NOVEL BIOLOGICAL ROLES FOR PYRIMIDINES

by

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Summary

The development of classical and lipophilic inhibitors of dihydrofolate reductase (DHFR) as antitumour agents is reviewed and the advantages and problems associated with each class are discussed. The antitumour activity, pharmacokinetics and metabolism of m-azidopyrimethamine (MZP), a novel lipophilic inhibitor, are considered and compared with metoprine, the prototype lipophilic antifolate. Evidence for a folate-independent target for lipophilic DHFR inhibitors is presented.

Synthetic studies centred on three principal objectives. Firstly a series of structural analogues of MZP were prepared encompassing alkoxy, chloro and alkylamino substituents and evaluated, as the ethanesulphonate salts, for activity against mammalian DHFR. Inhibitory constant (K_I) determinations were conducted by a Zone B analysis, the corresponding 4'-azido isomer of MZP proving more potent than the parent compound. Secondly, to facilitate metabolism and stability studies on MZP, a range of possible reference compounds were synthesised and characterised.

Finally, a series of diaminopyrimidine derivatives were synthesised embracing structural features incompatible with DHFR inhibitory activity, in order that such compounds may serve as biochemical probes for the unidentified folate-independent target for lipophilic diaminopyrimidines discussed previously. Inactivity against DHFR was achieved *via* introduction of an ionic or basic group into a normally hydrophobic region of the molecule and compounds were screened against mammalian DHFR and thymidylate synthase to confirm the abolition of activity. Several derivatives surprisingly proved potent inhibitors of DHFR exhibiting K_I values comparable to that of methotrexate.

Analogues were screened for antitumour activity *in vitro* and *in vivo* against murine leukaemia cell lines in order to identify potential lead compounds. Several derivatives virtually inactive against DHFR exhibited a disparate cytotoxicity and further biochemical studies are warranted.

The hitherto unreported debenzylation of 2,4-diamino-5-(N-alkyl-benzylaminophenyl)pyrimidines was discovered during the course of the synthetic studies, treatment of these compounds with nitrous acid affording the corresponding benzotriazoles.

Keywords: Dihydrofolate reductase
2,4-Diaminopyrimidines
m-Azidopyrimethamine
Folate-independent
Anti-cancer

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ABBREVIATIONS

br	Broad
CNS	Central nervous system
CRC	Cancer Research Campaign
D	Density
DHFR	Dihydrofolate reductase
DMF	N,N -Dimethylformamide
DMSO	Dimethylsulphoxide
d.UMP	Deoxyuridine monophosphate
ether	Diethyl ether
FH_2	Dihydrofolate
FH_4	Tetrahydrofolate
g.l.c.	Gas liquid chromatography
h	Hour(s)
h.p.l.c.	High performance liquid chromatography
i.p.	Intraperitoneal
i.r.	Infrared (spectroscopy)
min	Minute(s)
m.p.	Melting point
m.s.	Mass spectrum
MTX	Methotrexate
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NCI	National Cancer Institute
n.m.r.	Refers to ^1H-nuclear magnetic resonance
P	Partition coefficient
s	Second(s)
TFA	Trifluoroacetic acid
TFSA	Trifluoromethanesulphonic acid

THF	Tetrahydrofuran
t.l.c.	Thin layer chromatography
TS	Thymidylate synthase
UMP	Uridine monophosphate
u.v.	Ultraviolet (spectroscopy)
v/v	Volume/volume
water	Refers to double-distilled water
°	Degrees Celsius

Abbreviations employed in the kinetic equations

E	Enzyme
E_t	Total molar enzyme concentration
I	Inhibitor
I_t	Total molar inhibitor concentration
K_I	Inhibitor dissociation constant
K_{Iapp}	Apparent inhibitor dissociation constant
K_m	Michaelis constant
S	Substrate
V	Observed reaction velocity
Vi	Inhibited reaction velocity
V_{max}	Maximum reaction velocity
Vo	Uninhibited reaction velocity

'If a man will begin with certainties he shall end in doubts; but if he will be content to begin with doubts he shall end in certainties'.

FRANCIS BACON

SECTION 1
INTRODUCTION

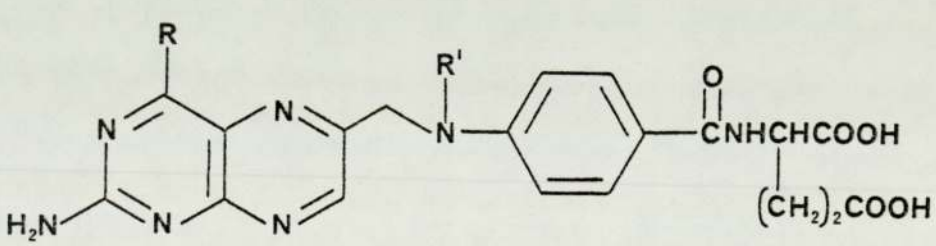
INTRODUCTION

CHAPTER 1

DIHYDROFOLATE REDUCTASE INHIBITORS AS ANTITUMOUR AGENTS

1.1 Introduction

The concept of antimetabolite chemotherapy propounded by Woods in 1940 catalysed an interest, motivated by the military situation, in applying this principle to folate metabolism. The subsequent synthesis of aminopterin[1] (2) and methotrexate[2] (3) as analogues of folic acid (1), and the diaminopyrimidines[3] originally as thymine antimetabolites signalled the advent of antifolate chemotherapy nearly a decade before dihydrofolate reductase (DHFR) was recognised as the target enzyme for these agents[4]. The intense chemical and biochemical interest generated by these discoveries has been the subject of several comprehensive reviews[5-7].



(1) folic acid R = OH R' = H [as 4(3H)-one tautomer]

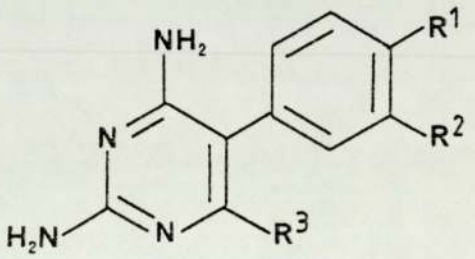
(2) aminopterin R = NH_2 R' = H

(3) methotrexate R = NH_2 R' = CH_3

Dihydrofolate reductase plays a pivotal role, via the provision of reduced folate, in the interconversion of one-carbon units essential for several anabolic processes, principally the de novo biosynthesis of nucleic acids and several essential amino acids[8]. Consequently, an inhibition of this enzyme results in the accumulation of metabolically inactive dihydrofolate and a decline in cellular metabolism, culminating ultimately in cell death.

Clinical evaluation of antifolates was initiated by Farber[9] who, in 1948, obtained temporary remissions in acute childhood leukaemia with aminopterin. Subsequent investigations with methotrexate proved the latter agent to be less toxic, and despite the evaluation of numerous analogues, methotrexate remains the antitumour antifolate of choice nearly forty years later.

Hitchings et al[10] reported that the cytotoxicity of a series of 2,4-diaminopyrimidines was also mediated through inhibition of folate metabolism and in 1954 metoprine (DDMP) (5) entered the clinic as a candidate antitumour agent[11] However, the severe toxicity associated with this agent precluded any further evaluation. Diaminopyrimidines were subsequently found to inhibit DHFR and extensive structure-activity studies have resulted in the development of analogues with inherent species selectivity as antibacterial and antimalarial agents[6,12,13].



(4) pyrimethamine R^1 = Cl R^2 = H R^3 = Et

(5) metoprine R^1 = Cl R^2 = Cl R^3 = Me

(6) etoprine R^1 = Cl R^2 = Cl R^3 = Et

The use of pyrimethamine (4) in 1971 to produce a temporary remission in a patient with meningeal leukaemia[14] stimulated a renewed interest in these agents. Hill et al[15] re-examined metoprine, a potent inhibitor of mammalian DHFR, for the treatment of methotrexate-resistant tumours and recognised the importance of concomitant folinic acid administration in alleviating toxicity[16]. However, other iatrogenic problems associated with metoprine and the closely related homologue etoprine (DDEP) (6), derive from effects unrelated to inhibition of folate metabolism, and this has generated a renewed interest in the development of less toxic successors.

It is evident that DHFR inhibitors may be considered under two categories:-

(i) The folate analogues or classical inhibitors

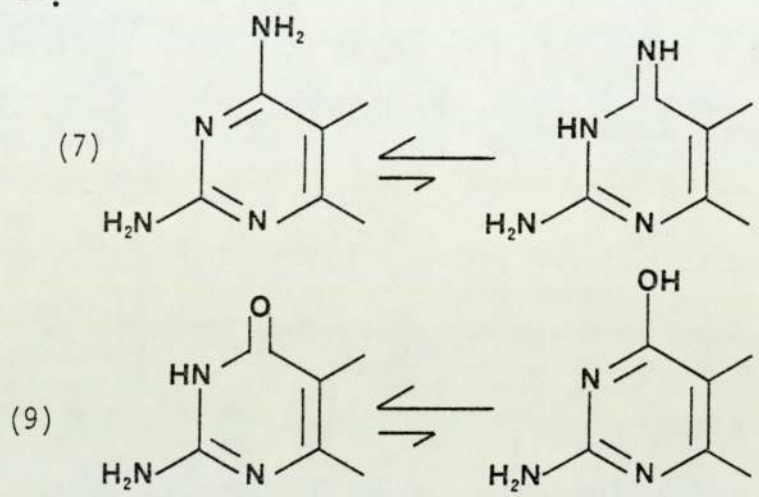
(ii) Small molecule, lipophilic, or non-classical inhibitors.

This thesis is concerned in part with the synthesis and evaluation of a series of novel lipophilic inhibitors, designed to overcome some of the problems associated with established agents. Thus a brief consideration of the characteristics of each class is appropriate.

1.2 Folate Analogues or Classical Inhibitors

Methotrexate, the prototype classical antifolate, is a potent 'pseudoirreversible' inhibitor of DHFR from all biological sources and this has been attributed to a close structural resemblance to the natural substrate[17]. Baker proposed that the isosteric replacement of a 4-hydroxy group by an amino substituent on the pteridine ring increased basicity, thus facilitating protonation and subsequent

association with an anionic site on the enzyme[18,19]. However, Zakrzewski[20] has argued that the predominant tautomer formed with a diaminopteridine (7) favours hydrogen bonding at the active site. More recent crystallographic evidence points to a combination of both factors contributing to the 'stoichiometric' binding of methotrexate to DHFR[21-23].



The polar nature of folate analogues (methotrexate log P -2.6) occurs as a consequence of the glutamate side-chain[24] and precludes any significant diffusion through cell membranes at normal concentrations. Some evidence has been obtained to suggest a limited diffusion process at the extracellular drug concentrations achieved with high-dose methotrexate regimes[25].

Chemical modification of the glutamate residue has been investigated independently in an attempt to facilitate the diffusion process[26,27] and several lipophilic methotrexate esters have been synthesised and evaluated[28,29]. The hydrophilic character of methotrexate also renders the drug inaccessible to fatty tissues, including the brain and cerebrospinal fluid; such compartmentation may provide a pharmacological sanctuary in which malignant cells are spared cytotoxic drug concentrations[30]. Intrathecal administration of methotrexate has become established practice in an attempt to

circumvent this problem.

Methotrexate gains access to cells *via* a specific transport mechanism, utilising a carrier system responsible for the transfer of reduced folate cofactors[31,32]. Evidence for a degree of selectivity in the transport (both influx and efflux) of folate analogues between normal and malignant cells has been adduced[33,34]. Since methotrexate competitively inhibits the uptake of reduced folates, including N^5-formyltetrahydrofolate, depletion of intracellular folates may constitute an alternative mechanism of action of the drug[35].

Folate analogues are recognised to undergo intracellular conversion to polyglutamyl derivatives, in a manner analogous to the natural folates, a process catalysed by folylpolyglutamate synthetase[36,37]. The polyglutamyl metabolites are retained intracellularly, in contrast to the unmetabolised drug[38] and bind to DHFR with at least an equal affinity to the parent drug[39] thus producing a prolonged inhibition of DNA synthesis and consequently increased cytotoxicity[40]. Matherly et al[41] have recently demonstrated the enhanced polyglutamation of aminopterin relative to methotrexate and suggest this as a contributory factor in the disparate potencies of these agents, aminopterin being the more toxic.

1.3 Resistance to Classical Antifolates

The emergence of resistance to methotrexate therapy was observed as a major obstacle to the successful clinical use of this agent shortly after its introduction and continues to overshadow this aspect of chemotherapy. Two forms of resistance have been described; *intrinsic* resistance, where little or no response is observed from

the outset and acquired resistance, where an initially sensitive tumour reproliferates and is refractive to subsequent chemotherapy with the same agent[42-44]. The possible factors responsible for each type of resistance are summarised (table 1.1).

Table 1.1

Possible causes of Resistance to Methotrexate (MTX)

Intrinsic	Acquired
Kinetic cells in G_0 or plateau phase	Reduced capacity for MTX transport
Low capacity for MTX poly-glutamate formation	Overproduction of DHFR (gene amplification)
Poor transport of MTX	Reduced affinity of DHFR for MTX
High DHFR levels	
Inadequate NADPH levels to facilitate MTX binding	Reduced capacity for polyglutamation
High intracellular folate levels	Opening of nucleoside salvage pathways
Rapid synthesis of DHFR	
Utilisation of salvage pathways	

A reduced capacity for transporting methotrexate is thought to contribute significantly to resistance in human cell lines[45] and this is substantiated by evidence of a concomitant decline in the transport of reduced folate cofactors observed with a methotrexate resistant hepatoma subline[46]. A related mechanism whereby exclusion from resistant cells is possible has been propounded on the basis that experimental elevation of the lipid composition of the cell membrane decreases methotrexate accumulation in L1210 murine leukaemia cells[47].

An induced overproduction of DHFR by mutant cells has been demonstrated in several resistant cell lines[48] and has been attributed to gene amplification[49]. The elevated enzyme concentration may constitute 10% of total intracellular protein[50].

Resistance to methotrexate by virtue of an alteration of the target DHFR enzyme has been documented and has led to the expression of an enzyme with a 10^5 fold decrease in affinity relative to the wild-type DHFR. Haber et al[51] evaluated the mutant and unmodified enzymes for sensitivity against a series of lipophilic inhibitors and concluded that the disparate inhibition observed, occurred as a consequence of structural differences associated with the p-amino-benzoyl binding region of the protein.

The aforementioned resistance mechanisms serve to increase effectively the concentration of available intracellular DHFR and the implications of this have been emphasised by Jackson et al[52] who estimate that 4% of intracellular enzyme activity may be sufficient to sustain adequate reduced folate levels. The development of resistance by more than one mechanism in a heterogenous population of tumour cells has been observed[53]. Cowen et al[54] have recently characterised a human breast cell line with multiple defects including a reduced capacity for methotrexate polyglutamation.

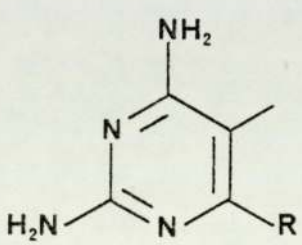
A tumour cell line resistant to methotrexate by virtue of a salvage pathway, whereby depleted nucleosides are replenished, has been described[55]. Dipyridamole, a non-specific nucleoside transport antagonist, has been shown to prevent the reversal, by thymidine, of methotrexate cytotoxicity[56].

1.4 Non-classical, Lipophilic or Small-molecule Inhibitors

As a class, these molecules are characterised by the absence of a glutamate residue and consequently are invariably lipophilic in nature. A rekindled interest in the use of metoprine (5) for the treatment of methotrexate-resistant tumours has stimulated the synthesis and clinical assessment of numerous candidate small-molecule DHFR inhibitors, including diaminopyrimidines[57,58] (11), dihydrotriazines[19] (12), quinazolines[59,60] (13) and pyrido-pyrimidines[61,62] (14) (fig 1.1). Of these, only the diaminopyrimidines will be considered in detail.

Intensive structure-activity studies have been implemented on diaminopyrimidine DHFR inhibitors and the chemical features implicit for activity are well established[7,12,19].

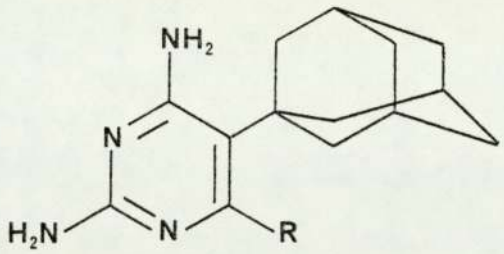
The 2,4-diamino-1,3-diazine moiety (15) provides the unifying feature of all lipophilic DHFR inhibitors and is a prerequisite for significant activity.



R = small alkyl

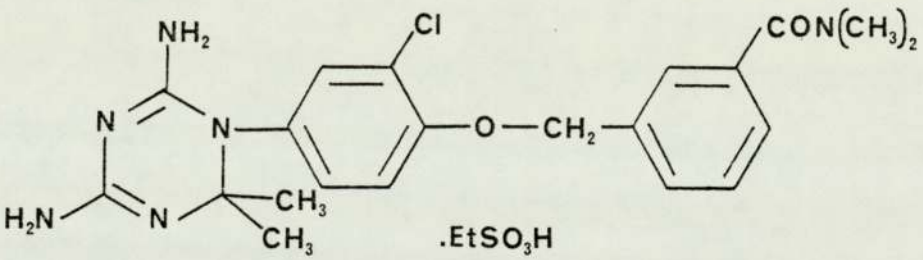
(15)

Figure 1.1 Non-classical, lipophilic or small-molecule DHFR inhibitors

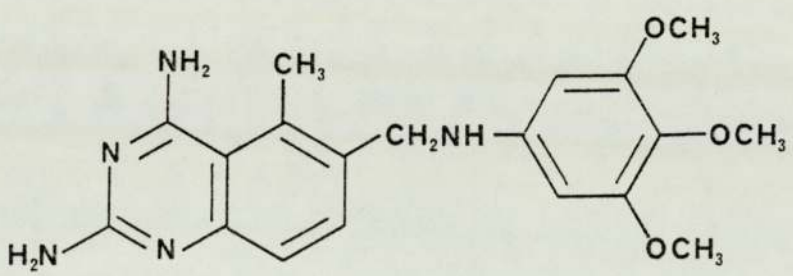


(11A): R = Me, DAMP

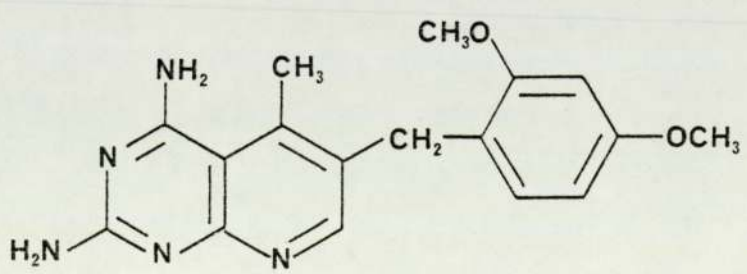
(11B): R = Et, DAEP



(12): Triazinate



(13): Trimetrexate



(14): BW301U

Favourable binding to the enzyme results from the introduction of a lipophilic group at position 5 of the pyrimidine ring. Ho et al[63] observed a correlation between the affinity of a series of 5-alkyldiaminopyrimidines for DHFR and their hydrophobicity and concluded that a bulky rigid substituent confers maximal activity. A relationship between lipophilicity, as measured by the octanol/water partition coefficient, and cellular uptake and cytotoxicity was also demonstrated[64]. Thus the 5-adamantyl analogues (11A and B) exhibit an activity against DHFR comparable to methotrexate[65] and this has been attributed to the occupation of a hydrophobic pocket at the enzyme active site by the lipophilic 5-substituent[66,67]. In addition, the conformation of the group at the 5-position is important since introduction of a lipophilic 1-naphthyl substituent (25) (see chapter 3) eliminates activity against DHFR. Computer modelling studies, using the co-ordinates of a Lactobacillus casei DHFR-methotrexate-NADPH ternary complex, reveal that a superimposition of the 1-naphthyl analogue into the methotrexate binding domain imposes severe steric interactions between the naphthyl group and side chains within the binding cleft. In contrast DAMP (11A) adopts a conformation facilitating association with the hydrophobic pocket[66].

Lipophilic diaminopyrimidines enter cells via a rapid passive, and possibly facilitated, diffusion process[68] and intracellular concentrations 30-100 fold that in the extracellular medium have been reported[64]. Consequently, tumours resistant to methotrexate due to a reduced capacity to transport the drug may exhibit a collateral sensitivity to metoprine[15,68-70], trimetrexate[71], BW301U[72] and other lipophilic inhibitors. Sirotnak et al[73] suggested, on the basis of evidence adduced with a methotrexate resistant L1210 cell line, that

collateral sensitivity may manifest as a consequence of a concomitant reduced rate of accumulation of 5-methyltetrahydrofolate into transport-resistant tumour cells.

Metoprine and etoprine bind extensively to plasma components, distribute rapidly throughout all body compartments including brain, pancreas and testes[74] and are effective against experimental brain tumours refractory to methotrexate[75,76].

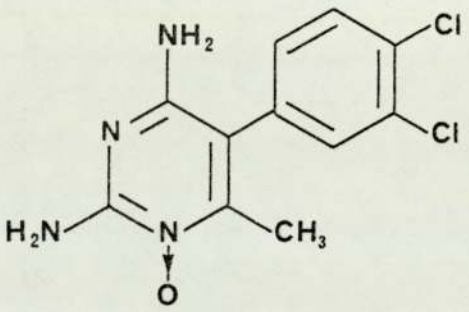
Non-classical inhibitors may have a role to play in the treatment of tumours resistant by virtue of an altered enzyme. Bertino[35] has argued that since specificity of inhibition may be achieved between mammalian and bacterial or protozoal DHFR, by analogy development of an inhibitor specific for the mutant enzyme is conceivable. Evidence to corroborate this has recently been reported by Hamrell[77] who demonstrated collateral sensitivity to several lipophilic inhibitors with a 3T6 murine cell line resistant to methotrexate, characterised by an enzyme with an altered affinity for the drug.

Browman et al[78] have developed an L1210 cell line resistant to metoprine without cross-resistance to methotrexate. Intracellular enzyme levels and affinity for metoprine were identical in mutant and wild-type cultures, as was accumulation of the drug, but the underlying resistance mechanism has not been established.

The absence of a glutamate residue precludes intracellular metabolism of metoprine and related agents to poly-γ-glutamyl derivatives, thus obviating resistance mediated through a mutant folylpolyglutamyltransferase[79]. In addition, the hepatotoxicity associated with chronic methotrexate administration for the treatment of psoriasis has been ascribed to polyglutamate formation[80] and substantiates the possibility of utilising lipophilic inhibitors in

psoriatic conditions. Unlike methotrexate lipophilic agents may be absorbed percutaneously[81].

The metabolism of lipophilic diaminopyrimidines has been investigated in the rat[82] and to a limited extent in man[74]. Metoprine is metabolised principally to the N-1-oxide (16) and its glucuronide conjugate, whilst pyrimethamine and etoprine predominate in the urine as the N-3-oxide hydroxylated on the ∝-position of the 6-ethyl substituent (17).



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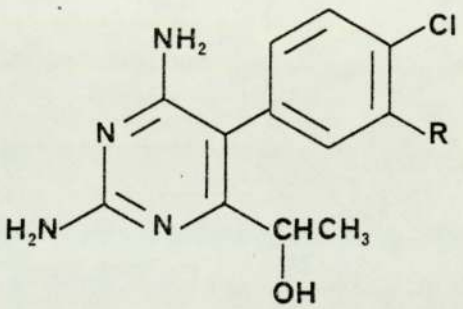
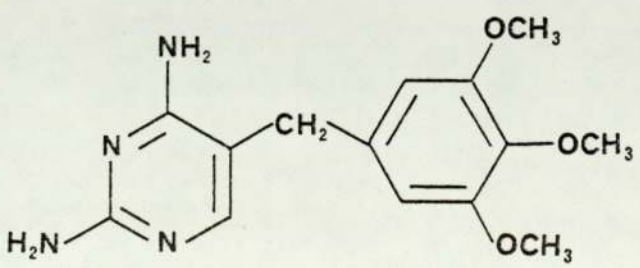


(17)

R = H Pyrimethamine metabolite

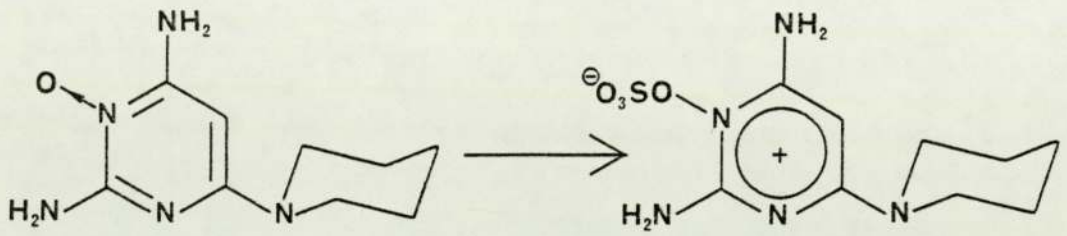
R = Cl Etoprine metabolite

Trimethoprim N-1-oxide, a metabolite of the antibacterial diaminopyrimidine trimethoprim (18), undergoes reduction to the parent compound and subsequent recycling in vivo[82]. Although a similar mechanism for metoprine or etoprine remains to be established, such recycling may influence the pharmacokinetics of lipophilic diaminopyrimidines in the body.



(18)

McCall et al[83] have recently described the biotransformation of a vasodilator minoxidil (2,4-diamino-6-piperidinylpyrimidine 3-oxide) (19) in vivo to the pharmacologically active 3-oxide sulphate (20), thus providing evidence for the further biological modification of diaminopyrimidine-N-oxides.



(19) (20)

Toxicity associated with the use of metoprine and related diaminopyrimidine antifolates have limited their wider therapeutic application. Metoprine has a protracted biological half-life in excess of 10 days in man[84] possibly as a consequence of extensive protein binding and distribution within lipid compartments[74]. Persistently high drug concentrations result in a cumulative toxicity as killing of resting cell populations occurs, principally in the bone marrow, leading to severe myelosuppression. Concurrent administration of folinic acid has proved of value in circumventing such toxicity[85]. A second form of toxicity, unrelated to folate metabolism, is mediated through the elevation of histamine by an inhibition of its metabolism. Metoprine administered at high doses to animals induces CNS stimulation, convulsions and death frequently within minutes[86]. Investigations by Cohn[87] and, more recently, Duch

et al[88-90], show metoprine to be a potent inhibitor of histamine-N-methyltransferase, elevating histamine levels in brain and other tissues. The pyridopyrimidine BW301U (14) has been promoted on the basis that minimal effects on histamine metabolism have been observed[62,91].

In summary, it is evident that both classical and lipophilic DHFR inhibitors have physico-chemical and pharmacological problems associated with their clinical use. The development of resistance to any novel folate analogue may be envisaged on the basis that transport into malignant cells will be subject to the vagaries of a carrier mechanism. Consequently, although analogues inherently more potent than methotrexate have been synthesised and evaluated few advantages over the parent compound have emerged. However, since the demonstrated therapeutic potential of metoprine, a prototype lipophilic inhibitor, has been overshadowed by toxicity associated with its use, the pursuit of novel non-classical DHFR inhibitors may thus be justified.

CHAPTER 2

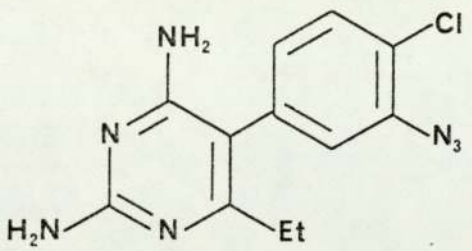
m-AZIDOPYRIMETHAMINE (MZP)

2.1 Introduction

Aromatic azides have found extensive use as photoaffinity reagents in several areas of molecular biology[92]. The azido group undergoes facile degradation to a nitrene intermediate on photolysis; the nitrene may subsequently react with a neighbouring group to form a covalent bond. Stevens and coworkers[93,94] have explored the possibility of adapting this principle for chemotherapeutic purposes on the basis that a molecule encompassing a strategically placed azido substituent might, on irradiation, or possibly biotransformation, associate irreversibly with a target macromolecule.

In one such study[93] a series of small-molecule DHFR inhibitors embracing an azido group, were synthesised as candidate 'active-site directed radioaffinity reagents'. However, although preliminary results show promise, the viability of this approach remains to be established.

2,4-Diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine (MZP) (21) an azido analogue of the antimalarial agent pyrimethamine, was developed to test the aforementioned hypothesis. However, the inherent antitumour activity of this agent, coupled with some intriguing physico-chemical characteristics, has warranted evaluation of MZP as a lipophilic DHFR inhibitor per se.



(21)

The following criteria serve to illustrate at least some of the properties required by a candidate lipophilic DHFR inhibitor:-

i) Potent inhibition of mammalian DHFR

ii) Adequate lipophilicity

iii) A pKa close to physiological pH, to optimise an equilibrium between the neutral transportable and active protonated species

iv) A therapeutically acceptable biological half-life

v) Weak inhibition of histamine-N-methyltransferase

vi) The absence of active metabolites

vii) Ease of synthesis and formulation.

The important characteristics of MZP have been summarised and compared with metoprine and methotrexate (table 2.1).

Table 2.1

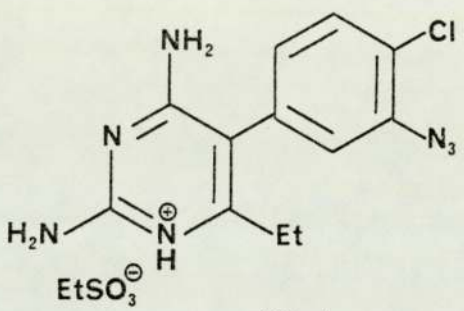
A comparison of the characteristics of MZP with methotrexate and metoprine

Property	Methotrexate	m-Azidopyrimethamine	Metoprine
Inhibition of DHFR (K_IM)	10^{-12}	10^{-9}	10^{-10}
Partition coefficient [a] (log P)	-1.85	2.94	2.82
Dissociation constant (pKa)	4.7	7.19	7.15
Biological half-life in humans (h)	12.20	4.6[b]	>200
Inhibition of histamine-N-methyltransferase (%)	Inactive	71 at 10^{-4} M	81 at 10^{-6} M
Solubility	Water	Lipid	Lipid
Formulation	Sodium salt	Ethanesulphonate (MZPES)	Lactate or Ethane-sulphonate salt

[a] octanol-water

[b] plasma half-life in mice

Clearly, MZP embraces many of the desirable characteristics of a lipid soluble inhibitor and yet in mice exhibits the short-lived pharmacokinetics of methotrexate, thus hopefully circumventing the problem of cumulative toxicity. As an inhibitor of histamine-N-methyltransferase MZP compares with the widely used antimalarial pyrimethamine[95], and consequently toxicity problems from this quarter are not envisaged. Bliss[93] reported a high-yielding three step synthesis from pyrimethamine and the ethanesulphonate salt (MZPES) (21a) was selected for formulation purposes.



(21a)

2.2 Antitumour Activity

The antitumour spectrum of activity for MZP against a wide variety of murine tumours is summarised (table 2.2)[96].

The cytotoxicity of MZP againt the L1210 leukaemia and B16 melanoma is particularly noteworthy since both are refractory to metoprine and this does not parallel the relative potencies of these agents against DHFR *in vitro*. The basis for this disparate sensitivity has not been established. In contrast, the M5076 tumour, which is naturally resistant to methotrexate, but highly responsive to metoprine[73], proved to be the most sensitive tumour to MZP.

In a preliminary study Hill[97] has demonstrated collateral sensitivity to MZPES with a methotrexate resistant L5178Y murine

leukaemia cell line, where resistance has been attributed to defective transport.

Table 2.2

Antitumour activity of MZP

Tumour	Optimum T/C (%) [a]	Assessment [b] (Metoprine in brackets)
P388 leukaemia	151	+ (+)
L1210 leukaemia	158	++ (+)
B16 melanoma	157	++ (-)
TLX5 lymphoma	135	+ (-)
M5076 reticulum cell sarcoma	174	++ (NT)
Lewis lung carcinoma	<140	- (-)
CD8F mammary	24	- (-)
Colon 38	42	- (-)

[a] The ratio of the median survival time (days) for treated (T) and control (C) animals expressed as a percentage value.

[b] NCI activity criteria in "Chemical Structures of Interest to the Division of Cancer Treatment", Vol III, (1983)

NT Not Tested

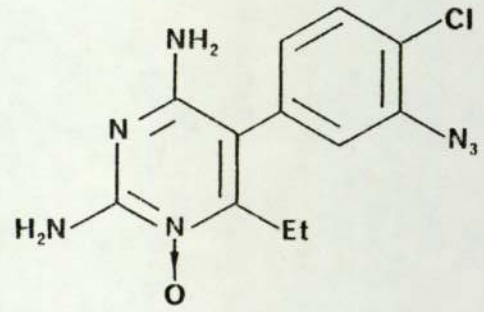
2.3 Pharmacokinetics and Metabolism

MZP has recently entered the clinic as a candidate lipophilic antifolate and is currently undergoing Phase I evaluation as the ethanesulphonate salt (MZPES). A sensitive and specific HPLC assay has been developed to allow determination of unchanged drug and metabolites in plasma and tissues[98]. Preliminary results in mice indicate a rapid absorption following oral and intraperitoneal administration, with subsequent distribution throughout the brain, lung and other tissues[99].

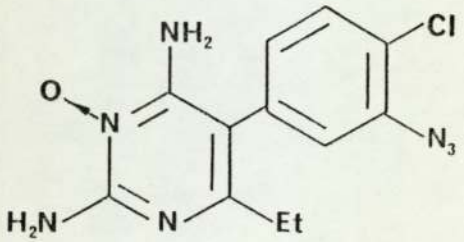
Little has been published regarding the metabolism of azides. The alkylazido substituent of azidomorphine, one of the few azides clinically evaluated in a human subject, is relatively resistant to biotransformation[100]. In contrast, a series of 4-azidobenzene-sulphonamides administered orally to rats, were detected in the urine principally as the appropriate arylamines[101]. Since aromatic azides are rapidly reduced by thiols at room temperature to afford arylamines[102,103], the corresponding reaction with glutathione *in vivo* may provide a mechanism for the biotransformation of aromatic azides.

m-Aminopyrimethamine (MAP) (22) has been identified in the urine of mice following administration of MZP, but together with unchanged drug, accounts for only 15% of the initial dose[98]. Biotransformation and subsequent elimination of MZP as the N-oxide or α-hydroxyethyl metabolite remains to be demonstrated. The possible biotransformation products of MZP are summarised in scheme 2.1.

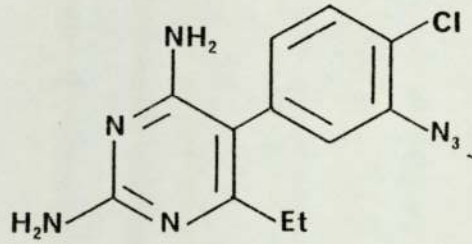
Scheme 2.1 Prospective biotransformation products of MZP



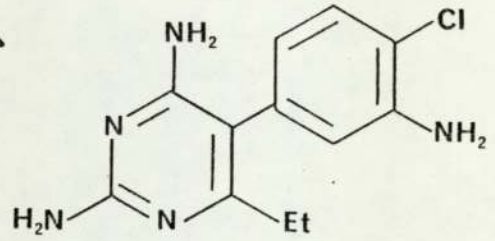
(77): MZP 1-oxide



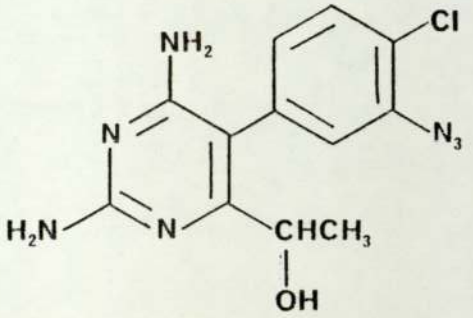
(78): MZP 3-oxide



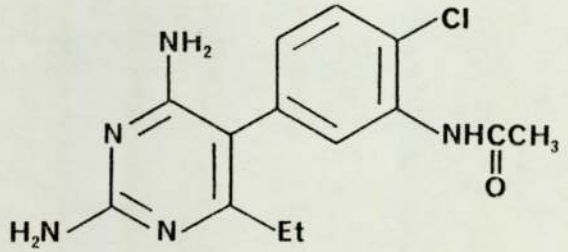
(21): MZP



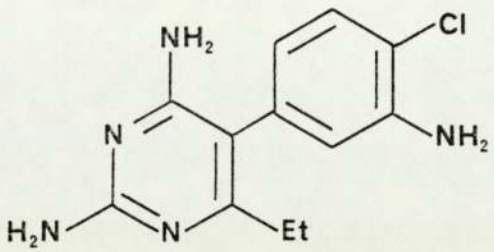
(22): MAP



α-hydroxy MZP



(90): N-acetyl MAP



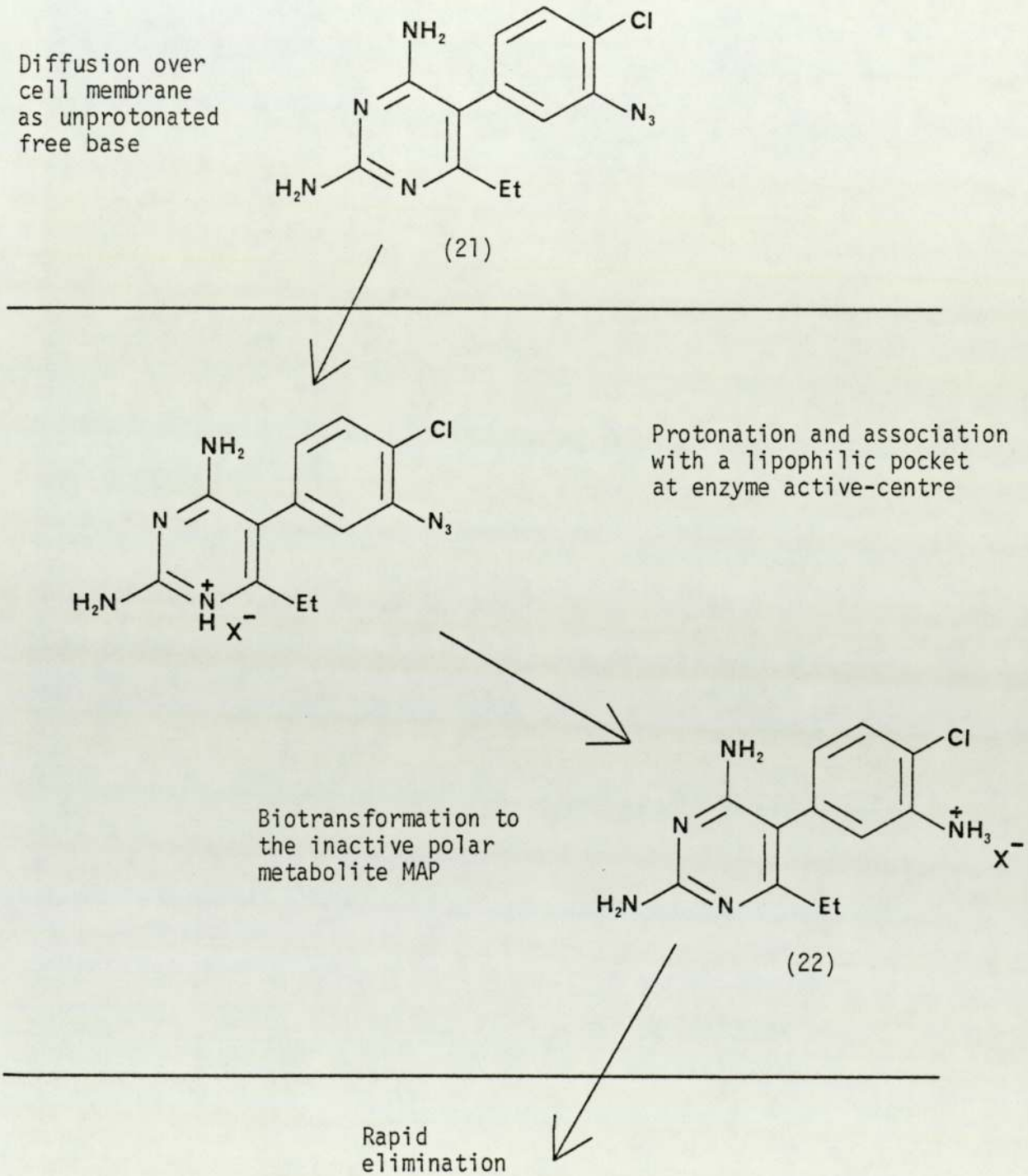
(22)

A structural similarity between MZP, metoprine and etoprine may be inferred on the basis that, with respect to size, electronic effects and lipophilicity, the azido group is bioisosteric with a bromo substituent[104,105]. Stevens has proposed a novel pro-drug role for the azido substituent in this context[106]. It is evident that introduction of this functional group into the molecule does not compromise or significantly alter its pharmacological character: consequently, diffusion through the cell membrane, protonation on the pyrimidine ring and association with a hydrophobic pocket within the enzyme active site is facilitated. However, subsequent bioreduction to the relatively polar arylamine (MAP) should preclude any association with a hydrophobic domain, culminating in an elimination of activity against DHFR. Moreover, the formation of a basic centre at a site remote from the pyrimidine ring may result in protonation on the aromatic ring, with a subsequent loss of activity (fig 2.1).

m-Aminopyrimethamine (MAP), the metabolite central to this hypothesis, exhibits physico-chemical characteristics favouring its rapid elimination[93] (log P 1.25, pKa 7.6). Subsequent acetylation or

glucuronide conjugation may account for the low levels identified in the urine of mice following MZP administration.

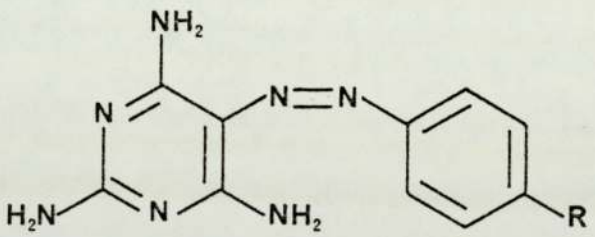
Figure 2.1 MZP as a pro-drug of MAP



CHAPTER 3

DIAMINOPYRIMIDINE CYTOTOXICITY UNRELATED TO INHIBITION OF DHFR

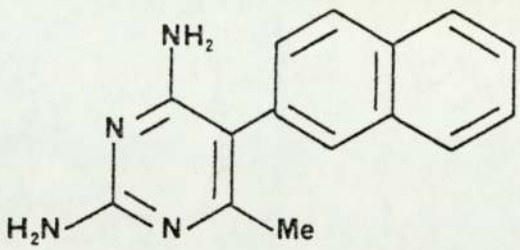
The possibility that some of the growth inhibitory effects of diaminopyrimidines might be mediated through association with a locus or mechanism of action unrelated to DHFR was recognised as early as 1952. Hitchings et al[107] observed that metoprine, in addition to inhibiting folate reduction by Streptococcus faecalis, also antagonised folinic acid utilisation by this organism. A series of arylazopyrimidines (23) synthesised by Hampshire and colleagues exhibited activity against several tumour lines, but no correlation between cytotoxicity and inhibition of DHFR was demonstrated[108].



(23)

R = SO_3H, SO_2NH_2, CO_2H, CHO

Kavai et al provided evidence that structurally related diaminopyrimidines may have different mechanisms of action[109]. A comparison of the in vitro cytotoxicity and DHFR inhibitory activities of two isomeric naphthylpyrimidines revealed a disparate relationship between these parameters. Thus, although the 2-naphthyl analogue (24) exhibited a cytotoxicity in accordance with activity against the enzyme, the 1-naphthyl congener (25) only a weak non-competitive inhibitor of DHFR, (chapter 1.4) is a relatively potent inhibitor of cell growth.



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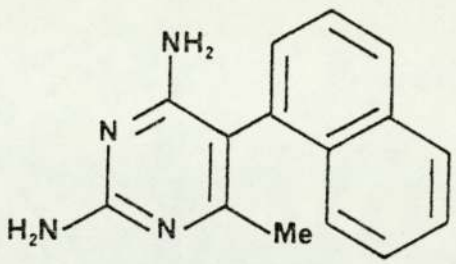


(25)

Several studies regarding the biochemical aetiology of these observations have been conducted. In one investigation the effect of equimolar concentrations of methotrexate and metoprine on the growth of a methotrexate resistant 3T6 murine fibroblast line was determined[110]. Resistance was ascribed to an elevated level of DHFR and not exclusion of the drug. At concentrations refractory to methotrexate an 80% inhibition of deoxyuridine incorporation with metoprine was reported. The authors propose that sensitivity to this agent is not fully accountable in terms of DHFR inhibition. However, a comparison of the enzyme from mutant and parent lines was not undertaken and the expression of a modified DHFR with a diminished affinity for methotrexate may account for the observed collateral sensitivity to metoprine.

Browman et al[111], in a subsequent investigation, conclude that metoprine and methotrexate have an identical intracellular mechanism of action and that any observed dissimilarities reflect the membrane transport and unique pharmacokinetic properties of the former. However, the possibility of a second low affinity locus for metoprine, not apparent at the concentrations utilised in this experiment, was not excluded.

The most compelling evidence in support of a folate-independent site is provided by Greco and Hakala[112,113] who, on the basis of earlier observations by Ho et al[65], examined the activity of a series of lipophilic diaminopyrimidines against several murine and human cell lines in culture. Addition of folinic acid to the culture medium only partially reversed diaminopyrimidine cytotoxicity in a non-competitive manner, in contrast to methotrexate where growth inhibition was completely reversed. More importantly, cells cultured under folate-independent conditions exhibited a sensitivity to the lipophilic inhibitors not evident with methotrexate and a similar potency was observed regardless of inhibitor type. This second folate-independent site was unrelated to depletion of methionine and did not involve nucleic acid polymerases.

More recent biochemical evidence for a folate-independent mechanism has been documented[91,114]. The cytotoxicity of BW301U (14) cannot be fully explained in terms of DHFR inhibition, since complete reversal of growth inhibition requires the addition of nucleosides to a medium supplemented with hypoxanthine, thymidine and glycine[62].

The aforementioned studies point to a second, relatively sensitive site or mechanism of action for diaminopyrimidines as yet to be characterised. Concurrent administration of folinic acid in order to circumvent the toxicity associated with metoprine and related compounds is well established. However, since it is evident that at higher concentrations, the cytotoxicity of diaminopyrimidines may be only partially reversed with folinic acid <u>in vitro</u>, a recognition of the implications of these observations would be prudent. Moreover, elucidation of the site or mechanism whereby folate-independent toxicity occurs is justified, as a basis for the rational design of novel potential antitumour agents acting predominately at this centre.

CHAPTER 4

RATIONALE AND OBJECTIVES

The previous chapters serve to illustrate some of the problems associated with antifolate chemotherapy as regards the use of DHFR inhibitors and the potential role of MZP as a candidate second-generation lipophilic inhibitor. Secondly, evidence favouring the existence of a folate-independent target for the small-molecule inhibitors has been presented. This thesis concerns two separate projects which overlap with regard to the chemical and biological techniques used and, in retrospect, with the results obtained:

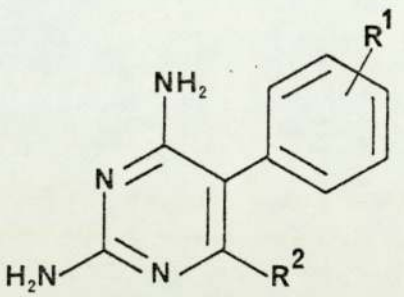
i) To investigate the chemistry and biochemistry of a series of azidopyrimidines, including MZP, and synthesise and evaluate the possible metabolites and degradation products of these compounds.

ii) To consider the possibility of developing modified diaminopyrimidines as potential molecular probes for the aforementioned alternative target, with a view to screening the most promising agents, for <u>in vitro</u> and <u>in vivo</u> antitumour activity.

4.1 <u>m-Azidopyrimethamine Derivatives</u>

It is evident from the comprehensive structure-activity studies implemented on 2,4-diaminopyrimidines[7], that both the potency and selectivity of DHFR inhibition may vary considerably with discrete

structural modification of the molecule. In the lipophilic 2,4-diamino-5-phenyl-6-alkylpyrimidine series (26) the most pronounced effects are observed with changes in the nature and position of aromatic substituents (R^1) and the magnitude of a 6-alkyl group (R^2).



(26)

Since diaminopyrimidines encompassing an azido substituent on the 5-phenyl ring constitute a novel class of DHFR inhibitor, a series of derivatives of MZP were synthesised and evaluated in an attempt to maximise activity against the target enzyme.

The unique pharmacokinetic properties of MZP have been attributed to the formation and subsequent elimination of the amine metabolite MAP. Introduction of an electron-donating substituent onto the 5-phenyl ring, ortho or para to the amino group, should increase the basicity of this group, facilitate protonation and thereby abolish activity against the enzyme. Thus several analogues of MZP embracing hydroxyl, alkoxy and substituted amino groups on the aromatic ring were prepared in order to investigate this hypothesis.

Crystallographic studies on lipophilic inhibitors of DHFR have revealed much as to the geometry of binding to the enzyme[66,115] and an investigation of the nature of MZP and its analogues in the crystalline state was warranted. Several salts suitable for this purpose were thus synthesised.

4.2 Putative Metabolites and Degradation Products of MZP

As discussed earlier (chapter 2.3) little is known regarding the biological fate of azides. Also information concerning the decomposition of these compounds under the conditions required for formulation, sterilisation and storage remains to be adduced. Consequently, a study of the metabolism, toxicology and stability characteristics of MZP was initiated; this required the synthesis of the appropriate reference compounds. Evidence for a rapid degradation of the drug *in vivo* would also substantiate the rationale central to its design.

The metabolism or degradation of MZP and its analogues may, in principle, occur by several mechanisms at a variety of sites within the molecule, providing a multitude of products. One objective therefore centred on the synthesis and characterisation of a comprehensive series of reference compounds. Furthermore, the evaluation of any putative metabolites for activity against DHFR was justified as a foundation on which to base an explanation for the short biological half-life of the drug.

Thus examples of six classes of possible products were synthesised (fig 4.1):

i) 2,4-diaminopyrimidines (27)

ii) 2-aminopyrimidin-4(3H)-ones (28)

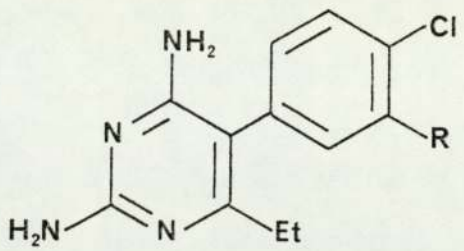
iii) 4-aminopyrimidin-2(1H)-ones (29)

iv) 2,4-diamino-6-(1-hydroxyethyl)pyrimidines (30)

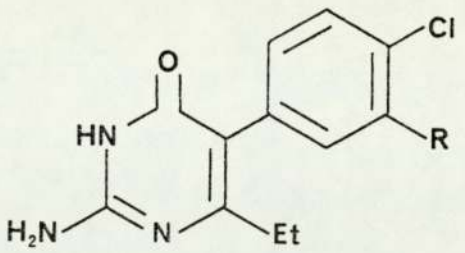
v) 2,4-diaminopyrimidine-1-oxides (31)

vi) 2,4-diaminopyrimidine-3-oxides (32)

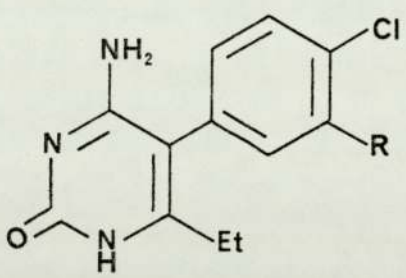
Figure 4.1 Reference compounds required for metabolic and stability studies on MZP



(27)



(28)



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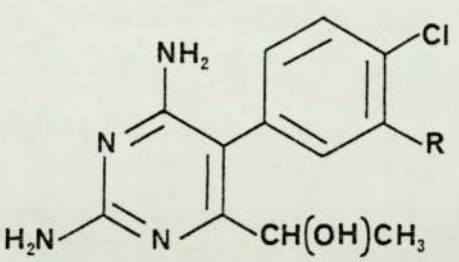
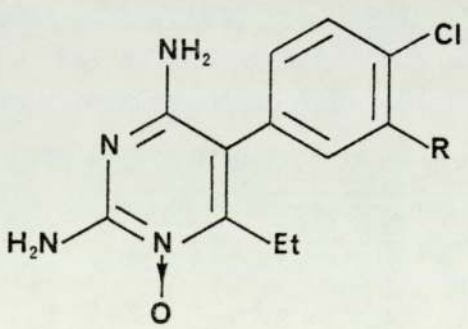


(30)



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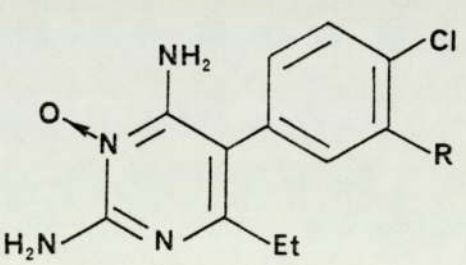


(32)

R = NO_2,NH_2,NHAc,N_3.

4.3 Diaminopyrimidines as probes for a target unrelated to DHFR

Chemical and biological studies on diaminopyrimidines have previously invariably centred around the development of better inhibitors of DHFR. Indeed, the term 'antifolate' has become immutably associated with an inhibitor of this enzyme[116]. Consequently, with several notable exceptions[37,117-119], the possibility of exploiting other potential target enzymes in the folate pathway remains largely unconsidered. Moreover, little has been documented regarding the development of 2,4-diaminopyrimidines as antitumour agents, where cytotoxicity occurs *via* a mechanism unrelated to folate metabolism. This notwithstanding strong evidence to support the existence of a folate-independent site for compounds of this nature.

