Heterocyclic Compounds and Biological Applications

Edited by M.R.Jayapal

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Preface

This text book is considered essential reading to all scientists involved in synthesis of heterocyclic compounds and biological applications who wish to keep abreast with the recent and important developments in the field. This innovative text is organized in a way that discourages rote memorization, by emphasizing what functional groups do rather than how they are made, highlighting mechanistic similarities and tying synthesis and reactivity together. The text balances coverage of traditional topics with organic chemistry, recognizing the importance of organic topics to today's students.

During the past decade, advances in synthetic chemistry have been one of the driving forces in the development of new classes of novel heterocyclic compounds for applications in biology and medicine. Despite the impressive scientific efforts towards the development of novel heterocyclic compounds, at the current time there is a tremendous need for standardizing cellular and molecular protocols used in biological applications. Since synthetic chemistry field is expanding and becomes part of the curricula in many universities, the present book with protocols will be extremely useful for the researchers, students and medical doctors.

In thinking about how reactions in nature could be integrated with modern synthetic chemistry, I came to see that another approach was required, so I decided to undertake the writing of a textbook that would differ from others in two ways. First, the reactions of organic molecules would be organized and presented by the mechanism of the transformation. Second, the reactions of metabolic and biosynthetic processes would be integrated with the reactions found in most other texts.

This book is an attempt to amalgamate biological, mechanistic and synthetic organic chemistry. It is written by a synthetic organic chemist who happens to also think deeply about mechanism and understands the importance of knowing

structure and reactivity to synthetic organic chemistry. I liked the project especially because I liked the book, and I thought way of dealing with synthesis and mechanism together was an approach sufficiently different that it might be the "whack on the side of the head" that could be useful in generating new thought patterns in students of organic chemistry.

At many points, we have tried to explain concepts from the very beginning level so that individuals who do not recall their basic chemistry can still develop insights into and understand the origin and limits of modeling calculations and correlation equations. We have also incorporated numerous references throughout the text to help people who want to follow particular topics further. Finally, by including many illustrative examples, we have attempted to show biological practitioners how to arrive at quantitative results for particular cases of interest to them. Hence, this book should serve as a text for introductory courses in organic chemistry. We hope that with this textbook, we can make a contribution to the education of synthetic scientists and biological scientist and, thus, to a better protection of our society.

Acknowledgements

Those who have ever written textbooks know that the authors are not the only ones who play an important role in the realization of the final product. Without the help of many of our co-workers, colleagues, it would have taken another millennium to finish this book.

I would like to thank Science Publishing group USA for enabling me to publish this book. God, for giving me the opportunity to live each day despite the adversities that are taken on the road. I don't have words to thank you despite your early absence always give me strength and energy to move forward. Above all I want to thank the my Parents M. Yesuratnam and M. Saramma and my family members who supported and encouraged me in spite of all the time it took me away from them. It was a long and difficult journey for them.

I would like to express my gratitude to whoever inspire to write for this book and many people who saw me through this book; to all those who provided support, talked things over, read, wrote, offered comments, allowed me to quote their remarks and assisted in the editing, proofreading and design.

M. R. Jayapal

Edior-in-Chief

Contents

Chapter 1

Biological Activity of Novel Ureas and Thioureas Containing Bioactive Heterocycles

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Abstract

Heterocyclic compounds are widely distributed in nature and, along with their synthetic analogues, play an important role in agriculture and pharmacy. The general strategy of our studies was to design new bioactive compounds based on the combination of two or three types of active structural units: a 5-or 6-heterocyclic nucleus, a urea/thiourea bridge and alkyleneureido/thioureido groups. The plant growth regulatory and stress-protective activities of newly synthesized compounds were studied to determine how different structural combinations could influence the type of activity and its intensity. To obtain more detailed information about the specific effects of the tested compounds on the physiological, biochemical, cellular and genetic processes, different test-systems (plants and human lymphocytes) and experimental conditions were used.

Depending on the chemical structure, each class of compounds manifests important and multiple biological effects, such as reduction of the genotoxic effect of ultraviolet C (UV-C) and gamma-radiation; protective effect against herbicides and oxidative stress inductors; stimulating effect on the micro-propagation of higher plants; anti-senescence, anti-phytoviral and plant growth-regulating activities.

These beneficial biological effects give us the basis to recommend the newly synthesized bioactive heterocyclic ureas/thioureas for further testing and use in practice.

Keywords

Cytokinins, Herbicides, Heterocycles, Plant Growth Regulators, Phytophores, Stress-Protectors, (Thio)Ureas

Abbreviations

RHE = relative herbicidal efficiency; Chl = chlorophyll; FAAC = free amino acid content; AsPO = ascorbate peroxidase activity; CAT = catalase activity;

GPO = guaiacol peroxidase activity; RNase = total ribonuclease activity, MDA = malondialdehyde

1.1 Introduction

The search for and the extraction/synthesis of new substances with new chemical and biological properties is a problem that has received a lot of attention over the years and continues to be of present interest. The synthetic modification of bioactive natural products is one of the powerful tools to discover new biologically active compounds. The structure-bioactivity relationships determined for different series of compounds can serve as a starting point for the synthesis of new compounds with optimal biological activity. A classical approach is the purposeful synthesis through incorporation of active structural units from active biomolecules into a single structure resulting in compounds ("bioactive xenobiotics") which may possess unusually high activity.

Ureas and thioureas are very important compounds that show a wide spectrum of biological activities [1, 2]. It is well known that a number of nitrogen-containing heterocyclic compounds are widely distributed in nature and essential to life in various ways, which makes them important in pesticide and pharmacological chemistry [3, 4]. The presence of a 5-or 6-member heterocyclic nucleus in the urea and/or thiourea molecules confers important and multiple biological properties with potential applications in agriculture and medicine, and is the basis for target-oriented synthesis. A series of hybrid compounds containing both a heterocyclic ring and a (thio)urea bridge have been synthesized and their biological activities demonstrated, such as pesticidal [5, 6, 7, 8], antimalarial [9], antiviral [10, 14], anticancer [11, 12, 13], plant growth regulating [15, 16, 17, 18], stress-protecting [19, 20], antioxidant [21, 22], antimicrobial [23] activities, antifungal activity against plant pathogens [24] and antiamoebic activities [25] etc.

In view of the above-mentioned observations and in continuation of our search for biologically active compounds, we designed a strategy to develop agrochemicals of high potency taking into account the importance of the biological activity of compounds containing a N-heterocyclic ring connected to different functionalities, such as carboxamide, urea, thiourea. We synthesized a series of new compounds that incorporate two or three types of active phytophores: a 5-or 6-member heterocyclic nucleus, (thio)urea bridge, ethylene group, diamines, in the hope that they may be biologically active. The biological activity tested mainly included plant growth regulatory and stress-protective activities to prove how different structural combinations could influence the type of activity and its intensity. To obtain more detailed information about the specific effects of the compounds on the physiological, biochemical, cellular and genetic processes, various test-systems (unicellular algae, plants and human lymphocytes) and experimental conditions were used.

1.2 Biological Activity of Novel Ureas and Thioureas Containing Bioactive Heterocycles

All ureas and thioureas synthesized by us in which the structural modifications involve a combination of two or three active structural moieties could be conventionally divided into three series: 1,1'-hexamethylenebis (3-substituted)ureas; N-aryl and alkyl-N'-(heterocycle)ureas and thioureas; and 1-methyl and acetyl-4-substituted piperazines.

1.2.1 Series One: 1,1'-Hexamethylenebis(3-substituted)ureas

This series includes four new compounds which had not been previously described in the literature as protective plant growth regulators.

Het-NHCONH-$(CH_2)_6$-NHCONH-Het

Het = 2-thiazolyl (2-Ts, 1); 4-picolyl (4-Pic, 2); 4-pyridyl (4-Pyr, 3); 3,5-dichloro-4-pyridyl (3,5-Cl_2-4-Pyr, 4)

These compounds contain three bioactive structural units: a heterocyclic ring, carbamoyl groups and diamine. The synthesis of the heterocyclic bis-ureas (compounds 1-4), their physical properties and anti-senescence effect were described earlier [26, 27]. The structure and purity of compounds were elucidated by means of elemental carbon (C), hydrogen (H), and nitrogen (N) CHN-analysis, infrared (IR) and ultraviolet (UV) spectra, thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) characteristics.

Biological Effect of the Compounds

i) Anti-Senescence Effect

Active phenylurea cytokinins, derivatives of natural N, N'-diphenylurea and aliphatic di-and polyamines possess a common physiological property: senescence-retarding action. Cytokinins and polyamines have been regarded as the most potent senescence-retarding hormones in plants and they play a significant role in the regulation of leaf senescence [28]. A wide variety of studies have shown that exogenous cytokinin and polyamine applications lead to dramatic senescence retardation in the plant leaves. This provoked us to synthesize organic compounds possessing a diamine moiety as well as a urea moiety in their molecules and to investigate the effect of the obtained compounds on a process controlled well by both polyamines and cytokinins. One such process is leaf senescence. The anti-senescent effect of heterocyclic bis-ureas by dark-induced senescence of both barley (*Hordeum vulgare* L.) and radish (*Raphanus sativus* L.) leaf tissues was established for the first time [27, 29].

It was found that the investigated compounds have behaviour like putrescine [Put] rather than like N, N'-diphenylurea [DPU] (standards) in terms of the

determined parameters of leaf senescence in both plant systems. The phenylurea-containing cytokinin DPUprotected more strongly against Chl degradation by high levels of peroxidase and catalase activities, whereas diamine Put protected against proteolysis by an increase in both these activities only up to 48th h. 1, 1'-Hexamethylenebis(3-heterocycles)ureas (after primary chlorophyll defense) protected against proteolysis in dark-incubated leaves, more effectively than putrescine, by moderate increase in peroxidase and inhibition of catalase activities. And the halogen-substituted bis-phenylureas prevented the chlorophyll loss in dark-incubated leaves like DPU, by a strong increase in antioxidant enzyme activities [29].

The anti-senescence effect was elucidated based on the kinetics of senescence-dependent changes in some biochemical (chlorophyll, protein, free amino acids) and enzymatic (catalase EC 1.11.1.6; guaiacol peroxidase EC 1.11.1.7; ribonuclease EC 3.1.27.5) characteristics (endpoints) over a period of 72 h treatment. Chl degradation and protein hydrolysis in the aging leaf segments did not pass to the same rate. The tested compounds almost completely inhibited both these processes in the early stages of senescence and, as senescence advanced, provided stronger protection against proteolysis by a significant increase in peroxidase activity until day 2 and a decline in catalase activity after day 1 of senescence. All compounds manifested a more rapid and higher effect in the monocotyledonous plant system [29].

The heterocyclic bis-urea derivatives of 1,6-diaminohexane showed pronounced anti-senescence effect and it depended on the nature of the 3-substituent (ring type and ring's substituents) in the urea moiety. The integrity of the heterocyclic rings was an essential feature for providing high anti-senescence activity, particularly for compound 4 (shown above). This compound showed interesting behaviour in dark-incubated barley and radish

leaves. It delayed senescence in radish leaves during the whole senescence period, protecting more strongly (after a lag-time of 24 h) against the process of Chl degradation by a concomitant sharp decrease in antioxidant enzyme activities. However, the same compound protected against both processes, Chl degradation and proteolysis, in an almost similar manner and enhanced the stabilization of the Chl/protein ratio in senescing barley leaf segments by decline in peroxidase, catalase and RNase activities.

The compound 1,1'-hexamethylenebis[3-(3,5-dichloro-4-pyridyl)]urea (5, 3 and 1 mM) was the most effective in arresting chlorophyll loss and protein breakdown in dark-incubated barley and radish leaves, possibly by controlling senescence-linked events which occur in darkness and inactivation of the relevant enzyme activities (see Table 1).

Table 1. *Effect of 1,1'-hexamethylenebis(3-heterocycle)ureas [compounds 1 - 4] on the time-dependent changes in Chl (a+b), soluble protein, free amino acid contents, specific catalase and guaiacol peroxidase activities and total ribonuclease activity in barley leaf segments induced to senescence in darkness.*

Compound / Concentration	**Chl (a+b) %**			**Soluble protein %**			**Free amino acid %**		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
Control	66	55	42	85	80	79	156	257	316
1 - 1.0 mM	79	73	54	97	136	174	140	167	242
1 - 0.1 mM	84	69	58	102	157	188	142	190	216
2 - 1.0 mM	82	67	50	74	97	176	147	186	248
3 - 1.0 mM	80	70	49	82	117	174	137	157	226
4 - 1.0 mM	91	95	85	184	181	180	83	63	24
4 - 3.0 mM	91	90	89	168	187	184	99	50	27
4 - 5.0 mM	92	88	88	157	146	173	106	67	24
Standards									
DPU-1 mM	75	62	44	68	63	71	109	133	194
Put - 5 mM	88	69	48	130	137	103	161	276	334

Table 1. *Continued.*

Compound / Concentration	C A T %			G P O %			RNase %		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
Control	176	133	123	403	408	597	112	123	133
1 - 1.0 mM	20	22	8	293	502	373	-	-	-
1 - 0.1 mM	15	14	13	407	438	372	-	-	-
2 - 1.0 mM	116	113	31	370	455	371	-	-	-
3 - 1.0 mM	216	96	77	403	348	368	-	-	-
4 - 1.0 mM	80	37	36	104	96	64	150	70	124
4 - 3.0 mM	90	31	34	98	89	54	149	141	109
4 - 5.0 mM	123	93	71	97	119	63	119	130	102
Standards									
DPU-1 mM	57	301	59	383	751	957	-	-	-
Put - 5 mM	47	34	35	248	359	451	86	139	107

Segments were floated on phosphate buffer (control) or on test solutions for 24, 48 and 72 h.

Data are expressed as % of the initial values: Chl (a+b) = 127±2.10 µg/segment; Protein = 318±2 µg/segment; Free amino acid content = 2.237±0.26 µmol leucine eqv/segment; CAT = 0.295±0.0072 µmol destr. H_2O_2 mg^{-1} protein min^{-1}; GPO = 2.503±0.35 µmol GDHP mg^{-1} protein min^{-1}; RNase = 18.610 segment$^{-1}h^{-1}$.

ii) Antioxidant, Anti-Cytotoxic and Anti-Genotoxic Potential of HMPU against Chemical Mutagens

Among the first series of compounds, 1,1'-hexamethylenebis [3-(3,5-dichloro-4-pyridyl)]urea (5, 3 and 1 mM) / HMPU / manifests multiple biological properties in the highest grade. Together with the high senescence-retarding effect, this compound also exhibits a potential to modify the clastogenic effect of some chemical mutagens.

Some aspects of the response to the diversity of biotic and abiotic stresses have been highly conserved from bacteria to humans. The signaling pathways involved in the initiation and maintenance of the hypersensitive response (HR) and

systemic acquired resistance (SAR) are still poorly understood. Rapid recognition of a potential invader is a prerequisite for the initiation of an efficient defense response. On the other hand, it is found that the pre-treatment of eukaryotic cells with some chemical compounds leads to increased cell tolerance to subsequent oxidative challenges [30]. Nicotinamide (NIC) and its structural analogue isonicotinamide (IND) are reported as stress-associated compounds that can induce and regulate the defensive and secondary metabolism in plants [31, 32]. Our research also showed that the treatment with HMPU increases greatly the antioxidant capacity of algal cells. The most rapidly appearing defense response was the strongly reduced MDA content in cells. The activities of H_2O_2-scavenging enzymes CAT and GPO increased and H_2O_2 accumulation decreased with increasing the post-treatment time. Therefore, the defense mechanisms in the treated cells are expressed at different times [33].

The problem of chemical protection of plants against the injuries induced by different environmental factors has been gaining importance over the past four decades. A large number of chemicals, mainly synthetic ones, used singly or in combination have been evaluated. The process of experimental search of effective compounds which can trigger a defense response can be significantly accelerated by the use of compounds that show protective action against the natural mutagenic process such as senescence. For that reason, the investigation on the senescence-retarding effect of the newly synthesized compounds is an important part and a prerequisite for success in our subsequent studies on their protective potential against some genotoxins in different test-systems.

Human lymphocytes are more sensitive than *Hordeum vulgare* root tip meristem cells. The genotoxic/clastogenic effect of 1,1'-hexamethylenebis [3-(3,5-dichloro-4-pyridyl)]-urea (HMPU) depended on the susceptibility of the test-systems used. *Hordeum vulgare* root tip meristem cells and human

lymphocytes showed different susceptibility. Taking into account the chemical structure of HMPU, we tested its potential to modulate the cytotoxic and genotoxic effects of two well-known experimental mutagens in various experimental schemes. Hydroxyurea (HU, an antimetabolite, anti-cancer agent used in chemotherapy) and N-methyl-N-nitroso-N′-nitroguanidine (MNNG, a carcinogen and mutagen which acts by adding alkyl groups to the O^6 of guanine and O^4 of thymine) were used [34]. HMPU triggered adaptive response (AR) in both test-systems assessed as a decrease in the induction of chromosome aberrations. The optimal effect was observed at 6-hour inter-treatment time between HMPU conditioning treatment and challenge application of the mutagen. The chromosome injuries were reduced 3-fold in barley and 2-fold in human lymphocytes (Figure 1a and Figure 1b)

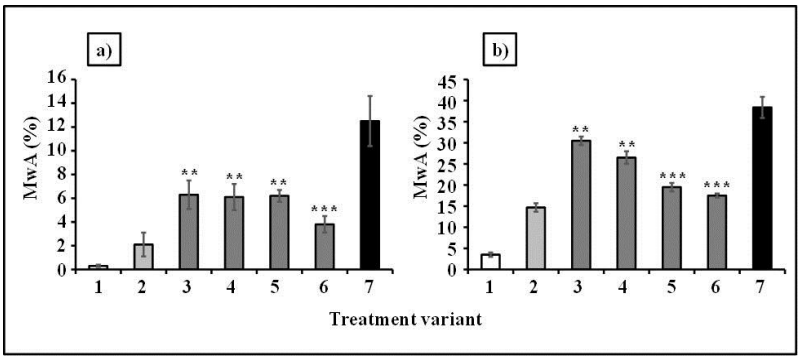


Figure 1. *Impact of HMPU conditioning treatment: (a) in barley root tip meristem cells prior to HU exposure; (b) in human lymphocytes in vitro prior to MNNG with different inter-treatment time (IT) detected as induction of chromosome aberrations.*
*** $p < 0.01$; *** $p < 0.001$.*

1- control
2- HMPU 7.5 x 10^{-3} M
3- HMPU 7.5 x 10^{-3} M - 2h IT - HU 3x10^{-2} M
4- HMPU 7.5 x 10^{-3} M - 3h IT - HU 3x10^{-2} M
5- HMPU 7.5 x 10^{-3} M - 4h IT - HU 3x10^{-2} M
6- HMPU 7.5 x 10^{-3} M - 6h IT - HU 3x10^{-2} M
7- HU 3x10^{-2} M

1- control
2- HMPU 10^{-5} M
3- HMPU 10^{-5} M - 1½h IT - MNNG 10^{-5} M
4- HMPU 10^{-5} M - 2½h IT - MNNG 10^{-5} M
5- HMPU 10^{-5} M - 4h IT - MNNG 10^{-5} M
6- HMPU 10^{-5} M - 6h IT - MNNG 10^{-5} M
7- MNNG 10^{-5} M

1.2.2 Series Two: N-aryl and alkyl-N'-(heterocycle)ureas and Thioureas

This series includes three subgroups with a total of 91 compounds (70 of which are new chemical substances) which had not been previously described in the literature as protective plant growth regulators.

R - NHCYNH - Het

Y = O or S;

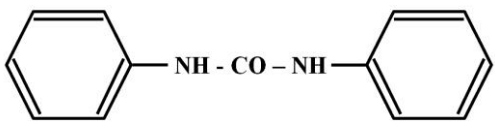
Het = 2-thiazolyl, 2-furfuryl; 2-, 3-, 4-pyridyl and substituted 2-pyridyl;

R = phenyl, 3- and 4-fluorophenyl, 3- and 4-chlorophenyl, 2,4-dichloro -phenyl, 4-bromophenyl, 2-chloroethyl, cyclohexyl, benzyl, 4-tolyl, 1-and 2-naphthyl; methyl, ethyl, n-butyl.

These compounds contained two bioactive structural units - a (thio)urea bridge and a 5-or 6-membered heterocyclic ring.

The synthesis of the different sets of compounds as well as their analytical characteristics and biological activity (herbicidal, growth inhibitory and stimulatory, anti-senescence and cytokinin-like activities) were earlier reported in a great number of articles [37, 39, 41, 42, 44, 45, 47, 48, etc]. The structure and purity of compounds were confirmed by means of elemental CHN-analysis, 1H NMR, 13C NMR, IR and UV spectra, TLC and HPLC characteristics.

The structural modifications of the parent molecule N, N'-diphenylurea (DPU) have different effects on the derivatives.



DPU

[Formula 1.1]

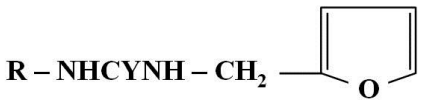
The substitution of one benzene ring with a heterocyclic ring (pyridyl, pyrazole, thiazole, thiadiazole and imidazole) on the one hand, and the presence of electron-acceptor substituents in the cycles, on the other hand, leads to increased biological activity. The five-membered heterocycles possessing adjacent nitrogen and sulfur atoms within the ring have received considerable attention in the fields of agricultural and medicinal chemistry.

Two basic groups of structurally different chemical substances show cytokinin effects; they are purine and non-purine compounds. The aromatic ureas, derivatives of N-phenyl-N'-(5-or 6-member heterocycle)ureas are the most significant group of non-purine cytokinins. Among them, there have been discovered compounds which manifest higher cytokinin activity than the classical purine cytokinins [15, 35, 36].

I. Biological Activity of N-aryl and alkyl-N'-(2-furfuryl and 2-thiazolyl)ureas and thioureas

The biological activity of two sets of N-aryl and alkyl-N'-(2-furfuryl and 2-thiazolyl)ureas and thioureas included herbicidal, root growth inhibitory and stimulatory activities (by root growth assay with wheat and cucumber seedlings) and cytokinin-like activity (by betacyanin and cotyledon enlargement assays). The structure-bioactivity relationships of the new compounds were also studied, focusing mainly on the substituent ® effect and the type of 5-member heterocycle [37]. The generally acknowledged high cytokinin activity of kinetin led us to synthesize the furfurylureas.

1- aryl and alkyl -3 - (2 – thiazolyl)ureas and thioureas



1- aryl and alkyl -3 - (2 – furfuryl)ureas and thioureas

[Formula 1.2]

In general, aryl substituents contributed greater activity than alkyl, 2-chloroethyl, or cyclohexyl substituents. The optimization in the benzene ring showed that compounds with an electronegative nonpolar substituent (F, Cl, Br) at the *meta* or *para* positions had the highest activity. In a series of halogenophenyl-ureas and thioureas, *meta*-and *para*-fluorophenyl derivatives had the highest herbicidal activity. Among fluorophenyl(thiazolyl and furfuryl)ureas, those having a fluorine in the *meta*-position of the aromatic ring tended to exhibit high cytokinin-like activity. The *para*-fluorophenyl substituent in the thiazolylthioureas resulted in a high cytokinin-like activity while in the furfurylthioureas, in a high herbicidal activity. Therefore, the 4-fluorophenyl substituent selectively influenced the herbicidal or cytokinin-like activities depending on the type of heterocyclic ring. The presence of two chlorine atoms on the benzene ring, of 4-tolyl and benzyl substituents did not significantly affect the herbicidal and cytokinin-like activities of the thiazolyl-and furfurylthioureas.

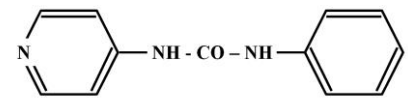
Alkyl (methyl, ethyl, n-butyl) derivatives of the thiazolylthioureas were at least 5-times more active herbicides than the aryl derivatives. The relationships between thiazolyl-and furfuryl-derivatives and their activity showed that the thiazol ring provided significantly enhanced activity, whereas the furan ring had

little effect, probably due to the separation of a furan ring from the urea/thiourea bridge by one methylene group.

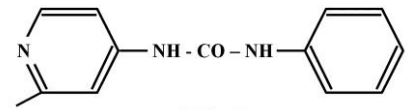
The ureas and thioureas containing thiazole nuclei were found to have high biological activity. The presence of an unsubstituted or *meta*-substituted (F or Cl) phenyl ring in these compounds increased the cytokinin-like activity, and alkyl groups markedly increased the herbicidal activity. The furfurylureas had only moderate cytokinin-like activity and the aryl(furfuryl)thioureas were completely inactive [37].

II. Biological Potential of N-(2-chloroethyl)-N'-(pyridyl)ureas

Among the aromatic ureas tested, N-phenyl-N'-(4-pyridyl)urea [4PU] exhibits strikingly high cytokinin activity comparable to N^6-benzylaminopurine [BAP]. Moreover, an electronegative chlorine atom introduced at the 2nd position of the pyridyl ring increases strongly the activity [4PU-30] and this activity is 10 times higher compared to that of purine cytokinins [36].



4PU



4PU – 30

[Formula 1.3]

It is well known that the 2-chloroethyl group included as a substituent to biologically active substrates (carriers) attributes different physiological action [38]. Thus, our following idea for target-oriented synthesis was to obtain novel N, N'-disubstituted ureas containing a pyridyl or a substituted-pyridyl ring connected to the one of the nitrogen atoms and a 2-chloroethyl group at the

other nitrogen atom, as well as to investigate the herbicidal and growth-regulating activities of the new compounds. In these urea derivatives, the chlorine atom introduced through the 2-chloroethyl group is at a stereochemical distance from the urea bridge (-NHCONH-) comparable to that in the N-phenyl-N'-[4-(2-chloro)pyridyl]urea (4PU-30).

[Formula 1.4]

At R = H, the amino group of the pyridyl nucleus is in 2nd, 3rd and 4th position;

At R = CH_3 or Cl, the amino group of the pyridyl nucleus is in 2nd position, and the CH_3 group is in 3rd, 4th, 5th and 6th position; di-CH_3 groups - in 4th and 6th positions; Cl - in 5th position; di-Cl - in 3rd and 5th positions [39, 41, 42].

