Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

The Synthesis and Testing of Inhibitors for the Enzyme GTP–Cyclohydrolase (I)

by

Mohammed Reza Ansari

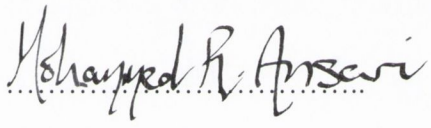
A thesis submitted to the University of Dublin for the degree of Doctor of Philosophy

Trinity College Dublin.

October 1999

Declaration

This thesis has not been previously submitted to this or any other university. Except where acknowledgement is given, all work in this thesis is entirely original and was carried out by the author alone. This author agrees that this thesis may be borrowed or copied only upon permission of the librarian.



Mohammed Reza Ansari

Acknowledgements

I would like to very much thank Dr. Peter Boyle for offering me the opportunity to work for a Ph.D. and his great support during the course of my study. I would like to also thank Dr. John O'Brien, Dr. Ken Robinson and also all the technical staff for their invaluable help and support, not to mention all the members of Lab. 0.14 past and present. I would also like to acknowledge Prof. A. Bacher, Dr. Andreas Bracher and Nicholas Schramek at the Technische Universität München for providing purified GTP-cyclohydrolase and useful technical information. My deepest thanks go to my parents for their love and support, for which I am forever grateful.

Abstract

This thesis investigates the design and synthesis of compounds to be tested as inhibitors of the enzyme guanosine triphosphate cyclohydrolase (I), an enzyme which plays an important role in the biosynthesis of almost all pteridines found in nature, including the folates. It catalyses the conversion of GTP to dihydroneopterin triphosphate through a number of intermediates. In chapter one a brief account of the importance of GTP-cyclohydrolase (I) is given in the introduction together with a description of the residues involved at the active site. On the basis of this information we identified several target compounds including 3-deazaguanosine, 8-aza-7-deazaguanosine and 8-azaguanosine. Chapter two of this thesis describes the synthesis of these molecules by selective coupling of the appropriate base with protected sugar derivatives, and also reports on other guanine analogues that were prepared for enzymatic experiments. Chapter three of this thesis describes the biological assay employed to evaluate the inhibitory activity of the target compounds on GTP-CH (I). Of the compounds tested, 8-azaguanine and 2,6-diamino-4(3*H*)-pyrimidinone were found to cause significant inhibition of the cyclohydrolase (I) reaction. Kinetic experiments suggested that 8-azaguanine is in fact a competitive inhibitor with a K_I of 0.21 mM. Similar experiments were carried out using 2,6-diamino-4(3*H*)-pyrimidinone which was also shown to exhibit competitive inhibition of the GTP-CH (I) reaction with a K_I value of 1.74 mM.

Abbreviations

Ala	Alanine
Arg	Arginine
BH_4	Tetrahydrobiopterin
BSA	Bistrimethyl silyl acatamide
CNS	Central nervous system
CSF	Cerebral spinal fluid
Cys	Cysteine
DCM	Dichloromethane
DIBAL-H	Diisobutylaluminium hydride
DHFR	Dihydrofolate reductase
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOPA	3,4-Dihydroxyphenylalanine
EtOAc	Ethyl acetate
FAB-MS	Fast atom bombardment mass spectroscopy
GDP	Guanosine diphosphate
Glu	Glutamic acid
GTP	Guanosine triphosphate
GTP–CH (I)	Guanosine triphosphate cyclohydrolase (I)
H_2-NTP	Dihydroneopterin triphosphate
H_2-biopterin	Dihydrobiopterin

H_2-neopterin	Dihyroneopterin
H_4-sepiapterin	Tetrahydrosepiapeterin
His	Histidine
HPLC	High performance liquid chromatography
IR	Infrared
Lys	Lysine
m.p	Melting point
NMR	Nuclear magnetic resonance
PAH	Phenylalanine hydroxylase
Phe	Phenyl alanine
PKU	Phenylketonuria
PPH_4	6-Pyruvyltetrahydrobiopterin
q-BH_2	Quinoniod dihydrobiopterin
Ser	Serine
TDA-1	Tri[2-(2-methoxyethoxy)ethyl]amine
R.T.	Room temperature
TCA	Trichloroacetic acid
Tris.	Tris (hydroxyaminomethane)
Tol	Toluene
TH	Tyrosine hydroxylase
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV	Ultra violet

Contents

1) Introduction

1.1 The discovery of pteridines 1

1.2 The biosynthesis of folic acid 3

1.3 The biosynthesis of pteridines 6

1.4 The biosynthesis of tetrahydrobiopterin 8

1.5 The dual nature of the *de novo* synthesis of tetrahydrobiopterin 11

1.6 Tetrahydrobiopterin metabolism in disease 15

1.7 Biosynthesis of molybdopterin 26

1.8 The mechanism of action of GTP–CH (I) 32

1.9 Inhibition of GTP–CH (I) 40

2) Design and synthesis of inhibitors of GTP–CH (I)

2.1 Review of the synthesis of 3-deazaguanine and 3-deazaguanosine 43

2.1.1 Synthesis of 3-deazaguanine and derivatives: Results and Discussion 53

2.2 Review of the synthesis of 8-aza-7-deazaguanine and 8-aza-7-deazaguanosine 57

2.2.1 Synthesis of 8-aza-7-deazaguanine and 8-azaguanosine: Results and Discussion 64

2.3 Review of the synthesis of 8-azaguanine 71

2.3.1 Synthesis of 8-azaguanosine: Results and Discussion 76

2.4 Synthesis of 9-D-Ribityl-guanine 80

2.4.1 9-D-Ribityl-guanine: Results and Discussion 82

2.5 Synthesis of 2-amino-5-formamido -6-D-ribityl-amino-4(3*H*)-pyrimidinone 84

2.5.1 Results and Discussion 85

3) Evaluation of compounds as inhibitors of GTP–CH (I) activity

3.1 Selection of compounds as inhibitors of GTP-CH (I) 87

3.2 Overview of the GTP-CH (I) assay 91

3.3 Enzyme kinetics 93

3.4 Results and discussion 98

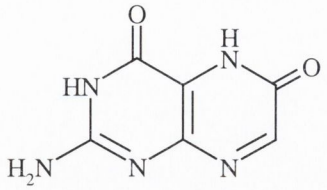
4) Experimental 105

5) Reference 126

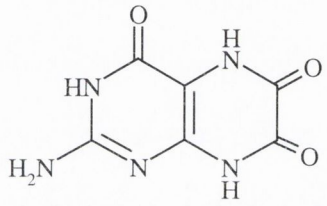
Introduction

1.1 The discovery of pteridines

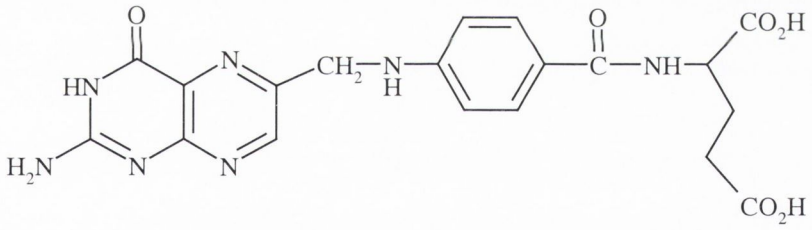
Pteridines are naturally occurring molecules, which play an important role in many biological systems. They were first isolated from the coloured pigments of the wings of certain butterflies by the English chemist F. Gowland Hopkins in 1889[1]; he was attracted by these coloured compounds and was very curious as to their chemical nature. Eventually after a few years he succeeded in isolating and partially characterising a yellow purine like pigment[2]. However it was not until the early part of the 20th century that these pigments were properly purified by the German chemists Heinrich Wieland and Clemens Schöpf[3], who named them after their colour and the source from which they were obtained. Their structure was elucidated by Robert Purrmann a student of Wieland who showed that the structure of xanthopterin **(1)** was 2-amino-4,6 (3*H*, 5*H*) pteridinedione[4] and that of leucopterin **(2)** was 2-amino-4,6,7 (3*H*, 5*H*, 8*H*) pteridinetrione[5].



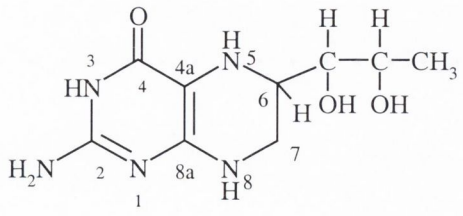
(1)



(2)



(3)



(4)

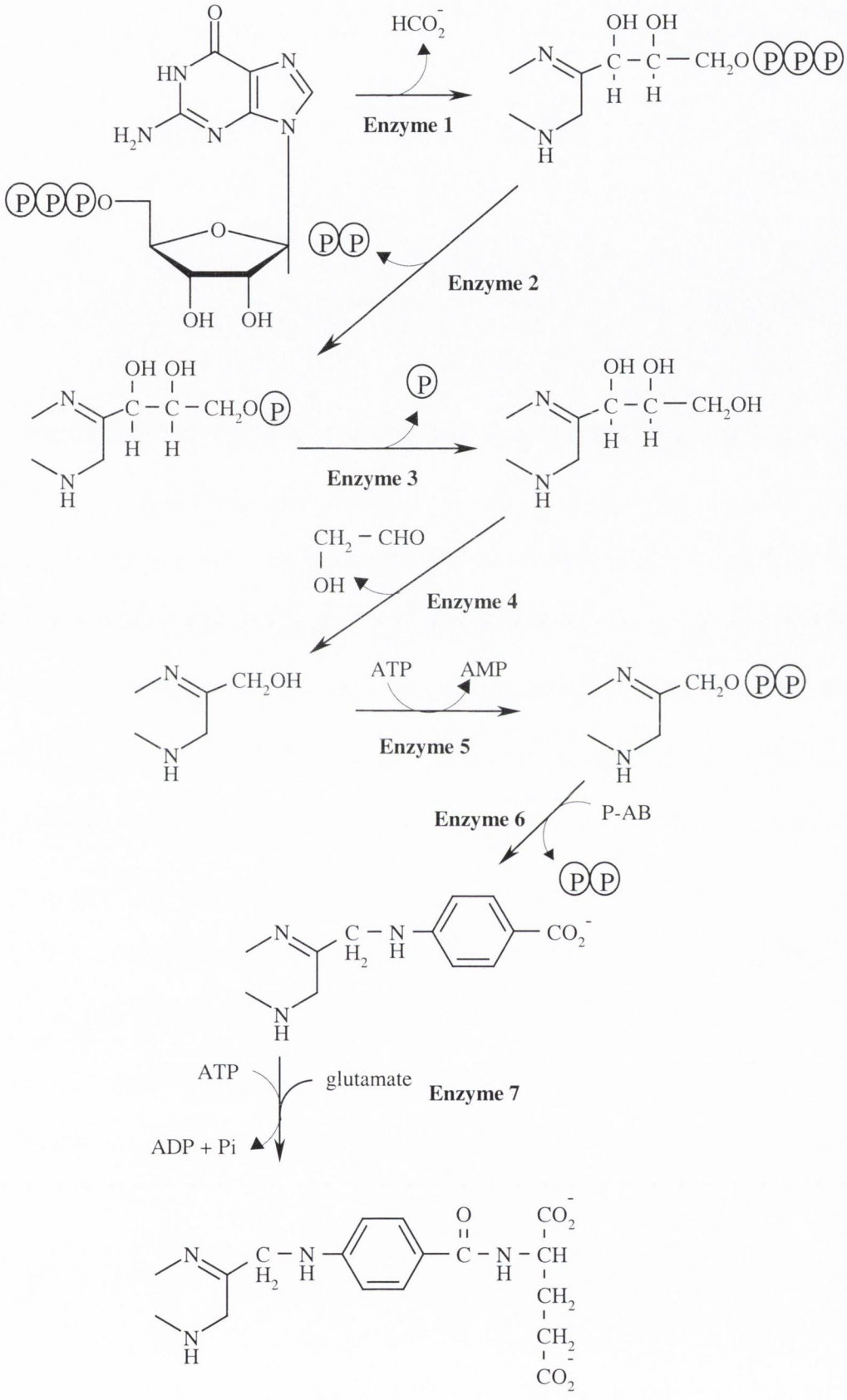
1.2 The biosynthesis of folic acid

Studies on the role played by folic acid in biological systems began in the early 1940 's when a group of researchers working on a microbiological assay for riboflavin discovered a previously unknown growth factor for *Lactobacillus casei*[6,7]. It seemed possible that the bacterial growth factor might also be nutritionally important in humans, hence research was directed towards establishing the identity of this unknown substance (*i.e.* folic acid)[8-10]. Finally in 1945 the structure of folic acid **(3)** was unequivocally assigned by its total synthesis[11]. As time passed by, further evidence accumulated as to the importance of folic acid in many biological systems[11-15]. At this point researchers began to focus on experiments to reveal the biosynthetic pathway to folic acid.

Successive experiments by Brown[16-18] during the early 1960's showed that cell-free extracts of *Escherichia coli* are able to catalyse the synthesis of the pteridine component of folic acid from guanosine triphosphate. Shiota and Plumbo[19] also came to the same conclusion after using a cell-free system from *Lactobacillus plantarum* as an enzyme source. The primary investigations, which elucidated the mechanism of folic acid biosynthesis, are reviewed in the works of Brown[20] and Shiota[21]. However a summary of their findings into the formation of H_2-folates is worth discussion. There are seven enzymes involved in the transformation of GTP to dihydrofolate **(scheme 1.1)** and of

these seven only three are relatively heat stable: GTP cyclohydrolase (I) **(enzyme 1)**, H_2-neopterin aldolase **(enzyme 4)** and CH_2OH pterin pyrophosphokinase **(enzyme 5)**. Among the seven enzymes involved in this pathway, GTP cyclohydrolase (I) is the most interesting because of the sequence of reactions it catalyses and also because this enzyme appears to be common to the biosynthesis of all other naturally occurring pterins with the possible exception of molybdopterin (see chapter 1.7).

In 1963 studies showed the involvement of another subclass of pterins in biological systems. The metabolic role of the group of unconjugated pterins that were originally discovered by Hopkins became established. It was found that the essential cofactor for phenylalanine hydroxylase (PAH) was tetrahydrobiopterin[22] **(4)**, which was later shown to play a major part in the biological functions of many organisms, and could no longer be looked upon as having minor significance. This discovery stimulated the growth of further research into the biosynthesis and biological roles of other pteridines. The findings of these studies however showed that the majority of pteridines played minor roles in the biology of the particular organism, such as pigments in the wings and eyes of insects, and the skin of fish, amphibians and reptiles[23,24,25]. However for many of these molecules the biological significance is still not fully understood.



Scheme 1.1 : *Enzymatic pathway for the synthesis H_2-Folate.*

1.3 The biosynthesis of pteridines

The early history of the unconjugated pterins revolved on the isolation of pigments from the wings of butterflies, the more restricted field of study on biopterin was nurtured by the growth in medium of the protozoon *Crithidia fasciulata*, Cowperthwaite *et al.*[26] observed that during attempts to grow insect trypanosomid in a chemically defined medium, exceptionally high concentrations of folic acid were required for maximal growth. This requirement for folic acid could be overcome if the insect was supplemented with certain nucleosides such as guanosine, adenosine and also by an unidentified compound in crude liver fractions.

The growth inducing activity (designated as the *Crithidia* factor) from the crude liver fractions was isolated by recrystallisation from boiling water. This left the task of identifying the unknown compound. It was found that the *Crithidia* factor was stable to boiling in 1 M HCl for one hour therefore ruling out the possibility that it was a purine nucleoside or a folic acid derivative. The researchers speculated that the factor was 2-amino-4-hydroxypteridine[27,28] with either a substituent at the 6 or 7 carbon, a conclusion which was later vindicated. A research group using the biological assay which first led to the discovery of the growth factor, purified the *Crithidia* factor from

4000 L of human urine and characterised it as 2-amino-4-hydroxy-6-(1,2-dihydroxy-propyl)-pteridine, and suggested it be called biopterin **(5)**[29].

As is often the case in scientific advances, biopterin was discovered independently and simultaneously by another group of scientists using a completely different approach[30]. This research involved the study of the eye pigments of the *Drosophila melanogaster* (fruit fly) using a remarkably simple chromatographic system to separate and estimate fluorescent eye pigments. During their studies of the *Drosophila* they isolated and characterised a number of pteridines, and one of these compounds, which was detected as a blue fluorescent spot in the Hadorn & Mitchell chromatographic system[31] was correctly characterised as biopterin **(5)**.

The importance of the discovery of biopterin was not fully realised until its tetrahydro-derivative (H_4-biopterin, BH_4) was shown to play a variety of roles in not only the biology of humans but also the majority of mammalian, insect and bacterial species found in nature. It was further established that tetrahydrobiopterin (H_4-biopterin) is produced from a *de novo* synthesis[32], rather than being obtained from a dietary source. As an outcome of the latter findings, attention was now diverted to studies on the mechanism of its biosynthesis.

1.4 The biosynthesis of tetrahydrobiopterin

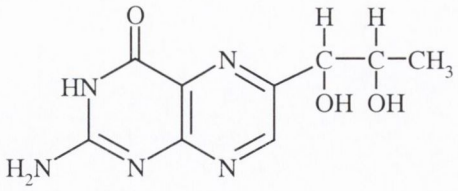
The tetrahydrobiopterin **(5)** ring system differs from the purine ring of guanine **(6)** by only an extra carbon atom, this led to the assumption that a guanine nucleoside maybe an immediate precursor of **(5)**. Investigations into the tadpole skin of *Rana catesbeiana*[33] provided the first evidence for the conversion a guanine derivative to a pteridine. Using an isotopically labelled precursor it was observed that carbon 2 and 4 but not carbon 8 of guanine are incorporated into biopterin. This observation was further supported by later studies of insects and bacterial models[34,35,36], where in a similar way it was found that carbon 8 of guanine was not incorporated into the H_4-biopterin moiety. The only question remaining was that of the origin of the extra carbon in the H_4-biopterin.

The enzyme called guanosine triphosphate cyclohydrolase (I) (GTP-CH (I)) catalyses the initial step in the production of tetrahydrobiopterin. The GTP-CH was first isolated from *Escherichi coli*[16] (*E. coli*) and was shown to convert guanosine triphosphate **(7)** into a pteridine derivative called dihydroneoptrin triphosphate **(8)** (H_2-NTP). All the carbon atoms of **(8)** were derived from the guanosine triphosphate precursor, with the concomitant loss of the guanine C-8 as a formate. The product of the cyclohydrolase reaction, namely dihydroneoptrin triphosphate has been shown to be crucially

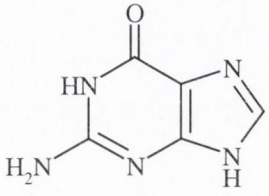
important in pteridine metabolism. Current evidence indicates that this is the precursor to all naturally occurring pterins with the possible exception of molybdopterin[37].

Through experimentation with the skin of *Rana catesbeiana*[38], Fukushima demonstrated that the carbon side chains of biopterin and sepiapterin **(9)** are also derived from GTP and also that there appeared to be a decrease in the amount of ^{14}C incorporation into biopterin from [u-^{14}C] GTP in the presence of unlabeled sepiapterin **(9).** These results appeared to indicate that sepiapterin might also be involved in the biosynthesis of H_4-biopterin. Fukushima proposed the following **(scheme 1.2)**: that H_2-neopterin triphosphate **(8)** was converted into H_2-neopterin **(10)** with the aid of the 6-pyruvoyl-7,8-dihydropterin synthase catalysed reaction. H_2-Neopterin would then be converted into sepiapterin **(9)**. From earlier work it was known that sepiapterin could be converted to dihydrobiopterin **(11)** by sepiapterin reductase and that dihydrobiopterin could than in turn be reduced to H_4-biopterin **(4)** with the aid of dihydrofolate reductase (**DHFR**) to complete the biosynthetic pathway.

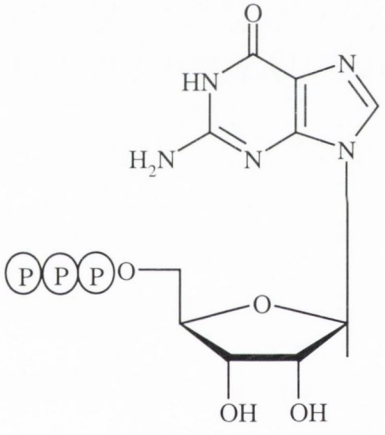
The proposed scheme was supported by the results of extensive studies of the synthesis of dihydrobiopterin carried out with various tissue preparations. The work of Tanka *et al.*[39] helped considerably, where it was found that chicken kidney extracts could be



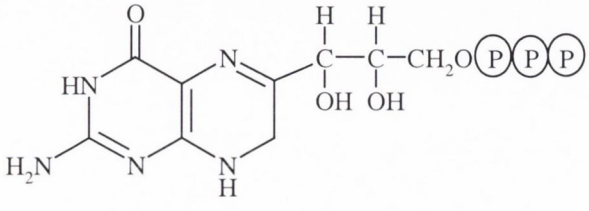
(5)



(6)



(7)



(8)

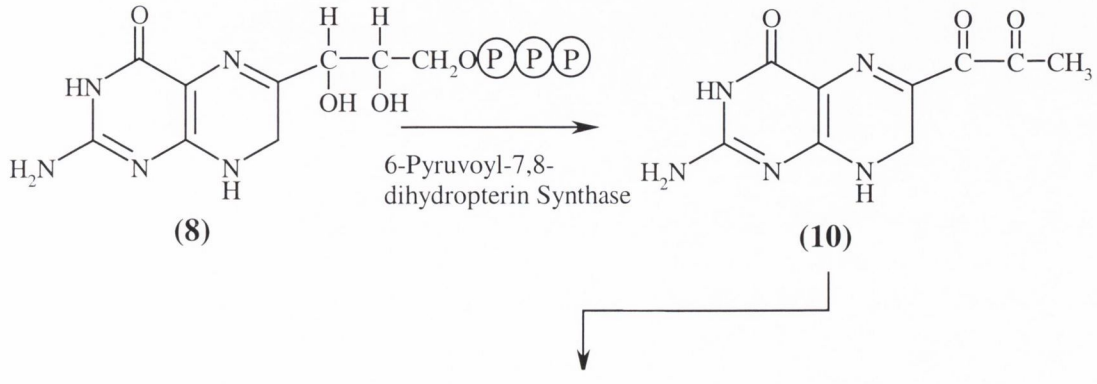
separated into three fractions and that they were all required for the synthesis of dihydrobiopterin from H_2-neopterin **(8)**. Furthermore it was found that in the absence of an external reducing agent, one of these fractions catalysed the conversion of H_2-neopterin to a labile intermediate. This step was found to require Mg^{2+}. On the basis of the important observation that this compound could breakdown to pterin and pyruvic acid, they proposed the labile intermediate was 6-(1,2-dioxopropyl)-7,8-dihydropterin. The other two fractions were found to contain different enzymes that catalysed the successive reductions of the two side-chain keto groups in the intermediate, the first converting it to sepiapterin **(9)** and the second, reducing sepiapterin to 7,8-dihydrobiopterin **(11)**.

1.5 The dual pathway for the *de novo* Synthesis of tetrahydrobiopterin

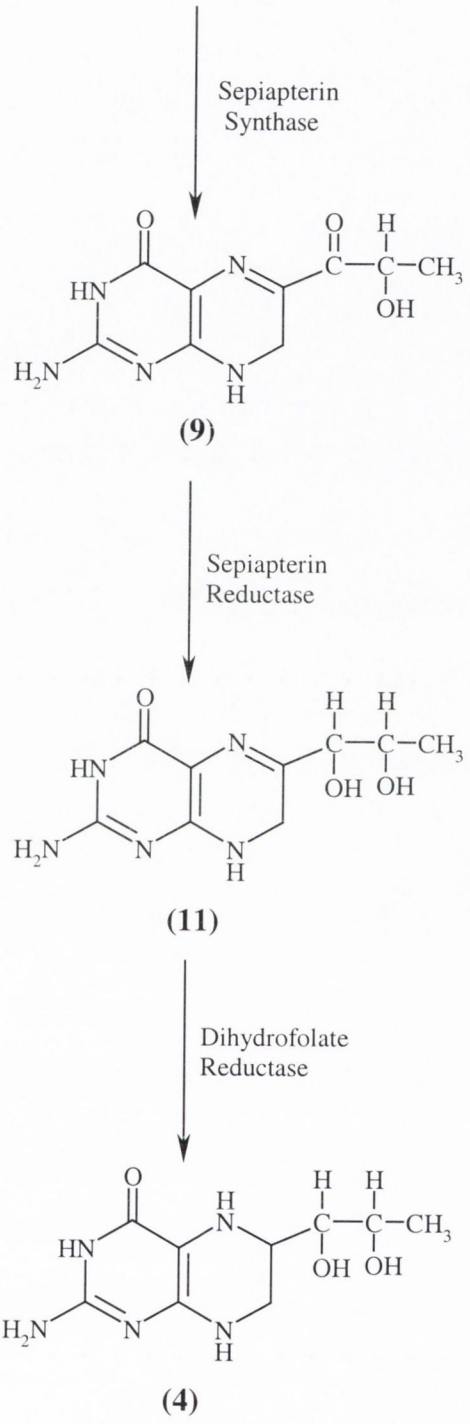
The previously outlined biosynthetic pathway to BH_4 was widely accepted, however there were discrepancies which could not be explained using the above scheme. Hence it became evident that the *de novo* synthesis of BH_4 had not been completely resolved and there might in fact be an alternative route for the synthesis of BH_4. Indeed the first indication of this became apparent after studies on the effect of the drug trimethoprim on hepatic levels of BH_4. This drug was believed to be a relatively selective inhibitor of the DHFR. It was found that when trimethoprim was administered to rats for three successive days, there was little or no effect on hepatic levels of BH_4[40]. However this

result was not conclusive enough to make the supposition that BH_4 could be synthesised by an alternative route, this was due to the lack of a positive control that the drug had actually reached the liver in sufficient amounts to inhibit the DHFR. The clinching argument against the involvement of DHFR in the pathway was the finding that in a Chinese hamster ovary mutant lacking this enzyme endogenous formation of BH_4 was normal[41].

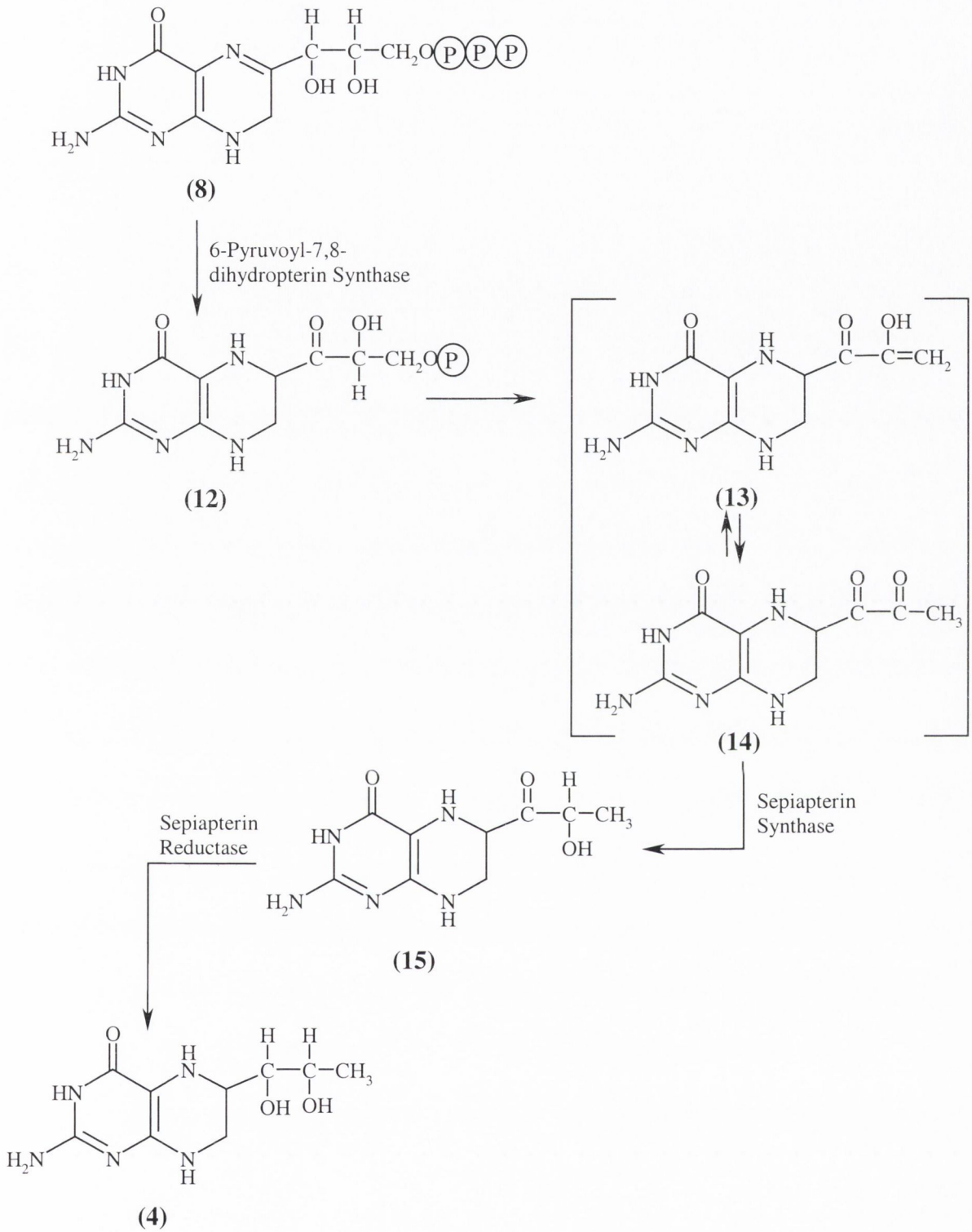
The elucidation of the alternative pathway to the synthesis of BH_4 resulted from the findings of Milstein and Kaufmann[42] indicating that there could be an alternative substrate involved in the *de novo* pathway. A likely candidate appeared to be tetrahydrosepiapterin (6-lactoyltetrahydrobiopterin) **(12)**, which upon reduction could yield BH_4. When tested with the adrenal preparation, this pterin was found to be converted to BH_4 significantly faster than either sepiapterin or H_2-neopterin-P_3, a result that suggested that 6-lactoyltetrahydrobiopterin is an intermediate in the synthesis of BH_4 **(scheme 1.3)**.



(8) **(10)**



Scheme 1.2 : *Biosynthetic pathway proposed by Fukushima for the synthesis of tetrahydrobiopterin*[38].



Scheme 1.3 : *Alternative biosynthetic pathway proposed by Milstein and Kaufmann* [42].

1.7 Tetrahydrobiopterin metabolism in disease

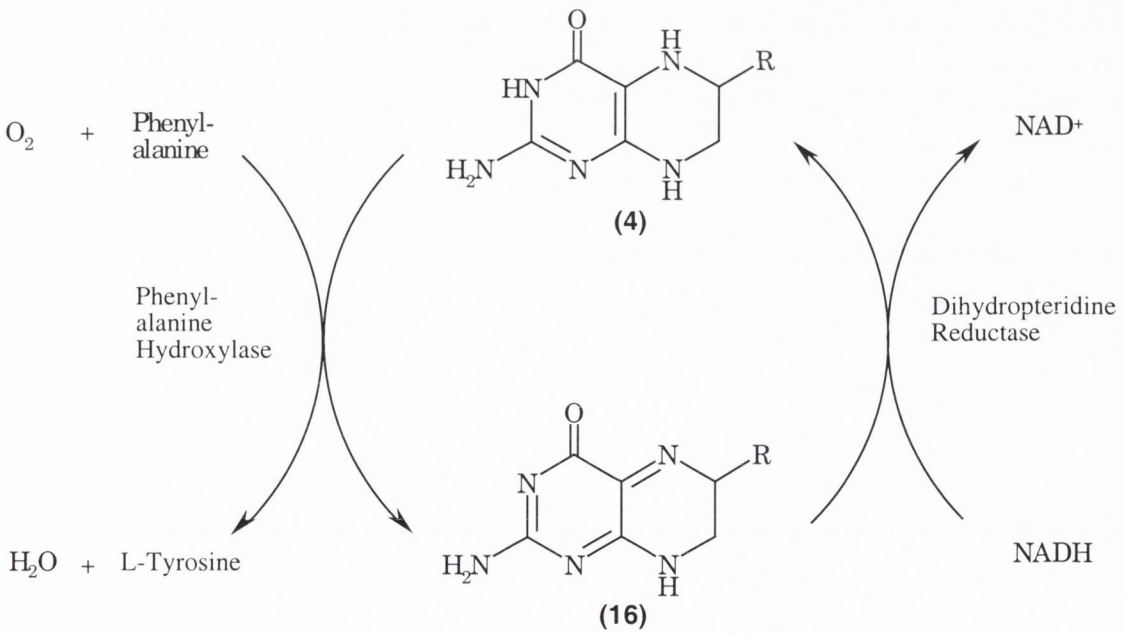
Tetrahydrobiopterin (BH_4) is found in many human body fluids and tissues at concentrations ranging from 2 μg / ml to 1 ng / ml. It has been detected in plasma, serum[43,44], urine[43,44], blood[45], cerebrospinal fluid[43,44], breast milk[44], liver[43], brain, gut, kidney, erythrocytes[45], breast tissue and tumours, colonic and rectal tumours and human placenta. The presence of BH_4 has also been found in foetal brain and liver at as early as 16 weeks gestation[43]. The only major role of BH_4 that has been firmly established is its function as a co-enzyme for the three amino acid hydroxylases (phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase) as well as nitric oxide synthase[46]. Almost every aspect of these enzymes from their structure and to the way they catalyse their reactions, to how their activities are regulated is determined by BH_4.

