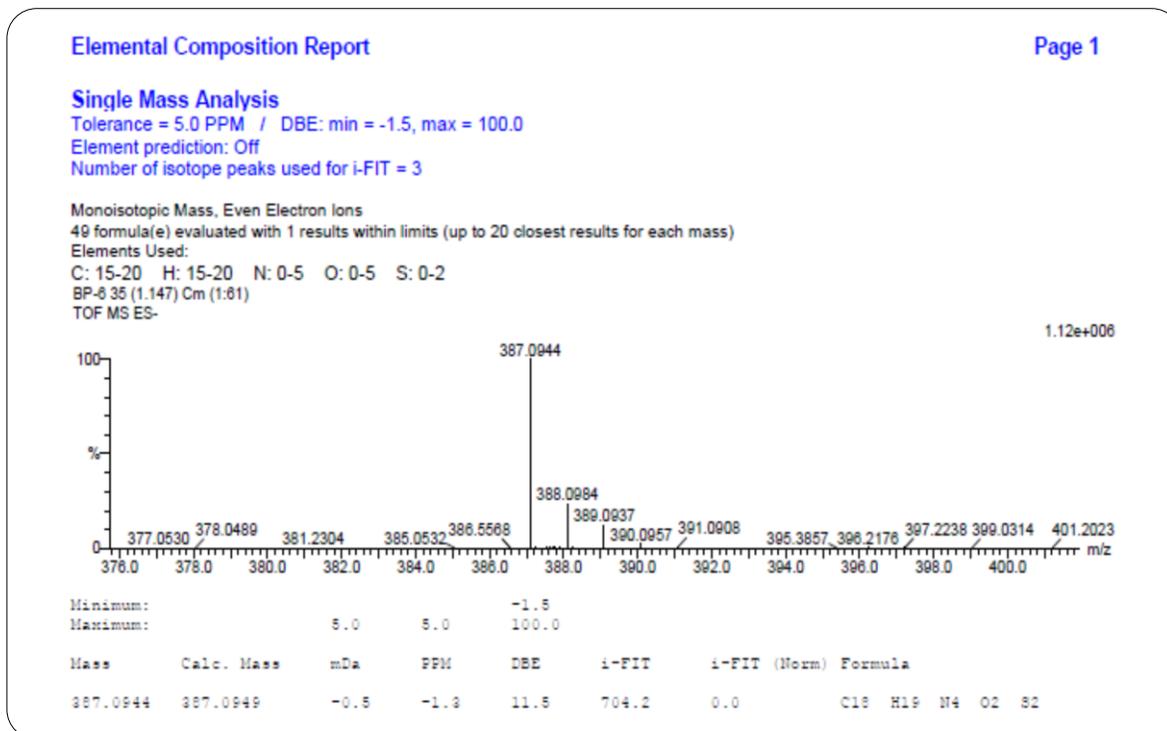
HRMS Spectrum of Compound 5b
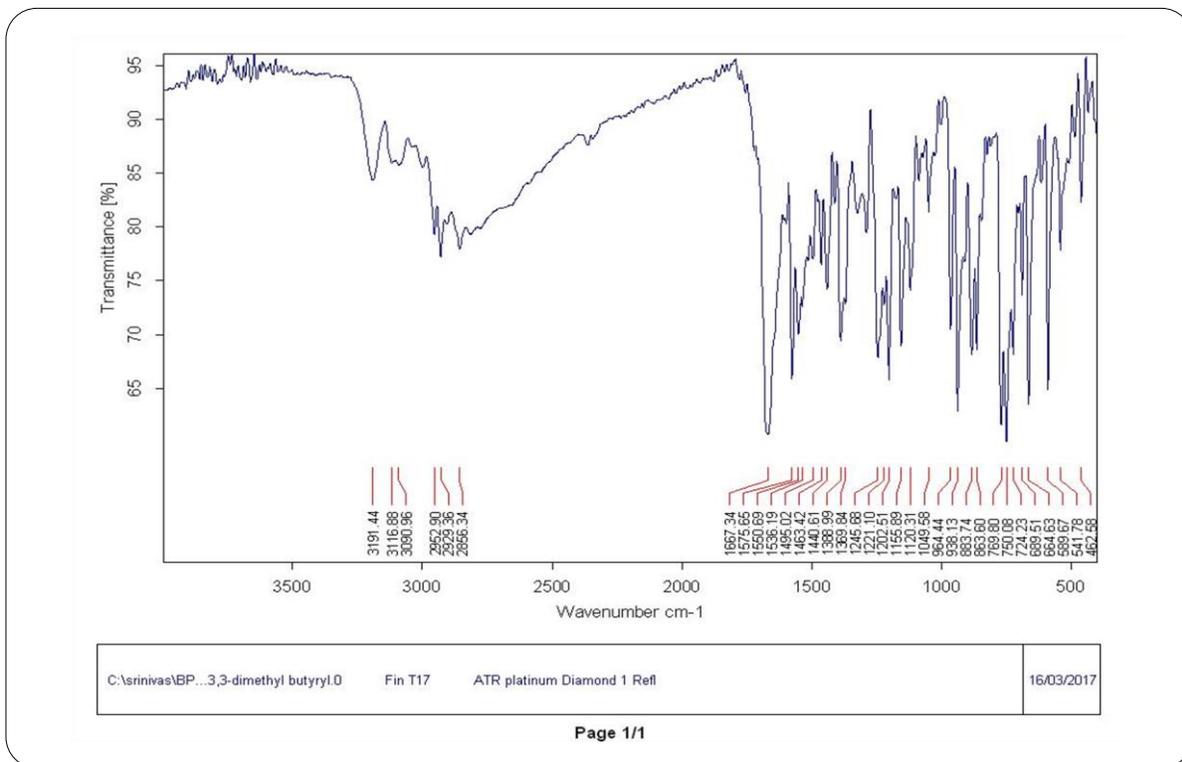
IR Spectrum of Compound 5c
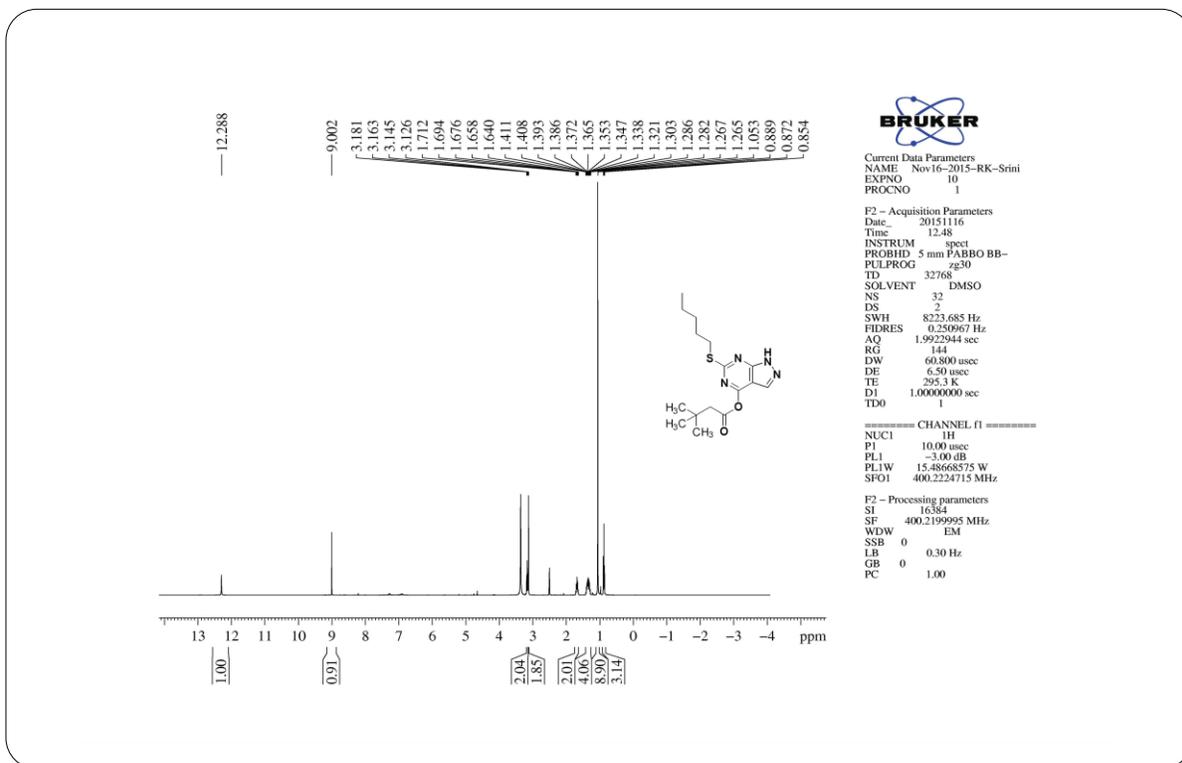

¹H NMR Spectrum of Compound 5c
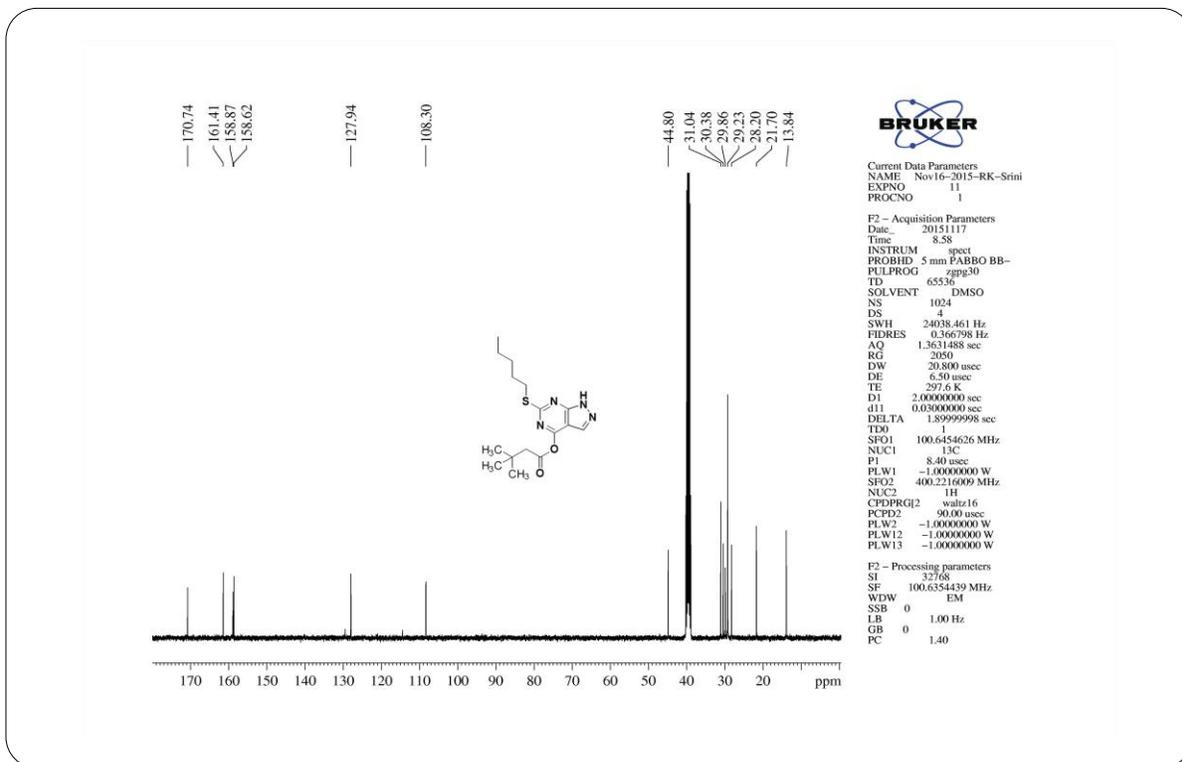
¹³C NMR Spectrum of Compound 5c
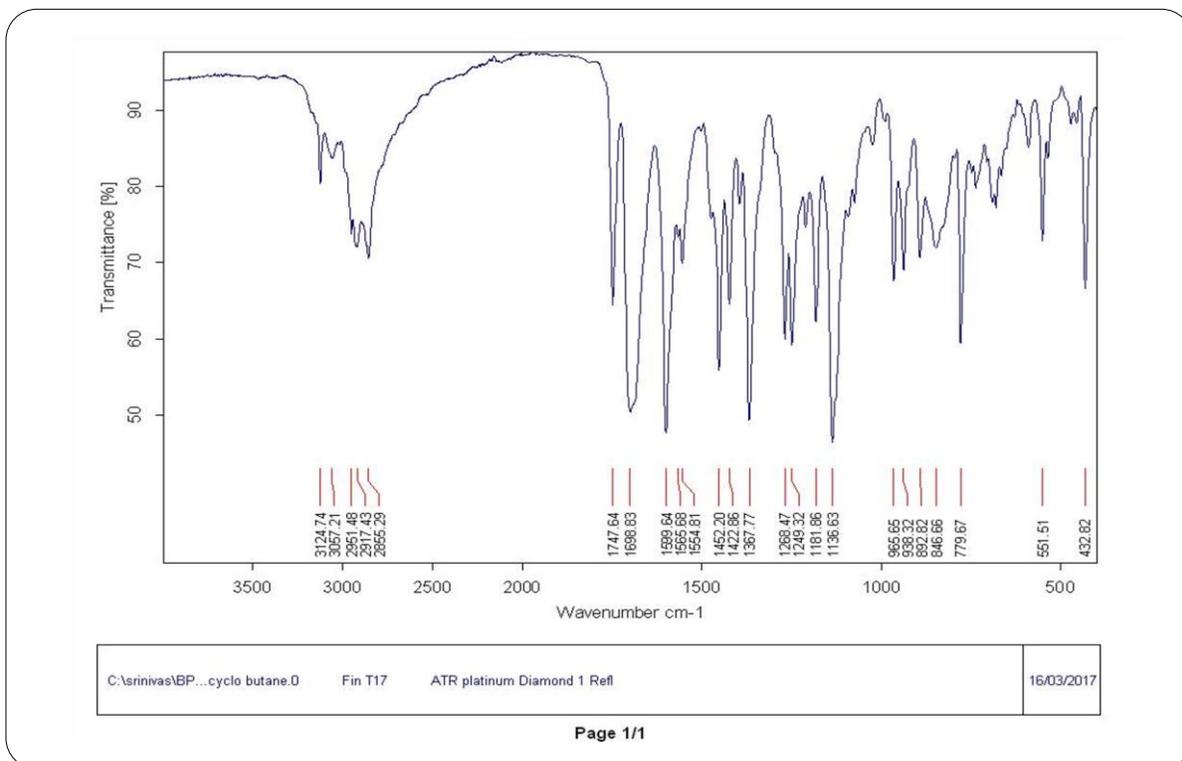
IR Spectrum of Compound 5d
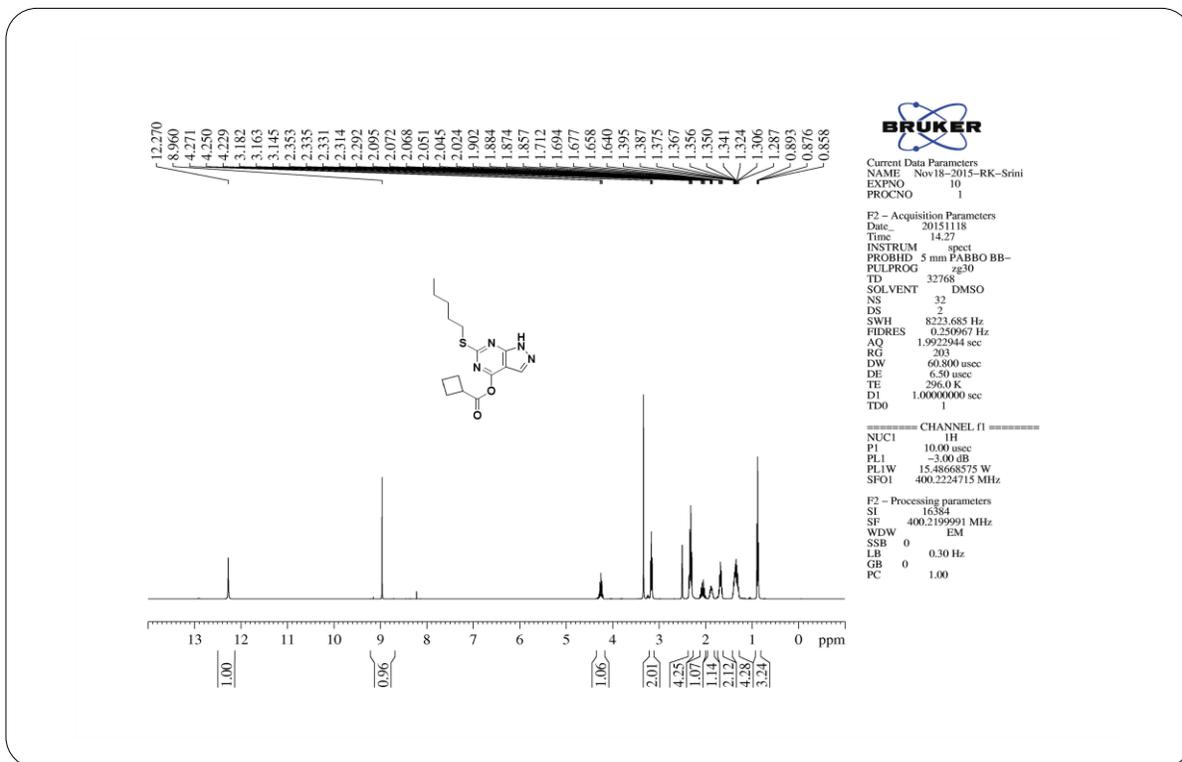
¹H NMR Spectrum of Compound 5d

¹³C NMR Spectrum of Compound 5d

C:\srinivas\BP...2-furoyl chloride.0   Fin T17   ATR platinum Diamond 1 Refl   16/03/2017

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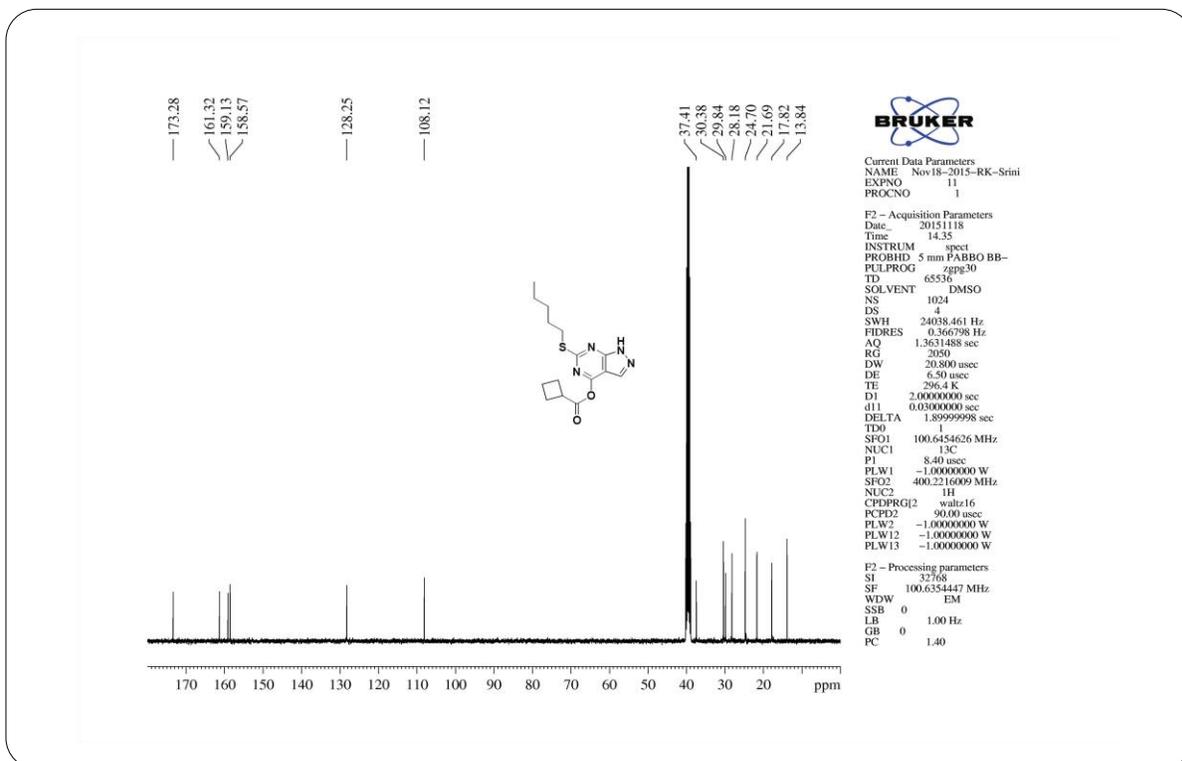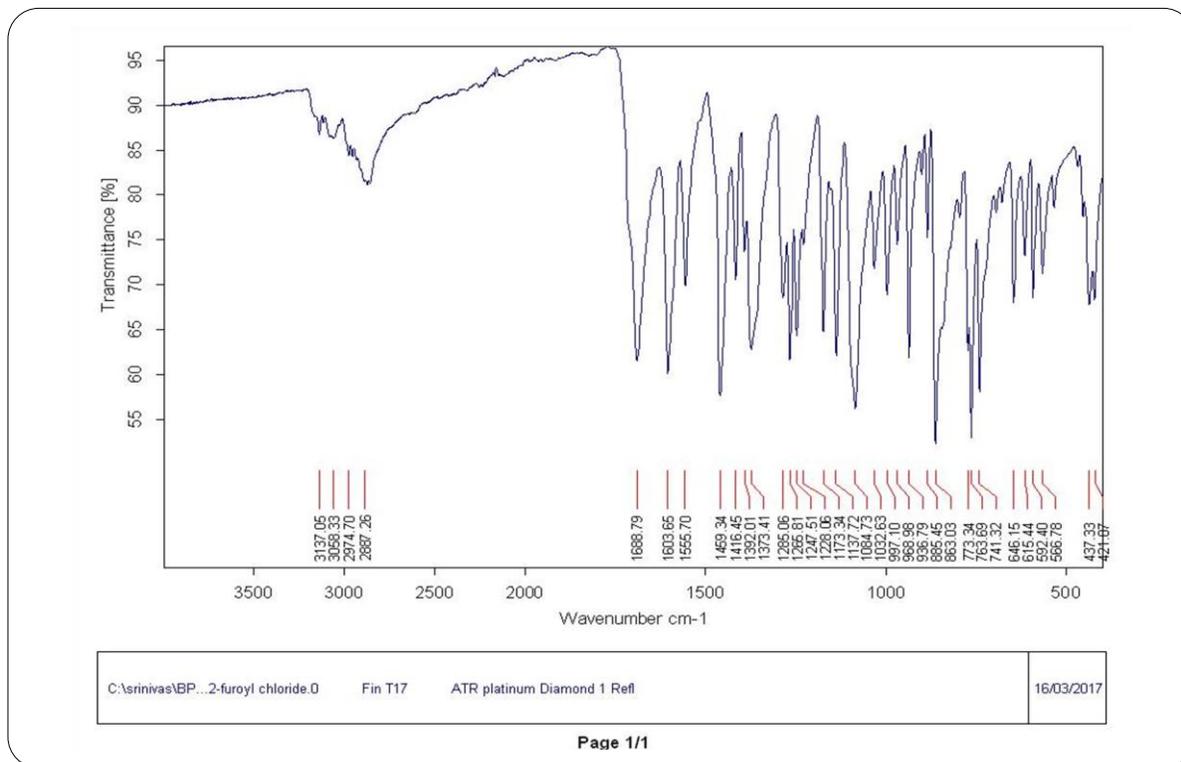
IR Spectrum of Compound 5e
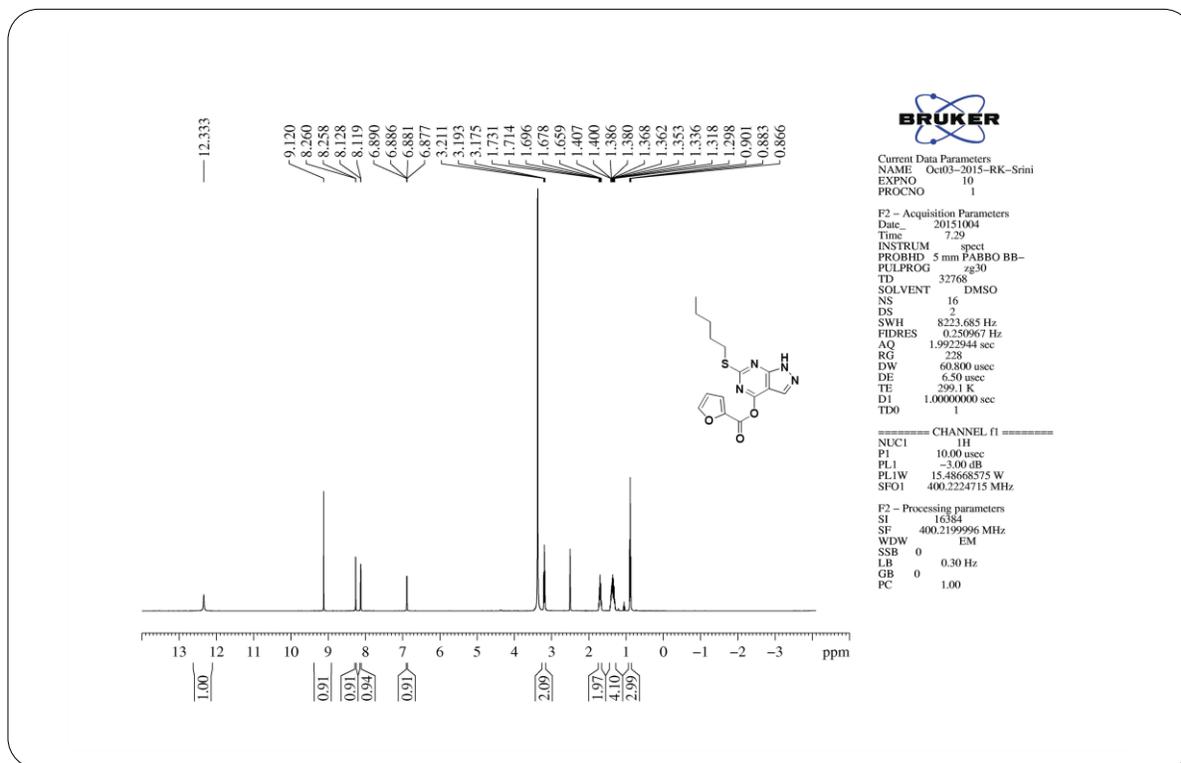
¹H NMR Spectrum of Compound 5e
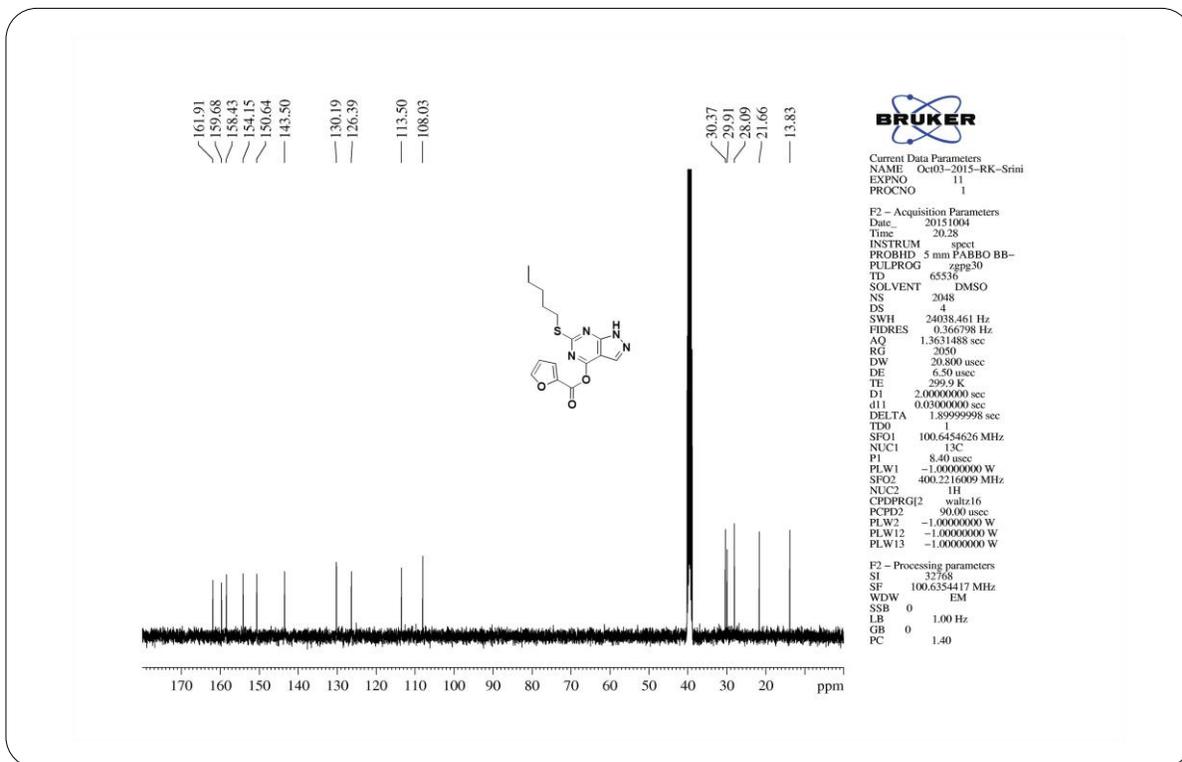
13C NMR Spectrum of Compound 5e
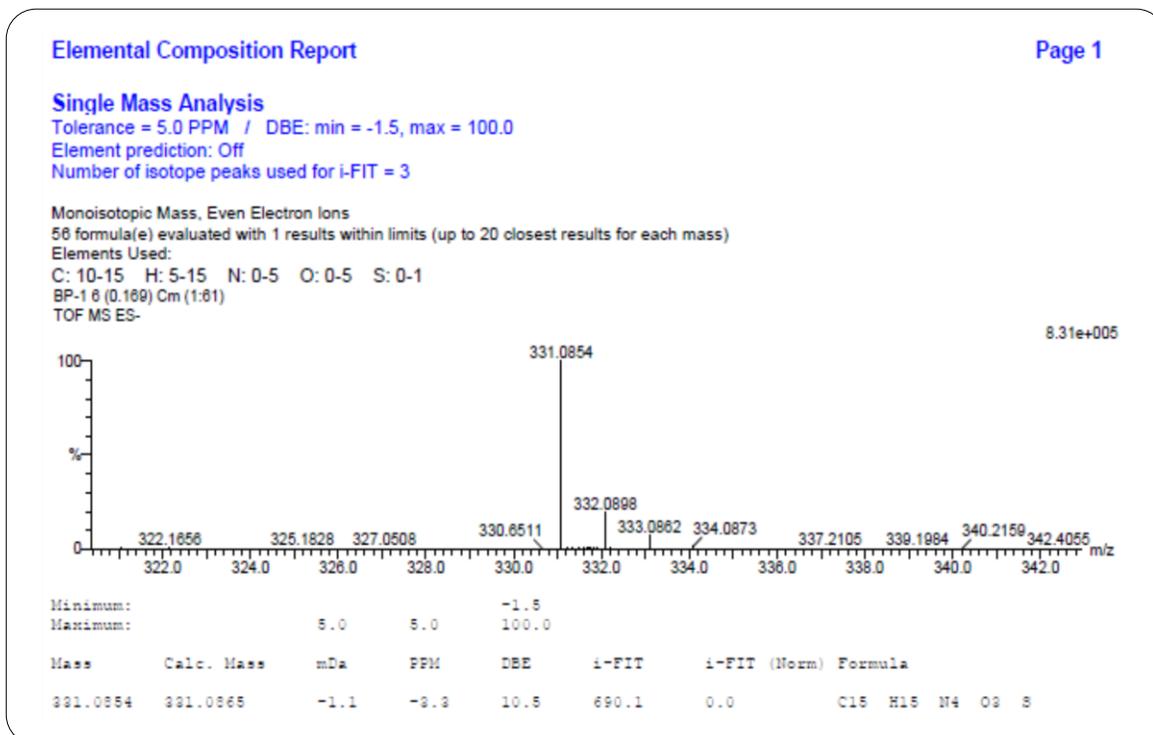
HRMS Spectrum of Compound 5e
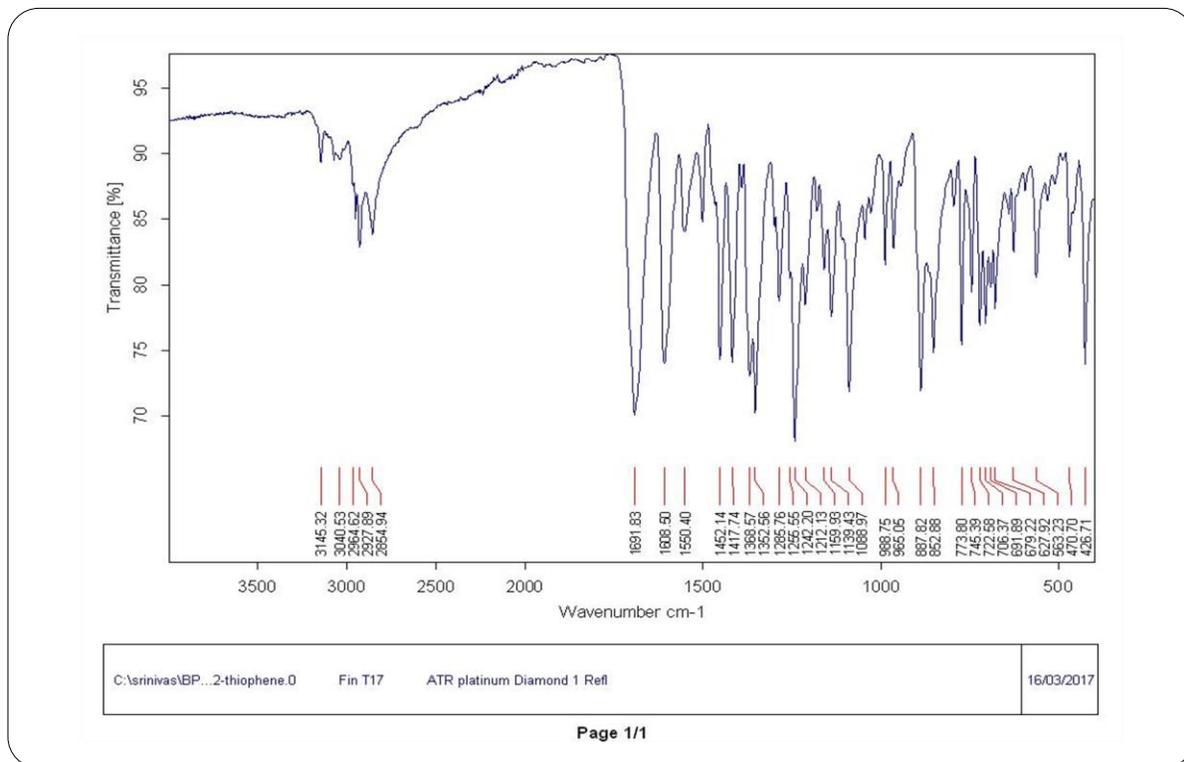
IR Spectrum of Compound 5f