The herbicidal activity of N-2-chloroethyl-N'-(pyridyl and methylpyridyl) ureas against both test plants (wheat and cucumber) was attributed to the aromatic (heterocyclic) unit rather than to the aliphatic unit (chloroethyl), which is in accordance with the conclusion that there is a direct proportion between the herbicidal activity and the lipophilic character of the compounds [40]. The N-2-chloroethyl-N'-4-pyridylurea [RHEx100=424] and N-2-chloroethyl -N'-[2-(6-methyl)pyridyl]urea [RHEx100=533] exerted the highest selective herbicidal activity towards wheat at the 1000 μM concentration and were substantially more herbicidally active than the standard Diuron [RHEx100=243]. However, the introduction of a 2-chloroethyl group at the first nitrogen atom leads to obtaining new highly active substances with cytokinin-like activity. We investigated the effect of all N-2-chloroethyl-N'-(pyridyland methylpyridyl) ureas on the betacyanin synthesis by Amaranthus bioassay [37]. The screening results showed that the above substances manifest considerably higher

cytokinin-like activity than the standard N, N'-diphenylurea (168% at 1000 μM, 151% at 100 μM and 133% at 10 μM) but are less active than the other standard, N-phenyl-N'-(4-pyridyl)urea (157% at 100 μM, 192% at 10 μM and 200% at 1 μM). Most active among the pyridyl isomers is the 2-isomer, N-2-chloroethyl-N'-2-pyridylurea - 210% at 1000 μM, 177% at 100 μM and 130% at 10 μM. Among the methylpyridyl derivatives, the compounds that displayed the highest cytokinin-like activity were N-2-chloroethy l-N'-2-(4-methyl)pyridylurea (240% at 100 μM and 186% at 10 μM) and N-2-chloroethyl-N'-2-(5-methyl)pyridylurea (182% at 100 μM and 133% at 10 μM).

i) Biological Activity of (2-pyridyl)-imidazolidine-2-one Derivatives

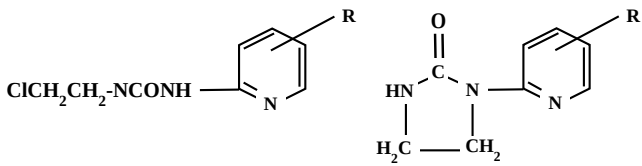
Two (2-pyridyl)-imidazolidine-2-one derivatives [B-1, B-2] comprising a cyclic ureido group were obtained for evaluation as plant growth regulators and were compared to the activity of (2-pyridyl)ureas possessing an acyclic ureido group. The literature survey revealed that linked bi-heterocyclic compounds containing hydrogenated NH-heterocycles are seldom reported to possess biological activity. In continuation of our studies on the synthesis of biologically active substances containing nitrogen heterocycles, we carried out synthesis of pyridines linked to a hydrogenated 5-member heterocycle (imidazolidine) as a result of the intramolecular cyclization of two (2-pyridyl)ureas: N-2-chloroethyl-N'-2-pyridylurea [R_1-Cl] and N-2-chloroethyl-N'-2-(4-methyl)pyridylurea [R_2-Cl] [41].

Both types of compounds, with and without a cyclic ureido group, had different behaviour in the bioassay systems employed for investigation of the herbicidal, root growth-regulating and cytokinin-like activities. The biheterocyclic compounds B-1 and B-2 did not have herbicidal activity towards either wheat or cucumber, but showed pronounced root growth stimulatory activity which well

correlated with their cytokinin-like activity at an optimal concentration of 10 μM. However, B-1 (153%) and B-2 (134%) are less active cytokinins than the corresponding R_1-Cl (130%) and R_2-Cl (186%) at 10 μM [41].

ii) Anti-senescence Effect of 2-pyridylureas with un-and/or cyclic-ureido Group

Dark-induced senescence of detached leaves or leaf segments is well suited to examine the effect of synthetic compounds on senescence as well as to study various physiological and biochemical changes associated with senescence. The potential anti-senescence effect of eight (2-pyridyl)ureas with an un-cyclic and cyclic ureido group was studied in excised barley (*Hordeum vulgare* L.) leaves which were induced to senescence by incubation in complete darkness [42].



1 – R = H (R_1 – Cl)
2 – R = 4 – Me (R_2 – Cl)
3 – R = 5 – Me
4 – R = 4,6 – Me_2
5 – R = 5 – Cl
6 – R = 3,5 – Cl_2

7 – R = H (B–1)
8 – R = 4 – Me (B-2)

[Formula 1.5]

The compounds possessing an uncyclic ureido group showed higher chlorophyll retention activity than those with a cyclic ureido group but this activity was lower compared to that of the standard 4PU at the end of the third day of aging. Treatment of leaf segments with R_1-Cl and B-2 led to increased carotenoids content after 48 h and it was higher than that in the 4PU-treated leaf tissues. The increased carotenoids content is an important response of cells to senescence or stress conditions. These effects of the compounds tested were

mediated by strongly increased H_2O_2-scavenging enzyme activities, the peroxisomal catalase activity being mainly affected. Among the compounds possessing one or two CH_3 groups, the most active member of this series was the 4-methyl isomer (R_2-Cl). Both compounds containing one or two Cl atoms on the pyridine ring were the most active compounds among all tested ones. The presence of two Cl atoms contributed to a long-term protective effect on chlorophyll degradation, while a single Cl atom induced a short-term effect. The highly activated state of the H_2O_2-scavenging enzymes contributed to the elimination of the consequences of the high extent of lipid peroxidation occurring in the tissues during day 1 of senescence [42].

Table 2. *Ratios of the activity of SOD relative to that of H_2O_2-scavenging enzyme during dark-induced senescence of barley leaf segments, 1 day, 2 days and 3 days after incubation.*

		SOD/CAT			SOD/AsPO			SOD/GPO		
Variants	**Concentration [(a)]**	**1day**	**2day**	**3day**	**1day**	**2day**	**3day**	**1day**	**2day**	**3day**
1	0.1	1.26	0.67	0.72	1.09	0.7	1.35	1.08	1.33	1.5
	1	0.9	0.47	0.65	0.71	0.74	1.48	1.02	1.5	1.55
2	0.1	0.7	0.62	0.6	0.49	0.89	1.03	1.1	1.11	1.13
	1	0.71	0.31	0.51	0.96	1.28	1.08	1.27	1.4	1.2
3	0.1	0.56	0.48	1.03	0.89	0.73	1.16	0.99	0.89	1.25
	1	0.41	0.27	0.85	0.75	0.75	1.64	0.99	0.88	1.41
4	0.1	0.76	0	0.71	0.83	0	1.34	1.07	0	1.27
	1	0.69	0	0.42	0.92	0	0.83	1.51	0	1
5	0.1	1.126	0.85	0.72	1.55	0.83	0.91	1.14	1.07	0.91
	1	0.36	0.48	0.46	0.59	0.62	0.56	0.51	0.88	0.73
6	0.1	1.04	0.61	0.69	1.28	1.21	1.17	1.52	1.22	0.92
	1	0.63	0.32	0.44	1.06	1.09	1.29	1.13	0.82	1.18
7	0.1	1.82	0.83	0.69	1.27	0.75	0.69	1.97	0.95	0.85
	1	1.8	0.6	0.51	1.23	0.49	0.45	2.16	0.59	0.63
8	0.1	0.82	0.85	0.91	0.68	0.76	0.84	1	1.08	1.16
	1	1.06	0.81	0.87	0.9	0.58	0.89	1.57	1.05	1.56
4PU[(b)]	0.1	1.22	0.83	0.85	1.07	0.85	0.87	1.34	0.73	0.92

a) concentration of test-compounds and 4PU in mM

Kanazawa et al. (2000) showed that the SOD/CAT ratio increased in the late stages of both natural and artificial senescence, whereas the SOD/AsPO and SOD/GPO ratios increased during artificial senescence but decreased during natural senescence [43]. We demonstrated that the activities of the antioxidant enzymes and the balance between them are important factors for the senescence-retarding effect of the tested urea compounds (see Table 2) [42]. The most active compound, 4PU, a well-known cytokinin used as a standard, was responsible for the balance between the H_2O_2-generating enzyme and H_2O_2-scavenging enzymes in the senescing segments, since the values of the three activity ratios were ~ 0.85 during the second and the third day of aging. The high anti-senescence effect of 4PU could be due to its cytokinin activity. The exogenous cytokinin can probably compensate the declined levels of the endogenous physiologically active cytokinins in senescing tissues. The cytokinin-like activity demonstrated by compound R_1-Cl in this and other cytokinin bioassays suggests that compounds 1-8 represent a new class of cytokinin mimics. In general, our findings showed that the cyclization of an ureido group in the imidasolidinone ring resulted in decreased chlorophyll retention activity, cytokinin-like activity, modified the level and mode of anti-senescence action compared to the compounds possessing anuncyclic ureido group. [42]

iii) Biological Properties of N – halogenophenyl - N'-(pyridyl)ureas and thioureas

The following group among the N-(pyridyl)ureas and thioureas contained halogenophenyl ring connected to the other nitrogen atom and the herbicidal and growth-regulating activities of the new compounds were investigated [44, 45, 46, 47, 48, 50, etc].

[Formula 1.6]

Y = O, S;

X = 3-and 4-F; 3-and 4-Cl;

R = H, the amino group of the pyridyl nucleus is in 2nd, 3rd and 4th position;

R = CH_3 or Cl, the amino group of the pyridyl nucleus is in 2nd position, and 3-, 4-, 5- and 6-CH_3; 4,6-di-CH_3; 5-Cl; 3,5-di-Cl.

Biological Activity of N-(3-and 4-fluorophenyl)-N'-pyridylureas and thioureas

N-(3-and 4-fluorophenyl)-N'-pyridyl and methylpyridyl-ureas/thioureas did not show herbicidal and root growth stimulatory activities but possessed high cytokinin-like activity (by the Amaranthus bioassay) with an optimal concentration of 10 μM. The more active ureas and thioureas started to affect amaranthin synthesis at a concentration of 0.1 μM and 1.0 μM, respectively. N-(4-fluorophenyl)-N'-[2-(4-methyl)pyridyl]thiourea was an exception. This compound started the amaranthin synthesis at 0.001 μM.

The cytokinin activity of N-(3-and 4-fluorophenyl)-N'-2-,3-and 4-pyridylureas/thioureas increased with the removal of the 3-and 4-fluorophenyl(thio)ureido group [3/4-FPhNHCYNH-] away from the heteroatom of the pyridyl cycle and the relative activity order is 4>3>2. Substitution in the pyridyl cycle with one methyl group reduced the cytokinin activity, as the degree of reduction depended on the CH_3-position in the 2-pyridyl ring toward the heteroatom and toward the urea/thiourea bridge. The 3-and 6-methyl-isomers (ortho-position) are usually less active, whereas the 4-and 5-methyl-isomers are highly active compounds. Therefore, the movement

of the methyl group away from the heteroatom and from the urea/thiourea bridge favors the manifestation of high cytokinin activity [44, 45].

We proved the cytokinin activity of N-(3-fluorophenyl)-N'-(2-pyridyl)urea in tobacco (CMS/81) and lucerne (74RS2) callus biotests as well. This substance (0.025 mg/l) possessed a cytokinin activity close to that of kinetin (0.5mg/l in tobacco and 0.2mg/l in lucerne biotests). [46].

Biological Evaluation of N-(3-and 4-chlorophenyl)-N'-pyridylureas

Only two compounds, N-(3-and 4-chlorophenyl)-N'-(4-pyridyl)ureas, displayed higher selective herbicidal activity against wheat [RHEx100=408 and 260, respectively] compared to the diuron standard [RHEx100=243]. The tested urea's compounds manifested high cytokinin activity at low concentrations of 1.0 μM to 100 μM, whereas DPU showed the highest cytokinin activity at 1000 μM.e structure-activity relationships in this group of substances were similar to those in the set of fluorophenyl-pyridylureas discussed above.

In general: 1) ureas with unsubstituted pyridyl or with 4-CH_3 monosubstituted 2-pyridyl rings showed considerable cytokinin activity; 2) the effect of substitution in the phenyl ring with one chlorine atom depended on its position toward the urea bridge; the 3rd position of chlorine atom was more favorable for the display of high growth-regulating and cytokinin activities [47].

We established that the nature of the halogen atom (F or Cl) in the benzene ring influences the biological activity of the above two sets of compounds. The N-3-chlorophenyl-N'-(methyl)pyridylureas had higher selective herbicidal activity and lower cytokinin activity compared to the N-3-fluorophenyl-N'-(methyl)pyridylureas. However, the N-3-fluorophenyl -N'-(2-pyridyl) urea showed a much weaker cytokinin effect on the growth of tobacco (*Nicotiana tabacum* L. CMS/81) callus compared to its chlorine analogue N-3-chlorophenyl-N'-(2-pyridyl)urea at the same concentration of

0.5 mg/l. The effect of all compounds depended on the specificity and susceptibility of the used test-system [48].

Antiphytoviral Activity of N-(3- and 4-chlorophenyl)-N'-Pyridylureas

Cytokinins such as kinetin and N^6-benzyladenine influence the augmentation of plant viruses. Correlative relations between the cytokinin and the antiphytoviral activities of adenine-and (thio)carbamoyl-compounds were established [49].

The antiphytoviral activity of 14 synthetic N-(3-and 4-chlorophenyl) -N'-pyridylureas and of seven initial amines (at $5x10^{-3}$M) was investigated by using the following "virus-host plant" systems: potato virus X strain H19 (PVX)-tobacco leaves (*Nicotiana tabacum* L. cv. Samsun NN); red clover mottle virus (RCMV)-peas leaves (*Pisum sativum* L. cv. Nadja). All compounds tested had positive I %-values, i.e. they inhibited the virus replication to a more or less marked degree. The highest effect in reducing the PVX concentration showed N-(3-chlorophenyl)-N'-(4-pyridyl)urea (80%). The starting compound 4-aminopyridine also had high inhibiting activity (73%); it increased weakly in the urea containing a 3-chlorophenyl group, whereas in the urea with a 4-chlorophenyl group, it was reduced to 50%. Among the ureas containing a 3-chlorophenyl group, four derivatives displayed superior PVX-inhibiting activity (50-80%) and only one derivative had a good RCMV-inhibiting activity (43%). Among the ureas containing a 4-chlorophenyl group, two derivatives had a good PVX-inhibiting activity (~50%). The potent compounds were those that contained an unsubstituted 4-pyridyl residue and/or one CH_3 group at the pyridyl nucleus in the 4th or 5th positions. The ureas with a 3-chlorophenyl group were more active than those with a 4-chlorophenyl group [50]. The same structure-activity relationships for the antiphytoviral and cytokinin activities of these compounds were observed. With regard to the problem of the increase in

plant tolerance to phytopatogenic viruses, our investigations lead to the design of new compounds with high antiphytoviral activity.

A series of novel derivatives of N-phenylurea containing a pyrimidine ring with two nitrogen atoms instead of a pyridyl ring were synthesized and their antiviral activity was evaluated. All of the target compounds exhibited good anti-TMV (tobacco mosaic virus) activity and two compounds had higher activity to virazole at $5.0x10^{-4}$ g/ml [10].

In addition, a series of N, N'-dipyridylthioureas inhibit the replication of HIV and other related viruses *in vitro*. The most active compound is reported to be N-[2-(2-pyridyl)ethyl]-N'-(5-bromo-2-pyridyl)thiourea [14].

[Formula 1.7]

Effect of the Synthetic Cytokinins of the Urea Type on the Growth and Development of Cytokinin-Dependent Tissue Cultures

The formation of active oxygen species plays an important role in cell division and can inhibit the morphogenesis of plant cells and tissues *in vitro* culture. Cytokinins have been found as effective free radical scavengers. The N^6-substituted adenine derivatives are the classical cytokinins which can induce callus growth in tissue cultures. However, the cytokinin-active phenylureas could substitute the adenine cytokinins in inducing callus growth and organogenesis in different plant species.

Among the three compounds, N-3-chlorophenyl-N'-(2-,3-and 4-pyridyl)ureas, which are positional isomers in relation to the pyridyl nucleus, the 2-isomer

showed cytokinin activity higher than that of kinetin, whereas the activity of the 3-and 4-isomers was equal to that of kinetin in tobacco (*Nicotiana tabacum* L. CMS/81) callus assay. The 4-isomer also manifested organogenic effect on the meristemal explants from cork oak (*Quercus suber* L.) and ash-tree (*Flaxinus excelsior* L.) which was most marked in the ash-tree [48].

The effect of two cytokinin-active compounds, N-3-chlorophenyl-N'-[2-and (4-methyl)-pyridyl)ureas, on *in vitro* shoot cultures and the physiological state of the micropropagated *Gypsophila paniculata* L. was studied and compared to that of the conventional purine cytokinin kinetin. It was found that the highest rate of shoot multiplication was in the explants grown on media containing the tested substances as compared to that in explants grown on media with kinetin. Moreover, the produced explants preserved this potential for growth of *Gypsophila* shoots on cytokinin-free medium. The physiological state induced by the cytokinins tested was characterized by an increased content of pigments, proline and enhanced invertase activity ensuring energy for the growth process of the explants cultivated *in vitro* [51]. The active phenylurea cytokinins tested in this research could find application in the micropropagation of flower species.

Protective Effects of N-(3-chlorophenyl)-N'-Pyridylureas

The growth and development of plants are determined by the interactions between their genome and the environment. To date, there is scant evidence in the literature for modulation and improvement in some basic physiological processes, such as photosynthesis, plant resistance etc., responsible for plant productivity.

It has been suggested that yield increases in crops might be at least partly accounted for by delayed leaf senescence and higher leaf net photosynthetic rates during the mid-to late grain-filling period [52]. In this respect, we investigated the effects of two active phenylurea derivatives,

N-(3-chlorophenyl)-N'-(2-pyridyl)urea [3CP-2PU] and N-(3-chlorophenyl) -N'-[2-(4-methyl)pyridyl]urea [3CP-4MPU] at 100 μM, on some photosynthetic parameters, such as stomatal conductance (Gs), net photosynthetic rate (Pn), enzyme activities of carbonic anhydrase (CA) and phosphoenolpyruvate carboxylase (PEPC), and chlorophyll and protein contents in wheat flag leaves. Wheat plants (*Triticum aestivum* L. cv. Sadovo-1) were grown under field conditions and the studied parameters were measured on the 7th and 14th days after single foliar spraying.

To the best of our knowledge, we demonstrated for the first time the ability of two phenylurea compounds to increase the photosynthetic capacity in wheat flag leaves during the early grain-filling stage [53]. The fact that they cause the opening of stomata suggests that the effect of the compounds is due to their function as cytokinins. This was associated with an increase in flag leaf chlorophyll, soluble protein, net photosynthesis rate and grain dry matter (see Table 3).

Table 3. *Effect of 3-CP-2PU and 3-CP-4MPU treatments on wheat grain yield in relation to number of grains per plant and 1000-grains weight (percent of control).*

Treatment	No. grains per plant[a]	1000-grain weight[b] (g)
control	100.00	100.00
3-CP-2PU	111.27	112.46
3-CP-4MPU	118.35	120.21

[a] control value = 60.13
[b] control value = 43.8 g

The treatment with both substances, 3-CP-2PU and 3-CP-4MPU, caused changes in the polypeptide patterns of soluble proteins from wheat flag leaves. They increased differentially the quantity and composition most individual polypeptides identified compared to the non-treated leaves. While 3-CP-2PU

only increased the polypeptide quantity, 3-CP-4MPU led to the appearance of a new 51 kDa polypeptide [54].

A positive effect of both substances, 3-CP-2PU and 3-CP-4MPU, on the photosynthetic potential, transpiration and green biomass production was also found in maize and sunflower plants [55].

A crucial problem in agriculture is to improve the stress- and disease-tolerance of cultivated plants, thus increasing crop yield. According to many studies, treatment with various natural and synthetic cytokinins can significantly contribute to prevent and reduce the damaging effect of various unfavorable environmental conditions.

Another field of great theoretical and practical importance in the era of atomic energy is the search for new, more effective radio-protectors for radiation protection. We examined the potential radio-protective effect of two active phenylurea derivatives, 3-CP-2PU and 3-CP-4MPU, which were applied as post-radiation (6 Gy) treatment of pea seeds (50 Gy) and oat plants during the vegetation period. The compounds exhibited a considerable radio-protective effect. The treatments resulted in significant reduction in the radiation-induced damage to the primary development of pea seeds and the survival and productivity (30% increased yield) of oat plants. The data from the anaphase analysis of pea plants indicated that the application of the novel compounds with protective effect increased the recombinant potential of the irradiated cells [56, 57].

The phenylurea derivative N-(3-chlorophenyl)-N'-[2-(5-chloro)pyridyl]urea (showing high cytokinin-like activity) induced protective effect against injury induced by the herbicide chlorsulfuron in *Nicotiana tabacum* callus cultures and young plants [58]. The safener-mediated induction of the herbicide-detoxifying enzyme glutathione S-transferase appears to be a major component of the stress

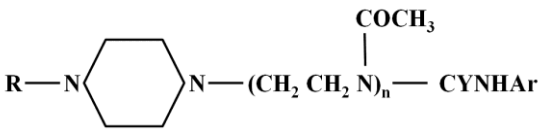
response. The treatment of young tobacco plants with the same compound resulted in enhanced tolerance to NaCl (85.5 mM). The protective effect was organ-specific, being more strongly pronounced on shoots than on roots (Yonova et al., unpublished data).

In addition, another one of our urea derivatives, N-(2-chloroethyl) - N'-[2-(4-methyl)pyridy]urea (with high cytokinin-like activity), manifested a protective effect against NaCl (up to 170 mM) in tobacco callus tissues. The protective potential was similar to that of 4PU-30 up to 85.5 mM of NaCl and equal to that of kinetin up to 126.5 mM of NaCl.

1.2.3 Series Three: 1-methyl and acetyl-4-substituted Piperazines

The series of 1-methyl and acetyl-4-substituted piperazines included 12 compounds (9 of which are novel chemical substances) which had not been previously described in the literature as protective plant growth regulators.

These compounds possess a hydrogenated NH-heterocycle [piperazine ring] and aryl(thio)carbamoyl groups connected directly or through an ethylene group and have the following general structure:

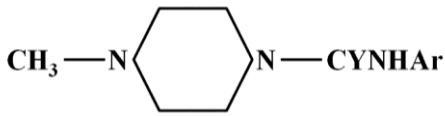


[Formula 1.8]

i) R = CH_3, n = 0, Ar = phenyl and halogenophenyl, Y = O, S [compounds 1 - 8]

ii) R = CH_3CO, n = 1, Ar = phenyl and halogenophenyl, Y = O, S [compounds 9 - 12]

Compounds 1-8 include a combination of two types of bioactive structural units (phytophores): aryl(thio)carbamoyl groups directly connected to the secondary nitrogen atom of the piperazine ring.



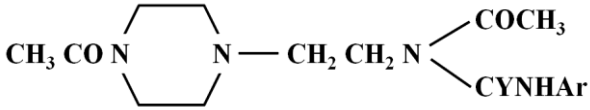
[Formula 1.9]

1-aryl(thio)carbamoyl-4-methyl-piperazines (i)

Y=O, Ar = Ph (1), 3-ClPh (2), 4-FPh (3), 4-ClPh (4);

Y=S, Ar = Ph (5), 4-FPh (6), 4-ClPh (7), 4-BrPh (8).

Compounds 9-12 combine three types of phytophores: aryl (thio)carbamoyl groups connected to the piperazine ring through an ethylene group.



[Formula 1.10]

1-[2-(acetylamino)ethyl]-4-acetyl-piperazines, N-aryl(thio)carbamoyl (ii)

Y = O, Ar = Ph (9), 4-FPh (10);

Y = S, Ar = Ph (11), 4-FPh (12).

The syntheses of all compounds as well as their analytical characteristics and biological activity (herbicidal, growth inhibitory, stimulatory and cytokinin-like activities) were reported by us [59]. The structure and purity of the compounds were confirmed by means of elemental CHN-analysis, 1H NMR, 13C NMR, IR and UV spectra, TLC and HPLC characteristics.

In spite of the scarce reports in the literature, it is interesting to note that some representatives of the groups of N-(phenyl or pyridyl)-N'-[(heterocycle or saturated NH-heterocycle)ethyl]-ureas and thioureas show important biological properties [14, 19, 60]. For instance, 1-phenyl-3-[2-(2-oxo-1-imidazolidinyl) ethyl]urea (EDU, ethylendiurea, containing an un-cyclic and a cyclic ureido group) displayed cytokinin-like activity; it is the most efficient synthetic protectant against acute ozone injury in a number of plant species, against UV-B radiation in soybean and also inhibits plant senescence.

EDU

[Formula 1.11]

I. Biological Activity of 1-methyl and acetyl-4-substituted Piperazines

The herbicidal, growth stimulatory and cytokinin-like activities of the two novel groups of aryl-ureas and thioureas possessing piperazine or 1-ethyl-piperazine ringswere studied [59].

In the first group of compounds (1-8), compounds 7 and 8, which possess 4-chloro-and bromo-phenylthiocarbamoyl groups, showed the best herbicidal activity as well as high selective herbicidal activity against wheat (RHE=316 and 438, respectively; in relation to chlorsulfuron, RHE=397). Stimulation of root growth both in wheat and cucumber was observed within the whole studied concentration range of compounds 1 and 6. Compounds 3, 4, 7 and 8 also exhibited high levels of selective stimulatory activity, more pronounced against wheat at 100, 10 and 1 μM (129-146%). Our results from the betacyanin bioassay of 1-aryl(thio)carbamoyl-4-methyl-piperazines (compounds 1-8) showed that the

compounds lacking herbicidal activity exhibited good cytokinin-like activity. Compound 1, which possesses an unsubstituted phenylcarbamoyl group, manifested the highest cytokinin-like activity at 1000 μM (like DPU). The introduction of a halogen atom at positions 3 or 4 of the phenyl ring in the 1-arylcarbamoyl-4-methyl-piperazines (compounds 1-4) tended to reduce the overall activity, whereas in the 1-arylthiocarbamoyl-4-methyl-piperazines (5-8) this led to a complete loss of activity.