Proteins in the body are synthesised from 20 different naturally occurring amino acids, 10 of which are classified as essential amino acids, as they must be obtained from a dietary source. Phenylalanine is one of these essential amino acids. It was known from as early as 1913 that tyrosine was formed from phenylalanine as demonstrated in perfused dog liver[47]. However investigations into the nature of the enzyme involved were largely neglected for the next 40 years, until in 1947 enzymologists discovered that the genetic disease phenylketonuria was caused by an inability to convert phenylalanine to tyrosine[48]. The later findings indicated that this single metabolic defect leads to severe mental

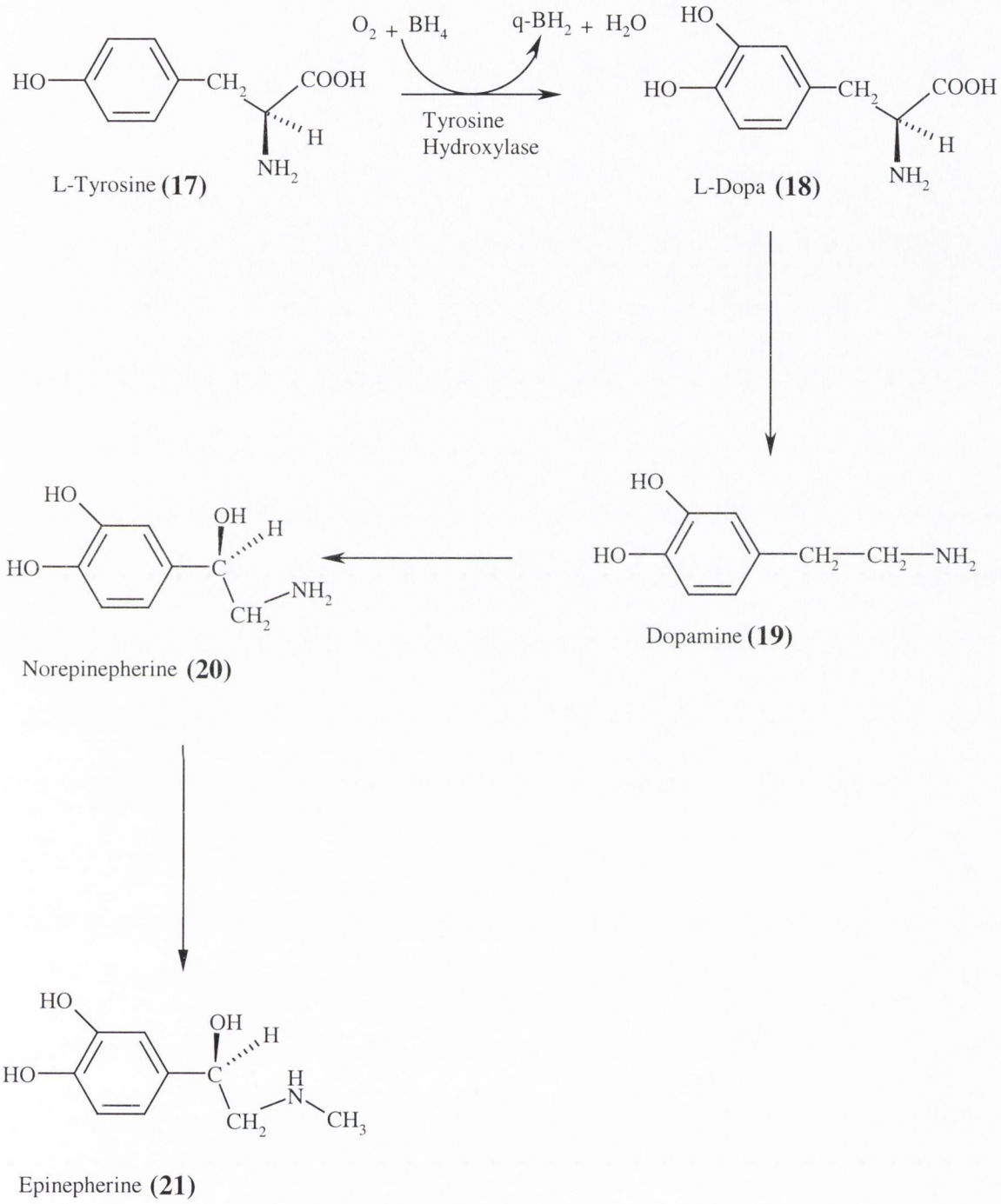
retardation. Consequently much attention was focused on elucidating the mechanism of phenylalanine hydroxylation[49-52] until finally in 1963 Kaufman[22] showed the crucial role BH_4 has to play as a cofactor.

The discovery of the essential role of unconjugated pterins in the enzymatic hydroxylation of phenylalanine[51,52] **(Scheme 1.4)** and the identification of the naturally occurring hydroxylating cofactor in the liver as tetrahydrobiopterin[22], naturally led researchers to ask themselves as to whether BH_4 is involved in other types of hydroxylation reactions. At the period before the discovery of phenylalanine hydroxylase, it was proposed that L-tyrosine **(17)** could be further hydroxylated to 3,4-dihydroxyphenylalanine[53] (L-dopa, **18**). The dopa molecule could then be decarboxylated to form dopamine **(19)** and subsequent hydroxylation at the β-position of the dopamine side chain would produce norepinephrine **(20)**, and epinephrine **(21)** upon methylation of the amino group of norepinepherine **(Scheme 1.5)**. Although the evidence in favour of the latter proposal increased[54-56] and it was widely accepted, the enzyme required for the ring hydroxylation remained elusive. In 1964, it was demonstrated that L-tryosine was converted to L-dopa through studies carried out on adrenal medulla[57], brain and other sympathetically innervated tissues[58] thus verifying the proposed biosynthetic pathway.

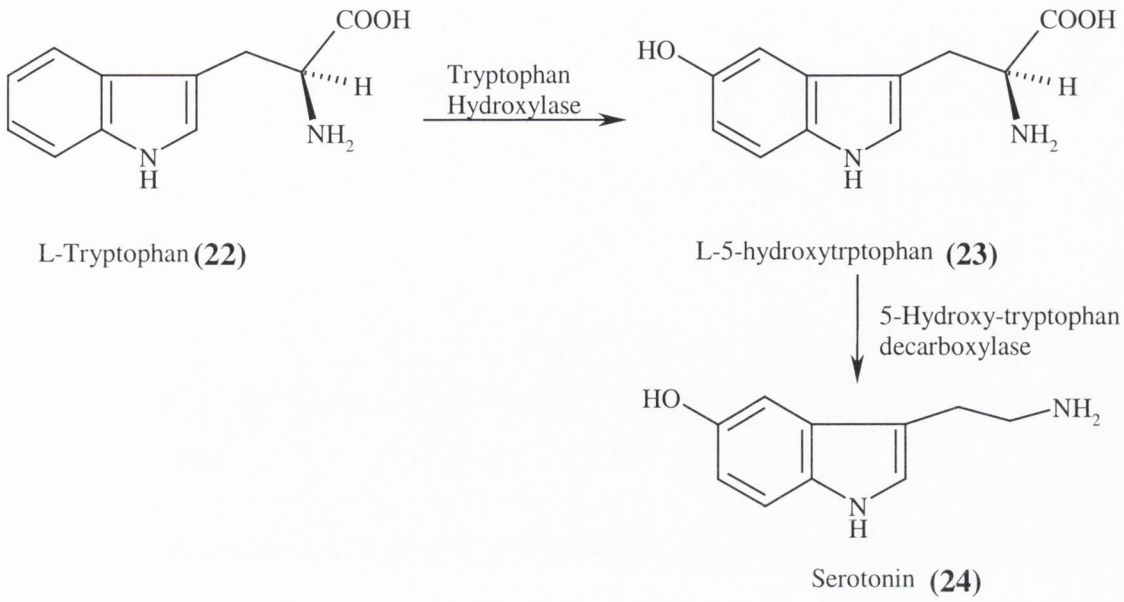
Considerably less is known about the third pterin dependent aromatic amino acid hydroxylase, namely tryptophan hydroxylase. This enzyme is believed to catalyse the rate limiting step **(Scheme 1.6)** in the biosynthesis of the putative neurotransmitter 5-hydroxytryptamine (serotonin, **24**). In light of the fact that there are only two consecutive enzymatic steps involved in synthesis of serotonin: I) hydroxylation of L-tryptophan **(22)** and followed by II) decarboxylation of L-5-hydroxy-tryptophan **(23)**, the proposal that tryptophan hydroxylation is the rate limiting step is attributed to the fact that the decarboxylase has a much higher *in vitro* activity than does the hydroxlase[59].



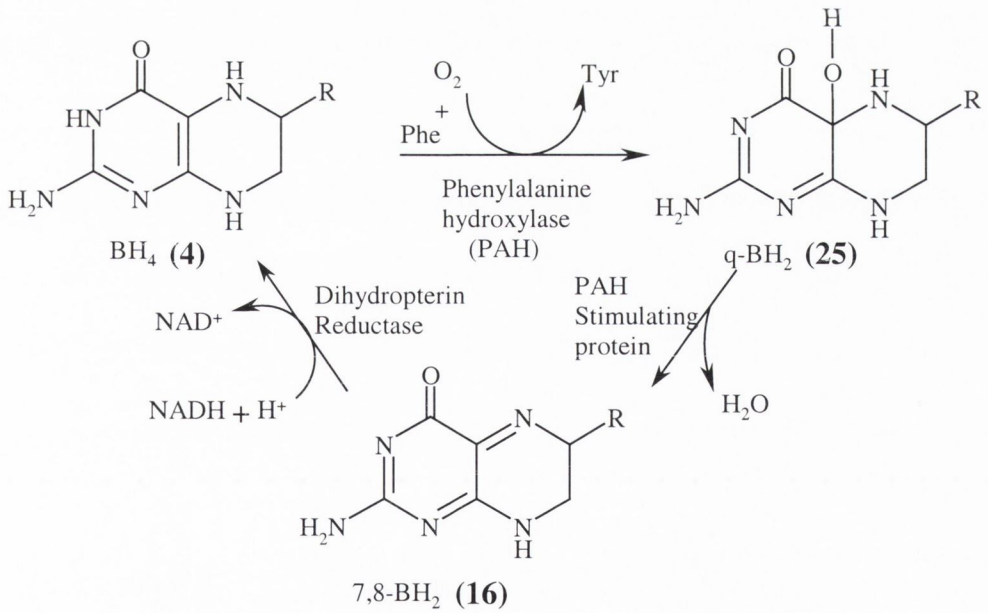
Scheme 1.4 : *The phenylalanine hydroxylating system, showing the reactions catalysed by phenylalanine hydroxylase and dihydropteridine reductase.*



Scheme 1.5: *The biosynthetic pathway for the conversion of tyrosine to Dopa catalysed by tyrosine hydroxylase and the synthesis of the essential catecholaminergic neurotransmitters.*



Scheme 1.6: *The biosynthesis of serotonin from trptophan catalysed by tryptophanhydroxylase and 5-hydroxy tryptophan decarboxylase. Note that the reaction catalysed by tryptophan hydroxylase is the rate limiting step which is controlled by the oxidation of tetrahydrobiopterin.*



Scheme 1.7: *Showing the conversion of phenylalanine to tyrosine, and the formation of the quinonoid dihydrobiopterin intermediate (q-BH_2), which is also considered to occur in a likewise manner in the tyrosine hyroxylating system.*

Tetrahydrobiopterin deficiencies

Gross deficiency of BH_4 occurs in three very rare genetic metabolic disorders, which occur at a frequency of one in every million births. The disease is referred to as malignant hyperphenylalanemia[60] and differs from classical phenylketonuria (PKU)[61] in that hydroxylation of all three amino acids is much reduced due to the BH_4 deficiency, where as in PKU it is only phenylalanine hyroxylase which fails. Children with untreated gross BH_4 deficiencies fail to meet developmental milestones and rarely survive much beyond two years[62]. Those that do survive suffer from mental retardation[62,63] which is attributed to the reduced levels of catecholamine neurotransmitters and serotonin, since the hydroxylases are involved in the first step in their formation.

The gross BH_4 deficiency can be divided into two categories I) Deficiency of DHFR enzyme where there is failure to salvage the cofactor oxidised during hydroxylase activity and II) Synthesis deficiency where *de novo* biosynthesis is not occurring[62]. The latter group can be further subdivided into GTP cyclohydrolase deficiency (GTP–CH)[64] and the absence of the phosphate eliminating enzyme (PEE, 6-pyruvoyl dihydropterin synthase)[65]. In BH_4 synthesis deficiency, urine and serum levels of total biopterins are much reduced compared to normal[66]. In subjects with the defect occurring at GTP–CH, urinary neopterin (a precursor of BH_4) is decreased, while in those with PEE deficiency urinary neopterin is elevated[64]. In both cases BH_4 synthesis is absent from the liver[67]. In DHPR

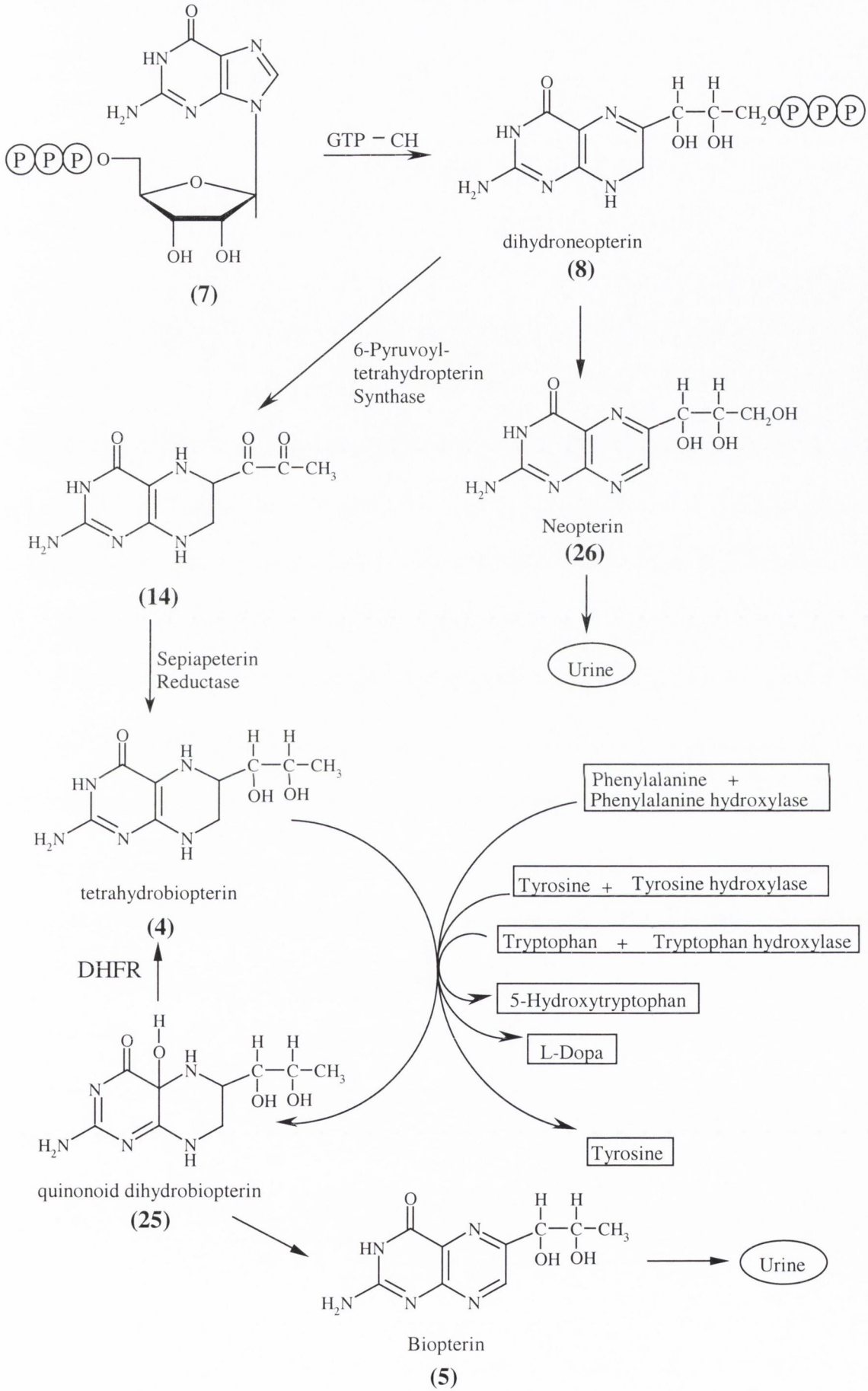
deficiency serum levels of total biopterin are much higher than normal and neopterin levels are also increased but remain in the normal range. It is important to note that in both synthesis deficiency and DHFR deficiency the levels of the associated neurotransmitters are much reduced[63,68].

Ageing and states of confusion

The biopterin serum levels in a healthy individual remain constant and do not show any diurnal rhythm. Analysis of a large group of people showed that the levels of biopterin increase significantly with age, suggesting depletion of cell BH_4 by loss of DHPR activity[69]. The levels of BH_4 in the cerebral spinal fluid decreases significantly with ageing as do those of homovanillic acid, a metabolite of dopamine[70]. Dopamine synthesis and BH_4 levels correlate each other closely and support the theory that in the central nervous system BH_4 controls dopamine formation. Reduced BH_4 in the central nervous system leads to lower dopamine levels, probably causing the decline in mental capacity[69].

Alzheimer's Disease

This disease is characterised by the decline in mental intellect and loss of memory. The levels of biopterin and related pterins have been found to be much reduced in patients suffering from senile dementia of the Alzheimer type (SDAT)[69]. In other studies carried



Scheme 1.8 : *Biochemical fate of pterins in the body.*

out at autopsy the total amount of biopterin was measured from patients with SDAT, and it was shown that the levels of biopterin were reduced whilst neopterin levels did not significantly alter[71,72]. These results are consistent with defects in biopterin biosynthesis similar to patients suffering from synthesis deficient malignant hyperalanaemia. This was correlated, by the observation that the human temporal lobe from SDAT age-matched controls taken at autopsy showed that biopterin biosynthesis was depressed, neopterin levels elevated and biopterin levels to be lowered[73]. DHFR activity was normal. It follows that from these observations that cellular BH_4 will be lowered and therefore neurotransmitter levels will be lower.

Down's Syndrome

Investigations carried out on a large number of patients with Down's syndrome revealed a significant increase in serum biopterin[74], whereas neopterin levels were found to be lower[75]. Brain samples from such patients had reduced DHFR activity and low levels of BH_4 biosynthesis[75]. It was not possible however to determine whether low levels of BH_4 synthesis in adults resulted from accelerated decline with age, or was a permanent defect throughout life. If the latter is the case, decreased BH_4 synthesis may contribute to the neurological deficit of Down's syndrome[75]. However, this deficit must only be expressed in the central nervous system, as such patients do not develop hyperalanaemia[76]. Depressed serum neopterin levels are consistent with reduced *de novo* BH_4 synthesis and

this is supported with experimental studies[76]. The elevated serum biopterin results are in line with DHFR activity. Thus in down's syndrome cellular BH_4 in the CNS may be much less because of both reduced synthesis and enhanced loss.

Parkinson's Disease

The deterioration of motor fibres and the degeneration of the central nervous system resulting in the loss of voluntary muscle movement are the symptoms of Parkinson's disease. In these patients it was found that in their cerebral spinal fluid (CSF) the BH_4 levels were significantly reduced compared with age-matched controls[70]. This observation was in correlation with homovanillic acid levels, which suggested that the reduced BH_4 levels may be limiting the synthesis of dopamine. Treatment of Parkinson patients with BH_4 has resulted in some positive responses[76,77].

Depression

The rationale for the implication that BH_4 may be involved in the pathogenesis of this kind revolves on the hypothesis that decreased synthesis of central monoamines such as serotonin, dopamine and norepinepherine may be a causal factor in the development of clinical depression[78]. The results of the measurement of BH_4 in tissues and fluids from depressed patients, however, were not as clear cut for a pathogenic relationship to be derived[79-82]. Although the results hardly add up to a compelling argument for a causal

role for BH_4 deficits in the development of this disease, several trials for BH_4 were carried out on a small scale. In one study carried out on patients suffering from depression, treatment was provided by the administration of a single oral dose of BH_4 (0.9-1.0 g) was found to provide temporary (4-5 hours) improvement in some clinical symptoms of a few patients and was ineffective in others[83].

Autism

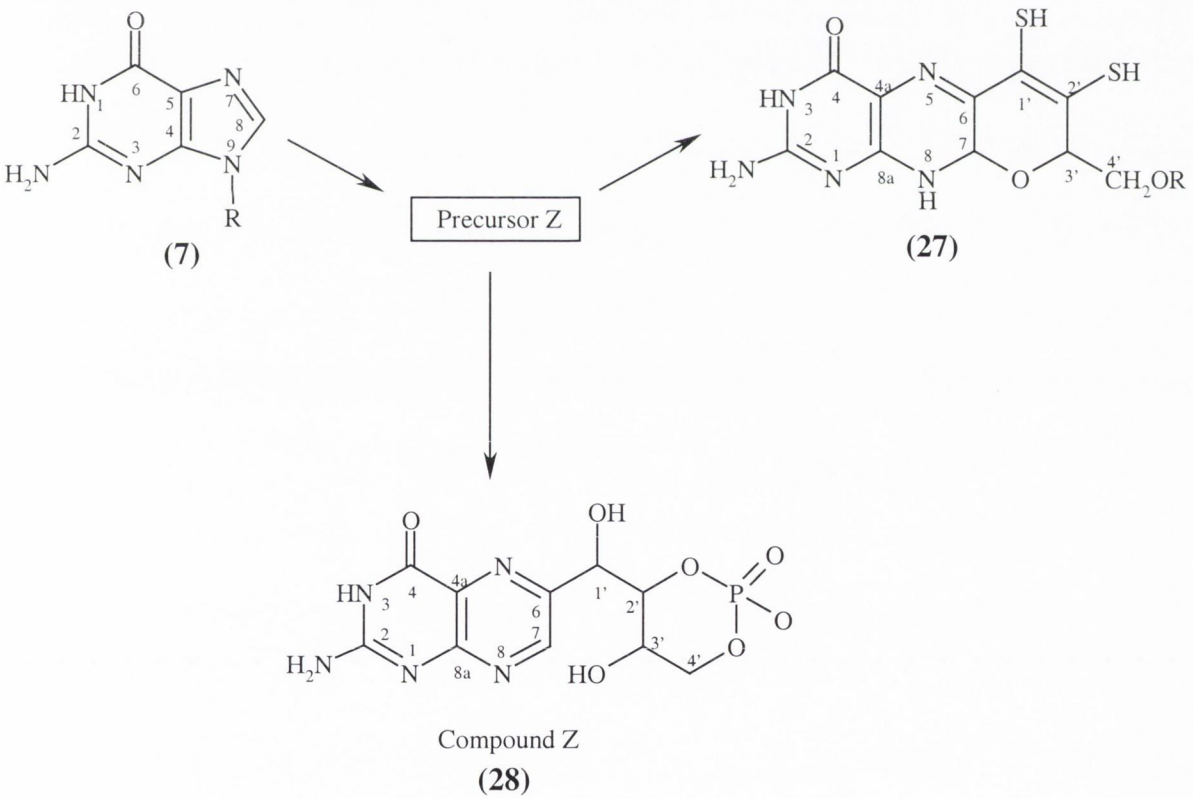
Autism impacts the normal development of the brain in the areas of social interaction and communication skills. Little is known about the origin of infantile autism, but evidence suggests that autistic patients may have a cellular defect in the cellular transport of aromatic amino acids[84]. In the trials conducted where BH_4 was administered to infants there was a definite improvement in more than half the patients, however the percentage of improvement on administration of a placebo was quite high, hence no definite conclusion could be derived from the study[84]. Clearly this type of work needs to be repeated to show a greater difference between the effects of BH_4 and the placebo. This study may be of a challenging nature, since there is data suggesting the hyper function of dopamine synthesis[85]. One reasonable explanation would be that the high turnover of dopamine, originating from some unidentified abnormality, exceeds the capacity for the synthesis or regeneration of BH_4.

1.7 Biosynthesis of molybdopterin

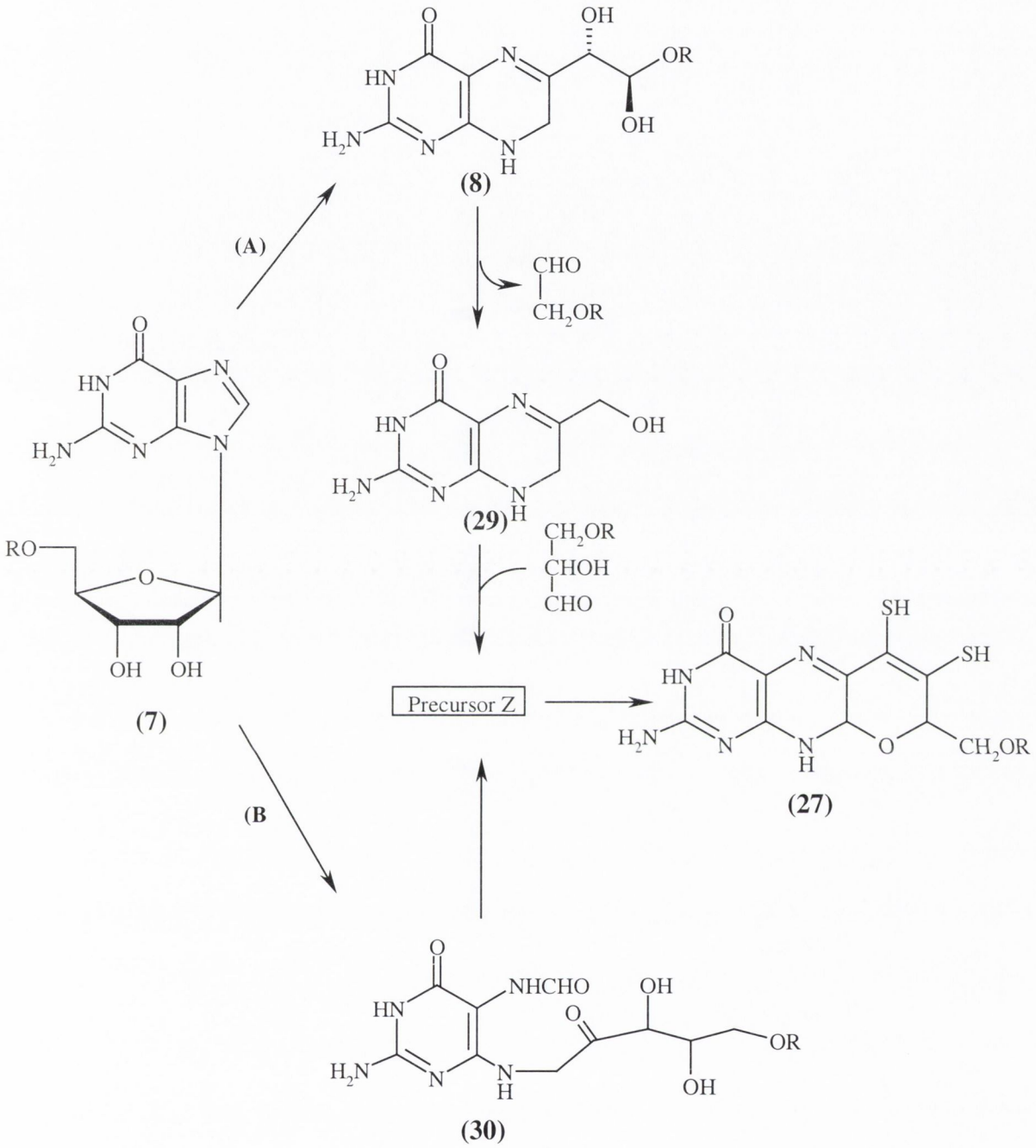
Molybdopterin **(27)** is a non conjugated tetrahydrobiopterin derivative that is complexed to a molybdenium metal ion[86]. This pterin derivative has been found to be a cofactor in a variety of enzyme catalysed redox reactions in microorganisms, plants and animals[87-89]. In microorganisms for example it is a cofactor for the enzymes nitrate reductase, sulfite oxidase and aldehyde oxido-reductase[90-92]. In vertebrates, molybdopterin acts as a cofactor for xanthine dehydrogenase and sulphite oxidase among others[93]. Genetic defects of the molybdopterin pathway are conducive to severe neurological deficiencies in man[94].

The direct isolation of molybdopterin **(27)** has not yet been achieved as it is very labile and is only found at very low concentrations in nature[95]. Experiments were also directed towards elucidating the structure of its precursor (referred to as precursor Z), however, similar difficulties were encountered[96]. The precursor Z was finally isolated when a mutant strain of *E. coli* was discovered to be unable to convert precursor Z to molybdopterin, and hence it accumulated. Due to its unstable nature precursor Z was oxidised prior to characterisation, yielding the more stable compound Z **(28, Scheme 1.9)**[97].

Experiments carried out using [5-^{3}H, U-^{14}C] guanosine, Irby and Adair[98] came to the conclusion that molybdopterin in yeast (*Pichia canadensis*) is formed *via* dihydroneopterin triphosphate **(8)**, the first key intermediate in the biosynthesis of tetrahydrofolate. It was proposed[98] **(Scheme 1.10)** that the carbons C-6 and C-7, and the side chain carbon C-1' of molybdopterin, are derived from the ribose side chain of the guanosine nucleotide **(7)**, whereas the side chain carbon atoms C-2'–C-4' stem from glyceraldehyde phosphate or a related carbohydrate.



Scheme 1.9 : *Conversion of a guanine derivative to molybdopterin via precursor Z, and stable compound Z.*



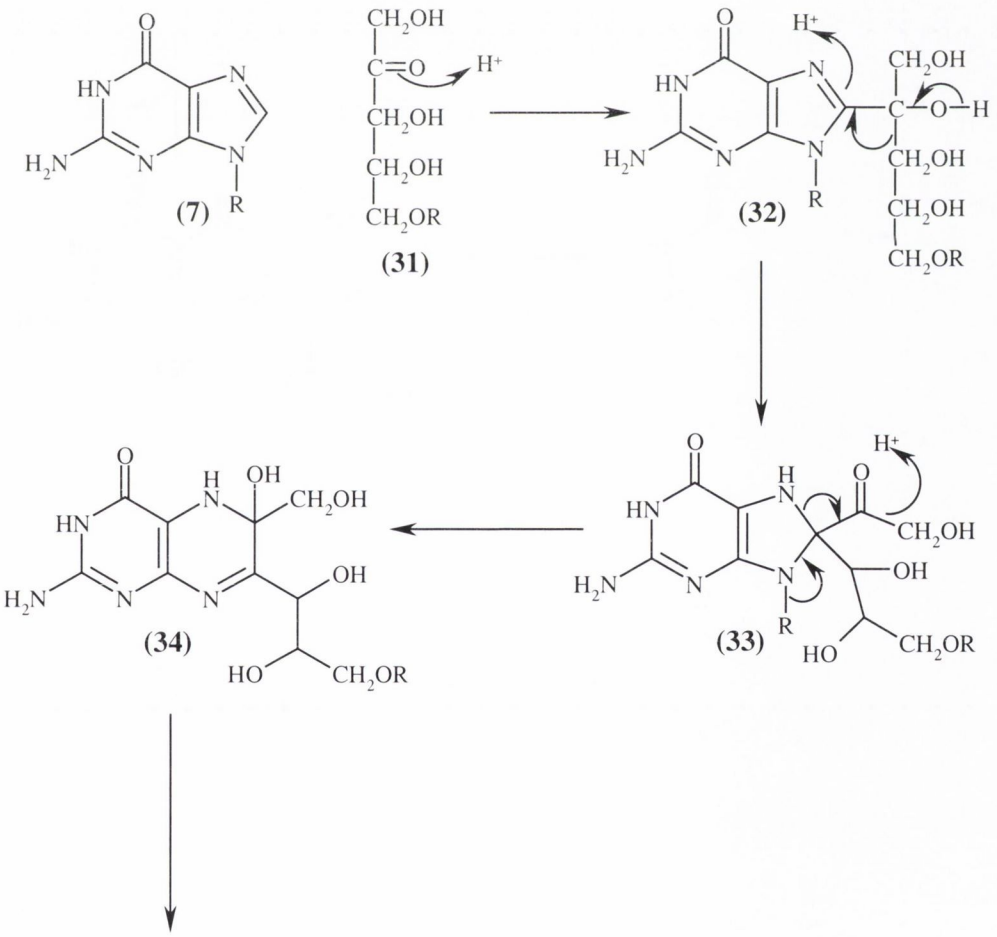
Scheme 1.10 : *Pathway proposed for the biosynthesis of molybdopterin, A) Via the folic biosynthesis intermediates H_2-neopterin triphosphate and 6-hydroxymethyldihydropterin B) by fragmentation and reutilisation of the ribose side chain.*

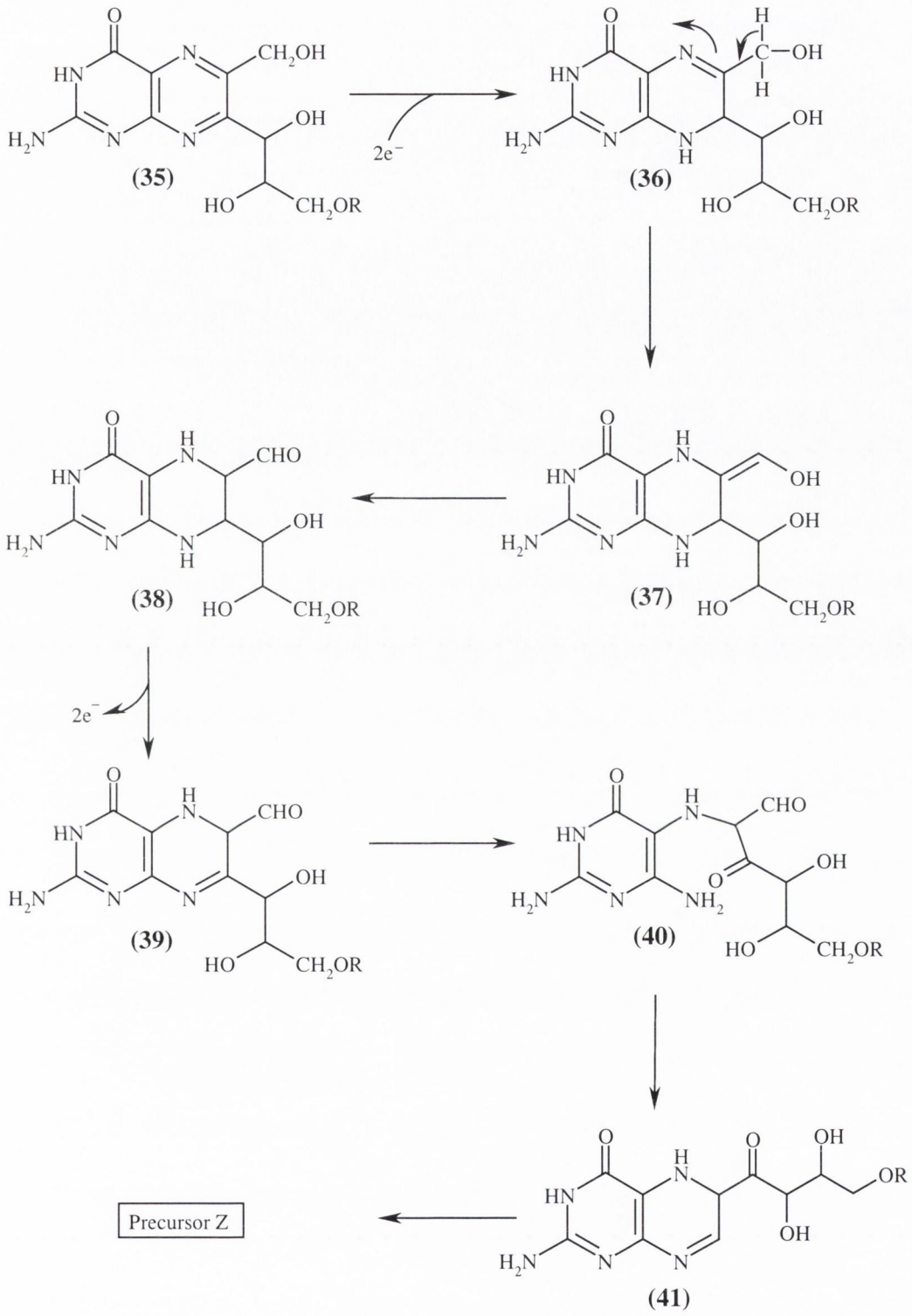
In contrast, Wuebbens and Rajagoplan[99] proposed a different mechanism **(Scheme 1.10B)**, on the basis of results obtained from experiments on *E. coli* that was supplied with ^{14}C labelled guanosine triphosphate. They found that the side chain carbon atoms C-2', C-3'and C-4' of molybdopterin, as well as the ring carbon atoms C-6 and C-7 are derived from the ribose side chain of guanosine. The side chain carbon atom of C-1'was proposed to be derived from the purine ring system.

The investigations conducted by Bacher and coworkers[37] gave results in close agreement with the work reported by Wuebbens and Rajagoplan[99]. It was found that the ^{14}C label from [8-^{14}C]-guanosine was transferred to C-1' of compound Z. Moreover, the data obtained by Bacher *et al.*[37] showed conclusively that all five carbon atoms from one specific ribose molecule are jointly diverted to compound Z by an intramolecular process, although at least one of the carbon-carbon bonds in the precursor molecule must be broken in the reaction pathway. Similarly, N-7 as well as C-8 of double-labelled guanine were jointly transferred to compound Z, but again the bond between the respective atoms was broken during its conversion to compound Z.

