SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL GLYCOSYLATED AND NON-GLYCOSYLATED *S*-NITROSO AMINO ACIDS AS POTENTIAL NITRIC OXIDE-DONOR COMPOUNDS

A thesis presented for the degree of Doctor of Philosophy
to the University of St. Andrews in May 2001

By
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DECLARATION

I, Francesca Ann Mazzei, hereby certify that this thesis, which is approximately 28,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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To Stefano

(* 23-1-1971 - † 31-5-1999)

He showed me how life should be lived

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Those who know me, know that family and friends are the most important things for me. I would like to thank my parents for believing in me, supporting me and being close to me even when 2000 miles away and my grandmother for looking after me, cooking for me and putting up with the piles of dirty washing. I would like to thank Lesley and Henry for all those Sunday roasts, the lovely afternoons spent chatting and the occasional lambing. I would also like to thank my best friend Giovanna for always being at the other end of the phone when I needed advice, support, conforting or just a laugh.

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CONTENTS

ABBREVIATIONS

RSH	General thiol
RSNO	General *S*-nitrosothiol
NOS	Nitric oxide synthase
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
sGC	Soluble guanylate cyclase
GTP	Guanosine triphosphate
cGMP	Cyclic guanosine monophosphate
Hb	Deoxyhaemoglobin
HbO_2	Oxyhaemoglobin
EDRF	Endothelium-derived relaxing factor
Hb-SNO	*S*-Nitrosohaemoglobin
PTBA	Percutaneous transluminal balloon angioplasty
SNAP	*S*-Nitroso-*N*-acetyl-D,L-penicillamine
GLACSNAP	*N*-(*S*-Nitroso-*N*-acetyl-D,L-penicillaminyl-1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose
SNAG	2,3,4,6-Tetra-*O*-acetyl-1-deoxy-*S*-nitroso-1-thio-β-D-glucopyranose
GSNO	*S*-Nitrosoglutathione
SNPP	*S*-Nitroso-*N*-propionyl-D,L-penicillamine
SNVP	*S*-Nitroso-*N*-valeryl-D,L-penicillamine
SNHP	*S*-Nitroso-*N*-heptanoyl-D,L-penicillamine
Boc	Butoxycarbonyl
SNBocP	*S*-Nitroso *N* butoxycarbonyl-D,L-penicillamine
Fmoc	9-Fluorenylmethoxycarbonyl
SNFmocP	*S*-Nitroso-*N*-9-fluorenylmethoxycarbonyl-D,L-penicillamine
SNPAP	*S*-Nitroso-*N*-phenylacetyl-D,L-penicillamine
CMEC	1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimidemetho-*p*-toluenesulfonate
NMM	*N*-Methylmorpholine
IBCF	*iso*Butylchloroformate

Glyco-SNAP-1	*N*-(*N*-Acetyl-*S*-nitroso-D,L-penicillaminyl)-1-amino-1-deoxy-β-D-glucopyranoside
Glyco-SNAP-2/ GLUSNAP	*N*-(*N*-Acetyl-*S*-nitroso-D,L-penicillaminyl)-2-amino-2-deoxy-α-D-glucopyranose
Glyco-SNAP-3	*N*-(*N*-Acetyl-*S*-nitroso-D,L-penicillaminyl)-3-amino-3-deoxy-α-D-glucopyranose
SNAPOA	*S*-Nitroso-*N*-acetyl-D,L-penicillamineoctyl amide
DCC	Dicyclohexylcarbodiimide
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
PyBOP	Benzotriazolyoxytris(pyrrolidino)phosphonium hexafluorophosphate
GTN	Glycerol trinitrite

ABSTRACT

This study focuses on the preparation and biological evaluation of novel NO-donating compounds, namely *S*-nitrosothiols. This class of molecules release nitric oxide and act as vasodilators and could therefore be adopted in the treatment of conditions such as angina or as post-operative vasodilators after by-pass surgery or balloon angioplasty.

The mechanism by which *S*-nitrosothiols bring about vasodilation is not fully understood, for this reason a number of *S*-nitrosothiols was prepared so that the structure-activity relationship for these compounds could be investigated. It was also our aim to determine whether *S*-nitrosothiol activity could be enzyme-mediated (as proposed by a number of groups).

All *S*-nitrosothiols prepared were based on the amino acid penicillamine. A number of modifications were carried out on the amino acid amine and the thiol moiety on the amino acid was then nitrosated. Penicillamine was *N*-acylated with a number of different groups, from simple, linear chains to *N*-protecting groups such as the butoxycarbonyl and 9-fluorenylmethoxycarbonyl groups. Penicillamine was also *N*-glycosylated with 2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose. This compound was found to be efficiently retained within the vessel wall of isolated rat femoral arteries and therefore a number of other *N*-glycosylated *S*-nitrosopenicillamines were prepared, using sugars such as methyl 6-amino-6-deoxy-β-D-galactopyranoside, 2-amino-1,5-anhydro-2-deoxy-D-glucitol and 3-aminopropyl-β-D-glucopyranoside.

