NON-CLASSICAL INHIBITORS OF DIHYDROFOLATE REDUCTASE

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by

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Summary

This thesis comprises two main objectives. The first objective involved the stereochemical studies of chiral 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines and an investigation on how the different conformations of these stereoisomers may affect their binding affinity to the enzyme dihydrofolate reductase (DHFR). The *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines were synthesised by the three component method. An *ortho*-substitution at the C6' position was observed when *meta*-azidocycloguanil was decomposed in acid. The *ortho*-substituent restricts free rotation and this gives rise to atropisomerism. *Ortho*-substituted 4,6-diamino-1-aryl-2-ethyl-1,2-dihydro-2-methyl-s-triazine contains two elements of chirality and therefore exist as four stereoisomers: (S,aR), (R,aS), (R,aR) and (S,aS). The energy barriers to rotation of these compounds were calculated by a semi-empirical molecular orbital program called MOPAC and they were found to be in excess of 23 kcal/mol. The diastereoisomers were resolved and enriched by C18 reversed phase h.p.l.c. Nuclear overhauser effect experiments revealed that (S,aR) and (R,aS) were the more stable pair of stereoisomers and therefore existed as the major component. The minor diastereoisomers showed greater binding affinity for the rat liver DHFR in *in vitro* assay.

The second objective entailed the investigation into the possibility of retaining DHFR inhibitory activity by replacing the classical diamino heterocyclic moiety with an amidinyl group. 4-Benzylamino-3-nitro-N,N-dimethyl-phenylamidine was synthesised in two steps. One of the two phenylamidines indicated weak inhibition against the rat liver DHFR. This weak activity may be due to the failure of the inhibitor molecule to form strong hydrogen bonds with residue Glu-30 at the active site of the enzyme.

Keywords: 4,6-Diamino-1-aryl-1,2-dihydro-*s*-triazines, Atropisomerism, 4-Benzylamino-3-nitro-N,N-dimethyl-phenylamidine, Chromatography, Dihydrofolate Reductase.

To my beloved parents

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Contents

List of figures, photographs, schemes and tables.

Abbreviations

ACHT	Alpha chymotrypsin
AGP	$Alpha_1$ glycoprotein
Asp	Aspartic acid
a.u.f.s.	Sensitivity (of u.v. detector)
br.	Broad
BSA	Bovine serum albumin
CSP	Chiral stationary phase
D	Right-handed
d.e.	Diastereoisomeric excess
DHFR	Dihydrofolate reductase
eg.	For example
equiv.	Equivalent
Glu	Glutamic acid
h	Hour(s)
HCl	Hydrochloric acid
h.p.l.c.	High performance liquid chromatography
Hz	Hertz
Ile	Isoleucine
ie.	That is
i.r.	Infra red (spectroscopy)
k	Capacity factor
L	Left-handed
Leu	Leucine
MCT	Microcrystalline cellulose triacetate
min	Minute(s)
m.p.	Melting point
MTX	Methotrexate
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate

NaOH	Sodium hydroxide
n.m.r.	Nuclear magnetic resonance (spectroscopy)
Phe	Phenylalanine
ppm	Parts per million (n.m.r. chemical shift unit)
Pro	Proline
R	Clockwise direction (rectus)
RP	Reversed phase
S	Anticlockwise direction (sinister)
sec	Second
Ser	Serine
t_R	Retention time
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetic anhydride
TFSA	Trifluoromethanesulphonic acid
Thr	Threonine
t.l.c.	Thin layer liquid chromatography
Tyr	Tyrosine
u.v.	Ultraviolet (spectroscopy)
v/v	Volume/volume
water	Double distilled water
(+)	Dextrorotatory
(-)	Levorotatory
λ	Wavelength
°C	Degree Celsius
°K	Degree Kelvin
α	Selectivity
Å	Angstron (unit)
ΔH	Heat of formation

Section 1: Introduction

Chapter 1

Drug stereochemistry

1.1 Early history of stereochemistry[1]

In the early nineteenth century, linear polarisation of light and the rotation of plane polarised light were the subjects of intensive study by French scientists. In 1815, at the College de France, a physicist by the name of Jean-Baptiste Biot, discovered the ability of a substance to rotate plane polarised light. Thus the concept of optical activity was born.

By 1848, at the Ecole Normale in Paris, Louis Pasteur observed optical activity in organic solutions. From his observations, Pasteur proposed that asymmetrical molecules which produce non-superimposable mirror image structures were optically active; and in the same year, Pasteur laboriously separated a quantity of the sodium ammonium salt of tartaric acid into two piles using a hand lens and a pair of tweezers. Of the two piles of crystals, one contained 'left-handed' crystals while the other contained 'right-handed' crystals. This was the first ever optical resolution of a racemic mixture.

Later in 1858, Kelule proposed the theory of the tetravalent carbon. By 1874, the theory of three-dimensional

organic structures was proposed independently by Jacob Henricus van't Hoff in Holland and Joseph Achille Le Bel in France. van't Hoff and Le Bel went on to propose that the four valences of carbon atom were not planar but were directed into a tetrahedral three-dimensional space. van't Hoff defined the asymmetric carbon as a carbon bearing four different groups (*asymmetrisch koolstof-atoom*). This asymmetric carbon would therefore be capable of existing in two distinctly different non-superimposable forms. He proposed that this asymmetric carbon was the cause of molecular asymmetry and optical activity in organic compounds.

In 1949, exactly hundred years after the first separation of (d,l)-tartaric acid by Pasteur, a Dutchman called Bijvoet determined the actual spatial arrangement of sodium rubidium salt of (+)-tartaric acid using X-ray diffraction. This was the first determination of the absolute configuration about asymmetric carbon.

Today, the concept of stereochemistry remains an important facet of organic chemistry. In particular, the stereochemistry of pharmaceuticals cannot be overlooked as this has serious implications in terms of drug efficacy and toxicity. In the chapters which follow, some basic stereochemistry of organic molecules will be reviewed; the impact of stereoisomerism on drug action will be discussed;

the various routes to optically pure stereoisomers will be explored and the use of stereoisomers as inhibitors of a target enzyme for chemotherapy will be evaluated.

1.2 Stereoisomerism in organic molecules

A molecule is chiral when its mirror-image is non-superimposable on itself. The non-superimposable mirror-images are termed enantiomers; alternatively they may also be called enantiomorphs, optical isomers or optical antipodes. Enantiomers of a compound share similar chemical and physical properties. However they can be distinguished from each other by their effects on plane polarised light. The enantiomer which rotates the light to the right is the (+)-isomer while the enantiomer which rotates the light to the left is the (-)-isomer. Enantiomers can also be distinguished by their different reactivity rates with chiral reagents. An equal mixture of the two enantiomers is optically inactive and is called a racemic mixture or a racemate. Stereoisomers which are not mirror-images are termed diastereoisomers. Diastereoisomers, unlike enantiomers, possess different chemical and physical properties.

Chiral compounds may arise from several different conditions. Compounds bearing one asymmetric carbon must be

optically active. Apart from carbon, a chiral molecule may possess other quadrivalent asymmetric atoms such as Silicon, Germanium, Tin or Nitrogen (in quarternary salts or N-oxides). Adamantanes bearing four different substituents at the bridgehead positions are also chiral.[2]

Molecules in which two ring systems are joined by a single bond may exhibit chirality when there is hindered rotation about the single bond due to steric effects. This will give rise to chiral atropisomerism. Helically shaped molecules are chiral as the molecules tend to be either left- or right-handed in orientation.

In all the examples mentioned above, the molecules lack a plane or a point of symmetry. Therefore, molecular dissymmetry is an important criterion for chirality in organic compounds.

1.3 Chiral drugs and their use in chemotherapy

Drugs which are derived from natural products are usually obtained in the optically active or pure form of a single stereoisomer. For instance, (-)-morphine is derived from the juice of the poppy plant, *Papaver somniferum* and (+)-digitoxin is extracted from the dried leaves of the foxglove plant, *Digitalis purpurea*. These naturally

occurring products are optically active because they are synthesised by enzymes which are themselves chiral in structure. However, drugs which are produced by organic synthesis are usually mixtures of equal parts of two or more stereoisomers depending on the number of asymmetric centres present.

Ariens[3,4,5] carried out a survey which revealed that most synthetic chiral drugs are sold as racemates rather than single enantiomers. Of the 1850 drugs marketed in the world today, 1327 of them are totally synthetic while 523 are natural or semi-synthetic. Out of these 1327 totally synthetic drugs, 528 are chiral and only 61 of these are marketed as single enantiomers while the other 467 are sold as racemates.

This survey clearly indicates the prevalence of chiral drugs in use for chemotherapy. Drug action has been shown to be stereoselective and frequently only one of a pair of enantiomeric drugs is active while the other may be inactive or contribute to adverse effects. In the following chapter, the impact of stereoisomerism on drug efficacy, metabolism and pharmacokinetics will be briefly dealt with. The legal and ethical aspects of using chiral drugs will also be discussed.

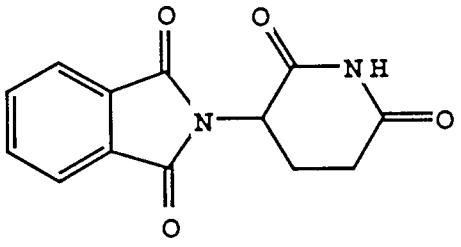
Chapter 2

The impact of stereoisomerism on drug action

2.1 Introduction

For drug molecules to exert a pharmacological response in man, they must interact with an enzyme, a receptor or the cell surfaces. All these constituents of the physiological environment are chiral in nature. Therefore, when enantiomeric drug molecules interact with these chiral biomolecules, they may exhibit differential behaviour.

The tragic incidence which connects the use of thalidomide racemate to the cause of prenatal deformities triggered the awareness of the importance of stereochemistry of drug molecules. Thalidomide **(1)** was marketed as a sedative in the early 1960s and it was commonly prescribed to pregnant women. Later it was discovered that the high incidence of phocomelia in neonates was linked to the use of thalidomide, especially in the first trimester of pregnancy. Phocomelia is a pathological condition of teratogenic malformation where the babies are born with arms and feet attached directly to their trunks, giving a seal-like appearance.[6] Further research showed that only the (R)-isomer of thalidomide has the favourable sedative effect while the (S)-isomer is teratogenic.[7,8]



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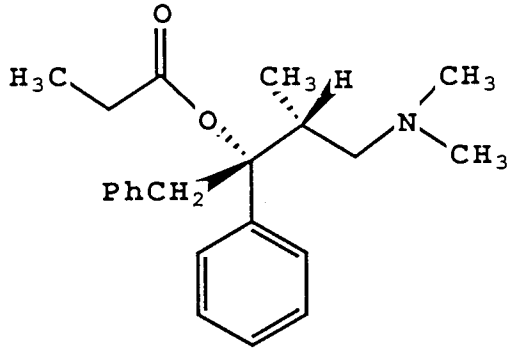
Enantiomeric drugs may exert their pharmacological activity *via* four different possibilities.[9] Firstly, all the pharmacological activity may reside in only one of the enantiomers while the other is considered 'inactive'. The enantiomers may, on the other hand, exhibit nearly identical qualitative and quantitative pharmacological activity. The isomers may show qualitatively similar pharmacological activity but differ in quantitative potencies. Finally, the isomers may exhibit totally different pharmacological activity. In the following sections, the pharmacodynamic and pharmacokinetic differences between drug stereoisomers will be illustrated with a few well studied examples.

2.2 Efficacy and toxicity of drug stereoisomers

As mentioned above, enantiomeric drugs may exhibit totally different biological activity. For example, α-dextropropoxyphene (Darvon) **(2)** has analgesic properties while α-levopropoxyphene (Novrad) **(3)** is devoid of any analgesic effects but is an antitussive.[10]



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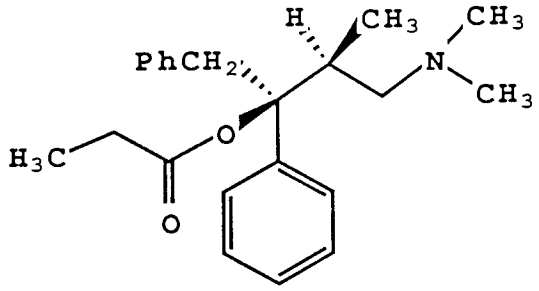

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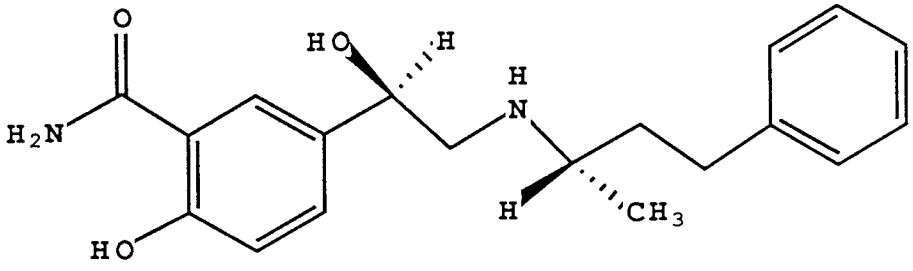
Labetalol possesses both α and β adrenoceptor blocking properties, and is used in the treatment of hypertension. Labetalol is a diastereoisomer that is commercially available as equal proportions of four stereoisomers. Non-specific β_1 and β_2 blocking activity is predominantly conferred by the (R,R)-isomer **(4)**, while α_1 blocking activity is produced by the (S,R)-isomer **(5)**. The other two isomers [S,S **(6)** and R,S **(7)**] probably contribute to the drug activity, but to a much lower extent.[11]



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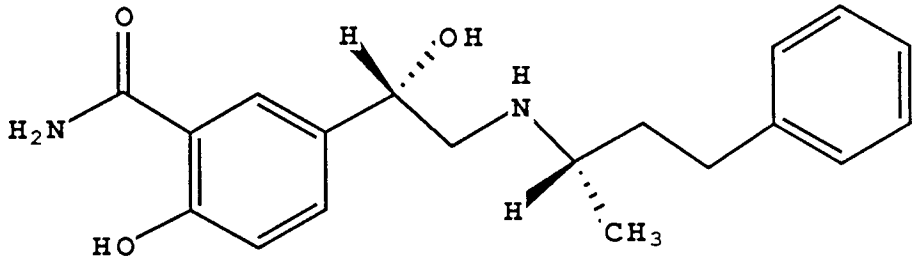

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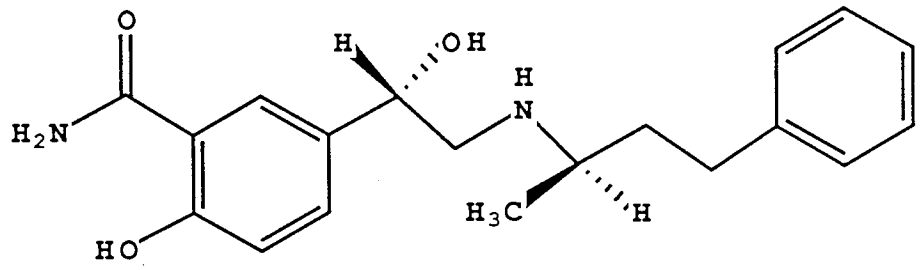

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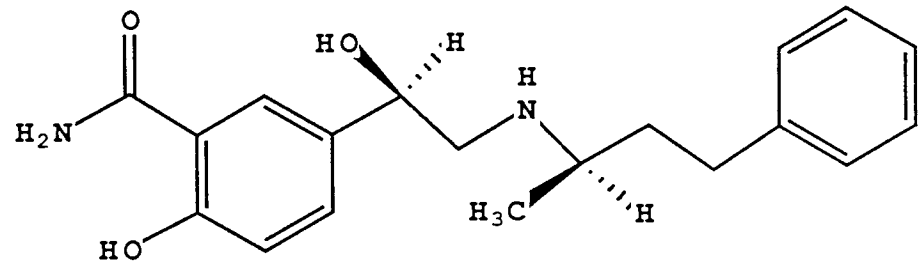

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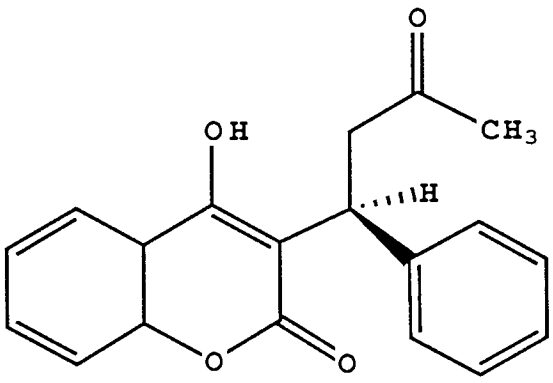
The anticoagulant drug warfarin is administered as a racemate. In humans, the hypoprothrombineamic effect of (S)-(-)-warfarin **(8)** is two to five times more potent than (R)(+)-warfarin **(9)**.[12]



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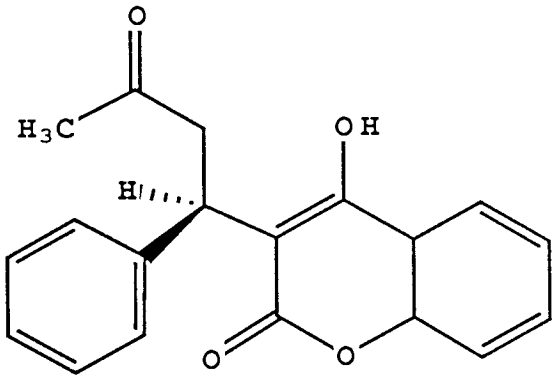

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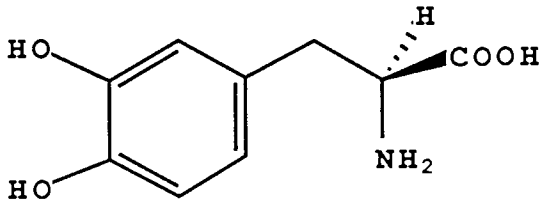
Patients suffering from Parkinsons Disease are prescribed L-dopa **(10)** to supplement their lack of dopamine production. This drug is sold exclusively in the L-enantiomeric form as the D-enantiomer can give rise to the condition granulocytopenia.[10]



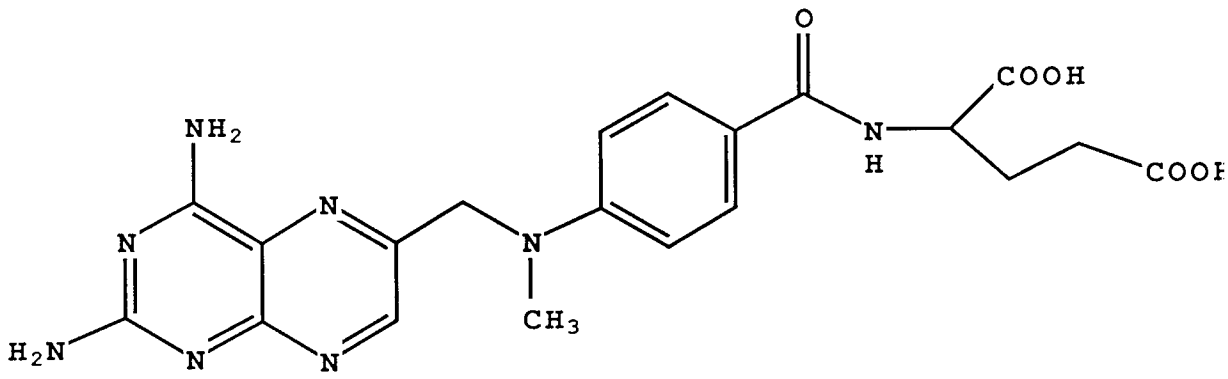
(10)

2.3. Pharmacokinetic differences between drug enantiomers in man

In man, the pharmacokinetics of an enantiomeric drug may be different because of stereoselectivity in processes such

as active transport, protein binding and metabolism. When racemic drugs are administered in equal proportions, the ratios of isomers at locations where they are measured (e.g. plasma, brain, urine or myocardium) are rarely close to unity. This variability of isomeric ratios suggests that each enantiomer behaves as a separate entity in the chiral physiological environment.

The oral bioavailability of D-methotrexate **(11)** is only 2.5% that of the L-isomer. This is due to the fact that the L-isomer is actively absorbed from the small intestine whilst the D-isomer diffuses passively across the intestinal epithelium.[10]



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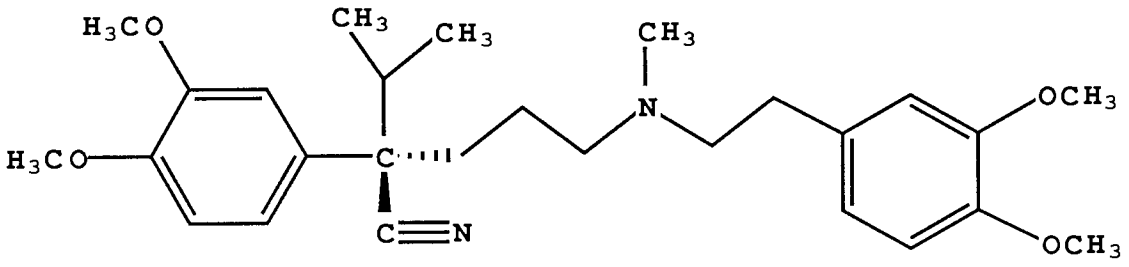
The enantiomeric components of a racemate may also be bound to plasma protein to different extents. For instance, the free fraction of (+)-warfarin is 0.012 while that of the (-)-warfarin is only 0.009.[13]

(+)-Verapamil **(12)** is less potent than (-)-verapamil **(13)** in blocking the slow inward calcium channels because the (+)-isomer is preferentially bound to plasma protein. The free fraction of the (+)-isomer is 0.064 compared to a value of 0.115 for the (-)-isomer.[14] (+)-Verapamil also undergoes pre-systemic metabolism, therefore its bioavailability is significantly less than that of the (-)-isomer.



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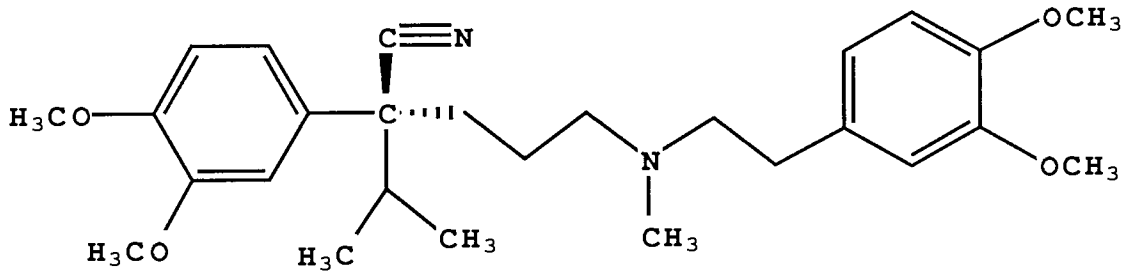

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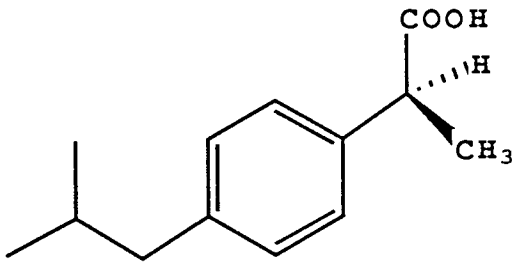
(S)-(+)-ibuprofen **(14)** is 160 times more potent as a prostaglandin synthase inhibitor *in vitro*, yet only 1.4 times as potent as (R)-(-)-ibuprofen **(15)** as an analgesic *in vivo*. Studies have shown that the 2-arylpropionic acids can undergo *in vivo* metabolic chiral inversion. It is found that the

(S)-(+)-isomer is inverted to the (R)-(-)-isomer during metabolism, thus this explains the drop in relative potency of the (S)-(+)-isomer *in vivo*.[10]



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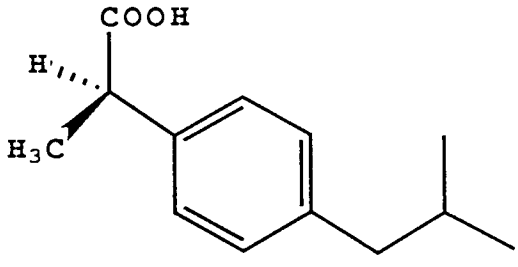

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2.4 Single isomers versus racemate in chemotherapy

Since the pharmacodynamics and pharmacokinetics of enantiomeric drugs can differ dramatically in man, there arises the dilemma of whether only single isomers or racemates should be used in chemotherapy.

The Food and Drug Administration (FDA) is expected, in the near future, to begin regulating the marketing of single enantiomers versus racemates. New FDA regulations will require new chiral drug submissions to accompany data on each enantiomer or diastereoisomer before approval is granted.[15] This will definitely put some pressure on pharmaceutical companies which will have to conduct exploratory studies on racemates before deciding whether to study the enantiomers.

On the other hand, medicinal chemists will have to devise chiral assays for the study of every promising compound.

Pharmacologists feel that if the activities of both the enantiomers were known, it would be sensible to use only the favourable stereoisomer so that undesirable effects could be avoided. Clinicians and pharmacokineticists also share the same views as the pharmacologists in using only single isomers. This is because the use of single isomers can help the clincians to design more effective drug regimens without the interference of the unfavourable isomer.

Therefore, the medicinal chemists are left with the formidable task of synthesising optically pure isomers for use in clinics. With the advent of modern technology and advances in synthetic organic chemistry, highly pure optical isomers can be synthesised in respectably high yields. In the next chapter, various routes to the synthesis of optically pure isomers will be explored and their feasibility to large scale manufacturing will be assessed.

Chapter 3

Routes to optically pure stereoisomers

3.1 Introduction

The medicinal chemist now has available a variety of synthetic tools for preparing enantiomerically pure compounds.[17] The concepts of enantiopreparation fall, generally, into three categories: resolution, asymmetric synthesis and the use of the chiral carbon pool. Each of these approaches will be briefly discussed in the following sections.

3.2 Optical resolution of chiral drugs

Since the day when Louis Pasteur separated the tartaric acid racemate by using a pair of tweezers and a hand lens, the art of optical resolution has advanced tremendously. Crystallisation is perhaps the most classical resolution technique known and is still widely used today. The principle behind this method is to convert the enantiomers into diastereoisomeric salts using optically active acids or bases. The relative solubilities of the pair of diastereoisomeric salts, in a suitable solvent, allow only one of the pair to crystallise.

The advantage of this method is that both the enantiomers may be obtained from the racemate. The optically pure reagents are readily available and they can be recycled for use again after each operation.

One of the disadvantages of the method is that the maximum obtainable yield is 50%. Several recrystallisations may be required before a fairly pure diastereoisomeric salt is obtained. The choice of optically active reagent and crystallisation solvent to be used are usually derived from trial and error. On a large scale basis, this technique is certainly not a cost-effective route as losses can be high.

An alternative resolution technique is to convert the enantiomers into diastereoisomeric derivatives. These derivatives are then separated by using either gas or liquid chromatography. After separation, the enantiomers are obtained by destroying the covalent linkage. With the advent of preparative chromatography, this method has gained much popularity. However, the possibility of racemisation during the recovery phase has often been the limiting factor for choosing this technique.

Direct separation of enantiomers by chiral liquid chromatography has revolutionised the art of optical resolution in the last decade. Today, both analytical and

preparative chiral stationary phase (CSP) columns are commercially available for the separation of a wide spectrum of chiral compounds.

There are many different types of CSP available and they include proteins like AGP[18] and BSA[19]; α- and β-cyclodextrin[20]; microcrystalline cellulose triacetate[21]; helical polymethacrylates, polyacylamides and metal co-ordinating ligands[22]. In 1979, Pirkle proposed the three-point rule[23] as the chiral selective mechanism in h.p.l.c.:

" That for chiral resolution to take place, there must be at least three simultaneous interactions between a CSP and one solute enantiomer, whereby one or more of the interactions involved in the chiral recognition process are π-π interactions, hydrogen bonds, dipole-dipole, hydrophobic and steric interactions."

The convenience of direct resolution by CSP is perhaps the strongest point for its use in chiral drug separation. However, the main disadvantage of this method is usually the high cost of the CSP columns.

3.3 Asymmetric synthesis for drug design

Asymmetric synthesis is defined as the chemical or biochemical conversion of a prochiral substrate to a chiral

product. In general, this involves reaction at an unsaturated site having prochiral faces (eg. C=C, C=N, C=O) to give one enantiomeric product in excess over the other.[24] Such reactions involve the use of homochiral reagents or catalyst which control the introduction of one or more asymmetric centres.[25] Although this technique is still developing, its potential in obtaining highly pure enantiomers has proved to be a favourable method in the synthesis of either intermediates or products. Asymmetric synthesis ,when applicable, is therefore a very valuable tool especially in the manufacture of chiral pharmaceuticals.

3.4 The chiral carbon pool.[24]

The third source of chiral drug synthesis involves using of naturally occurring chiral molecules as starting materials. Compounds which are commonly used include carbohydrates, amino acids, terpenes and smaller, microbiologically-derived compounds such as lactic acid and tartaric acid. The main concern in the use of the chiral carbon pool has to be the enantiomeric homogeneity of the material. Also, the availability of only one member of an enantiomeric pair may limit its use in some instances.

Chapter 4

Dihydrofolate reductase inhibitors as therapeutic agents

4.1 Biochemistry of dihydrofolate reductase inhibitors

Folic acid is a member of the vitamin B complex and is found abundantly in green vegetables, yeast and liver. It is an essential factor for normal haemopoiesis and cell division. Folate derivatives are coenzymes for the transfer, oxidation and reduction of single carbon units used for the biosynthesis of thymidylate, purine nucleotides, methionine, serine and glycine.

Dihydrofolate reductase (DHFR) is an important enzyme in the metabolism of folic acid. DHFR, together with the coenzyme NADPH, catalyses the reduction of 5,6-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid. Tetrahydrofolic acid, in the presence of serine hydroxymethyl transferase, is converted to N^5,N^{10}-methylene tetrahydrofolic acid. This in turn is used in the biosynthesis of thymidylate from deoxyuridine monophosphate by the enzyme thymidylate synthase.

Formyl transferase reactions utilising 10-

formyltetrahydrofolate are involved in the biosynthesis of formyl glycinamide ribonucleotide and formylamino-imidazole carboxamide nucleotide in the *de novo* pathway of purine synthesis.

N^5,N^{10}-Methylene tetrahydrofolic acid not only supplies the one carbon unit to the 5'-position of the uracil ring of dUMP but also serves as a reductant supplying a hydrogen from C6 of the reduced pyrazine ring of tetrahydrofolic acid, yielding the substrate dihydrofolic acid. Thus tetrahydrofolic acid must be regenerated *via* the DHFR-catalysed reaction in order to maintain the intracellular pool of tetrahydrofolic acid one-carbon derivatives for both dTMP and purine nucleotide biosynthesis.

4.2 The use of antifolates in chemotherapy

As the enzyme DHFR is involved intimately in the biosynthesis of thymidylate and purines, it serves as a vulnerable target site for chemotherapeutic agents. Inhibition of DHFR causes the accumulation of the metabolically inactive dihydrofolic acid and the depletion of the pool of tetrahydrofolic acid. This, indirectly, will result in a deficiency of the cellular pools of dTMP and purines and thus in a decrease in nucleic acid synthesis and cell death follows.

