Analogues of BH_4 and Nitric Oxide Synthase Activators

By

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Abbreviations

AcOH	acetic acid
ACh	acetylcholine
ADDP	6-acetyl-7,7-dimethyl-7,8-dihydropterin
aq.	aqueous
Arg	arginine
atm	atmospheres
BH_2	dihydrobiopterin
BH_4	tetrahydrobiopterin
Boc	*tert*-butyloxycarbonyl
$(Boc)_2O$	di-*tert*-butyl dicarbonate
BOP	benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate
B.P.	boiling point
br	broad
bsNOS	*Bacillus subtilis* nitric oxide synthase
Bu	butyl
^oC	degree(s) centigrade
CAM	calmodulin
Cat.	Catalyst
CHD	coronary heart disease
cGMP	cyclic guanosine monophosphate
Conc.	concentrated
d	doublet
DBU	diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DHF	dihydrofolic acid
DHFR	dihydrofolate reductase
DHPR	dihydropteridine reductase
DIPEA	*N*,*N*-diisopropylethylamine
DRD	dopa-responsive dystonia

DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
eq.	equivalent
eNOS	endothelial nitric oxide synthase
Et	ethyl
et al.	et alia
FAB	fast atom bombardment
FAD	flavin adenine dinucleotide
FLiNOS	full-length iNOS
FMN	flavin mononucleotide
g	grams
GTP	guanosine triphosphate
GTPCH	GTP cyclohydrolase
GSH	glutathione
h	hour
Hb	hemeoglobin
HDiNOS	heme domain iNOS
5-HIAA	5-hydroxy indole acetic acid
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
Hz	Hertz
HVA	homovanillic acid
iNOS	inducible nitric oxide synthase
IR	infrared (spectroscopy)
Jmod	J modulation experiment
lit.	literature
L-NAME	L-nitro-arginine methyl ester
LRMS	low resolution mass spectrometry
LUMO	lowest unoccupied molecular orbital
m	multiplet
M	molar
MCPBA	*m*-chloroperoxybenzoic acid

Me	methyl
metHb	methemoglobin
mg	milligrams
min	minute(s)
ml	millilitre
mmol	millimole(s)
mol	moles
mp	melting point
M.S.	molecular sieves
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NOHA	*N*-hydroxy-L-arginine intermediate
NEt_3	triethylamine
NMR	nuclear magnetic resonance
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
Nu	nucleophile
PAF	platelet activating factor
PCD	pterin-4-carbinolamine dehydratase
PDB	protein data bank
Ph	phenyl
PKU	phenylketonuria
PTPS	6-pyruvoyl-tetrahydropterin synthase
Pr	propyl
Py	pyridine
q	quartet
r.t.	room temperature
R_t	retention time
s	singlet
SIPBS	Strathclyde Institute of Pharmacy and Biomedical Sciences
sat.	saturated
SM	starting material
SNP	sodium nitroprusside

SR	sepiapterin reductase
SAR	structure activity relationship
TFA	trifluoroacetic acid
THF	tetrahydrofolic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
T3P	propylphosphonic anhydride
UV/vis	ultraviolet/visible

Abstract

Nitric oxide synthase (NOS) is an enzyme that catalyses the synthesis of nitric oxide (NO) from L-arginine. There are three kinds of nitric oxide synthase enzymes: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Nitric oxide is a biological messenger molecule and a potent vasodilator which controls many biological processes, such as hypertension, stroke, memory, learning disorders and many more. The Nobel Prize in Physiology and Medicine was granted for the discovery and identification of the endothelium-derived relaxing factor as nitric oxide. 5,6,7,8-Tetrahydrobiopterin (BH_4) is an essential cofactor of nitric oxide synthases (NOSs) and presumably is present in every cell or tissue of higher organisms. Oxidation of BH_4, in diabetes and in different chronic vasoinflammatory diseases can produce cofactor deficiency. This decreased level of BH_4 can result in uncoupling of endothelial nitric oxide synthase where this enzyme produces superoxide and thus nitric oxide production is suppressed. BH_4 in its active, reduced form is highly unstable and therefore not suitable for oral administration. BH_4 does not readily pass across the blood brain barrier; also it cannot be utilized to improve the central neurotransmitter insufficiency in case of BH_4 deficiency. This defect of BH_4 owes to its hydrophilic nature; however, lipophilic particles can readily pass this brain barrier. If we can have a molecule that is not easily oxidized, and is more lipophilic so that it can cross the blood brain barrier and is also a nitric oxide synthase activator, we can treat all diseases which are caused due to deficiency of nitric oxide especially in old age, when the body starts producing less of nitric oxide. In other words, we can find a cure for diseases which are caused by nitric oxide deficiency.

When I started my PhD, Prof Suckling group has already discovered an active pteridine called as WSG1002 **1.2** which is more stable to oxidation and has greater solubility than BH_4 **1.1**. It was an improvement but still not an ideal molecule; both stability and solubility needed to improve. We worked to develop an oxidatively stable and lipophilic pteridine molecule which can act as a cofactor for nitric oxide synthase (NOS) and can correct BH_4 deficiency in selected diseases.

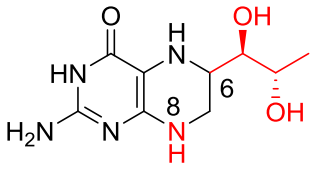
The following substitutions at WSG1002 (**1.2**) were considered for my research project.

i) N^8-deaza

ii) C^6-unsubstituted

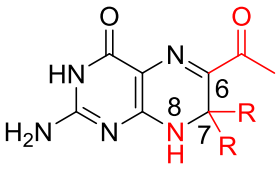
iii) 2H at C^7 instead of methyl groups.

iv) At C^6 methyl, hydroxymethyl, acetoxymethyl, acetyl and 1, 2-dihydroxypropyl.



(BH_4)

1.1



R=Me

1.2

This research project provided 8-alkyl and 8-deaza analogues for for nitric oxide synthase enzyme essay, the biological assays for these compounds will be discussed Chapter 3. The investigation of these compounds also made possible studies of the mechanism of action of NOS.

Chapter 1 describes nitric oxide and how it is formed from NOS enzymes along with NOS structures and mechanism. It also describes the role of BH_4 and its drawbacks. The stucture of the cofactor plays an important role for binding and NOS activity which has been discussed with examples along with requirements for a better molecule.

Chapter 2 is about synthesis of important molecules which can help us to understand the mechanism of NO formation and the possibility of finding an ideal drug with better medicinal properties. Although the reactions were challenging, we have prepared many new molecules, many them have been evaluated, and some of them were found to be active and oxidatively stable which is one of the problems with these molecules. 8-Alkyl and 8-deaza analogues have been successfully synthesized.

Chapter 3 describes biological results of our compounds which were screened for nitric oxide synthase assays in collaboration with Dr. Simon Daff at the University of Edinburgh. In addition to NOS activity, a number of molecues were also submitted for antibacterial and microbial activity in SIPBS and some of them were found to be active.

Chapter 4 describes the experiments for the synthesis of our desired compounds. The methods for the preparation and their characterisation have been given in detail.

The last Chapter lists the references.

1.0 Introduction

1.1 Nitric Oxide and Nitric Oxide Synthase

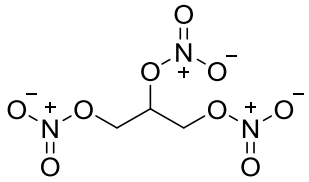
1.1.1 What is nitric oxide?

Nitric oxide is a free radical, and also a colourless gas that controls many biological processes. It is slightly soluble in water; moreover, it is a biological messenger molecule. Nitric oxide is a potent vasodilator with a short half-life of less than 5 seconds in biological tissues. Its short life restricts the availability of newly formed nitric oxide to neighbouring cells.[1] In many cases, nitric oxides biological effects are caused by activating an enzyme called guanylyl cyclase and enhancing cyclic guanosine monophosphate (cGMP) production from GTP. The cGMP is a cyclic nucleotide which causes relaxation of smooth muscles and hence vasodilation and more blood flow. There are a number of effects of nitric oxide, which do not depend upon cyclic GMP. For instance, nitric oxide can react with iron, thiol groups, other free radicals, superoxide anion, oxygen and unsaturated fatty acids. Nitric oxide can act as a neurotransmitter, an intracellular messenger, or as a signaling molecule.[2]

Nitric oxide is an endogenous gas that has important biological effects.[3] The chemical characteristics of nitric oxide (NO) have been investigated for more than two centuries. The initial study of nitric oxide gas started in 1772 when Joseph Priestley named this gas “nitrous air” as a colourless and toxic gas.[4]

1.1.2 Nitroglycerine and nitric oxide

The study of nitrate-containing compounds for medicinal use completed its 150 years in 1997. Nitroglycerine (glyceryl trinitrate) **1.3** was the initial compound of this group. On the other hand, it is only within the past two decades that the biological applications of this chemistry and enzymatic synthesis of nitric oxide have been studied.[5,6]



1.3

Nitroglycerine was used by Alfred Nobel for the production of dynamite. The effect of nitroglycerine for the symptoms of angina was discovered in Nobel's dynamite factories in the late 1860s. It was observed that the factory workers frequently experienced headaches on Monday morning that disappeared over the weekends. The second observation was that the factory workers suffering from chest pain or congestive cardiac failure felt relief from chest pain symptoms during working days but pain returned by the weekend. The nitroglycerine was working as a vasodilator in the factory workers to which physiologists and doctors quickly became aware in local communities. In the early 1980s, scientists found that the vasodilator action of nitroglycerine was due to nitric oxide.[7]

Alfred Nobel's physicians prescribed nitroglycerine for his heart complaint in 1890 when nitroglycerine was just about to be established as a cure for the treatment of angina.[8] Nobel refused to take it because from his work he was aware that it caused headaches. Alfred Nobel wrote to his friend Ragnar Sohlman in October 1896, just seven weeks before his death: [9] "Isn't it the irony of fate that I have been prescribed nitroglycerine, to be taken internally! They call it Trinitrin, so as not to scare the chemist and the public." It is possible that he had in his mind the observations of Sobrera, who was the first person to taste nitroglycerine in 1847 and was working for the company making commercially useful explosives established by Alfred Nobel. Sobrera wrote that great precaution should be used as he put upon his tongue a tiny quantity, which produced a violent headache for several hours.[9]

1.1.3 The discovery of nitric oxide role and Nobel Prize

Nitric oxide was found to be an intermediate of denitrification in the *Pseudomonas perfectomarinus,* a marine bacterium in 1967.[10] Nitric oxide was considered to be a toxic gas and air pollutant until 1987 when it became clear to scientists that nitric oxide plays an important role in regulation of blood pressure and heart diseases. It was found that macrophages use nitric oxide to kill bacteria and tumour cells.[11,12] In 1992, nitric oxide was

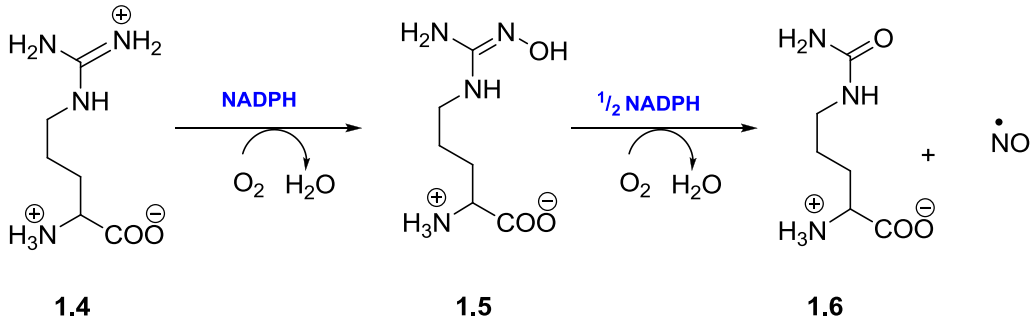
picked up as "Molecule of the Year" by Science magazine. The significance of the molecule was the first page news in 1998 when Louis J. Ignerro, Ferid Murad and Robert F. Furchgott were awarded the Nobel Prize in Physiology and Medicine for proving that nitric oxide is a signaling molecule in the vascular system.[13,14]

The Nobel Prize acknowledged three important experiments: (i) the finding that NO increased guanylate cyclase activity to cause smooth muscle cell relaxation,[15] (ii) the study that the endothelium cells released a diffusible factor that caused acetylcholine-induced vasodilation,[16] and (iii) the identification of endothelium-derived relaxing factor (EDRF) which is responsible for smooth muscle relaxation and is produced and released from blood vessels as a nitric oxide.[17] Nitric oxide (NO) protects body cells from harmful chemicals and stimuli.

The research of nitric oxide NO research has developed very fast in the late 20 years. The role of nitric oxide in physiology has been widely studied. The nitric oxide research has produced more than 80,000 publications in the field of NO signalling.[6]

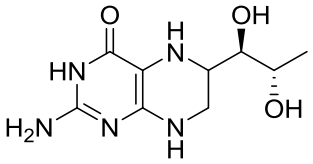
1.1.4 Formation of nitric oxide and its reactions in the living organisms

The oxidation of L-arginine **1.4** into L-citrulline **1.6** in the presence of nitric oxide synthase enzymes produces nitric oxide. Nitric-oxide synthases hydroxylate a guanidino nitrogen of L-arginine **1.4** to form *N*-hydroxy-L-arginine intermediate (NOHA) **1.5** and then oxidizes NOHA into to L-citrulline **1.6** and NO. These reactions utilize NADPH as a source of electrons while molecular oxygen acts as a co-substrate. Both reactions utilize one equivalent of O_2 while two equivalents of the NADPH are used in the first reaction, and one equivalent is used in the second reaction. Nitric oxide synthases require tetrahydrobiopterin (BH_4) as a redox cofactor, and their electron transfer reactions are controlled by calmodulin, which is a Ca^{2+} binding protein. In the absence of L-arginine or BH_4, neural nitric oxide synthase has been found to produce H_2O_2.[18, 19]



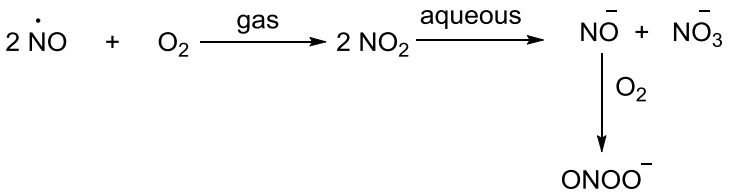
1.4 **1.5** **1.6**

Scheme 1.1: *Oxidation of L-arginine for NO synthesis*

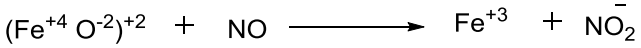


1.1 (BH_4)

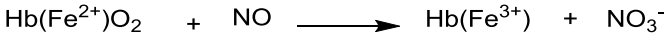
Nitric oxide reacts with superoxide to produce a strong oxidizing agent called peroxynitrite. Peroxynitrite is an oxidizing agent and is harmful for cells and tissues in living organisms and causes tissue and cellular injury.[13, 20, 21] Antioxidants preserve the level of nitric oxide in the body by preventing its oxidation.[22]



The main actions of nitric oxide can be split into its direct and an indirect effect. In direct effect, it plays a role in either protecting or damaging the nearby cells. In an indirect effect, the derivatives of nitric oxide formed, play an important role in biological reactions. The reactions of nitric oxide with metal ions or oxo complexes reduce them to a lower oxidation state.[13,23]



Nitric oxide reacts with oxyhaemoglobin and forms met-Hb and nitrate ion. The rate constant of this reaction is 3 x 10^7 M^{-1} s^{-1}. [13, 24]



1.2 Types of NOS enzymes

In mammals, three different isoforms of nitric oxide synthase have been characterized.[25,26] The neuronal NOS (NOS1 or nNOS) produces small amounts of nitric oxide for a short period of time. Its activity is dependent upon Ca^{2+} and calmodulin, and it is constitutively expressed, which means that the gene which produce nNOS is continuously transcribed. The nNOS (neuronal isoform) is found in neurons, epithelial cells, skeletal muscle, and is involved in neurotransmission, behavior and gastrointestinal motility. NOS II (NOS2 or iNOS) was originally found in cytokine-induced macrophages. It is found in large amounts, so it is called high-output NOS whose activity is largely Ca^{2+}–independent. This is not typically expressed unless the cells have been induced by particular stimuli such as microbes, or microbial products, hypoxia or cytokines, tumor or activated murine macrophages.[27] It takes part in immune response. It could be important to eliminate pathogens.[28] This enzyme has antiviral, antiparasitic and microbiocidal effects.[29,26]

NOS III (NOS3, eNOS) was originally found in endothelial cells. It is constitutively expressed and whose activity is regulated by Ca^{2+} and calmodulin. The nitric oxide produced by eNOS controls blood pressure, preventing platelet aggregation and relaxing the blood vessels.[27] It is also a low-output NOS.[26,30] The catalytic mechanism of NOSs is analogous to cytochrome P450 enzymes. Despite the similar structures of the three NOS isoforms, the nitric oxide produced by them has different biological activities.[31] The three types of NOS enzymes are products of different genes. The human gene for nNOS (neuronal isoform), iNOS (inducible isoform) and eNOS (endothelial isoform) are located on chromosome number 12, 17 and 7 respectively.[27, 30]

Inducible nitric oxide synthase (iNOS) can produce high amounts of nitric oxide that could be toxic and can cause cerebral ischemia. iNOS expression is one of the critical factors that affect the development of the brain damage that occurs after ischemia. The inhibition of iNOS could be a novel therapeutic approach that can be used particularly at the secondary development of ischemic brain injury.[32] iNOS is responsible for the production of nitric oxide at many places in the gastrointestinal tract, which is significantly increased in the colon of patients with collagenous and ulcerative colitis. iNOS expression is enhanced in active ulcerative colitis which might result in the production of enhanced reactive nitrogen species. The diarrhoea observed in patients with collagenous colitis could be due to increased formation of nitric oxide. [33, 34]

Endothelial and inducible NOS have been found to be present in adipose tissue which suggests that adipose tissue could be a possible source of NO production. The locally produced NO in adipose tissue could perform a part in the control of lipolysis and adipogenesis in addition to activation of glucose uptake by insulin.[35]

1.2.1 Nitric oxide synthase cofactors

All isoforms of nitric oxide synthase require L-arginine as a substrate and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) and molecular oxygen as co-substrates. Flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4) are cofactors of NOS. All isoforms of nitric oxide synthase require calmodulin and heme.[36]

1.2.3 Electrons shift between NOS domains and role of BH_4

NOS isoforms are homodimers (**Figure 1.1**). Efficient NOS transmit electrons from NADPH, through the flavins FAD plus FMN in the reductase domain, towards the heme in the oxygenase domain. The oxygenase domain binds to the substrate L-arginine, molecular oxygen and essential cofactor BH_4. The electrons are used at the heme site, to oxidize L-arginine to L-citrulline and NO and to reduce and activate O_2.[36] Calmodulin plays an important role in activating nitric oxide synthase enzyme because when it binds the enzyme,

it assists in the movement of electrons from NADPH which binds to the reductase domain, and to the heme in the oxygenase domain. [36, 37]

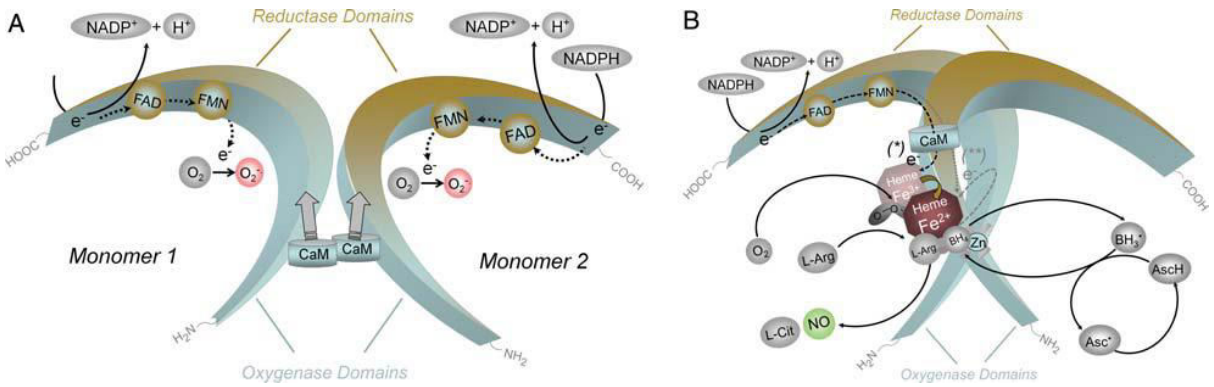


Figure: 1.1 *Structure and catalytic mechanisms of NOS. (**A**) NOS monomers shift electrons from reduced NADPH to FAD and FMN. (**B**) If heme is present, NOS can form a functional dimer.*[36]

Even though the precise mechanism for CaM action is not completely understood, some data is available. When CaM binds to nNOS, it changes the structural arrangement of the reductase domain, enhances the rate of electron transmissions into the flavins, and enhances the rate at which the reductase domain can transmit electrons to electron acceptors such as cytochrome c. When CaM binds to eNOS, the above mentioned fluctuations also take place but to a much lesser degree. [38]

An electron derived from NADPH is provided by NOS flavoprotein domain to the ferric heme (**Figure 1.2**) which is a very slow step of this biosynthetic reaction and forms a ferric heme-superoxy species (I) in the *N*-hydroxy-L-arginine intermediate (NOHA) or Arg reactions.[39, 40] Species I may accept an electron[41] from BH_4 or from the flavoprotein domain in the absence of BH_4.[42] The donation of an electron by BH_4 is another very slow step in this biosynthetic reaction. Well-timed electron transmission from BH_4 stops superoxide formation (**Figure 1.2**). BH_4 could as well provide an electron in the NOHA reaction. [39, 43]

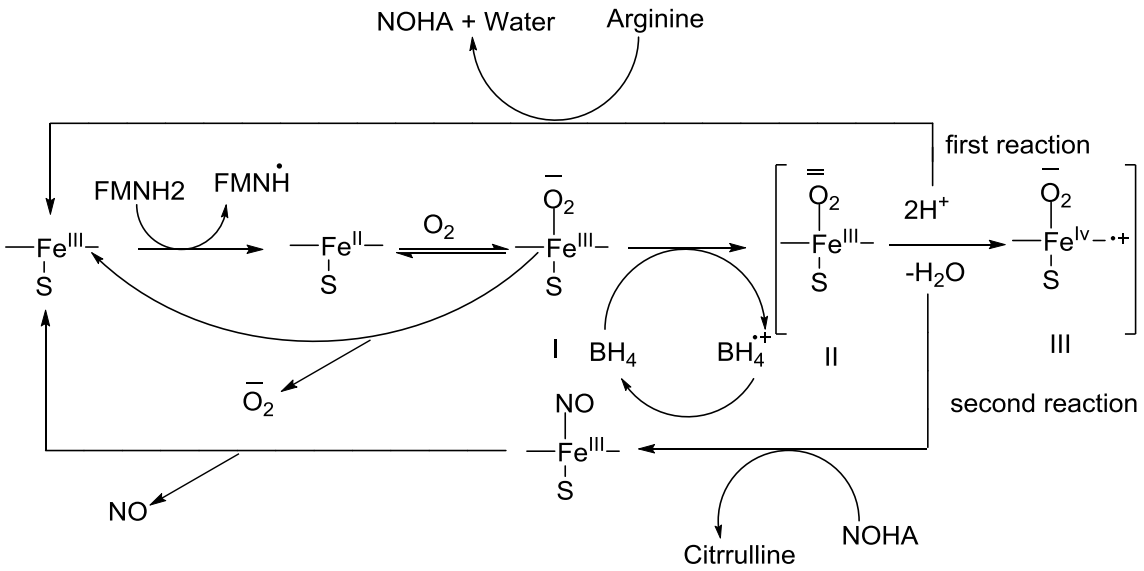


Figure: 1.2 *NO biosynthesis model*[44]

The electron-transfer pathway and structure of NOS are illustrated in **Figure 1.3.** It is noteworthy that electrons are transferred from Flavin mononucleotide (FMN) in one subunit to the heme of the other subunit.[45]

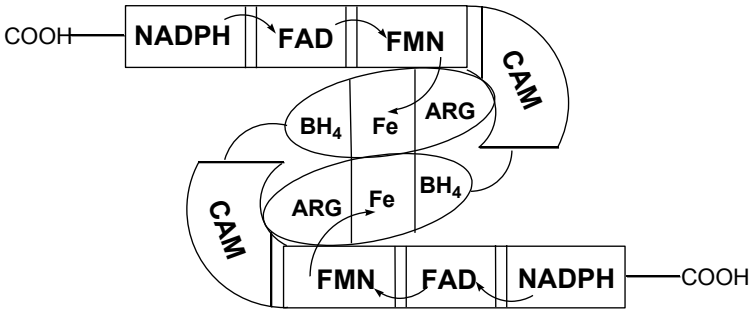


Figure: ***1.3*** *Electron-transfer pathway in NOS dimer.*[45]

Calmodulin is a calcium-binding messenger protein which binds between the reductase and oxygenase domains and promotes electron transfer between flavin and heme groups.[46] The arginine is nearby heme, although there is no direct interaction.[47] The procedure for the flow of electrons in the reductase domain is similar to cytochrome P450 reductase. The role of

calmodulin is to correctly line up nearby oxygenase and reductase domains in dimeric nNOS for electron transmission between these two domains, which results in the formation of nitric oxide by the heme.[46] (**Figure 1.3**).

Below is another diagram for the flow of electrons between the reductase domain and the oxygenase domain of NOS enzyme as explained by Alderton *et al.*[48] NADPH provides electrons to the reductase domain of the NOS enzyme where they pass through FAD, FMN and towards the oxygenase domain. After that the electrons are passed towards the heme iron and BH_4 in the reductase domain at the active site where they initiate the reaction of L-arginine with oxygen and produce the citrulline and nitric oxide.[48] The flow of electrons is controlled by the presence of Ca^2+/CaM as presented in **Figure 1.4**. The stuctures of NADPH, FAD and FMN will be discussed in section **1.4.0** along with structure of nitric oxide synthase enzyme.

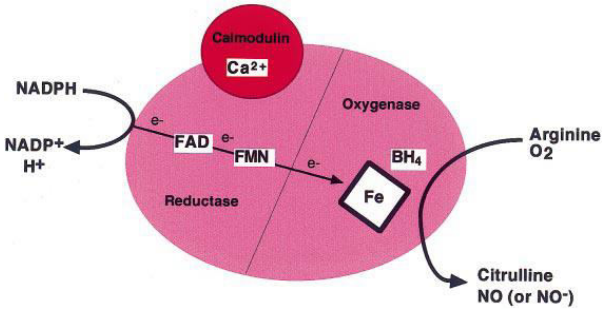


Figure: 1.4 *Overall reaction catalysed and cofactors of NOS.*[48]

In the O_2 activation step, there is a fundamental distinction between nitric oxide synthase and cytochrome P450 and the difference is that the nitric oxide synthase needs a cofactor which is the tetrahydrobiopterin (BH_4).[49] The BH_4 role is to give an electron to the oxy-complex to make a temporary pterin radical.[50] Sorlie *et al.*[51] suggested the role for BH_4 working as an electron and proton contributor. In P450, the electron is carried from the FMN of P450 reductase and FMN is also very close to the heme as shown in **Figure 1.5** whereas the proton is transferred from a nearby active site group, mostly water. The important variations in

mechanism between NOS and P450 are due to proximity of BH_4 to the heme in NOS enzyme.

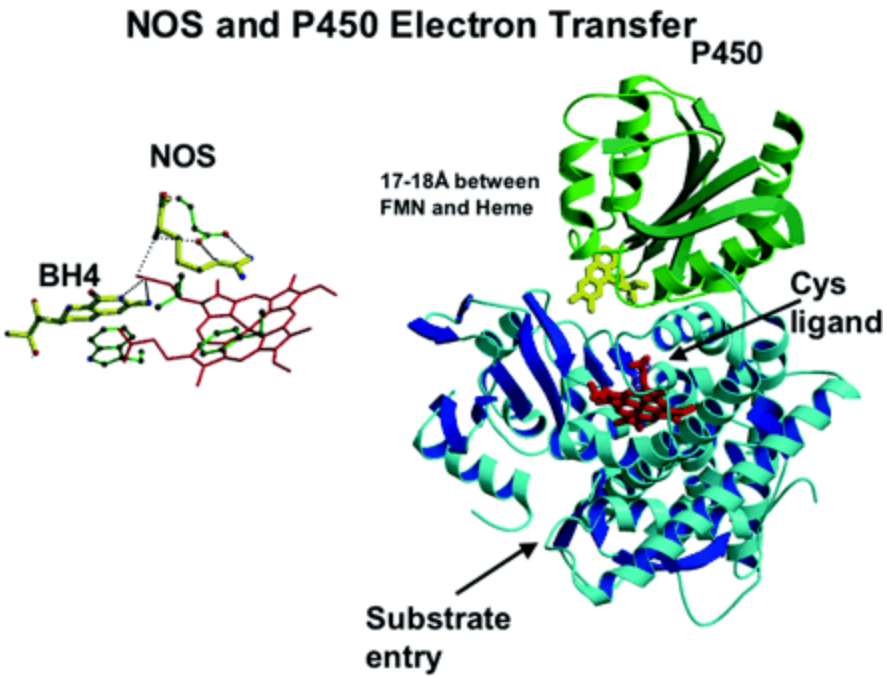


Figure: 1.5 This Figure shows the structure of the electron transfer complex produced by the FMN plus heme regions of P450 and NOS active site.[52] The distance between the heme and FMN is 17 to 18 Å while there is a direct H-bonded coupling between the BH_4 cofactor and the heme in NOS.[53]

1.3.0 Importance of nitric oxide in our body

Nitric oxide plays many roles in our body. One role that nitric oxide plays in our body is angiogenesis or vascularization which is the growth of new blood vessels from the pre-existing ones. The development of new blood vessels is vital during tissue healing, female reproductive cycle, and foetal development. Insufficient angiogenesis can result in coronary artery disease.[54] When a blood vessel becomes narrowed or clogged, the best therapy should be a medicine that produces new blood vessels in the affected tissue, without any undesired effects in the healthy tissues. If there is a clotting in the blood vessels in the brain, an ischemic stroke can occur. Hundreds of millions of people suffer from strokes. Therefore, we

need a drug that can promote new blood vessels formation in the ischemic tissues. Some drugs can cause angiogenesis in healthy tissues as well. When nitrite was administrated in mice for a long time, it was reduced to nitric oxide (NO), and it restored blood supply in ischaemic tissue as shown in **Figure 1.6**. It did not cause angiogenesis in healthy tissues.[55]

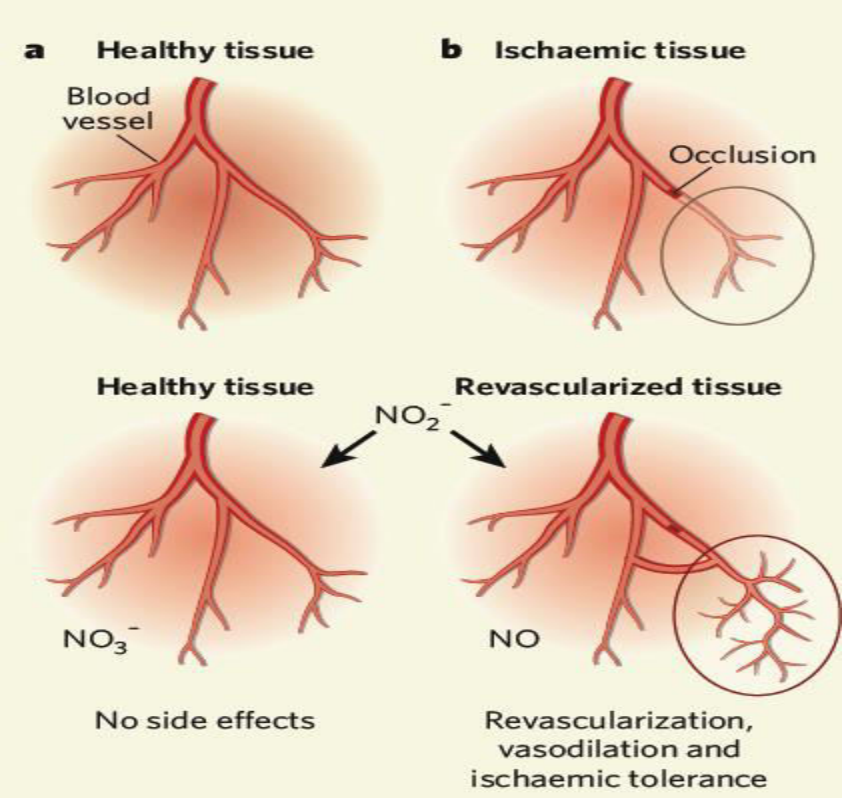


Figure: 1.6 *The effect of regular administration of nitrite in mice, (a) nitrite (NO_2^-) is changed to nitrate (NO_3^-) in tissues that contain normal blood vessels. (b), in ischaemic tissues, nitrite is changed into nitric oxide (NO), where it restores blood supply by formation of new blood vessels.*[55]

Nitric oxide is not only a powerful vasodilator; in addition, also it prevents platelet adhesion and aggregation, decreases adhesion of leukocytes to the vascular endothelium cells, and regulates proliferation of vascular smooth muscle cells. A reduced level of nitric oxide can cause a number of vascular disorders like atherosclerosis, hypertension, thrombosis and restenosis.[56, 57] During the period of physical exercise, cardiac performance is increased and

blood is supplied to the exercising muscles. The regular physical exercise has been linked to eNOS protein expression. If there is more physical activity, nitric oxide level also remains sufficient during rest. Furthermore, a single session of exercise causes a great increase in NO formation. The positive relationship between physical activity and NO formation could help to justify the useful effects of physical fitness on cardiovascular health. [58,59]

There is a significant body of data which proposes that nitric oxide's role is as a neurotransmitter in the nervous system of mammals.[60] Immunohistochemical studies revealed that NOS-containing neurones are found in the mammalian brain.[61] Nitric oxide controls neurogenesis in the growing and fully developed brain. Nitric oxide levels can control neuronal differentiation, cell reproduction and olfactory precursor cells.[62]

1.3.1 Dual nature of nitric oxide

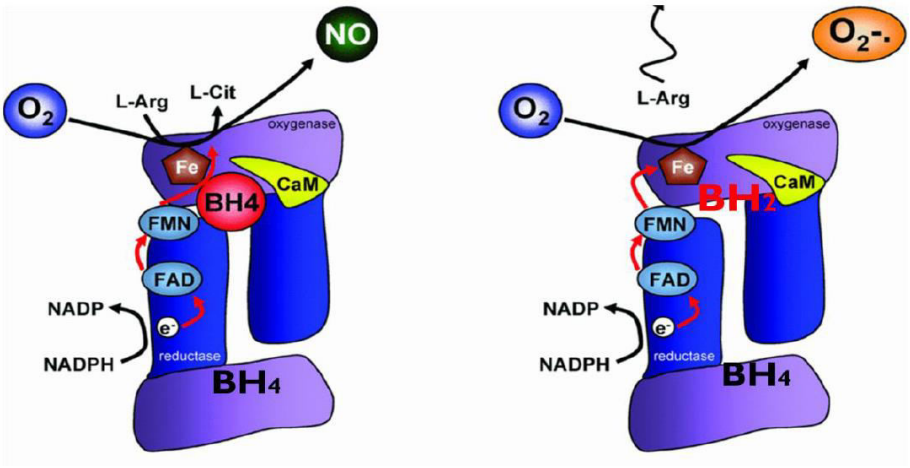
Nitric oxide has both harmful and beneficial impacts. Enhanced levels of circulating nitric oxide are correlated with vital vasodilation, hypotension, reduced tolerance to drugs which are used to raise blood pressure (vasopressor), changed circulation of blood flow, enhanced capillary leakage, and various organ dysfunctions.[63] In contrast, nitric oxide is associated with useful impacts involving increased macrophage action, enhanced oxygen distribution to ischemic muscle, increased free radical hunter capacities as well as diminished platelet cells adhesion.[64,65,66]

1.3.2 Nitric oxide role in regulatory functions and importance of BH_4

The nitric oxide (NO) is involved in a wide variety of critical regulatory functions *in vivo*, including regulation of vascular tone and host immune defense response. The metabolites of nitric oxide, such as nitrite or NO-derived peptides or proteins, could also play essential physiological roles. Too much endogenous and exogenous free radical formation and nitric oxide are involved in the development of cancer.[67] Tobacco smoke, which is rich in reactive oxygen species (ROS), is a well-known source of exogenous oxidants, is a significant risk factor of developing lung cancer.[68] The high levels of NOS expression (for instance triggered

by macrophages) could be cytostatic, or cytotoxic against tumor cells and microbes while low level activity might have the opposite effect and can support tumor growth.[69]

When endothelial-derived nitric oxide supply is decreased, endothelial dysfunction occurs, which triggers inflammation and increased leukocyte-endothelial interactions. Under normal physiological conditions, eNOS produces nitric oxide in the presence of BH_4 by catalyzing the reduction of molecular oxygen to L-arginine **1.4** oxidation including formation of L-citrulline. While uncoupled eNOS is a phenomenon (**Figure 1.7***)* where the electron transfer from the eNOS molecule becomes uncoupled from L-arginine oxidation and produces superoxide by the reduction of molecular oxygen, this happens when the BH_4 to BH_2 ratio is decreased. As a result the dihydrobiopterin (BH_2) to BH_4 ratio is raised.[70, 71] Oxidation of BH_4, in the context of diabetes including different continuing vasoinflammatory diseases, can produce cofactor deficiency as well as uncoupling of endothelial NOS (eNOS), and a switch from nitric oxide (NO) to superoxide formation. The cause of nitic oxide deficiency in diabetes is decreased BH_4:BH_2 ratio and not BH_4 depletion.[72]

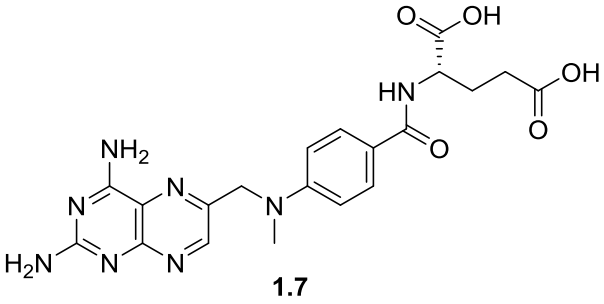


eNOS Coupling **eNOS Uncoupling**

Figure: 1.7 *Diagram of the coupled eNOS and uncoupled eNOS.*[71]

Endothelial BH_4 bioavailability is an important factor in controlling the balance between NO and superoxide production. The BH_4 level is changed by oxidation, producing 7,8-

dihydrobiopterin (BH_2), which causes eNOS uncoupling. Dihydrofolate reductase (DHFR) might control eNOS coupling *in vivo* as shown in **Figure 1.8**. Methotrexate (MTX) **1.7** can inhibit BH_4 recycling by DHFR; it was given to the wild-type, BH_4-deficient, and GTPCH-overexpressing mice. Administration of **1.7** induced a reduced BH_4/BH_2 proportion in the aortas of wild-type mice. These effects were greater in BH_4-deficient mice but lower in guanosine triphosphate (GTP) cyclohydrolase (GTPCH)-overexpressing mice. DHFR thus plays a key role in controlling the BH_4:BH_2 ratio. BH_4 administration protects against the adverse effects of DHFR inhibition **1.7**.[73]



1.7

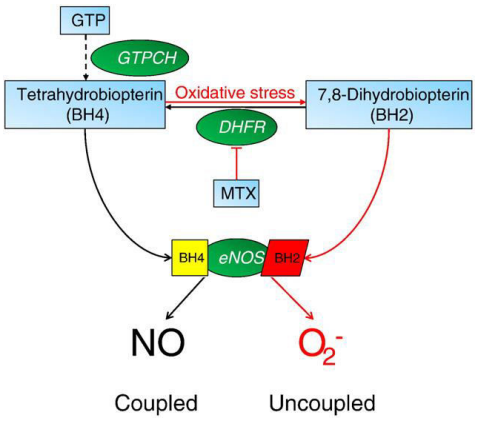


Figure: 1.8 *The diagram is a representation of the eNOS coupling and BH_4 recycling pathway. BH_4 is produced from GTP via a sequence of reactions requiring 6-pyruvoyltetrahydropterin synthase, GTP cyclohydrolase (GTPCH), and sepiapterin reductase. DHFR can restore BH_4 from BH_2, both BH_4 and BH_2 have equal affinity for binding with eNOS enzyme. BH_4-bound eNOS generates NO, while BH_2-bound eNOS causes uncoupling and eNOS-derived superoxide rather than NO.*[73]

The decreased bioavailability of NO could be because of reduced synthesis of NO and enhanced scavenging by reactive oxygen species and is related to endothelial dysfunction. When BH_4 bioavailability is low, eNOS does not produce nitric oxide but instead produces superoxide. In vascular disease conditions, reactive oxygen species cause degradation of BH_4. The regulation of BH_4 concentrations in vascular disease, by BH_4 supplementation, by reducing its oxidation or, by an increase in BH_4 biosynthesis, has been found to increase NO bioavailability.[72]

There is a noticeable direct relationship between cellular BH_4 stoichiometry and eNOS protein, When eNOS:BH_4 molar ratio >1, eNOS uncoupling occurs. In addition, in the presence of fixed eNOS:BH_4 ratio, increasing the intracellular BH_2 level was enough to cause eNOS-dependent superoxide production.[74]

Tetrahydrobiopterin is a member of a class of compounds that involves pterins, alloxazines, lumazines, riboflavin and folates. Two fused six-membered heterocyclic rings are a basic structure in these molecules. The nitrogen atoms are present at positions 1, 3, 5, and 8 (**Figure 1.9**).

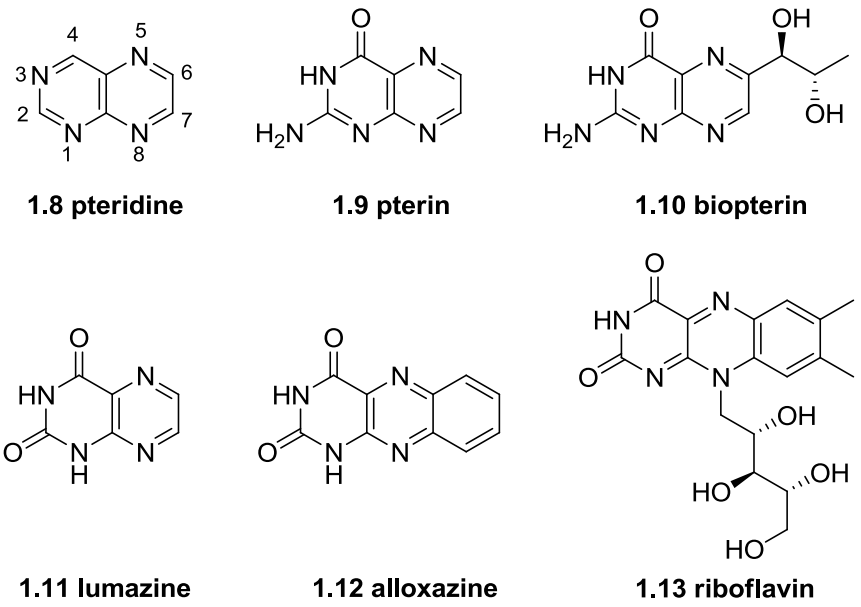


Figure: 1.9 *A few chemical structures of pteridines together with alloxazines.*[75]

When BH_4 bioavailability is sufficient within the active site of enzyme, the catalytic activity of eNOS would be reasonable; also there would be an adequate amount of nitric oxide in healthy vascular endothelium. In such cases, interaction of NO with superoxide to form peroxynitrite is considerably reduced (**Figure 1.10 A**).[76]

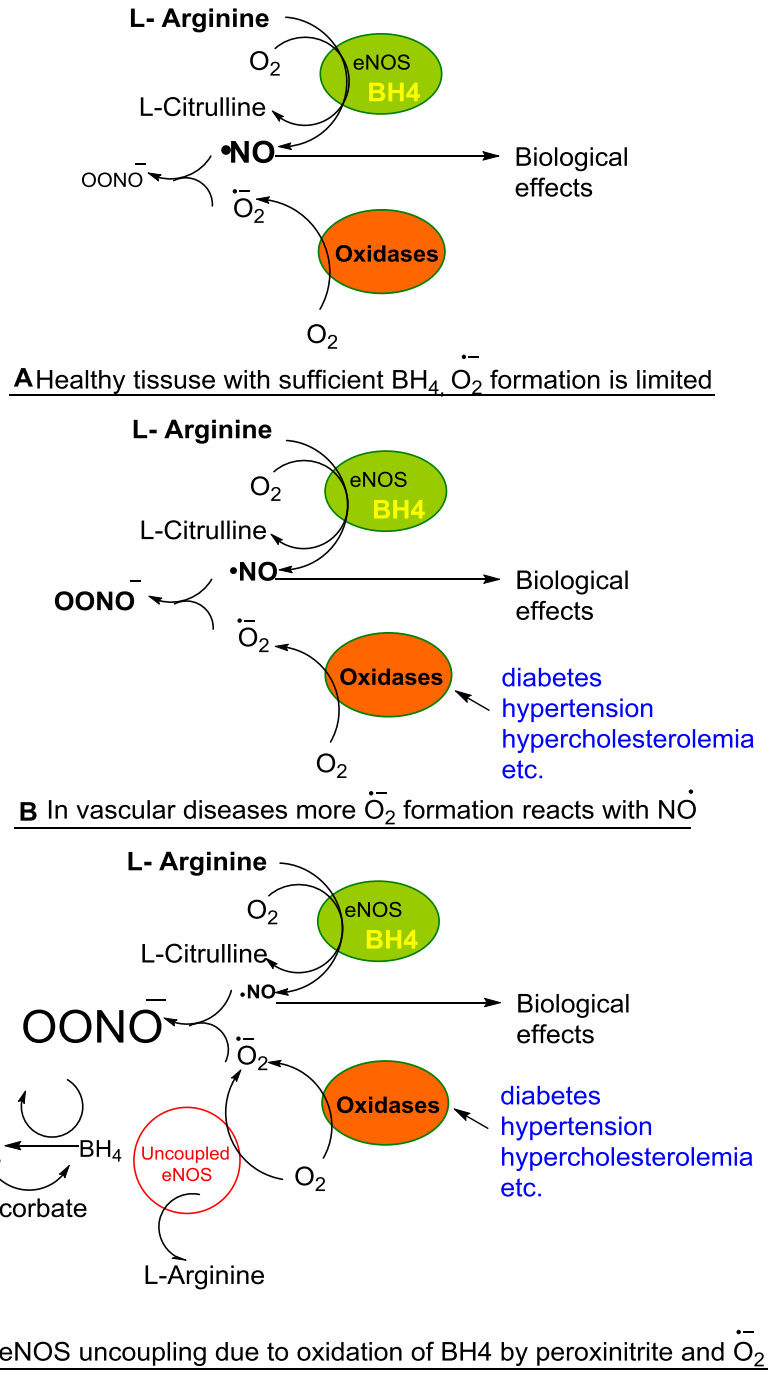


Figure: 1.10 *The function of BH_4 in eNOS activity in healthy vascular endothelium (A) and vascular disease conditions (B and C).*[76]

In vascular disorders, such as diabetes, hypercholesterolemia and hypertension, superoxide produced by oxidases such as NADPH oxidases will not change normal nitric oxide formation in the beginning. However, the produced nitric oxide will react with superoxide and form increased peroxynitrite which would cause loss of NO bioavailability for normal biological functions (**Figure 1.10** B).[76]

The reactive oxygen species including peroxynitrite rapidly oxidize BH_4 to the BH_3 radical. From extended oxidation of BH_3 radical, BH_2 and biopterin are formed which are unable to act as a cofactor and decrease BH_4 availability by competitive binding to eNOS at the active site causing eNOS uncoupling. The dissociation of the ferrous-dioxygen complex occurs and superoxide is formed from the oxygenase domain (**Figure 1.10 A** to **C**). eNOS produces only superoxide, but not nitric oxide which causes vascular oxidative stress and decreased NO bioavailabilty (**Figure 1.10** C).[76]

The amount of ROS affects the availability of nitric oxide in the body of living organism. The nitric oxide, cyclic guanosine-3',5'-monophosphate (cGMP) and soluble guanylate cyclase (sGC) pathway plays an important role in normalizing cardiovascular functions by forming new blood vessels, dilation of blood vessels, myocardial contraction and preventing platelet aggregation, and vascular smooth muscle growth (**Figure 1.11**). In patients with cardiovascular diseases, the NO, cGMP and sGC pathway is disrupted.[77,78] Reactive oxygen species (ROS) are suggested to cause the worsening of cardiac function in heart patients. Reactive oxygen species are enhanced in heart failure and are involved in myocardial ischemia, atherosclerosis, and heart failure.[78]

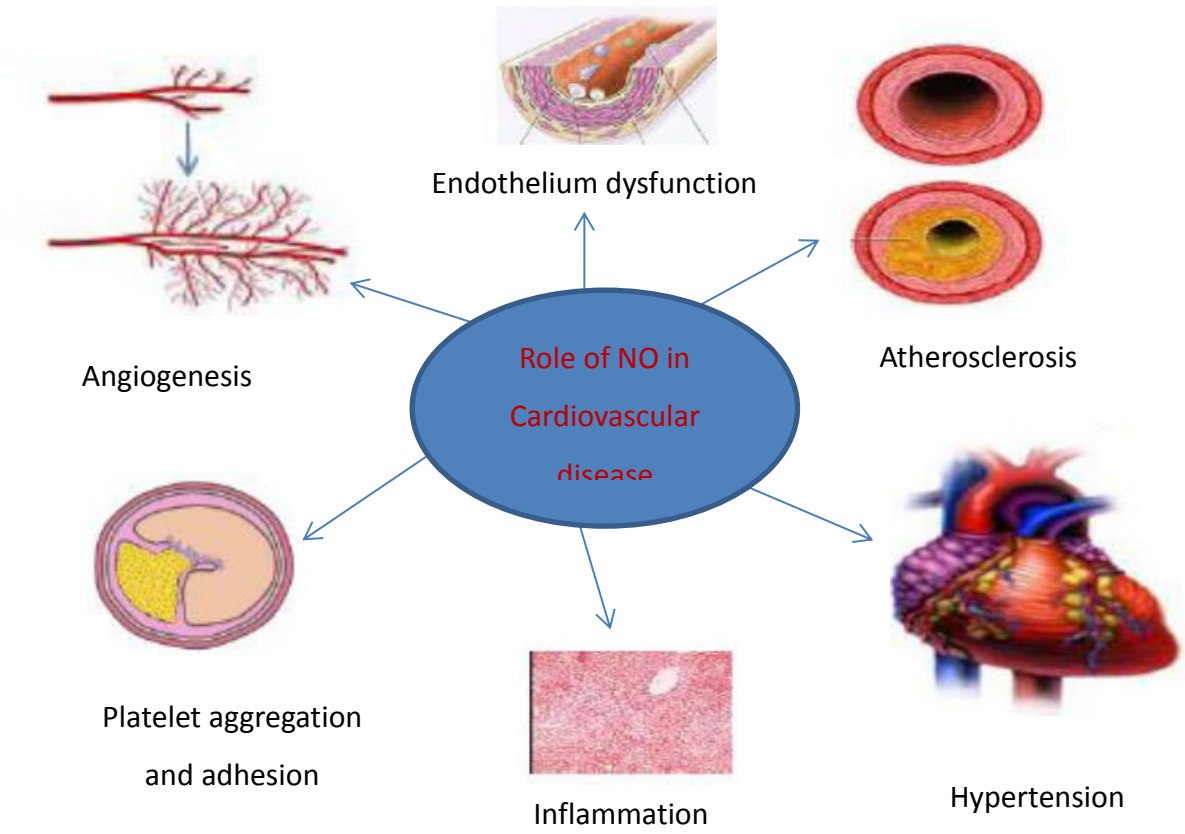


Figure: 1.11 *Nitric oxide role in various cardiovascular disorders.*[78]

Nitric oxide shows direct and indirect effects. The direct effects of NO include (i) stimulation of soluble guanylate cyclase to produce cGMP, (ii) binding to cytoglobin, myoglobin and hemeoglobin, subsequent NO breakdown, (iii) suppression of activity of cytochrome oxidase, and (iv) inhibition of catalase and breakdown of hydrogen peroxide. All of the above actions

are due to binding of NO to the heme of these proteins. [79] Further direct effects of NO are (v) reaction with oxygen to give NO_2 and N_2O_3, and (vi) reaction with superoxide to give peroxynitrite.[80] High levels of NO can cause a reduction in energy, due to inhibition of glycolysis and mitochondrial respiration. [81] It is interesting to know that a low level of nNOS has also found in the vascular smooth muscle cells, which have been found to cause some degree of vasodilation in case of eNOS uncoupling.[82]

1.4.0 Structure of nitric oxide synthase enzyme

All NOS isoforms are homodimers, featuring a C-terminal, flavin-containing reductase domain that binds FAD, FMN and NADPH and a N-terminal, oxidase domain that binds heme, L-arginine and BH_4. A calmodulin (CaM)-binding region is located at the junction between these two domains. Because of the reversible binding of calmodulin, the two isoforms (nNOS and eNOS) depend upon Ca^{2+} for their enzymatic activities.[83] iNOS has tightly bound CaM which is more or less calcium-independent.[84] The reductase domain shares sequence homology to NADPH-cytochrome P450.[85]

The three isoforms of NOS have been known as the products of distinct genes. These three isoforms share approximately 50–60% sequence homology and the same structural mechanisms, although they differ in their location within the cells, their functions and size. The molecular mass of nNOS is 165 kDa, it is found in neurons, in the brain at neuromuscular junctions. The molecular mass of eNOS is 133 kDa, it is found in vascular endothelial cells and is involved in vascular homeostasis. Nitric oxide produced by nNOS and eNOS is involved in signal transduction events that control neurotransmission and vascular tone.[86] The molecular mass of iNOS is 130 kDa; it is found in macrophages and is induced only in response to an inflammatory stimulus or bacterial endotoxin.[87] The stereo view of nNOS is shown in below in **Figure 1.12** along with the protein data bank (PDB) code.[89]

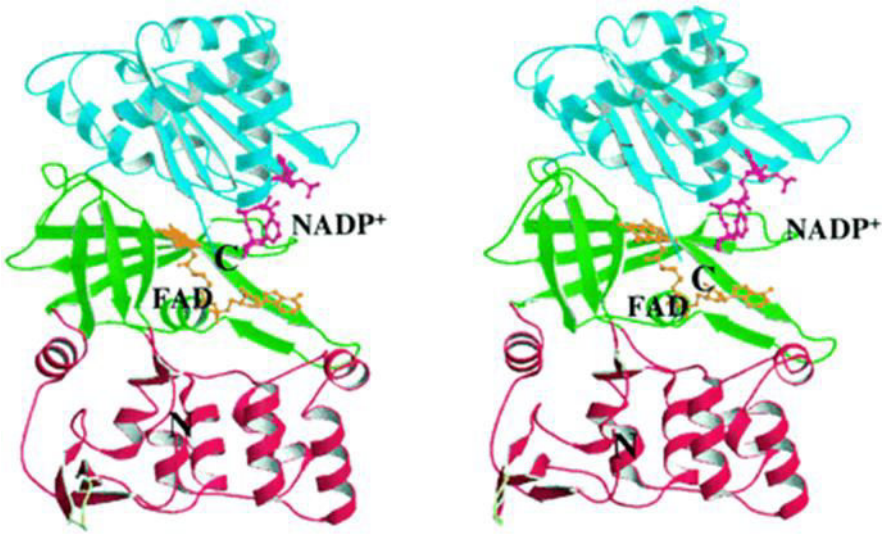
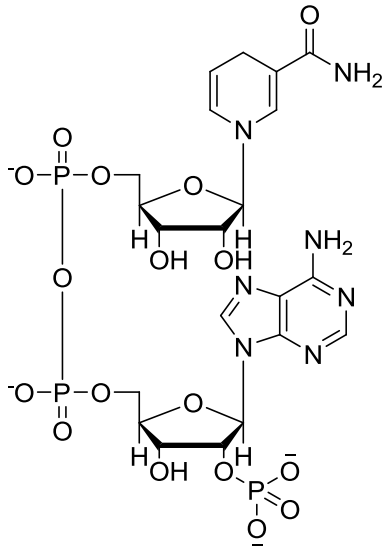


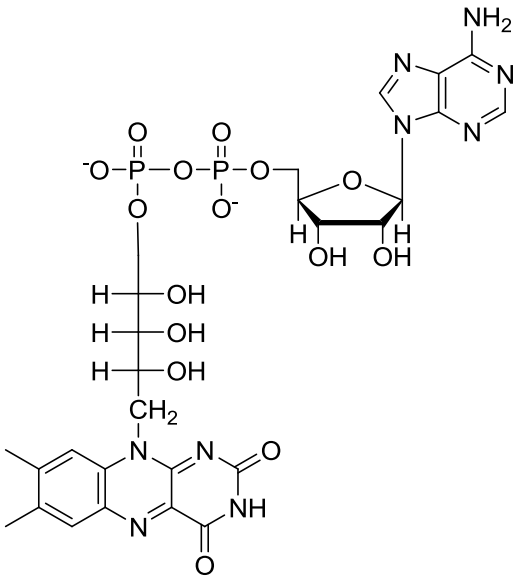
Figure: 1.12 *A stereo view of the nNOS-FAD/NADP structure, PDB code (1F20).*[88,89]

The N-terminus is at the bottom and C-terminus at the top, the connecting domain is *red* in colour, the FAD domain *(green),* and the NADPH domain (*blue)* are also shown. The $NADP^+$ and FAD has been indicated with the *ball* and stick model where $NADP^+$ is *red* in colour while FAD is *yellow* in colour.

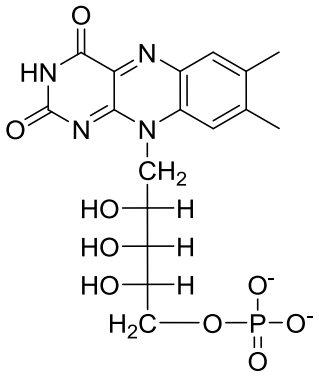
All isoforms of NOS use molecular oxygen[45] and L-arginine as substrates and require cofactors/coenzymes such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) **1.14**, flavin adenine dinucleotide (FAD) **1.15**, flavin mononucleotide (FMN) **1.16**, heme, calmodulin/Ca^{2+}, Zn^{2+} and 5,6,7,8-tetrahydrobiopterin (BH_4) **1.1**.[90]



1.14
NADPH



1.15
FAD



1.16
FMN

In a human, a single copy of each NOS isoforms exists in the haploid genome (**Figure 1.13**). The genomic structure of each NOS gene is the same, which suggests that there is a common ancestral NOS gene.[48]

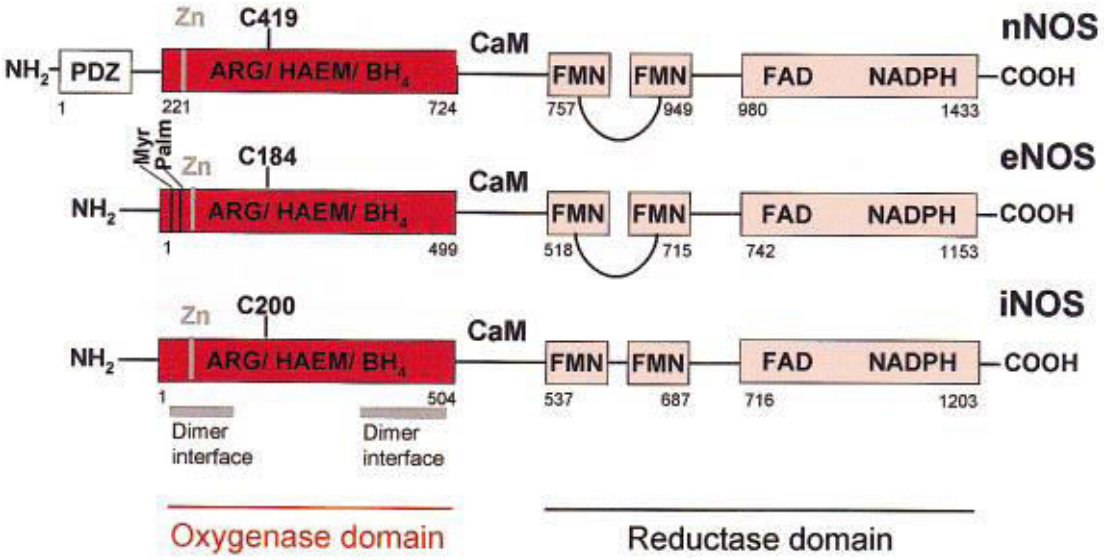


Figure: 1.13 *Oxygenase and reductase domains of NOS enzyme.*[48]

Nitric oxide synthases (NOSs) are proteins that contain metal atoms and are multidomain. The structure of nitric oxide synthase found in *bacillus subtilis* (a gram positive bacterium) and human inducible nitric oxide synthase (iNOS) is shown in **Figure 1.14**.[91]

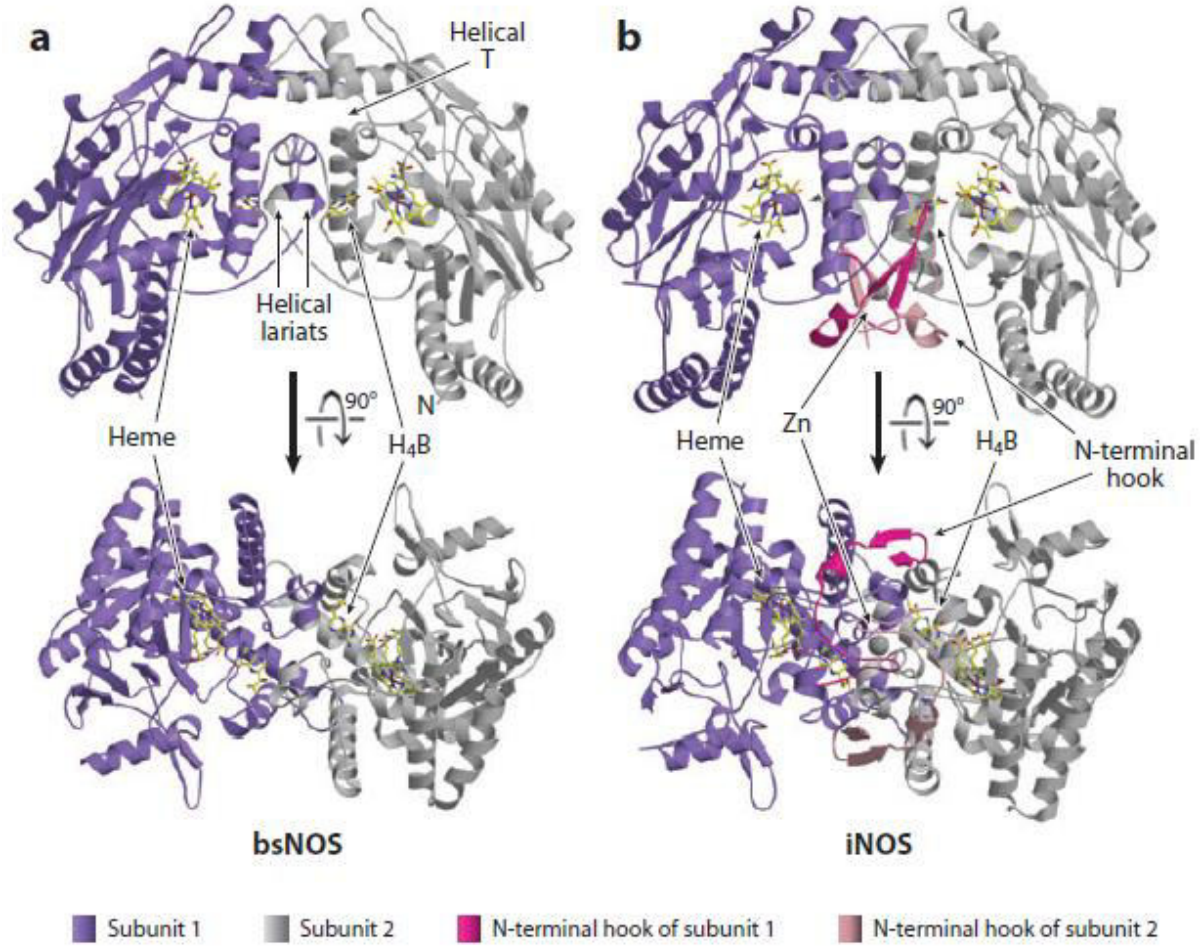


Figure: 1.14 *(**a**) Structures of the N-terminal heme oxygenase (NOSox) domains of B. subtilis nitric oxide synthase (bsNOS). (**b**) human inducible nitric oxide synthase (iNOS) with heme, Arg, and tetrahydrobiopterin (BH_4) bound, PDB code (2FLQ) .* [91]

The above structures are closely related with a difference of the absent *N*-terminal hook and zinc-binding area in bsNOS. The NOSox binds heme, Arg, and cofactor 6R-tetrahydrobiopterin (BH_4) and has the catalytic centre of the enzyme. NOSred has binding sites for NADPH, flavine mononucleotide (FMN) and flavine adenine dinucleotide (FAD) and works as a supply of reducing agent for oxygen activation at the heme center (**Figure 1.14**). A calmodulin connects NOSox to NOSred and controls the reduction of NOSox by NOSred in the presence of calcium. [91]

The figure below represents the role of calmodulin (CAM) in electrons transfer in neural nitric oxide synthase. The reductase domain and oxygenase domain are represented by two circles (**Figure 1.15**).[92]

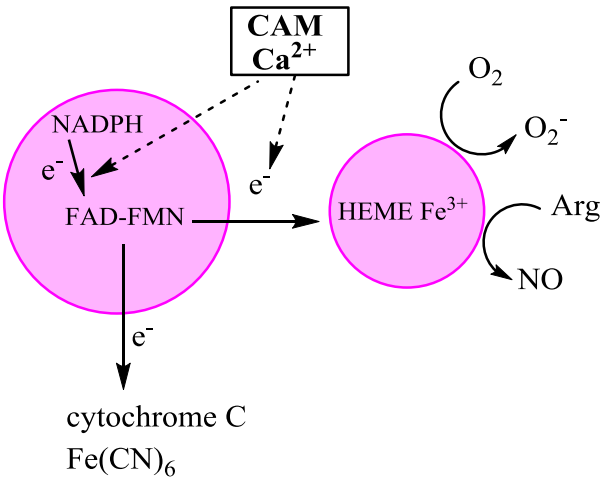


Figure: 1.15 *The hypothetical representation for calmodulin switch for electron transfer in neural NOS enzyme.*[92]

The dimerization of NOS enzyme is required to catalyse the reaction of L-arginine into citrulline and for NO catalytic activity. Other important factors for catalysis are FMN, FAD, BH_4 and heme. Only the homodimeric form of NOS can metabolize L-arginine and a single inter-subunit ZnS_4 cluster could be essential for dimerization and BH_4 binding site stability.[47,93]

1.4.1 X-ray structure of endothelial NOS and hydrogen bonding

Below is the X-ray structure of endothelial NOS, which shows the role of water at substrate binding sites and protein-cofactor. This structure gives information on protein–cofactor interactions and help to understand the high BH_4 binding affinity. Two molecules of BH_4 bind to each dimer in a similar fashion under crystallization conditions as shown in **Figure 1.16**.[94]

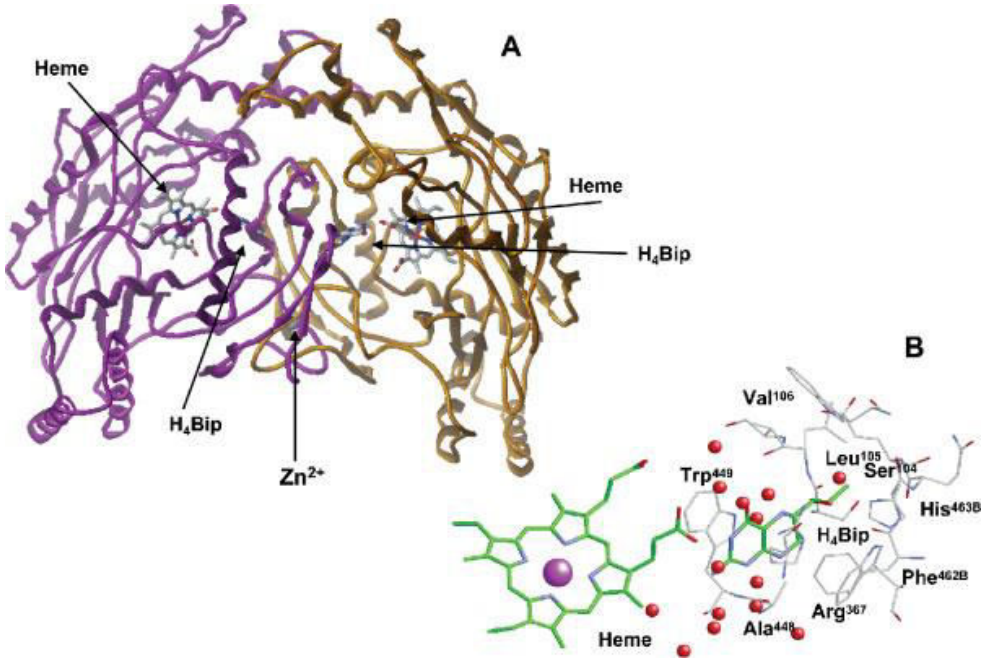


Figure: 1.16 *The quaternary structure, shown using a representative eNOS X-ray structure. Both monomer subunits, the position of BH_4 cofactor and heme are shown.* ***B****: BH_4 binding mode within the pterin binding site from eNOS is shown.*[94]

There is a wide range of hydrogen-bonding networks at the protein–cofactor interface with BH_4 as a cofactor in all NOS structures.[95, 96] In **Figure 1.17** below the dotted lines show hydrogen bonding interactions in an eNOS/BH_4 X-ray structure. Moreover there are also van-der Waals forces which help for holding the pterin and thus result in strong binding. The BH_4 binds perpendicular to the heme subunit with its 2-amino-4-hydroxypyrimidine moiety on the proximal side with a heme propionate side-chain.[94]

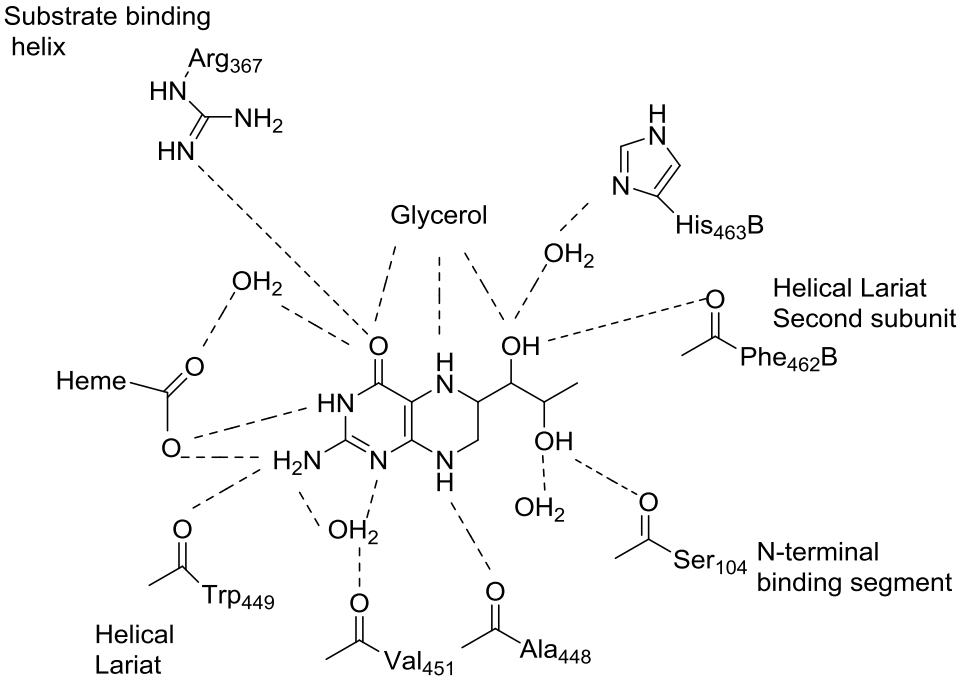


Figure: 1.17 *BH_4 and protein interactions within the pterin binding site of eNOS.* [94]

The heme propionate connections with the substrate arginine and pterin suggest that the pterin has a specific electronic effect on the heme-bound oxygen. The O^4 and N^5 heteroatoms in pterin are exposed to solvent and are bonded by hydrogen bonds to arginine. A number of water-mediated hydrogen bonds are formed between the binding site and pterin. [94]

1.4.2 Mechanism of NO synthesis

The mechanisms proposed for the formation of nitric oxide are similar to those for cytochromes P450s. In the oxygenase domain, electrons are shifted from NADPH by way of FAD and FMN to the heme. FAD takes two electrons from NADPH, and gives them singly to FMN semiquinone, which serves as a one-electron contributor to the heme.[97]

If there is molecular oxygen present, the decrease in oxidation state of ferric heme [Fe(III)] is accompanied by coupling of O_2 giving oxyferrous [Fe(II) O_2] or superoxy ferric complex [Fe(III) O_2^-] (**Figure 1.18(c)**). [98] The second electron is given by BH_4[99], which changes this intermediate into the superoxy ferrous [Fe(II) O_2^-] or peroxy ferric state [Fe(III) O_2^{2-}]

(**Figure 1.18**(**d**). The fast uptake of two protons and O-O bond cleavage forms a water molecule and an iron monooxide (FeO) (**Figure 1.18**(**e**)). This species is believed to extract hydrogen from the substrate arginine, the arginine binds at the active site to produce an intermediate (**Figure 1.18**(**f**)) which is not stable and is quickly transformed into ferric heme (**Figure 1.18**(**a**)) and Nω-hydroxy-L-arginine (**1.5**). [90,100]

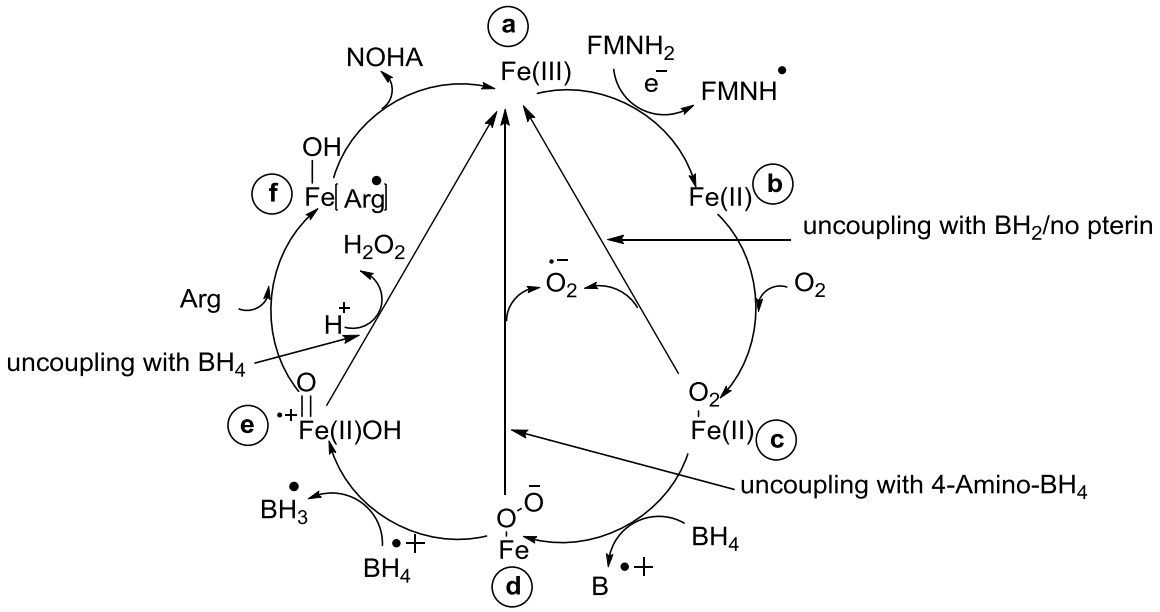


Figure: 1.18 *A series of oxygen processing steps at the NOS heme in connection to flow of electrons, oxidation of arginine, and formation of superoxide or* H_2O_2. [90,100]

In the next step, NOHA is oxidized to L-citrulline **1.6** and nitric oxide, using up 0.5 mol of NADPH in addition to 1 mol of O_2 (**Scheme 1.1**). The conversion of NOHA into nitric oxide and L-citrulline is considered to differ from the P450 sequence of reactions. Latterly it was published that this second step takes place in the absence of an enzyme and is thought to be accelerated by some reactive oxygen species (ROS) produced by the cytochrome P450-catalyzed conversion of O_2.[101] The NOS heme also binds self-produced NO as an essential characteristic of catalysis.[102,103]

1.4.3 Biological significance of tetrahydrobiopterin.[104]

Nitric oxide synthases are the only heme-containing enzymes that need the cofactor BH_4 for their activity. The role of BH_4 in NO biosynthesis is not fully understood.[104] About six roles

of BH_4 are known, however, some of them could be for a particular isoform.

1) BH_4 is involved in NADPH oxidation for NO biosynthesis.[105]
2) BH_4 protects NOS from inactivation.[106]
3) BH_4 causes a shift in the heme iron from low spin to high spin.[107]
4) BH_4 is involved in binding within the dimer interface and assists in stabilizing the quaternary structure of NOS by promoting and stabilizing dimerization.[108, 109,110]
5) BH_4 acts as a redox-active cofactor, analogous to aromatic amino acid hydroxylases.[111]
6) BH_4 stabilizes and increases L-arginine binding and thus functions as an allosteric modulator of substrate binding which means that it causes a shift in conformation in the protein structure.[112]

Some other important roles of BH_4 are described below:

i) As shown in **Figure 1.19** (steps 2 and 7), it is needed to serve as an electron donor during oxygen activation.[113, 39]
ii) It retakes an electron from the ferrous nitrosyl complex so as to cause NO liberation.[113]
iii) The activation of oxygen involves a two-electron reduction. It is only possible due to the presence of a high-potential one-electron donor and acceptor molecule. BH_4 gives an electron to activate dioxygen, and retakes it in the later cycle to produce nitric oxide **Figure 1.19** (Step 11).[113]
iv) The electron transfer step is slow as shown in **Figure 1.19** (Steps 2 and 3), in this process protonation of the peroxy-heme species occurs and oxyferryl complex is formed. [34]

In chemical mechanism of NO formation, steps 1 and 6 (**Figure 1.19**) shows formation of oxyferrous complex. One electron reduction of this oxyferrous complex occurs when BH_4 donates an electron (steps 2 and 7). The reduction of BH_4 radical results in the formation of nitric oxide from ferrous nitric oxide complex 11 (**Figure 1.19**).

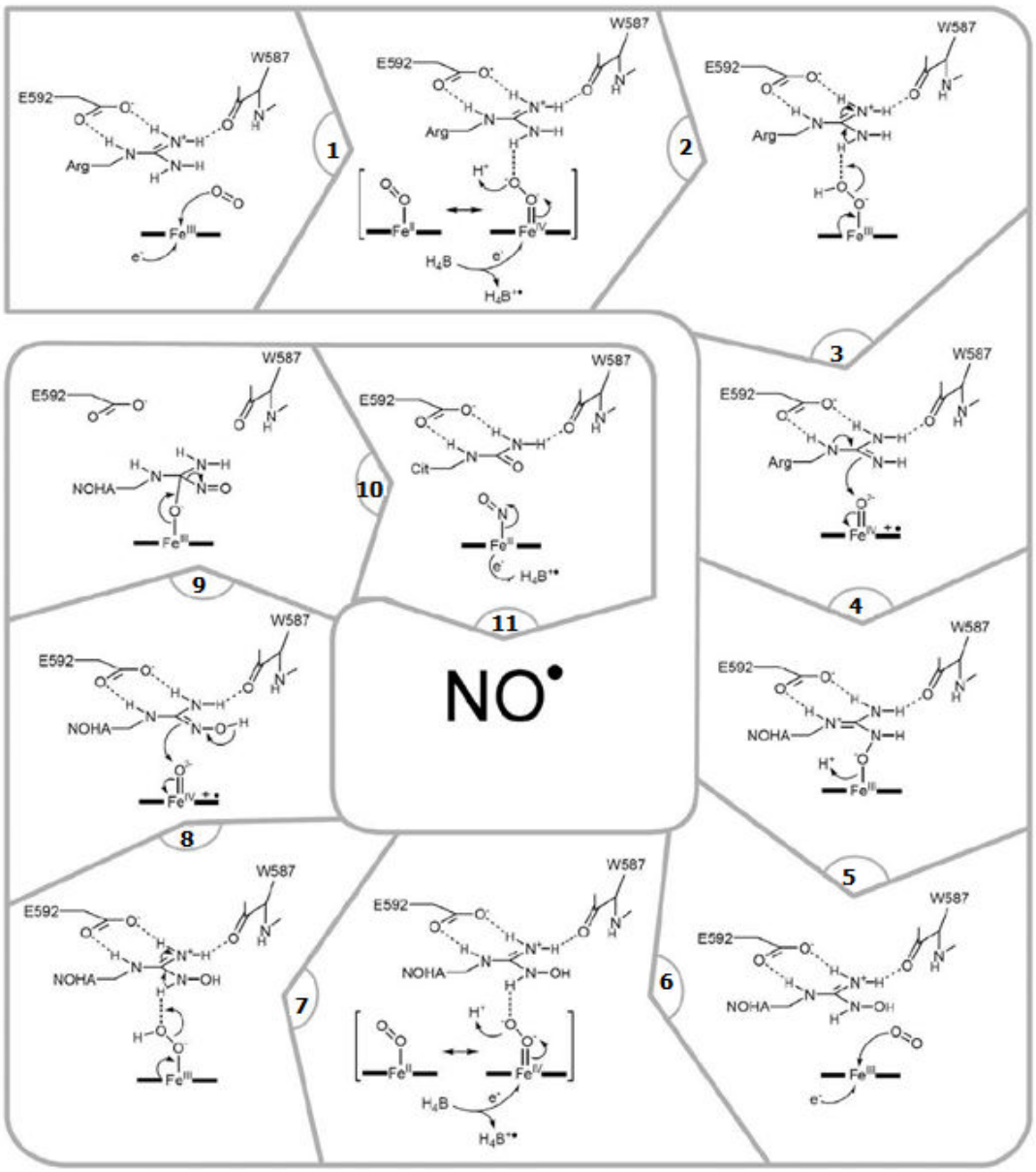


Figure: 1.19 *Chemical mechanism of NO synthesis postulated for both L-Arg and NOHA.**114***

The proposed function of BH_4 as a proton donor is based upon a hydrogen bonding which is found between the N^3 of BH_4 by a heme propionate to the substrate guanidinium group of arginine and this is questionable.[109, 115] The pterin can possibly transfer an electron and a proton at the same time to the oxygen bound to the heme. The pattern of pterin comparative to heme and substrate arginine shows an important hydrogen bonding interaction between N^3 of pterin together with heme propionate is shown in **Figure 1.20.**

The proton on N^4 of the pterin is solvent-friendly. The BH_4 will quickly get a positive charge due to electron transmission from BH_4 to heme. Then there will be a change in the equilibrium point of the H-bond in the direction of the heme propionate. However, for overall proton shift to happen the hydrogen-bond should be broken and the proton will be transferred across the active site, giving a BH_3 radical. Breaking of this hydrogen-bond would need energy. The proton shift model has been established due to the failure of 4-amino-BH_4 to give an electron to the heme. 4-Amino-BH_4 is able to donate an electron, but would not give up its proton as easily as BH_4.[51, 116,117]

Further it has been suggested that protonated form of 4-amino-BH_4 has an affinity for binding with the enzyme, [115] but its ability to transfer its electron will be decreased. [118] If it is true that the pterin site supports a positively charged cofactor, it will support electron shift to the heme, due to a decrease in reduction potential of the original pterin. The BH_4^+ would be more resistant to additional oxidation than the neutral radical.