¹H NMR Spectrum of Compound 5f

161.97
159.33
158.39
158.35
140.13
139.59
130.95
129.92
128.25
108.37
30.33
29.89
28.00
21.62
13.80

BRUKER

Current Data Parameters
NAME   Oct26–2015–RK–Srini
EXPNO   11
PROCNO   1

F2 – Acquisition Parameters
Date_   20151028
Time   21.48
INSTRUM   spect
PROBHD   5 mm PABBO BB–
PULPROG   zgpg30
TD   65536
SOLVENT   DMSO
NS   2048
DS   4
SWH   24038.461 Hz
FIDRES   0.366798 Hz
AQ   1.3631488 sec
RG   2050
DW   20.800 usec
DE   6.50 usec
TE   298.5 K
D1   2.00000000 sec
d11   0.03000000 sec
DELTA   1.89999998 sec
TD0   1
SFO1   100.6454626 MHz
NUC1   13C
P1   8.40 usec
PLW1   –1.00000000 W
SFO2   400.2216009 MHz
NUC2   1H
CPDPRG[2   waltz16
PCPD2   90.00 usec
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PLW12   –1.00000000 W
PLW13   –1.00000000 W

F2 – Processing parameters
SI   32768
SF   100.6354462 MHz
WDW   EM
SSB   0
LB   1.00 Hz
GB   0
PC   1.40

¹³C NMR Spectrum of Compound 5f

Elemental Composition Report   Page 1

Single Mass Analysis
Tolerance = 5.0 PPM   /   DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
71 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 10-15   H: 15-20   N: 0-5   O: 0-5   S: 0-2
BP-2 2 (0.034) Cm (1:61)
TOF MS ES-   9.56e+005

347.0634

348.0674 349.0624

339.2009 340.1971   343.1404   346.6589   351.0598   353.0925 354.0948   357.2039 359.8754 361.0818

Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
347.0634	347.0636	-0.2	-0.6	10.5	654.9	0.0	C15 H15 N4 O2 S2

HRMS Spectrum of Compound 5f

IR Spectrum of Compound 5g
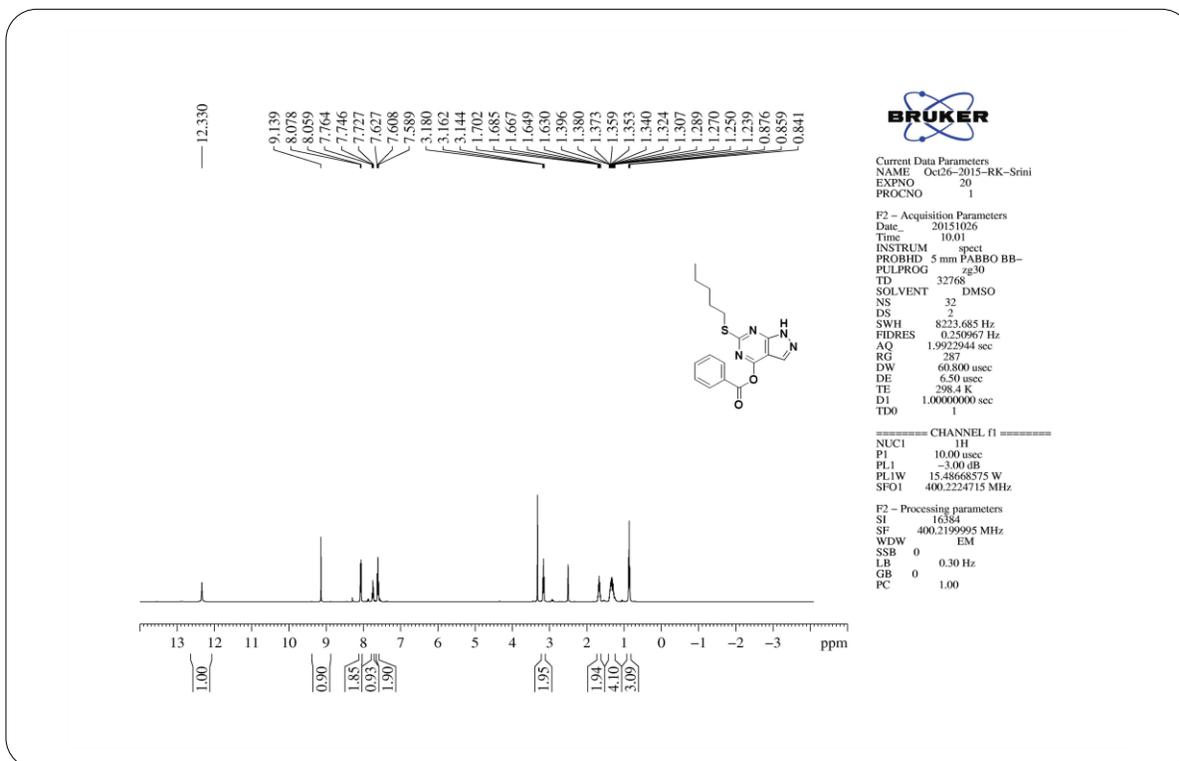
¹H NMR Spectrum of Compound 5g
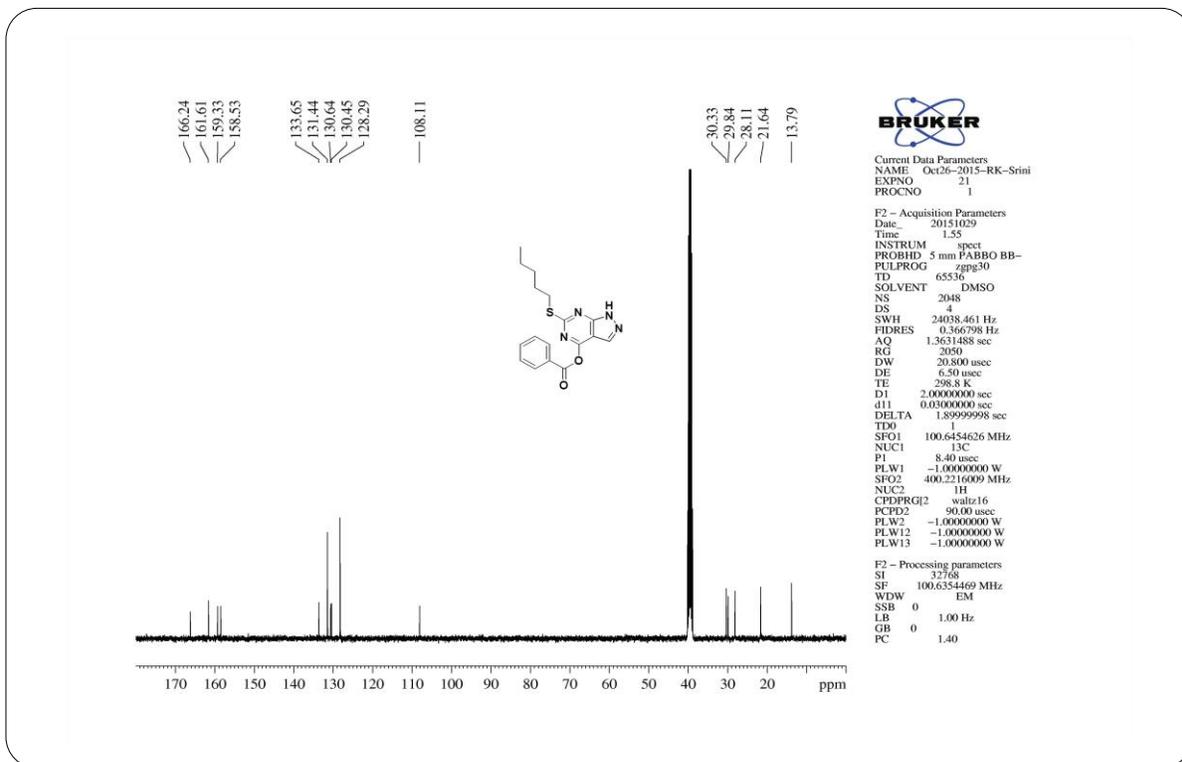
¹³C NMR Spectrum of Compound 5g
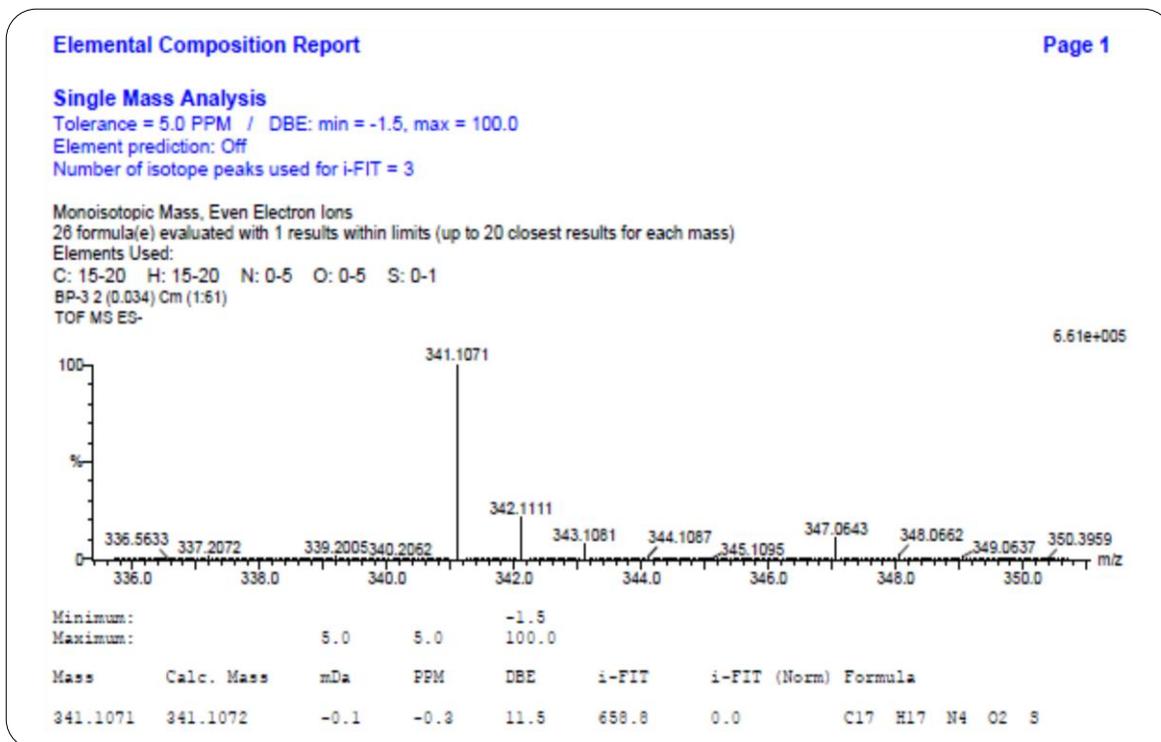
HRMS Spectrum of Compound 5g
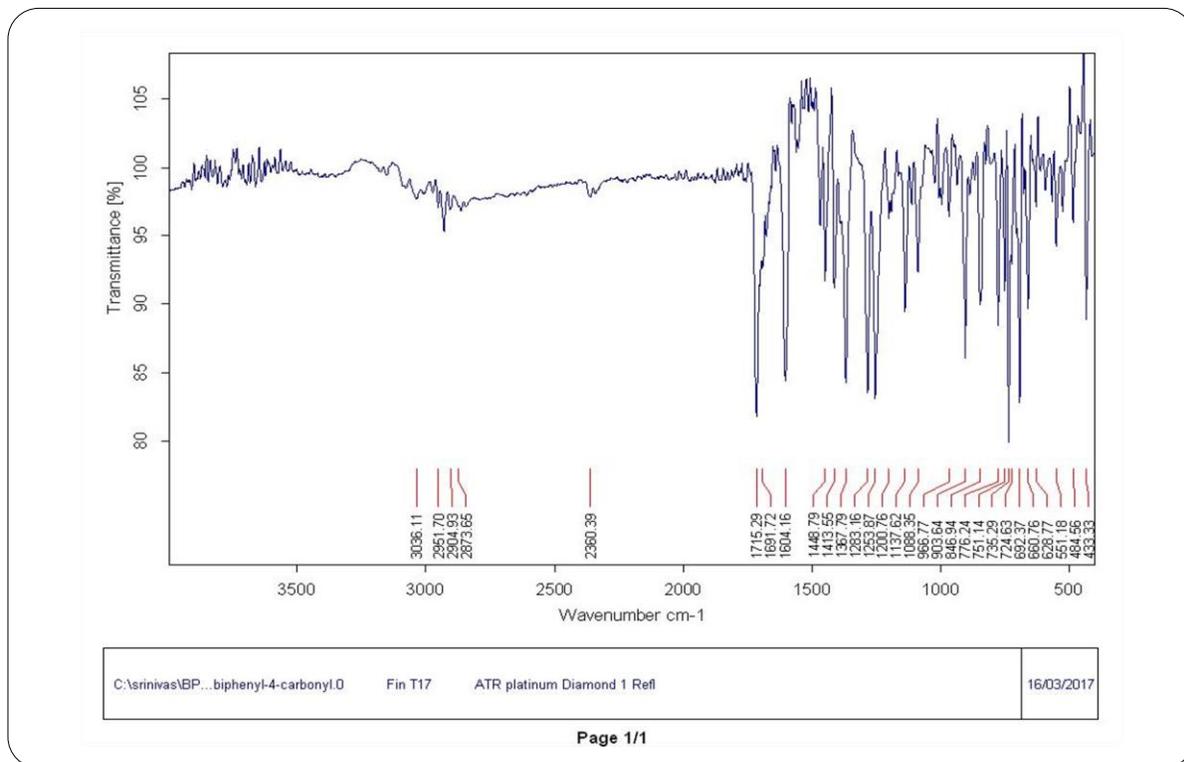
IR Spectrum of Compound 5h
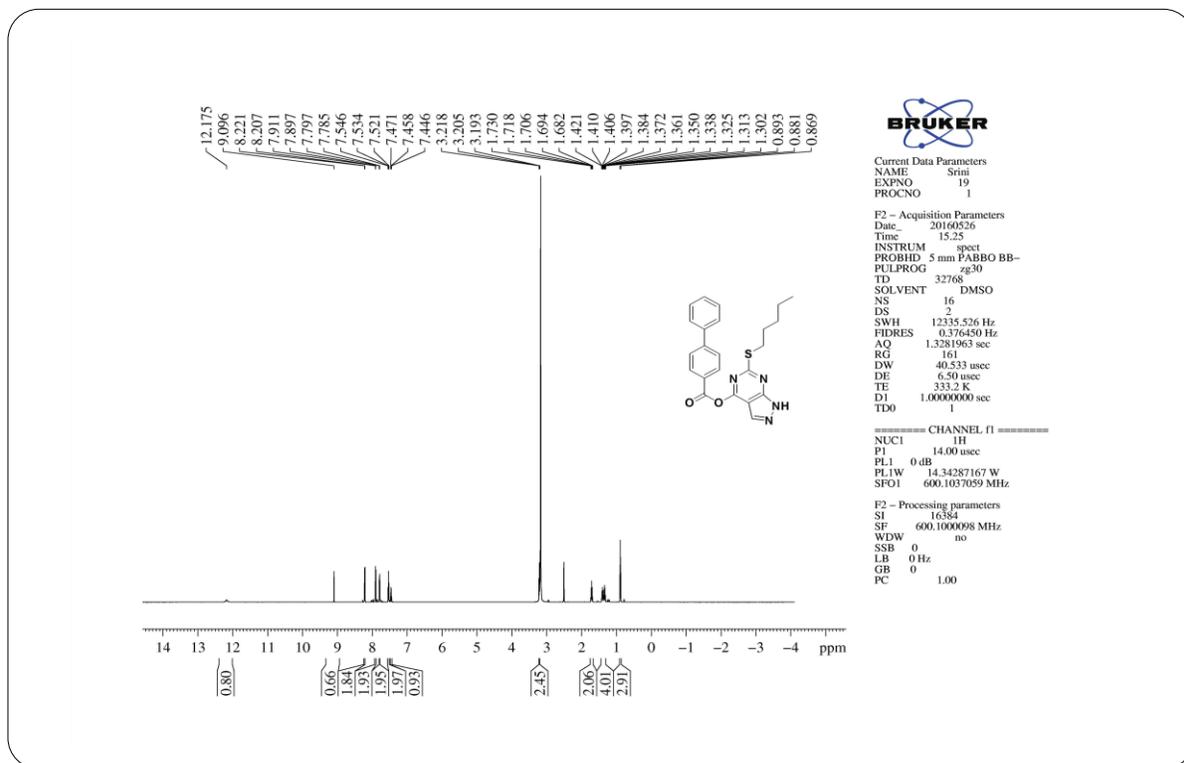
¹H NMR Spectrum of Compound 5h
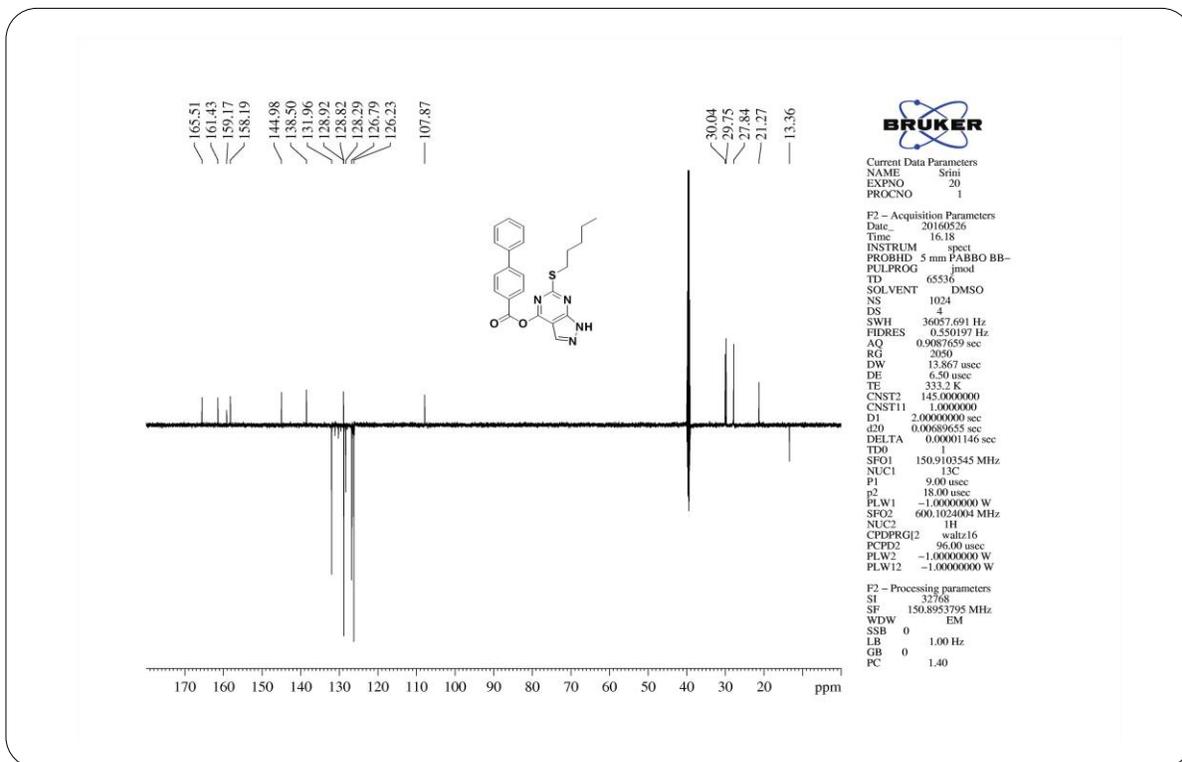
13C NMR Spectrum of Compound 5h
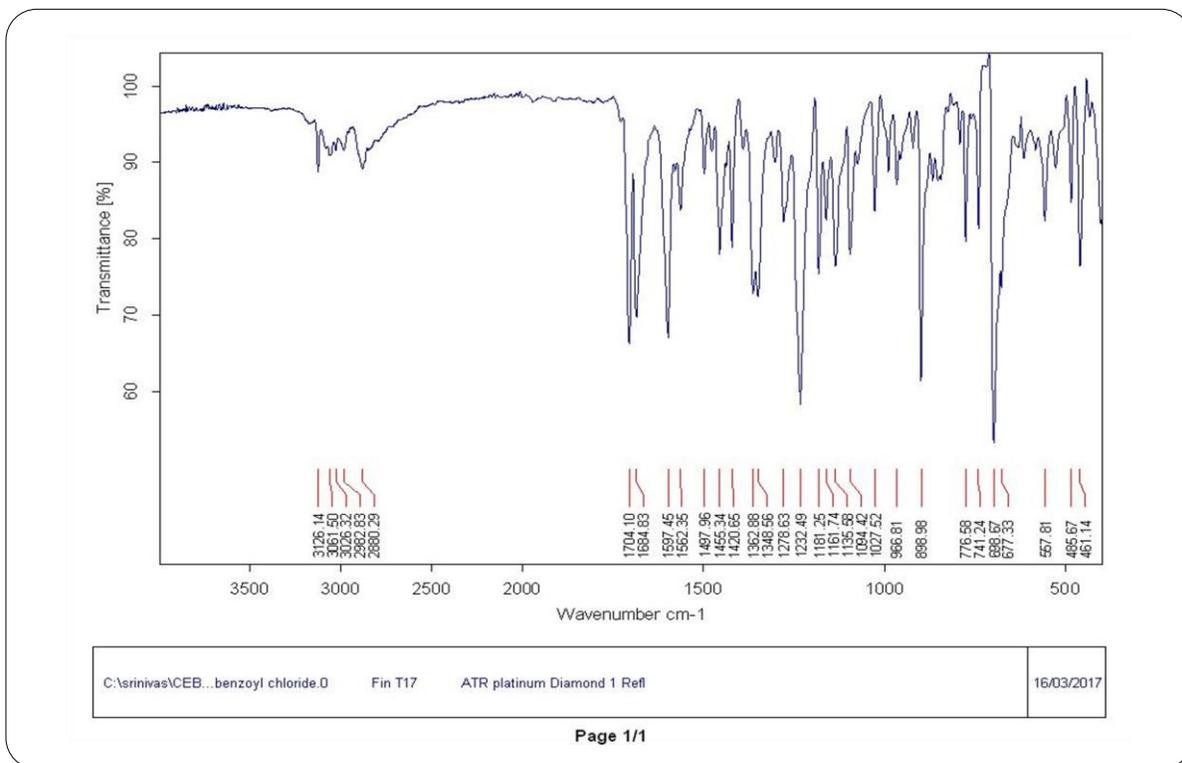
IR Spectrum of Compound 6a
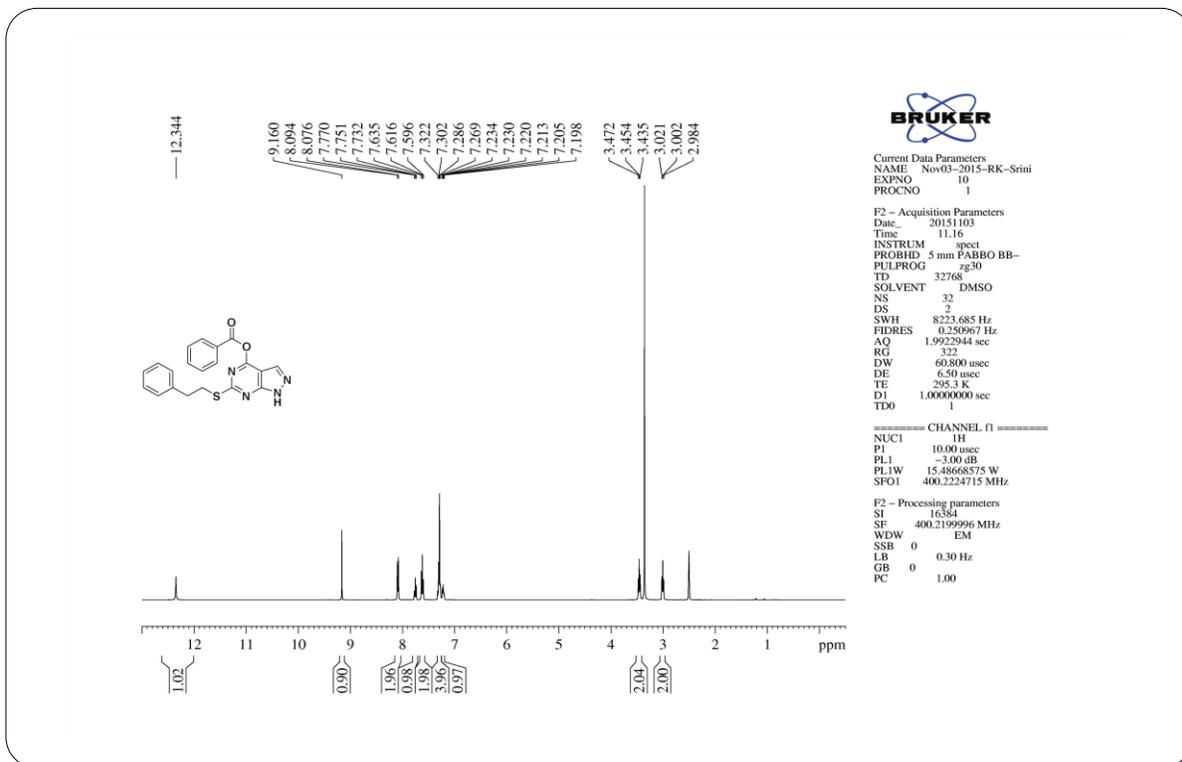
¹H NMR Spectrum of Compound 6a


¹³C NMR Spectrum of Compound 6a

Elemental Composition Report   Page 1

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
44 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 15-20   H: 15-20   N: 0-5   O: 0-5   S: 0-2
CEB-1 2 (0.034) Cm (1:61)
TOF MS ES-
                                                                    1.99e+005

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
375.0920	375.0916	0.4	1.1	15.5	592.3	0.0	C20 H15 N4 O2 S

Minimum: -1.5
Maximum: 5.0   5.0   100.0

HRMS Spectrum of Compound 6a

C:\srinivas\CEB....2-furoyl chloride.0   Fin T17   ATR platinum Diamond 1 Refl   16/03/2017