In experiments using [U-$^{13}C_5$]-ribulose triphosphate, it was shown that ^{13}C is incorporated into compound Z with higher efficacy than the ribose part of the nucleoside[37]. The higher enrichment of compound Z carbon atoms derived from pentulose 5-phosphate **(31)** in

comparison with the ribose side chain of guanosine **(7)** could indicate that the respective carbon atoms in compound Z are not directly derived from the side chain of the same guanosine molecule. This would implicate a biosynthetic pathway where guanine or a derivative such as the nucleoside react initially with a compound of the pentulose phosphate pool, e.g. ribulose 5-phosphate **(Scheme 1.11)**.



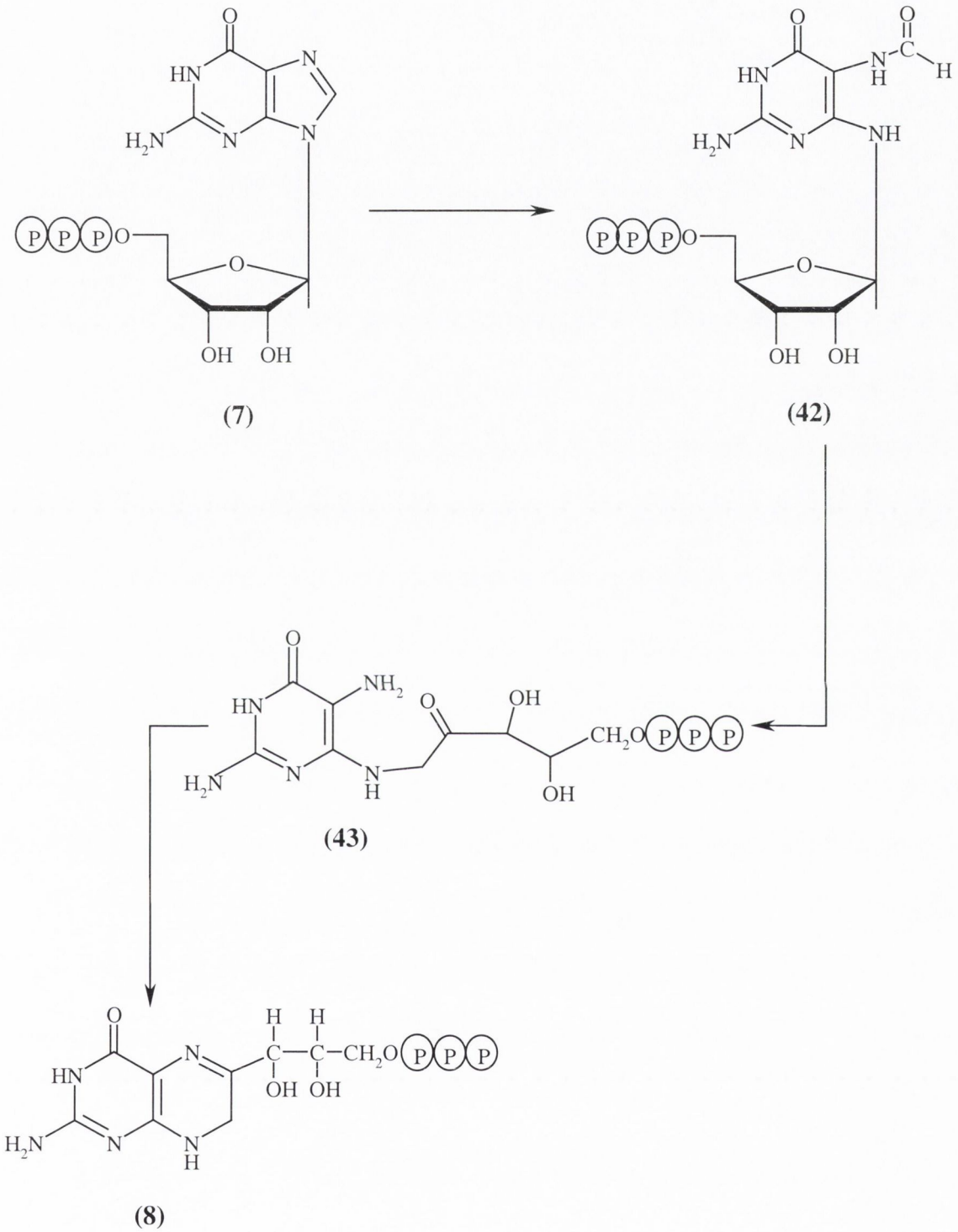


Scheme 1.11: *Hypothetical mechanism for the formation of precursor Z by condensation of guanine or a derivative with a ribulose moiety*[37].

1.8 The mechanism of action of GTP–CH (I)

As discussed earlier all naturally produced pteridines with the exception of molybdopterin[37] derive their skeleton from guanosine triphosphate, *via* the key intermediate dihydroneopterin triphosphate **(8).** To investigate the possible mechanism through which GTP cyclohydrolase (I) converts GTP into H_2-neopterin, an experiment was performed in which [U-^{14}C] GTP and [8-^{14}C]GTP were fed to the organism *Lactobacillus planarum*[19]. It was found that all the carbon atoms of GTP were incorporated into H_2-neopterin triphosphate with the exception of the carbon atom at the 8-position, which was lost as a formate. This was further demonstrated in studies carried out on other biological systems and led to the proposal that the imidazole portion in the purine ring of GTP was derived from a 2-amino-5-formylamino-6-ribofuranosyl amino 4(3*H*) pyrimidone 5'-triphosphate intermediate[20] **(42)**, although the positioning of the formyl group on the 5-amino group rather than on the 6-ribosoamino group was not proved absolutely **(Scheme 1.12)**.

It was proposed that the ribose moiety in **(42)** is then opened up and undergoes an Amadori type rearrangement to give **(43)**. At this stage there might also be a loss of formate, followed by the rearranged ribose **(43)** undergoing recyclisation, with the carbon 1 and 2 of the ribose completing the heterocyclic ring of dihydroneopterin



Scheme 1.12 : *Proposed mechanism for the transformation of GTP to H_2-neopterin*[19,20].

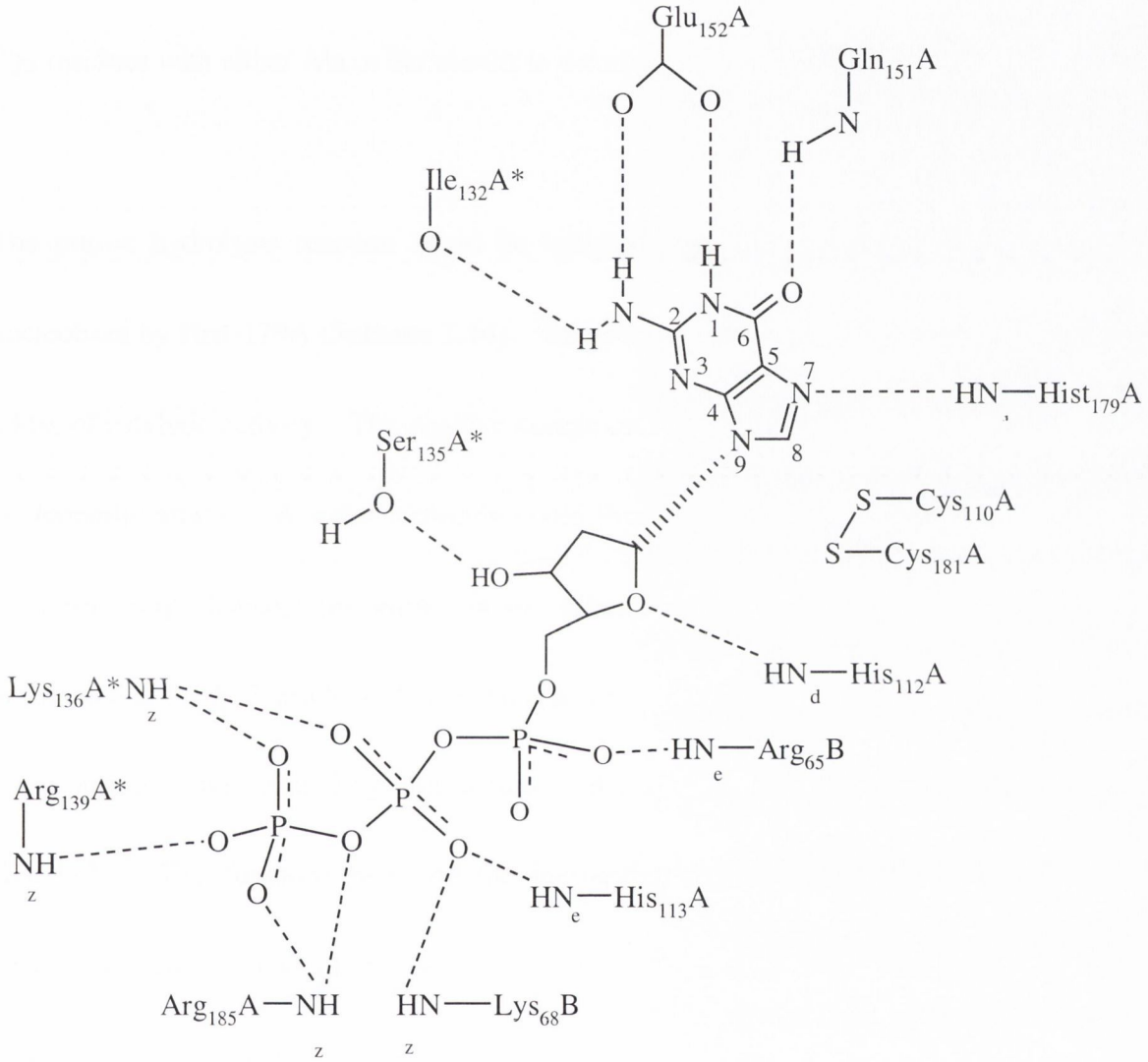
triphosphate **(8)**. Although this proposal is quite old, it has been widely accepted. However there has been little direct evidence for the existence of the intermediates shown above, and almost nothing is known about the mechanism of the reactions catalysed by the enzyme.

Advances in the field of molecular biology have recently allowed the successful cloning and sequencing of GTP cyclohydrolase (I) enzyme[100-106]. X-ray studies carried out on GTP–CH[107] from *E. coli* gave the following information: the protein is a doughnut shaped decamer with D_5-symmetry and an approximate 100 Å diameter and 65 Å height. The active site **(scheme 1.13)** is a cavity located at the interface of three adjacent subunits, A, A* and B (two from one pentamer and one from the other pentamer; the corresponding subunits are labelled by A, A*, B). Further information on the active site was obtained after crystals of the mutant GTP–CH, in which Hist-112 was converted to a serine residue, were successfully grown in the presence of the substrate analogue 2'-deoxyguanosine triphosphate. X-ray crystallography revealed that at the active site of the GTP-CH[107,108], the guanine moiety is fixed in a cleft constituted by Ile-132A*, Glu-152A, Gln-151A, Hist-179A, Hist-112A. The entrance to the active site pocket is lined by a cluster five basic amino acids residues Arg-65B, Hist-113A, Lys-68B, Arg-185A and Arg-139A*. The protein acceptor site for the substrate analogue contains a 10 Å-deep pocket, which is ideally suited to

receive the nucleoside triphosphate molecule. Hist-112A and Ser-135A* forms hydrogen bonds to the ribose unit.

The binding site for the pyrimidine portion of the purine ring system is located at the base of the active site cavity. Glu-152A forms a salt bridge with the guanidine moiety. Gln-151A forms a hydrogen bond with its amide hydrogen to the C-4 oxo group of the purine. Hydrogen bonding also occurs between the N-2 of the guanine moiety and the carbonyl oxygen of Ile-132A*. This pattern of recognition is specific for guanine and explains why the enzyme is selective for GTP rather than ATP. The inner wall that lies parallel to the pyrimidine ring is lined by non polar amino acids residues Val-150A and Leu-134B that provide a suitably hydrophobic environment[109]. The imidazole portion of the nucleobase is flanked by Cys-110A–Cys-181A and is hydrogen bonded to Hist-179A. There is space left on both side of the plane that might be occupied by solvent during catalysis.

A cluster of basic residues Hist-113A, Arg-185A, Lys-136A*, Arg-139A* and Lys-65B, at the pocket entrance binds the triphosphate group of the dGTP. In contrast to other nucleoside triphosphate binding proteins, Mg^{2+} is neither necessary nor realised in GTP–CH. The binding of the nucleobase deep in the active site pocket places the imidazole ring proton at C-8 in close proximity to the disulfide bridge Cys-110A–Cys-

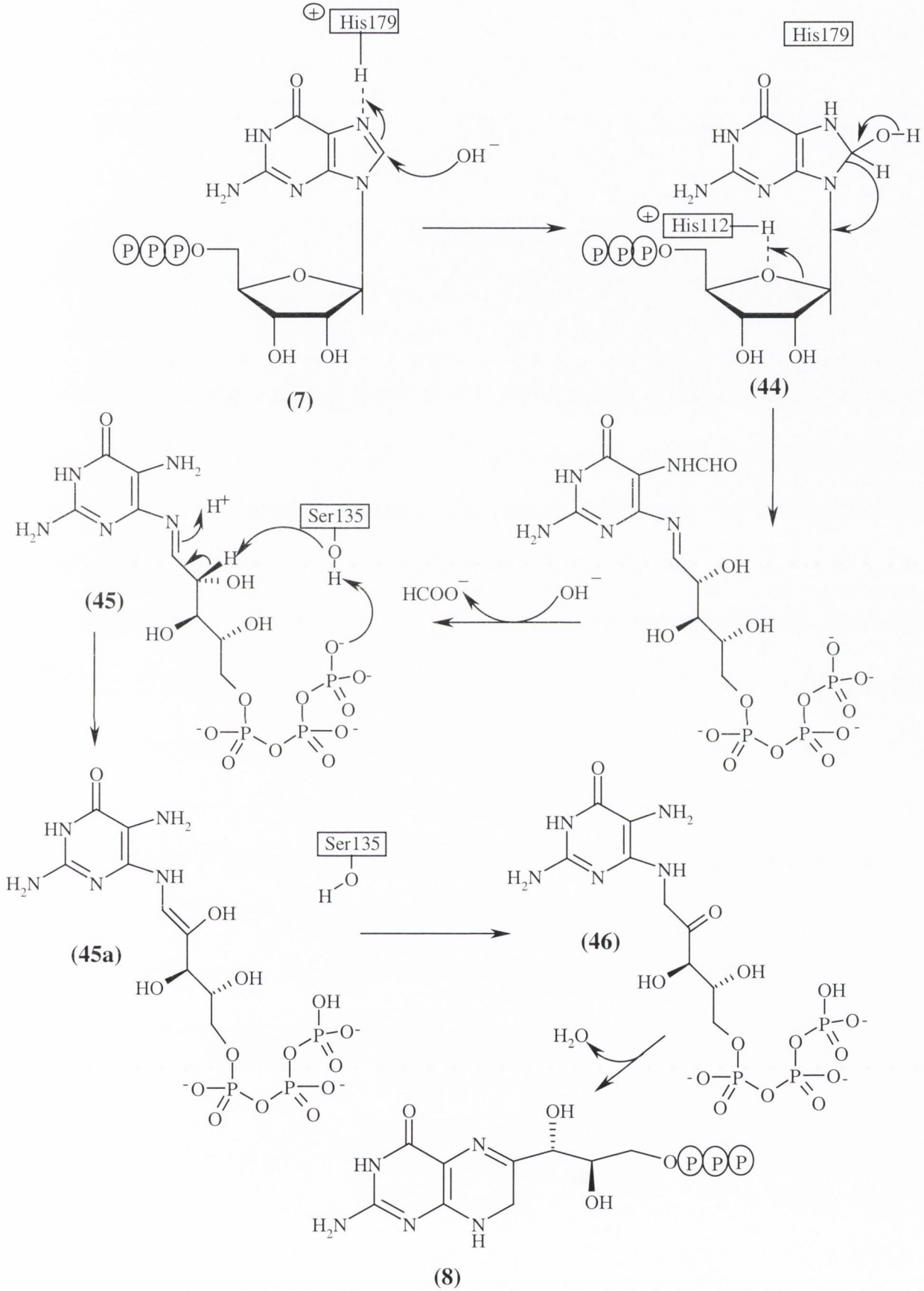


Scheme 1.13 : *represents the interactions of active site residues with 2'-deoxy-guanosine triphosphate*[107,108]. *A, A* and B denote the subunits to which the residues belong.*

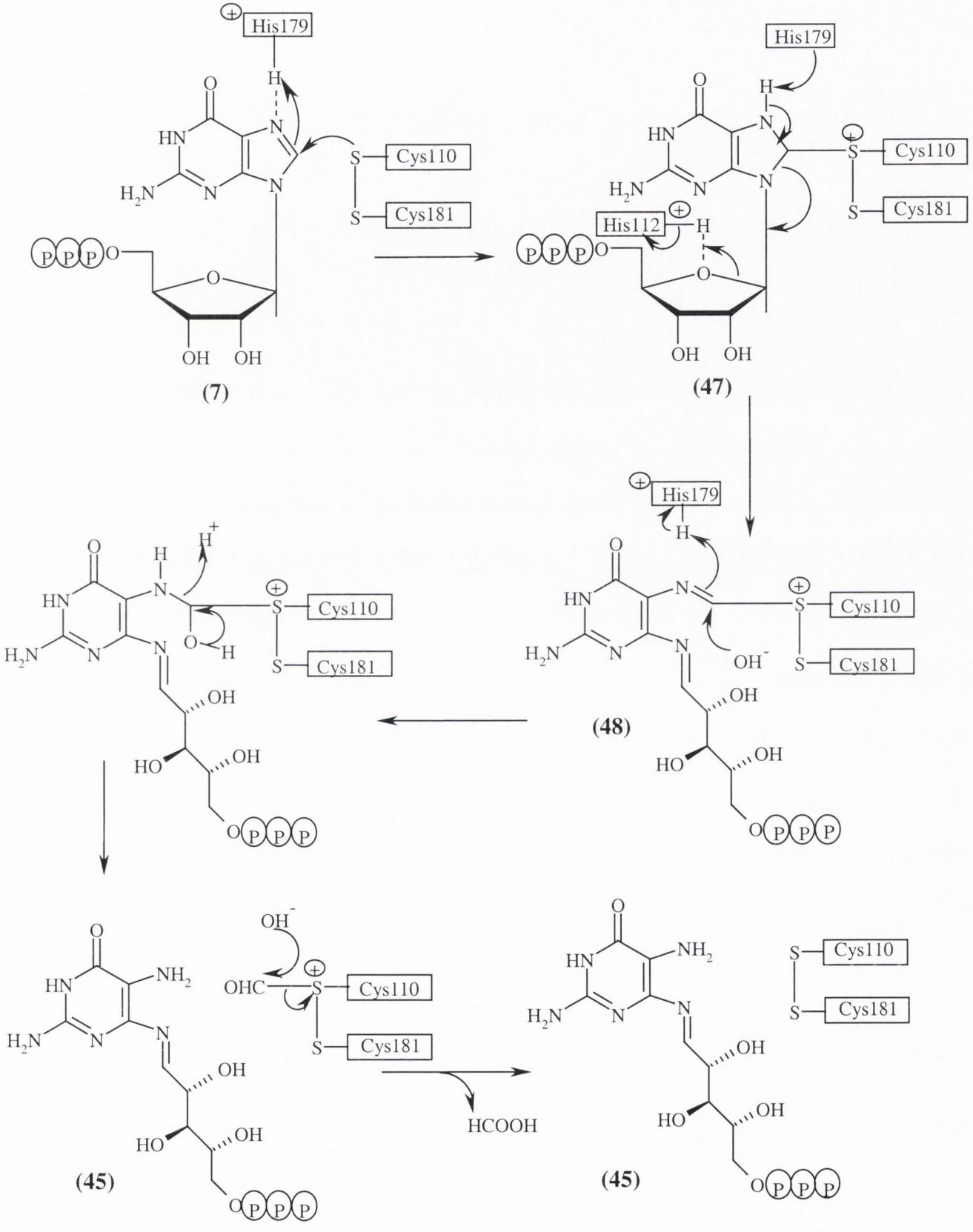
181A, which forms the central part of the active site structure. The distance between C-8 and the S of Cys-110A is 3.5 Å. Site directed mutagenesis experiments have been used to show that the elimination of the disulfide bridge by replacement of either of the Cys residues with either Ala or Ser results in a complete virtual loss of activity[110].

The purine hydrolysis reaction could be initiated by protonation at the N-7 of the nucleobase by Hist-179A **(Scheme 1.14)**. Replacement of Hist-179A by Phe results in a loss of catalytic activity. The positive charge on the imidazole C-8 makes it prone to nucleophilic attack. A water molecule could then act as a nucleophile to initiate the imidazole ring cleavage (as shown in the scheme below). Hydration of the GTP protonated at the N-7 would yield the intermediate **(44)**. The opening of the imidazole ring could then be assisted by protonation of the bridging O atom in the furanose by Hist-112. The furanose ring and the imidazole ring could both be opened in a concerted manner to yield the Schiff's base intermediate **(45)**. In line with this hypothesis, the exchange of Hist-112 for Phe yields enzyme of very low activity[109].

Alternatively, the cystine might be the agent that attacks the C-8 of the purine **(Scheme 1.15)**. Nucleophilic attack of the purine by the sulphur of Cys-110A would involve the formation of a thiasulfonium ion intermediate **(47)**, that would react with solvent to



Scheme 1.14 : *Hypothetical mechanism for GTP–CH proposed on basis of crystallographic and mutagenasis data*[109].



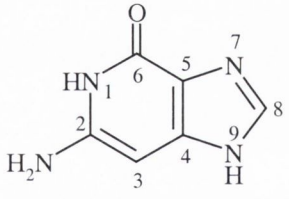
Scheme 1.15 : *Alternative hypothetical mechanism for opening of imidazole ring*[109].

restore the cystine. However, no precedent for this hypothetical reaction sequence is yet known. The Amadori rearrangement of intermediate **(45)** probably involves the loss of the C-2' proton and keto-enol tautomerisation to yield the C-1' methylene, C-2' intermediate **(45a and 46, Scheme 1.14)**. On the basis of the structure of the dGTP complex, the structure suggests a hydrogen bond between Ser-135A* acts as a base for C-2' proton abstraction. It is located in close proximity to the C-2'–C-3' bond of the sugar.

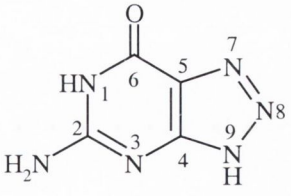
1.9 Inhibition of GTP–CH

The development of drugs for treating infectious diseases caused by bacteria and fungi has been largely successful, however it is often overlooked that micro-organisms are increasingly developing multi-drug resistance, for example several strains of bacteria have acquired immunity to penicillin. Hence it is crucially important that a new alternative approach is found to combat these pathogenic organisms. In the modern approach to drug design, an enzyme which catalyses an essential biochemical transformation in the pathogenic organism is targeted. There are several strategies for the development of enzyme inhibitors. One approach is to design mechanism based inactivators which can covalently modify amino acid residues at the active site, thereby blocking the targeted enzymatic transformation. Another way is to use transition state analogues, which will bind strongly to the active site preventing turnover of substrate.

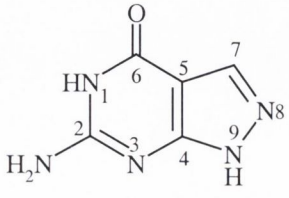
In our approach towards the synthesis of inhibitors for GTP–CH (I) careful consideration was given to the structure of the active site of the enzyme **(scheme 1.13)**. Initially three possible lead compounds were chosen as candidates for further study and biological evaluation. The nitrogen at the 3-position in the guanine moiety should not have any significant impact with the active site, and hence its replacement to give 3-deazaguanine **(49)** should not have any effect on its binding to the active site. The other chosen lead molecules for initial studies were 8-aza-7-deazaguanine **(50)** and 8-azaguanine **(51)** to observe whether or not modification in the imidazole portion of the guanine ring would give any inhibition.



(49)



(51)



(50)

Chapter 2

Design and synthesis of inhibitors for the enzyme GTP–CH (I)

2.1 *Review* : The synthesis of 3-Deazaguanine and 3-deazaguanosine

Biological Significance

3-Deazaguanine (6-amino-4-oxo-4,5-dihydroimidazo[4,5-c]pyridine) **(49)** is a structural analogue of guanine in which there is an isosteric replacement of the nitrogen at the purine 3-position by a carbon. The broad spectrum of antiviral activity against a variety of DNA and RNA viruses[111], as well as potent antitumour activity[112-115] of 3-deazaguanine and its metabolites 3-deazaguanosine (6-amino-4-oxo-4,5-dihydro-9-β-D-ribofuranosyl-imidazo[4,5-c]pyridine) **(52)** and 3-deazaguanylic acid (6-amino-4-oxo-4,5-dihydro-9-β-D-ribofuranosylimidazo[4,5-c]pyridine 5'-phosphate) **(53)** has been well documented by a number of laboratories[115-119].

Synthesis of 3-deazaguanine

The first reported attempt to prepare 3-deazaguanine **(49)**[120] was made starting from 4,6-dichloroimidazo-[4,5-c] pyridine **(56)** which was synthesised from 4-amino-2,6-dichloro-3-nitropyridine **(54).** The nitro group of this compound **(54)** was reduced, following which ring closure was accomplished by condensing the two amino groups of the resulting 3,4-diamino-2,6-dichloropyridine **(55)**[121] with 98% formic acid to give **(56)**. The emphasis of the synthesis was on the replacement of the chlorine atoms in the 4- and 6-

positions of **(56)** by a hydroxyl and an amino group respectively. To achieve this **(56)** was first heated with concentrated ammonia in a sealed tube at 180°C. The structure of the resulting compound proved to be 4-amino-6-chloroimidazo[4,5-c]pyridine **(57)** which upon hydrogenolysis resulted in the formation of 3-deazaadenine **(58)**[122]. The amino group was therefore introduced into the wrong position for the desired target molecule **(49)**, and all attempts to replace the chlorine atom in the 4-position of **(56)** by a hydroxyl group *via* a benzyloxyl or mercapto group were not successful. Also the replacement of the 6-chlorine atom in **(56)** by an amino group or a hydroxyl group *via* a mercapto group failed.

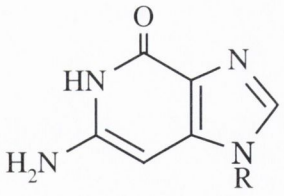
As a result of the lack of success of synthesising 3-deazaguanine **(49)** starting from a pyridine precursor, it became of interest whether the synthesis could be achieved starting from an imidazole precursor. This approach proved to be successful and the first route to the synthesis of 3-deazaguanine **(49)** and 3-deazaguanosine **(52)** was reported in 1976 by Robins and Cook *et al.*[123], which involved a base catalysed ring closure of a 5(4)-cyanomethylimidazole-4(5)-carboxamide **(60).** The latter **(60)** was prepared from the corresponding methyl 5(4)-cyanomethylimidazole-4(5)-carboxylate **(59)**[123,124].

Methyl 5(4)-cyanomethylimidazole-4(5)-carboxylate **(59)** and liquid ammonia when heated at 100°C for 8 days, provided 3-deazaguanine **(49)** in 77 % yield. The

intermediate methyl 5(4)-cyanomethylimidazole-4(5)-carboxamide **(60)** could also be obtained in 77% yield by interruption of the reaction of **(59)** and ammonia after 48 hours. Product **(60)** was smoothly cyclised to 3-deazaguanine **(49)** upon refluxing with 10% sodium carbonate. The disadvantage of this method is that it requires the use of high-pressure apparatus.

The other methods of synthesising 3-deazaguanine **(49)**, apart from one exception, are simply a modification of the approach by which the cyclisation is carried out starting from the key precursor, methyl 5(4)-cyanomethylimidazole-4(5)-carboxylate **(59)**[123]. These alterations made the synthesis of 3-deazaguanine more practical, as the use of high-pressure apparatus was no longer necessary. **Scheme 2.3** shows a further improvement in the synthesis[125] of 3-deazaguanine **(49)** from **(59)**, which proved to be more advantageous in terms of the purity of the final product.

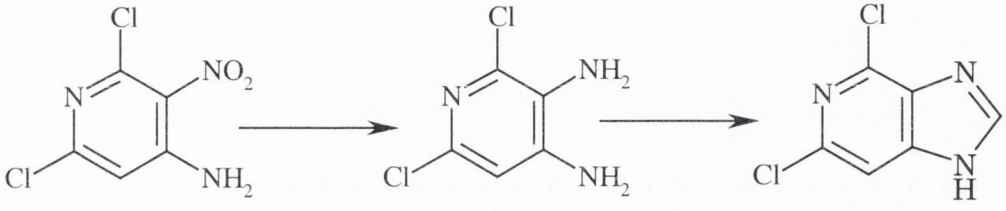
The key intermediate methyl 5(4)-cyanomethylimidazole-4(5)-carboxylate **(59)** was refluxed in ethanol with hydroxylamine, where the nitrile group readily reacted with hydroxylamine to form methyl 4(5)-carboxamidoximyl methylimidazole-5(4)- carboxylate **(62)**. Hydrogenation of derivative **(62)** in the presence of Raney nickel catalyst provided 3-deazaguanine **(49)** in 52% yield.



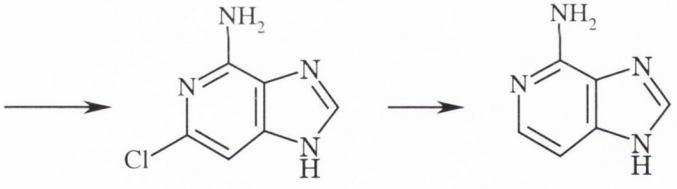
(49) R = H

(52) R = -*β*-D-Ribofuranose

(53) R = -*β*-D-Ribofuranosyl-5'-phosphate

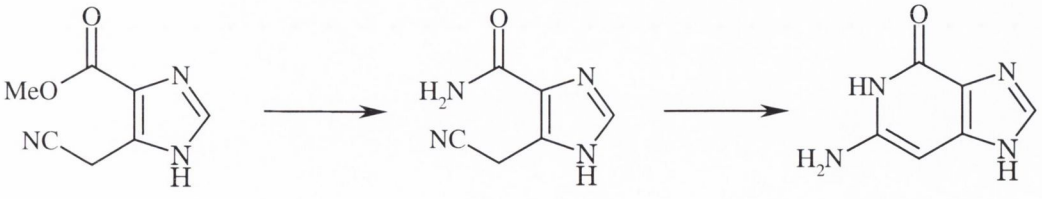


(54) **(55)** **(56)**



(57) **(58)**

Scheme 2.1

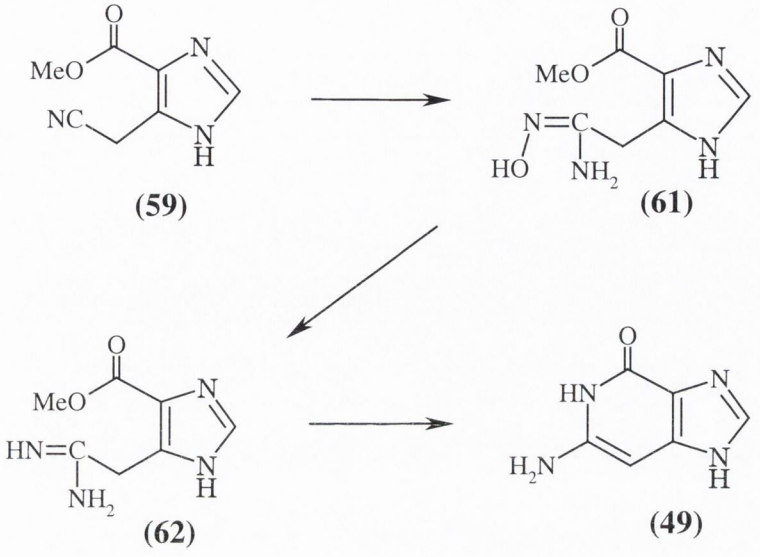


(59) **(60)** **(49)**

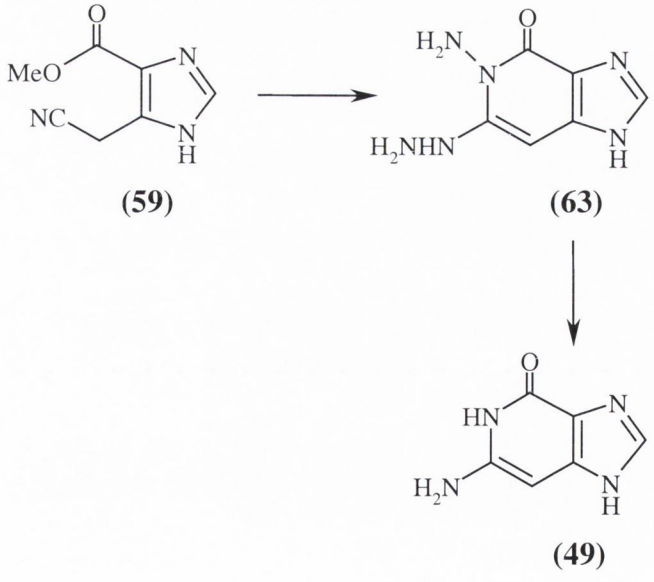
Scheme 2.2

Further to the cyclisation strategy already mentioned above of methyl 5(4)-cyanomethylimidazole-4(5)-carboxylate **(59),** it was found[126] that cyclisation of **(59)** could also be carried out upon treatment with pure hydrazine hydrate (99%) to give the cyclised adduct 6-hydrazino-1,5-dihydro[4,5-c]pyridin-4-one **(63)**, which on hydrogenation in the presence of Raney nickel catalyst gave 3-deazaguanine in 80% yield, which is a considerable improvement to the previous method[125] using hydroxylamine as already outlined, which gave an approximate yield of 52%.

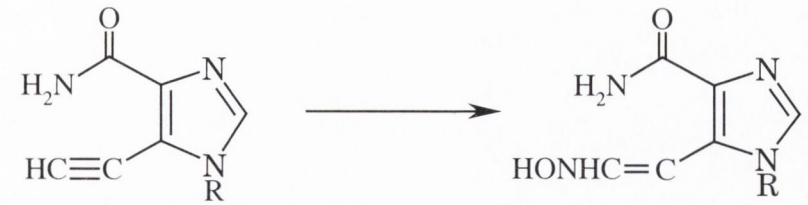
As a result of the problems associated with the classical condensation methods of regio and stereochemical disadvantages Minikawa and Matsuda[127] reported a new synthetic route towards 3-deazaguanine **(49)**. The latter approach involved the synthesis of 5-ethynylimidazole-4-carboxamide **(64)**[128] and its cyclisation to give the desired product **(49)**. Initially hydrobromination of 5-ethynylimidazole **(64)** was carried out using hydroxylamine, dimethylamine and acetic acid over several hours. The desired product 5-(2-hydroxyiminoethyl)-imidazole-4-carboxamide **(65)** was obtained in 91% yield. Treatment of **(65)** with phenyl isocyanate gave intermediate **(66)**, which was subsequently heated at 50°C to give 5-cyanomethylimidazole-4-carboxamide **(60)** in 66% yield. The ring closure of **(60)** was achieved using 5% aqueous sodium carbonate-ethanol under reflux to furnish the desired product 3-deazaguanine **(49)**.