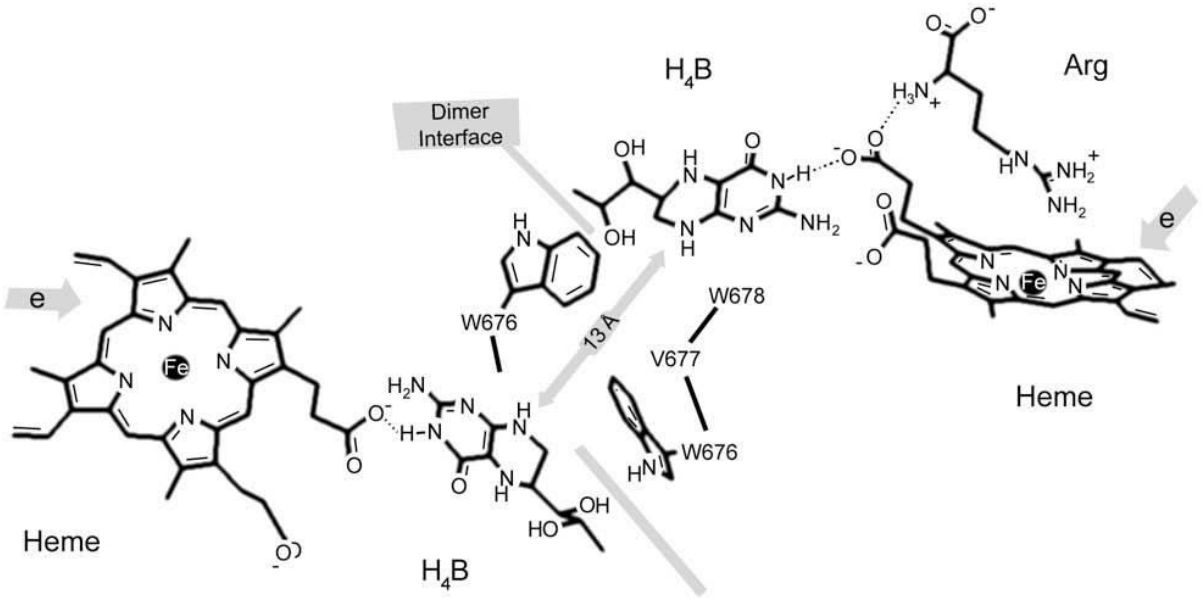


Figure: 1.20 *Fundamental arrangement of the BH_4 and heme cofactors throughout the surface of an nNOS oxy dimer (pdb 1ZVL).*[119]

An oxyferryl porphyrin radical cation as shown in **Figure 1.19** (intermediate structure 3) has been assumed to be the active species in the reaction with arginine which is similar to cytochrome P450s. In case of NO synthase, this intermediate should be formed within a hydrogen-bond of a BH_4 radical cation (**Figure 1.20**). The puzzle is whether this unusual intermediate can exist for sufficient time prior to breakdown by pterin due to heme electron shift. The reduction of the oxyferrous complexes of NO synthase in the presence of BH_4 may present a mechanism for approaching this puzzle. [120, 121]

1.4.4 Factors that Cause nitric oxide deficiency

Nitric oxide deficiency produces a significant increase in endothelial dysfunction. Preliminary studies have shown that decreased NO bioavailability could be due to following factors:

i) Due to co-factor or substrate insufficiency or the occurrence of increased nitric oxide degeneration by reactive oxygen containing compounds.[122, 123]

ii) The occurrence of decreased levels of nitric oxide generation.[123]

iii) The oxidative degeneration of BH_4 by reactive oxygen containing compounds.[76]

One possible cause for endothelial abnormality is inflammation.[124] Endothelial abnormality is found in many disorders linked with an enhanced cardiovascular risk, such as primary hypertension,[125] dyslipidaemia and emotional strain. The endothelium is a working check between the vessel wall and blood stream. Inflammation changes plasma protein synthesis which forms the endothelial lining of blood vessels and thus affects the endothelial formation and its role so it supports vascular disorders.[126]

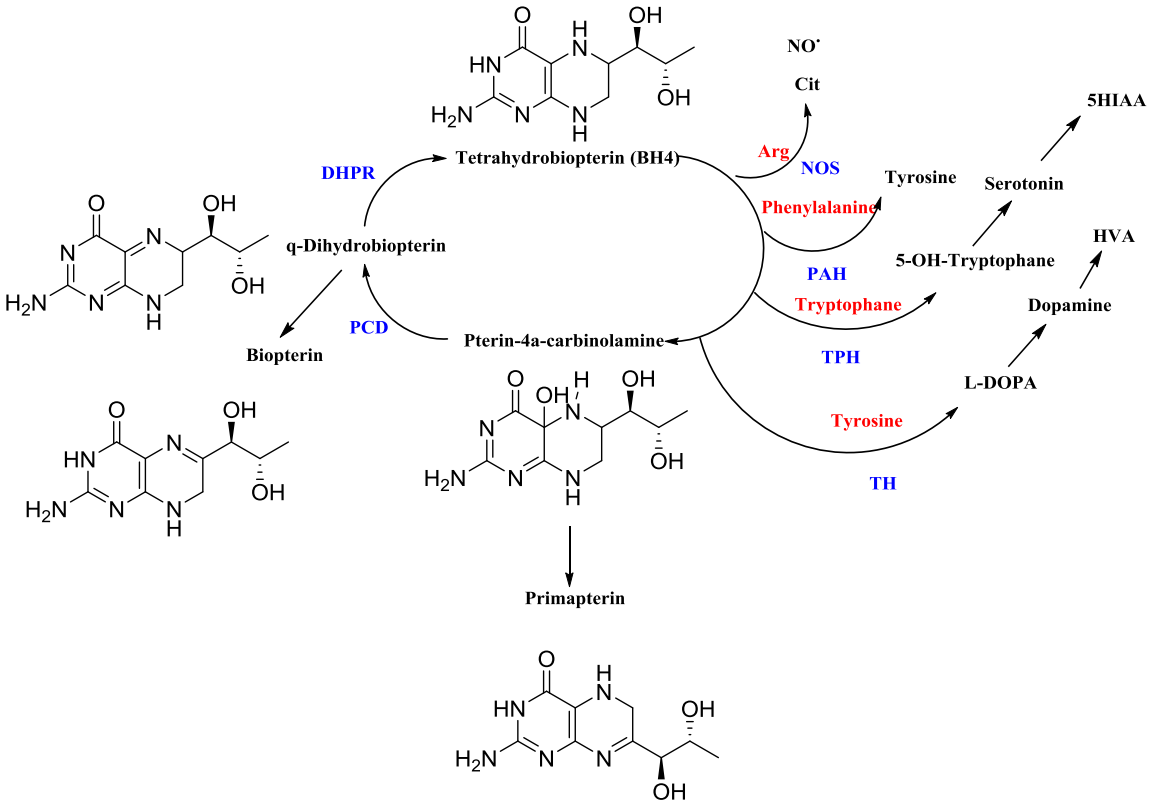
So we can say that restoration of BH_4 function and maintenance of NO levels are appropriate ways to tackle cardiovascular diseases.

1.5.0 Multifunctional BH_4 role

BH_4 is a crucial cofactor in a minimum of five enzymes:

i) ***Phenylalanine hydroxylase***, which catalyses the transformation of phenylalanine to tyrosine.
ii) ***Tyrosine hydroxylase*****,** which is essential for that metabolism of tyrosine to DOPA (a precursor of dopamine, noradrenaline plus adrenaline).
iii) ***Tryptophan hydroxylase***, which transforms tryptophan into serotonin and melatonin.
iv) ***Nitric oxide synthase,*** which is involved in the synthesis of nitric oxide (NO).
v) ***Alkylglycerol monooxygenase***, which regulates ether lipid metabolism.
Tyrosine 3-hydroxylase and tryptophan 5-hydroxylase are important enzymes in the biosynthesis of biogenic amines.[128]

BH_4 is a unique cofactor which controls the activity of different enzymes; hence it also controls the levels of essential neurotransmitters and hormones. The cofactor BH_4 reduces molecular oxygen in the hydroxylation of aromatic amino acids. BH_4 supplies electrons and as a result it is oxidized to BH_2.[127] The substrates (in red) together with corresponding enzymes (in blue) of all catalysed reactions are shown **Scheme 1.2.**[127]



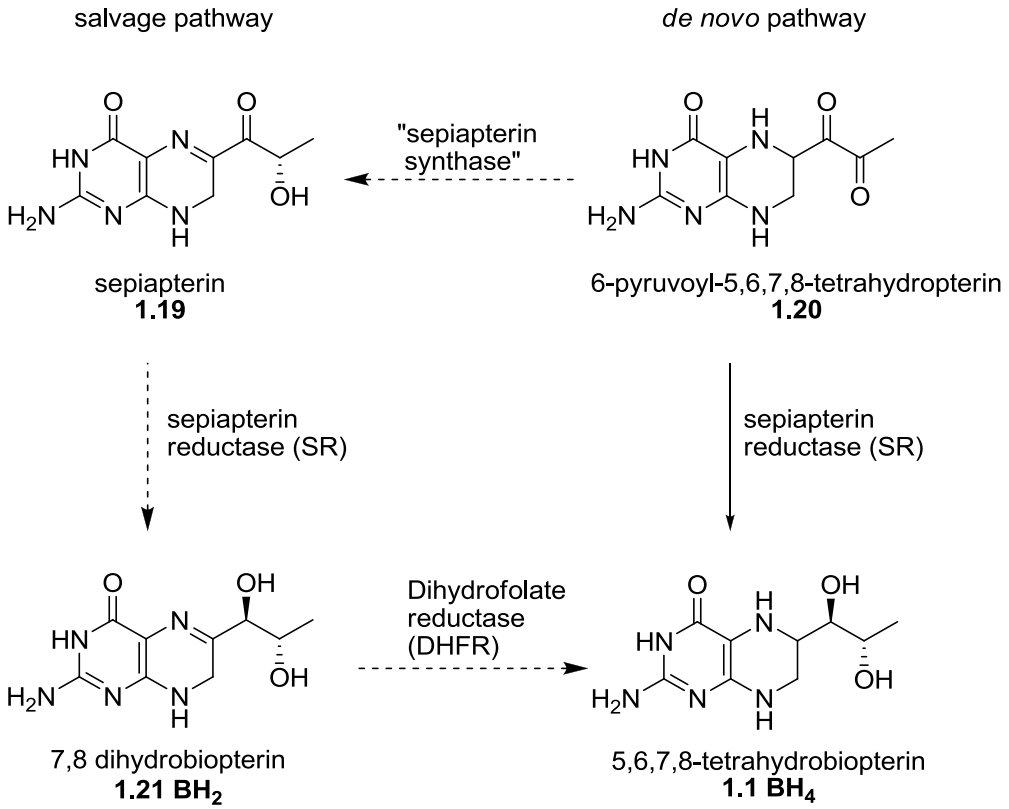
Scheme 1.2 *Different biochemical roles of BH_4.*[127] *Dihydropteridine reductase (DHPR) and pterin-4-carbinolamine dehydratase (PCD are enzymes which are involed in BH_4 biosynthesis. 5-Hydroxy indole acetic acid (5-HIAA) is formed by the breakdown of serotonin. Homovanillic acid (HVA) is formed from dopamine.*

The additional functions of BH_4 have been recognized: reduction of methemoglobin, hydroxylation of proline, and mitochondrial oxidoreduction. Renal tissue produces more terahydrobiopterin as compared to liver tissue.[127] The exact function of biopterin has not been established in kidney, it is possible that biopterin takes part in diuresis. The BH_4 cofactor is present in presumably in every cell or tissue of higher organisms.[128]

In recent times, scientists have begun to concentrate their study towards the BH_4 function in nitric oxide synthesis in endothelial cells. The entire present research acts as a guide to the significance of the investigation of the BH_4 biosynthesis together with its biochemistry in different metabolisms.

1.5.1 Biosynthesis of BH_4

BH_4 biosynthesis takes place by way of a *de novo* pathway. The reaction is dependent upon NADPH, Zn^{2+} and Mg^{2+}. The BH_4 biosynthesis starts from GTP.[128] There is another pathway for BH_4 synthesis as well, named the salvage pathway which is shown in **Scheme 1.3.** This pathway has been described in bacteria[129] and *Drosophila*.[130] In the salvage pathway, an enzyme called "sepiapterin synthase" converts 6-pyruvoyl tetrahydropterin into sepiapterin. Sepiapterin reductase and dihydrofolate reductase transform sepiapterin into 7,8-dihydrobiopterin and 5,6,7,8-tetrahydrobiopterin respectively.[76] The entire pathway is presented in **Scheme 1.3.**



Scheme 1.3 *Salvage pathway for BH_4 synthesis*[57]

1.5.2 BH_4 insufficiency and treatment with reduced pterins

Based on the thinking that pterins possibly will be adopted in the therapy of phenylketonuria (PKU), it was suggested that cases with BH_4 insufficiency might be treated by substitution with reduced pterins.[131] It was noted that intravenous treatment of synthetic BH_4 reduces the serum phenylalanine levels and therefore can consequently work as a cofactor substituting for hyperphenylalaninaemia (HPA). The treatment with carbidopa, L-DOPA and 5-hydroxytryptophan only or in mixture with BH_4, was noted to be beneficial for patients with different kinds of BH_4 insufficiency.[132] Oxidatively stable, non-toxic and NOS active reduced pterins can substitute BH_4 to correct nitic low level of nitric oxide.

1.5.3 BH_4 in therapy

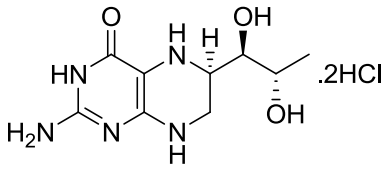
Formation of nitric oxide depends upon the presence of sufficient amounts of BH_4 cofactor. In nitric oxide formation by NOS enzyme, BH_4 donates an electron for the hydroxylation of L-arginine. It has been noted in cases of cardiovascular disorder that there is a decreased level of BH_4, which shows that deficiency of nitric oxide is responsible for the cardiovascular disorders related to deficiency of BH_4. This decreased level of BH_4 can result in uncoupling of endothelial nitric oxide synthase and thus decreased level of nitric oxide. This also causes magnified superoxide anion generation. The greater number of investigations has proved the useful impacts of BH_4 supplementation on vascular function *in vitro* and *in vivo*.[133, 134,135]

A high level of BH_4 causes local vasodilation *in vivo* in normal persons. At lower concentrations, BH_4 controls disordered NO-dependent vasodilation in cases with endothelial dysfunction. BH_4 has been involved in neurological dysfunctions, depression, Alzheimer's disorder, Parkinson's disorder, autism, endothelial dysfunction and hyperphenylalaninaemia.[136]

Some defects which are induced by the insufficiency of BH_4 and proof of effectiveness of therapy with BH_4 supplementation are outlined below.

1.5.3.1 BH_4 deficiency and phenylketonuria (PKU)

Phenylketonuria (PKU) is caused by a disorder in BH_4 metabolism;[137] if not treated the children can die at an early age.[138] The US Food and Drug Administration authorized sapropterin dihydrochloride **1.22** for the treatment of PKU in 2007.[139] PKU is a metabolic disorder in humans which is triggered by changes in the phenylalanine hydroxylase gene (*PAH*).[140,141]



1.22

Phenylalanine hydroxylase (PAH) enzyme requires BH_4 and molecular oxygen as substrates for the hydroxylation of L-phenylalanine to the L-tyrosine.[142] Since the start of infant screening programs and with immediate dietary intervention and therapy, babies born with PKU can now hope to lead comparatively healthy lives.[143]In BH_4 deficiency, the patients need drug treatment for their whole life to avoid any complications.[144] Hyperphenylalaninemia is a disease in which patients have raised levels of phenylalanine in their blood, though not as high as observed in cases with classic PKU.[145]It was found that some cases with PKU had a drop in blood phenylalanine after oral intake of BH_4. [146]

1.5.3.2 BH_4 role in dopa-responsive dystonia (DRD) and Parkinson's disease

Dopa-responsive dystonia (DRD) additionally recognized as Segawa syndrome is a genetic disease and described by low dopamine production.[147] These patients have low level of BH_4.[148] Tyrosine 3-hydroxylase is essential in dopamine synthesis. The dopamine is the principal neurotransmitter in major neural systems in the brain, and degeneration of this pathway results in very low level of dopamine and proceeds to Parkinson's disease.[149, 150] This

disease is treated by an administration of BH_4 and a rare substitution with neurotransmitter precursors (L-DOPA, 5-OH Trp.).[151,152]

1.5.3.3 BH_4 role in diabetes Mellitus

Numerous studies[153,154] in patients *in vitro* show that diabetes mellitus changes the metabolism and role of endothelium that could result in vascular injury. In diabetes mellitus, the role of endothelial nitric oxide synthase (eNOS) is changed such that the enzyme generates superoxide anion ($O_2^{-\bullet}$) rather than nitric oxide ($NO^{\bullet}$). This phenomenon is called eNOS "uncoupling." Tetrahydrobiopterin (BH_4), a crucial cofactor for eNOS, might perform an especially significant role in controlling NO and $O_2^{-\bullet}$ generation by eNOS.[155,156] BH_4 loss is responsible for eNOS uncoupling in diabetes mellitus,[157,158] since these irregularities were efficiently limited by co-administration of sepiapterin, a precursor for BH_4, in diabetic animals,[157] and patients with type II diabetes mellitus.[158]

The supplementation of BH_4 replenishes BH_4 levels and normalizes eNOS function.[155]The endothelial dysfunction is linked with diabetes mellitus and proceeds from the local generation of oxidants and free radicals in the region of the vascular endothelium.[154] Oxidative stress is the general feature in the pathogenesis of diabetic vascular disorder.[159]

1.5.3.4 BH_4 role in cirrhosis of the liver

In cirrhosis, endothelial dysfunction is one of the mechanisms suggested in the improved resistance to portal blood flow and hence in the increase of portal hypertension. Matei *et al.*[160] found that tetrahydrobiopterin supplementation enhanced the endothelial dysfunction of cirrhotic livers.[160]

1.5.3.5 BH_4 role in controlling high blood pressure (hypertension)

Therapy with oral BH_4 decreases vascular ROS production, raise NO production.[161] Fortepiani *et al.*[162] observed that BH_4 decreases blood pressure in male spontaneously hypertensive rats (SHR) by decreasing the biosynthesis of testosterone by boosting nitric oxide levels, as high levels of testosterone increases the rate of superoxide formation

1.5.3.6 Regulation of immune system.

Activation of the immune system causes a rise in GTP cyclohydrolase I (GTPCH I) activity which is a BH_4 rate-limiting enzyme.[163]

1.5.3.7 Cancer

Inducible nitric oxide (NO) synthase (iNOS) is involved in macrophage-mediated antitumor activity.[164,165]

1.5.3.8 Intestinal hypoperfusion and necrosis

Hypoperfusion is reduced blood flow through an organ, as in circulatory collapse; if continued, it may end in persistent cellular dysfunction and mortality. Tetrahydrobiopterin stops intestinal hypoperfusion and necrosis.[166]

1.5.3.9 Osteoporosis

Osteoporosis is a disease that thins bones, causing them to become brittle and also makes them more liable to break. The increase in NO has been found to enhance bone mass in trial animals and data indicates that agents that produce NO control bone turnover in man. The balance of NO is a novel target for the therapy and inhibition of bone disorders.[167]

1.5.3.10 Fibrosis and hypertrophy

Fibrosis is the development of extra fibrous connective tissue in an organ and hypertrophy is thickening of the muscles. Moens *et al*. found that after 4 weeks of aortic constriction in mice, oral BH_4 therapy was started for 5 more weeks, BH_4 reversed fibrosis and hypertrophy.[168]

1.5.3.11 Vitamin C and vascular health

Endothelial superoxide, which is formed when there is a low level of BH_4, and causes the uncoupling of eNOS, is deactivated by vitamin C and E. The protective function for vitamin C on NO and its biosynthetic pathway is confirmed by clinical data that treatment with vitamin C increases endothelium-dependent vasodilatation in patients with endothelial disorder.[169]

1.5.4 Some therapeutic approaches to treat cardiovascular diseases

One objective for a new drug for the treatment of cardiovascular diseases, should be protection or restoration of signalling pathways in arteries which are mediated by nitric oxide. Such therapeutic approaches may include the use of L-arginine, BH_4 gene transfer methods and antioxidants. Endothelium-derived NO is a powerful vasodilator that is formed from the oxidation of L-arginine. Endothelium dysfunction is an early sign in atherogenesis and heart disorder caused by disease of the coronary arteries. It was studied whether L-arginine could be a practical treatment to correct homeostatic functions of vascular endothelium in patients with progressive coronary heart disease (CHD). Patients were randomly allocated to L-arginine or control daily, after one month of oral treatment with L-arginine did not increase NO bioavailability in patients with coronary heart disease (CHD) under medical supervision.[170]

The preservation of sufficient BH_4 levels within the endothelium is very crucial in controlling eNOS coupling and, therefore, the balance between the formation of superoxide and eNOS-

derived NO. A convincing approach may be to regulate BH_4 levels in the endothelium by decreasing oxidative stress to protect against oxidation of tetrahydrobiopterin.[171]

BH_4 decreases blood pressure in male hypertensive rats (SHR) by boosting nitric oxide (NO). BH_4 treatment boosts the effects of eNOS gene transfer within the ischemic rat hind limb muscle. There was evidence of increased nitric oxide production, reduced necrosis and diminished oxidative stress with BH_4 treatment.[172]

1.5.5 Drawbacks of BH_4 therapy and nitric oxide disorders

The oral BH_4 therapy in coronary artery disease substantially increases plasma BH_4 levels, but there is a similar increase in plasma BH_2 and biopterin so that after treatment, the ratio of reduced to oxidized biopterins (BH_4/BH_2) in plasma remains the same. **Figure 1.21** shows an analysis of high-dose BH_4 therapy compared to placebo. The blood specimens were obtained at each time of coronary artery bypass graft surgery 12 to 16 hours after the last dose. The oral BH_4 treatment therapy in coronary artery disease was found more efficient at increasing arterial BH_2 levels than BH_4 levels. Increasing BH_4 concentarion from 400 mg per day to 700 mg per day to each patient had almost no effect upon the vasorelaxation of saphenous vein rings as shown in Figure **1.21**.[173] This could be because BH_2 compete with BH_4 for binding with NOS enzyme so an increase in BH_2 concentration in the blood resulted in an inhibition of NOS activity, as active site of NOS enzyme was also occupied by BH_2.

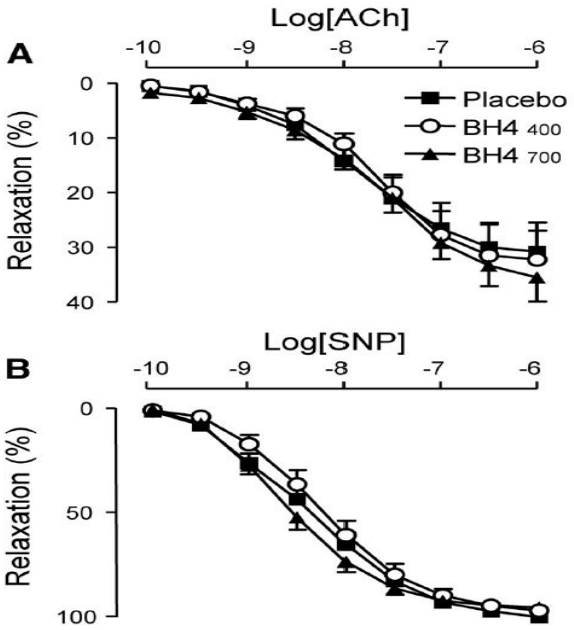


Figure: 1.21 *Effect of oral BH_4 therapy on endothelial function, the vasorelaxation of saphenous vein rings to acetylcholine (ACh) figure A and sodium nitroprusside (SNP) figure B in vivo. In the graph, placebo represents when no BH_4 was given, BH_4 400 reperesents when low dosage of 400 mg of BH_4 was given orally to each patient on daily basis while BH_4 700 represents a higher dosage of 700 mg per day.*[173]

Oral BH_4 treatment increases total biopterin levels in blood and vascular tissues in patients with coronary artery disease but has no clear effect on endothelial function, or vascular redox due to vascular oxidation of BH_4 to BH_2. The BH_2 does not have eNOS cofactor activity, so there was no net effect on eNOS coupling or endothelial function. BH_4 is still a rational therapeutic strategy in cardiovascular disease, but alternative approaches are necessary to target BH_4-dependent endothelial role in established vascular disease states.[173]

1.5.6 Problems with BH_4 as a drug

The preceding section shows a strong argument for therapeutic intervention with BH_4 to restore healthy conditions. Nevertheless, although treatment with BH_4 is capable of improving the dysfunction of endothelial system in some cases, it has limited physicochemical characteristics as a medicine: it can readily oxidise into aromatic biopterin, it has a large polarity, low solubility and is susceptible to side-chain cleavage. BH_4 is generally indicated as a therapeutic factor in cases with BH_4 insufficiencies and moderate kinds of PKU. Fiege *et al.*[174] calculated its plasma concentrations in patients with cardiovascular dysfunction. They concluded that, after oral treatment with BH_4 on patients with hyperphenylalaninemia and mild PKU plasma, BH_4 levels might not be sufficient to completely stimulate the liver phenylalanine hydroxylase and hence to lower blood phenylalanine levels. According to Lipinski's rule, an orally active drug having more than 5 hydrogen bond donors is not considered to be an ideal drug, however the BH_4 molecule has 7 hydrogen bond donors.

Several cases with a critical insufficiency of BH_4 have been given BH_4 treatment and have had their quality of life considerably changed, but the BH_4 uptake has not been significant. There has been little fundamental investigation on the permeability and the metabolic outcome of exogenously administered BH_4 as related to various organs of the body.[175]

BH_4 in its active, reduced form is very unstable and hence not suitable for oral treatment. Moreover, treatment of BH_4 in the presence of prolonged oxidative stress may result in accelerated oxidative degeneration of BH_4 and, therefore restrict continuation of the useful effects.[176]

Dumitrescu *et al.*[177] calculated cardiac BH_4 content including its degeneration after ischemia. BH_4, including its metabolites 7,8-dihydrobiopterin (BH_2), biopterin (B), pterin (P), and dihydroxanthopterin (XPH_2), were measured by HPLC with electrochemical detection (**Scheme 1.4**). Those investigations illustrate that BH_4 is consumed during ischemia (**Figure 1.22**). BH_4 levels remained to a small extent lower in hearts limited to 15 min of ischemia

matched with controls. But, ischemic durations longer than 15 min induced a sharp drop in BH_4 levels, which became nearly undetectable after 60 min of 37 °C, which is our body temperature.

The abrupt drop of BH_4 cardiac content with continued ischemia is paralleled by a sharp rise in the levels of a pterin, named XPH_2 in **Figure 1.22**, and proposed to be 6-hydroxy-5,6-dihydropterin **1.18**. From the data it can be proposed that the degeneration of the crucial cofactor BH_4 might induce a failure of NOS function. BH_4 was mainly oxidized to produce XPH_2 **1.18**, which can oxidize with side-chain cleavage, and there were no detectable levels of either BH_2 **1.21**, or B **1.10** that could be possibly reduced back to BH_4. Therefore, BH_4 is irreversibly degraded in this post-ischemic heart and results in a loss of NOS activity (**Scheme 1.4**).[177]

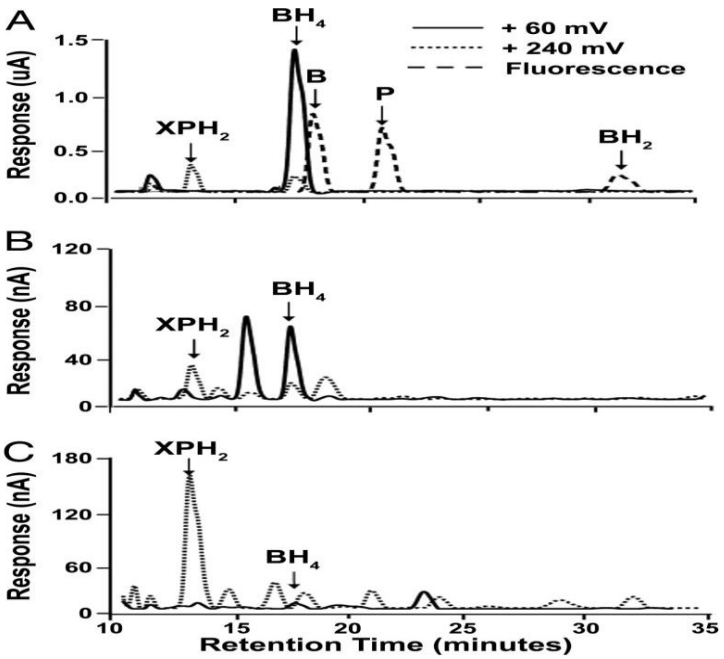
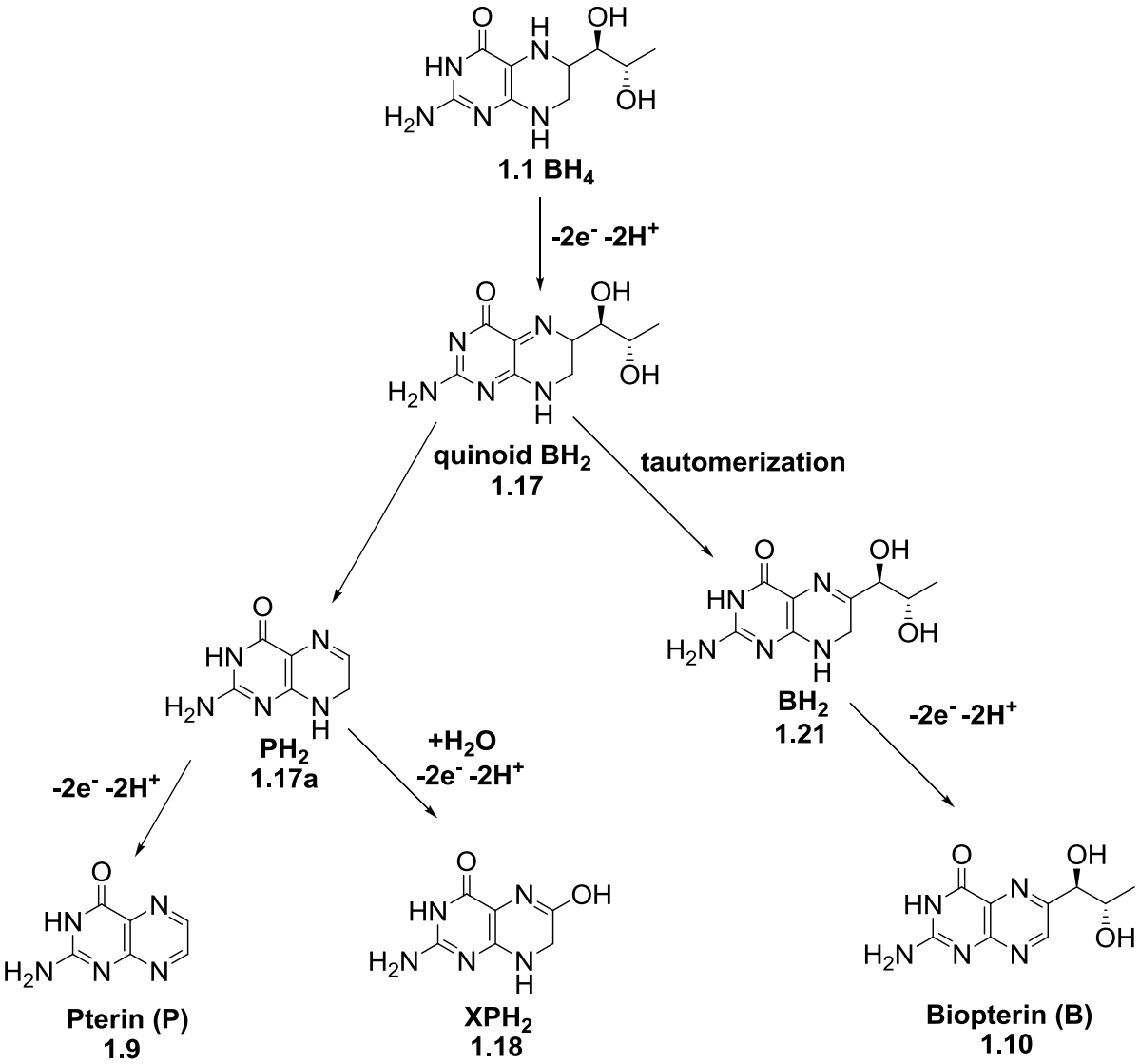


Figure: 1.22 *BH_4 degeneration during ischemia. Figure **A** represents the chromatogram of pterin (p) and biopterin (B). Figure **B** is the the chromatogram of homogenate of a nonischemic rat heart. Figure **C** shows chromatogram ischemic heart taken after 60 minutes which shows a significant loss in BH_4 level.*[177]



Scheme: 1.4 *BH_4 catabolic pathways. BH_4 is labile and quickly oxidized and degenerated to several biopterin and pterin derivatives through enzymatic or non-enzymatic methods.*[177]

BH_4 cannot be utilized to improve the central neurotransmitter insufficiency in either of the BH_4 insufficiencies.[178] This defect of BH_4 owes to its hydrophilic nature, however, lipophilic compounds can readily pass this brain barrier.

The genetic disease of tetrahydrobiopterin biosynthesis results in the insufficiency of dihydropteridine reductase (DHPR) which can cause mental retardation, microcephaly,

hyperphenylalaninemia, and convulsions. A case with DHPR insufficiency was treated with BH_4 supplementations (20 mg/ kg, two times a day). The plasma phenylalanine (Phe) concentration was found to be normal, and the patient had an increased phenylalanine tolerance as noted after 1 month of treatment. But the cerebral spinal fluid (CSF) neurotransmitter metabolites did not change. After one month of treatment with BH_4 supplementation (40 mg/kg, two times a day), no significant improvement of CSF neurotransmitters, BH_4 or BH_2 concentrations were found, although phenylalanine (Phe) level was normal.[179]

Low-dose oral antioxidants such as ascorbic acid seem to possess no important clinical advantage in patients suffering from cardiovascular disorder, and may be inadequate in changing redox signalling at the level of the vessel wall.[180] Together, the indicated defects lead to clinical requirement to produce an oxidatively stable cofactor with suitable physicochemical characteristics which can improve BH_4 insufficiency in chosen disorders.

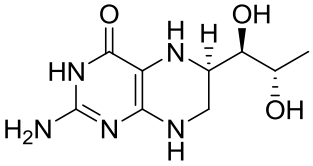
Although antioxidants may function in the protection against oxidative stress; clinical trials with antioxidants such as vitamins E and C were unsuccessful in improving cardiovascular health.[181]

1.5.7 The role of pterins for NADPH oxidation

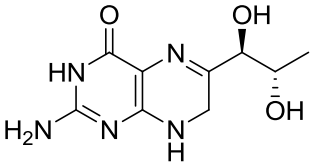
Presta *et al.*[182] described that NADPH oxidation was increased in response to L-arginine addition to iNOS saturated with any of four tetrahydropterin analogues mentioned below. While when L-arginine to iNOS saturated with any of the three dihydropterins, NADPH oxidation did not increase significantly. As shown in the **Table 1.1**, all tetrahydropterin analogues produced NO and synthesised citrulline or L-arginine. The rates were closer to those recorded for iNOS saturated with (*6R*)-BH_4 **1.24**. The three dihydropterins, BH_2, sepiapterin and deoxysepiapterin produced no measurable NO or citrulline with either L-arginine or NOHA.[182]

When either L-arginine or NOHA was added to iNOS in the absence of pterin, NADPH oxidation was increased, which indicated that substrates can increase iNOS NADPH

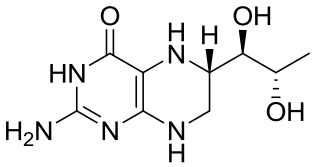
oxidation with bound pterin. Adding (*6R*)-BH_4 **(*6R*)-1.1** to iNOS without any substrate increased its NADPH oxidation to 245 nmol of NADPH min^{-1} (mg^{-1} of enzyme) (**Table 1.1, Entry 1**). While the tetrahydroneopterin (NH_4) **1.23**, 6-methyl terahydropterin (6-$MePH_4$) **1.25** and 6-hydroxymethyl tetrahydropterin (6-$OHMePH_4$) **1.26** (**Table 1.1, Entry 3, 4 & 5**) were not found to cause an increase in NADPH oxidation by iNOS over the basal level, (*6S*)-BH_4 **(*6S*)-1.1** only increased up to 97 nmol of NADPH min^{-1} (mg^{-1} of enzyme) (**Table 1.1, Entry 2**). The sepiapterin **1.19**, deoxy-sepiapterin **1.24** and dihydropterins BH_2 **1.21** each caused an increase in NADPH consumption by iNOS to levels comparable to that found with (*6R*)-BH_4 **(*6R*)-1.1**. When the substrate-based blocker of heme iron reduction (thiocitrulline) was added, NADPH oxidation associated with (*6R*)-BH_4 **(*6R*)-1.1**or BH_2 **1.21** saturation was blocked which suggested that NADPH oxidation due to these pterins is due to electron transfer to the iNOS heme iron.[182,183]



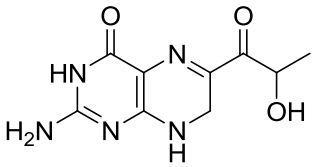
(6*R*)-Tetrahydrobiopterin
(*6R*)-BH_4
(*6R*)-1.1



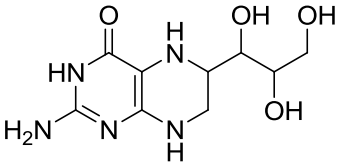
Dihydrobiopterin
BH_2
1.21



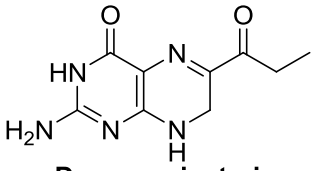
(6*S*)-Tetrahydrobiopterin
(*6S*)-BH_4
(*6S*)-1.1



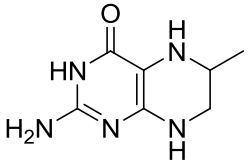
Sepiapterin
1.19



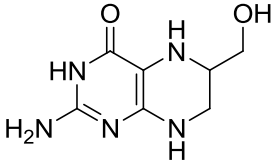
Tetrahydroneopterin
NH_4
1.23



Deoxysepiapterin
1.24



6-Methyl terahydropterin
6-MePH_4
1.25



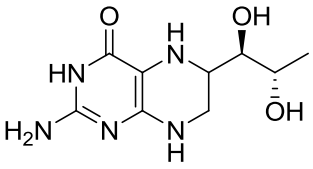
6-Hydroxymethyl tetrahydropterin
6-OHMePH_4
1.26

Entry	Compound number	Pterin	Substrate	NADPH oxidation (nmol min^{-1} mg^{-1})	NO synthesis (nmol min^{-1} mg^{-1})	Citrulline synthesis (nmol min^{-1} mg^{-1})
1	**(*6R*)-1.1**	(*6R*)-BH_4	none	245 ± 1	0	0
			L-arginine	1270 ± 140	219 ± 4	219 ± 7
			NOHA	390 ± 10	205 ± 3	210 ± 17
2	**(*6S*)-1.1**	(*6S*)-BH_4	none	97 ± 5	0	0
			L-arginine	1260 ± 140	97 ± 5	90 ± 9
			NOHA	331 ± 12	124 ± 3	124 ± 10
3	**1.23**	NH_4	none	60 ± 2	0	0
			L-arginine	1070 ± 120	223 ± 8	270 ± 20
			NOHA	415 ± 24	179 ± 7	190 ± 20
4	**1.25**	6-MePH_4	none	65 ± 1	0	0
			L-arginine	1200 ± 120	210 ± 8	197 ± 15
			NOHA	360 ± 22	163 ± 5	190 ± 20
5	**1.26**	6-OHMePH_4	none	56 ± 3		
			L-arginine	1320 ± 120	202 ± 9	195 ± 25
			NOHA	327 ± 6	200 ± 10	270 ± 20
6	**1.21**	BH_2	none	304 ± 7	0	0
			L-arginine	280 ± 30	0	0
			NOHA	291 ± 2	0	0
7	**1.19**	sepiapterin	none	131 ± 3	0	0
			L-arginine	281 ± 5	0	0
			NOHA		0	0
8	**1.24**	Deoxysepiapterin	none	240 ± 4	0	0
			L-arginine	290 ± 40	0	0
			NOHA		0	0

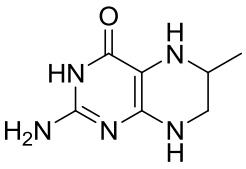
Table 1.1 *Enzymatic Activities of iNOS with Different Pterins.*[182]

1.5.8 The effect of structure of pterin on nitric oxide formation by NOS enzyme

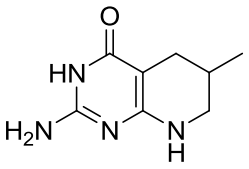
NO synthesis and the ability to enhance heme-dependent NADPH oxidation in response to substrates are two properties of iNOS which depend on fully reduced tetrahydropterins and do not depend upon side-chain structure. On the other hand, five properties are fully dependent on stereochemistry or pterin side-chain structure and are not dependent upon pterin oxidation state. These five properties are the ability of pterin to change the heme iron to its high-spin state, to stabilise the ferrous heme iron coordination structure, its binding affinity, heme iron reduction, and iNOS dimerization.[182]



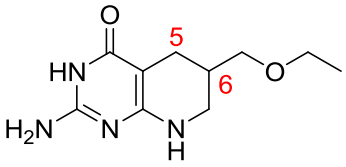
BH_4
1.1



6-MePH_4
1.25



DZPH_4
1.27



EtOMeDZPH4
1.28

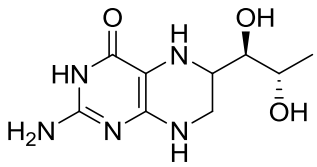
Rusche *et al.*[184] found that BH_4 binding to iNOS was not fast without the unique existence of a thiol in its reduced state. The thiol in reduced state is also called a free thiol, which is attached to an amino acid residue but is free on other side and does have its hydrogen atom, so it does not have a disulfide linkage between two amino acid residues. In the absence of thiol in reduced state, the spectral shifts and restoration of activity were not complete. The stoichiometric BH_4 binding was obtained in the presence of glutathione (GSH). The binding of BH_4 **1.1** was dependent upon the concentration of glutathione (GSH). The pterin-free NOS was allowed to bind to redox-stable deazapterins analogues **1.27** and 6-methylpterin **1.25**. Binding with the redox-stable pterin analogue was not time or thiol-dependent but was dependent upon concentration of analogue.[184]

The 200-fold reduction in the affinity of $DZPH_4$ **1.27** for NOS could be due to variations in the pterin side-chain. The 6-ethoxymethyl side chain of $EtOMeDZPH_4$ **1.28** brings about a 400-fold decrease in affinity as compared with BH_4 **1.1** The C^6 side-chain of the pterin therefore plays an important role in binding, which is due to multiple interactions of the biopterin side-chain with protein residues, as found in the NOS structures.[47,93,109]

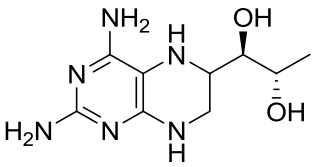
The structure of $DZPH_4$ **1.27** does not have an N^5 atom, so the differences in binding between $6\text{-}MePH_4$ **1.25** and $DZPH_4$ **1.27** are not due to a C^6 substituent, but $DZPH_4$ **1.27** lacks a hydrogen bond donor. The pattern of $DZPH_4$ **1.27** binding may be different from that of BH_4, **1.1**, due to different hydrogen bonding.[184]

1.5.9 Investigations for role of BH_4 in enzyme functions

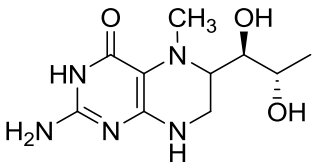
BH_4 is an important cofactor of nitric oxide synthases (NOSs), but its role in enzyme function is not fully understood. The binding of the pterin changes the electronic structure of the heme group which results in an increase in stabilization of the active homodimeric structure of the protein. On the other hand, these effects are also observed by the inhibitor of NOS, 4-amino-BH_4 **1.29**, so the natural cofactor BH_4 has another catalytic function which is not known yet. Riethmuller *et al.*[185] showed that the 5-methyl analogue of BH_4 **1.30** does not react with O_2 and is an active pterin cofactor of neuronal NOS.



BH_4
1.1



4-amino-BH_4
1.29



5-MeBH_4
1.30

The affinity of the 5-methyl compound **1.30** is three-fold less than that of BH_4. The BH_4 inactivates nitric oxide (NO) via nonenzymatic superoxide formation; 5-methyl derivative **1.30** did not react with O_2 and had no effect on NO concentrations. So the 5-methyl analogue of BH_4 **1.30** did not produce superoxide and just produced free NO as a NOS product which

shows that that reductive O_2 activation by the pterin cofactor is not important for NO biosynthesis. [185]

As compared with BH_4, the 5-methyl analogue **1.30** showed less activity toward O_2 as shown in **Figure 1.23.** This could be due to steric hindrance by the 5-methyl group, inhibiting the formation of the assumed hydroperoxide intermediate at the C^4 position of the pterin ring.[185]

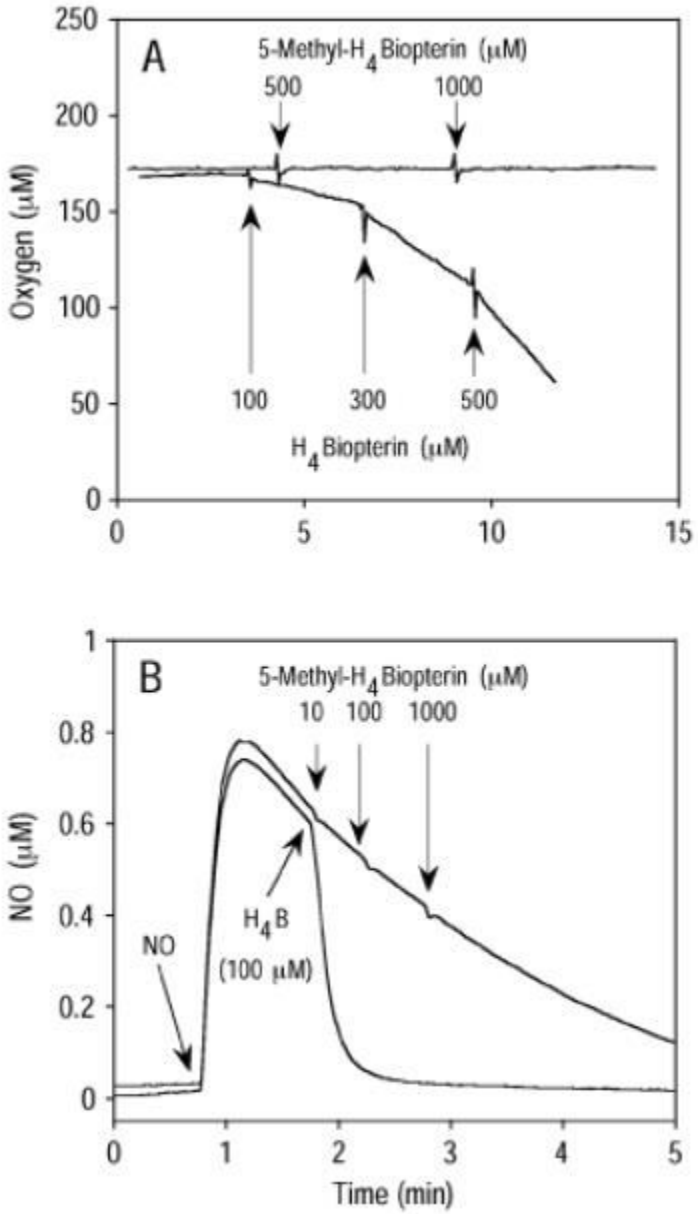


Figure: 1.23 *Oxygen depletion (**A**) Nitric oxide oxidation by BH_4 and 5-methyl analogue **1.30(B)**.*[185]

The nNOS produce free NO in the absence of scavengers when the enzyme is allowed to bind with the autoxidation-resistant 5-methyl analogue of BH_4 as a substitute of BH_4. The NO formation slowly increased when the concentration of 5-methyl-BH_4 was increased (**Figure 1.24**).[185]

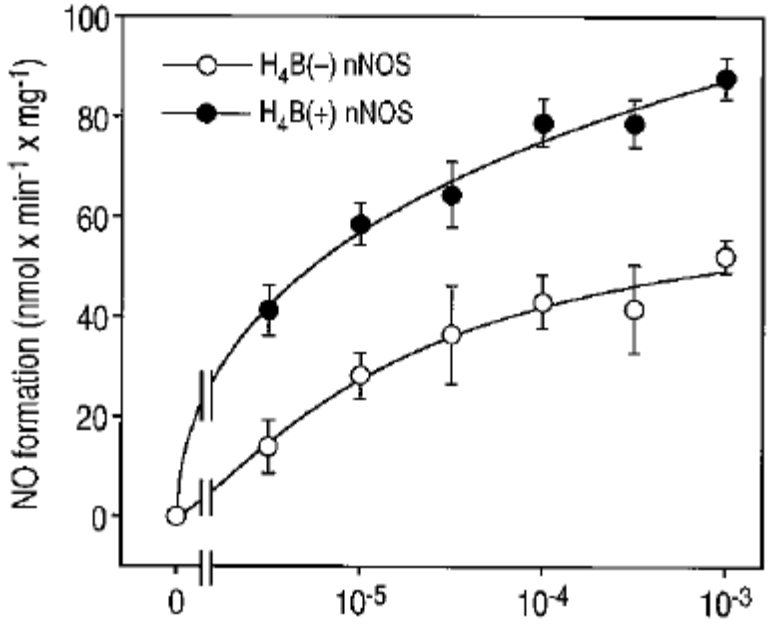
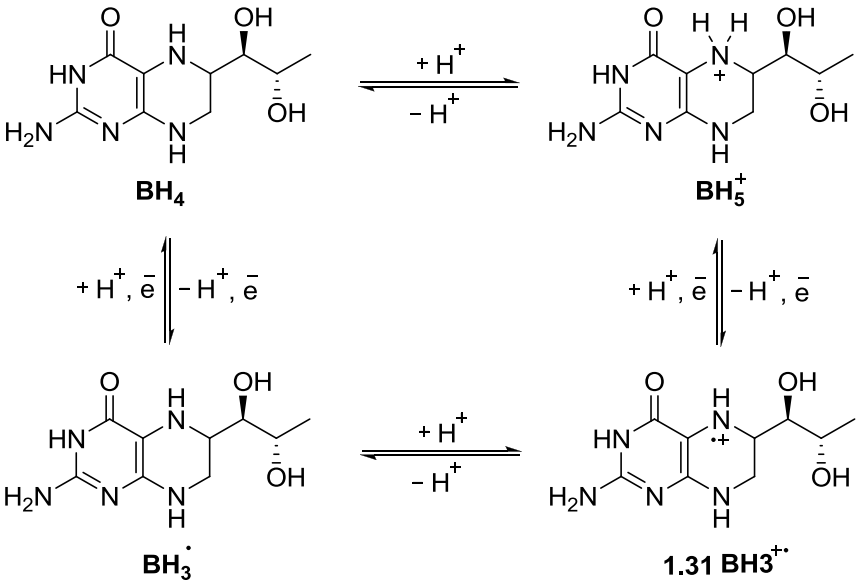


Figure: 1.24 *Effect of concentration of 5-methyl-BH_4* ***1.30*** *on nNOS activity.*[185]

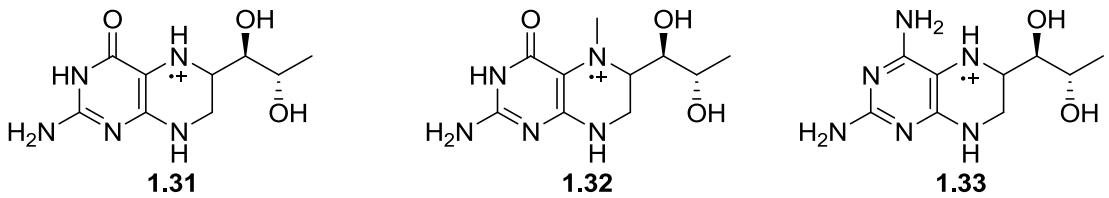
1.5.10 The effect of methyl group on stability of $^{\bullet}BH_3$ radical

Raman et al.[47] suggested that BH4 and $^{\bullet}BH_3$ can bind with eNOS heme domain in their protonated cationic states (BH_5^+ and $BH_4^{\bullet}+$ respectively, **Scheme 1.5**). Alderton et al.[104] also proposed that in NOS catalysis, BH_4 performs a redox role. BH_4 is converted into BH_5^+ by protonation which has bound to the NOS enzyme and provides an electron to the heme to give $BH_4^{\bullet}+$. In the reductase domain, $BH_4^{\bullet}+$ is later reversed to its reduced state by receiving an electron from a Flavin to form BH_5^+ as shown in **Scheme 1.5**.



Scheme: 1.5 *Structures of BH_4 and of the $BH_3^{\cdot}$ radical in protonated and unprotonated forms*[147].

Hurshman *et al.*[43] investigated the capability of 4-amino-BH_4 **1.29** and 5-methyl-BH_4 **1.30** bound heme domain enzyme (HDiNOS) and full-length enzyme (FLiNOS) to catalyze product formation from both arginine and NHA via a pterin radical formation. The kinetics of formation and decay of BH_4 and 5-methyl-BH_4 **1.30** radicals were assessed (**Entry 2** and **3** respectively, **Table 2.1**). Even though, the rate of radical formation for both was similar, the rate of degeneration of 5-methyl-$^{\cdot}BH_3$ **1.32** was 400-times slower than that of $^{\cdot}BH_3$ **1.33** (**Entry 3**, **Table 2.1**). The radical of 5-methyl-BH_4 is probably stabilised due to electron donating effects of the methyl substituent.[43]



5-Methyl-BH_4 **1.30** forms an extremely stable radical (5-methyl-$^{\cdot}BH_3$) **1.32** with the heme domain iNOS (HDiNOS) that builds up in the arginine reaction to approximately 60% of the heme domain iNOS (HDiNOS) (**Entry 1, Table 2.1**). In the NHA reaction, the amount of

radical (5-methyl- $^{\bullet}BH_3$) **1.32** formed is less than 3% of HDiNOS. A pterin radical (4-amino-$^{\bullet}BH_3$) **1.33** was not formed with 4-amino-BH_4 **1.29** with any substrate. The catalytic activity of 4-amino-BH_4-bound heme domain iNOS (HDiNOS) and full-length enzyme (FLiNOS) was similar to that of pterin-free iNOS. The hydroxylation of arginine was found to be less than 2% of that of BH_4-bound iNOS (**Entry 9, Table 2.1**). The binding of BH_4 is involved in stabilizing the NOS structure and its active site.[43]

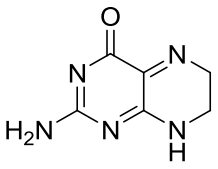
Entry	HDiNOS	BH_4	5-methyl-BH4	4-amino-BH_4
1	$^{\bullet}BH_3$ stoichiometry	0.8	0.6	0
2	$^{\bullet}BH_3$ $k_{formation}$ (s^{-1})	15-20	30	n/a
3	$^{\bullet}BH_3$ k_{decay} (s^{-1})	0.12	0.003	n/a
4	NHA stoichiometry	0.78 ± 0.11	0.47	0
5	NO formation	Yes	yes	no
	FLiNOS, oxyhaemoglobin assays			
6	arginine (%)	100	33.7	0.1
7	NHA (%)	100	38.0	0.8
8	amino acid products	NHA, citrulline	NHA, citrulline	citrulline
9	relative activity (%)	100	53.5 ± 8.5	1.6 ± 0.7
10	% citrulline	99.9 ± 0.2	98.6 ± 0.6	84.3 ± 10.3
11	relative activity (%)	100	48.4 ± 7.5	25.5 ± 5.9

Table 1.2 *Summary of activities of BH_4 and tetrahydrobiopterin analogue, the activity described is the relative amount of product produced in 2 h of reaction at 25°C.*[43]

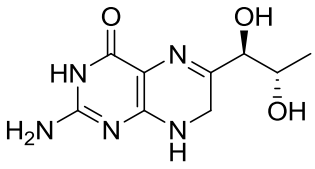
It is proposed that an electron given from BH_4 starts from the N^5 position as shown in structure of pterin radical **1.31**. It has been proposed that an analogue which will also promptly give an electron to the heme could enhance the rate of nitric oxide generation. But insufficient work has been conducted to evaluate the impact of a more stable radical at N^5 position.

Due to the prolonged stability of the radical **1.32** formed from 5-methyl-BH_4 **1.30**, it was observed that 5-methyl-BH_4 **1.29** gives an electron to the heme three times faster than BH_4.[43,186] This impact is seen at saturation levels of the pterin. In fact at similar concentrations to BH_4, 5-methyl-BH_4 **1.30** works as a poorer cofactor.

Addition of a methyl group at N^5 of BH_4 and other tetrahydropterins makes them more stable to autoxidation[186] and resists the formation of the 6,7-quinonoid dihydropterin **1.34** form which is the kinetically feasible product of pterin oxidation. The electrochemical properties of 5-methyl-BH_4 **1.30** shows that it takes part in one-electron redox cycling between tetrahydrobiopterin and trihydrobiopterin radical ($^{\bullet}BH_3$); on the other hand it does not take part in electrochemically reversible two-electron transitions to give dihydrobiopterin **1.21**.[187]



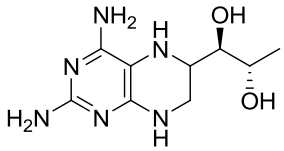
1.34



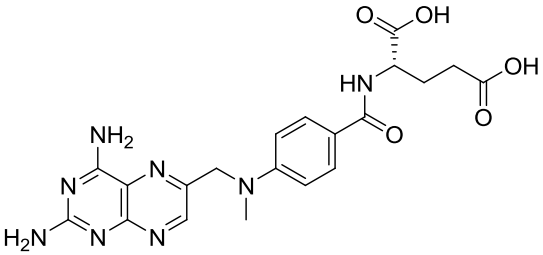
1.21 BH_2

1.5.11 Control of High level of nitric oxide by analogues of BH_4

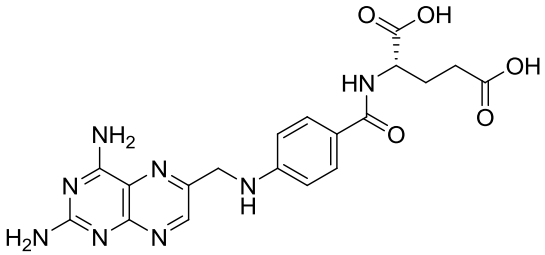
The high level of nitric oxide in stroke, sepsis and inflammation can be controlled by pharmacological agents that inhibit the nitric oxide synthase enzymes. One approach is to target the BH_4 binding site of NOS by suitable inhibitors. The first BH_4 based inhibitor of NOS was 4-amino derivative of BH_4 **1.29** which is similar to methotrexate **1.7**, an inhibitor of folic acid **1.35**.[94]



4-amino-BH_4
1.29



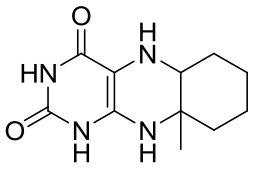
Methotrexate
1.7



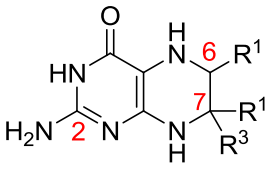
Folic acid
1.35

1.6.0 Discoveries at Strathclyde with WSG1002

Following the redesign and synthesis of 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase enzyme inhibitors, Suckling together with his co-workers prepared a range of pteridine compounds with modification at site 2, 6 and 7 positions (**1.36**, **1.37**) as represented below in **Table 1.1**. A few of these compounds seemed to possess antibacterial activity and hindered folic acid biosynthesis.[188]



1.36



1.37

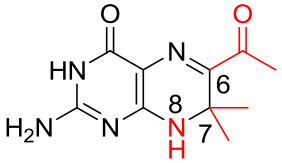
Compounds	R_1	R_2	R_3
1	CH_2OH	Me	Me
2	CH_2OH	Me	CH_2Ph
3	CH_2OH	Me	CH_2CH_2Ph
4	CH_2OH	Et	Et
5	CH_2OH		$-(CH_2)_5-$
6		$-(CH_2)_3-$	Me
7		$-(CH_2)_4-$	Me
8	Me	Me	Me
9	Et	Me	Me
10	*n*-Pr	Me	Me
11		$-CO(CH_2)_2-$	Me
12		$-CO(CH_2)_3-$	Me
13	COMe	Me	Me
14	CHO	Me	Me

Table 1.3 *Pteridine compounds with variations at different sites.*[188]

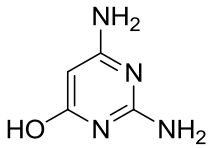
At this time (1980), the study on the use of BH_4 in aromatic amino acid hydroxylation was being revealed, [256, 257] but the study of nitric oxide synthesis by NOS including BH_4 as a cofactor was not completely founded. Hence, physiological evaluations with regard to aromatic amino acid hydroxylations or NO generation were not carried out. Current research in choosing these compounds as BH_4 replacements for the investigation of nitric oxide synthesis, showed that several compounds in the family supported NO production by NOS; the activity of greatest examined compound, WSG1002 **1.2**, is presented below.

1.6.1 Biological properties of WSG1002

WSG1002 (**W**ood, **S**uckling, **G**ibson) **1.2** also called ADDP (6-acetyl-7,7-dimethyl-7,8-dihydropterin) was taken for the primary tissue based research. 6-Acetyl-7,7-dimethyl-7,8-dihydropterin 1.2 has an acetyl side-chain at the C^6 position which is similar to that of the BH_4 C^6 side-chain, and that can help to bind with the active site of the enzyme by hydrogen bonding. The arterial rings taken from the rat lungs were pre-incubated along with an inhibitor of GTP-cyclohydrolase-1 i.e. 2,4-diamino-6-hydroxypyrimidine **1.38** (DAHP); later ascending concentrations of WSG1002 (**1.2**) were administered.[189]

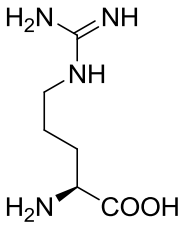


1.2

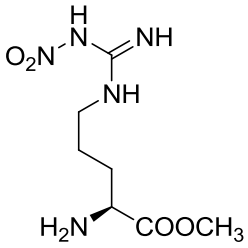


1.38

As a consequence, an actual relaxation of arteries was noted (**Figure 1.25**, lower trace). It was also noted that the relaxation induced by WSG1002 (**1.2**) is a consequence of nitric oxide generation in the same manner as BH_4. The association of NO was confirmed by incubating arterial loops with a non-specific nitric oxide synthase blocker L-nitro-arginine methyl ester (L-NAME) **1.39**; in this example no relaxation was noted (**Figure 1.25**, upper trace). L-arginine **1.4** supports nitric oxide production while L-NAME) **1.39** is an inhibitor.[189]



L-arginine
1.4



L-NAME
1.39

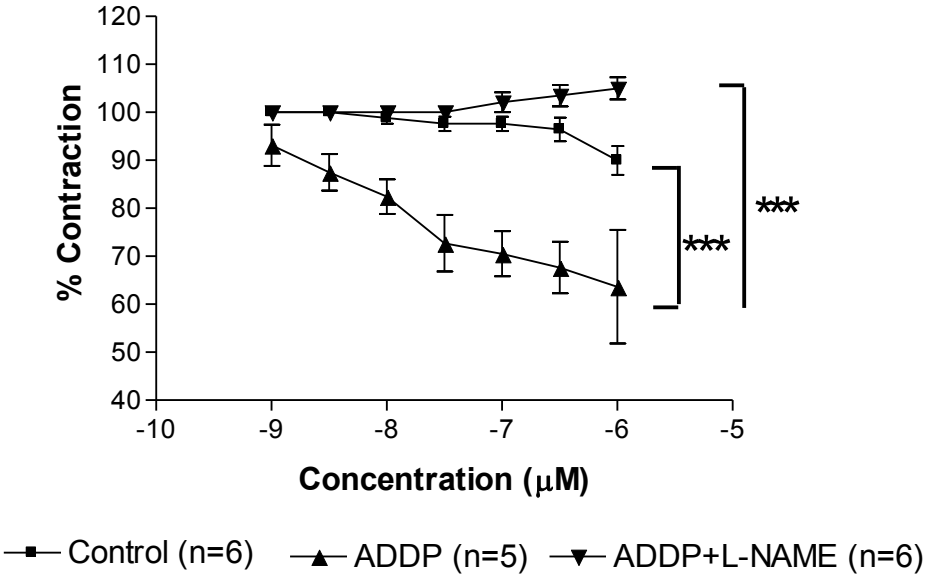


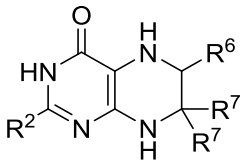
Figure: 1.25 *WSG1002 produced relaxation of pulmonary arteries (BH_4 consumed).*[189]

The graph shows that WSG1002 provided a sizeable relaxation in BH_4 depleted pulmonary arterial endothelium (eNOS). Just when eNOS activity was blocked out, there was none of that kind of relaxation with WSG1002. That provided evidence that the relaxation induced by WSG1002 happened due to NO formation by NOS. WSG1002 resists oxidation. The basic characteristics of WSG1002 which provide it a great chemical stability are the 7,7-dimethyl substituents together with the oxidized C^6 side chain. These gem-dimethyl substituents prevent the pyrizine ring being oxidized at the N^8 position and thus block it from aromatizing. That is why this is also called blocked dihydropteridine. There are several structural modifications feasible at those sites.

The oxidation states of WSG1002 and BH_4 are varied and so the mechanistic characterization could not be identical for the two compounds. WSG1002 was found not to be orally effective in mice; there was a problem of bioavailability. WSG1002 is a new compound to replace BH_4; different compounds were later examined to solve the bioavailability problem and to know the mechanism of action of further dihydropterins.

1.6.2 Some structural variations of WSG1002 that have been carried out by Suckling and his co-workers.

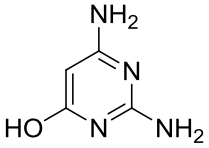
A few examples of tetrahydropterins that had been synthesized and were studied by Suckling and his co-workers in the primary investigations of NOS are given in **Figure 1.26**. This remains unpublished.



WSG	R^2	R^6	R^7
1001	NH_2	CH_2OH	H
1002	NH_2	COMe	CH_2Ph
1004	NH_2	CH_2OH	CH_2CH_2Ph
1005	NH_2	CO_2H	Et
1006	NH_2	Et	$-(CH_2)_5-$
1013	NH_2	Me	Me2
1014	NH_2	Me	Me
1016	NH_2	CHO	Me
1017	NH_2	$CH=CH-pC_6H_4Cl$	Me
1018	NH_2	CH_2OH	Me
1031	SCH_2Ph	CHO	Me

Figure: 1.26 Table of commonly named synthesised blocked dihydropterins.[258]

The indicated various compounds in **Figure 1.26** were examined in living systems, using macrophages that include the iNOS isoform with two types of cells; BH_4 depleted cells and BH_4 non-depleted cells. GTP-cyclohydrolase-1 is an important enzyme for BH_4 biosynthesis. 2,4-diamino-6-hydroxypyrimidine **1.38** (DAHP) is an inhibitor of GTP-cyclohydrolase enzyme, which was used to deplete BH_4 from an iNOS cell-based assay. The nitric oxide formation by these compounds using BH_4 depleted cells is represented in the blue bar, while with BH_4 non-depleted cells is represented in the maroon bar (**Figure 1.27**).



1.38 DAHP

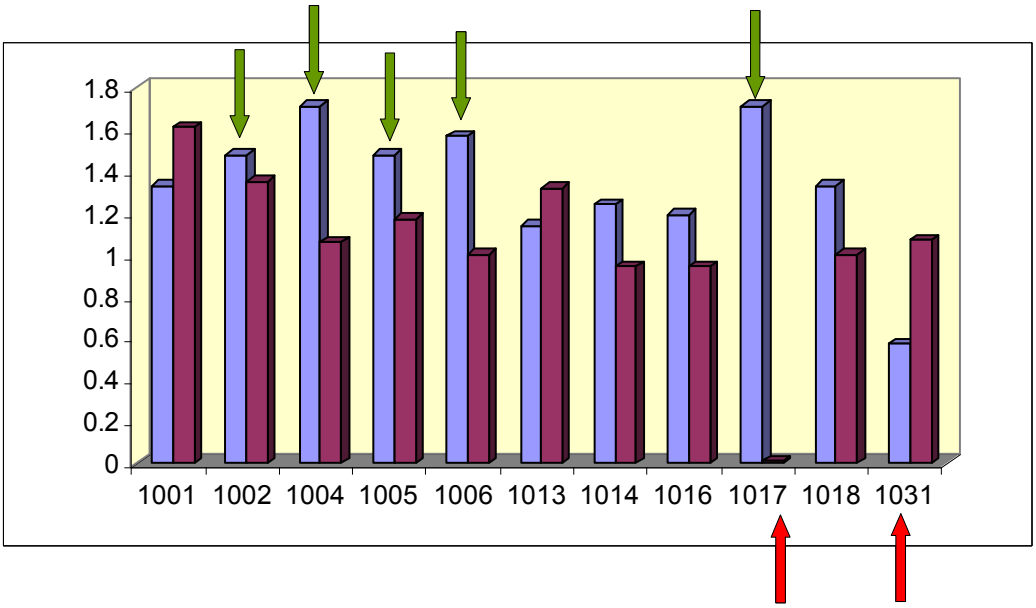
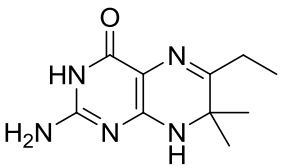
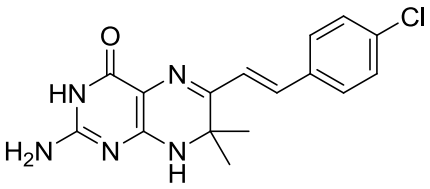


Figure: 1.27 *iNOS activity in macrophage screen*

NO production is greater with the compounds indicated by green arrows while compounds indicated by red arrows show an inhibition of enzyme. The blue bar indicates iNOS activity in DAHP ***1.38*** *treated (BH4 depleted cells) while the maroon bar indicates iNOS activity in non* BH_4 *depleted cells.*[258]



WSG1006
1.40

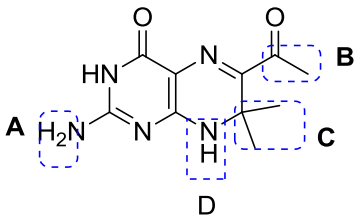


WSG1017
1.41

1.6.3 Structure-Activity Relationships

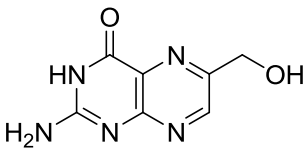
A few essential features were noticed based on the conclusions gathered from examining a short number of compounds also from molecular modelling investigations taken out by Prof. Suckling.

1) There are four potential changes in the structure of pteridine molecule, the indicated positions 2 (A), 6 (B), 7 (C) and 8 (D) are shown below.

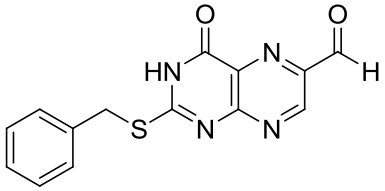


1.2

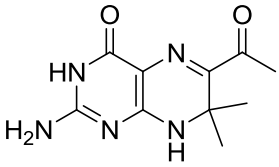
2) Unpublished results in the Suckling group, about NOS enzyme assay indicate that S-methyl **1.44** and **1.45** or S-benzyl substitution **1.43** at point 2 (A) produces inhibitors instead of activators.



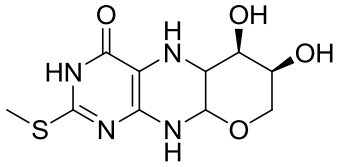
1.42 (WSG1001)



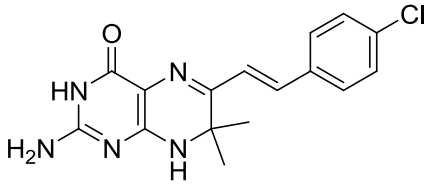
1.43 (WSG1031)



1.2 (WSG1002)

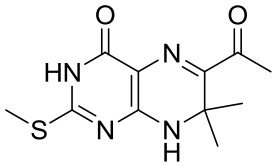


1.44



1.41 (WSG1017)

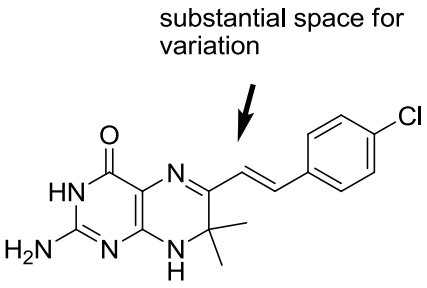
Activators



1.45

Inhibitors

3) Considerable space exists in the enzyme at C^6 (B) as pointed in **1.41**, which is required for binding at the active position of the enzyme.

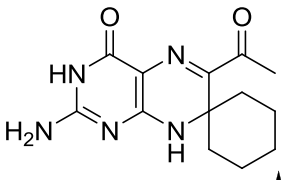


1.41 (WSG1017)

4) Elimination of one pyrimidine 'amino-oxo' functionality produces an inhibitor.

5) Electron-withdrawing groups at C^6 (B) supports NOS activation.

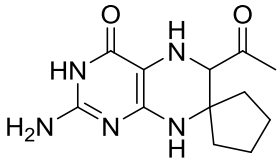
6) Any two groups bigger than methyl at C^7 (C) could be too large to fit into the active position of NOS.



1.46

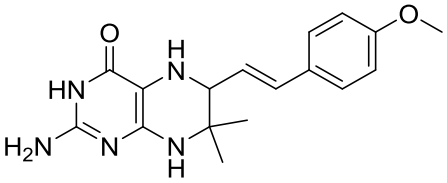
Two groups larger than Me could be big to bind NOS

Raghu *et al.*[190] from our research group found that tetrahydropteridine (**1.47**) is a NOS activator. There was one exception in that example that the reaction remained slow for some seconds and after this appeared to be fast. Therefore it was assumed that **1.47** required a little time to bind to the active site.[190]

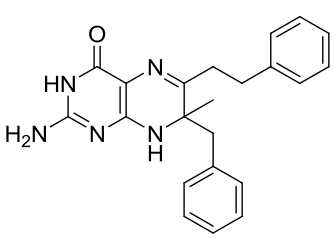


1.47

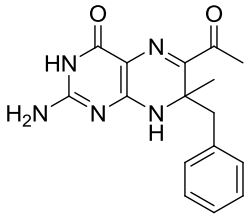
Raghu *et al.* [190] also found that the benzyl group present at C^7 in **1.48-1.53** is too big to go in the active site of nNOS but **2.203** gave 12 % activity.



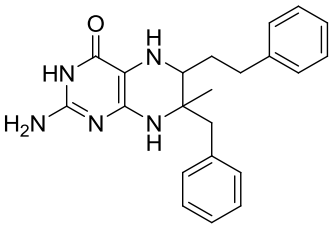
2.203



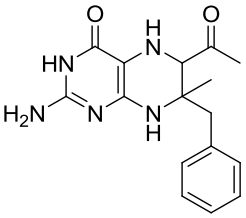
1.48



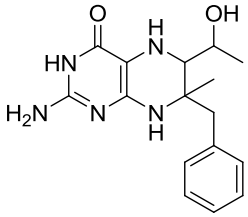
1.49



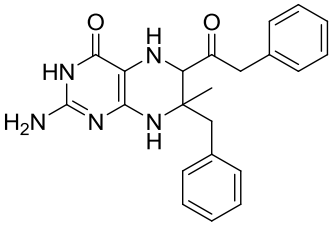
1.50



1.51

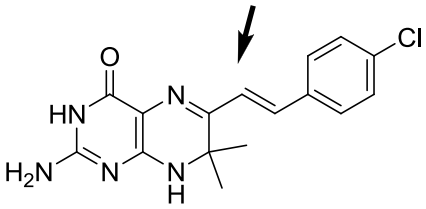


1.52



1.53

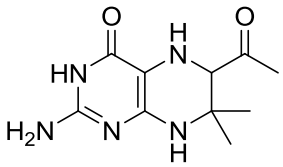
substantial space for variation



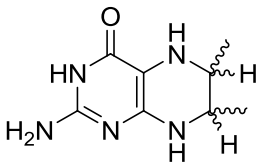
1.41 (WSG1017)

The fact that such a simple synthetic pteridine, which lacks side chain functional groups, can act as a coenzyme suggests that most of the important interactions for binding take place between the basic pteridine scaffold: this will be addressed later in **Section 6.7**.

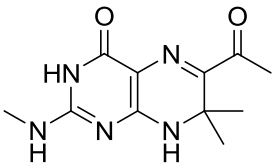
McInnes *et al.*[191] found that a racemic mixture of 5,6-dimethyltetrahydropterin **1.55** is a NOS activator. This simple synthetic pteridine does not have a side-chain and yet can function as a coenzyme. We can propose from this that nearly all of the significant interactions for binding take place between the primary pteridine structure and the active site of the NOS enzyme. The 2-*N*-methylaminopterin **1.56** did not bind with the enzyme, which indicates that extension at the C^2 site restricts the pterin from serving as a BH_4 analogue for NOS and which also shows that there is inadequate space accessible for binding. Furthermore, it explains that hydrogen bonding is very important at position 2 of the pteridine. On the other hand, high activity was found for pterins **1.57** and **1.58** as described in next paragraph.[191]



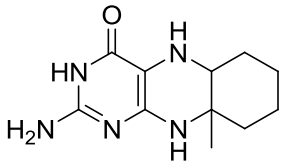
1.54 WSG1002



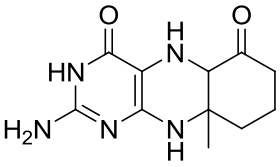
1.55



1.56



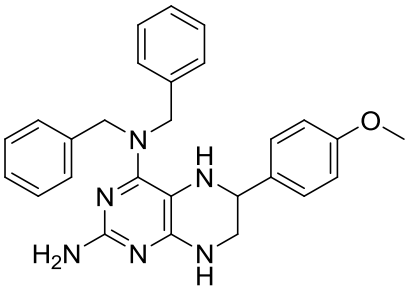
1.57



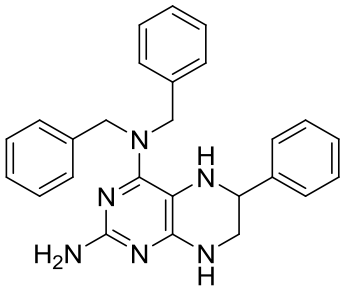
1.58

The tetrahydropterin **1.57** and **1.58** had basically equal affinity for nNOS. This could be due to the great correlation between these structures. The tetrahydropterin **1.57** showed a turnover rate (2.8 ± 0.40 min^{-1}), while the tetrahydropterin **1.58** showed an extraordinary turnover rate (9.61 ± 0.67 min^{-1}), which was very near to the rate obtained by BH_4 (13 min^{-1}). It was clear that it is an excellent example of a synthetic pteridine acting as a BH_4 analogue. [191]

Schmidt *et al.*[192] studied effects of pteridine derivatives on nNOS inhibition with different substituents at 2, 4, 6 and 7 positions. The substituents at the 2, 6 and 7 positions had no noticeable effect on NOS inhibition in the dihydro oxidation state of the pteridine. But, in the tetrahydro form, the pteridine derivatives with C^6-substituents were potent inhibitors. The IC_{50} for compound **1.59** was 5 µM and for compound **1.60**, IC_{50} was 3 µM while BH_4 had K_m of 1 µM).[193, 194]



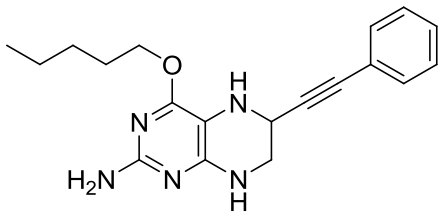
1.59



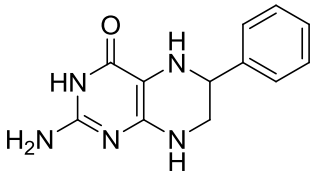
1.60

It was proposed that a significantly great amount of space is free at the 4-amino position of the pterin binding site due to large benzyl groups which were present in these potent inhibitor derivatives.

Schmidt *et al.*[194] studied another structure-activity relationship (SAR) with further modifications at the 2, 4, 5, 6 and 7 positions, principally concerned with 4-oxo substituents. The most effective inhibitors were **1.61** possessing an IC_{50} value of 8 μM and **1.62** with an IC_{50} of 7 μM.