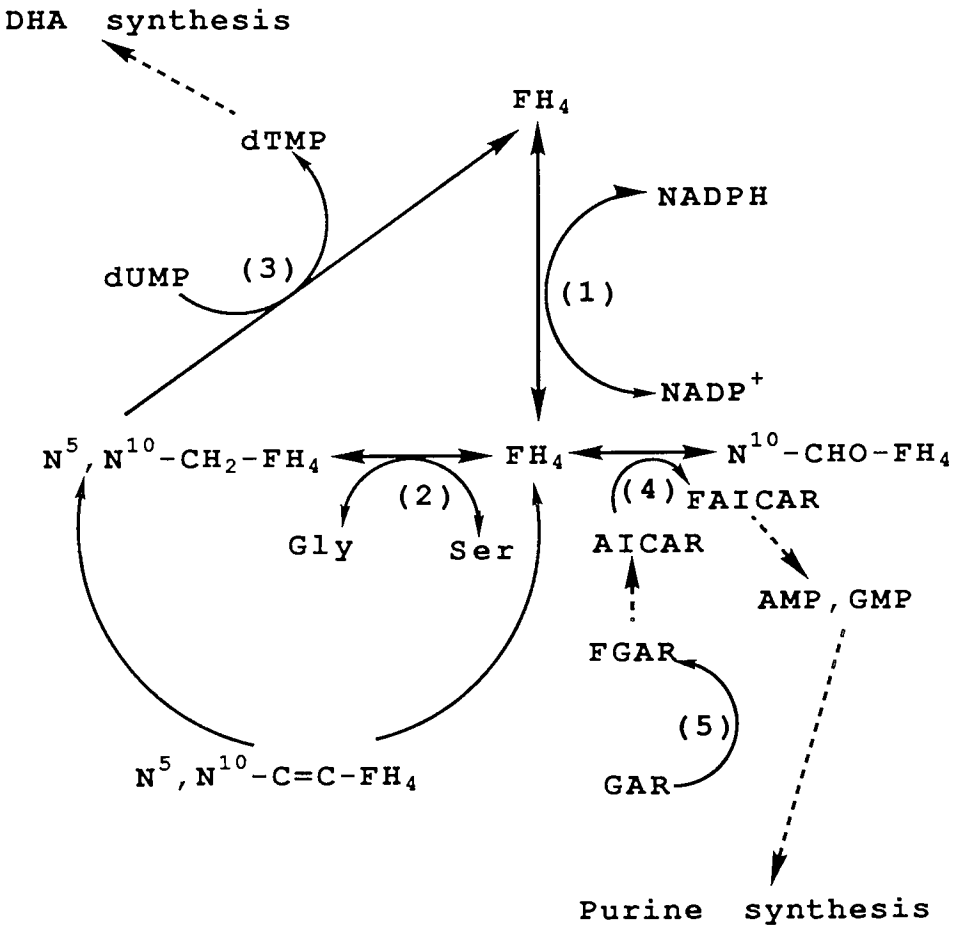
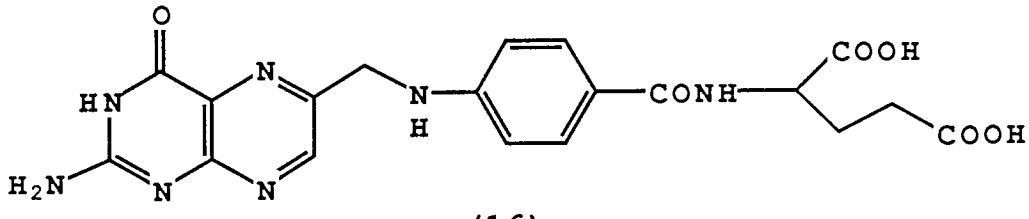


Figure 4.1: *Biochemical pathway involving DHFR.*
Key: FH_2 = Dihydrofolate; FH_4 = Tetrahydrofolate; N^5,N^{10}-CH_2-FH_4 = N^5,N^{10}-Methylenetetrahydrofolate; N^5,N^{10}-C=C-FH_4 = N^5,N^{10}-Methenyltetrahydrofolate; FGAR = Formylglycinamide ribonucleotide; GAR = Glycinamide ribonucleotide; AICAR = Amino-imidazolecarboxamide ribonucleotide; FAICAR = 5-Formamidoimidazole-4-carboxamide ribonucleotide; AMP = Adenosine monophosphate; GMP = Guanine monophosphate; 10-CHO-FH_4 = 10-Formyltetrahydrofolate; (1)DHFR; (2)Serine Hydroxymethyl Transferase; (3)Thymidylate Synthase; (4)Phosphoglutamate Methyltransferase; (5)Phosphoribosyl Glycinamide Formyltransferase.

After elucidating the structure of folic acid **(16)**[27], the Lederle group undertook its synthesis and that of a number of its analogues. Of these compounds synthesised, only aminopterin **(17)** and methotrexate **(11)**[30] showed significant inhibitory activity against the growth of *Streptococcus faecalis*. These compounds are known as 'classical' DHFR inhibitors as they have close resemblance to the enzyme substrate. Aminopterin produced temporary remission in acute leukaemia in children[29] while methotrexate is still one of the most effective antitumour agents after more than forty years of clinical use.

Substantial attention has also been accorded to the development of 'non-classical', simpler and more lipophilic analogues of folic acid for use as antifolates. This has led to the discovery of a variety of heterocycles which possess anti-metabolite properties.



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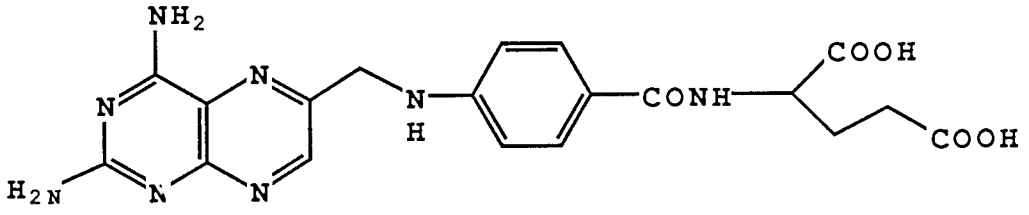


(17)

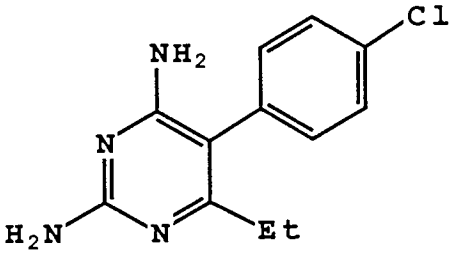
4.2.1 2,4-Diaminopyrimidines

This is by far the largest class of non-classical lipophilic antifolates studied. Hitching's[31] discovery of the important antimalarial pyrimethamine **(18)** provided strong impetus to the search for more potent antifolate drugs. The compound trimethoprim **(19)** also belongs to this class of antifolates and possesses anti-bacterial property. An analogue called metoprine **(20)** or DDMP was found to be active in both *in vitro* and *in vivo* tumour systems and later discovered to be effective against methotrexate-resistant tumour cells.

Meta-azidopyrimethamine (MZP) **(21)** is a non-classical antifolate which was developed by Stevens and co-workers to combine the potential advantages of lipid solubility and rapid metabolic deactivation for possible reduced toxicity.[32,33] Its short half-life relative to metoprine is promoted by the presence of the azido group which has the potential to be biologically transformed to the more polar and less active amine. Bliss has reported a high yielding three step synthesis of MZPES from pyrimethamine. This involved the nitration of pyrimethamine followed by the reduction to the amino analogue. The amine was diazotised and treated with sodium azide to produce MZP which was converted to the salt, MZPES, by using ethanesulphonic acid.

MZPES, in the form of the free base MZP, has shown *in vivo* activity against a range of murine tumour lines as well as cell line L5187Y which shows resistance to methotrexate due to impaired transport-mediated uptake.

In the phase I clinical trial, the dose of MZPES was increased from 5.4 mg/m^2 to 460 mg/m^2 given as a 1h infusion. The dose-limiting toxicity in the study comprised marked nausea, vomiting and subjective neurological disturbances. In view of the lack of antifolate and antitumour activity at toxic levels, no phase II clinical trial was proposed.



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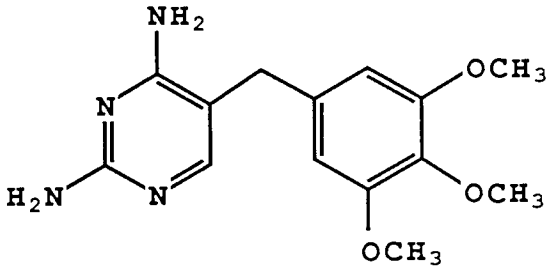

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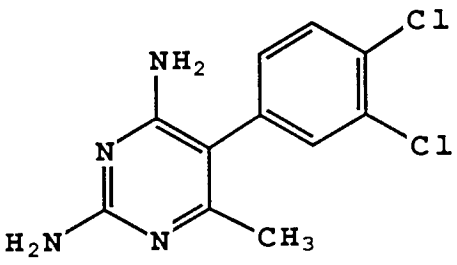

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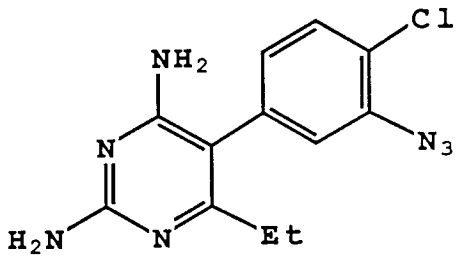
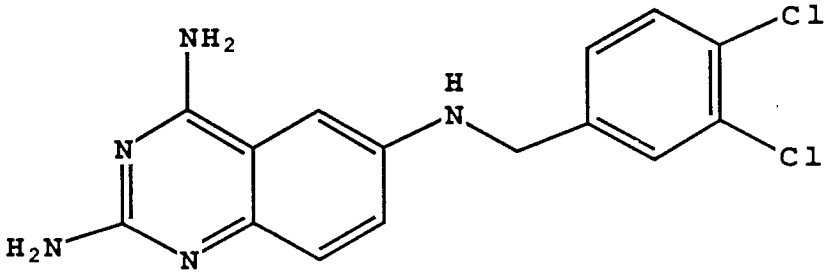


(21)

4.2.2 2,4-Diaminoquinazolines.

The discovery of significant anti-leukaemic activity in the 2,4-diaminoquinazolines prompted a study of their inhibitory action on DHFR. One of the early non-classical prototypes **(22)** displayed strong antimalarial activity in experimental infections, and work progressed from this point through a host of structural modulations, culminating with the sulphone analogue **(23)**.



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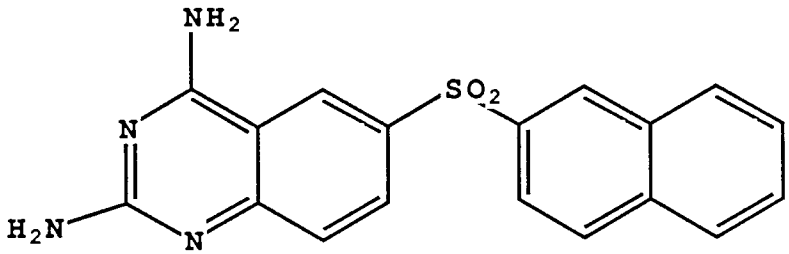
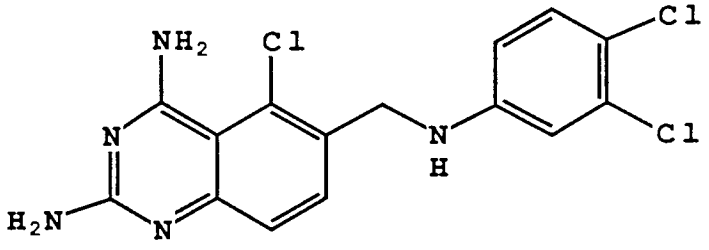


(23)

A variety of these structures was screened for antitumour activity. It was found that analogue **(24)** proved to be a potent inhibitor of DHFR from L1210 murine cell line (IC_{50} = 5.8×10^{-9} M).[34]

Another analogue called trimetrexate **(25)** stirred up a lot of excitement when it was shown to exhibit a broad spectrum of anti-tumour effects. Trimetrexate was active against B16 melanoma, colon carcinoma 26, colon carcinoma 38 and the L1210 tumour. Unlike methotrexate, it was found to be active against a broad spcetrum of murine solid tumours.[35,36]



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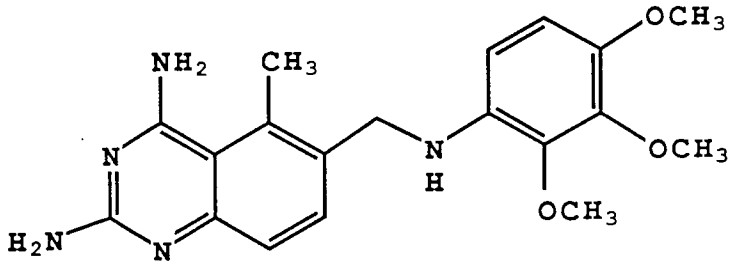


(25)

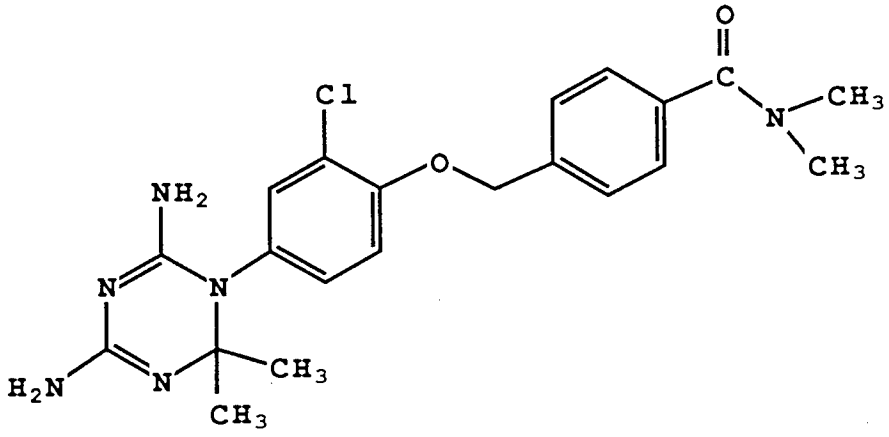
4.2.3 4,6-Diaminodihydrotriazines.

This class of compounds was first synthesised in 1952 by Edward J. Modest and his colleagues.[37] Later, B.R.Baker and his students carried out an extensive structure-activity relationship study on the 4,6-diaminodihydrotriazines.[38] They

introduced the novel concept of active-site-directed irreversible enzyme inhibition. This approach to achieving selective cytotoxicity to cancer cells was based on the assumption that isoenzymes from cancer cells differ in their secondary and tertiary structure from the analogous enzymes in normal mammalian cells.

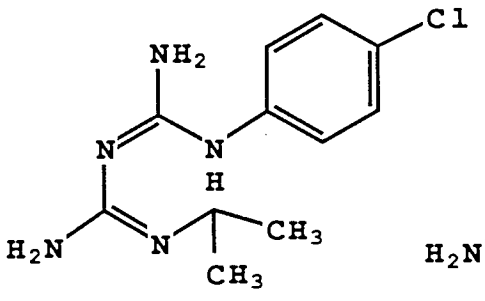
Baker initiated the preparation of inhibitors that contained groups capable of forming covalent linkages with nucleophilic centres near the enzyme active site. It was reasoned that if the location and/or nature of these nucleophilic groups of the cancer cell enzyme differ from the normal cell enzyme, selective cytotoxicity should result.

Out of Baker's study, perhaps NSC-139105 **(26)** was the antifolate which generated the most excitement. This triazinate was found to be a potent antitumour agent which is capable of penetrating the blood-brain-barrier to achieve significant concentration high enough to be effective against the acites from the murine Walker 256 carcinoma and the Dunning leukaemia.[39]



(26)

4,6-Diaminodihydotriazines were initially developed as antimalarial agents; the classical example being proguanil **(27)**. Proguanil, *in vivo*, undergoes metabolism to form the active cycloguanil **(28)** which inhibits the DHFR of plasmodia.[40]



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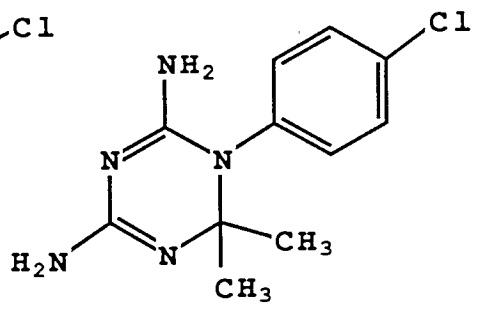


(28)

Apart from the examples given above, there are other

diamino heterocycles which were developed through the years primarily as potential antiprotozoal, antibacterial or antitumour agents. Some of these include the 2,4-diamino pyrido[2,3-d]pyrimidine[41,42], 2,4-diaminopyrroloquinazolines[43,44], pyrimido[4,5-b]thiazines[45] and 1-deaza-7,8-dihydropteridines[46].

4.3 Structure-activity relationship of DHFR inhibitors

The structure-activity relationship of DHFR inhibitors has been elucidated from the synthesis and biological evaluation of a wide variety of diamino heterocycles. The structure of folic acid has been divided into five regions which have been modified in structure in the attempt to develop better inhibitors of DHFR.

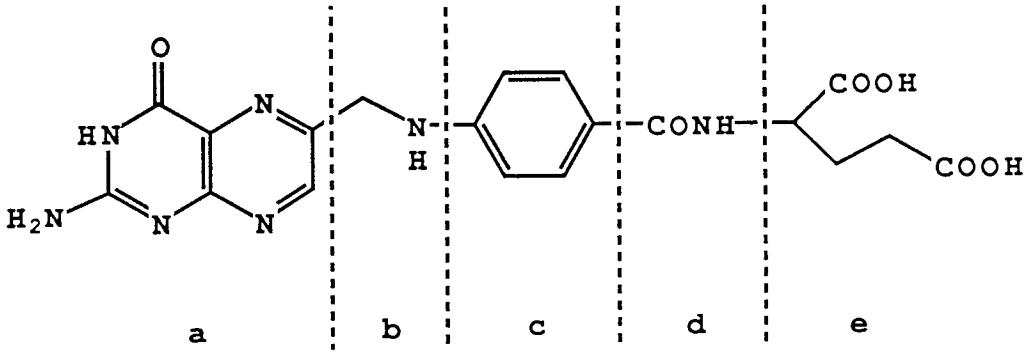
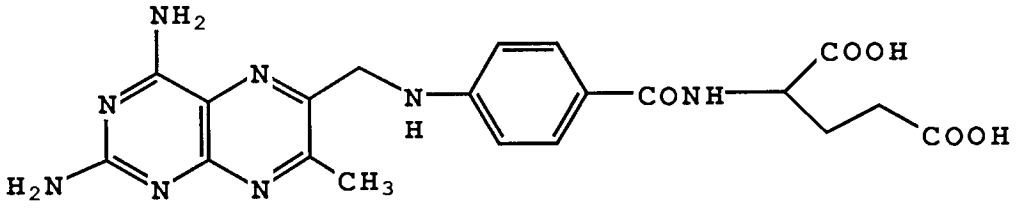


Figure 4.2: *Folic acid divided into its various structural components: (a) pteridine moiety (b) C-9,N-10 bridge (c) benzene ring (d) peptide linkage (e) glutamate moiety.*

4.3.1 The pteridine moiety

Some investigators[49,50] found that reduction of the pyrazine ring in aminopterin and methotrexate produced compounds with diminished growth-inhibitory properties.

It was found from the metabolic studies of methotrexate that it is hydroxylated at C-7 by aldehyde oxidase in the liver. Therefore, 7-methylaminopterin **(29)** and 7-methylmethotrexate **(30)** were found to be inactive against leukaemic L1210 cells.[51]



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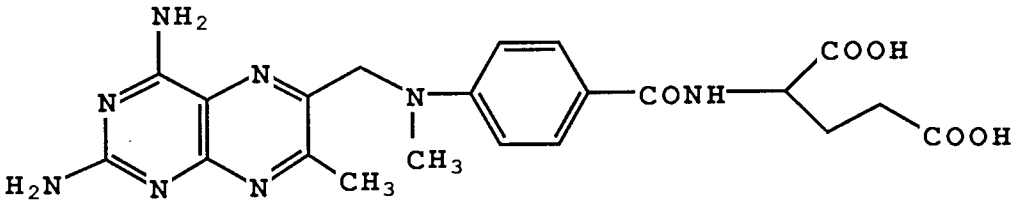
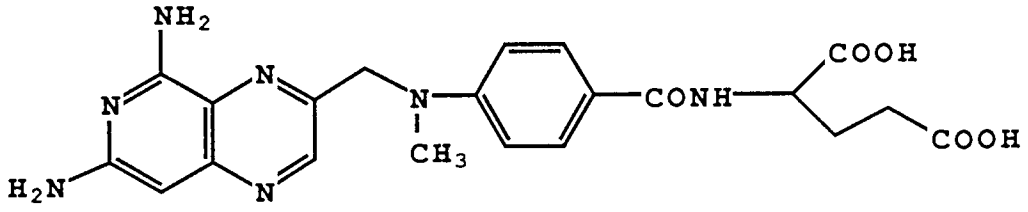


(30)

When diaminoquinazolines, particularly those in which the L-glutamate moiety was replaced by L-aspartate, were found to be quite active against leukaemia L1210 and several of its sublines.[52] This observation implies that not all the

ring nitrogen atoms in the pteridine ring are essential for antifolate activity. Montgomery and Elliott[53] synthesised and tested the compounds 1- and 3-deazamethotrexate **(31,32)**. It was revealed that the 3-deaza analogue **(32)** binds about 1/5 as well as methotrexate to pigeon DHFR, whereas the 1-deaza analogue **(31)** binds only about 1/400 as well. These data established the importance of N-1 in the binding of methotrexate to DHFR. These observations have been confirmed by X-ray[54,55] and n.m.r. studies[47] of the enzyme-inhibitor complex.



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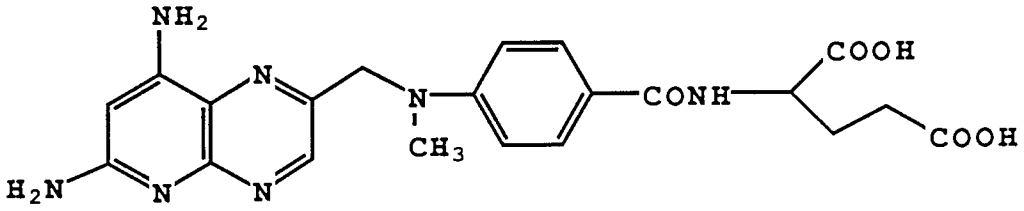


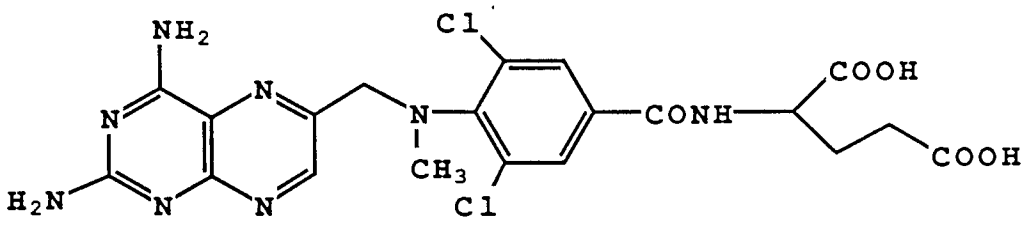
(32)

4.3.2 The C-9,C-10 bridge

Many variations have been made in the C-9,N-10 bridge of both folic acid and aminopterin but few of these have resulted in improved anti-cancer activity relative to methotrexate. Nair[48] reported in his studies that elongation of this bridge region resulted in a decrease in enzyme inhibition and transport, hence a lower antitumour activity was observed.

4.3.3 The benzene ring

Chlorination of the benzene ring as in 3',5'-dichloromethotrexate **(33)** led to a more active compound than methotrexate in the treatment of advanced leukaemia L1210.[56,57] Alkylbenzenes and a variety of heterocycles were substituted for benzene in folic acid but the resulting compounds showed only marginal activity. More drastic changes[58], like replacing the benzene ring with an alkyl group, produced inactive compounds.



(33)

4.3.4 The peptide linkage

Few modifications to the peptide linkage have been made to date. The insertion of one or two methylene groups between the benzene ring of the folic acid and the amide group gave structures with weak antiviral and no antitumour activity.[59] Substitution of a methylene group for the carbonyl group reduced the affinity for DHFR and resulted in inactivity.[60]

4.3.5 The glutamate moiety

A number of other amino acids and other groups have been substituted for the glutamic acid moiety of aminopterin or methotrexate.[61,62] None of these analogues has proved to be superior to the parent compounds in *in vivo* model systems and most of them are less active.

Chapter 5

Rationale and objectives

5.1 Introduction

It has been shown in the introductory chapters that the action of many drugs is very much influenced by the stereochemistry of the drug molecule. Without exception, the enzyme dihydrofolate reductase, too, shows stereoselectivity for its substrates[63] and inhibitor molecules. In addition, evidence from the structure-activity relationship studies of DHFR inhibitors have indicated that the protonation at N1 of the diamino heterocyclic rings is essential for directing the inhibitor molecule to the active site.[65] These observations serve as the foundations for two separate projects which are investigated in this thesis:

(a) To investigate the chemistry and synthesis of chiral 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines, and to observe how the different conformations of these stereoisomers may affect their binding affinity to the enzyme DHFR.

(b) To consider the possibility of retaining DHFR inhibitory activity by replacing the classical diamino heterocyclic groups with other simpler and basic functional groups such as the amidinyl or the guanidinyl groups.

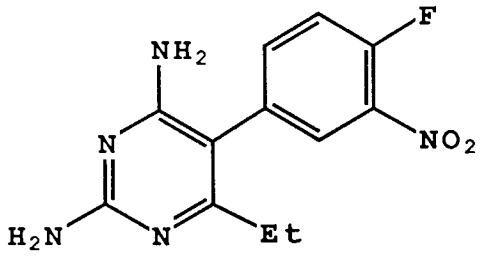
5.2 Stereochemistry of 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines

4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines belong to a class of non-classical lipophilic DHFR inhibitors. In his studies, Baker[38] has shown that *ortho*-substitution on the phenyl ring yielded analogues which were less active than the parent compounds. Recently, Kim and co-workers have shown that the *ortho*-SH analogue is ten times more active than its parent molecule with both bovine liver and murine L5178Y DHFR.[64] These observations could not be satisfactorily explained by either steric or electronic effects of the *ortho*-substituents.

The results of crystal structure determinations on a series of protonated 4,6-diamino-1-aryl-1,2-dihydro-2,2-dimethyl-*s*-triazine antifolates show that the *s*-triazine ring adopts a twist-sofa conformation with C2 nearly 0.5 Å above the plane of the ring. The 2,2-dimethyl groups are equatorial and axial in configuration. The phenyl ring is almost perpendicular to the *s*-triazine ring. This conformation is consistent with the minimum energy calculations[93] and with the structure of the inhibitor-enzyme ternary complex of chicken liver DHFR.[65]

In the study conducted by Tendler *et al*[67],

fluoronitropyrimethamine **(34)** was bound to the *Lactobacillus casei* DHFR as both the binary complex and the ternary complex. Using ^{19}F n.m.r., it was observed that the binary ligand-enzyme complex had two signals in addition to an intense peak referenced at 0 ppm which was due to the free ligand. The former two signals represented the bound state of the two conformers and they appeared in a ratio of 0.6:0.4. Restricted rotation in fluoronitropyrimethamine produces two conformers which are found to bind to the *Lactobacillus casei* DHFR with different affinities. In the two-dimensional ^{19}F NOESY/exchange experiment, cross peaks were observed between the signals for the free ligand and those for the conformers; however no cross peak was observed between the two conformers, thus indicating that the two conformers were not interconvertible when bound. When $NADP^+$ was added, the ratio of the two signals was changed from 0.6:0.4 in the binary complex to 0.3:0.7 in the ternary complex, indicating that the effect of the coenzyme was to reverse the preference the enzyme had for binding to each of the conformers. This study clearly highlights the stereoselectivity of the enzyme DHFR for its inhibitor molecules.



(34)

Enzyme/inhibitor docking experiments carried out on the *Escherichia coli* DHFR and a series of diaminopyrimidines have shown evidence that the binding affinity of the inhibitor is influenced by its stereochemistry. The results of these molecular modelling experiments[66] suggested that one of the conformers of *meta*-azidopyrimethamine **(21)** failed to bind to the active site because of unfavourable steric clashes, whilst the other conformers showed enhanced binding energy attributed to the occupation of a hydrophobic pocket by the azido group.

From the evidence presented by the aforementioned experiments, it may be inferred that the anomaly observed in the inhibitory activity of *ortho*-substituted 4,6-diamino-1-aryl-1,2-dihydro-s-triazines may be attributed to the preference of the enzyme to accommodate stereoselectively only one of the conformers. To examine this hypothesis, the following experiments were conducted:

(a) to synthesise *ortho*-substituted 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines,

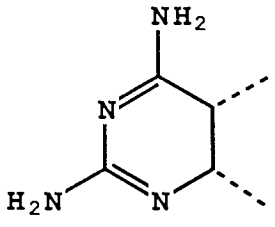
(b) to calculate the energy barrier to rotation of the chiral conformers and determine their stability at ambient conditions,

(c) to resolve the chiral conformers,

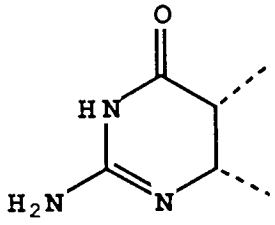
(d) to evaluate the inhibitory activity of each separated conformers against the rat liver DHFR.

5.3 Phenylamidines as potential DHFR inhibitors

Classical antifolates such as methotrexate **(11)** and aminopterin **(17)** show close structural resemblance to the substrate folic acid **(16)**. Structure-activity relationship studies have shown that a 2,4-diaminopyrimidine, 4,6-diamino-*s*-triazine, 2,4-diaminoquinazoline or 2,4-diaminopteridine ring is sufficient for tight binding to the DHFR active site from any species.[26] Although all the antifolates have different structures, however, they all share a common structural unit (I) replacing portion (II) of the pteridine ring of folic acid.[69]



(I)



(II)

Matthews *et al* have shown evidence which suggested that in methotrexate, N1 was protonated in the bound state. In the crystal structure of *Escherichia coli* methotrexate-enzyme complex , the proton at N1 and a proton from the 2-NH_2 group are observed to form two hydrogen bonds with the carboxylate group of Asp-27. This observation inferred that basicity of the ligand molecule is important for binding.[54]

In the development of novel non-classical lipophilic antifolates, methylbenzoprim **(35)** was found to exhibit excellent inhibitory activity against the rat liver DHFR.[71] In the initial screening for antifolate property, methylbenzoprim was found to possess a I_{50} value of 0.01 μM against the rat liver enzyme. *In vitro* cytotoxicity study against the L1210 cell line, showed that the IC_{50} value of methylbenzoprim was less than 0.001 μM. The binding of antifolates to DHFR is thought to involve association at a minimum of four regions within the enzyme active

site[72], namely:

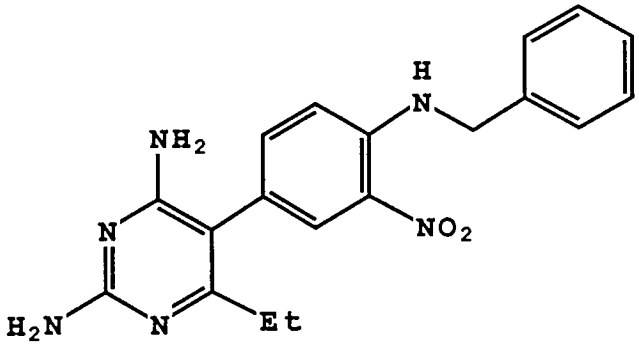
(a) a pteridinyl or pyrimidinyl region

(b) the hydrophobic domain

(c) a *para*-aminobenzoyl binding region

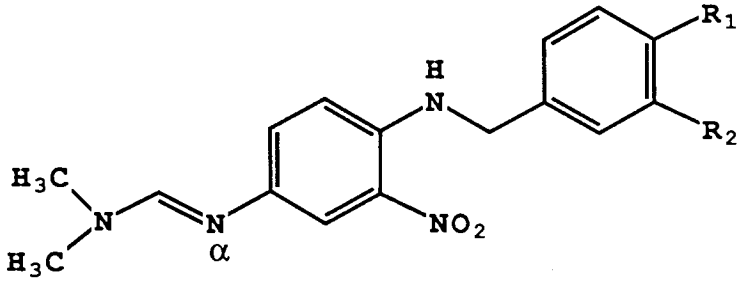
(d) the glutamate or polyglutamate region.

It is believed that methylbenzoprim can be contorted to associate with binding regions (a), (b) and (c) on the enzyme and thus exhibit properties intermediate between metoprine and methotrexate.[71]



(35)

The objective of this second project is to replace the diaminopyrimidine moiety in methylbenzoprim **(35)** with an amidinyl moiety, giving target molecules **(36)** and **(37)**. The basicity of the amidine allows its protonation at physiological pH and therefore binding by hydrogen bonds to the carboxylate group of Glu-30 in the rat liver enzyme may be possible. These target molecules when synthesised would be screened for antifolate activity against the rat liver DHFR in *in vitro* experiments.



Compounds	R_1	R_2
36	H	H
37	Cl	Cl

Section 2: Results and Discussion

Chapter 6

Chemistry of 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines.

