MANNICH BASES AND OTHER RELATED COMPOUNDS DESIGNED
PRINCIPALLY AS CANDIDATE CYTOTOXIC AND
ANTICANCER AGENTS

A Thesis Submitted
to the College of Graduate Studies and Research
in Partial fulfillment of the Requirements for
the Degree of Doctor of Philosophy
in Pharmacy

By
Sarvesh Chandra Vashishtha

Spring 1997

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Mannich bases and other related compounds designed principally as candidate cytotoxic and anticancer agents

The purpose of this study was to (i) search for original selectively cytotoxic and anticancer molecules by evaluating the theory of sequential cytotoxicity (ii) examine the antifungal activity of the proposed compounds and (iii) investigate the mode of cell death induced by a representative compound. In order to evaluate the theory, a number of Mannich bases of 1-aryl-1-ethanones, 4-aryl-3-buten-2-ones and their quaternary ammonium salts were synthesized, screened against P388 and L1210 leukemic cells, human T lymphocytes and a variety of human tumor cell lines and the stabilities of representative compounds under simulated physiological conditions was checked using TLC and \textsuperscript{1}H NMR spectroscopy. In general, the cytotoxicity results supported the theory in the important human tumor cell screen in contrast to evaluation using the murine leukemic cells where support for the hypothesis was marginal. Bioevaluation indicated that many of the compounds described in this study were potent cytotoxic agents and some selective cytotoxicity for certain tumor cell lines was revealed in the L1210 and human tumor assays. The in vivo activity of N,N-bis[5-(4-methylphenyl)-3-oxo-4-pentenyl] ethylamine hydrochloride against human colon tumors revealed it to be a promising lead compound. The cell death caused by this compound was found to be apoptotic in the human Jurkat T leukemia cell line. In vitro screening of the compounds against various pathogenic fungi revealed N,N-bis(5-aryl-3-oxo-4-pentenyl)-N-ethylamine hydrochlorides (MIC: 0.2-100 µM) as a novel class of potential antifungal agents. QSAR indicated a number of correlations between cytotoxicity or antifungal activity and various physicochemical constants of the aryl substituents which serve as pointers for future molecular modifications. At the end of study it was found [X-ray crystallographic and the proton NMR spectrum (500 MHz) data] that three compounds namely 4-chloro, 3,4-dichloro and 4-methyl derivatives in the N,N-bis(5-aryl-3-oxo-4-pentenyl)-N-ethylamine hydrochlorides series had undergone cyclization to form 4-hydroxy piperidinol derivatives. Though the theory of sequential cytotoxicity cannot be evaluated in the present scenario but it can be concluded that this study led to the identification of a novel class of alkylating and antifungal agents.
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Canada.
To my parents
and
wife Chhaya
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ABSTRACT

This study describes principally the synthesis of a number of Mannich bases of 1-aryl-1-ethanones, 4-aryl-3-buten-2-ones and their quaternary ammonium salts. These compounds were evaluated against P388 and L1210 leukemic cells, human T lymphocytes and a variety of human tumor cell lines. There were four reasons for undertaking the synthesis of these compounds. First, to examine the theory of sequential cytotoxicity which states that the successive release of two or more cytotoxic agents will cause greater damage to malignant rather than normal cells. The cytotoxicity data using human tumor cell lines were in accord with the hypothesis in general while use of murine leukemic cells supported this theory in one third to half of the cases. Another series of compounds namely azomethines (series 13,14) of certain cytotoxic Mannich bases, i.e., 1-(4-aryl)-3-dimethylamino-2-dimethylaminomethyl-1-propanone dihydrochlorides, discovered earlier in this laboratory and O or O,O'-bis Mannich bases of 4-hydroxy benzoyl hydrazine (compounds XX and XXII respectively), were designed on the basis of the known difference in pH values between cancer and normal cells, in order to evaluate the theory of sequential cytotoxicity. But difficulties in their synthesis aborted their evaluation.

The second reason was to search for new prototypic cytotoxic and anticancer molecules. The bioevaluation revealed a number of potent cytotoxic agents. In addition, N,N-bis[5-(4-methylphenyl)-3-oxo-4-pentenyl] ethylamine hydrochloride (XIIIId) reduced the size of human colon xenografts in athymic mice.

Third, quantitative structure-activity relationships (QSAR) were established between various physicochemical constants of the aryl substituents and cytotoxicity. Preliminary in vitro cytotoxicity results for one of the several compounds designed on the basis of QSAR results indicate that compound XIIIi has higher IC_{50} values and greater selectivity towards colorectal cancer as compared to the lead molecule (XIIIId). Thus, this particular class of compounds (series 7) has the capability to be developed as antineoplastic agents. In vivo testing of XIIIi in athymic mice is in progress. In addition,
the stabilities of representative compounds in solution using TLC and $^1$H NMR indicated that the mono Mannich bases underwent deamination extensively, the bis Mannich bases were much more stable while the quaternary ammonium salts decomposed completely to unidentified compounds. An attempt was made to explain these results by comparing the theoretical pKa values of the leaving groups. However the results of the stability studies indicated that much more experimentation is required in order to understand the decomposition pathways of a number of these compounds and the reasons for their fragilities.

Fourth, to screen the proposed compounds against pathogenic fungi from which N,N-bis(5-aryl-3-oxo-4-pentenyl)-N-ethyamine hydrochlorides (series 7) were revealed as a novel class of potential antifungal agents. These compounds (XIIIa-e) in general, had minimum inhibitory concentrations (MIC) figures of 0.2-100 µM against a variety of fungi and the antifungal activity seemed to be favored by hydrophobic, electron-attracting substituents in the aryl ring.

Finally, the mode of cell death induced by a representative Mannich base XIII\textsubscript{d}, in the human Jurkat T leukemia cell line was determined. Morphological evidences indicated that XIII\textsubscript{d} induced death in the cells by apoptosis.
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Figure A.1 Postulated mechanism for the intramolecular cyclization of XIIIb-e and related compounds
INTRODUCTION

The war on cancer is still not won. Cancer is responsible for about 500,000 deaths/year (20% to 50% of the total mortality) in the USA with about one million cases developing each year. The overall incidence and mortality rates in the USA between 1973 and 1990 have increased by 18.3 and 6.7 % respectively. The three most common cancers in men are lung, prostate, and colorectal cancers. In women, breast, colorectal and lung cancer are the most common tumors (Beardsley, 1994).

Vast efforts have been made in the last two decades in the treatment of cancer by means of chemotherapy either alone or in combination with other therapeutic modalities, e.g., surgery and radiation therapy. As a result, the five year survival rates of the disease have increased steadily to 55% for patients diagnosed as having cancer (Denny, 1995). Surgery and radiotherapy are effective only for localized tumors. They cannot cure neoplasms that have metastasized or invaded critical normal organs.

Chemotherapy, no doubt, is the major cancer modality for patients having a tumor that has metastasized to distant sites of the body at the time of diagnosis or relapsed at some time following primary surgery or radiation therapy. But the currently used drugs of which half belong to the class known as alkylating agents and most of them exert their cytotoxicity by interacting with DNA, have many limitations which should be overcome in order to find cures for cancer. The major limitations are (i) severe toxicity to fast growing normal cells in the bone marrow, hair follicles and gastrointestinal tract because of the inability of the presently available anticancer drugs to distinguish cancer cells from the normal cells and hence have a low therapeutic index, (ii) development of drug resistance [it has been estimated that more than 90% of all cancer deaths are in some measure influenced by the problem of drug resistance (Young, 1990)], (iii) narrow spectrum of clinical antitumor activity (ineffective against the slow growing, more common tumors of colon, prostate, pancreas, breast etc., as compared to the less common, fast growing, neoplasms such as leukemias or lymphomas e.g., Hodgkin's
tumor, Wilm’s tumor, Burkitt’s lymphoma) and finally (iv) mutagenicity and/or carcinogenicity [there are examples of secondary malignancy known in patients who have undergone treatment with alkylating agents (Boffetta and Kaldor, 1994)].

Thus, there is an urgent need for less toxic novel antineoplastic drugs which exert their cytotoxicity by mechanisms other than interaction with DNA and by virtue of their properties are selectively toxic to cancer cells particularly to metastatic solid tumors.

The development of such a drug is quite a challenge. Three major problems which may be the reasons why only five new anticancer drugs reached the US market in the 25 year span, 1965-1990 (Hollander and Gordon, 1990), are encountered in most of the anticancer drug discovery programs. First, the cost of bringing a drug from a laboratory to the market is very high. According to the 1990 statistics, it takes approximately 12 years, the synthesis of more than 10,000 compounds and an investment of $Cdn 277 million to develop a drug. Second, there is often the inability to identify and define an exploitable biochemical difference between normal human cells and invading cancer cells such as exist between mammalian and bacterial cells and on which the selective toxicity of highly effective antibacterial agents depend. If such a difference was known, drugs could be administered at higher doses and the toxic effects to the host would be minimized. Finally, the non-availability of a suitable screen capable of predicting clinical activity is a major problem (Connors, 1989). In the past, the wide use of the murine P388 lymphocytic and L1210 lymphoid leukemias and Lewis lung carcinoma as well as the Walker 256 tumor in rats has led to the identification of drugs active against fast growing tumors only. Novel screening models which can predict activity against human tumors, particularly the slow growing ones, are urgently needed. A new in vitro screen which employs a battery of human tumor cell lines was started in 1990 by the National Cancer Institute (NCI), USA. It hopes to predict disease specific compounds but its utility remains to be seen.

Despite these limitations, one can attempt to design novel strategies and approaches to combat the disease related problems such as lack of antitumor selectivity (i.e. preferential activity towards the neoplastic tissue without severe toxic effects on the
host) by taking advantage of the information which has accumulated over the years and particularly due to recent advances in the molecular biology of the contrasting biochemical features which distinguish tumors from normal tissues.

The theory of sequential cytotoxicity by Dimmock et al., (1993) is one of such approaches proposed with the objective of exerting selective toxicity to cancer cells by making use of the information pertaining to the differences in the glutathione levels and glutathione S-transferase isoenzymes between malignant and normal cells, and the biochemical properties of a novel class of alkylating agents namely Mannich bases which have been investigated extensively for their cytotoxic properties in this laboratory.

In the present investigation, the foremost objective was therefore to (i) evaluate and extend the scope of the theory of sequential cytotoxicity and (ii) search for original selectively cytotoxic and anticancer molecules capable of exerting bioactivity by a novel mechanism. The evaluation of the theory involved (i) the design and synthesis of various Mannich bases of 1-aryl-1-ethanones and conjugated styryl ketones and related derivatives and azomethines, (ii) the screening for cytotoxicity against various murine and human tumors, (iii) undertaking stability studies using simulated physiological conditions and finally (iv) an analysis of the biological and stability studies data in the light of the theory of sequential cytotoxicity.

In addition, the other objectives of the investigation were to (i) develop quantitative structure activity relationships (QSAR) between various physicochemical parameters and cytotoxicity in order to understand the molecular features/properties which were most important in determining activity and which can guide the optimization of biological activity within a series of compounds, (ii) screen the proposed compounds for antifungal activity and finally (iii) investigate the mode of cell death induced by a representative Mannich base.
2.1.0.0 Cancer

Cancer, a term often used synonymously with malignancy or neoplasia, is a group of over 100 types of diseases, characterized by autonomous, uncontrolled growth of abnormal cells which have the ability to invade adjacent normal tissues (invasiveness) and break away from the primary tumor, travel through the blood or lymph, and establish a new tumor at a different site in the body (metastasis). These cells are usually accompanied by a loss of differentiation and thus are unable to carry out the physiological functions of their differentiated (mature) counterparts. These cancer cells, described as tumors or neoplasms, may occur as benign (non-metastatic) or malignant (metastatic). The classification of malignant neoplasms is based primarily on the anatomical (organ) location and the type of cell from which the neoplasm develops. Solid tumors arising from epithelial cells are termed as carcinomas, whereas those arising from connective tissue and are often of a fibrous nature are termed sarcomas. Malignancies that arise from the hematopoietic system include the leukemias and lymphomas.

2.1.1.0 Carcinogenesis

The mechanism by which a normal cell is transformed into a cancer cell is not completely understood. Current evidence supports the role of a multistage mutation theory of carcinogenesis. According to this theory, the formation of a cancer is a result of a three step process. The first irreversible step in this process is initiation which involves the mutation of normal cells after exposure to a carcinogenic substance. The mutated cell in the second step called promotion which is reversible, grows preferentially over the normal cells and forms a clone of neoplastic cells. Depending upon the type of cancer, months to years may elapse between the two carcinogenic phases and the development of a clinically detectable cancer. The third stage of neoplastic growth, called progression involves further genetic changes leading to increased cell proliferation. The critical
elements of this phase include tumor invasion into local tissues and development of metastasis. At the molecular level, carcinogenesis is believed to be caused by activation of specific dominant growth genes called oncogenes or the loss of tumor suppressor genes.

Carcinogenic agents include those which are (i) physical - ultraviolet light and ionizing radiation (ii) chemical- arsenic, asbestos, aromatic amines (napthyl amines, benzidine etc.), drugs-alkylating agents (cyclophosphamide, melphalan), diethylstilbestrol etc. and (iii) biological agents like the Epstein-Barr virus, Burkitt’s lymphoma, and hepatitis B virus (hepatocellular cancer). All of these aforementioned carcinogens, as well as age, gender, diet, growth factors and chronic irritations, are among the factors promoting carcinogenesis.

2.2.0.0 Cancer chemotherapeutic drugs

The era of chemotherapy began with the identification of the clinical activity of the sulfur mustards in 1942. Since then many antineoplastic agents have been discovered, mostly by random screening. At present, there are about forty drugs approved for clinical use in the USA. The major antineoplastic drugs can be classified under three categories as given below.

(i) Natural products and related compounds
   (a) Vinca alkaloids e.g. vinblastine, vincristine and colchicine
   (b) Taxol, docetaxol
   (c) Antibiotics e.g. dactinomycin, mitomycin, pentostatin, daunorubicin, doxorubicin, bleomycin
   (d) Etoposide, teniposide, mitoxantrone
   (e) L-asparaginase

(ii) Antimetabolites
   (a) Folic acid antagonists e.g. methotrexate
   (b) Purine antagonists e.g. 6-mercaptopurine, thioguanine
   (c) Pyrimidine antagonists e.g. fluorouracil, cytarabine, fludarabine
(iii) Alkylating agents

(i) Nitrogen mustards: mechlorethamine hydrochloride, cyclophosphamide, chlorambucil, melphalan, ifosfamide

(ii) Ethyleneimines: thiotepa

(iii) Triazenes: dacarbazine

(iv) Nitrosoureas: carmustine, lomustine, semustine, streptozocin

(v) Alkyl sulfonates: busulfan

A summary of the mechanisms and sites of action of chemotherapeutic agents useful in neoplastic diseases is shown in figure 2.1.

2.3.0.0 Alkylating agents

Alkylating agents, the oldest and most useful among the antineoplastic agents, may be defined as compounds that can replace a hydrogen atom of a cellular constituent by an alkyl group under physiological conditions (Ross, 1962).

\[
\text{Alkyl} - \text{H} + \text{Nu}^- \xrightarrow{\text{pH 7.4}} \text{Alkyl} - \text{Nu}
\]

37°C

Although alkylating agents were synthesized as early as 1854, their clinical effectiveness was established in 1942 when clinical studies were initiated using nitrogen mustards. Since then, thousands of derivatives of nitrogen mustards and other chemical classes of alkylating agents have been prepared, but only a few have confirmed effectiveness in cancer treatment. There are five major classes of alkylating agents as indicated under 2.2.0.0. All of these drugs share the property of being the source of electrophiles that alkylate various nucleophilic groups such as phosphate, amino, sulfhydryl, hydroxyl, carboxyl and imidazole present in biomolecules such as proteins and nucleic acids. The order of preference for the alkylation of biological nucleophiles has been found to be thiols > amines > phosphates > carboxylates (Price, 1975). For DNA, the most reactive nucleophilic sites are N-7 of guanine > N-3 of adenine > N-1 of adenine > N-1 of cytosine (Lawley and Brooks, 1963).
Figure 2.1: Summary of the mechanisms and sites of action of chemotherapeutic agents useful in neoplastic diseases (From Calabresi and Chabner, 1995, reproduced with permission).
2.3.1.0 Mechanism of action of alkylating agents

The mechanism by which alkylating agents produces cytotoxicity may be illustrated using the example of mechlorethamine (Figure 2.2). The other alkylating agents react with DNA in a similar manner.

At neutral or alkaline pH, mechlorethamine undergoes a first order $S_N 1$ intramolecular cyclization, with the release of chloride ion and the formation of an aziridinium ion, which is a highly reactive and unstable species reacting with a variety of electron rich sites present in biomolecules, particularly the N-7 nitrogen of guanine by $S_N 2$ nucelophilic substitution with three important biological effects. First, mispairing of guanine with thymine may occur during DNA synthesis resulting in the substitution of an adenine-thymine base pair for a guanine-cytosine base pair. Second, alkylation of the N-7 nitrogen labilizes the imidazole ring making possible the opening of the imidazole ring or depurination by excision of guanine residues. Either of these processes seriously damages the DNA molecule and must be repaired. Third, as in the case of other bifunctional alkylating agents, crosslinking of two nucleic acid side chains or linking of a nucleic acid to a protein may cause a major disruption in nucleic acid function. The ultimate cause of death related to DNA damage is not known. Specific cellular responses include cell cycle arrest, DNA repair and apoptosis.

Although the most common site of binding of alkylating agents is the N-7 position of guanine, yet other atoms in the purine and pyrimidine bases of DNA e.g. the N-1 and N-3 nitrogens of adenine, the N-3 nitrogen of cytosine and the O-6 oxygen of guanine and also the phosphate atoms of the DNA chain and the proteins associated with DNA, may also be involved. Because alkylating agents can damage DNA during any phase of the cycle, they are considered cell cycle non-phase specific. The chemotherapeutic and cytotoxic effects are directly related to the alkylation of DNA (Fisher, 1994).
Figure 2.2: Alkylation reaction of mechlorethamine
2.3.2.0 Nitrogen mustards

2.3.2.1 Mechlorethamine

Mechlorethamine (1), the first nitrogen mustard to be introduced into clinical medicine, is highly reactive and is inactivated very fast by hydrolysis when injected into the body. Moreover, it is of value only in the treatment of certain cancers such as leukemias which can be attributed to the fact that such tumor cells grow morc rapidly than normal cells and hence are more easily affected. Therefore, several structural modifications have been attempted with the objective of producing compounds with greater selectivity (melphalan) and stability (chlorambucil) and therefore less toxicity.

\[
\text{CH}_3\text{N} \quad \text{Cl} \\
\text{Cl}
\]

(1)

2.3.2.2 Chlorambucil

Chlorambucil (2), in which the electron withdrawing phenyl ring reduces the rate of cyclization and carbonium ion formation, can reach distant sites in the body before reacting with components of blood and other tissues. Chlorambucil can therefore be given orally as compared to mechlorethamine which is given by injection.

\[
\text{HOOC} \quad \text{N} \quad \text{Cl} \\
\text{Cl}
\]

(2)

2.3.2.3 Melphalan

Another analog of mechlorethamine, melphalan (3), was developed in an attempt to cause selective toxicity to melanoma cells. Tumor cells, because of their higher
growth fraction, require phenylalanine in greater amounts than normal cells to synthesize melanin. Thus phenylalanine can act as a carrier for nitrogen mustards and penetrate preferentially into the melanoma cells to exert selective cytotoxicity.

2.3.2.4 Cyclophosphamide and Ifosfamide

Cyclophosphamide (4) is the most widely used alkylating agent. It was designed to release the nitrogen mustard preferentially in the tumor cells by the cleavage of the phosphoramide ring. It was thought that cyclophosphamide might selectively kill the tumor cells due to the higher quantities of the phosphoramide ring cleaving enzyme phosphoramidase, in the tumor cells. But, this did not happen. Instead, cyclophosphamide acts as a prodrug. It is activated by mixed hepatic oxidase enzymes to

form a number of metabolites (figure 2.3) of which phosphoramid mustard (6), acrolein (7) and nor-mechlorethamine (8) are toxic. Ifosamide (5), similar in structure and mechanism of action to cyclophosphamide, is activated more slowly compared to the latter compound.
Cytotoxic metabolites

**Figure 2.3:** Activation of cyclophosphamide *in vivo.*

### 2.3.3.0 Ethylenimines

Ethylenimines such as triethylentemelamine (TEM, 9) and triethylene thiophosphoramide (thiotepa, 10) exert their cytotoxicity in a similar fashion as nitrogen mustards but are activated at an acidic pH.
2.3.4.0 Triazenes

Dacarbazine (11), a triazene containing compound, is activated in the liver by cytochrome P-450 through a N-demethylation reaction. The mono methylated compound (12) thus formed undergoes nonenzymatic, spontaneous decomposition to release diazomethane which is the source of a highly reactive and electrophilic carbonium ion. This ion is responsible for the alkylation of cellular nucleophiles particularly at the N-7 position of guanine.

![Chemical Structure](image)

2.3.5.0 Nitrosoureas

Carmustine (13), lomustine (14) and semustine (15) are the most widely used nitrosoureas. These compounds exert their cytotoxicity through spontaneous, nonenzymatic decomposition to alkylation (2-chloroethyl carbonium ion) and carbamoylating (isocyanate) moieties. The carbonium ion can alkylate a variety of groups on the DNA molecule such as guanine, cytidine and adenine and produce intrastand and interstrand crosslinking of DNA. The latter reaction is supposed to be the major cause of cytotoxicity. Isocyanates can transfer carbamoyl groups to the lysine residues of proteins, a reaction that apparently can inactivate certain DNA repair
enzymes.

2.3.6.0 Busulfan

Busulfan (16) is a bifunctional alkylating agent that reacts with nucleophiles by a $S_N2$ mechanism. The alkyloxy bonds of busulfan rupture and react at the N-7 position of the guanine residues in DNA to form diguanyl derivatives.

In spite of the wide use of alkylating agents in the treatment of cancer (some cancers can be cured with high success rate e.g. a single dose of cyclophosphamide cures Burkitt’s lymphoma in 60% of the patients), these drugs should be used with care because of their carcinogenic, mutagenic and teratogenic nature. Additionally, these drugs are highly toxic, myelosuppressive and develop resistance. So, alternative tumor-selective alkylating agents which do not interact with nucleic acid should be sought.
2.4.0.0 α,β-Unsaturated ketones and Mannich bases as potential alkylating agents

α,β-Unsaturated ketones e.g. the styryl ketone 17, as well as Mannich bases of conjugated styryl ketones and 1-arylthianones e.g. 18, 19 which regenerate α,β-unsaturated ketones in vivo or in vitro, are a novel class of alkylating agents that are finding increasing interest, mainly in pharmaceutical academia, due to three reasons.

First, these compounds have diverse pharmacological activities. For example, a large number of them have been found to possess various bioactivities including cytotoxic, antifungal, antimicrobial, antimitotic, analgesic, antibiotic, psychotropic and anaesthetic properties as well as being used as bifunctional synthetic building blocks such as precursors in the preparation of α,β-unsaturated carbonyl compounds or γ amino alcohols (Reviews: Kleinman, 1991; Tramontini and Angiolini, 1994; Tramontini and Angiolini, 1990; Tramontini, 1973; Hellmann, 1960; Reichert, 1959; Schroter, 1957; Hellmann, 1956; Blicke, 1947; Brewster, 1953). Second, the pharmacokinetic properties namely solubility i.e. the hydrophilic-lipophilic balance of highly lipophilic pharmacologically active compounds are improved by introducing the hydrophilic amine hydrochloride groups. Third, these compounds act as alkylating agents exhibiting cytotoxicity by a novel mechanism i.e. alkylation of thiols such as glutathione, which does not involve interaction with DNA.
Several workers have reported the cytotoxic and antimicrobial activities of Mannich bases (Werner et al., 1970; Schoenenberger et al., 1969; Dimmock et al., 1989a-c; Chen et al., 1991a; Chen et al., 1994a; Erciyas et al., 1994) and natural products containing the α,β-unsaturated keto group such as α-methylene-γ-lactones e.g. various sesquiterpenes (Kupchan et al., 1971; Lee et al., 1972; Lee et al., 1973), compounds containing the α-methylene cyclopentanone nucleus e.g., diterpenes (Fujita et al., 1972; Fujita et al., 1973), sarkomycin (Marx et al., 1982), methylenemycin A (Wexler et al., 1982), xanthocidin (Govidan et al., 1983) and synthetic compounds (Rosowsky et al., 1974; Howie et al., 1974; Grieco et al., 1977; Cassady et al., 1978; Heindel et al., 1981).

2.4.1.0 Possible mode of action of α, β-unsaturated ketones and Mannich bases

The mechanism(s) by which α,β-unsaturated ketones and Mannich bases exert cytotoxicity is still not clear. Current evidence suggests the interplay of one or more factors namely (i) alkylation of thiols e.g. glutathione (ii) inhibition of mitochondria and (iii) inhibition of various glutathione S-transferases. Another recently discovered target of [(alkylamino)methyl] acrylophenones and (alkylamino) propiophenones is tyrosine and serine/threonine kinases including the epidermal growth factor-receptor protein tyrosine kinase (Traxler et al., 1995).

2.4.1.1 Alkylation of thiols

There are many thiol containing compounds present in the body either free or as a part of peptides and proteins. Glutathione (20) and cysteine are a few
examples of such compounds. Glutathione is an ubiquitous tripeptide (gly-cys-glu) which protects vital cellular constituents against reactive electrophilic groups by virtue of its nucleophilic sulfhydryl (SH) group. This group is highly ionized at body pH and hence reacts with a variety of xenobiotics including alkylating agents to form S-substituted glutathione adducts. This reaction may be catalyzed by a family of cytoplasmic enzymes called glutathione S-transferases. The biological activities such as cytotoxic (Mutus et al., 1989), antifungal (Baluja et al., 1964) and antimicrobial (Erciyas et al., 1994) of molecules containing the α,β-unsaturated carbonyl group have been attributed to reactivity toward cellular thiols such as glutathione (Koechel et al., 1977 and references cited therein; Lantz et al., 1976).