Among the second group of compounds (compounds 9-12), compound 10, which possesses a 4-fluorophenylcarbamoyl group connected to the piperazine ring through an ethylene group, was a highly active herbicide against both wheat and cucumber at 1000 μM. It showed very high selective herbicidal activity at 100 μM against wheat (RHE = 529), which exceeded that of chlorsulfuron (RHE=397 at 1000 μM). This group of compounds also produced a strong stimulating effect mostly on the wheat root growth at concentrations of 10^{-1} μM. However, this stimulating effect of compounds 9-12 did not correlate with their cytokinin-like activity. All compounds inhibited the pigment synthesis (7-24%) within the whole concentration range tested. Compound 10 was more active, showing ~ 40% inhibition at concentrations of 1 and 0.1 μM.

In general, our results showed that ureas in which the ethylene group was missing (compounds 1-4) exhibited relatively good cytokinin-like activity. Thioureas without the ethylene group possessing 4-chloro-and bromo-phenyl substituents (compounds 7, 8) manifested highly selective herbicidal activity against wheat. Among the ureas and thioureas possessing an ethylene group, compound 10 exhibited the highest herbicidal activity against *Triticum aestivum*. Whereas the presence of an ethylene group determined a different type of activity of the fluorinated urea derivatives 3 and 10, it did not affect any of the activities of the fluorinated thiourea derivatives 6 and 12. Thus, our results

complement the existing information about the structure-activity relationships of aryl-ureas and thioureas containing piperazine or 1-ethyl-piperazine rings.

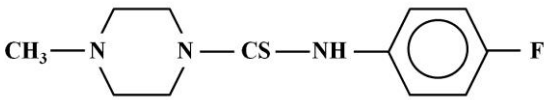
II. Protective Effect against Herbicides (Herbicide Antidotes)

A major problem associated with the use of herbicides for a long time is the emergence of herbicide-resistant weeds [61]. Consequently, there is a continuous need for development of new products with new modes of action. To stop the need for creation of an ever increasing number of chemicals, there are two alternative approaches: 1) to increase the herbicide selectivity, or 2) to increase the herbicide tolerance of sensitive crops. The latter may be achieved by using appropriate herbicide protectors (antidotes). Up to now, limited evidence has been reported on the effect of antidotes on the herbicidal activity of chlorsulfuron (CS) and paraquat (PQ).

We investigated the protective effect of two new compounds, urea and thiourea derivatives of 1-methylpiperazine (compounds 3 and 6), applied as pre-treatment against chlorsulfuron (10 μM) injury in the sensitive plant maize (*Zea mays* L.) and determined their effects (alone or in combination with the herbicide) on some parameters of the growth, the antioxidant system and the activity of acetolactate synthase and glutathione S-transferase [62, 63, 64]. The mode of action of chlorsulfuron appears to block the activity of acetolactate synthase (ALS, EC 4.1.3.18), a key enzyme in plants needed in the biosynthesis of the branched amino acids isoleucine, leucine and valine. The enzymatic conjugation of herbicide with glutathione and consequently its detoxification is mediated by glutathione S-transferases (GSTs, EC 2.5.1.18). These enzymes have the potential to decrease and/or eliminate the cytotoxic or genotoxic effects of compounds that can damage DNA, RNA and proteins.

To the best of our knowledge, we demonstrated for the first time the antidote activity of both the urea and thiourea derivatives of 1-methylpiperazine

(compounds 3 and 6) against chlorsulfuron in maize. It was demonstrated that the thiourea derivative showed higher protective effect against CS than the urea derivative at a concentration of 500 μM and IT=0 h (inter-treatment time). The amount of free amino acids in the combined variant was similar to that in the variant with the protector alone (38%), while this amount in the variant with chlorsulfuron alone was 63% over the control. Treatments with the herbicide and compounds 3 / 6 alone or in combination had no significant effect on the parameters of the antioxidative defense system tested.



FTMP

[Formula 1.12]

The antidote effect of the synthetic compound 6 (FTMP) (500 μM) against chlorsulfuron in maize plants was compared to the effect of the commercial herbicide safener 1,8-naphthalic anhydride (NA) (500 μM). Our results showed that both tested compounds manifested a specific effect on the changes in growth, in ALS and GST activities. The specificity of the action on ALS was supported by the lack of effect on the enzyme activity *in vitro*, i.e. we could suggested that the defense action of both compounds does not involve an effect on the enzyme synthesis. However, pre-treatment of seeds with both protectors overcame the chlorsulfuron-induced inhibition of ALS activity in leaves and roots, 8 and 12 days after treatment. The pre-treatment of seeds with NA and FTMP and following treatment with chlorsulfuron moderately increased the GST (CDNB) activity only in roots on the 8th day but the effect of FTMP was higher than that of NA on the 12^{th} day after the treatment.

We can conclude that FTMP was a more effective protector against CS injury in the roots, whereas NA, in aerial part of the maize plants.

The protective effect of FTMP was proved, to the best of our knowledge, for the first time against the herbicide paraquat in young barley (*Hordeum vulgare* L.) plants as well. Seeds with growing root meristems were used as an experimental material. Treatment with FTMP (5, 50, 500 and 1000 μM) prior to paraquat (10 μM) application was performed. Pre-treatment with FTMP reduced the inhibiting effect of PQ on the growth of shoots to 20% and of roots to 8.0% in 9-day-old barley plants, increased the chlorophyll content in leaves, decreased the level of oxidative stress (H_2O_2 and MDA) more greatly in leaves (except for 1000 μM) than in roots (only at 5 μM) compared to treatment with PQ only. A stimulation of both ascorbate-and guaiacol-peroxidases in the roots of plants pre-treated with PQ/FTMP along with an increase in the level of lipid peroxidation was found. The protective effect of FTMP against paraquat depended on the applied concentration. Among the four tested concentrations, the one that gave the best effect was 5 μM: it completely eliminated the paraquat-induced oxidative damages in leaves and roots of barley plants [65]. We suggest that the protective effect of FTMP against paraquat was mediated by the increased activities of antioxidant enzymes in the roots as an initial defense against PQ.

It is noteworthy that the tested compound FTMP may display antioxidant properties due to conversion of the thiourea [-N-C(=S)-NH-] group into an isothiourea group [-N-C(-SH)-N-]. Thus, we propose that FTMP contributes to the decrease in the oxidative potential in leaves and roots of PQ/FTMP pre-treated plants as a reducing agent.

In general, we showed that the newly synthesized compound FTMP is highly effective as a herbicide protector against chlorsulfuron in maize and against paraquat in barley plants. On the basis of the results obtained by us, it is difficult to explain the exact mechanism of the protective action of FTMP

against the herbicide-induced damage. Probably, the protective mechanism of FTMP depended on the mode of action of each herbicide.

III. Anti-cytotoxic and Anti-genotoxic Effects

The stress-induced cytotoxic and genotoxic effects in living cells continuously exposed to the action of mutagenic factors are mainly caused by the increased level of oxidative stress, leading to instability of the genome. The activity of the antioxidant defense machinery is often insufficient to neutralize the overproduced reactive oxygen species [66]. One of the modern approaches for reduction of the mutagenic burden in cells is the application of natural and synthetic compounds that have protective and anti-mutagenic potential.

In view of genome protection, we investigated the potential of some of our (thio)urea derivatives to mitigate the genotoxicity of "radiomimetics" (paraquat), UV-C and gamma-radiation.

The radio-protective effect of 1-(phenylthiocarbamoyl)-4-methylpiperazine (compdound 5) was demonstrated in oat plants. Post-radiation (6 Gy) treatment of oat plants during the vegetation period with this compound (at 1000 μM) modified the damaging effect of gamma-irradiation upon oat plants, which resulted in increased plant productivity (by 13%).

The radio-protective effect of the other investigated compound, 3-CP-4MPU, was even higher (30% increased yield) in oat plants [57].

The ability of 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine (FPTU) to reduce PQ-induced oxidative stress in *Hordeum vulgare* and human lymphocytes *in vitro* was proved by endpoints for cytoxicity (mitotic index, MI) and genotoxicity (induction of chromosome aberrations and micronuclei) [67]. The DNA protective potential of FTMP was manifested by decreasing both chromosome aberrations (MwA) and micronuclei (MN) in barley and human

lymphocytes (Figure 2). The obtained results are specific and they depend on the experimental test-system. The mitotic activity was not significantly influenced in barley, whereas in lymphocyte cultures *in vitro*, conditioning treatment with two different non-toxic FTMP concentrations prior to PQ challenge treatment significantly enhanced the mitotic activity ($p < 0.001$) (data are not shown).

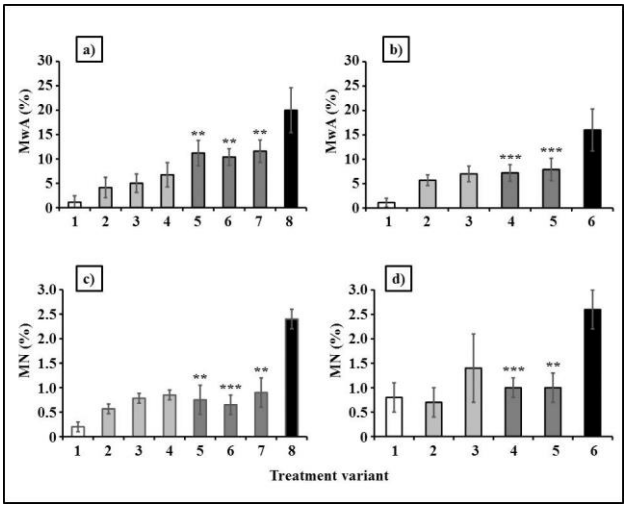


Figure 2. *Protective effect of FTMP against PQ applied as conditioning treatment at non-toxic concentrations, prior to experimental mutagen, assessed as induction of chromosome aberrations and micronuclei in barley root tip meristem cells (a, c) and in human lymphocytes in vitro (b, d). ** $p < 0.01$; *** $p < 0.001$*

1- Control
2- FTMP 10^{-6} M
3- FTMP 10^{-5} M
4- FTMP 10^{-4} M
5- FTMP 10^{-6} M - 4h IT - PQ 10^{-4} M
6- FTMP 10^{-5} M - 4h IT - PQ 10^{-4} M
7- FTMP 10^{-4} M - 4h IT - PQ 10^{-4} M
8- PQ 10^{-4} M

1- Control
2- FTMP 10^{-6} M
3- FTMP $5x10^{-6}$ M
4- FTMP 10^{-6} M - 4h IT - PQ 10^{-4} M
5- FTMP $5x10^{-6}$ M - 4h IT - PQ 10^{-4} M
6- PQ 10^{-4} M

In *Hordeum vulgare* root tip meristem cells, FTMP conditioning treatment prior to PQ resulted in a significantly decreased ($p < 0.01$) frequency of

chromosome aberrations in all effective concentrations of FTMP compared to the PQ-induced chromosome alterations (Figure 2a). Lymphocyte cultures were found to be more sensitive to FTMP than barley. Here, conditioning with FTMP prior to the PQ challenge resulted in a significant decrease ($p < 0.001$) in the structural chromosome disturbances, which were approximately three-fold lower compared to those induced by PQ alone (Figure 2b).

The other endpoint in our investigations, induced micronuclei, also revealed similarities in the trends for all exposure concentrations in both test-systems used in the study. In barley, conditioning treatment with FTMP and inter-treatment time of 4 h significantly reduced the yield of MN compared to the single PQ treatment ($p < 0.01$) (Figure 2c). The MN rate was significantly reduced in cultured lymphocytes when FTMP was applied in effective concentrations with an inter-treatment time of 4 h ($p < 0.001$), compared to the PQ treatment alone (Figure 2d).

The protective potential of FTMP was also examined against UV-C radiation. UV-C (200-280 nm), a component of sunlight, shows strong cytotoxic and genotoxic effects after irradiation in mammalian cells [68]. It induces photodimers in DNA, which, if not repaired, might cause DNA damage leading to skin photoaging and photocarcinogenesis [69]. FTMP applied to human lymphocytes *in vitro* as a conditioning treatment before UV-C (100-150 J/m^2) decreased slightly, but significantly, the harmful UV-C effect ($p < 0.05$) in the variants with 4-hour inter-treatment time, as assessed by mitotic activity and chromosome aberrations (Figure 3a, b). All these results demonstrate that the thiourea synthetic compound FTMP provide genomic protection against the oxidative stress inducer PQ as well as against UV-C radiation.

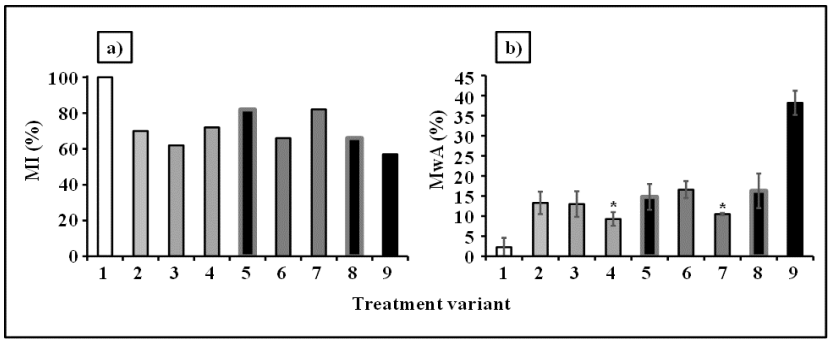


Figure 3. *Effect of FTMP conditioning treatment prior to UV-C exposure on mitotic activity (a) and chromosome aberration induction (b) in cultured human lymphocytes. * $p < 0.05$.*

1- Control
2- FTMP 10^{-6} M
3- FTMP 10^{-6} M - 1½h IT - UV-C 100 J/m^2
4- FTMP 10^{-6} M - 4h IT - UV-C 100 J/m^2
5- UV-C 100 J/m^2
6- FTMP 10^{-6} M - 1½h IT - UV-C 150 J/m^2
7- FTMP 10^{-6} M - 4h IT - UV-C 150 J/m^2
8- UV-C 150 J/m^2
9- MNNG 10^{-5} M

1.3 Conclusions

The novel effective ureas and thioureas containing bioactive heterocycles showed antisenescence effect, antioxidant and anti-genotoxic potential against various environmental genotoxins and stress factors.

The search for alternative protectants which would be safe is a rather urgent task. Such protectants could possibly be based on natural substances of plant origin. The structure of the series of synthetic compounds presented in this review includes structural units/groups from the natural phenylurea-type cytokinin N, N'-diphenylurea (DPU) and different N-containing heterocyclic compounds. The protective effect of our substances on the described plant species and stress factors was demonstrated in experimental studies on pre-treated seeds or vegetative propagules. This is technique, which does not have any impact on the environment, is highly effective and less costly than

post-treatment application. Besides the protective effects, the discussed substances also have other beneficial effects on sensitive crops, i.e. stimulate growth and development, enhance stress tolerance and have low toxicity to plants and human lymphocytes *in vitro*. It may be concluded that these synthetic compounds could be used for plant protection as well as for enhancement of plant productivity because they are considerably less expensive and toxic.

Thus, we developed new classes of agrochemicals that are promising and could be recommended for agricultural application as active ingredients of: 1) effective plant-growth regulators and pesticides; 2) means and ways for crop protection and creation of mutants with valuable agricultural qualities; 3) protocols for micropropagation of flower and woody species that are difficult to propagate.

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Chapter 2

Heterocyclic Compounds and Their Biological Applications of *Semecarpus anacardium* L.f

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Abstract

Heterocyclic compounds such as (1) 1H-Pyrazole, (2) 2, 6-Piperidinedione, (3) 13-Tetrade-cene-1-ol acetate, (4) 2-Methyl-3-(4-methoxybenzoyl)indole, (5) 5H-Naphtho [1,8-bc] thiophen-5-one]; Essential fatty acids and lipid-soluble bioactives, Glycolipids i.e. Monogala-ctosyldiacylglycerol (MGDG), Digalactosyldiacylglycerol (DGDG), Sulfoquinovosyldiacyl-glycerol (SQD), Steryl glucoside(SG), Acylated steryl glucoside (ASG)], Amino acids i.e. tryptophan, thiamine, riboflavine, histidine, nicotinic acid & Tocopherols [α, β and γ-Tocopherols], Flavanoids and Biflavanoids have been isolated in our laboratory from the *Semecarpus anacardium* L.f Nuts, Leaves, Flowers, Stem bark and Root bark. These were characterized through their chemical and spectral data. These were nutritionally considered as a new non-conventional supply for pharmaceutical industries and edible purposes. The knowledge concerning the composition and properties of *Semecarpus anacardium* L.f would assist majorly in efforts of nutritional and industrial, applications of this plant. To explore the binding mode and understanding of key active site residues of the extracted natural compounds such as fatty acids in the active site, docking studies were carried out by taking the crystal structure of human FAAH (PDB ID: 3K84). The Docking studies indicated the presence of 20(Amino acids) amino acids in the active site. Phytocomponents in the extract imply the phytopharmaceutical importance of the plant. Most of the chemical compounds can target both gram-positive and gram-negative bacteria, Anti-cancer activity, Radical Scavenging activity is being reported for the first time.

Keywords

Semecarpus anacardium L.f, Anacardiaceae Family, Glycolipids, Amino Acids, Tocopherols, Biflavonoids (Heterocyclic Compounds), Antimicrobial Activity, Anti-cancer Activity and Anti Oxidant Activity

2.1 Introduction

Semecrpus anacardium (SA) L.f (Family: Anacardiaceae) [1-2], Trees, up to 25 m height with grey bark exfoliating in small irregular flakes, leaves simple alternate, obviate-oblong, flowers are greenish white, in panicles and nuts is about 2.5 cm long, shining black when ripe, seated on an orange-colored receptacle form of the disk. The black corrosive juice of the pericarp contains tarry oil consisting of 90% of oxy-acid anacardic acid & 10% of higher nonvolatile alcohol called cardol, also contains catechol and a mono-hydroxy phenol called as anacardol. the most significant components of the *Semecarpus anacardium* L.f oil are phenolic compounds [3], on exposure to air, Phenolic compounds get oxidized to quinines, the oxidation process can be prevented by keeping the oil under nitrogen, is well known for its viceant liquid which causes severe burns and allergic edema in exposed parts is obviously due to the lipoid-soluble C_{15} chain present in the catechol, and it finds frequent application in Indian medicine in the treatment of gout, rheumatic pains and other ailments [4]. Hetero cyclic compounds are essential to life. These are present in a wide variety of many natural products of plant and marine origin. During our literature survey we found that, the Anacardiaceae family has 77 genera and 850 species, and contains several anticancer drugs isolated from Anacardiaceae family. We have selected *Semecarpus anacardium* L.f for its high medicinal value in ayurvedic and siddha systems and isolated several active constituents in our laboratory. These were isolated, purified and highly analyzed basing on FT-IR, ^{1}H NMR, ^{13}C NMR, MASS, NP-HPLC, GC & GC-MS Analysis.

2.2 Materials and Methods

2.2.1 Plant Material

All parts like Flowers, Leaves, Stem bark, Nuts, Root bark, were collected from field area nearer to the village Nandgaon which is situated at nearly 20-25 km. outskirts to Kolhapur city, Maharashtra, India (Figure 1). All plant material specimen's were identified by Dr Vatsavaya S. Raju, Former Head and Chairperson-BOS Plant Systematic Laboratory, Department of Botany, Kakatiya University, Warangal (A. P.), India and conformed as *Semecarpus anacardium* L.f. (syn: *Anacardium latifolium* Lam., *A. orientale* Steud.) of Anacardiaceae Family and plant specimen deposited at Kakatiya University Herbarium, Warangal (KUW) with accession number 1874. It is locally known as '*nalla jeedi*' and popularly known '*markingnut/dhobinut.*

Figure 1. *Images of plant material collection.*

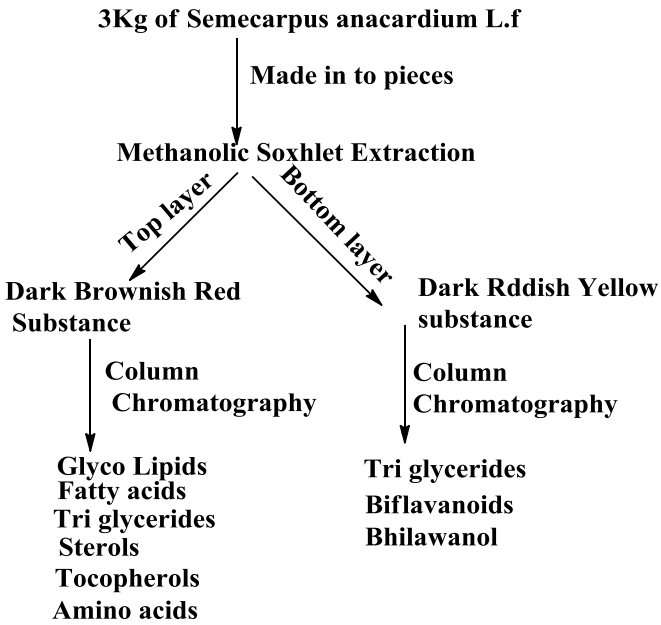


Figure 2. *Scheme of separation of compounds from Semecarpus anacardium* L.f.

2.2.2 Preparation of Plant Extract

Isolation: *Semecarpus anacardium* L.f. 3 kg Fruiting Nuts were extracted with 3 lit of methanol by soxhlet extraction for 72 hours, two layers observed dark brownish black substance at top layer and dark reddish yellow at bottom layer, These were separated by separating funnel and were concentrated by vacuum-evaporation and weighed. The crude extract of the two layers were subjected for column chromatography followed by TLC, Prep. TLC & by individual crystallization. Compounds were isolated, purified and highly analyzed basing on FT-IR, ^{1}H NMR, ^{13}C NMR, MASS, NP-HPLC, GC & GC-MS Analysis. Top layer contains, Triglycerides, sterols, Glycolipids, Tocopherols, Aminoacids and bottom layer contains, Triglycerides, Biflavonoids, and Bhilawanol (Figure 2).

2.3 Glycolipids

2.3.1 Column Chromatography and Thin-Layer Chromatography of Lipid Classes

The Total lipids were separated into different classes by elution with solvents of increasing polarity over a column packed with silica gel (100-200 mesh), the major portion of Glico lipids were eluted with excess volume of acetone, the composition was determined by GC/FID. By means of TLC on Silica gel plates a further characterization of Glico lipids were done, each spot was identified with lipid standards as well as their reported retention factor (Rf) values. Individual bands were visualized under UV light, scraped from the plate and recovered by extraction with chloroform/methanol (2:1, v/v), Monogalacto-syldiacylglycerol(MGDG), Digalactosyldiacylglycerol (DGDG), Sulfo - quinovosyldiacylglycerol (SQD), Steryl gluco side (SG), Acylated steryl glucoside (ASG) were obtained from *Semecarpus anacardium* L.f Nuts [5] (Figure 3).

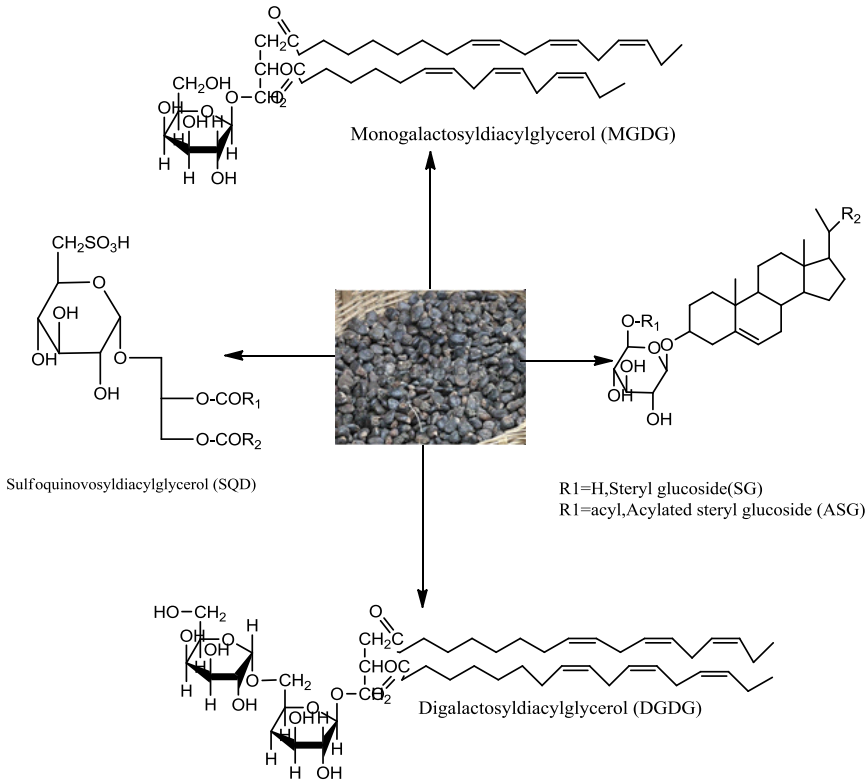


Figure 3. *Glycolipids from Semecarpus anacardium* L.f *Nuts.*

2.3.2 Tocopherols

Normal phase high performance liquid chromatography (NP-HPLC) analysis of tocopherols: NP-HPLC has advantage of allowing the resolution of the four isomers (α, β, γ, δ), RP-HPLC does not allow the complete resolution of β & γ isomers, and consequently, in RP-HPLC, these two vitamins were quantified together and also NP-HPLC was selected to avoid extra sample treatment (e.g., saponification), The analysis was performed with a solvent delivery LC-9A HPLC [6]. The chromatographic system included a model 87.00 variable wavelength detector and a 250×4 mm i.d. LiChrospher-Si 60, 5 μm column. The separations of tocopherols were based on isocratic elution when the solvent flow

rate was maintained at 1 mL min^{-1} at a column back-pressure of about 65-70 bar. The solvent system selected for elution was isooctane/ethyl acetate (96:4, v/v) with detection at 295 nm. Twenty µL of the diluted solution of TL in the mobile phase were directly injected into the HPLC. Tocopherols were identified by comparing their retention times with those of authentic samples (Figure 4).