The different *S*-nitrosopenicillamines were found to possess varying vasodilatory abilities and different thermal decomposition patters. The preliminary results obtained do not support or confute the involvement of an enzyme in the decomposition of *S*-nitrosothiols, however, despite the vast degree of variation in the structures of the compounds tested, their biological activities remained comparable.

Chapter 1: Introduction

Nitric oxide (NO) is a gas, particularly infamous for its presence in car exhausts and for its toxic effects. It is therefore quite extraordinary that this gas is present in the human body[1] and that it is involved in many different biological processes.[2] Major research effort in this area started around 15 years ago and much remains to be explained.[3] However, there are many areas in which NO has been identified as having an important role. The following sections contain a few representative examples.

1.1. Reactions of NO

Although a radical species, NO reacts readily with only a limited number of other molecules, principally other radicals.[4]

1.1.1. NO and oxygen

NO reacts with O_2 both in the gaseous and aqueous phases to yield NO_2 (Eqn. 1). This reaction is second order with respect to NO and first order with respect to O_2.[5]

$$2NO + O_2 \rightarrow 2NO_2 \qquad \text{(Eqn. 1)}$$

In aqueous solution, instead of dimerising to give N_2O_4 (Eqn. 2), NO_2 reacts with another molecule of NO, to give N_2O_3, a very good nitrosating agent (Eqn. 3).

$$2NO_2 \rightarrow N_2O_4 \qquad \text{(Eqn. 2)}$$

$$NO_2 + NO \rightarrow N_2O_3 \qquad \text{(Eqn. 3)}$$

N_2O_3 reacts with water to give nitrous acid as the sole product.

As a radical, NO reacts very rapidly with superoxide ($O_2^{\bullet -}$), yielding peroxynitrite ($ONOO^-$).[6] At physiological concentrations, the rate for this reaction is

3 times faster than that of superoxide dismutase (SOD) with superoxide. Peroxynitrite is a potent oxidant and has been found to react with many classes of biomolecules.[6]

1.1.2. NO and transition metals

Reactions of NO with iron-containing proteins are amongst the most common and fast reactions involving NO.[4] Oxyhaemoglobin (HbO_2) reacts rapidly both with $NO^{\bullet}$ and with NO_2^- to yield nitrate (NO_3^-) (Equations 4 and 5).[7]

$$HbO_2(Fe^{2+}) + NO \rightarrow Hb(Fe^{3+}) + NO_3^- \quad \text{(Eqn. 4)}$$

$$4HbO_2(Fe^{2+}) + 4NO_2^- + 4H^+ \rightarrow 4Hb(Fe^{+}) + O_2 + 2H_2O + 4NO_3^- \quad \text{(Eqn. 5)}$$

The relevance of these reactions, however, is not completely understood and their importance *in vivo* is still unclear, as will be outlined in section 1.4.4.3.
Reaction of NO with iron-sulfur clusters can result in sulfur oxidation or in the formation of iron-nitrosyl intermediates. Metals can, therefore, be involved in NO exchange with thiols (Eqn. 6).[8]

$$Fe^{+}(NO)_2(RS^-)_2 + H^+ \rightarrow Fe^{2+} + RSNO + NO + RSH \quad \text{(Eqn. 6)}$$

Although NO-iron interactions are by far the most important in biological systems, NO-copper interactions are also common.[4] NO can undergo a series of redox reactions with copper-containing proteins, mainly involving charge transfers and the formation of NO^+.[9]

1.1.3. NO and carbon or nitrogen

The most biologically important nitration at a carbon atom is that of the 3-position carbon of tyrosine residues, Scheme 1.1.

Tyrosine 3-Nitro-tyrosine

Scheme 1.1: Nitration of peptide-based tyrosine

Tyrosine nitration seems to be an important event during inflammation[10] and it might even contribute to some of the tissue damage. Several species can promote the nitration of the phenolic carbon, namely peroxynitrite ($ONOO^-$) and nitrogen dioxide ($NO_2^{\bullet}$).[11]

Reaction of NO with primary amines leads to deamination, whilst reaction with secondary amines leads to the formation of potentially mutagenic nitrosoamines.[12] *N*-Nitrosation can have devastating effects on DNA, potentially leading to cancer.[13]