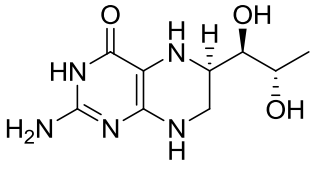
1.61



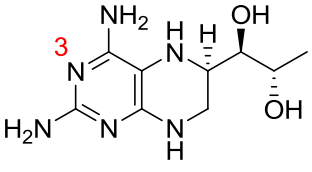
1.62

Schmidt *et al.*[108,194] also found another inhibitor **1.63** with IC_{50} value of 30 μM while their most active inhibitor was **1.64** had an IC_{50} value of 2 μM.

4-Amino-BH_4 **1.29** lacks the proton at N^3 and is an inhibitor of NO synthesis, which suggests that this particular proton plays a role in the molecular mechanism of NO synthesis.[195] 4-Amino-BH_4 **1.29** is possibly the best inhibitor of NOS, with an IC_{50} value of 1 μM, while BH_4 has a Km of 1 μM).[194,196,197]



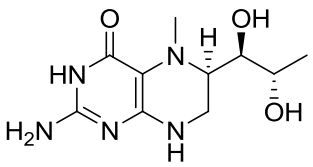
1.1 (BH_4)



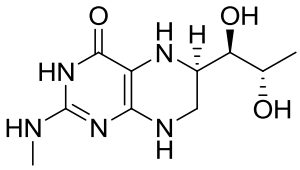
1.29

Werner and his co-workers reported that due to a positive inductive effect of methyl substituents, 5-methyl-BH_4 **1.30** and 2-*N*-methyl-BH_4 **1.65** have the capability to give an electron to NOS more efficiently than BH_4 **1.1**.[198]

It can be proposed that 5-methyl-BH_4 **1.30** and 2-*N*-methyl analogues **1.65** served as weak activators of NOS was because NOS "favours" BH_4 **1.1** over its 5-methyl analogue **1.30** and 2-*N*-methyl analogue **1.65** as a consequence of unfavourable steric interactions. [198]



1.30



1.65

5-Methyl-BH_4 **1.30** has approximately 20-fold lower affinity for NOS enzyme[199] and has been used in many mechanistic studies. **1.30** has also served to confirm the redox role of BH_4 **1.1** for NOS enzymes.[43, 186, 200, 201, 202, 203] If the enzyme is saturated with 5-methyl-BH_4 **1.30**, the rate of NO generation is in fact greater than with BH_4 **1.1**. When studying a merely electronic justification, we find that the greater the electron density in the pterin, the more easily it will donate an electron.

There is a current working assumption that inhibition of eNOS or nNOS induces illness. The initiation of inducible nitric oxide synthase (iNOS) is not good and distinct inhibition of this enzyme is useful.[204] The iNOS is continuously working and generates NO until the substrate is used.[205] A drug that decreases nitric oxide generation, not simply inhibits NO synthesis but also induces hypertension and lack of vasoconstrictor reaction. Therefore, this balance of NO in the body is one of the crucial parts for the medication for several vascular disorders. Hence, it is advantageous to have a molecule which can selectively activate eNOS in therapy for specific vascular disorders.

In summary, BH_4 is a crucial cofactor of various enzymes. BH_4 is essential in assuring that those enzymes continue their part in different biological functions. Hence, an inadequate amount of BH_4 might end in different diseases linked to regulative systems in the body such as many functions in the tissues and organs of the circulatory, neural and immune systems. Several investigations have emerged explaining that, this kind of diseases have been adequately reduced by BH_4 supplementation.[175] BH_4 in its effective, reduced form is very unstable and hence not appropriate for oral treatment. We need to develop an oxidatively stable and lipophilic pteridine molecule which can act as a cofactor for nitric oxide synthase (NOS) and can take us one step forward to open ways for the modern therapeutic possibilities of the disorders linked with BH_4 deficiency.

1.6.4 The possible electron transfer mechanism for blocked dihydrobiopterins WSG 1002

As mentioned in section 1.4.2, for hydroxylation of arginine to occur, BH_4 donates its electron from N^5 towards the dioxygen iron species (**Figure 1.18 c** and **d**). WSG 1002 (**1.2**) was found to be causing a significant relaxation in pulmonary arteries.[189] One possible hypothesis for this relexation was that WSG 1002 (**1.2**) binds to the active site of NOS enzyme and is involved in the captodative radical mechanism for electron transfer from N^5 towards heme. The possible electron transfer cycle of **1.2** is depicted in **Figure 1.28**. The term captor referes to the electron-withdrawing group and dative refers to the electron-donating group so in captodative effect, free radicals are stablised by nearby electron-withdrawing and electron-donating substituents.[259]

In **Figure 1.28** WSG 1002 (**1.2**) is shown to accept an electron from NADP to give an intermediate **2.1a**, which has a free radical at C^6. This intermediate bearing a free radical can exist in resonance with structure **2.1b**. By protonation **2.1a** and **2.1b** can give **2.1c** and by accepting an electron, **2.1c** can form **2.1d**. The deprotonation of **2.1d** can give back dihydropterine WSG 1002 (**1.2**) and all these steps are considered reversible.

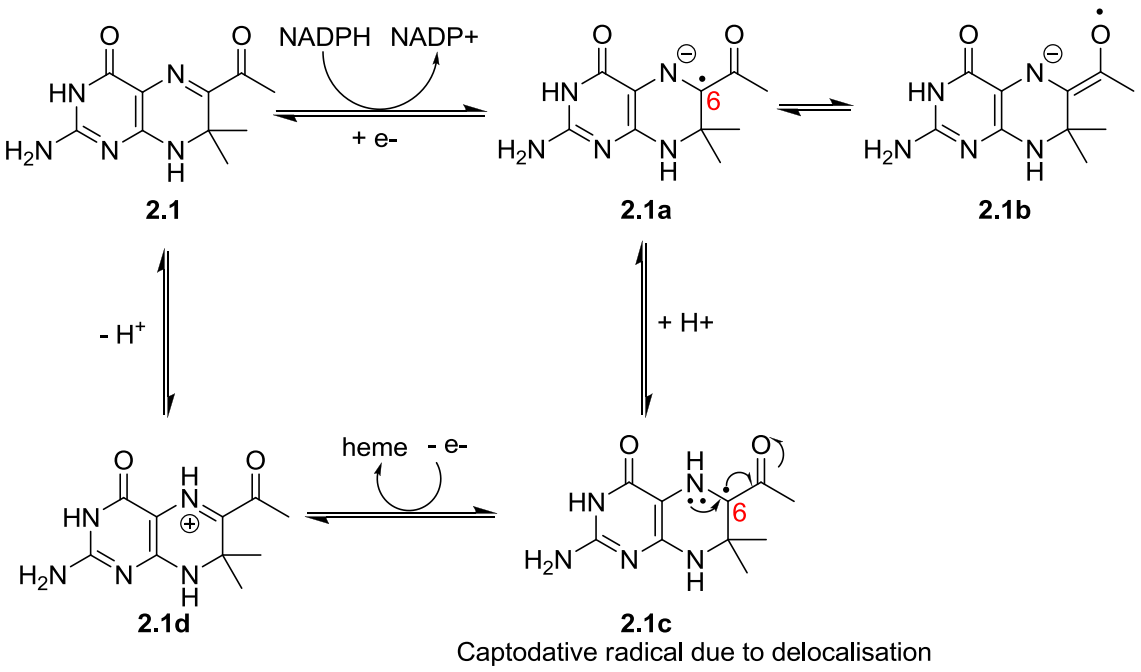


Figure: 1.28 *Hypothetical captodative radical mechanism of WSG1002 for a donation of electron to heme.*

1.7 Project aims

Deficiency of nitric oxide causes so many diseases which have been mentioned previously, but still there is no proper drug to prevent or treat these diseases. Tetrahydrobiopterin (BH_4) is an essential cofactor of NOS enzyme, without which nitric oxide generation stops. However BH_4 also has its drawbacks. It just acts as a cofactor of NOS enzyme in its tetrahydro form and it is not stable in tetrahydro form. It is a polar molecule with more hydrogen bonding, and so does not easily cross the blood-brain barrier and has a solubility problem. It also has a side-chain cleavage problem and all these characteristic of BH_4 cause a bioavailability problem for acting as a cofactor of NOS enzyme in various parts of the body which results in nitric oxide deficiency and diseases.

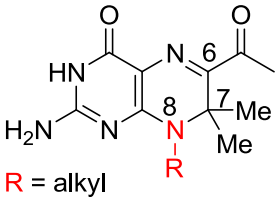
Suckling and his co-workers prepared a range of pteridine compounds with modifications at site 2, 6 and 7 in the 1980s.[190] In recent years, some of these compounds were chosen as BH_4 replacements for the investigation of nitric oxide synthesis. This investigation showed that several compounds in the family supported NO production by NOS. This investigation

showed that that WSG1002 **(1.2)** was able to simulate the effects of BH_4 in endothelial cells,[190] so a research project was started to study those pteridine compounds and to synthesise new derivatives with a hope to find the best BH_4 substitute. [190, 206, 207]

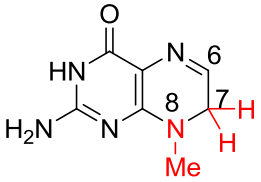
However all compounds tested so far have many H-bonding sites including the 2-amino, 4-oxo system and a proton at N^8. Each of these plays a part in making the compounds hydrophilic and possessing of a high melting point, which may not be desirable for a drug. So the structure of WSG1002 needs to be modified to get compounds more suitable to be drugs. But we can also ask detailed questions about the mechanism of action of BH_4 such as influence of substituents on N^2, C^4 and N^8. 4-Amino-7,8-dihydropteridines have the ability to inhibit NO synthase and can be can be utilized for the therapy of disorders which are induced by an enhanced nitric oxide level.[194]

The question arose of what might be better substitutes for BH_4, for example N^8 substituents **1.66**, **1.67** or 8-deaza compounds so my research project involved the synthesis of N^8-substituted and N^8-deaza analogue of BH_4 with some variations at C^6, **1.68**. As the mechanism of nitric oxide formation and role of BH_4 is not fully understood so we were also hoping that BH_4 analogues would improve our mechanistic understanding of NOS.

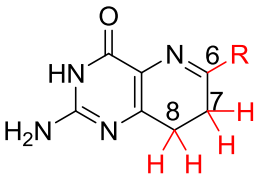
Putting an alkyl group at N^8 should make a dihydropterin more oxidatively stable, decrease hydrogen bonding, and make the compound more lipophilic. It was also of interest to investigate the effects of an alkyl group such as methyl at N^8 **1.66, 1.67** or just to substitute the nitrogen with carbon **1.68**, so analogues of BH_4 with methyl at N^8 and its N^8 deaza analogues were prepared. N^8 substituted analogues **1.66**, **1.67** would show that how much space is available for binding at active site of the enzyme and any effect of alkyl group for electron donation too. The N^8 deaza analogue **1.68** would show the role of nitrogen for binding and activating NOS enzyme.



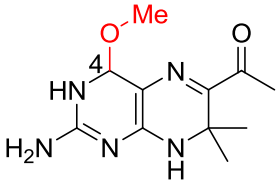
R = alkyl or aryl **1.66**



1.67



R = Me, hydroxy methyl acetoxy methyl **1.68**



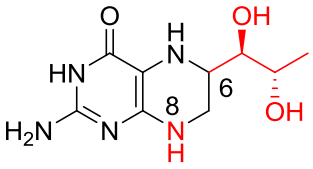
1.69

CHAPTER 2

Diversity at C^8 in Pteridines

2.1 Introduction

As described before in section 1.1, BH_4 is an essential cofactor for nitric oxide synthase enzyme. However, it has limited physicochemical characteristics as a medicine as it is very unstable in its active, reduced form and can easily be oxidized into its dihydro form (BH_2), which inhibits the enzyme activity.

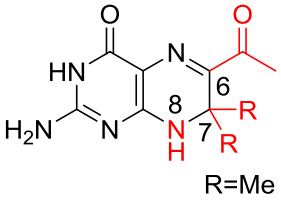


(BH_4)

1.1

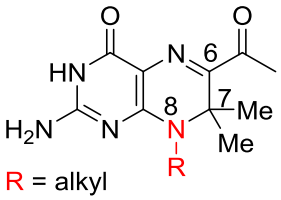
BH_4 is still the standard therapeutic approach in cardiovascular disease, but alternative strategies are required to target BH_4-dependent endothelial role to control vascular diseases.[173] The above-mentioned defects in BH_4 lead to a clinical requirement to design an oxidatively stable cofactor with proper physicochemical properties which can improve BH_4 insufficiency in BH_4-deficient patients.

As mentioned in section 1.6.1, WSG1002 (**W**ood, **S**uckling, **G**ibson) **1.2** was synthesised by Suckling and co-workers, and is an analogue of BH_4 and a NOS activator. This was a very encouraging result and it gave a motivation that a drug-like analogue of BH_4 could be found. The preparation of more suitable analogues with different electrochemical properties will further support the understanding of the specific mechanism of NOS action. While **1.2** is an effective compound, improvements could be approached by substituent modification.

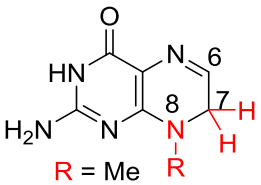


1.2

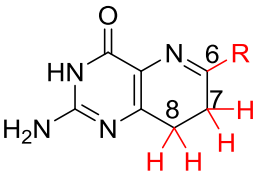
Addition of a methyl group at the N^5 position of BH_4, including other tetrahydropterins, makes them more stable towards autoxidation.[186] It was expected that placing an alkyl group at the N^8-position of **1.66** and **1.67** would result in oxidatively more stable analogues; that are more lipophilic and with decreased hydrogen bonding. We were interested in understanding the effect of placing an alkyl group, such as methyl, at N^8 (**1.66**, **1.67**) or replacing the nitrogen atom with a carbon as in **1.68**.



1.66

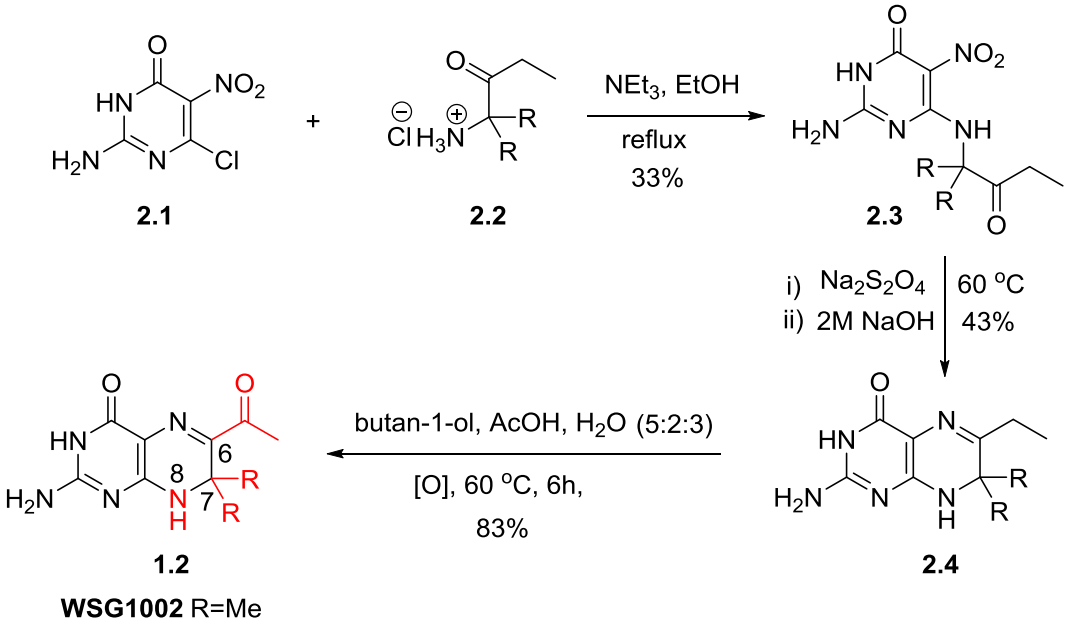


1.67



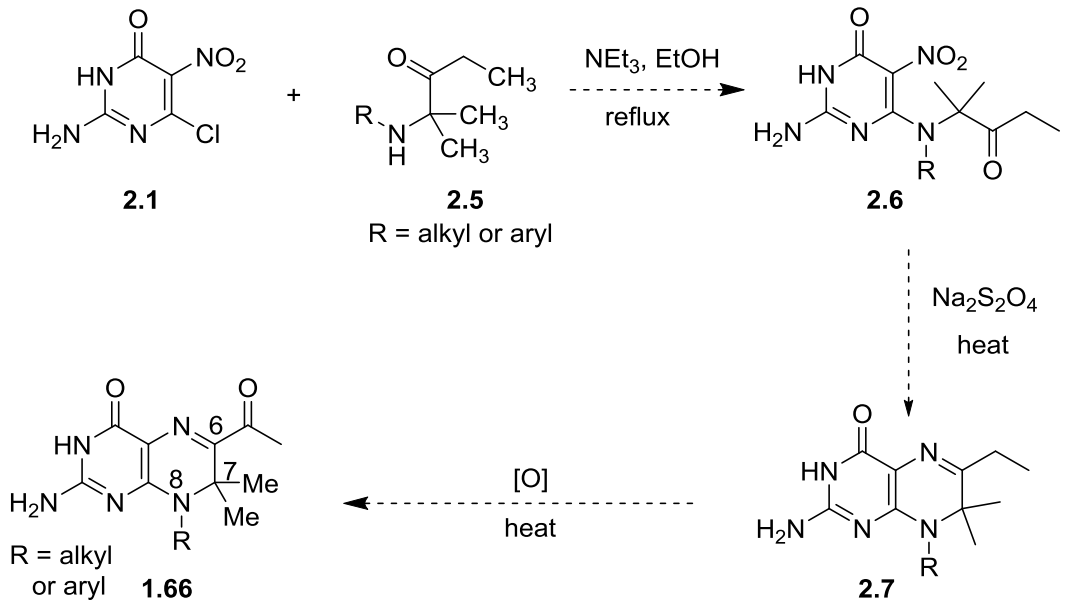
1.68
R = Me, hydroxymethyl
acetoxymethyl

The blocked dihydropterin WSG1002 (**1.2**) has two alkyl groups at the 7-position of the pteridine, which compelled it to persist at the dihydro oxidation state; therefore **1.2** was called blocked dihydropteridine. The synthesis of **1.2** involves preparation of 5-nitropyrimidine **2.1**, which was produced by nitration of 2-amino-6-chloropyrimidine. The condensation of this nitropyrimidine **2.1** with 2-methyl-3-oxo-2-pentanaminium chloride **2.2** provided 6-(substituted amino)-5-nitropyrimidine **2.3** in 33% yield**.** The 5-aminopyrimidine, produced by the reduction of the nitro group of **2.3** with sodium dithionite, participated in an intramolecular cyclisation to produce pteridine **2.4** in 43% yield. This pteridine on atmospheric oxidation produced (WSG1002) **1.2** in 83% yield.



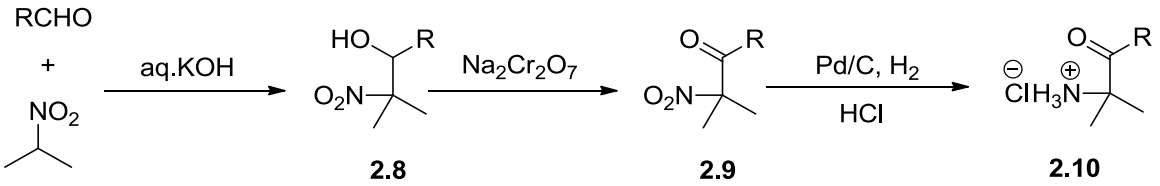
Scheme 2.1

As stated before in part **1.7**, the first aim of the project was to synthesise blocked dihydropterin **1.66**, the N^8-substituted analogue of parent blocked dihydropterin WSG1002 (**1.2**). The proposed synthetic strategy that would lead to new types of compounds **1.66**, with an additional alkyl or aryl group at N^8, is shown in Scheme **2.2**.



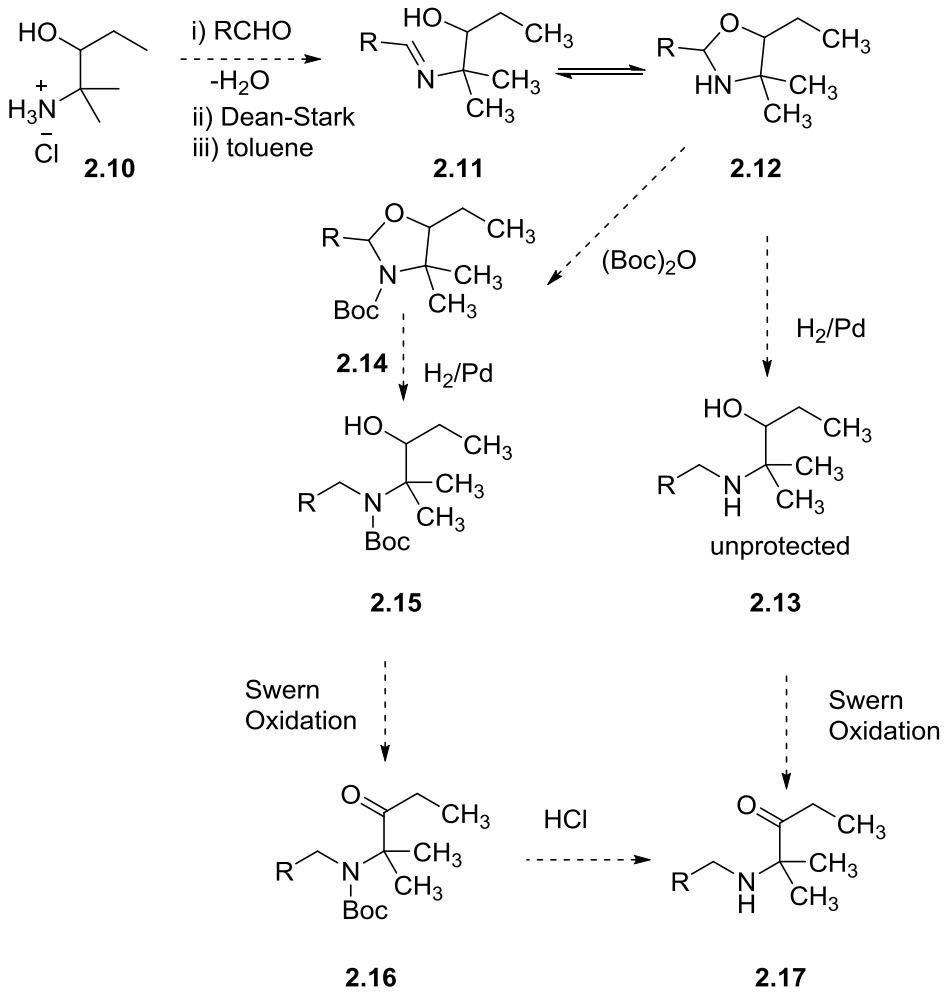
Scheme 2.2

Most of the reactions in **Scheme 2.2** followed earlier work carried out in our laboratory.[188, 208] Al-Hassan *et al*.[188] synthesised the blocked dihydropterins such as **1.2** by using aminoketones as intermediates to couple and cyclise with chloronitropyrimidine **2.1.** The established synthetic route for simple aminoketones **2.10** is given in **Scheme 2.3**.



Scheme 2.3

In order to obtain an 8-*N*-alkyl or aryl pteridine, the *N*-alkyl or aryl analogues of aminoketones **2.10** and **2.17** are required**.** The proposed synthesis for 2-(alkyl or phenyl)amino ketone **2.17** is shown in **Scheme 2.4**.

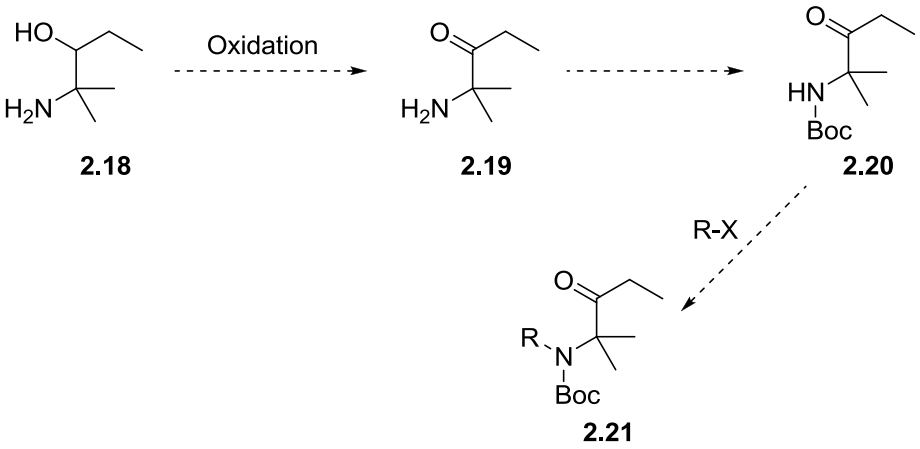


Scheme 2.4

Condensation of an amino alcohol with an aldehyde is understood to produce a product which exists in solution as a mixture of the required imine, together with the corresponding ring-closed oxazolidine.[209,210] Amino alcohols **2.13** and **2.15** can be converted into amino ketones **2.16** and **2.17** by Swern oxidation.

Another possible route for preparation of WSG 1002 (**1.2**) with an additional alkyl group at N^8 is without preparation of an oxazolidine**.** The carbonyl group present in the amino carbonyl compounds easily undergoes successive conversions.[211] By oxidation, amino alcohol **2.18** can be converted into amino ketone[212] **2.19** (**Scheme 2.5**). By partially protecting the amine and then by the reaction with an alkyl halide it could lead to *N*-substituted amino

ketone **2.21.** By *N*-Boc deprotection of **2.21**, aminoketone **2.17** can be obtained, which is an intermediate in the preparation of *N*-substituted analogues of WSG1002 (**1.2**).

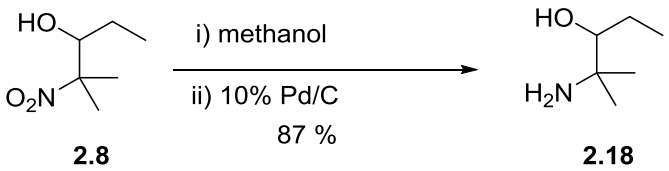


Scheme 2.5

2.2.0 Results and Discussion

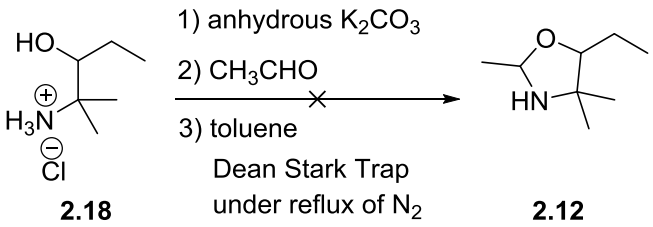
2.2.1 Approaches to the synthesis of N^8 substituted blocked dialkyl pterins through oxazolidine intermediates

2-Amino-2-methyl-pentan-3-ol **2.18** was required for the preparation of oxazolidine. It was prepared by the reduction of 2-methyl-2-nitro-pentan-3-ol **2.8** in methanol using palladium over charcoal in 87% yield, which was pure by TLC and NMR (**Scheme 2.6**).



Scheme 2.6

The preparation of oxazolidine **2.12** (Table 2.1, Entry 1) using amino-alcohol hydrochloride salt **2.18** and acetaldehyde in ethanol for 48 hours was not successful. A yellow oily material was produced which showed some of the expected signals by ^{1}H-NMR such as a quartet (δ 4.70) and a doublet (δ 1.27), however it decomposed during work up and purification by silica gel chromatography (**Scheme 2.7**).



Scheme 2.7

Entry	Salt of amino Alcohol 2.18	Acetaldehyde	Anhydrous K_2CO_3	Conditions	Reaction time
1	5.0 mmol, 1 equiv.	10.0 mmol, 2 equiv.	10.0 mmol, 2 equiv.	anhydrous K_2CO_3 in ethanol, room temperature	48 h
2	3.3 mmol, 1 equiv.	85.0 mmol, 26 equiv.	3.3 mmol, 1 equiv.	activated molecular sieves in a sealed tube, 40 ^oC	12 h
3	5.0 mmol, 1 equiv.	5.0 mmol, 1 equiv.	10.0 mmol, 2 equiv.	Dean Stark trap, toluene as a solvent, 120 ^oC	16 h

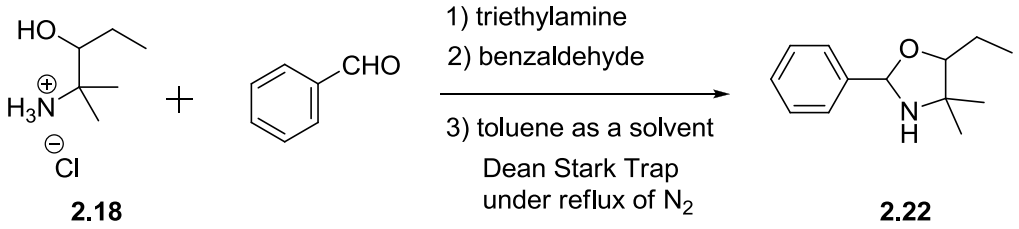
Table 2.1

Water is produced during oxazolidine formation, and various techniques for removal of this liberated water from the reaction mixture have been suggested.[213] Normally, azeotropic distillation[214] is applied, or molecular sieves are added or dehydrating agents such as $MgSO_4$ [215] are used. The preparation of oxazolidine **2.12**, using activated molecular sieves (Table 2.1, Entry 2) or using Dean Stark trap (Table 2.1, Entry 3) following the method of David *et al.*[216] were not successful.

It was decided to use benzaldehyde for the preparation of an oxazolidine because it is possible that it can form a more stable oxazolidine than acetaldehyde; a phenyl group is a weak electron-withdrawing group and also a larger group than ethyl, so purification of the product may have been easier.

The reaction of 3-hydroxy-2-methyl-2-pentanaminium chloride salt **2.18** with benzaldehyde (2.0 equiv.) in toluene and triethylamine, using activated molecular sieves in a sealed tube at 40 ^oC for 16 h (**Scheme 2.8**) (Entry 1, Table 2.2), gave oxazolidine **2.22** (15%). In the ^1H-

NMR spectrum there was a doublet of doublets at δ_H 3.46 which corresponded to O*C*<u>*H*</u>-CH_2, while the benzylic proton appeared at δ_H 7.90.



Scheme 2.8

Entry	Amino Alcohol **2.18**	Benzaldehyde	Triethylamine	Reaction time (h)	Yield (%)
1	3.26 mmol, 1 equiv (salt)	10.0 mmol, 2 equiv.	9.78 mmol, 1.5 equiv.	16	15
2	6.52 mmol, 1 equiv. (salt)	16.3 mmol, 2.5 equiv.	9.77 mmol, 1.5 equiv.	18	14
3	4.06 mmol, 1 equiv	4.06 mmol, 1equiv.	6.09 mmol, 1.5 equiv.	16	8

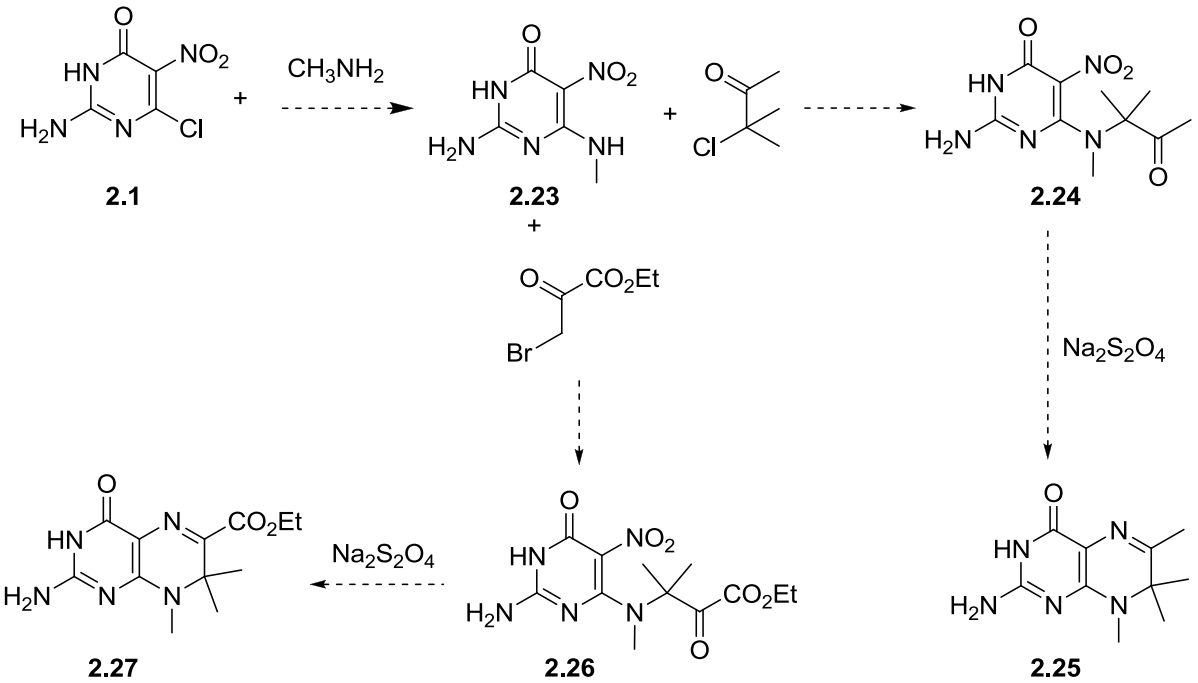
Table 2.2

When benzaldehyde (2.5 equiv.) was used at 120 ^oC for 18 h using a Dean Stark trap (Table 2.2, Entry 2), the yield of oxazolidine **2.22** was 14%. This oxazolidine was decomposing during purification and the yield could not be increased. Furthermore, it was found that the increase in molar concentration of benzaldehyde did not increase the yield of oxazolidine **2.22**.

Vega-Perez *et al.*[217] synthesised a related oxazolidine using magnesium sulfate as a dehydrating agent; the oxazolidine was characterized by its mass spectrum and was used without further purification because of its low stability. The instability of the oxazolidine

made it difficult to complete the planned synthetic route. It became obvious that it would not be worthwhile to spend more time on the preparation of this intermediate so this work was stopped and focus was directed towards the next target for preparing 8-*N*-alkyl or aryl pteridine.

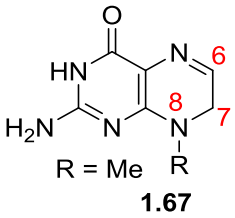
As a proposal for further work, a different path to the N^8 substituted analogue of WSG1002 (**1.2**) is presented in **Scheme 2.9**. The reaction of 6-chloro-5-nitropyrimidine **2.1** with methylamine would produce 6-methylamine pyrimidine **2.23**, which could be allowed to react with α-chloroketone or ethyl bromopyruvate using a suitable base to give *N*-substituted pyrimidine **2.24** or **2.26**. The reduction of 5-nitropyrimidines **2.24** or **2.26** using sodium dithionite would give 5-aminopyrimidine, enabling an intramolecular cyclisation to produce *N*-substituted pteridines **2.25** or **2.27**. This route would avoid the preparation of an unstable oxazolidine as an intermediate.



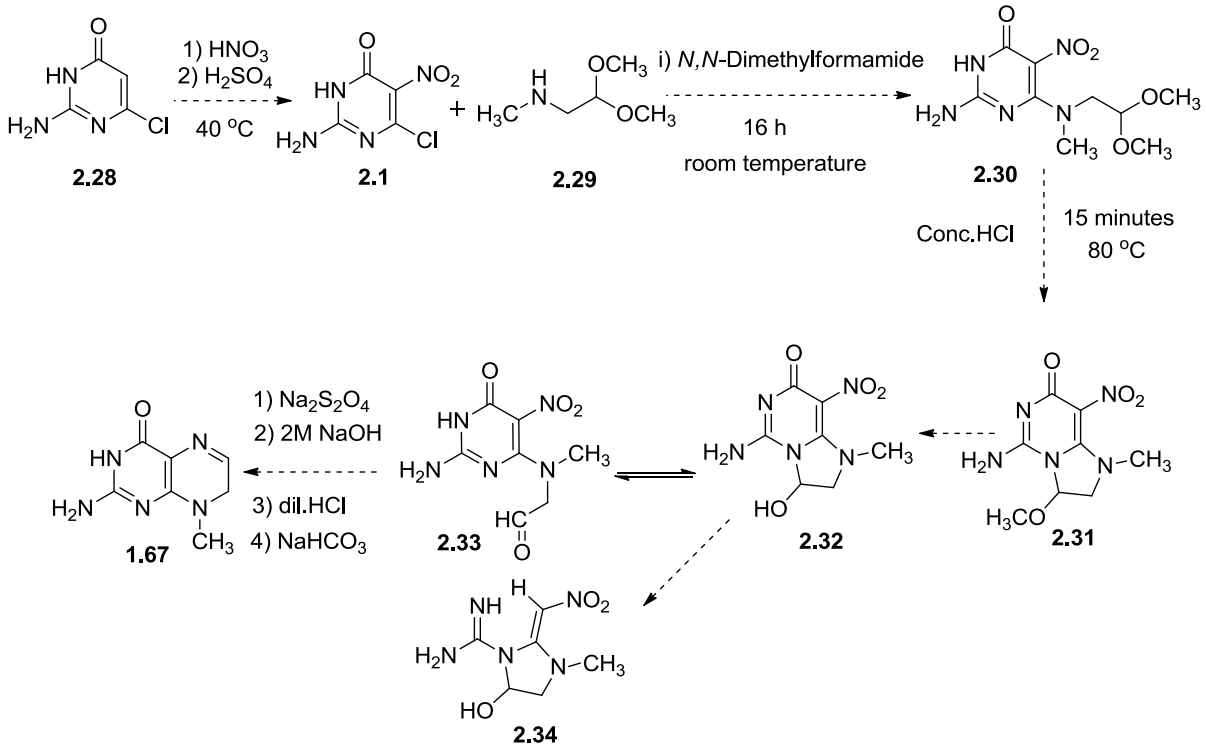
Scheme 2.9

2.3.0 The proposed synthesis of WSG1002 analogues, unsubstituted at C^7 and with variation at N^8.

As described in section 1.7, the second strategy for preparing 8-*N*-alkyl or aryl pteridine was to synthesise the equivalent compound (**1.67**), substituted at N^8 but unsubstituted at C^6 and C^7, with the purpose that compounds with variations at N^8 could be made and assessed.

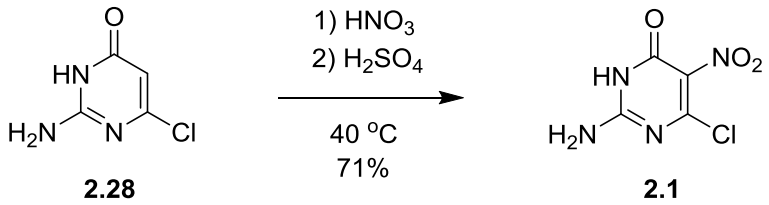


The synthetic procedure, as described by Brown *et al.*,[218] was followed for the preparation of 7,8-dihydro-8-methylpterin **1.67**. The proposed synthesis for target compound, 7,8-dihydro-8-methylpterin **1.67** is shown in **Scheme 2.10**.



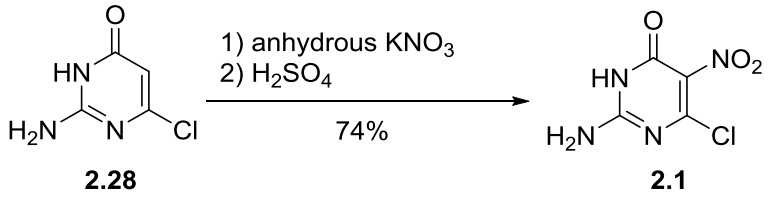
Scheme 2.10

The first step in this reaction scheme was the nitration of 2-amino-6-chloro-5-nitro-4(3*H*)-pyrimidinone **2.28**. This is an essential intermediate in many of the heterocyclic syntheses contained within this thesis and is one principal starting material for synthesis of pteridines**.** Many modifications for this reaction have been published. [219,220] The nitration of 2-amino-6-chloropyrimidine **2.28** using concentrated nitric and sulfuric acid for 2 hours at room temperature gave nitropyrimidine **2.1** in 71% yield, as shown in **Scheme 2.11**.



Scheme 2.11

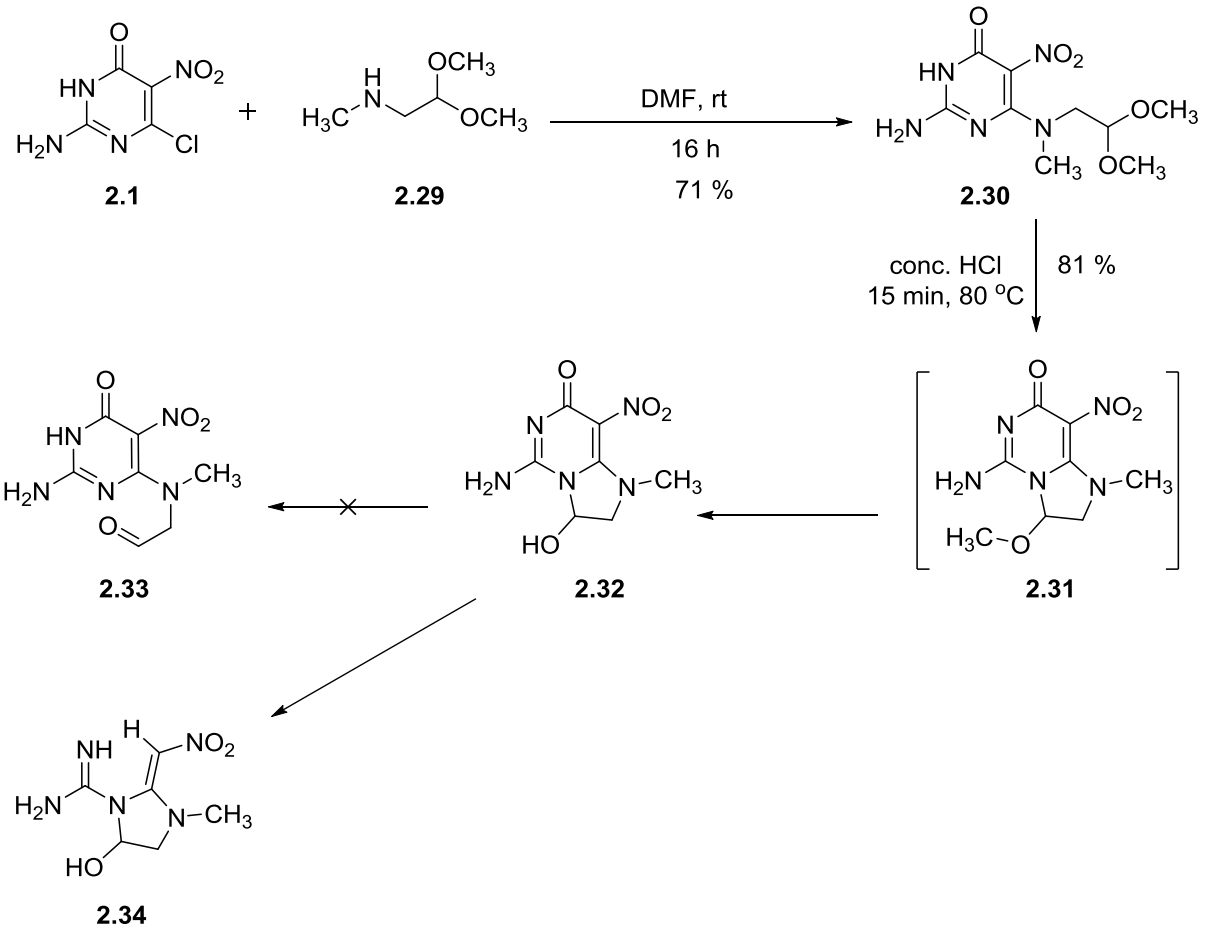
Boyle *et al.*[221] reported the nitration of 6-chloro-pyrimidinone **2.28** using potassium nitrate as a nitrating agent in concentrated sulfuric acid. It was described that after 3 hours reaction time, the product of nitration was 5-nitro-pyrimidinone **2.1**. This method of nitration, using one molar potassium nitrate and concentrated sulfuric acid, stirring for 2 hours at room temperature, resulted in the formation of chloronitropyrimidine **2.1** as a light-yellow solid in 74% yield (**Scheme 2.12**).



Scheme 2.12

2.3.1 Coupling of chloronitropyrimidine 2.1 with acetal 2.29 and hydrolysis of C^6-substituted pyrimidine 2.30

The nitro group increases the nucleophilic susceptibility of the C^6 position of pyrimidine **2.1** and supports the coupling with methylaminoacetaldehyde dimethyl acetal **2.29** by a nucleophilic substitution reaction. For the coupling, 2.5 equiv. of acetal **2.29** was used so that it can also act as a base. The expected product was precipitated cleanly by adding the reaction mixture to crushed ice to give the C^6-substituted pyrimidine **2.30**, as a light-yellow solid in 71 % yield (**Scheme 2.13**).

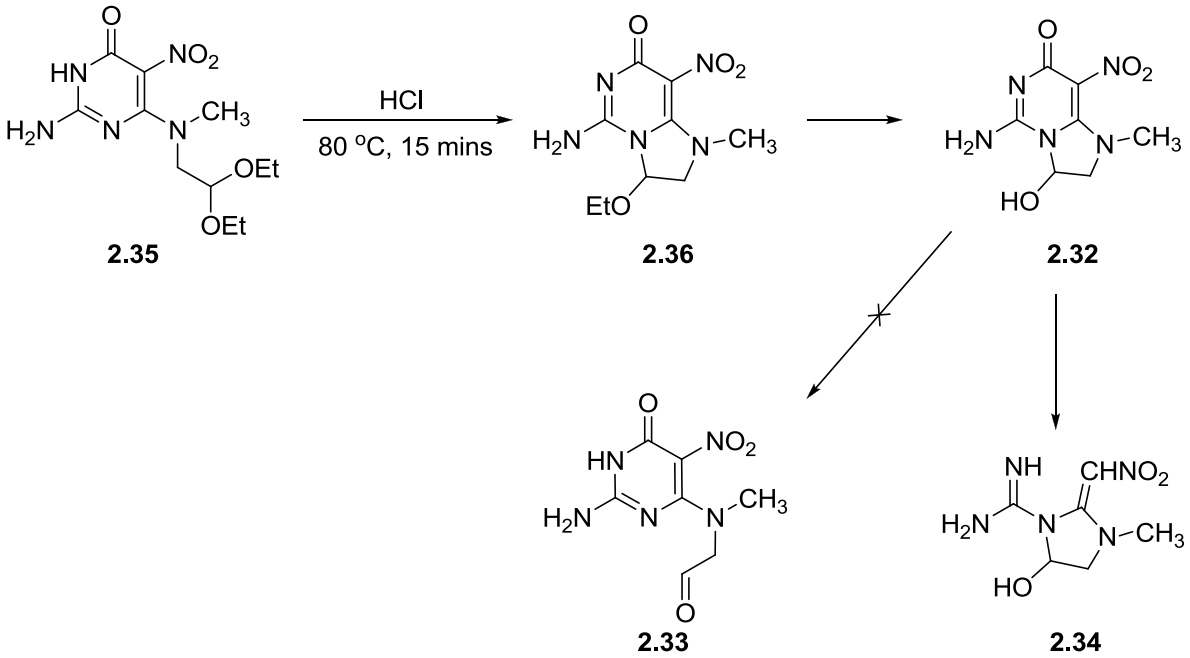


Scheme 2.13

The C^6-substituted pyrimidine **2.30** was subjected to acid hydrolysis to afford the corresponding aminoketone hemiacetal **2.32** as a yellow solid in 81 % yield. Based on the ^1H

NMR spectrum, the major product of this reaction was **2.32** having little side-products; imidazoline **2.34** and an intermediate **2.31** (**Scheme 2.13**). The ^{1}H NMR indicated that there was no resonance corresponding to the aldehyde of **2.33.** Two CH_2 protons next to CH-OH in **2.32** appeared as a doublet at δ 4.13 while CH-OH proton appeared as triplet at δ 6.20.

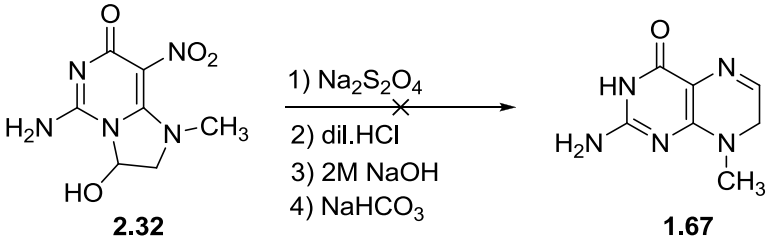
Pfleiderer and Zondler[222] could not get aldehyde **2.33** during acidic hydrolysis of C^6-substituted pyrimidine **2.35** due to a pyrimidine opening reaction occurring to give imidazoline **2.34**, as shown in **Scheme 2.14.**



Scheme 2.14

2.3.2 Cyclisation of substituted pyrimidine 2.32

Cyclisation of substituted pyrimidine **2.32** was attempted *in situ* by reduction using sodium dithionite to give a new class of blocked dihydropteridines (**1.67**) as shown in **Scheme 2.15**. After work up, it was found that desired product **1.67** could not be formed by this reaction.

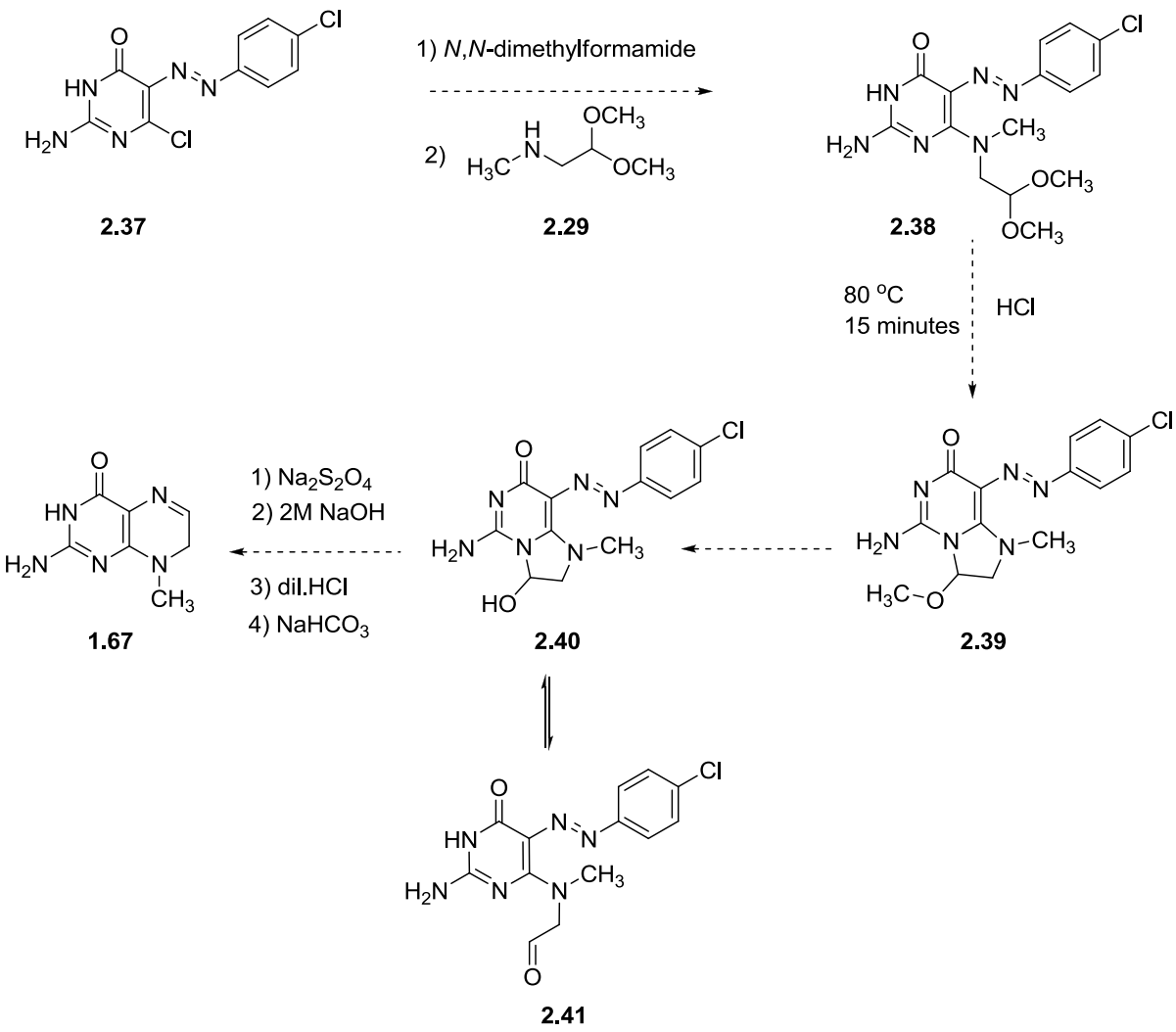


Scheme 2.15

The second attempt for reduction and cyclisation of substituted pyrimidine **2.32,** using an excess of sodium dithionite was also not successful and the product **1.67** was oxidized to an unidentified side-product. Therefore, it was decided to synthesise the substituted pyrimidine **2.32** by a variation of this route.

2.4.0 Alternative route for synthesising dihydropteridine 2.23

Brown *et al.*[218] synthesised dihydropteridines **1.67** by a comparable route *via* the 5-arylazopyrimidine **2.37**, which is not very sensitive to ring-opening during hydrolysis of the acetal **2.38** with concentrated hydrochloric acid**.** The proposed reaction scheme for the preparation of dihydropteridines **1.67** *via* the 5-arylazopyrimidine **2.37** is shown in **Scheme 2.16.**

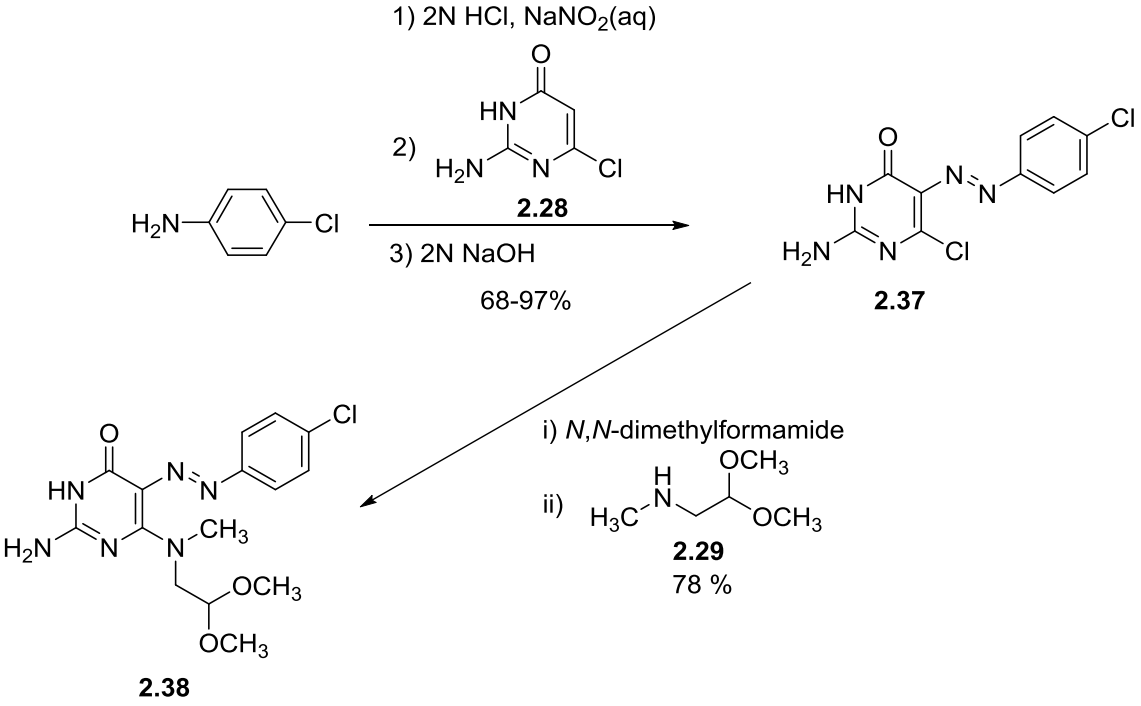


Scheme 2.16

2.4.1 Diazotisation of pyrimidine 2.28 and coupling of 5-arylazopyrimidine 2.37 with acetal 2.29.

As pure dihydropteridine **1.67** could not be prepared by the first strategy, a second strategy was considered. The first reaction of this scheme was to prepare 5-substituted pyrimidine **2.37**. If two electron-donating substituents, *e.g.,* NH, or OH, are present in pyrimidines, the electrophilic substitution at C^5 takes place easily, although one such substituent is enough for halogenation.[223] The diazotisation of pyrimidine **2.28** was successful every time (**Scheme**

2.17). As compared to nitration this reaction was very clean and the product was stable. The diazotisation of pyrimidine **2.28** was successful on 5 g scale as well; by lowering the temperature of the Dewar apparatus to -20 °C, the product **2.37** was successfully obtained in 68-97% yield on 1.0-5.0 g scale at -10 to -20 °C.



Scheme 2.17

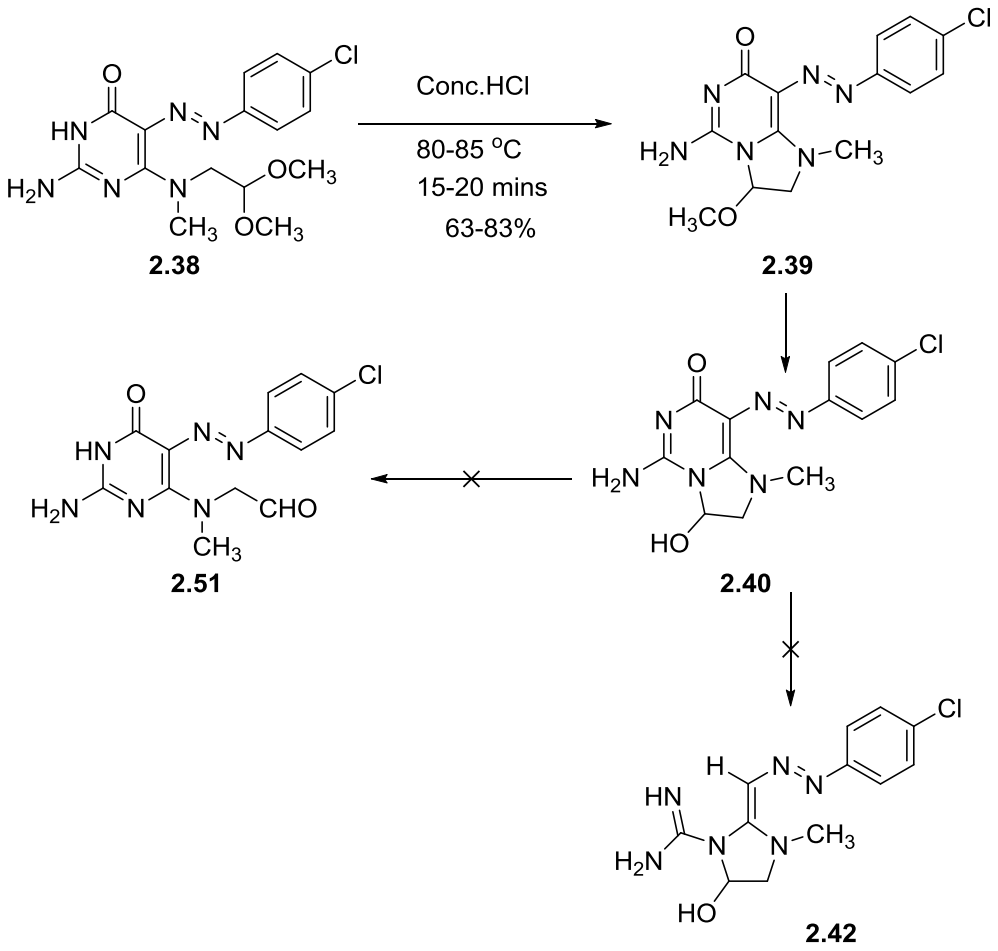
The azo group enhances the nucleophilic susceptibility at the C^6 position of pyrimidine **2.37** and supports coupling with methylaminoacetaldehyde dimethyl acetal **2.29** by a nucleophilic substitution reaction. 5-Chlorophenylazopyrimidine **2.37** was subjected to the coupling reaction with methylaminoacetaldehyde dimethyl acetal **2.29** in *N*,*N*-dimethylformamide under reflux conditions (**Scheme 2.17**). The product **2.38** was obtained successfully as an orange-coloured solid in 57-78% yield on 0.7-7.0 g scale at room temperature (Table 2.3).

Entry	Acetal 2.29	Reaction Time (h)	Mass of 2.38 (g)	Yield of 2.38 (%)
1	6.18 mmol, 2.5 equiv.	16	0.710	78
2	7.71 mmol, 2.5 equiv.	14	0.760	67
3	10.22 mmol, 2.5 equiv.	16	1.136	76
4	74.19 mmol, 3.0 equiv.	16	6.815	75
5	20.33 mmol, 3.0 equiv	16	1.421	57
6	27.14 mmol 3.0 equiv.	24	1.895	57
7	15.78 mmol 3.0 equiv.	18	1.375	71

Table 2.3: Conditions for coupling pyrimidine **2.37** with acetal **2.29.**

2.4.2 Hydrolysis of C^6-substituted pyrimidine 2.38 and synthesis of 2.40

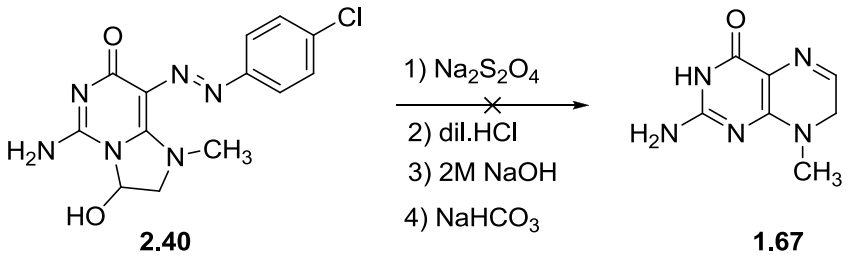
The C^6-substituted pyrimidine **2.38** was hydrolysed using concentrated HCl to afford the corresponding cyclic aminoketone hemiacetal **2.40** as an orange-coloured solid (**Scheme 2.18**). The product was identified as **2.40**, with no side-product **2.42** or intermediate **2.39** observed. The ^{1}H NMR spectrum showed a doublet corresponding to two CH_2 protons at δ 4.14 and a triplet for C<u>H</u>-OH proton at δ 5.5. The product **2.40** was obtained successfully in 63-83% yield on 0.6-6.1 g scale at 80-85^o C.



Scheme 2.18

2.4.3 Cyclisation of substituted pyrimidine 2.40.[218]

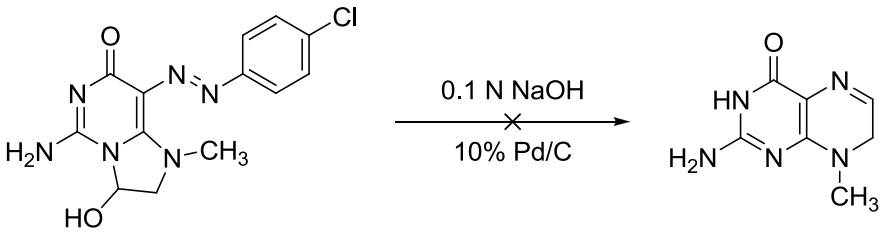
Cyclisation of substituted pyrimidine **2.40** using sodium dithionite in a minimum amount of water at room temperature did not give the desired product **1.67** in the required purity and quantity (**Scheme 2.19**). When cyclisation of **2.40** was performed with sodium dithionite[188] at 60 °C, it gave a dark-brown solid but it did not prove to be the desired blocked dihydropteridines **1.67** by characterization. In the next attempt, the temperature was raised to 100 °C but characterization data showed that dihydropteridine **1.67** was not present. The reductive cyclisation of substituted pyrimidine **2.40** in acetic acid/water (1:1) using Zn powder at 30-35 °C also did not give dihydropteridine **1.67**.



2.40 **1.67**

Scheme 2.19

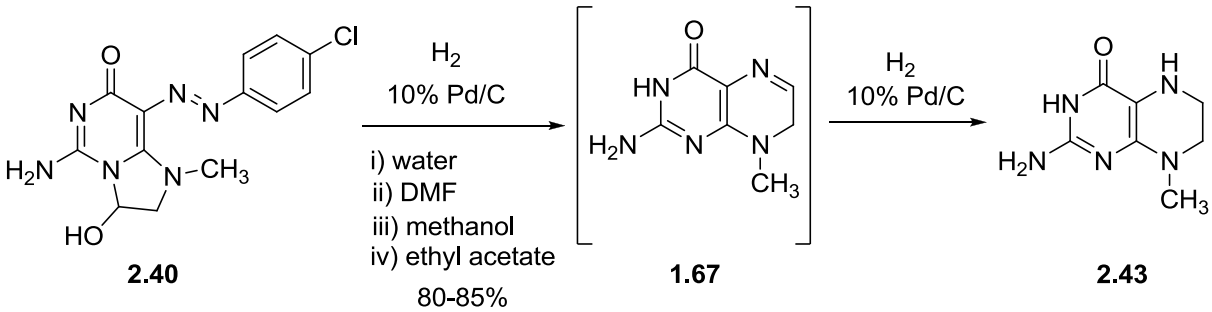
Substituted pyrimidine **2.40** was found to be only completely soluble in dilute aqueous sodium hydroxide, but hydrogenation using 10% Pd/C in dilute aqueous sodium hydroxide for 7 h did not give the dihydropteridine **1.67** (**Scheme 2.20**).



2.40 **1.67**

Scheme 2.20

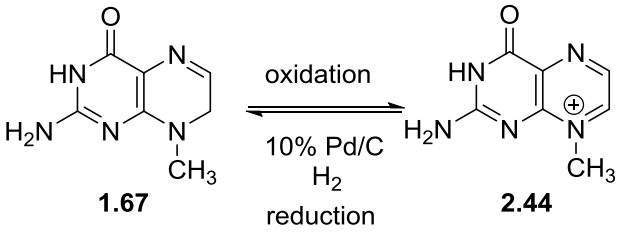
The reduction of substituted pyrimidine **2.40** using 10% Pd/C was successful under non-acidic conditions in a solvent mixture of water, methanol, DMF and ethyl acetate. Evidence for purity of dihydropteridine **1.67** came from TLC by using 1:1 water and 2-propanol as an eluent. It was purified by CG-50 (H^+) ion exchange resin. To obtain the highest purity, it was purified by HPLC. The product **2.43** was obtained successfully in 80-85% yield on 100-200 mg scale at room temperature and pressure (**Scheme 2.21**).



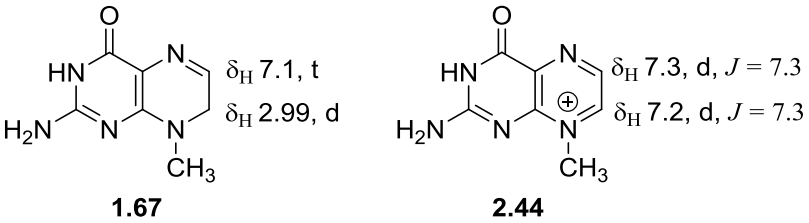
2.40 **1.67** **2.43**

Scheme 2.21

One property of dihydropteridine **1.67** is its tendency to oxidise to **2.44** during purification or exposure to air, which is a common property of dihydropteridines. Compound **2.44** was purified by column chromatography and then reduced to **1.67** by hydrogenation for one hour.



Scheme 2.22



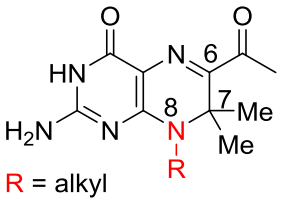
Proton NMR

From the ^{1}H NMR spectrum of compound **1.67** in DMSO-d_6, we could see a doublet at δ_H 2.99 and a triplet δ_H 7.1 , both with coupling constants of 6.8 Hz. The ^{1}H NMR spectrum of the oxidised dihydropteridine **2.44** shows two doublets close to each other at lower field. Mass spectra of oxidised dihydropteridine **2.44** corresponded to its exact mass.

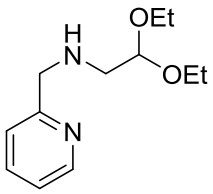
2.4.4 Diversity at N^{8} of dihydropteridine with an additional aryl group.

Some of the initial *N*-alkyl and *N*-benzyl targets (**1.66**) could not be prepared using oxazolidines **2.12** and **2.22** as an intermediate due to decomposition of these oxazolidines during purification and work up. However, using the diazo method, *N*-methyl dihydropteridine **1.67** was successfully prepared. If the acetal is available, for example **2.45**, **2.46** and **2.47**, more compounds could be prepared and these are analogues of the *N*-aryl

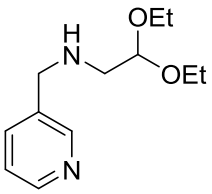
compound **1.66**. A route to several N^8 alkyl and benzyl dihydropterins had been found, which was one of the objectives.



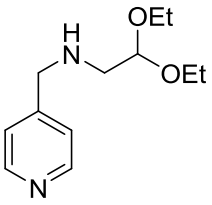
R = alkyl or aryl **1.66**



2.45

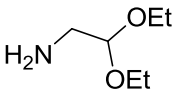


2.46

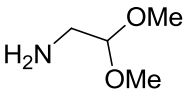


2.47

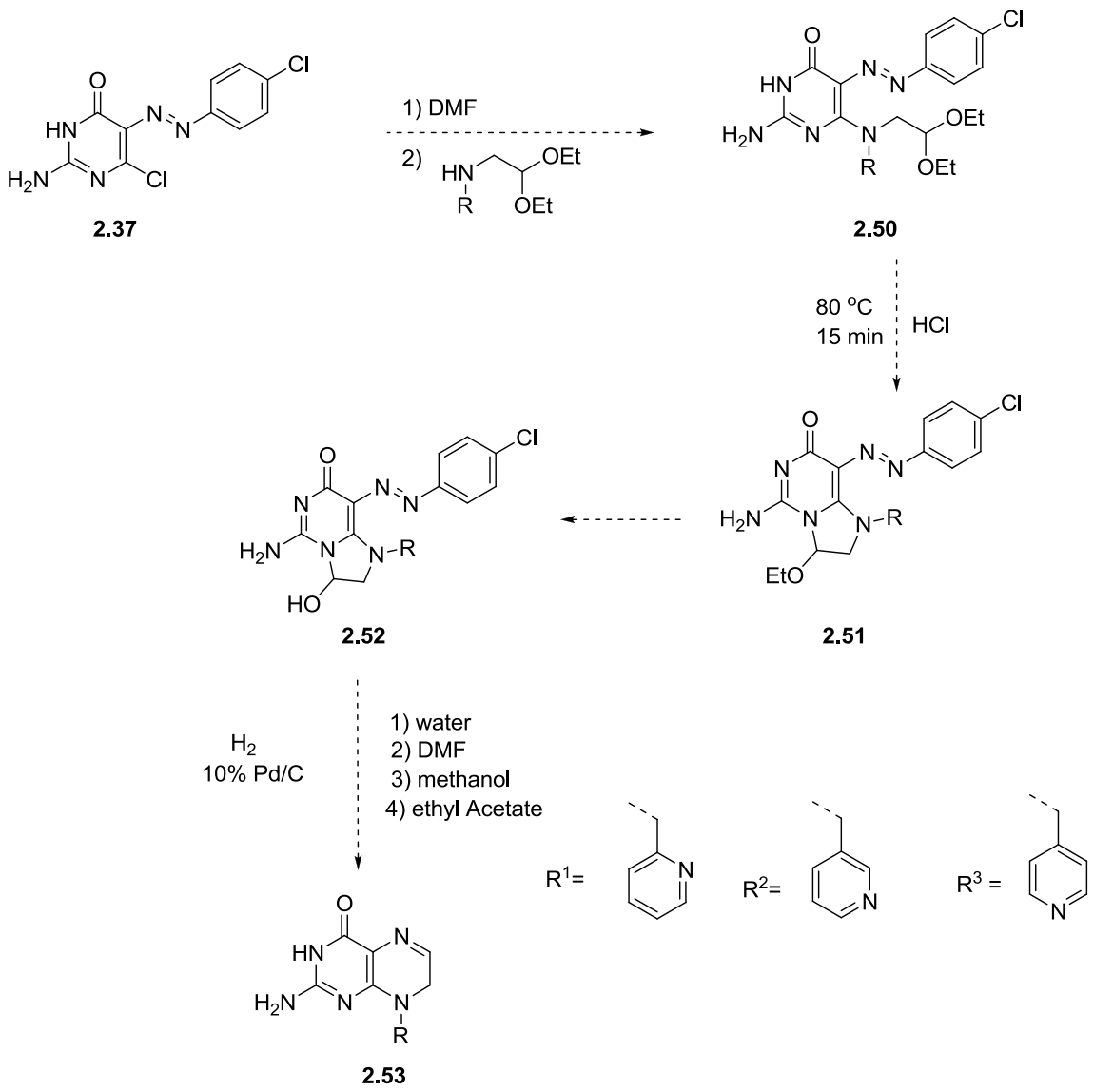
By heating a chloro- or bromo-acetal with an excess of appropriate primary amines, *N*-substituted aminoacetals can easily be synthesised. [223a] By reductive amination of aldehyde or ketone with amino acetal (**2.48** or **2.49**), different types of *N*-substituted acetals can be prepared, [224] which can be used for coupling with 5-substituted pyrimidine **2.37**. A possible route for the synthesis of different types of N^8 aryl-substituted dihydropteridine analogues is shown in **Scheme 2.23.**



2.48



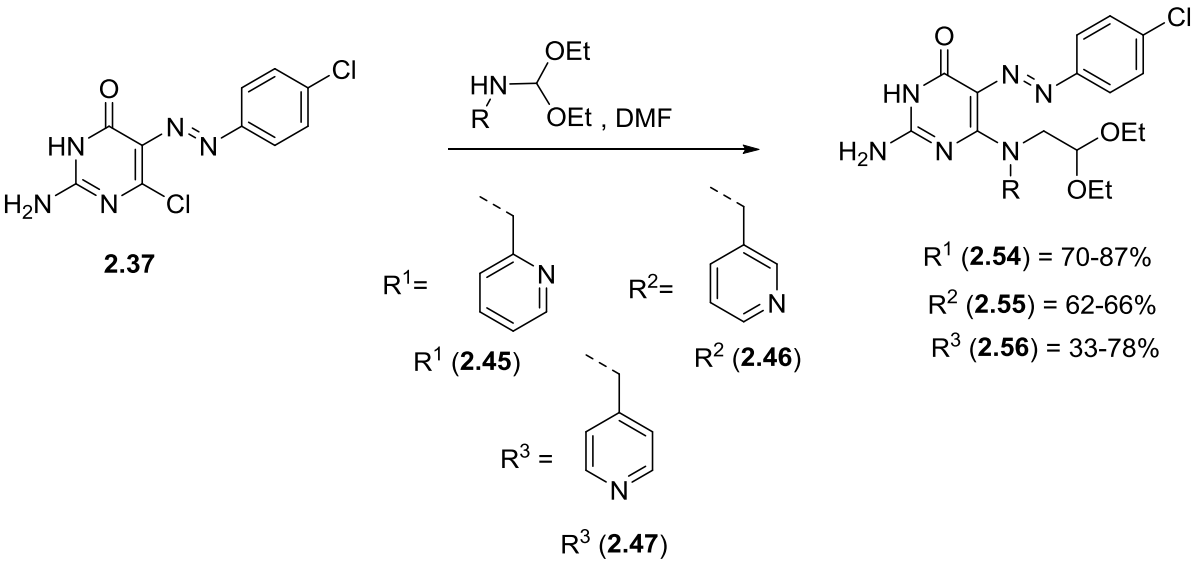
2.49



2.37 2.50 2.51 2.52 2.53

Scheme 2.23

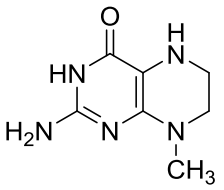
5-Chlorophenylazopyrimidine **2.37** was coupled (**Scheme 2.24**) with the commercially available 2-pyridyl acetal **2.45** in dimethylformamide under reflux conditions. The C^6 substituted pyrimidine **2.54** was obtained in 70-87% yield as a light-yellow solid.



Scheme 2.24

The coupling reaction of 3-pyridyl acetal **2.46** with 5-arylazopyrimidine **2.37** in dimethylformamide in the presence of triethylamine for 16 hours gave the coupled product **2.54** in 62-66% yield**.** Under the same conditions, 4-pyridyl acetal **2.47** was used to prepare coupled product **2.56** in 33-78% yield.

Although the products of nucleophilic substitution of 5-arylazopyrimidine **2.51** with pyridine acetals **2.45**, **2.46** and **2.47** (**Scheme 2.24**) were achieved, the final cyclisations were not performed as N^8-methyl analogue **2.43** had been tested at that time for nNOS enzyme activity. N^8-methyl substituent resulted decrease in nNOS activity so it was assumed that there is no sufficient space for binding within the active site of enzyme at N^8 position and any group bigger than methyl at this position will further decrease NOS activity. However the compounds prepared were available for biological evaluation in a number of screens.

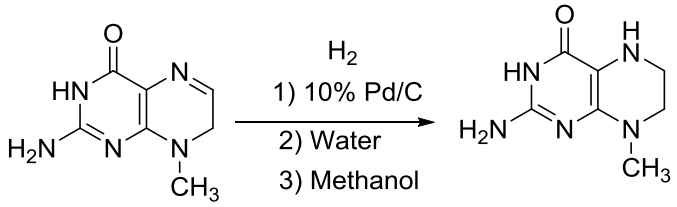


2.43

2.5.0 Reduction of pteridines

In order to investigate the mechanism behind the nitric oxide generation by blocked dihydropteridine WSG1002, as well as to discover the effect of N^8 and C^6 substitutions for nitric oxide synthase activity, the dihydropteridines **1.67** and **2.58** must be reduced by hydrogenation or with sodium cyanoborohydride to provide the corresponding tetrahydropteridines **2.57** and **2.59**. This oxidation state is needed for the activation of NOSs. The tetrahydropteridines were examined as substitutes for the BH_4 replacements in isolated nNOS at the University of Edinburgh. The tetrahydropteridines are supposed to be very susceptible to oxidation by air at ambient temperatures and light. The reduced pteridines were obtained by HPLC purification, aluminium foil was wrapped around the flasks and samples and each fraction was kept in liquid nitrogen during purification. The solvents were removed by freeze drying to yield pure analogues, in their tetrahydro-forms, as white solids. ^{1}H NMRs were taken as quickly as possible to avoid oxidation. These compounds (**1.67** and **2.57**) were taken to the University of Edinburgh in dry ice packs for testing for NOSs activity. Biological outcomes of above tetrahydropteridines are explained in Chapter 3.

The dihydropteridine **1.67** was reduced in methanol and water 1:1 by hydrogenation using 10 % Pd/C at atmospheric pressure for 6 h (**Scheme 2.25**). The tetrahydropteridine **2.57** was surprisingly found to be stable to oxidation to the dihydro form, determined by LRMS and ^{1}H NMR analysis. Even when left for several days both in solid and solution form at room temperature and exposed to air, no oxidation was observed. Evidence for the dihydropteridine **1.67** structure came from LRMS and ^{1}H NMR resonances at 2.99 and 7.10 ppm corresponding to the C^7 and C^6 protons respectively. Evidence for the tetrahydro structure came from LRMS and the ^{1}H NMR resonances at 2.42 and 2.65 ppm corresponding to the C^6 and C^7 protons respectively. Tetrahydropteridine **2.57** was obtained successfully in 89-92% yield on 40-50 mg scale at room temperature by hydrogenation as shown in Table 2.4. This was a very encouraging result. Tetrahydropteridine **2.57** was found to be stable to oxidation by LRMS and ^{1}H NMR if properly freeze-dried. After properly freeze-drying, tetrahydropteridine **2.57** could be exposed to air for a couple of days without much oxidation.



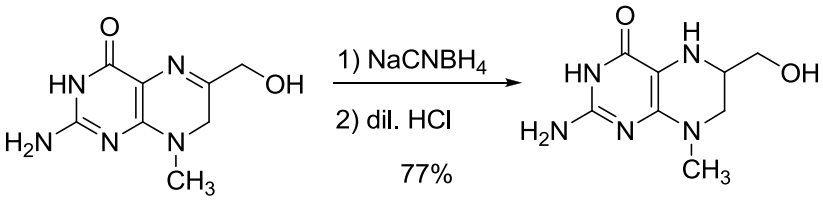
1.67 **2.57**

Scheme 2.25

Entry	Scale	Reaction Time	Yield
1	0.281 mmol	6 h	92%
2	0.281 mmol	4 h	89%
3	0.225 mmol	2 h	92%
5	0.281 mmol	2 h	90%

Table 2.4: Hydrogenation of **1.67** with 10% Pd/C to give tetrahydropteridine **2.57**.

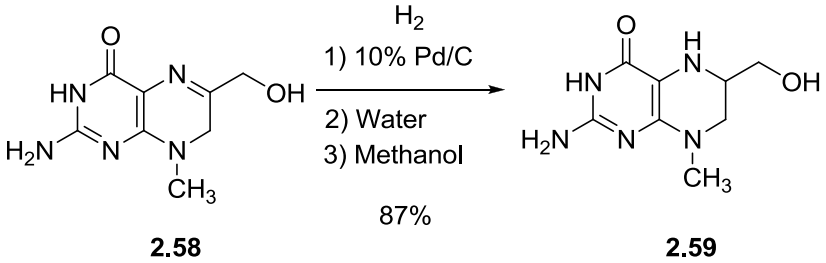
Dihydropteridine **2.58** was prepared by previous researchers within our group and was taken from our library of compounds. It has a side-chain similar to that of BH_4. 6-Hydroxymethyltetrahydropterin is an active analogue of BH_4.[149] Therefore, dihydropteridine **2.58** can tell us something about the acceptability or otherwise of an N^8 substituent. Dihydropteridine **2.58** was purified by HPLC and reduced with sodium cyanoborohydride in methanol, adding a few drops of dilute hydrochloric acid. After 4 hours reaction time at room temperature, tetrahydropterin **2.59** was obtained in 77% yield (**Scheme 2.26**).