It has been established that acyclic and cyclic α,β-unsaturated carbonyls may combine directly with glutathione in a Michael type 1,4 addition reaction as shown in figure 2.4 (Esterbauer et al., 1975).

![Figure 2.4: Michael addition of glutathione to an α,β-unsaturated ketone.](image)

Acrolein for example when incubated with liver microsomes reacted directly with GSH causing complete depletion of GSH (Fry et al., 1993). Nucleophilic attack by GSH on α,β-unsaturated compounds occurs because the olefinic group is rendered electron deficient by the electron-withdrawing carbonyl group. Substituents (e.g. methyl,
methoxy) which can donate electrons to the double bond will cause diminution or abolition of reaction with glutathione.

These compounds do not react with other nucleophiles found in various nucleic acids and proteins. For example, tenulin, an antitumor sesquiterpenoid lactone, was found to react with glutathione and L-cysteine only. No reactions with various oxygen and nitrogen containing nucleophiles such as methanol, ethanol, alanine, serine, histidine, adenine, adenosine, deoxyribonucleic acid etc. was found to occur (Waddell et al., 1983). Similarly, the Mannich bases (21) (Mutus et al., 1989) and (22) (Dimmock, 1988a) reacted preferentially with thiols in contrast to other protein functional groups.

Reversible reaction with small molecular weight thiols such as L-cysteine, glutathione, mercaptoethanol and irreversible reaction with oncomodulin, thioredoxin, glyceraldehyde-3-phosphate dehydrogenase, glutathione reductase and BSA was found to occur with the Mannich base (21). No reaction in either of the two cases with nitrogen or oxygen containing nucleophiles was noticed (Mutus et al., 1989).

The inability of these compounds to react with functional groups such as amino, hydroxyl, carboxyl, or phosphate groups, commonly found in nucleic acids indicates that these compounds may not be carcinogenic or mutagenic. In fact, a few representative Mannich bases were found to be non mutagenic in the Ames test (Dimmock et al., 1980a).
2.4.1.2 Inhibition of glutathione S-transferases (GSTs)

The reaction of many α,β-unsaturated ketones with glutathione has been shown to be catalyzed by different GST isoenzymes (Boyland et al., 1967; Alin et al., 1985; Ishikawa et al., 1986; Jenson et al., 1986; Wadleigh et al., 1987; Danielson et al., 1987). Many of these compounds have been studied as substrates or selective inhibitors of GST isoenzymes (Wadleigh et al., 1987; Miyamoto et al., 1987; Kubo et al., 1989). For example, trans-4-phenyl-3-buten-2-one was found to be a more potent inhibitor of the μ class relative to the α class of GST isoenzymes from rat liver (Chien et al., 1994).

Mannich bases have also displayed selective inhibition of glutathione S-transferase isoenzymes. The conjugation of compound 21 with GSH has been shown to be catalyzed specifically by the α class of GST isolated from horse liver (Sexton et al., 1993). The mode of antifungal action of some styryl ketone Mannich bases was shown to be due, at least partially, to inhibition of one or more of the following enzymes in the glutathione metabolic pathway namely glutathione S-transferase, glutathione reductase, γ-glutamyl transpeptidase and glutathione peroxidase (Dimmock et al., 1994a).

These observations are of particular interest, as potent and selective inhibitors will not only be useful tools in the elucidation of the role of different enzyme systems but also may have important clinical implications e.g. selective inhibitors of GST isoenzymes may help to (i) sensitize tumors with acquired multidrug resistance to cytostatic drugs by blocking the inactivation of these drugs by their increased level of GSH or GST’s and (ii) to achieve selective cytotoxicity.

2.4.1.3 Inhibition of mitochondria

Mitochondria are known as the power house of the cell. They supply the energy which is needed by the cell to carry out its normal functions. The chemical substances which inhibit respiration in mitochondria can cause toxicity to the cell. Several reports from this laboratory have shown that α,β-unsaturated ketones and Mannich bases cause inhibition of respiration in yeast mitochondria (Dimmock et al., 1976a) as well as in mitochondria isolated from the livers of mice (Dimmock et al., 1986a, 1989a), rats
(Dimmock et al., 1979a, 1983a, 1983b; Hamon et al., 1978, 1982) and Morris 5123 TCH hepatomas (Dimmock et al., 1979b, 1980a).

The suggestion was made therefore that the cytotoxicity and antifungal activity of compounds containing the α,β-unsaturated carbonyl group namely styryl ketones and the corresponding Mannich bases as well as compounds which produce such molecules in vivo or in vitro e.g. mono and bis Mannich bases of 1-aryl-1-ethanones, may be due to at least, in part, to interference with mitochondria (Dimmock et al., 1986a).

The structural features necessary for mitochondrial-inhibiting properties have been suggested (Dimmock et al., 1976a) and these are (i) the presence of an enone group and (ii) a basic center positioned between two carbon atoms from the carbonyl function. This may be one of the reasons that styryl ketones which do not possess a basic group are far less active in inhibiting mitochondria than Mannich bases.

According to Dimmock et al., (1989a), there may be two receptor sites in mitochondria for interaction with Mannich bases. While one receptor site is common for all Mannich bases, the other, a narrow hydrophobic binding area, can accommodate an n-butyl or higher alkyl groups, present in some Mannich bases. The sites of action of some of these compounds in the electron transport chain in mitochondria have been determined (Hamon et al., 1978; Hamon et al., 1982). These compounds probably block the electron transport chain prior to the sequence of cytochromes. This is because of their similarity in structure with coenzyme Q10 (the presence of α,β-unsaturated ketone groups), and hence they probably compete with coenzyme Q10 for the electrons produced from the various substrates (Hamon, 1978). That is why the inhibition of mitochondria was seen to be reversed partially by coenzyme Q10.

Furthermore, the mitochondria from Morris 512TCH heptomas has been reported, in general, to be more sensitive to Mannich bases than mitochondria from normal liver tissue (Dimmock et al., 1980a). This observation may be of importance in eliciting selective toxicity of Mannich bases towards malignant tissue.
2.5.0.0 Structure-activity relationships among α,β-unsaturated ketones and Mannich bases

Some salient features of structure-activity relationships among α,β-unsaturated ketones and Mannich bases are as follows.

1. Mannich bases possess greater cytotoxicity and mitochondrial-inhibiting properties than the corresponding α,β-unsaturated ketones. Several styryl ketones (23) were found to be inactive against L1210 lymphoid leukemia in mice. Some of the compounds were weakly active in the KB screen at concentrations of 20-30 μg/ml. The lack of antineoplastic activity of the unsaturated ketones was thought to be due to their poor water solubility. Due to their high lipophilicity, styryl ketones may have been sequestered into the fatty depots of the body and thus were unable to reach the site of action.

![Chemical structures](image)

Hence, to increase water solubility, Mannich bases (24) were synthesized (Dimmock et al., 1974) and found to be inactive towards L-1210 lymphoid leukemia in mice but they showed a significant increased level of cytotoxicity in the KB screen (1-3 μg/ml). In the P-388 leukemia screen, the 2,4-dichloro and 3,4-dichloro derivatives increased the mean survival time by 30 and 42% at doses of 18 and 6.25 mg/kg respectively. Murine toxicity increased at the same time as activity increased in the P388 screen.

In order to discern the reasons for the disparity in bioactivity between series 23 and 24, the second order rate constants for the reaction of a model nucleophile ethyl mercaptan, with representatives of both series of compounds were compared. This study showed that compounds from series 24 reacted approximately 240 times more
quickly than the corresponding enones 23 (Dimmock et al., 1980b). Probable contributing factors to this difference in chemical reactivities included the stabilization of the reaction intermediate (25) by the inductive effect of the quadrivalent nitrogen and also hydrogen bonding.

![Chemical Structure](image)

(25)

The basic group in the β-position of the keto function is crucial (Traxler et al., 1995). Replacement of the dialkylamino group by a hydroxy group (26) or chain extension to (aminoethyl) propiophenone (27) lead to inactive compounds.

![Chemical Structure](image)

(26)

![Chemical Structure](image)

(27)

2. The introduction of an olefinic group between the aryl ring and the keto group increases cytotoxicity considerably. e.g. compound 28 was found to be more potent than 29 in the L1210, WiDr, KB and NCI cytotoxicity screens (Dimmock et al., 1992).
There are various literature references (Lee et al., 1972; Abdullah et al., 1991; Nagaya et al., 1994; Chen et al., 1994b and the references cited therein) that suggest the importance of the \( \alpha,\beta \)-unsaturated keto group for cytotoxicity. Any chemical modification such as reduction of the double bond or keto group or loss of conjugation, e.g. isomerization of the conjugated double bond from an \( \alpha,\beta \)-position to a \( \beta,\gamma \)-position, leads to compounds with a loss of activity (Hejchman et al., 1995). Addition of nucleophiles e.g. thiols (Dimmock et al., 1990a; Krivobock et al., 1994), bisulphates (Hejchman et al., 1995), by Michael addition or the conversion of the keto group to hydrazones (Dimmock et al., 1988a, 1992), oximes (Dimmock et al., 1993) or ketals (Dimmock et al., 1980a) also led to decreases in activity.

It was shown by Dimmock et al., (1990b) that placement of electron-withdrawing groups at the C-3 carbon increases cytotoxicity considerably. Such groups facilitate the attack of thiols at the C-4 carbon atom by decreasing the electron density. Thus, compounds 30c,d were found to be more active than 30b in the L1210 screen while compound 30a was the least active.
Similarly, introduction of electron-withdrawing groups in the α methylene group in the styryl ketone 28 (Dimmock et al., 1983a, 1988b, 1989b) as well as in 1-aryl-1-ethanones 29 (Dimmock et al., 1983b, 1984a, 1987) led to increases in activity e.g. compounds belonging to series 31-34 were found to have higher cytotoxicity as well as increased thiol-depletion capabilities than their precursor styryl ketones and 1-aryl-1-ethanones. A representative compound in series 33 (R_1 = 4-CH_3; R_2 = H) was found to be more cytotoxic than BCNU in vitro towards P388 cells (Dimmock et al., 1987).
5. Compounds with one methylene group (35) are more cytotoxic than compounds with two methylene groups (36) (Dimmock et al., 1989c). In compounds such as 35, β elimination cannot occur but substitution by thiols is possible. Moreover, the electron-withdrawing quaternary nitrogen is now closer to the α,β-unsaturated carbonyl group, thereby facilitating 1,4-Michael addition with thiols.

6. Introduction of electron-withdrawing groups in the aryl ring which are capable of enhancing the reaction of α,β-unsaturated ketones with glutathione, does not always lead to active compounds. Similarly, linear relationships between the pKa of the leaving amino group and cytotoxicity are not always be observed.
2.6.0.0 Metabolism of \( \alpha, \beta \)-unsaturated ketones and Mannich bases

The majority of xenobiotic ketones are metabolized through reductive reactions in many mammalian tissues (Bachur, 1976; Felsted and Bachur, 1980). The resulting alcohols may be further metabolized to more hydrophilic conjugates which are readily excreted in the urine or bile. Since, \( \alpha,\beta \)-unsaturated ketones are electronically more stable and hence less prone to enzymatic reduction than their saturated counterparts (Ringold et al., 1964), the reduction of the carbonyl function of \( \alpha,\beta \)-unsaturated ketones is, in general, preceded by hydrogenation of the double bond. The resulting saturated ketone metabolite may undergo further enzymatic reduction to produce a saturated alcohol. This sequential enzymatic reduction of \( \alpha,\beta \)-unsaturated ketones has been considered an essential biotransformation pathway for a wide variety of endogenous 3-keto steroids in humans (Farnsworth and Brown, 1963) as well as in experimental animals (Berseus et al., 1967). Saturated ketones, reduced alcohol metabolites and an allyl alcohol of shogoal have been identified (Surh and Lee, 1994). The metabolism of a cytotoxic conjugated styryl ketone has been examined (Dimmock et al., 1976b).

2.7.0.0 Mannich bases: instability under physiological conditions and cytotoxicity

Mannich bases frequently are unstable. They undergo a variety of decomposition reactions e.g. deamination, deaminomethylation or intramolecular cyclization under reaction conditions of high pH, temperature etc. But on occasions these reactions have also been demonstrated to occur at physiological pH of 7.4 and at a temperature of 37 \(^\circ\)C which is of prime importance especially in relation to cytotoxicity.

2.7.1.0 Deaminomethylation of Mannich bases and cytotoxicity

The reversible nature of the Mannich reaction indicates that the deaminomethylation or retro Mannich reaction is theoretically possible.

\[
\text{R-CH}_2\text{-N(CH}_3)_2 \quad \longrightarrow \quad \text{R-H} \quad + \quad \text{HCHO} \quad + \quad \text{HN(CH}_3)_2
\]
Though this process has been demonstrated with a variety of Mannich bases under different reaction conditions, only two examples of C-Mannich bases are known where this reaction has been found to occur under physiological conditions (buffer, pH 7.4, 37 °C). Its importance in relation to antitumor activity has been discussed in a few reports (Weitzel et al., 1964; Werner et al., 1977).

The lack of antineoplastic activity of bis Mannich bases of 1-aryl-1-ethanones e.g. 37

and styryl ketones (39) was attributed to the formation of the respective mono Mannich bases (38,40) by a deaminomethylation reaction under physiological conditions. The mono Mannich bases were found to be inactive in the P388 mouse leukemia screen (Dimmock et al., 1986b; Dimmock et al., 1987).
2.7.2.0 Deamination of Mannich bases and cytotoxicity

Another type of instability i.e. the propensity for amine elimination has long been recognised especially for synthetic applications. Deamination of Mannich bases as illustrated in figure 2.5, involves the elimination of a primary or secondary amine from a secondary or tertiary Mannich base or a tertiary amine from quaternary ammonium salts (Liang et al., 1995; Dimmock et al., 1988). This type of decomposition occurs only if a proton is situated vicinal to the carbonyl group and appears to more prevalent when β-aminoketone salts or the free base form of β-aminocarboxylic acids are heated with water. Dimerization is a frequently seen irreversible process which forces the elimination reaction towards completion. Michael type addition reactions also can force an elimination reaction towards completion.

Several cytotoxic prodrugs of ketones have been designed by taking this property into consideration. Mono Mannich bases of styryl ketones (Dimmock et al., 1975; Dimmock et al., 1990c; Dimmock, et al., 1992), 1-aryl-1-ethanones (Dimmock et al., 1992), cyclohexanones (Dimmock et al., 1993) and bis Mannich bases of 1-aryl-1-
ethanones (Dimmock et al., 1983b, 1987) have been shown to undergo deamination to give the α,β-unsaturated ketones under physiological conditions.

Quaternary ammonium salts are more easily decomposed than the tertiary ammonium salts (Dimmock et al., 1984b). Some amines such as diethylamine are more prone to undergo deamination probably due to steric reasons (Miller et al., 1973; Grass, 1978).

2.7.3.0 Intramolecular cyclization of Mannich bases and cytotoxicity

Many Mannich bases have been reported to undergo cyclization reactions. On occasions, efforts have been made to correlate cytotoxicity and the extent of intramolecular cyclization (Dimmock et al., 1989b; Dimmock et al., 1990a). Evaluation of the compounds in series 31 and 32 versus EMT6 cells in vitro indicated that cytotoxicity did not appear to be influenced by the ratio of cyclic and acyclic species.
2.8.0.0 Approaches to increase the selective cytotoxicity of Mannich bases

$\alpha,\beta$-Unsaturated ketones, albeit their weak murine toxicity, are not potent enough to be useful as antineoplastic agents, whereas Mannich bases, despite their high cytotoxicity under in vitro conditions, are not markedly active in vivo in animals. The reason is that they are highly toxic. Moreover, efforts to augment potency by increasing the reactivity of Mannich bases towards thiols often result in an increased toxicity to the host also. Therefore, a number of strategies have been employed in the past to ameliorate mammalian toxicity and increase the selective tumor lethality of these Mannich bases.

2.8.1.0 Exploitation of a differential transport mechanism exhibited by sugars

Sugar derivatives can enter the cells by a facilitated diffusion process (Styrer, 1975) and this phenomenon has proved beneficial on occasions in the case of carbohydrate conjugates of cytotoxic agents (Honda et al., 1988; Ross, 1962).

![Chemical structure of a Mannich base](image)

(41)

But a similar strategy did not work in the case of two mono Mannich bases of 4-hydroxy acetophenone having a $\beta$-D-glucopyranosyloxy group in the ring e.g. 41 (Dimmock et al., 1990c). These sugar derivatives may be too large to pass via membranes using an activated transport mechanism and also they possessed increased polarity compared to the corresponding Mannich bases which may hinder their passage through membranes.
2.8.2.0 Prodrug approach

Since a number of tumors are more acidic than the corresponding normal tissues (Wike-Hooley et al., 1984), a number of acid sensitive prodrugs of ketones and Mannich bases namely hydrazones, oximes and oxime esters have been suggested in order to allow preferential release of ketones in malignant tissue.

Some Mannich bases have been converted into thioethers (e.g. compound 47), which may function as prodrugs because a few thiol adducts of α,β-unsaturated ketones have been demonstrated to undergo a retro-Michael reaction to release the precursor enones in vitro (Koechel et al., 1977).

2.8.2.1 Hydrazones

A number of hydrazones of Mannich bases were prepared as potential prodrugs (42,43) which were less potent but more selective than the precursor ketones in the NCI human tumor screen. High resolution $^1$H NMR spectroscopy indicated that these compounds were active per se since representative hydrazones did not regenerate the ketones or produced them only in minute quantities at pH values normally encountered in living tissues. (Dimmock et al., 1988a, 1992).

\[
\text{R} \begin{array}{c}
N \\
\text{R} \\
N \\
\text{HCl}
\end{array}
\]

(42)

\[
\text{R} \begin{array}{c}
N \\
\text{R} \\
N \\
\text{HCl}
\end{array}
\]

(43)

2.8.2.2 Oximes and their esters

Using the same strategy of acid sensitive prodrugs, oximes (45) of α,β-unsaturated ketones (44) were prepared. Since oximes contain a polar hydroxyl group which could impede penetration into cell membranes, their latentiated form namely oxime esters (46) were also synthesized (Dimmock et al., 1993).
But, both oximes and their esters were found to be less active than the corresponding ketones. Stability studies indicated that these compounds were stable under the conditions of bioevaluation and hence were active per se.

### 2.8.2.3 Thiol adducts

Mesna adducts of some styryl ketone Mannich bases (47) were found to be have increased selective cytotoxicity towards certain groups of human tumors and less potency than the corresponding enones (Dimmock et al., 1995a). These compounds as revealed by $^1$H NMR spectroscopic studies, release $\alpha,\beta$-unsaturated ketones by a retro Michael reaction under simulated physiological conditions and thus may act as prodrugs.

### 2.8.3.0 Sequential cytotoxicity

The theory of sequential cytotoxicity which states that the successive release of two or more cytotoxic compounds causes greater toxicity to malignant tissue
rather than normal cells was proposed by Dimmock et al., (1993) in an attempt to ameliorate the toxicity of Mannich bases. It is based on the selective inhibition (cancer vs normal cells) of glutathione, glutathione S-transferase isoenzymes and mitochondria by Mannich bases.

\[
\begin{align*}
\text{(48)} & \quad \text{(49)}
\end{align*}
\]

Based on this theory, Dimmock et al., (1995b) designed some mono and bis Mannich bases of cyclohexanone and cyclopentanone and found that the bis Mannich bases (49) with two potential sites for alkylation of cellular thiols were more active than the corresponding mono compounds (48) which had only one site for alkylation of cellular thiols. This observation was based on the cytotoxicity results carried out using murine P388 D1 lymphocytic leukemia cells.

2.9.0.0 Antineoplastic drugs and apoptosis

It is generally recognized that there are two distinct types of cell death; necrosis and apoptosis or programmed cell death (Kerr and Harmor, 1991; Wyllie, 1987). Apoptosis is an important mechanism that balances cell production with cell death and seeks to maintain the correct cell numbers in the body under physiological and pathological situations. Its impairment may lead to tumor progression and oncogenesis (Thompson, 1995).

Apoptosis is distinguished from necrotic cell death by several morphological and biochemical features (Kerr et al., 1994). The morphological changes in apoptosis are characterized by the condensation of the chromatin at the nuclear periphery, membrane blebbing but no loss of integrity, intact lysosomes, cell shrinkage,
loss of cell-cell contacts, and finally formation of apoptotic bodies (nuclear and cytoplasmic fragments enclosed in the plasma membrane). Apoptosis involves deletion of single cells which are phagocytosed by adjacent normal cells and some macrophages but there is no inflammatory response. In contrast, necrosis is characterized by cell swelling, loss of plasma membrane integrity, and finally spillage of the cell contents upon rupture of the cell and leakage of lysosomes resulting in an inflammatory response. The necrotic cells are then phagocytosed by macrophages.

A principal biochemical feature of apoptosis in most cell types is the cleavage of DNA into oligonucleosomal or nucleosomal sized fragments due to the activation of an endogenous endonuclease. This DNA fragmentation can be demonstrated by gel electrophoresis as multiples of 180-200 bp fragments forming a characteristic DNA ladder as opposed to the random DNA fragmentation seen in necrosis. Other important biochemical features which distinguish apoptosis from necrosis are as follows.

(i) Apoptosis is induced by physiological stimuli in contrast to necrosis which is evoked by non-physiological disturbances.

(ii) Apoptosis requires energy and synthesis of proteins and nucleic acids while necrosis does not have any of these requirements.

(iii) Finally, unlike necrosis, apoptosis involves de novo gene transcription.

Apoptotic cell death generally occurs in a tightly regulated manner whereas necrosis has been considered a passive phenomenon induced by direct toxic and physical injuries.

Several clinically used anticancer drugs have been shown to induce apoptosis (Amico and McKenna, 1994 and references cited therein) in a number of cell lines in vitro. These include DNA alkylating agents, cisplatin, etopside, teniposides, macromolecular synthesis inhibitors and topoisomerase inhibitors.

Cancer cell injury caused by common antineoplastic agents such as radiation and chemicals may provide the stimulus that initiates apoptosis. Critical regulators of cell death pathway have been established (Hickman, 1992) and these include p53, bcl-2 and c-myc oncogenes which have been found to be defective in human cancers resistant to antineoplastic agents. In vitro studies which stimulate the genetic regulators of apoptosis (e.g. antisense bcl-2) have been shown to reverse chemoresistance. Genes
such as myc (Shi et al., 1990; Evan et al., 1992) and p53 (Lowe et al., 1993) are known to drive the process of apoptosis whereas genes like bcl-2 (Hockenbery et al., 1990) inhibit apoptosis. All these genes can be useful drug targets. Manipulation of these novel targets may help solve the problem of drug resistance as well as cancer.
3.0.0.0 RATIONALE

Biochemical modulation refers to the pharmacological manipulation of intracellular metabolic pathways by an agent (the modulating agent) to the selective enhancement of the antitumor effect or the selective protection of the host from the anticancer agent (the effector agent). When two anticancer drugs interact through biochemical modulation, then by definition, the effect of the combination must either be enhanced or diminished. In simplistic terms, the former is called synergy and the latter antagonism. When two drugs in combination do not interact their effect is additive. The goal of biological modulation is to generate synergistic cytotoxicity. However, one should not assume that synergistic cytotoxicity will result in an improved therapeutic index. Synergistic cytotoxicity may affect both cancer and normal host cells alike and biomodulation may result in not only enhanced cancer cell kill but also unacceptable host toxicity. Thus, biomodulation is most useful when the modulation selectively enhances tumor cell cytotoxicity relative to normal cell cytotoxicity (i.e. selective synergy).

The theory of sequential cytotoxicity (Dimmock et al., 1993) states that the sequential release of two or more cytotoxic agents in vitro or in vivo causes greater toxicity to malignant rather than normal cells and is a new approach to biomodulation. The basic difference from the commonly used biomodulation approach is that the drugs are not administered as a combination of two or more drugs. Rather, it is given as a single compound that releases two or more cytotoxic compounds sequentially which need not to be different. The goal is identical i.e., to achieve selective synergy. This theory seeks to exploit the quantitative differences in the levels of key biochemical targets like glutathione, γ-glutamyl transpeptidase (γ-GGT), various glutathione S-transferase isoenzymes and inhibition of mitochondria, between normal and cancer cells. The next section presents the background and evidences to support the theory of sequential cytotoxicity.
3.1.0.0 Theoretical background for the theory of sequential cytotoxicity

3.1.1.0 Modulation of glutathione (GSH)

It has been demonstrated that lowering the concentration of glutathione, prior to the administration of various anticancer drugs, enhances the cytotoxicity of these drugs towards tumors rather than normal cells. Buthionine sulfoximine (BSO) (Griffith and Meister, 1979), which is a specific inhibitor of γ-GGT, can deplete intracellular GSH levels, up to 90% or more (Meister, 1991), and increased the cytotoxicity of melphalan, cisplatin, adriamycin, and radiation in human ovarian cancer cell lines selected in vitro for resistance to these treatments (Hamilton et al., 1985; Louie, 1985; Green et al., 1984; Batist et al., 1986; Behrens et al., 1987). These and other numerous reports support the role of BSO in increasing the sensitivity of tumor cells to a number of cytotoxic agents, as well as in reversing the acquired resistance to these agents (Robson et al., 1987; Meijer et al., 1990; Barranco et al., 1990; Lee, 1989; Teicher et al., 1991a; Cole et al., 1989; Chen, 1991a). In vivo studies in preclinical models showed that the treatment of nude mice bearing a human ovarian cancer cell line (OVCAR-3) with oral BSO reduced the tumor cell GSH levels by 90% and prolonged the survival of animals treated with melphalan plus BSO, compared to the untreated and melphalan only treated animal, (Ozols et al., 1987). In preliminary Phase I clinical trials, a decrease in GSH levels in peripheral mononuclear cells to 20% of the normal level was achieved in approximately half of the patients. (O’Dwyer et al., 1992). BSO itself was shown not to induce immediate life threatening side effects. The mechanism of enhanced cytotoxicity caused by BSO, towards cancer cells is not known. It is speculated that BSO selectively depletes the tumor cells of glutathione thereby making the cells more sensitive to the action of the antineoplastic agents. Loss of improved selectivity when BSO is given after administrating an antineoplastic agent supports this idea. Therefore, it can be expected that Mannich bases and related compounds that release two or more molecules of α,β-unsaturated ketones, may preferentially deplete glutathione from the cancer cells, thereby sensitizing them to the action of a second alkylating agent. Thus, Mannich bases may
exert selective toxicity by fulfilling the role of BSO and that of antineoplastic agents at the same time.