Page 1/1

IR Spectrum of Compound 6b

1H NMR Spectrum of Compound 6b
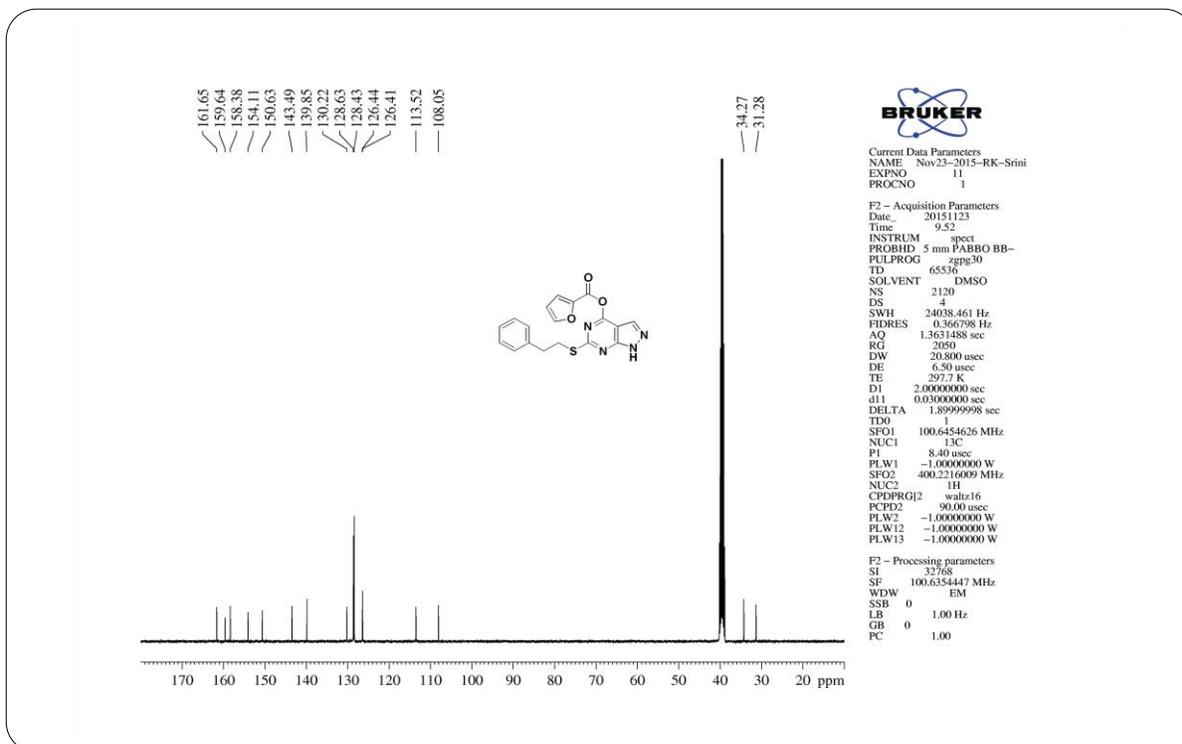
13C NMR Spectrum of Compound 6b
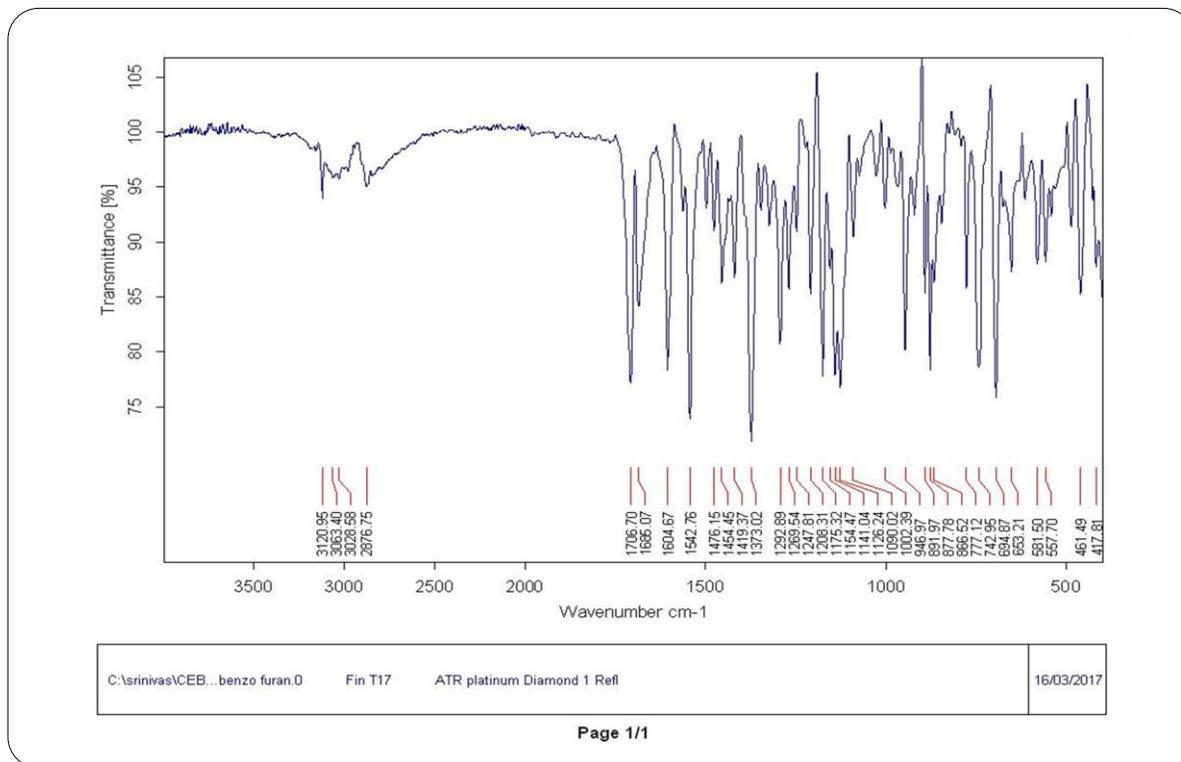
IR Spectrum of Compound 6c
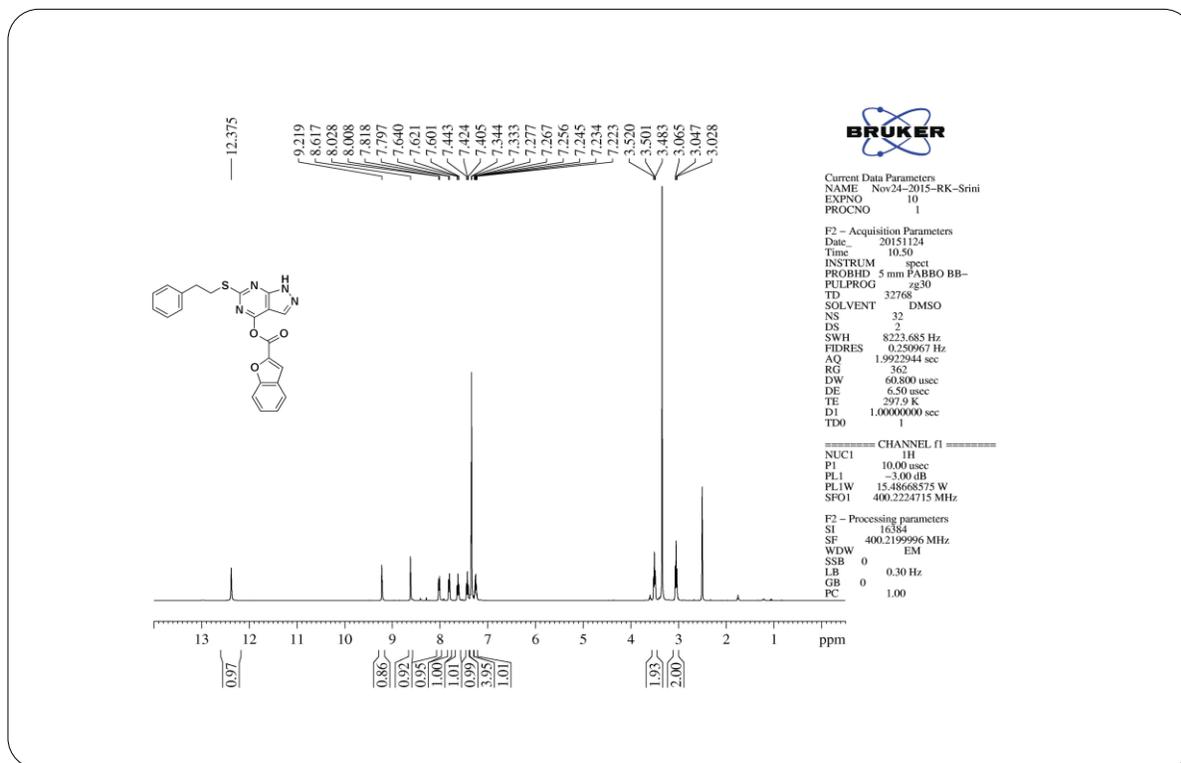
¹H NMR Spectrum of Compound 6c
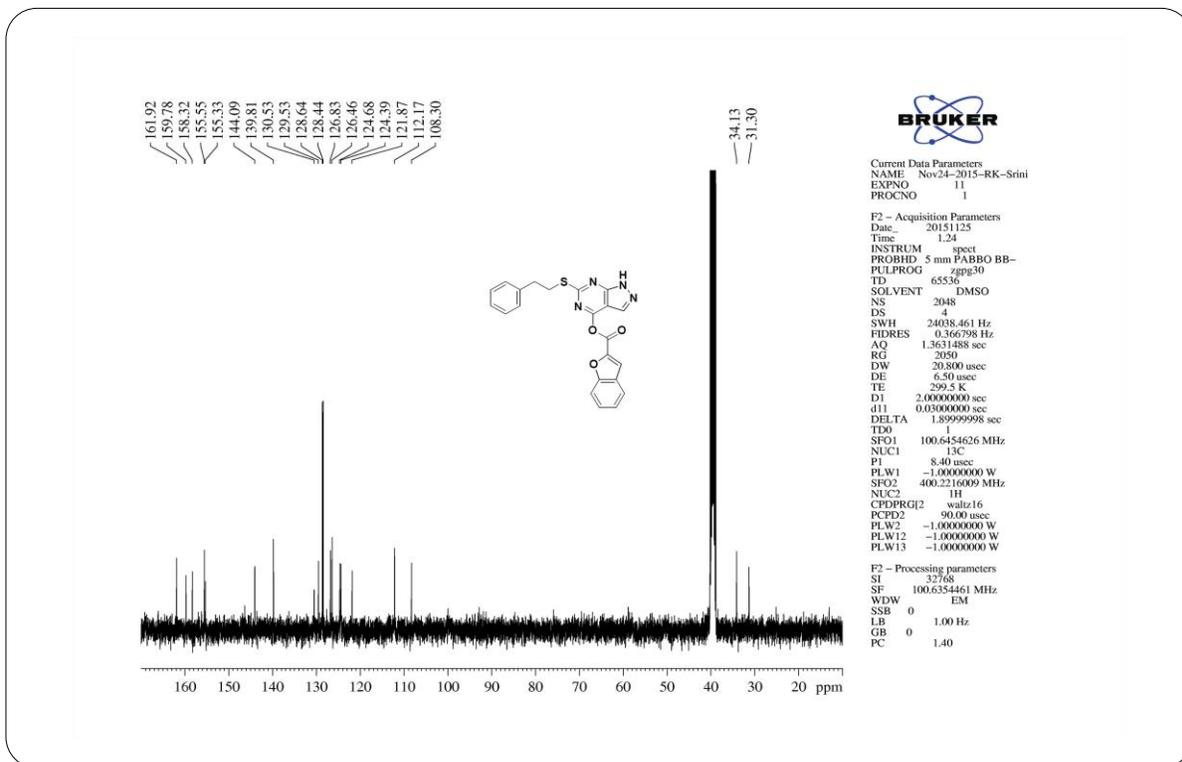
^{13}C NMR Spectrum of Compound 6c
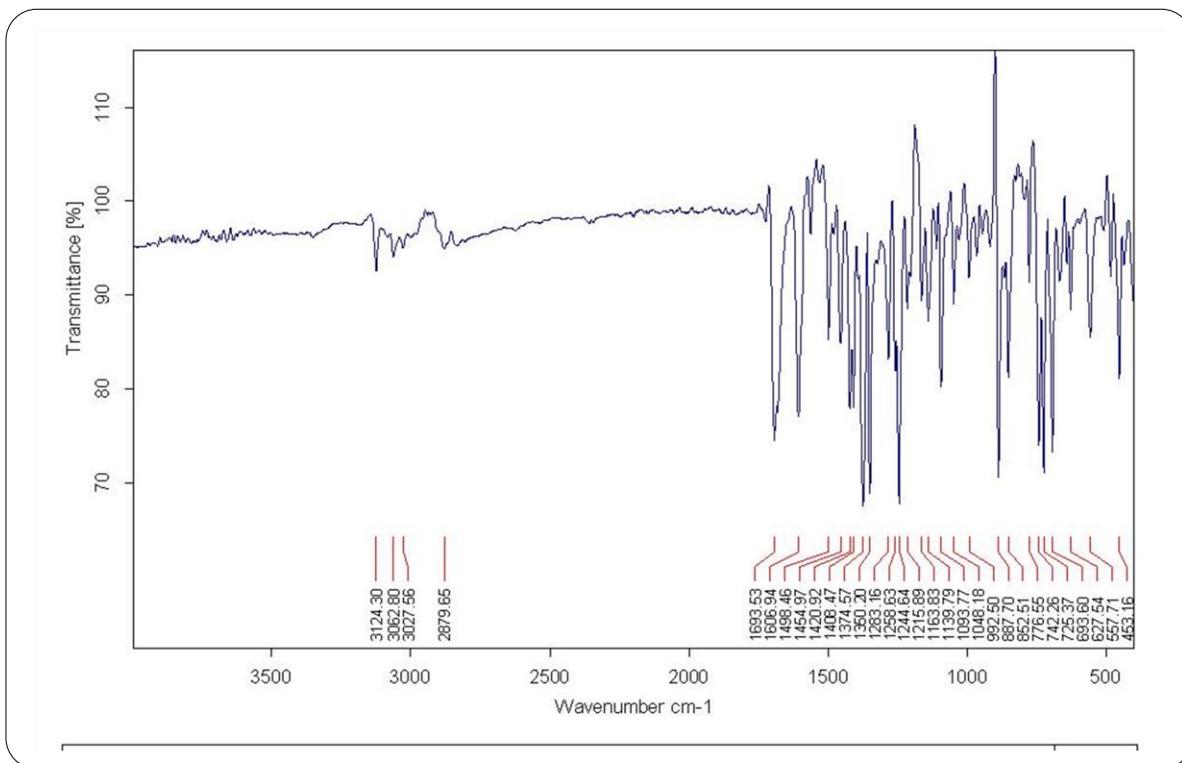
IR Spectrum of Compound 6d
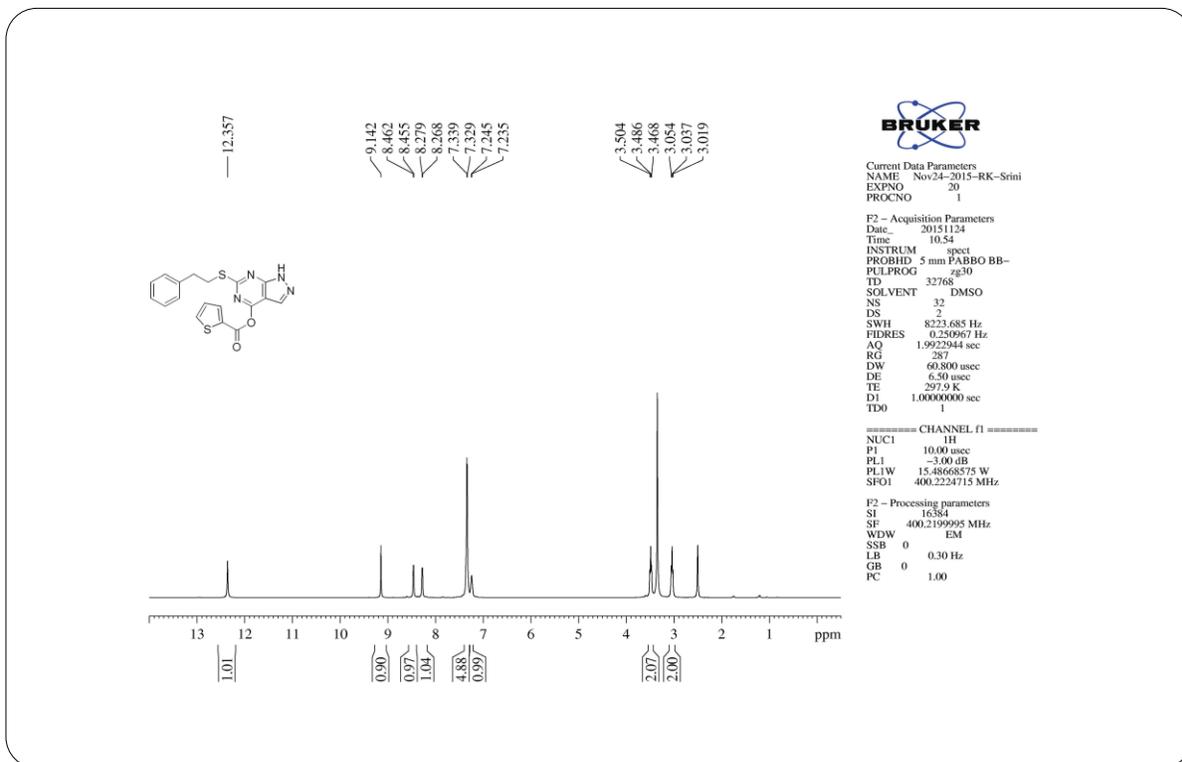
¹H NMR Spectrum of Compound 6d
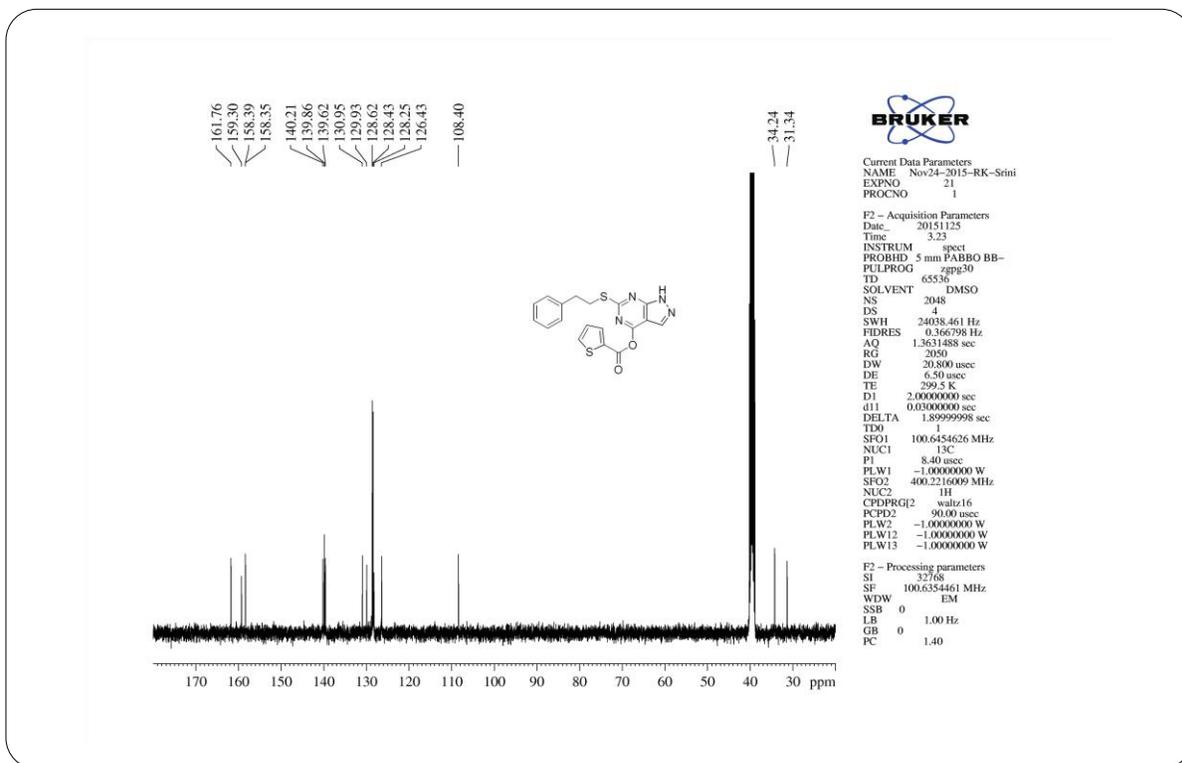
¹³C NMR Spectrum of Compound 6d
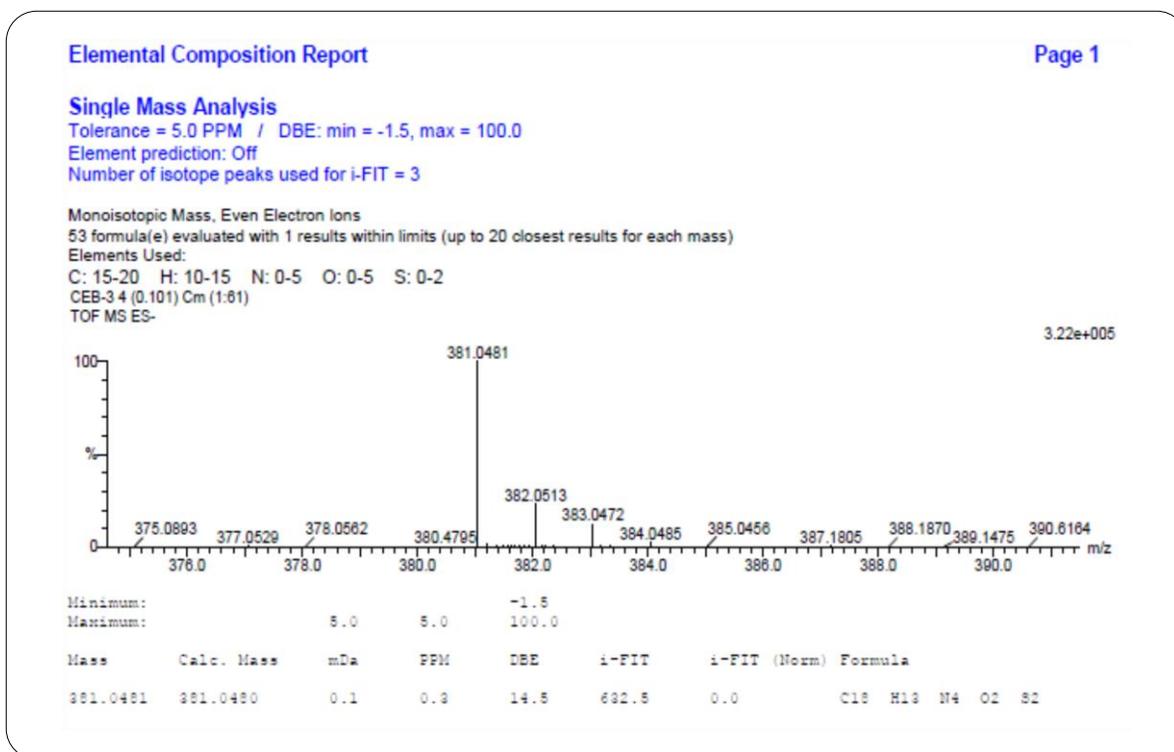
HRMS Spectrum of Compound 6d
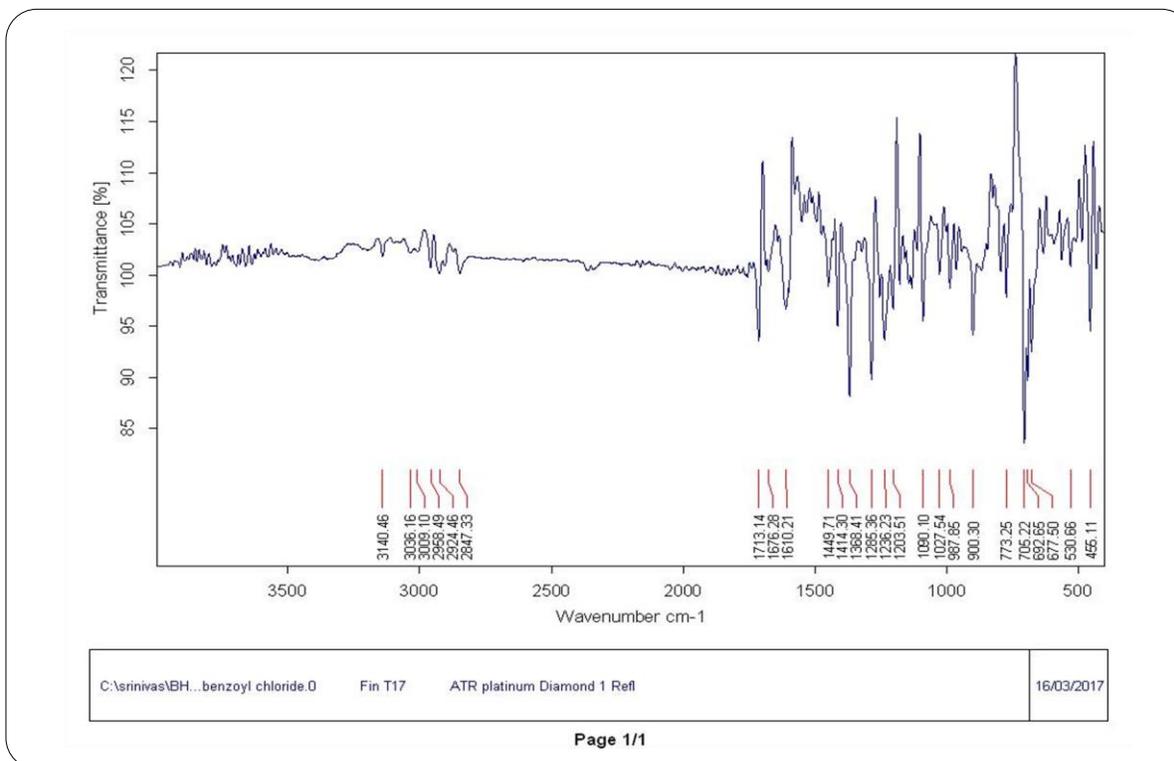
IR Spectrum of Compound 7a
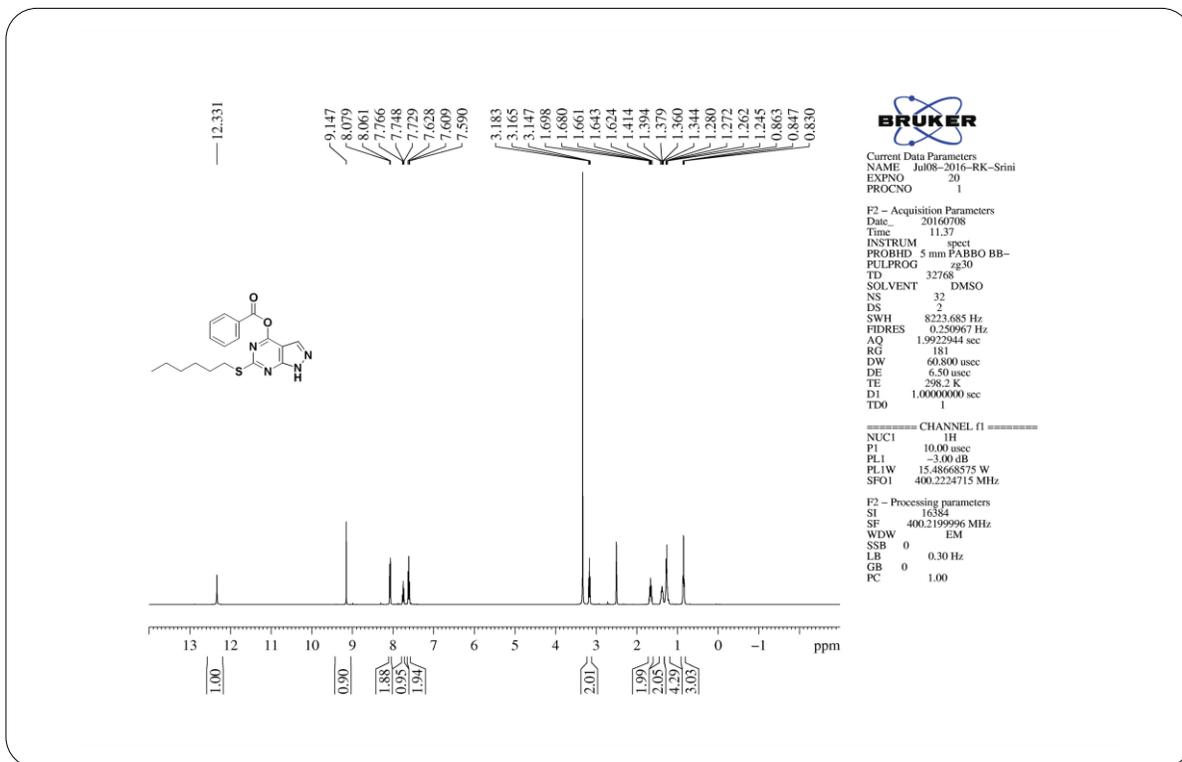
¹H NMR Spectrum of Compound 7a
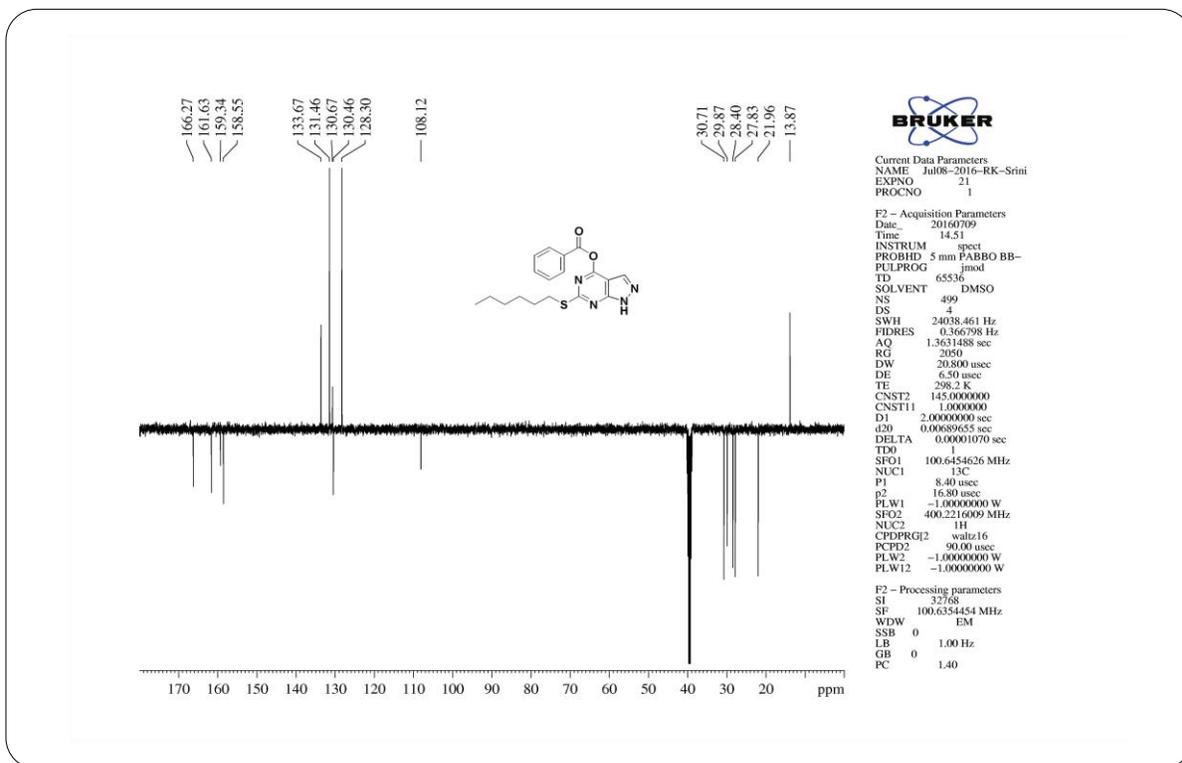
¹³C NMR Spectrum of Compound 7a
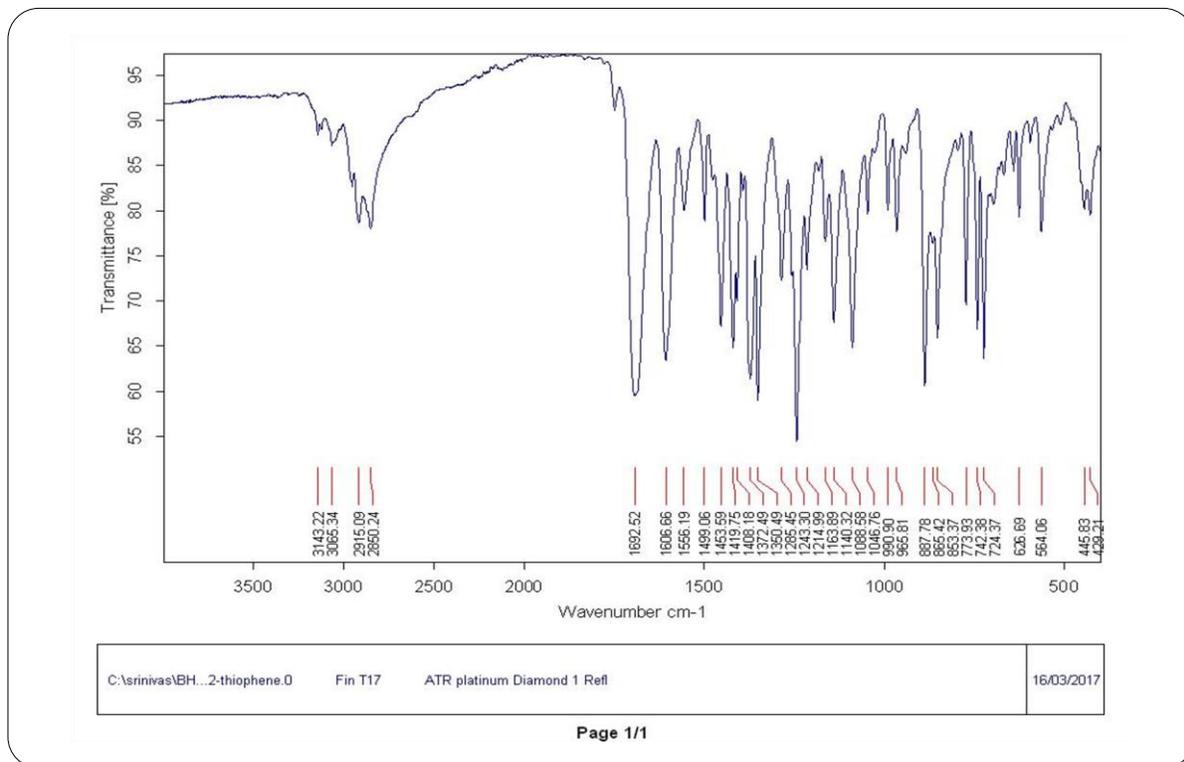
IR Spectrum of Compound 7b
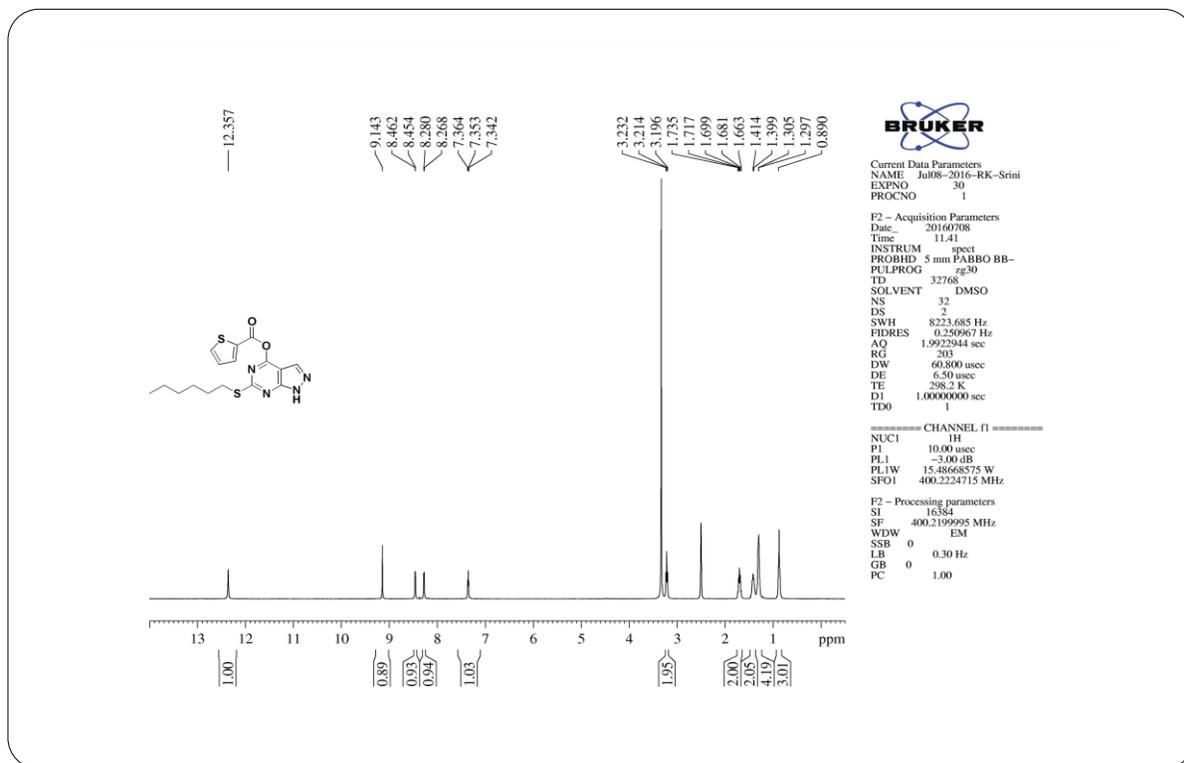
**¹H NMR Spectrum of Compound 7b**

¹³C NMR Spectrum of Compound 7b


**HRMS Spectrum of Compound 7b**

IR Spectrum of Compound 7c
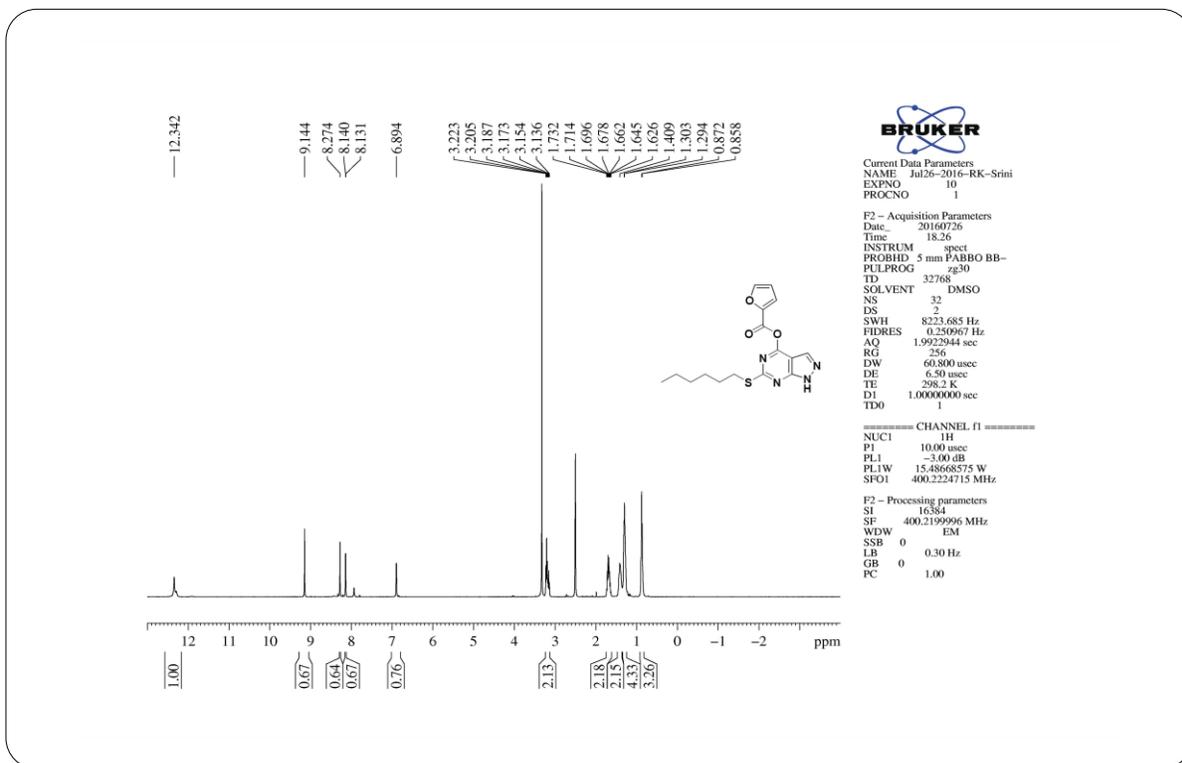
¹H NMR Spectrum of Compound 7c
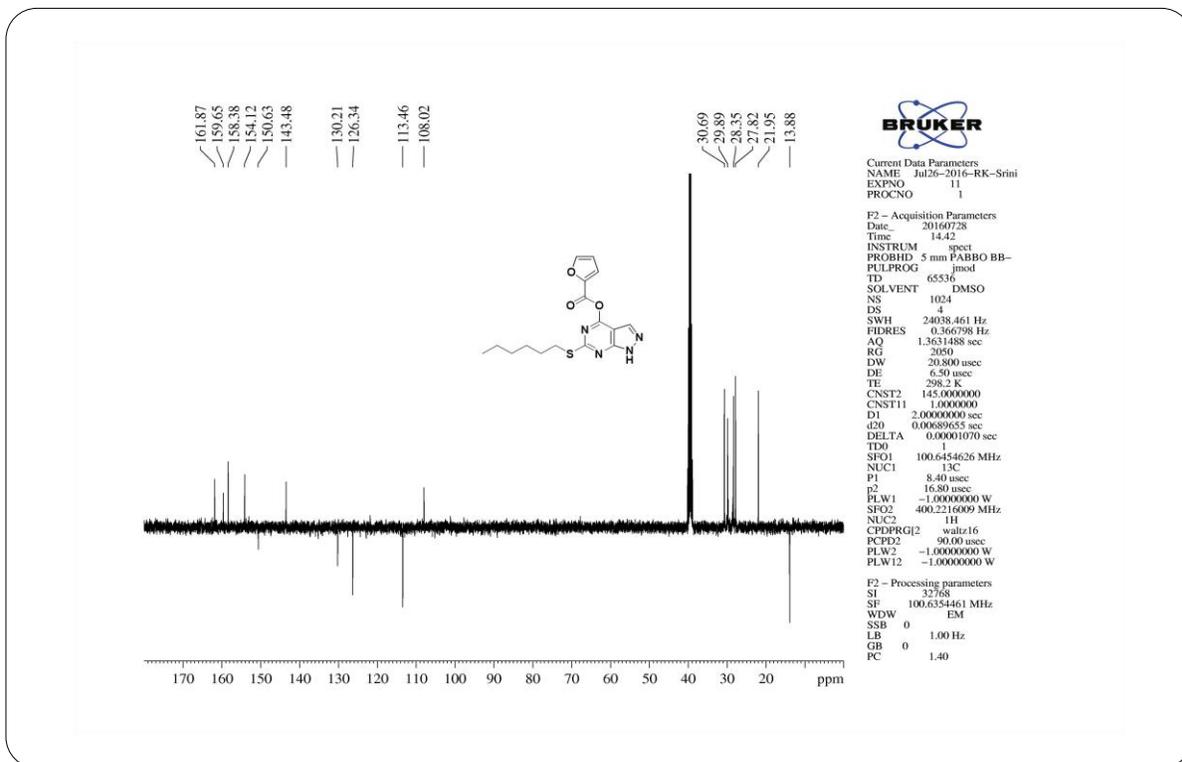
¹³C NMR Spectrum of Compound 7c
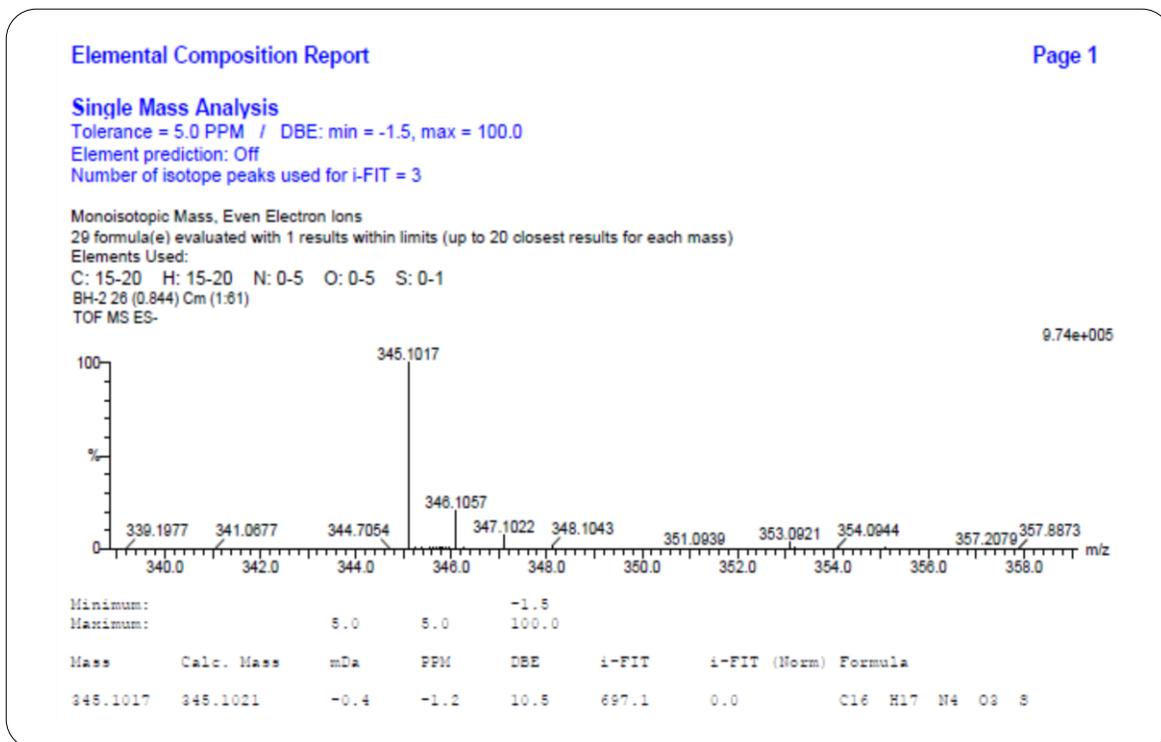
HRMS Spectrum of Compound 7c