2.58 **2.59**

Scheme 2.26

In another reduction experiment, the dihydropteridine **2.58** was purified by HPLC and then hydrogenated at room temperature for 4 hours. Tetrahydropteridine **2.59** was obtained as a white solid in 87% yield (**Scheme 2.27**).



Scheme 2.27

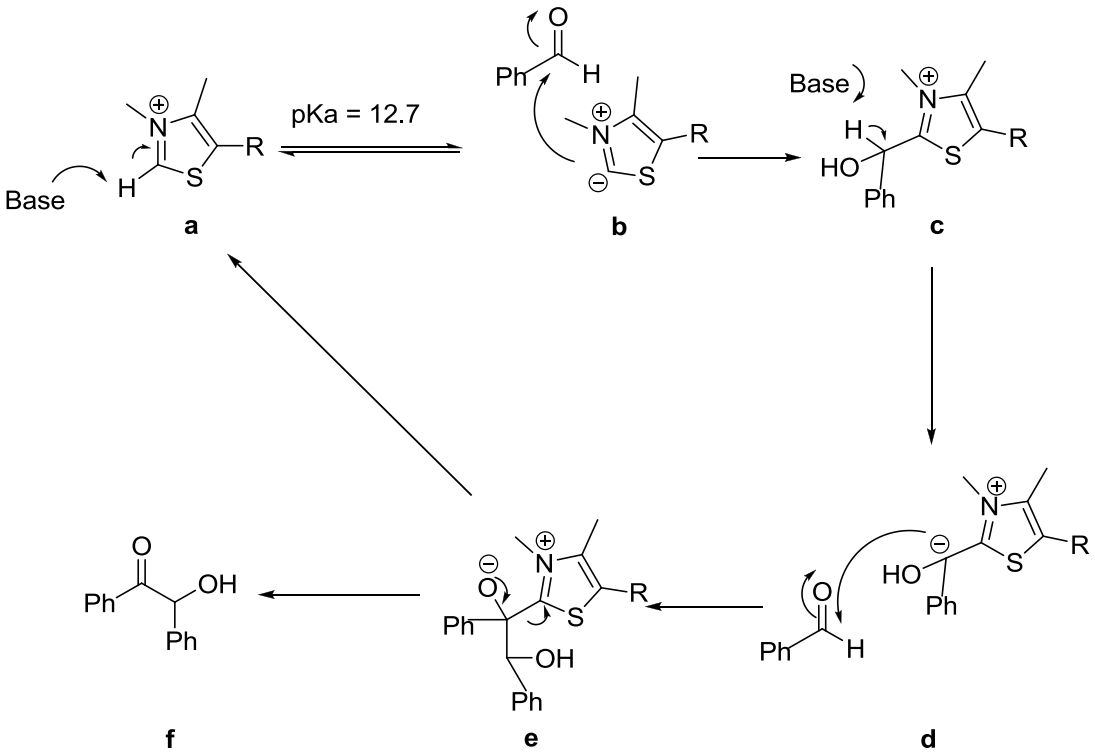
The tetrahydropteridines **2.57** and **2.59** were taken to Edinburgh University and these were fully evaluated in Dr. Daff's lab.

This synthetic route was successful in providing novel compounds with substituents at the N^8 position; these compounds do not possess a C^6 side-chain, which is an important feature of BH_4. Therefore for C^6 side-chain extension, a new strategy involving thiazoluim salt chemistry was considered.

2.6.0 Thiazolium Chemistry

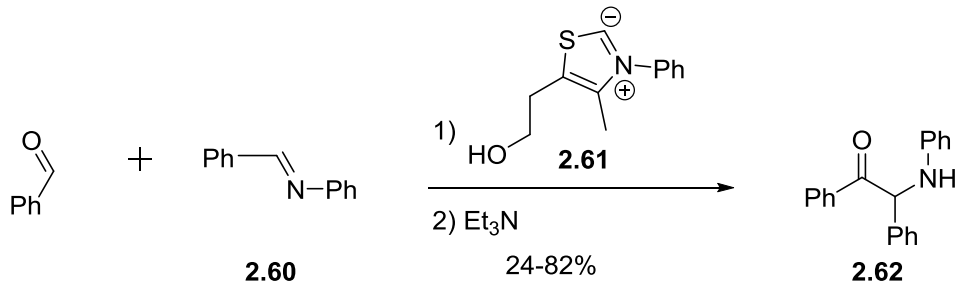
It has been recognized for a long time that thiazolium salts *(e.g. a)* and a base will catalyse the formation of acyloins[225] from aldehydes. The commonly supported mechanism, originally suggested by Breslow,[226] is presented in **Scheme 2.28**.

Deprotonation of the thiazolium salt **a** gives an active nucleophilic catalyst **b** which attacks the electrophilic aldehyde to give an adduct product **c**. Deprotonation of **c** results in the formation of an anion **d** which again attacks the aldehyde to form **e**. Re-formation of the carbonyl group gives corresponding acyloins (f) and regenerates the catalyst **a.**



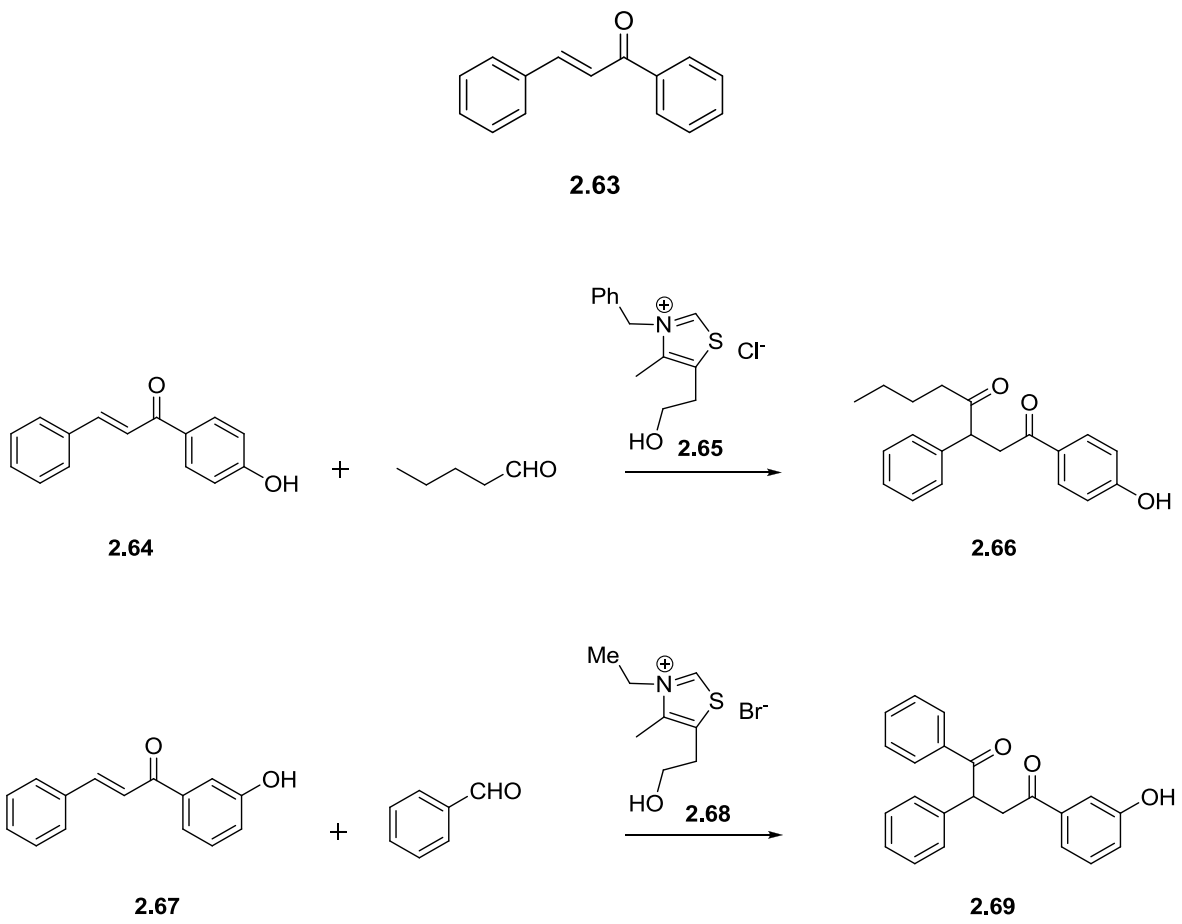
Scheme 2.28: The mechanism of the thiazolium salt-catalysed benzoin condensation

The reactivity of aldehydes can be reversed (Umpolung) by nucleophilic *N*-heterocyclic carbenes (NHC), which can give a unique path to a few significant target molecules. Various receptors, such as nitroalkenes, ketones and aziridines have been investigated.[227] To synthesise alpha-amino ketone, Gong-Oiang *et al.*[228] employed imines as a receptor for an acyl anion addition. In the presence of triethylamine and thiazolium salt **2.61**, the cross-coupling of benzaldehyde with *N*-benzylidenebenzenamine **2.60** was investigated. The coupled product **2.62** was obtained in a yield of 24-82% depending on solvent (**Scheme 2.29**).



Scheme 2.29

Chalcones (*e.g.,* **2.63**) are aromatic ketones and compounds with a chalcone-based structure are known to possess many significant biological activities such as nitric oxide inhibition, as well as anti-inflammatory, antiprotozoal and anticancer properties. [229] Raghavan *et al.*[230] used chalcones as Michael acceptors to prepare 1,4-diketones. Addition of aldehydes to chalcones, using an aldehyde with a large excess of chalcone and triethylamine as a base and above stoichiometric thiazolium salt in dioxane/ethanol solvent afforded the 1,4-diketone as shown in **Scheme 2.30**. As this reaction involves conjugate addition of an aldehyde to a chalcone, which is an α,β-unsaturated compound, and it requires thiazolium salt as a catalyst, this reaction can be named as a Stetter Reaction.

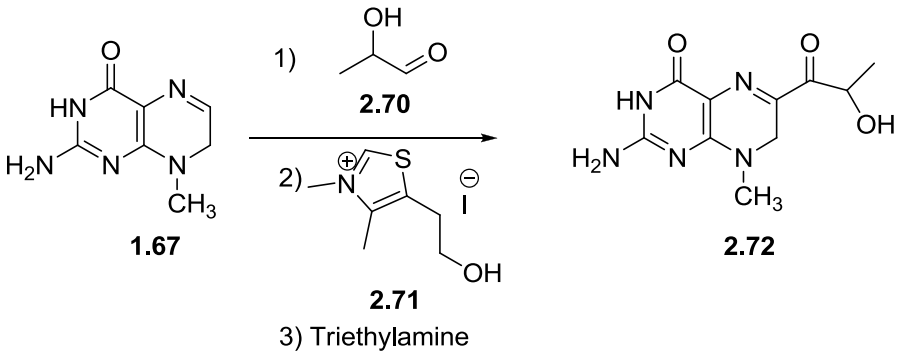


Scheme 2.30

Pteridines have not been used as the electrophile in thiazolium salt-promoted addition of carbonyl anions at the C^6 position, despite the evidence that C^6-substituted pteridines are

essential biological compounds. The thiazolium salt-promoted reactions may prove a great mechanism for the synthesis of new chemical compounds for drug discovery and for the synthesis of analogues of BH_4. The thiazolium salt chemistry can be a useful tool for the synthesis of C^6 subsituted analogous of BH_4.

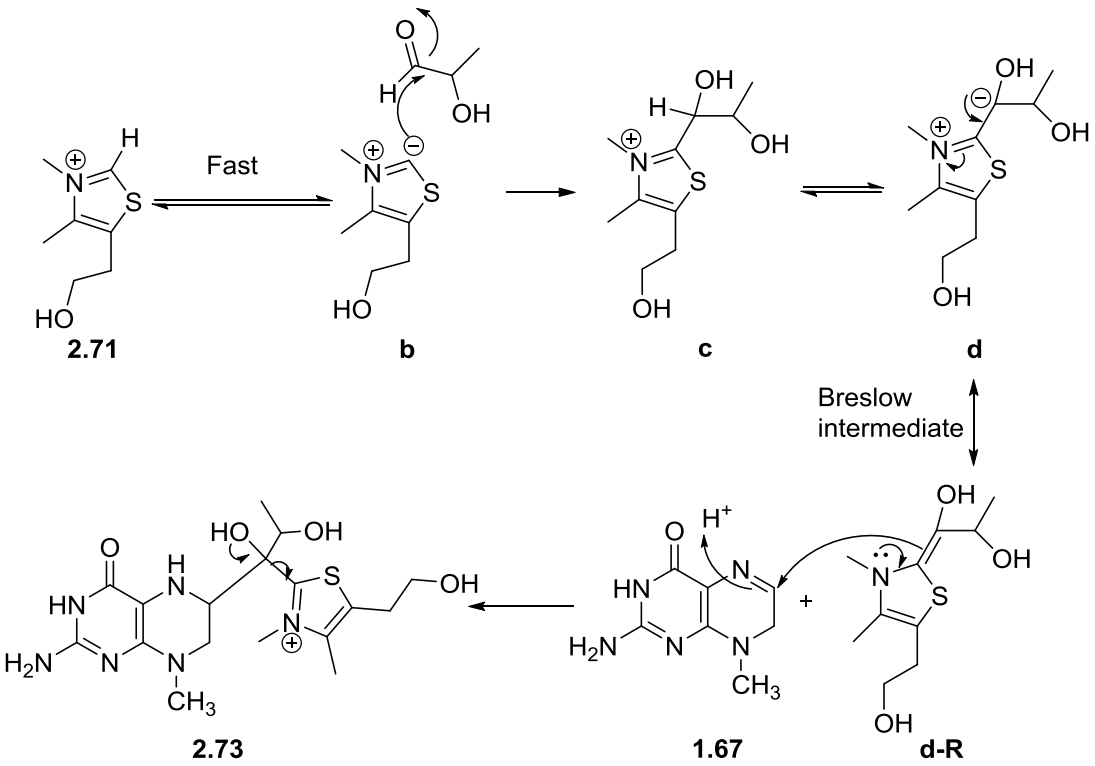
2.6.1 Reaction scheme and mechanism for the preparation of 8-methyl-6-substituted dihydropterins.



Scheme 2.31

Mechanism

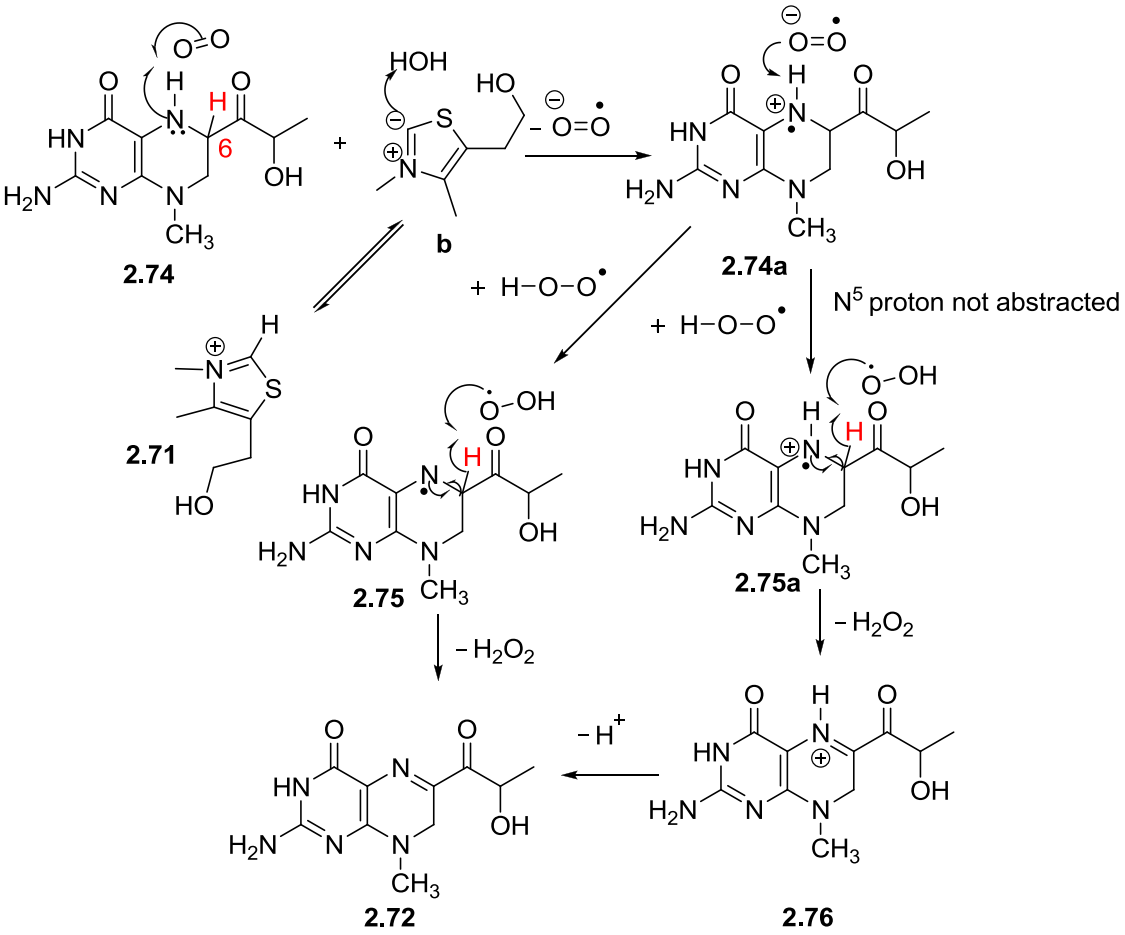
The ylide carbanion **b** is formed when a proton is removed by a base, it is stabilised by the adjacent positively charged nitrogen. The ylide carbanion **b** is good nucleophile, and so it attacks the electrophilic carbon of the aldehyde carbonyl group. The negative charge is shifted on that carbon as shown in complex **d** (**Scheme 2.31a).** This carbanion then acts as a good nucleophile, and it attacks the electrophilic carbon which is bonded covalently to an electronegative atom by a double bond. *e.g* C^6 of dihydropteridine **1.67**.



Coupling of thiazolim salt with pteridine 1.67

Scheme 2.31a

The hypothetical mechanism for the oxidation of tetrahydro analogue **2.74** into its dihydro analogue **2.72** is presented below in **Scheme 2.31b.** The N^8 electron of tetrahydro analogue **2.74** can get photochemically excited which will result in a positive charge on N^8 atom and that positive charge can be delocalised in the pyrazine ring which could be the driving force for this oxidation reaction.

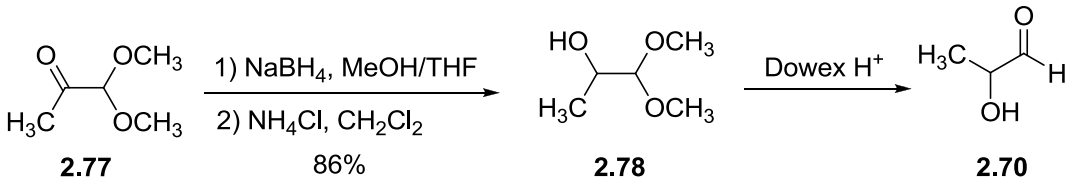


Hypothetical free radical mechanism for the oxidation of tetrahydro analogue 2.74

Scheme 2.31b

The photochemically excited free-radical reaction between tetrahydro analogue **2.74** and oxygen can give oxidized pteridine molecule **2.76** by abstraction of N^5 and C^6 H-atoms. The removal of one electron from **2.74** can give a radical cation **2.74a** and a peroxy anion. If the oxyanion species abstracts one proton from N^5 **2.74a**, it will give **2.75** and a hydroperoxy radical. The hydroperoxy radical can abstract C^6 hydrogen either from **2.75** or **2.75a** to give **2.72** or an oxidised product **2.76** respectively. The oxyanion species can also abstract a proton from solution to give hydroperoxy radical which can possibly abstract C^6 proton as shown in **2.75a** to give an aromatic cation **2.76**. This pathway could have more probability as aromatic cationic structure is possibly more stable intermediate and this structure **2.76** was found to be present in solution by mass spectra.

2-Hydroxypropanal **2.70** was required for the preparation of 8-methyl-6-substituted dihydropterins **2.72** by thiazolium salt reaction. For this purpose, pyruvic aldehyde dimethyl acetal **2.77** was reduced to 1,1-dimethoxy-2-propanol **2.78** using $NaBH_4$ in 86% yield as a colourless liquid which was stored in the freezer. 1,1-Dimethoxy-2-propanol **2.78** was treated with CG-50 (H^+) ion exchange resin (**Scheme 2.32**) to give aldehyde **2.70**, which was used immediately in the thiazolium salt reaction with dihydropteridine **1.67**.

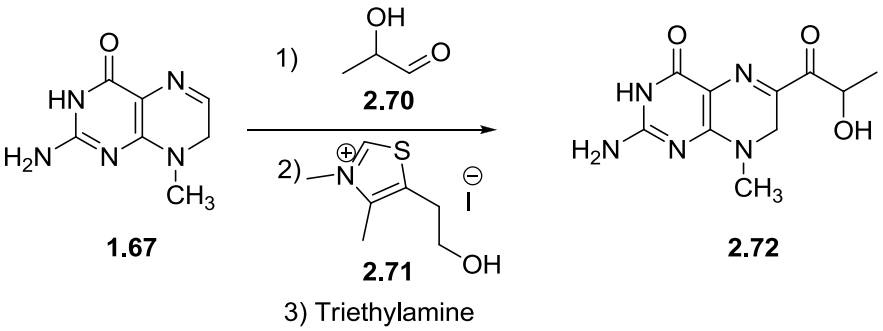


2.77 86% **2.78** **2.70**

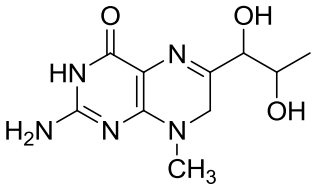
Scheme 2.32

2.6.2 The thiazolium salt promoted addition of 2-hydroxypropanal 2.70 to dihydropteridine 1.67

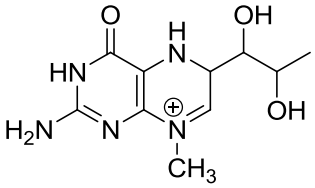
The reaction for the synthesis of 6-substituted dihydropterins **2.72** using thiazolium salt catalysis was carried out using dihydropteridine **1.67** with an excess of freshly prepared 2-hydroxypropanal solution **2.70**, followed by thiazolium salt (**Scheme 2.33**), (Table 2.5, Entry 1). From HPLC, the first fraction proved to be thiazolium salt **2.71**. The next fraction after thiazolium salt was **2.80** by LRMS along with 30% ions of **2.81**. From other fractions, the following C^6 substituted dihydropteridines, **2.79**, **2.80**, **2.81** and **2.82** were observed by LRMS but could not be confirmed by NMR. An intermediate **2.82** was also observed, however the desired product **2.72** could not be obtained.



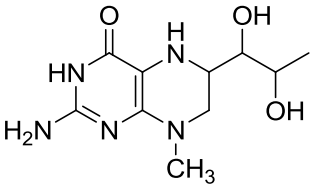
Scheme 2.33



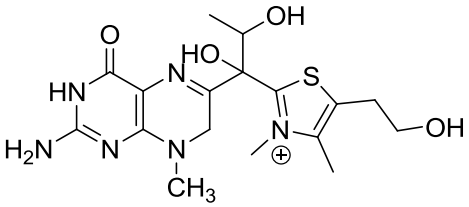
2.79



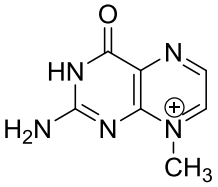
2.80



2.81



2.82



2.44

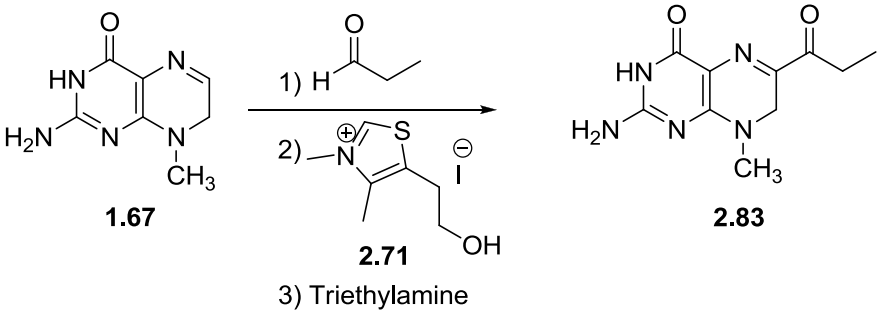
Entry	Scale (1.67)	Aldehyde	Thiazolium Salt (as compared to 1.67)	Reaction Time
1	0.56 mmol, 1 equiv.	in excess	0.28 mmol, 0.5 equiv	4 h
2	0.28 mmol, 1 equiv.	2 equiv.	0.028 mmol, 10 mol %	6 h

Table 2.5

In another attempt at the synthesis of 6-substituted dihydropterins **2.72** (Table 2.5, Entry 2), dihydropteridine **1.67** was first purified by HPLC and the reaction was performed using freshly prepared 2-hydroxypropanal **2.70**. Dihydropterin **1.67** was reacted with 2-hydroxypropanal in water using thiazolium salt as a catalyst. The crude material was purified by HPLC. The first fraction proved to be thiazolium salt **2.71.** The second fraction was found to be unreacted oxidized dihydropteridine **2.44** and **2.80** by LRMS. The desired product could not be found in any fraction by ^{1}H NMR. Because of difficulty in isolating products, we decided to study a simple aliphatic aldehyde, propanal, as a substrate.

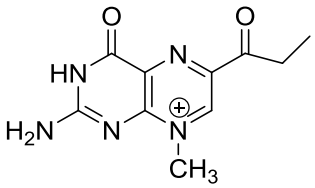
2.6.3 The thiazolium salt promoted addition of propionaldehyde to dihydropteridine 1.67

The C^{6}-substitution of dihydropteridine **1.67** was attempted using propionaldehyde and trimethylamine in an equimolar ratio and thiazolium salt **2.71** (10 mol%) in water at room temperature for 30 h (Table 2.6, Entry 1). From HPLC the first fraction which was eluted before the thiazolium salt **2.71**, proved to be **2.84** by mass spectrum**.** The formation of **2.84** suggested that the planned condensation had occurred but that oxidation had also taken place. The desired product **2.83** was also seen in LRMS but the peak was negligible compared to other fractions obtained by HPLC. The **2.85** was found as (MH^{+}) and (MNa^{+}) in LRMS. NMR analysis did not confirm the desired product **2.83** (**Scheme 2.34**). The possible mechanism for the formation of **2.85** will be described in section 2.6.4 (**Scheme 2.39**).



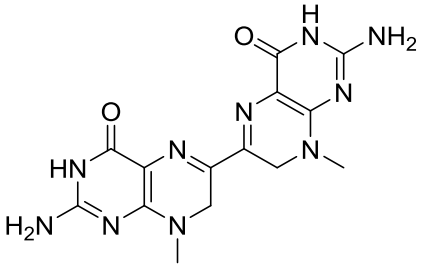
1.67 **2.71** **2.83**

Scheme 2.34



calculated mass: 234.10

2.84



calculated mass: 356.15
mass found 357.3

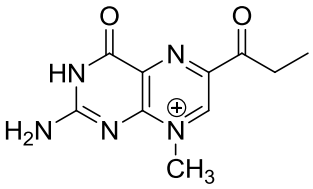
2.85

Entry	Triethylamine	Thiazolium Salt **2.71**	Reaction Time	Product
1	0.28 mmol, 1 equiv.	0.028 mmol, 10 mol % and after 20 h further 8 mg.	30 h	no required product
2	0.28 mmol, 1 equiv.	0.014 mmol, 5 mol % after every 10 h	30 h	no required product
3	1.44 mmol, 5 equiv.	0.014 mmol, 5 mol % after every 10 h	30 h	no required product

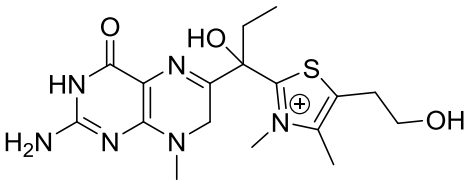
Table 2.6

In a second attempt at the synthesis of 6-substituted dihydropterins **2.83**, thiazolium salt **2.71** (5 mol%) was added after every 10 h for 30 h at room temperature. After work up and purification, the desired product in oxidized form **2.84** appeared to be eluting by LRMS (ESI), very close to thiazolium salt, however it decomposed in DMF (as a solvent) at room temperature. The LRMS (ESI) spectrum showed a pure compound but due to decomposition of this product, sufficient material for full characterization could not be obtained. The LRMS of another fraction corresponded to **2.86,** but material was not sufficient for ^{1}H NMR. A light-brown material was precipitated during filtration. From the LRMS of that material, it appeared that the required product **2.83** was coupling with pteridine to form **2.87**. LRMS was

clear showing following ions: (i) 393.17 for an intermediate **2.86** and (ii) 413.7, for a coupled product **2.87**. The ^{1}H NMR spectrum was complex, but it showed signals of substituted pteridine and thiazolium salt complex.

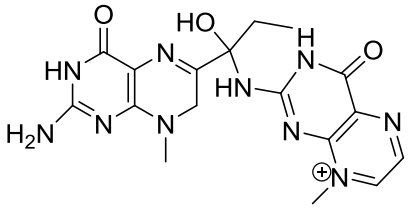


2.84



calculated mass: 393.17

2.86



calculated mass: 413.1793

2.87

It is apparent from the data that the expected reactions were taking place in the presence of thiazolium salts but at the same time other reactions such as free radical coupling could also be happening. Alkyl groups are known to stimulate free-radical reactions in substituted indoles and tetrahydropterins and it could be that *N*-methyl group in dihydropterin **1.67** was triggering free radical reaction by a similar kind of mechanism. Wilfred *et al.*[231] expressed a mechanism of oxidation of tetrahydropteridine **2.88** which involves free radical intermediates, the initiation step is given in the **Scheme 2.35**.

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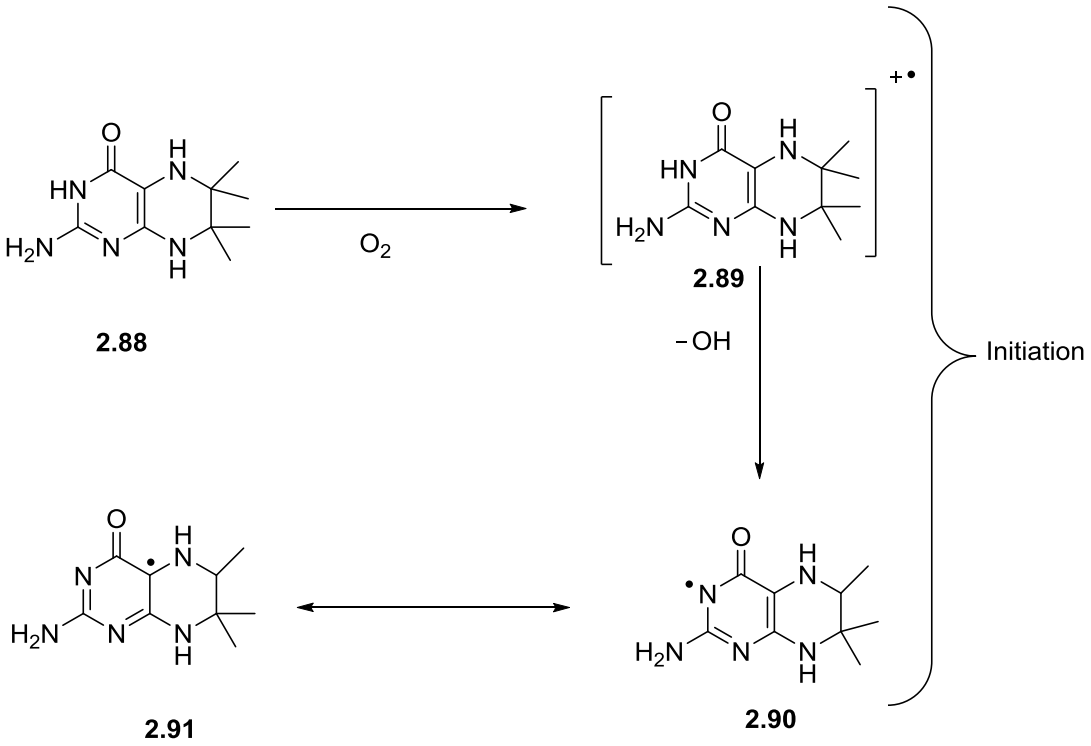
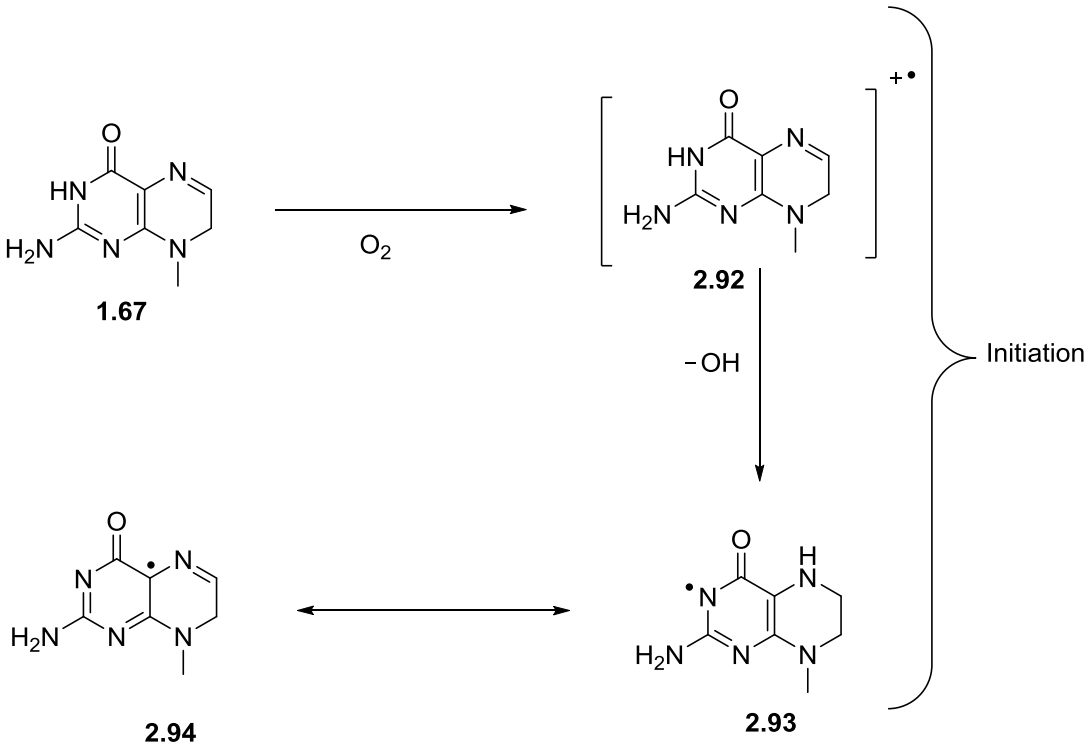


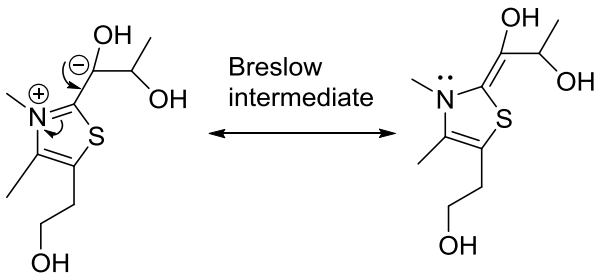
Scheme 2.35

The polar effects of a methyl group in stimulating free radical reactions may increase if the *N*-methyl group is on nitrogen atoms due to a part of the conjugated system. *N*-Methyl groups increase the basic strength by about 0.5 pKa units.[231] A mechanism can be suggested for the initiation step of free-radical formation in dihydropteridine **1.67** (**Scheme 2.36**). One plausible cause of dimerisation and polymerisation of dihydropteridine **1.67** and its substituted compounds may be that it formed free-radical intermediates.



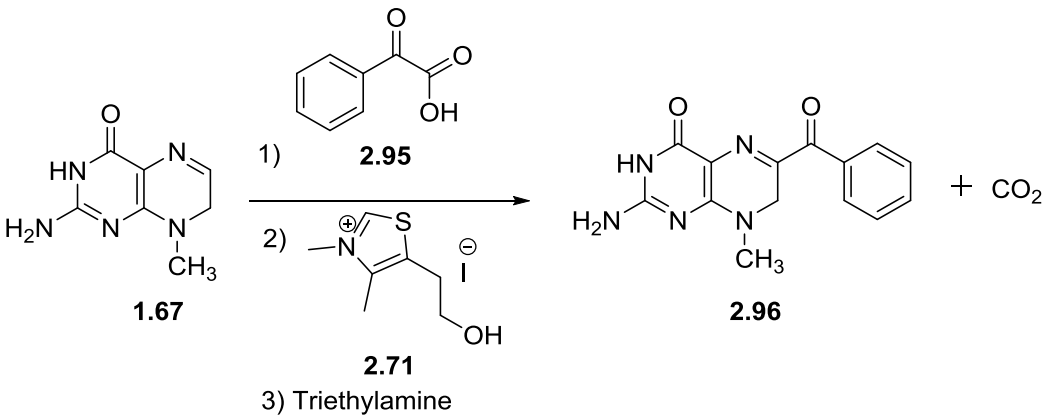
Scheme 2.36

The products are suggested to be made under kinetic control in the thiazolium salt reaction where the receptor (in the aforementioned discussed reactions, dihydropteridine **1.67**) should react more slowly towards the nucleophilic thiazolium salt catalyst and faster with the Breslow intermediate (**Scheme 2.31a**), rather than aldehyde as in the Stetter reaction. For both yield and reaction rate, choice of solvent and temperature performs an important role. [228,245] Some researchers did thiazolium salt-promoted nucleophilic reactions for two, three or four days. We have found that thiazolium salt-promoted addition of aldehyde to dihydropterine **2.23** gives products that are substituted at position 6, but the yield was very low. The change in concentration of aldehydes or thiazolium salt and reaction time could not give improved yields of the required substituted products. The pterin derivative **1.67** easily oxidises to **2.44** which has a double bond in between N^8 and C^7.



2.6.4 The thiazolium salt promoted addition of phenylglyoxylic acid and benzaldehyde to dihydropteridine 1.67

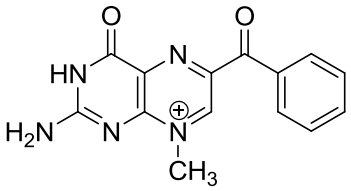
The reason to change the reagent for this thiazolium salt reaction was that a more non-polar side-chain would help the isolation of a product. Decarboxylation would drive the equilibrium towards product. The first reaction for the preparation of 6-substituted dihydropterins **2.96** was carried out using an equimolar ratio of dihydropteridine **1.67** and 2-oxo-2-phenylacetic acid **2.95** in water followed by the thiazolium salt for 30 hours at room temperature (**Scheme 2.37**), (Table 2.7, Entry 1). A light-brown solid was purified by silica gel column chromatography, eluting with 40 % EtOAc/methanol. It was further purified by HPLC.



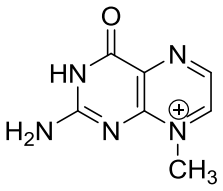
Scheme 2.37

From HPLC purification, one fraction was found to be **2.97** by LRMS. The major portion was oxidized starting material **2.44** and the yield of expected product **2.97** were so low from

HPLC purification that sufficient material for all characterization could not be obtained. The second attempt also did not give 6-substituted dihydropterins **2.96**.



2.97

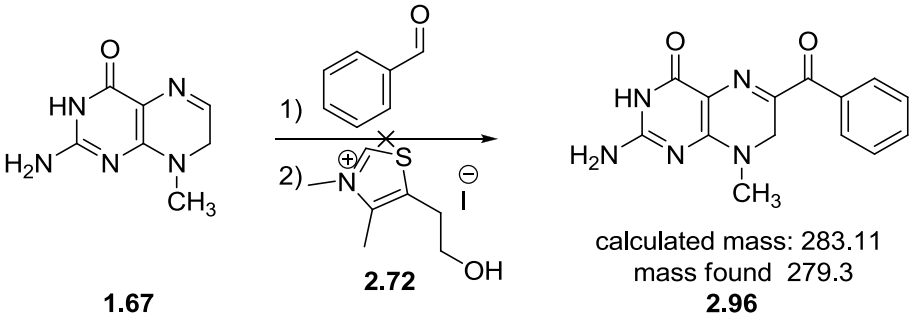


2.44

Entry	Triethylamine	Thiazolium Salt	Reaction Time	product
1	1.44 mmol, 5 equiv.	0.014 mmol, 5 mol % after every 10 h	30 h	**2.44** (major) and **2.97** (minor)
2	1.44 mmol, 5 equiv.	0.028 mmol, 10 mol %	30 h	**2.44** (major) and **2.97** (minor

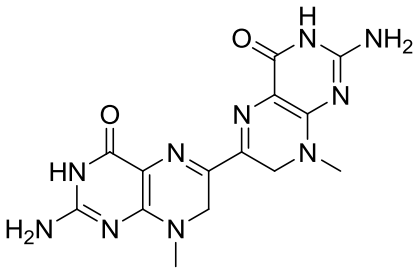
Table 2.7

The thiazolium salt reaction for the synthesis of 6-benzoyl substituted dihydropterins **2.96** was also carried out using an equimolar ratio of dihydropteridine **1.67**, benzaldehyde and trimethylamine in methanol, followed by thiazolium salt (10 mol %) (**Scheme 2.38**). The desired 6-substituted dihydropterins **2.96** could not be found in any fraction from HPLC. Only the pterin derivatives **2.85** and **2.107** were found.



1.67 **2.72** **2.96**

Scheme 2.38

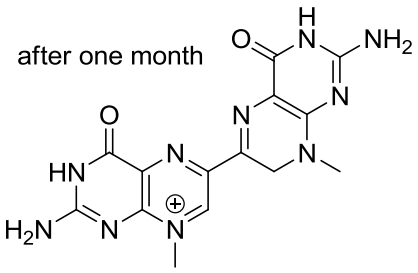


calculated mass: 356.15

mass found 357.3

2.85

after one month

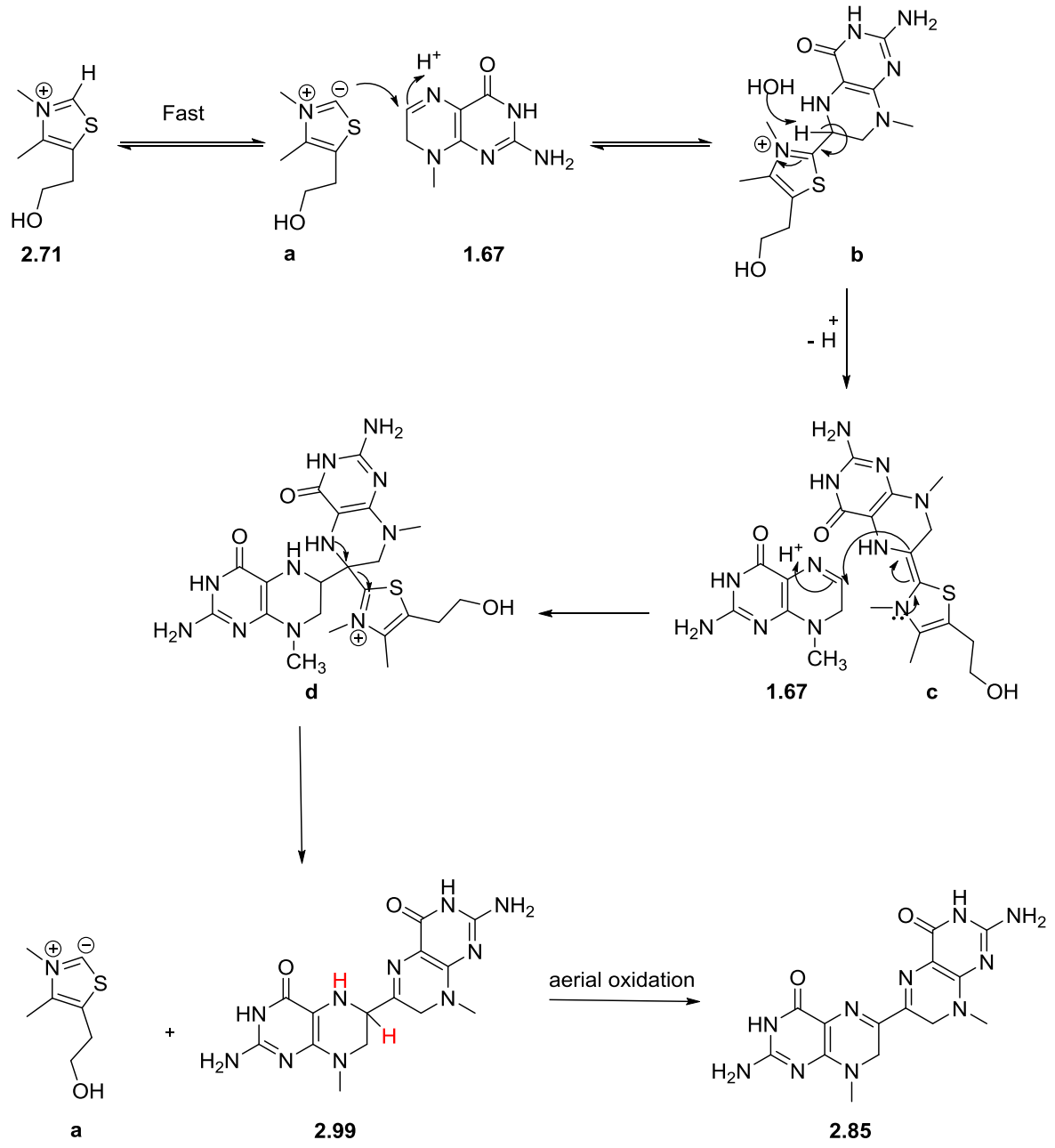


calculated mass: 355.14

mass found 355.3

2.98

The possible mechanism for the formation of product **2.85** is shown in **Scheme 2.39** in which ylide intermediate **a** attacks a molecule of dihydropteridine **1.67** to give an intermediate **b** in which proton next to nitrogen is very acidic. Deprotonation of this intermediate gives another intermediate **c,** which acts as a strong nucleophile and attacks another molecule of dihydropteridine **1.67** to give an intermediate **d** and then **2.99** which on aerial oxidation can give **2.85**. As dihydropteridine **1.67** quickly oxidizes to **2.44,** it can be proposed that **2.98** could have formed through the same mechanism.

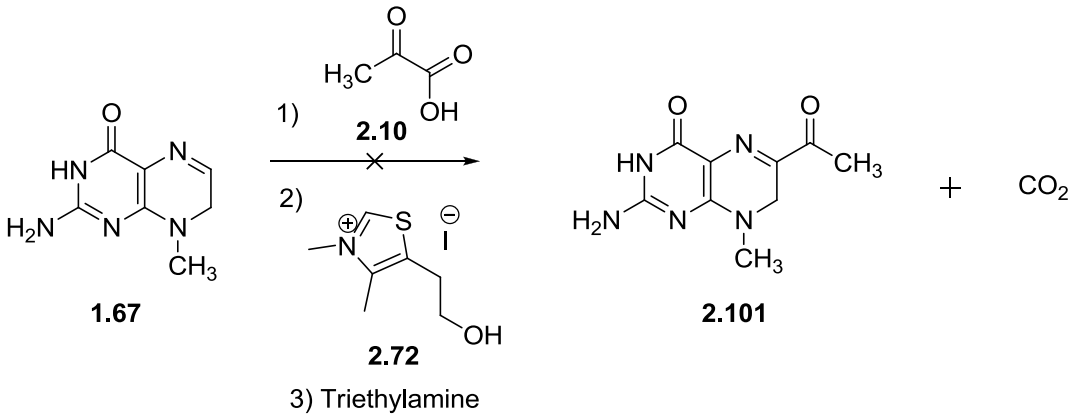


Scheme 2.39

2.6.5 The thiazolium salt promoted addition of pyruvic acid to dihydropteridine 1.67

For the synthesis of 6-substituted dihydropterins **2.101**, dihydropteridine **1.67** was reacted with pyruvic acid and thiazolium salt (10 mol %) in water, in the presence of triethylamine at

room temperature for 30 h. The solution looked light-red in colour suggesting that some reaction has occurred. A light-red solid was crystallized with ethanol and as a solution was purified by HPLC before LRMS analysis. The major portion was oxidized starting material **2.44** and the required product **2.101** could not be obtained.

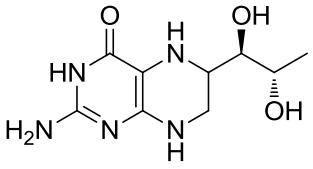


Scheme 2.40

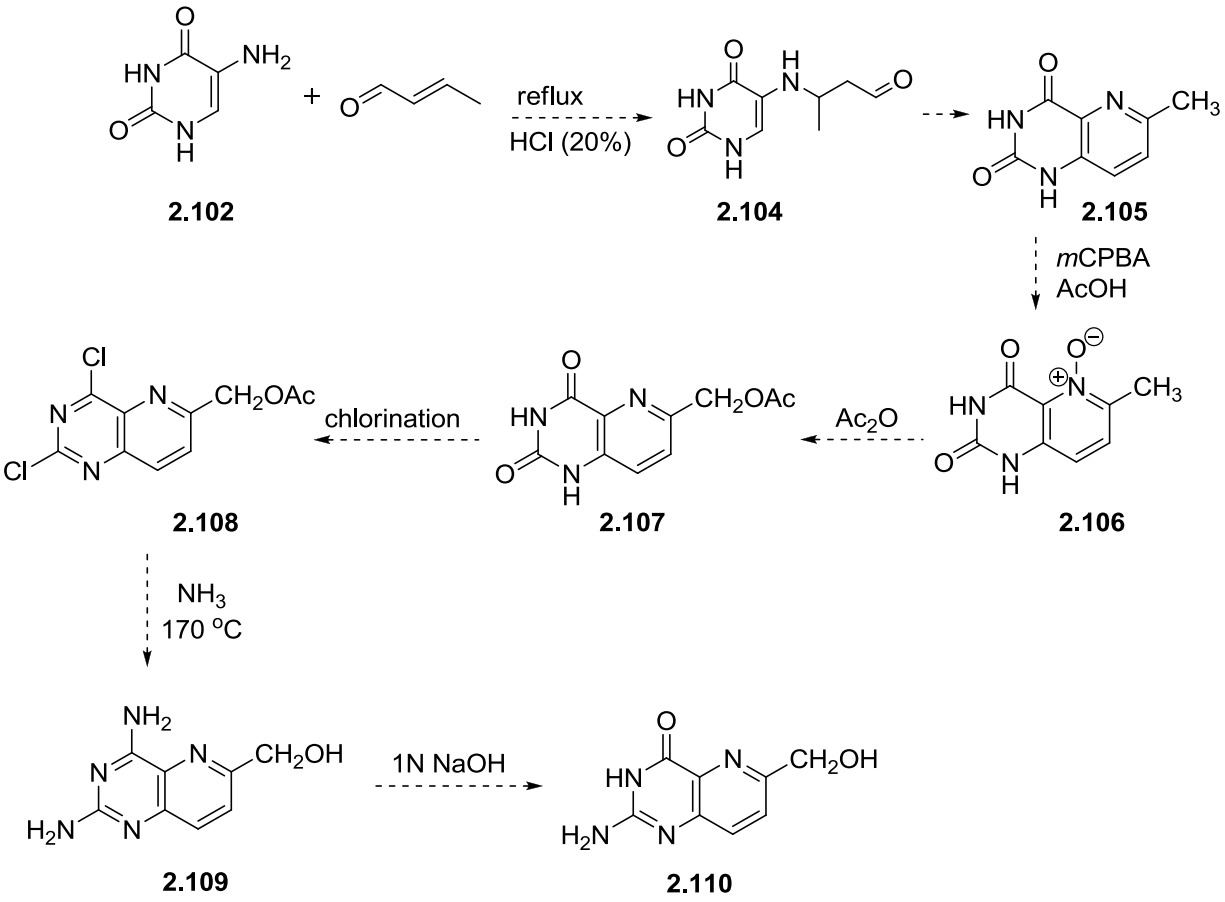
It was anticipated that thiazolium salts might give us the products we wanted with some substitutions at C^6 of pteridine. Although from the reactions described above there is evidence of thiazolium salt mediated coupling of pterins with aldehyde and ketones, a complex mixture was always obtained and work was stopped at this stage.

2.7.0 Diversity at N^8 of dihydropteridine, replacement of nitrogen with carbon.

In order to investigate the effect on nitric oxide synthase activity of replacement of N^8 of **1.1** (**BH_4**) with carbon, it was planned to synthesise 6-hydroxymethyl analogue **2.110** with a carbon atom at N^8 position. The proposed synthesis of target compound **2.110** is shown in **Scheme 2.41**.[232]

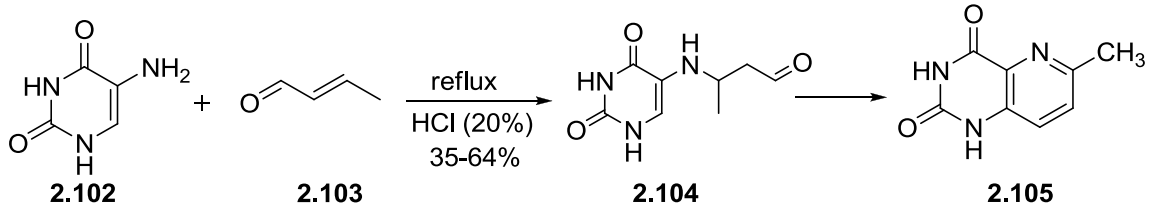


1.1 (BH_4)



Scheme 2.41

Irwin *et al.*[233] used 5-aminouracil **2.102** for the synthesis of some pyridopyrimidines by an intramolecular electrophilic cyclisation with crotonaldehyde in hydrochloric acid (20%). The pyridopyrimidine **2.105** was reported in 28% yield by trituration of the residue of the reaction with aqueous ammonia solution. It was found that pyridopyrimidine **2.105** was completely soluble in aqueous ammonia solution but in very dilute ammonia solution it precipitated in low yield and purity. A different strategy was developed to increase the yield and to synthesise pyridopyrimidine **2.105** with the highest purity.



2.102 2.103 2.104 2.105

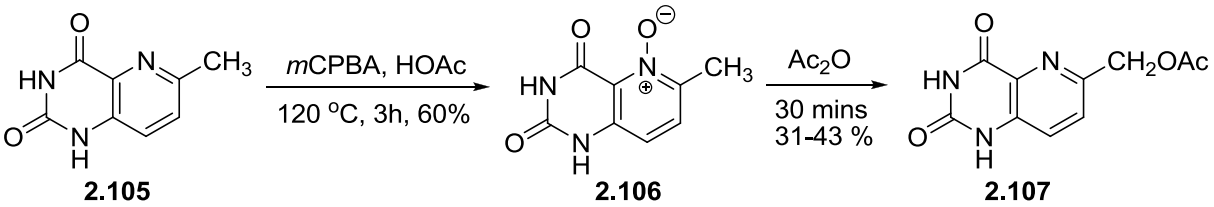
Scheme 2.42

The pyridopyrimidine **2.105** was an intermediate for the synthesis of our target molecule **2.110** (**Scheme 2.41**). The pyridopyrimidine **2.105** was obtained in 35-64% yield depending upon reaction conditions (Table 2.8, Entry 1-5) (**Scheme 2.42**).

Entry	Crotonaldehyde	Temperature	Reaction Time	Yield 2.105
1	3.93 mmol, 1.0 equiv	102 °C	1h	35%
2	15.74 mmol, 1.0 equiv	120 °C	1h	38%
3	27.54 mmol, 1.5 equiv	120 °C	1h	45%
4	118.05 mmol, 1.5 equiv	122 °C	1h	52%
5	1.77 mol, 1.5 equiv	120 °C	2h	64%

Table 2.8

The functionalisation of the 6-methyl group of **2.105** was carried out by oxidation of **2.105** with *m*-chloroperoxybenzoic acid in acetic acid.[232] N^5 is the only site available for oxidation due to the lactam structure of **2.105.** The reaction of **2.105** with *m*-chloroperoxybenzoic acid in glacial acetic acid at 122 °C for 3 h gave the N^5-oxide of 6-methylpyridopyrimidine **2.106** in 60% yield, as a light-yellow solid (**Scheme 2.43**).



Scheme 2.43

Two methods were used for the preparation of **2.107**. In the first method, a solution of **2.106** was heated under reflux in an acetic acid-acetic anhydride mixture for 30 min to give **2.107** as a light-brown solid (83%). The aromatic methyl resonance at δ_H 2.56 was replaced by the acetyl methyl resonance at δ_H 2.09, and a new two-proton signal for the methylene group appeared at δ_H 5.13.

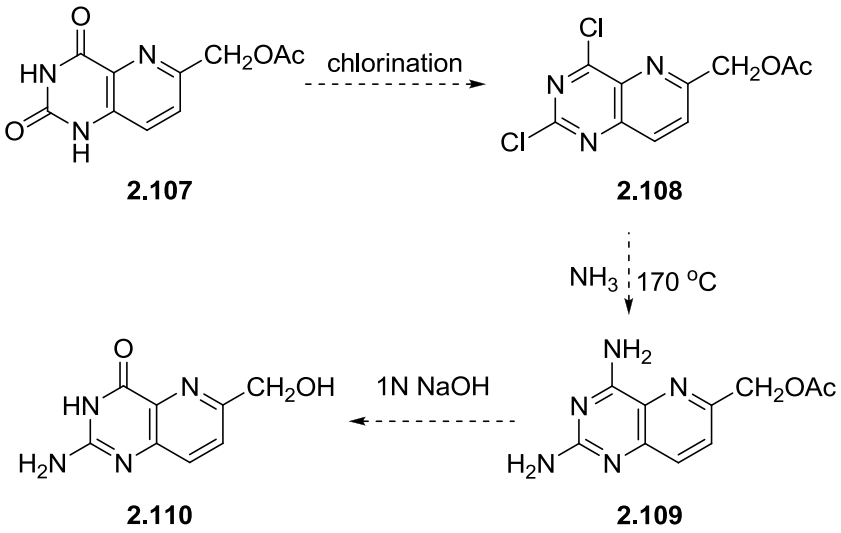
In a second method, **2.107** was prepared without the isolation of the *N*-oxide **2.106**. The hot reaction mixture of **2.106** and acetic anhydride was heated under reflux for a further 30 min. The product **2.107** was obtained in 31-43% yield under different reaction conditions (Table 2.9).

Entry	**Scale (g)**	***m*-CPBA (mmol)**	**Temperature (°C)**	**Reaction Time in HOAc (h)**	**Reaction Time with Ac_2O (min)**	**Yield 2.112 (%)**
1	3.0	59.20 , 3.5 equiv	120	3	30	31
2	4.0	56.45 , 2.5 equiv	120	3	30	40
3	3.6	50.80, 2.5 equiv	125	3	35	43
4	3.5	49.39 , 2.5 equiv	122	3	40	41

Table 2.9

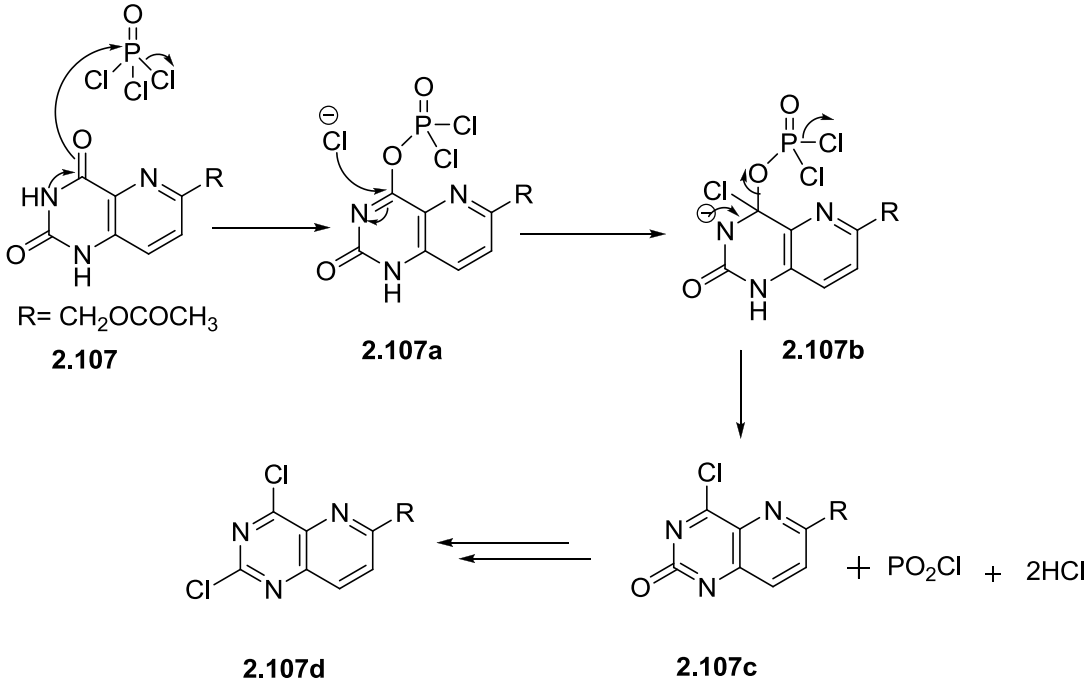
2.7.1 Diversity at C^2 of pyridopyrimidine 2.107 by activating the carbonyl by chlorination

The nucleophilic substitution at the C^2 and C^4 positions of pyridopyrimidine **2.107** requires activation of the carbonyl group. The first strategy considered was chlorination; the nucleophilic substitution using a suitable amine such as ammonia would give 2,4-diamino-substituted pyridopyrimidine **2.109** which, on hydrolysis with sodium hydroxide would give access to 2-amino pyridopyrimidine **2.110**, as shown in **Scheme 2.44**.



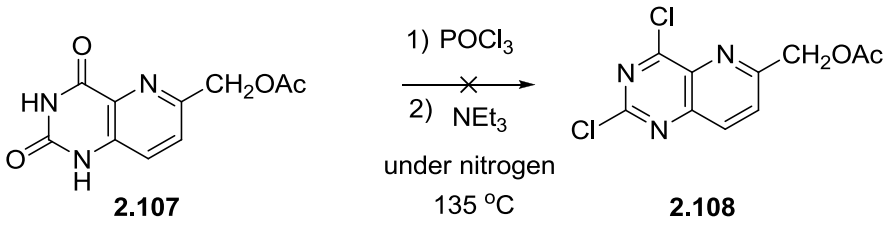
Scheme 2.44

The chlorination of oxo-compounds is usually accomplished under vigorous conditions using phosphorous-based chlorinating reagents such as phosphoryl chloride. The mechanism of chlorination for **2.107** using phosphoryl chloride is described in **Scheme 2.45**.



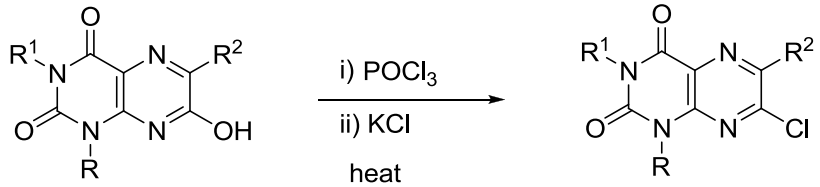
Scheme 2.45

Chlorination was attempted under several conditions. The first reaction for the chlorination of **2.107** was attempted using a procedure described by Srinivasan *et al.*[232] using freshly distilled phosphoryl chloride in the presence of triethylamine at 135 °C under nitrogen for 8 h (**Scheme 2.46**). After work up, the product **2.108** could not be obtained.



Scheme 2.46

Heckel *et al.*[245] reported the product of chlorination of 7-hydroxy-pteridines **2.111** as 7-chloropteridines **2.112** using an excess of phosphorus oxychloride and potassium chloride at 80-90 °C for 2-8 h reaction time in 57-92% yield (**Scheme 2.47**).



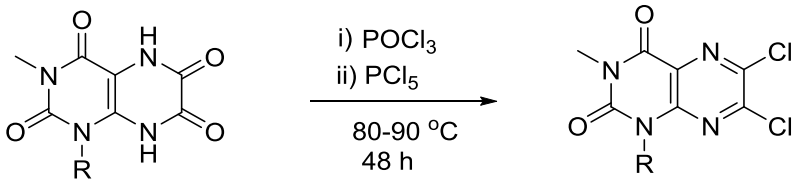
2.111

R, R^1, R^2 = CH_3 or H

2.112

Scheme 2.47

Heckel *et al.*[246] had also reported the product of chlorination of 1,3-dimethyllumazine **2.113** as 6,7-dichloropteridine **2.114**, using an excess of phosphorus oxychloride and phosphorus pentachloride at 80-90 ^{o}C for 48 hours in a 46% yield (**Scheme 2.48**).

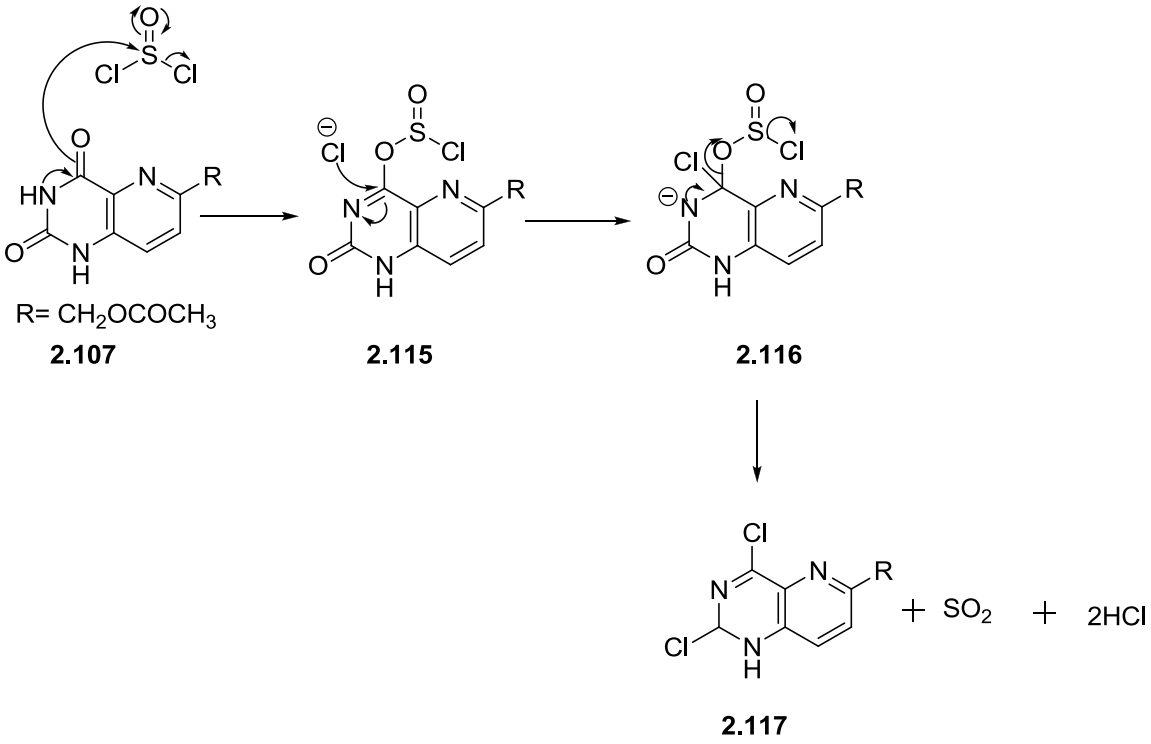


2.113 **2.114**

Scheme 2.48

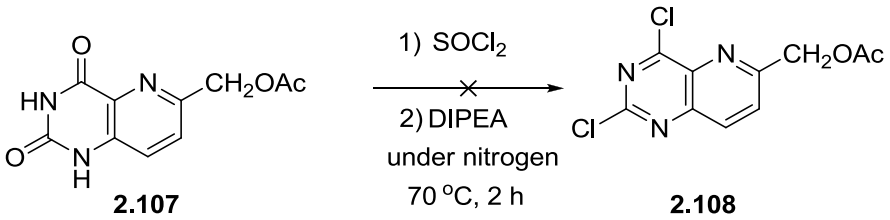
In view of the above reactions, another chlorination reaction was attempted using freshly distilled phosphoryl chloride, followed by phosphorus chloride and triethylamine at room temperature at 120 ^{o}C for 2 h. After work up, only 34% of the starting material was recovered.

The chlorination reaction of pyridopyrimidine **2.107** was also attempted using thionyl chloride**.** The proposed mechanism is shown in (**Scheme 2.49**)**.**



Scheme 2.49

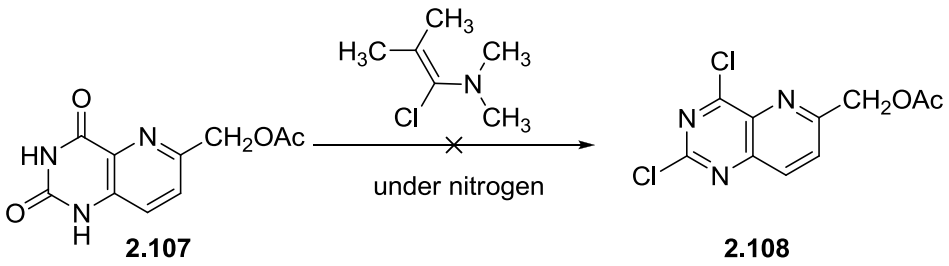
In an attempt to chlorinate pyridopyrimidine **2.107,** thionyl chloride was used under a nitrogen atmosphere in the presence of freshly distilled *N*,*N*-diisopropylethylamine at 70 ^{o}C for 2 hours (**Scheme 2.50**), however the desired chlorinated product **2.108** was not formed. Using DBU as a base, with an excess of thionyl chloride at 70 ^{o}C for 12 hours did not give any chlorinated product **2.108**.



Scheme 2.50

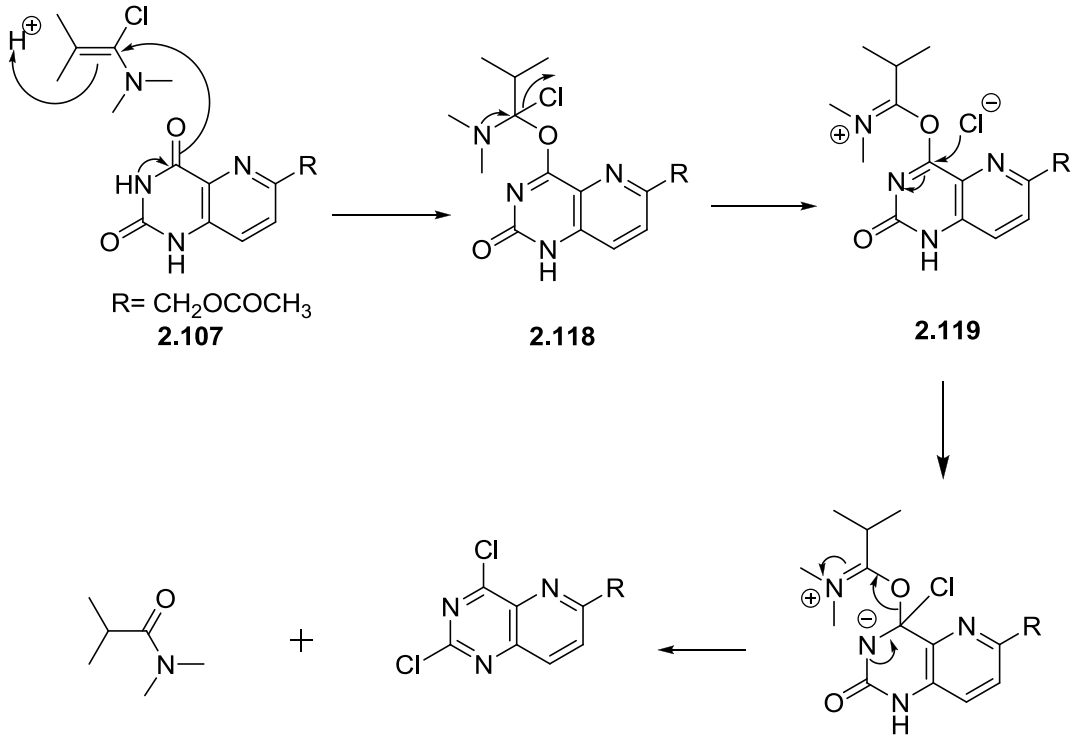
The chlorination of pyridopyrimidine of **2.107** was also attempted using a ten-fold excess of Ghosez's reagent in DCM at room temperature (**Scheme 2.51**); only starting material **2.107**

(67%) was isolated but none of the required dichloro product **2.108** was obtained. When the chlorination reaction was done under microwave conditions with excess of Ghosez's reagent at 130 ^{o}C for 15 min, just starting material **2.107** (50%) was again recovered.



Scheme 2.51

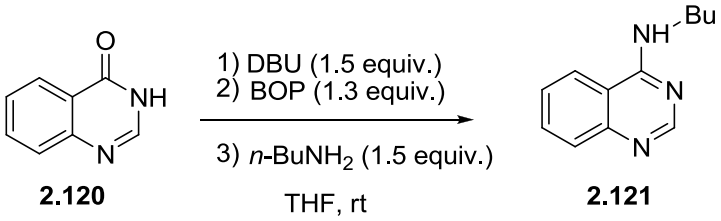
The proposed mechanism for chlorination of pyridopyrimidine **2.107** with Ghosez's reagent is shown in **Scheme 2.52**.



Scheme 2.52

2.7.2 The nucleophilic substitution reaction at 2 and 4 position of 2,4-dioxopyridopyrimidine 2.114 using BOP as a coupling reagent.[234]

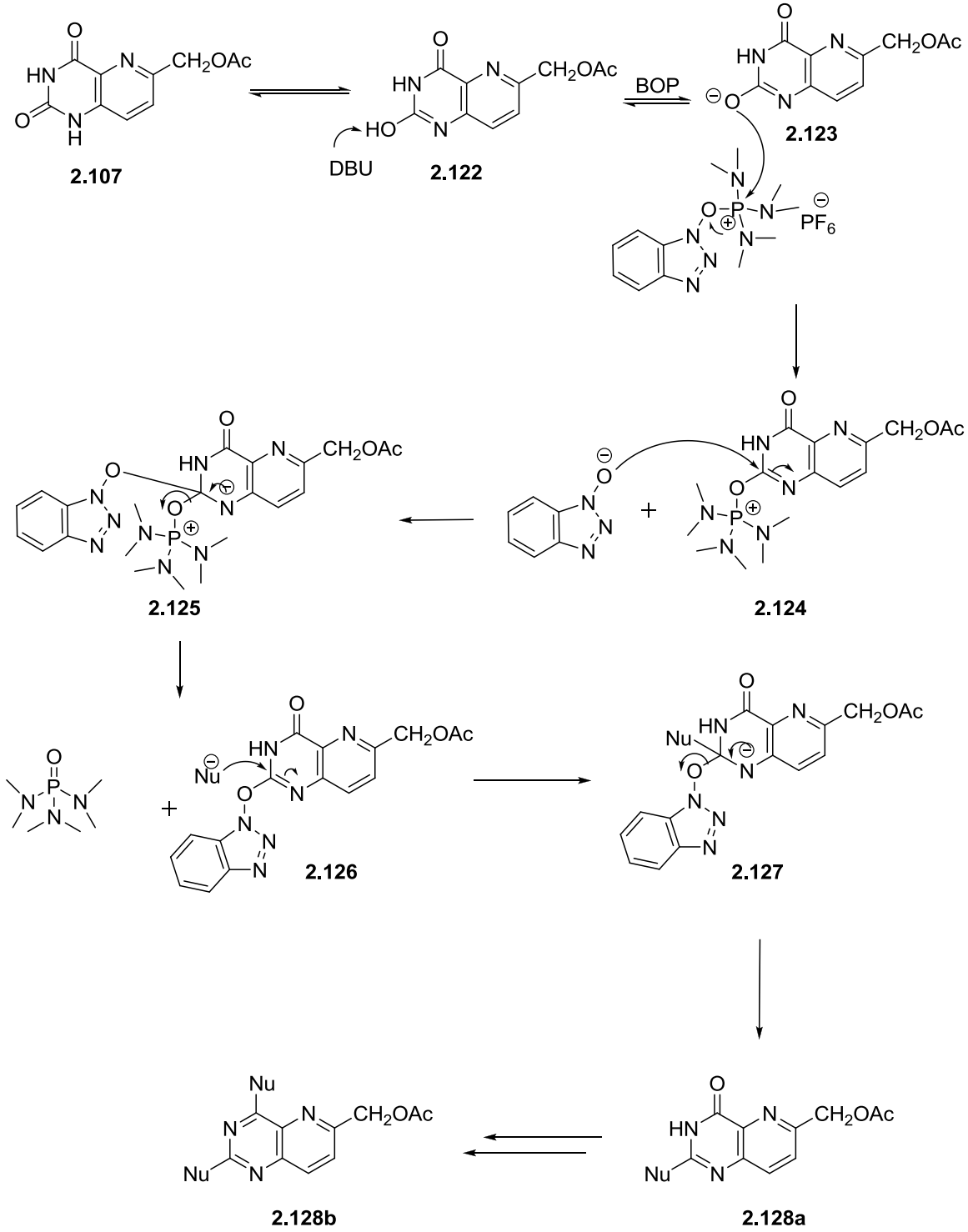
The amination of cyclic amides is usually achieved *via* S_NAr substitution. Using chlorinating reagents such as $SOCl_2$, PCl_5 and $POCl_3$, the carbonyl group is activated under harsh and acidic conditions which might result in loss of functional groups. By employing a nucleophilic substitution reaction under milder conditions, benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate (BOP) can be utilized for amine bond formation as shown in **Scheme 2.53**. [234]



2.120 **2.121**

Scheme 2.53

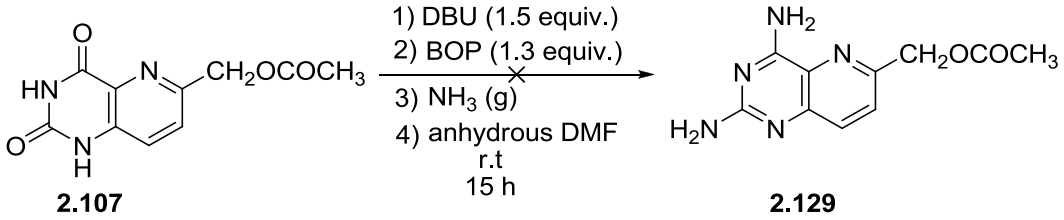
One possible mechanism for this reaction using BOP as a catalyst to activate the carbonyl is given in **Scheme 2.54**.



Scheme 2.54

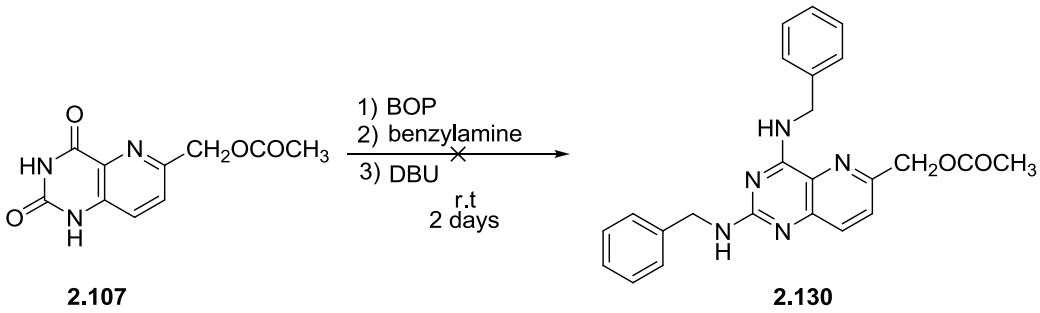
The first reaction for the preparation of **2.129**, (**Scheme 2.55**) was attempted using BOP and DBU in anhydrous DMF as a solvent, saturated with dry ammonia gas, for 15 hours at room

temperature under a nitrogen atmosphere. After work up, only starting material **2.107** (60%) was recovered and product **2.129** could not be found.



Scheme 2.55

The reaction of benzylamine using BOP in DMF/DMSO (1:1) and DBU as a non-nucleophilic base for 2 days at room temperature and then for 2 hours at 60 ^{o}C (**Scheme 2.56**) also did not give the expected product **2.130**.



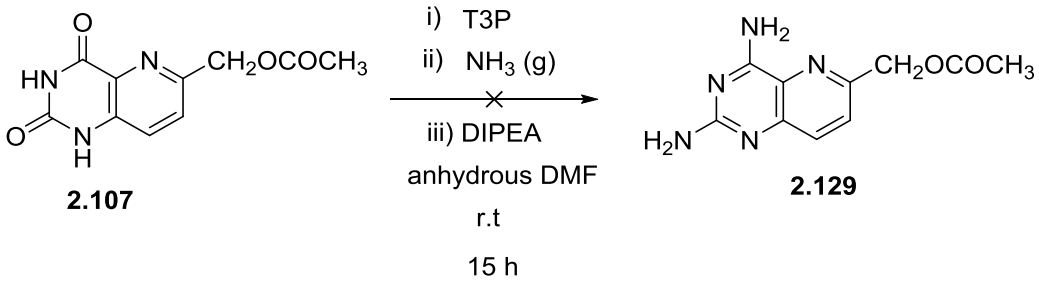
Scheme 2.56

2.7.3 The nucleophilic substitution at C^2 and C^4 positions of 2,4-dioxopyridopyrimidine 2.114 by the activation of carbonyl using T3P as a coupling reagent[235]

The activation of carbonyl group for nucleophilic attack at C^2 and C^4 positions of 2,4-dioxopyridopyrimidine **2.107** was attempted using T3P, however only starting material was recovered in 52-77% yield under different reaction conditions (Table 2.10, Entry 1-3), (**Scheme 2.57**).