3.1.2.0 Modulation of glutathione S-transferase isoenzymes (GST)

The qualitative and quantitative differences in the occurrence of GST isoenzymes in different organs are of particular interest, as they might cause differential susceptibility of tissues to the toxic effects of xenobiotics.

Some tumors express different isozymes of GST than normal tissues (Castro et al., 1990; Sako, 1989; Tsuchida and Sato, 1992) and on occasions mean GST activity is higher in malignant cells than the corresponding normal tissues (Clapper et al., 1991). There is also strong evidence that GSH and its associated enzymes play a role in cellular resistance to anticancer drugs (Black et al., 1991). Selective inhibition of tumor GST'S may therefore be a tool to increase the sensitivity of tumors to cytostatics.

Therefore, it is possible that initial formation of an alkylating agent i.e. an α,β-unsaturated ketone from a Mannich base may inhibit one or more of the isozymes of GST which could amplify the cytotoxic action caused by the release of a second thiol alkylator e.g. if a certain group of tumors had lower levels of the GST α isozyme than the corresponding normal cells and the initially released cytotoxic agent had high affinity for only this enzyme [Sexton et al., (1993) found that a Mannich base possessed high affinity for the α isozyme of GST but low and zero affinities for the π and μ isozyme forms] the tumors would be expected to be more sensitive to the second alkylating agent than the nonmalignant tissue. The evidences for this hypothesis are as follows. Ethacrynic acid (EA), a clinically used diuretic agent, inhibits GST either by covalent binding to the enzyme (Yamada, 1980) or through inhibition with an ethacrynic acid-glutathione conjugate (Ploeman et al., 1990). Preclinical studies demonstrated that EA when administered before chlorambucil was effective in sensitizing a rat breast carcinoma cell line and human colon cancer cell line to chlorambucil (Tew et al., 1988). Sensitization of cells to melphalan (Hansson et al., 1991), BCNU (Berhane et al., 1989), mitomycin C (Xu and Singh, 1992) and nitrogen mustards (Nagourney et al., 1990) has also been reported. A phase I clinical trial using ethacrynic acid as a modulating agent to assess
dose limiting toxicities for humans (O'Dwyer et al., 1991) reported diuresis as a major toxicity of ethacrynic acid. A case study described the partial reversal of chlorambucil resistance by ethacrynic acid in a chronic lymphocytic leukemia patient (Petrini et al., 1993).

Therefore, it can be concluded that the modulation of GSH levels and GST activity by Mannich bases may be of some value in exerting selective toxicity.

3.1.3.0 Sequential administration of mitochondrial inhibitors and cytotoxic agents

It was reported that the prior administration of the cellular energy metabolism inhibitor lonidamine potentiated the cytotoxicity of various antineoplastic agents (Teicher et al., 1991b; Ning and Hahn, 1990; Raaphorst et al., 1990). Since a number of Mannich bases of \( \alpha,\beta \)-unsaturated ketones are mitochondrial inhibitors (Dimmock et al., 1983a; Dimmock et al., 1989a), it is understandable that initial inhibition of mitochondria may lead to tumors having greater vulnerability than the corresponding normal cells to the subsequent release of an alkylating agent.

3.2.0.0 Design of compounds based on the sequential cytotoxicity theory

Various classes of compounds namely the Mannich bases of acetophenones, \( \alpha,\beta \)-unsaturated ketones, their quaternary salts and azomethines were designed by taking into consideration the concept of sequential cytotoxicity.

The Mannich bases, in general, were chosen as candidate cytotoxic and antineoplastic agents in this study for the following reasons.

1. These compounds represent a novel class of alkylating agents having different structures and mechanisms of action than the presently available antineoplastic agents. Hence they may be of use in treating neoplasms which are resistant to the drugs available now and also they may be useful in demonstrating activity towards certain tumors for which very few of today's drugs are currently effective.

2. Several Mannich bases have been shown to undergo deamination to give \( \alpha,\beta \)-unsaturated ketones and hence these compounds could exemplify the theory of sequential cytotoxicity.
Many of the Mannich bases have been shown to be thiol alkylators. By virtue of this property, Mannich bases may offer three potential advantages over other clinically used alkylating agents.

(a) First, they may be noncancerogenic and nonmutagenic because of their preferential reaction with thiols rather than amino or hydroxyl groups present in the nucleic acids.

(b) Second, they may show selective toxicity towards tumors which multiply faster than the corresponding normal tissues. The reason is that the rate of synthesis of thiols, e.g. glutathione, is higher just prior to and during mitosis in fast growing tumors (Emmelot, 1964; Schauenstein et al., 1985) and thus they are more susceptible to thiol alkylation. Moreover, many thiol alkylators have shown greater effects against tumors than normal tissues which has been attributed to preferential depression of various precursors into DNA, RNA and proteins in contrast to traditional alkylating agents and antimetabolites which usually depress the incorporation of substrates into these macromolecules more in normal tissues than cancers.

(c) Third, the association between enhanced levels of glutathione and glutathione-S-transferases and drug resistance against alkylating agents suggests that Mannich bases may be useful in overcoming the problem of drug resistance.

As is evident from a literature survey, Mannich bases of 1-aryl-1-ethanones, α,β-unsaturated ketones and their quaternary salts are not only highly cytotoxic but also possess a range of interesting biochemical properties e.g., they bind reversibly with low molecular weight thiols including cysteine and glutathione but irreversibly and selectively with GST isoenzymes and inhibit the power house of the cell namely the mitochondria.

Mannich bases, thus, according to the definition of biomodulation could serve the role of modulating agents as well as effector agents and hence represent a quite suitable class for the evaluation of the theory of sequential cytotoxicity.
3.2.1.0 Sequential cytotoxicity concept using Mannich bases of styryl ketones, acetophenones and their quaternary ammonium salts

In order to investigate the theory of sequential cytotoxicity, eight series of Mannich bases 1-8, each consisting of five compounds were proposed. The choice of Mannich bases is crucial here. The proposed compounds must undergo elimination by whatever mechanism, to generate α,β-unsaturated ketones which should be able to undergo Michael addition reaction with thiols. Since it is a well known fact the biological activity of a compound is mostly determined by its electronic, hydrophobic and steric properties, a Topliss analysis (Topliss, 1977) which initially allows selection of five substituents with divergent electronic, hydrophobic and steric properties in the aryl ring was employed. Thus, the five substituents [sigma (σ), pi (π) and molar refractivity (MR) values in parentheses respectively] chosen in each series are (a) R = H (0, 0, 1.03); (b) R = 4-Cl (0.23, 0.71, 6.03); (c) R = 3,4-Cl₂ (0.60, 1.42, 12.06); (d) R = 4-CH₃ (-0.17, 0.56, 5.65); (e) R = 4-OCH₃ (-0.27, -0.02, 7.87). The electronic properties of the substituents in the aryl ring denoted by the σ value will control the rate of release of the α,β-unsaturated ketone from the Mannich base as well as the reaction of the deaminated product with thiols, while the hydrophobic properties denoted by the π value will influence the absorption and distribution kinetics of the molecule. Molar refractivity, denoted by MR, will determine the effect of steric properties of the substituents on the biological activity. On the basis of the biological results, one can examine which physicochemical factor σ, π or MR or a combination of these three is controlling the bioactivity.

Series 1

![Chemical structure](image)

(a) R = H; (b) R = 4-Cl; (c) R = 3,4-Cl₂
(d) R = 4-CH₃; (e) R = 4-OCH₃

Same for series 1-10.
Series 2

Accordingly, the selection of further substituents can be made to optimize the biological activity. Series 1 and 2 are anticipated to give rise to 1-aryl-2-propen-1-ones which have one electrophilic site for thiol attack (figure 3.1). These mono compounds and their quaternary salts are not expected to exert selective cytotoxicity since they release only one molecule of alkylating agent.

Figure 3.1: Proposed deamination pathway for mono Mannich bases of acetophenones, their quaternary salts and thiolation of α,β-unsaturated ketones.
On the other hand, the bis Mannich bases 3 may produce the same $\alpha$, $\beta$-unsaturated ketones initially as well as the corresponding 3-ethylamino-1-aryl propanones.

**Series 3**

![Chemical structure of Series 3]

**Series 4**

![Chemical structure of Series 4]

These latter compounds could subsequently undergo deamination producing another molecule of thiol alkylator and ethylamine hydrochloride (figure 3.2).

Thus, the rate of release of the ketones in the case of bis compounds will depend not only on the type of substituent in the aryl ring but also on the $p$Ka of the leaving group thereby allowing the gradual release of the alkylating agent. Hence if the theory of sequential cytotoxicity is valid, series 3 should be more than twice as cytotoxic as series 1 and 2.

Quaternary ammonium salts of Mannich bases namely series 2 and 4, will deaminate in the same manner as their mono and bis counterparts but will differ in their elimination kinetics. These compounds may be useful against leukemia, since the presence of a charged group (as found in many cholinesterases) may assist in retaining the compounds in plasma. The compounds in series 4 were predicted to display greater cytotoxicity than series 1 and 2.
Figure 3.2: Proposed deamination pathway for bis Mannich bases of acetophenones, their quaternary salts and thiolation of α,β-unsaturated ketones.
To examine the theory of sequential cytotoxicity further, series 5-8 were proposed.

Series 5

A site available for initial attack by thiols is present in series 5 and 6 and the molecules and/or the thiol adducts can undergo deamination (figure 3.3) to provide a second site for thiol interactions. Hence series 5 and 6 should have in excess of twice the cytotoxicity of series 1 and 2. The bis unsaturated ketones 7 and 8 have two sites for initial thiol attack.

Series 7

Series 8
Figure 3.3: Deamination pathway for mono Mannich bases of styryl ketones, their quaternary salts and thiolation of α,β-unsaturated ketones.

In addition, deamination will lead to the formation of both a third alkylator and a secondary (series 7) or tertiary (series 8) amine which in turn could yield a fourth site of alkylation after the extrusion of the amine (figure 3.4). It is conceivable therefore that the cytotoxicity of series 7 and 8 will display more than twice the activity of the compounds in series 5 due to the greater avidity for thiols and successive release of two rather than one alkylating species.
Figure 3.4: Proposed deamination pathway for bis Mannich bases of styryl ketones, their quaternary salts and thiolation of \(\alpha,\beta\)-unsaturated ketones.
Finally, the rate of deamination in Mannich bases is inversely proportional to the basicity of the amines. Therefore, in the case of mono Mannich bases of styryl ketones, compounds 9a-e having different leaving groups were suggested. The pKa values of dimethylamine, diethylamine, piperidine, morpholine and pyrrolidine are 10.77, 10.97, 11.22, 8.70, 11.27 respectively (Albert et al., 1984a,b). Hence, these compounds will differ in their rate of deamination.

Series 9

\[
\begin{align*}
\text{R} & \quad \text{O} \\
\text{R} & \quad \text{R}_1 \quad \text{HCl} \\
\text{a. N(CH}_3)_2 & \quad \text{b. N(C}_2\text{H}_5)_2 \quad \text{c. N} \\
\text{d. N} & \quad \text{O} \quad \text{e. N} \\
\end{align*}
\]

Compounds 10a (series 10, R = H) or 11 were suggested since both deamination steps would be predicted to be faster than in the case of analogs 3a, 7a e.g., the pKa values of the nitrogen atoms in piperazine are 5.33 and 9.73 (Albert et al., 1984a) while the pKa figures for 3-ethylamino-1-phenyl-1-propanone and ethylamine (predicted to be released sequentially from 4a) were calculated to be 8.95 (Perrin et al., 1981) and recorded as 10.65 (Albert et al., 1984b).

Series 10

\[
\begin{align*}
\text{R} & \quad \text{O} \\
\text{N} & \quad \text{N} \quad \text{2HCl} \\
\text{R} & \quad \text{O} \\
\end{align*}
\]
**Compound 11**

![Chemical structure of Compound 11](image)

A comparison therefore of the cytotoxicites of these five compounds 9a-e as well as bis Mannich bases 10a,11,3a and 7a may indicate whether bioactivity is influenced by the rates of release of alkylating agents.

**Compound 12**

![Chemical structure of Compound 12](image)

Compound 12 was proposed in which the deamination process is prevented due to the presence of a geminal dimethyl group (Dimmock et al., 1995c). Hence the prediction is made that 5b should display greater bioactivity than compound 12.

### 3.2.2.0 Sequential cytotoxicity concept using azomethines

One of the most exploited biochemical differences employed to exert selective toxicity in the field of anticancer prodrug design, is the difference in pH between the cancer and normal cells. While the pH of most normal cells is 7.4, the extracellular and intracellular pH in tumors has been recorded as 7.0 and 6.5 respectively (Griffiths, 1991). The acidic pH of tumors is reported to be due to the increased rate of anaerobic glycolysis and hence increased production of lactic acid (Tannock et al., 1989). The low pH of the tumors can be used advantageously to design acid sensitive prodrugs, e.g., hydrazones of aldehydes and ketones. According to Greenfield et al. (1990) and Loewenthal (1973), compounds containing azomethine linkages are more prone to
undergo hydrolysis under acidic conditions than under neutral or alkaline conditions. One can therefore expect preferential regeneration of aldehydes and ketones in tumors compared to normal cells. Therefore, to extend the scope of the theory of sequential cytotoxicity to other chemical classes, a new series of compounds namely 3-dimethylamino-4-hydroxybenzoic acid hydrazide hydrazones (series 13) and 3,5-bis(dimethylamino)-4-hydroxybenzoic acid hydrazide hydrazones (series 14) of the cytotoxic compound 50 discovered earlier in this laboratory were designed by taking into account the differential pH between tumorous and cancer cells.

(series 13)

(series 14)

(50)
Under physiological conditions, the compounds in series 13 and 14 were predicted to break down in the acidic pH in tumors to regenerate the corresponding phenolic Mannich base (b) and the β amino ketone (c) (figure 3.5). The phenolic Mannich base (b) can undergo deamination to form quinone methides which are well known for their electrophilic nature and cytotoxic properties. The deamination kinetics of the β amino ketone (c) (Dimmock et al., 1987) has been studied earlier and it was shown to form the compound (e) that has potent cytotoxicity in the P388 lymphocytic leukemia screen. Both the breakdown products can undergo Michael addition reactions with thiols thereby lowering the thiol level in the tumor cells and hence making them more susceptible to the toxic effect of another molecule of the alkylating agent.

3.3.0.0 Selection of cytotoxicity screening models

Initially, many of the compounds prepared in the project were screened in vitro against mouse P388 D1 leukemia cells, mouse L1210 leukemic cells and human T lymphocytes (Molt 4/C8 & CEM) as well as examined in the NCI human tumor cell line panel. Based on the in vitro screening results, a few compounds were tested in vivo against murine tumors and/or human tumor xenografts in athymic mice. The reasons for choosing mouse P388 D1 leukemia and mouse L1210 leukemic screens are (i) the results are generated more rapidly than using the NCI protocols (ii) all compounds are evaluated in contrast to the NCI screen whereby a derivative may not be examined and (iii) these screens in the past have been employed as prescreens for a long time by NCI, USA and the review of screening data generated suggested that these screens were sensitive to most classes of clinically effective antineoplastic agents (Venditti, 1975a; Venditti, 1975b). The NCI in vitro human tumor screen, initiated five years ago employs approximately sixty cell lines representing eight or nine different types of tumors. Since these cell lines are more closely related to human cancers in histology, progression and growth kinetics than murine cell lines, this screen can give a better prediction about the sensitivity and disease specificity of the compounds to human cancers than the mouse screens. Since it was not feasible to evaluate every compound in vivo in animals which would definitely have been the most justified model to predict the selective toxicity of the
Figure 3.5: Proposed degradation pathway for azomethines under physiological conditions.
compound, normal human T lymphocytes (Molt 4/C8 & CEM) have been employed to
evaluate the theory. A comparision of the cytotoxicity results from the normal cells, and
the P388 and L1210 screens will give an indication of selective cytotoxicity. In addition,
all the compounds which were prepared in sufficient quantity were tested for
anticonvulsive activity in mice and often rats. These screens will indicate whether (i)
toxicity and lethality are present in rodents and (ii) whether penetration of the CNS
occurs.

3.4.0.0 Stability studies

If the theory of sequential cytotoxicity is to be validated in the present
case, then it must be proved that the proposed Mannich bases do break down under the
conditions utilized for the in vitro cytotoxicity studies to release two or more cytotoxic
alkylating species, namely acrylophenones and dienones, which as the literature reports
suggest, do alkylate thiols including glutathione. These studies will give an idea about the
extent of deamination i.e., whether the breakdown of the compounds is nonexistent,
minimal or extensive, as well as the number of alkylating species generated and also the
nature of the breakdown products. In addition, it can be proved by carrying out the
stability studies whether the cytotoxicity is due to the intact Mannich bases and related
quaternary ammonium compounds or due to their breakdown products. Thus, the
stability studies of representative compounds from each series under simulated
physiological conditions, buffer pH 7.4 at 37 °C for 48 hours were proposed. The time
and temperature chosen for these studies are the same as used for the P388 and human
tumor cell assays. Among the compounds in series 1,3,5 and 7 having the same aryl
substituents, the 3,4-dichloro and 4-methoxy analogs were chosen since they have the
highest and lowest Hammett σ constants respectively. It was of interest, therefore, to
observe whether the extent of decomposition would be affected by the electronic nature
of the aryl substituents.
3.5.0.0 Mode of cell death induced by XIIIId

Several chemotherapeutic agents commonly used in the treatment of cancer have been shown to be able to induce apoptosis (O'Connor et al., 1991; Barry et al., 1990; Bertrand et al., 1991; Delbino and Darzynkiewicz, 1991; Kaufman, 1989; Ling et al., 1993; Martin et al., 1990) in a number of cell lines in vitro. These include DNA alkylating agents, cisplatin, etoposide, teniposides, macromolecular synthesis inhibitors, dexamethasone and other topoisomerase I and II inhibitors. The significance of apoptosis in the etiology of cancer (Thompson, 1995) and the development of new cancer therapies (Amico and McKenna, 1994) has been discussed. It was therefore proposed to investigate the manner by which the Mannich bases kill the cancer cells in vitro, i.e. necrosis or apoptosis. Therefore, the mode of cell death, induced by one of the representative compounds XIIIId was investigated in human Jurkat T leukemia cell lines.

3.6.0.0 Reasons for screening the proposed compounds against fungi

1. First, the incidence of opportunistic fungal infections in humans have increased dramatically worldwide not only among cancer patients as they receive higher doses of chemotherapy treatments and undergo bone marrow transplantation and AIDS patients, but also due to the widespread use of indwelling intravenous catheters and increased use of potent, broad spectrum antimicrobial agents.

As a result, new antifungal agents are needed to address these critical problems. Existing antifungals can be used to effectively treat most cases of topical infection caused by the opportunistic pathogen Candida albicans, which is the principal agent of nosocomically acquired fungal infections. However life threatening, disseminated Candida infections are treated with modest success. Existing antifungals can be toxic or ineffective because of natural resistance or even reduced resistance.

2. Second, there are many known examples of styryl ketones (Okazaki et al., 1953; Baluja et al., 1964), β-amino ketones and Mannich bases as antifungals available in the literature. It can be inferred from these reports that the antifungal activity is due to the presence of an α,β-unsaturated keto group and it increases considerably when styryl ketones are converted into the corresponding Mannich bases. The mode of action of
Mannich bases, according to Dimmock et al. (1994a), is at least partly due to inhibition of mitochondria and various enzymes involved in the glutathione metabolic pathway such as GST, GSH reductase (GSSGR), γ glutamyl transpeptidase (γGGT) and GSH peroxidase (GP) enzymes. A Mannich base has also been found to inhibit the yeast to mycelium (Y to M) transition in C. albicans by thiol depletion (Thomas et al., 1991).

Hence, it was considered worthwhile to screen the proposed compounds against a variety of fungi.
4.0.0.0 EXPERIMENTAL

4.1.0.0 Materials and methods

4.1.1.0 Chemical

All of the solvents, either of analytical grade or glass distilled, were used as received unless otherwise indicated in the text. Thin layer chromatography using either silica gel 60 F254 precoated TLC plastic plates or Whatman C18 coated glass plates were employed to monitor the reactions and check the homogeneity of the compounds. Unless otherwise stated in the text, all the reported compounds were homogenous by TLC using solvent systems of hexane (7 ml) and methanol (3 ml) for the α,β-unsaturated ketones, chloroform (7ml) and methanol (3 ml) for the Mannich bases and chloroform (7ml), methanol (3 ml) and ammonium hydroxide (0.08 ml) for the quaternary ammonium salts. Chromatographic separations were performed using E Merck 0.03-0.200 mm (70-230 mesh ASTM) silica gel 60. Chromatograms were observed under ultraviolet light and/or in an iodine chamber. The compounds were dried under reduced pressure at 60 °C in a vacuum oven and stored in well closed containers or in a desiccator at room temperature. Melting points were determined with a Galenkamp MF-370 apparatus and are uncorrected. Infrared spectra were recorded with a Beckman Acculab TM spectrometer calibrated against a polystyrene standard. While 1H NMR spectra were recorded in the solvents indicated in the text on a Varian T-60 MHz, the stability studies were conducted using a Bruker AM-300 spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane or DSS as internal standards. Elemental analyses of the dry compounds, (dried for at least 3 hours in an Abderhalden drying pistol under reduced pressure), was undertaken on a Perkin-Elmer CHN analyzer in the Department of Chemistry, University of Saskatchewan.
4.1.2.0 Biological

Most of the compounds were screened in vitro for their cytotoxicity against mouse P388 D1 leukemia cells (Dr. T. M. Allen, Department of Pharmacology, University of Alberta), mouse L1210/0 leukemia cells, Molt4/C8 and CEM/0 human T lymphocytes (Dr. E. De Clercq, Rega Institute, Belgium) and a battery of human tumor cell lines (National Cancer Institute, Bethesda, USA). Two compounds namely XIIIId and XIVa were screened in vivo by the NCI, USA in nude mice against a variety of human tumor xenografts and murine P388 leukemia cells. Some of the selected compounds were screened for antimicrobial activity against a range of fungi and bacteria (Dr. E.K. Manavathu, Wayne State University, USA).

The mode of cell death induced by XIIIId was studied by the author using the human Jurkat T leukemia cell line. The human Jurkat T leukemia cell line was grown in RPMI 1640 medium, (Sigma), pH 7.4, supplanting with 10% fetal bovine serum and 0.01% gentamycin (Sigma) at 37 °C in a humidified 5% carbon dioxide atmosphere and was passaged three times a week. The stock solutions of XIIIId (1mM, 0.1 mM, 0.01 mM) were prepared in DMSO and stored at -20 °C. Freshly prepared solutions of Melphalan (Sigma) [1mM, 0.1 mM, 0.01 mM], dissolved in DMSO were used for the apoptosis studies.

A trypan blue [0.04% in phosphate buffer saline (PBS), pH 7.4] dye solution was used for counting living cells. A dye mix consisting of 100μg/ml acridine orange (Sigma) and 100μg/ml ethidium bromide (Sigma) both prepared in PBS was used for the identification of apoptotic cells on a fluorescent microscope (model 2071, American Optical). The agarose gel was run using a GIBCO BRL horizontal gel electrophoresis apparatus.
4.2.0.0 Synthesis of α,β-unsaturated ketones

4.2.1.0 General procedure for the synthesis of 4-aryl-3-buten-2-ones (Ib-k, table 4.1)

Compound Ia was obtained from the Aldrich company. The remaining styryl ketones Ib-k were prepared by a literature procedure (Edwards et al., 1983). An aqueous solution of sodium hydroxide (0.17 mole, 150 ml) was added over a period of half an hour to a mechanically stirred ice cold solution of an appropriate aldehyde (0.1 mole) in acetone (100 ml). The temperature was maintained below 10 °C. The mixture was stirred for 2-5 hours, acidified with 1N hydrochloric acid followed by extraction with chloroform (3 x 50 ml). The organic extract was washed with water and then dried over anhydrous magnesium sulfate. The solution was evaporated under reduced pressure to give an oil which solidified in the cases of Ib,c,e,k on cooling. The compounds Ib,c,e,k were purified by recrystallization from a suitable solvent while the compounds Id, f-j were purified by vacuum distillation. The physical data of the compounds is given in table 4.1. The spectroscopic data of a representative compound Ie are as follows.

$^1$HNMR (60 MHz, CDCl$_3$), δ: 2.3 (s, 3H, COCH$_3$), 3.8 (s, 3H, OCH$_3$), 6.4 - 6.6 (d, 1H, CH=CHCO, J = 18Hz), 6.7 - 7.5 (m, 5H, C$_6$H$_5$CH=CHCO).