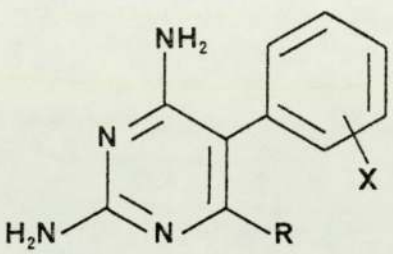
The primary objective of this work was based on observations that diaminopyrimidines encompassing structural features incompatible with activity against DHFR, may still inhibit cell growth at an unidentified locus elsewhere. In essence, a series of diaminopyrimidines were synthesised embracing structural modifications precluding inhibition of the enzyme, in order that such compounds might fulfil a role as biochemical probes for the aforementioned alternative target. Theoretically, a variety of chemical alterations would be predicted to abolish, or compromise, activity against DHFR, and these include:

i) hydrolysis, alkylation or acylation of the 2- or 4-amino substituent;

ii) oxidation or alkylation of the heteroatoms;

iii) introduction of an ionic group into the 5-position of the diaminopyrimidine.

In principle, several of the potential metabolites described previously (fig 4.1) may be considered in this context. However, it is proposed that the synthesis of a class of diaminopyrimidines bearing ionic substituents in the aromatic ring might represent a more novel strategy, since association with the hydrophobic region on the active site of DHFR, a prerequisite for activity, will be prevented and it is conceivable that pyrimidines incorporating a cationic group may have a membrane directed mechanism of action.



(33)

R = small alkyl group

X = basic or ionic substituent

A series of 2,4-diaminopyrimidines bearing a basic or cationic 5-substituent (33) was thus synthesised and screened against mammalian DHFR, in order to confirm that inhibition of this enzyme did not represent the primary mechanism of cytotoxicity.

Finally, the most promising compounds, ie those inactive against DHFR, were evaluated for <u>in vitro</u> and <u>in vivo</u> antitumour activity, in an attempt to select possible candidate biological probes for the folate-independent site under consideration.

SECTION 2

RESULTS AND DISCUSSION

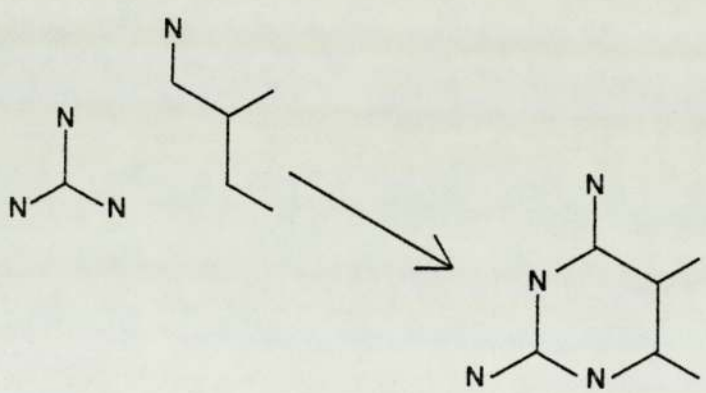
CHAPTER 5

SYNTHESES IN THE AZIDOPYRIMIDINE SERIES

5.1 2,4-Diaminopyrimidines

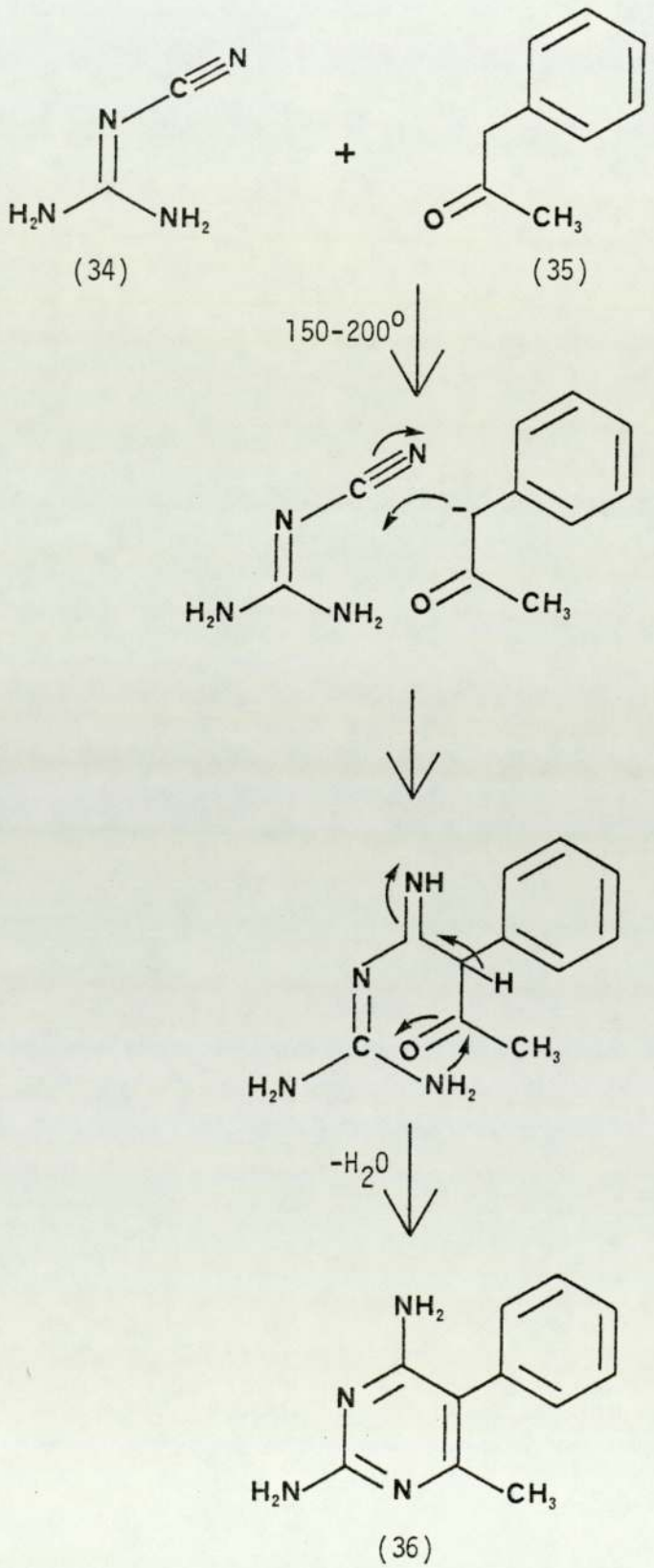
Recognition of the therapeutic potential of pyrimidines and their importance in biological systems has resulted in extensive studies regarding the synthesis and chemical properties of these molecules[120,121]. Several synthetic procedures for the preparation of diaminopyrimidines have been developed, the most useful of which involve a direct ring forming reaction usually *via* the condensation of guanidine with a 1,3-bifunctional 3 carbon fragment bearing the requisite substituents (scheme 5.1).

Scheme 5.1 Synthesis of the pyrimidine ring



Two principal synthetic methods have found widespread application for the preparation of diaminopyrimidines encompassing substituents necessary for activity against DHFR; the first, a one-step synthesis pioneered by Modest et al[122-124], entails heating a mixture of dicyandiamide (cyanoguanidine) (34) and a ketone (35) at

Scheme 5.2 Diaminopyrimidine synthesis (Modest et al)



150-200° for 2-44 h with the removal of water as formed. In some cases, yields may be improved by the use of a high-boiling solvent or addition of a base catalyst of the quaternary ammonium type. The reaction is thought to proceed *via* a slow decomposition of dicyandiamide to ammonia, base catalysed generation of a carbanion by proton loss from the α-carbon of the ketone, and subsequent cyclisation to the diaminopyrimidine (36) with elimination of water (scheme 5.2).

In the present work this method was adopted initially for the synthesis of 2,4-diamino-6-methyl-5-phenylpyrimidine (36) from dicyandiamide and phenylacetone. However, although the product obtained proved identical to that reported and to an authentic sample synthesised by the alternative method, yields of less than 10% were common and extensive purification was necessary to remove traces of melamine formed in a side reaction. Attempts at optimisation by inclusion of a solvent or base catalyst and extending the reaction time were unsuccessful, as was the addition of a molar excess of dicyandiamide which purportedly reduces the incidence of self-condensation reactions.

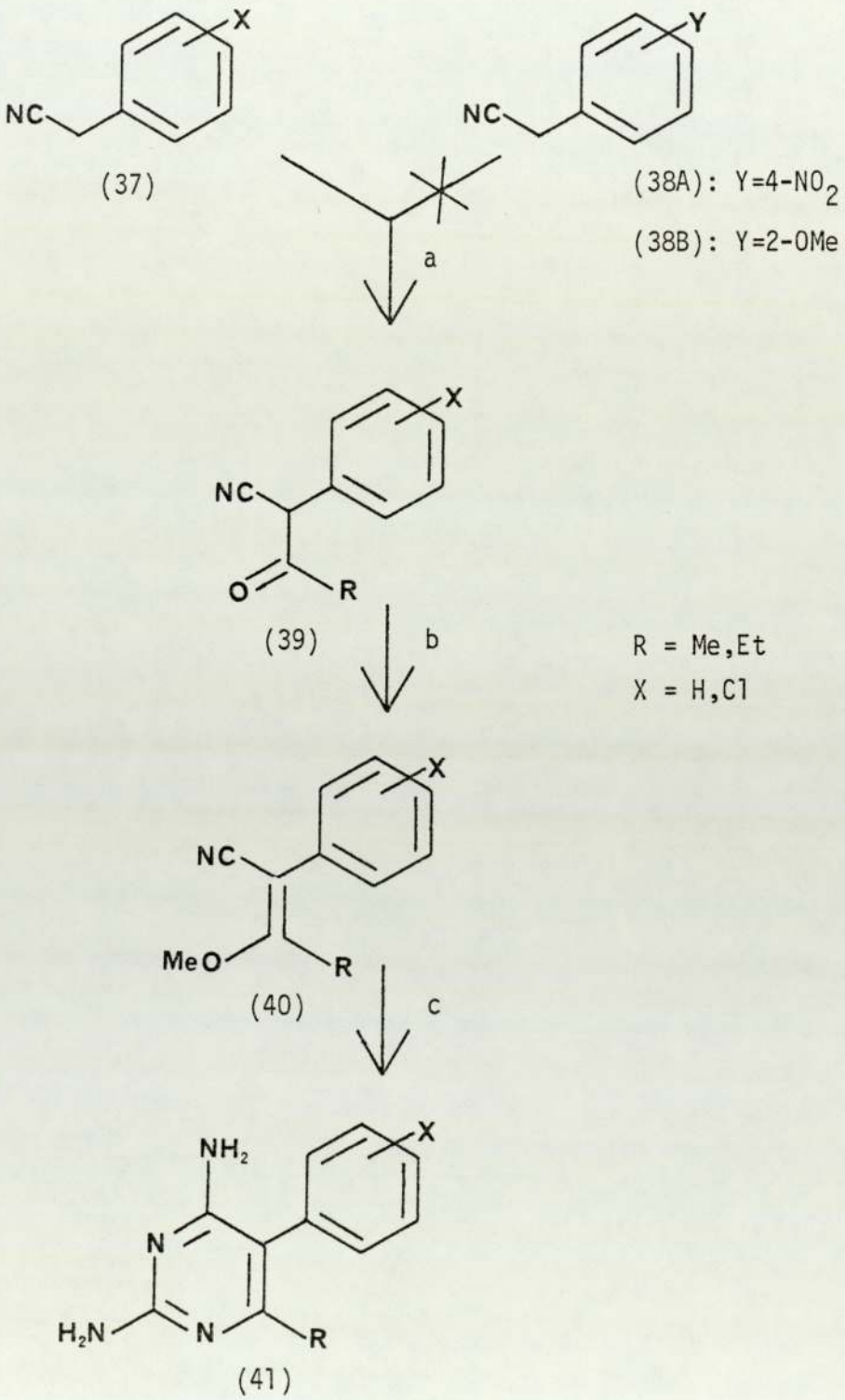
The second and most universally exploited route to diamino-pyrimidines involves three steps:-

(i) the base catalysed condensation of an arylacetonitrile (37) with an alkyl ester *via* a Claisen type reaction;

(ii) methylation of the β-ketonitrile (39) to furnish the corresponding methoxyacrylonitrile derivative (40);

(iii) condensation of (40) with guanidine in ethanolic sodium ethoxide to give the requisite diaminopyrimidine (41) (scheme 5.3).

Scheme 5.3 Diaminopyrimidine synthesis (Russell and Hitchings)



a. RCO_2Et in NaOEt/EtOH; reflux 4-6 h

b. CH_2N_2 in Et_2O; 20°; 12 h

c. $(NH_2)_2CNH$ in NaOEt/EtOH; reflux 10-15 h

Initial conversion to the methoxyacrylonitrile (40) is necessary since early attempts to condense directly the β-ketonitrile with guanidine afforded an aminotriazine[125] and several reagents including diazomethane[3] aliphatic orthoesters[126,127] and dimethyl sulphate[128] have been adopted for this purpose.

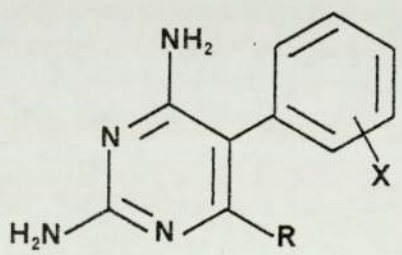
The requisite diaminopyrimidines were conveniently synthesised following the original literature method described by Russell and Hitchings[3]; thus methylation of the β-ketonitrile (39) prepared in moderate yield, with an excess of diazomethane in ether afforded the corresponding methoxyacrylonitrile (40) in good yield. Diazomethane was prepared immediately prior to use by the action of ethanolic potassium hydroxide on N-methyl-N-nitroso-p-toluenesulphonamide ('Diazald') and collected by codistillation in ether using fire-polished glassware[129]. The progress of the methylation reaction was not monitored but in all cases spectroscopic examination of the product (i.r. and n.m.r.) after 12 h showed an absence of reactants. In order to minimise explosion hazards excess diazomethane was destroyed by the dropwise addition of acetic acid to the reaction mixture until the yellow colouration had discharged, when removal of the solvent by vacuum evaporation was considered safe. The methoxyacrylonitrile derivatives were condensed with guanidine without further purification. In contrast to the literature method reaction times of 12-15 h gave improved yields of the diaminopyrimidines which invariably crystallised from the reaction mixture on cooling, thereby obviating the reported purification procedure.

The synthesis of 2,4-diaminopyrimidines bearing a nitro substituent on the 5-phenyl ring was desirable but all attempts at condensing 4-nitrophenylacetonitrile (38A) with ethyl propionate

under base catalysed conditions were unsuccessful, and furnished either starting materials or an intensely coloured tar. Similar efforts to prepare ∝-propionyl-2-methoxyphenylacetonitrile as a precursor of the target compound 2,4-diamino-5-(2-methoxyphenyl)-6-ethylpyrimidine (161) (chapter 10) failed and only starting materials were recovered following prolonged refluxing of the reactants at elevated temperatures. The reluctance of 2-methoxyphenylacetonitrile (38B) to condense with ethyl propionate may be attributable to electronic effects rather than steric hindrance, since 2-chlorophenylacetonitrile afforded the corresponding β-ketonitrile (39), albeit in meagre yield, on reaction with ethyl propionate.

Those diaminopyrimidines successfully synthesised are summarised in table 5.1. All structures were confirmed by i.r., n.m.r. and mass spectroscopy.

Table 5.1 Structures of the diaminopyrimidines



R	X	Compound Number
Et	4-Cl	4[a]
Me	H	36
Me	3-Cl	42
Me	4-Cl	43
Et	H	44
Et	2-Cl	45
Et	3-Cl	46

pyrimethamine [a]

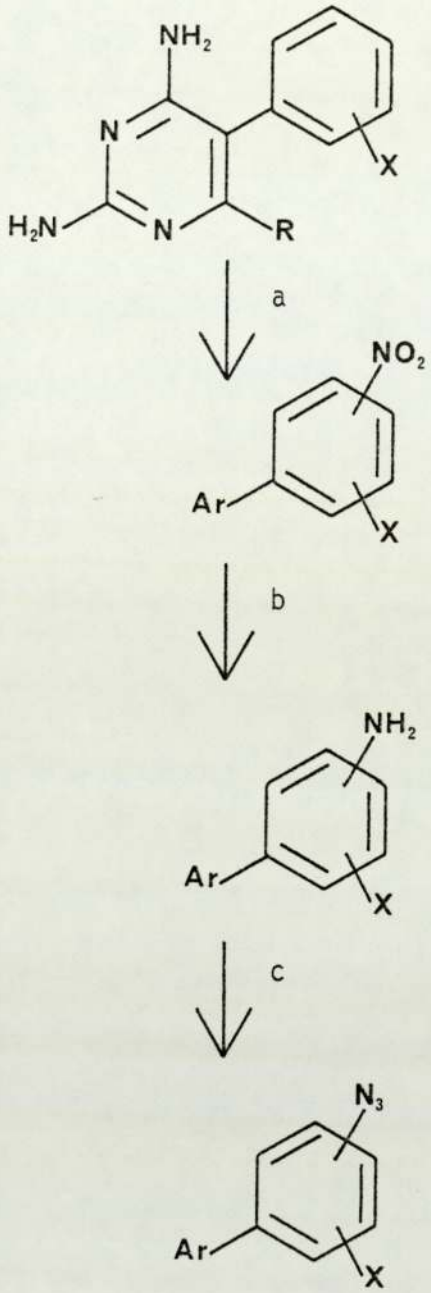
5.2 Introduction of the azido group

m-Azidopyrimethamine (MZP) (21) was synthesised from pyrimethamine (4) essentially following the method described by Bliss[93] and this approach (scheme 5.4) was utilised for the preparation of the novel MZP analogues. Such an approach was advantageous since the corresponding nitro and aminopyrimidines prepared as intermediates were required for subsequent studies on the metabolism and stability of the azidopyrimidines. In addition MNP (49) and iso-MNP (50) proved useful synthons for the development of potential molecular probes at a target unrelated to DHFR inhibition (chapter 7).

Thus pyrimethamine (4) was nitrated exclusively in the 3'-position on treatment with a mixture of concentrated nitric and sulphuric acid at 10° for 12 h and the product (49) was sufficiently pure in most cases for subsequent use without further recrystallisation. T.l.c. examination of pyrimethamine nitrated at temperatures above 40° showed the presence of a contaminant in addition to the desired product, possibly as a consequence of further nitration, or more probably due to oxidation. The treatment of MNP with a mixture of fuming nitric and concentrated sulphuric acid at 100° in an attempt to form the dinitro analogue afforded only an intractable gelatinous material.

In order to minimise the possibility of mixed product formation the chloro-substituted diaminopyrimidines synthesised previously (table 5.1) were nitrated with 1.1 mole equivalents of concentrated nitric acid in concentrated sulphuric acid at 10°. As expected the 4-chlorophenyl-6-methylpyrimidine (43) nitrated in the 3'-position in

Scheme 5.4 Introduction of the azido substituent: synthetic strategy



Ar = 2,4-diamino-6-alkylpyrimidin-5-yl

R = Me,Et

X = H,Cl

a. HNO_3/H_2SO_4; 12 h; 10°

b. Ni/N_2H_4; 65°

c. HNO_2/NaN_3; <5°

a manner analogous to pyrimethamine. Since for pyrimethamine the activity of the 3'-position has been attributed to an ortho activating effect of the chloro substituent and a meta directing effect due to the diaminopyrimidine moiety[93], it may be conjectured that the greater degree of coplanarity possible between the diaminopyrimidine and phenyl rings with a less bulky 6-methyl group might enhance the nitration reaction.

The 3-chlorophenylpyrimidines (42 and 46) nitrated predominantly in the 4'-position as required, although examination of the crude reaction products by t.l.c. demonstrated the formation of a second yellow component, possibly due to nitration in the 5'-position. These compounds (48 and 50) were conveniently purified by recrystallisation from aqueous ethanol and the position of nitration was established from the n.m.r. spectra with respect to that obtained with MNP (49). Thus a comparison of the aromatic splitting patterns of MNP (49) and iso-MNP (50) showed that the observed deshielding effects of the respective 3' and 4'-nitro substituents were in accordance with the appropriate 1,3,4-trisubstituted benzenoid structure (fig. 5.1). Similar patterns were observed with the corresponding 6-methyl compounds (47 and 48).

The unsubstituted diaminopyrimidines (36 and 44) were nitrated in an identical manner in an effort to prepare the corresponding 4-nitrophenyl-pyrimidines (51 and 52) and t.l.c. examination with several solvent systems suggested the formation of a single product in each case. However, although i.r. and mass spectral data were consistent with the proposed structures the n.m.r. spectra demonstrated the existence of a mixture of compounds in each case. Fractional crystallisation of the components of the mixture proved to be impracticable.

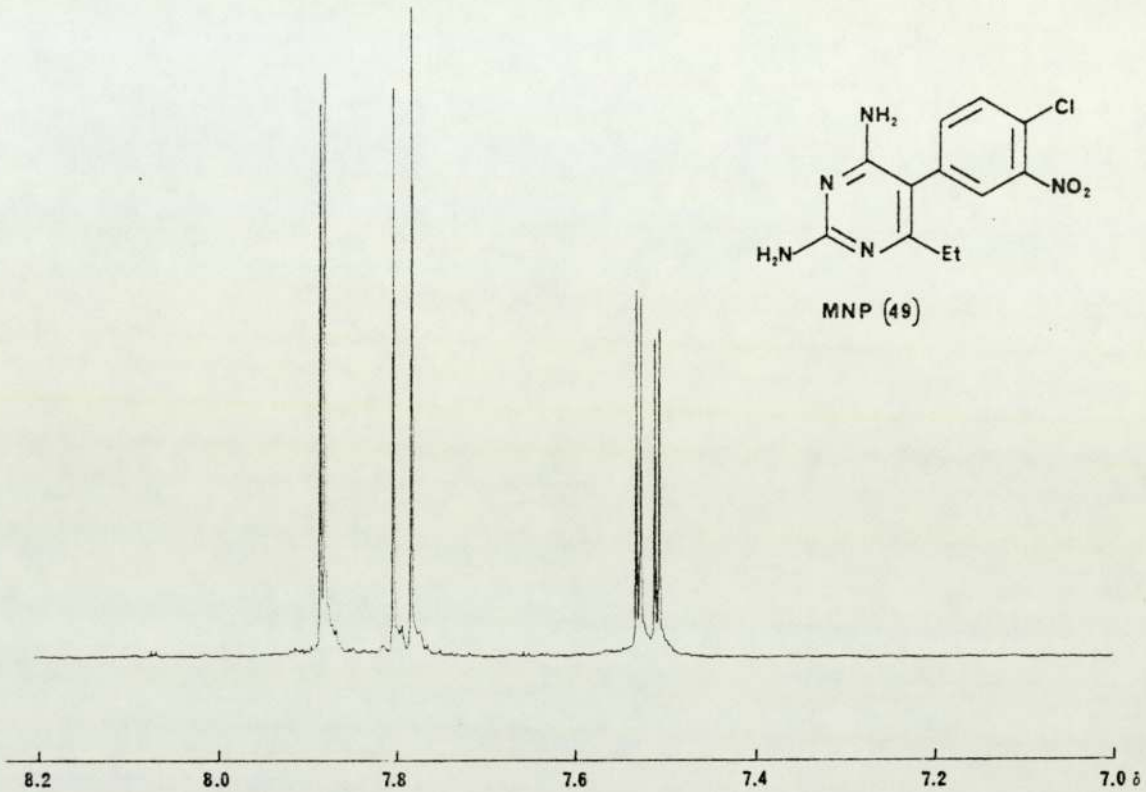
The synthesis of 2,4-diamino-5-(4-nitrophenyl)-6-methyl and 6-ethylpyrimidines (51 and 52) by nitration of the 5-phenyl group has been previously reported[3], utilising potassium nitrate in concentrated sulphuric acid, but the products were not characterised by n.m.r. and one attempt at nitrating the diaminopyrimidines by this method furnished mixtures identical to those described above.

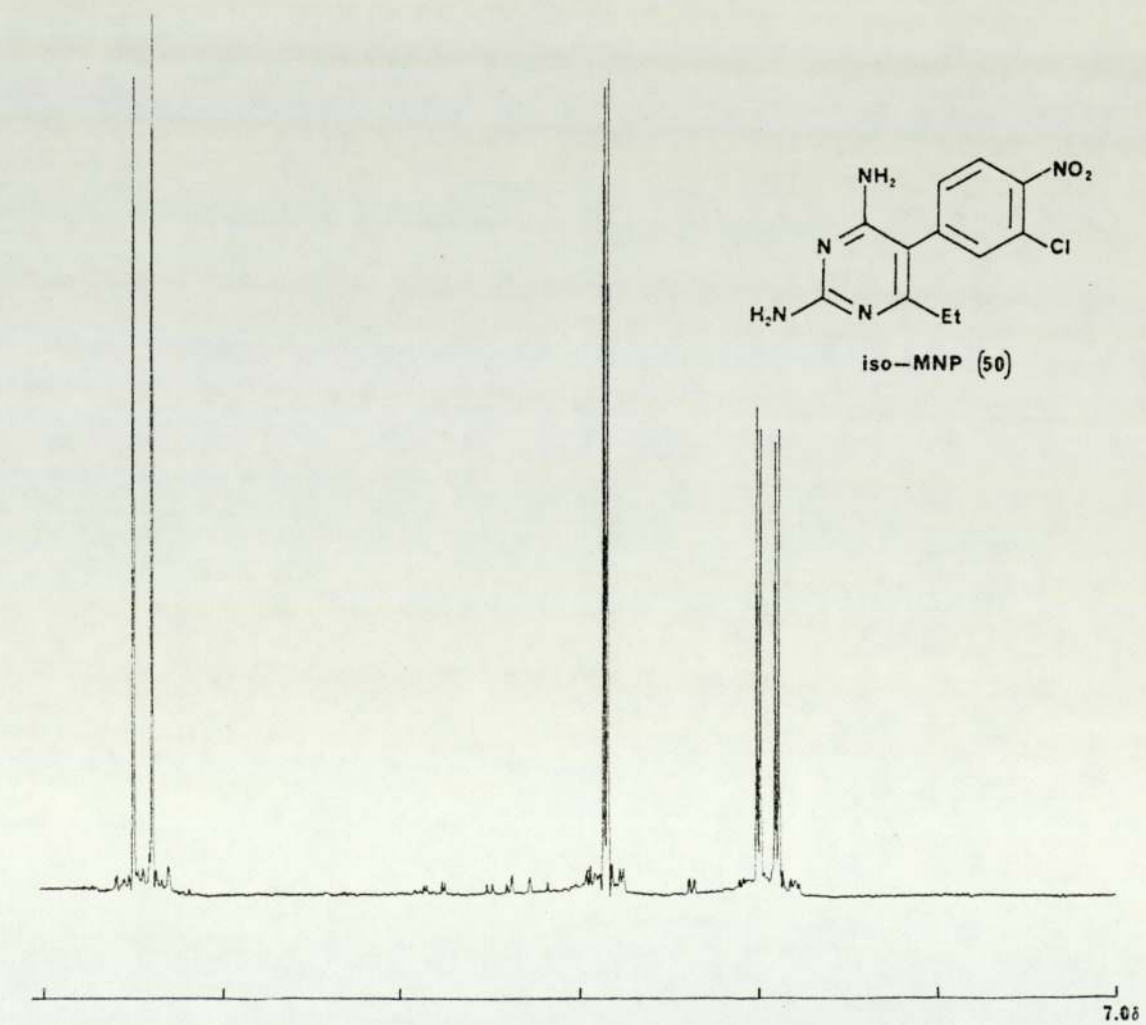
Introduction of a 3'-nitro group activates the 4'-chloro substituent of pyrimethamine towards nucleophilic substitution thus offering a synthetic route to the requisite 4'-alkoxyazidopyrimidine derivatives (scheme 5.5). Refluxing a suspension of MNP in methanolic sodium methoxide for 18 h afforded the methoxynitropyrimidine (53) in good yield *via* a Williamson type reaction. However, the corresponding reaction with sodium ethoxide required a reaction temperature of only 55-60° for 12 h and at higher temperatures the product (54) degraded to a tar.

Treatment of (49) with sodium *n*-butoxide in *n*-butanol furnished only starting materials at temperatures below reflux and although boiling the mixture for 1 h gave the requisite *n*-butoxyphenyl-pyrimidine (55), yields were disappointing and subsequent purification necessitated conversion to the corresponding ethanesulphonate salt (55a). Reaction times in excess of 1 h afforded a mixture of products as observed by t.l.c. *o*-Nitrophenylethers are known to cyclise under vigorous reaction conditions to form the appropriate benzoxazole derivatives[130] and this may account for the low yields obtained, and the formation of several products after extended reaction times.

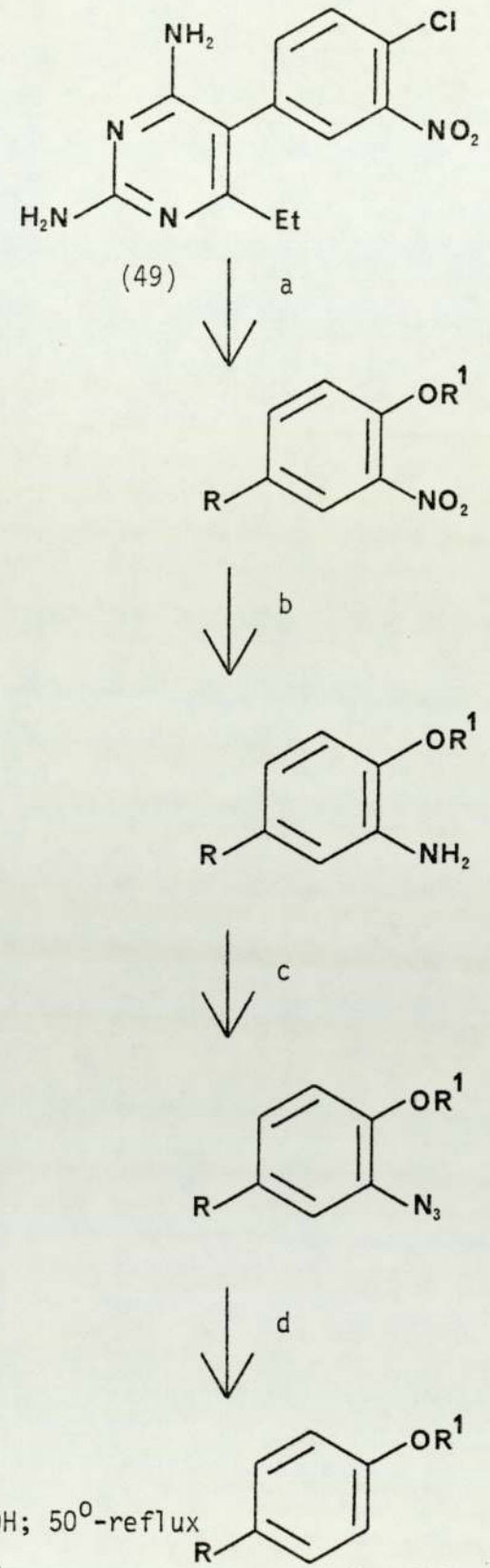
Attempts at preparing the phenol (56) by an analogous reaction of MNP with aqueous sodium hydroxide failed, and only a trace of highly coloured material was in evidence on examination by t.l.c. after boiling the mixture for 48 h. The structures of the aforementioned nitropyrimidines are detailed in table 5.2.

Figure 5.1 N.m.r. aromatic splitting patterns for MNP (49) and iso-MNP (50). Spectra recorded at 400 MHz in $[^2H_6]$DMSO as solvent.





Scheme 5.5 Synthesis of the alkoxyazidopyrimidines



a. $NaOR^1$ in R^1OH; 50°-reflux

b. Ni/N_2H_4; 65°

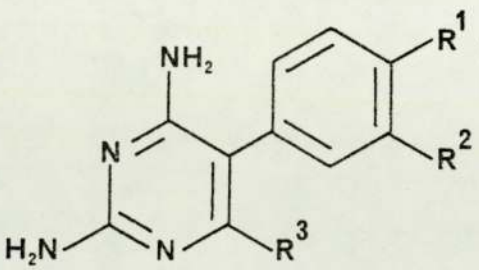
c. HNO_2/NaN_3 ; <5°

d. N_2H_4; reflux

R = 2,4-diamino-6-ethylpyrimidin-5-yl

R^1 = Me,Et,Bu^n

Table 5.2 Structures of the nitropyrimidines



R^1	R^2	R^3	Compound Number
Cl	NO_2	Me	47
NO_2	Cl	Me	48
Cl	NO_2	Et	49
NO_2	Cl	Et	50
NO_2	H	Me[b]	51
NO_2	H	Et[b]	52
OMe	NO_2	Et	53
OEt	NO_2	Et	54
OBu^n	NO_2	Et	55
OH	NO_2	Et[a]	56

[a] only starting materials obtained

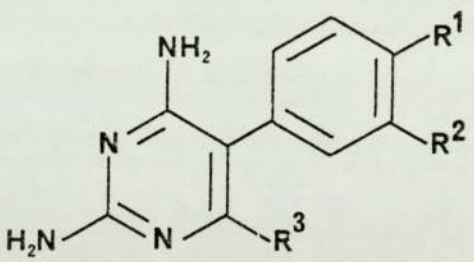
[b] only mixtures obtained in both cases

The nitropyrimidines were reduced with hydrazine hydrate and Raney nickel[131] in ethanol at 65° to give the corresponding amines (table 5.3) and this vigorous exothermic reaction was controlled by dilution of the hydrazine hydrate with ethanol prior to its addition. With the exception of (60), which required recrystallisation from dimethylformamide, the amines crystallised from aqueous ethanol as the corresponding monohydrates.

The reactivity of hydrazine both as a nucleophile[105] and ether-cleaving reagent[132,133] is well established; however, under the reaction conditions employed no other product formation was observed,

presumably due to the rapid catalytic decomposition of hydrazine on addition to the reaction mixture. Indeed more recent studies have shown that the reduction of aromatic nitro compounds by hydrazine-Raney nickel may be carried out selectively in the presence of other reducible or reactive substituents[134] and the partial reduction of dinitroarenes to nitroanilines by hydrazine has been reported[135].

Table 5.3 Structures of the aminopyrimidines

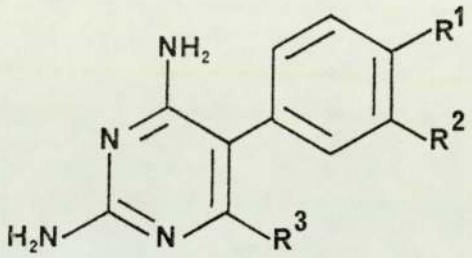


R^1	R^2	R^3	Compound Number
Cl	NH_2	Et	22
Cl	NH_2	Me	57
NH_2	Cl	Me	58
NH_2	Cl	Et	59
OMe	NH_2	Et	60
OEt	NH_2	Et	61

Subsequent diazotisation of the amines in 5M-hydrochloric acid at 0° and treatment with an excess of sodium azide furnished the novel azido analogues as candidate lipophilic DHFR inhibitors (table 5.4) in reactions monitored by t.l.c. when the azide rapidly degraded under u.v. light and observation of the characteristic sharp absorption band at $\sim$ 2170 cm^{-1} in the i.r. spectrum. The water soluble ethanesulphonate salts of these azides were prepared from the

base and aqueous ethanesulphonic acid without incident or evidence of any acid-catalysed degradation of the azido group.

Table 5.4 Structures of the azidopyrimidines



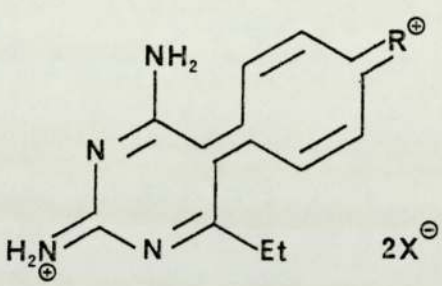
R^1	R^2	R^3	Compound Number
Cl	N_3	Et	21
Cl	N_3	Me	62
N_3	Cl	Me	63
N_3	Cl	Et	64
OMe	N_3	Et	65
OEt	N_3	Et	66

5.3 Deazidation of the alkoxyazidopyrimidines

Aromatic azides undergo reductive deazidation on treatment with hydrazine[136] or a solution of hydrazine in ethanol[137] and this reaction was used to establish the structures of the alkoxyphenylpyrimidine derivatives (65 and 66). The methoxyazide (65) underwent complete deazidation after 30 min as monitored by t.l.c. and the disappearance of the characteristic absorption in the i.r. spectrum, whilst in contrast the ethoxyazide (66) required a total reaction time in excess of 5 h.

The 4'-alkoxypyrimidines obtained (67 and 68) exhibited a poor solubility in chloroform or DMSO and consequently n.m.r. spectra were

determined in [^{2}H]trifluoroacetic acid, where intriguingly the colourless crystals dissolved to form intensely red solutions in each case. Both compounds were subsequently found to exhibit similar colour changes in concentrated, but not dilute, sulphuric and hydrochloric acids, suggesting the formation of an extended chromophore. Interestingly, the 4'-amine substituted pyrimidines (chapter 8) were identical in this respect and although speculative the presence of an electron donating (+M) substituent in the 4'-position may result in dication formation in strong acids, and a concomitant achievement of coplanarity between the phenyl and pyrimidine rings (69).



(69)

R=OMe,OEt,NMe_2 etc

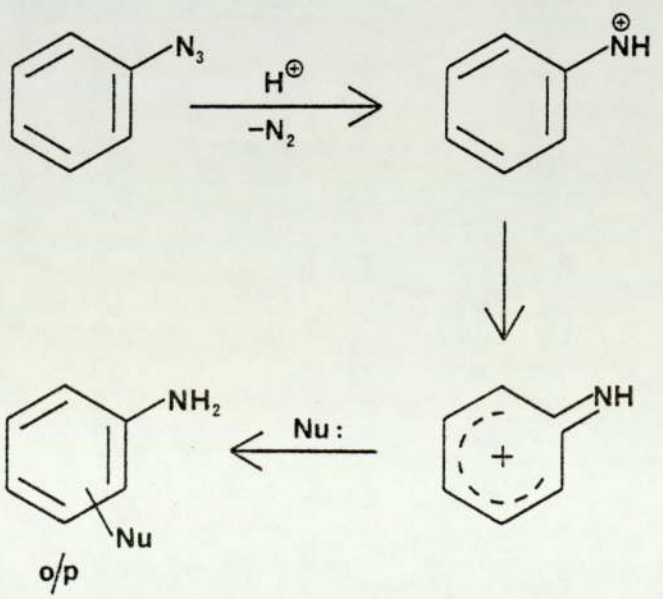
5.4 Acid catalysed decomposition of MZP

From discussions elsewhere (chapters 2.1 and 10.3) it is evident that the synthesis of analogues of MZP bearing an electron donating group para to the azido substituent was one objective of the present studies, since compounds of this nature may exhibit the pharmaco-

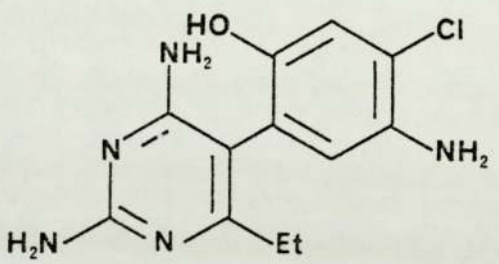
kinetic characteristics required for a short-acting lipophilic antifolate. Also the possible value of ortho substituted diaminopyrimidines as prospective stereoselective inhibitors of DHFR has been considered elsewhere (chapter 14.1a). However, synthetic efforts directed at the preparation of diaminopyrimidines embracing a 2'-methoxy substituent have proved unsuccessful (5.1).

The acid-catalysed decomposition of aryl azides, a reaction discovered over a century ago, has been extensively reviewed[138,139]. More recent studies have centred on the utility of the reaction from a synthetic viewpoint, particularly for the preparation of aromatic ring systems via intramolecular cyclisation[140,141]. The reaction proceeds via the intermediacy of a nitrenium ion following acid-catalysed loss of nitrogen and subsequent charge delocalisation to form a π-carbocation which reacts with the appropriate nucleophile to furnish a substituted aniline (scheme 5.6). The attacking nucleophile may be exogenous, the counterion of the acid or derive from an electron-rich site elsewhere on the molecule when cyclisation occurs.

Scheme 5.6 Acid-catalysed decomposition of aryl azides



The possibility of exploiting the above reaction for the direct introduction of a 2'-substituent onto the phenyl ring of MZP was thus investigated. Reaction of MZP (21) with trifluoroacetic acid (TFA) at room temperature gave only unchanged azide after 4 days, as observed by n.m.r., and consequently a solution of (21) in dry TFA at 0° was treated with trifluoromethanesulphonic acid (triflic acid, TFSA) when effervescence was observed and t.l.c. examination revealed the formation of a single product. Initial spectroscopic analysis (n.m.r. and m.s.) of the photosensitive microprisms deposited following crystallisation of the product from ethyl acetate - petroleum ether, suggested formation of a 2'-hydroxypyrimidine (70) presumably resulting from nucleophilic attack by water <u>para</u> to the nitrenium ion intermediate.

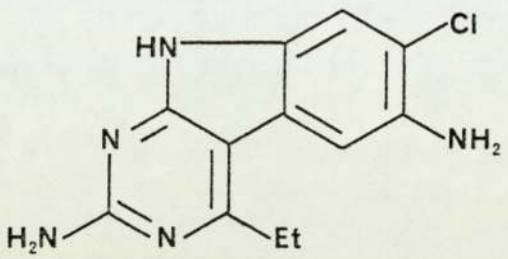


(70)

However, although the 60 MHz n.m.r. spectrum was consistent for a 1,2,4,5-tetra substituted benzenoid structure as shown (70) and the observed molecular ion (m/z = 279 [281]) was in agreement with the proposed product, only six exchangeable protons were evident on deuteration. Moreover, the product failed to develop a characteristic colour on treatment with ferric chloride solution and proved insoluble in aqueous sodium hydroxide solution.

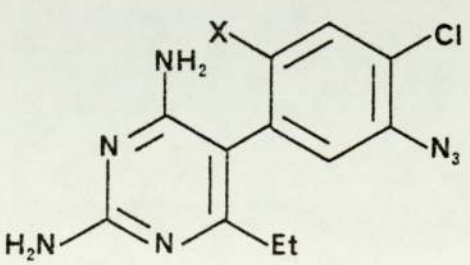
The possibility of an intramolecular cyclisation reaction by nucleophilic attack of the pyrimidine 4-amino substituent to furnish (71) was considered feasible[142] but unlikely in light of the

spectroscopic evidence obtained. Also the reactivity of the 2- and 4-amino substituents would almost certainly be reduced due to protonation of the pyrimidine ring nitrogens in such a strongly acidic environment.



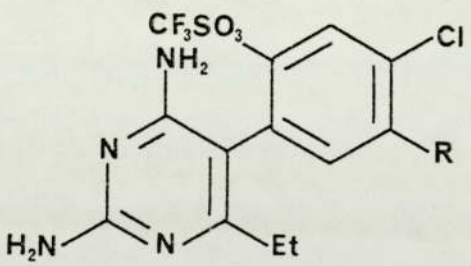
(71)

Diazotisation and subsequent treatment of the compound with an excess of sodium azide afforded a product that rapidly degraded under u.v. light and gave a characteristic absorption at 2170 cm^{-1} in the i.r. spectrum indicating azide formation. A downfield shift in absorbance of one of the aromatic protons relative to the corresponding amine was observed in the n.m.r. spectrum, consistent with the deshielding influence of a 5'-azido substituent, and a molecular ion (m/z) of 305 [307] with a distinctive M^+-28 fragment ($-N_2$) confirmed the product as the appropriate aromatic azide (72). Thus, as expected, a 5'-amino substituent was present in the original compound and the anomaly appears to reside at the the 2' position (X).



(72)

The precise structure of compounds (70 and 72) remains to be established and studies to this end are in progress. However, the microanalytical data obtained for each compound indicates the formation of a trifluoromethanesulphonate substitution product in each case and although the exact position of substitution has yet to be assigned unambiguously, the aforementioned evidence favours a 2'-trifluoromethanesulphonate as shown (73) (scheme 5.7). Interestingly, Abramovitch et al[141] have recently described the formation of an aromatic trifluoromethanesulphonate as one of several products identified following decomposition of an aryl azide with TFSA in trifluoroacetic anhydride.



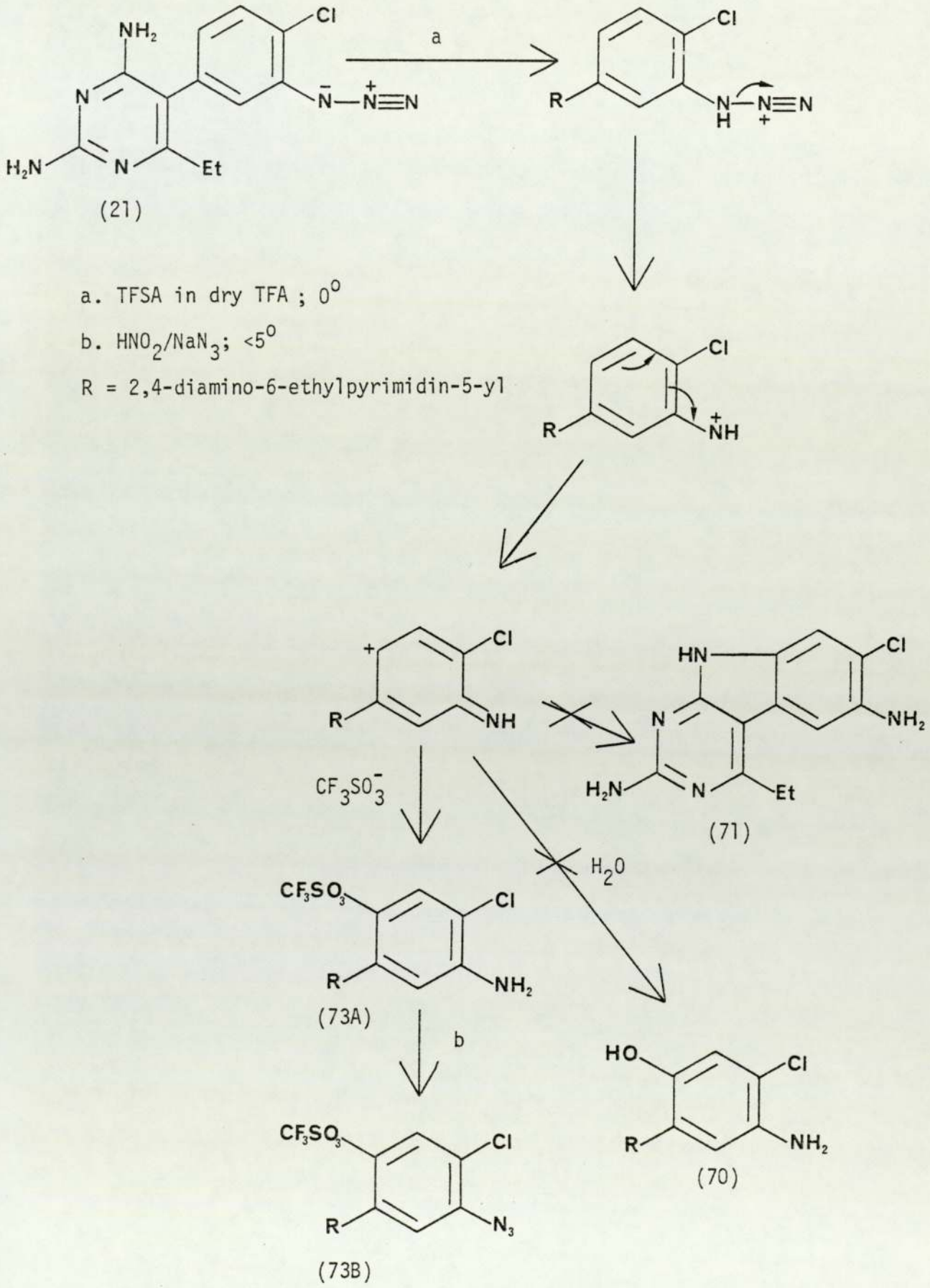
(73)

(73A): R=NH_2

(73B): R=N_3

Preliminary investigations as to the viability of utilising the above reaction for the introduction of a 2'-methoxy substituent were unsuccessful. Thus quenching the reaction mixture with methanol gave the identical product (73A), whilst an attempt at conducting the decomposition of MZP with TFSA in methanol as solvent furnished only unchanged MZP.

Scheme 5.7 The acid-catalysed decomposition of MZP by TFSA



CHAPTER 6

SYNTHESIS OF PUTATIVE METABOLITES AND DEGRADATION PRODUCTS OF M-AZIDOPYRIMETHAMINE (MZP)

6.1 2,4-Diaminopyrimidine N-oxides

Little has been published regarding the N-oxidation of 2,4-diaminopyrimidines. Ochia[143] has reported that aromatic diazines undergo N-oxidation and while pyrazines may form mono- or dioxide products, pyridazine and pyrimidine compounds invariably form only mono-N-oxides. Hubbell et al[82] have prepared synthetic N-oxides of diaminopyrimidines following the procedure of Rey-Bellett and Reiner[144] using m-chloroperoxybenzoic acid as oxidant, but more recent investigations indicate that higher yields of pyrimidine oxides may be obtained by oxidation with peracetic acid[145].

However, the attempted oxidation of nitropyrimethamine (49) in peracetic acid at 70° afforded only reactants after 12 h and subsequent refluxing of the mixture was without effect. The successful N-oxidation of (49) was achieved with performic acid at 25° when three components were detected by t.l.c. after 72 h, one of which co-chromatographed with the starting material. Increasing reaction temperature or time had no effect on the relative component proportions. Recrystallisation of the mixture of products from aqueous ethanol furnished a pure sample of the nitropyrimethamine 3-oxide (75) in moderate yield, whilst separation of the mixture by column chromatography gave unchanged (49), nitropyrimethamine 3-oxide (21%) (75) and a meagre yield of the N-1-oxide (9%) (74).

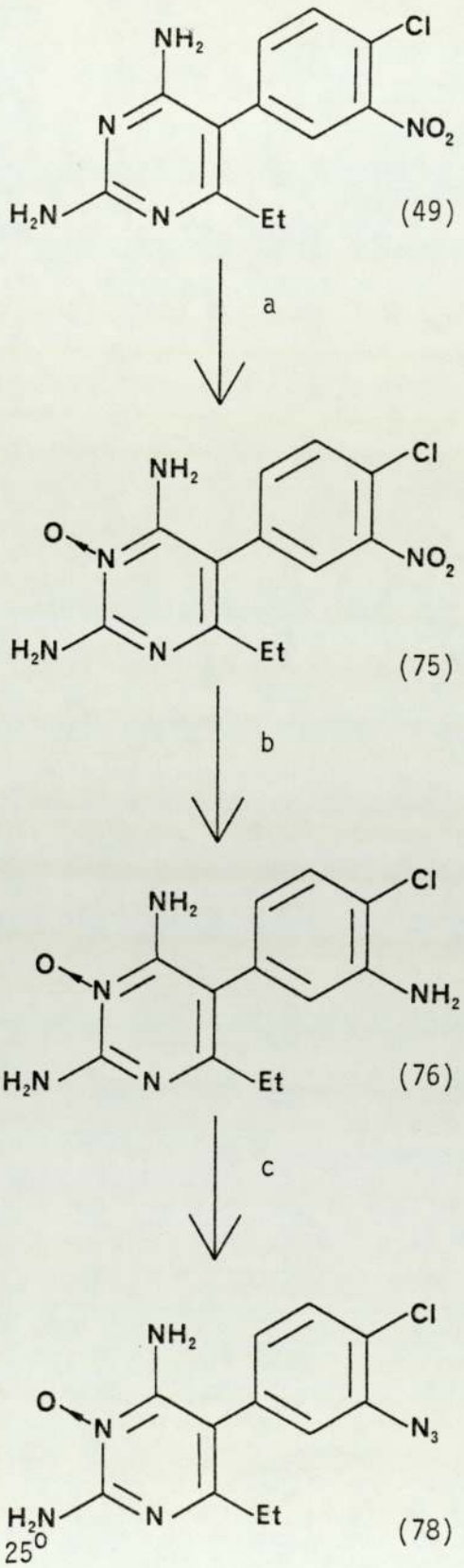
The nitropyrimethamine 3-oxide was reduced selectively by tin

(II) chloride dihydrate in refluxing ethanol to afford the 3'-aminopyrimidine 3-oxide (76) in moderate yield, and under these conditions concomitant reduction of the N-oxide was not observed. Recrystallisation of the m-aminopyrimethamine N-oxide (76) proved difficult due to formation of a persistent colloidal contaminant which necessitated filtration through Kieselguhr prior to crystallisation. Diazotisation and subsequent azidation of the aminopyrimethamine 3-oxide (76) by the usual method furnished 3'-azidopyrimethamine 3-oxide (78) without incident (scheme 6.1).

Direct oxidation of MZP (21) with performic acid as described above again gave a mixture of the photosensitive 1- and 3-oxides in a ratio of approximately 1:3, together with unchanged MZP and traces of a deep red compound. The mixture was separated by chromatography on silica gel. The azidopyrimethamine 3-oxide (78) proved identical (mixed m.p., i.r., n.m.r. and t.l.c.) to that obtained *via* the synthetic route described above. The decomposition of aromatic azides in peracetic acid with formation of azo and azoxy compounds has been documented[146] and may account for the small quantity of coloured material obtained, although insufficient of this contaminant was isolated to permit identification. m-Azidopyrimethamine 3-oxide (78) proved considerably more soluble in ethanol than the 1-oxide isomer (77); however, separation by fractional crystallisation was precluded by the presence of unreacted MZP.

Oxidation of 3'-aminopyrimethamine (22) with performic acid under the aforementioned reaction conditions was unsuccessful and only unchanged starting material was recovered. However, treatment of (22) in methanol with sodium tungstate and hydrogen peroxide at room temperature resulted in the formation of two discrete products as monitored by t.l.c. Intriguingly, subsequent chromatographic

Scheme 6.1 Synthesis of MZP N-3-oxide



a. H_2O_2/HCOOH; 25°

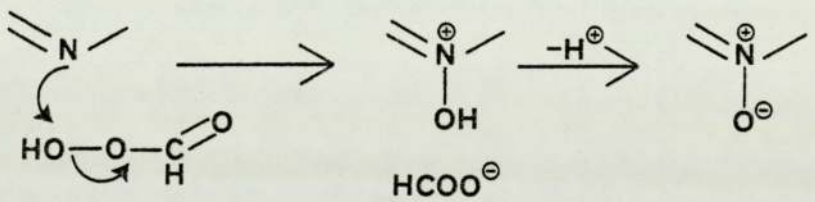
b. $SnCl_2$ in EtOH; reflux

c. HNO_2/NaN_3; <5°

separation on silica gel afforded nitropyrimethamine (49) and nitropyrimethamine 3-oxide (75) in approximately equal proportions and these compounds were characterised as being identical to those authentic samples prepared independently.