APPENDIX – III

SUPPLIMENTARY INFORMATION

CHAPTER 6

Design, synthesis and biological evaluation of novel pyrazolo[3,4-*d*]pyrimidine analogues as anticancer agents

Department of Pharmaceutical Chemistry, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa.
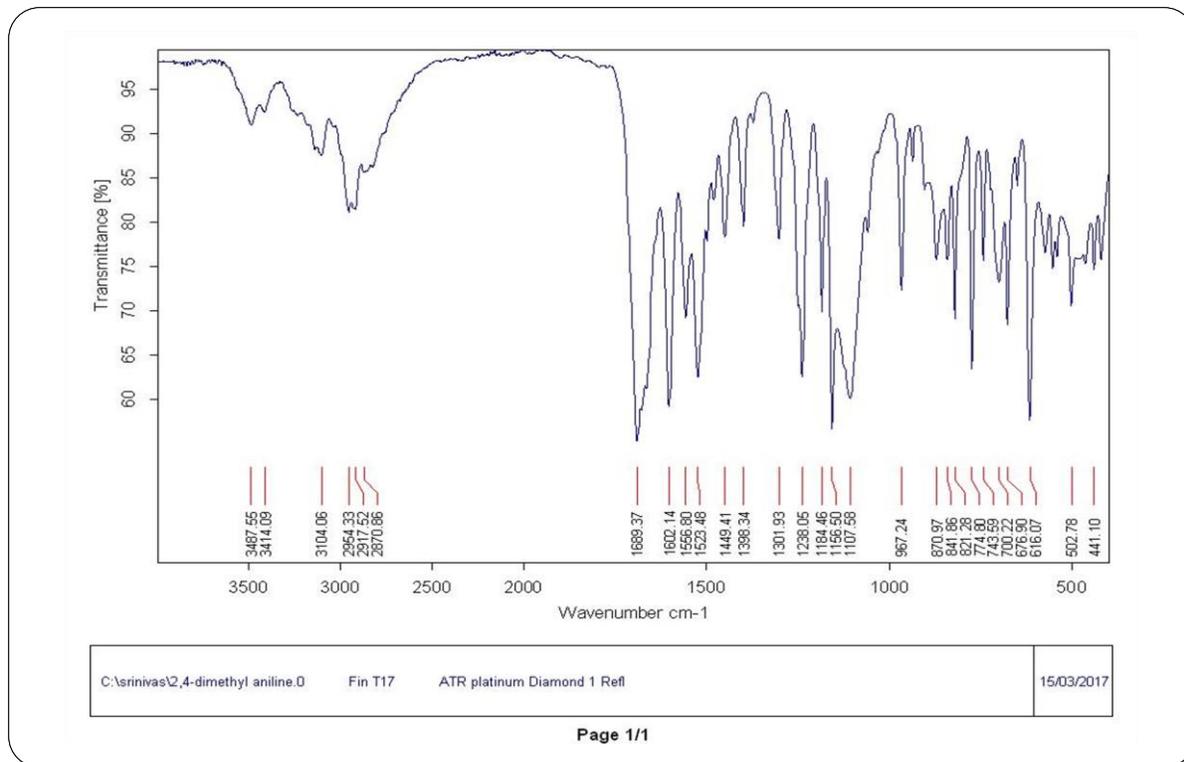
IR Spectrum of Compound 9a
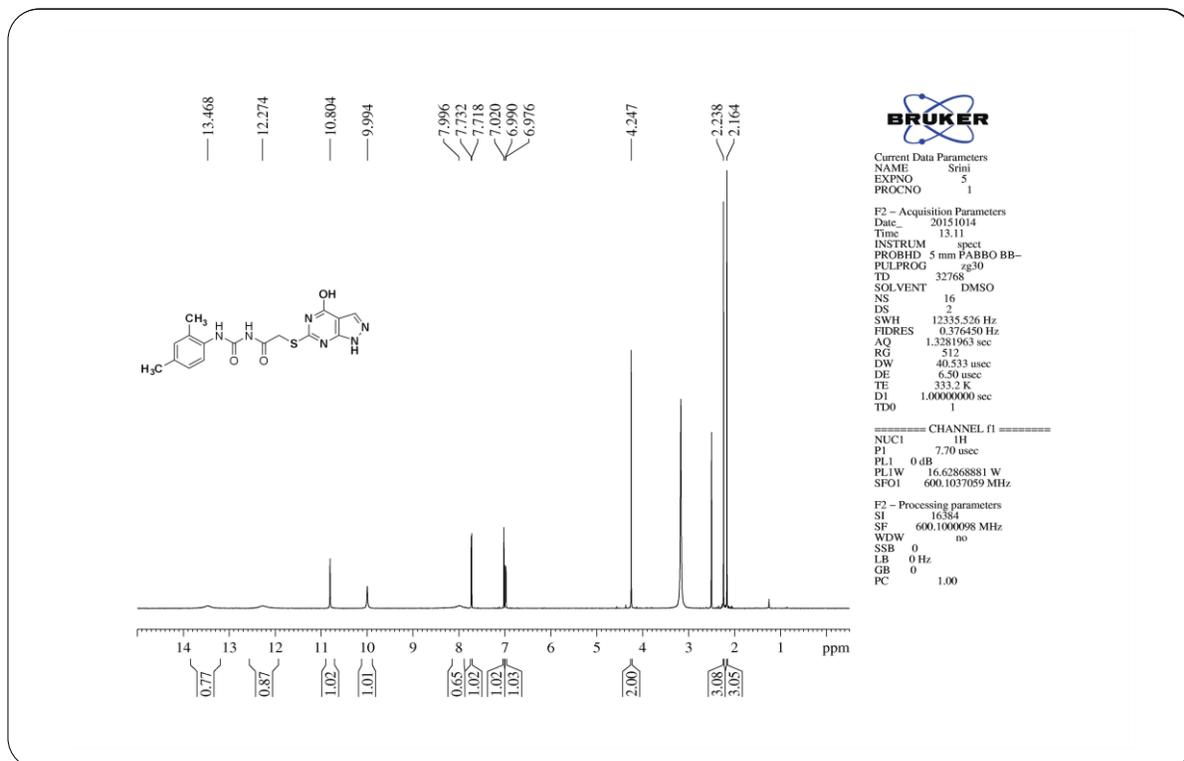
¹H NMR Spectrum of Compound 9a
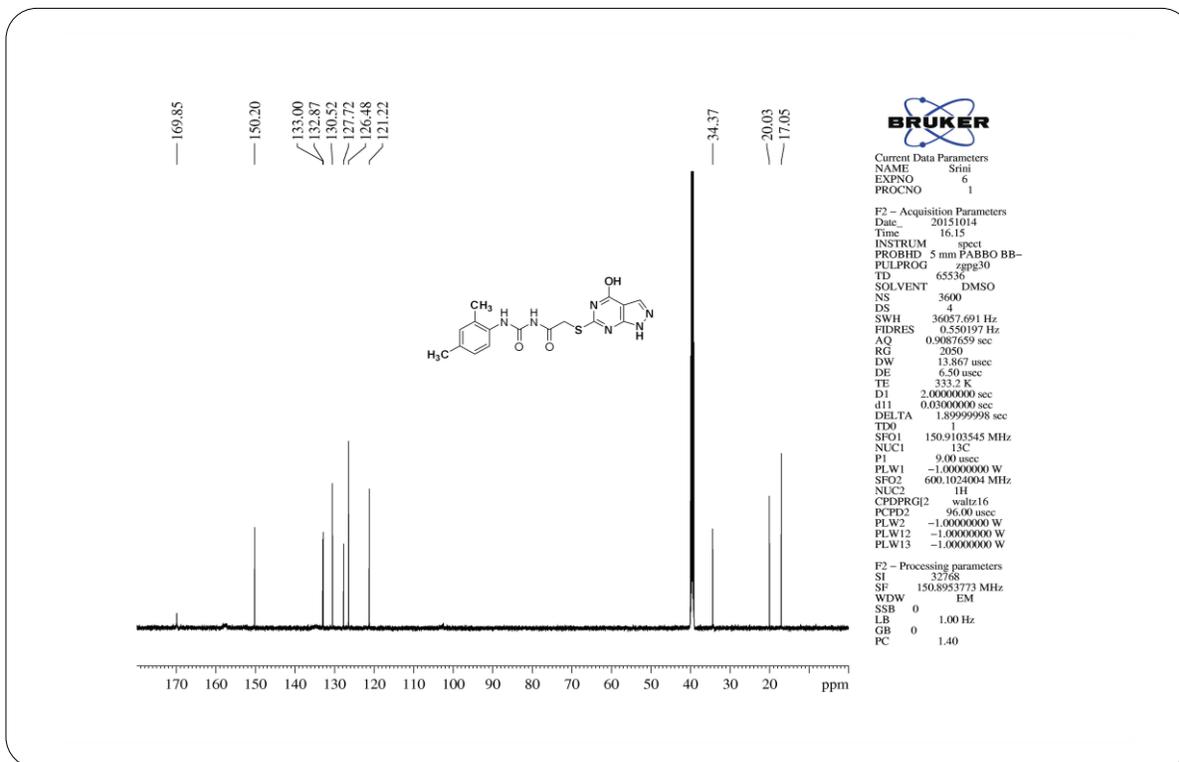
^{13}C NMR Spectrum of Compound 9a
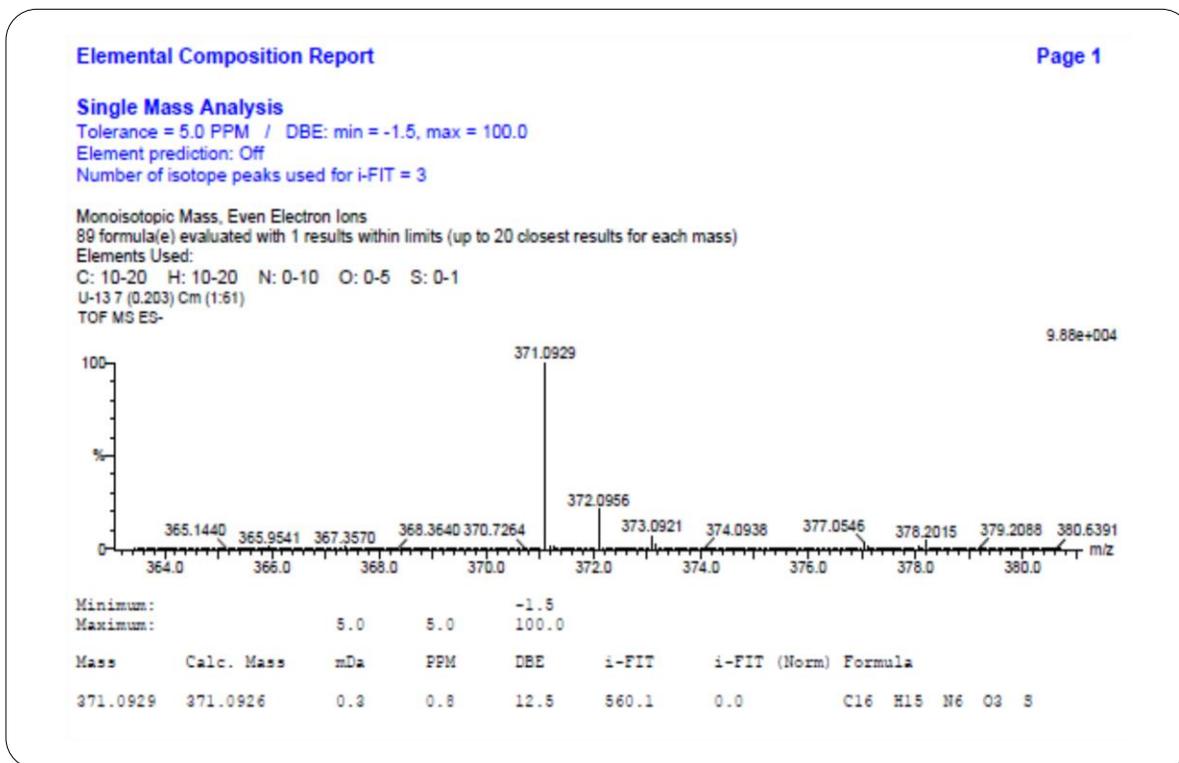
HRMS Spectrum of Compound 9a
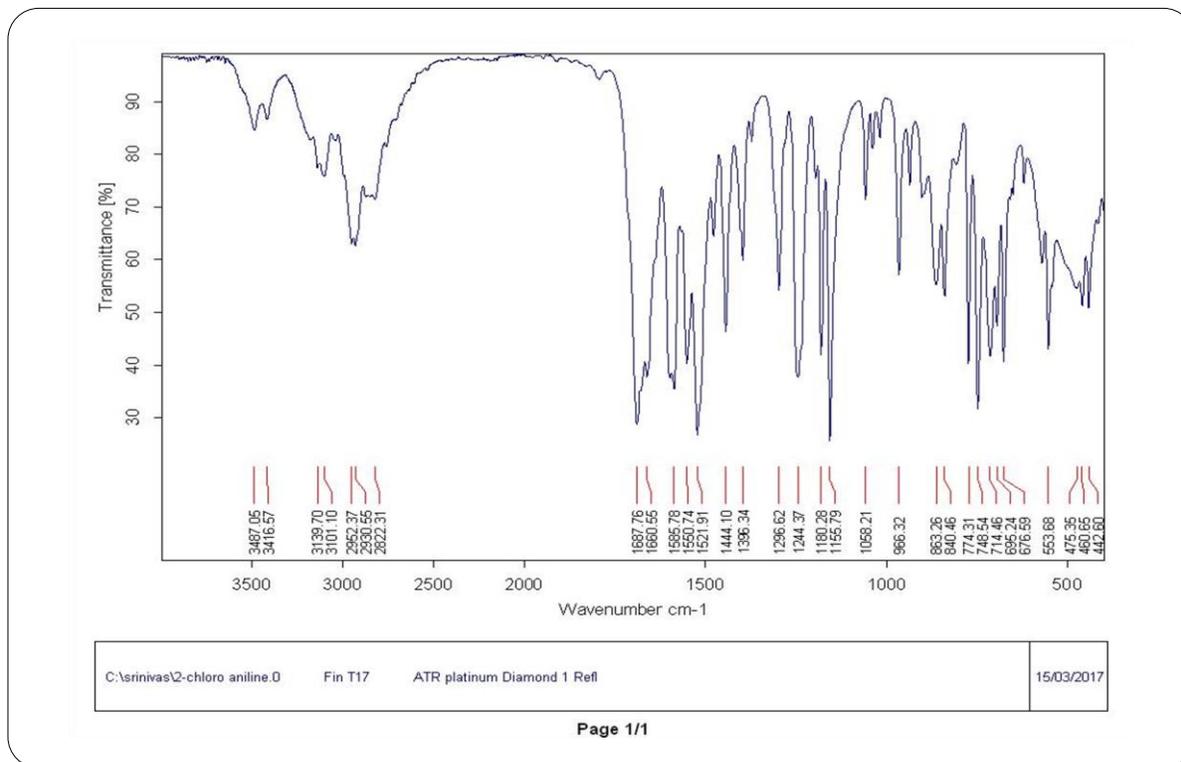
IR Spectrum of Compound 9b
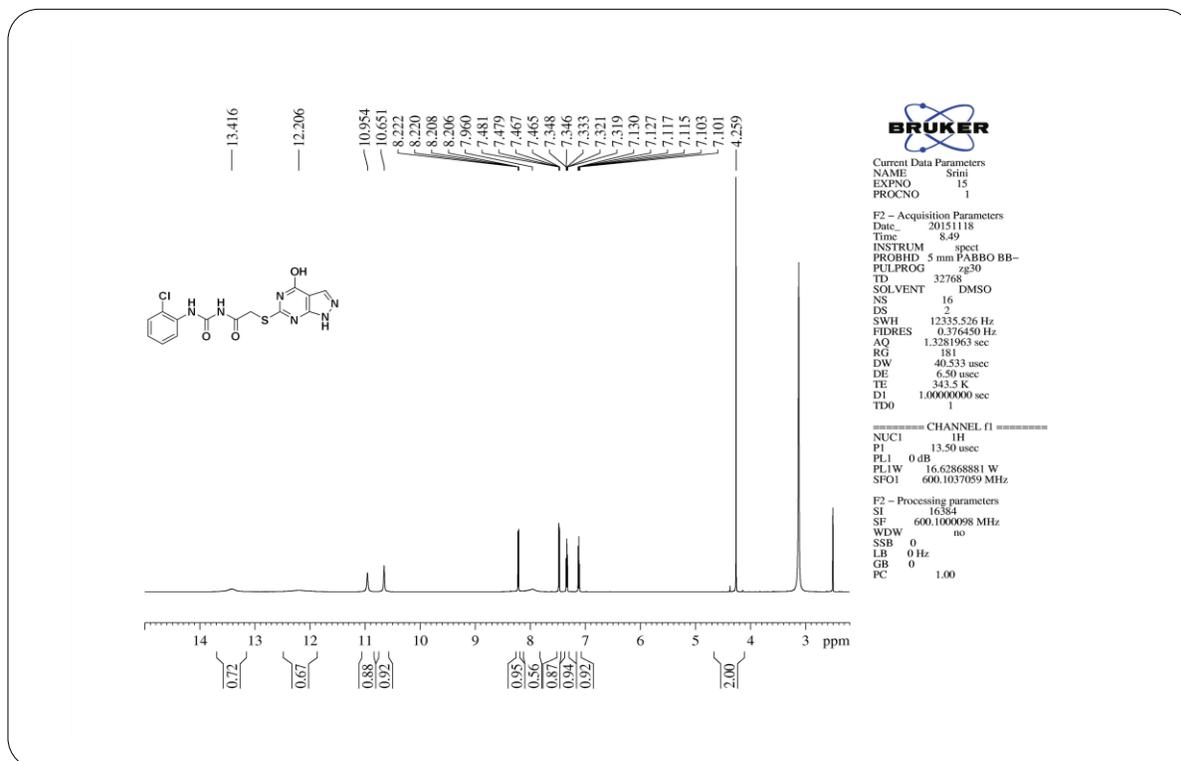

¹H MNR Spectrum of Compound 9b
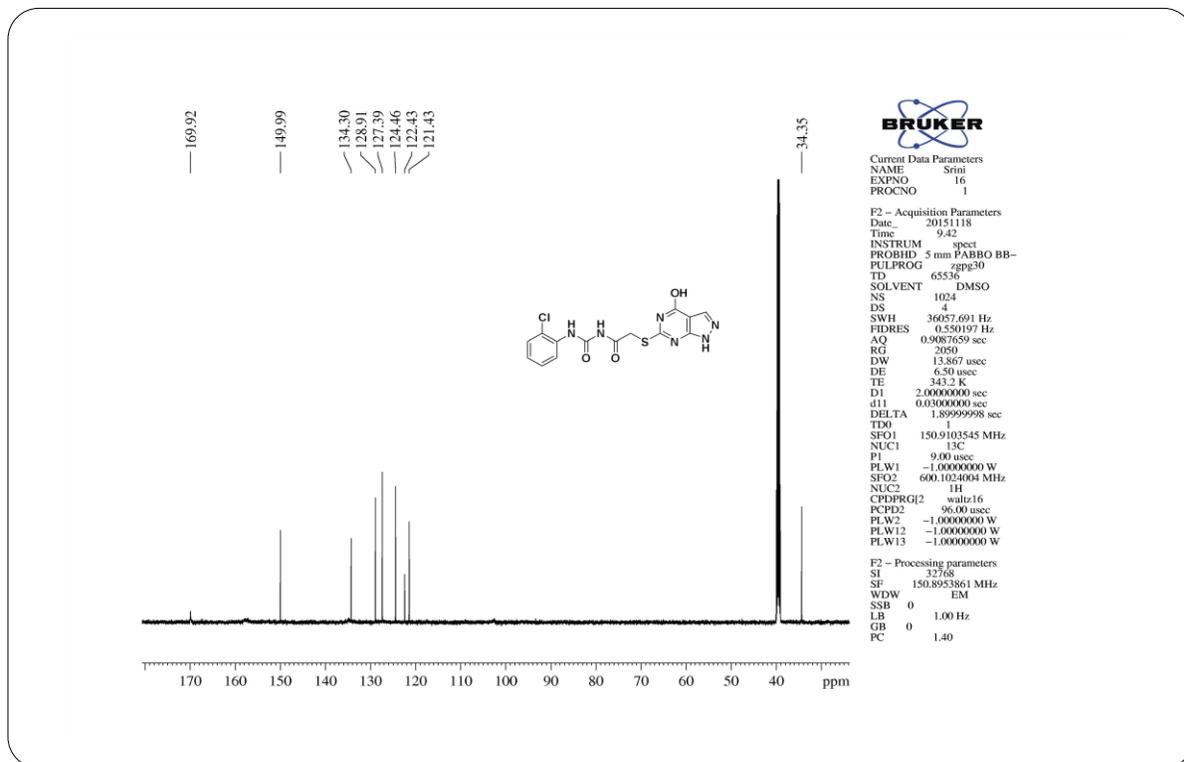
¹³C NMR Spectrum of Compound 9b
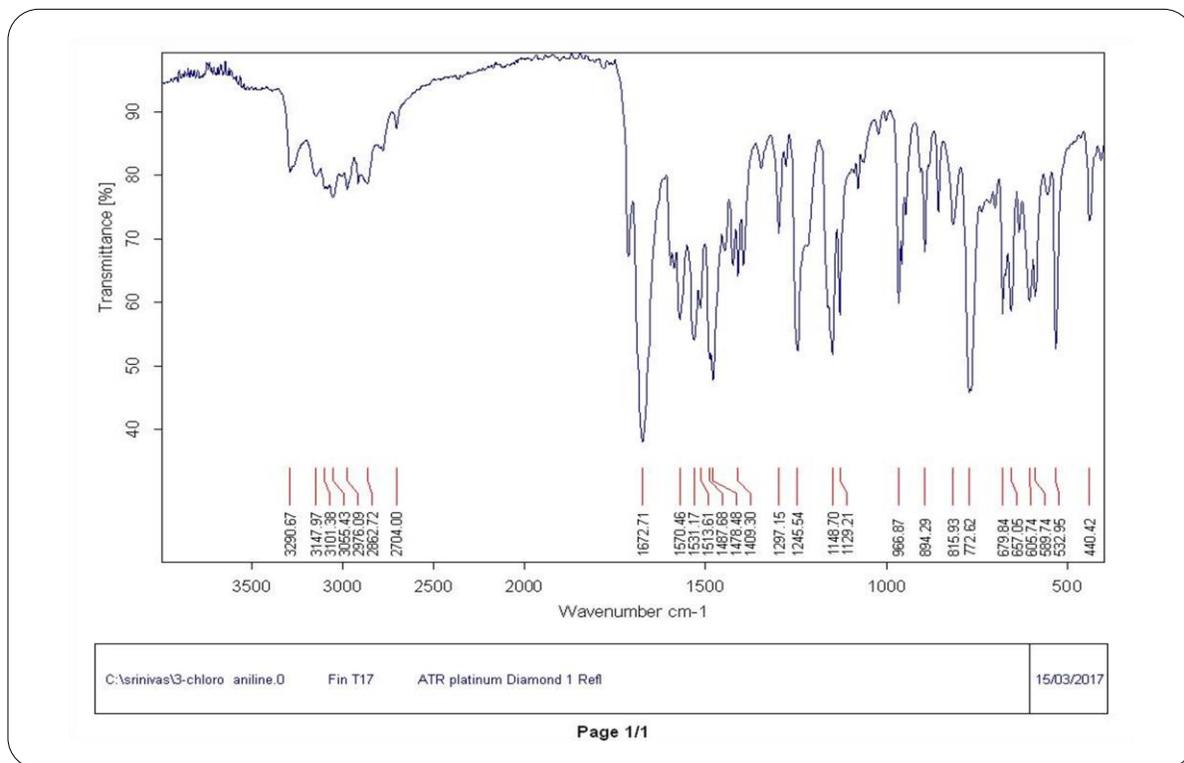
IR Spectrum of Compound 9c
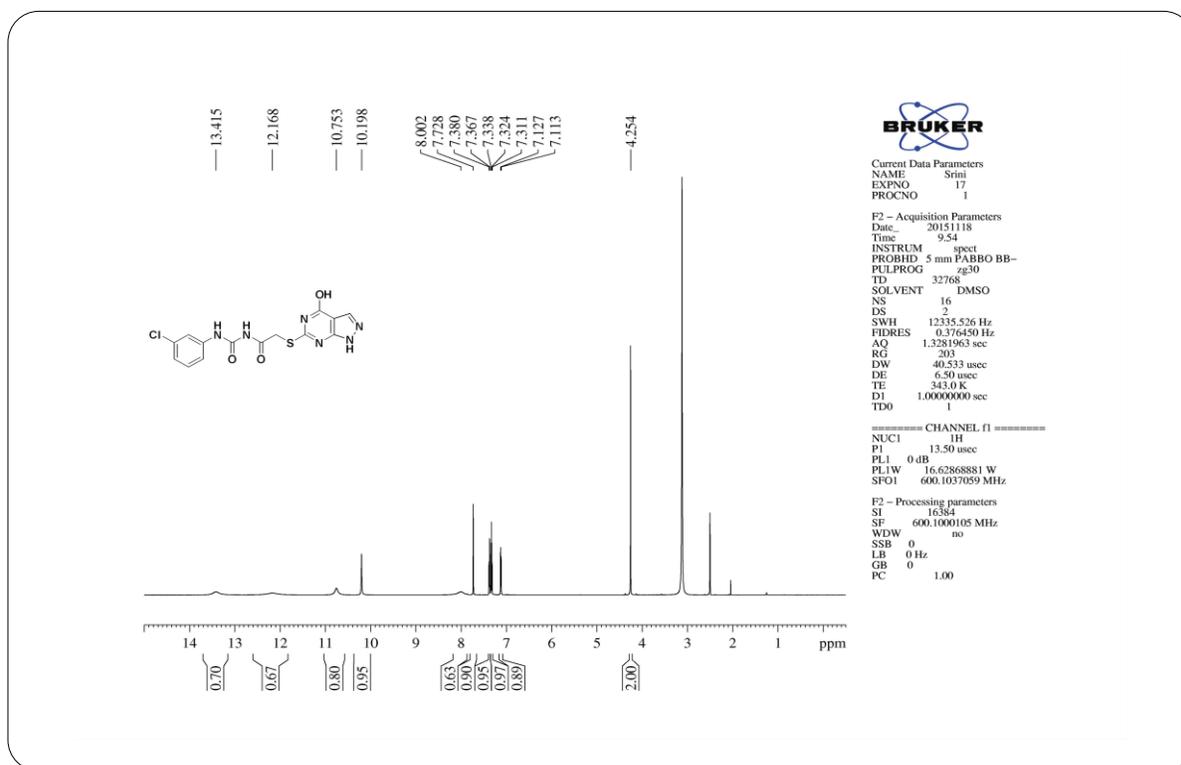
¹H NMR Spectrum of Compound 9c


¹³C NMR Spectrum of Compound 9c

Elemental Composition Report   Page 1

Single Mass Analysis
Tolerance = 5.0 PPM   /   DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
176 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 10-15   H: 10-15   N: 0-10   O: 0-5   S: 0-1   Cl: 0-1
U-5 2 (0.034) Cm (1:61)
TOF MS ES-
                                                                                                         1.57e+005

| Minimum: | | | -1.5 | | | |
| Maximum: | | 5.0 | 5.0 | 100.0 | | |

| Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
377.0226	377.0224	0.2	0.5	12.5	606.3	0.0	C14 H10 N6 O3 S Cl