IR (film): 1710 (C=0, s-trans), 1685 (C=0, s-cis), 1635 (C=C, aromatic, olefinic) cm$^{-1}$. 
Table 4.1: Physical data of 4-aryl-3-buten-2-ones

<table>
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<tr>
<th>Compound</th>
<th>R</th>
<th>Yield (%)</th>
<th>M.P. (°C) or B.P. (°C/mm Hg)</th>
<th>Recrystallization solvent</th>
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<td>Ia</td>
<td>H</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>petroleum ether&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>45</td>
<td>53-55</td>
<td>petroleum ether&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>85</td>
<td>70-72/1.0</td>
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<tr>
<td>Ie</td>
<td>4-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>65</td>
<td>72-74</td>
<td>toluene</td>
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<td>If</td>
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<td>78</td>
<td>135-37/0.5</td>
<td>---</td>
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<tr>
<td>Ig</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>89</td>
<td>123-125/0.5</td>
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<tr>
<td>Ih</td>
<td>4-C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>75</td>
<td>100-102/0.5</td>
<td>---</td>
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<tr>
<td>Ii</td>
<td>2,4-(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>78</td>
<td>80-82/0.6</td>
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<tr>
<td>Ij</td>
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<td>90-92/0.6</td>
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<td>Ik</td>
<td>4-Br</td>
<td>80</td>
<td>79-80</td>
<td>toluene</td>
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</table>

<sup>a</sup> Boiling point 30-60 °C
4.2.2.0 Synthesis of 1-(4-chlorophenyl)-4-methyl-1-penten-3-one (II)

Compound II was synthesized using a literature procedure (Smith et al., 1972). A mixture of 4-chlorobenzaldehyde (0.1 mole), 3-methyl-2-butanone (0.12 mole), sodium hydroxide (0.25 mol) and water (100 ml) was heated under reflux for 24 hours. The reaction mixture was cooled, acidified with 1N hydrochloric acid and extracted with chloroform. The organic extract was washed with water and then dried over anhydrous magnesium sulfate. The oil obtained by evaporation of the solvent was purified by vacuum distillation. The yield and b.p. (°C/mmHg) of II was 80% and 130/1.0 [literature value 132/2.5 °C/mmHg (Marcas et al., 1964) respectively.

Elemental analysis (%) : C H
Calculated (C_{12}H_{13}ClO) : 69.06 6.28
Found : 68.87 6.58

The spectroscopic data of II were as follows.

\[
\text{\text{(II) }}
\]

\(^1\text{H NMR (60 MHz, CDCl}_3\text{), }\delta: 1.6 \text{ [s, 6H, J_con = 8Hz, C(CH}_3\text{)_2]}, 2.25-2.7 \text{ [m, 1H, -CH (CH}_3\text{)_2]}, 6.5-7.8 \text{ [m, 6H, aryl and olefinic protons, J_bb = 16Hz].}
\]

IR (film): 1700 (-C=O, s-trans), 1685 (-C=O, s-cis), 1620 (C=C, aromatic, olefinic) cm\(^{-1}\).

4.2.3.0 Attempted synthesis of 4-(4-carboxyphenyl)-3-buten-2-one (III)

The synthesis of 4-(4-carboxyphenyl)-3-buten-2-one was initially attempted using the procedure of Edwards et al.,(1983) as described under 4.2.1.0. There was no reaction between the 4-carboxybenzaldehyde and acetone at 10 °C for 10 hours or at room temperature overnight. The reaction was repeated by passing dry hydrogen chloride gas (0.001mol) in a solution of the aldehyde (0.001mol) in acetone (25ml). Initially, the solution was colorless for 2 hours. Later, it turned to orange and finally to dark red. The
TLC showed at least six spots of equal intensity including some unreacted aldehyde. The reaction mixture was diluted with water and extracted with ether. The ethereal layer contained at least four compounds as revealed by TLC. No attempts were made to isolate and identify them.

A reaction of 4-carboethoxybenzaldehyde (0.001 mol, table 4.1) with acetone (25 ml) under the conditions described in 4.2.1.0 gave a complex mixture of products as indicated by TLC. No attempts were made to separate them. No reaction took place after passing hydrogen chloride into the solution of 4-carboethoxybenzaldehyde (0.001 mol) and acetone (25 ml) and stirring it for 24 hours.

Another route employed to synthesize the ketone involved the synthesis of the 4-bromostyryl ketone, protection of the keto group by ketal formation, a Grignard reaction and finally reaction with solid carbon dioxide and deprotection. The 4-bromostyryl ketone was synthesized as explained under 4.2.1.0. To prepare the isopropylene ketal of the ketone, a mixture of Ik (0.01 mol), isopropylene glycol (0.015 mol) and para toluenesulfonic acid (10 mg) in toluene was refluxed for 72 hours in a Dean and Stark apparatus. The reaction mixture was made alkaline with sodium bicarbonate solution and extracted with chloroform (3 x 25 ml). After drying the chloroform extract over magnesium sulfate, it was filtered, concentrated and purified by vacuum distillation. The structure of the protected ketone was confirmed by IR and $^1$H NMR spectroscopy. The infrared spectra showed the disappearance of the carbonyl peak. The NMR data for the ketal of Ik are as follows.

$^1$H NMR (60 MHz, CDCl$_3$), δ: 1.4 [d, 3H, CH(CH$_3$)$_2$], 1.6 [s, 3H, -CH=CH-CO-CH$_3$], 3.2-3.8 [br,m, 1H, CH(CH$_3$)$_2$], 3.9-4.5 [br,m, 2H, CH$_2$], 6.0-7.8 [m, 6H, C$_6$H$_4$-CH=CH-].

Repeated attempts to form the Grignard reagent of the protected 4-bromo styryl ketone were unsuccessful. No further attempts were made to prepare the desired ketone.
4.3.0.0 Synthesis of Mannich bases of acetophenones and styryl ketones and their methohalides

4.3.1.0 General procedure for synthesis of 1-aryl-3-diethylamino-1-propanone hydrochlorides (series 1, IV a-h, table 4.2)

A mixture of an appropriate 1-aryl-1-ethanone, paraformaldehyde and diethylamine hydrochloride in acetonitrile (100 ml, IV a,c,f-i), isopropanol (100 ml IV b, d, e), acidified with 2 drops of hydrochloric acid (37% w/v, 0.04 ml) was heated under reflux for different periods of time. The reaction mixture was evaporated under reduced pressure to give an oil which was triturated with anhydrous ether followed by acetone to remove the unreacted ketone and induce precipitation of the Mannich base hydrochloride. The precipitates were collected, washed with anhydrous ether and subjected to fractional crystallization from a suitable solvent in order to remove unreacted diethylamine hydrochloride. A satisfactory elemental analysis for IVi i.e. the 4-nitro derivative, could not be obtained even after repeated purification by crystallization or column chromatography. The compound decomposed on the silica gel column to give probably the deaminated product (Katrizky et al., 1990). The molar ratios of the ketone : paraformaldehyde : amine hydrochloride and reaction time (hours) were as follows. IVa: 0.04:0.03:0.01, 30; IVb: 0.025:0.03:0.01,17; IVc: 0.03:0.025:0.01, 24; IVd: 0.04:0.03:0.01, 42; IVe: 0.04:0.03:0.01, 42; IVf: 0.01:0.01:0.01, 5; IVg: 0.01:0.01:0.01, 5; IVh: 0.03:0.02:0.01, 24; IVi: 0.01:0.025:0.02, 12.

The physical data of the compounds are given in table 4.2. The spectroscopic data of a representative compound (IVa) are as follows.

$^1$H NMR (60 MHz, CDCl$_3$), δ: 1.2 - 1.6 (t, 6H, CH$_3$), 2.83 - 4.0 (m, 8H, CH$_2$), 7.13 - 8.0 (m, 5H, C$_6$H$_5$).

IR (nujol): 2200-2650 (b, C=N+ str), 1690 (s, C=O str)
Table 4.2: Physical data of 1-aryl-3-diethylamino-1-propanone hydrochlorides.

<table>
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<th>Compound</th>
<th>R</th>
<th>Yield (%)</th>
<th>M.P. (°C)</th>
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<td>C</td>
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<tr>
<td>IVa</td>
<td>H</td>
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<td>IVc</td>
<td>3,4-Cl₂</td>
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<td>131-133</td>
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<td>-</td>
<td>95% ethanol</td>
<td>-</td>
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</tr>
</tbody>
</table>
4.3.2.0 Synthesis of 1-phenyl-3-ethylamino-1-propanone hydrochloride (V)

A mixture of acetophenone (0.01 mol), paraformaldehyde (0.01 mol) and ethylamine hydrochloride (0.02 mol) in 95% ethanol (50 ml) was refluxed for 18 hours. TLC showed the presence of four compounds namely the mono (Rf = 0.28) and bis Mannich bases in addition to unreacted ketone and ethylamine hydrochloride. The oil obtained after concentrating the reaction mixture was triturated with petroleum ether to give a colorless semisolid that was washed 3-4 times with anhydrous ether to remove the unreacted ketone. The colorless semisolid was dissolved in acetone and kept at 4 °C overnight. Repeated recrystallizations of the impure material failed to remove completely the bis compound and ethylamine hydrochloride. Column chromatography of the crude material on silica gel using a solvent of dichloromethane:methanol (9:1) as an eluent led to the decomposition of the mono compound. However, flash chromatography was performed using the same conditions to obtain the pure compound whose identity and purity was confirmed by IR, NMR and elemental analysis. The melting point and yield of V were found to be 155-157 °C and 26% respectively.

Elemental analysis (%) : C H N
Calculated (C_{11}H_{16}ClNO) : 61.82 7.55 6.55
Found : 62.07 7.54 6.75

The spectroscopic data of V are as follows.

$^1$H NMR (300 MHz, CDCl$_3$), δ: 1.5 (t, 3H, CH$_3$), 3.1 (q, 2H, CH$_2$CH$_3$), 3.4 (t, 2H, COCH$_2$CH$_2$N+), 3.7 (t, 2H, COCH$_2$CH$_2$N+), 7.3-8.0 (m, 5H, C$_6$H$_5$), 8.7-10.9 (br, s, 2H, +NH$_2$),

IR (nujol): 2450, 2670 (s, C-N+ str), 1690 (s, C=O str)
4.3.3.0 Attempted synthesis of the 1-aryl-1-propanone-3-diethylamino methobromides (series 2, VI)

Solutions of the mono Mannich base hydrochlorides IV a-e (0.005 mol) in cold methanol (25 ml) were neutralized by adding a sufficient amount of aqueous ammonia with constant stirring so as to make the pH alkaline (pH = 8). The temperature of the solution was maintained below 5 °C. The mixture was extracted with ether (5 x 25 ml). TLC of the ethereal extract showed decomposition of IVa-e with the formation of an appreciable amount of a new compound which was probably the deaminated product. Ethereal extracts were dried over anhydrous magnesium sulfate and evaporated under reduced pressure to give oils which were dissolved in anhydrous ether (50 ml). An excess of methyl bromide (0.05 mole) was added to the solution of the Mannich base in anhydrous ether maintained at 0 °C and the reaction mixture was stirred for 6 hours. The precipitates were filtered, washed with dry ether and dried under vacuum. Repeated recrystallizations from ether/methanol or acetone failed to give pure products. Column chromatography led to further decomposition of the methobromide.
4.3.4.0 General procedure for the synthesis of N,N-bis(3-aryl-3-oxopropyl)-N-ethylamine hydrochlorides (series 3, VII a-j, table 4.3)

A mixture of the 1-aryl-1-ethanone, paraformaldehyde and ethylamine hydrochloride in acetonitrile (100 ml, VIIa,d,e,g-j), isopropanol (100 ml, VIIb,c,f,j), acidified with trifluoroacetic acid (0.04 ml, VIIa,d,e; 3 ml, 2f) or hydrochloric acid (37% w/v, 0.04 ml, VIIb,c,g-j) was heated under reflux for different periods of time. The colorless materials which precipitated in the case of VIIb,h at the end of the reactions were collected, washed with isopropanol and finally recrystallized from a suitable solvent. For the remaining compounds, the reaction mixture was evaporated under reduced pressure leading to oils which were treated with anhydrous ether to remove the unreacted ketones. The oil was dissolved in absolute ethanol and enough anhydrous ether was added to make the solution turbid. The solution was kept in the refrigerator (-4°C) for 2-3 days. The deposited material was collected and recrystallized 3-4 times from a suitable solvent to produce a pure compound.

The molar ratios of ketone:paraformaldehyde:amine hydrochloride and times of heating (hours) were VIIa: 0.04:0.04: 0.01, 20; VIIb: 0.03:0.025:0.01, 48; VIIc: 0.03:0.025:0.01, 24; VIId: 0.04:0.04:0.01, 20; VIIe: 0.04:0.04:0.01, 48; VIIf: 0.05:0.05:0.01, 48; VIIg: 0.04:0.04: 0.01, 48; VIIh: 0.04:0.04: 0.01, 48; VIIi: 0.04:0.04: 0.01, 55; VIIj: 0.04:0.04: 0.01, 19. The physical data of the compounds are given in table 4.3.

The spectroscopic data of a representative compound (VIIa) are given below.

$^1$H NMR (60 MHz, CDCl$_3$), δ: 1.2 - 1.66 (t, 3H, CH$_3$), 2.93 - 4.0 (m, 10H, CH$_2$), 7.0 - 8.16 (m, 10H, C$_6$H$_5$)

IR (nujol): 2460, 2520, 2630, 2700 (m, C-N+ str), 1690 (s, C=O, str).
Table 4.3: Physical data of N,N-bis(3-aryl-3-oxopropyl)-N-ethylamine hydrochlorides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Yield (%)</th>
<th>M.P. (°C)</th>
<th>Recrystallization Solvent</th>
<th>Elemental Analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Calculated Find</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>C          H          N          C          H          N</td>
</tr>
<tr>
<td>VIIa</td>
<td>H</td>
<td>46</td>
<td>208-210</td>
<td>ethanol (95%)</td>
<td>69.45      6.99      4.05      69.41      6.83       3.95</td>
</tr>
<tr>
<td>VIIb</td>
<td>4-Cl</td>
<td>70</td>
<td>202-203</td>
<td>ethanol (95%)</td>
<td>57.91      5.35      3.38      57.84      5.19       3.35</td>
</tr>
<tr>
<td>VIIc</td>
<td>3,4-Cl₂</td>
<td>69</td>
<td>185-187</td>
<td>methanol</td>
<td>49.66      4.17      2.90      49.41      4.12       2.73</td>
</tr>
<tr>
<td>VIId</td>
<td>4-CH₃</td>
<td>57</td>
<td>195-197</td>
<td>isopropanol</td>
<td>70.68      7.49      3.74      70.87      7.27       3.53</td>
</tr>
<tr>
<td>VIIe</td>
<td>4-OCH₃</td>
<td>56</td>
<td>178-179</td>
<td>methanol/ether</td>
<td>65.09      6.95      3.45      65.17      7.03       3.34</td>
</tr>
<tr>
<td>VIIf</td>
<td>4-OH</td>
<td>50</td>
<td>178-180</td>
<td>methanol</td>
<td>62.09      6.51      3.62      62.03      6.44       3.49</td>
</tr>
<tr>
<td>VIIg</td>
<td>2,4-Cl₂</td>
<td>50</td>
<td>214-215</td>
<td>acetone/methanol</td>
<td>49.66      4.17      2.90      49.81      3.96       2.74</td>
</tr>
<tr>
<td>VIIh</td>
<td>4-Br</td>
<td>20</td>
<td>212-213</td>
<td>methanol</td>
<td>47.69      4.40      2.78      47.62      4.45       2.74</td>
</tr>
<tr>
<td>VIIi</td>
<td>4-F</td>
<td>30</td>
<td>198-199</td>
<td>methanol/ether</td>
<td>62.91      5.61      3.67      62.72      5.60       3.67</td>
</tr>
<tr>
<td>VIIj</td>
<td>4-NO₂</td>
<td>24</td>
<td>200-202</td>
<td>methanol</td>
<td>52.88      5.29      9.26      52.95      5.11       9.09</td>
</tr>
</tbody>
</table>
4.3.5.0 General procedure for the synthesis of \( N,N\)-bis(3-aryl-3-oxopropyl)-\( N\)-ethylamine methobromides and methiodides (series 4, VIII a-i, table 4.4)

A solution of the bis Mannich base hydrochlorides VIIIa-e (0.01 mol) in 20% cold aqueous methanol (25 ml) was neutralized by adding a sufficient amount of aqueous ammonia with constant stirring so as to make the solution alkaline (pH = 8). The temperature of the solution was maintained below 10 °C. The mixture was extracted with ether (5x25 ml). The ether extracts were dried over anhydrous magnesium sulfate and evaporated under reduced pressure to give an oil which was dissolved in anhydrous ether (50 ml). An excess of methyl bromide (0.1 mole) or methyl iodide (0.1 mole) was added to the solution of the Mannich base in anhydrous ether cooled at 0 °C and the reaction mixture was stirred for 6 hours in the case of the methobromides and overnight for the methiodides at the same temperature (0 °C). The precipitates were filtered, washed with dry ether and recrystallized from a suitable solvent. The physical data of the compounds are given in table 4.4.

The spectroscopic data of a representative compound (VIIIa) are given below.

\(^1\)H NMR (60 MHz, DMSO), \( \delta \): 1.55 - 1.17 (t, 3H, N-CH\(_2\)-CH\(_3\)), 2.07 - 4.73 [m, 13H, CH\(_2\)CH\(_2\)N(CH\(_3\))(CH\(_2\)CH\(_3\)CH\(_2\),CH\(_3\))], 6.87 - 7.67 (m, 10H, C\(_6\)H\(_5\)).

IR (nujol): 1670 (s, C=O)
Table 4.4: Physical data of N,N-bis(3-aryl-3-oxopropyl)-N-ethylamine methohalides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>X'</th>
<th>Yield (%)</th>
<th>M.P. (°C)</th>
<th>Recrystallization Solvent</th>
<th>Elemental Analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td>Calculated</td>
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<td>Found</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>VIIId</td>
<td>4-CH₃</td>
<td>Br⁻</td>
<td>80</td>
<td>152-154</td>
<td>methanol/ether</td>
<td>62.72</td>
</tr>
<tr>
<td>VIIe</td>
<td>4-OCH₃</td>
<td>Br⁻</td>
<td>76</td>
<td>188-190</td>
<td>methanol/ether</td>
<td>59.48</td>
</tr>
<tr>
<td>VIIIf</td>
<td>4-Cl</td>
<td>I⁻</td>
<td>72</td>
<td>181-183</td>
<td>ethanol (95%)</td>
<td>48.48</td>
</tr>
<tr>
<td>VIIIf</td>
<td>3,4-Cl₂</td>
<td>I⁻</td>
<td>70</td>
<td>173-175</td>
<td>ethanol (95%)</td>
<td>42.81</td>
</tr>
<tr>
<td>VIIIf</td>
<td>4-CH₃</td>
<td>I⁻</td>
<td>85</td>
<td>177-178</td>
<td>ethanol (95%)</td>
<td>57.62</td>
</tr>
<tr>
<td>VIIIf</td>
<td>4-OCH₃</td>
<td>I⁻</td>
<td>80</td>
<td>167-169</td>
<td>ethanol (95%)</td>
<td>54.02</td>
</tr>
</tbody>
</table>

69
4.3.6.0 General procedure for the synthesis of 1-aryl-5-dialkylamino-1-penten-3-one hydrochlorides and related compounds (series 5, IX a-i, table 4.5)

A mixture of an appropriate 4-aryl-3-buten-2-one (0.03 mole), paraformaldehyde (0.03 mole), and an amine hydrochloride (0.01 mole) acidified with two drops of trifluoroacetic acid (IXa-e) in acetonitrile (IXa-e) or ethanol (IXf-i) was heated under reflux for 24 hours (IXb,c), 36 (IXa) and 48 (IXd-i) hours. The pure compound was isolated as explained under 4.3.1.0. The physical data of the compounds are given in table 4.5. The spectroscopic data of a representative compound (IXa) are given below:

\(^1\)H NMR (60 MHz, CDCl₃), \(\delta\) : 1.2 - 1.66 (t, 6H, CH₃), 2.83 - 3.6 (m, 8H, CH₂), 6.4 - 6.73 (d, 1H, -CH=CHCO-, Jₓᵧ = 16 Hz), 7.4 - 7.67 (d, 1H, PhCH=CH, Jₓᵧ = 16Hz), 7.0 -7.4 (m, 5H, C₆H₅).

IR (nujol): 2500, 2580, 2600, 2770 (m, C-N+ str), 1690 (C=O, s-trans), 1670 (C=O, s-cis).

4.3.7.0 Synthesis of 1-(4-chlorophenyl)-5-diethylamino-4,4-dimethyl-1-penten-3-one hydrochloride (X)

This compound was synthesized essentially in the same manner as described under series 4.3.6.0. A mixture of 1-(4-chlorophenyl)-4-methyl-1-penten-3-one (0.05 mole), paraformaldehyde (0.05 mole), diethylamine hydrochloride (0.01 mole), trifluoroacetic acid (0.04 ml) and solvent (95%ethanol, 100ml) was heated under reflux for 48 hours. Utilisation of the procedure described in section 4.3.6.0 led to the isolation of X in 61% yield, m.p. 161-162 °C after recrystallization from methanol/ether.

Elemental analysis (%): C H N
Calculated (C₁₇H₂₅Cl₂NO): 61.82 7.63 4.24
Found: 61.62 7.87 4.17

The spectroscopic data of X are as follows.

\(^1\)H NMR (60 MHz, CDCl₃), \(\delta\): 1.0-1.6 [m, 6H, N(CH₂CH₃)₂], 1.6 [s, 6H, C(CH₃)₂], 2.66-3.5 [m, 6H, -CH₂N(CH₂CH₃)₂], 7.06-7.33 (d, 1H, olefinic H, Jₓᵧ=16Hz), 7.33-7.93 (m, 4H, C₆H₄), 7.66-7.93 (d, 1H, olefinic H, Jₓᵧ=16Hz).

IR (film): 2980, 2740 (w, C-N+ str), 1695 (s, C=O).
### Table 4.5: Physical data of 1-aryl-5-dialkylamino-1-penten-3-one hydrochlorides and related compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Yield (%)</th>
<th>M.P. (°C)</th>
<th>Recrystallization Solvent</th>
<th>Elemental Analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td>Calculated</td>
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<td></td>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>IXa</td>
<td>H</td>
<td>N(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>60</td>
<td>130-132</td>
<td>acetone</td>
<td>67.27</td>
</tr>
<tr>
<td>IXb</td>
<td>4-Cl</td>
<td>N(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>67</td>
<td>150-152</td>
<td>methanol</td>
<td>59.60</td>
</tr>
<tr>
<td>IXc</td>
<td>3,4-Cl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>53</td>
<td>158-160</td>
<td>methanol/ether</td>
<td>53.51</td>
</tr>
<tr>
<td>IXd</td>
<td>4-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>N(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>47</td>
<td>156-158</td>
<td>methanol/ether</td>
<td>68.19</td>
</tr>
<tr>
<td>IXe</td>
<td>4-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>N(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>89</td>
<td>148-150</td>
<td>methanol/ether</td>
<td>64.52</td>
</tr>
<tr>
<td>IXf</td>
<td>H</td>
<td>N(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>70</td>
<td>180-182</td>
<td>ethanol/acetone</td>
<td>65.13</td>
</tr>
<tr>
<td>IXg</td>
<td>H</td>
<td></td>
<td>65</td>
<td>182-184</td>
<td>ethanol/acetone</td>
<td>68.68</td>
</tr>
<tr>
<td>IXh</td>
<td>H</td>
<td>N&lt;sub&gt;3&lt;/sub&gt;</td>
<td>52</td>
<td>175-176</td>
<td>ethanol/acetone</td>
<td>63.94</td>
</tr>
<tr>
<td>IXi</td>
<td>H</td>
<td>N&lt;sub&gt;3&lt;/sub&gt;</td>
<td>50</td>
<td>185-187</td>
<td>ethanol/acetone</td>
<td>67.79</td>
</tr>
</tbody>
</table>
4.3.8.0 Synthesis of 5-ethylamino-1-phenyl-1-penten-3-one hydrochloride (XI)

Compound XI was synthesized and purified in an identical manner as the analog V. A mixture of 4-phenyl-3-buten-2-one (0.01 mole), paraformaldehyde (0.02 mole), ethylamine hydrochloride (0.02 mole), was heated under reflux for 24 hours. Utilisation of the procedure described in section 4.3.2.0 led to the isolation of XI in 24% yield, m.p. 171-173 °C after recrystallization from acetone/methanol.

Elemental analysis (%) C H N
Calculated (C_{13}H_{18}ClNO) : 65.13 7.57 5.84
Found : 65.21 7.58 5.84

The spectroscopic data of XI are as follows.

$^1$H NMR (60 MHz, DMSO), δ: 1.2 [t, 3H, +NH(CH$_3$CH$_3$)], 2.4-3.6 [m, 6H, CH$_2$CH$_2$NH(CH$_2$CH$_2$)], 6.6-7.7 [m, 7H, C$_6$H$_5$CH=CH].
IR (film): 2700 (w, C-N+ str), 1680 (s, C=O) cm$^{-1}$.

