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New Studies in the Synthesis and Applications of Pteridines

by

Thomas Mc Donald

A thesis submitted to the University of Dublin for the degree of Doctor of Philosophy

Department of Chemistry
University of Dublin
Trinity College

October 2002

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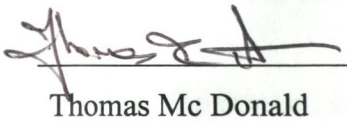

Thomas Mc Donald

October 2002

Acknowledgements

I would like to sincerely thank Dr. Peter Boyle for all his help and encouragement over the past four years; I would also like to thank him for giving me the chance to achieve this goal.

I would like to express my thanks to both Prof. Corish and Prof. Kelly for allowing me the use of department facilities. I am very grateful to the other members of staff, especially to Dr. John O' Brien for the supply of insightful and sometimes "interesting" NMR spectra and Mr. Fred Cowzer, the store man who can organise anything. I would like to thank the other technical staff including the two Brendan's, Martin, Patsy, Seamus, Peggy, John and Theresa.

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I want to thank the many people outside of Trinity that have helped me over the last few years, to name them all would bring me over the page limit for the thesis but I would like to thank some people in particular. I want to thank Pat Walsh, someone who played a big part in directing me onto this road. I also want to say a big thank you to Gandy and Gero for all their help and friendship over the last n years.

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Finally, to my mam and dad, whose understanding and encouragement contributed as much as any other factor to the completion of this work, I dedicate this thesis.

"The roots of education are bitter, but the fruit is sweet."

-Aristotle

Summary

The work described in this thesis involved the study of two distinct areas of research within pyrimidine and pteridine chemistry. The first of these involved investigation of the pteridine biosynthetic pathway within the zebrafish (*Danio rerio*). The use of zebrafish for the study of embryonic development in vertebrates is a relatively new area of research, but this species is fast replacing *Drosophila* as the genetic model of choice around the world today. The neural crest is responsible for some of the most important cells and developmental aspects of all vertebrates including humans. Included in the cells that originate in the neural crest are the pigment cells. It has been suggested that pteridines play a role in the formation of these cells within the zebrafish and this work was particularly interested in the formation of these pigment cells. In a programme of collaborative research with Dr. Imgard Ziegler (Munich) a series of pteridines were extracted from wildtype zebrafish (*Danio rerio*). These extracts were analysed by HPLC-MS and the pteridines present were identified by comparison with authentic samples. The pteridines present in these extracts were identified to be isoxanthopterin **(35)**, violapterin **(36)** and biopterin **(37)**. This work led to a suggested new biosynthetic route for the formation of pteridines within the zebrafish (*Danio rerio*).

The second part of the work described in this thesis investigated a potential new route for the synthesis of the pteridine ring system. While there are many known routes for the preparation of pteridines, one pathway still remains undiscovered. This work investigated the feasibility of this approach, namely intramolecular cyclisation of electrophilic nitrogen onto the 5-position of a pyrimidine ring system. This involved the synthesis of 2,4-dintrophenyl oximes of type **(251)**, with the hope that this system could be used a source of electrophilic nitrogen, which could

facilitate this electrophilic cyclisation. While efforts to prepare the desired pteridine product **(252)** were unsuccessful, some interesting new pyrimidine chemistry was developed. In particularly, some sigmatropic rearrangement reactions were uncovered which led to interesting heterocyclic cyclisations and a new source of pyrrolo[2,3-*d*]pyrimidine **(179)**, benzo[b]furan **(193)** and furano [3,4-*d*]pyrimidine **(201)** ring systems.

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Abbreviations and Notes

AIDS	Acquired Immune Deficiency Syndrome
AZT	Azidothymidine
br	Broad
Calc.	Calculated
$CDCl_3$	d-Chloroform
CNS	Central Nervous System
CNT	Cyanothymidine
Conc.	Concentrated
Cq	Quaternary Carbon
CSF	Cerebral Spinal Fluid
Cys	Cystein
d	Doublet
D_2O	Deuterium oxide
DCM	Dichloromethane
dd	Double Doublet
DEPT	Distortionless Enhancement by Polarisation Transfer
DHFR	Dihydrofolate reductase
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNP	Dinitrophenol
DOPA	3,4-Dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray Ionisation

Et	Ethyl
Et_2O	Diethyl ether
EtOAc	Ethylacetate
EtOH	Ethanol
Gln	Glycine
Glu	Glutamic Acid
GTP	Guanosine triphosphate
GTPCH	Guanosine triphosphate cyclohydrolase
H_2NTP	Dihydroneopterin triphosphate
H_4biopterin	5,6,7,8-tetrahydrobiopterin
HCl	Hydrochloric
Hex	hexane
His	Histidine
HIV	Human Immunodeficiency Virus
HPF	Hours Post Fertilisation
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectrum
Hz	Hertz
I.D.	Internal Diameter
I.U.P.A.C.	International Union of Pure and Applied Chemistry
ICU	Intensive Care Unit
Ile	Isoleucine
IR	Infrared
J	Coupling Constant
KBr	Potassium Bromide

LHS	Left Hand Side
m	Multiplet
m.p.	Melting Point
MeCN	Acetonitrile
MeOH	Methanol
min.	Minutes
MS	Mass Spectrometry
MSA	Migrating Staging Area
Mw	Molecular Weight
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NC	Neural Crest
NCC	Neural Crest Cells
NMR	Nuclear Magnetic Resonance
NT	Neural Tube
PAH	Phenylalanine Hydroxylase
PDA	Photodiode Array
PKU	Phenylketonuria
PM	Point Mutations
PNS	Peripheral Nervous System
PPH_4	6-Pyruvoyltetrahydropterin
ppm	Parts Per Million
PTPS	Pyruvoyltetrahydropterin synthase
R_f	Retention Factor
RF	Radio Frequency
RHS	Right Hand Side

RNA	Ribonucleic Acid
R_t	Retention Time
RT	Room Temperature
s	Singlet
SR	Sepiapterin reductase
t	Triplet
TEA	Triethylamine
TFA	Triflouroacetic Acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
TOF	Time Of Flight
UV	Ultra Violet
v/v	Volume Per Volume
Vis	Visible
w/v	Weight Per Volume

Note 1.: The isomeric nature (syn / anti) of all oximes discussed in this thesis has not been elucidated for this work. All oximes are drawn non-specifically and in no example where an oxime was synthesised and isolated, was there any evidence of a mixture of isomeric products (NMR).

Chapter One

Pteridines

Their importance to man and nature.

1.1 The history of pteridines

Over 100 years ago in 1889, Frederick Gowland Hopkins published his first work describing the isolation of pigments from various species of butterfly.[1] His initial work saw the isolation of a yellow pigment from the common English Brimstone butterfly (*Gonepteryx rhamni*, Zitronenfalter) and a white pigment from the Cabbage butterfly (*Catopsilia argante*, Südamerika). Wieland suggested that this group of pigments should be called 'pteridines', the name coming from the Greek word "pteron" meaning "wing".[2] From 1924 to 1926 these pigments were further purified by Schöpf and they were named xanthopterin **(1)** and leucopterin **(2)** according to their colours and appearance in nature.[3,4] It was not until 1940 however, that Purrmann was able to elucidate a structure for these pigment molecules when he showed they were derivatives of the pyrazino[2,3-*d*]-pyrimidine ring system[5].

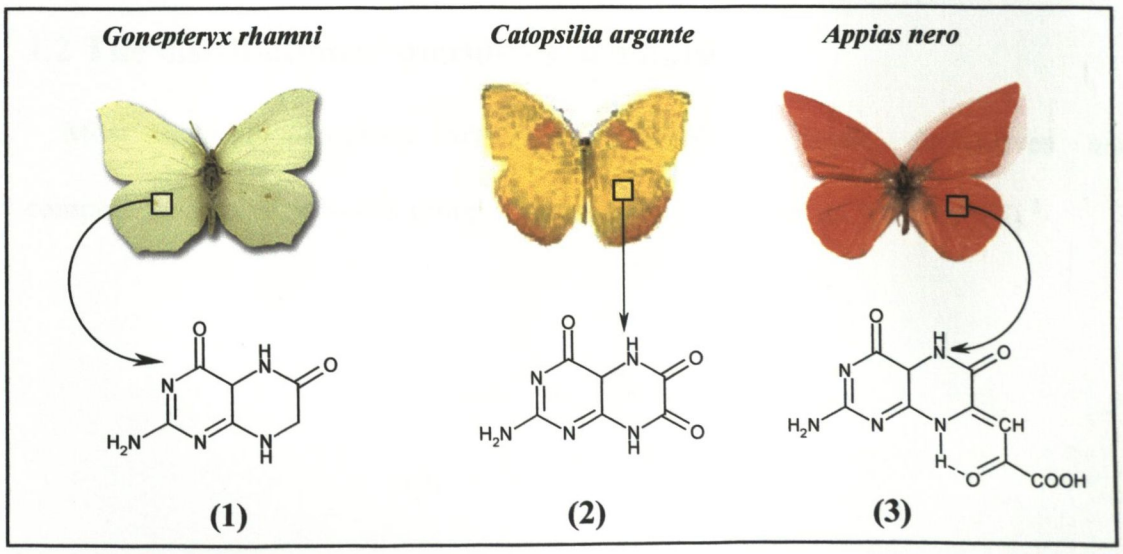
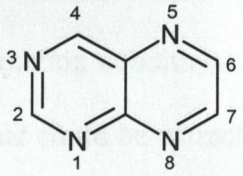


Figure 1.1 Butterflies and their corresponding pteridine pigments.

The numbering system normally used for pteridines **(4)** is based on the I.U.P.A.C. rules customarily applied to all other aza derivatives of naphthalene.[6]

Some earlier workers in the field[7] had used a numbering system **(5)** based on the purine ring system, but this has now been abandoned.



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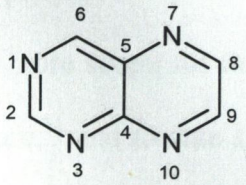
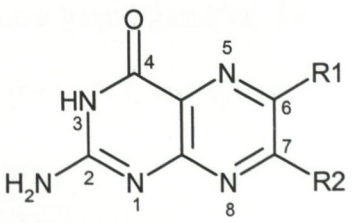


(5)

Investigation of different butterfly species was to yield more examples of pteridine pigments over the next twenty years. Tropical species were studied and these yielded more brightly coloured examples of pterins, such as the orange-red erythropterin **(3)**, isolated from the wings of the *Appias Nero* (Java).[8] These early discoveries led to a new interest in this type of chemistry, the work continued, and many more discoveries were made.

1.2 The distribution of pteridines in nature

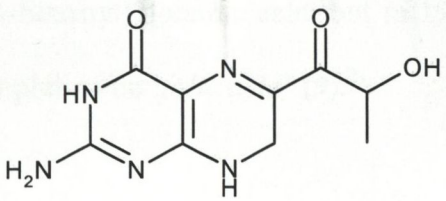
Most natural pteridines are 2-amino-4(3*H*)-pteridinone derivatives and compounds belonging to this group of substances are known as "pterins" **(6)**.[2]



(6)

Although pteridine **(4)** has a high solubility in water as well as in non-polar solvents, pterins **(6)** are poorly soluble in water and in organic solvents, for example the solubility in water at room temperature of xanthopterin **(1)** is 1 part in 40,000.[9]

These characteristics made the initial compounds very difficult to isolate and purify and very harsh conditions had to be employed. This made the work unsuitable for the detection of labile or sensitive pterins. By 1964 Watt[10] had developed milder extraction conditions. This gave more scope for the number and type of compounds that could be extracted from nature,[11] and to date a huge number of pteridines have been isolated from natural sources. For example, sepiapterin **(7)** was isolated from the wings of butterflies and also from the 'deep red' eye pigment of *Drosophila*.[12] This latter species was to prove very important, since it was shown later to contain a wide variety of pterins. Further extraction of *Drosophila* eyes gave a yellow pigment, isosepiapterin[13] **(8)** as well as red dimeric pterin pigments known as the drosopterins.[14]



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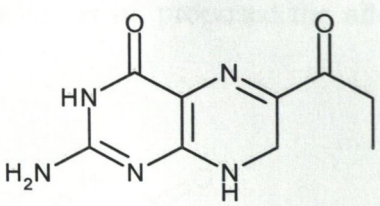
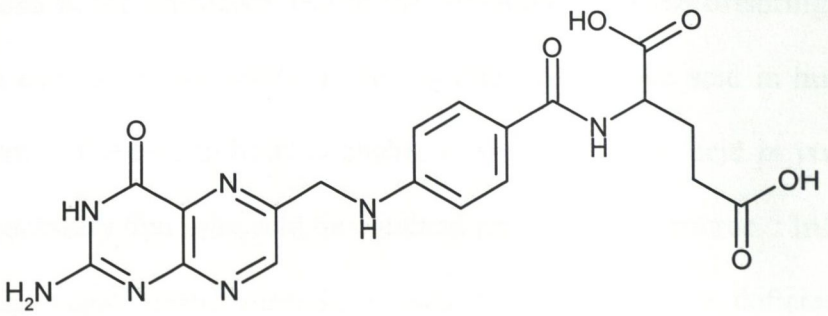


(8)

Several species of fish have been examined for the presence of pterins in their skin. For example, xanthopterin **(1)**, sepiapterin **(7)**, isosepiapterin **(8)** as well as several other violet fluorescing pterins not encountered in insects or amphibia have all been identified in species such as the red carp (*Cyprinus carpio*) and the goldfish (*Carassius auratus*).[15,16] Pteridines have also been isolated from the wings and eyes of other insects, from the skin of many other fish species, amphibia and reptiles.[17] The presence and role of pteridines in fish, particularly in the zebrafish (*Danio rerio*) will be discussed in more detail further on in this thesis.

During the same period that German chemists were isolating and characterising the pigments from butterfly wings and elucidating the structure of these compounds, a number of apparently unrelated studies on nutrition was about to lead to the discovery of the one of the best known and biologically important pteridines, namely folic acid. The first of these studies[18] reported how extracts from liver were effective in the treatment of tropical macrocytic anaemia. A second group of studies showed that certain wheat bran extracts stimulated the growth of chicks,[19] and studies on the bacterium *Streptococcus faecalis* showed that its growth was also stimulated by the same wheat bran extract. Purification of these extracts all yielded the same yellow, highly insoluble compound. Its structure **(9)** was solved in 1946 by Angier *et al.*,[20] who called it 'pteroylglutamic acid'. The Chemical Abstracts name for this compound is (N-[4-{[(2-amino-4-hydroxy-6-pteridinyl)methyl]-amino}-benzoyl]glutamic acid, but in 1947 Mitchell *et al.* proposed the alternative and simpler name '*folic acid*' **(9)**.[21]



(9)

1.3 The biology of pteridines

1.3.1 Introduction

Once identified in a species, in many cases it was difficult to assess whether a pteridine functioned as a pigment or not. For example, leucopterin **(2)** and

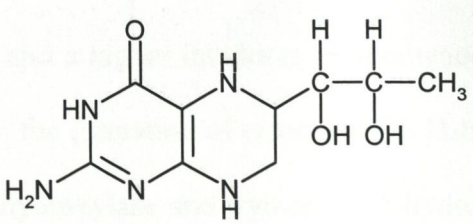
isoxanthopterin **(35)** are responsible for the white appearance of the wings of some butterflies, but yet in the eyes of *Drosophila,* they have no colouring effect.[22] The idea that pteridines were not just present for pigmentation in nature led to many more studies been carried out. The discovery of folic acid and its derivatives (often collectively referred to as the 'folates', from the Latin word for leaf, "*folium*") further encouraged these studies and it was at this time that the importance of pteridines in man began to unfold. Herbert[23] was the first to study the deficiency of folate in man. By excluding all folate derivatives from the diet of a healthy adult over five months he showed that some very serious disorders occurred. After three months folate deprivation the subjects were anaemic and by the fifth month they had developed sleeplessness, forgetfulness and irritability. These symptoms disappeared within 48 hours after oral therapy with folic acid was commenced, and were therefore considered to have resulted from folate deprivation.

Other studies of rat foetal development in the absence of folate showed many complications in the pregnancy and in the development of the offspring.[24] These studies pre-empted the discovery of the significance of folic acid in human foetal development. The human body is unable to synthesise folic acid *in vivo* and it is therefore necessary that folic acid be obtained from a dietary source. Initial studies showed that megaloblastic anaemia is caused by severe folate deficiency during pregnancy. If it is severe enough it can be fatal and in tropical countries, due to diet, it is responsible for a significant percentage of maternal mortality.[25]

Folate deficiency has become a major topic of conversation in the western world also due to its relationship with 'neural tube defects'. In the developing human embryo, the neural plate changes to form the neural tube between days 24 and 28 post conception.[26] This eventually leads to the formation of the spinal cord,

incomplete closure of which leads to spina bifida. Spina bifida is the opening of the spinal cord, resulting in a child with physical disabilities. These disabilities range from paralysis of the bladder and lower limbs to hydrocephalus, due to interruption of the flow of cerebrospinal fluid from the brain. These and similar conditions are collectively called 'neural tube defects'. It was found that when additional folic acid was introduced into the diet of women pre- and post-conception, then the incidence of neural tube defects in the offspring was dramatically reduced. This has now led to new legislation in the United States making it compulsory to add folic acid to some common foods such as flour, rice and cereals. Scott and Weir have recently reviewed these developments.[27]

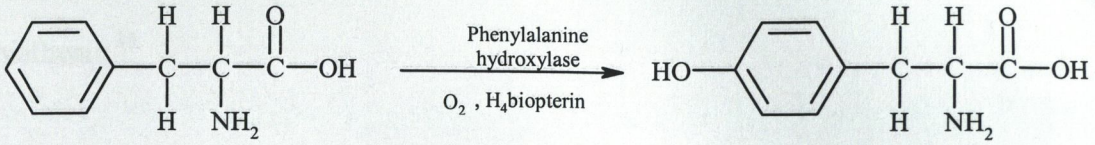
In 1963 it was discovered that the essential cofactor for phenylalanine hydroxylase (PAH) was a pteridine.[28] This was 5,6,7,8-tetrahydrobiopterin (H_4biopterin) **(10)**, which was later shown to play a vital role in the biology of many organisms and was not of only minor importance as had been previously believed.



(10)

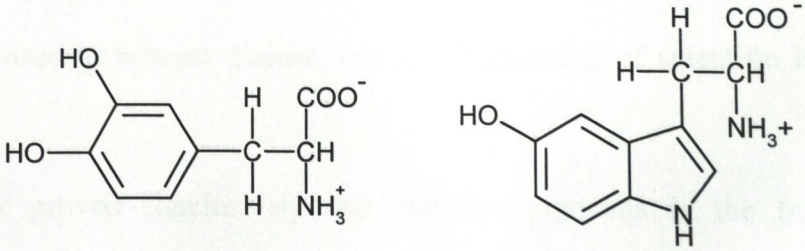
Phenylalanine **(11)** is an amino acid that is essential in the human diet, since it cannot be synthesised within the human body. It has been known since 1913 that the amino acid tyrosine **(12)** is produced by hydroxylation of phenylalanine **(11)**, and there was a great impetus to research on this reaction when it was discovered

that the disease phenylketonuria was caused by the inability of phenylalanine **(11)** to be hydroxylated.[29,30]



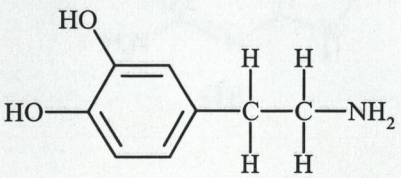
(11) **(12)**

Phenylketonuria (phenylketones in the urine) can be caused either by certain genetic defects that result in a deficiency in the amount of phenylalanine hydroxylase enzyme, "classical PKU", or by a deficiency in the H_4biopterin cofactor which is called "atypical PKU" or malignant hyperphenylalaninemia.[31] In classical PKU, phenylalanine levels are monitored in newborn babies, since treatment if necessary can prevent the chance of mental retardation occurring. Studies have suggested that these high levels of phenylalanine prevent the uptake of other essential amino acids required for brain protein synthesis.[32,33] Instead of affecting only the brain, hyperphenylalaniemia (atypical PKU) appears also to cause heart defects, lower birth weights and a higher incidence of spontaneous abortion.[34] As well as playing a vital role in the formation of tyrosine **(12)**, H_4biopterin **(10)** serves as a cofactor for tyrosine-3-hydroxylase and tryptophan-5-hydroxylase. These are key enzymes in the biosynthesis of DOPA **(13)** and 5-hydroxytrytophan **(14)** respectively.



(13) **(14)**

Since tyrosine **(12)** is the precursor of dopamine **(15)** and epinephrine (adrenaline) **(16)**, and since 5-hydroxytryptophan **(14)** is involved in the biosynthesis of serotonin **(17)**, H_4biopterin **(10)** indirectly controls neurotransmitter synthesis.[35]



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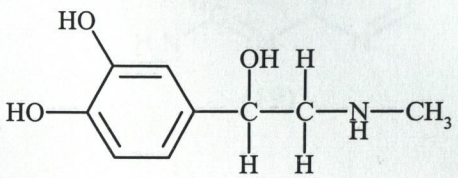

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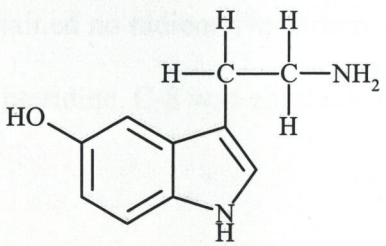


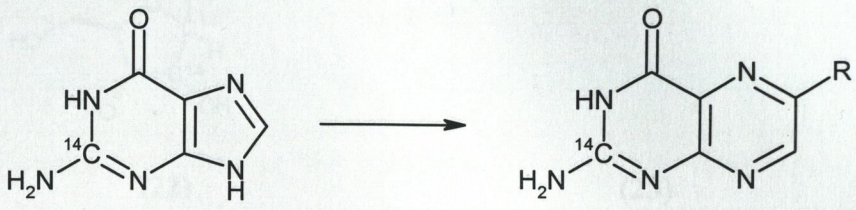
(17)

1.3.2 Initial studies on the biosynthesis of pteridines

Ever since Wills[18] first realised the importance of the liver extract that was to become folic acid, and ever since Patterson identified biopterin **(37)** after purification of 4000 litres of human urine,[36] a major research effort has been devoted to discovering how pteridines are formed in nature. Pterins were now becoming very important molecules and the fact that they play an important role in disease, and could be used to prevent disease, led to a burgeoning of scientific interest in them.

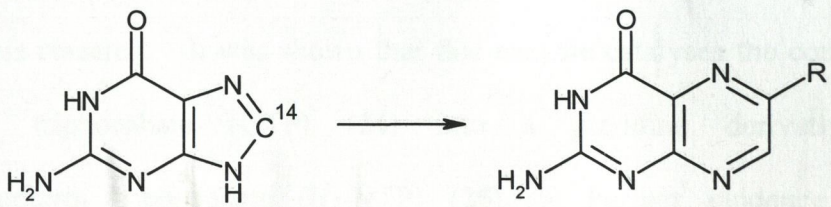
Early work proved conclusively that pteridine pigments in the frog (*Rana temporaria*) were formed from a purine precursor, namely guanine, since

radiolabelled pteridines **(19)** were isolated from frogs, which had been injected with guanine radiolabelled at C-2 **(18)**.



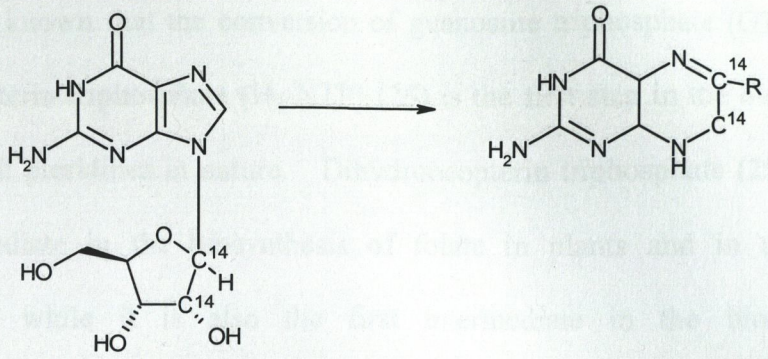
(18) **(19)**

Furthermore, if the guanine were labelled at C-8 **(20)** instead of at C-2 **(18)**, the pteridines isolated **(21)** contained no radioactive carbon. It followed that during the conversion of the purine to pteridine, C-8 was eliminated at some stage.[37]



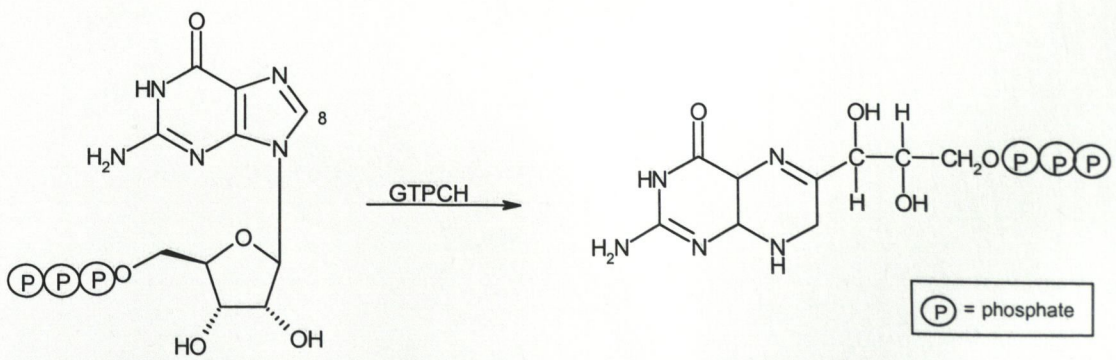
(20) **(21)**

Later work using radiolabelled guanosine **(22)** proved that C-6 and C-7 of the pteridine ring **(23)** were derived from C-1 and C-2 of the ribose moiety in the starting guanosine **(22)**. It became obvious from these results that a complex sequence of reactions must be occurring in the conversion of the purine nucleosides into the pteridine.[38,39,40]



(22) **(23)**

Weygand *et al.*[40] suggested a pathway that featured cleavage of the purine imidazole ring with loss of the carbon in position 8, followed by rearrangement of the ribose moiety to form the pteridine. Reynolds and Brown[41] observed after much work that an enzyme present in extracts of *Escherichia coli* could catalyse the imidazole cleavage reaction. This enzyme was guanosine triphosphate cyclo-hydrolase (GTPCH) and its discovery was one of the most important in pteridine biosynthesis research. It was shown that this enzyme catalyses the conversion of guanosine triphosphate (GTP) **(24)** into a pteridine derivative called dihydroneopterin triphosphate (H_2-NTP) **(25)**. Further evidence that this conversion was carried out by a single enzyme (GTPCH) was reported for *Drosophila* by Fan and Brown,[42] and for humans by Blau and Niederweiser.[43] The mechanism of this enzymatic reaction has been studied intensively ever since and a review of results to date is presented later in this chapter.

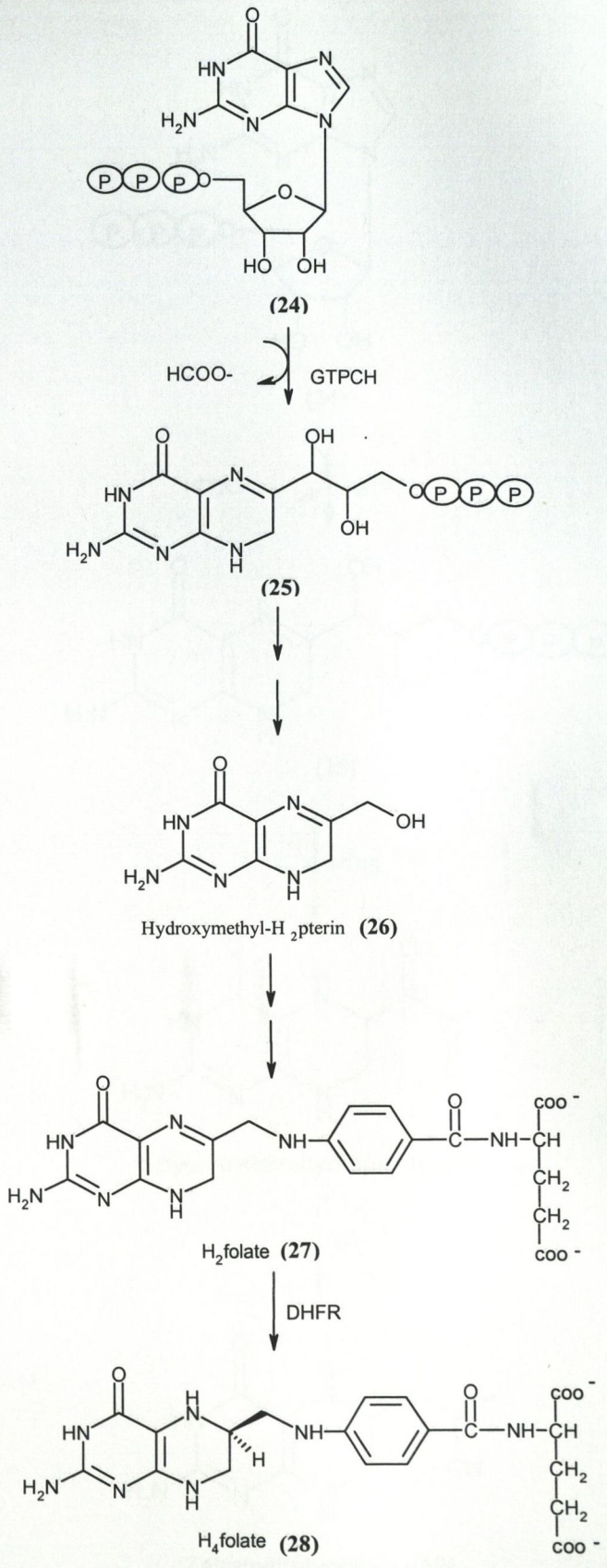


(24) **(25)**

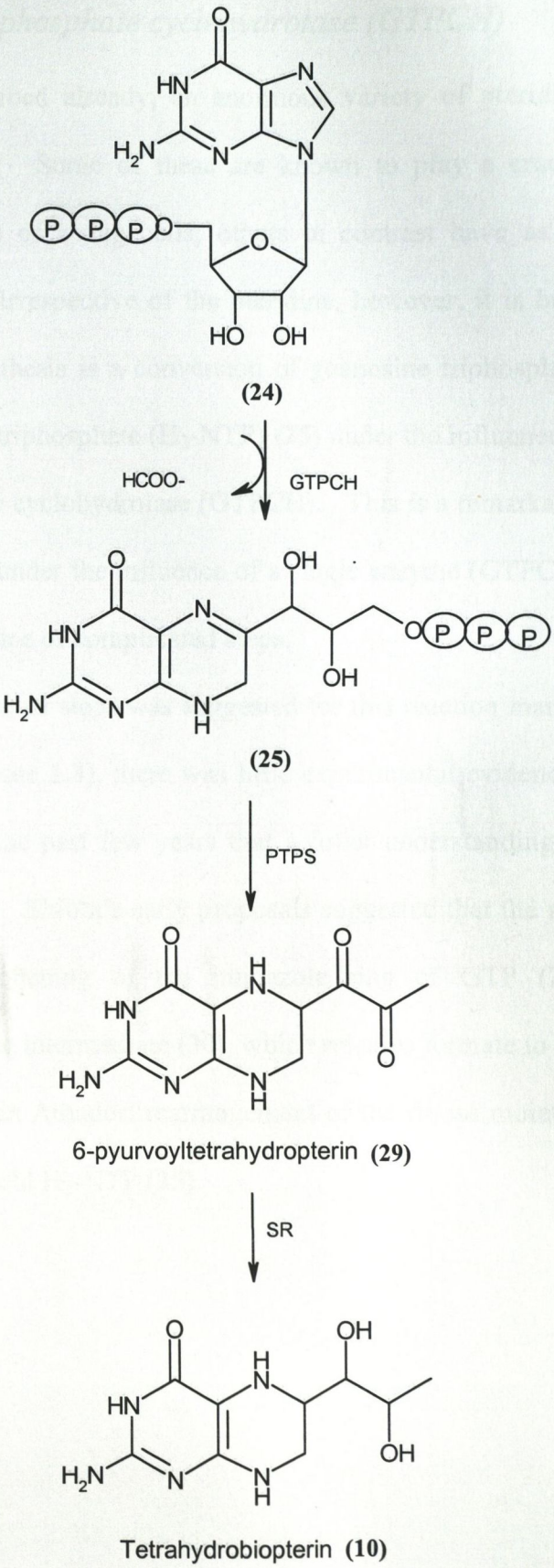
It is now known that the conversion of guanosine triphosphate (GTP) **(24)** into dihydroneopterin triphosphate (H_2-NTP) **(25)** is the first step in the biosynthesis of practically all pteridines in nature. Dihydroneopterin triphosphate **(25)** is thus the first intermediate in the biosynthesis of folate in plants and in many micro-organisms,[44] while it is also the first intermediate in the biosynthesis of tetrahydrobiopterin **(10)** in man and animals. This is shown in **Scheme 1.1** and **1.2**, which outline the currently accepted steps in the biosynthesis of tetrahydrofolate **(28)** and tetrahydrobiopterin **(10)** respectively.

As outlined in **Scheme 1.1**, dihydroneopterin triphosphate **(25)** proceeds via 6-hydroxymethyl-H_2pterin **(26)** to dihydrofolate **(27)**, which is reduced to give tetrahydrofolate **(28)**. A key enzyme, dihydrofolate reductase, catalyses the final reduction of the dihydro- **(27)** to the tetrahydrofolate **(28)**, and this enzyme is a major target in anticancer chemotherapy.[45]

Scheme 1.2 shows that in the formation of tetrahydrobiopterin **(10)**, where the dihydroneopterin triphosphate **(25)** retains its three carbon side chain, and is converted to 6-pyruvoyltetrahydropterin **(29)** under the influence of the enzyme 6-pyruvoyltetahydropterin synthase (PTPS). The 6-pyruvoyltetahydropterin **(29)** is converted to H_4biopterin **(10)** in a complicated series of intermediates involving the enzyme sepiapterin reductase (SR).[46,47]



Scheme 1.1 Biosynthetic pathway of tetrahydrofolate

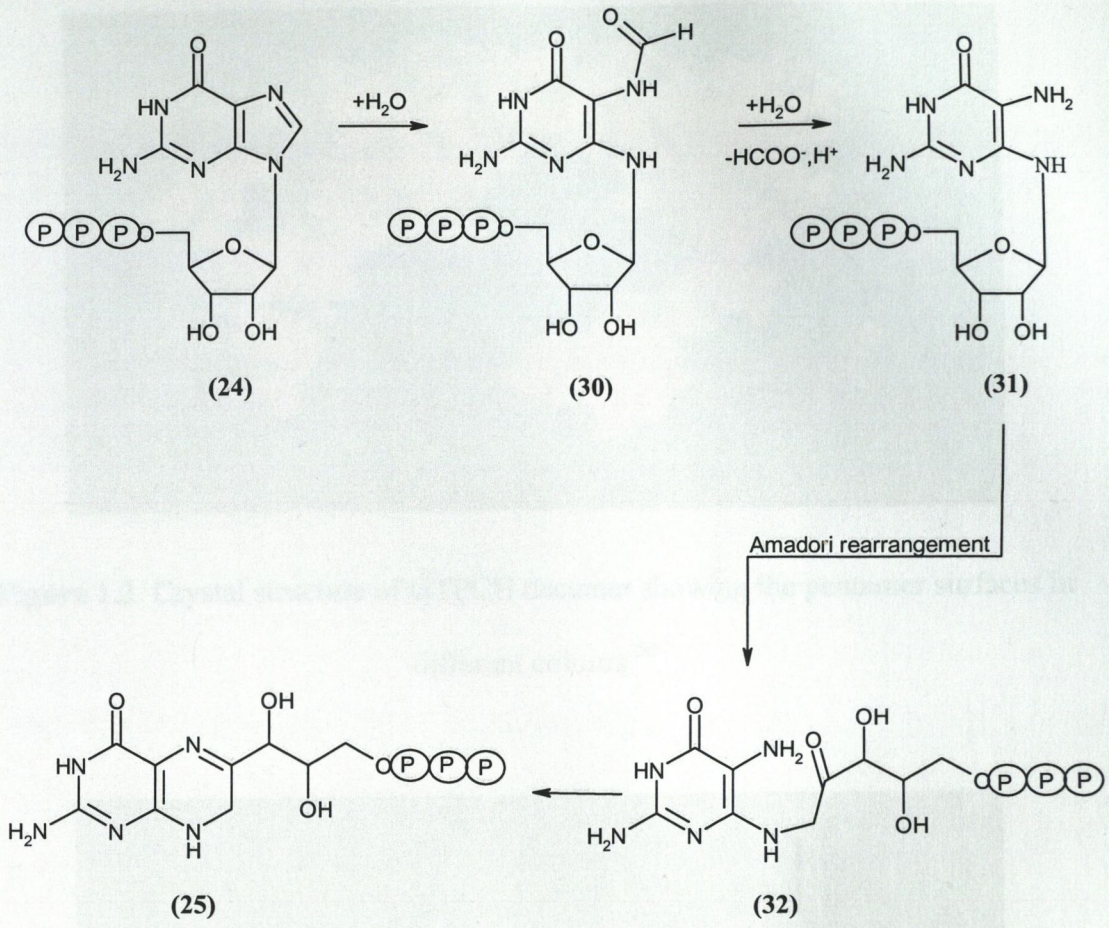


Scheme 1.2 The biosynthesis of H_4biopterin

1.3.3 Guanosine triphosphate cyclohydrolase (GTPCH)

As has been described already, an enormous variety of pteridines have been discovered in nature. Some of these are known to play a crucial role in the biochemical processes of living cells; others in contrast have as yet no known biological function. Irrespective of the pteridine, however, it is believed that the first step in its biosynthesis is a conversion of guanosine triphosphate (GTP) **(24)** into dihydroneopterin triphosphate (H_2-NTP) **(25)** under the influence of the enzyme guanosine triphosphate cyclohydrolase (GTPCH). This is a remarkable conversion, for although it occurs under the influence of a single enzyme (GTPCH), it is known to proceed *via* a sequence of complicated steps.

Although a sequence of steps was suggested for this reaction many years ago by Shiota *et al.*[48,49] (**Scheme 1.3**), there was little experimental evidence to support it, and it is only within the past few years that a fuller understanding of the process involved has emerged. Shiota's early proposals suggested that the starting point of the reaction is the opening of the imidazole ring of GTP **(24)** giving the formylaminopyrimidine intermediate **(30)**, which releases formate to give **(31)**. The latter then undergoes an Amadori rearrangement of the ribose moiety to form **(32)**, which re-cyclises to yield H_2-NTP **(25)**.



Scheme 1.3 Hypothetical pathway for the conversion of GTP to H2-NTP

A major step forward in our understanding of these processes was made when Bacher managed to obtain crystals of *Escherichia coli* GTPCH.[50] Bacher and Huber then succeeded in obtaining an X-ray diffraction crystal structure of the molecule.[50] The enzyme was shown to be a homodecameric complex (**Figure 1.2**) of molecular mass 24700 Da, in which two identical 5-fold symmetric pentameric subunits (**Figure 1.3**) are tightly associated to form a torus-shaped superstructure of 65Å x 100Å. A narrow pocket was found at the interface of the three subunits (two from one pentamer and one from the other pentamer) and this was thought to be the active binding site of the enzyme.

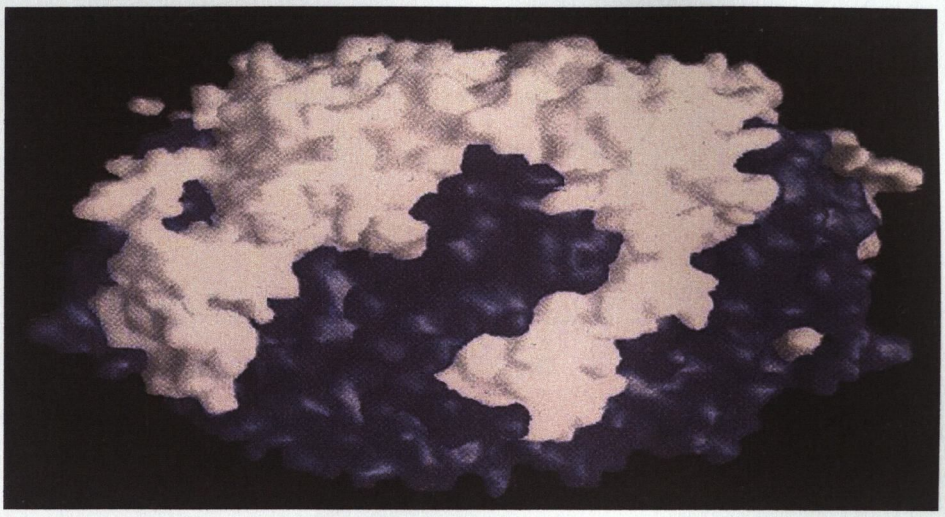


Figure 1.2 Crystal structure of GTPCH decamer showing the pentamer surfaces in different colours.[50]

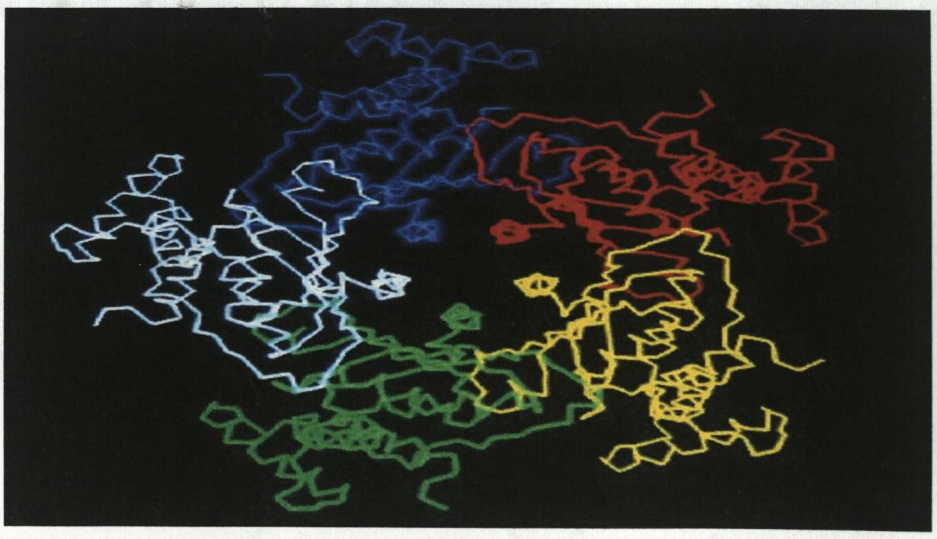
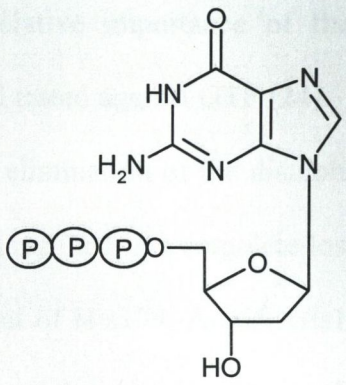


Figure 1.3 Crystal structure of GTPCH pentamer with the individual monomers are shown in different colours [50]

The location of the active site was confirmed when Huber *et al* managed to obtain crystals of a complex between deoxy-GTP **(33)** and GTPCH.[51] It was assumed that deoxy-GTP **(33)** would be incorporated in the same active site as GTP **(24)**.



(33)

These results confirmed the active site location, and due to the symmetry of the enzyme units it was shown that there were in fact ten active sites per macromolecule. A diagram of the active site of GTPCH containing deoxy-GTP **(33)** is shown in **Figure 1.4**, and this shows how **(33)** binds to the amino acids lining the cavity of the active site.

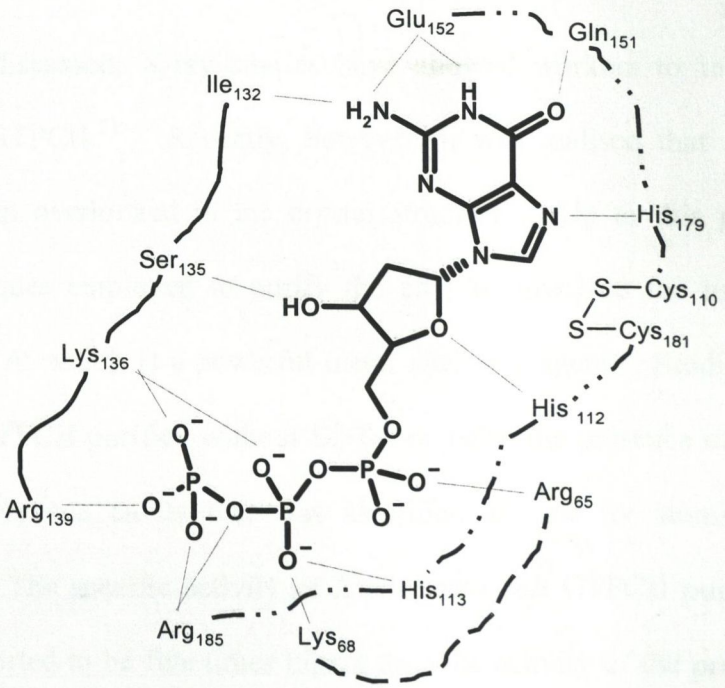


Figure 1.4 Interaction of GTPCH active site residues with deoxy-GTP[51]

To clearly define the relative importance of these residues, several mutant enzymes were generated and tested against GTP **(24)**. In particular, replacement of the histidine 179 residue or elimination of the disulphide bridge by replacement of cysteine 110 or 181 residues resulted in a complete loss of activity towards H_2-NTP **(25)** synthesis. Replacement of His179, Arg65, His112, His113, Ser135, Lys136, Glu152 or Arg139 also reduced the enzyme activity (**Figure 1.4**).[52] Over the next few years various studies were carried out to investigate the mechanism of this reaction and various proposals were made. However, in 2000 Bacher and co-workers[53] made a discovery that changed our understanding of the mechanism of the conversion of GTP **(24)** to H_2-NTP **(25)** and this work allowed the proposal of the most likely mechanism to date.

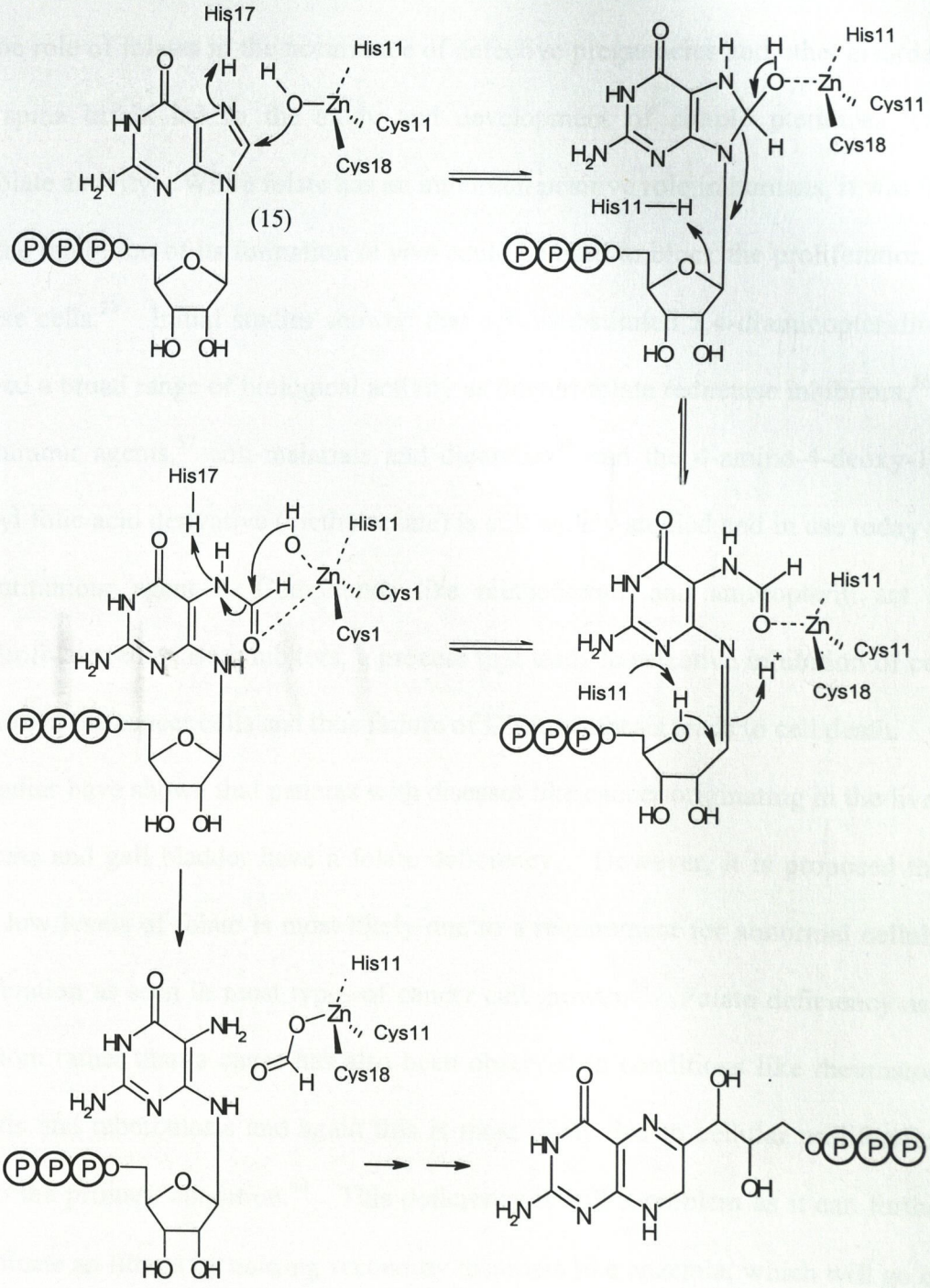
1.3.4 The mechanism of the conversion of GTP to H_2-NTP *under the action of GTPCH*

As already discussed, X-ray studies have allowed workers to investigate the active site of GTPCH.[51] Recently, however, it was realised that an important feature had been overlooked in the crystal structure. Up to this point, all the reported techniques employed to purify the enzyme involved the use of buffers containing EDTA, which is a powerful metal chelating agent. Studies on human and bacterial GTPCH purified without EDTA revealed the presence of a key metal ion in the active site cavity that was identified as zinc by atomic absorption spectrometry. The specific activity of *Escherichia coli* GTPCH purified without EDTA was reported to be five times higher than the activity of the previously used enzyme. It was found that in GTPCH of *Escherichia coli*, the zinc ion is bound to His113 and to the sulfhydryl groups of Cys110 and Cys181. The disulphide bridge

previously observed in GTPCH crystal structures had therefore been an oxidation side product of the two cysteine residues caused by the removal of the zinc ion by EDTA. Moreover, the mutant enzymes created by substitution of these amino acid residues involved in the zinc chelation did not possess the metal ion and failed to catalyse the reaction. Zinc was consequently pointed out as a key component of the GTPCH active site, and was shown to activate a water molecule that could generate a nucleophillic attack on the GTP imidazole ring. Closer studies of the original crystal structures with this in mind did show the presence of the metal and supported this new thinking about the mechanism, which is outlined in **Scheme 1.4**.

Finally, a series of quenched-flow and stopped-flow experiments was set up to identify the general kinetics and spectroscopic pattern of the enzymatic conversion of GTP **(24)** into H_2-NTP **(25)**.[54,55] These experiments provided the first direct evidence for the intermediates proposed in **Schemes 1.3** and **1.4**. The kinetic studies were conducted using quenched-flow experiments, in which a single turn-over enzyme-catalysed conversion of [8-^{14}C]GTP was allowed to proceed for 0.125 to 150 s, and was then quenched with trichloroacetic acid. GTP **(24)** and H_2-NTP **(25)** were identified and quantitatively assessed by spectroscopic data. The formylaminopyrimidine **(30)** and formate were monitored by radioactive measurements. Computer modelling to fit the experimental data that had been obtained with the quenched-flow experiments treated the kinetic equations derived from this sequence. Even if the experimental error combined with the simplification of the kinetic scheme achieved relative errors of 50%, the calculated rate constants clearly indicated that the rate-limiting step in the overall GTPCH sequence was the Amadori rearrangement (and/or ring closure) step and not the purine ring cleavage, as it was previously thought. More details on the reaction

sequence were obtained from advanced computer analysis of a single turnover stopped-flow experiment monitored by multi-wavelength ultraviolet spectroscopy. The three dimensional spectra (wavelength, time, absorbance) were submitted to background subtraction and singular value decomposition and this gave the first evidence for several of the other intermediates proposed in Schemes **1.3** and **1.4**. A simple kinetic model featuring five enzyme-catalysed reactions followed by product degradation was designed to model the experimental data. These results also provided evidence to show that the Amadori rearrangement of **(31)** to **(32)** was the rate limiting step of the whole biosynthetic sequence of GTP **(24)** to H_2-NTP **(25)**.



Scheme 1.4 Mechanism of the conversion of GTP into H_2-NTP

1.4. Folate and disease

The role of folates in the occurrence of defective pregnancies and other disorders like spina bifida led to the study and development of simple pteridines with antifolate activity. While folate has an important positive role in humans, it was felt that the inhibition of its formation *in vivo* could be used to block the proliferation of disease cells.[23] Initial studies showed that 6,7-disubstituted 2,4-diaminopteridines showed a broad range of biological activity as dihydrofolate reductase inhibitors,[56,23] anti-tumour agents,[57] anti-malarials and dieuretics[58] and the 4-amino-4-deoxy-10-methyl folic acid derivative (methotrexate) is still widely studied and in use today as an antitumour agent. Compounds like methotrexate and aminopterin act as dihydrofolate reductase inhibitors, a process that leads to selective inhibition of cell division in the cancer cells and thus failure of DNA synthesis leads to cell death.

Studies have shown that patients with diseases like cancer originating in the liver, pancreas and gall bladder have a folate deficiency. However, it is proposed that these low levels of folate is most likely due to a requirement for abnormal cellular proliferation as seen in most types of cancer cell growth.[59] Folate deficiency as a symptom rather than a cause has also been observed in conditions like rheumatoid arthritis and tuberculosis and again this is most likely due to cellular proliferation due to the primary condition.[60] This deficiency is still a problem as it can further complicate an illness by causing secondary disorders like anaemia, which will go on to weaken a patient and lower his/her natural defence against the primary disorder in question. In situations like this, methotrexate in low doses can be used to treat non-cancerous diseases like rheumatoid arthritis, asthma, lupas and psoriasis.[61]

The fact that a change in folate or pteridine concentration can occur when disease is present can be used as a diagnostic tool for various disorders.[62] For example, the

determination of neopterin levels in human body fluids offers a useful and innovative mechanism for monitoring diseases associated with the activation of cell-mediated immunity. Applications of this test include prognostication of HIV-infected patients, follow-up control of ICU and allograft transplantation patients, screening of blood donor samples and differentiation of viral from bacterial infections.[63] The discovery of this relationship has led to the development of many commercial pteridine diagnostic kits that are widely used today.

1.5 Tetrahydrobiopterin and disease

The human body relies on a *de nova* synthesis of H_4biopterin **(10)** and does not normally obtain this co-vitamin from dietary sources. H_4biopterin **(10)** imbalance as already discussed can lead to very serious disorders like phenylketonuria (PKU). It also plays a crucial role in the synthesis of neurotransmitters, and many studies have been carried out to investigate if a deficiency of H_4biopterin **(10)** has any role to play in neurological conditions.[64] In recent years, research has shown that a H_4biopterin **(10)** imbalance can either be the main or contributing factor for certain neurological disorders, as described below.

1.5.1 Dystonia

This disease causes involuntary muscle contractions, impaired motor control and some neural damage. When patients with dystonia were studied it was found that the levels of biopterin **(37)** and H_4biopterin **(10)** in the cerebral spinal fluid (CSF) were very low.[65] Treatment with H_4biopterin **(10)**, however, was only partially successful in alleviating their symptoms, suggesting that the low levels were only a contributing factor rather than a direct cause of the condition.[66] Initial studies also

proposed that altered levels of tyrosine hydroxylase were responsible for the occurrence of dystonia. Further analysis ruled this possibility out, however, though a recessive form of the disease has been traced to a mutation in the tyrosine hydroxylase gene.[67] Another genetic relationship was observed when it was noticed that the gene responsible for producing GTPCH is also found on the same chromosome as the point mutation is found in the recessive form of the disease. Also patients with the hereditary progressive form of dystonia have been found to contain a mutation in the gene responsible for GTPCH production.[68]

1.5.2 Parkinson's disease

This disease is characterised by loss of motor control with lack of voluntary movement and degeneration of the central nervous system (CNS). It was found that people suffering from Parkinson's disease had a defect in monoamine neurotransmitter metabolism and as a direct result had lowered levels of dopamine.[69] A reduced level of H_4biopterin **(10)** was also found in the CSF, although lower levels were also found in patients suffering from similar related conditions, perhaps indicating some unknown primary cause was responsible.[70]

1.5.3 Alzheimer's disease

It has been shown that patients suffering from senile dementia of the Alzheimer type have lower levels of H_4biopterin **(10)**, than that observed in the general population.[71] Levels of phenylalanine **(11)** in comparison to tyrosine **(12)** were found to be very high also, most likely due to lower levels of phenylalanine hydroxylase.[72] To date there is no evidence of a direct relationship between levels

of H_4biopterin **(10)** and this disease, but work continues to investigate any direct relationship.

1.5.4 Autism

Autism results in lowered intelligence and communication abilities. Currently very little is known about this condition, though recently it has been shown that patients suffering from autism have lowered levels of aromatic amino acids and that upon supplementation of H_4biopterin **(10)** some improvement was observed.[73] It is still not known whether patients with autism have lower levels of biopterin, though it is known that a lot of its symptoms are very similar to those of PKU.[74]

1.6 Work described in this thesis

The work described in this thesis involves the study of two distinct areas of research within pyrimidine and pteridine chemistry. The first of these involves investigation of the pteridine biosynthetic pathway within the zebrafish (*Danio rerio*). The study of this species is relatively a new area but the zebrafish (*Danio rerio*) is a fast replacing *Drosophila* as the genetic model of choice around the world today. While it has been suggested that pteridines play a role in the pigmentation of zebrafish, no real study of this has been carried out before. In a programme of collaborative research with Dr. Imgard Ziegler (Munich) a series of pteridines were extracted from wild type zebrafish (*Danio rerio*) with the goal of investigation of the biosynthetic pathway of the pterins present and their role in the development of the neural crest. The work describes the analysis of these extracts by HPLC-MS and confirmation of their structure by comparison to authentic samples. Once identified their formation could be monitored and the pteridine pathway could be identified.

The main part of the thesis concerns a study of 2,4-dinitrophenyl oximes as a potential source of electrophilic nitrogen. This could have led to a new route for the synthesis of pteridines. While this initial goal was not achieved some novel sigmatropic rearrangement reactions were uncovered which led to interesting new heterocyclic cyclisations.

Chapter Two

The Zebrafish
(*Danio rerio*)

Development of the pteridine pathway.

2.1 Why study the zebrafish?

2.1.1 Introduction

Well-defined traits like seed shape and seed colour in peas allowed Mendel to formulate the principles that form the basic foundation of understanding genetic inheritance.[75] Mendel's work allowed man to realise that certain traits were determined by those of the parent species, such as eye or hair colour in humans. An understanding of the roles of genes in development requires that we identify as many of the genes involved as possible. In an era where the solving of the human genome has been widely discussed, its beginnings originated with the detailed study of one species in particular; *Drosophila melanogaster* or the fruit fly, which is one of the most important organisms in biological research.

The discovery of a mutant strain of *Drosophila melanogaster* with white eyes instead of the normal red stimulated Morgan[76] and his collaborators to collect and study a large number of mutants with visible adult phenotypes.[77] Since then, a large number of mutations causing visible phenotypes in adult *Drosophila* have been accumulated and these have allowed the elucidation of its full genome.[78] An understanding of the cause and consequence of these mutations can be used as a model to study many aspects of human development.