1.1.4. NO and sulfur

NO is known to react rapidly with thiols, in the presence of an oxidant, to give *S*-nitrosothiols.[14] The mechanism that has been suggested involves transfer of the nitrosonium ion (NO^+) onto the thiol. N_2O_3 is a good nitrosating agent since it can be viewed as $NO^+ NO_2^-$. *S*-Nitrosothiols have been shown to occur endogenously in various tissues, including blood plasma, airway fluid, platelets and white blood cells.[5] Their role is not fully understood. They can easily *trans*-nitrosate with other thiol-containing entities, such as low molecular weight thiols or cysteine residues on proteins.[15] *S*-Nitrosothiol decomposition can be thermal, photochemical or copper-catalysed.[16] The decomposition was found to be catalysed by Cu^+ ions by the following mechanism:

$$Cu^{2+} + RS^- \leftrightarrow RSCu^+ \rightarrow RS^{\bullet} + Cu^+ \qquad \text{(Eqn. 7)}$$

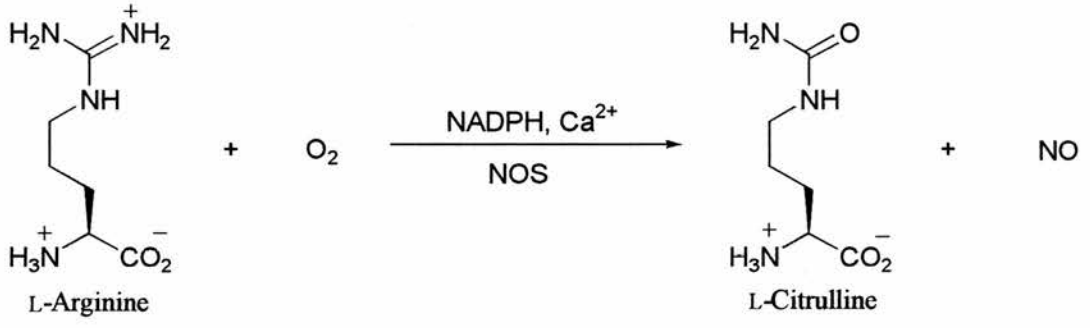
$$RSNO + Cu^+ \rightarrow RS^- + Cu^{2+} + NO \qquad \text{(Eqn. 8)}$$

Copper(II), abundant *in vivo*, is converted to copper(I) by thiolate anion (Eqn. 7), almost always present in conjunction with *S*-nitrosothiols. It is copper(I) that then catalyses the decomposition of the RSNOs (Eqn. 8), yielding NO, thiolate and copper(II), hence fuelling a chain reaction.

S-Nitrosothiols will be discussed in more depth in section 1.6.6.

1.2. NO biosynthesis

Endogenously, NO is produced by nitric oxide synthase (NOS). This enzyme has been found in a variety of cell types and it is expressed in two main forms: (1) a constitutive form, regulated by Ca^{2+} and calmodulin, and (2) a cytokine-inducible form.[17] All of the NOS enzymes require NADPH, tetrahydrobiopterin (BH4) FAD and FMN as cofactors[17] (Scheme 1.2).



Scheme 1.2: Enzymatic formation of NO

The enzyme shares a significant sequence homology to cytochrome P_{450} and it has been demonstrated that an iron-protoporphyrin IX similar to that of cytochrome P_{450} is involved in the conversion of arginine to citrulline.[18]

The fact that NOS has been isolated from a variety of cell types implies that the production of NO is vital for the maintenance of homeostasis of various organs and systems. Furthermore, since NOS has been identified in cells such as macrophages, it is clear that NO is also involved in the cellular response to xenobiotics.[18]

Three isoforms of NOS have been identified: an endothelial isoform (eNOS), a neuronal isoform (nNOS) and an inducible isoform (iNOS).[19] The neuronal and

endothelial forms are constitutive, whilst iNOS is absent in normal cells but is induced in response to bacterial products or immunoactive cytokines.[19] The three isoforms have similar biochemical properties and catalyse the same reaction, but differ in their primary structure and molecular weight. They all are homodimeric and their weight varies between 130 kDa (iNOS) and 160 kDa (nNOS). The main difference seems to be the method of regulation (Figure 1.1).