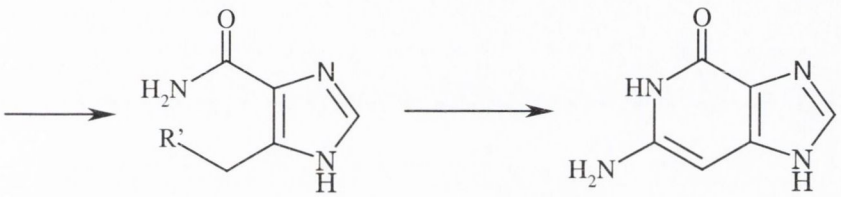
Scheme 2.3



Scheme 2.4



(64) **(65)**



(66) R' = CH=NOCONHPh **(49)**

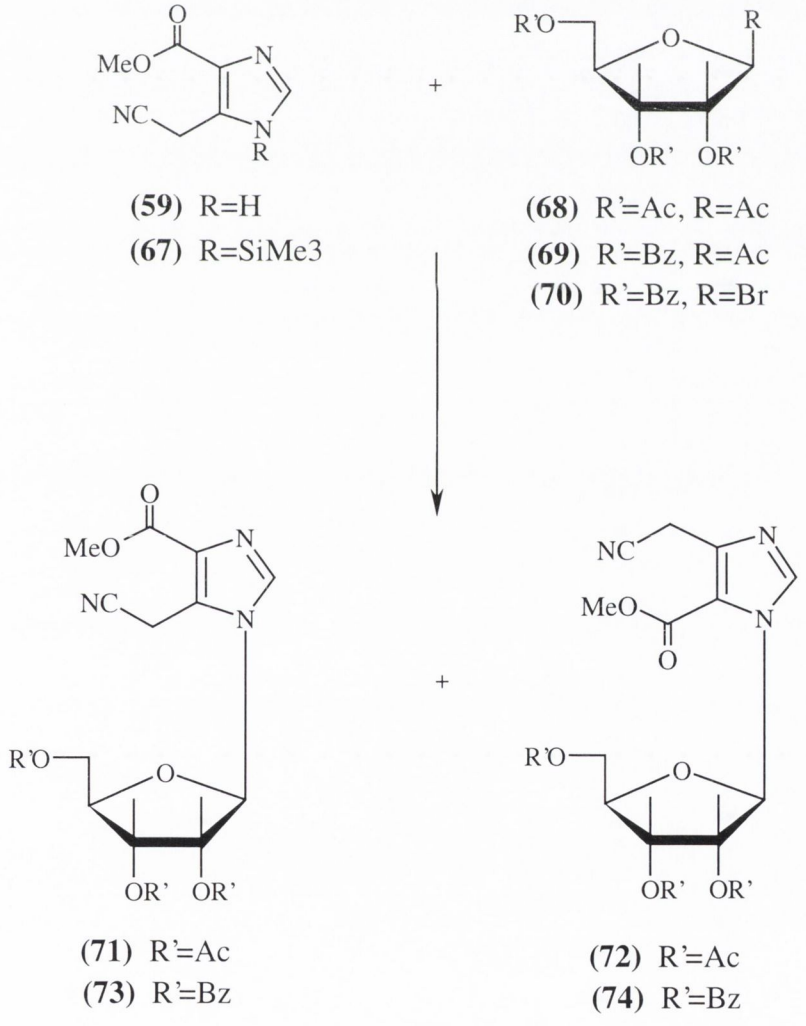
(60) R' = CN

(conversion of **66** to **60**)

Scheme 2.5

Synthesis of 3-deazaguanosine

The approach adopted for the synthesis of 3-deazaguanosine **(52)** thus far, regardless of the method used to synthesise the requisite 3-deazapurine **(49)** ring system, relies initially on the attachment of the protected ribosyl unit to the key precursor: methyl 5(4)-cyanomethylimidazole-4(5)-carboxylate **(59)** prior to the cyclisation to form the purine derivative **(52)**[123,125,126,127].



(59) R=H
(67) R=SiMe3

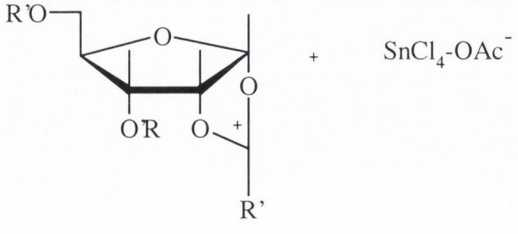
(68) R'=Ac, R=Ac
(69) R'=Bz, R=Ac
(70) R'=Bz, R=Br

(71) R'=Ac
(73) R'=Bz

(72) R'=Ac
(74) R'=Bz

Scheme 2.6

Ribosylation of **(59)** was approached by three methods: **(A)** Stannic chloride-catalysed condensation of the trimethylsilylated derivative **(67)** [methyl 5(4)-cyanomethyl-N-trimethylsilylimidazole-4(5)-carboxylate] with a fully acylated ribofuranose [1,2,3,5-tetra-*O*-acetyl-β-D-ribofuanose **(68)**] or 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose **(69)**[129], **(B)** Condensation of **(59)** with 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl bromide **(70)** in acetonitrile[130], and **(C)** acid-catalysed high temperature fusion of **(67)** with **(68)**[131,132]. In general, ribosylations of imidazoles provide a mixture of positional isomers (*i.e.*, a ribose on either the 1- or 3-nitrogen ring atoms, **71** or **72**, **73** or **74** respectively)[133]. This was the case with methods **(B)** and **(C)**; however, it was found that the yield and ratio of positional isomers in method **(A)** markedly depends on the ratio of stannic chloride to **(67)** and the blocked sugars, **(68)** or **(69)**. Thus, treatment of 1 equivalent of **(67)** in dichloroethane or acetonitrile with 1 equivalent of **(68)** or **(69)** and 1.44 equivalence of stannic chloride afforded a quantitative yield of methyl 5(4)-cyanomethyl-(3,3,5-tri-*O*-benzoyl- or acetyl-β-D-ribofuranosyl) imidazole-4(5)-carboxylate, **(71)** or **(73)**, respectively.



(75)

The intermediacy of a stannic chloride heterocycle **(75)** complex, which provides a regiospecific ribosylation of **(67)**, may be a plausible explanation for these results. However, although it is assumed that Lewis acids such as stannic chloride, promote the formation of the acyloxonium ions of fully acylated riboses, such as **(75)**, as noted by usually exclusive formation of β-nucleosides[129,134], no account of silylated heterocycles with stannic chloride or other Lewis acids has been reported.

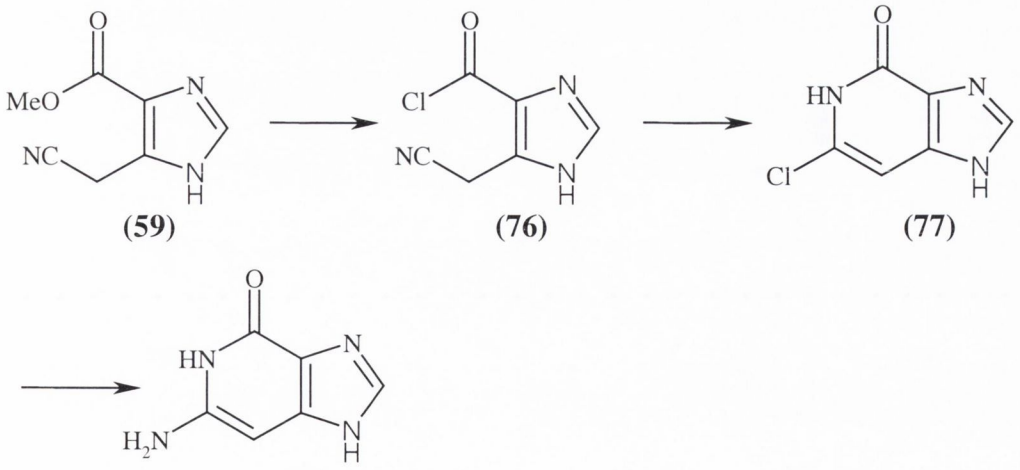
2.1.1 Synthesis of 3-deazaguanine: and derivatives: Results and Discussion

The initial goal of the project, besides trying to find inhibitors for the enzyme GTP cyclohydrolase (I), was to investigate whether a simpler route could be found towards the synthesis of 3-deazaguanine which would make it more readily available. A second objective was to see whether it was possible to produce 3-deazaguanosine selectively using an approach, which was reported to be successful for guanosine[135].

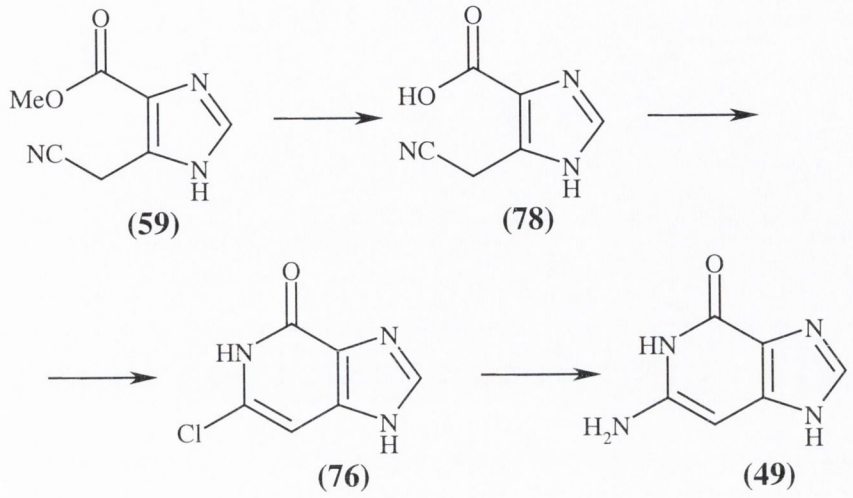
The literature report by Robins *et al.*[126] for the synthesis of the required 3-deazaguanine **(49)** appeared to be by far the best method in terms of yield and ease of isolation of the product. However in practice in our hands it was found otherwise on repeating this synthesis, the reactions were often found to be poor in terms of purity and yields throughout the synthesis, and in the final step, the desired catalytic hydrogenation of 6-hydrazino-1-amino-4-oxo-imidazo[4,5-c]pyridine **(63)** using Raney nickel did not generate the quantity of 3-deazaguanine **(49)** as quoted in the literature[126] and often gave no product at all. The starting material **(63)** appeared to be pure on analysis by both TLC and ^{1}H NMR.. Another possibility was that the commercially available Raney Ni was not of a high enough grade, so we investigated the preparation of fresh Raney Ni by a literature procedure[125]. This approach did not improve the yields of 3-deazaguanine obtained and

we therefore began to investigate for alternative strategies, which would allow the preparation of greater quantities of the desired product.

It was decided that methyl 5(4)-cyanomethylimidazole-4(5)-carboxylate **(59)** would be a good candidate for an alternative precursor of **(49)**. If the ester functionality on **(59)** could be transformed into an acid chloride **(76)**[136] using a chlorinating agent such as thionyl chloride, then there may be spontaneous cyclisation of **(76)** to give 6-chloro-4-oxo-imidazo[4,5-c]pyridine **(77).** Subsequent amination of the latter **(77)** could lead to the desired product **(49)**. Unfortunately the initial reaction of **(59)** with thionyl chloride did not lead to the desired transformation.

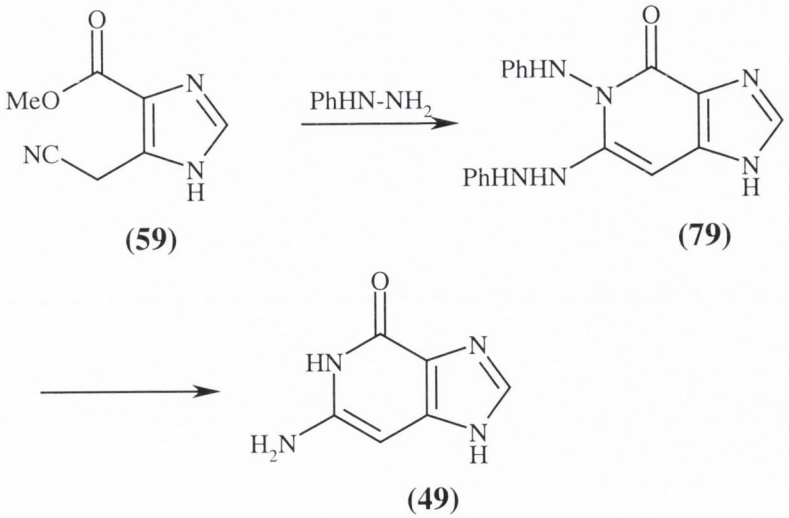


Scheme 2.7



(59) **(78)** **(76)** **(49)**

Scheme 2.8



(59) **(79)** **(49)**

Scheme 2.9

Another possibility was to see if the ester functionality of **(59)** would undergo hydrolysis to give the imidazolecarboxylic acid derivative **(78),** which could then be converted into the acid chloride derivative **(76)** and the synthesis mentioned above could still have been applicable. TLC analysis of the reaction mixture was promising. The NMR of the product could not be resolved however, hence any further work in this area was aborted. Another approach was to use phenyl hydrazine instead of hydrazine monohydrate in the condensation reaction of **(59)** to give a product **(79)** which could be more easily reduced, however this initiative came to no avail.

Our inability to produce substantial amounts of 3-deazaguanine meant that the synthesis of 3-deazaguanosine using the selective ribosylation[135] procedure could not be pursued further. As an alternative the procedure of Robins and Cook[123] was investigated. This method differed from the intended in that the protected ribose sugar unit was attached first to the precursor **(59)** prior to the cyclisation and formation of 3-deazaguanosine **(52)**. This latter method[123] proved successful giving the desired product 3-deazaguanosine **(52)** and enough material was obtained for biological evaluation (see chapter 3) as an inhibitor of GTP-CH.

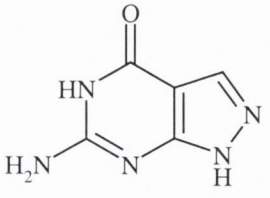
2.2 *Review*: The synthesis of 8-aza-7-deazaguanine and 8-aza-7-deazaguanosine

Interest in the synthesis of 8-aza-7-deazaguanine (6-amino-4-hydroxypyrazolo[3,4-d]pyrimidine) **(50)** stems from the antitumor activity of several other derivatives of the pyrazolo[3,4-d]pyrimidine ring systems[137,138,139].

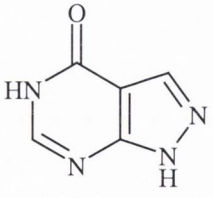
The first synthesis of 8-aza-7-deazaguanine **(50)** was achieved after the preparation of the appropriately substituted pyrazole ring system (3-amino-4-cyanopyrazole) **(80)**[139,140]. The hydrolysis of the nitrile group of **(80)** with concentrated sulphuric acid gave 3-amino-4-pyrazolecarboxamide **(81)** as a sulfate salt, fusion of this salt **(81)** with urea gave 4,6-dihydropyrazolo[3,4-d]pyrimidine **(82),** treatment of the latter compound **(82)** with phosphorus oxychloride[140] resulted in the formation of the 4,6-dichloropyazolo[3,4-d]pyrimidine **(83)**. Reaction of **(83)** with boiling sodium hydroxide gave 4-hydroxy-6-chloropyrazolo[3,4-d]pyrimidine **(84)**. Subsequent reaction of **(84)** with alcoholic ammonia at high temperature-pressure gave the desired compound **(50)**.

The synthesis mentioned above remained the standard procedure to be followed for making 8-aza-7-deazaguanine **(50)**, until only recently, when there was a renewed interest in the biologial activity of the pyrazolo[3,4-d]pyrimidine ring system[141,142]. A classical example of these biologically active purine analogues is allopurinol (1*H*-pyrazolo[3,4-d]pyrimidin-

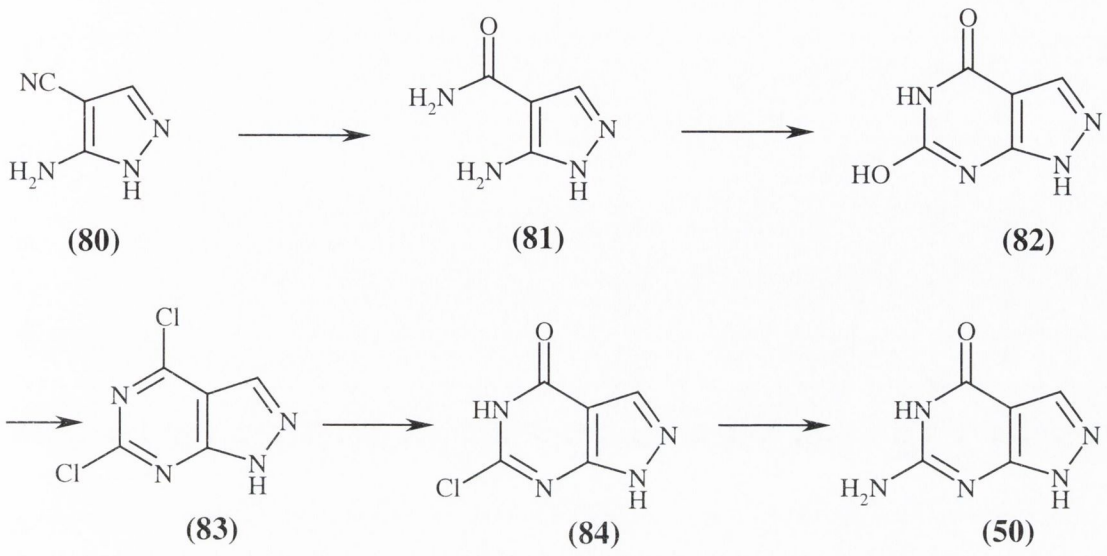
4(5*H*)-one) **(85)** which is the compound of choice for the treatment of gout[143]. In this new approach Seela and Steker[144] proposed to start with a pyrimidine derivative and then condense the pyrazole moiety onto it afterwards. Formylation of 2-amino-,6-hydroxy-pyrimidin-4-one **(86)** *via* a Vilsmeyer-Haack reaction yielded 2-amino-4,6-dichloro-5-formyl pyrimidine **(87)**. Treatment of **(87)** with aqueous hydrazine gave 6-amino-4-chloro-1*H*-pyrazolo[3,4-d]pyrimidine **(88)** and subsequent hydrolysis[145] of **(88)** produced the desired purine derivative **(50)**.



(50)



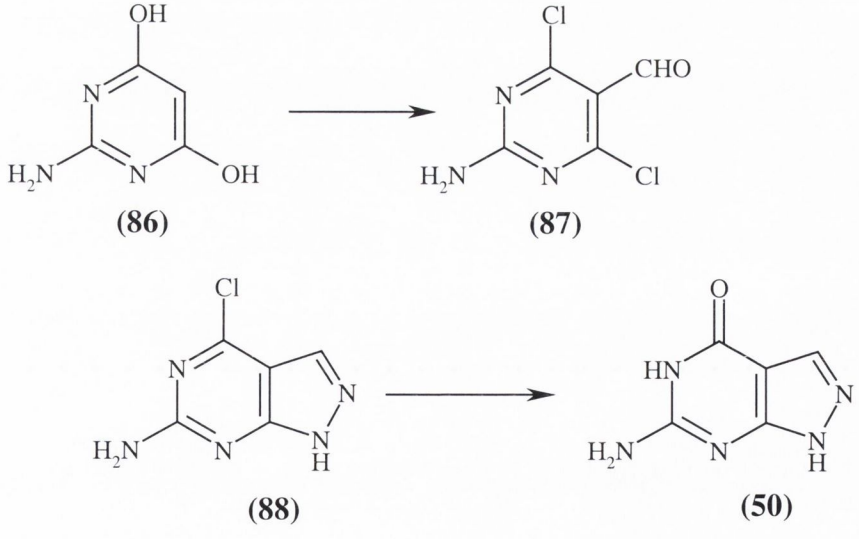
(85)



(80) **(81)** **(82)**

(83) **(84)** **(50)**

Scheme 2.10



(86) **(87)**

(88) **(50)**

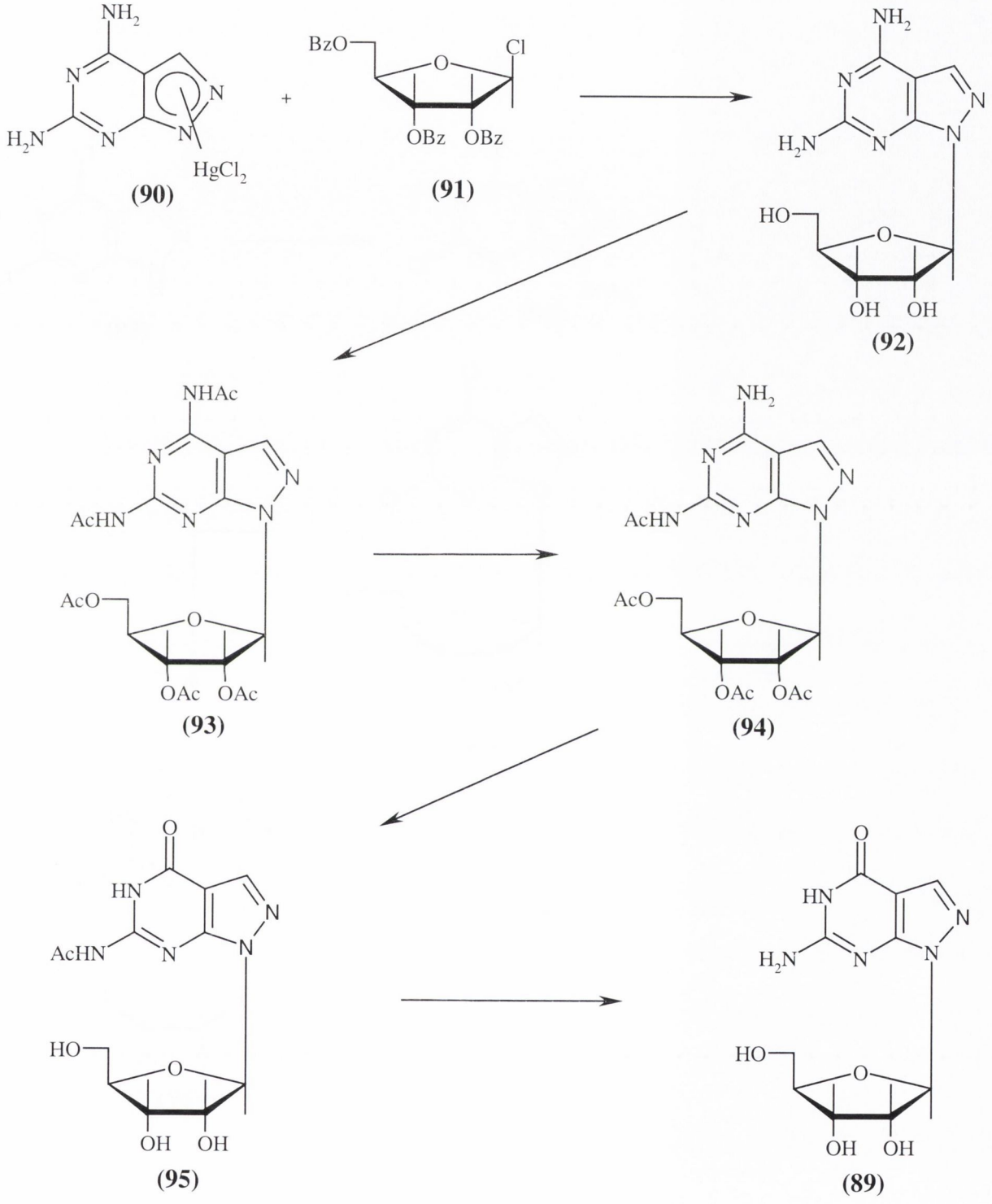
Scheme 2.11

Synthesis of 8-Aza-7-deazaguanosine

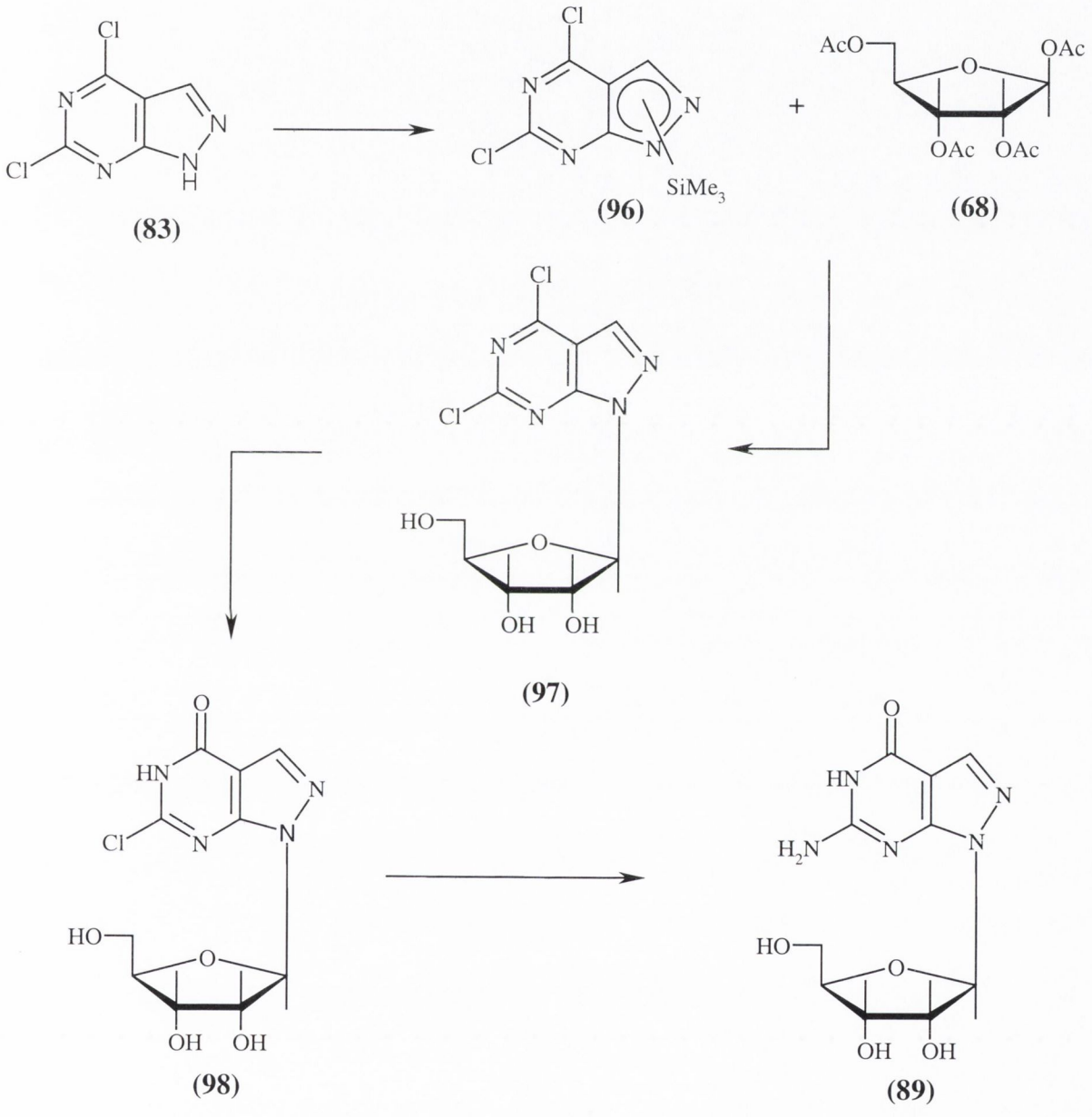
The first method for the synthesis of 8-aza-7-deazaguanosine (8-aza-7-deaza-9-β-D-ribofuranosylpyrazolo[3,4-d]pyrimidine) **(89)** was reported by Davoll and Kerridge[146]. In summary this method involved the condensation of the chloromercuric derivative of the 4,6-diamino-8-aza-7-deazaguanine **(90)** with tri-*O*-benzoyl-D-ribofuranosyl chloride **(91)**. Once the ribose protected sugar had been attached it was debenzoylated to give the selective product 4,6-diamino-9-β-D-ribofuranosylpyrazolo[3,4-d]pyrimidine **(92)** which was acetylated to give **(93)**, and subsequently selectively deprotected at one of the amino groups (4-position) to produce **(94)**. This was achieved through the treatment of **(93)** with aqueous sodium nitrite and acetic acid to produce 6-acetamido-4-hydroxy-1-β-D-ribofuranosyl-pyrazolo[3,4-d]pyrimidine **(95)**, which upon deacetylation using sodium methoxide gave the desired compound **(89)**.

The second method of synthesising 8-aza-7-deazaguanosine was reported by Robins *et al.*[147] following the general glycosylation procedure of Vorbrüggen and coworkers[148], which involved the coupling of 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuanose **(68)** with the requisite trimethylsilylated heterocyclic base in the presence of trimethylsilyl trifluormethane sulfonate ($Me_3SiOSO_2CF_3$) as a catalyst. The parent compound used for the latter coupling reaction was 4,6-dichloropyazolo[3,4-d]pyrimidine **(83)**[140] due to its availability.

The latter method involved the trimethylsilylation of 4,6-dichloropyazolo[3,4-d]pyrimidine **(83)** which was accomplished by heating with hexamethyldisilazane (HMDS) in the presence of $(NH_4)_2SO_4$ to give **(96)**. Treatment with one equivalent of 1,2,3,5-tetra-*O*-acetyl-*β*-D-ribofuanose **(68)** in the presence of 1.5 molar equivalent of trimethylsilyl triflate in dry acetonitrile at ambient temperature for 16 hours gave 4,6-dichloro-1-(2,3,5-tri-*O*-acetyl-*β*-ribofuranosyl)pyrazolo-[3,4-d]pyrimidine **(97)** as the only major nucleoside product[147]. The chlorine at the 4-position of **(97)** was displaced selectively using aqueous sodium hydroxide to give 6-chloro-4-hydroxy-(2,3,5-tri-*O*-acetyl-*β*-D-ribofuanosyl)-pyrazolo[3,4-d]pyrimidine **(98)**, which upon treatment with methanolic ammonia gave the requisite nucleoside **(89)**.


NH2
N
N
H2N
N
N
HgCl2
(90)
+
BzO
O
Cl
OBz OBz
(91)
NH2
N
N
H2N
N
N
HO
O
OH OH
(92)
NHAc
N
N
AcHN
N
N
AcO
O
OAc OAc
(93)
NH2
N
N
AcHN
N
N
AcO
O
OAc OAc
(94)
O
HN
N
AcHN
N
N
HO
O
OH OH
(95)
O
HN
N
H2N
N
N
HO
O
OH OH
(89)

Scheme 2.12


Cl
N
Cl
N
N
H
(83)
Cl
N
Cl
N
N
N
SiMe3
(96)
+
AcO
O
OAc
OAc
OAc
(68)
Cl
N
Cl
N
N
N
HO
O
OH
OH
(97)
O
HN
N
Cl
N
N
HO
O
OH
OH
(98)
O
HN
N
H2N
N
N
HO
O
OH
OH
(89)

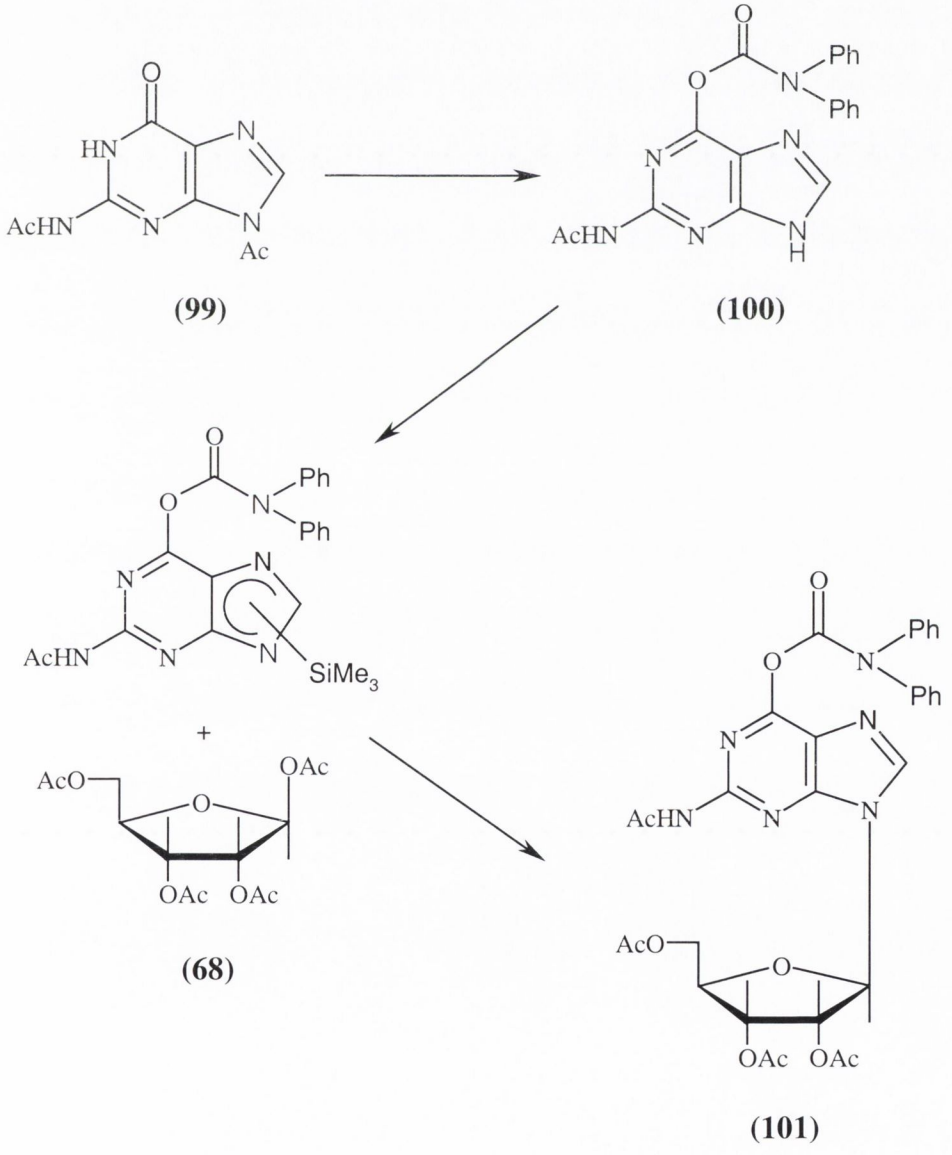
Scheme 2.13

2.2.1 Synthesis of 8-aza-7-deazaguanine and 8-aza-7-deazaguanosine: Results and discussion

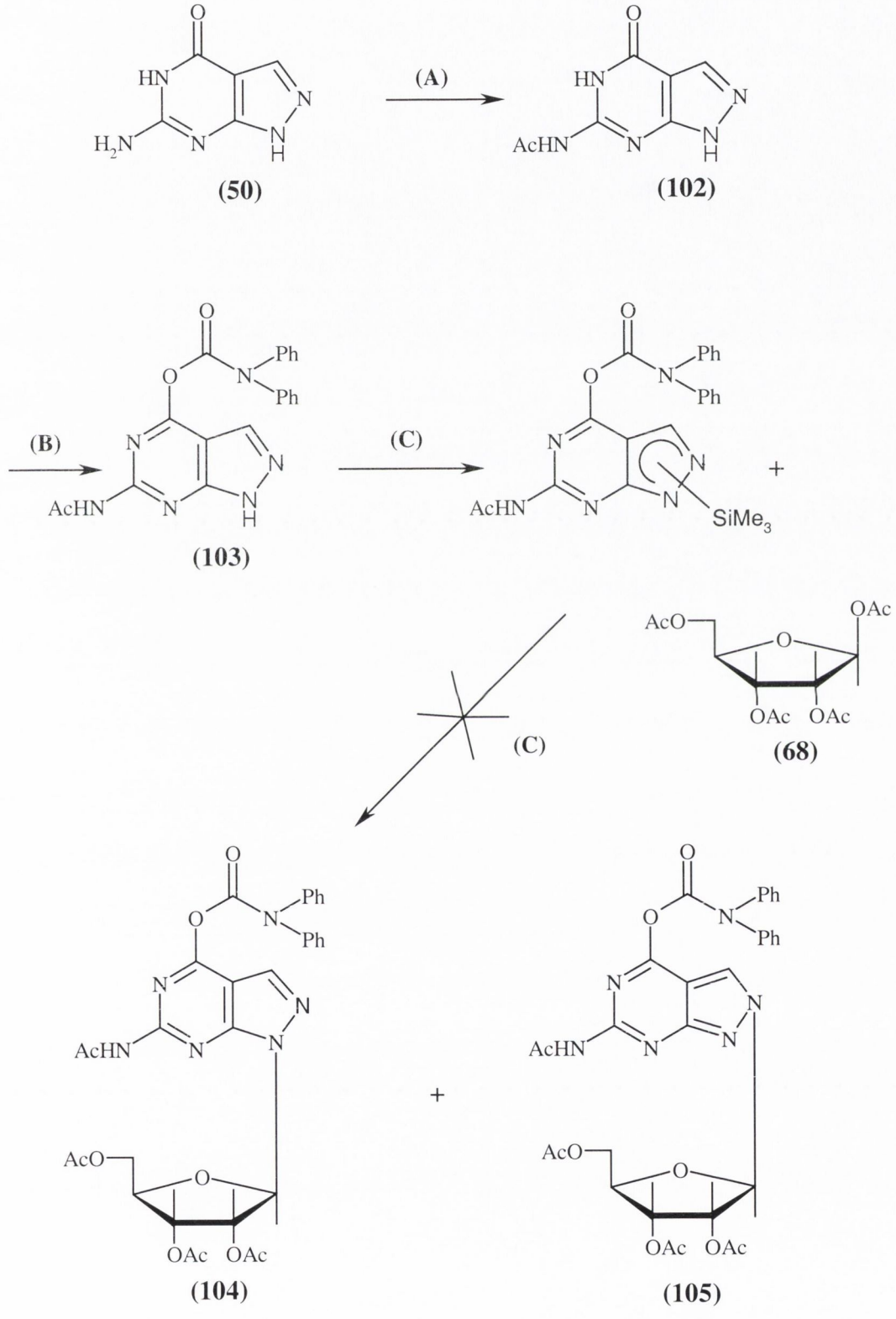
The synthesis of 8-aza-7-deazaguanine **(50)** proved to be quite successful and high yielding in comparison to that for the earlier heterocyclic system **(49)**. The procedure adopted for the synthesis of the pyrazolo[3,4-d]pyrimidine ring **(50)** was that of Seela and Steker[144] and the method for the hydrolysis of the chlorine at the 6-position of **(88)** was clearly outlined by Taylor and Petal[145]. Having been able to successfully synthesise the requisite purine derivative **(50)** attention could now be diverted towards the synthesis of its nucleoside, 8-aza-7-deazaguanosine **(89)**.