Peracid oxidation of heterocyclic tertiary amines is thought to involve formation of a relatively stable trialkylammonium peroxide which undergoes facile decomposition to the corresponding N-oxide under the reaction conditions employed[147,148]. Heterocyclic N-oxidation by peracids occurs via a mechanism analogous to the oxidation of primary aromatic amines with Caro's acid (H_2SO_5) (scheme 6.2).

Scheme 6.2



Jovanovic[145] has recently considered factors influencing the orientation of oxidation at the 1- and 3-positions of disubstituted pyrimidines, and concludes that the following criteria are of importance:

(i) the reactivity of a pyrimidine ring towards N-oxidation is increased by the presence of electron-donating groups;

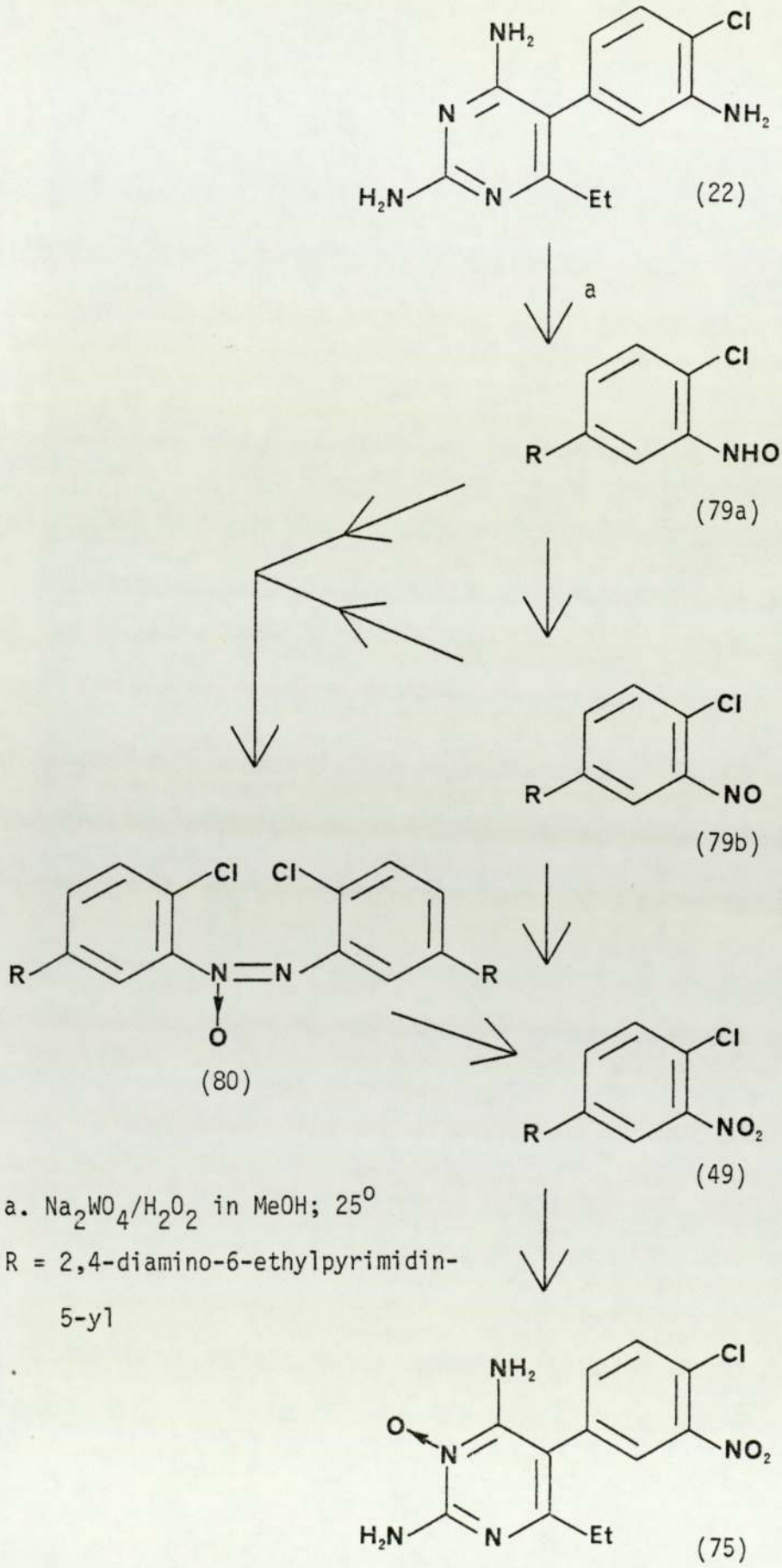
(ii) ring nitrogens ortho or para to electron-donating substituents will oxidise preferentially;

(iii) the reaction is sterically hindered by phenyl groups but not simple alkyl groups.

Any extrapolation of these observations to the more complex polysubstituted pyrimidines must be regarded with caution, but the predominant formation of 3-oxides with those diaminopyrimidines considered previously may be attributable to the presence of two activating ortho-amino substituents, and a 'para directing' 6-ethyl group. The isomeric 1-oxide presumably forms to a lesser extent as a consequence of the weaker ortho/para directing effect of the 2- and 4-amino groups on the pyrimidine ring and also since N-1 will be protonated in acid solution[149].

The sodium tungstate catalysed oxidation of m-aminopyrimethamine (22) by hydrogen peroxide to furnish nitropyrimethamine (49) and the corresponding 3-oxide (75) occurs via an unusual and complex mechanism[150] (scheme 6.3). The sodium tungstate was observed to slowly dissolve in methanolic hydrogen peroxide solution to develop an orange-yellow colouration due to formation of a tungstate peroxo anion (WO_8^{--}) which subsequently acts as oxidant. Thus oxidation of the aromatic amine (22) furnishes a hydroxylamine (79A) which oxidises further to the nitroso compound (79B). The nitroso derivative may again oxidise to form 3'-nitropyrimethamine (49) or react with (79A) forming the azoxy compound (80) that might conceivably undergo oxidative cleavage to yield (49). Subsequent N-oxidation of nitropyrimethamine affords the corresponding 3-oxide (75). The possibility of exploiting this reaction in order to obtain the required intermediates as possible degradation products of MZP (21) has yet to be investigated, but may provide a synthetic route to

Scheme 6.3 The sodium tungstate catalysed oxidation of MAP by hydrogen peroxide

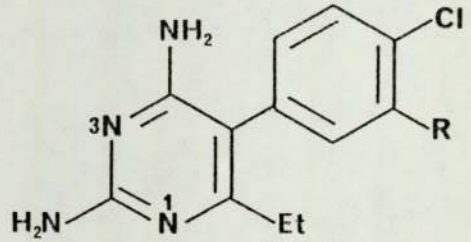


the requisite azoxy compound (80) under optimal conditions, perhaps involving phase-transfer catalysis[151].

The structures of the diaminopyrimidine N-oxides were confirmed by n.m.r., u.v. and mass spectroscopy. In all cases the molecular ion was observed with a characteristic M^+-16 fragment corresponding to loss of oxygen, and for the azidopyrimidines (77 and 78) this preceeded the distinctive M-28 fragment consequent to nitrogen elimination. Thus, following loss of oxygen, the mass spectra were identical to those observed with the unoxidised parent compounds. The position of oxidation was determined from analysis of the u.v. and n.m.r. spectra of each compound with respect to the parent molecule, on the basis of published evidence relating the spectral properties of trimethoprim (18), pyrimethamine (4) and metoprine (5) to the corresponding N-oxides[82].

Oxidation of the diaminopyrimidine at N1 or N3 reportedly elicits a bathochromic shift in the u.v. spectra particularly following oxidation at N1. However, in contrast to the published results, a small shift to longer wavelength was observed with the N-3-oxides and, surprisingly, the diaminopyrimidine 1-oxides exhibited a shift to shorter wavelength in both cases (table 6.1). Definitive information regarding the orientation of oxidation was provided by analysis of the n.m.r. spectra where a deshielding influence was observed on ring substituents adjacent to an N-oxide. Thus the overall deshielding effect of an N-oxide on the N-H protons of both the 2- and 4-amino groups, was particularly evident for the 2-amino substituent of a pyrimidine 1-oxide where a large downfield shift was observed. The methylene protons of a 6-ethyl substituent were also subject to a similar effect when juxtaposed to an N-oxide as for the diaminopyrimidine 1-oxides, a situation clearly not

Table 6.1 Spectral properties of the diaminopyrimidine N-oxides



Compound Number	Position of oxidation	R	^{1}H n.m.r. (δ values)[a] 2[c]-NH_2 brs	4[c]NH_2 brs	6-CH_2 q	U.V.[b] (n.m.)
49 (MNP)	-	NO_2	6.01	6.10	2.15	283
74	1	NO_2	7.92	7.08	2.46	250
75	3	NO_2	7.02	7.22	2.17	290
22 (MAP)	-	NH_2	5.69	5.90	2.18	284
76	3	NH_2	6.65	7.14	2.19	293
21 (MZP)	-	N_3	5.84	5.96	2.18	284
77	1	N_3	7.88	7.01	2.52	246
78	3	N_3	6.92	7.25	2.22	290

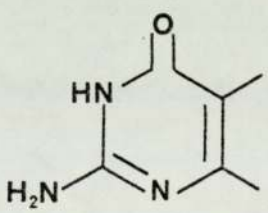
[a] Solvent [$^2H_6$]DMSO 1% TMS brs = broad singlet [c] amino groups assigned in accordance with literature[82]
[b] Solvent 95% ethanol q = quartet

apparent with the corresponding 3-oxides (table 6.1).

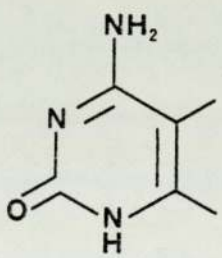
Of those diaminopyrimidine N-oxides described above MZP 1-oxide (77) and 3-oxide (78) and m-aminopyrimethamine 3-oxide (76) are of particular interest as prospective metabolites of the parent drug MZP (21). Unfortunately, synthetic efforts directed towards the preparation of the corresponding 1-oxide of m-aminopyrimethamine have been unsuccessful to date. However, conversion of m-nitropyrimethamine 1-oxide (75) or m-azidopyrimethamine 1-oxide (77) by established chemical methods may offer a future route to the requisite compound if sufficient of these materials are prepared.

6.2 Aminopyrimidinones

Substituted 2-aminopyrimidin-4(3H)-ones (81) and the isomeric 4-aminopyrimidin-2(1H)-ones (82) may be formally regarded as derivatives of isocytosine and cytosine respectively and consequently a wealth of information concerning the chemistry of such compounds has been published[120,121,152].



(81)



(82)

Each of the above structures may be considered to exist in seven tautomeric forms; that the predominant structures are as shown has been established by extensive spectroscopic and crystallographic

studies[153] although the importance of other tautomers in explaining the biological and chemical properties of aminopyrimidinones warrants emphasis.

Two principal synthetic methods have been adopted for the preparation of aminopyrimidinones[120,121]:

(i) the base-catalysed condensation of an appropriately substituted β-ketoester or β-ketonitrile with guanidine or urea respectively;

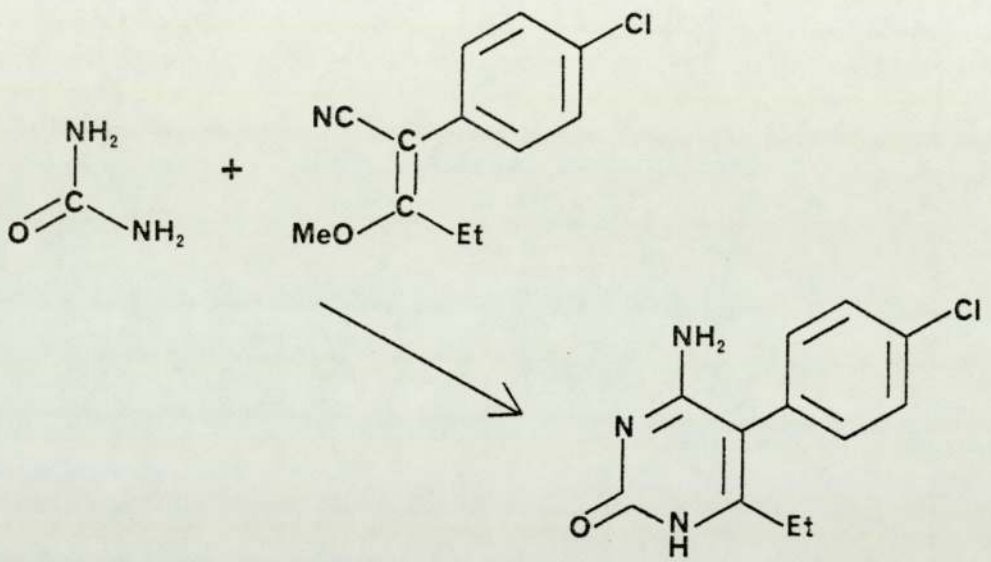
(ii) metathesis of a diaminopyrimidine, synthesised following a conventional route (chapter 5) by hydrolysis under acidic or basic conditions, or deamination with nitrous acid.

The 4-aminopyrimidin-2(1H)-one (83) derivative of pyrimethamine (4) was synthesised by reacting the corresponding methoxyacrylonitrile, prepared by the method described previously (chapter 5.1) with urea in sodium ethoxide solution (scheme 6.4). Formation of a persistent orange contaminant, probably due to polymerisation of the methoxyacrylonitrile, necessitated isolation of the pyrimidin-2-one (83) as a hydrochloride salt (83a) following repeated crystallisation from 2M-hydrochloric acid and subsequent precipitation with alkali to furnish a pure sample (t.l.c.) of the requisite free base.

This compound and other related pyrimidinones were found to exhibit unusual physical properties consistent with extensive intermolecular hydrogen bonding in a manner analogous to that documented for isocytosine[154]; thus a poor solubility in polar organic solvents, melting ranges often in excess of 10° and broad and indistinct spectroscopic characteristics (i.r. and n.m.r.) were

invariably observed. Several pyrimidinones retained variable amounts of solvent of crystallisation even at elevated temperatures (80°) *in vacuo*, and consequently elemental analysis often gave disappointing results. Compounds of this nature were characterised by n.m.r. and mass spectrometry.

Scheme 6.4 Synthesis of 4-amino-5-(4-chlorophenyl)-6-ethylpyrimidin-2(1H)-one (83)



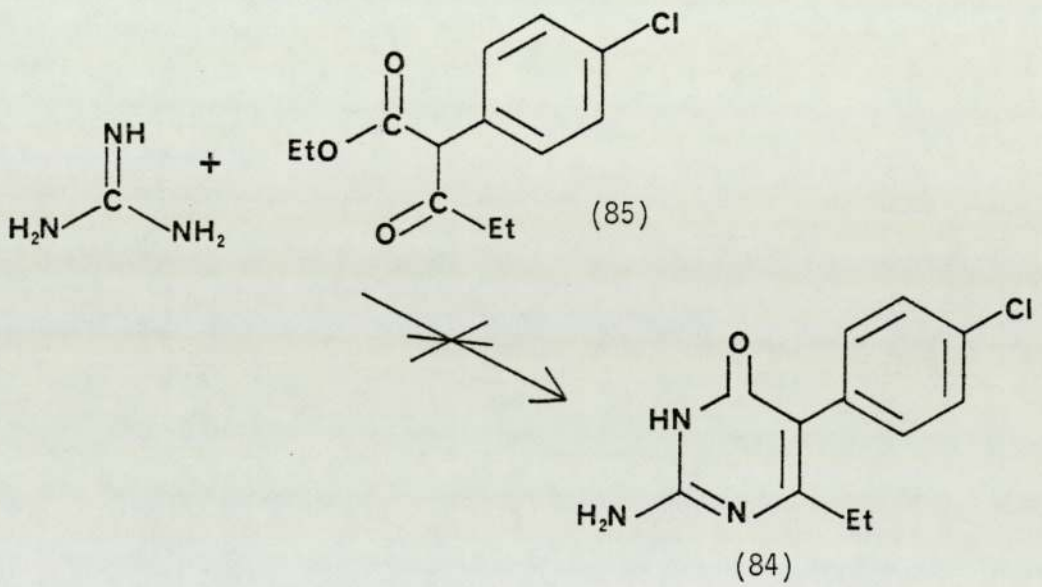
(83)

Synthesis of the corresponding 2-aminopyrimidin-4(3H)-one (84) was attempted by several methods described in the literature, where conflicting reports concerning the viability of preparing (84) by a condensation reaction analogous to that described above for the pyrimidin-2-one (83) were encountered (scheme 6.5). Russell et al[3] found that yields of 2-aminopyrimidin-4-ones were exceptionally poor by this route and failed entirely with α-phenyl-β-ketoesters of the type (85). However, a subsequent patent[155] described the facile synthesis of 2-amino-5-phenylpyrimidin-4(3H)-one from guanidine and the appropriate β-ketoester under base-catalysed conditions, where

the β-ketoester was not isolated.

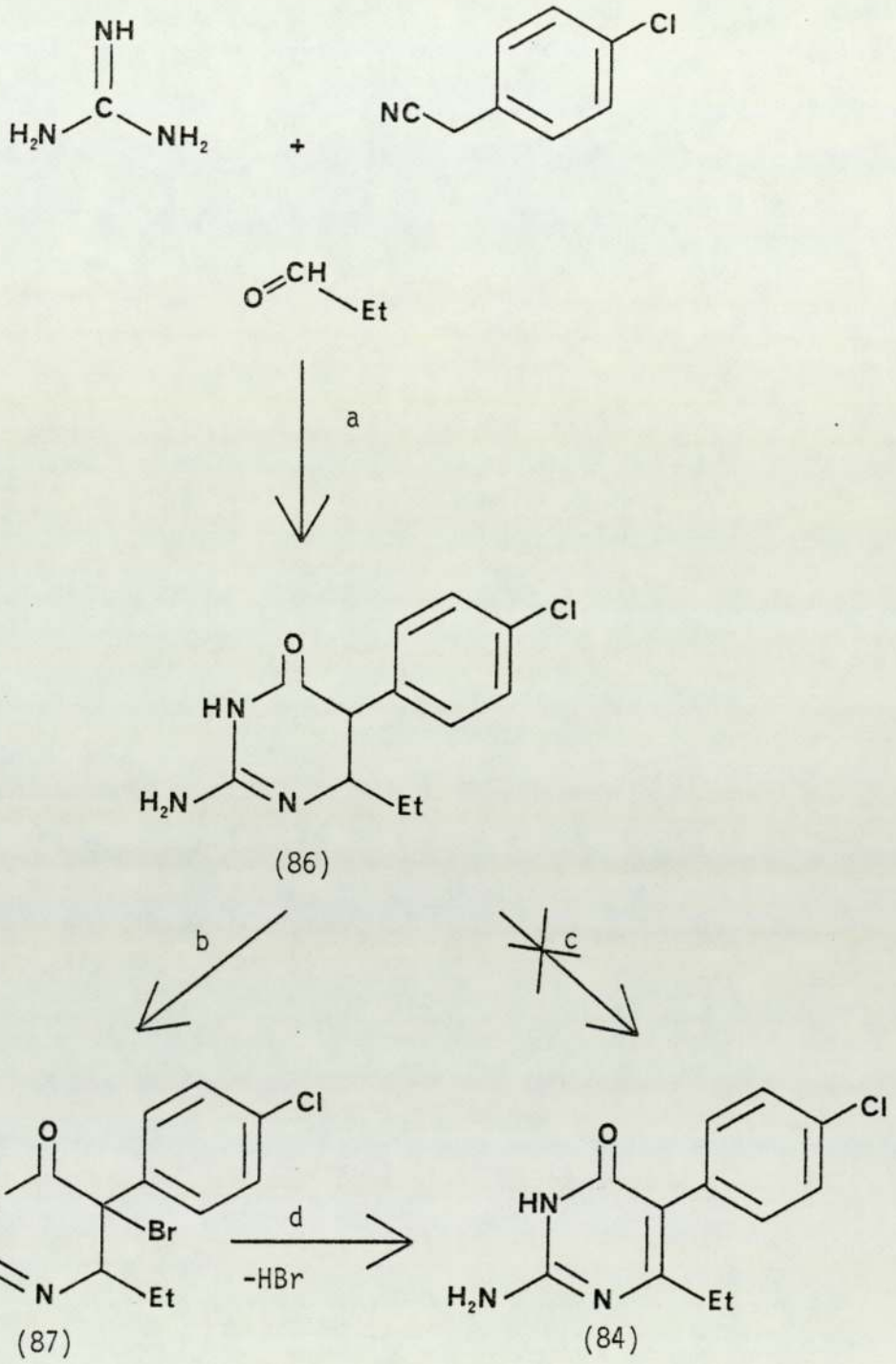
Thus a mixture of ethyl 4-chlorophenylacetate and ethyl propionate were treated with sodium in ether and the resulting oil was condensed with guanidine in ethanolic sodium ethoxide following the prescribed method. Examination of the reaction mixture by t.l.c. showed an absence of reactants but subsequent attempts at isolating a product foundered and this approach was discontinued. Interestingly, an attempt to repeat the reaction exactly as described with ethyl phenylacetate and ethyl formate was also unsuccessful.

Scheme 6.5 Attempted synthesis of 2-amino-5-(4-chlorophenyl)-6-ethylpyrimidin-4(3H)-one



A second synthetic approach[156] outlined in scheme 6.6 required the preparation of a dihydropyrimidinone (86) and subsequent dehydrogenation to furnish the pyrimidinone (84). Thus a mixture of guanidine, 4-chlorophenylacetonitrile and propanal in ethanolic sodium hydroxide were condensed smoothly, to give the dihydropyrimidinone (86) in moderate yield via a convenient 'one pot'

Scheme 6.6 Synthesis of the dihydropyrimidinone derivative of pyrimethamine



a. EtOH/NaOH; reflux

b. Br_2/AcOH; 65^o

c. i) sulphur; 200^o ii) MnO_2/acetic acid; reflux

d. within mass spectrometer beam

reaction. The reaction is thought to proceed with the intermediate formation of an iminoacylguanidine, hydrolysis to the acylguanidine preceeding ring closure. Recrystallisation from ethanol furnished the pure product (t.l.c.) which was characterised (i.r., n.m.r. and m.p.) as identical to that described in the literature. Subsequent dehydrogenation of (86) with sulphur at 200° as reported was fraught with difficulties; residual sulphur proved intransigent to repeated extraction with large volumes of carbon disulphide and the minute yield of compound obtained following multiple reprecipitation from alkali was characterised (m.s.) as unreacted dihydropyrimidine.

Other established dehydrogenation procedures proved almost as unsatisfactory; bromination of (86) in acetic acid[157] furnished a very poor yield of a yellowish powder giving a molecular ion at m/z 249 (251) corresponding to elimination of HBr from the 5-bromopyrimidinone (87), probably in the electron beam, with formation of the required pyrimidine (84). However, examination by t.l.c. demonstrated the presence of considerable quantities of unreacted starting material. Treatment of the dihydropyrimidinone (86) with activated manganese dioxide, an established aromatisation reagent, in refluxing acetic acid furnished a single low-melting product (t.l.c.) with a distinctive odour. However, further characterisation (n.m.r. and i.r.) proved unsuccessful although the material was clearly not the requisite aminopyrimidinone, and conducting the reaction at lower temperatures or with toluene as solvent gave only starting material.

The alternative approach to aminopyrimidinones <u>via</u> deamination of a diaminopyrimidine proved more rewarding. Trattner et al[158] investigated the effect of substitution on the deamination of a series of diaminopyrimidines and concluded that acid hydrolysis of pyrimethamine (4) furnished the 2-aminopyrimidin-4(3H)-one (84)

exclusively. However, treatment of (4) with boiling 6M-hydrochloric acid in the prescribed manner afforded a mixture of hydrochloride salts characterised (n.m.r. and h.p.l.c.) as the pyrimidin-4-one (84a) and the isomeric 4-aminopyrimidin-2(1H)-one (83a) in a product ratio 1:2[159]. Moreover, the component hydrochloride salts were separable by fractional crystallisation from 2M-hydrochloric acid and basification with ammonia solution liberated the corresponding aminopyrimidinones, one of which was identical (m.p., i.r., n.m.r., m.s.) with the authentic 4-amino-5-(4-chlorophenyl)-6-ethylpyrimidin-2(1H)-one (83) synthesised by the aforementioned method.

The characteristic shift of the methylene group at C(6) in the n.m.r. spectra ([$^2H_6$]DMSO) of each aminopyrimidinone free base (83 and 84) again proved useful in identifying the appropriate isomer (c.f. the pyrimidine N-oxides). Thus for the 2-aminopyrimidin-4(3H)-one (84) a quartet centred at δ2.24 (±0.04) was observed, in contrast to the 4-aminopyrimidin-2(3H)-one (83) where the corresponding absorption pattern appeared upfield at δ2.14 (±0.05) (figure 6.1).

Compelling evidence in corroboration of earlier observations that aminopyrimidinones may undergo extensive intermolecular hydrogen bonding has been adduced[159]; X-ray crystallographic studies on a mixture of the isomeric pyrimidinone free bases (83 and 84) following co-crystallisation from ethanol, show the formation of a hydrated duplex linked by three hydrogen bonds in a manner analogous to a Watson-Crick cytosine:guanine base pair (figure 6.2).

The possible biological implications of these observations form the subject of continuing investigations.

Nitration of the isomeric aminopyrimidinones (83 and 84) in sulphuric acid containing nitric acid (1.1 mol equivs.) at 25°, furnished the corresponding nitroaminopyrimidinones (88 and 89)

without incident. The 3'-nitropyrimidin-2(1H)-one (88) exhibited poor solubility in organic solvents by comparison to the isomeric (89) and was purified as a hydrochloride salt. Direct hydrolysis of nitropyrimethamine (49) in 6M-hydrochloric acid as for pyrimethamine furnished a mixture of nitroaminopyrimidinones in the ratio 1:1.2. The components were characterised by n.m.r. and comparison with the authentic samples prepared above, and shown to be the 2-amino-pyrimidin-4(3H)-one (89) and 4-aminopyrimidin-2(1H)-one (88) respectively.

6.3 Diaminopyrimidines

The syntheses of m-aminopyrimethamine (22) as a potential metabolite of MZP (21) and m-nitropyrimethamine as a possible oxidative degradation product, have been described previously (chapter 5). 3'-Acetylaminopyrimethamine (90) was synthesised from the corresponding amine (22) essentially following the method described by Bliss, who conducted the acetylation at 100° with acetyl chloride in pyridine[93]. However, at this reaction temperature yields were disappointing and the product was heavily contaminated with di- and triacetylated material, whereas an identical reaction conducted at 25° gave the 3'-acetylated derivative exclusively and in a higher yield.

Two synthetic strategies were adopted in addressing the problem of preparing the requisite 6-(1-hydroxyethyl)pyrimidines (30); the first involved a direct oxidation of the methylene group with potassium permanganate or activated manganese dioxide but was unsuccessful and gave either unchanged starting material or an intractable tar, probably due to oxidative degradation of the pyrimidine ring.

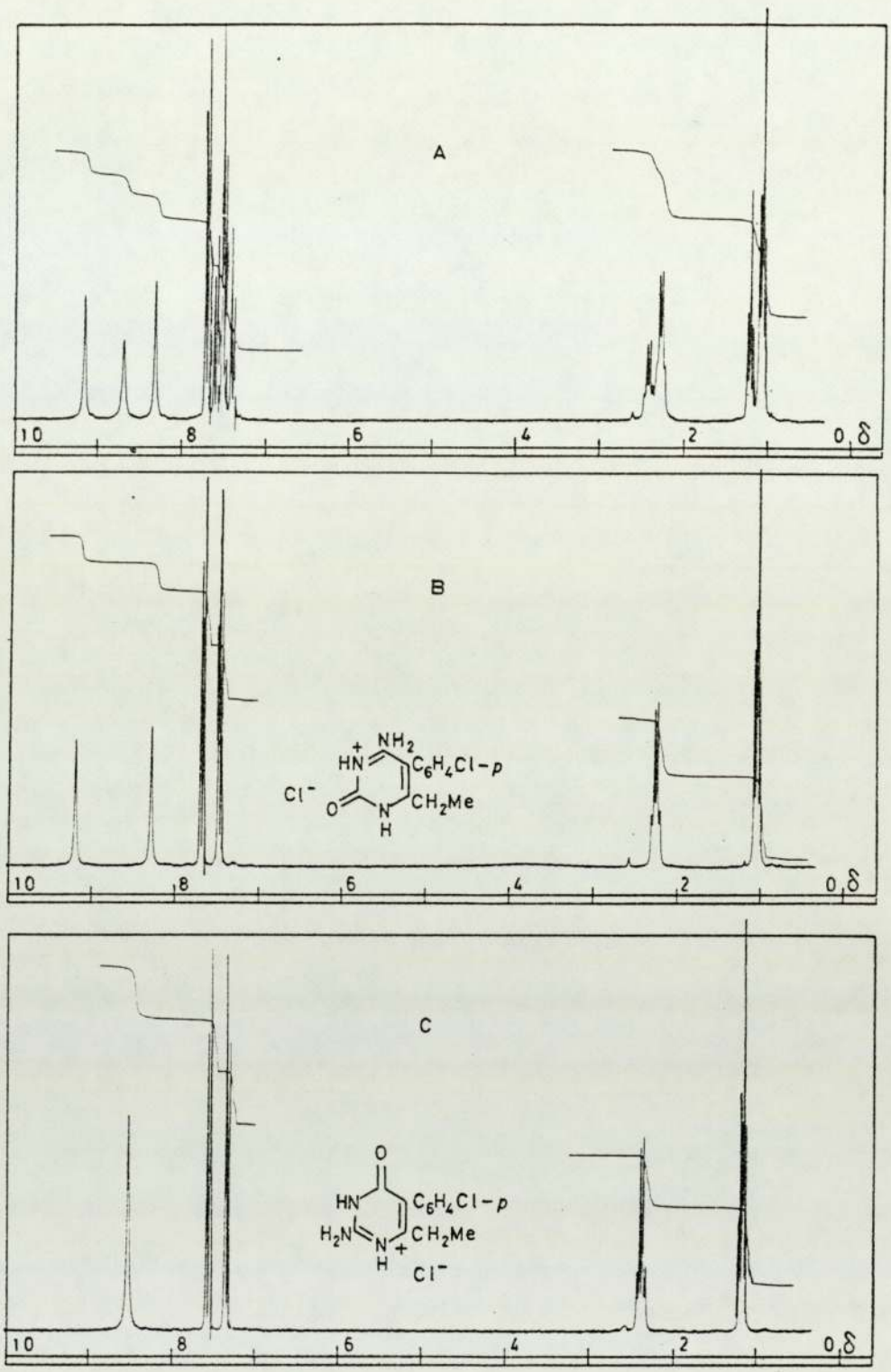
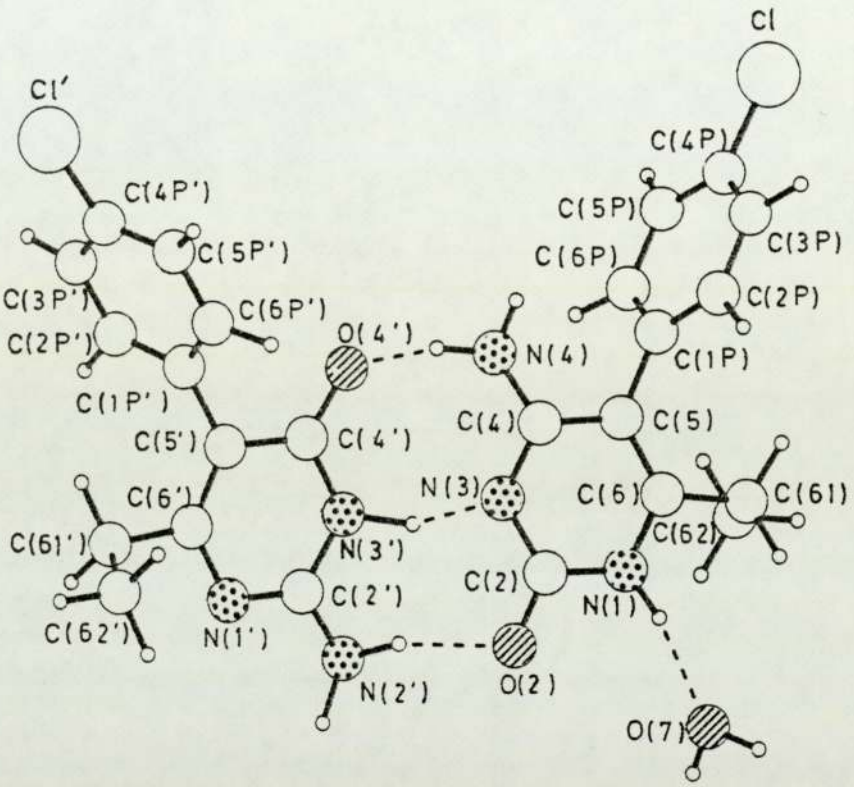
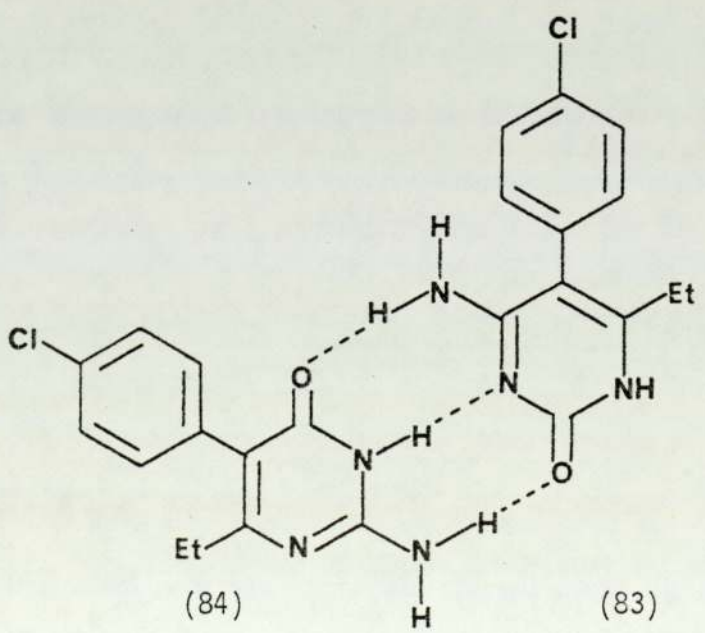


Figure 6.1 220 MHz ^{1}H-n.m.r. spectra of A: mixture of hydrochloride salts formed from the 6M-hydrochloric acid hydrolysis of pyrimethamine (4); B: authentic compound (83a); C: authentic compound (84a). All spectra were recorded in [$^{2}H_{6}$]DMSO.

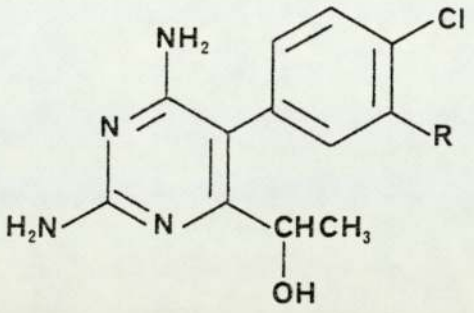
Figure 6.2 PLUTO drawing of the pyrimidinone hydrated duplex. Nitrogen atoms are stippled and oxygen atoms are hatched.



(84) (83)



(84) (83)

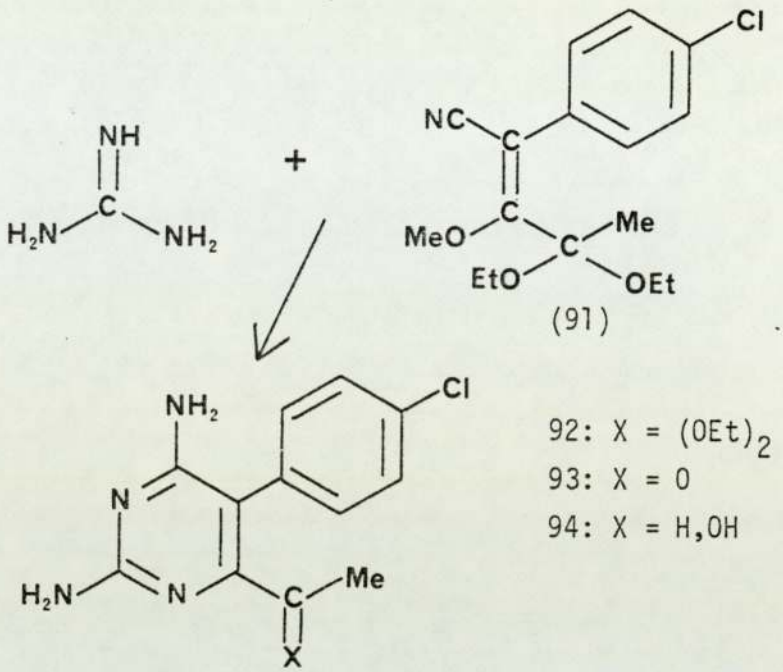


(30)

R = NO_2, NH_2, NHAc, N_3

The second approach entailed an initial synthesis of 6-(1-hydroxyethyl)-pyrimethamine (94) with the alcohol function suitably protected and the subsequent introduction of a 3'-nitro group by conventional methods. The 6-hydroxyethylpyrimidine (94) was prepared by the method of Rees and colleagues[160] (outlined in scheme 6.7) in a manner identical to that described for the synthesis of diaminopyrimidines described previously (chapter 5); thus ethyl 2,2-diethoxypropanoate was prepared and reacted with 4-chlorophenylacetonitrile in basic medium and the product treated with diazomethane to furnish the enol ether (91). Condensation of (91) with guanidine in ethanolic sodium ethoxide gave the diaminopyrimidine and subsequent hydrolysis and reduction by standard methods afforded the ketone (93) and racemic alcohol (94) respectively.

Scheme 6.7 Synthesis of 6-(1-hydroxyethyl)-pyrimethamine



Since pyrimethamine (4) nitrated exclusively in the 3'-position on treatment with nitric acid-sulphuric acid at room temperature (chapter 5), a similar reaction was attempted with the ketone (93) at 5° and with the alcohol (94) under identical conditions. Although, in both cases, t.l.c. examination of the reaction mixture indicated the complete consumption of starting material, product isolation or identification proved impossible. In retrospect it is probable that the alcohol (94) underwent an acid catalysed elimination under the reaction conditions employed, whereupon further degradation of the molecule might be expected. Treatment of the methyl ketone (93) with sodium nitrate in anhydrous trifluoracetic acid, a less vigorous nitrating agent, gave only starting materials and further attempts to synthesise the required 6-(1-hydroxyethyl)pyrimidines via this route were abandoned.

The photolytic and thermolytic degradation of aromatic azides in solution has been the subject of extensive investigations[139] and the formation of a variety of products including the amino, azo, azoxy

and nitro compounds has been reported. Synthetic efforts directed towards the preparation of authentic standards of the appropriate derivatives of MZP, as potential degradation products were limited to the amino (22) and nitro (49) compounds and the synthesis of intermediate products was without success.

Thermolysis of arylazides in an inert solvent often offers a route to the corresponding azo compound in acceptable yield. However, all attempts to prepare the appropriate azo derivative from MZP in this manner failed regardless of solvent used and an intractable black solid was isolated. Oxidative coupling of aromatic amines to furnish the azo compound is well established and several conventional reactions were attempted in order to couple (22) although only starting materials were recovered in all cases. A recent report documents the use of superoxide ion as oxidant[162] and this method entailed stirring a solution of the amine and potassium superoxide in dry pyridine for 12 h, whereupon a deep red colouration developed. However, t.l.c. examination of the mixture showed only a trace of new material and extending the reaction time was ineffective. An alternative approach *via* reduction of nitropyrimethamine (49) with zinc powder in methanolic sodium hydroxide was equally unsuccessful.

Synthesis of the phenylhydroxylamine (79A) by partial reduction of the nitro compound (49) was attempted following the standard method with zinc powder and ammonium chloride, but the poor solubility of (49) in aqueous solvent systems rendered this reduction ineffective. In a recent report, Ayangor et al[164] have described the facile reduction of nitroarenes to N-arylhydroxylamines with hydrazine and Raney nickel under phase-transfer conditions. Thus a suspension of (49) in ethanol-dichloroethane (1:1) was reduced at 0°

following the literature method. However, the product was identified as the corresponding amine (22) due to complete reduction of the nitropyrimethamine (49) despite the low reaction temperature employed.

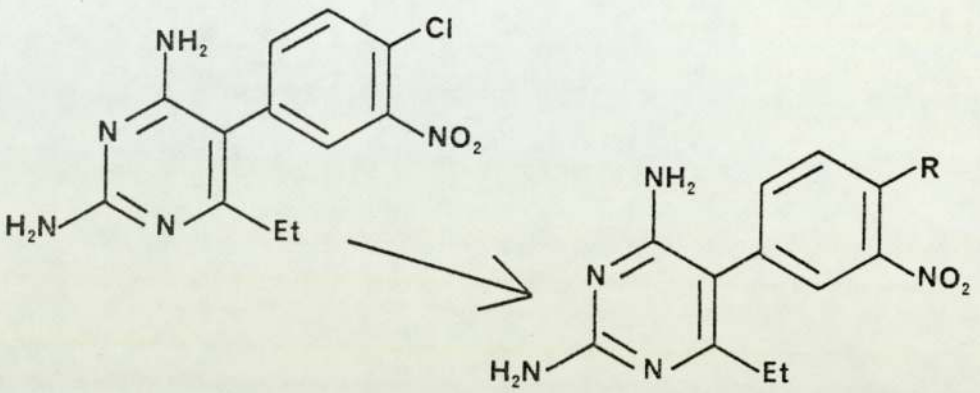
CHAPTER 7

SYNTHESES INVOLVING THE REACTION OF NITROPYRIMETHAMINE (MNP) AND *iso*-NITROPYRIMETHAMINE (*iso*-MNP) WITH AMINES

Inactivity against DHFR was a central precept in the development of modified 2,4-diaminopyrimidines as probes for a folate-independent target, and this was achieved by the synthesis of compounds encompassing a polar or ionic substituent in the 5-position such that association with the enzyme active site was precluded. As discussed previously (chapter 5) the 4'-chloro group of nitropyrimethamine (49) and indeed the 3'-chloro substituent of the isomeric 2,4-diamino-5-(3-chloro-4-nitrophenyl)-6-ethylpyrimidine (50) are activated to nucleophilic attack by virtue of the corresponding *ortho* nitro group and consequently introduction of the requisite polar species was achieved *via* nucleophilic aromatic substitution reactions. Thus the nitropyrimidines (49 and 50) served as starting materials for all subsequent synthetic reactions. Amines were invariably the nucleophile of choice in such reactions (tables 7.1 and 7.2) since, in addition to the inherent basicity of these compounds, subsequent chemical modification of the amine was possible after its introduction.

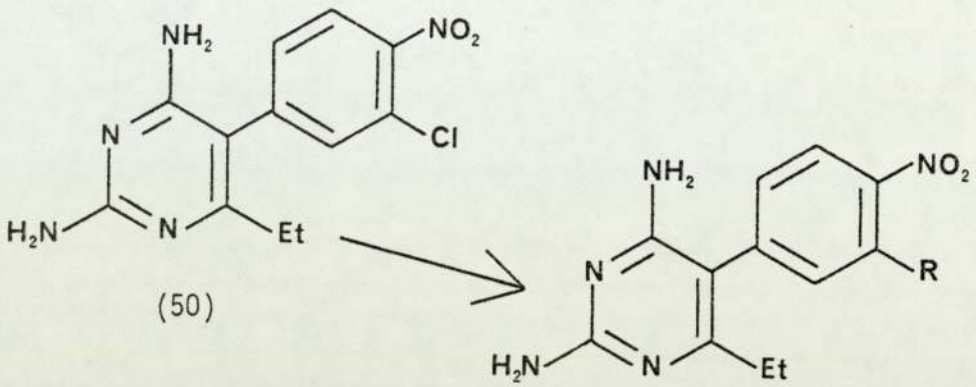
m-Nitropyrimethamine (MNP) (49) reacted slowly with aqueous solutions of the volatile simple aliphatic amines methylamine, dimethylamine and ethylamine to furnish the appropriate substitution products (95-97) in reactions hampered by the poor solubility of (49) in aqueous media and loss of amine under the reflux conditions employed. The progress of each reaction was monitored by t.l.c., complete consumption of reactant invariably necessitating reflux

Table 7.1 Structures of the amine-substituted nitropyrimethamine analogues



Compound Number	R
95	methylamino
96	dimethylamino
97	ethylamino
98	n-butylamino
99	piperidin-1-yl
100	piperazin-1-yl
101	4-methylpiperazin-1-yl
102	pyrrolidin-1-yl
103	4-morpholino
104	cyclohexylamino
105	N-methylcyclohexylamino
106	benzylamino
107	N-methylbenzylamino
108	N-ethylbenzylamino
109	(±)α-methylbenzylamino
110	dibenzylamino
111	phenethylamino
112	2-aminoethylamino
113	3-aminopropylamino

Table 7.2 Structures of the amine-substituted iso-nitropyrimethamine analogues



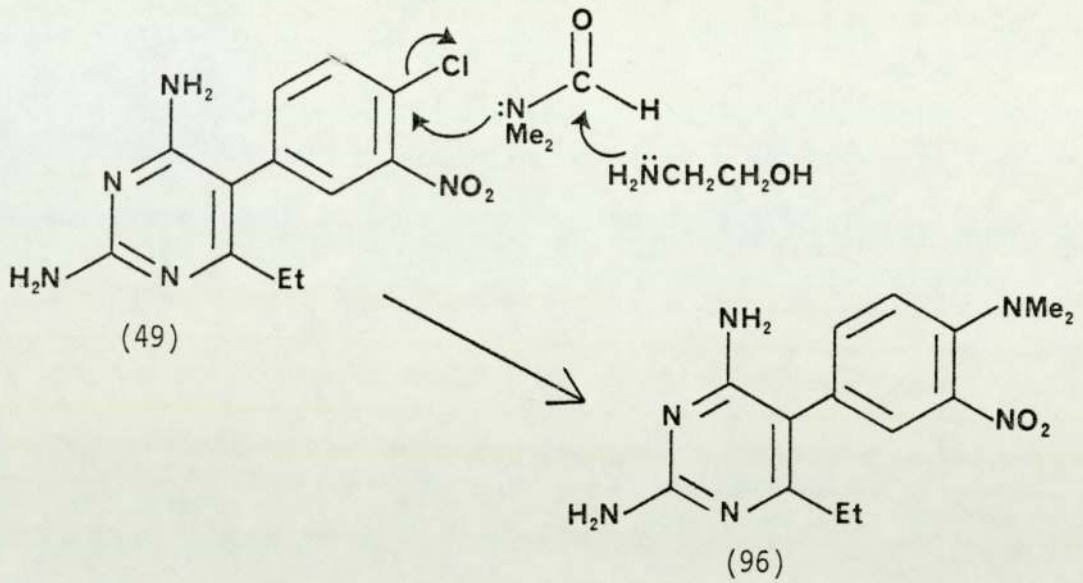
Compound Number	R
114	n-butylamino
115	benzylamino
116	N-methylbenzylamino

times in excess of 48 h and addition of a large excess of amine. Diethylamine failed to react with (49) despite protracted reaction times presumably due to steric factors and the use of DMSO, a promoter of nucleophilic aromatic substitution reactions, as solvent was without effect. Recrystallisation of the amine derivatives (95-97) from aqueous DMF afforded highly coloured crystalline compounds characterised by n.m.r. spectroscopy as being unsolvated.

The dimethylamino analogue (96) was also prepared by an interesting alternative synthesis; Yamamoto[165] described the replacement of labile aromatic chloro substituents by dimethylamino groups in a novel exchange reaction catalysed by ethylenediamine or ethanolamine and utilising DMF as solvent. Thus MNP (49) reacted under identical conditions in DMF containing ethanolamine (1.1 mole equivalents) at 90° to form a product identical (t.l.c., n.m.r. and mixed m.p.) with the dimethylaminopyrimidine (96) synthesised by the aforementioned conventional method.

The exact mechanism of the reaction has yet to be elucidated but is probably mediated through formation of a DMF-ethanolamine complex, where ethanolamine serves as a formyl acceptor (scheme 7.1). The dimethylamination is also catalysed to a lesser extent by longer chain diamines, but not triethylamine or ethylene glycol. Interestingly, an attempt to react MNP with hydroxylamine in DMF yielded traces of a red component that co-chromatographed with (96) but was not isolated. DMF slowly decomposes on prolonged boiling to form dimethylamine and carbon monoxide and the dimethylamination of 2-chloronitrobenzene under such conditions has been documented[166]. However, (49) was stable in DMF at 90° in the absence of ethanolamine, and no evidence of a reaction between (49) and ethanolamine was observed at lower temperatures as reported.

Scheme 7.1 The ethanolamine-catalysed dimethylamination of MNP



(49) (96)

Analogous reactions conducted with formamide, N-methylformamide and diethylformamide gave results paralleling those reported by the author, and although (49) reacted with N-methylformamide to form a trace of the methylaminopyrimidine (95), as monitored by t.l.c., formamide and diethylformamide were unreactive.

Amines boiling above 100° reacted readily with MNP (49), complete product formation invariably evident (t.l.c.) after refluxing for 2 h, although no reaction occurred at room temperature. Thus while propylamine gave only starting materials, n-butylamine reacted smoothly with (49) and also iso-MNP (50) to furnish the corresponding butylaminopyrimidines (98 and 114) in excellent yield and these crystallised from aqueous ethanesulphonic acid as the appropriate amine monoethanesulphonate salts (98a and 114a). In contrast sec-butylamine afforded a mixture of starting material and one new component (t.l.c.) in unacceptable yield regardless of the reaction conditions employed and the poor reactivity of this amine was attributed to steric factors.

Piperidine and other heteroalicyclic amines reacted efficiently with MNP (49) to form a single product (t.l.c.) in all cases and no evidence for the production of benzimidazole N-oxides by intervention of an ortho-nitro interaction[167] was adduced under such basic conditions. Limited solubility in ethanol or 2-ethoxyethanol invariably necessitated recrystallisation of the products from DMF often as a solvate. The structure of the piperidinopyrimidine (99) has been solved by X-ray crystallography as a monohydrochloride monohydrate[168] following treatment with hydrochloric acid in aqueous ethanol.

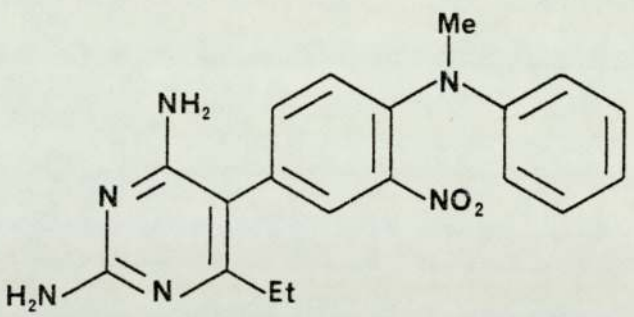
Piperazine, a crystalline solid at temperatures below 100°, failed to react with (49) in boiling diglyme or DMF at 100°. However, addition of (49) in portions to an excess of the amine at 125° was successful and the piperazinopyrimidine (100) deposited on cooling following dilution of the crimson melt with 2-ethoxyethanol.

Primary and secondary arylalkylamines also furnished the requisite substitution products after extended refluxing with MNP (49) or iso-MNP (50) in reactions monitored by t.l.c. as somewhat less facile than those described above. For those amines immiscible with water product isolation was effected by precipitation with ether, collection and subsequent trituration with water to minimise contamination with the corresponding amine hydrochloride, a by-product of the reaction.

One noteworthy observation arose from these predominantly unexceptional syntheses regarding the reaction of MNP with dibenzylamine, where a second, less abundant, component was detected (t.l.c.) in addition to the desired dibenzylaminopyrimidine, possibly due to a base catalysed cyclisation[167,169] of (110). Evidence in corroboration of this hypothesis was provided by refluxing a sample

of (110) in dibenzylamine for 12 h when starting material was consumed and a heavily contaminated product developed, which nevertheless gave a molecular ion at m/z 436 corresponding to the benzimidazole N-oxide (117) (scheme 7.2).

Preliminary investigations concerning the reaction of substituted anilines with MNP were disappointing; thus the nitropyrimidine (49) was recovered unchanged after boiling in benzylaniline (N-phenylbenzylamine) for 48 h, while N-methylaniline furnished a deep red product characterised (m.s.) as the anilinopyrimidine (118), but heavily contaminated (n.m.r., t.l.c.) despite attempted purification by column chromatography.