4.3.9.0 Attempted synthesis of 1-aryl-5-diethylamino-1-penten-3-one methobromides (series 6, XII)

The synthesis of the title compounds was attempted in the identical manner as described under 4.3.3.0. Similar kind of difficulties were experienced in this case.
4.3.10.0 General procedure for the synthesis of N,N-bis (5-aryl-3-oxo-4-pentenyl)-N-ethylamine hydrochlorides (series 7, XIII a-j, table 4.6)

A solution of an appropriate 4-aryl-3-buten-2-one, paraformaldehyde and ethylamine hydrochloride in molar ratios of 0.06 : 0.04 : 0.01 (XIIIa,b,d,f-j), 0.08 : 0.08 : 0.01 (XIIIc), 0.05 : 0.05 : 0.01 (XIIIe) in 95% ethanol (100 ml) acidified with two drops of hydrochloric acid was refluxed for 36 (XIIIa,f), 30 (XIIIb), 24 (XIIIc), 42 (XIIIc), 10 (XIIIe,g) and 72 (XIIIh-j) hours. The reaction mixture was evaporated under reduced pressure to give an oil which was triturated with ether to remove the unreacted ketone. The oil was dissolved in absolute ethanol (10 ml) and enough ethyl acetate was added to make the solution turbid. The solution was kept in a refrigerator (-30 °C) for 3-4 days. The deposited material was collected and recrystallized several times from a suitable solvent. The physical data of the compounds are given in table 4.6.

The spectroscopic data of a representative compound (XIIIa) are given below.

$^1$H NMR (60 MHz, CDCl$_3$), $\delta$ : 1.4 - 1.8 (t, 3H, CH$_3$), 3.0 - 3.6 (m, 10H, CH$_2$), 6.3 - 8.1 (m, 14H, 10 C$_6$H$_5$, 2 -CH=CHCO-, $J_{ab} = 16$ Hz).

IR (nujol): 2720, 2680, 2600, 2500, 2420 (w, C-NH+ str), 1690 (s, C=O) cm$^{-1}$. 
Table 4.6: Physical data of N,N-bis(5-aryl-3-oxo-4-pentenyl)-N-ethylamine hydrochlorides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Yield (%)</th>
<th>M. P. (°C)</th>
<th>Recrystallization Solvent</th>
<th>Elemental Analysis (%)</th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Calculated</td>
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<td>Found</td>
<td>Found</td>
<td>Found</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>H</td>
<td>N</td>
<td>C</td>
<td>H</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>XIIIa</td>
<td>H</td>
<td>56</td>
<td>194-196</td>
<td>ethanol (95%)</td>
<td>72.43</td>
<td>7.09</td>
<td>3.52</td>
<td>72.14</td>
<td>7.15</td>
<td>3.29</td>
<td></td>
</tr>
<tr>
<td>XIIIb</td>
<td>4-Cl</td>
<td>23</td>
<td>210-212</td>
<td>methanol/ether</td>
<td>61.74</td>
<td>5.61</td>
<td>3.00</td>
<td>61.64</td>
<td>5.83</td>
<td>2.87</td>
<td></td>
</tr>
<tr>
<td>XIIIc</td>
<td>3,4-Cl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>62</td>
<td>192-194</td>
<td>methanol (95%)</td>
<td>49.66</td>
<td>4.17</td>
<td>2.89</td>
<td>49.41</td>
<td>4.12</td>
<td>2.73</td>
<td></td>
</tr>
<tr>
<td>XIIIId</td>
<td>4-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>20</td>
<td>190-192</td>
<td>methanol/ether</td>
<td>73.30</td>
<td>7.57</td>
<td>3.29</td>
<td>73.18</td>
<td>7.49</td>
<td>3.17</td>
<td></td>
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<tr>
<td>XIIIe</td>
<td>4-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>24</td>
<td>180-182</td>
<td>methanol/ether</td>
<td>68.18</td>
<td>7.04</td>
<td>3.06</td>
<td>68.08</td>
<td>7.22</td>
<td>2.96</td>
<td></td>
</tr>
<tr>
<td>XIIIf</td>
<td>2-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>30</td>
<td>164-166</td>
<td>methanol</td>
<td>70.33</td>
<td>7.71</td>
<td>3.15</td>
<td>70.73</td>
<td>8.03</td>
<td>2.89</td>
<td></td>
</tr>
<tr>
<td>XIIIg</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>18</td>
<td>167-169</td>
<td>methanol/ether</td>
<td>70.39</td>
<td>7.73</td>
<td>3.16</td>
<td>70.25</td>
<td>7.67</td>
<td>3.12</td>
<td></td>
</tr>
<tr>
<td>XIIIh</td>
<td>2,4-(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>28</td>
<td>172-174</td>
<td>methanol/ether</td>
<td>74.06</td>
<td>7.99</td>
<td>3.08</td>
<td>73.82</td>
<td>7.90</td>
<td>3.05</td>
<td></td>
</tr>
<tr>
<td>XIIIi</td>
<td>4-C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>23</td>
<td>165-166</td>
<td>methanol/ether</td>
<td>71.24</td>
<td>8.11</td>
<td>2.97</td>
<td>71.50</td>
<td>7.82</td>
<td>2.98</td>
<td></td>
</tr>
<tr>
<td>XIIIj</td>
<td>4-CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>27</td>
<td>200-202</td>
<td>acetonitrile</td>
<td>74.74</td>
<td>8.36</td>
<td>2.91</td>
<td>74.90</td>
<td>8.02</td>
<td>2.68</td>
<td></td>
</tr>
</tbody>
</table>
4.3.11.0 General procedure for the synthesis of N,N-bis (5-aryl-3-oxo-4-pentenyl)-N-ethylamine methobromides or methiiodides (series 8, XIV a-f, table 4.7)

These compounds were synthesized by the same manner as outlined under 4.3.5.0 except that the bis Mannich bases used were XIIIa-e. TLC of the reaction products obtained in the case of the methobromides of 4-chloro and 4-methoxy bis Mannich bases (XIIIb,e) revealed the presence of an impurity. Repeated recrystallizations and column chromatography did not lead to the isolation of pure compounds. The physical data of the compounds are given in table 4.7.

The spectroscopic data of a representative compound (XIV a) are given below.

$^1$H NMR (60 MHz, CDCl$_3$), δ: 1.2 - 1.55 (t, 3H, N-CH$_2$CH$_3$), 2.1 - 4.7 (m, 13H, CH$_2$ and N-CH$_3$), 6.9 - 7.7 (m, 10H, C$_6$H$_5$).

IR (nujol): 1700 (s, C=O) cm$^{-1}$.

4.3.12.0 Synthesis of 2- dimethylaminomethyl acrylophenone hydrochloride (XV)

This compound was synthesized by a literature procedure (Lesieur et al., 1986). To a solution of acetophenone (0.01 mole) in acetic acid (20ml) was added trioxymethylene (0.02 mole) and dimethylamine hydrochloride (0.01 mole). The mixture was refluxed for 3 hours and the solvent was evaporated followed by the addition of acetone to the residual oil under stirring. The resulting solid was filtered, dried and recrystallized from acetone/methanol. The yield and melting point of XV were 50% and 156-158 °C [literature value: 159-160 °C, (Lesieur et al., 1986)] respectively.

<table>
<thead>
<tr>
<th>Elemental analysis (%)</th>
<th>C</th>
<th>H</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated (C$<em>{13}$H$</em>{18}$ClNO)</td>
<td>65.13</td>
<td>7.57</td>
<td>5.84</td>
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<tr>
<td>Found</td>
<td>65.43</td>
<td>7.50</td>
<td>5.48</td>
</tr>
</tbody>
</table>

The spectroscopic data of XV are as follows.

$^1$H NMR (60 MHz, CDCl$_3$), δ : 3.1 (s, 6H, CH$_3$), 4.1 (s, 2H, CH$_2$N), 6.15(s, 1H, C=CH$_2$), 6.59 (s, 1H, C=CH$_2$), 6.9-7.3 (m, 5H, C$_6$H$_5$).

IR (film): 2680, 2540, 2500 (w, C-N+ str), 1665 (s, C=O) cm$^{-1}$. 
Table 4.7: Physical data N,N-bis (5-aryl-3-oxo-4-pentenyl)-N-ethylamine methohalides

<table>
<thead>
<tr>
<th>Compound&lt;sup&gt;(i)&lt;/sup&gt;</th>
<th>R</th>
<th>Yield (%)</th>
<th>M.P. &lt;sup&gt;(°C)&lt;/sup&gt;</th>
<th>Recrystallization Solvent</th>
<th>Elemental Analysis (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>XIVa</td>
<td>H</td>
<td>77</td>
<td>178-179</td>
<td>ethanol (95%)</td>
<td>65.78</td>
<td>6.63</td>
<td>3.07</td>
<td>65.66</td>
<td>7.03</td>
<td>2.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIVb</td>
<td>4-Cl</td>
<td>70</td>
<td>198-200</td>
<td>ethanol (95%)</td>
<td>52.47</td>
<td>4.93</td>
<td>2.45</td>
<td>52.43</td>
<td>5.0</td>
<td>2.44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIVc</td>
<td>3,4-Cl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>69</td>
<td>202-204</td>
<td>ethanol (95%)</td>
<td>50.53</td>
<td>4.41</td>
<td>2.36</td>
<td>50.31</td>
<td>4.14</td>
<td>2.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIVd</td>
<td>4-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>78</td>
<td>222-224</td>
<td>methanol/ether</td>
<td>66.93</td>
<td>7.07</td>
<td>2.89</td>
<td>67.04</td>
<td>7.13</td>
<td>2.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIVe</td>
<td>4-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>77</td>
<td>189-191</td>
<td>ethanol/acetone</td>
<td>57.55</td>
<td>6.08</td>
<td>2.49</td>
<td>57.55</td>
<td>6.18</td>
<td>2.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIVf</td>
<td>4-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>80</td>
<td>200-202</td>
<td>ethanol (95%)</td>
<td>61.02</td>
<td>6.45</td>
<td>2.64</td>
<td>60.94</td>
<td>6.48</td>
<td>2.67</td>
<td></td>
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</tbody>
</table>

<sup>(i)</sup> XIVa-e: chlorides

XIVf: iodide
4.3.13.0 Synthesis of 1,4-bis(3-oxo-3-phenylpropyl) piperazine hydrochloride (XVI)

A mixture of acetophenone (0.06 mole), paraformaldehyde (0.06 mol) and piperazine dihydrochloride (0.01 mol) in 95% ethanol (100ml), acidified with four drops of concentrated hydrochloric acid was heated under reflux for 17 hours. The precipitated solid was filtered while the solution was hot followed by recrystallization from 70% ethanol. The yield and melting point of XVI were 41% and 198(dec) °C respectively.

Elemental analysis (%): C  H  N
Calculated (C<sub>22</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>3</sub>·1/2 H<sub>2</sub>O): 61.16  6.53  6.53
Found: 61.40  6.57  6.41

The spectroscopic data of XVI are as follows.

<sup>1</sup>H NMR (60 MHz, CDCl<sub>3</sub>), δ: 3.0-4.2 (m, 16H, CH<sub>2</sub>), 7.0-8.2 (m, 10H, C<sub>6</sub>H<sub>5</sub>).

IR (KBr disc): 3080, 3020 (m, phenyl C-H str), 2980, 2840 (s, aliphatic C-H str), 2720, 2680, 2500, 2400 (w, C-N+ str), 1670 (s, C=O str) cm<sup>-1</sup>.

4.3.14.0 General procedure for the synthesis 1,4-bis(5-aryl-3-oxo-4-pentenyl) piperazine dihydrochlorides (series 9, XVII a-c, Table 4.8)

A suspension of the appropriate styryl ketone (0.04 mol), paraformaldehyde (0.04 mol) and piperazine dihydrochloride (0.01 mol) in 95% ethanol (100ml) was heated under reflux for 2 (XVIIb), 4(XVIIa) and 8(XVIIc) hours. In all cases, the compound precipitated at the end of the reaction. The precipitates were filtered, washed with hot 95% ethanol and recrystallized 5-6 times from an appropriate solvent. The physical data of the compounds are given in table 4.8.

The spectroscopic data of a representative compound XVIIa are as follows.

<sup>1</sup>H NMR (60 MHz, CDCl<sub>3</sub>), δ: 2.7-4.3 (m, 16H, CH<sub>2</sub>), 6.4-7.8 (m, 14H, C<sub>6</sub>H<sub>5</sub> and olefinic H).

IR (nujol): 2200-2650 (s and br, C-N+ str), 1670 (s, C=O str) cm<sup>-1</sup>.
Table 4.8: Physical data of 1,4-bis(5-aryl-3-oxo-4-pentenyl) piperazine dihydrochlorides

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Yield (%)</th>
<th>M.P. (°C)</th>
<th>Recrystallization Solvent</th>
<th>Elemental Analysis (%)</th>
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<tbody>
<tr>
<td>XVIIa</td>
<td>H</td>
<td>61</td>
<td>234 dec.</td>
<td>60% ethanol</td>
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<tr>
<td>XVIIb</td>
<td>3,4-Cl₂</td>
<td>65</td>
<td>199 dec.</td>
<td>80% ethanol</td>
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</tr>
<tr>
<td>XVIIc</td>
<td>4-OCH₃</td>
<td>59</td>
<td>220 dec.</td>
<td>80% ethanol</td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
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<th>Found</th>
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<tbody>
<tr>
<td>C</td>
<td>H</td>
<td>N</td>
</tr>
<tr>
<td>C</td>
<td>H</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calculated</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>H</td>
</tr>
<tr>
<td>65.68</td>
<td>6.79</td>
</tr>
<tr>
<td>50.92</td>
<td>4.60</td>
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<tr>
<td>62.81</td>
<td>6.78</td>
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<tbody>
<tr>
<td>C</td>
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<td>65.38</td>
<td>7.10</td>
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<tr>
<td>51.17</td>
<td>4.59</td>
</tr>
<tr>
<td>63.03</td>
<td>6.50</td>
</tr>
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</table>
4.4.0.0 Synthesis of mono and bis Mannich bases of 4-hydroxy benzoyl hydrazine

4.4.1.0 Synthesis of a Mannich reagent, N,N-dimethyl(methylene)ammonium chloride (XVIII)

N,N-Dimethyl(methylene)ammonium chloride was prepared by a literature procedure (Bohme and Hartke, 1960). A solution of acetyl chloride (0.12 mol) in dry methylene chloride (50ml) was slowly added with stirring to an ice cold solution of N,N,N',N'-tetramethyldiaminomethane (0.10 mol) in dry methylene chloride (100ml). The precipitated colorless solid was filtered, washed quickly with dry methylene chloride, dried and stored in vacuo over calcium chloride. The Mannich reagent was used as such without any further purification.

4.4.2.0 Synthesis of methyl 3-dimethylaminomethyl-4-hydroxy benzoate hydrochloride (XIX)

To a suspension of potassium carbonate (0.015 mol) and methyl 4-hydroxy benzoate (0.01 mol) in dry dichloromethane (100ml), N,N-dimethyl(methylene)ammonium chloride (0.01 mol) was added and the suspension was stirred for 24 hours. The suspension was filtered, evaporated to give an oil that was purified by column chromatography on silica gel using dichloromethane:methanol (9:1) as an eluant. The low melting solid so obtained was dissolved in acetone and hydrogen chloride gas was passed through it. The precipitated solid was filtered, dried and recrystallized from ether/methanol. The yield and melting point of XIX were 42% and 168-169 °C respectively.

Elemental analysis (%) : C H N
Calculated (C<sub>11</sub>H<sub>16</sub>ClNO<sub>3</sub>) : 53.76 6.56 5.70
Found : 53.72 6.48 5.48

The spectroscopic data of XIX are as follows.
1H NMR (60 MHz, DMSO), δ: 2.4 [s, 6H, CH<sub>2</sub>N(CH<sub>3</sub>)<sub>2</sub>], 3.7 [s, 2H, CH<sub>2</sub>N(CH<sub>3</sub>)<sub>2</sub>], 3.9 [s, 3H, -COOC<sub>2</sub>H<sub>5</sub>], 6.7-6.9 [d, 1H, aryl], 7.6-8.0 [d, 2H, aryl].
IR (film): 2680, 2500 (C-N+ str), 1730 (C=O str) cm<sup>-1</sup>.
4.4.2.1 Synthesis of 3-dimethylamino-4-hydroxy benzoic hydrazide hydrochloride (XX)

An aqueous solution of methyl 3-dimethylaminomethyl-4-hydroxy benzoate hydrochloride (0.01 mole) was neutralized by adding an aqueous solution of sodium bicarbonate (10% w/v) followed by extraction with ether (5 X 25 ml). The ether extract was dried over anhydrous magnesium sulfate and the solvent was evaporated in vacuo to give an oil which was suspended in 25 ml of hydrazine hydrate (99%). Sufficient absolute alcohol (5ml) was added to make the solution clear. The solution was heated under reflux for 45 minutes and the solvent was evaporated in vacuo. The oil so obtained was triturated with petroleum ether (bp 30-60 °C) until the oil solidified. The colorless solid was dried in vacuo and then recrystallized from ethanol/hexane. Finally, hydrogen chloride was passed into the solution of the base in ethanol to form the corresponding hydrochloride salt. The yield and melting point of XX were 99 and 156-158 °C respectively.

Elemental analysis (%)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated (C₁₀H₁₅N₃O₂)</td>
<td>57.39</td>
<td>7.23</td>
<td>20.08</td>
</tr>
<tr>
<td>Found</td>
<td>57.54</td>
<td>7.20</td>
<td>20.11</td>
</tr>
</tbody>
</table>

The spectroscopic data of XX are as follows.

¹H NMR (60 MHz, DMSO), δ : 2.2 [s, 6H, +NH(CH₃)₂], 3.5 [s, 2H, CH₂N(CH₃)₂], 6.7-6.8 [d, 1H, aryl], 7.4-8.4 [m, 2H, aryl].

IR (film): 2740, 2680 (C-N+ str) cm⁻¹.

4.4.3.0 Synthesis of methyl 3,5-bis(dimethylaminomethyl)-4-hydroxy benzoate dihydrochloride (XXI)

A suspension of N,N-dimethyl(methylene)ammonium chloride (0.01 mol), methyl 4-hydroxy benzoate (0.04 mol) and potassium carbonate (0.01 mol) in dry acetonitrile (100 ml) was heated under reflux for 24 hours. Methanol (25ml) was added to dissolve the precipitates and the suspension was filtered to remove potassium carbonate. The solution was concentrated followed by the addition of acetone (50 ml). The white precipitates were filtered, dried and recrystallized from methanol/ether. The yield and melting point of XXI were 26 % and 242-244 °C respectively.
Elemental analysis (%)  C    H    N
Calculated (C₁₄H₂₄Cl₂N₂O₃) : 49.56  7.13  8.26
Found : 49.44  6.93  8.07

The spectroscopic data of XXI are as follows.

¹H NMR (60 MHz, D₂O), δ: 2.8 [s, 12H, -CH₂N(CH₃)₂], 3.9 [s, 3H, -COOCH₃], 4.4 [s, 4H, 3.5 -CH₂N(CH₃)₂], 8.1 [s, 2H, aryl].

IR (film): 2570, 2530, 2480 (w, C-N+ str), 1740 (C=O str) cm⁻¹.

4.4.3.1 Synthesis of 3,5-bis(dimethylaminomethyl)-4-hydroxy benzoic hydrazide hydrochloride (XXII)

An aqueous solution of methyl 3,5-bis(dimethylaminomethyl)-4-hydroxy benzoate dihydrochloride (0.01 mole, 10ml) was neutralized by adding an aqueous solution of sodium bicarbonate (10% w/v) followed by extraction with ether (5 x 25ml). The ethereal extract was dried over anhydrous magnesium sulfate and the solvent was evaporated in vacuo to give an oil which was suspended in 25 ml of hydrazine hydrate (99%). Sufficient absolute alcohol (5ml) was added to make the solution clear and the solution was then heated under reflux for 3.3 hours. The reaction mixture was evaporated in vacuo to give a viscous syrupy colorless liquid which, after repeated trituration with petroleum ether (boiling point: 30-60 °C) yielded a low melting solid which was dissolved in ethanol and hydrogen chloride was passed through it. The colorless solid obtained was recrystallized from methanol to give the desired compound in low yield.

The yield and melting point of XXII were 20% and 223-225 °C respectively.

Elemental analysis (%)  C    H    N
Calculated (C₁₃H₂₂N₄O₂) : 58.62  8.33  21.04
Found : 58.91  8.51  20.97

The spectroscopic data of XXII are as follows.

¹H NMR (60 MHz, DMSO), δ: 2.8 [s, 12H, -CH₂N(CH₃)₂], 4.5 [s, 4H, CH₂N(CH₃)₂], 8.1 [s, 2H, aryl].

IR (film): 2740, 2680 (C-N+ str) cm⁻¹.
4.4.4.0 Synthesis of 1-(4-methoxyphenyl)-3-dimethylamino-2-dimethylaminomethyl-1-propanone dihydrochloride (XXIII)

A mixture of 4-methoxyacetophenone (0.02 mol), aqueous solutions of formaldehyde (37% w/v, 0.06 mol) and dimethylamine (25% w/v, 0.06 mol) and ethanol (25 ml) was heated under reflux for 3 hours. The solvent was removed in vacuo and the mixture was extracted with ether (3 x 25 ml). The combined ethereal extracts were washed with water, dried and then the solvent was removed under reduced pressure to give the Mannich base, which was dissolved in acetone and treated with hydrogen chloride. The colorless solid which precipitated was removed by filtration, dried and recrystallized from methanol to give the compound in 25% yield. Melting point 172-174 °C (literature value 174-176 °C, Dimmock et al., 1984a). The spectroscopic data of XXIII are as follows.

$^1$H NMR (60 MHz, D$_2$O), δ: 2.95 [s, 12H, 2N(CH$_3$)$_2$], 3.2-3.9 [m, 5H, (CH$_2$)$_2$], 3.95 (s, 3H, OCH$_3$) and 7.2-8.1 (m, 4H, C$_6$H$_4$).

IR (film): 2400-2700 (C-N+ str), 1680 (C=O str) cm$^{-1}$.

4.5.0.0 Attempted synthesis of series 12 and 13

A mixture of mono (XX) or bis (XXII) Mannich base hydrazide (0.001 mol), compound dissolved in ethanol (25 ml) and acetic acid (0.1 ml) was stirred at room temperature. No reaction took place. The reaction mixture was heated under reflux for 5 hours. A complex mixture consisting of at least five compounds as shown by TLC was obtained. No attempts were made to separate them. Repetition of the reaction in methanol with or without acetic acid did not lead to the formation of the desired compound.
4.6.0.0 General procedure for the synthesis of N-arylidene-3-dimethylaminomethyl-4-hydroxybenzoic hydrazide hydrochloride (series 13, XXIV)

A solution of 3-dimethylaminomethyl-4-hydroxybenzoic hydrazide hydrochloride (0.002 mol) and an appropriate aryl aldehyde (0.002 mol) in methanol was heated under reflux for 3 (XXIVA) and 9 (XXIVB) hours. The reaction mixture was evaporated in vacuo followed by the recrystallization of the residue from ethyl acetate/ethanol (XXIVA) and acetone/ethanol (XXIVB). The physical data for both compounds are given below.

N-(4-Chlorophenylmethylene)-3-dimethylaminomethyl-4-hydroxybenzoic hydrazide hydrochloride (XXIVA)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.P.</td>
<td>190-192°C</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>71%</td>
</tr>
<tr>
<td>Elemental analysis (%)</td>
<td>C: 55.45, H: 5.20, N: 11.41</td>
</tr>
<tr>
<td>Calculated (C_{17}H_{19}Cl_{2}N_{3}O_{2})</td>
<td>55.45, 5.20, 11.41</td>
</tr>
<tr>
<td>Found</td>
<td>55.27, 5.04, 11.27</td>
</tr>
</tbody>
</table>

¹H NMR (60 MHz, DMSO), δ: 2.8 [s, 6H, -CH₂N(CH₃)₂], 3.4 [s, 2H, CH₂N(CH₃)₂], 4.3 [s, 1H, -CH==NNH], 6.9-8.2 [m, 7H, aryl].

IR (film): 3200-3700 (s, br, OH str, intramolecularly hydrogen bonded), 2700 (C-N+str), 1680 (m, C=N str) cm⁻¹.

N-(4-Methoxyphenyl methylene)-3-dimethylaminomethyl-4-hydroxy benzoic hydrazide hydrochloride (XXIVB)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.P.:</td>
<td>166-168 °C</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>61%</td>
</tr>
<tr>
<td>Elemental analysis (%)</td>
<td>C: 57.96, H: 6.21, N: 11.27</td>
</tr>
<tr>
<td>Calculated (C_{18}H_{22}ClN_{3}O_{3}, 1/2 H₂O)</td>
<td>57.96, 6.21, 11.27</td>
</tr>
<tr>
<td>Found</td>
<td>57.84, 6.02, 11.23</td>
</tr>
</tbody>
</table>

¹H NMR (60 MHz, DMSO), δ: 2.8 [s, 6H, -CH₂N(CH₃)₂], 3.4 [s, 2H, CH₂N(CH₃)₂], 3.8 [s, 3H, OCH₃], 4.3 [s, 1H, -CH==NNH], 6.7-8.2 [m, 7H, aryl].

IR (film): 3200-3600 (s, br, OH str, intramolecularly hydrogen bonded), 2700 (C-N+str), 1680 (m, C=N str) cm⁻¹.
4.7.0.0 In vitro cytotoxicity screening against mouse P388 D1 leukemia cells

The cytotoxic evaluation of compounds (Phillips et al., 1989) against P388 D1 leukemia cell line is briefly described below. The P388 D1 leukemia cells were cultivated in Fischer’s medium containing 10% heat inactivated horse serum at 37 °C in a humidified 5% carbon dioxide-air atmosphere. The cell suspension containing 10^5 viable cells/ml was treated with five different concentrations of the test compound namely 50, 10, 2, 0.5, and 0.125 μM, melphalan (positive control) dissolved in either ethanol or dimethylsulfoxide and the solvent (negative control) for 48 hours at 37 °C. The number of cells/ml was determined using a model ZF Coulter counter. All the tests were performed thrice. The percentage survival of the cells was calculated as follows.

\[
\% \text{ survival} = \frac{(T_{48} - T_0)}{(C_{48} - C_0)} \times 100
\]

where \(T_{48}\) is the mean of the number of living cells/ml for each concentration of the test compound at the end of 48 hours, \(T_0\) is the mean of the numbers of living cells/ml at time zero, \(C_{48}\) is the mean for the living cells in the control wells after 48 hours of incubation and \(C_0\) is the mean for the number of cells in control wells at time zero (usually \(C_0 = T_0 = 10^5\) cells/ml). The IC_{50} (50% inhibitory concentration) values were calculated graphically and are the concentrations of the compound required to inhibit the growth of the cells to 50% of the controls.