One of the main reasons why *Drosophila* is studied in such detail is the fact that it has polytene chromosomes or 'magic markers'. These allow the study of the species chromosomes under a microscope.[79] This characteristic also makes invertebrates like *Caenorhabditis elegans* (ring worm) good genetic model organisms.[80] Vertebrates are not as amenable to genetic analysis, as traditionally their development is not as easy to study. The classical vertebrate used for genetic analysis has been the mouse and to a lesser extent the chicken. Unlike the fruit fly,

however, their development takes place in the womb. This limits study, as most analysis has to be carried out postnatal and thus does not supply information on mutations that affect the earlier stages of development. From a practical point of view these are high maintenance animals, which leads to higher costs, and a longer time needed to obtain the same level of information in comparison to species like *Drosophila.*[81]

The solution to this problem is the use of a vertebrate species that has external development, a fast developmental time scale and is cheap to maintain. One theoretical animal model that would allow study of all aspects of genetic development in vertebrates was first proposed by Sive.[82] The "fromoshken", as shown in **Figure 2.1**, is a frog-mouse-fish-chicken combination.

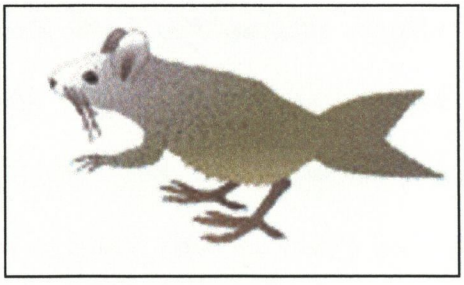


Figure 2.1 The 'Fromoshken' a vertebrate study model.

While the fromoshken is an unrealistic option, its design was spurred by the demand for a species that allows the study of all aspects of embryonic development in vertebrates. Studies of the individual species only allow fragments of information to be obtained, but one species, the zebrafish (*Danio rerio*) shown in **Figure 2.2**, is fast becoming the model of choice for post fertilisation study of vertebrates.

Figure 2.2 The zebrafish (*Danio rerio*)

2.1.2 Characteristics of the zebrafish (Danio rerio)

In 1981, Streisinger[83] recognised that the zebrafish had many of the advantages observed in invertebrates like *Caenorhabditis elegans* and *Drosophila*. These included high fecundity (mature females lay several hundred eggs at weekly intervals), short generation time (3-4 months), rapid development, external fertilisation, translucent embryos, and practically they are cheap and easy to feed and maintain.

High fecundity allows high output per parent fish, which allows many hybrid studies to be carried out over a short space of time. The ability to produce so many embryos is also a big advantage over other models used. A mouse for example will produce on average only twelve embryos a year. The rapid development observed in zebrafish makes it ideal for genetic studies by use of point mutations (PM). These allow the study of all aspects of its development through hybridisation and study of the consequential change in the offsprings physical attributes. A generation time of 3-4 months allows a large number of genetically different species

to be studied over a short space of time. For example, the zebrafish's development over three days is equivalent to that observed over three months in humans, and its development is up to fifty times faster than that observed in the mouse. In the zebrafish, most major organ systems have developed within 24 hours post fertilisation (HPF). **Figure 2.3** illustrates how the zebrafish develops from four cells (1 HPF) to a swimming larva within five days.[84]

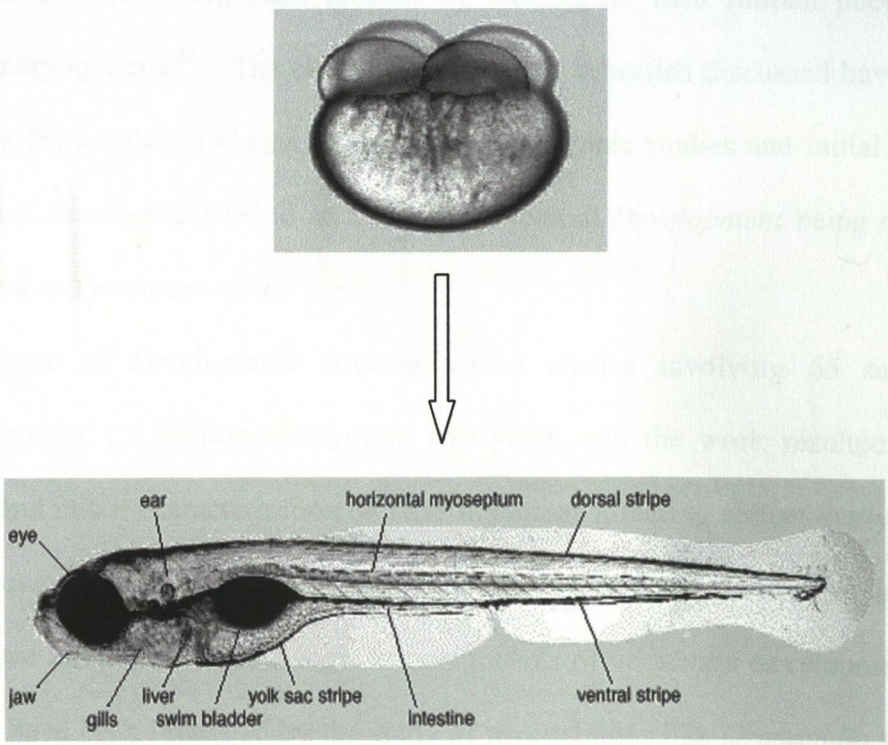


Figure 2.3 Development of the zebrafish (one hour to five days)

Perhaps the biggest advantage of the zebrafish is the fact that its development is external and this development occurs within translucent embryos. This feature allows the study of the major organ development through the transparent skin, even cells developing deep within the embryo can be observed using relatively low magnification microscopes.[85] This also allows individual cells to be labelled so

their development can be monitored, understanding how mutations affect embryonic cell fates.[86]

Many of the fundamental principles of vertebrate embryology were elucidated by classical experiments in amphibia embryos. However, an understanding of the underlying mechanisms has been hampered by the inability to carry out genetic studies in these organisms. Using early studies it was realised that the most efficient way to find genes that have key regulatory functions in vertebrate development, is to screen them directly by looking for their mutant phenotypes during embryogenesis.[85] The characteristics of the zebrafish discussed have led it to become the species of choice for vertebrate embryonic studies and initial studies of zebrafish development led to an issue of the journal *Development* being devoted to this work in December 1996.[87]

This issue of *Development* covered screen studies involving 65 scientists examining over 1.5 million embryos in two years, and the work resulted in the isolation and initial characterisation of 1858 mutations affecting almost every aspect of embryonic development within the zebrafish. Mutations discovered in these studies have already revealed a logic to components of vertebrate development that could not have been predicted by the study of invertebrates like *Drosophila* or even in vertebrates like the mouse.[88] These studies involved chemically induced point mutations being studied under a stereomicroscope in order to examine what alterations occurred in the morphological features of the resultant fish. The studies were designed to cover many developmental features including maternal effects,[89] establishing the body axis[90] and the central nervous system.[91] The most important aspects of this work , however, was the study of the neural crest.

2.1.3 The Neural Crest

Thorogood[92] once described the neural crest as "the only interesting thing about vertebrates" as it is responsible for some of the most important cells and developmental aspects of all vertebrates including humans. It is the favourite system for studying cell fate specification since it begins as a population of cells that branch out to form a diverse range of systems which control many aspects of the body.[93]

Neural crest cells (NCC) originate early in vertebrate development. As shown in **Figure 2.4**, these cells originate in the neural tube (NT) where they then move to the migration staging area (MSA) and migrate along defined pathways in the embryo.

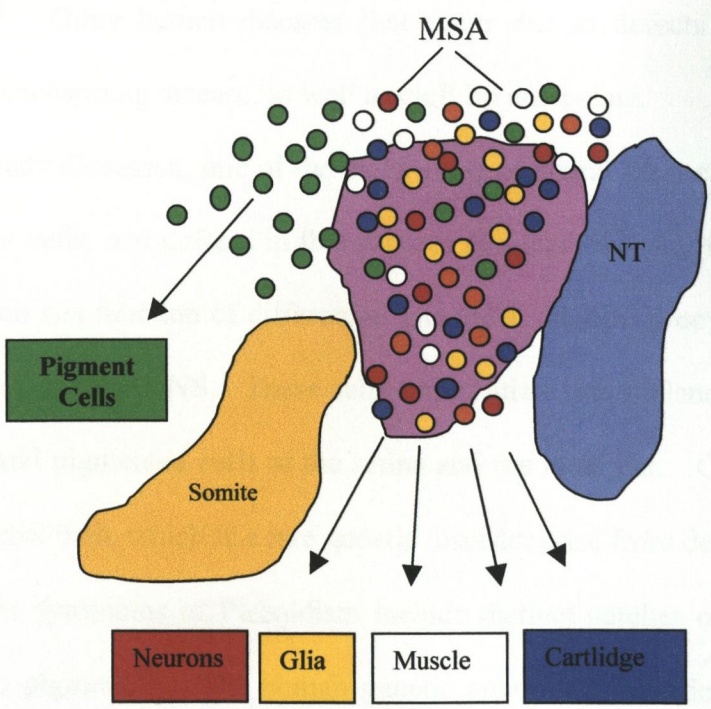


Figure 2.4 Migration of the neural crest cells from the migration staging area.

In the trunk, these cells differentiate into melanocytes (pigment cells in the skin) and cells of the peripheral nervous system. In the developing cranial region these cells contribute to the formation of cell types including cartilage and bone giving rise to the upper and lower jaw and inner and outer ear as well as neurons, glia and smooth muscle.[94]

Considering that the neural crest contributes to so many different functions in a species, its study can provide an understanding of many aspects of human development and disease. Neural crest cells (NCC) are known to be involved in cardiovascular development, through their control of aortic artery development. From this a whole series of human cardiac defects known as 'neural crest-associated' have been discovered. Study of these cells is also used to investigate cardiac inflow and outflow anomalies, and conditions that can occur in the aorta in humans.[95] Other human diseases that occur due to defects in the neural crest include Hirschsprung disease, as well as cleft lip and palate.

As already discussed, one of the many roles of neural crest cells is the formation of pigment cells, and defects in this process can lead to many different conditions. Myelination is a function of differential glia cells: oligodendrocytes in the CNS and Schwann cells in the PNS. These cells differentiate into melanocytes (that pigment the skin) and pigmented cells of the retina and the inner ear. Conditions including human Piebaldism, which is a rare genetic disorder arise from defects in the pigment cells. The symptoms of Piebaldism include distinct patches of skin and hair that contain no pigment.[96] The human genetic condition, Waardenburg syndrome, is another condition caused by a defect in pigment cell production. This causes deafness with a defect in the pigmented cells and can cause non-development of the

retina within the womb. Studies of the zebrafish NCC have been used to investigate this condition in particular.[97]

In humans, epidermal cells have the role of neutralising any radiation the skin is exposed to (particularly from the sun) before it reaches the deeper layers of tissue. The epiderm uses the melanocytes to perform this role. As stated these melanocytes originate in the neural crest and in mammals melanin is the main pigment, present as eumelanin and pheomelanin, responsible for brown-black and orange-red colour respectively.

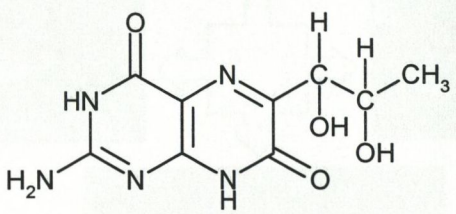
Pigment cells like many other cells in our bodies are prone to abnormal growth patterns generally referred to as tumours. Non-cancerous or benign tumours originating from abnormal pigment cell growth exist as moles, something most people have. The cancerous forms of these tumours are called malignant melanoma, which is the most aggressive form of skin cancer. This has a very high probability of spreading to other organs of the body like the lymph nodes, lung, liver brain or bone, if it is not diagnosed and treated early. [98] Statistical data from America shows that the incidence of malignant melanoma has doubled in Caucasians from 1970 to 1996 and this trend is expected to continue to rise.[99] This emphasises the need for a better understanding of this disease and study of the development of pigment cells originating in the neural crest.

2.1.4 Pigments in the zebrafish

To date, it has only been suggested that pteridines act as pigments in zebrafish, with their biosynthesis patterns changing according to mutation.[100] Within general cyprinid fish there are three types of pigment cell types. Firstly there are the Iridophores, which are silver in colour, and consist of cells that are packed with

purine crystal's identified as guanine and hypoxanthine.[101] Secondly there are the black melanophores, which are responsible for the synthesis of melanin from DOPA **(13)**, the source of DOPA being tyrosine **(12)**. H_4biopterin **(10)** may therefore be required as a cofactor for the hydroxylation of phenylalanine **(11)** within these species of fish.[28] As early as 1963, Ziegler[102] had suggested that H_4biopterin **(10)** was responsible for this reaction in melanophores. More importantly it has since been shown that it plays a role in the synthesis of tyrosine **(12)** in human epidermis, this then goes on to form the melanin in human skin.[103]

The final type of pigment found in cyprinid fish is the xanthophores, in which pteridines serve directly as pigments. Recent research has suggested that the yellow appearance of these cells is due directly to the presence of sepiapterin **(7)**.[100] 7-oxobiopterin (ichthyopterin) **(34)** may also be present in xanthophores.[104]



(34)

These pigment cells are a prominent and easily visible neural crest derivative. As part of the screening process that led to the 1996 issue of 'Development'[87] 258 mutations in genes were performed affecting embryonic pigmentation patterns.[97,100] The ability to transplant genes controlling pigments in zebrafish is at this stage a well developed area, and has allowed the production of various species with different pigment phenotypes. This in turn has allowed a deeper understanding of the role of specific genes in forming different pigmentation patterns in the zebrafish.

In the original 'wildtype' zebrafish, *Danio rerio* (**1**, **Figure 2.5**), the colour pattern is made up of several well defined, horizontal dark melanophore stripes with intervening, light interstripe regions, explaining the naming process. Some examples of the mutant fish that were obtained after point mutations (P.M.) are shown in **Figure 2.5** (**D-F**) and the pictures show that by affecting melanophore and xanthophore development, visibly different species are obtained.[105] It remains true, however, that in spite of all that has been discovered in this area, the chemical constitution of the pigments of the zebrafish is still largely unknown.

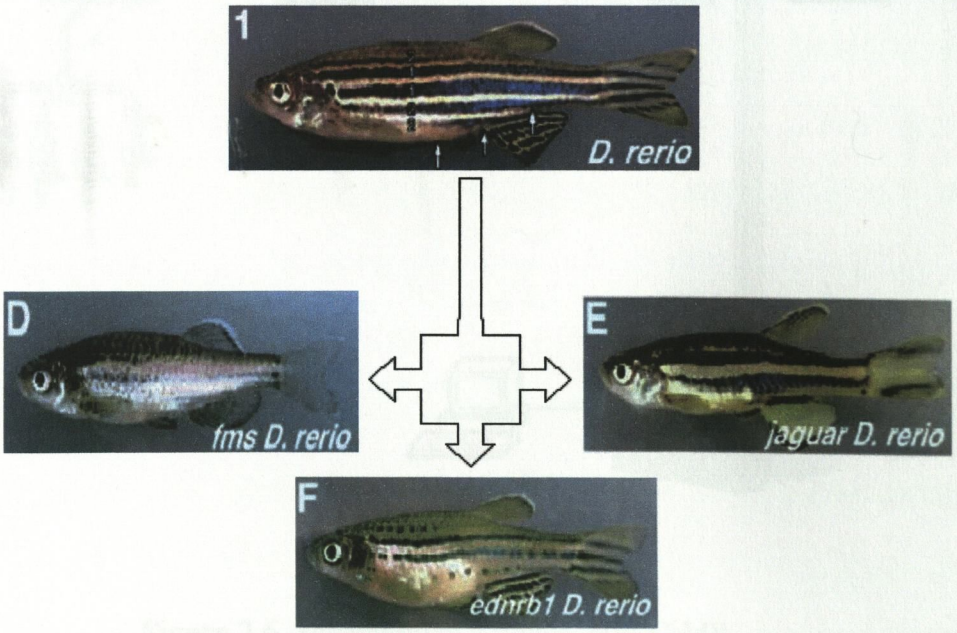


Figure 2.5 *Danio* pigment patterns observed after point mutations of 'wild type' *Danio rerio*

2.1.5 Objectives of the work

In collaboration with Dr. Ziegler of the GSF Institute, Munich, we set out to determine the biosynthetic pathway of the pteridines in the zebrafish (*Danio rerio*). Our role in this project was to analyse by HPLC-MS, various organic extracts that were isolated from the zebrafish by Dr. Ziegler.

2.2 HPLC-MS

2.2.1 HPLC analysis

In this work, reverse phase HPLC columns were used, together with a photo diode array (PDA) detector. The latter allowed UV spectra of individual chromatographic peaks to be obtained. A typical HPLC-MS system is shown in **Figure 2.6**.

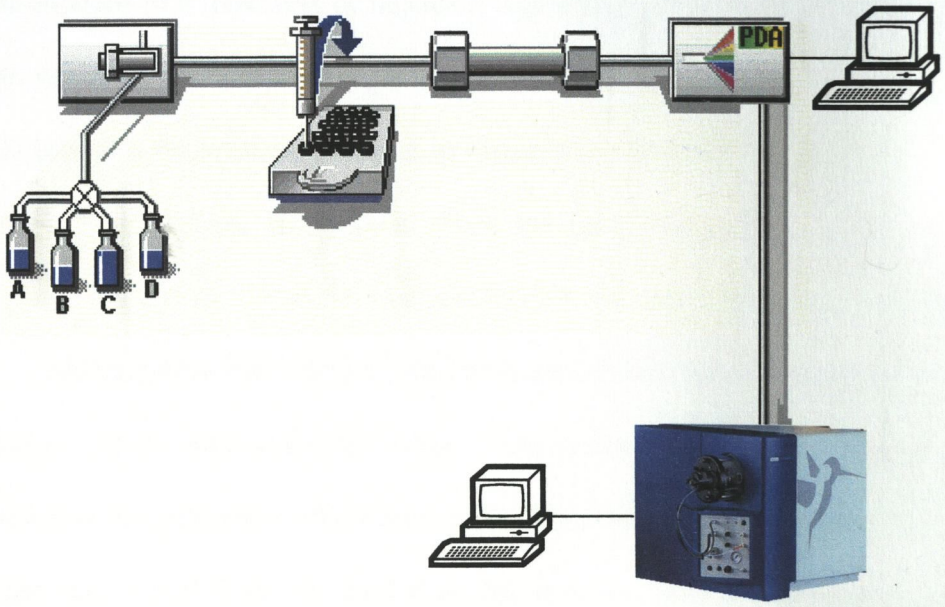


Figure 2.6 Schematic of a typical HPLC-MS

2.2.2 Electrospray Mass Spectrometry

The mass spectrometer used in this work is a Micromass® LCT electrospray Time of Flight mass spectrometer. The typical feature of an electrospray ionisation (ESI) spectrum is that it does not normally show the fragmentation pattern typical of electron ionisation (EI) or fast atom bombardment (FAB) mass spectra. Instead, the main peak in an ESI spectrum corresponds to the molecular weight of the sample

plus a positive ion, usually H^+ or Na^+. It is also possible to obtain negative ion ESI spectra in which the main peak corresponds to a negative ion, usually the molecular weight minus H^+.

Figure 2.7 shows, in schematic form, a Micromass® LCT electrospray mass spectrometer. In this, the mobile phase containing the sample is pumped through a fine capillary tube into the ionisation chamber. Here with the aid of nitrogen as a nebuliser gas, a cone like spray is created ('Taylor cone effect'). At this stage the spray is exposed to a (positives or negative, depending on mode of analysis) voltage of up to 4000v at the 'ion source', which ionises the molecules present. Nitrogen gas also acts as a desolvation gas, and in conjunction with the high temperatures of the chamber the solvent is removed from the ions of interest. The ions are transferred to the time of flight analyser (TOF) *via* two Radio Frequency (RF) lenses. After entering the analyser, the ion beam is focussed into the pusher by the acceleration, focus, steer and tube lenses. The pusher then pulses a section of the beam towards the reflectron, which then reflects ions back to the detector.[106]

As the ions travel from the pusher to the detector they are separated in mass according to their flight times, with ions of the highest mass to charge ration (m/z) arriving later in the spectrum. The pusher may be operated at repetition frequencies of up to 20 KHz, resulting in a full spectrum being recorded by the detector every 50 milliseconds. The ions leave the TOF analyser and are detected by a dual microchannel plate detector and ion counting system.

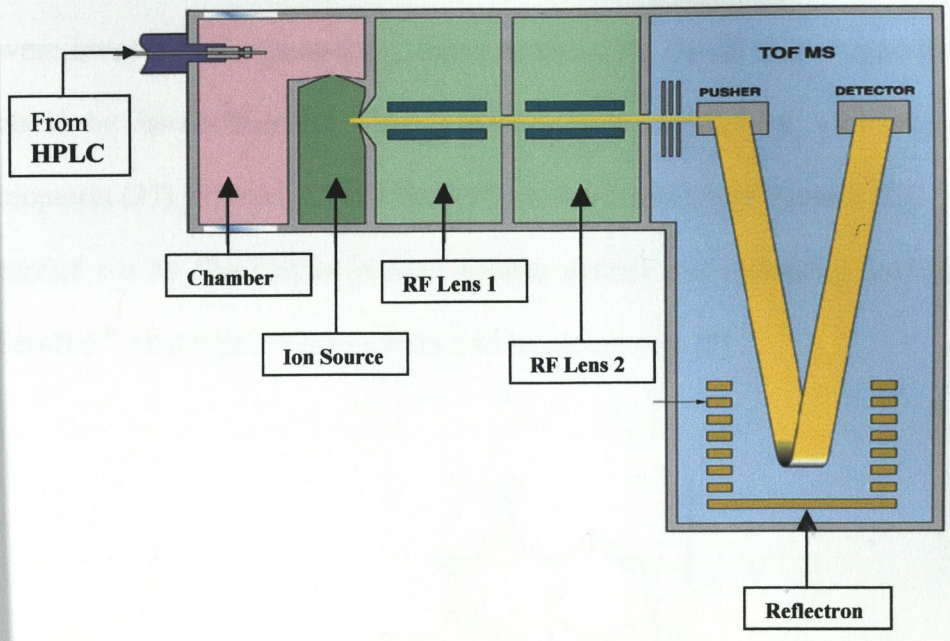
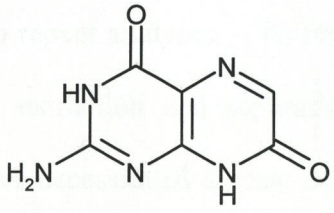


Figure 2.7 Schematic of the Micromass® LCT electrospray Time of Flight mass spectrometer

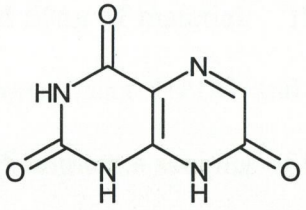
2.3 Analysis of zebrafish extracts

The animals used for this study were wild type zebrafish and were raised in the zebrafish facility of the Institute of Mammalian Genetics, Munich. Embryos were obtained from natural spawning and were grouped according to the number of hours postfertilisation (HPF) at 28.5°C. The samples to be analysed were collected in batches of 10-20 animals, immediately frozen, and kept at -85°C. Batches of 50 animals (90-120 HPF) were extracted by Dr. Ziegler using homogenisation with a Wheaton Teflon homogeniser in Tris buffer (50mM, pH 6.9) and centrifuged at 13,000xg for 10 minutes. The extract was oxidised by acidic iodine, deproteinised by trichloroacetic acid, and purified by cation exchange chromatography. The lyophilised material was pre-fractionated by reverse phase HPLC, using 3% methanol in water and a fluorescence detector (Shimadzu RF535) and the fractions

were concentrated in vacuum. This work yielded three main fractions (I-III), which were investigated separately. Initial analysis by Dr. Ziegler suggested that these could be isoxanthopterin **(35)** (Fraction I), a mixture of violapterin **(36)** and biopterin **(37)** (fraction II) and finally 7-oxobiopterin **(34)** (fraction III). Other work carried out by Dr. Ziegler yielded another extract and following purification using Sep-Pak® cartridges, this was shown to be sepiapterin **(7)**.



(35)



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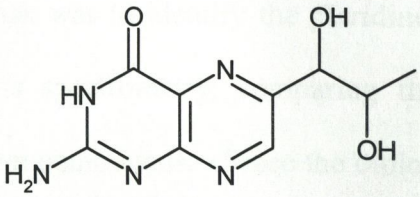

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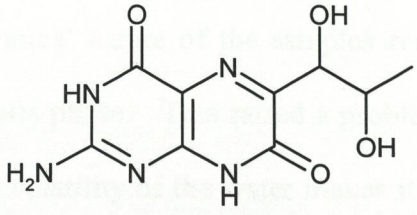
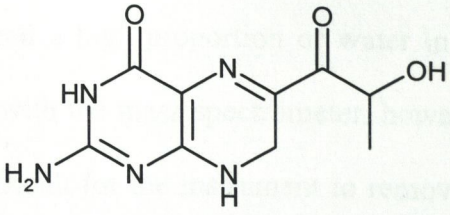


(34)



(7)

The goal of this present work was to identify the pteridines in fraction I, and II, using the HPLC-MS technique described.

2.3.1 Method development

The main difficulty in this work was the tiny amount of biological sample available for analysis. It was estimated by UV absorption that the total amount of pteridine contained in each of the fractions obtained by Dr. Ziegler was only 60 ng, and this raised two main problems. Firstly, all work had to be carried out at the limit of detection of our instruments. Secondly, since the total amount of pteridine in each fraction was of the order of only 60 ng, each sample was very precious, so that it was not possible to do repeat analyses. To repeat the analysis again would have required the breeding, extraction and separation of further batches of the zebrafish, and this would have necessitated a great deal of work on the part of our German partners. The biological extracts supplied to us by Dr. Ziegler for analysis contained two samples of fraction I, and two samples of fractions II, each containing an estimated 60ng of material. The task was to identify the pteridines in each of these fractions using HPLC and mass spectrometry, comparing the biological samples with authentic samples of known compounds. Since the biological sample contained so little material, all method development was carried out using authentic pteridine samples at a concentration of 2 ng/μl.

The chemical nature of the samples required a high proportion of water in the HPLC mobile phase. This raised a problem with the mass spectrometer, however, since the involatility of the water makes it difficult for the instrument to remove in the ionisation chamber. Another problem was that the difficulty in removing the water, required slow flow rates through the MS, which in turn led to in ordinarily long retention times (R_t) on the HPLC column. These problems necessitated a great deal of work in finding HPLC experimental conditions that would suit the mass spectrometer, while allowing efficient HPLC analysis of the fractions. One useful

technique was to employ narrow bore HPLC columns, which allowed for slower flow rates without significantly affecting the resolution or increasing the retention times (R_t).

As with the HPLC system, the analysis conditions of the mass spectrometer were developed using authentic pteridine samples at a concentration of 2 ng/µl. This was achieved using the continuous flow system shown in **Figure 2.8**.

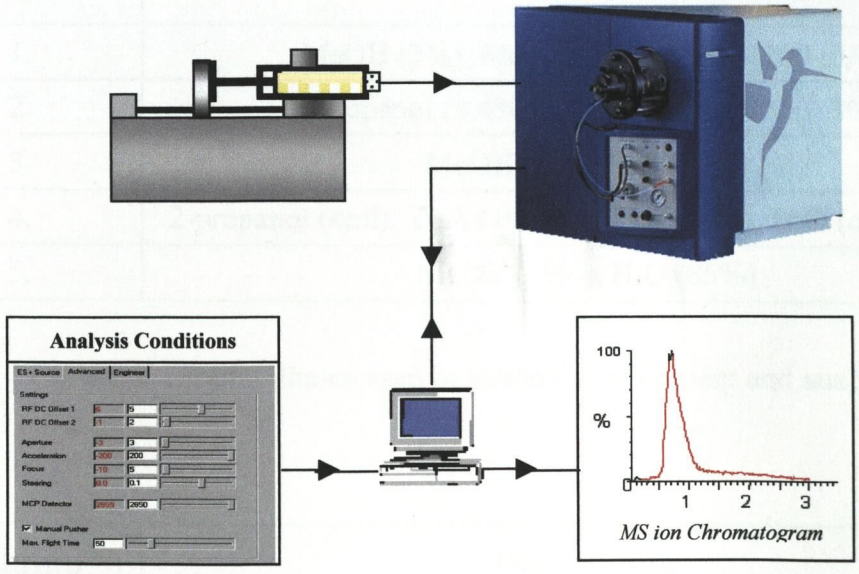


Figure 2.8 Continuous flow apparatus for Mass Spec. sensitivity development

Using this set-up (**Figure 2.8**), much work was done to improve the sensitivity of the mass spectrometer for each of the pteridines to be analysed. This involved changing the instrument parameters, including the capillary voltage, desolvation and source temperatures, acceleration, focus, time of flight tube, detector and physical variables like the position of the probe in the chamber and monitoring the effect of this change on sensitivity. Using the syringe pump, a solution of the compound to be analysed was supplied to the MS at a constant flow rate. By monitoring the MS ion chromatogram for the compound in question, the effect of changing any variable

was monitored, and adjusted to optimise the sensitivity of the system. Optimisation of the MS system for each molecule studied in this work was particularly important, considering the amount of material available to analyse. In the case of a mixture, however, the average optimum system had to be used and thus the sensitivity of the system to certain parts of the mixture was compromised.

System	Composition
1.	MeOH (3%), MeCN (1%), H_3PO_4 (200 µl/l)
2.	2-propanol (0.4%), TEA (1%), H_3PO_4 (0.3%)
3.	MeOH (3%), H_2O (97%)
4.	2-propanol (4ml), TEA (10ml), H_3PO_4 (pH7), H_2O (ca. 983 mls)
5.	MeOH (15%), H_2O (85%)

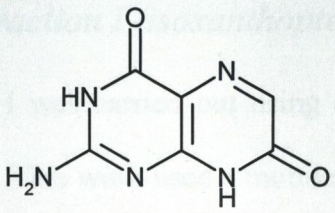
Table 2.1 Mobile Phases used in method development and analysis

Column	Description
A.	Hypersil ODS; 250 x 4.6mm I.D
B.	Nucleosil 10; 250 x 4.0 mm I.D.
C.	Hypersil H5ODS; 250 x 3.2mm I.D

Table 2.2 HPLC columns used in method development and analysis

2.4 Analysis and identification of Fraction I (isoxanthopterin)

Initial analysis by Dr. Ziegler of fraction I in comparison with standard pteridines suggested that the main constituent of the fraction may be isoxanthopterin (7-oxopterin) **(35)**.



(35)

Firstly, HPLC analysis of this fraction using a fluorescence detector showed one main fluorescence emission at 410 nm, identical to authentic isoxanthopterin **(35)**. Secondly, UV-Vis analysis of the fraction yielded identical spectra in both 0.1M HCl and NaOH, with those obtained for authentic isoxanthopterin **(35)**. These results suggested that the first pteridine identified in developing zebrafish was isoxanthopterin **(35)**. This was confirmed, however, using HPLC-MS as described below. The HPLC-MS analysis conditions employed were developed and optimised using an authentic sample of isoxanthopterin **(35)** supplied by Dr. Ziegler. As discussed already, the solubility of pterins is very poor,[9] and this created problems since isoxanthopterin **(35)** proved to be insoluble in all organic solvents. Due to this, 0.1 M ammonium hydroxide was used as the dissolution solvent. This in turn, however, led to a different problem since the MS (ESI) system is sensitive to the presence of salts, which can disrupt the results giving rogue peaks in the final spectrum. This in turn reduces the overall sensitivity of the system. This problem was overcome by using a 'stripping' function within the MS software. This allowed us to subtract a standard ammonium hydroxide spectrum from the final sample spectrum, similar to running a background scan.

2.4.1 HPLC analysis of Fraction I (isoxanthopterin)

Initial analysis of fraction I was carried out using a Shimadzu HPLC analysis system with a PDA detector. This work used a mobile phase of 85:15 H_2O:MeOH at a flow rate of 0.35 ml/min, this system was developed using authentic isoxanthopterin **(35)**. Authentic isoxanthopterin **(35)** under these conditions was found to have a retention time (R_t) of 5.43 minutes. Its UV spectrum under these conditions is shown in **Figure 2.9**.

The zebrafish extract, while not completely pure, was shown to contain one main compound by HPLC analysis, and this compound had an identical retention time (R_t) (5.43 min.) and an identical UV spectrum (λmax 203, 229 and 334 nm) with that of authentic isoxanthopterin **(35)**, as already described. Analysis of the zebrafish extract (fraction I), spiked with a standard solution of isoxanthopterin **(35)** showed co-elution of the two samples, causing the main signal (R_t 5.43 min.) to grow in intensity. The peak was shown to be homogeneous (UV absorption) across its width, confirming that fraction I was isoxanthopterin **(35)**.

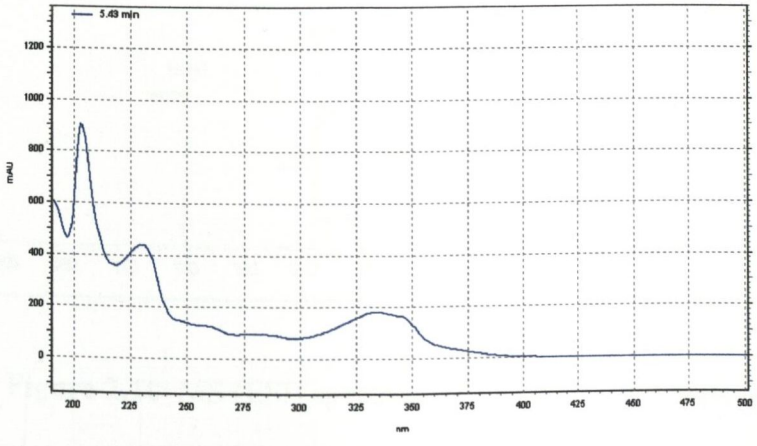


Figure 2.9 UV spectrum of authentic isoxanthopterin

2.4.2 HPLC-MS analysis of Fraction I (isoxanthopterin)

HPLC-MS analysis of fraction I was carried out using mobile phase system 5 (**Table 2.1**) and HPLC column C (**Table 2.2**). Column C was a reverse phase narrow bore column (I.D. 3.2 mm), and this allowed a flow rate of 0.2 ml/min to be used. As described already, the HPLC-MS conditions were optimised using a sample of authentic isoxanthopterin **(35)**. Using these conditions, a solution of authentic isoxanthopterin **(35)** (2 ng/μl in 0.1 M NH_4OH) was examined by HPLC. The UV chromatogram showed one main peak. The total ion chromatogram also showed one main peak. The corresponding mass spectrum was very crude because of the presence of the ammonium hydroxide. Software subtraction of a standard ammonium hydroxide spectrum, however, gave the mass spectrum shown in **Figure 2.10**. This is the mass spectrum of authentic isoxanthopterin **(35)** and contained three main peaks corresponding to $M+H^+$ (m/z 180), $M+Na^+$ (m/z 202) and $M+K^+$ (m/z 218). The other minor peaks present within the spectrum are most likely due to the ammonium hydroxide.

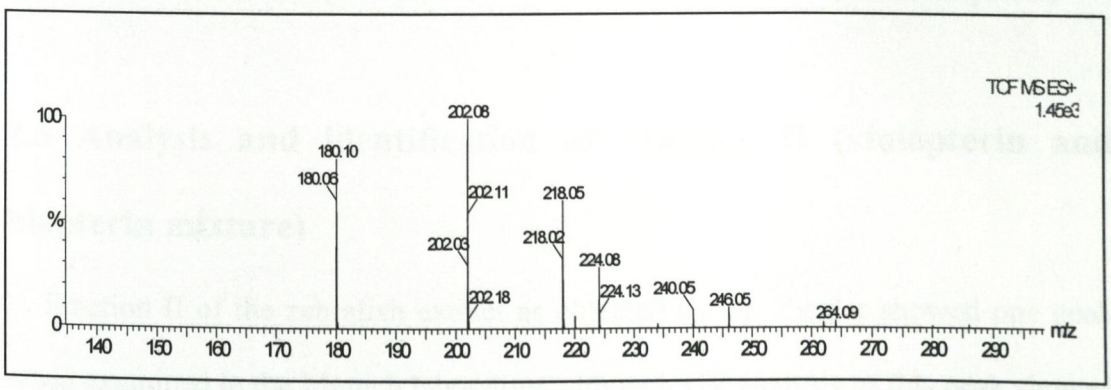


Figure 2.10 MS (ESI) spectrum of authentic isoxanthopterin

The zebrafish extract (fraction I) was examined by HPLC-MS using the same conditions as were used for the authentic isoxanthopterin **(35)**. The total ion

chromatogram obtained for fraction I, showed one main peak with a retention time (R_t) corresponding to that observed for the authentic isoxanthopterin **(35)**. The crude mass spectrum of this peak was subjected to software subtraction of a standard ammonium hydroxide spectrum to give the mass spectrum shown in **Figure 2.11**. This spectrum exhibited only two main peaks at m/z 180 and m/z 218, and these peaks correspond to isoxanthopterin **(35)**, where m/z 180 corresponds to $M+H^+$ and m/z 218 corresponds to $M+K^+$. These results confirm that the pteridine contained in the zebrafish extract, fraction I, was isoxanthopterin **(35)**

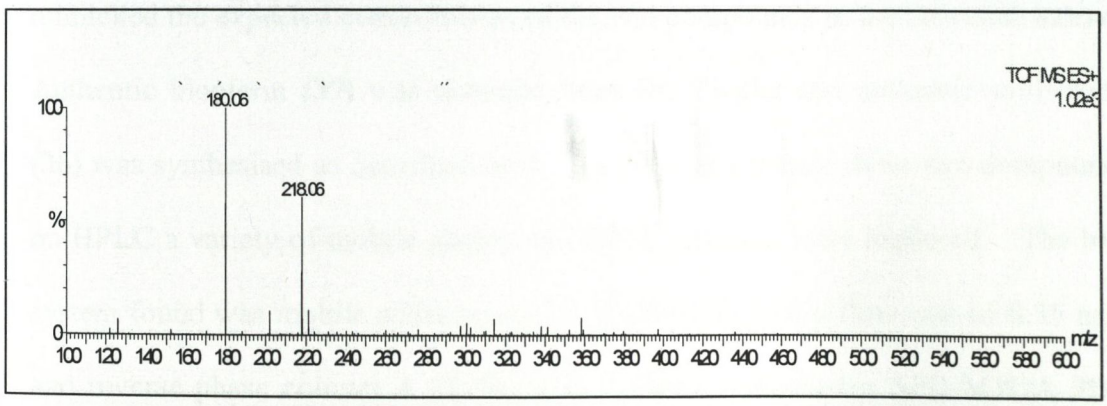
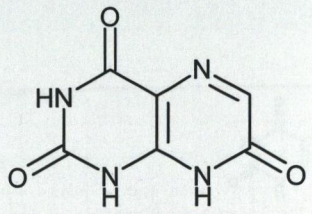


Figure 2.11 MS (ESI) spectrum of zebrafish extract, fraction I (isoxanthopterin)

2.5 Analysis and identification of Fraction II (violapterin and biopterin mixture)

Fraction II of the zebrafish extract as obtained by Dr. Ziegler showed one peak when examined in the Munich laboratory, although UV analysis of this peak showed that it was not homogeneous, and suggested it could contain a mixture of violapterin (2,4,7-trioxopteridine) **(36)** and biopterin **(37)**. In the present work, the zebrafish extract (fraction II) was examined further both by HPLC and HPLC-MS.



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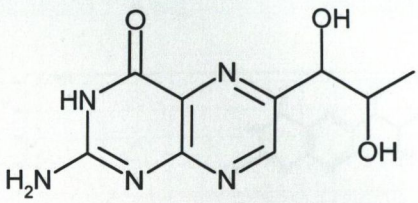


(37)

2.5.1 HPLC analysis of fraction II (violapterin and biopterin)

Initial work in our laboratory used a 50:50 mixture of authentic violapterin **(36)** and biopterin **(37)** at a final concentration of 1ng/µl each. This concentration mimicked the expected concentration of the two compounds in the zebrafish extract. Authentic biopterin **(37)** was obtained from Dr. Ziegler and authentic violapterin **(36)** was synthesised as described later. In order to separate these two compounds on HPLC a variety of mobile phases and HPLC columns were explored. The best system found was mobile phase system 5 (**Table 2.1**) with a flow rate of 0.35 min. and reverse phase column A (**Table 2.2**). Using a Shimadzu SPD-M1OA PDA detector, violapterin **(36)** and biopterin **(37)** were clearly resolved into two peaks (R_t 2.99 and 4.52 min. respectively), and from these UV absorption spectra for both authentic violapterin **(36)** and biopterin **(37)** were obtained, as shown in **Figure 2.12**.

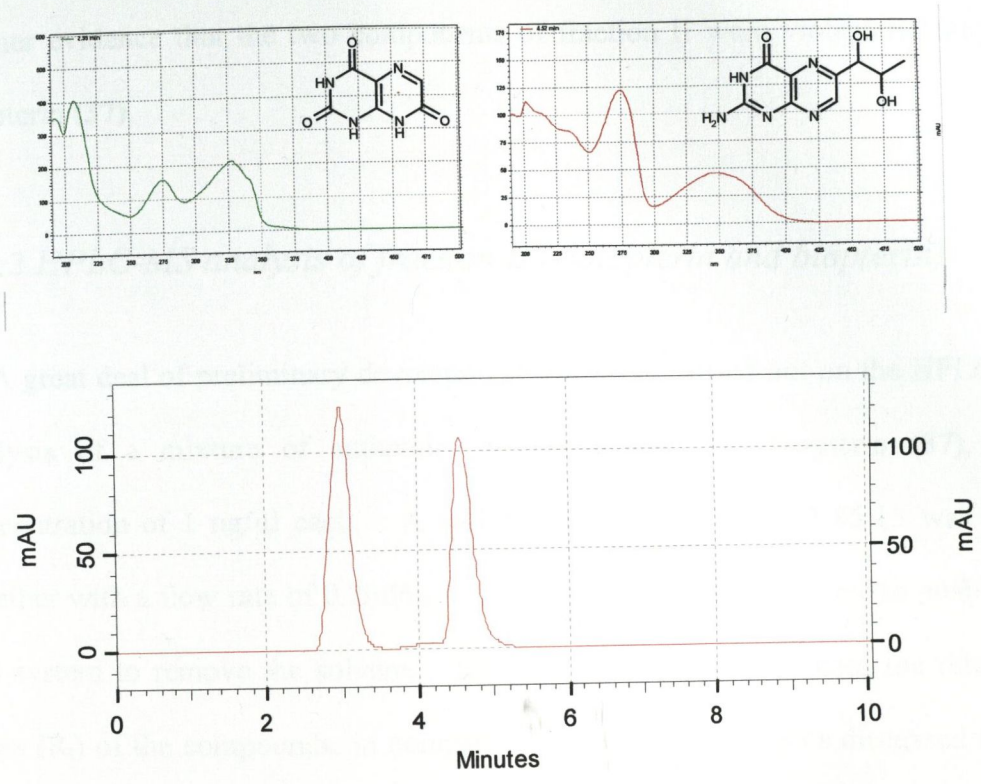


Figure 2.12 HPLC-UV analysis of a mixture of authentic violapterin and biopterin

HPLC analysis of the zebrafish extract, fraction II, was carried out as described above. This extract was shown to contain two main components, although at very low concentrations. The UV chromatogram showed two peaks. The first peak had a retention time (R_t) of 2.99 minutes and UV absorption maxima at 207, 275 and 327. These values are identical with the retention time (R_t) and UV maxima observed for authentic violapterin **(36)**. The second peak had a retention time (R_t) of 4.52 minutes and UV absorption maxima at 202, 274 and 346 nm. These values are identical with the retention time (R_t) and UV maxima observed for authentic biopterin **(37)**.

HPLC analysis of the zebrafish extract (fraction II) spiked with a mixture of authentic violapterin **(36)** and biopterin **(37)**, showed the same two signals present

(R_t 2.99 min. and 4.52 min.), although at greater intensity. These results provide further evidence that the two components of fraction II were violapterin **(36)** and biopterin **(37)**.

2.5.2 HPLC-MS analysis of fraction II (violapterin and biopterin)

A great deal of preliminary development work was carried out on the HPLC-MS analysis of a mixture of authentic violapterin **(36)** and biopterin **(37)**, at a concentration of 1 ng/µl each. A mobile phase of H_2O:MeOH 85:15 was used, together with a flow rate of 0.2ml/min. A low flow rate was required to enable the MS system to remove the solvent. However, this in turn increased the retention times (R_t) of the compounds, in comparison to the HPLC analysis discussed above in section *2.5.1*. Furthermore, development of the MS analysis conditions for a mixture of compounds meant that the sensitivity of the system was compromised for each part of the mixture. This was especially true in the case of analysis of violapterin **(36)**, the spectra for which were not as clear as those obtained for isoxanthopterin **(35)** (**Figure 2.11**).

Analysis of an authentic mixture of violapterin **(36)** and biopterin **(37)** by HPLC-MS gave a UV chromatogram containing two peaks, and a total ion chromatogram that also contained two peaks. The first peak (R_t 5.26 min.) observed in the UV chromatogram was violapterin **(36)**, with analysis of this peak yielding a UV spectrum for violapterin **(36)**, identical to that shown in **Figure 2.12**. The first peak observed in the total ion chromatogram yielded the mass spectrum shown in **Figure 2.13**. As expected this mass spectrum had signals corresponding to violapterin **(36)** with peaks at m/z 181 ($M+H^+$) and m/z 203 ($M+Na^+$), the remainder of the peaks were taken to be from the background.

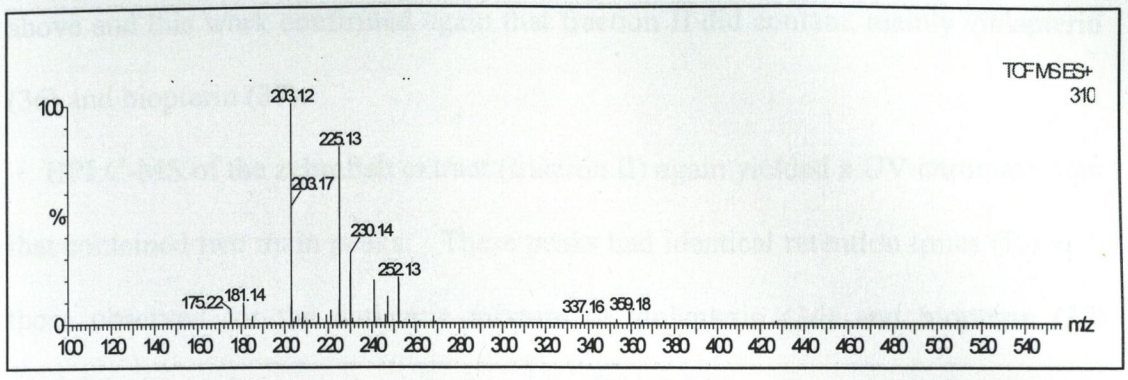


Figure 2.13 MS (ESI) spectrum of authentic violapterin

The second peak observed in the UV chromatogram (R_t 10.5 min.) of the authentic mixture of violapterin **(36)** and biopterin **(37)** was that of the biopterin **(37)**, as shown by its UV spectrum (identical with that shown in **Figure 2.12**). The second peak in the total ion chromatogram was also due to biopterin **(37)**, as shown by its mass spectrum. This contained three peaks corresponding to $M+H^+$ (m/z 238), $M+Na^+$ (m/z 260) and $M+K^+$ (m/z 276), as expected for biopterin **(37)**. This spectrum is shown in **Figure 2.14**.

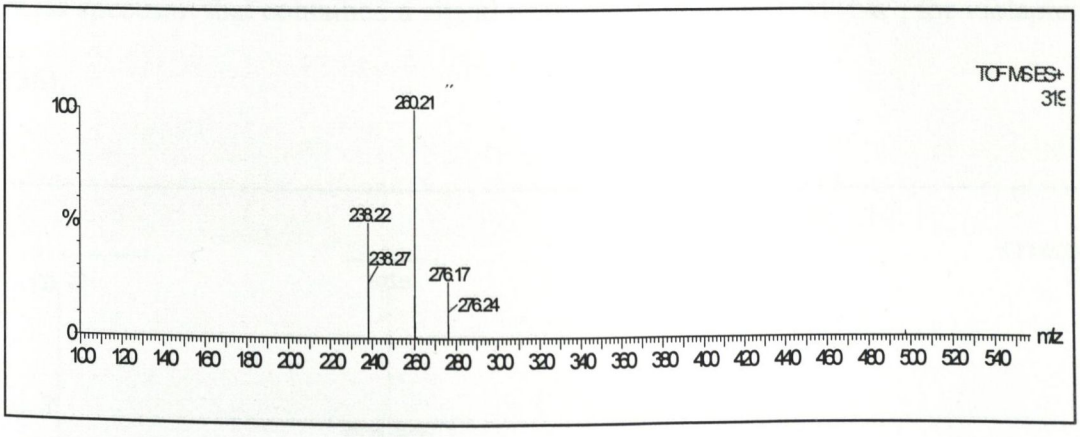


Figure 2.14 MS (ESI) spectrum of authentic biopterin

Attention was turned next to an analysis of the zebrafish extract (Fraction II). HPLC-MS analysis of this fraction was carried out using the conditions described

above and this work confirmed again that fraction II did contain, mainly violapterin **(36)** and biopterin **(37)**.

HPLC-MS of the zebrafish extract (fraction II) again yielded a UV chromatogram that contained two main peaks. These peaks had identical retention times (R_t) with those observed for the authentic mixture of violapterin **(36)** and biopterin **(37)** (R_t 5.26 and 10.5 minutes), as analysed by HPLC-MS. The two peaks gave UV absorption spectra, which were identical to those observed for authentic violapterin **(36)** and biopterin **(37)** **(Figure 2.12).** The MS ion chromatogram also showed the presence of two peaks. Analysis of the first peak in the ion chromatogram yielded the mass spectrum shown in **Figure 2.15**. This spectrum, while being of low intensity, did contain a peak at m/z 203 that corresponded to violapterin **(36)** ($M+Na^+$). The main peak in this spectrum, m/z of 365, was not identified and most likely represents a background signal due to the low concentration of violapterin **(36)**. The absence of a $M+H^+$ peak (m/z 181) for violapterin was not unexpected. Analysis of authentic violapterin **(36)**, prepared as described later, also yielded a mass spectrum that contained a signal peak corresponding to $M+Na^+$, for violapterin **(36)**.

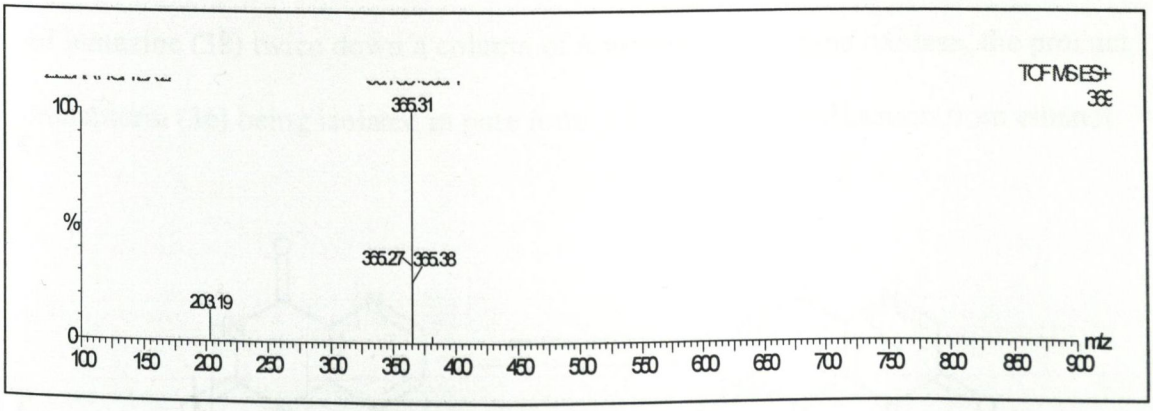


Figure 2.15 MS (ESI) spectrum of violapterin in fraction II

Analysis of the second peak observed in the ion chromatogram for fraction II yielded the mass spectrum shown in **Figure 2.16**. This contained three peaks that corresponded to biopterin **(37)**. These were observed at m/z 238 ($M+H^+$), m/z 260 ($M+Na^+$) and m/z 276 ($M+K^+$).

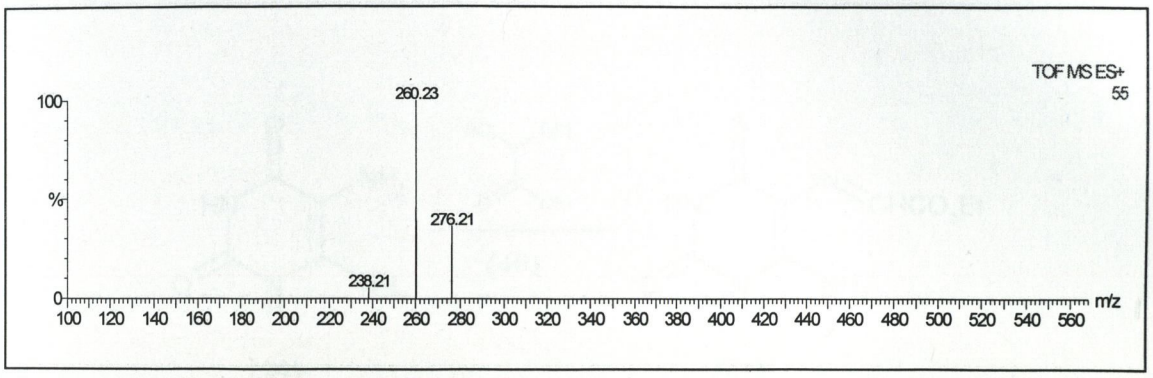
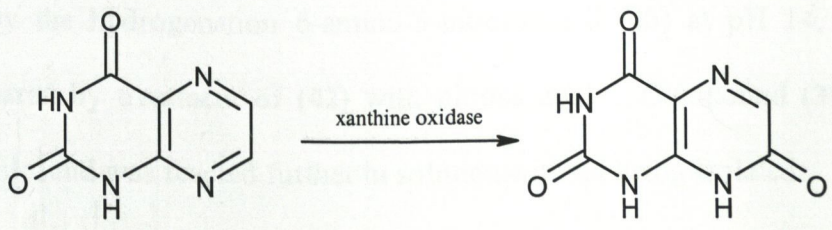


Figure 2.16 MS (ESI) spectrum of biopterin in fraction II.

Considering the evidence discussed above, it is evident that fraction II isolated from the zebrafish, did contain a mixture of violapterin **(36)** and biopterin **(37)**.

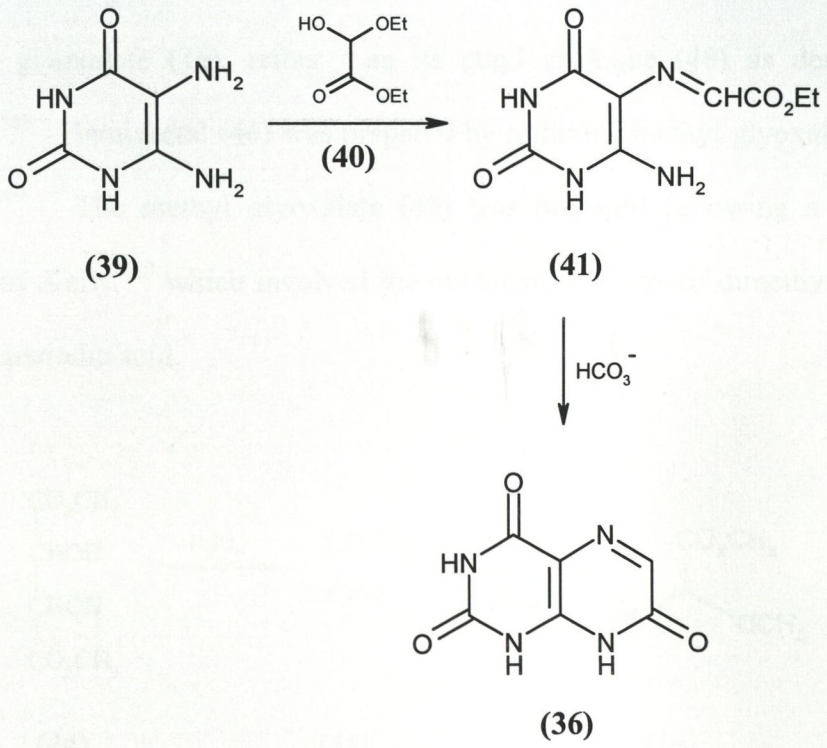
2.5.3 Preparation of violapterin

Davis[107] reported that violapterin **(36)** can be prepared by passing a basic solution of lumazine **(38)** twice down a column of immobilised xanthine oxidase, the product violapterin **(36)** being isolated in pure form following recrystallisation from ethanol.



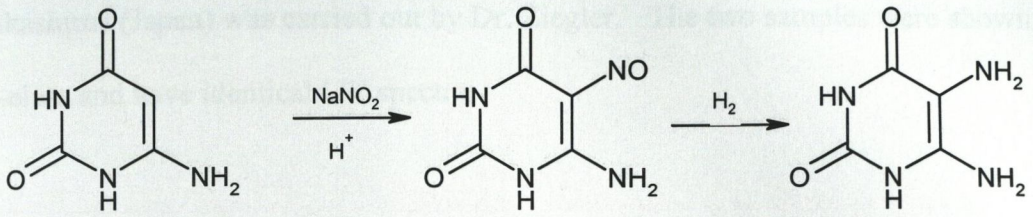
(38) **(36)**

A chemical synthesis of violapterin **(36)** was reported, by Pfleiderer.[108] As shown in **Scheme 2.1**, this synthesis involved the treatment of 5,6-diaminouracil **(39)** with the hemiacetal of ethyl glyoxalate **(40)** to give the open chain intermediate **(41)**. Treatment of **(41)** with aqueous bicarbonate yielded violapterin **(36)** in good yield.



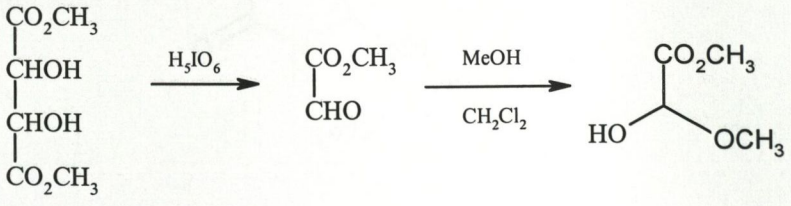
Scheme 2.1 Synthetic pathway for violapterin **(36)**[108]

This synthesis was modified in the present work. 5,6-Diaminouracil **(39)** was prepared by the hydrogenation 6-amino-5-nitrosouracil **(43)** at pH 14, the latter being prepared by treatment of **(42)** with nitrous acid. Compound **(39)** is very oxygen labile, and was reacted further in solution without being isolated.



(42) **(43)** **(39)**

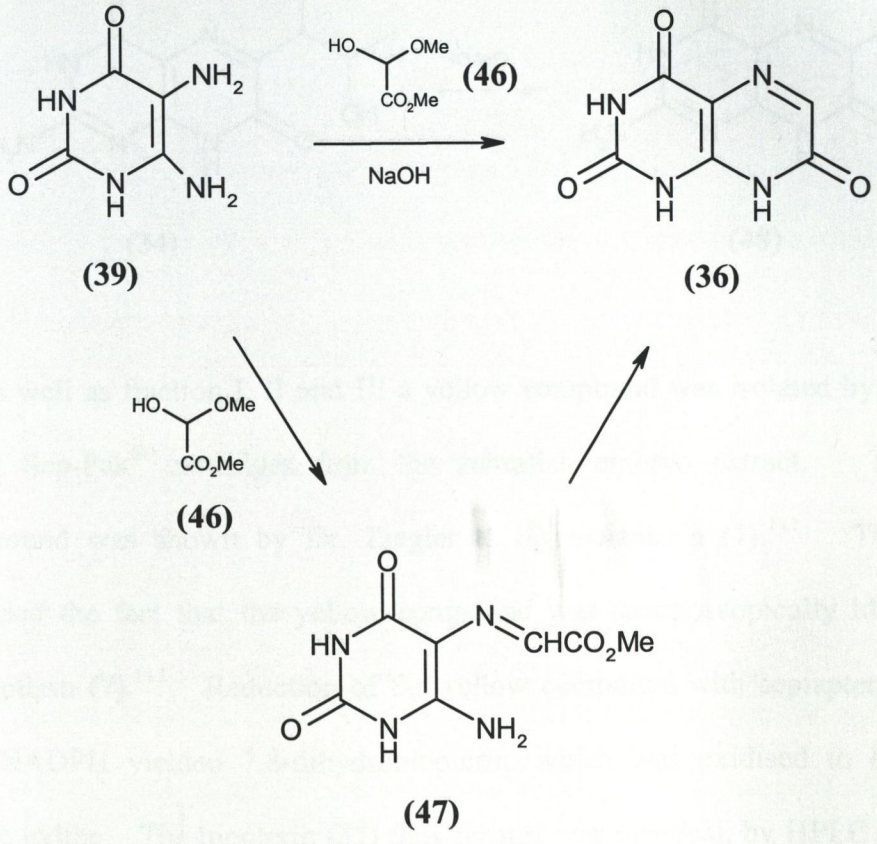
As shown in **Scheme 2.2**, 5,6-diaminouracil **(39)** was treated with the hemiacetal of methyl glyoxalate **(46)**, rather than its ethyl analogue **(40)** as described by Pfleiderer.[108] Hemiacetal **(46)** was prepared by refluxing methyl glyoxalate **(45)** in methanol.[109] The methyl glyoxalate **(45)** was prepared following a procedure described by Kelly,[110] which involved the oxidative cleavage of dimethyl L-tartrate **(44)** using periodic acid.