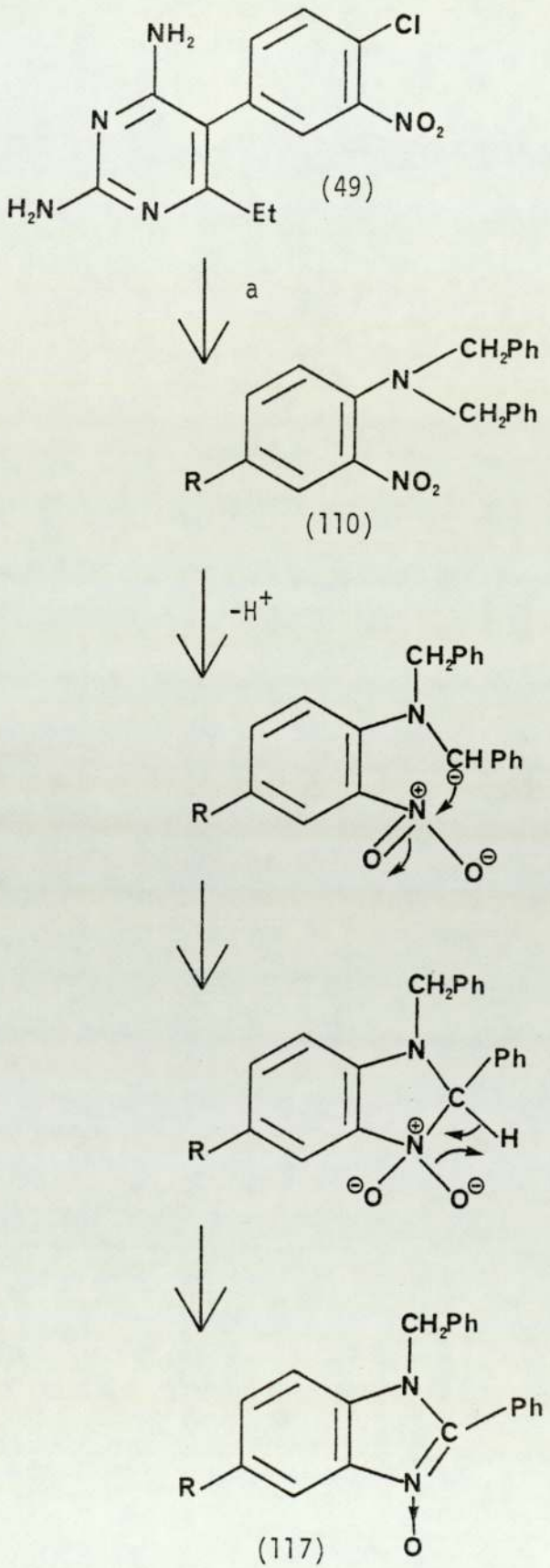


(118)

The preparation of (118) was desirable since subsequent treatment with triethyl phosphite or triphenyl phosphine would conceivably represent a route to an interesting phenazine derivative.

The aminoethylamino- (112) and aminopropylaminopyrimidines (113) were synthesised from (49) and an excess of corresponding diamine in the usual manner, and no evidence suggesting dimer formation was adduced. Indeed, refluxing a sample of (112) in excess ethylenediamine for 12 h furnished only unchanged (112) as evident on t.l.c. examination. In contrast, the homologous diamines, putrescine, diaminohexane and diaminododecane invariably gave several polar products regardless of repeated attempts under various conditions of temperature, reflux time and solvent used and numerous

Scheme 7.2 Cyclisation of compound (110)

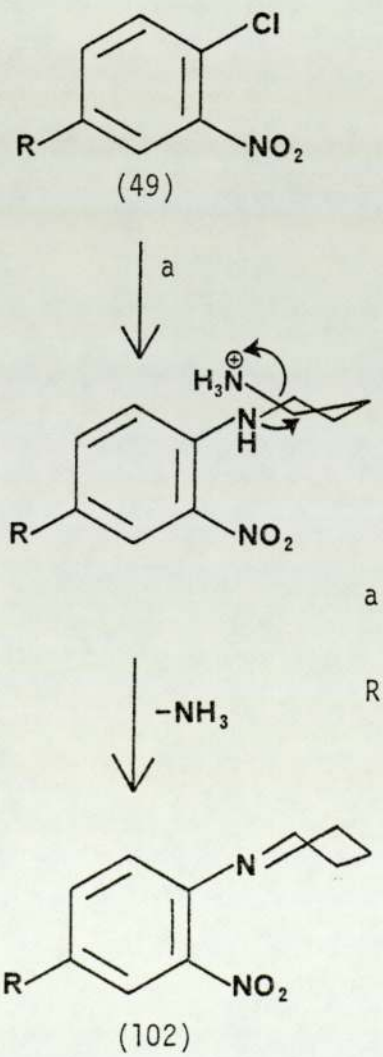


a. excess dibenzylamine; reflux 12 h

R = 2,4-diamino-6-ethylpyrimidin-5-yl

efforts to purify the mixtures by fractional crystallisation or reprecipitation were abortive. Diaminobutane (putrescine) readily cyclises under acid catalysed conditions to form pyrrolidine[170]; MNP (49) reacted with diaminobutane to yield an orange waxy solid characterised by t.l.c. as comprising of three components, one of which co-chromatographed with the pyrrolidinopyrimidine (102) synthesised independently. The obvious implications of such an observation should be regarded as speculative but it is possible that formation of (102) may account for one component of the mixture (scheme 7.3).

Scheme 7.3 Cyclisation of the putrescine analogue of MNP; possible mechanism



a. putrescine in EtOH; reflux, 4 h

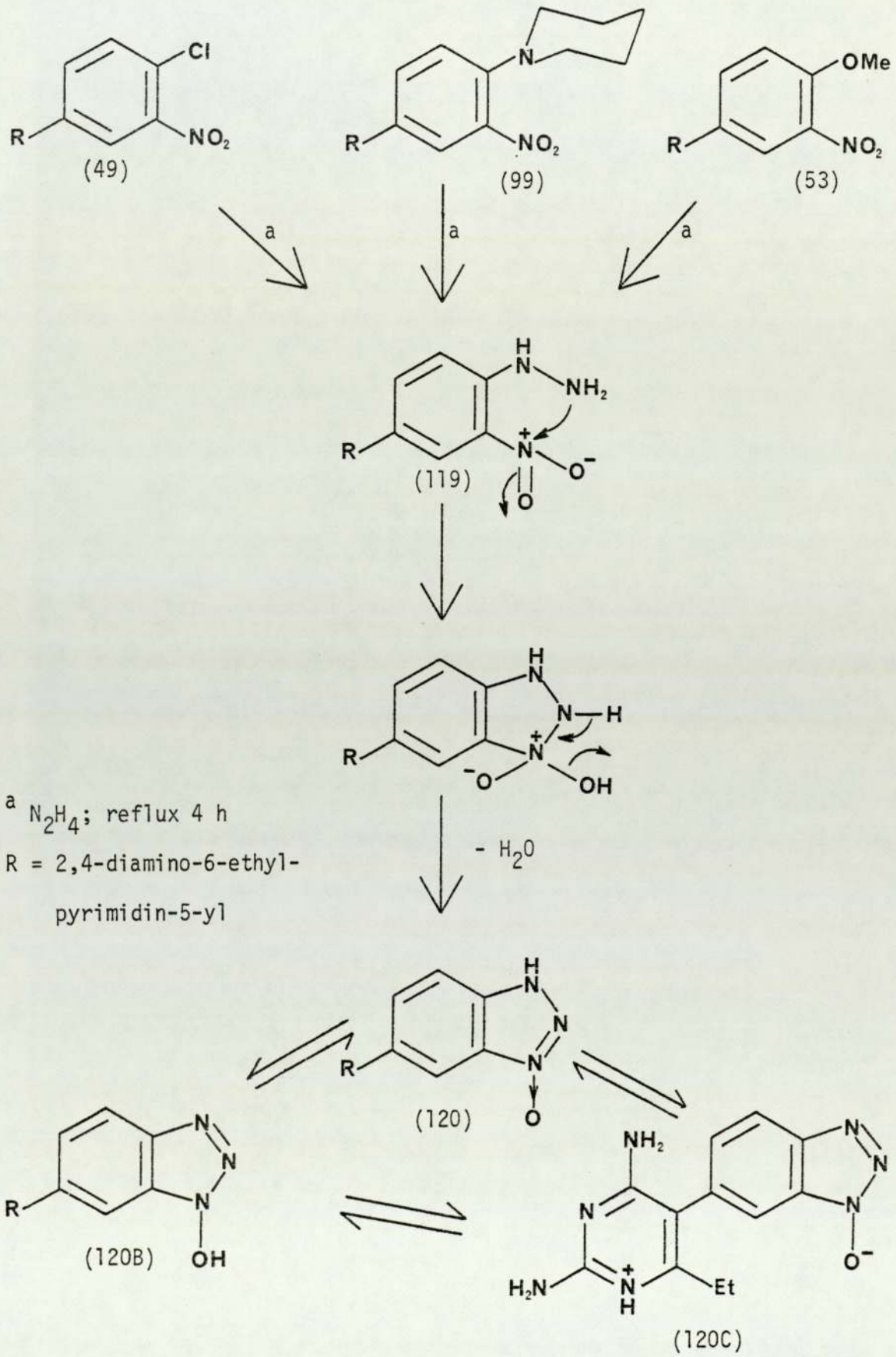
R = 2,4-diamino-6-ethyl-pyrimidin-5-yl

Replacement of the chloro group of MNP (or iso-MNP) by a hydrazino substituent would be desirable since, in addition to abolishing activity against DHFR, the ortho-nitrophenylhydrazine derivative (119) would offer a route to the corresponding ortho-nitrophenylazide following treatment with nitrous acid. However, isolation of (119) proved impossible even when the reaction with hydrazine was conducted in ethanol as solvent, and treatment of (49) with hydrazine hydrate furnished the hydroxybenzotriazole (120) as a hydrazinium salt via cyclisation of an intermediate o-nitrophenylhydrazine (119) under base catalysed conditions.

The well documented[148,167] formation of 1-hydroxy-1,2,3-benzotriazoles from o-halonitroarenes and hydrazine has also been reported for the corresponding o-methoxy-[171] and o-piperidino-[172] compounds. Consequently, the methoxynitropyrimidine (53) and piperidinonitropyrimidine (99) afforded, on treatment with hydrazine, products characterised as identical to that prepared from (49) (scheme 7.4). Purification of (120) proved singularly difficult and was finally achieved by conversion to a hygroscopic monomethanesulphonate salt (120a) and crystallisation from ethanol-ethyl acetate. The extraordinary insolubility of the hydroxybenzotriazole (120) in water or organic solvents may be attributable to formation of a stable zwitterionic tautomer (120C) in addition to those established tautomeric species (120 and 120B) although spectroscopic data has yet to confirm this possibility.

Phenylhydrazine, either undiluted or as a solution in ethanol, reacted in an analogous manner with the nitropyrimidine (49) to yield an N-phenylbenzotriazole N-oxide (122) presumably through the intermediacy of an o-nitrohydrazobenzene derivative (121) (scheme 7.5). Clearly, formation of a zwitterion as described above,is

Scheme 7.4 Reaction of MNP and derivatives with hydrazine hydrate



[a] N_2H_4; reflux 4 h

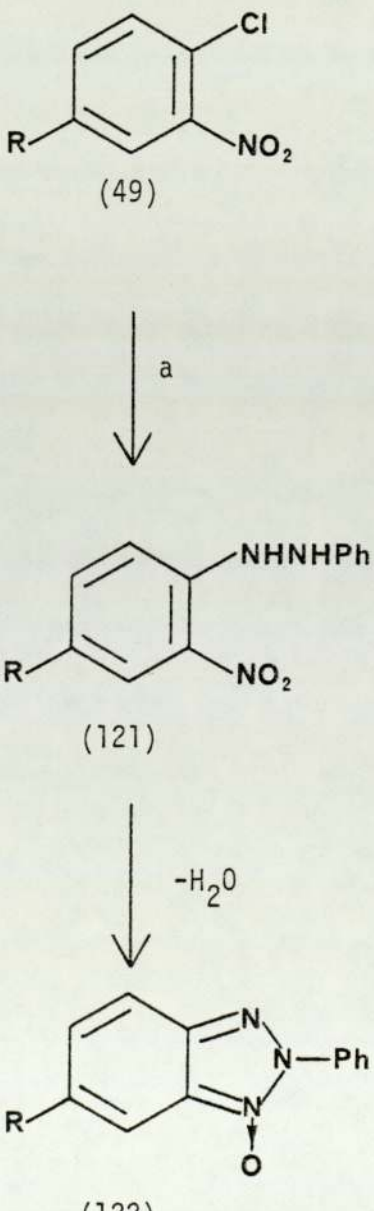
R = 2,4-diamino-6-ethyl-pyrimidin-5-yl

precluded by the absence of a mobile proton, perhaps accounting for the observed increased solubility in organic solvents. Phenylhydrazine decomposes at its boiling point forming aniline, benzene and phenyldiazene, presumably explaining the moderate yield of (122) obtained and the recovery of considerable quantities of an ether-soluble red contaminant, probably azobenzene.

Scheme 7.5 Reaction of phenylhydrazine with nitropyrimethamine

a. $PhNHNH_2$; reflux 4 h

R = 2,4-diamino-6-ethylpyrimidin-5-yl



CHAPTER 8

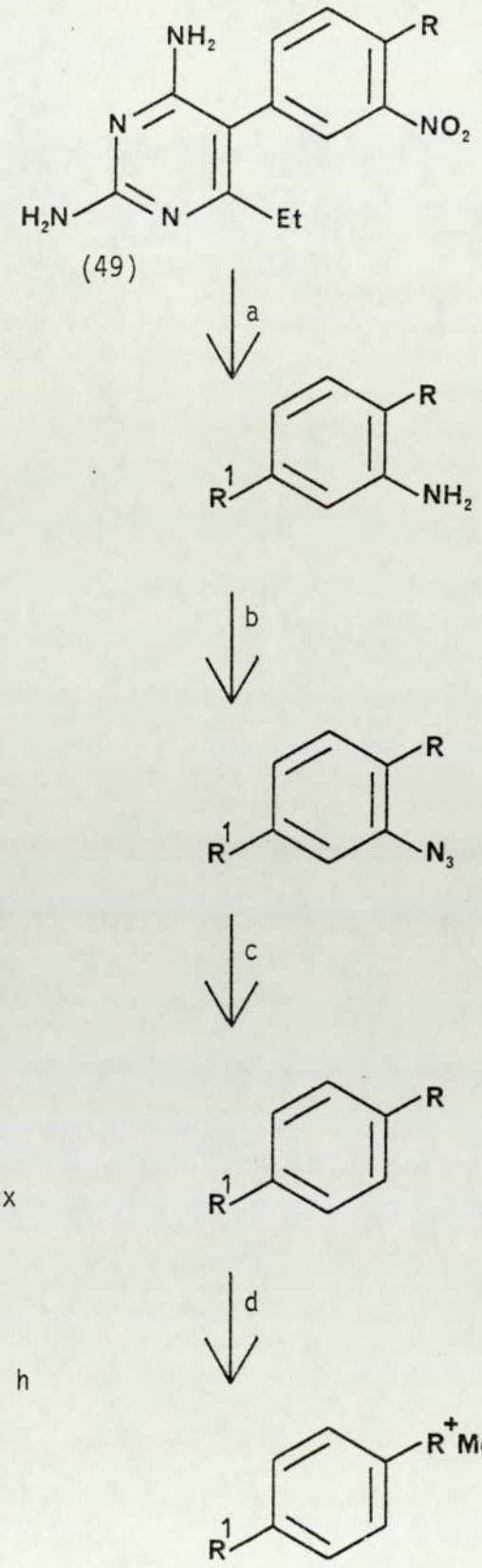
FURTHER REACTIONS IN THE NITROPYRIMETHAMINE SERIES

Functionalisation and subsequent replacement of the lipophilic chloro substituent of pyrimethamine (4) by a polar amine group cannot be achieved directly and was accomplished following nitration of the diaminopyrimidine as discussed previously (chapter 7). The compounds synthesised in this manner (tables 7.1 and 7.2) were regarded as prospective 'non-inhibitors' of DHFR by virtue of the positioning of a basic substituent in a former hydrophobic domain. These compounds were screened as inhibitors of rat liver enzyme, hopefully to confirm their lack of activity.

However, the ortho-nitro substituent present in all the aforementioned analogues may militate against the desired effect of the amine group, the electron withdrawing nature of the nitro substituent weakening the basicity of the amine. Also the inherent lipophilicity of an aromatic nitro group may counteract the influence of a polar species thereby restoring activity against DHFR. Moreover, subsequent chemical transformation and ultimate removal of the nitro group would afford a series of intermediate analogues, any of which might conceivably fulfil a role as probes for the alternative target under consideration.

The synthetic strategy adopted was essentially identical to that utilised for the preparation of compounds in the azidopyrimidine series (chapter 5), removal of the azido group and final quaternisation of the 4'-amine substituent being effected in two additional steps (scheme 8.1).

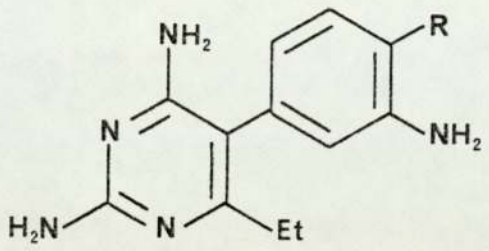
Scheme 8.1 Further reactions in the nitropyrimethamine series: synthetic strategy



a. $SnCl_2$/EtOH; reflux
or N_2H_4/Ni; 65°
b. HNO_2/NaN_3; <5°
c. N_2H_4; reflux
d. MeI/MeOH; 40°, 12 h

R = primary, secondary or tertiary amino group
R^1 = 2,4-diamino-6-ethylpyrimidin-5-yl

Table 8.1 Structures of the amine-substituted aminopyrimethamine derivatives



Compound Number	R
123	methylamino
124	dimethylamino
125	ethylamino
126	n-butylamino
127	piperidin-1-yl
128	piperazin-1-yl
129	4-methylpiperazin-1-yl
130	pyrrolidin-1-yl
131	4-morpholino
132	cyclohexylamino
133	N-methylcyclohexylamino
134	benzylamino
135	N-methylbenzylamino
136	N-ethylbenzylamino
137	(±)∝-methylbenzylamino
138	phenethylamino
139	2-aminoethylamino
140	3-aminopropylamino

The corresponding 3'-aminopyrimidines (table 8.1) were synthesised by reduction of the appropriate nitropyrimidines with hydrazine and Raney nickel or tin (II) chloride in ethanol, in reactions critically dependent on the choice of reagent used. Thus although several of the 3'-nitropyrimidines reduced smoothly on treatment with Raney nickel and hydrazine in ethanol, the majority were accompanied by formation of a persistent green contaminant and subsequent purification by recrystallisation or reprecipitation invariably proved abortive, usually resulting in complete degradation of the product. Moreover, several of the nitro analogues exhibited a poor solubility in ethanol or other acceptable solvents, the morpholinonitropyrimidine (103) being a notable example and consequently removal of Raney nickel by filtration after reduction proved impracticable.

In contrast, reductions conducted with tin (II) chloride in ethanol furnished the required amines in excellent yield often as crystalline solids, following liberation from the amine-stannicomplex with alkali, and solubility problems were not encountered. Bellamy et al[173] have described the selective reduction of nitroarenes in the presence of other reducible substituents with stannous chloride in ethanol or ethyl acetate. Recrystallisation of the amine derivatives was without incident with the exception of the 3'-amino-N-ethylbenzylaminopyrimidine (136) which had an uncharacteristically low melting point (<40°) necessitating purification as a diethanesulphonate salt (136a). All attempts at reducing the dibenzylaminonitropyrimidine (110) were unsuccessful due to the formation of two products (t.l.c.) regardless of the conditions employed and this presumably occurred as a consequence of reductive debenzylation rather than thermal decomposition, since prolonged boiling of the

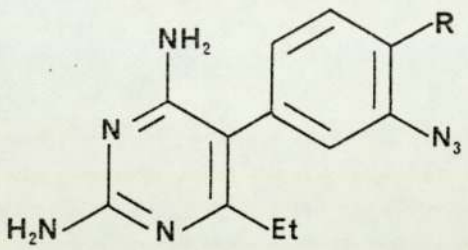
mixture in nitrobenzene was ineffectual. Catalytic hydrogenation in acetic acid afforded an identical mixture and the insolubility of (110) in suitable organic solvents precluded any further attempts.

The appropriate azide analogues (table 8.2) were synthesised by diazotisation of the corresponding amines in 5M-hydrochloric acid and treatment with an excess of sodium azide in the usual manner. Those derivatives that cyclised upon diazotisation to furnish benzotriazoles form the basis of a discussion elsewhere (chapter 9).

Formation of a diazonium salt was evident in all cases by the development of a golden yellow solution and the progress of azide formation was monitored by t.l.c. and appearance of the characteristic sharp absorption band at ~ 2150 cm^{-1} in the i.r. spectra. With the exception of the N-methylcyclohexylaminopyrimidine (133) and the pyrrolidinopyrimidine (130), azidation proceeded smoothly with no evidence of interference by the 4'-amine substituent and after precipitation with ammonia solution the photosensitive azide free bases were crystallised from aqueous ethanol.

All attempts at diazotising (133) failed due to a rapid degradation of the amine in acid even at low temperatures and this synthesis was abandoned. Diazotisation of the pyrrolidinopyrimidine (130) in the normal manner was uneventful and an aliquot gave a deep red colour on reaction with alkaline β-naphthol solution, confirming the formation of a diazonium salt. However, the dark precipitate formed on basification was characterised by t.l.c. as comprising in excess of six components and subsequent attempts at optimising the reaction were unsuccessful.

Table 8.2 Structures of the amine-substituted azidopyrimethamine derivatives



Compound Number	R
141	dimethylamino
142	piperidin-1-yl
143	piperazin-1-yl
144	4-methylpiperazin-1-yl
145	4-morpholino

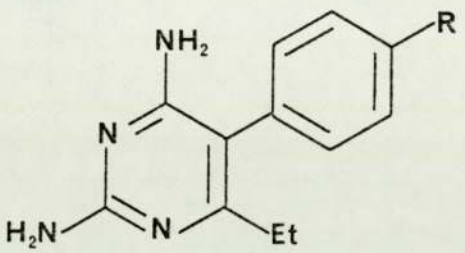
Removal of the azido substituent by reductive deazidation in hydrazine hydrate was accomplished in a manner essentially identical to that adopted for the alkoxyazidopyrimidines (65 and 66) (chapter 5). The vigorously effervescent reactions invariably deposited starting material on the walls of the condenser, necessitating removal by the cautious addition of ethanol, and product formation was determined chromatographically (t.l.c.) and by the absence of a

characteristic azide inflection in the i.r. spectrum. The requisite 4'-aminopyrimidines (table 8.3) obtained in moderate yield on dilution of the reaction mixture with water, resembled the alkoxy-pyrimidines (67 and 68) in exhibiting an intense red or purple colouration on dissolution in concentrated acids presumably attributable to formation of a dicationic species (chapter 5.4)

The nucleophilic displacement by hydrazine of amino groups in π-deficient heterocycles has been reported by Bliss[93] who prepared dihydrazinoquinazoline from diaminoquinazoline in refluxing hydrazine. However, no evidence to support the analogous formation of 2,4-dihydrazinopyrimidine derivatives via a similar mechanism was adduced in the present study.

Unfortunately, insufficient of the 4'-morpholinoazidopyrimidine (145) was available to render a deazidation reaction practicable at the time of writing. However, a reaction conducted on a small scale (50 mg) indicated complete deazidation after 2 h (t.l.c. and i.r.) and microanalytical data is awaited.

Table 8.3 Structures of the amine-substituted pyrimethamine derivatives



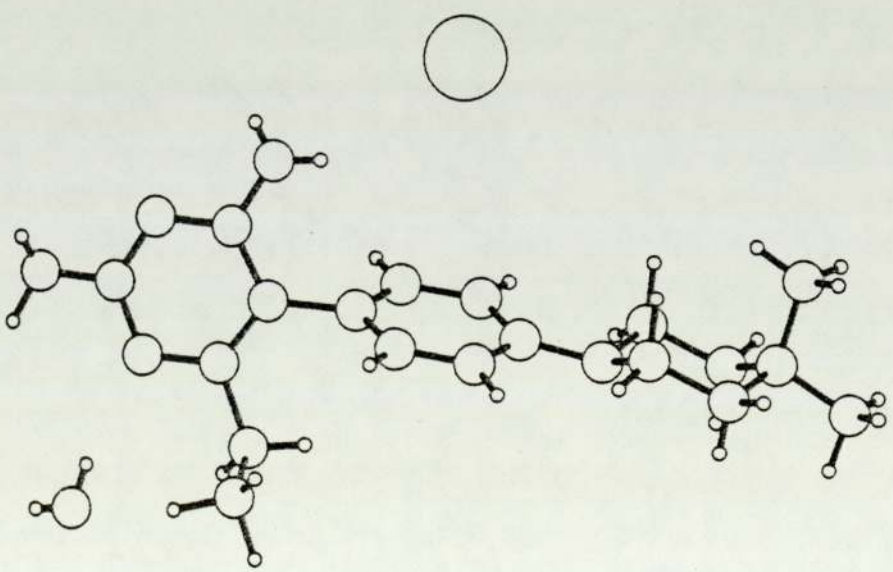
Compound Number	R
146	dimethylamino
147	piperidin-1-yl
148	piperazin-1-yl
149	4-methylpiperazin-1-yl

Conversion of the 4'-aminopyrimidines prepared above to the corresponding quaternary ammonium iodides was conveniently achieved with an excess of methyl iodide in dry methanol at 40° and a control reaction with pyrimethamine under identical conditions discounted any

possibility of methylation elsewhere since, in the latter case, only starting materials were recovered. Unfortunately, insufficient of the piperidinopyrimidine (147) was obtained from the deazidation stage to render the quaternisation reaction feasible, while the piperazino- (148) and 4-methylpiperazinopyrimidines (149) afforded hygroscopic colourless prisms characterised (t.l.c., m.s. and n.m.r.) as identical (150). In each case the mass spectra of the quaternary iodides did not give a molecular ion but furnished a prominent M^+-CH_3 peak characteristic of such compounds at an m/z value corresponding to the respective tertiary amine.

The structure of the pyrimidinodimethylpiperazinium iodide (150) has been solved by X-ray crystallography[174] (fig. 8.1).

Figure 8.1 PLUTO drawing of the crystal structure of compound (150)



(150)

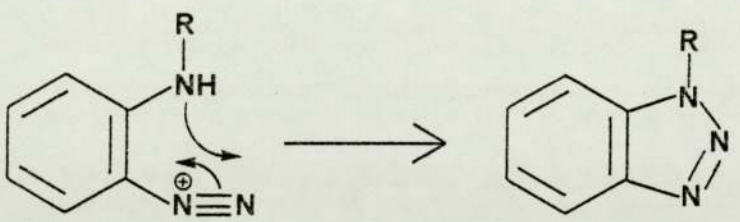
CHAPTER 9

SYNTHESES IN THE BENZOTRIAZOLE SERIES

9.1 Cyclisation of o-phenylenediamine derivatives

Benzotriazoles are readily obtained following diazotisation of o-phenylenediamines or appropriately substituted derivatives (scheme 9.1) and numerous examples of compounds prepared by this route have been documented[175-177].

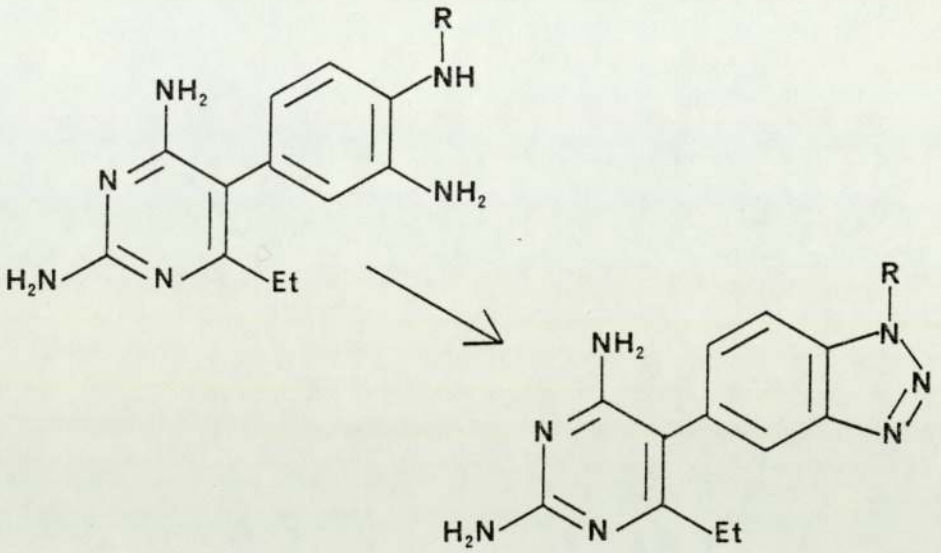
Scheme 9.1



R = alkyl or aryl

Thus treatment of those diaminopyrimidines prepared previously (chapter 8) encompassing an o-phenylenediamine structure with nitrous acid afforded the corresponding 1,5-disubstituted benzotriazoles (table 9.1) in reactions that frequently went to completion within 1 h (t.l.c.). Yields were satisfactory although the presence of a coloured contaminant, probably due to azo dye formation, occasionally necessitated reprecipitation as the hydrochloride salt from water.

Table 9.1 Structures of the benzotriazoles

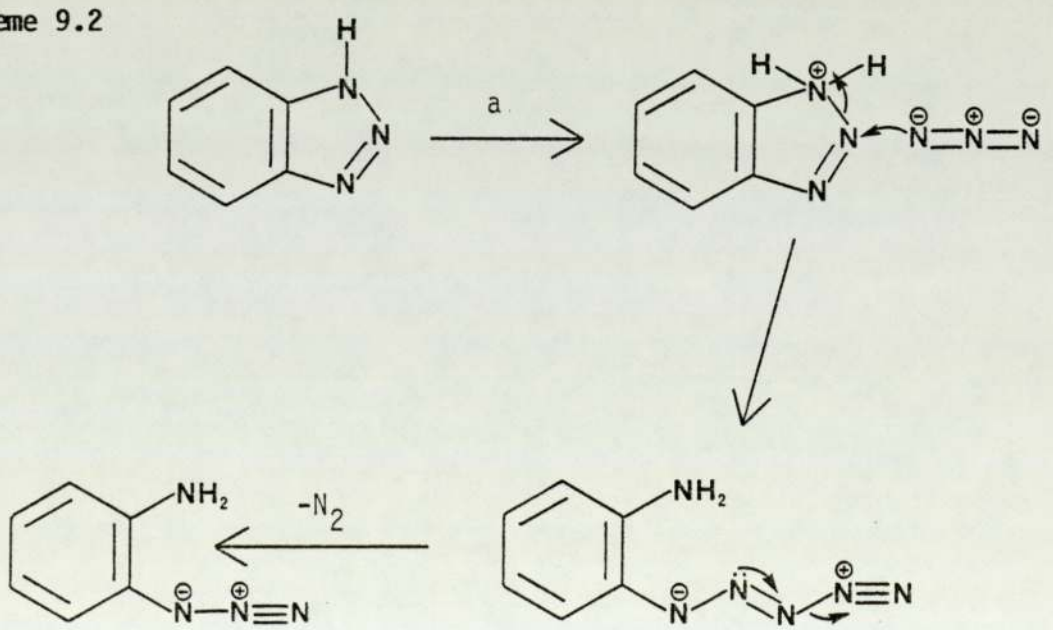


Compound Number	R
152	methyl
153	ethyl
154	n-butyl
155	cyclohexyl
156	benzyl
157	phenethyl

Although the benzotriazole derivatives prepared above constitute a novel series of substituted diaminopyrimidines and consequently warrant evaluation, synthesis of the appropriate 3'-azidopyrimidines

was a primary objective and the possibility of achieving this was explored *via* several approaches. Dissolution of the methylamino-pyrimidine (123) in concentrated sulphuric acid prior to diazotisation was attempted in an effort to suppress benzotriazole formation and facilitate pre-emptive azidation with an excess of sodium azide, but was without success and only the corresponding N-methylbenzotriazole (152) was obtained. Smith et al[178] have described the preparation of *o*-azidoaniline from benzotriazole on reaction with sodium azide in acetic acid at 50-55°. However, treatment of (152) in an identical manner furnished only starting materials (t.l.c. and i.r.). Since the reaction is thought to proceed *via* initial protonation of the benzotriazole at N1 and subsequent ring-opening on nucleophilic attack by azide ion (scheme 9.2), the weaker basicity of the N-methyl nitrogen may preclude initial protonation and formation of the reactive intermediate species.

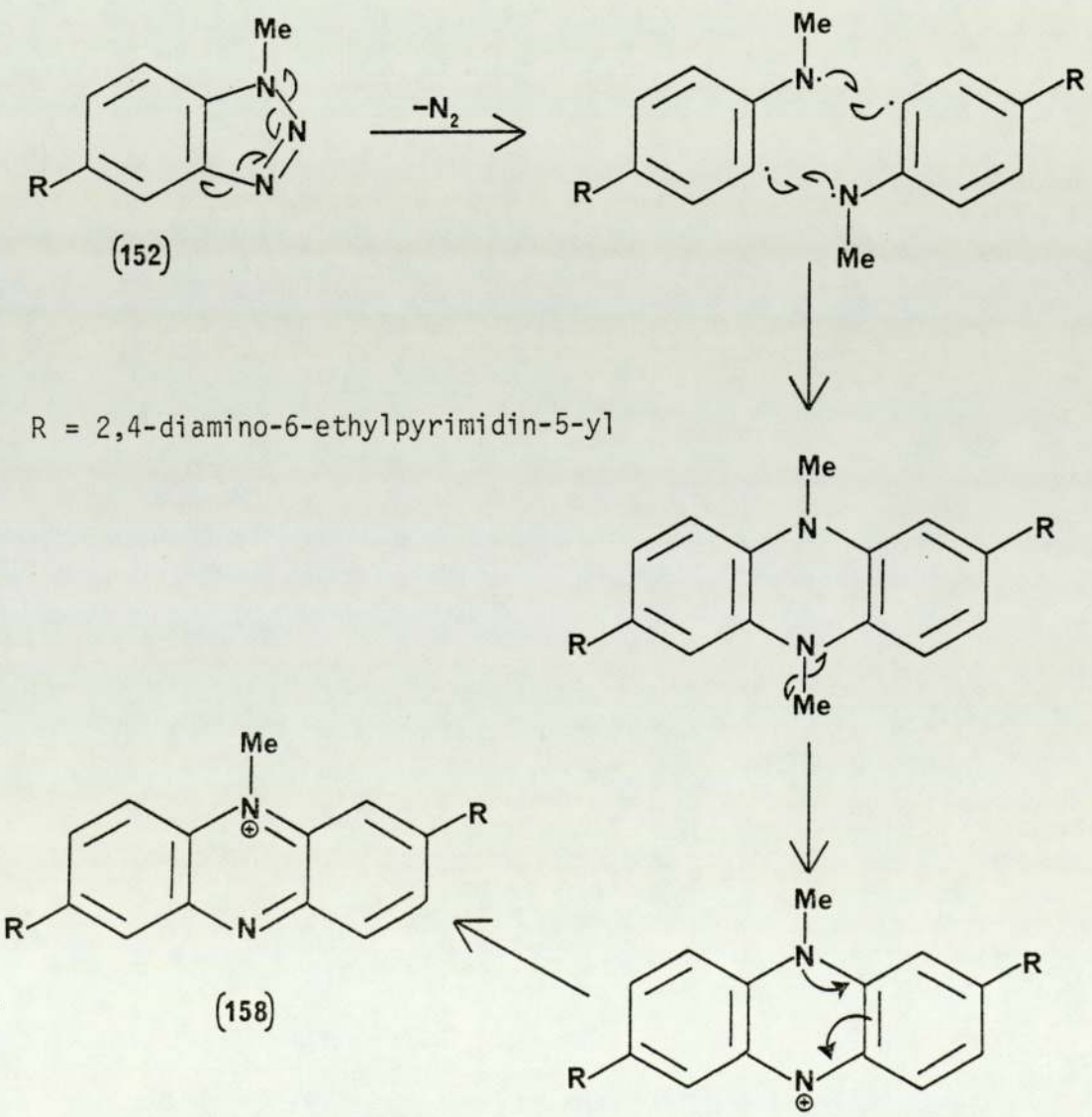
Scheme 9.2



a. NaN_3 in AcOH; 50-55°

Reactions involving the thermal or photolytic decomposition of benzotriazoles are well established and often involve an elimination of nitrogen from the molecule[177]. Pyrolysis of (152) in diethyl phthalate at 300° and removal of the solvent by steam distillation gave a cream solid which proved insoluble in all common organic solvents. Mass spectral analysis furnished a molecular ion (M^+) of m/z = 467 and although speculative this may correspond to formation of a phenazine derivative (158) arising via the mechanism proposed in scheme 9.3.

Scheme 9.3 Possible mechanism for the pyrolysis of the N-methyl-benzotriazole (152)



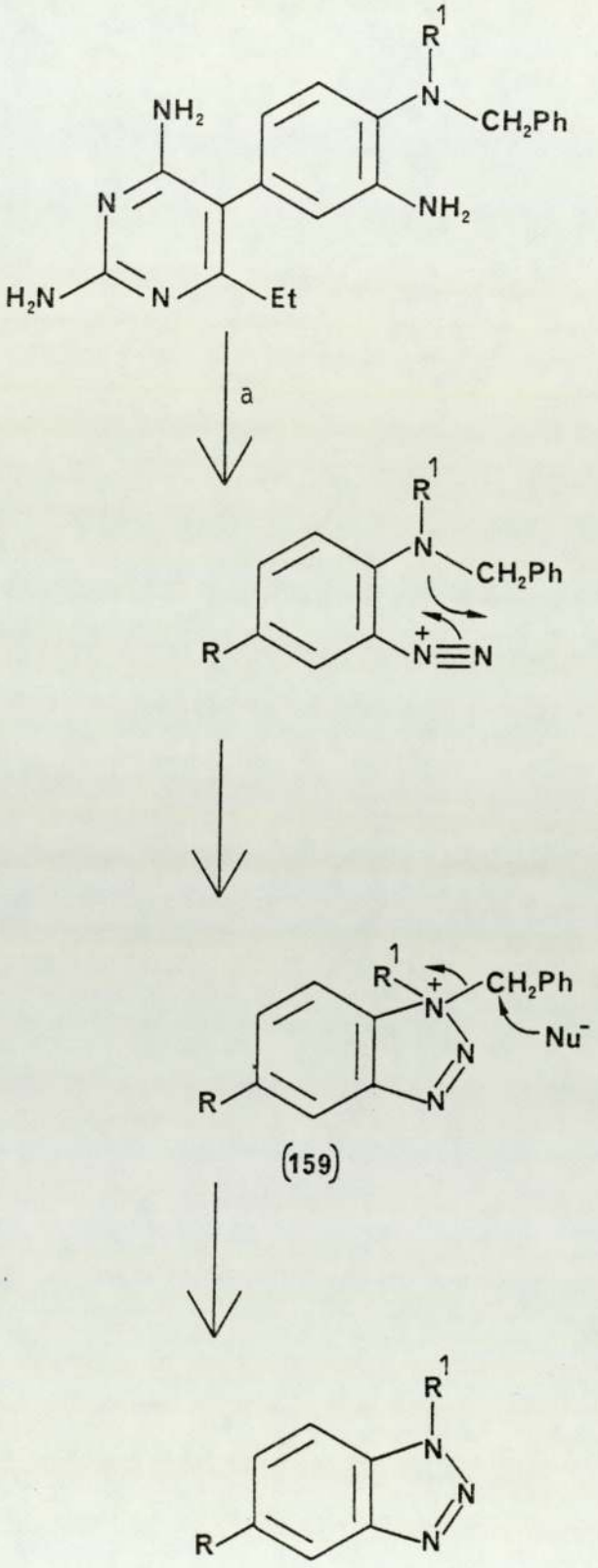
9.2 Debenzylation of alkylbenzylaminopyrimidines

Although unsubstituted or monosubstituted o-phenylenediamines of the type discussed above underwent facile cyclisation to furnish benzotriazole derivatives, it was assumed that the corresponding N,N-disubstituted compounds, or those with a heteroalicyclic substitutent, could not and these compounds were consequently diazotised and converted to the appropriate azido analogues (chapter 3). However, treatment of the N-methylbenzyl for N-ethylbenzyl-pyrimidines (135 and 136) with nitrous acid at 0° as usual and subsequent addition of sodium azide, gave products in almost quantitative yield which surprisingly were not the expected azide analogues (i.r.) and formed equally well in the absence of azide ion.

The compounds were subsequently characterised by high resolution ^{1}H-n.m.r. and mass spectrometry as being the N-methyl and N-ethylbenzotriazoles respectively and were identical (t.l.c., mixed m.p. and i.r.) to the appropriate benzotriazole derivatives (152 and 153) synthesised by the alternative route. The decomposition of 2-benzyl-1-methylbenzotriazolium salts in alkali to give 1-methylbenzotriazole has been reported[179] but to my knowledge the synthesis of 1-alkylbenzotriazoles from N-alkylbenzylamine derivatives has not been described previously and constitutes a novel reaction.

The mechanism probably involves initial formation of an N-alkyl-N-benzylbenzotriazolium intermediate (159) with subsequent nucleophilic attack at the benzylic carbon by chloride, nitrite or water to furnish the corresponding benzotriazole and benzyl chloride, benzyl alcohol or benzyl nitrite (scheme 9.4). Evidence in support of this hypothesis was provided subjectively by the characteristic odour of benzyl chloride emanating from the reaction mixture and more

Scheme 9.4 Probable mechanism for the debenzylation of N-alkylbenzylaminopyrimidines



a. HNO_2; $<5^{o}$

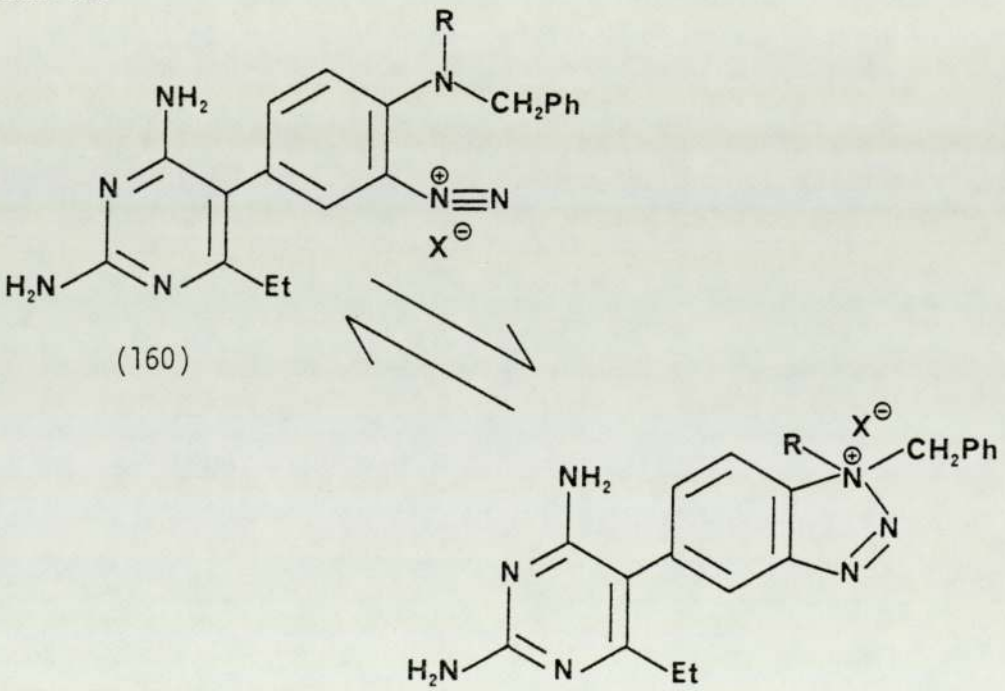
R = 2,4-diamino-6-ethyl-pyrimidin-5-yl

R^1 = Me,Et

precisely following extraction of the reaction mixture with dichloromethane and analysis by g.l.c., when three components were detected, two of which co-chromatographed with authentic samples of benzyl alcohol and benzyl chloride. The yellow oil remaining after evaporation of dichloromethane was identified by spectroscopic analysis (i.r., n.m.r. and m.s.) as benzyl alcohol possibly due to a rapid hydrolysis of the other components formed initially.

Should the debenzylation reaction proceed via the proposed mechanism, it is conceivable that diazotisation with exactly one mole equivalent of sodium nitrite in a relatively non-nucleophilic acid may afford the benzotriazolium species (159), possibly in equilibrium with a diazonium salt (160) (scheme 9.5).

Scheme 9.5



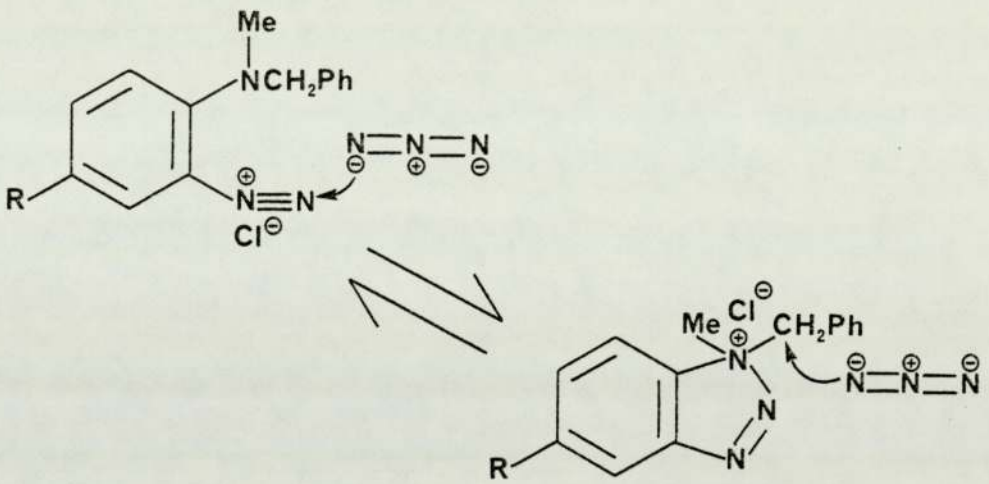
(160)

(159)

In an effort to substantiate this possibility, a solution of the N-methylbenzylaminopyrimidine (135) in glacial acetic acid was added to a solution of sodium nitrite (1 mole equiv.) in sulphuric acid at

0°, according to the method of Hodgson and Walker[180]. Addition of an aliquot taken after 30 min to an alkaline solution of β-naphthol resulted in the formation of a deep red precipitate indicating the presence of a diazonium salt. Unfortunately, addition of an excess of sodium azide to the reaction mixture was to no avail and the N-methylbenzotriazole (152) was again the only product observed, perhaps suggesting that attack by azide ion occurs at the benzylic carbon rather than at the diazonium nitrogen (scheme 9.6).

Scheme 9.6



R = 2,4-diamino-6-ethylpyrimidin-5-yl

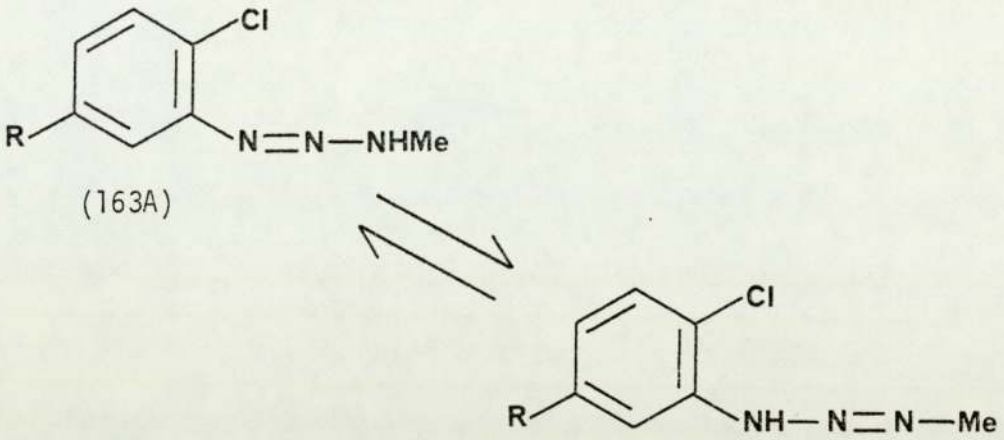
9.3 Attempted cyclisation of a monomethyltriazene derivative

The monomethyltriazene analogue of pyrimethamine (163) was synthesised by Stevens[181] as a candidate irreversible DHFR inhibitor and screened as such for activity against the enzyme (chapter 10.5).

In common with other monoalkyltriazenes compound (163) exists in a tautomeric equilibrium (163A) $\rightleftharpoons$ (163B) the position of which is both solvent-dependent and influenced by aromatic substituents, and for (163) n.m.r. evidence suggests (163A) as the predominant tautomer

in [$^2H_6$]DMSO.

R = 2,4-diamino-6-ethylpyrimidin-5-yl



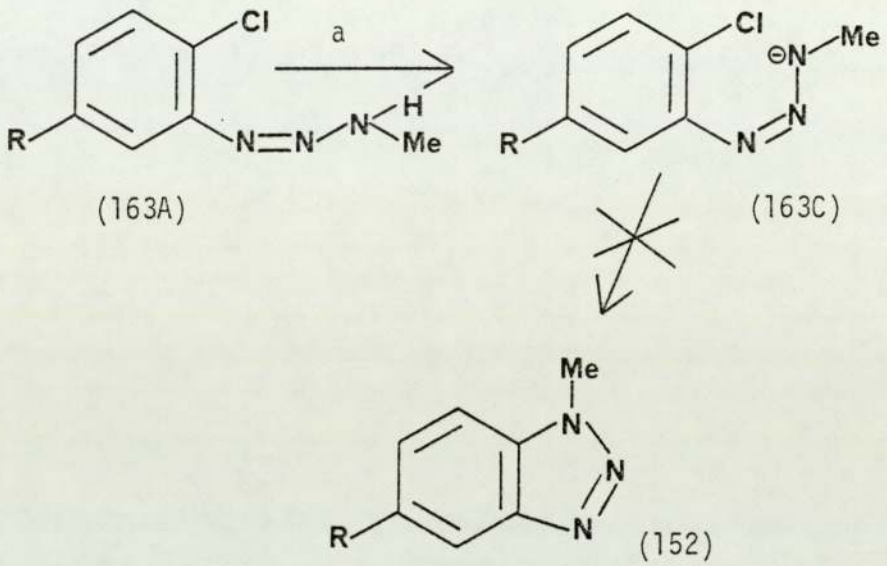
(163A) (163B)

3-Alkyl-aryltriazenes with an ortho carboxamide or nitrile substituent readily undergo cyclisation to furnish 1,2,3-benzotriazines in reactions catalysed by protic solvents and alumina[182]. However, the analogous formation of 1,2,3-benzotriazoles via a similar mechanism has not been reported. It may be conjectured that in the predominant tautomeric form indicated (163A) the ortho-chloro substituent of (163) may be sufficiently activated by the triazenyl group to facilitate nucleophilic replacement. Furthermore, the base-catalysed generation of an anion by removal of the triazene proton would conceivably render an intramolecular cyclisation possible with formation of the corresponding N-methylbenzotriazole (152) (scheme 9.7). Such a reaction would offer an interesting alternative synthetic route to the novel diaminopyrimidine derivative (152).

Scheme 9.7 Attempted cyclisation of the monomethyltriazene (163)

a. $([CH_3]_3Si)_2NLi$/THF; 20°

R = 2,4-diamino-6-ethylpyrimidin-5-yl



(163A) (163C)

(152)

Treatment of a solution of (163) in dry THF at room temperature with lithium bis(trimethylsilyl)amide gave an immediate yellow colouration indicating formation of the requisite anion (163C), although, disappointingly, examination of the mixture by t.l.c. after 12 h showed only starting materials and subsequent refluxing of the mixture was without effect. Further investigations utilising polar, aprotic solvents and alternative base catalysts are planned.

CHAPTER 10

PRELIMINARY SCREENING OF COMPOUNDS FOR ACTIVITY AGAINST DHFR AND THYMIDYLATE SYNTHASE

10.1 Introduction

The catalytic reduction of folate or dihydrofolate by DHFR occurs *via* a mechanism which, despite intensive studies conducted over some two decades, remains to be fully elucidated. However, it is established that both substrate (folate or dihydrofolate) and cofactor (NADPH) must bind to the enzyme for hydride transfer to occur, that either may bind first and products may leave in any order. This 'random bi bi' model for DHFR is further complicated by evidence suggesting that the reduction product tetrahydrofolate may act as an inhibitor of the enzyme *per se* and also bind at the NADPH binding site. Consequently, DHFR cannot be defined in simple kinetic terms and any inhibitory studies must be interpreted with caution[183,184].

In the present study, two series of structurally related diaminopyrimidines were evaluated as inhibitors and 'non-inhibitors' of the DHFR reaction. The initial reaction velocity was monitored by a decrease in absorbance at 340 nm as NADPH and FH_2 are converted to $NADP^+$ and FH_4 respectively[185] and the diminution in velocity at a particular inhibitor concentration was expressed as a percentage of the uninhibited reaction rate. The I_{50} value is defined as the final concentration of inhibitor in the assay system necessary to reduce the reaction rate to 50% of the uninhibited rate, and was obtained graphically from a plot of $\log_{10}$[inhibitor] versus percentage

inhibition of enzyme activity. The limitations and pitfalls associated with the use of I_{50} values are well established. However, for an enzyme from a single source and under identical conditions (fixed substrate and cofactor concentration and incubation time), the I_{50} value provides a useful estimate of the relative inhibitory potencies for a given series of prospective inhibitors[186], assuming competitive inhibition in all cases.

10.2 Inhibitor protocol

As discussed previously (chapter 4) two principal objectives were central to the synthetic rationale forming the basis of the studies under consideration:

(i) to synthesise and evaluate some potentially interesting analogues of MZP as prospective inhibitors of mammalian DHFR;

(ii) to prepare a series of modified diaminopyrimidines as putative non-inhibitors of DHFR.

Thus, in screening the compounds in each category for activity against the enzyme, the level of success will depend on enhanced inhibition of DHFR for those compounds in the first category and an abolition of inhibitory activity for those comprising the second.

A total of eighty six compounds were screened for activity against the rat liver enzyme. These consisted of fourteen analogues of MZP, fifty six potential non-inhibitors and sixteen miscellaneous compounds. Each compound was initially tested in duplicate at a

final concentration of 25 μM and, depending on the result obtained, ranked according to the following criteria:-

(a) inactive - those compounds exhibiting less than 50% inhibition at 25 μM;

(b) active - compounds inhibiting DHFR activity by greater than 50% at 25 μM.

Those compounds designated as active (b above) were subsequently subjected to an I_{50} determination by conducting inhibitory assays in duplicate at four inhibitor concentrations estimated to reduce DHFR activity by approximately 20, 40, 60 and 80% of control respectively. If necessary, the degree of inhibition at additional drug concentrations was evaluated.

Finally, ten compounds were selected for K_I determination on the basis of the I_{50} values obtained from the above and three for comparison with literature K_I values for MZP, pyrimethamine and metoprine, in order to validate the assay procedure and provide an approximate frame of reference for comparison with published data.

10.3 Results of initial screening and I_{50} determinations for compounds in the azidopyrimidine series

These values are summarised in table 10.1.