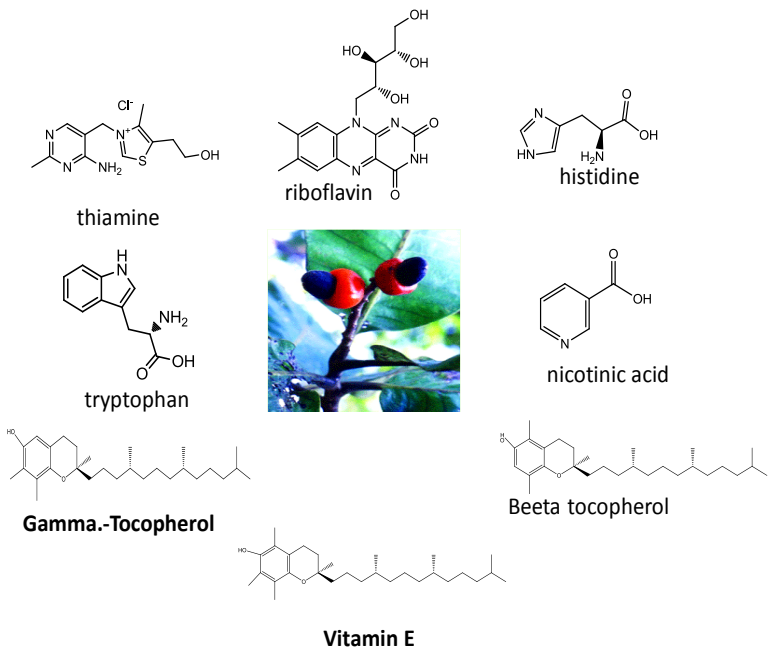


Figure 4. *Amino Acids and Tocopherols from SA Fruiting Nuts.*

RP-HPLC with UV-detector on a Lichrosolv C-18 [7] column with methanol in Na-Phosphate buffer gradient elution used For Amino acids. Amino acids play central roles both as building blocks of proteins and as intermediates in metabolism. The 20 amino acids that are found within proteins convey a vast array of chemical versatility. The 10 amino acids that we can produce are

alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine and tyrosine. Tyrosine is produced from phenylalanine, so if the diet is deficient in phenylalanine, tyrosine will be required as well. The essential amino acids are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Humans can produce 10 of the 20 amino acids. The others must be supplied in the food.

To explore the binding mode and understanding of key active site residues of the extracted natural compounds such as fatty acids in the active site docking studies were carried out by taking the crystal structure of human FAAH (PDB ID: 3K84). Docking studies indicated the presence of 20 (Amino acids) amino acids in the active site.

2.4 Heterocyclic Compounds from SA Flowers & Leaves

Semecarpus anacardium L.f. Shade dried Leaves, Flowering Buds 3 kg were extracted with 4 lit. of each polar and non polar solvent by soxhlet extraction for 72 hours. These extracts were concentrated and analyzed additionally by using Gas Chromatography-Mass Spectrometry. GC-MS analysis of phyto constituents in plants gives a clear picture of the pharmaceutical value of that plant (Figure 5 & Figure 6). The mass spectrum of the hexane extract of *Semecarpus anacardium* L.f. was compared with the available library sources (NIST08 LIB, WILEY8 LIB) it was found that *Semecarpus anacardium* Flowers revealed the presence of 1H-Pyrazole2.15%, γ-Tocopherol 0.96%, γ-Tocopherol1.33%, Vitamin E2.5% 2,6-Piperidinedione 2.14% and Leaves contains 2-Methyl-3-(4-methoxybenzoyl) indole 0.22%, 5H-Naphtho [1,8-bc] thiophen-5-one1.59%, γ-Tocopherol1.68% & Vitamin E2.88% (See Table 1 & 2) & (Figure 7).

Table 1. *Heterocyclic compounds and their medicinal properties from Semecarpus anacardium* L.f *Flowers.*

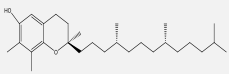
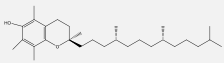
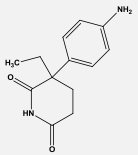
S.NO	Chemical Name	Chemical Structure	Chemical Formula	Molecular Weight
1	1H-Pyrazole		$C_3H_4N_2$	68.0773
2	γ-Tocopherol both leaves and flowers		$C_{28}H_{48}O_2$	416.68
3	Vitamin E Both leaves and flowers		$C_{29}H_{50}O_2$	430.71
4	2,6-Piperidinedione		$C_{13}H_{16}N_2O_2$	232.3

Table 1. *Continued.*

S.NO	R.T/Min	Area %	Medicinal Uses
1	16.44	2.15	Anti inflammatory, anti diabetic and antibacterial activity [8].
2	24.55	0.96	Preventing diseases of the heart and blood vessels including hardening of the arteries, heart attack, chest pain, leg pain due to blocked arteries, and high blood pressure [9].
3	25.18	2.55	Breast cancer, and breast cysts preventing diseases of the heart and blood vessels including hardening of the arteries, heart attack, chest pain, leg pain due to blocked arteries, and high blood pressure [9].
4	29.46	2.14	Immunomodulatory agent with antineoplastic and antimitogenic properties [10].

Table 2. *Heterocyclic Compounds and Their Medicinal Properties from Semecarpus Anacardium* L.f *Leaves.*

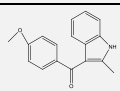
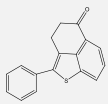
S.NO	Chemical Name of the Compound	Chemical Structure	Chemical Formula	Molecular Weight
1	2-Methyl-3-(4-methoxybenzoyl)indole		$C_{17}H_{15}NO_2$	265.3116
2	5H-Naphtho[1,8-bc]thiophen-5-one		$C_{17}H_{12}OS$	264.05

Table 2. *Continued.*

S.NO	R.T/Min	AREA %	Medicinal Uses
1	19.879	0.22	It is used in the study of CB_2 mediated responses and has been used to investigate the possible role of CB_2 receptors in the brain [11].
2	21.926	1.59	Anti inflammatory, anti diabetic and antibacterial activity [12].

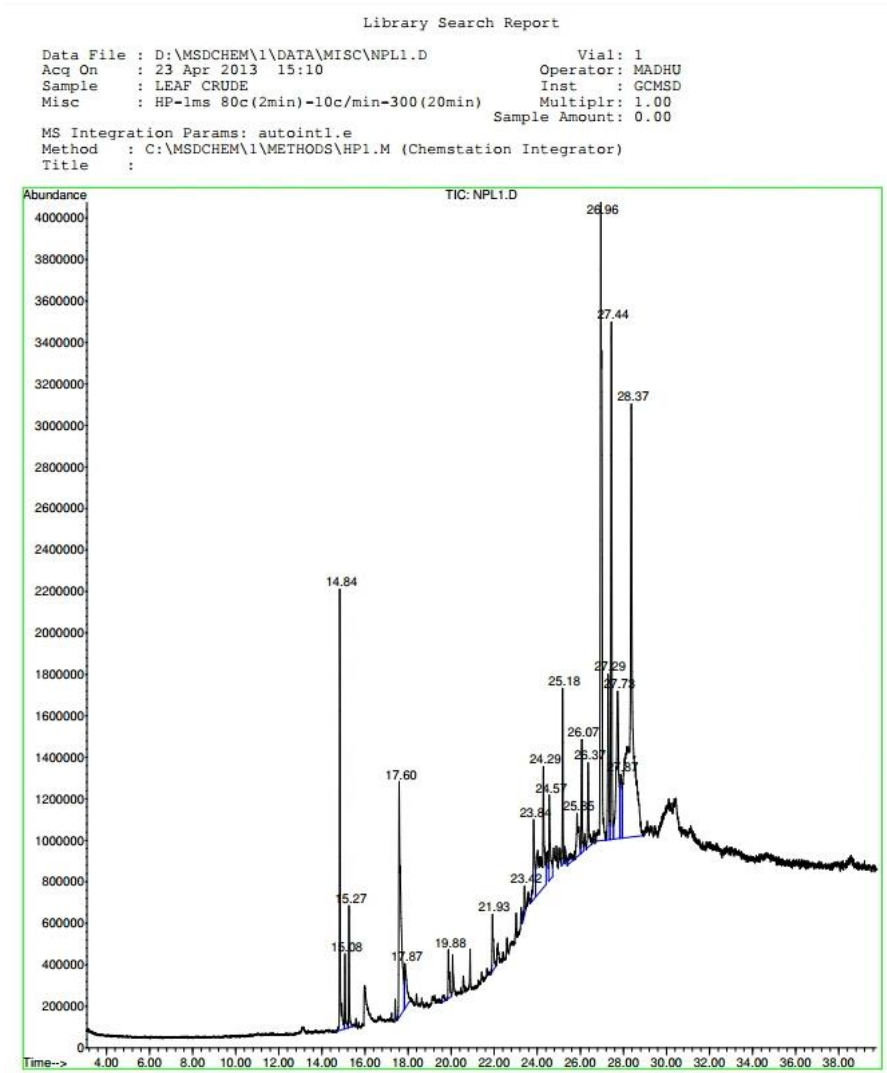


Figure 5. *GCMS reports of SA Leaves & Flowers (1).*

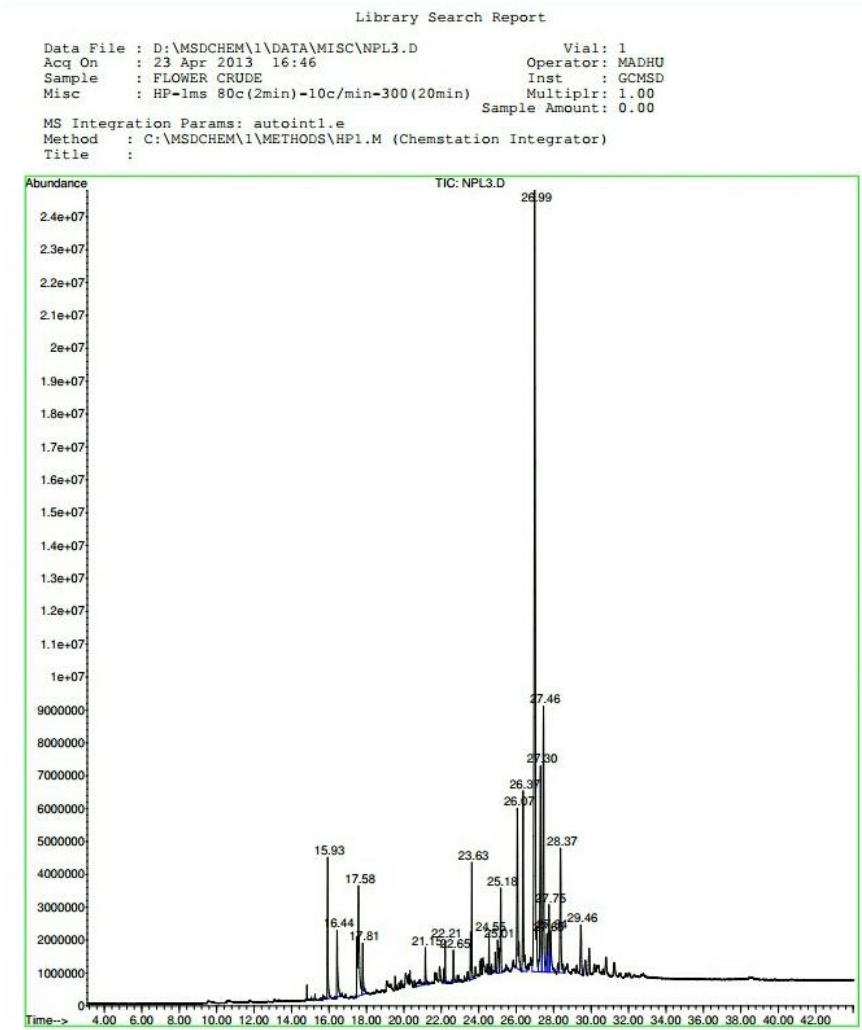


Figure 6. *GCMS reports of SA Leaves & Flowers (2).*

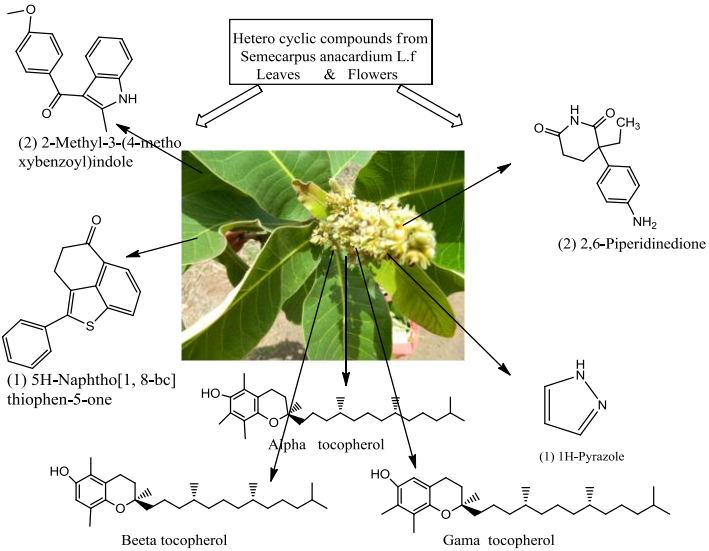


Figure 7. *Heterocyclic compounds from SA Leaves & Flowers.*

2.5 Isolation and Purification of Biflavonoids from SA Nuts, Leaves and Root Bark

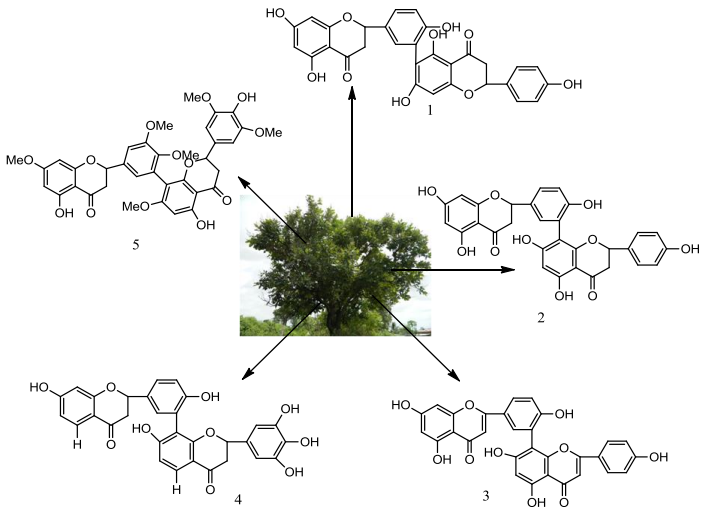


Figure 8. *The various compounds isolated from Semecarpus anacardium*
1. Tetrahydrorubustaflavone, 2. Tetrahydroamentoflavanone, 3. Amentoflavone, 4. Semecarpu flavanone, 5. Nallaflavanone.

Semecarpus anacardium L.f. 3 kg nuts were collected and shade dried, The nuts were directly percolated with cold petroleum ether 5-6 times, filtered and concentrated by vacuum evaporation. Then the nuts were made in to small pieces and percolated with petroleum ether 5-6 times, filtered and concentrated by vacuum evaporation. Again the powdered nuts were percolated with hot petroleum ether 5-6 times, filtered, and concentrated by vacuum evaporation.

After that, the same powdered nuts were extracted with cold acetone by changing the solvent for 3 hours in three intervals, filtered and concentrated by vacuum evaporation.

After cold acetone percolation, nuts were subjected to hot acetone extract, and concentrated by vacuum evaporation.

The above fourth concentrate was then fractionated using silica gel column with appropriate solvent gradient (Benzene: Acetone 9:1) and the Biflavanoids obtained from the fraction of 13 (Benzene: Acetone; 9:1) were rechromatographed and purified. Leaves and Root bark were extracted individually with 4 lit. of each polar and non polar solvents by soxhlet extraction for 72 hours, was concentrated by vacuum-evaporation, and submitted for spectral analysis, Spray reagents were used for the detection of Biflavonoides Thin layer plates Ceric sulphate (70% in con H_2SO_4) Ref: cambie and James, 1967, Kawano et., al.1964. Biflavonoids gave a greenish-violet ferric reaction, A pinkish-red color with sodium borohydride -hydrochloric acid, Mg-Hcl and an Orange-red color with $NaBH_4$-Hcl, Spectral data of compounds 1-5 were consistent with the reported literature, Therefore, the structure of these compounds were determined as follows:

Compound 1: It appears as a yellow needles, mp 251 ℃, Its UV absorption in methanol are at λmax (nm) 289, 224 and 211, its IR absorptions shows at Hydroxyl (3394 cm^{-1}), conjugated carbonyl (1643), and aromatic rings

(1597, 1516, 1493 and 1458 cm^{-1}, The negative ESI-MS at m/z 541[M-H]$^-$, Thus the molecular formula was deduced to be $C_{30}H_{22}O_{10}$, ^{1}H NMR (Acetone d6, 400 MHz): δ 12.38 (1H, *s*, OH), 12.16 (1H, *s*, OH), 9.62 (1H, *br s*, OH), 7.35 (2H), 7.29 (1H, *m*), 7.13 (1H, *d*, *J* =2.3 Hz, H-2'), 6.88(1H, *d*, *J* = 8.4 Hz, H-5'), 6.81 (2H), 6.04 (1H, *s*), 5.89 (1H), 5.88 (1H), 5.47 (1H), 5.43 (1H), 3.32 *(*1H), 3.26(1H), 2.72 (1H), 2.66 (1H).

^{13}CNMR (acetone-*d6*): CH_2(43), CH(127.4), CH(126.3), CH(116.1), CH(120.1)

CH(127.4), CH(128.3), CH(116.1), CH(95.1), C(131.1), C(115), C(95.8), C(102.8), C(101.9), C(157), C(164), C(160.2), C(116), C(196.3), CH(82), CH(82.8), C(163.6), C(162.5) δppm. Spectral data of compounds 1 were consistent with the reported literature, Therefore, the structure of compound 1 was determined to be as tetra hydrorobustaflavone.

Compound 2: It appears as yellow amparphous substance, mp 235 ℃. Its UV absorption in methanol are at λmax (nm) 285, 225(sh), 330(sh)., its IR absorptions shows at Hydroxyl group 3323.88, benzene rings at 1636.74, 1344.05, 1219.65, 772.64. The negative ESI-MS at m/z 541.1[M-H]$^-$, Thus the molecular formula to be $C_{30}H_{22}O_{10}$, ^{1}H NMR (Acetone d6, 400 MHz): δ 12.28 (1H, s, OH), 12.17(1H, s, OH), 7.21 (4H, m), 6.85 (1H, d, *J* = 8.19), 6.71 (2H, d, *J* = 8.04), 6.05 (1H, s), 5.88 (2H, s), 5.44 (2H, m), 3.16 (2H, brm), 2.77 (2H, br m).

^{13}CNMR (acetone-*d6*): CH_2 (43.46), CH(120), CH(120), CH(128.3), CH(116.1), CH(95.1), CH(95.6), CH(94.6), C(130.9), C(131.2), C(105.9), C(102.8),

C(101.6), C(157.4), C(162.7), C(160.2), C(16 4.7), C(163.8), C(196.8), CH(82.8), C(163.6), C(155.9) δppm. Spectral data of compounds 2 was consistent with the reported literature. Therefore, the structure of compound 2 was determined to be as tetrahydroroamento flavone.

Compound 3: It appears as yellow cubes, mp 235℃, UV λmax (nm) 285, 223(sh), 330(sh). The positive ESI-MS at m/z 538.46, its molecular formula is $C_{30}H_{18}O_{10}$, its IR absorptions shows at Hydroxyl group 3400, benzene rings at 1605, 1445, 1310, 1235, 1148, 1078, 820, ^{1}H NMR (Acetone d6, 400 MHz) δ 12.28 (1H, s, OH), 12.17(1H, s, OH), 7.21 (4H, m), 6.85 (1H, d, J = 8.19), 6.71 (2H, d, J = 8.04), 6.05 (1H, s), 5.88 (2H, s), 5.44 (2H, m), 3.16 (2H, brm), 2.77 (2H, br m), ^{13}CNMR (acetone-*d6*): CH_2 (43), CH(126.3), CH(120.1), CH(120), CH(128.3), CH(116.2), CH(114.2), CH(95.1), CH(95.6), CH (94.6), C(131.1), C(131.2), C(105.1), C(102.8), C(101.6), C(164.9), C(146.2), C(145.9), C(160.2), C(164.77), C(163.8), C(196.8), C(196.8), CH(82.8), CH (83.1), C(163.6), C(155.9) δppm. Spectral data of compounds 3 was consistent with the reported literature, Therefore, the structure of compound 3was determined to be as amentoflavone.

Compound 4: It appears as yellow amarphous substance, mp 249℃, UV λmax (nm) 291, 296(sh), The positive ESI-MS at m/z 543.2, so the molecular formula is$C_{30}H_{22}O_{10}$, its IR absorptions shows at Hydroxy291, 296(sh), 338(sh), ^{1}H NMR (Acetone d6, 400 MHz) δ3.08 (2H, m, trans), 5.42 (2H, d d, J = 4.0, 12.0Hz), 7.15 (l H, *d, J*= 8.5 Hz), 7.37 (l H, *d, J* = 2.0 Hz) and 7.46 (l H, *d d, J= 2.0, 8.5* Hz), at 6.34 (l H, *d, J* = 8.0 Hz), 6.14 (l H, *d d, J= 2.0, 8.0* Hz) and 6.22 (l H, *d, J = 2.0* Hz), 6.84 *(d, J = 2.0* Hz), 6.92 *(d, J= 2.0* Hz), 7.26 (2H, s), 7.64 (2H, s), 7.76 (l H, s) and 8.50 (l H, s), 6.52 (l H, *d, J* = 8.0 Hz) and 6.72(l H, *d, J* = 8.0 Hz). ^{13}CNMR (acetone-*d6*): CH_2(42.7), CH(126.3), CH(120.1), CH(109.2), CH(128.3), CH(106.8), CH(107.4), CH(106.8), CH(102), CH(129.7), CH(130.8), C(131.2), C(136), C(114.3), C(115.1), C(113.3), C(113.4), C(163.5), C(146.4), C(160.2), C(164.4), C(146.4), C(134.9), C(190.9), CH(82.8), CH(83.4), C(164), C(153.9) δppm. Spectral data of compounds 4 was consistent with the reported literature, Therefore, the structure of compound 4 was determined to be as Semecarpuflavanone.

Compound 5: It appears as yellow amarphous substance, mp 249 ℃, UV λmax (nm) 296, 296(sh), 338(sh). The positive ESI-MS at m/z 674.65, so the molecular formula is $C_{36}H_{34}O_{13}$, Its IR absorptions shows at Hydroxylgroup (3450.12); broad), methoxy groups(2830), Chelated flavanonecarbonyl

(1650) and benzene rings(1590.9). ^{1}H NMR (Acetone d6, 400 MHz) δ 2.78(dd,2H, J=4,17H, cis-protons), 5.32(dd,2H, J=4,12Hz), 6.10(d, 1H, J=2Hz), 6.18(d, 1H, J=2Hz), 6.78(d, 1H, J=2Hz)6.90(d, 1H, J=2Hz), 7.30(d, 1H, J=2Hz), 7.40(d, 1H, J=2Hz), 3.48, 14.28(s, 1H) and 14.44(s, 1H), 8.74(s,1H). ^{13}CNMR (acetone-*d6*): OH(5.35), CH(5.51), CH(6.22), CH(6.18), CH(6.24), CH(6.99), CH(6.53), CH(7.37), CH_2(3.38), CH_3(3.83),

CH_3(55.8), CH_3(56.1), CH_3(60.6), CH_3(56.1), CH_2(43), CH(120), CH(102.1), CH(108.3), CH(94), CH(94.8), C(134.4), C(135.2), C(126.8), C(102.4), C(101.3), C(162.3), C(163.4), C(136.3), C(165.8),

C(147.6), C(154), C(164.1), C(151.1), C(147.6), C(196.8), CH(83.1), CH(83.4), C(163.2), C(158.3) δppm. Spectral data of compounds 5 was consistent with the reported literature, Therefore, the structure of compound 5 was determined to be as Nallaflavanone.

2.6 Docking Studies

In the present study, the molecular docking study has been conducted with 5 Biflavonoids on PTB1B targets. It also regulates the hepatocyte growth factor receptor signaling pathway through dephosphorylation of MET. From the docking studies, we have explored different probable binding pockets, putative active site residues, binding modes of extracted natural compounds from plants in different targets according to their mechanism of action. The molecular docking studies could provide substantial design clues for the development of novel, potent inhibitors for PTBIB targets. All computations and molecular

modeling studies were carried out on Schrodinger software. A dataset comprising of 5 Biflavonoids were drawn in ChemDraw and converted into 3D-molecules with all possible tautomers and chiral centers. The converted 3D-molecules were minimized with OPLS-2005 force field using water as solvent in the GB/SA continuum solvation model. The probable binding modes of best docked compounds are shown in Figures 9-13 and its interaction profile is shown in Table 3. The docking parameters and physicochemical properties of Biflavonoids in PTP1B targets are shown in Table 4.

2.6.1 SCF in the Active Site of PtP1B

The two hydroxyl groups in catechol moiety showed hydrogen bond interactions with Asp236. The hydroxyl group of hydroxyl chromanone ring connected to catechol interacts with Glu200. The carbonyl group of dihydroxy chromanone ring showed hydrogen bond interaction with Asn193 and hydroxyl group's hydrogen bond interaction with Ser190 and Glu276. BF1 showed hydrophobic interactions with Phe196, Ile281, Phe280, Leu192 and Ala189.

2.6.2 THAF in the Active Site of PtP1B

The two hydroxyl groups in catechol moiety showed hydrogen bond interactions with Asp236. The hydroxyl group of hydroxyl chromanone ring connected to catechol interacts with Glu200. The carbonyl group of dihydroxy chromanone ring showed hydrogen bond interaction with Asn193 and hydroxyl groups hydrogen bond interaction with Ser190 and Glu276. PBF1 showed hydrophobic interactions with Phe196, Ile281, Phe280, Leu192 and Ala189.

