ANTITUMOUR IMIDAZOTETRAZINES: PROBES FOR THE MAJOR GROOVE OF DNA.

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Antitumour imidazotetrazines: probes for the major groove of DNA.

by

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The antitumour imidazotetrazinones are believed to act as prodrugs for the triazene series of alkylating agents, showing a marked preference for the alkylation of the middle guanine residue in a run of three or more contiguous guanines. However, the exact nature of the interactions of imidazotetrazinones within the micro-enviroment of DNA are as yet unknown. In order to examine such interactions a three pronged approach involving molecular modelling, synthetic chemistry and biological analysis has been undertaken during the course of this project.

Molecular modelling studies have shown that for the 8-carboxamido substituted imidazotetrazinones antitumour activity is dependent upon the presence of a free NH group which can be involved in the formation of both intramolecular and intermolecular hydrogen bonds, and the presence of a non-bulky substituent with a small negative potential volume. Modelling studies involving the docking of mitozolomide into the major groove of DNA in the region of a triguanine sequence has shown that a number of hydrogen bonding interactions are feasible.

A series of 8-substituted carboxamide derivatives of mitozolomide have been synthesised *via* the 8-acid chloride and 8-carboxylic acid derivatives including a number of peptide analogues. The peptide derivatives were based upon the key structural features of the helix-turn-helix motif of DNA-binding proteins with a view to developing agents that are capable of binding to DNA with greater selectivity. An examination of the importance of intramolecular hydrogen bonding in influencing the antitumour activity of the imidazotetrazinones has led to the synthesis of the novel pyrimido[4´,5´:4,3]pyrazolo[5,1-d]-1,2,3,5-tetrazine ring system. In general, *in vitro* cytotoxicity assays showed that the new derivatives were less active against the TLX5 lymphoma cell line than the parent compound mitozolomide despite an increased potential for hydrogen bonding interactions.

Due to the high reactivity of the tetrazinone ring system it is difficult to study the interactions between the imidazotetrazinones and DNA. Consequently, a number of structural analogues that are stable under physiological conditions have been prepared based upon the 1,2,3-triazin-4(3H)-one ring system fused with both benzene and pyrazole rings. Although the 3-methylbenzotriazinones failed to antagonise the cytotoxic activity of temozolomide encouraging results with a 3-methylpyrazolotriazinone may suggest the existence of an imidazotetrazinone receptor site within DNA.

The potential of guanine rich sequences to promote the alkylating selectivity of imidazotetrazinones by acting as a catalyst for ring cleavage and thereby generation of the alkylating agent was examined. Experiments involving the monitoring of the rate of breakdown of mitozolomide incubated in the presence of synthetic oligonucleotides did not reveal any catalytic effect resulting from the DNA. However, it was noted that the breakdown of mitozolomide was dependent upon the type of buffer used in the incubations and this may indeed mask any catalysis by the oligonucleotides.

Keywords: imidazotetrazinones, triazenes, triazines, sequence-specific, alkylating agents.

To the memory of my father.

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CONTENTS. Page No.

CONTENTS.

LIST OF SCHEMES.

LIST OF FIGURES.

LIST OF TABLES.

LIST OF ABBREVIATIONS.

A	Adenine.
AIC	5-aminoimidazole-4-carboxamide.
Ala	Alanine.
Asn	Asparagine.
BCNU	1,3-Bis(2-chloroethyl)-nitrosourea.
C	Cytosine.
5´CMP	Cytosine-5´-monophosphate.
2D COSY	Two-dimensional correlation spectroscopy.
dec.	Decomposed.
DMF	Dimethylformamide.
DMSO	Dimethylsulphoxide.
DTIC	5-(3,3-Dimethyltriazen-1-yl)imidazole-4-carboxamide.
G	Guanine.
Gln	Glutamine.
5´GMP	Guanosine-5´-monophospate.
HPLC	High Pressure Liquid Chromatography.
Ile	Isoleucine.
i.v.	Intravenous.
lit.	Literature.
MCTIC	5-[3-(2-Chloroethyl)triazen-1-yl]imidazole-4-carboxamide.
MTIC	5-(3-Methyltriazen-1-yl)imidazole-4-carboxamide.
NCI	National Cancer Institute.
RPMI	Rosewell Park Memorial Institute.
Ser	Serine.
T	Thymine.
Thr	Threonine.
Val	Valine.

INTRODUCTION.

1.1 Foreword.

The word **cancer** has strong emotive connotations, for it is the second most frequent cause of death in the U.K., and to many people remains incurable. Moreover, despite the synthesis and testing of 1000's of compounds no "magic bullet" has yet been discovered. No single agent has been found to be active against all tumour types and indeed many tumours have developed forms of resistance to cytotoxics. Furthermore, all the compounds that have reached the clinic have deleterious side-effects mainly associated with lack of selectivity between neoplastic and normal cells, especially normal proliferative tissues of the skin, bone-marrow, hair and intestinal lining. Toxicity is often a limiting factor in drug treatment, and so the ultimate goal of the design of a tumour specific agent is much sought after.

In order to produce better agents there is a need for a greater understanding of the biochemistry of tumour and normal cells, as well as a knowledge of how existing drugs act, especially why some drugs are ineffective in certain cases.

What has become obvious is that there are few defined biochemical differences between normal and cancerous cells that are capable of being exploited. The heterogeneous mixture of cells in many tumours, with some cells being drug resistant, often leads to resistant sub-populations of cells which are able to re-establish the tumour after the sensitive population has been destroyed. To further complicate therapy a proportion of all tumour tissue will be quiescent and so not vulnerable to drug treatment, while metatases lead to dissemination of the tumour throughout the body, often to sites which are difficult to reach with chemotherapy. Solid tumours present special problems, not least because their poor vascularisation prevents the effective distribution of the drug to the target area.

Despite the problems, success has been achieved against certain cancers, most notably choriocarcinoma, Hodgkin's disease and childhood lymphatic leukaemia. The use of combination therapy (drug : drug, surgery : drugs and radiotherapy : drugs) offers new and exciting possibilities for the use of the existing clinical agents.

Most antitumour agents may be broadly classified as:[1]

(i) alkylating agents - compounds which lead to replacement of hydrogen atoms of the DNA bases with alkyl groups to give covalent linkages, disrupting the ability of DNA to act as a template e.g. nitrogen mustards.

(ii) antimetabolites - compounds which interrupt the supply of precursors necessary for DNA synthesis e.g. methotrexate.

(iii) intercalating agents - compounds which insert themselves between DNA bases leading to distortion of the helix and a disruption of DNA synthesis e.g. adriamycin.

(iv) vinca alkaloids - naturally occurring compounds which damage the mitotic spindle preventing normal cell division e.g. vinblastine.

1.2 The design of alkylating agents.

One of the main aims of this project is to synthesise imidazotetrazinones with a greater selectivity for their ultimate target - the DNA of tumour cells. The following section deals with the advances that have been made in the preparation of tumour specific alkylating agents, and is by no means a comprehensive review of all molecules that interact with DNA.

Ever since the discovery of the cytotoxicity of sulphur mustard,[2] alkylating agents have been intensively investigated. Their importance should not be underestimated as they currently make up over half of the drugs in clinical use. It is fair to say that alkylating agents have developed in three distinct phases:[3]

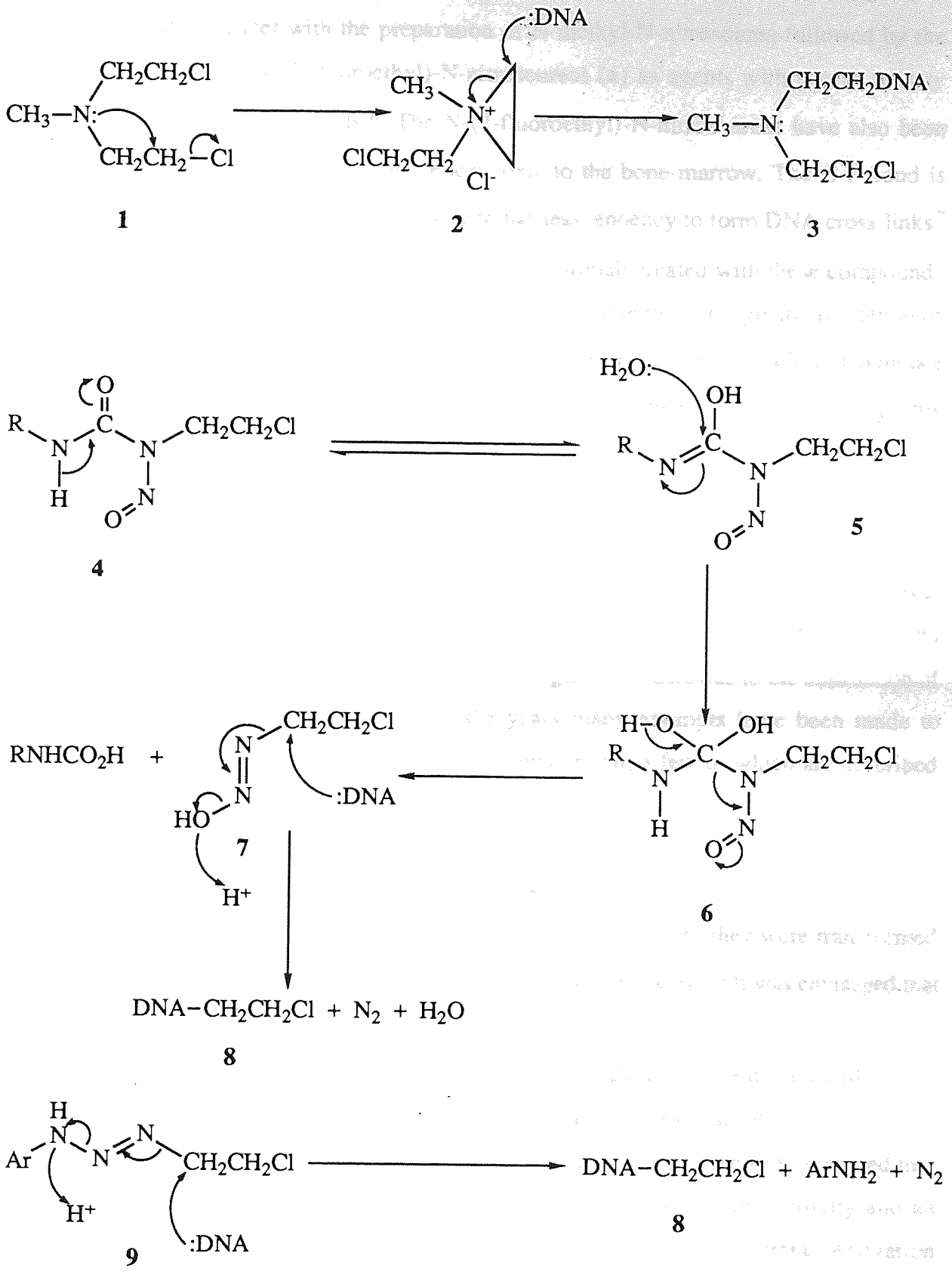
(a) the synthesis of novel alkylating functions leading to the preparation of the first generation clinical agents.

(b) the search for structures with greater tumour selectivity utilising theoretical principles e.g. latent activity and carrier molecules, leading to the second generation agents.

(c) the search for tumour directed agents using newly developed techniques, such as mono-clonal antibodies, and DNA binding groups capable of recognising specific base sequences.

1.2.1 Alkylating functionalities.

An immense number of moieties have been prepared that are capable of acting as alkylating agents, however remarkably few have proved sufficiently active against tumour cells to warrant further investigation (Scheme 1.1). The first type of agent to prove of benefit in cancer chemotherapy was nitrogen mustard (**1**),[4] the N,N-bis(2-chloroethyl)amino group has subsequently proved to be a potent alkylating function and has been shown to exert its alkylating activity *via* the formation of the positively charged



Scheme 1.1: Common alkylating functions and their modes of action.

aziridinium ion (**2**).[1] Johnston *et al.*[5] indicated the potential of the N-nitrosoureido group in the treatment of cancer with the preparation of N-methyl-N-nitrosourea followed by the identification of the N-(2-chloroethyl)-N-nitrosoureas (**4**) as agents with potent activity against murine tumour models.[6] The N-(2-fluoroethyl)-N-nitrosoureas have also been shown to possess good activity but are less toxic to the bone-marrow. The C-F bond is more stable than its chloro counterpart and so has less tendency to form DNA cross-links[7] (see page 47), however the mean survival time of animals treated with these compounds was significantly less than that of the chloro derivatives due to the enzymatic formation of the highly toxic fluoroacetate from 2-fluoroethanol.[8] The nitrosoureas are believed to initiate cell death by the generation of an alkyldiazo hydroxide alkylating species (**7**), although this is far from certain. The final type of alkylating species that has shown some promise in the treatment of cancer are the triazene (**9**) series of agents, of which DTIC has achieved extensive use in the clinic.[9]

A major problem with the alkylating agents that were first developed was their lack of selectivity for tumour cells over normal cells, a problem that was often manifested in the form of life-threatening haematological disorders due to the destruction of the rapidly dividing bone-marrow. Over the years many attempts have been made to improve the selectivity of these agents for the tumour cell, a few of which are described below.

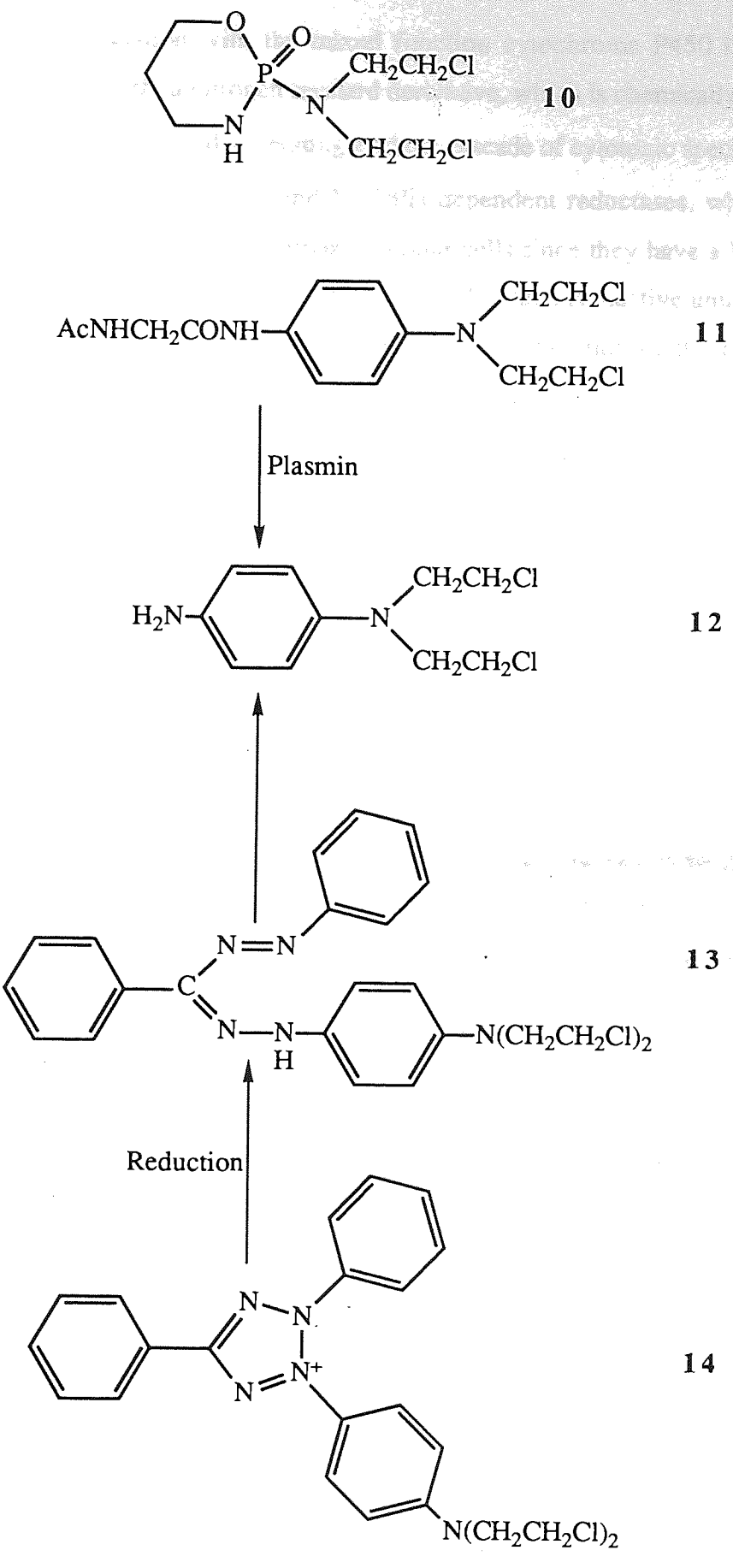
1.2.2 Alkylating agents with latent activity.

The concept of using agents that were inactive until they were transformed *in vivo* to a highly reactive species was first postulated by Druckrey.[10] It was envisaged that selectivity could be improved as a result of:

(a) the achievement of a greater concentration of the active alkylating agent in the cell.

(b) selective activation within the tumour cell to release the alkylating agent.

Consequently a range of compounds (Scheme 1.2) have been prepared that are usually characterised by a low molecular weight, an optimum lipophilicity and an unionised form thereby allowing passive transport across the cell membrane. Activation may occur *via* three mechanisms:



Scheme1.2: Latent alkylating agents.

(a) microsomal oxidation with the mixed function cytochrome P450 isoenzymes e.g. cyclophosphamide (**10**), a nitrogen mustard derivative, which is chemically inactive ($t_{1/2}$ > 7 days), but which is oxidised generating a whole cascade of cytotoxic species.[3]

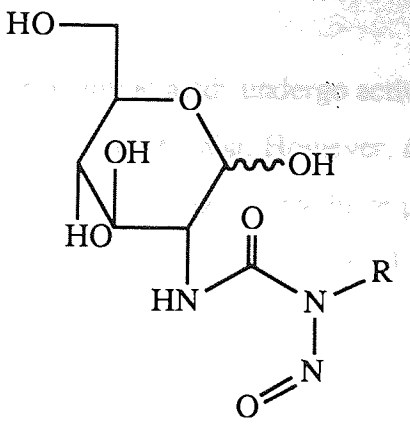
(b) reduction *via* nitroreductases and NADPH dependent reductases, which was hoped would induce selective killing of hypoxic tumour cells since they have a higher reducing potential e.g. the tetrazolium mustard derivative (**14**) which is inactive until reduced to the unstable formazan derivative (**13**). Spontaneous decomposition of the formazan group yields the highly toxic ρ-phenylenediamine nitrogen mustard (**12**).[11]

(c) intra- or extra-cellular enzymatic hydrolysis utilising enzymes that are specific for, or at least more abundant in, neoplastic cells e.g. the nitrogen mustard derivative (**11**) which is inactive except in the presence of plasmin. In some tumours high local concentrations of plasmin have been found, due to the over secretion of plasminogen activator, which is responsible for generating the ρ-phenylenediamine nitrogen mustard (**12**) by cleavage of the amide bonds.[11]

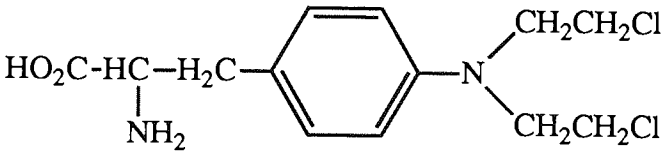
1.2.3 Alkylating agents linked to carrier molecules.

This idea was based on the theory that certain molecules accumulate, against a gradient, in tumour cells to a greater extent than normal cells, a phenomenon known as active transport. By coupling alkylating agents to naturally occurring molecules it was hoped to take advantage of tumour specific active transport systems. A wide range of physiological molecules have been used in an attempt to achieve these goals (Figure 1.1):

(a) carbohydrates: it was anticipated that the differential energy requirements that are seen with tumour cells could be exploited by coupling alkylating agents to sugar moieties. Although several interesting compounds have been prepared, the most notable being the nitrosoureas streptozocin (**15**) and chlorotozocin (**16**), none of these compounds have yet enjoyed widespread use. Indeed, there is no correlation between their activity and active transportation. Streptozocin and chlorotozocin have proved interesting since they have significantly less myelosuppressive tendencies which is not seen with other sugar derivatives. The reason for this is unclear, however, myelosuppression has been found not to be a function of alkylating activity, carbamoylating activity or hydrophilicity and appears to be more mediated by the glucose residue directing alkylation to different sites in the bone-



R = Me (Streptozocin) **15**
CH_2CH_2Cl (Chlorotozocin) **16**



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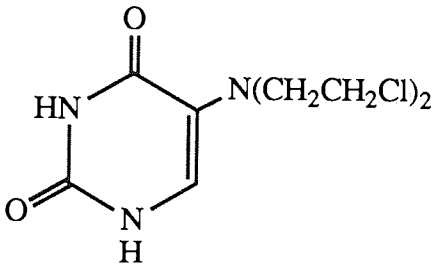

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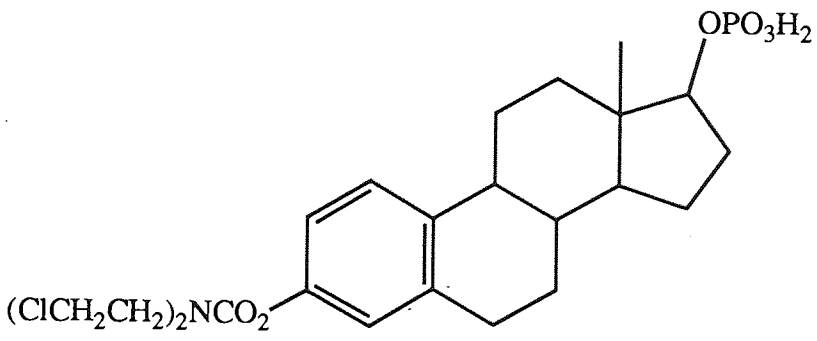


19

Figure 1.1: Carrier linked alkylating agents.

marrow chromatin.[12]

(b) amino acids: a number of amino acids undergo active transport into cells, a factor which was not missed by the medicinal chemist. However, despite the synthesis of hundreds of alkylating agents as amino acid analogues few have proved effective and in cases where agents such as L-PAM (**17**) have reached the clinic selectivity was disappointing. Analogously to the linking of carbohydrates no direct evidence has been found to indicate that active transportation of these agents occurs *in vivo*, although the observation that the activity of L-PAM is enantio-specific, the D-isomer is inactive, lends hope to this theory.[3]

(c) purines and pyrimidines: the knowledge that rapidly dividing cells have a greater requirement for nucleotides suggested that analogues based on the naturally occurring bases would lead to selective uptake into tumour cells. Compounds such as uracil mustard (**18**) were developed, which although it possessed good activity proved to have serious side-effects preventing its clinical use.[3]

(d) steroids: several types of cancers have been shown to be hormone dependent, a factor that has been shown to be mediated *via* specific oestrogen (ER) and progesterone (PR) receptors on the cell membrane. Alkylating agents have been linked to the steroid nucleus in order to exploit this unique active transport mechanism. Early attempts produced compounds that were highly active but which displayed little selectivity. However, the nitrogen mustard derivative of 17-β-oestradiol (**19**) has displayed some affinity for the ER receptor and is selectively active against prostatic carcinoma. It is interesting to note that this compound could also be classified as a latent alkylating agent since the nitrogen mustard is included in a urethane type linkage which requires enzymatic hydrolysis in order to produce activity.[3]

(e) liposomes: these are phospholipid vesicles which enclose an aqueous environment. A great deal of attention has been placed on the use of liposomes as vehicle for drug delivery, the active ingredient being incorporated in the aqueous space if hydrophilic, or in the lipid bilayer if it is hydrophobic.[13] Their role in cancer chemotherapy is still under investigation, however, as yet there appears to be no reason to suspect that drugs in liposomes are selectively accumulated in tumour cells.[11] Indeed, they have been shown to have the potential to accumulate in the bone-marrow an organ in which the release of alkylating

agents is anything but desired.[13] However, the advent of antibody technology could spark a whole new revolution in the targeting of liposomes to tumour cells.

As it has been outlined many attempts have been made to induce greater selectivity in alkylating agents with only modest success. The advent of the third generation agents currently under development may prove that at last we are now closer to the preparation of tumour specific compounds.

1.2.4 The third generation?

Advances in molecular biology and synthetic chemistry have now opened up a whole new area for the design of selective antitumour agents. The following approaches are currently receiving much attention:

(a) the use of antibodies that are generated to specific tumour associated antigens (TAA). A number of drugs, including the alkylating agent chlorambucil, and toxins, have been linked to monoclonal antibodies with reasonable success.[14] However, even with this approach problems have become evident, not least the observation that tumours are capable of shedding their antigenic sites (a protective mechanism preventing attack by the host), while only small amounts of the drug can be delivered to the site of action requiring substances of high potency, such as diphtheria or ricin toxins. A recent development that may prove interesting is the use of antibody directed enzyme/prodrug therapy (ADEPT) which involves the targeting of enzymes responsible for the selective activation of specific antitumour prodrugs to localised sites on the cell membrane or to extracellular sites around tumour cells. It is anticipated that it should be possible to direct to tumour targets an enzyme that matches a particular prodrug thereby overcoming the need for a natural, favourable, distribution of the enzyme.[15]

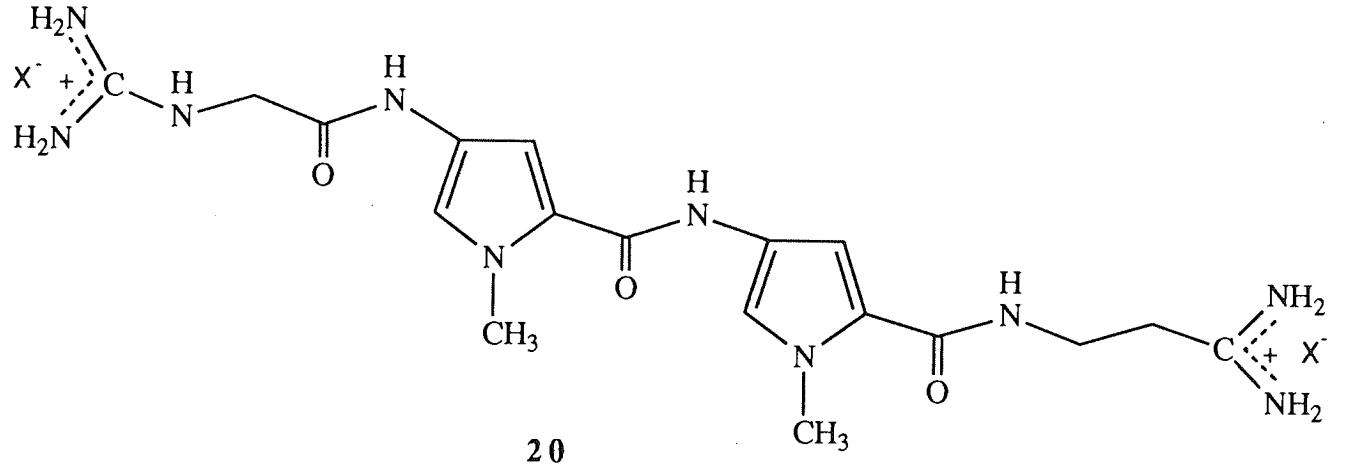
(b) a variation on the carrier principle is the design of molecules with a high sequence-specific affinity for DNA, to which alkylating agents could be linked in order to react with designated targets, such as oncogenes. In military terms this represents a guided missile with the alkylating agent as the warhead. To date three main approaches have been used which are outlined in the following sections:

(i) groove binders:[16] Nature has provided us with two distinct examples of sequence-specific groove-binders. The first involves the binding of the proteins which regulate gene expression to the major groove of DNA e.g. promoters and repressor proteins which have well defined primary and secondary structures enabling them to bind specific sequences of DNA. Little or no attempt has been made to utilise this natural phenomenon in the development of specific alkylating agents, probably because such molecules would be extremely complex and rational design is difficult since there is no simple code that determines which amino acids recognise which DNA bases.

The second example are the antiviral antitumour antibiotics netropsin (**20**) and distamycin (**21**) (Figure 1.2). These agents have been shown to bind in the minor groove of DNA specifically at runs of A-T sequences. Electrostatic attraction between the amidine function of (**20**) and (**21**) promotes the initial binding, then hydrogen bonding of the amide linkages orientates the drug in the proper reading frame. Krowicki and Lown[17] have shown that replacement of the pyrrole ring by an imidazole ring produces analogues with a greater preference for G-C regions. This has led to the proposition that the recognition of any DNA sequence could be achieved by the construction of suitably phased A-T and G-C recognising units - the so-called lexitropsins or information reading oligopeptides.

Baker and Dervan[18] have incorporated the bromoacetyl alkylating function into the tripeptide tris-(N-methyl)-pyrrolecarboxamide unit of distamycin (**22**) and obtained selective alkylation of the N3-position of adenine adjacent to the binding site, suggesting that such compounds have great promise in directing DNA alkylation.

However, this approach is not devoid of problems. Despite valiant attempts to produce specific G-C recognition units, those that have been prepared do not have a high affinity for G-C sites in comparison with their A-T binding counterparts. Moreover, in order to bind a unique cellular control sequence (approx. 15-17 base pairs) a large lexitropsin would be required with all the problems of delivery to the cell that it entails. While it remains to be seen whether the minor groove of DNA inherently has sufficient sequence specificity information to give rise to the level of selectivity required to successfully 'read' DNA, nature by its protein-DNA interactions, would suggest the major groove is a better target.



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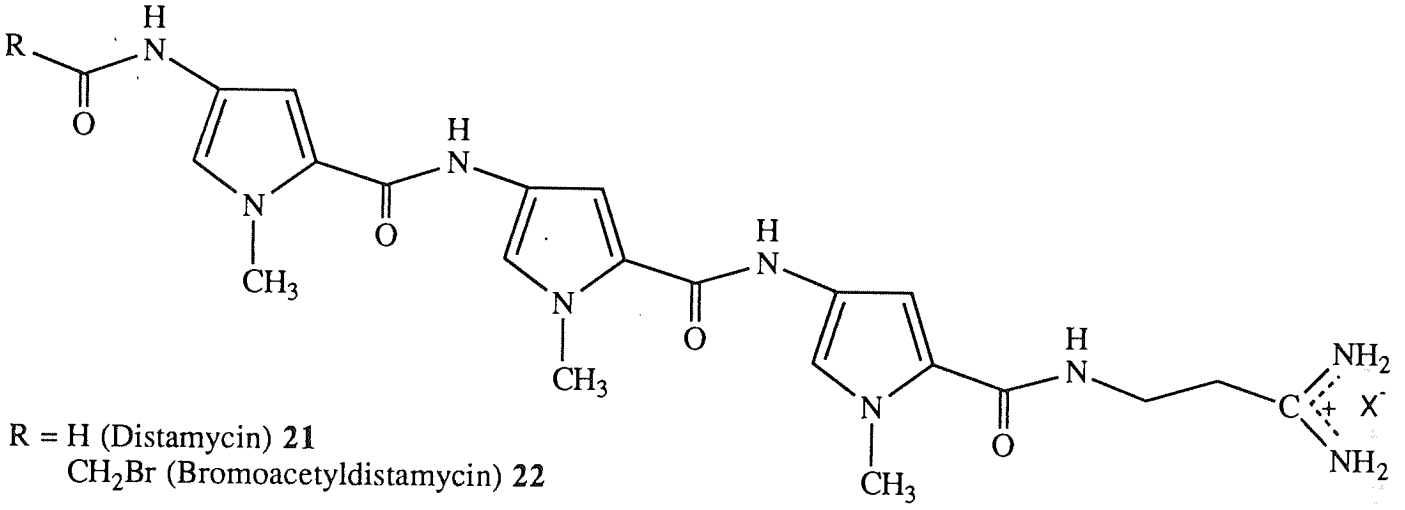
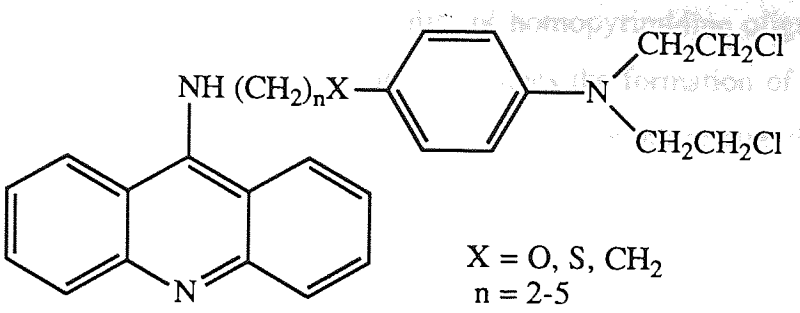


R = H (Distamycin) **21**
CH_2Br (Bromoacetyldistamycin) **22**

Figure 1.2: Minor groove binding antibiotics.

(ii) intercalating analogues (Figure 1.3): intercalators are ligands which have a strong, but reversible, affinity for DNA by the insertion of a flat aromatic chromophore between base pairs and as such can be said to be DNA selective. As early as 1972 Creech and his colleagues[19] showed that heterocyclic components of simple mono- and bifunctional alkyl mustards had a potentiating influence on activity against ascites tumour. This was shown to be a result of the high affinity of the chromophore for DNA, since changes in the carrier which would be expected to increase DNA binding resulted in enhanced potency.[20] Gourdie *et al.*[21,22] have since synthesised a series of aniline mustards linked by a flexible alkyl chain to the DNA-intercalator 9-aminoacridine (**23**). The DNA targeted mustards proved more potent *in vitro* and to some extent *in vivo* than either the corresponding untargeted mustards or chlorambucil. A striking feature of these molecules was the fact that a change in the length of the flexible alkyl chain allowed significant degrees of alkylation of the N7 position of specific adenine residues.[23] A similar degree of sequence discrimination was observed with quinacrine mustard (**24**) which only alkylated guanine residues flanked on the 3′ side by either a guanine or thymine, and then by a purine[24] indicating that the future may see the development of agents capable of directing alkylation to any desired base sequence.

(iii) oligonucleotide analogues: oligonucleotides have been demonstrated to bind to complementary sequences of single-stranded DNA and RNA through hydrogen bonding of the base pairs - otherwise known as complementary address.[25] Belikova[26] over 20 years ago suggested that oligonucleotides had conceivable chemotherapeutic applications in directing DNA reactive functions. Early studies used simple oligonucleotides with N-2-chloroethyl-N-methylaminophenyl (**25**) as the alkylating group, however, initial results were disappointing probably because the oligonucleotide sequences were too short to enable stable hybridisation of the duplexes. The stability of small duplexes was improved by the incorporation of the phenazinium intercalating group (**26**) at the opposite end of the oligonucleotide from the alkylating function enabling alkylation of residues adjacent to the region of complementary address.[27] Kutyavin and co-workers[27] also showed that such analogues were capable of directing alkylation within the stems of hairpin structures of single-stranded DNA indicating that reaction with double-stranded DNA was possible.



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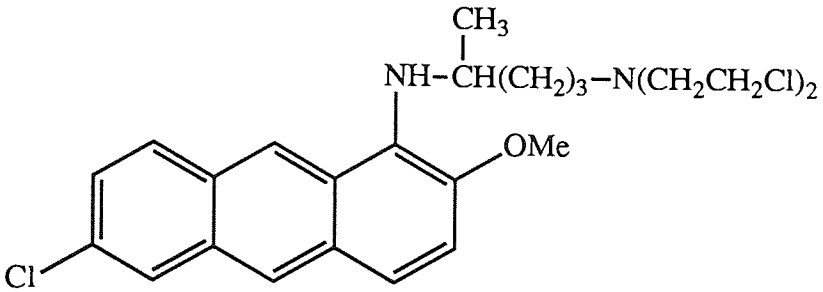

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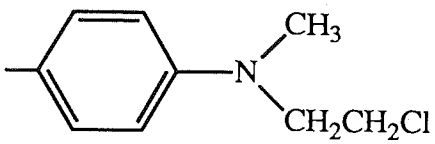

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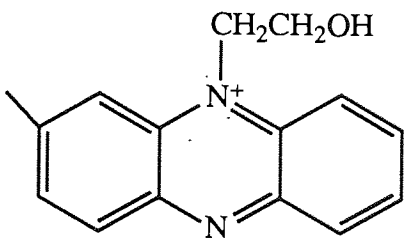


26

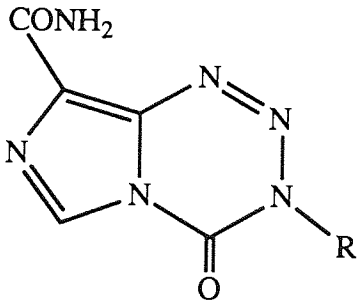
Figure 1.3: Targeting groups used in the selective modification of DNA.

Recent studies[28] have shown that selective alkylation of sites in double-stranded DNA can be achieved using the ability of homopyrimidine oligonucleotides to recognise homopurine sequences. Recognition involves the formation of a triple helical structure with the oligonucleotide chain binding parallel to the purine strand *via* Hoogsteen hydrogen bonds.[29]

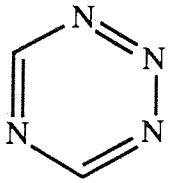
The clinical development of oligonucleotide derivatives is fraught with problems mainly associated with delivery into the target cell.[25] Oligonucleotides with the natural phosphate linkage between the sugars are susceptible to the nuclease group of enzymes, leading to rapid degradation in serum. Linkages resistant to nucleases have been prepared. The phosphate linkage can be replaced with a methylphosphonate linkage, but this introduces chirality into the system which for long oligonucleotides gives a large number of diastereomers each with a different binding propensity. Changing the sugar conformation from the natural β to the unnatural α form tends to concentrate the oligonucleotide in the cytoplasm rather than the nucleus.

1.3 Imidazotetrazinones.

The imidazotetrazinones are a novel group of agents which were synthesised in the hope that they would possess potent antitumour activity. The lead compound in this series, mitozolomide or 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (**27**) was synthesised at Aston in late 1980 by Stone and Stevens.[30] Mitozolomide was shown to have excellent antitumour activity in both *in vitro* and murine *in vivo* tests, which led to phase I and II clinical trials. Unfortunately, the production of a prolonged and marked thrombocytopenia thwarted further clinical development. However, the second generation agent temozolomide (**28**) has now reached phase II clinical trials with encouraging results against the melanoma and glioma tumour types. This thesis represents an attempt to synthesise, in a rational manner, new imidazotetrazinone derivatives with improved DNA specificity and to increase the general understanding of how these compounds exert their biological effects.



R = CH_2CH_2Cl (**27**)
CH_3 (**28**)



29

1.3.1 Synthesis of the 1,2,3,5-tetrazine system (Scheme 1.3).

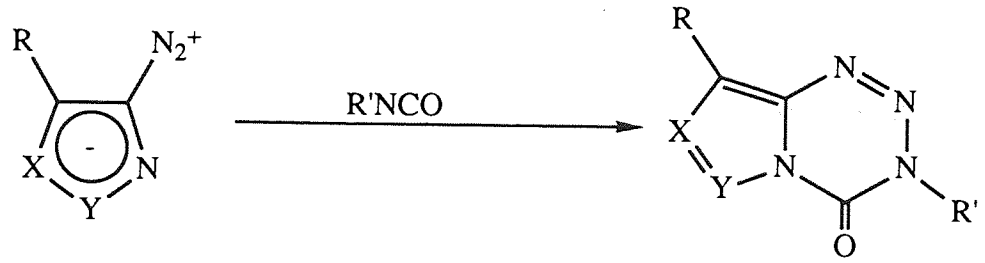
The previously unknown 1,2,3,5-tetrazine ring (**29**) system was first reported by Ege and Gilbert in their preparation of pyrazolo[5,1-d]-1,2,3,5-tetrazin-4(3H)-ones (**35**) by the cycloaddition of an isocyanate with a diazopyrazole (**31**).[31,32] Stone *et al.*[30,33] utilised this reaction in the synthesis of a series of imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-ones (**34**) with the intention that the inherent instability of the ring system would lead to degradation and the production of an array of products with potential antitumour activity. For example cleavage of the 2,3 and 4,5 bonds of mitozolomide would lead to the release of 2-chloroethyl isocyanate, while nucleophilic attack at C4 would cause the breakage of the 3,4 and 4,5 bonds leading to the generation of the antitumour triazene MCTIC (see Scheme 1.5, page 34).

Pyrazolotetrazinones (**35**) were subsequently synthesised *via* two new distinct routes:[34]

(i) diazotisation of N-carbamoylaminopyrazoles (**38**).

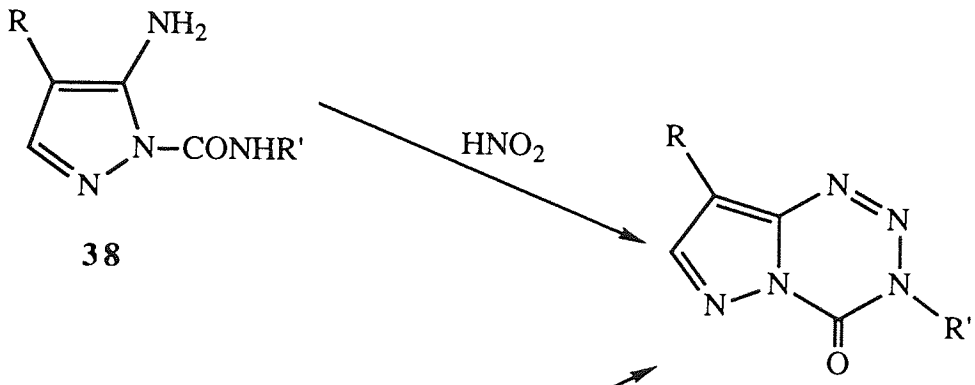
(ii) insertion of the C4 carbonyl moiety by the reaction of the triazene (**39**) with phosgene or its equivalents.

The tetrazinone ring has since been prepared fused with triazolo (**36**)[34] and indazolo (**37**)[35] ring systems, although the potentially interesting pyrrolo- and tetrazolo-tetrazinones remain unknown. Claims have been made for the preparation of the 1,2,3,5-tetrazine ring without fusion to an azole. However, the literature contains only one definite report of a 2,4,5,6-tetrasubstituted 1,2,3,5-tetrazine (**41**) prepared by the cycloaddition of



X = N, Y = CH (**30**)
X = CH, Y = N (**31**)
X = Y = N (**32**)
X and R = -CH=CH-CH=CH-, Y = N (**33**)

X = N, Y = CH (**34**)
X = CH, Y = N (**35**)
X = Y = N (**36**)
X and R = -CH=CH-CH=CH-, Y = N (**37**)



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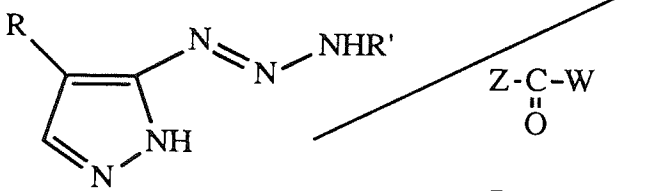

Z = W = Cl, $OCCl_3$, Imidazole

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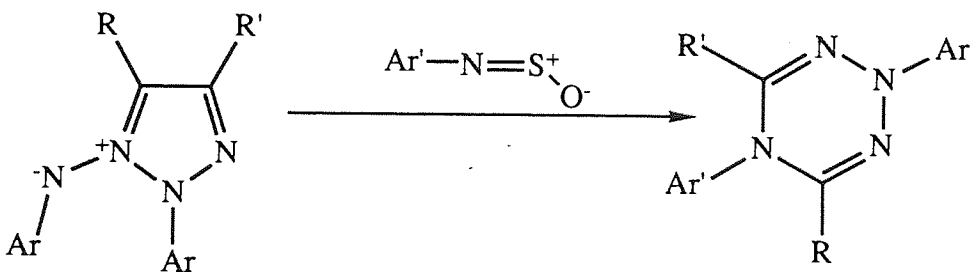

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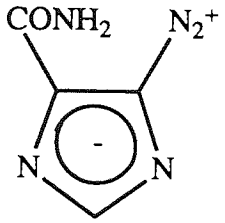
Scheme 1.3: Synthetic routes to the 1,2,3,5-tetrazine ring system.

1,2,3-triazolium imides (**40**) with aryl-N-sulphinylamines.[36]

1.4 Synthesis of imidazotetrazinones.

Mitozolomide (**27**) was originally synthesised by the reaction of 5-diazoimidazole-4-carboxamide (**42**) with 2-chloroethyl isocyanate, in a dry non-hydroxylic solvent, in the dark, at room temperature.[30] Strict observance of the conditions were required since the prolonged reaction times could lead to the formation of 2-azahypoxanthine (**43**) in the presence of water and UV light.[30]



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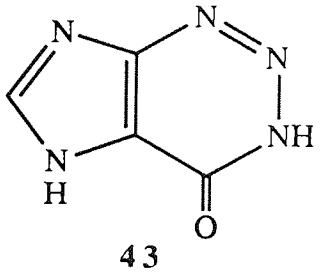

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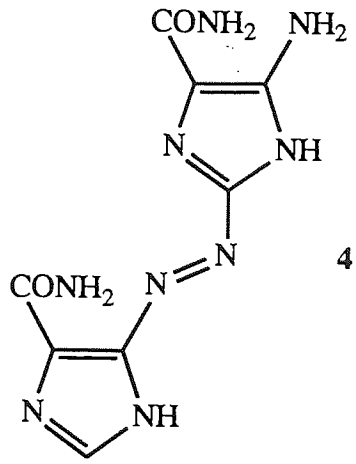
The reaction proved rewardingly adaptable to exploitation with a range of diazoimidazoles and isocyanates. However, no tetrazinones were formed with cyclohexyl, n-butyl, t-butyl, n-tridecyl and n-pentadecyl isocyanates probably due to steric hindrance.[30] Diazoimidazoles also failed to react with other heterocumulenes such as phenyl-(ρ-tolyl)-isothiocyanate, N,N´-diphenyl, N,N´-di-(ρ-tolyl) and N,N´-dicyclohexylcarbodiimides.[30]

Yields, in general, from this reaction were good except in the case of diazoimidazoles with a bulky substituent in the 2-position where the steric bulk of the substituent presumably hinders reaction with the isocyanate. Crude imidazotetrazinones were often contaminated with a maroon pigment which was shown to be the imidazoylazoimidazole (**44**) formed by the reaction of the 5-diazoimidazole (**42**) with some contaminating 5-aminoimidazole.[30,33] Purified tetrazinones were cream or pastel coloured solids that were soluble in DMF, DMSO and 1-methylpyrrolidin-2-one, sparingly soluble and unstable in alcohols.[30] Imidazotetrazinones could be stored without deterioration for several months provided they were kept dry and protected from light.[30]



44

The mechanism for the formation of the tetrazinones has yet to be fully explained, however, three possible pathways have been suggested (Scheme 1.4):[30,37,38]

(i) a concerted [4+2] cycloaddition - however, Gilchrist and Storr[39] have rejected concerted mechanisms in the reaction of heterocumulenes as dipolarophiles.

(ii) a [3+2] cycloaddition *via* an unstable spirobicycle (**47**), with a [1,5] sigmatropic shift - an analogous mechanism has been postulated for the reaction of diazopyrazoles with 1,1-dimethoxyethene.[40]

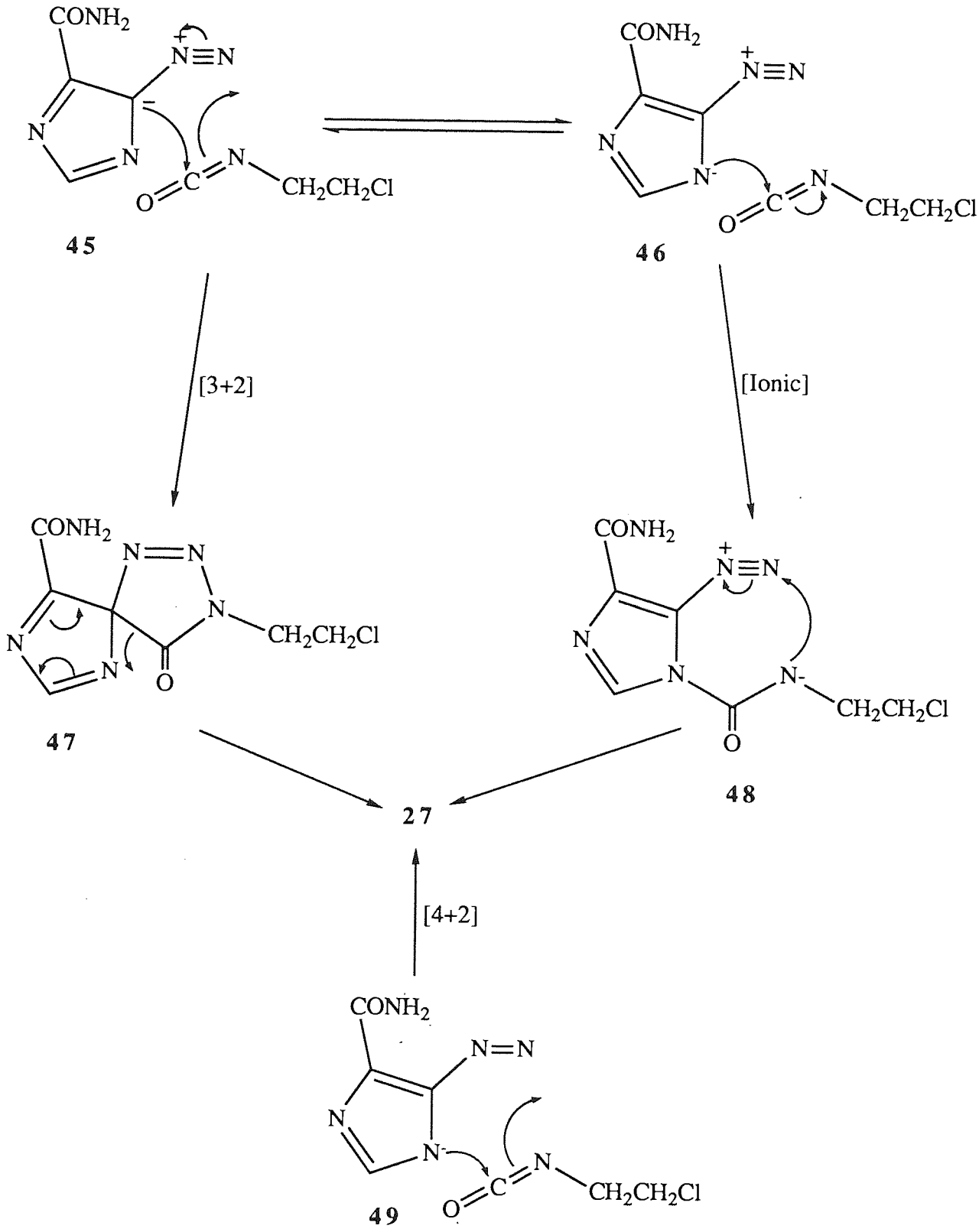
(iii) an ionic mechanism, involving nucleophilic attack at the electrophilic isocyanate carbon, followed by ring closure of the dipolar intermediate (**48**). The observation that the reaction rate was increased when hexamethyltriphosphoramide (HMPA) was used as the reaction medium led Stone to suggest this was the most conceivable mechanistic route.[30]

1.5 Chemical properties of the imidazotetrazinones.

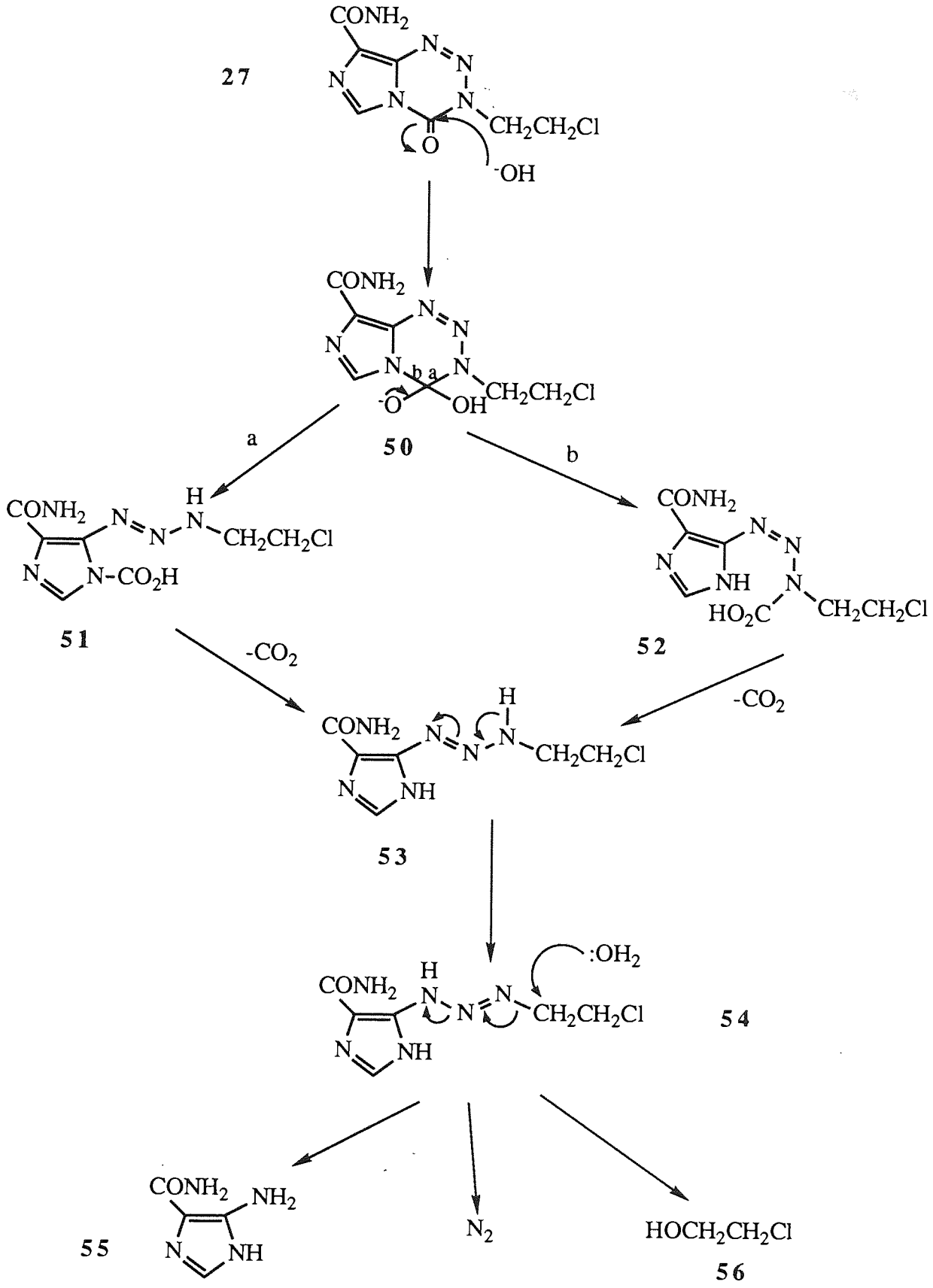
The chemical reactions of imidazotetrazinones can be neatly classified into two main groups:[38]

(i) reactions in which fragmentation of the tetrazinone ring occurs with the generation of highly reactive intermediates.