6.1 Three component synthesis of *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines.

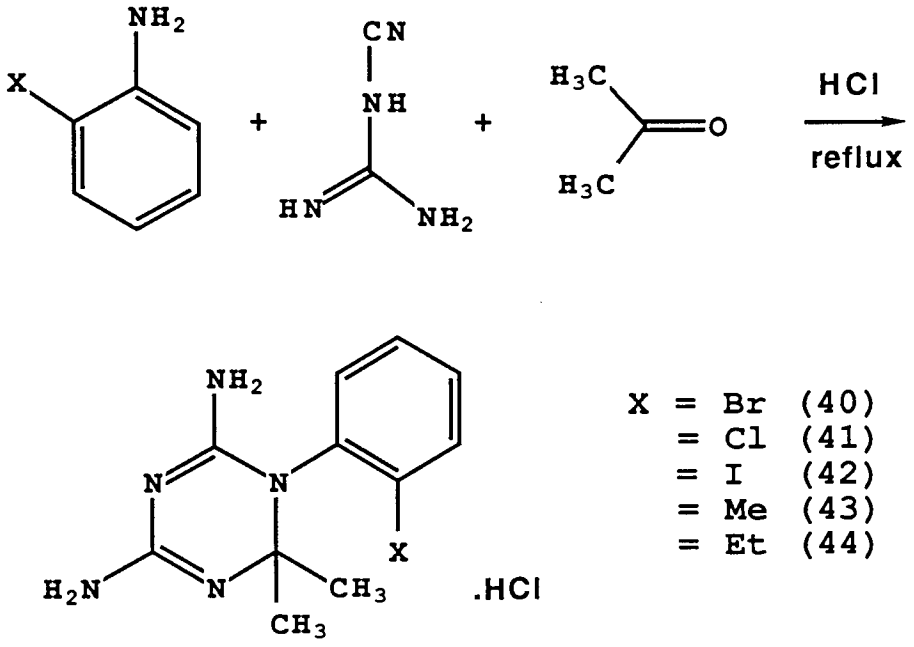
All the *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines were synthesised by Modest's three component synthetic method.[73] This synthesis involved refluxing a mixture of an *ortho*-substituted aniline, cyanoguanidine, acetone and hydrochloric acid for several hours **(Scheme 6.1)**. In some cases, the products were precipitated during the reflux, while in other cases, the products would only crystallise when the reaction mixture was allowed to cool. The products obtained from these reactions were generally quite pure and needed only one recrystallisation from aqueous ethanol or isopropanol to yield crystals of analytical quality.

Aromatic aldehydes and ketones can be used in the three component synthesis to produce different analogues of *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines. Benzaldehyde has been used to synthesise the 2-phenyl analogue, however, the yield obtained was relatively poor. This is attributed to the unfavourable steric hindrance between the 2-phenyl group

and the *ortho*-substituent. Apart from acetone, 2-butanone was the other linear ketone which produced *ortho*-substituted analogues in reasonably high yields. These syntheses produced a series of 2-ethyl-2-methyl analogues which will be dealt with in more details in later chapters. When larger ketone homologues were used, no formation of 1-aryl-1,2-dihydro-*s*-triazines was observed. However, cyclic ketones such as cyclohexanone and cyclopentanone were used successfully with the three component synthesis.

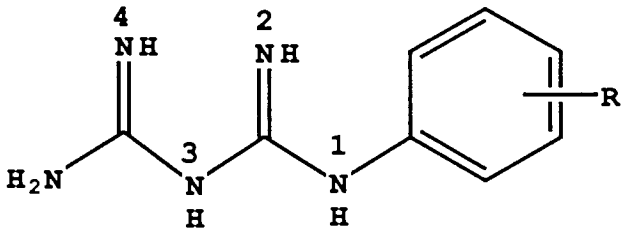
The following anilines were used in the synthesis of the 1-aryl-1,2-dihydro-*s*-triazines: 2-chloroaniline, 2-bromoaniline, 2-iodoaniline, 2-methylaniline and 2-ethylaniline. These syntheses were generally clean and the yields ranged between 61% and 83%. There was a correlation between the yields obtained and the size of the *ortho*-substituents. When the size of the substituent was large, as in the iodo group, the yield was only 61%. On the other hand, smaller substituent like the chloro group, produced a 83% yield. Anilines such as 2-nitroaniline and anthranilic acid were used without success in the "one-pot" synthesis. This is probably due to the strongly electron-withdrawing nature of the *ortho*-substituent which can exert a (-M) mesomeric effect on the lone pair of the arylamino group, thus reducing its nucleophilicity. However, 1-aryl-1,2-dihydro-*s*-triazines were synthesised successfully from 3-

nitroaniline and 4-chloro-3-nitroaniline. This is because the *meta*-substituted nitro group cannot participate in the similar (-M) mesomeric effect on the arylamino group.



Scheme 6.1: *Synthesis of ortho-substituted 1-aryl-1,2-dihydro-s-triazines by the three component synthetic method.*

Modest has proposed the formation of an arylbiguanide **(45)** as an intermediate in the three-component synthesis.[73]



(45)

In the cyclisation of 1-aryl-1,2-dihydro-*s*-triazines, a molecule of water is lost between the oxygen atom of acetone and one hydrogen atom each of N1 and N4 of the arylbiguanide intermediate.

The 1-aryl-1,2-dihydro-*s*-triazines were distinguished from the arylbiguanides by the failure of the 1-aryl-1,2-dihydro-*s*-triazines to form coloured copper complexes with cuprammonium ion.[73]

6.2 Dimroth rearrangement

The hydrochloride salts of 1-aryl-1,2-dihydro-*s*-triazines are quite stable under ambient temperatures. However, the free bases of these compounds are less stable and can undergo a Dimroth rearrangement to form the isomeric anilinodihydrotriazines.[73] The conditions which can bring about this isomeric rearrangement are listed as follows:

(a) by treatment of the hydrochloride salt with heat and strong alkali.

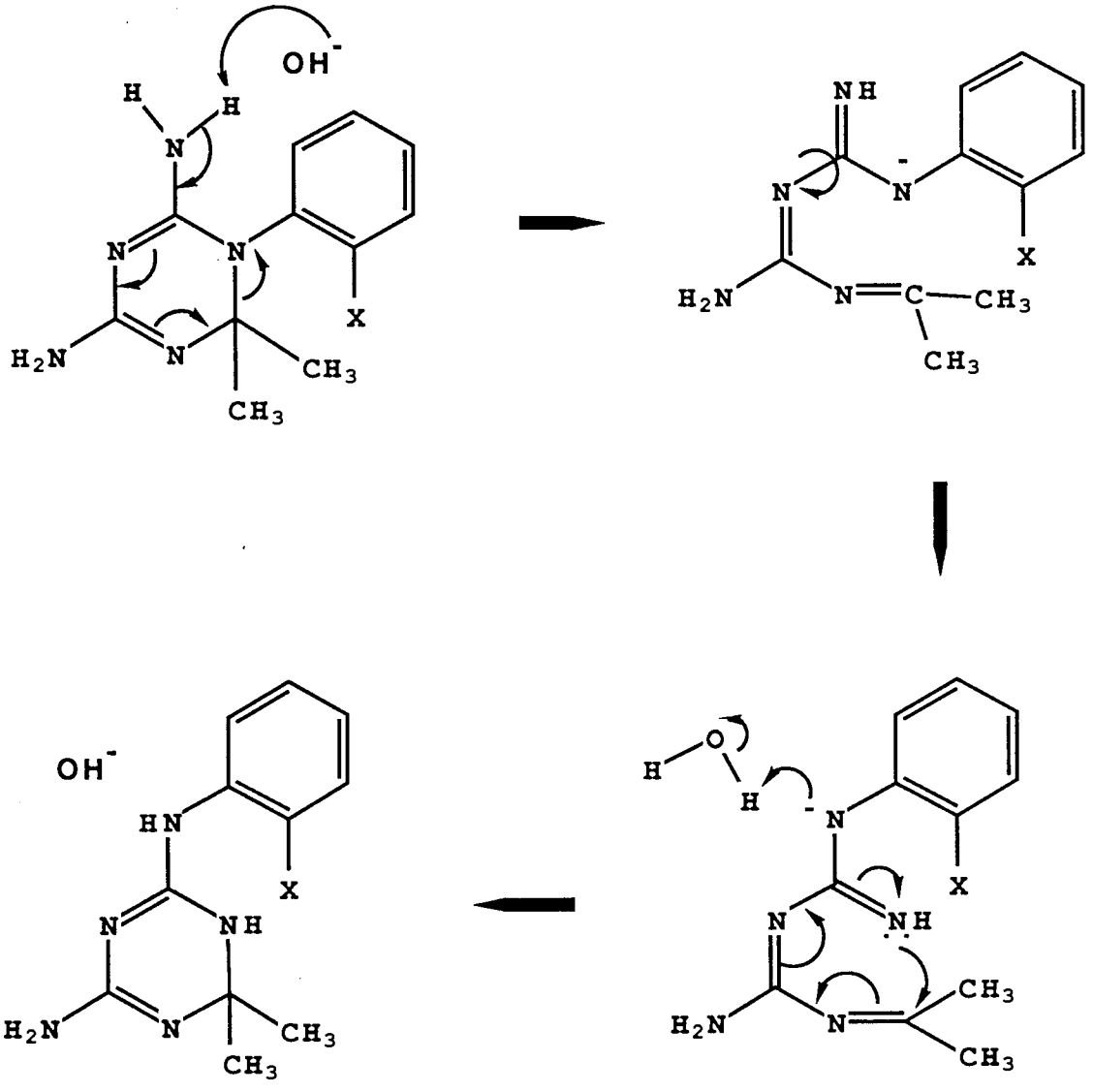
(b) by the action of mild heat on the free bases

(c) by the prolonged action of alkali on the free base in aqueous solution at room temperature.

(d) by thermal rearrangement of the salt or free base in the dry state.

The mechanism[73] of the base-catalysed Dimroth rearrangement was proposed in Modest's work **(Scheme 6.2)**. The alkali abstracts a proton from C6-amino group. The electron shift encourages ring-opening at position C2 of the heterocycle. The lone pair of electrons which resides at C6-amino group flips over by a bond rotation and ring-closes at the C2 position to form the isomeric anilinodihydrotriazine.

All the *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines were rearranged by refluxing the salts in a solution of sodium hydroxide for one hour. The reaction mixture was allowed to stand in the refrigerator overnight and crystals of the anilino-isomer were formed.



Scheme 6.2: *Mechanism of Dimroth rearrangement in alkaline aqueous condition.*

In an attempt to study the mechanism of Dimroth rearrangement in 1-aryl-1,2-dihydro-*s*-triazines, bases such as hydrazine hydrate, piperidine and morpholine were used in the present work. The purpose of using these bases was

firstly to serve as catalysts to initiate the rearrangement and secondly to serve as a probe for reactive intermediates of the rearrangement process. Cycloguanil **(28)** was used as the candidate for this experiment. In all cases, the cycloguanil was rearranged without the formation of new products. This result suggests that the opening and closing of the ring is a concerted event which did not involve any reactive intermediates which can participate in intermolecular reactions with the added bases.

All the anilinodihydrotriazines exhibited bathochromic shifts in their λ_{max} values compared to their parent isomers. In the anilino-isomers the phenyl ring is able to adopt a co-planar conformation, with the heterocyclic ring, which is stabilised by conjugation, hence a red shift is observed in the λ_{max} values. Whereas in the parent 1-aryl-1,2-dihydro-*s*-triazines such conjugative-stabilisation cannot be achieved because steric hindrance prohibits the attainment of ring coplanarity. In addition, the lone pair of electrons on N1 is out of plane with the π electrons of the phenyl ring, therefore stabilisation by resonance cannot be achieved.

^1H n.m.r. spectroscopy was found to be a useful means to study the Dimroth rearrangement in *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines. The dimethyl groups in 1,2-dihydro-*s*-triazines are diastereoisotopic and therefore appear as two distinct singlets of equal intensity in the ^1H

n.m.r. spectrum. The *ortho*-substituent restricts free rotation about the pivotal bond, N1-C1', and gives rise to two enantiomeric rotamers. The dimethyl groups are considered diastereoisotopic because replacing either one of them will generate a chiral centre at C2 and the molecule becomes diastereoisomeric.

When a 1,2-dihydro-*s*-triazine rearranges to the isomeric anilinodihydrotriazine, it loses its enantiomerism and the dimethyl groups become enantiotopic instead. Enantiotopic protons are not distinguishable in ^{1}H n.m.r. spectroscopy except when they are placed in a chiral environment. Hence the two singlets of the dimethyl groups now appear as only one singlet instead **(Figure 6.1)**. Therefore this conversion from two signals to one signal of the dimethyl groups, can be used as a marker for monitoring Dimroth rearrangement in *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines.

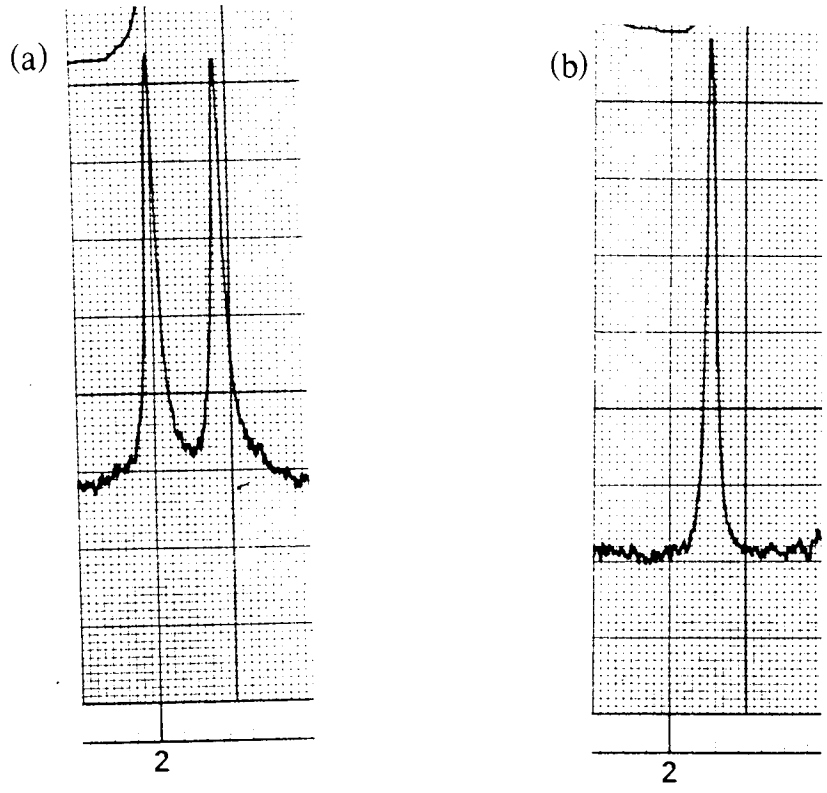


Figure 6.1: *(a) ^{1}H n.m.r. of 1,2-dihydro-s-triazine **(41)** showing the diastereotopic 2,2-dimethyl groups (b) ^{1}H n.m.r. of the 2,2-dimethyl groups after Dimroth rearrangement*

6.3 Alternative route to *ortho*-substitution in 1-aryl-1,2-dihydro-*s*-triazines.

As discussed, *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines can be synthesised directly by using the appropriate 2-substituted aniline. Here, an alternative route to *ortho*-substitution *via* the decomposition of an aryl azide is employed.

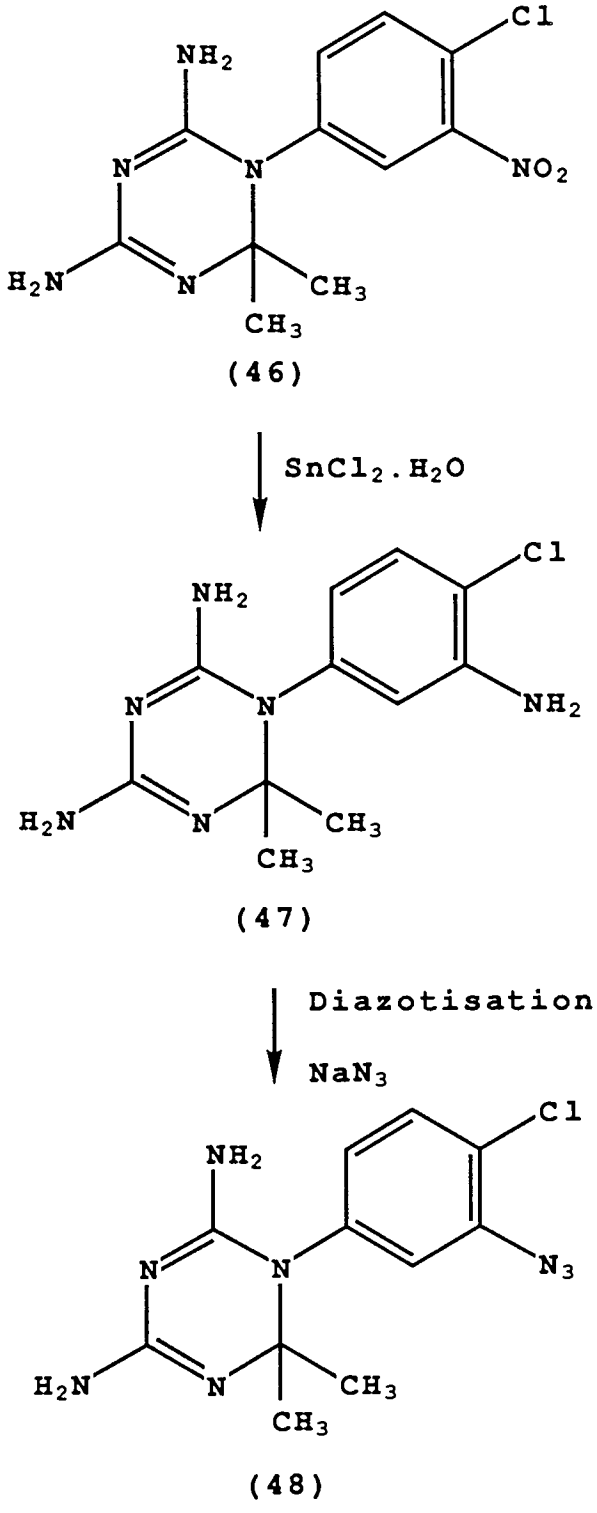
Over a century ago, Griess reported that acid-catalysed decomposition of aryl azides gave *ortho*- and *para*-substituted anilines.[74,75] Takeuchi and Koyama studied the

thermolysis and photolysis of phenyl azide in acetic acid and found that a singlet phenylnitrene was first formed followed by its protonated by acetic acid to give a phenylnitrenium ion pair.[76,77] This ion pair may react with a variety of nucleophiles present in, or added to, the solution to form substituted anilines. The decomposition of phenyl azide in the presence of TFA and/or TFSA at room temperature produces unsubstituted phenylnitrenium ions.[78] Those phenylnitrenium ions with an electron-withdrawing group will undergo N-substitution whereas those with an electron-donating group will undergo C-substitution. The potential of these synthetic mechanisms were exploited in the decomposition of *meta*-azidocycloguanil.

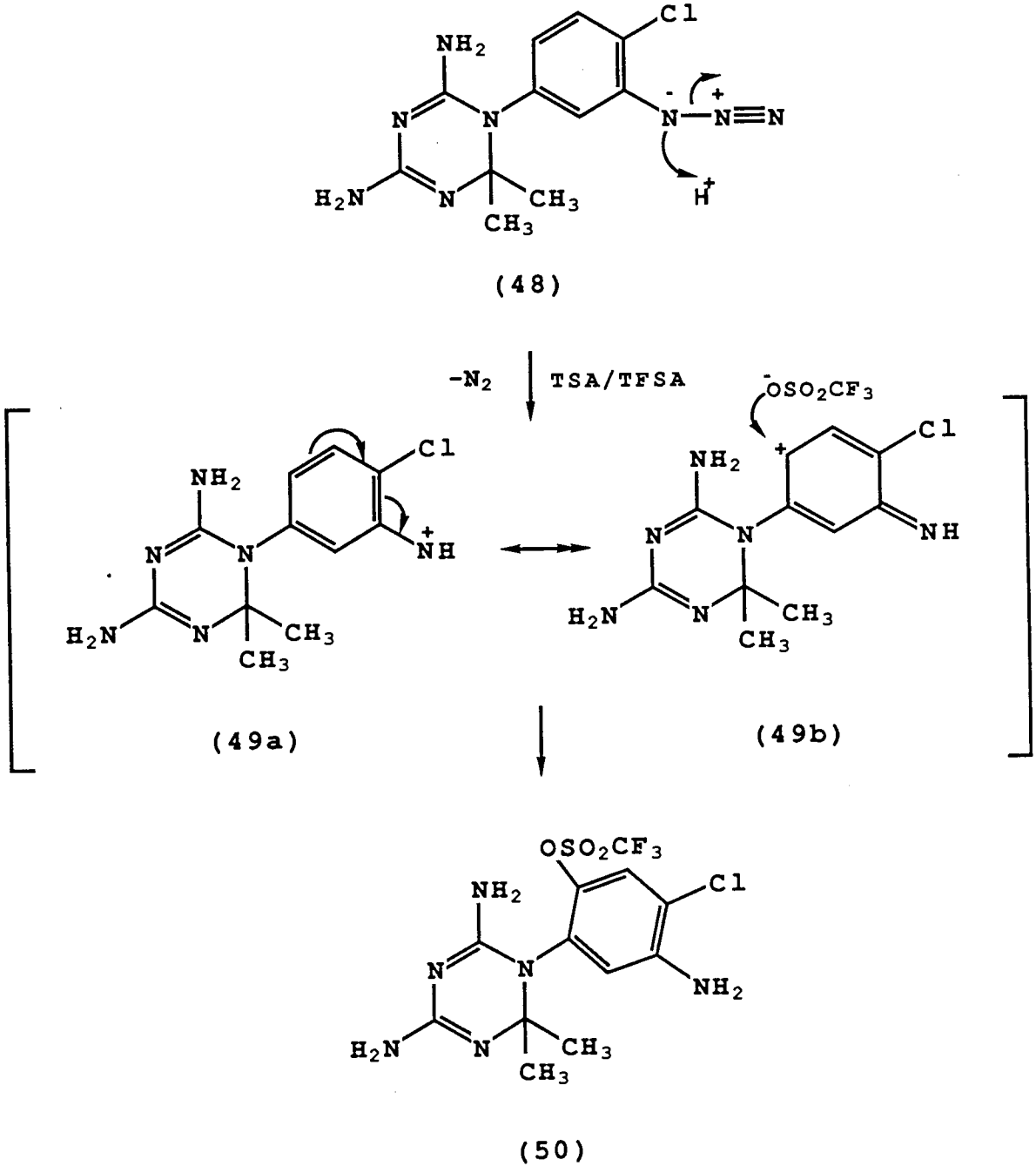
Compound **(46)** was synthesised by the three-component synthesis using 4-chloro-3-nitroaniline, cyanoguanidine, acetone and hydrochloric acid. The *meta*-nitrocycloganil was reduced by stannous chloride to give *meta*-aminocycloguanil **(47)**.[82] This amine was diazotised and treated with four equivalents of sodium azide to yield *meta*-azidocycloguanil **(48)** in about 71% yield **(Scheme 6.3)**.

When *meta*-azidocycloguanil was added to a cold (0°C) mixture of TFA(8ml), TFAA(1ml) and TFSA(3ml)[79], there was an effervescence of nitrogen gas and product **(50)** was formed with a yield of 97.5%. The ^{1}H n.m.r. spectrum of product

(50) consists of the following signals: 7.92 (2H,br.s,$N\underline{H}_2$), 7.47 (1H,s,Ar$\underline{H}$), 7.06 (2H,br.s,$N\underline{H}_2$), 6.95 (1H,s,Ar$\underline{H}$), 6.06 (2H,br.s,$N\underline{H}_2$), 1.51 (3H,s,$C\underline{H}_3$) and 1.28 (3H,s,$C\underline{H}_3$). The two singlets in the aromatic region belong to the protons on C2' and C5' of the phenyl ring. This infers that a *para*-substitution has occurred. Had there been an *ortho*-substitution, the protons on C5' and C6' would appear as doublet of doublets. The absence of this doublet of doublets in the aromatic region of the spectrum, suggests that C-substitution has occurred exclusively at the C6' position. Results from mass spectroscopic analysis show the presence of the fragment *m/z* 149 which belongs to the triflate (OSO_2CF_3) moiety. There are also fragments *m/z* 223 and 225 (3:1 ratio) which when added to *m/z* 149 give the molecular ions of the compound **(50)**. Thus these results indicate that when the aryl azide **(48)** was decomposed in the presence of TFSA/TFA, the hydrogen at position C6' of the phenyl ring was substituted by a triflate anion. The *meta*-nitrenium moiety and the 2,2-dimethyl groups on the triazine ring rendered position C2' inaccessible to the bulky triflate anion, therefore nucleophilic substitution occurred exclusively at position C6'. The mechanism of this aryl azide decomposition is illustrated in Scheme 6.4.

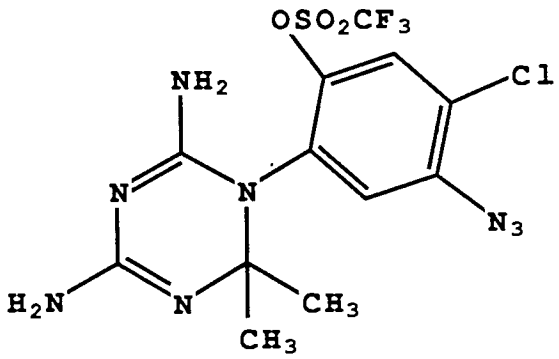


Scheme 6.3: *Synthesis of meta-azidocycloguanil.*



Scheme 6.4 : *Nucleophilic substitution by acid-catalysed decomposition of meta-azidocycloguanil.*

Compound **(50)** was diazotised and converted to the azido analogue **(51)** successfully. However, an attempt to substitute a second triflate on the phenyl ring by decomposing the azide in TFSA and TFA only led to tar formation.



(51)

The highly successful introduction of an *ortho*-substituent in *meta*-azidocycloguanil prompted the study of substitution of other nucleophiles such as Br^-,Cl^- or I^- *via* the same synthetic route. It has been found that attack at either the *ortho*- or *para*- positions of the phenylnitrenium ion, generated from a phenyl azide in the presence of sulphuric acid or hydrogen halides, leads eventually to a ring substituted aniline.[77,80,81] Starting material such as hydrogen halides, sodium bromide and potassium bromide were used as sources of nucleophiles in the decomposition of aryl azide **(48)**. However, nucleophilic substitution was not observed in any of the attempts carried out.

Chapter 7

Stereochemistry of 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines.

7.1 Atropisomerism in *ortho*-substituted 1,2-dihydro-*s*-triazines.

The stereochemistry of 1-aryl-1,2-dihydro-*s*-triazines has been of interest because of an apparent relationship between molecular configuration and inhibitory activity against the enzyme DHFR. Compounds containing an *ortho*-substituted phenyl ring were found to exhibit lower inhibitory activity than the *meta*- or *para*- substituted isomers with *in vitro* models.[64] Baker[83] has proposed the hypothesis that steric interference between bulky *ortho*-substituents and groups at positions C2 and C6 of the triazine ring inhibits the attainment of coplanarity which is a requisite for maximal biological activity. However, Volz *et al*[84] have reported from their X-ray studies on the avian DHFR-phenyltriazine-NADPH ternary complex that when phenyltriazine is bound to the active site of the enzyme, its principal torsion angle between the two rings is about 88°. This observation seems to dispute the hypothesis proposed by Baker.[83]

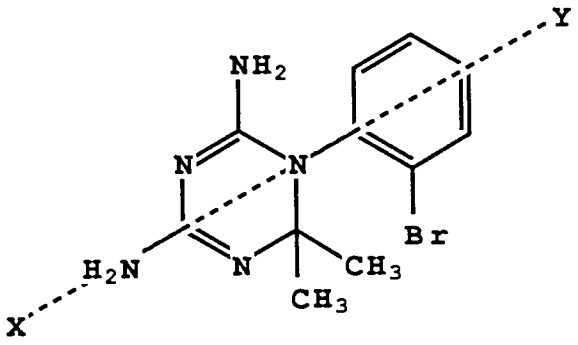
Conformational analyses on a series of protonated 1-aryl-1,2-dihydro-*s*-triazines[85] have shown that the triazine

ring is puckered at position C2 and the dimethyl groups are equatorial and axial. The triazine ring adopts a twist-sofa conformation with C2 nearly 0.5 Å above its plane and the N1-phenyl ring is almost perpendicular to the triazine ring **(Photographs 7.1 and 7.2)**.

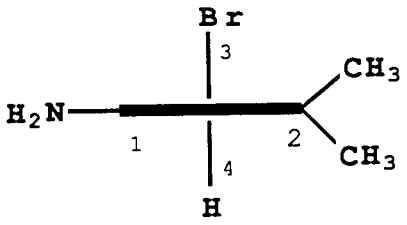
Atropisomerism is a form of isomerism which results from restricted rotation about single bonds. *Ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines exhibit atropisomerism which is very similar to that observed in the 2,2'6,6'-substituted biphenyls.[86] The conventional rules for stereochemical nomenclature in biphenyls are adopted for the highly hindered 1-aryl-1,2-dihydro-*s*-triazines.

4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(*ortho*-substituted phenyl)-*s*-triazines are considered asymmetric because they do not possess any of the following elements of symmetry: namely, axes of rotation (C_n), planes of symmetry (σ) and centres of symmetry (i). In addition, there is a chiral axis (XY) which runs along the pivotal bond between the triazine ring and the phenyl ring. The determination of (R) and (S) configuration of a chiral axis follows the usual sequence rules together with an additional rule which states that the two near groups precede the two far groups. When compound **(40)** is viewed from the X end of the chiral axis **(Figure 7.1)**, the two near groups: the amino group and 2,2-dimethyl groups are given the numbers 1 and 2 respectively

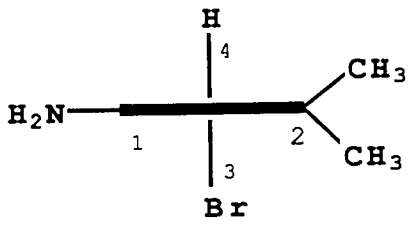
according to the sequence rule. The two far groups: bromine and hydrogen are assigned with the numbers 3 and 4 respectively. These numbers are transformed into a two dimensional model (III). Viewing the model from the side remote from 4 results in the numbers 1,2,3 tracing an anti-clockwise path, therefore this rotamer is assigned the configuration (S). While the other rotamer where the bromo-substituent lies below the plane of the triazine ring gives model (IV) and is therefore assigned the (R) configuration.



(40)



(III)



(IV)

Figure 7.1: *Models (III) and (IV) --- Projections of compound (40) along the XY axis.*

In the case of 4,6-diamino-1-(bromophenyl)-2-ethyl-1,2-dihydro-2-methyl-*s*-triazine **(58)**, there are two elements of chirality. There is a centre of chirality at C2 of the triazine ring and a chiral axis (XY) which runs along the pivotal bond. These two elements of chirality give rise to four stereoisomers (2^n, where n is the number of chiral

elements). The four stereoisomers are assigned the following configurations: (R,aR), (S,aS), (R,aS), (S,aR) where "a" refers to the stereochemical descriptor of the chiral axis. The photographs **(Photographs 7.3-7.6)** in the following pages illustrate the configurations of the four stereoisomers. From the assigned configurations, it can be seen that of the four stereoisomers, there exist two pairs of enantiomers, ie. (R,aR), (S,aS) and (R,aS),(S,aR); and the first pair of enantiomers is diastereomeric to the second pair of enantiomers. In the pair of enantiomers (R,aR) and (S,aS), the *ortho*-substituent is *transoid* to the C2 methyl group on the triazine ring, while in the other pair of enantiomers (R,aS) and (S,aR), the *ortho*-substituent is *cisoid* to the C2 methyl group.

The 1,2-dihydro-*s*-triazine atropisomers experience two opposing forces, namely, resonance-stabilisation favouring a planar conformation; and steric interactions between ortho-substituents and C2,C6-groups on the triazine ring which favours perpendicular conformation. The bulkiness of these *ortho*-substituents contribute immensely to the energy barrier to rotation of these atropisomers. Therefore large *ortho*-substituents will confer a higher energy barrier and hence more stable atropisomers can be formed. The resolvability of these chiral atropisomers is dependent on the magnitude of the energy barrier.