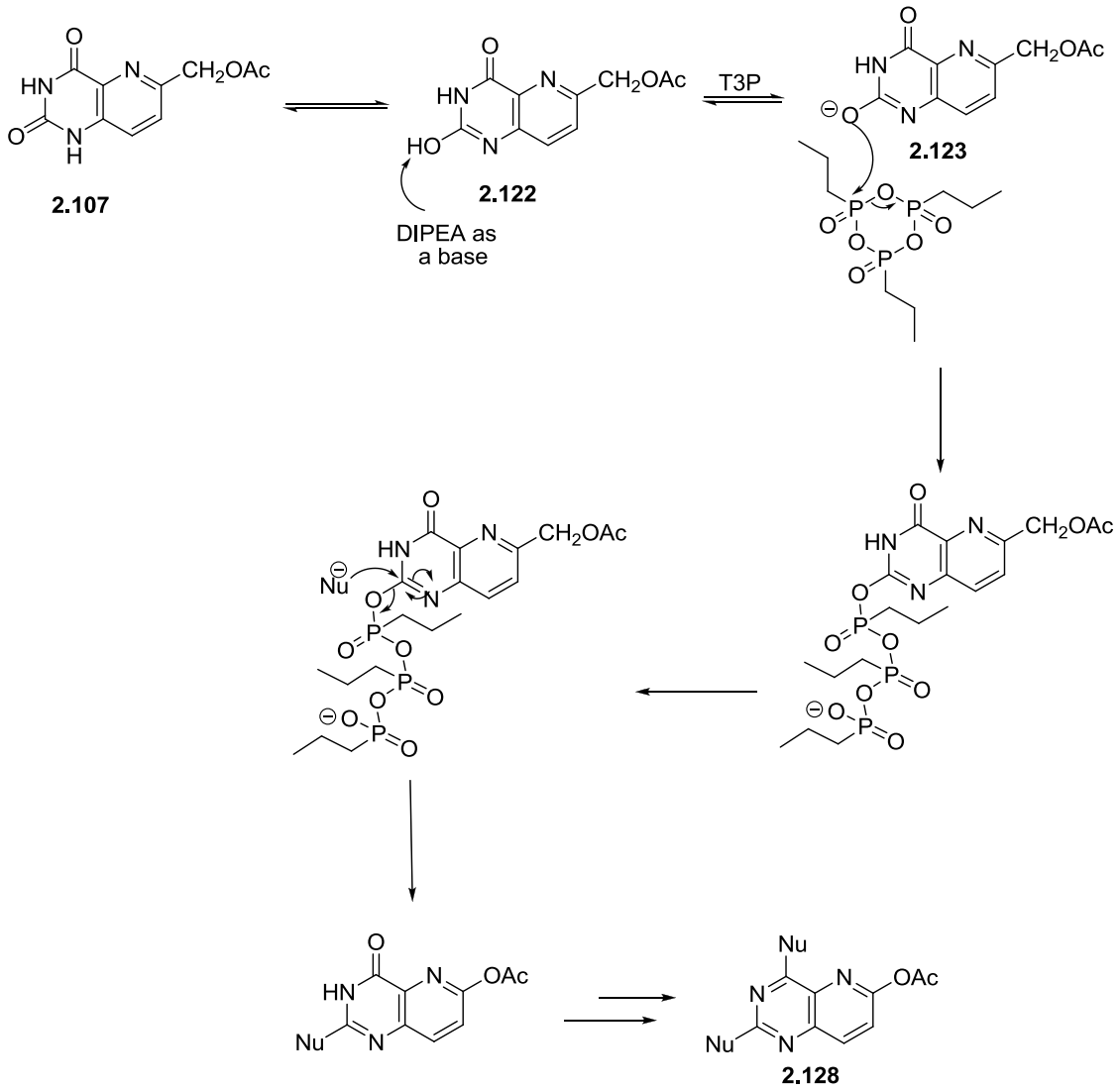
Entry	Nucleophile	T3P	Base (DIPEA)	Anhydrous Solvent	Reaction time (h)	Starting material recovered (%)
1	$NH_{3\,(g)}$ in excess	2.0 equiv.	1.5 equiv.	DMF	15	77
2	Ammonia solution in ethanol (2M)	2.2 equiv.	8.0 equiv.	THF	12	68
3	$NH_{3\,(g)}$ in excess	2.0 equiv.	2.7 equiv.	DMF	12	52

Table 2.10



Scheme 2.57

The proposed mechanism for coupling using T3P is shown in **Scheme 2.59.**

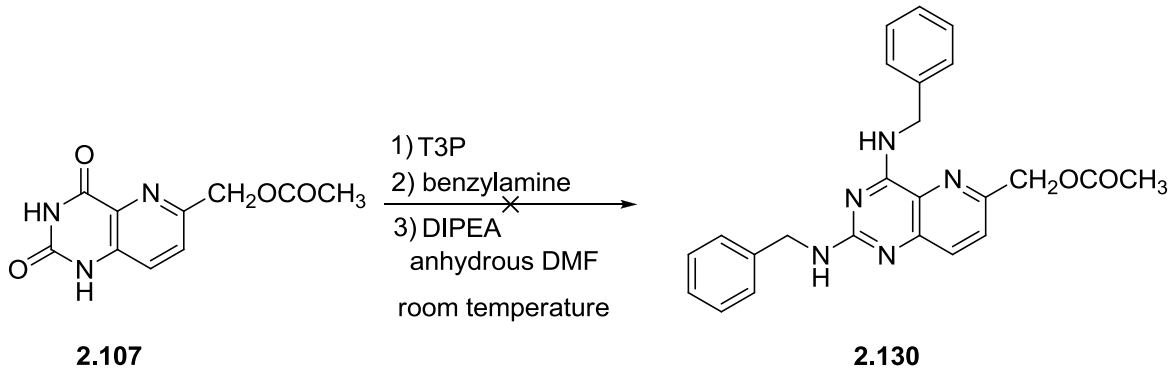


Scheme 2.58

The coupling reaction was also attempted using benzylamine as a nucleophile instead of ammonia. The disubstituted product **2.130** would be different in polarity as compared to starting material **2.107** and would likely be simpler to purify. But after the reaction, only starting material was recovered in 63-84% yield (Table 2.11, Entry 1-3), (S**cheme 2.59**).

Entry	Nucleophile (Benzylamine)	T3P	Base	Anhydrous solvent	Reaction time (h)	Starting material recovered
1	2.0 equiv.	2.2 equiv.	DIPEA 2.2 equiv.	DMF	16	84%
2	2.0 equiv.	2.0 equiv.	DBU 6.2 equiv.	THF	12	68%
3	2.2 equiv	2.0 equiv.	DIPEA 8.0 equiv.	DMF	15	63 %

Table 2.11

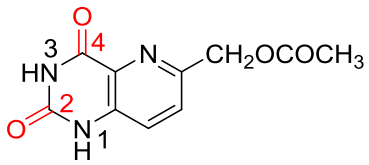


2.107 **2.130**

Scheme 2.59

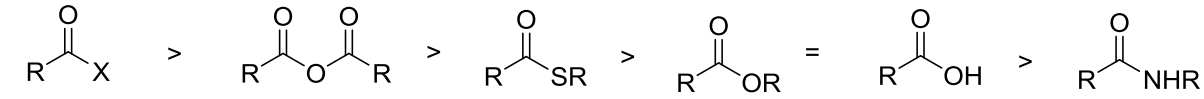
2.8.0. Some drawbacks with (2,4-dioxo-1,2,3,4-tetrahydropyrido[3,2-*d*]pyrimidin-6-yl)methyl acetate and new synthetic plan

There were some drawbacks with **2.107**; it has an ester functionality which is acid- and base-sensitive under vigorous conditions. Therefore, in most of the reactions attempted, hydrolysis of the ester was possible to some extent, reducing the recovery of starting material **2.107.** At high temperatures, in acidic and basic conditions almost complete hydrolysis of the ester was observed. Nucleophilic attack at the C^2 and C^4 positions in pyrimidones does not occur without activation. The 2,4-dioxopyridopyrimidine **2.107** has a uracil carbonyl group at the C^2 position and an amide carbonyl at the C^4 position and both carbonyls are unreactive without some sort of activation.



2.107

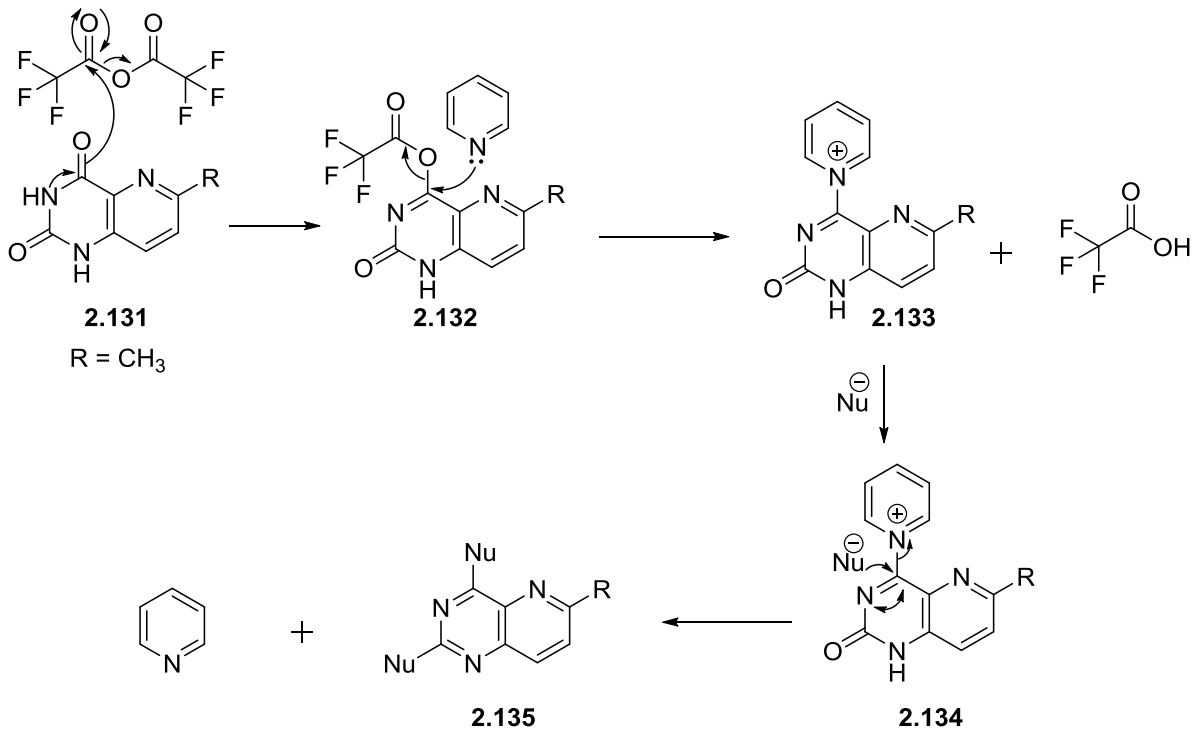
The amides are the least reactive of carboxylic acid derivatives towards nucleophilic attack. The order of reactivity of carboxylic acid derivatives is shown below. The amide carbonyl carbon is less electron-deficient when compared to an ester carbonyl carbon because the lone pair on the nitrogen in an amide group is more likely to be delocalized onto the nearby carbonyl as compared to non-bonding electrons on oxygen in an ester group.



X = Cl, Br

Reactivity of carboxylic acid derivatives towards nucleophilic attack

The activation of the amide carbonyl carbon was successful with trifluoroacetic anhydride in anhydrous pyridine at room temperature, which will be discussed in section 2.13 and 2.13.1. The proposed mechanism for nucleophilic reaction with trifluoroacetic anhydride and pyridine is shown in **Scheme 2.60**.



Scheme 2.60

The rate-limiting step in this carbonyl activation reaction is the reaction of the keto and enol tautomers with the electron-deficient electrophile generated from TFA anhydride during the course of the reaction. Although the keto form is more stable than the enol form, the enol form could be more important than the keto form for such a carbonyl activation reaction. The pyridine could have played a role in stabilizing that electron-deficient electrophile (**Scheme 2.61**).

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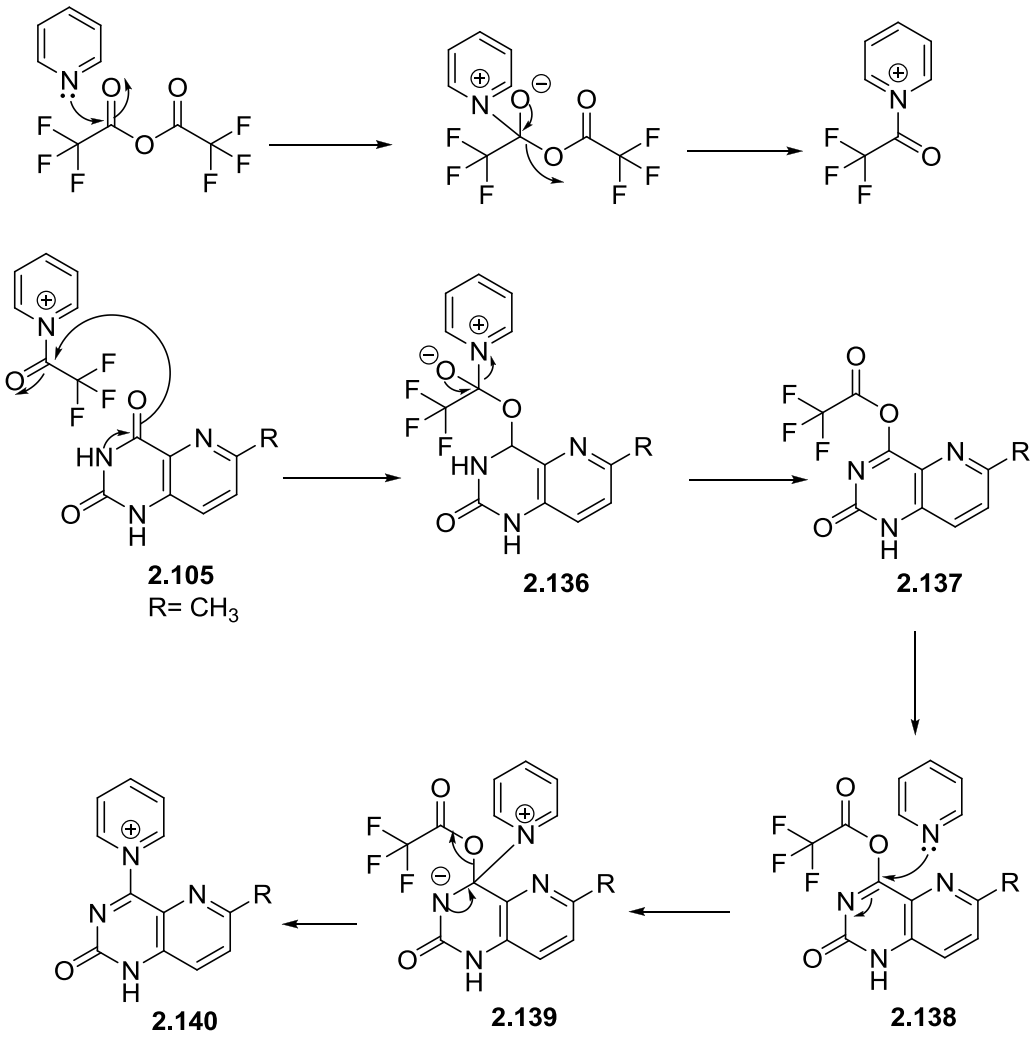
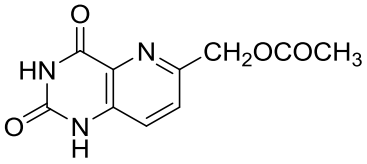


Scheme 2.61

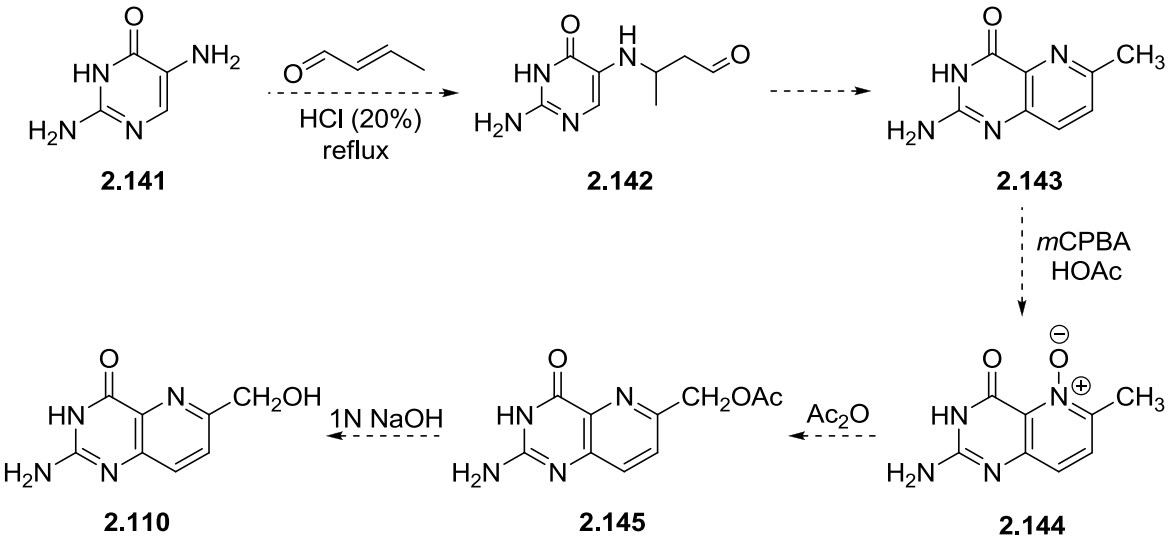
2.9.0. New synthetic plan for C^2 substituted analogue of 2,4-dioxopyridopyrimidine 2.107

Whilst the approach to **2.110** through the 2,4-dioxo intermediate **2.107** would in principle give access to several different compounds of interest as NOS activators with different substituents at C^2 and C^4, the substitution step was not successful.



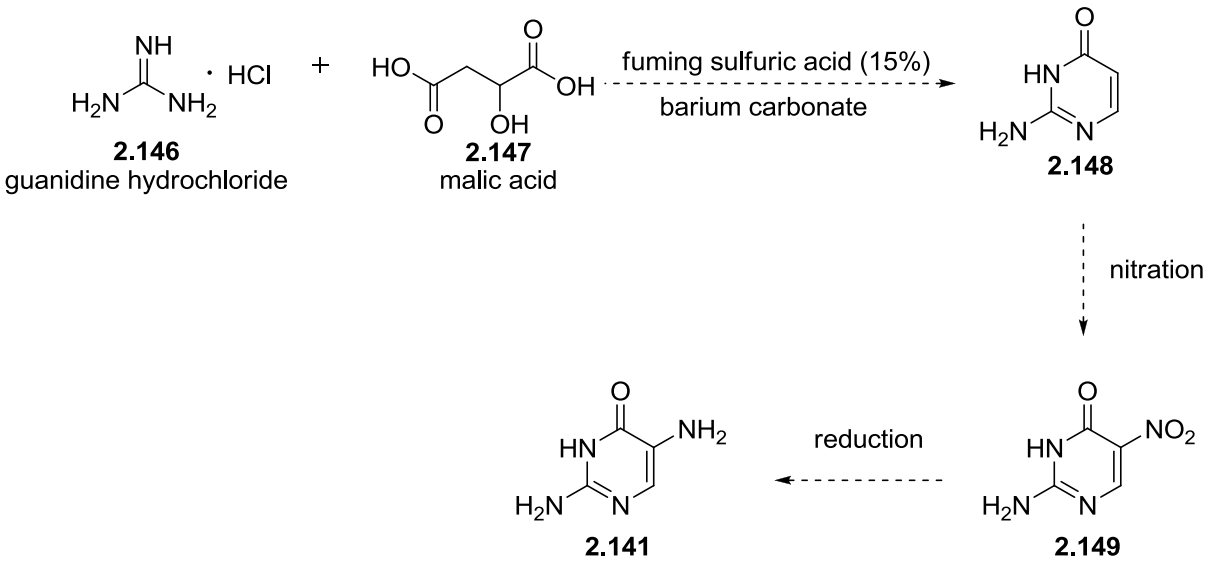
2.107

It was therefore decided to synthesise 2,5-diamino-2,3-dihydro-4(1*H*)-pyrimidinone **2.141**, which can be used for the synthesis of **2.110** as shown in **Scheme 2.62**.[236]



Scheme 2.62

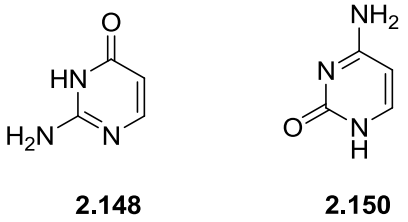
2,5-Diamino-2,3-dihydro-4(1*H*)-pyrimidinone **2.141** is available from some chemical companies but only as custom synthesis. 2-Amino-2,3-dihydro-4(1*H*)-pyrimidinone **2.148** can be used for the preparation of **2.141** by its nitration and subsequent reduction, so the first step was to synthesise isocytosine **2.148.** Isocytosine **2.148** can be prepared by the reaction of guanidine hydrochloride (2.146) and malic acid (2.147|) in fuming sulfuric acid (15%).[237] The full proposed synthesis of 2,5-diamino-2,3-dihydro-4(1*H*)-pyrimidinone **2.141** is shown in **Scheme 2.63**.



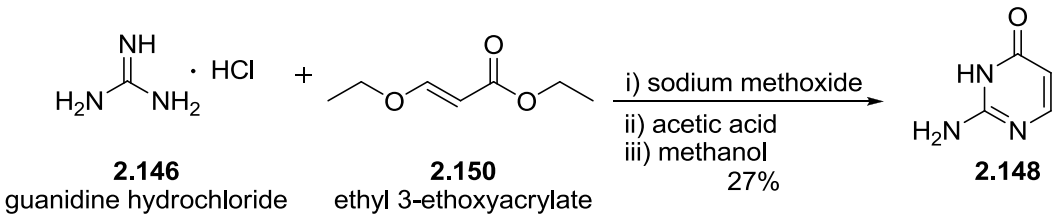
Scheme 2.63

2.9.1 Synthesis of 2-amino-4(3*H*)-pyrimidinone 2.148

2-Amino-4(3*H*)-pyrimidinone **2.148**, also called isocytosine, is a structural isomer of cytosine **2.150**.[238] Cytosine is one of the four principal bases found in DNA and for RNA, these four bases are adenine, guanine, thymine and uracil. Cytosine is a derivative of pyrimidine. The use of isocytosine for the synthesis of antiviral compounds has been published.[239,240] Isocytosine played in important role for the preparation of a biologically important compound **2.110**.



Doskocil *et al.*[241] synthesized isocytosine **2.148** by the reaction of guanidine hydrochloride **2.146** with an excess of ethyl 3-ethoxyacrylate **2.150** and sodium methoxide (1 M) in methanol under reflux conditions for 5 h in 27% yield. The reaction is shown in **Scheme 2.64**.



2.146
guanidine hydrochloride

2.150
ethyl 3-ethoxyacrylate

2.148

Scheme 2.64

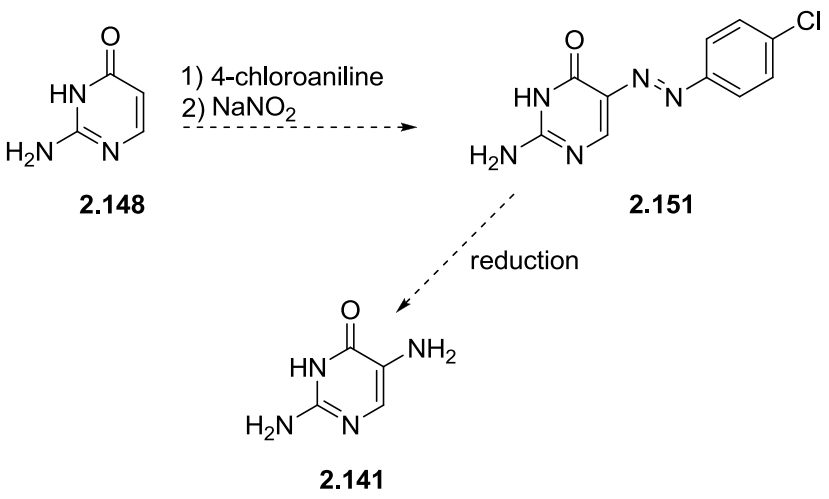
Caldwell *et al.*[237] synthesized isocytosine **2.148** using guanidine hydrochloride **2.146** (1 equiv.), malic acid **2.147** (0.71 equiv.) and fuming sulfuric acid (15%) in 23% yield. This procedure was followed, but with some variations in reaction conditions. The reaction of guanidine hydrochloride and malic acid in an excess of concentrated sulfuric acid at room temperature showed just starting material by TLC, so the minimum temperature and reaction time was explored for the reaction to occur. When the reaction was heated at 105 ^{o}C, two proton signals as doublets for CH=CH were observed in the ^{1}H NMR spectrum, at δ_H 5.23 and δ_H 7.36. The isocytosine **2.148** was obtained in 34-58% yield as a white solid on a 5.0-24.0 g scale (Table 2.12).

Entry	Scale (g)	Temperature (^{o}C)	Reaction time (h)	Yield (%)
1	5	105	2	34
2	24	105	8	49
3	24	105	24	58

Table 2.12

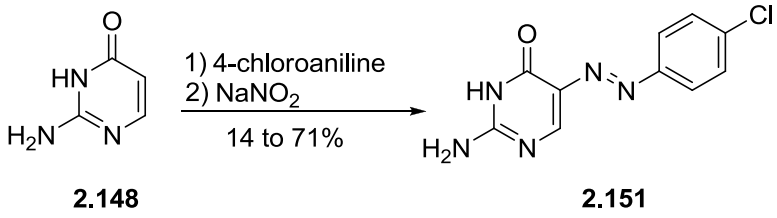
2.9.2 Preparation of 2-amino-5-[(*E*)-(4-chlorophenyl)diazenyl]-4(3*H*)-pyrimidinone 2.151

The first strategy for the preparation of 2,5-diamino-4(3*H*)-pyrimidinone **2.141** was considered by synthesising 5-substituted azopyrimidine **2.151** as shown in **Scheme 2.65**.



Scheme 2.65

For the synthesis of azopyrimidine **2.151**, the reaction mixture was allowed to stir for 15 minutes after coupling with diazonium salt and kept overnight for precipitation. TLC showed remaining starting material and formation of a product. The recovery of a brick-red solid product **2.151** was 14% and starting material **2.148** (85%) was also recovered.

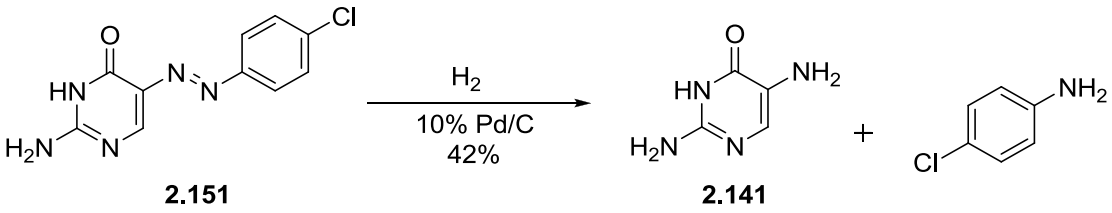


Scheme 2.66

In another experiment, the reaction mixture was allowed to stir for 30 min after coupling with diazonium salt and left overnight for precipitation. The recovery of the brick-red solid product **2.151** was 48%. TLC showed starting material in filtrate and recovery of the starting material **2.148** as a white solid was 50%. When azopyrimidine **2.151** was allowed to react for 4 h, the complete conversion of isocytosine into the azo product was observed by TLC. The 5-azopyrimidine **2.151** was obtained as a brick-red solid in 71% yield. The product **2.151** was confirmed by ^{1}H NMR, HSQC NMR, LRMS and HRMS analyses but it was sparingly

soluble in DMSO and precipitated in the NMR tube, so a ^{13}C NMR spectrum could not be obtained.

2.9.3 Preparation of 2,5-diamino-4(3*H*)-pyrimidinone 2.141 by the reduction of 5-azopyrimidine 2.151

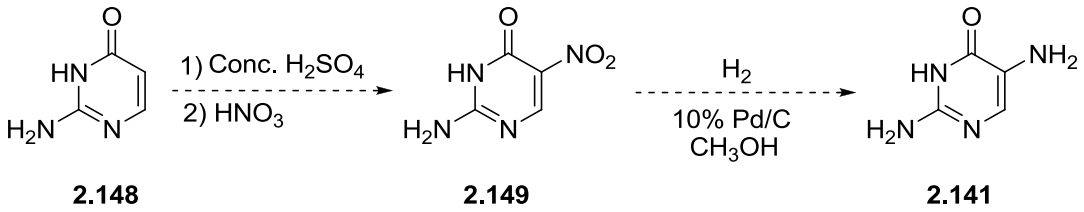


Scheme 2.67

The reduction of 5-diazopyrimidine **2.151** was performed by hydrogenation using 10% Pd/C in methanol for 12 h to give **2.141** as a light-red solid in 42% yield. The reduction of **2.151** was also performed in a mixture of methanol, water, ethyl acetate and DMF using 10% Pd/C by hydrogenation at room temperature for 12 h as it was found not to be completely soluble in methanol and water. After work up, a dark brick-red solid was recovered that proved to be decomposed starting material. From ^{1}H NMR analysis, product **2.141** was found to be impure, also the yield was poor, so the decision was made to synthesise compound **2.141** by the reduction of 2-amino-5-nitro-4(3*H*)-pyrimidinone **2.149.**

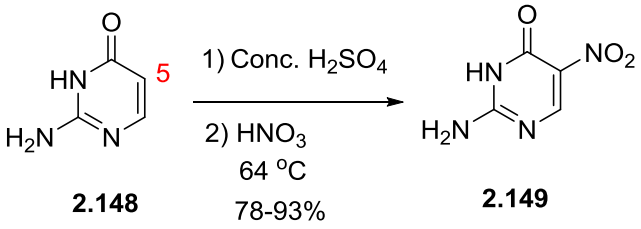
2.9.4 Preparation of 2,5-diamino-4(3*H*)-pyrimidinone 2.141 by the reduction of 2-amino-5-nitro-4(3*H*)-pyrimidinone 2.149

As the reduction of 5-diazopyrimidinine **2.151** did not give us the pure reduced product 2,5-diamino-4(3*H*)-pyrimidinone **2.141** in a good yield, it was planned to obtain 2,5-diamino-4(3*H*)-pyrimidinone **2.141** through nitration of 2-amino-4(3*H*)-pyrimidinone **2.148** and subsequent reduction of 5-nitro pyrimidine **2.149** as shown in **Scheme 2.68**.



Scheme 2.68

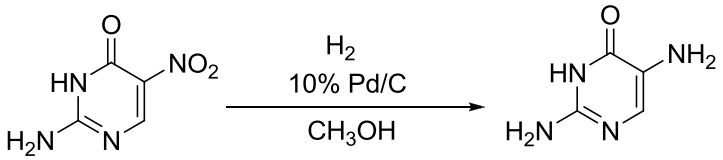
The first step was the preparation of 2-amino-5-nitro-4(3*H*)-pyrimidinone **2.149**. The nitration of 2-amino-4(3*H*)-pyrimidinone **2.148** did not occur at room temperature using concentrated sulfuric acid and 70 % nitric acid or fuming nitric acid for 24 h. Therefore, the minimum temperature was investigated for nitration of **2.148** which was found to be 64 ^{o}C. The complete conversion of 2-amino-4(3*H*)-pyrimidinone **2.148** into nitro product **2.149** was observed using concentrated sulfuric acid and 70 % nitric acid at 64 ^{o}C for 24 h. In the ^{1}H NMR spectrum, a resonance at δ_H 5.23 due to C^5 proton was completely lost which confirmed the completion of the reaction. The product was soluble in acidified water but it precipitated from solution when the reaction mixture was added slowly to dry ice cooled methanol or diethyl ether. The product **2.149** was obtained successfully in 78-93% yield on 1.0-4.0 g scale (**Scheme 2.69**).



Scheme 2.69

The next step was the reduction of 2-amino-5-nitro-4(3*H*)-pyrimidinone to 2,5-diamino-4(3*H*)-pyrimidinone **2.141**. Initially, the reduction of 2-amino-5-nitro-4(3*H*)-pyrimidinone **2.149** was carried out by hydrogenation using 10% Pd/C in methanol for 12 h at room temperature (atmospheric pressure) to give **2.141** as a light-brown solid (85%). One proton signal at C^6 next to nitro group which appeared at 8.80 ppm in the ^{1}H NMR spectrum of the

starting material **2.149** was replaced with a proton signal at 6.87 ppm and two more proton signals were observed due to NH_2 at 6.10-6.30 ppm. The product **2.141** was obtained successfully in 73-92% yield on 0.1-2.4 g scale as shown in **Scheme 2.70**.



2.149 **2.141**

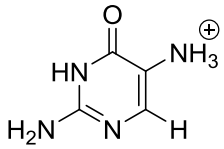
Scheme 2.70

The starting material **2.149** was not completely soluble in methanol, so a large volume of methanol was required, however product **2.141** was soluble in methanol. The yield of product **2.141** was increased by increasing the volume of methanol, but increasing the volume of HCl (1N) had almost no effect upon the yield of product **2.141**, as shown in Table 2.13. This was due to increased solubility of starting material **2.149** in HCl (1N).

Entry	Solvent	Concentration of 2.224 (g/mL) ratio	Time (h)	Yield
1	CH_3OH	0.7/0.25 = 2.80	12	85%
2	CH_3OH	1.0/0.17 = 5.88	12	58%
3	CH_3OH	1.0/0.30 = 3.33	12	76%
4	CH_3OH	0.5/0.52 = 0.96	30	81%
5	HCl (1N)	0.4/0.10 = 4.00	18	84%
6	HCl (1N)	1.0/0.20 = 5.00	12	89%
7	HCl (1N)	0.9/0.20 = 4.50	12	87%
8	HCl (1N)	2.0/0.03 = 66.67	12	84%
	HCl (1N) and methanol by V/V 30/100	2.4/0.13 = 18.46	12	92%

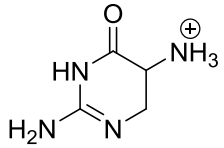
Table 2.13

When HCl (1N) was used as a solvent, two types of compounds, the product **2.152** and side-product **2.153** were found in crude material. The product **2.152** was precipitated in methanol, the C^6 proton which appeared at 8.80 ppm in the ^{1}H NMR spectrum of the starting material **2.149** was replaced with a proton signal at 7.23 ppm and one peak integrating for 3H was observed at 7.79 ppm due to protonation of the NH_2.



2.152

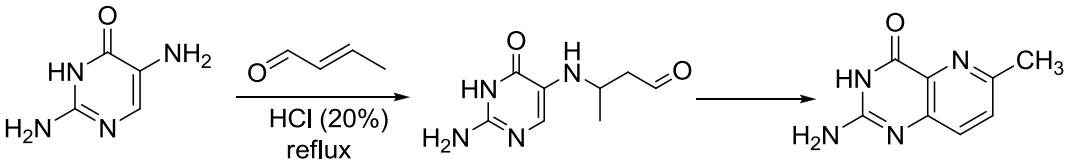
The second compound **2.153** was a light-cream coloured solid and was obtained by adding diethyl ether to the filtrate. The C^6 proton which appeared at 8.80 ppm in ^{1}H NMR spectrum of the starting material **2.149** was replaced with two proton signals as a multiplet in the range of 3.54-3.91 ppm, while one peak integrating for 3H was found at 7.79 ppm due to protonation of the NH_2. LRMS analysis was consistent with the structure of over-reduced pyrimidine **2.153**.



2.153

2.9.5 Preparation of 2-amino-6-methylpyrido[3,2-*d*]pyrimidin-4(3*H*)-one 2.144.[236]

Kelley *et al*.[236] reported the synthesis of 6-methylpyrido[3,2-*d*]pyrimidin-4(3*H*)-one **2.228** by an intramolecular electrophilic cyclisation of intermediate **2.227**, derived from 2,5-diamino-4(3*H*)-pyrimidinone **2.226.**



2.142 **2.143** **2.144**

Scheme 2.71

The pyridopyrimidine **2.144** was an intermediate for the synthesis of our target molecule **2.110**, as shown before in **Scheme 2.62**. The product **2.144** was soluble in water and impurities were separated by filtration. The light-yellow solid was purified by column chromatography (50 % ethyl acetate/methanol). The product **2.144** was white fluorescent on TLC and in solution. In solution it was also white fluorescent if viewed under UV in longer wavelength light.

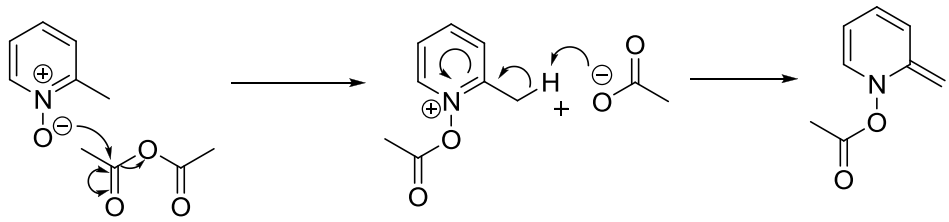
After 2 h reaction time at 120 ^{o}C, it was found that starting material **2.142** was left in the reaction mixture. Different reaction conditions were investigated to improve the yield of **2.144**. With a decrease in temperature the yield of **2.144** was decreased**.** The product **2.144** was obtained successfully in 32-55% yield on 0.12-3.4 g scale (Table 2.14).

Entry	Crotonaldehyde	Temperature (^{o}C)	Reaction Time (h)	Yield of 2.228 (%)
1	3.65 mmol, 1.5 equiv.	122	2	48
2	2.97 mmol, 1.5 equiv.	122	2	50
3	1.82 mmol, 1.9 equiv.	122	2	42
4	6.08 mmol, 2.1 equiv.	120	3	46
5	18.22 mmol, 2.9 equiv.	120	3	44
6	2.43 mmol, 1.9 equiv.	120	4	55
7	8.50 mmol, 1.9 equiv. 8.50 mmol, 1.9 equiv.	50 85 120	3.5 2 3	43
8	32.20 mmol, 1.2 equiv. 32.20 mmol, 1.2 equiv.	105 120	2 5	32
9	11.90 mmol, 1.5 equiv.	105	2	34
10	13.09 mmol, 1.5 equiv.	96	14	36
11	24.33 mmol, 1.2 equiv.	96	14	32

Table 2.14

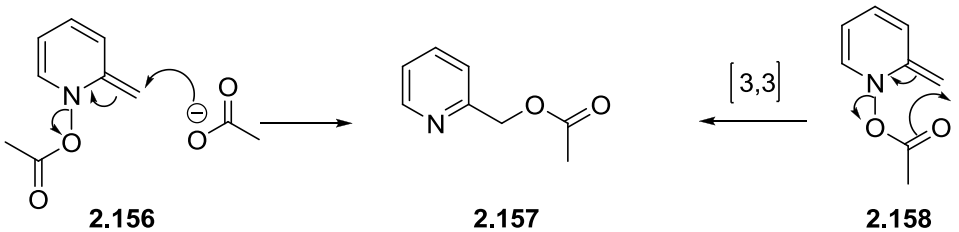
2.9.6 Preparation of [2-acetylamino)-4-oxo-3,4-dihydropyrido[3,2-*d*]pyrimidin-6-yl]methyl acetate 2.159

m-CPBA (*meta*-chloroperoxybenzoic acid) is more reactive than peroxybenzoic acid. Pyridine can be oxidized to pyridine *N*-oxide with reagents such as *m*-CPBA or H_2O_2 in acetic acid. These *N*-oxides are stable dipolar species with the electrons on oxygen delocalized round the pyridine ring. Pyridine *N*-oxides are reactive towards both electrophilic and nucleophilic substitution. If there is an alkyl group at the 2-position of pyridine *N*-oxide, nucleophilic addition at a more distant site is possible on reaction with acid anhydrides. In the first step, acylation occurs on oxygen and a proton is lost from the side-chain to give the uncharged intermediate **2.156**.



2.154 **2.155** **2.156**

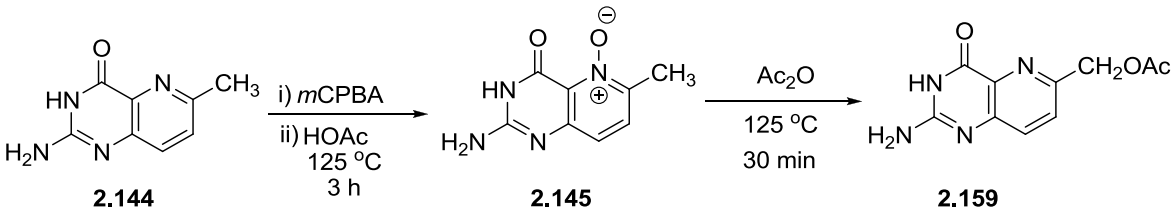
Compound **2.156** rearranges with migration of the acetate group to the side-chain and the restoration of aromaticity to give **2.157**. This may be an ionic reaction or a [3,3]-sigmatropic rearrangement.



2.156 **2.157** **2.158**

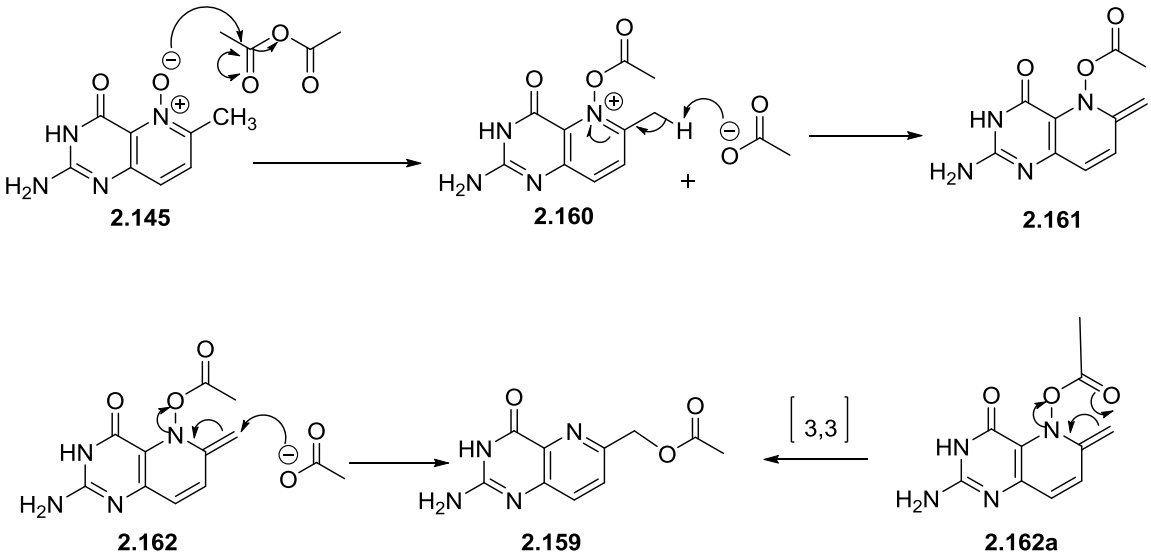
The functionalization of the 6-methyl group of **2.144** was carried out by oxidation of **2.144** with *m*-chloroperoxybenzoic acid in acetic acid at 125 ^{o}C for 3 h and subsequent treatment of the reaction mixture with acetic anhydride at the same temperature for 30 minutes. After

work up and HPLC purification, the 6-methyl acetate pyridopyrimidine **2.159** was obtained as a light-brown solid in 33% yield (**Scheme 2.72**).



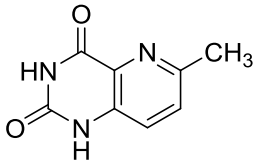
Scheme 2.72

The proposed reaction mechanism for the synthesis of **2.159** from **2.145** is shown in **Scheme 2.73**.

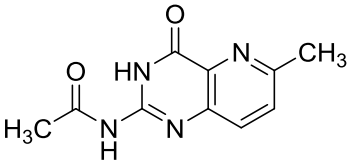


Scheme 2.73

Although product **2.159** was the major fraction from HPLC, there was also *N*-oxide **2.145** occupying 5 % area and **2.105** occupying 10 % area of the HPLC peaks. A less polar white solid obtained by precipitation using methanol and excess diethyl ether proved to be **2.163**.



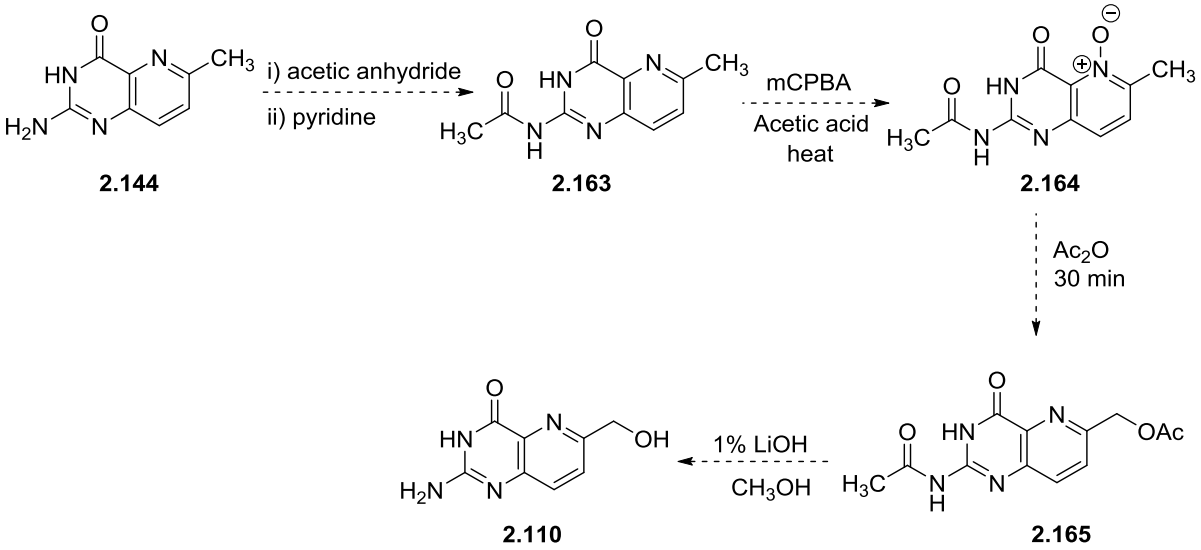
2.105



2.163

2.9.7 Preparation of 6-hydroxymethyl-pyridopyrimidine 2.110 by the protection of amino group of 2-amino-6-methylpyridopyrimidine 2.144 with acetic anhydride

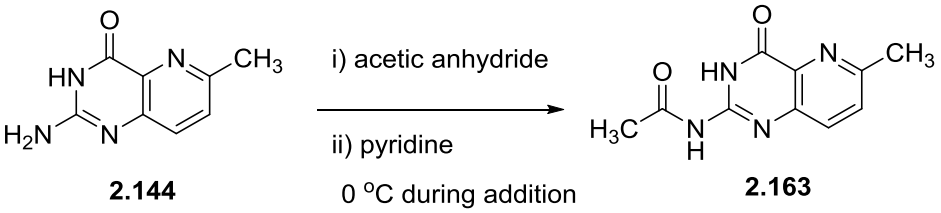
The protected compound could be more easily handled. Also as mentioned earlier in section **2.9.6**, during oxidation of **2.144** with *m*-CPBA, a portion of **2.144** was hydrolysed to 2,4-dioxopyridopyrimidine **2.105.** Also the yield of 6-methyl acetate pyridopyrimidine **2.159** was low, which is a precursor for the preparation of 6-hydroxymethyl-pyridopyrimidine **2.110**. We decided to protect the amino group of pyridopyrimidine **2.144** with an acetyl group, which could then be subjected to *N*-oxide formation to give **2.164**. Subsequent acetic anhydride treatment for ester functionality at the C^6 position to give pyridopyrimidine **2.165**. The basic hydrolysis of this pyridopyrimidine **2.165** would provide the required 6-substituted pyridopyrimidine **2.110** as shown in **Scheme 2.74**.



2.144 2.163 2.164 2.165 2.110

Scheme 2.74

The first step was the acetyl protection of **2.144**, which was accomplished using pyridine and acetic anhydride in an equimolar ratio at room temperature. For *N*-protected product **2.163** in a reasonable quantity, the reaction was repeated by changing the reaction conditions and the acetyl protected pyridopyrimidine **2.163** was obtained in 36-66% yield (Table 2.15), (**Scheme 2.75**).



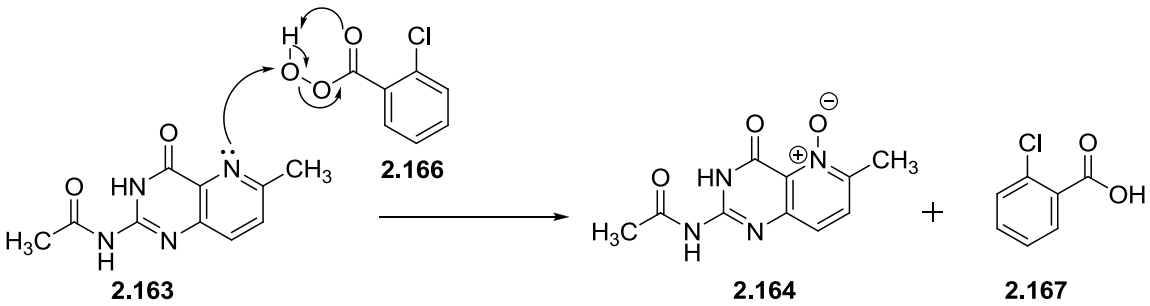
Scheme 2.75

Entry	Compound 2.144 (mmol)	Pyridine (no of equiv.)	Ac_2O (no of equiv.)	Reaction time	Yield 2.163 (%)
1	0.57	36	36	16 h	64
2	2.79	15	15	16 h	61
3	0.43	36	36	18 h	60
4	0.98	17	22	72 h	58
5	0.57	22	22	24 h	36
6	0.28	37	37	16 h	61
7	0.28	37	37	16 h	61
8	7.89	13	13	5 days	66
9	1.14	36	36	24 h	38

Table 2.15

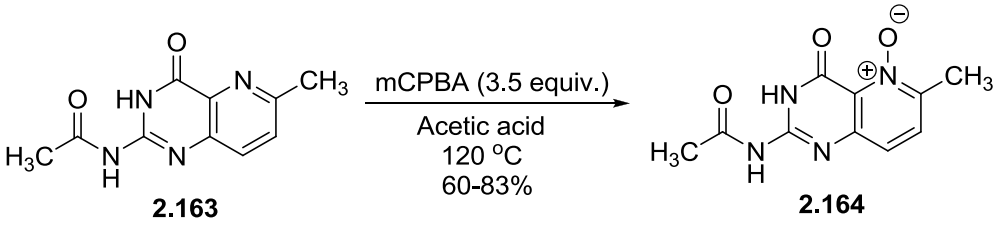
During work up, when product **2.163** was washed with an excess of ether, some of the product was also washed away and was found in filtrate which was recovered by precipitation with a mixture of ethyl acetate, methanol and diethyl ether to give more **2.163** (Table 2.15, Entry 5, 7 and 9).

The next step was the formation of *N*-oxide **2.164**. The mechanism involves nucleophilic attack of the pyridine moiety of **2.163** on the *m*CPBA **2.166**, which was converted into *m*-chlorobenzoic acid **2.167** during the course of the reaction (**Scheme 2.76**).



Scheme 2.76

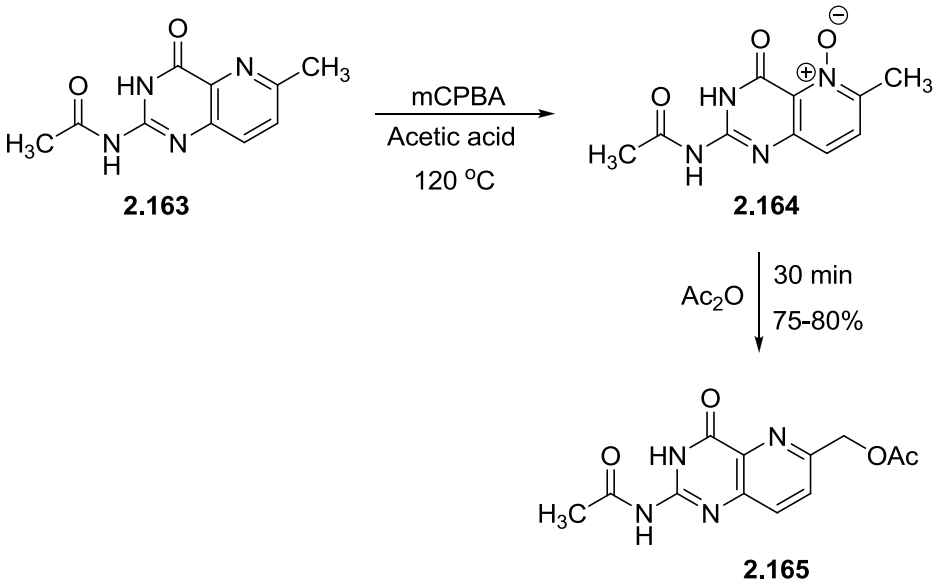
The reaction of **2.163** with *m*-CPBA (3.5 equiv) for 3 h at 120 °C gave *N*-oxide **2.164** successfully in 60-83% yield on 50-120 mg scale (**Scheme 2.77**).



Scheme 2.77

For the preparation of **2.165,** two methods were used. In the first method, a solution of **2.164** was heated under reflux in an acetic acid-acetic anhydride mixture for 30 min. After work up, a light-brown residue was crystallized from methanol and diethyl ether to give **2.165** as a light- brown solid in 75-80% yield on 35-67 mg scale. The product **2.165** was further purified by HPLC to afford **2.165** in 45 % yield. The acetyl methyl resonance from ester group

appeared in the ^{1}H NMR spectrum at δ_H 2.18 and a new, two-proton signal for the methylene group appeared at δ_H 5.21.

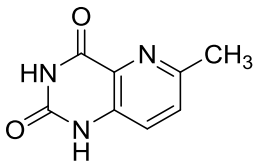


2.163 **2.164** **2.165**

Scheme 2.78

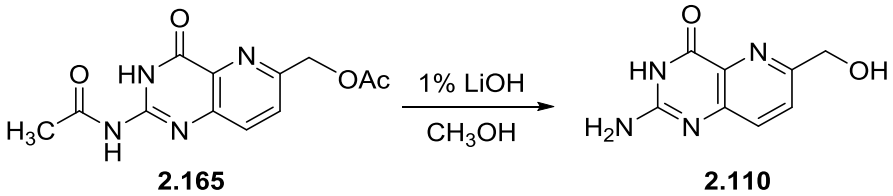
In the second method, **2.165** was prepared without the isolation of the *N*-oxide **2.164**. After removing unreacted *meta*-chlorobenzoic acid with diethyl ether, a light-brown solid was purified to give the desired product **2.165** as a cream-coloured solid in 30-37% yield on 35-90 mg scale.

There were two other fractions found from HPLC, the first fraction occupied area 20%, proved to be **2.105**. The second fraction occupied area 26% in HPLC chromatogram and corresponded to starting material **2.163**.



2.105

The final step was the preparation of 2-amino-6-(hydroxymethyl)pyrido[3,2-*d*]pyrimidin-4(3*H*)-one **2.110** by the hydrolysis of the ester group and deprotection of amine functionality from **2.165** (**Scheme 2.79**).



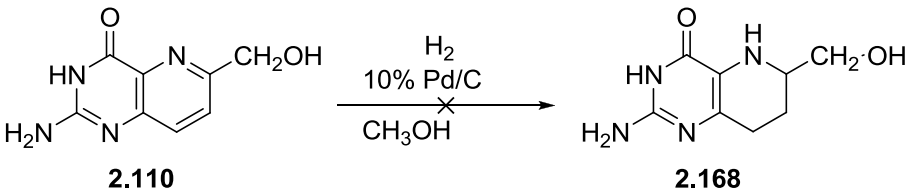
2.165 **2.110**

Scheme 2.79

The hydrolysis of **2.165** was accomplished with 1% LiOH solution in methanol at room temperature for 12 h. A light-brown solid was obtained after work up which was purified by HPLC to give **2.110** as a light-brown solid in 52% yield.

2.10.0 Attempted reduction of 2-amino-6-hydroxymethyl-pyridopyrimidine 2.110 by hydrogenation

2-Amino-6-hydroxymethyl-pyridopyrimidine **2.110** was subjected to hydrogenation over Pd/C in methanol at room temperature (atmospheric pressure) for 12 h (**Scheme 2.80**). After work up, the light-brown solid was purified by HPLC, however just starting material **2.110** was obtained and the reduction did not work.

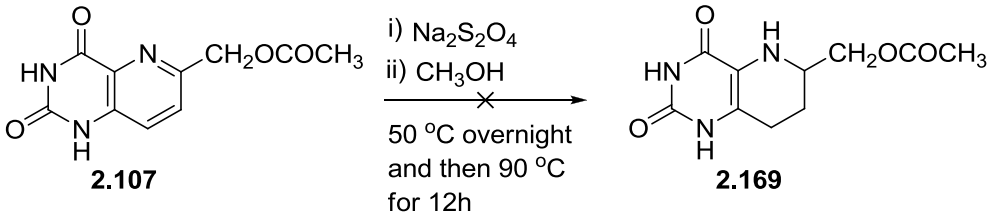


2.110 **2.168**

Scheme 2.80

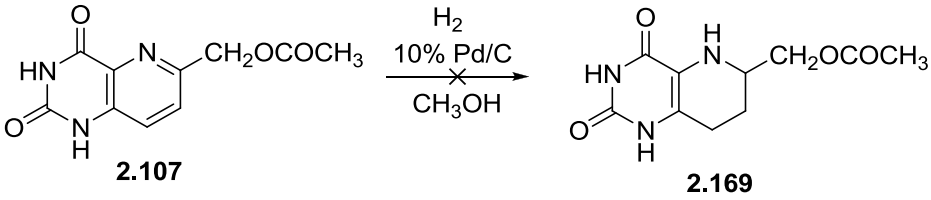
2.10.1 Attempted preparation of 2,4-dioxo-tetrahydropyridopyrimidine 2.169 by reduction

The reduction of 2,4-dioxo-pyridopyrimidine **2.107** using an excess of sodium dithionite in methanol at 50 °C for 12 h (**Scheme 2.81**) gave only starting material. An excess of sodium dithionite was added again, and the reaction mixture was further heated at 90 °C for 12 h. After work up, the light-brown solid residue was purified by HPLC, but no product **2.169** was found and just starting material **2.107** was collected.



Scheme 2.81

The reduction of 2,4-dioxo-pyridopyrimidine **2.107** was also attempted in methanol by hydrogenation over Pd/C at room temperature (atmospheric pressure) for 12 h (**Scheme 2.82**). After HPLC purification, the product **2.169** could not be found and again, only starting material **2.107** was collected.

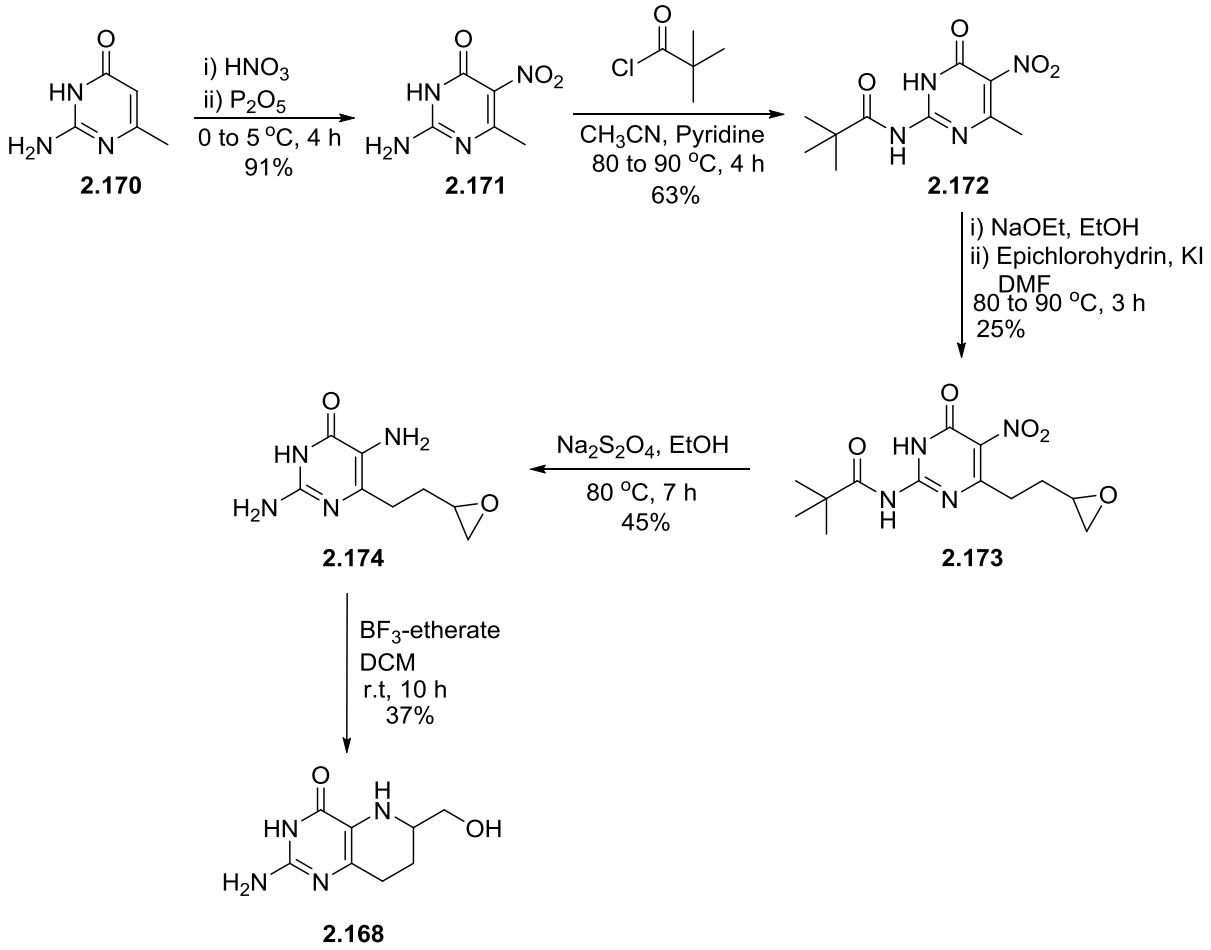


Scheme 2.82

2.11 Preparation of 2-Amino-6-(hydroxymethyl)-5,6,7,8-tetrahydropyrido[3,2-*d*]pyrimidin-4(3*H*)-one 2.168[242]

Zhang *et al.*[242] reported the synthesis of 6-substituted 8-deazatetrahydropteridine **2.168** starting from commercially available 2-amino-6-methyluracil **2.170** (**Scheme 2.83**). 2-Amino-5-nitro-6-methylpyrimidin-4-one **2.171** was prepared by reaction of 2-amino-6-

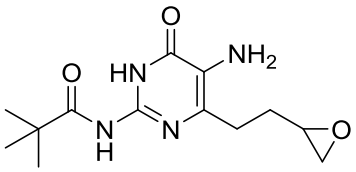
methyluracil **2.170** with HNO_3 and P_2O_5 at 0 to 5 °C. 2-Amino-5-nitro-6-methylpyrimidin-4-one **2.171** was protected with a stable pivaloyl group at the 2-position using pivaloyl chloride in acetonitrile and pyridine to give the 2-pivaloylamide **2.172**. This was then epoxyalkylated at the 6 position with 3-chloro-1,2-epoxypropane and KI in ethanolic sodium ethoxide to give 2-pivaloylamino-5-nitro-6-(3,4-epoxybutyl)uracil **2.173**. This was reduced by sodium dithionite to afford 2,5-diamino-6-(3,4-epoxybutyl)uracil **2.174**. 2-Amino-6-(hydroxymethyl)-5,6,7,8-tetrahydropyrido[3,2-*d*]pyrimidin-4(3*H*)-one **2.168** was obtained in 37% yield by cyclisation of **2.174** with BF_3-etherate in dichloromethane.



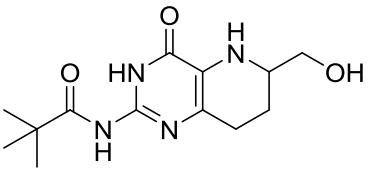
Scheme 2.83

2-Amino-6-(hydroxymethyl)-5,6,7,8-tetrahydropyrido[3,2-*d*]pyrimidin-4(3*H*)-one **2.168** was prepared in six steps with some variations in reaction conditions and methods of purifications. 2-Amino-6-methyl-5-nitro-4(3*H*)-pyrimidinone **2.170** was nitrated using

concentrated sulfuric acid and fuming nitric acid at 0 °C. When 2-pivaloylamino-5-nitro-6-(3,4-epoxybutyl)uracil **2.173** was reduced with sodium dithionite, compound **2.176** was obtained (**Scheme 2.87**). With BF_3-etherate in dichloromethane, compound **2.177** was obtained in 49 % yield (**Scheme 2.88**). The target molecule 8-deazatetrahydropteridine **2.168** was obtained in 89 % yield by the hydrolysis of **2.177** with 0.01 % solution of LiOH in methanol. The different reactions performed for the synthesis of **2.168** are described below.

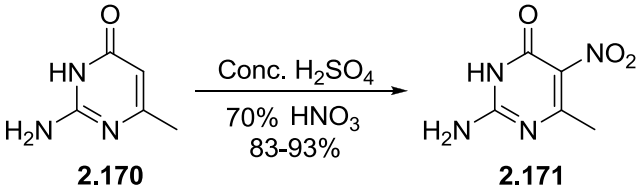


2.176



2.177

The nitration of 2-amino-6-methyl-4(3*H*)-pyrimidinone **2.170** was accomplished using concentrated sulfuric acid and nitric acid at -5 to -10 °C for 30 min and then at room temperature for 2 h and 30 min (**Scheme 2.84**).



2.170 **2.171**

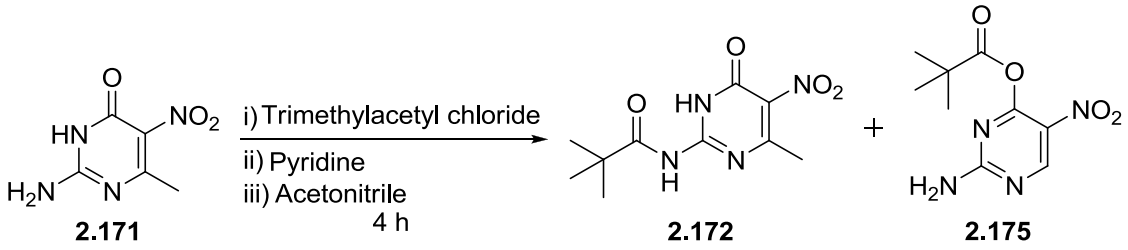
Scheme 2.84

The reaction was monitored by TLC and ^{1}H NMR. The ^{1}H NMR signal at δ_H 5.40 (CH_3-C=C*H*) was completely lost, which confirmed completion of reaction. The product was soluble in acidified water and therefore the work-up used organic solvents for precipitation. The nitro product **2.171** was obtained as a white solid in 83-93% yield on 2.0-6.0 g scale (Table 2.16).

Entry	Scale	H_2SO_4	HNO_3	Temp. (^OC)	Reaction Time (h)	Yield (%)
1	2.0 g	Conc. sulfuric acid	Fuming HNO_3	-5 to -10	30 min at low temperature and 2.5 h at r.t.	93
2	5.0 g	=	=	=	30 min at low temperature, 2 h at r.t.	91
3	6.0 g	=	=	=	30 min at low temperature and 1.5 h at r.t.	83

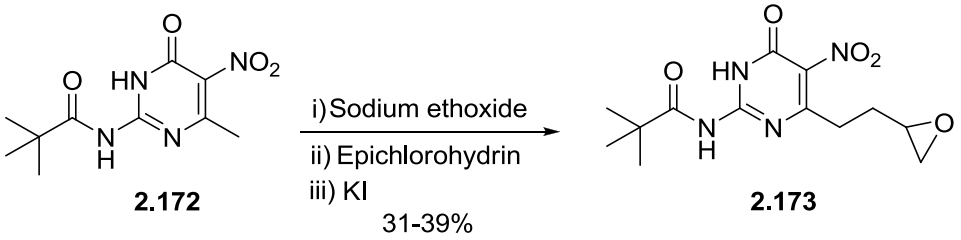
Table 2.16

Pivaloyl protection of the nitropyrimidine **2.171** was accomplished using pivaloyl chloride in acetonitrile solution with pyridine as base under reflux conditions for 4 h. Two products, the *N*-pivaloyl pyrimidinyl **2.172** and *O*-pivaloyl pyrimidine **2.175** were obtained. The *N*-pivaloyl pyrimidinyl **2.172** was obtained as a brown-yellow solid in 70-72% yield on 1.5-5.0 g scale (**Scheme 2.85**).



Scheme 2.85

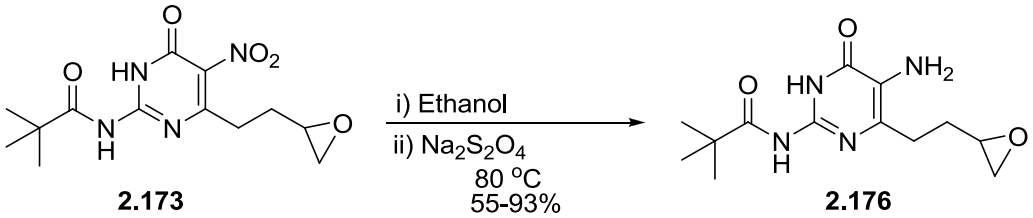
The next step was the preparation of **2.173**, which was done by treating *N*-pivaloyl pyrimidinyl **2.172** with ethanolic sodium ethoxide (1N) in dry ethanol under reflux conditions for half an hour, and then treating the resulting residue with 3-chloro-1,2-epoxypropane and KI in anhydrous DMF at 160 ^oC for 3 h (**Scheme 2.86**).



2.172 **2.173**

Scheme 2.86

After work up, a yellow oily residue was purified by silica gel chromatography and eluting with ethyl acetate and *n*-hexane (2:1). The product **2.173** was eluted before impurities. The product **2.173** was collected as yellow oil in 31-39% yield on 1.3-4.8 g scale. In the next step, the nitro compound **2.173** was reduced with an excess of sodium dithionite at 80 ^{o}C for 7 h in 95% ethanol, after purification by silica gel chromatography, the reduced product **2.176** was obtained in 55% yield.

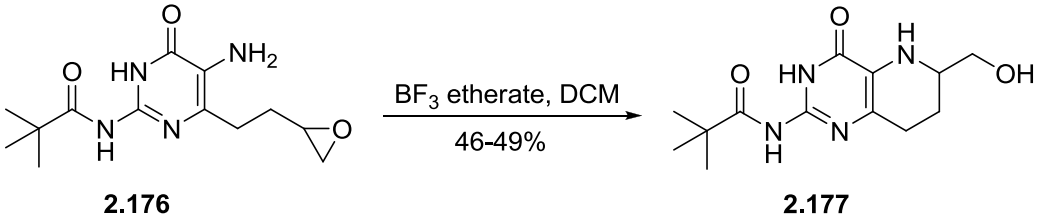


2.173 **2.176**

Scheme 2.87

The reduction of **2.173** on 2.1 g scale, in 95% ethanol with an excess of sodium dithionite, at 80 ^{o}C for 48 h gave 5-amino-6-oxo-1,6-dihydro-2-pyrimidinyl-2,2-dimethylpropanamide **2.176** in 93% yield.

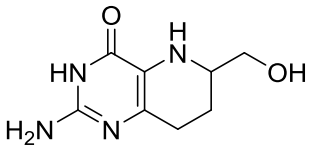
For the next step of reaction, when **2.176** was reacted with boron trifluoride diethyl etherate in dry dichloromethane for 16 h, the epoxide ring opening of **2.176** occurred successfully. The product **2.177** was obtained by purifying on silica gel chromatography and eluted with ethyl acetate and methanol (9:1) and then by HPLC to give the required compound **2.177** in 46-49% yield on 0.1g-2.0 g scale (**Scheme 2.88**).



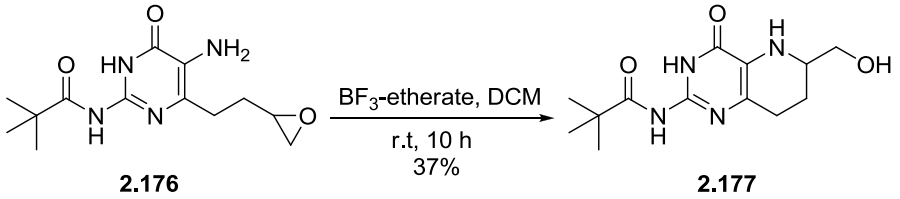
2.176 **2.177**

Scheme 2.88

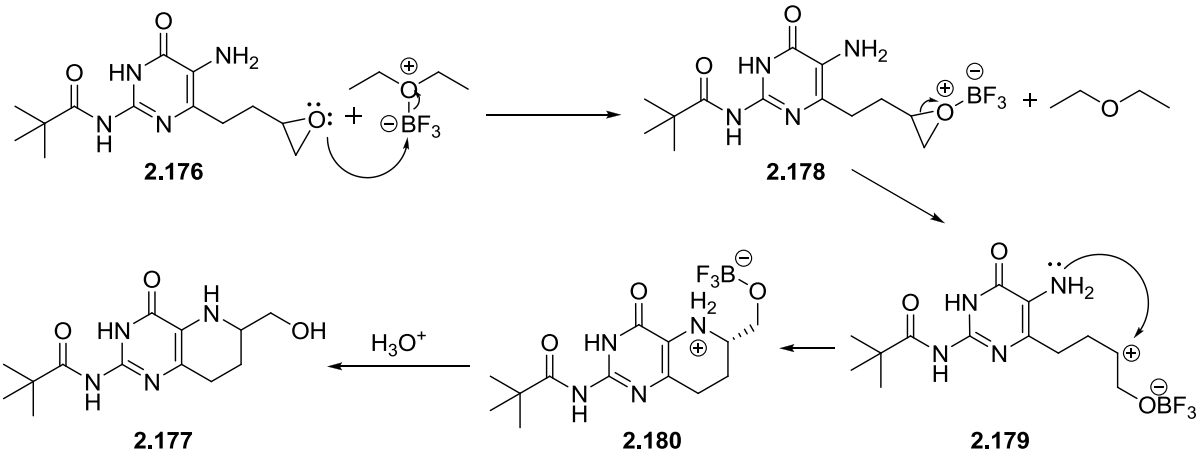
There were two other peaks from HPLC in addition to product **2.177**. The first peak had occupied area 12% and proved to be **2.168**. The second peak was from starting material **2.176** which occupied 31 % area of HPLC chromatogram. The proposed mechanism for epoxide ring opening in **2.176** with BF_3 etherate, to give the cyclised product **2.177**, is shown below in **Scheme 2.89**.



2.168



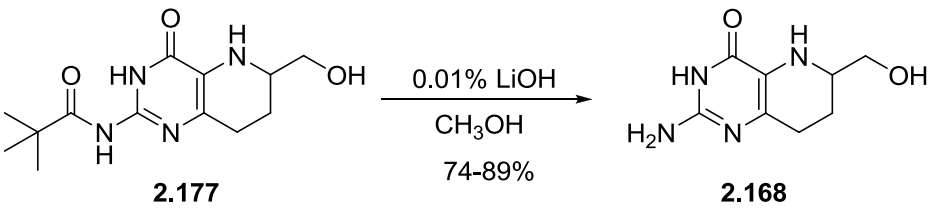
2.176 **2.177**



2.176 **2.178** **2.179** **2.180** **2.177**

Scheme 2.89

The pivaloyl protecting group in **2.177** was successfully hydrolysed using LiOH solution (0.01%) for 12 h under nitrogen. The residue was acidified with acetic acid and purified to give the required product **2.168** as a colourless oil in 74-89% yield on 45 mg-100 mg scale (**Scheme 2.90**).

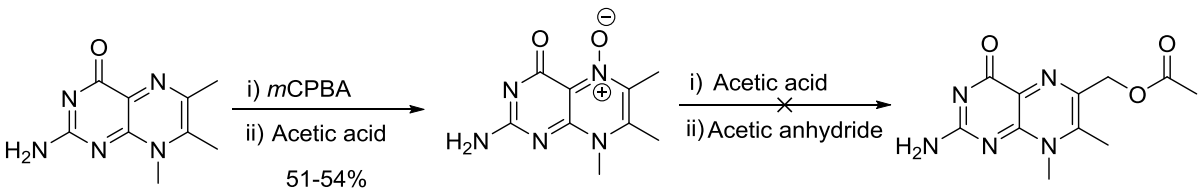


2.177 **2.168**

Scheme 2.90

2.12 Preparation of (2-amino-7,8-dimethyl-4-oxo-4,8-dihydro-6-pteridinyl)methyl acetate

In order to produce more analogues of BH_4 for testing for NOS activity, the synthesis of **2.183** was attempted. 2-Amino-6,7,8-trimethyl-4(8*H*)-pteridinone 5-oxide **2.182** was an intermediate for the preparation of **2.183**. Oxidation of **2.181** was successful with *m*CPBA in glacial acetic acid at 120-138 ^{o}C for 2-3 h (**Scheme 2.91**). However treatment of this reaction mixture with acetic anhydride, followed by purification of the yellow residue by HPLC just gave the *N*–oxide **2.182** as a bright-yellow solid in 51-54% yield on 100 to 160 mg scale (Table 2.17) and unfortunately **2.183** could not be obtained by this reaction.



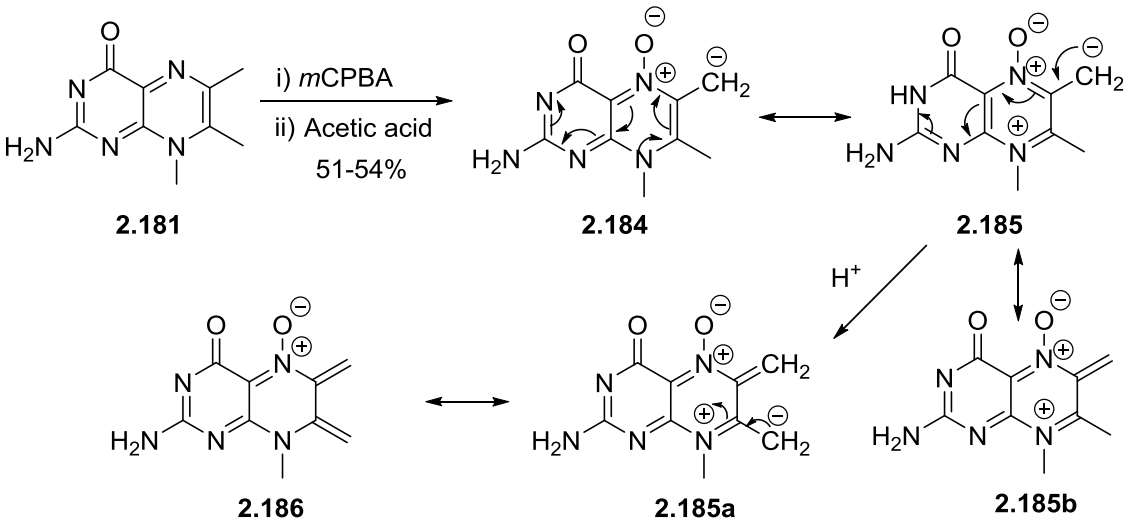
2.181 **2.182** **2.183**

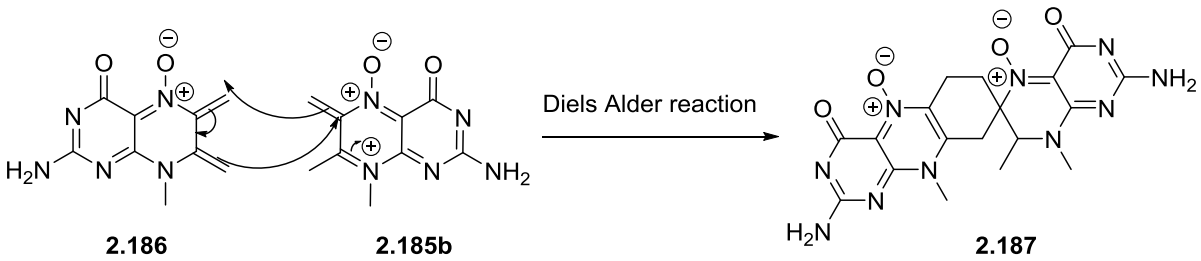
Scheme 2.91

Entry	Scale	*m*CPBA	Temperature	Reaction Time in HOAc	Reaction Time with Ac_2O	Yield 2.182	Yield 2.183
1	0.49 mmol	3.5 equiv	122 °C	3 h	-	52 %.	-
2	0.49 mmol	3.5 equiv	122 °C	3 h	1 h	51 %	0 %.
3	0.78 mmol	3.5 equiv	138 °C	2 h	1 h	53 %	0 %.
4	0.74 mmol	3.5 equiv	120 °C	2 h	1 h and 40 min	54 %	0 %.

Table 2.17

When a methyl group was present at the C^7 positon, as in **2.181**, the expected reaction did not occur. Formation of the *N*-oxide **2.184** using *m*-CPBA proceeded smoothly, however the presence of the two methyl groups could lead to formation of the diene **2.186**. This could participate in a Diels-Alder dimerisation to give the side-product **2.187**.