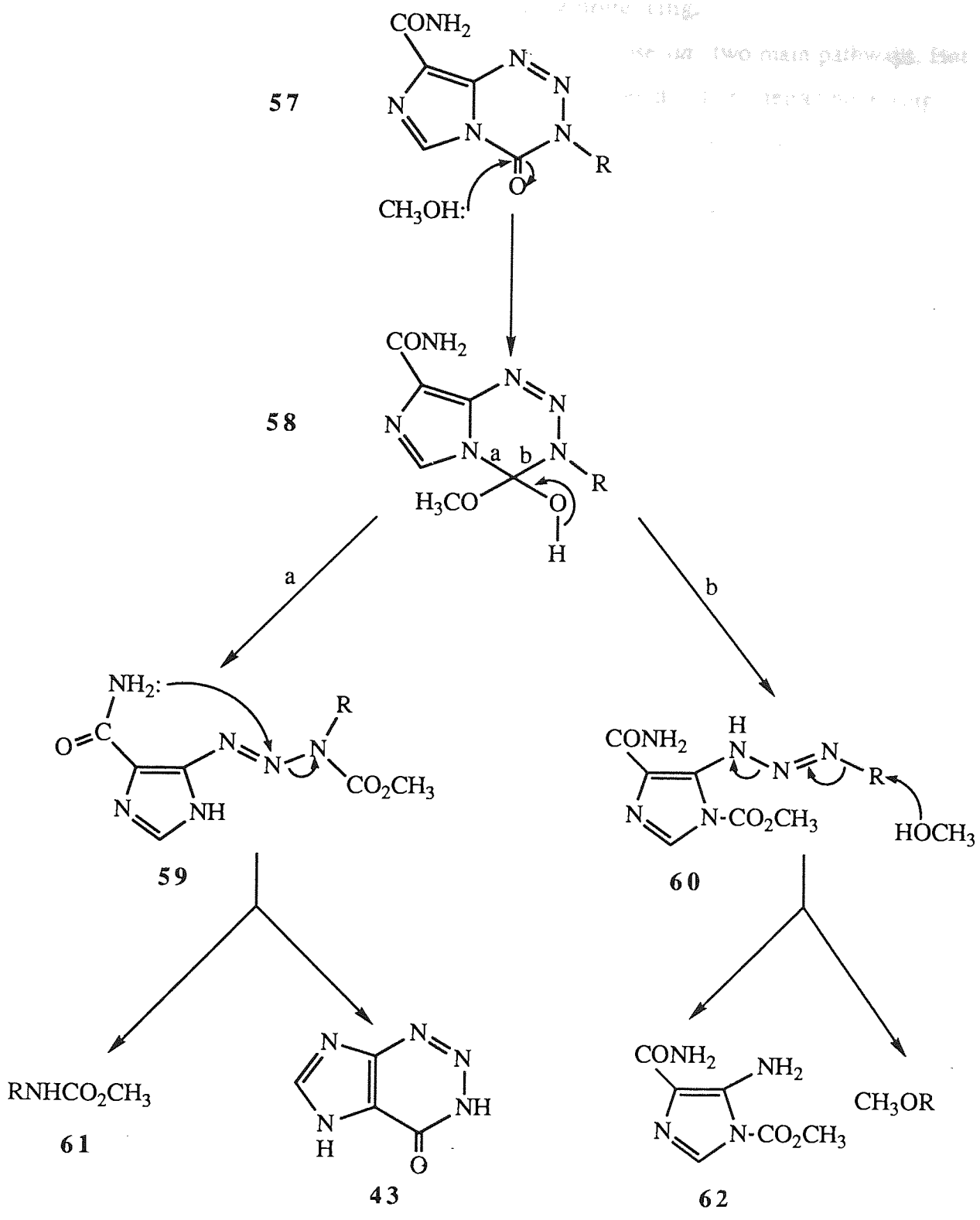
(ii) reactions in which the tetrazinone ring is retained with the preparation of novel analogues.



Scheme 1.4: Possible mechanisms for the formation of the tetrazinone ring system.



Scheme 1.5: Mechanism for the hydrolysis of mitozolomide.



Scheme 1.6: The decomposition of imidazotetrazinones in methanol where R = -CH_2CH_2Cl, -Me and -Et.

1.5.1 Reactions involving loss of the tetrazinone ring.

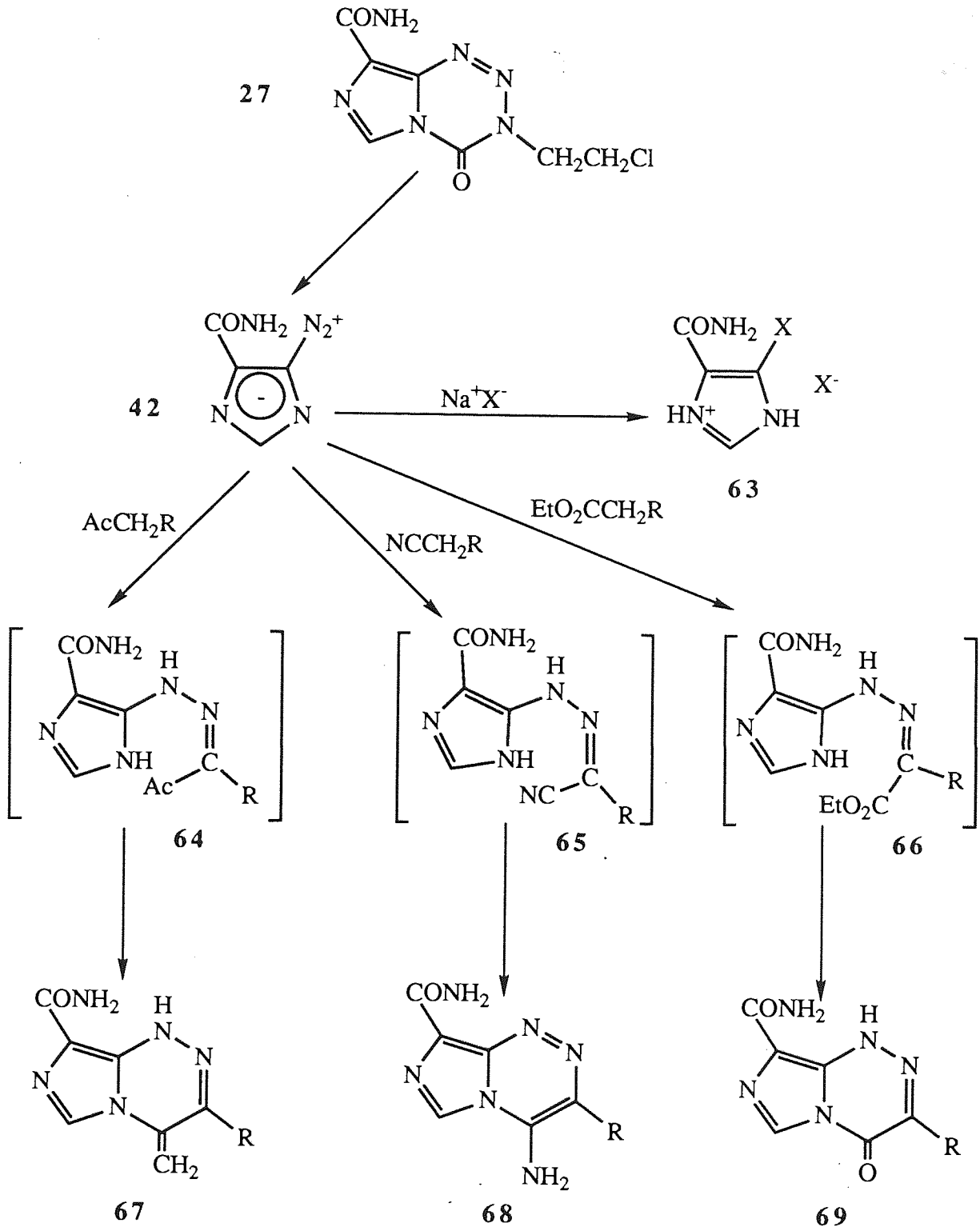
Mitozolomide (**27**) was found to decompose *via* two main pathways. Hot acetonitrile led to fragmentation of the 2,3 and 4,5 bonds of the tetrazinone ring to regenerate 5-diazoimidazole-4-carboxamide (**42**) and 2-chloroethyl isocyanate.[33] The mechanism for this reversal of the synthetic procedure probably involves a [1,5] sigmatropic shift *via* an unstable spirobicycle (c.f. mechanisms for the formation of the tetrazinone ring, Scheme 1.4). In contrast the aqueous stability of the imidazotetrazinones is very much pH dependent. The tetrazinone ring is remarkably stable under acidic conditions ($t_{1/2}$ at pH 4 is 240 hours)[41] and indeed mitozolomide can be obtained unchanged from concentrated sulphuric acid at 60-65°C. However, at physiological pH, degradation of the tetrazinone ring is initiated by nucleophilic attack by water at C4 to form (**50**). Decomposition of (**50**), by cleavage of either the 3,4 or 4,5 bonds might then give the unstable carbamic acids (**51** and **52**); loss of CO_2 then gives the unstable triazenes (**53** and **54**). Alkylation of a water molecule, by (**54**), leads to the formation of 5-aminoimidazole-4-carboxamide (**55**), nitrogen and 2-chloroethanol (**56**) (Scheme 1.5).[33]

Baig and Stevens[42] have shown that in the presence of oxygen and nitrogen containing nucleophiles initial attack also occurs at C4 to give a tetrahedral adduct, for example the reaction with methanol (Scheme 1.6). Further decomposition was dependent upon the nature of the 3-substituent. Mitozolomide (3-CH_2CH_2Cl) appears to cleave exclusively at the 4,5 bond (route a), whereas temozolomide (3-Me) and the 3-ethyl analogue undergo cleavage at the 4,5 bond and to a minor extent at the 3,4 bond (route b).

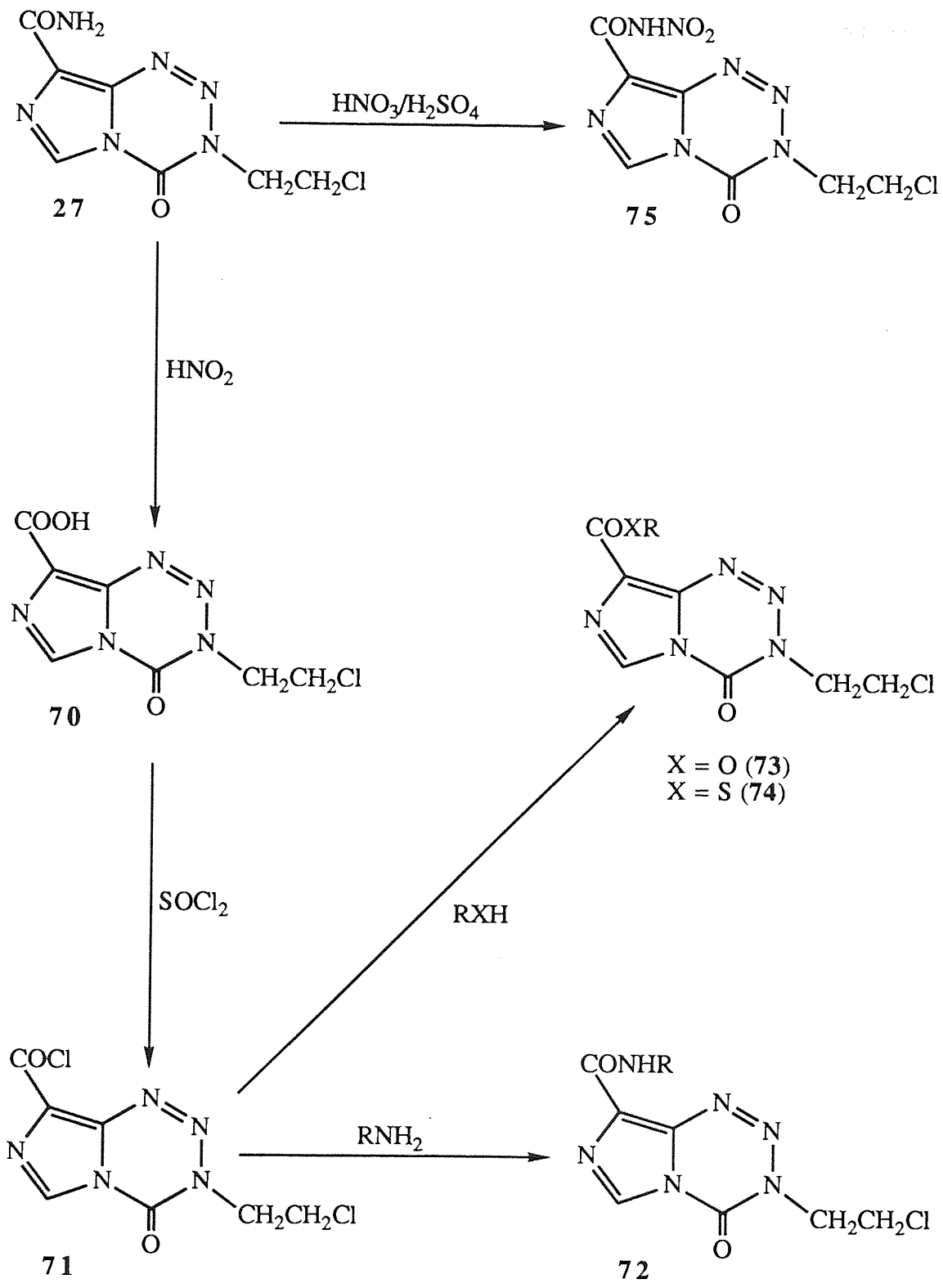
In hot acetic acid mitozolomide decomposed to give the parent 5-diazoimidazole-4-carboxamide (**42**) which was treated with halide ions in a Sandmeyer type reaction to give the 5-haloimidazoles (**63**), and with methylenic substrates to give the imidazo-1,2,4-triazines (**67-69**), reactions which were not seen with the 3-methyl or 3-ethyl analogues (Scheme 1.7).[42]

1.5.2 Reactions with retention of the tetrazinone ring.

Despite the instability of the tetrazinone ring under basic conditions treatment of mitozolomide with commercial bleach was found to give a moderate yield of the free acid



Scheme 1.7: Non-aqueous decomposition of mitozolomide.



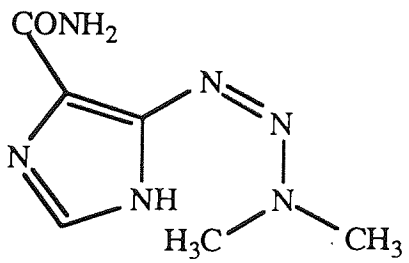
Scheme 1.8: Reactions of mitozolomide at C8.

(**70**).[43] The acid (**70**) was subsequently prepared in a higher yield by using nitrosylsulphuric or nitrosyltrifluoroacetic acid.[37,43] Horspool *et al.*[43] utilised the acid (**70**) in the preparation of the 8-carbonyl chloride (**71**) which was found to react preferentially with nucleophiles at the 8-position rather than C4 allowing the synthesis of a wide range of novel analogues (**72-74**) (Scheme 1.8). However, attempts to prepare the potentially interesting 8-amino and the 8-homologous acid derivatives *via* a Curtius rearrangement and Wolff degradation respectively failed. The acid stability of the tetrazinone ring is further exemplified by the nitration of mitozolomide, not in the expected 6-position, but in the 8-carboxamide substituent to give the 8-(N-nitrocarbamoyl)imidazotetrazinone (**75**).[43]

1.6 Antitumour evaluation of imidazotetrazinones.

1.6.1 Antitumour activity of mitozolomide.

Mitozolomide exhibited broad and potent antitumour activity against a variety of murine tumour models,[44,45] showing curative action against L1210, P388 leukaemias, solid tumour types colon 38, M5076 sarcoma and ADJ/PC6A plasmacytoma. Significant activity was also displayed against TLX5 lymphoma, B16 melanoma, Lewis lung carcinoma and colon 26 tumour.[45] Mitozolomide compared favourably with widely used clinical agents in the now redundant NCI tumour panel being equi-potent with cisplatin, BCNU, cyclophosphamide and adriamycin, while proving superior to methotrexate and DTIC (**76**) (Table 1.1).[46]



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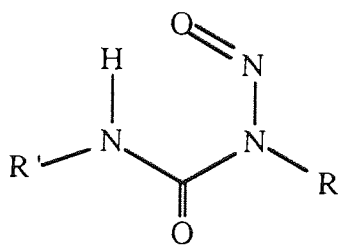


77

Considering the structural resemblance of the imidazotetrazinones to the nitrosoureas (**77**) and the triazene DTIC (**76**), it was not surprising that all three types displayed similar observed patterns of resistance. Cross resistance studies showed

Table 1.1: Activity of mitozolomide and other drugs in the National Cancer Institute murine tumour panel.[46]

Drug	L1210 leukaemia	P388 leukaemia	B16 melanoma	LL carcinoma	Colon 26 tumour	C38 adeno-carcinoma	$CD8F_1$ mammary
Mitozolomide	++	++	++	++	++	++	+
Nitrogen mustard	++	+	++	inactive	++	+	+
BCNU	++	++	++	++	++	+	+
DTIC	++	++	++	++	inactive	+	+
Cisplatin	++	++	++	++	++	+	++
Cyclophosphamide	++	++	++	++	++	++	++
Methotrexate	++	++	inactive	+	inactice	inactive	+
Adriamycin	++	++	++	++	++	inactive	++
% T/C activity criteria							
(++)	>150	>175	>150	>150	>150	<10	<10
(+)	>125	>120	>125	>140	>130	<42	<42

mitozolomide was inactive against L1210 leukaemia and TLX5 lymphoma with derived resistance to BCNU and a dimethyltriazene respectively. Activity was maintained against cyclophosphamide-resistant L1210 leukaemia. However, it was clear that mitozolomide had clear advantages over these earlier classes of antitumour agents in terms of activity, oral bioavailability, pharmacokinetics and, with respect to the triazenes, greater photostability.[47]

The chemosensitivity of human tumour xenografts to mitozolomide has been studied. Pronounced activity was shown against human sarcoma, colon cancer and melanoma xenografts,[48] while at best, marginal activity was observed against human gestational choriocarcinoma.[49]

As a result of these studies mitozolomide entered phase I clinical trials in 1983. Newlands *et al.*[50] established that mitozolomide possessed good oral bioavailability and in general was well-tolerated with only minor nausea and vomiting. However, myelosuppression was found to be the dose-limiting toxic effect, manifested by a marked and prolonged thrombocytopenia lasting up to 8 weeks, at doses > 115mg/m^2. An abbreviated phase I clinical trial of i.v. mitozolomide led to the recommendation of a maximum i.v. dose of 100mg/m^2 in previously treated patients with a reduction to 90mg/m^2 in patients with a history of prior treatment.[51]

Phase II clinical trials[52] indicated promising response rates against small cell lung carcinoma (28%), while response rates in malignant melanoma[53] were comparable with those of the most active established drugs. However, further evaluation was abandoned due to the severe and unpredictable thrombocytopenia that was encountered despite dosage reduction in some cases as far as 70mg/m^2.

1.6.2 Antitumour activity of other imidazotetrazinones.

The exciting antitumour potential of mitozolomide prompted the synthesis and testing of a large number of new imidazotetrazinones with various substituents in the 3, 6, and 8-position.[35,43,47] No derivative emerged with an outstanding improvement in therapeutic index, although the 3-methyl analogue (temozolomide) possessed qualitative differences in activity from mitozolomide that merited further exploration.

Table 1.2: Activity of temozolomide against murine survival-time models, including resistant lines.[35]

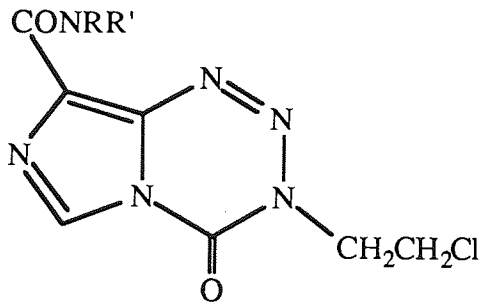
Tumour	Schedule (day of injection)	Optimum dose (mg/kg/day)	Optimal T/C (%)	Assessment[a]
P388 leukaemia	1	200	143	+[b]
	1-4	100	214	++[b]
	1-5	200	>214	+++[b]
P388/mitozolomide	1	200	110	-
	1-4	100	112	-
L1210 leukaemia	1	200	149	+
	1-4	100	200	++
L1210/DTIC	1	200	116	-
	1-4	100	108	-
L1210/BCNU	1	200	148	+
	1-4	100	175	++
B16 melanoma	1-9	100	181	++
M5076 reticulum sarcoma	1,5,9,13	200	170	++
TLX5 lymphoma	3	160	151	++
	3,6,9	80	154	++
	3-7	40	181	++

a. Antitumour assessment shown represents the optimal result on the treatment schedule indicated. Activity was rated according to the following scale: +++, T/C > 150% with cures at one or more dose levels; ++, T/C > 150% with no cures; +, T/C > 125%; -, T/C < 125%.

b. For the P388 tumour: +++, T/C > 175% with cures at one or more dose levels; ++, T/C > 175% with no cures; +, T/C > 120%.

Temozolomide displayed potent activity against L1210, P388 leukaemias, M5076 sarcoma, B16 melanoma and ADJ/PC6A plasmacytoma.[35] The antitumour activity of temozolomide was improved further when used in a divided dose schedule, in contrast to mitozolomide whose maximum efficacy was obtained on a single dose regime (Table 1.2).[35]

Temozolomide was inactive against mitozolomide-resistant P388 leukaemia, while cross-resistance with DTIC-resistant L1210 leukaemia indicated that the activity of temozolomide shared a common pathway with DTIC. Comparison with DTIC against a range of tumour models established temozolomide as the superior agent, a potential that is currently being investigated in clinical trials (Table 1.3).[35]



(a) R = R' = Me (**78**)

(b) R = H, R' = Me (**79**)

An interesting analogue is the 8-N,N-dimethylcarbamoyl derivative (**78**) of mitozolomide which was shown to be inactive against TLX5 lymphoma *in vitro* when compared with mitozolomide, but which displayed a significant increase in activity when incubated with hepatic microsomes. Indeed, *in vivo* studies showed that 89% of the administered dimethyl analogue underwent oxidative N-demethylation to the N-methyl analogue (**79**) which was responsible for the antitumour activity.[54]

1.7 Mode of action of imidazotetrazinones.

Much of the initial work on the mechanistic studies of imidazotetrazinone-mediated cytotoxicity was conducted using mitozolomide. Theoretically fragmentation of the 1,2,3,5-tetrazinone ring was capable of generating a cascade of potential antitumour species. However, chemical studies had demonstrated that under physiological conditions mitozolomide acted as a pro-drug for the alkylating agent MCTIC.[33,35]

Table 1.3: Comparison of temozolomide and DTIC against murine tumour systems.[35]

Model	Tumour	Optimal T/C or Therapeutic Index Temozolomide	DTIC
Murine ascitic survival time	TLX5 lymphoma[a]	181	180
	L1210 leukaemia[a]	200	160
	P388 leukaemia[a]	>254	166
	B16 melanoma[a]	181	145[d]
	M5076 reticulum cell sarcoma[a]	200	NT[e]
Murine solid	M5076 reticulum cell sarcoma[b]	6.5	8.1
	ADJ/PC6A plasmacytoma[b]	>8.0	50
Murine human xenograft	MX-1 mammary[c]	15	37[d]

a. Increase in survival time.

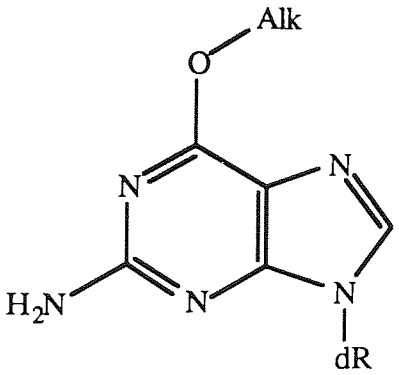
b. Therapeutic Index, LD_{50}/ID_{50}

c. Tumour volume change.

d. Data from Goldin *et al.*[46]

e. Not tested.

Alkylation reactions involve the formation of covalent linkages to a variety of C, O, N, S and P containing nucleophiles *via* an S_N2 nucleophilic substitution. Two main sites are readily accessible to alkylation in DNA - O6 (**80**) and N7 (**81**) positions of guanine residues.



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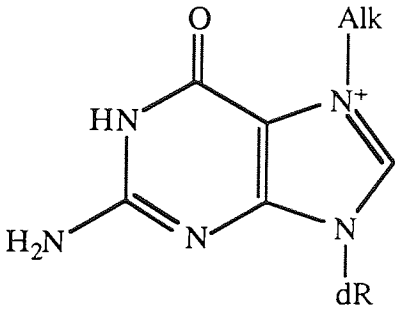


81

In common with other alkylating agents mitozolomide was shown to extensively alkylate the N7 position of guanine with a greater preference for the inner guanine in a run of three or more contiguous bases, but with less selectivity than the nitrosoureas.[55] However, although most alkylating agents extensively attack the N7 position of guanine there is no clear evidence that such reactions are responsible for their cellular toxicity. N7 alkylation results in the production of apurinic sites, the loss of the guanine base leading to single strand breaks in DNA.[56]

The alkylation of O6 is a pro-mutagenic lesion resulting in the interpretation of the guanine as an adenine base and consequently leads to misreading of the genetic code.[57,58,59] It is now generally considered that such events have a crucial role in mediating the cytotoxicity of alkylating agents.[60,61,62,63] Studies involving chloroethylnitrosoureas suggested that the formation of DNA interstrand cross-links following alkylation at the O6 position were implicated in initiating cell death.[64,65] Indeed, Gibson *et al.*[66] showed that in L1210 leukaemic cells mitozolomide and MCTIC were equi-potent with the nitrosourea CNU at producing interstrand cross-links. Suggestions arose that these agents shared a common mechanism of cellular toxicity by inducing these cross-linked adducts.

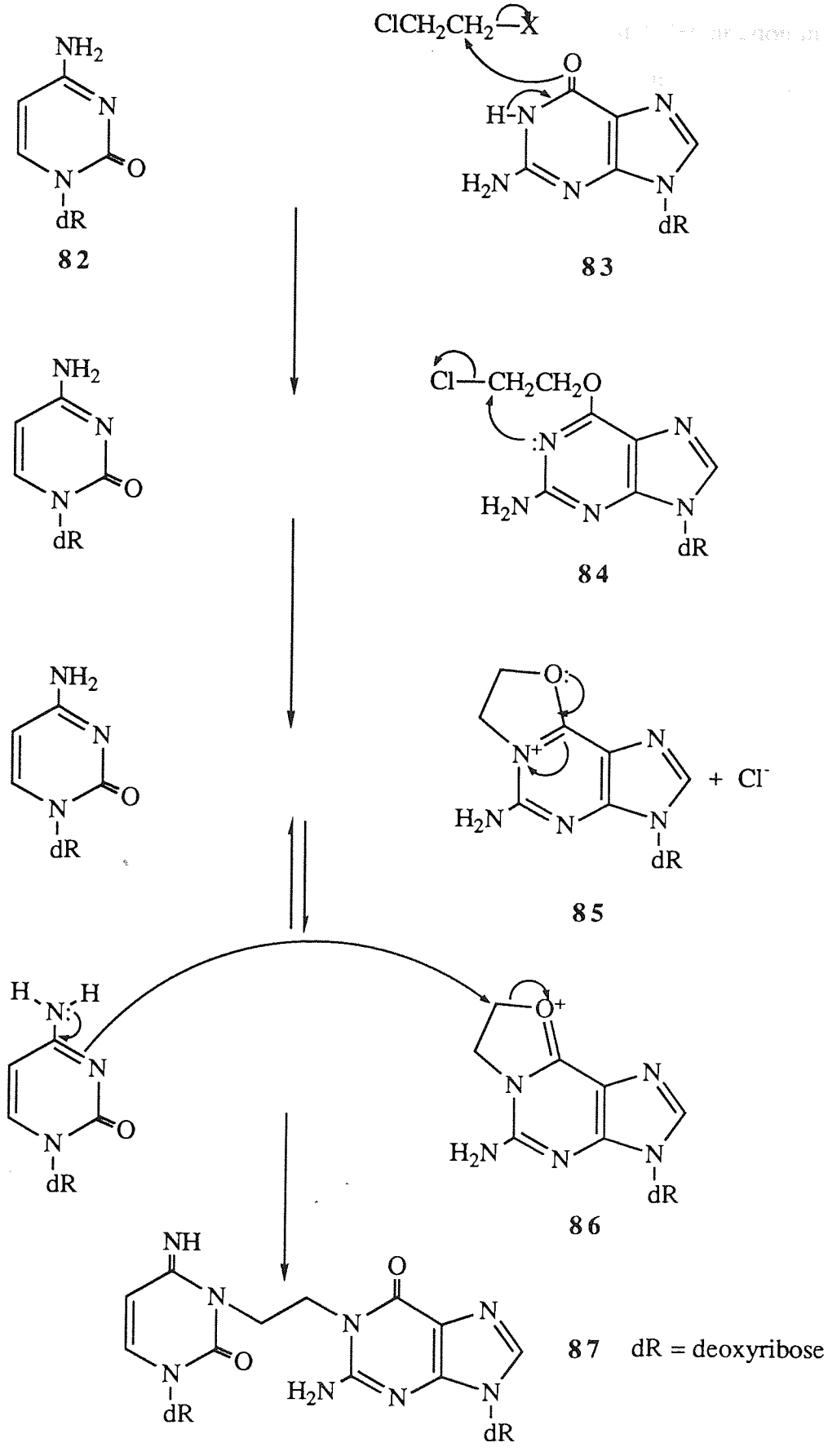
Tong *et al.*[64] has proposed a possible mechanism for the formation of

interstrand cross-links by chloroethylating agents (Scheme 1.9). Initial alkylation occurs at the O6 position of a guanine residue (**83**). The disruption in the hydrogen bonding of the G-C base pair would leave the adjacent N1 position of guanine open to undertake intramolecular nucleophilic attack (**84**) with subsequent loss of a chloride ion generating the resonance-stabilised N1, O6-ethanoguanine (**85-86**). Nucleophilic attack by the N3 position of the cytosine on the opposite strand results in cleavage of the ethanoguanine moiety (**86**) and formation of a N1-guanine-N3-cytosine interstrand cross-link (**87**).

Further evidence for the role of cross-linking in mediating the toxicity of mitozolomide and MCTIC came from studies comparing the activity of these agents against cells with an O6-alkyltransferase repair facility (Mer+ cells) and cells deficient in this repair process (Mer- cells). It was argued that Mer- cells should exhibit greater sensitivity to the action of mitozolomide and MCTIC because they were unable to remove the initial O6 chloroethyl adduct and thereby prevent cross-linking. Gibson *et al.*[67] demonstrated that for mitozolomide and MCTIC there was a linear correlation between the formation of cross-links and log cell kill in SV-40 transformed embryo cells (Mer-), while there were insignificant amounts of cross-linking in the IMR-90 (Mer+) cell line and no correlation with cell death. It was suggested that by analogy with the nitrosoureas mitozolomide exerted its antitumour activity by the formation of cross-links in DNA *via* the hydrolytic generation of a chloroethyldiazo species i.e. MCTIC.[68]

Subsequent research has called into question the role of DNA-interstrand cross-links in initiating cell death. Using a series of alkyltriazenylimidazoles against HT-29 colon carcinoma cells (Mer+) and BE colon cells (Mer-) Gibson and his co-workers[69] found that chloroethyl and monomethyl analogues both produced differential toxicity in the Mer+ and Mer- cell lines. However, unlike the chloroethyl derivatives the methyltriazenes are unable to cross-link DNA suggesting that alkylation at O6 of guanine is sufficient in itself to cause cell death, leaving the role of cross-links open to debate.

Tisdale *et al.*[70] have since examined the alkylation of the O6 position of guanine by temozolomide and the 3-ethyl analogue in comparison with mitozolomide. It would appear that temozolomide, *via* MTIC, displays the same ability as mitozolomide to alkylate the O6 position of guanine; however, the 3-ethyl analogue is interesting in that its lack of activity has been attributed to the formation of an, as yet unknown, alternative



Scheme 1.9: Mechanism for the formation of cross-linked DNA.

cytotoxic lesion.

Temozolomide has been found to promote erythroid differentiation in K562 human erythroleukaemic cells, a property not shared by mitozolomide, suggesting that the methylating agent is more effective in altering gene expression, an hypothesis supported by the observation that there is a decrease in the amount of 5-methylcytosine in temozolomide-treated cells.[71]

Although it is theoretically possible for mitozolomide to break down generating 2-chloroethyl isocyanate this has not been shown to occur under physiological conditions.[33] Nevertheless chloroethylnitrosoureas have been shown to yield isocyanates, which react with intracellular proteins in a carbamoylation reaction with amino acids such as lysine and arginine.[72] This has been attributed to be of only minor importance since the nitrosoureas ACNU and chlorozotocin (**16**) have negligible carbamoylating properties and yet have excellent antitumour activity.[72] Tisdale and Horgan[73] examined the ability of mitozolomide to act as a carbamoylating agent by comparing the inhibition of the isocyanate-sensitive enzymes γ-glutamylpeptidase, glutathione reductase and α-chymotrypsin. Mitozolomide and MCTIC were found not to produce any significant inhibition of any of the enzymes, even after 24 hours incubation. The nitrosourea BCNU, in contrast, produced complete inhibition of these enzymes within 2 hours. These results indicated that isocyanates did not mediate mitozolomide-induced cellular toxicity. However, Dive *et al.*[74] produced an interesting result using flow cytoenzymlogical analysis suggesting that mitozolomide did indeed have a degree of carbamoylating activity, although to a much lesser extent than that shown by nitrosoureas.

Despite these findings it is now widely accepted that the imidazotetrazinones act as prodrugs of the monoalkyltriazenes which are responsible for the covalent modification of the O6 position of guanine residues in DNA resulting in cell death.

1.8 Structure-activity relationships in azolotetrazinones.

The search for analogues of mitozolomide with improved therapeutic indices has led to the preparation of a large number of novel compounds, for whom structure-activity relationships have been examined.[35,37,43]

For the imidazotetrazinone series of compounds analogues were produced that had various substituents in the 3, 6, and 8-positions. *In vivo* tests indicated that the substituent of choice in the 3-position was a 2-chloroethyl group.[35] Activity was retained with the 3-methyl group, however, an increase in the size of the alkyl residue reduced antitumour activity, while substitution with aromatic groups completely abolished activity. This is an interesting observation which closely parallels that of other alkylating agents such as the alkyltriazenes, nitrosoureas and hydrazines, an explanation of which is still required since the substituent at the 3-position is responsible for the alkylating properties.[35]

Imidazotetrazinones with alkyl substituents in the 6-position gradually experienced a decline in activity with an increase in chain length. Several reasons for this have been proposed:[47]

(a) an increased π value altering transport characteristics.

(b) failure of the enlarged molecule to fit an unknown binding site.

(c) a steric inhibition of hydrolysis at C4, preventing ring opening to the active triazene.

The substituent at the 8-position in imidazotetrazinones has been shown to have a crucial effect upon activity. An element of this thesis is to extend our knowledge of how the 8-substituent is involved in modifying antitumour activity and so will be discussed in a later chapter.

A number of tetrazinones fused to a variety of azoles have also been prepared (see section 1.3). The activity of the isomeric pyrazolotetrazinones paralleled that of its imidazotetrazinone counterparts with the 8-carbamoyl, 8-NN-dimethylcarbamoyl and 8-methylsulphonyl[47] all possessing significant activity, however, the indazolotetrazinone proved to be inactive.[35]

1.9 Current investigations.

The long term objective of the present studies into this class of molecules is the development of a third generation of imidazotetrazinones that are capable of functioning as probes for the major groove of DNA, in particular exploiting their ability to preferentially alkylate sites in guanine residues that are exposed in the major groove.[55] The major groove is responsible for containing most of the genetic information involved in cellular control and

is a focal point for the cellular control systems.[75] Furthermore, the presence of GGG regions in the major groove of oncogenes (genes which have been implicated in promotion of the cancerous state) offers an attractive target for potential antitumour agents.[76]

To date, the history of drug design has been a mixture of intuition, experience and serendipity. However, today, medicinal chemistry along with molecular pharmacology and computer assisted drug design form the basis for a new era of rational drug design. In order to facilitate such rational design processes it is crucial to have an in-depth understanding of how drug entities interact with their macromolecular target, or where this is not possible an hypothesis based upon sound scientific fact. Our knowledge of the mode of action of imidazotetrazinones has already been reviewed (section 1.7) and it is widely considered that these compounds act as prodrugs for triazene alkylating agents. However, two important features have yet to be fully explained. Firstly, what if any is the exact role of the C-8 substituent which has been shown to have such a profound effect upon antitumour activity: and secondly, which molecule actually enters the major groove of DNA, is it the ring intact tetrazinone or is it the ring opened triazene form?

As a result of these unanswered questions the present studies have been based upon a working hypothesis as to the mode of action of the imidazotetrazinones within the micro-enviroment of DNA. It is our belief that it is the ring intact tetrazinone form which reaches the major groove where hydrogen bonding interactions *via* the C-8 substituent anchors the molecule in the locality of G-C base pairs. It is then envisaged that nucleophilic cleavage of the tetrazinone ring occurs, not *via* direct attack by the sterically constrained nucleic acid bases, but by a greatly more mobile 'activated' water molecule (activated by the high electronegativity associated with G-C regions) leading to the generation of the triazene. Therefore, the overall impression is of a prodrug activated at its site of action akin to the suicide substrates that have become commonplace in enzyme inhibition.

To test this hypothesis a three pronged approach involving molecular modelling, biological analysis and synthetic chemistry has been used, the details of which are discussed in the following sections.

RESULTS AND DISCUSSION.

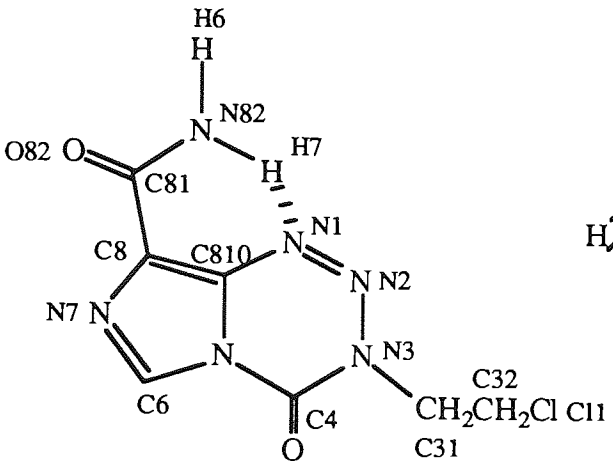
CHAPTER 2 : MOLECULAR MODELLING STUDIES.

2.1 Aims and objectives.

As part of the approach to testing the proposed hypothesis on the mechanism of action of the imidazotetrazinones it was envisaged that molecular modelling techniques could be utilised to examine the key structural features, especially with regard to the C-8 substituent, which were involved in the recognition of G-C sequences within DNA.

2.2 The nature of the C-8 substituent.

2.2.1 Geometry of the C-8 position.



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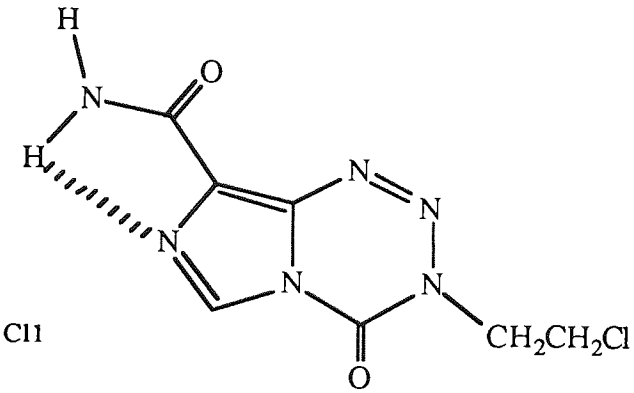

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Both mitozolomide and temozolomide contain carboxamide groups at the 8 position which from the crystal structure of mitozolomide are capable of intramolecular hydrogen bonding generating two rotamers in which there is hydrogen bonding of the carboxamide -NH_2 to either the N1 (**88**) or N7 (**89**) positions.[77] However, the crystal structure of temozolomide although it shows two distinct molecules per unit cell, both represent the NH_2---N7 rotamer.[78] In order to examine the importance of intramolecular hydrogen bonding in the carboxamide group both rotamers of temozolomide were constructed and optimised within Chem-X.[79] The energy difference between the rotamers was calculated by *ab initio* Gaussian 80 quantum mechanics[80] to be 2.4kJ/mol in favour of

the NH_2---N7 rotamer.

The rotation barrier about C8 for the more stable NH_2---N7 rotamer was studied by altering the value of the torsion angle about the C8-C81 bond in steps of 15° between 0° and 90° and calculating the single point *ab initio* energy of each conformation. The energy barrier to rotation was found to be 27.4kJ/mol suggesting that in solution under physiological conditions interconversion between both rotamers is possible. As this energy difference is greater than the mean energy of a hydrogen bond (16kJ/mol) it is likely that hydrogen bonding interactions will take place without disruption of the coplanarity of the carboxamide group.

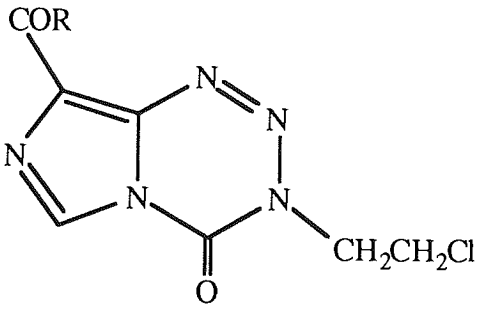
2.2.2 Structure-activity relationships at C8.

The observed antitumour activity of a number of imidazotetrazinones had indicated that the effect of structure upon activity was more complex at position 8 than for substituents elsewhere in the tetrazinone ring. For the carboxamido series of compounds it appeared that small N-alkyl groups could be tolerated without a loss in activity while bulkier N-substituents such as the phenyl group virtually abolished *in vivo* potency. The poor activity of the 8-ester, 8-nitro, 8-phenyl and 8-cyano derivatives suggested that an NH moiety capable of acting as an hydrogen bond donor was required for optimal activity. Indeed, it was suggested by Stevens that the preferred substituents at the 8-position were the carboxamido, and interestingly, the sulphonamido or alkylsulphonyl groups.[35]

In an attempt to rationalise these observations a number of electrostatic and geometric properties for a small series of mitozolomide derivatives were used to generate a structure-activity relationship with their known activity against murine TLX5 lymphoma. A series of imidazotetrazinones containing a carboxyl functionality at the 8-position were constructed, from the crystal structure of mitozolomide solved by Lowe *et al.*[77], using standard bond lengths and angles within the Chem-X molecular modelling package. Each structure was optimised using the semi-empirical molecular orbital program MOPAC[81] ensuring the coplanarity of the carboxyl group with the tetrazinone ring. For each analogue the following electrostatic and geometric properties were calculated within Chem-X (Table 2.1):

(a) sum of the atoms in the C-8 substituent (Sum At.).

Table 2.1: The electronic and geometric properties calculated for a series of mitozolomide derivatives within Chem-X used in the generation of structure-activity relationships.



R	Sum At.	Sum Ch.	VDW-Surf.	VDW-Vol.	Vol-[a]	Vol+[b]	TLX5 Act.[c]
NH_2	7	-0.253	65.07	56.52	1477.5	0	458
NHMe	10	-0.318	79.27	76.92	1449.8	0	555
NHPh	17	-0.262	120.48	134.97	2322.6	0	103
NHOMe	11	-0.262	87.99	77.63	1891.0	0	325
$NHNO_2$	9	-0.294	77.29	75.20	1836.4	7.92	354
$NH(CH_2)_2Cl$	13	-0.258	109.31	110.47	1871.2	2.25	362
$NHCH_2CO_2Et$	19	-0.262	131.17	143.67	2395.5	3.34	139
OH	6	-0.218	60.95	48.18	742.2	0	223
OMe	9	-0.267	73.58	73.38	1430.9	0	140
OEt	12	-0.300	86.26	84.34	1067.9	0	137

a. represents the volume in which the energy of the proton probe is less than -20kcal/mol ($Å^3$).

b. represents the volume in which the energy of the proton probe is less than +20 kcal/mol ($Å^3$).

c. defined as the maximum T/C (%).

Table 2.2: A comparison of the correlation coefficients (r) for the structure-activity relationships generated within Chem-DBS and ChemStat for all derivatives (n=10) and for only those compound containing a carboxamide NH (n=7).

Property.	n=10 Correlation (r).	n=7 Correlation (r).
Sum At.	-0.47497	-0.85282
Sum Ch.	-0.31266	-0.52696
VDW-Surf.	-0.41227[a]	-0.83499[a]
VDW-Vol.	-0.44373[a]	-0.84191[a]
Vol-.	-0.14042	-0.97862
Vol+	-0.08402	-0.12148

a. rejected due to correlation with the Sum At. property.

(b) sum of the atomic charges of the C-8 substituent (Sum Ch.).

(c) van der Waals surface of the C-8 substituent (VDW-Surf.).

(d) van der Waals volume of the C-8 substituent (VDW-Vol.).

(e) negative potential volume of the C-8 substituent (Vol-.).

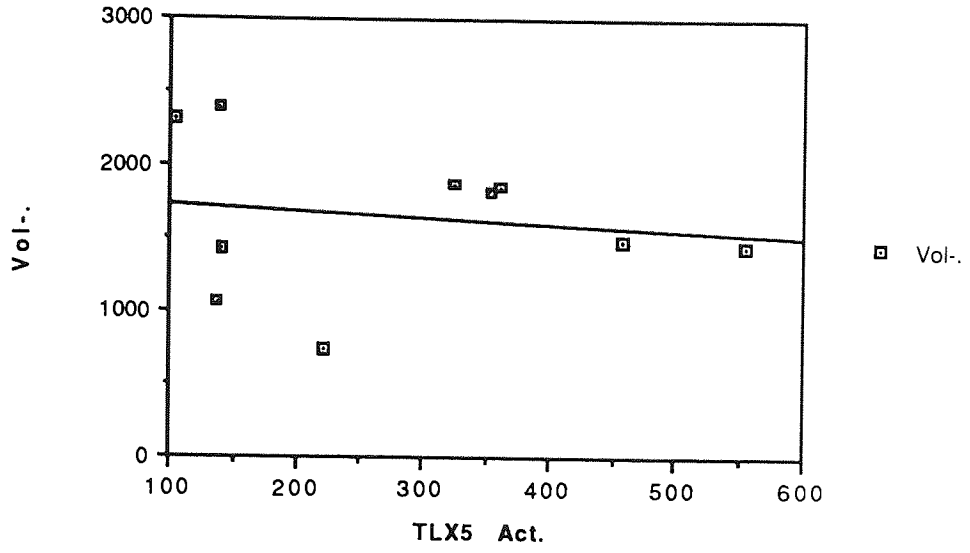
(f) positive potential volume of the C-8 substituent (Vol+.).

The ChemDBS-1 and ChemStat routines were used to generate the structure-activity relationships (Table 2.2).

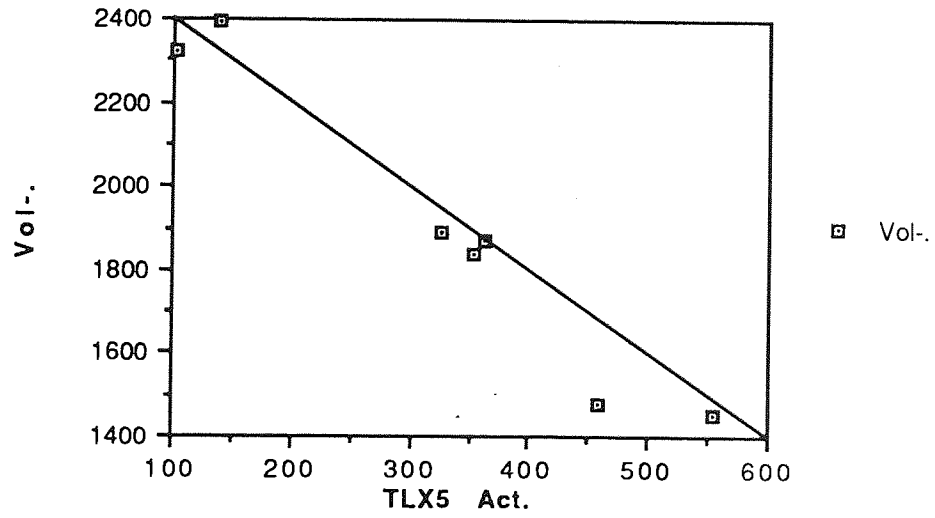
Graphical analysis of the results (Figure 2.1) from the calculations involving all ten analogues revealed that those compounds containing the carboxamido group lay closer to the line of best fit. This appeared to be in accordance with structure-activity relationships that had been generated by Horspool which had implied that for optimal activity there was a requirement for the presence of an NH group.[37] Indeed, when the calculations were repeated without the 8-acid and ester congeners much better correlations could be obtained (Table 2.2). From these calculations two significant factors can been seen. Firstly there is a strong negative correlation between antitumour activity and the size of the negative potential lobe associated with the carboxyl portion of the carbamoyl group. Secondly, a significant negative correlation was also seen with the total number of atoms in the C-8 substituent. The

Figure 2.1: Plots of negative potential volume (Vol-) versus antitumour activity against TLX5 lymphoma (TLX5 Act.) for:

(a) all derivatives.



(b) -NH containing derivatives only



structure-activity relationships presented here and those calculated by Horspool[37] clearly indicate that for the carboxy series of imidazotetrazinones optimal activity is dependent on the presence of a N-H group. Combined with the observation of increased stability within the molecule when coplanarity of the carboxamide group, and therefore the ability to form intramolecular hydrogen bonds, is conserved this clearly indicates a significant hydrogen bonding role for the C-8 substituent. This is an interesting comparison, and indeed appears to contradict the structure-activity relationships that have been calculated for the triazene series - the active component of the imidazotetrazinones. Connors *et al.*[82] calculated for a series of 1-phenyl-3,3-dialkyltriazenes that the effect of substituents in the aromatic ring upon activity are negligible and seems to be independent of whether the group is electron withdrawing, electron donating, hydrophilic or hydrophobic. Indeed Lin *et al.*[83] have suggested that the carboxamide group in phenyl triazenes can be replaced with an ester group without a detrimental effect upon activity and the position such groups occupy in the ring (i.e *ortho*, *meta* or *para* to the triazene) does not alter activity, an effect that was also noted by Hansch *et al.*[84] in the imidazole-3,3-dialkyltriazene series of compounds. The activity of the 3,3-dialkyltriazenes has been expressed to closely correlate with their partition coefficient with an optimum log P of approximately 1, in contrast to the imidazotetrazinones which have a greater dependence upon the presence of an N-H group and as has been indicated in this study a low negative potential volume associated with the C-8 substituent; while Horspool[37] has shown only a minor effect attributed to the partition coefficient of the tetrazinones. Such qualitative differences in the structure-activity relationships between the imidazotetrazinones and 3,3-dialkyltriazenes suggests that there may be an important biological role fulfilled by the ring intact tetrazinone over and above that of acting as a source of monoalkyltriazenes. However, it must be stressed that although both the imidazotetrazinones and 3,3-dialkyltriazenes are prodrug forms for the same alkylating species they require quite different methods of activation and as such may not be directly comparable.