Table 3. *The key active site residues in PTP1B targets around 5Å.*

Target	Active site (5Å)
PTP1B	SER187, PRO188, ALA189, LEU192, ASN193, LEU195, PHE196, LYS197, GLU200, LEU232, GLU276, GLY277, ALA278, LYS279, PHE280, ILE281 and MET282

Table 4. *The docking parameters and physicochemical properties of flavonoids in PtpIB target.*

Flavonoid	gscore	evdw	ecoul	energy	emodel	Mol.wt	logP	PSA
SCF	-7.42	-39.36	-11.14	-50.51	-62.3	542.5	1.7	197.14
THAF	-6.9	-39.86	-4.08	-43.94	-61.18	542.5	2.76	193.26
NF	-6.59	-34.83	-5.88	-40.71	-59.33	674.66	5.46	172.91
A	-6	-33.03	-13.07	-46.1	-64.47	538.47	2.62	192.59
THRF	-5.68	-45.04	-7.88	-52.91	-63.71	542.5	3.13	194.07

3D Structures of Biflavonoids from SA Nuts, Leaves and Root Bark:

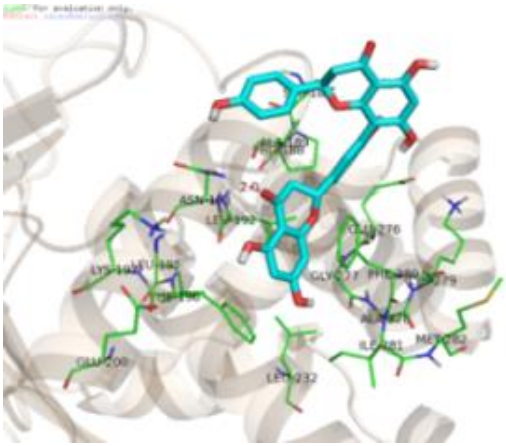


Figure 9. *THRF.*

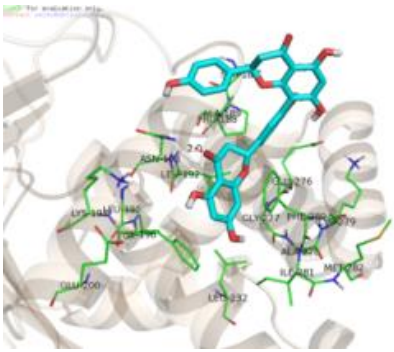


Figure 10. *THAF.*

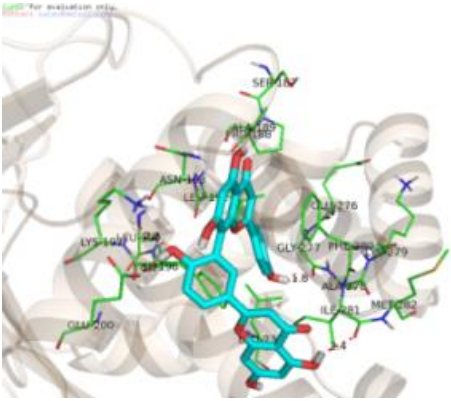


Figure 11. *AF.*

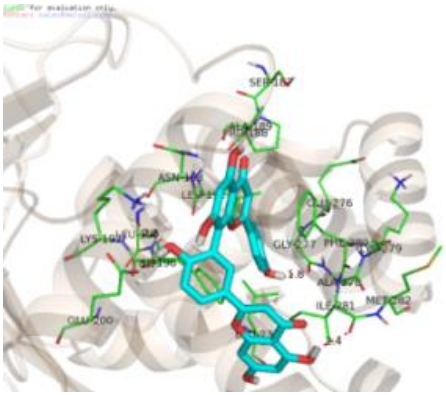


Figure 12. *SCF.*

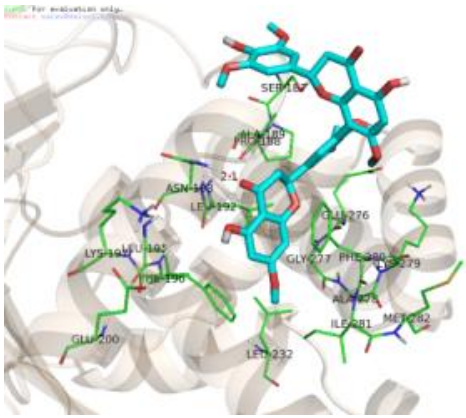


Figure 13. *NF.*

2.7 Antimicrobial Activity of the Isolated Compounds

The antimicrobial activity of the isolated compounds and their derivatives were determined by using well diffusion method against different pathogenic reference strains procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. The pathogenic bacteria and *Candida* reference strains were seeded on the surface of the media Petri plates, containing Muller-Hinton agar with 0.1 ml of previously prepared microbial suspensions individually containing 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland). Wells of 6.0 mm diameter were prepared in the media Petri plates using a cork borer and the isolated compound and their derivatives at a dose range of 300 - 1.4 µg $well^{-1}$ was added in each well under sterile conditions in a laminar air flow chamber. Standard antibiotic solutions of Neomycin and Miconazole at a dose range of 300-1.4 µg $well^{-1}$ and the well containing methanol served as positive and negative controls, respectively. The plates were incubated for 24 h at 30 ℃ and the well containing the least concentration showing the inhibition zone was considered as the minimum inhibitory concentration.

Table 5. *Anti microbial Activity of Biflavonoids.*

COMPOUNDS	Staphylococcus aureus MTCC 96	Klebsiella planticola MTCC 530	Bacillus subtilis MTCC 121	S.aureus MLS16 MTCC 2940
THRF	9.37	18.75	9.37	9.37
THAF	18.75	9.37	9.37	9.37
AF	9.37	18.75	18.75	--
SCF	9.25	--	18.75	--
NF	18.75	--	--	18.75
Neomycin	18.75	18.75	18.75	18.75
Miconazole	--	--	--	--

Table 5. *Continued*

COMPOUNDS	Micrococcus luteus MTCC 2470	Escherechia coli MTCC 739	Pseudomonas aeruginosa MTCC 2453	Candida albicans MTCC 3017
THRF	18.75	--	9.37	18.77
THAF	9.37	--	9.37	18.77
AF	9.37	9.37	18.75	9.37
SCF	9.37	0	9.75	18.75
NF	--	9.75	9.75	--
Neomycin	18.75	18.75	18.75	--
Miconazole	--	--	--	9.37

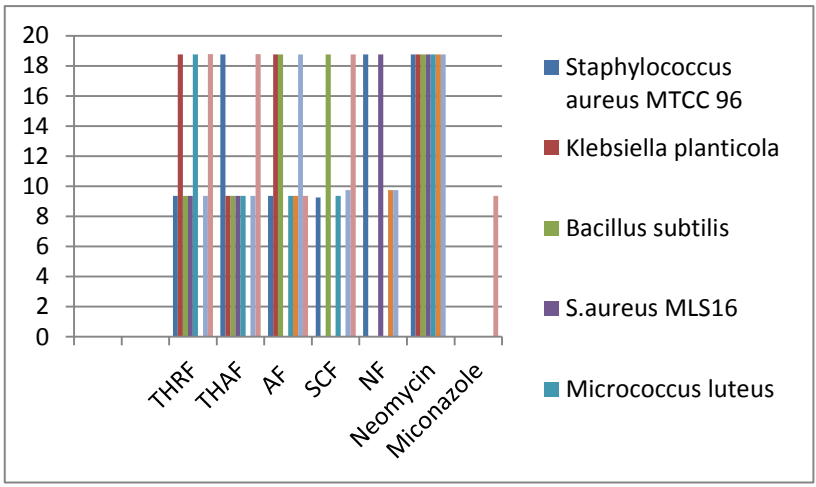


Figure 14. *Graphical representation of Antimicrobial activity of Biflavonoids from SA.*

2.8 Radical Scavenging Activity Using DPPH Method

The free radical scavenging power of the extracts was determined by the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical-scavenging method. Aliquots of 0.2 ml of the extracts (1 mM) were mixed with 2 ml of 0.1 mM methanolic DPPH. The volume was made up to 3 ml with methanol. The solutions were incubated in dark at room temperature for 40 min. Absorbance was read at 517 nm using methanol as a blank and methanolic DPPH as control. Methanolic solution of tert-butyl hydroxy anisole (BHA) at 1 mM was taken as reference. The scavenging activity was calculated using the following equation:

(%) Free radical scavenging activity = Absorbance of DPPH - Absorbance of sample X 100

Table 6. *Radical Scavenging Activity of Biflavonoids from SA.*

Sample Name	% Free radical Scavenging activity
BHA(1Mm)Reference antioxidant.	93.467
THRF	72
THAF	72
AF	70
SCF	71
NF	65

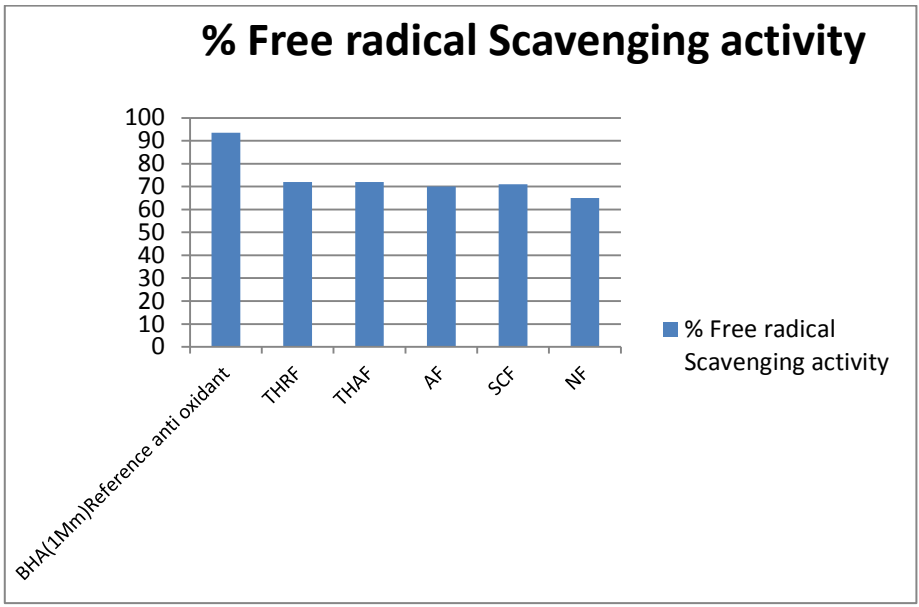


Figure 15. *Graphical representation of Radical Scavenging activity of Biflavonoids from SA.*

2.9 Conclusion

The present study is the first report on the *Semecarpus anacardium* L.f Nuts, Leaves & Flowers which is a good source of essential fatty acids and lipid-soluble bioactivities. Amino acids & Tocopherols were nutritionally considered as a new non-conventional source to supply for pharmaceutical industries and edible purposes. Tocopherols also have great utility in preserving the taste and preventing the oxidation or rancidity of many foods that contain oils and fats. Docking studies indicated the presence of 20 (Amino acids) amino acids in the active site. Phyto components present in the extract shows the phyto pharmaceutical importance of the plant [13-16]. To explore the binding mode and understanding of key active site residues of the extracted natural compounds such as fatty acids in the active site docking studies were carried out by taking the crystal structure of human FAAH (PDB ID: 3K84) [17] Most of

the chemical compounds isolated from Semecarpus anacardium can target both gram-positive and gram-negative bacteria, Anti cancer activity, Radical Scavenging activity.

Acknowledgements

We would like to thank all the faculty members & administrative staff of the Dept. of Chemistry, Acharya Nagarjuna Univrsity for providing infra structure facilities and Sri Dr. S. Chandrasekhar Director of IICT-CCMB, Sri Dr. G. Naraharisastry, Head, and Center for Molecular Modeling, IICT, and Hyderabad Dr. SridharaJanardhan for their kind cooperation and help during the tenure of this research.

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Chapter 3

1,3,5-Triazine Based Compounds: Synthesis and Anti-Cancer Activities

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Abstract

The emergence of heterocyclic compounds containing one or more nitrogen atoms has gained much attention owing to their various biological activities. Among different nitrogen containing heterocyclic compounds, compounds with 1,3,5-triazine motif have recently gained a special attention for their potent anti-proliferative activities. In this focus book chapter, we highlight the recent advance in the development of 1,3,5-triazine based anti-cancer compounds reported since the year 2010.

Keywords

1,3,5-Triazine, Anti-Cancer Agents, Heterocycles,
Biologically Active Molecules, Chemotherapy, Drugs

3.1 Introduction

Cancer is one of the leading causes of death worldwide, accounting for 8.2 million deaths in 2012 [1]. The most common forms of cancer are lung cancer (1.59 million deaths), liver cancer (745,000 deaths), colorectal cancer (694,000 deaths), stomach cancer (723,000 deaths), breast cancer (521,000 deaths), and esophageal cancer (400,000 deaths) [1]. Cancer is also a leading cause of death in children ages 5-14 [2]. It is estimated that 1 in 285 children is diagnosed with cancer before the age of 20 in United States [3].

Among different techniques used to cure cancer, chemotherapy (use of drugs to kill cancer cells) is most popular and well-studied [4]. Although a plenty of compounds have been discovered as effective anti-cancer agents so far, the search of safer, cheaper, and more efficient anti-cancer drugs is still one of the hottest topics of research worldwide.

In the recent past, compounds containing one or more heterocyclic rings have shown promising anti-cancer and other biological activities [5-12]. Especially, compounds possessing triazine ring in their structures have shown promising anti-cancer activities.

Triazines are a class of nitrogen-containing heterocycles with molecular formula $C_3H_3N_3$. Triazines exist in three isomeric forms i.e. 1,2,3-triazine, 1,2,4-triazine, and 1,3,5-triazine (Figure 1).

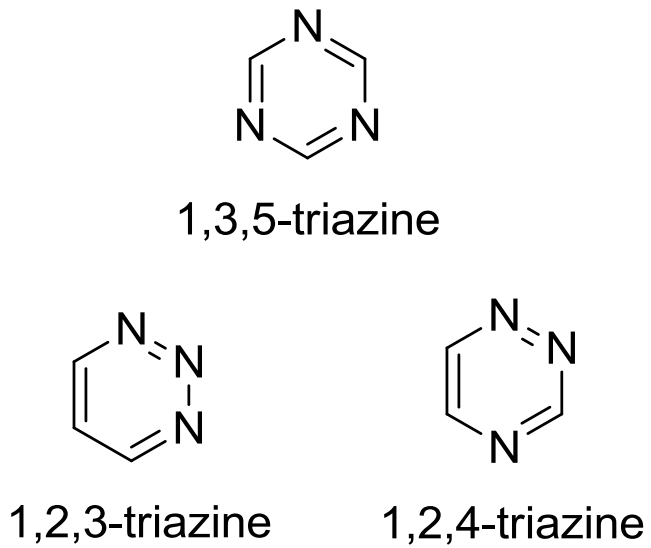
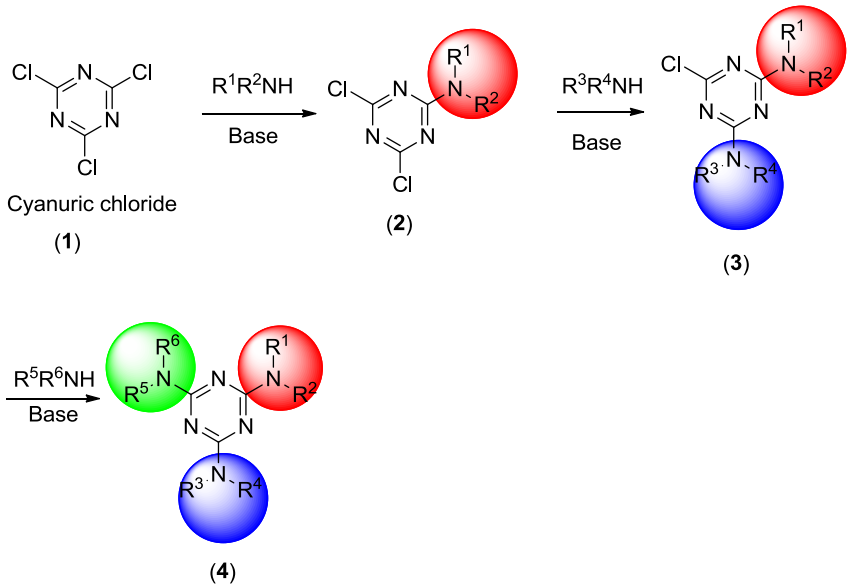


Figure 1. *Structures of three isomeric forms of triazine.*

Among three isomers, 1,3,5-triazine is most common and studied one. The common method of synthesis of 1,3,5-triazine derivatives is starting from cyanuric chloride, a chlorinated derivative of 1,3,5-triazine [13]. The ease of displacement of chlorine atoms of cyanuric chloride by a variety of nucleophiles in the presence of different bases has made this reagent very useful for selective preparation of mono-, di-, and tri-substituted-1,3,5-triazines. The substitution pattern also depends upon the structure of nucleophiles, strength of base used, steric factors, substituents in the triazine ring, and solvents. Scheme 1 depicts the general scheme of synthesis of 1,3,5-triazine derivatives using amines as nucleophilic species.



Scheme 1. *Synthesis of different 1,3,5-triazine derivatives.*

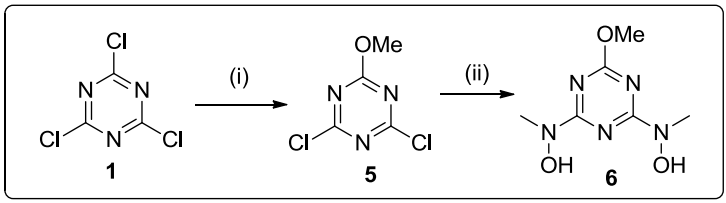
Because of significant amount of work done in this area, it is important to focus on those recent reports in which 1,3,5-triazine based compounds have been discovered as anti-cancer agents. In this focus book chapter, we highlight the recent advance in the development of 1,3,5-triazine based anti-cancer compounds reported since the year 2010.

3.2 Synthesis and Anti-Cancer Actvities of 1,3,5-Triazine Derivatives

Sun and co-workers synthesized compound 6 by monosubstitution of a chlorine atom of 1 with a methoxy group, followed by treatment with an excess of *N*-methylhydroxylamine (Scheme 2). Compound 6 displayed potent ant-cancer activities against MiaPaCa and MDA-MB-231 cell lines (IC_{50} values of 0.6 and 5 μM against MiaPaCa and MDA-MB-231 cell lines, respectively) [14]. The activity of compound 6 was much better than standard drug DFX (Scheme 2).

Kumar and co-workers and co-workers synthesized some 1,3,5-triazine based tetrahydro-β-carbolines (9-11) by using the synthetic strategy depicted in Scheme 3 [15].

(A)



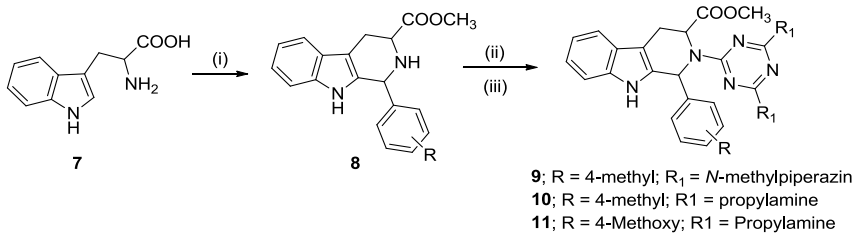
(B)

Cytotoxicity, IC_{50} (μM) of **6**		
MiaPaCa	MDA-MB-231	Foreskin fibroblasts
0.6 ± 0.2	5.0 ± 2.0	100 ±10

Cytotoxicity, IC_{50} (μM) of Deferasirox		
MiaPaCa	MDA-MB-231	Foreskin fibroblasts
4 ± 1	10 ± 2.5	100 ±10

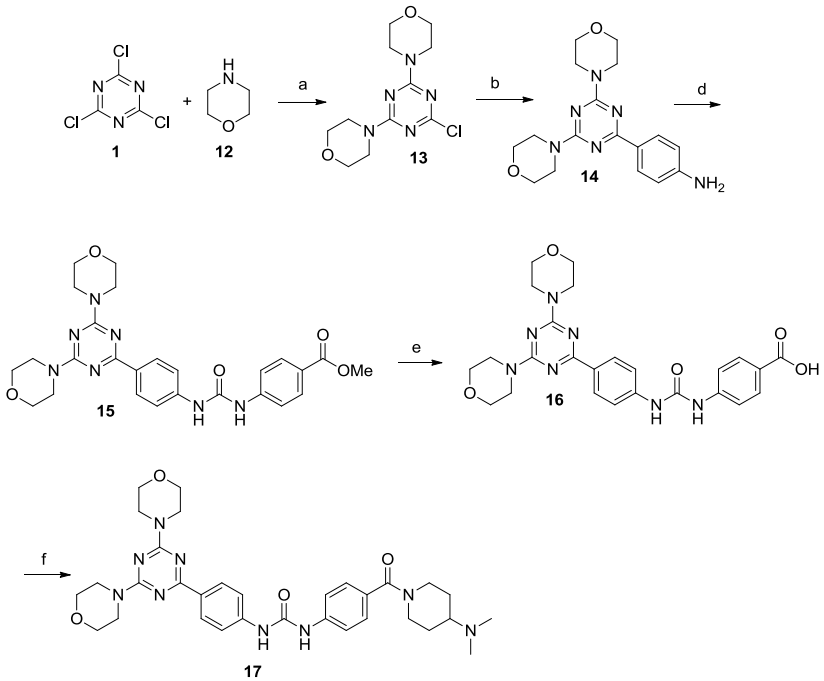
Scheme 2. *(A) Reagent and conditions: (i) MeOH, 2,6-lutidine; (ii) MeNHOH, excess; (B) Anti-cancer activities of compound 6 and Deferasirox.*

First, the tetrahydro-β-carbolines (8) were synthesized *via* Pictet-Spengler cyclization of methyl ester of tryptophan with different substituted benzaldehydes, followed by nucleophilic substitution of one chloro group of cyanuric chloride and then displacement of the remaining two chloro groups with different amines as shown in scheme 3. All compounds were then assayed for their anti-proliferation effects against eight human cancer cell lines and normal human fibroblasts (NIH3T3) [15]. Among all compounds screened, 9, 10, and 11 displayed promising cytotoxic effects against KB (oral cancer) cell lines with IC_{50} values of 105.8, 664.7, and 122.2 nM, respectively.



Scheme 3. *Reagents and Conditions: (i) Thionyl chloride, MeOH, various benzaldehydes, (ii) Cyanuric chloride, K_2CO_3, THF, 0 ℃ - r.t. (iii) Amines, K_2CO_3, THF, Reflux.*

Venkatesan and co-workers synthesized compound 17 by using the synthetic route shown in scheme 4 [16]. First, the chlorines atoms of cyanuric chloride 1 were replaced with 2 equivalents of morpholine 12 to yield 13, followed by displacement of third chlorine by a 4-aminophenyl group employing Suzuki coupling to give compound 14. The compound 14 was then reacted with methyl 4-isocyanatobenzoate in DCM to give compound 15 in a quantitative yield, followed by ester group hydrolysis to yield compound 16. The acid derivative 16 was then reacted with *N*(Me)2-piperidnine in the presence of HOBt/EDCI and triethylamine to furnish compound 17 in 52% yield. The compound 17 showed excellent *in vitro* inhibiting potential against PI3KR, PI3Kγ, and mTOR along with good microsomal stability (Table 1). The compound 17 was also evaluated against a panel of 236 other human protein kinases at 10 μM concentration, and was found to be highly selective for PI3K and mTOR.

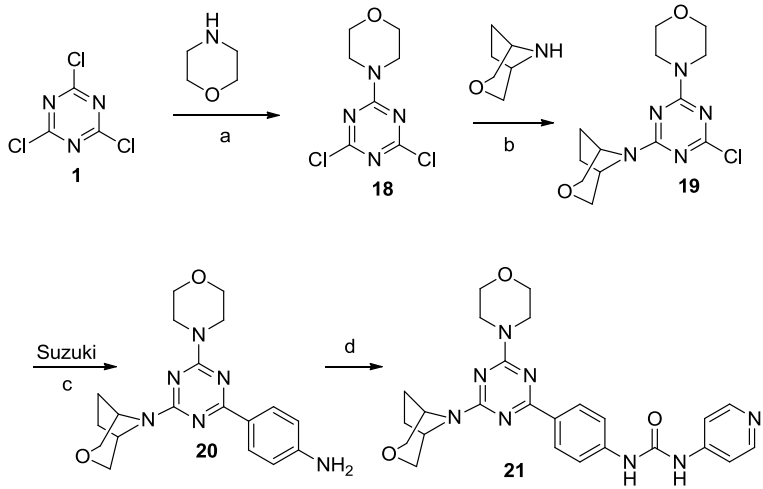


Scheme 4. *Reagents and conditions. (a) Et_3N (5q.), acetone, crushed ice, -20 ℃-0 ℃; (b) 4-Aminophenylboronic acid, pinacol ester (1.2 eq), $Pd(Ph_3P)_4$ (5 mol%), DME, 2M Na_2CO_3, reflux, 8h; (d) Methyl-4-isocyanatobenoate (1.1 eq), CH_2Cl_2, RT; (e) 5N NaOH (3 eq), MeOH, THF, 70 ℃, 12h; (f) Amines (2 eq), HOBt (1.5 eq), Et_3N (2 eq), THF, rt, 12h.*

Table 1. *In vitro activity of compound 17.*

IC_{50} (nM)				
PI3K-α	**PI3K-γ**	**PI3K-γ**	**MDA-361**	**PC3-MM2**
0.4	5.4	1.6	4.0	13.1

Later on, the same group discovered compound 21 bearing a 3-oxa-8-azabicyclo[3.2.1]octane moiety to be potent and orally efficacious dual PI3K/mTOR inhibitor displaying an excellent *in vitro* cell activity (table 2) and a good *in vivo* efficacy in the MDA-361 xenograft model [17]. The synthesis of compound 21 is displayed in scheme 5.