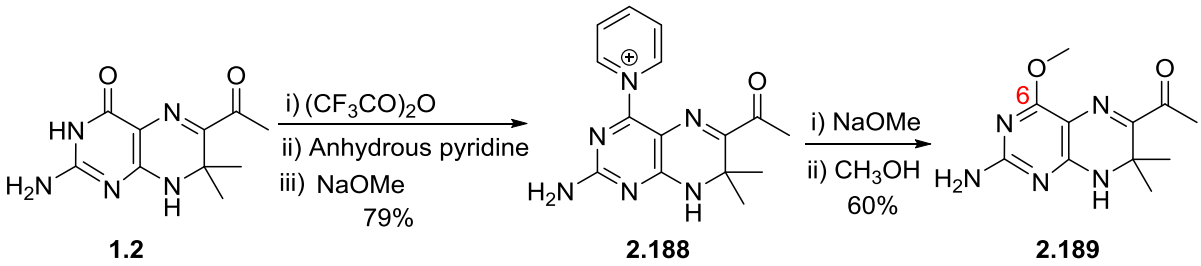




Scheme 2.92

2.13.0 The nucleophilic substitution reaction at the 4 position of 6-acetyl-2-amino-7,7-dimethyl-7,8-dihydro-4(3*H*)-pteridinone 1.2

For the preparation of the 4-methoxy analogue of **1.2** which can be tested for NOS activity, 6-acetyl-2-amino-7,7-dimethyl-7,8-dihydro-4(3*H*)-pteridinone **1.2** was treated with trifluoroacetic anhydride in anhydrous pyridine at 0 ^{o}C, followed by addition of NaOMe/MeOH at room temperature overnight. After work up, the crude yellow material was purified by HPLC to give an intermediate salt **2.188.**

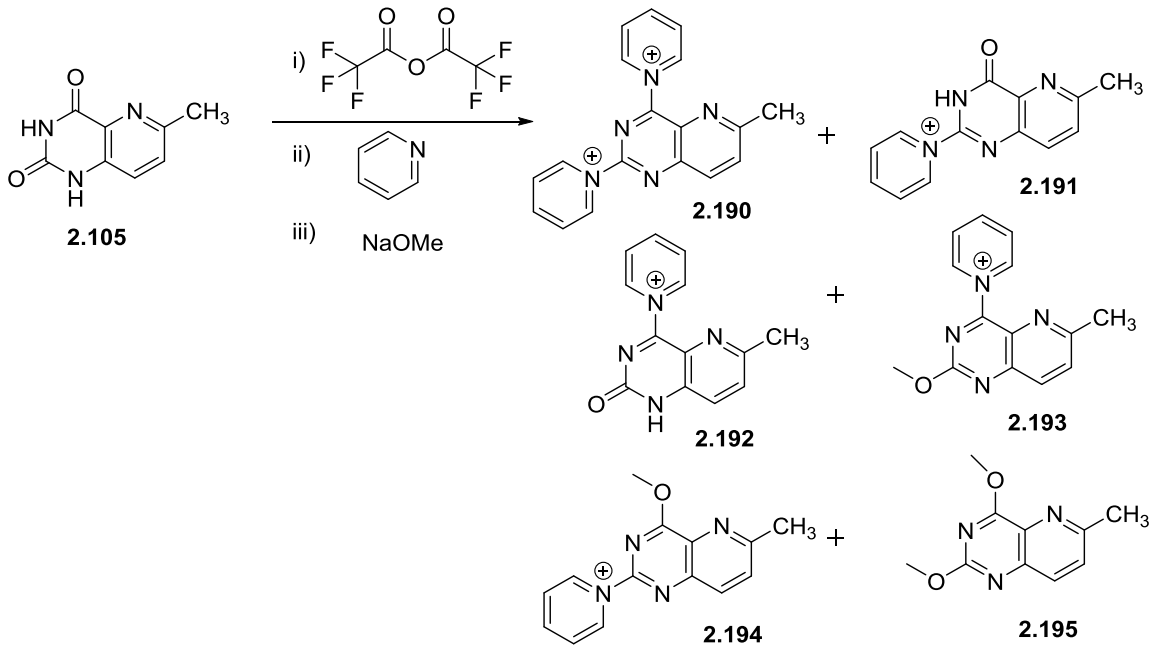


Scheme 2.93

The pyridine group at the 4-position of **2.188** was easily substituted by a nucleophilic substitution by a methoxy group using sodium methoxide. For this purpose, the compound **2.188** was reacted with sodium methoxide in dry methanol under nitrogen overnight. The required analogue **2.189** was obtained by purification with silica gel chromatography, eluting with ethyl acetate and methanol (2:1) and then by HPLC purification, as a yellow solid in 60 % yield. The ^{1}H NMR spectrum of **2.189** showed a singlet at δ_H 3.84 due to the C^{6} methoxy and in the HSQC NMR spectrum, these proton signals were in correlation with a carbon signal at δ_c 53.71.

2.13.1 The nucleophilic substitution reaction at the 2 and 4 position of pyridopyrimidine 2.105

As mentioned before in section, activation of the 2- and 4-positions of **2.105** was not successful by some chlorinating reagents and coupling reagents. But the activation of these positions (uracil and amide carbonyl) was successful with trifluoroacetic anhydride in anhydrous pyridine at 0-5 ^{o}C for 2.5 h (**Scheme 2.94**).



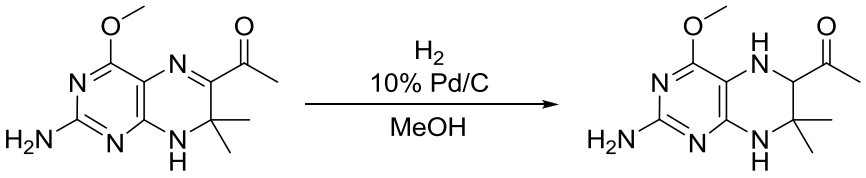
Scheme 2.94

Sodium methoxide in methanol was used as a nucleophile overnight at room temperature, to give the methoxy-substituted product **2.195**. After work-up, the product solution, purple in colour, was purified by silica gel chromatography, eluting with methanol and ethyl acetate (9:1). The methoxy substituted product **2.193** or **2.194** was eluted before the pyridine-substituted product **2.251** or **2.192.** The dark-purple solid residue from HPLC purification also confirmed that the first fraction, which occupied area 59% of HPLC chromatogram, corresponded to **2.193** or **2.194.** The second fraction (occupying an area of 28%) belonged to **2.191** or **2.192**. The third fraction (occupying an area of 2%) corresponded to **2.191** or **2.192**. The last fraction (occupying an area of 4%) corresponded to **2.193** or **2.194.** The mass spectra

for these HPLC fractions were very clean but due to limited time available, these fractions were not fully characterized. This reaction indicated that the activation of uracil and amide functionality at pyridopyrimidine **2.105** is possible with trifluoroacetic anhydride and using different nucleophiles, many 2,4-disubstituted compounds can be prepared.

2.14 Preparation of some tetrahydro analogues for NOS assay by reduction.

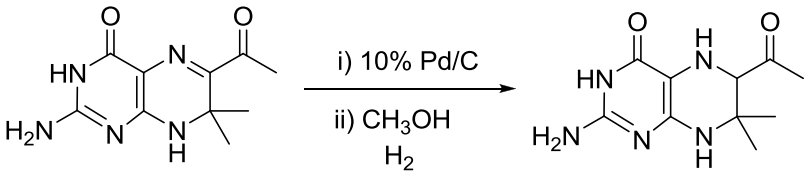
The reduction of 1-(2-amino-4-methoxy-7,7-dimethyl-7,8-dihydro-6-pteridinyl)ethanone **2.189** was accomplished in methanol by hydrogenation using 10 % Pd/C overnight. By HPLC purification, the product **2.196** was obtained as a light-yellow solid in 33% yield (**Scheme 2.95**).



2.189 **2.196**

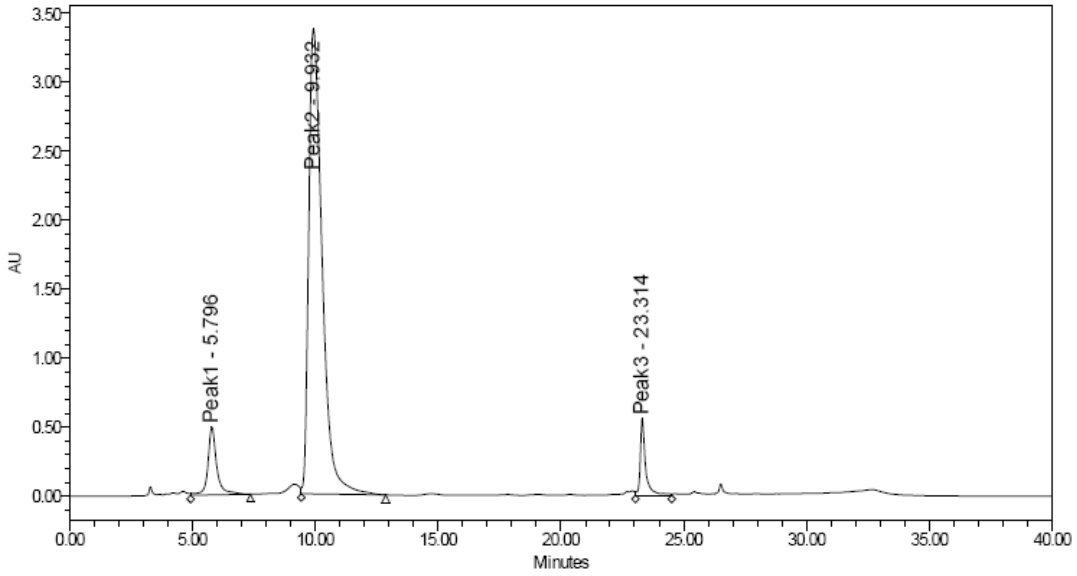
Scheme 2.95

6-Acetyl-2-amino-7,8-dihydro-7,7-dimethylpteridine-4(3*H*)one **2.189** was reduced by hydrogenation in methanol using 10 % Pd/C as a catalyst for 12 h. After work up, the residue was purified by HPLC to give the required tetrahydro analogue **2.197** as a white solid in 54% yield (**Scheme 2.96**).



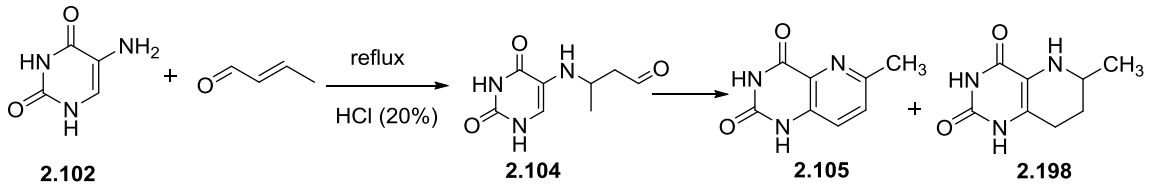
1.2 **2.197**

Scheme 2.96



HPLC chromatogram showing 2.197, eluting at 9.932 minutes

2.15 Preparation of 6-methyl-5,6,7,8-tetrahydropyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione



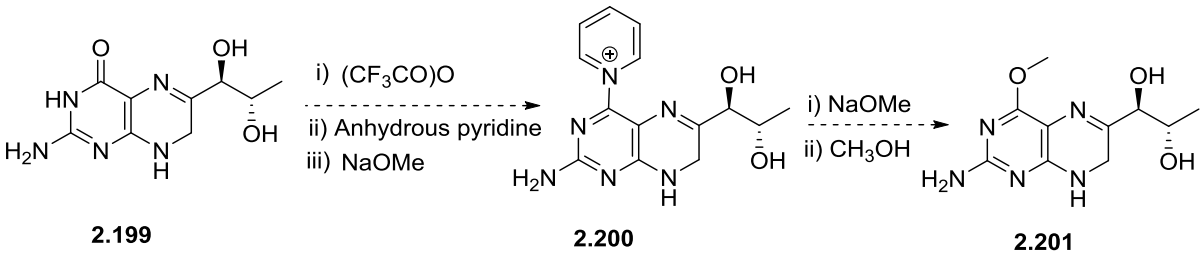
2.102 **2.104** **2.105** **2.198**

Scheme 2.97

5-Amino-2,4(1*H*,3*H*)-pyrimidinedione **2.102** was reacted with crotonaldehyde in hydrochloric acid (20%) at 120 ^{o}C for 2 h. After work up and crystallisation from methanol, a cream-coloured solid **2.105** was precipitated. By decreasing the volume of filtrate, a further two crops of **2.105** were obtained. Further evaporation of solvents and by adding some diethyl ether, the product **2.198** was precipitated, which was filtered and dried under reduced pressure to give the side-product **2.198** as a pure white solid (0.48%).

2.16 Conclusion and further work

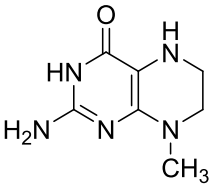
As described in section 2.9.13 and 2.9.13.1, the nucleophilic substitution reaction at the 4 position of 6-acetyl-2-amino-7,7-dimethyl-7,8-dihydro-4(3*H*)-pteridinone **2.5** was successful using trifluoroacetic anhydride in anhydrous pyridine and NaOMe/MeOH mixture. The rationale for putting a methyl or methoxy group at different positions of our analogues of BH_4 is due to the electron-donation effect of these groups, which contributes towards the HOMO energy level of these molecules. The role of our analogues of BH_4 is like BH_4 itself i.e. to donate an electron to the heme group of nitric oxide synthase enzyme for the production of nitric oxide. BH_4 is already a safe medicine which is being prescribed to patients, so putting a small group like methoxy at the 4 position of BH_4 might show beneficial results and can give a more lipophilic molecule; a drawback might be that the hydroxy groups at the 7 and 8 positions of **2.199** would also come under nucleophilic attack.



2.199 **2.200** **2.201**

Scheme 2.98

A number of *N*-substituted compounds for biological evaluation have been prepared as shown below. These compounds can help us to understand the role of N^8 for binding with nitic oxide enzyme and its activity. The detail will be discussed in Chapter 3 for biological results. In addition these compounds were available for screening for different types of antibacterial and antimicrobial activity.



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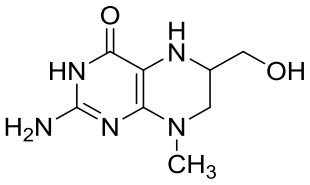
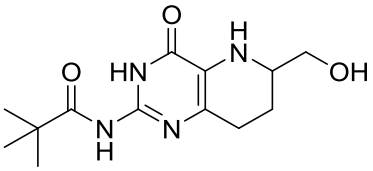
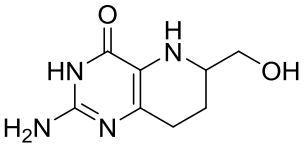


2.59



2.177



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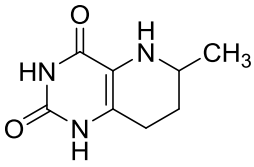

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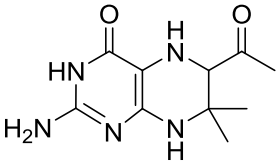

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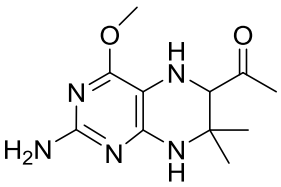

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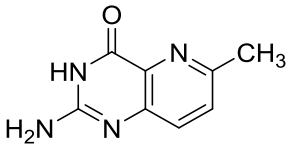

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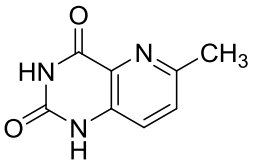
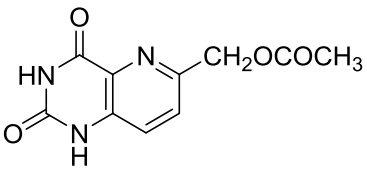


2.105



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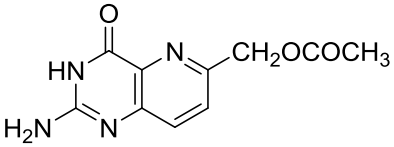

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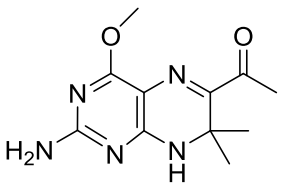
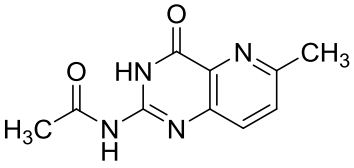
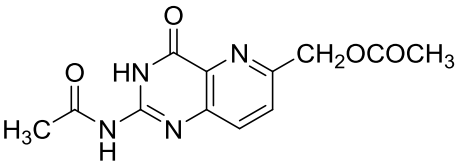


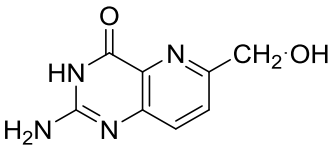
2.189



2.163



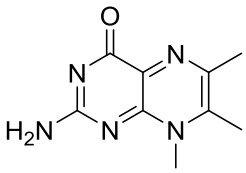
2.165



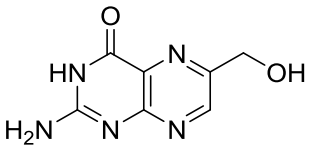
2.110

List of newly prepared compounds

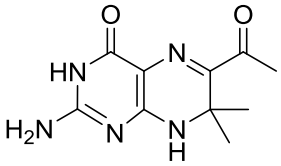
These compounds were available to us from WSG collection of compounds.



2.181



2.110

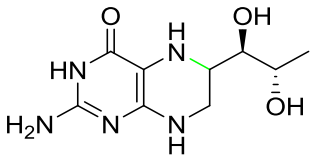


1.2

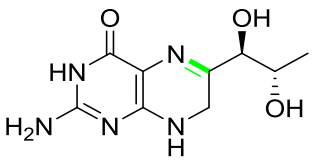
3.0 BIOLOGICAL RESULTS

3.1 Background

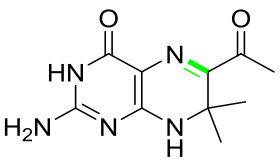
As mentioned earlier in section 1.1, oxidised forms of BH_4 (BH_3 and BH_2) compete with available BH_4 for binding to NOS and mimic the effects of BH_4.[243] BH_4 is easily oxidised to BH_2, but it can not act as a cofactor for NOS enzymes. The blocked dihydropteridine **1.2** was found to induce relaxation in the pulmonary arteries of rats in *in vitro* experiments; the arteries were depleted of BH_4. The blocked dihydropteridine **1.2** has different substituents at C^6 and C^7 as compared to BH_2 and it can be speculated that it could activate nitric oxide synthase enzymes by donating an electron through a capto-dative radical intermediate as shown in **Figure 3.1**. One possibility was that the enzymes such as dihydropteridine reductase and dihydrofolate reductase could have reduced this blocked dihydropteridines in rat tissues. This caused us to think about the possibility of investigating the replacement of natural cofactor BH_4 **1.1** with blocked dihydropteridines in *in vitro* experiments to find out if these compounds were NOS activators. Another possibility was to obtain tetrahydropteridines from the chemical reduction of blocked dihydropteridines, which could then be used to replace BH_4 in NOS assays.



BH_4 (1.1)



BH_2 (1.21)



1.2 (WSG 1002)

It has been discussed in sections **1.1.3** and **1.1.4** that BH_4 gives an electron from N^5 to the iron dioxygen species (Fig 1.6 c and d) to start arginine hydroxylation. However, for dihydropterins like **WSG 1002**, the capto-dative radical mediated electron shift is hypothesized. The possible electron transfer cycle for **1.2** is shown in **Figure 3.1**.

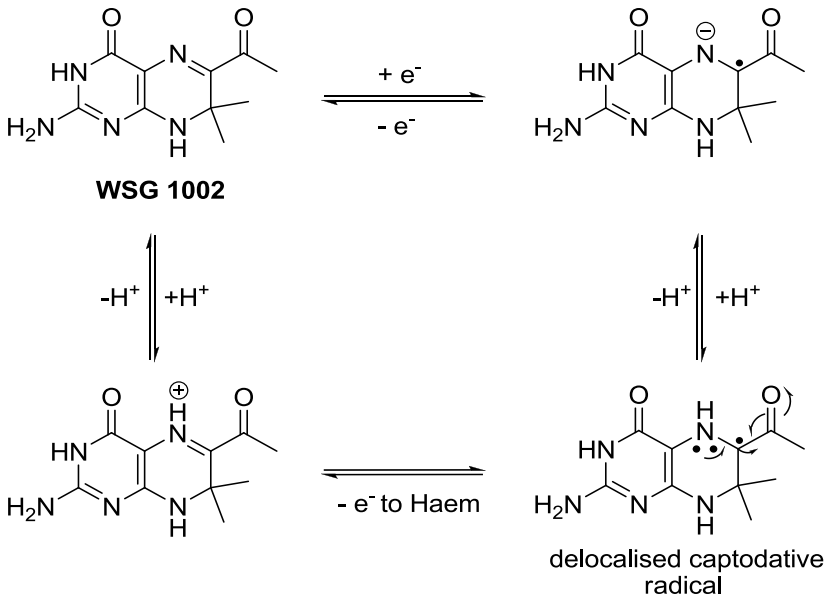


Figure 3.1 Hypothetical electron transfer cycle of of **WSG 1002**.

The major aim of this project was the modification, synthesis and evaluation of analogues of BH_4 as NOS activators. It was hoped that testing these compounds will help in investigating how much free space is available within the BH_4 binding pocket, aswell as the effect of the electron density of pteridine on its ability to function as a cofactor of NOS enzyme. The question was whether the analogue would be active for NOS enzyme if these regions were expanded into. Previously in the Suckling group, it was established by testing these compounds in Dr. Daff's laboratory that BH_4 analogues are only active in the tetrahydro form.[248,249] Therefore, these compounds were reduced by sodium cyanoborohydride or hydrogenation and their activity tested for nNOS enzyme activity. Nitric oxide synthase assays were performed in collaboration with Dr. Daff at the University of Edinburgh. If there is any activity resulting from these tetrahydro analogues, it will explain the initial cell- and tissue-based investigations that other enzymes reduce dihydropteridines before entering to the NOS active sites. While the eNOS enzyme was also of interest, work was focussed mainly on nNOS as Dr Daff and co-workers had isolated the BH_4-free nNOS enzyme which was available for assay.

Nitric oxide is a gaseous free radical; it interacts with molecular oxygen and generates nitrogen oxides such as NO_2 and N_2O_3. These reactive species either generate nitrite (NO_2^-) or

nitrate (NO_3^-) by hydrolysis[242] or react with amines and thiols *in vivo*. Superoxide ($O_2^{\cdot -}$) reacts with NO to generate peroxynitrite (ONO_2^-) (equation 3.1).[244]

$$\bullet NO + O_2^{\bullet -} \longrightarrow ONOO^- \quad \textbf{(3.1)}$$

$$ONOO^- + H^+ \rightleftharpoons ONOOH \longrightarrow HNO_3 \rightleftharpoons H^+ + NO_3^- \quad \textbf{(3.2)}$$

This peroxynitrite *via* its conjugate acid produces nitrate (equation 3.2). The peroxynitrite also reacts with NO and generates nitrogen dioxide (NO_2) and nitrosating agent (N_2O_3) (Equations 3.3 and 3.4).[244]

$$ONOO^- + NO \longrightarrow NO_2 + NO_2^- \quad \textbf{(3.3)}$$

$$NO_2 + NO \rightleftharpoons N_2O_3 \quad \textbf{(3.4)}$$

In addition to these nitric oxide interactions with molecular oxygen and its derivatives, nitric oxide which is generated in the active pocket also reacts with heme to form nitrosylhemoglobin [$Hb(Fe3^+)NO$] (equation 3.5).[244] Nitric oxide also reacts with oxyhaemoglobin and forms methemoglobin [$Hb(Fe3^+)$] or (metHb) and nitrate (NO_3^-) (equation 3.6).

$$Hb(Fe^{2+}) + NO \longrightarrow Hb(Fe^{3+})NO \quad \textbf{(3.5)}$$

$$\underset{\text{Oxyhaemoglobin}}{Hb(Fe^{2+})O_2} + NO \longrightarrow \underset{\text{Methemoglobin}}{Hb(Fe^{3+})} + NO_3^- \quad \textbf{(3.6)}$$

Among all the equations from 3.1 – 3.6, the highest probability of interaction of NO is with oxyhaemoglobin, rather than with molecular oxygen or its metabolites, because the interaction of NO with oxyhaemoglobin is faster than the auto-oxidation of NO even in a saturated oxygen environments. This is very important for the excretion of excess of NO

from the body.[24] The oxidation state of iron determines the rate at which NO reacts with heme. The rate of reaction of NO with oxyhaemoglobin (Fe^{2+}) is 3.7 x 10^7 $M^{-1}s^{-1}$, as compared to the rate of reaction of NO with methaemoglobin [Hb(Fe^{3+})], where the rate can vary from 10^2 –10^7 $M^{-1}s^{-1}$.[250,251,252]

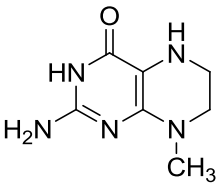
The reaction of nitric oxide with superoxide (equation 3.1) to form peroxynitrite is very fast, i.e. 7 x 10^9 $M^{-1}s^{-1}$ as compared to the reaction of NO with OxyHb. That is why the rate of MethHb formation cannot be measured accurately if superoxide is present in the assay. Therefore superoxide dismutase (SOD) was added in the assay because it completely removes all superoxide before NO formation, as it reacts with superoxide at a rate of 2 x 10^9 $M^{-1}s^{-1}$.

UV-Vis spectrometry at 401 nm is used for testing these tetrahydro compounds in the *in vitro* assay, which is based on the principle of change of oxyhaemoglobin to methaemoglobin caused by NO (equation 3.6). The concentration of metHb indicates how much nitric oxide is formed in the system, so if we can measure the concentration of metHb it will be proportional to the amount of nitric oxide produced in the system; more absorption means more conversion of oxyHb to metHb.[253,254,255] In *in vitro* experiments, there will be no neighbouring cells present where nitric oxide can diffuse so all the NO produced by NOS will react with oxyHb to form metHb.

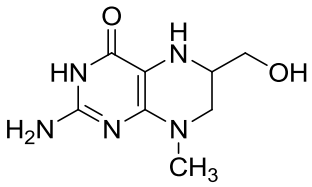
3.2 Enzyme Assay

Escherichia Coli BL21 (DE3) strain was used to express rat nNOS wild type by Dr Daff's group at the University of Edinburgh. It was purified before the enzyme assay. Dihydropteridines were reduced to tetrahydropteridines by hydrogenation or by using sodium cyanoborohydride in acidic medium (discussed in section 2.5.0). HPLC (waters, Vydac protein and peptide C18 column, 254 nm) with gradient elution water/acetonitrile (0.1 % trifluoroacetic acid) and flow rate of 6 ml/min was used to purify these reduced compounds. The tetrahydropteridines were protected from light by aluminium foil and stored in the freezer. Ice packs were used to carry these terahydropteridines to Edinburgh for the assays.

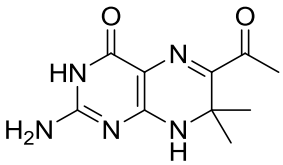
The stock solutions were freshly prepared by Ben Gazur, a PhD student from Dr Daff's group. The buffer (50mM Tris/HCl) was used to dissolve tetrahydropteridines analogues for assays. Dithiothreitol (DTT) (0.2 M) was also added to the buffer to prevent autoxidation before the assay, and stored in ice during the experiment. The tetrahydropteridines (**2.57**, **2.59**) were reduced and purified by myself and **1.2** and **1.58** were reduced and purified by Craig McInnes.



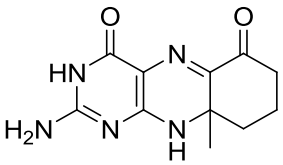
2.57



2.59



WSG 1002
1.2



WSG 1060
1.58

The composition of the assay mixture is shown in **Table 3.1**. The stock solution was prepared by adding NADPH, calmodulin and SOD/catalase to the buffer solution. This stock solution was used to add 920 µl in a cuvette. OxyHb, nNOS, BH_4 and Arg were added sequentially to this cuvette for enzyme assay.

Entry	Reagents	Volume in Cuvette	Final Concentration
1	Tris/HCl buffer (KCl + DTT) pH 7.5	920 µl	50 mM
2	NADPH	10 µl	100 µM
3	Calmodulin/Ca^{2+}	10 µl	500 µg/ml and 5 mM
4	SOD/Catalase	2 µl	20 µg/ml
5	Oxyhaemoglobin	45 µl	not specific
6	NOS	5 µl	5 µM
7	BH_4 or its analogue/DTT	1 µl	20 µM /200 µM
8	Arginine	10 µl	20 mM

Table 3.1 *NOS assay conditions*

NO-mediated conversion of oxyHb to metHb was determined by UV-1601 Shimadzu Spectrophotometer at 401 nm by applying metHb minus oxyHb extinction coefficient ($\Delta\varepsilon_{401nm}$) of 49 mM^{-1} cm^{-1}. All the tetrahydro compounds along with dithiothreitol (DTT) were dissolved in stock solution. For reliable results, the ingredients of the assay were mixed together, except arginine, and left to equilibrate for 30 - 60 seconds at room temperature. The absorbance of the UV-Vis spectrophotometer was set to zero. Arginine was added and assay was run for 100 seconds. Three readings for each sample were taken. From the initial part of the spectrum the gradient of the slope was measured which was used to calculate the rate of reaction for each analogue, in collaboration with Ben Gazur from the University of Edinburgh (equation 3.7).

3.3 Results and discussion

Initially, all the tetrahydropteridines (**2.57**, **2.59**, **1.2** and **1.58**) were screened for activity. When the BH_4 analogue was added last, the reaction rate was initially slow during the first few seconds, but after that it rapidly increased. It was thought that the analogue needed some

time to bind to the active site so BH_4/DDT was pre-mixed before arginine was added. The assay was fine, it was run for 100 seconds and the resulting curves were linear.

For each analogue, the rate of NO formation was obtained by using the following formula:

$$k = (\Delta \text{ Absorbance/min}) / (\varepsilon \times [\text{NOS}]) \qquad \textbf{(3.7)}$$

Where k is the rate of formation of metHb or NO while ε is the extinction coefficient at 401 nm (metHb minus oxyHb): 49000 M^{-1} cm^{-1}. [NOS] is the final concentration of nNOS which was 500 nM. The rate of NO formation for the first batch of tetrahydropteridine is shown in **Table 3.2**.

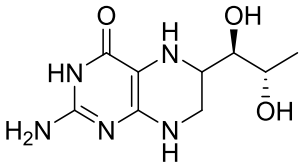
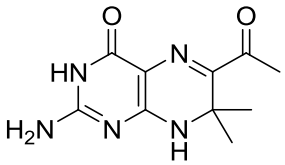
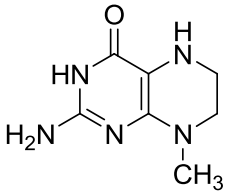
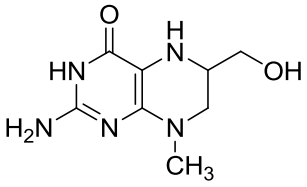
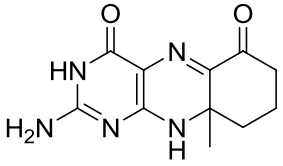
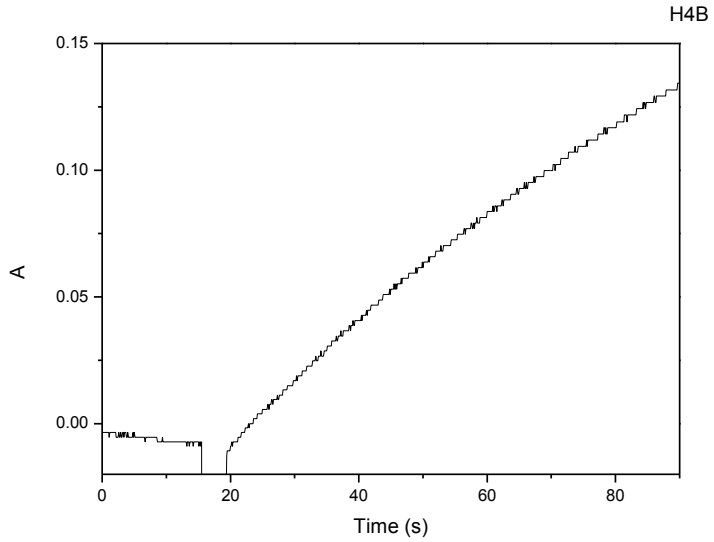
Entry	Compound	Rate of NO formation (k) min^{-1}
1	 **1.1 (BH_4)**	~18
2	 **WSG 1002** **1.2**	3.2
3	 **2.57**	2.8
4	 **2.59**	2.8
5	 **WSG 1060** **1.58**	9.8

Table 3.2 *Rate of NO formation with nNOS.*

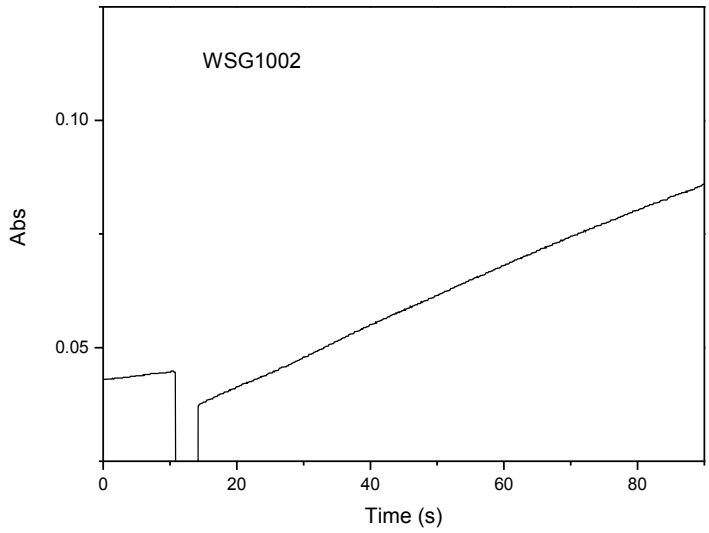
The rate of NO formation should be positive, but in some assays the rate was negative. This could be due to an absence of an active analogue which resulted in the disruption of the haem complexes in NOS. The rates of analogues of BH_4, along with their computer plotted graphs of absorbance are shown (**Fig 3.2-3.6**).



NOS assay for **BH_4**

NO rate ~18 min^{-1}

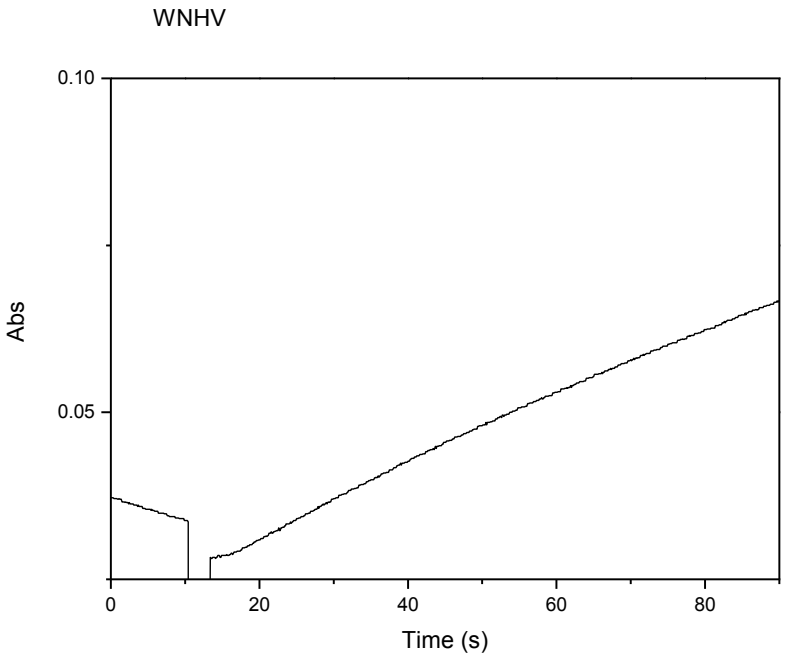
Figure 3.2



NOS assay for **WSG1002 (1.2)**

NO rate = 3.2 min^{-1}

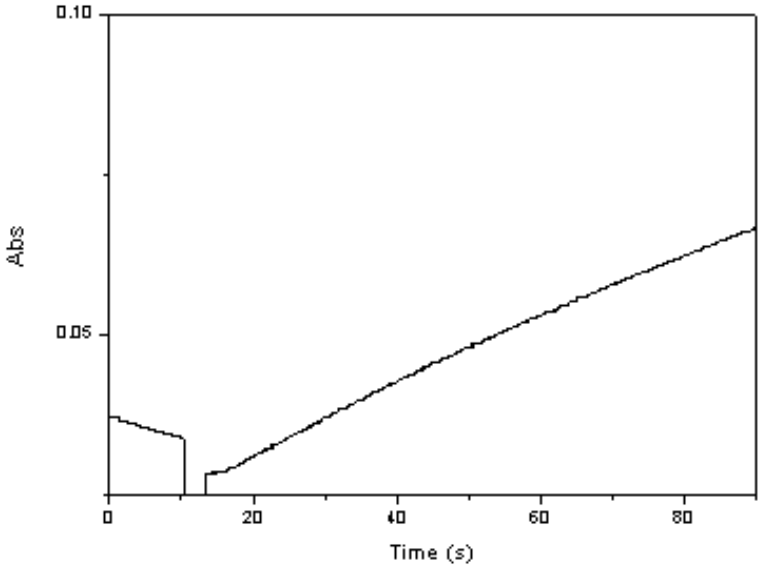
Figure 3.3



NOS assay for **2.57**

NO rate = 2.8 min^{-1}

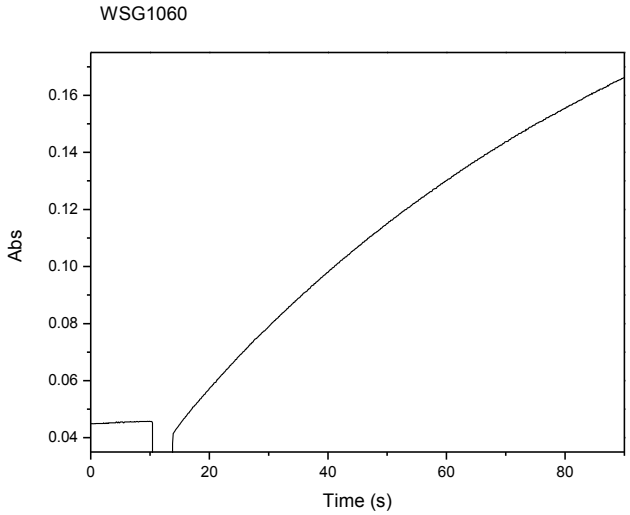
Figure 3.4



NOS assay for **2.59**

NO rate = 2.8 min^{-1}

Figure 3.4

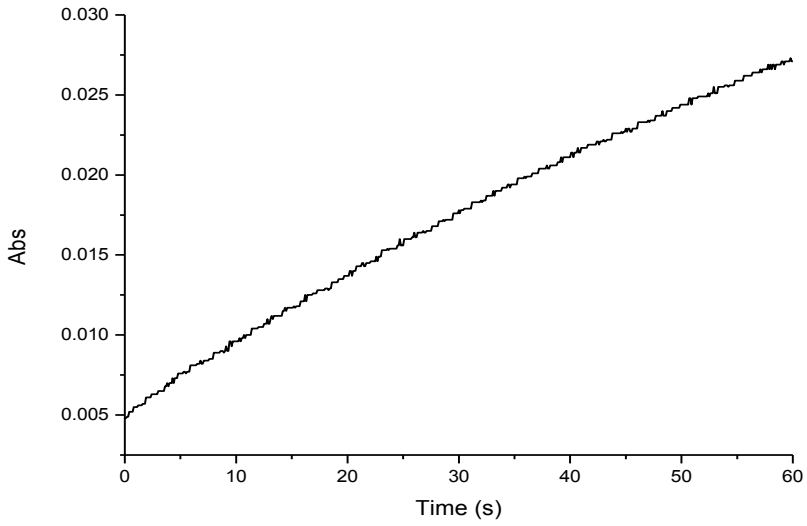


NOS assay for **WSG1060 (1.58)**

NO rate = 2.8 min^{-1}

Figure 3.5

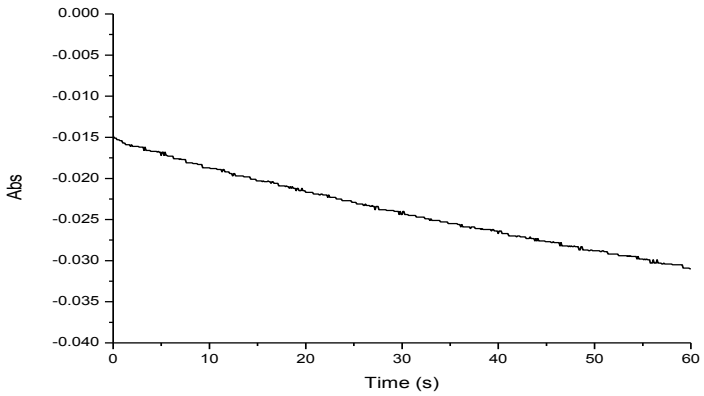
In the second batch, some more tetrahydro compounds were tested for nNOS assay. Their enzyme assay results with computer plotted graphs, along with their rates for NO formation**,** are shown in **Figures 3.6-3.10** below:

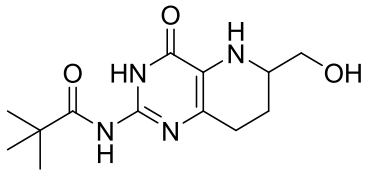


NOS assay for **BH_4**

NO rate = 22.20, 21.81 and 21.14 min^{-1}

Figure 3.6



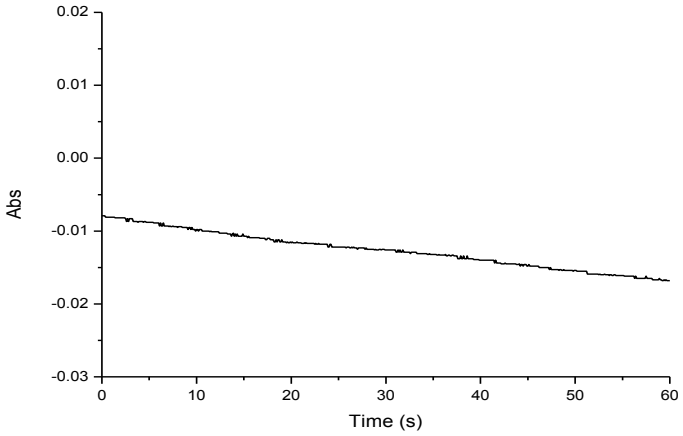


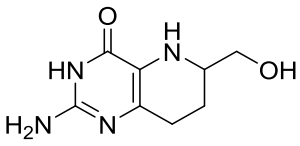
2.177

NOS assay for **2.177**

NO rate = -15.01min^{-1}

Figure 3.7



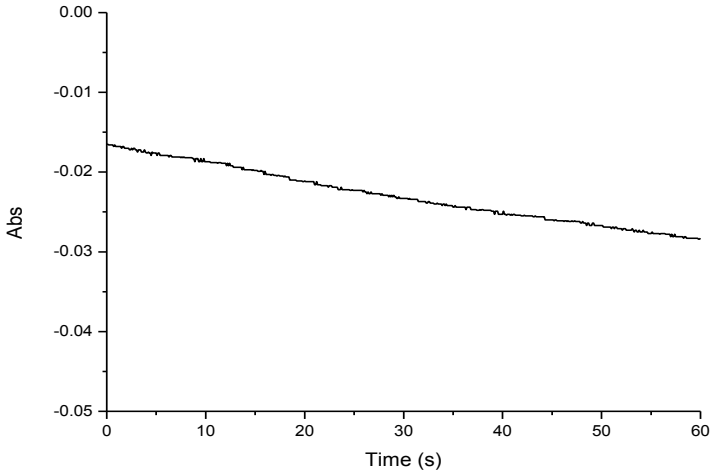


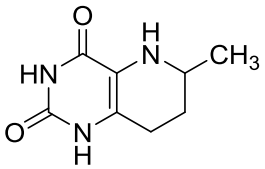
2.168

NOS assay for **2.168**

NO rate = -6.914 and -12.00 min^{-1}

Figure 3.8



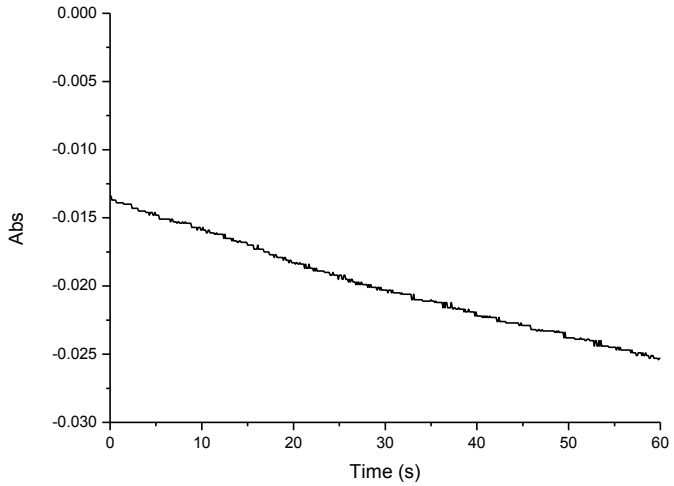


2.198

NOS assay for **2.198**

NO rate = -11.74 and -11.11 min^{-1}

Figure 3.9

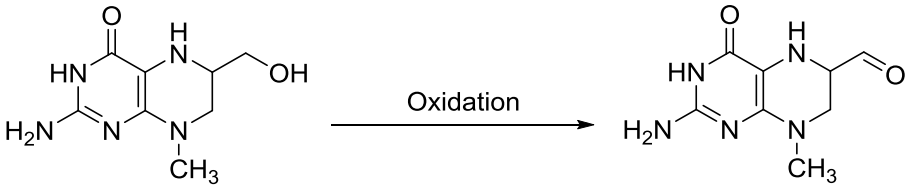


Another NOS assay for **2.198**

NO rate = -11.13 and -11.49 min^{-1}

Figure 3.10

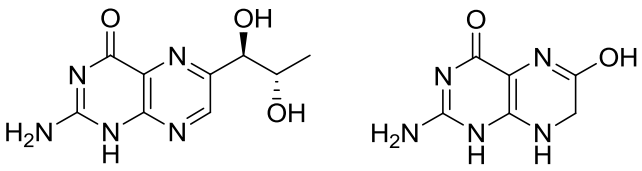
It was found that **2.57**, which has a methyl group at N^8 of BH_4 **1.1** but no side-chain at C^6, is more stable in the reduced form than other tetrahydropteridines. It has no solubility problem and is lipophilic but has nearly 10% activity as compared to BH_4. The analogue **2.57** is without the side-chain at the C^6 position and this side-chain could have a role in binding at the active site of the enzyme (NOS) by hydrogen bonding. Based on the results obtained from testing a small number of compounds and from molecular modelling studies carried out by Prof. Suckling, it has been found that an electron withdrawing group at C^6 favours NOS activation. The analogue of BH_4 **2.59** can be converted into aldehyde **3.1** by oxidation (**Scheme 3.1**), which can be used to extend the side-chain at the C^6 position.



2.59 **3.1**

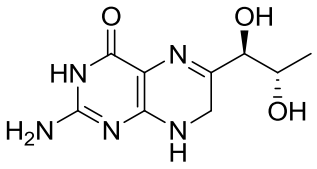
Scheme 3.1

As discussed in chapter 2 - section 2.16, a number of compounds were prepared for testing as NOS activators or inhibitors. The tetrahydro compounds **2.57**, **2.59**, **2.177**, **2.168**, **2.198** and **2.197** have been tested. The rest of the compounds are available for assays. The dihydro compounds also give information about their binding ability at the active site of the NOS enzyme if they are added with BH_4 analogue in NOS assay, as dihydro compounds can compete for binding with BH_4. It has been found in *in vivo* experiments that BH_4 oxidises mainly to 6-hydroxy-5,6-dihydropterin **1.18** after just 15 minutes and after one hour there was no detectable level of BH_2 or biopterin **1.10**.[177] So that is why the aim of research was to find a molecule which can resist oxidation, but at the same time is an activator of NOS enzyme.

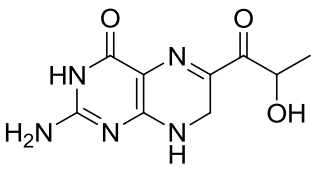


Biopterin (B) **1.10** **XPH_2** **1.18**

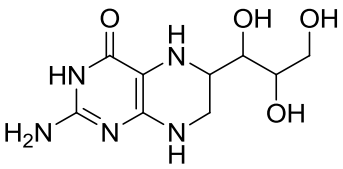
Presta *et al.*[182] suggested in their experiments that that NADPH oxidation by pterins is due to electron transfer to the iNOS heme iron. They found that when iNOS was saturated with 6-tetrahydroneopterin (NH_4) **1.23, 6-**$MePH_4$ **1.25** or 6-$OHMePH_4$ **1.26**, the rate of NADPH oxidation or the ability to donate electrons for these pterins were closer to BH_4. While three dihydropterins, BH_2, sepiapterin and deoxysepiapterin were not active and did not produce nitric oxide.



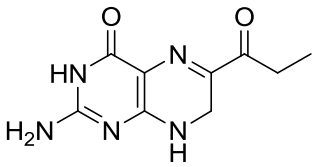
Dihydrobiopterin BH_2
1.21



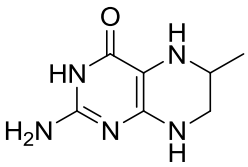
Sepiapterin
1.19



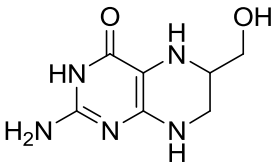
Tetrahydroneopterin NH_4
1.23



Deoxysepiapterin
1.24

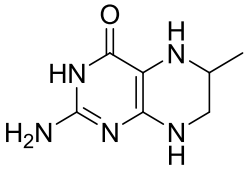


6-Methyltetrahydropterin
6-$MePH_4$ 1.25

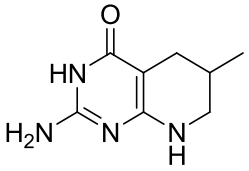


6-Hydroxymethyltetrahydropterin
6-$OHMePH_4$ 1.26

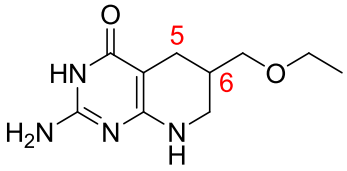
Heme dependent NADPH oxidation and nitric oxide formation is dependent upon fully reduced state pterins while binding affinity, ability of pterin to change the heme iron to its high-spin state and dimer stablisation depend upon pterins side structures.[182] The binding affinity of $DZPH_4$ **1.27** for NOS is 200 times less than BH_4, while 6-ethoxymethyl $EtOMeDZPH_4$ **1.28** has 400-times decrease in affinity as compared with BH_4 **1.1** which shows that the N^5 and C^6 side-chains play an important role for binding with protein residues of NOS.[47,93,109]. The binding of 6-methylpterin **1.25** is time and thiol dependent while the binding of redox-stable deazapterins analogues, **1.27** and **1.28** depend just upon concentration of analogue and is independent of time or thiol- concentration. The difference between these two analogues is only the N^5 nitrogen.[184]



6-Me-PH$_4$
1.25

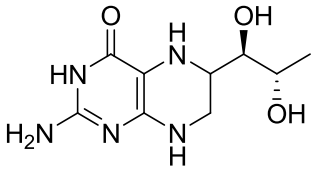


DZPH$_4$
1.27

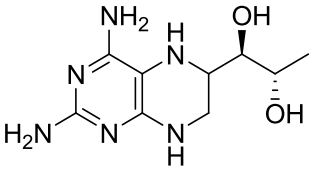


EtOMeDZPH4
1.28

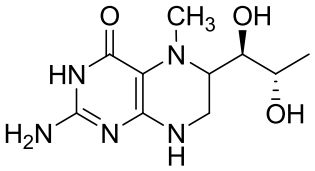
The 5-methyl analogue of BH_4 **1.30** does not react with O_2 and is an active pterin cofactor of neuronal NOS, however, the binding affinity of the 5-methyl compound **1.30** is three-fold lower than that of BH_4, which could be due to steric hindrance by the 5-methyl group. But if the stability of the radicals are compared, the degeneration of 5-methyl-$^{\bullet}BH_3$ **1.35** is 400-times slower than that of $^{\bullet}BH_3$ **1.31**. The stability of 5-methyl-BH_4 radical **1.31** is possibly due to electron donating effects of the methyl substituent.[43]



BH$_4$
1.1

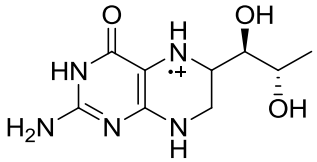


4-amino-BH$_4$
1.29

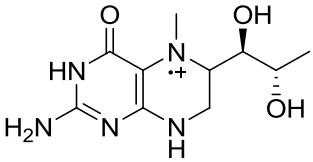


5-methyl-BH$_4$
1.30

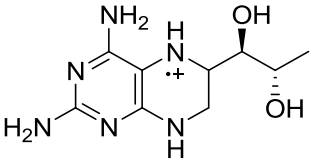
It is considered that the electron donation of BH_4 starts from the N^5 position, so it can be proposed that an analogue with greater electron donating ability to the heme would increase NO formation rate. However there has not been sufficient work done in this field to evaluate the impact of a more stable radical at the N^5 position.



1.31



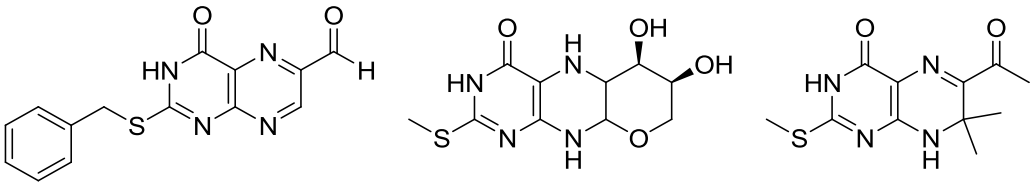
1.32



1.33

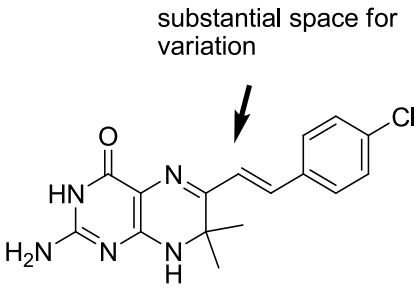
It was discovered in WSG1002 (**1.2**) that 7,7-dimethyl substituents, together with the oxidized C^6 side-chain, provide great chemical stability as 7,7-dimethyl substituents prevent it from being oxidized at the N^8 position. Several structural modifications are still possible.

A number of compounds have been examined within the Suckling group and based upon that knowledge it is known that S-methyl **1.44** and **1.45** or S-benzyl substitution **1.43** at position 2 of pteridine produce inhibitors instead of activators, while just a methyl group was enough to make our tetrahydropteridine inactive for NOS activity.



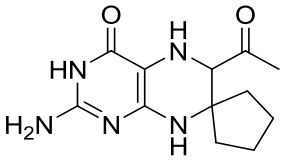
1.43 WSG1031 Inhibitor **1.44 Inhibitor** **1.45 Inhibitor**

But considerable space exists at C^6, as **1.41** was found to be active.



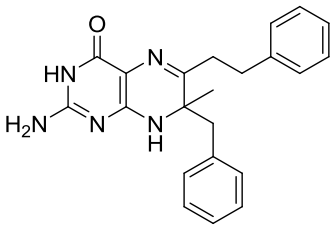
1.41 (WSG1017)

Amino functionality at position 4 produces inhibitors, while any two groups bigger than methyl at C^7 could be too sterically encumbered to fit into the active position of NOS. Raghu *et al.*[190] from the Suckling group found that tetrahydropteridine (**1.47**) is an NOS activator.

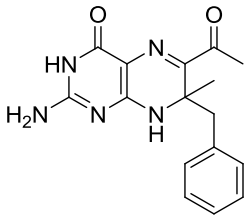


1.47

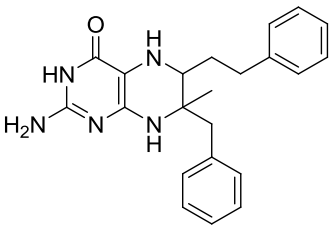
Raghu *et al.*[190] also found that the benzyl group present at C^7 in **1.48-1.53** as shown below is too large to be accommodated in the active site of nNOS, but a tetrahydropteridine of **1.41** as shown above gave 12 % activity.



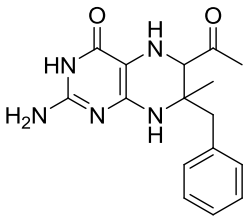
1.48



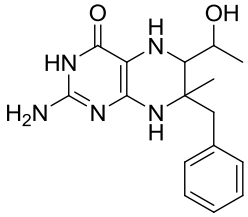
1.49



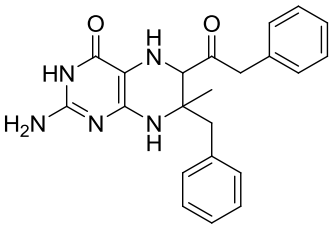
1.50



1.51

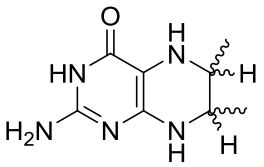


1.52

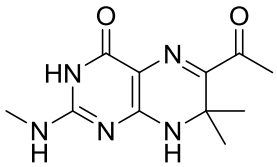


1.53

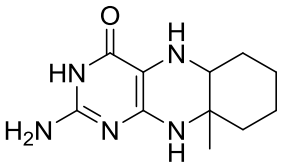
5,6-Dimethyltetrahydropterin **1.55** has been found to be an NOS activator. *N*-Methylaminopterin **1.56** did not bind with the enzyme, from which it can be speculated that there is inadequate space for extension from the C^2 position for binding for a methyl group, it also explains the role of hydrogen bonding. The pterins **1.57** and **1.58** had equal affinity for nNOS and were found to be very active.[191]



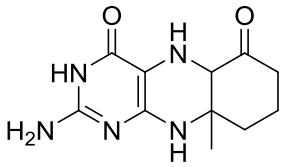
1.55



1.56

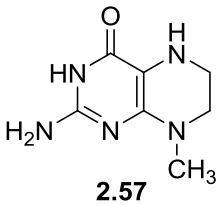


1.57

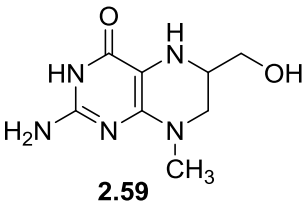


1.58

Analogues **2.57** and **2.59** were found to be active, which shows that a methyl group at N^8 is tolerated, but activity was low. Compound **2.57** was found to be very stable in methanol at room temperature; it did not oxidize for several weeks as compared to BH_4 which oxidizes at room temperature in an hour. This is a more lipophilic molecule which was one of the targets of the project. All the 8-deazo analogues did not demonstrate any activity.



2.57



2.59

3.4: Conclusion:

The summary of this investigation is that any extension at C^2 does not support any NOS enzyme activity, while amino substituent at 4-oxo is an inhibitor of NOS enzyme . There is little space available at C^7 for binding. Although putting a methyl group at the N^8 position gave an oxidatively stable pterin. Tetrahydropterin **1.58** showed a rate closer to that obtained by BH_4, making it an ideal molecule for acting as a BH_4 analogue. The C^6 side-chain is very important for binding and NOS activity, so the real opportunity exists at the C^6 position to design a better analogue.

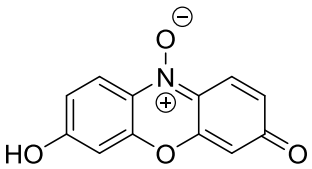
3.5 Antimicrobial activities of the analogues.

Antimicrobial agents are one of the most essential medicines used to destroy microorganisms. However due to the extreme and incorrect use of antibiotics, drug-resistance has developed.[260] Thus it's important to discover novel antimicrobial agents that might help to treat infectious diseases triggered by resistant microorganisms. Previously, we discussed and evaluated the potency optimisation of BH_4 small molecule analogues as NOS activators. In this section we aimed to detect and characterise the antimicrobial activity of these analogues.

These assays were performed in the Strathclyde Institute of Pharmacy and Biomedical Science (SIPBS). In this assay, an *in vitro* antimicrobial efficacy test was used, which is designed to assess small molecules potency against microbial infection. The antimicrobial activity of the analogues was assessed using a number of different Gram-positive bacteria, Gram-negative bacteria and parasites. One of the tests for antimicrobial assay was against *Methicillin resistant Staphylococcus aureus* (*MRSA*) as gram-positive bacterium which causes bacterial infection that mainly causes skin infection in several parts of the body and is resistant to a wide variety of antibiotics.[261] The analogues were also tested against *Escherichia coli* (*E. Coli*), a Gram-negative bacterium. *E. Coli* is commonly found in human and animal intestines and mainly causes gastroenteritis and urinary tract infections.[262]

Our synthetic analogues' activity was also tested against *Mycobacterium marinum* (*M. marinum)* which is not classified as either a Gram-positive or a Gram-negative bacterium. *M. marinum* is found in non-chlorinated water and commonly causes skin infections in human by direct contact with contaminated water.[262] Finally it was also decided to test compounds activity against a parasite; for this purpose *Trypanosoma brucei* (*T. b. brucei*) was used. *T. b. brucei* is found in specific types of flies and insects and causes vector-borne disease in human and animal.[263]

In order to measure the activity of small molecules, solutions of each compound at different concentrations were prepared by dilution from a concentrated stock solution to determine minimum inhibitory concentrations. 2 mg of each analogue was dissolved in 10 mL of sterile water.[264]Initially, 96 well microtiter plate was plated with 100 µL of culture medium containing 100 µM concentration of best selected growth microorganisms (except for *T. b. brucei* which was 20 µM) and incubated at 37 °C. The solution of each analogue was then added to 96 well plate which contained 100 µL of culture medium, taking into account some wells were left as control (plain medium was added). The plate was then incubated and visually monitored for any microorganism growth indication. Growth indicator called resazurin **3.2** was then added and the microorganism's growth was then assessed by colour change.[265]



resazurin
3.2

A noticeable colour change from blue to red was recorded when microorganisms' growth occurred. Resazurin indicator **3.2** is a purple dye in its oxidised stable state and becomes pink and red fluorescent in colour in its reduced form, called resorufin. It is used as an oxidation-reduction indicator to monitor cell viability for bacteria and mammalian cells, to determine bacterial contamination and to assess minimum inhibitory concentration (MIC) for number of different antibiotics.[266] The **Tables (3.3-3.6)** illustrates the *in vitro* assessment of synthesised BH_4 analogues against a number of different microorganisms.

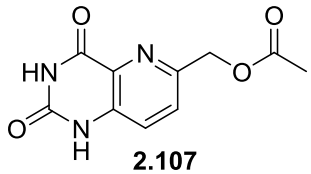
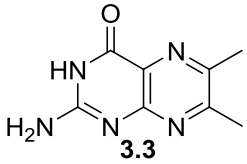
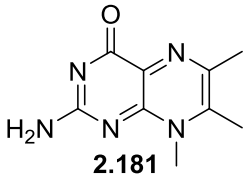
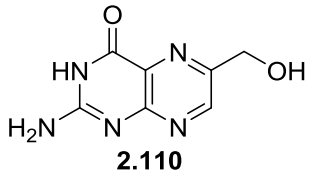
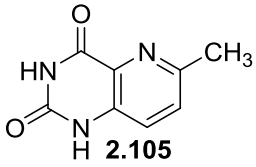
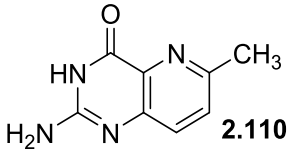
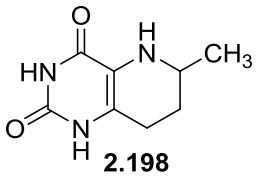
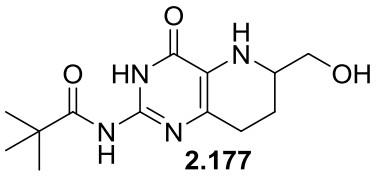
Structure	100µM EMRSA 16 % of control	100µM SMRSA 106 % of control	100µM *E.coli ATCC.8739* % of control	20µM *T.b.brucei* % of control or MIC (µM)	100µM *M.marinum ATCC BAA535* % of control or MIC (µM)
 2.107	97.1	102.6	86.5	97.9	90.1
 3.3	95.7	101.7	73.4	97.5	86.2
 2.181	94.5	97.5	84.2	103.7	83.6
 2.110	96.2	102.0	83.9	105.8	77.1
 2.105	90.3	100.5	81.6	102.0	77.3
 2.110			65.9	109.0	43.6
 2.198	92.3	101.8	79.0	103.9	88.2
 2.177	84.5	101.6	88.0	106.8	104.4

Table 3.3 Antimicrobial activities of the analogues

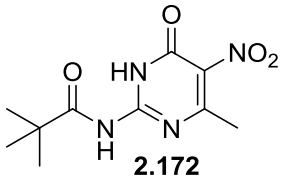
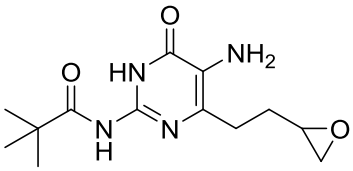
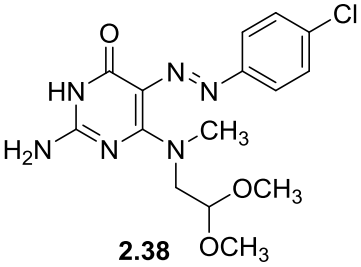
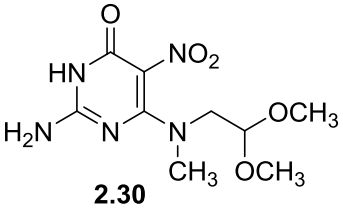
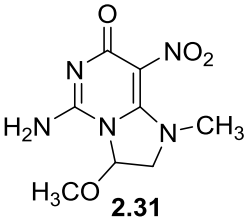
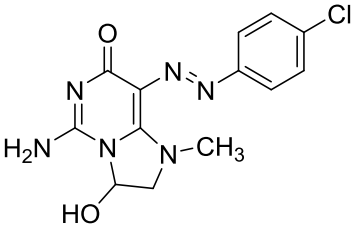
Structure	100µM EMRSA 16 % of control	100µM SMRSA 106 % of control	100µM *E.coli ATCC.8739* % of control	20µM *T.b.brucei* % of control or MIC (µM)	100µM *M.marinum ATCC BAA535* % of control or MIC (µM)
 **2.172**	83.4	100.2	94.0	103.7	112.4
 **2.176**	95.7	95.7	93.0	97.8	114.3
 **2.38**	93.6	79.1	66.9	72.1	24.0
 **2.30**	87.8	72.8	70.3	107.1	59.1
 **2.31**	74.4	62.2	66.2	108.0	52.7
 **2.40**	87.1	57.4	63.6	**99.5**	**1.0 MIC 25**

Table 3.4 Antimicrobial activities of the analogues

Results obtained from this assay indicated the effectiveness of these analogues as antimicrobial agents. Most of these compounds showed a modest effect against microorganisms; however some analogues showed a tremendous antimicrobial activity against bacteria and other microorganisms. As shown in the **Table 3.4**, analogue **2.40** showed its highest effect against *Mycobacterium marinum* with minimum inhibitory concentration at 1.0 µM. The analogue **2.46** (**Table 3.5**) displayed its highest efficacy against *Trypanosoma brucei* parasite with minimum inhibitory concentration at 0.7 µM and *M. marinum* with minimum inhibitory concentration at 3.3 µM.

The highest efficacy against *Trypanosoma brucei* parasite in this assay was obtained when testing analogue **2.47** (**Table 3.5**) with minimum inhibitory concentration at 0.5 µM. The analogue **2.47** also displayed a significant effect against *Mycobacterium marinum* with minimum inhibitory concentration at 2.0 µM. Also analogues **2.37** and **2.38** (**Table 3.6**) showed activity against *Mycobacterium marinum* with minimum inhibitory concentration at 0.9 µM and 1.3 µM respectively.

It is worth to mention that all of the most active molecules in this series have the same substructures, which might be responsible for the antimicrobial activity; however more investigation needed in this particular area because only a small number of compounds were tested. The presence of chlorophenyl, aminopyrimidine and diazenyl groups in all of these molecules might explain the observed effect against the microorganisms tested.

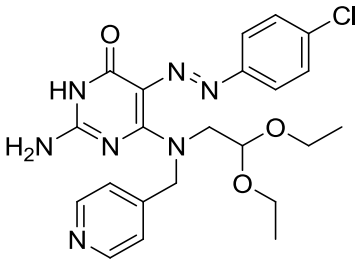
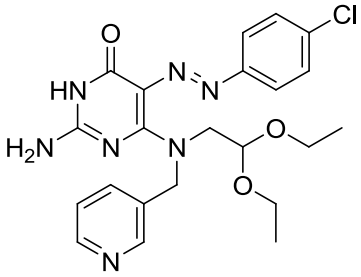
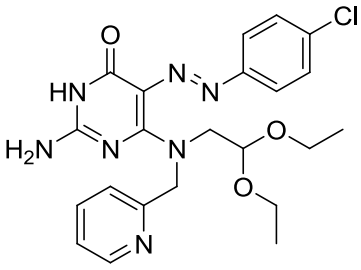
Structure	100µM EMRSA 16 % of control	100µM SMRSA 106 % of control	100µM *E.coli ATCC.8739* % of control	20µM *T.b.brucei* % of control or MIC (µM)	100µM *M.marinum ATCC BAA535* % of control or MIC (µM)
 **2.45**	66.2	73.8	53.4	94.9	**40.3**
 **2.46**	99.0	123.0	66.9	**0.7 MIC (µM) 25**	**3.3**
 **2.47**	96.6	68.7	71.7	**0.5 MIC (µM) 12.5**	**2.0**

Table 3.5 Antimicrobial activities of the analogues

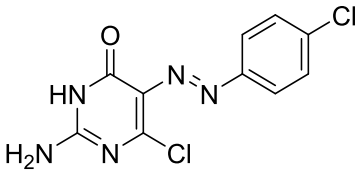
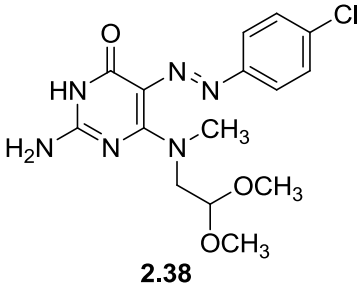
Structure	100µM EMRSA 16 % of control	100µM SMRSA 106 % of control	100µM *E.coli ATCC.8739* % of control	20µM *T.b.brucei* % of control or MIC (µM)	100µM *M.marinum ATCC BAA535* % of control or MIC (µM)
 **2.37**	**101.1**	**86.6**	**78.1**	42.5	**0.9**
 **2.38**	**96.9**	**86.9**	**74.4**	69.9	**1.3**

Table 3.6 Antimicrobial activities of the analogues

4.0 EXPERIMENTAL

4.1 Instrumental

Rotary evaporator was used to remove the solvents by evaporation under reduced pressure (*ca* 20 mmHg) if not specified.

Reichert 7905 hot stage melting point apparatus was used to check melting points which are uncorrected.

Pre-coated silica plates (Alugram® Sil G/UV_{254}) were used for thin layer chromatography (TLC). UV (254 nm) and 2% aqueous potassium permanganate were used to visualise TLC plates.

Nicolet Impact 400 D FTIR spectrometer was used for recording Infra-red (IR) where KBr discs were used in case of solids and NaCl plates for oily compounds.

A Bruker Spectrospin NMR spectrometer with 400 MHz or 500 MHz operating frequency was used for ^{1}H spectra and at 100 MHz or 125 MHz for ^{13}C spectra. Chemical shifts (δ) are measured relative to the residual proton or carbon from the solvent and quoted in ppm. Coupling constants (J) are calculated in Hertz (Hz). Most commonly used abbreviations are as follows: s = singlet; d = doublet; dd = doublet of doublets; t = triplet; q = quartet; m = multiplet and br = broad.

Perkin Elmer 2400, analyser series 2 was used for micro analysis by technical staff at the University of Strathclyde. Tungsten trioxide was added as a catalyst with some pyrimidines and pteridines for satisfactory results.

JEOL JMS AX505 mass spectrometer was used at the University Strathclyde for low resolution mass spectrometry (LRMS). Jeol JMS-700 M Station high resolution magnetic sector spectrometer was used for recording high resolution mass spectra (HRMS) by

technical staff at University of Glasgow and the Strathclyde Institute of Pharmacy and Biomedical Sciences.

HPLC purification was carried out by HPLC [Waters 1525 binary HPLC pump, Waters 2487 dual λ absorbance detector with the help of Breeze software at 254 nm. The reverse phase column, Vydac protein and peptide CI8 was used, gradient elution with water/acetonitrile containing 0.1% TFA. The most commonly used gradient elution is shown in **Table 4.1.**

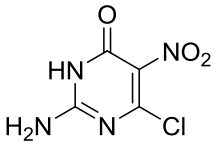
Entry	**Time (min)**	**Flow rate mL/min**	**Water (%)**	**Acetonitrile (%)**	**Curve[a]**
1	00.00	6	90	10	6
2	12.00	6	90	10	6
3	18.00	6	50	50	6
4	25.00	6	50	50	6
5	30.00	6	50	50	6
6	35.00	6	90	10	6
7	40.00	0	90	10	11
8	40.10	0	90	10	11

Table 4.1 HPLC gradient elution of solvents. [a]Curve 6 is the command to continue the HPLC run while 11 is a stop command.

Shimadzu UV-2101PC was used for biological spectrophotometry.

4.2 Synthesis

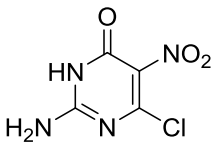
2-Amino-6-chloro-5-nitropyrimidin-4(3*H*)-one[221]



2.1

2-Amino-6-chloropyrimidin-4(3*H*)-one **2.28** (1.00 g, 6.87 mmol) was added to concentrated sulfuric acid (3 mL) at 0 °C and stirred at room temperature until it dissolved. It was added dropwise, with stirring, with 70 % nitric acid (2.5 mL) at 0 °C. The resulting solution was stirred for 2 h, and was then poured onto crushed ice (40 g), and filtered. The solid obtained was washed with cold water (10 mL), cold ethanol (10 mL), and cold ether (10 mL), then dried under reduced pressure overnight at 40 °C to give the chloronitropyrimidine **2.1** as a yellow solid (0.98 g, 75 %). mp >320 °C; ν_{max} (KBr, cm^{-1}) 3593, 3510, 3406, 3132, 3035, 1674, 1585, 1485, 1406, 1043, 927, 663; ^{1}H NMR (DMSO-d_6) δ_H 6.90-8.80 (2H, br, *NH*$_2$), 12.15 (1H, s, *NH*); ^{13}C NMR (DMSO-d_6) δ_c 150.6 (-*C*-NO_2), 153.1 (-*C*-Cl), 154.2 (-*C*-NH_2), 155.0 (-*C*=O); LRMS: found (MH^-) 189.1, $C_4H_3ClN_4O_3$ requires (MH^-) 190.0.

2-Amino-6-chloro-5-nitropyrimidin-4(3*H*)-one by using anhydrous potassium nitrate[221]

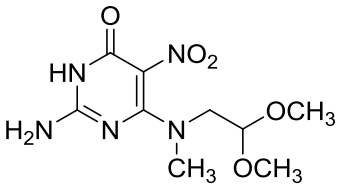


2.1

2-Amino-6-chloropyrimidin-4(3*H*)-one **2.28** (1.00 g, 6.87 mmol, 1 equiv.) was added to concentrated sulphuric acid (3 mL) at 0 °C and stirred at room temperature until it dissolved. The reaction mixture was cooled to -5 °C using dry ice in acetone. Anhydrous KNO_2 (963 mg, 6.87 mmol, 1 equiv.) was added in small portions during 10 min and the reaction mixture

was stirred for 2 h at room temperature. It was poured onto crushed ice (45 g), and filtered. The solid obtained was washed with cold water (10 mL), cold ethanol (25 mL), and cold ether (25 mL), and then dried under reduced pressure overnight at 40 °C to give the title compound **2.1** as a light-yellow solid (0.97 g, 74 %). mp >320 °C; ^{1}H NMR (DMSO-d_6) δ_H 6.90-8.70 (2H, br, *NH*$_2$), 12.15 (1H, s, *NH*); ^{13}C NMR (DMSO-d_6) δ_c 150.6 (-*C*-NO$_2$), 153.1 (-*C*-Cl), 154.2 (-*C*-NH$_2$), 155.0 (-*C*=O); LRMS: found (MH^-) 189.0, $C_4H_3ClN_4O_3$ requires (MH^-) 189.0.

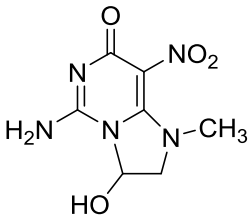
2-Amino-6-[(2,2-dimethoxyethyl)(methyl)amino]-5-nitro-4(3*H*)-pyrimidinone[218]



2.30

To an ice-cooled, stirred mixture of nitrochloropyrimidine **2.1** (0.955 g, 5.03 mmol) and dimethylformamide (5 mL) was added methylaminoacetaldehyde dimethyl acetal **2.29** (2.5 equiv., 1.59 mL) over 5 min and stirring was continued at room temperature overnight. No solid was observed in the product mixture but when the mixture was poured onto ice and a precipitate was formed. It was filtered, washed with ether and dried under reduced pressure overnight at 40 °C to give 2-amino-6-[(2,2-dimethoxyethyl)(methyl)amino]-5-nitro-4(3*H*)-pyrimidinone **2.30** as a light-yellow solid (0.97 g, 71 %). mp 212 °C; ν_{max} (KBr, cm^{-1}) 3407, 3313, 2941, 1677, 1643, 1583, 1456, 1408, 1365, 1290, 1225, 1070, 787, 667; ^{1}H NMR (DMSO-d_6) δ_H 2.82 (3H, s, -N-*CH*$_3$), 3.29 (6H, s, 2 x *OCH*$_3$), 3.65 (2H, d, *J* = 5.4, -N-*CH*$_2$-CH), 4.60 (1H, t, *J* = 5.4, -*CH*- 2 x CH$_3$), 6.70-7.50 (2H, br, *NH*$_2$), 10.60 (1H, s, *NH*); ^{13}C NMR (DMSO-d_6) δ_C 40.1 (-N-*CH*$_3$), 52.4 (N-*CH*$_2$-CH), 55.2 (-2 x *OCH*$_3$), 102.5 (-*C*-2 x OCH$_3$), 112.3 (-*C*-NO$_2$), 152.6 (-*C*-NH$_2$), 156.8 (-*C*O-), 159.6 (=N-*C*-N-); LRMS: found (MH^-) 272.1, HRMS (FAB): found (MH^+) 274.1148, $C_9H_{15}N_5O_5$ requires (MH^+) 274.1146.

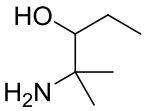
5-Amino-3-hydroxy-1-methyl-8-nitro-2,3-dihydroimidazo[1,2-*c*]pyrimidin-7(1*H*)-one[218]



2.32

2-Amino-6-[(2,2-dimethoxyethyl)(methyl)amino]-5-nitro-4(3*H*)-pyrimidinone **2.30** (0.944 g, 3.45 mmol) was dissolved in cold concentrated HCl (5 mL) and stirred continuously for 5 min. After that it was stirred at 80 °C for 15 min. The conc. HCl was evaporated by using the rotary evaporator and the yellow solid material was dried under reduced pressure at 40 °C to give the title compound **2.32** as a yellow solid (0.632 g, 81 %). mp 171 °C (decomp) (lit.[111] 171 °C); ν_{max} (KBr, cm^{-1}) 3119, 2758, 1643, 1461, 1416, 1326, 1292, 1221, 1121, 1034, 975, 924, 853, 773, 638; ^{1}H NMR (DMSO-d_6) δ_H 2.97 (3H, s, -N-CH_3), 3.40 (1H, s, -C-*OH),* 4.13 (2H, d, *J* = 4.0, -N-CH_2), 6.20 (1H, t, *J* = 4.0, >C*H*-OH), 8.13 (2H, br, NH_2) ; ^{13}C NMR (DMSO-d_6) δ_C 52.5 (-N-CH_3), 60.4 (>N-CH_2-OH), 78.9 (-*C*-OH), 109.6 (-C-NO_2), 148.5 (-*C*-NH_2), 149.8 (-*CO*-), 154.1 (N-*C*-N) ; LRMS: found (MH^+) 228.0, $C_7H_9N_5O_4$ requires (MH^+) 228.1, HRMS (FAB): found (MH^+) 228.0729, $C_7H_9N_5O_4$ requires (MH^+) 228.0727.

2-Amino-2-methyl-3-pentanol

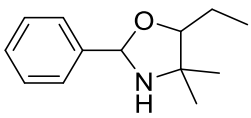


2.18

To a stirred solution of 2-methyl-2-nitro-3-pentanol **2.8** (1.00 g, 6.80 mmol) in methanol (10 mL) was added 10 % Pd/C (0.5 g) at 0 °C and hydrogenated using a balloon of H_2 at room temperature (atmospheric pressure) for 16 h. The solvent was removed under reduced

pressure, amino alcohol was applied on TLC and it was observed that there was only one spot on TLC.The amino alcohol was dissolved in DCM (10 mL), dried ($MgSO_4$), some solid material was precipitated out which was separated by filtration. The solvent was evaporated under reduced pressure to give amino alcohol **2.18** as a yellow oily liquid (0.692 g, 87%). ^{1}H NMR (DMSO-d_6) δ_H 1.04 (3H, t, -CH_2-$C\underline{H}_3$), 1.13 (6H, s, 2 x CH_3), 1.24-1.57 (2H, m, -C$\underline{H}_2$-CH_3), 1.80-2.20 (2H, b, NH_2), 3.12 (1H, dd, J = 2.1 & 10.4, -C$\underline{H}$OH), 3.49 (1H, s, -*OH*); LRMS: Found (MH^+) 118.11, $C_6H_{15}NO$ requires 118.12.

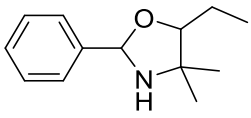
5-Ethyl-4,4-dimethyl-2-phenyl-1,3-oxazolidine using molecular sieves[216]



2.22

3-Hydroxy-2-methyl-2-pentanaminium chloride **2.8** (0.500 g, 3.26 mmol, 1 equiv.) was dissolved in toluene (10 mL), it was cooled in an ice bath and then triethylamine (686 µl, 9.78 mmol, 1.5 equiv.) was added, followed by benzaldehyde (346 mg, 334 µl 3.26 mmol, 1 equiv.). Activated molecular sieves were added in sealed tube. The mixture was stirred at 40 ^{o}C for 16 h. A full face shield was place in front of the sealed tube. Bubbles were constantly coming from the liquid and a foamy material was being formed on the walls of the tube. The solvent was evaporated under reduced pressure and the residue was filtered, dissolved in ethyl acetate in a separating funnel and the salt of triethylamine with HCl was precipitated out. The ethyl acetate containing oxazolidine was evaporated under reduced pressure to give a yellow oily residue (0.321 g) which on purification by silica gel column afforded oxazolidine **2.22** (0.099 g, 15%). ^{1}H NMR (DMSO-d_6) δ_H 1.15 (3H, s, CH_3), 1.17 (3H, t, J = 7.12, $C\underline{H}_3$-CH_2),), 1.24 (3H, s, CH_3), 1.55-1.29 (2H, m, -OCH-$C\underline{H}_2$-CH_3), 3.45 (1H, dd, J_1 = 3.9, J_2 = 4.6 -O$C\underline{H}$-CH_2-), 5.48 (1H, s, *NH*), 7.30-7.60 (5H, m, C_6H_5) 7.90 (1H, s, Ar-CH); LRMS: Found (MH^+) 206.2, $C_{13}H_{19}NO$ requires 206.1.