Recently, Pullman and Pullman[85] have calculated the electrostatic potential associated with the individual components that make up DNA together with the potentials of the double helix itself. They have shown that G-C regions within the major groove of DNA

generate areas of the greatest negative electrostatic potential in the double helix, an observation that may help to explain why imidazotetrazinones with a large negative lobe at the C-8 position have considerably less antitumour activity. It is conceivable that as either the imidazotetrazinone or its active triazene counterpart enter the major groove of DNA they experience repulsive interactions within the area of G-C target regions which prove to be stronger than those associated with potential hydrogen bonding interactions between the 8-carbamoyl group and the base pairs; this suggests in part that there does exist a degree of attraction between DNA and either the tetrazinone or the active triazene. It is hoped that by incorporating substituents such as small peptides into the molecule it may be possible to overcome such repulsive interactions by improving their hydrogen bonding capability and thereby open the door to an improvement in sequence selectivity. However, the increase in size of such C-8 substituents when the tetrazinone is linked to structural fragments conferring additional recognition capability could engender difficulties in the design of gene sequence specific alkylating agents considering the significant negative correlation that was observed between antitumour activity and the size of the C-8 substituent.

2.3 Electronic and geometric properties of C-4.

Calculation of the partial charges for both possible rotamers of mitozolomide (Table 2.3) showed that C-4 is the most electropositive atom and therefore the most likely target for nucleophilic attack. This is in agreement with the observations of chemical degradation of mitozolomide in the presence of oxygen and nitrogen containing nucleophiles.[42]

The proposed mechanism of action of the imidazotetrazinones requires attack by a water molecule at the C-4 position leading to the formation of the unstable diol which decomposes with the loss of carbon dioxide to form the monoalkyltriazene. To test this hypothesis the proposed water adduct was constructed from the optimised structure of temozolomide and fragments of a tetrahedral diether from the Cambridge Crystallographic Database and re-optimised using the MOPAC program within Chem-X while maintaining the coplanarity of the carboxamide group. The most significant feature of the adduct was the lengthening of the N3-C4 and C4-N5 bonds, giving a weakly bonded geometry in which the C4 atom remains tetrahedral and significantly positively charged indicating that the proposed

Table 2.3 : Partial charges in mitozolomide: non-hydrogen atoms.

Atom	NH_2--N1 Rotamer Partial charge	NH_2--N7 Rotamer Partial charge
N1	-0.11	-0.08
N2	0.05	0.05
N3	-0.12	-0.13
C4	0.44	0.44
N5	-0.09	-0.10
C6	0.13	0.15
N7	-0.17	-0.19
C8	-0.04	-0.05
C810	0.14	0.15
C31	0.10	0.11
C32	0.03	0.02
Cl1	-0.14	-0.14
C81	0.35	0.35
N82	-0.28	-0.26
O82	-0.35	-0.32

mechanism for the ring opening of the imidazotetrazinones appears to be energetically favourable.

2.4 Modelling of the imidazotetrazinones in the major groove of DNA.

A crucial part of the proposed hypothesis is that the imidazotetrazinones are capable of recognising G-C sequences in the major groove of DNA *via* interactions, probably hydrogen bonding, involving the C8 amide substituent. Modelling studies involving mitozolomide and temozolomide, in isolation, had highlighted a key role for the C8 group especially with regard towards its ability to participate in hydrogen bonding interactions. However, an important question that needed to be answered was could such hydrogen bonding interactions be extended to involve those required for the binding and recognition of DNA sequences?

In an attempt to answer this question preliminary modelling studies have been conducted to examine potential interactions between imidazotetrazinones and G-C sequences in DNA. The two rotamers of mitozolomide (**88**) and (**89**) were docked on to a portion of DNA, constructed from data obtained from the Cambridge Crystallographic Database, at a site containing 3 contiguous guanine residues. The resulting complexes were examined for potential stabilising attractive interactions (Figure 2.2 and 2.3). Although it is important to stress that the results from these studies are preliminary and further detailed studies are required, several observations are worth noting for these complexes:

(a) the formation of hydrogen bonds *via* the C8 amide substituent indicates that the NH_2---N7 rotamer (**89**) has the potential to form three hydrogen bonds with cytosine residues on the adjacent strand; *via* the H6 amidic proton in mitozolomide interacting with the pyrimidine nitrogen N3 and the carbonyl group at O2 in the 5′ cytosine residue 2C, and *via* the carbonyl group of the carboxamide group in mitozolomide acting as an hydrogen bond acceptor to the amino function of the cytosine residue 3C (Figure 2.2).

(b) the NH_2---N1 rotamer (**88**) can form only one hydrogen bond, which has a considerably greater degree of geometric strain, with adjacent cytosine residues *via* the H6 amidic proton of mitozolomide and the pyrimidine N3 nitrogen in the cytosine residue 3C. However, rotamer (**88**) has the potential to form two hydrogen bonds with adjacent guanine residues; *via* the H7 amidic proton of mitozolomide and the carbonyl group at O6 of the 5′ guanine residue 3G and *via* the carbonyl group of the carboxamide group in mitozolomide acting as an hydrogen bond acceptor to the amino function of the 3′ guanine residue 2G (Figure 2.3). This would appear to be in accordance with observation that rotamer (**89**) also is the most stable form of the molecule indicating a potential role for the pseudo 'tricyclic' structure of (**89**) in recognising DNA sequences.

(c) the aromatic tetrazinone ring of both rotamers lies parallel and approximately halfway between two of the guanine residues which could lead to the possibility of π-π stacking interactions of the ring systems and consequently a stabilisation of the hydrogen bonding interactions.

(d) the 2-chloroethyl side chain in mitozolomide projects away from the plane of the G-C base pairs and as such has neither any attractive or repulsive interactions within DNA.

(e) the 6-position in the tetrazinone ring is orientated such that substitution with large alkyl

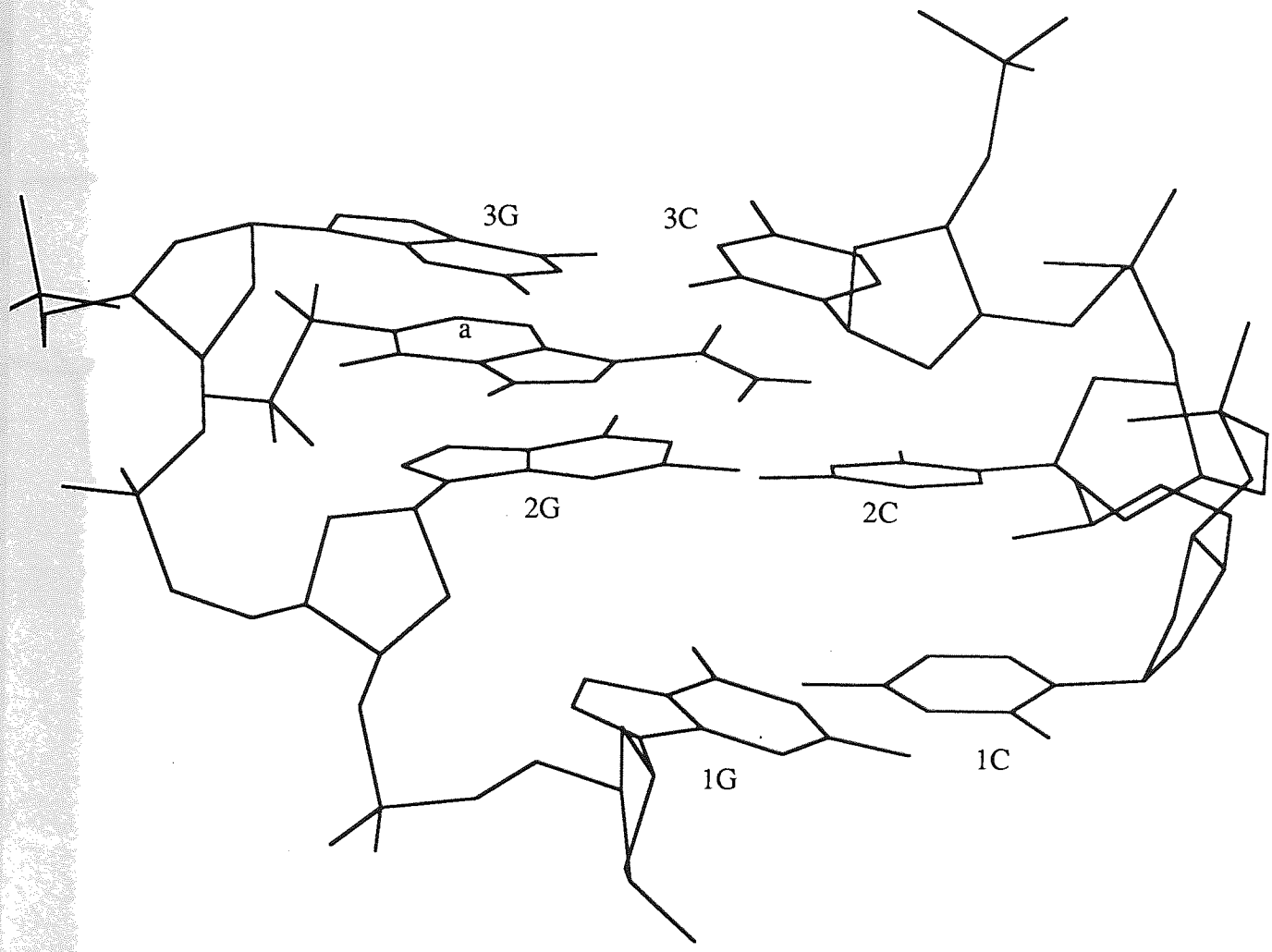


Figure 2.2: A representation of the binding of the NH2---N7 rotamer (a) in the major groove of DNA in the region of G-C base pairs.

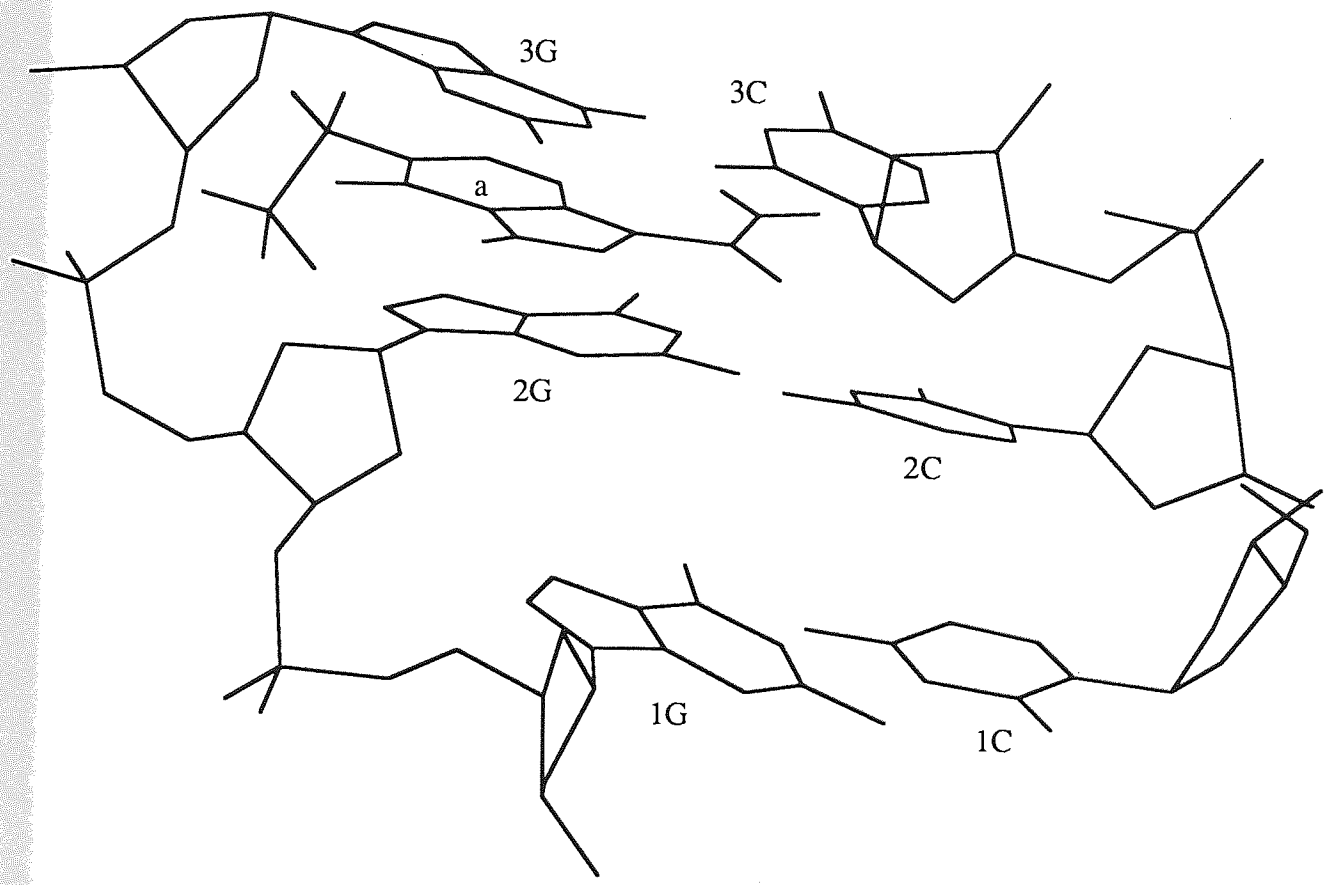


Figure 2.3: A representation of the binding of the NH2---N1 rotamer (a) in the major groove of DNA in the region of G-C base pairs.

groups is unlikely to affect the binding, as depicted, of the tetrazinone ring which would imply that the reason for the observed lack of antitumour activity of such derivatives results purely from steric crowding of the C4 position making it less liable to nucleophilic attack, and not as suggested earlier from the prevention of binding to specific sites in DNA.

(f) the C4 position of the tetrazinone ring is within a 2Å distance of the N7 position of the middle guanine residue, the preferred site of alkylation by mitozolomide. Therefore, it may be argued that within the highly hydrated environment of the major groove of DNA a water molecule hydrogen bonded to the N7 position of the guanine would be in a position for the required orthogonal attack on C4, thereby generating the active triazene alkylating agent at its site of action in accordance with the proposed hypothesis on the mechanism of action of the tetrazinones.

CHAPTER 3 : CHEMICAL SYNTHESES.

3.1 Synthesis of 1,2,3,5-tetrazinones.

3.1.1 Aims and objectives.

The main synthetic aim of this project was the preparation of novel 8-substituted mitozolomide derivatives with a greater propensity to bind to DNA in a sequence specific manner. Preliminary molecular modelling studies had indicated that the antitumour activity of the 8-carboxamido derivatives decreased with an increase in the size of the 8-substituent, probably as a result of repulsive interactions within the major groove of DNA for either the tetrazinone or its active triazene counterpart. It was hoped that such interactions could be overcome by the use of substituents with an improved capability for hydrogen bonding.

Two avenues for synthetic investigation were considered:

(i) increasing the hydrogen bonding potential of compounds that are known to be inactive in order to examine the effect of hydrogen bonding upon activity: for example, by substitution of exocyclic carboxamide groups on to the N-phenyl derivative (**103**).

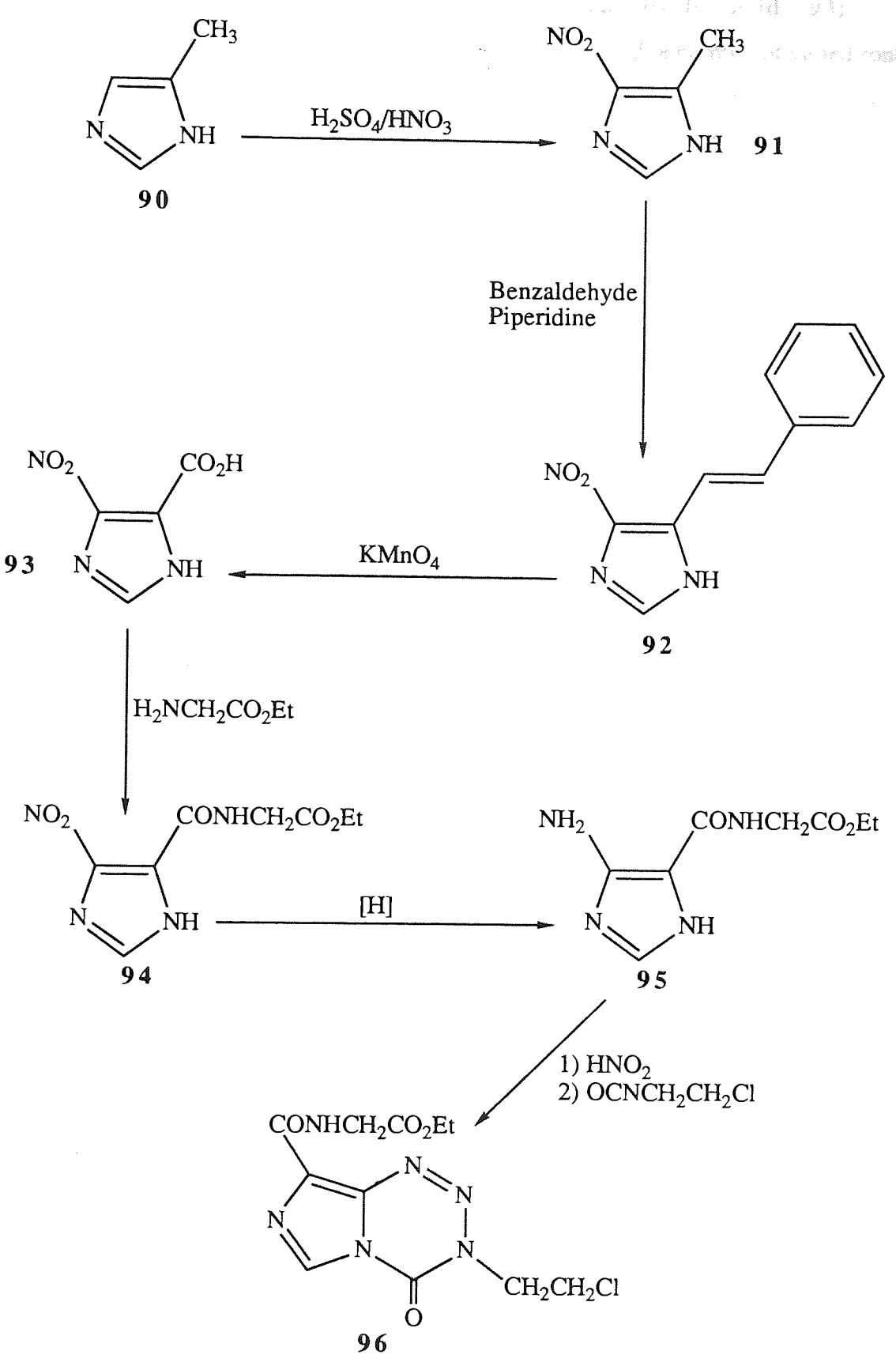
(ii) the preparation of amino acid/peptide analogues may improve hydrogen bonding potential with the added advantage that such derivatives may be capable of reading base sequences within DNA, analogously to the major groove binding proteins. Moreover, the development of the chemistry allowing the coupling of peptides could lead on to the synthesis of tetrazinones linked to monoclonal antibodies.

The synthesis of 8-substituted imidazotetrazinones was reviewed earlier (Chapter 1, page 31 and 36) and involves two distinct routes:

(i) the diazotisation of the appropriate AIC derivative.

(ii) formation of the 8-carboxylic acid (**70**) and acid chloride (**71**) from mitozolomide and their reaction with nucleophiles.

The use of the former method appeared the more appealing since it avoided the need to work with large quantities of the highly toxic mitozolomide.



Scheme 3.1: Envisaged synthesis of the glycine ethyl ester derivative of mitozolomide (**96**).

3.1.2 Attempted syntheses *via* 5-nitroimidazole-4-carboxylic acid (93).

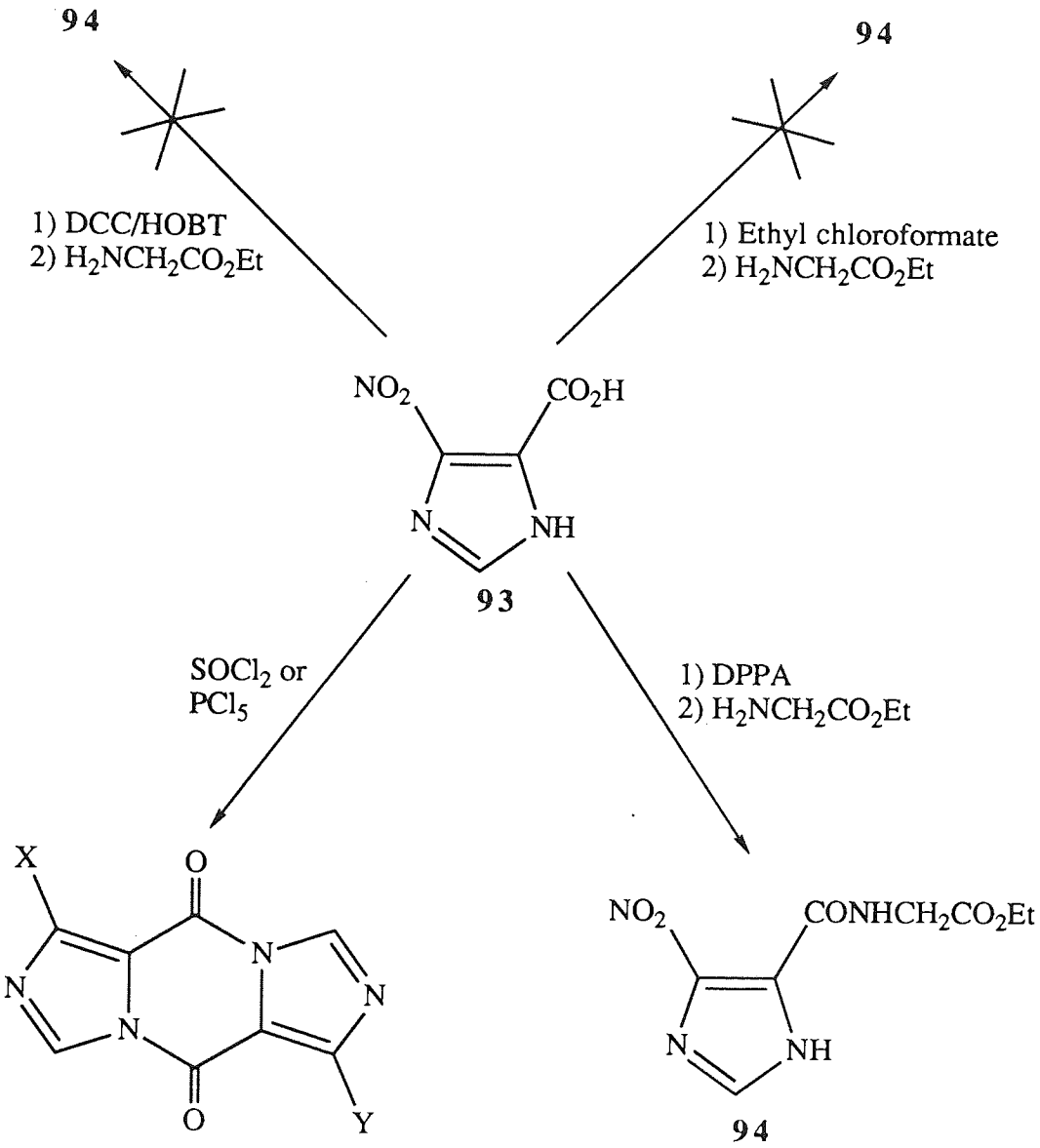
AIC and derivatives have been prepared through a number of varied routes many of which involved the preparation of 5-nitroimidazole-4-carboxylic acid (**93**) as a key intermediate (for a review see Shealey[86]). The preparation of (**93**) followed by the appropriate modification of the carboxylic acid group and reduction of the nitro group was envisaged as a possible route to AIC precursors for imidazotetrazinone synthesis (Scheme 3.1).

The nitro acid (**93**) was successfully synthesised according to the method of Windaus and Langenbeck[87] in an average yield of 11% from the commercially available 4-methylimidazole (Scheme 3.1). Conversion of (**90**) to the nitro derivative (**91**) and the styryl derivative (**92**) proceeded smoothly in reasonable yields of 77% and 56% respectively, with excellent purity. However, oxidation of the styryl compound gave variable, but consistently poor, yields of the nitro acid (**93**) ranging from 12% to 24%. Attempts to improve the yield of the acid (**93**) were unsuccessful.

There are a large number of reactions reported in the literature[88] that are capable of converting carboxylic acids and primary amines to the respective carboxamides. However, a major synthetic aim of this project was the preparation of amino acid or peptide derivatives which require mild conditions at or below room temperature for their formation. As a result a number of reactions were tried in order to synthesise a model compound derived from the amino acid analogue, glycine ethyl ester (Scheme 3.2), which could ultimately be converted to the previously characterised imidazotetrazinone (**96**).[37,43]

An important feature of the chemistry of the nitro acid (**93**) was discovered by Yasuda and his coworkers.[89] When (**93**) was treated with phosphorus pentachloride the dimeric lactam (**97**) was formed. The high reactivity of (**97**) was utilised by Lunt *et al.*[47] who found that in the presence of primary and secondary amines cleavage of the lactam occurred to give 5-nitroimidazole-4-carboxamides, reduction of the nitro group yielding AIC or its derivatives. The use of the dimeric lactam (**97**) appeared to be a mild procedure applicable to the preparation of amino acid or peptide derivatives.

The attempted preparation of (**97**) by the reaction of the acid (**93**) with phosphorus pentachloride or thionyl chloride proved remarkably unsuccessful yielding a



X = Y = NO_2 (**97**)
X = NO_2 (Cl) Y = Cl (NO_2) (**98**)
X = Y = Cl (**99**)

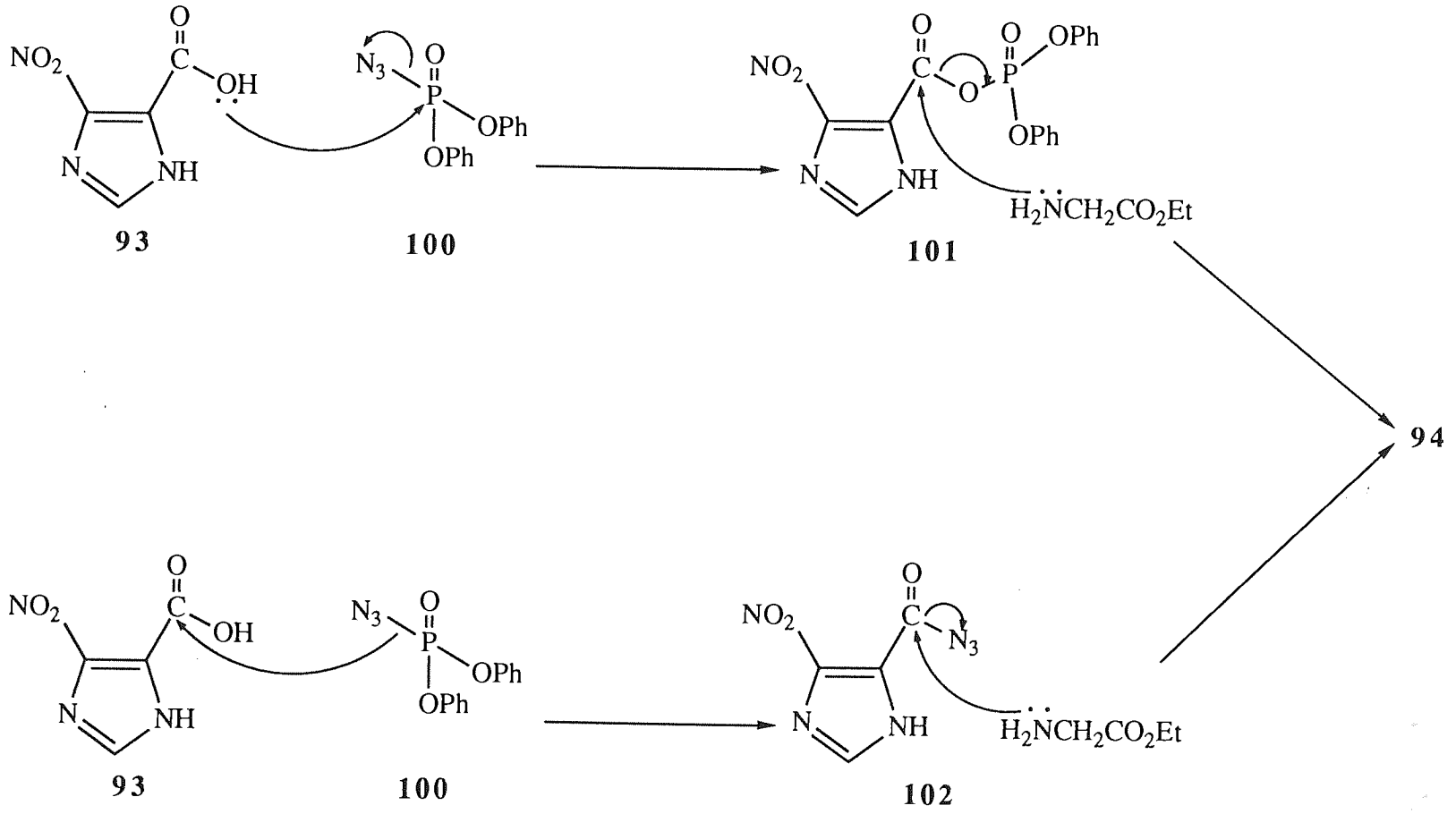
Scheme 3.2: Reactions of 5-nitroimidazole-4-carboxylic acid (**93**).

mixture of products which were identified by mass spectrometry as the desired dinitro lactam (**97**) together with significant amounts of the chloro-nitro lactam (**98**) and the dichloro lactam (**99**). Interestingly, a similar nitro group displacement was also seen when a nitro group was attached to a pyrazole nucleus during the preparation of intermediates for the synthesis of pyrazolotriazinones, a feature which is discussed in more detail later (page 114). Attempts to prevent displacement of the nitro group by reducing the reaction temperature to 60°C and by the use of the mild chlorinating agent dimethylformamidinium chloride failed to effect ring closure and starting material was recovered unchanged.

Subsequently, attempts were made to couple glycine ethyl ester to the nitro acid (**93**) using procedures that had been developed in peptide chemistry. Glycine ethyl ester was successfully coupled to (**93**) using diphenylphosphoryl azide (DPPA) (**100**) as the condensing agent to give (**94**) in a 43% yield. The mechanism of DPPA mediated coupling is unclear and has been suggested to occur *via* the classical transfer of the azido group to the carboxylic acid to give the acyl azide (**102**) which then undergoes nucleophilic attack by the amine. Alternatively a more concerted approach has been reasoned involving the mixed anhydride (**101**) which undergoes selective substitution at the carbonyl group (Scheme 3.3).[90]

A number of alternative methods were also tried based upon traditional carbodiimide and mixed anhydride chemistry but which failed to give products that were consistent with that produced using the DPPA method. TLC examination of the products in all cases showed complex unidentified mixtures. The reasons for this may possibly be attributed to the tendency of the imidazole ring to catalyse acyl transfers between N and O residues a feature that has been shown to occur with the imidazole containing amino acid histidine when the ring nitrogens are unprotected.[91]

The poor yields of the starting material nitro acid (**93**) and the problems encountered with the coupling of a simple amino acid derivative, such as glycine ethyl ester, to the carboxylic acid moiety suggested that this route would not be best suited to the preparation of more complex derivatives, especially considering the observation that in some cases the carboxamide substituent had to be protected during diazotisation of the amino function in order to prevent intramolecular cyclisation side reactions.[47]



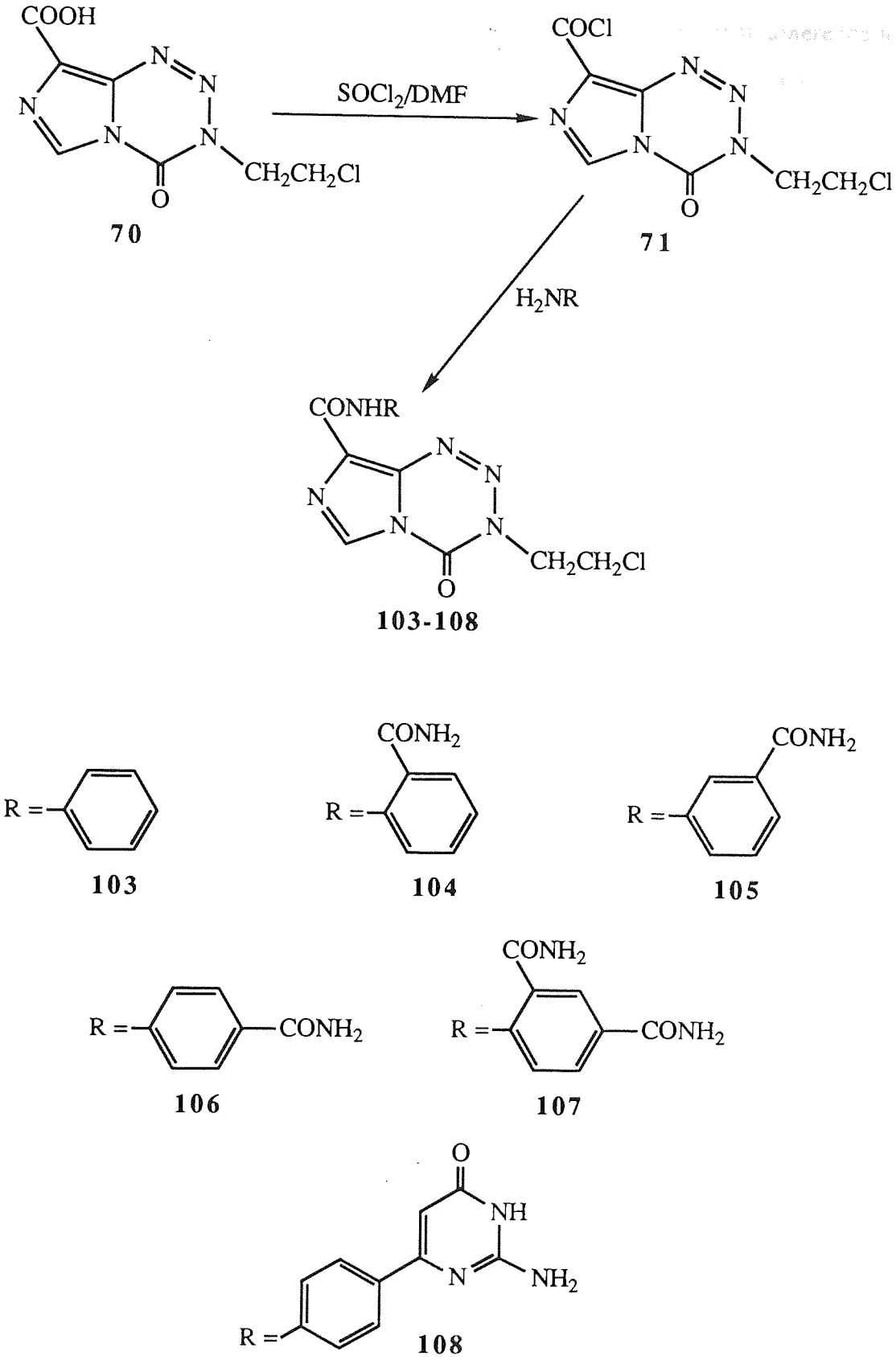
Scheme 3.3: Possible mechanisms for the formation of amides using DPPA.

3.1.3 Syntheses *via* 3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl chloride (71).

The failure of the synthetic routes utilising the nitro acid (**93**) prompted a return to the use of the 8-carboxylic acid derivative of mitozolomide as a precursor to further 8-substituted congeners. The reaction of the acid (**70**) with the chlorinating agent dimethylformamidinium chloride had been used by Horspool *et al.*[37,43] to prepare the acid chloride (**71**) which was shown to react preferentially with nucleophiles at the C8 rather than C4 positions. This method was successfully applied to the preparation of a series of 8-substituted carboxamides based on the inactive N-phenylcarbamoyl derivative (**103**), but which hopefully would possess better antitumour activity due to a greater potential for forming hydrogen bonding interactions.

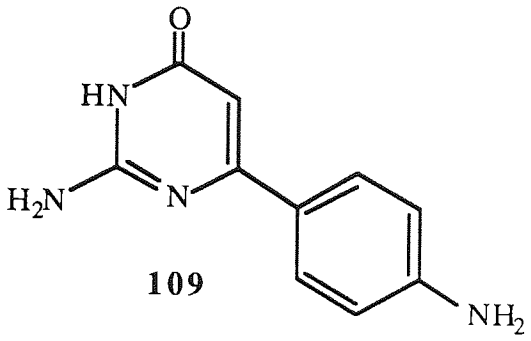
A series of aminobenzamide derivatives were prepared using the acid chloride procedure. Typically a solution of the appropriate aminobenzamide (1 equivalent) and triethylamine (1 equivalent) was added dropwise to a solution of the acid chloride (1 equivalent) (**71**) at room temperature (Scheme 3.4). Precipitation of the imidazotetrazinone was achieved using dilute HCl, which also served to remove any reacted aminobenzamide and generated an acidic environment in which the tetrazinones have considerably greater stability. The compounds synthesised *via* this procedure were frequently contaminated with coloured by-products.

Purification of the aminobenzamide derivatives proved troublesome due to a combination of the inherent instability of the tetrazinone ring system, their marked insolubility in volatile solvents and their polar nature. These derivatives were found to be soluble only in DMSO, DMF and 1-methylpyrrolidin-2-one. Indeed attempts to recrystallise the derivative (**104**) from the usual solvent, acetone:water (9:1), failed. Increasing the water content allowed dissolution but also led to a marked breakdown in the tetrazinone ring as evidenced by TLC. Significant amounts of impurities could be removed by repeated precipitation of a solution of the tetrazinone in 1-methylpyrrolidin-2-one with anhydrous ether. However solids generated this way were found by NMR analysis to contain varying degrees of solvation with 1-methylpyrrolidin-2-one although this proved to be the only satisfactory method for the purification of the dicarbamoylaniline derivative (**107**) which was obtained as a buff solid solvated with 0.5 moles of 1-methylpyrrolidin-2-one.



Scheme 3.4: The synthesis of anilide derivatives of mitozolomide *via* the acid chloride method.

Purification by flash chromatography was also investigated, the tetrazinones being stable on the acidic silica. However, their polar nature meant that adherence to the silica combined with their poor solubility in the eluting solvent caused considerable loss of material which explains the poor yields that were obtained in these reactions.



109

The acid chloride of mitozolomide (**71**) was also reacted with the pyrimidine (**109**) to give the pyrimidoaniline derivative (**108**). Compounds of this type have been widely studied at Aston and have been shown to possess interesting physical characteristics. The crystal structure of the bromo analogue revealed the existence of two tautomeric forms, the 1H (**111**) and 3H (**110**) forms, which are capable of forming 3 intermolecular hydrogen bonds (Figure 3.1).[92] This observation closely resembles the hydrogen bonding pattern of a cytosine-guanine base pair (**112**) in DNA and so it may be possible for this type of compound to improve DNA binding and recognition.

In common with the benzamide derivatives (**104-107**), (**108**) has limited solubility in organic solvents. Purification by repeated precipitation from 1-methylpyrrolidin-2-one with ether gave (**108**) as an orange solid which by NMR analysis was shown to be solvated with a single molecule of 1-methylpyrrolidin-2-one.

3.1.4 Syntheses using 8-carboxy-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (70).

The glycine ethyl ester analogue of mitozolomide (**96**) had previously been synthesised *via* the acid chloride route. However, the reaction could not be extended to the preparation of glycine, serine benzyl ester, phenylalanine and glycinamide derivatives. Reaction of the acid chloride in the presence of diphenylphosphoryl azide (DPPA) with phenylalanine ethyl ester, glycine ethyl ester and serine benzyl ester were also

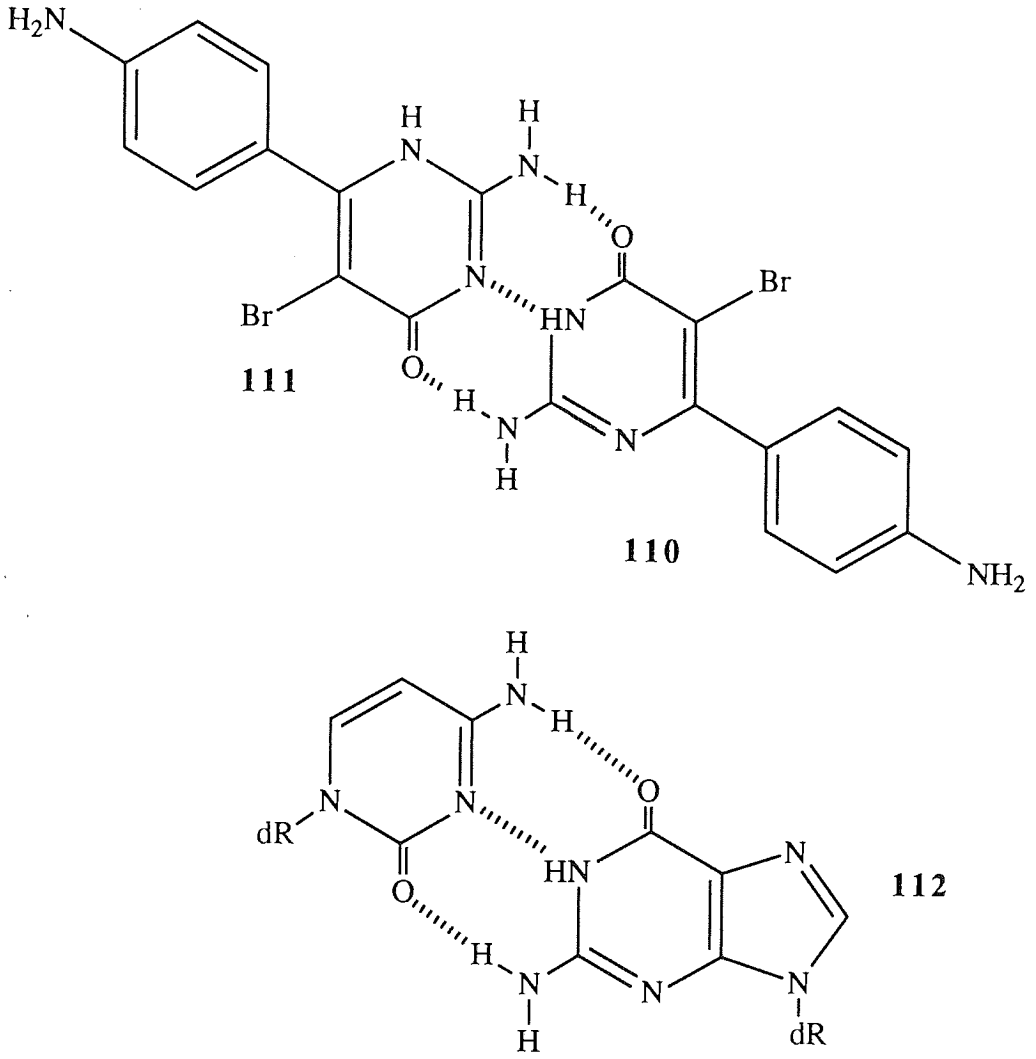


Figure 3.1: A comparison of the crystal structure of 2-amino-5-bromo-6-(4-aminophenyl)-pyrimidin-4-one (**110-111**) with a guanine-cytosine base pair (**112**).

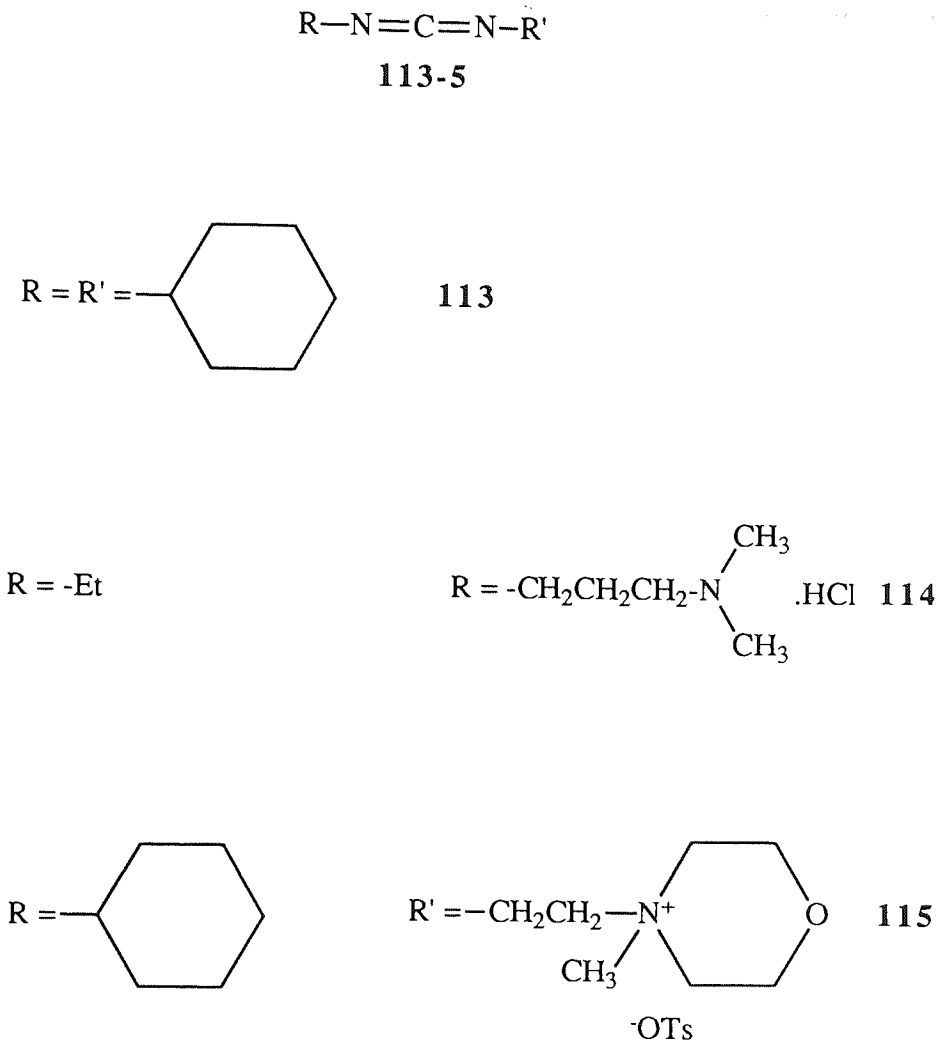
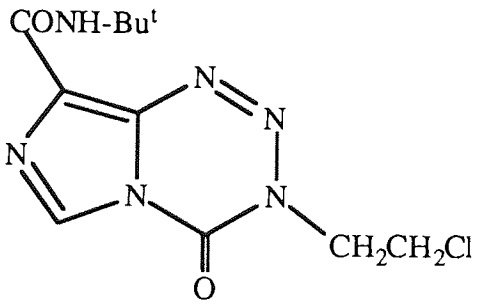


Figure 3.2: Reagents used in the formation of peptide bonds.

unrewarding.[37] As a result a search was conducted for an alternative synthetic procedure.

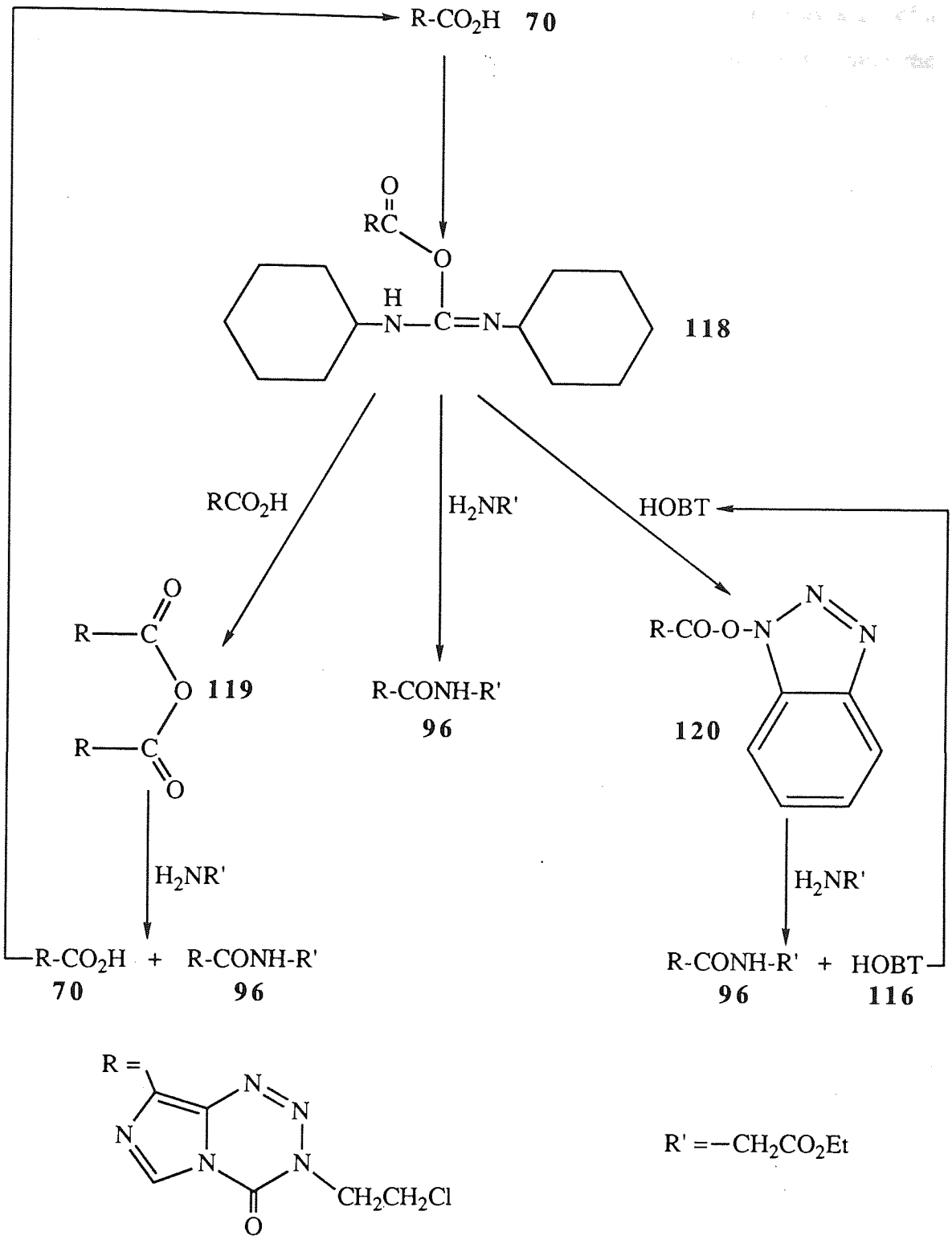
Sheehan and Hess first reported the use of dicyclohexylcarbodiimide (DCC) (**113**) in the formation of the peptide bond during 1955.[93] DCC was subsequently employed in the synthesis of a number of biologically active peptides e.g. oxytocin and vasopressin.[94] Carbodiimide mediated coupling has since become the mainstay of peptide synthesis with virtually every solid phase synthesis and many solution syntheses employing carbodiimides either directly or through the use of active esters.

DCC was selected for the initial attempts to couple t-butylamine to the acid derivative (**70**) of mitozolomide. Although not an amino acid, t-butylamine is a strongly basic nucleophile and as such would test, to the maximum extent, the ability of the carbodiimide method to activate the carboxylic acid to nucleophilic substitution in preference to C4 of the tetrazinone ring.



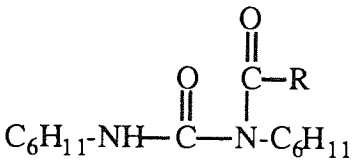
117

It soon became apparent that the order of mixing of the reagents was crucial to the reaction proceeding in the desired direction. Using the standard procedure of treating a solution of the acid (**70**), 1-hydroxybenzotriazole (HOBT) (**116**) and t-butylamine at 0°C with DCC led to a rapid darkening of the reaction mixture with the evolution of heat and gaseous products characteristic of the cleavage of the tetrazinone ring. The procedure was modified by pretreatment of a solution of the acid and HOBT at 0°C with DCC for 1 hour prior to the addition of the t-butylamine. Work-up of the reaction mixture gave a colourless solid, which following purification by flash chromatography gave the imidazotetrazinone (**117**) identical with that reported by Horspool using the acid chloride method.[43] The use of DCC as a coupling agent was extended to the preparation of Horspool's glycine ethyl ester derivative (**96**) as a colourless solid identical to that reported.[37,43]



Scheme 3.5: The cascade type mechanism for the formation of a peptide bond in the presence of DCC and HOBT.

The nature of the reaction conditions clearly suggests that preformation of a C8-activated intermediate was essential in allowing preferential attack at C8 over the tetrazinone ring. The reaction mechanism for the coupling is complex and involves a number of discrete activated intermediates which ultimately lead to the formation of a peptide bond, giving the appearance of a cascade type mechanism (Scheme 3.5).[91] The key initial step in this mechanism is the formation of the O-acylisourea (**118**), a highly reactive species which is capable of reacting with all the components of the reaction mixture. Reaction of (**118**) with a second molecule of the carboxylic acid (**70**) leads to the formation of the symmetrical anhydride (**119**) which undergoes nucleophilic attack by the amino component to generate one equivalent of the peptide (**96**) and one equivalent of the carboxylic acid (**70**) for recycling into the reaction. The O-acylisourea (**118**) may also react with the auxiliary nucleophile HOBT to give the active ester (**120**) which in turn reacts with the amino component leading to formation of the peptide and regeneration of HOBT. Finally the isourea (**118**) can react directly with the amino component to form the peptide bond in (**96**).