(44) **(45)** **(46)**

Reaction of 4,5-diaminouracil **(39)** with hemiacetal **(46)** was carried out at pH 14 to give violapterin **(36)** in good yield. This route was more convenient than the original described by Pfleiderer,[108] which would have involved isolation of the intermediate **(47)**, as shown in **Scheme 2.2**. A high resolution mass spectrum (HRMS) of the product violapterin **(36)**, contained a single peak at 203.0187 corresponding to $M+Na^+$(Calc. 203.0181) for violapterin **(36)**. A HPLC comparison of the product **(36)**, with an authentic sample of violapterin **(36)** supplied by Dr.

Nakashima (Japan) was carried out by Dr. Ziegler. The two samples were shown to co-elute and have identical UV spectra.

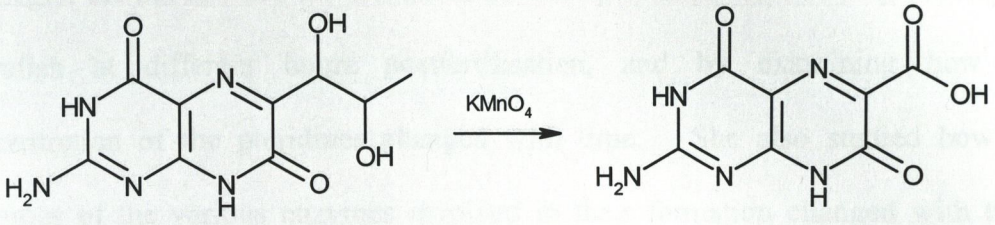


Scheme 2.2 Alternative synthetic route for violapterin

2.6 Analysis and identification of Fraction III (7-oxobiopterin) and of sepiapterin

Using HPLC, the zebrafish extract (fraction III) was shown by Dr. Ziegler[111] to co-elute with 7-oxobiopterin **(34)**. This fraction (III) showed only one peak on HPLC analysis using a fluorescence detector, and the fluorescent intensity of this peak at 420nm was 1.5 times the intensity of that at 450 nm. This is characteristic of a 7-oxopteridine. Further evidence of identity came from the fact that alkaline

permanganate oxidation of the compound in fraction III gave 6-carboxy-7-oxopterin **(48)**.



(34) **(48)**

As well as fraction I, II and III a yellow compound was isolated by Dr. Ziegler using Sep-Pak® cartridges from the zebrafish embryo extract. This yellow compound was shown by Dr. Ziegler to be sepiapterin **(7)**.[111] The evidence included the fact that the yellow compound was spectroscopically identical with sepiapterin **(7)**.[112] Reduction of the yellow compound with sepiapterin reductase and NADPH yielded 7,8-dihydrobiopterin, which was oxidised to biopterin by acidic iodine. The biopterin **(37)** thus formed was identical, by HPLC comparison, with an authentic sample of biopterin **(37)**.

As described in the foregoing sections, analysis of extracts obtained from zebrafish embryos allowed the pteridines in these extracts to be identified as biopterin **(37)**, sepiapterin **(7)**, 7-oxobiopterin **(34)**, 2,4,7-trioxopteridine (violapterin) **(36)** and isoxanthopterin **(35)**. The work up used in obtaining the extracts involved an oxidation, so that biopterin **(37)**, which was identified in fraction II would have been present originally in the zebrafish as its biologically active reduced form, 5,6,7,8-tetrahydrobiopterin **(10)**. Once the pteridines in the zebrafish embryo were identified, it then became possible for the first time to propose a new biosynthetic pathway for the formation of pteridines in these animals.

2.7 The proposed biosynthetic pathway of pteridines in zebrafish (*Danio rerio*)

Ziegler set out to trace the formation of these pteridines by studying developing zebrafish at different hours postfertilisation, and by examining how the concentration of the pteridines changed with time. She also studied how the activities of the various enzymes involved in their formation changed with time. This new data led to a new biosynthetic scheme for the formation of pteridines in zebrafish (*Danio rerio*).

2.7.1 Time sequence of pteridine formation in the developing zebrafish embryo

By studying the fish at different times postfertilisation, Ziegler was able to show that H_4biopterin **(10)** and sepiapterin **(7)** were the first pteridines to form, appearing within 24 hours postfertilisation. After 72 hours development H_4biopterin **(10)** reached a maximum concentration of *ca.* 7 pmol/animal. Sepiapterin **(7)** continued to rise in concentration after this time, reaching a concentration of *ca.* 80 pmol/animal at 120 hours postfertilisation. The three 7-oxopteridines (7-oxobiopterin **(34)**, 2,4,7-trioxopteridine (violapterin) **(36)** and isoxanthopterin **(35)** began to form only after 24 hours development. Their concentrations then increased dramatically to reach a combined concentration of *ca.* 140 pmol/animal after 120 hours development. These results are summarised in **Figure 2.17**.[111] The lag time of 24 hours in formation of these 7-oxopteridines gave the first indication of what pathway might be involved.

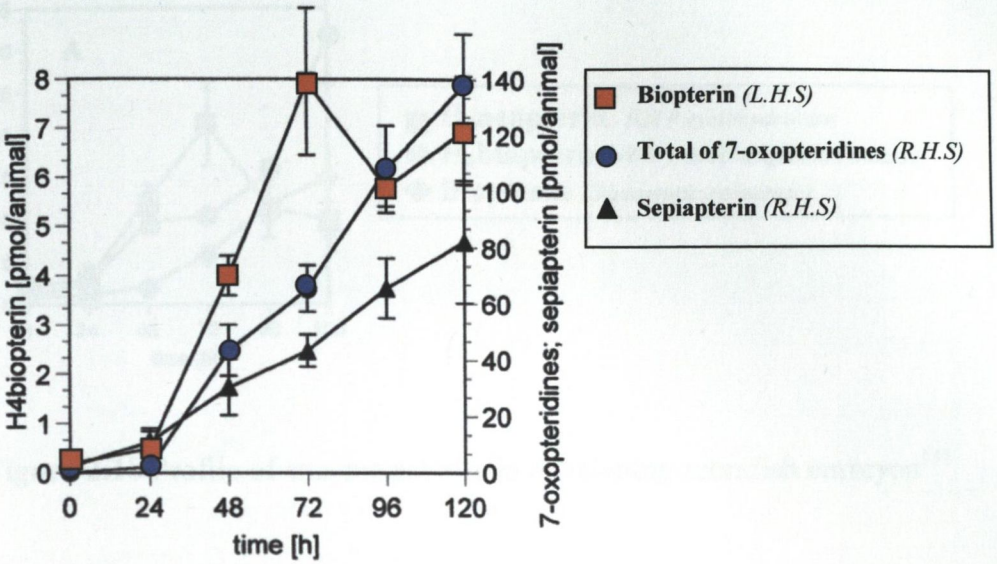


Figure 2.17 Profile of pteridine formation in developing zebrafish (*Danio rerio*)[111]

2.7.2 Time sequence of enzyme activity in the developing zebrafish embryo

To understand how these pteridines were forming it was necessary to understand what enzymes were playing a role in the process. The three enzymes that catalyse the biosynthesis of H_4biopterin **(10)**, namely GTP cyclohydrolase, 6-pyruvoyl-H_4pterin synthase and sepiapterin reductase, were all studied. The level of H_2neopterin present was used as an indicator of GTP cyclohydrolase activity, and the levels of H_4biopterin **(10)** and biopterin **(37)** were used as indicators of 6-pyruvoyl-H_4pterin synthase and sepiapterin reductase activity respectively. It was shown that the three enzymes become active within the first 24 hours of development. After 72 hours it was shown that their activity diverged, with GTPCH reaching maximum activity at this time. Significantly both 6-pyruvoyl-H_4pterin synthase and sepiapterin reductase continued to increase after this time. These results are summarised in **Figure 2.18**.[111]

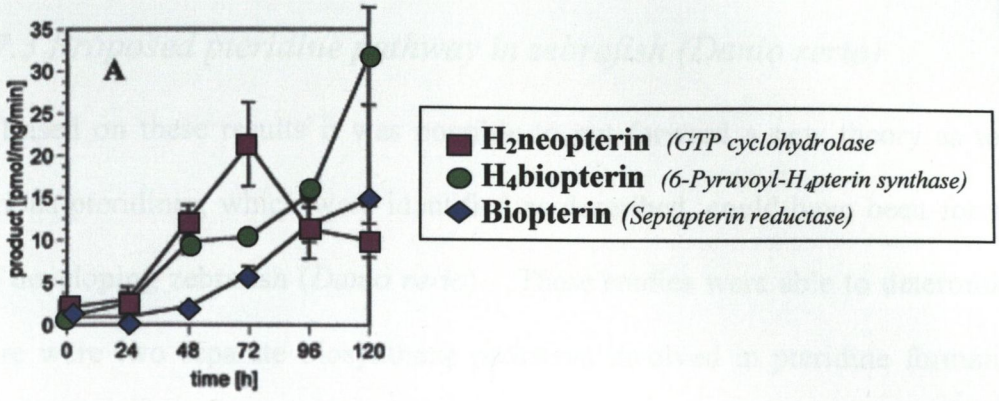


Figure 2.18 Profile of enzyme activity in developing zebrafish embryos[111]

From studies already documented [113,114] it is known that the enzyme xanthine oxidoreductase efficiently coverts pterin to isoxanthopterin, suggesting that in the zebrafish, this enzyme was responsible for the formation of the three 7-oxo pteridines shown to be present (7-oxobiopterin **(34)**, 2,4,7-trioxopteridine (violapterin) **(36)** and isoxanthopterin **(35)**). While this was shown to be true for the formation of 2,4,7-trioxopteridine (violapterin) **(36)** and isoxanthopterin **(35)**, a new variant of xanthine oxidoreductase was responsible for the formation of 7-oxobiopterin **(34)**. These results are summarised in **Figure 2.19**.

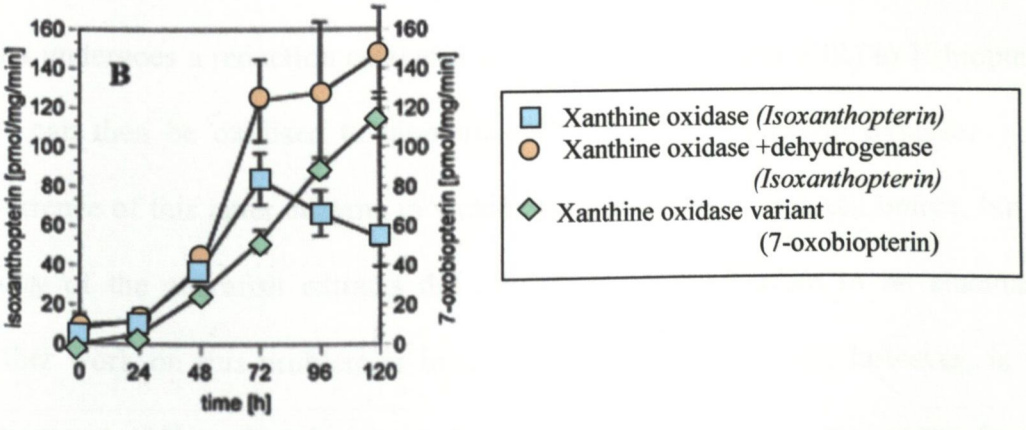


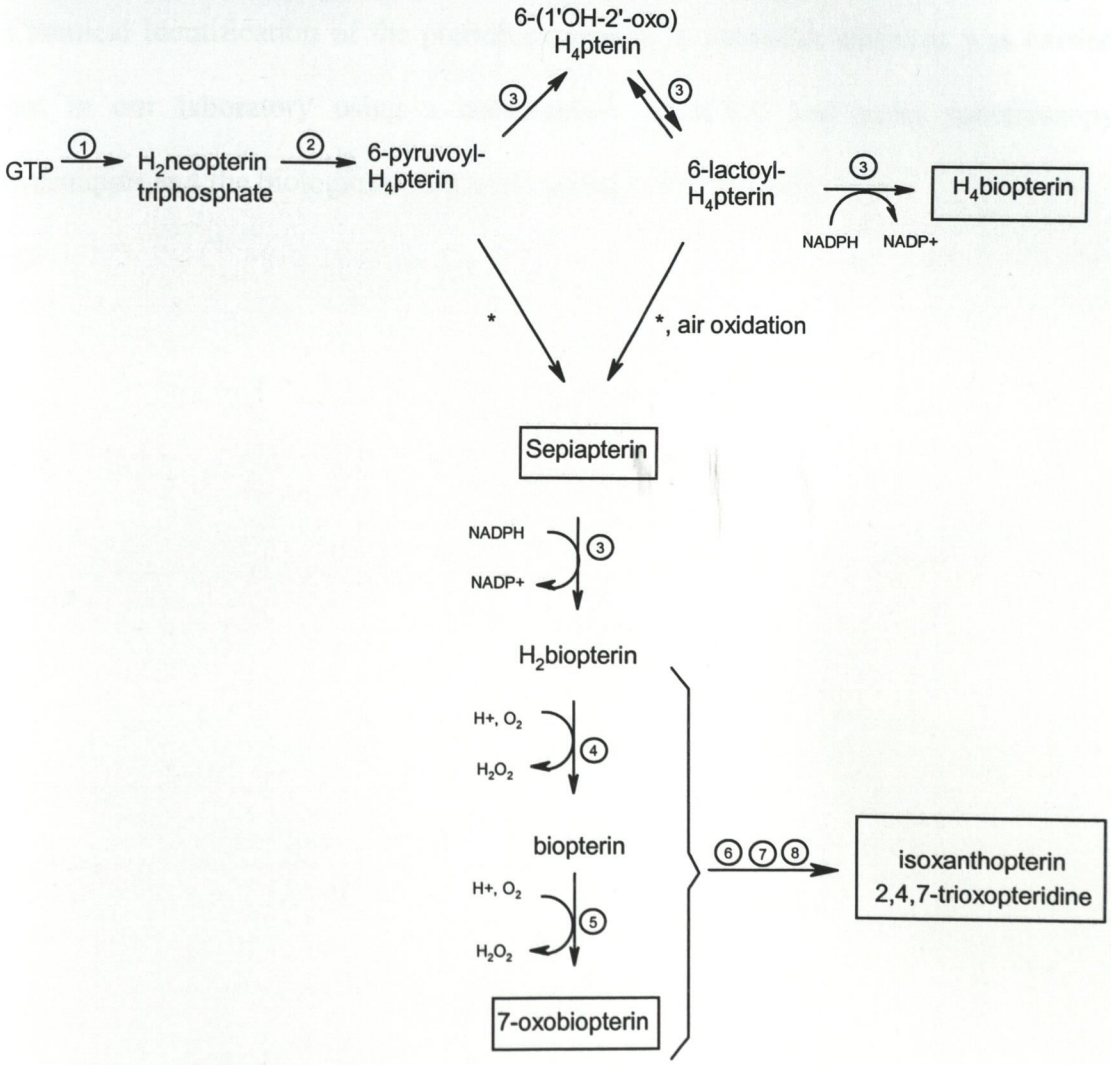
Figure 2.19 Study of enzyme levels involved in 7-oxopteridine formation in developing zebrafish embryos[111]

2.7.3 Proposed pteridine pathway in zebrafish (Danio rerio)

Based on these results it was possible to put forward a new theory as to how various pteridines, which were identified as described, could have been formed in the developing zebrafish (*Danio rerio*). These studies were able to determine that there were two separate biosynthetic pathways involved in pteridine formation in zebrafish (*Danio rerio*). The first pathway involves the formation of H_4biopterin **(10)** from GTP **(24)** under the influence of GTPCH; this pathway is well understood, as already discussed in Chapter one. The second route uses H_2biopterin and biopterin **(37)** as a source of the 7-oxopteridines present in the zebrafish. The first pteridine required for this sequence of reactions is sepiapterin **(7)**. How the latter is formed in zebrafish is still not very clear, but its presence has been confirmed by the analysis already described. Studies in *Drosophila melanogaster* have proposed that sepiapterin **(7)** could be formed by air oxidation of 6-lactoyl-H_4pterin, which is a product of the action of 6-pyruvoyl-H_4pterin synthase on H_2neopterin triphosphate.[115] A second possibility is that sepiapterin is formed by enzymatic action on 6-lactoyl-H_4pterin, these ideas remain to be clarified further, however

Ziegler was able to show that the sepiapterin **(7)** that accumulates in the first 24 hours undergoes a reduction catalysed by sepiapterin reductase (SR) to H_2biopterin. This can then be oxidised to biopterin **(37)** by dihydrobiopterin oxidase. The occurrence of this latter enzyme in vertebrates has not been reported before, but the quality of the zebrafish extracts did not allow the mechanism to be elucidated. Further work on this problem is in progress. What is known, however, is that H_4biopterin **(10)** and/or biopterin **(37)** are the biosynthetic precursors for the formation of the 7-oxopteridines. It was shown, for example, that a new xanthine oxidase variant was responsible for the formation of 7-oxobiopterin **(34)**. This

latter pteridine could then possibly be the source of 7-oxopterin (isoxanthopterin) **(35)** and 2,4,7-trioxopteridine (violapterin) **(36)**. The proposed biosynthetic pathways are summarised in **Scheme 2.20**.



1. GTP cyclohydrolase
2. 6-pyryuvoyl-H_4pterin synthase
3. Sepiapterin reductase
4. H_2pterin oxidase
5. Xanthine oxidase variant
6. Pterin deaminase
7. Xanthine oxidoreductase
8. Nonenzymatic side chain cleavage

*. Hypothetical enzymatic reaction

Figure 2.20 Proposed pathway leading to the zebrafish pteridines

2.8 Conclusion

In collaboration with Dr. Ziegler of the GSF Institute in Munich, the work described in this chapter led to the proposal of a new biosynthetic scheme by which pteridines are formed in the developing embryo of the zebrafish (*Danio rerio*). Chemical identification of the pteridines present in zebrafish embryos was carried out in our laboratory using a combination of HPLC and mass spectroscopy techniques and the biological work was carried out in Munich.

Chapter Three

Synthesis of Pteridines

A new approach

3.1 Introduction

The pteridine ring system **(4)** consists of a pyrimidine ring and a pyrazine ring fused together. From a retro-synthetic point of view, therefore, two main approaches offer themselves for the synthesis of a pteridine. The first of these approaches involves construction of a pyrazine ring onto a pre-existing pyrimidine ring. The alternative approach involves construction of a pyrimidine ring on to a pyrazine ring. These two approaches are illustrated in **Figure 3.1**. Since pyrimidines are very easily accessible, the former pathway is by far the more important. However, approaches using pyrazine starting materials have also been developed.

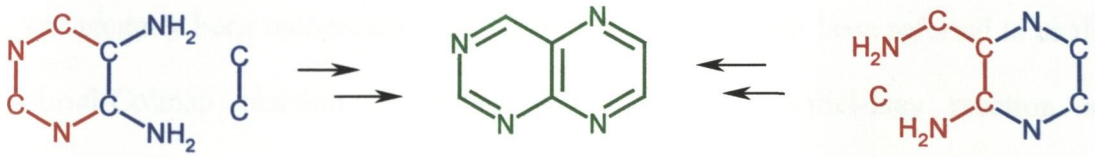
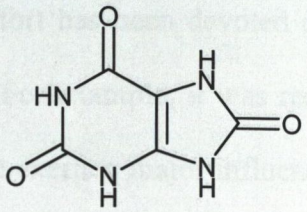
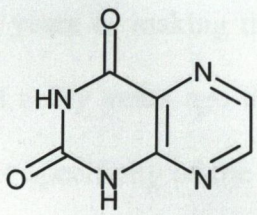


Figure 3.1 Synthetic routes of the pteridine ring system

The first laboratory synthesis of a pteridine was achieved before Hopkins reported his discovery of pteridines in butterflies in 1889,[1] although the significance of the discovery was not appreciated at the time. Wöhler[116] and Hlasiwetz[117] reported this early synthesis in 1858 when they isolated yellow products after prolonged heating of uric acid **(49)** in water above 100°C in a sealed tube. One hundred years later in 1959,[118] Pfleiderer showed that these original yellow products were in fact derivatives of lumazine **(38)**.



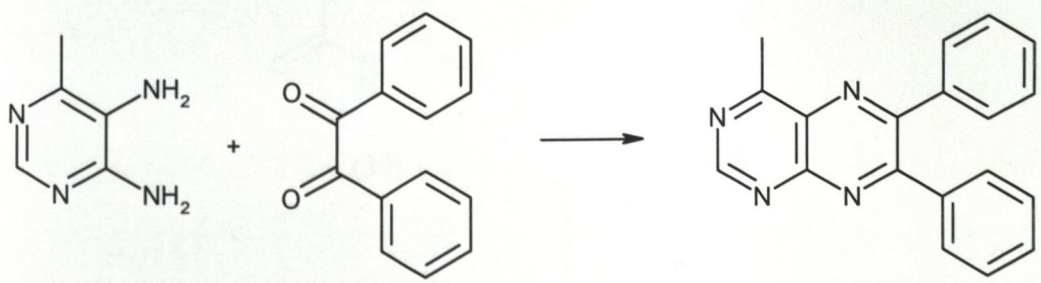
(49)



(38)

3.1.1 Synthesis of pteridines from pyrimidines

Gabriel and Colman[119] were the first to describe a general pteridine synthesis, when in 1901 they prepared 4-methyl-6,7-diphenylpteridine **(52)** by the condensation of 5,6-diamino-4-methylpyrimidine **(50)** with benzil **(51)**. Five years later, in 1906, Isay[120] reported the same reaction, which for many years was referred to as the Isay synthesis of pteridines. The prior claim by Gabriel and Colman has more recently been recognised, however, and the reaction has been referred to as the Gabriel-Colman reaction by Brown,[121] and as the Gabriel-Isay reaction by Pfleiderer.[122]

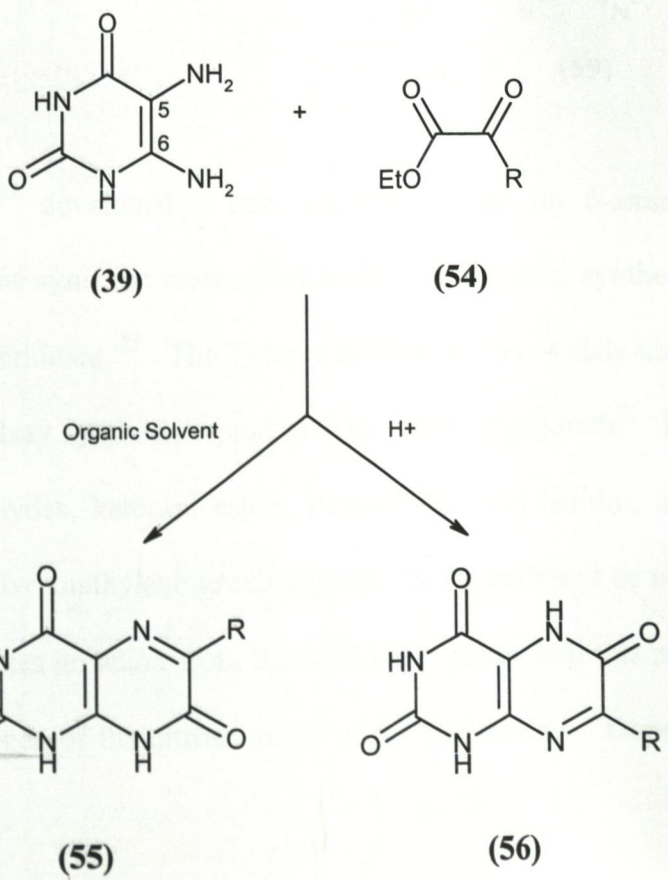


(50) (51) (52)

The reaction in general involves the condensation of 5,6-diaminopyrimidines with 1,2-dicarbonyl compounds. Problems with regioselectivity occur, however, when unsymmetrical dicarbonyl reagents such as α-keto aldehydes or α-keto acids are used, since a mixture of 6- and 7- substituted regioisomers can be obtained. A

great deal of effort has been devoted over the years to making this reaction more regiospecific. For example, it was recognised many years ago that the pH of the reaction mixture exerts a major influence on the specificity of the reaction and that exploiting the differences in the basicity and nucleophilic reactivity of the 5- and 6-amino groups can allow the control of product formation.[123]

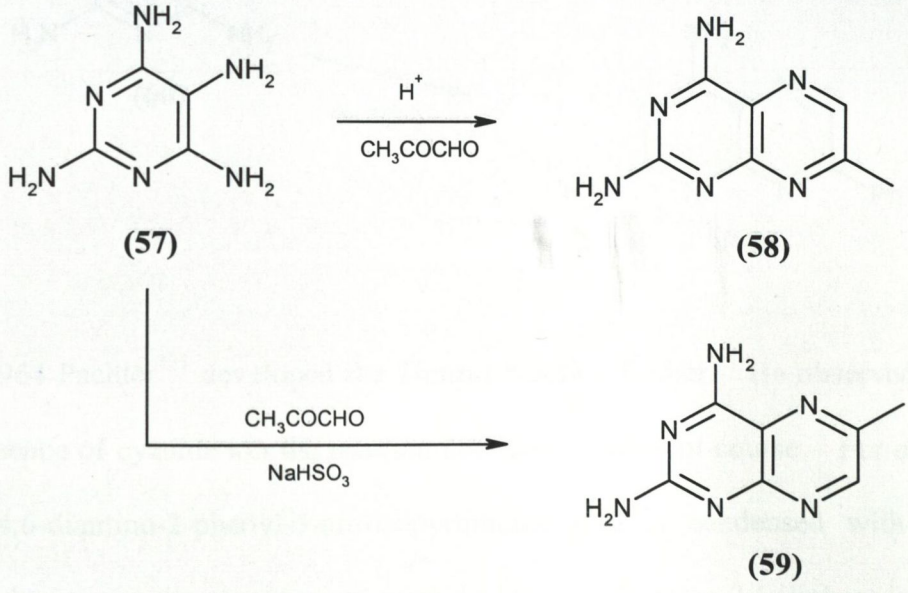
An example of this selectivity is seen in the reaction of 5,6-diaminouracil **(39)** with α-ketoesters **(54)**. In neutral or weakly acidic media, as well as in organic solvents, the formation of 7-pteridinones **(55)** is favoured. This occurs because in neutral solution the more basic 5-amino group of the pyrimidine **(39)** preferentially attacks the ketone group in the α-ketoester **(54)**. In strongly acidic media on the other hand, the more basic 5-amino group is protonated, so that it is the 6-amino group of the pyrimidine **(39)** which reacts preferentially, leading to the 6-pteridinone **(56)** with the opposite orientation of the substituents.[124,125,126]



(39) **(54)**

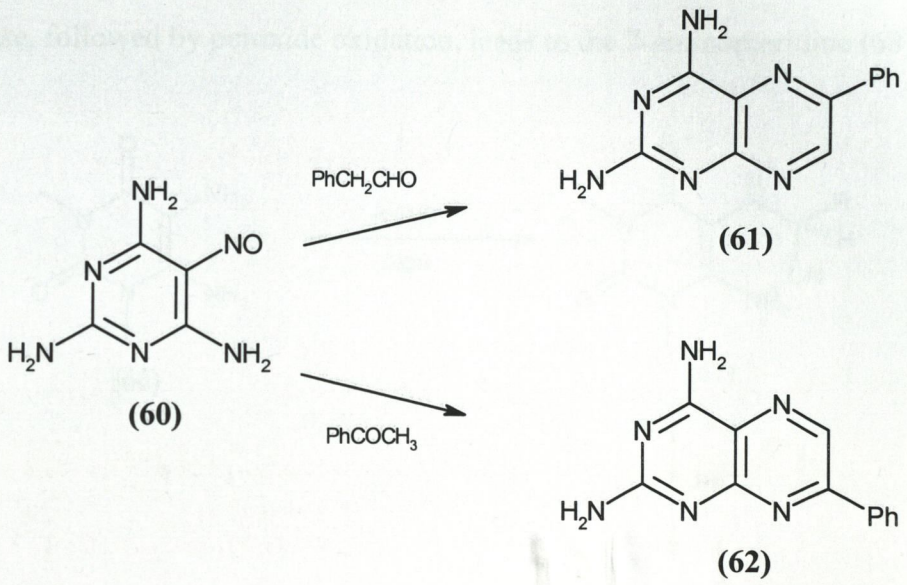
(55) **(56)**

Another way of overcoming this problem of regiospecifity involves the use of aldehyde and ketone binding reagents such as sodium hydrogen sulphite. This tends to direct the alkyl group to the 6-position of the pteridine being formed. For example, condensation of 2,4,5,6-tetraaminopyrimidine **(57)** with pyruvaldehyde in the presence of strong acid gives the 7-methyl product **(58)**, whereas in the presence of sodium hydrogen sulphite it gives the 6-methyl product **(59)**.[127]

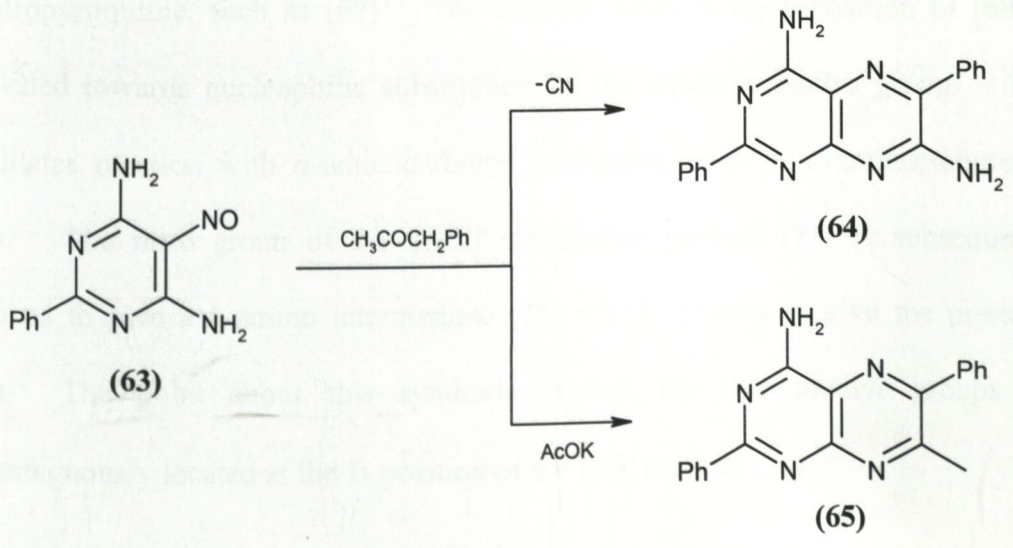


In 1949 Timmis[128] developed a new synthesis based on 6-amino-5-nitroso pyrimidines. This new synthetic method led to the unambiguous synthesis of either 6- or 7- substituted pteridines.[129] The Timmis reaction is now widely used for cases in which the Gabriel-Isay synthesis would give mixtures of products. The reaction is applicable to aldehydes, ketones, esters, nitriles and acyl halides, all of which should possess an active methylene group adjacent to the carbonyl or nitrile group. Base treatment generates an anion from the methylene group, and this anion attacks the electrophilic nitrogen of the nitroso group of the pyrimidine. Depending upon

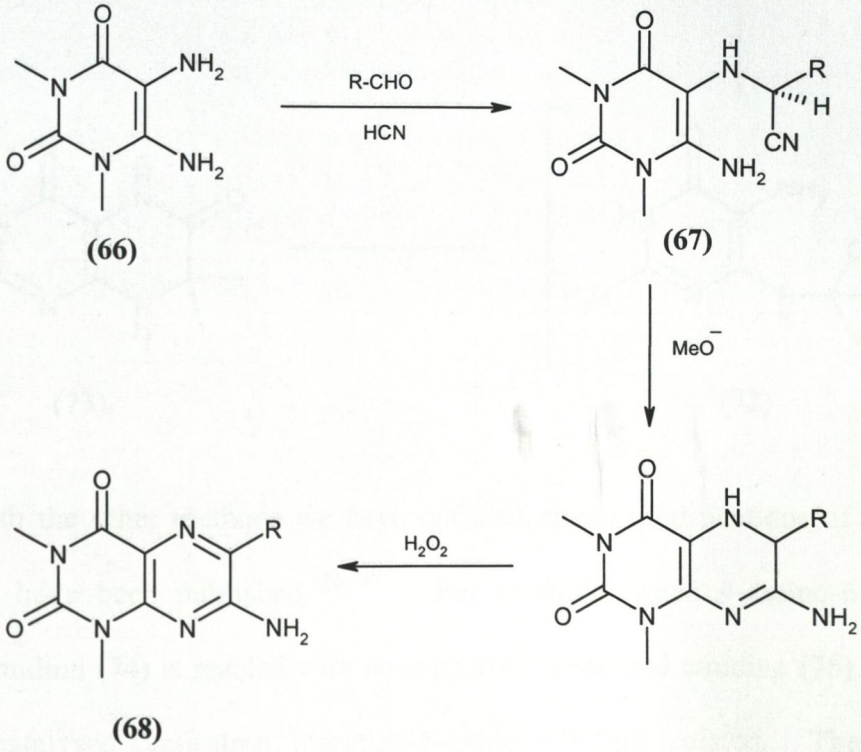
the precise reagent used, either a 6- **(61)** or a 7-substituted pteridine **(62)** may be prepared from a starting 5-nitroso pyrimidine, such as **(60)**. [130]



In 1964 Pachter[131] developed the Timmis reaction further. He observed that in the presence of cyanide ion the reaction may take a different course. For example, when 4,6-diamino-2-phenyl-5-nitrosopyrimidine **(63)** is condensed with benzyl methyl ketone in the presence of cyanide ion, 4,7-diamino-2,6-diphenylpteridine **(64)** is obtained. In the absence of cyanide on the other hand, the expected Timmis product, 4-amino-7-methyl-2,6-diphenylpteridine **(65)** is formed.

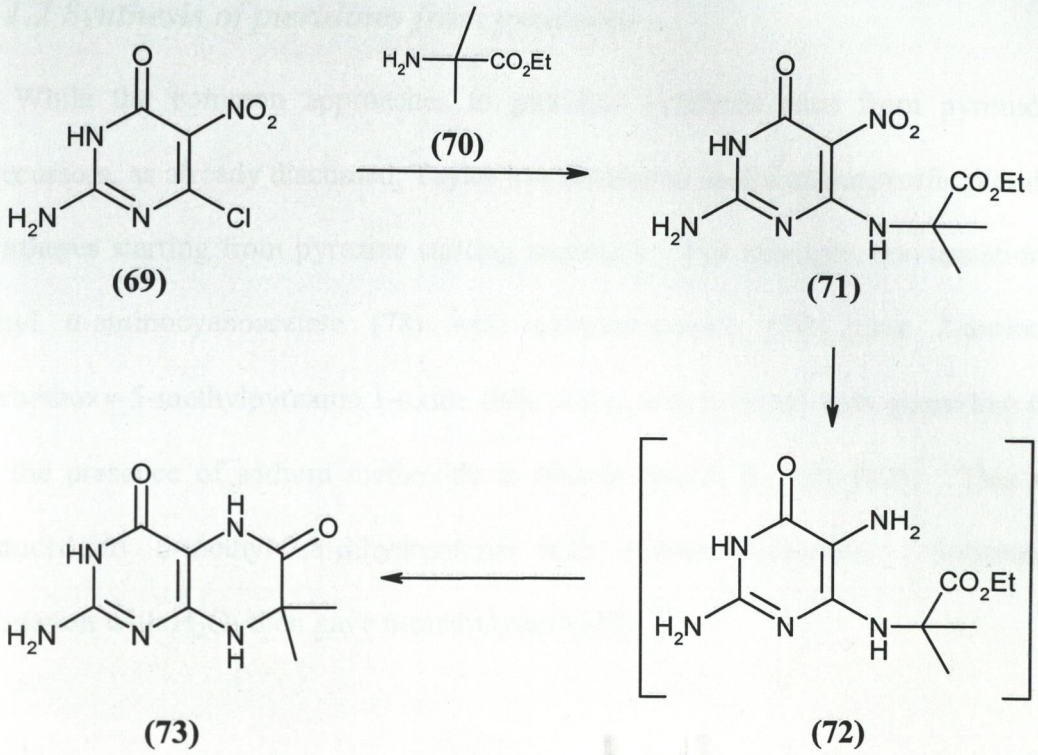


Another synthesis is the Bliche-Pachter[132] method, which involves the initial formation of an aminonitrile **(67)** from a 5,6-diaminopyrimidine **(66)** on treatment of the latter with an aldehyde and hydrogen cyanide. Cyclisation of intermediate **(67)** with base, followed by peroxide oxidation, leads to the 7-aminopteridine **(68)**.

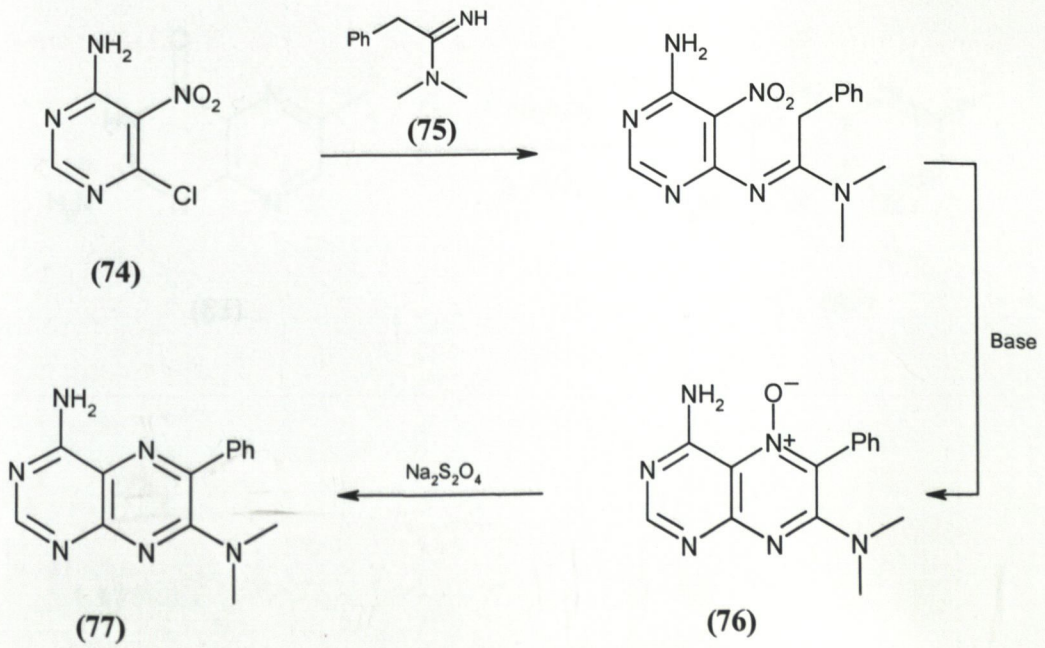


(66) **(67)** **(68)**

Perhaps the most important unambiguous pteridine synthesis was that developed by Polonovski[133] and Boon.[134] The starting material for this synthesis is a 6-chloro-5-nitropyrimidine, such as **(69)**. The chlorine atom in the 6-position of **(69)** is activated towards nucleophilic substitution by the adjacent 5-nitro group. This facilitates reaction with α-aminocarbonyl molecules, such as α-aminoisobutyrate **(70)**. The nitro group of the initial substitution product **(71)** is subsequently reduced to give a 5-amino intermediate **(72)** which cyclises to give the pteridine **(73)**. The point about this synthesis is that the two methyl groups are unambiguously located at the 7- position of the final product **(73)**.[135]

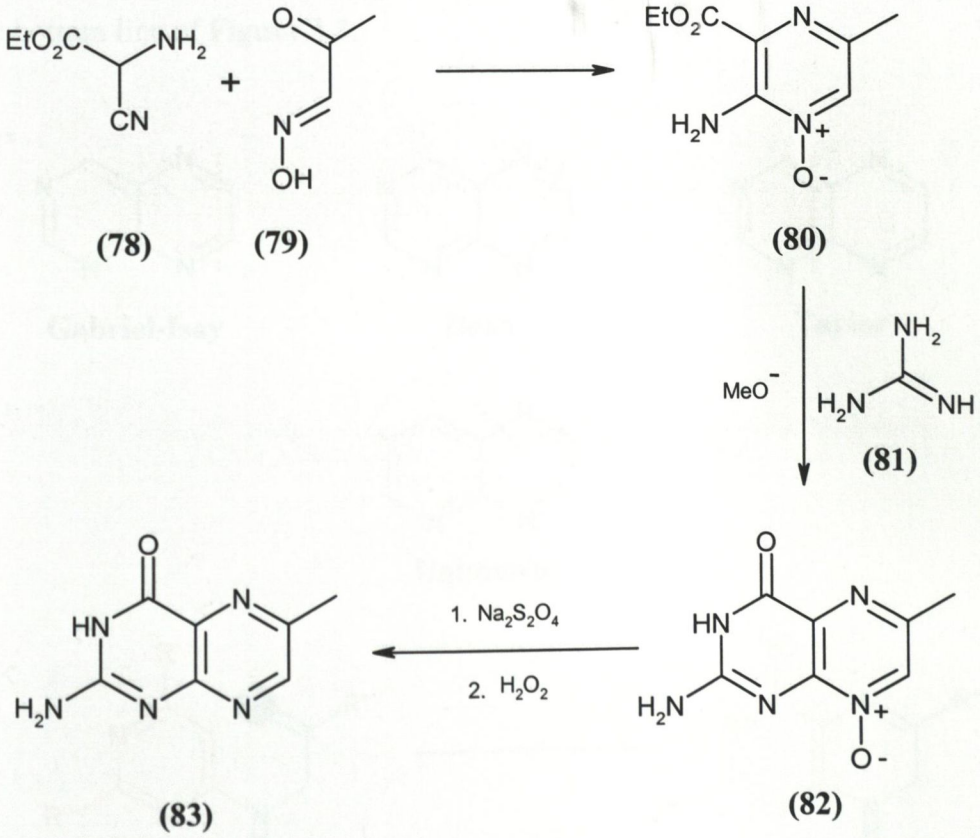


As with the other methods we have outlined, many modifications of the Boon synthesis have been published.[136,137] For example, when 4-amino-6-chloro-5-nitropyrimidine **(74)** is reacted with an α-phenyl substituted amidine **(75)**, followed by base catalysed cyclisation, pteridine-5-oxides **(76)** are isolated. These can be reduced with sodium dithionite to the parent pteridine molecule **(77)**.[138]



3.1.2 Synthesis of pteridines from pyrazines

While the common approaches to pteridine synthesis start from pyrimidine precursors, as already discussed, Taylor has developed useful regiospecific pteridine syntheses starting from pyrazine starting materials. For example, condensation of ethyl α-aminocyanoacetate **(78)** with oximinoacetone **(79)** gave 2-amino-3-carbethoxy-5-methylpyrazine 1-oxide **(80)**, which was cyclised with guanidine **(81)** in the presence of sodium methoxide to 6-methylpterin 8-oxide **(82)**. This was reduced to 6-methyl-7,8-dihydropterin with sodium dithionite. Subsequent oxidation with H_2O_2 then gave 6-methylpterin **(83)**.[139]



(78) **(79)** **(80)** **(81)** **(82)** **(83)**

3.2 Pteridine synthesis *via* electrophilic nitrogen

3.2.1 Introduction

Various approaches to pteridine synthesis are outlined in **Figure 3.2**. Of these, the first three have been widely investigated, and examples of them have already been discussed in the previous section. To date, however, no pteridine synthesis has been achieved using the fourth approach shown, for the obvious reason that this would involve intramolecular cyclisation onto the 5-position of a pyrimidine ring by an electrophilic nitrogen atom, and nitrogen as it occurs in common functional groups is normally a nucleophilic element. The work to be described in this chapter explores the feasibility of an approach involving electrophilic nitrogen, as outlined in the bottom line of **Figure 3.2**.

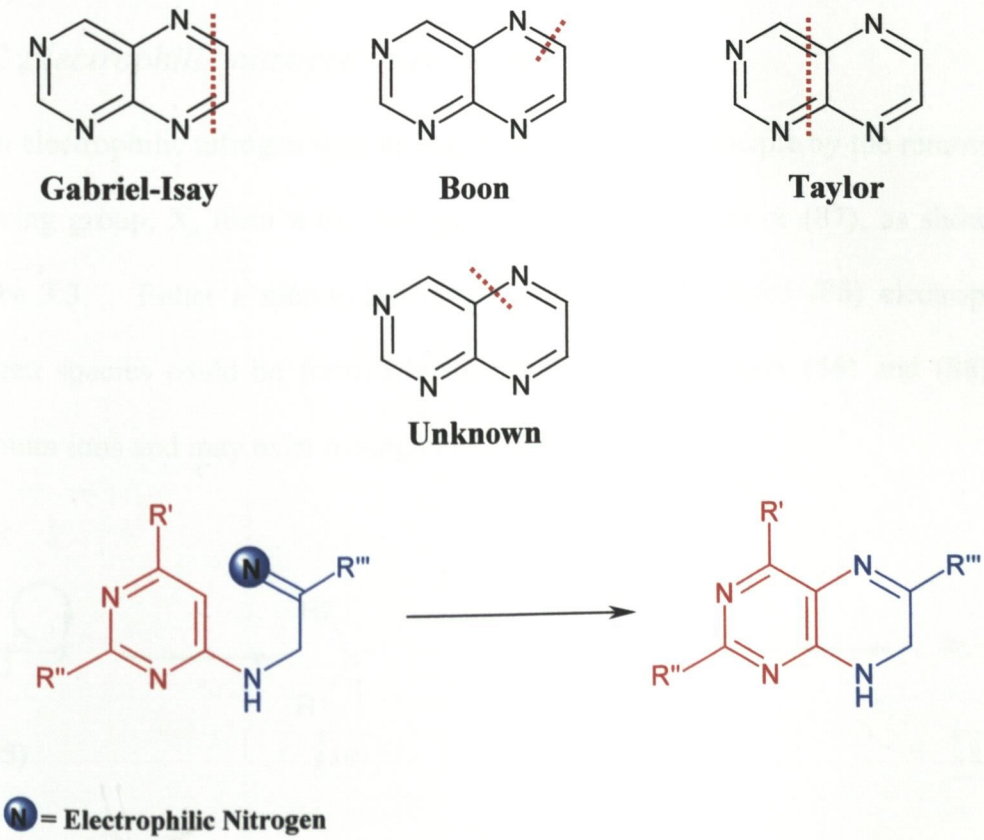
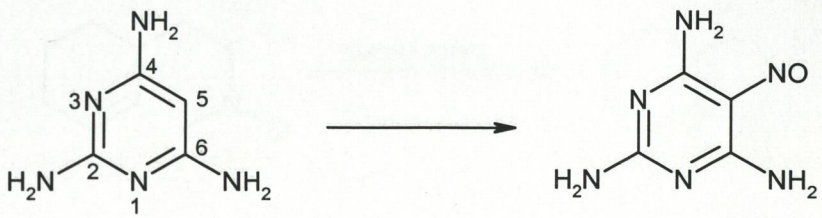


Figure 3.2 New and old approaches for the synthesis of pteridines

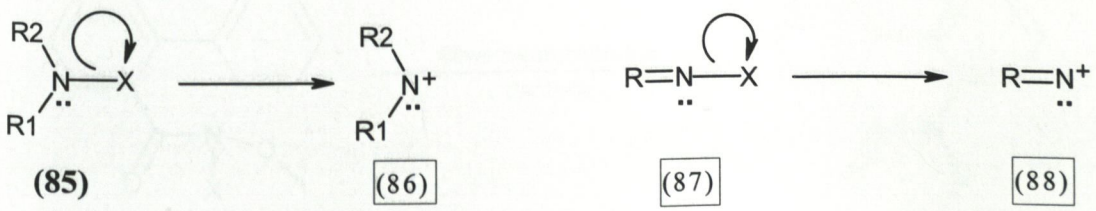
These ideas depend on the fact that the 5-position of a pyrimidine is exceptional in that it is not normally electron deficient, and may even become electron rich if there is electron donating groups at the 2-, 4- or 6- positions. As a result, the 5-position of substituted pyrimidines may undergo electrophilic substitution. A simple example is the nitrosation of 2,4,6-triaminopyrimidine **(84)**, to give 2,4,6-triamino-5-nitrosopyrimidine **(60)**.



(84) **(60)**

3.2.2 Electrophilic nitrogen intermediates

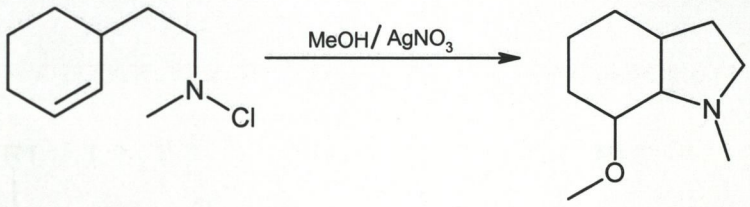
An electrophilic nitrogen species may be generated in principle by the removal of a leaving group, X, from a trivalent nitrogen precursor, **(85)** or **(87)**, as shown in **Figure 3.3**. Either a monosubstituted **(88)** or a disubstituted **(86)** electrophilic nitrogen species could be formed by this method. Structures **(86)** and **(88)** are nitrenium ions and may exist in singlet or triplet states.[140]



(85) (86) (87) (88)

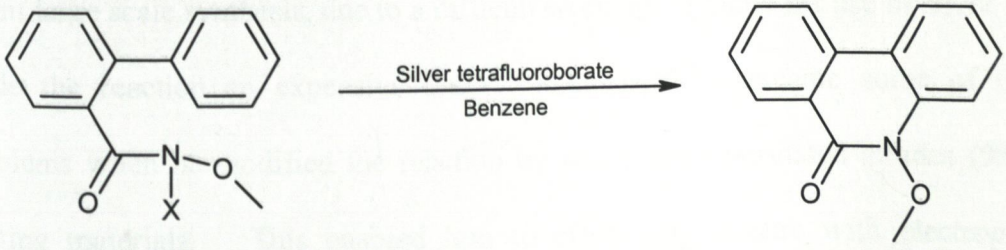
Figure 3.3 Formation of nitrenium ions

Early work in this area was published by Patterson who generated an electrophilic nitrogen species, which he believed to have free radical character.[141] A little later Barton and Beckwith[142] showed how cyclisation of some *N*-iodoamides could be effected *via* an electrophilic nitrogen species, which they suggested was a nitrenium ion. Mokotoff[143] also suggested a nitrenium ion as an intermediate in the preparation of **(90)** from **(89)**.



(89) **(90)**

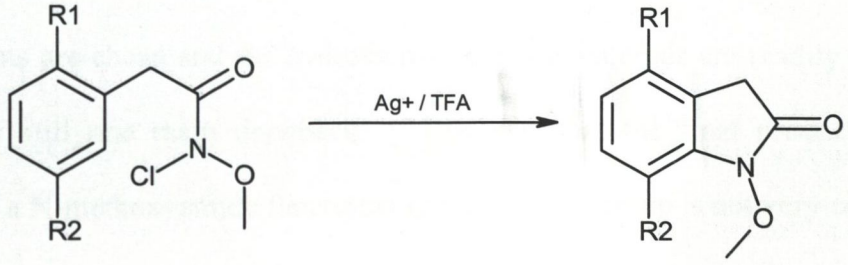
The main problem with much of this work was that the desired nitrenium ions were generated from *N*-chloroamine precursors such as **(89)** and this severely limited the scope of the reaction. In 1984, however, Glover[144] reported successful intramolecular electrophilic aromatic substitution reactions using *N*-halogeno-*N*-methoxybiphenyl-2-carboxyamides such as **(91)** to produce *N*-methoxyphenanthridinones **(92)**.



X = Cl, Br, I

(91) **(92)**

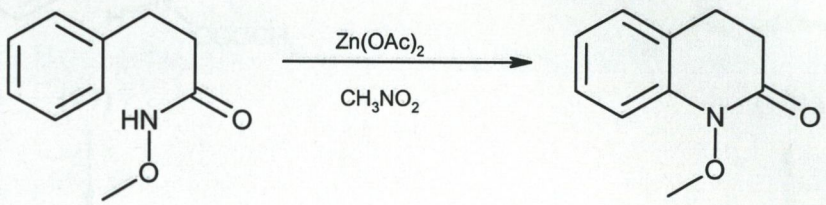
Around the same time, Kikugawa and Kawase also investigated the use of nitrenium ions for the synthesis of nitrogen heterocycles.[145] They showed that when N-chloro-N-methoxyphenylacetamides **(93)** were treated with silver salts and trifluoroacetic acid, their corresponding cyclised products, 1-methoxyindoles **(94)**, were isolated. They found that the highest yields could be obtained under acidic conditions at room temperature. The desired ring closure was almost quantitative with various different R groups present, and the reaction was complete within 1-2 hours.



(93) **(94)**

This new method was very successful, although it did present some disadvantages. For example, chlorination of the amides to give the chloroamide precursors **(93)** was found to produce other chlorinated aryl side products. In addition, the method was not suitable for use with acid sensitive molecules, nor did it suit large scale synthesis, due to a difficult work up. Finally the use of silver salts made the reaction an expensive one. Kikugawa[146] overcame some of these problems when he modified the reaction by using non-chlorinated amides **(95)** as starting materials. This enabled him to effect ring closure with electrophilic nitrogen using anhydrous zinc acetate in refluxing nitromethane. An example is

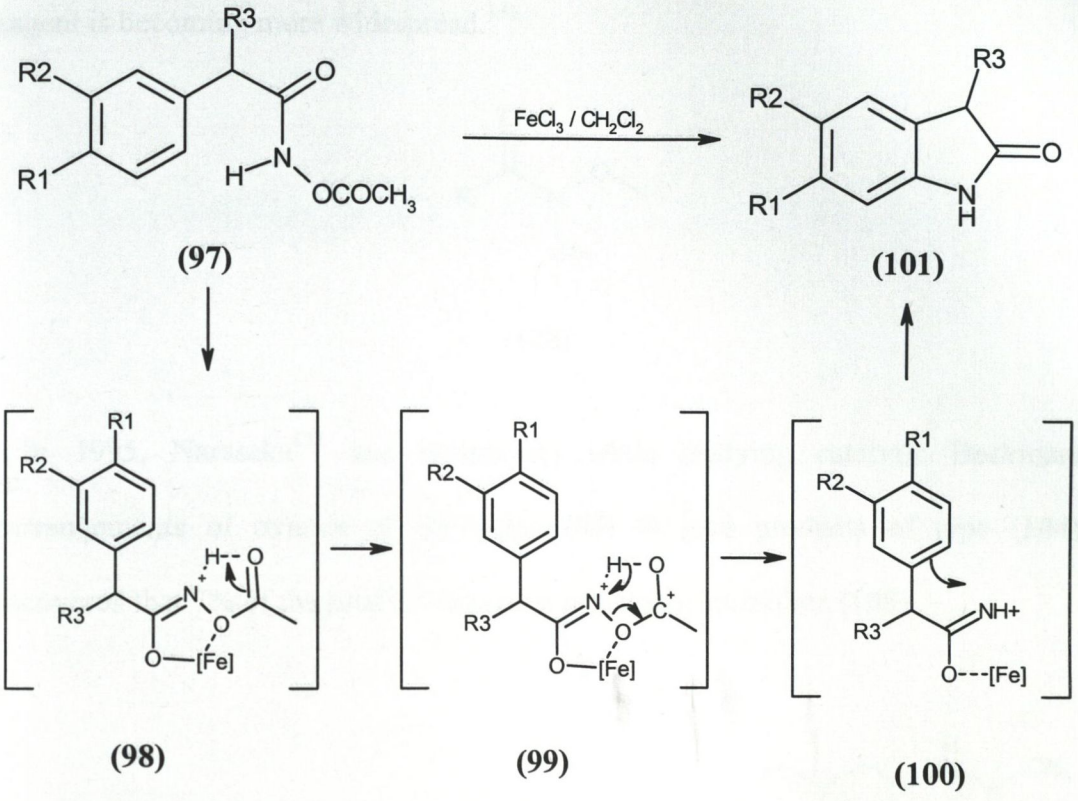
shown in the conversion of **(95)** into **(96)**. Both Fe(II) and Pd(II) ions were also found to be effective under these conditions.[146]



(95) **(96)**

While this modification improved the synthetic potential of these reactions in that the reagents are cheap and the hydroxamate starting materials are readily available, there was still one main drawback. This was that the final products always contained a N-methoxyamide functional group and this group is not very common in natural products. Fisher[147] went some way towards improving matters by developing a method for the conversion of these N-methoxy amides into amides by treatment with Ti(III) chloride in ethanol. Unfortunately, this meant an extra step in the overall synthetic scheme.

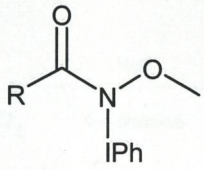
In 1989 Cherest[148] reported a new method that started with a different hydroxamic acid derivative, namely an N-acetyloxyamide **(97)**, obtainable in high yields from the hydroxamic acid itself. He showed how oxindoles **(101)** with different patterns of substitution on the benzene ring could be obtained by treatment of suitable N-acetyloxyamides with $FeCl_3$ in dichloromethane. He suggested that the reaction proceeded *via* intermediates **(98)** and **(99)** and **(100)**. The complexation of the enol form of **(98)** makes the amidic proton more acidic, thus promoting intramolecular protonation of the acetoxy group, making it a good leaving group.



This reaction solved a lot of the problems already discussed, especially the fact that chlorination of an amide is not necessary and that the products isolated did not contain a methoxy group. It was found that, however, that the presence of the amidic proton in the starting material **(97)** was essential for the reaction to proceed. When the reaction was attempted with corresponding N-methyl derivative, cyclisation did not occur.

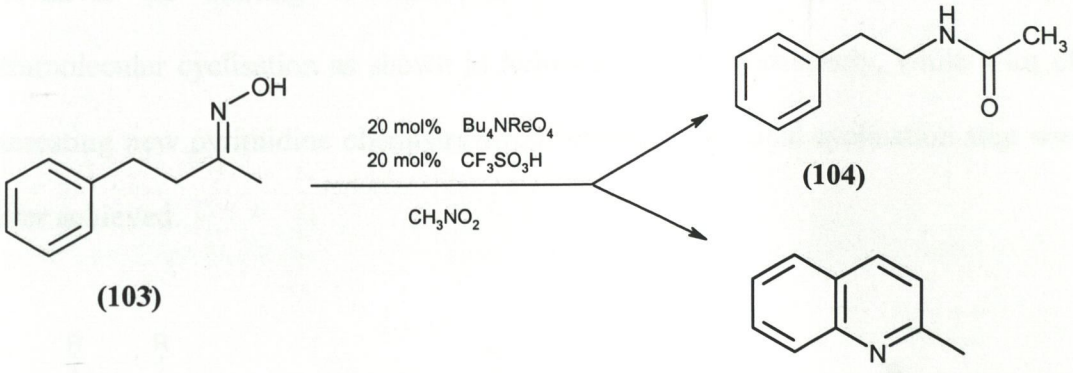
In 1990, Kikugawa[149] reported a new method for the improved cyclisation of *N*-methoxyamides using hypervalent iodine. The reaction is believed to proceed through the intermediate **(102)**. This method also avoids the need for chlorination of an amide. Using reagents such as $PhI(CO_2CF_3)_2$ Kikugawa[149] showed how starting materials such as **(95)** could afford product **(96)** in good yield. A variety of hypervalent reagents were investigated for use in this reaction. While the use of

hypervalent iodine as an oxidising reagent is well known,[150] its use as a cyclisation reagent is becoming more widespread.[151]



(102)

In 1995, Narasaka[152] and co-workers while studying catalytic Beckmann rearrangements of oximes of the type **(103)** to give products of type **(104)**, discovered that 7% of the product formed was 2-methylquinoline **(105)**.