The established DHFR inhibitors, pyrimethamine (4) and metoprine (5), were included as standard diaminopyrimidines and exhibited I_{50} values of 1.4 and 0.1 μM respectively. The approximate I_{50} value for the tight binding inhibitor MTX (3) was found to be 1.9 nM. Although

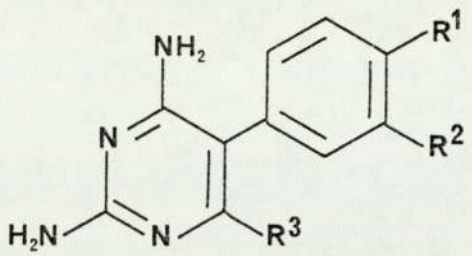
any comparison with data reported elsewhere must be regarded as tenuous, these results fall within the same order of magnitude as those reported elsewhere[187] with rat liver DHFR under identical assay conditions and the approximate relative potencies observed for each inhibitor are in agreement with those documented for mammalian enzyme from other biological sources[13].

As a measure of the precision of the assay method, the I_{50} value for pyrimethamine was determined on three independent occasions to give values of 1.8, 1.2 and 1.3 μM respectively and an average I_{50} of 1.4 μM. The first and last values were determined with the same enzyme preparation after an interval of some 12 months.

As expected, nitropyrimethamine (49), a potent inhibitor of DHFR from L1210 cells[93], exhibited a high level of activity against rat liver enzyme as the ethanesulphonate salt (49a), in contrast to the corresponding amine derivative (22) which was virtually inactive against the enzyme but warranted an I_{50} evaluation for comparison with MZP (21). Both (49) and (22) were synthesised some 15 years ago and screened for DHFR inhibitory activity as candidate antitrypanosomal agents[188]. However, the in vivo toxicity of (49) overshadowed any initially encouraging results obtained.

The introduction of an azido substituent onto the 3 position of the phenyl ring of pyrimethamine to furnish MZP (21) had no significant effect on inhibitory activity. However, m-aminopyrimethamine (22), a putative metabolite of MZP, showed a fifty-fold decrease in activity with respect to the parent compound while the N-acetyl derivative (90) was inactive against DHFR. These observations provide evidence in support of the possibility of regarding MZP as a pro-drug of MAP (22) and corroborate the potential role of the azidopyrimidine as a short acting lipophilic antifolate. The N-oxides of

Table 10.1 Inhibition of rat liver DHFR[a] by MZP and analogues



Compound Number	R^1	R^2	R^3	Solvent	I_{50} (μM)
5[b]	Cl	Cl	Me	B	0.10
4[b]	Cl	H	Et	B	1.40[c]
49a	Cl	NO_2	Et	A	0.08
22	Cl	NH_2	Et	B	62.0
21a[b]	Cl	N_3	Et	A	1.30
64a[b]	N_3	Cl	Et	A	0.34
62a[b]	Cl	N_3	Me	A	3.20
63a[b]	N_3	Cl	Me	A	1.00
53	OMe	NO_2	Et	B	0.18
60	OMe	NH_2	Et	B	1.80
65a[b]	OMe	N_3	Et	A	0.66
54	OEt	NO_2	Et	B	0.14
61	OEt	NH_2	Et	B	15.0
66a[b]	OEt	N_3	Et	A	1.60
55a[b]	OBu^n	NO_2	Et	A	0.06
90	Cl	NHAc	Et	B	>25.0

Solvents: A = water; B = 0.1M-hydrochloric acid; C = ethanol

[a] under identical assay conditions MTX gave an I_{50} of 1.9×10^{-9} M.

[b] compounds selected for K_I determination

[c] I_{50} the average of 3 independent evaluations

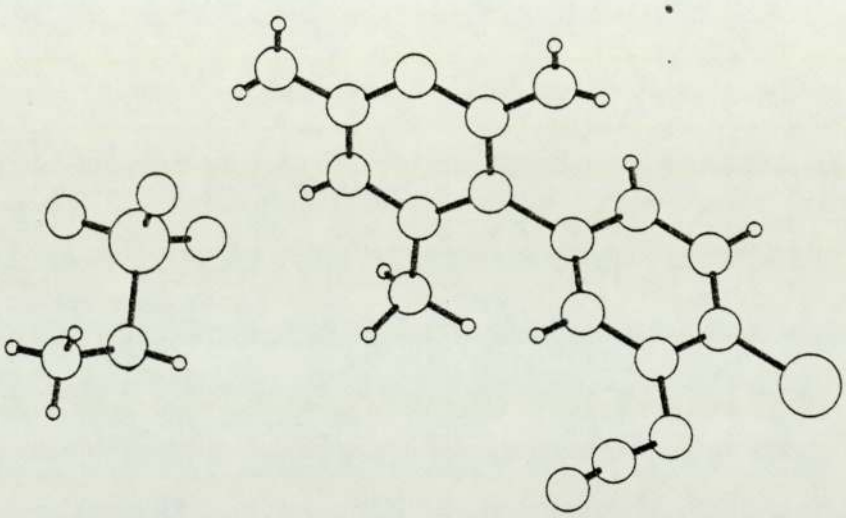
MZP (77 and 78) were also devoid of inhibitory activity at the concentrations tested (table 10.4) and although the corresponding m-aminopyrimethamine N-oxides may be construed as representing the more likely metabolite *in vivo*, by implication these compounds should also exhibit little, if any, activity against mammalian DHFR.

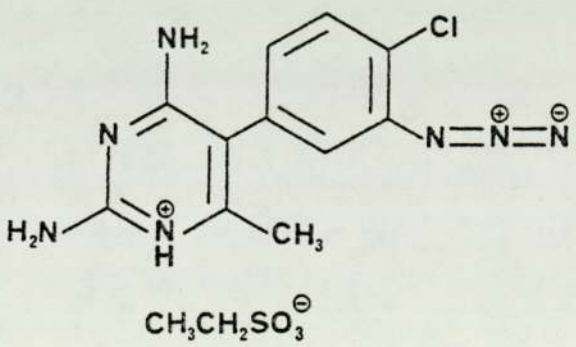
In the azidopyrimidine series (62-66) replacement of the 6-ethyl substituent by a methyl group (62 and 63) failed to enhance activity against the enzyme and the 6-methyl MZP analogue (62) was marginally less active with respect to the parent compound (21). Presumably, the conformational constraints imposed on the 5-phenyl ring by a methyl or ethyl substituent are not reflected as significantly different with regard to the association with the hydrophobic domain of DHFR (fig. 10.1).

A transposition of the chloro and azido substituents of MZP (21) and 6-methyl MZP (62) resulted in an increase in activity of approximately four and three fold respectively for the corresponding isomeric azidopyrimidines (63 and 64). However, such observed differences in inhibitory activity are small and may possibly be attributable to the experimental error inherent to any biological assay procedure. Unfortunately, a pure sample of the potential metabolite of *iso*-MZP (64) *iso*-MAP (59) had not been characterised when the enzyme assays were conducted since a comparison of the differential activities of each compound would have been of interest.

For compounds in the alkoxypyrimidine series, the overall pattern of activity paralleled that observed with the chlorophenylpyrimidines. Thus the methoxy and ethoxynitropyrimidines (53 and 54) were the most potent inhibitors and the corresponding aminopyrimidines (60 and 61) the least active, with the methoxy and ethoxyazidopyrimidines (65 and 66) occupying an intermediate

Figure 10.1 PLUTO drawing of the crystal structure of 2,4-diamino-5-(3-azido-4-chlorophenyl)-6-methyl-pyrimidine ethanesulphonate salt (62)





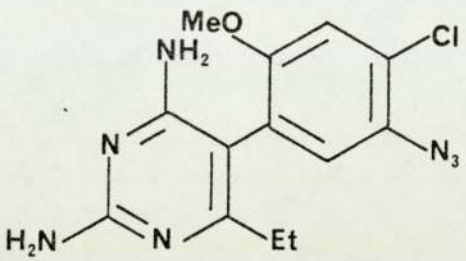
(62)

position. However, although the replacement of a chloro group by a methoxy or ethoxy substituent was tolerated without compromising activity as witness the I_{50} values of (65 and 66), the marked decrease in activity (fifty-fold) between MZP and MAP was not apparent for the alkoxyazides and the corresponding alkoxyamines (60 and 61). Indeed, only a two-fold reduction of inhibition was observed between the methoxyazide (65) and its putative bioreduction product (60), while the ethoxyamine (61) was approximately ten times less active than the parent inhibitor (66). Any structure-activity correlations based on these observations are tentative.

The alkoxy groups were introduced ortho to the azido substituent in an effort to reinforce the basicity of the amine metabolite thereby facilitating its protonation and inactivity against DHFR. Surprisingly, the converse situation occurred, the alkoxyamines (60 and 61) proving to be better inhibitors of the enzyme than MAP. In retrospect these effects may be rationalised by a comparison of the pKa values (conjugate acids at 25°) of the analogous arylamines aniline, o-anisidine and p-anisidine[189]. Thus the basicity of aniline (pKa = 4.63) is in fact reduced on introduction of an ortho methoxy group as for o-anisidine (pKa = 4.52) due to the predominant electron withdrawing inductive effect (-I) of oxygen over a less pronounced electron donating mesomeric effect (+M).

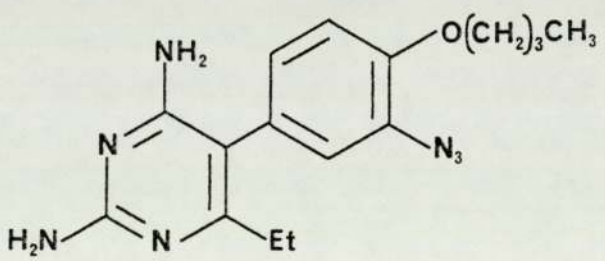
The base-strengthening influence of a para methoxy group as evident with p-anisidine (pKa = 5.34) where the mesomeric effect prevails, suggests that an MZP analogue encompassing a 2'-methoxy substituent (161) would furnish, on bioreduction, an amine metabolite with the requisite basicity. Of course, such a substituent would certainly impart a restricted rotation about the pyrimidine-phenyl ring bond, with a consequent effect on enzyme activity, and this

factor would merit consideration. Moreover, although the aforementioned predictions based on an extrapolation from simple aromatic amines may be valid, pKa determinations conducted on the diaminopyrimidines under consideration would be of greater relevance.



(161)

Compound (55) was synthesised as a consequence of the encouraging preliminary antitumour activity observed for the n-butylaminopyrimidine derivative (98) (chapter 13), in order to assess the possibility of replacing the amine function by an ether substituent. Subsequent transformation of the 3'-nitro group of (55) to an azido group by the usual method, a conversion not possible with (98) due to benzotriazole formation, would conceivably furnish an interesting lipophilic DHFR inhibitor (166).



(166)

The n-butoxynitrophenylpyrimidine ethanesulphonate salt (55a) proved to be a potent DHFR inhibitor, some three times more active than the analogous amine (98) as such warranting a K_I determination.

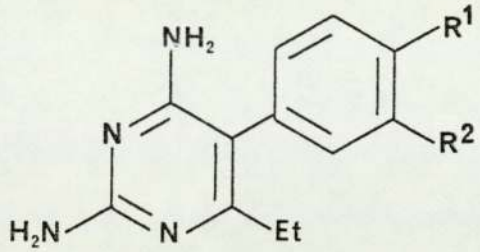
10.4 Results of initial screening and I_{50} determinations for compounds designed as non-inhibitors of DHFR

These are outlined in table 10.2.

In considering the effect of introducing a polar amine substituent onto the 4'-position of the 5-phenyl ring of diamino-nitrophenylpyrimidines, a comparison with the inhibitory activity of nitropyrimethamine (49) as the parent compound was conducted, in order that an estimation of the relative contribution of the 3'-nitro group to the overall activity of each molecule was possible. Replacement of the 4'-chloro group of nitropyrimethamine by a mono or dialkylamino, or a monoheteroalicyclic amino substituent furnished analogues (95-99) approximately equiactive with the parent compound and exhibiting I_{50} values of 0.1-0.3 μM. Such substituents would impose negligible steric hindrance within the hydrophobic region of the enzyme and the electron withdrawing nature of the 3'-nitro group (-I and -M) will render the amino function effectively non-basic. For the n-butylamino derivative (98) the inherent hydrophobicity of the alkyl side chain may be conjectured to facilitate association with a hydrophobic pocket within the enzyme active site.

Those compounds embracing a second polar species at a site remote to the 3'-nitro substituent, either within a heteroalicyclic structure (100-103) or at the terminal carbon of an alkyl chain (112 and 113) showed an activity against DHFR at least ten-fold lower than nitropyrimethamine and comparable in potency to pyrimethamine. Compounds (104 and 105) were also similar to pyrimethamine in terms of activity and this was surprising in the light of the above observations with monoalkylamino derivatives.

Table 10.2 Inhibition of rat liver DHFR by the amine-substituted diaminopyrimidines



Compound	R^1	R^2	Solvent	I_{50} (μM)
95	methylamino	NO_2	B	0.15
123	methylamino	NH_2	B	15.0
96	dimethylamino	NO_2	B	0.25
124	dimethylamino	NH_2	B	67.0
141[a]	dimethylamino	N_3	A	1.60
146	dimethylamino	H	B	6.20
151	trimethylammonium iodide	H	A	>25.0
97	ethylamino	NO_2	B	0.16
125	ethylamino	NH_2	B	>25.0
98[a]	n-butylamino	NO_2	B	0.17
126	n-butylamino	NH_2	B	>25.0
99	piperidin-1-yl	NO_2	B	0.62
127	piperidin-1-yl	NH_2	B	>25.0
142	piperidin-1-yl	N_3	B	8.50
147	piperidin-1-yl	H	B	>25.0
100	piperazin-1-yl	NO_2	B	3.10
128	piperazin-1-yl	NH_2	B	>25.0
143	piperazin-1-yl	N_3	B	>25.0
148	piperazin-1-yl	H	B	>25.0

Table 10.2 continued

101	4-methylpiperazin-1-yl	NO_2	B	3.90
129	4-methylpiperazin-1-yl	NH_2	B	>25.0
144	4-methylpiperazin-1-yl	N_3	B	>25.0
149	4-methylpiperazin-1-yl	H	B	>25.0
150	4-(1,1-dimethylpiperazinium)iodide	H	A	>25.0
102	pyrrolidin-1-yl	NO_2	B	1.70
130	pyrrolidin-1-yl	NH_2	B	>25.0
103	4-morpholino	NO_2	B	8.00
131	4-morpholino	NH_2	B	>25.0
145	4-morpholino	N_3	B	9.40
104	cyclohexylamino	NO_2	B	1.20
132	cyclohexylamino	NH_2	B	>25.0
105	N-methylcyclohexylamino	NO_2	B	2.50
133	N-methylcyclohexylamino	NH_2	B	8.50
106	benzylamino	NO_2	B	0.01
134	benzylamino	NH_2	B	0.50
107[a]	N-methylbenzylamino	NO_2	B	0.01
135	N-methylbenzylamino	NH_2	B	0.19
108[a]	N-ethylbenzylamino	NO_2	B	0.02
136	N-ethylbenzylamino	NH_2	B	4.00
109	(±)α-methylbenzylamino	NO_2	B	0.18
137	(±)α-methylbenzylamino	NH_2	B	35.0
110	dibenzylamino	NO_2	C	0.26
111	phenethylamino	NO_2	C	0.07
138	phenethylamino	NH_2	B	1.90
112	2-aminoethylamino	NO_2	B	2.60
139	2-aminoethylamino	NH_2	B	>25.0

Table 10.2 continued

113	3-aminopropylamino	NO_2	B	1.00
140	3-aminopropylamino	NH_2	B	1.30
115	NO_2	benzylamino	C	9.20
116	NO_2	N-methyl-benzylamino	C	0.44

Solvents: A = water

B = 0.1 M-hydrochloric acid

C = ethanol

[a] compounds selected for K_I determination

As expected, a profound decline in activity resulted from reduction of the 3'-nitro group and the products (123-133) showed virtually no activity against the rat liver enzyme, presumably due to the effective introduction of two polar and basic centres onto the phenyl ring. An unusual result was obtained with the 3-aminopropylaminophenylpyrimidine (113) where, intriguingly, the reduction product (140) was equipotent with the parent compound, both having an I_{50} of approximately 1 μM, and this discrepancy was not apparent with the related 2-aminoethylaminopyrimidines (112 and 139) where an abolition of activity accompanied reduction of the aromatic nitro group. One possible but as yet unsubstantiated explanation for such an anomaly may be that the compound (140) inhibits DHFR in a non-competitive manner.

In common with the pattern of activity observed for the azidopyrimidine series discussed previously (10.3) conversion of the reduced analogues to the corresponding azides (141-145) furnished compounds intermediate in inhibitory activity between the nitro and amino derivatives. Compound (141) exhibited significant inhibition at concentrations comparable to those evident with MZP whereas the potential amine metabolite (124) was virtually inactive. Consequently, the further evaluation of (141) as a candidate lipophilic DHFR inhibitor was considered necessary.

Predictably, the deazidation products (147-149) and the quaternary ammonium derivatives (150 and 151) were inactive against rat liver enzyme at 25 μm while the 4'-dimethylaminopyrimidine (146) showed only marginal activity, which nevertheless was some ten times that observed for the corresponding 3'-amine derivative (124), and deviated from the established activity trend in this respect. Thus the inactivity of compounds (123-133) and (147-151) against DHFR

rendered these diaminopyrimidines suitable for evaluation as prospective probes for an alternative target elsewhere.

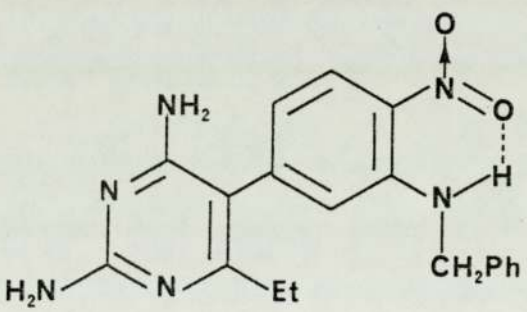
Synthetic efforts directed towards the development of analogues devoid of DHFR inhibitory activity entailed the preparation of numerous compounds *via* the reaction of nitropyrimethamine with amines and surprisingly several of these by contrast proved powerful inhibitors of the enzyme, notably those encompassing a 4'-benzylamino or N-alkylbenzylamino group (106-108). The I_{50} values of 10-20 nM obtained for compounds of this nature were intermediate between those observed for metoprine (5) and MTX (3). A further evaluation (K_I) of the N-methyl (107) and N-ethylbenzylamino (108) analogues was conducted in order to ascertain an unambiguous indication of the potency of such tight-binding inhibitors. The (±) ∝-methylbenzylamino and phenethylamino derivatives (109 and 111) exhibited activity against DHFR comparable to that observed with nitropyrimethamine and some ten-fold less than that for the benzylamino (106) or N-methylbenzylamino (107) compounds.

Of the three isomeric compounds synthesised *via* the reaction of *iso*-nitropyrimethamine (50) with amines only the 3'-benzylamino- and 3'-N-methylbenzylaminopyrimidines (115 and 116) were evaluated for DHFR inhibitory activity since, unfortunately, a pure sample of the 3'-*n*-butylaminopyrimidine (114) had not been characterised when the assays were conducted. A comparison of the respective I_{50} values of the 4'-benzylamino analogue (106) and the isomeric 3'-benzylamino compound (116) revealed approximately a thousand-fold reduction in activity against the enzyme following a transposition of the benzylamino and nitro groups in this manner. In contrast the 3'-N-methylbenzylamino congener (116) was only some forty times less active than the 4'-N-methylbenzylamino compound (107) and remained a potent inhibitor of DHFR.

The virtual abolition of inhibitory activity observed upon

translocating the benzylamino substituent from a 4' to a 3' position may possibly be attributable to a lack of bulk tolerance of the enzyme active site to a benzylamino group as compared to a nitro substituent. However, the introduction of an N-methylbenzylamino group onto the 3' position of the phenyl ring as for (116) should impose similar steric constraints with a concomitant decline in activity, although clearly this was not the case. Effects due to the greater basicity of the benzylamino group relative to the N-methylbenzylamino group are unlikely since any differences would also reflect in the 4' position and were not observed.

A consideration of the intramolecular hydrogen bonding capacity of each molecule may provide one possible explanation for the aforementioned anomalous results obtained. Thus the 3'-benzylaminopyrimidine (115) may form an intramolecular hydrogen bond <u>via</u> the NH of the benzylamino group and an oxygen atom on the adjacent nitro group.



(115)

In this event the barrier to rotation about the 3'-C-N bond imparted by the electron withdrawing <u>ortho</u>-nitro substituent, in conjunction with the conformational restraint of an intramolecular hydrogen bond, may force the benzyl group into a position incompatible with association at the enzyme active site and hence compromise activity against DHFR. In contrast the corresponding 3'-N-methylbenzylaminopyrimidine (116) cannot form an intramolecular

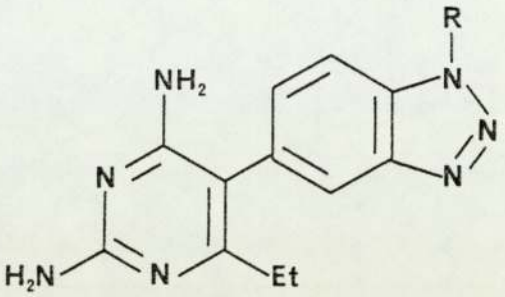
hydrogen bond and presumably the bulky benzyl substituent may orient to adopt a conformation facilitating association with the hydrophobic domain of the enzyme active site. Although similar interactions may be envisaged to occur with the appropriate 4'-derivatives (106 and 107), such factors may be conjectured as of less importance since the compounds were equipotent against DHFR. Crystallographic studies on compounds (106) and (115) are in progress in an effort to confirm the validity of the aforementioned hypothesis.

In the benzotriazole series (table 10.3) it is evident that conversion of the appropriate amine to a benzotriazole invariably restores some activity against DHFR. With the exception of (157), the derivatives exhibited only moderate inhibitory activity and these compounds were considered as generally unsuitable for further consideration as candidate molecular probes.

10.5 Results of initial screening and I_{50} determinations for miscellaneous compounds and those obtained as gifts (table 10.4)

From table 10.4 it can be seen that those compounds in the aminopyrimidinone series (83, 84, 88, 89 and 165) proved inactive against the enzyme as expected, since amino substituents in the 2 and 4 positions are a prerequisite for significant activity. The isocytosine derivative 2-amino-5-bromo-6-phenylpyrimidin-4(3H)-one (ABPP) (162) is currently undergoing evaluation as an antitumour agent and has interferon inducing properties[190]. Since the demonstrated antitumour activity of ABPP has yet to be correlated with the immunomodulatory properties of this agent[191], a preliminary assay for DHFR inhibitory activity was conducted in order to

Table 10.3 Inhibition of rat liver DHFR by compounds in the benzotriazole series



Compound Number	R	Solvent	I_{50} (μM)
152a[a]	Me	A	8.60
153	Et	B	5.00
154	n-butyl	B	2.10
155	cyclohexyl	B	>25.0
156	benzyl	B	8.00
157	phenethyl	B	0.47

[a] ethanesulphonate salt

Solvents: A = water

B = 0.1 M hydrochloric acid

Table 10.4 Miscellaneous compounds and those obtained as gifts: activity against rat liver DHFR

Compound Number	Solvent	I_{50} (μM)
75	B	2.50
77	B	>25.0
78	B	>25.0
83	B	>25.0
84	B	>25.0
88	B	>25.0
89	B	>25.0
120a[a]	A	>25.0
122a[a]	A	2.70
162	D	>25.0
163	B	2.50
164	B	3.30
165	B	>25.0

Solvents: A = water

B = 0.1 M-hydrochloric acid

C = ethanol

D = 0.1 M-sodium hydroxide solution

[a] methanesulphonate salt

ascertain that inhibition of this enzyme did not represent a primary locus for cytotoxicity. As predicted the drug was totally devoid of activity as an inhibitor of DHFR at a concentration of 25 μM.

The pyrimethamine derivatives embracing a mono- or dimethyltriazene moiety (163 and 164) were synthesised as prospective irreversible inhibitors of the enzyme, on the basis that alkyltriazenes have an established propensity to react with biological nucleophiles, for example at the active site of an enzyme[182]. From the results obtained it is evident that an alkyltriazene group is tolerated on the 3' position of pyrimethamine since both compounds have I_{50} values comparable to that observed for the parent compound. However, the question of whether these compounds bind reversibly or irreversibly to the enzyme remains to be answered and would necessitate a substrate reversibility study at several inhibitor concentrations.

The observed inactivity of the hydroxybenzotriazole derivative (120) against DHFR rendered this compound of some interest for further consideration. Surprisingly, in contrast the 2-phenylbenzotriazole-1-oxide analogue (122) was about ten times more active than (120), being approximately equipotent to pyrimethamine. These disparate activities are presumably a reflection of the relative solubility of each compound in organic solvents, the hydroxybenzotriazole (120) being the more polar (chapter 7).

10.6 Results of screening for activity against thymidylate synthase (TS)

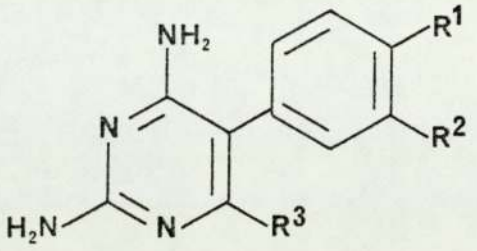
Since the azidopyrimidine analogues (62-66) constitute a novel series of lipophilic antifolates, it was desirable to establish DHFR

as the principal locus of cytotoxicity. Thus these compounds were assessed, as the ethanesulphonate salts (62a-66a) for TS inhibitory activity. In addition, a representative cross-section of those compounds designed as probes for a folate-independent target were screened as possible TS inhibitors, in order to confirm that any subsequent cytotoxicity exhibited by these derivatives was unrelated to activity against TS.

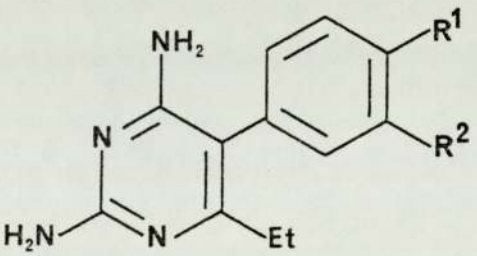
Those compounds selected for evaluation as possible TS inhibitors are summarised in table 10.5.

The fourteen diaminopyrimidines assayed against TS showed no significant activity at a final concentration of 400 μM. Under identical conditions the established quinazoline TS inhibitor CB3717 gave an I_{50} of 40 nM in agreement with the reported value[192].

Table 10.5 Compounds screened as TS inhibitors



Compound Number	R^1	R^2	R^3	Solvent
21a	Cl	N_3	Et	A
62a	Cl	N_3	Me	A
63a	N_3	Cl	Me	A
64a	N_3	Cl	Et	A
65a	OMe	N_3	Et	A
66a	OEt	N_3	Et	A



96	dimethylamino	NO_2	-	B
100	piperazin-1-yl	NO_2	-	B
101	4-methylpiperazin-1-yl	NO_2	-	B
106	benzylamino	NO_2	-	B
113	3-aminopropylamino	NO_2	-	B

Table 10.5 continued

144	4-methylpiperazin-1-yl	N_3	-	B
148	piperazin-1-yl	H	-	B
149	4-methylpiperazin-1-yl	H	-	B

Solvents: A = water

B = 0.01 M hydrochloric acid

CHAPTER 11

INHIBITION CONSTANT (K_I) DETERMINATIONS FOR SELECTED COMPOUNDS AGAINST RAT LIVER DHFR

11.1 Rationale for compound selection

K_I determinations are time-consuming and relatively expensive to conduct and, consequently, only those compounds warranting a more detailed kinetic analysis were selected for this purpose on the basis of the preliminary screening results (chapter 10).

Compounds in the azidopyrimidine series (62-66) comprise a group of novel structurally similar potential lipophilic inhibitors of DHFR and a consideration of the respective K_I values was regarded as necessary for comparison with the parent compound MZP (21) and as a basis for subsequent structure-activity and antitumour investigations. The dimethylaminoazidopyrimidine (141) synthesised initially as a candidate 'non-inhibitor', clearly exhibited activity against the enzyme comparable to those azidopyrimidines in the above series, thereby justifying inclusion for kinetic analysis as a potential short-acting lipophilic inhibitor. This decision was augmented by the forty-fold decrease in activity shown by the amine derivative (124), a potential metabolite of (141).

Pyrimethamine and metoprine were included as standard inhibitors in order to allow comparison with the compounds under consideration and with results documented elsewhere.

Compounds (107 and 108) although discovered fortuitously proved sufficiently potent as inhibitors of DHFR to warrant evaluation *per se* as potential novel antifolates. For reasons discussed below

(11.2), the relevance of I_{50} values determined for compounds exhibiting tight-binding characteristics is questionable, and since some correlation between potency against DHFR and observed antitumour activity was desired these agents were included for kinetic analysis.

The rationale central to the synthesis of the n-butyloxynitropyrimidine (55) has been outlined previously (chapter 10.3) and this compound was also selected in order to allow a comparison with the analogous n-butylaminonitropyrimidine (98).

11.2 The kinetics of tight-binding inhibitors

The majority of studies regarding the kinetics of reversible competitive enzyme inhibition have been conducted under steady-state conditions, where for the reaction:

$$E + I \rightleftharpoons EI$$

the rate of equilibrium establishment between enzyme (E), inhibitor (I) and the enzyme-inhibitor complex (EI) is rapid and the molar enzyme concentration is very much lower than that of the inhibitor. Under these conditions the assumption that the fraction of enzyme-bound inhibitor is negligible compared with the total inhibitor concentration is valid and a conventional double reciprocal plot by the method of Lineweaver and Burke will be linear, in accordance with equation 1[193].

$$\frac{1}{V} = \frac{K_m(1 + \frac{[I]}{K_I})}{V_{max}[S]} + \frac{1}{V_{max}} \qquad (1)$$

Such Zone A conditions are generally considered applicable to weak or moderate reversible competitive inhibitors of DHFR.

However, for tight-binding inhibitors the dissociation constant of an enzyme-inhibitor complex is very low and inhibition of the enzyme will occur at inhibitor concentrations comparable to that of the enzyme. Clearly the steady-state assumption is not valid under such conditions and an allowance must be made for the reduction of inhibitor concentration subject to formation of the enzyme-inhibitor complex, when Zone B kinetics apply[194]. The pseudolinear equation 2 takes such factors into account and allows a determination of $K_{I\ app}$, the apparent dissociation constant, from which, assuming competitive inhibition, the true dissociation constant (K_I) may be calculated (equation 3)[195-197].

$$\frac{I_t}{I - \frac{Vi}{Vo}} = K_{I\ app}\left(\frac{Vo}{Vi}\right) + E_t \qquad (2)$$

$$K_{I\ app} = K_I\left(1 + \frac{S}{K_m}\right) \qquad (3)$$

Hart[52,198] has written a modification of a FORTRAN computer program developed by Henderson[199] which utilises the pseudolinear expression (2) to generate estimates of the $K_{I\ app}$ by a statistical method, and hence the determination of K_I values from equation 3 is possible. This statistical method allows the calculation of 95% confidence limits on the $K_{I\ app}$ and K_I values as recommended by Dietrich et al[200] for comparative work.

Thus estimates of K_I together with 95% confidence limits were obtained for each compound under consideration from the inhibition data, by the aforementioned procedure.

11.3 Results of the K_I determinations (table 11.1)

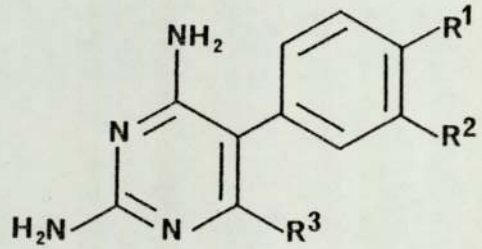
The inhibitory constant (K_I) values obtained for pyrimethamine, metoprine and MZP are in agreement with values quoted in the literature[93] for similar determinations against mammalian DHFR. In general, the results parallel those observed for the I_{50} values with respect to the effects of structural modification on activity against the enzyme, although for the methoxy and ethoxyazido derivatives (65 and 66) the two-fold increased activity of (65) over (66) was not evident. Since I_{50} determinations are critically dependent on several parameters including the enzyme concentration, such anomalies are unexceptional and highlight the limitations of I_{50} interpretation for compounds of similar potency.

The necessity for conducting a precise kinetic analysis on those compounds warranting consideration as tight-binding inhibitors is also evident for the n-butylamino- and n-butoxynitropyrimidine analogues (98 and 55). Examination of the I_{50} values would suggest an approximately three-fold differential activity, whereas a comparison of the respective K_I values shows no significant difference in activity between the two compounds in terms of the derived 95% confidence limits.

As expected the N-methyl and N-ethylbenzylaminopyrimidines (107 and 108) gave K_I values consistent with potent inhibition of rat liver enzyme, each exhibiting activity only some ten times less than that reported for MTX and such molecules certainly merit classification as tight-binding inhibitors.

The precise nature of the molecular geometry of binding to DHFR with compounds (107 and 108) remains to be determined and forms the subject of continuing studies. Although speculative, the high

Table 11.1 Kinetic data for selected diaminopyrimidine DHFR inhibitors



Compound Number	R_1	R_2	R_3	I_{50} (μM)	$K_I$app (μM)[a]	K_I(nM)[b]
4 (pyrimethamine)	Cl	H	Et	1.80	1.50 ± 0.18	2.60 ± 0.31[c]
5 (metoprine)	Cl	Cl	Me	0.10	0.07 ± 0.02	0.12 ± 0.04
62a	Cl	N_3	Me	3.20	1.47 ± 0.43	2.60 ± 0.76
63a	N_3	Cl	Me	1.00	0.46 ± 0.01	0.82 ± 0.01

Table 11.1 continued

21a (MZPES)	Cl	N_3	Et	1.30	0.91 ± 0.21	1.60 ± 0.38
64a (iso-MZPES)	N_3	Cl	Et	0.34	0.21 ± 0.07	0.38 ± 0.12
65a	OMe	N_3	Et	0.66	0.97 ± 0.20	1.72 ± 0.34
66a	OEt	N_3	Et	1.60	0.98 ± 0.13	1.73 ± 0.23
141	dimethylamino	N_3	Et	1.60	1.68 ± 0.12	3.00 ± 0.22
98	n-butylamino	NO_2	Et	0.17	0.11 ± 0.03	0.19 ± 0.05
55a	n-butoxy	NO_2	Et	0.06	0.05 ± 0.03	0.08 ± 0.06
107	N-methylbenzylamino	NO_2	Et	0.01	0.005 ± 0.001	0.009 ± 0.002
108	N-ethylbenzylamino	NO_2	Et	0.02	0.021 ± 0.02	0.04 ± 0.03

[a] $K_I$app values obtained from kinetic data analysis;

[b] $$K_I = \frac{K_I app}{\frac{1+[FH_2]}{K_m(FH_2)}}$$

$[FH_2] = 1.13 \times 10^{-4}$ M. K_m taken as 0.20 μM for FH_2.

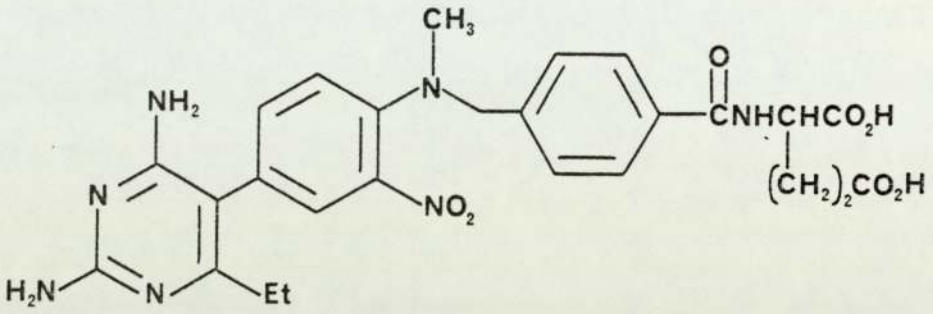
[c] 95% confidence limits

affinity for DHFR exhibited by these compounds may be attributable to a degree of structural homology with the natural substrate dihydrofolate and by definition with MTX also. The binding of folates to DHFR is thought to involve association at a minimum of four regions within the enzyme active site[12]:

(i) a pteridyl or pyrimidine region
(ii) the hydrophobic domain
(iii) a p-aminobenzoyl binding region
(iv) the glutamate or polyglutamate region.

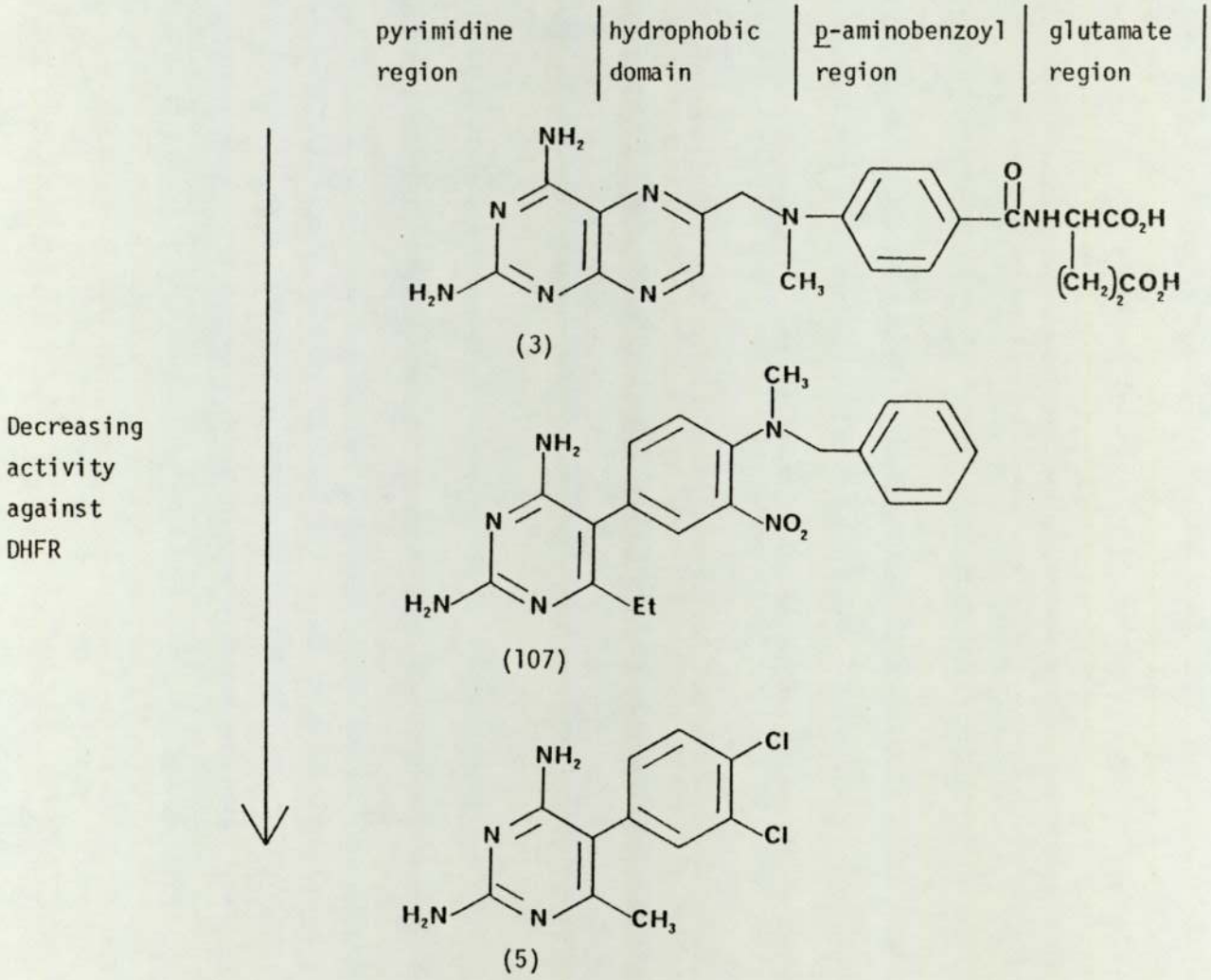
The high activity and lack of species selectivity of MTX has been attributed to association with all four binding regions, whilst in contrast metoprine may bind only to regions (i) and (ii). The N-alkylbenzylaminopyrimidines (107 and 108) may be contorted to associate with binding regions (i), (ii) and (iii) on the enzyme and thus exhibit properties intermediate between metoprine and MTX. Although axiomatic this explanation is consistent with the observed activity of these molecules with respect to metoprine and MTX (fig. 11.1). The bioisosteric transposition $-CH_2-NH-$ to $-NH-CH_2-$ is well established as an approach for the development of substrate analogue inhibitors and may be envisaged as a contributory factor in the binding of (107 and 108) to DHFR.

On the basis of the above hypothesis, an analogue of the N-methylbenzylamino derivative (107) embracing a p-glutamate substituent would be of interest, since such a compound would be predicted to exhibit activity against DHFR comparable to MTX and thus represent a structurally novel folate-analogue inhibitor (167).



(167)

Figure 11.1 Structural similarities between MTX, compound (107) and metoprine



CHAPTER 12

PRELIMINARY STUDIES ON THE *IN VITRO* CYTOTOXICITY OF SELECTED COMPOUNDS AGAINST L1210 MURINE LEUKAEMIA CELLS

12.1 Introduction

From the results obtained for the screening of compounds for activity against rat liver DHFR (chapters 10 and 11), it is evident that in several cases a more extensive biological and biochemical evaluation was justified in order to assess candidate lipophilic DHFR inhibitors as antitumour agents and also to allow the identification of lead compounds exhibiting cytotoxicity unrelated to inhibition of this enzyme. Although a more comprehensive study regarding the *in vitro* cytotoxicity of the appropriate compounds characterised previously is proposed, the present investigation was conducted in order to ascertain the viability of such an approach and the relationship between activity against DHFR and *in vitro* cytotoxicity.

Eight compounds were selected for preliminary cytotoxicity studies and these comprised of the two lipophilic DHFR inhibitors, MZPES (21a) and *iso*-MZPES (64a), the potent 4'-benzylaminopyrimidine (106) and its virtually inactive 3'-benzylamino isomer (115), the corresponding 4'- and 3'-*n*-butylaminopyrimidine ethanesulphonate salts (98a and 114a) and the quaternary ammonium compounds (150 and 151). The compound numbers, solvent used and structures of the appropriate compounds are summarised in table 12.1.

12.2 Results of the in vitro cytotoxicity studies

Several disadvantages are associated with the use of cell suspensions for the evaluation of cytotoxicity, including a substantial error factor and a poor correlation with solid tumour models. Moreover, the L1210 line has a tendency to select for hydrophilic agents[201] and Sirotnak et al[73] have demonstrated that murine tumour lines sensitive to MTX, a relatively hydrophilic compound, are often refractory to lipophilic inhibitors. However, for preliminary comparative estimates of drug cytotoxicity growth inhibition assays on cell suspensions provide a rapid and convenient method, although the limitations associated with any conclusions drawn from the relatively small number of results obtained in the present study are recognised.

The IC_{50} value of a drug was defined as the final concentration necessary to reduce the 72 h cell count to 50% of control, and this value was estimated from a conventional plot of molar drug concentration against percentage growth inhibition (fig. 12.1). From table 12.1 it is apparent that in several cases a greater than 100% inhibition of cell growth occurred. This anomaly is inherent to the assay procedure and arises as a consequence of an overall decrease in the number of viable cells after 72 h relative to the initial control cell count at time zero.

iso-MZPES (64a) was selected for comparison with the parent compound MZPES (21a), as the most promising of the azidopyrimidines evaluated for activity against DHFR. Clearly, the four-fold higher activity of (64a) against rat liver enzyme was reflected in the relative potency of each compound against L1210 cells, iso-MZPES being the more cytotoxic agent.

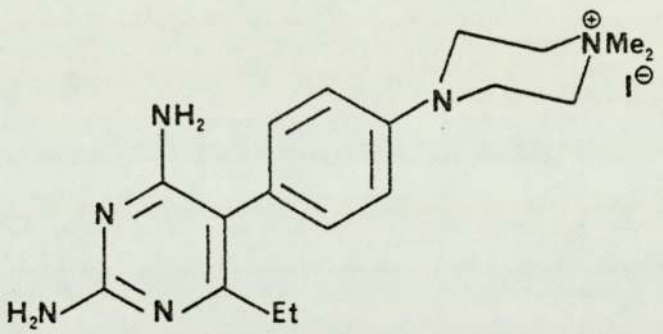
Compounds (98a, 114a, 106 and 115) were evaluated for several reasons; the 4'-benzylaminopyrimidine (106) warranted consideration as a potential cytotoxic agent by virtue of the high observed activity against DHFR and indeed proved to be the most cytotoxic of the eight compounds tested. The 4'-n-butylaminopyrimidine (98a) was screened for *in vitro* activity as a result of the considerable *in vivo* antitumour activity reported for this compound (chapter 13), which intriguingly did not correlate with activity against DHFR. Surprisingly, (98a) was only a moderate inhibitor of cell growth *in vitro* although the use of the ethanesulphonate salt, necessary due to the low solubility of the free base (98) in DMSO, may have influenced the result obtained.

The isomeric 3' derivatives (114a and 115) were included for comparison with the 4' compounds (98a and 106) in order to relate the disparate activity against rat liver enzyme, particularly evident for the two benzylaminopyrimidines, to growth inhibition *in vitro*, since any differences may indicate cytotoxicity at a locus unrelated to inhibition of DHFR. It is evident that the two isomeric benzylaminopyrimidines (106 and 115) exhibited a differential potency against L1210 cells paralleling the respective activities observed against rat liver enzyme, the 4'-benzylaminopyrimidine being approximately a thousand-fold more active in both cases.

Unfortunately, the activity of the 3'-n-butylaminopyrimidine (114a) against DHFR has yet to be determined before any comparison between this compound and the isomeric congener (98a), with respect to cytotoxicity and enzymic activity is possible.

For reasons outlined in the introduction (section 1) the quaternised derivatives (150 and 151) were considered to represent the most promising candidate probes for a folate-independent target,

and as predicted failed to inhibit DHFR at pharmacologically relevant concentrations. However, disappointingly, neither compound proved significantly cytotoxic in vitro, although the phenyltrimethylammonium analogue (151) with an IC_{50} of approximately 10 μM may warrant further consideration over a more comprehensive concentration range.



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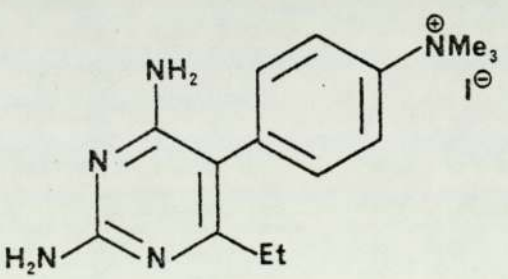
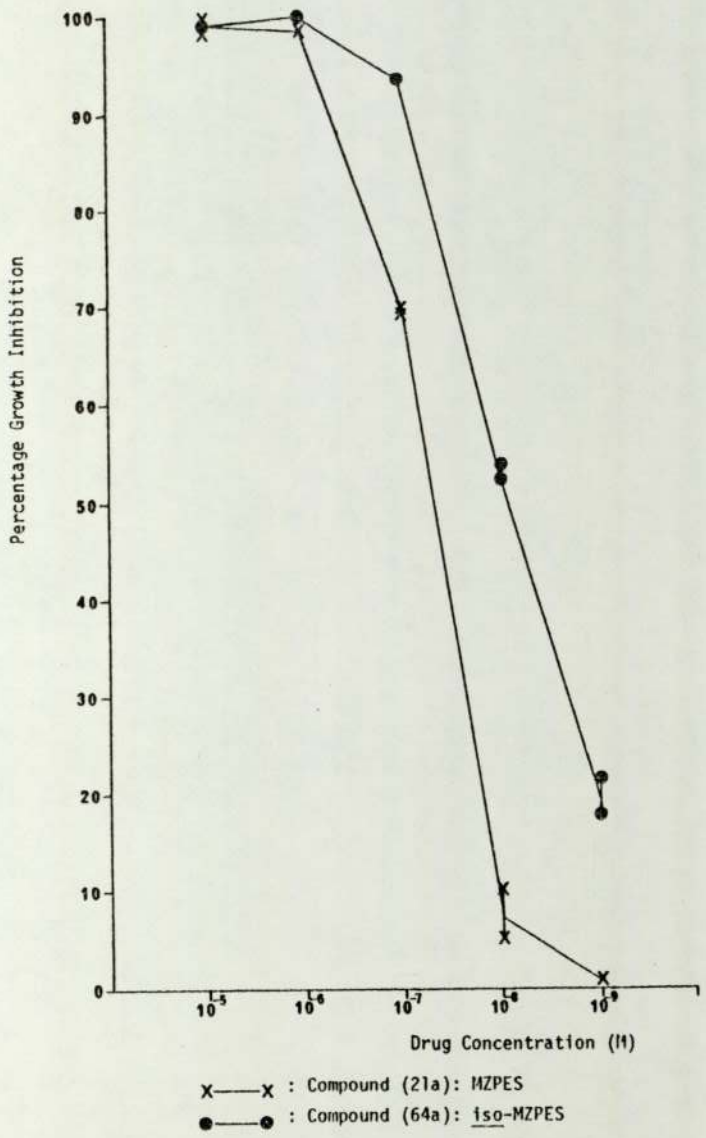
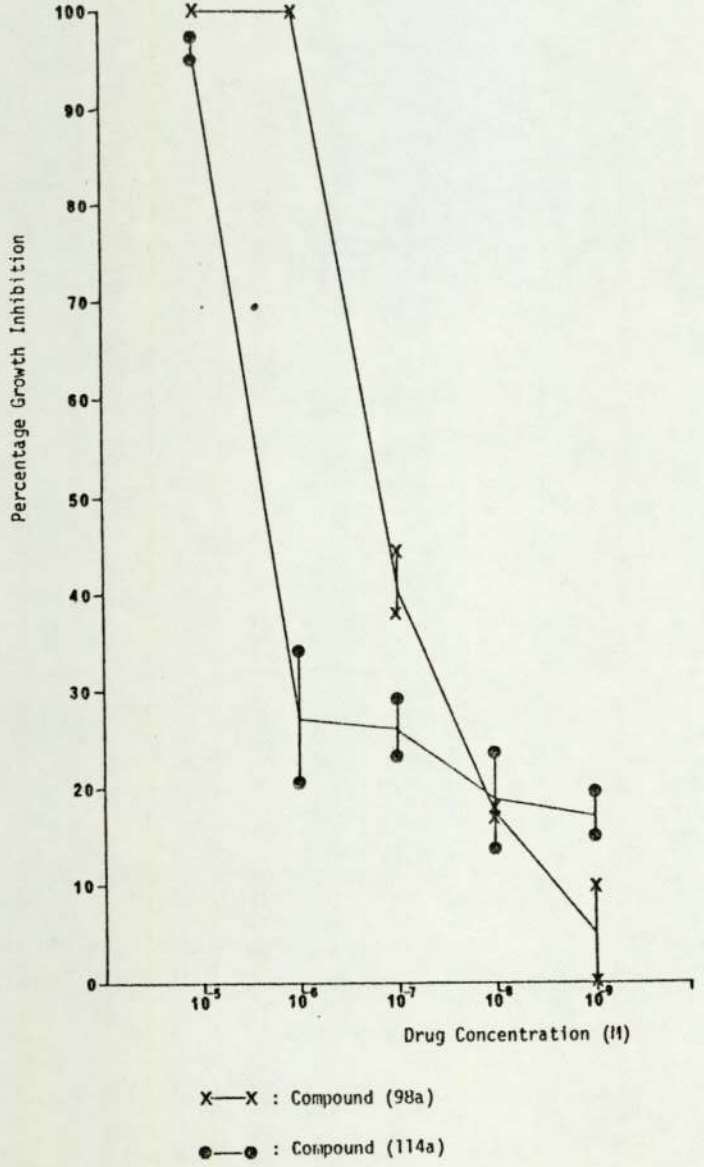


(151)

Figure 12.1 Cytotoxicity of selected diaminopyrimidines against L1210 cells in vitro.