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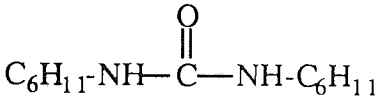


122

Although DCC is an efficient coupling agent it is not without problems, many of which are reduced by the use of HOBT as an auxiliary nucleophile. The O-acylisourea intermediate (**118**) is prone to undergo intramolecular side reactions the most notable being an O-N shift generating N-acylurea or ureide derivatives (**121**) which often prove difficult to separate from the main product. HOBT reacts so rapidly with the O-acylisourea that such competing intramolecular reactions are not seen. When DCC is used alone the coupling reaction is often associated with a considerable degree of racemisation. With HOBT as an auxiliary nucleophile low levels of racemisation are seen without a reduction in coupling efficiency probably in part due to the conversion to the active ester (**120**) which is less conducive to racemisation.

However, a significant disadvantage in using DCC was removal of the major by-product dicyclohexylurea (DCU) (**122**). Although predominately insoluble in most

organic solvents (except alcohols) some remains in solution especially in the presence of other dissolved material. Even after flash chromatography NMR spectra showed small quantities of DCU in the final products.

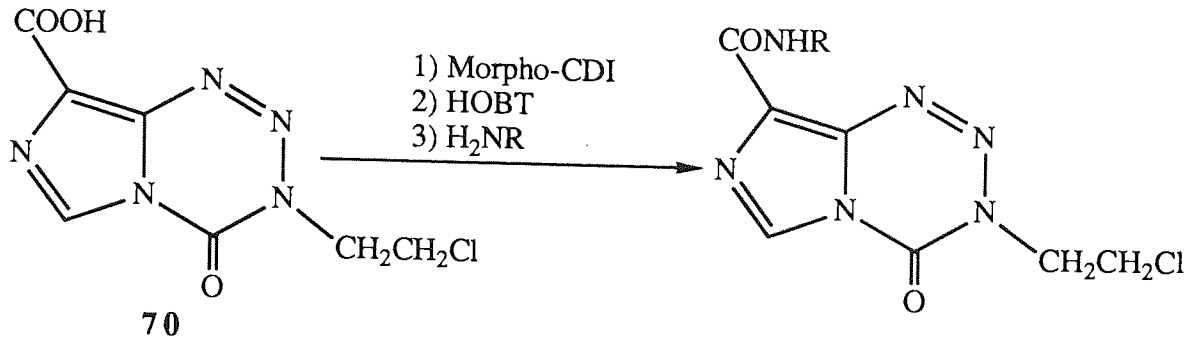
Sheehan and Hlavaka[95] had reported the synthesis and the use of carbodiimides containing amino and quaternary centres. The use of such reagents was appealing since the urea by-products are water or acid soluble and as such may be entirely removed during the working up procedure. Two such carbodiimides are commercially available; 1-ethyl-3-(3´-dimethylaminopropyl)carbodiimide hydrochloride (WSC.HCl) (**114**) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-ρ-toluene sulphonate (Morpho-CDI) (**115**) (Figure 3.2).

Attempts to prepare the glycine ethyl ester derivative (**96**) with WSC.HCl using the same procedure as above led to the rapid production of a red colour indicative of tetrazinone ring cleavage. The reason for this is unclear and awaits explanation since it would not be expected that the highly hindered and protonated tertiary amino function within the carbodiimide would directly attack the tetrazinone ring, yet nucleophilic attack obviously takes place.

Morpho-CDI, which contains a non-nucleophilic quaternary ammonium centre, was not expected to directly attack the tetrazinone ring. Indeed, it was possible to prepare the glycine ethyl ester derivative (**96**) free of urea by-products in a 35% yield. Furthermore, unlike coupling with DCC it was established that pretreatment with the carbodiimide was not necessary, in fact the reagents could be mixed as one in acetonitrile at room temperature without significant breakdown of the tetrazinone ring. There may be two reasons for this observation:

(a) the amino acid derivative, glycine ethyl ester (pK_a 9.7), is a much weaker base than alkylamines such as t-butylamine (pK_a 10.8) and as such is far less nucleophilic.

(b) Kunz[96] reported that under aqueous conditions Morpho-CDI in the presence of HOBT produces a solution with an initial pH *ca.* 4.5 changing to 3.5 at the conclusion of the reaction. The small amount of water present in the reaction mixture may lead to a slightly acidic solution which would be beneficial in preventing nucleophilic attack at the tetrazinone ring.



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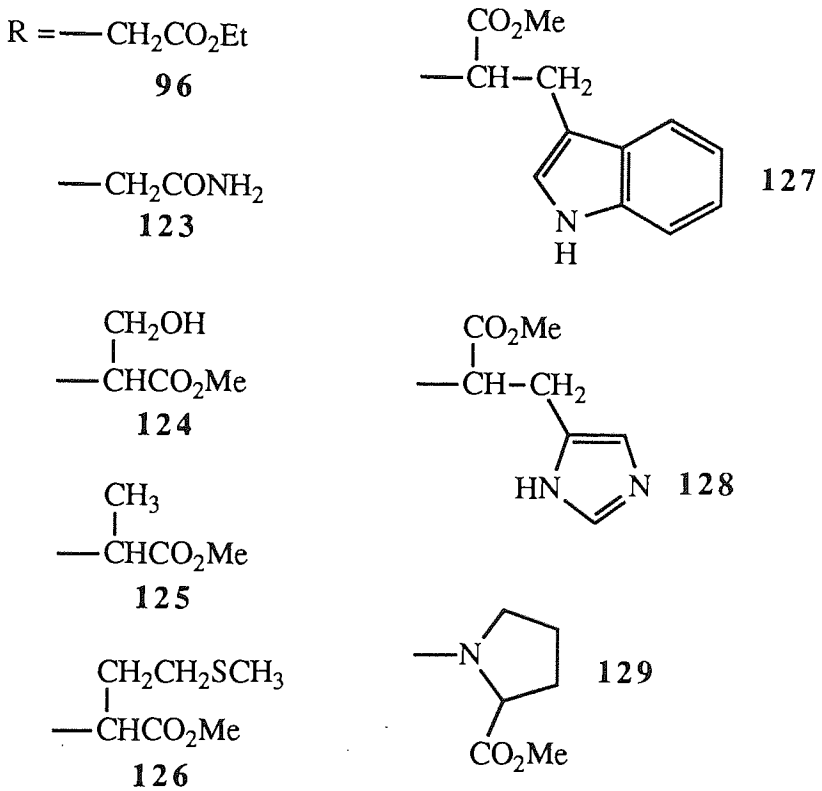


Scheme 3.6: The synthesis of amino acid derivatives of mitozolomide.

The use of Morpho-CDI was extended to include the reaction of the acid (**70**) with derivatives of several amino acids. A number of interesting analogues were prepared in reasonable yields ranging from 30% to 74% (Scheme 3.6).

The serine methyl ester derivative (**124**) is unusual in that it is a compound in which there is a potential nucleophilic alcohol group within a molecule susceptible to nucleophilic attack and known to be unstable in alcohols. The tryptophan derivative (**127**) was prepared following the observation that tryptophan is capable of participating in aromatic π-π orbital stacking interactions with 7-methylguanine[97] and so may possibly improve binding to DNA G-C rich regions. The glycinamide derivative (**123**) was an attempt to improve the activity of the glycine ethyl ester analogue by incorporating a remote carboxamide group thereby increasing its hydrogen bonding potential.

However, the histidine methyl ester derivative (**128**) could not be isolated even though TLC examination indicated that some reaction had taken place. This would be an interesting derivative to pursue since there is some evidence to indicate that the imidazole ring may be involved in the recognition of cytosine and/or guanine residues in protein-DNA interactions.[98] Likewise, it was not possible to isolate the proline methyl ester derivative (**129**) in a pure form. The crude compound was isolated as a cream solid which rapidly darkened to give a pinky-red resinous material when attempts were made to purify it by precipitation from ethyl acetate with ether, or flash chromatography.

3.1.5 The synthesis of peptide analogues of mitozolomide.

There are numerous reports of antitumour agents linked to peptide moieties in an effort to improve cellular transport and selectivity.[99-101] The successful coupling of the acid derivative (**70**) to individual amino acids opened up the possibilities of peptide congeners of mitozolomide.

The strategy for the preparation of peptide analogues was based on mimicking the α-helical structure of DNA-binding proteins. The helix-turn-helix motif of DNA-binding proteins comprises two α-helical subunits, one known as the 'recognition helix' which fits neatly into the major groove of DNA, while the other lies across the major groove stabilising the complex *via* interactions with the phosphate backbone (Figure 3.3).

The amino acid sequences of many DNA-binding proteins are known and

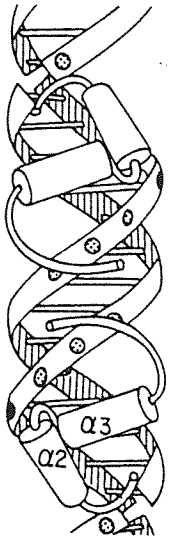


Figure 3.3: The helix-turn-helix binding motif of proteins used in the recognition of DNA.[102]

have recently been reviewed.[103-4] Analysis of these sequences indicated that the key structural requirements for the recognition helix was a valinyl or isoleucinyl residue flanked on the carboxy terminus by an hydroxy containing residue followed by a residue such as glutamine or asparginine.

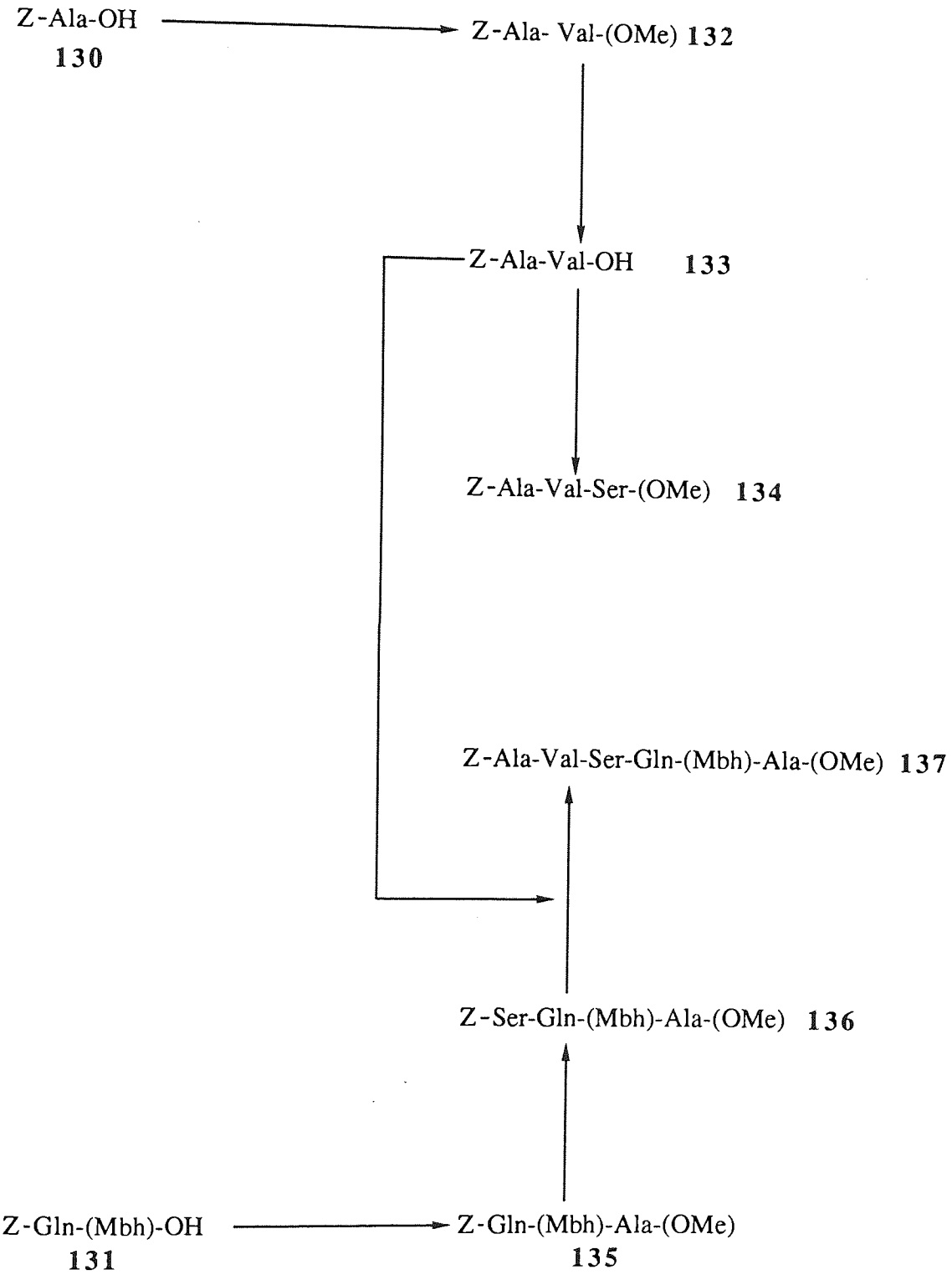
Armed with this information a series of four residue sequences were constructed within the Protelyze® molecular modelling package.[105] Garnier-Osgoodthorpe analysis of these sequences gave results for the tendency to adopt an α-helical configuration (Table 3.1). The most promising results were generated with the Ala-Val-Ser-Gln and Ala-Ile-Ser-Gln sequences, both having 75% predicted α-helical characteristics.

The Ala-Val-Ser-Gln sequence was chosen for further analysis, with several derivatives being constructed with additional amino acids at the carboxy terminus (Table 3.1). The sequence Ala-Val-Ser-Gln-Ala was calculated as possessing a 100% tendency to adopt an α-helical configuration and as a consequence was chosen as the final synthetic target. Moreover, the intention was to construct mitozolomide analogues in which the amino acids were incorporated stepwise to determine the effect each amino acid had upon antitumour activity.

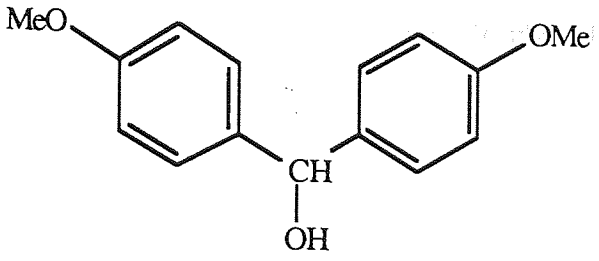
The peptides (**132-137**) were prepared using the standard carbodiimide

Table 3.1: The tendency of a range of small peptides to adopt α-helical or β-sheet conformations as predicted by Garnier-Osgoodthorpe calculations (the most favourable sequences for the helix conformation are shown in bold type).

Peptide fragment.	%-α-helix.	%-β-sheet.
Gly-Val-Ser-Gln.	0	25
Ala-Val-Ser-Gln.	**75**	**25**
Phe-Val-Ser-Gln.	0	75
Gly-Ile-Ser-Gln.	0	25
Ala-Ile-Ser-Gln.	**75**	**25**
Phe-Ile-Ser-Gln.	0	25
Gly-Val-Thr-Gln.	0	75
Ala-Val-Thr-Gln.	50	50
Phe-Val-Thr-Gln.	0	100
Gly-Ile-Thr-Gln.	0	75
Ala-Ile-Thr-Gln.	50	50
Phe-Ile-Thr-Gln.	0	100
Gly-Val-Ser-Asn.	0	0
Ala-Val-Ser-Asn.	0	50
Phe-Val-Ser-Asn.	0	50
Gly-Ile-Ser-Asn.	0	0
Ala-Ile-Ser-Asn.	0	50
Phe-Ile-Ser-Asn.	0	50
Gly-Val-Thr-Asn.	0	0
Ala-Val-Thr-Asn.	0	75
Phe-Val-Thr-Asn.	0	75
Gly-Ile-Thr-Asn.	0	0
Ala-Ile-Thr-Asn.	0	75
Phe-Ile-Thr-Asn.	0	75
Ala-Val-Ser-Gln-Gly.	0	40
Ala-Val-Ser-Gln-Ala.	**100**	**0**
Ala-Val-Ser-Gln-Phe.	60	40
Ala-Val-Ser-Gln-Cys.	40	40
Ala-Val-Ser-Gln-His.	80	0
Ala-Val-Ser-Gln-Lys.	80	0
Ala-Val-Ser-Gln-Pro.	0	40
Ala-Val-Ser-Gln-Trp.	20	20
Ala-Val-Ser-Gln-Asp.	40	20



Scheme 3.7: The strategy employed in the synthesis of the pentapeptide (**137**).

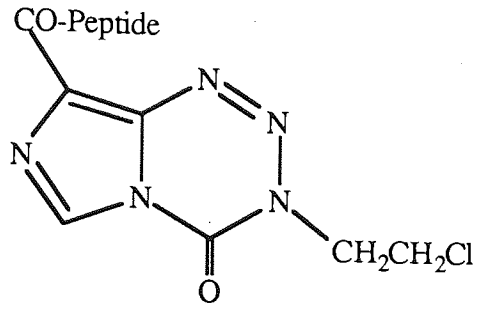


138

condensation with WSC.HCl as the carbodiimide and HOBT as the auxiliary nucleophile in yields ranging from 51% to 76%. The terminal amino groups were protected with the benzyloxycarbonyl (Z) group which was readily removed by catalytic hydrogenation and the deprotected peptide coupled without isolation or purification. Terminal carboxy groups were protected as the methyl esters and where necessary deprotected with methanolic sodium hydroxide. The hydroxyl group of the serine residue was not protected as the ether since previous reactions had shown that this amino acid could be incorporated trouble free into the tetrazinone ring system. Early on it became clear that the incorporation of the glutamine residue induced marked insolubility into the peptides. For this reason the ω-carboxamide group in glutamine was protected with the 4,4-dimethoxybenzhydrol group (Mbh) (**138**) and so thereby increasing the lipophilicity of the peptides. The Mbh group is acid sensitive being cleaved by trifluoroacetic acid.[106] Base cleaved protecting groups could not be used since this would lead to concomitant breakdown of the tetrazinone ring during deprotection. The strategies used in the preparation of the peptides are shown in Scheme 3.7.

A range of N-deprotected peptides were coupled, without isolation, to the acid (**70**) using the carbodiimide method outlined earlier (Table 3.2). Unfortunately, the pentapeptide derivative (**141**) was not sufficiently pure for elemental analysis due to the presence of a dark grey 'colloidal' material, formed during the hydrogenation of the Z-protected peptide precursor (**137**) in DMF, contaminating the final product. Attempted purification *via* column chromatography with neutral alumina failed due to cleavage of the tetrazinone ring while the poor solubility of the peptide in volatile organic solvents prevented the use of flash chromatography. However, I.R. and NMR analysis produced spectra consistent with the preparation of (**141**) while the presence of $(M+H)^+$ and $(M+Na)^+$ in the FAB mass spectrum confirmed the synthesis of (**141**). Consequently, it would appear that the synthesis of larger peptidic derivatives of the imidazotetrazinones will require the use of

Table 3.2: Peptide derivatives of mitozolomide prepared using Morpho-CDI and their yields.



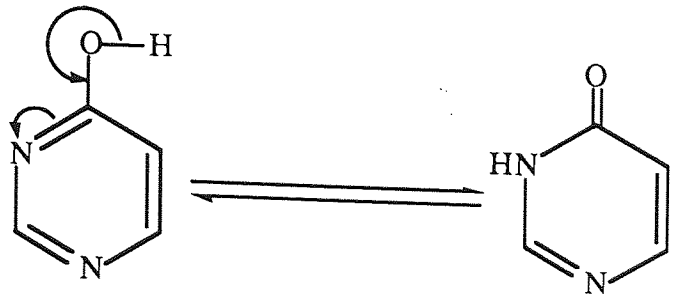
Compound No.	Peptide.	Yield (%).
139	Ala-Val-(OMe).	48
140	Ala-Val-Ser-(OMe).	6
141	Ala-Val-Ser-Gln-(Mbh)-Ala-(OMe).	-[a]
142	Gln-(Mbh)-Ala-(OMe).	22

a. not sufficiently pure to allow calculation of an accurate yield.

more complicated purification techniques, probably involving HPLC.

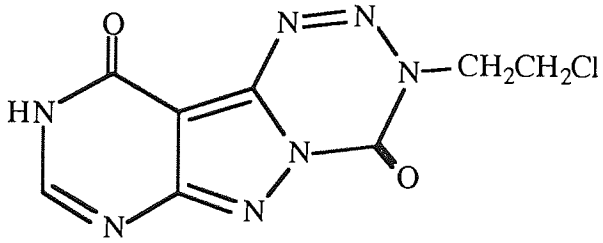
3.1.6 Synthesis of the pyrimido[4´,5´:3,4]pyrazolo[5,1-d]-1,2,3,5-tetrazine ring system.

All the mitozolomide derivatives synthesised to date can be considered to contain linear carboxamide groups at the C8 position. However, molecular modelling studies have indicated that the preferred orientation of the carboxamide group in mitozolomide involved an intramolecular hydrogen bond with the imidazole nitrogen at the N7 position. It was suggested that it would be interesting to synthesise compounds in which the carboxamide group was constrained into the preferred orientation within a cyclic system. The preparation of such a system requires a carbon at the 7-position, a criterion not fulfilled by the imidazotetrazinones, but fortunately is feasible with the isomeric pyrazolotetrazinones - a series of compounds that have been shown to possess comparable antitumour activity.



143 144

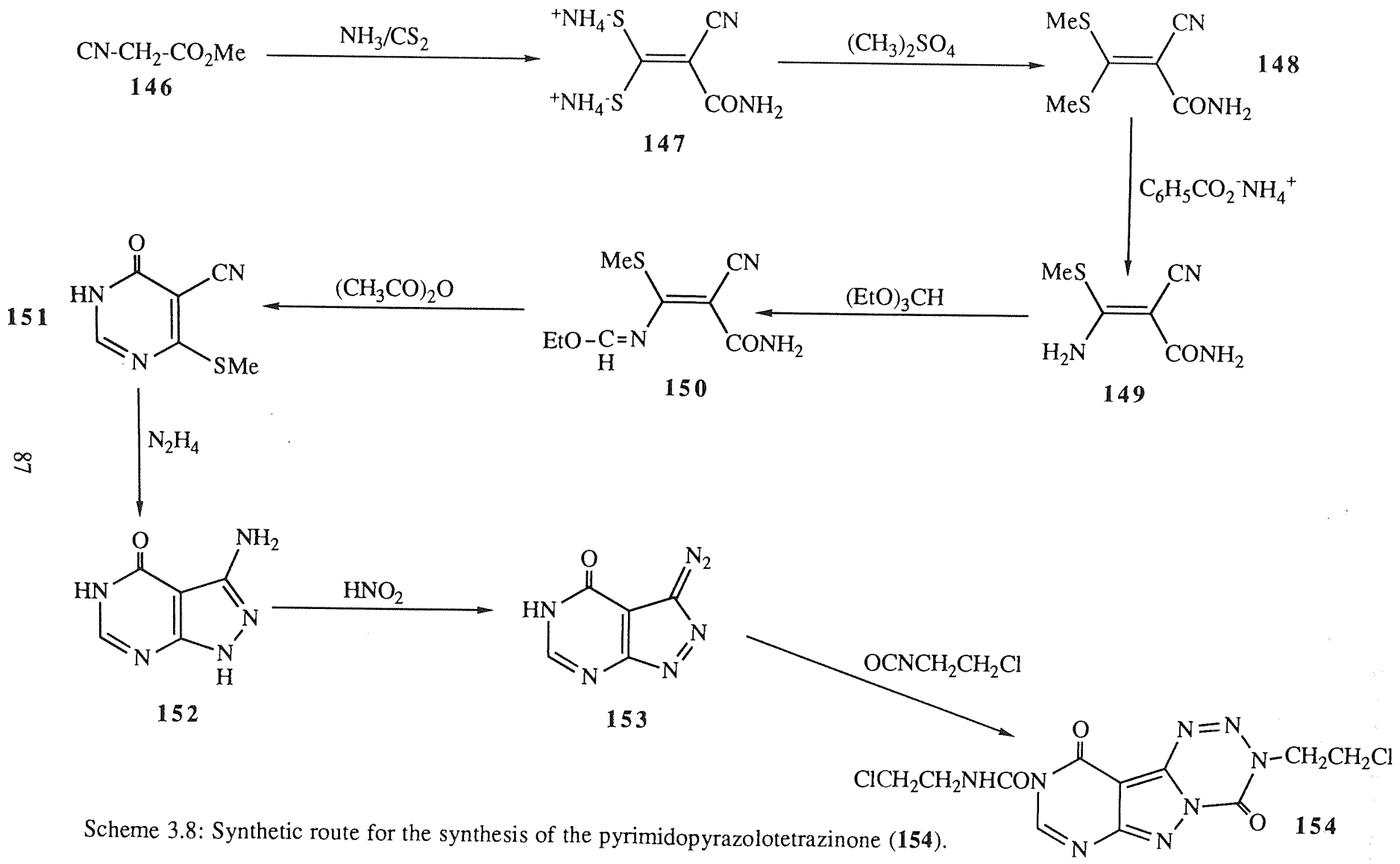
The naturally occurring 4-hydroxypyrimidine ring system (**143**), in which the preferred tautomer is the pyrimidin-4(3H)-one form (**144**), contains what can be considered a carboxamide group in a cyclic form. Moreover, this ring system is an important component of nucleosides and as such could be imagined as participating in the formation of hydrogen bonds within DNA, while the formation of a tricyclic aromatic system may possibly induce a significant degree of π-π stacking interactions, enabling a degree of intercalating activity to become evident in the molecule. As a result of these considerations it was decided to attempt the synthesis of a model pyrimidopyrazolotetrazinone (**145**).



145

It was envisaged that (**145**) could be prepared *via* the standard method of the addition of an isocyanate to the diazo compound (**153**). Although the diazo compound is not reported in the literature there is one report of the synthesis of the amino precursor 3-aminopyrazolo[4,3-d]pyrimidin-6(1H)-one (**152**) by Dornow and Dehmer (Scheme 3.8).[107]

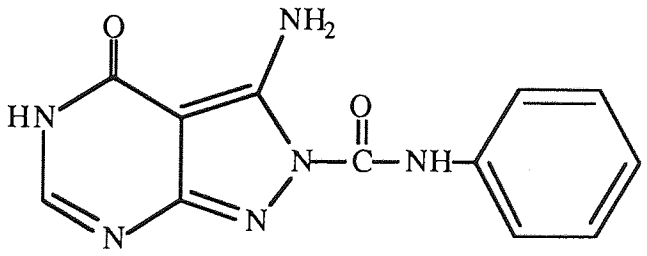
The amine (**152**) was successfully prepared *via* the method illustrated in Scheme 8. Formation of the olefinic bond which ultimately is destined to become the C5-C6 double-bond in the pyrimidine (**151**) was formed by the addition of CS_2 to the active



Scheme 3.8: Synthetic route for the synthesis of the pyrimidopyrazolotetrazinone (**154**).

hydrogens of methyl cyanoacetate (**146**).[108] Methylation of (**147**) using dimethyl sulphate,[108] followed by the nucleophilic displacement of one of the methylthio residues in (**148**) by ammonia gave the cyanoacrylamide (**149**).[109] Compounds of this type are capable of undergoing intramolecular cyclisation to the pyrimidine (**151**) in the presence of triethyl orthoformate and acetic anhydride *via* the ethoxymethyleneamino intermediate (**150**). The methylthio substituent of the pyrimidine (**151**) undergoes nucleophilic displacement reactions with a range of reagents. When the reagent was hydrazine displacement occurred with concomitant intramolecular cyclisation to the required aminopyrazole (**152**).

Diazotisation of the the amine (**152**) gave a yellow solid, which did not melt on gentle heating but which exploded violently when subjected to shock or rapid heating and was identified, by its infrared spectra, as the diazo compound (**153**). Attempts to prepare the tetrazinone by the addition of 2-chloroethyl isocyanate and stirring the reaction mixture at room temperature failed to yield the tetrazinone (**145**) even after a prolonged reaction period of 30 days. However, when a suspension of the diazo compound (**153**) and 2-chloroethyl isocyanate were heated under reflux in dichloromethane for 5 days a pale yellow solid was isolated that was identified as the tetrazinone (**154**) (Scheme 3.8). Evidently the excess isocyanate required to drive the reaction towards formation of the tetrazinone also acts as a carbamoylating agent towards the N1 position of the pyrimidine ring. The side reaction, and poor yield (17%) of the addition reaction prompted a search for a new synthetic pathway.



155

Ege and his co-workers[34] had successfully prepared pyrazolotetrazinones *via* the diazotisation of N-carbamoylpyrazoles (see Scheme 1.3, page 30), examples of which had been prepared by the condensation of semicarbazides with cyanoketones. Furthermore, the earlier observation that the methylthio group in the pyrimidine (**151**) underwent nucleophilic displacement by hydrazine to yield the pyrazolopyrimidine (**152**)

suggested that displacement would also occur with semicarbazides which should give the N-carbamoylpyrazolopyrimidine (**155**). This reaction was tried with the commercially available 4-phenylsemicarbazide under the reaction conditions, refluxing with the pyrimidine (**151**) in pyridine, used for the cyclisation with hydrazine. Unfortunately, although cyclisation did occur to the pyrazolopyrimidine the isolated compound proved to be identical with a sample of the 3-aminopyrazolopyrimidine (**152**) prepared previously. Evidently base induced degradation of the N-carbamoylpyrazolopyrimidine (**155**) had occurred, a feature that has also been observed with the N-carbamoylpyrazoles.[34] The use of 2-ethoxyethanol in preference to pyridine as the reaction solvent failed to induce any reaction between the individual components.When ethanol was used as the solvent a buff coloured solid was produced, although only in a poor yield of 10%, which had the spectroscopic characteristics of (**155**) but for which satisfactory elemental analysis could not be obtained. However, by manipulation of the reaction conditions it would appear that this reaction could be encouraged to proceed in the desired direction allowing the preparation of tricyclic compounds of considerable interest.

3.1.7 Physical properties of 1,2,3,5-tetrazinones.

In common with the previously reported imidazotetrazinones the compounds prepared in this study were white or pastel coloured solids freely soluble in DMF, DMSO or 1-methylpyrrolidin-2-one. The incorporation of polar residues, such as carboxamide groups, into the molecules led to a decrease in their solubility in volatile organic solvents with a concomitant increase in their melting points. Upon heating all the derivatives decomposed usually with effervescence to give deep red resins.

The spectroscopic properties of the imidazotetrazinones are particularly characteristic and are worth noting:

(a) uv-visible absorption: all the compounds prepared to date possessed a strong characteristic absorption in the range 320-350nm.

(b) I.R. absorption: all the compounds exhibited carbonyl stretching frequencies at 1730-1750cm^{-1} which was indicative of the presence of the tetrazinone ring. Where the molecule also contained an aliphatic ester group, as in the amino acid and peptide derivatives, it was generally impossible to distinguish the absorbances due to the C=O of the tetrazinone ring

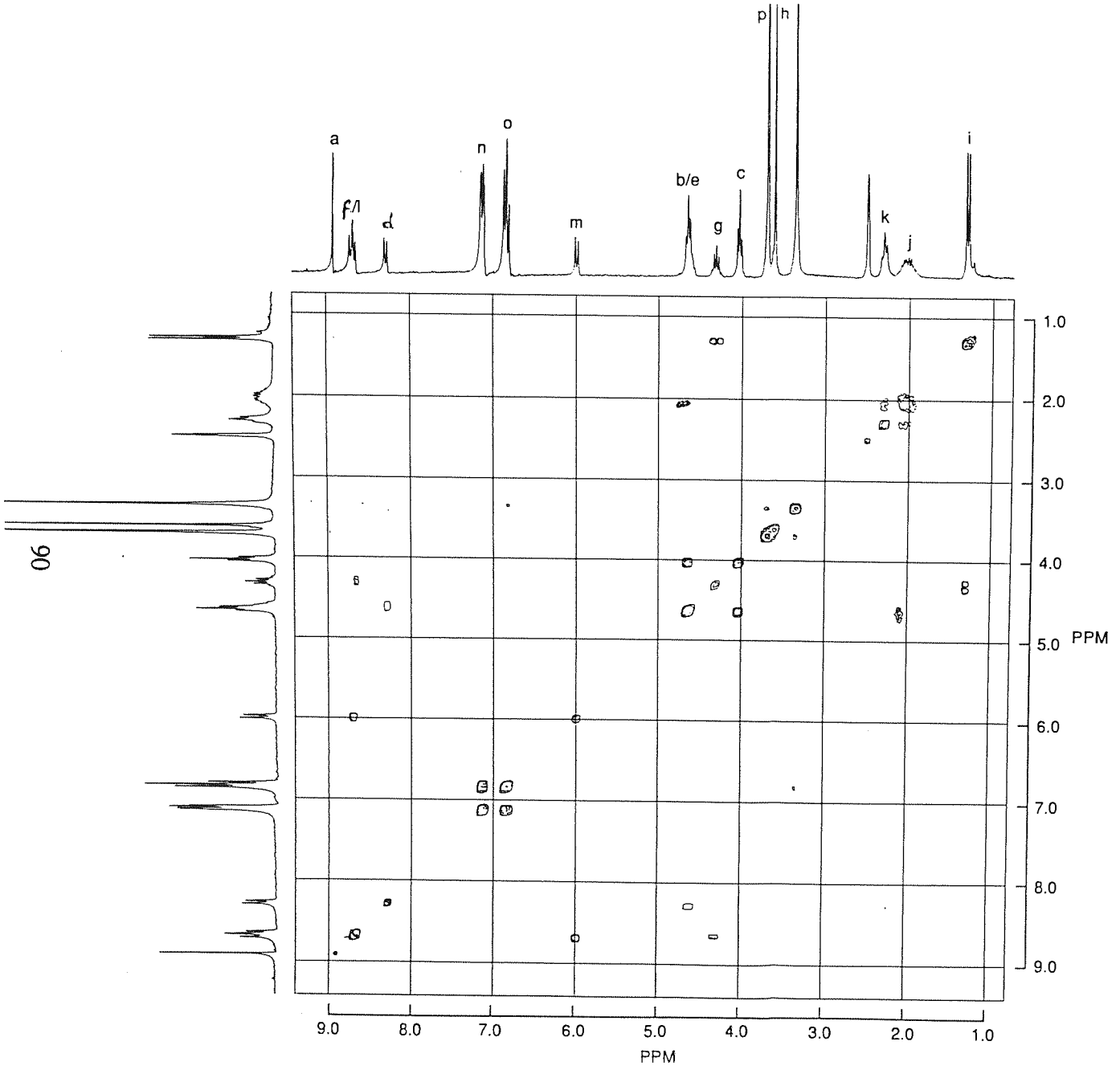
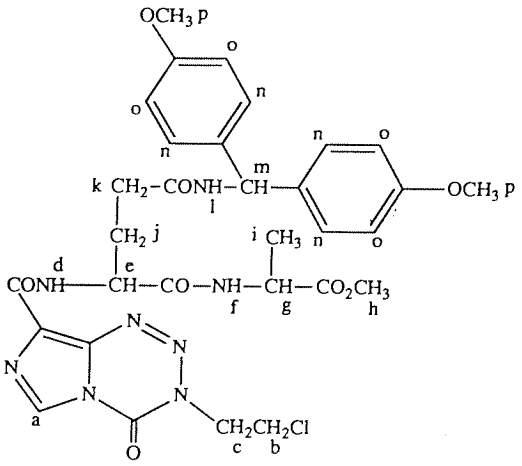


Figure 3.4: 2D–COSY spectrum for the dipeptide derivative of mitozolomide (142)



and the ester moiety. The tetrazinones also possessed amide carbonyl stretching frequencies between 1650 and 1680 cm^{-1} representing the C8 substituent.

(c) NMR spectra: confirmation of the presence of the tetrazinone ring could be obtained by ^1H NMR which showed characteristic triplets at 4.7-4.6 and 4.1-4.0 ppm representing the 2-chloroethyl CH_2Cl and N-CH_2 protons respectively. The H6 imidazole proton was seen at 9.1-8.8 ppm as a sharp singlet. For the more complex compounds, such as the dipeptide derivative (**142**) where resonances overlap it was possible to assign all the signals to their respective protons by the use of 2D COSY experiments. (Figure 3.4). A large proportion of the signals in Figure 3.4 can be assigned from the one-dimensional spectrum printed along the axis of the two-dimensional contour plot. Analysis of the chemical shifts enables the clear assignment of the signals from protons (a), (b), (c), (h), (i), (m), (n), (o) and (p). the data in the two-dimensional spectra showed eight cross peaks which were symmetrically placed with respect to the diagonal. Interpretation of the cross peaks and the signals they are connected to allowed the following assignment of the remaining signals:

(i) the signal from the alanyl β-CH_3 (i) has a cross peak connecting it to the quintet at 4.31 ppm which therefore, must be from the α-CH (g) of the alanine residue.

(ii) the signal from the multiplet (j) has a cross peak connecting it to a signal under the triplet at 4.65 ppm indicating that the signal from the α-CH of the glutamine residue (e) is hidden and that by definition the multiplet (j) refers to the β-CH_2 protons and multiplet (k) refers to the γ-CH_2 protons of the glutamine residue, which themselves show cross peak connections.

(iii) the signal assigned to the α-CH of the alanine residue (g) has a cross peak connecting it to the triplet at 8.67 ppm which also has a cross peak connecting it to the doublet at 5.98 ppm indicating that the triplet arises from the imposition of the signals from the amide protons (f) and (l).

(iv) the signal assigned to the α-CH of the glutamine residue (e) has a cross peak connecting it to a doublet at 8.27 ppm which must therefore refer to the amide proton (d).

(d) mass spectra: the fragmentation pathway for the electron impact mass spectrum of the imidazotetrazinone (**96**) is shown in Figure 3.5. The main characteristic to note is the absence of a molecular ion with cleavage of the tetrazinone ring and the loss of 2-chloroethyl isocyanate generating the principal high molecular weight fragment (**156**) (i.e. M+ -

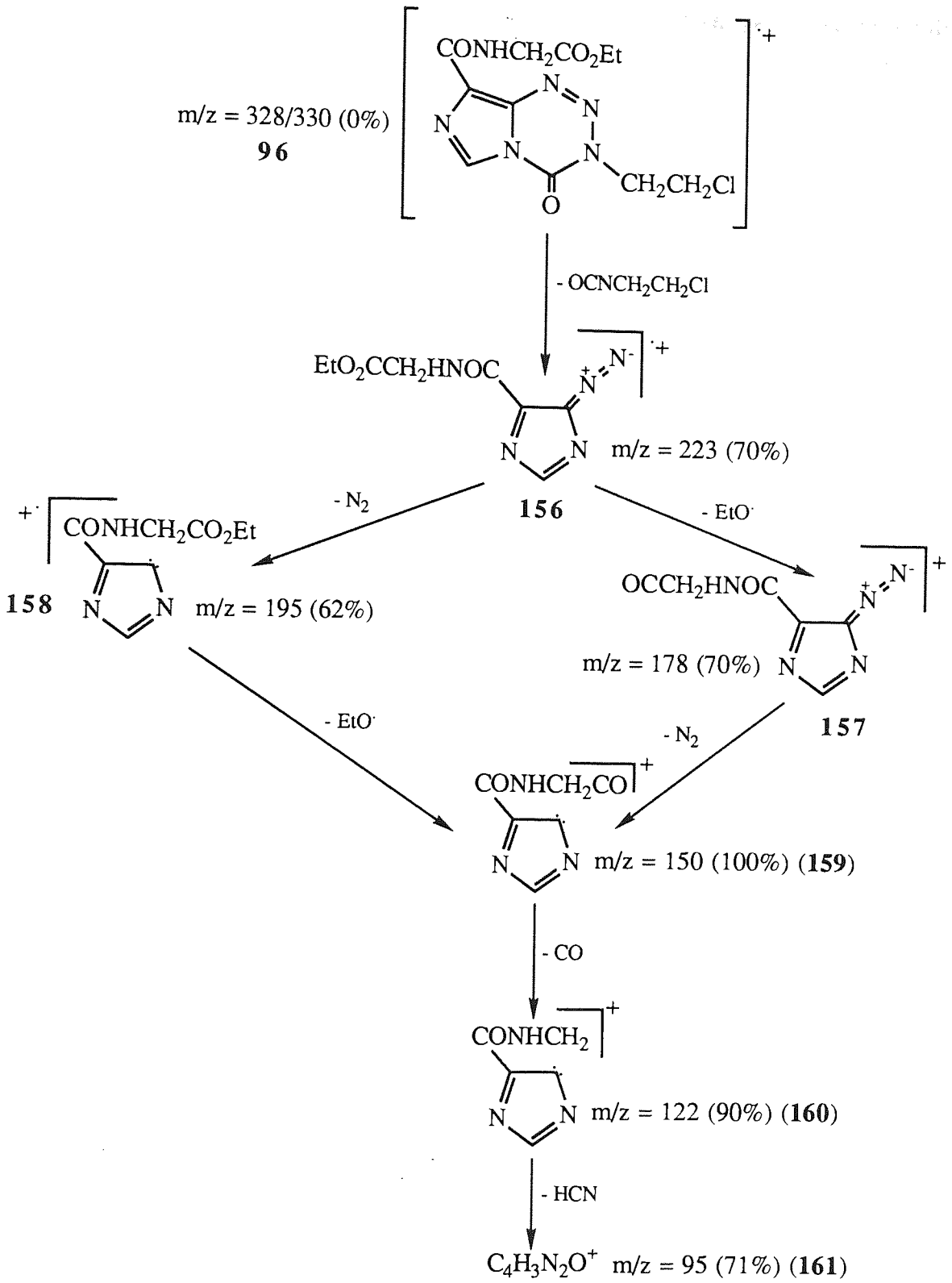


Figure 3.5: The major fragmentation products for the electron impact mass spectrum of the glycine ethyl ester derivative of mitozolomide (**96**).

105/107). Subsequent fragmentation is characteristic of the diazo function and the glycinyl substituent of (**156**). Loss of nitrogen from the diazo species (**156**) generates the highly reactive carbene species (**158**). Fragmentation of the glycine ester portion of the molecular *via* the loss of an ethoxy radical and carbon monoxide to give (**159**) and (**160**) respectively, represents the other characteristic fragments. The fragmentation pattern of the other C8 substituted imidazotetrazinones are essentially similar with minor variations resulting from the nature of the nuclear substituent.

In contrast imidazotetrazinones analysed using Fast Atom Bombardment (FAB) spectroscopy showed the increased stability of the tetrazinone ring when using this technique by the presence of $(M+H)^+$ and $(M+Na)^+$ ions in the characteristic 3:1 ratio of chlorine containing compounds. The fragmentation pattern of the tripeptide derivative (**140**) is shown in Figure 3.6. Even electron ions, such as the $(M+H)^+$ ion (**162**), are more stable than their radical counterparts produced in electron impact spectroscopy undergoing less fragmentation and decomposing almost exclusively by the ejection of neutral molecules to give other even electron ions. It is noticeable that with FAB the peptide substituent fragments, in preference to the tetrazinone ring, from the C-terminus in a sequence which confirms the structure of the molecule. The $(M+H)^+$ ion (**162**) can fragment at the amide bonds (a or b) with the charge retained by the C-terminal fragments to give either (**163**) or (**164**). The ionic species (**164**) may also arise from the fragmentation of (**163**). Loss of carbon monoxide from (**164**) generates the secondary cation (**165**) which is further stabilised *via* an ability to form resonance stabilised structures. The presence of the signal at m/z 219 (**166**), of unknown structure, indicates that cleavage of the tetrazinone ring also occurs.

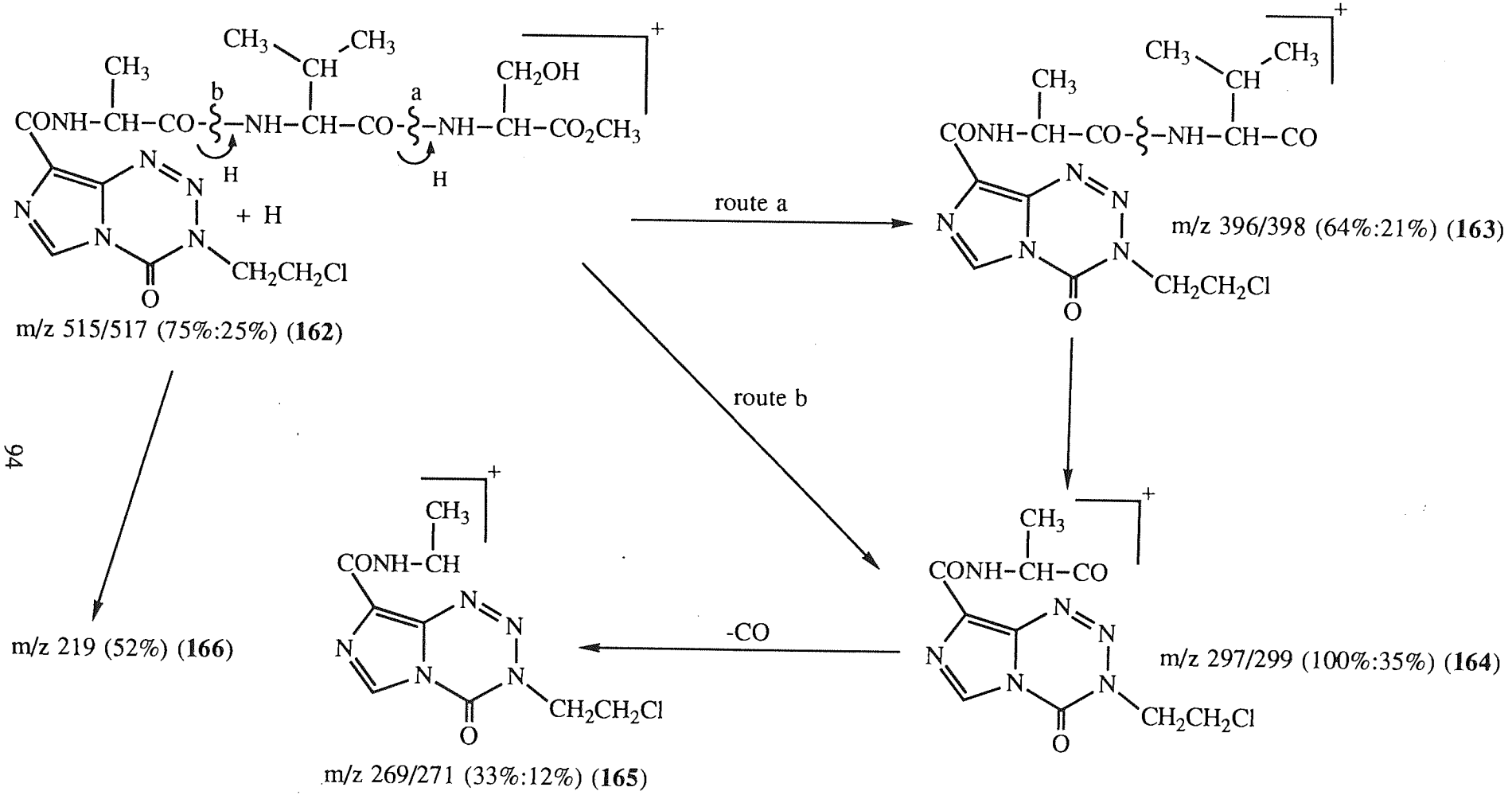
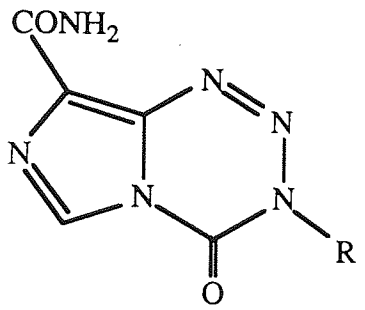


Figure 3.6: The major fragmentation products for the FAB mass spectrum of the tripeptide derivative of mitozolomide (**140**).

3.2 Synthesis of 1,2,3-Benzotriazin-4(3H)-ones.

The inherent instability of the 1,2,3,5-tetrazin-4(3H)-one ring system towards nucleophiles means the study of the interactions between imidazotetrazinones and DNA is plagued with difficulties. However, it was considered that by the use of molecules which contained the structural features of the tetrazinones, but which were less susceptible to ring cleavage, a model for such interactions could be elucidated.



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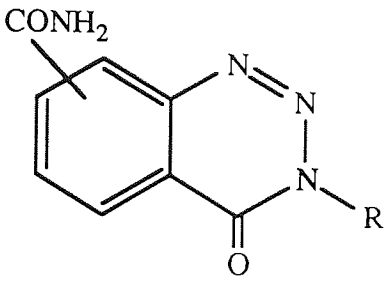
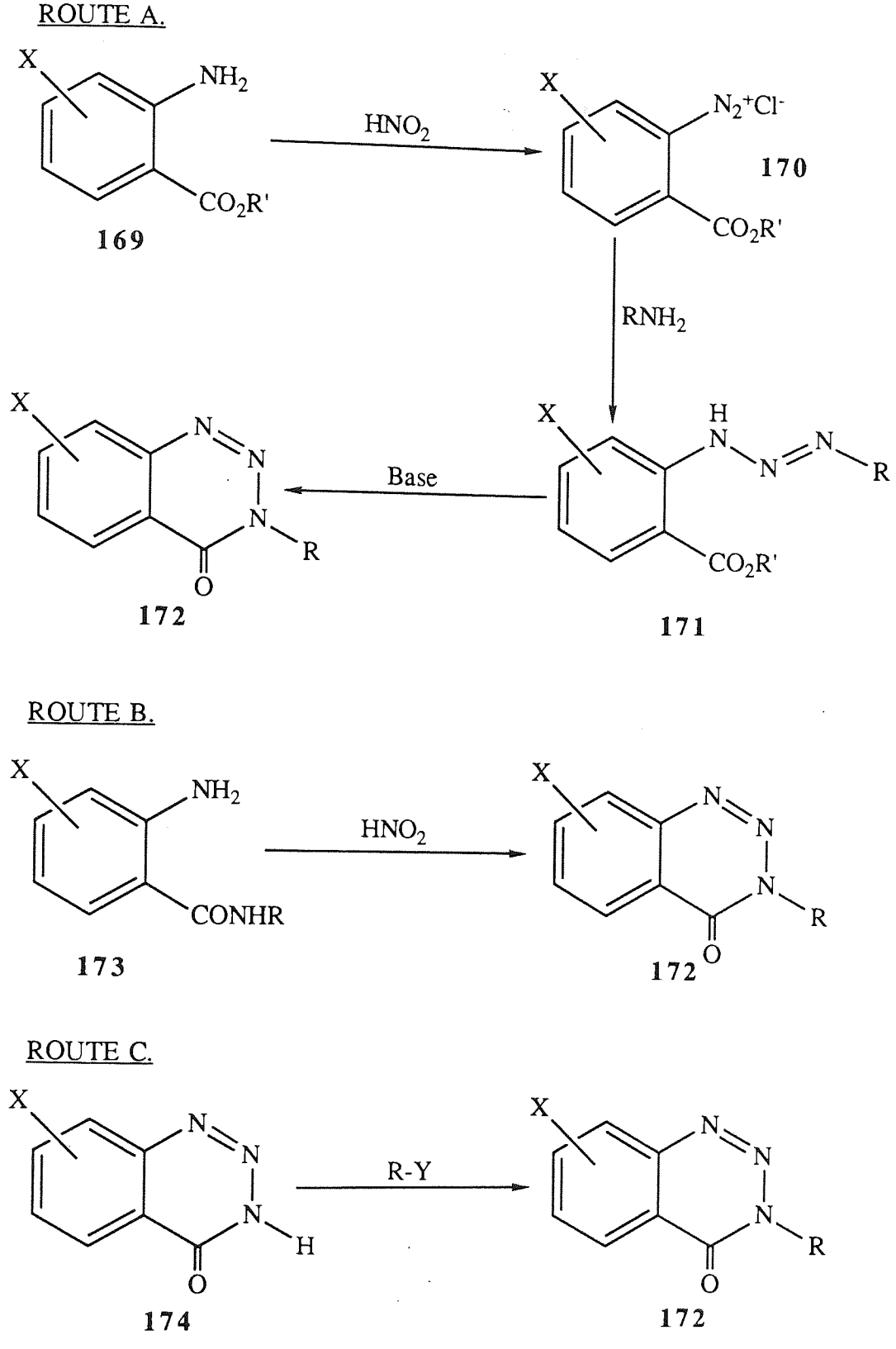


168

3-Substituted-1,2,3-benzotriazin-4(3H)-ones are well known and have been extensively studied by workers at Aston. Furthermore, benzotriazinones contain the triazine linkage, 4-carbonyl moiety and a co-planar fused aromatic residue characteristic of the imidazotetrazinones. Replacement of the the imidazole ring by a benzene ring changes the nitrogen bridgehead in (**167**) to a carbon bridgehead in (**168**), thereby reducing the susceptibility of the 3,4 and 4,4a bonds to nucleophilic attack. It was anticipated that the synthesis of 3-substituted-1,2,3-benzotriazin-4(3H)-ones with a 7- or 8-carboxamide substituent in the benzene ring would give compounds that have a propensity to bind noncovalently to DNA in a similar manner to the tetrazinones.

Surprisingly, a review of the literature revealed that, although a large number of 1,2,3-benzotriazin-4(3H)-ones have been synthesised, there are no known derivatives that contain a carboxylic acid or its derivatives located in the benzene ring. The majority of the substituents seen in the aromatic nucleus were alkyl, halogen, nitro or methoxy groups, although there were reports of sulphonamide ($-SO_2NH_2$) substituted benzotriazinones.[110]

The preparation of 3-substituted-1,2,3-benzotriazin-4(3H)-ones has been extensively reviewed.[111-13] Three general synthetic routes were available (Scheme 3.9):



Scheme 3.9: Routes for the synthesis of 3-substituted-1,2,3-benzotriazin-4(3H)-ones.