HRMS Spectrum of Compound 9c

IR Spectrum of Compound 9d
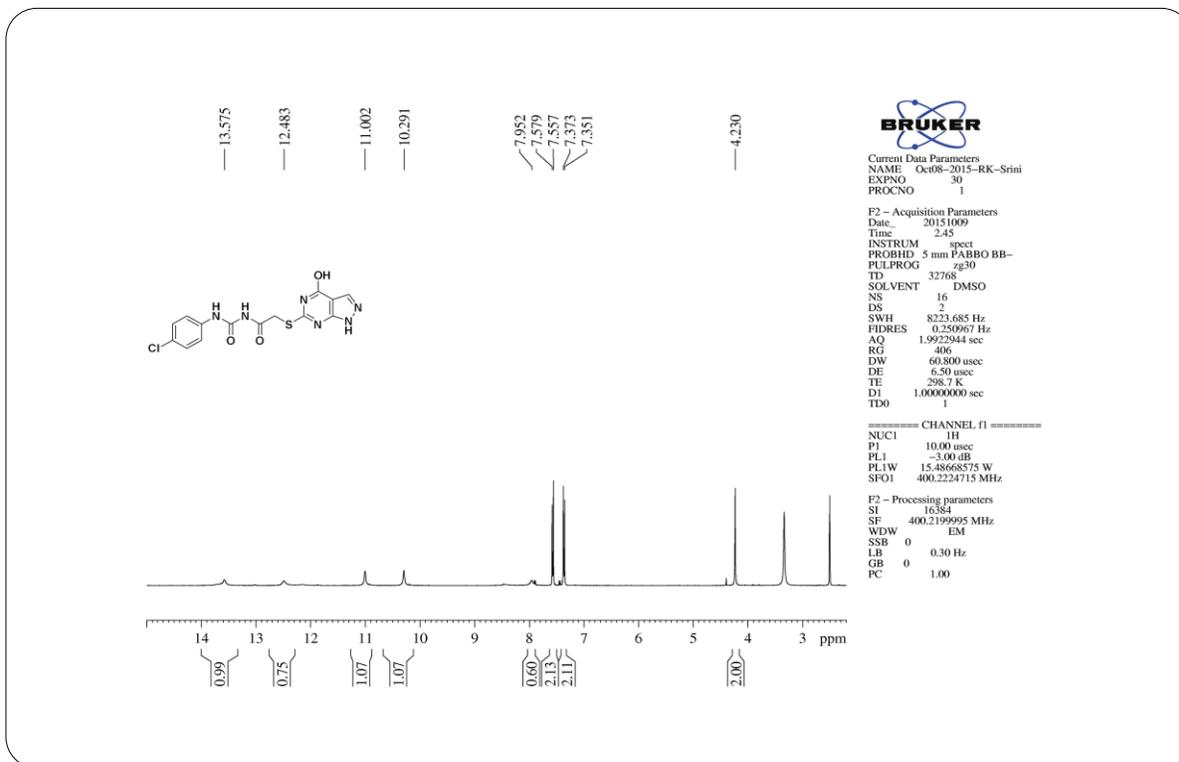
¹H NMR Spectrum of Compound 9d
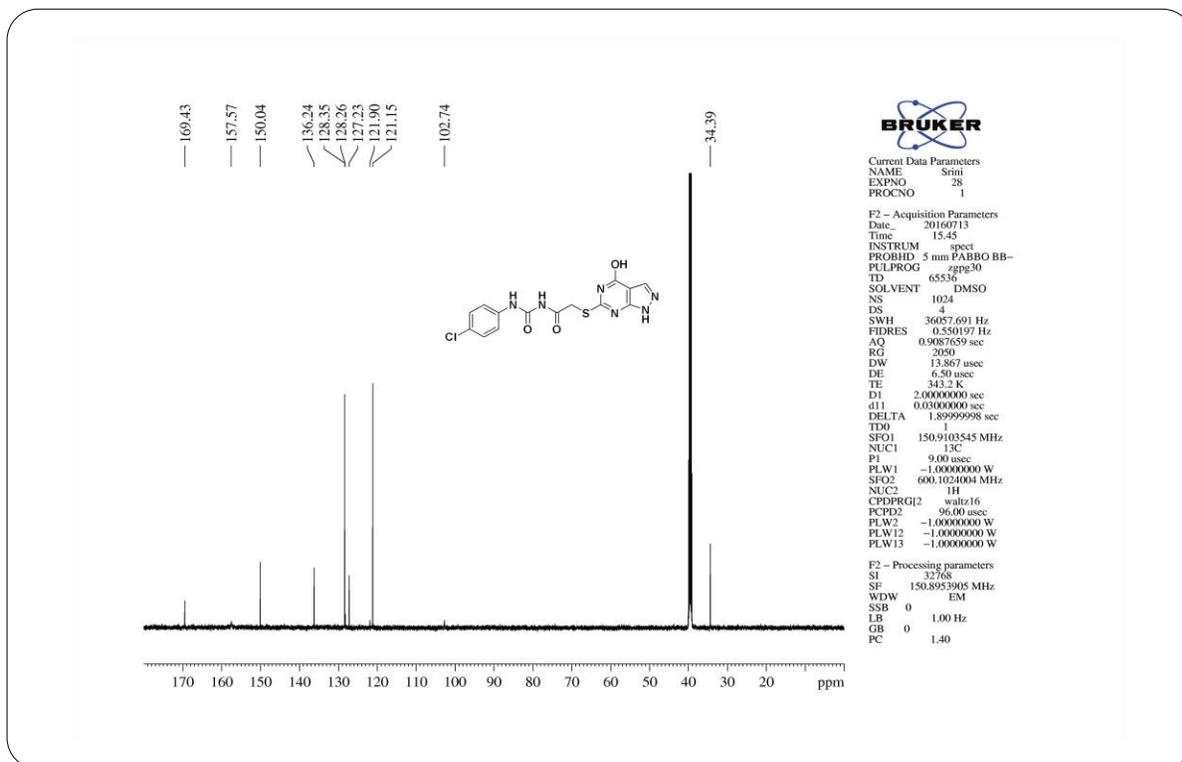
¹³C NMR Spectrum of Compound 9d
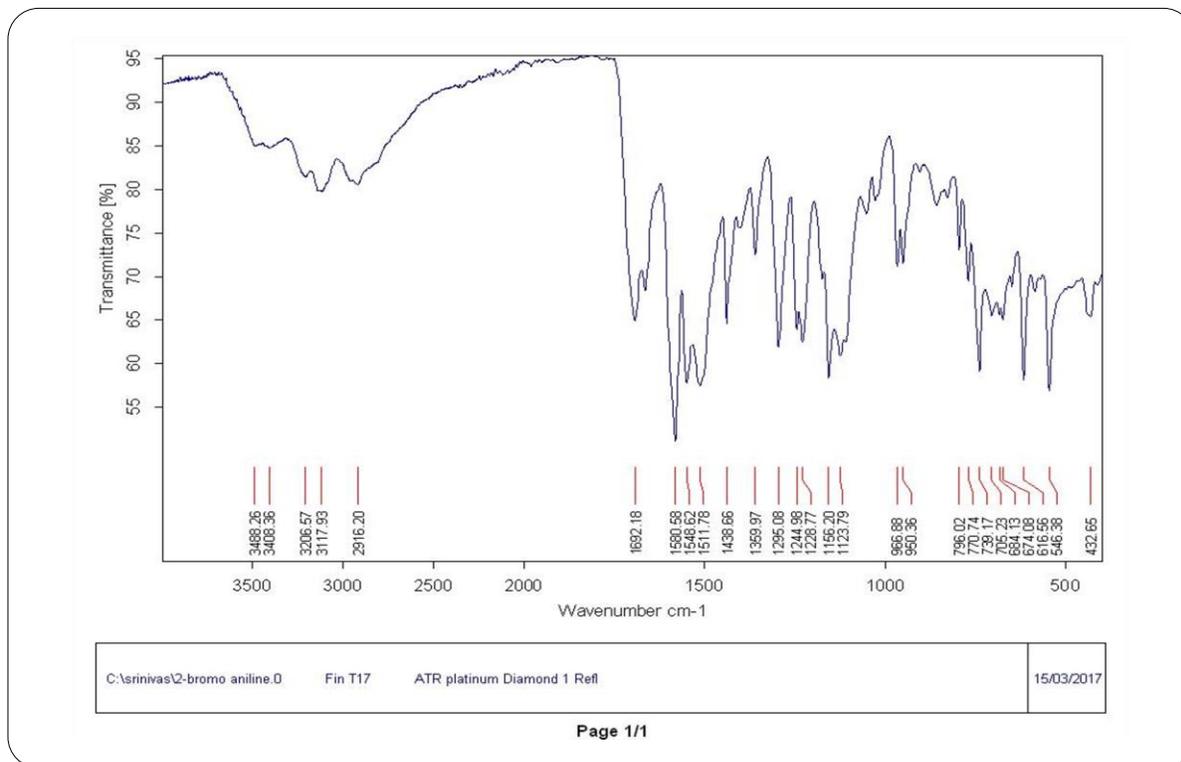
**IR Spectrum of Compound 9e**
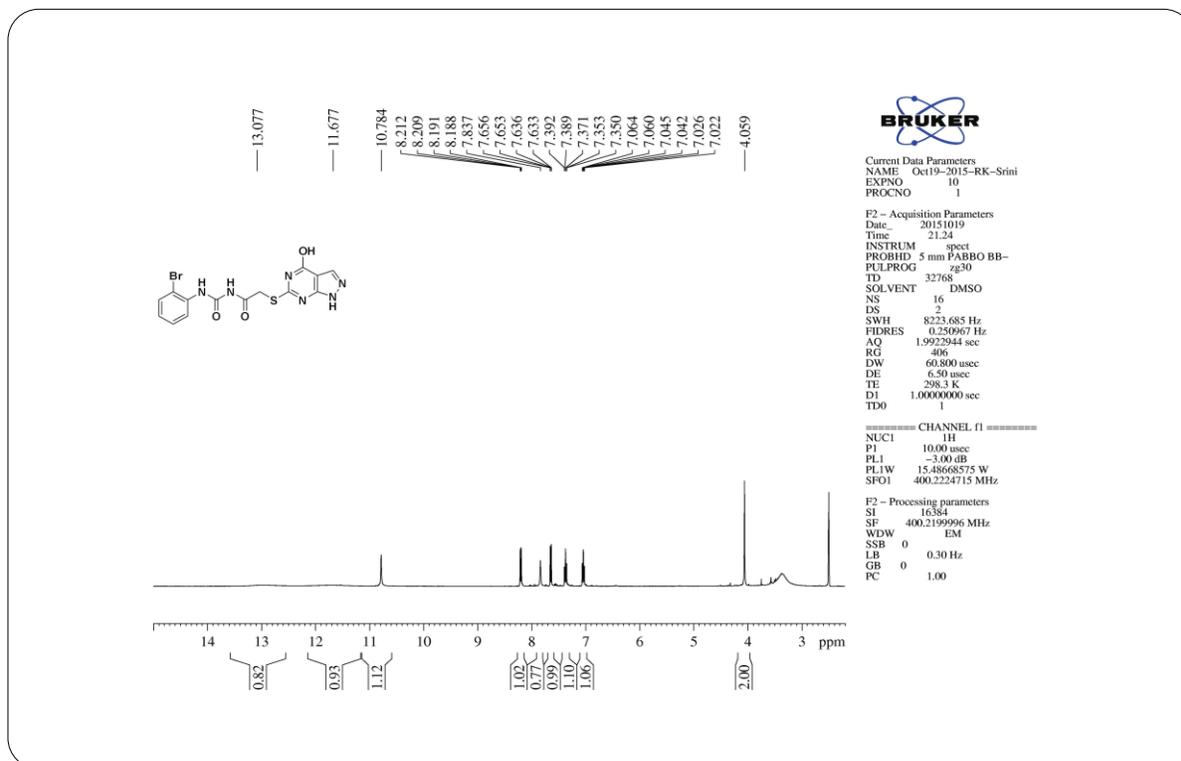

^{1}H NMR Spectrum of Compound 9e
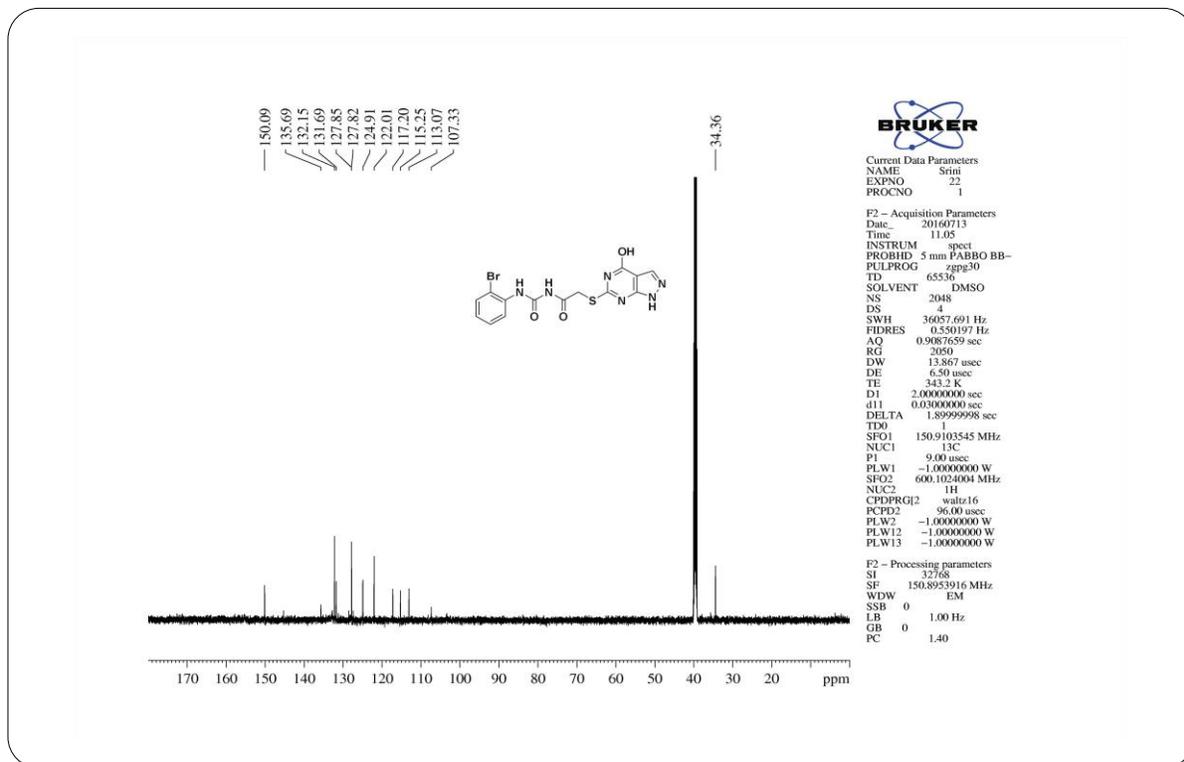
¹³C NMR Spectrum of Compound 9e
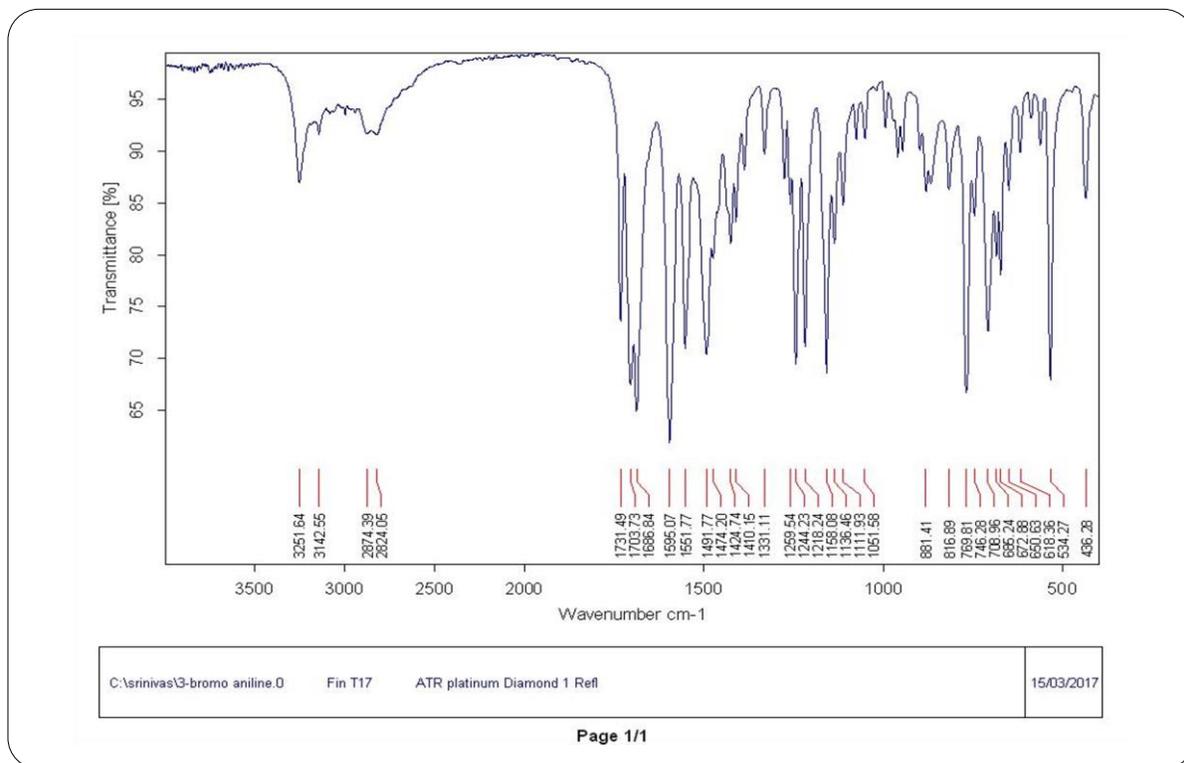

IR Spectrum of Compound 9f
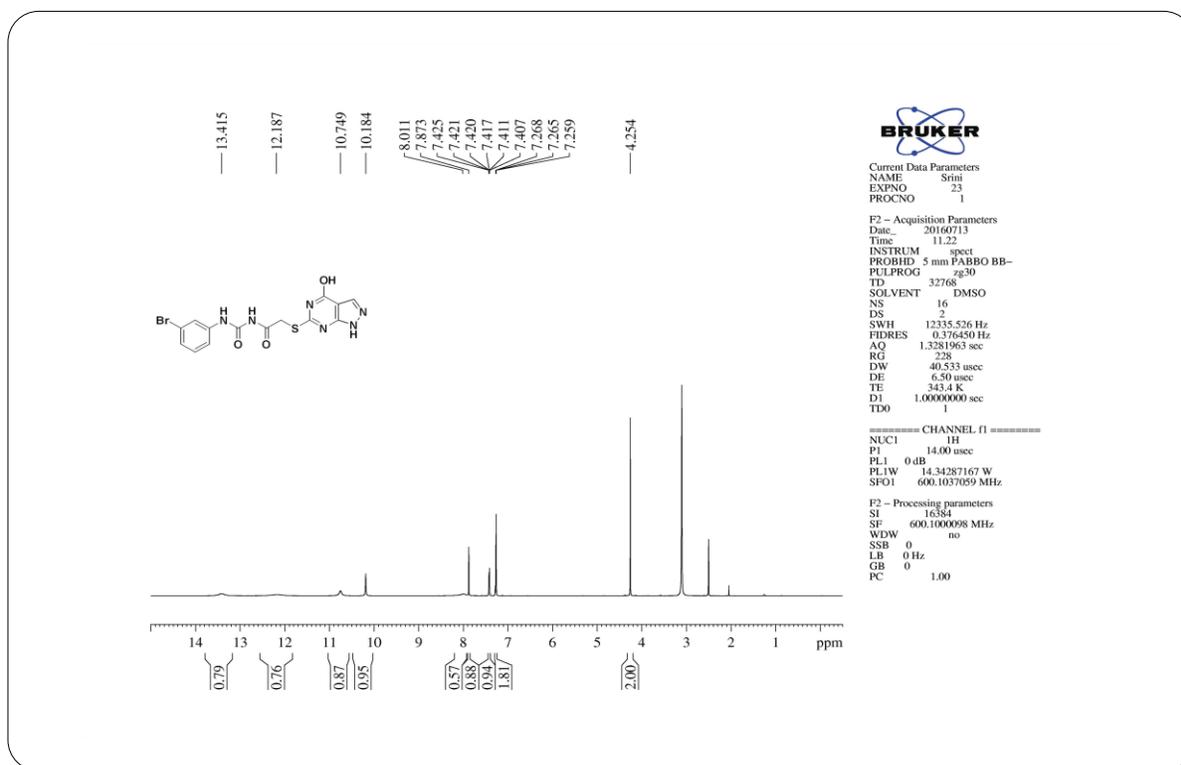
**¹H NMR Spectrum of Compound 9f**
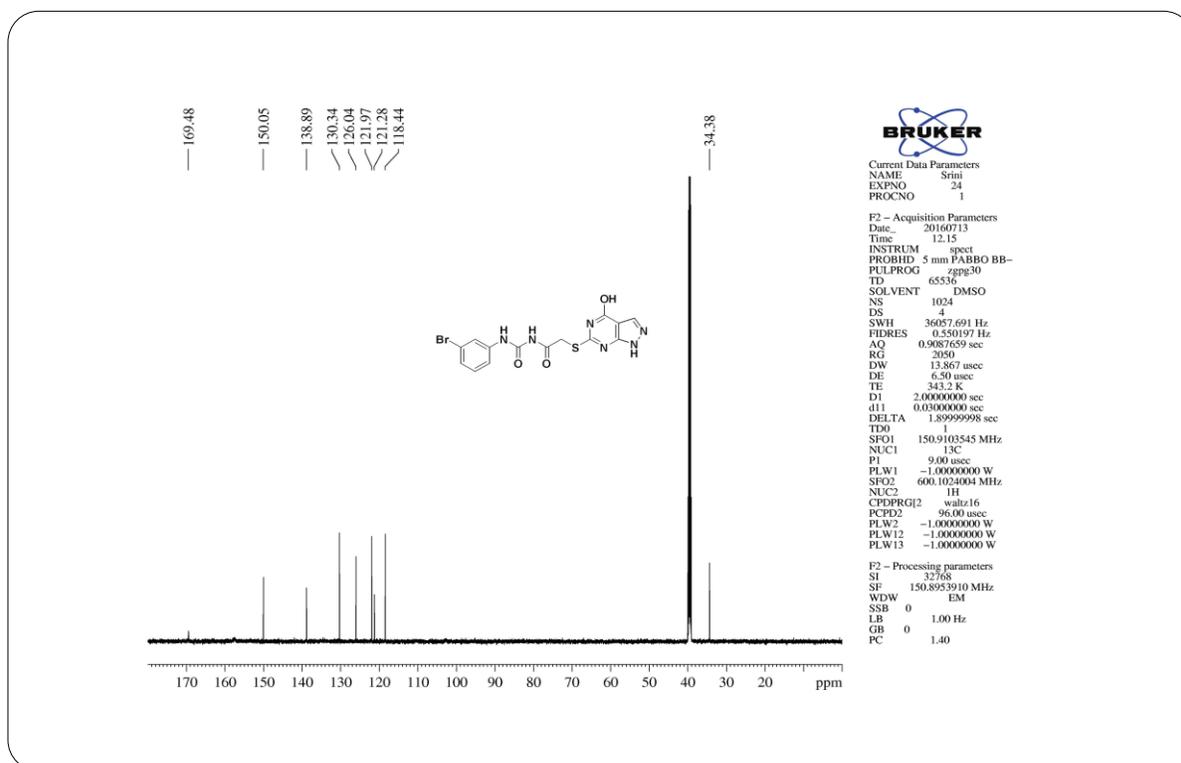
¹³C NMR Spectrum of Compound 9f
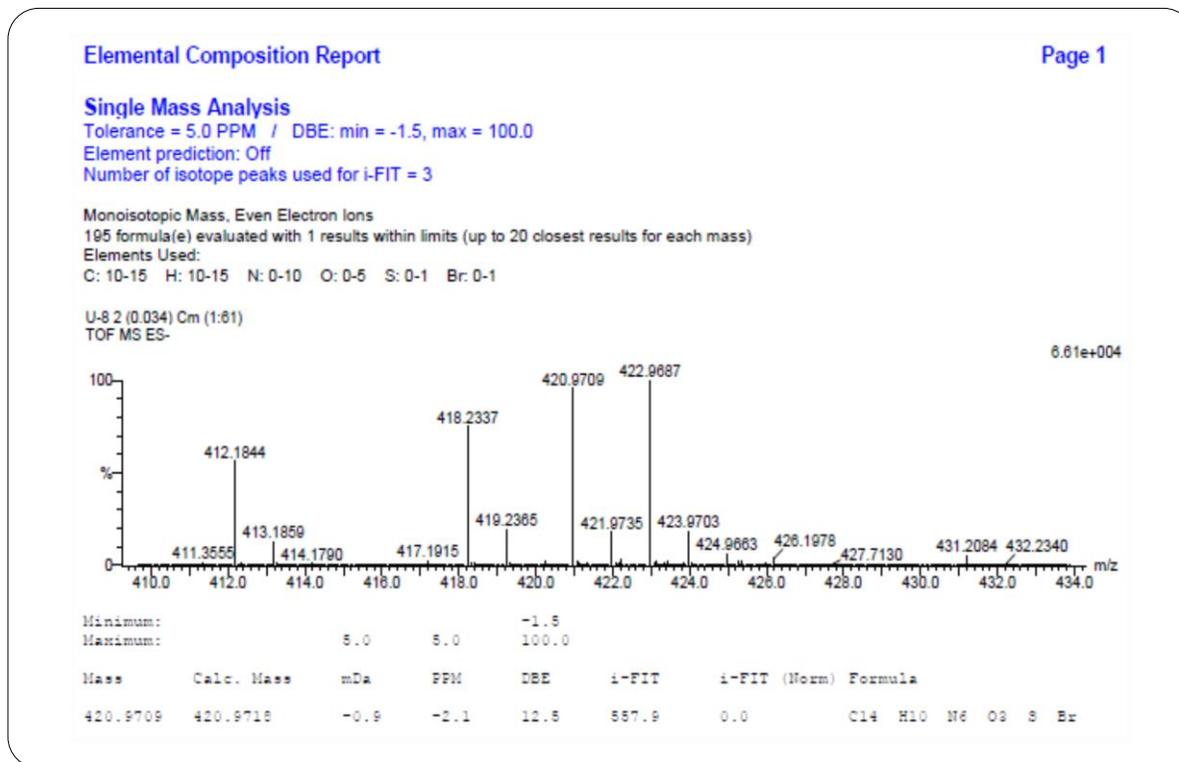
HRMS Spectrum of Compound 9f
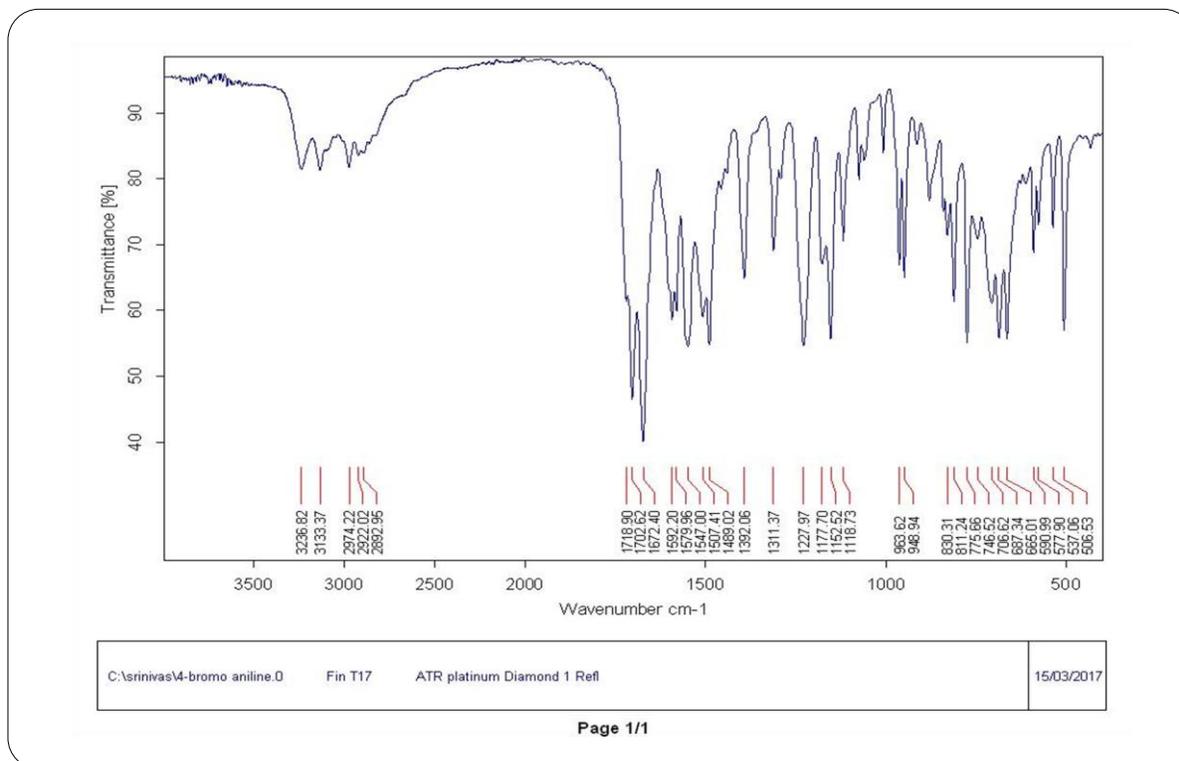
IR Spectrum of Compound 9g
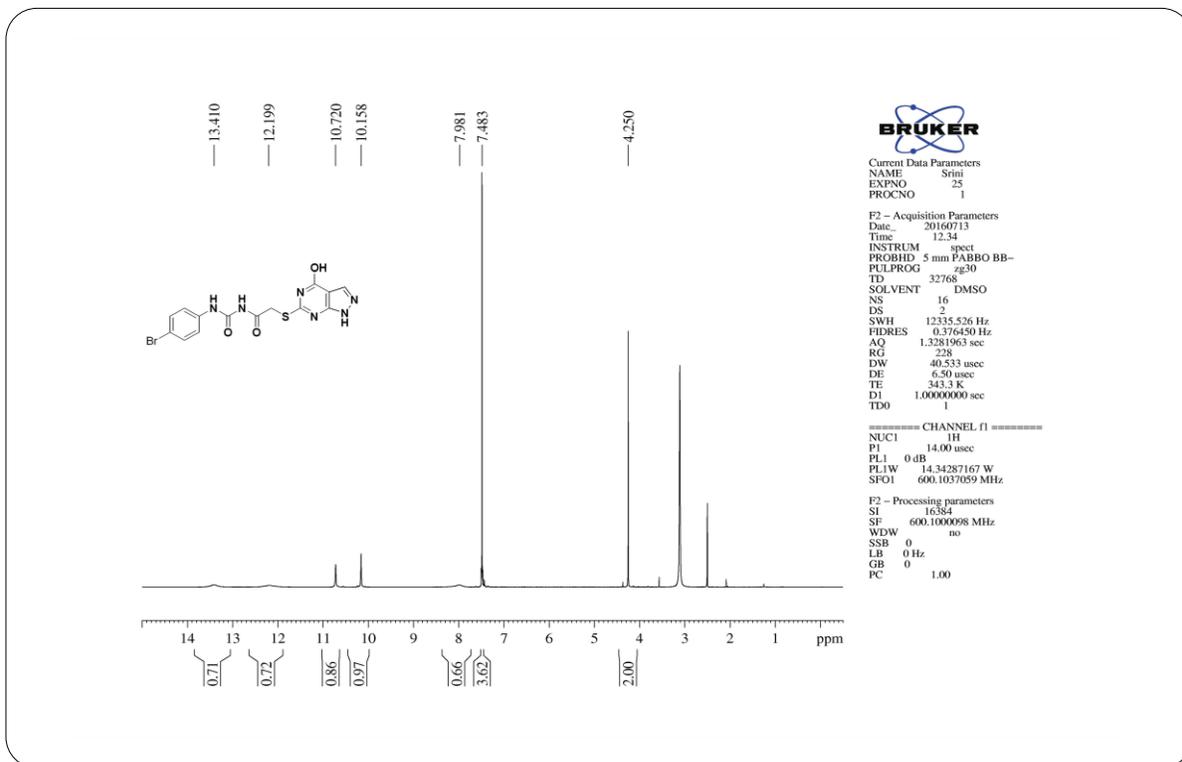
¹H NMR Spectrum of Compound 9g
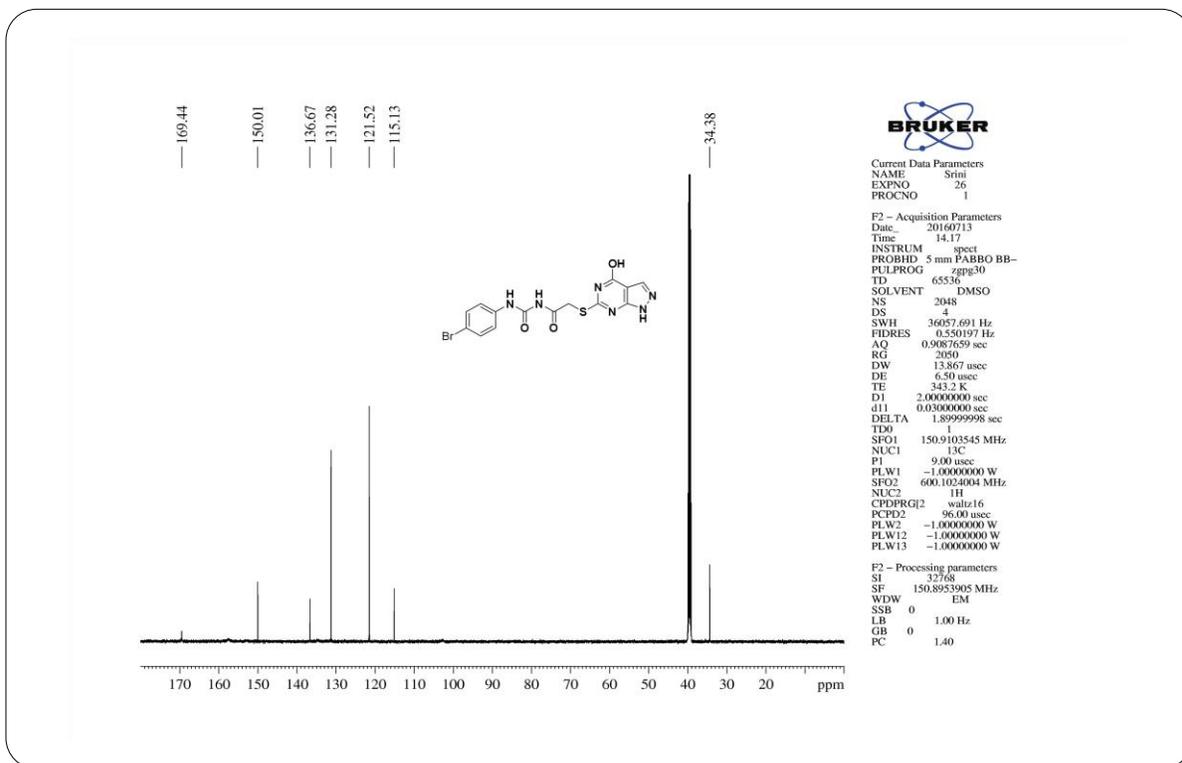
¹³C NMR Spectrum of Compound 9g
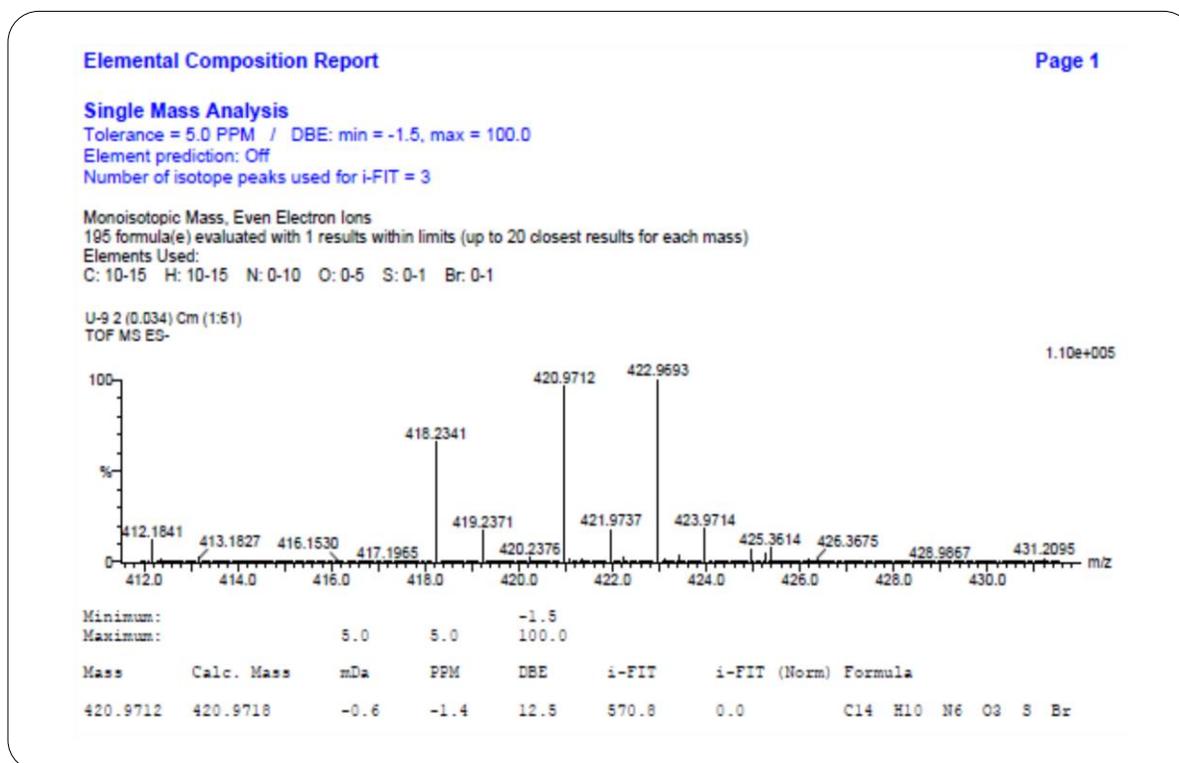
HRMS Spectrum of Compound 9g
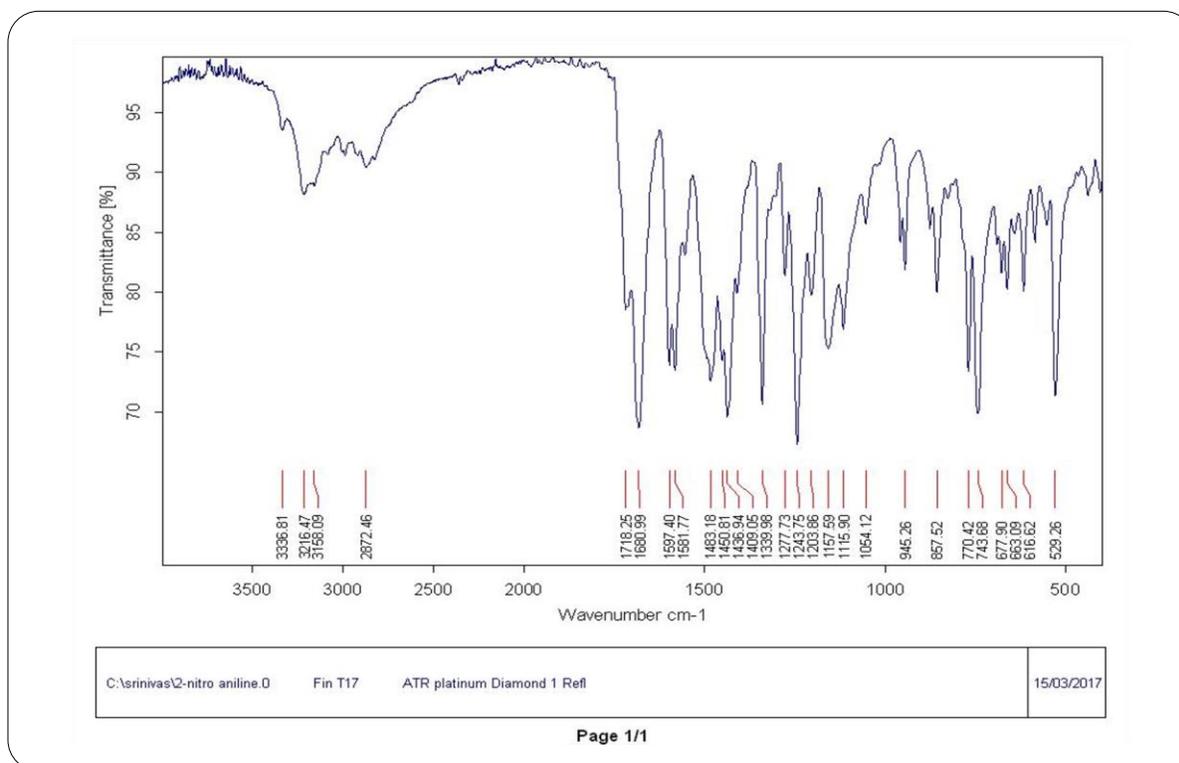
IR Spectrum of Compound 9h
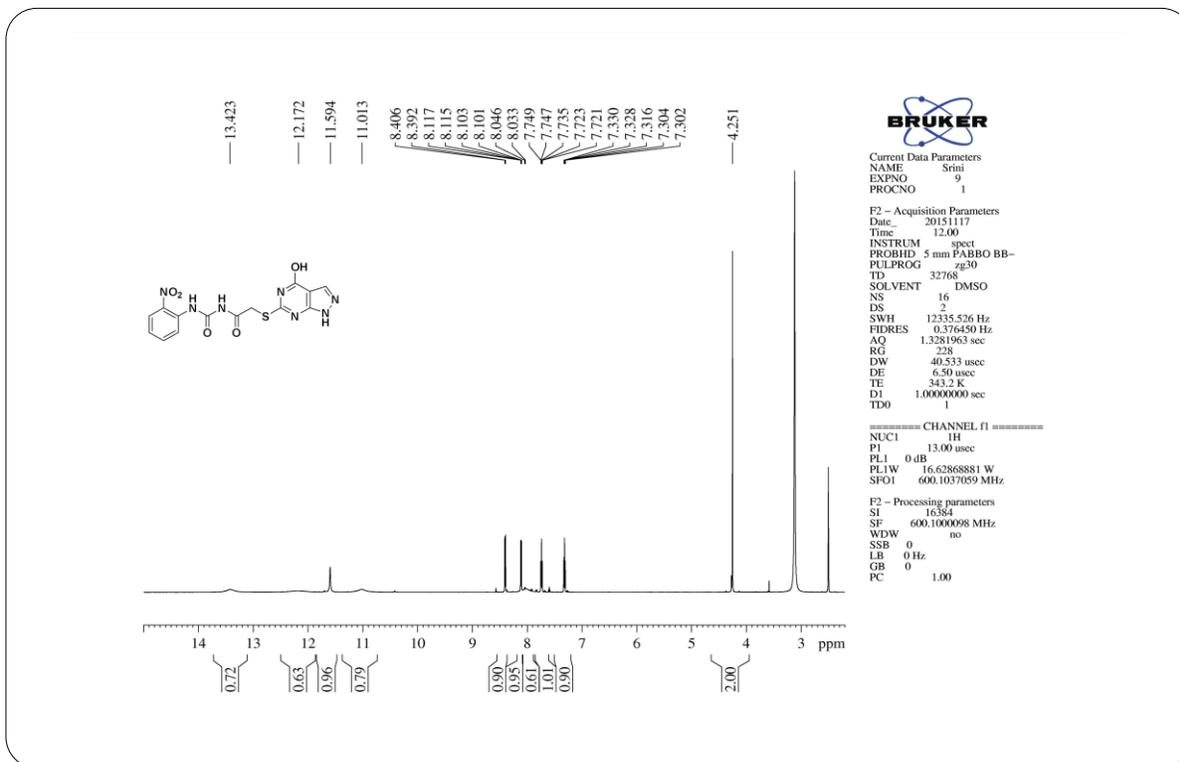
¹H NMR Spectrum of Compound 9h
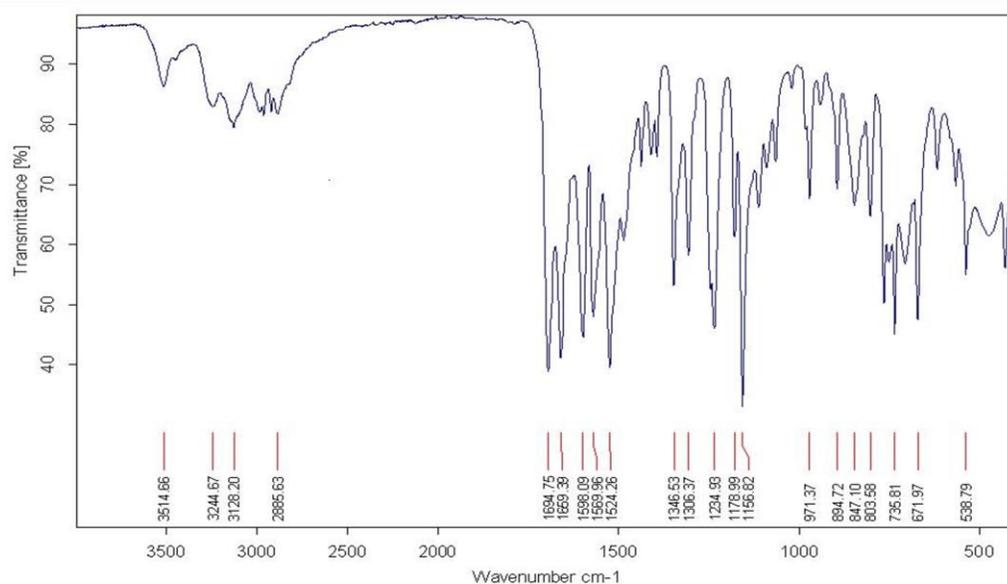
¹³C NMR Spectrum of Compound 9h