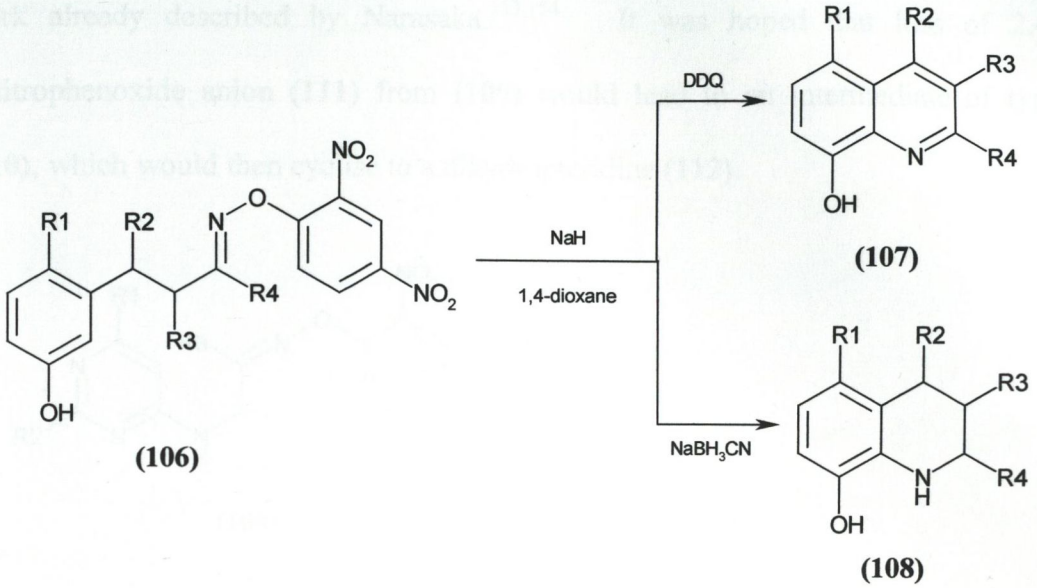


(103)

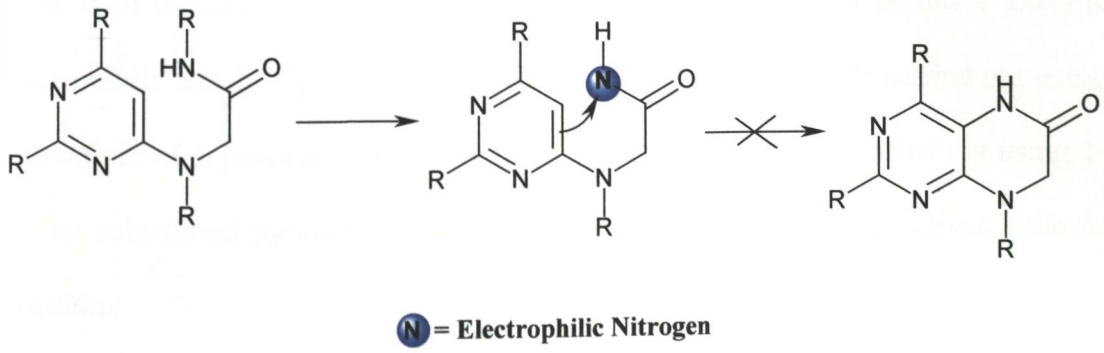
(104)

(105) 7%

Narasaka investigated this unusual source of electrophilic nitrogen further as a potential new synthetic method for quinolines. He studied several different types of oxime as the starting materials for the reaction, and proposed 2,4-dinitrophenyloximes of type **(106)** to be most suitable.[153,154] He showed that it was possible to isolate either the fully oxidised **(107)** or the reduced form **(108)** of the quinoline product.[155]



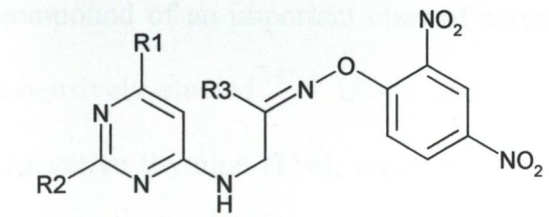
Past studies in this laboratory by Duff[156] looked at pyrimidine hydroxamic acid derivatives as starting materials for the synthesis of pteridines through intramolecular cyclisation as shown in **Scheme 3.1**. Unfortunately, while a lot of interesting new pyrimidine chemistry was discovered, the final cyclisation step was never achieved.



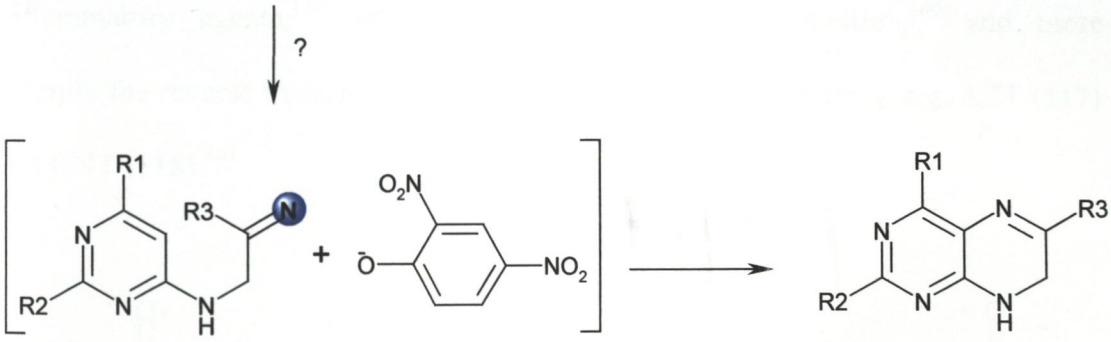
Scheme 3.1 Attempted pteridine synthesis from hydroxamic derivatives

The work described in the following sections of this chapter extends these studies further and pyrimidine 2,4-dinitrophenyl oxime derivatives of type **(109)**, were synthesised as precursors for the generation of electrophilic nitrogen, based on the

work already described by Narasaka.[153,154] It was hoped that loss of 2,4-dinitrophenoxide anion **(111)** from **(109)** would lead to an intermediate of type **(110)**, which would then cyclise to a dihydropteridine **(112)**.

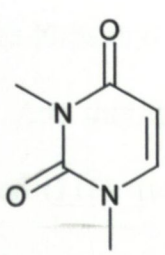


(109)

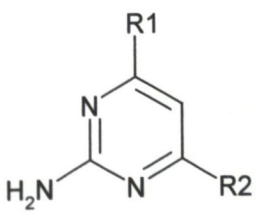


(110) **(111)** **(112)**

The substitution pattern of the pyrimidine moiety of the starting material in this route is of importance, for it is necessary to have the 5-position as active towards electrophilic attack as possible. The first series of reactions were carried out using derivatives of 1,3-dimethyluracil **(113)**. Experiments were also carried out using 2-amino substituted pyrimidines **(114)**, in which the 2-amino group activates the 5-position.



(113)

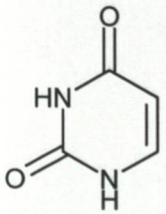


(114)

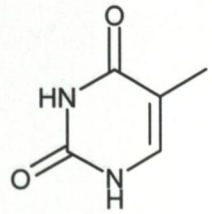
3.3 Reactions of 1,3-dimethyluracil derivatives.

3.3.1 Introduction

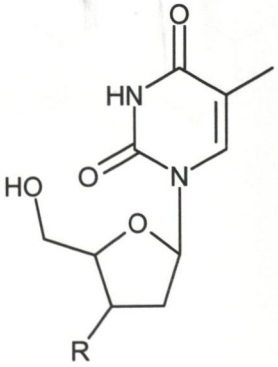
2,4-(1*H*,3*H*)-Pyrimidinedione **(115)**, normally referred to as uracil, is the parent compound of an important class of naturally occurring pyrimidines that have been extensively studied.[157] Uracil itself is a constituent of RNA, while its 5-methyl derivative thymine **(116)**, replaces it in DNA. The use of uracil based drugs is widespread, with activities that cover many disorders. These include anti-inflammatory agents,[158] anti-cancer agents,[159] thyroid inhibitors,[160] and more recently the reverse transcriptase inhibitors used in AIDS treatments, e.g. AZT **(117)** and CNT **(118)**.[161]



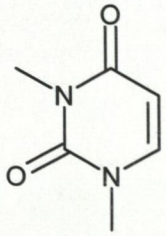
(115)



(116)



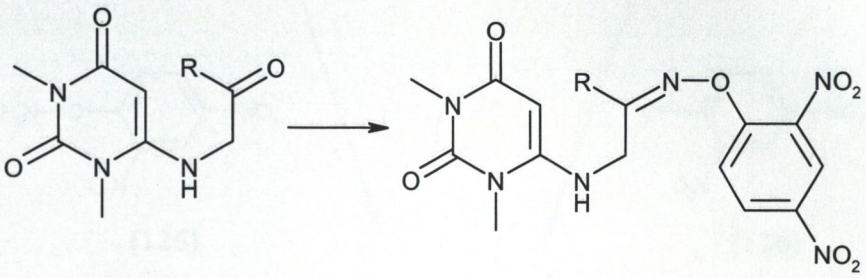
(117) R = N_3
(118) R = CN



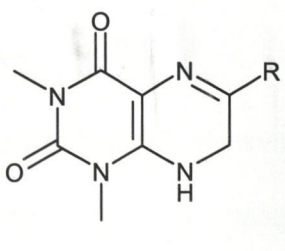
(119)

In the present work, N,N-dimethyluracil **(119)** was chosen as the pyrimidine derivative used for most experiments. In the first place, the 5-position of the molecule **(119)** is known to be very susceptible to electrophilic substitution.[162] Secondly, the two N-methyl groups should prevent unwanted cyclisation on to the ring nitrogens. And thirdly, the dimethyl- derivative **(119)** is significantly more soluble than uracil **(115)** itself, thus facilitating experimental work. The general strategy followed was to synthesise 2,4-dinitrophenyl oximes of the type **(121)**, from

a dimethyluracil aldehyde or ketone derivative of type **(120)**, and then to search for conditions under which **(121)** would cyclise to the dihydropteridine **(122)**.

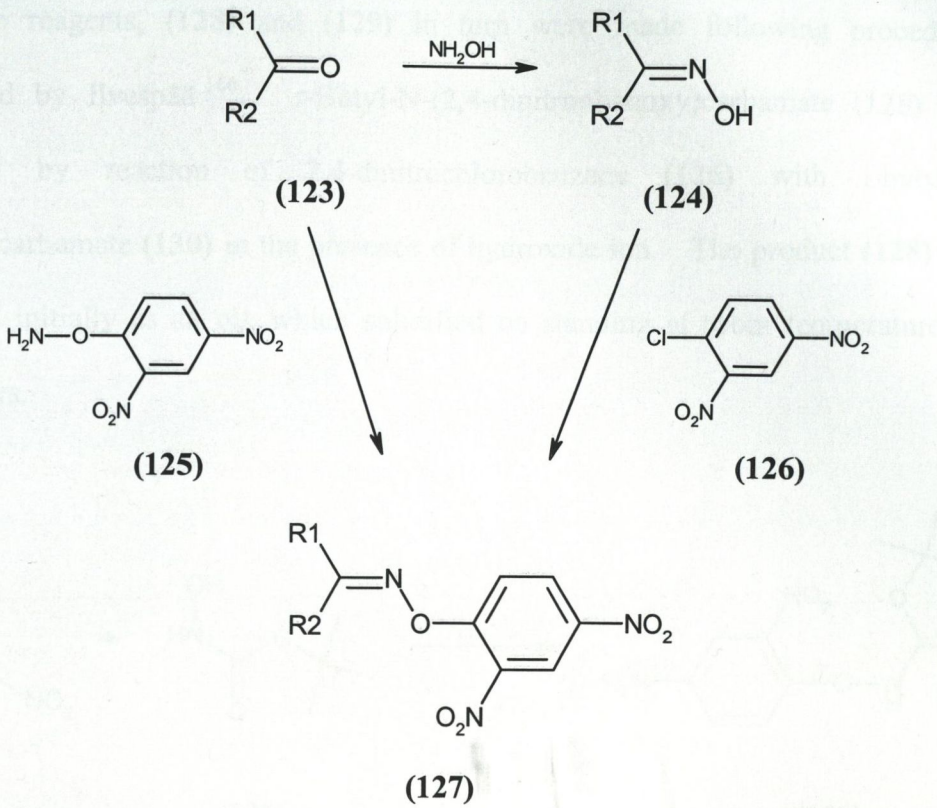


(120) **(121)**

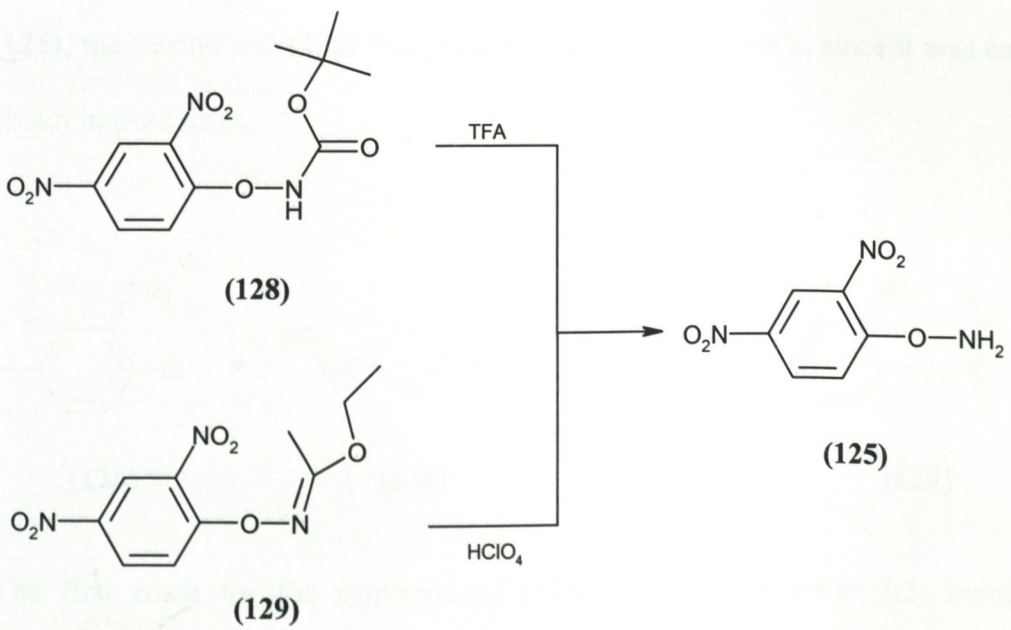


(122)

Two general methods have been reported in the literature for the preparation of 2,4-dinitrophenyl oximes. The first of these involves initial preparation of the simple oxime **(124)** from either an aldehyde or ketone **(123)** starting material. Reaction of oxime **(124)** with 2,4-dinitrochlorobenzene **(126)** gives the desired dinitrophenyl oxime **(127)**.[163] The alternative route generates the dinitrophenyl oxime **(127)** in one step, by reaction of the aldehyde or ketone **(123)** with O-(2,4-dintrophenyl)-hydroxylamine **(125)**.[164]

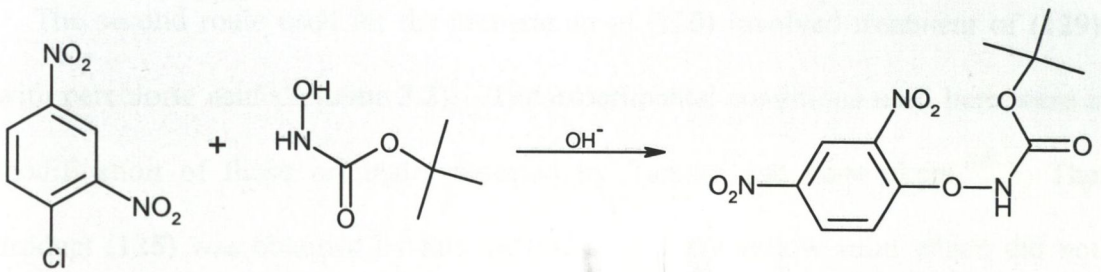


As shown in **Scheme 3.2**, the O-(2,4-dinitrophenyl) hydroxylamine reagent **(125)** was prepared from either *t*-butyl-N-(2,4-dinitrophenoxy)carbamate **(128)** or O-(2,4-dinitrophenyl)acetohydroxamate ethyl ester **(129)**.[165]



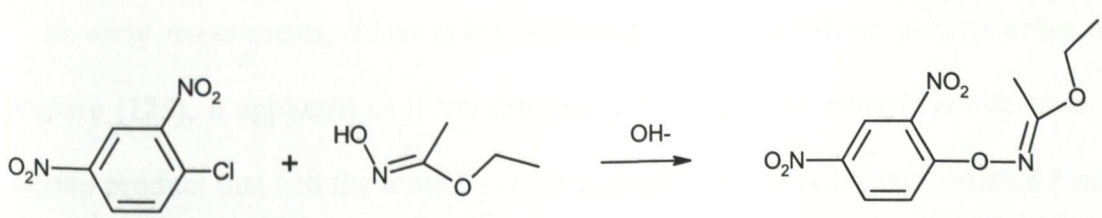
Scheme 3.2 Preparation of O-(2,4-dinitrophenyl)-hydroxylamine

These reagents, **(128)** and **(129)** in turn were made following procedures described by Ilvespää.[166] *t*-Butyl-N-(2,4-dinitrophenoxy)carbamate **(128)** was obtained by reaction of 2,4-dinitrochlorobenzene **(126)** with t-butyl-N-hydroxycarbamate **(130)** in the presence of hydroxide ion. The product **(128)** was obtained initially as an oil, which solidified on standing at room temperature for three days.



(126) **(130)** **(128)**

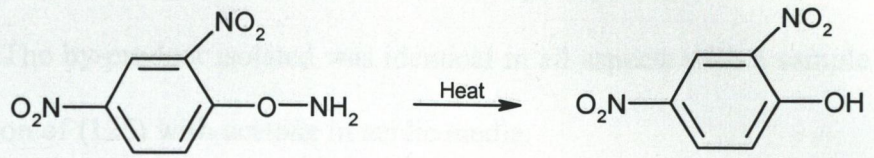
The second reagent, O-(2,4-dinitrophenyl)-acetohydroxamate ethyl ester **(129)**,[166] was made by reaction of 2,4-dinitrochlororbenzene **(126)** with ethyl acetohydroxamate **(131)**. Of the two possible starting materials for the preparation of **(125)**, the second one **(129)** was preferred in the present work, since it was easier to obtain in pure form.



(126) **(131)** **(129)**

The first route for the synthesis of **(125)** shown in **Scheme 3.2**, involved treatment of the carbamate **(128)** with TFA. The product **(125)** was isolated initially as a crude oil which took several days to solidify, and was very difficult to

purify. Efforts to crystallise the solid **(125)** led to significant thermal decomposition of it to 2,4-dinitrophenol **(132)**.

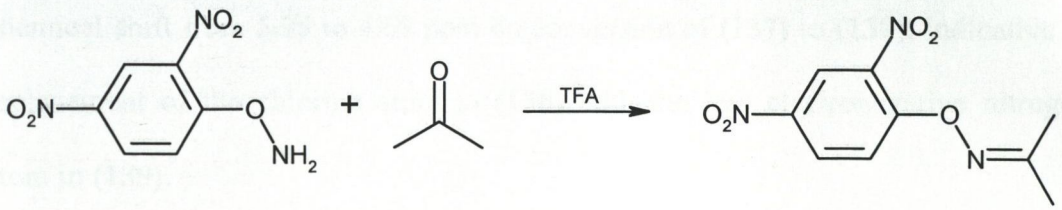


(125) **(132)**

The second route used for the preparation of **(125)** involved treatment of **(129)** with perchloric acid (**Scheme 3.2**). The experimental conditions used here were a modification of those originally reported by Tamara and co-workers.[165] The product **(125)** was obtained by this method as a fluffy yellow solid which did not require further purification, and which could be used directly as a reagent. This was the method of choice for the preparation of **(125)**, firstly because the precursor **(129)** was easier to make than precursor **(128)**, and secondly because reactions using **(129)** led to a purer product. As already mentioned, the O-(2,4-dinitrophenyl)-hydroxylamine **(125)** was heat labile, and decomposed on standing at room temperature. It was therefore stored at 4□C and used as quickly as possible after preparation.

In early experiments, when **(129)** was treated with perchloric acid in order to prepare **(125)**, it appeared as if the reaction had not gone to completion because a second product that had the same R_f as the starting material **(129)** was isolated from the reaction mixture. In the event, however, this product proved not to be unreacted starting material, but rather was shown to be the 2,4-dinitrophenyloxime of acetone **(133)**. It is believed that this was formed due to traces of acetone that were present in the ethanol used for the reaction, for when the reaction was repeated with HPLC grade ethanol, no trace of product **(133)** was observed. The ^{1}H NMR spectrum of

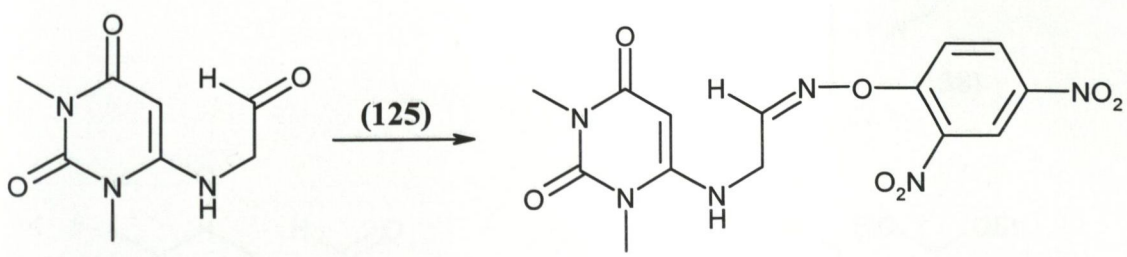
this product **(133)** showed three aromatic proton signals at 7.95, 8.42 and 8.88 ppm as well as two CH_3 singlets at 2.12 and 2.21 ppm. Its IR spectrum displayed a sharp band at 1606 cm^{-1}, corresponding to the stretching frequency of the oxime functional group. The by-product isolated was identical in all aspects with a sample prepared by reaction of **(125)** with acetone in acidic media.



(125) **(133)**

3.3.2 Reactions starting from 6-chloro-1,3-dimethyluracil

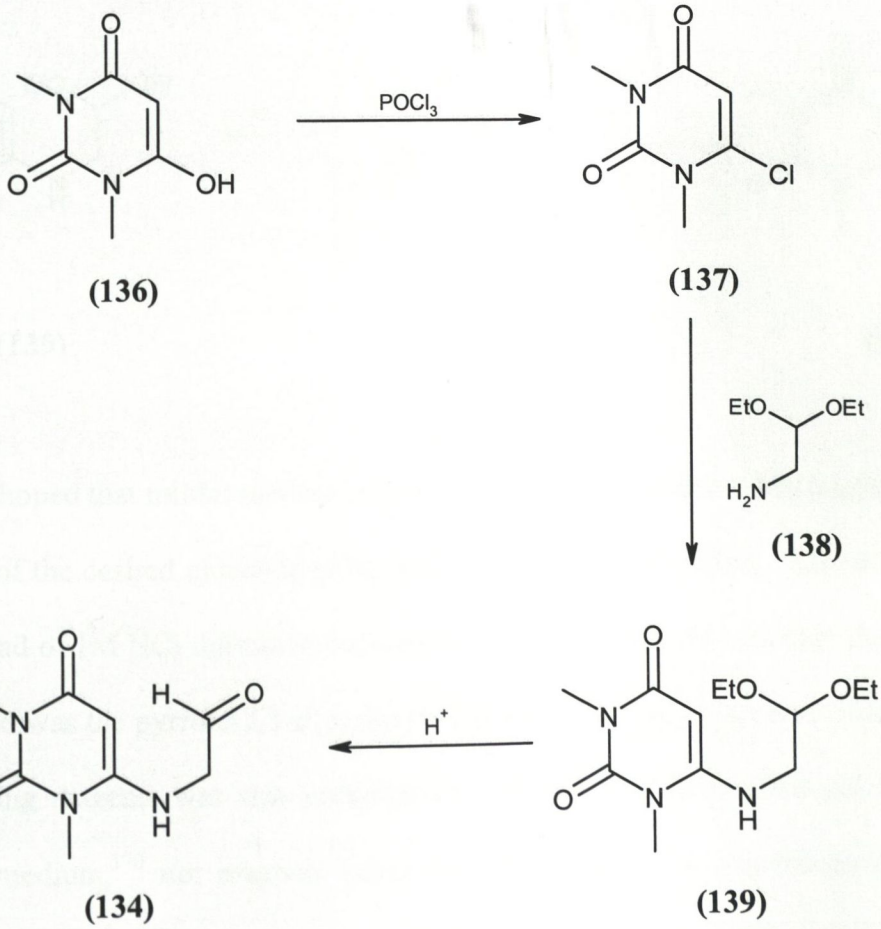
The 1,3-dimethyluracil derivative **(134)**, was required as a precursor for the preparation of the desired oxime **(135)**.



(134) **(135)**

In the event, it did not prove possible to obtain **(134)**, since the latter was found to cyclise spontaneously to a pyrrolo[2,3-*d*]pyrimidine. Three different approaches were used in the effort to prepare **(134)**. In the first of these, the starting material 1,3-dimethylbarbituric acid **(136)** was chlorinated following a procedure described

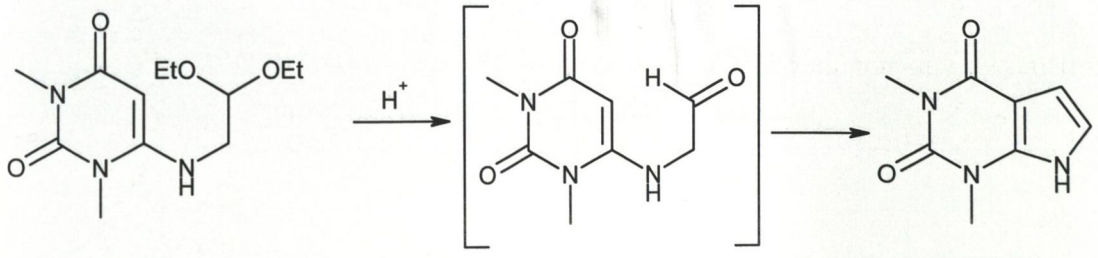
by Pfleiderer[167] using $POCl_3$. This gave 6-chloro-1,3-dimethyluracil **(137)** in good yield, with analytical data identical to those described by Pfleiderer. Reaction of the chloropyrimidine **(137)** with the diethyl acetal of aminoacetaldehyde **(138)** led to the product **(139)** in good yield, by nucleophilic displacement of chlorine.[168] ^1H and ^{13}C NMR spectra of **(139)** clearly showed the presence of the diethyl acetal group. The proton at 5-position of the starting material **(137)** moved up field in chemical shift from 5.95 to 4.69 ppm on conversion of **(137)** to **(139)**, indicative of replacement of the chlorine atom in **(138)** with the less electronegative nitrogen atom in **(139)**.



(136) **(137)** **(138)** **(139)** **(134)**

In order to obtain aldehyde **(134)**, by deprotection of its acetal **(139)**, the latter was stirred at room temperature in 1M HCl. This treatment gave a white solid

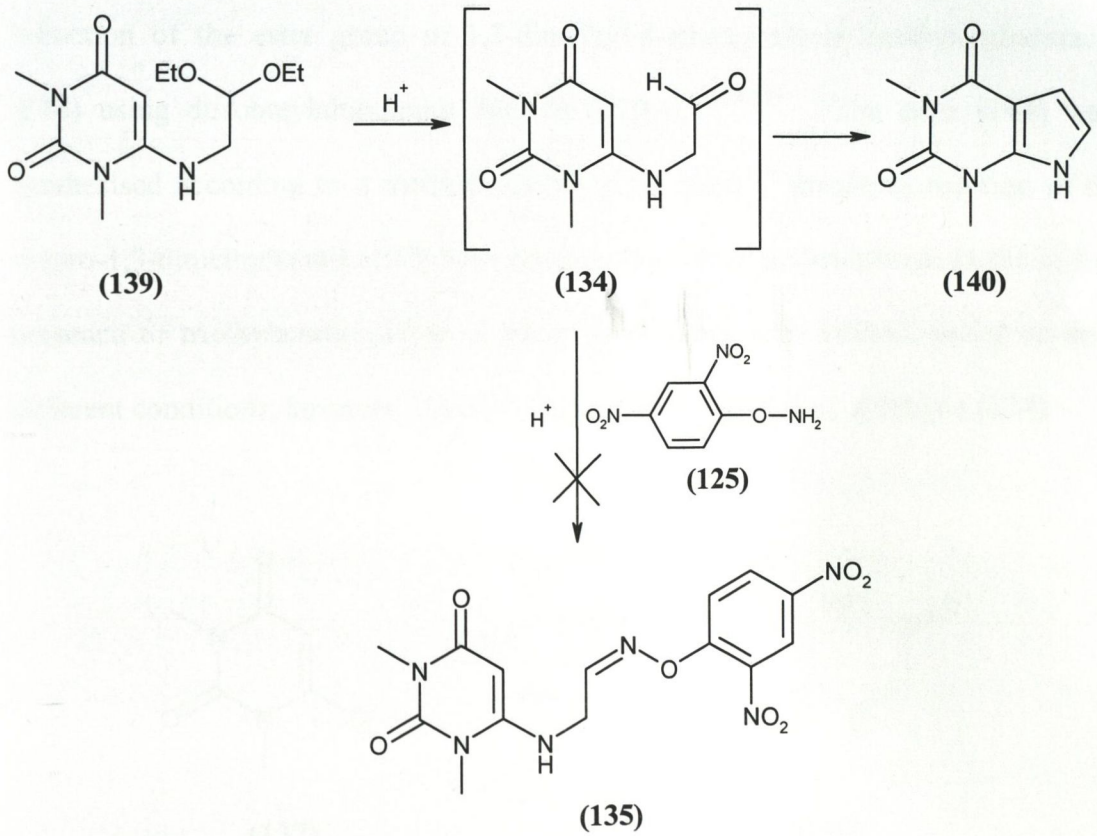
product, which was shown to be pure by TLC. An electrospray mass spectrum of the product, however, showed a peak at m/z 180, instead of an $M+H^+$ peak at m/z 198, expected for the desired aldehyde **(134)**. The ^1H NMR spectrum of the product showed neither an expected low field aldehyde signal, nor any signal that could be assigned to a pyrimidine 5-proton. The product isolated, was in fact, the pyrrolo[2,3-*d*]pyrimidine[169] **(140)**, formed presumably by spontaneous cyclisation of the initially formed aldehyde **(134)**. The two adjacent protons on the pyrrole ring of **(140)** appeared as two doublets at 6.36 and 6.77 ppm respectively in the ^1H NMR spectrum of **(140)**.



(139) **(134)** **(140)**

It was hoped that milder methods of deprotection of the acetal **(139)** might enable isolation of the desired aldehyde **(134)** without causing it to cyclise. Use of 0.01M HCl instead of 1M HCl did cause deprotection, but again the only product that could be isolated was the pyrrolo[2,3-*d*]pyrimidine **(140)**. The use of several silica based deprotecting reagents was also investigated. However, neither silica gel itself in aqueous medium,[170] nor reagents based on ferric chloride or aluminium chloride adsorbed on silica,[170] gave any deprotection of the acetal **(139)**, only starting material **(139)** being isolated in every case.

Finally, several attempts were made to deprotect acetal **(139)** in the presence of the hydroxylamine reagent **(125)**, in the hope of trapping the aldehyde **(134)** before it could cyclise. Under mild conditions, however, only the starting material **(139)** was isolated, whereas under more vigorous conditions cyclisation of **(134)** to **(140)** occurred. In no case could the desired oxime **(135)** be isolated.

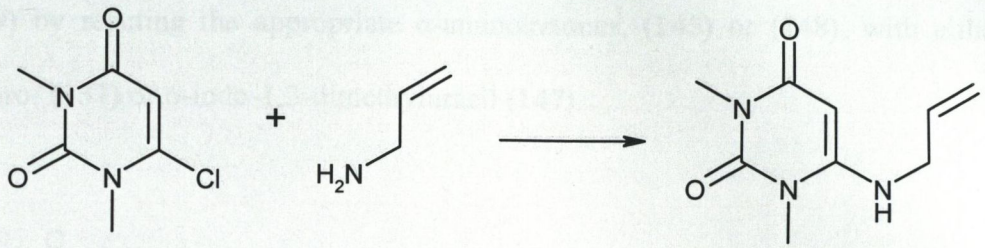


(139) **(134)** **(140)**

(125)

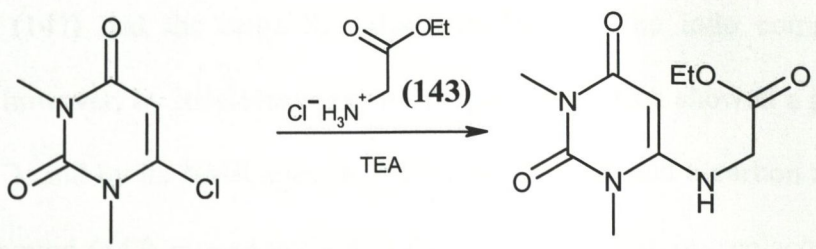
(135)

Another attempt was made to prepare aldehyde **(134)** by ozonolysis of the double bond in the 6-allylaminopyrimidine **(142)**. The latter was prepared from 6-chloro-1,3-dimethyluracil **(137)** by reaction with allylamine.[171] Ozonolysis of **(142)** was carried out at -10□C, followed by treatment with dimethylsulfide to reduce the intermediate ozonides formed. [172,173] A complex mixture of products was obtained, however, from which none of the desired aldehyde **(134)** could be isolated.



(137) (141) (142)

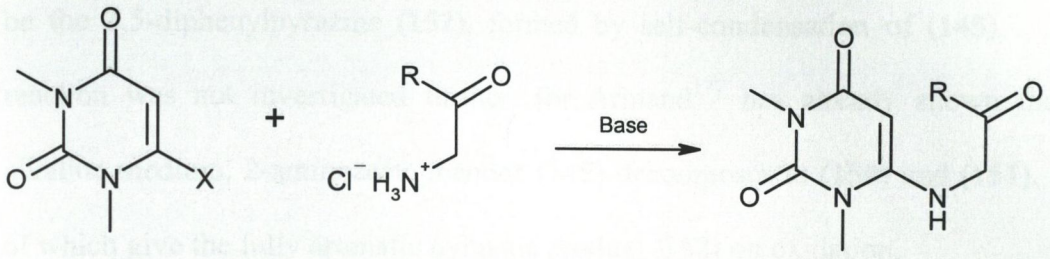
A final approach used to obtain the aldehyde **(134)** involved the selective reduction of the ester group in 1,3-dimethyl-4-ethoxycarbonylmethylaminouracil **(144)** using diisobutylaluminium hydride (DIBAL).[174,175] The ester **(144)** was synthesised according to a method described by Duff,[156] involving reaction of 6-chloro-1,3-dimethyluracil **(137)** with glycine ethyl ester hydrochloride **(143)** in the presence of triethylamine (TEA). Reaction of **(144)** with DIBAL under several different conditions, however, failed to afford any of the desired aldehyde **(134)**.



(137) (144)

Since it was not possible to prepare the aldehyde **(134)**, attention was turned to the preparation of the aryl ketones **(146)** and **(149)**. The ketone carbonyl atoms in both **(146)** and **(149)**, should be less electrophilic than the aldehyde carbonyl carbon in **(134)**. It was hoped, therefore, that **(146)** and **(149)** might show a lesser tendency toward unwanted cyclisation. Attempts were made to prepare ketones **(146)** and

(149) by reacting the appropriate α-aminoketones, **(145)** or **(148)**, with either 6-chloro- **(137)** or 6-iodo-1,3-dimethyluracil **(147)**.



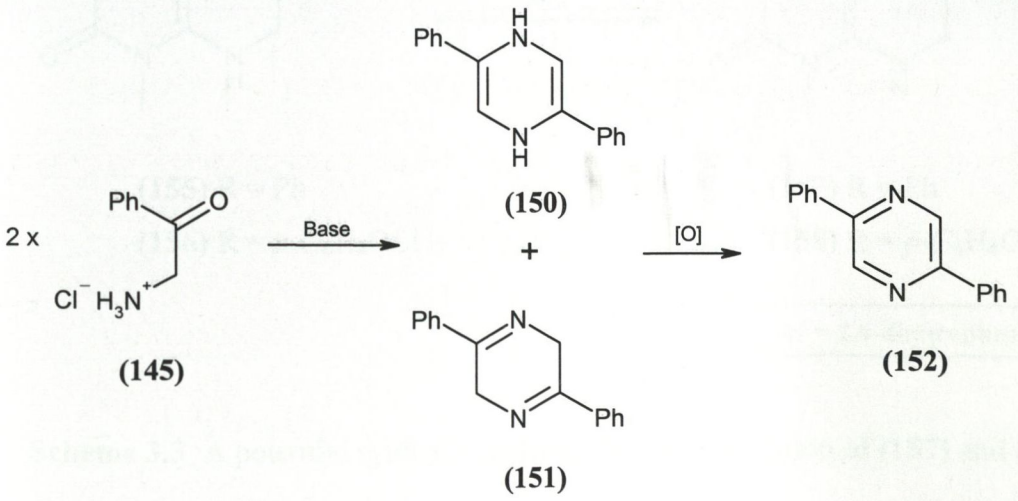
(137) X = Cl **(145)** R = Ph **(146)** R = Ph

(147) X = I **(148)** R = *p*-$C_6H_4OCH_3$ **(149)** R = *p*-$C_6H_4OCH_3$

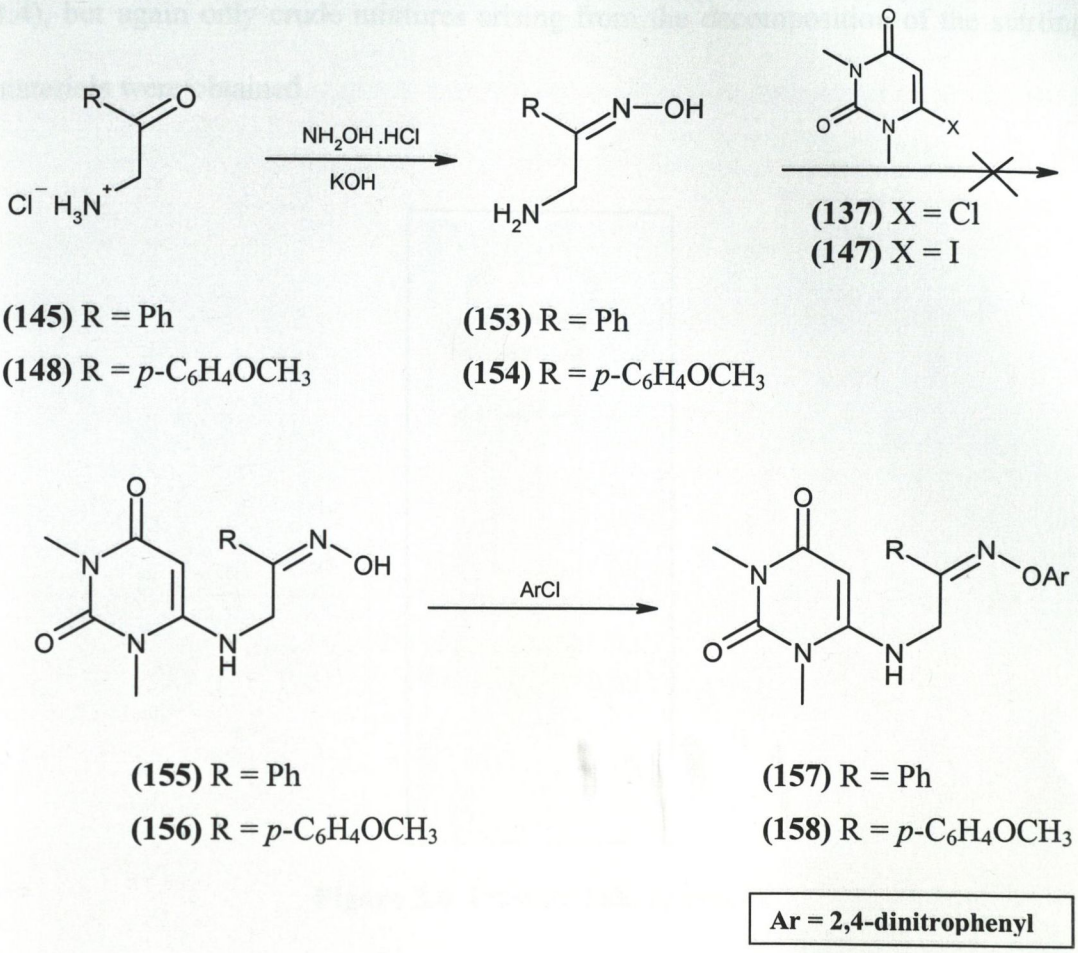
6-Chloro-1,3-dimethyluracil **(137)** was prepared by chlorination of dimethyl-barbituric acid **(136)** as already described.[167] 6-Iodo-1,3-dimethyluracil **(147)** was obtained from the chloro compound **(137)** following a procedure described by Pfleiderer,[176] which involved refluxing **(137)** in DMF with sodium iodide. Both **(137)** and **(147)** had the same R_f values on TLC. The iodo compound was identified, however, by its electrospray mass spectrum, which showed a peak at m/z 267 ($M+H^+$), and by its NMR spectra. Both the 5-proton and 6-carbon atom of the iodo- compound **(147)** moved upfield in the NMR spectrum, on replacing 6-chloro with 6-iodo.

Unfortunately, no reaction conditions could be found under which either of the 6-halopyrimidines **(137)** or **(147)** could react with either of the amino ketones **(145)** or **(148)**. The latter were used as their HCl salts and initial attempts used the weak base, $NaHCO_3$, in order to minimise self-condensation of the aminoketones. Many different reaction conditions were used. At room temperature or lower, only starting material could be recovered from the reaction, irrespective of whether the chloro- **(137)** or the iodo- **(147)** pyrimidine was used. Use of higher temperatures

or stronger bases (TEA or NaOH) also gave none of the desired products, **(146)** or **(149)**, but rather led to decomposition of the ketone starting materials. In using 2-aminoacetophenone hydrochloride **(145)** a product was isolated, but it turned out to be the 2,5-diphenylpyrazine **(152)**, formed by self-condensation of **(145)**. This reaction was not investigated further, for Armand[177] has already shown that in alkaline medium, 2-aminoacetophenone **(145)** decomposes to **(150)** and **(151)**, both of which give the fully aromatic pyrazine product **(152)** on oxidation.



Yet another synthetic approach towards the desired 2,4-dinitrophenylketone oximes **(157)** and **(158)** is outlined in **Scheme 3.3**, as shown on the next page. Conditions have already been described in the literature[178] for the successful conversion of **(145)** and **(148)** into their corresponding simple oximes **(153)** and **(154)**, without self-condensation of the aminoketones taking place.



(145) R = Ph
(148) R = *p*-$C_6H_4OCH_3$

(153) R = Ph
(154) R = *p*-$C_6H_4OCH_3$

(137) X = Cl
(147) X = I

(155) R = Ph
(156) R = *p*-$C_6H_4OCH_3$

(157) R = Ph
(158) R = *p*-$C_6H_4OCH_3$

Scheme 3.3 A potential synthetic pathway for the preparation of **(157)** and **(158)**

Both oximes, **(153)** and **(154)** were therefore synthesised according to procedures described by Gnichtel,[178] which involved condensation of the ketones with hydroxylamine hydrochloride in aqueous potassium hydroxide. Both products, **(153)** and **(154)** were obtained pure, and all spectroscopic data were identical with those previously reported.[178] Unfortunately, all attempts to react the oximes **(153)** and **(154)** with 6-chloro- **(137)** and 6-iodo-1,3-dimethyluracil **(147)** were unsuccessful, although a wide variety of reaction conditions was used. For example, a series of different bases was employed, including TEA, DMAP and KOH, in combination with various solvents and temperatures. Higher temperatures and pressures were achieved by carrying out the reactions in a pressure tube (**Figure**

3.4), but again only crude mixtures arising from the decomposition of the starting materials were obtained.

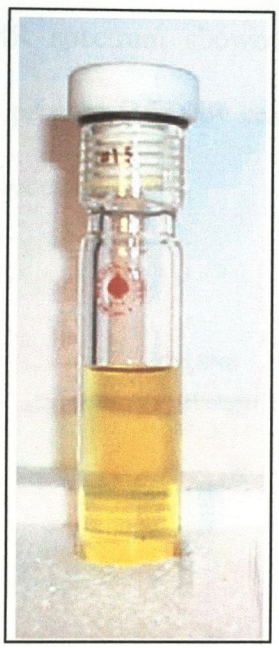
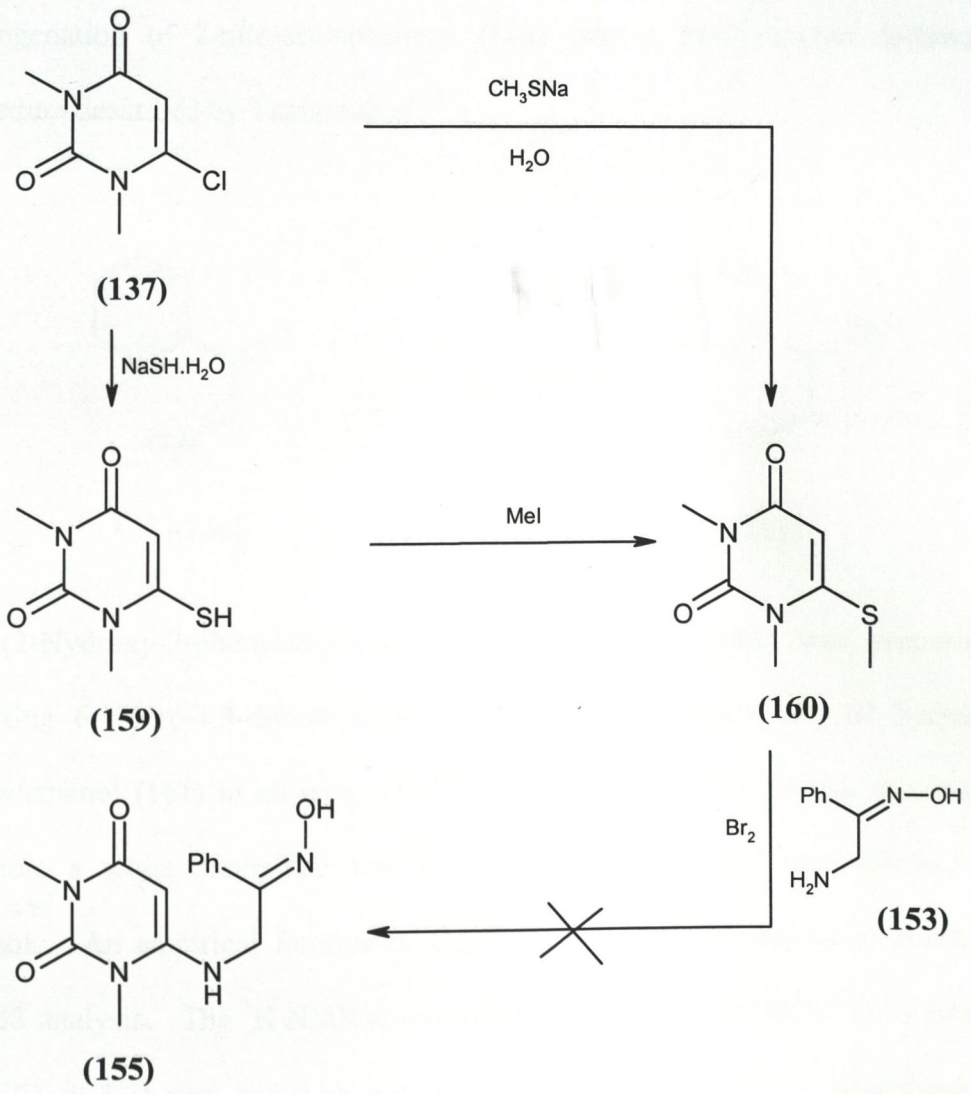


Figure 3.4 Pressure tube apparatus

It is clear from the above experiments that the electron attracting carbonyl group in the α-aminoketones **(145)** and **(148)** has so reduced the nucleophilicity of the adjacent α-amino group, that substitution of the 6-halo uracils **(137)** and **(147)** is energetically unfavourable. The amino group in the α-amino oximes **(153)** and **(154)** is similarly reduced in reactivity. Another problem is that the 6-halo uracils **(137)** or **(147)** are also not particularly active towards nucleophilic substitution.

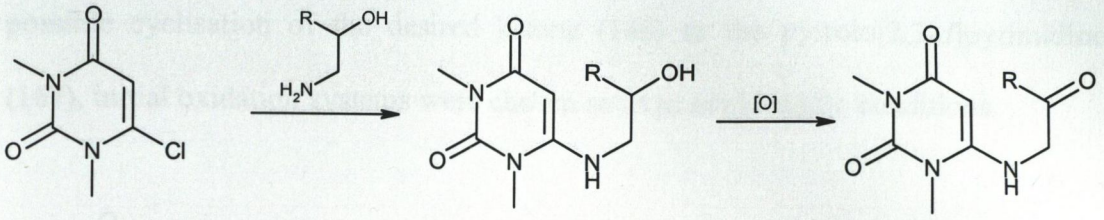
It is known that with some heterocyclic systems, a methylthio group in the presence of bromine acts a better leaving group than chlorine or iodine.[179,180] Accordingly, 6-methylthio-1,3-dimethyluracil **(160)** was prepared from 6-chloro-1,3-dimethyluracil **(137)** *via* the mercapto intermediate **(159)**. A much better route, however, was the direct conversion of **(137)** to **(160)** using an aqueous solution of

sodium thiomethoxide. This latter approach yielded **(160)** as a clean white precipitate, which was isolated by filtration. This product was shown to be pure by TLC, and both elemental analysis and HRMS confirmed an empirical formula of $C_7H_{10}N_2O_2S$. The ^1H NMR spectrum showed a methyl peak at 2.49 ppm. Unfortunately, all attempts to prepare **(155)** by reaction of **(160)** with **(153)** in the presence of bromine were unsuccessful.



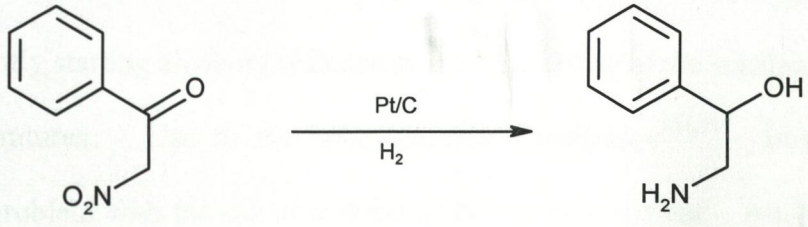
(137) **(159)** **(160)** **(153)** **(155)**

A final attempt to prepare pyrimidine ketones, such as **(146)** and **(165)** was made by initial preparation of the corresponding alcohols **(162)** and **(164)**. It was hoped to obtain the ketones by oxidation of the alcohols.



(137) **(161)** R = Ph **(162)** R = Ph **(146)** R = Ph
(163) R = CH_3 **(164)** R = CH_3 **(165)** R = CH_3

Alcohol **(163)** was available commercially. Alcohol **(161)** was prepared by hydrogenation of 2-nitroacetophenone **(166)** over a Pt/C catalyst following a procedure described by Tamura *et al.*[181]

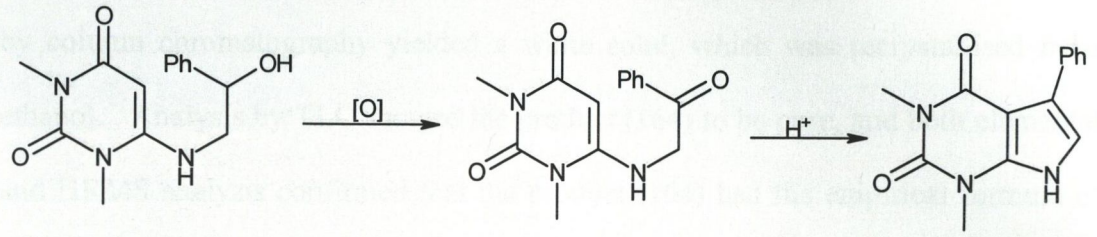


(166) **(161)**

6-(2-Hydroxy-2-phenylethylamino)-1,3-dimethyluracil **(162)** was prepared by refluxing 6-chloro-1,3-dimethyluracil **(137)** with two equivalents of 2-amino-1-phenylethanol **(161)** in ethanol, with TEA as the base. On cooling the resultant solution, a white precipitate was isolated by filtration and recrystallised from ethanol. An empirical formula of $C_{14}H_{17}N_3O_3$ was confirmed by elemental and HRMS analysis. The ^{1}H NMR spectrum of the product **(162)** showed the presence of a CH_2 at 3.21 ppm and a phenyl group at 7.35 ppm, as well as an exchangeable NH group (D_2O) at 6.72 ppm, confirming the structure of the product as **(162)**.

A major problem encountered in efforts to oxidise alcohol **(162)** to its corresponding ketone **(146)** was the insolubility of the alcohol. In order to inhibit

possible cyclisation of the desired ketone **(146)** to the pyrrolo[2,3-*d*]pyrimidine **(167)**, initial oxidation systems were chosen so as to avoid acidic conditions.

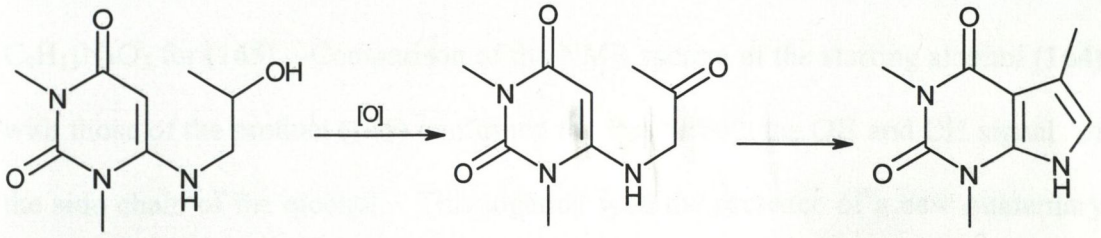


(162) **(146)** **(167)**

However, attempted oxidation of **(162)** to **(146)** using pyridinium chlorochromate[182] (PCC) was not successful, yielding a crude mixture each time. An attempt to repeat this oxidation using the Swern[183] oxidation yielded predominantly starting alcohol **(162)** due to the insolubility of the starting material at low temperatures. Use of the Jones oxidation technique[184,185] overcame this solubility problem with the use of acetone as the reaction solvent. Analysis by MS (ESI) of the crude reaction solution obtained by treating alcohol **(162)** under Jones conditions, suggested that the ketone had been formed in good yield, indicated by a strong signal at m/z 274 ($M+H^+$). Despite the low pH of the reaction medium, there was no evidence of the pyrrolo[2,3-*d*]pyrimidine **(167)** in the mass spectrum. Workup of the reaction, however, gave a crude mixture, from which no pure product could be isolated after column chromatography. Attempts to trap the ketone **(146)**, following the oxidation of **(162)**, were made by reacting hydroxylamine hydrochloride with the crude reaction mixture, after the chromium salts were removed by filtration. This failed to give any of the expected simple oxime product.

Due to the difficulties in obtaining the desired phenyl ketone **(146)**, attention was next turned to the preparation of its methyl analogue, 6-(2-oxo-propylamino)-1,3-

dimethyluracil **(165)**. The alcohol precursor **(164)** required for the preparation of **(165)** was prepared by refluxing 6-chloro-1,3-dimethyluracil **(137)** with ten equivalents of 1-amino-2-propanol **(163)**. Purification of the reaction product **(164)** by column chromatography yielded a white solid, which was recrystallised from ethanol. Analysis by TLC showed the product **(164)** to be pure, and both elemental and HRMS analysis confirmed that the product **(164)** had the empirical formula of $C_9H_{15}N_3O_3$. Its ^1H NMR spectrum showed the presence of a CH at 3.84 ppm, a new CH_3 doublet at 1.08 ppm and the hydroxyl proton at 7.23 ppm.



(164) **(165)** **(168)**

Oxidation of **(164)** to the ketone **(165)** was attempted using PCC in DCM,[186] with the reagent adsorbed onto alumina, but this reaction yielded a mixture of products. Analysis of the mixture by MS (ESI) showed two main peaks at m/z 194 and 212. This suggested that both the ketone **(165)** and its corresponding pyrrolo[2,3-*d*] pyrimidine **(168)** had been formed. Neither product could be isolated from the reaction mixture, however.

Attention was then switched to the Swern oxidation method. This had the advantage of being non-acidic, although the low temperature of the reaction (-60°C) led to solubility problems with the starting alcohol **(164)**. Analysis of the Swern reaction mixture by MS (ESI) again indicated that both **(165)** and **(168)** had been formed. Both the ketone product **(165)** and its pyrrolo derivative **(168)** were isolated in pure form by column chromatography, but the yield of **(165)** was very

low. It was found that agitation of the desired ketone **(165)** over silica in ethanol caused complete conversion of it to its cyclised derivative **(168)** in a very short time. It is clear, therefore, that most of the ketone **(165)** formed in the reaction was converted into **(168)** during efforts to isolate it by column chromatography on silica. Efforts were also made to purify **(165)** on a column of basic alumina, but they too were unsuccessful.

This instability made characterisation of the ketone difficult. Nevertheless it was found possible to obtain satisfactory NMR spectra, as well as a high resolution mass spectrum. The accurate mass spectrum confirmed an empirical formula of $C_9H_{13}N_3O_3$ for **(165)**. Comparison of the NMR spectra of the starting alcohol **(164)** with those of the product **(165)** confirmed the loss of both the OH and CH signals of the side chain of the alcohol. This together with the presence of a new quaternary carbon peak at 200.36 ppm in the ^{13}C spectrum, confirmed the formation of **(165)** from **(164)**.

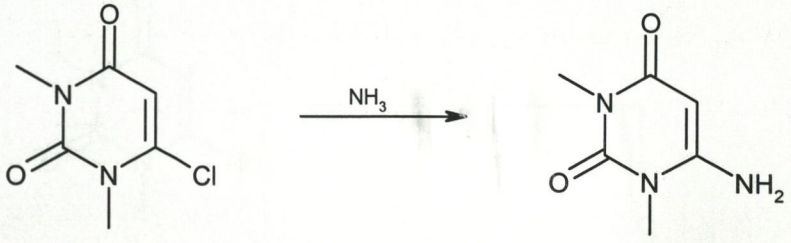
The structure of the cyclised product **(168)** was confirmed by spectroscopic characterisation. Both ^{1}H and ^{13}C NMR analysis showed the loss of the uracil 5-CH as well as the loss of the CH_2 of the side chain in **(165)**. HRMS confirmed an empirical formula of $C_9H_{11}N_3O_2$ for **(168)**. These data corresponded to those reported by Kawahara *et al.* for 1,3,5-trimethylpyrrolo[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)dione **(168)**.[187]

In spite of all these efforts no suitable 2,4-dinitrophenyl oximes of type **(109)** could be prepared. Starting from 6-chloro- **(137)** or 6-iodo-1,3-dimethyluracil **(147)** a major problem was the ease with which the desired ketone **(165)** decomposed to **(168)**, especially under acidic conditions. It was considered unlikely, therefore, that **(165)** could be converted to its corresponding 2,4-

dinitrophenyl oxime, since oxime formation is usually carried out under acidic conditions.[188] An alternative approach starting from 6-amino-1,3-dimethyluracil **(169)** did lead to some interesting new results, however, and is described in the next section of this chapter.

3.3.3 Reactions starting from 6-amino-1,3-dimethyluracil

6-Amino-1,3-dimethyluracil **(169)** was obtained in good yield by amination of 6-chloro-1,3-dimethyluracil **(137)** with ammonia.