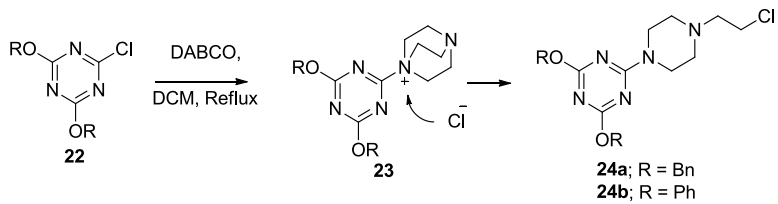


Scheme 5. *Reagents and conditions: (a) morpholine (1.1 equiv), Et_3N (2 equiv), acetone, crushed ice 20 ℃ to 0 ℃; (b) 1.1 equiv of 5, Et_3N (2 equiv), CH_2Cl_2, room temperature; (c) 4-aminophenylboronic acid, pinacol ester (1.2 equiv), $Pd(Ph_3P)_4$ (5 mol %), DME, 2 M Na_2CO_3, 120 ℃/30 min, microwave irradiation; (d) triphosgene (0.6 equiv), Et_3N (3 equiv), CH_2Cl_2, room temperature, 15 min, then 4-aminopyridine (5 equiv), 2-6 h.*

Table 2. *In vitro enzyme inhibition and cell proliferation inhibition IC50 (nM) values and calculated c log P values of 21.*

PI3K-α	PI3K-γ	mTOR	MDA361	PC3	c log P
8	74	0.42	22	29	2.12

Kolesinska and co-workers synthesized compounds 24a and 24b by replacement of chlorine of corresponding starting triazines by DABCO as shown in scheme 6.



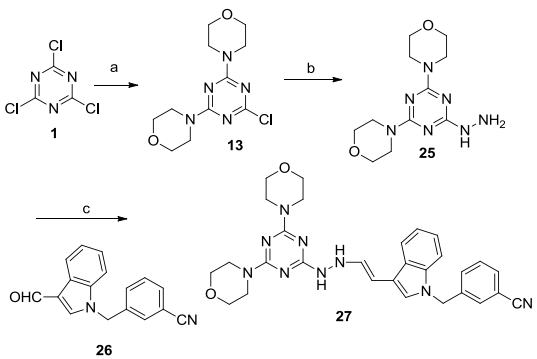
Scheme 6. *Reagent and conditions. DABCO (10 mmol), DCM (10 mL), Reflux.*

Both compounds showed good tendency to inhibit different cancer cell lines such as breast cancer T47D, prostate cancer LNCaP, colorectal cancer SW707, lung cancer A549, and lymphoblastic leukemia Jurkat [18]. The IC_{50} values of both compounds against these cancer cell lines are given in table 3.

Table 3. *IC50 values of compound 24a and 24b against different cancer cell lines.*

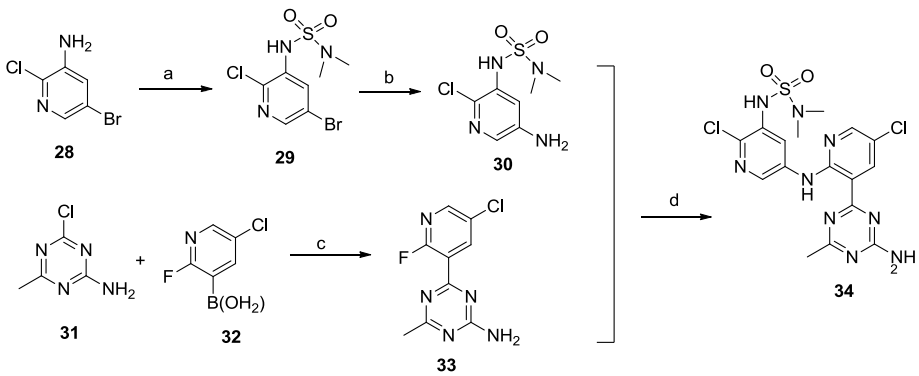
Compd	**Cell line/IC_{50} [µg/mL] mean ± SD**				
	breast cancer T47D	**prostate cancer LNCaP**	**colorectal cancer SW707**	**lung cancer A549**	**lymphoblastic leukemia Jurkat**
24a	1.40 ± 0.33	0.99 ± 0.52	3.45 ± 0.28	2.06 ± 0.66	0.62 ± 0.15
24b	2.60 ± 0.99	1.47 ± 0.95	2.93 ± 0.81	2.67 ± 1.33	2.93 ± 0.36

Zhu and co-workers synthesized compound 27 by using the synthetic strategy shown in scheme 7. First, the replacement of two chlorine atoms of cyanuric chloride with morpholine, followed by substitution with hydrazine gave compound 25 as a pale yellow solid [19]. Condensation of compound 25 with aldehyde 26 in EtOH/acetic acid afforded the target compounds 27 in a quantitative yield. Compound 27 was then evaluated for its anti-proliferative effects against five cancer cell lines (H460, HT-29, MDA-MB-231, U87MG, and H1975). It showed strong anti-proliferative activity against H460, HT-29, and MDA-MB-231 cell lines with IC_{50} values of 0.05, 6.31, and 6.50 µM, respectively.



Scheme 7. *Reagents and conditions. (a) Morpholine (2eq), TEA (3 eq), Acetone/Water, -10 ℃, 1h, rt; (b) 80% $NH_2NH_2.H_2O$, 80 ℃; (c) CH_3COOH (cat.), EtOH, rt.*

Wurtz and co-workers synthesized compound 34 by using the synthetic strategy shown in Scheme 8 [20]. Compound 30 was prepared from commercially available 3-amino-5-bromopyridine 28 by its treatment with dimethylsulfamoyl chloride to afford compound 29 which was then converted into compound 30 by palladium-catalyzed cross-coupling followed by hydrolysis with 1 N HCl. On the other hand, compound 33 was synthesized by Suzuki coupling of 31 with boronic acid 32. Base-promoted SNAr displacement of the 2-fluoropyridine 33 with compound 30 afforded desired compound 34 in quantitative yield.



Scheme 8. *Reagents and conditions: (a) $N(Me)_2SO_2Cl$, pyridine, DMAP; (b) (i) $Pd_2(dba)_3$ (5 mol %), Xantphos (10 mol %), benzophenone imine (1.1 equiv), NaO-t-Bu (4 equiv), DMF, microwave, 140 ℃, 20 min; (ii) THF, 1 N HCl, RT, 30 min; (c) $(4\text{-}NMe_2C_6H_4Pt\text{-}Bu_2)_2PdCl_2$ (Amphos, 5 mol %), KOAc (3 equiv), 100 ℃, 1,4-dioxane, 16 h; (d) LiHMDS or NaHMDS (4 equiv.), DMF or THF, 0 ℃ to rt.*

Compound 34 was found to be promising PI3K [21] inhibitor displaying moderate selectivity over the mammalian target of rapamycin (mTOR) in the enzyme assay. In a U87 MG cellular assay measuring phosphorylation of Akt, compound 34 displayed potent IC_{50} value of 24 nM along with good oral bioavailability in rats (F = 63%) (Tables 4 and 5).

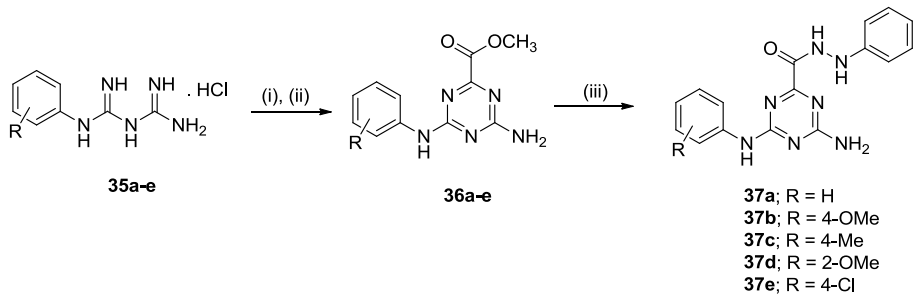
Table 4. *Enzyme and cellular assay of compound 34 against PI3K.*

Compd	PI3K Ki (nM)	pAkt (U87 MG) IC_{50} (nM)	cLogP
34	12	24	2.1

Table 5. *Pharmacokinetic properties of compound 34.*

Compd	*In vivo* rat PK				
	i.v.			po	
	CL (L/h/ kg)	Vdss (L/ kg)	MRT (h)	%F	AUC (ng h/mL)
34	0.39	0.97	2.5	63	3360

Kothayer and co-workers synthesized 4-amino-6-(arylamino)-*N*-phenyl-1,3,5-triazine-2-carbohydrazides (37a-e) in two steps from commercially available arylbiguanide hydrochloride salts (35a-e) (Scheme 9) [22]. First, neutralization of the arylbiguanide hydrochloride salt with sodium methoxide/methanol was performed, followed by treatment with dimethyloxalate to afford methyl 4-amino-6-(arylamino)-1,3,5-triazine-2-carboxylates (36a-e) in a good range of yields. Reaction of intermediates (36a-e) with phenylhydrazine afforded corresponding triazine carbohydrazides (37a-e) in 91-96% yield (Scheme 9).



Scheme 9. *Synthesis of 4-amino-6-(arylamino)-N-phenyl-1,3,5-triazine - 2-carbohydrazides (3a-e). Reagents and conditions: (i) $NaOCH_3$, CH_3OH, rt, 3 h; (ii) dimethyloxalate, CH_3OH, reflux, 4 h; (iii) phenylhydrazine, AcOH, EtOH, reflux, 12 h.*

Compounds 37a-e were tested for their *in vitro* anti-cancer activity against MDA-MB-231 and MCF-7 cell lines (Table 6). All compounds showed potent

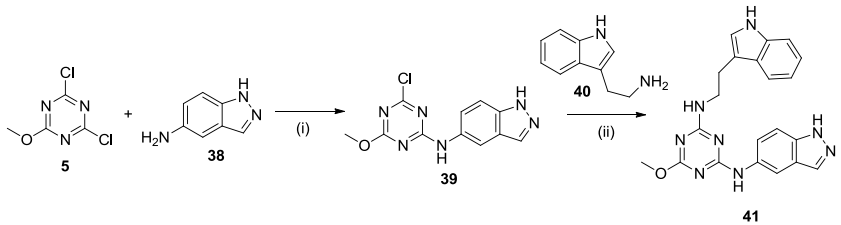
activity against MDA-MB-231 cell lines, whereas moderate activity was observed against MCF-7 cell lines (Table 6).

Table 6. *Growth inhibitory activity of compounds 37a-e against different cell lines.*

Compound	MDA-MB-231	MCF-7	MCF10A
37a	3.67 (0.46)	31.3 (3.1)	>100
37b	4.79 (0.40)	38.0 (2.5)	>100
37c	4.65 (0.13)	16.6 (0.2)	>100
37d	2.71 (0.21)	53.2 (4.8)	>100
37e	2.48 (0.72)	25.5 (5.2)	>100

Results are expressed as triplicate mean values (standard deviation in parentheses).

Ryu and co-workers synthesized compound 41 from 2,4-dichloro -6-methoxy-1,3,5-triazine (5) by using the synthetic strategy shown in scheme 10 [23]. Treatment of triazine 5 with 5-aminoindazole (38) using Et_3N as a base gave triazinyl indazolamine 39. Further reaction of compound 39 with tryptamine (40) in methanol afforded 41 in 72% yield.



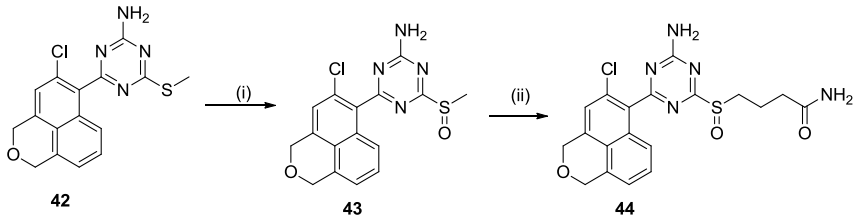
Scheme 10. *Reagent and Conditions: (i) Et_3N (1.35 eq.), MeOH, rt, overnight, 99%; (ii) Et_3N (1.35 eq.), MeOH, reflux, 4h, 72%.*

Compound 41 showed a good tendency to inhibit PAK4 activity along with moderate activity against LNCap and PC-3 cells lines (table 7). The activity against LNCap cell lines was about 3 times over PC-3 cells lines.

Table 7. *Anti-cancer activity of compound 41.*

Compd	IC_{50} (µM)			
	LNCap cells		PC-3 cells	
	Viability	AR luciferase	Viability	AR luciferase
41	14.47 ±1.26	2.50 ±0.28	47.47 ±0.92	1.96 ±0.50

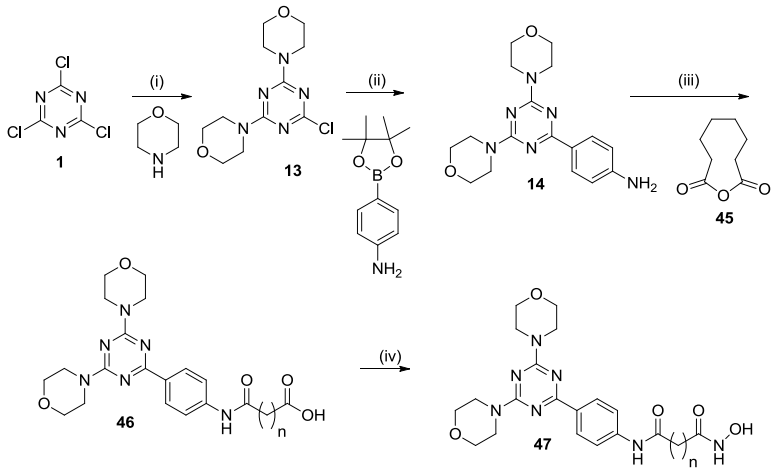
Suda and co-workers synthesized compound 44 in two steps synthesis as shown in scheme 11 [24].



Scheme 11. *Reagents and conditions: (i) mCPBA, CH_2Cl_2, rt, 3 h, 78%; (ii) 2-aminoethan-1-ol, HOBT, EDC.HCl, DIPEA, DMF, rt.*

Compound 44 showed a high binding affinity for *N*-terminal Hsp90a (K_d = 0.52 nM) along with strong *in vitro* cell growth inhibition of HCT116 (IC_{50} = 0.098 µM) and NCI-N87 (IC_{50} = 0.066 µM) cell lines. Compound 44 also displayed high oral bioavailability in mice (F = 44.0%) along with satisfactory anti-tumor efficacy in a human NCI-N87 gastric cancer xenograft model (tumor growth inhibition = 136%).

Zhao and co-workers synthesized compound 47 by using the synthetic strategy outlined in Scheme 12 [25]. The replacement of two chlorine atoms of cyanuric chloride with 2 equivalents of morpholine gave compound 13, followed by replacement of third chloride atom by 4-aminophenyl groups using Suzuki coupling to afford compound 14. Compound 14 was then reacted with suberic anhydride to give acid 8 which on condensation with hydroxylamine in the presence of isobutyl chloroformate gave desired hydroxamic acid 47.



Scheme 12. *Reagents and conditions: (i) Et_3N, CH_3COCH_3; (ii) $Pd(Ph_3)_4$, K_2CO_3, DMF; (iii) THF, 0 ℃; (iv) $NH_2OH.HCl$, ClCOOBu-i, THF.*

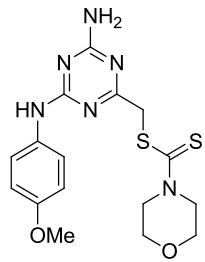
Compound 47 was then evaluated for its potential of inhibiting human histone deacetylases and anti-proliferative effect against HCT-116, MCF-7, and HeLa cancer cell lines. The results are illustrated in table 8. DNA flow cytometric analysis showed that compound 47 could induce apoptosis and cell cycle arrest at G2/M phase in HCT-116 cells.

Table 8. *In vitro enzyme inhibition and cell proliferation inhibition IC_{50} (nM) values and calculated c log P values of 47.*

Compd	HDACs (% of inhibition at 1 μM)	HDACs IC_{50} (M)a	HCT-116 IC_{50} (M)	MCF-7 IC_{50} (M)	HeLa IC_{50} (M)
47	77.6	0.31 x 10-6	0.7 x 10-6	7.9 x 10-6	12.6 x 10-6
SAHA	90.2	0.06 x 10-6	1.4 x 10-6	1.5 x 10-6	1.6 x 10-6

Pogorelcnik and co-workers discovered that triazine based compound 48 could show moderate *in vitro* inhibitory activity against human DNA topoisomerase Iiα with an IC_{50} value of 229 μM (Figure 2) [26]. Compound 48 also displayed moderate anti-proliferative activity against hepatocellular

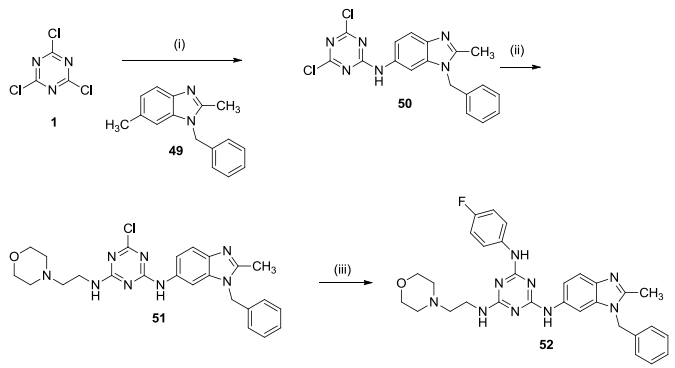
carcinoma (HepG2) and human umbilical vein endothelial (HUVEC) cell lines with IC_{50} values of 20.53 and 122 µM, respectively.



48

Figure 2. *Structure of compound 48.*

Singla and co-workers synthesis of compound 52 by using the synthetic strategy depicted in scheme 13 [27]. First, one chlorine atom of 2,4,6-trichloro-1,3,5-triazine (1) was replaced by 3-benzyl-2-methyl -3H-benzimidazol-5-ylamine (49) to afford compound 50. Second chlorine atom displacement of compound 50 by 2-aminoethylmorpholine afforded compound 51 which was then converted into compound 52 by replacement of third chlorine atom by *p*-fluoro aniline (Scheme 13).



Scheme 13. *Synthesis of monosubstituted, disubstituted and trisubstituted triazines. Reagents and conditions: (i) 10% $NaHCO_3$, THF, 0-5 ℃; (ii) 2-Aminoethylmorpholine, 10% $NaHCO_3$, THF, room temperature; (iii) p-fluoro aniline, K_2CO_3, 1,4-dioxane, 110 ℃.*

Compound 52 displayed potent anti-tumor activity with a GI_{50} value of 2.87 μM. Compounds 52 was also displayed inhibition of dihydrofolate reductase with an IC_{50} value of 2.0 nM (Table 9).

Table 9. *Median growth inhibitory (GI_{50}, μM), total growth inhibitory (TGI, μM) and median lethal concentrations (LC_{50}, μM) of compound 52 against different cancer cell lines.*

Compd	Cancer Cell lines	GI50	TGI	LC50
52	I	1.96	6.82	55.0
	II	3.21	18.5	38.5
	III	2.60	8.51	24.0
	IV	2.72	12.0	15.3
	V	1.91	4.08	24.2
	VI	4.01	27.4	62.3
	VII	3.03	19.3	14.6
	VIII	4.41	43.9	62.5
	IX	2.04	8.99	20.6
	MIG-MIDa	2.87	16.6	35.2

I, leukemia; II, non-small cell lung cancer; III, colon cancer; IV, CNS cancer; V, melanoma; VI, ovarian cancer; VII, renal cancer; VIII, prostate cancer; IX, breast cancer.

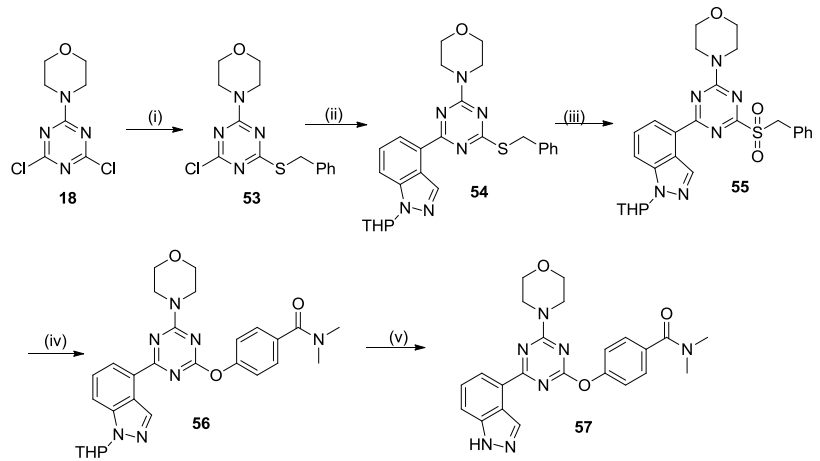
[a] Full panel mean-graph midpoint (μM).

Dugar and co-workers synthesized compound **57** from compound **18** by using the synthetic route shown in scheme 14 [28]. Replacement of one chlorine atom of **18** with benzyl mercaptan, followed by Suzuki coupling of resulting compound **53** with aryl borate afforded compound **54**. Compound **54** was then oxidized into compound **55** using ozone as an oxidizing agent. Replacement of sulfonyl group by 4-hydroxy-*N*, *N*-dimethylbenzamide, followed by deprotection of THP group afforded the desired compound **57**.

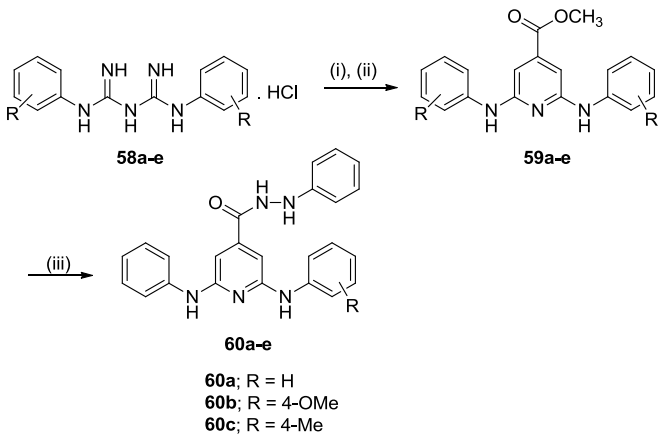
Compound **57** displayed potent inhibition of PI3Kα with an IC_{50} value of 60 nM. Compound **57** also potently inhibited the ovarian cancer (A2780) cell

lines with an EC_{50} value of 500 nM. Compound 57 showed good oral bioavailability with an AUC of 5.2 μM at a dose of 3 mpk in mice.

Kothayer and co-workers synthesized *N*'-phenyl-4,6-bis (arylamino) -1,3,5-triazine-2-carbohydrazides (60a-c) from bisarylbiguanide hydrochloride salts [29] (58a-e) by using the synthetic route depicted in scheme 15 [30]. First, the neutralization of the bis-arylbiguanide hydrochloride salt was performed with sodium methoxide/methanol, followed by reaction with dimethyloxalate in refluxing methanol yielding methyl 4,6-bis(arylamino)-1,3,5-triazine-2 -carboxylates (59a-c) in good yield following recrystallization from methanol. Further treatments of compounds (59a-e) with phenylhydrazine in refluxing ethanol catalyzed by glacial acetic acid afforded the desired new triazines (60a-c) in good moderate to good yields following recrystallization from ethanol-water (3:1) (Scheme 15).



Scheme 14. *Reagents and conditions: (i) Benzyl mercaptan, DIPEA, morpholine, THF, 0 ℃, 5 h; (ii) aryl borate, $Pd(PPh_3)_4$, Na_2CO_3, DME/water (4:1), 90 ℃, 18 h; (iii) oxone, THF/water (1:1), 0°C−RT, 25 h; (1v) RX, DMF, K_2CO_3, RT, 24 h; (v) methanesulfonic acid, methanol/ water (2:1), 55 ℃, 1 h.*



Scheme 15. *Reagents and conditions: (i) $NaOCH_3$, CH_3OH, room temp 3 h; (ii) dimethyloxalate, CH_3OH, $NaOCH_3$ reflux, 12 h; (iii) phenylhydrazine, EtOH, AcOH, reflux 18 h.*

Compounds 60a-c showed moderate to potent activities against different cancer cell lines as shown in table 10. All three compounds showed most potent activities against MDA-MB231 cell lines (Table 10).

Table 10. *Activity of compounds 60a-c against different cancer cell lines.*

Cell lines	**IC_{50} (µM)**		
	60a	**60b**	**60c**
OV 90	8	12	5
A2780	7.1	6.3	3.6
MCF-7	6	7.2	4.2
MDA-MB231	2.5	4.2	3.5
A549	14.6	10.8	11.6
H1299	11	5	22
HT-29	9.5	5.8	5.2

3.3 Conclusion

Collectively, 1,3,5-triazine motif has emerged as a valuable scaffold for potent anti-cancer activities displaying IC_{50} values in nano to micromolar

concentration range against a wide variety of cancer cell lines. Some of these compounds have also shown promising oral bioavailability (%F) which further highlights the extra advantage of three nitrogen atoms present in the triazine ring. Moreover, the synthesis of most of these compounds is straightforward and economical usually from commercially available cyanuric chloride. It is believed that the information in this book chapter will be very much help for medicinal chemists in designing and synthesizing more potent anti-cancer drugs based upon 1,3,5-triazine scaffold in future.

Acknowledgements

This work was supported by a grant of Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2015M3A6A4065734 and 2014R1A2A2A01005455), Republic of Korea.