A procedure for selective ribosylation of guanine has been described by Zou and Robins[135] **(Scheme 2.14)**. In summary their work involved: **A)** Diacetylation of guanine to yield **(99)** **B)** protection of carbonyl group by carbamoylation using diphenylcarbamoyl chloride to give **(100)** and **C)** the silylation of **(100)** and the catalytic cross coupled reaction of the product with 1,2,3,5-tetra-*O*-acetyl-*β*-*D*-ribofuranose **(68)** and trimethyl-silyltriflate to give the guanosine derivative **(101).** It was hoped that this procedure could be adopted for the glycosylation of 8-aza-7-deazaguanine **(Scheme 2.15)**, although it was anticipated that the selectivity may be reduced with the formation of two possible products **(104)** and **(105)**.

The procedure of Zou and Robins[135] was applied in an attempt to synthesis 8-aza-7-deazaguanosine **(Scheme 2.15)** but proved to be unsuccessful and all attempts at altering the reaction conditions came to no avail (i.e. no glycosylation could be observed by TLC or NMR of the crude reaction mixture). Clearly an alternative approach needed to be adopted and the general glycosylation procedure of Vorbrüggen and coworkers[148] was investigated **(Scheme 2.16)**.



Scheme 2.14

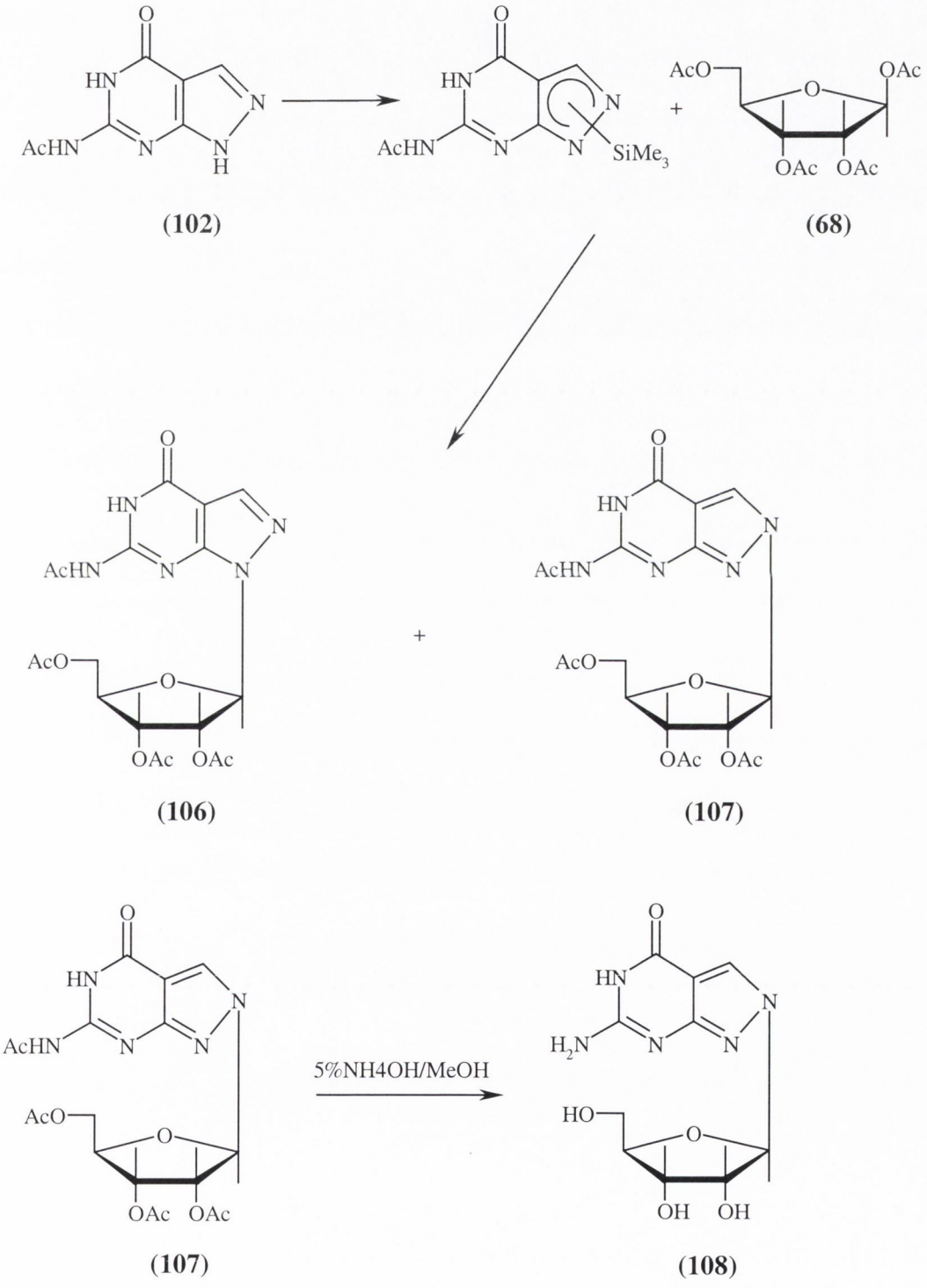


Scheme 2.15

2-N-Acetyl-8-aza-7-deazaguanine **(102)** was silylated using *bis*-trimethylsilylacetamide (BSA) in dichloroethane by refluxing over a two hour period. The excess reagents were removed under reduced pressure. Acetonitrile was added to the yellow oil followed by 1,2,3,5-tetra-*O*-acetyl-*β-D*-ribofuranose **(68)** and 1.5 equivalents of the trimethylsilyl-triflate as the Lewis acid catalyst. Monitoring by TLC showed that no reaction had occurred possibly because the Lewis acid was not strong enough to induce glycosylation. Hence we investigated the addition of tin-(IV)-chloride to the mixture. TLC of the reaction the following day showed that a new higher running spot had been formed. After work-up and evaporation the crude solid product was purified using flash silica and the higher running spot was successfully isolated. Evaporation of the solvent gave a white solid and analysis by TLC showed only one spot. Subsequent analysis by NMR showed only one C-3 proton signal, suggesting that only one of the isomeric nucleosides **(106)** or **(107)** had been formed.

The deprotection strategy of Zou and Robins[135] was adopted for removal of the acetate protecting groups. The protected nucleoside was heated in 5% NH_4OH/MeOH over a 24 hour period at 65°C. White precipitate began to form after 2 hr and by the end of the reaction the solution appeared cloudy. Upon allowing the reaction to cool and settle the white solid precipitate deposited at the bottom of the flask and was filtered. Comparison with literature[144] data for the 2'-deoxy-β-D-ribofuranosyl derivative of 8-aza-7-

deazaguanine, which had previously synthesised by an alternative method indicated that nucleoside **(108)** had been formed.



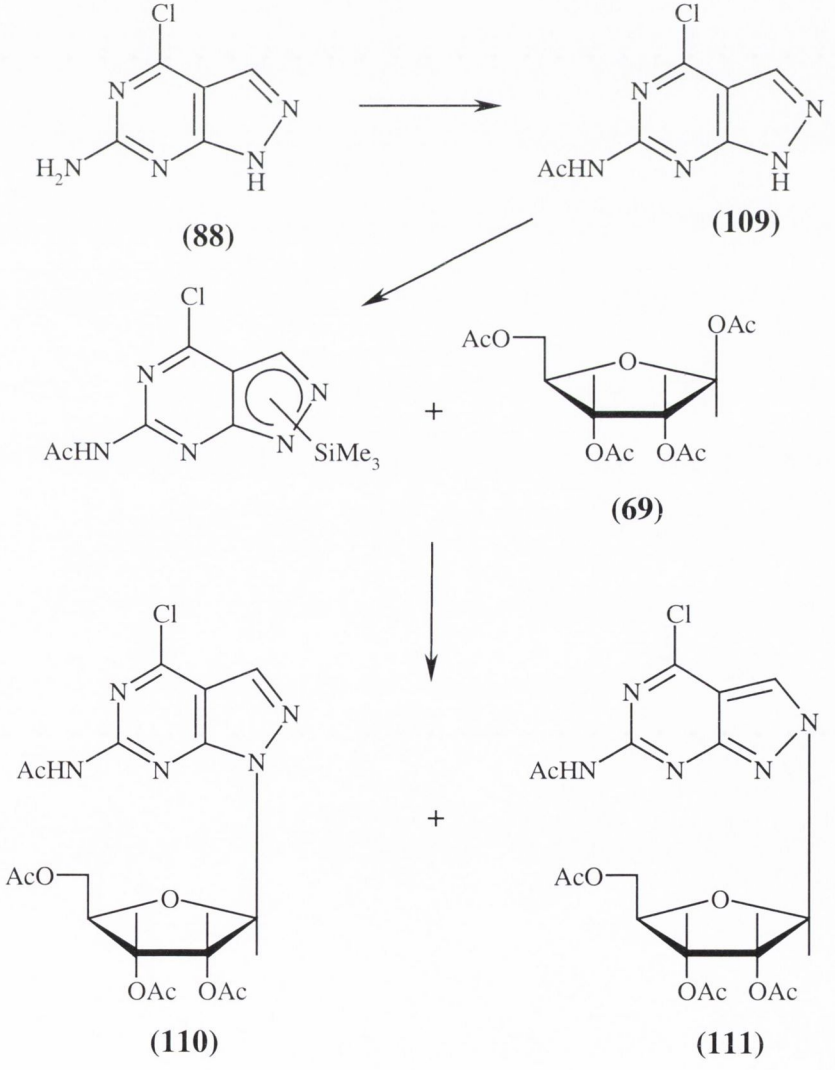
(102) **(68)**

(106) **(107)**

(107) **(108)**

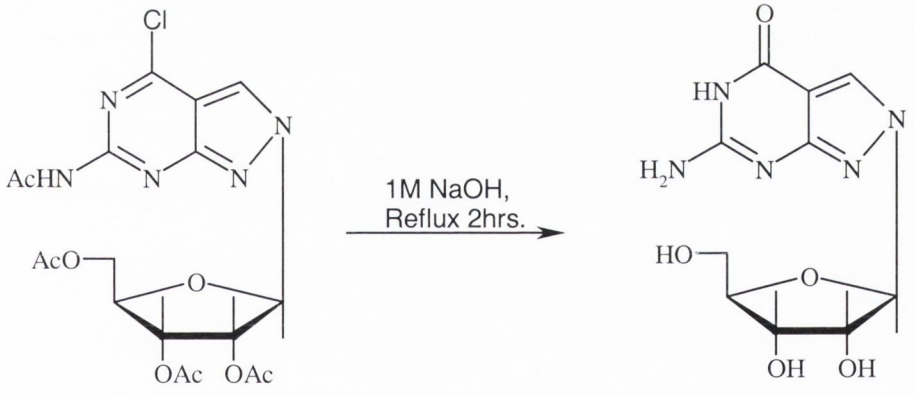
Scheme 2.16

In a new attempt to synthesis the desired nucleoside **(106)** a different strategy was adopted using 2-amino-6-chloro-8-aza-7-deazaguanine **(88)**[144]. The latter seemed a good candidate for our glycosylation procedure, for two reasons : I) to see if the different electronic distribution in the pyrazolo[3,4-d]pyrimidine ring would influence the selectivity of glycosylation (N-9 or N-8 position) and II) the chlorine could be simultaneously hydrolysed in the final acetate deprotection step to give the desired nucleoside **(89)** hence reducing the number of steps by one to the target molecule.



Scheme 2.17

The strategy outlined in **Scheme 2.17** was attempted after the successful synthesis of the required 2-N-acetyl-6-chloro-8-aza-7-deazaguanine **(109)**. Glycosylation of compound **(109)** was successful, although the selectivity was uncertain since the compounds **(110** and **111)** had not been made or characterised. Upon hydrolysis of the chlorine and deprotection of the acylated sugar moiety, crude NMR of the reaction mixture and UV measurements indicated that undesired nucleoside **(107)** had been formed, (This nucleoside had previously been characterised by Seela and Steker[144]).



(111) **(108)**

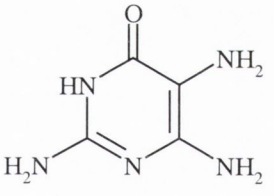
2.3 *Review* : Synthesis of 8-azaguanosine

The first reported synthesis of 8-azaguanine **(51)** (triazolo[3,4-*d*]pyrimidine) was that of Roblin *et al.*[149], which was achieved through the diazotisation of 4,5-diaminopyrimidine **(112)**. The synthesis of 8-azaguanosine **(113)** however remained elusive until 1958 when Davoll[150] reported the synthesis of the desired nucleoside **(113)** from the glycosylation of the chloromercuric derivative of the triazolo[3,4-*d*] pyrimidine **(114)** to give the glycoside **(116)** and its hydrolysis to give the desired glycoside **(113)**.

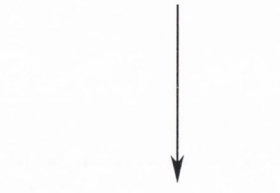
Further developments towards the synthesis of 8-azaguanosine followed shortly in the 1970's. One approach was that of Robins *et al.*[151], **(Scheme 2.19)** where direct glycosylation of 8-azaguanine was attempted. This procedure involved the silylation of 8-azaguanine **(51)** directly without prior protection of the purine derivative. After removal of the excess reagents a solution of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β -D-ribofuranose (**69**) in 1,2-dichloroethane was added to the semi-solid syrup at a low temperature in the presence of an acid catalyst (HBr) which resulted in the formation of more than one anomer of **(117)**. The other approach adopted was that of Montgomery and Elliott[152], **(Scheme 2.20)** which involved the non selective glycosylation of 7-(methylthio)-3*H*-1,2,3,triazolopyrimidine-5-amine **(118)**. The derivative **(118)** was first acyl protected before being glycosylated with tri-*O*-acetylribofuranosyl chloride **(115)** in 1,2-

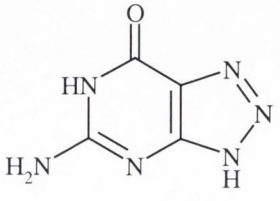
dichloroethane containing Linde-4Å-molecular sieves. The reaction gave rise to a mixture of three products, one of which was isolated and shown to be the desired product **(113)**.

More recently during the 1990's Seela and Lampe[153] were interested in synthesising different glycosides of 8-azaguanine (*e.g.* 2-deoxy-8-azaguanosine) for biological reasons. Similarly their procedure was non-selective and a large number of undesired glycosidic products resulted **(Scheme 2.21)**, although the desired product was obtained after successive chromato-graphic separation, the method seemed poor in terms of yield and reproducibility.

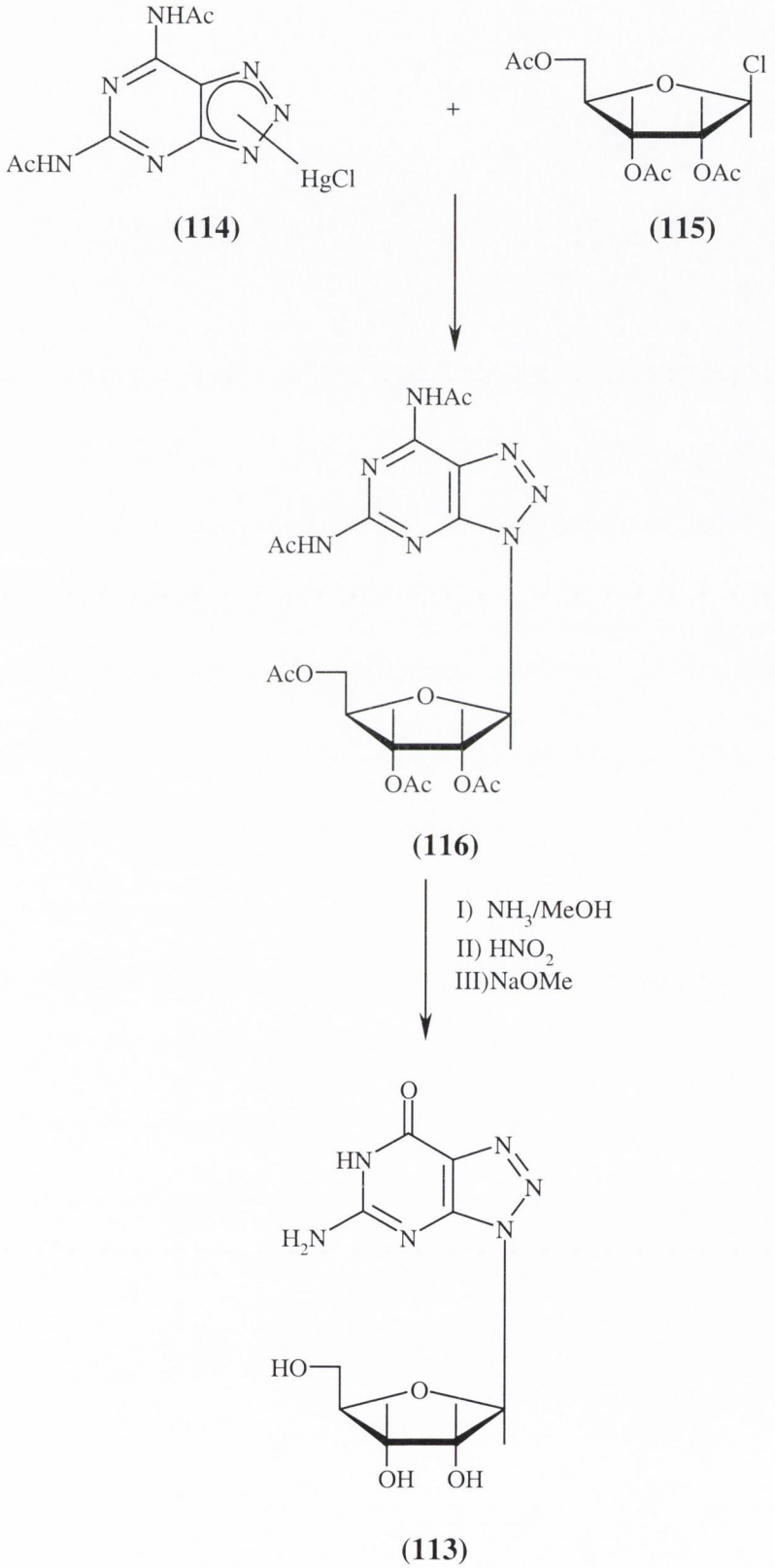


(112)

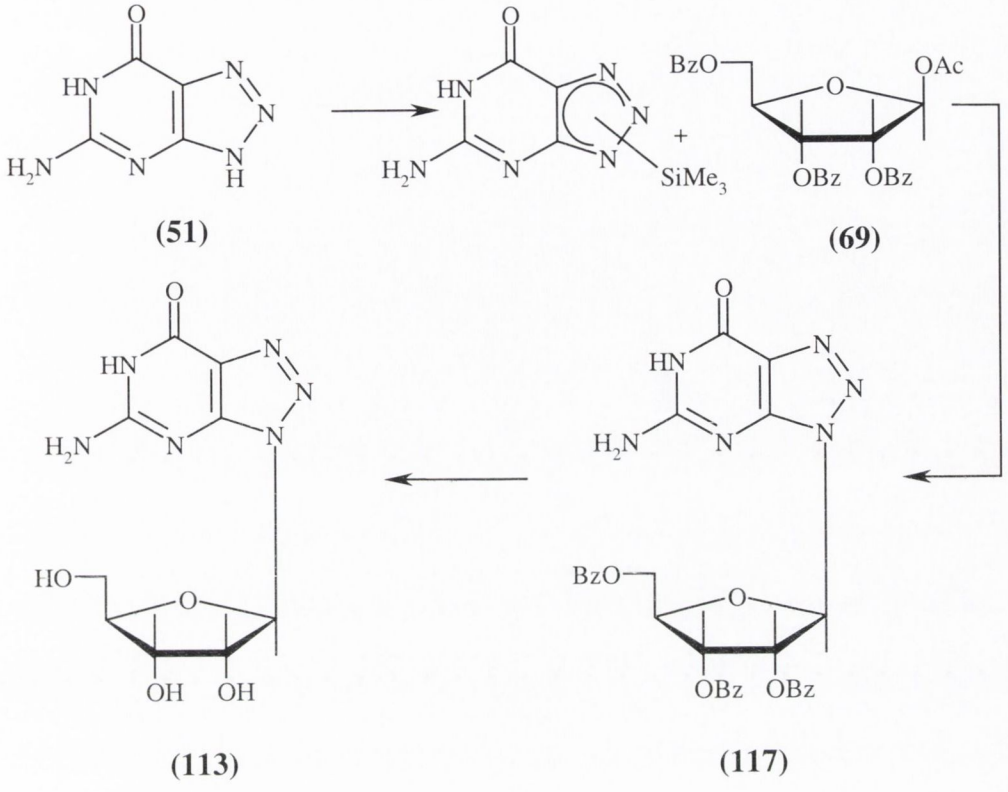




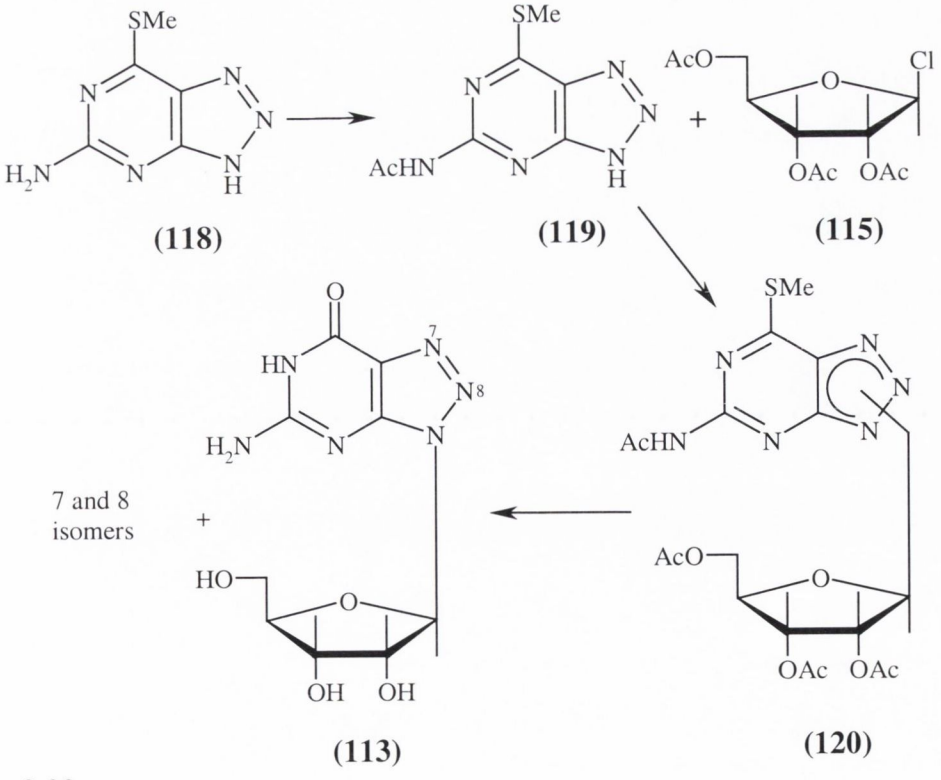
(51)


NHAc
AcHN
HgCl
AcO
Cl
OAc OAc
(114)
(115)
(116)
I) NH3/MeOH
II) HNO2
III)NaOMe
H2N
HO
OH OH
(113)

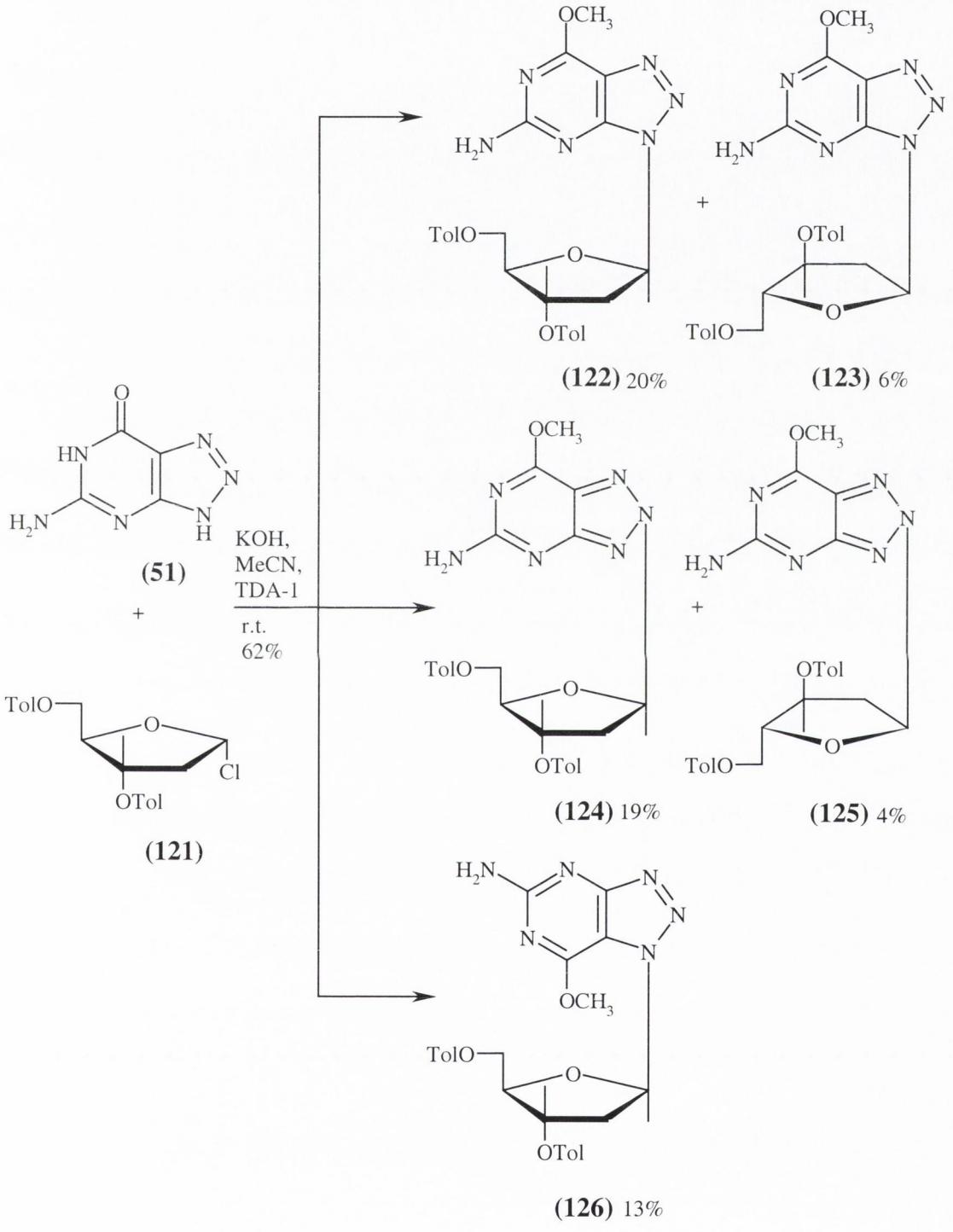
Scheme 2.18



Scheme 2.19



Scheme 2.20



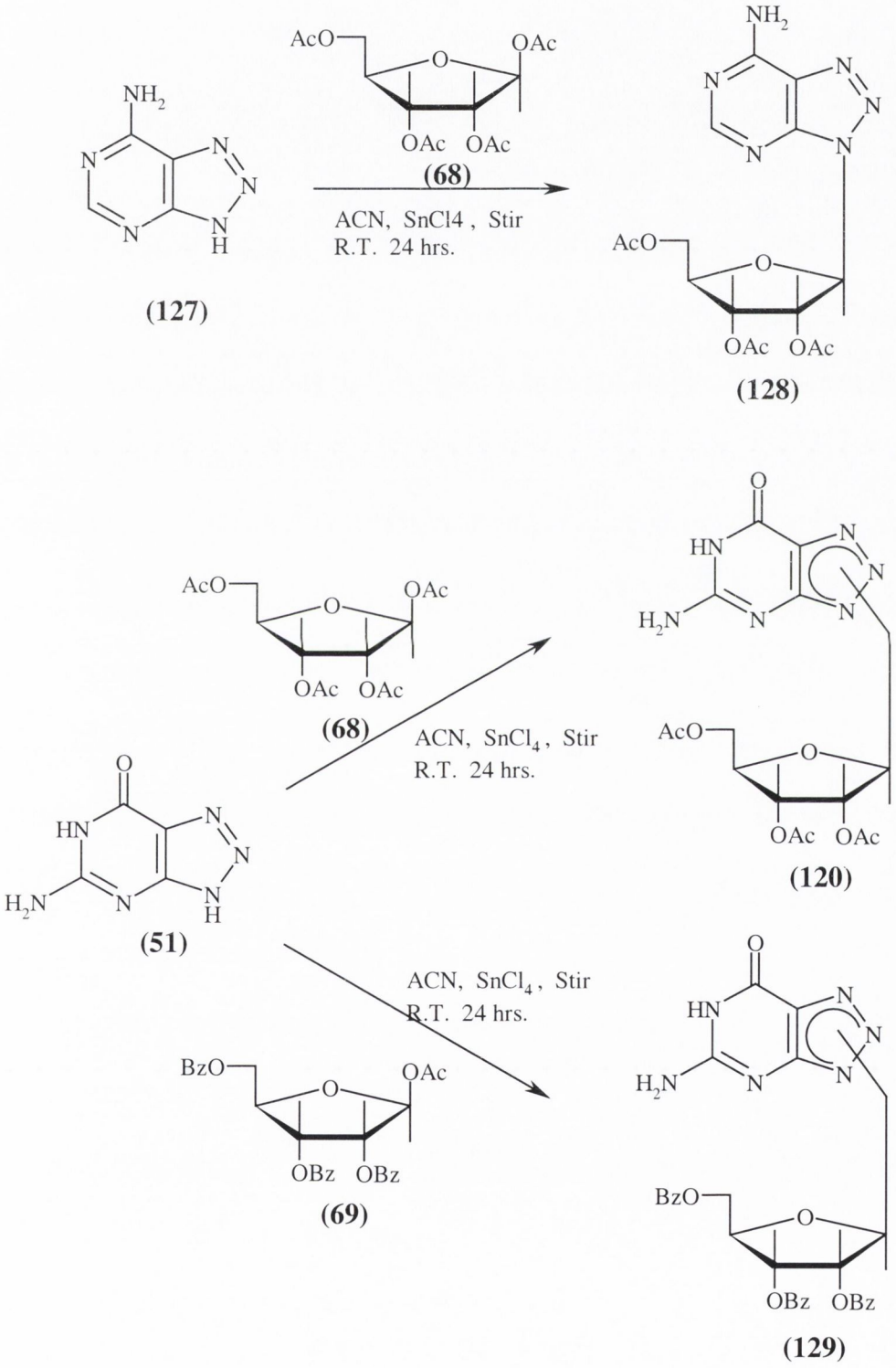
Scheme 2.21

2.3 Synthesis of 8-azaguanosine: Results and Discussion

For the selective synthesis of 8-azaguanosine **(113)** a simple, easily applicable synthetic approach was desired that would avoid tedious preparative and separative techniques. A literature search was conducted on the parent ring system of 8-azaguanine [i.e. 1,2,3-triazolo[3,4-d]pyrimidine) and the work of Seela *et al.*[154] on the glycosylation of 8-azaadenine **(127)** attracted our attention. In summary the procedure was as follows: to a suspension containing 1 equiv. of 8-azaadenosine and 1 equiv. of the acyl protected ribose sugar **(68)** in acetonitrile, stannic-(IV)-chloride was added to the reaction and allowed to stir for 24 hr under argon atmosphere. After routine work-up they obtained one out of the three possible isomeric products, which was shown to be glycosylated at the N-9 position **(128)**.

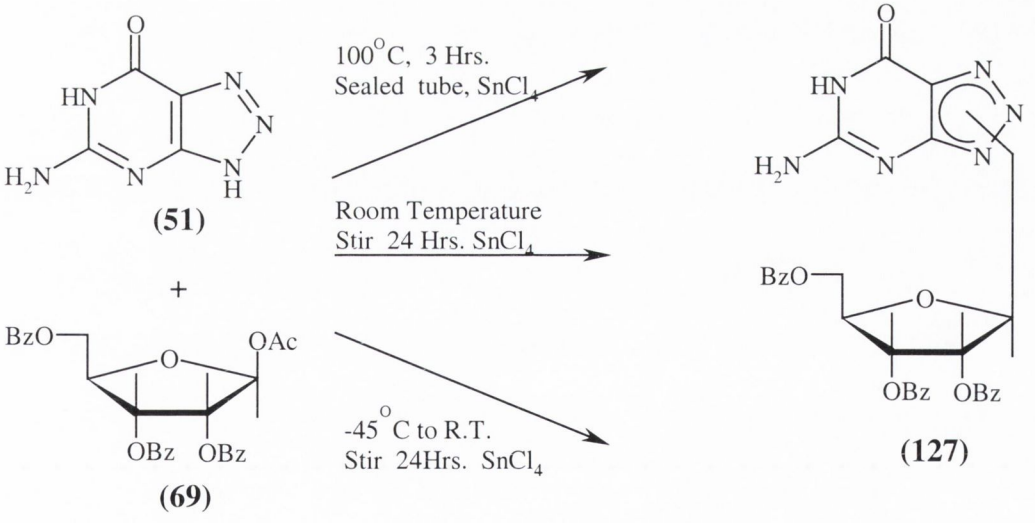
Initial trials of this reaction with 8-azaguanine and tetra-*O*-acetyl-β-D-ribofuranose were attempted, but with limited success. The NMR data of the products obtained did not show clearly whether the sugar had been attached or not. To overcome this problem and to ease assignment and characterisation of the product, the benzoate protected ribofuranose sugar **(69)** derivative was used. The NMR of the latter reaction appeared clearer and it could be deduced that glycosylation had occurred and that one product had been formed. However the position at which glycosylation had occurred could not be

distinguished by NMR, and could only be achieved by comparing the UV of the nucleoside after hydrolysis with that of the known literature[150].