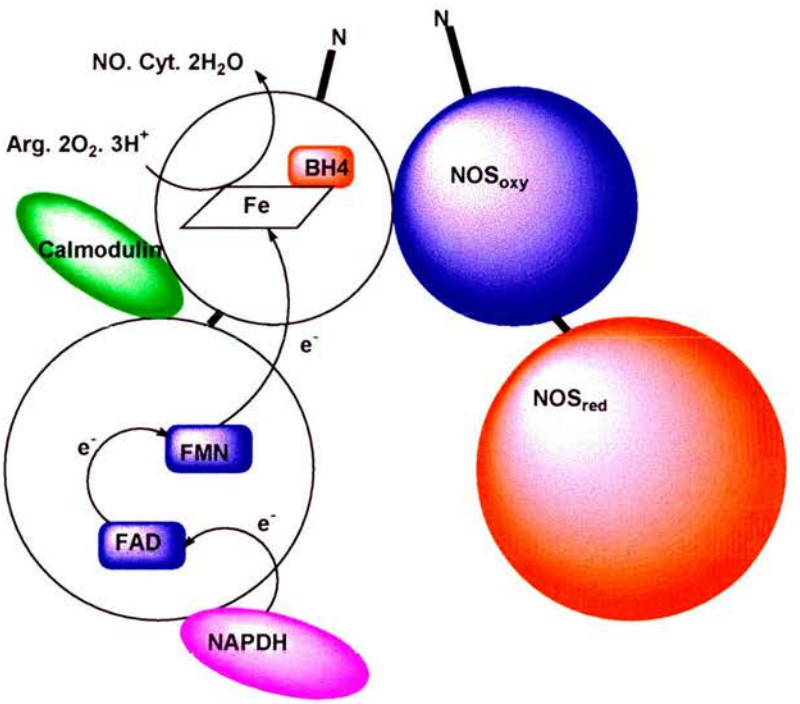


Figure 1.1: Schematic diagram of NOS

The constitutive forms are strictly calcium and calmodulin dependent and are activated by hormones and neurotransmitters that open channels that allow the influx of calcium ions into the cell.[19] In contrast, the activity of iNOS is independent of calcium levels, as the enzyme binds calmodulin even in the absence of calcium ions.[20] Once expressed, this isoform is permanently switched on and provides a non-specific source of NO at destructive concentrations. Regulation of iNOS occurs through a combination of mechanisms, such as control of gene expression and substrate/cofactor availability[19]

The activity of eNOS is implicated in vasodilation and maintenance of vascular tone as well as inhibition of platelet aggregation and adhesion and inhibition of smooth muscle cell proliferation.[18] Deficiency in this enzyme is linked to hypertension and other vascular diseases.[21] The activity of nNOS is associated with signal transduction in central and peripheral neurons.[22]

1.3. NO's biological target: soluble Guanylate Cyclase (sGC)

Soluble guanylate cyclase (sGC) was first identified as a constituent of mammalian cells in the late 1960s.[23,24] A decade later, a number of groups found that sGC was activated by NO[25] and that thiols seemed to be necessary to aid activation by such NO-donors as nitroprusside and nitrosoguanidine.[26] In 1982, Ignarro and co-workers successfully managed to purify sGC in the presence of its haem moiety, a task that had eluded many groups up to then.[27] This finally proved that the haem was necessary for the activation of sGC by NO.

It is now known that sGC is a heterodimer, with the α subunit weighing 73-88 kDa and the β subunit weighing 70 kDa.[28] Each subunit has three domains (Figure 1.2).

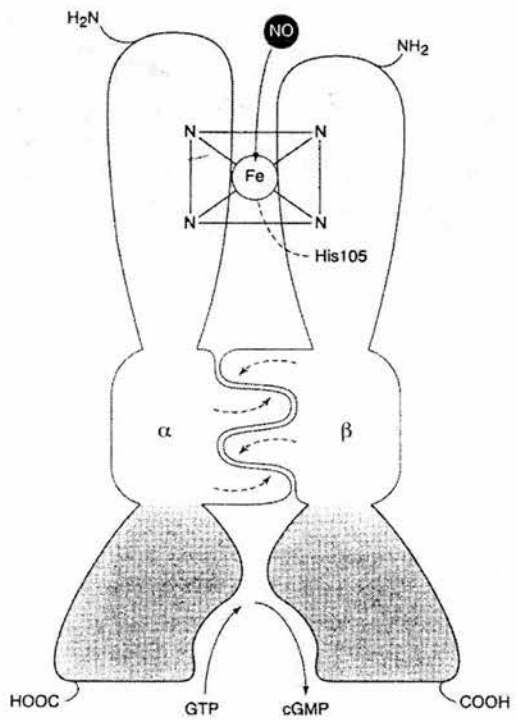


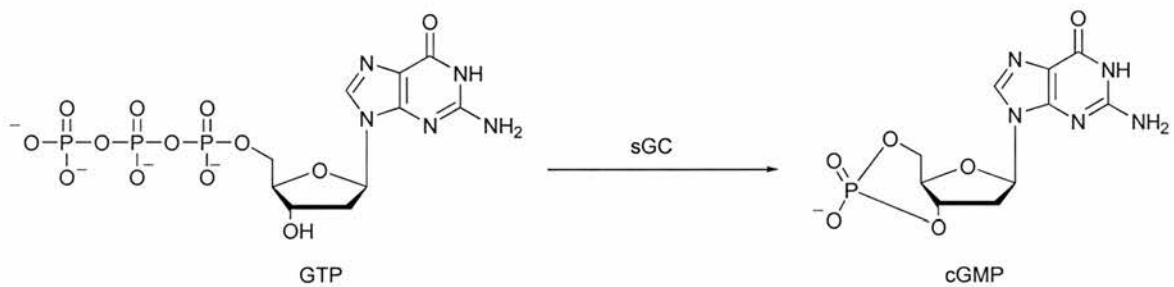
Figure 1.2: Schematic representation of NOS. (Diagram from A. J. Hobbs, 1997[29])