(i) cyclisation of *ortho*-triazenobenzoate esters. (Route A).

(ii) diazotisation of substituted anthranilamides. (Route B)

(iii) alkylation of 1,2,3-benzotriazin-4(3H)-ones or by modification of substituents already bound to the benzotriazinone nucleus. (Route C).

3.2.1 Synthesis *via ortho*-triazenobenzoate esters.

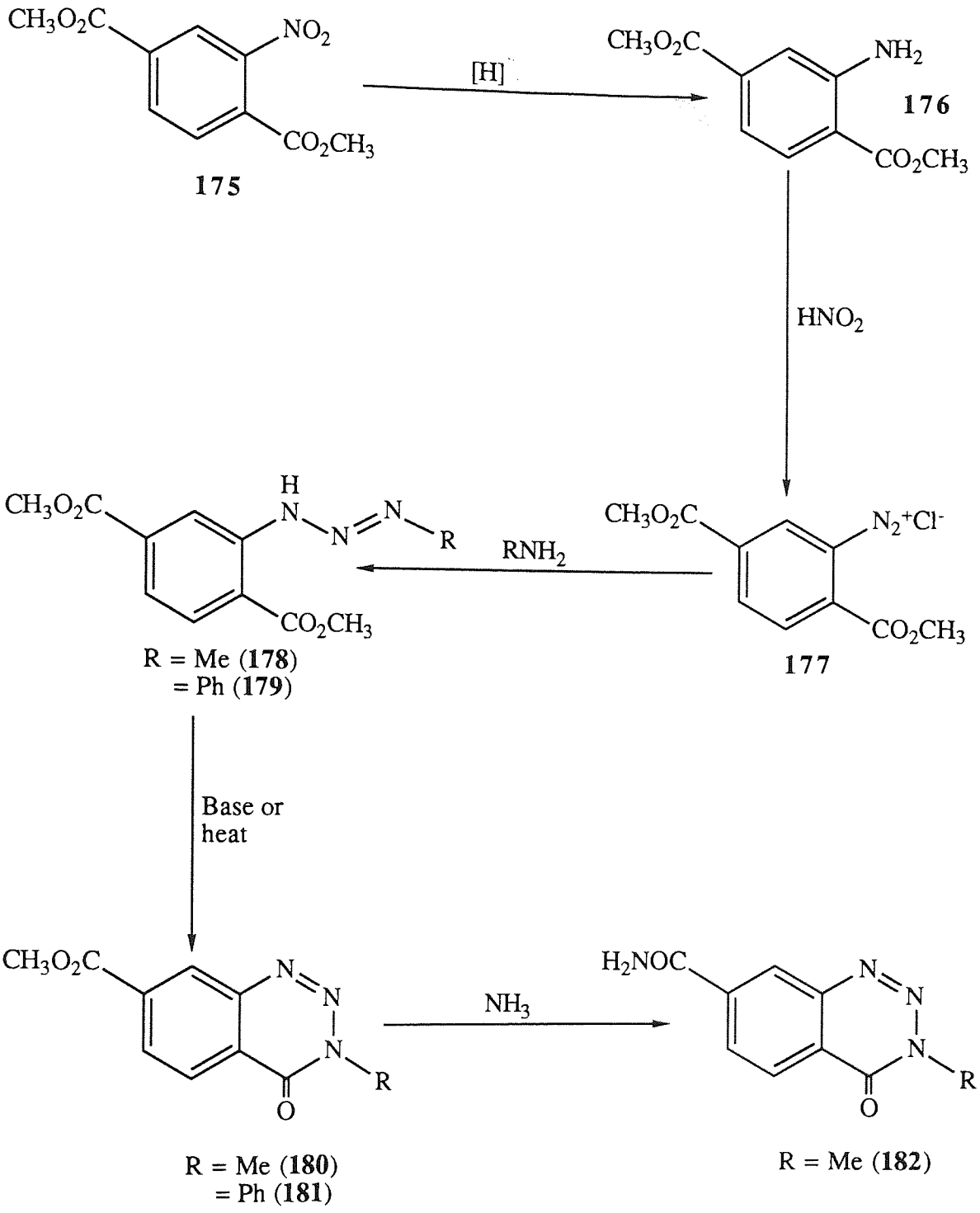
3-Substituted-benzotriazin-4(3H)-ones with a 7-carboxamide group were selected for initial synthesis since the nitro diester (**175**) (Scheme 3.10) was commercially available.

Catalytic reduction of the nitro group over palladium/charcoal proceeded smoothly to give the amine (**176**) in near quantitative yield. Diazotisation, followed by sequential treatment with sodium acetate and aqueous methylamine gave the 3-methyl-1,2,3-benzotriazin-4(3H)-one-7-methyl ester (**180**) as a yellow solid in a yield of 42%. The ester function was readily converted to the carboxamide (**182**) with methanolic ammonia.

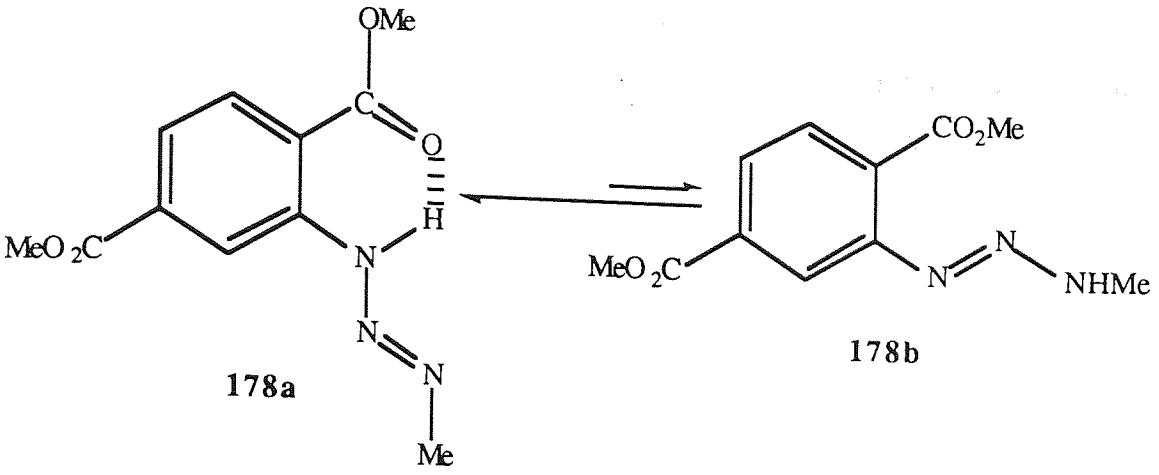
The primary reaction product of the coupling of a diazonium salt with an aliphatic amine has been shown to be the triazene, which subsequently cyclises to the benzotriazinone. Indeed LeBlanc and Vaughan[114] have successfully isolated a range of semi-stable 1-aryl-3-alkyltriazenes in excellent yields. Using the method of LeBlanc and Vaughan[114] it was possible to isolate the triazene intermediate (**178**) as yellow needles in a yield of 70%. Attempted recrystallisation led to extensive degradation, mainly to the amino diester starting material (**176**). Recrystallisation of the triazene (**178**) from 95% ethanol led to cyclisation to the 3-methyl-1,2,3-benzotriazin-4(3H)-one (**180**) in a 75% yield.

Although triazenes have a potential for tautomerism the NMR spectra of (**178**) showed the N-CH_3 protons as a singlet (3.60 ppm) indicative of the existence of the triazene in its unconjugated tautomer (**178a**). This is a common feature of triazenes with an *ortho* substituent capable of hydrogen bonding in which they exhibit a complete preference for the intramolecularly hydrogen bonded form (**178a**).

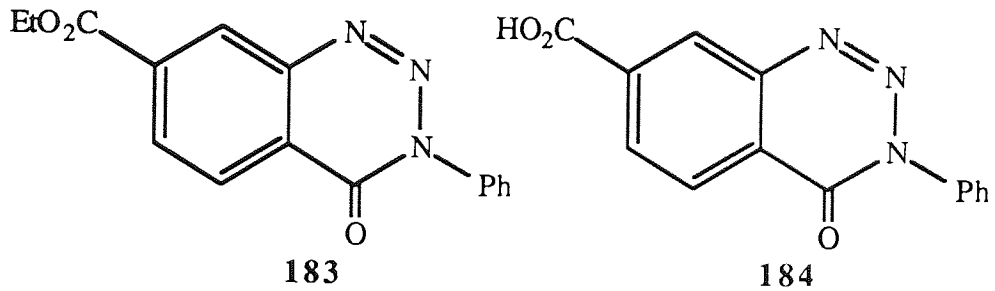
The diazonium salt (**177**) was also coupled with aniline to give the considerably more stable 1,3-diaryltriazene (**179**). Heating the triazene (**179**) in 70% aqueous ethanol led to the expected cyclisation to 3-phenyl-1,2,3-benzotriazin-4(3H)-one-7-



Scheme 3.10: The preparation of 3-substituted-1,2,3-benzotriazin-4(3H)-one-7-carboxamides *via ortho*-triazenobenzoate esters



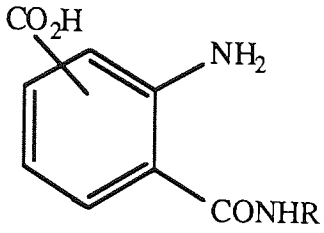
methyl ester (**181**). Cyclisation of (**179**) according to the method of Stevens *et al.*[115], using 2% piperidine in ethanol gave the transesterified benzotriazinone (**183**). The use of methanol in place of ethanol allowed cyclisation with retention of the methyl ester. This interesting observation of cyclisation with concomitant base-catalysed transesterification was extended to include saponification. When the triazene (**179**) was heated under reflux in a mixture of ethanol/water/piperidine (70:28:2) the free acid (**184**) was produced in a 44% yield.



Although the use of route A (Scheme 3.9) was found to work on a small scale, attempts to scale-up (2-5g size) led to a decrease in the yield of the 3-methyl derivative and gave material contaminated with the aminodiester starting material (**176**), which was difficult to remove. Therefore, a better general synthetic route was required.

3.2.2 Synthesis *via ortho*-aminobenzamides.

A large number of substituted 1,2,3-benzotriazin-4(3H)-ones have been prepared in high yields and excellent purity *via* the diazotisation of *ortho*-aminobenzamides (Route B, Scheme 3.9). The use of route B necessitated the preparation of compounds of the general type (**185**), in which there was a free carboxylic acid and a substituted amide within the same molecule.

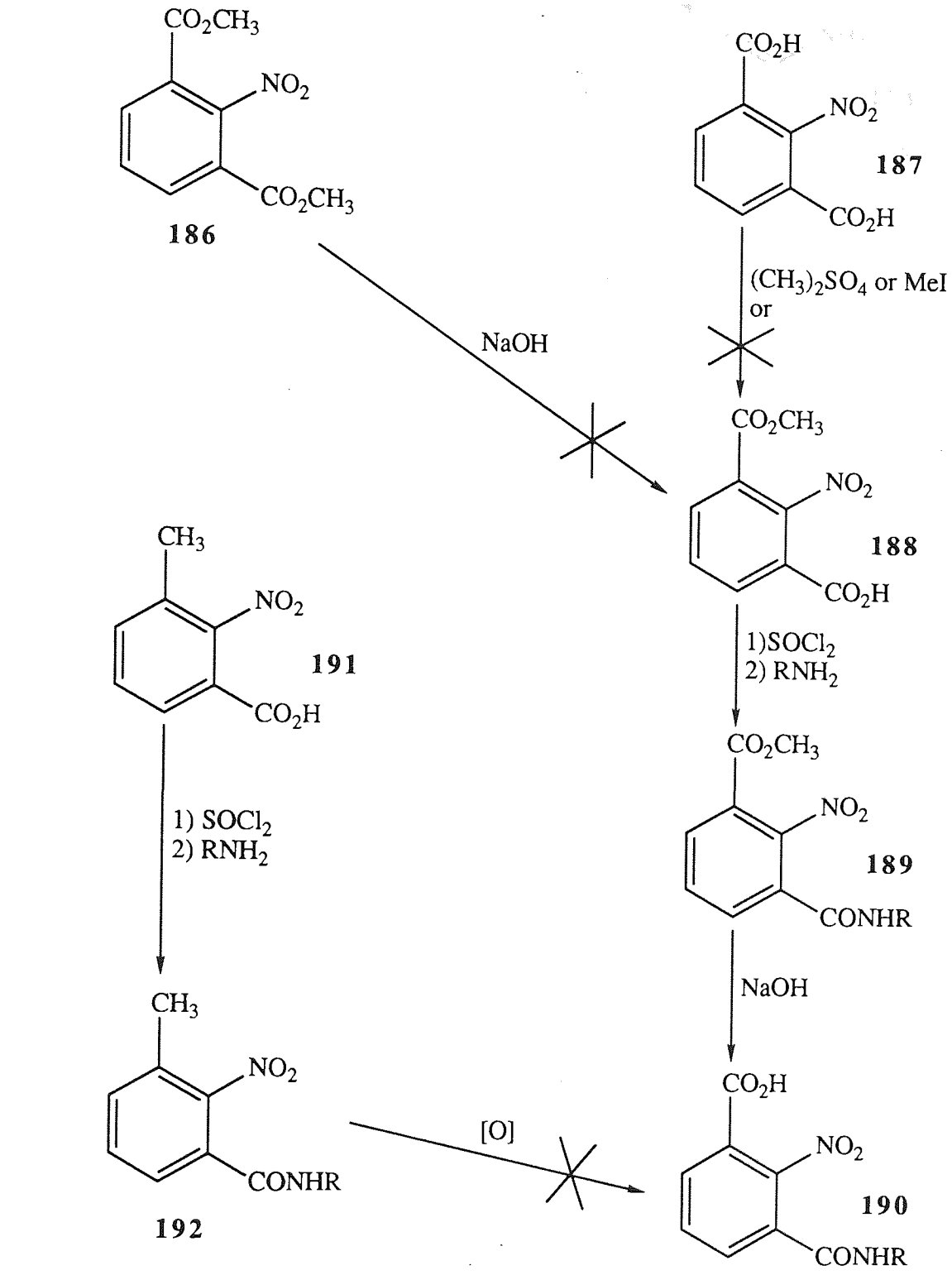


185

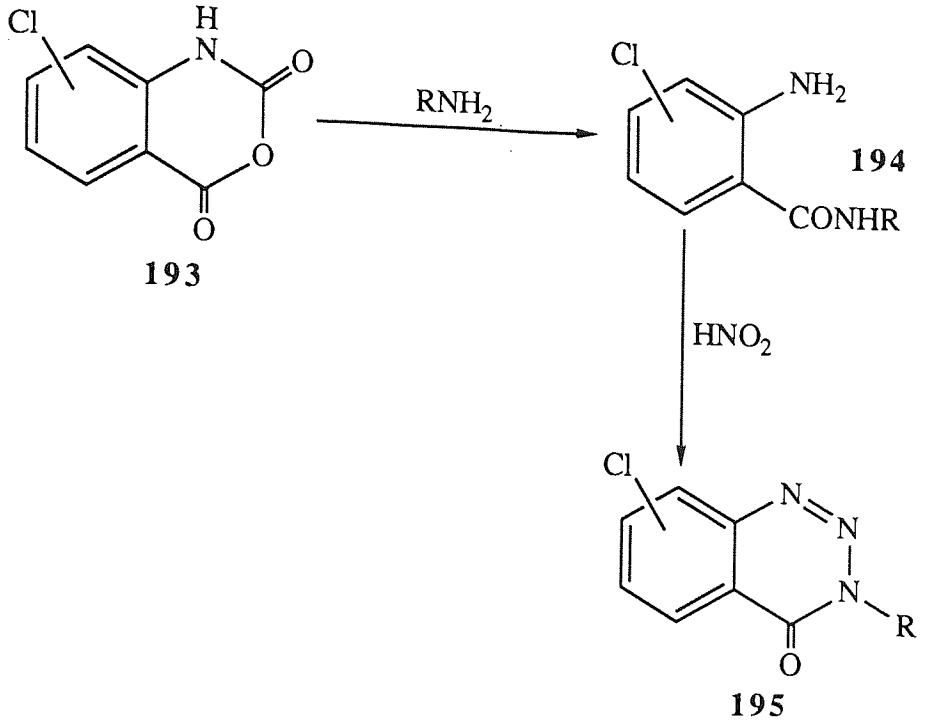
Wohl[116] had synthesised the monoamide of isophthalamic acid (**190** R=H) *via* partial saponification of the dimethyl ester (**186**) in an overall yield of 40% (Scheme 3.11). Attempts to repeat Wohl's method only gave the monoester (**188**) in a low yield, with significant amounts of the diacid and diester starting material as contaminants. This route was not explored any further, and the attempts to obtain the monoester by selective esterification with dimethyl sulphate, methyl iodide and N,N-dimethylformamide dimethyl acetal (Scheme 3.11) also failed. Presumably, the equivalence of the carboxylic acid groups in the symmetrical 2-nitroisophthalic acid (**187**) prevented both selective saponification and esterification to any significant extent.

An alternative route for the preparation of the monoamide (**190** R=Me) was direct oxidation of a methyl side chain in (**192** R=Me) to the carboxylic acid (Scheme 3.11). However, neutral potassium permanganate and chromium trioxide/sulphuric acid both failed to give the mono-amide (**190** R=Me) under the conditions used.

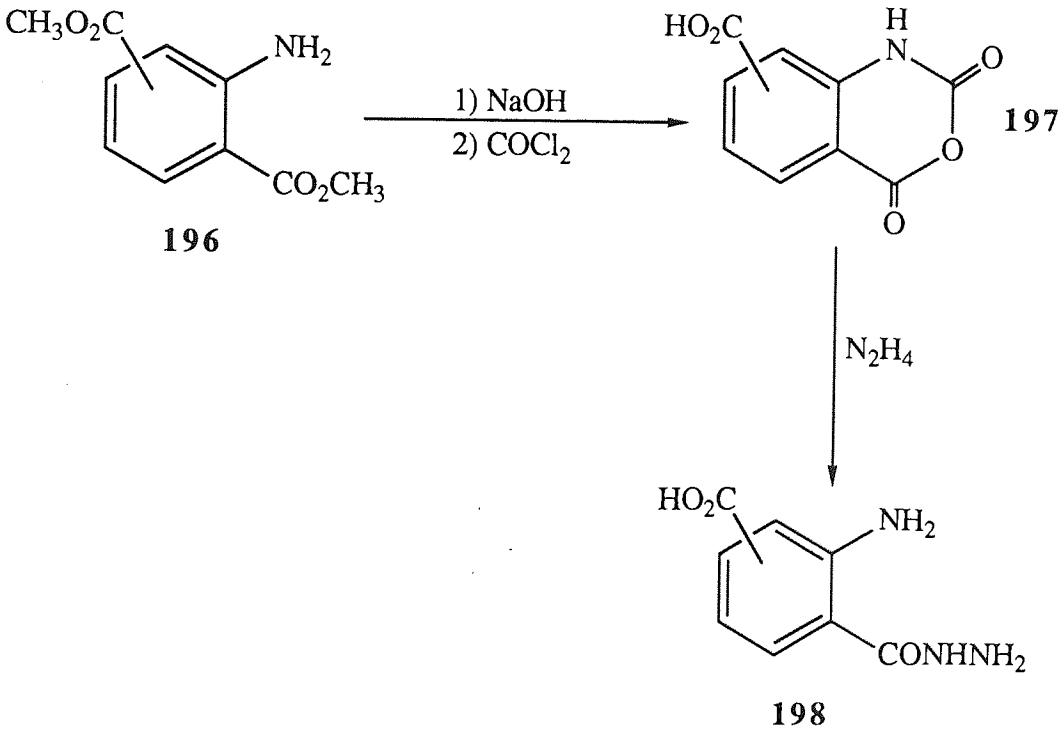
Gadeker and Ross[117] reported the preparation of chloro-substituted 1,2,3-benzotriazin-4(3)-ones (**195**) *via* a substituted isatoic anhydride (Scheme 3.12). The use of the analogous carboxylic acid substituted isatoic anhydrides appeared to offer an attractive route to the synthesis of the general molecules (**185**), since it allowed activation of only one of the carboxylic acid groups to attack by amines. Moreover, Sircar *et al.*[118] had previously



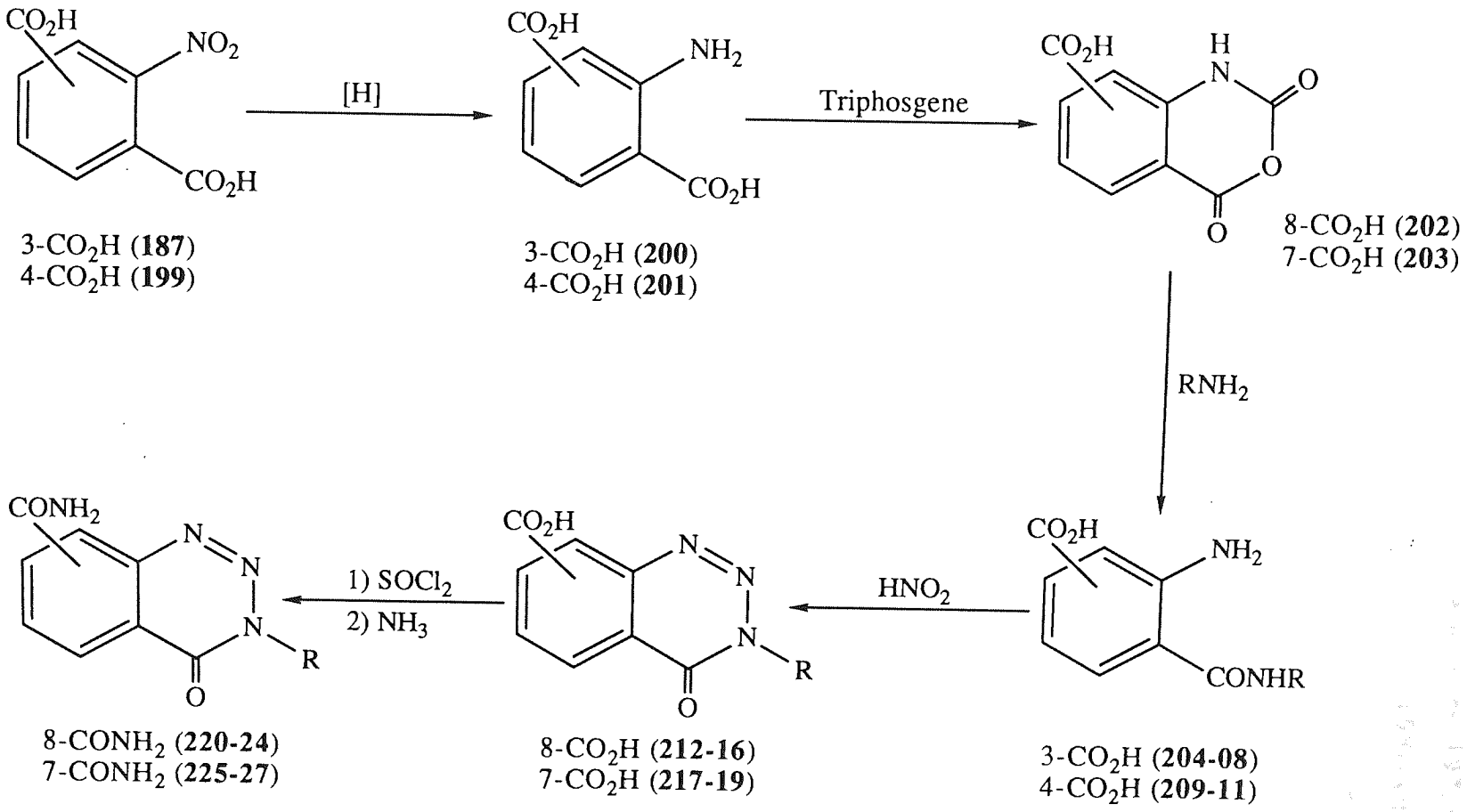
Scheme 3.11 : Synthetic avenues for the preparation of 2-nitroisophthalamic acid derivatives.



Scheme 3.12 : Synthesis of chloro-substituted benzotriazinones *via* isatoic anhydrides.



Scheme 3.13 : Synthesis and reactions of carboxyisatoic anhydrides.



3-CO_2H (**187**)
4-CO_2H (**199**)

3-CO_2H (**200**)
4-CO_2H (**201**)

8-CO_2H (**202**)
7-CO_2H (**203**)

3-CO_2H (**204-08**)
4-CO_2H (**209-11**)

8-CO_2H (**212-16**)
7-CO_2H (**217-19**)

8-$CONH_2$ (**220-24**)
7-$CONH_2$ (**225-27**)

Scheme 3.14 : Synthesis of 3-substituted-1,2,3-benzotriazin-4(3H)-one 7- and 8-carboxamides (see Table 3.4 for R)

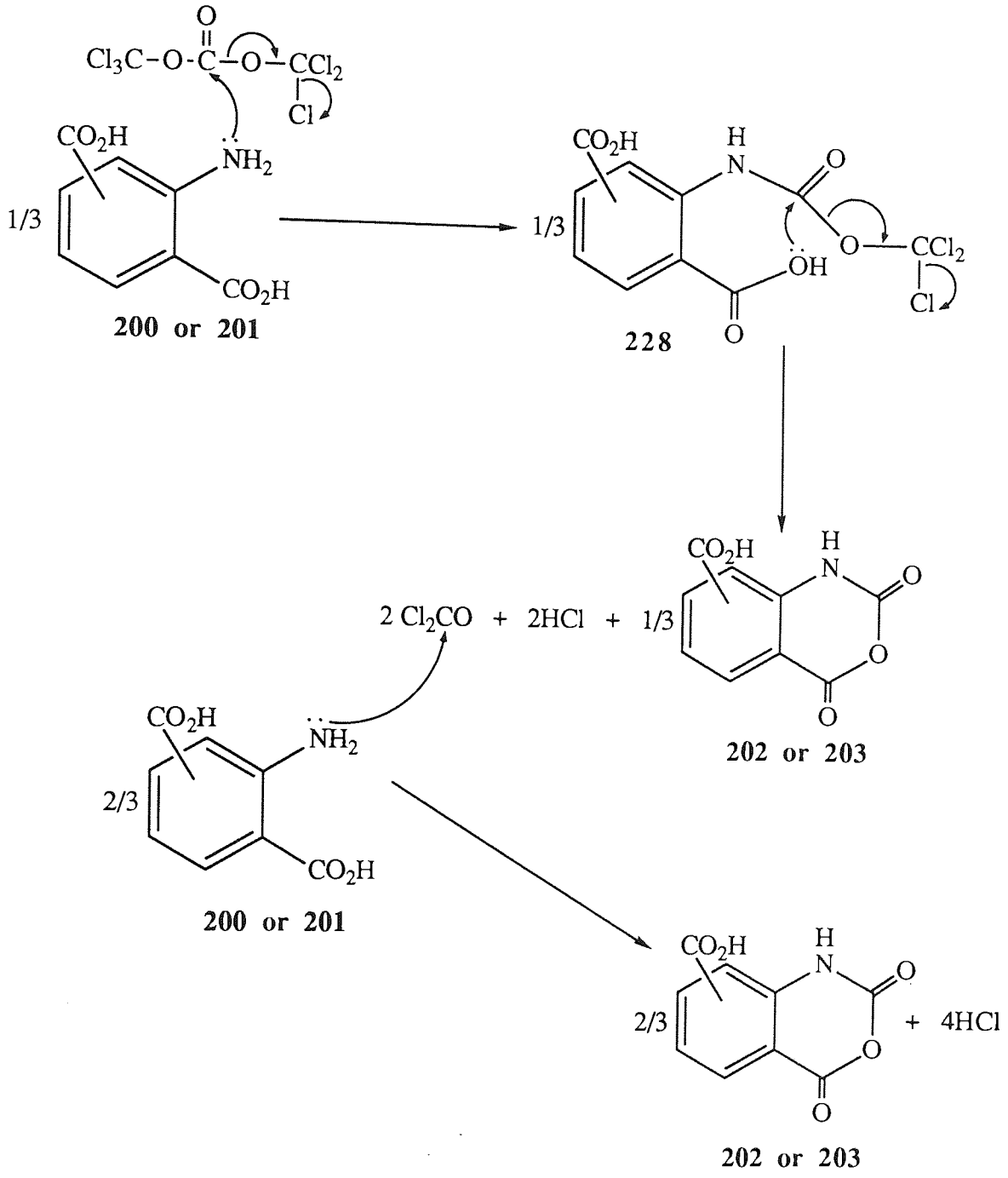
reported the synthesis of carboxy-substituted isatoic anhydrides (**197**) with phosgene and their reaction with hydrazine to give the mono acid-hydrazides (**198**) (Scheme 3.13).

Although phosgene has been widely used in the preparation of isatoic anhydrides there are problems with the metering of the highly toxic gas and so maintaining the proper stoichiometric balance. Eckert and Foster[119] recently outlined the general utility of triphosgene ($Cl_3COCO_2CCl_3$) as a phosgene substitute, while Daly and Poche[120] have successfully prepared N-carboxyanhydrides of amino-acids using triphosgene. Triphosgene is a crystalline solid which can be safely handled and exact amounts can be weighed easily, so avoiding side reactions associated with excess phosgene.

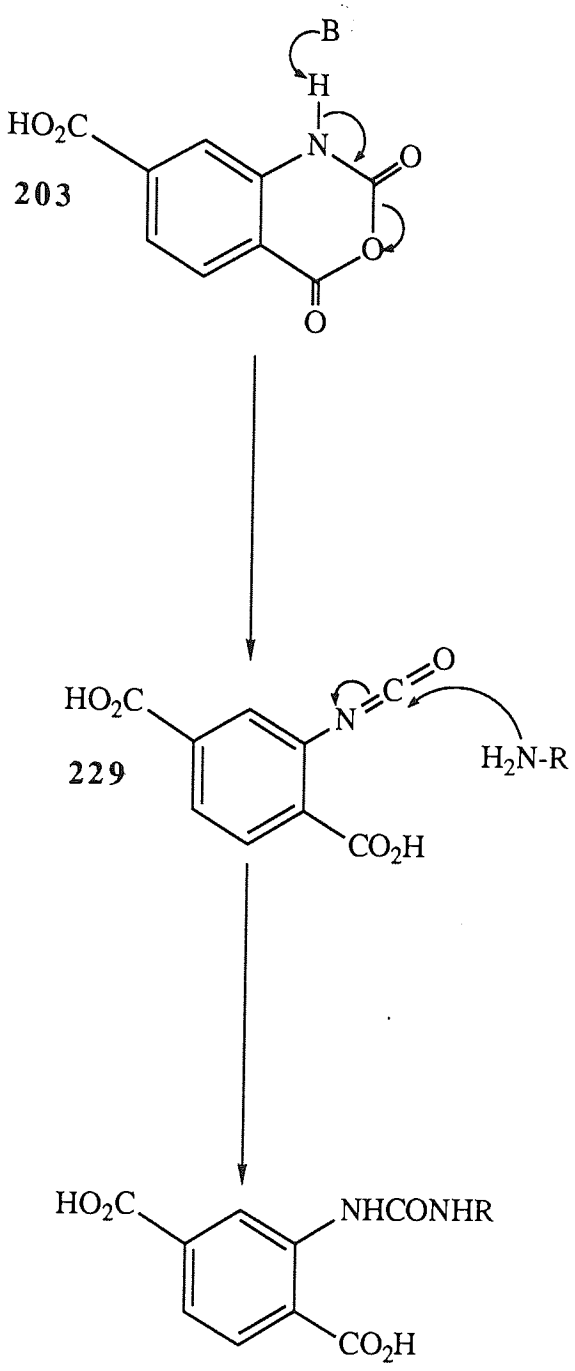
Treatment of the respective aminobenzene dicarboxylic acids with 1/3 equivalent of triphosgene yielded the isatoic anhydrides (**202** and **203**) in greater than 90% yields (Scheme 3.14). These derivatives were sufficiently pure for use in the next stage. No catalyst was required since the aminobenzene dicarboxylic acid acted as the nucleophile in generating the three equivalents of phosgene (Scheme 3.15). The low basicity and hindered nature of 2-aminoisophthalic acid (**200**) prevented its reaction with the HCl by-product. The less sterically hindered 2-aminoterephthalic acid (**201**) reacted slightly with HCl to give the hydrochloride salt, reducing its nucleophilicity and solubility. However, periodic sparging with nitrogen improved the yield by driving the HCl from the reaction medium.

The isatoic anhydrides reacted with primary amines under aqueous conditions (except aniline which was heated with the anhydride in 1,4-dioxane) with the evolution of carbon dioxide to give the *ortho*-aminobenzamides (**204-211**) in 70-80% yields (Table 3.3).

The reaction of 7-carboxyisatoic anhydride (**203**) with methylamine and ethylamine was unusual, in that not only were the expected *ortho*-aminobenzamides produced, but the ureides (**230**) and (**231**) were identified as side products by NMR spectroscopy. Staiger and his co-workers[121-2] studied the reaction of amines with isatoic anhydride and found that the proportions of the 'normal' benzamide to the 'abnormal' ureide varied with the structure of the amines and their molar ratio with the isatoic anhydride. The ureides have been proposed to occur *via* the formation of an intermediate isocyanate (**229**), which is capable of undergoing nucleophilic attack by amines to yield the side products (Scheme 3.16). The reaction of 7-carboxyisatoic anhydride (**203**) with aniline failed to give



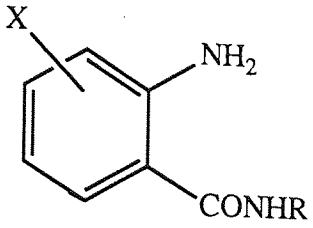
Scheme 3.15 : Mechanism for the reaction of triphosgene with aminobenzene dicarboxylic acids.



R = Me (**230**) and Et (**231**)

Scheme 3.16: Proposed mechanism for the formation of ureides from the reaction of 7-carboxyisatoic anhydrides with methylamine (R = $-CH_3$) and ethylamine (R = $-C_2H_5$).

Table 3.3: Substituted 2-aminobenzamides prepared according to Scheme 3.14

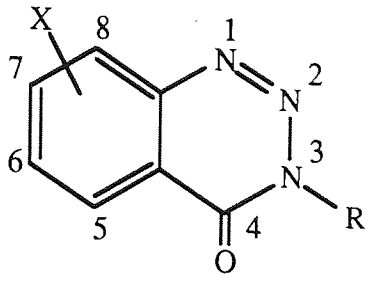


Compound No.	X	R
204	3-CO_2H	Me
205		Et
206		Ph
207		$(CH_2)_2Cl$
208		CH_2CO_2Me
209	4-CO_2H	Me
210		Et
211		Ph

any of the corresponding ureido product, probably because aniline is not a sufficiently strong base to abstract the -N-H proton necessary for the formation of the isocyanate species (**229**). 8-Carboxyisatoic anhydride (**202**) did not give any ureido type side products in its reaction with primary amines, possibly as result of the hindered nature around the -N-H and/or intramolecular hydrogen bonding preventing proton abstraction.

The monoamides (**204-11**) were cyclised in 60-70% yields to the corresponding 7- and 8-carboxy-1,2,3-benzotriazin-4(3H)-ones (**212-19**) using an inverted diazotisation technique. The free carboxylic acid in the monoamides was utilised in reaction with sodium carbonate to generate the water soluble sodium salts. Freshly prepared sodium nitrite solution was added and the mixture immediately poured on to a mixture of ice and 2M-hydrochloric acid. This procedure allowed rapid formation of the diazonium salt without precipitation of the sparingly water soluble monoamides, and as a consequence, without formation of azobenzenes, which is often seen as a competing reaction with weakly basic arylamines.

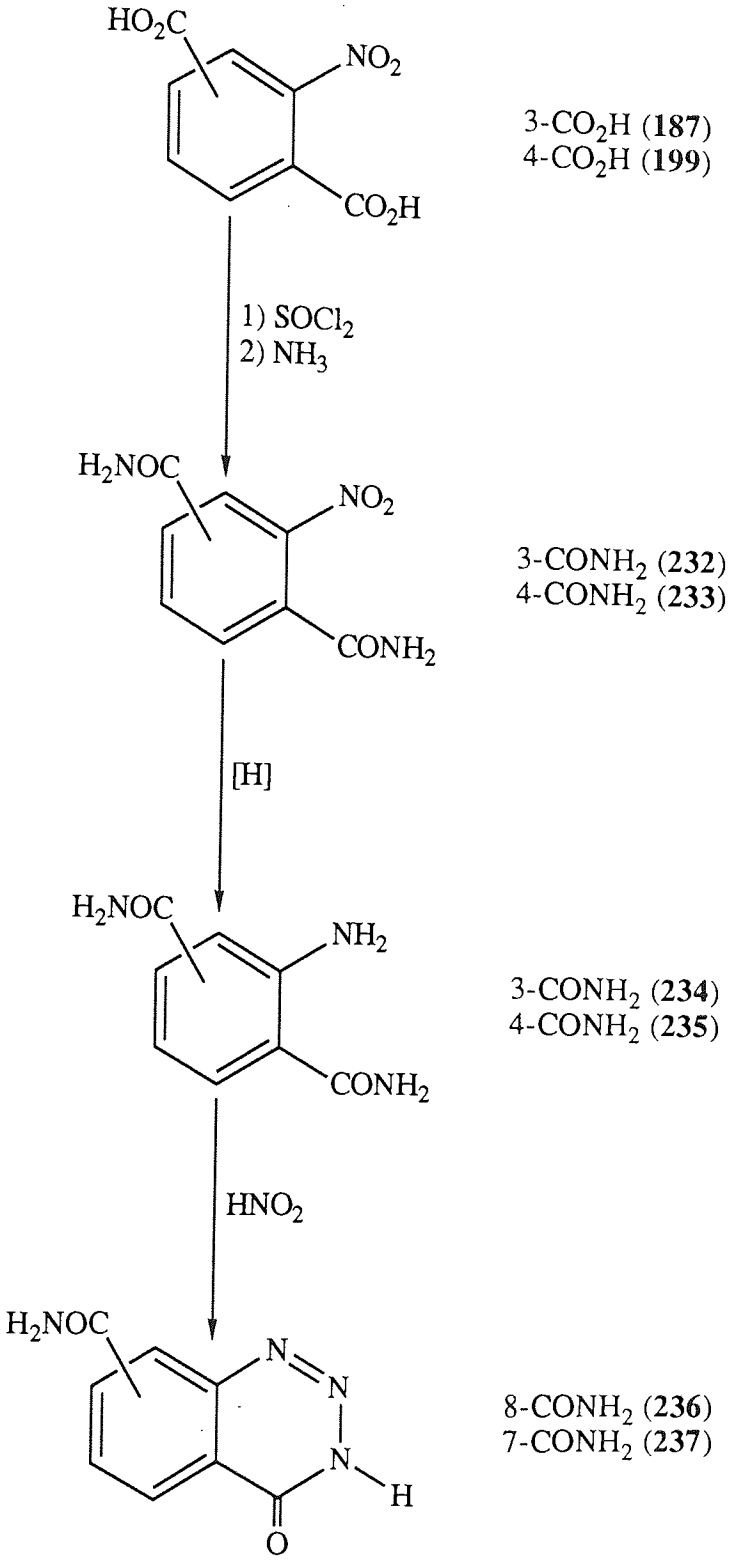
Table 3.4: Substituted 1,2,3-benzotriazin-4(3H)-ones prepared according to Scheme 3.14



Compound No.	X	R
212	8-CO_2H	Me
213		Et
214		Ph
215		$(CH_2)_2Cl$
216		CH_2CO_2Me
217	7-CO_2H	Me
218		Et
219		Ph
220	8-$CONH_2$	Me
221		Et
222		Ph
223		$(CH_2)_2Cl$
224		CH_2CONH_2
225	7-$CONH_2$	Me
226		Et
227		Ph

The carboxy-substituted 1,2,3-benzotriazin-4(3H)-ones (**212-19**) were converted to the corresponding amides (**220-27**) by reaction with thionyl chloride/DMF to give the acid chloride followed by treatment with ammonia (Scheme 3.14). The methyl ester moiety of the glycinyl derivative (**216**) was also aminated during this reaction sequence to give the dicarboxamide derivative (**224**).

The 1,2,3-benzotriazin-4(3H)-one 7- and 8-carboxamides (**236** and **237**) unsubstituted at the 3-position were prepared by diazotisation of the aminobenzene

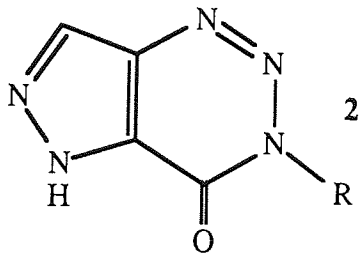


Scheme 3.17: Synthesis of 1,2,3-benzotriazin-4(3H)-one 7- and 8-carboxamides.

dicarboxamides (**234** and **235**). The dicarboxamides were conveniently prepared from the nitrobenzenedicarboxylic acids (**187** and **199**) as shown in Scheme 3.17.

3.3 Synthesis of pyrazolo[4,3-d]-1,2,3-triazin-4(3H)-ones.

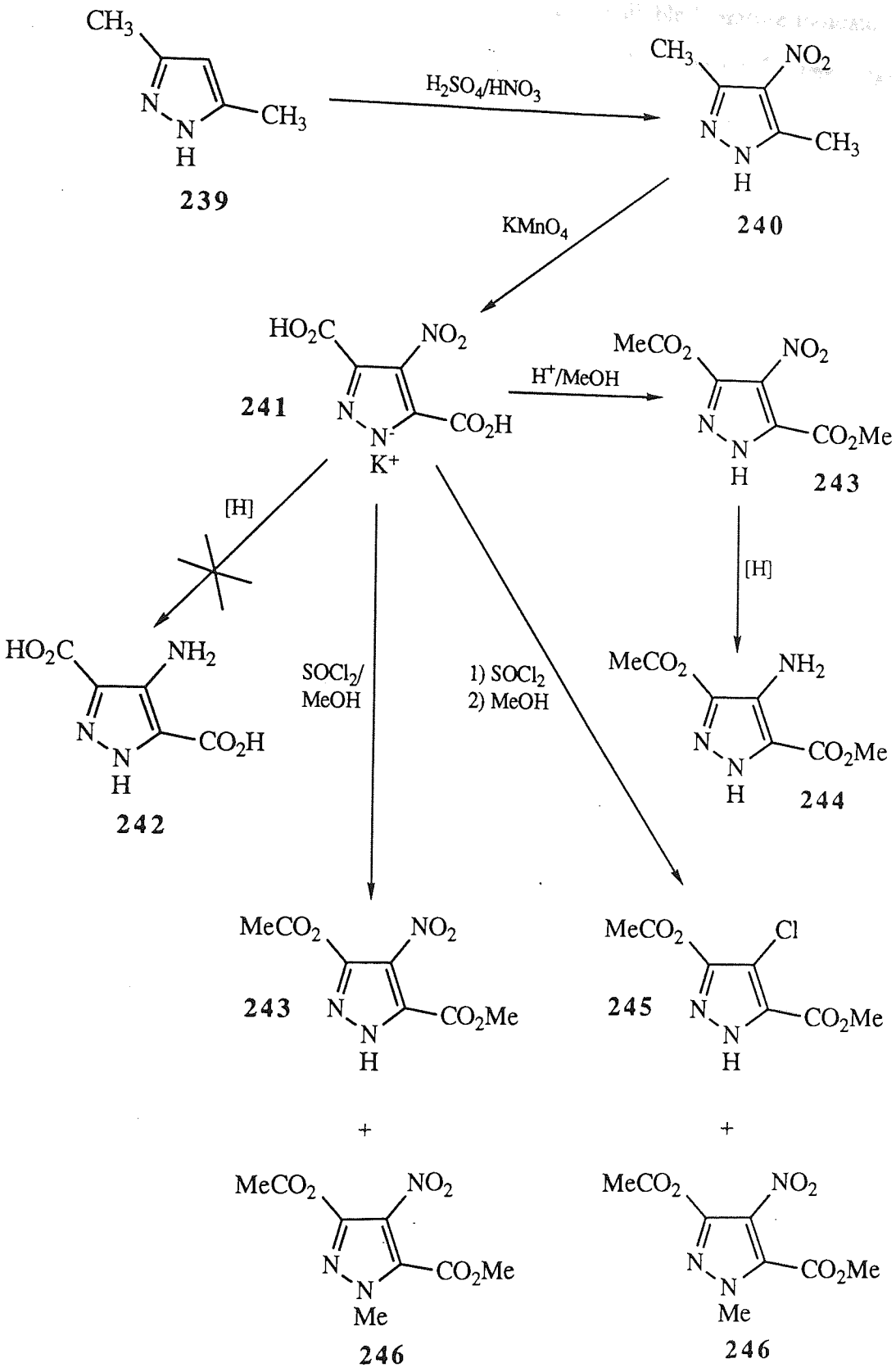
Although the 1,2,3-benzotriazinones contain the important structural elements of the tetrazinone ring system they are far from ideal models (see biological results). A significant feature of the imidazotetrazinones is the fused imidazole ring. Indeed, as previously highlighted molecular modelling studies indicated that the most stable tautomer of mitozolomide involved an intramolecular hydrogen bond to the N7 position in the imidazole ring. Such a potential to adopt a similar 'tricyclic' conformation is noticeably absent in the benzotriazinones. Therefore, there was considered a need to prepare triazinones fused to a N-containing heterocycle with the potential to participate in intramolecular hydrogen bonding. The synthesis of the appropriately substituted pyrazolotriazinone ring system offered the opportunity to prepare an isomer of the imidazotetrazinones with all the required structural features while remaining incapable of ring opening.



238

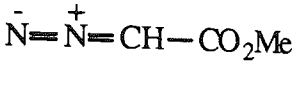
The literature contains only one report of the synthesis of the pyrazolo[4,3-d]triazin-4(3H)-one ring system (**238**) prepared *via* the diazotisation of a 4-aminopyrazole-3-carboxamide.[123] It was envisaged that suitably substituted 4-aminopyrazole-3-carboxamides could be prepared using the analogous N-carboxyanhydride route that had been successful in the synthesis of the benzotriazinones, and diazotisation according to the literature method would generate the pyrazolotriazinone.

3,5-Dimethylpyrazole (**239**), which is commercially available, was nitrated following the method of Morgan and Ackerman[124] to give the nitro compound (**240**). Permanganate oxidation of the aromatic methyl groups proceeded smoothly to give 4-nitropyrazole-3,5-dicarboxylic acid as the monopotassium salt (**241**).[125] However, attempts to prepare the amino acid (**242**) by catalytic hydrogenation and reduction with stannous chloride dihydrate were not successful, although there were indications of some reaction



Scheme 3.18: The preparation and reactions of 4-nitropyrazole-3,5-dicarboxylic acid monopotassium salt (**241**).

taking place (Scheme 3.18). Surprisingly a review of the available literature indicated the acid (**242**) has not been reported, yet the diester (**244**) is well known.[126-8] Interestingly, a similar situation is seen in the case of 5-aminoimidazole-4-carboxylic acid, which is unstable under acidic and neutral conditions readily undergoing decarboxylation to the 5-aminoimidazole, while a range of esters have been characterised.[86]



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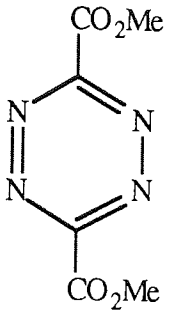


248

Upon the failure of the N-carboxyanhydride route attentions were turned to the use of the amino diester as a precursor to the formation of the triazinone [*via* the triazene, a pathway previously discussed for the preparation of benzotriazinones, Scheme 3.9 , route A]. The preparation of the amino diester (**244**) had previously been reported using two distinct routes. The first involved the cycloaddition of methyldiazoacetate (**247**) with a cyanoacetate which suffered from the need for carefully controlled conditions and complex working up procedures resulting in a poor yield.[126] The second route involved a cycloaddition of the 1,2,4,5-tetrazine derivative (**248**) with an isocyanide.[127] However, the initial synthesis of the tetrazine was complex and fraught with difficulties.[128]Therefore it was considered that conversion of the nitro acid potassium salt (**241**) that been prepared in good yields during attempts to synthesise the free amino acid (**242**) offered a convenient route to amino diester (**244**) *via* esterification and reductive processes (Scheme 3.18).

Initial attempts to esterify the acid salt (**241**) centred around the use of the chlorinating agent thionyl chloride; the author had earlier esterified 2-nitrobenzene-1,3-dicarboxylic acid (**187**) in excellent yields by refluxing the acid in a mixture of methanol and thionyl chloride. However, when the acid salt (**241**) was esterified using this method a mixture of the desired nitro diester (**243**) and the N-methyl nitro diester (**246**) was obtained. The N-methyl derivative (**246**) may arise from the alkylation of the pyrazole N-H

by dimethyl sulphite $(CH_3O)_2SO$ formed by the nucleophilic substitution of the chlorines in thionyl chloride with methanol, which is present in the reaction mixture in excess.

The conditions were modified by refluxing the acid salt (**241**) in thionyl chloride prior to the addition of methanol. The reaction yielded two compounds, separated by flash chromatography, the first was identical to the N-methyl nitro diester (**246**) prepared above. The second compound, and the major product of the reaction (75%), was identified by mass spectrometry as the chloro diester (**245**) (Scheme 3.18), an interesting observation that closely resembles that seen with the nitroimidazole carboxylic acid (**93**) earlier (section 3.1.2, page 68).

The replacement of aromatic nitro groups by halogens is not unknown, both phosphorus pentachloride and thionyl chloride, as well as other halogenating agents, have been reported to replace nitro with chloro in bromo- or chloronitrobenzenes at high temperatures (e.g.200°C).[129] However, such side reactions have not been shown to occur with nitrobenzoic acids under the relatively mild conditions used in acid chloride formation. Therefore it would appear that both 4-nitropyrazole-3,5-dicarboxylic acid (**241**) and 5-nitroimidazole-4-carboxylic acid (**93**) possess common features that are responsible for the activation of the usually stable nitro group to nucleophilic substitution. Four such factors may be considered crucial to inducing nitro group mobility of which (a) and (b) are the major contributors:[130]

(a) electronic activation to substitution - the presence of electron withdrawing substituents in the ring can induce or activate mobility in certain groups. A general order for the ability of groups to activate nucleophilic displacement of substituents is :

$$N_2^+ > CR_2^+ > 4° \text{ Hetero N} > N{=}O > NO_2 > \text{Hetero N} > CH_3SO_2 >$$

$$(CH_3)_3N^+ > CF_3 > RCO > CN > COOH > SO_3^- > \text{Halogen} > COO^- > \text{Ph.}$$

From this order it can been seen that the sp^2 (pyridine) type nitrogen atoms in both imidazole and pyrazole rings have a strong activation ability, especially on groups in the 3 position relative to the nitrogen atom. Furthermore, alkylation or acylation of the hetero nitrogen greatly augments its activating power, an effect that is also produced by protonation under acidic conditions such as those produced during the formation of acid chlorides with phosphorus pentachloride or thionyl chloride. It is also important to note the activation

potential of carboxylic acid groups, although this is less marked than that of the hetero nitrogens an additive effect probably operates making the nitro group even more mobile in the presence of the two activating functions.

(b) steric hindrance with activating groups - bulky *ortho*-substituents can have profound effects on the electronic effects of adjacent groups. In order for groups such as the nitro group to exert their electronic effects they must participate in resonance structures which are double-bonded to the aromatic rings. Such structures require that the group remains co-planar with the aromatic ring, a feature that can be diminished by the presence of bulky *ortho* groups (such as carboxylic esters in **241**) with a subsequent decrease in the electronic effects of the nitro group. Indeed, steric interference of this kind is responsible for the ease of displacement of the 1-nitro group from 2,6-disubstituted nitrobenzenes.

(c) steric hindrance of reagent approach - theoretical considerations advanced by Cowdrey *et al.*[131] indicated that for nucleophilic substitution to occur the reagent must approach from a direction lateral to the plane of the aromatic ring. Therefore it was anticipated that the presence of bulky *ortho*-subtituents, such as the carboxylic acid and esters in the above cases, has little steric effect on the reaction, a fact that has subsequently been corroborated by experimental evidence. This is in contrast to other nucleophilic reactions involving aromatic systems such as benzoic ester saponification which has great sensitivity towards the size of the *ortho*-substituent.

(d) influence of the displaced group - there exists a general relationship between the replaceability of a substituent and its capacity to exist as a stable anion, a feature shown by the nitrite ion which is capable of resonance stabilisation in solution. The general order of mobility of aromatic substituents has been reported as $F > NO_2 > Cl,Br,I > N_3$. Indeed a nitro group is regularly displaced in preference to a similarly activated chlorine especially where the electronic effects of the nitro group are reduced due to the presence of *ortho*-substituents.