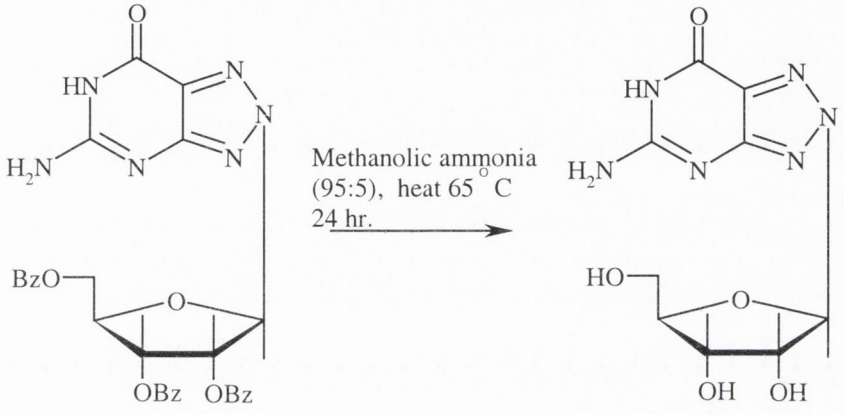
Scheme 2.22

In considering the thermodynamic aspects of the reaction and the preferential formation of one particular isomeric product we embarked upon a series of experiments to investigate the effect of changing the temperature at which glycosylation was carried out. Three temperature variations were chosen: I) elevated temperature 100°C, II) room temperature and III) low temperature (-45°C to room temperature). The reactions were carried out at the three stated temperatures and in each case the glycosylated base was isolated as a white solid upon concentrating the ethyl acetate solvent used to extract the desired product from the solid precipitate obtained from the reaction.



It was found that the room temperature reaction gave only one UV active spot by TLC which was shown to be a single isomeric product by NMR (^{1}H and ^{13}C) of the crude mixture. After removal of the benzoate protecting groups[135] the product of this reaction

was assigned the structure **(130)** by comparison with known literature data[150]; characteristic UV absorption of N-8 glycoside = 286 nm and N-9 isomer = 245 nm.



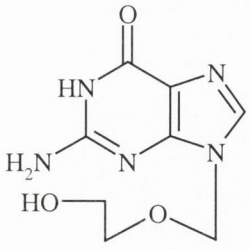
(130) **(131)**

The crude NMR spectra of the low temperature product suggested that the same product had been formed as the reaction carried out at room temperature. However the crude NMR of the high temperature product showed that a mixture of two products had been formed. All attempts at separating the glycosides failed.

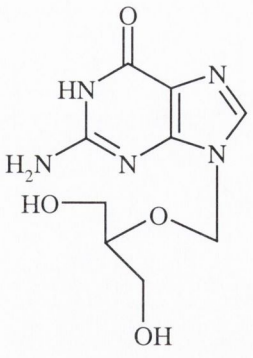
2.4 Synthesis of 9-D-ribitylguanine

The history of the synthesis of acyclonucleosides have been well documented[155], due to their antiviral properties and a variety of strategies have been devised to design nucleoside analogues. Acyclonucleosides such as acyclovir[156] (**132**) and granciclovir[157] (**133**) have been developed for the treatment of certain herpes virus infections. Most synthetic methods developed for the preparation of such acyclonucleosides involves the condensation of a nucleobase moiety with the appropriate side chain moiety[158].

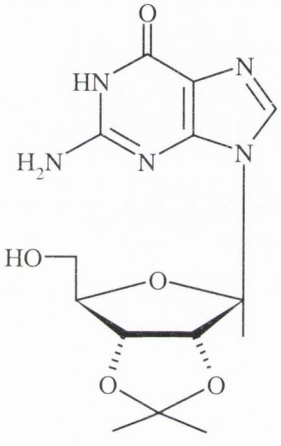
Until recently synthetic methods starting from commercially available nucleosides such as adenosine and guanosine have been largely limited only to the oxidative cleavage of 2', 3'-*cis*-diol portion of ribonucleosides with $NaIO_4$[159]. In 1997, however, when Hirota and coworkers[160] during the course of investigations on the synthesis of 1,6-dihydropurine nucleosides using various reducing agents found that when purine nucleosides **(134)** were reacted with diisobutylaluminium hydride (DIBAL-H), selective cleavage of the C-1'–O-4' bond in the ribose ring occurred to give the corresponding 9-D-ribityl purine **(135)**. Although it was not reported by the authors[160], it did seem that it should be possible to remove the isopropylidene group by weak acid hydrolysis, to give a molecule **(136)** which could be a good analogue to one of the intermediates in the GTP-CH (I) reaction.



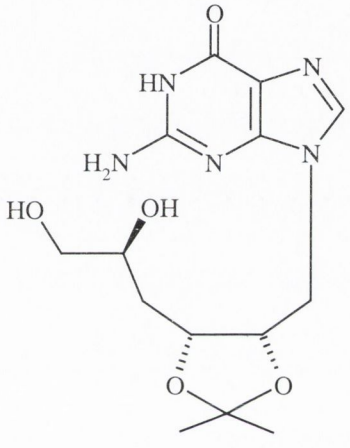
(132)



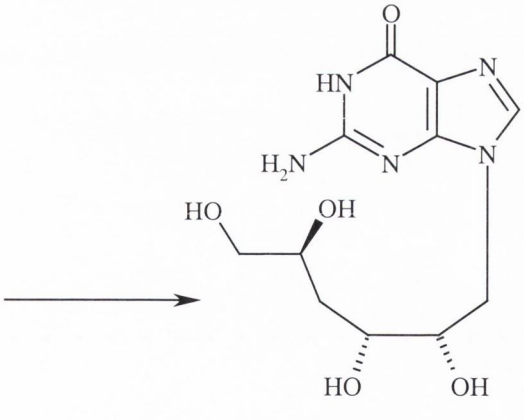
(133)



(134)



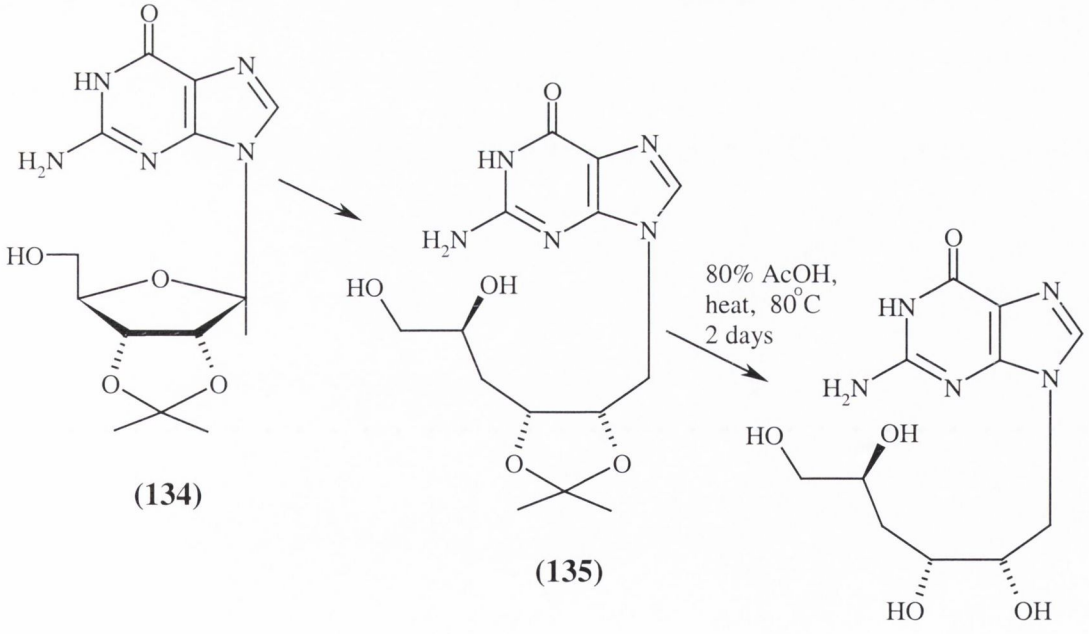
(135)



(136)

2.4 Synthesis of 9-D-ribitylguanine: Results and Discussion

The reductive cleavage of isopropylideneguanosine **(134)** was successfully repeated by the method of Hirota and coworkers[160] using DIBAL-H. Problems were encountered in getting the reaction to go to completion and initial attempts resulted in the isolation of a mixture of unreacted starting material **(134)** and the desired product **(135)**. This was overcome by allowing the reaction to run for longer periods of time. The isopropylidene protecting group in **(135)** was removed to give the target molecule **(136)** by heating in 80% AcOH for 2 days at 80°C. This material was used directly for biological tests without further purification.

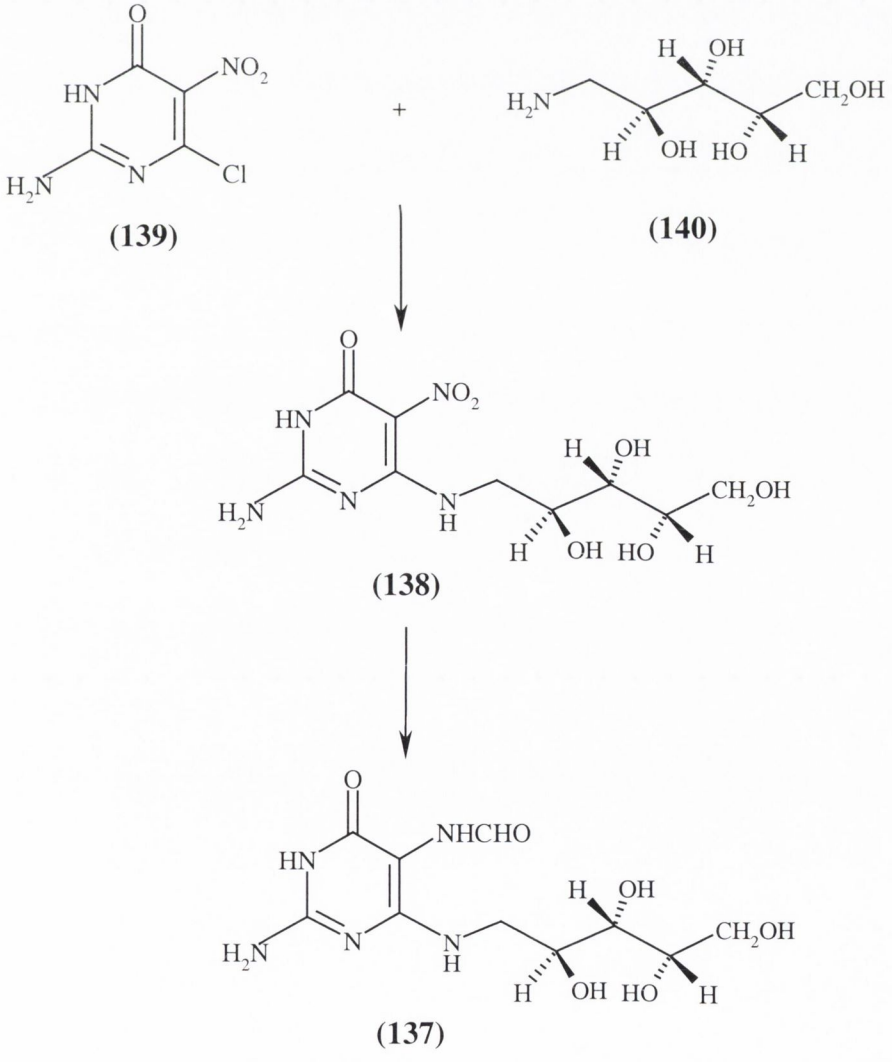


(134) **(135)** **(136)**

The 9-D-ribitylguanine compound was found upon HPLC analysis with fluorescence detection that it contained several fluorescent impurities, which would have made the task of testing the compound for enzymatic assay impossible, as it would have masked the desired enzyme activity. These impurities probably originate from the metal hydride reaction which gave the product **(135)** as a greenish / yellow solid. Ion exchange chromatography was attempted as a method for purification of the final product **(136)**, where it was found that the coloured impurities stuck to the resin (DOWEX 50W x 81200), but very little product was recovered from the eluent. Another purification strategy was to use flash chromatography. These problems were eventually solved when it was found that recrystallisation of the protected 9-D-ribitylguanine **(135)** and treatment with activated charcoal removed more or less, all fluorescent impurities as observed by HPLC.

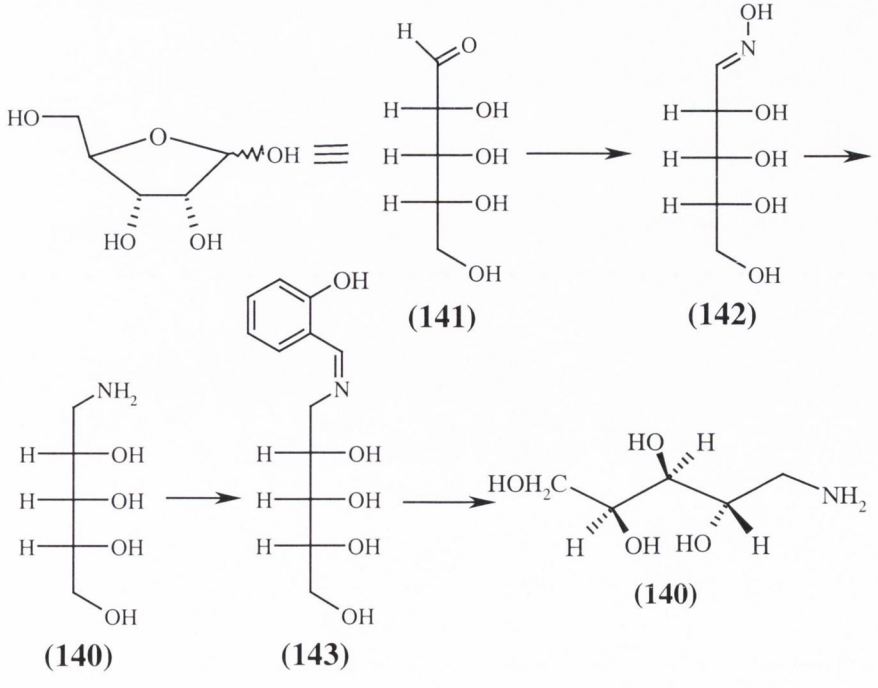
2.5 Synthesis of 2-amino-5- formamido-6-D-ribityl-amino-4(3*H*)-pyrimidinone (137)

The first reported synthesis of 2-amino-5-nitro-6-D-ribityl-amino-4(3*H*)-pyrimidinone **(138)**, the direct precursor of the desired compound **(137),** was by authors Davoll and Evans[161]. This work entailed the synthesis of 2-amino-6-chloro-5-nitro-4(3*H*)-pyrimidinone **(139)** and its condensation with 1-amino-1-deoxyribityl **(140)**[162]. In view of the synthetic methodology that already exists we decided to adopt a similar approach.

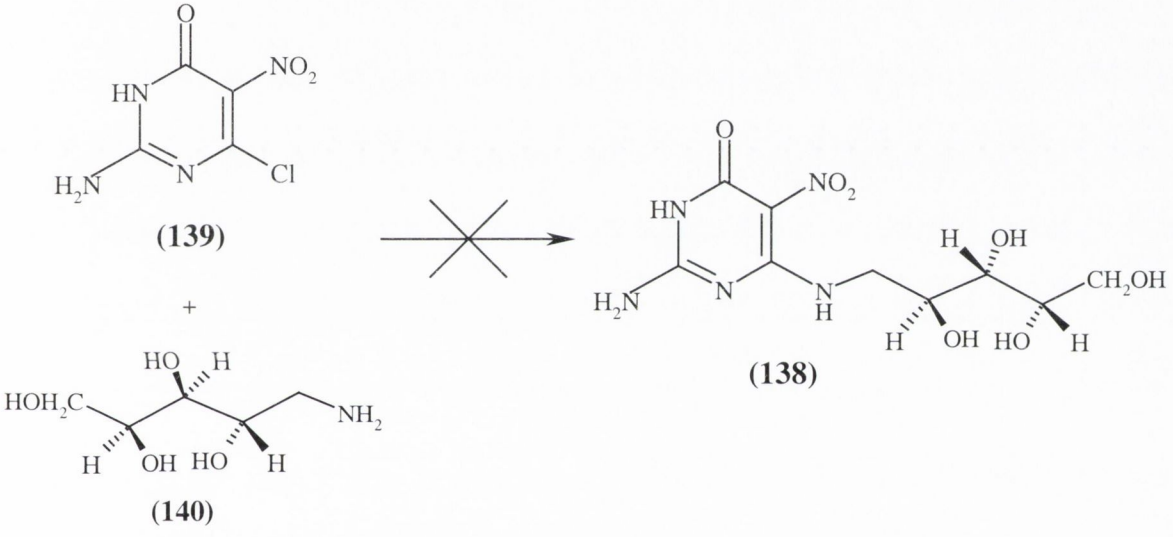


2.5 Synthesis of 2-amino-5-Formamido-6-D-ribityl-amino-4(3*H*)-pyrimidinone: Results and Discussion

In preparing the 1-amino-1-deoxyribitol **(140)** starting material for the proposed synthesis the method reported by R. Barker *et al.*[163] was investigated. This work involved the synthesis of the oxime **(142)** of D-ribose **(141)** using hydroxylamine and subsequently reducing it by catalytic hydrogenation using platinum oxide to give **(140)**. The practical application of this method however in our hands was met with limited success. The major problem with the synthesis was the removal of the finely powdered platinum oxide catalyst from the reaction mixture. Contrary to the suggestion made by the authors[163] the use of a celite pad and ion exchange were of no use in removing the metal catalyst. Note also that the only alterations made in our reaction conditions compared to that reported in the literature[163] was that the use of high pressure was avoided.



In view of the problems mentioned above, we decided to modify the latter synthetic approach[163] by changing the catalyst used at the hydrogenation stage. The use of Raney nickel catalyst seemed to be a good alternative instead of platinum oxide. This modification resulted in the successful synthesis of 1-amino-1-deoxyribitol, which was isolated as the salicylaldehyde derivative **(143)**.



The proposed synthesis of 2-amino-5-nitro-6-D-ribityl-amino-4(3*H*)-pyrimidinone **(138)** was attempted according to the literature procedure[161] but the anticipated product was not obtained, due to time limitation the proposed synthesis could not be investigated further.

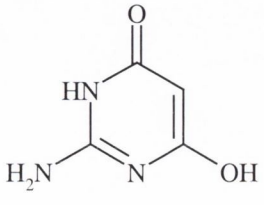
Chapter 3

Evaluation of compounds as inhibitors of GTP-CH (I)

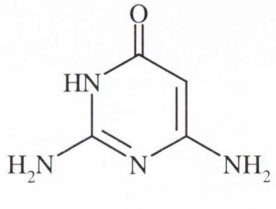
3.1 Selection of compounds as inhibitors of GTP-CH (I)

In deciding what compound is most likely to produce an effect on the enzyme activity, two approaches may be considered: I) The first is to select lead compounds which resemble the natural substrate for enzyme recognition II) The second requires an understanding of the mechanistic properties of the enzyme which can be used for the synthesis of transition state inhibitors, (i.e. the lead compound chosen may resemble a transition state intermediate of the natural substrate which the enzyme can not convert any further, hence it will remain bound to the active site and inhibit the enzyme activity). In following the first approach to finding a potential lead inhibitory compound for the enzyme GTP–CH (I), three class of purine derivatives were focused upon: 3-deazaguanine **(49)**, 8-aza-7-deazaguanine **(50),** 8-azaguanine **(51)**. A number of other compounds were also targeted for analysis. One of these compounds, 2,6-diamino-4(3*H*)-pyrimidinone **(144)**, has already been well established as an inhibitor of GTP–CH (I)[164]. One reason why this molecule may be an effective inhibitor is because it can mimic the pyrimidine portion of the GTP, alternatively the small size of the molecule may allow it to bind to a different region on the enzyme than the active site. From this study it was decided to concentrate on simple purine and pyrimidine ring based compounds **(144**, **86)**, before expanding to more complex leads which would more closely resemble the natural substrate of the enzyme.

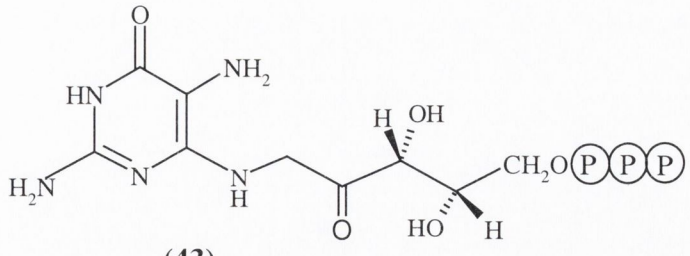
Later experiments were directed towards the synthesis of stable compounds which could mimic the proposed intermediates of the enzymatic reaction, but could not be turned over in the same way as the natural substrate / intermediate. Another possibility in following this type of approach is that the enzyme may be able to convert the synthetic intermediate into some other product, which may not have been realised with the natural substrate. One target molecule, which particularly interested us for this type of experiments was 9-D-ribitylguanine **(136)** or its triphosphate derivative **(145)** which closely resembles intermediate **(43)** in the proposed GTP–CH (I), enzymatic pathway. It was rationalised that 9-D-ribitylguanine **(136)** would bind to the active site of the enzyme with similar affinity as the natural intermediate to the active site of the enzyme and may proceed through the first committed step in the biosynthetic pathway, the opening of the imidazole ring portion of the purine moiety with the concomitant loss of formate to give derivative **(146)**. However the enzyme could not take the derivative **(146)** any further because unlike in the Amadori rearranged product **(43)** there is no carbonyl function present at C-2' of the sugar side chain to undergo cyclisation.



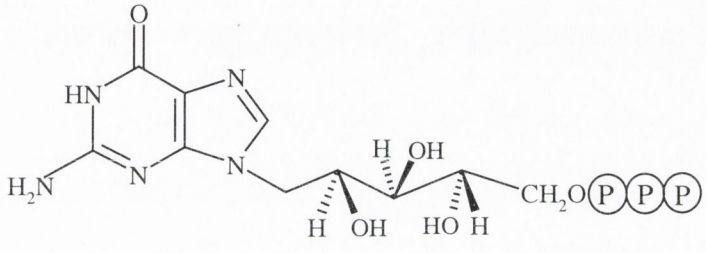
(86)



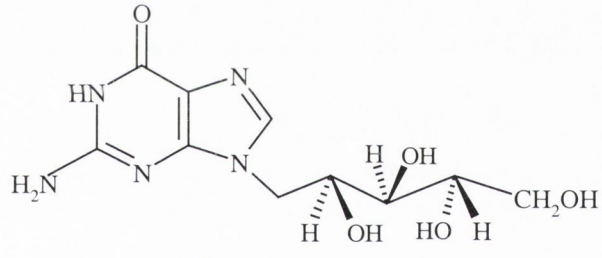
(144)



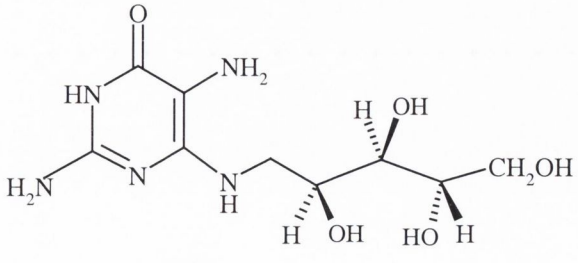
(43)



(145)



(136)

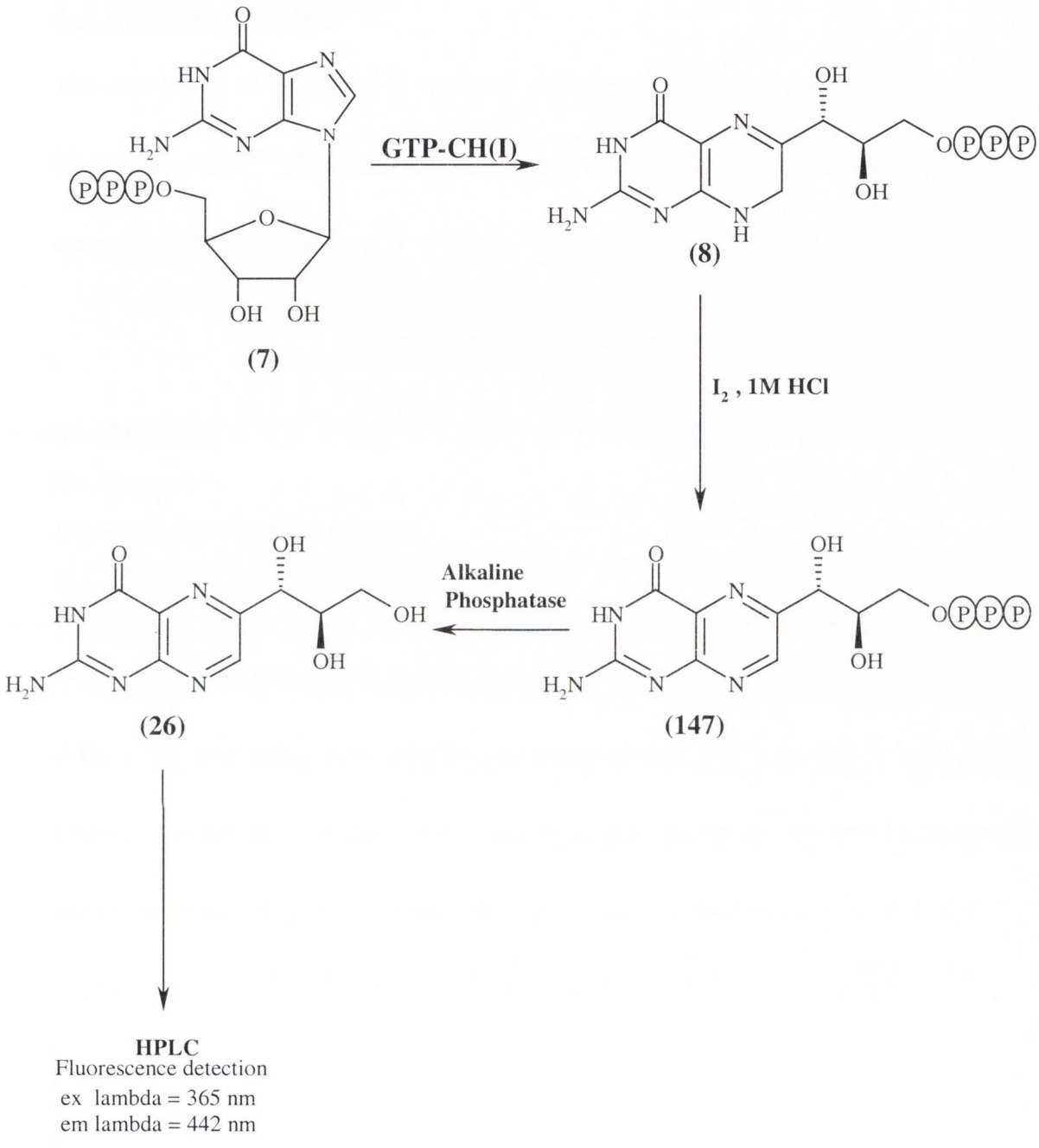


(146)

3.2 Overview of the GTP-CH (I) assay

GTP-CH (I) was isolated and purified by our German partners [Prof. A. Bacher and N. Schramek (Technische Universität München)] from recombinant *E. coli* cells, modified to express the desired enzyme. The enzyme was received in a powder form and was re-suspended in distilled water, to give a stock containing 4 mg / ml GTP-CH, 10 mM Tris. / HCl (pH 8.5), 50 mM KCl, 0.01% NaN_3. The activity of the enzyme was retained for approximately 3-4 months when stored in single use aliquots kept at –20 °C (activity of the enzyme may be prolonged upon storage at 4 °C under an inert atmosphere which became the preferred method of storage).

The biological evaluation of the compounds selected for GTP-CH inhibitory studies was carried out using the assay reported Bacher *et al*[109]. In this procedure the product of the cyclohydrolase reaction, dihydroneopterin triphosphate **(8)** is oxidised by iodine to neopterin triphosphate **(147)**, which can be detected by fluorescence. To ease characterisation of this product the triphosphate function was hydrolysed using alkaline phosphatase to give neopterin **(26)**, which produces a single detectable peak on examination by HPLC using fluorescence detection. The amount of neopterin **(26)** produced in the assay could be quantified by comparison with samples of commercial neopterin of known concentration.



Scheme 3.1: *Summary of the GTP-CH (I) assay.*

3.3 Enzyme kinetics[165,166]

The activity of an enzyme (*i.e.* turnover, velocity) can be expressed using the Michaelis-Menten postulate[166] **(Equation 3.1)**, which is applicable for a one-substrate, one-product enzyme catalysed reaction.

$$S + E \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_3} E + P \qquad \textbf{Equation 3.1}$$

S = Substrate.

E = Enzyme.

ES = Enzyme-Substrate complex.

K_1, K_2 and K_3 are rate constants.

P = Product

When a graph of the enzyme velocity (V) against substrate concentration is plotted **(Figure 3.1)**, it can seen that at low substrate concentrations [S], the velocity (V) of the enzyme is directly proportional to [S], with a decreasing response as substrate concentration is increased until saturation is achieved. The shape of this plot can be described by the mathematical relationship, known as the Michaelis-Menten equation **(Equation 3.2)**.

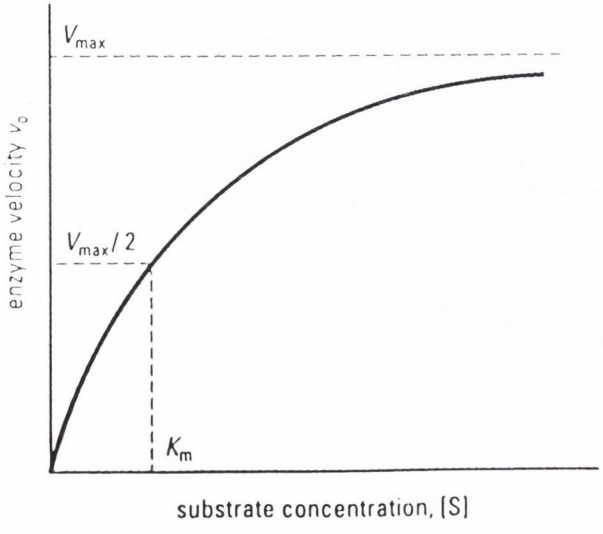


Figure 3.1: *Effect of substrate concentration on enzyme activity.*

$$V = \frac{V_{max}\,[S]}{K_m + [S]}$$

Equation 3.2: *Michaelis-Menten equation.*

The equation makes use of two kinetic constants:

I) V_{max}, the maximum rate of the reaction (at infinite substrate concentration)

II) K_m, the Michaelis constant, is the substrate concentration where $V = ½\ V_{max}$.

V_{max} is a function of the amount of enzyme and is the appropriate rate to use when determining the specific activity of the purified enzyme. The Michaelis constant K_m is expressed in terms of substrate concentration (mol l^{-1}) and is independent of the substrate concentration. K_m is derived from the individual rate constants of the reaction represented by **Equation 3.1**:

$$S + E \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_3} E + S$$

Equation 3.1

The Michaelis constant can be expressed as:

$$K_m = \frac{K_2 + K_3}{K_1}$$

Equation 3.3

For many enzymes, $K_3 << K_2$ and the equation above simplifies to:

$$K_m = \frac{K_2}{K_1} = \frac{[E][S]}{[ES]}$$

Equation 3.4

When this applies **(Equation 3.4)**, K_m provides a measure of the affinity of an enzyme for the substrate. To determine the kinetic constants for a particular enzyme, the rates of reaction at several substrate concentrations need to be measured. The data is then transformed by a linear transformation of **Equation 3.2** to give the Lineweaver-Burk plot[168] **(Figure 3.2)**:

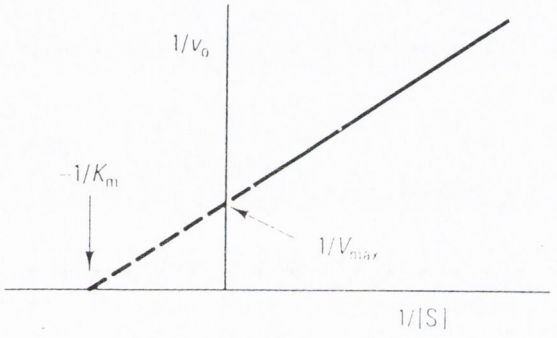


Figure 3.2: *LineWeaver-Burk plot showing the graphical transformation for determining the kinetic constants of an enzyme.*

The LineWeaver-Burk plot is a graph of the reaction rate $1/V$ against the reciprocal of the substrate concentration (1/[S]) which gives $-1/K_m$ as the intercept of the x-axis and $1/V_{max}$ as the intercept of the y-axis. The slope of the graph is most affected by the least accurate values, i.e. those measured at low substrate concentrations.

Using enzyme inhibition kinetics to identify the type of inhibitor

Enzyme kinetics can also be used to determine the different forms of inhibition observed and to provide information on the effectiveness of various inhibitors, by allowing the dissociation constant of the enzyme–inhibitor complex, K_I, to be determined.

Competitive inhibition

In this category the inhibitor present reversibly competes with the natural substrate for the active site of the enzyme, and where the inhibitor-enzyme complex EI is formed the enzyme becomes inactive and rendered from its normal turnover of substrate.

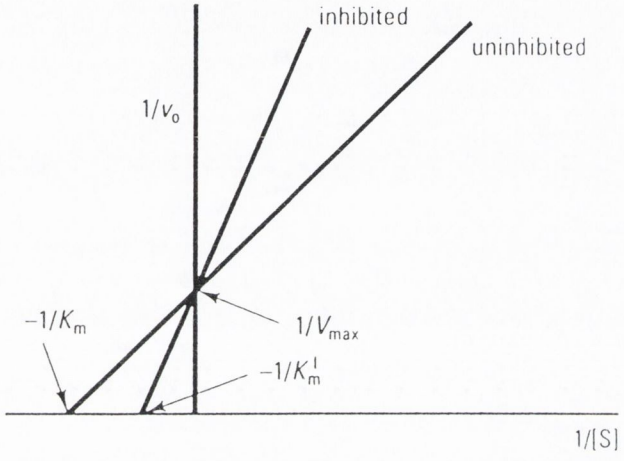


Figure 3.3: *LineWeaver-Burk plot in the presence and absence of a competitive inhibitor.*

The LineWeaver-Burk plot of a competitive inhibitor is shown in **Figure 3.3**. Where this type of inhibition is occurring the lines in the presence and absence of inhibitor intersect the y-axis and the inhibitory effect disappears at high substrate concentrations (when 1/[S] = 0, [S] = ∞). The value K_I can be calculated either from knowledge of the K_m values obtained in the presence and absence of inhibitor (intercept on the x-axis), since the following relationship applies **(Equation 3.5)**:

$$\text{Slope}_{\text{inhibited}} = \text{Slope}_{\text{uninhibited}} \frac{(1+[I])}{k_I} \qquad \textbf{Equation 3.5}$$

Non competitive inhibition

In this case the inhibitor present not only binds to the free enzyme but also to the substrate-enzyme complex [ES] to give the inactive complex [ESI]. In the simplest case the binding of the inhibitor has no effect on the binding of the substrate, hence the dissociation constant of [ESI] is the same as that of [ES].