4.8.0.0. In vitro cytotoxicity screening against mouse L1210/0 leukemic cells and human T lymphocytes (Molt 4/C8 and CEM/0)

The screening was undertaken using a procedure (De Clercq et al., 1981) as outlined below. The mycoplasma-free mouse L1210/0 leukemic cells and human T lymphocytes (Molt 4/C8 & CEM/0) were cultivated in Eagle’s essential medium, supplemented with 10% (v/v) inactivated fetal calf serum, 2 mM glutamine, sodium bicarbonate (0.075% w/v) and 25 units/ml of nystatin at 37 °C in a humidified 5% carbon dioxide-air atmosphere. The cell suspension containing 5 x 10^4 cells was incubated with different concentrations of the test compound, melphalan (the standard drug) and the solvent (control) for 48 hours at 37°C in a humidified carbon dioxide controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter
counter and the number of dead cells was evaluated by staining with trypan blue. The IC$_{50}$ (50% inhibitory concentration) defined as the concentration of the compound that reduced the number of living cells by 50% was determined graphically.

4.9.0.0 In vitro cytotoxicity screening against human tumor cell lines (NCI in vitro screen)

Briefly, the NCI screening procedure (Monks et al., 1991) involves plating the cells (5000-40,000 cells per well based on the growth characteristics) for 24 hours, followed by a 48 hours continuous drug exposure over a broad concentration range (at least five 10 fold dilutions, i.e., $10^{-4}$ - $10^{-8}$ M) against a panel of ~60 cell lines comprising eight tumor types - leukemia, melanoma, non small cell lung, small cell lung, colon, central nervous system, ovarian and renal cancer in a 5% carbon dioxide atmosphere and 100% humidity. The cytotoxicity was assessed with sulphorhodamine B (SRB) protein assay (Rubenstein et al., 1990; Skehan et al., 1990). Three evaluation parameters were established, namely (a) the drug concentration which inhibits growth by 50% (GI$_{50}$), (b) the drug concentration which inhibits growth by 100% (total growth inhibition or TGI) and (c) the drug concentration which yields a 50% reduction in the amount of proteins at the end of the experiment in comparison with the amount at the start of the test (LC$_{50}$) indicating a net loss of cells after the drug treatment. The results were presented in the form of mean graphs (Paull et al., 1989) in which the drug effects were calculated as positive or negative deviations from the average sensitivity of the entire cell line panel. This generates a characteristic cell line “fingerprint” for each individual test compound.

4.10.0.0 Antitumor activity of XIIId and XIVa against P388 murine leukemia and human tumor xenografts in vivo

On the basis of the in vitro results, two compounds, XIIId and XIVa, were selected for in vivo antitumor assays. Compound XIIId was tested against P388 murine leukemia, SC NCI-H522 non-small cell lung tumor, SW-620 colon tumor, LOX 1MV1 melanoma and Colo 205 colon tumor xenografts in athymic mice. Compound XIVa was
tested against P388 murine leukemia in CD2F1 mice and the KM1 colon tumor, Colo 205 colon, CAK1-1 renal tumor xenografts in athymic mice.

The experiments involving P388 murine leukemia were initiated by implanting $10^6$ leukemic cells intraperitoneally into CDF1 mice. Each compound-treated group consisted of 6 mice and control groups were composed of 10 mice. The experiment was terminated on day 30. Mice alive at the end of experiment were autopsied and judged to be cured if no signs of disease were visible. Each compound in the experiment was evaluated at three dose levels for each treatment schedule used. The treatment schedules used in this experiment were either single injections on day 1 or five consecutive daily injections beginning on day 1. Mice were observed daily, and the antitumor activity of each tested material was determined based upon the median extension of lifespan in treated mice (T) compared to control mice (C) expressed as % T/C. A compound was considered active if it produced a T/C > 125%. The compound-treated mice dying prior to day 5 were presumed to have died from treatment-associated toxicity and were excluded from calculations of % T/C. No results were used in which deaths were attributed to material toxicity exceeding 17% in the treated group.

The experiments involving the determination of antitumor activity of the test compounds against murine and human tumor xenografts in athymic mice is briefly explained below. Murine or human tumor fragments (30mg), established previously from the respective tumors, through in vivo passages in similar mice are implanted subcutaneously into the axillary region of pathogen free athymic (nude) mice weighing 20g. The tumor is allowed to grow and the animal weights and tumor sizes are recorded daily according to the NCI in vivo cancer model protocols (Dykes et al., 1992). Briefly, caliper measurements of tumor dimensions were recorded in millimeters and tumor net weights were obtained using the formula for prolate ellipsoids i.e., $(L \times W^2)/2$ where $L$ is the longer of two measurements. Toxic doses were determined by prior studies by giving control mice a series of three consecutive daily injections of different doses of compound and recording deaths, survivors and white blood cell counts at the end of one week after the initial dose.
There were six mice in each treatment and control group. Treatment with compounds is started on a day when tumors are reliably measurable. Treatment consists of three intraperitoneal injections of varying concentrations of compounds, used from fresh stocks prepared in saline and Tween 80 (0.05%). Control animals (negative control) received saline and Tween 80 (0.05%) or melphalan (positive control). The antitumor activity of each tested compound is determined on the basis of the optimum % T treated/C control expressed in days. A T/C % greater than 40 is considered inactive.

4.11.0.0 Antifungal evaluation of Mannich bases in vitro

The antifungal activity of selected Mannich bases against C. albicans strains 3153A and B311 was determined according to the procedures of Espinet-Ingroff et al., (1995).

**Determination of minimum inhibitory and minimum fungicidal concentration**

The minimum inhibitory concentrations (MICs) of various compounds for the yeasts were determined using a broth dilution technique in RPMI 1640 (except in the case of Cryptococcus neoformans, for which PYG medium was used) according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 1992). Briefly, fresh conidia were collected from A. fumigatus and A. niger, and resuspended in PYG medium at a density of 2 x 10^4 conidia per ml. Two times the required concentrations of the drugs were prepared in the PYG medium (0.5ml) by serial dilution in sterile 6-ml polystyrene tubes and inoculated with an equal volume (0.05 ml) of the conidial suspension. The tubes were incubated at 35 °C for 48 hours and scored for visible growth after vortexing the tubes gently, or scraping the walls of the tube followed by vortexing. The MIC was defined as the lowest concentration of the drug in which no visible growth occurred.

To determine the minimum fungicidal concentrations (MFCs), the entire cell suspension from tubes that contained the drugs equal to and greater than the MIC was spread on PYG agar (0.01 ml per plate) and incubated at 30 °C for 2 days for growth. The concentration of the drug that completely inhibited growth was considered as the MFC.
4.12.0.0 *In vitro* evaluation of mode of cell death induced by XIIIId

4.12.1.0 Effect of XIIIId and melphalan on human Jurkat leukemia T cells *in vitro*

The tissue culture flasks containing a suspension of \( \sim 10^6 \) cells/ml in 10 ml RPMI medium were treated with different concentrations of XIIIId (1.0, 2.0, 4.0, 6.0 and 8.0 \( \mu \)M). Similarly the cells were treated with different concentrations of melphalan (0.125, 0.5, 2.0, 10.0 and 50.0 \( \mu \)M). A flask containing \( \sim 10^6 \) cells/ml and 50 \( \mu \)l of DMSO was treated as a control. All the flasks were incubated for 48 hours at 37 °C in a 5% humidified carbon dioxide atmosphere. After 48 hours, cells were counted using a haemocytometer in the following manner. An aliquot of the cell suspension (25\( \mu \)l) was mixed with trypan blue dye solution (0.04%, 25\( \mu \)l) for a minute. Then 10\( \mu \)l of this suspension was placed on the hemocytometer. Live (colorless) and dead (blue) cells were counted in all the 64 squares of the hemocytometer. The percentage of growth inhibition was calculated as follows: (C-T/C) 100, where C is the mean cell number in the control and T is the mean cell number in each treatment. The concentration needed to reduce the growth of cells in culture to 50% of control values (IC\(_{50}\)) was determined for XIIIId and melphalan from the graph drawn between the percentage cell growth inhibition as a function of dose. The experiment was performed in triplicate.

4.12.2.0 Induction of apoptosis by XIIIId and melphalan in human Jurkat leukemia T cells

Initially, the cell death in human Jurkat leukemia T cells caused by three different concentrations of XIIIId and melphalan was investigated. At the end of 48 hours, no morphological evidence supporting apoptosis was found. So the effect of one concentration of XIIIId and melphalan at various time intervals namely 5, 8, 11, 14, and 17 hours was investigated. The procedure followed is given below. Tissue culture flasks containing \( \sim 10^6 \) cells/ml in 10ml RPMI medium, an appropriate concentration of the test compound or melphalan or DMSO (control) were incubated at 37°C in a 5% humidified carbon dioxide atmosphere. At the end of 5, 8, 11, 14 and 17 hours, a 25\( \mu \)l cell suspension from each flask was mixed with 1\( \mu \)l of dye mix in a glass tube. Of this mixture, 10\( \mu \)l was
placed in a clean haemocytometer covered with a cover slip and 200 cells were then counted with a 100X dry objective using epiillumination and a filter combination suitable for observing fluorescein. The number of normal versus apoptotic nuclei were recorded. The percentage of apoptotic cells (apoptotic index) was calculated as follows.

\[
\% \text{ Apoptotic cells} = \frac{\text{total number of cells with apoptotic nuclei}}{\text{total number of cells counted}} \times 100
\]

The normal as well as apoptotic cells were then photographed using a Polaroid camera.

4.12.2.1 Isolation of DNA

Treated or untreated flask cell suspensions (0.5 ml) were placed in a 1.5 ml microcentrifuge tube labeled B and centrifuged for 10 min at 200Xg (1000rpm in IE CCRU-5000) and 4°C. The culture supernatant was transferred carefully to a 1.5 ml microcentrifuge tube labeled S and set aside. 0.5 ml TTE (appendix II) solution was added to cell pellet in tube B and vortexed vigorously followed by microcentrifuging for 10min at 13000 rpm and 4°C. The supernatant was then carefully transferred to a tube labeled T. 0.5ml TTE solution was added to the very small pellet remaining in B tube followed by the addition of 0.1 ml of ice cold 5M sodium chloride to each tube labeled as B, S and T. Each tube was vortexed and ice cold isopropanol (0.7 ml) was added. Again, these were vortexed vigorously and placed at -20 °C overnight to precipitate the DNA. The suspensions were microcentrifuged for 10min at 13000 g and 4°C. The supernatant was removed carefully by rapidly inverting the tubes. Each tube was then half filled with ice cold 70% ethanol and microcentrifuged for 10min at 13000g. The supernatant was removed carefully followed by placing all the tubes in an inverted position for 30min over adsorbent paper to allow as much remaining supernatant as possible to drain away. After 30 min, each tube was placed upright and allowed to air dry for 3 to 4 hours. Finally, 20 μl TE buffer (appendix II) was added to the DNA pellet and incubated for 72 hours at 37 °C.

4.12.2.2 Gel electrophoresis of the fragmented DNA

Electrophoresis of the fragmented DNA was carried out in 1% and 1.5% agarose gels. Before electrophoresis, a loading buffer (appendix II) was added to each
sample in a 1:5 ratio. The samples were then heated to 65 °C for 10 min in a water bath. Solutions of size markers (Lambda BSTE II and Lambda Hind III) in glycerol were applied. After electrophoresis, DNA was visualized by soaking the gel in TBE (appendix II) containing 1μg/ml ethidium bromide and destaining briefly in this buffer.

4.13.0.0 Stability studies

The procedure for the examination of the stability of representative compounds of series II-VII using thin layer chromatography and 1H NMR spectroscopy is presented below.

Stability studies using TLC

Solutions of IVc,e, VIIc,e, IXc,e, and XIIIc,e in a mixture of phosphate buffer saline (PBS), pH 7.4 and dimethylsulfoxide (55:45) were incubated for 48 hours at 37 °C. At the end of 48 hours, solutions were extracted with chloroform. The chloroform extract was spotted on silica gel plates and the plates were developed using a chloroform: methanol (7:3) solvent system and examined under U.V. light and exposure to iodine.

Stability studies using 1H NMR spectroscopy

1H NMR spectroscopy of representative compounds was undertaken using 1mM solutions of IVa, VIIa, VIIIa, IXa and XIVa in deuterated PBS, pD 7.4 (PBS-d). The lack of aqueous solubility of the bis Mannich base, XIIIa, necessitated the use of a mixture of PBS-d and deuterated dimethylsulfoxide (3:1). A solution of PBS-d was prepared as follows. Both sodium chloride and sodium phosphate were dried overnight at 110 °C. The pH of a solution of sodium chloride (0.9 g) and sodium phosphate (2.12 g) in deuterated water (100 ml) was adjusted using a glass electrode calibrated with aqueous buffers to 7.0 with deuterium chloride. The pD (negative logarithm of deuterium ion activity) is now 7.4, since it has been shown that pD = pH + 0.4 (Glasoe and Long, 1960). Spectra were recorded as rapidly as possible after addition of the solvent to the compounds (t0) and after 48 hours incubation at 37 °C (t48).
5.0.0.0 RESULTS AND DISCUSSION

5.1.0.0 Synthesis of (E)-4-aryl-3-buten-2-ones (1b-k, table 4.1)

These conjugated styryl ketones, 1b-k, table 4.1 were synthesized by a Claisen-Schmidt condensation reaction as exemplified by the reaction of benzaldehyde with acetone to form 4-phenyl-3-buten-2-one (Ia).

\[
\text{PhCHO} + \text{CH}_3\text{COCH}_3 \xrightarrow{10\% \text{ NaOH}} \text{PhC} = \text{CHCOCH}_3 \hspace{1cm} (\text{Ia})
\]

This reaction is a variation of the aldol condensation and in general involves the reaction of an aromatic aldehyde having no α hydrogen atom with an aliphatic ketone in the presence of aqueous alkali to form a β-hydroxy ketone which usually undergoes dehydration to form an α,β-unsaturated ketone. Since the aldehydes used in this study had no α hydrogen atoms, they cannot undergo the aldol condensation. The formation of dibenzalacetone (51)

\[
\text{2PhCHO} + \text{CH}_3\text{COCH}_3 \xrightarrow{10\% \text{ NaOH}} \text{PhC} = \text{CHCOCH}_3 \text{Ph} \hspace{1cm} (51)
\]

was prevented by using excess of acetone and a low reaction temperature (<10 °C). The possibility of a Cannizzaro reaction with the aromatic aldehyde was kept low by using a dilute solution of sodium hydroxide.
The self condensation of acetone under the reaction conditions was too slow to be a significant competitor. The Claisen-Schmidt condensation involves the nucleophilic addition of a carbanion to a carbonyl group and can be catalyzed by acid or base. The generally accepted mechanism for the base catalyzed condensation is illustrated in figure 5.1, using the example of benzaldehyde and acetone.

The base, usually a hydroxide ion, abstracts an acidic $\alpha$ hydrogen atom from the ketone (acetone) to form a carbanion which is in resonance with the enolate anion. The enolate ion attacks the electropositive carbon of the carbonyl compound i.e.

\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{CH}_2(e) \\
\text{O} & \quad \text{H}_3\text{C} \quad \text{CH}_2
\end{align*}
\]

benzaldehyde, to form an alkoxide which abstracts a hydrogen atom from water to form a $\beta$-hydroxyketone and regenerates the hydroxide ion catalyst. The $\beta$-hydroxy ketone so formed is very susceptible to dehydration because the intermediate produced is more stable due to the presence of a conjugated $\alpha,\beta$-unsaturated carbonyl group. Thus the $\beta$-hydroxy ketone undergoes dehydration to form the desired $\alpha,\beta$-unsaturated ketone. All the steps are reversible. The hydroxide ion is not a strong enough base to convert substantially all of a ketone molecule into the corresponding enolate ion. The equilibrium lies well to the left. Nevertheless enough enolate ion is present for the reaction to proceed. In order to have a favorable equilibrium, the acidity of the carbonyl compound should be higher than the conjugate acid of the corresponding base.

The ambivalent nature of the enolate makes possible the occurrence of C- as well as O-alkylation. Since oxygen is more electronegative than carbon, the negative charge is concentrated more on the oxygen than carbon atoms. But in polar solvents, enolate ions do not exist in the free form and hence they cannot function as effective nucleophiles. They form ion pairs with solvent molecules. Therefore C-alkylation predominates.
But in aprotic solvents, where enolate ions are free, O-alkylation predominates.

1. Enolization

\[
\text{CH}_3\text{COCH}_3 + \text{OH}^- \rightleftharpoons \text{CH}_3\text{COCH}_2^- + \text{H}_2\text{O}
\]

2. Nucleophilic addition

3. Proton transfer

4. Dehydration

**Figure 5.1:** Mechanism of the base catalyzed Claisen-Schmidt condensation.
The aldol reaction is stereoselective. The reaction leads to the formation of the thermodynamically stable E isomer. The exclusive formation of the E isomer can be explained by observing the Newman’s projection formulae of the β-hydroxy ketones corresponding to the Z and E isomers (figure 5.2). In the Newman’s projection of the β-hydroxy ketone leading to the formation of the Z isomer, two bulky groups namely the phenyl and acetyl functions are closer relative to that of the E isomer where such groups are as far apart as possible. Therefore, due to steric reasons, the energy of (a) is higher than that of (b). Hence the E isomer was formed predominately.

![Figure 5.2: Stereochemical course of the Claisen-Schmidt condensation.](image_url)
The existence of the E configuration in all of the compounds Ib-k was confirmed by the determination of the coupling constant (J) which was 18Hz.

In the IR spectra of Ie, three strong intensity peaks were observed at 1635, 1685 and 1710 cm\(^{-1}\). As suggested by Dimmock et al., (1968), the 1635 cm\(^{-1}\) absorption, which had several shoulders, was due to a combination of the olefinic double bond vibration and the C=C in-plane vibrations of the aromatic ring. The remaining two bands at 1685 and 1710 were due to the s-cis and s-trans rotational isomers respectively as shown below.

![Chemical structures](image)

**s-trans rotamer**

**s-cis rotamer**

### 5.1.1.0 Synthesis of (E)-1-(4-chlorophenyl)-4-methyl-1-penten-3-one (II)

As compared to acetone in which all the six hydrogens are chemically equivalent, 3-methyl-2-butanone (52) has two sets of acidic \(\alpha\) hydrogens, namely three hydrogens on C-1 and one hydrogen on C-3. Hence two kinds of carbanions are possible namely 52a and 52b formed by abstraction of the hydrogen atoms on C-1 and C-3 respectively. Therefore, 4-chlorobenzaldehyde can undergo condensation via either of the anions 52a and 52b to form the hydroxyketones 52c and 52d respectively. But compound 52c predominated as was evident by the formation of II. There may be two reasons. First, the hydrogens on C-1 are more acidic than the hydrogens on C-3 since in the latter, there are two electron releasing methyl groups. Second, the base may have difficulty in abstracting the C-3 hydrogen due to the presence of two methyl groups as compared to the C-1 hydrogens which are not sterically hindered. Thus, due to a combination of these two factors, the carbanion 52a predominates and II is formed exclusively. In general, the
carbanion component from an unsymmetrical ketone is that derived from the less highly alkylated carbon atom.

Figure 5.3: Synthesis of 1-(4-chlorophenyl)-4-methyl-1-penten-3-one (II).
5.1.2.0 Attempted synthesis of (E)-4-(4-carboxyphenyl)-3-buten-2-one (III)

The retrosynthetic analysis of III shown in figure 5.4 suggested the following synthetic routes for its synthesis. The direct reaction between 4-carboxy benzaldehyde and acetone under acidic and basic conditions was not successful. Under basic conditions, formation of the sodium salt of the acid might have prevented its reaction with acetone.

\[
\text{HOOC-} \quad \text{(III)} \quad \text{O}
\]

\(\text{(iii) } \text{CO}_2\)
\(\text{(ii) } \text{H}^+\)

\[
\text{BrMg}\quad \text{HOOC-} \quad \text{CHO} + \text{CH}_3\text{COCH}_3
\]

\[
\text{Mg, dry ether}\quad \text{isopropylene glycol, PTSA}\quad \text{O}
\]

\[
\text{Br-} \quad \text{Br-} \quad \text{CHO} + \text{CH}_3\text{COCH}_3
\]

Figure 5.4: Retrosynthetic analysis of 4-(4-carboxyphenyl)-3-buten-2-one.
Under acidic conditions, cross condensation might be slower compared to other side reactions e.g. self condensation of acetone. An alternative strategy as depicted above was attempted but it did not work.

5.2.0.0 Synthesis of Mannich bases

A variety of β-amino ketones were prepared by the Mannich reaction. According to the mechanism (figure 5.5) proposed for the acid catalyzed Mannich

![Chemical diagram](image_url)

**Figure 5.5:** Mechanism of the acid catalyzed Mannich reaction.
reaction (Cummings and Shelton, 1960), it is the free amine, not the salt that reacts, even in acid solution. The free amine reacts with formaldehyde in the presence of acid to give an adduct which eliminates water to form an electrophile and the acid also catalyses the conversion of acetone into its enolic tautomer. The enol then reacts with the electrophile and the resulting adduct tautomerizes to the amine salt.

5.2.1.0 Synthesis of the mono Mannich bases (IVa-h, IXa-i, X and XV)

The Mannich reaction, an imino analogue of the aldol condensation, was carried out by heating under reflux an active methylene compound (a variety of acetophenones, styryl ketones and phenols), a non-enolizable aldehyde (paraformaldehyde or trioxymethylene was used as a source of formaldehyde) and an amine hydrochloride (a variety of secondary amine hydrochlorides or a primary amine hydrochloride i.e., ethylamine hydrochloride) in a suitable solvent, catalyzed by hydrochloric acid or trifluoroacetic acid for a few hours to a couple of days.

The synthesis of mono Mannich bases of acetophenone (IV a-h) and styryl ketones (IXa-i) as shown in figure 5.6, posed a number of problems such as polyaminomethylation, deamination and deaminomethylation. First, all the acetophenones and styryl ketones except III have three $\alpha$ acidic hydrogens which conceptually can be replaced by three aminomethyl groups. Practically, it was observed that the reaction did not go beyond the second substitution stage probably due to steric reasons. For example, in the reaction of the unsubstituted acetophenone ($R = H; X = \cdot\cdot\cdot$) with dimethylamine hydrochloride and formaldehyde (figure 5.6), mono and bis aminomethylated compounds were isolated, the latter in low yield. The bis compound can undergo deamination to form the more stable conjugated compound (d) as well as the deaminomethylated compound (b) by a retro-Mannich reaction as explained earlier. Formation of the bis aminomethylated compound (c) and the acrylophenone (d) was prevented by carrying out the reaction in ethanol or isopropanol and stopping it before completion. Second, there are a few reports which state that the bulkier diethylamine need more stringent reaction conditions than dimethylamine or other less bulky amines. For example, it is reported that the typical Mannich reaction does not take place with ethyl methyl ketone, diethylamine
If $X = \cdots$, the compound is an acetophenone derivative, else if $X = -\text{CH}=\text{CH}$- then it is a styryl ketone derivative.

**Figure 5.6:** Synthesis of mono Mannich bases of acetophenones and styryl ketones and the possible side reactions.

and formaldehyde. On the other hand, formaldehyde and this amine do give normal products with acetone or acetophenone (Kermack and Muir, 1931). Here also, it was observed that reaction of acetophenones and styryl ketones with dimethylamine hydrochloride required 3-4 hours while use of diethylamine required 20 hours or more and yields were lower. The reaction was attempted in a variety of solvents including methanol, ethanol, isopropanol and acetonitrile. In methanol, the reaction was too slow while in acetonitrile a number of side products were formed. Therefore ethanol or isopropanol were the most suitable solvents. In all cases, the reaction did not go to completion. At the end of the reaction, the mixture contained not only unreacted ketone, and amine hydrochloride but also the desired Mannich bases and traces of the bis aminomethylated compounds. Diethylamine hydrochloride and Mannich bases often
cocrystallized, so fractional crystallization was employed to remove the amine hydrochloride. Attempts to isolate Mannich bases by basification, extraction with ether followed by passing hydrogen chloride gas failed because the Mannich base underwent elimination to give the acrylophenone. It was observed that in PBS, pH 7.4, 37 °C, the mono Mannich base (IVa) was completely decomposed by elimination in 15 minutes. This observation is supported by a report which states that due to steric reasons, some particular amines such as diethylamine are more suited to the deamination process (Miller and Smith, 1978). Attempts to purify the compounds on silica gel or alumina also failed as the compounds underwent elimination. This type of difficulty has also been reported by other workers (Katritzky et al., 1990).

A satisfactory elemental analyses for IVi could not be obtained even after subjecting the compound to repeated recrystallizations or other purification techniques as described above.

In the case of compound X, although polyaminomethylation could not take place, due to the presence of two methyl groups at the α position, long reaction times were required. Probably, the resulting carbanion might not be as stable as the ones generated from normethylated styryl ketones.

To synthesize XV, the reaction had to be carried out in acetic acid. In ethanol, the major product was β-aminopropiophenone (b) and XV was formed only in trace amounts.