HRMS Spectrum of Compound 9h

IR Spectrum of Compound 9i
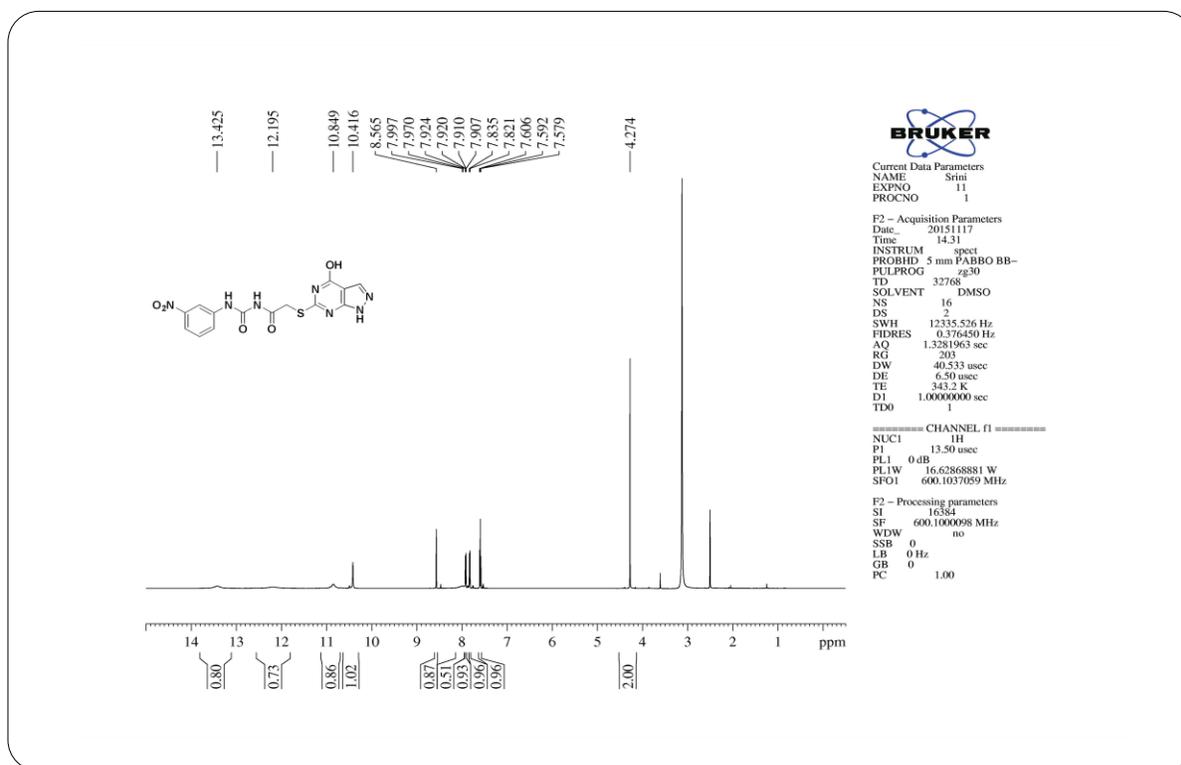
¹H NMR Spectrum of Compound 9i
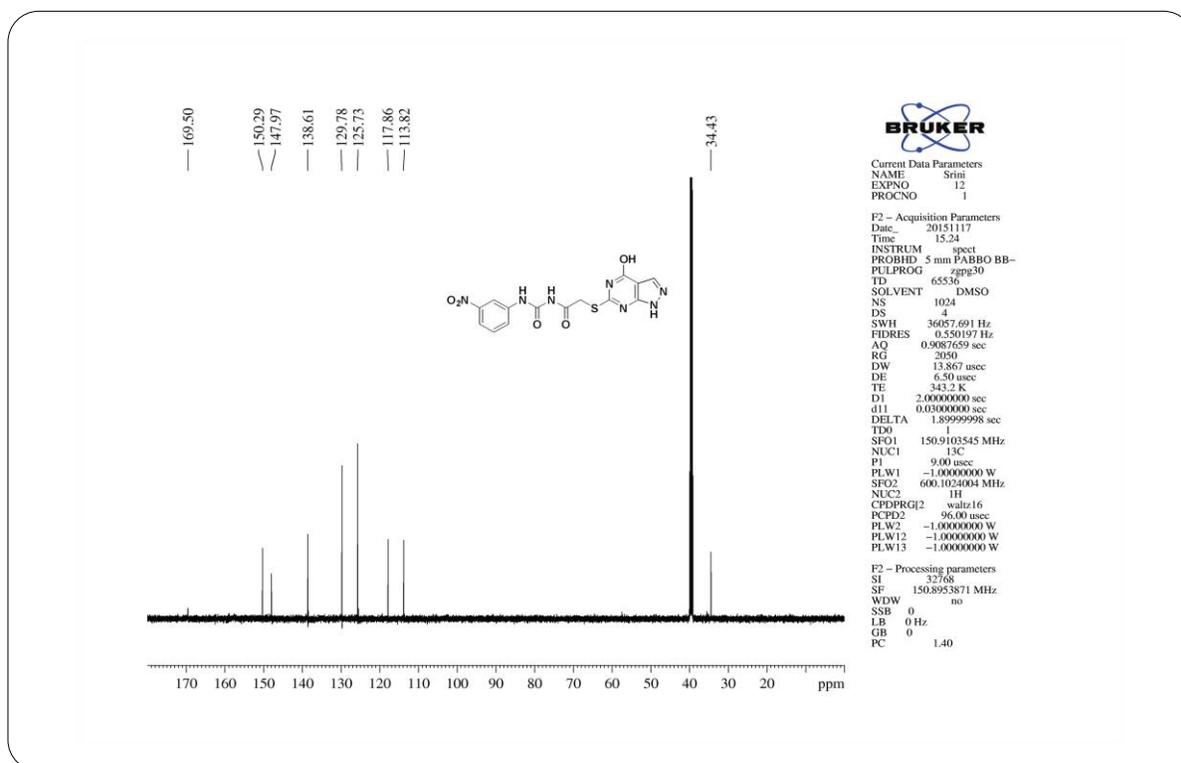
¹³C NMR Spectrum of Compound 9i
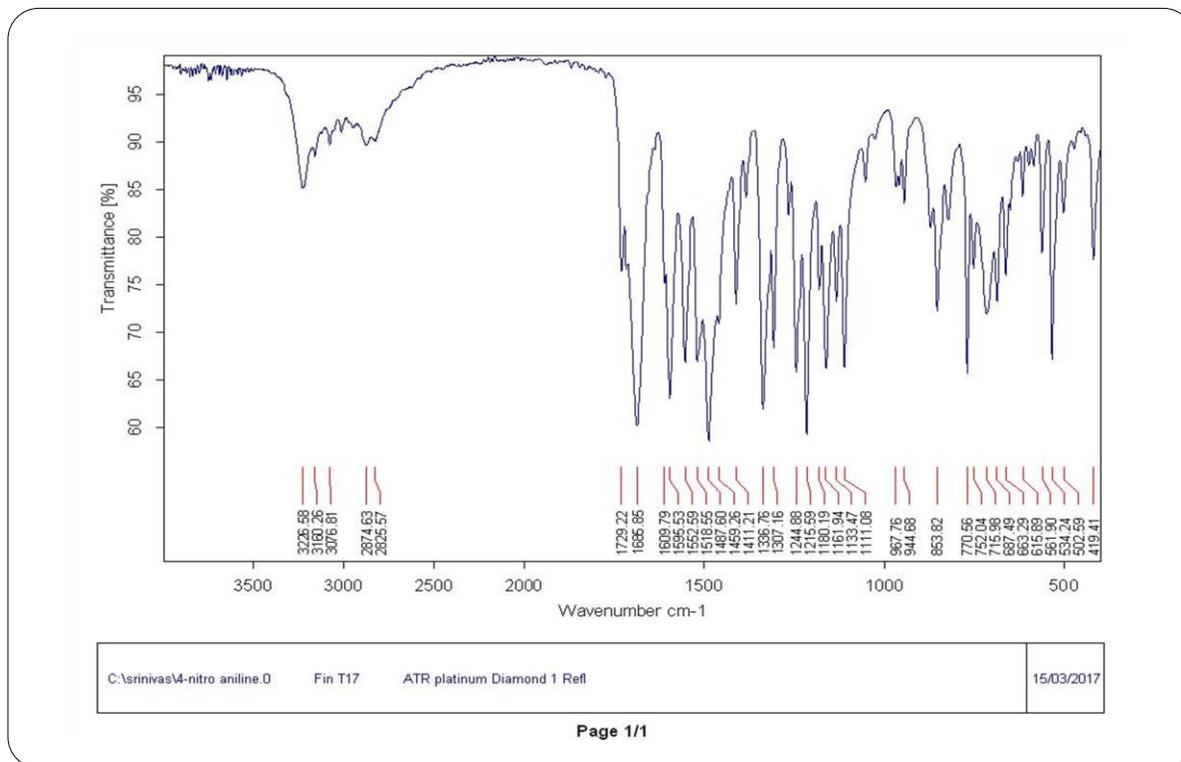
IR Spectrum of Compound 9j
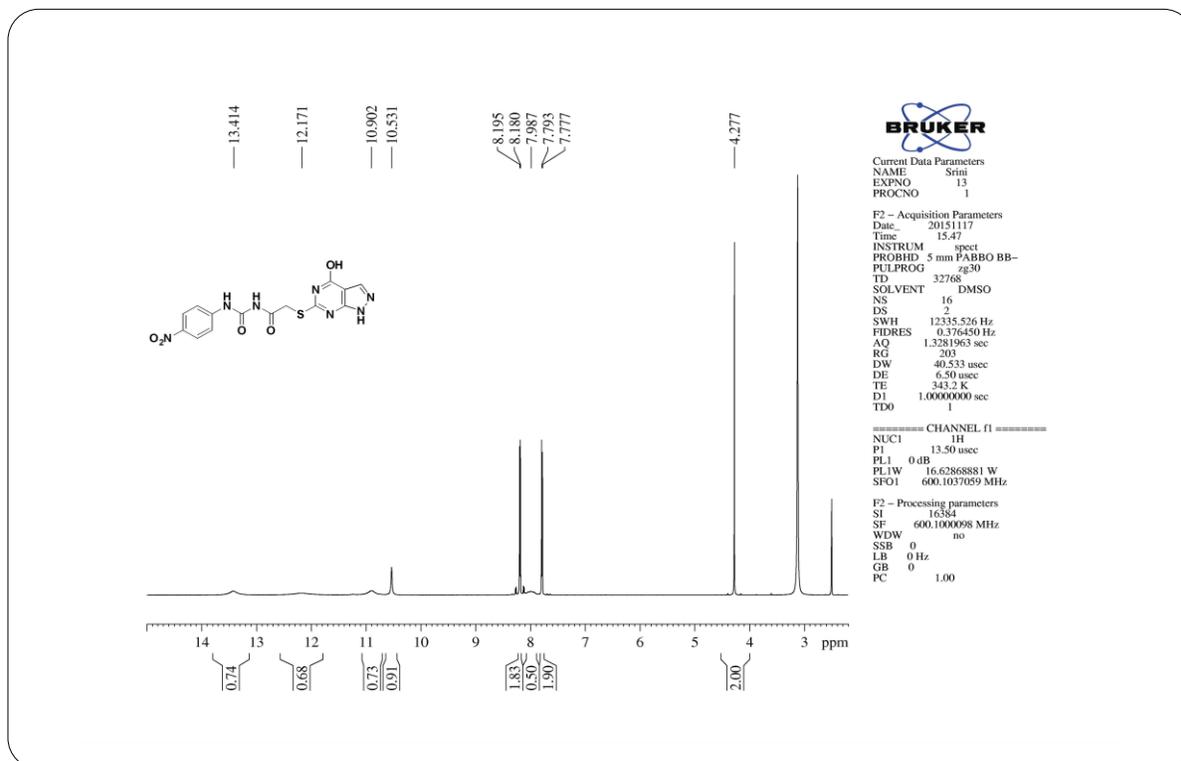
¹H NMR Spectrum of Compound 9j
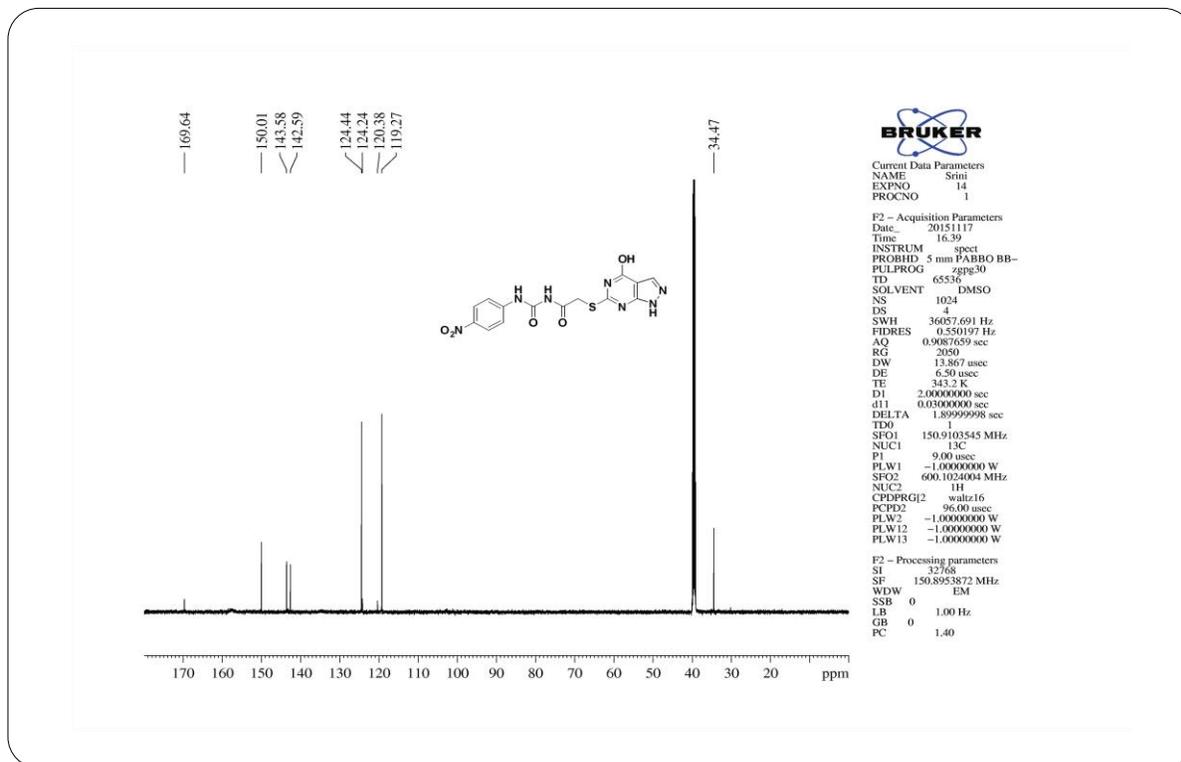
¹³C NMR Spectrum of Compound 9j
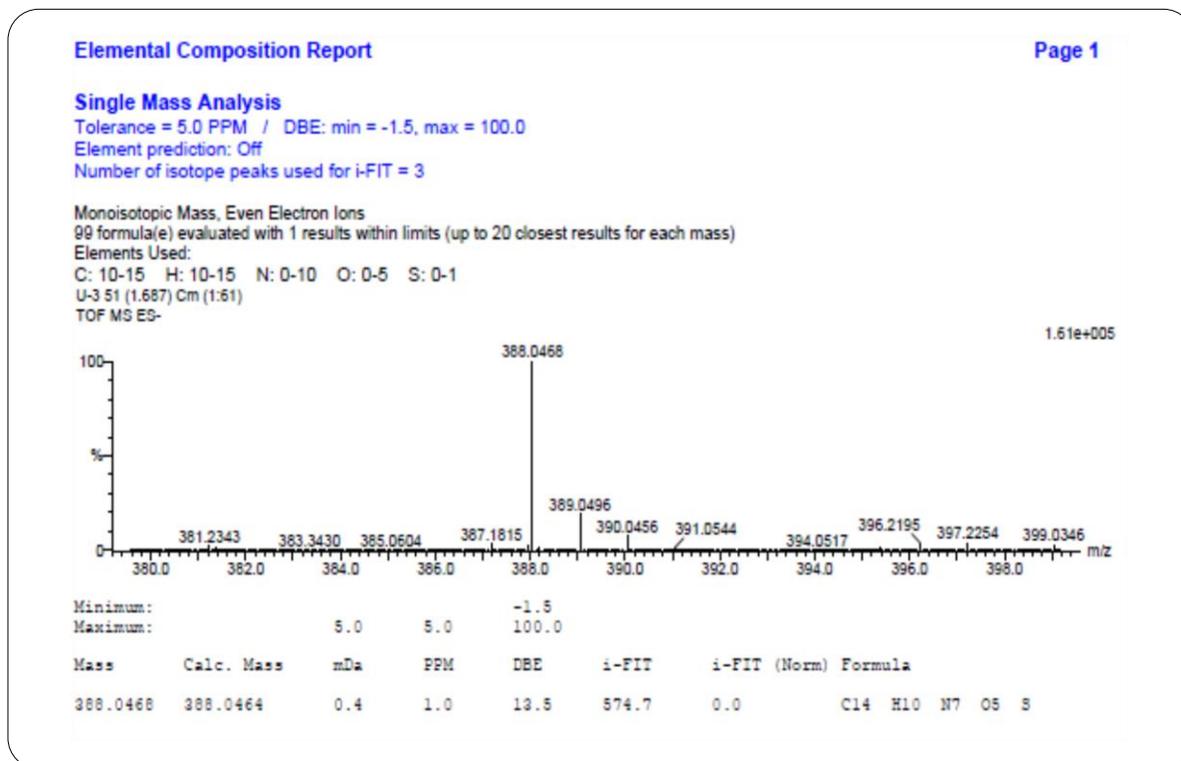

**HRMS Spectrum of Compound 9j**
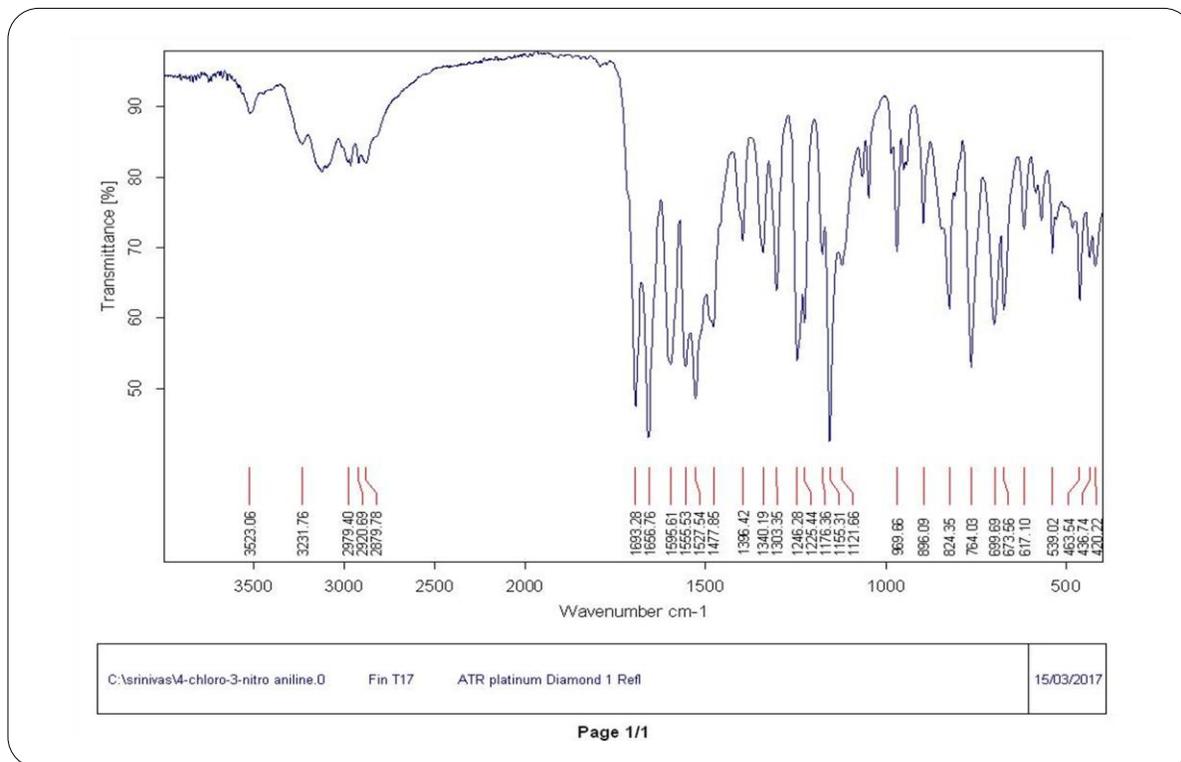
IR Spectrum of Compound 9k
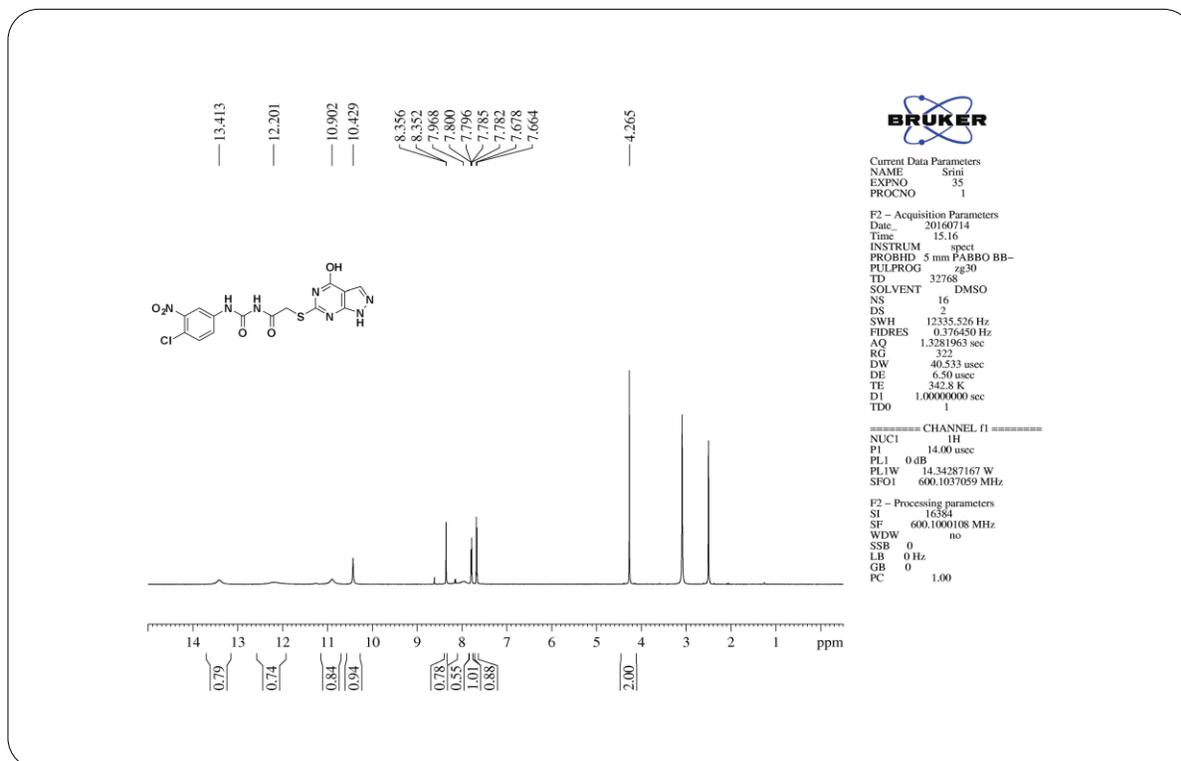