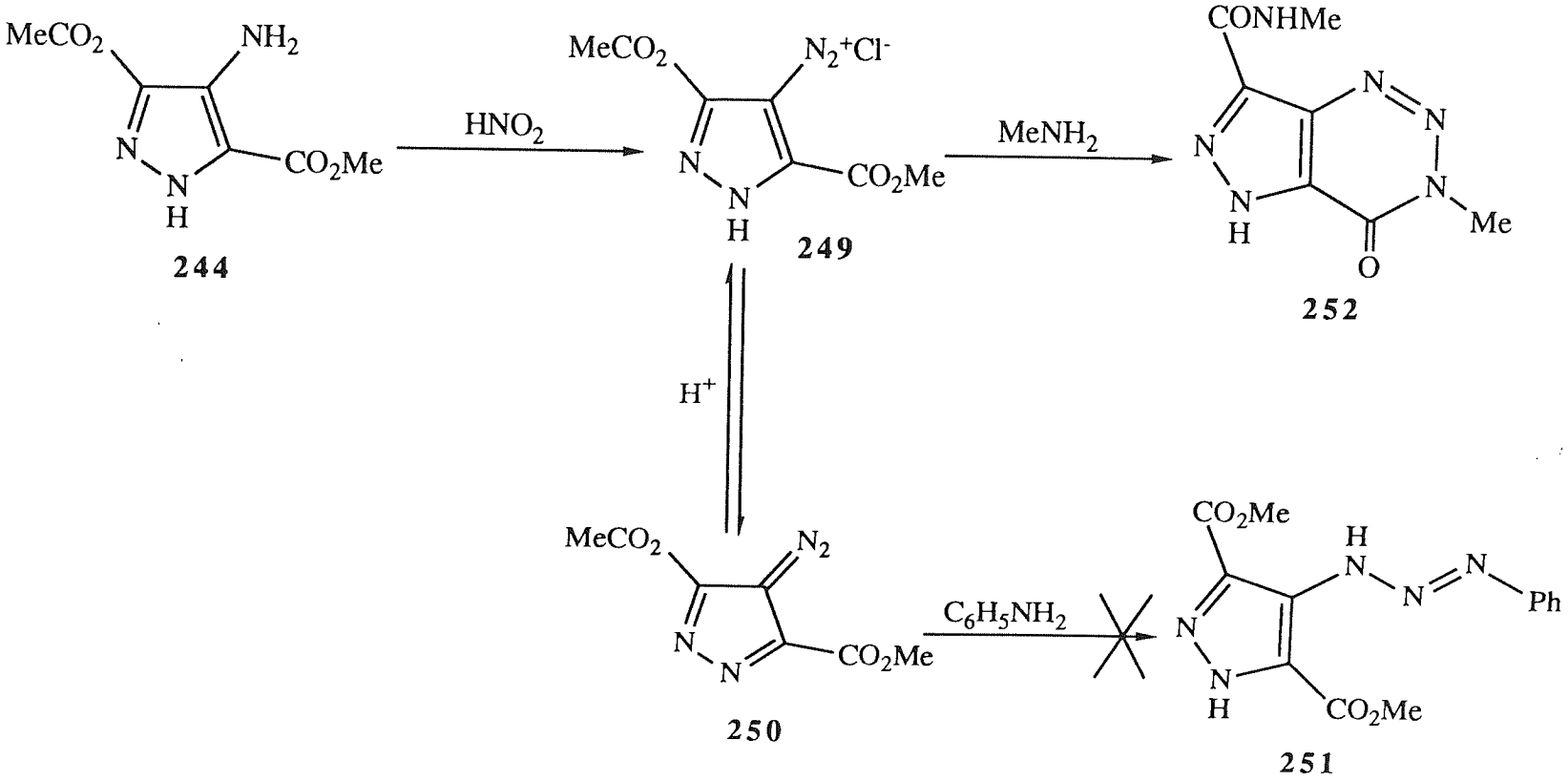
Therefore, considering the factors outlined above it is no surprise that the nitro groups in (**241**) and (**93**) are displaced. It would appear that for the imidazole derivative (**93**) that factor (a) is the most influential, since the presence of only one *ortho*-substituent does not facilitate steric crowding. While, for the pyrazole analogue (**241**) it is likely that both factor (a) and factor (b) play crucial roles in inducing the mobility of the nitro

group.

A sample of the nitro diester (**243**) was finally prepared according to the method of Makabe *et al.*[132] by refluxing the acid salt (**241**) in methanol saturated with HCl gas and purification of the residue by flash chromatography. The nitro diester (**243**) was readily reduced by catalytic hydrogenation to the amino diester (**244**) identical to that reported in the literature (Scheme 3.18).

A solution of the amino diester (**244**) was diazotised under aqueous conditions with nitrous acid. Treatment of the diazotised solution with aniline did not lead to the precipitation of the expected phenyltriazene (**251**) (Scheme 3.19). In fact extraction of the alkaline reaction mixture with chloroform followed by dissolution of the residue in ethanol and trituration with hexane led to the precipitation of a colourless crystalline solid, which was stable to light and mild heating. Infrared analysis of this solid showed the presence of a diazo peak, indicating that the isolated compound was the diazo diester (**250**). A common feature of pyrazole diazonium salts (**249**) is their ability to undergo deprotonation to the diazo form (**250**) under basic conditions, a feature which has probably occurred here when the diazotised solution was treated with aniline. The treatment of the diazo diester (**250**) with aniline in ethanol for 5 days also failed to give any of the phenyltriazene (**251**), probably as a result of steric hindrance produced by the surrounding ester groups preventing the weakly basic and bulky aniline molecule from reaction with the diazo centre. This is in accordance with the observation that the equally sterically hindered 3,5-dimethyl-4-pyrazolediazonium chloride fails to yield triazenes.[124]

A solution of the amino diester (**244**) was diazotised and the acidic aqueous solution was extracted with ethyl acetate to ensure that the diazotised compound was present as the diazonium salt (**249**). The resulting solid was treated with a saturated solution of methylamine in ethyl acetate at room temperature. The reaction mixture slowly deposited a white solid which upon analysis by NMR and IR indicated the presence of two compounds, neither of which was starting material (Scheme 3.19). Attempts to separate the compounds by fractional recrystallisation failed. However, it proved possible to isolate one of the compounds by flash chromatography and recrystallisation from methanol gave colourless needles which were identified as the pyrazolotriazinone (**252**). Evidently the stronger basicity and reduced bulkiness of methylamine allowed coupling with the diazonium group



Scheme 3.19: The synthesis of pyrazolo[4,3-d]-1,2,3-triazin-4(3H)-ones.

to take place with the formation of the triazene, which ultimately underwent cyclisation to the triazinone. Moreover, the excess methylamine in the reaction mixture was responsible for amination of the methyl ester function to give the N-methylcarbamoyl substituent.

3.4 Physical properties of 1,2,3-triazinones.

The triazinones prepared above were either colourless or cream crystalline solids which decomposed upon heating. They were soluble in DMF, DMSO and hot ethanol; partially soluble in cold ethanol, acetone, ethyl acetate and insoluble in water. The spectroscopic properties of triazinones, especially 1,2,3-benzotriazin-4(3H)-ones, have been extensively reported[111-13] and the compounds prepared in this study do not deviate essentially from those reviewed except for minor variations resulting from the nature of the nuclear substituents. However, several characteristic features are worth noting:

(a) I.R. absorption: the I.R. spectra showed the usual carbonyl stretching frequencies between 1640-1700cm^{-1}, although the spectra are unexpectedly complicated probably as a result of hydrogen bonding involving the carboxylic acid and carboxamide substituents therefore, in many cases, preventing a clear assignment of the triazinone carbonyl stretching frequency.

(b) NMR spectra: the NMR spectra were consistent with those expected for the individual groups in the molecule, however, the carbamoyl derivatives gave two signals for the $-NH_2$ protons clearly showing the restricted rotation about the amide bond.

(c) mass spectra: the main characteristic of the electron impact mass spectra of the triazinones was the production of an abundant molecular ion, usually the base peak and in marked contrast to the imidazotetrazinones. Fragmentation of the molecular ion was essentially the same as for those reported.[111-13]

CHAPTER 4 : HYDROLYSIS OF MITOZOLOMIDE.

4.1 Aims and objectives.

The essence of the proposed hypothesis upon the ring opening of imidazotetrazinones *in vivo* (section 1.9) is that G-C rich regions in DNA act as a catalyst in promoting the rate of ring cleavage, not directly, but *via* the intermediacy of a water molecule. Therefore, selectivity is induced by the binding affinities of the tetrazinone ring to such sequences and also as a result of kinetic control in which ring cleavage occurs faster in the presence of G-C sequences than under any other conditions. It was envisaged that such a catalytic effect would be easily discernible using experiments in which the rate of disappearance of mitozolomide in the presence of synthetic nucleic acids was monitored.

4.2 Hydrolysis in phosphate buffer at pH 7.4.

Initial experiments were conducted in which the hydrolytic degradation of mitozolomide was examined in different solutions at pH 7.4 and 37°C which contained the following:

(a) phosphate buffer (0.1M, pH 7.4) which acted as a positive control in the measurement of the rate of pH dependent hydrolysis (k_{pH}).

(b) a solution of guanosine-5´-monophosphate/cytidine-5´-monophosphate (1:1) which acted as a positive control to ensure that any observed catalytic effect resulted from a function of the structure of DNA and not its individual components.

(c) a solution of d(A)-d(T) homopolymer which was used as a source of synthetic double stranded A-T DNA and acted as a positive control to ensure that the catalytic effect was confined only to G-C sequences and not DNA as a whole.

(d) a solution of d(G)-d(C) homopolymer which was used as a source of synthetic double stranded G-C DNA.

The reaction mixtures had a total volume of 3ml and were constituted as shown in Table 4.1. Mitozolomide was incubated with two different concentrations of the oligonucleotide solutions, an equi-molar concentration with mitozolomide and also a tenfold excess of the oligonucleotide which ensured that the natural situation occurring in the nucleus, where the molar concentration of nucleotide bases would greatly exceed that of

Table 4.1: Composition of incubation mixtures used to examine the hydrolysis of mitozolomide at pH 7.4 in phosphate buffer at 37°C.

Component solutions.	Volume of solutions added (μL).					
	1	2	3	4	5	6
Mitozolomide stock solution in DMSO (200mg/ml).	50	50	50	50	50	50
5´GMP stock solution in phosphate buffer (0.1M, pH 7.4) (380mg/2.5ml).	-	125	-	-	-	-
5´CMP stock solution in phosphate buffer (0.1M, pH 7.4) (302mg/ml).	-	50	-	-	-	-
d(A)-d(T) stock solution in phosphate buffer (0.1M, pH 7.4) (10units/5ml).	-	-	100	-	500	-
d(G)-d(C) stock solution in phosphate buffer (0.1M, pH 7.4) (10units/5ml).	-		-	100	-	500
Phosphate buffer (0.1M, pH 7.4).	2950	2775	2850	2850	2450	2450

mitozolomide, was imitated. The disappearance of the tetrazinone ring from the reaction mixtures was monitored using a slightly modified HPLC method that had been originally devised by Slack *et al.*[41] and utilised by Horspool.[37] Aliquots were taken from the reaction mixture and injected directly on to the column. The eluent was analysed by UV absorbance at 325nm which prevented interference from the other constituents of the reaction mixture.

The hydrolysis of mitozolomide in all solutions was found to follow first order kinetics and the rate constants (k) were obtained from plots of log[mitozolomide peak area] versus time that were linear to 3 half lives (Figure 4.1). Values for k and also the half lives ($t_{1/2}$) are shown in Table 4.2.

A comparison of the rate constants given in Table 4.2 clearly show that there was no significant difference in the rate of hydrolysis of mitozolomide in phosphate buffer compared with degradation in the presence of the added nucleotides or oligonucleotide

Table 4.2: A comparison of the first order rate constants (k) and half lives ($t_{1/2}$) for the hydrolysis of mitozolomide in phosphate buffer at pH 7.4 and 37°C in the presence of nucleotides and synthetic nucleic acids.

Incubation solutions.	Rate constant k x 10^{-3} ($mins^{-1}$.)[d]. 1	2	3	Mean	Mean half life $t_{1/2}$ (mins.)[e].
Phosphate buffer	9.74	9.51	9.82	9.69 +/- 0.18	71.51
5´GMP/5´CMP (1:1)[a].	9.79	9.86	9.72	9.79 +/- 0.07	70.80
d(A)-d(T) (1:1)[b].	8.95	9.34	9.15	9.15 +/- 0.20	75.75
d(A)-d(T) (10:1)[c].	9.23	9.30	9.38	9.32 +/- 0.09	74.37
d(G)-d(C) (1:1)[b].	9.39	9.30	9.68	9.40 +/- 0.28	73.27
d(G)-d(C) (10:1)[c].	8.68	9.29	9.35	9.11 +/- 0.43	76.08

a. represents equi-molar ratios of 5´GMP to 5´CMP.

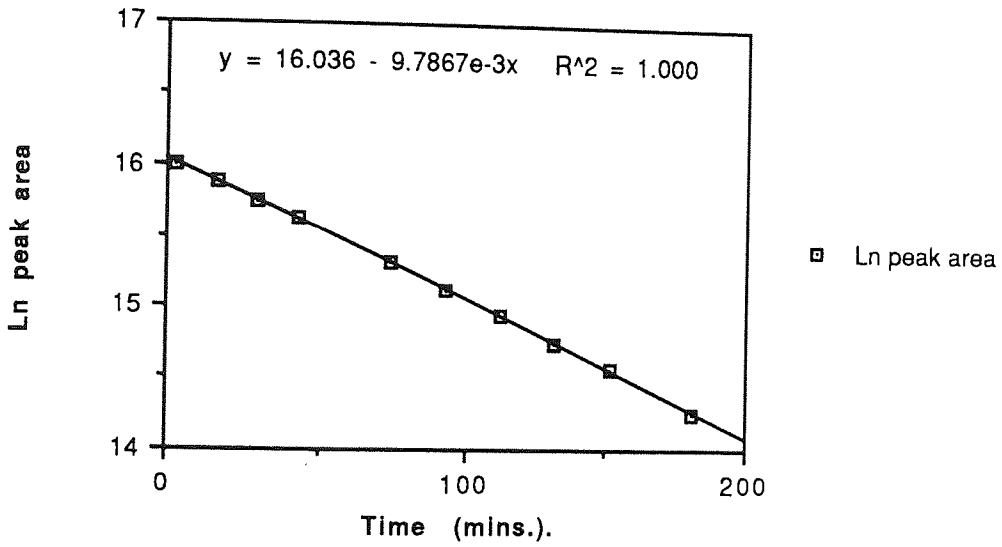
b. represents equi-molar ratios of mitozolomide to nucleotide bases in the oligonucleotide.

c. represents a tenfold molar excess of nucleotide bases in the oligonucleotides compared to mitozolomide.

d. calculated from plots of log[mitozolomide peak area] versus time with a minimum of 10 data points.

e. calculated from the mean rate constant using the equation $t_{1/2} = 0.693/k$.

Figure 4.1 An example of a plot of log[mitozolomide peak area] versus time for the hydrolysis of mitozolomide in the presence of 5´GMP/5´CMP (1:1) mixture in phosphate buffer (0.1M, pH 7.4) at 37°C.



fragments (there was less than +/- 5% deviation from the mean of the rate constants). Furthermore, there was no obvious difference in the rates of hydrolysis for mitozolomide between the A-T oligonucleotide and the G-C oligonucleotide. Although the results from these initial studies were disappointing it could be argued that the lack of any observable catalytic effect was due to:

(a) the ring opening of mitozolomide was not catalysed by G-C sequences in DNA which would be in contradiction to the proposed hypothesis indicating that there was no kinetic control dictating the site of DNA alkylation.

(b) at pH 7.4 the pH dependent hydrolysis saturates or dominates any effect associated with the presence of the oligonucleotide i.e. $k_{pH} >> k_{G\text{-}C}$.

(c) the absence of any observed difference between the hydrolysis of mitozolomide in the presence of the A-T oligonucleotide compared with the G-C oligonucleotide was surprising considering that G rich regions are the preferred site of alkylation for this series of compounds. This could possibly suggest that the reaction mixture contained a stronger nucleophile than the nucleic acids which was initiating ring cleavage.

4.3 Hydrolysis in phosphate buffer pH 6.5.

In order to examine the possibility of the pH dependent hydrolysis dominating any effect due to the oligonucleotides it was considered, that by lowering the pH of the reaction medium it should be possible to increase the stability of the tetrazinone ring towards pH dependent hydrolysis thereby reducing k_{pH} such that $k_{G\text{-}C}$ became more evident. As a consequence the experiments were repeated in phosphate buffer at pH 6.5 and 37°C. Although pH 6.5 does not represent a typical physiological model it does, however, represent a comprise between the generally more acidic confines of the micro-enviroment of DNA likely to be encountered by mitozolomide and a pH at which the tetrazinone ring possesses significantly greater stability while allowing retention of the structural integrity of the oligonucleotides (DNA begins to decompose rapidly below pH 5.5).

The incubation mixtures were set up as shown in Table 4.3. It was not possible to prepare a sufficiently concentrated stock solution of 5´GMP at pH 6.5, therefore the 5´GMP/5´CMP control was omitted from these experiments, while only the incubation of mitozolomide with the tenfold molar excess of the oligonucleotides was repeated. The

Table 4.3: Composition of incubation mixtures used to examine the hydrolysis of mitozolomide at pH 6.5 in phosphate buffer.

Component solutions.	Volume of solutions added (μL). 1	2	3
Mitozolomide stock solution in DMSO (200mg/ml).	50	50	50
d(A)-d(T) stock solution in phosphate buffer (0.1M, pH 6.5) (10units/5ml).	-	500	-
d(G)-d(C) stock solution in phosphate buffer (0.1M, pH 6.5) (10units/5ml).	-	-	500
Phosphate buffer (0.1M, pH 6.5).	2950	2450	2450

results from these experiments are shown in Table 4.4.

A comparison of the results shown in table 4.4 clearly indicate that as at pH 7.4 there was no significant difference between the rate of degradation of mitozolomide in the presence of buffer alone or when oligonucleotide solutions were present (less than a +/- 1% deviation from the mean) suggesting that k_{pH} does not mask any catalysis by G-C oligonucleotides (i.e. k_{G-C}).

4.4 Hydrolysis in sodium cacodylate buffer at pH 7.4 and 6.5.

The suggestion that the reaction mixtures used so far contained a more potent nucleophile than the nucleic acids was examined. The only other constituent in these solutions was the buffer salts and it seemed possible that the highly charged and polar phosphate anion could be acting as the nucleophile since it was present in a much greater molar concentration than the nucleic acids. A simple experiment to examine the effect of the phosphate buffer upon the hydrolysis of mitozolomide was to change the type of buffer used. Sodium cacodylate/HCl has been widely used as a buffer in the characterisation of the reaction of alkylating agents with DNA and was chosen as a replacement for phosphate

Table 4.4: A comparison of the first order rate constants (k) and half lives ($t_{1/2}$) for the hydrolysis of mitozolomide in phosphate buffer at pH 6.5 and 37°C in the presence of nucleic acids.

Incubation solutions.	Rate constant k x 10^{-3} ($mins^{-1}$.)[b]. 1	2	3	Mean	Mean half life $t_{1/2}$ (mins.)[c].
Phosphate buffer	1.52	1.53	1.51	1.52 +/- 0.01	456.02
d(A)-d(T) (10:1)[a].	1.54	1.54	1.53	1.54 +/- 0.01	450.10
d(G)-d(C) (10:1)[a].	1.54	1.55	1.55	1.55 +/- 0.01	447.20

a. represents a tenfold molar excess of nucleotide bases in the oligonucleotides compared to mitozolomide.

b. calculated from plots of log[mitozolomide peak area] versus time with a minimum of 10 data points.

c. calculated from the mean rate constant using the equation $t_{1/2} = 0.693/k$.

Table 4.5: A comparison of the first order rate constants (k) and half lives ($t_{1/2}$) for the hydrolysis of mitozolomide in sodium cacodylate buffer at pH 7.4 and 6.5 at 37°C in the presence of nucleic acids.

Incubation mixture.	Rate constant k x 10^{-3} ($mins^{-1}$)[b]. 1	2	Mean	Mean half life t1/2 (mins.)[c].
Sodium cacodylate buffer pH 7.4.	12.5	12.2	12.4 +/- 0.2	55.89
d(A)-d(T) (10:1)[a] in buffer pH 7.4.	11.0	13.4	12.2 +/- 1.2	56.80
d(G)-d(C) (10:1)[a] in buffer pH 7.4.	11.7	12.7	12.2 +/- 0.5	56.80
Sodium cacodylate buffer pH 6.5.	2.01	2.05	2.03 +/- .02	341.45
d(A)-d(T) (10:1)[a] in buffer pH 6.5.	2.02	1.97	2.00 +/- .03	346.57
d(G)-d(C) (10:1)[a] in buffer pH 6.5.	2.01	2.00	2.01 +/- .01	344.85

a. represents a tenfold molar excess of nucleotide bases compared with mitozolomide.
b.calculated from plots of log[mitozolomide peak area] versus time with a minimum of 10 data points.
c. calculated from the mean rate constant using the equation $t_{1/2}=0.693/k$.

buffer in duplicate experiments conducted at pH 7.4 and 6.5.

The reaction mixtures were set up as before (Table 4.3) except that sodium cacodylate buffer (0.1M, pH 7.4 or 6.5) was used in place of the phosphate buffer. The results for this series of experiments are shown in Table 4.5. A comparison of the results for the degradation of mitozolomide in sodium cacodylate buffer at pH 7.4 and 6.5 again show that there was no difference (less than +/- 1% deviation from the mean) between the

degradation in buffered solution alone or in the presence of oligonucleotides confirming the observations seen with phosphate buffer that the rate of pH dependent hydrolysis does not mask any significant catalytic effect exerted by a solution of the G-C oligonucleotide. However, there was a significant increase in the rate constants for the degradation of mitozolomide in cacodylate buffer (+21% and +25% at pH 7.4 and 6.5 respectively) compared with those in phosphate buffer, clearly indicating that the buffer salts are capable of acting as potent nucleophiles towards the tetrazinone ring. Indeed, it would appear that the large molar excess of buffer salts employed in these reactions could be masking any effect due to the presence of oligonucleotides in the reaction solutions.

CHAPTER 5 : BIOLOGICAL ACTIVITY.

5.1 Aims and objectives.

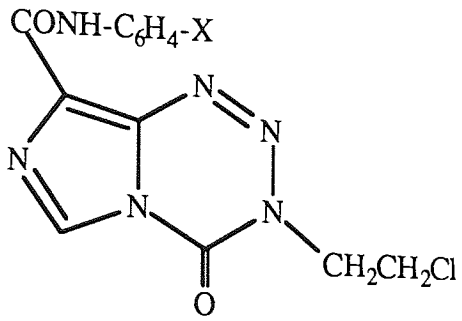
The cytotoxicity of the imidazotetrazinones prepared during the course of this study was investigated using the murine TLX5 lymphoma cell line. The TLX5 cell line has been extensively investigated at Aston and is known to be sensitive to the effects of imidazotetrazinones. Moreover, Horspool[37] tested a wide range of tetrazinones using an *in vitro* assay against the TLX5 cell line, thereby allowing comparisons to be made within this class of compounds.

5.2 Cytotoxicity of 1,2,3,5-tetrazinones.

5.2.1 Cytotoxicity of the anilide derivatives of mitozolomide.

The series of anilide derivatives (**103-108**) were synthesised in the belief that the lack of antitumour activity of the 8-(N-phenyl)carbamoyl derivative of mitozolomide (**103**) could be improved upon by increasing the hydrogen bonding potential of novel derivatives *via* the inclusion of exocyclic carboxamide groups. The results obtained from the *in vitro* cytotoxicity assay for compounds (**103-108**) are shown in Table 5.1. From these results it is clear that the incorporation of a single carboxamide group into the 8-(N-phenyl) ring leads to a sharp decrease in the cytotoxicity when compared with the unsubstituted control (**103**). However, there was a marked difference in the cytotoxicity of the 3-$CONH_2$ (**105**) and 4-$CONH_2$ (**106**) derivatives when compared with the 2-$CONH_2$ derivative (**104**). This is an interesting observation that closely parallels the order for the degree of solvation of these molecules when they were purified by precipitation from 1-methylpyrrolidin-2-one with ether, the 2-$CONH_2$ derivative (**104**) remaining solvated to a greater extent than the other derivatives, perhaps indicating the greater hydrogen bonding capacity of (**104**) brought about by the presence of carboxamide groups in an *ortho* relationship to each other. The cytotoxicity of the dicarboxamide derivative (**107**) which also contains a carboxamide group in the 2 position of the phenyl ring was disappointing and can be considered essentially non-cytotoxic in comparison with the monocarboxamide and unsubstituted derivatives. However, this probably reflects a problem in delivery to the target

Table 5.1: *In vitro* cytotoxicity of the substituted anilide derivatives of mitozolomide (**103-108**) against the TLX5 lymphoma cell line.



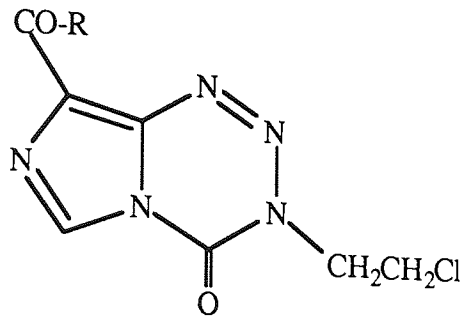
Compound No.	X	IC_{50} (μM) 1	2	Mean
103	H	1.5	1.0	1.25 +/0.25
104	2-$CONH_2$	11.0	15.0	13.0 +/- 2.0
105	3-$CONH_2$	40.0	30.0	35.0 +/- 5.0
106	4-$CONH_2$	39.0	35.0	37.0 +/- 2.0
107	2,4-$(CONH_2)_2$	375.0	-	375.0
108	Pyrimidone	25.0	25.0	25.0 +/- 0.0

cell as a result of the marked insolubility of the compound in the cell culture medium from which it readily precipitates out of solution, a problem that is also present, but to a lesser degree, with the monocarboxamide derivatives.

5.2.2 Cytotoxicity of amino acid and peptide derivatives of mitozolomide.

The results obtained for the *in vitro* cytotoxicity of a number of amino acid and peptide derivatives of mitozolomide are given in Table 5.2. In general most derivatives were less active than mitozolomide with the exception of the alanyl methyl ester derivative (**125**) which possessed a marginal improvement in activity and the trytophan methyl ester derivative (**127**) which was equi-potent. Attempts to improve the antitumour activity of the glycinyl ester derivative (**96**) by conversion to the to the amide derivative (**123**) with a

Table 5.2: *In vitro* cytotoxicity of amino acid and peptide derivatives of mitozolomide against the TLX5 lymphoma cell line.



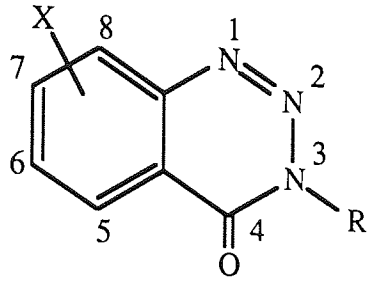
Compound No.	R	IC_{50} (μM) 1	2	Mean
27	NH_2	2.5	-	2.5
96	Gly-(OEt)	30.0	20.0	25.0 +/- 5.0
123	Gly-(NH_2)	35.0	32.5	33.7 +/- 1.3
124	Ser-(OMe)	5.0	5.0	5.0 +/- 0.0
125	Ala-(OMe)	2.0	1.0	1.5 +/- 0.5
126	Met-(OMe)	75.0	25.0	50.0 +/- 25.0
127	Trp-(OMe)	2.5	5.0	3.7 +/- 1.3
139	Ala-Val-(OMe)	7.0	6.8	6.9 +/- 0.1
142	Gln(Mbh)-Ala-(OMe)	150.0	106.0	128.0 +/- 22.0

greater potential for forming hydrogen bonds failed, both compounds exhibiting approximately the same degree of cytotoxicity.

5.2.3 Cytotoxicity of the pyrimido[4´,5´:3,4]pyrazolo[5,1-d]-1,2,3,5-tetrazinone (154).

The synthesis of the novel pyrimido[4´,5´:3,4]pyrazolo[5,1-d]-1,2,3,5-tetrazine ring system was an attempt to examine the extent to which intramolecular hydrogen bonding to the N7 position of the imidazotetrazinones, by constraining the carboxamide

Table 5.3: *In vitro* cytotoxicity of substituted 1,2,3-benzotriazin-4(3H)-ones against the TLX5 lymphoma cell line.



Compound No.	X	R	$IC_{50}(\mu M)$
236	8-$CONH_2$	H	400
220		Me	750
221		Et	325
222		Ph	650
223		$(CH_2)_2Cl$	225
224		CH_2CONH_2	1000
226	7-$CONH_2$	Et	108
227		Ph	140

group into a cyclic system, contributed to their antitumour activity. The pyrimidopyrazolotetrazinone (**154**) was tested *in vitro* against the murine TLX5 lymphoma and found to possess a mean IC_{50} value of 89μM clearly indicating a lack of antitumour activity. This result is not that surprising when it is considered that the tetrazinone (**154**) does not possess a free NH group in the pyrimidine ring and in essence is more akin to the N,N-disubstituted carbamoyl imidazotetrazinones which have previously been shown to be inactive. Unfortunately it was not possible to synthesise the desired compound (**145**) unsubstituted at the pyrimidine N1 position

5.3 Cytotoxicity of the triazinones.

The 1,2,3-triazinones were synthesised in the hope that they would retain the structural features of the tetrazinones but with increased stability thereby being unable to act

Table 5.4: A comparison of the *in vitro* cytotoxicities of 3-methyl-1,2,3-benzotriazinones and the imidazotetrazinone temozolomide against the Raji and GM892A cell lines.

Compound No.	Cell line IC_{50}(μM) (+/- SEM). Raji	GM892A
28 (temozolomide)	160 +/- 3.88	7.1+/- 2.1
220	980 +/- 59	785 +/- 32
225	541 +/- 52	-[a]

a. due to the insolubility of this compound insufficient concentrations for IC_{50} determination were attained however, at 500μM 35% inhibition of growth was seen.

as alkylating agents. Indeed, *in vitro* cytotoxicity assays against the murine TLX5 lymphoma cell line for a number of the 8- and 7-carbamoyl benzotriazinones indicated a lack of any antitumour potential, the results of which are shown in Table 5.3. *In vitro* cytotoxicity assays were also performed for the 3-methyl-substituted benzotriazinones (**220**) and (**225**) and the 3-methyl-substituted pyrazolotriazinone (**252**) against the GM892A (Mer+) and Raji (Mer-) cell lines (Table 5.4). A comparison of the IC_{50} values for the benzotriazinones (**220**) and (**225**) against the 3-methylimidazotetrazinone temozolomide (**28**) clearly show the lack of cytotoxicity exhibited by this class of compounds. The poor solubility of the pyrazolotriazinone (**252**) in the culture medium meant that insufficient concentrations for IC_{50} determination were attained, however, at a concentration of 500μM 37.5% and 31.1% growth inhibition were seen against the Raji and GM892A cell lines respectively.

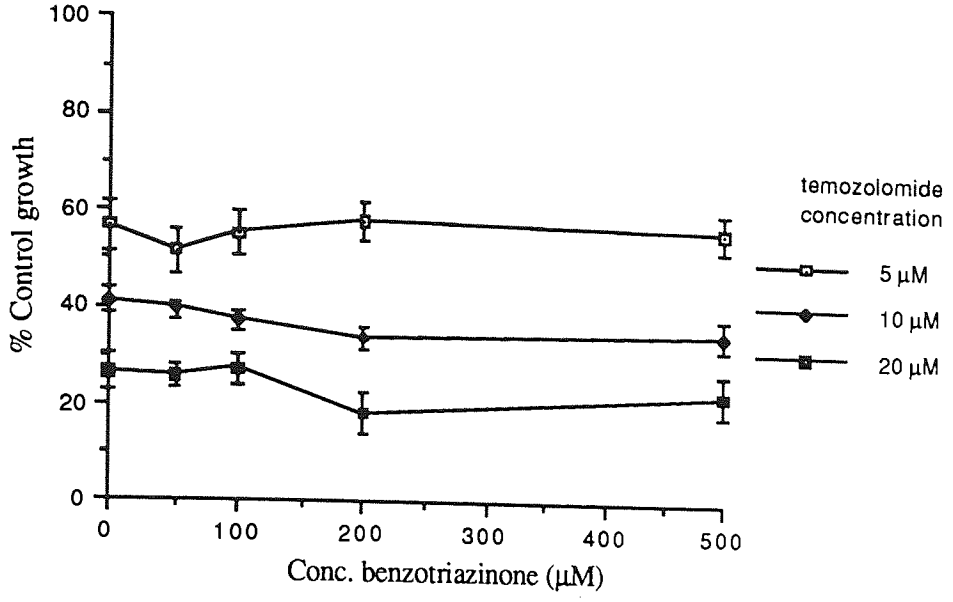
5.4 Competition experiments between 3-methyl substituted triazinones and temozolomide.

It was anticipated that in the triazinone series molecules suitably substituted with a carboxamide residue might be able to bind noncovalently to DNA in a similar manner to the tetrazinones and would therefore, in part, allow the study of the interactions between imidazotetrazinones and DNA. Competition assays were performed, using the 3-methyl

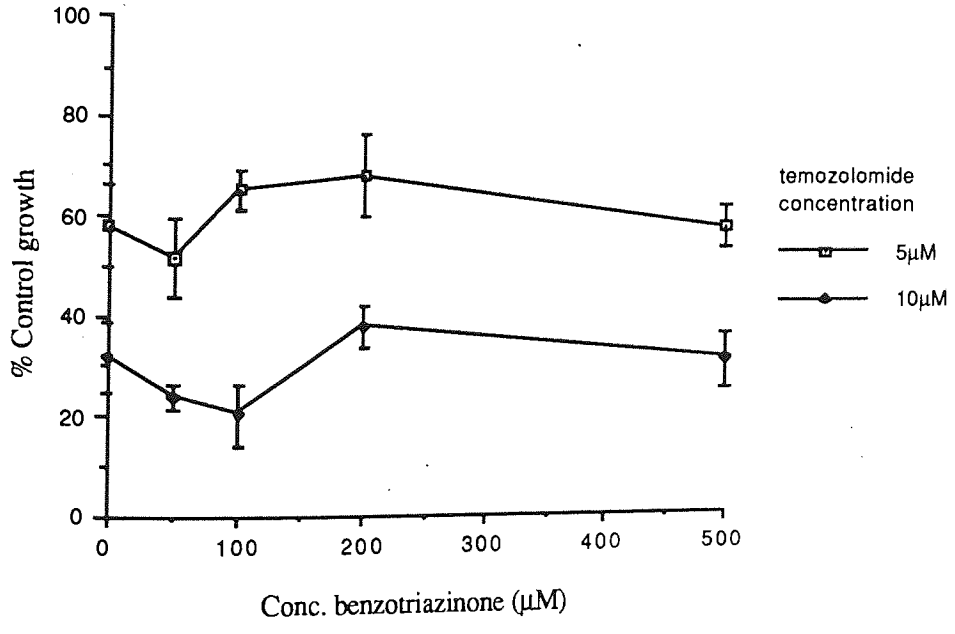
substituted derivatives (**220** and **225**), to assess their ability to inhibit the cytotoxic action of temozolomide which would act as an indication that the triazinones and the imidazotetrazinones had similar binding properties and occupied the same 'receptor' site in DNA. The results obtained from these assays are presented graphically in Figure 5.1. Pre-incubation of the GM892A cell line with the 7- and 8- substituted benzotriazinones over a range of concentrations did not appear to inhibit the cytotoxic properties of temozolomide indicating that they do not compete with temozolomide for binding sites in DNA. However, preliminary results from competition studies involving the 3-methylpyrazolotriazinone (**252**) at a single concentration of 500μM indicated a significant decrease in the cytotoxicity of temozolomide, with the GM892A cell line showing 93.1% control growth in the presence of (**252**) compared with 69.9% control growth in untreated cells at a 5μM concentration of temozolomide, while a concentration of 10μM of temozolomide produced a reduction in control growth to 42% which in the presence of (**252**) was only reduced to 64.5% control growth. Unfortunately, the poor yield of the pyrazolotriazinone (**252**) did not allow more than one experiment to be performed. Based on this evidence there may well exist an imidazotetrazinone specific binding region or 'receptor' which merits further investigation and the synthesis of new pyrazolo[3,4-d]-1,2,3-triazinones.

Figure 5.1: Effect of the pretreatment of the GM892A cell line with the benzotriazinones (**225**) and (**220**) on the growth inhibitory effect of temozolomide.

(a) 1,2,3-benzotriazinone (**225**).



(b) 1,2,3-benzotriazinone (**220**).



CHAPTER 6 : CONCLUSIONS.

In common with other alkylating agents e.g. nitrogen mustards, nitrosoureas and dialkyltriazenes the tetrazinones have been shown to have a marked preference for the alkylation of guanine rich sequences of DNA, especially the N7 and O6 positions exposed in the major groove. The structural diversity of these agents suggest that a number of factors are important in conferring sequence specificity. The sequence selectivity of covalent DNA modification can in principle be summarised in the terms of the generalised kinetic scheme (equation 1) which contains both noncovalent binding and covalent binding or bonding terms.[133]

$$\text{drug} + \text{DNA} \underset{}{\overset{K_b}{\rightleftharpoons}} (\text{drug-DNA})_{\text{noncov}} \xrightarrow{k_r} (\text{drug}-\text{DNA})_{\text{cov}}$$

Equation 1: Generalised kinetic scheme for covalent DNA modification

Inspection of the terms in equation 1 allows three interpretations to be made as to the factors that may be involved in the sequence selective recognition processes:

(i) the binding step is sequence selective when different DNA sequences have differing binding affinities for the drug (i.e. different values for K_b).

(ii) the covalent bonding step is sequence selective when the rate constant for the formation of the covalent adduct (i.e k_r) varies with different DNA sequences.

(iii) both the binding step and the covalent bonding step induce sequence selectivity by a combination of the mechanisms highlighted in (i) and (ii). Indeed, the proposed mode of action of the imidazotetrazinones involves an initial binding step *via* interactions between the C8 substituent and G-C base pairs followed by a covalent bonding step in which the rate of ring cleavage, and thereby generation of the active alkylating agent, is a function of the nucleotide sequence surrounding the molecule.

During the research conducted in the course of this thesis it has not been possible to establish, conclusively, the validity of the hypothesis under test. However, a number of interesting conclusions can been drawn with regard to the ability of imidazotetrazinones to induce sequence selective modifications of DNA which can be neatly separated into the two categories outlined by equation 1:

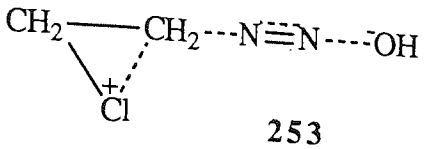
(i) the participation of imidazotetrazinones in an initial noncovalent binding step.

(ii) selectivity derived from the covalent bonding reactions.

6.1 Factors contributing to a possible binding potential of imidazotetrazinones in the major groove of DNA.

The essence of this approach relies on the likelihood that all DNA bases no matter what their surrounding sequences are equally reactive to alkylating agents, but their reaction becomes sequence specific as a result of the relative frequency of alkylation being determined by local variations in concentration (the so-called mass action effect) resulting from noncovalent binding of the agent to specific preferred sequences present in the DNA structure. The likelihood of small molecules such as alkylating agents possessing such targeting abilities would on the face of it seem small. However, Kohn *et al.*[24] have shown that the selectivity of nitrogen mustards for alkylating the middle guanine in a run of three or more contiguous guanines correlated closely with the negative molecular electrostatic potential (MEP) exhibited by the DNA sequences. This observation was rationalised by the consideration that the positively charged aziridinium intermediate (**2**) generated by nitrogen mustards was drawn selectively towards the regions with a greater negative electrostatic potential giving the overall picture of a charge attraction leading to an increase in the local concentration of the drug at specific DNA sequences. A similar theory has been advanced for the similar sequence selectivity of chloroethylnitrosoureas *via* the generation of a partial chloronium ion (**253**).[134] The inhibition of guanine alkylation by salt or cationic DNA binders which weaken the negative MEP led to further evidence for a charged attractive interaction.[135] However, positively charged species are not generated by the triazene and imidazotetrazinone series of alkylating agents which would suggest that factors other than charge attraction are responsible for promoting the binding of these agents to guanine rich regions of DNA.

The results generated both in the present work and those reported previously clearly indicate an important biological role for the C8 substituent in the tetrazinone series of antitumour agents. Unlike the 1-aryl-3,3-dialkyltriazene series of agents the effect of the partition coefficient (logP) upon the antitumour activity of the imidazotetrazinones is considerably less marked and of only relatively minor importance.[37] While the effect of the nature of the 8-substituent upon the degree of susceptibility, and the rate, of tetrazinone ring



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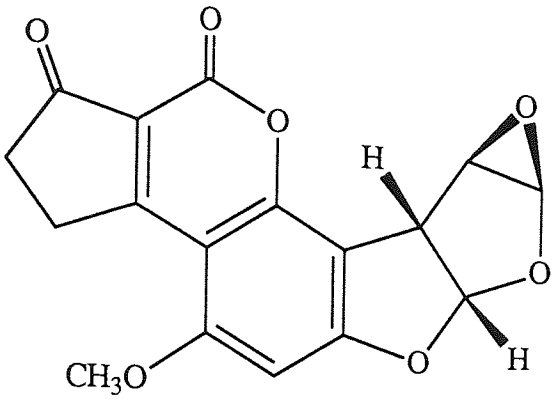

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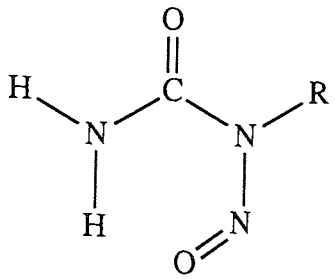
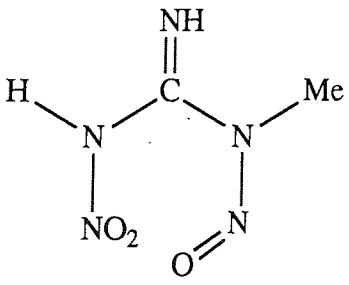


R = Me (**255**)
= Pr (**256**)



257

Figure 6.1: Structural formulae of alkylating agents selective for the major groove of DNA..

cleavage (i.e. the ability of the tetrazinone to generate the active triazene species) has been shown by chemical hydrolysis studies not to be important in determining antitumour activity with all the compounds in the homologous 3-(2-chloroethyl) series showing comparatively similar half-lives under aqueous conditions. Indeed, if antitumour activity was reflected by the ability to undergo ring cleavage the 8-cyano derivative which possesses a markedly shorter half-life[35] would be expected to have a greater antitumour potential than is in fact observed with this compound. The qualitative differences in the factors relating structure and antitumour activity would suggest that although the tetrazinones and the dialkyltriazenes ultimately yield the same class of cytotoxic species their different modes of activation - the dialkyltriazenes requiring metabolic demethylation to the monoalkyltriazene to induce activity while the tetrazinones require hydrolytic activation - does not allow much more than a cursory comparison of the structural facets which dictate activity.

The obvious requirement for a free NH group in the 8-carbamoyl series of compounds would tend to suggest that the ability to participate in hydrogen bonding interactions was crucial for maintaining antitumour activity. The geometry of the tetrazinones allows both intramolecular and intermolecular hydrogen bonding interactions, both of which should be considered when trying to establish the mode of action of these compounds:

(a) intramolecular hydrogen bonding: the nature of the intramolecular hydrogen bonding of the imidazotetrazinones has been revealed by X-ray crystallographic studies. In general all the 8-carbamoyl substituted compounds showed a preference for the conformation involving hydrogen bonding between the C8 NH group and the imidazole nitrogen at N7. It was envisaged that the ability of the 8-carbamoylimidazotetrazinones to form the pseudo 'tricyclic' structure (**89**) may be important in modulating antitumour activity. Unfortunately attempts to synthesis the tricyclic tetrazinone (**145**), which would mimic this system, were unsuccessful. Interestingly, 8-carbamoylpyrazolotetrazinones, in which the nitrogen at the 7 position is replaced by a CH group, cannot form the 'tricyclic' structure highlighted above and yet remain equally as active as their imidazotetrazinone counterparts. However, the pyrazolotetrazinones can participate in hydrogen bonding interactions with the tetrazine nitrogen at N1, a situation that is also exhibited in the crystal structure of mitozolomide, but not by any other of the imidazotetrazinones. Although quantum mechanics calculations

suggested that the NH_2---N7 rotamer was the most favourable conformation the rotation barrier about the carbamoyl group is small enough to allow free interconversion between rotamers under physiological conditions. Hydrogen bonding interactions between the amidic NH and the tetrazine nitrogen would also lead to the generation of a pseudo 'tricyclic' species (**88**) common to both the pyrazolo- and imidazotetrazinones. Imidazotetrazinones with large bulky substituents at the 8 position would be less likely, for steric reasons, to favour the formation of an NH_2---N1 rotamer and instead adopt the more open and favourable NH_2---N7 conformation which could explain their poor antitumour activity. Such a feature may well be worth further study possibly by the synthesis of compounds which mimic the NH_2---N1 tricyclic system, with particular reference to molecules which are unreactive, enabling them to act as a model for the study of the conformational features important to the imidazotetrazinones.

(b) intermolecular hydrogen bonding: a major component of the proposed mechanism of action of the imidazotetrazinones is the importance of intermolecular hydrogen bonding as a recognition process. Intermolecular hydrogen bonding interactions have long been considered important in the recognition by both drugs and endogenous substances of their biological target, indeed a number of antitumour agents have important hydrogen bonding interactions with DNA bases crucial to their sequence selectivity. Examples include the minor groove binders netropsin and distamycin and more interestingly from the point of major groove specific agents uracil mustard (**18**). Kohn has shown that uracil mustard had enhanced reactivity with 5´PyG*C sequences while for other mustards the presence of a 3´ cytosine residue greatly reduced the reactivity of the target guanine residue.[24] Modelling studies indicated that hydrogen bonding interactions between the uracil O4 and the cytosine NH_2 resulted in displacement of the guanine residue towards the phosphate backbone resulting in an improved negative electrostatic potential for the guanine residue. A number of interesting observations can be made with regard to the potential intermolecular hydrogen bonding of the tetrazinones:

(i) molecular modelling studies: preliminary modelling of mitozolomide in the major groove of DNA around the site of GGG bases shows encouraging hydrogen bonding patterns

although more work is needed especially to rule out similar binding in the region of AT sequences. Of particular interest is the observation that the NH_2---N1 rotamer in mitozolomide produced more geometrically strained interactions with DNA, which would tend to suggest that the NH_2---N7 rotamer has greater potential than the NH_2---N1 rotamer for DNA binding interactions (c.f. intramolecular hydrogen bonding above). The ability to visualise the binding of mitozolomide in the major groove of DNA, albeit at present only in a superficial way, adds credence to the theory of a DNA receptor site for the imidazotetrazinones. The existence of clearly defined binding sites for alkylating agents in the major groove of DNA is not unknown, in fact Muench *et al.*[136] have postulated a binding site for the mutagenic and carcinogenic epoxide of the mycotoxin aflatoxin B_1 (**254**). The epoxide (**254**) exhibits a marked preference for alkylation of the N7 position of certain guanines relative to others in double-stranded DNA but not in single-stranded DNA, while an inhibition of alkylation by nonreactive structural analogues suggests the presence of well defined areas of binding to DNA. Analogously, it was hoped that the synthesis of the nonreactive benzotriazinones (**220-227**) and the pyrazolotriazinone (**252**) would provide firm experimental evidence for the existence of an imidazotetrazinone receptor, however, experimental results have proved discouraging with little or no inhibition of temozolomide mediated cell death in the presence of the 3-methyl-1,2,3-benzotriazinones, although, encouraging preliminary results have been obtained with the pyrazolotriazinone (**252**). Attempts to analyse potential binding sites for temozolomide in DNA using footprinting studies have recently begun, however, initial results suggest that the small size, a paucity of strong interactions and the high reactivity of this compound may prevent a successful study. The endonucleases used in DNA footprinting studies splice from the minor groove side of DNA and it may turn out that such a technique will not identify major groove binding.[137]

(ii) attempts to improve the activity of inactive imidazotetrazinones by the incorporation of additional hydrogen bonding acceptor and donator functionalities did not lead to an improvement in antitumour activity, indeed the reverse situation has manifest, with a dramatic decrease in the *in vitro* activity of a series N-phenyl carboxamide analogues against the TLX5 lymphoma, although poor solubility of these compounds in the culture medium may explain these results.

(iii) the synthesis of amino acid and small peptide derivatives with particular reference to

residues that may be implicated in promoting DNA binding have also been disappointing. Although the chemistry now exists to prepare almost any peptide analogue of the imidazotetrazinones as the number of residues increase not only is purification more problematical; antitumour activity decreases rapidly, with again solubility factors masking a critical appraisal of the potential for hydrogen bonding to elicit an improvement in antitumour activity.

The poor antitumour activity exhibited by many of the tetrazinones synthesised during the course of this project would tend to suggest that these compounds are less, or at best no more, specific than the parent compound mitozolomide. However, in many cases the poverty of antitumour activity probably reflects the problem of cellular delivery due to the poor solubility of these compounds. Therefore, instead of using cytotoxicity assays as an indication of improved sequence specificity it may be more productive to examine the alkylation patterns of these compounds using tests such as the Maxam and Gilbert sequencing method[138] and thereby investigate any changes in the alkylation pattern across this class of compounds resulting from the modification of the C8 position.

6.2 Factors contributing to the selective covalent bonding reactions of imidazotetrazinones.

It has been suggested that the fact that many agents differ significantly in the structure of their non-alkylating residues and yet show the same sequence preference argues against a role for the specific binding interactions of the non-alkylating moieties in influencing selectivity, especially when it is considered that few small molecular weight alkylating agents have been shown to possess significant noncovalent DNA binding capabilities other than an apparent charge attraction. Consequently Warpehoski and Hurley[133] have indicated that sequence selectivity may result from the covalent bonding step shown in equation 1 which would help to reconcile the following observations:

(a) methylnitrosourea (**255**) displays a similar pattern of sequence selectivity for the N7 position of guanine, preferably where they occur in clusters, exhibited by chloroethylnitrosoureas and yet it is difficult to postulate a positive ion intermediate which induces selectivity *via* charged attractions, while the lack of any marked hydrogen bonding

potential makes a specific binding interaction unlikely.

(b) the actual effect of an increase in the salt concentration upon the alkylation pattern of nitrogen mustards is a reduction in the magnitude of alkylation without a difference in sequence preference.

Warpehoski and Hurley have suggested that selectivity may result from the ability of specific nucleotide sequences to stabilise an incipient positive charge on the electrophilic substrate in the transition state, thereby reducing the relative free energy of activation of the bonding reaction with a concomitant increase in favourability of the alkylation reaction.[133] The N7 and O6 positions of runs of guanine residues possess the properties of the highest electron densities and thereby nucleophilicities of the DNA bases while also being the most exposed sites in the major groove of DNA to the alkylation reaction, factors which may be important in directing the sequence selectivity of alkylating agents. Consequently, the overall effect is to imply that DNA sequences with significant negative electrostatic potential act to catalyse the alkylation reaction. Although such a catalytic effect is difficult to demonstrate due to the highly reactive nature of many alkylating species a few examples have been noted:

(i) aflatoxin oxide (**254**) alkylates the N7 position of guanine in double stranded DNA much more readily than that of guanosine.[139]

(ii) the rate of hydrolysis of n-propylnitrosourea (**256**) is 3-fold lower than that for its rate of alkylation of calf thymus DNA.[140]

(iii) N´-methyl-N-nitro-N´-nitrosoguanidine (MNNG) (**257**) does not alkylate guanosine but readily alkylates DNA.[133]

Unfortunately, attempts to show a catalytic effect of synthetic G-C rich double stranded DNA in the hydrolysis of mitozolomide failed to yield any conclusive results with the observation that hydrolysis appeared to be more dependent upon the type of buffer used in the experiments. Although these results were disappointing it may be worth persevering with this approach, either by using unbuffered reaction conditions or by the, potentially more useful, determination of the rate of hydrolysis of mitozolomide/MCTIC compared with their rate of alkylation of the N7 position of guanine residues in natural DNA.

6.3 Summary.

Most alkylation reactions show a degree of sequence selectivity, for example simple chemical reagents such as dimethyl sulphate exhibit a preference for alkylating the N7 position of guanine. This is a result of guanine possessing the highest reactivity with, and also accessibility to, electrophilic reagents. However, the observation that subtle changes in the alkylation patterns for distinct guanine sites in double stranded DNA between, and also more importantly within, the common classes of alkylating agents indicates an important role for the non-alkylating or 'carrier' moiety.

The molecular mechanisms involved in the covalent modification of DNA involves, in principle, an initial noncovalent attractive step followed by a covalent modification step. The participation of the imidazotetrazinones in both of these steps has yet to be fully explained. While it was originally expected that a degree of sequence selective binding affinity would have a predominant effect upon the observed alkylation pattern this has yet to be firmly established. Clearly their antitumour activity does have a crucial reliance upon the nature of the C8-substituent with a marked preference for the presence of an NH group, but here as elsewhere, exceptions to the rule are found most notably the excellent activity of the 8-sulphone derivatives. The role of the C8-carbamoyl group has been postulated purely upon theoretical grounds using molecular modelling techniques which although reasonable, require corroboration with solid experimental data, a situation which has proved difficult to achieve due to the high reactivity of the tetrazinone ring. However, the synthesis of closer related structural analogues stable under physiological conditions (e.g. the pyrazolotetrazinone (**252**)) offers new hope in this area.