5.2.2.0 Synthesis of bis Mannich bases (VIIa-j, XIIIa-j, XVI and XVIIa-c)

When a primary amine salt is reacted with formaldehyde and a ketone in a Mannich reaction, the first product is a secondary amine but this compound often reacts with more of the reagents to give a tertiary amine (figure 5.7). The synthesis of bis Mannich bases XIIIa-e, VIIa-j having ethylamine hydrochloride as the amine component required excess of ketones and long reaction hours in order to drive the reaction in the forward direction. The reaction never proceeded to completion. In contrast, the synthesis of XVI and XVIIa-c having piperazine dihydrochloride as the amine was faster. The reaction mixture in all the cases contained mono and bis Mannich bases, unreacted ketone
Figure 5.7: Synthesis of bis Mannich bases of acetophenones and styryl ketones and their quaternary salts.

and some unknown products. The reaction proceeded better when formaldehyde was added in portions instead of a one time addition. The yields were quite poor especially in the case of bis Mannich bases of styryl ketones. The reaction mixture often led to oils which were repeatedly triturated with petroleum ether as well as subjected to multiple recrystallizations. Column chromatography could not be carried out as the compounds decomposed on silica gel or alumina.
5.3.0.0 Synthesis of quaternary compounds (VI, VIIIa-i, XII and XIVa-f)

Quaternization of tertiary amines (the Menschutkin reaction, Bottini, 1972) as illustrated below, is an example of a nucleophilic substitution reaction in which the tertiary nitrogen atom with its lone pair of electrons acts as a nucleophile, attacking the methyl halide and displaces the halide ion.

\[ R_3N: + CH_3X \rightarrow R_3N\text{-CH}_3X^- \]

Since, Mannich bases have a tendency to undergo deamination under alkaline conditions,

\[ \text{O} \]
\[ \text{R} \]
\[ \text{X} \]
\[ \text{N} \]
\[ \text{HCl} \]
\[ \downarrow \text{(i) NaHCO}_3 \]
\[ \text{(ii) CH}_3\text{Br} \]
\[ \text{O} \]
\[ \text{R} \]
\[ \text{X} \]
\[ \text{N}^+ \text{Br}^- \]
\[ \text{deamination} \]
\[ \text{O} \]
\[ \text{R} \]
\[ \text{X} \]
\[ \text{N} \text{HBr} \]

**Figure 5.8:** Quaternization of mono Mannich bases and the deamination reaction.

The temperature and pH were maintained below 10 °C and 8 respectively in order to suppress the degradation reactions. Still, some decomposition was unavoidable. The quaternization of the Mannich bases IVa-e and IXa-e with methyl bromide as shown in figure 5.8 was not successful. This was probably due to the nitrogen atom in the
quaternary salts being highly strained. This strain is relieved with the elimination of diethyl methylammonium bromide and the formation of acrylophenone and dienones in the cases of acetophenone and styryl ketone quaternary salts respectively. The reaction is facilitated because the resulting product is more stable due to conjugation. Although some quaternary salts such as 53 are

\[
\text{\begin{tikzpicture}
  \node (a) at (0,0) {\text{O}};
  \node (b) at (1,0) {\text{\textbf{+}}};
  \node (c) at (2,0) {\text{\textbf{Br}}};
  \node (d) at (0,-1) {\text{R}};
  \node (e) at (1,-1) {\text{N}};
\end{tikzpicture}}
\]

reported, they required careful handling in isolation despite the fact that the leaving group is less bulky (Edwards et al., 1983). It was reported that in the recrystallization of compound 53, if heating was involved, a product contaminated with trimethylammonium iodide was formed. In the case of the reaction of the morpholino Mannich base with methyl iodide only starting materials were isolated and no quaternary derivative was formed (Edwards et al., 1983). In fact, quaternary salts of Mannich bases have been used as latent sources of \( \alpha,\beta \)-unsaturated carbonyl compounds required for condensation reactions. Thus for a base catalyzed condensation utilizing methyl vinyl ketone, it is better to employ the quaternized Mannich base from acetone, from which the unsaturated ketone is generated in situ by the action of base, than to use the free ketone, since this readily polymerizes. The best known example of this application is the Robinson ring extension used in building the ring system of steroids. The tertiary Mannich bases obtained from the hydrochlorides VIIa-j and XIIIa-i were also quaternized with methyl iodide or methyl bromide. The 4-chloro and 4-methyl derivatives (quaternary bromide salts) could not be purified despite repeated crystallizations and because of their tendency to undergo decomposition on silica gel. The corresponding iodide salts were synthesized successfully.
5.4.0.0 Attempted synthesis of azomethines (Series 13, 14)

The retrosynthetic analysis of the target compounds in series 13 and 14 revealed many reaction schemes some of which are shown in figure 5.9. Compound 50 was synthesized by a literature procedure (Dimmock et al., 1983b) as described in section 4.4.0.0. The synthesis of the compounds from series 13 and 14 was attempted by treating 4-hydroxy benzoic hydrazide with stoichiometric amounts of either dimethylamine and aqueous formaldehyde or the Mannich reagent (a) as shown in figure 5.10. A complex mixture of at least 5 compounds which could not be resolved, was obtained in each case. An alternative strategy was successfully adopted. Methyl 4-hydroxy benzoate was reacted with the Mannich reagent by a known procedure (Pochini et al., 1983) which led to the formation of the mono compound with one mole of Mannich reagent. The preparation of the bis compound required a little excess of two moles of Mannich reagent and an aprotic solvent i.e. acetonitrile, otherwise in dichloromethane, only minute quantities of the bis compound were formed. The reaction conditions employed were typical of solid liquid phase transfer catalysis with the phenol dissolved in an organic solvent (dichloromethane or toluene) and stirring at room temperature in the presence of the solid base potassium carbonate and a solid methylene imminium salt (a).

A mechanism (figure 5.10) was suggested according to which a soluble and reactive ion pair (b) might form, by gegenion exchange from potassium phenolate and methyleneimminium salts and which eventually collapsed to give the ortho substituted product XIX. The mildness of bases and low polarity of the solvent used probably played an important role in avoiding polyalkylation (Pochini et al., 1983). The reaction of the mono or bis Mannich base with hydrazine was quite unpredictable. The reaction did not progress at all in ethanolic or methanolic solutions of hydrazine.
Figure 5.9: Retrosynthetic analysis of series 13.
(CH₃)₂N — N(CH₃)₂ + CH₃COCl $\rightarrow$ (CH₃)₂N$^+$—CH₂ + CH₃CON(CH₃)₂

(a)

OH

\begin{align*}
\text{C}_6\text{H}_4\text{COOCH}_3 + \text{K}_2\text{CO}_3 & \xrightarrow{\text{CH}_2\text{Cl}_2} \text{C}_6\text{H}_4\text{COOCH}_3 + \text{KHCO}_3 \\
\text{COOCH}_3
\end{align*}

(b)

Figure 5.10: Synthesis of mono and bis phenolic Mannich bases.
It worked only when the mono or bis Mannich base was reacted with hydrazine (99%). The reaction of compounds in series 12 and 13 with compound 50, led to a complex mixture which could not be resolved. However the reaction with aryl aldehydes which are more reactive than ketones was successful. None of the compounds, either the intermediates namely esters, hydrazides or azomethines of aryl aldehydes were active (>50 μM) in the P388 or human tumor cytotoxicity screens. Stability studies on the phenolic Mannich bases indicated that these compounds did not form quinone methides as predicted earlier under simulated physiological conditions. This could be one of the reasons why these compounds were inactive.

5.5.0.0 In vitro cytotoxicity screening

All the compounds were examined against murine P388 lymphocytic leukemia cells. In addition, approximately three quarters of the Mannich bases and quaternary ammonium salts were evaluated against L1210 cells, human T lymphocytes and human tumor cell lines. These data for all screens are presented in Table 5.1 and 5.2. Since the desired azomethines could not be obtained, discussion will be primarily concerned with the compounds in the acetophenone and styryl ketone series. In the P388 screen all the compounds had IC\textsubscript{50} figures of less than 10μM except V, XI, and XIX-XXII, XXIVa,b. Calculations of the average IC\textsubscript{50} data in series 1, 3, 4, 5, 7 and 8 revealed that the order of potencies was 5>8>4>1>7>3 indicating that both the mono Mannich bases (1,5) and the quaternary ammonium compounds (4,8) were more cytotoxic than the bis analogues 3 and 7. Representative compounds were also screened against murine L1210 lymphocytic leukemia cells and the IC\textsubscript{50} figures ranged from 5.3 (series 7, compound XIIIa) to 61.2 (series 5, compound IXc) μM. In all cases, these compounds were less active towards L1210 cells than the P388 leukemia cell line. The average potencies of the compounds in the different series revealed a potency order of 7>8>3>1>4>5 i.e. in this case the bis compounds were among the most potent agents. The L1210 and human tumor assays were designed not only to detect compounds with significant bioactivity but to find novel derivatives with selective toxicity to malignant cells. Thus, in the case of the L1210 test, the differential in cytotoxicity between L1210
Table 5.1: *In vitro* screening data of the Mannich bases against murine leukemia P388, L1210, and human lymphocytes Molt4/C8 and CEM cells.

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<th>P388 Cells</th>
<th>L1210 cells and human lymphocytes</th>
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<td></td>
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<td>IC$_{50}$ (µM)</td>
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<td>Molt4/C8 cells</td>
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<td>IVd</td>
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<tr>
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<td>NA</td>
</tr>
<tr>
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</tr>
<tr>
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<td>IC₅₀ (µM)</td>
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### Table 5.2: *In vitro* evaluation of compounds against various human tumors.

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<th>TGI (μM)</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>Selectivity</th>
<th>Cell line</th>
<th>Parameter</th>
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cells and human T lymphocytes was noted. In 68% of the comparisons, the therapeutic index (TI) figures were greater than one and TI values of greater than 4 were noted for VIIb,f, and XIIIa,b,d which are useful prototypic molecules for analog development.

Approximately three quarters of the compounds were selected by the NCI for screening using approximately 55 human tumor cell lines from eight or nine different neoplastic diseases namely leukemia, non-small cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate and breast cancers. The data from this assay were made available in several formats, but the mean graph formats shown for the compounds XIIIa,i and XIVa in figures 5.11, 5.12 and 5.13 respectively are highly informative (Paull et al., 1989). In the mean graph, the dose response data for a compound are plotted on a logarithmic scale as horizontal bars to the right (more active) or to the left (less active) than the mean value for the activity of the compound against all the cell lines after 48 hours of exposure. This method of presentation allows one to quickly ascertain the activity of a given compound against specific tumor cell lines. Compounds XIIIa,i showed high selective activity towards leukemia and colorectal cell lines using the GI₅₀ level but lower activity with the TGI and LC₅₀ levels. Compound XIVa showed high selective activity towards leukemia using the GI₅₀ level. The remaining compounds were either not selective or not potent enough despite some selectivity.

The data in Table 5.2 indicate a range of IC₅₀ from 0.38 (XIIic) to 91.2 (XXIVb) and 78% of the compounds have lower IC₅₀ values than melphalan. The potency order of 8>5>7>4>1>3 revealed that greater cytotoxicity was displayed by compounds containing olefinic linkages (5,7,8). Examination of the data in both groups of compounds namely 1,3,4 as well as 5,7,8 revealed that the quaternary ammonium salts had greater activity than the mono Mannich bases which were more cytotoxic than the bis compounds. Selective toxicity towards leukemia was observed for four of the seven
quaternary ammonium salts examined in the screen namely VIIa,b,e and XIVa; this property was also noted with IXa and XIIIe. In addition, XIIId,i had preferential cytotoxicity towards human colon cells.

Evaluation of the P388 and L1210 results did not reveal whether the presence of a geminal dimethyl group (X) or a piperazine ring (XVI, XVIIa) had greater cytotoxicity than the analogs IXb, VIIa, XIIIa respectively. Thus in the P388 screen, IXb>X, VIIa >XVI and XVIIa>XIIIa while the L1210 test indicated that X>IXb and XVI, XVIIa had similar potencies as VIIa, XIIIa respectively.
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Figure 5.11: Selected growth inhibition data (XIIIa) for four tumor types (leukemia, non-small cell lung cancer, colon and renal cancer from the National Cancer Institute in vitro screen. These results are an abridged version of the complete data set which include results from ~60 human tumor cell lines.
Figure 5.12: Selected growth inhibition data (XIIIi) for five tumor types (leukemia, non-small cell lung cancer, colon, CNS and renal from the National Cancer Institute in vitro screen. These results are an abridged version of the complete data set which include results from ~60 human tumor cell lines.
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**Figure 5.13:** Selected growth inhibition data (XIVa) for six tumor types (leukemia, non-small cell lung cancer, colon, melanoma, ovarian and renal) from the National Cancer Institute in vitro screen. These results are an abridged version of the complete data set which include results from ~60 human tumor cell lines.
5.6.0.0 Stability studies

The stability of solutions of representative compounds was examined with a view to understanding their possible behavior under conditions akin to those used in the bioevaluations. Cytotoxicity will be dependent upon the extent of deamination whereby thiol-alkylators are liberated i.e. whether the breakdown of the compounds is nonexistent, minimal or extensive, as well as the number of alkylating species generated. In addition, cytotoxicity may be caused by the intact Mannich bases and related quaternary ammonium compounds. Evaluation of the theory of sequential cytotoxicity would be simplified if there is quantitative release of all of the predicted alkylating species during the time frame of the bioevaluation.

Due to the lack of aqueous solubility of the compounds, preliminary experiments led to the utilization of a mixture of phosphate buffered saline (PBS) pH 7.4 and dimethylsulfoxide (55:45). Among the compounds in series 1, 3, 5, 7 having the same aryl substituents, the 3,4-dichloro and 4-methoxy analogs were chosen since they have the highest and lowest Hammett $\sigma$ constants respectively. Solutions of IVc,e, VIIc,e, IXc,e, XIIIc,e were incubated for 48 hours at 37 °C, which were the time and temperature used in the P388 and human tumor assays. Thin layer chromatography revealed that the mono Mannich bases (IVc,e, IXc,e) were either completely decomposed (IVc,e, IXc) or only approximately 20% of the original compound remained (IXe). On the other hand, the bis Mannich bases were either stable (VIIc) or had decomposed by 10-15% (VIIe, XIIIc,e). Hence, the stability of the compounds in this solvent system is influenced by their general structures rather than the nature of the aryl substituents. The Mannich base (X) was essentially stable with two very minor additional compounds (~10%) appearing after 48 hours incubation at 37 °C. Solubilization of XVIIa required the dimethylsulfoxide concentration to rise to 70%. Both compounds (XVI, XVIIa) were stable upon incubation.

$^1$H NMR spectroscopy of representative compounds was undertaken using 1mM solutions of IVa, VIIa, VIIIa, IXa, XIIIa and XIVa in deuterated PBS, pH 7.4 (PBS-d). Spectra were recorded as rapidly as possible after addition of the solvent to the compounds ($t_0$) and after 48 hours incubation at 37 °C ($t_{48}$). Spectra were obtained
with solutions of IVa, VIIa, VIIIa, IXa, XIVa but the lack of aqueous solubility of the bis Mannich base XIIIa necessitated the use of a mixture PBS-d and deuterated dimethylsulfoxide (3:1). The spectra of the mono Mannich bases IVa, IXa revealed that the compounds broke down in solution liberating the corresponding amine and 1-phenyl-2-propen-1-one and 1-phenyl-1,4-pentadien-3-one respectively. At time t₀, the ratios of Mannich bases and diethylamine hydrochloride were 82:18 and 80:20 for IVa and IXa respectively while the t₄₈ spectra revealed the ratios to be 12:88 for both compounds. Examination of the t₀ and t₄₈ spectra of the bis Mannich bases VIIa, XIIIa indicated that the spectra of VIIa were identical and the presence of minor peaks in the olefinic proton region of the t₄₈ spectrum of XIIIa indicated that minimal decomposition had occurred. After 48 hours incubation at 37 °C, solutions of the quaternary ammonium compounds VIIIa, XIVa revealed that complete decomposition had occurred although the products formed were not identified. After incubating solutions of VIIIa, XIVa in PBS, pH 7.4 at 37 °C for 48 hours, TLC revealed the absence of the quaternary ammonium compounds and in each case, three new products which were fluorescent under ultraviolet light were formed. No additional compounds were detected after exposure of the chromatogram in an iodine chamber suggesting the absence of N-methyllethylamine hydrobromide.

5.7.0.0 Evaluation of the sequential cytotoxicity theory in light of the stability and biological data

The results are discussed below in regard to the theory of sequential cytotoxicity. A number of correlations were predicted if the compounds released various thiol alkylating species in vitro and in vivo. These postulated relationships are summarized in the Table 5.3. In order to examine whether the theory of sequential cytotoxicity was fulfilled or not from the biodata generated, comparisons of the IC₅₀ figures in different series were made as follows. Compounds with the same aryl substituents were compared with each other and a positive correlation in the table meant that the majority of comparisons between analogs in the two series favored the theory while a negative correlation meant that most comparisons did not support the hypothesis. In order to illustrate this approach, the last entry in table in regard to P388 cells will be
Table 5.3: Evaluation of the cytotoxicity data in the light of the theory of sequential cytotoxicity.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>P388 cells</th>
<th>L1210 cells</th>
<th>Human tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>3&gt;1</td>
<td>-</td>
<td>=</td>
<td>-</td>
</tr>
<tr>
<td>4&gt;1</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7&gt;5</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8&gt;5</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5&gt;1</td>
<td>=</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7&gt;3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8&gt;4</td>
<td>+</td>
<td>-</td>
<td>=</td>
</tr>
</tbody>
</table>

* See the Discussion section regarding the procedure adopted for making comparisons. In the case of series 8, comparisons were made with IXa,c,d and VIIIa,c,d. The symbols + and - indicate validation and negation of the theory while = means that half of the compounds were in accord with the hypothesis.

considered. In the case of the compounds in series 8, reaction with four molar equivalents of thiols both directly and after deamination has occurred is theoretically possible. On the other hand, a maximum of two olefinic bonds can be generated from the quaternary ammonium salts 4. Thus if the theory of sequential cytotoxicity is valid, the IC_{10} figures in 4 should be more than twice those of the analogs in series 8. The data in the Table indicate the potencies of XIVa-c (series 8) are 0.57, 3.59 and 3.39 times that of VIIIa,c,d (series 4) respectively and hence for the majority of compounds, the hypothesis is vindicated. The results indicate that 64% of the predictions made if the theory of sequential cytotoxicity is valid were fulfilled in the case of the human tumor cell lines. On average, compounds were screened against 55 different tumor cells from eight or nine neoplastic diseases. Hence, a substantial number of individual assays were undertaken for each compound tested and the fact that support for the theory was observed in two thirds of the comparisons is noteworthy. On the other hand, the data from the L1210 and P388 murine leukemic cell lines revealed that the theory was fulfilled in 50% and 36% of the comparisons made. In regard to the stabilities of these compounds, {1H NMR
spectroscopy of selected compounds in buffered solution revealed that the mono Mannich bases (series 1, 5) gave rise to the predicted unsaturated ketones resulting from liberations of diethylamine hydrochloride. Similarly, the quaternary ammonium compounds (series 4, 8) were unstable in solution although in this case, the products were not identified. On the other hand, the bis Mannich bases 3, 7 were much more stable under the conditions employed. If one assumes that deamination is the first step in the decompositions of the quaternary ammonium salts prior to the formation of other compounds, then the relative rates of deamination among series 1, 3, 4 was 4>1>3 and among series 5, 7, 8, it was 8>5>7.

Deamination is influenced by the basicity of the amino groups. The pKa value of diethylamine is 10.84 (Albert et al., 1984a) and for 3-ethylamino-1-phenyl-1-propanone and 3-N-ethyl-N-methyl-1-phenyl-1-propanone, the pKa figures were calculated to be 8.95 and 8.10 respectively (Albert et al., 1984b). Hence the predicted rates of the initial decomposition of Ia, IIIa, IVa, is IVa> IIIa >Ia whereas in fact the relative ease of decomposition observed was IVa> Ia >IIIa. Furthermore while a solution of X was essentially stable upon incubation as expected, the lack of breakdown of solutions of XV, XVIa was surprising. The results from this study indicate that while the Mannich bases Ia and IXa (and presumably other members of series 1 and 5) deaminate as predicted, much additional experimentation is required in order to understand the factors controlling the stabilities of bis Mannich bases, the corresponding quaternary ammonium salts and related compounds in solution.

5.8.0.0 In vivo antitumor screening

Four factors namely (i) disease panel specificity or cell line selectivity, (ii) uniqueness in chemical structure, (iii) novel “compare pattern” that does not match with agents with known mechanisms of action and (iv) availability, are considered for selecting a compound for in vivo evaluation. On the basis of this criteria, two compounds namely, XIIIId and XIVa were selected for in vivo antitumor activity assays in nude mice bearing those human tumors representing the most sensitive in vitro tumor cell lines from the screen. Therefore, XIIIId and XIVa were examined in the subcutaneous minimal stage
Table 5.4: Antitumor activity of XIIIId against human tumor xenografts in vivo.

<table>
<thead>
<tr>
<th>Tumor type/Line (host)</th>
<th>Route&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Dose (mg/kg)</th>
<th>% ILS&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Opt. T/C %&lt;sup&gt;d&lt;/sup&gt; (day)</th>
<th>Median days to reach (x gm)&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Growth delay %</th>
<th>T/C&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine leukemia P388, (Athymic nude mice)</td>
<td>IP QDX5, day 9</td>
<td>120 80 54</td>
<td>5</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Human non-small cell lung cancer SC, NCI-H522</td>
<td>IP QDX5, day 1 control</td>
<td>120 80 54</td>
<td>107 77(26)</td>
<td>14.8 15.1 14.6</td>
<td>4 2 5</td>
<td>92(19)</td>
<td>15.4 (0.8)</td>
</tr>
<tr>
<td>Human colon SW-620</td>
<td>IP QDX5, day 9 control</td>
<td>120 80 54</td>
<td>78(16) 68(16) 73(14)</td>
<td>16.5 16.6 15.9</td>
<td>9 9 5</td>
<td>15.2 (1.5)</td>
<td></td>
</tr>
<tr>
<td>Human colon Colo 205</td>
<td>IP Q4DX3, day 3 control</td>
<td>200 134 90</td>
<td>53(22) 68(22) 87(33)</td>
<td>29.6 28.3 22.8</td>
<td>18 13 -9</td>
<td>25.0 (0.8)</td>
<td></td>
</tr>
<tr>
<td>Human colon Colo 205</td>
<td>IP Q4DX5, day 8 control</td>
<td>200 134 90</td>
<td>32(23) 107(23) 75(27)</td>
<td>24.5 17.7 18.4</td>
<td>28 -7 -4</td>
<td>19.1 (0.5)</td>
<td></td>
</tr>
<tr>
<td>Human melanoma LOX IMVI</td>
<td>IP QDX5, day 7 control</td>
<td>120 80 54</td>
<td>74(14) 65(20) 78(16)</td>
<td>15.1 15.4 14.8</td>
<td>10 12 8</td>
<td>13.7 (1.5)</td>
<td></td>
</tr>
<tr>
<td>See footnotes under Table 5.5.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.5: Antitumor activity of XIVa against human tumor xenografts in vivo.

<table>
<thead>
<tr>
<th>Tumor type/Line</th>
<th>Route(^{a})/ Schedule(^{b})</th>
<th>Dose (mg/kg)</th>
<th>% ILS(^{c})</th>
<th>Opt. T/C (^{d}) (day)</th>
<th>Median days to (x gm)(^{e})</th>
<th>Growth delay (^{f}) T/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine</td>
<td>IP</td>
<td>15</td>
<td>11.0</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Leukemia</td>
<td>QDX5, day 1</td>
<td>10</td>
<td>11.0</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>P388</td>
<td>control</td>
<td>6</td>
<td>12.0</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Human Colon</td>
<td>IP</td>
<td>25.0</td>
<td>81(28)</td>
<td>28.9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Colo205</td>
<td>Q4DX3, day 15</td>
<td>16.80</td>
<td>80(31)</td>
<td>30.2</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>11.20</td>
<td>94(31)</td>
<td>26.8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Human Colon</td>
<td>IP</td>
<td>24.0</td>
<td>72(35)</td>
<td>23.9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Colo205</td>
<td>QDX5, day 12</td>
<td>16.0</td>
<td>109(35)</td>
<td>20.9</td>
<td>-8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>10.80</td>
<td>100(19)</td>
<td>22.4</td>
<td>-1</td>
<td></td>
</tr>
<tr>
<td>Human renal</td>
<td>IP</td>
<td>25.0</td>
<td>86(23)</td>
<td>23.9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>CAKI-1</td>
<td>Q4DX3, day 13</td>
<td>16.80</td>
<td>56(40)</td>
<td>24.7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>11.20</td>
<td>114(23)</td>
<td>21.8</td>
<td>-6</td>
<td></td>
</tr>
<tr>
<td>Human renal</td>
<td>IP</td>
<td>24.0</td>
<td>50(19)</td>
<td>23.8</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>CAKI-1</td>
<td>QDX5, day 14</td>
<td>16.0</td>
<td>73(26)</td>
<td>22.3</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>10.0</td>
<td>39(33)</td>
<td>21.5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Route of drug administration is IP (intraperitoneal).