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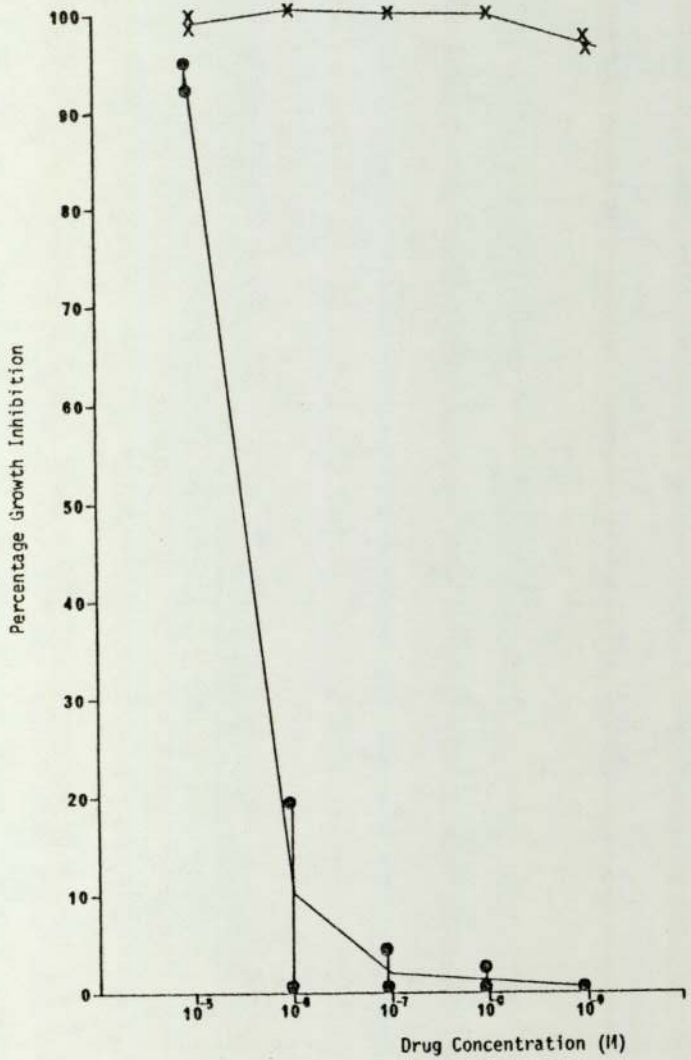
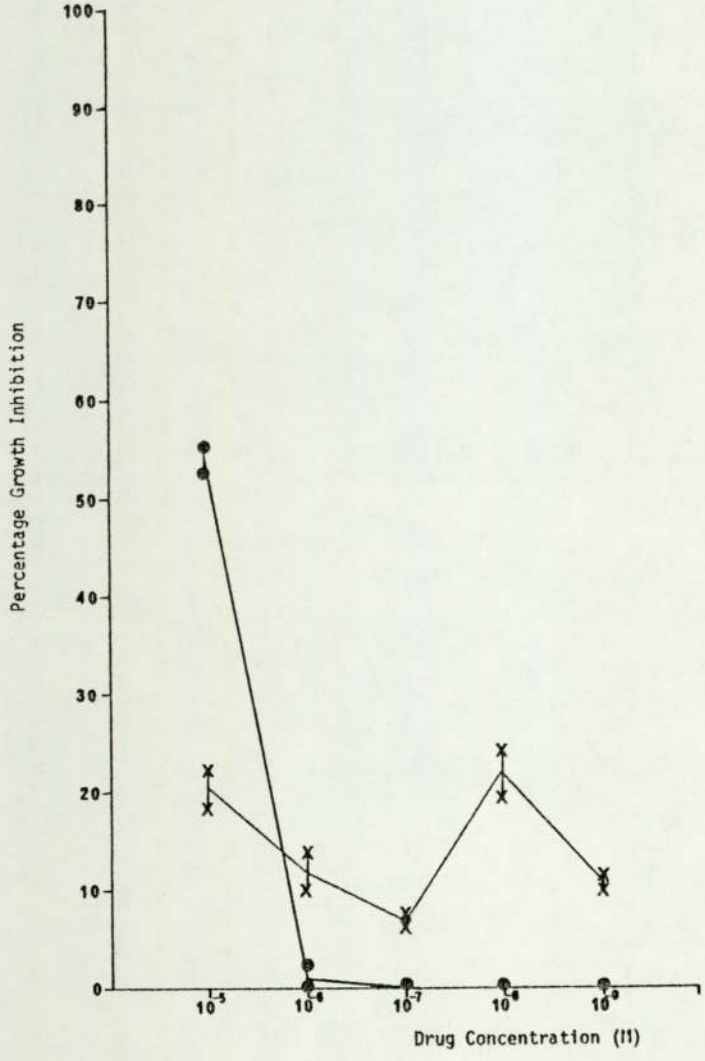


x——x : Compound (106)

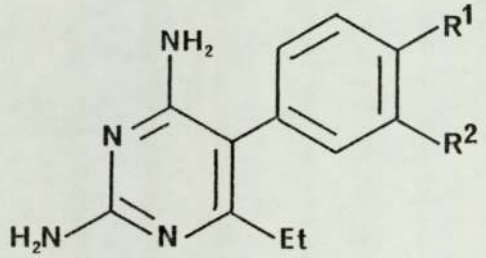
●——● : Compound (115)



x——x : Compound (150)

●——● : Compound (151)

Table 12.1 Cytotoxicity of selected diaminopyrimidines against L1210 cells *in vitro*



Compound Number	R^1	R^2	Concentration (M)	Growth	Percentage Inhibition[d]	I.C.$_{50}$[a] (μM)
21a	Cl	N_3	0		0	0.07
(MZPES)			10^{-5}	98.2	99.6	
			10^{-6}	98.6	98.8	
			10^{-7}	70.4	68.6	
			10^{-8}	5.8	10.1	
			10^{-9}	(+6.7)[b]	3.1	

Table 12.1 continued

64a	N_3	Cl	0		0	0.008
(iso-MZPES)			10^{-5}	98.1	98.5	
			10^{-6}	98.4	99.5	
			10^{-7}	93.9	93.7	
			10^{-8}	53.3	52.6	
			10^{-9}	21.3	17.9	
98a[c]	n-butylamino	NO_2	0		0	0.2
			10^{-5}	>100	>100	
			10^{-6}	99.4	99.5	
			10^{-7}	38.4	44.7	
			10^{-8}	19.3	18.1	
			10^{-9}	(+4.3)[b]	12.2	

Table 12.1 continued

114a[c]	NO_2	n-butylamino	0		0	3.0
			10^{-5}	95.8	97.3	
			10^{-6}	33.5	21.7	
			10^{-7}	29.8	22.9	
			10^{-8}	22.5	12.5	
			10^{-9}	18.5	13.6	
106[c]	benzylamino	NO_2	0		0	<0.001
			10^{-5}	98.5	99.5	
			10^{-6}	>100	>100	
			10^{-7}	>100	>100	
			10^{-8}	>100	>100	
			10^{-9}	97.2	96.4	

Table 12.1 continued

115 [c]	NO_2	benzylamino	0		0		5.0
			10^{-5}	92.6		95.2	
			10^{-6}	0		19.1	
			10^{-7}	0		4.9	
			10^{-8}	3		0	
			10^{-9}	0.7		0	
150	4-(1,1-dimethyl-	H	0		0		>10.0
	piperazinium)iodide		10^{-5}	18.3		22.1	
			10^{-6}	14.5		11.0	
			10^{-7}	6.6		7.3	
			10^{-8}	19.9		24.0	
			10^{-9}	12.1		10.0	

Table 12.1 continued

151	trimethylammonium iodide	H	0		0	10.0
			10^{-5}	57.1	52.9	
			10^{-6}	2.8	(+1.4)[b]	
			10^{-7}	0	0	
			10^{-8}	0	0	
			10^{-9}	0	0	

[a] the drug concentration necessary to reduce the 72 h cell count to 50% of control

[b] no inhibition of cell growth evident

[c] prepared as a 1 mM solution in DMSO and diluted immediately prior to use. All other compounds were prepared as aqueous solutions (1 mM) and diluted to the requisite concentration in the same manner

[d] counts conducted in duplicate

CHAPTER 13

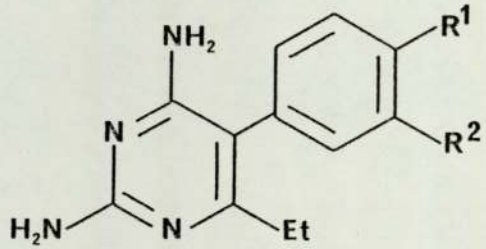
SCREENING OF SELECTED COMPOUNDS FOR ANTITUMOUR ACTIVITY *IN VIVO*

13.1 Introduction

The biochemical studies described previously (chapters 10-12) centred on the identification of compounds suitable for further evaluation as possible lipophilic DHFR inhibitors and also analogues that, by virtue of their inactivity against the aforementioned enzyme, may qualify as molecular probes for a target elsewhere. Fortuitously, several compounds designed to fulfil the latter role may merit consideration under the former category. Those compounds showing a high activity against mammalian DHFR were deemed suitable for antitumour screening as prospective novel antifolates *per se*, or were tested for comparison with the parent azidopyrimidine MZP (21).

Analogues identified as 'non-inhibitors' of DHFR were assessed for *in vivo* antitumour activity, on the basis that any observed response might be mediated through association with a locus of cytotoxicity unrelated to inhibition of this enzyme, although the shortcomings of such an extrapolation were appreciated. On a more fundamental level the screening of any novel, structurally interesting compound for antitumour activity is prudent and thus, where practicable, the maximum number of analogues were submitted to the NCI (USA) for routine appraisal. In several cases, insufficient compound (<0.5 g) was available, or was not of a suitable purity for this purpose and consequently agents of this nature have yet to be submitted for screening.

Table 13.1 Antitumour activity for selected compounds screened by the NCI[a]



Compound Number	R^1	R^2	Optimum Dose ($mg.kg^{-1}$)	Optimum[b] T/C (%)	NCI [c] Status
53	OMe	NO_2	120	189	active
60	OMe	NH_2	200	144	active
64	N_3	Cl	100	160	active
65	OMe	N_3	240	117	inactive
95	methylamino	NO_2	100	182	active
96	dimethylamino	NO_2	100	145	active
98	n-butylamino	NO_2	240	206	active

Table 13.1 continued

99	piperidin-1-yl	NO_2	200	125	inactive
100	piperazin-1-yl	NO_2	120	112	inactive
101	4-methylpiperazin-1-yl	NO_2	200	130	active
102	pyrrolidin-1-yl	NO_2	200	110	inactive
103	4-morpholino	NO_2	100	116	inactive
104	cyclohexylamino	NO_2	60	101	inactive
105	N-methylcyclohexylamino	NO_2	480	168	active
106	benzylamino	NO_2	240	126	inactive
107	N-methylbenzylamino	NO_2	240	185	active
108	N-ethylbenzylamino	NO_2	200	144	active
109	(±) ∝-methylbenzylamino	NO_2	30	132	active
111	phenethylamino	NO_2	200	142	active
112	2-aminoethylamino	NO_2	240	116	inactive
113	3-aminopropylamino	NO_2	120	129	presumptive[d] active

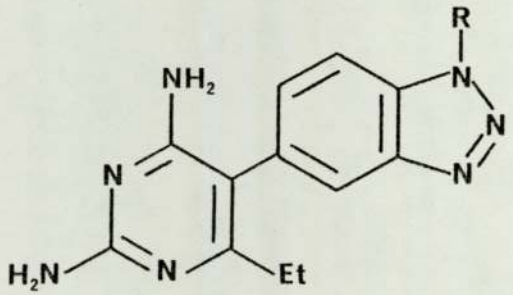
Table 13.1 continued

124	dimethylamino	NH_2	100	96	inactive
127	piperidin-1-yl	NH_2	50	100	inactive
129	4-methylpiperazin-1-yl	NH_2	50	108	inactive
130	pyrrolidin-1-yl	NH_2	50	100	inactive
131	4-morpholino	NH_2	200	98	inactive
135	N-methylbenzylamino	NH_2	200	103	inactive
138	phenethylamino	NH_2	200	100	inactive
139	2-aminoethylamino	NH_2	50	107	inactive
140	3-aminopropylamino	NH_2	50	110	inactive
141	dimethylamino	N_3	60	113	inactive
142	piperidin-1-yl	N_3	50	100	inactive
144	4-methylpiperazin-1-yl	N_3	200	130	presumptive[d] active
145	4-morpholino	N_3	6.25	100	inactive
146	dimethylamino	H	12.5	98	inactive

Table 13.1 continued

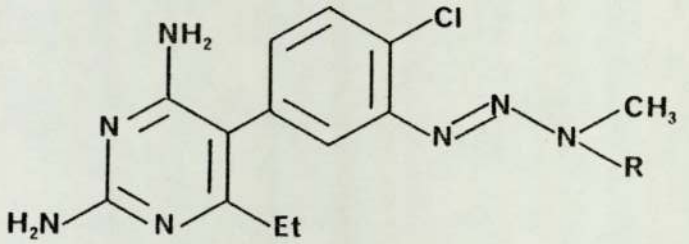
147	piperidin-1-yl	H	7.5	112	inactive
148	piperazin-1-yl	H	200	101	inactive
149	4-methylpiperazin-1-yl	H	50	103	inactive
150	4-(1,1-dimethylpiperazinium) iodide	H	0.96	119	inactive
151	trimethylammonium iodide	H	25	103	inactive

Table 13.1 continued



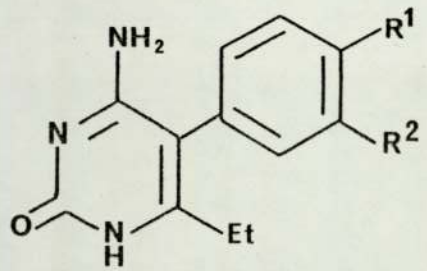
Compound Number	R	Optimum Dose ($mg.kg^{-1}$)	Optimum[b] T/C (%)	NCI[c] Status
152	Me	100	104	inactive
154	n-butyl	200	89	inactive
155	cyclohexyl	100	100	inactive
157	phenethyl	50	99	inactive

Table 13.1 continued



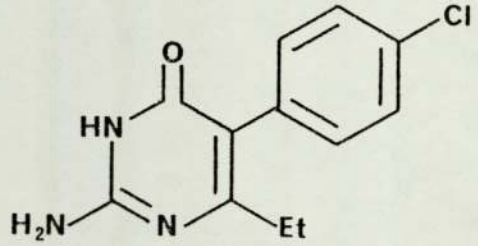
Compound No	R			
163	H	100	112	inactive
164	Me	200	105	inactive

Table 13.1 continued



Compound Number	R^1	R^2	Optimum Dose ($mg.kg^{-1}$)	Optimum[b] T/C (%)	NCI[c] Status
83a (hydrochloride)	Cl	H	62.5	96	inactive
165	piperidin-1-yl	NO_2	240	97	inactive

Compound Number Structure



84a (hydrochloride)			26.25	96	inactive

Table 13.1 continued

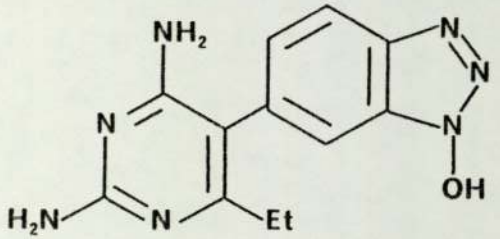
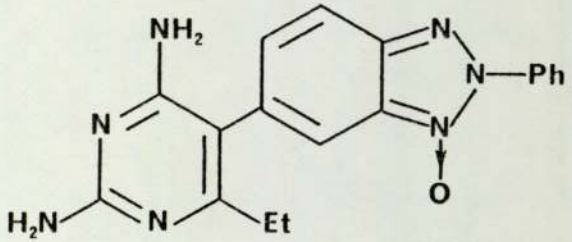
120a[e]		200	105	inactive
122a[e]		200	142	active

Table 13.1 continued

[a] all compounds administered to mice *via* the intraperitoneal route; for further details regarding the NCI screening protocol, see reference 202.

[b] antitumour activity was evaluated against the P388 lymphocytic leukaemia. Activity is expressed as a ratio (%) of the median survival time (days) for treated animals (T) to that of control animals (C).

[c] for the P388 cell line a minimal reproducible T/C of >120 implies activity. A T/C of 288 is a maximum value for this system

[d] awaiting retest.

[e] methanesulphonate salt.

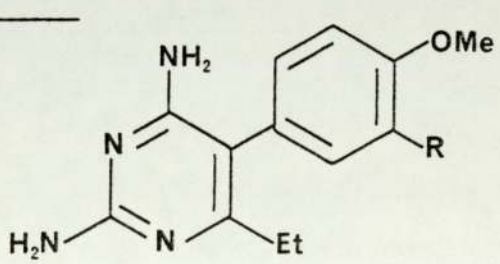
13.2 Results of the antitumour screening

The results of the preliminary screening of fifty one compounds against the P388 lymphocytic leukaemia in mice are summarised in table 13.1. Of those compounds tested, fifteen were found to exhibit significant activity.

In the azidopyrimidine series the methoxynitropyrimidine (53) was found by the NCI to be active at an optimum dose of 120 $mg.kg^{-1}$. Although the parent compound nitropyrimethamine (49) exhibited comparable antitumour activity at a lower dose (T/C=163% at 12.5 $mg.kg^{-1}$)[93] against the identical tumour model toxicity occurred with (49), whereas no toxic effects were observed with (53) at the aforementioned dose. Surprisingly, the corresponding methoxyamine (60) derived from (53), although a ten-fold weaker inhibitor of DHFR was also found to be active, and yet the methoxyazide (65), a marginally better inhibitor of DHFR than (53) proved inactive in vivo (table 13.2).

Table 13.2 DHFR inhibition and antitumour activity of the alkoxypyrimidines

Compound Number	R	I_{50} (μM)	Optimum T/C (%)
53	NO_2	0.18	189
60	NH_2	1.80	144
65	N_3	0.66	117



Any speculation regarding the above results must be considered as tentative since toxicological and pharmacokinetic factors may be involved. However, if the antitumour activity of (60) is mediated through inhibition of DHFR then, in keeping with earlier observations (eg. the MZP series) the more lipophilic methoxyazidopyrimidine (65) should also be active *in vivo*. Clearly this is not the case.

iso-MZP (64), the most potent of the azidopyrimidines synthesised as lipophilic DHFR inhibitors showed significant antitumour activity in the preliminary screen and in this respect compares favourably with the 'lead' compound MZP (21) (best T/C = 151% at 200 $mg.kg^{-1}$)[93]. The NCI have requested further quantities of *iso*-MZP for a more comprehensive evaluation. Interestingly, MZPES (21a), the ethanesulphonate salt of MZP, was found to be inactive against the P388 model following intraperitoneal administration possibly due to retention within the peritoneal cavity, and a sample of *iso*-MZPES (64a) has since been submitted for assessment in order to establish the generality of this phenomenon. Dilution of an aqueous solution of MZPES with normal (0.9%) saline elicits an immediate precipitation of the sparingly soluble hydrochloride salt, and a similar effect *in vivo* may be responsible for the reported inactivity of MZPES *via* the intraperitoneal route.

With several notable exceptions the antitumour activity displayed by the remainder of the compounds tested correlated reasonably with activity against DHFR, and inactivity against the enzyme invariably reflected as an absence of cytotoxicity *in vivo*.

Of those nitropyrimethamine analogues encompassing a 4'-amine substituent the *n*-butylamino-, N-methylcyclohexylamino and N-methylbenzylaminopyrimidines (98, 105 and 107) were the most active and further quantities of these compounds have been despatched to the

NCI. Interestingly, the benzylaminonitropyrimidine (106) proved inactive as an antitumour agent in contrast to the N-methylbenzylamino homologue (107), this notwithstanding the identical DHFR inhibitory activities (I_{50}=1.0x10^{-8} μM) observed and the potent cytotoxicity of (106) against L1210 cells in vitro (chapter 12). Although this pronounced difference as of yet remains unexplained, such a disparate relationship serves to illustrate the limitations of extrapolation from enzyme to animal tumour model, and may reflect the bioavailability of each molecule.

Two other compounds worthy of emphasis are the aminopropylaminonitropyrimidine (113) and the N-methylpiperazinoazidopyrimidine (144), both of which may warrant further investigation. Structurally, the aminopropylamino derivative (113) may be regarded as similar to the corresponding aminoethylamino analogue (112) and indeed both compounds are equipotent as DHFR inhibitors (I_{50} 1 μM). However, (113) shows marginal antitumour activity in contrast to (112) which is inactive against the P388 model and in vitro biochemical studies directed at identifying the locus of cytotoxicity of (113) are in progress. Initial results indicate that the N-methylpiperazinoazidopyrimidine (144) exhibits some antitumour activity and evidence to confirm this is awaited with interest, since (144) was designated as inactive as an inhibitor of DHFR.

The two quaternary ammonium compounds (150 and 151) were found to be inactive by the NCI and the dimethylpiperazinium iodide (150) proved exceedingly toxic at doses in excess of 2 mg.kg^{-1}, probably emanating from neurological effects.

CHAPTER 14

CONCLUSIONS AND POSSIBLE FUTURE OBJECTIVES

14.1 Compounds in the azidopyrimidine series

Synthetic studies directed towards the development of more effective analogues of an established lead compound invariably involve a degree of empiricism regarding the most appropriate structural modifications required. However, although in principle numerous derivatives of the prototype azidopyrimidine antifolate MZP (21) are possible, in practice any structural changes were restricted to the 5-phenyl ring or the 6-alkyl substituent, since the 2,4-diaminopyrimidine moiety is a prerequisite for activity against DHFR.

Of those analogues of MZP synthesised and screened for activity against the enzyme only the 4'-azidopyrimidine iso-MZP (64) proved significantly more active than the parent compound, an effect also reflected by the superior *in vitro* and *in vivo* antitumour activity observed for (64). Consequently, iso-MZP has been selected for further evaluation as the free base and as the ethanesulphonate salt (64a), although preliminary investigations indicate that formulation problems arising from the poor aqueous solubility of (64a) may be encountered, perhaps necessitating the use of a co-solvent system.

A short biological half life was implicit to the rationale of introducing an azido substituent onto the pyrimethamine molecule and it is therefore imperative that iso-MZP embraces similar pharmacokinetic properties to MZP *in vivo*, since a protracted biological half life would overshadow any potential advantages conferred by the superior antitumour activity observed. Moreover, the para-

positioning of the azido group of iso-MZP precludes any possibility of enhancing bioinactivation by the introduction of an electron-donating substituent, as was proposed for MZP.

In view of the above possibilities the following studies are planned:

(i) In order to facilitate a more relevant comparison of iso-MZP with the parent compound MZP it is necessary to screen both the free bases (21 and 64) and the respective ethanesulphonate salts for activity against a panel of tumour models (eg. table 2.2). It would also be of interest to evaluate each isomer for activity against several methotrexate resistant cell lines in order to identify the most suitable analogue for subsequent therapeutic studies, especially for the treatment of methotrexate resistant malignancies.

(ii) A determination of the DHFR inhibitory activity of the corresponding 4'-amine derivative (59) of iso-MZP would be prudent, prior to the initiation of a comprehensive pharmacokinetic study on the azidopyrimidine, since the inactivity of (59) against the enzyme may substantiate the possibility of rapid bioinactivation of iso-MZP in vivo.

(iii) X-ray crystallographic investigations on iso-MZP are warranted in order that the enhanced activity against DHFR with respect to MZP, may be rationalised in terms of association of the azido substituent with a

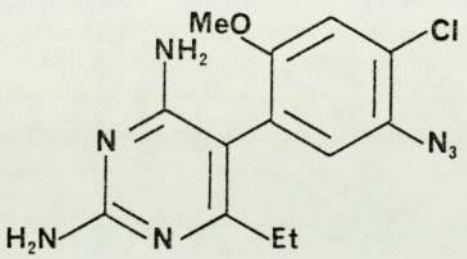
hydrophobic domain at the enzyme active-site. Apparently, a 4'-azido substituent may interposition within the hydrophobic pocket more readily than an azido group in the 3' position and, intriguingly, evidence to corroborate this observation was provided by the benzylaminopyrimidine analogues (106 and 115) where a transposition of the bulky benzylamino group from a 4' to a 3' position severely compromised activity against DHFR (chapter 10).

Recent pharmacokinetic data for MZPES obtained from a Phase 1 clinical trial currently in progress[203] indicates that for human subjects the elimination half life is considerably longer than that of 4-6 h reported for mice, and a value of 32-43 h appears more appropriate. Consequently, although the drug is eliminated from the body at a ten-fold faster rate than that documented for metoprine in patients, toxicity may still be manifested at higher doses and the importance of developing an analogue exhibiting the pharmacokinetics of methotrexate (12-20 h in man) is evident.

An MZP analogue embracing an electron-donating substituent <u>para</u> to the 3'-azido group would conceivably possess the physico-chemical characteristics facilitating rapid bioreduction and subsequent elimination of the drug. However, synthetic efforts directed towards the preparation of a compound of this nature have to date proved unsuccessful.

The preparation of an azidopyrimidine bearing a 2'-methoxy substituent was attempted by the conventional approach (chapter 5) but failed due to the reluctance of 2-methoxyphenylacetonitrile (38B) to condense with ethyl propionate under the reaction conditions

employed. DeGraw et al[204] have developed an alternative synthesis for the preparation of β-ketonitriles employing sodamide in liquid ammonia to force the condensation reaction and this method may represent a route to the requisite intermediate, although subsequent introduction of the chloro and azido groups to furnish the target compound (161) may present difficulties.



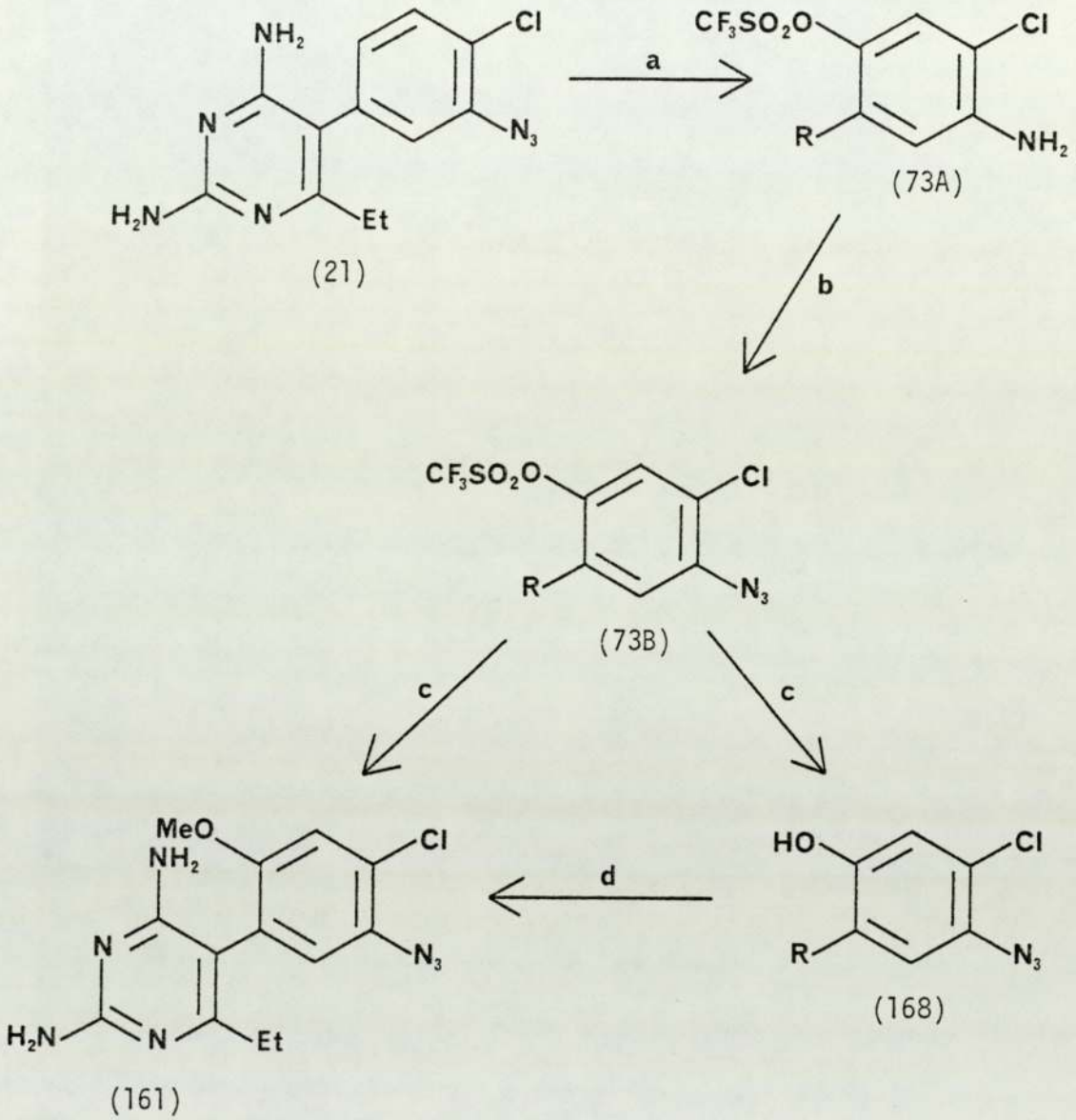
(161)

The direct introduction of a methoxy group para to the azido substituent of MZP would circumvent problems associated with the above approach and preliminary studies to this end utilising the acid-catalysed decomposition of azidopyrimidines are encouraging. Thus treatment of MZP with TFSA afforded the aryl triflate (73A) in excellent yield (chapter 5.4) from which the corresponding 5'-azide (73B) was prepared without incident. Since aryl triflates are susceptible to nucleophilic attack both at sulphur and at the phenyl-carbon atom in reactions dependent on the conditions employed[205,206], treatment of (73B) with the appropriate reagent, for example sodium methoxide, may furnish (161) in a single step or more probably the phenol (168). Alkylation of the phenol (168) should proceed without difficulty to yield the requisite diaminopyrimidine (161) (scheme 14.1).

Analogous reactions conducted with azidopyrimidines other than MZP (62-66 and 141-145) would also be of interest from a synthetic

viewpoint, since preliminary studies suggest that the ease of acid catalysed decomposition varies with the nature of substituents on the aromatic ring, the methoxyazide (65) decomposing rapidly in TFA at room temperature in the absence of TFSA.

Scheme 14.1 Possible further reactions involving aryl triflates: synthetic strategy



R = 2,4-diamino-6-ethylpyrimidin-5-yl

a. TFSA/TFA; 0°

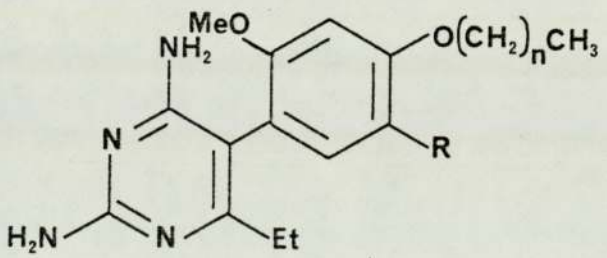
b. HNO_2/NaN_3; <5°

c. NaOMe in MeOH

d. $Me_2SO_4/NaOH$

Replacement of the chloro group of nitropyrimethamine by an n-butoxy substituent afforded a derivative (55a) with a high affinity for mammalian DHFR (K_I = 8.0x10^{-11} M). Subsequent transformation of the nitro group to an azido group should proceed without incident and the product (166) would be an interesting candidate lipophilic DHFR inhibitor. Moreover, decomposition of (166) in TFA or TFSA as discussed previously and reconversion to the azide by the conventional method to afford the azidopyrimidine (170) would be desirable, since such a compound may exhibit potent inhibitory activity against DHFR and yet undergo facile biotransformation *in vivo* to the inactive amine derivative (169).

An optimum balance between potency against DHFR and inactivation on bioreduction might be achievable by varying the size (n) of the 4'-alkoxy side-chain, and synthetic efforts to this end are proposed.



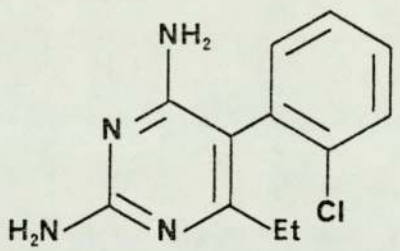
(169): R = NH_2 n=3

(170): R = N_3 n=3

14.1a Chiral diaminopyrimidines

The novel pyrimethamine isomers (45 and 46) are at present the subject of crystallographic studies to determine the influence of a chloro substituent on rotation about the pyrimidine-phenyl ring C-C bond and the overall conformational restraints imposed on the

molecules as a result. Moreover, for the 2'-chloro derivative (45) in particular it is conceivable that the barrier to rotation may be sufficient for chirality to occur. Optical isomerism in biphenyls is well established and is possible because the conformation imparting a plane of symmetry is unfavourable due to steric repulsion between ortho-substituents on adjacent rings. Consequently, non-coplanar conformations are favoured and for a barrier to rotation about the C-C bond exceeding approximately 80 KJ. mol^{-1}, isolation of two enantiomers by conventional methods of resolution may be practicable[207].



(45)

Resolution of the two optical isomers of 'o-chloropyrimethamine' (45) would be of interest since, in common with many drug-receptor interactions, one enantiomer may exhibit a greater affinity for DHFR than the other, and provide useful information regarding the stereo-specificity of binding of substrates and inhibitors to this enzyme. Charlton et al[208] have recently elucidated the stereochemistry of reduction of folic acid by DHFR using ^{1}H-nmr techniques, but to my knowledge the development of chiral small-molecule DHFR inhibitors as probes for the enzyme is unprecedented.

Preliminary studies have provided evidence to substantiate the existence of two enantiomeric forms of (45). Although a high resolution ^{1}H-nmr experiment conducted with a chiral shift reagent was unsuccessful due to line broadening and loss of resolution, in

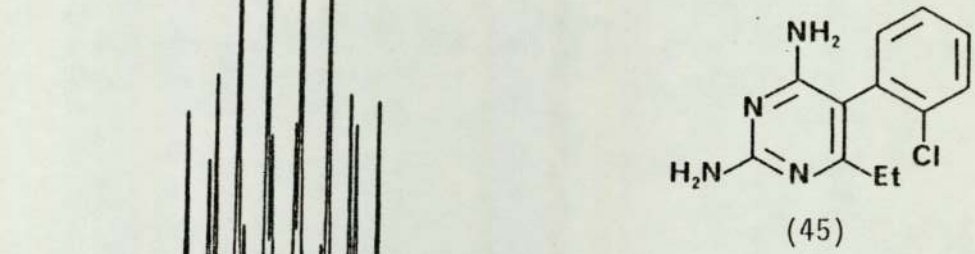
the absence of a shift reagent the methylene group of the 6-ethyl substituent of (45), which appears as a quartet centred at δ2.12 for pyrimethamine (4), was observed as a sixteen line multiplet at δ2.21 (fig. 14.1). Stereoisomerism about the pyrimidine-phenyl ring C-C bond would imply prochirality at the 6-ethyl methylene group rendering the methylene protons non-equivalent and consequently the observed multiple splitting pattern at this centre results from chirality within the molecule as predicted.

The possibility of resolving the respective enantiomers of (45) forms the subject of current investigations. Treatment of the racemic mixture with a chiral acid (d-10-camphorsulphonic acid monohydrate) in boiling water furnished a sparingly soluble salt and separation of the respective diastereoisomers by fractional crystallisation from water has been attempted, although polarimetric analysis of the components has yet to be conducted.

14.2 Metabolites and degradation products of MZP

With the exception of the 6-(1-hydroxyethyl)-derivatives (30) at least one example of each of the six categories of compounds considered in chapter 4 has been synthesised, and in this respect the objectives of this project have essentially been realised. In retrospect, nitration of 6-(1-hydroxyethyl)pyrimethamine, the key step in the preparation of the requisite 6-(1-hydroxyethyl)azidopyrimidines, should have been conducted on the protected derivative (92) since, in the absence of water, hydrolysis of the diethylacetal protecting group would not be envisaged. Future investigations will centre on the evaluation of anhydrous nitrating agents utilising pyrimethamine as the model substrate, and subsequent nitration of

Figure 14.1 400 MHz ^{1}H-n.m.r. spectrum of compound (45) recorded in $CDCl_3$ as solvent. Inset the observed multiplet derived from the methylene protons of the 6-ethyl substituent (enlarged x 7).



(45)

(92) under the least vigorous conditions possible, may furnish the required intermediate from which the corresponding amino and azido-pyrimidines would be available *via* conventional chemical transformations.

Studies conducted *in vivo* regarding the metabolic fate of MZP have yet to establish the nature and extent of the biotransformation processes and the principle route of elimination of the drug. Consequently, a future project of immediate importance will centre on the synthesis of radiolabelled MZP in order to facilitate detailed investigations of the pharmacokinetics, distribution, metabolism and elimination of the drug. The synthesis of pyrimethamine with a ^{14}C label at C-2 has been reported by Murray[209] who condensed commercially available ^{14}C-guanidine with the appropriate enol-ether *via* a method essentially identical to that described previously (chapter 5.1).

Subsequent introduction of the 3'-azido substituent by the usual procedure should furnish the desired ^{14}C-MZP in good yield, from which the appropriate radiolabelled metabolites may, in principle, be available if necessary.

The desired pharmacokinetic properties of MZP depend on rapid bioinactivation of the drug, possibly *via* reduction to the corresponding amine (MAP) and subsequent elimination. Moreover, aryl azides react readily with thiols under mild conditions forming arylamines[102,103] and an *in vivo* conversion of MZP to MAP by glutathione has been suggested as a possible mechanism for inactivation of the drug.

Should glutathione play a significant role in the biotransformation of MZP it is conceivable that modifying the endogenous levels of glutathione may influence the pharmacokinetics of the drug

and perhaps represent a means of modulating the biological half life *in vivo*. Thus depleting endogenous glutathione levels should increase the biological half life of MZP, while an elevation of the peptide levels would conceivably have the converse effect. Furthermore, it has been demonstrated that a depletion of intracellular glutathione reserves may inhibit the essential polyglutamation of folate cofactors and methotrexate[210].

It may be conjectured that depleting endogenous glutathione reserves prior to treatment with MZP may elicit a synergistic antitumour effect, subsequent elevation of glutathione levels promoting a rapid inactivation and elimination of the drug. Preliminary experiments designed to establish the practicality of such an approach are planned utilising the glutathione depleting agent buthionine sulphoximine[211].

Methotrexate and related folate antagonists have been used extensively, both systemically and *via* topical administration, for the amelioration of psoriatic conditions, but toxicity problems associated with the chronic use of these agents have invariably arisen. However, despite the demonstrated clinical efficacy of topical pyrimethamine for the treatment of psoriasis[81], little has been published regarding the evaluation of lipophilic small-molecule inhibitors as candidate antipsoriatic agents. One future objective therefore will centre on the assessment of the azidopyrimidine analogues, notably MZP (21) and *iso*-MZP (64), as prospective topical antipsoriatic agents, possibly in conjunction with a suitable absorption enhancer, for example decylmethylsulphoxide or azone.

Systemic toxicity following the topical administration of an azidopyrimidine analogue should not pose a serious problem since polyglutamation and retention within hepatocytes, the principal

toxicological problem associated with methotrexate therapy, cannot occur.

One intriguing possibility stems from the previous consideration regarding the importance of endogenous glutathione for MZP bio-inactivation; an elevation of circulating glutathione levels *in vivo* by the administration of dietary glutathione precursors prior to treatment with topical MZP should, in principle, restrict cyto-toxicity to the cutaneous layers of the skin, since any drug entering the systemic circulation would undergo rapid biodegradation. This hypothesis has yet to be substantiated and must be regarded as speculative.

14.3 Diaminopyrimidines as probes for a target unrelated to inhibition of DHFR

The concept of suppressing or abolishing the activity of a recognised enzyme inhibitor by modification of the chemical structure, such that cytotoxicity at a site elsewhere may predominate, is an unorthodox one. Not least, the obscurity of the alternative target under consideration engenders problems as to the most appropriate biochemical system for the identification of candidate lead compounds generated in this fashion. Consequently, in the present study, prospective marker compounds synthesised by the aforementioned method, were selected for further biochemical evaluation on the basis of a disproportionate cytotoxicity *in vitro* and *in vivo* with respect to observed activity against DHFR.

Synthetic efforts directed at the introduction of a polar or ionic substituent onto the 5-phenyl ring of diaminopyrimidines were successful in that activity against the enzyme was reduced or

effectively eliminated as required. Furthermore, of those compounds designated as non-inhibitors of DHFR (123-133 and 147-151) several exhibited marginal antitumour activity *in vivo*, thus qualifying for a more comprehensive biochemical evaluation in order to elucidate the precise mechanism of cytotoxicity.

The synthetic route adopted whereby activation and nucleophilic substitution of the 4'-chloro substituent of pyrimethamine (4) by the appropriate amine, preceeded removal of the activating 3'-nitro substituent, was useful. Each intermediate prepared *via* this procedure was assessed for activity against DHFR and ranked according to the nature of the 3'-substituent. Thus, for a particular analogue, potency against DHFR decreased in the order $NO_2 > N_3 > NH_2 > H$. Consequently, allowing for pharmacokinetic or metabolic factors, if inhibition of DHFR constituted the sole locus of cytotoxicity then the *in vivo* antitumour activity of the analogues under consideration should follow a similar trend and compounds exhibiting anomalous antitumour activity warranted further evaluation. For example, the methoxyamine (60) discussed earlier (chapter 13.2) was unusual in proving more active against the NCI screen than the corresponding methoxyazide (65), although (65) was a more potent DHFR inhibitor, and further studies on (60) are proposed.

A major disadvantage associated with the introduction of ionic substituents as a means of eliminating DHFR inhibitory activity, stems from the pharmacokinetic consequences of such a modification. Although binding to the enzyme active site will be compromised, the overall polar character of the molecule may preclude passive diffusion across the cell membrane resulting in a cellular exclusion of the compound. Should the alternative target in question reside on the surface of the cell membrane, intracellular accumulation of the

diaminopyrimidine derivative would not be necessary and, indeed, exclusion may be advantageous in divorcing activity against DHFR from cytotoxicity at this site. However, only those derivatives embracing an element of inherent lipophilicity would be predicted to gain access to an intracellular independent target and ionic or highly polar compounds may be rendered inactive as a result.

An alternative mechanism whereby inactivity against DHFR was achieved without significantly reducing the essential lipophilic nature of the diaminopyrimidine became apparent during the course of these investigations. Thus for the benzylaminopyrimidine analogues (106 and 115) a transposition of the benzyl substituent from the 4' to the 3' position reduced activity against the enzyme by a factor of 10^3 (chapter 10.4) presumably due to steric incompatibility at the active site, since in terms of solubility and chromatographic character the two isomers were of similar polarity. On the basis of this evidence it is conceivable that analogues encompassing bulky 3'-substituents may serve as probes for an intracellular folate independent target, since diffusion across cell membranes should be unaffected while activity against DHFR may be abolished.

It would be of interest in a future study, therefore, to synthesise the corresponding 3' isomers of 4'-amine substituted derivatives characterised as potent inhibitors of DHFR (table 10.2) using iso-MNP (50) as starting material. Those compounds subsequently identified as inactive against the enzyme may nevertheless prove cytotoxic <u>in vitro</u> or <u>in vivo</u> and qualify for further biochemical evaluation as probes for the elusive alternative target in question. Further evidence to substantiate the rationale for designing 'steric non-inhibitors' has been considered elsewhere (chapter 3) where two isomeric naphthylpyrimidines were found to

differ with regard to DHFR inhibition and cytotoxicity[66,109].

The alternative, folate-independent mechanism or site of action of lipophilic diaminopyrimidine DHFR inhibitors remains to be identified and utilising those derivatives selected previously as candidate probes by virtue of their inactivity against DHFR, the following experiments are planned:

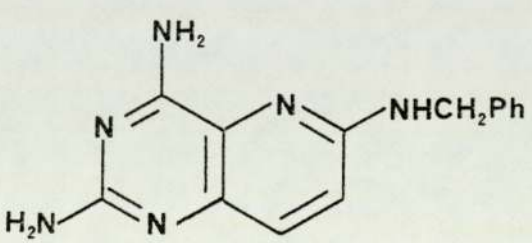
Firstly, compounds should be screened for cytotoxicity in vitro under both folate dependent and independent conditions of cell growth, by a procedure essentially identical to that described in the literature, such that analogues inhibiting cell growth under folate independent conditions are identified.

Secondly, it will be necessary to conduct more comprehensive biochemical studies on the most promising of those analogues characterised above in order to ascertain the exact locus of cytotoxicity. Active compounds encompassing an azido substituent may additionally serve as photoaffinity labels for the target under consideration, the introduction of a suitable radiolabel facilitating subsequent detection of the appropriate analogue-target complex.

The use of mutant cell-lines may represent an alternative approach for the selection of modified diaminopyrimidines cytotoxic by virtue of activity at a folate-independent site. The metoprine resistant L1210 tumour line reported by Browman et al[78] remained sensitive to methotrexate, and it would be of interest to investigate the generality of this phenomenon since, for a cell line resistant to lipophilic antifolates, any observed cytotoxicity may manifest as a consequence of an effect unrelated to inhibition of folate metabolism. More recently, Kamen et al[212] have demonstrated a lack

of DHFR in several human tumour lines *in vivo*. Should these tumours prove refractory to lipophilic DHFR inhibitors as predicted, it is conceivable that analogous cell lines established *in vitro* may serve as preliminary screens for the identification of modified diaminopyrimidine probes, since any observed inhibition of cell growth would not be attributable to activity against DHFR.

The N-alkylbenzylaminopyrimidines (106-108) discovered fortuitously during the course of this project constitute a novel series of potent DHFR inhibitors and may merit further evaluation as antitumour or antibacterial agents. Interestingly, in a recent report, Colbry et al[213] have described the synthesis of a series of antifolate 2,4-diaminopyridopyrimidines (171) bearing a benzylamino or N-alkylbenzylamino substituent in the 6-position, as prospective antimalarial agents. A structural similarity between these compounds and the aforementioned benzylaminopyrimidines is evident and consequently, it would be of interest to investigate the practicality of preparing analogues devoid of the 3'-nitro substituent as possible antimalarial agents.



(171)

SECTION 3
MATERIALS AND METHODS

Experimental: Chemistry

Notes

1. All melting points are reported uncorrected.

2. 'Ethanol' refers to 95% ethanol.
   'Acetic acid' refers to 100% acetic acid.

3. Infra red spectra were recorded on a Pye-Unicam SP200 spectrometer in potassium bromide discs.

4. Ultra violet spectra were recorded on a Pye Unicam SP8000 recording spectrophotometer.

5. The mass spectra were recorded on a Micromass 12B single focusing mass spectrometer.

6. Microanalyses were conducted by Elemental Microanalysis Limited and by the Department of Chemistry, Aston University.

7. The t.l.c. systems employed Kieselgel $60F_{254}$ (0.25 mm) as adsorbent and either butanol-acetic acid-water (2:1:1), chloroform-methanol (5:1) or toluene-acetone-ethanol (7:5:3) as developing solvents.

8. Analytical data for the appropriate compounds are presented at the conclusion of each chapter.

CHAPTER 15

SYNTHESES IN THE AZIDOPYRIMIDINE SERIES

2,4-Diamino-6-methyl-5-phenylpyrimidine (36)

Method A

A mixture of dicyandiamide (20 g) and phenylacetone (35 g) was heated with agitation for 6 h at 185° (bath temperature) in a flask fitted with an air condenser. The reaction mixture was cooled and the resultant solid triturated with acetone (50 ml) and collected. Repeated crystallisation from methanol furnished an off-white powder (3.7 g, 7.8%), m.p. 250-253 °C, (lit[3], m.p. 249-250°).

Method B

A solution of α-acetylphenylacetonitrile (22 g) in ether (250 ml) was treated with a solution of diazomethane (10 g) in ether (500 ml) over 15 min. The mixture frothed vigorously and was stirred at 10° overnight. Excess diazomethane was destroyed by the dropwise addition of acetic acid and the ether was evaporated to give the methoxyacrylonitrile as a yellow syrup (22.6 g). Sodium (4 g) was dissolved in ethanol (100 ml) and a solution of guanidine hydrochloride (16 g) in ethanol (50 ml) added, whereupon the mixture was stirred for 5 min and sodium chloride removed by filtration. The filtered solution was added to a solution of the methoxyacrylonitrile in ethanol (50 ml), and the mixture was refluxed for 12 h. After cooling and concentrating the dark mixture to half volume, the

product crystallised and was collected. A sample recrystallised from ethanol as colourless needles of the diaminopyrimidine (36) which was identical (m.p., i.r. and n.m.r.) to the compound obtained by method A above.

The following compounds were synthesised by the literature method[3] and treated in the aforementioned manner (method B) to furnish the appropriate diaminopyrimidines (table 15.1):

∝-acetyl-3-chlorophenylacetonitrile

m.p. 83-84° (lit[3], m.p. 84-86°)

∝-acetyl-4-chlorophenylacetonitrile

m.p. 124-125° (lit[3], m.p. 124-125°)

∝-propionylphenylacetonitrile

m.p. 72-73° (lit[3], m.p. not quoted)

∝-propionyl-2-chlorophenylacetonitrile

m.p. 41-43°

∝-propionyl-3-chlorophenylacetonitrile

m.p. 51-52°

∝-propionyl-4-chlorophenylacetonitrile

m.p. 51-53° (lit[3], m.p. 50-52°).

Attempted synthesis of ∝-acetyl-4-nitrophenylacetonitrile (38A).

A mixture of 4-nitrophenylacetonitrile (5 g) and ethyl acetate (2.8 g) was added to a stirred solution of sodium (2 g) in ethanol

(40 ml) and the solution was boiled for 5 h, cooled and poured into water (200 ml). The resultant dark green precipitate resisted all attempts at purification and was discarded.

The above reaction was repeated using 2,6-lutidine and triethylamine respectively as base. In both cases only starting materials were recovered in almost quantitative yield, after refluxing for time periods in excess of 48 h.

Attempted synthesis of α-propionyl-2-methoxyphenylacetonitrile (38B)

A mixture of 2-methoxyphenylacetonitrile (5 g) and ethyl propionate (4.1 g) was refluxed in an ethanolic solution of sodium ethoxide as described above. The compound obtained following purification by the literature method[3], was identical (i.r., n.m.r. and t.l.c.) with the starting material.

The above reaction was repeated using sodium (0.9 g) in n-butanol (35 ml) in an attempt to elevate the reaction temperature. Again only starting materials were recovered after refluxing the mixture for 36 h.

2,4-Diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (49)

Pyrimethamine (4) (100 g) was added in portions over 1 h to a stirred mixture of nitric acid (D. 1.42; 300 ml) and sulphuric acid (300 ml) at a temperature maintained below 5°. The mixture was stirred for a further 12 h at room temperature, poured onto ice and basified with concentrated aqueous ammonia. The yellow solid was collected and washed with water. A sample crystallised from aqueous ethanol as yellow microrosettes, m.p. 204-205° (lit[93], m.p. 203-205°) (Found: M^+, 293 [295]. $C_{12}H_{12}ClN_5O_2$ requires M, 293 [295]); ν_{max} 3450, 3290 and 3100 br (NH), 1530 and 1340 cm^{-1} (NO_2).

The ethanesulphonate salt of (49) was prepared by boiling a solution of the base in aqueous ethanesulphonic acid from which pale yellow rosettes were deposited on cooling, m.p. 260-262 (decomp.) (Found: C, 41.9; H, 4.5; N, 17.4; M^+, 293 [295]. $C_{14}H_{18}ClN_5O_5S$ requires C, 41.6; H, 4.5; N, 17.3%; M, 293 [295] [free base]); δ 1.03 (3H,t,CH_3), 1.13(3H,t,$\underline{CH_3}CH_2SO_3^-$), 2.25 (2H,q,$CH_2$), 2.60 (2H,q,$CH_3\underline{CH_2}SO_3^-$), 7.5-7.7 (2H,m,5'-$H_1$ and 6'-H_1), 8.0 (1H,d,2'-H_1), 7.5-8.5 (4H,NH), ([$^2H_6$]DMSO).

2,4-Diamino-5-(3-chloro-4-nitrophenyl)-6-ethylpyrimidine (50)

The 2,4-diaminopyrimidine (46) (9.0 g) was dissolved in sulphuric acid and stirred at a temperature maintained below 5°. Nitric acid (D. 1.42; 3.6 g) was added over 1 h and the mixture was stirred at room temperature for 12 h, poured onto ice and basified with concentrated aqueous ammonia as described previously. Recrystallisation from aqueous ethanol afforded yellow microprisms of the 4'-nitropyrimidine (table 15.2).

Nitration of the 6-methyldiaminopyrimidines (42 and 43) in an identical manner afforded the requisite products without incident (table 15.2), whilst compounds (36 and 44) gave a mixture (t.l.c.) of two products in each case. Nitration of (36 and 44) following the literature method[3] again yielded a mixture identical to that obtained above.

2,4-Diamino-6-ethyl-5-(4-methoxy-3-nitrophenyl)pyrimidine (53)

Nitropyrimethamine (49) (2 g) was added to a solution of sodium (1.0 g) in dry methanol (50 ml) and the suspension was refluxed for 18 h. After cooling and concentrating the pale green mixture to half volume, water (50 ml) was added and the yellow solid collected. Crystallisation from DMF gave a yellow amorphous solid (table 15.2).

2,4-Diamino-5-(4-ethoxy-3-nitrophenyl)-6-ethylpyrimidine (54)

To a solution of sodium (1.0 g) in ethanol (50 ml), nitropyrimethamine (49) (2 g) was added and the suspension stirred for 12 h at 55-60°. The mixture was cooled and poured into water (50 ml), whereupon a cream precipitate developed and was collected. A sample recrystallised from aqueous ethanol as a pale yellow amorphous solid (table 15.2).

2,4-Diamino-5-(4-n-butoxy-3-nitrophenyl)-6-ethylpyrimidine (55)

To a solution of sodium (0.17 g) in n-butanol (30 ml) was added nitropyrimethamine (49) (2.0 g) and the mixture was refluxed for 1 h, when t.l.c. examination showed the absence of starting materials and formation of a single product. The olive green mixture was cooled and diluted with water (50 ml) whereupon a brown amorphous solid deposited and was collected (0.8 g, 35%). Repeated recrystallisation from aqueous ethanesulphonic acid furnished hygroscopic microprisms of the 4'-butoxypyrimidine monoethanesulphonate (55a) (table 15.2).

2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine (22)

A suspension of the nitro analogue (49) (20 g) in ethanol (250 ml) was stirred at 60-65° and Raney nickel (c.a. 20 g) was added. Hydrazine hydrate (75 ml) was added dropwise over 2 h as a solution in ethanol (75 ml) at a rate such that the temperature did not exceed 70°. When effervescence had subsided the solution was filtered hot through a Kieselguhr pad and on evaporation of the solvent a cream solid remained which was triturated with water and collected. Crystallisation from 50% ethanol afforded pale yellow needles of the amine monohydrate, m.p. 217-219° (lit[93], m.p. 215-217°) (Found: M+ 263 [265]. $C_{12}H_{14}N_5Cl$ requires M, 263 [265]); λ_{max}

1602, 3180 br, 3330, 3480 cm^{-1} (NH_2).

2,4-Diamino-5-(4-amino-3-chlorophenyl)-6-ethylpyrimidine (59)

The nitrophenylpyrimidine (50) (9 g) was stirred as a suspension in ethanol (300 ml) at 70° and tin (II) chloride dihydrate (34.6 g) was added in portions over 10 min. All solids rapidly dissolved and the pale yellow solution was stirred for a further 12 h. After cooling, ethanol was evaporated to leave a yellow syrup which was redissolved in hot water (200 ml), cooled and basified to pH 12 with 10 M-sodium hydroxide solution. The microcrystalline precipitate was collected, washed with water and recrystallised from aqueous ethanol to yield cream needles. Identical reductions implemented on the other nitropyrimidines furnished the corresponding amines as required (table 15.3).