Abbreviations

1. AUC = Area Under the curve
2. DABCO = 1,4-Diazabicyclo[2.2.2]octane
3. DCM = Dichloromethane
4. DFX = Deferasirox
5. DME = 1,2-Dimethoxyethane
6. EDCI = 1-Ethyl-3-(3-dimethylaminopropyl)

   Carbodiimide
7. Et_3N = Triethylamine
8. %F = % Fraction

9. GI = Gross inhibition
10. HOBT = Hydroxybenzotriazole
11. HUVEC = Human umbilical vein endothelial cell
12. i.v. = Intravenous
13. K_d = Dissociation constant
14. LiHMDS = Lithium bis(trimethylsilyl)amide
15. m-TOR = Mammalian target of rapamycin
16. MCF-7 Cells = Human breast adenocarcinoma cell line
17. MIA PaCa = Miapaca pancreatic cancer
18. NaHMDS = Sodium bis(trimethylsilyl)amide
19. PC = Prostate Cancer
20. PI3K = Phosphoinositide 3-kinase
21. LiHMDS = Lithium bis(trimethylsilyl)amide
22. TGI = Total growth inhibition

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Chapter 4

Neocarzinostatin - New Promises in Anticancer Activities

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4.1 Introduction

Medically cancer is a malignance, a broad cluster of assorted disease. The malignancy may spread to more expelled parts of the body through the lymphatic structure or circulatory framework. While trying to battle uncontrolled development cells, researchers are making an endeavor to seek out molecules to arrest formation of the malignant tumors. These molecules are referred to as anticancer antibiotics. Chemotherapy is typically the primary selection for the treatment of the many cancer sorts. Enediynes antibiotics potential natural toxins that possess potent medicinal drug, malignant tumor activities due to their distinctive molecular structure mode of action, currently the foremost promising leaders within the malignant tumor medical aid and tried clinical affectivity [1]. Among 20 distinct enediynes, the foremost necessary Neocarzinostatin (NCS) in cancer treatment and a potent tumour, drug actions [2] and exerted by DNA cleavage. DNA harming movement essentially in single-strand DNA cuts related yield through an O_2subordinate reaction [3], Thiols [4] and UV radiation [5] enormously upgrade DNA-severing properties of NCS. This Chapter offers a high level read of NCS, synthesis, mode of action and efforts undertaken to vogue artificial enediyne-related DNA cleaving agents.

4.2 Neocarzinostatin Chromophore

NCS is the first member of enediyne antibiotic class [6], isolated from *Streptomycescarzinostaticus* Var. F-41reported by Ishida *et al* in 1965 [7]. In 1966, the complementary chromophore binding protein (apo-NCS) isolated [8] and following Maeda‘s studies primary sequence of protein was published [9], later revised [10] and confirmed by NMR studies [11]. NCS is an acidic protein, MW 10,700, 1:1 non-covalently associated mixture of a protein component (NCS apoprotein) and a chromophoric molecule (NCS chromophore, Figure 1).

It is the in freestanding state is highly unstable upon exposure to heat, high pH or UV-light irradiation.

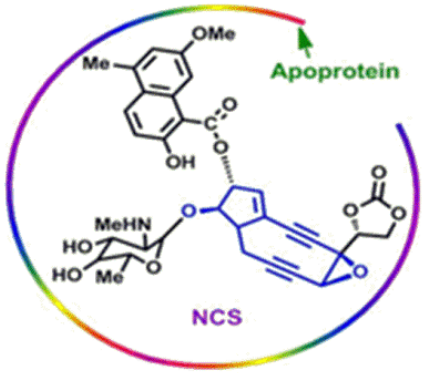
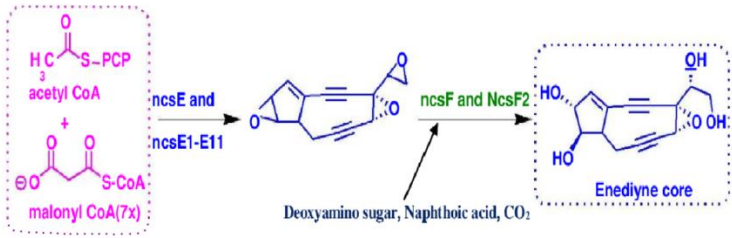


Figure 1. *Neocarzinostatin chromophore (peptide loop composed of 113 amino acids).*

4.3 Enediyne Core of NCS Biosynthesis

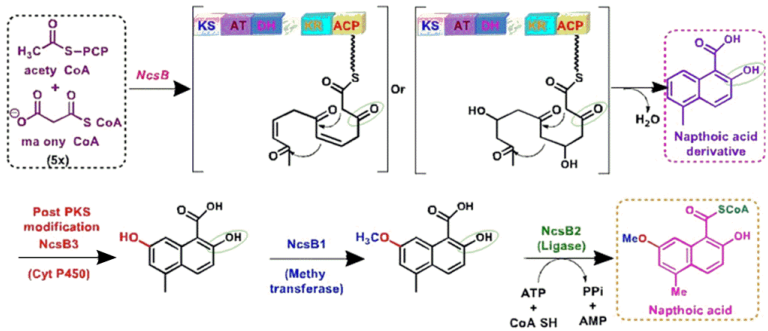
14 genes identified within*ncs* gene (*ncsE*to*ncsE11*and*ncsF1*to*ncsF2*) cluster, important role in the NCS core biosynthesis [12]. The enediyne core previously predicted to be synthesized by an iterative type Ipolyketide synthase (PKS) with five domains, of which keto-synthase (KS), acyltransferase (AT), ketoreductase (KR), and dehydratase (DH) are characteristic of known type I PKSs [13, 14, 15, 16, 17]. NcsE shows head-to-tail sequence homology to the SgcE [13] and CalE8 [14] enediyne PKSs. Consequently, it proposed that NcsE, in a mechanistic analogy to other enediyne PKSs, catalyzes the formation of the nascent linear polyunsaturated intermediate from one acetyl CoA and seven malonyl CoAs in an iterative manner, which processed to form the enediyne core by several gene products, including NcsE1-E11 and epoxide hydrolases F1 and F2 (Scheme 1).



Scheme 1. *Biosynthetic hypothesis of the enediyne core of NCS.*

4.4 Naphthoic Acid Biosynthesis

Naphthoic acid moiety synthesis from polyketidechain of sixhead-to-tail acetate units are disclosed by isotopic labeling experiments. In NCS gene cluster, major enzymesare (a) an iterative PKS (NcsB), (b) a CoA ligase and (c) several ancillary enzymes. Naphthoic acid synthesis starts with NcsB, an iterative PKS that contain domains (a) the keto-acyl synthase (KS), (b) acyltransferase (AT), (c) keto-reductase (KR), (d) dehydratase and (e) acyl carrier protein domain (ACP) and a core domain with unknown function. NcsB uses acetyl coenzyme A (CoA) as stating material and malonyl-CoA as extender to assemble a nascent hexaketide with reduction and dehydration of the keto groups at C5 and C9 (Scheme 2).

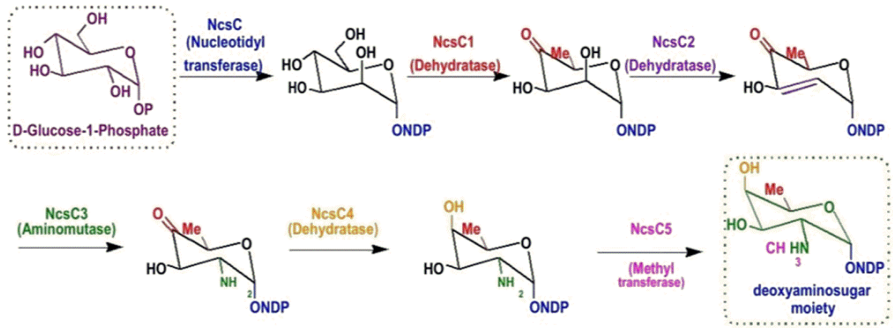


Scheme 2. *Possible biosynthesis mechanism for the naphthoic acid moiety of neocarzinostatin.*

The hexaketide intermediate then undergoes aromatization by intramolecular aldol condensation to furnish the naphthoic acid moiety. The post-PKS modification of the naphthoic acid starts with the incorporation of a OH group at C8 carbon which catalyzed by the cytochrome P450 hydroxylase NcsB3. Ultimatelymethylation of OH group catalyzed by an S-adenosylmethionine (SAM) dependent O-methyltransferase(NcsB1). Then NcsB2 ligase catalyzes the adenylation of 2-hydroxy-7-methoxy-5-methyl-1-naphthoic acid to form its CoA derivative. Finally, putative acyl transferase (NcsB4) responsible for transfer of naphthoic group onto enediyne core.

4.5 Deoxyamino Sugar Biosynthesis

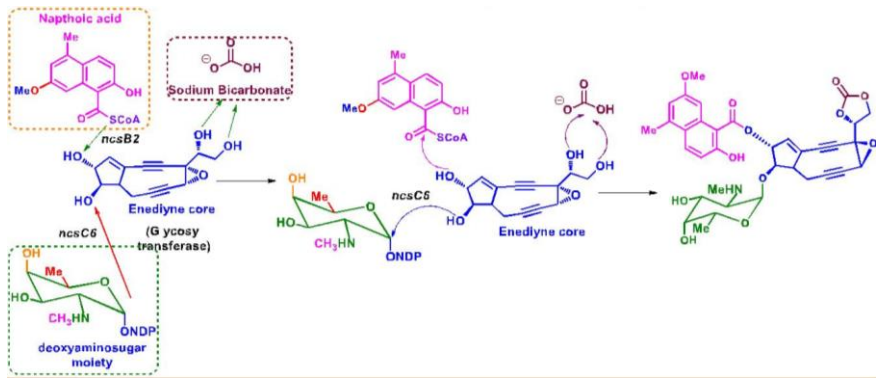
The deoxy amino sugar moiety biosynthesis start with activation of monosaccharide as its nucleotide diphospho (NDP) derivative by nucleotidyltransferase NcsC (Scheme 3). Several gene products have been proposed for the activation of sugar ring via formation of a 4-keto intermediate, deoxygenation at C6 and installation of amino group at C2. The isotopic labeling experiments with [methyl-3H] methionine revealed that the N-methyl of deoxy amino sugar originates from methionine of SAM. The methylation catalyzed by methyltransferase NcsC5, whereas glycosyltransferase NcsC6 may transfer sugar moiety onto enediyne core.



Scheme 3. *Possible mechanism of biosynthetic hypothesis of deoxyaminosugar moiety.*

4.6 Biosynthesis of NCS by Joining Together Peripheral Moieties to Enediyne Core

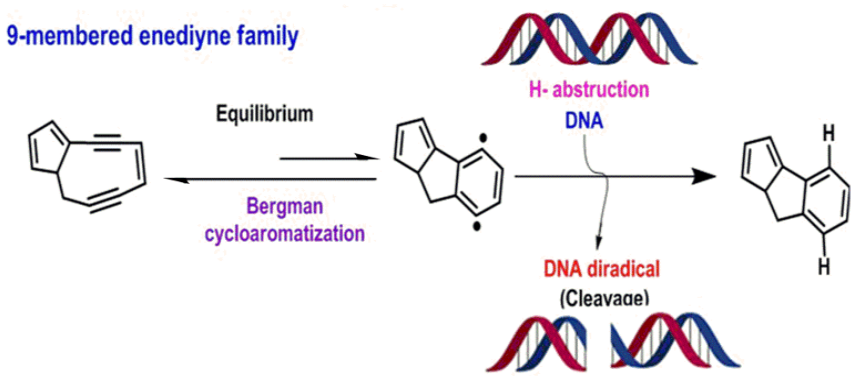
A convergent strategy could be envisaged for the assembly of the NCS chromophore from three individual building blocks of deoxy amino sugar, naphthoic acid, and enediyne core (Scheme 4). The coupling between dNDP-sugar and enediyne core catalyzed by NcsC6 glycosyltransferase while the other coupling between naphthoyl-S-NcsB and enediyne core catalyzed by NcsB2 CoA ligase. Although the cyclic carbonyl carbon of NCS previously shown to originate from carbonate, no obvious candidate catalyzing the attachment of carbonate could be identified within the gene cluster.



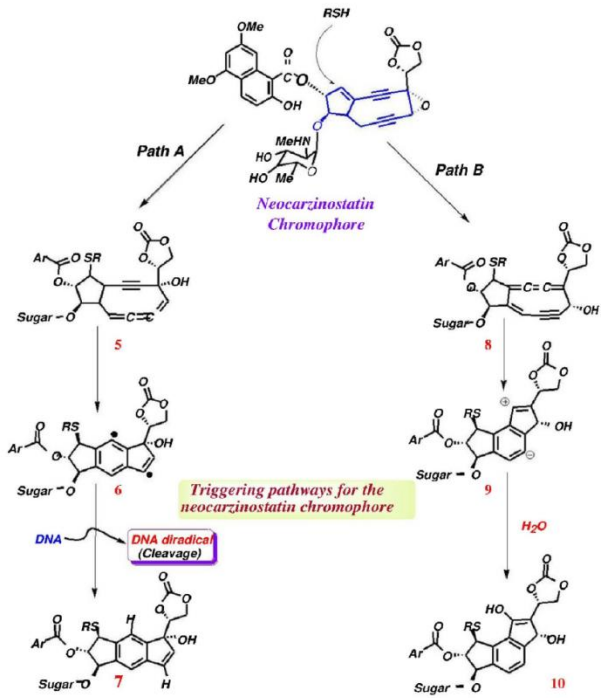
Scheme 4. *Biosynthesis of enediyne core of NCS with peripheral moieties.*

4.7 Mechanism of Action of NCSon Cancer Cell

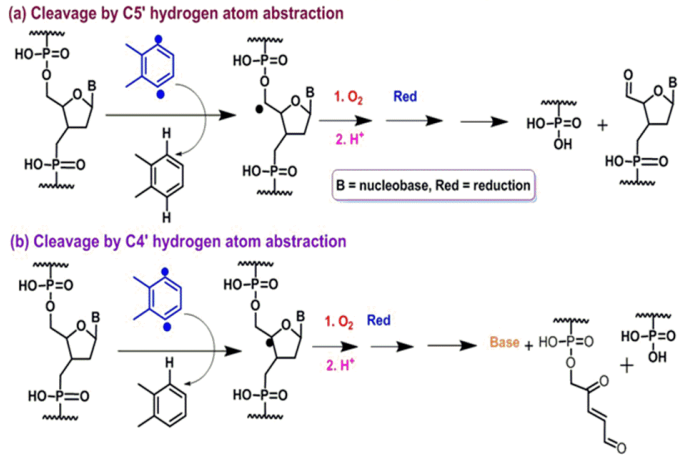
High-resolution X-ray diffraction, NMR techniques, together with thermodynamic studies and molecular modeling disclosed basic principles in DNA-drug interaction [18-20] and NCS-DNA mechanism first reported in 1987 [21]. In pathway A (Schemes 5 & 6), DNA damage initiated by stereospecific nucleophilic attack at C-12. This triggering reaction accompanied by rearrangement of ring skeleton with epoxide opening and formation of cumulene observed by NMR at low temperature [22] in 5 as shown in Scheme 6. Then this reactive intermediate undergoes a rapid cycloaromatization to form diradical in 6, and then which proceeds to attack DNA by removing hydrogen atoms in **7** and this scenario provided by using $HSCH_2CO_2Me$ [21-24], $NaBH_4$ [24] as nucleophiles in *in vitro* experiments. The methyl thioglycolate isolated, fully characterized and the evidence of the basic methyl amino side chain on the sugar residue assists the thiol addition at C-12 through base catalysis provided by Myers [22, 25]. The additional information provided by three-dimensional structure of intact NCS [26] - the amino methyl group of sugar forced into close proximity to C-12(4.3Å) due to a salt bridge with Asp33, suggesting that nucleophilic attack at C-12 assisted by nitrogen, and together with additional steric hindrance at C-12 from the side chains of Ser98, Asp33, Phe52 and the positioning of epoxide in a hydrophobic pocket away from an acid catalyst, this indicates that how apoprotein serves to stabilize the chromophore. In pathway B cycloaromatization, NCS chromophore incubated with 2-mercaptoethanol in the presence of apoprotein in which the zwitterionic intermediate 9 (Scheme 6) is indicated [27, 28], although this mechanism probably does not operate for the free chromophore. Since it is thought that dissociation of NCS chromophore from apoprotein and subsequent DNA binding precedes activation of chromophore, the biological relevance of this second mechanism seems dubious, and this pathway is not responsible for DNA cleavage reported by Chin and Goldberg in 1993 [29].



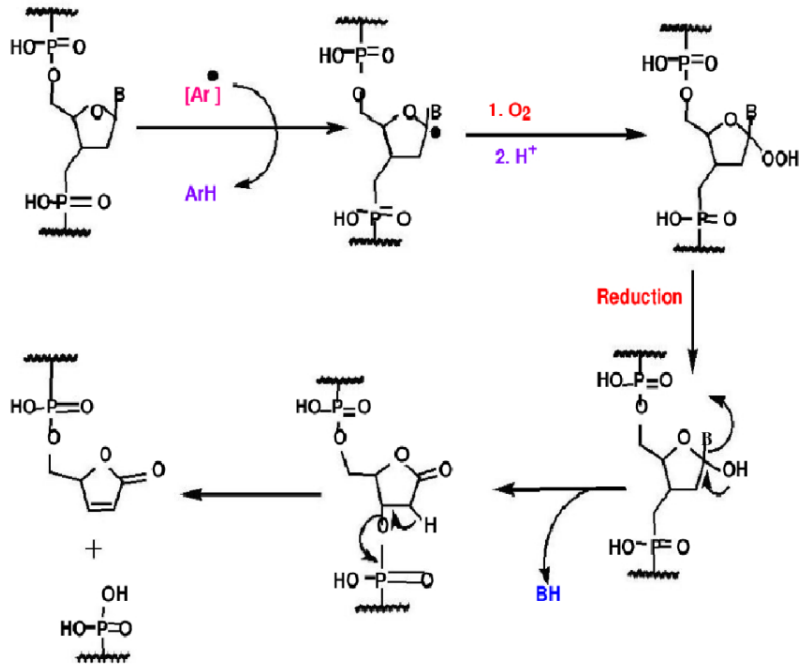
Scheme 5. *General mechanism of action of enediyne anticancer antibiotics: DNA cleavage.*



Scheme 6. *Mechanism of action of enediyne anticancer antibiotics: DNA cleavage initiated by C4'- or C5'-hydrogen atom abstraction.*



Scheme 7. *Mechanism of action of enediyne anticancer antibiotics: DNA cleavage initiated by (a) C4' or (b) C5' hydrogen atom abstraction.*



Scheme 8. *Mechanism of action of enediyne anticancer antibiotics: DNA cleavage initiated by C1'- hydrogen atom abstraction.*

Several scientists worked out to disclose the details of DNA damage by NCS chromophore diradical 6. Itdemonstrated that at least 80% of DNA cleavage leads to 5'-aldehyde of A and T residues selectively [30]. These cleaves involves hydrogen atom abstraction from C-5' of deoxyribose and reaction with molecular oxygen as showed in scheme 7. Less than 20% of strand breaks result from hydrogen atom abstraction at C-4'[31-35] and C-1' [32] (Schemes 7 & 8). The radical at C-2 of 6 particularly susceptible to both internal and external quenching up to 70% under physiological conditions reported by Goldberg [36]. A convincing explanation that the NCS chromophore effects primarily single-stranded DNA cuts by C-6 radical at C-5' of deoxyribose, whereas those double stranded lesions are involved hydrogen abstraction by C-2 radical from C-1' or C-4' of deoxyribose on complementary strand. Further insight into the interaction of NCS chromophore with DNA, recent observation made clear that a thiol independent cleavage mode is possible with single-stranded DNA bulges, the regions where double-stranded structuresgenerated intra molecularly [37]. These logical consequences indicated that DNA is an active participant in its own destruction, since DNAs containing point mutations which disrupt the bulge are not cleavage substrates.

4.8 Conclusion and Future Prospects

The studies described above indicate that how the mechanistic and synthetic challenges resulting from discovery of the neocarzinostatin antibiotic have been approached. Designed enediynes demonstrated abilities to cleave DNA and exhibited selective cytotoxicity against tumor cells versus normal cells [38, 39]. Enediynes have been implicated in the puzzling but important phenomenon of programmed cell death (apoptosis) [40] and the total synthesis of prominent and complex member of enediyne class has been achieved [41]. The most studied systems relate to neocarzinostatin, perhaps because this available for longest

period of time. This compound shown to possess antitumor activity in patients with liver cancer, bladder cancer, stomach cancer, and leukemia as well as in various animal tumors [42]. Polystyrene-co-maleic acid-NCS shown high antitumor activity in animal models following oral administration [43, 44]. Immuno-conjugates of neocarzinostatin such as A7-NCS [45] showing increased survival times when administered to postoperative cancer patients (both with and without metastases) when compared with other chemotherapies. Therefore these novel natural products with their unprecedented modes of action are clearly more than a scientific curiosity, and it remains to be seen whether enediynes, either natural or designed, will become useful additions to the arsenal of chemotherapies available to clinicians for the treatment of cancer.

The next phase of research in enediyne field will undoubtedly include further synthetic attempts at naturally occurring targets, new designed enediynes with sophisticated mechanisms of *in vitro* and *in vivo* activation, and attachment of these systems to suitable delivery systems. Targeting devices may include antibodies, oligonucleotides, oligosaccharides, peptides and proteins, DNA intercalators, DNA groove binders, hormones, and other ligands. Hybrid molecules between enediyne "molecular warheads" and such delivery systems should provide new insights into biological phenomena and may facilitate drug design and development.

We thank our many talented associates, whose names appear in the original articles cited below, for their invaluable contributions to this research effort. Our work was financially supported by School of Medicine, Research Division, Debre Markos University, Ethiopia.

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Chapter 5

Synthetic and Biological Applications of Benzothiazole Phosphonates

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Abstract

Benzothiazole derivatives have attracted considerable attention over the years as useful biological and pharmacological agents. The benzothiazole scaffold is one of the most frequently encountered heterocyclic moieties in many marine, as well as plant and natural products. Taking Nature's lead, the benzothiazole moiety provides a versatile bicyclic ring system that can be easily modified synthetically in the laboratory. Its synthetic derivatives are known to exhibit a wide range of useful medicinal and therapeutic properties: anticancer, antiviral, antimicrobial, antidiabetic, anti-inflammatory, anticonvulsant and antitubercular. Since the phosphinic acid moiety P(O)OH can mimic carboxylic acids, its incorporation into heterocyclic compounds has stimulated considerable interest in the possibility of producing unique chemical/biological properties for benzothiazole phosphonate derivatives. The pharmacological significance of these compounds in the field of medicinal chemistry could be substantial and this chapter will summarize the development and current status for the synthesis of new benzothiazole phosphonate compounds and report on biological aspects of these compounds that offer the promise of truly useful drugs for treating various maladies.

Keywords

α-Aminophosphonates, Benzothiazole Phosphonates, Biological Applications, Kabachnik-Fields Reaction, Organophosphorus Chemistry

5.1 Introduction

Naturally occurring organophosphorus compounds are not only essential for life but manmade organophosphous species have important applications in medicine, agriculture, industry and technology. Figure 1 illustrates the diversity of organophosphorus compounds. They occur naturally as fundamental building blocks of life itself. And manmade organophosphorus compounds have found use as nerve gases and organic synthetic reactants. [1-6] Phosphorus compounds in general, and phosphonates in particular, are a cornerstone of pharmaceutical drugs. [7-10] Many of these compounds exhibit antifungal [11-13], antiviral [14, 15], antibacterial [16-18], antioxidant [19-21], anticancer [22-25] and significant analgesic/anti-inflammatory properties [26-28]; several examples are shown in Figure 2.

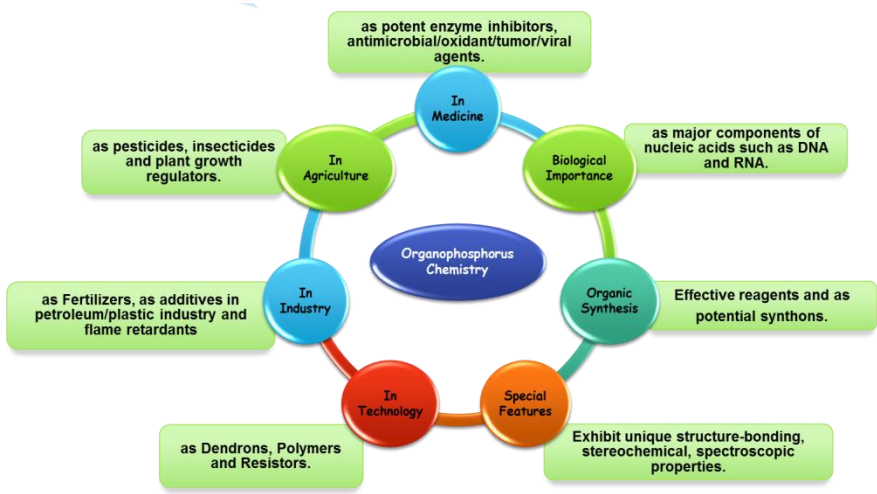


Figure 1. *Applications of Organophosphorus Compounds.*

Heterocyclic chemistry is a complex and fascinating branch of organic chemistry. Naturally occurring compounds containing heterocyclic fragments

are also fundamental to life. They are components of a broad spectrum of biological systems, have diverse applications and can evoke a wide range of chemical and physiological responses. [29-41] Heterocyclic compounds are active components of many drugs, agrochemicals, additives and modifiers used in a variety of industrial applications including cosmetics, reprography, information storage and plastics [42-45].