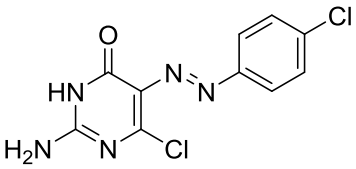
5-Ethyl-4,4-dimethyl-2-phenyl-1,3-oxazolidine[216]



2.22

3-Hydroxy-2-methyl-2-pentanaminium chloride **2.8** (1.00 g, 6.52 mmol, 1 equiv.) was dissolved in toluene (10 mL), it was cooled in an ice bath and then triethylamine (686 µl, 9.77 mmol, 1.5 equiv.) was added, followed by benzaldehyde (1.73 g, 16.3 mmol, 2.5 equiv.). The reaction mixture was stirred at 120 ^{o}C for 18 h under a nitrogen atmosphere using a Dean Stark Trap. The apparatus was allowed to cool and the solvent was evaporated under reduced pressure to give an oily brown liquid which was dissolved in ethyl acetate. The salt of triethylamine precipitated out which was separated by filtration. The solvent was evaporated under reduced pressure to give a yellow oily residue (0.543 g) which on purification by silica gel column gave oxazolidine **2.22** (0.185 g, 14%). ^{1}H NMR (DMSO-d_6) δ_H 1.16 (3H, s, *CH*$_3$), 1.17 (3H, t, J = 7.12, *CH*$_3$-CH$_2$), 1.24 (3H, s, CH$_3$), 1.56-1.30 (2H, m, -OCH-*CH*$_2$-CH$_3$), 3.44 (1H, dd, J_1 = 3.9, J_2 = 4.6 -O*CH*-CH$_2$-), 5.48 (1H, s, *NH*), 7.3-7.5 (5H, m, C$_6$H$_5$) 7.89 (1H, s, Ar-CH); LRMS: Found (MH$^+$) 206.1, $C_{13}H_{19}NO$ requires 206.1.

2-Amino-6-chloro-5-[(*E*)-(4-chlorophenyl)diazenyl]-4(3*H*)-pyrimidinone

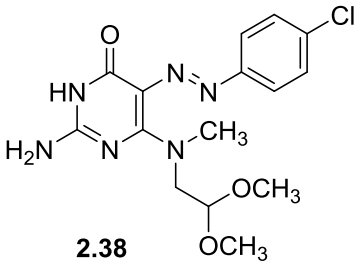


2.37

4-Chloroaniline (0.58 g, 1 equiv.) was dissolved in hydrochloric acid (2M, 20 mL) and this solution was cooled to -5 ^{o}C by using a dry ice and acetone bath. A solution of sodium nitrite (0.32 g, 1.1 equiv.) in water (10 mL) was added dropwise with stirring. After completion of the addition, the reaction mixture was stirred for 15 min at -5 to -2 ^{o}C. 2-Amino-6-chloro-5-nitropyrimidin-4(3*H*)-one **2.1** (1.00 g, 2.14 mmol) was dissolved in 2N NaOH (20 mL) at room temperature and this solution was cooled to -5 to -2 ^{o}C causing a small amount of a dark-yellow compound to precipitate. To this suspension, the cold diazonium salt solution was added slowly so that the reaction temperature did not rise above -5 ^{o}C. The temperature

was reduced to -20 °C, stirring continuously. The reaction mixture was allowed to warm to between -5 to -0 °C and stirred for 30 min, then neutralised with dilute acetic acid to give a yellow solid which was filtered off, washed with water (50 mL) and ether (50 mL), and dried under reduced pressure to give title compound **2.37** as a dark-yellow solid (1.32g, 68%). mp <220 °C (dec.); ν_{max} (KBr, cm^{-1}): 3138, 2775, 1682, 1493, 1427, 1279, 1199, 1158, 1089, 1016, 929; ^{1}H NMR (DMSO-d_6) δ_H 7.56 (2H, d, *J* = 8.72, 2×-C*H*-C-N=), 7.68 (2H, d, *J* = 8.7, 2×-C*H*-C-Cl), 7.80-8.60 (2H, b, -NH_2) 11.79 (1H, s, NH); ^{13}C (DMSO-d_6) δ_C 99.3, (C-N=N), 118.4, 119.0, 129.6, 130.7, 140.2, 153.0 (-*C*-aromatic), 155.5, (-C-NH_2), 157.9, (-CO-), 159.7, (-N-*C*-Cl); LRMS found (MH^+) 284.1, $C_{10}H_7Cl_2N_5O$ requires 284.0, HRMS (FAB): found (MH^+) 284.0110, $C_{10}H_7Cl_2N_5O$ requires (MH^+) 284.0106.

2-Amino-5-[(*E*)-(4-chlorophenyl)diazenyl]-6-[(2,2-dimethoxyethyl)(methyl)amino]-4(3*H*)-pyrimidinone[218]

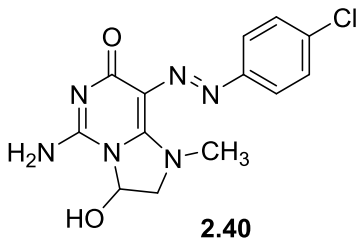


2.38

To an ice-cooled stirred mixture of 2-amino-6-chloro-5-[(*E*)-(4-chlorophenyl)diazenyl]-4(3*H*)-pyrimidinone **2.37** (0.70 g, 2.47 mmol) and dimethylformamide (5 mL) was added methyl amino acetaldehyde dimethyl acetal (780 µl, 6.18 mmol, 2.5 equiv.). The reaction mixture was stirred under nitrogen at room temperature overnight . The mixture was filtered and the orange-coloured solid was washed with dimethylformamide (15 mL) and then with ether (30 mL). The solid was dried under vacuum at 40 °C to give the title compound **2.38** as an orange solid (0.71 g, 78%). mp 227 °C; ν_{max} (KBr, cm^{-1}) 3407, 3313, 2941, 1677, 1643, 1583, 1456, 1408, 1365, 1290, 1225, 1070, 787, 667; ^{1}H NMR (DMSO-d_6) δ_H 3.39 (6H, s, -2xOC*H*$_3$), 3.42 (3H, s, -N-*CH*$_3$), 3.95 (2H, d, *J* = 5.3, -N-C*H*$_2$-CH), 4.68 (1H, t, *J* = 5.3, -*CH*- 2xOCH$_3$), 6.9-7.1 (2H, br, NH_2), 7.55 (2H, d, *J* = 8.8, 2×-C*H*-C-N=), 7.67 (2H, d, *J* = 8.8, 2×-C*H*-C-Cl), 7.95 (1H, s, NH); ^{13}C NMR (DMSO-d_6) δ_C 40.1 (N-*CH*$_3$), 54.0 (N-*CH*$_2$-), 54.2 (2×O*CH*$_3$), 99.2 (*C*-N=N-), 102.5 (-*CH*=2×OCH$_3$), 118.1, 119.9, 128.9, 129.5, 141.7,

155.4 (-*C*-aromatic), 159.5 (C-NH_2), 162.5 (-CO-), 164.0 (=N-C-N); LRMS: found (MH^+) 367.2, HRMS (FAB): found (MH^+) 367.1274, $C_{15}H_{19}ClN_6O_3$ requires (MH^+) 367.1285.

5-Amino-8-[(*E*)-(4-chlorophenyl)diazenyl]-3-hydroxy-1-methyl-2,3-dihydroimidazo[1,2-*c*]pyrimidin-7(1*H*)-one[218]

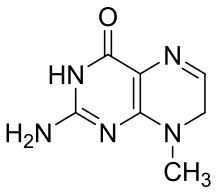


2.40

2-Amino-5-[(*E*)-(4-chlorophenyl)diazenyl]-6-[(2,2-dimethoxyethyl)(methyl)amino]-4(3*H*)-pyrimidinone **2.38** (0.59 g, 1.61 mmol) was dissolved in cold concentrated HCl (6 mL) by stirring continuously for 5 min. The reaction was then stirred at 80 °C for 15 min. During the first 5 min the title compound began to crystallize from solution. After cooling, the orange solid was collected and stirred with water for 2 h. The resulting yellow solid was filtered off and washed with water to obtain the hydrochloride of 2-amino-5-[(*E*)-(4-chlorophenyl)diazenyl]-6-[(2,2-dimethoxyethyl)(methyl)amino]-4(3*H*)-pyrimidinone, which was dried under reduced pressure at 40 °C to give an orange solid (0.56g, 83 %). mp 240 °C (decomp) (lit.[2] 240 °C). For crystallization, the product was dissolved in a minimum amount of water and boiled for 3 h. On cooling crystals were collected, dried under reduced pressure at 40 °C to afford dark-orange crystals (0.06 g). ν_{max} (KBr, cm^{-1}) 3071, 1605,1616, 1572, 1496, 1440, 1410, 1273, 1087; ^{1}H NMR (DMSO-d_6) δ_H 3.52 (1H, s, -C-*OH),* 3.60 (3H, s, -N-*CH*$_3$), 4.14 (2H, d, J_1 = 5.8, >N-C*H*$_2$-C-OH), 5.50 (1H, t, J = 5.8, HO-*CH*<), 7.56 (2H, d, J = 8.9, 2×-*CH*-C-N=), 7.68 (2H, d, J = 8.9, 2×-*CH*-C-Cl), 8.15 (2H, br, NH_2); ^{13}C NMR (DMSO-d_6) δ_C 54.6 (-N-*CH*$_3$), 60.6 (>N-C*H*$_2$-CHOH), 79.2 (>N-CH$_2$-*CH*OH), 82.1 (-CO-*C*-N=), 119.8, 121.4, 129.2, 129.9, 131.8, 144.3 (-*C*-aromatic), 152.1 (-*C*-NH_2), 157.9 (-*CO*-),

159.2 (=N-*C*-N); $C_{13}H_{13}ClN_6O_2$ requires (%) C, 42.64; H, 4.13; N, 22.95; found C, 42.70; H, 3.97; N, 22.11; LRMS: found (MH^+) 321.1, $C_{13}H_{13}ClN_6O_2$ requires (MH^+) 321.1, HRMS (FAB): found (MH^+) 321.0872, $C_{13}H_{13}ClN_6O_2$ requires (MH^+) 321.086

2-Amino-8-methyl-7,8-dihydro-4(3*H*)-pteridinone

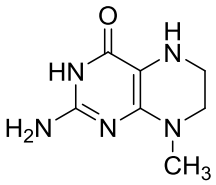


1.67

5-Amino-8-[(*E*)-(4-chlorophenyl)diazenyl]-3-hydroxy-1-methyl-2,3-dihydroimidazo[1,2-*c*]pyrimidin-7(1*H*)-one **2.40** (200 mg, 0.624 mmol) was dissolved in a mixture of water (25 mL), methanol (25 mL), DMF (10 mL) and ethyl acetate (10 mL) and stirred for 10 min. The flask was heated at 65 ^oC and stirred for further 5 min because it was not completely soluble at room temperature. The flask was cooled in an ice bath at 0 ^oC, 10% Pd/C (100 mg) was added to the flask during 10 min; the reaction mixture was stirred for 5 min and hydrogenated using a balloon of H_2 at room temperature (atmospheric pressure) for 4 h. The resulting yellow solution was filtered through celite (during filtration, the colourless solution turned brown) and the solvent was evaporated under reduced pressure at 50 ^oC to give a viscous brown oil. Ether (10 mL) was added and scratched to separate the dark-brown oily product, which was filtered off, washed with excess ether and recrystallised from EtOH to give the title compound as a shiny dark-brown solid. It was applied on TLC by using 1:1 water and 2-propanol as eluent. The spot for pteridine was near the base line showing very nice fluorescence (white in colour) under long wavelength in UV light. It was purified by CG-50 (H^+) ion exchange resin, the height of column was kept small (8 cm) and same eluent was used as for TLC. The ion exchange resin was washed with excess of acetic acid (0.5 N) and

stored. The solvents were evaporated under reduced pressure at 50 ^{o}C to offer a dark brown oily product. It was further purified by HPLC (water/acetonitrile, flow rate = 3mL/min, R_t = 15.73 min). The combined fractions were freeze dried to give title compound **1.67** as a fluffy yellow solid (52 mg, 46 %). mp >215 ^{o}C (dec.); ^{1}H NMR (DMSO-d_6) δ_H 2.71 (3H, s, -N-*CH*$_3$), 2.99 (2H, d, *J* = 6.8, -C*H*$_2$-CH), 6.99 (2H, br, N*H*$_2$), 7.10 (1H, t, *J* = 6.8, -C*H*-CH$_2$), 7.95 (1H, s, N*H*); ^{13}C NMR (DMSO-d_6) δ_C 41.2 (-N-*CH*$_3$), 52.4 (-*C*H$_2$-CH), 129.0 (-*C*-CO), 149.2 (-*C*-NH$_2$), 151.1 (=N-*C*-N<), 156.1 (-*C*O-), 158.0 (CH$_2$-*CH*=N-); LRMS: found (MH$^+$) 180.10, (MNa$^+$) 202.10, $C_7H_9N_5O$ requires (MH$^+$) 180.08, (MNa$^+$) 202.07, HRMS (FAB): found (MH$^+$) 180.0881, $C_7H_{10}N_5O$ requires (MH$^+$)180.0885.

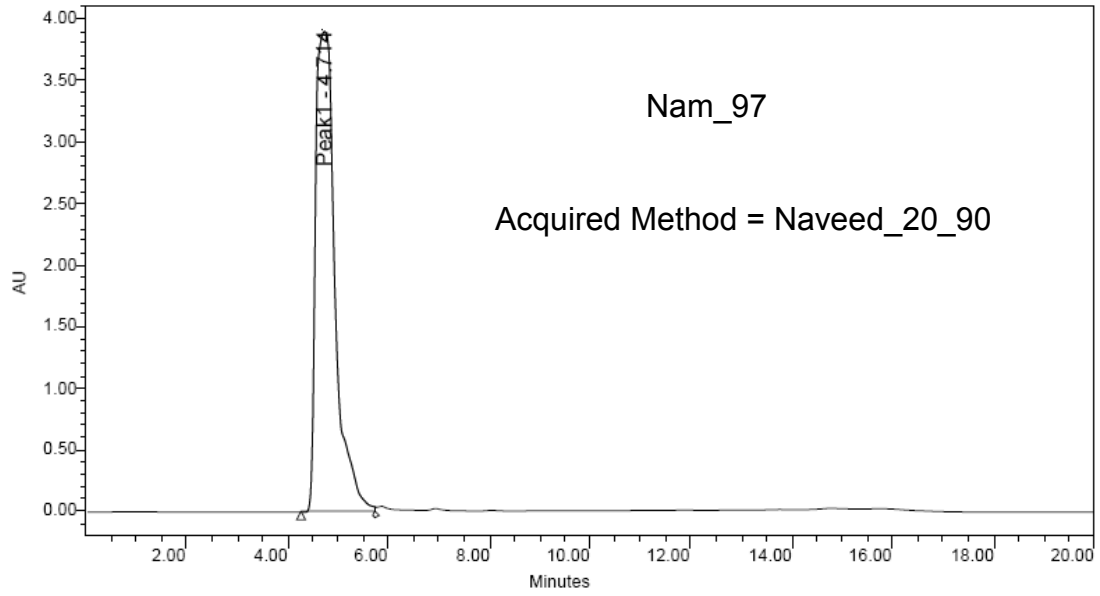
2-Amino-8-methyl-5,6,7,8-tetrahydro-4(3*H*)-pteridinone



2.43

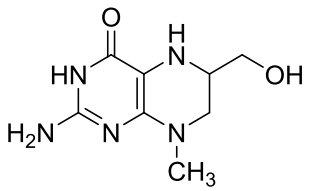
To the stirred solution of 2-amino-8-methyl-7,8-dihydro-4(3*H*)-pteridinone **1.67** (0.05 g, 0.281 mmol) in water (10 mL) and methanol (10 mL) was added 10 % Pd/C (25 mg) at 0 ^{0}C and hydrogenated using a double balloon of H_2 at room temperature (atmospheric pressure) for 6 h. The resulting colourless solution was filtered through a pad of kieselghur under suction through a glass sintered funnel. The filtrate flask was cooled in liquid nitrogen, aluminium foil was wrapped around the flask and the light was turned off during filtration. The solvents were removed by freeze drier to give a light-green residue which was purified by HPLC (water (95%)/acetonitrile (5%)), flow rate = 1 mL/min for first 8 min and then gradually changed to 6 mL/min , R_t = 5.64 min). The product was eluted at R_t = 4.71 min with (water (90%)/acetonitrile (10%)), flow rate = 1 mL/min as shown below in chromatogram. The solvents were removed by freeze drier to afford title compound **2.43** as a colourless solid (47 mg, 92%). mp >190 ^{o}C (dec.); ^{1}H NMR (DMSO-d_6) δ_H 2.42 (2H, t, *J* = 6.8 -NH-C*H*$_2$-CH$_2$), 2.65 (2H, t, *J* = 6.8, -NH-CH$_2$-C*H*$_2$), 2.72 (3H, s, -N-*CH*$_3$), 6.98 (2H, br, N*H*$_2$), 7.94 (1H, s, N*H*); ^{13}C NMR (DMSO-d_6) δ_C 41.1 (-N-*CH*$_3$), 52.5 (-NH-*C*H$_2$-CH$_2$), 63.1

(-NH-CH_2-$\underline{C}H_2$), 102.3 (-$\underline{C}$-CO), 139.2 (=N-*C*-N<), 150.3 (-*C*-NH_2), 157.6 (-CO-); LRMS: found (MH^+) 182.10, $C_7H_{11}N_5O$ requires (MH^+) 182.10.



HPLC chromatogram for the purification of 2.43

2-Amino-6-(hydroxymethyl)-8-methyl-5,6,7,8-tetrahydro-4(3*H*)-pteridinone

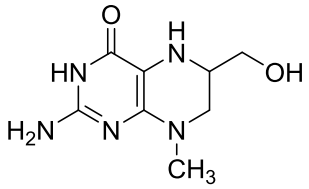


2.59

2-Amino-6-(hydroxymethyl)-8-methyl-7,8-dihydro-4(3*H*)-pteridinone **2.58** (100 mg, 0.48 mmol) was purified by HPLC (water/acetonitrile, flow rate = 6 mL/min, R_t = 8.14 min). The combined fractions were freeze-dried to give starting material **2.58** as a fluffy yellow solid (36 mg, 36 %). 2-Amino-6-(hydroxymethyl)-8-methyl-7,8-dihydro-4(3*H*)-pteridinone **2.58** (30 mg, 0.14 mmol) was suspended in methanol (10 mL). The suspension was treated with sodium cyanoborohydride (23 mg, 0.36 mmol, 2.5 equiv.) and followed by dilute

hydrochloric acid (2N, 3 drops) very quickly. Care was taken that sodium cyanoborohydride should not touch any part of flask other than solution. The reaction mixture was stirred for 4 h. The solvent was evaporated under reduced pressure at room temperature and the product was purified by HPLC (water/acetonitrile with 0.1 % TFA, flow rate = 6 mL/min R_t = 8.16 min). The solvents were evaporated using a freeze-drier to afford the corresponding tetrahydropteridine **2.59** as a colourless fluffy solid (27 mg, 77%). mp >200 ^{0}C (dec.); ^{1}H NMR (DMSO-d_6) δ_H 2.83 (2H, m, -N-C*H*$_2$-CH), 2.87 (1H, m, -NH-C*H*-CH$_2$-), 2.95 (3H, s, -N-*CH*$_3$), 3.98 (2H, m, -CH-C*H*$_2$-OH), 5.70 (1H, br), 6.59-6.87 (2H, br, -N*H*$_2$), 7.09 (1H, br), 10.88 (1H, br, -N*H*-CO-); ^{13}C NMR (DMSO-d_6) δ_C 42.1 (-N-*CH*$_3$), 49.1 (-N-*C*H$_2$-CH), 64.4 (-NH-*C*H-CH$_2$), 93.5 (*C*H$_2$-OH), 101.5 (-*C*-CO), 150.6 (=N-*C*-N<), 154.7 (-*C*-NH$_2$), 158.6 (-CO-); LRMS: found (MH$^+$) 212.10, $C_7H_{11}N_5O$ requires (MH$^+$) 212.11.

2-Amino-6-(hydroxymethyl)-8-methyl-5,6,7,8-tetrahydro-4(3*H*)-pteridinone

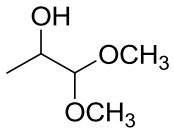


2.59

2-Amino-6-(hydroxymethyl)-8-methyl-7,8-dihydro-4(3*H*)-pteridinone **2.58** (100 mg, 0.48 mmol) was purified by HPLC (water/acetonitrile, flow rate = 6 mL/min, R_t = 8.15 min). The combined fractions were freeze-dried to give **2.58** as fluffy ayellow solid (35 mg, 35 %). To a solution of this purified starting material **2.58** (30 mg, 0.14 mmol) in water (5 mL) and methanol (5 mL) was added 10 % Pd/C (15 mg) at 0 ^{0}C and hydrogenated using a balloon of H_2 at room temperature (atmospheric pressure) for 4 h. The resulting colourless solution was filtered through a celite pad. The filtrate flask was cooled in liquid nitrogen, aluminium foil was wrapped around the flask and light was turned off during filtration. The solvents were removed using a freeze-drier and the residue was purified by HPLC (water (90%)/acetonitrile (10%)), flow rate = 1 mL/min for first 8 min and then gradually changed to 6 mL/min, R_t = 5.61 min). The solvents were again evaporated by freeze-drier to afford the title compound

2.59 as a colourless fluffy solid (26 mg, 87%). mp >200 ^{0}C (dec.); ^{1}H NMR (DMSO-d_6) δ_H 2.84 (2H, m, -N-C$\underline{H_2}$-CH), 2.87 (1H, m, -NH-C$\underline{H}$-CH$_2$-), 2.94 (3H, s, -N-CH_3), 3.98 (2H, m, -CH-C$\underline{H_2}$-OH), 5.71 (1H, br), 6.58-6.86 (2H, br, -NH_2), 7.10 (1H, br), 10.82 (1H, br, -NH-CO-); ^{13}C NMR (DMSO-d_6) δ_C 42.1 (-N-CH_3), 49.2 (-N-$\underline{C}H_2$-CH), 64.4 (-NH-$\underline{C}H$-CH$_2$), 93.5 (CH_2-OH), 101.4 (-$\underline{C}$-CO), 150.6 (=N-C-N<), 154.7 (-C-NH$_2$), 158.6 (-CO-); LRMS: found (MH$^+$) 212.10, $C_7H_{11}N_5O$ requires (MH$^+$) 212.11

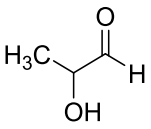
1,1-dimethoxy-2-propanol



2.78

To a solution of pyruvic aldehyde dimethyl acetal **2.77** (5.91 g, 50.0 mmol) in MeOH/THF (25 mL/ 25 mL) was added $NaBH_4$ (1.89 g, 50 mmol, 1.0 equiv) in portions over 10 min at 0 ^{0}C. The mixture was then allowed to warm to room temperature and was stirred for 30 min. The mixture was then poured into saturated aqueous NH_4Cl solution (25mL) and the aqueous layer was extracted with Et_2O (3 × 50 mL). The combined organic layers were concentrated by evaporation under reduced pressure. The residue was dissolved in CH_2Cl_2 (75 mL), which was washed with brine (25 mL) and then was dried (Na_2SO_4), and filtered. The solvent was removed under reduced pressure to give the crude product, which was purified by Kugelrohr distillation to afford the title compound **2.78** (10.1 g, 86%) as a colourless liquid, which was kept in the freezer. ν_{max} (NaCl, cm^{-1}) 3436, 2976, 2938, 2835, 1639, 1453, 1381, 1270, 1193, 1112, 1077, 979, 941, 920; ^{1}H NMR ($CDCl_3$) δ_H 1.09 (3H, d, J = 6.2 $C\underline{H_3}$-CH), 2.54 (1H, s, OH), 3.36 (6H, d, J =11.6 -2×OCH_3), 3.70 (1H, quintet, J = 6.2 CH$_3$-C$\underline{H}$-CH), 4.00 (1H, d, J = 6.2 CH$_3$-CH-C$\underline{H}$); ^{13}C NMR ($CDCl_3$) δ_C 17.3 ($\underline{C}H_3$-C-OH), 54.8 (-2×OCH_3), 67.2 ($\underline{C}$-OH), 107.8 ($\underline{C}$×2OCH$_3$); LRMS: found (MNa$^+$) 143.0, $C_5H_{12}O_3$ requires (MNa$^+$) 143.1.

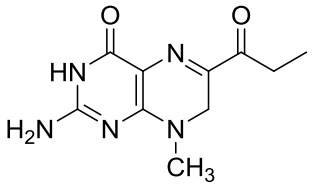
Hydroxypropanal



2.70

1,1-Dimethoxy-2-propanol **2.78** (1.50 g, 12.5 mmol) was dissolved in distilled water (15 mL) and treated with CG-50 (H^+) ion exchange resin (1.5 g) until pH 2.8. The mixture was heated at 50 ^oC for 8 h, and the resin was filtered off and washed with water (2 × 5 mL). The pH was adjusted to 7.0 by using a solution of sodium hydroxide (0.2 N) and product was used immediately for thiazolium salt promoted addition of aldehyde **2.70** to 2-amino-8-methyl-7,8-dihydro-4(3*H*)-pteridinone **1.67**.

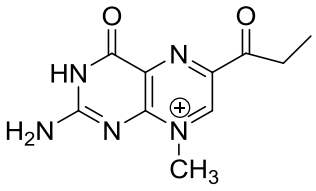
2-amino-8-methyl-6-propionyl-7,8-dihydro-4(3*H*)-pteridinone



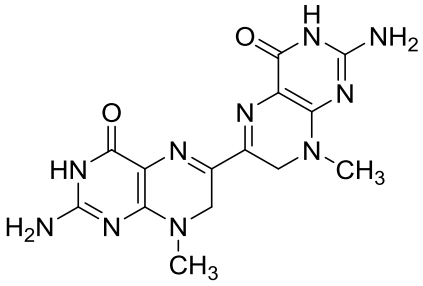
2.83

2-Amino-8-methyl-7,8-dihydro-4(3*H*)-pteridinone **1.67** (50 mg, 0.28 mmoles, 1 equiv.) was dissolved in water (10 mL), propionaldehyde (16 mg, 0.28 mmol, 1 equiv.) was added followed by triethylamine (28 mg, 39 µl, 0.28 mmol, 1 equiv.). The reaction mixture was cooled at 0 ^oC, stirred and thiazolium salt **2.81** (4.0 mg, 0.014 mmol, 5 mol%) was added over 5 min. The reaction mixture was stirred for 30 h at room temperature and after 10 h thiazolium salt **2.81** (4.0 mg, 0.014 equiv.) was added. The solvent was removed by freeze drier to give a brown oily material (55 mg) which was purified by silica gel using water as an eluent. The fractions were filtered through a pad of kieselghur under suction through a glass sintered funnel to remove any silica gel. The water was removed by freeze drier to give the brown oily material (20 mg) which was purified by HPLC. Four fractions were obtained; the LRMS of first fraction corresponded to **2.84**, it was very close to second fraction of thiazolium salt **2.81** and it decomposed in DMF at room temperature. No doubt LRMS was

very clean but due to decomposition of this product in DMF overnight, sufficient material for full characterization could not be obtained. LRMS for that fraction was found (M^+) 234.1, $C_{10}H_{12}N_5O_2^+$ requires (M^+) 234.1 corresponds to **2.84**.



2.84

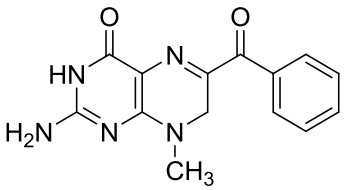


2.85

Most of the material was left on silica gel column which was separated by dissolving in ethyl acetate. The solution was filtered through a pad of kieselghur under suction through a glass sintered funnel to remove any silica gel. The ethyl acetate was evaporated under reduced pressure to give a light-brown material (25 mg). From the LRMS and proton NMR of that material, product **2.85** was supposed to be dimer of starting material **1.67**. It was third fraction from HPLC, LRMS found (MH^+) 357.4, $C_{14}H_{16}N_{10}O_2$ requires (MH^+) 357.2 corresponds to **2.85**.

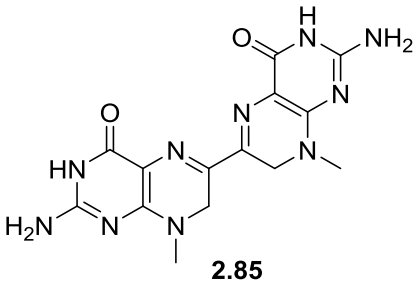
Fraction No.	Flow Rate mL/min	Retention Time min	LRMS
1	6	2.3	234.1
2	6	3.1	158.1
3	6	3.9	357.4
4	6	10.1	430.6

2-amino-6-benzoyl-8-methyl-7,8-dihydropteridin-4(*3H*)-one 2.96

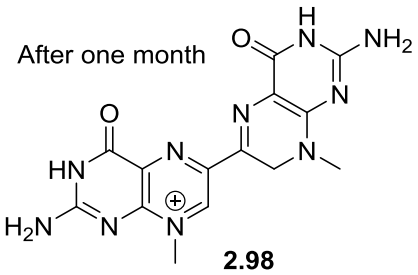


2.96

2-Amino-8-methyl-7,8-dihydro-4(3*H*)-pteridinone **1.67** (50 mg, 0.28 mmoles, 1 equiv.) was dissolved in methanol (10 mL) and the solution was cooled at 0 °C, stirred gently and thiazolium salt **2.81** (13 mg, 0.047 mmol, 17 mol%) during 10 min, triethylamine (28 mg, 39 µl, 0.28 mmols, 1 equiv.) were added, followed by benzaldehyde (30 mg, 29 µl, 0.28 mmols, 1 equiv.) and the reaction mixture was stirred for 2 days at 70 °C under an atmosphere of nitrogen. The solvent was removed under reduced pressure to give a residue of a dark-brown solid which was crystallized with methanol to give a brown solid (68 mg), was characterized and subjected to HPLC purification. The product was a dimer of **1.67** by all characterization. There was a further evidence of dimerization after one month, the dimer oxidized similarly to the parent dihydropterin **1.67**. ^{1}H NMR (DMSO-d_6) δ_H 3.04 (4H, =N-C*H*$_2$-), 3.34 (6H, s, =N-C*H*$_3$), 6.69-7.58 (4H, br, 2 x *NH*$_2$), 8.64 (2H, s, 2 x *NH*); LRMS for dimer was found (MH^+) 357.3, $C_{14}H_{16}N_{10}O_2$ requires (MH^+) 357.2 corresponds to **2.85**. LRMS of oxidized dimer was found (M^+) 355.3, $C_{14}H_{15}N_{10}O_2$+ requires (M^+) 355.14 corresponds to **2.98**.

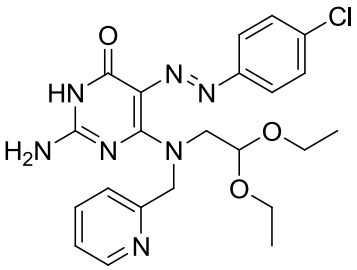


2.85



2.98

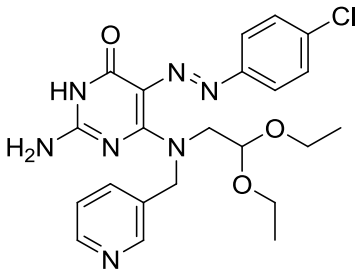
(*E*)-2-Amino-5-((4-chlorophenyl)diazenyl)-6-((2,2-diethoxyethyl)(pyridin-2-ylmethyl)amino)pyrimidin-4(3*H*)-one



2.55

To an ice-cooled stirred mixture of 2-amino-6-chloro-5-[(*E*)-(4-chlorophenyl)diazenyl]-4(3*H*)-pyrimidin-4(3*H*)-one **2.37** (305 mg, 1.07 mmol, 1.2 equiv.) in dimethylformamide (2 mL), 2,2-diethoxy-*N*-(2-pyridinylmethyl)ethanamine **2.47** (200 mg, 0.89 mmol, 1 equiv.) and triethylamine (151 µl, 1.2 equiv.) were also added. The reaction mixture was stirred under nitrogen at room temperature for 24 h. The solvent was removed under reduced pressure. The light-yellow solid was suspended in methanol and the insoluble solid that separated was transferred to a test tube and triturated. The process was repeated until the solvent was clear. The solid was filtered through celite and was washed with methanol (15 mL) and then dried under vacuum overnight at 40 ^{o}C to give the title compound **2.55** as a light-red solid (379 mg, 72%). mp >320 ^{o}C; ν_{max} (KBr, cm^{-1}) 3407, 3313, 2941, 1677, 1643, 1583, 1456, 1408, 1365, 1290, 1225, 1070, 787, 667; ^{1}H NMR (DMSO-d_6) δ_H 1.10 (6H, t, J = 7.2, -2 x CH_2-C$\underline{H}_3$), 2.66 (2H, d, J = 5.2, -$C\underline{H}_2$-CH(O$_2$)), 3.45 (2H, m, $C\underline{H}_2$-CH$_3$), 3.58 (2H, m, $C\underline{H}_2$-CH$_3$), 3.85 (2H, s, =N-CH_2-C-aromatic), 4.60 (1H, t, J= 5.2, -$C\underline{H}$-CH$_2$-N=), 6.73 (1H, s, -*NH*-), 7.20-8.50 (8H, m, 2 x 4 *CH*-aromatic), 8.07 (2H, br, -NH_2); ^{13}C NMR could not be obtained due to poor solubility; LRMS: found (MH$^+$) 472.4 (100%), 474.3 (32.0%), (MNa$^+$) 494.3 $C_{22}H_{27}ClN_7O_3$ requires (MH$^+$) 472.2 (100%), 474.2 (32.0%), (MNa$^+$) 494.2, HRMS (FAB): found (MH$^+$) 472.1861 (100%), 474.1843 (36.3%), $C_{22}H_{26}{}^{35}ClN_7O_3$ requires (MH$^+$) 472.1864 (100%), 474.1844 (36.2%).

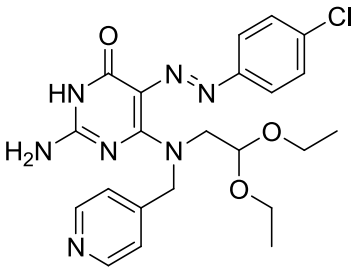
(*E*)-2-Amino-5-((4-chlorophenyl)diazenyl)-6-((2,2-diethoxyethyl)(pyridin-3-ylmethyl)amino)pyrimidin-4(3*H*)-one



2.54

2-Amino-6-chloro-5-[(*E*)-(4-chlorophenyl)diazenyl]-pyrimidin-4(3*H*)-one **2.37** (127 mg, 0.45 mmol, 1 equiv.) was dissolved in dimethylformamide (2 mL) at 0 ^{o}C, 2,2-diethoxy-*N*-(3-pyridinylmethyl)ethanamine **2.46** (100 mg, 0.45 mmol, 1 equiv.) and triethylamine (63 µl, 1 equiv.) were added. The reaction mixture was stirred under a nitrogen atmosphere at room temperature for 16 h. The solvent was removed under reduced pressure. The light-green solid residue was suspended in methanol and the insoluble solid that settled down was transferred into a test tube and was triturated. The process was repeated until the solvent was very light in colour. The solid was filtered through celite and was washed with methanol (10ml) and then dried under vacuum overnight at 40 ^{o}C to give the title compound **2.54** as a light-green solid (139 mg, 66%). mp >320 ^{0}C; ν_{max} (KBr, cm^{-1}) 3407, 3313, 2941, 1677, 1643, 1583, 1456, 1408, 1365, 1290, 1225, 1070, 787, 667; The product was sparingly soluble in DMSO in the presence of DMF. ^{1}H NMR (DMSO-d_6) δ_H 1.09 (6H, t, J = 7.04, -2 x CH_2-C$\underline{H_3}$), 2.79 (2H, d, J = 5.2, -C$\underline{H_2}$-CH(O_2)), 3.45 (2H, m, C$\underline{H_2}$-CH_3), 3.55 (2H, m, C$\underline{H_2}$-CH_3), 4.54 (2H, s, =N-CH_2-C-aromatic), 4.60 (3H, t, J = 5.2 , -$\underline{CH}$-CH_2-N=), 6.72 (1H, s, -*NH*-), 7.34-7.80 (6H, m, 6 x *CH*-aromatric), 8.07 (2H, br, -NH_2); 8.47 (1H, d, J = 1.1 , -N=*CH*-aromatic); The product precipitated in the NMR tube so ^{13}C NMR could not be obtained; LRMS: found (MH$^+$) 472.4 (100%), 474.3 (32.0%), 476.3 (32.0%), (MNa$^+$) 494.3 $C_{22}H_{26}ClN_7O_3$ requires (MH$^+$) 472.2 (100%), 474.2 (32.0%), (MNa$^+$) 494.2, HRMS (FAB): found (MH$^+$) 472.1852 (100%), 474.1828 (36.2%) $C_{22}H_{26}{}^{35}ClN_7O_3$ requires (MH$^+$) 372.1864 (100%), 474.1844 (36.2%).

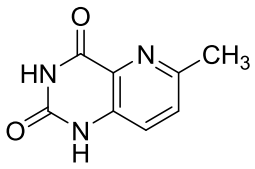
(*E*)-2-amino-5-((4-chlorophenyl)diazenyl)-6-((2,2-diethoxyethyl)(pyridin-4-ylmethyl)amino)pyrimidin-4(3*H*)-one



2.56

To an ice-cooled, stirred mixture of 2-amino-6-chloro-5-[(*E*)-(4-chlorophenyl)diazenyl]pyrimidin-4(3*H*)one **2.37** (348 mg, 1.26 mmol, 1.1 equiv.) in dimethylformamide (2 mL), was added 2,2-diethoxy-*N*-(4-pyridinylmethyl)ethanamine **2.45** (250 mg, 1.12 mmol. 1 equiv.) followed by triethylamine (151 µl, 1.2 equiv.). The reaction mixture was stirred under nitrogen at room temperature for 24 h. The solvent was removed under reduced pressure. The light-yellow solid residue was suspended in methanol and the insoluble solid that settled at the bottom was transferred into a test tube and was triturated. The process was repeated until the solvent was clear. The solid was filtered through celite and was washed with methanol (10 mL) and then dried under vacuum overnight at 40 ^{o}C to give the title compound **2.56** as a yellow solid (339 mg, 78%). mp >320 ^{o}C; ν_{max} (KBr, cm^{-1}) 3407, 3313, 2941, 1677, 1643, 1583, 1456, 1408, 1365, 1290, 1225, 1070, 787, 667; ^{1}H NMR (DMSO-d_6 ^{1}H NMR (DMSO-d_6) δ_H 1.09 (6H, t, J = 7.04, 2 x CH_2-C$\underline{H_3}$), 2.66 (2H, d, J = 5.2, -C$\underline{H_2}$-CH(O_2)), 3.44 (2H, m, C$\underline{H_2}$-CH_3), 3.57 (2H, m, C$\underline{H_2}$-CH_3), 3.85 (2H, s, =N-CH_2-C-aromatic), 4.60 (3H, t, J = 5.2 , -C$\underline{H}$-CH_2-N=), 6.73 (1 H, s, -*NH*-), 7.24-8.5 (8H, m, 2 x 4 *CH*-aromatric), 8.07 (2H, br, -NH_2); ^{13}C NMR could not be obtained due to poor solubility; LRMS: found (MH^+) 472.4 (100%), 474.3 (32.0%), (MNa^+) 494.3 $C_{22}H_{26}ClN_7O_3$ requires (MH^+) 472.2 (100%), 474.2 (32.0%), (MNa^+) 494.2, HRMS (FAB): found (MH^+) 372.1865 (100%), 474.1839 (37.3%), $C_{22}H_{26}{}^{35}ClN_7O_3$ requires (MH^+) 372.1864 (100%), 474.1844 (36.24%).

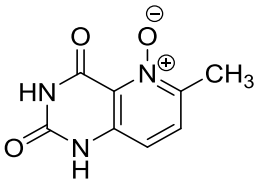
6-Methylpyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione[233]



2.105

5-Amino-2,4(1*H*,3*H*)-pyrimidinedione **2.102** (2.33 g, 18.4 mmol, 1 equiv), hydrochloric acid (38 mL, 20%), and crotonaldehyde (2.26 ml, 27.5 mmol, 1.5 equiv) were heated together under reflux for 1 h. The solution was evaporated to dryness and the residue was dissolved in methanol (20 mL). After an hour, the cream-coloured solid was filtered, washed with methanol (15 mL) and dried under vacuum overnight at 40 ^{o}C to give the title compound **2.105** as a cream-coloured solid (1.44 g, 44%). mp >340 ^{o}C (decomp.) (lit.[233] above 350 ^{o}C (decomp.)) ; ν_{max} (KBr, cm^{-1}): 3185, 2900, 2033, 1989, 1860, 1657, 1630, 1552, 1439, 1358, 1296, 1255, 1223, 1155, 1028, 999, 933, 843, 809, 744, 671; ^{1}H NMR (DMSO-d_6) δ_H 2.56 (3H, s, C$\underline{H_3}$), 7.66 (1H, d, J = 8.4, $C\underline{H}$-CH-C-CH$_3$), 7.79 (1H, d, J = 8.4, CH-$C\underline{H}$-C-CH$_3$), 11.58 (1H, s, CO-$N\underline{H}$-C), 11.64 (1H, s, CO-$N\underline{H}$-CO); ^{13}C NMR (DMSO-d_6) δ_C 22.2 (C-$\underline{C}$H$_3$), 126.7 (-$\underline{C}$H-C-NH), 128.4 (-$\underline{C}$H-C-CH$_3$), 129.8 (>C=$\underline{C}$-CO-), 136.8 (>$\underline{C}$=C-CO-), 149.5 ($\underline{C}$-CH$_3$), 152.5 (-NH-*C*O-NH-) 160.3 (NH-$\underline{C}$O-C); LRMS: found (MH$^+$) 178.20, (MNa$^+$) 200.07 $C_8H_7N_3O_2$ requires (MH$^+$) 178.05, (MNa$^+$) 200.04, HRMS (ESI): found (MH$^+$) 178.0610 $C_8H_7N_3O_2$ requires (MH$^+$) 178.0611.

6-Methylpyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione 5-oxide[232]

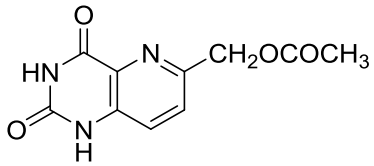


2.106

To a suspension of 6-methylpyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione **2.105** (0.9 g, 5.1 mmol, 1 equiv.) in glacial acetic acid (50 mL) was added *m*-chloroperoxybenzoic acid (3.08 g, 17.9 mmol, 3.5 equiv.) and the reaction mixture was heated under reflux at 122 ^{o}C for 3 h. Acetic acid was evaporated under reduced pressure and the yellow solid material was stirred with ether (100 mL) and filtered. The yellow solid was dissolved in glacial acetic acid (10

mL). The cream-coloured material was precipitated and filtered by sintered funnel. The soluble material was precipitated by reducing the volume of glacial acetic acid. The solid was dissolved in dilute $NaHCO_3$ solution and organic layer was extracted using ethyl acetate (3 × 50 mL). The organic layer was dried under reduced pressure to give the title compound **2.106** as light-yellow solid (0.59 g, 60%). mp >300 °C (lit.[138] >300 °C) ; ^{1}H NMR (DMSO-d_6) δ_H 2.26 (3H, s, C*H*$_3$), 6.89 (1H, d, *J* = 8.6, *CH*-CH-C-CH$_3$), 7.57 (1H, d, *J* = 8.6, CH-*CH*-C-CH$_3$), 11.12 (1H, s, -CO-*NH*-C), 11.24 (1H, s, -CO-*NH*-CO-); LRMS: found (MH^+) 194.20, (M^+-16) 177.20, (MNa^+) 216.13, $C_8H_7N_3O_3$ requires (MH^+) 194.05, (M^+-16) 177.05, (MNa^+) 216.05.

(2,4-Dioxo-1,2,3,4-tetrahydropyrido[3,2-*d*]pyrimidin-6-yl)methyl acetate[232]



2.107

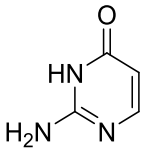
Two methods were used for the preparation of (2,4-dioxo-1,2,3,4-tetrahydropyrido[3,2-*d*]pyrimidin-6-yl)methyl acetate **2.107**.

Method A. 6-Methylpyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione-5-oxide **2.106** (0.90 g, 4.66 mmol) in a mixture of glacial acetic acid (10 mL) and acetic anhydride (10 mL) was heated under reflux for 30 min. The clear-brown solution was evaporated to dryness and the residue was crystallized from methanol to give the title compound **2.107** as a light-brown solid (0.91 g, 83%). mp 285-286 °C (lit.[137] 288-289 °C) ; ν_{max} (KBr, cm^{-1}): 3195, 3067, 2844, 2225, 2063, 1709, 1608, 1420, 1475, 1366, 1293, 1155, 1058, 923, 841, 754, 682; ^{1}H NMR (DMSO-d_6) δ_H 2.09 (3H, s, C*H*$_3$), 5.13 (2H, s, -*CH*$_2$-O-COCH$_3$), 7.57 (1H, d, *J* = 8.4, *CH*-CH-C-CH$_2$), 7.64 (1H, d, *J* = 8.4, CH-*CH*-C-CH$_2$), 11.23 (1H, s, CO-*NH*-C), 11.48 (1H, s, -CO-*NH*-CO-); ^{13}C NMR (DMSO-d_6); δ_C 20.6 (CH_3), 66.0 (CH_2), 124.7 (*C*H-CH-C-CH$_2$), 127.4 (CH-*C*H-C-CH$_2$), 130.7 (-CO-*C*-N=), 137.6 (-NH-*C* CH=), 149.7 (-N=*C*-CH$_2$-), 150.5 (-NH-*C*O-NH-), 161.0 (-NH-*C*O-C), 170.1 (-*C*O-CH$_3$); LRMS: found (MH^+) 236.13, (MNa^+)

258.20, $C_{10}H_9N_3O_4$ requires (MH^+) 236.07, (MNa^+) 258.07, HRMS (ESI): found (MH^+) 236.0668, (MNa^+) 258.0489 $C_{10}H_9N_3O_4$ requires 236.0666, (MNa^+) 258.0485.

Method B. 6-Methylpyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione **2.105** (3.00 g, 16.9 mmol, 1 equiv.) was added in glacial acetic acid (135 mL) with stirring, *m*-chloroperoxybenzoic acid (10.22 g, 59.2 mmol, 3.5 equiv) was added portionwise. A safety shield was placed in front of the reaction flask. The temperature of the oil bath was raised and reaction mixture was heated under reflux and stirred for 3h. Acetic anhydride (400 mL) was added to the hot reaction mixture and the heating was continued for another 30 min. The clear-brown solution was evaporated to dryness and the solid mass was stirred with ether (260 mL). It was filtered through celite and was washed using ether (50 mL). The solid material was then dissolved in methanol (200 mL) and was heated up to 50 ^oC and stirred. The insoluble, cream-coloured, grain like impurity (20 mg) was precipitated and separated by filtration. After cooling the filtrate was further filtered and a black solid impurity (35 mg) was separated. The volume of methanol was reduced to 5 mL under reduced pressure and was stirred by adding diethyl ether (100 mL). The stirred mixture was allowed to stand at room temperature, the product was precipitated as a light-brown solid which was washed with ether (20 mL) and dried under vacuum overnight at 40 ^oC to give the title compound **2.107** as light-brown solid (1.22 g, 31%), identical in all respects with the one obtained in procedure **A**.

2-Amino-4(3*H*)-pyrimidinone[237]

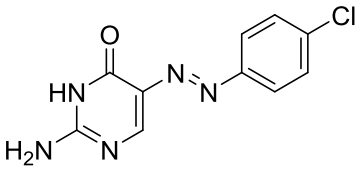


2.148

Guanidine hydrochloride **2.146** (24.0 g, 0.25 mol, 1 equiv.) was added slowly to a well-stirred concentrated sulfuric acid solution (100 mL, 1.87 mol) at -5 ^oC. While guanidine hydrochloride was being added, the temperature was further decreased to -10 ^oC. Malic acid **2.147** (33.6 g, 0.25 mol, 1 equiv.) was added and the mixture was heated at 105 ^oC for 24 h. It

was cooled to room temperature and poured over ice (330 g). The reaction mixture was cooled to -5 °C and barium carbonate paste (368 g, 1.87 mol) was added in small portions; addition was continued until the reaction mixture was slightly basic which was stirred for overnight and allowed to stand for 2 h. Barium carbonate and barium sulphate were removed by filtration. Acetone (200 mL) was added to filtrate and a cream-coloured solid precipitate was observed which was separated by filtration, further crops of product were obtained by adding methanol (100 mL) while shaking and allowing filtrate to stand for 4 h. Barium carbonate and barium sulphate were washed with methanol and further product was observed in that filtrate, however salt was also found in that filtrate. All crops of product were first crystallised from methanol and then recrystallized from hot water. The solid precipitate was separated by filtration and dried under reduced pressure to afford the title compound **2.148** as a white solid (16.2 g, 58%). mp 276 °C (lit.[142] 276 °C); ν_{max} (KBr, cm^{-1}): 3140, 2828, 2739, 1969, 1819, 1677, 1608, 1518, 1474, 1445, 1409, 1393, 1372, 1318, 1301, 1232, 1204, 1156, 1082, 1021, 981, 920, 809, 784, 762, 632, 589, 568, 548; ^{1}H NMR (DMSO-d_6) δ_H 5.23 (1H, d, J = 6.4, CO-C*H*-CH), 6.70-7.00 (2H, br, -NH_2-), 7.36 (1H, d, J = 6.4, -CH-C*H*-N=), 11.14 (1H, s, -N*H*-CO-); ^{13}C NMR (DMSO-d_6) δ_c 104.4 (-C=*C*-CO-), 141.7 (-C=*C*-N=), 153.3 (-N=*C*-NH_2), 159.8 (-*C*=O); LRMS: found (MH^+) 112.20, (MNa^+) 134.27, $C_4H_5N_3O$ 111.04 requires (MH^+) 112.04, (MNa^+) 134.27. HRMS (ESI): found (MH^+) 112.0502, (MNa^+) 134.0321, $C_4H_5N_3O$ requires (MH^+) 112.0505, (MNa^+) 134.0325.

2-Amino-5-[(*E*)-(4-chlorophenyl)diazenyl]-4(3*H*)-pyrimidinone 2.230

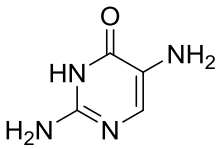


2.151

4-Chloroaniline (2.20 g, 17.3 mmol, 1.2 equiv.) was dissolved in hydrochloric acid (2M, 32 m1) and this solution was cooled to -5 °C by using acetone and dry ice. Sodium nitrite (1.19 g, 17.3 mmol, 1.2 equiv.) in water (16 mL) was added dropwise with stirring. After completion of the addition, the reaction mixture was stirred for 15 min at -5 °C. 2-Amino-4(3*H*)-pyrimidinone (1.6 g, 14.4 mmol, 1 equiv.) was dissolved in 2N NaOH (32 mL) at

room temperature and this solution was cooled to -5 °C. To this suspension, the above diazonium salt solution was added slowly so that the reaction temperature did not rise above -5 °C. After stirring for 4 h, complete conversion of isocytosine into diazo product was observed by TLC. The reaction mixture was left overnight without stirring. In the morning the solid precipitate was filtered off, washed with water and ethyl acetate, dried under reduced pressure to give the title compound **2.151** as a brick-red solid (2.55 g, 71%). mp >300 °C (dec.); ^{1}H NMR (DMSO-d_6) δ_H 7.53 (2H, d, J = 8.8, 2×-C<u>H</u>-C-N=), 7.67 (2H, d, J = 8.8, 2×-C<u>H</u>-C-Cl), 7.70-7.90 (2H, br, -NH_2), 8.27 (1H, s, =N-<u>CH</u>=), 11.67 (1H, s, NH); HSQC NMR showed following δ_H to δ_c relationship 7.53, 129.24 (2H, d, J = 8.8, 2×-C<u>H</u>-C-N=), 7.67, 123.30 (2H, d, J = 8.8, 2×-C<u>H</u>-C-Cl), 7.70-7.90 (2H, br, -NH_2), 8.27, 150.9 (1H, s, =N-<u>CH</u>=) , 11.67 (1H, s, NH); Product was sparingly soluble in DMSO and precipitated in NMR tube so ^{13}C NMR could not be obtained. LRMS found (MH^+) 250.27, $C_{10}H_8ClN_5O$ requires (MH^+) 250.04, HRMS (ESI): found (MH^-) 248.0347 $C_{10}H_8ClN_5O$ requires (MH^-) 248.0345

2,5-diamino-4(3*H*)-pyrimidinone 2.226 from 2-amino-5-[(*E*)-(4-chlorophenyl)diazenyl]-4(3*H*)-pyrimidinone 2.142

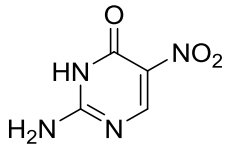


2.142

2-Amino-5-[(*E*)-(4-chlorophenyl)diazenyl]-4(3*H*)-pyrimidinone **2.151** (100 mg, 0.40 mmol) was dissolved in methanol (100 mL), cooled to 0 °C. It was sparingly soluble. 10% Pd/C (60 mg) was added little by little and hydrogenated for 12 h. Pd/C was removed by filtering the solution through a pad of kieselguhr under suction through a glass sintered funnel. The solvent was evaporated under reduced pressure. The solid crude material was dissolved in methanol and allowed to stand for 2 h, a brick-red solid precipitate (5 mg) was found but it was found to not be the product. Diethyl ether was added for further precipitation. The precipitate was filtered off, washed with ethyl acetate and diethyl ether, dried under reduced pressure at 40 °C to give the product **2.142** as a light-red solid (21 mg, 42%). mp >250 °C (dec.); ν_{max} (KBr, cm^{-1}): 3382, 3328, 3039, 2932, 2797, 2610, 2540, 2009, 1752, 1708, 1595,

1560, 1541, 1489, 1405, 1346, 1330, 1262, 1224, 1176, 1142, 1096, 1072, 995, 981, 954, 879, 839, 803, 784, 712, 667, 599, 556, 519; ^{1}H NMR (DMSO-d_6) δ_H 6.10-6.30 (4H, br, -2×*NH*$_2$), 6.89 (1H, s, =N-*CH*=), 11.58 (1H, s, NH); LRMS found (MH^+) 127.10, $C_4H_6N_4O$ requires (MH^+) 127.06.

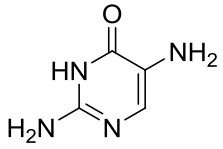
2-Amino-5-nitro-4(3*H*)-pyrimidinone



2.149

2-Amino-4(3*H*)-pyrimidinone **2.148** (1.0 g, 9.0 mmol) was dissolved in concentrated sulphuric acid (3 mL) at 0 °C and treated dropwise, with stirring, with 70 % nitric acid (4 mL). The resulting solution was stirred for 24 h at at 64 °C. It was neutralised at 0 °C with NH_4OH solution and the precipitate was separated by filtration, washed with cold water (25 mL), methanol (5 mL) and diethyl ether (50 mL) and dried under reduced pressure at 40 °C overnight to give the title compound **2.149** as a cream-coloured solid (1.2 g, 82%). mp 300 °C (dec.); 3463, 3348, 3178, 2764, 2427, 2095, 1986, 1725, 1685, 1590, 1557, 1590, 1557, 1508, 1487, 1433, 1384, 1356, 1303, 1117, 996, 850, 831, 810, 779, 732, 704, 639, 618; ^{1}H NMR (DMSO-d_6) δ_H 7.00-7.30, 8.40-8.70 (2H, br, -NH_2), 8.80 (1H, s, -*CH*-C-NO_2), 11.72 (1H, s, NH); ^{13}C NMR (DMSO-d_6) δ_c 125.7 (-*C*-NO_2), 141.7 (-C=*C*-N=), 153.3 (-N=*C*-NH_2), 159.8 (-*C*=O); LRMS found (MH^+) 157.13, (MNa^+) 179.13, (MH^-) 155.07, $C_4H_4N_4O_3$ requires (MNa^+) 179.02, (MH^-) 155.02, HRMS (ESI): found (MH^+) 157.0351, (MNa^+) 179.0170, $C_4H_4N_4O_3$ requires (MH^+) 157.0356, (MNa^+) 179.0176.

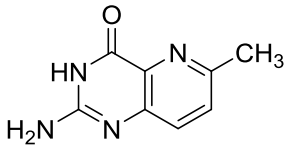
2,5-diamino-4(3*H*)-pyrimidinone 2.141



2.141

To a stirred solution of 2-amino-5-nitro-4(3*H*)-pyrimidinone **2.149** (0.70 g, 4.5 mmol) in methanol (250 mL) was added 10 % Pd/C (350 mg) at 0 ^{o}C and hydrogenated using a balloon of H_2 at room temperature (atmospheric pressure) for 12 h. The resulting light-yellow solution was filtered through a pad of kieselghur under suction through a glass sintered funnel. The solvent was evaporated under reduced pressure to give the title compound **2.141** a light-brown solid (0.48 g, 85%). mp >250 ^{o}C (dec.); ν_{max} (KBr, cm^{-1}): 3369, 3138, 2959, 2740, 2009, 1752, 1710, 1559, 1540, 1488, 1411, 1392, 1346, 1330, 1291, 1257, 1224, 1174, 1150, 1096, 1069, 995, 982, 954, 898, 879, 839, 818, 804, 755, 713, 635, 599, 556; ^{1}H NMR (DMSO-d_6) δ_H 6.10-6.30 (4H, br, -2×*NH*$_2$), 6.87 (1H, s, =N-*CH*=), 11.26 (1H, s, NH); ^{13}C NMR (DMSO-d_6) δ_c 102.1 (=*CH*-N=), 125.3 (-CO-*C*-NH2), 149.9 (-N=*C*-NH$_2$), 162.0 (-*C*=O); LRMS found (MH$^+$) 127.20, (MNa$^+$) 149.20, (2MH$^+$) 253.27, (2MNa$^+$) 275.27, (MH$^-$) 125.27, $C_4H_6N_4O$ requires (MH$^+$) 127.06, (MNa$^+$) 149.04, (2MH$^+$) 253.12, (2MNa$^+$) 275.10, (MH$^-$) 125.04, HRMS (ESI): found (MH$^+$) 127.0614, (2MH$^+$) 253.1154, $C_4H_6N_4O$ requires (MH$^+$) 127.0614, (2MH$^+$) 253.1156.

2-Amino-6-methylpyrido[3,2-*d*]pyrimidin-4(3*H*)-one[236]

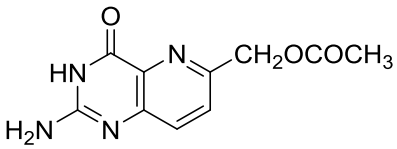


2.144

2,5-Diamino-4(3*H*)-pyrimidinone **2.141** (300 mg, 2.38 mmol, 1 equiv), hydrochloric acid (6.0 mL, 20%), and crotonaldehyde (300 µl, 3.65 mmol, 1.53 equiv) were heated together under reflux at 122 ^{o}C for 2 h. The solution was evaporated to dryness under reduced

pressure and the black solid residue was dissolved in water and stirred for 2 h. The impurities were insoluble in water and were separated by filtration. The filtrate was evaporated under reduced pressure to give light-yellow solid which was purified by column chromatography (50 % ethyl acetate/methanol). The product **2.144** was white fluorescent on TLC and in solution it was also white fluorescent if viewed under UV in longer wavelength light. Solvents were evaporated under reduced pressure and the resulting solid was suspended in methanol and collected by filtration to give the title compound **2.144** as white solid (201 mg, 48 %). mp >300 ^{o}C (dec.) (lit.[141] >300 ^{o}C (decomp.)); ν_{max} (KBr, cm^{-1}): 3445, 3251, 3082, 3036, 2018, 1969, 1729, 1688, 1637, 1529, 1495, 1147, 1379, 1320, 1302, 1252, 1233, 1153, 1091, 1054, 1027, 974, 917, 871, 823, 787, 716, 670, 631, 605, 568; ^{1}H NMR (DMSO-d_6) δ_H 2.56 (3H, s, C*H*$_3$), 7.66 (1H, d, *J* = 8.4, *CH*-CH-C-CH$_3$), 7.87 (1H, d, *J* = 8.4, CH-*CH*-C-CH$_3$), 8.40-8.60 (2H, br, -*NH*$_2$), 11.39 and 11.57 (1H, s, *NH*); ^{13}C NMR (DMSO-d_6) δ_C 23.3 (*C*H$_3$-C=N), 126.9 (CH-*C*H-C-CH$_3$), 129.7 (*C*H-CH-C-CH$_3$), 131.1 (N-*C*-CH-CH-C-CH$_3$), 135.3 (*C*-CO), 151.1 (N=*C*-CH$_3$), 155.4 (NH$_2$-*C*=N), 157.6 (-*C*O-); LRMS found (MH$^+$) 177.13, (MNa$^+$) 199.13, (2MH$^+$) 353.20, (2MNa$^+$) 375.07, $C_8H_8N_4O$ requires (MH$^+$) 177.08, (MNa$^+$) 199.06, (2MH$^+$) 353.15, (2MNa$^+$) 375.13, HRMS (ESI): found (MH$^+$) 177.0770, (MNa$^+$) 199.0589, $C_8H_8N_4O$ requires (MH$^+$) 177.0771, (MNa$^+$) 199.0590.

2-Acetylamino)-4-oxo-3,4-dihydropyrido[3,2-*d*]pyrimidin-6-yl methyl acetate 2.151

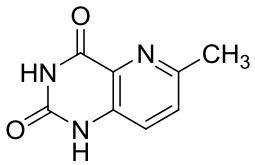


2.151

2-Amino-6-methylpyrido[3,2-*d*]pyrimidin-4(3*H*)-one **2.144** (100 mg, 0.57 mmol. 1 equiv.) was dissolved in acetic acid (5 mL). The flask was cooled with ice, and *m*-chloroperoxybenzoic acid (245 mg, 1.42 mmol, 2.5 equiv) was added in small portions. A safety shield was placed in front of the reaction flask. After completion addition of *m*-CPBA, the reaction mixture was heated under reflux at 125 ^{o}C for 3 h. Acetic anhydride (4 mL) was added to the hot reaction mixture and it was further heated at the same temperature for

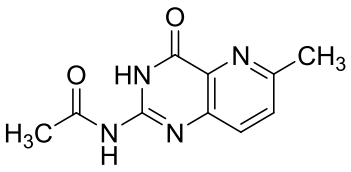
another 30 min. The solvents were removed under reduced pressure at 55 °C and residue was stirred with diethyl ether (70 mL) and filtered through a sintered funnel under reduced pressure, the less polar impurities were washed with ether. The solid precipitate was further washed with diethyl ether and dried under reduced pressure at 40 °C to give a light-brown solid (95 mg) which was purified by HPLC (water (90%)/acetonitrile (10%)), flow rate = 6 mL/min, R_t = 14.49 min). The brown solution was evaporated to dryness and the residue was crystallized from methanol to give title compound **2.151** as a light-brown solid (44 mg, 33%). mp >310 °C (dec.); ν_{max} (KBr, cm^{-1}): 3443, 3386, 3193, 2998, 1874, 1718, 1685, 1658, 1610, 1542, 1384, 1354, 1299, 1263, 959, 896, 854, 802, 769, 707, 615, 578; ^{1}H NMR (DMSO-d_6) δ_H 2.18 (3H, s, C$\underline{H_3}$), 5.22 (2H, s, -$C\underline{H_2}$-O-$COCH_3$), 7.74 (1H, d, J = 8.4, CH-$\underline{CH}$-C-CH_2), 7.86 (1H, d, J = 8.4, $\underline{CH}$-CH-C-CH_2), 11.23 (1H, s, CO-$N\underline{H}$-C), 11.50 (1H, s, -CO-$N\underline{H}$-CO-); LRMS: found (MH^+) 235.13, (MNa^+) 257.20, $C_{10}H_{10}N_4O_3$ requires (MH^+) 235.08, (MNa^+) 257.06.

Although product **2.151** was the major fraction from hplc, there was also *N*-oxide **2.145** eluting at R_t = 12.13 min, occupying 5 % area, LRMS: found (MH^+) 193.13, (MNa^+) 215.20, $C_8H_8N_4O_2$ requires (MH^+) 193.07, (MNa^+) 215.05 and **2.105** eluting at R_t = 9.92 min and occupying 10 % area, LRMS: found (MH^+) 177.3, (MNa^+) 200.07, ($2MH^+$) 355.40 ($2MNa^+$) 376.93, $C_8H_7N_3O_2$ requires (MH^+) 177.06, (MNa^+) 200.04, ($2MH^+$) 355.12 ($2MNa^+$) 377.10.



2.105

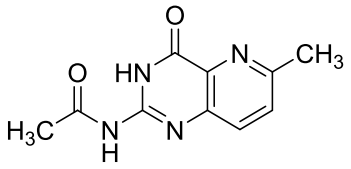
A less polar white solid (25 mg) obtained by precipitation using less methanol and more diethyl ether proved to be **2.163**



2.163

^{1}H NMR (DMSO-d_6) δ_H 2.18 (3H, s, C$\underline{H_3}$), 2.56 (3H, s, -$\underline{CH_3}$-CO-NH), 7.74 (1H, d, J = 8.4, $\underline{CH}$-C-CH$_3$), 7.88 (1H, d, J = 8.4, $\underline{CH}$-CH-C-CH$_3$), 11.68 (1H, s, -CO-$\underline{NH}$-C-), 12.21 (1H, s, CH$_3$-CO-$\underline{NH}$); LRMS: found (MH$^+$) 219.20, (MNa$^+$) 241.20, $C_{10}H_{10}N_4O_2$ requires (MH$^+$) 219.09, (MNa$^+$) 241.07; HRMS (ESI): found (MH$^+$) 219.0877, (MNa$^+$) 241.0694 $C_{10}H_{10}N_4O_2$ requires (MH$^+$) 219.0882, (MNa$^+$) 241.0696.

***N*-(6-Methyl-4-oxo-3,4-dihydropyrido[3,2-*d*]pyrimidin-2-yl)acetamide**

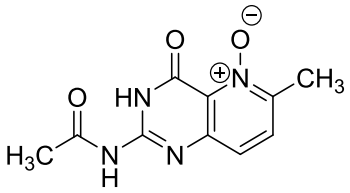


2.163

2-Amino-6-methylpyrido[3,2-*d*]pyrimidin-4(3*H*)-one **2.144** (100 mg, 0.57 mmol) was dissolved in pyridine (168 µl, 1.63 g, 20.6 mmol) and cooled in an ice-salt bath. Acetic anhydride (2.0 mL, 2.1 g, 20.6 mmol) was added dropwise with stirring. After 2 h, the ice bath was removed and reaction mixture was stirred for 16 h at room temperature. The solvents were evaporated under reduced pressure at 50-60 ^{o}C to give a light-brown residue which was dissolved in MeOH (5 mL), a solid precipitate was observed which was separated by filtration under reduced pressure, washed with diethyl ether (25 mL) and dried under reduced pressure at 40 ^{o}C to give the title compound **2.163** as a cream-coloured solid (83 mg, 64%). mp >220 ^{o}C; ν_{max} (KBr, cm^{-1}): 3401, 1737, 1699, 1699, 1644, 1557, 1490, 1449, 1408, 1337, 1300, 1116, 917, 879, 841, 640, 609, 497; ^{1}H NMR (DMSO-d_6) δ_H 2.12 (3H, s, C$\underline{H_3}$), 2.56 (3H, s, -$\underline{CH_3}$-CO-NH), 7.66 (1H, d, J = 8.4, $\underline{CH}$-C-CH$_3$), 7.85 (1H, d, J = 8.4, $\underline{CH}$-CH-C-CH$_3$), 11.68 (1H, s, -CO-$\underline{NH}$-C-), 12.21(1H, s, CH$_3$-CO-$\underline{NH}$); ^{13}C NMR (DMSO-d_6) δ_C 23.8 ($\underline{CH_3}$-CO-NH), 23.9 ($\underline{CH_3}$-C=N), 129.2 (CH-$\underline{CH}$-C-CH$_3$), 134.2 ($\underline{CH}$-CH-C-CH$_3$), 135.5 (N-$\underline{C}$-CH-CH-C-CH$_3$), 144.1 ($\underline{C}$-CO), 146.5 (-N=$\underline{C}$-NH-), 155.9 (N=$\underline{C}$-CH$_3$), 158.5 (-NH-$\underline{C}$O-C), 173.7 (-$\underline{C}$O-CH$_3$); LRMS: found (MH$^+$) 219.23, (MNa$^+$) 241.23, $C_{10}H_{10}N_4O_2$ requires

(MH^+) 219.09, (MNa^+) 241.07; HRMS (ESI): found (MH^+) 219.0877, (MNa^+) 241.0694, ($2MH^+$) 437.1678, ($2MNa^+$) 459.1498, $C_{10}H_{10}N_4O_2$ requires (MH^+) 219.0882, (MNa^+) 241.0696, ($2MH^+$) 437.1680, ($2MNa^+$) 459.1500.

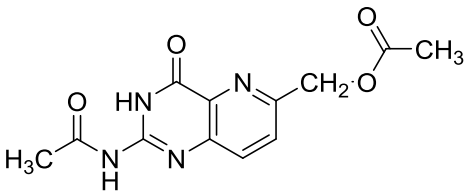
_N_-(6-Methyl-5-oxido-4-oxo-3,4-dihydropyrido[3,2-*d*]pyrimidin-2-yl)acetamide



2.164

To a suspension of *N*-(6-methyl-4-oxo-3,4-dihydropyrido[3,2-*d*]pyrimidin-2-yl)acetamide **2.163** (50 mg, 0.23 mmol, 1 equiv.) was added glacial acetic acid (2.5 mL). The flask was cooled in an ice bath and *m*-chloroperoxybenzoic acid (138 mg, 0.80 mmol, 3.5 equiv) was added portionwise. A safety shield was placed in front of the reaction flask. The oil bath temperature was raised and the reaction mixture was heated under reflux and stirred for 3 h at 120 ^oC. Acetic acid was evaporated under high vacuum at 60 ^oC and the resulting brown residue was stirred with diethyl ether (50 mL). The solid precipitate was filtered off and washed with excess ether and dried under reduced pressure to give the title compound **2.164** as light-yellow solid (0.43 mg, 81%). mp >220 ^oC (dec.); ν_{max} (KBr, cm^{-1}): 3422, 3090, 2506, 1682, 1475, 1434, 1352, 1329, 1304, 1208, 1049, 1023, 924, 841, 799, 752, 722, 673, 619, 518, 501; LRMS: found (MH^+) 235.47, MNa^+ 257.47, (MH^-) 233.20 $C_{10}H_{10}N_4O_3$ requires (MH^+) 235.08, (MNa^+) 257.06, (MH^-) 233.07.

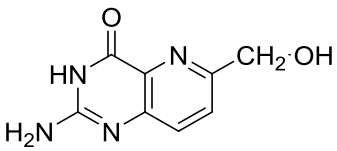
[2-(Acetylamino)-4-oxo-3,4-dihydropyrido[3,2-*d*]pyrimidin-6-yl]methyl acetate



2.165

N-(6-Methyl-5-oxido-4-oxo-3,4-dihydropyrido[3,2-*d*]pyrimidin-2-yl)acetamide **2.164** (36 mg, 0.15 mmol) in a mixture of glacial acetic acid (2.5 mL) and acetic anhydride (2 mL) was heated under reflux for 30 min. The light-brown solution was evaporated to dryness under high vacuum at 50 ^{o}C and the residue crystallized from methanol and diethyl ether to give the title compound **2.165** as a light-brown solid (34 mg, 80%). mp >250 ^{o}C (dec.); ν_{max} (KBr, cm^{-1}): 3422, 2924, 2612, 1695, 1638, 1482, 1384, 1236, 1128, 1046, 914, 825, 620; ^{1}H NMR (DMSO-d_6) δ_H 2.12 (3H, s, CH_3-CO-NH), 2.18 (3H, s, CH_3-CO_2-CH_2), 5.21 (2H, s, -CH_2-O-$COCH_3$), 7.74 (1H, d, J = 8.4, CH-CH-C-CH_2O), 7.89 (1H, d, J = 8.4, CH-CH-C-CH_2O), 11.69 (1H, s, -CO-NH-C-), 12.21 (1H, s, CH_3-CO-NH); LRMS: found (MH^+) 277.13, $MNa^{+)}$ 299.13, $C_{12}H_{12}N_4O_4$ requires (MH^+) 277.09, (MNa^+) 299.08; HRMS (ESI): found (MH^+) 277.0931, $C_{12}H_{12}N_4O_4$ requires (MH^+) 277.0937.

2-Amino-6-(hydroxymethyl)pyrido[3,2-*d*]pyrimidin-4(3*H*)-one

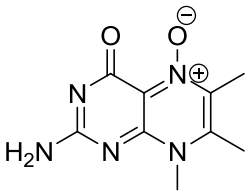


2.110

2-(Acetylamino)-4-oxo-3,4-dihydropyrido[3,2-*d*]pyrimidin-6-yl methyl acetate **2.165** (22 mg, 0.08 mmol) was added to 1% LiOH solution in methanol (10 mL) and stirred at room temperature for 12 h. The solvent was evaporated under reduced pressure and the residue was added into water (2 mL) and acidified with acetic acid. A solid precipitate was collected by filtration and dried under reduced pressure to give a light-brown solid (13 mg 84%) which was purified by HPLC (water/acetonitrile, flow rate = 6 mL/min, R_t = 5.77 min). The solvents were freeze dried to afford the title compound **2.110** as a light-brown solid (8 mg, 52

%). mp >320 °C (dec.); LRMS found (MH^+) 193.13, (MNa^+) 215.13, $C_8H_8N_4O_2$ requires (MH^+) 193.07, (MNa^+) 215.05, HRMS (ESI): found (MH^+) 193.0719, (MNa^+) 215.0536, $C_8H_8N_4O_2$ requires (MH^+) 193.0720, (MNa^+) 215.0539,

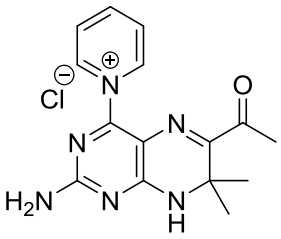
2-Amino-6,7,8-trimethyl-4(8*H*)-pteridinone 5-oxide



2.182

To a suspension of 2-amino-6,7,8-trimethyl-4(8*H*)-pteridinone **2.181** (100 mg, 0.49 mmol, 1 equiv.) in glacial acetic acid (29 mL) was added *m*-chloroperoxybenzoic acid (294 mg, 1.71 mmol, 3.5 equiv.) and reaction mixture was stirred at 122 °C for 3 h. Acetic acid was evaporated under reduced pressure and the yellow residue was stirred with ether (50 mL). The solid precipitate was filtered under reduced pressure to give a yellow solid which was purified by HPLC (water/acetonitrile, flow rate = 6 mL/min, R_t = 9.64 min). The solvent was removed by freeze drier to give the title compound **2.182** as a bright-yellow solid (108 mg, 52%). mp >240 °C (dec.); ν_{max} (KBr, cm^{-1}): 3424, 3145, 3035, 2945, 2476, 1719, 1688, 1628, 1599, 1512, 1484, 1461, 1407, 1377, 1259, 1203, 1136, 1043, 816, 771, 721, 569, 654, 600, 538, 517; ^{1}H NMR (DMSO-d_6) δ_H 2.18 (3H, s, *C*$\underline{H_3}$-C-N-O), 2.47 (3H, s, *C*$\underline{H_3}$-C-N-CH_3), 3.59 (3H, s, CH_3-C-N-*C*$\underline{H_3}$), 11.75, 12.04 (2H, 2s, NH_2); ^{13}C NMR (DMSO-d_6) δ_C 13.1 (*C*$\underline{H_3}$-C-N-O), 23.8 (*C*$\underline{H_3}$-C-N-CH_3), 28.9 (N-*C*$\underline{H_3}$), 116.9 (CH_3-*C*-N-O), 147.7 (CO-*C*=N-O), 148.1 (CH_3-*C*-N-CH_3), 149.3 (*C*-NH_2), 153.8 (N=*C*-N-CH_3), 173.5 (*C*=O); LRMS: found (MH^+) 222.07, (MNa^+) 244.00, $C_9H_{11}N_5O_2$ 111.04 requires (MH^+) 222.10, (MNa^+) 244.10; HRMS (ESI): found (MH^+) 222.0986, (MNa^+), 244.0806, ($2MNa^+$), 465.1720, $C_9H_{11}N_5O_2$ requires (MH^+) 222.1986, (MNa^+), 244.0805 ($2MNa^+$), 465.1718.

1-(6-acetyl-2-amino-7,7-dimethyl-7,8-dihydro-4-pteridinyl)pyridinium chloride

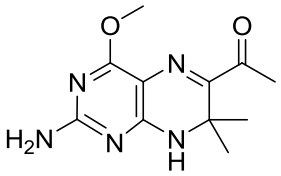


2.188

6-Acetyl-2-amino-7,7-dimethyl-7,8-dihydro-4(3*H*)-pteridinone **2.5** (50 mg, 0.21 mmol, 1 equiv.) was suspended in anhydrous pyridine (1 mL). The mixture was cooled to 0 ^{o}C in an ice bath. Trifluoroacetic anhydride (4.56 mL, 32.8 mmol, 150 equiv.) was added dropwise. After 1.5 h NaOMe/MeOH (0.08 mol/L, 0.432 mg/100mL, 63 mL) was added. The reaction mixture was stirred at room temperature overnight. The solvents were removed under reduced pressure. The crude yellow material was acidified with HCl (2N) and was purified by HPLC. With 90% water and 10% acetonitrile, flow rate 6m/min, the yellow product was eluted at 23.26 min, occupying area 72 %, starting material was eluted at 23.50 min, occupying area 7 %. The solvents were removed using the freeze-drier to give the title compound **2.188** as a yellow solid (50 mg, 79 %). mp >220 ^{o}C (dec.); ν_{max} (KBr, cm^{-1}) 3385, 3196, 1679, 1627, 1530, 1467, 1414, 1285, 1204, 1187, 1135, 1051, 833, 804, 782, 720, 613; ^{1}H NMR (DMSO-d_6) δ_H 1.55 (6H, s, -2×*CH*$_3$), 2.12 (3H, CH_3), 7.30-7.50 (2H, br, *NH*$_2$), 8.33, 127.6 (2H, t, *J* = 7.5, 2×*CH*-aromatic), 8.41 (1H, s, *NH*), 8.86-8.90, 144.9 (1H, t, *J* = 1.5, -*CH*-aromatic), 9.35-9.37,149.6 (2H, dd, *J*$_1$ = 1.0, *J*$_2$ = 1.0, 2×*CH*-aromatic); HSQC NMR showed following δ_H to δ_c relationship 1.55, 29.04 (6H, s, -2×*CH*$_3$), 2.12, 26.6 (3H, *CH*$_3$), 7.30-7.50 (2H, br, *NH*$_2$), 8.33 (2H, t, *J* = 7.5 , 2×*CH*-aromatic), 8.41 (1H, s, *NH*), 8.86-8.90 (1H, ttt, *J*$_1$ = 1.5, *J*$_2$ = 1.0, *J*$_3$ = 1.0, -*CH*-aromatic), 9.35-9.37 (2H, dd, *J*$_1$ = 1.0, *J*$_2$ = 1.0, 2×*CH*-aromatic); ^{13}C NMR (DMSO-d_6) δ_C 26.6 (2×*C*H$_3$), 29.0 (*C*H$_3$-C=O), 55.4 (NH-*C*-2CH$_3$), 106.6 (N-*C*-C-Py), 127.6 (2×*CH* aromatic), 150.0 (2×*CH*=N aromatic), 149.6 (*CH* aromatic), 154.7 (*C*-Py), 156.0 (CO-*C*=N), 156.3 (NH-*C*-N=) 163.0 (*C*-NH$_2$), 197.6 (*C*=O) ; ^{13}C NMR jmod (DMSO-d_6) δ_C 26.56 (2×*C*H$_3$) downward signal, 29.04 (*C*H$_3$-C=O) downward signal, 55.38 (NH-*C*-2CH$_3$) upward, 106.59 (N-*C*-C-Py) upward, 127.64 (2×*CH* aromatic) downward, 144.99 (2×*CH*=N aromatic) downward, 149.60 (*CH* aromatic) downward, 154.66 (*C*-Py) upward, 156.01 (CO-*C*=N) upward, 156.29 (NH-*C*-N=) upward, 162.97 (*C*-NH$_2$) upward, 197.60 (*C*=O) upward; LRMS: found (M$^+$) 297.13, $C_{15}H_{17}N_6O$+

requires (M^+) 297.15; HRMS (ESI): found (M^+) 297.1454, $C_{15}H_{17}N_6O+$ requires (M^+) 297.1458.