¹H NMR Spectrum of Compound 9k
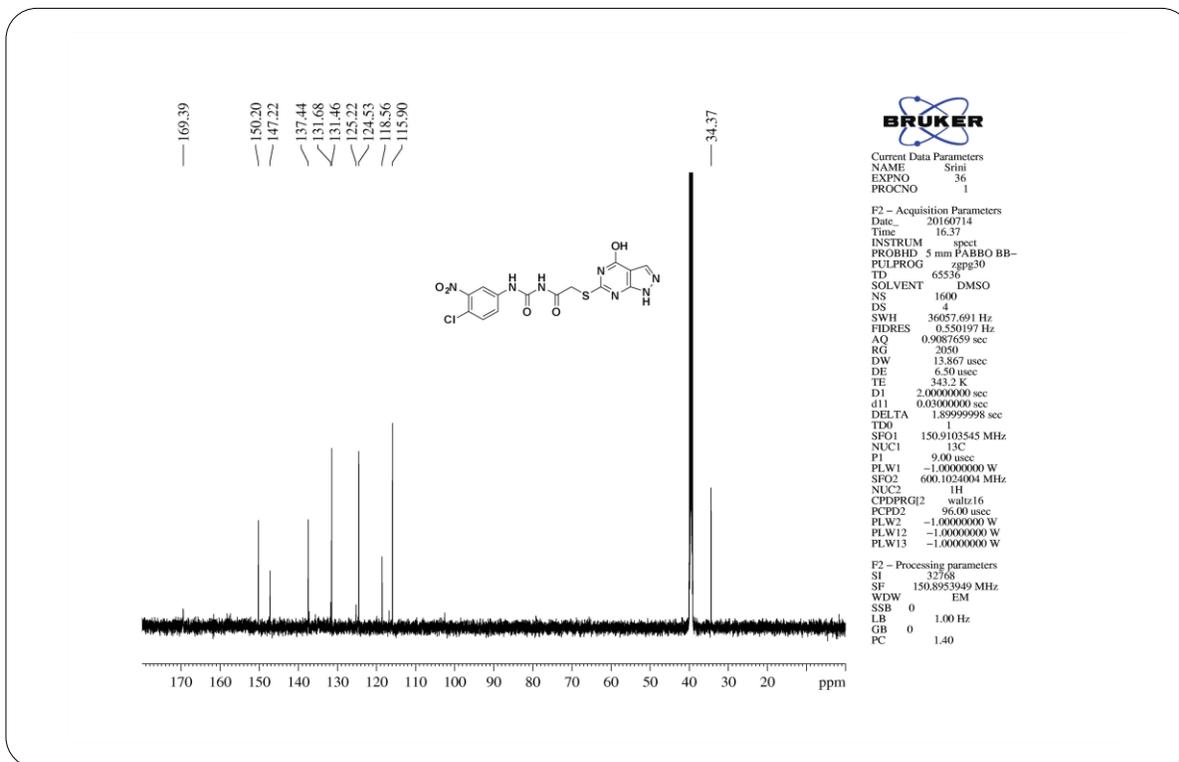
¹³C NMR Spectrum of Compound 9k
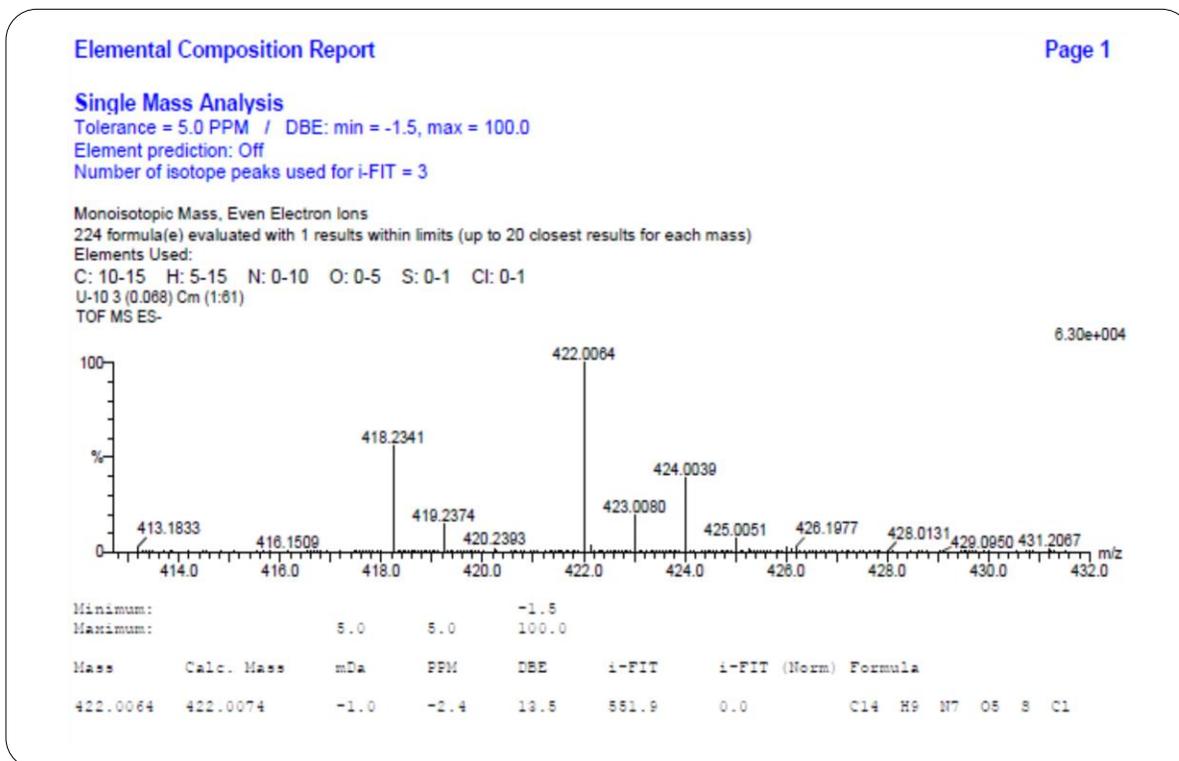
HRMS Spectrum of Compound 9k
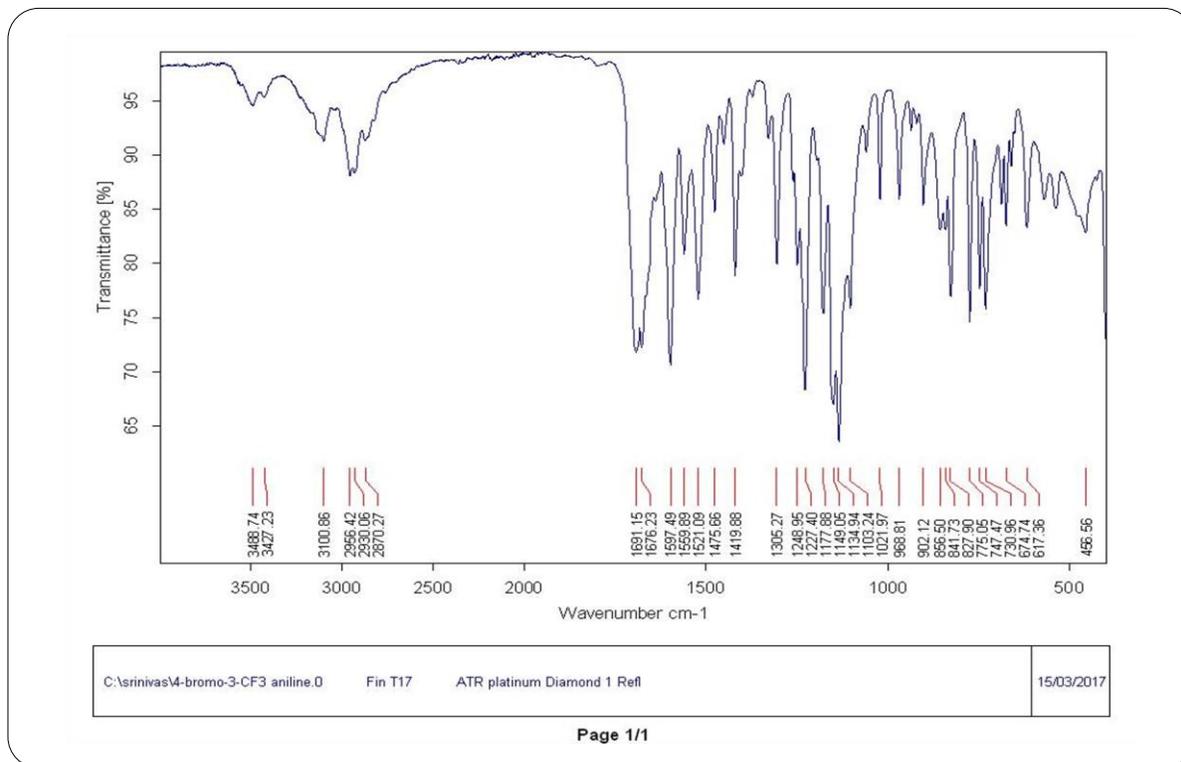
IR Spectrum of Compound 9l
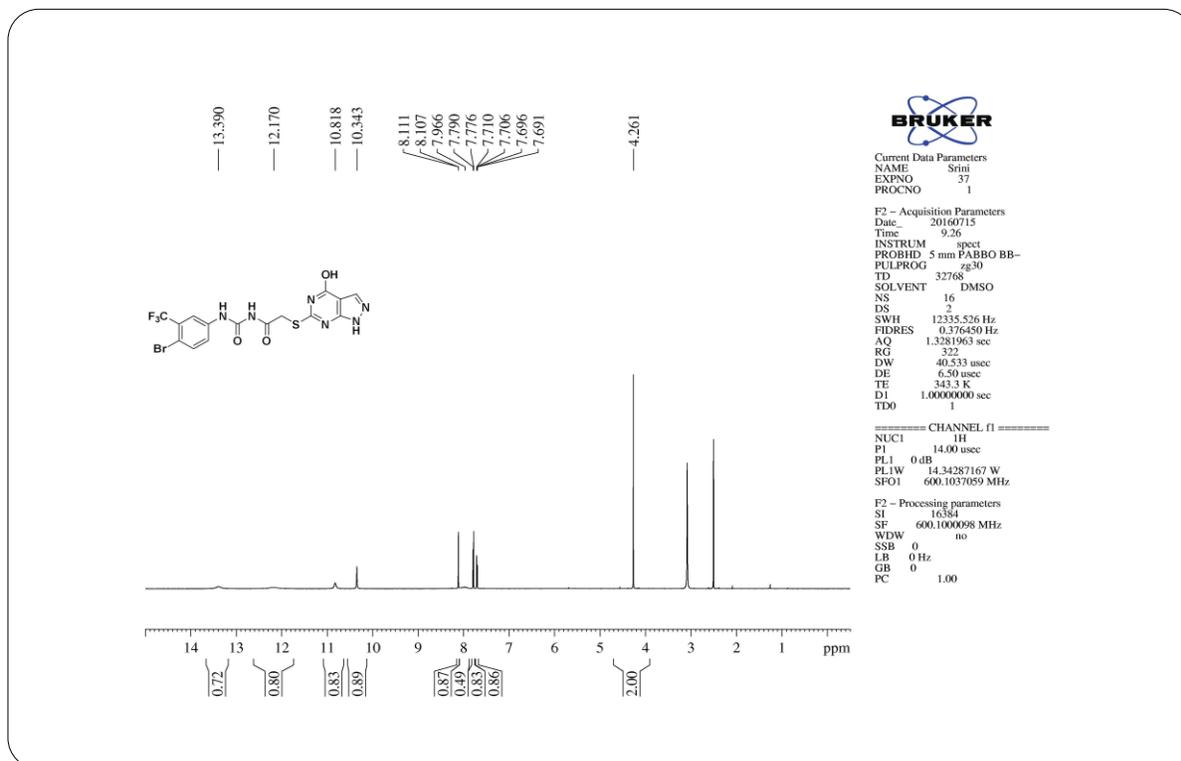
¹H NMR Spectrum of Compound 9l
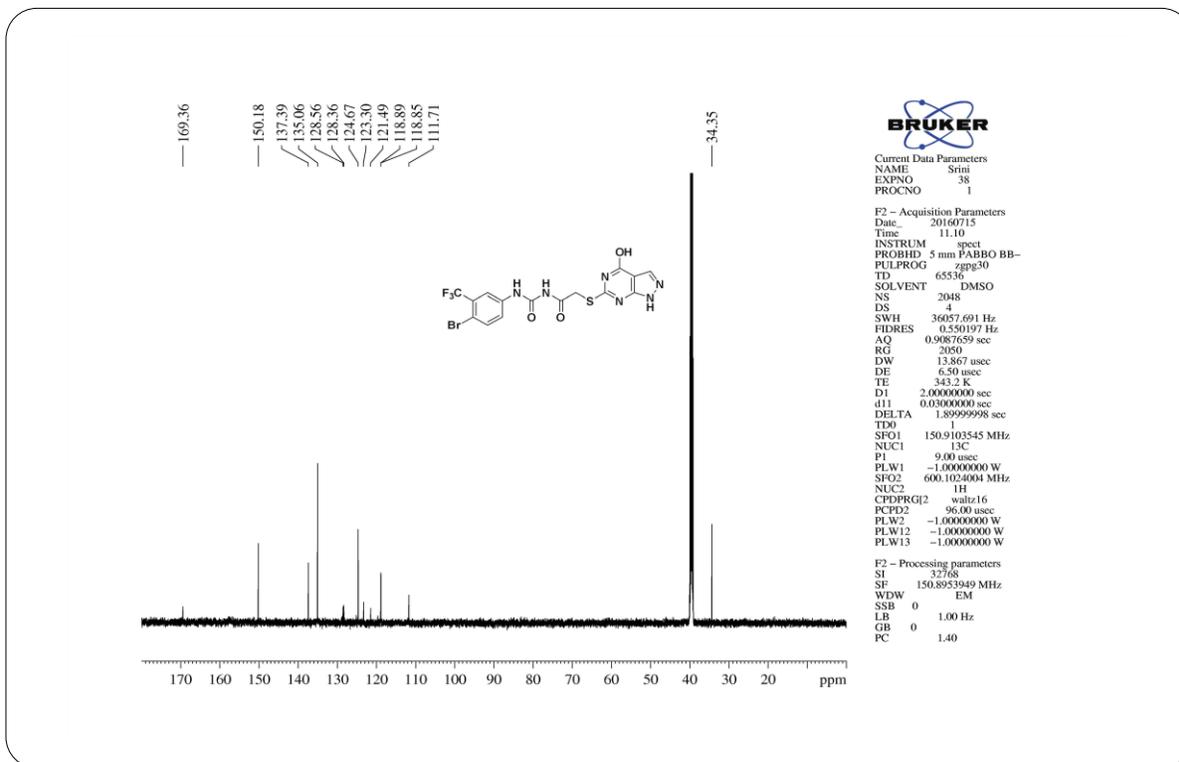
¹³C NMR Spectrum of Compound 9l
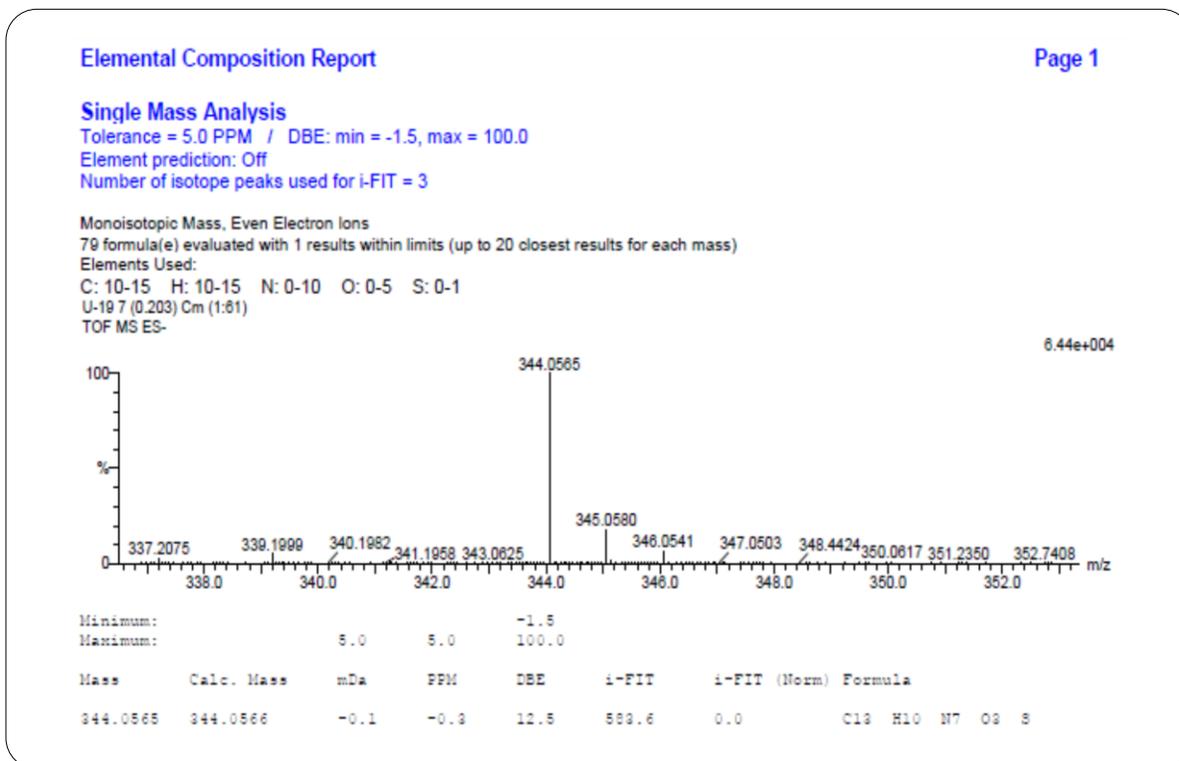
HRMS Spectrum of Compound 9l
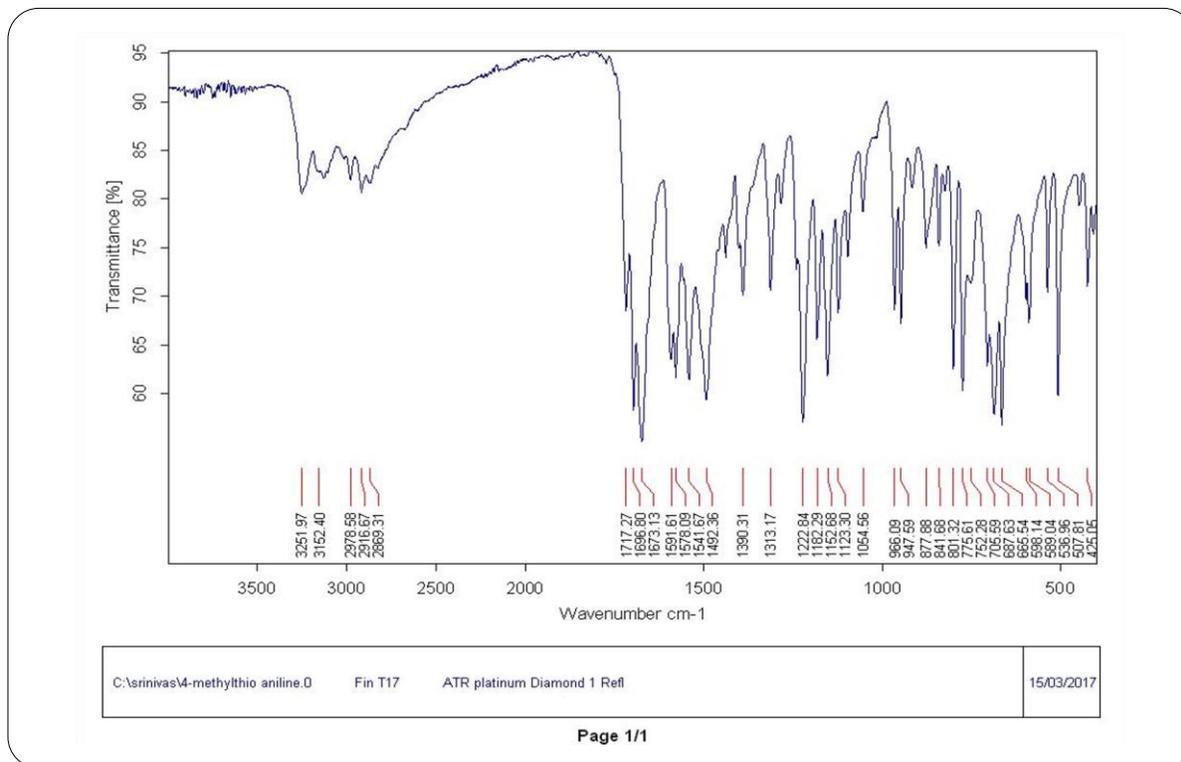
IR Spectrum of Compound 9m
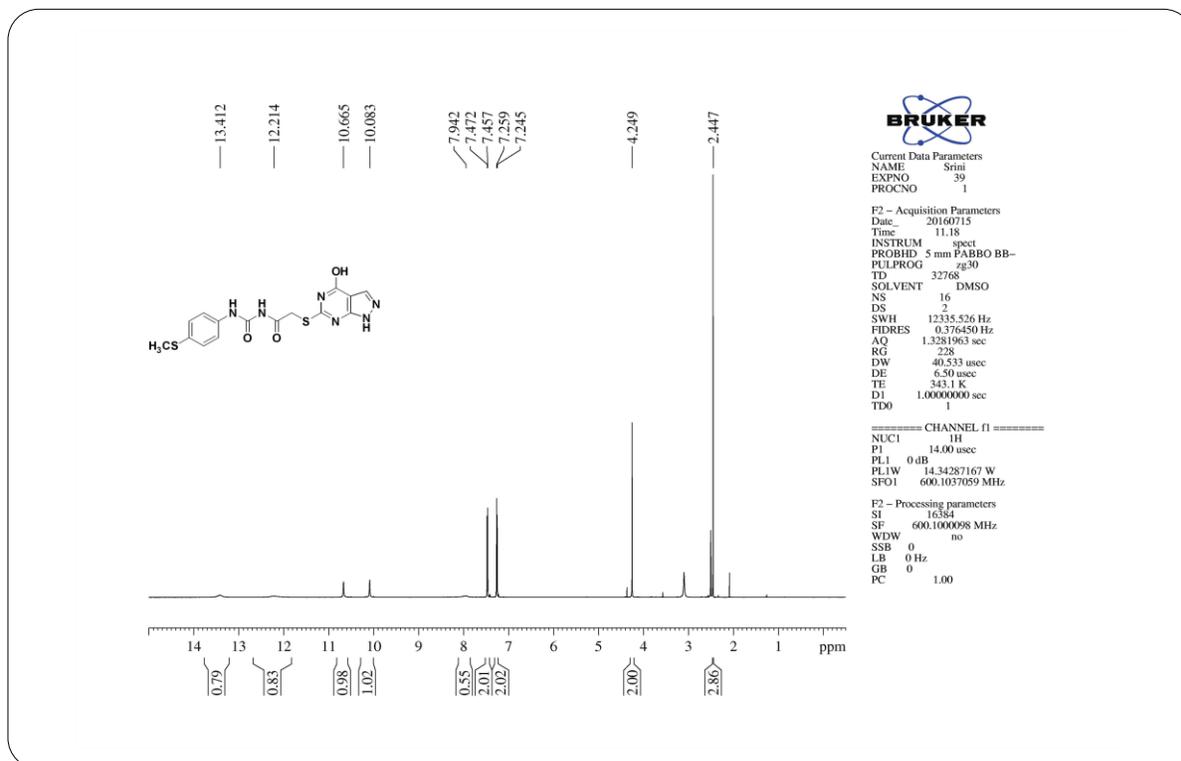
^{1}H NMR Spectrum of Compound 9m
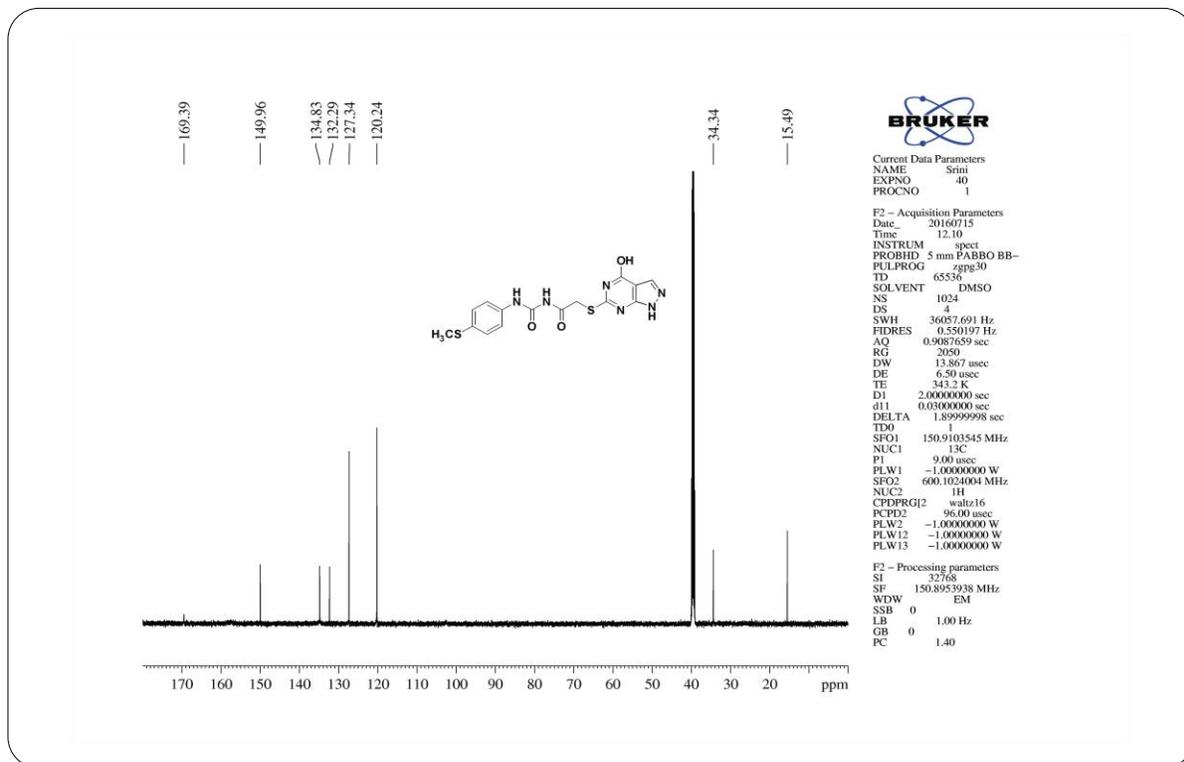
^{13}C NMR Spectrum of Compound 9m
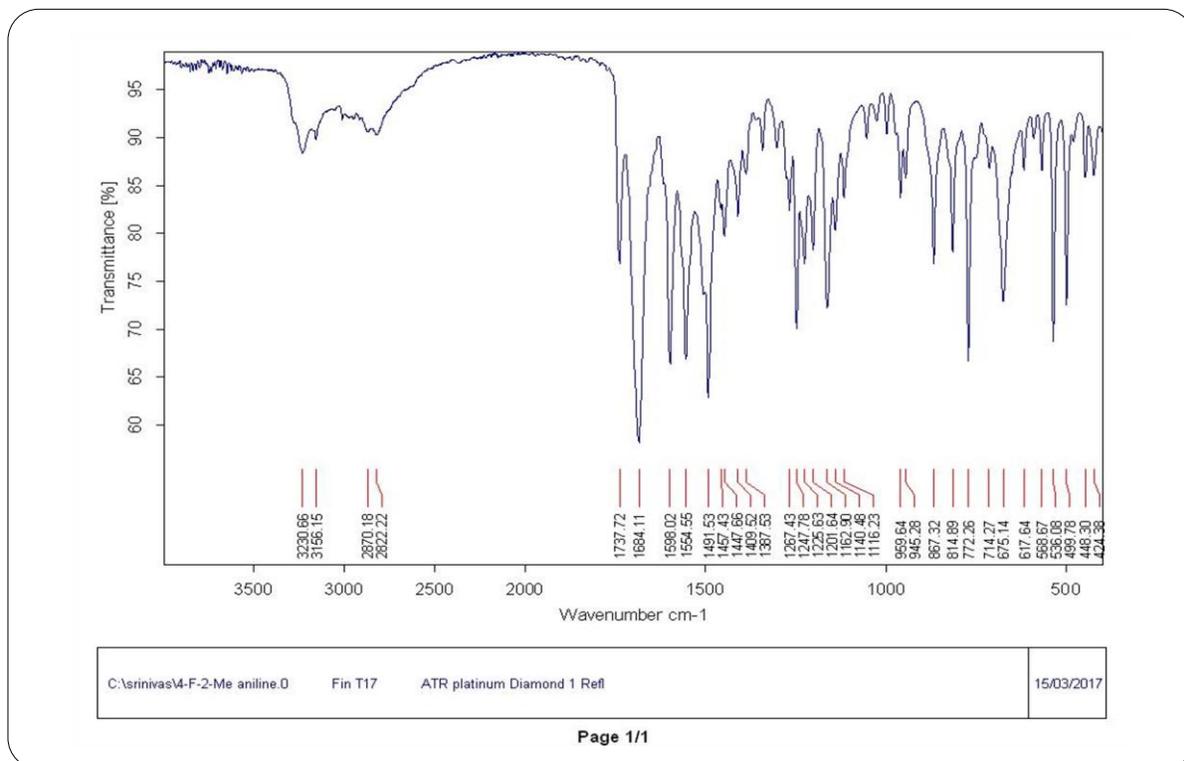
IR Spectrum of Compound 9n
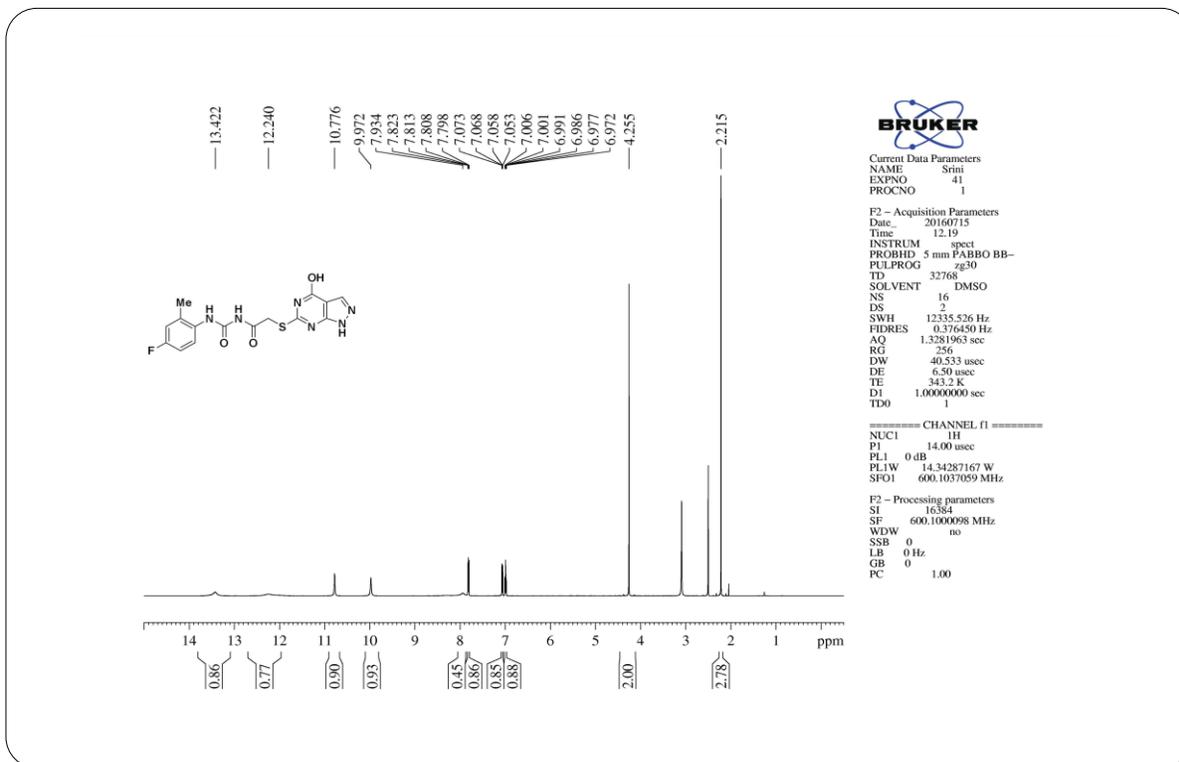
^{1}H NMR Spectrum of Compound 9n

^{13}C NMR Spectrum of Compound 9n

HRMS Spectrum of Compound 9n

IR Spectrum of Compound 9o

¹H NMR Spectrum of Compound 9o
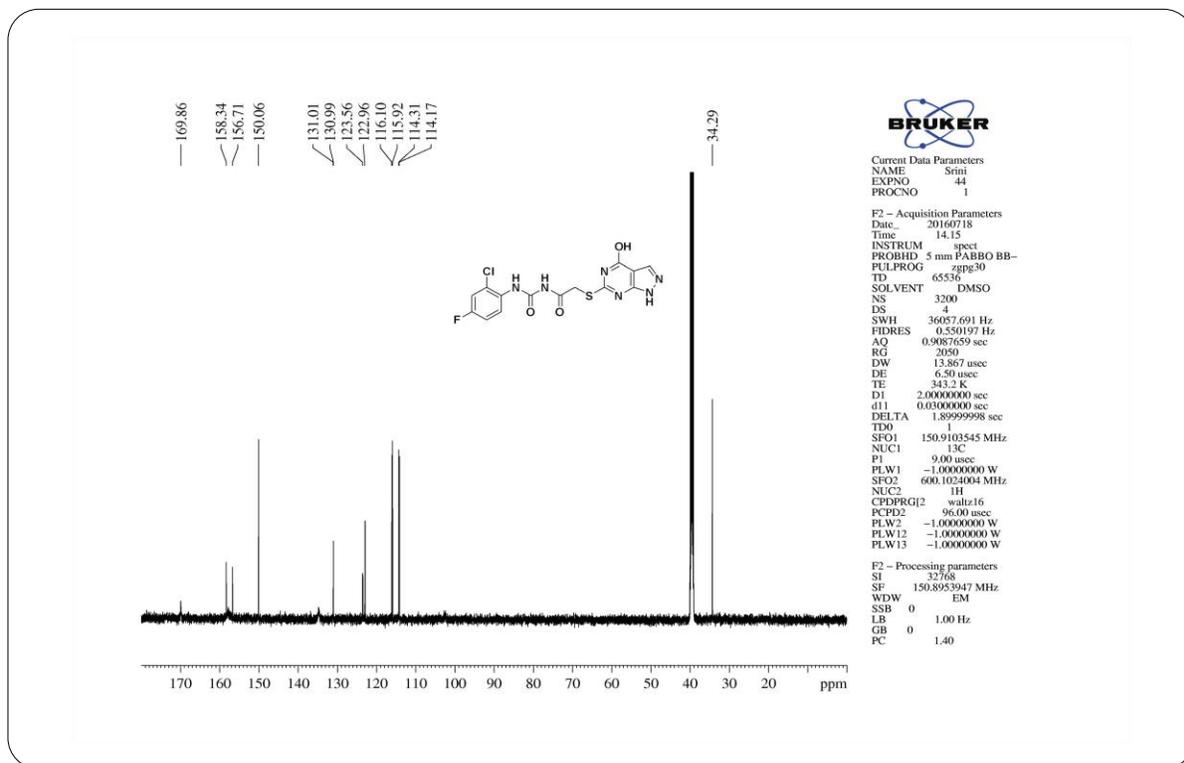
¹³C NMR Spectrum of Compound 9o

Elemental Composition Report   Page 1

Single Mass Analysis
Tolerance = 5.0 PPM   /   DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
416 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 10-15   H: 5-15   N: 0-10   O: 0-5   F: 0-1   S: 0-1   Cl: 0-1
U-14 56 (1.855) Cm (1:61)
TOF MS ES-
                                                                                              1.40e+005
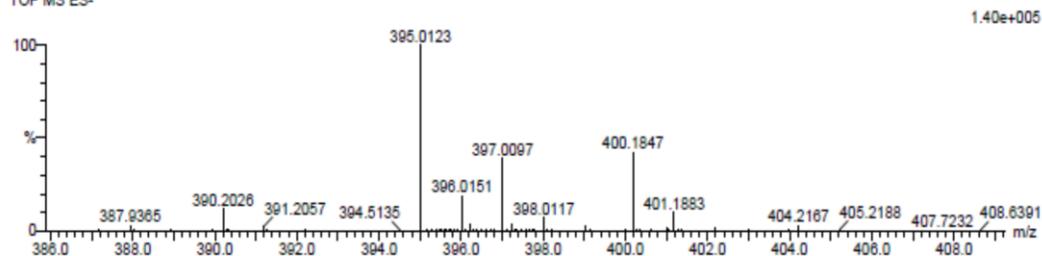
Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
395.0123	395.0129	-0.6	-1.5	12.5	547.3	0.0	C14 H9 N6 O3 F S Cl