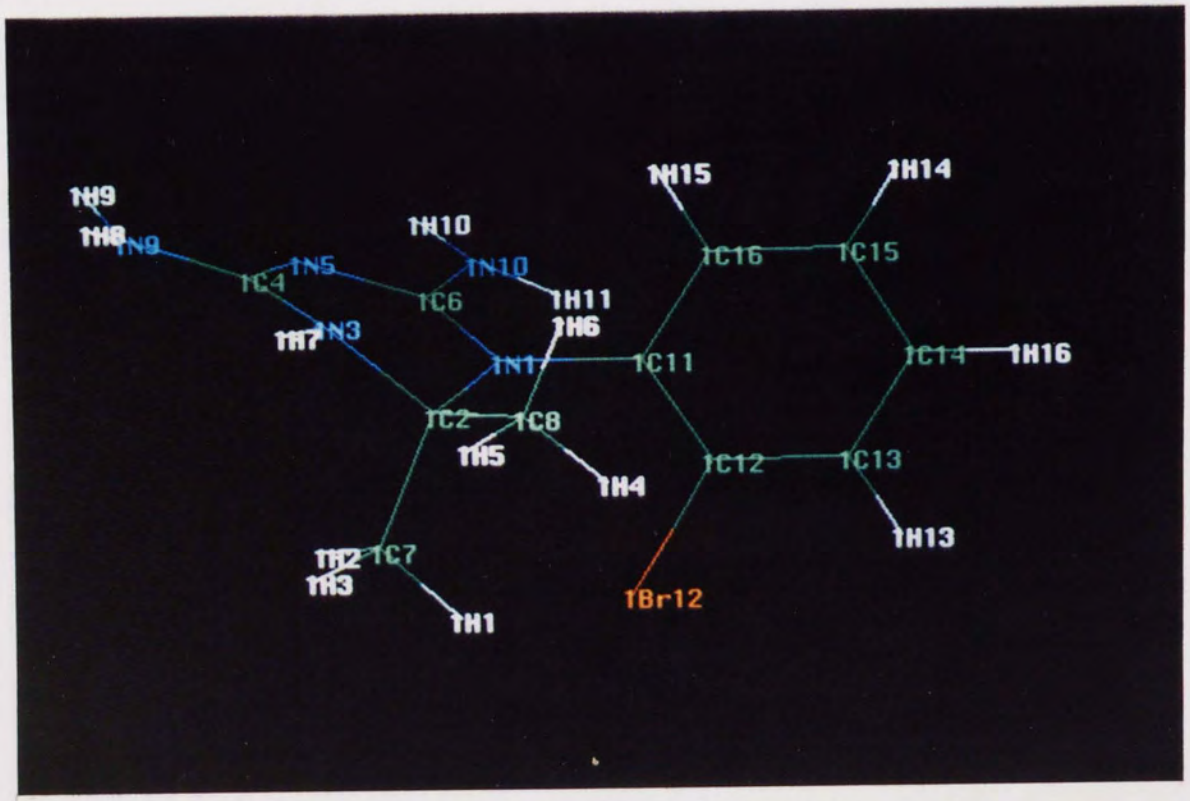


Photo 7.1: *Molecular model of (R)-4,6-diamino-1-(2-bromophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine*

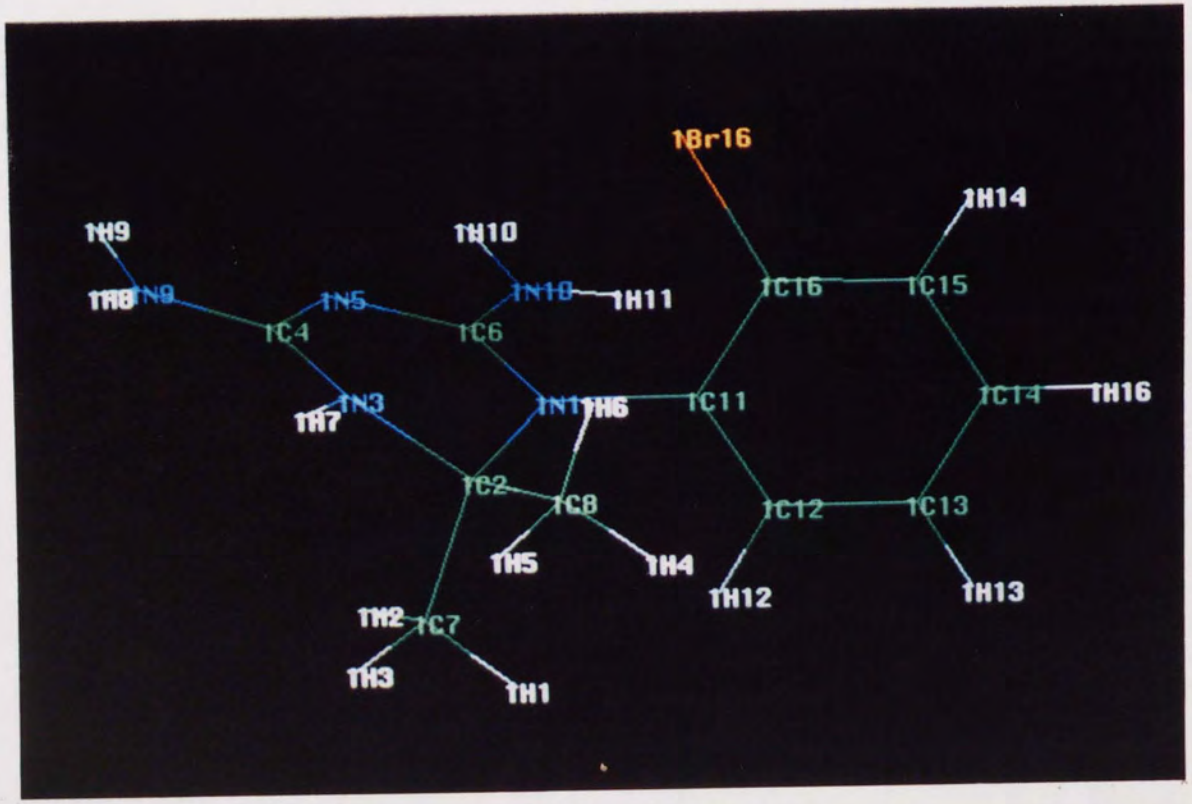


Photo 7.2: *Molecular model of (S)-4,6-diamino-1-(2-bromophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine*

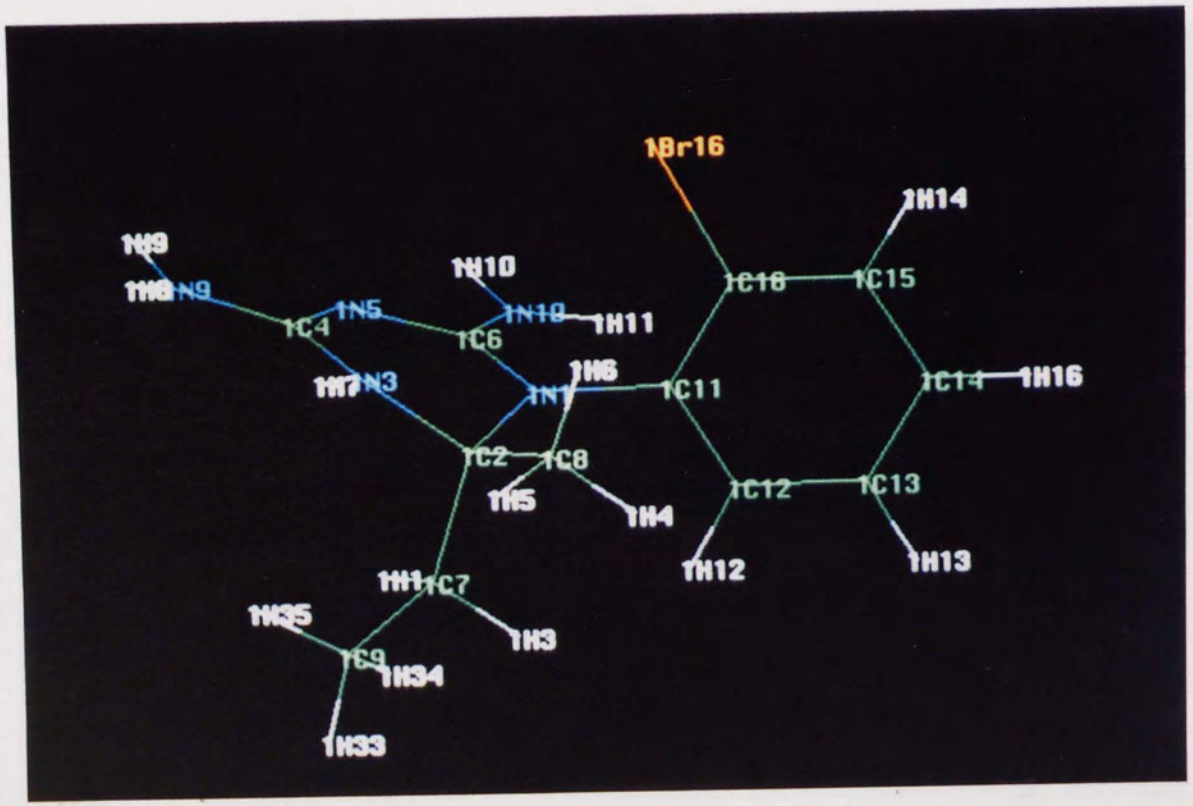


Photo 7.3: *Molecular model of (cisoid)-(R,aS)-4,6-diamino-1-(2-bromophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine*

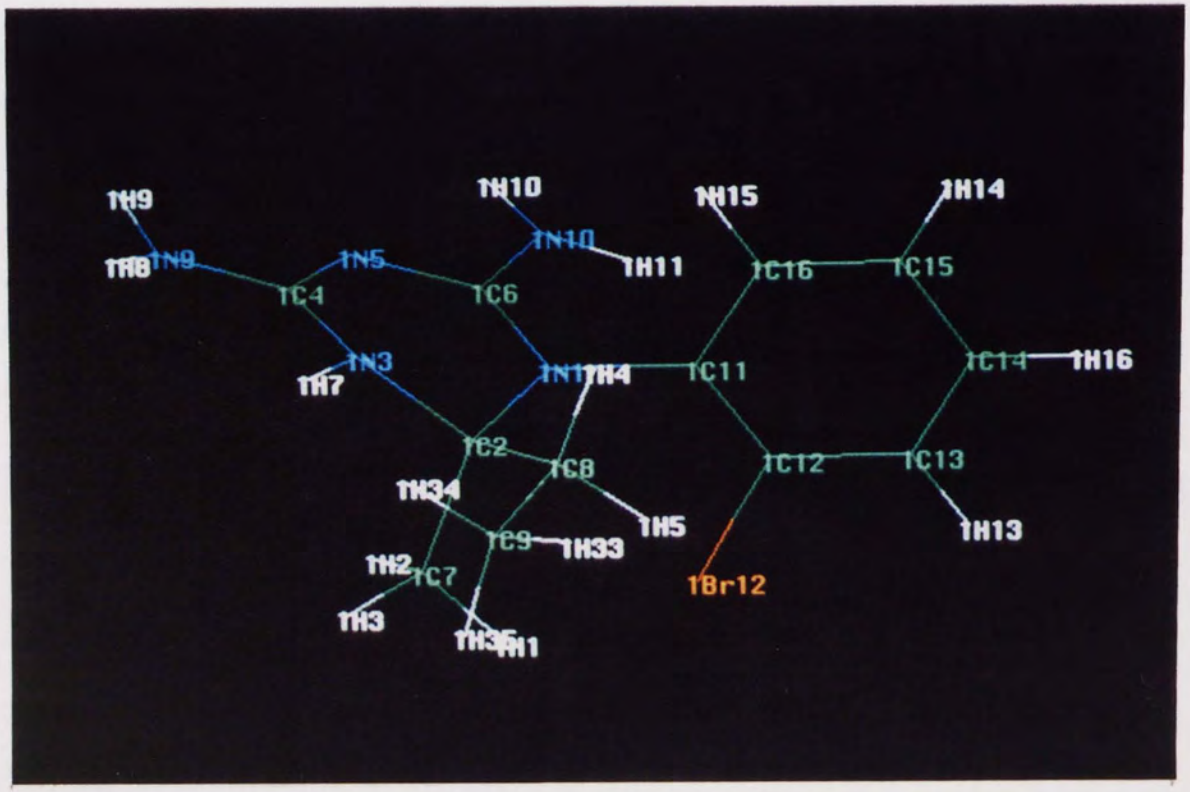


Photo 7.4: *Molecular model of (cisoid)-(S,aR)-4,6-diamino-1-(2-bromophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine*

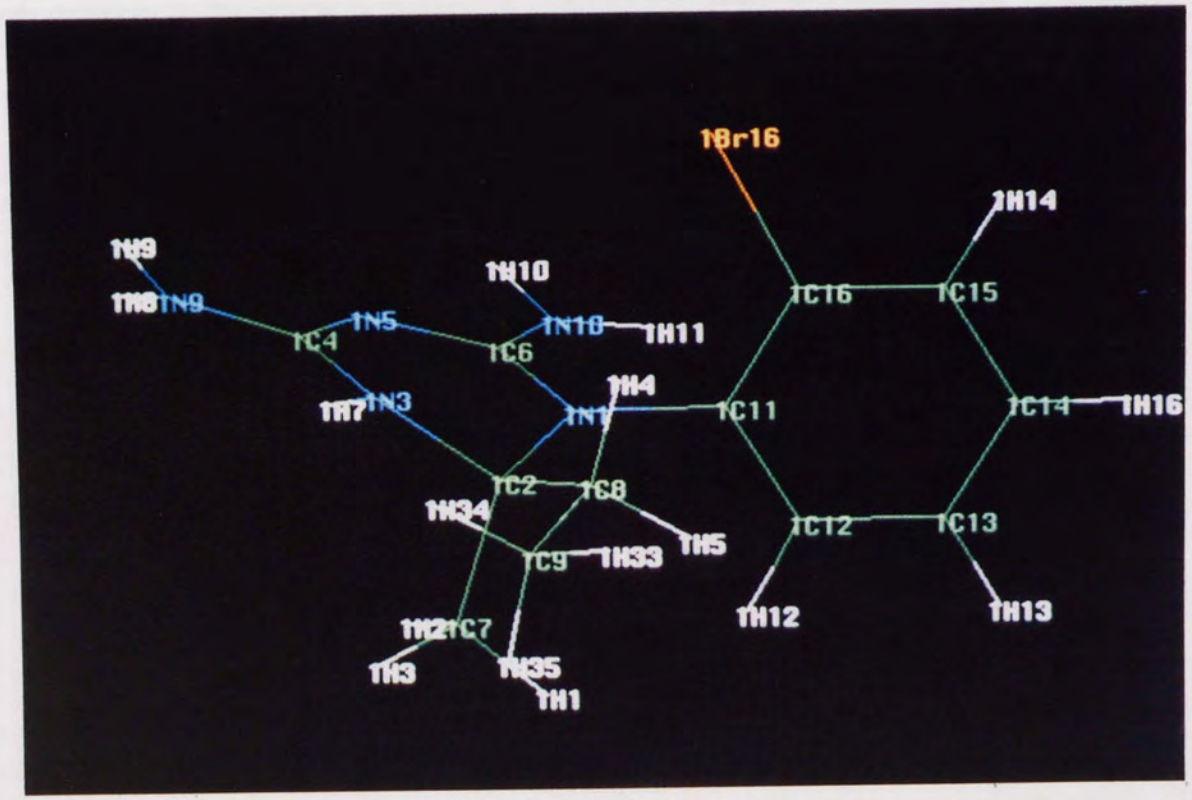


Photo 7.5: *Molecular model of (transoid)-(S,aS)-4,6- diamino-1-(2-bromophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine*

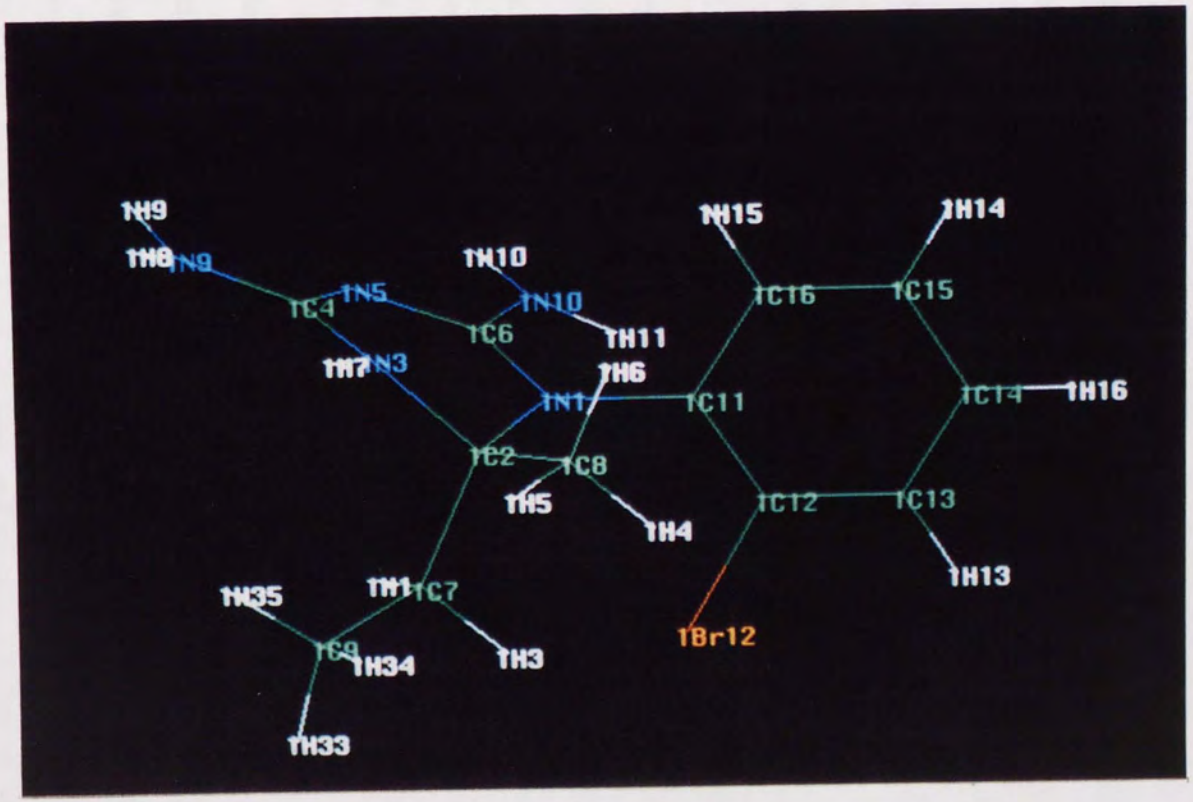


Photo 7.6: *Molecular model of (transoid)-(R,aR)-4,6-diamino-1-(2-bromophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine*

7.2 Determination of energy barriers in atropisomers

Energy barriers to rotation in a series of 2-methyl and 2,2-dimethyl-1,2-dihydro-*s*-triazines have been determined by Colebrook and co-workers.[87] In most of the 2,2-dimethyl-compounds, the barriers were too high to be measured by the proton n.m.r. lineshape method and they were reported to be greater than 20 kcal/mol, while the barriers for 2-methyl compounds were found to be between 22 and 24 kcal/mol.

In the present work, proton n.m.r. lineshape analysis was attempted to determine the energy barriers of 4,6-diamino-2-ethyl-1,2-dihydro-2-methyl-1-(*ortho*-substituted phenyl)-*s*-triazines. However, Dimroth rearrangement was observed in all cases when the temperature was raised to coalescence point. This method was thus abandoned and a molecular modelling technique was employed instead.

The semi-empirical molecular orbital program, MOPAC,[88] was used to calculate the heat of formation (ΔH) for a series of different conformations defined by the dihedral angles. The dihedral angle (Θ) is defined by the angle formed between the triazine and phenyl dummy planes. When the dihedral angle is 0°, the triazine ring and the phenyl ring are co-planar and the *ortho*-substituent lies on the same side as the 2-ethyl and the 2-methyl groups. At 90°,the *ortho*-substituent lies below the plane of the triazine ring

while at 270°, the *ortho*-substituent is located above the triazine plane. The calculated ΔH values were plotted against the dihedral angles to give the energy profile of the atropisomer. The difference in ΔH values between the local maxima and the local minima was taken to be the energy barrier of that atropisomer.

Atropisomers can be resolved if their lifetime is of the order of several hours, which is equivalent to a rate constant for interconversion of $k < 10^{-4}$ sec^{-1}. For separability at room temperature (T = 298°K) it thus follows from the Eyring equation (see below) that the free energy of activation ΔG must be larger than 23 kcal/mol **(Figure 7.2)**.[89,90]

$$k = \kappa \frac{RT}{hN} \exp\left(-\frac{\Delta G^{\ddagger}}{RT}\right)$$

κ = transmission coefficient (taken as unity)
R = gas constant
T = absolute temperature
h = Planck's constant
N = Avogadro's number

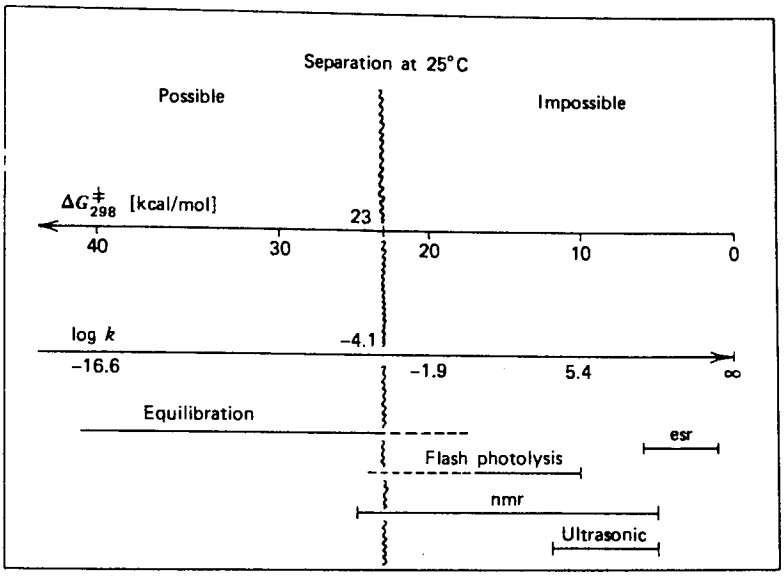
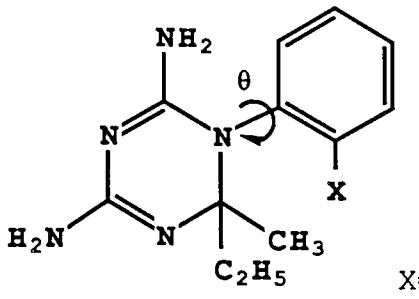


Figure 7.2: *Kinetic limitations of experimental methods for determination of interconversion rates.*

Tables 7.1 - 7.3 show the calculated values of ΔH for atropisomers **(58)**, **(59)** and **(60)**. The ΔH values were plotted against the dihedral angles to give the energy profile of each atropisomer **(Figures 7.3 - 7.5)**. Each of the three energy profiles shows two maxima and two minima . There is a correlation between the maximal ΔH values and the size of the *ortho*-substituents. The maximal ΔH for X=Cl is 223.52 kcal/mol, X=Br is 233.41 kcal/mol and X=I is 244.34 kcal/mol. It is also interesting to note that the minimal energy conformation for all the three atropisomers falls between Θ = 240°-255°. This shows that the two rings are not exactly perpendicular to each other at the lowest energy conformation. This result is consistent with the

conformational analysis and X-ray crystallographic studies carried out by Cody[85], Schwalbe[91], Hunt[92] and Hopfinger[93]. From the energy plots it can be seen that there are two possible energy barriers because the phenyl ring can rotate either in a clockwise or anti-clockwise direction. The smaller of the two values is taken to be the energy barrier of the compound. Hence the calculated energy barrier for compounds **(58)**, **(59)** and **(60)** are 24.2, 24.5 and 25.1 kcal/mol respectively. These high values of energy barriers suggest that these three atropisomers have low interconversion rates and therefore resolvable at ambient conditions. In the next chapter, the three approaches used in the attempt to resolve *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazine atropisomers will be discussed.

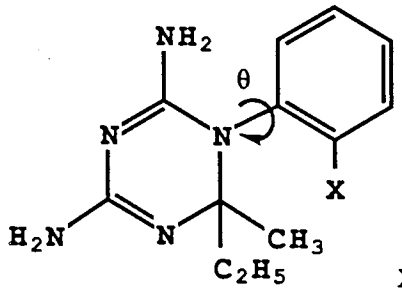
Table 7.1: *Calculated ΔH values for compound* ***(58)*** *.*



X=Br

Dihedral angles, Θ (degrees)	ΔH (kcal/mol)
0	226.12
5	224.12
10	222.22
15	220.40
30	218.68
45	214.20
60	210.31
75	206.56
105	209.16
120	215.26
135	218.59
140	220.79
145	223.09
155	227.94
160	230.44
165	229.06
180	221.22
195	214.84
210	209.90
225	206.89
240	204.83
255	204.69
270	204.99
285	207.38
300	211.07
315	216.05
330	222.20
345	233.30
350	230.41
355	228.21

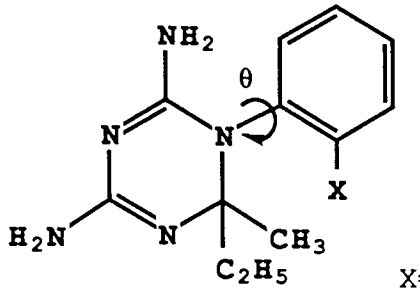
Table 7.2: *Calculated values of ΔH for compound* ***(59)*** *.*



X=Cl

Dihedral angles, Θ (degrees)	ΔH (kcal/mol)
0	223.52
5	216.15
10	214.07
15	212.09
60	197.76
75	194 95
90	193.95
105	195.18
120	198.29
150	208.99
155	211.25
160	213.60
165	216.01
170	218.45
175	217.55
180	214.76
195	207.32
210	201.39
240	193.72
255	192.29
270	192.74
285	195.00
300	198.73
330	209.70
345	216.26
350	218.51
355	220.76

Table 7.3: *Calculated values of ΔH for compound* ***(60)*** *.*



X=I

Dihedral angles, Θ (degrees)	ΔH (kcal/mol)
0	240.89
5	238.63
10	236.48
15	234.42
75	216.24
80	215.57
85	215.13
90	214.93
95	214.97
100	215.28
105	215.83
165	237.45
170	240.07
175	238.94
180	236.08
195	228.41
210	222.28
240	214.31
255	212.96
270	213.65
285	216.37
300	220.73
345	239.73
350	242.04
355	244.34

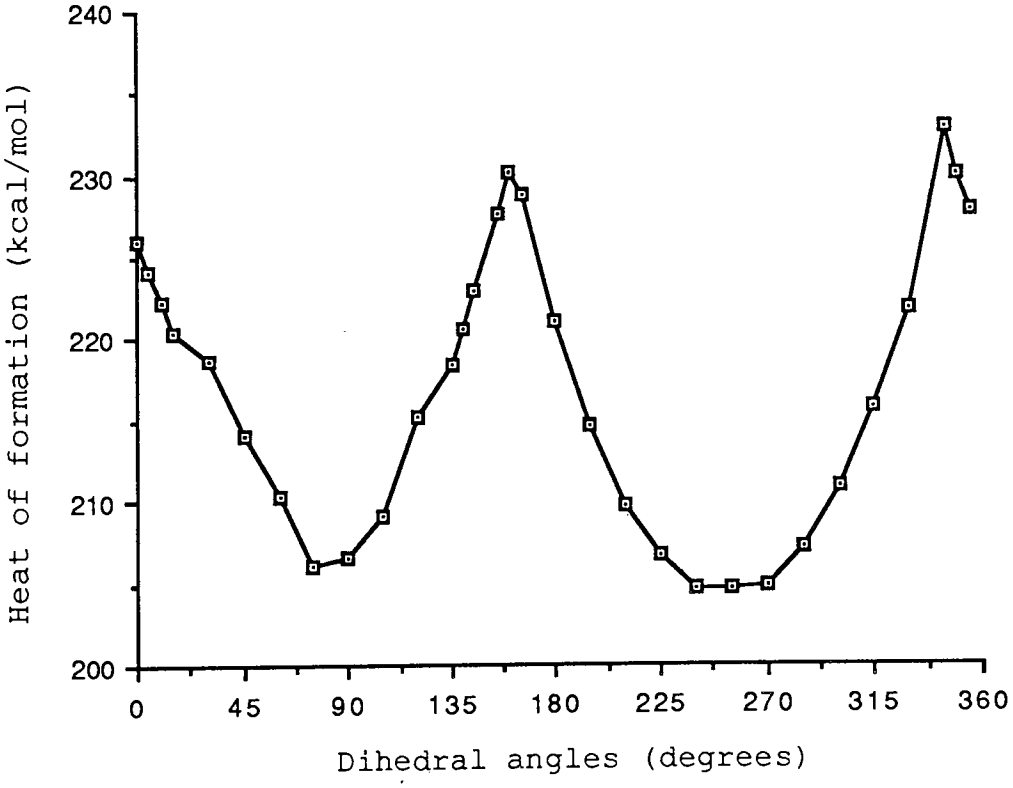


Figure 7.3: *Energy profile of compound* ***(58)****, X = Br.*

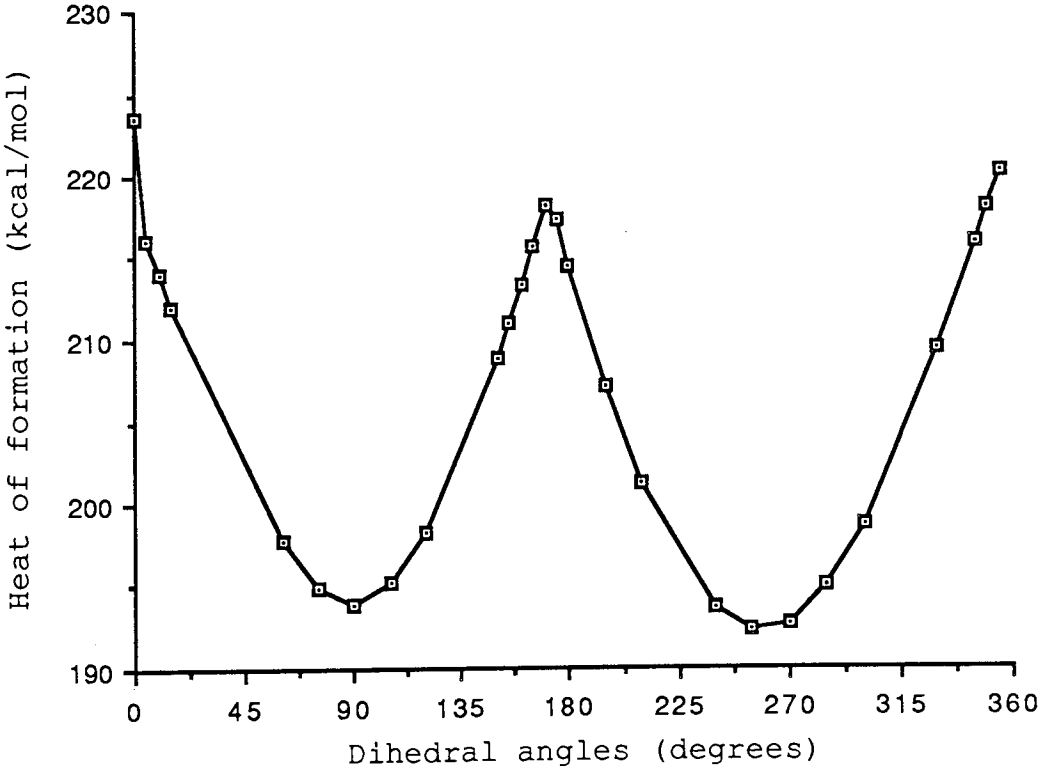


Figure 7.4: *Energy profile of compound* ***(59)****, X = Cl.*

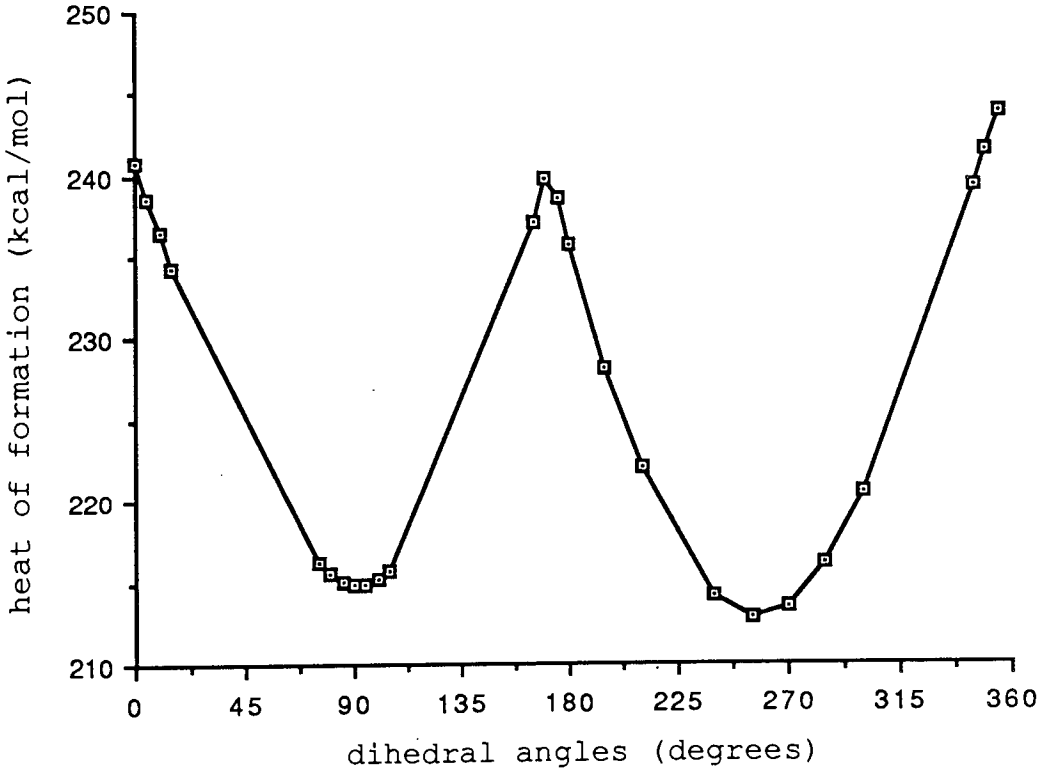


Figure 7.5: *Energy profile of compound* ***(60)****, X = I.*

Chapter 8

Optical resolution of chiral 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines.