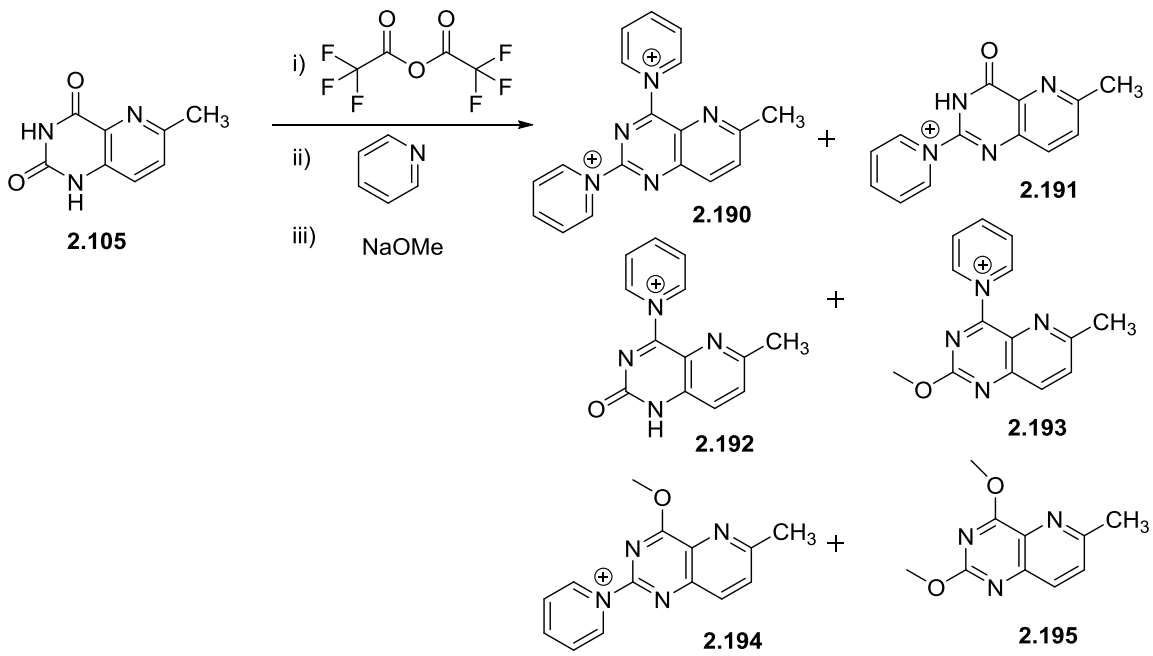
1-(2-amino-4-methoxy-7,7-dimethyl-7,8-dihydro-6 pteridinyl) ethanone



2.189

1-(6-Acetyl-2-amino-7,7-dimethyl-7,8-dihydro-4-pteridinyl)pyridinium **2.188** (30 mg, 0.10 mmol) was dissolved in dry methanol (30 mL) under nitrogen. Sodium methoxide (300 mg) was added and reaction mixture was stirred overnight. Methanol was removed under reduced pressure. The crude brown material was dissolved in a small volume of methanol and unreacted sodium methoxide was separated by filtration. It was purified by silica gel chromatography eluting with ethyl acetate and methanol (2:1). The solvents were evaporated to give a brown solid (26 mg). The brown material was dissolved in DMF and was acidified by adding HCl and purified by HPLC using 90% water and 10% acetonitrile, flow rate 6mL/min. The product was eluted at 25.0 min. The solvents were removed using the freeze-drier to give the title compound **2.189** as a yellow solid (15 mg, 60 %). mp >225 ^oC (dec.); ^1H NMR (DMSO-d_6) δ_H 1.44 (6H, s, -2×CH_3), 2.30 (3H, CH_3), 3.84 (3H, OCH_3), 6.50-6.90 (2H, br, NH_2), 7.50 (1H, s, NH); HSQC NMR showed following δ_H to δ_c relationship 1.44, 28.8 (6H, s, -2×CH_3), 2.30, 26.4, (3H, CH_3), 3.84, 53.7 (3H, OCH_3), 6.50-6.90 (2H, br, NH_2), 7.50 (1H, s, NH); LRMS: found (MH^+) 250.13, (MNa^+) 272.07, $C_{11}H_{15}N_5O_2$ requires (MH^+) 250.12, (MNa^+) 272.11; HRMS (ESI): found (MH^+) 250.1296, (MNa^+) 272.1116, $C_{11}H_{15}N_5O_2$ requires (MH^+) 250.1299, (MNa^+) 272.1118.

The nucleophilic substitution reaction at 2nd and 4th position of 6-methylpyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione 2.105

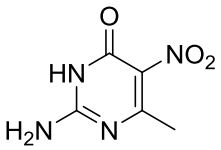


6-Methylpyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione **2.105** (1.00 g, 5.65 mmol, 1 equiv.) was suspended in anhydrous pyridine (8 mL). The mixture was cooled to 0 °C in an ice bath. Trifluoroacetic anhydride (30 mL, 216 mmol, 38.2 equiv.) was added dropwise. It was stirred at 0 °C for 1.5 h at 5 °C. A solution of sodium methoxide in methanol (0.148 mol/L, 2.0 g/250 mL, 250 mL) was added. The reaction mixture was stirred at room temperature overnight. The solvents were removed under reduced pressure. The crude material was stirred with methanol and allowed to stand for 20 min, a solid precipitate of starting material (90 mg) was separated by filtration, by adding diethyl ether a further three crops of solid precipitate were separated, from those three crops, two precipitate (201 mg) were from starting material and one was from pyridinium salt (0.294 g). The purple filtrate was purified by silica gel chromatography, eluting with methanol and ethyl acetate (9:1). The Product **2.193** or **2.194** which was substituted with methoxy group at C^2 or C^4 of the starting material **2.105** was eluted but product **2.191** or **2.192** was stuck on silica gel column. Sodium methoxide (550 mg was added in methanol (100 ml), eluted through column four times and was left in column for 48 h. The column was washed with methanol and solvents were evaporated under reduced pressure to give a dark-purple solid residue (850 mg) from which 50 mg was purified by HPLC (water (90%)/acetonitrile (10%), with 0.1% TFA, flow rate = 6 ml/min, R_t = 12.58 min, occupied area 59.25%). The solvents were removed using the freeze-drier to give the

substituted product as a dark-purple colour solid **2.193** or **2.194** (27 mg). mp > 250 ^{o}C; ν_{max} (KBr, cm^{-1}): 3421, 3118, 3062, 2957, 2925, 2855, 1688, 1617, 1595, 1581, 1532, 1493, 1358, 1286, 1203, 1022, 955, 904, 833, 799, 721, 670, 644, 568, 521; ^{1}H NMR (DMSO-d_6) δ_H 2.78 (3H, s, C$\underline{H_3}$), 4.41 (3H, s, OC$\underline{H_3}$), 8.04 (1H, d, J = 8.5, $C\underline{H}$-CH-C-CH$_3$), 8.38, (2H, t, J = 7.5 , 2×*CH*-aromatic), 8.43 (1H, d, J = 8.5, CH-$C\underline{H}$-C-CH$_3$), 8.93 (1H, t, J = 7.5, -*CH*-aromatic), 10.17 (2H, dd, J_1 = 1.0, J_2 = 1.0, 2×*CH*-aromatic); HSQC NMR showed following δ_H to δ_c 2.78, 24.8 (3H, s, C$\underline{H_3}$), 4.41, 56.2 (3H, s, OC$\underline{H_3}$), 8.04, 132.1 (1H, d, J = 8.5, $C\underline{H}$-CH-C-CH$_3$), 8.38, (2H, t, J = 7.5 , 2×*CH*-aromatic), 8.43, 136.4 (1H, d, J = 8.5, CH-$C\underline{H}$-C-CH$_3$), 8.93, 150.1 (1H, t, J = 7.5, -*CH*-aromatic), 10.18, 141.9 (2H, dd, J_1 = 1.0, J_2 = 1.0, 2×*CH*-aromatic); LRMS found (M$^+$) 253.13, $C_{14}H_{13}N_4O^+$ requires (M$^+$) 253.11, HRMS (ESI): found (M$^+$) 253.1083, $C_{14}H_{13}N_4O^+$ requires (M$^+$) 253.1084.

There were three other fractions eluted from HPLC, the first second major fraction was eluted at 6.11 min (water (90%)/acetonitrile (10%), with 0.1% TFA, flow rate = 6 ml/min, occupied area 27.99%). LRMS found (M$^+$) 239.10, $C_{13}H_{11}N_4O^+$ requires (M$^+$) 239.09 could be **2.191** or **2.192**. The second fraction was eluted at 8.61min (water (90%)/acetonitrile (10%), with 0.1% TFA, flow rate = 6 ml/min, occupied area 2.11%). LRMS found (M$^+$) 239.10, $C_{13}H_{11}N_4O^+$ requires (M$^+$) 239.09 could be **2.191** or **2.192**. The last fraction was eluted at 18.37 min (water (90%)/acetonitrile (10%), with 0.1% TFA, flow rate = 6 ml/min, occupied area 4.17%), LRMS found (M$^+$) 253.13, $C_{14}H_{13}N_4O^+$ requires (M$^+$) 253.11, could be **2.193** or **2.194.**

2-Amino-6-methyl-5-nitro-4(3*H*)-pyrimidinone

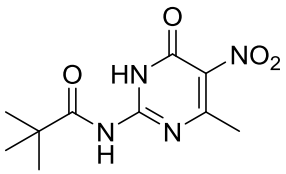


2.171

2-Amino-6-methyl-4(3*H*)-pyrimidinone **2.170** (2.00 g, 16.0 mmmol) was dissolved in concentrated sulphuric acid (6 mL) at 0 ^{o}C, it was further cooled at -5 to -10 ^{o}C and treated dropwise with stirring, with fuming nitric acid (6 mL). The resulting solution was stirred for

30 min at low temperature and then at room temperature for 2.5 h. The reaction was monitored by TLC and ^{1}H NMR. A few drops of the reaction mixture were taken out from the flask, added to cold diethyl ether (20 mL), stirred for 10 min and then left without stirring. The settled product was separated by filtration, and the solvent removed under reduced pressure to give a white solid (10 mg). In ^{1}H NMR, the proton signal at δ_H 5.40 (CH_3-C=*C<u>H</u>*) was completely lost which confirmed completion of reaction. The product was soluble in acidic water and therefore the work-up used organic solvents for precipitation. Diethyl ether (250 mL) was cooled to -20 ^oC in a 500 mL flask and reaction mixture was added dropwise into it while stirring. The solid precipitate was formed which was separated by filtration, added into cold methanol (50 mL) and stirred for 10 min. After standing overnight, the product precipitated, which was separated by filtration, washed with excess cold diethyl ether and cold water (10 mL) and dried under reduced pressure to give the title compound **2.171** as a white solid (2.53 g, 93%). mp > 250 ^oC; (lit.[143] 250 ^oC (dec.); ν_{max} (KBr, cm^{-1}): 3341, 2986, 1695, 1535, 1505, 1415, 1363, 1168, 1148, 1067, 1032, 1009, 972, 847, 778, 732, 667, 613; ^{1}H NMR (DMSO-d_6) δ_H 2.27 (3H, s, *CH_3*), 6.60-6.90 (2H, br, -*NH_2*), 11.61 (1H, s, *NH)*; ^{13}C NMR (DMSO-d_6) δ_c 22.0 (*CH3*), 128.3 (-*C*-NO_2), 154.7 (-*<u>C</u>*-CH_3), 155.0 (=*<u>C</u>*-NH_2), 164.0 (-*C*=O); LRMS found (MH^+) 171.00, (MNa^+) 193.00, $C_5H_6N_4O_3$ requires (MH^+) 171.05, (MNa^+) 193.03; HRMS (ESI): found (MH^+) 171.0510, $C_5H_6N_4O_3$ requires (MH^+) 171.0513.

2,2-Dimethyl-*N*-(4-methyl-5-nitro-6-oxo-1,6-dihydro-2-pyrimidinyl)propanamide[242]

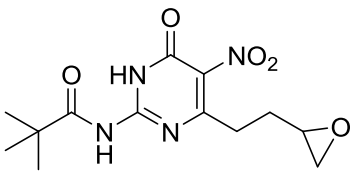


2.172

2-Amino-6-methyl-5-nitro-4(3*H*)-pyrimidinone **2.171** (1.50 g, 8.82 mmol, 1 equiv.) was dissolved in acetonitrile (15 mL) and pyridine (6 mL) by stirring, pivaloyl chloride (3.80 mL, 30.8 mmol, 3.5 equiv.) was also added under nitrogen atmosphere and the reaction mixture

was heated under reflux for 4 h. Volatile solvents were removed under reduced pressure to give a dark-brown residue. TLC showed two new spots less polar than starting material. The residue was stirred with water (50 mL) for 1 h; dark-brown coloured beads of solid material were formed which were separated by filtration. The HPLC showed two new peaks in the light- yellow water filtrate, a fraction eluted at R_t = 28.02 min (water (95%)/acetonitrile (5%), flow rate = 6 mL /min) showed LRMS (MH^+) 255.07, MNa^+) 277.00, $C_9H_{12}N_4O_4$ requires (MH^+) 255.11, MNa^+) 277.09 was suggested **2.175** Another fraction eluted at R_t = 31.07 min (water (95%)/acetonitrile (5%), flow rate = 6 mL /min) was from product which was left in the light- yellow water filtrate. The dark-brown coloured solid beads separated by filtration were stirred in methanol. The solid material was precipitated in methanol which was separated by filtration and dried under reduced pressure to give the title compound **2.172** as a brownish- yellow solid (1.60 g, 72%). mp 186-194 ^oC; (lit.[143] 211-214 ^oC); ν_{max} (KBr, cm^{-1}): 3243, 2981, 2938, 2876, 2631, 2451, 1670, 1608, 1562, 1442, 1386, 1366, 1229, 1146, 1103, 1012, 942, 918, 843, 819, 757, 696, 612; ^1H NMR (DMSO-d_6) δ_H 1.24 (9H, s, 3×*CH*$_3$), 2.35 (3H, s, *CH*$_3$), 11.61 (1H, s, -CO-*NH*), 12.52 (1H, s, $C(CH_3)$ -CO-*NH*); ^{13}C NMR (DMSO-d_6) δ_c 20.9 (*CH3*), 25.9 (3×*CH*$_3$), 40.2 ($(CH_3)_3$-*C*-CO), 133.4 (-*C*-NO_2), 151.2 (N-*C*-CH_3), 152.9 (N=*C*-NH), 161.4 (-*C*=O), 181.8 (NH-*CO*-C-$(CH_3)_3$); LRMS found (MH^+) 255.07, (MNa^+) 277.07, $C_{10}H_{14}N_4O_4$ requires (MH^+) 255.11, (MNa^+) 277.09; HRMS (FAB): found (MH^+) 255.1084, ($2MNa^+$) 531.1920, $C_{10}H_{14}N_4O_4$ requires (MH^+) 255.1088, ($2MNa^+$) 531.1922.

2,2-Dimethyl-*N*-{5-nitro-4-[2-(2-oxiranyl)ethyl]-6-oxo-1,6-dihydro-2-pyrimidinyl}propanamide[242]

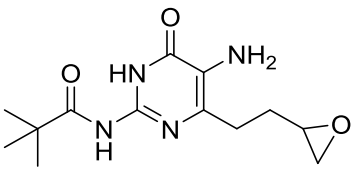


2.173

2,2-Dimethyl-*N*-(4-methyl-5-nitro-6-oxo-1,6-dihydro-2-pyrimidinyl)propanamide **2.172** (1.30 g, 5.11 mmol) was dissolved in dry ethanol (11 mL), ethanolic sodium ethoxide (5.5 mL, 1N) was also added and the reaction mixture was heated under reflux for 30 min. The solvent was removed under reduced pressure and the residue was added to anhydrous DMF

(9.4 mL). To this solution was added 3-chloro-1,2-epoxypropane (0.548 mL, 7.01 mmol) and KI (0.49 g, 2.95 mmols). The resulting solution was heated under reflux under nitrogen at 160 ^{o}C for 3 h. The solvents were removed under reduced pressure and the residue was suspended in water and extracted with ethyl acetate. The organic layers were pooled and solvent was evaporated under reduced pressure to give a yellow oily residue, which was dried under anhydrous calcium chloride and reduced pressure. It was purified by silica gel chromatography eluted with ethyl acetate and *n*-hexane (2:1). The product was eluted before impurities. The product was eluted from HPLC at R_t = 27.43 min (water (70%)/acetonitrile (30%), with 0.1 % TFA, flow rate = 6 mL/min). The solvents were evaporated to give the title compound **2.173** as yellow oil (0.496 g, 31%). ν_{max} (KBr, cm^{-1}): 2977, 2879, 2729, 1736, 1679, 1628, 1529, 1462, 1435, 1361, 1284, 1189, 1143, 1032, 962, 864, 830, 799, 775, 746, 723, 651, 588; ^{1}H NMR ($CDCl_3$) δ_H 1.20 (9H, s, 3×*CH*$_3$), 2.26-2.42 (2H, m, C=C-CH$_2$-*CH*$_2$), 3.63-3.74 (2H, m, C=C-*CH*$_2$-CH$_2$), 4.07-4.63 (3H, m, *CH*O*CH*$_2$), 9.60-10.8 (2H, br, 2×*NH*); ^{13}C NMR (DMSO-d_6) δ_c 20.3 (C=C-*CH*$_2$), 26.9 (3×*CH*$_3$), 38.9 (C=C-CH$_2$-*CH*$_2$), 45.8 ((CH$_3$)$_3$-*C*-), 53.0 (-*CH*$_2$-O-), 64.5 (-*CH*-O-), 130.5 (-*C*-NO$_2$), 152.7 (N=*C*-NH), 155.4 (N-*C*-CH$_2$-), 160.5 (-*C*=O), 178.0 (NH-*CO*-C-(CH$_3$)$_3$); LRMS found (MH$^+$) 311.07, (MNa$^+$) 333.07, $C_{13}H_{18}N_4O_5$ requires (MH$^+$) 311.14, (MNa$^+$) 333.12; HRMS (ESI): found (MH$^+$) 311.1343, (2MH$^+$) 621.2628, $C_{13}H_{18}N_4O_5$ requires (MH$^+$) 311.1350, (2MH$^+$) 621.2627.

_N_-{5-Amino-4-[2-(2-oxiranyl)ethyl]-6-oxo-1,6-dihydro-2-pyrimidinyl}-2,2-dimethylpropanamide[242]

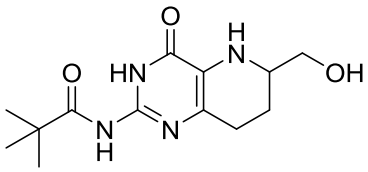


2.176

2,2-Dimethyl-*N*-{5-nitro-4-[2-(2-oxiranyl)ethyl]-6-oxo-1,6-dihydro-2-pyrimidinyl}propanamide **2.173** (280 mg, 0.902 mmol,) was dissolved in 95% ethanol (15 mL), sodium dithionite (1.68 g, 9.65 mmol,) was added and reaction mixture was heated at 80

°C for 7 h. It was cooled to room temperature and filtered. The filtrate was evaporated under reduced pressure and the brownish-yellow residue was purified by silica gel chromatography eluted with ethyl acetate and *n*-hexane (4:1). The starting material was eluted before product, when all starting material was eluted (39 mg) and product started to elute, the solvent system was changed to ethyl acetate and methanol (1:1). The product was further purified by HPLC (water (70%)/acetonitrile (30%), with 0.1 % TFA, flow rate = 6 mL/min, R_t = 23.80 min). The starting material was eluted from HPLC (water (70%)/acetonitrile (30%), with 0.1 % TFA, flow rate = 6 mL/min, R_t = 27.36 min). The solvents were removed using the freeze-drier to afford title compound **2.176** as a yellow oil. ν_{max} (KBr, cm^{-1}): ν_{max} (KBr, cm^{-1}): 3434, 2980, 1722, 1656, 1608, 1482, 1399, 1224, 1111, 1017, 923, 791, 627, 588; ^{1}H NMR ($CDCl_3$) δ_H 1.17 (9H, s, $(CH_3)_3$), 2.10-2.40 (2H, m, C=C-CH_2-CH_2), 3.62-3.71 (2H, m, C=C-CH_2-CH_2), 4.10-4.62 (3H, m, $CHOCH_2$), 5.46-5.49 (2H, br, NH_2), 11.20-11.40 (2H, br, 2×*NH*); LRMS found (MH^+) 281.13, MNa^+) 303.07, $C_{13}H_{20}N_4O_3$ requires (MH^+) 281.16, MNa^+) 303.14; HRMS (ESI): found (MH^+) 281.1600, (2×MH^+) 561.3134, $C_{13}H_{20}N_4O_3$ requires (MH^+) 281.1608, (2×MH^+) 561.3144.

***N*-[6-(Hydroxymethyl)-4-oxo-3,4,5,6,7,8-hexahydropyrido[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide**

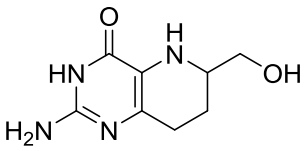


2.177

N-{5-Amino-4-[2-(2-oxiranyl)ethyl]-6-oxo-1,6-dihydro-2-pyrimidinyl}-2,2-dimethylpropanamide **2.176** (2.00 g, 7.13 mmol) was dissolved in dry dichloromethane (166 mL) in a dry and nitrogen flushed flask, boron trifluoride diethyl etherate (916 µl, 7.16 mmol) was added dropwise. The aluminum foil was wrapped around the flask and reaction

mixture was stirred for 16 h under nitrogen at room temperature. It was quenched by adding water, the solvents were evaporated under reduced pressure and residue was purified by silica gel chromatography eluted with ethyl acetate and methanol (9:1). Out of 115 fractions, the fraction ≠ 6-15 proved to nitro material **2.246,** fraction ≠ 16-37 was starting material **2.247** and fraction ≠ 39-115 showed required product **2.177** was further purified by HPLC (water (90%)/acetonitrile (10%), with 0.1 % TFA, flow rate = 6 ml/min, R_t = 10.00 min, occupied area 57.33 %). The solvents were removed using the freeze-drier to give the title compound **2.177** as a white solid (981 mg, 49%). mp 174-176 ^oC; ν_{max} (KBr, cm^{-1}): 3421, 3128, 2979, 2091, 1732, 1714, 1669, 1525, 1440, 1400, 1334, 1286, 1186, 1162, 1083, 921, 844, 814, 760, 725, 674; ^1H NMR ($CDCl_3$) δ_H 1.10 (9H, s, $(CH_3)_3$), 2.09-2.12 (2H, m, CH_2-$C\underline{H_2}$-CH-NH), 3.42-3.49 (2H, m, $C\underline{H_2}$-CH_2-CH-NH), 3.41-3.60 (1H, br), 3.42-3.49 (1H, m, $C\underline{H}$-NH), 4.01-4.11 (2H, m, $C\underline{H_2}$-OH), 5.00-5.20 (1H, br), 8.50-8.80 (2H, br, NH_2), 11.30-11.70 (2H, br, 2×*NH*); ^{13}C NMR ($CDCl_3$) δ_c 14.5 ($\underline{C}H_2$-CH-NH), 26.9 (3×$\underline{C}H_3$), 38.9 (C=C-$C\underline{H_2}$-CH_2), 46.1 ($(CH_3)_3$-$\underline{C}$-), 53.6 (CH_2-$\underline{C}H$-NH), 64.5 ($\underline{C}H_2$-OH), 121.9 (C=$\underline{C}$-CH_2-CH_2), 127.0 (C=$\underline{C}$-NH-CH), 150.1 (N=$\underline{C}$-NH), 155.5 (-*C*=O), 177.8 (NH-$\underline{C}O$-C-$(CH_3)_3$); LRMS found (MH^+) 281.13, (MNa^+) 303.07, $C_{13}H_{20}N_4O_3$ requires, (MH^+) 281.16, (MNa^+) 303.14; HRMS (ESI): found (MH^+) 281.1604, ($2MH^+$) 561.3142 $C_{13}H_{20}N_4O_3$ requires (MH^+) 281.1608, ($2MH^+$) 561.3144.

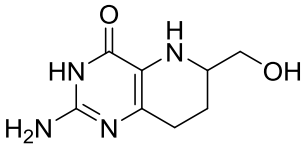
There were two other peaks from HPLC except product. The first peak proved to be **2.168** (water (90%)/acetonitrile (10%), with 0.1 % TFA, flow rate = 6 ml/min, R_t = 2.51 min, occupied area 12.07 %). The solvents were removed using the freeze-drier to give **2.168** as colourless semisolid (142 mg). ^1H NMR ((DMSO-d_6) δ_H 2.10-2.20 (2H, m, CH_2-$C\underline{H_2}$-CH-NH), 3.46-3.52 (2H, m, $C\underline{H_2}$-CH_2-CH-NH), 3.88-3.92 (1H, m, $C\underline{H}$-NH), 3.64-3.71 (IH, br, OH), 4.08-4.30 (2H, m, $C\underline{H_2}$-OH), 5.00-5.20 (1H, br), 8.40-8.70 (2H, br, NH_2); HSQC NMR showed following δ_H to δ_c relationship 2.28-2.43, 18.6 (2H, m, CH_2-$C\underline{H_2}$-CH-NH), 3.40-3.45, 45.1 (2H, m, $C\underline{H_2}$-CH_2-CH-NH), 3.72-3.77, 54.2 (1H, m, $C\underline{H}$-NH), 4.08-4.30, 62.5 (2H, m, $C\underline{H_2}$-OH); LRMS found (MH^+) 197.13, (MNa^+) 219.00, $C_8H_{12}N_4O_2$ requires (MH^+) 197.10, (MNa^+) 219.09.



2.168

The second peak was from starting material **2.176** (water (90%)/acetonitrile (10%), with 0.1 % TFA, flow rate = 6 ml/min, R_t = 22.33 min, occupied area 30.60 %). The solvents were removed using the freeze-drier to give the starting material **2.176** as a brownish-yellow solid (543 mg). ^{1}H NMR ($CDCl_3$) δ_H 1.17 (9H, s, $(CH_3)_3$), 2.10-2.40 (2H, m, C=C-CH_2-CH_2), 3.62-3.71 (2H, m, C=C-CH_2-CH_2), 4.10-4.62 (3H, m, $CHOCH_2$), 5.46-5.49 (1H, br, *NH*), 11.20-11.40 (2H, br, 2×*NH*); LRMS found (MH^+) 281.13, (MNa^+) 303.07, $C_{13}H_{20}N_4O_3$ requires, (MH^+) 281.16, (MNa^+) 303.14.

2-Amino-6-(hydroxymethyl)-5,6,7,8-tetrahydropyrido[3,2-*d*]pyrimidin-4(3*H*)-one

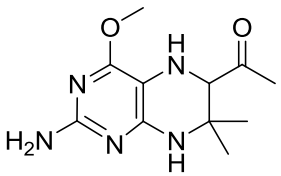


2.168

N-[6-(hydroxymethyl)-4-oxo-3,4,5,6,7,8-hexahydropyrido[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide **2.177** (45 mg, 0.16 mmol) was dissolved in LiOH solution (0.01%, 10 mL), aluminium foil was wrapped around the flask and reaction mixture was stirred under nitrogen for 12 h. The mixture was slightly acidified with acetic acid, the solvents were evaporated under reduced pressure and the residue was purified by HPLC (water (99%)/acetonitrile (1%), with 0.1 % TFA, flow rate = 6 ml/min, R_t = 5.08 min, occupied area 98 %). The solvents were removed using the freeze-drier to give the title compound **2.168** as a colourless oil (28 mg, 89 %). ^{1}H NMR ((DMSO-d_6) δ_H 2.09-2.23 (2H, m, CH_2-CH_2-CH-NH), 3.42-3.49 (2H, m, CH_2-CH_2-CH-NH), 3.87-3.90 (1H, m, *CH*-NH), 3.64-3.71 (IH, br, OH), 4.03-4.11 (2H, m, CH_2-OH), 5.00-5.20 (1H, br), 8.40-8.70 (2H, br, NH_2); HSQC NMR showed following δ_H to δ_C relationship 2.29-2.44, 18.6 (2H, m, CH_2-CH_2-CH-NH), 3.40-3.45,

45.1 (2H, m, *C*$\underline{H_2}$-CH_2-CH-NH), 3.72-3.77, 54.2 (1H, m, *C*$\underline{H}$-NH), 4.08-4.30, 62.5 (2H, m, *C*$\underline{H_2}$-OH), ^{13}C NMR ($CDCl_3$) δ_c 19.07 (CH_2-*C*$\underline{H_2}$-CH-NH), 45.58 (*C*$\underline{H_2}$-CH_2-CH-NH), 54.75 (*C*$\underline{H}$-NH), 63.01 (*C*$\underline{H_2}$-OH), 119.5 (C=*C*-CH_2-CH_2), 125.01 (C=*C*-NH-CH), 154.23 (N=*C*-NH_2), 157.05 (-*C*=O); LRMS found (MH^+) 197.13, (MNa^+) 219.13, $C_8H_{12}N_4O_2$ requires (MH^+) 197.10, (MNa^+) 219.09, HRMS (ESI): found (MH^+) 197.1029, $C_8H_{12}N_4O_2$ requires (MH^+)197.1033

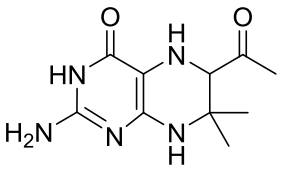
1-(2-Amino-4-methoxy-7,7-dimethyl-5,6,7,8-tetrahydropteridin-6-yl)ethanone



2.196

1-(2-Amino-4-methoxy-7,7-dimethyl-7,8-dihydro-6-pteridinyl)ethanone **2.189** (15 mg, 0.06 mmol) was dissolved in methanol (10 ml), cooled to 0 ^oC, 10 % Pd/C (15 mg) was added in portions, stirred for 10 min, and hydrogenated overnight. The solution was filtered through a pad of silica gel under suction through a glass sintered funnel and Pd/C was separated. The solvent was evaporated under reduced pressure and the residue was purified by HPLC. The product was eluted at R_t = 4.27 min (water 70%/acetonitrile 30%, with 0.1% TFA, flow rate = 6 ml/min, occupied area 34%). The solvents were removed using the freeze-drier to give the title compound **2.196** as a light-yellow solid (5 mg, 33%). LRMS: found (MH^+) 252.00, (MNa^+) 274.00 $C_{11}H_{17}N_5O_2$ requires (MH^+) 252.15, (MNa^+) 274.13.

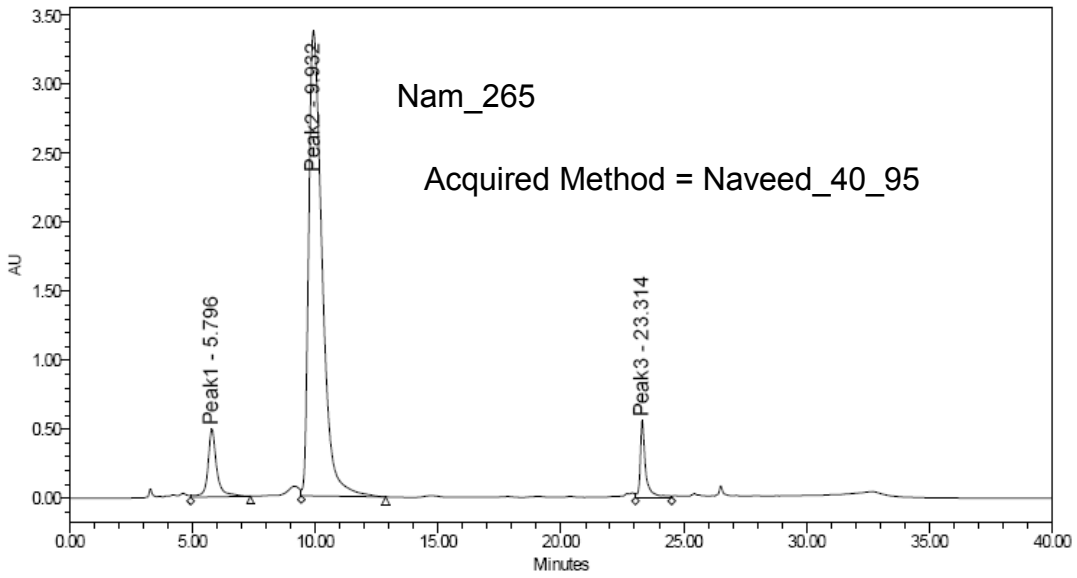
6-Acetyl-2-amino-5,6,7,8-tetrahydro-7,7-dimethylpteridin-4(3*H*)-one



2.197

6-Acetyl-2-amino-7,8-dihydro-7,7-dimethylpteridine-4(3*H*)one **2.5** (30 mg, 0.15 mmol) was dissolved in methanol (10 ml) and cooled to 0 °C. 10 % Pd/C (30 mg) was added in portions while stirring and hydrogenated for 12 h. Pd/C was separated by filtering the solution through a pad of kieselghur under suction through a glass sintered funnel. The solvent was evaporated under reduced pressure at room temperature and residue was purified by HPLC (water 95%/acetonitrile 5%, with 0.1% TFA, flow rate = 6 ml/min, R_t = 9.93 min occupied area 86.15%). The solvents were removed using the freeze-drier to give the title compound **2.197** as a white solid (19 mg, 54%); mp >145 °C (dec.); ^{1}H NMR (DMSO-d_6) δ_H 1.30 (3H, s, >C(C*H*$_3$)$_2$), 1.32 (3H, s, >C(C*H*$_3$)$_2$), 2.20 (3H, s, *CH*$_3$), 3.45-3.65 (1H, br), 4.47-4.49 (1H, m, -NH-*CH*-CO-), 6.53 (2H, br, N*H*$_2$), 7.29 (1H, br, N*H*), 10.28 (1H, br, N*H*); LRMS: found (MH$^+$) 238.00, (MNa$^+$) 260.07 $C_{10}H_{15}N_5O_2$ requires (MH$^+$) 238.13, (MNa$^+$) 260.11.

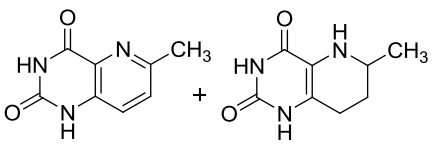
The starting material **2.5** eluted at 23.31 min (water 95%/acetonitrile 5%, with 0.1% TFA, flow rate = 6 ml/min, occupied area 4.58%). LRMS: found (MH$^+$) 236.07 $C_{10}H_{13}N_5O_2$ requires (MH$^+$) 236.11.



Time (min)	Water (%)	Acetonitrile (%)
00.00	95	5
12.00	95	5
18.00	50	50
25.00	50	50
30.00	50	50
35.00	95	5
40.00	95	5

HPLC Procedure for purification of 2.197

6-Methyl-5,6,7,8-tetrahydropyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione A



2.105 **2.198**

5-Amino-2,4(1*H*,3*H*)-pyrimidinedione **2.102** (15.0 g, 0.118 mol, 1 equiv), hydrochloric acid (243 mL, 20%), and crotonaldehyde (14.6 ml, 1.77 mol, 1.5 equiv) were heated together under reflux at 120 ^{o}C for 2 h. The solution was evaporated to dryness and the residue was

dissolved in methanol (150 ml) and kept overnight. It was then heated up to 50 ^{o}C and filtered immediately, the impurities were separated. The volume of the filtrate was reduced and a solid was precipitated, which was separated by filtration and washed with methanol (50 mL), dried under reduced pressure to give a cream-coloured solid **2.105** (5.13 g). The volume of filtrate was reduced by evaporation and a second crop of **2.105** (6.26 g) was obtained after washing with methanol. The third crop of **2.105** (1.95 g) was obtained by reducing the volume of methanol by evaporation. By further evaporation of solvents and by adding some diethyl ether, the product **2.198** was precipitated, which was filtered and dried under reduced pressure to give the side product, the title compound **2.198** as a white solid (102 mg, 0.48%). mp >220 ^{o}C (decomp.). ν_{max} (KBr, cm^{-1}): 3210, 3002, 2900, 2789, 2685, 2627, 2479, 2408, 2074, 2006, 1924, 1895, 1735, 1678, 1547, 1514, 1451, 1432, 1424, 1407, 1397, 1370, 1356, 1313, 1280, 1236, 1220, 1156, 1121, 1106, 1055, 1027, 974, 962, 954, 846, 826, 788, 755,667. 643; ^{1}H NMR (CD_3OD) δ_H 1.48 (3H, d, J = 6.56, CH-*C*$\underline{H_3}$), 1.81-1.91 (1H, m, CH_2-*C*$\underline{H_2}$-CH-CH_3), 2.18-2.24 (1H, m, CH_2-*C*$\underline{H_2}$-CH-CH_3), 2.61-2.67 (1H, m, *C*$\underline{H_2}$-CH_2-CH-CH_3), 2.72-2.81 (1H, m, *C*$\underline{H_2}$-CH_2-CH-CH_3), 3.36 (1H, br), 3.47-3.56 (1H, m, CH_2-CH_2-*C*$\underline{H}$-CH_3) 11.58 (1H, s, CO-*N*$\underline{H}$-C), 11.64 (1H, s, CO-*N*$\underline{H}$-CO); ^{13}C NMR (CD_3OD) δ_C 16.1 (CH-$\underline{C}$*H*$_3$), 22.0 ($\underline{C}$*H*$_2$-CH_2-CH-CH_3), 24.9 (CH_2-$\underline{C}$*H*$_2$-CH-CH_3), 50.1 (CH_2-CH_2-*C*$\underline{H}$-CH_3) 104.7 (C=$\underline{C}$-CO), 148.2 (C=$\underline{C}$-NH), 149.7 (NH-$\underline{C}$*O*-NH), 159.3 (NH-$\underline{C}$O); LRMS: found (MH^+) 182.07, (MNa^+) 203.93, $C_8H_{11}N_3O_2$ requires (MH^+) 181.09, (MNa^+) 204.07; HRMS (ESI): found (MH^+) 182.0924, ($2MH^+$) 363.1785 $C_8H_{11}N_3O_2$ requires (MH^+) 182.0924, ($2MH^+$) 363.1775.

References

(1) L. J. Ignarro, *Hipertensión,* **1990**, *16*, 477-483.

(2) F. Murad, *Angew. Chem. Int. Ed*., **1999**, *38*, 1856-1868

(3) R. Sorelle, *Circulation*, **1998**, *98*, 2365-2366.

(4 W. D. A. Smith, *Br. J. Anaesth*., **1965**, *37*, 790-798.

(5) H. Date, A. N. Triantafillou, E. P. Trulock, M. S. Pohl, J. D. Cooper and G. A. Patterson, *J. Thorac. Cardiovasc. Surg.,* **1996**, *111*, 913-919.

(6) Okamoto, Masumi, K. Tsuchiya, Y. Kanematsu, Y. Izawa, M. Yoshizumi, S. Kagawa, and Toshiaki, *Am. J. Physiol. Renal. Physiol.,* **2005**, *288*, 182-187.

(7) L. J. Ignarro, *Proc. Natl. Acad. Sci. USA.,* **2002**, *99,* 7816-7817.

(8) J. C. Liao and L. Kuo, *Am. J. Physiol*., **1997**, *272,* H1571-H1581.

(9) E. Braunwald, *Circ Res*., **2010**, *107*, 3-5.

(9) A. J. M. Cornelissen, *Med. Biol. Eng. Comput*., **2011**, *49*, 627-629.

(10) J. M. Barbaree and W. J. Payne, *Marine Biol*., **1967**, *1*, 136-139.

(11) P. Vallance and I Charles*, Gut*, **1998**, *42*, 313-314.

(12) R. S. Dykhuizen, A. Fraser, H. McKenzie, M. Golden, C. Leifert and N Benjamin, *Gut,* **1998**, *42*, 334-337.

(13) S. Habib and A. Ali, *Ind. J. Clin. Biochem*., *2011, 26*, 3-17.

(14) A. J. Gow*, Proc. Am. Thorac. Soc.,* **2006***, 3,* 150-152.

(15) W. P. Arnold, C. K. Mittal, S. Katsuki and F. Murad, *Proc. Natl. Acad. Sci. USA.,* **1977**, *74***,** 3203-3207.

(16) R. F. Furchgott and J. V. Zawadzki, *Nature,* **1980**, *288***,** 373-376.

(17) L. J. Ignarro, G.M. Buga, K. S. Wood, R. E. Byrns and G. Chaudhuri, *Proc. Natl. Acad. Sci. USA.,* 1**987**, *84*, 9265-9269.

(18) P. Klatt, K. Schmidt, G. Uray and B. Mayer, *J. Biol. Chem.,* **1993**, *268*, 14781-14787.

(19) H. G. Korth, R. Sustmann, C. Thater, A. R. Butler and K. U. Ingold, *J. Biol. Chem.,* **1994**, *269*, 17776-17779.

(20) M. N. Hughes, H. G. Nicklin and W. A. C. Sackrule, *J. Chem. Soc. (A)*, **1971**, *23*, 3722-3725.

(21) D. D. Thomas, L. A. Ridnour, J. S. Isenberg, W. F. Santana, C. H. Switzer, S. Donzellie, P. Hussain, C. Vecoli, N. Paolocci, S. Ambs, C. Colton, C. Harris, D. D. Roberts and D. A. Wink, *Free Radic. Biol. Med*., **2008**, *45*, 18-31.

(22) A. Carr and B. Frei, *Free Radic. Biol. Med*., **2000**, *28*, 1806-1814.

(23) D. A. Wink and J. B. Mitchell, *Free Radic. Biol. Med*., **1998**, *25*, 434-456.

(24) J.G. Andrew, P.L. Benjamin, R.P. John, J.S. David and S.S. Jonathan, *Proc. Natl. Acad. Sci. USA* (*Biochemistry*), **1999**, *96*, 9027-9032.

(25) R. G. Knowles and S. Moncada, *Biochem. J.,* **1994**, *298*, 249-258.

(26) H. H. H. W. Schmidt, J. S. Pollock, M. Nakane, L. D. Gorsky, U. Forstermann and F. Murad, *Proc. Natl. Acad. Sci. USA*., **1991**, *88*, 365-369.

(27) A. K. Nussler and T. R. Billiar, *J. Leukoc. Biol.,* **1993**, *54*, 171-178.

(28) U. Forstermann, J. S. Pollock, H. H. Schmidt, M. Heller and F. Murad, *Proc. Natl. Acad. Sci. U.S.A.,* **1991**, *88*, 1788-1792.

(29) A Pautz, J. Art, S. Hahn, S. Nowag, C. Voss and H Kleinert, *Nitric Oxide*, **2002**, *23*, 75–93.

(30) U. Forstermann and H. Kleinert, *N-S. Arch. Pharmacol*., **1995**, *352*, 351-364.

(31) U. Forstermann, J. Boissel, and H. Kleinert*, J. Fed. Am. Soc. Exp. Bio.,* **1998**, *12*, 773-790.

(32) C. Iadecola, F. Zhang, R. Casey, M. Nagayama and M. E. Ross, *J. Neurosci*., **1997**, *17*, 9157-9164.

(33) G. Kolios, N. Rooney, C. T. Murphy, D. A. F. Robertson and J. Westwick, *Gut*, **1998**, *43,* 56-63.

(34) A. Perner, L. Andresen, M. Normark, B. Fischer-Hansen, S. Sorensen, J. Eugen-Olsen and J. Rask-Madsen, *Gut,* **2001**, *49*, 387-394.

(35) S. Engeli, J. Janke, K. Gorzelniak, J. Bohnke, N. Ghose, C. Lindschau, F.C. Luft and A. M. Sharma, *J. Lipid. Res.,* **2004**, *45*, 1640-1648.

(36) U. Forstermann and W. C. Sessa, *Eur. Heart J*., **2012**, *33*, 829–837.

(37) H. M. Abu-Soud, L. L. Yoho and D. J. Stuehr, *J. Biol. Chem*., **1994**, *269*, 32047-32050.

(38) D. J. Stuehr, *Biochimica et Biophysica Acta,* **1999,** *1411,* 217-230.

(39) C.C. Wei, Z. Q.Wang, C. Hemann, R. Hille and D. J. Stuehr, *J. Biol. Chem*., **2003**, *278*, 46668-46673.

(40) A. P. Ledbetter, K. McMillan, L. J. Roman, B. S. Masters, J. H. Dawson and M. Sono, *Biochemistry,* **1999**, *38*, 8014-8021.

(41) C.C. Wei, Z. Q.Wang, C. Hemann, R. Hille, E. D. Getzoff and D. J. Stuehr, *Biochemistry*, **2003**, *42*, 1969-1977.

(42) K. M. Rusche, M. M. Spiering and M. A. Marletta, *Biochemistry,* **1998**, *37*, 15503-15512.

(43) A. R. Hurshman, C. Krebs, D. E. Edmondson and M. A. Marletta, *Biochemistry,* **2003**, *42*, 13287-13303.

(44) D. J. Stuehr, J. Santolini, Z. Q. Wang, C.C. Wei and S. Adak, *J. Biol. Chem.,* **2004**, *279,* 36167-36170.

(45) C. Wei, R. B. Crane and D. J. Stuehr, *Chem. Rev*., **2003**, *103*, 2365-2383.

(46) K. Panda, S. Ghosh and D. J. Stuehr, *J. Biol. Chem.,* **2001**, *276*, 23349-23356.

(47) C. S. Raman, H. Li, P. Martasek, V. Kral, B. S. S. Masters and T. L. Poulos, *Cell*, **1998**, *95*, 939-950.

(48) W. K. Alderton, C. E. Cooper and R. G. Knowles, *Biochem. J.,* **2001**, *357*, 593-615.

(49) M. A. Tayeh and M. A. Marletta, *J. Biol. Chem.,* **1989**, *264,* 19654-19658.

(50) A. R. Hurshman, C. Krebs, D. E. Edmondson, B. H. Huynh and M. A. Marletta, *Biochemistry,* **1999**, *38,* 15689–15696.

(51) M. Sorlie, A. C. F. Gorren, S. Marchal, T. Shimizu, R. Lange, K. K. Andersson and B. Mayer, *J. Biol. Chem.,* **2003**, *278,* 48602-48610.

(52) I. F. Sevrioukova, H. Li, H. Zhang, J. A. Peterson and T. L. Poulos, *Proc. Natl. Acad. Sci. USA.,* **1999**, *96*, 1863-1868.

(53) T. L. Poulos, *Drug. Metab. Dispos.,* **2005**, *33*, 10-18.

(54) N. M. Pandya, N. S. Dhalla and D. D. Santani, *Vasc. Pharmacol*., **2006,** *44,* 265-274.

(55) D. Kumar, *Proc. Natl. Acad. Sci. USA.,* **2008**, *105,* 7540-7545.

(56) J. P. Cooke and V. J. Dzau, *Annu. Rev. Med.*, **1997**, *48,* 489-509.

(57) T. Nakaki, M. Nakayama and R. Kato, *Eur. J. Pharmacol.,* **1990**, *189***,** 347- 353.

(58) J. A. Berlin and G. A. Colditz, *Am. J. Epidemiol.,* 1990, *132*, 612-628.

(59) L. Jungersten, A. Ambring, B. Wall and A. Wennmalm, *Appl. Physiol.*, **1997**, *82*, 760-764.

(60) S. Moncada, R. M. J. Palmer and E. A. Higgs, *Pharmacol. Rev.,* **1991**, *43*, 109-142.

(61) D. C. Bredt, C. E. Glatt, P. M. Hwang, M. Fothui, D. L. Dawson and S. H. Synder, *Neuron,* **1991**, *7*, 615–624.

(62) L. Sulz, G. Astorga, B. Bellette, R. Iturriaga, A. Mackay-Sim and J. Bacigalupo, *Nitric Oxide*, **2009**, *20*, 238-252.

(63) J. L. Vincent, H. Zhang , C. Szabo and J. C. Preiser, *Am. J. Respir. Crit. Care Med.*, **2000**, *161*, 1781–1785.

(64) C. J. Lowenstein, J. L. Dinerman and S. H. Snyder, *Ann. Intern. Med*., **1994**, *120*, 227-237.

(65) P. Vallance, N. Benjamin and J. Collier, *Eur. J. Clin. Pharmacol*., **1992**, *42*, 37-41.

(66) O. V. Evgenov and L. J. Bjertnaes, *Crit. Care Med*., **2003**, *31,* 1601–1602.

(67) H. Wiseman and B. Halliwell, *Biochem. J.,* **1996,** *313,* 17-29.

(68) R. D. Hurt and J. O. Ebbert, *Clin. Chest. Med.,* **2002**, *23*, 27-36.

(69) K. Xie and S. Huang, *Free Radic. Biol. Med.,* **2003**, *34*, 69-986.

(70) D. Adlam, J. K. Bendall, J. P. De Bono, N. J. Alp, J. Khoo, T. Nicoli, M. Yokoyama, S. Kawashima and K. M. Channon, *Exp. Physiol.,* **2006**, *92*, 119-126.

(71) T. S. Schmidt and N. J. Alp, *Clinical Science*, **2007**, *113*, 47-63.

(72) J. Mark, Crabtree, L. Caroline, Smith, G. Lam, S. Michael. Goligorsky and S. S. Gross, *Am. J. Physiol. Heart. Circ. Physiol.,* **2008**, *294*, 1530-1540.

(73) M. J. Crabtree, A. B. Hale and K. M. Channonin, *Free Radic. Biol. Med*., **2011**, *50*, 1639-1646.

(74) M. J. Crabtree, A. L. Tatham, Y. Al-Wakeel, N. Warrick, A. B. Hale, S. Cai, K. M. Channon and N. J. Alp, *J. Biol. Chem.,* **2009***, 284,* 1136-1144.

(75) W. Kaim, B. Schwederski, O. Heilmann and F. M. Hornung, *Coordination Chemistry Reviews,* **1999**, *182,* 323-342.

(76) J. A. Nicholas and K. M. Channon, *Arterioscler. Thromb. Vasc. Biol.,* **2004,** *24***,** 413-420.

(77) S. Umar, A. van der Laarse, *Mol. Cell Biochem*., **2010**, *333*, 191-201.

(78) S. Mattapally and S. K. Banerjee, *Institute of Integrative Omics and Applied Biotechnology Journal (IIOAB)*., **2011**, *2*, 29-38.

(79) T. C. Bellamy, C. Griffiths and J. Garthwaite, *J. Biol. Chem*., **2002**, *277*, 31801-31807.

(80) J. R. Lancaster, *Chem. Res. Toxicol.,* **2006**, *19*, 1160-1174.

(81) G. C. Brown, *Nitric Oxide*, **2010**, 23, 153–165.

(82) P. M. Schwarz, H. Kleinert and U. Forstermann, *Arterioscler. Thromb. Vasc. Biol*., **1999**, *19***,** 2584-2590.

(83) D. S. Bredt and S. H. Snyder, *Proc. Natl. Acad. Sci. U. S. A.,* **1990**, *87,* 682-685.

(84) H. J. Cho, Q. W. Xie, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee and C. Nathan, *J. Exp. Med.,* **1992**, *176*, 599-604.

(85) D. C. Bredt, C. E. Glatt, P. M. Hwang, M. Fothui, D. L. Dawson and S. H. Synder, *Neuron,* **1991**, *351*, 714-718.

(86) H. Drexler, *Circulation*, **1999**, *99*, 2972-2975.

(87) J. Zhang, P. Martàsek, R. Paschke, T. Shea, B. S. S. Masters and J. P. Kim, *J. Biol. Chem.,* **2001**, *276*, 37506-37513.

(88) J. Kraulis, *J. Appl. Crystallogr.,* **1991**, *24***,** 946-950.

(89) E. A. Merritt and M. E. P. Murphy, *Acta. Crystallogr.,* **1994**, *50***,** 869-873.

(90) R.T. Miller, P. Martasek, C. S. Raman and B. S. S. Masters, *J. Biol. Chem*., **1999**, *274*, 14537-14540.

(91) B. R. Crane, J. Sudhamsu and B. A. Patel, *Annu. Rev. Biochem*., **2010**, *79*, 445-470.

(92) H. M. Abu-Soud, L. L. Yoho and D. J. Stuehr, *J. Biol. Chem.,* 1994*, 269*, 32047-32050.

(93) T.O. Fischmann, A. Hruza, X. D. Niu, J. D. Fossetta, C. A. Lunn, E. Dolphin, A. J. Prongay, P. Reichert, D. J. Lundell, S. K. Narula and P. C. Weber, *Nat. Struct. Biol*., **1999**, *6*, 233-242.

(94) H. Matter and P. Kotsonis, *Med. Res. Rev*., **2004**, *24*, 662-684.

(95) H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov and P. E. Bourne, *Nucleic Acids Res*., **2000**, *28*, 235-242.

(96) A. Presta, U. Siddhanta, C. Q. Wu, N. Sennequier, L. X. Huang , H. M. Abu-Soud, S. Erzurum and D. J. Stuehr, *Biochemistry*, **1998**, *37*, 298-310.

(97) M. A. Nobel, A. W. Munro, S. L. Rivers, L. Robledo and S. K. Chapman, *Biochemistry,* **1999**, *38*, 16413-16418.

(98) H. M. Abu-Soud, R. Gachhui, F. M. Raushel and D. J. Stuehr, *J. Biol. Chem*., **1997**, *272*, 17349-17353.

(99) C. Wei, Z. Wang, A. Meade, J. McDonald and D. J. Stuehr, *J. Inorg. Biochem*., **2002**, *91*, 618-624.

(100) A. C. F. Gorren, S. Marchal, M. Sørlie, K. K. Andersson, R. Lange and B. Mayer, *Biochim. Biophys. Acta*, **2006**, *1764*, 578-585.

(101) A. Jousserandot, J. L. Boucher, Y. Henry, B. Niklaus, B. Clement and D. Mansuy, *Biochemistry,* **1998**, *37*, 17179-17191.

(102) H. M. Abu-Soud, K. Ichimori, H. Nakazawa and D. J. Stuehr, *Biochemistry,* **2001**, *40*, 6876-6881.

(103) Z. Q. Wang, C. C. Wei and D. J. Stuehr, *J. Biol. Chem.,* **2002**, *277*, 12830-12837.

(104) W. K. Alderton, C. E. Cooper and R. G. Knowles, *Biochem. J*., **2001**, 357, 593–615.

(105) J. Vasquez-Vivar, B. Kayanaraman, P. Martasek, N. Hogg, B. S. Masters, H. Karoui, P. Tordo and K. A. Pritchard, *J. Proc. Natl. Acad. Sci. USA*., **1998**, *95*, 9220-9225.

(106) P. Kotsonis, L. G. Frohlich, Z. V. Shutenko, R. Horejsi, W. Pfleiderer and H. H. H. W. Schmidt*, Biochem. J*., **2000**, 346,767-776.

(107) K. McMillan and B. S. Masters, *Biochemistry,* **1993**, *32*, 9875-9880.

(108) H. Matter, P. Kotsonis, O. Klingler, H. Strobel, L. G. Frohlich, A. Frey, W. Pfleiderer and H. H. H. W. Schmidt, *J. Med. Chem.,* **2002**, *45,* 2923-2941.

(109) B. R. Crane, A. S. Arvai, D. K. Ghosh, C. Q. Wu, E. D. Getzoff, D. J. Stuehr and J. A. Tainer, *Science,* **1998**, *279*, 2121-2126.

(110) P. Klatt, K. Schmidt, D. Lehner, O. Glatter, H. P. Bachinger and B. Mayer, *EMBO J*., **1995**, *14*, 3687-3695.

(111) N. Bec, A. C. Gorren, C. Voelker, B. Mayer and R. Lange, *J. Biol. Chem.,* **1998***, 273,* 13502-13508.

(112) P. Klatt, M. Schmidt, E. Leopold, K. Schmidt, E. R. Werner and B. Mayer, *J. Biol. Chem.,* **1994***, 269,* 13861-13866.

(113) D. J. Stuehr, J. Santolini, Z. Q. Wang, C. C. Wei and S. Adak, *J. Biol. Chem*., **2004**, *279,* 36167-36170.

(114) S. Daff, *Nitric Oxide*, **2010**, *23*, 1-11.

(115) B. R. Crane, A. S. Arvai, S. Ghosh, E. D. Getzoff, D. J. Stuehr and J. A. Tainer, *Biochemistry*, **2000**, *39,* 4608-4621.

(116) D. K. Menyhard, *J. Phys. Chem. B*., **2009**, *113*, 3151-3159.

(117) I. Morao, G. Periyasamy, I. H. Hillier and J. A. Joule, *Chem. Commun*., **2006**, 3525-3527.

(118) K.B. Cho, M.A. Carvajal and S. Shaik, *J. Phys. Chem. B*., **2009**, *113*, 336–346.

(119) H. Matter, H. S. A. Kumar, R. Fedorov, A. Frey, P. Kotsonis, E. Hartmann, L.G. Frohlich, A. Reif, W. Pfleiderer, P. Scheurer, D. K. Ghosh, M. Schlichting and H. Schmidt, *J. Med. Chem*., **2005**, *48,* 4783-4792.

(120) D. Papale, C. C. Miles and S. N. Daff, *J. Biol. Inorg. Chem*., **2007**, *12*, S199.

(121) J. S. Tejero, A. Biswas, Z. Q. Wang, R. C. Page, M. M. Haque, C. Hemann, J. L. Zweier, S. Misra and D. J. Stuehr, *J. Biol. Chem*., **2008**, *283*, 33498-33507.

(122) B. Clapp, A. D. Hingorani, R. K. Kharbanda, V. Mohamed-Ali, J. W. Stephens, P. Vallance and R. J. MacAllister, *Cardiovasc. Res.,* **2004**, *64*, 172-178.

(123) R. Wever, P. Boer, M. Hijmering, E. Stroes, M. Verhaar, J. Kastelein, K. Versluis, F. Lagerwerf, H. van Rijn, H. Koomans and T. Rabelink, *Arterioscler. Thromb. Vasc. Biol.,* **1999**, *19*, 1168-1172. Arterioscler. Thromb. Vasc. Biol.

(124) P. Stenvinkel, *Nephrol. Dial. Transplant.,* **2001**, *16,* 1968-1971.

(125) J. S. Wang, H. Singh, F. Zhang, T. Ishizuka, H. Deng, R. Kemp, M. S. Wolin, T. H. Hintze, N. G. Abraham, A. Nasjletti and M. Laniado-Schwartzman, *Circ. Res.,* **2006**, *98,* 962-969.

(126) G. A. Kaysen and J. P. Eiserich, *Seminars In Dialysis*, **2003**, *16*, 438-446.

(127) G. Bjelakovic, T. J. Stoimenov, B. Bjelaković and I. Stojanovic, *Medicine and Biology*, **2004**, *11*, 49-54.

(128) B. Thony, G. Auerbach and N. Blau, *Biochem. J.,* **2000**, *347,* 1-16.

(129) H. J. Woo, J. Y. Kang, Y. K. Choi and Y. S. Park, *Appl. Environ. Microbiol*., **2002**, *68*, 3138–3140.

(130) G. G. Krivi and G. M. Brown, *Biochem. Genet*., **1979**, *17*, 371–390.

(131) I. Smith, B. E. Clayton and O. H. Wolff, *Lancet*, **1975**, *1*, 1108-1111.

(132) K. Bartholome and D. J. Byrd, *Lancet*, **1975**, *2*, 1042-1043.

(133) Z. S. Katusic, *Am. J. Physiol. Heart. Circ. Physiol.,* **2001***, 281,* 981-986.

(134) S. Verma, A. Dumont and A. Maitland, *Heart*, **2001**, 86, 706-708.

(135) S. Verma, A. Maitland, R. D. Weisel, P. W. Fedak, N. C. Pomroy, S. H. Li, D. A. Mickle, R. K. Li and V. Rao, *J. Thorac. Cardiovasc. Surg.,* **2002***, 123*, 1074-1083.

(136) R. Walter, N. Blau, A. Schaffner, M. Schneemann, R. Speich, R. Stocker, B. Naujeck and G. Schoedon, *Am. J. Respir. Crit. Care. Med*., **1997**, *156*, 2006-2010.

(137) I. Smith and J. Lloyd, *Arch. Dis. Child*., **1974**, *49*, 245.

(138) D. M. Danks, *J. Inher. Metab. Dis*., 1987, *1*, 47-48.

(139) B. K. Barbara, D. J Adams, D. K. Grange, J. I. Malone, E. Jurecki, H. Bausell, K. D. Marra, L. Sprietsma and K.T. Swan, *J. Pediatr*., **2011**, *158*, 410-415.

(140) H. L. Levy, A. Milanowski, A. Chakrapani, M. Cleary, P. Lee, F. K. Trefz, C. B. Whitley, F. Feillet, A. S. Feigenbaum, J. D. Bebchuk, H. Christ-Schmidt and A. Dorenbaum, *Lancet*, **2007**, *370,* 504-510.

(141) A. L. Pey and A. Martinez, *Lancet*, **2007**, *370,* 462-463.

(142) A. L. Pey, M. Ying, N. Cremades, A. Velazquez-Campoy, T. Scherer, B. Thony, J. Sancho and A. Martinez, *J. Clin. Invest.,* **2008***, 118*, 2858-2867.

(143) R. A. Williams, C. D. S. Mamotte and J. R. Burnett, *Clin Biochem. Rev.,* **2008**, 29, 31-41.

(144) H. Shintaku and M. Ohwada, *Brain & Development*, **2013**, 35*,* 406-410.

(145) A. Daniele, G. Cardillo, C. Pennino, M. T. Carbone, D. Scognamiglio, L. Esposito, A. Correra, G. Castaldo, A. Zagari and F. Salvatore, *Biochim. Biophys. Acta*., **2008**, *1782*, 78-384.

(146) K. Michals-Matolon, *Expert Opin. Investing Drugs*, **2008**, *17*, 245-251.

(147) K. Yo-Sik, C. Yong-Bock, L. Jeong-Hwa, Y. Sei-Hoon, C. Ji-Hyun, S. Chang-Ho, L. Sang-Do, P. Moon-Kee and H. Kyeong-Man, *Exp. Mol. Med*., **2008**, *40*, 271-275.

(148) B. N. R. Cheyette, S. N. R. Cheyette, K. Cusmano-Ozog and G. M. Enns, *Pediatric Neurology*, **2008**, *38*, 273-275.

(149) Y. X. Jia, K. Sekizawa, T. Ohrui, K. Nakayama and H. Sasaki, *Am. J. Physiol. Regul. Integr. Comp. Physiol*., **1998***, 274,* 76-80.

(150) A. S. Hui, J. B. Striet, G. Gudelsky, G. K. Soukhova, E. Gozal, D. Beitner-Johnson, S. Z. Guo, L. R. Sachleben, J. W. Haycock, D. Gozal and M. F. Czyzyk-Krzeska, *Hypertension,* **2003,** *42*, 1130-1136.

(151) P. L. Pearl, P. K. Capp, E. J. Novotny and K. M. Gibson, *Clinical Biochemistry,* **2005**, *38*, 1051-1058.

(152) C. Sumi-Ichinose, F. Urano, R. Kuroda, T. Ohye, M. Kojima, M. Tazawa, H. Shiraishi, Y. Hagino, T. Nagatsu, T. Nomura and Hiroshi Ichinose**,** *J. Biol. Chem.,* **2001**, 276, 41150-41160.

(153) U. Forstermann and T. Munzel, *Circulation,* **2006**, *113,* 1708–1714.

(154) M. Brownlee, *Diabetes,* **2005**, *54,* 1615-1625.

(155) V. Matei, A. Rodriguez-Vilarrupla, R. Deulofeu, D. Colomer, M. Fernandez, J. Bosch and J. C. Garcia-Pagan, *Hepatology,* **2006**, *44*, 44-52.

(156) N.J.Alp, S. Mussa, J. Khoo, S. Cai, T. Guzik, A. Jefferson, N. Goh, K.A. Rockett and K. M. Channon, *J. Clin. Invest*., **2003**, *112,* 725-735.

(157) J. W. Elrod, M.R. Duranski, W. Langston, J. J. Greer, L. Tao, T. R. Dugas, C. G. Kevil, H. C. Champion and D. J. Lefer, *Circ. Res*., **2006**, 99, 78-85.

(158) E. Stroes, J. Kastelein, F. Cosentino, W. Erkelens, R. Wever, H. Koomans, T. Luscher and T. Rabelink, *J. Clin. Invest*., **1997**, *99,* 41-46.

(159) J. Xu, Y. Wu, P. Song, M. Zhang, S. Wang and Z. Ming-Hui, *Circulation*, **2007**, *116*, 944-953.

(160) V. Matei, A. Rodrıguez-Vilarrupla, R. Deulofeu, D. Colomer, M. Fernandez, J. Bosch and J. Garcia-Pagan, *Hepatology*, **2006**, *44*, 44-52.

(161) E. Podjarny, G. Hasdan, J. Bernheim, G. Rashid, J. Green, Z. Korzets and J. Bernheim, *Nephrol. Dial. Transplant*, **2004**, *19,* 2223-2227.

(162) **A.** L. Fortepiani and J. F. Reckelhoff, *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **2005***, 288,* 733-736.

(163) Y. Kondo, T. Ishikawa, K. Yamaguchi, T. Yada and M. Fujisawa, *J. Androl., 29*, 153-163.

(164) X. Le, D. Wei, S. Huang, J. R. Lancaster and K. Xie, *Cancer Research,* **2005**, 102, 8758-8763.

(165) A. G. Sikora, A. Gelbard, M. A. Davies, D. Sano, S. Ekmekcioglu, J. Kwon, Y. Hailemichael, P. Jayaraman, J. N. Myers, E. A. Grimm and W. W. Overwijk, *Clin. Cancer Res.,* **2010**, *16*,1834-1844.

(166) X. W. Qu, L. G. Thaete, R. A. Rozenfeld, Y. Zhu, I. G. De Plaen, M. S. Caplan and W. Hsueh, *Crit Care Med.,* **2005**, *33*, 1050-1056.

(167) R. J. van't Hof and S. H. Ralston*, Immunology*, **2001**, *103*, 255-261.

(168) A. L. Moens, E. Takimoto, C. G. Tocchetti, K. Chakir, D. Bedja, G. Cormaci, E. A. Ketner, M. Majmudar, K. Gabrielson, M. K. Halushka, J. B. Mitchell, S. Biswal, K. M.

Channon, M. S. Wolin, N. J. Alp, N. Paolocci, H. C. Champion and D. A. Kass, *Circulation,* **2008**, *117*, 2626-2636.

(169) Y. Li and H. E. Schellhorn**,** *J. Nutr., 137*, 2171-2184.

(170) C. Napoli and L. J. Ignarro, *Nitic Oxide*, **2001**, *5,* 88-97.

(171) M. J. Crabtree and K. M. Channon, *Nitric Oxide,* **2011***, 25*, 81-88.

(172) J. Yan, G. Tie, A. Hoffman, Y. Yang, P. T. Nowicki and L. M. Messina, *Mol. Ther*., **2010** *18*, 1482-1489.

(173) C. Cunnington, T. V. Assche, C. Shirodaria, I. Kylintireas, A. C. Lindsay, J. M. Lee, C. Antoniades, M. Margaritis, R. Lee, R. Cerrato, M. J. Crabtree, J. M. Francis, R. Sayeed, C. Ratnatunga, R. Pillai, R. P. Choudhury, S. Neubauer and K. M. Channon, *Circulation*, **2012**, *125*, 1356-66.

(174) B. Fiege, D. Ballhausen, L. Kierat, W. Leimbacher, D. Goriounov, B. Schircks, B. Thony and N. Blau, *Molecular Genetics and Metabolism,* **2004**, *81*, 45-51.

(175) K. Sawabe, K. O. Wakasugi and H. Hasegawa, *J. Pharmacol. Sci*., **2004**, *96*, 124 - 133.

(176) M. C. Verhaar**,** P. E. Westerweel**,** A. J. V. Zonneveld and T. J .Rabelink, *Heart,* **2004**, *90,* 494-495.

(177) C. Dumitrescu, R. Biondi, Y. Xia, A. J. Cardounel, L. J. Druhan, G. Ambrosio and J. L. Zweier, *Proc. Natl. Acad. Sci. U.S.A*., **2007**, *104*, 15081-15086.

(178) K. Hyland, *J. Nutr*., **2007**, *137*, 1568-1572.

(179) C. R. Coughlin II, K. Hyland, R. Randall and C. Ficicioglu, *JIMD Rep*., **2013**, *10*, 53-56.

(180) K. M. Channon, *Trends. Cardiovas. Med*., **2004**, *14*, 323-327.

(181) U. Forstermann, *Pflugers Arch – Eur. J. Physiol*., **2010**, *459,* 923-939.

(182) A. Presta, U. Siddhanta, C. Wu, N. Sennequier, L. Huang, H. M. Abu-Soud, S. Erzurum and D. J. Stuehr, *Biochemistry,* **1998**, *37*, 298-310

(183) H. M Abu-Soud, P. L. Feldman, P. Clark and D. J. Stuehr, *J. Biol. Chem*., **1994**, *269*, 2318-32326.

(184) K. M. Rusche and M. A. Marletta, *J. Biol. Chem.,* **2001***, 276,* 421-427.

(185) C. Riethmuller, A. C. F. Gorren. Antonius, E. Pitters, B. Hemmens, H. J. Habisch, S. J. R. Heales, K. Schmidt, E. R. Werner and B. Mayer, *J. Biol. Chem.,* **1999***, 274,* 16047-16051.

(186) C. Riethmuller, A. C. Gorren, E. Pitters, B. Hemmens, H. J. Habisch, S. J. Heales, K. Schmidt, E. R. Werner and B. Mayer, *J. Biol.Chem.,* **1999***, 274, 16047-16051.*

(187) A. C. Gorren, A. J. Kungl, K. Schmidt, E. R, Werner and B. Mayer*, Nitric Oxide,* **2001***, 5,* 176-186.

(188) S. S. Al-Hassan, R. Cameron, A. W. C. Curran, W. J. S. Lyall, S. H. Nicholson, D. R. Robinson, A. Stuart, C. J. Suckling, I. Stirling and H. C. S. Wood, *J. Chem. Soc. Perkin Trans. 1,* **1985**, 1645-1659.

(189) S. P. Kunuthur, P. H. Milliken, C. L. Gibson, C. J. Suckling and R. M. Wadsworth, *Eur. J. Pharmacol.,* **2011**, *650*, 371-377.

(190) C. J. Suckling, C. L. Gibson, J. K. Huggan, R. R. Morthala, B. Clarke, S. Kununthur, R. M. Wadsworth, S. Daff and D. Papale, *Bioorg. Med. Chem. Lett.*, **2008**, *18*, 1563-1566.

(191) C. R. McInnes, C. J. Suckling, C, L, Gibson, R. R. Morthala, S. Daff and B. Gazur, *Pteridines*, **2009**, *20*, 27-35.

(192) L. G. Fröhlich, P. Kotsonis, H. Traub, S. Taghavi-Moghadam, N. Al-Masoudi, H. Hofmann, H. Strobel, H. Matter, W. Pfleiderer and H. H. H. W. Schmidt, *J. Med. Chem.*, **1999**, *42*, 4108-4121.

(193) P. Kotsonis, L. G. Fröhlich, C. S. Raman, H. Li, M. Berg, R. Gerwig, V. Groehn, Y. Kang, N. Al-Masoudi, S. Taghavi-Moghadam, D. Mohr, U. Münch, J. Schnabel, P. Martasek, B. S. S. Masters, H. Strobel, T. Poulos, H. Matter, W. Pfleiderer and H. H. H. W. Schmidt, *J. Biol. Chem.*, **2001**, *276*, 49133-49141.

(194) E. R. Werner, E. Pitters, K. Schmidt, H. Wachter, G. Werner-Felmayer and B. Mayer, *Biochem. J.*, **1996**, *320*, 193-196.

(195) C. C. Wei, B. R. Crane and D. J. Stuehr, *Chem. Rev.*, **2003**, *103*, 2365-2384.

(196) B. Mayer, C. Wu, A. C. F. Gorren, S. Pfeiffer, K. Schmidt, P. Clark, D. J. Stuehr and E. R. Werner, *Biochemistry*, **1997**, *36*, 8422-8427.

(197) S. Pfeiffer, A. C. F. Gorren, E. Pitters, K. Schmidt, E. R. Werner and B. Mayer, *Biochem. J.*, **1997**, *328*, 349-352.

(198) E. R. Werner, H. J. Habisch, A. C. F. Gorren, K. Schmidt, L. Canevari, G. Werner-Felmayer and B. Mayer, *Biochem. J.*, **2000**, *348*, 579-583.

(199) M, Demosthenous, C. Antoniades, D. Tousoulis, M. Margaritis, K. Marinou and C. Stefanadis, *Artery Res.*, **2011**, *5*, 37-49.

(200) D. J. Stuehr, C. C. Wei, Z. Wang and R. Hille, *Dalton Trans.*, **2005**, 3427-3435.

(201) P. P. Schmidt, R. Lange, A. C. F. Gorren, E. R. Werner, B. Mayer and K. K. Andersson, *J. Biol. Inorg. Chem.*, **2001**, *6*, 151-158.

(202) A. C. F. Gorren, N. Bec, A. Schrammel, E. R. Werner, R. Lange and B. Mayer, *Biochemistry,* **2000,** *39*, 11763-11770.

203) D. K. Menyhard, *J. Phys. Chem. B*, **2009***, 113*, 3151-3159.

(204) P. Kubes, *Gut,* **2000**, *47*, 6-9.

(205) M. J. Hickey, D. N. Granger and P. Cubes, *Acta. Physiol. Scand.*, **2001**, *173*, 119-126

(206) S. P. Kunuthur, P. H. Milliken, C. L. Gibson, C. J. Suckling and R. M. Wadsworth, *Eur. J. Pharmacol*., **2011**, *650*, 371-377.

(207) R. Wadsworth, C. Suckling and C. Gibson, U.S. Pat. Appl. Publ., **2006**. 20060194800.

(208) D. Guiney, C. L. Gibson and C. J. Suckling, *Org. Biomol. Chem*., **2003**, *1*, 664-675.

(209) S. M. Allin, C. J. Northfield, I. M Page and A. M. Z. Slawin, *Tetrahedron Lett.,* **1997**, *38*, 3627-3630.

(210) M.A. Scialdone and A. I. Meyers, *Tetrahedron Lett.,* **1994**, *35,* 7533-7536.

(211) L. D. Luca, G. Giacomelli and A. Porcheddu, *Org. Lett.,* **2001**, *3*, 3041-3043.

(212) H. C. Brown, Chandra, C. P. Garg and K. T. Liu, *J. Org. Chem.*, **1971**, *36*, 387-390.

(213) N. A. Keiko, E. A. Funtikova, L. G. Stepanova, Yu. A. Chuvashev and L. I. Larina, *Russ. J. Org. Chem.,* **2003**, *39*, 1477-1483.

(214) Fulop, F. Pihlaja, K. Neuvonen, K. Bernath, G.Argay, and A. Kalman, *J. Org. Chem.,* **1993**, *58*, 1967-1969.

(215) C. Adami and T. Rizk, *Tetrahedron,* **1985**, *41*, 537-540.

(216) E. David, Fenton and G. Papageorgiou, *Tetrahedron,* **1996**, *52*, 5913-5928.

(217) M. J. Vega-Perez, M. Vega, E. Blanco, and F. Iglesias-Guerra, *Tetrahedron Asymmetry,* **2001**, *12,* 3189–3203.

(218) R. Brown, M. Joseph, T. Leigh and M. L. Swain, *J. Chem. Soc. Perkin Trans. I,* **1977**, *9*, 1003-1009.

(219) J. Davoll and D. D. Evans, *J. Chem. Soc*., **1960**, 5041-5049.

(220) D. T. Browne, J. Eisinger and N. J. Leonard, *J. Am. Chem. Soc.,* **1968**, *90*, 7302-7323.

(221) P. H. Boyle, K. M. Daly, F. Leurquin, J. K. Robinson and D. T. Scully, *Tetrahedron Lett.,* **2001**, *42*, 1793–1795.

(222) W. Pfleiderer and H. Zondle, *Chem. Ber.,* **1966**, *99,* 2984.

223) J. H. Chesterfield, D. T. Hurst, J. F. W. McOmie andM. S. Tute, *J. Chem. Soc*., **1964**, 100-105.

(223a) R. G. Jones, E. C. Kornfeldk, K. C. Mclaughlin and R. C. Anderson, *J. Am. Chem. Soc.*, **1949**, *71*, 4000-4002.

(224) F. Ahmed, Abdel-Magid, K. G. Carson, D. Bruce, Harris, A. Cynthia, Maryanoff and R. D. Shah, *J. Org. Chem.*, **1996**, *61,* 3849-3862.

(225) L. Roland, Knight and J. F. J. Leeper, *Tetrahedron Lett.,* **1997**, *38*, 3611-3614.

(226) R. Breslow, *J. Am. Chem. Soc.,* **1958**, *80*, 3719-3726.

(227) A. E. Mattson, A.M. Zuhl, T. E. Reynolds and K. A. Scheidt, *J. Am. Chem. Soc*., **2006**, 1*28*, 4932-4933.

(228) Li. Gong-Qiang, D. Li-Xin, and Y. Shu-Li, *Chem. Commun.,* **2007**, *7***,** 852-854.

(229) S. F. Nielsen, T. Boesen, M. Larsen, K. Schonning and H. Kromann, *Bioorgan. Med. Chem., **2004***, 12, 3047-3054.

(230) S. Raghavan and K. Anuradha, *Tetrahedron Lett.,* **2002***, 43,* 5181-5183.

(231) L. F. Wilfred, Armarego and P. Waring, *J. Chem. Soc., Perkin. Trans.* II, **1982**, 1227-1233.

(232) A. Srinivasan and A. D. Broom, *J. Org. Chem*., **1981**, *46,* 1777-1781.

(233) W. J. Irwin and D. G. Wibberley, *J. Chem. Soc. C*, **1967**, 1745-1750.

(234) Z-K. Wan, S. Wacharasindhu, E. Binnun and T. Mansour, *Org. Lett.,* **2006**, *8*, 2425-2428.

(235) J. Klose, M. Bienert, C. Mollenkopf, D. Wehle, C. W. Zhang, L. A. Carpinoc and P. Henklein, *Chem. Commun.*, **1999**, 1847-1848.

(236) J. L. Kelley and E. W. Mclean, *J. Heterocyclic Chem.,* **1981***, 18,* 671-673.

(237) W. T. Caldwell and H. B. Kime, *J. Am. Chem. Soc.*, **1940**, *62*, 2365.

(238) M. Dracinsky, P. Jansa, K. Ahonen and M. Budesinsky, *Eur. J. Org. Chem.*, **2011**, *8,* 1544–1551.

(239) L. M. Beauchamp, B. L. Dolmatch, H. J. Schaeffer, P. Collins, D. J. Bauer, P. M. Keller and J. A. Fyfe, *J. Med. Chem.*, **1985**, *28*, 982–987.

(240) L. M. Beauchamp, B. L. Serling, J. E. Kelsey, K. K. Biron, P. Collins, J. Selway, J. C. Lin and H. J. Schaeffer, *J. Med. Chem.*, **1988**, *31*, 144–149.

(241) J. Doskocil, A. Holy and J. Filip, *Nucleic Acids Res.*, **1974**, *1*, 1209-1220.

(242) Z. Zhang, J. Liu, W. Liu, X. Wang, Z. Cheng and J. Liu, *J. Heterocyclic Chem.,* **2009**, *46,* 1151-1153.

(243) E. S. Denmark and S. M. Yang, *J. Am. Chem. Soc*., **2002**, *124*, 2102-2103.

(244) M. Kelm, R. Dahmann, D. Winks and M. Feelisch, *J. Biol. Chem*., **1997**, *272*, 9922-9932.

(245) J. A. Murry, D. E. Frantz, A. Soheili, R. Tillyer, E. J. J. Grabowski and P. J. Reider, *J. Am. Chem. Soc*., **2001**, *123*, 9696-9697.

(246) A. Heckel and W. Pfleiderer, *Helv. Chim. Acta*, *69*, **1986**, 1095-1106.

(247) A. Heckel and W. Pfleiderer, *Helv. Chim. Acta*, *69*, **1986**, 708-717.

(248) R. R. Morthala, *PhD Thesis, The University of Strathclyde*, **2007**.

(249) H. C. S. Wood, *Chem. Br.*, **1966**, *2*, 536-541.

(250) J. G. Andrew, P. L. Benjamin, R. P. John, J. S. David and S. S. Jonathan, *Proc. Natl. Acad. Sci. USA* (*Biochemistry*), **1999**, *96*, 9027-9032.

(251) R. Cassoly and Q. H. Gibson, *J. Mol. Biol., 91,* **1975**, 301-313.

(252) R. F. Eich, T. Li, D. D. Lemon, D. H. Doherty, S. R. Curry, J. F. Aitken, A. J. X. J. K. Mathews, R. D. Smith, G. N. J. Phillips and J. S. Olson, *Biochemistry,* **1996**, *35*, 6976–6983.

(253) I. Hamon, H. Gauthier-Moulinier, E. Grelet-Dessioux, L. Storme, J. Fresson and J. M. Hascoet, *Acta Paediatr.*, **2010**, *99*, 1467-1473.

(254) C. De Labra, C. Rivadulla, N. Espinosa, M. Dasilva, R. Cao and J. Cudeiro, *Front. Syst. Neurosci.*, **2009**, *3*, 1-9.

(255) J. L. González-Mora, F. A. Martín, D. Rojas-Díaz, S. Hernández, I. Ramos-Pérez, V. D. Rodríguez and M. A. Castellano, *J. Neurosci. Methods*, **2002**, *119*, 151-161.

(256) T. Fukushima and J.C. Nixon, Anal Biochem., **1980**, *102*, 176-188.

(257) T. Nagatsu, T. Yamaguchi, T. Kato, T. Sugimoto, S. Matsuura, M. Akino, I. Nagatsu, R. Iizuka and H. Narabayashi, Clin. Chim. Acta, **1981**, *109*, 305-311.

(258) C. J. Suckling, B. Clark and R. M. Wadsworth, unpublished results.

(259) H. G. Viehe, Z. Janousek, R. Merenyi and L. Stella, 1985, *18*, 148-154.

(260) R. D. L. Fuente, N. D. Sonawane, D. Arumainayagam and A. S. Verkman, Br. J. Pharmacol.,2006, 149, 551-559.

(261) M. Z. David and R. S. Daum, *Clin. Microbiol. Rev*., **2010**, *23*, 616-687.

(262) J. P. Cabral, Int. J. Environ. Res. Public Health, **2010**, *7*, 3657-3703.

(263) T. Y. Sia, S.Taimur, D. M. Blau, J. Lambe, J. Ackelsberg, K.Yacisin, J. Bhatnagar, J. Ritter, W. J. Shieh, A. Muehlenbachs, K. Shulman, D. Fong, E. Kung and S. R. Zaki, Clin. Infect. Dis., **2016**, *62*, 590-595.

(264) K. Stuart, R. Brun, S. Croft, A. Fairlamb, R. E. Gürtler, J. McKerrow, S. Reed and R. Tarleton, *J. Clin.Invest*., **2008**, *118*, 1301-1310.

(265) A. I. Khalaf, A. H. Ebrahimabadi, A. J. Drummond, N. G. Anthony, S. P. Mackay, C. J. Suckling and R. D. Waighb, *Org. Biomol. Chem*., **2004**, *2*, 3119-3127.

(266) N. K. Taneja and J. S. Tyagi, *J. Antimicrob. Chemother*., **2007**, *60*, 288-293.