To date, most of the research into covalent DNA modification has centred around the identification of the alkylating specificities of alkylating agents in synthetic or partially purified DNA. However, the subtle differences in antitumour activity and variations in the side-effects of antitumour agents may lie not at the level of pure DNA but could depend more on the nature of the interaction with chromosomal DNA, with its complexities of secondary and tertiary structure, and the presence of structural complexes such as those with proteins and histones. For example, the lack of myelosuppression seen with the nitrosoureas streptozocin (**15**) and chlorotozocin (**16**) has been attributed to the the mediation of the glucose residue in directing alkylation to different sites in bone-marrow

chromatin[12]. It is not inconceivable that the difference in tumour specificity (i.e.the fact that temozolomide has been shown to be clinically specific for glioma and melanoma tumours) may result form the so called 'carrier' groups directing alkylation to guanine rich sequences at distinct parts of the chromatin some of which are lethal to the tumour while others have no detrimental effect. Such a theory may help to explain the differences in the clinical activity of agents which have been identified as attacking the same sites in DNA.

Many questions remain to be answered regarding both the tetrazinones and the other classes of alkylating agents which attack the major groove of DNA, especially a clear separation of the extent that the binding and covalent bonding steps are responsible for dictating activity. Only when answers to this question are forthcoming can the realisation of the rational design of a sequence selective alkylating agent be accomplished. Until such a time the rewards of selective cancer chemotherapy remain open to us all.

CHAPTER 7 : EXPERIMENTAL.

7.1 Molecular modelling methods.

As mentioned in Chaper 2, the coordinates of the imidazotetrazinones used in this study were based on the X-ray crystal structure of mitozolomide[77], the derivatives being generated using standard bond lengths and angles in the molecular modelling package Chem-X.[79] The initial geometries of mitozolomide and temozolomide were optimised using the MOPAC molecular orbital program.[81] The following molecular properties were calculated: partial charges by the molecular orbital program CNINDO using the CNDO/2 parameter set;[141] potential energy maps by Chem-X; single point energies of temozolomide by the *ab initio* quantum mechanics program Gaussian 80[80] using the STO-3G basis set. The ChemDBS-1 and ChemStat routines within Chem-X were used in the generation of the structure-activity relationships. All the above operations were performed on a VAX 8650 computer. The modelling of mitozolomide in the major groove of DNA was conducted using Quanta 2.1[142] on a Silicon Graphics IRIS 3120 work station.

7.2 Chemical methods.

Chemicals used in the course of this work were, in general, purchased from The Aldrich Chemical Co. Ltd., Gillingham with the exception of: mitozolomide which had been donated to this department by May and Baker Ltd., Dagenham, England, Morpho-CDI was purchased from Fluka, Glossop, England; the protected amino acids and WSC.HCl were purchased from Novobiochem, Nottingham, England and 2-amino-5-bromo-6-(4-aminophenyl)-pyrimidin-4-one which was synthesised in this department by Dr R.T. Wheelhouse.

All melting points (M.pt.) were measured on an Electrothermal Digital Melting Point Apparatus and are uncorrected.

Infrared spectra were recorded using KBr discs, unless stated otherwise, on either a Mattson 2020 Galaxy Series F.T. Infrared Spectrophotometer equipped with Fourier Infrared Software Tools (FIRST) or a Perkin-Elmer 1310 Infrared Spectrophotometer.

NMR spectra were recorded on Bruker 250MHz AC Spectrometer. All signals were assigned with reference to tetramethylsilane (TMS) as the internal standard. Abberviations used in the interpretation of the NMR spectra are:

br = broad, s = singlet, d = doublet, d.d = double doublets, d.q = double quartets, t = triplet, q = quartet, m = multiplet.

Electron impact mass spectra were measured at 70eV on a V.G. Micromass 12B single focussing spectrometer with a variable temperature source and my thanks go to Mrs K. Farrow, Mrs D. Jones and Mr C. Bache, Aston University, for supplying this data. FAB mass spectra were supplied by the SERC Mass Spectrometry Service Centre, Chemistry Department, University College of Swansea, Singleton Park, Swansea SA2 8PP. M^+ signifies the observed molecular ion peak, while m/z refers to peaks other than the true molecular ion.

UV-Visible spectra were recorded upon a Cecil CE 5095 High Performance Scanning Spectrophotometer with a CE 500 Control-Record Module using matched quartz cells with a pathlength of 1cm.

Flash column chromatography was performed using Sorbsil C60 silica gel and TLC using Kieselgel 60 silica plates containing a fluorescent indicator. Spots were visualised under UV light at 254nm or with the aid of iodine.

C, H, N analyses were performed by Butterworth Laboratories Ltd., 54-56 Waldegrave Road, Teddington, Middlesex TW11 8LG.

Attempted synthesis of 1,6-dinitro-diimidazo[1,5-a:1′,5′-d]pyrazine-5,10-dione (97).

Method A: a mixture of 5-nitroimidazole-4-carboxylic acid[87] (600mg,3.8mmol), anhydrous toluene (8ml), and thionyl chloride (1.3ml,17mmol) was heated under reflux for 3 hours. The reaction mixture was evaporated under reduced pressure, the residue triturated with toluene:petroleum ether (60-80°C) 1:1 (10ml), filtered and dried in *vacuo* to give a brown solid (425mg) identified as mixture of (**97**) and the chloro derivatives (**98**) and (**99**).

M.pt. 247-249°C. (lit 249-251°C).[47] I.R. v cm^{-1} 1750 (C=O), 1540 (NO_2 asym str.), 1350 (NO_2 sym str.). M^+ 278, 267/269 (3:1), 256/258/260 (9:6:1).

Method B: an intimate mixture of 5-nitroimidazole-4-carboxylic acid[87] (600mg,3.8mmol) and phosphorus pentachloride (940mg,4.5mmol) was heated at 120°C for 1 hour. The mixture was evaporated at 60°C(1mmHg) for 1 hour to remove phosphorus oxychloride and then the residue was washed with toluene:petroleum ether (60-80°C) 1:1 (10ml) to give a brown solid (410mg) containing the same mixture of components as the sample produced in method A.

N-[5-nitroimidazole-4-carbonyl]-glycine ethyl ester (94).

5-Nitroimidazole-4-carboxylic acid[87] (500mg,3.2mmol) and glycine ethyl ester hydrochloride (530mg,3.8mmol) were dissolved in DMF (30ml) and cooled to 0°C. To the stirred solution was added a precooled solution of diphenylphosphoryl azide (1.05g,3.8mmol) in DMF (10ml) followed by triethylamine (710mg,7mmol). The reaction mixture was stirred for 6 hours at 0°C and at room temperature overnight. The DMF was removed under reduced pressure and the residue washed with 0.5M $NaHCO_3$ to give a cream solid. Recystallisation from ethyl acetate gave (**94**) as colourless plates (330mg,43%).

M.pt. 210-212°C. I.R. ν cm^{-1} 3350 (NH), 1730 (O-C=O), 1640 (N-C=O), 1550 (NO_2 asym str.), 1350 (NO_2 sym str.). ^1H NMR ($CDCl_3$) δ 14.05 (1H, br s, NH), 9.20 (1H, s, NH-C=O), 7.89 (1H, s, H2), 4.10 (2H, s, N-CH_2), 4.08 (2H, q, J=7Hz, O-CH_2), 1.19 (3H, t, J=7Hz, CH_3). M^+ 242. Found C 39.58, H 4.19, N 23.38, $C_8H_{10}N_4O_5$ requires C 39.67, H 4.16, N 23.13.

General procedure A: 3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-[N-(2-carbamoylphenyl)]carboxamide (104).

A solution of the acid chloride[37] (**71**) (525mg,2mmol) in anhydrous THF (20ml) was treated dropwise with a solution of 2-aminobenzamide (275mg,2mmol) and triethylamine (202mg,2mmol) in dry DMF (5ml) at room temperature. The reaction mixture was stirred for 30 minutes at room temperature and then poured onto a solution of 2M HCl (25ml) in water (50ml). The precipitated imidazotetrazinone was filtered and washed with water. The crude residue was purified by flash chromatography using ethyl

acetate:acetonitrile 1:1 as the eluent to give (**104**) as a pale yellow powder (105mg,15%). M.pt. 179-181°C (dec.). I.R. ν cm^{-1} 3525-3100 br (NH), 1742 (C=O), 1685 (NH-C=O), 1642 (NH-C=O). ^1H NMR (DMSO-d_6) δ 12.89 (1H, br s, CONH-Ph), 8.97 (1H, s, H6), 8.74 (1H, d, J_{ortho}=6.9Hz Ph H3), 8.31 (1H, br s, CONH), 7.84 (1H, d, J_{ortho}=8.1Hz Ph H6), 7.71 (1H, br s, CONH), 7.59 (1H, t, J_{ortho}=8.3Hz and 7.9Hz, Ph H5), 7.20 (1H, t, J_{ortho}=7.4Hz and 7.6Hz Ph H4), 4.68 (2H, t, J=6Hz, CH_2Cl), 4.05 (2H, t, J=6Hz, NCH_2). λ_{max} 340nm (DMF). m/z 256 (M^+ - $NCO(CH_2)_2Cl$). Found C 46.68, H 3.51, N 26.88, $C_{14}H_{12}N_7O_3Cl$ requires C 46.48, H 3.34, N 27.10.

3-(2-Chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-[N-(3-carbamoylphenyl)]carboxamide (105).

A mixture of the acid chloride (**71**)(525mg,2mmol) and 3-aminobenzamide (275mg,2mmol) was reacted according to general procedure A. The crude residue was purified by flash chromatography using ethyl acetate:acetonitrile 1:1 as the eluent to give (**105**) as a cream powder (152mg,21%).

M.pt 250-252°C (dec.). I.R. ν cm^{-1} 3435 (NH), 3368 (NH), 3187 br (NH), 1740 (C=O), 1661 (NH-C=O). ^1H NMR (DMSO-d_6) δ 10.55 (1H, br s, CONH-Ph), 9.01 (1H, s, H6), 8.36 (1H, t, J_{meta}=1.7Hz, Ph H2), 7.96 (1H, br s, CONH), 7.65 (1H, d.d, J_{ortho}=7.1Hz and J_{meta}=2.1Hz, Ph H4), 7.62 (1H, d, J_{ortho}=7.8Hz, Ph H6), 7.44 (1H, t, J_{ortho}=7.9Hz, Ph H5), 7.38 (1H, br s, CONH), 4.67 (2H, t, J=6Hz, CH_2Cl), 4.04 (2H, t, J=6Hz, NCH_2). λ_{max} 336nm (EtOH). m/z 256 (M^+ - $NCO(CH_2)_2Cl$). Found C 46.65, H 3.47, N 27.35, $C_{14}H_{12}N_7O_3Cl$ requires C 46.48, H 3.34, N 27.10.

3-(2-Chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-[N-(4-carbamoylphenyl)]carboxamide (106).

A mixture of the acid chloride (**71**) (525mg,2mmol) and 4-aminobenzamide (275mg,2mmol) was reacted according to general procedure A. The crude imidazotetrazinione was purified by flash chromatography using ethyl acetate:acetonitrile 1:1 as the eluent to give (**106**) as a cream powder (175mg,24%).

M.pt. 249-250°C (dec.). I.R. ν cm^{-1} 3378 br (NH), 3168 br (NH), 1734 (C=O), 1677

(NH-C=O), 1655 (NH-C=O). ^{1}H NMR (DMSO-d_6) δ 10.65 (1H, br s, CONH-Ph), 9.03 (1H, s, H6), 7.97 (2H, d, J_{ortho}=8.8Hz Ph H3 and H5), 7.90 (1H, br s, CONH), 7.88 (2H, d, J_{ortho}=8.8Hz, Ph H2 and H6), 7.30 (1H, br s, CONH), 4.67 (2H, t, J=6Hz, CH_2Cl), 4.05 (2H, t, J=6Hz, NCH_2). λ_{max} 340nm (DMF). m/z 256 (M^+ - $NCO(CH_2)_2Cl$). Found C 46.79, H 3.39, N 26.39, $C_{14}H_{12}N_7O_3Cl$ requires C 46.48, H 3.34, N 27.10.

3-(2-Chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-[N-(2,4-dicarbamoylphenyl)]carboxamide (107).

A mixture of the acid chloride (**71**) (525mg,2mmol) and 2-aminobenzene-1,4-dicarboxamide (358mg,2mmol) was reacted according to general procedure A. The crude residue was purified by repeated precipitation from 1-methylpyrrolidin-2-one with dry ether to give (**107**) as a buff solid (290mg,32%) solvated with 0.5 molar equivalents of 1-methypyrrolidin-2-one (NMP).

M.pt. 292-295°C (dec.). I.R. ν cm^{-1} 3378 br (NH), 3192 br (NH), 1741 (C=O), 1675 (NH-C=O), 1654 (NH-C=O). ^{1}H NMR (DMSO-d_6) δ 12.82 (1H, s, CONH-Ph), 9.17 (1H, d, J_{meta}=1.4Hz, Ph H3), 8.98 (1H, s, H6), 8.40 (1H, br s, CONH), 8.13 (1H, br s, CONH), 7.87 (1H, d, J_{ortho}=8.1Hz, Ph H6), 7.80 (1H, br s, CONH), 7.64 (1H, d.d, J_{ortho}=8.1Hz and J_{meta}=1.4Hz, Ph H5), 7.56 (1H, br s, CONH), 4.67 (2H, t, J=6Hz, CH_2Cl), 4.05 (2H, t, J=6Hz, NCH_2), 3.30 (1H, t, J=7Hz, NMP H5), 2.69 (1.5H, s, NMP N-CH_3), 2.17 (1H, t, J=7Hz, NMP H3), 1.91 (1H, m, NMP H4). λ_{max} 343nm (DMF). m/z 299 (M^+ - $NCO(CH_2)_2Cl$). Found C 46.41, H 3.91, N 26.84, $C_{15}H_{13}N_8O_4Cl.1/2(C_5H_9NO)$ requires C 46.26, H 3.88, N 26.21.

3-(2-Chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-[N-(4-pyrimidin-6(1H)-one)phenyl]carboxamide (108).

A mixture of the acid chloride (**71**) (525mg,2mmol) and the aniline (**109**) (405mg,2mmol) was reacted according to general procedure A. The crude residue was purified by repeated precipitation from 1-methylpyrrolidin-2-one with dry ether to give (**108**) as an orange powder (295mg,28%) solvated with 1 molecule of 1-methylpyrrolidin-2-one (NMP).

M.pt. decomposes without melting < 320°C. I.R. ν cm^{-1} 3500-3000 br (NH), 1746 (C=O), 1690 (C=O), 1654 (C=O). ^1H NMR (DMSO-d_6) δ 12.40 (1H, br s, pyrim CONH), 10.84 (1H, br s, CONH-Ph), 9.06 (1H, s, H6), 8.20 (2H, br s, NH_2), 8.09 (2H, d, J_{ortho}=8.7Hz, Ph H2 and H6), 7.98 (2H, d, J_{ortho}=8.7Hz, Ph H3 and H5), 6.36 (1H, s, pyrim H5), 4.68 (2H, t, J=6Hz, CH_2Cl), 4.05 (2H, t, J=6Hz, NCH_2), 3.30 (2H, t, J=7Hz, NMP H5), 2.69 (3H, s, NMP N-CH_3), 2.17 (2H, t, J=7Hz, NMP H3), 1.90 (2H, m, NMP H4). λ_{max} 349nm (DMF). m/z (FAB, NOBA matrix) 450/452 (3:1) (M^+ + Na), 428/430 (3:1) (M^+ + H). Found C 50.48, H 4.43, N 26.18, $C_{17}H_{14}N_9O_3Cl.C_5H_9NO$ requires C 50.14, H 4.40, N 26.58.

3-(2-Chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-(N-phenyl)carboxamide (103).

A mixture of the acid chloride (**71**) (525mg,2mmol) and aniline (186mg,2mmol) was reacted according to general procedure A. The crude residue was purified by recrystallisation from acetone:water 9:1 to give (**103**) as yellow crystals (331mg,52%).

M.pt. 162-163°C (lit. 166-167°C).[43] I.R. ν cm^{-1} 3379 (NH), 3118 (NH), 1736 (C=O), 1679 (NH-C=O). ^1H NMR (DMSO-d_6) δ 10.45 (1H, br s, CONH), 9.01 (1H, s, H6), 7.87 (2H, d, J_{ortho}=8Hz, Ph H2 and H6), 7.37 (2H, t, J_{ortho}=7.1Hz and 7.8Hz, Ph H3 and H5), 7.12 (1H, t, J_{ortho}=7.1Hz, Ph H4), 4.67 (2H, t, J=6Hz, CH_2Cl), 4.05 (2H, t, J=6Hz, NCH_2). λ_{max} 335nm (EtOH).

General procedure B: N-(benzyloxycarbonyl)-*L*-alanyl-*L*-valyl methyl ester (132).

A mixture of Z-*L*-alanine (4.46g,20mmol), *L*-valine methyl ester hydrochloride (5.03g,30mmol), triethylamine (3.03g,30mmol), 1-hydroxybenzotriazole (2.70g,20mmol), WSC.HCl (4.60g,24mmol) and acetonitrile (100ml) was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, the residue taken up into ethyl acetate, washed with 1M HCl, water, 1M $NaHCO_3$, brine and dried over Na_2SO_4. Precipitation from ethyl acetate with petroleum ether (60-80°C) gave (**132**) as

colourless needles (4.83g,73%).

M.pt. 84-85°C. I.R. ν cm^{-1} 3311 br (NH), 1738 (O-C=O), 1685 (Z NH-C=O), 1657 (NH-C=O). ^1H NMR ($CDCl_3$) δ 7.33 (5H, s, Z Ar-H), 6.74 (1H, br d, J=8.3Hz, CONH-Val), 5.62 (1H, br d, J=7.6Hz, CONH-Ala), 5.10 (2H, s, Z CH_2-Ph), 4.53 (1H, q, J=4.7Hz 8.3Hz, Val α CH), 4.36 (1H, d.q, J=7Hz 7.6Hz, Ala α CH), 3.70 (3H, s, $COOCH_3$), 2.21 (1H, m, Val CH), 1.35 (3H, d, J=7Hz, Ala CH_3), 0.93 (3H, d, J=7Hz, Val CH_3), 0.83 (3H, d, J=7Hz, Val CH_3). M^+ 336. Found C 60.65, H 7.14, N 8.45, $C_{17}H_{24}N_2O_5$ requires C 60.71, H 7.19, N 8.33.

N^2-(benzyloxycarbonyl)-N-[bis(4-methoxyphenyl)methyl]-*L*-glutamyl-*L*-alanyl methyl ester (135).

Z-N-[bis(4-methoxyphenyl)methyl]-*L*-glutamine[105] (1.01g,2mmol) was coupled to *L*-alanine methyl ester hydrochloride (280mg,2mmol) according to the general method B. Precipitation from ethyl acetate with hexane gave (**135**) as a cream solid (760mg,64%).

M.pt. 209-212°C. I.R. ν cm^{-1} 3296 br (NH), 1741 (O-C=O), 1689 (Z NH-C=O), 1651 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.62 (1H, br d, J=8.7Hz, CONH-Mbh), 8.34 (1H, br d, J=6.9Hz, CONH-Ala), 7.41 (1H, br d, J=8.1Hz, CONH-Glu), 7.34 (5H, m, Z Ar-H), 7.14 (4H, d, J_{ortho}=8.5Hz, Mbh 2xH2 and H6), 6.86 (4H, d, J_{ortho}=8.5Hz, Mbh 2xH3 and H5), 5.99 (1H, br d, J=8.6Hz, MbH CH), 5.01 (2H, s, Z CH_2-Ph), 4.26 (1H, d.q, J=7.2Hz 7Hz, Ala α CH), 4.01 (1H, d.q, J=6.2Hz 8.6Hz, Glu α CH), 3.71 (6H, s, Mbh 2xOCH_3), 3.65 (3H, s, $COOCH_3$), 2.25 (2H, m, Glu γ CH_2), 1.85 (2H, m, Glu β CH_2), 1.27 (3H d J=7.2Hz Ala CH_3). m/z (FAB NOBA matrix) 614 (M^++Na), 592 (M^++H). Found C 65.06, H 6.28, N 7.03, $C_{32}H_{37}N_3O_8$ requires C 64.96, H 6.30, N 7.10.

N-(benzyloxycarbonyl)-*L*-alanyl-*L*-valine (133).

To a solution of Z-*L*-alanyl-*L*-valyl methyl ester (4,35g, 13mmol) in methanol (30ml) at 0°C 1M NaOH (16ml,16mmol) was added slowly with stirring. The reaction mixture was stirred for 1 hour at 0°C and then the methanol removed under reduced pressure. Water was added to the residue and the resulting solution filtered to remove any

unreacted starting material. The cooled solution was carefully acidified with 1M HCl and left in the refrigerator (4°C) overnight to give (**133**) as a white solid (2.15g,51%) which was filtered, washed with water and dried in *vacuo* over P_2O_5.

M.pt. 149-150°C. I.R. ν cm^{-1} 3300 br (NH), 3250-2750 br (OH), 1714 (HO-C=O), 1693 (Z NH-C=O), 1656 (NH-C=O). ^1H NMR ($CDCl_3$) δ 11.84 (1H, br s, CO_2H), 7.33 (5H, s, Z-Ar-H), 6.94 (1H, br d, J=8.3Hz, CONH), 5.59 (1H, br d, J=7.6Hz, CONH), 5.11 (2H, s, Z CH_2-Ph), 4.53 (1H, d.d, J=4.7Hz 8.6Hz Val α CH), 4.34 (1H, d.q, J=6.9Hz 7.6Hz, Ala α CH), 2.20 (1H, m, Val CH), 1.36 (3H, d, J=6.9Hz, Ala-CH_3), 0.91 (6H, t, J=6.6Hz, Val 2xCH_3). M^+ 322. Found 59.52, H 6.97, N 8.67, $C_{16}H_{22}N_2O_5$ requires C 59.61, H 6.88, N 8.69.

N-(benzyloxycarbonyl)-*L*-alanyl-*L*-valyl-*L*-seryl methyl ester (134).

Z-*L*-alanyl-*L*-valine (403mg,1.25mmol) was coupled to *L*-serine methyl ester hydrochloride (390mg,2.5mmol) according to the general method B. The crude residue was washed directly with 1M HCl, water, 1M $NaHCO_3$, water and acetone. Precipitation from a minimum volume of DMF with water gave (**134**) as a white powder (405mg,76%).

M.pt. 213-215°C. I.R. ν cm^{-1} 3530-3200 br (OH), 3296 (NH), 1739 (O-C=O), 1685 (Z NH-C=O), 1641 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.31 (1H, br d, J=7.3Hz, CONH-Ser), 7.68 (1H, br d, J=9Hz, CONH-Val), 7.52 (1H, br d, J=7.8Hz, CONH-Ala), 7.35 (5H, s, Z-Ar-H), 5.07 (1H, br s, Ser OH), 5.02 (2H, s, Z CH_2-Ph), 4.31 (2H, m, Ser α CH and Val α CH), 4.12 (1H, d.q, J=6.9Hz 7.3Hz Ala α CH), 3.68 (2H, m, Ser CH_2), 3.61 (3H, s, $COOCH_3$), 1.98 (1H, m, Val CH), 1.18 (3H, d, J=7.1Hz, Ala CH_3), 0.86 (3H, d, J=6.9Hz, Val CH_3), 0.83 (3H, d, J=6.8Hz, Val CH_3). m/z 305 (M^+ - serinyl methyl ester). Found C 56.76, H 6.68, N 9.59, $C_{20}H_{29}N_3O_7$ requires C 56.73, H 6.90, N 9.92.

General procedure C: the removal of the N-benzoxycarbonyl- (Z) protecting group.

A solution of Z-*L*-alanyl-*L*-valyl methyl ester (1.68g,5mmol) in ethanol (50ml) was treated with palladium-on-charcoal 10% (168mg) and hydrogenated until there

was no further uptake of hydrogen. The mixture was filtered through Celite and the filtrate evaporated to dryness under reduced pressure to give the peptide ester as a white solid which was used directly in the coupling stage without isolation or purification.

The following peptides (and the solvents used) were deprotected according to the general method C:

Z-Gln(Mbh)-Ala(OMe) (**135**).	methanol.
Z-Ala-Val-Ser(OMe) (**134**).	methanol.
Z-Ser-Gln(Mbh)-Ala(OMe) (**136**).	ethanol/DMF.
	methanol.
Z-Ala-Val-Ser-Gln(Mbh)-Ala(OMe) (**137**).	DMF.

General procedure D: N-(benzyloxycarbonyl)-*L*-seryl-*L*-glutamyl-N-[bis(4-methoxyphenyl)methyl]-*L*-alanyl methyl ester (136).

A mixture of Z-*L*-serine (263mg,1.1mmol), *L*-glutamyl-N-[bis-(4-methoxyphenyl)methyl]-*L*-alanyl methyl ester [prepared from the dipeptide (**135**) (650mg,1.1mmol) according to the general method C], 1-hydroxybenzotriazole (150mg,1.1mmol), WSC.HCl (250mg,1.3mmol) and acetonitrile (20ml) was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure and the residue was washed directly with 1M HCl, water, 1M $NaHCO_3$ and water. Precipitation from a minimum volume of DMF with water gave (**136**) as a cream solid (520mg,69%). M.pt. 210-214°C. I.R. ν cm^{-1} 3550-3210 br (OH), 3292 br (NH), 1741 (O-C=O), 1691 (Z NH-C=O), 1641 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.64 (1H, br d, J=8.7Hz, CONH-Mbh), 8.31 (1H, br d, J=6.9Hz, CONH-Ala), 8.07 (1H, br d, J=7.7Hz, CONH-Glu), 7.34 (6H, s, Z Ar-H and CONH-Ser), 7.15 (4H, d, J_{ortho}=8.6Hz, Mbh 2xH2 and H6), 6.85 (4H, d, J_{ortho}=8.6Hz, Mbh 2xH3 and H5), 6.01 (1H, br d, 8.7Hz, Mbh CH), 5.01 (3H, m, Z CH_2-Ph and Ser OH), 4.26 (2H, m, Glu α CH and Ala α CH), 4.12 (1H, m, Ser α CH), 3.71 (6H, s, Mbh 2xOCH_3), 3.59 (3H, s, $COOCH_3$), 3.57 (2H, m, Ser-CH_2), 2.25 (2H, m, Glu γ CH_2), 1.85 (2H, m, Glu β CH_2), 1.26 (3H, d, J=7.3Hz, Ala-CH_3). m/z (FAB NOBA matrix) 701 (M^++Na), 679 (M^++H). Found C 61.38, H 6.37, N 8.10, C $_{35}H_{42}N_4O_{10}$ requires C 61.94, H 6.24, N 8.25.

N-(benzyloxycarbonyl)-*L*-alanyl-*L*-valyl-*L*-seryl-*L*-glutamyl-N-[bis(4-methoxyphenyl)methyl]-*L*-alanyl methyl ester (137).

Z-*L*-alanyl-*L*-valine (242mg,0.75mmol) was coupled to *L*-seryl-*L*-glutamyl-N-[bis(4-methoxyphenyl)methyl]-*L*-alanyl methyl ester [prepared from the tripeptide (**136**) (545mg,0.75mmol) according to the general method C] according to the general method D. A solution of the peptide in DMF (30ml) was passed through a small column of neutral alumina. The filtrate was concentrated under reduced pressure and treated with water to give (**137**) as cream plates (478mg,75%).

M.pt. 244-247°C. I.R. ν cm^{-1} 3510-3180 br (OH), 3284 br (NH), 1737 (O-C=O), 1685 (Z NH-C=O), 1637 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.62 (1H, br d, J=8.6Hz, CONH-Mbh), 8.30 (1H, br d, J=6.8Hz, CONH-Ala), 7.98 (2H, br t, J=7.2Hz, CONH-Glu and CONH-Val), 7.72 (1H, br d, J=8.9Hz, CONH-Ala), 7.52 (1H, br d, J=7.8Hz, CONH-Ser), 7.33 (5H, s, Z Ar-H), 7.13 (4H, d, J_{ortho}=8.6Hz, Mbh 2xH2 and H6), 6.85 (4H, d, J_{ortho}=8.6Hz, Mbh 2xH3 and H5), 6.00 (1H, br d, J=8.7Hz, Mbh CH), 5.05 (1H, t, J= Ser OH), 5.00 (2H, s, Z CH_2-Ph), 4.22 (5H, m, 2xAla, Val, Ser and Glu α CH), 3.70 (6H, s, Mbh 2xOCH_3), 3.58 (3H, s, $COOCH_3$), 3.56 (2H, m, Ser-CH_2), 2.25 (2H, m, Glu γ CH_2), 1.97 (2H, m, Glu β CH_2), 1.76 (1H, m, Val CH), 1.24 (3H, d, J=7.3Hz, Ala CH_3), 1.18 (3H, d, J=7.1Hz, Ala CH_3), 0.81 (6H, t, J=7.5Hz, Val 2xCH_3). m/z (FAB NOBA matrix) 871 (M^++Na), 849 (M^++H). Found C 61.21, H 6.47, N 9.31, $C_{43}H_{56}N_6O_{12}$ requires C 60.84, H 6.65, N 9.90.

8-(N-tert-butylcarbamoyl)-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (117).

A solution of the carboxylic acid (**70**)[43] (1.0g,4mmol) and 1-hydroxybenzotriazole (542mg,4mmol) in dry DMF (30ml) was stirred at 0°C while a solution of DCC (870mg,4.2mmol) in dry DMF (5ml) was added. The mixture was stirred at 0°C for 1 hour and then treated with tert-butylamine (293mg,4mmol). Stirring was continued for 1 hour at 0°C and at room temperature for a further hour. The mixture was filtered to remove DCU and the filtrate quenched with 1M HCl to give a colourless solid. Purification by flash chromatography using ethyl acetate as the eluent gave (**117**) as colourless needles (460mg,49%) identical with those reported.

M.pt. 122-124°C (lit. 126-128°C)[43] I.R. ν cm^{-1} 1750 (C=O), 1675 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.85 (1H, s, H6), 7.60 (1H, br s, CONH), 4.65 (2H, t, J=6Hz, CH_2Cl), 4.03 (2H, t, J=6Hz, NCH_2), 1.42 (9H, s, 3xCH_3). λ_{max} 328nm (EtOH).

N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-glycine ethyl ester (96).

A solution of the carboxylic acid (**70**) (1.25g,5mmol) and 1-hydroxybenzotriazole (680mg,5mmol) in anhydrous DMF (25ml) was stirred at 0°C while a solution of DCC (1.03g,5.5mmol) in dry DMF (5ml) was added. The mixture was stirred at 0°C for 1 hour and then treated with a mixture of glycine ethyl ester hydrochloride (700mg,5mmol) and triethylamine (506mg,5mmol) in dry DMF (10ml). Stirring was continued at 0°C for 1 hour and then the mixture was allowed to warm to room temperature overnight. The mixture was filtered, the filtrate quenched with 1M HCl (50ml), and concentrated under reduced pressure. The residue was triturated with 1M HCl to give a cream solid which was extracted into dichloromethane, washed with water and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue purified by flash chromatography using ethyl acetate:petroleum ether (60-80°C) 8:2 to give (**96**) as a white solid (690mg,42%) identical with that reported below.

General procedure E: N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-glycine ethyl ester (96).

A mixture of the carboxylic acid (**70**)[43] (660mg,2.5mmol), glycine ethyl ester hydrochloride (360mg,2.5mmol), triethylamine (253mg,2.5mmol), 1-hydroxybenzotriazole (340mg,2.5mmol), Morpho-CDI (1.27g,3mmol) and acetonitrile (50ml) was stirred at 0°C, in the dark, for 6 hours. The reaction mixture was allowed to warm to room temperature and stirred for a further 48 hours. The solvent was removed under reduced pressure and the residue was taken up into ethyl acetate washed with 1M HCl, water and dried over Na_2SO_4. The solution was concentrated under reduced pressure and purified by flash chromatography using ethyl acetate:petroleum ether (60-80°C) 8:2 as the eluent to give (**96**) as a white powder (290mg,35%).

M.pt. 122-124°C (dec.). (lit. 114°C).[43] I.R. ν cm^{-1} 3446 (NH), 3318 (NH), 1751 (C=O),

1667 (NH-C=O). ^{1}H NMR (DMSO-d_6) δ 8.92 (1H, s, H6), 8.83 (1H, br t, J=5.7Hz, CONH), 4.64 (2H, t, J=6Hz, CH_2Cl), 4.12 (2H, q, J=7.1Hz, O-CH_2), 4.03 (2H, t, J=6Hz, NCH_2), 4.03 (2H, d, J=5.9Hz, Gly CH_2), 1.20 (3H, t, J=7.1Hz, CH_3). λ_{max} 329nm (EtOH). m/z 223 (M^+ - $NCO(CH_2)_2Cl$). Found C 39.87, H 4.00, N 25.81, $C_{11}H_{13}N_6O_4Cl$ requires C 40.19, H 3.99, N 25.57.

N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-glycinamide (123).

The carboxylic acid (**70**) (660mg,2.5mmol) and glycinamide hydrochloride (280mg,2.5mmol) were coupled according to the general method E. The solvent was removed under reduced pressure and the residue washed directly with 1M HCl and water. Recrystallisation from acetone:water 9:1 gave (**123**) as a white powder (330mg,44%).

M.pt 146-148°C (dec.). I.R. ν cm^{-1} 3619 (NH), 3473 (NH), 3390 br (NH), 3150 br (NH), 1737 (C=O), 1665 (NH-C=O), 1639 (NH-C=O). ^{1}H NMR (DMSO-d_6) δ 8.91 (1H, s, H6), 8.46 (1H, t, J=5.4Hz, CONH), 7.46 (1H, br s, CONH), 7.13 (1H, br s, CONH), 4.63 (2H, t, J=6Hz, CH_2Cl), 4.02 (2H, t, J=6Hz, NCH_2), 3.89 (2H, d, J=5.5Hz, Gly CH_2). λ_{max} 329nm (EtOH). m/z 194 (M^+ - $NCO(CH_2)_2Cl$). Found C 34.36, H 3.42, N 30.55, $C_9H_{10}N_7O_3Cl.H_2O$ requires C 34.02, H 3.81, 30.86.

N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-*L*-serine methyl ester (124).

The carboxylic acid (**70**) (660mg,2.5mmol) and *L*-serine methyl ester hydrochloride (390mg,2.5mmol) were coupled according to the general method E. The residue was taken up into dichloromethane washed with 1M HCl, water and dried over Na_2SO_4. Addition of petroleum ether (60-80°C) gave (**124**) as a white powder (258mg,30%).

M.pt. 119-121°C (dec.). I.R. ν cm^{-1} 3550-3250 br (OH), 3470 (NH), 3402 (NH), 1743 (C=O), 1656 (NH-C=O). ^{1}H NMR (DMSO-d_6) δ 8.94 (1H, s, H6), 8.41 (1H, br d, J=7.5Hz, CONH), 5.31 (1H, br t, J=5.6Hz, Ser OH), 4.62 (3H, m, CH_2Cl and Ser α CH), 4.03 (2H, t, J=6Hz, NCH_2), 3.93 (2H, m, Ser CH_2), 3.67 (3H, s, $COOCH_3$). λ_{max}

328nm (EtOH). m/z 239 (M^+ - $NCO(CH_2)_2Cl$). Found C 38.06, H 3.52, N 24.15, $C_{11}H_{13}N_6O_5Cl$ requires C 38.32, H 3.80, N 24.38.

N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-*L*-alanine methyl ester (125).

The carboxylic acid (**70**) (660mg,2.5mmol) and *L*-alanine methyl ester hydrochloride (350mg,2.5mmol) were coupled according to the general method E. Purification by flash chromatography using ethyl acetate as eluent gave (**125**) as a white powder. Precipitation from ethyl acetate with petroleum ether (60-80°C) gave (**125**) as colourless needles (550mg,67%).

M.pt. 106-108°C (dec.). I.R. ν cm^{-1} 3392 (NH), 3112 (NH), 1746 (C=O), 1675 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.93 (1H, s, H6), 8.80 (1H, br d, J=7.4Hz, CONH), 4.65 (2H, t, J=6Hz, CH_2Cl), 4.57 (1H, d.q, J=7.2Hz and 7.4Hz, Ala α CH), 4.03 (2H, t, J=6Hz, NCH_2), 3.66 (3H, s, $COOCH_3$), 1.43 (3H, d, J=7.2Hz, Ala CH_3). λ_{max} 328 nm (EtOH). m/z 223 (M^+ - $NCO(CH_2)_2Cl$). Found C 40.19, H 3.74, N 25.39, $C_{11}H_{13}N_6O_4Cl$ requires C 40.19, H 3.99, N 25.57.

N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-*L*-methionine methyl ester (126).

The carboxylic acid (**70**) (660mg,2.5mmol) and *L*-methionine methyl ester hydrochloride (500mg, 2.5mmol) were coupled according to the general method E. Purification by flash chromatography using ethyl acetate as the eluent gave (**126**) as a colourless oil which slowly solidified to yield a cream solid (505mg,52%).

M.pt. 93-95°C (dec.). I.R. ν cm^{-1} 3390 br (NH), 3127 (NH), 1739 (C=O), 1675 (NH-C=O). ^1H NMR ($CDCl_3$) δ 8.94 (1H, s, H6), 7.91 (1H, br d, J=8Hz, CONH), 4.98 (1H, m, α CH), 4.74 (2H, t, J=6Hz, CH_2Cl), 3.98 (2H, t, J=6Hz, NCH_2), 3.79 (3H, s, $COOCH_3$), 2.56 (2H, m, CH_2S), 2.31 (1H, m, β CH), 2.15 (1H, m, β CH), 2.09 (3H, s, SCH_3). λ_{max} 326nm (EtOH). m/z 283 (M^+ - $NCO(CH_2)_2Cl$). Found C 40.40, H 4.53, N 21.50, $C_{13}H_{17}N_6O_4SCl$ requires C 40.16, H 4.41, N 21.61.

N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-*L*-tryptophan methyl ester (127).

The carboxylic acid (**70**) (660mg,2.5mmol) and *L*-tryptophan methyl ester hydrochloride (640mg,2.5mmol) were coupled according to the general method E. The residue was washed directly with 1M HCl, water, acetonitrile and dried in *vacuo* over P_2O_5 to give (**127**) as a bright yellow powder (660mg,74%).

M.pt 181-183°C (dec.). I.R. ν cm^{-1} 3407 (Indole NH), 3345 br (NH), 3133 (NH), 1751 (C=O), 1737 (C=O), 1663 (NH-C=O). ^1H NMR (DMSO-d_6) δ 10.96 (1H, br s, Indole NH), 8.89 (1H, s, H6), 8.53 (1H, br d, J=7.8Hz, CONH), 7.51 (1H, d, J_{ortho}=7.7Hz, Trp H4), 7.33 (1H, d, J_{ortho}=7.9Hz, Trp H7), 7.20 (1H, d, J=2.2Hz, Trp H2), 7.08-6.93 (2H, m, Trp H5 and H6), 4.84 (1H, d.q, J=7.8Hz and 6.1Hz, α CH), 4.63 (2H, t, J=6Hz, CH_2Cl), 4.01 (2H, t, J=6Hz, NCH_2), 3.65 (3H, s, $COOCH_3$), 3.36 (2H, m, β CH_2). λ_{max} 328nm (DMF). m/z 338 (M^+ - $NCO(CH_2)_2Cl$). Found C 51.74, H 4.20, N 22.28, $C_{19}H_{18}N_7O_4Cl$ requires C 51.42, H 4.09, N 22.09.

General procedure F: N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-*L*-alanyl-*L*-valyl methyl ester (139).

A mixture of the carboxylic acid (**70**) (660mg,2.5mmol), *L*-alanyl-*L*-valyl methyl ester [prepared from the dipeptide (**132**) (840mg,2.5mmol) according to the general method C], 1-hydroxybenzotriazole (340mg,2.5mmol), Morpho CDI (1.27g,3mmol) and acetonitrile (50ml) was stirred at 0°C, in the dark, for 6 hours. The reaction mixture was allowed to warm to room temperature and stirred for a further 48 hours. The solvent was removed under reduced pressure and the residue was taken up into dichloromethane washed with 1M HCl, water and dried over Na_2SO_4. The solution was concentrated under reduced pressure and purified by flash chromatography using ethyl acetate as the eluent to give (**139**) as white crystalline solid (520mg,48%).

M.pt. 75-77°C (dec.). I.R. ν cm^{-1} 3420-3230 br (NH), 3107 br (NH), 1743 (C=O), 1662 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.43 (1H, s, H6), 7.84 (1H, br d, J=7Hz, CONH), 6.65 (1H, br d, J=7Hz, CONH), 4.80 (1H, m, Ala α CH), 4.76 (2H, t, J=6Hz, CH_2Cl), 4.54 (1H, d.d, J=4.8Hz and 8.7Hz, Val α CH), 3.99 (2H, t, J=6Hz, NCH_2), 3.76 (3H, s,

$COOCH_3$), 2.19 (1H, m, Val CH), 1.54 (3H, d, J=6.9Hz, Ala CH_3), 0.92 (3H, d, J=6.8Hz, Val CH_3), 0.89 (3H, d, J=6.9Hz, Val CH_3). λ_{max} 330nm (EtOH). m/z 322 (M^+ - $NCO(CH_2)_2Cl$). Found C 44.75, H 5.00, N 22.60, $C_{16}H_{22}N_7O_5Cl$ requires C 44.92, H 5.18, N 22.92.

N^2-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-N-[bis(4-methoxyphenyl)methyl]-*L*-glutamyl-*L*-alanylmethyl ester (142).

A mixture of the carboxylic acid (**70**) (154mg,0.6mmol) and *L*-glutamyl-N-[bis(4-methoxyphenyl)methyl]-*L*-alanyl methyl ester [prepared from the dipeptide (**135**) (350mg,0.6mmol) according to the general method C] was reacted according to the general method F. The residue was purified by precipitation from ethyl acetate with ether to give (**142**) as a buff solid (125mg,22%).

M.pt. 193-194°C (dec.). I.R. ν cm^{-1} 3320 br (NH), 1740 (C=O), 1653 (NH-C=O), 1637 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.91 (1H, s, H6), 8.67 (2H, br t, J=9.2Hz, CONH-Ala and CONH-Mbh), 8.27 (1H, br d, J=8.1Hz, CONH-Glu), 7.11 (4H, d.d, J_{ortho}=8.5Hz, Mbh 2xH2 and H6), 6.83 (4H, d.d, J_{ortho}=8.5Hz, Mbh 2xH3 and H5), 5.98 (1H, d, J=8.6Hz, Mbh CH), 4.65 (3H, m, CH_2Cl and Glu α CH), 4.31 (1H, d.q, J=7.1Hz and 7.3Hz, Ala α CH), 4.03 (2H, t, J=6Hz, NCH_2), 3.70 (6H, s, Mbh 2xOCH_3), 3.61 (3H, s, $COOCH_3$), 2.27 (2H, m, Glu γ CH_2), 2.05 (2H, m, Glu β CH_2), 1.30 (3H, d, J=7.3Hz, Ala CH_3). λ_{max} 328nm (EtOH). m/z (FAB NOBA matrix) 705/707 (3:1) (M^++Na), 683/685 (3:1) (M^++H). Found C 54.74, H 5.03, N 16.78, $C_{31}H_{35}N_8O_8Cl$ requires C 54.51, H 5.16, N 16.40.

N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-*L*-alanyl-*L*-valyl-*L*-seryl methyl ester (140).

A mixture of the carboxylic acid (**70**) (154mg,0.6mmol) and *L*-alanyl-*L*-valyl-*L*-seryl methyl ester [prepared from the tripeptide (**134**) (250mg,0.6mmol) according to the general method C] was reacted according to the general method F. The residue was purified by flash chromatography using ethyl acetate as the eluent to give (**140**) as a white

powder (19mg,6%).

M.pt. 211-212°C (dec.). I.R. ν cm^{-1} 3600-3150 br (OH and NH), 1741 (C=O), 1641 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.93 (1H, s, H6), 8.45 (1H, br d, J=7.7Hz, CONH), 8.35 (1H, br d, J=7.2Hz, CONH), 8.08 (1H, br d, J=9Hz, CONH), 5.06 (1H, t, J=5.9Hz Ser OH), 4.63 (3H, m, CH_2Cl and α CH), 4.30 (2H, m, 2xα CH), 4.02 (2H, t J=6Hz, NCH_2), 3.70 (2H, m, Ser CH_2), 3.68 (3H, s, $COOCH_3$), 1.95 (1H, m, Val CH), 1.35 (3H, d, J=6.9Hz, Ala CH_3), 0.87 (6H, t, J=7.1Hz, Val 2xCH_3). λ_{max} 329nm (EtOH). m/z (FAB NOBA matrix) 537/539 (3:1) (M^++Na), 515/517 (3:1) (M^++H). Found C 44.56, H 5.38, N 21.23, $C_{19}H_{27}N_8O_7Cl$ requires C 44.32, H 5.29, N 21.76.

N-[3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one-8-carbonyl]-*L*-alanyl-*L*-valyl-*L*-seryl-*L*-glutamyl-N-[bis-(4-methoxyphenyl)methyl]-*L*-alanyl methyl ester (141).

A mixture of the carboxylic acid (**70**) (102mg,0.4mmol) and *L*-alanyl-*L*-valyl-*L*-seryl-*L*-glutamyl-N-[bis-(4-methoxyphenyl)methyl]-*L*-alanyl methyl ester [prepared from the pentapeptide (**137**) (350mg,0.4mmol) according to the general method C] was reacted according to the general method F. The addition of water to the reaction mixture gave crude (**141**) as a grey solid.

M.pt. 197-201°C (dec.). I.R. ν cm^{-1} 3280 br (NH), 1743 (C=O), 1676, 1664, 1645 br (NH-C=O). ^1H NMR (DMSO-d_6) δ 9.27 (1H, s, H6), 8.92 (1H, br d, J=8.6Hz, CONH), 8.43 (4H, m, CONH), 8.36 (1H, br d, J=7.4Hz, CONH), 7.14 (4H, d, J_{ortho}=8.5Hz, Mbh 2xH2 and H6), 6.85 (4H, d, J_{ortho}=8.5Hz, Mbh 2xH3 and H5), 5.98 (1H, d, J=8.6Hz, Mbh CH), 5.07 (1H, br s, Ser OH), 4.64 (3H, m, CH_2Cl and α CH), 4.24 (4H, m, 4x α CH), 4.05 (2H, t, J=6Hz, NCH_2), 3.71 (6H, s, Mbh 2xOCH_3), 3.59 (5H, s + m, $COOCH_3$ and Ser CH_2), 2.25 (2H, m, Glu γ CH_2), 1.98 (2H, m, Glu β CH_2), 1.76 (1H, m, Val CH), 1.36 (3H, d, J=7.3Hz, Ala CH_3), 1.26 (3H, d, J=7Hz, Ala CH_3), 0.87 (6H, t, J=7.4Hz, Val 2xCH_3). λ_{max} 331nm (DMF). m/z (FAB NOBA matrix) 963/965 (3:1) (M^++Na), 941/943 (3:1) (M^++H).

3-Diazopyrazolo[3,4-d]pyrimidin-4-one (153).

A stirred suspension of 3-aminopyrazolo[3,4-d]pyrimidin-4-one (**152**)[106] (960mg,6.4mmol) in 5% hydrochloric acid at 0°C was treated portionwise with solid sodium nitrite (480mg,7mmol) such that the temperature of the mixture did not rise above 5°C. A rapid yellow colour was produced and the suspension was stirred for 30 minutes at 0°C. The solid was filtered, washed with water, cold methanol and allowed to air dry to give (**153**) as a yellow crystalline solid (840mg,82%).

CAUTION: shock sensitive when dry, handle with care.

M.pt. decomposes with effervescence without melting < 260°C. I.R. (Nujol) ν cm^{-1} 2190 (N_2), 1700 (C=O).

3-(2-chloroethyl)-9-[N-(2-chloroethyl)carbamoyl]-3,4,9,10-tetrahydropyrimido[4´,5´:3,4]pyrazolo[5,1-d]-1,2,3,5-tetrazine-4(3H),10(9H)-dione (154).

A suspension of 3-diazopyrazolo[3,4-d]pyrimidin-4-one (500mg, 3.1mmol) in dry dichloromethane (20ml) containing 2-chloroethyl isocyanate (1.6g,15mmol) was heated under reflux, in the dark, for 5 days. The suspension was filtered to give a yellow residue which was identical with the starting material and a yellow-orange filtrate. The filtrate was triturated with dry ether to give (**154**) as a pale yellow solid (140mg,17%) which was filtered washed with dry ether and dried in *vacuo* over P_2O_5.

M.pt. 260-262°C (dec.). I.R. ν cm^{-1} 3413 br (NH), 1743 (Tetrazine C=O), 1696 (Pyrimidone C=O), 1623 (C=O). ^1H NMR (DMSO-d_6) δ 9.47 (1H, br t, J=5.5Hz, CONH), 8.95 (1H, s, H8), 4.80 (2H, t, J=6Hz, Tetrazine CH_2Cl), 4.09 (2H, t, J=6Hz, Tetrazine NCH_2), 3.81 (2H, t, J=5.4Hz, CH_2Cl), 3.68 (2H, m, NCH_2). λ_{max} 316nm (EtOH). m/z (FAB NOBA matrix) 373/375/377 (9:6:1) (M^++H). Found C 35.63, H 2.61, N 29.55, $C_{11}H_{10}N_8O_3Cl_2$ requires C 35.41, H 2.70, N 30.03.

Attempted synthesis of 3-amino-2-(N-phenylcarbamoyl)pyrazolo[3,4-d]pyrimidin-4(5H)-one (155).

Method A: a mixture of 5-cyano-4-methylthiopyrimidin-6(1H)-one[106] (250mg,1.5mmol)

and 4-phenylsemicarbazide (275mg,1.8mmol) was heated under reflux in pyridine (15ml) for 24 hours. Initially the reaction generated an orange-brown solution which slowly deposited a cream solid. The solid was filtered washed with water, methanol and dried in *vacuo* over P_2O_5 to give a cream solid (90mg,22%) which was identical with a sample of 3-aminopyrazolo[3,4-d]pyrimidin-4-one (**152**) prepared according to the method of Dornow and Dehmer.[106]

M.pt. > 340°C (lit. >360°C).[106] I.R. ν cm^{-1} 3475, 3408 (NH_2), 3292 br (NH), 3219 br (NH), 1670 (C=O). Found C 39.67, H 3.39, N 45.86, $C_5H_5N_5O$ requires C 39.74, H 3.33, N 46.34.

Method B: a mixture of 5-cyano-4-methylthiopyrimidin-6(1H)-one (200mg,1.2mmol) and 4-phenylsemicarbazide (215mg,1.4mmol) in 2-ethoxyethanol (20ml) was heated under reflux for 24 hours to give a orange-brown solution which comprised of unreacted starting materials. Heating under reflux for a further 72 hours did not induce any reaction.

Method C: a mixture of 5-cyano-4-methylthiopyrimidin-6(1H)-one (167mg,1mmol) and 4-phenylsemicarbazide (152mg,1mmol) in absolute ethanol (10ml) was heated under reflux for 5 days during which time a buff solid was slowly deposited. The solid was filtered washed with water, cold methanol and dried in *vacuo* over P_2O_5.

M.pt. 135-138°C. I.R. ν cm^{-1} 3473, 3412 (NH_2), 3219 br (NH), 1742 (C=O), 1722 (C=O). ^1H NMR (DMSO-d_6) δ 8.77 (1H, s, H6), 7.97 (1H, s, Pyrimidine NH), 7.48 (2H, d, J_{ortho}=8Hz, Ph H2 and H6), 7.24 (2H, t, J_{ortho}=8Hz and J_{ortho}=7.5Hz, Ph H3 and H5), 6.94 (1H, t, J_{ortho}=7.5Hz, Ph H4).

Dimethyl 2-aminobenzene-1,4-dicarboxylate (176).

A mixture of dimethyl 2-nitrobenzene-1,4-dicarboxylate (**175**) (2.39g,10mmol), stannous chloride dihydrate (11.28g,50mmol) and ethyl acetate (100ml) was heated under reflux, under N_2, for 1 hour. The solvent was removed under reduced pressure, the residue treated with 1M sodium hydroxide solution (100ml) and extracted with chloroform. The combined chloroform extracts were washed with water, brine and dried over Na_2SO_4. Removal of the solvent under reduced pressured gave a yellow solid which

was recrystallised from methanol to give (**176**) as yellow needles (1.95g,93%).

M.pt. 133-134°C. (lit. 135°C)[143]. I.R. ν cm^{-1} 3465, 3355 (NH_2), 1705 (C=O), 1685 (C=O). ^1H NMR ($CDCl_3$) δ 7.89 (1H, d, J_{ortho}=8.3Hz, H6), 7.35 (1H, d, J_{meta}=1.5Hz, H3), 7.23 (1H, d.d, J_{ortho}=8.3Hz and J_{meta}=1.5Hz, H5), 5.84 (2H, br s, NH_2), 3.90 (3H, s, $COOCH_3$), 3.89 (3H, s, $COOCH_3$). M^+ 209.

Methyl 3-methyl-1,2,3-benzotriazin-4(3H)-one-7-carboxylate (180).

Dimethyl 2-aminobenzene-1,4-dicarboxylate (**176**) (520mg,2.5mmol) was suspended in a solution of 2M hydrochloric acid (4ml) and water (16ml) at 0°C, and treated dropwise with sodium nitrite (194mg,2.8mmol) in water (1ml). The reaction mixture was stirred at 0°C for 2 hours and then filtered to remove any unreacted amino component. The solution was stirred at 0°C, neutralised with 1M sodium acetate solution and then treated with 40% aqueous methylamine solution (1.2ml,15mmol). A precipitate soon developed which was filtered, washed with water and allowed to air dry. Recrystallisation from aqueous ethanol gave (**180**) as a yellow needles (230mg,42%).