8.1 Formation of diastereoisomeric salts.

The conversion of enantiomeric 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(*ortho*-substituted phenyl)-*s*-triazines to diastereoisomers by salt formation was the first approach to optical resolution of such chiral antifolates. Rather than choosing randomly a single resolving agent to form the diastereoisomeric salt, systematic trials[94] using a range of optically active acids were carried out instead. The procedures of the trials comprise the following steps:

(a) combining 0.1 to 1 mM of the racemate with one equivalent of optically active acid in the presence of 1ml of a suitable solvent.

(b) when a crystalline salt was formed, it was isolated, dried and weighed. The weight of the crystals should be less than half the total weight of the two diastereoisomeric salts.

(c) after having converted the salts to their free bases, optical rotation of the free bases was measured by polarimetry.

One of the advantages of the systematic trials is the need for only small amounts of substrate and resolving agent. In addition, they facilitate the testing of a wide range of

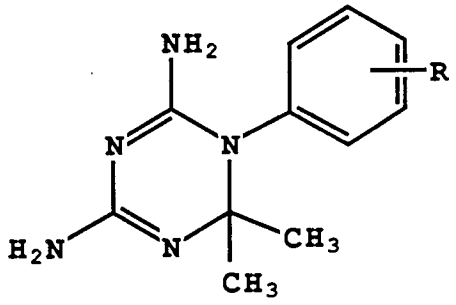
resolving agents and substrates in a short period of time. The most suitable resolving agent and solvent are then chosen from these trials and the resolution is conducted on a larger scale.

Eight optically active acids were used in three systematic trials involving compounds **(40)**, **(44)** and **(50)**. These optically pure acids were chosen from the Tables of Resolving Agents and Optical Resolution.[95] These are resolving agents which are commonly used for the resolution of amines and basic heterocycles. The eight resolving agents are: (-)malic acid, (R)-(-)mandelic acid, (+)-d-tartaric acid, (+)-o,o'-dibenzoyltartaric acid, (-)-di-o-isopropylidene-2-keto-L-gulonic acid, (+)-camphor-10-sulphonic acid, (+)-3-bromocamphor-9-sulphonic acid and (R)-(-)-2,2'-(1,1'-binaphthyl)-phosphoric acid. Absolute ethanol was used with all the three compounds as it was used in the crystallisation of all the hydrochloride salts of the 1-aryl-1,2-dihydro-*s*-triazines.

Table 8.1 summaries the results of the three systematic trials conducted. The bases which formed crystalline salts with the optically active acids were designated the symbol (*) or (Δ). The crystalline salts were collected, dried and weighed. The enantiomers were retrieved from the salts by adding sodium hydroxide solution to a solution of the salt in water. The optical rotations of the free bases were measured by a digital polarimeter. In all the cases, no optical

activity was observed in any of the free bases isolated. This suggests that there had been no separation between the enantiomers from crystallisation. One possible explanation for this observation may be the formation of solid solutions during co-crystallisation of the diastereoisomeric salts. Another likely explanation is that differential crystallisation was not observed because of insufficient difference in the solubilities of the pair of diastereoisomeric salts.

Table 8.1: *Resolution trials for ortho-substituted 1-aryl-1,2-dihydro-s-triazines.*

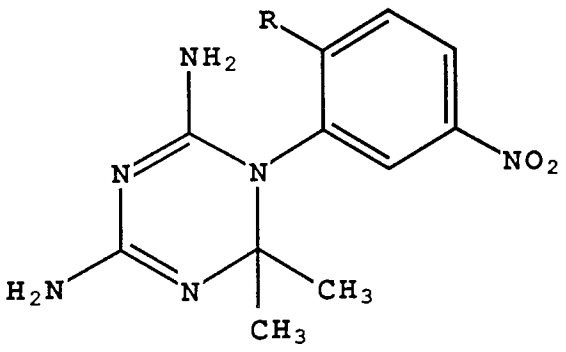


R	1	2	3	4	5	6	7	8
2-Br						Δ(±)	*(±)	
2-Et	*(±)	*(±)				*(±)	Δ(±)	
3-NH_2-4-Cl-6-OSO_2CF_3	Δ(±)	*(±)	Δ(±)	Δ(±)			Δ(±)	

*Key: (1) (-)-malic acid, (2) (R)-(-)-mandelic acid, (3) (+)-d-tartaric acid, (4) (+)-o,o-dibenzoyltartaric acid, (5) (-)-di-o-isopropylidene-2-keto-L-gulonic acid, (6) (+)-camphor-10-sulphonic acid, (7) (+)-3-bromocamphor-9-sulphonic acid, (8) (R)-(-)-2,2'-(1,1'-binaphthyl)-phosphoric acid; Δ = crystalline salt with less than 50% yield; * = crystalline salt with more than 50% yield; (±) = optically inactive.*

8.2 Diastereoisomeric derivatisation.

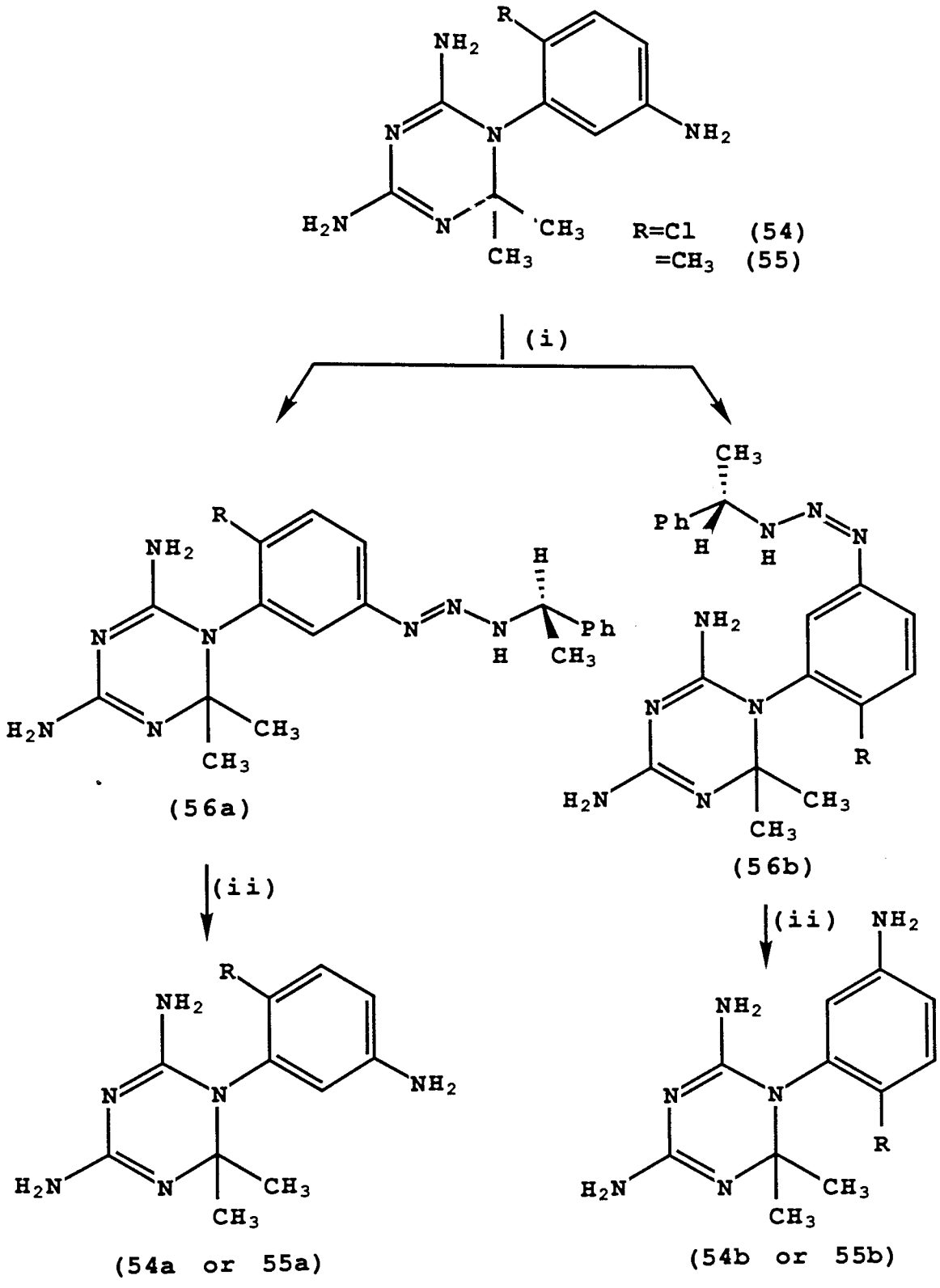
In this second approach, 1-aryl-1,2-dihydro-*s*-triazines were derivatised to form covalent diastereoisomers. Such diastereoisomeric derivatives possess different physical properties and therefore are separable by conventional liquid chromatographic techniques. For example, the diastereoisomers of (6RS,αS)-folinic acid have been separated by liquid chromatography and crystallisation.[63] To derivatise covalent diastereoisomers, the following *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines have to be synthesised:



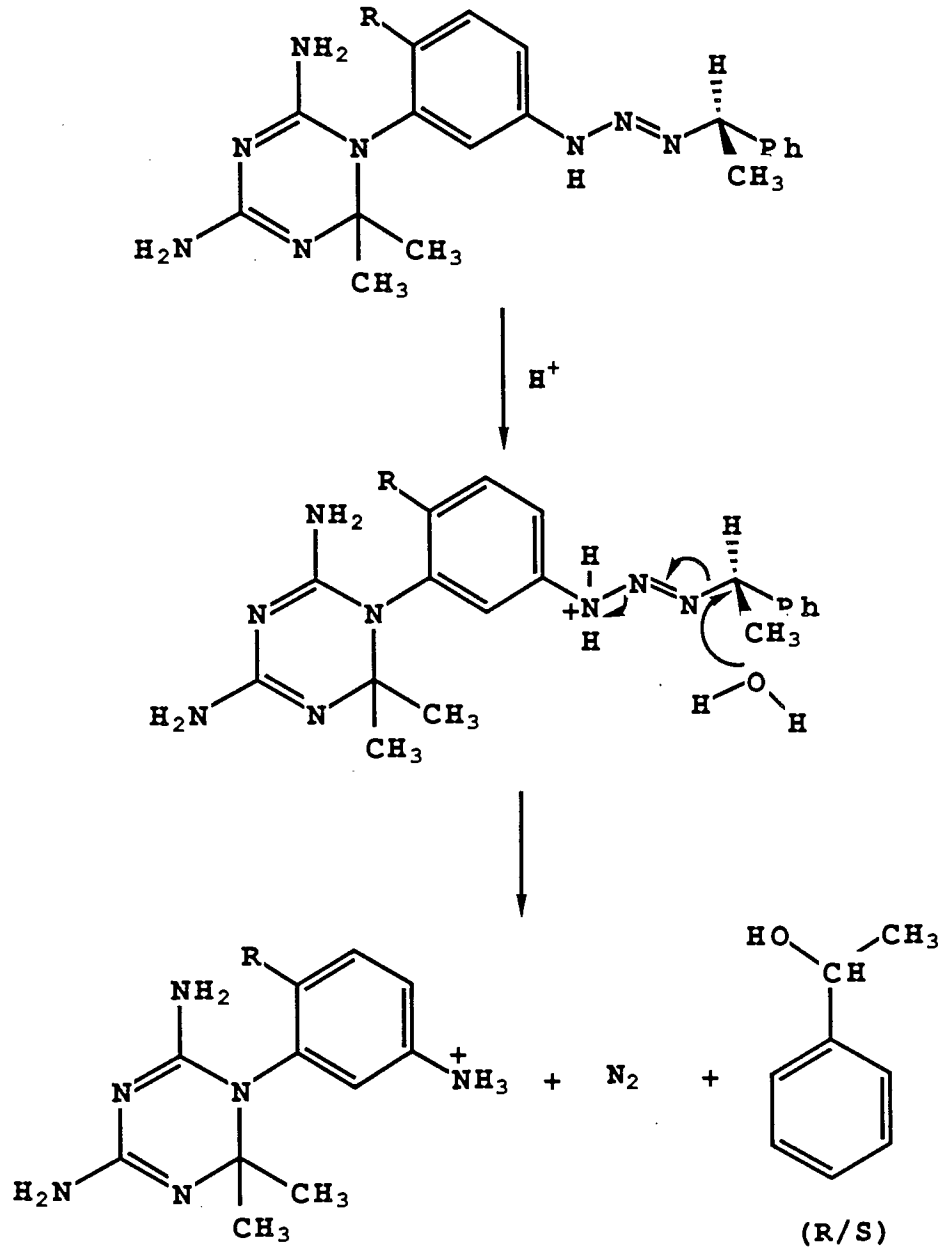
R = Cl (52) or CH_3 (53).

The *meta*-nitro group was reduced to the amino function by catalytic hydrogenation to give compounds **(54)** and **(55)**. This step was essential because the amino group served as a site for covalent derivatisation. The formation of amide and carbamate derivatives was considered not practical because it was thought that the hydrolysis of these functions required conditions which might racemise the atropisomers during the

recovery phase. Alkaline hydrolysis would also initiate the Dimroth rearrangement in these compounds. It was decided that formation of a monoalkyltriazene[96,97,98] would be more practical because triazenes can be readily hydrolysed in mild acidic condition **(Scheme 8.2)**. The strategy is illustrated in Scheme 8.1. This method was later abandoned due to unsatisfactory synthesis of the monoalkyltriazene.



Scheme 8.1: *Diastereoisomeric derivatisation of compounds (54) and (55); (i) diazotisation, (R)-(+)-(α)-methylbenzylamine; (ii) acid hydrolysis.*



Scheme 8.2: *Mechanism of acid hydrolysis of monoalkyltriazene.*

8.3 Chromatographic resolution of diastereoisomeric atropisomers.

A second element of chirality can be introduced into the *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines by converting C2 in the triazine ring into an asymmetric carbon. This was done by substituting acetone with 2-butanone in the three component synthesis. As described in chapter 7, these 1-aryl-1,2-dihydro-*s*-triazines are made up of a mixture of four stereoisomers, of which there exist two pairs of enantiomers. Each enantiomeric pair shares a diastereoisomeric relationship with the other enantiomeric pair, therefore the diastereoisomeric pair should be separable by achiral liquid chromatography.

Several h.p.l.c. methods for the analysis of proguanil and cycloguanil[99,100,101] were considered. Two h.p.l.c. methods which were developed for the analysis of *meta*-azidopyrimethamine[102,103,104] were selected as models for the separation of chiral 1-aryl-1,2-dihydro-*s*-triazines. Both methods used C18 Lichrosorb-RP Select B column as the stationary phase, however the mobile phases were different. In the first method, the mobile phase was made up of methanol : 0.02M potassium dihydrogen phosphate buffer, pH 3, containing 0.02M sodium dodecyl sulphate (70:30, v/v). The mobile phase of the second method comprised methanol : 0.05M potassium dihydrogen phosphate buffer, pH 7 (65:35,v/v).

Preliminary analyses showed the possibility of resolving the diastereoisomeric atropisomers on a C18 reversed-phase achiral column using the aforementioned mobile phases. The chromatograms obtained from both methods indicated that the mixture of diastereoisomeric atropisomers was made up of a major isomer and a minor isomer. The following modifications of the model experiments were necessary to suit the requirements of the present investigation:

(a) To develop a suitable procedure for isolating the separated atropisomer from the mobile phase. Low temperature and non-alkaline conditions are essential for this extraction process to avoid racemisation of the purified atropisomers and Dimroth rearrangement.

(b) To achieve basal resolution by optimising the chromatographic conditions. Basal resolution is essential because an automated fraction collector would be used for the collection of the individual peaks.

The constituents of the mobile phases in both the model methods were not compatible with the DHFR enzyme inhibition assay. Therefore it was necessary to isolate the atropisomers from the mobile phase and reconstitute in water for use in the bioassay. A straightforward way would be to extract the atropisomers with an organic solvent. However, the diastereoisomeric atropisomers did not extract efficiently in a range of organic solvents. An attempt was made to use only methanol and water, adjusted to pH 4 by glacial acetic acid, in the mobile phase. This mobile phase

would be ideal because the atropisomers could be retrieved readily by evaporating the mobile phase. However, such a mobile phase was not selective enough to render separation of the atropisomers. This observation suggested that an ionic component in the mobile phase was essential for the separation of the mixture. When ammonium acetate was added to the mobile phase separation of the atropisomers was again observed. Ammonium acetate was chosen because it could be removed readily by evaporation under high vacuum. To avoid fluctuation in temperatures, the mobile phase was removed by freeze drying rather than rotary evaporation.

The next modification was to derive an ideal mobile phase composition that would bring about basal resolution of the diastereoisomeric mixture. The atropisomers were not retained at all when the methanol : ammonium acetate buffer composition was 50:50,v/v. Partial separation was observed when the mobile phase composition was changed to 30:70,v/v. Good resolution of the two components of the diastereoisomeric mixture was achieved when the mobile phase was adjusted to 25:75,v/v.

It was deduced from the chromatographic conditions that the separation of the diastereoisomeric atropisomers was dependent on the ionic strength and the composition of the mobile phase. At pH 4, all the 1,2-dihydro-*s*-triazine molecules would be charged and they would not have any affinity for the lipophilic stationary phase; hence no

separation was observed. When an ionic species was added, the ionic strength of the mobile phase was increased. This had the effect of 'salting out' the charged atropisomers and forcing these molecules to reside on the stationary phase. The thermodynamic differences in the interaction between the diastereoisomeric atropisomers and the stationary phase was believed to be the primary cause of the separation of the atropisomers. This hypothesis was supported by the fact that when the methanolic content of the mobile phase was relatively high (as in 50:50,v/v) no separation was observed, whereas a lower methanolic content (as in 25:75,v/v), produced the best resolution of the atropisomers.

Five diastereoisomeric 1-aryl-1,2-dihydro-*s*-triazines were successfully resolved by using the optimised chromatographic conditions. Their chromatograms are presented in **(Figures 8.1-8.5)**. Table 8.2 summaries all the chromatographic parameters of the separated atropisomers.

Enrichment of the diastereoisomeric atropisomers was carried out by using a semi-preparative μBONDAPAK^{TM} C18 column (25mm x 10cm). The usual chromatographic conditions were used with the exception of the flow rate, which was increased to 8ml/min. An Advantec SF2120 super fraction collector was programmed to collect each peak in separate bottles over a duration of five injections. The contents of each bottle were concentrated by rotary evaporation under high vacuum. The concentrates were then freeze-dried

overnight. The residue obtained from this operation was used directly in both the n.m.r. analyses and the enzyme inhibition assay. The diastereoisomeric excess percentages of the enriched atropisomers are summarised in Table 8.3.

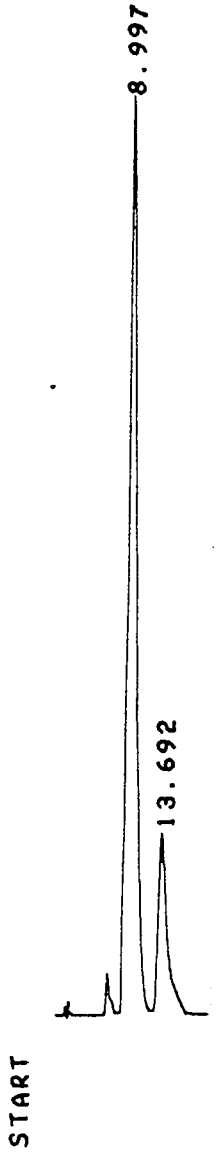


Figure 8.1: *Chromatogram showing the separation of diastereoisomeric 4,6-diamino-1-(2-bromophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine*

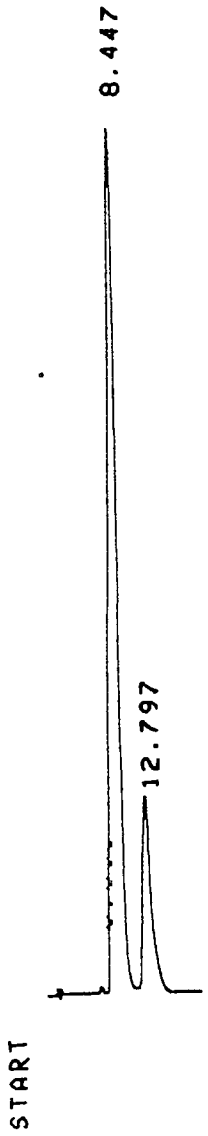


Figure 8.2: *Chromatogram showing the separation of diastereoisomeric 4,6-diamino-1-(2-chlorophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine*

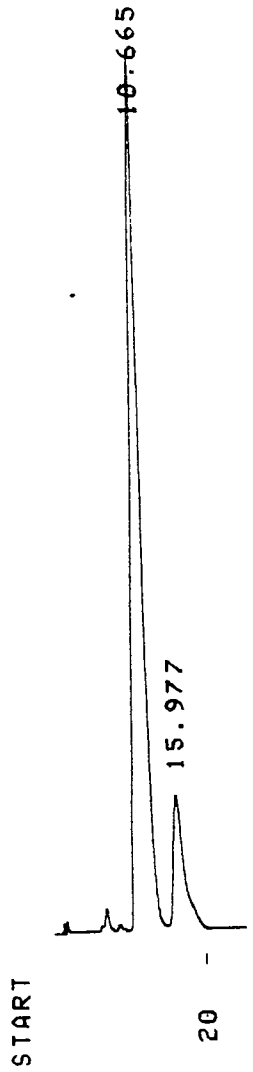


Figure 8.3: *Chromatogram showing the separation of diastereoisomeric 4,6-diamino-2-ethyl-1,2-dihydro-1-(2-iodophenyl)2-methyl-s-triazine*

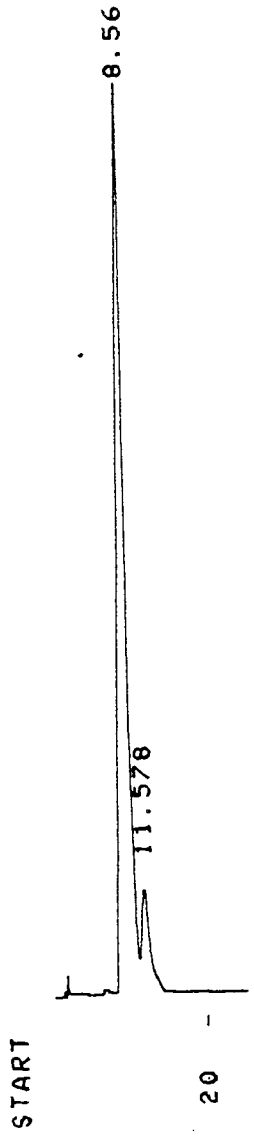


Figure 8.4: *Chromatogram showing the separation of diastereoisomeric 4,6-diamino-2-ethyl-1,2-dihydro-2-methyl-1-(2-methylphenyl)-s-triazine*

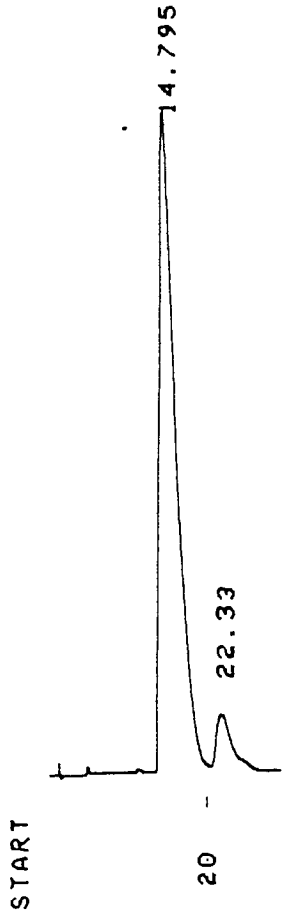
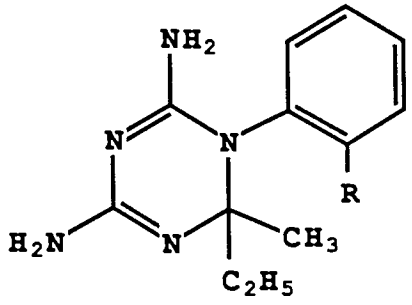


Figure 8.5: *Chromatogram showing the separation of diastereoisomeric 4,6-diamino-2-ethyl-1-(2-ethylphenyl)-1,2-dihydro-2-methyl-s-triazine*

Table 8.2: *Chromatographic parameters of five diastereoisomeric 1-aryl-1,2-dihydro-s-triazines.*



R		yield(%)	d.e.(%)	t_R (min)	k	α
Br	(major)	78.9	99	8.997	6.42	1.60
	(minor)	17.8	74	13.692	10.30	
Cl	(major)	79.9	98	8.447	5.92	1.60
	(minor)	18.7	87	12.797	9.49	
I	(major)	83.0	98	10.665	7.76	1.56
	(minor)	14.8	88	15.977	12.12	
Me	(major)	87.7	-	8.560	5.99	1.41
	(minor)	11.1	-	11.578	8.45	
Et	(major)	92.6	-	14.795	11.11	1.55
	(minor)	6.5	-	22.330	17.27	

Key: d.e. = diastereoisomeric excess, after enrichment; t_R= retention time; k = capacity factor; α= selectivity.

8.4 Analysis of the enriched atropisomers by ^1H n.m.r. spectroscopy.

All the chromatograms showed that the diastereoisomeric mixtures could be separated into two components. However,

this evidence alone was not sufficient to prove that the two peaks in each chromatogram were in fact the diastereoisomeric atropisomers. Therefore positive identification of each peak was essential.

During the preliminary studies, an important experiment was conducted on the separated components. The major peak of one of the diastereoisomers was collected and the fraction was concentrated by rotary evaporation. When this concentrate was injected onto the column, a single peak was observed at the same retention time. The concentrate was then subjected to heat at 75°C for 15mins and the cooled concentrate was re-injected. This time, a peak for the minor diastereoisomer appeared with the isolated major peak. The retention time of the anilino-isomer was longer than that of the two peaks, suggested that Dimroth rearrangement did not occur during heating. This observation shows that the two peaks are interconvertible by thermal activation; hence it suggests strongly that the two peaks may belong to the atropisomers.

The major and minor atropisomers are distinguishable by ^{1}H n.m.r.. Figures 8.6-8.8 show the ^{1}H n.m.r. spectra of three diastereoisomeric atropisomers **(59), (58) and (60),** prior to enrichment by semi-preparative h.p.l.c. Table 8.3 summarises the chemical shifts and splitting pattern of the compounds.