The *N*-terminus has a haem-binding domain. This region is not very well conserved, with the exception of His 105 in the β subunit, the axial ligand to the haem iron. It is not known whether there are one or two haems per dimer, since there does not appear to be a conserved His in the α subunit that would mirror the function of His 105 on the β subunit.[29] The C-terminus consists of a catalytic domain. This region has a very high degree of homology with the catalytic domain of adenylate cyclase.[29] Between the

N- and the *C*-termini, there is a dimerisation domain, the region of the protein that holds the two subunits together. Both the catalytic and the haem-binding domains are necessary for the activity of sGC.[29]

The haem is known to have a 5-coordinate iron that is high spin both in the ferrous and ferric states. His 105 is the sole axial ligand.[28] Interestingly, sGC does not have a high affinity for oxygen, but a very strong one for NO. The reason for this is that upon binding of NO to iron, the bond to the axial His is broken, hence restoring the iron to its 5-coordinated state. The binding of oxygen, or carbon monoxide, does not seem to bring about the breakage of the iron-His bond, hence leaving the iron in a 6-coordinated state, which is, presumably, unfavourable.[28]

sGC catalyses the conversion of guanosine-5'-triphosphate (GTP) to cyclic guanosine-3',5'-monophosphate (cGMP) (Scheme 1.3).



Scheme 1.3: Conversion of GTP to cGMP by sGC

This conversion was found to be dependent on Mn^{2+} or Mg^{2+} ions.[29] The exact role for these cations is not yet fully understood. It is also known that Cu^{2+} and Cu^{+} are present and they are thought to aid the decomposition of *S*-nitrosothiols.[29] Not much is known about the activation of sGC, but the deactivation of the enzyme seems to take only a few seconds, which does not correlate to the kinetic data that shows the dissociation of NO from a 5-coordinated haem-iron can take up to 4 min at physiological pH and temperature. However, Sharma and co-workers, have demonstrated that, in the presence of Mg^{2+} and GTP, the dissociation of NO from 5-coordinated iron is very fast, with the species having a half life of 5 seconds at 37°C.[30] This data correlates exactly with the proposed half life of activated sGC.

1.4. NO in the body

NOS has been found to be present throughout the body, hence suggesting that NO has a role to play in a number of biological functions. The following are a few examples of NO function in various physiological contexts.

1.4.1. NO and the nervous system

The clearest role for NO within the nervous system has been found in the peripheral autonomic nervous system which regulates involuntary bodily functions and more specifically in the non-adrenergic-non-cholinergic (NANC) nerves.[31] These are important in the innervation of many organs. The nature of the transmitter has been much debated, but it is now widely accepted to be NO. This means that the NANC nerves make use of NO as a neurotransmitter. For example, in the gastrointestinal tract NO seems to mediate many forms of muscle relaxation, including stomach dilation following the ingestion of food,[32] and intestine muscle relaxation in peristalsis.[33] Therefore, the tone of the gastrointestinal tract seems to be critically dependent on NO.

NO is also responsible for the dilation of blood vessels and smooth muscle of the corpus cavernosum and hence development of penile erection.[34] Evidence of NO-containing nerves has been found in penile tissue[34] and other work has indicated a dysfunction of the NO system as a possible cause of male impotence.[35] Studies on both rat and canine tissue have shown that NOS is widely distributed throughout the urogenital tract, especially in the pelvic ganglia, the urethra, the bladder neck and the penis,[36] suggesting that NO may be important in the regulation of urine continence. The widespread system of NANC nerves in the body, correlates with the use of NO as a signal for muscle relaxation.