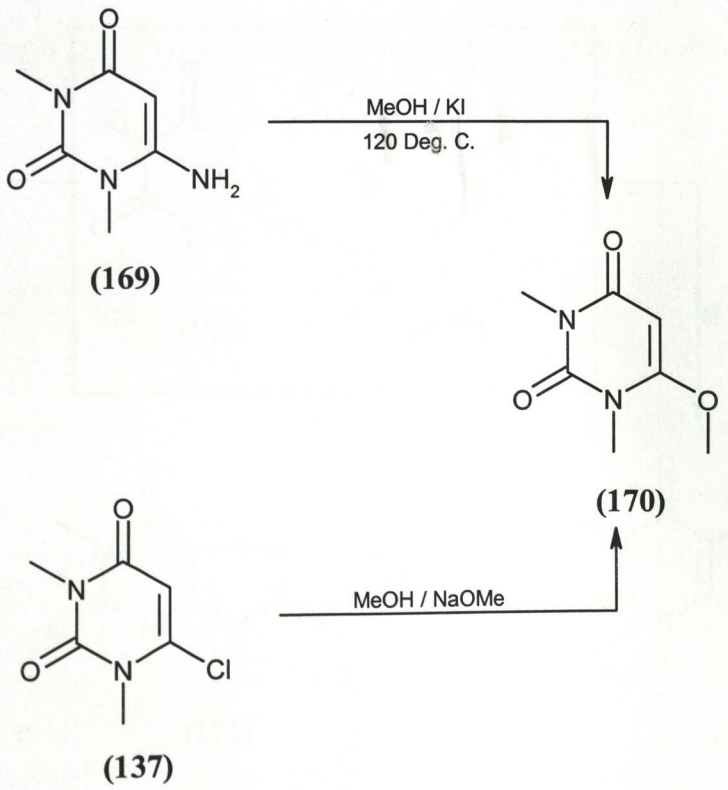


(137) **(169)**

Treatment of **(169)** with chloroacetone under a variety of reaction conditions showed no sign of any reaction, with only starting materials being isolated from the reaction mixtures. Reactions conditions used, included a variety of bases (TEA, NaOH, DMAP and NaH), as well as a variety of different solvents and reaction temperatures. Some reactions were also carried out at elevated temperatures and pressure in a pressure tube. Addition of a catalytic amount of KI had no effect on the results of these reactions, again only starting materials being isolated.

Iodoacetone was prepared from chloroacetone using the Finckelstein reaction.[189] Because of its extreme lachrymatory properties, the product was not purified and was used as a crude oil for all reactions. Treatment of 6-amino-1,3-dimethyluracil **(169)** with iodoacetone at temperatures up to 100°C in refluxing water yielded no

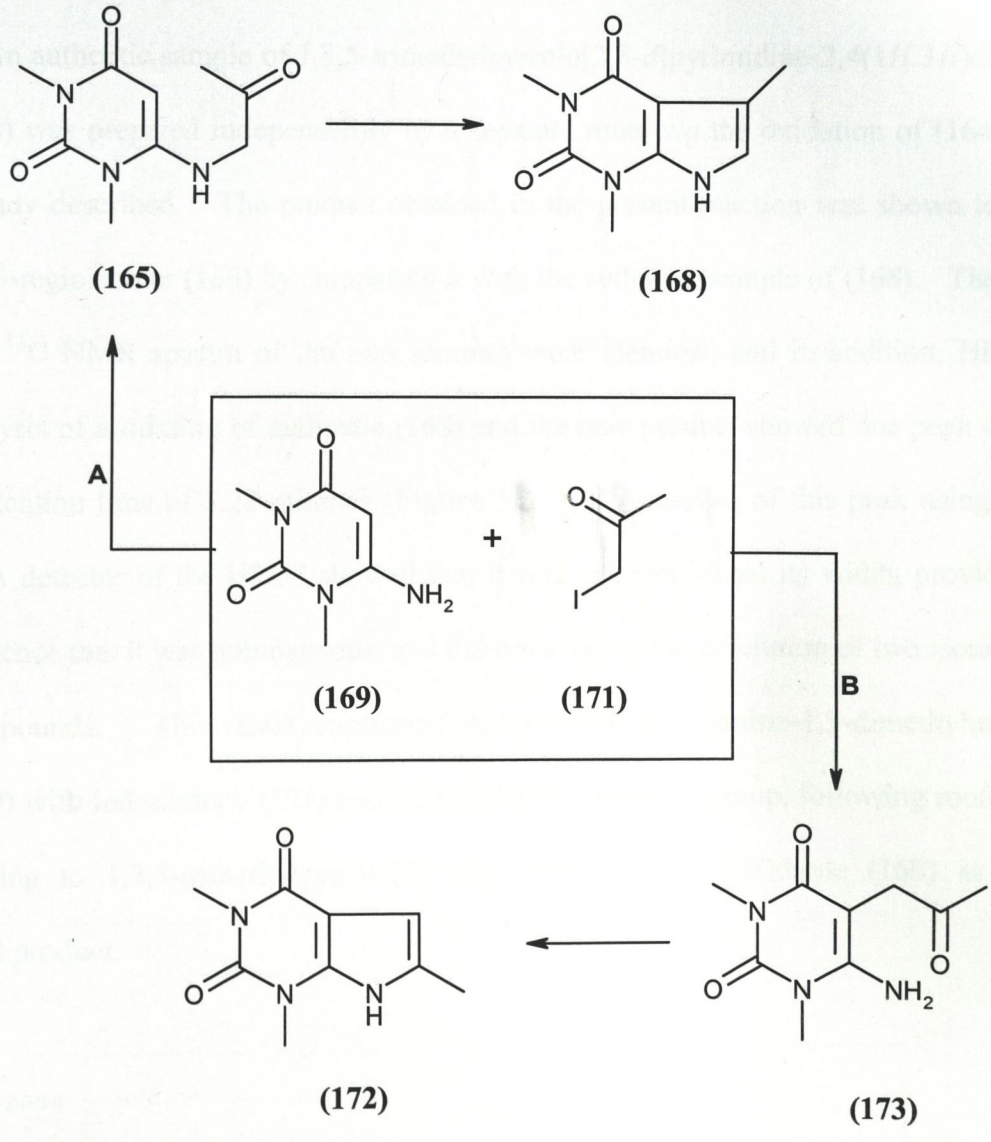
products. However, when **(137)** was treated with iodoacetone **(171)** in MeOH at 120°C in a pressure tube, TLC analysis of the reaction solution showed the formation of two new products, which were isolated by column chromatography. The first product from the reaction was shown to be 6-methoxy-1,3-dimethyluracil **(170)**. Firstly, a high resolution mass spectrum showed a peak at 171.1758 ($M+H^+$), corresponding to a empirical formula of $C_7H_{10}N_2O_3$. Secondly, the NMR spectra of the product were identical with those of an authentic sample of **(170)**, made by reaction of 6-chloro-1,3-dimethyluracil **(137)** with sodium methoxide in methanol.



(169) **(170)** **(137)**

A high resolution mass spectrum of the second product, showed a peak at m/z 194.2131 ($M+H^+$), corresponding to an empirical structure of $C_9H_{11}N_3O_2$. The ^1H NMR spectrum showed the absence of a pyrimidine 5-H, the presence of a methyl peak at 2.28ppm and a low field aromatic type proton at 6.13 ppm. These results are compatible with a structure of either 1,3,5-trimethylpyrrolo[2,3-*d*]pyrimidine-

2,4-(1*H*,3*H*)dione **(168)** or 1,3,6-trimethylpyrrolo[2,3-*d*]pyrimidine-2,4(1*H*,3*H*) dione **(172)** for the new product, either of which could be formed as shown in **Scheme 3.4**.



Scheme 3.4 Alternative reaction pathways for the reaction of **(169)** with **(171)**

Nucleophilic replacement of the iodine in **(171)** by the 6-amino group of the pyrimidine **(169)**, following route A in **Scheme 3.4**, would lead to the ketone **(165)**. Subsequent cyclisation would form 1,3,5-trimethylpyrrolo[2,3-d]pyrimidine-2,4(1*H*,3*H*)dione **(168)**. The alternative pathway following route B in **Scheme 3.4**, would give the regioisomeric product 1,3,6-trimethylpyrrolo[2,3-*d*]pyrimidine-

2,4(1*H*,3*H*)dione **(172)**, by initial electrophilic substitution of the iodoacetone **(171)** onto the 5-position of the pyrimidine **(169)**. Reactions of this type are well documented in the literature.[190,191] The intermediate **(173)** thus formed would readily cyclise to the bicyclic product **(172)**.

An authentic sample of 1,3,5-trimethylpyrrolo[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)dione **(168)** was prepared independently by a separate route *via* the oxidation of **(164)** as already described. The product obtained in the present reaction was shown to be the 5-regioisomer **(168)** by comparing it with the authentic sample of **(168)**. The ^{1}H and ^{13}C NMR spectra of the two samples were identical, and in addition, HPLC analysis of a mixture of authentic **(168)** and the new product showed one peak with a retention time of 5.23 minutes (**Figure 3.5**). UV analysis of this peak using the PDA detector of the HPLC showed that it was uniform across its width, providing evidence that it was homogenous, and did not arise by the co-elution of two isomeric compounds. This result confirmed that reaction of 6-amino-1,3-dimethyluracil **(169)** with iodoacetone **(171)** had occurred at the 6-amino group, following route A, leading to 1,3,5-trimethylpyrrolo[2,3-*d*]pyrimidine2,4-(1*H*,3*H*)dione **(168)** as the final product.

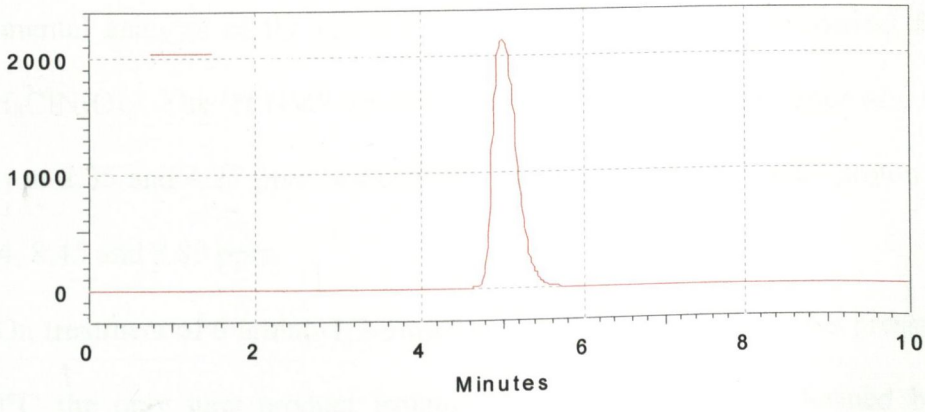
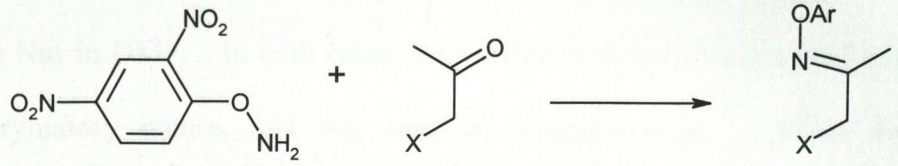
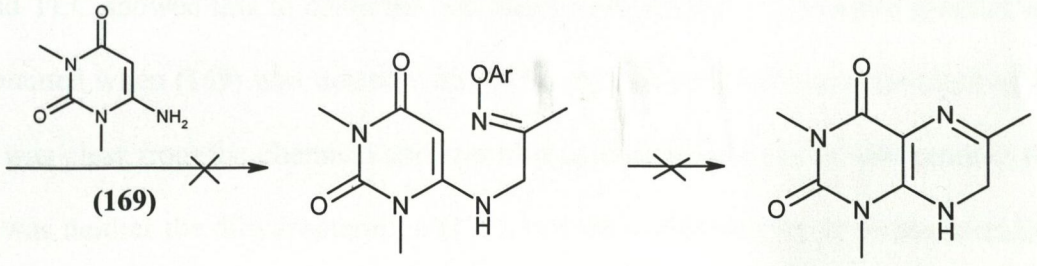


Figure 3.5 HPLC analysis of 1,3,5-trimethyl-1,7-dihydro-pyrrolo[2,3-*d*]pyrimidine

Following on from this result, it was expected that reaction of 6-amino-1,3-dimethyluracil **(169)** with either **(175)** or **(176)** in a pressure tube would form the dinitrophenyloxime **(177)**, which could cyclise *in situ* to give the dihydropteridine **(178)**.



(125) **(174)** X = Cl; **(171)** X = I **(175)** X = Cl; **(176)** X = I



(169) **(177)** **(178)**

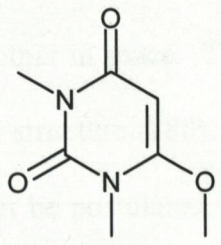
Ar = 2,4-dinitrophenyl

1-Chloropropan-2-one (2,4-dinitro-phenyl)-oxime **(175)** was prepared by reaction of chloroacetone **(174)** with O-(2,4-dinitrophenyl)-hydroxylamine **(125)** in TFA. Elemental analysis of the crystallised product confirmed an empirical formula of $C_9H_8ClN_3O_5$. The ^1H NMR spectrum of **(175)** showed the presence of a CH_2 and a CH_3 at 2.35 and 4.27 ppm respectively, as well as three aromatic proton signals at 7.94, 8.45 and 8.89 ppm.

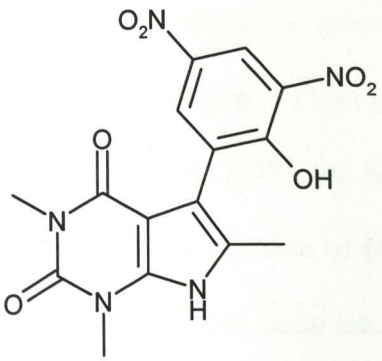
On treatment of 6-amino-1,3-dimethyluracil **(169)** with **(175)** in a pressure tube at 120°C the only new product isolated was 2,4-dinitrophenol, formed by thermal decomposition of **(175)**. Unreacted **(169)** was recovered on cooling of the reaction solution. The same result was obtained when the reaction was repeated using a

variety of different experimental conditions including the refluxing of both reactants, **(169)** and **(175)**, in various solvents, both with and without a catalytic amount of KI or NaI.

1-Iodopropan-2-one O-(2,4-dinitro-phenyl)-oxime **(176)** was obtained either by reaction of iodoacetone **(171)** with **(125)** in acidic media, or by treatment of **(175)** with NaI in DMF. In both cases, the product was not characterised because of its lachrymatory nature, and was used as a crude solid. When 6-amino-1,3-dimethyluracil **(169)** was reacted with **(176)** in a pressure tube at 120°C for twelve hours in methanol, a dark orange precipitate formed on cooling the reaction solution, and TLC showed this to comprise one major new product. The same product was obtained when **(169)** was treated with **(175)** in the presence of one equivalent of KI. It was clear from the chemical and spectroscopic properties of the new product that it was neither the dihydropteridine **(178)**, nor the corresponding aromatic pteridine. Neither was it the trivial product 1,3-dimethyl-6-methoxyuracil **(170)**, which could have been formed by reaction of **(169)** with the solvent methanol, as already described. The new product, in fact, turned out to have the completely unexpected structure shown in **(179)**.



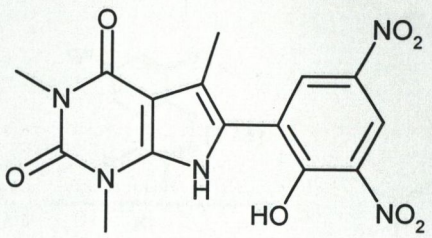
(170)



(179)

High resolution mass spectral analysis of the new product showed a single peak at m/z 376.0895 ($M+H^+$), indicating an empirical structure of $C_{15}H_{14}N_5O_7$. The ^1H NMR spectrum unexpectedly showed only two aromatic protons at 7.79 ppm and 8.65 ppm. The appearance of these as two doublet signals with coupling constants of $J = 3$ Hz, suggesting two *meta* aromatic protons. The presence of a low field exchangeable signal (D_2O) at 11.76 ppm was indicative of a phenolic OH, and this was supported by an OH stretching band at 3401 cm^{-1} in the IR spectrum. In agreement with the above the ^{13}C NMR spectrum showed only two aromatic CH signals at 123.41 ppm and 128.83 ppm. Together, these results suggested the presence of a 2-hydroxy-3,5-dinitrophenyl moiety in the new molecule. The ^1H NMR spectrum also showed three methyl groups at 2.05, 2.51, and 3.14 ppm. Significantly, there was no signal that could have been attributed to a pyrimidine 5-H. Other important features observed in the NMR spectra were the exchangeable NH signal and the nine quaternary carbon signals. All of these data are in agreement with the proposed structure **(179)**.

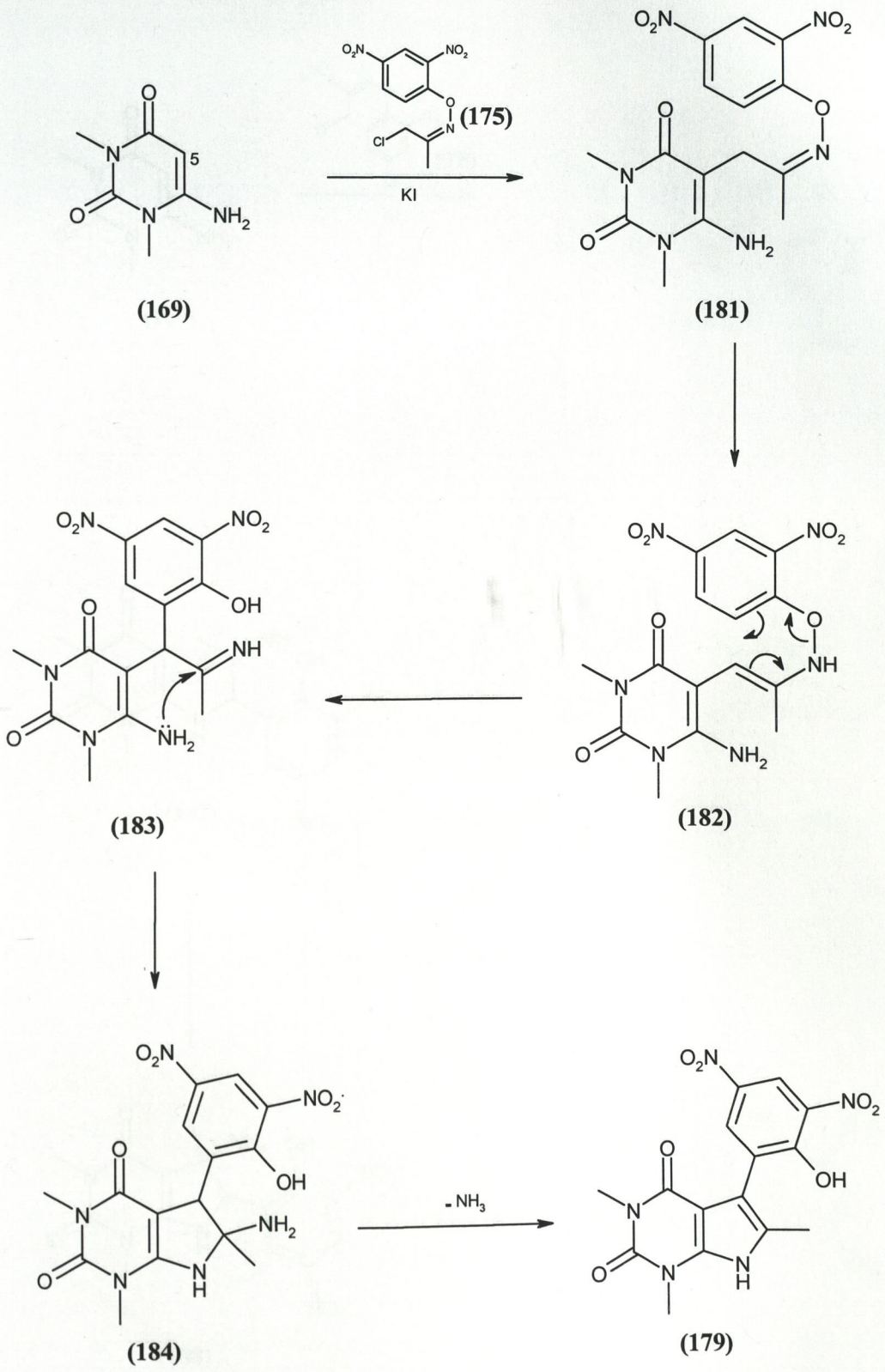
These data are also consistent with the regioisomeric structure **(180)**. This structure was ruled out, however, on the basis of NOE results, which showed a positive increase in intensity of the pyrrolo CH_3 when the NH signal at 7.46 ppm was irradiated. When the pyrrolo CH_3 was irradiated, the same response was observed for the amine group, thus showing that both groups are in close proximity to each other in space. This result is compatible with structure **(179)**, but not for the isomeric structure **(180)**. Various other mechanisms for the reaction of **(169)** with **(176)** can be postulated. However, the resultant products from these can be ruled out when spectroscopic data of the product obtained and reactivity of the starting pyrimidine **(169)** are considered.



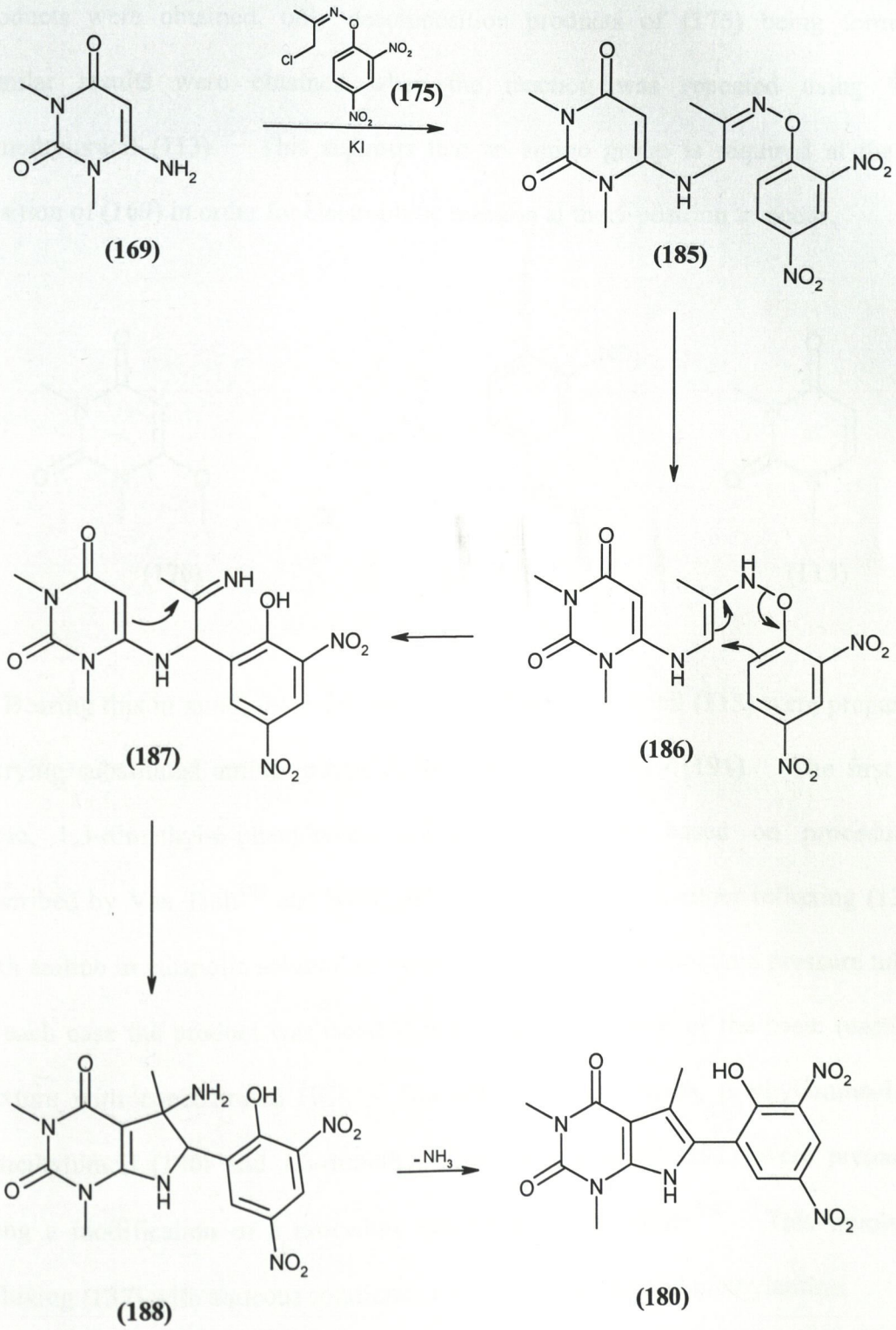
(180)

A possible mechanism for the formation of **(179)** is outlined in **Scheme 3.5**. The first step involves electrophilic substitution at the 5-position of the starting pyrimidine **(169)**, to give intermediate **(181)**. It is noteworthy that electrophilic attack takes place on the pyrimidine ring of **(169)** rather than at the 6-amino group. A double bond shift leads from **(181)** to **(182)**, and sets the system up for a Claisen type sigmatropic rearrangement in which **(182)** is converted into intermediate **(183)**. An intramolecular nucleophilic attack on the imine carbon centre in **(183)** by the nitrogen of the 6-amino group leads to ring closure, giving the fused ring compound **(184)**. Loss of ammonia from **(184)**, then gives the fully aromatic final product **(179)**.

Formation of the alternative isomer **(180)** would involve nucleophilic displacement of the halogen atom in **(175)** by the 6-amino group of the starting pyrimidine **(169)**, as shown in **Scheme 3.6**. After double bond migration leading from **(185)** to **(186)** the system is again set up for a sigmatropic rearrangement to give **(187)**. The main difference now, in contrast to the reaction shown in **Scheme 3.5**, is that the intramolecular cyclisation could occur by electrophilic substitution of the pyrimidine 5-position in **(187)** to give **(188)**. Loss of ammonia from **(188)** could then lead to structure **(180)**.

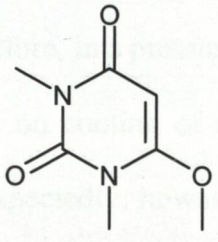


Scheme 3.5 Proposed mechanism for the formation of **(179)**

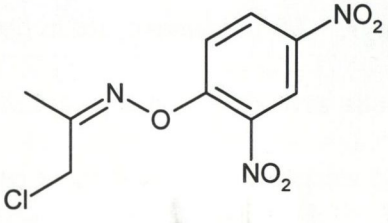


Scheme 3.6 Proposed mechanism for the formation of **(180)**

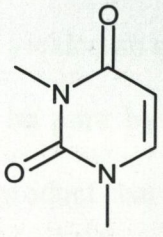
When 6-methoxy-1,3-dimethyluracil **(170)** instead of the 6-amino compound **(169)** was reacted with **(175)** under the same reaction conditions as before, no new products were obtained, only decomposition products of **(175)** being formed. Similar results were obtained when the reaction was repeated using 1,3-dimethyluracil **(113)**. This suggests that an amino group is required at the 6-position of **(169)** in order for electrophilic reaction at the 5-position to occur.



(170)

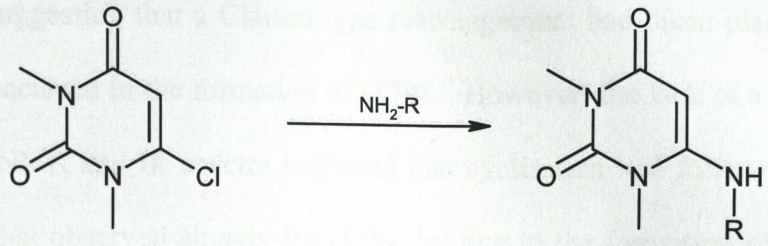


(175)



(113)

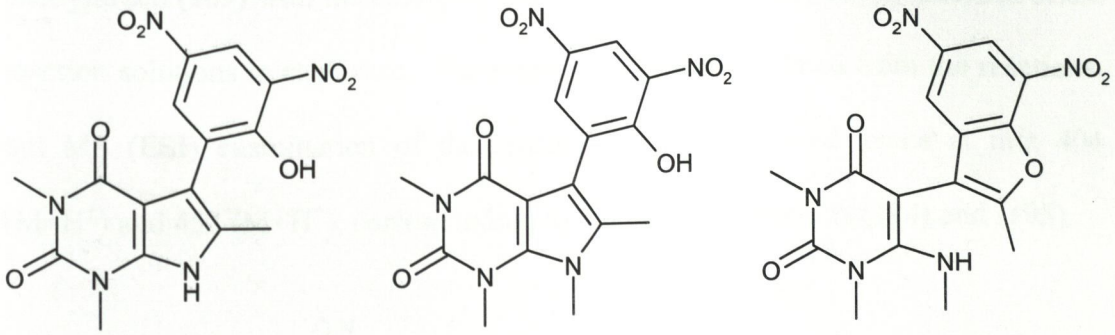
Bearing this in mind, three derivatives of 1,3-dimethyluracil **(113)** were prepared carrying substituted amino groups at the 6-position, **(189)** - **(191)**. The first of these, 1,3-dimethyl-6-phenyluracil **(189)**, was prepared based on procedures described by Van Tinh[192] and Nishigaki.[193] These involved either refluxing **(137)** with aniline in ethanolic solution or by heating **(137)** with aniline in a pressure tube. In each case the product was isolated following acidification of the basic reaction mixture with concentrated HCl. The other two derivatives, 6-ethylamino-1,3-dimethyluracil **(190)** and 1,3-dimethyl-6-methylaminouracil **(191)**, were prepared using a modification of a procedure described by Pfleiderer.[194] This involved refluxing **(137)** with aqueous solutions of either ethylamine and methylamine.



(137)

(189) R = C_6H_5
(190) R = C_2H_5
(191) R = CH_3

Reaction of **(169)** with 1,3-dimethyl-6-methylaminouracil **(191)** was carried out as before, in a pressure tube and in the presence of KI. Again this yielded an orange solid on cooling of the reaction solution. This was shown to be pure by TLC. Unexpectedly, however, it was clear from the properties of this product that it did not have structure **(192)**, which is the methyl derivative of the previously obtained product **(179)**, and which has already been discussed in detail. The structure of the new product, in fact, turned out to be that shown in **(193)**, which is a dinitro-benzo[*b*]furan.



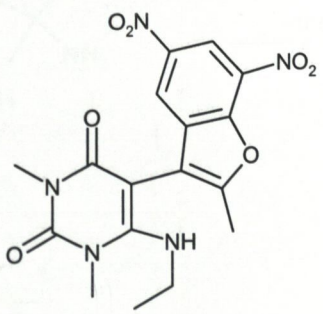
(179) **(192)** **(193)**

HRMS analysis of the product confirmed that it had an empirical formula of $C_{16}H_{15}N_5O_7$, by the presence of a peak at m/z 408.0976 ($M+H^+$). The ^1H NMR spectrum again showed the presence of only two aromatic proton signals at 8.16 and

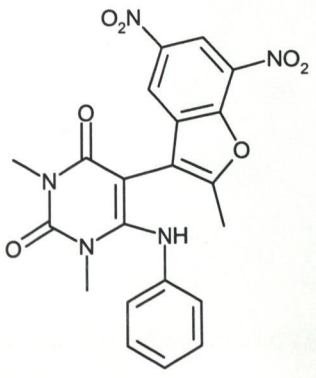
8.72 ppm, suggesting that a Claisen type rearrangement had taken place, similar to that which occurred in the formation of **(179)**. However, the lack of a phenolic OH in both the NMR and IR spectra indicated that cyclisation had followed a different route from that observed already for **(179)**, leading to the formation of a furan ring instead of a pyrrolo ring in the final product. This result was confirmed by the ^{13}C NMR spectrum of the product, which showed two aromatic CH signals. The ^{1}H spectrum also showed three methyl singlets, as well as one methyl doublet, the latter being assigned to the 6-N-Me group of **(193)**.

It is assumed that formation of **(193)** follows a pathway (**Scheme 3.7**) similar to that already described (**Scheme 3.5**) for the formation of **(179)**. The Claisen rearrangement intermediate **(196)**, however, now carries a substituted 6-amino group, and this appears to promote preferential intramolecular cyclisation on to the phenolic oxygen, leading to **(197)**. This with loss of ammonia gives the fully aromatic benzo[*b*]furan product **(193)**

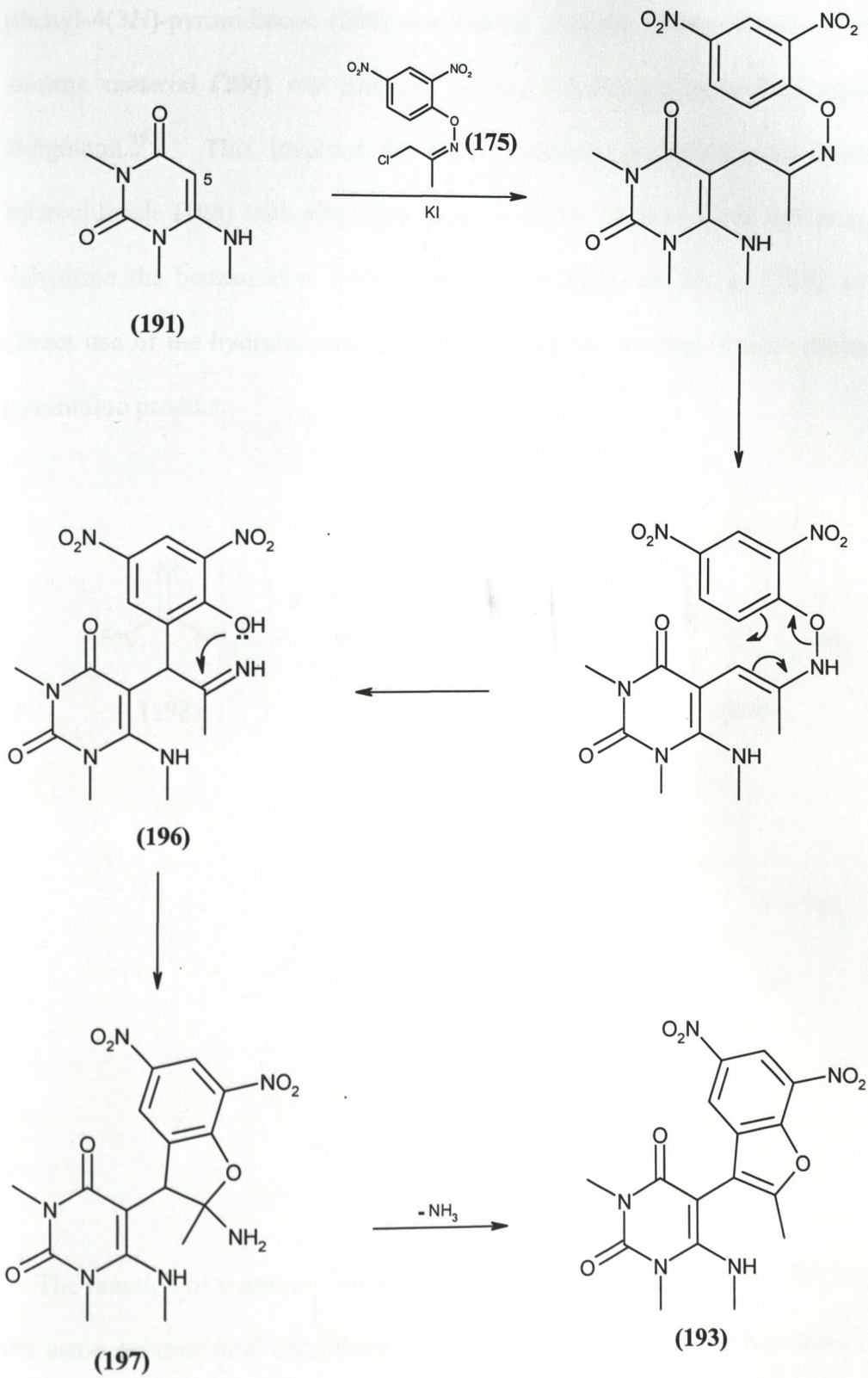
Reaction of both 6-ethylamino-1,3-dimethyluracil **(190)** and 1,3-dimethyl-6-phenyluracil **(189)** with the chloro oxime **(175)** in the presence of KI, afforded crude reaction solutions in each case. Pure products were not isolated from the reactions, but MS (ESI) examination of the reaction solutions showed peaks at m/z 404 ($M+H^+$) and 452 ($M+H^+$), corresponding to the expected products **(194)** and **(195)**.



(194)

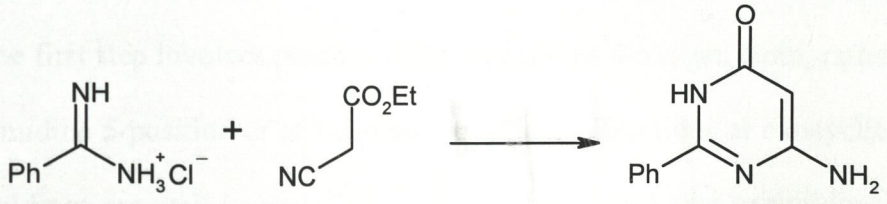


(195)

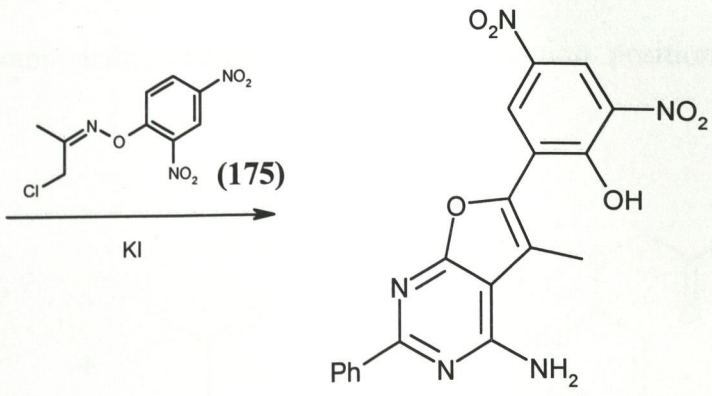


Scheme 3.7 Proposed mechanism for the formation of **(193)**

Yet another variation of this type of reaction was observed when 6-amino-2-phenyl-4(3*H*)-pyrimidinone **(200)** was reacted with the chloro oxime **(175)**. The starting material **(200)** was prepared readily following a procedure reported by Bergmann.[195] This involved the base catalysed condensation of benzamidine hydrochloride **(198)** with ethyl cyanoacetate **(199)**. It was found advantageous to dehydrate the benzamidine hydrochloride by heating the salt at 120°C *in vacuo*. Direct use of the hydrated salt as sold commercially resulted in poor yields of the pyrimidine product.



(198) **(199)** **(200)**

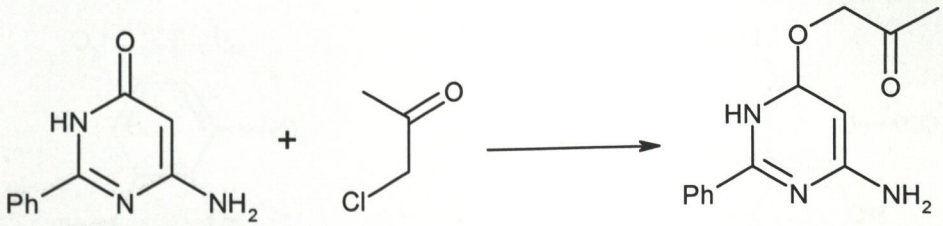


(201)

The reaction of 6-amino-2-phenyl-4(3*H*)-pyrimidione **(200)** with **(175)** was under the same experimental conditions as already discussed for the preparation of **(179)** and **(193)**, namely, in methanol solution in a pressure tube at 120°C with one equivalent of KI. The precipitate that formed on cooling the reaction solution was purified by column chromatography to give a product, which is believed to have

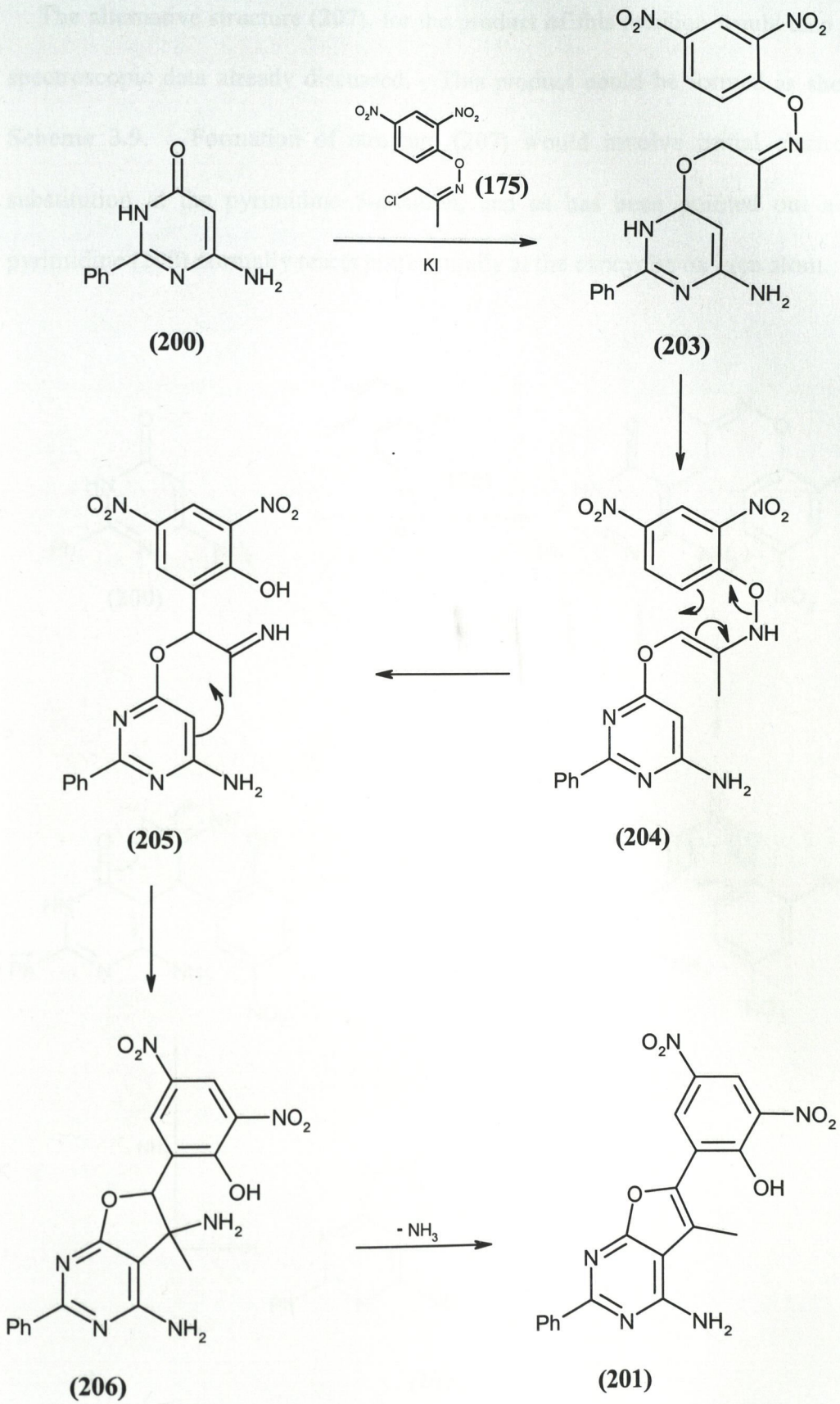
structure **(201)**. High resolution mass spectral analysis of the new product showed a single peak at m/z 408.0976 (MH^+), indicating an empirical formula of $C_{19}H_{13}N_5O_6$. The ^1H NMR spectrum of the product showed characteristic peaks at 8.14 and 8.65 ppm corresponding to pair of *meta* aromatic protons in **(201)**. Significantly, the 6-NH_2 was shown to be present and a low field peak at 12.06 ppm signified the presence of the OH group. Both the ^1H and ^{13}C spectra showed the presence of a phenyl group and a CH_3 group. Again, no signal that could have been attributed to a pyrimidine 5-CH group was observed in the spectra.

The suggested mechanism for the formation of **(201)** is shown in **Scheme 3.8**, in which the first step involves reaction at the pyrimidine 4-oxygen atom, rather than at the pyrimidine 5-position or at the 6-amino group. Reactions at exocyclic oxygen in pyrimidines are well known,[196,197] and Duff has shown that pyrimidine **(200)** in particular, preferentially reacts at oxygen. For example reaction of **(200)** with chloroacetone **(174)** gives product **(202)**.[156] To our knowledge, there are no examples of halo-compounds reacting at the 5-C or 6-amino position of this pyrimidine **(200)**.



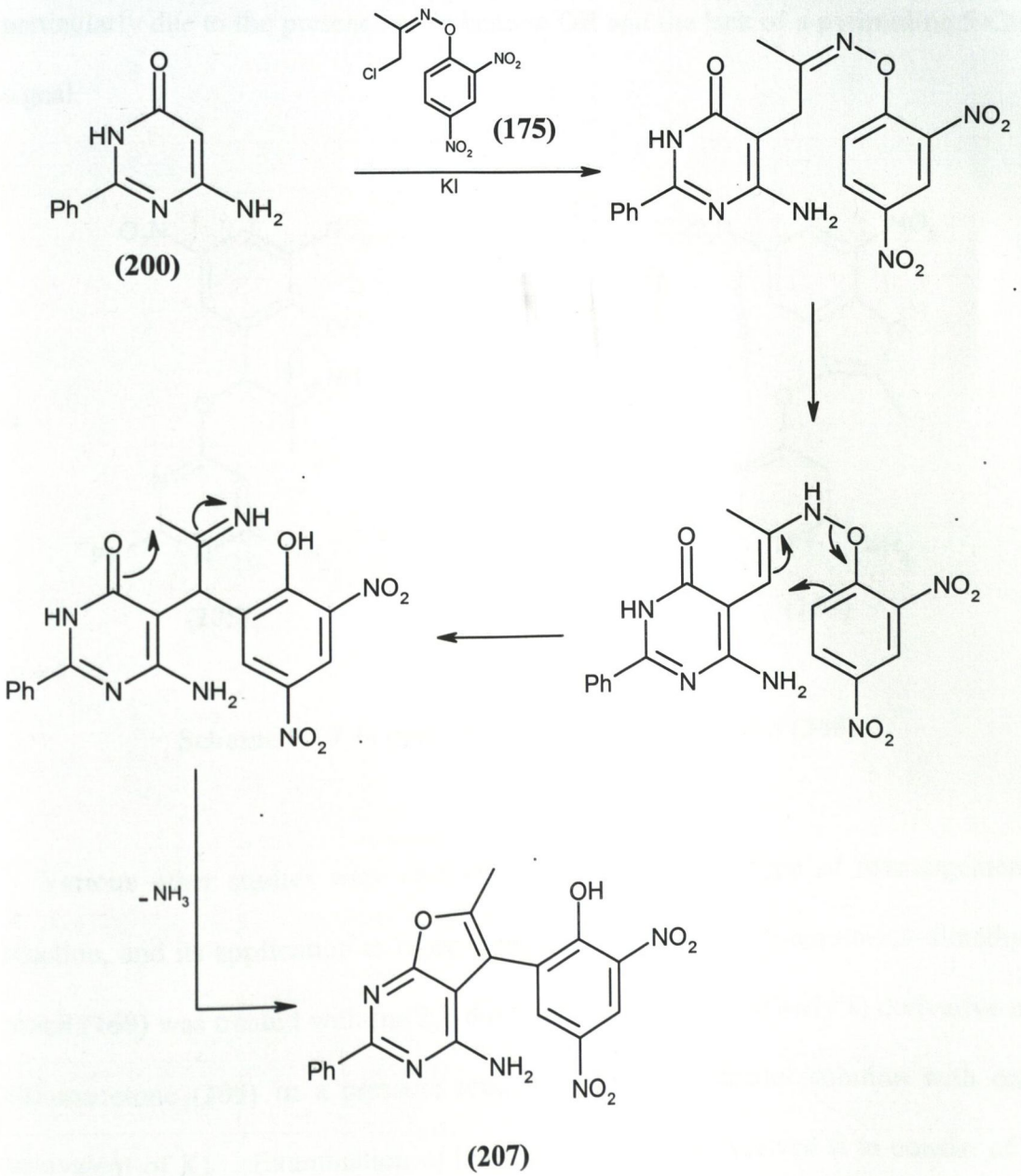
(200) **(174)** **(202)**

The reaction **Scheme 3.8** shows how the first formed intermediate **(203)** could be converted into the final product **(201)**, *via* a double bond shift **(204)**, Claisen type rearrangement **(205)**, cyclisation onto the nucleophilic pyrimidine 5-position **(206)**, and finally loss of ammonia.



Scheme 3.8 Proposed mechanism for the formation of **(201)**

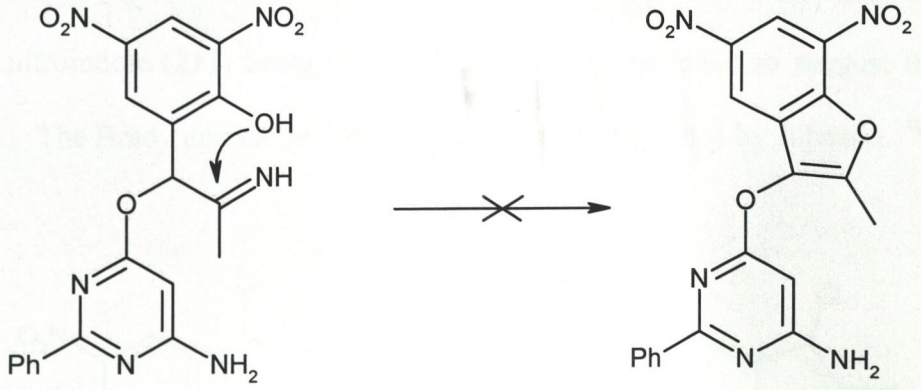
The alternative structure **(207)**, for the product of this reaction would also fit the spectroscopic data already discussed. This product could be formed as shown in **Scheme 3.9**. Formation of structure **(207)** would involve initial electrophilic substitution at the pyrimidine 5-position, and as has been pointed out already, pyrimidine **(200)** normally reacts preferentially at the exocyclic oxygen atom.



(207)

Scheme 3.9 Proposed mechanism for the alternative product **(207)**

Several other schemes are formally possible for the reaction of **(200)** with **(175)**. However, they all lead to structures that are incompatible with the spectroscopic data observed for the isolated product. For example, intermediate **(205)** in **Scheme 3.8** could have undergone intramolecular cyclisation involving the phenolic OH group to give the product **(208)**, as shown in **Scheme 3.10**. This structure, however, is incompatible with the observed spectroscopic data for the isolated product, particularly due to the presence of a phenolic OH and the lack of a pyrimidine 5-CH signal.

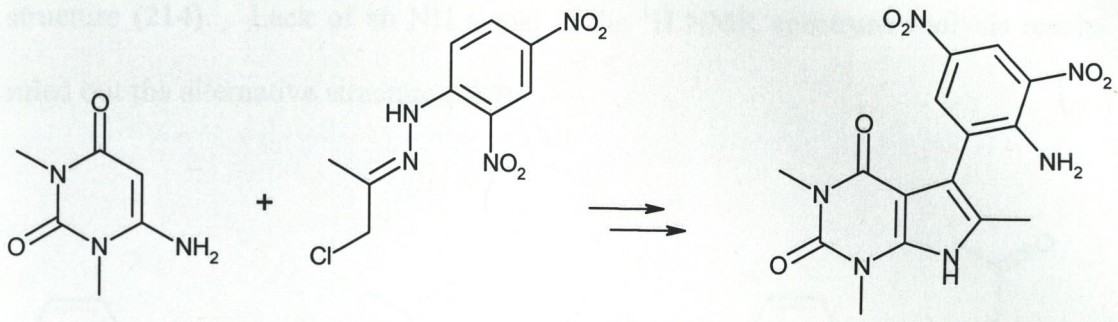


(205) **(208)**

Scheme 3.10 Formation of the alternative product **(208)**

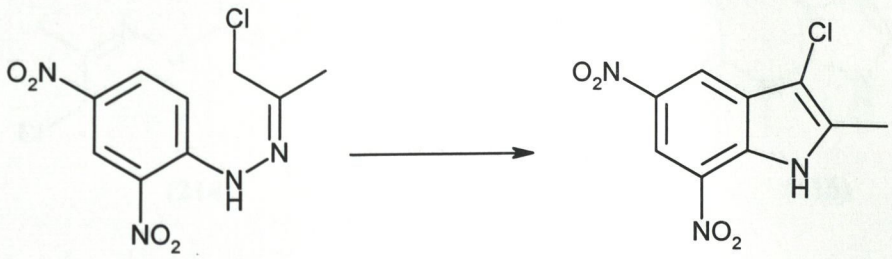
Various other studies were carried out to examine this type of rearrangement reaction, and its application to other systems. For example, 6-amino-1,3-dimethyl uracil **(169)** was treated with the 2,4-dinitrophenylhydrazone (Brady's) derivative of chloroacetone **(209)** in a pressure tube at 120°C in methanol solution with one equivalent of KI. Examination of the product, however, showed it to consist of a complex mixture, which included significant amounts of the starting material **(169)**.

MS (ESI) examination of the mixture showed no trace of any species corresponding to product of type **(210)**.



(169) **(209)** **(210)**

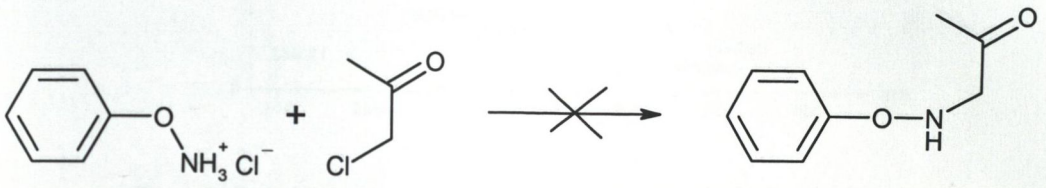
While it is possible that a Fischer indole reaction could have led to small amounts of a dinitroindole **(211)** being formed, no evidence was found to suggest that this was so. The Brady derivative **(209)** was prepared as described by Johnson.[198]



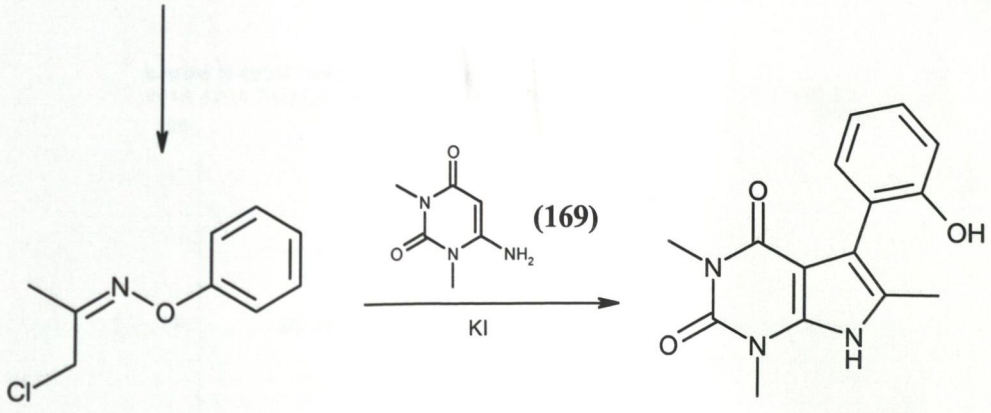
(209) **(211)**

We also set out to investigate the role of the nitro groups in the reactions that have already been discussed. Reaction of phenyl-hydroxylamine hydrochloride **(212)** with chloroacetone **(174)** afforded a product, which after purification by column chromatography, was isolated as a clear oil. HRMS analysis showed that this product had an empirical formula of $C_9H_{10}ClNO$. Its ^1H NMR spectrum showed signals corresponding to a phenyl group, as well as CH_3 and CH_2 groups at

2.22 and 4.25 ppm respectively. An IR spectrum showed a stretching band at 1590 cm^{-1} as would be expected for an oxime. These data showed the product to have structure **(214)**. Lack of an NH signal in the ^1H NMR spectrum analysis results ruled out the alternative structure **(213)**.



(212) **(174)** **(213)**



(214) **(215)**

Reaction of **(214)** with 2-amino-1,3-dimethyluracil **(169)** in a pressure tube in the presence of KI yielded a crude reaction mixture. All attempts to isolate a pure product from this reaction mixture were unsuccessful. However, analysis of the mixture by MS (ESI) showed peaks at m/z 284 (negative mode; **Figure 3.6)** and at m/z 286 (positive mode; **Figure 3.7**) and these peaks do correspond to a product of structure **(215)**. Although further work needs to be done on this aspect of these reactions, this result suggests that the aromatic nitro groups are not necessary in reactions of this type.

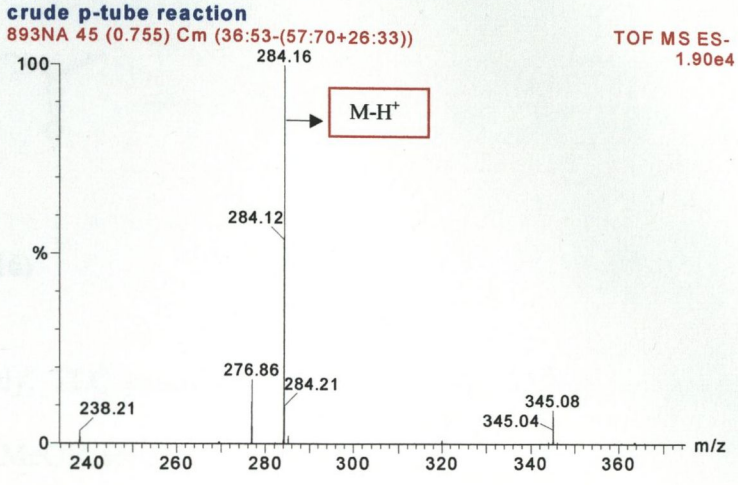


Figure 3.6 Negative mass spectrum (ESI) of **(214)** and **(169)** crude reaction mixture.

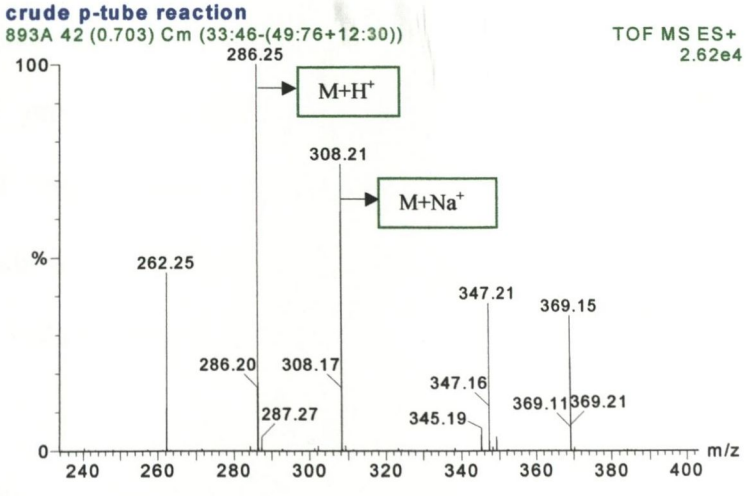
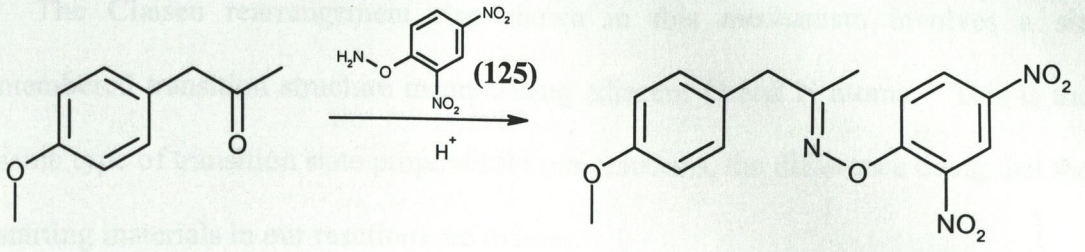


Figure 3.7 Positive mass spectrum (ESI) of **(214)** and **(169)** crude reaction mixture.