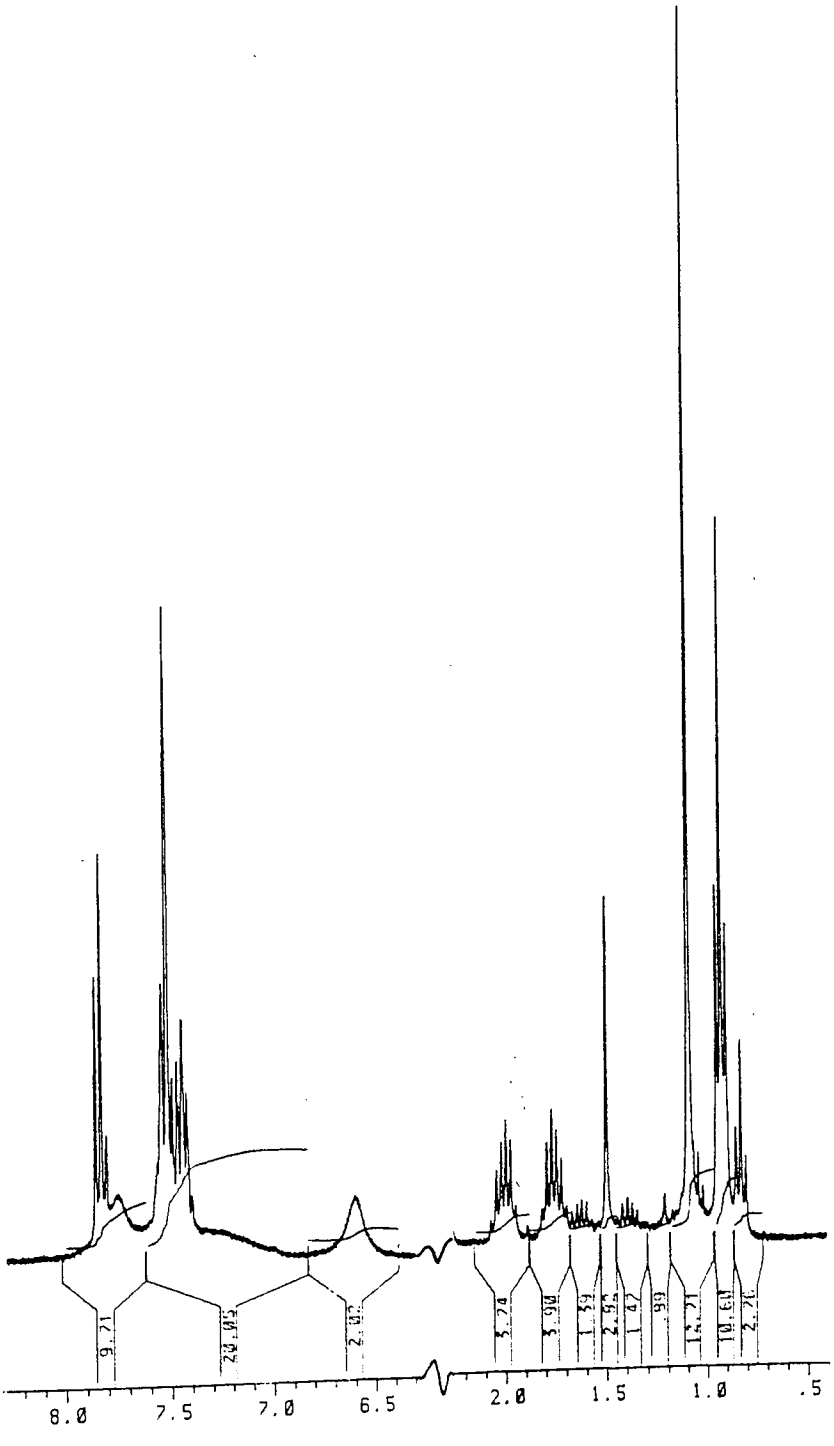


Figure 8.6: *^{1}H n.m.r. spectrum of 4,6-diamino-1-(2-bromophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine* ***(58)***

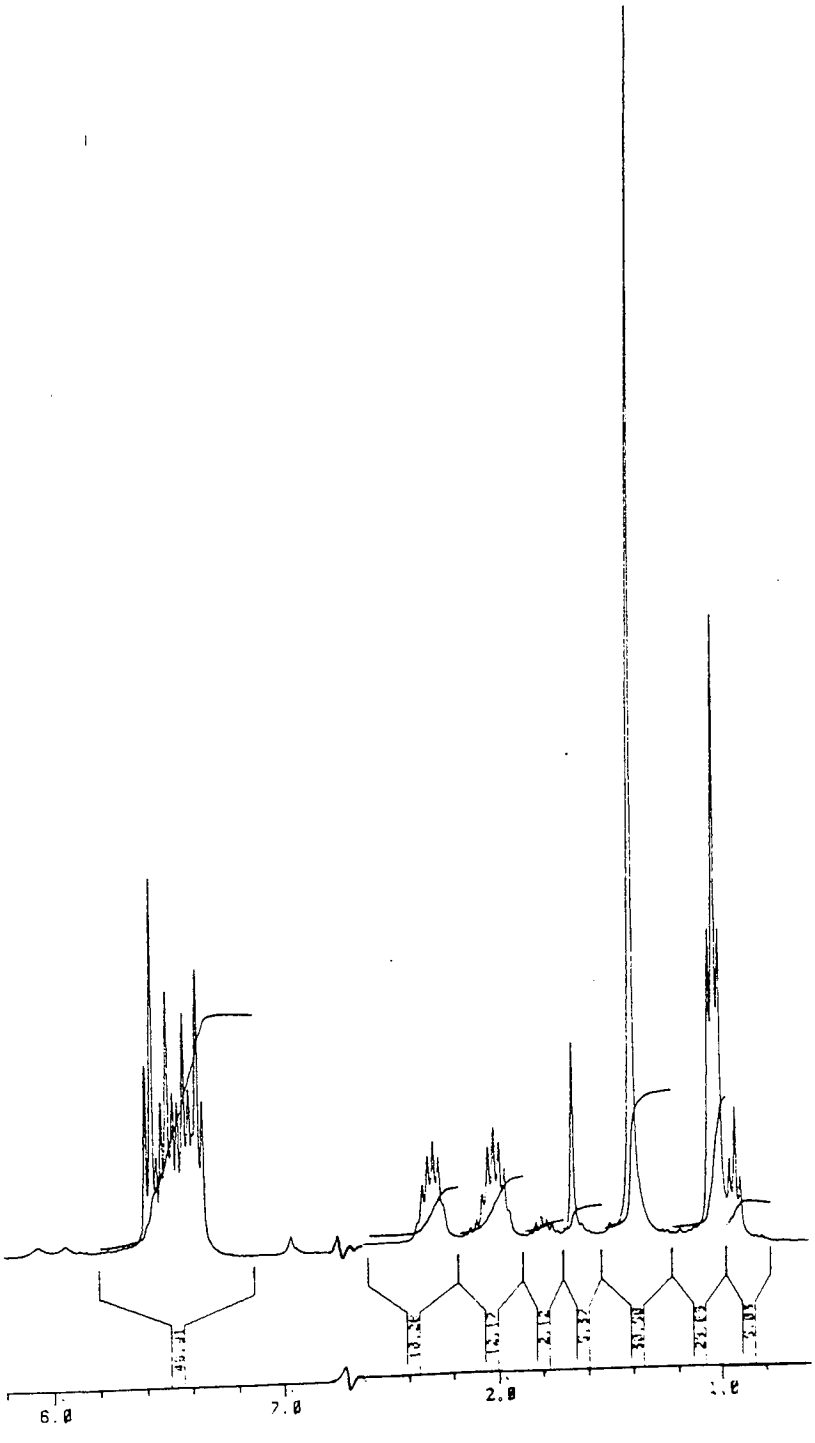


Figure 8.7: *^{1}H n.m.r. spectrum of 4,6-diamino-1-(2-chlorophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine* ***(59)***

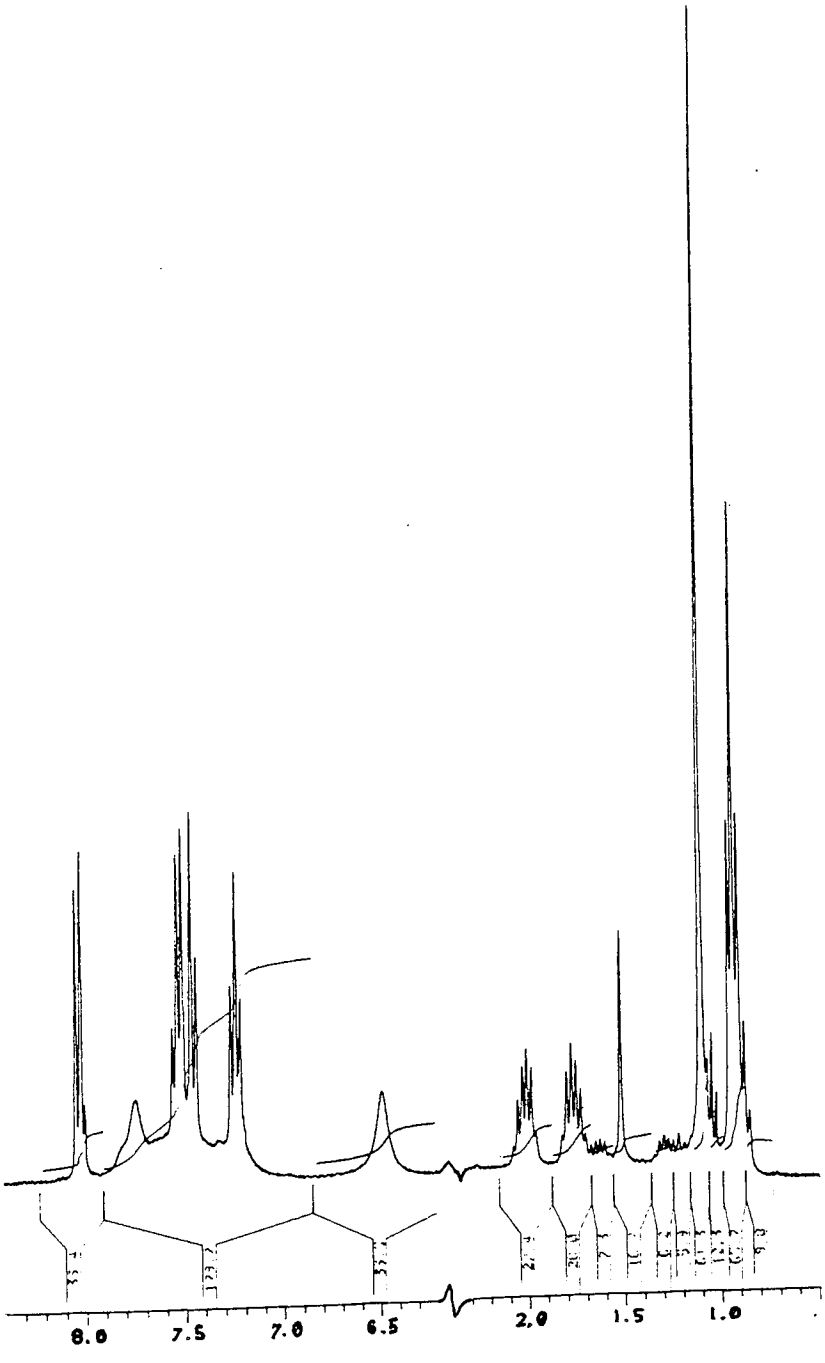
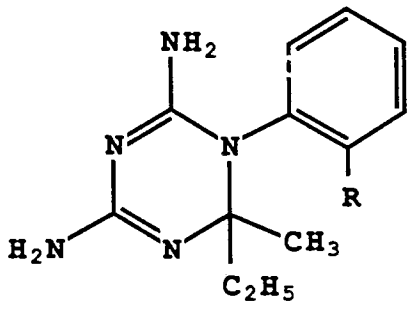


Figure 8.8: *^{1}H n.m.r. spectrum of 4,6-diamino-2-ethyl-1,2-dihydro-1-(2-iodophenyl)2-methyl-s-triazine* ***(60)***.

Table 8.3: *Chemical shifts and splitting patterns of 4,6-diamino-2-ethyl-1,2-dihydro-2-methyl-1-(ortho-substituted phenyl)-s-triazine hydrochlorides.*



R	^{1}H Chemical shifts (ppm)	
	major	minor
Br[b]	7.83-7.40 (4H,br.m,Ar<u>H</u>)	
	2.01 (1H,dq,C<u>H</u>$_2$CH$_3$)	1.67-1.57 (1H,br.m,C<u>H</u>$_2$CH$_3$)
	1.76 (1H,dq,C<u>H</u>$_2$CH$_3$)	1.42-1.35 (1H,br.m,C<u>H</u>$_2$CH$_3$)
	1.09 (3H,s,C<u>H</u>$_3$)	1.50 (3H,s,C<u>H</u>$_3$)
	0.92 (3H,t,CH$_2$C<u>H</u>$_3$)	0.83 (3H,t,CH$_2$C<u>H</u>$_3$)
Cl[a]	7.89-7.44 (4H,m,Ar<u>H</u>)	
	2,29 (1H,dq,C<u>H</u>$_2$CH$_3$)	2.10 (1H,dq,C<u>H</u>$_2$CH$_3$)
	2.09 (1H,dq,C<u>H</u>$_2$CH$_3$)	1.88 (1H,dq,C<u>H</u>$_2$CH$_3$)
	1.50 (3H,s,C<u>H</u>$_3$)	1.75 (3H,s,C<u>H</u>$_3$)
	1.12 (3H,t,CH$_2$C<u>H</u>$_3$)	1.02 (3H,t,CH$_2$C<u>H</u>$_3$)

I[b]	8.04-7.25 (4H,m,Ar<u>H</u>)		
	2.02 (1H,dq,C<u>H</u>$_2$CH$_3$)	1.68-1.60(1H,br.dq,C<u>H</u>$_2$CH$_3$)	
	1.76 (1H,dq,C<u>H</u>$_2$CH$_3$)	1.32-1.19 (1H,br.dq,C<u>H</u>$_2$CH$_3$)	
	1.10 (3H,s,C<u>H</u>$_3$)	1.52 (3H,s,C<u>H</u>$_3$)	
	1.05 (3H,t,CH$_2$C<u>H</u>$_3$)	0.93 (3H,t,CH$_2$C<u>H</u>$_3$)	
CH_3[b]	7.43-7.33 (4H,br.m,Ar<u>H</u>)		
	2.18 (3H,s,ArC<u>H</u>$_3$)	2.50 (3H,s,ArC<u>H</u>$_3$)	
	2.02 (1H,dq,C<u>H</u>$_2$CH$_3$)	1.57-1.48 (1H,br.m,C<u>H</u>$_2$CH$_3$)	
	1.74 (1H,dq,C<u>H</u>$_2$CH$_3$)	1.39-1.35 (1H,br.m,C<u>H</u>$_2$CH$_3$)	
	1.28 (3H,s,C<u>H</u>$_3$)	1.02 (3H,s,C<u>H</u>$_3$)	
	0.94 (3H,t,CH$_2$C<u>H</u>$_3$)	0.82 (3H,t,CH$_2$C<u>H</u>$_3$)	

Key: (a) CF_3COOD was used as the solvent for this compound. (b) DMSO-d_6 was used as the solvent for these compounds.

The aromatic protons of all the compounds appear as a multiplet. There are two populations of aliphatic protons signals, each belonging to one of the diastereoisomeric atropisomers; this observation is consistent with the results obtained from chromatographic resolution. The 2-methyl group appears as a singlet while the 2-ethyl group appears as a triplet and two multiplets. The two multiplets belong to the diastereotopic methylene protons in the ethyl substituent. Each multiplet is in fact a quartet which has been split up by geminal coupling (geminal coupling constant is on the average about 14.3 Hz). It should be mentioned that within each diastereoisomeric signal there exists a pair of

enantiomeric atropisomers which are not distinguishable by achiral ^{1}H n.m.r. spectroscopy.

Figure 8.7 shows the ^{1}H n.m.r. spectrum of compound **(59)**. In the mixture spectrum, the major and minor diastereoisomeric atropisomers appear as two populations of signals. In the enriched diastereoisomeric atropisomer spectra, only one set of the two populations of signals is seen **(Figure 8.9)**. These results confirm positively that the two components of the chromatograms are in fact the diastereoisomeric atropisomers.

Both chromatographic resolution and ^{1}H n.m.r. studies have shown that the each compound is made up of a pair of diastereoisomeric atropisomers of which one is more stable than the other at thermal equilibrium. In order to identify the more stable atropisomer, a Nuclear Overhauser Effect (NOE) experiment was carried out using compound **(61)**.

In the first step of the experiment, an off resonance irradiation was made at frequency 6267 Hz. Then the singlet which belongs to $ArCH_3$ at δ 2.18 was irradiated at frequency 6535 Hz. The spectra were superimposed and the signals of the former spectrum was subtracted from the signals of the latter spectrum. The resultant spectrum shows the enhancement of the signal belonging to a group of protons which lies very closely in space to the irradiated protons. In this case, a 10% enhancement was observed at δ 1.03 which

belong to the 2-methyl group **(Figure 8.10)**. This result shows that the 2-methyl group must lie very closely in space to the $ArC\underline{H}_3$ group. Therefore, the more stable atropisomer adopts the *cisoid* conformation. In a reciprocal experiment in which the 2-methyl group at δ1.02 was irradiated, a 10% enhancement was observed in the singlet at δ 2.18 **(Figure 8.10)**. Thus this second observation confirms the fact that the *cisoid* form is more stable. In conclusion, the atropisomers bearing the configurations (R,aS) and (S,aR) are more stable than the (R,aR) and (S,aS) atropisomers and therefore exist in greater proportion.

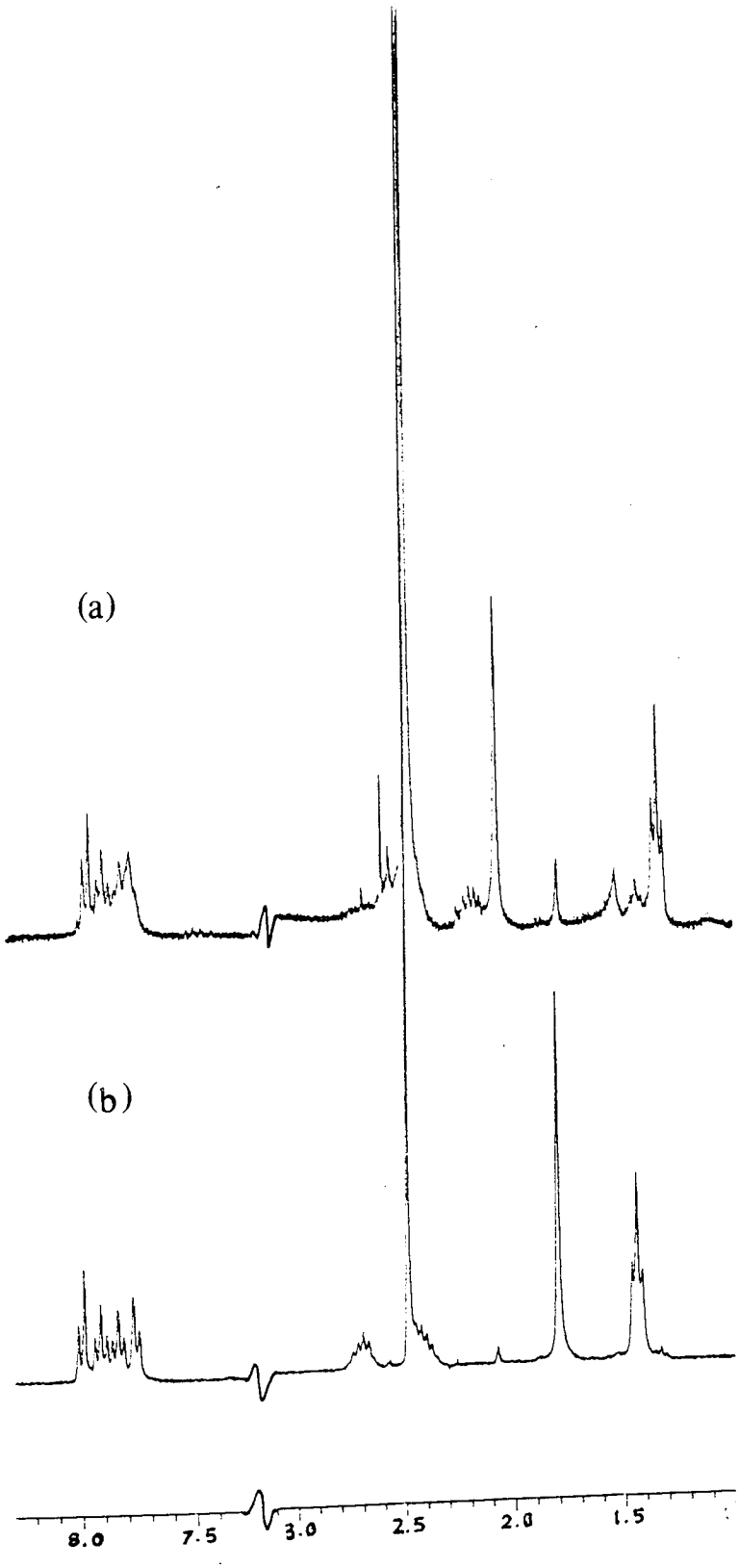


Figure 8.9: *(a) ^{1}H n.m.r. spectrum of the enriched minor component of compound* ***(59)*** *(b) ^{1}H n.m.r. spectrum of the enriched major component of compound* ***(59)***

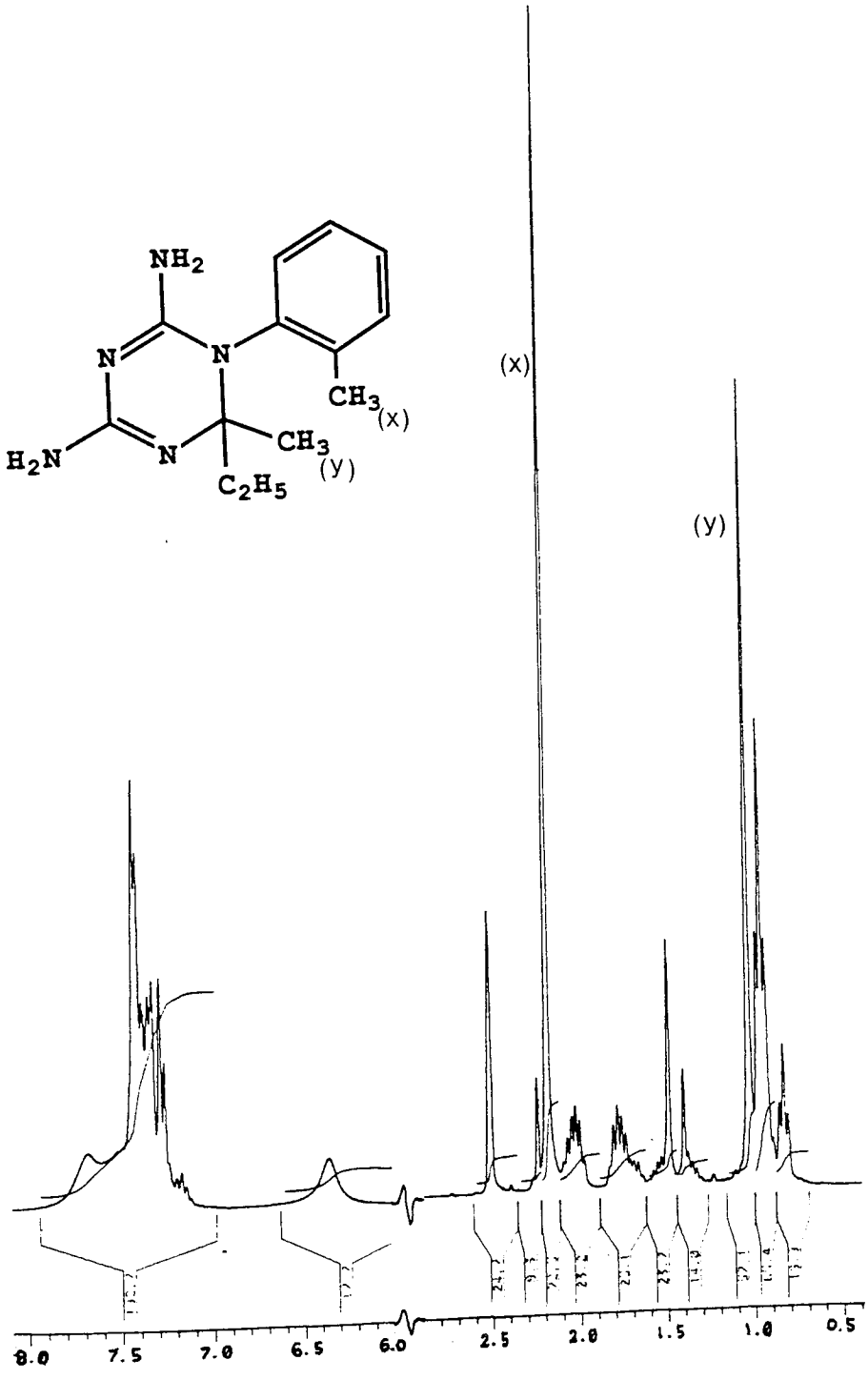


Figure 8.10: *(a) ^{1}H n.m.r. spectrum of 4,6-diamino-2-ethyl-1,2-dihydro-2-methyl-1-(2-methylphenyl)-s-triazine*

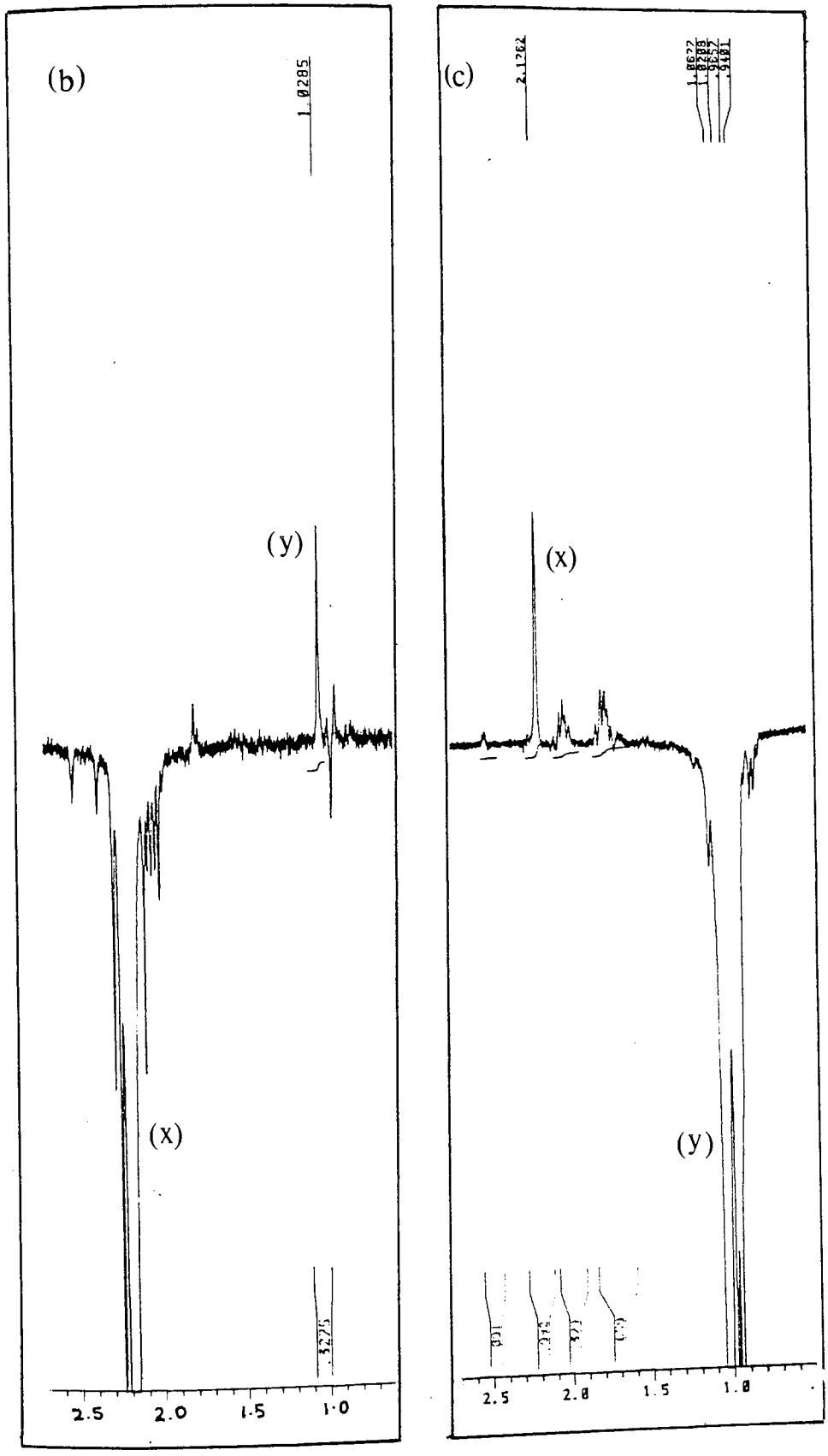


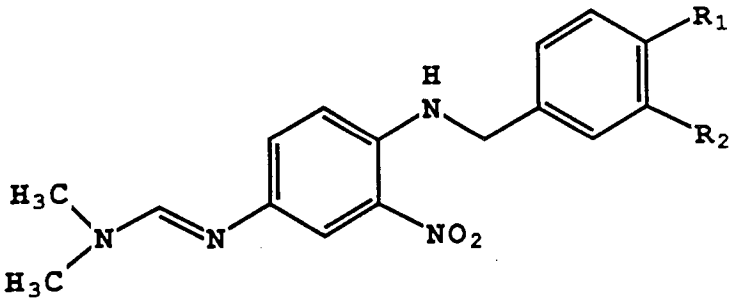
Figure 8.10: *(b) 10% enhancement of signal at δ1.0285 (c) 10% enhancement of signal at δ2.1762*

Chapter 9

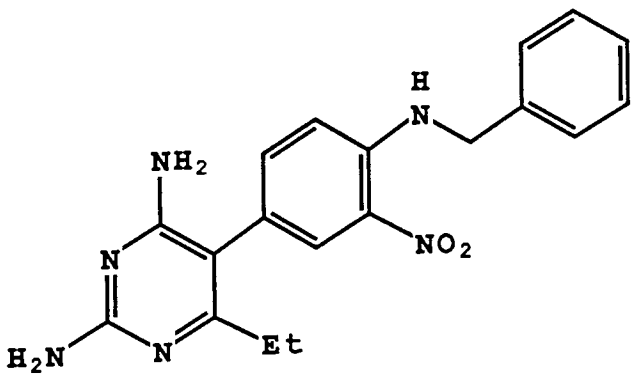
Synthesis of novel phenylamidines as potential DHFR inhibitors.

9.1 Introduction

The objective of this section of work is to investigate the possibility of retaining DHFR inhibitory activity by replacing the classical 2,4-diamino heterocyclic moiety with an amidinyl group. Target molecules (36) and (37) were designed from the lead compound, methylbenzoprim (35).

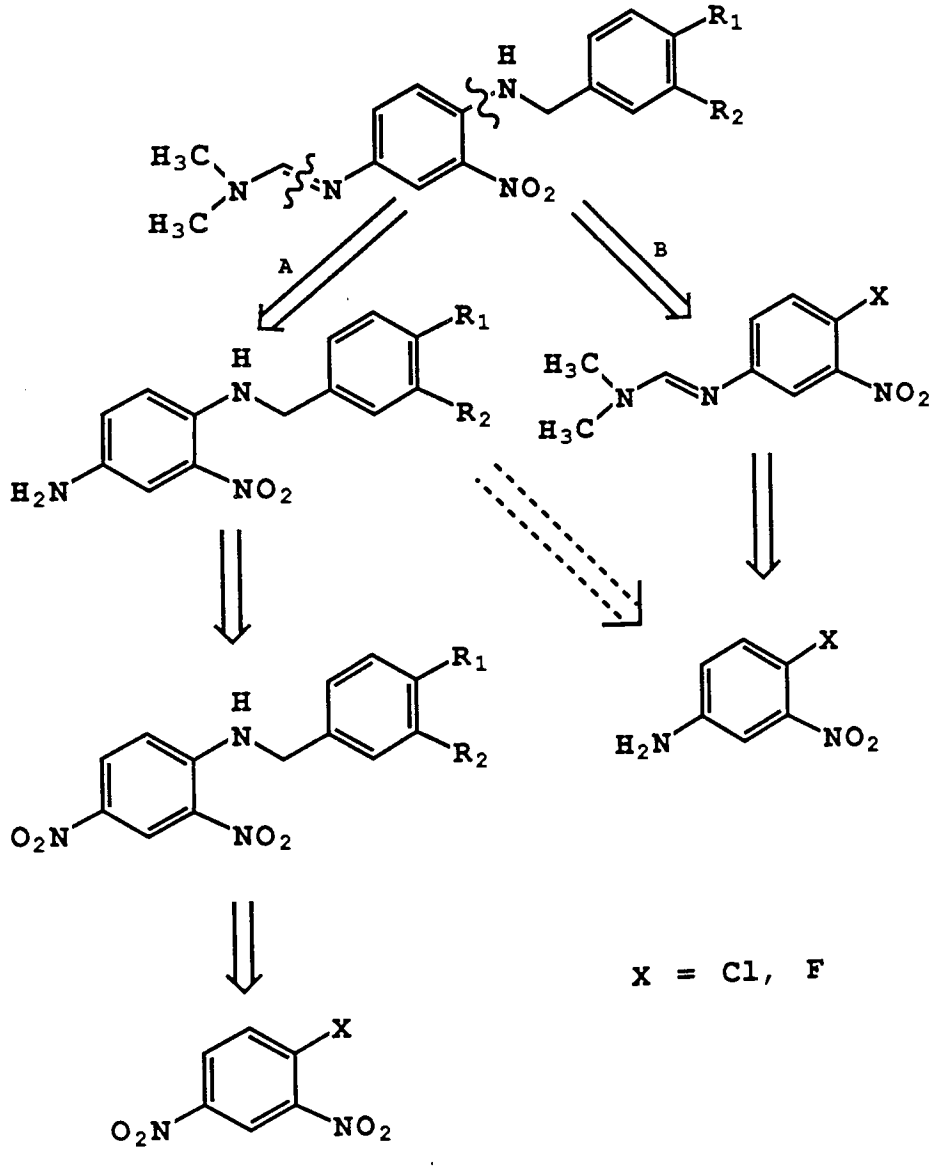


$R_1 = R_2 = H$ (36)
$R_1 = R_2 = Cl$ (37)



(35)

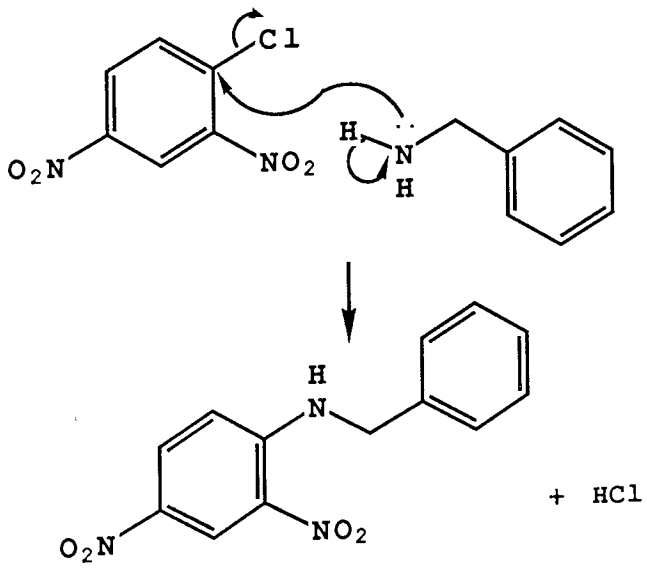
The following scheme illustrates the retrosynthesis of the target molecules. The target molecules can be disconnected at positions A or B; hence this suggests two possible synthetic routes. Both synthetic pathways A and B were attempted and the results of each synthesis are discussed in the sections which follow.