2,4-Diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine (21)

A solution of the amine (22) (8 g) in 5 M-hydrochloric acid (150 ml) was diazotised at 0° by the addition of sodium nitrite (2.1 g) as a solution in water (10 ml) over 30 min. After stirring for a further 30 min, sodium azide (7.2 g) was added in portions over 1 h the agitation being maintained. The mixture was stirred for a further 1 h, diluted with water (200 ml) and basified with concentrated aqueous ammonia, whereupon the product precipitated and was collected. A sample crystallised from aqueous ethanol as photosensitive microprisms of the azide monohydrate, m.p. 197-198° (Found: M^+ 289 [291]. $C_{12}H_{12}ClN_7$ requires M 289 [291]); ν_{max} 1450, 1564 br, 1639 br, 2150 (N_3), 3140 br, 3300 and 3460 cm^{-1} (NH_2).

The appropriate amines were diazotised and treated with sodium

azide as described above to give the corresponding azidopyrimidine derivatives (table 15.4).

2,4-Diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine ethanesulphonate salt (21a)

To a stirred suspension of the azide base (21) (6.0 g) in water (60 ml) ethanesulphonic acid (2.5 g) was added over 5 min and the mixture was boiled until all solids dissolved. Following filtration the pale yellow solution was allowed to cool and the product collected. Recrystallisation from water furnished pale yellow prisms of the azide monoethanesulphonate.

The requisite azidopyrimidine ethanesulphonate salts were prepared from the appropriate azide bases in an identical manner, and subsequently recrystallised from water (table 15.5).

2,4-Diamino-6-ethyl-5-(4-methoxyphenyl)pyrimidine (67)

A suspension of the methoxyazidopyrimidine (65) (0.5 g) in hydrazine hydrate (20 ml) was stirred at 50° for 30 min and finally boiled with vigorous effervescence for 30 min. The solution was cooled, diluted with water (50 ml) and stood at 4° overnight, whereupon a cream coloured microcrystalline precipitate developed and was collected (0.31 g, 72.4%). A sample recrystallised from aqueous 2-ethoxyethanol as pale yellow flakes of the methoxyphenylpyrimidine, m.p. 267-269° (Found: C, 63.6; H, 6.8; N, 23.0; M^+, 244. $C_{13}H_{16}N_4O$ requires C, 63.9; H, 6.6; N, 23.0%; M, 244); ν_{max} 3480, 3390 and 3180 br cm^{-1} (NH); δ 1.5 (3H,t,CH_3) 2.55 (2H,q,CH_2), 4.02 (3H,s,OCH_3), 7.35 (4H,s,C_6H_4), ([^{2}H]TFA).

2,4-Diamino-5-(4-ethoxyphenyl)-6-ethylpyrimidine (68)

The ethoxyazidopyrimidine (66) (0.6 g) was treated with hydrazine hydrate (20 ml) as above. However, examination by i.r. and t.l.c. after 2 h showed the presence of starting materials and the mixture was refluxed for a further 3 h when deazidation was complete. The product (0.2 g, 38.6%) crystallised from aqueous 2-ethoxyethanol as colourless needles of the ethoxyphenylpyrimidine, m.p. 251-253° (Found: C, 65.3; H, 7.3; N, 21.8; M^+, 258. $C_{14}H_{18}N_4O$ requires C, 65.12; H, 7.0; N, 21.7%; M, 258); ν_{max} 3490, 3390 and 3150 br cm^{-1} (NH); δ 1.25 (3H,t,CH_3), 1.50 (3H,t,$OCH_2\underline{CH_3}$), 2.55 (2H,q,CH_2), 4.25 (2H, q, OCH_2CH_3), 7.25 (4H,s,C_6H_4), ([^{2}H]TFA).

Decomposition of m-azidopyrimethamine (21) with trifluoromethanesulphonic acid (TFSA)

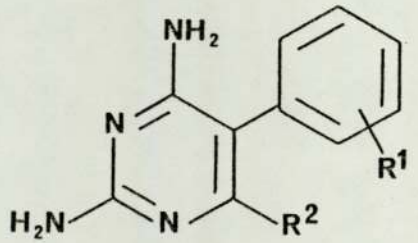
To a stirred mixture of trifluoracetic acid (10 ml) and trifluoroacetic anhydride (1 ml) at 0° was added trifluoromethanesulphonic acid (3 ml), with the exclusion of water. The solution was maintained at 0° for a further 30 min and MZP (21) (3.5 g) was added cautiously in portions over 2 h, the agitation being maintained, when some frothing was observed. The yellow solution was stirred overnight at room temperature, poured onto ice and basified with concentrated aqueous ammonia, whereupon a cream precipitate deposited and was collected (3.8 g). Recrystallisation from ethyl acetate-petroleum ether (60-80°) gave pale pink microprisms (73A), m.p. 177-178° (decomp.) (Found: C, 40.8; H, 4.2; N, 14.3; M^+, 279 [281]. $C_{13}H_{13}ClF_3N_5O_3S$. 1 EtOAc requires C, 40.4; H, 4.1; N, 14.8%; M-CF_3SO_3H, 279 [281]); ν_{max} 3455, 3300 and 3100 br cm^{-1} (NH); δ 1.16 (6H,m, CH_3 + $CH_3CO_2CH_2\underline{CH_3}$), 2.0 (3H,s,$\underline{CH_3}CO_2Et$), 2.20 (2H,q,$CH_2$), 4.10 (2H,q,$CH_3CO_2\underline{CH_2}CH_3$), 5.50 (2H,NH),

5.80 (4H,NH), 6.77 (1H,s,6'-H_1), 7.22 (1H,s,3'-H_1).

Preparation of the 5'-azido derivative of (73A)

A solution of (73A) (1.0 g) in 5M-hydrochloric acid (50 ml) was diazotised at 0° by the dropwise addition of sodium nitrite (0.27 g) as a solution in water (5 ml). The yellow solution was stirred for a further 30 min and sodium azide (0.9 g) was added such that the temperature did not exceed 5° (20 min), whereupon the mixture frothed vigorously and was stirred for a further 2 h. The suspension was poured onto ice (10 g) and basified with 50% aqueous ammonia solution when a cream precipitate formed and was collected (0.9 g). Crystallisation from aqueous ethanol afforded cream felted needles of the azide (73B), m.p. 161-162° (decomp.) (Found: C, 35.7; H, 2.5; N, 22.4; M^+, 305 [307]. $C_{13}H_{11}ClF_3N_7O_3S$ requires C, 36.0; H, 2.6; N, 22.2%; M- CF_3SO_3H, 305 [307]; ν_{max} 3140, 3300 and 3460 br (NH), 2160 cm^{-1} (N_3); δ 1.10 (3H,t,CH_3), 2.25 (2H,q,CH_2), 5.76 (4H,NH), 7.25 (1H,s,3'-H_1?), 7.43(1H,s,6'-H_1?), ([$^2H_6$]DMSO).

Table 15.1 Analytical data for compounds in the pyrimidine series



R^1	R^2	Compound[a] (Formula)	Yield (%)	mp (°C) (Lit[3], mp)	m/z (M^+)	Found (%) (Required) C	H	N
H	Me	36	61	250-251	200	-	-	-
		($C_{11}H_{12}N_4$)		(249-250)				
3-Cl	Me	42	54	221-222	234[236]	-	-	-
		($C_{11}H_{11}ClN_4$)		(219-220)				
4-Cl	Me	43	61	282-284	234[236]	-	-	-
		($C_{11}H_{11}ClN_4$)		(264-265)				
H	Et	44	60	243-244	214	-	-	-
		($C_{12}H_{14}N_4$)		(237-240)				

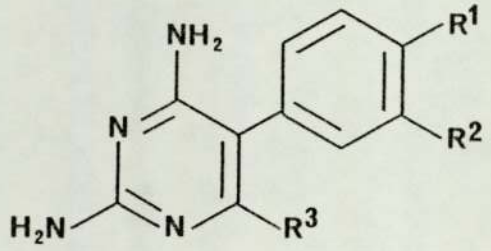
Table 15.1 continued

2-Cl[b]	Et	45	39	200-201	248[250]	58.4	5.3	22.7
		($C_{12}H_{13}ClN_4$)				(58.0)	(5.2)	(22.5)
3-Cl[b]	Et	46	39	211-213	248[250]	57.6	5.2	22.6
		($C_{12}H_{13}ClN_4$)				(58.0)	(5.2)	(22.5)

[a] all compounds were recrystallised from aqueous ethanol

[b] novel compounds

Table 15.2 Analytical data for compounds in the nitropyrimidine series



R^1	R^2	R^3	Compound (Formula)	Yield (%)	Mp (°C)	m/z (M^+)	Found (%) (Required) C	H	N
Cl	NO_2	Me	47	93	259-260	279[281]	47.3	3.7	25.3
			($C_{11}H_{10}ClN_5O_2$)		(sinters)		(47.2)	(3.6)	(25.0)
NO_2	Cl	Me	48	83	251-253	279[281]	47.2	3.9	25.0
			($C_{11}H_{10}ClN_5O_2$)		(sinters)		(47.2)	(3.6)	(25.0)
Cl	NO_2	Et	49	95	204-205	293[295]	-	-	-
			($C_{12}H_{12}ClN_5O_2$)						
NO_2	Cl	Et	50	95	266-267	293[295]	49.1	4.1	23.9
			($C_{12}H_{12}ClN_5O_2$)		(decomp)		(49.1)	(4.1)	(23.9)

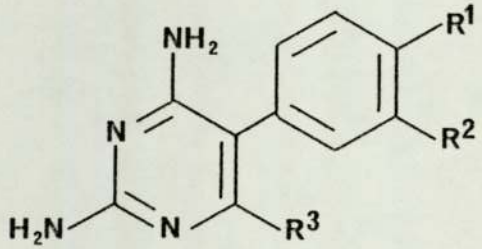
Table 15.2 continued

MeO	NO_2	Et	53[a]	92	277-278	288	53.7	5.1	24.5
			($C_{13}H_{15}N_5O_3$)		(decomp)		(54.0)	(5.2)	(24.2)
EtO	NO_2	Et	54	82	266-267	303	55.1	5.7	22.6
			($C_{14}H_{17}N_5O_3$)				(55.4)	(5.6)	(23.1)
OBu^n [b]	NO_2	Et	55a	55	260-262	331	49.0	6.3	15.9
			($C_{18}H_{27}N_5O_6S$)		(decomp)		(49.0)	(6.1)	(15.9)

[a] recrystallised from DMF; all other compounds were recrystallised from ethanol

[b] ethanesulphonate salt

Table 15.3 Analytical data for compounds in the aminopyrimidine series



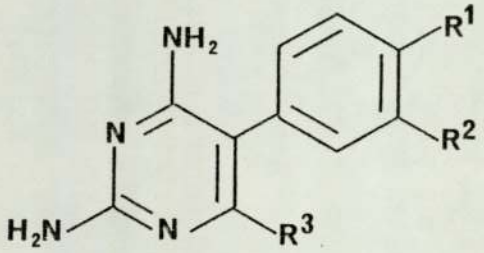
R^1	R^2	R^3	Compound (Formula)	Yield (%)	Mp (°C)	m/z (M^+)	Found (%) (Required) C	H	N
Cl	NH_2	Et	22	90	217-219	263[265]	-	-	-
			($C_{12}H_{14}ClN_5$)						
Cl	NH_2	Me	57	84	242-244	249[251]	52.5	4.9	28.0
			($C_{11}H_{12}ClN_5$)				(52.9)	(4.8)	(28.1)
NH_2	Cl	Me	58	81	205-206	249[251]	52.8	4.8	28.3
			($C_{11}H_{12}ClN_5$)		(sinters)		(52.9)	(4.8)	(28.1)

Table 15.3 continued

NH_2	Cl	Et	59	87	189-190	263[265]	54.3	5.2	26.2
			($C_{12}H_{14}ClN_5$)				(54.7)	(5.3)	(26.6)
OMe	NH_2	Et	60[a]	95	264-265	259	60.4	6.0	27.2
			($C_{13}H_{17}N_5O$)		(decomp)		(60.7)	(5.8)	(27.2)
OEt	NH_2	Et	61	78	176-177	273	61.2	7.1	25.5
			($C_{14}H_{19}N_5O$)				(61.5)	(7.0)	(25.6)

[a] DMF used as recrystallising solvent. All other recrystallisations were conducted from aqueous ethanol.

Table 15.4 Analytical data for the azidopyrimidines



R^1	R^2	R^3	Compound[a] (Formula)	Yield (%)	mp[b] (°C)	m/z (M^+)	Found (%) (Required) C	H	N
Cl	N_3	Et	21 ($C_{12}H_{12}ClN_7$)	94	197-198	289[291]	-	-	-
Cl	N_3	Me	62 ($C_{11}H_{10}ClN_7$)	92	198-200	275[277]	47.9 (47.9)	3.6 (3.6)	35.4 (35.6)
N_3	Cl	Me	63 ($C_{11}H_{10}ClN_7$)	90	160-162	275[277]	47.4 (47.9)	3.5 (3.6)	35.4 (35.6)

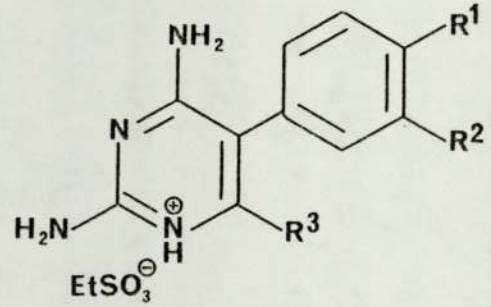
Table 15.4 continued

N_3	Cl	Et	64	88	186-187	289[291]	49.7	4.1	33.7
			($C_{12}H_{12}ClN_7$)				(49.7)	(4.2)	(33.9)
MeO	N_3	Et	65	91	184-185	285	54.6	5.3	34.7
			($C_{13}H_{15}N_7O$)				(54.7)	(5.3)	(34.4)
EtO	N_3	Et	66	90	182-183	299	56.3	5.7	32.4
			($C_{14}H_{17}N_7O$)				(56.2)	(5.7)	(32.8)

[a] all compounds recrystallised from aqueous ethanol

[b] decomposes

Table 15.5 Analytical data for the azidopyrimidine ethanesulphonate salts



R^1	R^2	R^3	Compound[b] (Formula)	Yield (%)	Mp[a] (°C)	m/z (M^+)	Found (%) (Required) C	H	N
Cl	N_3	Et	21a	75	191-192	289[291]	41.9	4.6	24.5
			($C_{14}H_{18}ClN_7O_3S$)				(42.1)	(4.5)	(24.6)
Cl	N_3	Me	62a	52	202-203	275[277]	40.4	4.1	25.6
			($C_{13}H_{16}ClN_7O_3S$)				(40.5)	(4.2)	(25.4)
N_3	Cl	Me	63a	64	184-185	275[277]	40.0	4.1	25.0
			($C_{13}H_{16}ClN_7O_3S$)				(40.5)	(4.2)	(25.4)

Table 15.5 continued

N_3	Cl	Et	64a	63	196-197	289[291]	42.4	4.4	24.3
			($C_{14}H_{18}ClN_7O_3S$)				(42.0)	(4.5)	(24.5)
MeO	N_3	Et	65a	43	272-274	285	45.6	4.9	24.5
			($C_{15}H_{21}N_7O_4S$)				(45.6)	(5.3)	(24.8)
EtO	N_3	Et	66a	38	173-174	299	47.4	5.6	23.7
			($C_{16}H_{23}N_7O_4S$)				(46.9)	(5.6)	(24.0)

[a] decomposes

[b] all compounds recrystallised from water

CHAPTER 16

SYNTHESIS OF PUTATIVE METABOLITES AND DEGRADATION PRODUCTS OF M-AZIDOPYRIMETHAMINE

16.1 Synthesis of 2,4-diaminopyrimidine N-oxides (table 16.1)

N-Oxidation of 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (49)

Hydrogen peroxide solution (30% v/v; 2.8 g) was added dropwise over 1 h to a stirred solution of nitropyrimethamine (49) (1.0 g) in formic acid (90%; 20 ml) and the mixture was stirred for 72 h at room temperature. Following the addition of water (50 ml), the pale yellow solution was basified with concentrated aqueous ammonia and the cream solid collected. Recrystallisation from ethanol gave orange needles of 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine 3-oxide (75).

In addition the crude product was subjected to chromatography on silica gel with chloroform-methanol (4:1) as eluant to give the pyrimidine N-3-oxide (75) and a meagre yield of the isomeric 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine 1-oxide (74).

Oxidation of 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine (22)

A solution of the aminopyrimethamine (22) (1.0 g) in methanol (100 ml) was stirred at room temperature and hydrogen peroxide solution (30% v/v; 50 g) was added over 30 min. Sodium tungstate (0.5 g) was added and the mixture was stirred for 12 h, diluted with water (100 ml) and extracted with chloroform (3 x 50 ml). The

chloroform layer was dried over sodium sulphate and evaporated under reduced pressure to afford an intensely yellow crystalline solid. Chromatography on silica gel with chloroform-methanol (9:1) as eluant gave 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (49) (0.27 g, 24%) and the corresponding pyrimidine 3-oxide (75) (0.24 g, 20%) as the only products.

A pure sample of the pyrimidine 3-oxide was also obtained following recrystallisation of the crude reaction product from ethanol.

2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine 3-oxide (76)

Tin (II) chloride dihydrate (3.3 g) was added to a solution of the nitropyrimethamine N-3-oxide (75) (1.1 g) in ethanol (40 ml) and the mixture was refluxed for 1 h, concentrated to a quarter volume and basified to pH 12 with 10M-sodium hydroxide solution. The cream precipitate was collected, washed with water and recrystallised from ethanol to give the aminopyrimethamine 3-oxide in moderate yield, after removal of a persistent colloidal material by filtration through a pad of Kieselguhr.

2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine 3-oxide (78)

A solution of the amine 3-oxide (76) (2 g) in 5M-hydrochloric acid (50 ml) was diazotised at 0° by the addition of sodium nitrite (0.54 g) in water (2 ml) over 20 min with agitation. Sodium azide (1.4 g) was added in portions over 15 min and the mixture was stirred for a further 1 h at 10°, diluted with water (50 ml) and basified to pH 9 with concentrated aqueous ammonia. The cream product was collected and recrystallised from aqueous ethanol to furnish photosensitive microprisms of the azidopyrimidine 3-oxide

Oxidation of 2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine (21)

A solution of m-azidopyrimethamine (21) (2 g) in formic acid (90%, 40 ml) was oxidised by the addition of hydrogen peroxide (30% v/v; 6 g) in portions over 30 min. The yellow solution was protected from light and stirred for a further 12 h at room temperature. After dilution with water (50 ml) the mixture was basified with concentrated aqueous ammonia and the precipitate collected. Chromatography on silica gel with chloroform-methanol (9:1) as eluant furnished m-azidopyrimethamine 3-oxide (78) and the isomeric azidopyrimidine 1-oxide (77) in moderate yield. Subsequent crystallisation from aqueous ethanol gave analytical samples of each compound.

16.2 Synthesis of 2,4-diaminopyrimidine derivatives

The synthesis of nitropyrimethamine (49) and aminopyrimethamine (22) has been described previously. (chapter 15).

2,4-Diamino-5-(3-acetylamino-4-chlorophenyl)-6-ethylpyrimidine (90)

Acetyl chloride (1.8 g) was added dropwise over 15 min to a solution of aminopyrimethamine (22) (5.0 g) in dry pyridine (50 ml) and the mixture was stirred at room temperature for 3 h. Water (100 ml) was added and, after stirring for a further 30 min, the solvent was evaporated to leave a yellow syrup that crystallised on trituration with ethanol. Recrystallisation from methanol afforded colourless microprisms of the acetylamino derivative m.p. 263-265° (lit[93], m.p. 257-259° from ethanol) (Found: M^+, 305 [307].

$C_{14}H_{15}ClN_5O$ requires M, 305 [307]); ν_{max} 3450, 3330 and 3180 br (NH), 2950, 1670, 1640, 1618, 1578, 1450, 1280 cm^{-1}.

Attempted synthesis of 2,4-diamino-5-(4-chloro-3-hydroxylamino-phenyl)-6-ethylpyrimidine (79A).

Raney nickel (c.a. 0.2 g) was added to a suspension of 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (49) (1.0 g) in ethanol-dichloroethane (1:1, 20 ml) and the mixture was stirred at 0°. Hydrazine hydrate (0.4 ml) was added dropwise over 20 min, whereupon a yellow to grey colour transition occurred and subsequent t.l.c. examination after 1 h showed an absence of starting material. The white solid was collected, washed with water and recrystallised from ethanol to afford cream needles of a compound identical (m.p., i.r. and t.l.c.) with 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine (22).

Attempted nitration of 2,4-diamino-5-(4-chlorophenyl)-6-(1-hydroxyethyl)pyrimidine (94)

The following compounds were synthesised by a literature method[160]:

2,4-diamino-5-(4-chlorophenyl)-6-pyrimidinyl methyl ketone diethyl acetal (92) m.p. 253-254° (lit[160], m.p. 250-255°)

2,4-diamino-5-(4-chlorophenyl)-6-pyrimidinyl methyl ketone (93) m.p. 203-205° (lit[160], m.p. 206-208°)

2,4-diamino-5-(4-chlorophenyl)-6-(1-hydroxyethyl)pyrimidine (94) m.p. 187-189 (lit[160], m.p. 187-188°).

(i) The pyrimidinyl methyl ketone (93) (0.2 g) was dissolved in sulphuric acid (5 ml) and the pale yellow solution cooled to 0°. Nitric acid (D. 1.42; 75 mg) was added and after stirring for 12 h at 5° the orange mixture was poured onto ice (10 g) and basified to pH 9 with concentrated aqueous ammonia, whereupon a dark tarry material deposited. T.l.c. examination of the product showed an absence of starting material and the formation of three new components. However, all subsequent attempts at purification failed and the mixture was discarded.

(ii) A solution of the ketone (93) (0.2 g) in trifluoroacetic acid (5 ml) containing trifluoroacetic anhydride (0.5 ml) was cooled to -5° and sodium nitrate (65 mg) was added. The mixture was stirred for 12 h at 10°, poured onto ice (10 g) and neutralised with saturated sodium bicarbonate solution. The yellow solid was collected and found to consist predominantly of starting material as detected by t.l.c.

(iii) A solution of the ∝-hydroxypyrimethamine (94) (0.2 g) in concentrated sulphuric acid (5 ml) was nitrated as for (i) above. The green solid deposited on basification again proved impossible to purify or characterise.

3. Syntheses in the aminopyrimidinone series

4-Amino-5-(4-chlorophenyl)-6-ethylpyrimidin-2(1H)-one (83)

To a stirred solution of α-propionyl-4-chlorophenylacetonitrile (18 g) in ether (200 ml) was added diazomethane (8 g), as a solution in ether (500 ml) in portions over 10 min. The mixture frothed vigorously and was stirred overnight at 10°. Excess diazomethane was destroyed by the dropwise addition of acetic acid and the ether was evaporated to give the methoxyacrylonitrile as a yellow syrup (21 g). The methoxyacrylonitrile was dissolved in ethanol (50 ml) and added to a stirred solution of urea (5.1 g) in ethanolic sodium ethoxide, prepared from sodium (4 g) and ethanol (100 ml). After refluxing the mixture for 5 h excess ethanol was vacuum evaporated to leave an orange syrup which solidified on cooling. The product was triturated with water and collected. Repeated crystallisation from 2M-hydrochloric acid furnished the hydrochloride salt (83a) as colourless prisms, m.p. 310° (decomp.) (Found: C, 50.3; H, 4.5; N, 14.6. $C_{12}H_{13}Cl_2N_3O$ requires C, 50.4; H, 4.6; N, 14.5%); ν_{max} 3350 and 3160 (NH), 2950-2600 br (bonded NH), 1715 cm^{-1} (C=O); λ_{max} (2M-hydrochloric acid) 219 and 286 nm.

The free base of (83a) was prepared from the hydrochloride salt and aqueous ammonia and recrystallised from ethanol as colourless leaves, m.p. 295-310° (decomp.) (Found: M^+, 249 [251]. $C_{12}H_{12}ClN_3O$ requires M, 249 [251]); ν_{max} 3500, 3350 and 3170 br (NH), 3000-2750 br (bonded NH), 1640 br cm^{-1} (C=O); λ_{max} (EtOH) 213, 222 infl., 242 infl., 278 nm.

Hydrolysis of 2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidine (4)

A solution of the pyrimidine (4) (10 g) in 6M-hydrochloric acid (400 ml) was refluxed for 18 h, cooled and allowed to stand at 4° for 6 days. The product (9.4 g), a mixture of colourless prisms and flakes was collected and fractionally crystallised from 2M-hydrochloric acid (300 ml) to afford, after 16 h at 25°, colourless flakes (3 g) of the hydrochloride salt of 2-amino-5-(4-chlorophenyl)-6-ethylpyrimidin-4(3H)-one (84a).

Repeated crystallisation from 2M-hydrochloric acid furnished the analytical sample, m.p. 290-305° (decomp.) (Found: C, 50.1; H, 4.4; N, 14.5. $C_{12}H_{13}Cl_2N_3O$ requires C, 50.35; H, 4.55; N, 14.7%); λ_{max} 3400, 3270 and 3170 (NH), 3000-2750 (bonded NH), 1705 and 1685 cm^{-1} (C=O); λ_{max} (2M-hydrochloric acid) 233 and 265 nm.

The free base of (84a) prepared from the hydrochloride salt and aqueous ammonia, crystallised from ethanol as colourless flakes, m.p. 285-295° (sinters 265°) (Found: M^+, 249 [251]. $C_{12}H_{12}ClN_3O$ requires M, 249 [251]); λ_{max} 3450-3300 br and 3170 br (bonded NH), 1660 cm^{-1} (C=O); λ_{max} (EtOH) 215, 250 infl., 296 nm.

The 2M-hydrochloric acid mother liquors remaining after removal of (84a) were chilled at 4° overnight, whereupon colourless prisms deposited (4.75 g) and were collected. Recrystallisation from 2M-hydrochloric acid furnished a sample identical (m.p., i.r. and n.m.r.) with the authentic sample prepared above of the hydrochloride salt of 4-amino-5-(4-chlorophenyl)-6-ethylpyrimidin-2(1H)-one (83a).

2-Amino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidin-4(3H)-one (89)

2-Amino-5-(4-chlorophenyl)-6-ethylpyrimidin-4(3H)-one (84) (0.4 g) was stirred at 0° as a solution in concentrated sulphuric acid (10 ml), and nitric acid (D. 1.42; 0.9 g) was added dropwise

over 30 min. The mixture was stirred for a further 12 h at 25°, poured onto ice and basified to pH 7.5 with dilute aqueous ammonia. The precipitated nitrophenylpyrimidine was collected and recrystallised from ethanol-acetone to afford cream microrosettes in poor yield (0.1 g, 21%), m.p. 261-263° (Found: M^+, 294 [296]. $C_{12}H_{11}ClN_4O_3$ requires M, 294 [296]); λ_{max} 3470, 3350, 3120 br (NH), 3000-2750 br (bonded NH), 1670 (C=O), 1630 (C=N), 1535 and 1340 cm^{-1} (NO_2); λ_{max} (EtOH) 215, 253 and 297 nm; λ_{max} (2M-hydrochloric acid) 231 and 265 nm.

4-Amino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidin-2(1H)-one (88)

Nitration of 4-amino-5-(4-chlorophenyl)-6-ethylpyrimidin-2-(1H)-one (83) with a nitric acid-sulphuric acid mixture in the manner described above gave the nitrophenylpyrimidinone (95%), which was purified as follows:

10M-hydrochloric acid (0.4 g) was added to a suspension of (88) (1.0 g) in water (20 ml) whereupon all solids dissolved. After evaporation of water under reduced pressure, the creamy residue was recrystallised from acetone-water, and subsequently reprecipitated from water as the free base with 2M-sodium hydroxide solution. The cream product crystallised from ethanol as flakes, m.p. 320° (decomp.) (Found: M^+, 294 [296]. $C_{12}H_{11}ClN_4O_3$ requires M, 294 [296]): λ_{max} 3450, 3100 br (NH), 1640 (C=O and C=N), 1540 and 1355 cm^{-1} (NO_2); λ_{max} (EtOH) 217, 250 infl., 280 infl., nm; λ_{max} (2M-hydrochloric acid) 218, 284 nm.

Attempted synthesis of 2-amino-5-(4-chlorophenyl)-6-ethylpyrimidin-4(3H)-one (84)

(i) An intimate mixture of ethyl-4-chlorophenylacetate (10 g) and ethyl formate (5.4 g) was added in portions over 15 min to a suspension of sodium (1.4 g) in dry ether (100 ml) and the mixture was kept for 18 h at 25°. Excess ether was evaporated to leave an orange syrup (11.7 g) which was heated for 5 h with guanidine (5.8 g, as the hydrochloride salt) in ethanolic sodium ethoxide prepared from sodium (1.4 g) and ethanol (150 ml). The pale yellow solution was cooled, poured into water (500 ml) and acidified with acetic acid. However, no precipitation of product occurred after standing the mixture for several days at 4°. Subsequent examination of the reaction mixture by t.l.c. showed the absence of starting material, but no product formation was evident.

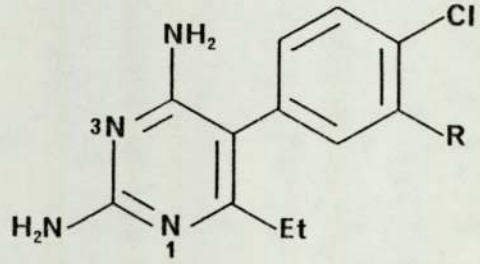
(ii) To a solution of guanidine hydrochloride (11.8 g) in ethanol (300 ml) at 0° was added 4-chlorophenylacetonitrile (30 g) and propanal (11.6 g). Sodium hydroxide (8 g) in water (20 ml) was added and the mixture stirred for 1 h at 0°, 2 h at 30° and finally refluxed for 3 h. The red mixture was cooled, poured into water (700 ml) and the cream product was collected and recrystallised twice from ethanol to give 2-amino-5-(4-chlorophenyl)-5:6-dihydro-6-ethyl-4-hydroxypyrimidine (86) (8 g) as a white amorphous powder, m.p. 272-273°, (lit[156], m.p. 273°).

(a) Subsequent treatment of the dihydropyrimidine from (ii) above with sulphur as described in the literature method[156] gave only starting materials, recovered in a meagre yield.

(b) Bromine (0.8 g) was added dropwise over 30 min to a solution of (86) (1.0 g) in acetic acid (20 ml) and the solution was stirred for 2 h at 65-70°. Vacuum evaporation of the solvent left a yellow oil which solidified on trituration with water and recrystallised from ethanol to furnish colourless microprisms (0.1 g). Examination of the product by t.l.c. showed a mixture of starting material and one other unidentified component. Extending the reaction time did not increase the yield of this product and subsequent attempts at fractional crystallisation were unsuccessful.

(iii) To a solution of the dihydropyrimidine (86) (0.5 g) in acetic acid (10 ml) was added activated manganese dioxide (0.5 g) and the mixture was refluxed for 12 h when t.l.c. examination showed the formation of a less polar product and the presence of a trace of starting material. Filtration of the mixture through a pad of Kieselguhr afforded a green solution which, after dilution with water (50 ml), was adjusted to pH 7.0 with dilute aqueous ammonia. The brown solid was collected and recrystallised from ethanol to give a cream powder (20 mg) m.p. 75-80°. The product was clearly not a diaminopyrimidine (i.r. and t.l.c.) and was not examined further.

Table 16.1 Analytical data for the diaminopyrimidine N-oxides



Position of N-oxidation	R	Compound Number (Formula)	Yield (%)	mp (°C)	m/z (M^+)	Found (%) (Required) C	H	N
1	NO_2	74	9	264-266	309[311]	46.7	3.9	22.2
		($C_{12}H_{12}ClN_5O_3$)				(46.5)	(3.9)	(22.6)
3	NO_2	75	57(20)[a]	250-252	309[311]	46.8	3.7	22.5
		($C_{12}H_{12}ClN_5O_3$)				(46.5)	(3.9)	(22.6)

Table 16.1 continued

3	NH_2	76	62	288-290	279[281]	51.6	5.3	24.8
		($C_{12}H_{14}ClN_5O$)		(decomp.)		(51.5)	(5.0)	(25.0)
1	N_3	77	11	210-212	305[307]	47.4	4.2	32.3
		($C_{12}H_{12}ClN_7O$)		(explosive decomp.)		(47.1)	(3.9)	(32.1)
3	N_3	78	24(72)[b]	189-190	305[307]	47.7	4.1	32.2
		($C_{12}H_{12}ClN_7O$)		(decomp.)		(47.1)	(3.9)	(32.1)

[a] prepared *via* oxidation of *m*-aminopyrimethamine

[b] prepared from the corresponding *m*-aminopyrimethamine3-oxide (76)

CHAPTER 17

DIAMINOPYRIMIDINES AS PROBES FOR A FOLATE-INDEPENDENT TARGET; REACTION OF NITROPYRIMETHAMINE AND ISO-NITROPYRIMETHAMINE WITH AMINES

2,4-diamino-5-(4-dimethylamino-3-nitrophenyl)-6-ethylpyrimidine (96)

Method A

A suspension of nitropyrimethamine (49) (10 g) in aqueous dimethylamine solution (40%; 200 ml) was refluxed for 48 h with the further addition of dimethylamine solution (100 ml) after 24 h and 36 h. An orange colour slowly developed and the consumption of starting material was monitored by t.l.c. After cooling and dilution with water, the crystalline product was collected and recrystallised from aqueous DMF to yield orange prisms of the dimethylaminopyrimidine.

Treatment of nitropyrimethamine (49) with methylamine (40% aqueous solution) and ethylamine (70% aqueous solution) in an identical manner furnished the corresponding products (95 and 97) in comparable yields (table 17.1). Diethylamine gave only a trace of a coloured material (t.l.c.) after refluxing with (49) for 96 h, and the use of DMSO as a solvent was without effect.

Method B

To a solution of nitropyrimethamine (49) (2 g) in DMF (10 ml) at 94° (bath temperature) was added 2-aminoethanol (0.84 g) dropwise over 5 min, and the mixture was stirred overnight at the same temperature. The deep red mixture was cooled, diluted with water (50 ml) and allowed to stand at 4° overnight, when a red crystal mass

deposited and was collected and recrystallised from aqueous DMF to give a product identical (mixed m.p., i.r. and n.m.r.) with (96) synthesised by method A above.

Analogous reactions utilising formamide or diethylformamide as solvent failed to give the appropriate products and in both cases only starting materials were obtained. N-methylformamide furnished a mixture of the methylaminopyrimidine (95) and starting material in approximately equal proportions (t.l.c.) regardless of the reaction conditions employed.

2,4-Diamino-5-(4-n-butylamino-3-nitrophenyl)-6-ethylpyrimidine (98)

A suspension of the nitropyrimidine (49) (10 g) in n-butylamine (30 ml) was refluxed for 4 h. The deep red liquor was cooled, diluted with water and stood for 12 h, whereupon red crystals deposited and were collected. Recrystallisation from aqueous 2-ethoxyethanol gave crimson plates of the n-butylaminonitropyrimidine (table 17.1).

Treatment of 2,4-diamino-5-(3-chloro-4-nitrophenyl)-6-ethylpyrimidine (50) with n-butylamine in an identical manner gave the isomeric 2,4-diamino-5-(3-butylamino-4-nitrophenyl)-6-ethylpyrimidine (114) in comparable yield.

The ethanesulphonate salts of (98) and (114) were prepared by dissolving the corresponding amine in boiling water containing 1.1 mole equivalents of ethanesulphonic acid, from which orange plates of the appropriate amine monoethanesulphonate deposited on cooling (98a and 114a).

Attempted synthesis of 2,4-diamino-5-(4-sec-butylamino-3-nitrophenyl)-6-ethylpyrimidine

A suspension of 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (49) (1.0 g) in sec-butylamine (30 ml) was boiled for 12 h, cooled and the dark mixture was diluted with water and kept for 12 h at 4°. The red precipitate was collected and recrystallised from ethanol to give a red amorphous powder. T.l.c. examination showed a mixture of starting material and an unidentified second component, in proportions that remained unchanged after extended refluxing in an excess of sec-butylamine.

2,4-Diamino-6-ethyl-5-(3-nitro-4-[piperidin-1-yl]phenyl)pyrimidine (99)

A mixture of nitropyrimethamine (49) (5 g) and piperidine (20 ml) was boiled for 5 h, cooled and diluted with water (100 ml). The nitropiperidinopyrimidine crystallised from aqueous DMF as red needles of the trisdimethylformamide solvate.

Identical reactions conducted with (49) and the appropriate amine gave the corresponding products in excellent yield (table 17.1).

2,4-Diamino-6-ethyl-5-(3-nitro-4-[piperazin-1-yl]phenyl)pyrimidine (100)

Nitropyrimethamine (49) (5 g) was added in portions over 30 min to anhydrous piperazine (15 g) at 125° with vigorous agitation and the mixture was refluxed for 2 h. The viscous red mixture was diluted with 2-ethoxyethanol (50 ml) and allowed to cool. Subsequent trituration with water liberated the nitropiperazinopyrimidine which crystallised from aqueous DMF as red needles (table 17.1).

2,4-Diamino-5-(4-benzylamino-3-nitrophenyl)-6-ethylpyrimidine (106)

A solution of the nitropyrimidine (49) (2 g) in benzylamine (20 ml) was boiled for 4 h, cooled and poured into ether (50 ml). The precipitate was washed with ether, and then water and subsequently recrystallised from 2-ethoxyethanol to give red microprisms of the benzylaminonitropyrimidine.

Those amines immiscible with water were reacted with nitropyrimethamine as above and the corresponding products were isolated in an identical manner (table 17.1).

Treatment of the iso-nitropyrimethamine (50) with benzylamine and N-methylbenzylamine under identical reaction conditions afforded the respective 3'-amine substituted derivatives (115 and 116) as the only products, as monitored by t.l.c. and these were precipitated from the reaction mixtures by the aforementioned method.

Attempted synthesis of 3-benzyl-6-(2,4-diamino-6-ethylpyrimidin-5-yl)-2-phenylbenzimidazole 1-oxide (117)

A suspension of the dibenzylaminopyrimidine (110) (0.2 g) in dibenzylamine (10 ml) was boiled for 12 h when t.l.c. examination of the red mixture showed the formation of one predominant component and three minor products. The yellow precipitate deposited after cooling the mixture and adding ether (10 ml) was collected (40 mg), but resisted all further attempts at purification. However, preliminary spectroscopic examination (i.r. and m.s.) indicated formation of the benzimidazole N-oxide (Found: M^+, 436. $C_{26}H_{24}N_6O$ requires M, 436).

Attempted synthesis of 2,4-diamino-6-ethyl-5-(4-N-methylanilino-3-nitrophenyl)pyrimidine (118)

A suspension of 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (49) (2.0 g) in N-methylaniline (10 ml) was refluxed for 12 h, cooled and the intensely red syrup poured into ethyl acetate (200 ml). The red precipitate (0.8 g) was collected and washed thoroughly with ether. Examination of the product by t.l.c. showed the absence of starting material but the presence of five components, one as a major product. Chromatographic separation on silica gel with chloroform-methanol (9:1) as eluant, afforded an orange tar (0.2 g) that crystallised on trituration with ether, m.p. 83-84° (Found: M^+, 364. $C_{19}H_{20}N_6O_2$ requires M, 364); ν_{max} 3450, 3290 and 3100 br (NH), 1530 and 1340 cm^{-1} (NO_2). Subsequent examination by t.l.c. revealed the presence of a major product and a persistent contaminant and all further attempts at purification failed.

2,4-Diamino-5-(4[2-aminoethylamino]-3-nitrophenyl)-6-ethylpyrimidine (112)

Ethylenediamine (20 ml) was added to nitropyrimethamine (49) (2 g), whereupon the solid rapidly dissolved to form a red solution. After boiling for 4 h the syrupy mixture was cooled and water (50 ml) was added with the evolution of heat. The mixture was stirred for a further 1 h and allowed to stand overnight at 4°. Recrystallisation of the precipitate from aqueous DMF afforded bright red microprisms of the aminoethylaminonitropyrimidine (table 17.1)

2,4-diamino-5-(4[3-aminopropylamino]-3-nitrophenyl)-6-ethylpyrimidine (113)

Nitropyrimethamine (49) (5 g) was treated with diaminopropane (40 ml) as above and a sample of the product crystallised from a large volume of DMF over 5 days as orange microprisms of the aminopropylaminonitropyrimidine.

Nitropyrimethamine (49) reacted with the homologous diamines, diaminobutane, diaminohexane and diaminodocecane to form orange highly polar mixtures of at least three products in all cases (t.l.c.) and attempts at optimising the reactions by varying the reaction conditions or including a solvent were abortive.

1-Hydroxy-6-(2,4-diamino-6-ethylpyrimidin-5-yl)benzotriazole (120)

Nitropyrimethamine (49) (2 g) was added to a stirred solution of hydrazine hydrate (5 ml) in ethanol (25 ml) and the suspension was refluxed for 4 h, whereupon the solid dissolved to form a yellow solution. On cooling a creamy precipitate developed that redissolved on dilution with water (50 ml). The alkaline solution was adjusted to pH 7 by the addition of dilute hydrochloric acid and the cream product was collected. All attempts at recrystallising this material proved unsuccessful.

The hydroxybenzotriazole (0.5 g) was added to a solution of methanesulphonic acid (0.2 g) in ethanol (10 ml) and the mixture was gently boiled for 15 min. The amorphous cream solid formed on cooling was collected and recrystallised from ethanol-ethyl acetate to furnish pale pink microcrystals of the monomethanesulphonate (120a), m.p. 240-241° (decomp.) (Found: C, 42.8; H, 4.70; N, 26.8; M^+, 271. $C_{13}H_{17}N_7O_4S$ requires C, 42.5; H, 4.7; N, 26.7%; M, 271); $\nu_{max.}$ 3480, 3400 and 3250 cm^{-1} (NH); δ 1.03 (3H,t,CH_3), 2.22

(2H,q,CH_2), 2.43 (3H,s,$\underline{CH_3}SO_3^-$), 6.95 (1H,dd,6'-H_1), 7.67 (2H,NH), 7.70 (1H,d,2'-H_1), 8.10 (1H,d,5'-H_1), 8.16(1H,NH), 12.30 (1H, brs, NH^+?), 13.0-14.0 (1H,brs,OH?), ([$^2H_6$]DMSO).

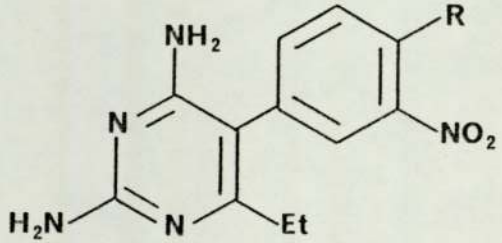
Treatment of the methoxynitropyrimidine (53) and the nitropiperidinopyrimidine (99), prepared previously, with hydrazine hydrate under identical reaction conditions gave products which, after conversion to the methanesulphonate salts, were characterised as identical (m.p., n.m.r., i.r. and m.s.) to the hydroxybenzotriazole (120) synthesised above.

2-Phenyl-6-(2,4-diamino-6-ethylpyrimidin-5-yl)-benzotriazole 1-oxide (122)

A suspension of the nitropyrimidine (49) (5 g) in phenylhydrazine (20 ml) was refluxed for 4 h when t.l.c. examination confirmed an absence of starting material. The red mixture was cooled, poured into ethyl acetate and the precipitate collected. Repeated washing with ethyl acetate left a creamy solid which recrystallised from ethanol in meagre yield as a sandy amorphous powder.

The monomethanesulphonate salt of (122) was prepared by dissolving the base in a hot ethanolic solution of methanesulphonic acid (1.1 mole equivalents) from which colourless prisms deposited after allowing the solution to stand overnight, m.p. 253-254° (Found: C, 51.3; H, 4.9; N, 22.6; M^+, 347. $C_{19}H_{21}N_7O_4S$ requires C, 51.5; H, 4.7; N, 22.1%; M, 347); ν_{max} 3470, 3420 and 3190 cm^{-1} (NH); δ 1.06 (3H,t,CH_3), 2.25 (2H,q,CH_2), 2.40 (3H,s,$\underline{CH_3}SO_3^-$), 5.54 (2H,NH), 6.41 (1H,dd,6'-H_1), 6.63 (1H,d,2'-H_1), 7.29 (1H,d,5'-H_1), 7.52 (2H, NH), 12.11 (1H, NH^+), ([$^2H_6$]DMSO).

Table 17.1 Analytical data for the amine-substituted nitropyrimidines



R	Compound (Formula)	Solvent[b]	Yield (%)	Mp (°C)	M/z (M^+)	Found (%) (Required) C	H	N
methylamino	95	aq. DMF	92	262-265	288	53.9	5.9	29.3
	($C_{13}H_{16}N_6O_2$)					(54.2)	(5.6)	(29.2)
dimethylamino	96	aq. DMF	68(87)[c]	256-257	302	55.6	5.9	27.4
	($C_{14}H_{18}N_6O_2$)					(55.6)	(5.9)	(27.8)

Table 17.1 continued

ethylamino	97	aq. DMF	87	275-276	302	55.6	5.9	28.0
	($C_{14}H_{18}N_6O_2$)					(55.6)	(6.0)	(27.8)
n-butylamino	98	aq. ethoxyethanol	95	252-254	330	58.2	6.8	25.5
	($C_{16}H_{22}N_6O_2$)					(58.2)	(6.7)	(25.5)
n-butylamino[a]	98a	water	75	250-252	330	49.3	6.5	19.3
	($C_{18}H_{28}N_6O_5S$)			(sinters)		(49.1)	(6.4)	(19.1)
piperidin-1-yl	99	DMF	90	243-245	342	59.8	6.7	25.0
	($C_{17}H_{22}N_6O_2$)					(59.7)	(6.4)	(24.6)
piperazin-1-yl	100	aq. ethoxyethanol	86	260-261	343	55.7	6.4	28.3
	($C_{16}H_{21}N_7O_2$)					(56.0)	(6.1)	(28.6)

Table 17.1 continued

4-methyl-piperazin-1-yl	101 ($C_{17}H_{23}N_7O_2$)	EtOH	93	233-234	357	57.2 (57.1)	6.4 (6.4)	27.3 (27.5)
pyrrolidin-1-yl	102 ($C_{16}H_{20}N_6O_2$)	DMF	89	233-234	328	58.4 (58.5)	6.3 (6.1)	25.5 (25.6)
4-morpholino	103 ($C_{16}H_{20}N_6O_3$)	ethoxyethanol	97	246-247	344	55.7 (55.8)	5.8 (5.8)	24.4 (24.4)
cyclohexylamino	104 ($C_{18}H_{24}N_6O_2$)	ethoxyethanol	91	252-253	356	60.7 (60.7)	7.2 (6.7)	24.0 (23.6)
N-methylcyclo-hexylamino	105 ($C_{19}H_{26}N_6O_2$)	aq. EtOH	67	256-260	370	61.6 (61.5)	7.4 (7.3)	22.3 (22.6)

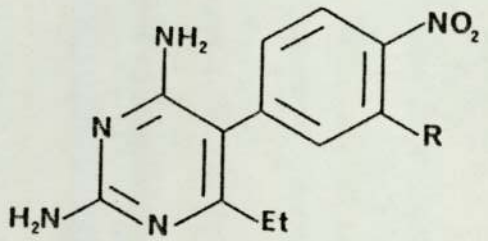
Table 17.1 continued

benzylamino	106	ethoxyethanol	73	253-255	364	63.0	5.8	23.3
	($C_{19}H_{20}N_6O_2$)					(62.6)	(5.5)	(23.1)
N-methylbenzyl-	107	aq. ethoxyethanol	91	210-211	378	63.5	6.1	22.3
amino	($C_{20}H_{22}N_6O_2$)					(63.5)	(5.8)	(22.2)
N-ethylbenzyl-	108	aq. ethoxyethanol	86	214-216	392	64.2	6.2	21.1
amino	($C_{21}H_{24}N_6O_2$)					(64.3)	(6.1)	(21.4)
(±)∝-methyl-	109	AcOEt	85	208-210	378	63.5	5.9	22.1
benzylamino	($C_{20}H_{22}N_6O_2$)					(63.5)	(5.8)	(22.2)
dibenzylamino	110	aq. ethoxyethanol	49	197-199	454	68.6	5.6	18.3
	($C_{26}H_{26}N_6O_2$)					(68.7)	(5.7)	(18.5)

Table 17.1 continued

phenethylamino	111	aq. ethoxyethanol	70	222-225	378	63.7	6.2	22.4
	($C_{20}H_{22}N_6O_2$)			(sinters)		(63.5)	(5.8)	(22.2)
2-aminoethyl-	112	aq. DMF	83	240-241	317	53.4	6.1	31.4
amino	($C_{14}H_{19}N_7O_2$)					(53.0)	(6.0)	(30.9)
3-aminopropyl-	113	DMF	62	238-240	331	54.1	6.7	30.0
amino	($C_{15}H_{21}N_7O_2$)					(54.4)	(6.3)	(29.6)

Table 17.1 continued



R	Compound (Formula)	Solvent	Yield (%)	Mp (°C)	M/z (M^+)	Found (%) (Required) C	H	N
n-butylamino	114	ethoxyethanol	45	271-273	330	58.7	6.8	25.7
	($C_{16}H_{22}N_6O_2$)					(58.2)	(6.7)	(25.5)
n-butylamino[a]	114a	water	56	270	330	49.5	6.3	19.4
	($C_{18}H_{28}N_6O_5S$)			(sinters)		(49.1)	(6.4)	(19.1)

Table 17.1 continued

benzylamino	115	aq. ethoxyethanol	45	247-248	364	62.9	5.5	22.8
	($C_{19}H_{20}N_6O_2$)					(62.6)	(5.5)	(23.1)
N-methylbenzyl-	116	aq. ethoxyethanol	31	189-190	378	63.2	5.8	21.9
amino	($C_{20}H_{22}N_6O_2$)					(63.5)	(5.8)	(22.2)

a ethanesulphonate salt

b crystallising solvent

c yields from methods A and B respectively

CHAPTER 18

FURTHER REACTIONS IN THE NITROPYRIMETHAMINE SERIES

2,4-Diamino-5-(3-amino-4[4-methylpiperazin-1-yl]phenyl)-6-ethyl-pyrimidine (129)

A suspension of the N-methylpiperazinonitropyrimidine (101) (9 g) in ethanol (200 ml) containing Raney nickel (c.a. 3 g) was stirred at 60-65° and a solution of hydrazine hydrate (24 ml) in ethanol (24 ml) was added dropwise over 2 h, at such a rate that the temperature did not exceed 70°. The mixture was maintained at 60-65° for a further 1 h, filtered hot through a Kieselghur pad and the solvent was evaporated under reduced pressure to leave a grey solid. A sample recrystallised from aqueous ethanol as cream flakes of the aminophenylpyrimidine. Similar reductions conducted on a series of the nitropyrimidine derivatives (99, 100, 104 and 106) gave the corresponding amines in moderate yield (table 18.1). The presence of a green contaminant invariably necessitated repeated crystallisation from ethanol with consequent loss of product.

2,4-Diamino-5-(3-amino-4-[morpholin-4-yl]phenyl)-6-ethylpyrimidine (131)

Tin (II) chloride dihydrate (3.3 g) was added in small portions over 15 min to a stirred suspension of the morpholinonitropyrimidine (103) (1 g) in ethanol (20 ml) at 50°. The red solid rapidly dissolved to form a yellow solution which was refluxed for a further 3 h, allowed to cool and the solvent was evaporated to leave the stannicomplex as a yellow syrup. A solution of the complex in hot water was basified to pH 12 with 10M-sodium hydroxide solution. The

white solid was collected, washed with water and crystallised from 2-ethoxyethanol to yield colourless prisms of the anhydrous base.

Reduction of the appropriate nitro derivatives in the manner described above furnished the requisite aminopyrimidines in good yield (table 18.1).

Attempted synthesis of 2,4-diamino-5-(3-amino-4-dibenzylaminophenyl)-6-ethylpyrimidine

A fine suspension of the dibenzylaminonitropyrimidine (110) (1 g) in ethanol (50 ml) was treated with tin (II) chloride dihydrate (2.5 g) as described previously, and the microcrystalline precipitate that deposited on basification with 10M-sodium hydroxide solution was collected and dried at 25°. T.l.c. examination showed the formation of two products in proportions that remained unchanged after refluxing for 3 h in nitrobenzene and subsequent efforts to separate the respective components by fractional crystallisation were unsuccessful.

2,4-Diamino-5-(3-azido-4[4-methylpiperazin-1-yl]phenyl)-6-ethylpyrimidine (144)

A stirred solution of the amine (129) (2.5 g) in 5M-hydrochloric acid (30 ml) was diazotised at 0° by the addition of sodium nitrite (0.6 g) as a solution in water (3 ml) over 30 min. After stirring for a further 30 min, sodium azide (2 g) was added in portions over 15 min and the mixture frothed vigorously. The creamy suspension was maintained at 5° with agitation for a further 2 h and following dilution with water (200 ml) the resultant slurry was basified to pH 9 with concentrated aqueous ammonia. The precipitate was collected, washed with water and recrystallised from aqueous ethanol to yield cream rosettes of the N-methylpiperazinoazidopyrimidine.

Under identical reaction conditions amines (124, 127, 128 and 131) were smoothly converted to the appropriate azido derivatives in good yield (table 18.2).

Attempted synthesis of 2,4-diamino-5-(3-azido-4-[pyrrolidin-1-yl]phenyl)-6-ethylpyrimidine

A solution of the aminopyrrolidinopyrimidine (130) (1 g) in 5M-hydrochloric acid (10 ml) was diazotised as previously described and treated with sodium azide (0.9 g) in the same manner. However, on neutralisation of the mixture with concentrated aqueous ammonia a purple solid formed and was collected. Repeated crystallisation from ethanol afforded pale pink microcrystals which were characterised by t.l.c. as consisting of at least six products, one of which photolysed under u.v. light. Further purification of this mixture proved impracticable.

2,4-Diamino-6-ethyl-5-(4[4-methylpiperazin-1-yl]phenyl)pyrimidine (149)

A suspension of the azide (144) (4 g) in hydrazine hydrate (30 ml) was stirred at room temperature and brought to reflux over 30 min. The mixture frothed vigorously and undissolved solids were washed from the condenser walls with ethanol (20 ml total volume). When effervescence had subsided (3 h) the yellow solution was allowed to cool, diluted with water (50 ml) and kept overnight at 4°. The crystalline product was collected, washed with water and recrystallised from ethanol to afford colourless prisms of the N-methylpiperazinopyrimidine. The product developed a pink colouration on subsequent exposure to light.