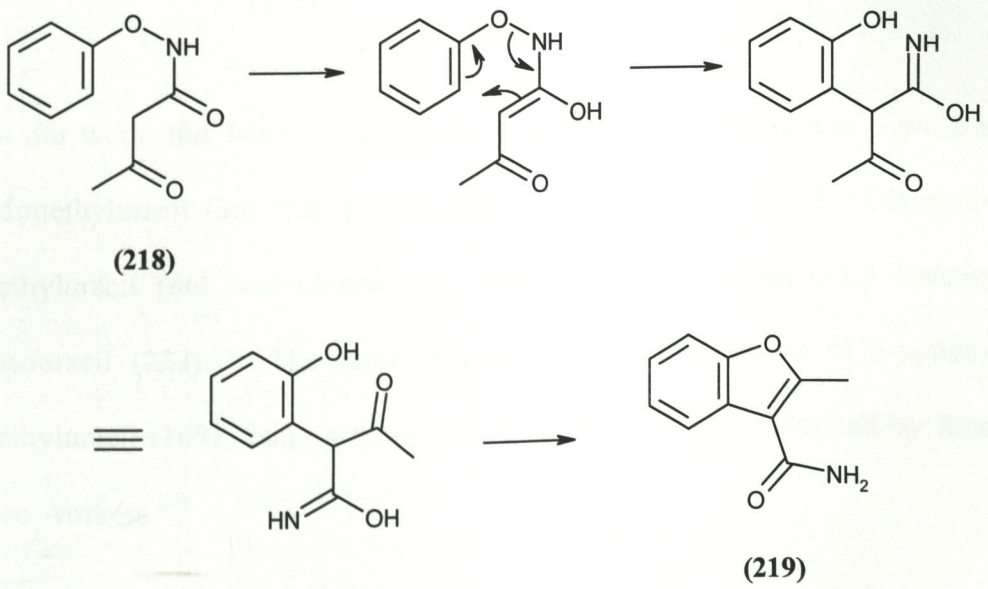
Finally, compound **(217)** was prepared in order to see whether reactions of the type already described would proceed with a benzene ring instead of a pyrimidine ring. Preparation of **(217)** was carried out by reaction of **(216)** with **(125)** in acidic media. The oxime **(217)** was isolated as a crystalline solid following recrystallisation from alcohol, and its structure was evident from its spectroscopic properties and from micro-analytical data.



(216) **(217)**

Unfortunately, TLC examination of the reaction mixture obtained when **(217)** was heated in MeOH solution in a pressure tube at 120°C revealed the presence of mainly starting material **(217)**, together with some 2,4-dinitrophenol **(132)**. When the reaction was repeated in the presence of acid the same result was observed.

To our knowledge, sigmatropic rearrangement reactions of 2,4-dinitrophenyl oximes as described here have not been reported previously. Many years ago Sheradsky[199] did report a Fischer type reaction of O-phenyloximes to give benzofurans, and more recently Shudo and co-workers[200] found that aryl hydroxamates such as **(218)** under strongly acidic conditions rearranged to a benzofuran **(219)**, following the mechanism suggested in **Scheme 3.11**.



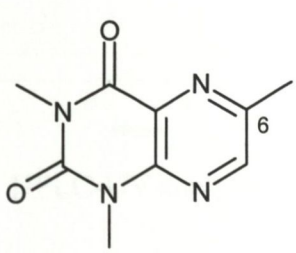
(218) **(219)**

Scheme 3.11 Benzofuran formation from an aryl hydroxamate

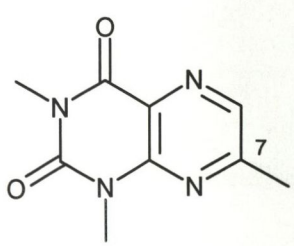
The Claisen rearrangement step shown in this mechanism involves a six membered transition structure incorporating adjacent O and N atoms. This is the same type of transition state proposed for our reactions, the difference being that the starting materials in our reactions are oximes.

3.4 Preparation of authentic samples of (220) and (221)

In the course of the work already described, authentic samples of 1,3,6-trimethyl-(1*H*, 3*H*)-pteridine-2,4-dione **(220)** and 1,3,7-trimethyl-(1*H*, 3*H*)-pteridine-2,4-dione **(221)** were required for comparison purposes. These compounds have been synthesised before, the 7-isomer **(221)** being prepared by Pfleiderer[201] and co-workers, and the 6-isomer **(220)** by Tada and co-workers.[202]

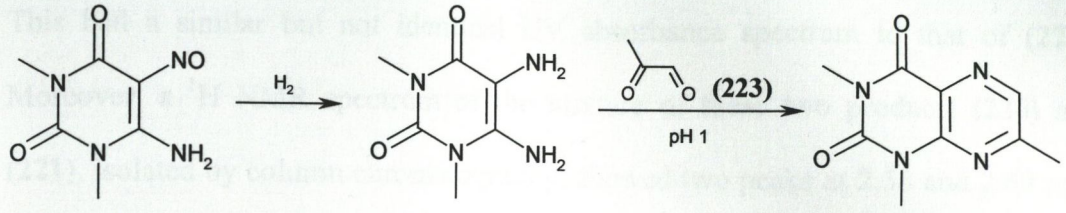


(220)



(221)

In our work, the 7-isomer **(221)** was prepared by condensation of 5,6-diamino-1,3-dimethyluracil **(66)** with pyruvic aldehyde **(223)** at pH 1. 5,6-Diamino-1,3-dimethyluracil **(66)** was obtained by hydrogenation of 6-amino-1,3-dimethyl-5-nitrosouracil **(222)**. The latter was prepared by nitrosation of 6-amino-1,3-dimethyluracil **(169)**, using a modification of the procedure described by Sirsakar and co-workers.[203]



(222) (66) (221)

Comparison of the spectroscopic data obtained for the product **(221)** with those reported in the literature for both **(221)**[201] and **(220)**[202] confirmed that the 7-methyl isomer **(221)** had been obtained. HPLC analysis of this product yielded the chromatogram (R_t 8.25 min.) and corresponding UV spectrum shown in **Figure 3.8**.

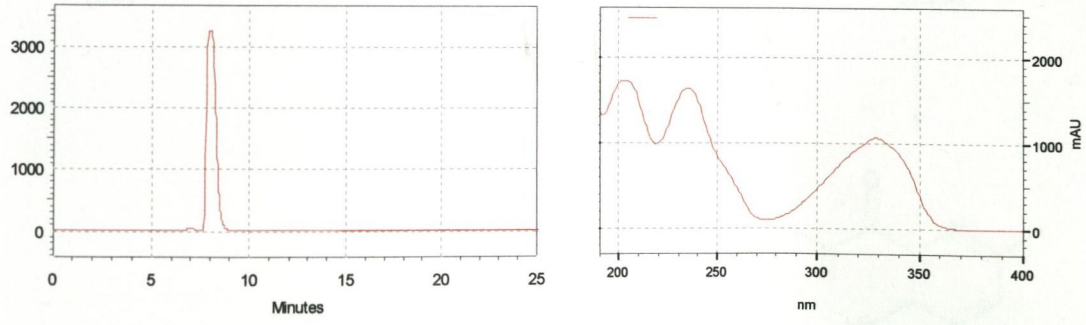
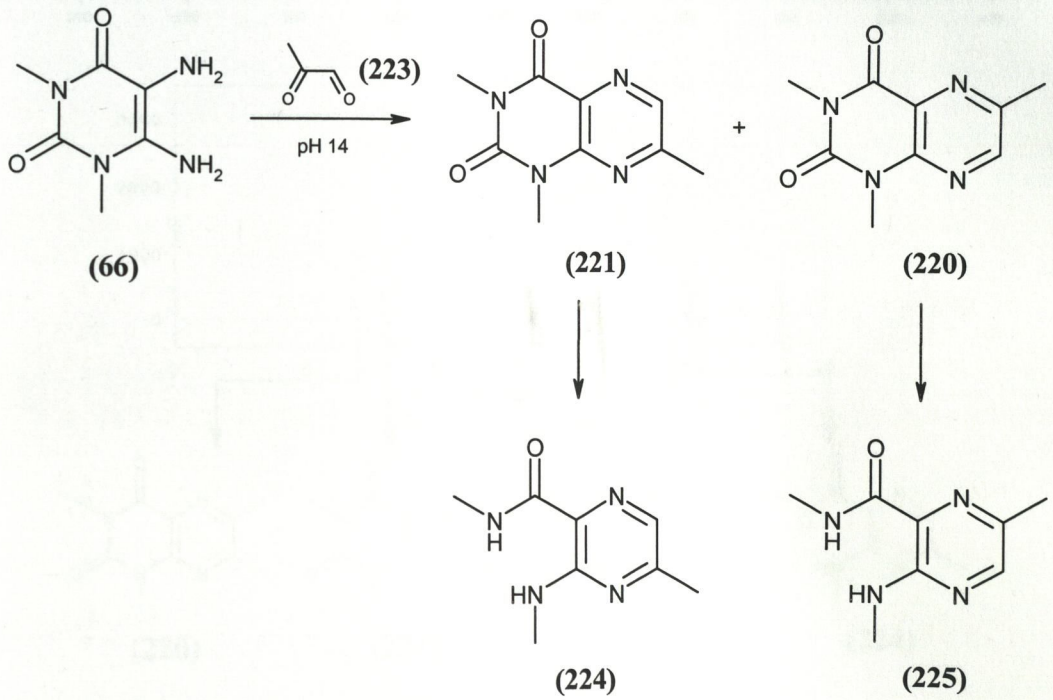


Figure 3.8 HPLC-UV analysis of 1,3,7-trimethyl-1*H*,3*H*)-pteridine-2,4-dione

The reaction of **(66)** with **(223)** was repeated with the pH of the solvent at pH 14, in an effort to obtain the regio isomeric product **(220)**. HPLC analysis of the reaction mixture (**Figure 3.9**) showed it to contain four major components, with retention times (R_t) of 7.09, 8.25, 15.80 and 19.25 minutes, on a C-18 column, eluting with a mobile phase of 85% water : 15% methanol. The peak eluting at 8.28 min. was taken to be 1,3,7-trimethyl-1*H*, 3*H*)-pteridine-2,4-dione **(221)**, since it co-eluted with an authentic sample of **(221)**, prepared as described as above and since it had an identical UV absorption spectrum. The peak eluting at 7.09 min. was taken to be the isomeric compound, 1,3,6-trimethyl-(1*H*,3*H*)-pteridine-2,4-dione **(220)**.

This had a similar but not identical UV absorbance spectrum to that of **(221)**. Moreover, a ^1H NMR spectrum of the mixture of these two products **(220)** and **(221)**, isolated by column chromatography, showed two peaks at 2.58 and 2.69 ppm which compared to the known chemical shifts of the 6- and 7-methyl groups, in compounds **(220)** and **(221)**, respectively.[202, 201]



(66) **(221)** **(220)**

(224) **(225)**

Column chromatography of the reaction mixture also yielded two pure products, corresponding to the HPLC peaks at 15.80 and 19.25 minutes (**Figure 3.9**). The two products were shown to have structure **(224)** and **(225)**, formed by base hydrolysis of the pteridine products **(221)** and **(220)** respectively. HRMS and elemental analysis confirmed that both **(224)** and **(225)** had an empirical structure of $C_8H_{12}N_4O$. The NMR spectra of both products were very similar, each containing signals due to three methyl groups, one low field aromatic type proton, and two NH groups. The UV absorption spectra of the two products were also very similar (**Figure 3.9**). The product eluting at 19.25 min. was shown to be **(224)** by

comparing it with an authentic sample of the material, prepared by hydrolysis of **(221)** with aqueous sodium hydroxide.

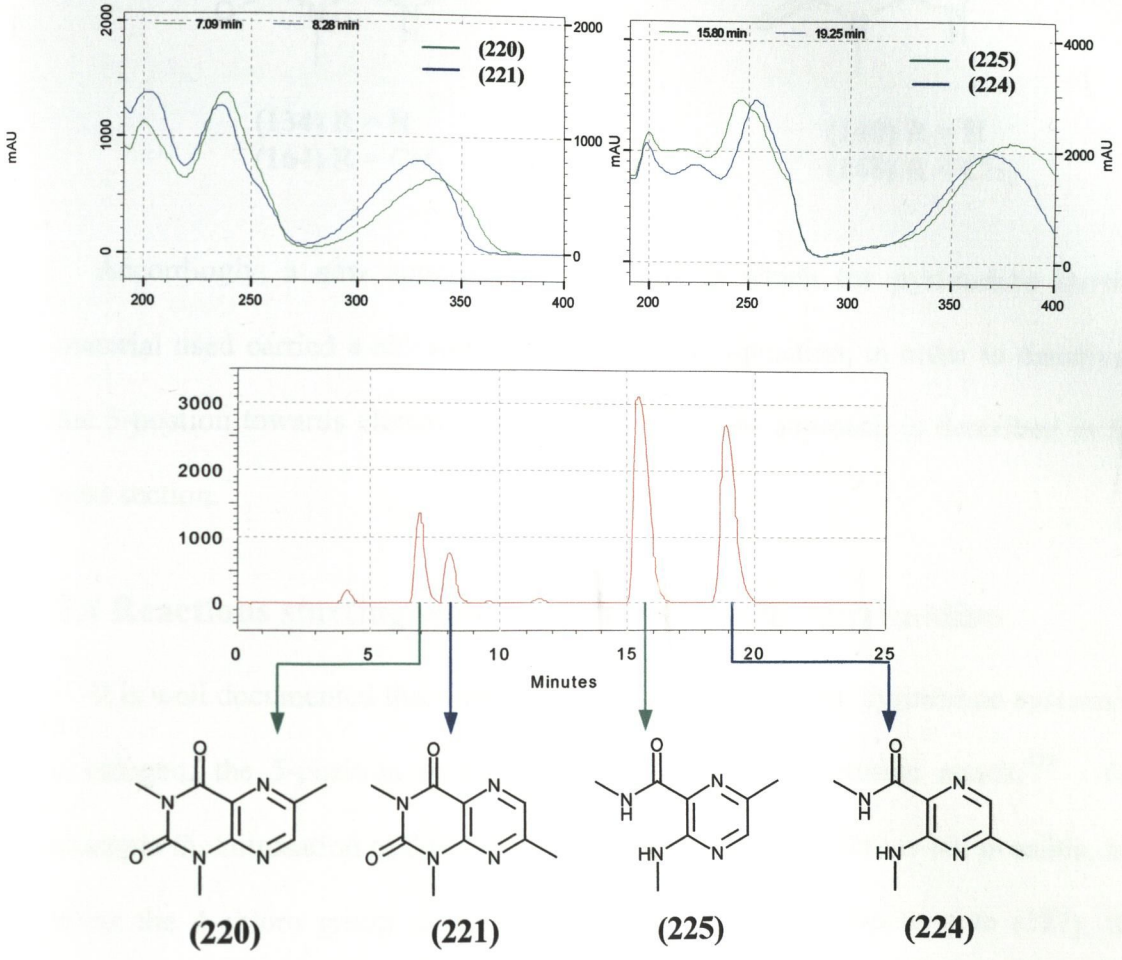
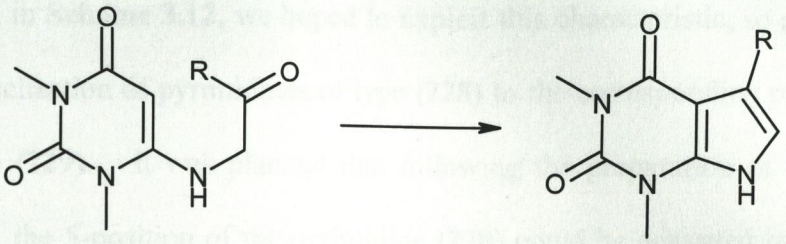


Figure 3.9 HPLC-UV of **(220)** and **(221)** and their respective hydrolysis products **(225)** and **(224)**

The reactions of 1,3-dimethyluracil derivatives discussed in the foregoing sections allowed us to develop some interesting aspects of pyrimidine chemistry. Unfortunately, however, the major difficulties encountered in the work was the extreme ease with which the oxime precursors **(134)** and **(164)** underwent intramolecular cyclisation to give the corresponding pyrrolo[2,3-*d*]pyrimidines **(140)** and **(168)**. This ready cyclisation depended upon the nucleophilicity of pyrimidines **(134)** and **(164)** at postion-5.



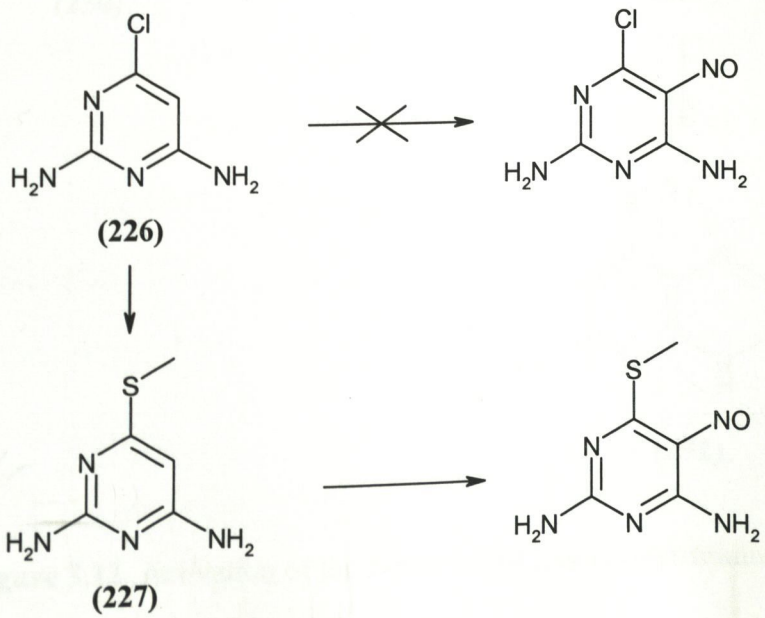
(134) R = H
(164) R = CH_3

(140) R = H
(168) R = CH_3

Accordingly, a new approach was adopted in which the pyrimidine starting material used carried a chlorine substituent at the 4-position, in order to deactivate the 5-postion towards electrophilic attack. This new approach is described in the next section.

3.4 Reactions starting with 2-amino-4,6-dichloropyrimidine

It is well documented that when the 4-substituent of certain pyrimidine systems is a halogen, the 5-position is not as active towards electrophilic attack.[179] For example the nitrosation of 2,6-diamino-4-chloropyrimidine **(226)** is not possible, but when the 4-chloro group is replaced with a methylthio group to give **(227)**, the reaction proceeds with ease.[180]



(226)

(227)

As shown in **Scheme 3.12**, we hoped to exploit this characteristic, so as to avoid premature cyclisation of pyrimidines of type **(228)** to the corresponding pyrrolo[2,3-*d*]pyrimidine **(229)**. It was planned that following the preparation of the dinitro oxime **(230)**, the 5-position of the pyrimidine **(230)** could be activated by replacing the 4-chlorine with an electron donating group, e.g. a methylthio group. Following loss of 2,4-dinitrophenoxide to give **(231)**, electrophilic cyclisation could yield the pteridine **(232)**.

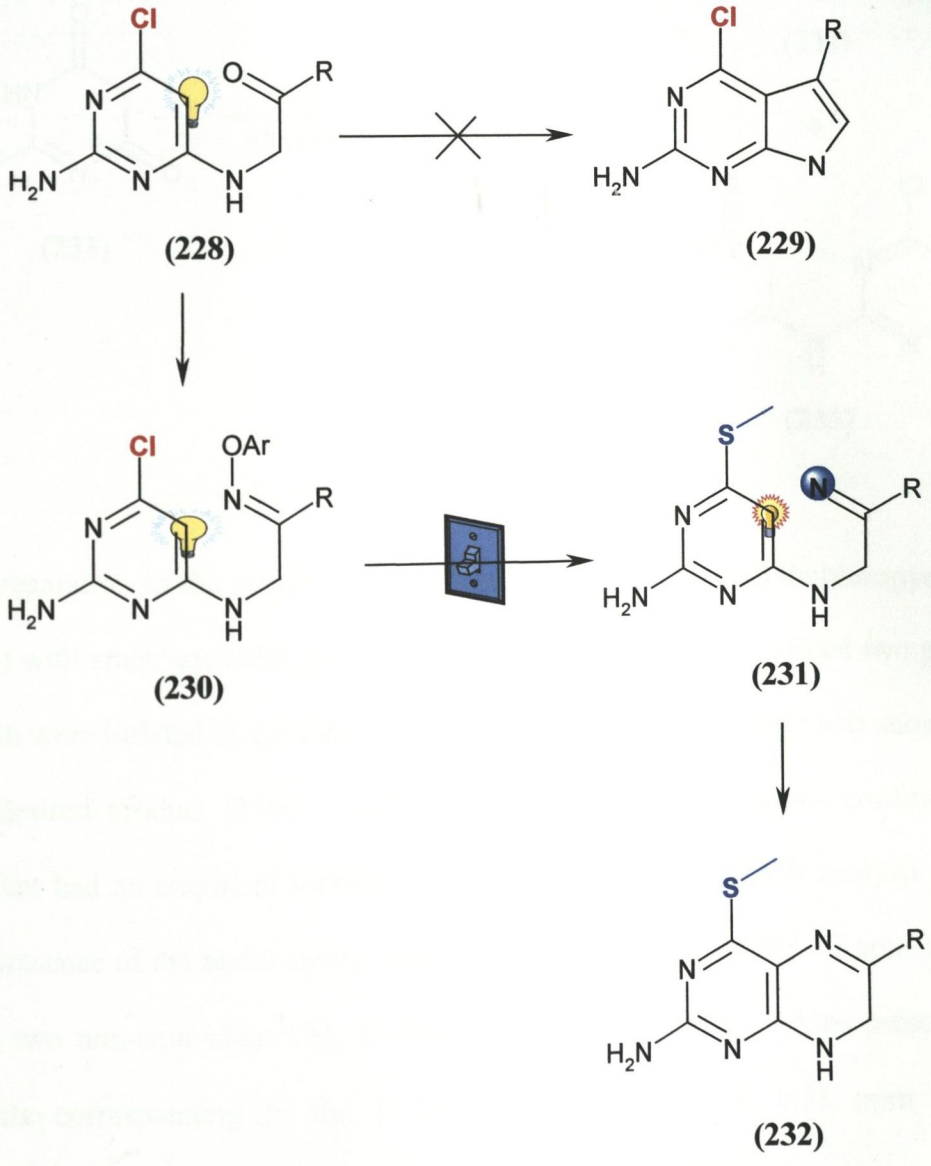
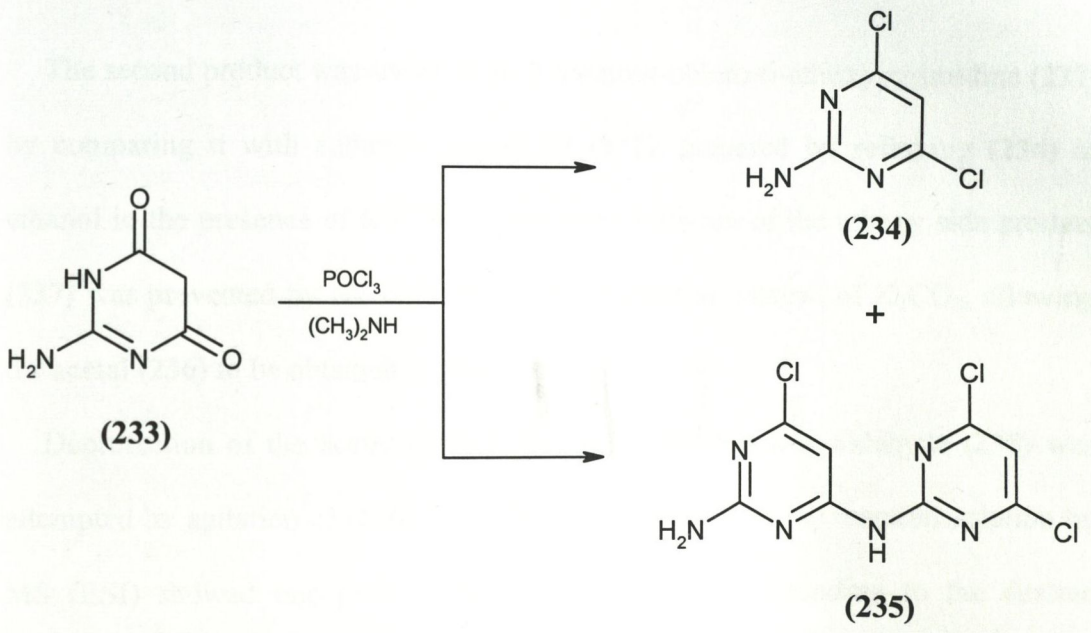
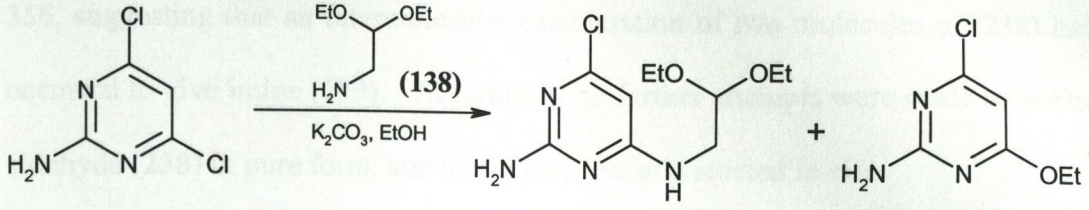


Figure 3.12 Activation of the 5-position of 2-aminopyrimidines

2-Amino-4,6-dichloropyrimidine **(234)** may be prepared by the chlorination of 2-amino-(1*H*,5*H*)-4,6-pyrimidinedione **(233)** with $POCl_3$ in the presence of dimethylamine.[204] This procedure, however, consistently yields a second product, now known to be the dimer compound **(235)**.[205]



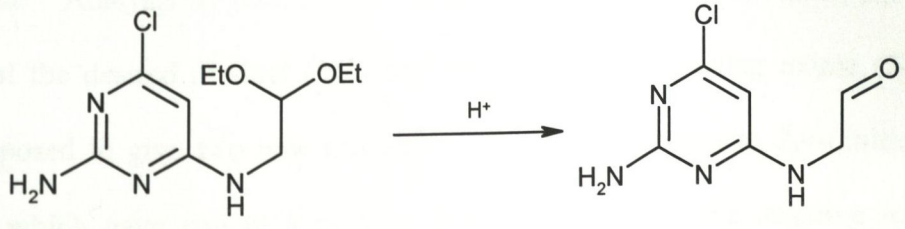
Preparation of the acetal **(236)**, by treatment of 2-amino-4,6-dichloropyrimidine **(234)** with aminoacetaldehyde **(138)** in the presence of K_2CO_3 yielded two products which were isolated by column chromatography. The first of these was shown to be the desired product **(236)**. Both HRMS and elemental analysis confirmed the product had an empirical formula of $C_{10}H_{11}ClN_4O_2$ and ^1H NMR analysis showed the presence of the acetal methyl groups as a six-proton triplet at 1.12 ppm, together with two non-equivalent CH_2 groups at 3.48 and 3.58 ppm. Also present were signals corresponding to the 5-CH of the pyrimidine at 6.38 ppm and an exchangeable (D_2O) signal at 6.34 ppm for the 2-amino group.



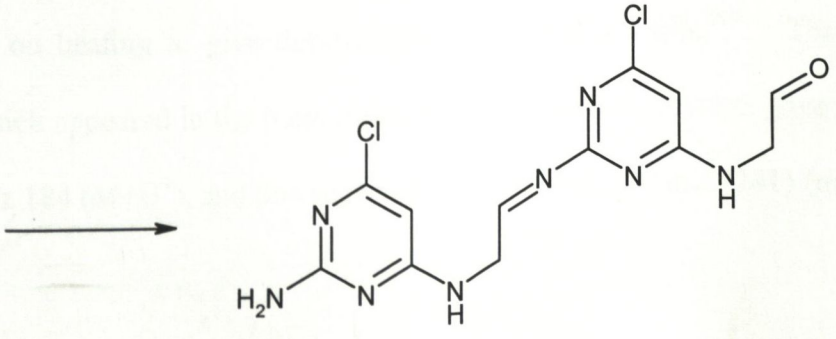
(234) **(236)** **(237)**

The second product was shown to be 2-amino-4-chloro-6-ethoxypyrimidine **(237)** by comparing it with authentic sample of **(237)**, prepared by refluxing **(234)** in ethanol in the presence of K_2CO_3. Later, the formation of the ethoxy side product **(237)** was prevented by use of $NaHCO_3$ in the reaction instead of K_2CO_3, allowing the acetal **(236)** to be obtained in good yield.

Deprotection of the acetal **(236)** to give the corresponding aldehyde **(238)** was attempted by agitation of **(236)** in 1M H_2SO_4. Analysis of the reaction solution by MS (ESI) showed one peak at m/z 187 ($M+H^+$), corresponding to the desired aldehyde **(238)**. All attempts to isolate it, however, led to decomposition.



(236) **(238)**

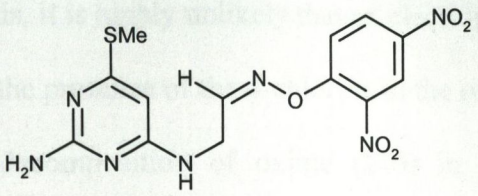


(239)

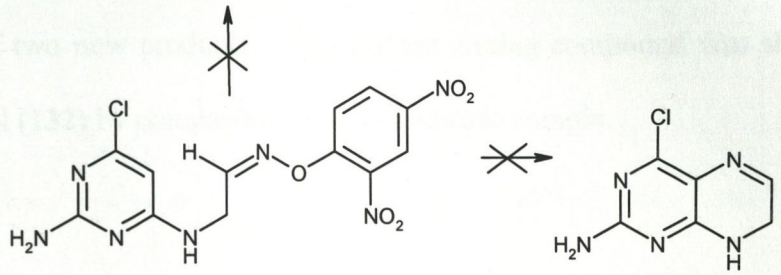
An MS (ESI) spectrum of the decomposed aldehyde showed a new peak at m/z 356, suggesting that an intermolecular condensation of two molecules of **(238)** had occurred to give imine **(239)**. Accordingly no further attempts were made to isolate aldehyde **(238)** in pure form, and it was prepared and reacted *in situ*.

(2-amino-4-chloropyrimidin-4-ylamino)acetaldehyde O-(2,4-dinitrophenyl) oxime **(240)** was prepared by treatment of an acidic solution of the aldehyde **(238)**, (formed *in situ*. by deprotection of **(236)** in aqueous sulphuric acid) with O-(2,4-dinitrophenyl)-hydroxylamine **(125)**. It was obtained as a cream coloured precipitate, which was isolated by filtration. A high resolution mass spectrum of this product **(240)** confirmed an empirical formula of $C_{12}H_{11}ClN_7O_5$, by the presence of a peak at m/z 368.0527 ($M+H^+$) (calculated m/z 368.0510).

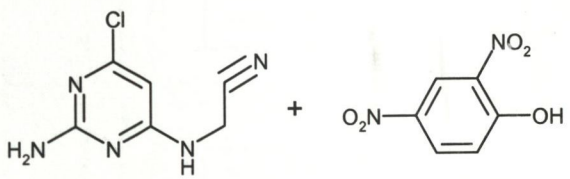
Unfortunately, the dinitrophenyl oxime **(240)** proved to be unstable in solution, and it was not possible to convert it to its methylthio derivative **(242)**. When **(240)** was warmed in water with sodium thiomethoxide the solution immediately changed in colour to dark yellow. It was not possible to isolate pure material from the solution. Analysis by both positive and negative MS (ESI), however, showed no trace of the desired product **(242)** and revealed that the starting oxime **(240)** had decomposed to give two new products. The first of these was 2,4-dinitrophenol **(132)**, which gave rise to a peak at m/z 183 ($M-H^+$) in the negative ion mass spectrum. It is well known that dinitrophenyl oximes of aromatic aldehydes may decompose on heating to give dinitrophenol **(132)** and a nitrile.[206] The second product, which appeared in the mass spectrum of the reaction mixture, gave rise to a signal at m/z 184 ($M+H^+$), and this suggested the presence of nitrile **(241)** (mw 183).



(242)



(240) (243)



(241) (132)

Analysis of the oxime **(240)** by NMR in d_6-DMSO yielded spectra, which showed that decomposition of **(240)** was occurring on dissolution in the solvent.

The ^{1}H NMR spectrum of the mixture contained three aromatic proton signals at 7.31, 8.36 and 8.69, identical in chemical shift with those observed for authentic 2,4-dinitrophenol **(132)**. The remaining peaks in the ^{1}H NMR spectrum, two singlets at 4.35 and 6.31 ppm corresponded to a CH_2 and CH respectively. These peaks again suggested formation of the nitrile **(241)**. The NMR and mass spectrum data are also compatible with structure **(243)**, which is a 7,8-dihydropteridine, and which could possibly have formed by ring closure of electrophilic nitrogen onto the pyrimidine 5-position. This is considered unlikely, however, because it is well known that dihydropteridines are extremely susceptible to oxidation by air, and it is unlikely that a product such as **(243)** could have survived under the reaction conditions used.

As well as this, it is highly unlikely that an electrophilic cyclisation could have taken place due to the presence of the 4-chlorine in the oxime **(240)**.

Thermal decomposition of oxime **(240)** in methanol solution at 37°C was followed by HPLC analysis as shown in **Figure 3.10**, which shows clearly the formation of two new products. The earliest eluting compound was shown to be dinitrophenol **(132)** by comparison with an authentic sample.

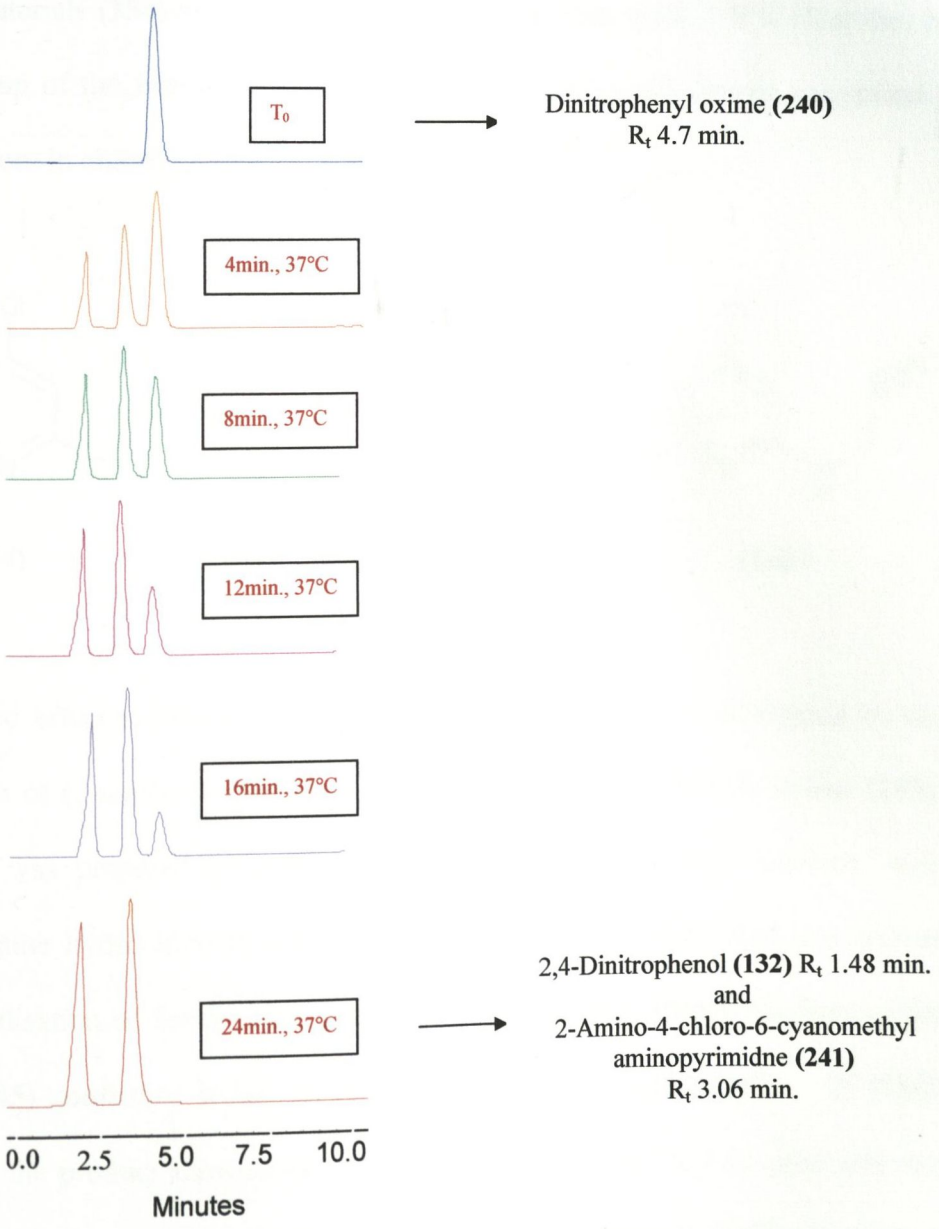
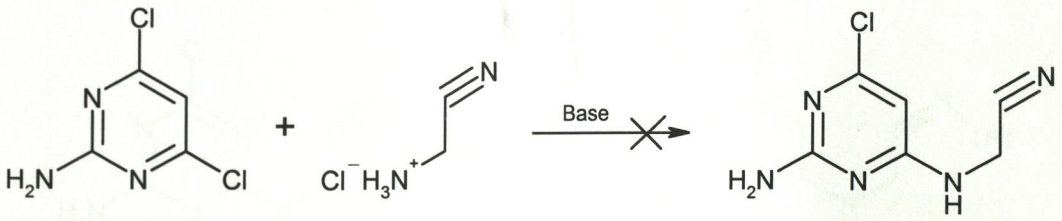


Figure 3.10 Decomposition of dinitrophenyl oxime **(240)** at 37°C, monitored by HPLC *(C-18 column; 85%MeOH: 15% H_2O)*

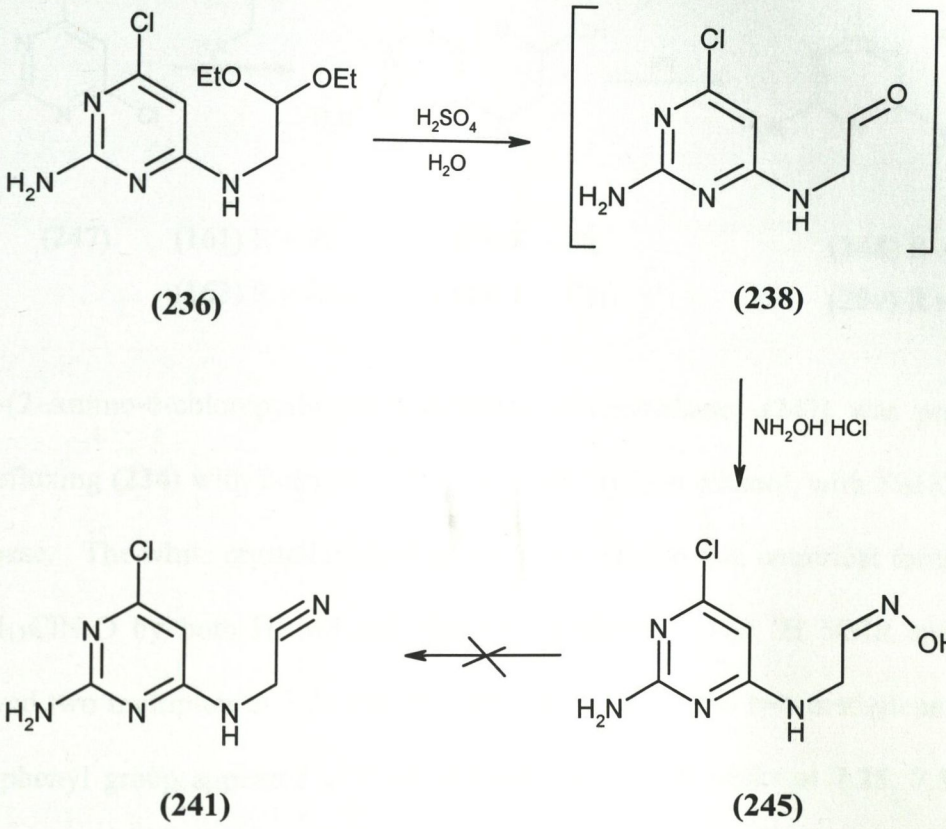
Some work was carried out to try and synthesise an authentic sample of **(241)** for comparative purposes. Reaction of 2-amino-4,6-dichloropyrimidine **(234)** with **(244)** proved unsuccessful, however, despite using many sets of reaction conditions. These included a variety of different reaction solvents and bases ($NaHCO_3$, KOH), as well as carrying out the reactions in a pressure tube elevated temperatures and pressure. In no case was there any evidence of reaction, with predominantly starting materials **(234)** and **(244)** being recovered in high yield. It is clear that he amino group of the aminonitrile **(244)** is not sufficiently nucleophilic to replace a chlorine atom in chloropyrimidine **(234)**.



(234) **(244)** **(241)**

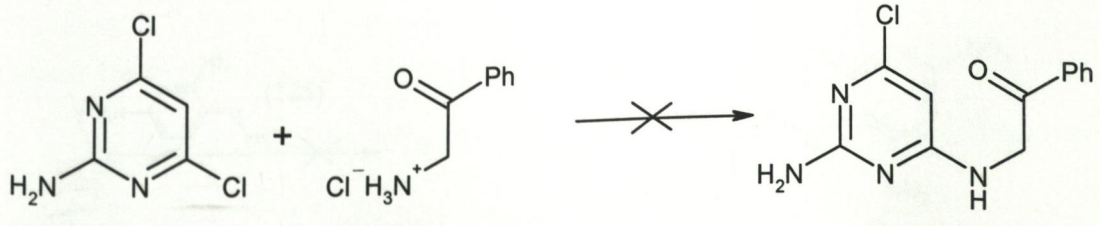
A second effort to prepare an authentic sample of **(241)** was attempted by the dehydration of (2-amino-4-chloro-pyrimidin-6-ylamino)-acetaldehyde oxime **(245)**. The latter was prepared by treatment of **(238)** in aqueous acid solution, with hydroxylamine hydrochloride, and was isolated as a white solid that precipitated after neutralisation of the reaction solution. Elemental and HRMS analysis of the product **(245)** confirmed it had an empirical formula of $C_6H_8ClN_5O$. ^1H NMR analysis of the product showed the presence of a CH_2 group at 3.95 ppm and two CH's at 5.78 and 6.44 ppm. The exchangeable oxime alcohol in **(245)** was evident by the presence of an exchangeable peak at 10.74 ppm. Unfortunately, all attempts

to dehydrate **(245)** to give **(241)** were unsuccessful. Reagents used included SeO_2 and $POCl_3$[207]



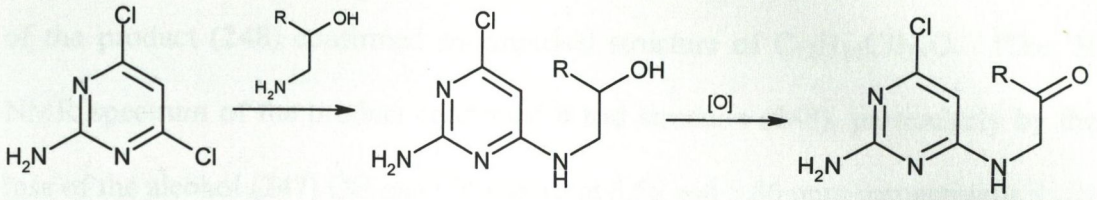
(236) **(238)** **(241)** **(245)**

Due to sensitivity of oxime **(240)**, attention was turned next to the preparation of ketones of type **(246)**. Attempts were made to prepare the ketone **(246)** by reacting **(234)** with 2-aminoacetophenone **(145)**. However, once again the α-amino group of **(145)** proved to be too unreactive, and no new products were formed.



(234) **(145)** **(246)**

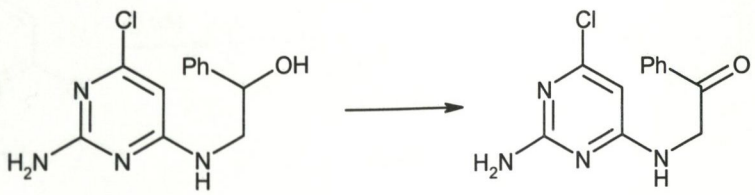
It was hoped the ketones **(248)** and **(250)** could be prepared by oxidation of their alcohol derivatives, **(247)** and **(249)** respectively.



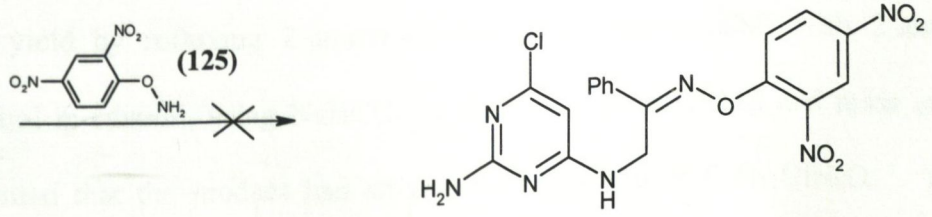
(247) **(161)** R = Ph **(247)** R = Ph **(248)** R = Ph

(163) R = CH_3 **(249)** R = CH_3 **(250)** R = CH_3

2-(2-Amino-6-chloropyrimidin-4-ylamino)-1-phenylethanol **(247)** was prepared by refluxing **(234)** with 2-amino-1-phenylethanol **(161)** in ethanol, with $NaHCO_3$ as the base. The white crystalline product was shown to have an empirical formula of $C_{12}H_{13}ClN_4O$ by both HRMS and elemental analysis. The ^1H NMR spectrum showed two multiplets at 3.22 and 3.57 ppm arising from the two methylene peaks The phenyl group appeared as three aromatic CH signals peaks at 7.25, 7.34 and 7.41 ppm. The exchangeable signals of the OH group and NH_2 groups appeared as two broad singlets at 5.56 ppm and 6.45 ppm.



(247) **(248)**

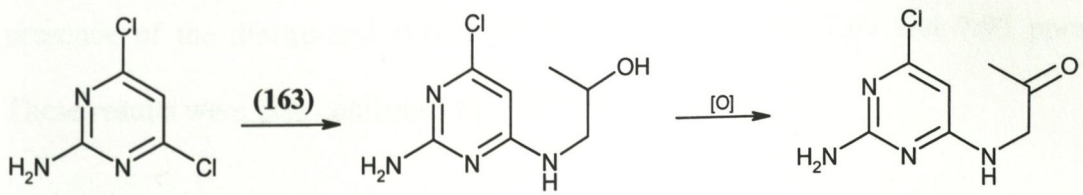


(251)

The alcohol **(247)** was oxidised successfully on treatment with Jones reagent. The product **(248)** was isolated after treatment of the crude reaction solution with charcoal, and shown to be pure by TLC. HRMS (m/z 263.7056) and mass analysis of the product **(248)** confirmed an empirical structure of $C_{12}H_{11}ClN_4O$. The ^1H NMR spectrum of the product confirmed it had structure **(248)**, particularly by the loss of the alcohol **(247)** OH and CH signals at 5.56 and 5.86 ppm respectively.

Attempts to prepare the oxime **(251)** by reaction of **(248)** with 2,4-dinitrophenyl hydroxylamine **(125)** were unsuccessful, however. When **(248)** was reacted with **(125)** at room temperature, MS (ESI) analysis of the reaction mixture showed no traces of the desired oxime **(251)**. When the reaction solution was heated, the only new product that was observed was dinitrophenol, formed by the thermal decomposition of **(125)**.

Because of this unreactivity of the phenyl ketone **(248)**, attention was turned next to the preparation of its methyl analogue **(250)**. It was hoped that the carbonyl group of this ketone **(250)** would be more reactive than that in **(248)**.



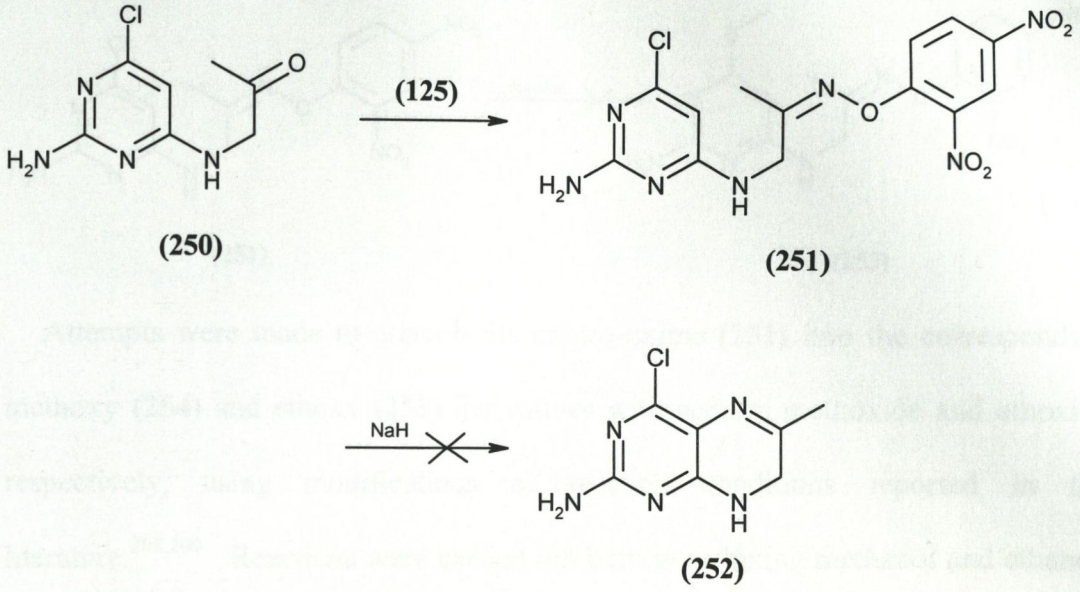
(234) **(249)** **(250)**

The alcohol precursor **(249)** required for the preparation of **(250)** was prepared in good yield by refluxing 2-amino-4,6-dichloropyrimidine **(234)** with 2-amino-1-propanol in ethanol, using $NaHCO_3$ as the base. Both HRMS and mass analysis confirmed that the product had an empirical structure of $C_7H_{11}ClN_4O$. The ^1H

spectrum of the product **(249)** confirmed the presence of a CH, CH_2, and CH_3 signal, as well as the exchangeable signals of the NH and OH groups.

Oxidation of the alcohol **(249)** to its ketone derivative **(250)**, was achieved successfully by the use of Jones reagent. The product was isolated as a white solid after treatment of the reaction solution with charcoal. Analysis of this solid by HRMS and elemental analysis confirmed the product had an empirical formula of $C_7H_9ClN_4O$. Comparison of the ^1H NMR spectrum of this product **(250)** with that of the alcohol **(249)** starting material showed the loss of the hydroxy proton signal at 4.75 ppm and CH signal at 3.71 ppm.

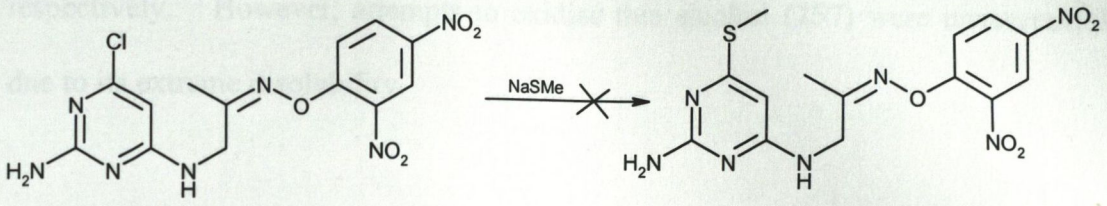
1-(2-amino-4-chloropyrimidn-6-ylamino)propan-2-one O-(2,4-dinitrophenyl)-oxime **(251)** was prepared by reaction of the methyl ketone **(250)** with O-(2,4-dinitrophenyloxime)-hydroxylamine **(125)** in acidic methanol solution. The product was shown to have an empirical formula of $C_{13}H_{16}ClN_7O_5$ by both mass and HRMS analysis. NMR analysis of the product showed the presence of only one isomer, although the nature of this was not elucidated. The ^1H NMR spectrum of **(251)** showed the presence of a CH_3 at 2.51 ppm and a CH_2 at 4.85 ppm as well as the presence of the dinitro-aryl system as three signals at 7.46, 7.69 and 7.93 ppm. These results were also confirmed by ^{13}C NMR analysis.



(250) **(251)** **(252)**

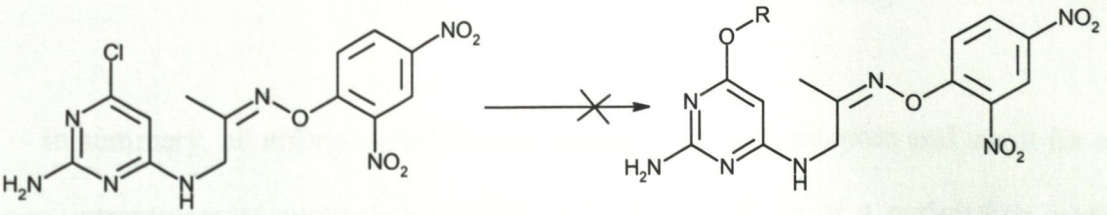
This oxime **(251)** was refluxed in 1,4-dioxane in the presence of NaH, following a procedure used successfully by Narasaka[153,154] for the cyclisation of a 2,4-dinitrophenyl oxime onto a benzene ring to give the quinoline product **(107)**. Only crude reaction mixtures were obtained, however, and no useful products could be identified.

In order to make the 5-position in oxime **(251)** more nucleophilic, thus favouring intramolecular cyclisation, attempts were made to replace the electronegative chlorine atom in **(251)** with an electron donating group. The oxime **(251)** was treated with sodium thiomethoxide in aqueous solution at 50°C. Only starting material **(251)** could be isolated from the reaction mixture, however. At higher temperatures the oxime **(251)** decomposed. Analysis of the reaction mixtures by MS (ESI) showed no trace of the expected product **(253)**.



(251) **(253)**

Attempts were made to convert the chloro-oxime **(251)** into the corresponding methoxy **(254)** and ethoxy **(255)** derivatives with sodium methoxide and ethoxide respectively, using modifications of reaction conditions reported in the literature.[208,209] Reactions were carried out both in refluxing methanol and ethanol, as well as at 120°C in a pressure tube. In all cases, TLC and MS (ESI) analysis of the reaction mixtures showed that no reaction had occurred.



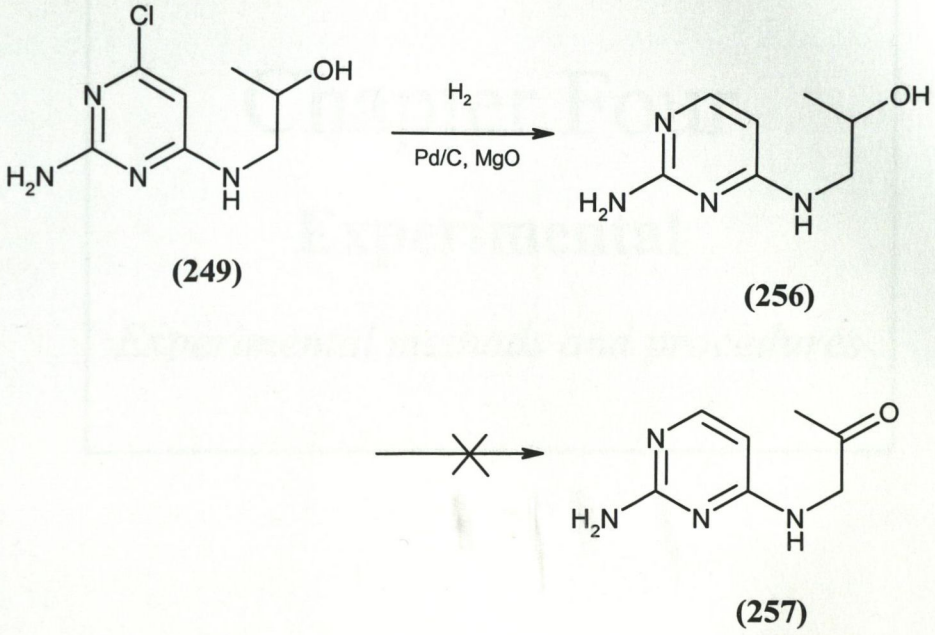
(251)

(254) R = Me

(255) R = Et

A final attempt to overcome these problems involved in the replacement of the chlorine atom in **(249)** by hydrogen, to give 1-(2-amino-pyrimidin-6-ylamino)-propan-2-ol **(256)**. It was hoped that the presence of a proton at the 4-position in **(256)** would prevent premature cyclisation but yet at the same time would retain a sufficiently active 5-position, for facilitating attack by an electrophilic nitrogen. The alcohol **(256)** was prepared by the hydrogenation of **(249)** over a Pd/C catalyst in the presence of MgO. HRMS and elemental analysis of the product showed it had an empirical formula of $C_7H_{12}ClN_4O$. The ^1H NMR spectrum showed the presence of the 4- and 5- protons in **(256)** as two doublets at 6.21 and 7.67 ppm

respectively. However, attempts to oxidise this alcohol **(257)** were unsuccessful due to its extreme insolubility.



(249)

(256)

(257)

In summary, all efforts to facilitate an electrophilic nitrogen atom and use it for a new intramolecular cyclisation reaction on to the 5-position of a pyrimidine were unsuccessful. On the other hand, the work did lead to the development of this approach and makes the approach more feasible in the future. The work also led to the discovery of a hitherto unobserved type of Claisen allyl ether rearrangement, which took place with 2,4-dinitrophenyl oxime of chloroacetone **(175)** involved a sigmatropic rearrangement in which two of the six atoms were nitrogen and oxygen. The initially formed product for this Claisen type reaction was found to undergo intramolecular cyclisation in a variety of ways leading to different ring systems including a pyrrolo[2,3-*d*]pyrimidine (7-deazapurine), a benzo[b]furan and a furano[3,4-*d*]pyrimidine.

Chapter Four

Experimental

Experimental methods and procedures

4.1 Materials and Methods used in Chapter Two

4.1.1 Animals used

Wild type zebrafish were used in this work were. These were raised in the zebrafish facility of the Institute of Mammalian Genetics, Neuherberg, and all embryos were obtained from natural spawning. They were grouped according to number hours postfertilisation hours at 28.5°C and the samples were collected in batches of 10-20 animals, immediately frozen, and kept at -85°C.

4.1.2 Identification of the Pteridine pattern

The samples (batches of 50 animals, 90-120 HPF) were extracted by homogenisation with a Wheaton Teflon homogeniser in 100ml of Tris buffer (50mM, pH6.9) and centrifuged at 13,000xg for 10 minutes. The extract was oxidised by acidic iodine, deproteinised by trichloroacetic acid, and purified by cation exchange chromatography. The lyophilised material was pre-fractionated by reverse phase HPLC, using 3% methanol in water and a fluorescence detector. The fractions were concentrated in vacuum.

4.1.3 HPLC analysis of zebrafish extracts

HPLC analysis of Fraction I and II was carried out using a Shimadzu HPLC system. This incorporated a SCL-10AVP system controller, a SPD-M10AVP PDA detector, a LC-10ADVP liquid chromatograph controller and a SIL-10ADVP Auto Injector. This analysis was carried out using a mobile phase of Water:MeOH 85:15 on a Hichrome HD05S reverse phase column at a flow rate of 0.35 ml/min.

4.1.4 HPLC-MS analysis of zebrafish extracts

HPLC-MS analysis of the fraction I and II was carried out using a Perkin Elmer HPLC system coupled with a Micromass LCT electrospray mass spectrometer. The HPLC incorporated a series 200 LC pump with a 235C PDA detector. Analysis was carried out using Hypersil-ODS C-18 columns with a mobile phase of 15% methanol in water. A flow rate of 0.3 and 0.2 ml/min was used, for analysis of fraction I and II respectively.

A Micromass LCT instrument with positive electrospray ionisation and time of flight analysis system was used for identification of the compounds present in both fractions. MS analysis conditions were optimised for each part of the mixture as described in section 2.3.1. Fraction I was dissolved in 30µl 0.1M ammonium hydroxide and Fraction II was dissolved in 30µl of mobile phase.

4.2 Materials and Methods used in Chapter three

4.2.1 Reagents

All reagents not prepared in the laboratory were purchased from either, divisions of the Aldrich Chemical Company or from Lancaster synthesis Ltd. (UK). All solvents used were sourced from the University of Dublin Hazardous Materials Facility.

4.2.2 Thin layer and column chromatography

Thin layer chromatography (TLC) was carried out using Merck (silica gel 60) TLC aluminium sheets, unless otherwise indicated. Visualisation was achieved by the use of either UV light or by development over iodine adsorbed onto silica.