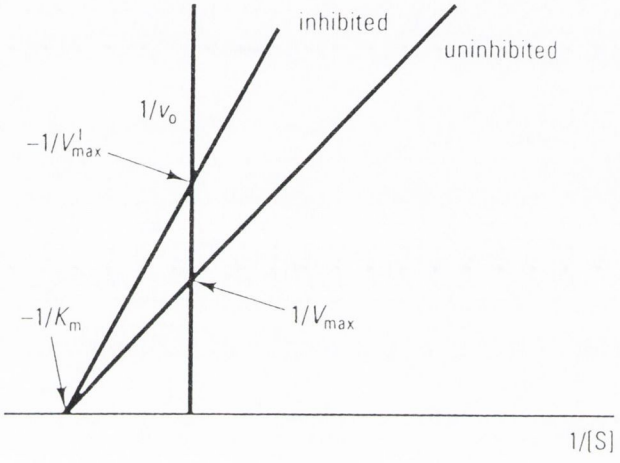


Figure 3.4: *LineWeaver-Burk plot in the presence and absence of a non competitive inhibitor.*

The LineWeaver-Burk plot for a non competitive inhibitor is shown **(Figure 3.4)**, the graph indicates that this type of inhibitor decreases V_{max} but does not effect K_m. Effectively this means that the inhibitor removes a certain fraction of active enzyme from operation, no matter what the concentration of the substrate. The V_{max} changes by a factor of $(1+[I])/K_I$ and K_I can be determined by comparison of the slopes obtained with the uninhibited enzyme and the inhibited enzyme using **equation 3.5**.

3.4 Results and Discussion for chapter 3

Before testing compounds as inhibitors, kinetic experiments were carried out with a view to measuring the K_m (Michaelis constant) for the GTP-CH (I) under our assay conditions. This involved setting up incubations at a range of GTP concentrations and measuring the initial rates of reaction in each case. It was found that the formation of neopterin was exceptionally high especially at the lower GTP concentrations. Careful examination of control incubations containing no GTP revealed that the quantity of neopterin measured in the assay reflected not only the neopterin formed as a result of the incubation but also that formed from the dihydroneopterin that was present in the enzyme preparation prior to the incubation. The values of the initial rates were therefore adjusted to take this into account. A LineWeaver-Burk plot was constructed **(Figure 3.5)** to give a K_m value of 0.8 μM which was within the range quoted by our German partners.

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

Equation 3.2: *Michaelis-Menten equation.*

The enzymatic assay described above was then applied to our target compounds. When running an assay to test the activity of a compound for GTP-CH inhibition, a 1ml assay would be typically set up containing approximately 10 μM GTP, 0.2 U of GTP-CH and the maximum concentration of inhibitor that solubility would allow (generally 1-10 mM).

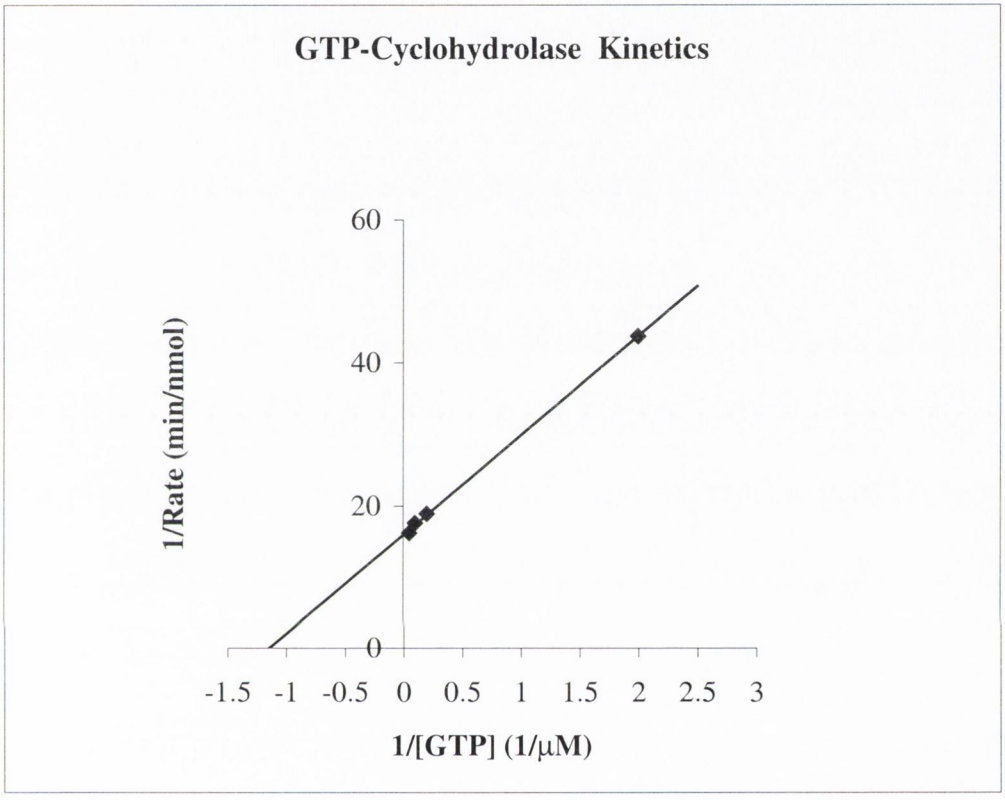


Figure 3.5: Graph depicting the LineWeaver-Burk plot obtained from the assay carried out to determine K_m of GTP-CH (I).

Two incubations were set up at the same time, one being the control, with no inhibitor present and the other containing the compound to be tested for inhibitory properties. It was found that 3-deazaguanine **(49)**, 2-amino-6-hydroxy-4(3*H*)-pyrimidinone **(86)** and 9-D-ribitylguanine **(136)** did not cause any inhibition as the amount of neopterin measured in the control and the vial containing the compound being tested were identical. Slight inhibition was observed when 8-aza-7-deazaguanine **(50)** was tested but this was not significant enough for further experiments. The best inhibition was observed when 8-azaguanine **(51)** was tested in our assay, more than 40% inhibition was observed as compared to the control incubation as the level of the neopterin measured was dramatically reduced. 2,6-Diamino-4(3*H*)-pyrimidinone **(144)** was also was found to be an effective inhibitor of GTP-CH (I) as had been reported[164] **(Table 3.1)**.

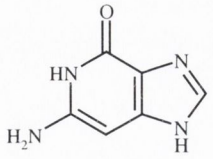
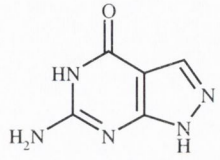
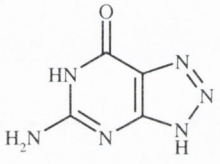
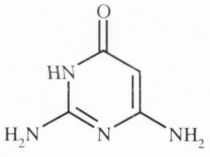
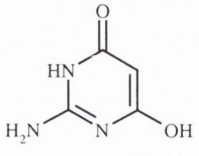
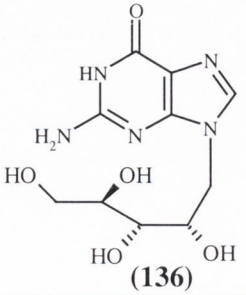
Name of Compound	Structure	Inhibition
3-deazaguanine	 **(49)**	**No inhibition**
8-aza- 7-deazaguanine	 **(50)**	**Slight inhibition**
8-azaguanine	 **(51)**	**K_I = 0.21 mM**
2,6-diamino- 4(3*H*)-pyrimidinone	 **(144)**	**K_I = 1.74 mM**
2-amino-6-hydroxy- 4(3*H*)-pyrimidinone	 **(86)**	**No inhibition**
9-D-ribitylguanine	 **(136)**	**No inhibition**

Table 3.1: *Summary of results obtained from the enzymatic assay of the compounds tested for GTP-CH (I) activity, where K_I represents the inhibition constant measured for the compounds where strong inhibition was observed.*

Measuring the K_I of 8-azaguanine and 2,6-diamino-4(3*H*)-pyrimidinone

Assays were set up at a range of GTP concentrations and a fixed concentration of 8-azaguanine **(51)** 1.33 mM. At the same time a similar range of control assays were carried out in which no inhibitor was present. The results of these incubations were then transformed as before into a LineWeaver-Burk plot (1/[GTP] against 1/[Rate]), where it was found that two sets of best fit lines could be drawn for each set of incubation carried out (i.e. control-no inhibitor, inhibitor) as shown in **Figure 3.6**. It was found that the two slopes one being the control and the other containing the inhibitor converged and intersected at the y-axis, which strongly suggested that 8-azaguanine is a competitive inhibitor with a K_I of 0.21 mM and provides direct evidence that this molecule causes inhibition by binding at the enzyme active site. A similar set of experiments were performed to ascertain the nature of inhibition caused by the presence of 2,6-diamino-4(3*H*)-pyrimidinone **(144)**, these results are shown in **Figure 3.7**. Again it appears that this compound is a competitive inhibitor with a calculated K_I value of 1.74 mM.

In conclusion these experiments demonstrate the importance of the pyrimidine function in binding and recognition at the GTP-CH (I) active site. It is therefore highly desirable that any future synthetic targets for inhibition studies contain this functionality. The inhibition of GTP-CH caused by 8-azaguanine in our experiments suggests that further modification at the 8-position and may provide good candidates for future experiments.

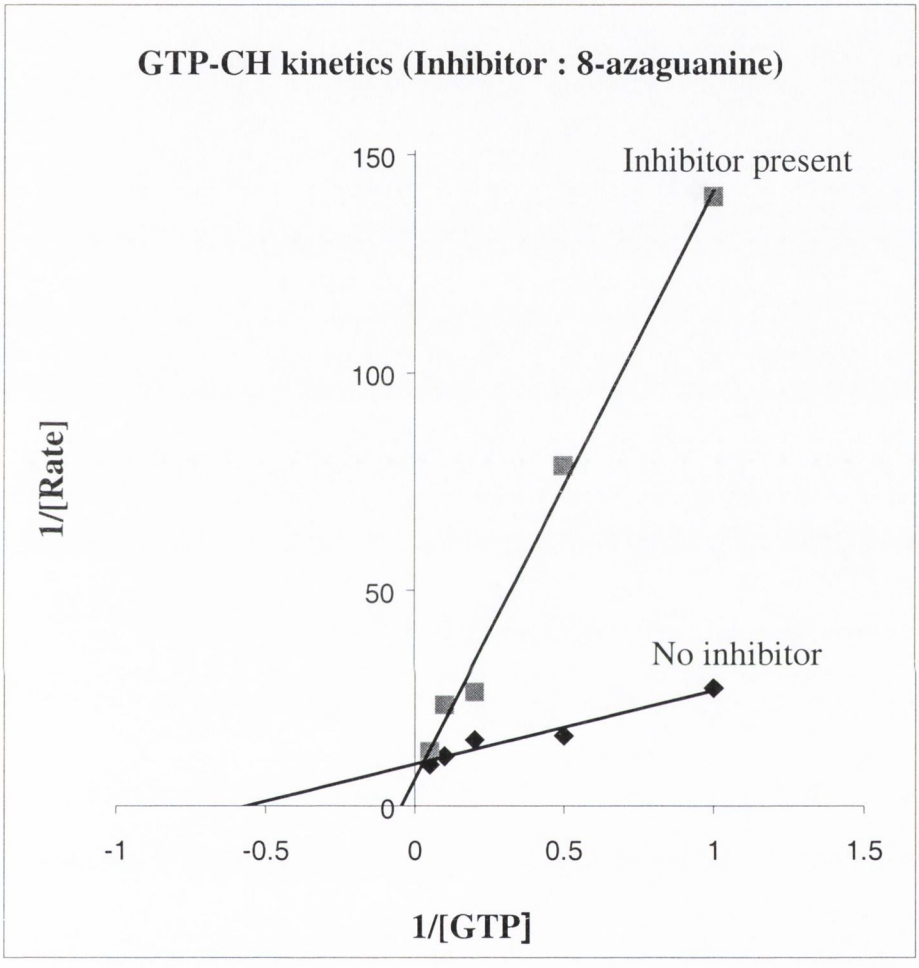


Figure 3.6: Graph depicting the LineWeaver-Burk plot obtained from the assay conducted to determine K_I of the inhibitor 8-azaguanine.

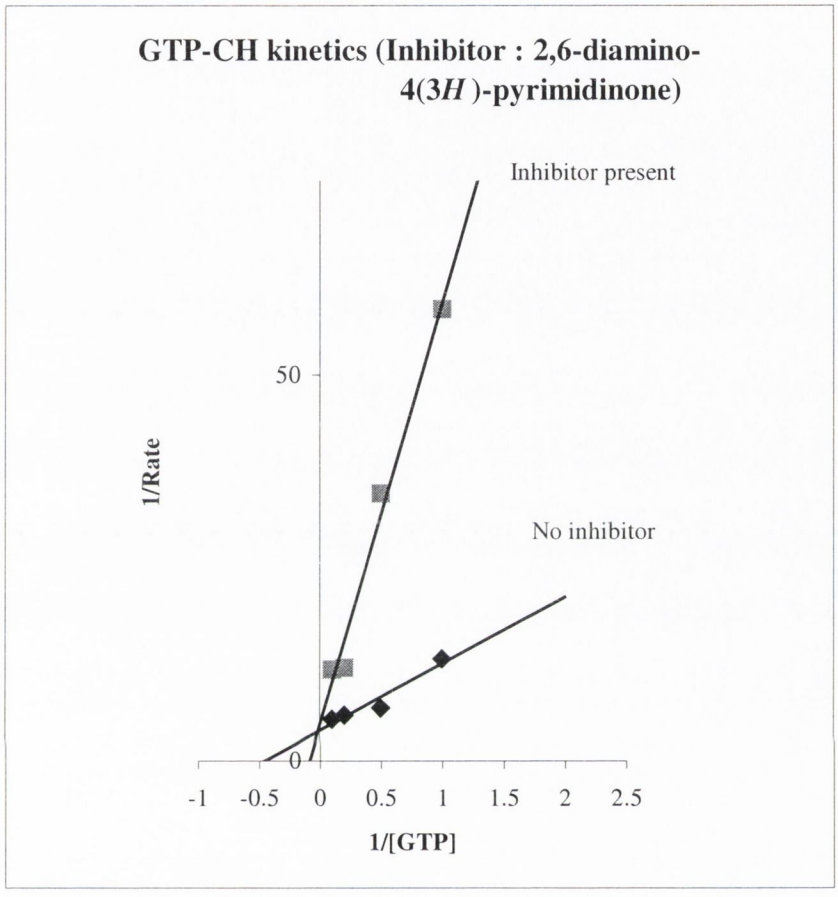


Figure 3.7: Graph shows the LineWeaver-Burk plot obtained from the assay carried out to determine the K_I value of the inhibitor 2,6-diamino-4(3*H*)-pyrimidinone.

Chapter 4

Experimental

Experimental

HPLC analysis for the GTP-CH assay was performed on a Perkin Elmer 200 Ic machine with a Nucleosil C18 reverse phase column (250 x 4 mm) connected to a fluorescence detector [Schoeffel Instruments FS 950 Fluoromat with an excitation wavelength of 365 nm giving an emission at 442 nm]. The output from the fluorescence detector was recorded using a Waters 746 Data Module Integrator. Chemicals were purchased from Aldrich / Sigma and were used without further purification unless otherwise noted. Flash chromatography was performed using the method of Still[169]. Acetonitrile was dried over CaH_2. 1,2-Dichloroethane was similarly dried over CaH_2. Dry diethyl ether was prepared by first drying over CaH_2 and then distilling from sodium wire and benzophenone. Dry methanol was prepared by distilling from Mg and I_2. Pyridine was dried over KOH overnight and then distilled from CaH_2. THF was dried first by allowing to stand overnight in KOH and than distilling it from CaH_2. A Perkin Elmer Paragon 1000 infrared spectrometer was used for all infrared analysis. Ultraviolet spectroscopy was performed on a ATI Unicam UV4 ultraviolet spectrometer. NMR analysis was done on a Bruker DPX 400 spectrometer. Coupling constants are quoted in Hertz. Chemical shifts are reported in ppm. Chemical shifts of ^{1}H and ^{13}C are reported relative to tetramethylsilane (TMS). All TLC analysis was done using alumina sheets silica gel 60 F_{254} unless otherwise mentioned. Elemental analyses were carried out at the Microanalytical Laboratory, University College Dublin.

Experimental :

3-Deazaguanine (49)

To a boiling solution of 6-hydrazino-1,5-dihydroimidazo[4,5-c]pyridin-4-one **(63)**[126] (1 g, 6.7 mmol) dissolved in water (20 ml) Raney nickel catalyst (≈1 g) was added and the mixture refluxed for ½ hr. More Raney nickel (≈1 g) was added to the reaction mixture and refluxing continued for a further 1 hr. The metal catalyst was removed by filtration and the resulting orange solution was treated with activated charcoal. The solution was concentrated to approximately 5 ml. Yellow crystals of 3-deazaguanine were collected by filtration (yield = 300 mg, 44%).

δ_H (400 MHz, Me_2SO - d_6) : 5.46 (1 H, s, H-7), 5.54 (2 H, s, NH_2), 7.69 (1 H, s, H-2), 10.48 (1 H, s, NH), 12.06 (1 H, s, NH). δ_C (100 MHz, Me_2SO - d_6) : 122.56 (CH-8), 137.11, 137.51, 143.12, 156.39 (quaternary C), 146.98 (CH-3). UV (MeOH) 264 nm.

2-N-Acetyl-8-aza-7-deazaguanine (102)

Finely powdered 8-aza-7-deazaguanine **(50)** (300 mg, 1.99 mmol) was refluxed for approximately 4 hr in a solution of dry pyridine (3 ml) and freshly distilled acetic anhydride (150 ml) under an atmosphere of nitrogen. The reaction mixture was evaporated to dryness to give a pale yellow coloured solid. Recrystallisation from methanol gave **(102)** as a white solid (Yield = 197 mg, 51 %).

δ_H (400 MHz, Me_2SO - d_6) : 2.16 (3 H, s, CH_3), 7.98 (1 H, s, H-7), 11.55 (1 H, s, NH), 11.77 (1H, s, NH), 13.47 (1H, s, NH-9). δ_C (100 MHz, Me_2SO - d_6) : 23.82 (CH_3), 173.71, 135.41, 149.25, 153.5, 156.16 (quaternary C), 102.12 (CH-7). $C_7H_7N_5O_2$ requires: C 43.53, H 3.65, N 36.26, O 16.57; found: C 43.55, H 3.85, N 36.30. UV (MeOH) 260 nm.

2-N-Acetyl-6-*O*-diphenylcarbamoyl-8-aza-7-deazaguanine (103)

2-N-Acetyl-8-aza-7-deazaguanine **(102)** (100 mg, 0.52 mmol) was suspended in dry pyridine (2 ml) and ethyl diisopropylamine (0.148 ml). Diphenylcarbamoyl chloride (0.108 g, 0.053 mmol) was added to the suspension and the mixture was stirred at room temperature for 1 hr. Water (0.4 ml) was added to the solution, which was stirred for a further 10 minutes and then evaporated to dryness. Toluene (3 ml) was added to the residue and was then removed *in vacuo*. This was repeated a further two times. The solid product obtained was heated at 80°C in 50 % aqueous ethanol (100 ml) for 1.5 hr. After allowing to cool in ice the beige coloured crystals were collected and washed successively with ethanol and ether and dried (Yield = 90 mg, 45 %).

δ_H (400 MHz, Me_2SO - d_6) : 2.12 (3 H, s, CH_3), 7.28 (5 H, m, Ar-H), 7.36 (5 H, m, Ar-H), 8.86 (1 H, s, H-7), 11.54 (1H, s, NH-6), 11.77 (1H, s, NH-9). δ_C (100 MHz, Me_2SO - d_6) :

23.84 (CH_3), 126.67, 127.13, 129.34, 131.53 (3Ar-CH, C-7), 105.85, 142.67, 149.85, 150.21, 157.42, 157.72, 173.63 (quaternary C).

Attempted synthesis of 2-N-acetyl 6-*O*-diphenylcarbamoyl-8-aza-7-deazaguanosine 2,3,5 triacetate (104)[47]

2-N-Acetyl-6-O-diphenylcarbamoyl-8-aza-7-deazaguanine **(103)** (388 mg / 0.885 mmol) was suspended in dry 1,2-dichloroethane (8.85 ml) and to it *bis*-trimethylsilylacetamide (0.442 ml, 1.79 mmol) was added. After stirring for 1.5 hr at 80° C the clear solution was evaporated, and a viscous brown oil was isolated. A solution of 1,2,3,5 tetra-*O*-acetyl-β-*D*-ribofuranose **(68)** (377.9 mg, 1.06 mmol) in dry toluene (4.4 ml) was added followed by trimethylsilyltriflate (0.222 ml, 1.15 mmol) and the mixture stirred at 80°C for 1hr. After cooling, ethyl acetate (50 ml) was added and the organic phase was washed with a saturated solution of $NaHCO_3$, brine, and H_2O. Drying over $MgSO_4$ was followed by removal of solvent *in vacuo* to leave brown foam. TLC and NMR analysis of the crude mixture showed that no reaction had occurred.

Attempted synthesis of 2-N-Acetyl -8-aza-7-deazaguanosine 2',3',5' triacetate (106)[148]

2-N-Acetyl-8-aza-7-deazaguanine **(102)** (100 mg, 0.66 mmol) was suspended in 1,2-dichloroethane (2 ml) and *bis*-trimethylsilylacetamide (0.3 ml, 1.18 mmol) was added. The resulting solution was refluxed for 2 hr and the excess reagents were removed under reduced pressure. Acetonitrile (2 ml) was added to the viscous oil followed by 1,2,3,5

tetra-*O*-acetyl-*β*-*D*-ribofuranose **(69)** (150 mg) and trimethylsilyltriflate (0.15 ml). The reaction mixture was allowed to stir at ambient temperature for approximately 20 hr under an atmosphere of nitrogen. No reaction was detected by TLC analysis.

Synthesis of 2-N-acetyl -8-aza-7-deazaguanosine 2',3',5' triacetate (107)

2-N-Acetyl-8-aza-7-deazaguaine **(102)** (100 mg, 0.52 mmol) was suspended in 1,2-dichloroethane (2 ml) and *bis*-trimethylsilylacetamide (0.3 ml, 1.18 mmol) was added. The resulting suspension was refluxed for 2 hr and the excess reagents were removed under reduced pressure. 1,2-Dichloroethane (2 ml) was added to the resulting oil followed by 1,2,3,5 tetra-*O*-acetyl-*β*-*D*-ribofuranose **(68)** (166 mg, 0.52 mmol) and tin-(IV)-chloride (0.3 ml). The reaction mixture was allowed to stir at ambient temperature for approximately 20 hrs under an atmosphere of nitrogen. Ethyl acetate (15 ml) was added to the reaction and the mixture was poured into a stirred solution of $NaHCO_3$ (sat., 8 ml). The organic layer was separated and washed with water (2 x 2 ml). The dried (Na_2SO_4) organic layer was evaporated and the residue was purified by flash chromatography [(EtOAc / Hexane), 2:1, R_f = 0.3] to give **(107)** a white solid (yield = 130 mg, 56 %).

δ_H (400 MHZ, $CDCl_3$) : 2.07 (3 H, s, CH_3), 2.1 (3 H, s, CH_3), 2.13 (3 H, s, CH_3), 2.4 (3 H, s, CH_3), 8.32 (1 H, s, H-7)), 11.17 (1 H, s, NH), 12.01 (1H, s, NH), 5.96 (1 H, d, H-

1'), 5.78 (1H, m, H-2'), 5.55 (1H, t, H-3'), 4.42 (2 H, m, H-5'), 4.24, 4.23 (1H, m, H-4'). δ_C (100 MHz, $CDCl_3$) : 19.93, 20.18, 24.13, 29.81 (4 x CH_3), 62.9 (CH_2), 70.28, 74.33, 80.13, 92.52 (4 x C'H), 128.51 (CH-7), 105.23, , 149.99, 158.13, 156.16 158.13, 168.78, 168.96, 169.9, 173.1 (quaternary C).

2-N-Acetyl-6-chloro-pyrazolo[3,4-*d*]pyrimidine (109)

Finely powdered 2-amino-6-chloro-pyrazolo[3,4-*d*]pyrimidine **(88)** (300 mg) was refluxed for approximately 4 hrs in a solution of dry pyridine (3 ml) and freshly distilled acetic anhydride (150 ml) under an atmosphere of nitrogen. The reaction mixture was evaporated to dryness and the lightly yellow coloured solid was recrystallised from methanol to leave **(109)** as a beige coloured solid (yield = 150 mg, 43 %).

δ_H (400 MHz, Me_2SO - d_6) : 2.17 (3 H, s, CH_3), 7.97 (1 H, s, H-7), 11.57 (1 H, s, NH), 11.57 (1 H, s, NH), 11.79, 13.47(1H, 2 geometric isomers, NH). δ_C (100 MHz, Me_2SO - d_6) : 23.84 (CH_3), 135.85, 149.33, 153.44, 156.09, 173.72 (quaternary C), 102.12 (CH-7).

2-N-Acetyl -6-chloro-8-aza-7-deazaguanosine 2',3',5' triacetate (111)

Bis-trimethylsilylacetamide (0.3 ml, 1.18 mmol) was added to a suspension of 2-N-Acetyl-6-chloro-8-aza-7-deazaguanine **(109)** (100 mg, 0.42 mmol) in 1,2-dichloroethane (2 ml). The mixture was refluxed for 2 hr and the excess reagents were removed under reduced pressure. 1,2-Dichloroethane (2 ml) was added to the residue followed by the addition of 1,2,3,5 tetra-*O*-acetyl-*β*-*D*-ribofuranose **(68)** (150 mg, 0.42 mmol) and tin-(IV)-chloride (0.24 ml). The reaction mixture was allowed to stir at ambient temperature for approximately 20 hr under an atmosphere of nitrogen. Ethyl acetate (15 ml) was added and the mixture was then poured into a stirred solution of $NaHCO_3$ (sat., 8 ml). The organic layer was separated and washed with water (2 x 2 ml). The dried (Na_2SO_4) organic layer was evaporated and the residue purified by flash chromatography [(EtOAc / Hexane), 2:1, R_f = 0.2)] to give **(111)** as a white solid (yield = 45 mg, 10 %).

δ_H (400 MHz, Me_2SO - d_6) : 2.09, 2.12, 2.37 (12 H, s, 4 x CH_3), 2.75(3 H, s, CH_3), 8.29 (1 H, s, H-7)), 11.04 (1 H, s, NH), 11.97 (1H, s, NH), 5.95 (1 H, d, H-1'), 5.77 (1H, m, H-2'), 5.56 (1H, t, H-3'), 4.45 (2 H, m, H-5'), 4.24, 4.22 (1H, m, H-4').

δ_C (100 MHz, Me_2SO - d_6) : 20.34, 20.92, 23.04, 29.84 (4 x CH_3), 62.9 (CH_2), 70.33, 74.37, 80.19, 92.52 (4 x C'H), 128.78 (C-7). 105.65, , 150.15, 158.71, 169.16, 169.35, 170.32, 173.42 (quaternary C). UV (MeOH) 273 nm.

Synthesis of 8-aza-7-deazaguanosine (108) from 2-N-acetyl-8-aza-7-deazaguanosine 2',3',5' triacetate (107)

2-N-Acetyl-6-chloro-8-aza-7-deazaguanosine 2',3',5' triacetate **(107)** (98 mg, 0.22 mmol) was dissolved in methanolic ammonia (40 ml, MeOH / NH_3 0.88 (95 :5)) and the solution stirred at 65°C for 24 hr. After removal of the solvent *in vacuo*, the residue was washed with DCM, EtOH and ether to give **(108)** as a white solid (45 mg, 73 %).

δ_H (400 MHz, Me_2SO - d_6): 6.32 (2 H, s, NH_2), 8.55 (1 H, s, H-7)), 10.52 (1 H, s, NH), 5.70 (1 H, d, H-1'), 5.5 (1H, s, OH), 5.13 (1 H, s, OH), 5.01 (1H, t, H-3'), 4.33 (1 H, s, OH), 4.16 (1H, s, OH), 3.96 (2 H, m, H-5'), 3.41-3.68 (2H, m, H-4'). δ_C (100 MHz, Me_2SO - d_6) : 61.36 (CH_2), 70.02, 74.77, 85.10, 94.06 (4 x C'H), 128.78 (C-7), 102.24, 153.66, 159.39, 160.9, (quaternary C). UV (MeOH) 278 nm.

Synthesis of 8-Aza-7-deazaguanosine (108) from 2-N-acetyl -6-chloro-8-aza-7-deazaguanosine 2',3',5' triacetate (111)

2-N-Acetyl-6-chloro-8-aza-7-deazaguanosine 2',3',5' triacetate **(111)** (65 mg, 0.14 mmol) was suspended in NaOH (1 M, 1 ml) and the solution refluxed for 2 hr with stirring. The resulting clear solution was cooled (5-10°C) and adjusted to pH 5 by slow addition of amberlite IRC-50 (H^+). The amberlite resin was removed by filtration and washed with

hot water (3 x 5 ml). The combined filtrate and washings were evaporated to provide **(108)** as a white solid (yield 20 mg, 53 %).

δ_H (400 MHz, Me_2SO - d_6): 7.04 (2 H, s, NH_2), 8.45 (1 H, s, H-7)), 5.65 (1 H, d, H-1'), 4.8 (1 H, s, OH), 4.27 (1 H, H-2'), 4.13 (1 H, m, H-3'), 3.64 (2 H, m, H-5'), 3.43 (1 H, m, H-4'). δ_C (400 MHz, Me_2SO - d_6) : 61.36 (CH_2), 70.03, 74.93, 85.20, 94.26 (4 x C'H), 127.7 (CH-7), 102.4, 154.8, 160.27, 161.21, (quaternary C). UV (MeOH) 274 nm.

8-Azaguanosine 2,3,5-tribenzoate (130)

To a suspension of 8-azaguanine **(51)** (100 mg, 0.66 mmol) and 1 -*O*-acetyl-2,3,5-tri-*O*-benzoyl-*β*-*D*-ribofuranose **(69)** (333 mg, 0.66 mmol) in acetonitrile (4 ml) was added stannic-(IV)-chloride (0.3 ml) under an atmosphere of nitrogen. The reaction mixture was allowed to stir at room temperature for 24 hr. The clear solution was then poured into a saturated solution of $NaHCO_3$ (15 ml). The solid precipitate was filtered and allowed to dry after which it was transferred to a flask and stirred thoroughly with hot ethyl acetate. The solid was filtered and the solvent was evaporated to leave **(130)** as a light white solid behind (yield = 90 mg, 27 %). TLC [(EtOAc / Hexane), 9:1, R_f = 0.4].

δ_H (400 MHz, Me_2SO - d_6) : 6.61 (2H, s, NH_2), 7.85-8.0 (7H, m, Ar-H), 7.7-7.59 (4H, m, Ar-H), 7.4-7.52 (8H, m, Ar-H), 6.89 (1H, d, H-1'), 6.44-6.43 (1H, m, H-2'), 6.20 (1H,

t, H-3'), 4.94 (1H, m, H-4'), 4.66-4.58 (2H, m, H-5'). δ_C (100 MHz, Me_2SO - d_6) : 63.3 (CH_2), 70.98, 73.96, 79.52, 92.52 (4 x C'H), 128.25, 128.49, 128.60, 128.66, 128.73, 128.78, 129.1, 129.26, 129.36, 129.42 (Ar-CH), 113.23, 155.79, 156.5, 161.91, 164.4, 164.61, 165.37 (quaternary C). UV (MeOH) 283 nm.

8-Azaguanosine 2,3,5-tribenzoate

To a suspension of 8-azaguanine **(51)** (100 mg, 0.66 mmol) and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-*β*-*D*-ribofuranose **(69)** (333 mg, 0.66 mmol) in acetonitrile (4 ml) stannic-(IV)-chloride (0.3 ml) was added under nitrogen atmosphere in a pressure tube. Once the addition of the Lewis acid catalyst was complete the pressure tube was quickly sealed shut and the reaction mixture was allowed to stir at 100°C temperature for 3 hr. The dark solution was than poured into a saturated solution of $NaHCO_3$ (15 ml). The solid precipitate was filtered and allowed to dry after which it was transferred to a flask and stirred with hot ethyl acetate. The solid was filtered and the solvent was evaporated to leave a white coloured solid behind (yield = 110 mg, 30 %). NMR analysis of the solid showed the presence of a mixture of products.

8-Azaguanosine 2,3,5-tribenzoate (130)

To a suspension of 8-azaguanine **(51)** (100 mg, 0.66 mmol) and 1 -*O*-acetyl-2,3,5-tri-*O*-benzoyl-*β*-*D*-ribofuranose **(69)** (333 mg, 0.66 mmol) in acetonitrile (4 ml) stannic-(IV)-chloride (0.3 ml) was added under nitrogen atmosphere at –45°C in a pressure tube. Upon the addition of the Lewis acid catalyst the reaction mixture was allowed to stir to room temperature for a further 20 hr. The clear yellow solution was than poured into a saturated solution of $NaHCO_3$ (15 ml). The solid precipitate was filtered and allowed to dry after which it was transferred to a flask and stirred thoroughly with hot ethyl acetate. The solid was filtered and the solvent was evaporated to leave a light beige coloured solid behind. (yield = 100 mg, 28 %). NMR analysis of the solid showed the same product **(130)** had been formed as when the reaction was done at room temperature.

Synthesis of 8-azaguanosine (131)

8-Azaguanosine 2',3',5' tri-*O*-benzoate **(130)** (97 mg, 0.16 mmol) was dissolved in methanolic ammonia (25 ml, MeOH / NH_3 0.88 (95 :5)) and the solution stirred at 65°C for 24 hr. After removal of the solvent *in vacuo*, the residue was washed with DCM, EtOH and ether to give **(131)** as a white solid (30 mg, 66 %).

δ_H (400 MHz, Me_2SO - d_6) : 6.59 (1H, bs, NH_2), 10.99 (1H, bs, NH), 3.48 (1H, m, 2'-H), 3.57 (1H, m, 2'-H), 3.98 (1H, m, 4'-H), 4.23 (1H, m, 3'-H), 4.49 (2H, m, 5'-H),

4.78 (1H, bs, OH), 5.24 (1H, bs, OH), 5.60 (1H, bs, OH), 5.87 (1H, t, J = 6.0 Hz, 1'-H). δ_C (100 MHz, Me_2SO - d_6): 62.06 (CH-5'), 70.65 (CH-3'), 74.39 (CH-2'), 86.07 (CH-4'), 96.34 (CH-1'), 138.84, 154.39, 156.71, 159.49 (quaternary C). UV (MeOH) 297 nm.

9 -(2',3'-*O*-diisopropylidene-D-ribityl)guanine (135)[160]

To a suspension of 2',3'-*O*-diisopropylideneguanosine **(134)** (300 mg, 0.93 mmol) in anhydrous THF (60 ml) at room temperature was added DIBAL-H (6 ml, 6 mmol) drop wise under nitrogen atmosphere. After allowing to stir for 50 hr at room temperature, the reaction mixture was quenched with a saturated aqueous solution of potassium sodium tartrate (15 ml) at 0°C and stirred at room temperature for a further 24 hr. The mixture was concentrated and the solution triturated with small portions of water. The solid residue was separated by filtration and washed with Et_2O and recrystallised from H_2O.

δ_H (400 MHz, Me_2SO - d_6): 1.22 and 1.42 (6H, 2s, 2xCH_3), 3.31 (1H, m, 5'-H), 3.58-3.61 (2H, m, 4' and 5'-H), 3.99-4.06 (2H, m, 1' and 3'-H), 4.32 (1H, dd, J = 14.2 and 2.9 Hz, 1'-H), 4.49 (1H, m, 2'-H), 4.64 (1H, m, 5'-OH), 5.09 (1H, d, 4'-OH), 6.51 (2H, bs, NH_2), 7.63 (1H, s, 8-H), 10.55 (1H, bs, NH).