As far as the central nervous system is concerned, neurotransmission by agents such as acetylcholine and glutamate has long been known to be associated with elevated levels of cGMP in the brain, particularly in the cerebellum.[37] In 1977, NO was shown to stimulate soluble guanylate cyclase in rat cerebellum.[38] Since then, cytosolic preparations of various types of brain tissue showed that NOS is present in the brain, with the highest concentrations being found in the cerebellum.[39] The precise role for NO

in the brain is still not entirely clear. However, some findings suggest an involvement in seizures.[37] NO is also thought to be involved in synaptic plasticity,[40] a process by which neuronal connections are re-enforced or altered. NO is thought to be involved in a number of cerebral functions such as memory and complex automated motor functions. Part of this process involves a retrograde messenger able to return from the post-synaptic neurone to the pre-synaptic one. This messenger has been postulated to be NO.[40] (Figure 1.3)

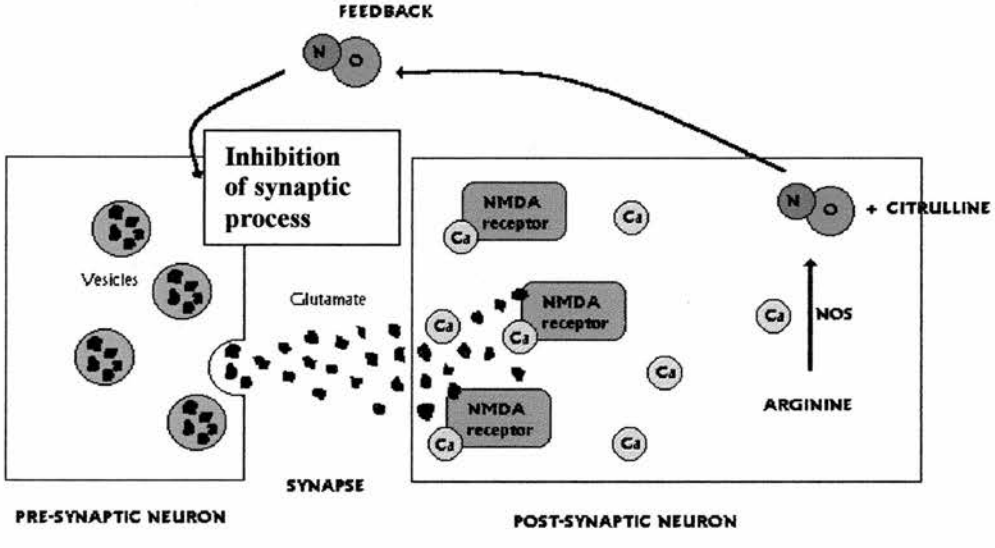


Figure 1.3: The role of NO in neural transmission

NO generated in the brain may be linked to an increase in cerebral blood flow,[41] however, it could also be associated with neurodegeneration. Overproduction of NO in the brain may be linked with senility and it has also been implicated in brain tissue damage that occurs after strokes.[1] During a stroke, the brain is starved of oxygen, so the arginine-NO pathway is blocked. The body then reacts by increasing the concentration of NOS in the brain. When reoxygenation occurs, there is a sudden burst of NO and other cytotoxic agents and this causes the death of brain tissue.[1] Inhibition of NOS before reperfusion would significantly decrease the damage, provided that the inhibition was not total and that it did not affect vital NO-dependent mechanisms such as cerebral blood vessel dilation and platelet de-aggregation.

1.4.2. NO and the respiratory system

Work with NO in the respiratory system provides the most direct evidence of the presence of NO in humans.[42,43] Using gas chromatography linked to mass spectrometry (GC-MS), scientists have discovered that there is NO in human breath.[42] It has also been possible to demonstrate that the levels of NO in human breath increase during physical exercise. The physiological and cardiovascular changes during exercise are profound and increased blood flow and reduction of oxygen levels both contribute to the elevation of NO release by the endothelium.[44,45]

The discovery of NO in human breath has led to the study of asthma and pulmonary hypertension to determine the role of NO in such conditions. Asthmatics exhale larger amounts of NO than normal and they express inducible NOS in lung tissue.[42] The implications of these findings are not clear, but it is thought that they are a reflection of a generalized inflammatory response in the lungs.

Treatment of patients with pulmonary hypertension or asthma often involves the administration of vasodilators. Their effect is to dilate all pulmonary arteries, hence a generalized reduction of systemic blood pressure is often a possible side effect. Prolonged administration of NO gas, followed by withdrawal has been found to successfully improve lung function and augment blood flow and oxygen exchange.[42]

1.4.3. NO and the immune system

The immune system provides a wide range of defense mechanisms against any xenobiotic agents. It has been discovered that NO is also involved in these mechanisms.[46] The first indication of this was the discovery that high levels of nitrates were found in people suffering from gastroenteritis.[47] It was then found that macrophages (one of the main groups of cells involved in immune response) also produce nitrates,[48] and that this production is dependent on the availability of arginine. These observations led to the conclusion that the arginine-NO pathway was present in macrophages. NO is therefore a natural cytotoxic agent and is synthesised as a defense mechanism against foreign or abnormal cells in the body.[48]