\(^{b}\) The schedule is shown in an abbreviated form. For interpretation, the Q represents "every" and H represents "hours" and a D represents "days". The day following the comma defines the day on which the first treatment was administered. Thus, Q4D X 3, Day 1 is read as "every four days for a total of three treatments with the first treatment given on day one."
c  % ILS: Percent increase in life span is calculated as follows.
   % ILS = [(T-C)/C] x 100 where T and C are the median days of
d  death for the treated and control groups respectively. Percent ILS provides an indication
  of tumor response. A compound showing ILS>20% is considered as active.
  Opt. % T/C (day): Percent treated/control is calculated by dividing treated tumor
  weight by the median control tumor weight on each observation day and multiplying by
  100. This calculation is performed each day the tumor are measured and the optimum
  value (minimum) obtained after the first course of treatment is presented. The day on
  which this optimum T/C occurs is shown in parenthesis. A T/C % of greater than 40 is
  considered as inactive.
e  Median day to x mg: Median of the times (days) required for the treated tumor
weights to obtain a specified size (e.g. 1000mg). Selection of the latter is dependent on
f  the tumor growth rate and treatment period.
  Growth delay: Expressed as the percentage by which the treated group median
  tumor weight is delayed in achieving the specified tumor size compared to the controls
  using the formula [(T-C)/C x 100] where T and C are the median times to x mg for the
  treated and control groups respectively. A positive value indicated that the treated tumor
  reached x mg more slowly than did the control tumor. The greater the positive value, the
  longer the delay in the treated tumor reaching x mg.

xenograft model for initial in vivo testing. Both the compounds were also tested in vivo
using the older murine P388 model in order to compare the results with the newer NCI
models involving human tumor xenografts. Another compound XIIIi was selected for
testing in a new in vivo hollow fibre assay. This assay consists of 12 selected human
tumor cell lines encased in hollow fibres which are implanted into athymic nude mice. Six
to eight days after administration of the test compound to the mice, the fibres are
collected, the cells removed and the growth inhibition is measured using the MTT assay.
Compounds which produce promising results in the assay may be selected for further in
vivo evaluation using xenografts models. The activity of these two compounds, XIIIId
and XIVa, towards several xenografts is summarized in Tables 5.4 and 5.5.

Both the compounds were found to be non toxic to mice at the doses
administered. Doses of 120 (XIIIId) and 24 (XIVa) mg/kg for five consecutive days
(total of 600 and 120 mg/kg respectively) were well tolerated. The data for %ILS in the
case of murine P388 leukemia and optimum T/C in the case of the rest of the tumors
suggested that these compounds were not active except on one occasion when XIIIId, at
a dose level of 200mg/kg every fourth day for five treatments, had 32% T/C value in
mice having a xenograft of human colon COLO 205 tumor. Thus, molecular modification of XIIIId should be considered with a view to obtaining a series of novel colon specific anticancer drugs.

5.9.0.0 Quantitative structure activity relationships

In order to seek correlations between the cytotoxicity data and the electronic, hydrophobic and steric properties of the aryl substituents, linear and semilogarithmic plots between the IC<sub>50</sub> values and the Hammett σ, Hansch π and molar refractivity (MR) constants in each of the series were made, providing that screening results were available for at least three members of a particular series. The test for zero correlation (Bolton, 1984) was applied at the 95% and 90% significance levels. In cases where good correlations were noted, the data was further evaluated revealing p values of less than 0.05. The significant relationships which were obtained are summarized in table 5.6. The data in the table reveal eleven correlations between cytotoxicity and the σ, π and MR constants in both the series of mono Mannich bases (series 1, 5) and the bis Mannich bases (series 7). No correlations were discerned in the other three series of compounds namely 3, 4 and 8. The relationships between cytotoxicity and the MR, π and σ values of the aryl substituents were noted in five, four and two cases respectively. Thus, where correlations were detected, differences in the sizes and hydrophobic properties of the aryl groups influence activity more than their chemical reactivity. For the purpose of subsequent drug design, development of each of the series (1, 5, 7) will use such correlations. However the test system being utilized needs to be considered since as Table 5.6 indicates, positive correlations were noted with the P388 screen, negative relationships were found in the L1210 test and both positive and negative correlations were obtained using the human tumor assay. For example, for future expansion of series 1, an increase in the size of the aryl substituent would be predicted to increase cytotoxicity in the P388 screen. On the other hand, a reduction in the MR value of the aryl group is expected to increase activity in the L1210 and human tumor assays. Similarly for series 5, while increases in the size of the aryl substituents would be expected to increase activity in the P388 screen, compounds containing aryl substituents
Table 5.6: Correlations between the sigma (σ), pi (π) and molar refractivity (MR) constants in the P388, L1210 and human tumor screens.

<table>
<thead>
<tr>
<th>Screen</th>
<th>Series</th>
<th>Aryl substituent</th>
<th>Plot&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Correlation&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P388</td>
<td>1</td>
<td>MR</td>
<td>lin, log</td>
<td>&lt;0.1, &lt;0.05</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>MR</td>
<td>lin, log</td>
<td>&lt;0.05, &lt;0.1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>σ</td>
<td>lin, log</td>
<td>&lt;0.1, &lt;0.005</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>π</td>
<td>lin, log</td>
<td>&lt;0.1, &lt;0.05</td>
<td>+</td>
</tr>
<tr>
<td>L1210</td>
<td>1</td>
<td>MR</td>
<td>log</td>
<td>&lt;0.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>π</td>
<td>lin, log</td>
<td>&lt;0.1, &lt;0.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>MR</td>
<td>lin, log</td>
<td>&lt;0.025, &lt;0.025</td>
<td>-</td>
</tr>
<tr>
<td>Human tumors</td>
<td>1</td>
<td>σ</td>
<td>log</td>
<td>&lt;0.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>π</td>
<td>lin, log</td>
<td>&lt;0.1, &lt;0.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>MR</td>
<td>lin, log</td>
<td>&lt;0.1, &lt;0.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>π</td>
<td>log</td>
<td>&lt;0.1</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Both linear (lin) and semilogarithmic (log) plots were made.
<sup>b</sup> When two values are quoted, they refer to correlations obtained from the linear and logarithmic plots respectively.
<sup>c</sup> Positive (+) correlations indicate that cytotoxicity rose as the σ, π and MR values are increased while negative (-) correlations reveal that increases in bioactivity occurs with diminishing σ, π, and MR figures.

with small MR values would be predicted to display increased cytotoxicity in the L1210 test. No correlations between IC<sub>50</sub> values and σ or π constants in series 1, 3 in the L1210 and P388 screens were found. But cytotoxicity results for these compounds in the above-mentioned screens indicated that lower IC<sub>50</sub> values were found in compounds with electron-attracting rather than electron-releasing substituents. Thus, compounds IVF-h and VILg-j having electron-attracting groups in the para position of the aryl ring were synthesized.
A positive correlation between cytotoxicity and \( \pi \) values in the P388 and human tumor screens indicates that bioactivity increases as the \( \pi \) value of the substituent in the para position is increased. Therefore, compounds XIIIi and XIIIj with 4-ethyl and 4-isopropyl substituents respectively, having higher \( \pi \) values (\( \pi_{\text{ethyl}} = 1.00; \pi_{\text{isopropyl}} = 1.30 \)) than the lead compound XIIId (\( \pi_{\text{methyl}} = 0.50 \)) were synthesized. The *in vitro* cytotoxicity results for XIIIi revealed higher cytotoxicity in both the P388 leukemia screen (1.45\( \mu \)M vs 2.1\( \mu \)M) and human tumor screen (3.63\( \mu \)M vs 5.01\( \mu \)M) and colon cancer selectivity than the lead compound XIIId. This compound has been selected for in vivo testing in mice (hollow fibre assay). In order to find the effect of variation in the interplanar angles between the aryl ring and adjacent ethylene linkage (C=C) on bioactivity, compounds XIII f,g,h were synthesized. The order of activity among the positional isomers of XIIId in P388 screen was found to be \( \text{m}_{\text{CH}_3} > \text{o}_{\text{CH}_3} > \text{p}_{\text{CH}_3} \). In the human tumor screen, the results are available for two compounds only namely XIIId and XIIIi which reveal that the latter compound is almost twice as active than the former but it is less selectively toxic to colon cancer cell lines. Once all the results are obtained, decisions can be made about the choice of analogs to be synthesized with a view to increased selectivity and potency.

5.10.0.0 Cell death

The end result of cell death whether apoptotic or necrotic is the loss of viability which is often defined as the loss of membrane integrity and is conveniently measured by the uptake of certain dyes such as trypan blue, eosin, ethidium bromide or propidium iodide or lactate dehydrogenase. In growth inhibition studies, the trypan blue exclusion test was used to determine the IC\(_{50}\) value against human Jurkat leukemia T cells. This test is based on the principle that live cells possess intact cell membranes that exclude certain dyes such as trypan blue and eosin whereas dead cells do not. Thus viable cells with clear cytoplasm and non viable cells having blue cytoplasm were seen and counted under a light microscope. As is apparent from the graph (Figures 5.14 and 5.15), compound XIIId and melphalan showed good dose dependent cytotoxicity. The IC\(_{50}\) (\( \mu \)M) of XIIId and melphalan was found to be 3.46 and 1.16 \( \mu \)M respectively. The determination of whether a cell dies by apoptosis as opposed to necrosis is best made...
Figure 5.14: Effect of XIIIId on human Jurkat T leukemia cell line.

Figure 5.15: Effect of melphalan on human Jurkat T leukemia cell line.
on the basis of distinct structural changes in the cell's chromatin which occur prior to the lysis of membranes. These changes can be assessed by light or electron microscopy and also by DNA fragmentation as detected in sedimentation assays or by gel electrophoresis. Fluorescent microscopy was employed to distinguish apoptotic cells from necrotic cells. The cell suspension was mixed with a mixture of fluorescent DNA binding dyes namely acridine orange and ethidium bromide because acridine orange alone cannot differentiate between viable and non-viable cells. The differential uptake of these two dyes allows the identification of viable and non viable cells. Both live and dead cells take up acridine orange which intercalates into DNA making it appear green and when binding to RNA, the staining it red. Thus, a viable cell has bright green chromatin in its nucleus and red cytoplasm. Ethidium bromide is only taken up by the non-viable cells. It intercalates into DNA making it appear orange but binds weakly to RNA which may appear slightly red. Thus a dead cell will have a bright orange chromatin and its cytoplasm if it has any contents remaining will appear dark red. In essence, both normal or apoptotic nuclei will fluorescence bright green and bright orange in live and dead cells respectively.

In the experiment involving measurement of the percentage of apoptotic cells at different time intervals, only green colored cells could be seen during the time frame of 18 hours. It means there were no non-viable cells during this period. Green colored cells were of two types as shown in figures 5.16 and 5.17. These were viable cells with normal nuclei (bright green chromatin with organized structure, figure 5.16) and viable cells with apoptotic nuclei (bright green chromatin which is highly condensed or fragmented, figure 5.17). In most of the apoptotic cells, the entire nucleus was present as one or a group of featureless bright spherical beads. Some cells had lost DNA or fragmented into apoptotic bodies and the overall brightness was less than that of normal cells. This stage is characterized as advanced apoptosis. Other types of cells, namely non viable cells with normal nuclei (bright orange chromatin with organized structure) and non viable cells with apoptotic nuclei (bright orange chromatin which is highly condensed or fragmented), were not seen. As is evident from figure 5.18, the magnitude of apoptosis increases with time and also with concentration. At the end of 48 hours, no sign of apoptosis was evident with both the compounds. No live cells could be seen.
Figure 5.16: Morphological appearance of cells from untreated cultures illustrating the morphology of human Jurkat T leukemia cells. Original magnification X 100.

Figure 5.17: Morphological appearance of cells from XIIId treated cultures illustrating the morphology of apoptotic human Jurkat T leukemia cells with their highly condensed chromatin and fragmented nuclei. Original magnification X 100.
Figure 5.18: Effect of time and concentration on the magnitude of apoptosis in the human Jurkat T leukemia cell line induced by XIIId and melphalan.

All the cells were broken into small fragments. The other biochemical event characteristic of apoptosis is a distinctive DNA ladder pattern consisting of multiples of ~180-bp (bp: base pair) subunit. This can be demonstrated by isolating DNA from cells undergoing apoptosis and subjecting them to gel electrophoresis. Two main fractions were generated from the cell suspension which had undergone apoptosis. The top (T) fraction contained fragmented DNA released from the nuclei of cells undergoing apoptosis that failed to sediment with intact chromatin when subjected to centrifugation and the bottom fraction (B) contained predominately very large DNA molecules. The DNA was then isolated, concentrated from both fractions and analyzed by agrose gel electrophoresis. Agrose gel electrophoresis is a simple and highly effective method for separating, identifying and purifying 0.5-25 kb DNA fragments. Voltage applied at the end of an agrose gel generates an electric field with the strength defined by the length and the potential difference at the ends (V/cm). DNA molecules exposed to this electric field migrate
towards the anode due to the negatively charged phosphate groups along the DNA backbone. The migration velocity is limited by the frictional force imposed by the gel matrix, while charge and/or size can affect the rate at which the macromolecules will pass through the gel. The charge to mass ratio is the same for DNA molecules of different lengths. Therefore it is the size of the DNA that determines the rate at which it passes through the gel thereby allowing an effective separation: of the DNA fragment length mixture by electrophoresis. Despite repeated attempts, the DNA ladder pattern could not be obtained in case of XIll'd. Only a very faint DNA ladder could be seen in the case of melphalan.

5.11.0.0 Antifungal screening

Initially, the effect of some Mannich bases and related compounds on the growth of two pathogenic fungi namely Candida albicans and Aspergillus fumigatus was examined. The data in table 5.7 revealed the following correlations.

1. On the basis of the limited biological data, it can be said that the quaternary ammonium salts in both the acetophenone and styryl ketone series were the most active followed by the bis and mono compounds. In fact the mono compounds (series 5 and IVa,b) were inactive (MIC>100 μM).

2. No relationship between antifungal activity and the pKa values of different leaving groups was observed. All five compounds with different leaving groups (IXa, f-i) were inactive.

3. The Mannich bases of styryl ketones and their quaternary salts possessed greater antifungal activities than their acetophenone counterparts. For example, XIll'b, a bis Mannich base of 4-chlorostyryl ketone was found to be 16 times more active than the bis Mannich base of acetophenone (VIIb). Similarly, XIVa, quaternary salt of a styryl ketone Mannich base was 4 times more active than the corresponding acetophenone quaternary salt (VIIIa).

4. While the piperazine bis Mannich base of a styryl ketone Ia was as active as XIVa, the corresponding analog derived from acetophenone XVI was inactive.
5. Among the compounds in table 5.7, XIIIb-e seem to be most promising ones. Therefore, these compounds were tested for their inhibitory effects against a variety of bacteria, yeasts and two filamentous fungi. The results are presented in table 5.8. Compounds XIIIb,c inhibited the growth of all of the fungi at low concentrations whereas XIIIa,d,e had significantly reduced potencies towards the microorganisms. The average MIC values of XIIIa-e were >100, 5.25, 16.3, 85 and 100 μM respectively.

Analysis of these results in the context of the Topliss potency order table approach (Topliss, 1977) revealed no correlation between the MIC values and σ, π or their various combinations. But a clearer relationship can be discerned by applying the Topliss decision tree approach (Topliss, 1972). In this series, the 4-chloro derivative is more than 40 times as active as the unsubstituted analog which suggests that the activity is increased with a rise in σ or π values. But the next analog, namely the 3,4-dichloro derivative which has still higher σ and π values is four times less active than the 4-chloro derivative. This could be due to either an unfavorable steric effect from the 3 position or the values of σ or π exceeding the optimum. Therefore, new analogs such as 4-bromo, 4-trifluoromethyl, 4-iodo etc. with intermediate hydrophobicity and chemical reactivity should be prepared. Another important finding is that the minimum fungicidal concentration (MFC) of XIIIb and c are in general either the same or two fold higher than the MIC values. Therefore these compounds could be useful antimycotic agents. Hence future work designed to increase antifungal potency should be directed to the placement of groups on the aryl ring with moderate hydrophobicity and chemical reactivities.
Table 5.7: Susceptibility of fungi to Mannich bases.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum inhibitory concentration (µM)</th>
<th>Compound</th>
<th>Minimum inhibitory concentration (µM)</th>
</tr>
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<tr>
<td></td>
<td>C. albicans</td>
<td>A. fumigatus</td>
<td>C. albicans</td>
</tr>
<tr>
<td>Ia</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>XIII d</td>
</tr>
<tr>
<td>Ib</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>XIII e</td>
</tr>
<tr>
<td>IV a</td>
<td>500</td>
<td>---</td>
<td>XIV a</td>
</tr>
<tr>
<td>IV b</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>XIV b</td>
</tr>
<tr>
<td>VII a</td>
<td>500</td>
<td>---</td>
<td>XIV c</td>
</tr>
<tr>
<td>VII b</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>XIV e</td>
</tr>
<tr>
<td>VIII a</td>
<td>250</td>
<td>---</td>
<td>XIV f</td>
</tr>
<tr>
<td>VIII f</td>
<td>100</td>
<td>100</td>
<td>XV</td>
</tr>
<tr>
<td>VIII g</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>XVI</td>
</tr>
<tr>
<td>VIII h</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>XVII a</td>
</tr>
<tr>
<td>VIII i</td>
<td>100</td>
<td>100</td>
<td>XVII b</td>
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<tr>
<td>IX a</td>
<td>500</td>
<td>---</td>
<td>XVII c</td>
</tr>
<tr>
<td>IX b</td>
<td>125</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>IX c</td>
<td>125</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>IX d</td>
<td>&gt;500</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>IX e</td>
<td>500</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>IX f</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>IX g</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>IX h</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>IX i</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>X</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>XIII a</td>
<td>250</td>
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<tr>
<td>XIII b</td>
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<td>6.25</td>
<td></td>
</tr>
<tr>
<td>XIII c</td>
<td>25</td>
<td>25</td>
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**Table 5.8:** Susceptibility of bacteria and fungi to Mannich bases (XIII b-e).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Minimum inhibitory concentration (µM)</th>
<th>XIIIb</th>
<th>XIIIc</th>
<th>XIIIId</th>
<th>XIIIe</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans B311</td>
<td>6.25 (12.5)</td>
<td>25 (50)</td>
<td>100 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>C. albicans 3153 A</td>
<td>6.25 (12.5)</td>
<td>25 (50)</td>
<td>100 (ND)</td>
<td>&gt;100 (ND)</td>
<td></td>
</tr>
<tr>
<td>C. albicans 6711</td>
<td>6.25 (12.5)</td>
<td>50 (50)</td>
<td>100 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>C. albicans 6714</td>
<td>6.25 (12.5)</td>
<td>25 (50)</td>
<td>100 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>C. albicans DC</td>
<td>12.5 (12.5)</td>
<td>25 (50)</td>
<td>100 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>C. krusei</td>
<td>3.12 (6.25)</td>
<td>12.5 (25)</td>
<td>100 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>T. glabrata</td>
<td>1.56 (3.12)</td>
<td>6.25 (6.25)</td>
<td>50 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>C. lusitaniae</td>
<td>3.12 (6.25)</td>
<td>12.5 (25)</td>
<td>100 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>Crypto. neoformans</td>
<td>0.78 (0.78)</td>
<td>3.12 (6.25)</td>
<td>50 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>0.19 (0.19)</td>
<td>3.12 (3.12)</td>
<td>50 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>6.25 (25)</td>
<td>25 (50)</td>
<td>100 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>12.5 (25)</td>
<td>25 (50)</td>
<td>100 (ND)</td>
<td>100 (ND)</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>&gt;100 (ND)</td>
<td>&gt;100 (ND)</td>
<td>&gt;100 (ND)</td>
<td>&gt;100 (ND)</td>
<td></td>
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<tr>
<td>Enterobacter cloaceae</td>
<td>&gt;100 (ND)</td>
<td>&gt;100 (ND)</td>
<td>&gt;100 (ND)</td>
<td>&gt;100 (ND)</td>
<td></td>
</tr>
<tr>
<td>S. aureus 675 (MRSA)</td>
<td>12.5 (50)</td>
<td>50 (100)</td>
<td>100 (ND)</td>
<td>&gt;100 (ND)</td>
<td></td>
</tr>
<tr>
<td>S. aureus 1199 (MSSA)</td>
<td>12.5 (25)</td>
<td>50 (50)</td>
<td>100 (ND)</td>
<td>&gt;100 (ND)</td>
<td></td>
</tr>
<tr>
<td>E. faceium LC51</td>
<td>12.5 (25)</td>
<td>50 (100)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E. faceium LC56</td>
<td>6.25 (12.5)</td>
<td>50 (50)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E. faceium LC53</td>
<td>12.5 (12.5)</td>
<td>50 (100)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E. faceium LC55</td>
<td>6.25 (6.25)</td>
<td>50 (100)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E. faceium LC58</td>
<td>12.5 (25)</td>
<td>50 (50)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Note: Minimum fungicidal concentrations and minimum bactericidal concentrations are shown in parenthesis. ND = not determined.
6.0.0.0 CONCLUSIONS

In general, the cytotoxicity results obtained for the Mannich bases and related quaternary ammonium salts support the theory of sequential cytotoxicity in the important human tumor screen in contrast to evaluation using the murine leukemia cells where support for the hypothesis was marginal. Bioevaluation indicated that many of the compounds described in this study are potent cytotoxic agents and some selective cytotoxicity for certain tumor cell lines was revealed in the L1210 and human tumor cell assays. The in vivo activity of XIIIId against human colon tumors revealed it to be a promising lead compound. The cell death caused by this compound was found to be apoptotic in the human Jurkat T leukemia cell line. But additional studies are required in order to understand how this compound induces apoptosis at the molecular level.

QSAR indicated a number of correlations between cytotoxicity and various physicochemical constants of the aryl substituents which serve as pointers for future molecular modifications. In addition, this study has revealed the need for additional investigations with a view to understanding the stabilities in solution of a number of the groups of compounds described herein.

Finally, in vitro screening of the compounds described herein against various pathogenic fungi revealed a few potent molecules which have significant potential for further development as candidate antifungal drugs.
APPENDIX I

Preliminary data from the X-ray crystallographic analysis of compound XIII\textsubscript{d} revealed the cyclic structure shown below. The proton NMR spectrum (500 MHz)

![Chemical Structure](image)

of this compound in deuterated chloroform also indicated that XIII\textsubscript{d} had undergone cyclization. Till now, three compounds namely XIII\textsubscript{b}, XIII\textsubscript{e} in addition to the above-mentioned compound have been found to be in the cyclic form. The postulated mechanism for the formation of the cyclic compounds is presented in figure A\textsubscript{1}. Under acidic conditions, it is quite probable that one of the carbonyl groups may undergo enolization to form I which is conjugated. The other carbonyl group may also enolize to form the allylic carbocation III which can undergo an intramolecular aldol cyclization in two ways as depicted in the figure. Formation of the eight membered ring is energetically unfavorable compared to a six membered ring and thus the latter is obtained. Also, hydrogen bonding between the hydroxyl and keto functions may further facilitate the formation of this ring.

None of the $\beta$-hydroxy ketones examined so far by $^1$H NMR spectroscopy showed any sign of dehydration, a reaction often seen with these kinds of molecules. Two of these compounds namely XIII\textsubscript{c}, XIII\textsubscript{e} were also stable in aqueous buffer, pH 7.4 and temperature $37^\circ$C. However attempts to isolate these compounds using silica gel or alumina columns were aborted as these compounds decomposed. It is likely that these cyclic compounds might have dehydrated (use of silica gel to effect dehydration of $\beta$-hydroxy ketones is quite common) to form either of two regioisomers (a or b), though the
Figure A.1: Postulated mechanism for the intramolecular cyclization of Xllib-e and related compounds.
latter may predominate due to its extended conjugation. Besides their complex stereochemical features (two adjacent chiral centers, four possible stereoisomers), these compounds preserved two important attributes of acyclic Mannich bases. First, the presence of two electrophilic sites which are chemically different namely an α,β-enone group and an isolated alkene system for alkylation. Second, these compounds retained another important feature of the Mannich base structure i.e., the presence of a β-amino ketone group. Probably, the presence of these two characteristics may be responsible for the observation of the high cytotoxicity seen in the NCI screen.

One may tentatively assume that all the bis Mannich bases and the corresponding quaternary salts have cyclized to form the corresponding 4-piperidinols. In order to evaluate the theory of sequential cytotoxicity therefore, one would like to compare the cytotoxicity data generated for the compounds which release two or more but structurally similar alkylation groups successively with those releasing only one or no alkylation groups. Since cyclic compounds, though having two sites for alkylation are quite different from the mono acyclic Mannich bases in terms of structure and stability, (cyclic compounds will not release alkylation agents), it would not be reasonable as per the definition of the theory of sequential cytotoxicity to compare cytotoxicity data for these two classes of compounds. But these cyclic compounds seemed to be more favorable than their acyclic counterparts as far as alkylation capabilities are concerned since cyclic compounds have two electron-deficient centers having different alkylation properties. For example, in the cyclic compounds, an initial sensitization of the tumor cells may occur by
rapid reaction of GSH with more reactive $\alpha,\beta$-unsaturated group, followed by delayed cytotoxicity caused by alkylation with the less reactive alkene portion of the molecule.

Thus it can be concluded that this study led to the identification of a novel class of alkylating agents which like various clinically used alkylating agents share the property of inducing apoptosis in vitro. Some of these compounds have been shown to possess selective cytotoxicity against a few human tumors in the *in vitro* studies. QSAR studies have revealed many correlations which could serve as the basis for future molecular modifications.
APPENDIX II

1. TE buffer
   10mM Tris (hydroxymethyl) aminomethane [tris. Cl], pH 7.4
   1mM Ethylenediaminetetraacetic acid (EDTA).

2. TBE electrophoresis buffer (10X stock solution)
   108g Tris base (89mM)
   55g Boric acid (89mM)
   40ml 0.5M EDTA, pH 8.0 (2mM).

3. TTE solution
   TE Buffer, pH 7.4 containing 0.2% Triton X-100.

4. 10 X loading buffer
   20% Ficoll 400
   0.1 Disodium ethylenediaminetetraacetic acid (Na₂EDTA), pH 8.0
   1.0% Sodium dodecyl sulphate (SDS)
   0.25% Bromophenol blue
   0.25% Xylene cyanol.
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