Column chromatography was carried out using manually prepared columns containing 'flash' silica gel, which was packed under pressure. Separation of mixtures on these columns was carried out at atmospheric pressure.

4.2.3 Elemental analysis

Elemental analysis was carried out in the microanalytical laboratory, Department of Chemistry, University College Dublin using a Carlo Erba 1006 automatic analyser. Expected range C, H, N ± 0.4%.

4.2.4 Melting point analysis

All melting points are uncorrected and were measured on an Electrothermal 9100 heating ramp.

4.2.5 Nuclear Magnetic Resonance

NMR spectra were recorded on a Bruker DPX 400 instrument, operating at 400.14 MHz for ^{1}H analysis and 100.14 MHz for ^{13}C analysis. Samples were analysed dissolved in an appropriate deuterated solvent (see experimental section for details) and were referenced against tetramethylsilane (TMS). Chemical shifts are reported in ppm with coupling constants in hertz

4.2.6 Infrared spectroscopy

Infrared spectra were recorded in the range 4000-600 cm^{-1} on a Perkin-Elmer 1600 series FTIR. Samples were run as 8mm diameter potassium bromide pellets (unless otherwise indicated) prepared using a punch and dye, under pressure.

4.2.7 Electrospray mass spectroscopy

Electrospray mass spectroscopy analysis of organic samples prepared in the laboratory was carried out using a Micromass LCT® in either positive or negative mode. Samples were dissolved in HPLC grade methanol at a concentration of ~2ng/L and analysed using a mobile phase of 50:50 methanol:water, unless otherwise stated. Analysis by high resolution mass spectroscopy was carried out as above, except all masses were standardised against an internal standard of leucine enkephalin (Aldrich) dissolved in the solvent used.

4.2.8 UV-Vis spectroscopy

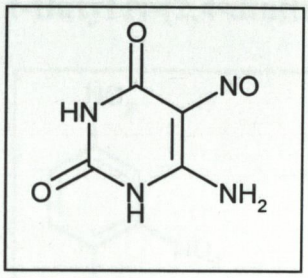
All UV analysis, other than that carried out as part of HPLC analysis was carried out in HPLC grade methanol (unless otherwise stated) using a Varian Cary 300 *UV-Vis spectrophotometer*

4.2.9 HPLC Analysis

HPLC analysis of Fraction I and II was carried out using a Shimadzu HPLC system. This incorporated a SCL-10AVP system controller, a SPD-M10AVP PDA detector, a LC-10ADVP liquid chromatograph controller and a SIL-10ADVP Auto Injector. This analysis was carried out using a mobile phase of Water:MeOH 85:15 on a Hichrome HD05S reverse phase column at a flow rate of 0.35 ml/min.

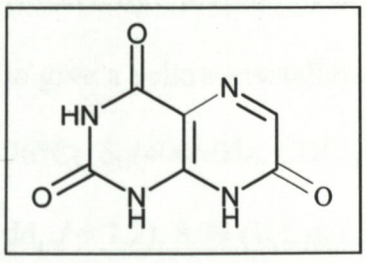
4.3 Experimental

6-Amino-5-nitrosouracil (43)



6-Aminouracil **(42)** (1.0g; 7.85 mmol) was dissolved in water (10 ml) that contained glacial acetic acid (3 ml). Sodium nitrite (0.8125g; 11.8 mmol) in water (3 ml) was added to the flask over 20 minutes. The white suspension turned pink immediately and was stirred at room temperature over-night. The pink suspension was further suspended in 50 ml of methanol, and a pink solid (1.19g; 98%) was isolated by filtration, washed with methanol and dried. R_f (DCM:MeOH) 0.85; m.p. >250°C; m/z (ESI) 156.02 ($M+H^+$).

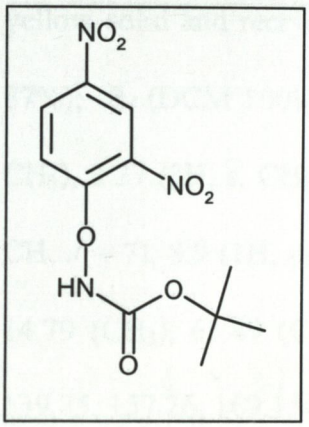
2,4,7-Trioxopteridine (Violapterin) (36)[108]



A suspension of 6-amino-5-nitrosouracil **(43)** (1.0g; 6.4 mmol) in degassed water (100 ml) containing Raney nickel (1 cm^3) and sodium hydroxide (0.26g; 6.4 mmol) was stirred vigorously under an atmosphere of H_2 for 15 hours. The catalyst was removed by filtration over celite® and under nitrogen to give a clear solution, which was filtered onto the hemiacetal of methyl glyoxalate **(46)** (1.572g; 12.8 mmol). This was stirred in the dark at room temperature over night. The water was removed under reduced pressure and a dark yellow solid was obtained. This was recrystallised from water following a hot charcoal filtration and a light yellow solid was obtained (1.051g; 91%). R_f (DCM:MeOH, 7:3) 0.42; m.p. >250 °C; λ_{max} (pH1)/nm 268, 324; m/z (ESI) 181.12

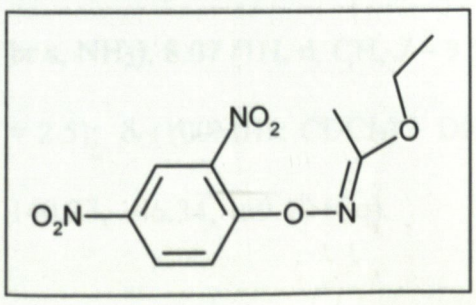
$(M+H^+)$, 203.16 $(M+Na^+)$; HRMS/ESI (Found; $M+Na^+$, 203.0187. Calc. For $C_6H_4N_3O_3$ $M+Na^+$, 203.0181).

t-Butyl N-(2,4-dinitrophenoxy) carbamate (128)[165]



Potassium hydroxide (0.84g; 15 mmol) and t-butyl-N-hydroxycarbamate **(130)** (2g; 15 mmol) were dissolved in ethanol under N_2 to give a clear solution. 2,4-dinitro-chlororbenzene **(126)** (3.03g; 15 mmol) was added to this solution in aliquots to give a red suspension, which was agitated at room temperature for 2 hours. The suspension was pH adjusted with glacial acetic acid to pH 6 and added to water (40 ml) to yield an oily product. The aqueous solution containing the oil was allowed to sit at RT for three days at which time the product had solidified and a crude yellow solid was isolated by filtration. The crude product was recrystallised from DCM:Hex at -4°C to give a yellow crystalline solid (3.1g; 70%); R_f (DCM:EtOH, 9:1) 0.86; m.p. 75-76°C; δ_H(400MHz; $CDCl_3$) 1.45 (9H, s, 3x CH_3), 7.73 (1H, d, CH, $J = 8$), 8.52 (1H, dd, $J = 7.2$), 8.79 (1H, d, CH, $J = 2.5$) and 11.5 (1H, s, NH); δ_C(100MHz; $CDCl_3$) + DEPT 27.67 (3 x CH_3), 82.39 (Cq), 116.16 (CH), 121.56 (CH), 129.71 (CH) and 136.28, 141.18, 155.87, 157.37, (Cq); m/z (ESI) 300.08 $(M+H^+)$.

O-(2,4-Dinitrophenyl)-acethydroxamate-ethylester (129)[165]

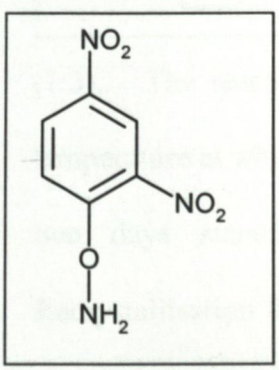


Ethyl acetohydroxamate **(131)** (2.02g; 19.6 mmol) was dissolved in ethanol:water (20:1), containing KOH (1.32g; 23.54 mol). The solution was cooled to -5°C in a 'brine bath'

and 2,4-dinitrochlorobenzene **(126)** (4g; 19.76 mmol) dissolved in EtOH (60 ml) was added slowly to the reaction, always keeping the reaction temperature at or below -5°C. Once the addition was complete the reaction was left to agitate for 45 min. and a dark red suspension formed. Filtration of this suspension yielded a yellow solid and recrystallisation of this from EtOH yielded a cream solid (4.56g; 87%); R_f (DCM 100%) 0.93; m.p. 111-112°C; δ_H(400MHz; $CDCl_3$) 1.43 (3H, t, CH_3), 2.27 (3H, s, CH_3), 4.25 (2H, q, CH_2), 7.90 (1H, d, CH, J = 9), 8.43 (1H, dd, CH, J = 7), 8.9 (1H, d, CH, J = 2.5); δ_C (100MHz; $CDCl_3$) + DEPT 13.74 (CH_3), 14.79 (CH_3), 63.49 (CH_2), 116.22 (CH), 121.63 (CH), 128.92 (CH) and 136.21, 139.75, 157.26, 169.154 (Cq).

Method A

O-(2,4-Dinitrophenyl)hydroxylamine (125)[164]



O-(2,4-Dinitrophenyl)-acethydroxamate ethylester **(129)** (0.5g; 1.86 mmol) was dissolved in 1,4-dioxane (2.5 ml) at room temperature with agitation. To this yellow solution was added drop wise, perchloric acid (70%) (1 ml). The solution was stirred at RT for 30 min. and then added to ice (15g) with vigorous agitation, which gave a yellow solid. The solid was isolated by filtration, washed with EtOH and dried (0.3g; 82%). R_f (DCM; 100%) 0.57; m.p. 113°C; ν_{max} (KBr)/cm^{-1} 3327, 3249, 1606, 1518 and 1343; δ_H (400MHz; $CDCl_3$) 6.42 (2H, br s, NH_2), 8.07 (1H, d, CH, J = 9.5), 8.45 (1H, dd, CH, J = 7.5), 8.82 (1H, d, CH, J = 2.5); δ_C(100MHz; $CDCl_3$) + DEPT 115.91 (CH), 121.43 (CH), 128.81 (CH) and 140.23, 146.34, 159.10 (Cq).

Method B

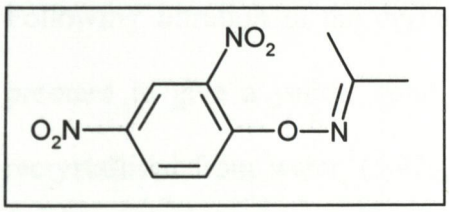
O-(2,4-Dinitrophenyl)hydroxylamine (125)[164]

t-butyl N-(2,4-dinitrophenoxy) carbamate **(128)** (1g; 3.34 mmol) was dissolved in triflouroacetic acid (15 ml) with the evolution of CO_2 gas. The yellow solution was agitated for 30 min. and poured onto ice (20g) giving a crude yellow oil. This was left to sit at room temperature for one week at which time a crude yellow solid was isolated by filtration, washed with water, ethanol and dried (0.35g; 52%).

Note: The product has to be stored in the dark and under N_2 to avoid decomposition.

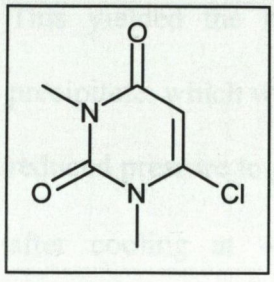
All analysis results as above.

Propan-2-one O-(2,4-dinitro-phenyl)-oxime (133)



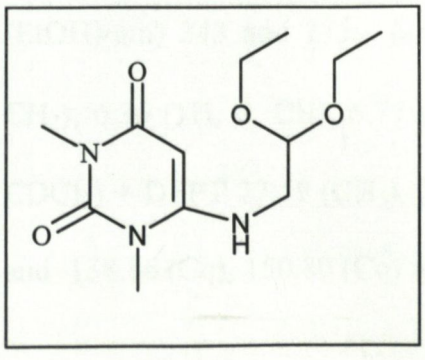
O-(2,4-Dinitrophenyl)hydroxylamine **(125)** (0.1g; 0.5mmol) was dissolved in TFA (10ml) and added to an acetone:ethanol solution (4ml) (1:3). The reaction solution was allowed to agitate for twenty minutes at room temperature at which time it was added to ice to give an oil. The oil solidified after two days standing at room temperature, and was isolated by filtration. Recrystallisation from ethanol yielded a white crystalline product, (0.9g; 75%). R_f (100% DCM) 0.93; m.p. 88-90°C; (KBr)/cm^{-1} 3127, 1606, 1517, 1342, 1279, 1241, 1133 and 1062; δ_H(400 MHz; $CDCl_3$) 2.12 (3H, s, CH_3), 2.21 (3H, s, CH_3), 7.96 (1H, d, CH, J = 9.5), 8.42 (1H, dd, CH, J = 6.5) and 8.88 (1H, d, CH, J = 3); δ_C(100 MHz; $CDCl_3$) + DEPT 16.87 (CH_3), 21.02 (CH_3), 116.72 (CH), 121.54 (CH), 128.79 (CH) and 135.36, 140.03, 156.93, 163.96 (Cq).

6-Chloro-1,3-dimethyluracil (137)[167]



1,3-Dimethylbarbituric acid **(136)** (5g; 32 mmol) was suspended in $POCl_3$ (40ml). To this was added water (4ml) drop wise over five minutes with agitation. **Note:** Addition of H_2O to $POCl_3$ should be carried out with great care as the reaction is very exothermic and evolves HCl gas. Once the vigorous reaction with water had subsided, the suspension was heated to reflux for 40 minutes. Three quarters of the $POCl_3$ was removed by distillation to give a brown oily residue and addition of this oil to ice water yielded a cream suspension. The solid was isolated by filtration and the aqueous mother liquor was extracted with dichloromethane, from which the organic layers were combined and dried over anhydrous $MgSO_4$. Following filtration of the drying agent, the solvent was removed under reduced pressure to give a yellow solid. Both batches of product were combined and recrystallised from water. (5.42g, 97%); m.p. 112-113°C; R_f (EtOAc:Hex, 8:2) 0.53; δ_H(400 MHz; $CDCl_3$) 3.34 (3H, s, CH_3), 3.58 (3H, s, CH_3) and 5.95 (1H, s, CH); δ_C(100 MHz; $CDCL_3$) + DEPT 27.83 (CH_3), 33.13 (CH_3), 101.24 (CH), 145.49, 150.75, 160.31 (Cq); m/z (ESI) 175.47 ($M+H^+$) and 198.34 ($M+Na^+$).

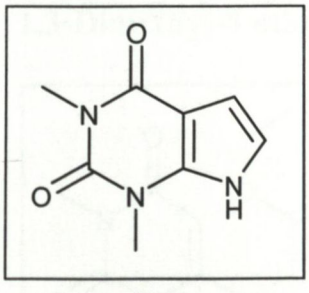
6-(2,2-Diethoxy-ethylamino)-1,3-dimethyl-1*H*-pyrimidine-2,4-dione (139)[168]



6-Chloro-1,3-dimethyluracil **(137)** (1g: 5.73 mmol) was suspended in aminoacetaldehyde diethyl acetal **(138)** (1.73 ml; 1.59g; 12.01 mmol) and water (2.86 ml). This suspension was heated at 100°C for one hour to form a clear solution. At this time the water was removed under reduced pressure to give a brown viscous

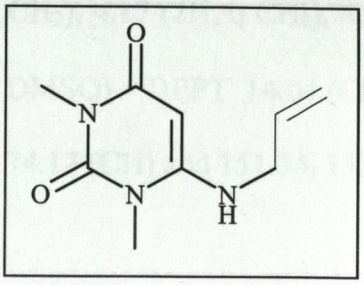
liquid, which was dissolved in ethyl acetate (20 ml) and cooled at -4°C over night. This yielded the hydrochloride salt of aminoacetaldehyde diethyl acetal as a precipitate, which was removed by filtration. The solvent was removed again under reduced pressure to give a brown residue. This was dissolved in toluene (10ml) and after cooling at -4°C gave a white crystalline product (1.3g; 84.5%); R_f (DCM:MeOH, 7:3) 0.56; m.p. 189-190; λ_{max} (MeOH)/nm) 267; δ_H(400 MHz; $CDCl_3$) 1.22 (6H, t, CH_3), 3.19 (2H, t, CH_2), 3.29 (3H, s, CH_3), 3.39 (3H, s, CH_3), 3.62 (4H, m, CH_2), 4.69 (1H, t, CH), 4.77 (1H, br s, NH) and 4.84 (1H, s, CH); δ_C(100 MHz; $CDCl_3$) + DEPT. 15.20 (CH_3), 27.65 (CH_3), 28.37 (CH_3), 45.28 (CH_2), 75.75 (CH) and 95.19 (CH) and 151.41, 152.42, 162.44 (Cq); m/z (ESI) 271.34 ($M+H^+$) and 293.41 ($M+Na^+$).

1,3-Dimethyl-1,7-dihydro-pyrolo [2,3-*d*] pyrimidine (140)[169]



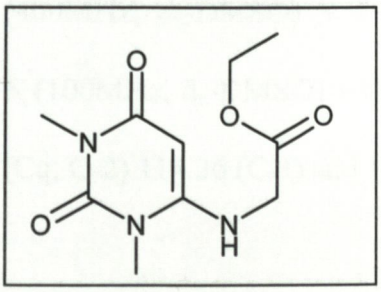
6-(2,2-Diethoxy-ethylamino)-1,3-dimethyl-1H-pyrimidine-2,4-dione **(139)** (0.1g; 0.37 mmol) was dissolved in 1M aqueous HCl (10ml) and agitated for three hours at room temperature. After this time a suspension formed and from this a white precipitate was isolated by filtration, washed with cold water and dried over P_2O_5 over night (0.5g; 78%); m.p. >250 °C; R_f (DCM:MeOH, 9:1) 0.3; λ_{max} (EtOH)/nm) 243 and 275; δ_H(400 MHz; $CDCl_3$) 3.21(3H, s, CH_3), 3.43(3H, s, CH_3), 6.36 (1H, d, CH), 6.77 (1H, d, CH) and 11.67 (1H, s, NH); δ_C(100 MHz; $CDCl_3$) + DEPT 27.59 (CH_3), 30.47 (CH_3), 98.52 (Cq), 103.84 (CH), 116.73 (CH) and 138.86 (Cq), 150.80 (Cq) and 158.50 (Cq). m/z (ESI) 180.21 ($M+H^+$).

6-Allylamino-1,3-dimethyl-1*H*-pyrimidine-2,4-dione (142)[171]



6-Chloro-1,3-dimethyluracil **(137)** (1g; 5.73 mmol) was suspended in water (3ml). To this was added allyl amine **(141)** (1.29ml; 17.184 mmol) and the cream suspension was heated at 50°C with agitation. After 1.4 hours a white precipitate was isolated, and recrystallised from EtOH to give a white crystalline product (0.76g; 65%); m.p. 163-164°C; R_f (DCM:MeOH:DIPEA, 9:0.9:0.1) 0.54; δ_H(400 MHz; d_6-DMSO) 3.09 (3H, s, CH_3), 3.30 (3H, s, CH_3), 3.73 (2H, s, CH_2), 4.60 (1H, s, CH), 5.16 (1H, d, CH), 5.24 (1H, d, CH), 5.79, (1H, m, CH), 7.00 (1H, br s, NH); δ_C(100 MHz; d_6-DMSO) + DEPT 27.05 (CH_3), 29.21 (CH_3), 44.43 (CH_2), 74.01 (CH), 116.14 (CH_2), 133.89 (CH) and 151.49, 153.19, 161.53 (Cq); m/z (ESI) 196.02 ($M+H^+$).

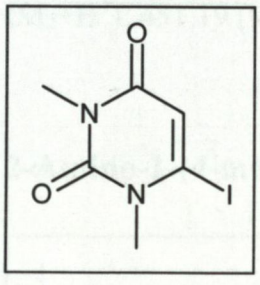
1,3-Dimethyl-4-ethoxycarbonylmethylaminouracil (144)[156]



6-Chloro-1,3-dimethyluracil **(137)** (1.47g; 8.4 mmol) was dissolved in water (40 ml) with heat. Glycine ethyl ester hydrochloride **(143)** (4.1g; 29.4mmol) was added and the solution was heated for 15 min at 50°C. Triethylamine (6ml; 43.5 mmol) was added and the solution was refluxed over night. Half the water was removed under reduced pressure and saturated aqueous NaCl (15 ml) was added. The aqueous phase was extracted with EtOAc; the organic layers were combined and dried over $MgSO_4$. The organic phase once dry was reduced to give a crude yellow solid which was recrystallised from EtOAc:Hex to give a white crystalline product (1.38; 68%). R_f (DCM:MeOH; 9:1) 0.84; m.p. 178-180°C; ν_{max} (KBr)/cm^{-1} 3316, 1740, 1696, 1636, 1581 and 1211; δ_H(400 MHz;

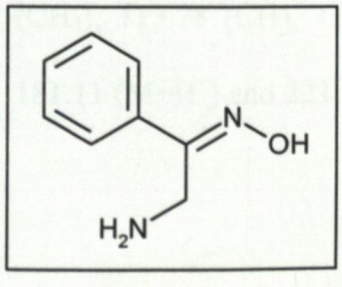
d-DMSO) 1.23 (3H, t CH_3), 3.12 (3H, s, CH_3), 3.35 (3H, s, CH_3), 3.96 (2H, d, CH_2), 4.17 (2H, q CH_2), 4.57 (1H, s, CH, 5-H), 7.23 (1H, t, NH); δ_C(100 MHz; d-DMSO) + DEPT 14.04 (CH_3), 27.09 (CH_3), 29.33 (CH_3), 43.76 (CH_2), 60.79 (CH_2), 74.17 (CH) and 151.34, 153.46, 161.46, 169.23 (Cq); m/z (ESI) 241.16 ($M+H^+$).

6-Iodo-1,3-dimethyluracil (147)



6-Chloro-1,3-dimethyluracil **(137)** (2g; 11.45 mmol) was dissolved in DMF (20ml) with agitation. To this yellow solution was added sodium iodide (6.87g; 45.87 mmol) and the reaction mixture was refluxed for 3 hr. At this stage the majority of the DMF was removed by vacuum distillation to give a brown residue. Triply distilled water (35 ml) was added to the residue to yield a precipitate, which was isolated by filtration. Recrystallisation of this from EtOH yielded a white crystalline solid, (2.16g; 71%); R_f (EtOAc:Hex, 8:2) 0.56; m.p. 173-174°C; δ_H (400MHz; d_6-DMSO) 3.32 (3H, s, CH_3), 3.69 (3H, s, CH_3), 6.50 (1H, s, CH); δ_C(100MHz; d_6-DMSO) + DEPT 27.89 (CH_3), 41.40 (CH_3), 101.2 (Cq, C6), 111.65 (Cq, C-2) 114.26 (CH) and 160.31 (Cq, C-4); m/z (ESI) 267.14 ($M+H^+$).

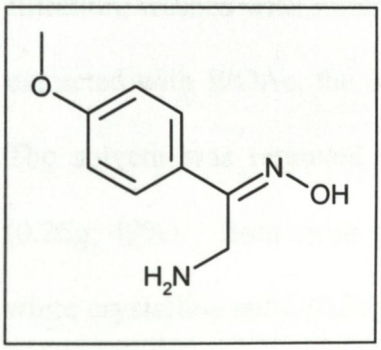
2-Amino-1-phenyl-ethanone oxime (153)[178]



Hydroxylamine hydrochloride (1.62g; 23.28 mmol) was dissolved in water (5 ml) and the flask was cooled to 0°C. To this was added aqueous potassium hydroxide (6.42ml; 30% w/v). Once this had equilibrated, 2-amino-acetophenone hydrochloride **(145)** (1g; 5.82mmol) as an aqueous solution (6 ml) was added dropwise over 10 min. On addition of the ketone a thick cream

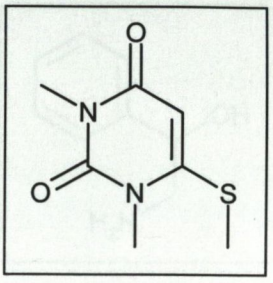
suspension formed which was agitated for a further 45 min. The product was isolated by filtration and recrystallised from EtOAc, giving a white crystalline solid (0.68g; 78%). R_f (DCM:MeOH, 8:2) 0.48; m.p. 142-144°C; δ_H(400 MHz; d_6-DMSO) 3.78 (2H, s, CH_2), 6.82 (2H, br s, NH_2), 7.37 (2H, d, 2 x CH), 7.64 (2H, d, 2 x CH); δ_C(100 MHz; d_6-DMSO) + DEPT 36.28 (CH_2), 126.16 (CH), 127.13 (Cq), 128.32 (CH), 128.49 (CH) and 135.7 (Cq); m/z (ESI) 151.05 ($M+H^+$), 301.11 (M_2+H^+), 451.19 (M_3+H^+).

2-Amino-1-(4-methoxy-phenyl)-ethanone oxime (154)[178]



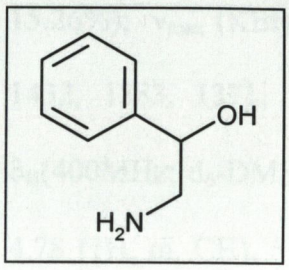
Hydroxylamine hydrochloride (0.45g; 6.45 mmol) and 2-amino-4'-methoxyacetophenone hydrochloride **(148)** (1g; 4.96mmol) were dissolved in water (10 ml). To this was added aqueous potassium hydroxide (2.5ml; 30% w/v) to neutralise the salts. After 15 minutes agitation a thick cream suspension had formed and this product was isolated by filtration and recrystallised from EtOAc to give a white crystalline solid (0.63g; 71%). R_f (DCM:MeOH, 8:2) 0.22; m.p. 142-144°C; λ_{max} (EtOH)/nm 262; δ_H(400 MHz; d_6-DMSO) 3.76 (2H, s, CH_2), 3.83(3H, s, CH_3), 6.94 (2H, d, 2 x CH), 7.62 (2H, d, 2 x CH); δ_C(100 MHz; d_6-DMSO) + DEPT 36.30 (CH_2), 55.15 (CH_3), 113.78 (CH), 127.47 (CH) and 128.05, 157.51, 159.61 (Cq); m/z (ESI) 181.11 ($M+H^+$) and 221.15 ($M+K^+$).

1,3-Dimethyl-6- methylthiouracil (160)



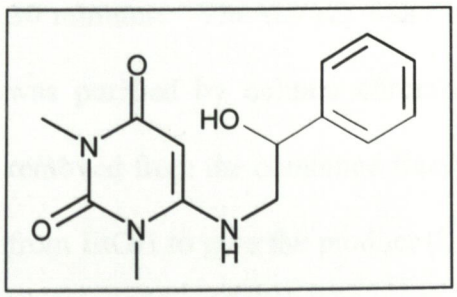
6-Chloro-1,3-dimethyluracil **(137)** (0.5g 2.9 mmol) was dissolved in water (15 ml) at 60°C to give a yellow solution. To this, sodium thiomethoxide (1.06ml; 21% aq. solution; 3.16 mmol) was added drop wise, and the reaction mixture was agitated for 15 minutes at 60°C. The reaction was allowed to cool to room temperature at which time a white suspension formed, and the reaction was agitated for a further 30 minutes at 0°C to optimise precipitation. The solid was shown to be pure and a new product by TLC analysis and thus the white solid was isolated by filtration, washed with water and cold EtOH (0.19g; 35%). The aqueous phase was extracted with EtOAc, the organic extracts were combined and dried over $MgSO_4$. The solvent was removed under vacuum to give another crop as a white solid (0.26g; 49%). Both crops were combined and recrystallised from EtOAc to give a white crystalline solid (0.38g; 71%). R_f (DCM:EtOH, 9:1) 0.73; m.p. 127-129°C; (Found C, 45.12; H, 5.39; N, 14.95; $C_7H_{10}N_2O_2S$ requires C, 45.15; H, 5.41; N, 15.04%); ν_{max} (KBr)/cm^{-1} 2988, 2942, 1702, 1688, 1635, 1508, 1456, 1431, 1382, 1362, 1281, 1174, 1030; λ_{max} (MeOH)/nm 220 and 280; δ_H-(400MHz; d_6-DMSO) 2.49 (3H,s, CH_3, CH_3-S), 3.14 (3H,s, CH_3), 3.34 (3H,s, CH_3), 5.49 (1H,s, CH); δ_C(100MHz; d_6-DMSO) + DEPT 14.79 (CH_3-S), 27.35 (CH_3), 31.56 (CH_3), 94.17 (CH) and 151.08, 157.44, 159.97 (Cq); m/z (ESI) 187.41 ($M+H^+$); HRMS/ESI (Found; $M+H^+$, 187.0541. Calc. For $C_7H_{11}N_2O_2S$ $M+H^+$, 187.0537).

2-Amino-1-phenyl-ethanol (161)[181]



2-Nitroacetophenone **(166)** (0.5g; 3mmol) was dissolved in EtOH: HCl (20:1 v/v) to give a light yellow solution. To this was added Pt/C (5%) catalyst and the suspension was agitated at 50°C under an atmosphere of H_2 over night. Once H_2 uptake had ceased, the catalyst was removed over celite® and the mother liquor reduced to dryness under vacuum. An oil was obtained, which was crystallised by addition of acetone (15ml) to give a white solid. This was dissolved in water (10 ml) and neutralised with aqueous sodium hydroxide to give the free base. The aqueous phase was extracted with EtOAc, dried over $MgSO_4$ and reduced to dryness to give a white solid (0.27g; 51%). R_f (DCM:MeOH, 9:1) 0.31; m.p. 56-58°C; δ_H(400 MHz; $CDCl_3$) 2.74 (2H, m, CH_2); 4.52 (1H, m, CH), 7.26 (5H, m, Ph) δ_C(100 MHz; $CDCl_3$) + DEPT 49.24 (CH_2), 74.23 (CH), 125.46 (CH), 126.91 (CH), 126.76 (CH) and 142.57 (Cq); m/z (ESI) 138.42 ($M+H^+$).

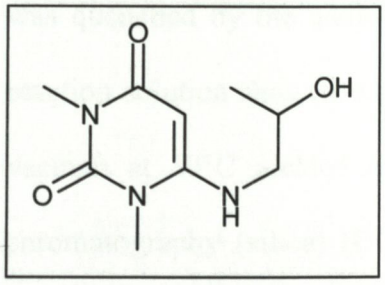
6-2-(Hydroxy-2'-phenyl-ethylamino)-1.3-dimethyluracil (162)



6-Chloro-1,3-dimethyluracil **(137)** (1g; 5.73 mmol) was dissolved in ethanol (20ml) with agitation. To the solution, 2-amino-1-phenyl-ethanol **(161)** (1.57g; 11.46 mmol) was added and the reaction was heated to 50°C to aid dissolution. Once a solution was obtained triethylamine (0.8ml; 5.73 mmol) was added and the reaction was refluxed for twelve hours. A white precipitate formed on cooling of the reaction solution, which was isolated by filtration and recrystallised from ethanol to give a white crystalline solid (1.38g; 87%). R_f (DCM:EtOH, 9:1) 0.48; m.p. 174-175°C;

(Found C, 60.84; H, 6.16; N, 15.19; $C_{14}H_{17}N_3O_3$ requires C, 61.08; H, 6.22; N, 15.26%); ν_{max} (KBr)/cm^{-1} 3342, 3212, 1697, 1627, 1603, 1553, 1498, 1473, 1451, 1432, 1383, 1371, 1362, 1299, 1174, 1059; λ_{max} (MeOH)/nm 208 and 268; δ_H(400MHz; d_6-DMSO) 3.21 (2H, d, CH_2), 3.28 (3H, s, CH_3), 3.36 (3H, s, CH_3), 4.78 (1H, m, CH), 5.63 (1H, s, CH), 6.72 (1H, br s, NH), 7.35 (5H, m, CH, Ph.); δ_C(100MHz; d_6-DMSO) + DEPT 27.50 (CH_3), 29.59 (CH_3), 50.70 (CH_2), 69.96 (CH), 74.16 (CH), 126.37 (CH), 127.63 (CH), 128.52 (CH) and 143.07, 151.5, 153.22, 161.59 (Cq); m/z (ESI) 258.15 ($M-H_2O$), 276.16 ($M+H^+$) and 298.14 ($M+Na^+$); HRMS/ESI (Found; $M+H^+$, 276.1352. Calc. For $C_{14}H_{17}N_3O_3$ $M+H^+$, 276.1348).

6-(2-Hydroxy-propylamino)-1,3-dimethyluracil (164)



6-Chloro-1,3-dimethyluracil **(137)** (3g; 17.18 mmol) was suspended in water (8 ml) with agitation. To this was added 1-amino-2-propanol **(163)** (13.2ml, 171.8 mmol) and the reaction was heated at 100°C for 30 minutes. The solvent was removed under vacuum to give a yellow oil, which was purified by column chromatography (DCM:EtOH, 7:3). The solvent was removed from the combined fractions to give a white solid. This was recrystallised from EtOH to give the product (2.9g; 79%). R_f (DCM:EtOH, 7:3) 0.41; m.p. 132-134°C; (Found C, 50.56; H, 7.01; N, 19.62; $C_9H_{15}N_3O_3$ requires C, 50.69; H, 7.09; N, 19.71%); ν_{max} (KBr)/cm^{-1} 3426, 3315, 1683, 1598, 1548, 1467, 1429, 1379, 1340, 1305, 1240, 1116; λ_{max} (MeOH)/nm 203 and 267; δ_H (400MHz; d_6-DMSO) 1.08 (3H, d, CH_3), 2.98 (2H, m, CH_2), 3.09 (3H, s, CH_3), 3.30 (3H, s, CH_3), 3.84 (1H, m, CH), 4.72 (1H, s, CH), 6.61 (1H, br s, NH) and 7.23 (1H, br s, OH);

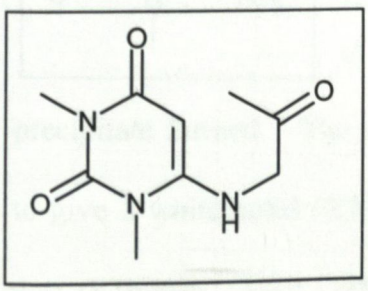
δ_C(100MHz; d_6-DMSO) + DEPT 20.98 (CH_3), 27.05 (CH_3), 50.01 (CH_2) 63.58 (CH), 73.50 (CH) and 151.51, 153.67, 161.57 Cq; m/z (ESI) 214.23 ($M+H^+$) and 236.18 ($M+Na^+$); HRMS/ESI (Found; $M+H^+$, 214.1190. Calc. For $C_9H_{16}N_3O_3$ $M+H^+$, 214.1192).

Swern oxidation of 6-(2-hydroxy-propylamino)-1,3-dimethyluracil (164)

Oxalyl chloride (488µl; 5.61 mmol) was dissolved in dry DCM (7ml) at -60°C (Acetone/ solid CO_2 bath) under N_2. To this, a dry DCM solution (7ml) of DMSO (639µl; 11.2 mmol) was added drop-wise over ten minutes. 6-(2-hydroxy-propylamino)-1,3-dimethyluracil **(164)** (1g; 4.65 mmol) was dissolved in dry DCM (40ml) under N_2 and added slowly to the reaction, maintaining a temperature of -55°C. The reaction solution was allowed to agitate for 30 minutes at which time it was quenched by the addition of TEA (3.3ml; 23.5mmol). TLC analysis of the reaction solution showed two main spots. Removal of the reaction solvents under vacuum at 30°C yielded a crude yellow solid, which was purified by column chromatography (silica) (DCM:EtOH, 9:1 – 8:2) yielding two products, analysis details of which are described below

Product 1

6-(2-Oxo-propylamino)-1,3-dimethyluracil (165)

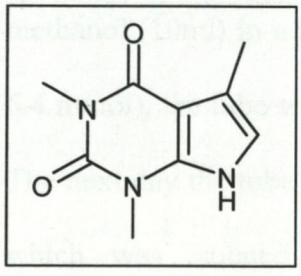


The product was isolated as a cream solid (0.106g; 10.7%). R_f (DCM:EtOH, 9:1) 0.48; δ_H(400MHz; $CDCl_3$) 2.33 (3H, s, CH_3), 3.31 (3H, s, CH_3), 3.47 (3H, s, CH_3), 3.97 (2H, d, CH_3), 5.30 (1H, s, CH), 4.48 (1H, br s, NH); δ_C(100MHz; $CDCl_3$) + DEPT 27.26 (CH_3), 27.70 (CH_3), 28.42 (CH_3),

52.19 (CH_2), 76.27 (CH) and 151.13, 153.45, 162.39, 200.36 (Cq); m/z (ESI) 212.12 ($M+H^+$); HRMS/ESI (Found; $M+H^+$, 212.2281. Calc. for $C_9H_{14}N_3O_3$ $M+H^+$, 212.2285)

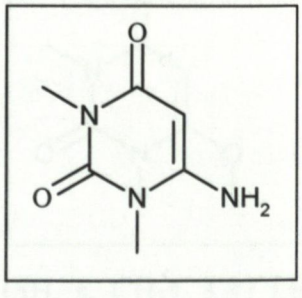
Product 2

1,3,5-Trimethyl-1,7-dihydro-pyrolo [2,3-*d*] pyrimidine (168)



The product was isolated as a white solid, which was recrystallised from EtOH to give white crystals (0.635g; 70%). R_f (DCM:EtOH, 9:1) 0.68; m.p. >250°C; ν_{max} (KBr)/cm^{-1} 3154, 1688, 1623, 1548, 1436; δ_H(400 MHz; $CDCl_3$) 2.28 (3H, s, CH_3), 3.35 (3H, s, CH_3), 3.50 (3H, s, CH_3), 5.89(1H, br s, NH) and 6.13 (1H, s, CH); δ_C(100 MHz; $CDCl_3$) + DEPT 10.87 (CH_3), 26.56 (CH_3), 29.07 (CH_3), 98.28 (Cq), 99.99 (CH) and 127.23, 151.10, 159.60, 160.32 (Cq); m/z (ESI) 194.21 ($M+H^+$) and 216.24 ($M+Na^+$); m/z (ESI) 194.21 ($M+H^+$); HRMS/ESI (Found; $M+H^+$, 194.2131. Calc. for $C_9H_{12}N_3O_2$ $M+H^+$, 194.2133).

6-Amino-1,3-dimethyluracil (169)



6-Chloro-1,3-dimethyluracil **(137)** (0.3g; 1.71 mmol) was suspended in 37% aqueous ammonia (15ml) and heated at 55°C over night. The yellow reaction solution was allowed to cool to room temperature at which time a white precipitate formed. The solid was isolated by filtration and washed with ice water to give a white solid (0.21g; 79.2%); m.p. >250 °C; R_f (DCM:EtOH, 9:1) 0.26; ν_{max} (KBr)/cm^{-1} 3396, 3351, 3230, 2949, 1657, 1614, 1496, 1450, 1213; δ_H(400 MHz; d_6-DMSO) 3.07 (3H, s, CH_3), 3.24 (3H, s, CH_3), 4.70 (1H, s, CH) and 6.75

(2H, s, NH_2); δ_C(100 MHz; d_6-DMSO) + DEPT 27.02 (CH_3), 29.26 (CH_3), 74.96 (CH) and 151.61, 154.90, 161.45 (Cq); m/z (ESI) 156.12 ($M+H^+$) and 195.16 ($M+K^+$).

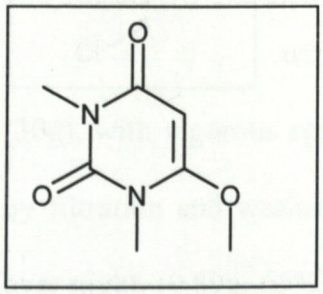
Reaction of 6-amino-1,3-dimethyluracil (169) with iodoacetone (171)

6-Amino-1,3-dimethyluracil **(169)** (1g; 6.4 mmol) was suspended in HPLC grade methanol (10ml) in a pressure tube. To this was added iodoacetone **(171)** (512 µl; 6.4 mmol), the tube was sealed and the suspension was heated over night at 120°C. The next day the tube was allowed to cool to RT at which time a precipitate formed which was isolated by filtration. Purification of the solid by column chromatography (DCM:EtOH, 9.5:0.5) yielded two pure products, **(168)** and **(170)**. The former **(168)** was isolated as a white solid, recrystallised from MeOH to give white crystals (0.34g; 40%).

All analytical data for (168)were as above.

Product 2.

1,3-Dimethyl-6-methoxyuracil (170)



This product was isolated as a white solid. This was recrystallised from MeOH to give a crystalline solid (0.21g; 38.5%). R_f (DCM:MeOH, 9:1) 0.72; m.p. 165°C; δ_H(400 MHz; $CDCl_3$) 3.30 (3H, s, CH_3), 3.33 (3H, s, CH_3), 3.87 (3H, s, CH_3) and 5.11 (1H, s, CH); δ_C(100 MHz; $CDCl_3$) + DEPT 27.35 (CH_3), 28.25 (CH_3), 56.39 (CH_3), 77.21 (CH, C-5) and 150.90, 160.88, 163.54 (Cq); m/z (ESI) 171.1 ($M+H^+$)and 193.1 ($M+Na^+$); HRMS/ESI (Found; $M+H^+$, 171.1758. Calc. for $C_7H_{10}N_2O_3$ requires $M+H^+$, 171.1760).

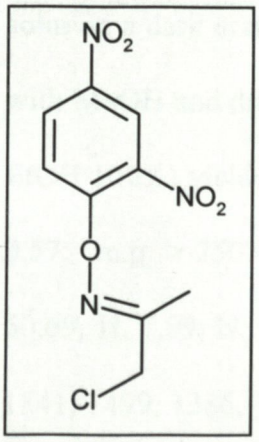
Method B

1,3-Dimethyl-6-methoxyuracil (170)

Sodium metal (0.05g; 2.2 mmol) was added to MeOH (15ml) with agitation to form a solution of sodium methoxide. To this solution was added 6-chloro-1,3-dimethyluracil **(137)** (0.2g; 1.1 mmol) and the reaction mixture was refluxed for two hours. The solvent was removed under vacuum to give a salt. This was dissolved in water and neutralised to pH 7 with aqueous HCl. The aqueous phase was extracted with ethyl acetate and cooling of the organic phase at -4°C yielded a white fluffy solid (0.18; 74%).

All analytical data were as above.

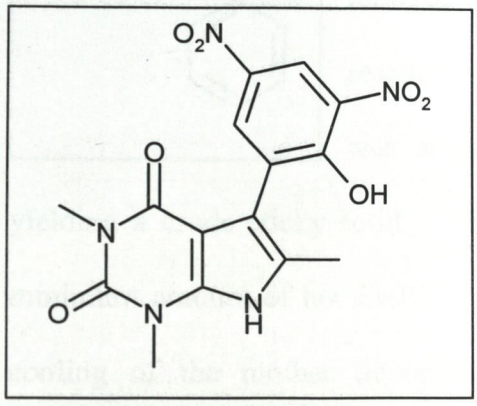
1-Chloro-propan-2-one O-(2,4-dinitro-phenyl)-oxime (175)



Chloroacetone **(174)** (322 µl; 5 mmol) was dissolved in EtOH (2ml) with agitation. To this was added a TFA solution (15ml) of O-(2,4-Dinitrophenyl)hydroxylamine **(125)** (1g; 5 mmol), drop wise over ten minutes with agitation. This yielded a dark yellow solution, which was left to stir for 30 min. at room temperature. The reaction solution was added slowly to ice (30g), with vigorous agitation to give a yellow precipitate. The solid was isolated by filtration and washed with water, dried and recrystallised from EtOH at –4 °C over night, (0.89g; 65%). R_f (DCM, 100%) 0.73; m.p. 93-95°C; (Found C, 39.23; H, 2.77; N, 15.16; $C_9H_8ClN_3O_5$ requires C, 39.51; H, 2.95; N, 15.36%); ν_{max} (KBr)/cm^{-1} 3099, 1602, 1525, 1471, 1340, 1303, 1264, 1234, 1142, 1130, 1067; λ_{max} (MeOH)/nm 212 and 271; δ_H(400MHz; $CDCl_3$) 2.35 (3H, s, CH_3), 4.27 (2H, s, CH_2); 7.94 (1H, d, CH, J = 9.5), 8.45 (1H, dd, CH, J = 7), 8.88 (1H, d, CH, J = 2.5);

δ_C(100MHz; $CDCl_3$) + DEPT 13.90 (CH_3), 43.67 (CH_2), 116.80 (CH), 121.57 (CH), 128.85 (CH) and 140.78, 154.23, 156.19, 162.11 (Cq).

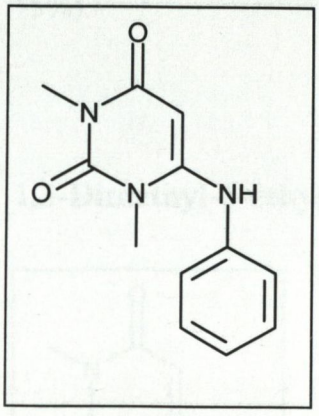
5-(2-Hydroxy-3,5-dinitro-phenyl)-1,3,6-trimethyl-1,7-dihydro-pyrolo[2,3-*d*]pyrimidine-2,4-dione (179)



6-Amino-1,3-dimethyluracil **(169)** (0.12g; 0.78 mmol) was suspended in MeOH (5ml) in a pressure tube. To this was added 1-chloro-propan-2-one O-(2,4-dinitro-phenyl)-oxime **(175)** (0.22g; 0.78 mmol) and KI (0.13g; 0.78mmol) as a methanol (5ml) suspension. The tube was sealed and allowed to heat at 120°C overnight. On cooling of the solution a dark orange precipitate was isolated by filtration. This solid was washed with MeOH and dried. Purification by column chromatography (DCM:EtOH, 9:1 – EtOH 100%) yielded a pure (TLC) orange solid (0.16; 56%). R_f (DCM:EtOH, 9:1) 0.57; m.p. > 250°C; (Found C, 50.56; H, 7.01; N, 19.62; $C_9H_{15}N_3O_3$ requires C, 50.69; H, 7.09; N, 19.71%); ν_{max} (KBr)/cm^{-1} 3401, 3132, 3046, 2948, 1684, 1635, 1541, 1499, 1386, 1335, 1249, 1233, 1158, 1098, 1055; λ_{max} (MeOH)/nm 204, 266 and 376; δ_H(400MHz; d_6-DMSO) 2.05 (3H, s, CH_3), 2.51 (3H, s, CH_3), 3.14 (3H, s, CH_3), 7.46 (1H, br s, NH), 7.79 (1H, d, CH, J = 3), 8.65 (1H, d, CH, J = 3) and 11.76 (1H, br s, OH); δ_C(100MHz; d_6-DMSO) + DEPT 11.71 (CH_3), 27.42 (CH_3), 30.43 (CH_3), 97.24 (Cq), 112.83 (Cq), 123.41 (CH), 125.05 (Cq), 126.99 (Cq), 128.83 (CH) and 130.33, 136.27, 137.98, 150.58, 157.86, 168.59 (Cq); m/z (ESI) 376.12 $M+H^+$); HRMS/ESI (Found; 376.0895 ($M+H^+$), Calc. For $C_{15}H_{14}N_5O_7$ requires ($M+H^+$), 376.0893).

Method A

1,3-Dimethyl-6-phenylaminouracil (189)



6-Chloro-1,3-dimethyluracil **(137)** (1g; 5.73 mmol) was suspended in EtOH (10 ml) and to this, was added aniline (1.33g; 14.33 mmol). The reaction suspension was refluxed for twelve hours. Following this the brown reaction solution was poured onto ice (15g) and the pH was adjusted to pH 1 with concentrated HCl (37%) yielding a crude sticky solid. This was isolated by filtration and dissolved in a minimum amount of hot EtOH. A hot charcoal filtration was carried out and on cooling of the mother liquor to room temperature; a white crystalline solid precipitated, (0.65g, 50%). R_f (DCM:EtOH, 9:1) 0.71; m.p. 236-237°C; δ_H(400MHz; $CDCl_3$) 3.29 (3H, d, CH_3), 3.54 (3H, s, CH_3), 4.98 (1H, s, CH), 6.64 (1H, br s, NH), 7.14 (2H, d, 2 x CH, J = 8), 7.25 (1H, dd, CH, J = 8), 7.35 (2H, t, 2 x CH, J = 8); δ_C(100MHz; $CDCl_3$) + DEPT 27.40 (CH_3), 28.99 (CH_3), 78.44 (CH), 125.41 (CH), 126.12 (CH), 129.24 (CH) and 136.60, 151.55, 152.47, 162.65 (Cq); m/z (ESI) 232.47 ($M+H^+$) and 463.48 (M_2+H^+).

Method B

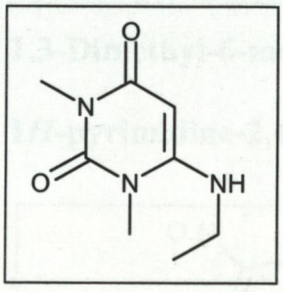
1,3-Dimethyl-6-phenylaminouracil (189)

6-Chloro-1,3-dimethyluracil **(137)** (1g; 5.73 mmol) was suspended in aniline (10 ml) in a pressure tube. The tube was sealed and the reaction mixture heated at 180°C for three hours. Following this the brown reaction solution was poured onto ice (15g) and the pH was adjusted to pH 1 with concentrated HCl (37%) yielding a light purple suspension. A dark purple solid was isolated by filtration and dissolved in a

minimum amount of hot EtOH. A hot charcoal filtration was carried out and on cooling of the mother liquor at -4°C; a white crystalline solid precipitated, (0.99g, 75%).

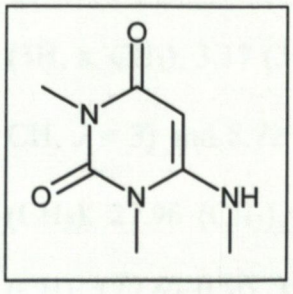
All analytical data were as above.

1,3-Dimethyl-6-ethylaminouracil (190)



6-Chloro-1,3-dimethyluracil **(137)** (1g; 5.73 mmol) was suspended in water (20 ml) To this, was added aqueous ethyl amine (4.3 ml; 30% w/v). The reaction suspension was refluxed for one hour and cooled to room temperature. The solvent was removed under vacuum, using EtOH and Et_2O to chase off all traces of the water. Once dry, a crude white solid was obtained. Recrystallisation from MeOH yielded a white product (0.61g; 58%); R_f (DCM:EtOH, 9:1) 0.51; m.p. 164 -165°C; δ_H(400MHz; $CDCl_3$) 1.15 (3H, t, CH_3), 2.96 (2H, m, CH_2), 3.09 (3H, s, CH_3), 4.64 (1H, s, CH), 6.69 (1H, br s, NH); δ_C(100MHz; $CDCl_3$) + DEPT 13.44 (CH_3), 27.03 (CH_3), 29.17 (CH_3), 37.11 (CH_2), 73.21 (CH) and 151.50, 153.13, 161.67 (Cq); m/z (ESI) 184.49 ($M+H^+$) and 206.49 ($M+Na^+$).

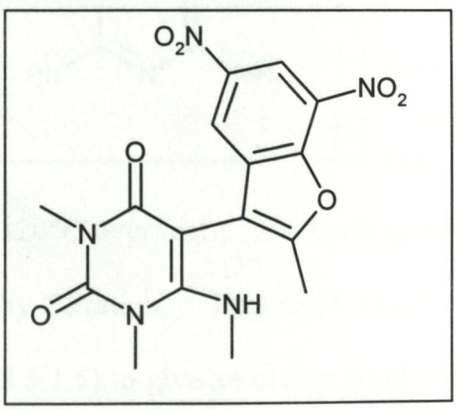
1,3-Dimethyl-6-methylaminouracil (191)



6-Chloro-1,3-dimethyluracil **(137)** (1g; 5.73 mmol) was suspended in water (20 ml), to this aqueous methyl amine (2.25 ml; 40% w/v) was added. The reaction suspension was refluxed for one hour, at which time most of the water was removed under vacuum. On cooling of the aqueous solution a white precipitate formed, this was isolated by filtration and washed with water. Recrystallisation

from EtOH yielded a white fluffy product (0.729; 75%); R_f (DCM:EtOH, 9:1) 0.38; m.p. 245-246°C; δ_H(400MHz; $CDCl_3$) 2.86 (3H, d, CH_3), 3.33 (3H, s, CH_3), 4.75 (3H, s, CH_3), 4.75 (1H, br s, NH), 4.85 (1H, s, CH); δ_C(100MHz; $CDCl_3$) + DEPT 27.29 (CH_3), 28.01 (CH_3), 29.37 (CH_3), 74.78 (CH) and 151.24, 153.46, 161.24 Cq; m/z (ESI) 170.24 ($M+H^+$).

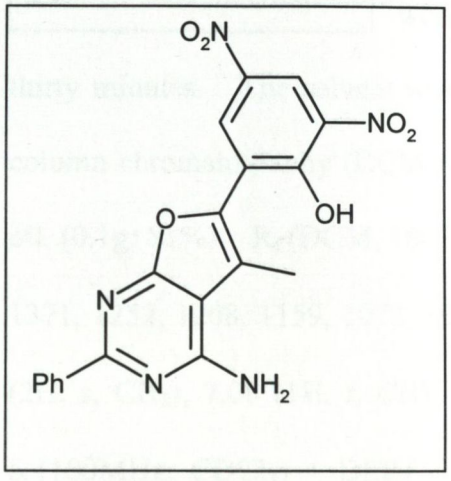
1,3-Dimethyl-6-methylamino-5-(2-methyl-5,7-dintro-3-benzo-furanyl)-1*H*-pyrimidine-2,4-dione (193)



1,3-Dimethyl-6-methylaminouracil **(191)** (0.12g; 0.73mmol) was suspended in MeOH (5ml) in a pressure tube. To this was added 1-chloropropan-2-one O-(2,4-dinitro-phenyl)-oxime **(175)** (0.20g; 0.73mmol) and KI (0.12g; 0.73mmol) as a methanol (5ml) suspension. The tube was sealed and allowed to heat at 120°C over night. On cooling of the solution a dark orange precipitate formed and was isolated by filtration. The solid was washed with MeOH and dried (0.14; 49%); R_f (DCM:EtOH, 9:1) 0.48; m.p. > 250°C; ν_{max} (KBr)/cm^{-1} 3102, 1686, 1615, 1561, 1534, 1458, 1385, 1315, 1265, 1174, 1100; λ_{max} (MeOH)/nm 219, 286 and 375; δ_H(400MHz; d_6-DMSO,) 2.09 (3H, s, CH_3), 3.17 (3H, d, CH_3), 3.78 (3H, s, CH_3), 3.85 (3H, s, CH_3), 8.16 (1H, d, CH, J = 3) and 8.72 (1H, d, CH, J = 3); δ_C(100MHz; d_6-DMSO) + DEPT 10.75 (CH_3), 27.96 (CH_3), 32.52 (CH_3), 33.13 (CH_3), 98.26 (Cq), 108.65 (Cq), 120.11 (CH), 127.67 (Cq), 128.87 (Cq), 131.34 (CH) and 136.57, 137.53, 138.73, 151.39, 155.796, 157.55, 178.71 (Cq); m/z (ESI) 390.52 ($M+H^+$), 412.09 ($M+Na^+$) and

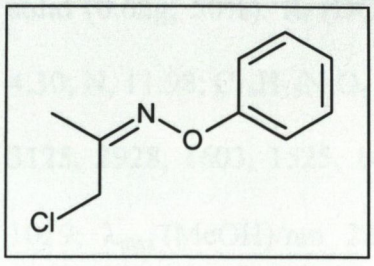
801.20 (M_2+H^+); HRMS/ESI (Found; 390.1067 M+H^+, Calc. For $C_{16}H_{16}N_5O_7$ requires M+H^+, 390.1050).

2-(4-Amino-2-phenyl-furo[2,3-*d*]pyrimidin-6-yl)-4,6-dinitrophenol (201)



6-Amino-2-phenyl-4(3*H*)-pyrimidione **(200)** (0.11g; 0.58mmol) was suspended in MeOH (6ml) in a pressure tube. To this was added 1-chloropropan-2-one O-(2,4-dinitro-phenyl)-oxime **(175)** (0.16g; 0.58mmol) and KI (0.10g; 0.58mmol) as a methanol (5ml) suspension. The tube was sealed and allowed to heat at 120°C over night. On cooling of the solution a dark brown precipitate was isolated by filtration. This solid was purified by column chromatography (DCM:MeOH, 8.5:1.5) to give an orange solid (0.08g; 36%); R_f (DCM:EtOH, 9:1) 0.39; m.p. 184-186°C; δ_H(400MHz; d_6-DMSO,) 3.17 (3H, s, CH_3), 6.84 (2H, br s, NH_2), 7.53 (2H, m, Ph), 8.11 (3H, s, CH_3), 8.14 (1H, d, CH, J = 3) and 8.65 (1H, d, CH, J = 3) and 12.06 (1H, br s, OH); δ_C(100MHz; d_6-DMSO) + DEPT 30.61 (CH_3), 104.92 (Cq), 109.55 (Cq), 121.06 (Cq), 127.23 (CH), 128.55 (CH), 127.21 (CH; Ph), 128.6 (CH; Ph) and 131.22, 132.91, 137.24, 147.94, 150.24, 159.01, 206.5 (Cq); m/z (ESI) 408.21 (M+H^+), 430.09 (M+Na^+); HRMS/ESI (Found; 408.0976 M+H^+, Calc. For $C_{19}H_{14}N_5O_6$ M+H^+, 408.0944).

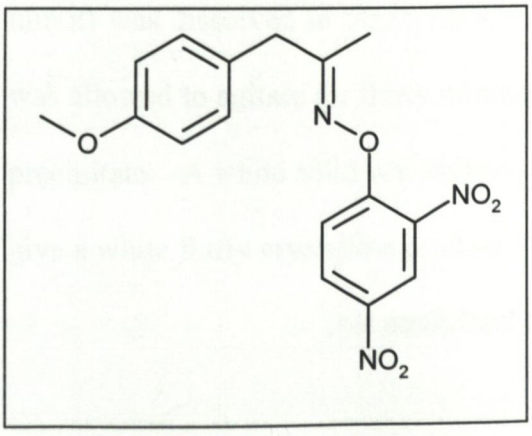
1-Chloropropan-2-one phenyl oxime (214)



Phenyl hydroxylamine hydrochloride **(212)** (0.1g; 0.69 mmol) was dissolved in EtOH (1.5 ml) at room temperature. Chloroacetone **(174)** (82 μl; 1.03 mmol) was added and the solution was heated at 40°*C* for thirty minutes. The solvent was removed to give a dark oil which as purified by column chromatography (DCM 9:1). The product was obtained as a clear yellow oil, (0.1g; 81%). R_f (DCM, 100%) 0.86; ν_{max} (Neat)/cm^{-1} 3040, 2950, 1590, 1487, 1371, 1252, 1208, 1159, 1072, 1023; δ_H(400MHz; $CDCl_3$) 2.22 (3H, s, CH_3), 4.25 (2H, s, CH_2), 7.08 (1H, t, CH), 7.24 (2H, d, 2 x CH) and 7.36 (2H, t, 2 x CH); δ_C(100MHz; $CDCl_3$) + DEPT 13.11 (CH_3), 55.05 (CH_2), 114.78 (CH), 122.56 (CH), 129.29 (CH) and 148.54, 156.53, 158.94 (Cq); m/z (ESI) 184.38 ($M + H^+$); HRMS/ESI (Found; $M+H^+$, 184.6448. Calc. For $C_9H_{11}ClNO$ requires $M+H^+$, 184.6451).