Initially it was suggested that the cytotoxicity of NO was derived solely from its radical character, but, unlike most radicals, it is not very reactive.[2] NO produced by

macrophages reacts with superoxide to give the peroxynitrite ion (Eqn. 9).[49] This ion is proposed to be the cytotoxic agent used by macrophages. It is partially protonated at physiological pH (Eqn. 10) to give peroxynitrous acid (pKa = 6.80) which then decomposes to give nitrogen dioxide and cytotoxic hydroxyl radicals (Eqn. 11).[6]

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^- \qquad \text{(Eqn. 9)}$$

$$ONOO^- + H^+ \rightarrow ONOOH \qquad \text{(Eqn. 10)}$$

$$ONOOH \rightarrow NO_2^{\bullet} + HO^{\bullet} \qquad \text{(Eqn. 11)}$$

However, not all experimental data supports this theory. It has been suggested that peroxynitrite directly interacts with cellular targets rather than producing hydroxyl radicals.[50] Another possibility is that NO directly reacts with iron in haemoglobin to give a range of iron-nitrosyl species.[51] This reaction could lead to the destruction of iron-sulfur clusters either in the respiratory system of bacteria or in various cellular proteins. Many bacteria and pathogenic microorganisms have been found to be more sensitive to N_2O_3 and $ONOO^-$ rather than to $NO^{\bullet}$ itself.[52]

1.4.4. NO in the vasculature

The role of NO in the vasculature is very complex. It is known to prevent platelet aggregation and smooth muscle cell proliferation and to be the main agent in causing the relaxation of smooth muscle in blood vessels, hence being an important regulator of blood flow. It is also known to interact very strongly with haemoglobin, the consequences of which are not yet fully understood.

1.4.4.1. NO and platelet aggregation

Platelets are free-flowing sub-cellular fragments found in plasma. They do not possess a nucleus but they are capable of a complex variety of reactions that are vital for

the healing of damaged blood vessels.[53] They are produced in the bone marrow and are about 2μm in diameter and contain mitochondria, tubules, granules and lysosomes. Their plasma membrane has an external glycoprotein coat.[53]

When an artherosclerotic plaque is ruptured or a vessel is cut or injured, the endothelium is damaged and the sub endothelial tissue is exposed. This layer consists mainly in collagen. Platelets interact strongly with collagen, which induces a shape change, causing them to adhere and become activated. Activation consists in the secretion of a variety of substances that cause fibrin to aggregate over the platelets. This process leads to the closure of the cut in the vessel, or, in the case of the artherosclerotic plaque, to a dangerous constriction in the vessel[53] (Figure 1.4).

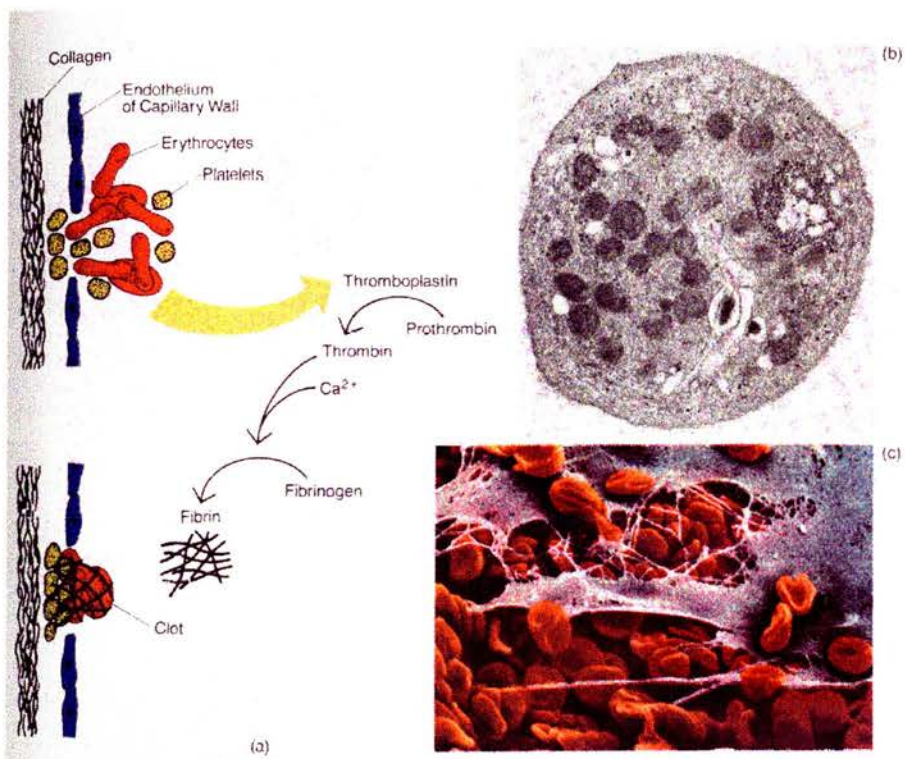


Figure 1.4: (a) schematic of the events that lead to the formation of a clot. (b) electron micrograph of a platelet (22,000 magnification). (c) a network of fibrin (1,250 magnification). (Diagram from Wessels and Hopson, *Biology*, Random House, New York, 1988; reproduced with permission by McGraw-Hill Publishers)