HRMS Spectrum of Compound 9o
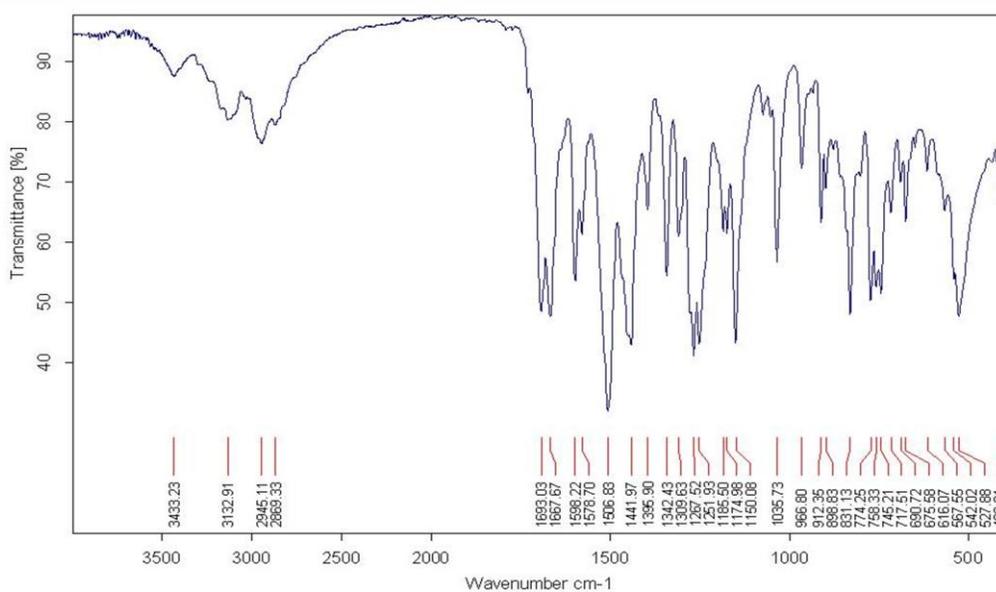
C:\srinivas\4-methoxy-2-nitro aniline.0   Fin T17   ATR platinum Diamond 1 Refl   15/03/2017

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IR Spectrum of Compound 9p
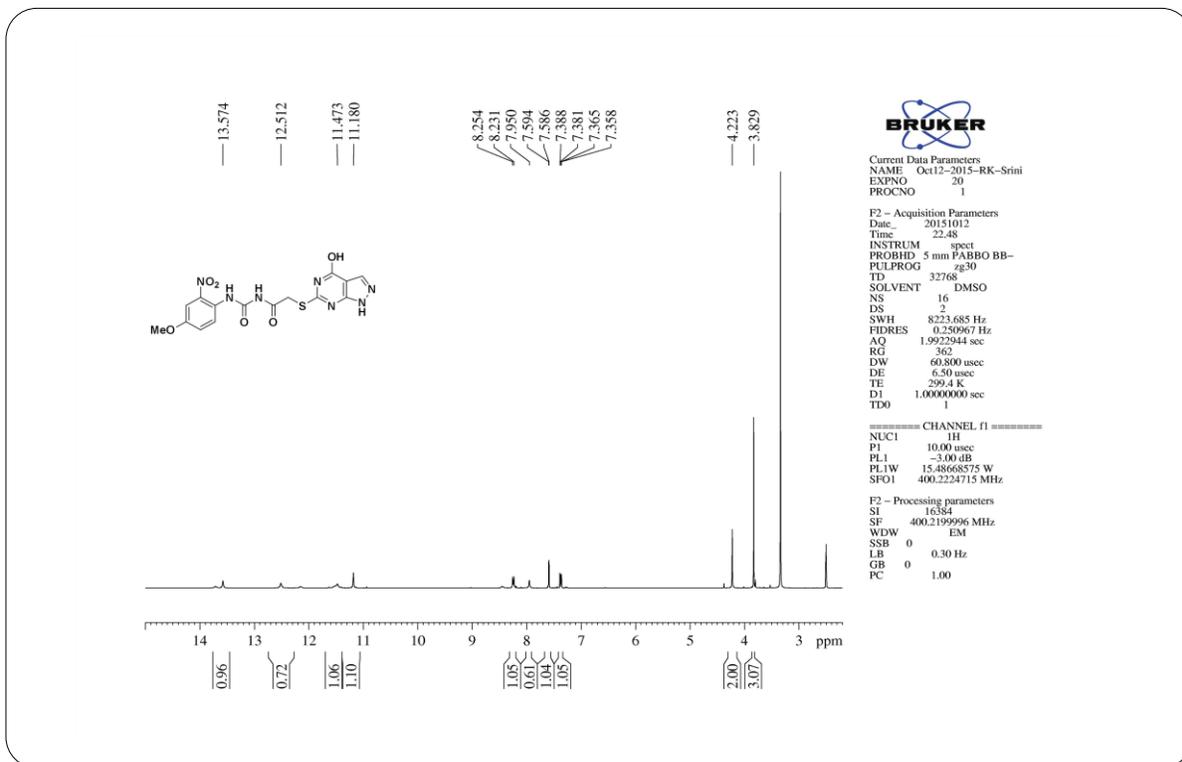
¹H NMR Spectrum of Compound 9p
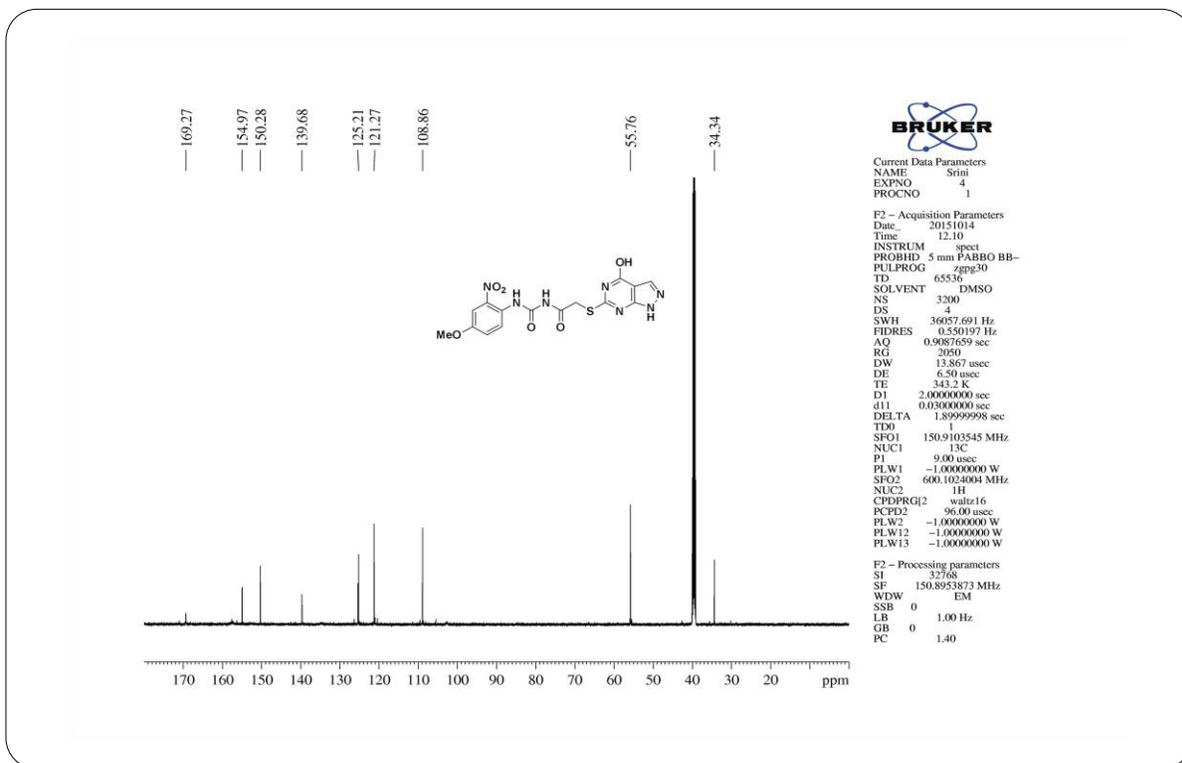

¹³C NMR Spectrum of Compound 20
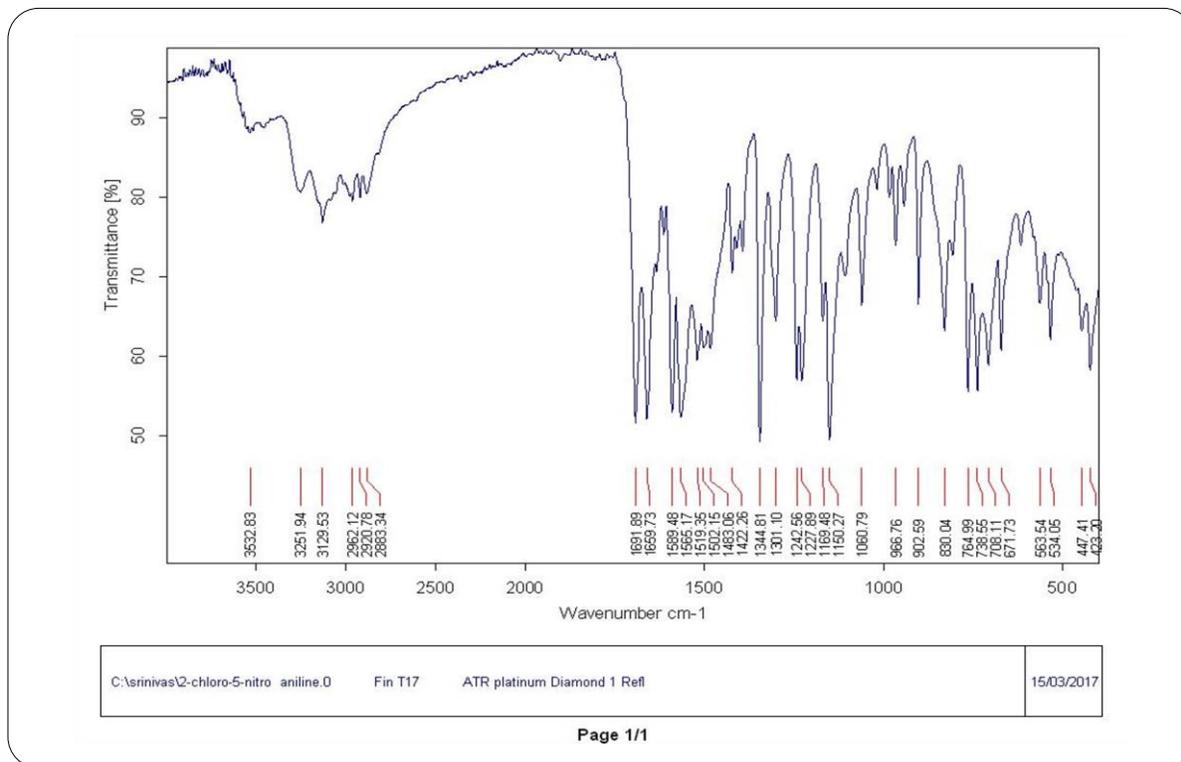
IR Spectrum of Compound 9q
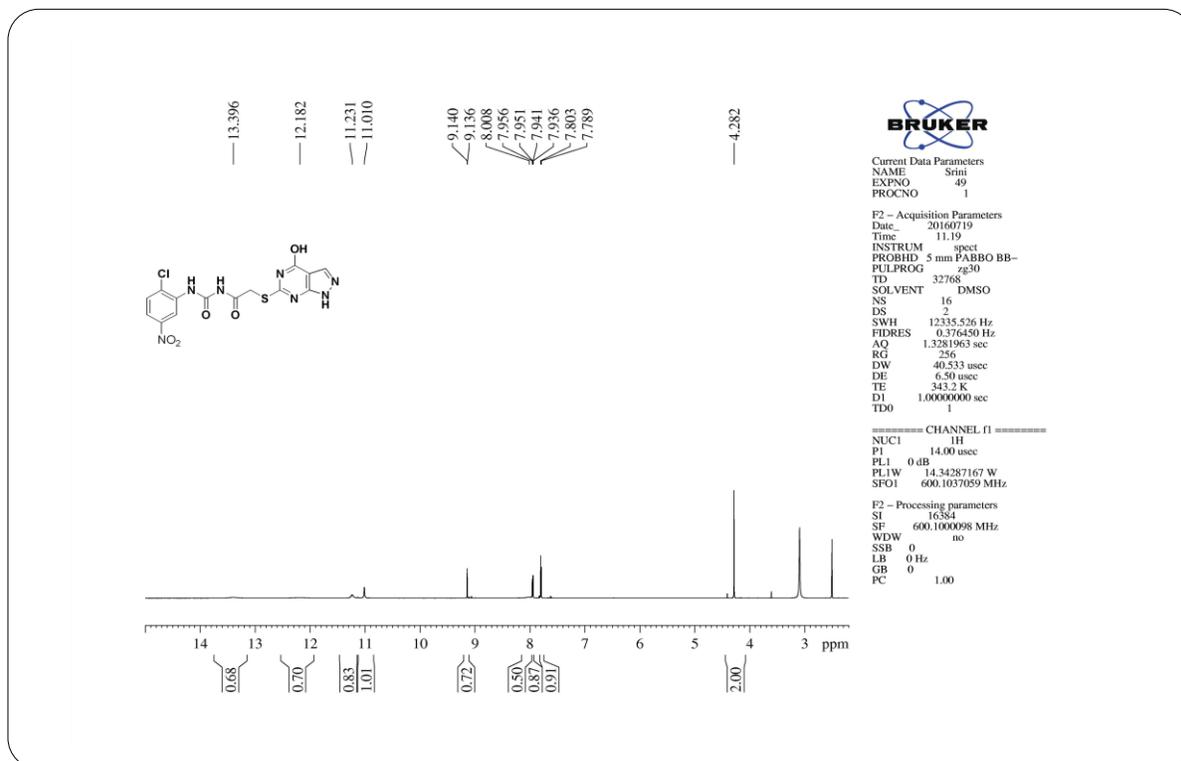

**¹H NMR Spectrum of Compound 9q**
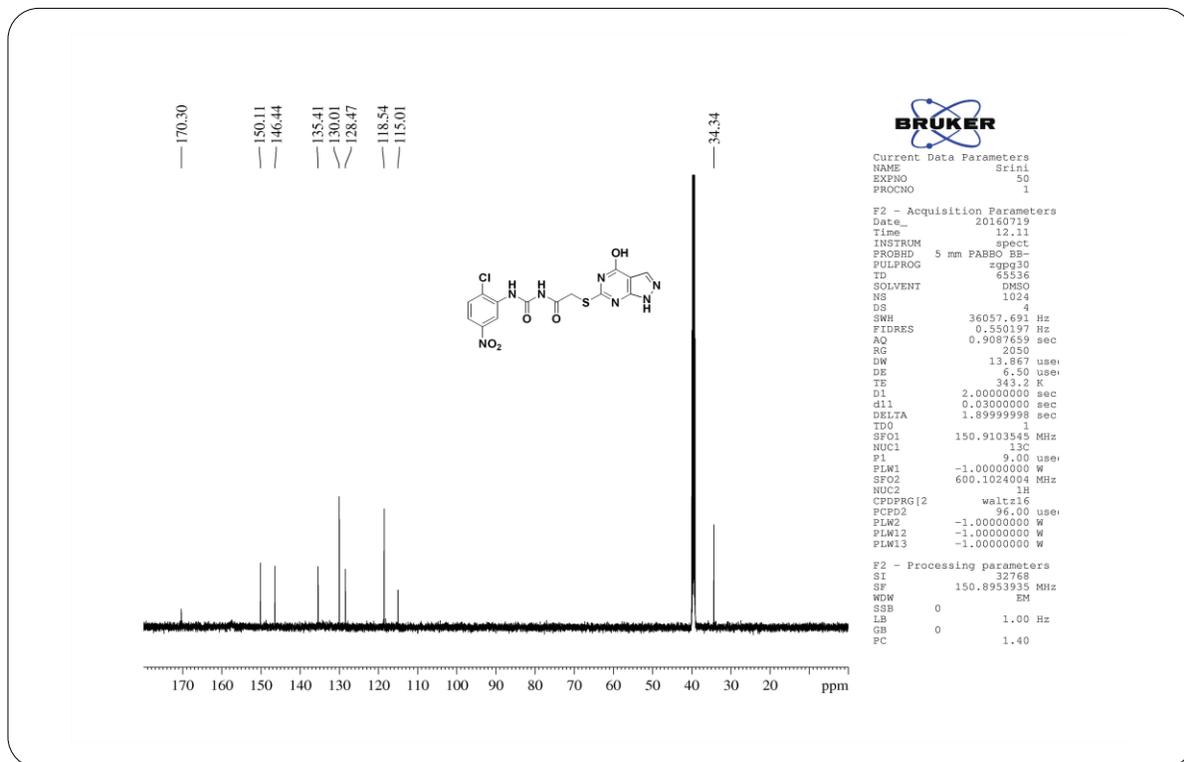
**¹³C NMR Spectrum of Compound 9q**
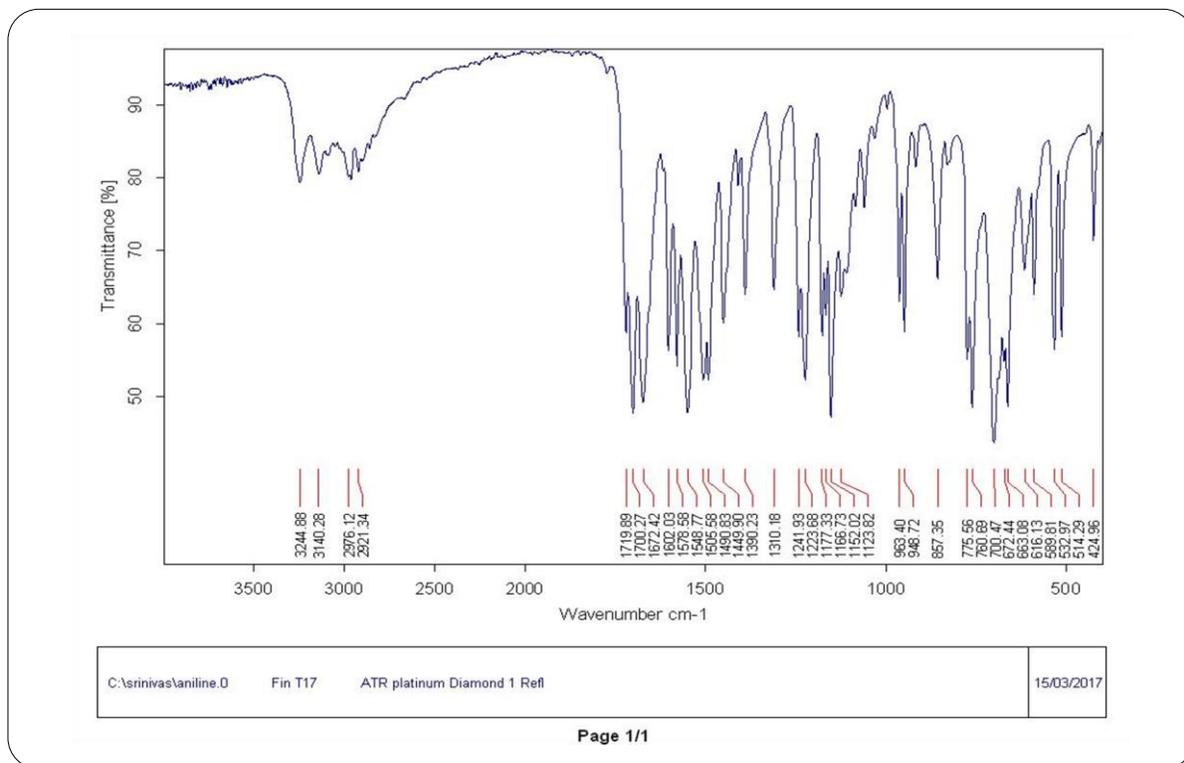
IR Spectrum of Compound 9r
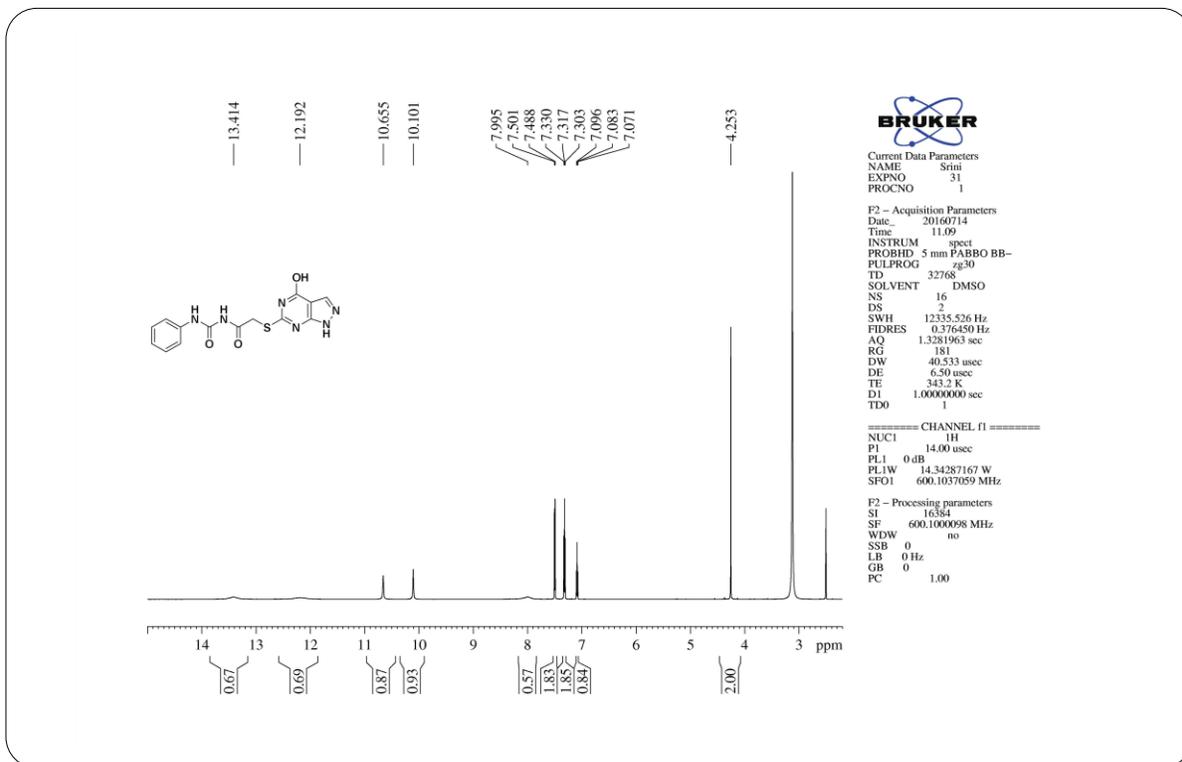
¹H NMR Spectrum of Compound 9r
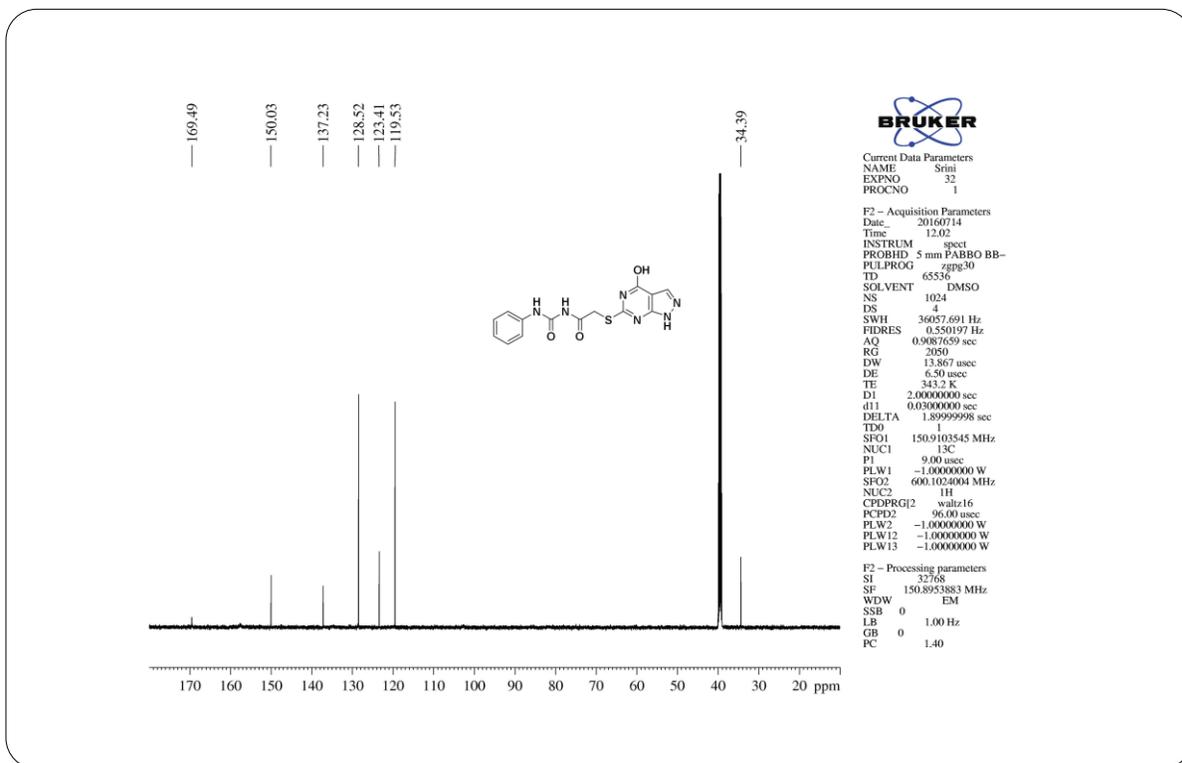

¹³C NMR Spectrum of Compound 9r
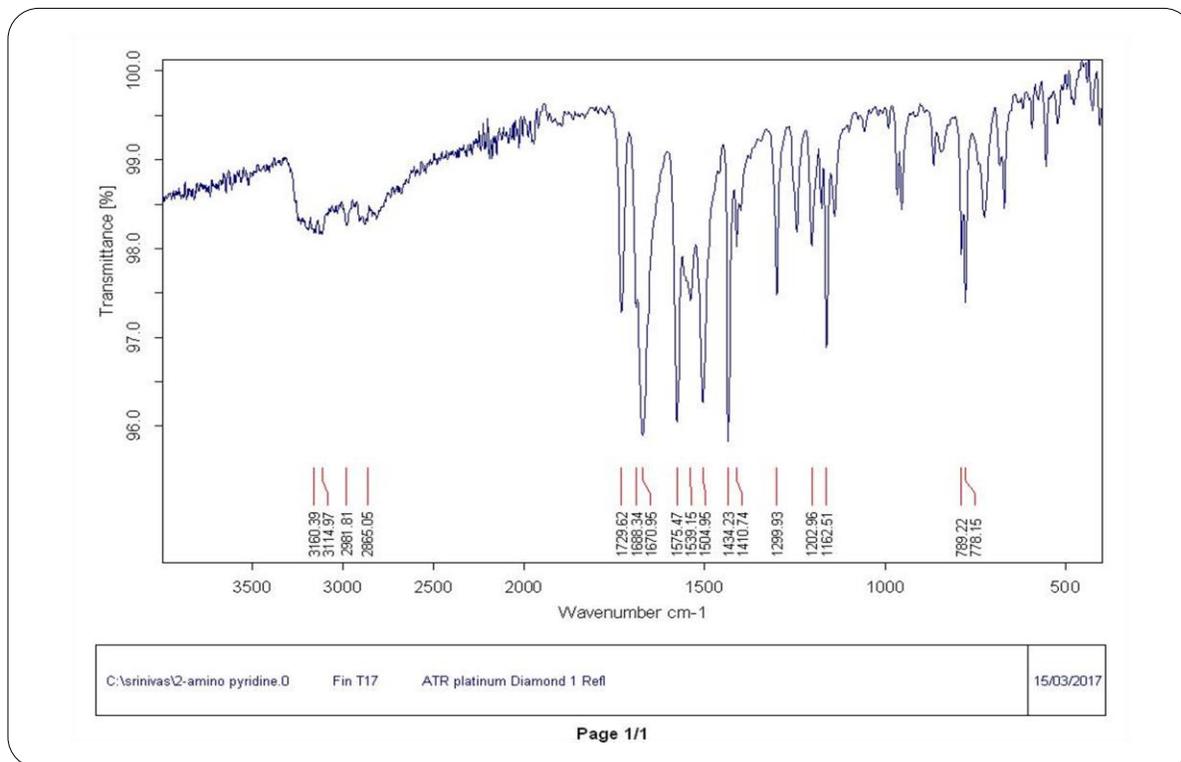
IR Spectrum of Compound 9s
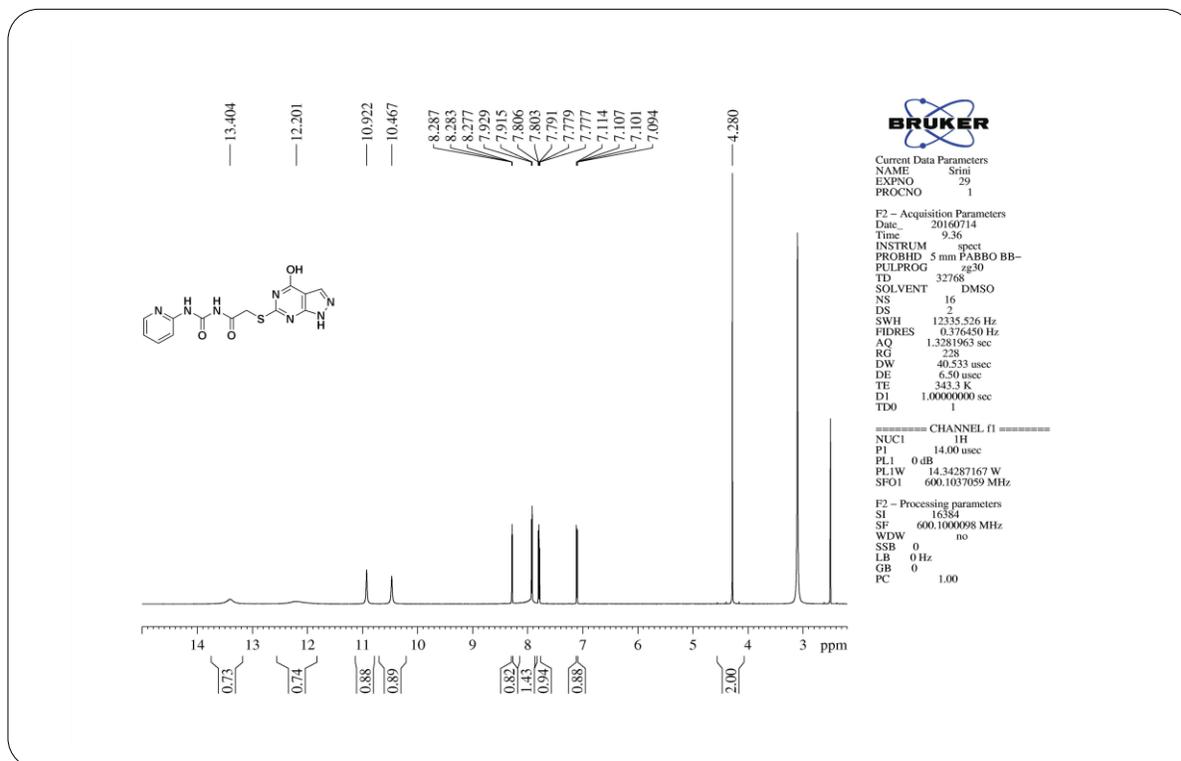
¹H NMR Spectrum of Compound 9s
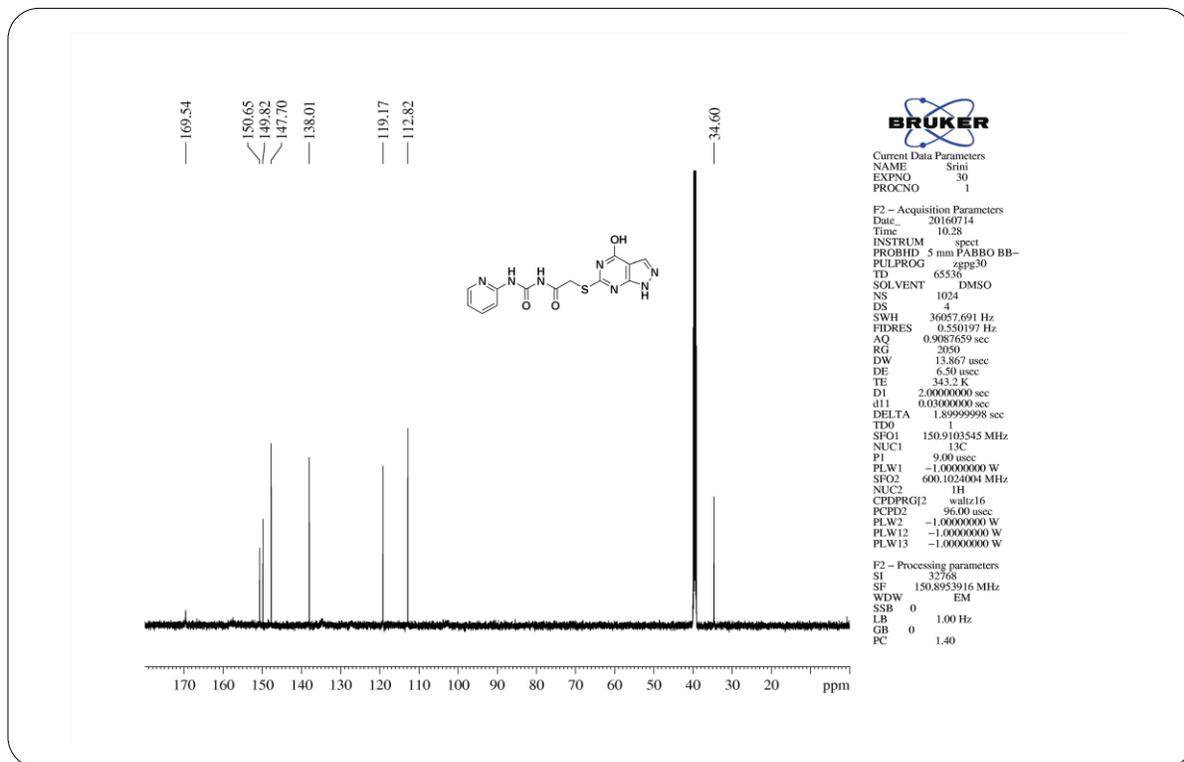
¹³C NMR Spectrum of Compound 9s

HRMS Spectrum of Compound 9s