δ_C (100 MHz, Me_2SO - d_6) : 25.94 (CH_3), 28.35 (CH_3), 43.88 (CH_2), 69.72, 75.22, 76.36 (3xC'-H), 138.53 (CH-8), 153.46, 146.92, 170.54 (quaternary C).

9-D-Ribitylguanine (136)

9 -(2',3'-*O*-diisopropylidene-D-ribityl)guanine **(135)** (58 mg) was heated at 70°C in 80% acetic acid for two days. The clear solution was evaporated to dryness and analysed by NMR.

δ_H (400 MHz, Me_2SO - d_6): 3.41-3.43 (1H, m, 2-H), 3.58 (2H, m, 5-H_2), 3.82-3.90 (2H, m, 1-H, 3-H), 4.19 (1H, dd, J = 14.2 and 2.3 Hz, 1-H), 4.44 (1H, bs, 3-OH), 4.68 (1H, bs, 5-OH), 4.94 (1H, s, 4-methylene), 5.03 (1H, s, 4-methylene), 6.44 (2H, bs, NH_2), 7.58 (1H, s, 8-H). δ_C (100 MHz, Me_2SO - d_6) : 45.79 (CH_2-1'), 62.85 (CH_2-4'), 70.33, 72.49, 73.30 (CH-2', CH-3', CH-5'), 116.26 (CH-8), 138.40, 153.33, 170.54 (quaternary C). UV (254 nm).

Synthesis of 1-Amino-1-deoxyribitol (139)

To a solution of hydroxylamine sulphate (1.12 g, 6.8 mmol) dissolved in in water (8 ml) D-ribose (2 g, 13.4 mmol) was added. A solution of ammonium hydroxide (3 M) was added dropwise to the reaction mixture to maintain a pH of 4.6. The pH reached this

constant value after 1.5 hr, indicating the reaction had reached completion. After which Raney nickel (0.6 g) was added and the reaction was a stirred at room temperature and pressure for 24 hr under an atmosphere of hydrogen. The mixture was filtered through celite and passed over a column containing Dowex (50W x 8 (H^+). The column was washed with water until the eluate was neutral, and these washings were discarded. The column was then eluated with ammonium hydroxide (5 M) and the eluate concentrated to dryness at 30°C. The eluate was taken to dryness several times from absolute ethanol, leaving the crude amine as a syrup. The amine was purified by conversion into the salicylidene derivative. To a solution of the amine syrup (0.8412 g) in water (0.84 ml) and ethanol (3 ml) was added salicylaldehyde (0.525 ml). The mixture was refluxed for 30 min. and than concentrated by air evaporation. The creme solid deposited was collected and washed with ether to remove any traces of the salicylaldehyde starting material (yield = 70mg, 5%). TLC analysis [(DCM / MeOH), 5:1, R_f = 0.43].

δ_H (400 MHz, Me_2SO - d_6): 3.62-3.64 (5H, m), 3.86 (1H, m), 3.98 (1H, m), 4.87 (1H, m), 5.12-5.17 (3H, m). δ_C (100 MHz, Me_2SO - d_6) : 65.26 (CH_2-5), 66.45, 66.56, 67.67, 71.28 (3xCH-OH), 96.18 (CH=N), 118.37, 132.84, 134.13, 142.66.

GTP-CH Assay

The reagents listed below were added to a 1.5 Eppenorf tube while storing on ice. Reactions were mixed by pipetting after addition of the enzyme and were immediately transferred to a water bath at 37°C.

	Volume (μl)	Final Conc.
distilled water (d H_2O)	X	
1M Tris. HCl pH 8.5	100	100mM
1M KCl	100	100mM
0.5M EDTA	5	2.5mM
2mM GTP	Y	0.5-100 μM
Inhibitor	Z	1-10 mM
GTP-CH	2.5	0.2 U / ml
	1ml (total)	

In experiments set up to test compounds for the ability to inhibit GTP-CH activity the compound being tested was first dissolved in the minimum amount of water. 1 M Tris. HCl (pH 8.5) was sometimes added to increase the final concentration of the test compound when it was found to be sparingly soluble in water.

At set times 100μl aliquots were removed for quenching, (usually 3, 6, 12 min.) by addition to 1% I_2 / 2% KI in 1M HCl (30 μl) followed by incubation at R.T. in the dark for 30 min. Excess I_2 was destroyed by the addition of 2% abscorbic acid (15μl) and added $MgCl_2$ 50 mM / $ZnCl_2$ 65 mM (3.3 μl) was added followed by alkaline phosphotase (0.5 μl, 2 U) and the mixture was incubated for 1hr at 37°C. Proteins was precipitated from the sample by the addition of 100% TCA (28μl) and cooling on ice (15 mins.). The precipitated protien was removed after centrifuging and transferring the supernatant to a fresh tube. Samples were stored at -20°C if not analysed immediately. HPLC analysis was carried out using a reverse phase (C-18) column, using 30 mM formic acid / 7% MeOH at a flow rate of 1.5 ml / min. The amount of neopterin formed in the assay was estimated by comparison with standard samples of neopterin of known concentration.

The concentration of the inhibitors in the incubations were as follows: I) 8-aza-7-deazaguanine **(50)** (2.3 mM), II) 8-azaguanine **(51)** (1.3 mM), III) 3-deazaguanine **(49)** (2.2 mM) IV) 2,6-diamino-4(3*H*)-pyrimidinone **(143)** (9.5 mM), V) 2-amino-6-hydroxy-4(3*H*)-pyrimidinone **(85)** (3.75 mM) and VI) 9-D-ribitylguanine **(135)** (5.52 mmol).

Kinetic Experiments:

Measuring K_m for the enzyme GTP-CH (I)

A number of incubations were set up at a range of concentrations of GTP (0.5 μM, 5 μM, 10 μM, 20 μM) in order to measure the K_m for the GTP-CH (I) enzyme. Each set of incubations were prepared as described previously and quenched after 3, 6 min. The amount of neopterin formed in each assay was estimated by comparison with samples of neopterin of known concentration. The data obtained was converted into the LineWeaver-Burk plot, taking into account also the neopterin present prior to the incubation in the enzyme preparation (page 93). The values obtained for the initial rates are shown in the table below.

Measuring K_m of GTP-CH (I)

[GTP] (μM)	**Rate** (n mol min.$^{-1}$)	**1/ [GTP]** (μM^{-1})	**1/ Rate** (n mol^{-1} min.)
0.5	0.080	2	43.75
5	0.185	0.2	18.92
10	0.199	0.1	17.59
20	0.216	0.05	16.20
40	0.220	0.025	15.91

Table showing the calculated reciprocal rates and the reciprocal concentration of GTP, values of which were used to determine the K_m value of the enzyme GTP-CH (I).

Measuring the K_I value of 8-azaguanine (51)

In order to determine the inhibition constant of 8-azaguanine (i.e. K_I value) on the enzyme GTP-cyclohydrolase (I), two sets of incubations were prepared at a range of GTP concentrations. One set was the control and the other set containing the inhibitor (8-azaguanine). The concentration of GTP chosen for the incubations were as follows: 1 μM, 2 μM, 5 μM, 10 μM, 20 μM. The inhibitor and control incubations were quenched after 3 min. and processed as described above.

Measuring the K_I value of 2,6-diamino-4(3*H*)-pyrimidinone (144)

To determine the inhibition constant of 2,6-diamino-4(3*H*)-pyrimidinone (i.e. K_I value) on the enzyme GTP-cyclohydrolase (I), two sets of incubations were prepared at a range of GTP concentrations. One set was the control and the other set containing the inhibitor (8-azaguanine). The concentration of GTP chosen for the incubations were as follows: 1 μM, 2 μM, 5μM, 10μM. The inhibitor and control incubations were quenched after 3 min. by the procedure as described above.

Determining the K_I value of the inhibitor 8-azaguanine (51)

Control Incubations (no inhibitor present)

[GTP] (μM)	**Rate** (n mol min.$^{-1}$)	**1/ [GTP]** (μM^{-1})	**1/ Rate** (n mol^{-1} min.)
1.0	0.037	1	27.0
2.0	0.062	0.5	16.1
5	0.065	0.2	15.4
10	0.087	0.1	11.5
20	0.105	0.05	9.5

Inhibitor incubations (conc. of 8-azaguanine 1.33 mM)

[GTP] (μM)	**Rate** (n mol min.$^{-1}$)	**1/ [GTP]** (μM^{-1})	**1/ Rate** (n mol^{-1} min.)
1.0	0.007	1	142.9
2.0	0.012	0.5	83.3
5	0.039	0.2	25.6
10	0.043	0.1	23.3
20	0.080	0.05	12.5

Determining the K_I value of the inhibitor 2,6-diamino-4(3*H*)-pyrimidinone (144)

Control Incubations (no inhibitor present)

[GTP] (μM)	**Rate** (n mol $min.^{-1}$)	**1/ [GTP]** (μM^{-1})	**1/ Rate** (n mol^{-1} min.)
1.0	0.076	1	13.25
2.0	0.147	0.5	6.82
5	0.170	0.2	5.88
10	0.185	0.1	5.39

Inhibitor incubations (conc. of 2,6-diamino-4(3*H*)-pyrimidinone 9.5 mM)

[GTP] (μM)	**Rate** (n mol $min.^{-1}$)	**1/ [GTP]** (μM^{-1})	**1/ Rate** (n mol^{-1} min.)
1.0	0.017	1	58.4
2.0	0.029	0.5	34.70
5	0.083	0.2	12.05
10	0.085	0.1	11.82

Chapter 5

References

References

1) F.G. Hopkins, *Nature*, 1889, **40**, 355.

2) F.G. Hopkins, *Proc. Royal Soc.* 1942, **130**, 359.

3) H. Wieland and C. Schöpf, *Liebigs Annalen der Chemie*, 1940, **544**, 163.

4) R. Purrmann, *Liebigs Annalen der Chemie*, 1940, **546**, 98.

5) R. Purrmann, *Liebigs Annalen der Chemie*, 1941, **548**, 284.

6) E.E. Snell and F.M. Strong, *Ind. Eng. Chem. anal. Ed.*, 1939, **11**, 346.

7) E.E. Snell and W.H. Patterson, *J. Bacteriol.*, 1940, **39**, 273.

8) E.L.R. Stokstad, B.L. Hitchings and Y. SubbaRowa, *J. Am. Chem. Soc.*, 1948, **70**, 3.

9) E.L.R. Stokstad, B.L. Hitchings, J.H. Mowat, J.H. Boothe, C.W. Waller, R.B. Angier, J. Semb and Y. SubbaRowa, *J. Am. Chem. Soc.*, 1948, **70**, 5.

10) B.L. Hitchings, E.L.R. Stokstad, N. Bohonos N.H. Sloan and Y. SubbaRowa, *J. Am. Chem. Soc.*, 1948, **70**, 1.

11) R.B. Angier, J.H. Boothe, B.L. Hutchings, J.H. Mowat, J. Semb, E.L.R. Stokstad, Y. SubbaRowa, C.W. Waller, D.B. Cosulich, M.J. Hultquist, E. Kuh, E.H. Northey, D.R. Seeger, J.P. Sickles and J.M. Smith, *Science*, 1945, **102**, 227.

12) E.E. Snell and H.K. Mitchell, *Proc. Natl. Acad. Sci. USA*, 1941, **27**, 1.

13) M.R. Stetten and C.L. Fox, *J. Biol. Chem.*, 1945, **161**, 333.

14) W.W. Shive, *Ann. NY Acad. Sci.*, 1950, **52**, 1212.

15) J.A. Brockman, B. Roth, H.P. Broquist, M.E. Hultquist, J.M. Smith, M.J. Farenbach, D.B. Cosulich, R.B. Parker, T.H. Jukes, *J. Am. Chem. Soc.*, 1950, **72**, 4325.

16) J.J. Reynolds and G.M. Brown, *J. Biol. Chem.*, 1962, **237**, 2713.

17) J.J. Reynolds and G.M. Brown, *J. Biol. Chem.*, 1964, **239**, 317.

18) A.W. Burg and G.M. Brown, *Biochim. Biophys. Acta*, 1966, **117**, 275.

19) T. Shiota and M.P. Plumbo, *J. Biol. Chem.*, 1965, **240**, 4449.

20) G.M. Brown, In: *Chemistry and Biology of Pteridines*, (K. Iwai, M. Goto and Y. Iwanami, eds.), International Academic Printing Co. Ltd., 1970, 243.

21) T. Shiota, R. Jackson and C.m. Baugh, *Chemistry and Biology of Pteridines*, (K. Iwai, M. Goto and Y. Iwanami, eds.), International Academic Printing Co. Ltd., 1970, 265.

22) S. Kuafman, *Proc. Natl. Acad. Sci. USA*, 1963, **50**, 1085.

23) I. Ziegler-Günder, H. Simon and A.Z. Wacker, *Naturforsch., Pt. B*, 1956, **11**, 82.

24) C.C. Levy, *J. Biol. Chem.*, 1964, **239**, 560.

25) W.B. Watt, *J. Biol. Chem.*, 1967, **242**, 565.

26) J. Cowperthwaite, M.M. Weber, L. Packer and H.H. Hutner, *Ann. NY Acad. Sci.*, 1953, **56**, 972.

27) H.A. Nathan and J. Cowperthwaite, *J. Protozool.*, 1955, **2**, 37.

28) E.L. Patterson, H.P. Boquist, A.M. Albercht, M.H. von Saltza and E.L. Stokad, *J. Am. Chem. Soc.*, 1956, **78**, 5868.

29) E.L. Patterson, M.H. von Saltza and E.L. Stokad, *J. Am. Chem. Soc.*, 1956, **78**, 5871.

30) I. Ziegler-Günder and E. Hadron, *Z. Vererbungslelehre*, 1958, **89**, 235.

31) E. Hadron and H.K. Mitchell, *Proc. Natl. Acad. Sci. USA*, 1951, **37**, 57.

32) T. Hama and M. Obika, *Nature*, 1960, **187**, 326.

33) H.L. Levy, *J. Biol. Chem.*, 1964, **239**, 560.

34) R. Dalal and J.S. Gots, *Biochim. Biophys. Res. Commun.*, 1965, **20**, 509.

35) H. Mitsuda, Y. Suzuki, K. Tadera and F. Kawai, *J. Vitaminol.*, 1966, **12**, 192.

36) B. Levenberg and D.K. Kaczmarek, *Biochim. Biophys. Acta*, 1966, **117**, 275.

37) C. Rieder, W. Eisenreich, J. O'Brien, G, Richter, E. Götze, P. Boyle, S. Blanchard, A. Bacher and H. Simon. *Eur. J. Biochem.*, 1998, **255**, 24.

38) T. Fukushima, *Arch. Biochem. Biophys.*, 1970, **139**, 361.

39) K. Tanaka, M. Akino, Y. Hagi, M. Soi and T. Shiota, *J. Biol. Chem.*, 1981, **256**, 2963.

40) K.J. Stone, *J. Biochem.*, 1976, **157**, 105.

41) C.A. Nichol, C.L. Lee, M.P. Edelstein, J.Y. Chao and D.S. Duch, *Proc. Natl. Acad. Sci. USA*, 1983, **80**, 1546.

42) S. Milstein and S. Kaufman, *Biochem. Biophys. Res. Commun.*, 1983, **115**, 888.

43) R.J. Leeming and J.A. Blair, *Clinica Chemica Acta*, 1980, **108**, 103.

44) J.L. Dhondt, Z. Bellahsene, C. Largilliere, J. Bonneterre, P. Vanhille, C. Noel, P. Choteau and J.P. Farrieaux, In: *Chemistry and Biology of Pteridines*, edited by J.A. Blair (Berlin:Walter de Gruyter), 1983, 845.

45) P.A. Barford, J.A. Blair, C. Eggar and C. Hamon, In: *Biochemical Aspects of Pteridines*, edited by H. C. Curtius, W. Pflieder and H. Watcher (Berlin:Walter de Gruyter), 1983, **2**, 303.

46) R.R. Cubberley, W.K. Alderton, A. Boyhan, I.G. Charles, P.N. Lowe and R.W. Old, *J. Biochem.* 1997, **323**, 141.

47) G. Embden and K. Baldes, *Biochem.*, 1913, **55**, 301.

48) G.A. Jervis, *J. Biol. Chem.*, 1947, **169**, 651.

49) S. Undenfriend, J.R. Cooper, C.T. Clark and J.E. Baer, *Science*, 1952, **117**, 663.

50) S. Kaufman, *J. Biol. Chem.* 1957, **226**, 511.

51) S. Kaufman, *J. Biol. Chem.* 1958, **230**, 931.

52) S. Kaufman and B. Levenberg, *J. Biol. Chem.* 1959, **234**, 2683.

53) H.J. Blaschko, *J. Physiol.*, 1939, **96**, 50.

54) S. Undenfriend and J.B. Wyngaarden, *Biochim. Biophys. Acta*, 1956, **20**, 48.

55) M. Goodall and N. Kirshner, *J. Biol. Chem.* 1957, **226**,213.

56) S. Kaufman, *J. Biol. Chem.*, 1964, **239**, 332.

57) T. Nagatsu, M. Levitt and S. Udenfriend, , *J. Biol. Chem.*, 1964, **239**, 2910.

58) A. Ichiyama, S. Nakamura, Y. Mishizuka and O. Hayaishi, *Adv. Pharmacol.* 1968, **64**, 5.

59) S. Kaufman, N. Holtzman, S. Milstein, I. Butler and A. Krumholz, *New England Journal of Medicine*, 1975, **293**, 785.

60) R.J. Leeming, J.A. Blair, AA. Green and D.N. Raine, *Archives of Diseases of Childhood*, 1976, **51**, 771.

61) A) D.M. Danks, K. Bartholm´e, B.E. Clayton, H.Ch. Curtius, H. Grobe, S. Kaufman, R.J. Leeming, R.J. Pflieder, H. Rembold and F. Rey, *J. Inherited Metabolic Disease*, 1978, **1**, 49. B) In : *Tetrahydrobioptrin, Basic Biochemistry and Role in Human Disease*, S. Kaufman. The John Hopkins University Press.

62) J.H. Young, V. Walter, P.A. Tippett, R.M. Veall and B.E. Clayton, *J. Inherited Metabolic Disease*, 1983, **6**, 111.

63) A. Niederwieser, N. Blau, M. Wang, P. Jollier, M. Atares and J. Cardessa-Garcia, *European Journal of Pediatrics*, 1984, **141**, 208.

64) A. Niederwieser, W. Leimbacher, H. Ch. Curtius, A. Ponzone, K. Rey and D. Leupold, 1984, **144**, 13.

65) R.J. Leeming, A.E. Pheasant and J.A. Blair, *Journal of Mental Deficiency Research*, 1981, **25**, 231.

66) F. Rey, J.P. Harpey, R.J. Leeming, J.A. Blair, J. Aicardi and J. Rey, *Archives Francaises de Pédiatrie*, 1977, **34**, 9.

67) K. Hyland, I. Smith, D.W. Howels, P.T. Clayton and J.V. Leonard, In *Biochemical and Clinical Aspects of Pteridines*, 1985, **4**, 85.

68) R.J. Leeming and J.A. Blair, *Clinica. Chemica. Acta.*, 1980, **108**, 103.

69) W. Lovenberg, R.A. Leving and D.S. Robinson, *Science*, 1979, **204**, 624.

70) C. Morar, S.B. Whitburn, J.A. Blair, R.J. Leeming and G.K. Wilcock, *Journal of Neurology, Neurosurgery and Psychiatry*, 1983, **46**, 582.

71) J.H. Young, B. Kelly and B.E. Clayton, *Journal of Clinical and Experimental Gerontology*, 1982, **4**, 389.

72) P.A. Barford, J.A. Blair, C. Eggar, C.G.B. Hamon, C. Morar and S.B. Whitburn, *Journal of Neurology, Neurosurgery and Psychology*, 1984, **47**, 736.

73) A. Aziz, J.A. Blair, R.J. Leeming and P.E. Sylvester, *Journal of Mental Deficiency Research*, 1983, **26**, 57.

74) J.A. Blair, C. Morar, C.G.B. Hamon, F.G. Al –Salihi, P.A. Barford, A.E. Pheasent and S.B. Whitburn, In: *Biochemical and Clinical Aspects of Pteridines*, edited by H. C. Curtius, W. Pflieder and H. Watcher (Berlin:Walter de Gruyter), 1984, **3**, 93.

75) R.A. Levine, D. Heintel, W. Leimbacher, A. Neiderwieser, H. Ch. Curtius and A. Ghisla, In *Biochemical and Clinical Aspects of Pteridines*, 1983, **2**, 325.

76) H. C. Curtius, H. Muldner and A. Niederweieser, *Journal of Neural Transmission*, 1982, **55**, 301.

77) H.M. Van Praag, *Lancet*, 1982, **4**, 1259.

78) J.A. Blair, P.A. Bradford, C. Morar, A.E. Pheasent, C.G.B. Hamon, S.B. Whitburn, R.J. Leeming, G.P. Reynolds and A. Coppen, *Lancet*, 1984, **2**, 163.

79) C.H. Kellner, R.M. Rakita, D.A. Rubinow, P.W. Gold, J.CC.. Ballenger and R.M. Post, *Lancet*, 1983, **2**, 55.

80) J.C. Garbutt, D.S. Duch, C.A. Nichol and J.H. Woolf, *Psychiatry Res.*, 1985, **16**, 181.

81) R. Hashimato, M. Mizutani, T. Ohta, Y. Kasahara, N. Kaneda and T. Nagatsu, *Neuropsychobiol.*, 1987, **17**, 176.

82) W. Lovenberg and R.A. Levine, *Topics in Neurochemistry and Neuro-pharmacology*, In: Unconjugated Pterins in Neurobiology Basic & Clinical Aspects, series editors: W. Lovenberg, M.B.H. Youdim and K.F. Tipton, 1987, **1**, 173.

83) H. Naruse, T. Hayashi, M. Takesada, A. Nakane, K. Yamazuki, T. Noguchi, Y. Watanabe and O. Hayaishi, *Proc. Jpn. Acad.*, 1987, **63**, 231.

84) C. Gillberg and L. Svennerholm, *Br. J Psychiatry*, 1987, **151**, 89.

85) R.A Levine, L.P. Miller and W. Lovenberg, *J. Neuro. Chem.*, 1981, **214**, 919.

86) R. Hille, *Chem. Rev.*, 1996, **96**, 2757.

87) M.J. Romão, M. Archer, I. Moura, J.J.G. Moura, J. LeGall, R. Engh, M. Scheinder, P. Hof and R. Huber, *Science*, 1995, **270**, 1170.

88) H. Schindelin, C. Kisker, J. Hilton, K.V. Rajagopalan and D.C. Rees, *Science*, 1996, **272**, 1615.

89) J.C.C. Boyington, V.N. Gladyshev, S.K. Khangulov, T.C. Stadtman and P.D. Sun, *Science*, 1997, **275**, 1305.

90) M.K. Chan, S. Mukund, A. Kletzin, M.W.W. Adams and D.C. Rees, *Science*, 1995, **267**, 1463.

91) O. Meyer, S. Jacobitz and B. Krüger, *FEMS Microbiol. Rev.*, 1986, **39**, 161.

92) D.J. Eaves, T. Palmer and D.H. Boxer, *Eur. J. Biochem.*, 1997, **246**, 690.

93) C. Kisker, H. Schindelin and D.C. Rees, *Annu. Rev. Biochem.*, 1997, **66**, 233.

94) J.L. Johnson and S.K. Wadman, In *The Metabolic Basis of Inherited Disease*, 1989, **1**, 1463.

95) S.P. Kramer, J.L. Johnson, A.A. Ribeiro, D.S. Millington and K.V. Rajagopalan, *J. Biol. Chem.*, 1987, **262**, 16357.

96) K.V. Rajagopalan, *Cellular and Molecular Biology*, 1996, **1**, 674.

97) J.L. Johnson, M.M. Wuebbens and K.V. Rajagopalan, *J. Biol. Chem.*, 1989, **264**, 13440.

98) R.B. Irby and W.L. Jr. Adair, *J. Biol. Chem.*, 1994, **269**, 23981.

99) M.M. Wuebbens and K.V. Rajagopalan, *J. Biol. Chem.*, 1995, **270**, 1082.K. Hatakeyama, Y. Inoue, T. Harada and H. Kagamiyama, *J. Biol. Chem.*, 1991, **266**, 765.

100) K. Hatakeyma, Y. Inoue, T. Harada and H. Kagamiyama, *J. Biol.Chem.*, 1991, **266**, 765.

101) G. Katzenmeier, C. Schmid, J. Kellermann, F. Lottspeich and A. Bacher, *Biol. Chem. Hoppe Seyler*, 1991, **372**, 991.

102) P. Babitzke, P. Gollnick and C. Yanofsky, *J. Bacteriol.*, 1992, **174**, 2059.

103) A. Togari, H. Ichinose, S. Matsumoto, K. Fujita and T. Nagatsu, *Biochem. Biophys. Res. Commun.*, 1992, **187**, 359.

104) J.R. McLean, S. Krishnakumar and J.M. O'Donnel, *J. Biol. Chem.*, 1993, **268**, 27191.

105) T. Nomura, H. Ichinose, C. Sumi-Ichinose, H. Nomura, Y. Hagino, K. Fujita and T. Nagatsu, *Biochem. Biophys. Res. Commun.*, 1993, **191**, 523.

106) S.A. Lacks, B. Greenberg and P. Lopez, *J. Bacteriol.*, 1995, **177**, 66.

107) W. Meining, H. Nar, C. Schmid, A. Bacher, L. Bachmann and S. WeinKauf, *Pteridines*, 1994, **5**, 77.

108) W. Meining, A. Bacher, L. Bachmann, C. Schmid, S. WeinKauf, S. Huber and H. Nar, *J. Mol. Biol.*, 1995, **253**, 208.

109) 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.

110) H. Nar, R. Huber, W. Meining, C. Schmid, C. Weinkauf and A. Bacher,

Structure, 1995, **3**, 459.

111) L.B. Allen, J.H. Huffman, P.D. Cook, R.K. Robins and R.W. Sidwell, *Antimicrob. Agents Chemotherapy*, 1977, **12**, 114.

112) T.A. Khwaja, L. Kigwana and R.B.Meyer, *Am. Assoc. Cancer Res.*, 1975, **16**, 162.

113) T.A. Khawja and J.C. Varen, *Proc. Am. Assoc. Cancer Res.*, 1975, **17**, 200.

114) P. Schwartz, D, Hammond and T.A. Khwaja, *Proc. Am. Assoc. Cancer Res.*, 1977, **18**, 153.

115) R.S. Rivest, D. Irwin and H.G. Mendel, *Proc. Am. Assoc. Cancer Res.*, 1980, **21**, 279.

116) D.G. Streeter and H.H.P. Koyama, *Biochem. Pharmacol.*, 1976, **25**, 2413.

117) P.P. Saunders, L.Y. Chao, T.I. Leo and R.K. Robins, *Biochem. Pharmacol.*, 1981, **30**, 2374.

118) T.A. Kwaja, L. Momparler, J.C. Vervan and A.M. Mains, *Proc. Am. Assoc. Cancer Res.*, 1979, **20**, 152.

119) R. Hilf, *In Prediction of Response in Cancer Therapy, Will the Best Model of Breast Cancer Please come forward, NCI Monograph* 1971, **34**, 43.

120) R. de Bode and C.A. Salemink, *Rec. Trav. Chem.*, 1974, **93**, 3.

121) R.J. Rousseau, R.J. Robins and J. Robins, *J. Heterocyl. Chem.*, 1965, **2**, 196.

122) C.A. Salemink and G.M. Vanderwant, *Rec. Trav. Chem.*, 1949, **68**, 1013

123) P.D. Cook, R.J. Rousseau, A.M. Main, P. Dea, R.B. Meyer and R.K. Robins, *J.*

Amer. Chem. Soc., 1976, **98**, 1492.

124) R.K. Robins, J.M. Horner, C.V. Greco, C.W. Noel and C.R. Beames, Jr., *J. Org. Chem.*, 1963, **28**, 3041.

125) a) R.K. Robins, *J. Heterocycl. Chem.*, 1979, **16**, 1063. b) A.I. Vogel, In: *A Text Book of Practical Organic Chemistry*, 3rd edition, 1956, 870.

126) G.R. Revankar, P.K. Gupta, A.D. Adams, N.K. Dalley, P.A. Mc Kernan, P.D. Cook, P.G. Canonico and R.K. Robins, *J. Med. Chem.*, 1984, **27**, 1389.

127) N. Minakawa and A. Matsuda, *Tetrahedron*, 1993, **49**, 557.

128) A.Matsuda, N. Minakawa, T. Sasaki and T. Ueda, *Chem. Pharm. Bull.*, 1988, **36**, 2730.

129) N. Niebella and H. Vorbürggon, *J. Org. Chem.*, 1974, **39**, 3654. F.W. Lichtentaler, P. Voss and A. Heerd, *Tetrahedron Lett.*, 1974, 2141.

130) M.W. Winkley and R.K. Robins, *J. Chem. Soc.* 1969, **Vol. C**, 791.

131) J.T. Witkowski, R.K. Robins, R.W. Sidwell and L.N. Simon, *J. Med. Chem.*, 1972, **15**, 1150.

132) T. Sato, T. Shimadate and Y. Ishido, *Nippon Kogaku Zasshai*, 1960, **81**, 1440.

133) L.B. Townsend, *Chem. Rev.*, 1967, **67**, 533.

134) (a) J.J. Fox and L. Wempen, *Adv. Carbohyr. Chem.*, 1958, **14**, 282; (b) J.A. Montgomery and H.J. Thomas, *Adv. Carbohyr. Chem.*, 1961, **17**, 301; (c) A.M. Michelson, *"The Chemistry of Nucleosides and Nucleotides"*, Academic Press

New York, N.Y. 1963; (d) K.A. Watanabe, D.H. Hollenberg and J.J. Fox, *J. carbohydr., nucleosides, nucleotides*, 1974, **1**, 1. (e) M. Goodman, in "Basic Principles in Nucleic Acid Chemistry", **Vol. 1**, P.O.P. Ts'O, ed., Academic press, New York, 1974, p122.

135) R. Zou and M.J. Robins, *Can. J. Chem.* 1987, **65**, 1436.

136) Patai series, In: *The Chemistry of carboxylic acids and derivatives.*

137) H.E. Skipper, R.K. Robins and J.R. Thomson, *Proc. Soc. Exp. Biol. and Med.,*. 1955, **89**, 594.

138) H.E. Skipper, R.K. Robins,. J.R. Thomson, C.C. Cheng, R.W. Brockman and F.M. Schabel, Jr., *Cancer Research*, 1957, **17**, 579.

139) R.K. Robins, *J. Amer. Chem. Soc.*, 1956, **78**, 784.

140) R.K. Robins, *J. Amer. Chem. Soc.*, 1957, **79**, 6407.

141) D.J. Nelson, S.W. LaFon, J.V. Tuttle, W.H. Miller, R.L. Miller, T.A. Krenitsky, G.B. Eilon, R.L. Berens and J.J. Marr, *J. Biol. Chem.* 1979, **254**, 11544.

142) R.L. Berens, J.J. Marr, D.J. Nelson and S.W. LaFon, *Biochem. Pharmacol.* 1980, **29**, 2397.

143) G.B. Elion, S.W. Callahan, H. Nathan, S. Bieber, R.W. Rundles and G.H. Hitchings, *Biochem. Pharmacol*, 1963, **12**, 85.

144) F. Seela and H. Steker, *Helvetica Chimica Acta*, 1986, **69**, 1602.

145) E.C. Taylor and H.H. Petal, *Tetrahedron*, 1992, **48**, 37.

146) J. Davoll and K.A. Kerridge, *J. Chem. Soc.*, 1961, 2589.

147) H.B. Cottom, G.R. Revankar and R.K. Robins, *Nucleic Acid Research*, 1993, 871.

148) H. Vorbrüggon, K. Krolikiewicz and B. Bennua, *Chem. Ber.* 1980, **114**, 1234.

149) R.O. Roblin, Jr., J.O. Lampen, J.P. English, Q.P. Cole, J.R. Vaughan, *J. Amer. Chem. Soc.*, 1945, **67**, 290.

150) J. Davoll, *J. Chem. Soc.*, 1961, 1593.

151) W. Hutzenlaub, R.L. Tolman and R.K. Robins, *J. Med. Chem.*, 1972, **15**, 1279.

152) R.D. Elliott and J.A. Montgomery, *J. Med. Chem.*, 1976, **19**, 1126.

153) F. Seela and S. Lampe, *Helvetica Chimica Acta*, 1993, **76**, 2388.

154) F. Seela, I. Münster, U. Löchner and H. Rosemeyer, *Helvatica Chimica Acta*, 1998, **81**, 1130.

155) a) E. De Clereq, R.T. Walker, In *Antiviral Drug Development: A Multidisciplinary Approach*, Plenum Press: New York, 1988. b) E. De Clereq, In *Advances in Antiviral Drug Design*; JAI press Inc. Greenwhich, Connecticut, 1996, **2**.

156) a) G.B. Elion, P.A. Furman, J.A. Fyfe, P. Miranda, L. Beauchamp and H.J. Schaeffer, *Proc. Natl. Acad. Sci. USA*, 1977, **74**, 5716.

b) H.J. Schaefer, L. Beauchamp, P. Miranda, G.B. Elion, D.J. Bauer and P. Collins, *Nature*, 1978, **272**, 583.

157) a) J.C. Martin, C.A. Dovak, D.F. Smee, T.R. Mathews and J.P.H. Verheyden, *J. Med. Chem.*, 1983, **26**, 759. b) K.K. Ogilivie, U.O. Cheriyan, B.K. Radatus, K.O.

Smith, K.S. Galloway and W.L. Kennell, *Can. J. Chem.*, 1982, **60**, 3005.

158) a) C.K. Chu and S.J. Cutler, *J. Heterocycl. Chem.* 1986, **23**, 289. b) E.S.H. El Ashry and Y. El Kilany, In *Advances in Heterocycl. Chemistry*, 1997, **67**, 391.

159) a) J. Nemec and J.M. Rhoades, *Nucleosides, Nucleotides*, 1983, **2**, 99. b) L.M. Lerner, *Carbohydr. Res.*, 1984, **127**, 141. c) G.I. Birnbaum, R. Stolarski, Z. Kazimierczuk and D. Shugar, *Can. J. Chem.*, 1985, **63**, 1215.

160) K. Hirota, Y. Monguchi, Y. Kitade and H. Sajiki, *Tetrahedron*, 1997, **53**, 16683.

161) J. Davoll and D. D. Evans, *J. Chem. Soc.*, 1960, 5041.

162) F.W. Holly, E.W. Peel, R. Mozingo and K. Folkers, *J. Amer. Chem. Soc.*, 1950, **72**, 5416.

163) D.D. Heard, B.G. Hudson and R. Barker, *J. Org. Chem.*, 1970, **35**, 464.

164) E.M. Gál, J.M. Nelson and A.D. Sherman, *J. Neurochem.*, 1979, **32**, 179.

165) R. Reed, D. Holmes, J. Weyers and A. Jones, In: *Practical Skills in Biomolecular Sciences*, Addison Wesley Longman Limited, England, 1998.

166) C.K. Mathews, K.E. Van Holde, In: *Biochemistry*, 2nd *ed.*, Benjamin and cumming publicating, USA, 1997.

167) I.E. Segal, In: *Enzymatic Kinetics,* Wiley, New York, 1975.

168) C.T. Walsh, In: *Enzymatic Reaction Mechanisms*, W.H. Freeman, New York, 1977.

169) W.C. Still, M. Khan and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.