Deazidation of the azide derivatives (146-148) by the

aforementioned method furnished the corresponding monosubstituted 2,4-diamino-5-phenylpyrimidines in moderate yield (table 18.3).

4-(4-[2,4-diamino-6-ethylpyrimidin-5-yl]phenyl)-1,1-dimethyl-piperazinium iodide (150)

2,4-Diamino-6-ethyl-5-(4[4-methylpiperazin-1-yl]phenyl)-pyrimidine (149) (1 g) was dissolved in dry methanol (10 ml) and stirred at 35°. Iodomethane (0.5 g) was added dropwise over 15 min, whereupon the mixture was stirred for a further 12 h, concentrated to half volume and allowed to cool. The product was collected and a sample recrystallised from aqueous ethanol as hygroscopic colourless prisms of the quaternary amine monohydrate, m.p. 278-279° (decomp.) (Found: C, 45.4; H, 6.3; N, 18.0; M^+-15, 312. $C_{18}H_{27}IN_6 . 1.0\ H_2O$ requires C, 45.8; H, 6.1; N, 17.8%; M-15, 312); ν_{max}. 3420, 3350 and 3190 br cm^{-1} (NH); δ 0.96 (3H,t,CH_3), 2.13 (2H,q,CH_2), 3.22 (4H,s,$[CH_2]_2$), 3.39 (4H,brs,$[CH_2]_2$), 3.57 (6H,s,$N^+[CH_3]_2$), 5.43 (2H,NH), 5.88 (2H,NH), 7.09 (4H,s,C_6H_4), ($[^2H_6]$DMSO).

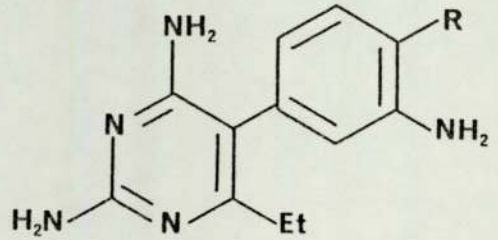
The piperazinopyrimidine (148) was subjected to exhaustive methylation with iodomethane in a similar manner, to furnish a product identical (mixed m.p., i.r., n.m.r. and m.s.) to (150) synthesised above.

4-(2,4-Diamino-6-ethylpyrimidin-5-yl)-phenyltrimethylammonium iodide (151)

A suspension of 2,4-diamino-5-(4-dimethylaminophenyl)-6-ethylpyrimidine (146) (0.75 g) in dry methanol (15 ml) was stirred at 40° and iodomethane (1.7 g) was added over 15 min. The flocculant mixture was stirred for a further 12 h when examination by t.l.c. showed an absence of starting materials. Following the evaporation

of solvent, the pink solid was recrystallised twice from aqueous methanol to yield hygroscopic colourless microprisms of the trimethylammonium iodide, m.p. 215-216° (Found: C, 42.3; H, 5.7; N, 16.7; M^+-15, 257. $C_{15}H_{22}IN_5$. 1.5 H_2O requires C, 42.3; H, 5.9; N, 16.4%, M-15, 257); $\nu_{max.}$ 3420, 3350 and 3190 br (NH), 1660 cm^{-1} (H_2O); δ 1.33 (3H,t,CH_3), 2.63(2H,q,CH_2), 3.84 (9H,s,$N^+[CH_3]_3$), 7.80(d) and (8.15(d), AA'BB', (TFA).

Table 18.1 Analytical data for the amine-substituted aminopyrimidines



R	Compound (Formula)	Solvent[a]	Yield (%)	Mp (°C)	M/z (M^+)	Found (%) (Required) C	H	N
methylamino	123 ($C_{13}H_{18}N_6$)	aq. EtOH	94	231-233	258	60.1 (60.4)	6.9 (7.0)	32.1 (32.5)
dimethylamino	124 ($C_{14}H_{20}N_6$)	aq. EtOH	89	188-189	272	61.4 (61.7)	7.8 (7.4)	31.1 (30.9)

Table 18.1 continued

ethylamino	125	EtOH	89	210-211	272	61.9	7.4	30.4
	($C_{14}H_{20}N_6$)					(61.8)	(7.4)	(30.9)
n-butylamino	126	aq. EtOH	93	191-193	300	63.8	8.1	27.9
	($C_{16}H_{24}N_6$)					(64.0)	(8.0)	(28.0)
piperidin-1-yl[c]	127	-	75	-	312	-	-	-
	($C_{17}H_{24}N_6$)							
piperazin-1-yl	128	aq. EtOH	88	210-211	313	61.0	7.7	31.1
	($C_{16}H_{23}N_7$)			(sinters)		(61.3)	(7.4)	(31.3)
4-methylpiper-	129	aq. EtOH	89	241-243	327	62.4	7.8	30.0
azin-1-yl	($C_{17}H_{25}N_7$)					(62.4)	(7.7)	(30.0)

Table 18.1 continued

pyrrolidin-1-yl	130	aq. EtOH	77	208-210	298	64.3	7.4	28.2
	($C_{16}H_{22}N_6$)					(64.4)	(7.4)	(28.2)
4-morpholino	131	aq. ethoxyethanol	77	235-237	314	60.9	7.2	27.0
	($C_{16}H_{26}N_6O$)			(sinters)		(61.1)	(7.1)	(26.7)
cyclohexylamino[c]	132	-	87	-	326	-	-	-
	($C_{18}H_{26}N_6$)							
N-methylcyclo-hexylamino[c]	133	-	87	-	340	-	-	-
	($C_{19}H_{28}N_6$)							
benzylamino	134	aq. EtOH	76	202-204	334	68.3	7.0	24.7
	($C_{19}H_{22}N_6$)					(68.3)	(6.6)	(25.1)

Table 18.1 continued

N-methylbenzyl-amino	135 ($C_{20}H_{24}N_6$)	aq. EtOH	94	206-208	348	68.9 (69.0)	6.7 (6.9)	24.4 (24.1)
N-ethylbenzyl-amino[b]	136a ($C_{25}H_{38}N_6O_6S_2$)	EtOH-EtOAc	88	211-212	362	51.1 (51.5)	6.3 (6.5)	14.3 (14.4)
(±)∝-methyl-benzylamino	137 ($C_{20}H_{24}N_6$)	aq. EtOH	87	250-253 (decomp)	348	68.9 (69.0)	6.6 (6.9)	24.1 (24.1)
phenethylamino	138 ($C_{20}H_{24}N_6$)	aq. EtOH	87	175-177	348	68.9 (69.0)	7.2 (6.9)	24.2 (24.1)
2-aminoethyl-amino	139 ($C_{14}H_{21}N_7$)	aq. EtOH	62	241-243	287	58.3 (58.5)	7.5 (7.3)	34.3 (34.2)

Table 18.1 continued

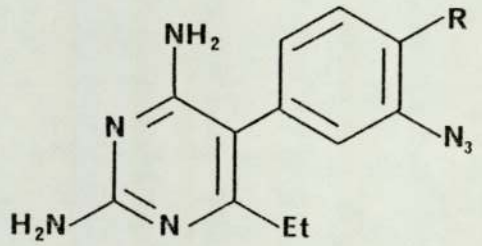
3-aminopropyl-	140	aq. EtOH	69	195-197	301	59.9	8.0	32.3
amino	($C_{15}H_{23}N_7$)					(59.8)	(7.6)	(32.6)

[a] recrystallising solvent

[b] diethanesulphonate salt

[c] not analysed since impure and degraded on all attempts at crystallisation

Table 18.2 Analytical data for the amine-substituted azidopyrimidines



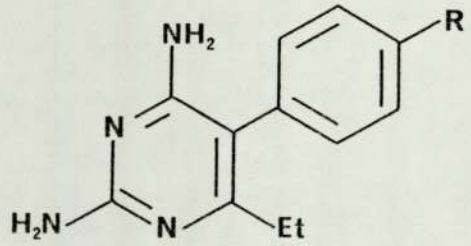
R	Compound[a] (Formula)	Yield (%)	Mp (°C)	M/z (M^+)	Found (%) (Required) C	H	N
dimethylamino	141	91	158-159	298	56.4	5.9	37.8
	($C_{14}H_{18}N_8$)				(56.4)	(6.0)	(37.6)
piperidin-1-yl	142	82	122-123	338	60.7	6.5	33.6
	($C_{17}H_{22}N_8$)				(60.4)	(6.5)	(33.1)

Table 18.2 continued

piperazin-1-yl	143	95	120-121	339	56.4	6.2	37.5
	($C_{16}H_{21}N_9$)				(56.6)	(6.2)	(37.2)
4-methylpiper-	144	60	124-126	353	57.5	6.9	35.7
azin-1-yl	($C_{17}H_{23}N_9$)				(57.8)	(6.5)	(35.7)
4-morpholino	145	90	211-212	340	56.3	5.9	32.8
	($C_{16}H_{20}N_8O$)				(56.5)	(5.9)	(32.9)

[a] all compounds recrystallised from aqueous ethanol

Table 18.3 Analytical data for the amine-substituted pyrimidines



R	Compound[a] (Formula)	Yield (%)	Mp (°C)	M/z (M^+)	Found (%) (Required) C	H	N
dimethylamino	146	78	237-239	257	65.4	7.5	27.5
	($C_{14}H_{19}N_5$)				(65.4)	(7.4)	(27.2)
piperidin-1-yl	147	72	183-185	297	68.8	7.9	23.5
	($C_{17}H_{23}N_5$)		(sinters)		(68.9)	(7.7)	(23.6)

Table 18.3 continued

piperazin-1-yl	148	64	250-252	298	64.1	7.5	28.2
	($C_{16}H_{22}N_6$)		(decomp)		(64.4)	(7.4)	(28.2)
4-methylpiper-	149	76	235-236	312	65.4	8.1	26.9
azin-1-yl	($C_{17}H_{24}N_6$)				(65.4)	(7.7)	(26.9)

[a] all compounds recrystallised from aqueous ethanol

CHAPTER 19

SYNTHESES IN THE BENZOTRIAZOLE SERIES

1-Methyl-5-(2,4-diamino-6-ethylpyrimidin-5-yl)benzotriazole (152)

Method A

2,4-Diamino-5-(3-amino-4-methylaminophenyl)-6-ethylpyrimidine (123) (1 g) was dissolved in 5M-hydrochloric acid (30 ml) and the solution was cooled to 0° whereupon a microcrystalline precipitate of the hydrochloride salt deposited. The suspension was diazotised by the addition of sodium nitrite (0.3 g), as a solution in water (3 ml) in portions over 30 min and the mixture was stirred for a further 30 min. After dilution with water (100 ml) the yellow solution was basified with concentrated aqueous ammonia to pH 9 and the brown solid collected. A sample was recrystallised from 2-ethoxyethanol to give a meagre yield of the benzotriazole as an off-white amorphous powder. The product was dissolved in 1M-hydrochloric acid and reprecipitated by the addition of 1M-sodium hydroxide solution to furnish pink microcrystals.

In an identical manner a series of amines were diazotised and converted to the appropriate benzotriazole (table 19.1). Yields were generally good and reprecipitation unnecessary since in all cases examination of the reaction mixture by t.l.c. showed an absence of starting material and formation of a single product after 30 min.

Method B

A fine suspension of 2,4-diamino-5-(3-amino-4-N-methylbenzylaminophenyl)-6-ethylpyrimidine (135) (3 g) in 5 M-hydrochloric acid

(100 ml) was diazotised at 0° by the addition of sodium nitrite (0.7 g) in water (5 ml) over 15 min. The resulting bright orange solution was stirred for a further 1 h, diluted with water (100 ml) and basified to pH 9 with concentrated aqueous ammonia. The creamy precipitate was collected and washed with water and dichloromethane (20 ml). Recrystallisation from aqueous 2-ethoxyethanol gave the N-methylbenzotriazole as an amorphous white powder, identical (t.l.c., i.r. and n.m.r.) to the authentic sample of (152) synthesised by method A.

The monomethanesulphonate of (152) was prepared by dissolving the base in hot 95% ethanol containing methanesulphonic acid (1 mole equivalent) from which cream microprisms deposited over 3 days, m.p. 281-283° (decomp.) (Found: C, 45.4; H, 5.3; N, 26.1. $C_{14}H_{19}N_7O_3S$. 0.3 x H_2O requires C, 45.3; H, 5.3; N, 26.4%; ν_{max} 3520, 3320 and 3150 br cm^{-1} (NH).

The aqueous filtrate remaining from the initial diazotisation reaction above was extracted with dichloromethane (3 x 100 ml) and the dichloromethane fractions and washings were bulked. After washing with water and drying over sodium sulphate, the solvent was evaporated to leave a pale yellow oil with a characteristic odour. G.l.c. analysis of the oil showed the presence of 3 components, 2 of which co-chromatographed with benzyl alcohol and benzyl chloride. Spectroscopic examination after 24 h (m.s., i.r. and n.m.r.) revealed only one compound, identical in character to benzyl alcohol.

1-Ethyl-5-(2,4-diamino-6-ethylpyrimidin-5-yl)benzotriazole (153)

A solution of 2,4-diamino-5-(3-amino-4-N-ethylbenzylamino-phenyl)-6-ethylpyrimidine (136) (3 g) in 5M-hydrochloric acid (30 ml) was diazotised with sodium nitrite (0.8 g) in water (3 ml) as above.

The yellow solid formed on basifying the mixture was collected and recrystallised from aqueous ethanol to yield the N-ethylbenzotriazole as photosensitive yellow microprisms. This compound again proved identical (t.l.c., m.p., i.r., n.m.r. and m.s.) with (153) synthesised by method A.

Extraction of the filtrate from above with dichloromethane as described previously and subsequent g.l.c. analysis of the oily residue showed the presence of three components, characterised as identical to the mixture obtained on formation of the N-methylbenzotriazole (152).

Attempted synthesis of 2,4-diamino-5-(3-azido-4-methylaminophenyl)-6-ethylpyrimidine

(i) The amine (123) (0.5 g) was dissolved in sulphuric acid (10 ml) and diazotised at -5° by the addition of sodium nitrite (0.13 g) in portions over 15 min. After stirring for a further 30 min, sodium azide (0.4 g) was added cautiously over 20 min. The mixture frothed vigorously and after 1 h was poured onto ice (20 g) and basified with concentrated aqueous ammonia. The cream product was collected and characterised (m.p., i.r. and n.m.r.) as identical to the benzotriazole (152) prepared previously.

(ii) A solution of the N-methylbenzotriazole (152) (0.3 g) in acetic acid was stirred at 50° and sodium azide (0.2 g) was added. Examination of the mixture by t.l.c. after a further 7 h showed only the presence of starting material and subsequent refluxing for 12 h had no effect. The recovered compound proved identical (i.r.) to the starting material.

Attempted synthesis of 2,4-diamino-5-(3-azido-4-N-methylbenzylamino-phenyl)-6-ethylpyrimidine

To a stirred solution of sodium nitrite (44 mg) in sulphuric acid (5 ml) at 0°, was added a solution of 2,4-diamino-5-(3-amino-4-N-methylbenzylaminophenyl)-6-ethylpyrimidine (135) (0.2 g) in glacial acetic acid (10 ml) dropwise over 15 min. A deep yellow colour developed and the mixture was maintained at 5° for a further 30 min. An aliquot was taken from the reaction mixture and added to an alkaline solution of β-naphthol, whereupon a deep red colour developed, indicating the presence of a diazonium salt. Sodium azide (0.2 g) was added in portions over 15 min, the agitation being maintained and after a further 2 h at 5° the mixture was poured onto ice water (50 g) and basified with concentrated aqueous ammonia. The cream solid was collected and found to be identical (i.r. and t.l.c.) to the benzotriazole (152) prepared previously.

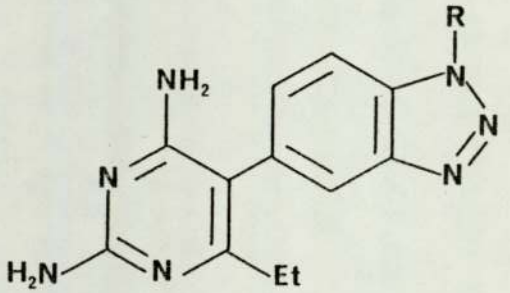
Pyrolysis of 1-methyl-5-(2,4-diamino-6-ethylpyrimidin-5-yl)benzotriazole (152)

A suspension of the benzotriazole (100 mg) in diethyl phthalate (5 ml) was boiled for 1 h and the dark mixture stood overnight to cool. Removal of diethyl phthalate by steam distillation afforded a brown tar, that solidified on trituration with petroleum ether (b.p. 60-80°) to form a brown powder (60 mg) (M^+ 467). The subsequent purification or further characterisation of the intractable brown material proved impossible and no further examination was undertaken.

Attempted cyclisation of the monomethyltriazene derivative (163)

Lithium bis(trimethylsilyl)amide (0.12 g, as a 1M solution in THF) was added to a stirred solution of (163) in dry THF at room temperature. A deep yellow colour immediately developed and the mixture was stirred for a further 12 h when inspection by t.l.c. showed only starting materials and no evidence for formation of the requisite benzotriazole (152). Subsequent refluxing of the reaction mixture had no effect and again only reactants were identified.

Table 19.1 Analytical data for compounds in the benzotriazole series



R	Compound[a] (Formula)	Solvent	Yield (%)	Mp (°C)	M/z (M^+)	Found (%) (Required) C	H	N
Me	152	ethoxyethanol	76(90)[b]	255-256	269	58.0	5.3	36.9
	($C_{13}H_{15}N_7$)			(sinters)		(58.0)	(5.6)	(36.8)
Et	153	aq. EtOH	87(92)[b]	265-267	283	59.8	6.1	34.5
	($C_{14}H_{17}N_7$)			(sinters)		(59.4)	(6.0)	(34.6)

Table 19.1 continued

n-butyl	154	aq. ethoxyethanol	96	253-255	311	61.7	6.8	31.4
	($C_{16}H_{21}N_7$)					(61.7)	(6.8)	(31.5)
cyclohexyl	155	aq. ethoxyethanol	90	250-253	337	64.0	6.9	29.2
	($C_{18}H_{23}N_7$)			(sinters)		(64.1)	(6.8)	(29.1)
benzyl	156	EtOH	82	262-263	345	66.2	5.5	28.2
	($C_{19}H_{19}N_7$)					(66.1)	(5.5)	(28.4)
phenethyl	157	aq. ethoxyethanol	89	210-212	359	67.0	5.8	27.5
	($C_{20}H_{21}N_7$)					(66.9)	(5.9)	(27.3)

[a] all compounds recrystallised from aqueous ethanol; [b] prepared from the corresponding N-alkylbenzylaminopyrimidines

CHAPTER 20

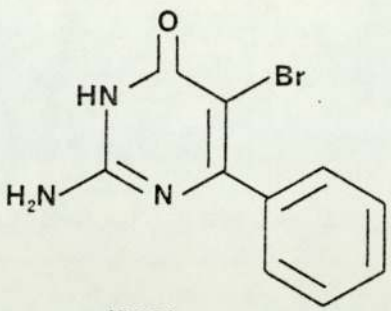
BIOLOGICAL MATERIALS AND METHODS

20.1 Compounds obtained as gifts

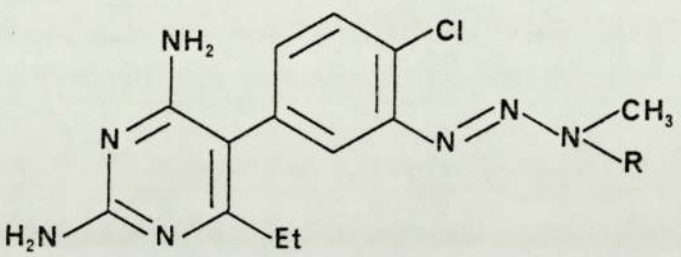
Methotrexate (3) and CB3717, a thymidylate synthase inhibitor used to validate the assay procedure (20.5), were provided by the Biochemical Pharmacology Section of the Institute of Cancer Research, Sutton, Surrey. Pyrimethamine (4) and metoprine (5) were obtained from The Wellcome Foundation Ltd, Dartford, Kent.

In addition to those compounds synthesised as described previously (chapters 15-19) and evaluated against DHFR, the compounds summarised in fig. 20.1 were also screened for activity against the enzyme. 2-Amino-5-bromo-6-phenylpyrimidin-4 (3H)-one (ABPP) (162) was supplied by Dr E N Gate and the three pyrimidine derivatives (163-165) were synthesised by Professor M F G Stevens. The mono- and dimethyltriazene analogues of pyrimethamine were of particular interest as prospective irreversible inhibitors of mammalian DHFR and as such warranted preliminary evaluation in order to establish activity against the enzyme.

Figure 20.1 Compounds obtained as gifts

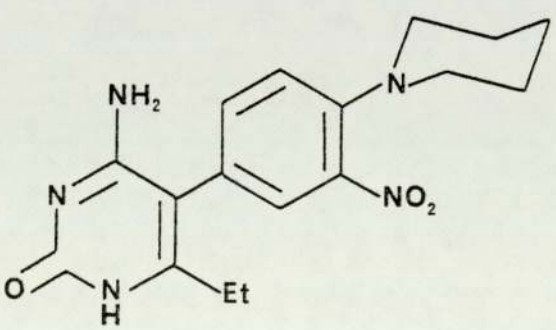


(162)



(163): R=H

(164): R=CH_3



(165)

20.2 Source of reagents and enzymes used in the assays

The following reagents were purchased from the sources indicated:

BDH Chemicals Ltd, Atherstone

Potassium dihydrogen orthophosphate

Potassium hydroxide

2-Mercaptoethanol

Dithiothreitol

Formaldehyde (37% w/v in water)

Sigma Chemical Company, Poole, Dorset

Dihydrofolate

Tetrahydrofolate

NADPH (tetrasodium salt)

d.UMP

The Radiochemical Centre, Amersham, Bucks

(5-^{3}H) d.UMP

Source of enzymes

Partially purified DHFR was prepared from rat liver by the procedure described below (20.3a). TS was prepared from L1210 cells by a previously published method[214] and used as such.

20.3 Compound dissolution

Stock inhibitor solutions were prepared by dissolving the appropriate compound in water, 0.1 M-hydrochloric acid or ethanol to give a final concentration of 1×10^{-3} M. Where difficulties in dissolving the compound were encountered the mixture was warmed in a water bath at 40° before dilution to the requisite volume. All solutions were prepared on the day previous to the experiment and stored in the dark at 4° prior to use.

The stock solutions were diluted, as necessary, immediately before use to produce the required inhibitor concentration. Following further dilution in the final reaction mixture, the highest solvent concentrations were 5×10^{-3} M and 1.1×10^{-2} M for hydrochloric acid and ethanol respectively. No effect on DHFR or TS activity was observed at these solvent concentrations.

20.4 The DHFR assays

20.4(a) **Preparation of partially purified DHFR**

Partially purified enzyme was prepared by the method of Bertino and Fischer[215] as follows: Two male Wistar rats were killed and the livers were removed rapidly and washed with water to remove blood. All further manipulations were performed at 4°; the livers (30.7 g) were transferred to a Waring blender to which approximately 8 volumes of water was added (final volume c.a. 300 ml), whereupon the mixture was homogenised for 2 min in periods of 30s, allowing 30s between each run to minimise heating. Liver homogenate was centrifuged at approximately 20 000 g for 20 min and the pellet was discarded. The supernatant liquid was adjusted to pH 5.1 with dilute acetic acid

(1.0 M initially and 0.1 M finally) and after centrifugation at 27000 g for a further 20 min, the clear wine-red solution was transferred to visking tubing (2.5 cm diameter) and dialysed for 12 h against 0.01 M sodium acetate buffer solution. The dialysate was examined and if turbid, centrifuged again at 27000 g for 20 min. The clear preparation was transferred to sterile plastic stoppered tubes (10 ml) and stored at -10° prior to use. Enzyme preparations stored frozen in this manner exhibited no significant decrease in DHFR activity after 12 months.

20.4(b) **Preparation of reagent solutions**

0.15 M Phosphate buffer (pH 7.0) was used for all DHFR assays and was prepared by dissolving potassium dihydrogen orthophosphate (10.21 g) in water (c.a. 300 ml), adjusting the solution to pH 7.0 with potassium hydroxide, and diluting to 500 ml with water. The buffer was kept at 4° to prevent bacterial growth and discarded after 3 days.

2-Mercaptoethanol solution (0.25 M) was prepared by dissolving 2-mercaptoethanol (1.75 ml) in water (to 100 ml) and the solution was stored in the dark at 4° prior to use.

A solution of dihydrofolate (1 $mg.ml^{-1}$, 2 mM) was prepared immediately before use by suspending dihydrofolate in 0.25 M - 2-mercaptoethanol solution and adding 1M-sodium hydroxide solution dropwise with vigorous agitation until dissolution had occurred. The solution was maintained at 0° and protected from light.

An aqueous solution of NADPH (2 $mg.ml^{-1}$, 2.0 mM) was prepared immediately prior to use and again maintained at 0°.

20.4 (c) **Assay for inhibitor activity**

The spectrophotometric assay was carried out with a Cary Model 16KC spectrophotometer fitted with a thermostatted rotating cell compartment capable of accommodating 5 sample and 5 reference cuvettes. Reaction rates were recorded on a Varian Model G2500 chart recorder, at a chart speed of 1 $cm.min^{-1}$ and a full scale deflection of 0.1 absorbance units and the cuvettes were read sequentially at 10 second time intervals. Plastic disposable cuvettes were used throughout.

The assay was carried out as follows:
NADPH (0.1 ml) and the enzyme preparation (0.1 ml) were incubated at 30° for 5 min in phosphate buffer (total volume 1.9 ml). The reaction was initiated by addition of dihydrofolate (0.1 ml) and monitored by following the decrease in absorbance at 340 nm. Parallel assays were implemented where the reaction mixture contained 0.1 ml of the inhibitor at the required concentration and the volume of buffer was adjusted accordingly to give a final volume of 2 ml after addition of dihydrofolate.

Reference cuvettes were set up containing NADPH, buffer, inhibitor if appropriate and dihydrofolate, but without enzyme (table 20.1).

Enzyme activity was estimated from the slope of the change of absorbance with time and this was arbitrarily designated as 100% for the uninhibited enzyme. The observed decrease in activity in the presence of inhibitor was expressed as a percentage of the uninhibited enzyme activity.

Table 20.1 DHFR assay; volumes of reagents, inhibitor and enzyme employed

Nature of Assay	NADPH[a] (ml)	Enzyme (ml)	Buffer (ml)	Inhibitor[b] (ml)	Dihydrofolate[c] (ml)	Final volume (ml)
Uninhibited enzyme	0.1	0.1	1.7	-	0.1	2
Reference	0.1	-	1.8	-	0.1	2
Inhibited enzyme	0.1	0.1	1.6	0.1	0.1	2
Reference	0.1	-	1.7	0.1	0.1	2

[a] 1×10^{-4} M final concentration (saturating)

[b] The highly coloured nature of several inhibitors necessitated the use of a reference cuvette.

[c] 1×10^{-4} M final concentration

Activity against DHFR was initially measured at a final inhibitor concentration of 2.5×10^{-5} M, in duplicate. Compounds producing an inhibition of less than 50% at this concentration were considered to be inactive. The remaining inhibitors were selected for I_{50} determinations and inhibitory activity was evaluated in duplicate at a minimum of four inhibitor concentrations, from which the I_{50} value was determined by a graphical method (chapter 11.2).

The thirteen compounds selected for inhibition constant (K_I) determinations were assayed following an identical method to that described above except that inhibitory activity was measured at a minimum of ten inhibitor concentrations, again in duplicate, from which the appropriate K_I values were determined by a Zone B analysis as described in chapter 11.2. A K_m value of 0.2 μM for dihydrofolate[216] was adopted for the calculation of K_I.

20.5 Assay for thymidylate synthase (TS) inhibition

Preparation of 5,10-methylenetetrahydrofolate

Tetrahydrofolate (7 mg) was suspended in aqueous dithiothreitol solution (100 mM) (c.a. 1 ml) and to the mixture was added 1M-sodium hydroxide solution (2 drops) and formaldehyde solution (7.8 μl). The mixture was gently agitated until all solids dissolved and diluted to a final volume of 5 ml with dithiothreitol solution. This reagent was prepared immediately prior to use and protected from light.

Thymidylate synthase activity was assayed isotopically by a modification[214,217] of the method originally described by Roberts[218]. Briefly, a 0.5 ml reaction mixture containing 50 μM [5-^{3}H]-d.UMP (24mCi.mmole^{-1}), 10 mM dithiothreitol, 10 mM 5,10-methylenetetrahydrofolate, 400 μM inhibitor and 0.2 ml of buffered

enzyme preparation was incubated at 37° for 1 h. The reaction was stopped by adding iced water (1 ml) and, following separation of the products by column chromatography, activity was determined in an Intertechnique SL30 liquid scintillation counter.

All assays were performed in triplicate and control reactions were set up to account for any effects due to solvent. The inhibitory activity of a quinazoline TS inhibitor (CB 3717) was evaluated in a parallel assay in order to confirm the viability of the method.

20.6 In vitro cytotoxicity studies

Cultures of L1210 murine leukaemia cells were grown as a suspension in RPMI 1640 medium (with 25 mM hepes and L-glutamine) and 10% horse serum (Gibco Ltd, Paisley, Scotland). Cells were seeded routinely every 72 h at 10^4 cells.ml^{-1} and counted at 10^6 cells.ml^{-1} prior to each experiment and counts were performed following incubation (5% CO_2 in air at full humidity) for 72 h at 37°, with the appropriate concentration of drug. All incubations were carried out in duplicate and the increase in cell number in the presence of inhibitor after 72 h was expressed as a percentage of the control cell count.

Appendices 15.1 to 19.1

^{1}H-nmr spectra

Notes

1. Spectra were recorded on a Varian EM 360 A (60 MHz), a Perkin Elmer R34 (220 MHz) or a Bruker WH400 (400 MHz) spectrometer.

2. Signals were assigned in ppm downfield of tetramethylsilane (δ).

3. Solvents A, [$^{2}H_{6}$] DMSO; B, [^{2}H] TFA.

4. Unless specified otherwise, all NH signals appeared as broad singlets exchangable with D_2O and are unassigned.

5. For clarity, signals derived from ethanesulphonic acid are omitted but in all cases appeared at approximately 1.14 (3H,t) and 2.58 (2H,q).

6. t = triplet, q = quartet, d = doublet, dd = double doublet, m = multiplet, brs = broad singlet.

Appendix 15.1 ^{1}H-nmr spectra (δ values) of the 2,4-diamino-5-chlorophenyl-6-ethylpyrimidines

Compound	Solvent	CH_3 ^{t}	CH_2	H'-2	H'-3	H'-4	H'-5	H'-6	Other
4 (pyrimethamine)	A	0.96	2.12q	———	7.25(d) and 7.53(d) AA' BB'			———	5.62(2H,NH) 5.91(2H,NH)
45	A	1.05	2.21m[a]	-	———	7.22 - 7.53 m		———	4.43(2H,NH) 4.85(2H,NH)
46	A	0.97	2.11q[b]	———	———	7.38 m	———	———	5.70(2H,NH) 5.94(2H,NH)

[a] sixteen line multiplet at 400 MHz

[b] lines appear blurred but multiplicity not evident

Appendix 15.2 - 15.5 ^{1}H-nmr spectra (δ values) of the nitro, amino and azidopyrimidines

Compound	Solvent	CH_3	CH_2 q	H-2'	H-5'	H-6'	Other
47	A	1.88s	-	7.88d	7.78d	7.52dd	5.94(2H,NH) 6.04(2H,NH)
48	A	1.90s	-	7.60d	8.10d	7.42dd	6.01(2H,NH) 6.12(2H,NH)
49	A	1.00t	2.15	7.94d	7.86d	7.59dd	6.01(2H,NH) 6.10(2H,NH)
50	B	1.20t	2.50	7.35d	8.08d	7.52dd	a
53	B	1.30t	2.70	8.05d	—— 7.63m ——		4.15(3H,s,OCH_3)

Appendix 15.2 - 15.5 continued

54	B	1.30t	2.65	8.02d	—— 7.55m ——		1.57(3H,t,$OCH_2\underline{CH_3}$) 4.35(2H,q,$O\underline{CH_2}CH_3$)
55	A	0.7-1.3 m	2.20	—— 7.40 m and 7.75 m ——			0.7-1.3(3H,m,CH_3) 1.65(4H,m,$[CH_2]_2$) 3.50(2H,q,OCH_2) 7.0(2H,NH) 7.0-8.0(2H,NH)
57	B	2.25s	-	7.75d	7.85d	7.50dd	a
58	A	1.88s	-	6.96d	—— 6.83 brs ——		5.20(2H,NH) 5.90(4H,NH)

Appendix 15.2 - 15.5 continued

22	A	1.00t	2.18	6.65d	7.25d	6.38dd	5.35(2H,NH) 5.69(2H,NH) 5.90(2H,NH)
59	A	1.00t	2.20	6.96d	——— 6.84 brs ——		5.13(2H,NH) 5.64(2H,NH) 5.77(2H,NH)
60	B	0.95t	2.20	6.40d	6.90d	6.70dd	4.12(3H,s,OMe)
61	A	1.00t	2.15	6.30d	6.90d	6.52dd	1.37(3H,t,$OCH_2\underline{CH_3}$) 4.08(2H,q,$OCH_2\underline{CH_3}$) 4.76(2H,NH) 5.60(2H,NH) 5.88(2H,NH)

Appendix 15.2 - 15.5 continued

62	B	2.35s	-	7.15d	7.65d	7.10dd	a
63	B	2.35s	-	———	7.40 brs	———	a
21 (MZP)	A	1.00t	2.18	7.19d	7.55d	7.00dd	5.84(2H,NH) 5.96(2H,NH)
64	A	0.96t	2.10	7.29d	7.46d	7.21dd	5.68(2H,NH) 5.90(2H,NH)
62a	A	2.03s	-	7.45d	7.69d	7.17dd	7.06(1H,NH) 7.82(2H,NH) 8.18(1H,NH)

Appendix 15.2 - 15.5 continued

63a	A	2.00s	-	7.53d	7.63d	7.40dd	7.04(1H,NH) 7.83(2H,NH) 8.16(1H,NH) 12.56(1H,NH^+)
21a (MZPES)	A	1.08t	2.26	7.39d	7.65d	7.12dd	7.02(1H,NH) 7.85(2H,NH) 8.19(1H,NH)
64a	A	1.04t	2.25	7.54d	7.63d	7.40dd	7.03(1H,NH) 7.83(2H,NH) 8.20(1H,NH)

Appendix 15.2 - 15.5 continued

65a[b]	A	1.05t	2.23	7.03d	7.30d	7.12dd	3.94(3H,s,OCH_3) 6.88(1H,NH) 7.73(2H,NH) 8.15(1H,NH)
66a[b]	A	1.04t	2.26	6.97d	7.27d	7.10dd	1.40(3H,t,$OCH_2\underline{CH_3}$) 4.22(2H,q,$OCH_2\underline{CH_3}$) 6.86(1H,s,NH) 7.72(2H,NH) 8.14(1H,NH)

[a] all NH protons fully exchanged

[b] the corresponding azide free bases (65 and 66) proved poorly soluble in DMSO and decomposed in TFA

Appendix 16.1 ^{1}H-nmr spectra (δ values) of the diaminopyrimidine N-oxides

Compound	Solvent	$CH_3{}^t$	$CH_2{}^q$	H-2'^{d}	H-5'^{d}	H-6'^{dd}	Other
49[a] (MNP)	A	1.00	2.15	7.94	7.86	7.59	6.01(2H,NH) 6.10(2H,NH)
74	A	1.05	2.46	7.90	8.05	7.65	7.08(2H,NH) 7.92(2H,NH)
75	A	0.99	2.17	7.98	7.84	7.59	7.02(2H,NH) 7.22(2H,NH)
22[a] (MAP)	A	1.00	2.18	6.65	7.25	6.38	5.35(2H,NH) 5.69(2H,NH) 5.90(2H,NH)

Appendix 16.1 continued

76	A	1.00	2.19	6.64	7.26	6.39	5.46(2H,NH) 6.65(2H,NH) 7.14(2H,NH)
21[a] (MZP)	A	1.00	2.18	7.19	7.55	7.00	5.84(2H,NH) 5.96(2H,NH)
77	A	1.06	2.52	7.48	7.69	7.19	7.01(2H,NH) 7.88(2H,NH)
78	A	1.00	2.22	7.34	7.63	7.11	6.92(2H,NH) 7.25(2H,NH)

[a] included for comparison

Appendix 16.3 ^{1}H-nmr spectra (δ values) of the aminopyrimidinones

Compound	Solvent	$CH_3{}^t$	$CH_2{}^q$	H-2'	H-3'	H-5'	H-6'	Other
4[a] (pyrimethamine)	A	0.96	2.12	———	7.25(d) and 7.53(d):AA'BB'	———		5.62(2H,NH) 5.91(2H,NH)
4a[a] (hydrochloride)	A	1.00	2.27	———	7.38(d) and 7.63(d):AA'BB'	———		6.95(1H,NH) 7.81(2H,NH) 8.22(1H,NH) 13.2(1H,NH^+)
83	A	0.99	2.10	———	7.31(d) and 7.55(d):AA'BB'	———		5.9(3H,NH)
83a (hydrochloride)	A	1.04	2.26	———	7.46(d) and 7.67(d): AA'BB'	———		3.5-5.0(1H,NH) 8.29(1H,NH) 9.20(1H,NH) 12.39(1H,NH^+)

Appendix 16.3 continued

84	A	1.04	2.23	——— 7.27(d) and 7.47(d): AA'BB' ———				6.72(2H,NH) 10.95(1H,NH)
84a (hydrochloride)	A	1.16	2.39	——— 7.35(d) and 7.57(d): AA'BB' ———				8.52(2H,NH) 12.90(2H,NH,NH^+)
49[a] (nitropyrimethamine)	A	1.00	2.15	7.94d	-	7.86d	7.59dd	6.01(2H,NH) 6.10(2H,NH)
88	A	1.07	2.28	7.95d	-	7.80d	7.60dd	6.79(3H,NH)
89	A	1.00	2.13	7.98d	-	7.85d	7.61dd	6.2(1H,NH) 7.15(2H,NH)

[a] included for comparison

Appendix 17.1 ^{1}H-nmr spectra (δ values) of the amine-substituted nitropyrimidines

Compound	Solvent	$CH_3{}^t$	$CH_2{}^q$	H-2'	H-5'	H-6'	Other
95	A	0.97	2.14	7.81d	7.05d	7.35dd	2.99(3H,d,NH[$\underline{CH_3}$]) 5.71(2H,NH) 5.86(2H,NH) 8.22(1H,q,$\underline{NH}$[CH_3])
96	A	0.98	2.15	7.59d	7.28d	7.36dd	2.53(6H,d,N[CH_3]$_2$) 5.71(2H,NH) 5.90(2H,NH)
97	A	1.01	2.18	7.85d	7.12d	7.34dd	1.26(3H,t,N[H]$CH_2\underline{CH_3}$) 3.43(2H,q,N[H]$\underline{CH_2}CH_3$) 6.32(2H,NH) 6.43(2H,NH) 8.18(1H,t,$\underline{NH}$[Et])

Appendix 17.1 continued

98	A	0.98	2.15	7.82d	7.12d	7.33dd	0.94(3H,t,CH_3) 1.40(2H,m,CH_2) 1.64(2H,m,CH_2) 3.39(2H,q,CH_2) 5.91(2H,NH) 6.06(2H,NH) 8.21(1H,t,NH)
98a	A	1.04	2.25	7.93brs	7.39d	7.17dd	0.94(3H,t,CH_3) 1.41(2H,m,CH_2) 1.64(2H,m,CH_2) 3.40(2H,q,CH_2) 6.5-8.5(1H,NH) 7.60(3H,NH) 8.28(1H,t,NH)

Appendix 17.1 continued

99	A	0.98	2.14	7.58brs	———	7.37 m ——	1.60(6H,brs,$[CH_2]_3$)
(3 x DMF solvate)							2.74(9H,s,3xNCH_3)
							2.90(9H,s,3xNCH_3)
							3.02(4H,brs,$[CH_2]_2$)
							5.69(2H,NH)
							5.88(2H,NH)
							7.98(3H,s,3x$HCONMe_2$)
100	A	1.00	2.16	7.60brs	———	7.35 brs ——	2.50(4H,brs,$[CH_2]_2$)
							3.20(4H,brs,$[CH_2]_2$)
							5.82(2H,NH)
							5.95(2H,NH)

Appendix 17.1 continued

101	A	0.97	2.15	7.56d	7.31d	7.35dd	2.23(3H,s,NCH_3) 2.44(4H,t,$[CH_2]_2$) 3.02(4H,t,$[CH_2]_2$) 5.67(2H,NH) 5.88(2H,NH)
102	A	0.97	2.14	7.51brs	——— 7.18 m ———		1.94(4H,brs,$[CH_2]_2$) 3.21(4H,brs,$[CH_2]_2$) 5.62(2H,NH) 5.84(2H,NH)
103	A	0.99	2.15	7.63brs	——— 7.42 m ———		3.06(4H,brs,$[CH_2]_2$) 3.45(4H,m,$[CH_2]_2$) 5.72(2H,NH) 5.92(2H,NH)

Appendix 17.1 continued

104	A	0.97	2.14	7.81d	7.17d	7.31dd	1.25(1H,t,CH_2) 1.41(4H,m,$[CH_2]_2$) 1.60(1H,t,CH_2) 1.71(2H,m,CH_2) 1.98(2H,m,CH_2) 3.66(1H,brs,CH) 5.80(2H,NH) 5.94(2H,NH) 8.08(2H,d,NH)
105	A	0.97	2.12	7.48s	—— 7.26s ——		1.12(1H,m,CH_2) 1.29(2H,q,CH_2) 1.54(3H,m,CH_2+CH) 1.74(4H,brs,$[CH_2]_2$) 2.63(3H,s,NCH_3)

Appendix 17.1 continued

105							3.26(1H,t,CH) 5.89(2H,NH) 5.94(2H,NH)
106	A	0.96	2.14	7.91d	7.08d	7.35dd	4.71(2H,d,CH_2Ph) 5.73(2H,NH) 5.89(2H,NH) 7.47(5H,m,C_6H_5) 8.77(1H,t,NH)
107	A	1.00	2.16	7.55brs	—— 7.26brs ——		2.70(3H,s,NCH_3) 4.40(2H,s,CH_2Ph) 5.85(4H,NH) 7.26(5H,brs,C_6H_5)

Appendix 17.1 continued

108	A	0.96	2.10	7.53d	—— 7.31m ——		1.02(3H,t,$NCH_2\underline{CH_3}$) 3.06(2H,q,$NC\underline{H_2}CH_3$) 4.30(2H,s,$C\underline{H_2}Ph$) 5.74(2H,NH) 5.91(2H,NH) 7.31(5H,m,C_6H_5)
109	A	0.97	2.15	7.90d	6.80d	7.15dd	1.65(3H,d, CH_3) 4.80(1H,q,$C\underline{H}Ph$) 5.65(4H,NH) 7.35(5H,m,C_6H_5) 8.40(1H,d,NH)

Appendix 17.1 continued

110	A	0.94	2.07	7.58d	—— 7.27m ——		4.23(4H,s,2x$\underline{CH_2}$Ph) 5.93(2H,NH) 6.12(2H,NH) 7.27(10H,m,2xC_6H_5)
111	A	0.98	2.15	7.81d	7.19d	7.33m	2.98(2H,t,CH_2) 3.64(2H,q,CH_2) 5.99(2H,NH) 6.14(2H,NH) 7.33(5H,m,C_6H_5) 8.21(1H,t,NH)

Appendix 17.1 continued

112	A	0.98	2.15	7.81d	7.12d	7.32dd	1.4-1.8(2H,NH_2)
							2.87(2H,q,$\underline{CH_2}NH_2$)
							3.40(2H,m,$NHC\underline{H_2}$)
							5.69(2H,NH)
							5.86(2H,NH)
							8.45(1H,t,$N\underline{H}CH_2$)
113	A	0.98	2.17	7.78d	7.20d	7.41dd	1.75(2H,m,CH_2)
							2.71(2H,m,$\underline{CH_2}NH_2$)
							3.49(2H,q,$NHC\underline{H_2}$)
							5.73(2H,NH)
							5.89(2H,NH)
							8.44(1H,$N\underline{H}CH_2$)

Appendix 17.1 continued

114a[a]	A	1.08	2.28	6.99d	8.15d	6.55dd	0.91(3H,t,CH_3)
							1.37(2H,m,CH_2)
							1.59(2H,m,CH_2)
							3.35(2H,q,CH_2)
							7.01(1H,NH)
							7.76(2H,NH)
							8.15(1H,NH)
							8.21(1H,NH)
115	A	0.74	1.90	6.65d	8.11d	6.48dd	4.63(2H,brs,$\underline{CH_2}$Ph)
							5.67(2H,NH)
							5.93(2H,NH)
							7.33(5H,m,C_6H_5)
							8.73(1H,t,NH)

Appendix 17.1 continued

116	A	0.87	2.00	6.93d	7.82d	6.75dd	2.73(3H,s,NCH_3) 4.42(2H,brs,$\underline{CH_2}Ph$) 5.71(2H,NH) 5.93(2H,NH) 7.26(5H,m,C_6H_5)

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[a] the corresponding amine free base (114) proved insoluble in DMSO and was not analysed as such

Appendix 18.1 ^{1}H-nmr spectra (δ values) of the amine-substituted aminopyrimidines

Compound	Solvent	$CH_3{}^t$	$CH_2{}^q$	H-2'	H-5'	H-6'	Other
123	A	0.97	2.16	6.37d	6.33d	6.44dd	2.74(3H,d,NCH_3) 4.53(2H,NH) 4.64(1H,brs,$\underline{NH}[CH_3]$) 5.36(2H,NH) 5.78(2H,NH)
124	A	0.98	2.15	6.48d	6.94d	6.35dd	2.60(6H,s,$N[CH_3]_2$) 4.77(2H,NH) 5.45(2H,NH) 5.81(2H,NH)

Appendix 18.1 continued

125	A	0.97	2.15	6.36d	6.46d	6.30dd	1.23(3H,t,$NCH_2\underline{CH_3}$)
							3.06(2H,m,$NCH_2\underline{CH_3}$)
							4.40(1H,t,NH)
							4.59(2H,NH)
							5.00-5.70(2H,NH)
							5.76(2H,NH)
126	A	0.98	2.18	6.37d	6.46d	6.32dd	0.94(3H,t,CH_3)
							1.45(2H,m,CH_2)
							1.62(2H,m,CH_2)
							3.04(2H,t,CH_2)
							4.43(1H,brs,NH)
							4.30-4.95(2H,NH)
							5.40-5.85(2H,NH)
							6.01(2H,NH)

Appendix 18.1 continued

127	A	0.99	2.18	6.55d	6.96d	6.41dd	1.55(2H,brs,CH_2)
							1.68(4H,brs,$[CH_2]_2$)
							2.81(4H,brs,$[CH_2]_2$)
							4.77(2H,NH)
							5.55(2H,NH)
							5.88(2H,NH)
128	A	0.98	2.14	6.49d	6.90d	6.35dd	2.74(4H,brs,$[CH_2]_2$)
							2.84(4H,m,$[CH_2]_2$)
							4.75(2H,NH)
							5.25-5.75(3H,NH)
							5.84(2H,NH)

Appendix 18.1 continued

129	A	0.98	2.17	6.56d	6.97d	6.41dd	2.24(3H,s,NCH_3)
							2.52(4H,m,$[CH_2]_2$)
							2.87(4H,s,$[CH_2]_2$)
							4.77(2H,NH)
							5.49(2H,NH)
							5.85(2H,NH)
130	A	0.99	2.19	6.54d	6.95d	6.39dd	1.87(4H,s,$[CH_2]_2$)
							3.03(4H,s,$[CH_2]_2$)
							4.69(2H,NH)
							5.46(2H,NH)
							5.82(2H,NH)

Appendix 18.1 continued

131	A	0.98	2.16	5.55 d	6.97 d	6.41 dd	2.86(4H,m,$[CH_2]_2$)
							3.78(4H,m,$[CH_2]_2$)
							4.85(2H,NH)
							5.40(2H,NH)
							5.82(2H,NH)
134	A	0.98	2.18	6.46d	7.46m	6.30 dd	4.34(2H,d,$\underline{CH_2}$Ph)
							5.17(2H,NH)
							5.50(2H,NH)
							5.83(2H,NH)
							7.40(5H,m,C_6H_5)
							7.40(1H,m,NH)

Appendix 18.1 continued

135	A	1.00	2.20	6.56d	6.96d	6.38dd	2.53(3H,s,NCH_3) 3.98(2H,s,$\underline{CH_2}Ph$) 4.73(2H,NH) 5.48(2H,NH) 5.73(2H,NH) 7.30(5H,brs,C_6H_5)
138	A	0.98	2.17	6.40d	6.55d	6.34dd	2.92(2H,t,CH_2Ph) 3.29(2H,m,CH_2) 4.57(3H,NH) 5.42(2H,NH) 5.78(2H,NH) 7.26(5H,m,C_6H_5)
139	B	1.25	2.60	———	7.10-7.60 m	———	3.65(4H,brs,$[CH_2]_2$) [a]

Appendix 18.1 continued

140	A	0.97	2.16	6.36d	6.48d	6.31dd	1.69(2H,m,CH_2)
							2.68(2H,t,CH_2)
							3.08(2H,t,CH_2)
							1.90-3.40(2H,NH)
							4.55(3H,NH)
							5.41(2H,NH)
							5.76(2H,NH)

[a] all NH protons fully exchanged

Appendix 18.2 ^{1}H-nmr spectra (δ values) of the amine-substituted azidopyrimidines

Compound	Solvent	CH_3 ^{t}	CH_2 ^{q}	H-2'	H-5'	H-6'	Other
141	A	0.98	2.13	6.84d	7.07d	6.91dd	2.74(6H,s,N[CH_3]$_2$) 5.63(2H,NH) 5.86(2H,NH)
142	A	1.00	2.16	6.88d	7.17d	6.98dd	1.58(2H,brs,CH_2) 1.71(4H,brs,[CH_2]$_2$) 2.98(4H,brs,[CH_2]$_2$) 5.71(2H,NH) 5.94(2H,NH)
143	A	0.96	2.15	6.80d	7.34d	7.00dd	1.60(6H,brs,[CH_2]$_2$+CH_2) 2.93(4H,brs,[CH_2]$_2$) 5.50-5.80(4H,NH)

Appendix 18.2 continued

144	A	0.97	2.13	6.88d	7.15d	6.95dd	2.23(3H,s,NCH_3) 2.54(4H,brs,$[CH_2]_2$) 3.02(4H,brs,$[CH_2]_2$) 5.58(2H,NH) 6.86(2H,NH)
145	A	0.97	2.12	6.87d	7.11d	6.94dd	3.00(4H,brs,$[CH_2]_2$) 3.75(4H,m,$[CH_2]_2$) 5.58(2H,NH) 5.87(2H,NH)

Appendix 18.3 ^{1}H-nmr spectra (δ values) of the 4'-amine-substituted pyrimidines

Compound	Solvent	$CH_3{}^t$	$CH_2{}^q$	H-2'	H-3'	H-5'	H-6'	Other
146	A	0.96	2.15	———	6.78(d) and 6.98(d):AA'BB'		———	2.92(6H,s,N[CH_3]$_2$) 5.39(2H,NH) 5.72(2H,NH)
147	A	0.98	2.17	———		7.06s	———	1.64(6H,brs,[CH_2]$_3$) 3.22(4H,brs,[CH_2]$_2$) 5.40(2H,NH) 5.82(2H,NH)
148	A	0.97	2.16	———		7.04s	———	2.89(4H,brs,[CH_2]$_2$) 3.13(4H,brs,[CH_2]$_2$) 3.30(1H,NH) 5.40(2H,NH) 5.80(2H,NH)

Appendix 18.3 continued

149	A	0.97	2.15	7.07s		2.26(3H,s,NCH_3) 2.50(4H,brs,$[CH_2]_2$) 3.22(4H,brs,$[CH_2]_2$) 5.40(2H,NH) 5.81(2H,NH)

Appendix 19.1 ^{1}H-nmr spectra (δ values) of compounds in the benzotriazole series

Compound	Solvent	CH_3 ^{t}	CH_2 ^{q}	H-2'	H-5'	H-6'	Other
152[a]	A	1.03	2.21	—— 7.99m ——		7.42dd	2.45(3H,s,$\underline{CH_3}SO_3^-$)
							4.36(3H,s,NCH_3)
							6.89(1H,NH)
							7.67(2H,NH)
							8.15(1H,NH)
							12.28(1H,NH^+)
153	A	0.95	2.10	7.81d	7.93dd	7.31dd	1.55(3H,t,$NCH_2\underline{CH_3}$)
							4.75(2H,q,$NCH_2\underline{CH_3}$)
							5.70(2H,NH)
							5.93(2H,NH)

Appendix 19.1 continued

154	A	0.95	2.10	7.79s	7.91d	7.30dd	0.92(3H,t,CH_3) 1.31(2H,m,CH_2) 1.92(2H,m,CH_2) 4.71(2H,t,$NC\underline{H_2}[CH_2]_2CH_3$) 5.58(2H,NH) 5.85(2H,NH)
155	A	0.95	2.10	7.79s	7.98d	7.29 dd	1.37(1H,m,CH_2) 1.55(2H,m,CH_2) 1.74(1H,m,CH_2) 1.91(2H,m,CH_2) 2.01-2.12(4H,m,$[CH_2]_2$) 4.89(1H,m,CH) 5.65(2H,NH) 5.91(2H,NH)

Appendix 19.1 continued

156	A	0.94	2.08	7.83s	7.91d	7.31dd	5.77(2H,NH) 5.99(2H,NH) 5.99(2H,brs,CH_2Ph) 7.39(5H,m,C_6H_5)
157	A	0.99	2.13	———	7.87d ——	7.19dd	3.28(2H,m,CH_2) 4.98(2H,m,CH_2) 6.41(2H,NH) 6.64(2H,NH) 7.23(5H,m,C_6H_5)

[a] as the methanesulphonate

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