Scheme 9.1: *Retrosynthesis of N-benzylamino phenylamidines (36) and (37).*

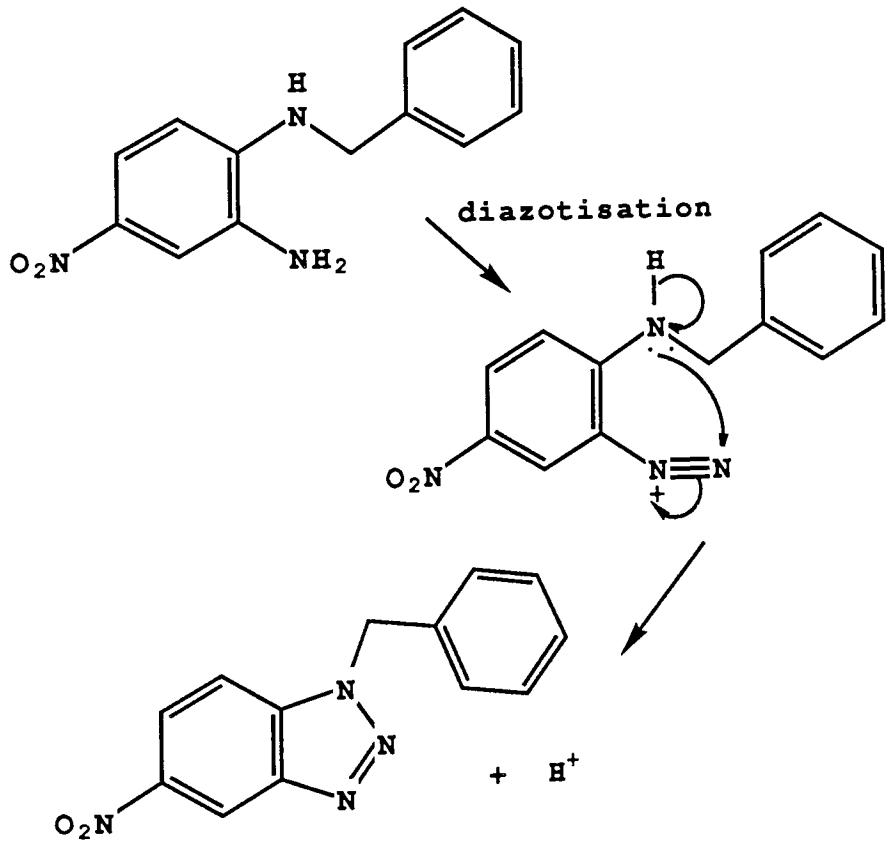
9.2. Synthetic scheme A

In the first step of synthetic Scheme A, 1-chloro-2,4-dinitrobenzene was reacted with benzylamine to give the product N-benzyl-2,4-dinitroaniline **(62)**. This aromatic nucleophilic substitution is activated by the two nitro groups on the benzene ring. The electron-withdrawing nature of the nitro groups exerts a -M effect which has the ability to stabilise the intermediate carbanion and facilitate the nucleophilic substitution. This reaction was found to be more favourable in a polar solvent such as 2-ethoxyethanol than in a less polar solvent such as toluene. Despite the use of excess benzylamine and 3,4-dichlorobenzylamine, the yields obtained were only 52.7% and 52.6% respectively. A slightly lower yield (39.0%) was obtained when N-methylbenzylamine was used. This may be due to the steric hindrance of the *ortho*-nitro substituent with the N-methyl group.



(62)

Selective reduction of the nitro groups was achieved by the use of sodium hydrosulphide.[105] However, when the reduced product was diazotised and treated with β-naphthol, there was no azo dye formation. An explanation for this observation is that the *ortho*-nitro group is reduced rather than the *para*-nitro group. When the diazotised solution was neutralised with sodium acetate, the *ortho*-diazonium ion ring closed to form a benzotriazole compound **(63)**. The mass spectral analysis of compound **(63)** shows a molecular ion of 254 which supports the identity of the benzotriazole. Scheme A was therefore abandoned since the *para*-nitro group could not be reduced selectively.

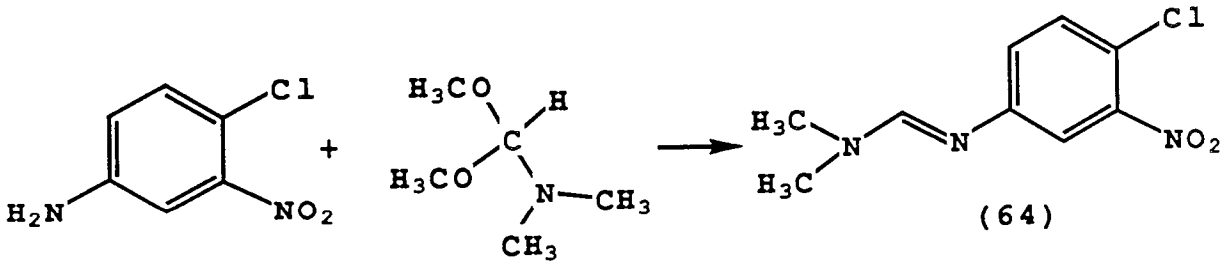


(63)

Scheme 9.3: *Benzotriazole formation from the diazotisation reaction.*

9.3 Synthetic scheme B

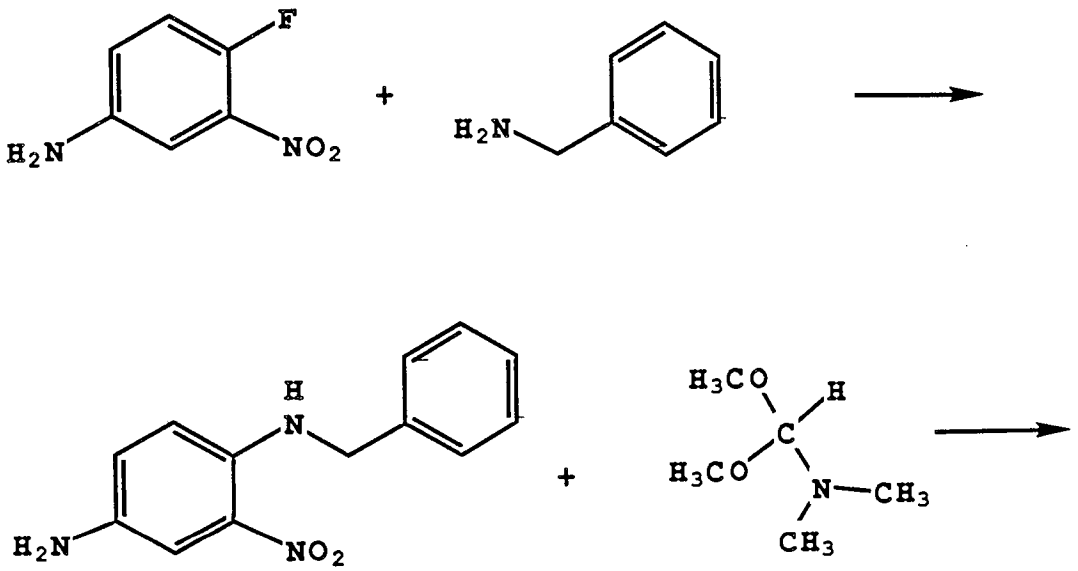
It was found that an almost quantitative yield (98.9%) of dimethylformamidine **(64)** was obtained when N,N-dimethylformamide dimethyl acetal was reacted with 4-chloro-3-nitroaniline. Compound **(64)** was then used in the substitution reaction with benzylamine to obtain target molecule **(36)**. However this reaction produced a low yield and some of the starting material was hydrolysed to 4-chloro-3-nitroaniline.



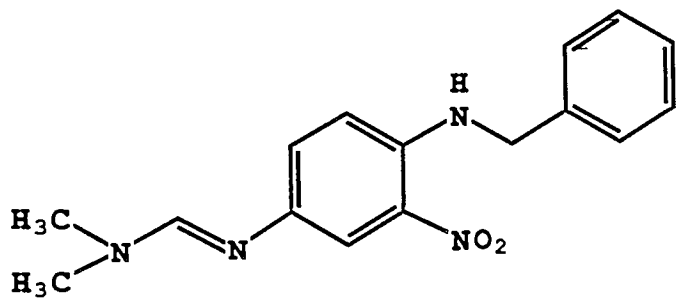
Scheme 9.4: *Phenylamidine synthesis using N,N-dimethylformamide dimethyl acetal as starting material.*

An alternative route **(Scheme 9.1, dotted arrow)** was to first react 4-fluoro-3-nitroaniline with benzylamine to yield 4-benzylamino-3-nitroaniline **(65)**. This compound when purified produced needle-shaped red crystals which gave a positive azo dye test. The amine was then refluxed in N,N-dimethylformamide dimethyl acetal to form the target molecule **(36)** which was a red oil. The hydrochloride salt of this

compound was formed by adding concentrated HCl to a solution of compound **(36)** in isopropanol. The bright orange salt was used in the enzyme inhibition assay. Target molecule **(37)** was also synthesised successfully by this route.



(65)



(36)

Scheme 9.5: *Synthesis of 4-benzylamino-3-nitro-N,N,-dimethylphenylamidine.*

Chapter 10

Dihydrofolate reductase inhibition assay

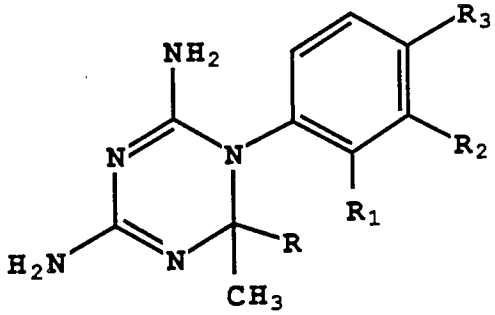
10.1 Inhibitory activity of 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines against the rat liver DHFR.

1-Aryl-1,2-dihydro-*s*-triazines are found to be potent inhibitors of vertebrate DHFR but are only weakly active against the bacterial enzyme. For the assay in this study, DHFR extracted from the liver of Wistar rats was employed.[107] The analogues of cycloguanil were tested against the rat liver DHFR enzyme for inhibitory activity. The enriched diastereoisomeric atropisomers were tested for differential affinity for the rat liver DHFR using the the same assay. The results of the assays conducted are summarised in Table 10.1.

Cycloguanil **(28)** is a potent inhibitor of DHFR and in this particular assay it was used as the reference compound. The I_{50} value for cycloguanil is 0.16 μM. When the *para*-chloro group is removed, there is a decrease in activity and the I_{50} value becomes 1.49 μM. There is a postulation that the active site cleft of the mammalian DHFR is larger along the heterocycle-phenyl axis of the inhibitor, and therefore can accommodate a bulky *para* substituent better. Indeed, the crystal structure of the active site cleft in chicken liver DHFR[84] is found to be 1.5-2.0 Å wider than that of the

bacterial enzyme. Matthews and co-workers[108] have reported that in addition to the several polar interactions between phenyltriazine and DHFR, numerous van der Waal contacts occur as well. When a phenyltriazine is bound to a vertebrate DHFR active site, the positioning of the phenyl ring is apparently influenced by three residues in helix αC, namely Thr-56, Ser-59 and Ile-60 **(Figure 10.1)**. Perhaps the *para*-chloro substituent interacts with these residues on helix αC via van der Waal forces to give the inhibitor a better holdfast in the active site.

Table 10.1: *I_{50} values of some 4,6-diamino-1-aryl-1,2-dihydro-s-triazines against the rat liver DHFR.*



Compound No.	R	R_1	R_2	R_3	I_{50} (μM)
38	CH_3	H	H	H	1.49 [a]
39	CH_3	H	NO_2	H	0.26
28	CH_3	H	H	Cl	0.16
46	CH_3	H	NO_2	Cl	0.003
48	CH_3	H	N_3	Cl	0.55
57	C_2H_5	H	H	Cl	2.20
58	C_2H_5	Br	H	H	1103.0
59	C_2H_5	Cl	H	H	1183.0
60	C_2H_5	I	H	H	553.0

a: Data from ref 64.

Figure 10.1: *Schematic representation of 1-aryl-1,2-dihydro-s-triazine binding to chicken DHFR.*[108]

The absence of the chloro substituent in compound **(38)**, therefore causes the inhibitor molecule to lose this contact and together with it, some degree of binding affinity. However, when the *meta* position is substituted with a nitro group, as in compound **(39)**, there is a gain in activity (I_{50} value is 0.26 μM). This result suggests that the *meta*-nitro group must have some contact with the protein residue such that the binding affinity of the inhibitor molecule is enhanced. Indeed it was found that a large substituent at the C3' position of the phenyl ring does bind in a narrow channel which leads from the extreme upper position of the active site to the protein surface.[108] This channel is

bounded primarily by five bulky hydrophobic residues (Tyr-31, Phe-34, Ile-60, Pro-61 and Leu 67) which account for most of the direct protein ligand interactions with large C3' substituent. When positions C3' and C4' are substituted with NO_2 and Cl respectively, the molecule becomes a very potent DHFR inhibitor and it has an I_{50} value of 0.003 μM. This must be due to the additive effects of the interaction of positions C3' and C4' with the protein residues in the enzyme. In compound **(48)**, the *meta*-substituent is replaced by an azido function which is rigid and almost linear in configuration[66]. This azido group must encounter unfavourable contact with the narrow channel and activity is thus slightly compromised.

When one of the 2,2-dimethyl groups was replaced by an ethyl group, as in compound **(57)**, the activity of the new compound is reduced about 14 fold. Compound **(57)** is enantiomeric and the ethyl substituent at C2 can adopt either an equatorial or an axial configuration. When the ethyl substituent is equatorial, it points downwards towards the carboxamide group of the coenzyme NADPH and this gives rise to unfavourable steric hindrance. On the other hand, when the methyl group is equatorial, this steric clash is avoided. This shows that the enzyme exhibits preferential binding to one of the two enantiomers hence the inhibitory activity of compound **(57)** is reduced.

Ortho-substituted compounds **(58-60)** show a marked decrease in activity compared with the *para*-substituted triazine **(57)**. These observations are very similar to the *ortho*-substituted 2,2-dimethyl triazines which Baker and co-workers[64] have reported from their studies. These observations may be explained by the following reasons.

X-ray crystallographic studies have shown that when 1-aryl-1,2-dihydro-*s*-triazine binds to the active site of the avian DHFR[84], the triazine ring and the phenyl ring are nearly perpendicular to each other. The triazine ring lies in the same position as does the pyrimidine ring of methotrexate. The phenyl ring, on the other hand, occupies the same position as the pyrazine ring of methotrexate which is just outside the pyrimidine pocket. The *ortho*-substituent on the phenyl ring collides with the nicotinamide moiety of NADPH.[26] In addition, the side chain of Leu-22 is positioned adjacent to the lower edge of the inhibitor's phenyl group and this may present steric hindrance with the *ortho*-substituent as well.[108] These unfavourable contacts cause the inhibitor molecule to bind less tightly to the active site and as a result, inhibitory activity is reduced.

Moving the halide substituent from the *para*-position to the *ortho*-position on the phenyl ring also shortens the heterocycle-phenyl axis. This modification, as discussed above, causes the inhibitor molecule to lose some binding

energy hence activity is reduced as observed.

Another explanation for this anomalous observation is the fact that each of these compounds is made up of four stereoisomers. These four stereoisomers may exhibit differential affinity for the enzyme; that is, the enzyme may stereoselectively accommodate only one of the four stereoisomers.

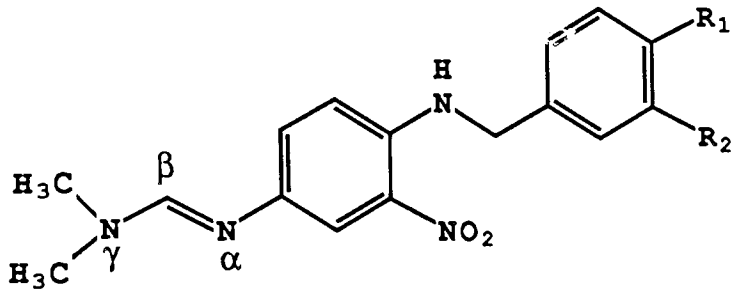
The inhibitory activity of the enriched diastereoisomeric atropisomers against the rat liver DHFR was investigated using the same assay. Due to the low solubility of the salt and the small quantity obtained from the collection, only two concentrations of the inhibitor were employed. These concentrations were extrapolated from the calibration curves of each compound . The enzyme assay results show that, in all cases, the minor diastereoisomeric atropisomers have greater affinity for the enzyme than the major atropisomers. However, it cannot be explained rationally why the minor atropisomers are relatively more active than the major atropisomers. It is noteworthy that the enriched diastereoisomeric atropisomers each contains a pair of enantiomeric atropisomers. The assay employed does not have the ability to distinguish the activity of these enantiomers, hence no conclusion can be made as regards to the activity among the four stereoisomers.

10.2 Screening of novel phenylamidines for DHFR inhibitory activity

The two novel phenylamidines were screened for DHFR inhibitory activity using the same enzyme assay as above. Compound **(36)** did not show any inhibition at the final concentration of about 333 μM. and is therefore considered non-active against the rat liver DHFR. However, compound **(37)** did indicate some degree of inhibition against the rat liver enzyme and its I_{50} value was found to be 167 μM.

The lead compound methylbenzoprim **(35)** has been shown to adopt a perpendicular conformation when it binds to the active site of *Lactobacillus casei* DHFR.[68] However, these phenylamidines cannot adopt this conformation because of resonance stabilisation. In fact, the phenyl ring and the amidinyl group must be coplanar in conformation. The planar phenyl ring is, presumably, unable to interact favourably with the hydrophobic domain hence tight binding is compromised. On the other hand, the 3,4-dichlorobenzyl moiety in compound **(37)** may still be able to interact with the *para*-aminobenzoyl binding region of the enzyme. This weak interaction may be sufficient to support the molecule in the active site cleft to cause a small degree of inhibition.

Table 10.2: *I_{50} values of phenylamidines **(36)** and **(37)** against the rat liver DHFR.*



Compound no.	R_1	R_2	I_{50} (μM)
36	H	H	-
37	Cl	Cl	167

At physiological pH, it is probable that protonation occurs at γN. Protonated N1 atom and the 2-NH_2 group in 2,4-diamino heterocycles are known to form a salt bridge with Glu-30 in vertebrate DHFR.[69] This is an essential interaction and it is believed to occur in all antifolates when they bind to the enzyme DHFR. The γN of the phenylamidines is believed to mimic this N1 atom, however salt bridge formation is weak because only one hydrogen bond can be formed with Glu-30. Presumably it is this weak ionic interaction which is responsible for the inactivity of compound **(36)** and the marginal inhibitory activity of compound **(37)**. In addition, the bulk of the dimethyl groups attached to the γN may

displace the molecule laterally so that hydrogen bonding with Glu-30 is less probable. This inability to form strong hydrogen bonds with the carboxylate group of Glu-30 may be the other attribute to the inactivity observed.

Chapter 11

Conclusion and possible future work

11.1 Optical resolution of enantiomeric 1,2-dihydro-*s*-triazines by chiral liquid chromatography.

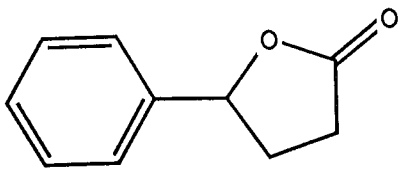
The work in this thesis has shown that diastereoisomeric 4,6-diamino-2-ethyl-1,2-dihydro-2-methyl-1-(*ortho*-substituted phenyl)-*s*-triazines are resolvable using C18 reversed phase h.p.l.c.. However, attempts to resolve the enantiomeric 2,2-dimethyl-1,2-dihydro-*s*-triazines did not proceed successfully *via* diastereoisomeric salt formation. Perhaps the use of chiral liquid chromatography is the solution to this problem.

There are a wide variety of chiral stationary phases (CSP) available for the resolution of enantiomers. The choice of CSP to use depends very much on the structure and the physico-chemical properties of the enantiomers in question. The application of microcrystalline cellulose triacetate[109,110] (MCT) in the resolution of twisted molecules[111] has been well documented.

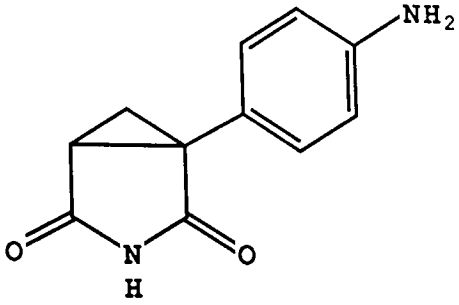
Cellulose is an unbranched polymer of D-(+)-glucose residues joined by α-1,4-linkages. These chains lie side by side in bundles and are held together by hydrogen bonds between the numerous neighbouring hydroxyl groups. These bundles become twisted together to form rope-like helical

structures into which enantiomers can partition and interact stereospecifically. It was found that triacetate derivatives of cellulose show good chiral recognition when used in its microcrystalline form[112]. Okamoto found that when MCT was deposited on silica gel, its chiral recognition property was retained.[113] Such MCT stationary phase is used successfully for the separation of enantiomeric molecules. Enantiomers bearing an aromatic moiety are likely to be separated by MCT stationary phase. The 'fit' of the aromatic moiety in the helical cavity needs to be reasonably tight. In addition, one of the substituents on the chiral centre needs to be able to interact with the steric environment just outside the cavity.

Twisted molecules such as γ-phenyl-γ-butyrolactone **(68)**, 1-(4-aminophenyl)-1,2-cyclopropanedicarboximide **(69)**[109] and sterically hindered N-aryl-4-pyridones **(70)** have been resolved by the use of MCT-CSP. It is therefore highly possible that *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines may be separable by MCT-CSP.



(68)



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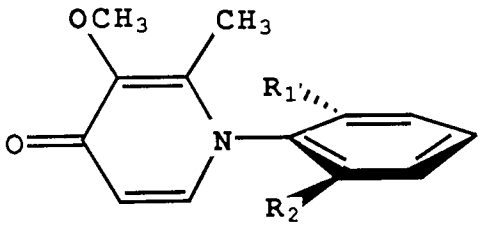


(70)

11.2 H.p.l.c. chiral stationary phase based on immobilised DHFR

α-Chymotrypsin (ACHT) is an enzyme which is composed of 245 L-amino acids. This enzyme contains a distinct active centre which is made up of a hydrophobic pocket and a hydrolytically active site. The binding site has a very specific three-dimensional steric structure. The binding and hydrolysis of a substrate at the active site of ACHT involve combination of hydrophobic, hydrogen bonding and stereochemical interactions.

ACHT has been successfully immobilised on a silica-based support containing covalently bonded glutaraldehyde.[114] This

immobilisation produces a stationary phase which is capable of binding and hydrolysing substrates of ACHT such as L-amino acid amides and esters. Substrate such as DL-tryptophanamide has been separated using this ACHT-CSP; the L-enantiomer is hydrolysed to the free amino acid, while the D-enantiomer is eluted unchanged. The chiral recognition mechanism involves one of the following: (i) chiral separation by diastereoisomeric association with the enzyme and (ii) chiral separation due to preferential hydrolysis of one of the enantiomers.

Perhaps DHFR can be immobilised on silica gel in a similar manner and the DHFR-CSP can then be used to separate enantiomeric DHFR inhibitors. Such DHFR-CSP can also serve as a chiral bioassay for screening enantiomeric antifolates for inhibitory activity.

11.3 Crystal structure of DHFR containing a chiral 1,2-dihydro-*s*-triazine.

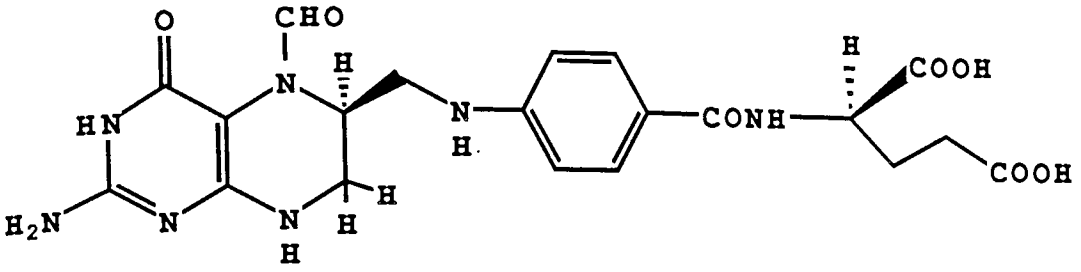
The crystal structure of chicken liver DHFR in a ternary complex with NADPH and 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(4-methoxyphenyl)-*s*-triazine has been determined by Volz and co-workers.[84] In this study, the chicken liver DHFR was isolated, purified and crystallised. Selected crystals were then soaked in a solution containing 0.05M Tris-HCl, 40% ethanol, 1mM NADPH and 3mM of the 1-aryl-1,2-dihydro-*s*-

triazine at pH 8.0 to prepare the ternary complex. This crystalline complex was used in the data collection and structure determination.

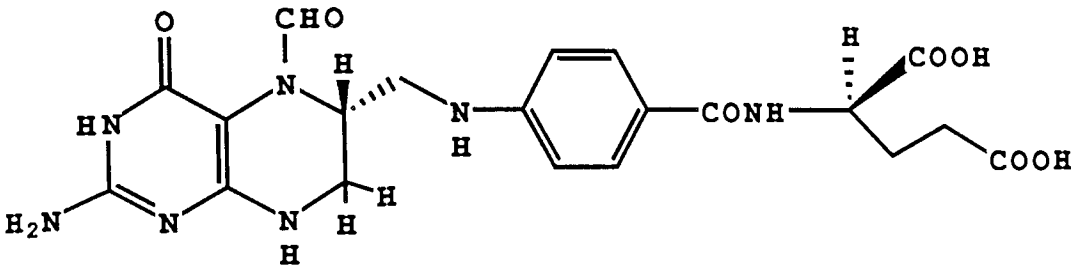
A similar experiment may be carried out using instead an *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazine to form the ternary complex. A 2'-iodo analogue would be preferable because the heavy atom would provide the required additional phasing information for X-ray crystallography. If both the enantiomers were to bind to the enzyme, there would be two populations of ternary complex crystals. The ratio between these two populations would reflect the binding affinity of each enantiomer.

11.4 Separation of the diastereoisomers of *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines

The two diasteroisomers of (6RS,αS)-folinic acid were separated by chromatography and crystallisation.[63] Though both the isomers of folinic acid bind to DHFR, the natural (6S,αS)-isomer **(72)** binds more tightly ($K_a=1x10^8\ M^{-1}$) than the (6R,αS)-isomer **(71)** ($K_a=1x10^4\ M^{-1}$).[63]

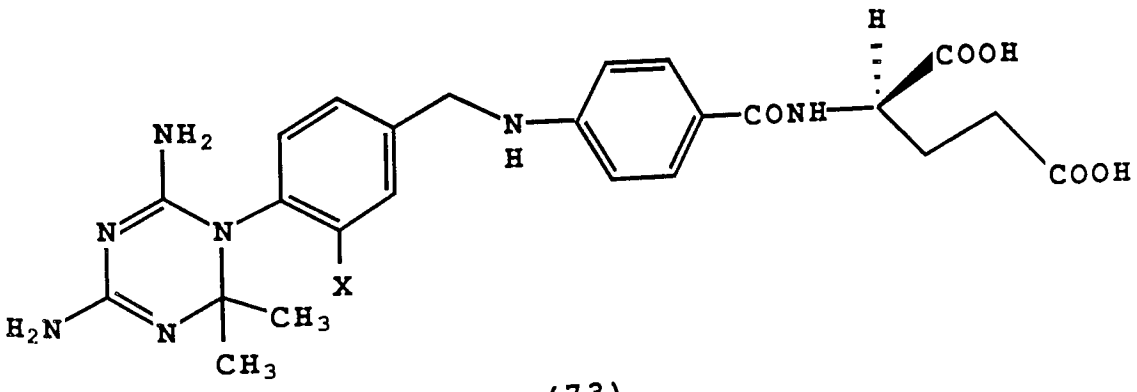


(71)



(72)

It is axiomatic that *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines have reduced inhibitory activity against the enzyme DHFR. However, this lack of activity may be compensated by incorporating the benzoyl moiety and the (αS)-glutamate moiety to the 1-aryl-1,2-dihyro-*s*-triazine molecule, thus giving the following structure:



(73)

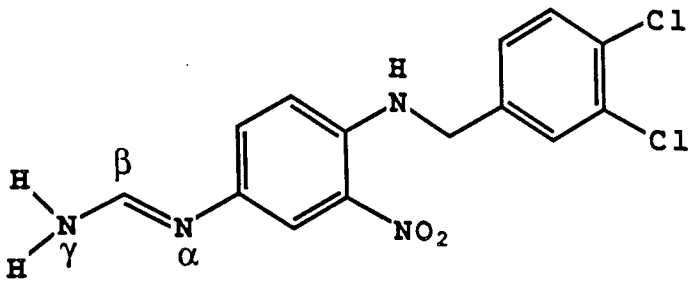
This new atropisomeric molecule will be diastereoisomeric and

therefore should be separable by achiral chromatography. Once resolved, the individual diastereoisomer can be used directly in the enzyme inhibition assay. The use of optically pure (αS)-glutamate eliminates the complexity of producing four stereoisomers, therefore only two diastereoisomers are involved in this case.

11.5 Modifications to phenylamidines

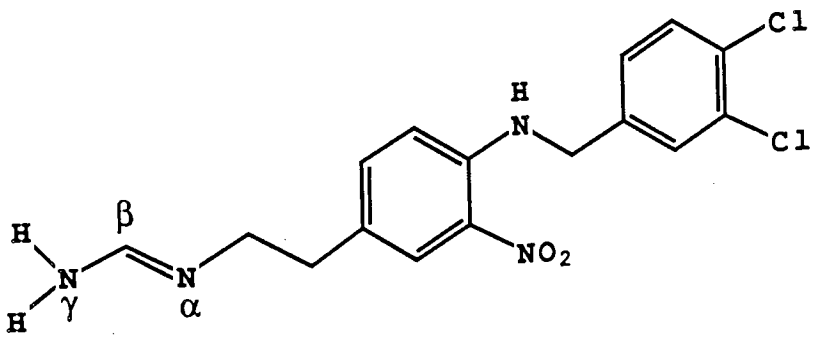
Results from the enzyme inhibition assay indicated that compound **(36)** is inactive while compound **(37)** shows weak inhibition against the rat liver DHFR. This lack of activity is postulated to be the inability of the inhibitor molecules to form strong hydrogen bonds with the residue Glu-30 at the active site. The following modifications to the the existing phenylamidines are warranted.

Firstly, the N,N-dimethyl groups on the amidinyl function may be removed to give the unsubstituted phenylamidine. The elimination of this bulk may enable the molecule to slide further into the active site cleft such that stronger ionic interaction with Glu-30 can occur.



(74)

Since the γN atom in compounds **(36)** and **(37)** is believed to mimic the N1 atom of the classical 2,4-diamino heterocycle, presumably this allows only one, if any, hydrogen bond to be formed at the carboxylate group of Glu-30. Perhaps an introduction of a diatomic spacer group such as $-(CH_2)_2-$, may enable both the αN and γN atoms to reside at the appropriate positions for the formation of the essential salt bridge with the residue Glu-30. Hence it would be worthwhile to synthesise **(75)** and evaluate its inhibitory activity against the rat liver DHFR.



(75)

Section 3: Materials and Methods

Chapter 12

Chemistry of 4,6-diamino-1-aryl-1,2-diamino-1,2-dihydro-*s*-triazines

All m.ps. were measured on an electrothermal digital melting point apparatus and were uncorrected. I.r. spectra were recorded on a Perkin-Elmer 1310 infrared spectrophotometer for potassium bromide discs. U.v. scans were recorded on a Unicam SP 8000 ultraviolet recording spectrophotometer. Mass spectra were recorded on a V.G. Micromass 12 Instrument at 70eV and source temperature of 300°C. ^{1}H n.m.r. spectra were recorded on a Varian EM-360 60 MHz spectrometer with TMS as reference; high resolution ^{1}H n.m.r. spectra were recorded on a 300 MHz Bruker AC spectrometer. T.l.c. were performed using Kieselgel 60 F_{254} as adsorbent and the developing solvent used was methanol:chloroform (1:9; v/v), unless otherwise stated. Elemental analyses stated were within $\pm$0.3% of the theoretical values.

12.1 The three component synthesis

4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(ortho-substituted phenyl)-s-triazine hydrochlorides (40-44)

A mixture of *ortho*-substituted aniline (1.0mole), cyanoguanidine(8.4g,1.0mole), concentrated HCl(5ml,0.055mole) and acetone(100ml) was refluxed for 14h. The precipitate

formed was collected, washed with acetone and dried. The crude products were recrystallised from aqueous ethanol. *4,6-diamino-1-(2-bromophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine hydrochloride (40)*; yield 65.0%; m.p. 212-216°C (lit.217°C); λ_{max}(water) 242nm . *4,6-diamino-1-(2-chlorophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine hydrochloride (41)*; yield 83.5%; m.p. 216-221°C (lit.217-221°C); λ_{max}(water) 241nm. *4,6-diamino-1-(iodophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine hydrochloride (42)*; yield 52.7%; m.p. 204-206°C (lit.207-209°C); λ_{max}(water) 242nm. *4,6-diamino-1-(ethylphenyl)-1,2-dihydro-2,2-dimethyl-s-triazine hydrochloride (43)*; yield 62.0%; m.p.220-221°C (lit.198-199°C); λ_{max}(water) 242nm. *4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2-methylphenyl)-s-triazine hydrochloride (44)*; yield 84.3%; m.p. 224-225°C(lit.223-224°C); λ_{max}(water) 241nm .