Method A

1-(4-Methoxy-phenlyl)-propan-2-one O-(2,4-dinitro-phenyl)-oxime (217)



4-Methoxyphenylacetone **(216)** (0.6g; 3.65 mmol) was dissolved in EtOH (1 ml) and to this was added O-(2,4-dinitro-phenyl)hydroxylamine **(125)** dissolved in a minimum amount of TFA. The brown reaction solution was allowed to agitate for thirty minutes and it was then added to ice with vigorous agitation. This yielded a brown oil which was left at room temperature for two days to solidify. A crude

brown solid was isolated by filtration and a hot charcoal filtration yielded a white solid (0.62g; 50%). R_f (DCM 100%) 0.8; m.p. 112-114°C; (Found C, 55.51; H, 4.30; N, 11.98; $C_{16}H_{15}N_3O_6$ requires C, 55.65; H, 4.38; N, 12.17%); ν_{max} (KBr)/cm^{-1} 3125, 2928, 1603, 1525, 1473, 1342, 1298, 1249, 1175, 1145, 1130, 1107, 1060, 1029; λ_{max} (MeOH)/nm 224 and 292; δ_H(400MHz; d_6-DMSO) 2.02 (3H, s, CH_3), 3.66 (2H, s, CH_2), 3.74 (3H, s, CH_3), 6.92 (2H, d, 2 x CH, J = 8.52), 7.24 (2H, d, 2 x CH, J = 8.56), 7.95 (1H, d, CH, J = 8.52), 8.56 (1H, dd, CH, J = 9.56), 8.81 (1H, d, CH, J = 3.04) δ_C(100MHz; d_6-DMSO) + DEPT 15.19 (CH_3), 40.01 (CH_2), 55.03 (CH_3), 114.16 (CH), 121.87 (2 x CH), 121.88 (2 x CH), 127.00 (Cq), 130.04 (CH), 130.17 (CH) and 135.76, 140.36, 156.35, 158.37, 166.99 (Cq); m/z (ESI) 346.38 ($M+H^+$); HRMS/ESI (Found; $M+H^+$, 346.3189. Calc. For $C_{16}H_{15}N_3O_6$ $M+H^+$, 346.3196).

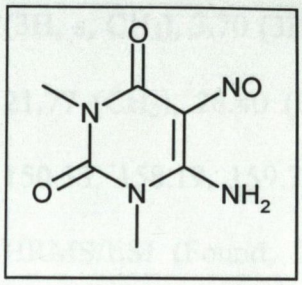
Method B

1-(4-Methoxy-phenlyl)-propan-2-one O-(2,4-dinitro-phenyl)-oxime (217)

4-Methoxyphenylacetone **(216)** (0.5g; 3.04 mmol) was dissolved in EtOH (2 ml) and to this was added O-(2,4-Dinitrophenyl)hydroxylamine **(125)** (0.64g; 3.04 mmol) was dissolved in 50:50 EtOH:H_2SO_4 (97%). The brown reaction solution was allowed to agitate for thirty minutes at room temperature, which yielded a thick precipitate. A white solid was isolated by filtration and recrystallised from EtOH to give a white fluffy crystalline product (0.75g; 72%).

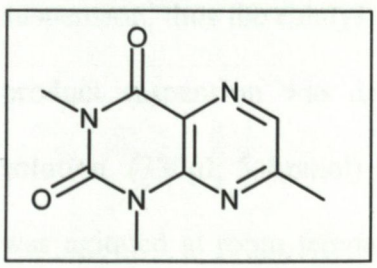
All analytical data were as above.

6-Amino-1,3-dimethyl-5-nitrosouracil (222)



6-Amino-1,3-dimethyluracil **(169)** (1.5g; 9.7 mmol) was suspended in water (15 ml), to which was added glacial acetic acid (2.5 ml). To this was added an aqueous solution (3ml) of sodium nitrite (1.07g, 15.5 mmol), which caused the reaction suspension to turn bright purple. The reaction was agitated for three hours at room temperature, at which time a purple solid was isolated by filtration, washed with EtOH and dried. (1.75g; 98%). R_f (DCM:EtOH, 8:2) 0.31; m.p. 239°C; λ_{max} (MeOH)/nm 227 and 318; δ_H (400MHz; d_6-DMSO) 3.28 (3H, d, CH_3), 3.35 (3H, s, CH_3), 9.05 (1H, s, CH); δ_C(100MHz; d_6-DMSO) + DEPT 27.84 (CH_3), 28.67 (CH_3) and 139.14, 146.09, 149.38, 160.21 (Cq); m/z (ESI) 185.34 ($M+H^+$).

1,3,7-Trimethyl-1*H*-pteridine-2,4-dione (221)



6-Amino-1,3-dimethyl-5-nitrosouracil **(222)** (0.3g; 1.62 mmol) was suspended in degassed water (30ml). To this was added Raney Nickel® (1ml), and the suspension was agitated under an atmosphere of hydrogen until gas uptake ceased. The reaction remained as a suspension, thus the catalyst was allowed to settle on the bottom of the flask and the product suspension was decanted off. This was added to 1M HCl (15ml) containing pyruvic aldehyde (198µl; 3.24mmol) and the reaction was allowed to agitate for four hours at room temperature in the dark. The aqueous reaction solution was extracted with DCM and the organic extracts were combined and dried over $MgSO_4$. The solvent was removed under vacuum to give a cream solid. This was recrystallised from EtOH to give white needles (0.28g; 84%). R_f (DCM:EtOH, 9:1) 0.57; m.p. 150-152; λ_{max}

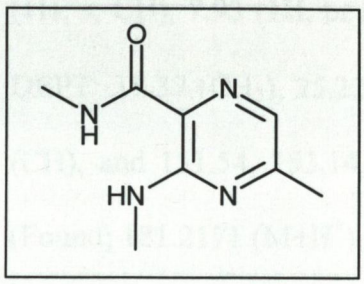
(MeOH:H2O, 4:6)/nm 238 and 329; δ_H(400MHz; $CDCl_3$) 2.69 (3H, s, CH_3), 3.51 (3H, s, CH_3), 3.70 (3H, s, CH_3), 8.436 (1H, s, CH); δ_C(100MHz; $CDCl_3$) + DEPT 21.77 (CH_3), 28.40 (CH_3), 28.66 (CH_3), 124.73 (Cq), 139.88 (CH) and 147.04, 150.33, 158.19, 159.72 (Cq); m/z (ESI) 207.3 ($M+H^+$) and 229.15 ($M + Na^+$); HRMS/ESI (Found; 207.2118 ($M+H^+$), Calc. For $C_9H_{11}N_4O_2$ requires ($M+H^+$), 207.2121).

Reaction of 5,6-Diamino-1,3-dimethyluracil (66) with pyruvic aldehyde (223) in aqueous sodium hydroxide

6-Amino-1,3-dimethyl-5-nitrosouracil **(222)** (1g; 5.14 mmol) was suspended in degassed water (40ml), which contained sodium hydroxide (0.21g; 5.4mmol). To this Raney Nickel® (1.5ml) was added and the reaction was agitated under an atmosphere of hydrogen, till hydrogen uptake ceased. The reaction remained as a suspension, thus the catalyst was allowed to settle on the bottom of the flask and the product suspension was decanted off. Pyruvic aldehyde **(223)** as a 40% aq. solution. (330µl; 5.4mmol) was added to the decanted suspension and the reaction was agitated at room temperature for three hours. The aqueous reaction solution was extracted with DCM and the organic extracts were combined and dried over $MgSO_4$. The solvent was removed under vacuum to give a cream solid, TLC analysis showed three main spots. This mixture was purified by column chromatography (DCM:EtOH, 9.7:0.3) to give a mixture of **(220)** and **(221)**, and **(224)** and **(225)** in pure form.

Product 1

7-Methyl-2-methylaminopyrazine-3-carboxylic acid methylamide (224)



R_f (DCM:EtOH, 9:1) 0.89; (Found C, 53.12; H, 6.68; N, 30.97; $C_8H_{12}ClN_4O$ requires C, 53.32; H, 6.71; N, 31.09%); m.p. 79-80°C; λ_{max} (MeOH:H2O, 4:6)/nm 215, 254 and 382; δ_H(400MHz; $CDCl_3$) 2.43 (3H, s, CH_3), 2.96 (3H, d, CH_3), 3.03 (3H, d, CH_3), 7.50 (1H, s, CH), 7.81 (1H, br s, NH) and 8.51 (1H, br s, NH); δ_C(100MHz; $CDCl_3$) + DEPT 21.407 (CH_3), 25.23 (CH_3), 26.61 (CH_3), 123.50 (Cq), 128.12 (CH) and 154.13, 155.79, 166.99 (Cq); m/z (ESI) 181.31 ($M+H^+$); HRMS/ESI (Found; 181.2169 ($M+H^+$), Calc. For $C_8H_{13}N_4O$ requires ($M+H^+$), 181.2175).

Method B

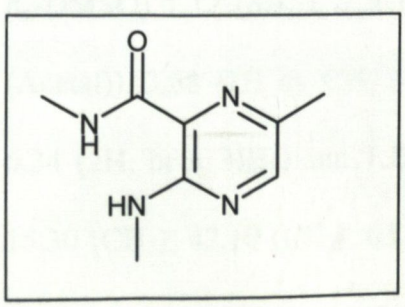
7-Methyl-2-methylaminopyrazine-3-carboxylic acid methylamide (224)

1,3,7-trimethyl-1*H*-pteridine-2,4-dione (0.25g; 1.2 mmol) **(221)** was dissolved in aqueous sodium hydroxide (0.08g; 2.42mmol, 5ml water) and agitated over night. The next day, a white precipitate was isolated by filtration, washed with water and dried, (0.17g; 78%).

All analytical data as above

Product 2

5-Methyl-2-methylaminopyrazine-3-carboxylic acid methylamide (225)

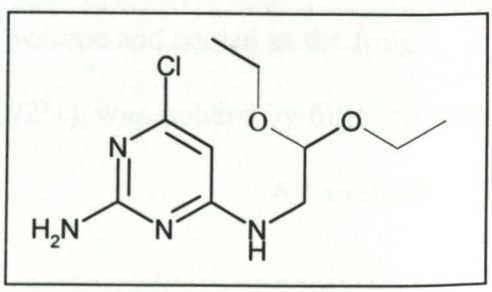


The product was isolated as a cream solid. R_f (DCM:EtOH, 9:1) 0.76; (Found C, 53.37; H, 6.72; N, 31.11; $C_8H_{12}ClN_4O$ requires C, 53.32; H, 6.71;

N, 31.09%); m.p. 80-81°C; λ_{max} (MeOH:H2O, 4:6)/nm 227, 261 and 371; δ_H(400MHz; $CDCl_3$) 2.36 (3H, s, CH_3), 2.95 (3H, d, CH_3), 3.01 (3H, d, CH_3), 7.28 (1H, s, CH), 7.93 (1H, br s, NH) and 8.36 (1H, br s, NH); δ_C(100MHz; $CDCl_3$) + DEPT 19.37 (CH_3), 25.22 (CH_3), 26.86 (CH_3), 121.86 (Cq), 135.84 (Cq), 145.242 (CH), and 151.54, 153.14, 166.84 (Cq); m/z (ESI) 181.31 ($M+H^+$); HRMS/ESI (Found; 181.2171 ($M+H^+$), Calc. For $C_8H_{13}N_4O$ requires ($M+H^+$), 181.2175).

Method A

2-Amino-4-chloro-6-(2,2-diethoxy-ethylamino)-pyrimidine (236)



2-Amino-4,6-dichloropyrimidine **(234)** (0.3g; 1.83 mmol) was dissolved in EtOH (10 ml) with heat. To this was added, aminoacet-aldehyde diethyl acetal **(138)** (266 µl) and K_2CO_3 (0.25g 1.83 mmol) to give a suspension, which was refluxed for one hour. The insoluble base was removed over celite® and the mother liquor was cooled at -4°C to give a batch of white solid, later identified to be **(237)**. On cooling of the mother liquor for a further 12 hours at -4°C, a second crop of white solid was the target molecule **(236)**was isolated by filtration (0.27g; 58%). R_f (DCM:EtOH, 9.5:0.5) 0.52; m.p. 143°C; (Found C, 45.85; H, 6.55; N, 21.35; $C_{10}H_{17}ClN_4O_2$ requires C, 46.07; H, 6.57; N, 21.49%); ν_{max} (KBr)/cm^{-1} 3319, 2978, 2891, 1649, 1595, 1475, 1327, 1248, 1115, 1042,; λ_{max} (MEOH)/nm 238 and 284; δ_H(400MHz; d_6-DMSO) 1.12 (6H, t, 2 x CH_3 (Acetal)), 3.30 (2H, s, CH_2), 3.48 (2H, m, CH_2 (Acetal)), 3.58 (2H m, CH_2 (Acetal)), 4.54 (1H, t, CH (Acetal)), 6.38H, s, 5-CH), 6.34 (2H, br s, NH_2) and 7.13 (1H, br s, NH); δ_C(100MHz; d_6-DMSO) + DEPT 15.30 (CH_3), 42.10 (CH_2), 61.57 (CH_2), 87.62 (CH), 100.21 (CH, 5-C) and 146.50,

162.86, 164.10 (Cq); m/z (ESI) 261.35, 263.34 ($M+H^+$); HRMS/ESI (Found; $M+H^+$, 261.7309. Calc. For $C_{10}H_{17}ClN_4O_2$ requires $M+H^+$, 261.7313).

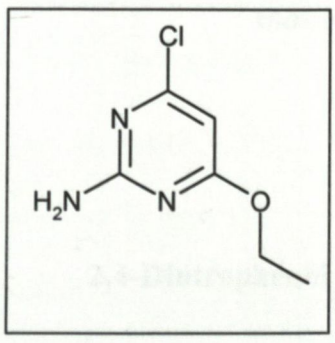
Method B

2-Amino-4-chloro-6-(2,2-diethoxy-ethylamino)-pyrimidine (236)

2-Amino-4,6-dichloropyrimidine **(234)** (0.5g; 3.05 mmol) was dissolved in EtOH (15 ml) with heat. To this was added, $NaHCO_3$ (0.25g 1.83 mmol) and aminoacetaldehyde diethyl acetal **(138)** (798 µl; 9.15 mmol) and the reaction suspension was refluxed for three hours. The insoluble base was removed over celite® while the solution was still boiling. The mother liquor was reduced in volume and cooled in the fridge. After three hours a white crystalline solid, (0.57g; 72%), was isolated by filtration, washed with EtOH and Et_2O and dried.

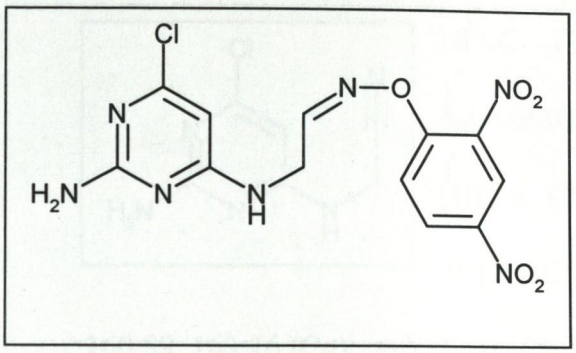
All analysis results were identical to above

1,3-Dimethyl-6-ethoxyuracil (237)



2-Amino-4,6-dichloropyrimidine **(234)** (0.5g; 3.05 mmol) was dissolved in EtOH (15 ml) with heat. NaOH (0.07g, 1.83 mmol) was added to the solution and the reaction was refluxed for three hours. The solvent was removed under vacuum to give a crude white solid; recrystallisation of this from EtOH gave transparent crystals, (0.47g; 89%). R_f (DCM:EtOH, 9.5:0.5) 0.33; m.p. 89-90°C; δ_H(400MHz; d_6-DMSO) 1.32 (3H t, CH_3), 4.25 (2H q, CH_3), 5.78 (2H br s, NH_2) and 6.03 (1H, s, CH); δ_C(100MHz; d_6-DMSO) + DEPT 13.81 (CH_3), 62.18 (CH_2), 96.14 (CH_2) and 106.08, 162.15, 170.68 (Cq); m/z (ESI) 174.34, 176.34 ($M+H^+$).

(2-Amino-4-chloro-pyrimidin-4-ylamino)acetaldehyde-O-(2,4-dinitro-phenyl)-oxime (240)

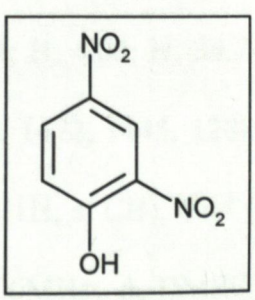


2-Amino-4-chloro-6-(2,2-diethoxy-ethylamino)-pyrimidine **(236)** (0.2g; 0.77 mmol) was suspended in 1M H_2SO_4 (15 ml) and agitated over night at room temperature to yield **(238)** *in situ.* To the clear solution that formed was added slowly, 2,4-dinitrophenyl hydroxylamine **(125)** (0.15g; 0.77 mmol) as a 5M H_2SO_4 (5 ml) solution. The dark yellow reaction solution was allowed to agitate at room temperature over night, at which time a cream precipitate was isolated by filtration, washed with water and EtOH and dried (0.17g; 60%). R_f (DCM:EtOH, 7:3) 0.73; m/z (ESI) 368.08 ($M+H^+$); HPLC (85:15 H_2O:MeOH) R_t (4.7 min.); HRMS/ESI (Found; 368.0527, $M+H^+$, Calc. For $C_{12}H_{11}ClN_7O_5Cl$ requires $M+H^+$, 368.0510).

NOTE: *NMR analysis of this compound in d_6-DMSO showed it had decomposed to two new products.*

Product 1

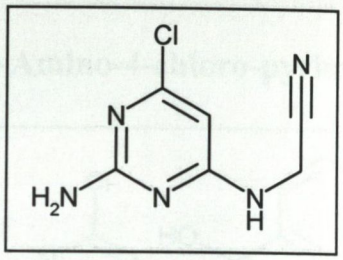
2,4-Dintrophenol (132)



HPLC (H_2O:MeOH, 85:15) R_t 1.48 min.; δ_H(400MHz; d_6-DMSO) 7.31 (1H, d, CH), 8.36 (1H, dd, CH), 8.69 (1H, d, CH); δ_C(100MHz; d_6-DMSO) + DEPT 119.65 (CH), 121.83 (CH), 129.44 (CH) and 136.56, 138.49, 157.21 (Cq); m/z (ESI) 183.27 ($M-H^+$).

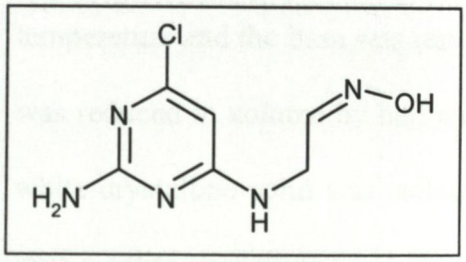
Product 2

(2-Amino-4-chloro-pyrimidn-6-ylamino)-acetonitrile (241)



HPLC (H_2O:MeOH, 85:15) R_t 3.06 min; δ_H(400MHz; d_6-DMSO) 4.35 (2H, d, CH_2), 6.01 (1H, s, CH); δ_C(100MHz; d_6-DMSO) + DEPT 28.68 (CH_2), 93.76 (CH) and 117.61, 149.52, 160.89, 163.16 (Cq); m/z (ESI) 184.24, 186.26 ($M+H^+$).

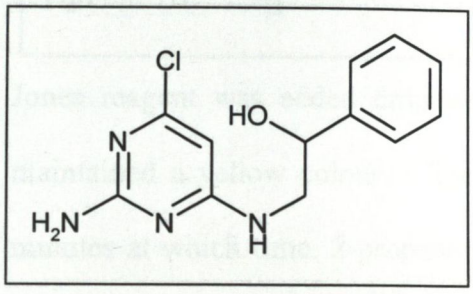
(2-Amino-4-chloro-pyrimidin-4-ylamino)-acetaldehyde oxime (245)



2-Amino-4-chloro-6-(2,2-diethoxy-ethylamino)-pyrimidine **(236)** (1g; 3.85mmol) was agitated overnight in 1M H_2SO_4 (15ml) to yield **(238)** *in situ*. The next day hydroxylamine hydrochloride (0.53g; 7.7mmol) was added to the acidic reaction solution with agitation. The reaction was agitated at room temperature for 45 minutes and neutralised to pH 7 with 3M NaOH. On reaching pH 7 a white solid precipitated which was isolated by filtration, washed with EtOH and dried. The solid was recrystallised from EtOAc to give a white crystalline solid (0.52g; 67%). R_f (DCM:EtOH, 9.5:0.5) 0.24; m.p. 190-193°C; (Found C, 35.69; H, 4.02; N, 34.82; $C_6H_8ClN_5O$ requires C, 35.74; H, 4.00; N, 34.74%); ν_{max} (KBr)/cm^{-1} 3501, 3300, 3155, 2852, 2782, 1637, 1541, 1472, 1445, 1288, 1247, 1163; δ_H(400MHz; d_6-DMSO) 3.95 (2H, s, CH_2), 5.78 (1H, s, CH), 6.44 (1H, s, CH), 7.32 (2H, br s, NH_2) and 10.74 (1H, br s, OH); δ_C(100MHz; d_6-DMSO) + DEPT 39.24 (CH_2), 92.60 (CH), 146.39 (CH) and 162.85, 163.99, 167.08 Cq; m/z (ESI) 202.64 ($M+H^+$) and 234.38 ($M+Na^+$);

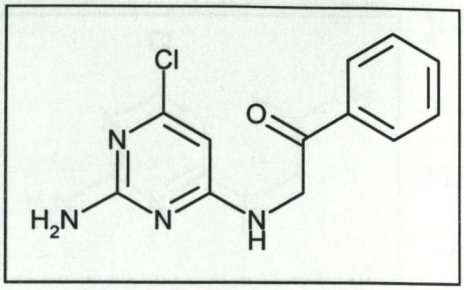
HRMS/ESI (Found; $M+H^+$, 202.6229. Calc. For $C_6H_8ClN_5O$ requires $M+H^+$, 202.6232).

2-(2-Amino-4-chloro-pyrimidin-6-ylamino)-1-phenyl-ethanol (247)



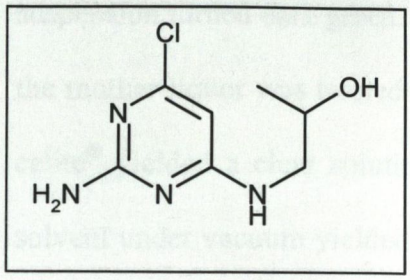
2-Amino-4,6-dichloropyrimidine **(234)** (1.5g; 9.34 mmol) was dissolved in EtOH (30 ml) at 50°C. $NaHCO_3$ (0.77g; 9.34 mmol) and 2-amino-1-phenylethanol (2.51 g; 18.3 mmol) as an EtOH suspension (5ml) were added with agitation, and the reaction suspension was refluxed for five hours. The reaction solvent was allowed to cool to room temperature and the base was removed by filtration over celite®. The mother liquor was reduced in volume by half and the solution was cooled over night at –4°C. A white crystalline solid was isolated by filtration and recrystallised form ethanol to give a white crystalline solid (2.2g; 89%). R_f (DCM:EtOH, 9.5:0.5) 0.49; m.p. 163-165°C; (Found C, 52.47; H, 4.69; N, 21.51; $C_{12}H_{13}ClN_4O$ requires C, 54.45; H, 4.95; N, 21.16%); ν_{max} (KBr)/cm^{-1} 3420, 3326, 3242, 3098, 1645, 1608, 1541, 1456, 1409, 1359, 1280, 1213, 1163, 1857, 1030; δ_H(400MHz; d_6-DMSO) 3.22 (1H, m, CH, N-*CH*), 3.57 (1H, m, CH, N-*CH*), 4.73 (1H, d, CH), 5.56 (1H, d, OH), 5.86 (1H, s, CH), 6.45 (2H, br s, NH_2), 7.25 (2H, t, 2 x CH), 7.34 (2H, t, 2 x CH) and 7.41 (1H, d, CH); δ_C(100MHz; d_6-DMSO) + DEPT 48.26 (CH_2), 71.17 (CH), 92.95 (CH), 126.02 (CH), 127.03 (CH), 128.01 (CH) and 143.93, 157.24, 162.85, 164.10 (Cq); m/z (ESI) 265.34, 267.37 ($M+H^+$) and 529.14, 531.12 (M_2+H^+); HRMS/ESI (Found; $M+H^+$, 265.7217. Calc. for $C_{12}H_{13}ClN_4O$ $M+H^+$, 265.7222).

2-(2-Amino-4-chloro-pyrimidin-6-ylamino)-1-phenyl-ethanone (248)



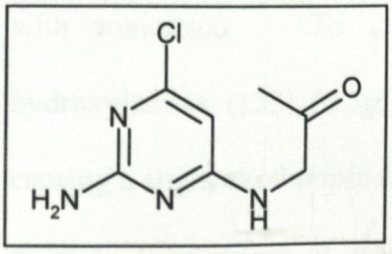
2-Amino-4-chloro-6-(Hydroxy-2'-phenyl-ethylamino)-1,3-dimethyluracil **(247)** (1g; 3.78 mmol) was dissolved in acetone (40 ml) at room temperature with agitation. To this, Jones reagent was added drop wise with agitation until the suspension formed, maintained a yellow colour. The reaction solution was agitated for a further 15 minutes at which time, 2-propanol was added until the reaction turned dark green. The chromate salts were removed by filtration over celite®, and the mother liquor was treated with charcoal at 60°C. Removal of the charcoal over celite® yielded a clear solution, which was dried over $MgSO_4$. Removal of the solvent, under vacuum yielded a white solid and this was recrystallised from EtOH to give the product (0.53g; 53%). R_f (DCM:EtOH, 9.5:0.5) 0.53; m.p. 186-188°C; (Found C, 55.02; H, 4.28; N, 21.49; $C_{12}H_{11}ClN_4O$ requires C, 54.87; H, 4.22; N, 21.33%); ν_{max} (KBr)/cm^{-1} 3193, 1688, 1654, 1566, 1469, 1449, 1410, 1341, 1248, 1162; λ_{max} (MeOH)/nm 239 and 285; δ_H(400MHz; d_6-DMSO) 3.33 (2H, s, CH_2), 5.97 (1H, s, CH), 6.52 (2H, br s, NH_2), 7.45 (2H, t, 2 x CH), 7.66 (2H, t, 2 x CH) and 8.02 (1H, d, CH); δ_C(100MHz; d_6-DMSO) + DEPT 50.41 (CH_2), 71.17 (CH), 93.14 (CH), 126.94 (CH), 128.04 (CH) and 145.52, 158.41, 163.19, 164.67, 171.34 (Cq); m/z (ESI) 263.68, 265.71 ($M+H^+$); HRMS/ESI (Found; $M+H^+$, 263.7056. Calc. for $C_{12}H_{11}ClN_4O$ $M+H^+$, 263.7063).

1-(2-Amino-4-chloro-pyrimidin-6-ylamino)-propan-2-ol (249)



2-Amino-4,6-dichloropyrimidine **(234)** (3g; 18.27 mmol) was dissolved in EtOH (60 ml) at 50°C. $NaHCO_3$ (1.5g; 18.27 mmol) and 2-amino-1-propanol (1.88 ml; 73.08 mmol) were added and the reaction was refluxed for five hours. The solvent was allowed to cool to room temperature and the base was removed by filtration over celite®. The mother liquor was reduced in volume by half and the solution was cooled overnight at –4°C. A white crystalline solid was isolated by filtration and recrystallised from EtOH (2.8g; 76%). R_f (DCM:EtOH, 9.5:0.5) 0.53; m.p. 140-142°C; (Found C, 41.52 H, 5.35; N, 27.42; $C_7H_{11}ClN_4O$ requires C, 41.49; H, 5.47; N, 27.65%); ν_{max} (KBr)/cm^{-1} 3389, 3319, 3162, 2971, 2924, 2872, 1649, 1547, 1480, 1450, 1357 1306, 1266, 1129; λ_{max} (MEOH)/nm 238 and 284; δ_H(400MHz; d_6-DMSO) 1.04 (3H, d, CH_3), 3.12 (1H, br s, NH-*CH*), 3.24 (1H, br s, NH-*CH*), 3.71 (1H, m, CH), 4.75 (1H, br s, OH), 5.81 (1H, s, CH), 6.38 (1H, s, CH), 7.14 (1H, br s, NH); δ_C(100MHz; d_6-DMSO) + DEPT 21.22 (CH_3), 47.57 (CH_2), 65.02 (CH), 92.84 (CH) and 157.05, 162.84, 164.17 (Cq); m/z (ESI) 185.38 ($M-H_2O$), 203.38, 205.38 ($M+H^+$); HRMS/ESI (Found; $M+H^+$, 203.6509. Calc. for $C_7H_{11}ClN_4O$ $M+H^+$, 203.6513).

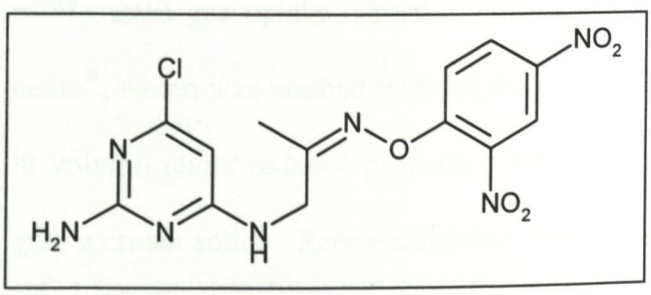
1-(2-Amino-4-chloro-pyrimidin-6-ylamino)-propan-2-one (250)



1-(2-Amino-6-chloro-pyrimidin-4-ylamino)-propan-2-ol **(249)** (1g; 4.93 mmol) was dissolved in acetone (60 ml) with sonication. To this, Jones reagent was added drop wise with agitation until the suspension that formed remained as a yellow colour. The reaction solution was agitated for a

further 15 minutes at which time, 2-propanol was added until the reaction suspension turned dark green. The chromate salts were filtered off over celite®, and the mother liquor was treated with charcoal at 60°C. Removal of the charcoal over celite® yielded a clear solution, which was dried over $MgSO_4$. Removal of the solvent under vacuum yielded a white solid, which was recrystallised from EtOH to give the product, (0.68g; 69%). R_f (DCM:EtOH, 9.5:0.5) 0.65; m.p. 141-143°C; (Found C, 41.37; H, 4.48; N, 27.82; $C_7H_9ClN_4O$ requires C, 41.49; H, 4.52; N, 27.93%); ν_{max} (KBr)/cm^{-1} 3155, 1728, 1697, 1577, 1463, 1408, 1355, 1237, 1160, 1133; δ_H(400MHz; d_6-DMSO) 2.12 (3H, s, CH_3), 3.38 (2H, s, CH_2), 4.37 (2H,br s, NH_2), 6.27 (1H, s, CH) and 7.93 (1H, br s, NH); δ_C(100MHz; d_6-DMSO) + DEPT 31.14 (CH_3), 52.88 (CH_2), 93.15 (CH) and 135.60, 138.39, 148.33, 156.06 (Cq); m/z (ESI) 201.71, 203.68; HRMS/ESI (Found; $M+H^+$, 201.6351. Calc. for $C_7H_9ClN_4O$ $M+H^+$, 201.6354).

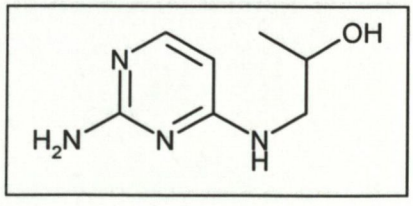
1-(2-Amino-4-chloro-pyrimidin-6-ylamino)-propan-2-one O-2,4-dinitro-phenyl)-oxime (251)



1-(2-Amino-4-chloro-pyrimidin-6-ylamino)-propan-2-one **(250)** (0.5g; 2.5 mmol) was dissolved in HPLC grade MeOH (10ml) with sonication. To this clear solution was added O-(2,4-dinitrophenyl)-hydroxylamine **(125)** (0.5g; 2.5 mmol), dissolved in 50:50 MeOH:H_2SO_4 (3 ml) causing a slight exothermic reaction. The reaction solution was agitated overnight at room temperature at which time a suspension had formed. The solid was removed by filtration, washed with EtOH and dried under vacuum, (0.43g; 45%).

R_f (DCM:EtOH, 9.5:0.5) 0.81; m.p. 176-178°C; (Found C, 41.01; H, 3.21; N, 25.72; $C_{13}H_{12}ClN_7O_5$ requires C, 40.90; H, 3.17; N, 25.68%); ν_{max} (KBr)/cm^{-1} 3135, 1672, 1607, 1533, 1457, 1342, 1315, 1182, 1069, 1046, 1010; λ_{max} (MeOH)/nm 237 and 289; δ_H(400MHz; d_6-DMSO) 2.51 (3H, s, CH_3), 4.85 (2H, s, CH_2), 5.71 (1H, s, CH), 7.46 (1H, d, CH), 7.69 (1H, dd, CH), 7.93 (1H, d, CH); δ_C(100MHz; d_6-DMSO) + DEPT 47.06 7.46 (CH_3), 61.12 (CH_2), 94.68 (CH), 124.83 (CH), 128.74 (CH), 134.42 (CH) and 138.24, 149.93, 153.41, 163.42, 165.17, 168.42, 171.10 (Cq); m/z (ESI) 382.76, 384.69 ($M+H^+$); HRMS/ESI (Found; $M+H^+$, 382.7429. Calc. for $C_{13}H_{12}ClN_7O_5$ requires $M+H^+$, 382.7431).

1-(2-Amino-pyrimidin-6-ylamino)-propan-2-ol (256)



2-Amino-4-chloro-6-(2-hydroxy-propylamino)-1,3-dimethyluracil **(249)** (0.5g; 2.48 mmol) was dissolved in degassed EtOH (40ml). To this solution was added water (20ml), magnesium oxide (0.2g; 4.95 mmol) and 10% Pd/C catalyst (0.05g). The reaction suspension was agitated under an atmosphere of H_2, until gas uptake ceased. The reaction suspension was then filtered over celite®, which was washed with EtOH (2 x 25ml). The mother liquor was reduced in volumn under reduced pressure, with traces of water azeotroped using DCM to give a cream solid. Recrystallisation from EtOAc yielded a white crystalline solid (0.28g; 67%). R_f (DCM:EtOH, 9:1) 0.41; m.p.193-195°C; (Found C, 51.26; H, 7.42; N, 33.64; $C_7H_{12}N_4O$ requires C, 49.99; H, 7.19; N, 33.31%); ν_{max} (KBr)/cm^{-1} 3183, 2871, 1670, 1590, 1519, 1445, 1289, 1151, 1076; λ_{max} (MeOH)/nm 238 and 267; δ_H(400MHz; d_6-DMSO) 1.06 (3H, d, CH_3), 3.22 (1H, m, CH, NH-*CH*), 3.33 (1H, m, NH-*CH*), 3.79 (1H, q, CH), 5.00 (1H, br s, OH), 6.21 (1H, d, CH), 7.67 (1H,

d, CH), 7.84 (2H, br s, NH_2), 9.01 (1H, d, NH); δ_C(100MHz; d_6-DMSO) + DEPT 21.15 (CH_3), 47.89 (CH_2), 64.34 (CH), 140.04 (CH), and 155.33, 162.73 (Cq); m/z (ESI) 169.26 ($M+H^+$); HRMS/ESI (Found; $M+H^+$, 169.2061. Calc. for $C_7H_{12}ClN_4O$ $M+H^+$, 169.2065).

Bibliography

[1] F.G. Hopkins, *Nature (London),* 1889, **40**, 335.

[2] H.Wieland and C.Schöpf, *Chem. Ber.*, 1925, **58**, 2178.

[3] C. Schöpf, R. Reichert and K. Riefstahl, *Liebigs Ann. Chem.*, 1941, **548**, 284.

[4] C. Schöpf and H.Wieland, *Chem. Ber.*, 1926, **59**, 2067.

[5] R. Purrmann, *Liebigs Ann. Chem.*, 1940, **544**, 182.

[6] R. Kuhn and A. H. Cook, *Ber. Deut. Chem. Ges.*, 1937, **70**, 761.

[7] C. Schöpf, R. Reichert and K. Riefstahl, *Liebigs Ann. Chem.*, 1941, **548**, 82.

[8] W. Pfleiderer, *Chem. Ber.*, 1962, **95**, 2195.

[9] A. Albert, D.J. Brown and G. Cheeseman, *J. Chem. Soc.*, 1952, 4219.

[10] W.B. Watt, *Nature*, 1964, **201**, 1326.

[11] W.B. Watt and S.R. Bowden, *Nature*, 1966, **210**, 304.

[12] S. Nawa, *Bull. Chem. Soc. Jpn.*, 1960, **33**, 1555.

[13] M. Viscontini and E. Möhlmann, *Helv. Chim. Acta*, 1959, **42**, 836.

[14] M. Viscontini, E. Hadorn and P. Karrer, *Helv. Chim. Acta,* 1957, **40**, 579.

[15] T. Hama, *Ann. N.Y. Acad. Sci.*, 1963, **100**, 977.

[16] Y. Mori, J. Matsumoto and T. Hama, *Vergleich. Physiol.*, 1961, **43**, 531.

[17] I. Ziegler, *Ergebnisse der Physiologie*, 1965, **56**, 1. K. Kramer ed.; Springer Verlag, Berlin.

[18] L. Wills, *Brit. Med. J.*, 1931, 1059.

[19] A.G. Hogana nd E.M. Parrott, *J. Biol. Chem.*, 1940, **132**, 507.

[20] R.B. Angier, J.H. Boothe, B.L. Hutchings, J.H. Mowat, J. Semb, L.R. Stockstad, Y. SubbaRow, C.W. Waller, D.B. Cosulich, M.J. Fahrenbach, M.E. Hultquist, E. Kuh, E.H. Northey, D.R. Seeger, J.P. Sickels and J.M. Smith, *Science*, 1946, **103**, 667.

[21] H.K. Mitchell, E.E. Snell and J.R. Williams, *J. Am. Chem. Soc.*, 1947, **63**, 2248.

[22] I. Ziegler and R. Harmsen, *Advances in Insect Physiology*, 1969, **6**, 139. Academic Press, London.

[23] R. Blakley, *The Biochemistry of Folic Acid and Related Pteridines*, 1969, 410. North Holland, Amsterdam.

[24] M.M. Nelson, H.V. Wright, C.D.C. Baird and H.M. Evans, *Proc. Soc. Exp. Biol. Med.*, 1956, **92**, 554.

[25] J.B. Lawson, *Ghana Med. J.*, 1962, **1**, 31.

[26] L.B. Bailey, *Folate in Health and Disease*, 1995, 329. Mercel Dekker Inc., New York.

[27] J.M. Scott, *Proc. Nutr. Soc.*, 1999, **58**, 441.

[28] S. Kaufman, Proc. Natl. Acad. Sci. U.S.A., 1963, **50**, 1085.

[29] G. Embden and K. Baldes, *Biochemistry*, 1913, **55**, 301.

[30] G. Jervis, *J. Biol. Chem.*, 1947, **169**, 651.

[31] D. Wellner, A. Meister, *Annu. Rev. Biochem.*, 1981, **50**, 91.

[32] T. Andersen and l. Avins, *Arch. Neurol.*, 1976, **33**, 684.

[33] J. Hughes and T. Johnson, *J. Neurochem.*, 1977, **25**, 117.

[34] R. Lenke and H. Levy, *N. Eng. J. Med.*, 1980, **303**, 1202.

[35] R.A. Levine, W. Lovenberg, H. Curtius and A. Niederwieser, *Chemistry and Biology of Pteridines*, 1983. J. Blair ed; Charles de Gruyetr, Berlin.

[36] E. Patterson, M. Von Saltza and E. Stokstad, *J. Am. Chem. Soc.*, 1956, **78**, 5871.

[37] I. Ziegler, H. Simon and A. Wacker, *Zeitschrift für Naturforschung*, 1956, **11b**, 82.

[38] O. Brenner-Holzach and F. Leuthardt, *Helv. Chim. Acta*, 1959, **42**, 2254.

[39] O. Brenner-Holzach and F. Leuthardt, *Helv. Chim. Acta*, 1961, **44**, 1480.

[40] F. Weygand, H. Simon, G. Dahms, H. Waldschmidt, H.J. Schliep and H. Wacker, *Angew. Chem. Int. Ed.,* 1961, **73**, 402.

[41] J.J. Reynolds and G.M. Brown, *J. Biol. Chem.*, 1962, **237**, 2717.

[42] C.L. Fan and G.M. Brown, *Biochim. Genet.*, 1976, **14**, 371.

[43] N. Blau and A. Niederwieser, *Anal. Biochem.*, 1983, **128**, 446.

[44] G.M. Brown and J.M. Williamson, *Escherichia coli and Salmonella typhimurium*, **1**, 521. American Society for Microbiology, Washington DC.

[45] J.M. Green, B.P. Nichols and R.G. Matthews, *Escherichia coli and Salmonella typhimurium*, **1**, 665. American Society for Microbiology, Washington DC.

[46] S. Katoh, T. Sueoka and S. Yamada, *Biochim. Biophys. Res. Commun.,* 1982, **105**, 75.

[47] D.S. Dutch and G.K. Smith, *J. Nutr. Biochem.*, 1991, **2**, 411.

[48] T. Shiota, C. W. Baugh and J. Myrick, *Biochim. Biophys. Acta.*, 1969, **192**, 205.

[49] T. Shiota, M. P. Palumbo and T. Tsai, *J. Biol. Chem.,* 1967, **242**, 1961.

[50] H. Nar, R. Huber, W. Meining, C. Schmid, S. Weinkauf and A. Bacher, *Structure*, 1995, **3**, 459.

[51] H. Nar, R. Huber, G. Auerbach, M. Fischer, C. Hösl, H. Ritz, A. Bracher, W. Meining, S. Eberhardt and A. Bacher, *Proc Natl. Acad. Sci. USA*, 1995, **92**, 12120.

[52] H. Nar, R. Huber, W. Meining, A. Bracher, M. Fischer, C. Hösl, H. Ritz, C. Schmid, S. Weinkauf and A. Bracher, *Biochem. Soc. Trans.*, 1996, **24**, 37.

[53] G. Auerbach, A. Herrmann, A. Bracher, G. Bader, M. Fischer, M. Gütlich, M. Neukamm, M. Garrido-Franco, J. Richardson, H. Nar, R. Huber, *Proc Natl. Acad. Sci. USA*, 2000, **97**, 13567.

[54] N. Schramek, A. Bracher and A. Bacher, *J. Biol. Chem.*, 2001, **276**, 2622.

[55] A. Bracher, N. Schramek and A. Bacher, *J. Biochem.*, 2001, **40**, 7896.

[56] J. Mc Cormac and J. Jaffe, *J. Med. Chem.*, 1969, **12**, 662.

[57] R. Schnitzer and F. Hawking, *Experimental Chemotheraphy*, 1966. Academic Press, New York.

[58] T. Osdene, P. Russel and L. Rane, *J. Med. Chem.*, 1967, **10**, 431.

[59] R. W. Heinle and A. D. Welch, *J. Clin. Invest.*, 1948, **27**, 539.

[60] D. J. Deller, E. Urban, R. N. Ibbotson, J. Horwood, S Milazzo and H. N. Brit, *Med. J.*, 1966, 755.

[61] S. L. Morgan and J. E. Baggot, Folate in Health and Disease, 1995, 405. Marcel Dekker Inc., New York.

[62] G. Werner-Felmayer, E.R. Werner, D. Fuchs, A. Hausen, G. Reibnegger, K. Schmidt, G. Weiss and H. Wachter, *J. Biol. Chem.*, 1993, **268**, 1842.

[63] H. Rokos, *Pteridines*, 1992, **3**, 79.

[64] A. Williams, R. Eldridge, R. Levine, W. Lovenberg and G. Paulson, *Lancet 2*, 1979, 410.

[65] P. Le Witt, R. Newman, L. Miller, W. Lovenberga nd M. Hallett, *Neurology*, 1988, **38**, 707.

[66] J. Fink, P. Ravin, C. Argoff, R. Levine, R. Brady, M. Hallett and N. Barton, *Neurology*, 1989, **39**, 1393.

[67] B. Ludecke, B. Dworniczak, K. Barthlolmè, *Hum. Genet.*, 1995, **95**, 123.

[68] H. Ichinose, T. Ohye and T. Takahashi, *Nature Genet.*, 1994, **8**, 236.

[69] W. Lovenberg, R. Levine, D. Robinson, M. Erbert, A. Williams and D. Calne, *Science*, 1979, **204**, 624.

[70] A. Williams, R. Levine, T. Chase, W. Lovenberg and D. Calne, *J. Neurol. Neurosurg. Psychiatry*, 1980, **43**, 735.

[71] R. Leeming and J. Blair, *Clin. Chim. Acta*, 1980, **108**, 103.

[72] R. Leeming and J. Blair, *Biochim. Med.*, 1980, **23**, 1223.

[73] H. Naruse, T. Hayashi, M. Takesada, A. Nakane, K. Yamazaki, T. Noguchi, Y. Watanabe and O. Hayaishi, *Proc. Jpn. Acad.*, 1987, **63**, 231.

[74] C. Chen and K. Hsiao, *J. Psychiatry*, 1989, **155**, 251.

[75] L.G. Mitchell, R. Mitchell and N.A. Campbell, *Biology*, 1998, 239. Addison Wesley Longman, California.

[76] T.H. Morgan, *Science*, 1910, **32**, 120.

[77] T.H. Morgan, A.H. Sturtevant, H.J. Muller and C.B. Bridges, *The Mechanisms of Mendelian Heredity*, 1915. Reinhart and Winston, New York.

[78] D.L. Lindsley and G.G. Zimm, *The Genome of Drosophila melanogaster,* 1992. Academic Press, San Diego.

[79] P. Lawrence, *The making of a fly*, 1992. Blackwell Scientific, London.

[80] W.B. Wood, *The Nematode Caenorhabditis elegans*, 1988. Cold Spring Harbour Press, New York.

[81] W. Driever and M.C. Fishman, *Development*, 1996, **123**, 37.

[82] W. Roush, *Science*, 1996, **272**, 1103.

[83] G. Streisinger, C. Walker, N. Dower, D. Knauber and F. Singer, *Nature*, 1981, **291**, 293.

[84] P. Haffter, *Development*, 1996, **123**, 1.

[85] J.S. Eisen, *Cell*, 1996, **87**, 969.

[86] A.E. Melby, R.M. Warga and C.B. Kimmel, *Development*, 1996, **122**, 2225.

[87] *Development*, 1996, **123**.

[88] M.C. Fishman, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 10554.

[89] M.C. Mullins, M. Hammerschmidt, D.A Kane, J. Odenthal, M. Brand, F. J. M. van Eeden, M. Furutani-Seiki, M. Granatio, P. Haffter, C.P. Heisenberg, Y.J. Jiang, R.N. Kelsh, C. Nüsslein-Volhard, *Development*, 1996, **123**, 81.

[90] L. Solnica-Krezel, D.L. Stemple, E. Mountcastle-Shah, Z. Rangini, S.C.F. Neuhauss, J. Malicki, A.F. Schier, D.Y.R. Stainier, F. Zwartkruis, S. Abdelilah and W. Driever, *Development*, 1996, **123**, 67.

[91] Y.J. Jiang, M. Brand, C.P. Heisenberg, D. Beuchle, M. Furutani-Seiki, R.N. Kelsh, R.M. Warga, M. Granato, P. Haffter, M. Hammerschmidt, D.A. Kane, M.C. Mullins, J. Odenthal, F.J.M. van Eeden, C. Nüsslein-Volhard, *Development*, 1996, **123**, 205.

[92] P. Thorogood, *TINS*, 1989, **12**, 38.

[93] D.J. Anderson, *TIG*, 1997, **13**, 276.

[94] R. I. Dorsky, R.T. Moon and D.W. Raible, *BioEssays*, 2000, **22**, 708.

[95] T.L. Creazzo, R.E. Godt, L. Leatherbury, S. J Conway and M.L. Kirby, *Annu. Rev. Physiol.*, 1998, **60**, 267.

[96] T. Horikawa, Y. Mishima, K. Nishino, M. Ichihashi, *Pigment Cell Res.* 1999, **123**, 175.

[97] R.N. Kelsh, M. Brand, Y.J. Jiang, C.P. Heisenberg, P. Haffter, J. Odenthal, M.C. Mullins, F.J.M. van Eeden, M. Furutani-Seiki, M. Granatio, M. Hammerschmidt, D.A Kane, R.M. Warga, D. Beuchle, E. Vogelsang and C. Nüsslein-Volhard, *Development*, 1996, **123**, 369.

[98] I. Meyer, *Malignant melanoma*, 1981. Prentice-Hall, London.

[99] B. Bishop, *A Ttime To Heal: triumph over cancer, the theraphy of the future*, 1996. Penguin Arkana, London.

[100] J. Odenthal, K. Rossnagel, P. Haffter, R.N. Kelsh, E. Vogelsang, M. Brand, F.J. M. van Eeden, M. Furutani-Seiki, M. Granato, M. Hammerschmidt, C.P. Heisenberg, Y.J. Jiang, D.A. Kane, M.C. Mullins and C. Nüsslein-Volhard, *Development*, 1996, **123**, 391.

[101] I. Ziegler, *Z. Vgl. Physiol.*, 1956, **39**, 163.

[102] I. Ziegler, *Z. Naturforsh.* 1963, **18b**, 551.

[103] K.U. Schallreuter, J.M. Wood, M.R. Pittelkow, M. Gütlich, R. Lemke, W. Rödl, N.N. Swanson, K. Hitzemann,and I. Ziegler, *Science*, 1994, **263**, 1444.

[104] T. Kauffmann, *Justus Liebigs Ann.*, 1959, **625**, 133.

[105] D.M. Parichy and S.L. Johnson, *Dev. Genes Evol.*, 2001, **211**, 319.

[106] Micromass®, *Training publication*, 2001.

[107] M.D. Davis, *J. Biol. Chem.*, 1982, **257**, 14730.

[108] W. Pfleiderer, *Chem. Ber.*, 1957, **90**, 2588.

[109] A. Walderner, *Tetrahedron Lett.*, 1986, **27**, 6059.

[110] T.R. Kelly, *Synthesis*, 1972, **544**, 1977.

[111] I. Ziegler, T. Mc Donald, C. Hesslinger, I. Pelletier and P. Boyle, *J. Biol. Chem.*, 2000, **275**, 18926.

[112] W. Pfleiderer, *Chem. Ber.*, 1979, **112**, 2750.

[113] J.S. Beckman, J.S. Parks, D.A. Pearson, J.D. Marshall, P.A. Marshall and B.A. Freeman, *Free Radic. Biol.*, 1989, **6**, 607.

[114] H.S. Forrest, E. Glassmann and H.K. Mitchell, *Science*, 1956, **124**, 725.

[115] A.C. Switchenko and G.M. Brown, *J. Biol. Chem.*, 1985, **260**, 2945.

[116] F. Wöhler, *Justus Liebigs Ann. Chem.*, 1857, **103**, 117.

[117] H. Hlasiwetz, *Justus Liebigs Ann. Chem.*, 1857, **103**, 200.

[118] W. Pfleiderer, *Chem. Ber.*, 1959, **92**, 2468.

[119] S. Gabriel and J. Colman, *Ber. Dtsch. Chem. Ges.*, 1901, **34**, 1234.

[120] O. Isay, *Ber. Dtsch. Chem. Ges.*, 1906, **39**, 250.

[121] D.J. Brown, *The chemistry of heterocyclic compounds*, 1988, **24**, No. 3. John Wiley and sons, New York.

[122] W. Pfleiderer, *Comprehensive heterocyclic chemistry II*, 1996, **7**, 679. Pergamon, U.K.

[123] G.B. Elion, G.H. Hitchings and P.B. Russell, *J. Am. Chem. Soc.*, 1950, **72**, 78.

[124] W. Pfleiderer, *Chem Ber.*, 1957, **90**, 2588.

[125] W. Pfleiderer, *Chem Ber.*, 1957, **90**, 2604.

[126] W. Pfleiderer, *Chem Ber.*, 1957, **90**, 2617.

[127] D.R. Seeger, D.B. Cosulich, J.M. Smith Jr. and M.E. Hultquist, *J. Am. Chem. Soc.*, 1949, **71**, 1753.

[128] G.M. Timmis, *Nature (London)*, 1949, **164**, 139.

[129] R.G.W. Spickett and G.M. Timmis, *J. Chem. Soc.*, 1954, 2887.

[130] D.G.I Felten, T.S. Osdene and G.M.Timmis, *J. Chem. Soc.*, 1954, 2895.

[131] I.J. Pachter, *Pteridine Chemistry*, 1964, 47. ed. W. Pfleiderer and E.C. Taylor. Pergamon, Oxford.

[132] F.F Bliche and H.G Godt Jr., *J. Am. Chem. Soc.*, 1954, **76**, 2798.

[133] M. Polonovski and H. Jerome, *C.R. Seances Acad. Sci.*, 1950, **230**, 392.

[134] W.R. Boon, W.G.M Jones and G.R. Ramage, *J. Chem. Soc.*, 1951, 96.

[135] A. Stewart, D.W. West and M.C.S. Wood, *J. Chem. Soc.*, 1964, 4769.

[136] W. Pfleiderer and H. Deiss, *Chem. Ber.*, 1971, **104**, 770.

[137] T. Sugimoto and S. Matsuura, *Bull. Chim. Soc. Jpn.*, 1980, **53**, 3385.

[138] G. Tennant and C.W. Yacomeni, *J. Chem. Soc., Chem. Commun.*, 1975, 819.

[139] E.C. Taylor, K.L. Perlman, I.P Sword, M. Séquin-Frey and P.A. Jacobi, *J. Am. Chem. Soc.*, 1973, **95**, 6407.

[140] P.G. Gassmann, *Acc. Chem. Res.*, 1970, **3**, 26.

[141] C.R. Patterson, *J. Am. Chem. Soc.*, 1964, **86**, 1648.

[142] D.H.R. Barton and A.J.L. Beckwith, *J. Chem. Soc.*, 1965, 181.

[143] M. Mokotoff, *Tetrahedron*, 1974, **30**, 2623.

[144] S.A. Glover, *J. Chem. Soc.*, 1984, 2255.

[145] Y. Kikugawa and M. Kawase, *J. Am. Chem. Soc.*, 1984, **106**, 5728.

[146] Y. Kikugawa, *Chem. Lett.*, 1987, 1771.

[147] L.E. Fischer, *J. Org. Chem.*, 1993, **58**, 3643.

[148] M. Cherest and X. Lusinchi, *Tetrahedron Lett.*, 1989, **30**, 715.

[149] Y. Kikugawa, *Chem. Lett.*, 1990, 581.

[150] A. Varvoglis, *Synthesis*, 1984, 709.

[151] A. Varvoglis, *Tetrahedron*, 1997, **53**, 117.

[152] H. Kusama, Y. Yamashita and K. Narasaka, *Chem. Lett.*, 1995, 5.

[153] H. Kusama, K. Uchiyama, Y. Yamashita and K. Narasaka, *Chem. Lett.*, 1995, 715.

[154] K. Uchiyama, Y. Hayashi and K. Narasaka, *Synlett.*, 1997, 445.

[155] A. Ono, K. Uchiyama, Y. Hayashi and K. Narasaka, *Chem. Lett.*, 1998, 437.

[156] T.D. Duff, *Ph.D. Thesis*, University of Dublin, 2000.

[157] H. Wamhoff and J. Dzenis, *Adv. Heterocycl. Chem.*, 1992, 55.

[158] S. Senda, H. Izumi and H. Fujimura, *Arzneim-Forsch*, 1967, **17**, 1519.

[159] T.Y. Shen, H.W. Lewis and V. Ruyle, *J. Org. Chem.*, 1965, **30**, 835.

[160] P. Liberti and J.B. Stanbury, *Annu. Rev. Pharmacol.*, 1971, **11**, 113.

[161] S. Broder, *Med. Res. Rev.*, 1990, **10**, 419.

[162] C. Lee, Y. Kim, *Tetrahedron Lett.*, 1991, **32**, 2401.

[163] H.P. Fischer and C.A. Grob, *Helv. Chim. Acta*, 1962, **45**, 2528.

[164] Z. Rappoport and T. Sheradsky, *J. Chem. Soc.*, 1967, 413.

[165] Y. Tamara, J. Minamikawa, K. Sumoto, S. Fujii and M. Ikeda, *J. Org. Chem.*, 1973, **38**, 1239.

[166] A.O. Ilvespää and A. Marxer, *Helv. Chim. Acta*, 1963, **46**, 2009.

[167] W. P. Pfleiderer and Karl Heinz Schünedhütte, *Chem. Ber.*, 1958, 612, 158

[168] A. Rybár and W. Pfleiderer, *Collect. Czech. Chem. Commun.*, 1987, **52**, 2722.

[169] F. Seela and U. Kretschmer, *J. Heterocycl. Chem.*, 1990, **27**, 479.

[170] K.S. Kim, Y.H. Song, B.H. Lee and C.S. Hahn, *J. Org. Chem.*, 1986, **51**, 404.

[171] T. Itoh, I. Tsuneo, O. Toshihiko, K. Haruo, N. Norio and K.A Watanabe, *Chem. Pharm. Bull.*, 1985, **33**, 1375.

[172] T. Odinokov, *Russ. Chem. Rev. (Engl. Transl.)*, 1981, **50**, 636.

[173] J.P. Pappas, W.P. Keaveney, E. Gancher and M. Berger, *Tetrahedron Lett.*, 1996, **36**, 4273.

[174] R.V. Stevens, P.M. Lesko and R. Lapalme, *J. Org. Chem.*, 1975, **40**, 3495.

[175] P. Garner and J. Min Park, *J. Org. Chem.*, 1987, **52**, 2361.

[176] W. Pfleiderer and H. Deiss, *Isr. J. Chem.*, 1968, **6**, 603.

[177] J. Armand, K. Chekir and J. Pinson, *Can. J. Chem.*, 1974, **52**, 3971.

[178] H. Gnichtel, *Chem. Ber.*, 1970, **103**, 3442.

[179] P.H. Boyle and R.J. Lockhart, *Tetrahedron*, 1984, **40**, 879.

[180] P.H. Boyle and R.J. Lockhart, *J. Org. Chem.*, 1985, **50**, 5127.

[181] R. Tamura, D. Oda and H. Kurokawa, *Tetrahedron Lett.*, 1986, **27**, 493.

[182] E.J. Corey and J.W. Suggs, *Tetrahedron Lett.*, 1975, **31**, 2647.

[183] A.J Mansusio and D. Swern, *Synthesis*, 1981, **24**, 165.

[184] R.F. Church and R.E Ireland, *Tetrahedron Lett.*, 1961, 493.

[185] K.E. Harding, L.M. May and K.F.Dick, *J. Org. Chem.*, 1975, **40**, 1664.

[186] W.H. Pearson, K. Lín and V. Poon, *J. Org. Chem.*, 1989, **54**, 5814.

[187] N. Kawahara, T. Nakajima, T. Itoh and H. Ogura, *Chem. Pharm. Bull.*, 1985, **3**, 4740.

[188] Z. Rappoporta and T. Sheradsky, *J. Chem. Soc.*, 1967, 898.

[189] D.J. McLennan and A. Pross, *J. Chem. Soc., Perkin Trans. 2*, 1984, **6**, 981.

[190] H. Ogura, M. Sakaguchi and K. Takeda, *Chem. Pharm. Bull.*, 1972, **2**, 404.

[191] J.A. Secrist and P.S. Liu, *J. Org. Chem.*, 1978, **43**, 3937.

[192] D.Van Tinh, M. Fischer and W. Stadlbauer; *J. Heterocycl. Chem.* 1996, **33**, 905.

[193] S. Nishigaki, *Chem. Pharm. Bull.*, 1980, **28**, 142.

[194] P. Pfleiderer and K. H. Schünedhütte; *Liebigs Ann. Chem.*, 1958, **612**, 158

[195] F. Bergmann and A. Kalmas, *J. Chem. Soc.*, 1963, 3729.

[196] T.R. Jones and F.L. Rose, *J. Chem. Soc. Perkin Trans 1.*, 1987, 2585.

[197] S. Shimizu and m. Ogata, *J. Org. Chem.*, 1988, **53**, 5160

[198] G.D. Johnson, *J. Am. Chem. Soc.*, 1951, **73**, 5888.

[199] T. Sheradasky, *Tetrahedron Lett.*, 1966, **43**, 5225.

[200] Y. Endo, K. Namikawa and K. Shudo, *Tetrahedron Lett.*, 1986, **27**, 4209.

[201] R. Baur, E. Kleiner and W. Pfleiderer, *Liebigs Ann. Chem.*, 1984, 1798.

[202] M. Igarashi and M. Tada; *J. Heterocycl. Chem.*, 1995, **32**, 807.

[203] U.V.K. Shirsagar and V.G. Bhave, *J. Indian Chem. Soc.*, 1997, **74**, 649.

[204] C. McKee, *J.Amer.Chem.Soc.*, 1951, **73**, 3862.

[205] A. Lemaçon, *M.Sc. Thesis*, University of Dublin, 2000.

[206] B.R. Cho, K.D Kim, J.C. Lee and N.S. Cho, *J. Am. Chem. Soc.*, 1988, **110**, 6145.

[207] K. Sosnovsky, *Synthesis*, 1978, 703.

[208] E.C. Taylor, P. Gillespie and M. Patel, *J. Org. Chem.*, 1992, **57**, 3218.

[209] F. Fidler, *J. Chem. Soc.*, 1957, 4157.