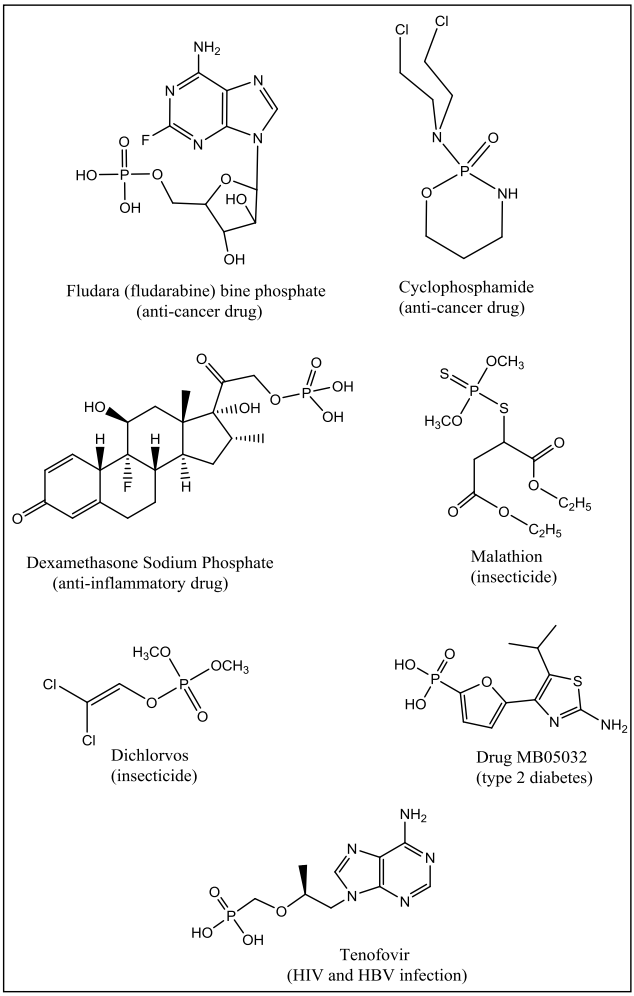


Figure 2. *Examples of Organophosphorus Compounds and Their Applications.*

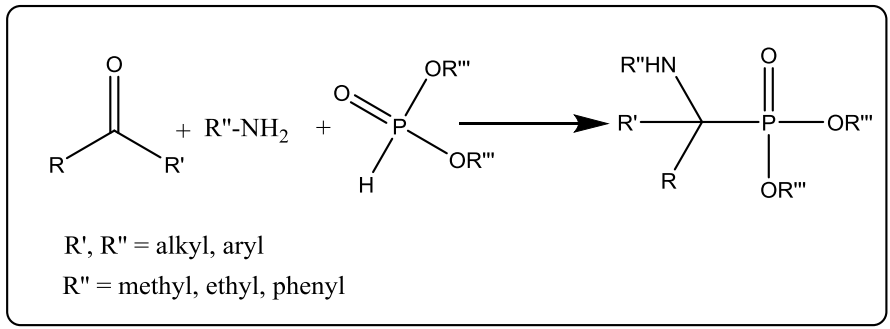
One particularly useful group of heterocyclic compounds, those based on the benzothiazole scaffold, have proved to have special significance in synthetic and pharmaceutical chemistry as drugs because of the broad spectrum of medicinal applications they seem to target. [46-50] Recently, there has been increased interest in the biological activities of benzothiazole phosphonate derivatives because the phosphinic acid moiety $P(O)(OH)_2H$ can mimic carboxylic acids and impart useful solubility (and other) properties to benzothiazole-based drugs. [51, 52] This chapter will focus on recent research involving this new class of potentially useful compounds - benzothiazole phosphonates.

Interest in developing benzothiaole-based drugs has recently heightened due to FDA approval of the benzothiazoyl urea drug, Frentizole, for treating rheumatoid arthritis and systemic lupus erythematosus. Valasani et al. performed structure-activity relationship studies of frentizole derivatives and identified a benzothiazole urea compound with a 30-fold improved potency in inhibiting the enzyme amyloid beta binding alcohol dehydrogenase (ABAD) that is associated with Alzheimer's disease. [53-55] Phosphonates of benzothiazole were synthesized in order to enhance the ability of benzothiazoyl urea compounds to cross the blood brain barrier and reach target organs. Phosphonate derivatization improves the solubility of the benzothiazole moiety, decreases the adverse effects of the drug and enhances the sustained delivery to the target organs. [21, 25, 53-56]. Given the drug like properties of the benzothiazole phosphonates, many researchers have attempted to synthesize these compounds and test their therapeutic potential. Herein, we have attempted to summarize the novel synthetic pathways for benzothiazole phosphonates and their biological applications.

5.2 Synthetic Pathways of Benzothiazole Phosphonates

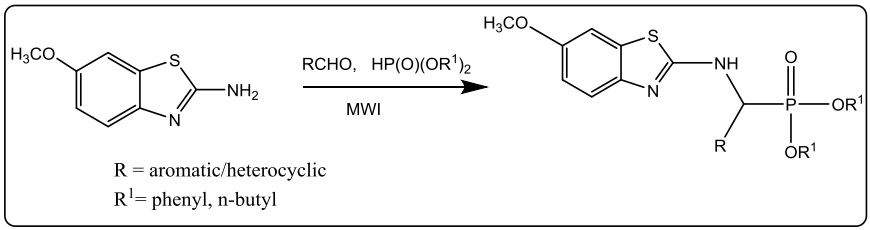
5.2.1 Kabachnik-Fields Reaction

Although numerous synthetic methodologies of benzothiazole α-aminophosphonates exist, the most noteworthy and remarkable one is probably the Kabachnik-Fields reaction that generally uses amines, dialkyl phosphites and carbonyl compounds as the reactants [57, 58] in an organic solvent system under high temperature (Scheme 1). The previous protocols for the synthesis of benzothiazole α-aminophosphonates mainly used simple starting reactants, but the recent approaches favor the use of even sterically-demanding starting materials.



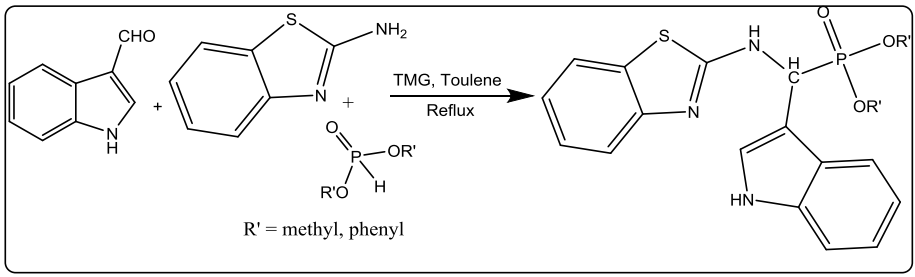
Scheme 1. *General scheme for the synthesis benzothiazoleα-aminophosphonates.*

Rao and coworkers [18] have reported the synthesis of various substituted benzothiazole aminophosphonates by the reaction of substituted aromatic/heterocyclic aldehydes, 2-amino-6-methoxy benzothiazole and dibutyl/diphenyl phosphites *via* the Kabachnik-Fields reaction under microwave irradiation (MWI) conditions (Scheme 2). The synthesized benzothiazole aminophosphonate derivatives were found to possess antimicrobial and antioxidant properties.



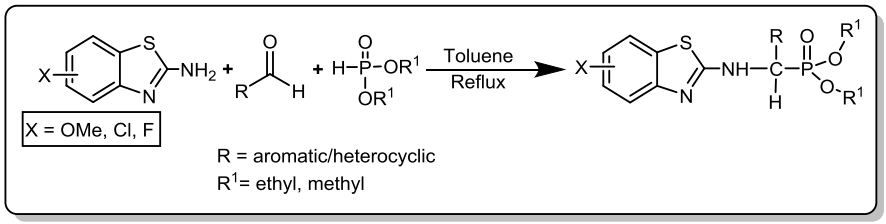
Scheme 2. *Kabachnik-Fields reaction with microwave irradiation.*

Reddy and coworkers [59] havesynthesized α-aminophosphonates by the Kabachnik-Fields reaction of dialkyl-or diphenyl-phosphite, indole-3-carb oxaldehyde and various heterocyclic-, cyclic-or other primary amines in the presence of tetramethylguanidine (TMG) as catalyst in toluene at reflux temperature (Scheme 3). The compounds possessed antimicrobial activity.



Scheme 3. *TMG catalyzed one-pot synthesis of α-aminophosphonates.*

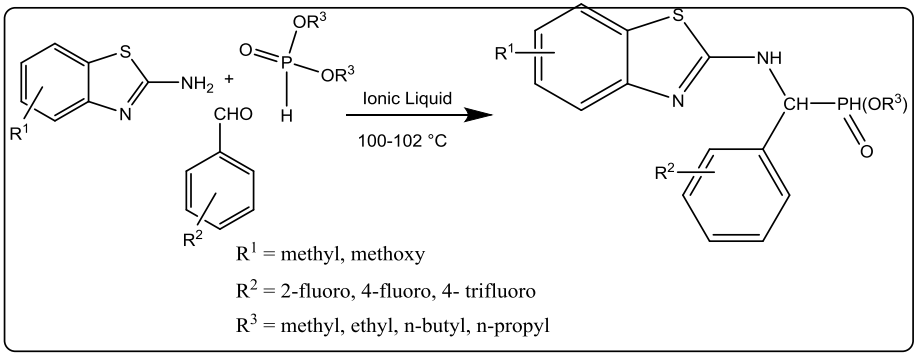
Valasani *et al*. [60] reported the Kabachnik-Fields synthesis of benzothiazole aminophosphonate derivatives using a three-component reaction of equimolar quantities of various 6-methoxybenzo[d]thiazol-2-amines, aromatic/heterocyclic aldehydes and dimethyl-or diethyl- phosphate in the presence of $Mg(ClO_4)_2$ in anhydrous toluene under reflux conditions (Scheme 4). Some of the resulting N-C-P benzothiazole phosphonate derivatives showed potent amyloid beta (Aβ) binding alcohol dehydrogenase enzyme inhibition.



Scheme 4. *Benzothiazole phosphonate derivatives containing the N-C-P scaffold.*

5.2.2 Mannich-Type Addition

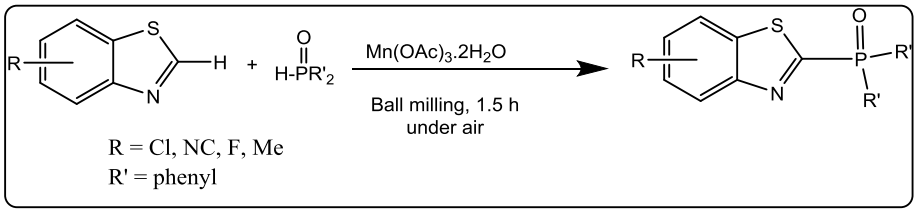
Jinand coworkers [61] have synthesized α-aminophosphonates containing benzothiazole and a fluorine-containing moiety by Mannich-type addition in ionic liquid media with short reaction times and high yields (Scheme 5). The synthesized N-C-P compounds were found to have antitumor activities.



Scheme 5. *Synthesis of N-(benzothiazole-2-yl)-1-(fluorophenyl)-O, O-dialkyl- α -aminophosphonates.*

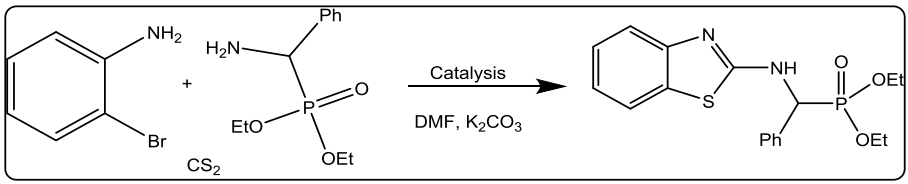
5.2.3 Direct Phosphonylation of Benzothiazole

Liang and coworkers [62] have synthesized structurally diverse C2-phosphonylated benzothiazole/thiazole derivatives with remarkable functional group tolerance and excellent yields by using organophosphorus compounds including phosphinate ester, phosphine oxides, and phosphonate diester promoted by $Mn(OAc)_3$ $2H_2O$ using a ball-milling technique (Scheme 6).



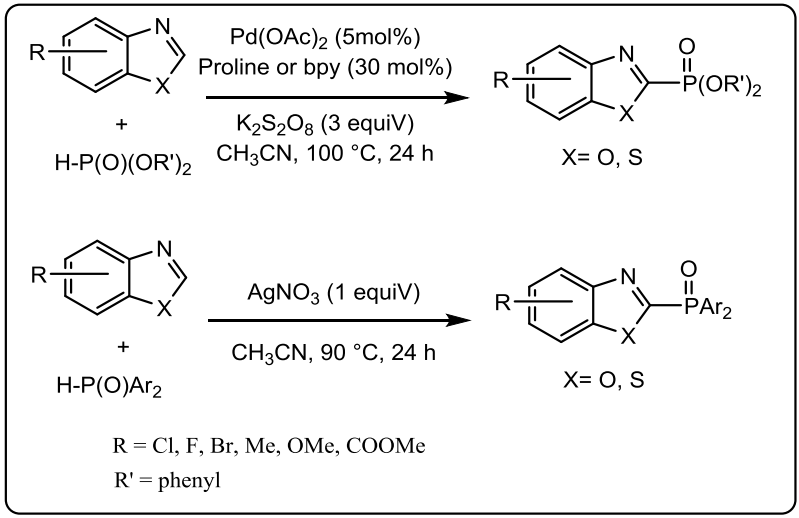
Scheme 6. *Ball-milling conditioned manganese (III) acetate-promoted cross-coupling reaction of benzothiazole/thiazole derivatives with organophosphorus compounds.*

Guand coworkers [63] have synthesizedN-C-Pα-aminophosphonates containing the benzothiazole moiety via a cascade three-component reaction (Scheme 7). The antitumor activities of the target compounds were evaluated against HL-60. One compound showed good cancer inhibitory activity against the tested cell line.



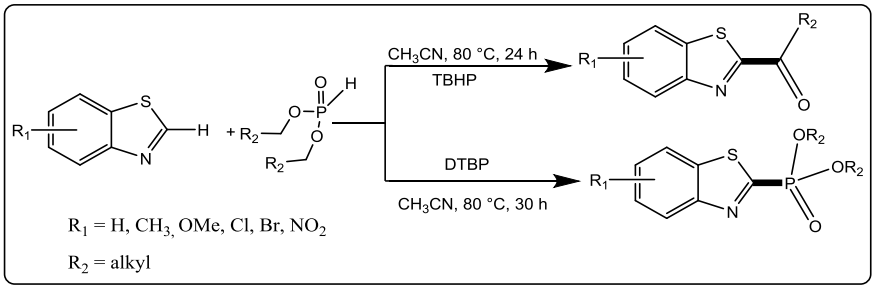
Scheme 7. *CS_2 mediated coupling reaction to synthesize benzothiazole α-aminophosphonates.*

Hui-Jun Zhang [64] and coworkers have synthesized variousbenzo[d] thiazol-2-yldiarylphosphine oxides through silver mediated direct phosphorylation of benzothiazoles and thiazoles. This method is similar to reported Pd-catalyzed reactions which may produce a more convenient synthetic route to a series of novel P, N-ligands (Scheme 8).



Scheme 8. *Direct phosphorylation of benzothiazoles and thiazoles.*

Xiao-Lan Chen and coworkers [65] have developed mild and metal-free methods for the preparation of two kinds of important benzothiazole derivatives, 2-acylbenzothiazoles and dialkyl benzothiazol-2-ylphosphonates. The dialkyl H-phosphonate $(RO)_2P(O)H$ exists in equilibrium with its tautomer dialkylphosphite $(RO)_2POH$. The final product depends on which tautomer reacts: tert-butyl hydroperoxide (TBHP) triggered α-carbon-centered phosphite radical formation, whereas di-tert-butyl peroxide (DTBP) triggered phosphorus-centered phosphonate radical formation. The two types of radicals led respectively to two different reaction processes, the direct C2-acylation of benzothiazoles and C2-phosphonation of benzothiazoles (Scheme 9).



Scheme 9. *Metal-free methods for preparation of 2-acylbenzothiazoles and dialkylbenzothiazol-2-ylphosphonates.*

5.3 Biological Applications of Benzothiazole Phosphonates

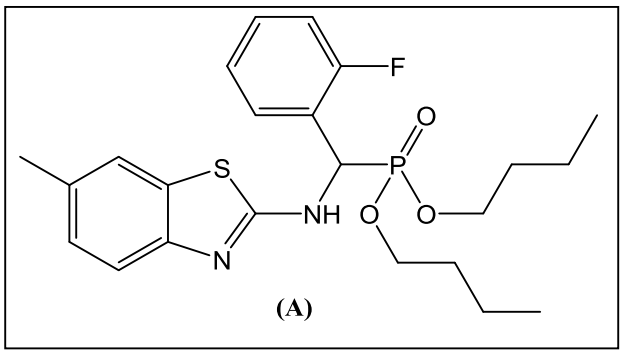
Benzothiazole phosphonates are found to possess a number of useful biological activities such as antitumor activity, antimicrobial activity, antioxidant activity and anti-Alzheimer activity.

5.3.1 Antitumor Activity

Cancer is a serious global health problem impacting millions of people and severely challenging the scientific community to develop new treatment strategies. The current major treatment strategies can be categorized into targeted therapies which attack the cancer cells and their signaling pathways directly and immunotherapies which try to harness the potential of the immune system to battle cancer. [66] The use of benzothiazole phosphonates and their derivatives are considered to betargeted therapy andsome of these compounds have been found to possess antitumor activity.

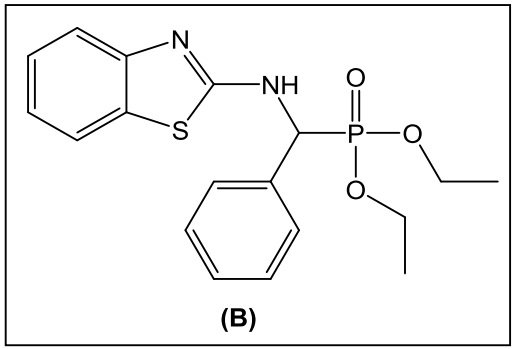
Jin et al. [61] first discovered the antitumor potential of these compounds in *in vitro* studies against a wide range of cancer cell lines such as PC3 (prostate cancer), A431 (human melanoma), A375 (uterus cancer), and Bcap-37(breast cancer) cells. They synthesized several derivatives of α-aminophosphonates

containing a benzothiazole moiety (Scheme 5) by a Mannich-type addition in ionic liquid media and tested their potential in inhibiting proliferation of cancer cell lines in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. MTT assay measures the cellular cleavage of tetrazolium salt (MTT) into formazan which has an absorbance at 550 nm that increases proportionately with increased formazan concentration in living cells. Compound A showed highest tumor inhibitory activity with 89% inhibition of prostate cancer cells (PC3) and 72% inhibition of human melanoma cells (A431) at 10 μM concentration. The nature of fluorine and alkyl at R^2 and R^3 positions are found to influence the antitumor potential of benzothiozole aminophosphonates as only 2-F at R^2 and n-Bu at R^3 showed great antitumor activity while many other groups showed less antitumor activity. While good antitumor potential was observed in PC3 and A431 cell lines, a poor-to-moderate tumor inhibition was noticed in A375 and Bcap-37 cell lines.



(A)

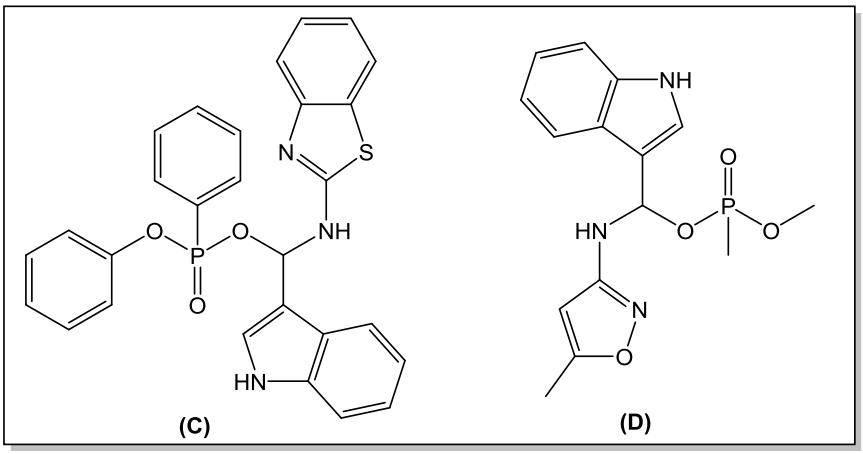
Liju*et al*. [63] synthesized a variety of benzothiazole phosphonate derivatives and screened them for antitumor activities against human acute promyelocytic leukemia cell line (HL-60) in MTT tests. Compound B, O, O'-Diethyl-α-(benzothiazole-2-yl) amino-(4- nitrophenylmethyl)phosphonate, possessed high antitumor activity and inhibited the proliferation of HL-60 with an IC50 value of 8.2 μmol/L.



(B)

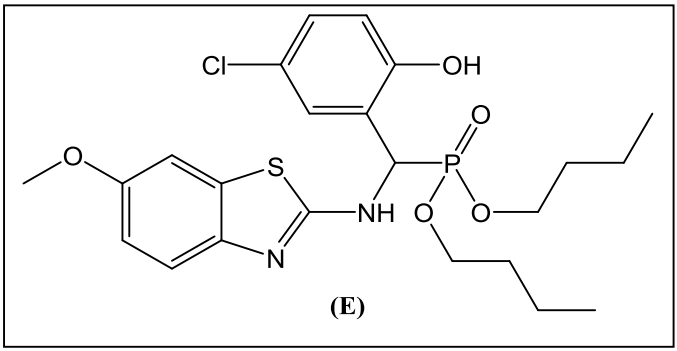
5.3.2 Antimicrobial Activity

Increasing resistance of bacteria and fungi to current antimicrobial compounds has dramatically increasedthe need for developing new compounds to treat bacterial and fungal infections [67-69]. Benzothionate aminophosphonates and their derivatives are one such group of compounds with antimicrobial potential. Two compounds, diphenyl (benzo[d]thiazol-2-ylamino)(1H-indol-3-yl)methyl phosphonate(C) and diphenyl (5-methylisoxazol-3-ylamino) (1H-indol-3-yl)methyl phosphonate (D)were found to be more effective than penicillin in inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli* [59]. Several derivatives of benzothionateaminophosphonates showed antifungal properties against *Aspergillus niger* and *Helminthosporium oryzae* and are found to be more efficient than a standard antifungal Griseofulvin. Compound C is more effective than Griseofulvin for inhibiting *Aspergillus niger* and D is more effective against *Helminthosporium oryzae*.



5.3.3 Antioxidant Activity

Rao *et al.* [18] designed a simple and efficient synthesis of α-aminophosphonates using the reaction between amino-6-methoxy-benzothiazole and dibutyl/diphenyl phosphite with microwave irradiation. The compounds were tested for antioxidant and antimicrobial properties. The antioxidant property was evaluated by estimation of ferric thiocyanate using linoleic acid emulsion. The oxidation of linoleic acid releases peroxidases that oxidize ferrous ions to ferric ions leading to formation of a complex ferric thiocyanate. Compound E, dibutyl (5-chloro-2-hydroxyphenyl) (6-methoxybenzo [d] thiazol-2-ylamino) methylphosphonate, yielded more ferric thiocyanate than the ferric thiocyanate in presence of vitamin C and is therefore considered a promising antioxidant. Some fragments such as 2-amino-6-methoxy benzothiazole and bromo/chloro/nitro salicylaldehyde attached to diphenyl phosphite were found to have both antimicrobial and antioxidant properties.

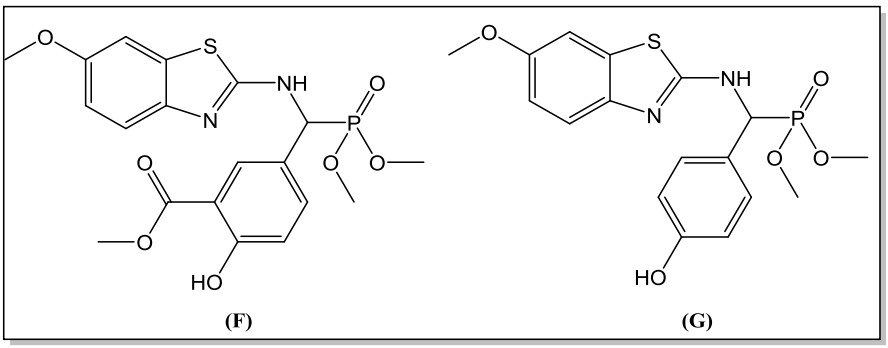


(E)

5.3.4 Benzothiazole α-Aminophosphonate Derivatives for Treating Alzheimer's Disease

Alzheimer's disease is a type of dementia in adults caused by neuronal stress and neuronal cell death that leads to poor cognitive ability and memory. [70-74] Mitochondrial and synaptic dysfunctions are common in patients with Alzheimer's disease and are caused by interaction of amyloid beta (Aβ) protein with amyloid beta binding alcohol dehydrogenase (ABAD). Valasani *et al*. [60] used surface plasmon resonance (SPR) screening to show that several benzothiazole α-aminophosphonate derivatives bind to ABAD and therefore potentially prevent the Aβ-ABAD interactions [75]. The compounds that were shown to bind to ABAD were subsequently examined for improvement in mitochondrial functions. Compounds F and G showed improvements in mitochondrial functions such as increased levels of ATP and Cytochrome C Oxidase, anenzyme associated with the mitochondrial respiratory chain. Besides being shown to inhibit Aβ-ABAD interactions, benzothiazole phosphonate derivatives also possess the ability to cross the blood-brain barrier in *in vivo* mice studies and thus hold great potential for AD therapy [56]. This general procedure also offers great promise for identifying and synthesizing more powerful drugs for treating other neurological diseases like amyotrophic lateral

sclerosis (ALS) that are presently being treated with benzothiazole based drugs like riluzole. [76-79]



5.4 Conclusions

This chapter highlighted synthetic routes and biological applications of benzothiazole phosphonate compounds. These compounds combine the desirable characteristics of the phosphonic acid moiety and the benzothiazole heterocyclic to produce species with enhanced chemical and biological properties that can hopefully be used as improved therapeutic anticancer, antitumor, antioxidant, antimicrobial and anti-Alzheimer's agents. The anti-Alzheimer'scompounds are highly soluble, cross the blood-brain barrier and are found to be less toxic in *in vitro* and *in vivo* mouse studies than their nonphosphorylated analogues. The clinical potential of these compounds as effective and safe drugs for human use is currently being explored. Besides producing more effective species through phosphorylation of known drugs, it is hoped that even more powerful molecules can be developed by comparing structure-activity relationship studies of known benzothiazole phosphonates with those for appropriately modified derivatives.

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