4,6-diamino-1-(2-chloro-5-nitrophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine hydrochloride (52)

A mixture of 2-chloro-5-nitroaniline (8.6g,0.05mole), cyanoguanidine (4.6g,0.055mole), concentrated HCl (5ml,0.055mole) and acetone (50ml) was refluxed overnight. A pale yellow precipitate was collected, washed with acetone and dried. The crude product was recrystallised in aqueous isopropanol; yield 75.4%; m.p. 210-212°C; ν_{max} 3300 br. (N-H),3160 br.,1600,1530 (NO_2,asym), 1340 (NO_2,sym); λ_{max}(water) 245nm; δ_H (300MHz,DMSO-d_6) 9.49 (1H,s,N^+-H), 8.38

(1H,d,J_{meta}=2.6Hz,Ar<u>H</u>),8.34(1H,dd,J_{meta}=2.6Hz,J_{ortho}=10.4Hz, Ar<u>H</u>), 8.09 (1H,d,J_{ortho}=10.4Hz,Ar<u>H</u>), 1.58 (3H,s,C<u>H</u>$_3$), 1.22 (3H,s,C<u>H</u>$_3$); M^+ 281[283] (3:1).

4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2-methyl-5-nitrophenyl)-s-triazine hydrochloride (53)

A mixture of 2-methyl-5-nitroaniline (15.2g,0.1mole), cyanoguanidine (8.4g,0.1mole), concentrated HCl (10ml,0.1mole) and acetone (100ml) was refluxed overnight. A colourless crystalline precipitate was collected, washed with acetone and dried. Recrystallisation from aqueous isopropanol gave yellow plate-shaped crystals; yield 80.0%; m.p. 220-222°C; ν_{max} 3300 br.(N-H),3100 br.,1640,1520 (NO_2,asym),1340(NO_2,sym); λ_{max}(water) 243nm; δ_H (300MHz,DMSO-d_6) 9.45 (1H,br.s,N^+-<u>H</u>), 8.27 (1H,d,J_{ortho}=8.5Hz,Ar<u>H</u>), 8.08 (1H,s,Ar<u>H</u>), 7.72 (1H,d,J_{ortho}=8.5Hz,Ar<u>H</u>), 2.28 (3H,s,ArC<u>H</u>$_3$), 1.58 (3H,s,C<u>H</u>$_3$),1.15 (3H,s,C<u>H</u>$_3$); M^+ 276.

4,6-amino-1-(5-amino-2-methylphenyl)-1,2-dihydro-2,2-dimethyl-s-triazine hydrochloride (55)

A catalytic amount of palladium on activated carbon was added to a methanolic solution of compound **(53)** (1.5g) . The suspension was allowed to shake in a hydrogenator at atmospheric pressure. At the end of the reaction, the carbon was filtered and the filtrate was rotary evaporated to dryness. The colourless residue was the reduced product and was used directly in the diastereomeric derivatisation with

(R)-(+)-(α)-methylbenzylamine; yield 95.6%; m.p. 223-224°C; M^+246.

12.2 Synthesis of *meta*-azidocycloguanil

4,6-diamino-1-(4-chloro-3-nitrophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine hydrochloride (46)

A mixture of 4-chloro-3-nitroaniline (8.6g,0.05mole), cyanoguanidine (4.6g,0,055mole), concentrated HCl (5ml) and acetone (100ml) was refluxed overnight. The mixture dissolved into a dark brown solution and a crystalline precipitate was formed after 0.5h. Filtration, washing with acetone and recrystallisation in aqueous ethanol yielded pale yellow needle-shaped crystals; yield 75.0%; m.p. 216-219°C; ν_{max} 3620 (N-H), 3460 (N-H), 1520 (NO_2,asym), 1340 (NO_2,sym) cm^{-1}; λ_{max}(water) 239nm; δ_H (300 MHz,TFA-*d*) 8.13 (1H,br.s,Ar<u>H</u>), 7.92 (1H,br.d,J_{ortho}=8.2Hz,Ar<u>H</u>), 7.72 (1H,br.d,J_{ortho}=8.2Hz,Ar<u>H</u>), 1.84 (3H,s,C<u>H</u>$_3$), 1.78 (3H,s,C<u>H</u>$_3$); M^+ 296[298] (3:1) (Found C,39.72; H,4.02; N,24.96 $C_{11}H_{14}N_6O_2Cl_2$ requires C,39.65; H,4.24; N,25.23).

4,6-diamino-1-(3-amino-4-chlorophenyl)-1,2-dihydro-2,2-methyl-s-triazine (47)

Compound **(46)** (0.5mole) was reduced, under a nitrogen atmosphere, using 5 equiv. of hydrated stannous chloride in absolute ethanol. When the reaction was complete, the ethanol was evaporated and the residue was dissolved in ice

water. 10M NaOH solution was used to basify the solution and the precipitated free base amine was collected, washed with a little water and dried under vacuum at room temperature. The colourless needle-shaped crystals were used directly in the synthesis of compound **(48)**; yield 75.0%; m.p. 124-125°C; ν_{max} 3450 (N-H), 3350(N-H) cm^{-1}; λ_{max}(water) 237nm; δ_H(300 MHz,CF_3COOD) 8.94(2H,s,$N\underline{H}_2$), 7.63(1H,d,J_{ortho}=8.4Hz,Ar$\underline{H}$), 7.49(1H,d,J_{meta}=2.3Hz,Ar$\underline{H}$),7.20(1H,dd,J_{ortho}=8.5Hz,J_{meta}= 2.3Hz,Ar$\underline{H}$),1.38(3H,s,$C\underline{H}_3$), 1.36 (3H,s,$C\underline{H}_3$); M+ 266[268](3:1).

4,6-diamino-1-(3-azido-4-chlorophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine (48)

Compound **(47)** (13.3g,0.05mole) was diazotised by using sodium nitrite (3.7g,0.055mole). Sodium azide (3.0g,0.2mole) was added, in small portions, and stirred for 0.5h. The solution was basified with iced aqueous ammonia. The cream-coloured solid was collected, washed with water and dried under vacuum at room temperature; yield 75.0%; m.p. 130°C; ν_{max} 3440 (N-H), 3300(N-H), 2100(N_3) cm^{-1}; λ_{max}(water) 246nm; δ_H (60MHz, CD_3COOD) 7.58 (1H,d,J_{ortho}=8.4Hz,Ar$\underline{H}$), 7.28 (1H,dd,J_{meta}=2Hz,J_{ortho}=8.3Hz,Ar$\underline{H}$), 7.08 (1H,d, J_{meta}=2.0Hz,Ar$\underline{H}$), 1.57 (6H,$C\underline{H}_3$); M+ 292[294]. The ethanesulphonate salt was prepared by adding 1 equiv. of ethanesulphonic acid to a solution of the azide in isopropanol. The salt was recrystallised in aqueous isopropanol.

12.3 Acid-catalysed decomposition of aryl azide

1-(3-amino-4-chloro-6-trifluoromethanesulphonyl oxyphenyl)-4,6-diamino-1,2-dihydro-2,2-dimethyl-s-triazine (50)

Compound **(48)** (1.0g) was added, in small portions, to a cold (0°C) mixture of TFA (8ml), TFAA (1ml), and TFSA (3ml). When evolution of nitrogen has stopped, the solution was left to stir overnight at room temperature. This solution was basified by pouring into iced aqueous ammonia. The cream coloured precipitate was collected and dried under vacuum at room temperature; yield 97.5%; m.p. 175-177°C (decomposed); ν_{max} 3450 (N-H), 3360 (N-H) cm^{-1}; λ_{max}(water) 242nm; δ_H (300 MHz,DMSO-d_6) 7.92 (2H,br.s,NH_2), 7.47 (1H,s,ArH), 7.06 (2H,br.s,NH_2), 6.95 (1H,s,ArH), 6.06 (2H,br.s,NH_2), 1.51 (3H,s,CH_3), 1.28 (3H,s,CH_3); *m/z* 149,223[225] (3:1).

4,6-diamino-1-(3-azido-4-chloro-6-trifluoro methanesulphonyloxyphenyl)-1,2-dihydro-2,2-dimethyl-s-triazine (51)

Compound **(50)** (1.0g,0.003mole) was diazotised with sodium nitrite (0.18g,0.003mole). Sodium azide was added in small portions, and stirred for 0.5h. The solution was basified with iced aqueous ammonia. The cream coloured solid was collected, washed with water and dried under vacuum at room temperature; yield 75.0%; m.p. 160-161°C (decomposed); ν_{max} 3450 (N-H), 3350 (N-H), 2100 (N_3) cm^{-1}; δ_H (60 MHz,DMSO-

d_6) 7.54 (1H,s,ArH), 7.02 (1H,s,ArH), 1.52 (3H,s,,CH_3), 1.3 (3H,s,CH_3).

12.4 Synthesis of diastereoisomeric atropisomers

4,6-diamino-1-(2-chlorophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine hydrochloride (59)

A mixture of 2-chloroaniline (6.4g,0.05mole), cyanoguanidine (4.5g,0.0535mole), concentrated HCl (5ml,0.055mole) and 2-butanone (50ml) was refluxed for 14h. The mixture became a clear solution after a few minutes and a crystalline material was precipitated after 1h. The precipitate was collected, washed with 2-butanone and dried in an oven. Recrystallisation in aqueous ethanol yielded colourless crystals; yield 72.2%; m.p. 213-216°C; λ_{max}(water) 241nm; ν_{max} 3260 br. (N-H), 3100,1630,1520 cm^{-1}; δ_H (300 MHz,CF_3COOD) 7.44-7.89(4H,m,ArH),2.29 (80%,1H,dq,J=7.6Hz,J_{gem}= 14.2Hz,CH_2CH_3), 2.10 (19%,1H,dq,J=6.9Hz,J_{gem}=14.2Hz,CH_2CH_3), 2.09 (80%,2H,dq,J=7.4Hz,J_{gem}=14.1Hz,CH_2CH_3), 1.88 (19%,1H,dq, CH_2CH_3), 1.75 (19%,3H,s,CH_3), 1.50 (80%,3H,s,CH_3), 1.12 (80%,3H,t,J=6.6Hz,CH_2CH_3), 1.02 (19%,3H,t,J=7.0Hz,CH_2CH_3); M^+ 265[267] (3:1) (Found C,47.46; H,5.57; N,23.23 $C_{12}H_{17}N_5Cl_2$ requires C,47.68; H,5.68; N,23.18).

4,6-diamino-1-(2-bromophenyl)-2-ethyl-1,2-dihydro-2-methyl-s-triazine hydrochloride (58)

A mixture of 2-bromoaniline (8.6g,0.05mole),

cyanoguanidine (4.5g,0.535mole), concentrated HCl (5ml,0.055mole) and 2-butanone (50ml) was refluxed overnight. The reaction mixture dissolved into a clear solution and a crystalline precipitate was formed after 1.5h. The product was collected, washed with 2-butanone and dried. Recrystallisation in aqueous ethanol yielded colourless plate-shaped crystals; yield 70.2%; m.p. 211-214°C; λ_{max}(water) 242nm; ν_{max} 3260 br. (N-H),3100,1640 cm^{-1}; δ_H (300 MHz, DMSO-d_6) 7.83 (1H,t,J_{ortho}=7.9Hz, Ar$\underline{H}$), 7.75 (2H,br.s,N$\underline{H}_2$), 7.57-7.40 (3H,br.m,Ar$\underline{H}$),6.60(2H,br.s,N$\underline{H}_2$),2.01 (79%,1H,dq,J=7.2Hz,J_{gem}=14.3Hz,C$\underline{H}_2$CH$_3$),1.76(79%,1H,dq,J=7.5Hz ,J_{gem}=14.6Hz,C$\underline{H}_2$CH$_3$), 1.67-1.57 (18%,1H,br.m,C$\underline{H}_2$CH$_3$), 1.50 (18%,3H,s,C$\underline{H}_3$), 1.42-1.35 (18%,1H,br.m,C$\underline{H}_2$CH$_3$), 1.09 (79%,3H,s,C$\underline{H}_3$), 0.92 (79%,3H,t,J=7.4Hz,CH$_2$C$\underline{H}_3$), 0.83 (18%,3H,t,J=7.4Hz,CH$_2$C$\underline{H}_3$); M^+ 310[312] (Found C,41.58; H,5.01; N,19.96 $C_{12}H_{17}N_5BrCl$ requires C,41.57; H,4.95; N,20.21).

4,6-diamino-2-ethyl-1,2-dihydro-1-(2-iodo phenyl)-2-methyl-s-triazine hydrochloride (60)

A mixture of 2-iodoaniline (8.0g,0.037mole), cyanoguanidine (3.5g,0.04mole), concentrated HCl (5ml,0.05mole) and 2-butanone (40ml) was refluxed overnight. A dark coloured crystalline precipitate was formed. This was collected, washed with 2-butanone and dried. The crude product was recrystallised in aqueous ethanol to yield pale brown crystals; yield 28.4%; m.p. 206-208°C; λ_{max}(water)

242nm; ν_{max} 3300 br. (N-H), 3120, 1630 cm^{-1}; δ_H (300 MHz, DMSO-d_6) 9.41 (83%,1H,s,N^+-H), 9.21 (15%,1H,s,N^+-H), 8.04 (1H,d,J_{ortho}=7.8Hz,ArH), 7.54 (1H,t,J_{ortho}=7.6Hz,ArH), 7.46 (1H,d,J_{ortho}=7.8Hz,ArH), 7.25 (1H,t,J_{ortho}=7.8Hz,ArH), 2.02 (83%,1H,dq,J=7.4Hz,J_{gem}=14.2Hz,CH_2CH_3),1.76(83%,1H,dq,J=7.1Hz ,J_{gem}=14.3Hz,CH_2CH_3),1.68-1.60 (15%,1H,br.dq,CH_2CH_3), 1.52 (15%,3H,s,CH_3) 1..32-1.19 (15%,1H,br.dq,CH_2CH_3), 1.10 (83%,3H,s,CH_3), 1.05 (83%,3H,t,J=7.1Hz,CH_2CH_3), 0.93 (15%,3H,t,J=7.2Hz,CH_2CH_3); M^+ 357 (Found C,36.69; H,4.49; N,17.50 $C_{12}H_{17}N_5ICl$ requires C,36.61; H,4.36; N,17.79).

4,6-diamino-2-ethyl-1,2-dihydro-2-methyl-1-(2-methylphenyl)-s-triazine hydrochloride (61)

A mixture of 2-methylaniline (5.4g,0.05mole), cyanoguanidine (4.5g,0.0535mole), concentrated HCl (5ml,0.05mole) and 2-butanone (50ml) was refluxed overnight. The mixture dissolved into a clear solution and a colourless crystalline precipitate was formed after 1h. The crude product was collected, washed and dried. Recrystallisation in aqueous ethanol yielded plate-shaped colourless crystals; yield 62.5%; m.p. 220-221°C; λ_{max}(water) 242nm; ν_{max} 3330 (N-H), 3100,1640cm^{-1}; δ_H (300 MHz, DMSO-d_6) 9.35 (2H,br.s,NH_2), 7.68 (2H,br.s,NH_2), 7.43-7.33 (4H,br.m,ArH), 2.50 (11%,3H,s,$ArCH_3$), 2.18 (88%,3H,s,$ArCH_3$), 2.02(88%,1H,dq,J=7.2Hz,J_{gem}=13.8Hz,CH_2CH_3),1.74(88%,1H,dq,J=7.2Hz,J_{gem}=14.1Hz,CH_2CH_3),1.57-1.48 (11%,1H,br.m, CH_2CH_3),

1.39-1.35 (11%,1H,br.m,$C\underline{H}_2CH_3$), 1.28 (11%,3H,s,$C\underline{H}_3$), 1.02 (88%,3H,s,$C\underline{H}_3$),0.94(88%,3H,t,J=7.2Hz,$CH_2C\underline{H}_3$),0.82(11%,3H,t,J= 7.2Hz, $CH_2C\underline{H}_3$); M^+ 245 (Found C,55.21; H,7.09; N,24.88 $C_{13}H_{20}N_5Cl$ requires C,55.40; H,7.17; N,24.86).

12.5 Dimroth rearrangement in 1,2-dihydro-*s*-triazines

1.0g of a 4,6-diamino-2,2-dialkyl-1,2-dihydro-1-(substituted phenyl)-*s*-triazine hydrochloride was dissolved in 20ml of 50% aqueous methanol. The solution was made to pH 11 by NaOH solution. This solution was refluxed for 1h. The reaction mixture was left to stand in a refrigerator overnight. The crystalline product was filtered, washed and dried. The yield of the rearranged isomers ranges between 80-90%.

12.6 Synthesis of novel phenylamidines

N-Benzyl-2,4-dinitroaniline (62)

A mixture of 1-chloro-2,4-dinitrobenzene (10.1g,0.05mole) and benzylamine (10.7g,0.10mole) was refluxed in 2-ethoxyethanol. The yellow precipitate was filtered and washed with hot water to dissolve any hydrochloride salt of benzylamine. Recrystallisation of the crude product from ethanol/toluene yielded bright yellow needle-shaped crystals; yield 52.7%; m.p. 107-110°C;

λ_{max}(methanol) 260,347nm; ν_{max} 3380(N-H), 3060, 1560(NO_2,asym), 1320(NO_2,sym), 1260br.cm^{-1}; δ_H(60 MHz,$CDCl_3$) 9.02(1H,d,J_{meta}=2Hz,ArH), 8.87 (1H,br.s,ArNHCH$_2$), 8.13 (1H,dd,J_{meta}=2Hz,J_{ortho}=10Hz,ArH), 7.30 (5H,s,ArH), 6.85 (1H,d,J_{ortho}=10Hz,ArH), 4.60 (2H,d, J=8Hz,NHCH$_2$); M^+ 273.

N-(3,4-dichlorobenzyl)-2,4-dinitroaniline (76)

A mixture of 1-chloro-2,4-dinitrobenzene (6.1g,0.03mole) and 3,4-dichlorobenzylamine (5.8g,0.033mole) was refluxed in toluene overnight. When the reaction was stopped, the yellow precipitate was collected, washed with hot water and dried. The crude product was recrystallised from toluene; yield 52.6%; m.p. 171°C; λ_{max}(methanol) 347nm; ν_{max} 3380(N-H),3080,1610, 1580 (NO2,asym), 1320 (NO_2,sym) cm^{-1}; δ_H(60 MHz,$CDCl_3$) 9.02(1H,d,J_{meta}=2Hz,ArH), 8.87 (1H,br.s,ArNHCH$_2$), 8.13 (1H,dd,J_{meta}=2Hz,J_{ortho}=10Hz,ArH), 7.60 (3H,m,ArH), 6.85 (1H,d,J_{ortho}=10Hz,ArH), 4.60 (2H,d, J=8Hz,NHCH$_2$); M+ 342[344,346] (9:6:1).

4-Benzylamino-3-nitroaniline (65)

A mixture of 4-fluoro-3-nitroaniline (2g,0.013mole) and benzylamine (1.5g, *ca.* 1.1equiv.) was refluxed overnight. The reaction mixture was purified by flash chromatography with 20% methanol in chloroform as the eluting solvent. The purified fraction was rotary evaporated and needle-shaped dark red crystals were obtained; yield 72.8%; m.p. 216°C; δH (300MHz,DMSO-d_6)8.73(1H,t,J=5.6Hz,ArNHCH$_2$),8.67(1H,s,ArH),

7.72(1H,d,J_{ortho}=9.2Hz,ArH), 7.37-7.24 (5H,m,ArH),7.00 (1H,d,J_{ortho}=9.3Hz,ArH), 4.65 (2H,d,J=5.7Hz,ArNHCH_2); M^+ 243.

4-Benzylamino-N,N-dimethyl-3-nitrophenylamidine (36)

A mixture of 4-benzylamino-3-nitroaniline (1g,0.004mole) and N,N-dimethylformamide dimethyl acetal (3g) was refluxed until the reaction was complete. The crude product was purified using flash chromatography with 20% methanol in chloroform as the eluting solvent. The eluted fraction was rotary evaporated to yield a residual red oil. The hydrochloride salt was prepared by adding concentrated HCl to a solution of the red oil in isopropanol until the pH was neutral. A bright orange precipitate was collected and dried. Recrystallisation in aqueous isopropanol yielded needle-shaped crystals; yield 58%; m.p. 239-241°C; δH (300 MHz, DMSO-*d*6) 11.85 (1H,br.s,N^+-H), 8.73 (1H,t,J=5.6Hz,ArNHCH_2), 8.67 (1H,s,ArH), 8.29 (1H,s,NCHN), 7.72 (1H,d,J_{ortho}=9.2Hz,ArH), 7.37-7.24 (5H,m,ArH),7.00 (1H,d,J_{ortho}=9.3Hz,ArH), 4.65 (2H,d,J=5.7Hz,ArNHCH_2),3.38 (6H,d,N[CH_3]$_2$). M+ 298, *m/z* 207,91 (Found C,56.88; H,5.69; N,16.36 $C_{16}H_{19}N_4O_2Cl$ requires C,57.39; H,5.73; N,16.74).

3,4-dichlorobenzylamino-3-nitroaniline (77)

4-Fluoro-3-nitroaniline (2g,0.013mole) was refluxed for 2h with 3,4-dichlorobenzylamine (4g,0.023mole). The reaction mixture was allowed to cool and was poured into iced water.

Dark red crystals were collected and dried; yield 90%; m.p. 158-160°C; δ_H (DMSO-*d6*) 8.78 (1H,t,J=6.3Hz,ArNHCH$_2$), 8.58 (1H,s,ArH),7.66 (2H,d,ArH). 7.63(1H,d,J_{ortho}=8.6Hz,ArH),7.36 (1H,dd,J_{ortho}=8.3Hz,J_{meta}=2.0Hz,ArH),6.9(1H,d,J_{ortho}=8.6Hz, ArH), 4.65(2H,d,J=6.2Hz, NHCH$_2$); M^+ 312[314,316](9:6:1).

4-(3,4-dichlorobenzylamino)-N,N-dimethyl-3-nitrophenyl amidine (37)

2g (0.006mole) of compound **(77)** and an excess of N,N-dimethylformamide dimethyl acetal was refluxed until the reaction was complete. The crude product was purified by flash chromatography using 20% methanol in chloroform as the eluting solvent. The purified fraction was rotary evaporated to give a residual red oil. The hydrochloride salt was prepared by adding concentrated HCl dropwise to solution of the free base in acetone. An orange precipitate was collected and dried. Recrystallisation from aqueous ethanol yielded needle-shaped crystals; yield 73%; m.p. >280°C; δ_H (60 MHz,DMSO-*d6*) 11.40(1H,br.s,N^+-H),8.78 (1H,t,J=6.3Hz,ArNHCH$_2$), 8.58(1H,s,ArH),8.20(1H,s,NCHN),7.66(2H,d,ArH),7.63(1H,d, J_{ortho}=8.6Hz,ArH),7.36(1H,dd,J_{ortho}=8.3Hz,J_{meta}=2.0Hz,ArH), 6.9(1H,d,J_{ortho}=8.6Hz,ArH), 4.65(2H,d,J=6.2Hz, NHCH$_2$), 3.24 (6H,d,N[CH$_3$]$_2$); M^+ 367[369,371] (9:6:1) (Found C,47.43; H,4.16; N,13.59 $C_{16}H_{17}N_4O_2Cl_3$ requires C,47.60; H,4.25; N,13.88).

12.6.1 Selective reduction of aromatic compounds using alkaline sulphides

A solution of sodium hydrosulphide (1g, *ca.* 2 equiv.) in aqueous methanol was added to a boiling solution of the dinitro compound in methanol. The mixture was refluxed until reduction was complete [t.l.c. ethyl acetate:n-hexane (3:7,v/v)]. Most of the methanol was removed by rotary evaporation and the residual suspension was added to ice water. A deep red product was collected and dried.

1-Benzyl-5-nitrobenzotriazole (63)

2-(N-benzylamino)-5-nitroaniline (1g) was diazotised with 1.1 equiv. of sodium nitrite in 3N HCl. Sodium acetate was added to basify the reaction mixture and this was allowed to stir for 1h. The product formed was collected,washed and dried; yield 65.0%; m.p.110-112°C ; ν_{max} 1530 (NO_2,sym), 1350(NO_2,sym)cm^{-1}; δ_H(60 MHz,$CDCl_3$) 8.87(1H,d,J_{meta}=2Hz,Ar<u>H</u>), 8.20 (1H,dd,J_{meta}=2Hz,J=8Hz,Ar<u>H</u>), 7.50(1H,d,J_{ortho}=8Hz,Ar<u>H</u>), 7.25 (5H,s,Ar<u>H</u>), 5.83 (2H,s,NC<u>H_2</u>Ar); M^+ 254.

Chapter 13

Molecular modelling of *ortho*-substituted 1-aryl-1,2-dihydro-*s*-triazines

The co-ordinates from the crystal structure of cycloguanil hydrochloride were used as the basis of building the new inhibitor molecules. The new molecules were built by using the structure-building facilities available in the Chem-X molecular modelling package running on a VAX 8650 computer. The geometry of each new inhibitor molecule was optimised by using the semi-empirical molecular orbital program called MOPAC.

MOPAC is a general-purpose, semi-empirical molecular orbital program for the study of chemical reactions involving molecules; ions and linear polymers. It implements the semi-empirical Hamiltonians MNDO, AM1, MINDO/3, MNDO-PM3 and combines the calculations of vibrational spectra, thermodynamic qualities, isotopic substitution effects and force constants in a fully integrated program. There are four distinct methods available within MOPAC: namely MINDO/3, MNDO, AM1 and PM3. In this section of work, the method MNDO was used. These methods are all self-consistent field (SCF) methods, they take into account electrostatic repulsion and exchange stabilisation and, in them, all calculated integrals are evaluated by approximate means. The level of precision in the work carried out by MOPAC depends on two principal

operations, namely passing the SCF criterion and satisfying the geometry optimisation. In all the calculations conducted here, the ΔH values of each conformation have satisfied either of these operations.

The triazine ring of the inhibitor molecule is puckered at position C2 and this presented a problem in the definition of the dihedral angle. Therefore a least square plane for each ring was used instead. A dummy atom which represents the centre of the least square plane was used to define the dihedral angles during data acquisition .

Preliminary calculations were carried out at 15° intervals. Around the vicinity of the maxima and minima of the energy plot, more precise calculations were carried out at 5° interval. All calculations were done in GEO-OK and PRECISE. Each conformation was optimised by keeping a constant geometry at the N1-C11 bond while allowing all other geometric parameters to vary.

The calculated heat of formation was plotted against the dihedral angles to give the energy profile of the inhibitor molecule. The difference in value between the maxima and the minima was taken to be the energy barrier to rotation.

Chapter 14

Optical resolution of diastereoisomeric atropisomers by liquid chromatography

14.1 Equipment and chromatographic conditions

The liquid chromatographic system consists of the Shimadzu LC-6A Pumps, the SCL-6B System Controller and the SIL-6B Auto-injector. A Lichrosorb RP-Select B stainless steel column (125 x 4 mm) was used for the preliminary analysis work. For the collection of the diastereoisomeric atropisomers on a semi-preparative scale, a μBONDAPAK^{TM} Precolumn Module containing μBONDAPAK^{TM} C18 inserts were used as guard columns. The column was linked to a Shimadzu SPD-6A UV Spectrophotometric Detector which was operated at 242nm at a sensitivity of 0.16 a.u.f.s. during the analytical work and 2.56 a.u.f.s. during the sample collection. An Advantec SF 2120 Super Fraction Collector was programmed to collect the two separated diastereomeric atropisomers.

The mobile phase used in the h.p.l.c. experiments was 0.05M ammonium acetate buffer (pH 4) : methanol (75:25,v/v) and was degassed by filtration through a No. 4 sintered glass funnel. The buffer solution was prepared by dissolving 1.925g of ammonium acetate in 500ml distilled water and adjusted to pH 4 with glacial acetic acid. The flow rate for the analytical work was 1.5 ml/min while the flow rate

for sample collection was 8 ml/min. The experiments were conducted at ambient temperature.

14.2 Chemicals

H.p.l.c. grade methanol, ammonium acetate and glacial acetic acid were obtained from FSA Laboratory Supplies.

The 4,6-diamino-2-ethyl-1,2-dihydro-2-methyl-1-(*ortho*-substituted phenyl)-*s*-triazines were synthesised as described in the previous chapter.

14.3 Treatment of enriched fractions

The methanol in each collected fraction was removed by high vacuum rotary evaporation. The resultant concentrate was frozen and allowed to to evaporate to dryness on a freeze dryer. The residue was sent for high resolution ^{1}H n.m.r. spectroscopic analysis.

A stock solution was made by dissolving the residue in 0.4ml of distilled water. A spectrum of different concentrations was made from the stock solution by serial dilution. These solutions were used directly in the DHFR enzyme inhibition assay. The concentration of the stock solution was obtained from the calibration curves of each inhibitor.

14.4 Calibration curves

Calibration curves were prepared by analysing a series of standard solutions containing the diastereoisomeric atropisomers mixture and *meta*-nitrocycloguanil hydrochloride as internal standard. The concentration range studied was 1.0-20.0mg/ml. Area ratios of each atropisomer to the internal standard was plotted against concentrations.

Chapter 15

Dihydrofolate reductase inhibition assay

15.1 Preparation of partially purified DHFR and reagent solutions

Partially purified enzyme was prepared by the method of Bertino and Fischer.[107] Two male Wistar rats were killed and the livers were removed and washed with water to remove blood. All further manipulations were performed at 4°C; the livers (30.7g) were blended with approximately 8 volumes of water (final volume *ca.* 300ml). The mixture was homogenised for 2 min and the pellet was discarded. The supernatant liquid was adjusted to pH 5.1 with dilute acetic acid (1.0 M initially and 0.1 M finally) and after centrifugation at 27000 g for 20 min, the clear wine-red solution was transferred to a visking tubing (2.5cm diameter) and dialysed for 12h against 0.01 M sodium acetate buffer solution. The dialysate was examined and if turbid, centrifuged again at 27000 g for 20 min. The clear preparation was transferred to sterile plastic stoppered tubes (10ml) and stored at -10°C prior to use. Enzyme preparations stored frozen in this manner exhibited no significant decrease in DHFR activity after 12 months.

0.15M Phosphate buffer (pH 7) was used for all the DHFR assays and was prepared by dissolving potassium dihydrogen

orthophosphate (10.21g) in water (*ca.* 300 ml), adjusting the solution to pH 7 with potassium hydroxide, and diluting to 500 ml with water. The buffer was kept at 4°C to prevent bacterial growth and discarded after 3 days.

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

An aqueous solution of NADPH (2 mg/ml, 2 mM) was prepared immediately prior to use and again maintained at 0°C.

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

15.2 Assay for DHFR inhibition

The assay was carried out by using a Cecil CE 377 linear grating spectrophotometer, at wavelength 340 nm and full scale deflection of 2.0 absorbance units. Readings of absorbance were taken sequentially at time intervals of 1 min, 5 min, 10 min and 15 min. A period of not more than 10

sec was allocated for each reading. Plastic disposable cuvettes were used throughout the assay.

The assay was conducted by first mixing the appropriate volumes of phosphate buffer, NADPH, dihydrofolate, DHFR and the inhibitor in the cuvettes **(Table 15.1)**. The reduction in absorbance was recorded at the time intervals as mentioned above. Any endogenous reduction of dihydrofolate, as observed in control I, was accounted for in the calculations. The percentage activity of each inhibitor was calculated by using the following formulae:

$$\text{(i)} \quad \text{activity} = \frac{\text{slope of inhibited enzyme} \times 100\%}{\text{slope of uninhibited enzyme}}$$

$$\text{(ii)} \quad \text{inhibition} = 100\% - \text{activity}$$

A graph of percentage inhibition against the logarithmic concentration (μM) was plotted for each compound and the I_{50} value was taken to be the concentration (μM) at which 50% inhibition was observed.

Table 15.1: *Volumes of reagents, inhibitor and enzyme used in the assays.*

Nature of assay	NADPH (ml)	Enzyme (ml)	DHF (ml)	Buffer (ml)	Inhibitor (ml)	Final vol(ml)
Control I	0.1	0.1	-	2.8	-	3.0
Control II	0.1	-	0.1	2.8	-	3.0
uninhibited enzyme	0.1	0.1	0.1	2.7	-	3.0
inhibited enzyme	0.1	0.1	0.1	2.6	0.1	3.0

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