M.pt.144-145°C (dec.). I.R. ν cm^{-1} 1710 (C=O), 1680 (triazinone C=O). ^1H NMR ($CDCl_3$) δ 8.76 (1H, d.d, J_{meta}=1.3Hz and J_{para}=0.8Hz, H8), 8.37 (2H, m, H5 and H6), 4.05 (3H, s, N-CH_3), 4.00 (3H, s, $COOCH_3$). M^+ 219. Found C 54.65, H 4.09, N 19.34, $C_{10}H_9N_3O_3$ requires C 54.79, H 4.14, N 19.17.

3-Methyl-1,2,3-benzotriazin-4(3H)-one-7-carboxamide (182).

The benzotriazinone (**180**) (219mg,1mmol) was dissolved in warm methanol (20ml), treated with methanolic ammonia (5ml) and shaken at room temperature for 48 hours. The mixture was filtered, the residue washed with chloroform and recrystallised from aqueous ethanol to give (**182**) as a pink solid (120mg,59%).

M.pt. 277-280°C (dec.). I.R. ν cm^{-1} 3420 br (NH), 3300 br (NH), 1680 (triazinone C=O), 1655 (C=O). ^1H NMR (DMSO-d_6) δ 8.61 (1H, s, H8), 8.44 (1H, br s, CONH), 8.29 (2H, s, H5 and H6), 7.85 (1H, br s, CONH), 3.92 (3H, s, CH_3). M^+ 204. Found C 52.99, H 4.06, N 27.45, $C_9H_8N_4O_2$ requires C 52.94, H 3.94, N 27.44.

1-Methyl-3-(2,5-bis-carbomethoxyphenyl)-triazene (178).

A suspension of dimethyl 2-aminobenzene-1,4-dicarboxylate (500mg,2.4mmol) in 2M HCl (3.5ml) and iced water (10ml) was stirred at 0°C and dropwise with sodium nitrite (190mg,2.8mmol) in water (1ml). The reaction mixtu stirred at 0°C for 2 hours and then filtered to remove any unreacted amino compone solution of the diazonium salt was stirred with ice at 0°C and treated with 40% a methylamine solution (0.7ml,90mmol). The precipitate that developed was imme filtered, washed once with water, taken up into chloroform and dried over Na_2SC solvent was removed under reduced pressure to give (**178**) as a pale yellow (420mg,70%).

M.pt. 116-118°C (dec.). I.R. ν cm^{-1} 3265 (NH), 1725 (C=O), 1695 (C=O). ^1H ($CDCl_3$) δ 11.90 (1H, br s, NH), 8.35 (1H, s, H6), 7.93 (1H, d, J_{ortho}=6.6Hz, H3 (1H, d, J_{ortho}=6.6Hz, H4), 3.93 (3H, s, $COOCH_3$), 3.90 (3H, s, $COOCH_3$), 3.60 (N-CH_3). m/z 208 (M^+-MeN_2). Found C 52.83, H 5.07, N 15.95, $C_{11}H_{13}N_3O_4$ requ 52.59, H 5.22, N 16.27.

Cyclisation of 1-methyl-3-(2,5-bis-carbomethoxyphenyl)-triazene (178)

The triazene (**178**) (100mg,0.4mmol) was suspended in 95% ethanol (and heated under reflux for 4 hours. Upon cooling yellow needles of methyl 3-m 1,2,3-benzotriazin-4(3H)-one-7-carboxylate (**180**) (65mg,74%) were deposited proved identical with the sample prepared above.

1-Phenyl-3-(2,5-bis-carbomethoxyphenyl)-triazene (179).

A suspension of dimethyl 2-aminobenzene-1,4-dicarboxylate (1.05g,5mmol) in 2M HCl (7.5ml) and iced water (20ml) was stirred at 0°C while sc nitrite (392mg,5.5mmol) in water (2ml) was added dropwise. The reaction mixtur stirred at 0°C for 2 hours and then filtered to remove any unreacted amino componen solution of the diazonium salt was neutralised with 1M sodium acetate solution and tr with aniline (470mg,5mmol). The reaction mixture was stirred at 0°C for a furth minutes and then the triazene (**179**) was removed by filtration and washed with v Recrystallisation from 95% ethanol gave (**179**) as yellow needles (1.25g,80%).

M.pt. 155-156°C (dec.). I.R. ν cm^{-1} 3225 (NH), 1690 br (C=O). ^1H NMR ($CDCl_3$) δ 10.60 (1H, br s, NH), 8.73 (1H, s, H6), 8.36 (2H, s, H3 and H4), 7.60-7.23 (5H, m, Ph-H), 3.96 (3H, s, $COOCH_3$), 3.90 (3H, s, $COOCH_3$). Found C 61.51, H 4.82, N 13.19, $C_{16}H_{15}N_3O_4$ requires C 61.34, H 4.83, N 13.41.

Cyclisation of 1-phenyl-3-(2,5-bis-carbomethoxyphenyl)-triazene (179).

Method A: the triazene (**179**) (500mg,1.6mmol) was suspended in 70% aqueous ethanol (10ml) and heated under reflux for 2 hours. Upon cooling crystals of methyl 3-phenyl-1,2,3-benzotriazin-4(3H)-one-7-carboxylate (**181**) were deposited, filtered and dried in *vacuo* to give (**181**) as yellow needles (340mg,76%).

M.pt. 148-149°C (dec.). I.R. ν cm-1 1715 (C=O), 1695 (triazinone C=O). ^1H NMR ($CDCl_3$) δ 8.87 (1H, d, J_{meta}=1Hz, H8), 8.48 (2H, m, H5 and H6), 7.61-7.50 (5H, m, Ph-H), 4.05 (3H, s, $COOCH_3$). M^+ 281. Found C 64.12, H 3.95, N 14.95, $C_{15}H_{11}N_3O_3$ requires C 64.05, H 3.94, N 14.94.

Method B: the triazene (**179**) (500mg,1.6mmol) was suspended in 2% piperidine in ethanol (10ml) and heated under reflux for 1 hour. Upon cooling crystals identified as ethyl-3-phenyl-1,2,3-benzotriazin-4(3H)-one-7-carboxylate (**183**) were deposited, filtered and dried in *vacuo* to give (**183**) as yellow needles (311mg,66%).

M.pt. 156-158°C (dec.). I.R. ν cm^{-1} 1720 (C=O), 1680 (triazinone C=O). ^1H NMR ($CDCl_3$) δ 8.87 (1H, d, J_{meta}=1.45Hz, H8), 8.47 (2H, m, H5 and H6), 7.46-7.76 (5H, m, Ph H), 4.49 (2H, q, J=7Hz, CH_2), 1.47 (3H, t, J=7Hz, CH_3). M^+ 295. Found C 65.13, H 4.42, N 14.38, $C_{16}H_{13}N_3O_3$ requires C 65.08, H 4.44, N 14.23.

Method C: the triazene (**179**) (500mg,1.6mmol) was suspended in 2% piperidine in methanol (10ml) and heated under reflux for 1 hour. Upon cooling yellow needles of methyl 3-phenyl-1,2,3-benzotriazin-4(3H)-one-7-carboxylate (**181**) (370mg,82%) were deposited, filtered and dried in *vacuo*, which proved identical with the sample prepared by method A.

3-Phenyl-1,2,3-benzotriazin-4(3H)-one-7-carboxylic acid (184).

The triazene (**179**) (500mg,1.6mmol) was suspended in a mixture of ethanol: water : piperidine (70:28:2) (10ml) and heated under reflux for 1 hour. The cooled reaction mixture was treated with a two phase mixture of ethyl acetate / 1M $KHSO_4$ (1:1) (20ml) and shaken to ensure complete mixing. The ethyl acetate layer was removed and the aqueous phase extracted with further aliquots of ethyl acetate. The combined ethyl acetate extracts were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was triturated with ether to give a white solid which was filtered and allowed to dry. Recrystallisation from aqueous ethanol gave (**184**) as a white solid (190mg,45%).

M.pt. 237-239°C (dec.). I.R. ν cm^{-1} 3250-2400 br (OH), 1694 (C=O). ^1H NMR (DMSO-d_6) δ 8.66 (1H, s, H8), 8.42 (2H, s, H5 and H6), 7.64-7.58 (5H, m, Ph H). M^+ 267. Found C 63.15, H 3.54, N 15.84, $C_{14}H_9N_3O_3$ requires C 62.92, H 3.39, N 15.72.

2-Aminobenzene-1,3-dicarboxylic acid (2-aminoisophthalic acid) (200).

2-Nitrobenzene-1,3-dicarboxylic acid (**187**) (2.11g,10mmol) was dissolved in ethanol (100ml), treated with palladium-on-charcoal 10% (200mg) and hydrogenated until there was no further hydrogen taken up. The suspension was treated with ether (100ml) and filtered through Celite. The filtrate was evaporated under reduced pressure to give a yellow-green solid. Recrystallisation from absolute ethanol gave (**200**) as pale yellow plates (1.67g,92%).

M.pt. subl. 260-265°C. (lit. subl. 267-269°C)[143] I.R. ν cm^{-1} 3460, 3346 (NH_2), 3100-2390 br (OH), 1685 (C=O). ^1H NMR ($CDCl_3$+DMSO-d_6) δ 11.95 (2H, br s, 2xCO_2H), 8.26 (2H, br s, NH_2), 8.00 (2H, d, J_{ortho}=7.7Hz, H4 and H6), 6.46 (1H, t, J_{ortho}=7.7Hz, H5). M^+ 181.

2-Aminobenzene-1,4-dicarboxylic acid (2-aminoterephthalic acid) (201).

2-Nitrobenzene-1,4-dicarboxylic acid (**199**) (2.11g,10mmol) was dissolved in ethanol (100ml), treated with palladium-on-charcoal 10% (200mg) and hydrogenated until there was no further hydrogen taken up. The suspension was treated with acetone (200ml) and filtered through Celite. The filtrate was evaporated under reduced pressure to give a deep

yellow solid. Recrystallisation from water gave (**201**) as yellow needles (1.70g,94%). M.pt. 321-322°C. (lit. 324-325°C.)[143] I.R. ν cm^{-1} 3506, 3389 (NH_2), 3200-2400 br (OH 1685 (C=O). ^1H NMR ($CDCl_3$+DMSO-d_6) δ 11.90 (2H, br s, $2xCO_2H$) 7.76 (1H, c J_{ortho}=8.2Hz, H6), 7.38 (1H, s, H3), 7.01 (1H, d, J_{ortho}=8.2Hz, H5), 5.50 (2H, br NH_2). M^+ 181.

General procedure G: 8-carboxyisatoic anhydride (202).

The amino acid (**200**) (5.0g,27.6mmol) was suspended in dry THF (150ml and warmed to 40-50°C on a water bath. The suspension was treated portionwise wit triphosgene (2.73g,9.2mmol). Stirring was continued for a further 3 hours with occasiona sparging of the reaction mixture with nitrogen if the hydrochloride salt precipitated out. Th reaction mixture was filtered and the isatoic anhydride (**202**) precipitated out with hexane a a white solid (5.60g,98%) pure enough for use in the next stage.

M.pt. subl. 245-250°C. I.R. ν cm^{-1} 3252 (NH), 1791 (O-C=O), 1707 (C=O), 1660 (NH C=O). ^1H NMR (DMSO-d_6) δ 11.08 (1H, br s, NH), 8.32 (1H, d.d, J_{meta}=1.5Hz anc J_{ortho}=7.8Hz, H7), 8.20 (1H, d.d, J_{meta}=1.5Hz and J_{ortho}=7.8Hz, H5), 7.36 (1H, t J_{ortho}=7.8Hz, H6). M^+ 207. Found C 52.75, H 2.61, N 6.76, $C_9H_5NO_5$ requires C 52.19 H 2.43, N 6.76.

7-Carboxyisatoic anhydride (203).

The amino acid (**201**) (1.50g,8.4mmol) and triphosgene (820mg,2.8mmol) were reacted according to the general method G. Precipitation from THF with hexane gave (**203**) as a buff solid (1.67g,97%).

M.pt. 320-322°C. I.R ν cm^{-1} 3227 (NH), 3200-2400 br (OH), 1784 (O-C=O), 1707 (C=O), 1687 (NH-C=O). ^1H NMR (DMSO-d_6) δ 9.15 (1H, br s, NH), 8.00 (1H, d, J_{ortho}=8.1Hz, H5), 7.69 (1H, d.d, J_{meta}=1.1Hz and J_{ortho}=8.1Hz, H6), 7.66 (1H, d, J_{meta}=1.1Hz, H8). M^+ 207.

General procedure H: 3-(N-methylcarbamoyl)-2-aminobenzoic acid (204).

8-Carboxyisatoic anhydride (**202**) (910mg,4.5mmol) was added portionwise to a stirred solution of 40% aqueous methylamine (1.5ml,18mmol) in water (25ml). The reaction was characterised by the evolution of carbon dioxide and was essentially complete once the isatoic anhydride had dissolved. The reaction mixture was stirred at ambient temperature for a further 15 minutes. Acidification with 2M HCl led to the precipitation of the amide, which was filtered and washed with water. Recrystallisation from aqueous ethanol gave (**204**) as colourless needles (690mg,79%).

M.pt. 222-224°C. I.R. ν cm^{-1} 3439, 3370 (NH_2), 3295 br (NH), 3150-2450 br (OH), 1694 (C=O), 1678 (NH-C=O). ^1H NMR (DMSO-d_6) δ 8.36 (1H, br d, J=4.4Hz, CONH), 7.88 (1H, d.d, J_{meta}=1.4Hz and J_{ortho}=7.8Hz, H6), 7.66 (1H, d.d, J_{meta}=1.4Hz and J_{ortho}=7.8Hz, H4), 6.55 (1H, t, J_{ortho}=7.8Hz H5), 2.73 (3H, d, J=4.5Hz, CH_3). M^+ 194. Found C 55.78, H 5.15, N 14.37, $C_9H_{10}N_2O_3$ requires C 55.67, H 5.19, N 14.43.

3-(N-ethylcarbamoyl)-2-aminobenzoic acid (205).

8-Carboxyisatoic anhydride (**202**) (2.07g,10mmol) was treated with 70% aqueous ethylamine (2.6ml,40mmol) according to the general method H. Recrystallisation from water gave (**205**) as colourless plates (1.77g,85%).

M.pt. 233-235°C I.R. ν cm^{-1} 3464, 3352 (NH_2), 3316 br (NH), 3200-2400 br (OH), 1674 (C=O). ^1H NMR (DMSO-d_6) δ 8.40 (1H, br t, J=5.4Hz, CONH), 7.88 (1H, d.d, J_{meta}=1Hz and J_{ortho}=7.6Hz, H6), 7.68 (1H, d.d, J_{meta}=1Hz and J_{ortho}=7.6Hz, H4), 6.56 (1H, t, J_{ortho}=7.6Hz, H5), 3.25 (2H, d.q, J=5.4Hz and 7.2Hz, CH_2), 1.14 (3H, t, J=7.2Hz, CH_3). M^+ 208. Found C 57.66, H 5.75, N 13.42, $C_{10}H_{12}N_2O_3$ requires C 57.68, H 5.81, N 13.45.

3-(N-phenylcarbamoyl)-2-aminobenzoic acid (206).

A mixture of 8-carboxyisatoic anhydride (**202**) (2.07g,10mmol), aniline (1.67g,40mmol) and dioxane (20ml) was warmed on a steam bath. The solvent was removed under reduced pressure and the residue triturated with 1M HCl (30ml), filtered and the residue washed with water. Recrystallisation from aqueous ethanol gave (**206**) as cream

needles (1.59g,62%).

M.pt. 248°C. I.R. ν cm^{-1} 3464, 3345 (NH_2), 3295 br (NH), 3150-2350 br (OH), (C=O), 1680 (NH-C=O). ^1H NMR (DMSO-d_6) δ 10.22 (1H, s, CONH), 7.96 (1H J_{meta}=1.5Hz and J_{ortho}=7.8Hz, H6), 7.83 (1H, d.d, J_{meta}=1.5Hz and J_{ortho}=7.8Hz, 7.71 (2H, d, J_{ortho}=7.6Hz, Ph H2 and H6), 7.34 (2H, t, J_{ortho}=7.6Hz and 7.3Hz, H and H5), 7.09 (1H, t, J_{ortho}=7.3Hz, Ph H4), 6.65 (1H, t, J_{ortho}=7.8Hz, H5). M^+ Found C 66.04, H 4.78, N 10.91, $C_{14}H_{12}N_2O_3$ requires C 65.62, H 4.72, N 10.93.

3-[N-(2-chloroethyl)carbamoyl]-2-aminobenzoic acid (207).

8-Carboxyisatoic anhydride (**202**) (2.07g,10mmol) was treated w solution of 2-chloroethylamine, prepared by the addition of triethylamine (4.04g,40mm ethanol (5ml) to 2-chloroethylamine hydrochloride (4,64g,40mmol) in water (2 according to the general method H. Recrystallisation from aqueous acetone gave (**20** fine white needles (1.41g,58%).

M.pt. 200-202°C. I.R. ν cm^{-1} 3475, 3364 (NH_2), 3308 br (NH), 3150-2400 br (OH), (C=O). ^1H NMR (DMSO-d_6) δ 8.65 (1H, br t, J=5.4Hz, CONH), 7.91 (1H, J_{meta}=1.4Hz and J_{ortho}=7.7Hz, H6), 7.71 (1H, d.d, J_{meta}=1.4Hz and J_{ortho}=7.7Hz, 6.57 (1H, t, J_{ortho}=7.7Hz, H5), 3.72 (2H, t, J=6Hz, CH_2Cl), 3.54 (2H, d.q, J=5.4Hz 6Hz, NCH_2). M^+ 242/244 (3:1). Found C 49.57, H 4.57, N 11.50, $C_{10}H_{11}N_2$ requires C 49.50, H 4.57, N 11.54.

N-[(2-amino-3-carboxy)benzoyl]glycine methyl ester (208).

8-Carboxyisatoic anhydride (**202**) (2.07g,10mmol) was treated wi solution of glycine methyl ester, prepared by the addition of triethylamine (4.04g,40m in ethanol (5ml) to glycine methyl ester hydrochloride (5.04g,40mmol) in water (25 according to the general method H. Recrystallisation from water gave (**208**) as colou needles (1.69g,67%).

M.pt. 192-193°C. I.R. ν cm^{-1} 3448, 3433 (NH_2), 3292 br (NH), 3120-2400 br (OH), 1 (O-C=O), 1685 (C=O). ^1H NMR (DMSO-d_6) δ 8.87 (1H, br t, J=5.7Hz, CONH), 7 (1H, d.d, J_{meta}=1.5Hz and J_{ortho}=7.8Hz, H6), 7.76 (1H, d.d, J_{meta}=1.5Hz

J_{ortho}=7.8Hz, H4), 6.58 (1H, t, J_{ortho}=7.8Hz, H5), 3.96 (2H, d, J=5.7Hz, CH_2), 3.66 (3H, s, $COOCH_3$). M^+ 252. Found C 52.77, H 4.80, N 11.09, $C_{11}H_{12}N_2O_5$ requires C 52.38, H 4.80, N 11.11.

4-(N-methylcarbamoyl)-2-aminobenzoic acid (209).

7-Carboxyisatoic anhydride (**203**) (2.07g,10mmol) was treated with 40% aqueous methylamine solution (3.4ml,40mmol) according to the general method H. The crude solid was stirred with warm ethyl acetate to remove the ureido side product and filtered. Recrystallisation from aqueous ethanol gave (**209**) as yellow plates (1.20g,62%).

M.pt. 217-218°C. I.R. ν cm^{-1} 3471, 3398 (NH_2), 3270 br (NH), 3100-2450 br (OH), 1703 (C=O), 1630 (C=O). ^1H NMR (DMSO-d_6) δ 8.33 (1H, br d, J=4.4Hz, CONH), 7.51 (1H, d, J_{ortho}=8.1Hz, H6), 7.32 (1H, d, J_{meta}=1.6Hz, H3), 7.02 (1H, d.d, J_{meta}=1.6Hz and J_{ortho}=8.1Hz, H5), 6.55 (2H, br s, NH_2), 2.73 (3H, d, J=4.4Hz, CH_3). M^+ 194. Found C 55.94, H 5.27, N 14.21, $C_9H_{10}N_2O_3$ requires C 55.67, H 5.19, N 14.43.

4-(N-ethylcarbamoyl)-2-aminobenzoic acid (210).

7-Carboxyisatoic anhydride (**203**) (2.07g,10mmol) was treated with 70% aqueous ethylamine solution (2.6ml,40mmol) according to the general method H. The crude solid was stirred with warm ethyl acetate to remove the ureido side product and filtered. Recrystallisation from water gave (**210**) as yellow needles (1.06g,51%).

M.pt. 200-201°C. I.R. ν cm^{-1} 3478, 3405 (NH_2), 3298 br (NH), 3050-2500 br (OH), 1694 (C=O), 1637 (C=O). ^1H NMR (DMSO-d_6) δ 8.35 (1H, br t, J=5.3Hz, CONH), 7.52 (1H, d, J_{ortho}=8.2Hz, H6), 7.31 (1H, d, J_{meta}=1.6Hz, H3), 7.02 (1H, d.d, J_{meta}=1.6Hz and J_{ortho}=8.2Hz, H5), 6.52 (2H, br s, NH_2), 3.24 (2H, d.q, J=5.4Hz and 7.2Hz, CH_2), 1.10 (3H, t, J=7.2Hz, CH_3). M^+ 208. Found C 57.94, H 5.74, N 13.30, $C_{10}H_{12}N_2O_3$ requires C 57.68, H 5.81, N 13.45.

4-(N-phenylcarbamoyl)-2-aminobenzoic acid (211).

A mixture of 7-carboxyisatoic anhydride (**203**) (2.07g,10mmol), aniline (1.67g,40mmol) and dioxane (20ml) was warmed on a steam bath for 30 minutes. The

solvent was removed under reduced pressure and the residue triturated with 1M HCl (3(filtered and the residue washed with water. Recrystallisation from aqueous ethanol (**211**) as a yellow powder (1.66g,65%).

M.pt. 231-233°C. I.R. ν cm^{-1} 3472, 3398 (NH_2), 3290 (NH), 3100-2400 br (OH), (C=O), 1665 (C=O). ^1H NMR (DMSO-d_6) δ 10.23 (1H, br s, CONH), 7.76 (1H J_{ortho}=8.1Hz, H6), 7.60 (1H, s, H3), 7.40-6.95 (6H, m, H5 and Ph H), 6.65 (2H, NH_2). M^+ 256. Found C 65.91, H 4.79, N 10.89, $C_{14}H_{12}N_2O_3$ requires C 65.62, H 4 N 10.93.

General procedure I: 3-Methyl-1,2,3-benzotriazin-4(3H)-one-8-carbox acid (212).

The monoamide (**204**) (600mg,3.1mmol) and anhydrous sodium carbo (173mg,1.6mmol) were warmed in water (15ml) until all the amide had dissolved. solution was allowed to cool to ambient temperature when a solution of sodium nit (270mg,3.7mmol) in water (1ml) was added. The resulting solution was immedia poured onto a mixture of 2M HCl (7ml), and iced water (20ml). The mixture was stirre 0°C for 30 minutes, basified with aqueous ammonia and stirred at room temperature for minutes. Upon acidification (pH 2) with 1M $KHSO_4$ the benzotriazinone was precipita which was filtered and washed with water. Recrystallisation from aqueous ethanol g (**212**) as colourless needles (490mg,77%).

M.pt. 188-189°C (dec.). I.R. ν cm^{-1} 3250-2500 br (OH), 1703 br (C=O). ^1H NM (DMSO-d_6) δ 8.43 (1H, d.d, J_{meta}=1.2Hz and J_{ortho}=7.7Hz, H7), 8.21 (1H, d J_{meta}=1.2Hz and J_{ortho}=7.7Hz, H5), 7.94 (1H, t, J_{ortho}=7.7Hz, H6), 3.94 (3H, s, CH M^+ 205. Found C 52.45, H 3.58, N 20.17, $C_9H_7N_3O_3$ requires C 52.69, H 3.44, 20.48.

3-Ethyl-1,2,3-benzotriazin-4(3H)-one-8-carboxylic acid (213).

The monoamide (**205**) (1.00g,4.8mmol) was cyclised according to t general method I. Recrystallisation from water gave (**213**) as colourless plat (780mg,74%).

M.pt. 145-146°C (dec.). I.R. ν cm^{-1} 3250-2400 br (OH), 1685 (C=O). ^1H NMR (DMSO d_6) δ 8.35 (1H, d.d, J_{meta}=1Hz and J_{ortho}=7.4Hz, H7), 8.22 (1H, d.d, J_{meta}=1Hz and J_{ortho}=7.4Hz, H5), 7.96 (1H, t, J_{ortho}=7.4Hz, H6), 4.43 (2H, q, J=7Hz, CH_2), 1.41 (3H t, J=7Hz, CH_3). M^+ 219. Found C 55.09, H 4.21, N 19.18, $C_{10}H_9N_3O_3$ requires C 54.79, H 4.14, N 19.17.

3-Phenyl-1,2,3-benzotriazin-4(3H)-one-8-carboxylic acid (214).

The monoamide (**206**) (2.00g,7.8mmol) was cyclised according to the general method I. Recrystallisation from aqueous ethanol gave (**214**) as colourless needles (1.71g,82%).

M.pt. 185°C (dec.). I.R. ν cm^{-1} 3200-2450 br (OH), 1701 (C=O), 1689 (C=O). ^1H NMR (DMSO-d_6) δ 8.41 (1H, d.d, J_{meta}=1.4Hz and J_{ortho}=7.7Hz, H7), 8.27 (1H, d.d, J_{meta}=1.4Hz and J_{ortho}=7.7Hz, H5), 8.00 (1H, t, J_{ortho}=7.7Hz, H6), 7.68-7.62 (5H, m, Ph H). M^+ 267. Found C 63.40, H 3.55, N 15.71, $C_{14}H_9N_3O_3$ requires C 62.92, H 3.39, N 15.72.

3-(2-Chloroethyl)-1,2,3-benzotriazin-4(3H)-one-8-carboxylic acid (215).

The monoamide (**207**) (500mg,2.1mmol) was cyclised according to the general method I. Recrystallisation from water gave (**215**) as colourless plates (378mg,71%).

M.pt. 163-165°C (dec.). I.R. ν cm^{-1} 3250-2400 br (OH), 1689 (C=O). ^1H NMR (DMSO-d_6) δ 8.38 (1H, d.d, J_{meta}=1Hz and J_{ortho}=7.6Hz, H7), 8.25 (1H, d.d, J_{meta}=1Hz and J_{ortho}=7.6Hz, H5), 7.99 (1H, t, J_{ortho}=7.6Hz, H6), 4.74 (2H, t, J=6Hz, CH_2Cl), 4.11 (2H, t, J=6Hz, NCH_2). M^+ 253/255 (3:1). Found C 47.65, H 3.32, N 16.53, $C_{10}H_8N_3O_3Cl$ requires C 47.35, H 3.18, N 16.57.

3-(Methoxycarbonylmethyl)-1,2,3-benzotriazin-4(3H)-one-8-carboxylic acid (216).

The monoamide (**208**) (1.00g,4mmol) was cyclised according to the general method I. Recrystallisation from water gave (**216**) as colourless plates (620mg,59%).

M.pt. 112-115°C (dec.). I.R. ν cm^{-1} 3100-2600 br (OH), 1741 (O-C=O), 1701 (C=O). ^1H NMR (DMSO-d_6) δ 8.37 (1H, d.d, J_{meta}=1.1Hz and J_{ortho}=7.6Hz, H7), 8.26 (1H, d.d, J_{meta}=1.1Hz and J_{ortho}=7.6Hz, H5), 8.02 (1H, t, J_{ortho}=7.6Hz, H6), 5.30 (2H, s, CH_2), 3.74 (3H, s, $COOCH_3$). M^+ 263. Found C 50.72, H 3.49, N 16.03, $C_{11}H_9N_3O_5$ requires C 50.20, H 3.45, N 15.96.

3-Methyl-1,2,3-benzotriazin-4(3H)-one-7-carboxylic acid (217).

The monoamide (**209**) (1.00g,5.2mmol) was cyclised according to the general method I. Recrystallisation from aqueous ethanol gave (**217**) as colourless plates (865mg,81%).

M.pt. 245-247°C (dec.). I.R. ν cm^{-1} 3250-2400 br (OH), 1688 (C=O). ^1H NMR (DMSO-d_6) δ 8.53 (1H, s, H8), 8.31 (2H, s, H5 and H6), 3.93 (3H, s, CH_3). M^+ 205. Found C 52.84, H 3.49, N 20.61, $C_9H_7N_3O_3$ requires C 52.69, H 3.44, N 20.48.

3-Ethyl-1,2,3-benzotriazin-4(3H)-one-7-carboxylic acid (218).

The monoamide (**210**) (250mg,1.2mmol) was cyclised according to the general method I. Recrystallisation from aqueous ethanol gave (**218**) as colourless plates (197mg,75%).

M.pt. 232-235°C (dec.). I.R. ν cm^{-1} 3220-2600 br (OH), 1692 (C=O). ^1H NMR (DMSO-d_6) δ 8.59 (1H, s, H8), 8.36 (2H, s, H5 and H6), 4.43 (2H, q, J=7.2Hz, CH_2), 1.41 (3H, t, J=7.2Hz, CH_3). M^+ 219. Found C 54.49, H 4.08, N 19.10, $C_{10}H_9N_3O_3$ requires C 54.79, H 4.14, N 19.17.

3-Phenyl-1,2,3-benzotriazin-4(3H)-one-7-carboxylic acid (219).

The monoamide (**211**) (1.00g,3.9mmol) was cyclised according to the general method I. Recrystallisation from aqueous ethanol gave (**219**) as colourless plates (720mg,69%) identical with the sample that was prepared earlier by the cyclisation of the triazene (**179**).

General procedure J: 8-carbamoyl-3-methyl-1,2,3-benzotriazin-4(3H)-one (220).

A mixture of the benzotriazinone (**212**) (450mg,2.2mmol), thionyl chloride (5ml) and DMF (1 drop) was heated under reflux for 3 hours. The residual thionyl chloride was removed under reduced pressure, the residue dissolved in benzene (5ml) and re-evaporated to give the acid chloride which was dissolved in dry THF (10ml), dropped cautiously into 28% aqueous ammonia (5ml) and stirred at room temperature for 12 hours. The resulting solid was filtered and washed with water. Recrystallisation from aqueous ethanol gave (**220**) as colourless needles (310mg,69%).

M.pt. 218-220°C (dec.). I.R. ν cm^{-1} 3363 br (NH), 3207 br (NH), 1699 (C=O), 1679 (C=O), 1652 (C=O). ^1H NMR (DMSO-d_6) δ 8.45 (1H, br s, CONH), 8.32 (2H, m, H7 and H5), 7.96 (2H, t+br s, J_{ortho}=7.7Hz, H6 and CONH), 3.96 (3H, s, CH_3). M^+ 204. Found C 52.84, H 3.97, N 27.23, $C_9H_8N_4O_2$ requires C 52.94, H 3.95, N 27.44.

8-Carbamoyl-3-ethyl-1,2,3-benzotriazin-4(3H)-one (221).

The benzotriazinone (**213**) (500mg,2.3mmol) was reacted according to the general method J. Recrystallisation from water gave (**221**) as colourless plates (361mg,72%).

M.pt. 196°C (dec.). I.R. ν cm^{-1} 3426 br (NH), 3157 br (NH), 1689 (C=O), 1654 (C=O). ^1H NMR (DMSO-d_6) δ 8.43 (1H, br s, CONH), 8.34 (2H, m, H7 and H5), 7.97 (2H, t+br s, J_{ortho}=7.7Hz, H6 and CONH), 4.44 (2H, q, J=7.2Hz, CH_2), 1.42 (3H, t, J=7.2Hz, CH_3). M^+ 218. Found C 55.40, H 4.56, N 25.68, $C_{10}H_{10}N_4O_2$ requires C 55.04, H 4.62, N 25.68.

8-Carbamoyl-3-phenyl-1,2,3-benzotriazin-4(3H)-one (222).

The benzotriazinone (**214**) (970mg,3.6mmol) was reacted according to the general method J. Recrystallisation from aqueous ethanol gave (**222**) as cream needles (630mg,66%).

M.pt. 234-236°C (dec.). I.R. ν cm^{-1} 3407 br (NH), 3201 br (NH), 1702 (C=O), 1665 (C=O), 1631 (C=O). ^1H NMR (DMSO-d_6) δ 8.42-8.33 (3H, m+br s, H7 H5 and CONH),

8.02 (2H, t+br s, J_{ortho}=7.7Hz, H6 and CONH), 7.68-7.52 (5H, m, Ph H). M^+ 266. Found C 63.26, H 3.76, N 20.88, $C_{14}H_{10}N_4O_2$ requires C 63.15, H 3.79, N 21.04.

8-Carbamoyl-3-(2-chloroethyl)-1,2,3-benzotriazin-4(3H)-one (223).

The benzotriazinone (**215**) (254mg,1mmol) was reacted according to the general method J. Recrystallisation from aqueous ethanol gave (**223**) as cream needles (151mg,60%).

M.pt. 211-212°C (dec.). I.R. ν cm^{-1} 3338 br (NH), 3164 br (NH), 1696 (C=O), 1684 (C=O), 1673 (C=O), 1654 (C=O). ^1H NMR (DMSO-d_6) δ 8.34 (2H, d.d+br s, J_{meta}=1Hz and J_{ortho}=7.9Hz, H7 and CONH), 8.30 (1H, d.d, J_{meta}=1Hz and J_{ortho}=7.9Hz, H5), 8.00 (2H, t+br s, J_{ortho}=7.9Hz, H6 and CONH), 4.76 (2H, t, J=6Hz, CH_2Cl), 4.12 (2H, t, J=6Hz, NCH_2). M^+ 252/254 (3:1). Found C 47.72, H 3.54, N 22.03, $C_{10}H_9N_4O_2Cl$ requires C 47.54, H 3.59, N 22.17.

8-Carbamoyl-3-(carbamoylmethyl)-1,2,3-benzotriazin-4(3H)-one (224).

The benzotriazinone (**216**) (263mg,1mmol) was reacted according to the general method J. Recrystallisation from aqueous ethanol gave (**224**) as a white powder (140mg,56%).

M.pt. 286°C (dec.). I.R. ν cm^{-1} 3374 br (NH), 3344 br (NH), 3208 br (NH), 1701, 1693, 1685, 1674 (C=O). ^1H NMR (DMSO-d_6) δ 8.41 (1H, br s, CONH), 8.32 (2H, m, H7 and H5), 7.98 (1H, t+br s, J_{ortho}=7Hz, H6 and CONH), 7.81 (1H, br s, CONH), 7.40 (1H, br s, CONH), 5.01 (2H, s, CH_2). M^+ 247. Found C 48.84, H 3.63, N 27.87, $C_{10}H_9N_5O_3$ requires C 48.59, H 3.67, N 28.33.

7-Carbamoyl-3-methyl-1,2,3-benzotriazin-4(3H)-one (225).

The benzotriazinone (**217**) (500mg,2.4mmol) was reacted according to the general method J. Recrystallisation from aqueous ethanol gave (**225**) as colourless needles (348mg,71%) identical to the sample prepared earlier *via* the triazene (**178**).

7-Carbamoyl-3-ethyl-1,2,3-benzotriazin-4(3H)-one (226).

The benzotriazinone (**218**) (150mg,0.7mmol) was reacted according to the general method J. Recrystallisation from aqueous ethanol gave (**226**) as colourless plates (101mg,66%).

M.pt. 260-262°C (dec.). I.R. ν cm^{-1} 3375 br (NH), 1698 (C=O), 1658 (C=O). ^1H NMR (DMSO-d_6) δ 8.65 (1H, s, H8), 8.52 (1H, br s, CONH), 8.37 (2H, s, H5 and H6), 8.00 (1H, br s, CONH), 4.42 (2H, q, J=7Hz, CH_2), 1.40 (3H, t, J=7Hz, CH_3). M^+ 218. Found C 55.00, H 4.52, N 25.54 $C_{10}H_{10}N_4O_2$ requires C 55.04, H 4.62, N 25.68.

7-Carbamoyl-3-phenyl-1,2,3-benzotriazin-4(3H)-one (227).

The benzotriazinone (**219**) (500mg,1.9mmol) was reacted according to the general method J. Recrystallisation from aqueous ethanol gave (**227**) as a white powder (270mg,54%).

M.pt. 266-267°C (dec.). I.R. ν cm^{-1} 3407 br (NH), 1701 (C=O), 1665 (C=O). ^1H NMR (DMSO-d_6) δ 8.76 (1H, s, H8), 8.50 (1H, br s, CONH), 8.39 (2H, s, H5 and H6), 7.91 (1H, br s, CONH), 7.65-7.40 (5H, m, Ph H). M^+ 266. Found C 63.10, H 3.90, N 20.71, $C_{14}H_{10}N_4O_2$ requires C 63.15, H 3.79, N 21.04.

General procedure K: 2-nitrobenzene-1,3-dicarboxamide (232).

A mixture of 2-nitrobenzene-1,3-dicarboxylic acid (**187**) (1.68g,8mmol), thionyl chloride (10ml) and DMF (5 drops) was heated under reflux for 12 hours. The thionyl chloride was evaporated under reduced pressure, the residue treated with toluene (10ml) and re-evaporated. The resulting solid was added portionwise to 28% aqueous ammonia and stirred at room temperature for 24 hours. The reaction mixture was filtered and the residual dicarboxamide washed with water. Recrystallisation from water gave (**232**) as colourless needles (540mg,32%).

M.pt. 288-290°C (dec.) (lit. 278-280°C (dec.)).[144] I.R. ν cm^{-1} 3400 (NH), 3320 br (NH), 3250 br (NH), 1664 (C=O), 1533 (NO_2 asym str.), 1354 (NO_2 sym str.). ^1H NMR (DMSO-d_6) δ 8.27 (2H, br s, 2xCONH), 7.76 (2H, br s, 2xCONH), 7.70 (3H, m, H4, H5 and H6). M^+ 209.

2-Nitrobenzene-1,4-dicarboxamide (233).

2-nitrobenzene-1,4-dicarboxylic acid (**199**) (2.11g,10mmol) was reacted according to the general method K. Recrystallisation from water gave (**233**) as yellow needles (1.28g,61%).

M.pt. 262-264°C. I.R. ν cm^{-1} 3420 (NH), 3364 br (NH), 3196 (NH), 1664 (C=O), 1533 (NO_2 asym str.), 1348 (NO_2 sym str.). ^1H NMR (DMSO-d_6) δ 8.45 (1H, s, H3), 8.34 (1H, br s, CONH), 8.24 (2H, m, H5 and CONH), 7.80 (1H, br s, CONH), 7.78 (1H, br s, CONH), 7.72 (1H, d, J_{ortho}=7.9Hz, H6). M^+ 209. Found C 45.78, H 3.36, N 20.11, $C_8H_7N_3O_4$ requires C 45.94, H 3.37, N 20.09.

General procedure M : 2-aminobenzene-1,3-dicarboxamide (234).

2-Nitrobenzene-1,3-dicarboxamide (**232**) (500mg,2.4mmol) was dissolved in DMF (20ml), treated with palladium-on-charcoal 10% (50mg) and hydrogenated until there was no further uptake of hydrogen. The suspension was filtered through a column of neutral alumina to give a colourless solution. The solvent was removed under reduced pressure and the residue recrystallised from water to give (**234**) as colourless crystals (283mg,66%).

M.pt. 290-292°C. I.R. ν cm^{-1} 3470 (NH_2), 3389 br (NH_2 and NH), 3176 br (NH), 1660 (C=O), 1641 (C=O). ^1H NMR (DMSO-d_6) δ 7.98 (2H, br s, NH_2), 7.85 (2H, br s, 2xCONH), 7.66 (2H, d, J_{ortho}=7.7Hz, H4 and H6), 7.24 (2H, br s, 2xCONH), 6.50 (1H, t, J_{ortho}=7.7Hz, H5). M^+ 179. Found C 53.71, H 5.10, N 23.45, $C_8H_9N_3O_2$ requires C 53.63, H 5.06, N 23.45.

2-Aminobenzene-1,4-dicarboxamide (235).

2-Nitrobenzene-1,4-dicarboxamide (**233**) (500mg,2.4mmol) was reacted according to the general method M. Recrystallisation from water gave (**235**) as yellow plates (265mg,62%).

M.pt. 275-277°C. I.R. ν cm^{-1} 3489 (NH_2), 3375 br (NH_2 and NH), 3188 br (NH), 1655 (C=O), 1649 (C=O). ^1H NMR (DMSO-d_6) δ 7.86 (2H, br s, 2xCONH), 7.56 (1H, d, J_{ortho}=8.2Hz, H6), 7.31 (1H, br s, CONH), 7.20 (1H, br s, CONH), 7.16 (1H, d,

J_{meta}=1.5Hz, H3), 6.92 (1H, d.d, J_{meta}=1.5Hz and J_{ortho}=8.1Hz, H5), 6.66 (2H, br s, NH_2). M^+ 179. Found C 53.69, H 5.05, N 23.46, $C_8H_9N_3O_2$ requires C 53.63, H 5.06, N 23.45.

General procedure N: 8-carbamoyl-3H-1,2,3-benzotriazin-4-one (236).

The dicarboxamide (**234**) (250mg,1.4mmol) was dissolved in a minimum volume of DMF and added to a freshly prepared solution of sodium nitrite (120mg,1.7mmol) in 2M HCl (3ml), and iced water (10ml) at 0°C. The reaction mixture was stirred at 0°C for 2 hours, basified with aqueous ammonia and then re-acidfied (pH 2) with 1M $KHSO_4$. The solid was filtered and washed with water. Recrystallisation from aqueous ethanol gave (**236**) as a white powder (200mg,75%).

M.pt. 254-256°C (dec.). I.R. ν cm^{-1} 3388 (NH), 3108 br (NH), 1685 (C=O), 1657 (NH-C=O). ^{1}H NMR (DMSO-d_6) δ 15.19 (1H, br s, NH), 8.48 (1H, br s, CONH), 8.32 (2H, m, H5 and H7), 7.95 (2H, t+br s, J_{ortho}=7.9Hz, H6 and CONH). M^+ 190. Found C 50.74, H 3.15, N 29.19 $C_8H_6N_4O_2$ requires C 50.53, H 3.18, N 29.46.

7-Carbamoyl-3H-1,2,3-benzotriazin-4-one (237).

The dicarboxamide (**235**) (250mg,1.4mmol) was cyclised according to the general method N. Recrystallisation from aqueous ethanol gave (**237**) as colourless needles (186mg,70%).

M.pt. 273-275°C (dec.). I.R. ν cm^{-1} 3369 br (NH), 3189 br (NH), 1692 (C=O), 1673 (NH-C=O). ^{1}H NMR (DMSO-d_6) δ 15.38 (1H, br s, NH), 8.63 (1H, s, H8), 8.44 (1H, br s, CONH), 8.29 (2H, s, H5 and H6), 7.83 (1H, br s, CONH). M^+ 190. Found C 50.69, H 3.18, N 28.92 $C_8H_6N_4O_2$ requires C 50.53, H 3.18, N 29.46.

Esterification of 4-nitropyrazole-3,5-dicarboxylic acid monopotassium salt (241).

Method A: a suspension of the carboxylic acid (**241**)[125] (500mg,2.1mmol) in methanol (20ml) containing thionyl chloride (2ml) was heated under reflux for 72 hours. The solvent was evaporated under reduced pressure to give a cream solid which was taken up into ethyl

acetate, filtered, applied to a small column of silica and eluted with ethyl acetate to give a white solid (1 spot Rf=0.45, TLC silica gel with ethyl acetate) which upon analysis was shown to be a mixture of the diesters (**243**) and (**246**).

Method B: a suspension of the carboxylic acid (**241**) (1.0g,4.2mmol) in thionyl chloride (5ml) was heated under reflux for 12 hours. The excess thionyl chloride was removed under reduced pressure to give a white solid which was treated with methanol (20ml) and the mixture heated under reflux for 24 hours. The solvent was removed under reduced pressure to give a white solid. TLC examination using ethyl acetate:petroleum ether (60-80°C) 1:1 on silica gel showed 2 spots Rf=0.45 (minor) and Rf=0.2 (major). Separation of the components using flash chromatography with the above solvents as eluent gave:

(a) a white solid (Rf=0.45) identified as dimethyl 1-methyl-4-nitropyrazole-3,5-dicarboxylate (**246**) (10mg,1%).

M.pt. 134-136°C. I.R. v cm^{-1} 1727 (C=O), 1719 (C=O). ^1H NMR ($CDCl_3$) δ 4.23 (3H, s, N-CH_3), 3.98 (3H, s, $COOCH_3$), 3.96 (3H, s, $COOCH_3$). M^+ 243.

(b) a white solid (Rf=0.20) identified as dimethyl 4-chloropyrazole-3,5-dicarboxylate (**245**) (750mg, 82%).

M.pt. 160-162°C. I.R. v cm^{-1} 3283 br (Pyrazole NH), 1741 (C=O), 1697 (C=O). ^1H NMR ($CDCl_3$) δ 12.00 (1H, br s, NH), 3.97 (6H, s, 2x$COOCH_3$). M^+ 218/220 (3:1). Found C 38.27, H 3.22, N 12.97, $C_7H_7N_2O_4Cl$ requires C 38.46, H 3.23, N 12.82.

Method C: A suspension of the carboxylic acid (**241**) (1.0g,4.2mmol) in methanol saturated with HCl gas (30ml) was heated under reflux for 4 hours. The solvent was removed under reduced pressure and the residue purified by flash chromatography using ethyl acetate as the eluent to give (**243**) as a white solid (420mg,47%).

M.pt. 108-109°C. I.R. v cm^{-1} 3231 br (NH), 1715 (C=O). ^1H NMR ($CDCl_3$) δ 11.85 (1H, br s, NH), 3.98 (6H, s, 2x$COOCH_3$). M^+ 229. Found C 36.57, H 3.09, N 18.43, $C_7H_7N_3O_6$ requires C 36.69, H 3.08, N 18.34.

Dimethyl 4-aminopyrazole-3,5-dicarboxylate (244).

A solution of dimethyl 4-nitropyrazole-3,5-dicarboxylate (**243**) (300mg,1.3mmol) in methanol (20ml) was treated with palladium-on-charcoal 10% (30mg) and hydrogenated until there was no further uptake of hydrogen. The mixture was filtered through Celite and the filtrate evaporated to dryness under reduced pressure to give (**244**) as a greyish solid. Recrystallisation from ethyl acetate gave (**244**) as a off-white needles (230mg,88%).

M.pt. 181-183°C (lit. 185°C)[126]. I.R. ν cm^{-1} 3515, 3394 (NH_2), 3246 br (NH), 1701 (C=O), 1679 (C=O). ^1H NMR ($CDCl_3$) δ 10.63 (1H, br s, NH), 6,54 (2H, br s, NH_2), 3.97 (6H, s, 2x$COOCH_3$). M^+ 199.

Attempted preparation of dimethyl 4-(3-phenyltriazen-1-yl)pyrazole-3,5-dicarboxylate (251).

Method A: a solution of the aminopyrazole (**244**) (200mg,1mmol) in 2M HCl (2ml) and water (13ml) was stirred at 0°C and treated dropwise with a solution of sodium nitrite (86mg,1.2mmol) in water (1ml). The reaction mixture was stirred for 30 minutes at 0°C and then neutralised with 1M sodium acetate. Ice was added to the reaction mixture followed by aniline (93mg,1mmol) and the mixture stirred vigourously for 1 hour. No precipitate was formed and the reaction mixture was extracted with chloroform. The chloroform extracts were combined and dried over Na_2SO_4. The solvent was removed under reduced pressure to give a yellow oil which was taken up into ethanol and triturated with hexane to give a colourless solid (80mg,38%) which was filtered and allowed to air dry. Analysis indicated that the compound isolated was the diazo species (**250**).

M.pt. 129-130°C (dec.). I.R. ν cm^{-1} 2196 (N_2), 1749, 1713 (C=O). ^1H NMR ($CDCl_3$) δ 4.05 (6H, s, 2x$COOCH_3$).

Method B: a solution of the diazopyrazole (**250**) (50mg,0.25mmol) (formed in method A) in ethanol (10ml) was treated with aniline (111mg,1.2mmol) and stirred at room temperature for 30 days to give a yellow solution. The solvent was evaporated under reduced pressure and the residue triturated with hexane to give an orange solid whose I.R. spectra was identical to that of the diazo species (**250**) described above.

Diazotisation of Dimethyl 4-aminopyrazole-3,5-dicarboxylate and coupling with methylamine.

A solution of the aminopyrazole (**244**) (200mg,1mmol) in 2M HCl (2ml) and water (13ml) at 0°C was treated dropwise with a solution of sodium nitrite (86mg,1.2mmol) in water (1ml) and stirred at 0°C for 30 minutes. The solution was extracted with ethyl acetate (4x50ml) and the combined extracts were briefly dried over Na_2SO_4. Removal of the solvent gave a white solid which was added to a saturated solution of methylamine in ethyl acetate (10ml) at room temperature. The reaction mixture was stirred for 24 hours at room temperature during which time a white solid was slowly deposited. The solid was removed by filtration washed with ethyl acetate and allowed to air dry. TLC examination (silica gel and ethyl acetate) revealed the presence of two compounds Rf=0.24 and Rf=0.14. Separation of the components by flash chromatography with ethyl acetate as eluent gave a white solid (Rf=0.14), which on recrystallisation from methanol gave colourless needles of (**252**) (10mg,5%).

M.pt. 179-181°C. I.R. ν cm^{-1} 3430 br (NH), 3127 br (NH), 1703 (C=O), 1672 (NH-C=O). ^1H NMR (DMSO-d_6) δ 12.05 (1H, br s, NH), 8.06 (1H, br d, CONH), 3.83 (3H, s, N-CH_3), 2.84 (3H, d, J=4.8Hz, NH-CH_3). M^+ 208. Found C 40.12, H 3.86, N 40.54, $C_7H_8N_6O_2$ requires C 40.39, H 3.87, N 40.37.

7.3 Hydrolysis of mitozolomide in the presence of nucleic acids.

The synthetic nucleic acid oligomers used in this study were purchased from Sigma Chemical Co., Poole, England and the HPLC grade solvents were purchased from Fisons, Loughborough, England. Phosphate and sodium cacodylate buffers at pH 7.4 and 6.5 were prepared as stated in *Data for Biochemical Research.*[145]

High performance liquid chromatography was performed using a Waters 600E gradient solvent delivery system fitted with a Merck 250 Lichrosorb RP select B column, Lichrocart reversed-phase C-18 endcapped guard column and monitored by UV at λ_{max} 325nm. Hydrolyses were performed in glass screw-capped vials in a total volume of 3ml, the various compositions of which can be found in Tables 4.1 and 4.3, and monitored

for approximately 2-3 half-lives. At the appropriate time aliquots were removed from the incubation mixture and injected (10µl) directly onto the column using a Waters WISP 710B Automatic Injection Sampler and eluted with a mobile phase of acetic acid (0.5%) in water/methanol 70:30 v/v at a flow rate of 1.0ml/min for 10mins, mitozolomide typically had a retention time of 4.8 minutes. Chromatograms were recorded using the Base software package.

7.4 Biological testing methods.

7.4.1 *In vitro* cytotoxicity assays against TLX5 lymphoma.

TLX5 lymphoma cells were maintained in exponential phase at $2x10^4$ cells/ml in RPMI 1640 media supplemented with 15% foetal calf serum. 2ml aliquots of the TLX5 cells were plated out into 6 wells of multi-well dishes and treated with 10µl of the required drug solutions (in DMSO), with three replicates for each drug concentration. The control incubates were composed of cells treated with 10µl of DMSO. After incubation for 72 hours at 37°C in a humid atmosphere of 5% CO_2 the cells were counted using a Coulter Laboratories ZM electronic coulter counter (settings: current 200, lower threshold 12 and attenuation 8). The cytotoxicity was calculated as % inhibition of TLX5 cell growth by expressing the number of cells remaining in the test wells as a percentage of the number of control cells remaining after 72 hours incubation.

Many thanks to Miss H. Hussey, CRC Laboratories, Aston University, for performing these tests.

7.4.2 *In vitro* cytotoxicity assays against Raji and GM892A cell lines.

Raji and GM892A cells growing in exponential phase were seed at $8x10^4$ cells/ml in Nunc 24 well plates and treated with a range of drug concentrations (in DMSO such that the concentration of DMSO did not exceed 0.5%). Control incubates were composed of cells treated with DMSO. After incubation for 72 hours at 37°C under an atmosphere of 5% CO_2 the cells were counted using a Coulter Laboratories ZM coulter counter, as above. The cytotoxicity was calculated as % inhibition of TLX5 cell growth by expressing the number of cells remaining in the test wells as a percentage of the number of

control cells remaining after 72 hours incubation.

Many thanks to Mr. B Deans, Aston University, for performing these tests.

7.4.3 Triazinone and temozolomide competitive binding studies.

GM892A cells were seeded at $8x10^4$ cells/ml in T25 flasks and treated with a range of concentrations of a triazinone (in DMSO). After incubation for 2 hours at 37°C the cells were centrifuged, washed with phosphate buffered saline, resuspended in media and then aliquoted into Nunc 24 well plates. Temozolomide (in DMSO) was added, the cells incubated for 3-4 days at 37°C under an atmosphere of 5% CO_2 and the cells counted as above. The ability of temozolomide to inhibit the growth of cells treated with the triazinones was compared with inhibition in solvent treated controls.

Many thanks to Mr. B Deans, Aston University, for performing these tests.

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