The process of aggregation has to be finely controlled by the vessel to avoid excessive clotting that might lead to the blockage of the vessel. The endothelial cells lining the vessel secrete substances that inhibit platelet aggregation. One of these is NO.[54]

NO has been shown to inhibit platelet aggregation *via* a cGMP-dependent mechanism.[55] It was also discovered that NO inhibits platelet adhesion to collagen fibrils and endothelial cells.[55,56] It was originally believed that NO triggered cGMP activation and thus prevented aggregation. However, studies showed that platelet aggregation is accompanied by an increase of cGMP levels within the platelets themselves, even in the absence of any exogenous NO.[57] It has become clear that platelets do themselves generate NO *via* the arginine-NO pathway as a feedback mechanism to regulate aggregation.[58]

1.4.4.2. NO as a vasodilator

Moncada and Ignarro demonstrated that NO is produced in endothelial cells of blood vessels and that it diffuses into smooth muscle cells where it causes relaxation.[59,60] They also discovered that the way NO acts as a secondary messenger is by the activation of guanylate cyclase.[61] This enzyme is found in the smooth muscle cells in arteries where it is involved in the production of cGMP, this in turn triggers arterial smooth muscle relaxation *via* a series of protein phosphorylations.[62] In doing so, Moncada and Ignarro helped clarify the overall mechanism by which arterial relaxation occurs. The process by which acetylcholine (or any endogenous, endothelium-dependent vasodilator) causes the relaxation of vascular smooth muscle is shown in Figure 1.5.[63,64]

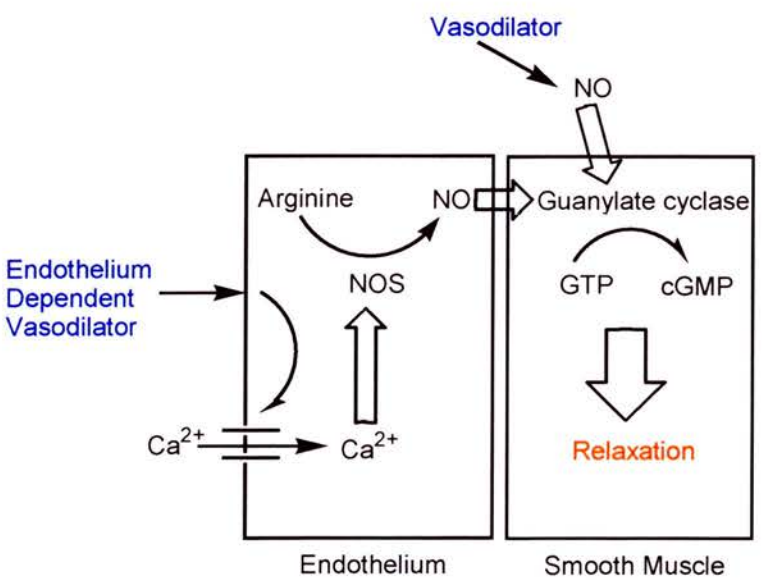


Figure 1.5: Mechanism for arterial smooth muscle relaxation

Acetylcholine released in the body stimulates receptors in endothelial cells inside the artery and a channel is opened to allow the uptake of Ca^{2+} ions. This activates NOS, which converts L-arginine into L-citrulline and NO. The latter diffuses into the smooth muscle layer and binds strongly to the haem group in guanylate cyclase. NO-activated guanylate cyclase converts GTP into cGMP. Conversion of GTP into cGMP is the first step towards muscle relaxation.[61,62] An exogenous vasodilator (an NO-donating compound) facilitates the direct endothelium-independent activation of guanylate cyclase.

It appears that the clinical consequences of a defect in NO production in the circulatory system are two-fold. The overproduction of NO can cause an overall progressive reduction in systemic blood pressure, one of the consequences of septic shock. This condition can be fatal, especially since it is also characterised by resistance to exogenously administered vasoconstrictors.[1,61] Conversely, reduced production of NO can lead to vasoconstriction, platelet aggregation and arteriogenesis and hence to life-threatening conditions such as high blood pressure, angina and myocardial infarction.[1,61]

1.4.4.3. NO, haemoglobin and blood flow

It has been known ever since NO was found to be involved in vessel dilation, that it reacts rapidly with haemoglobin (Hb).[65] Indeed, experiments on isolated arteries often involve perfusion with oxyhaemoglobin (HbO_2) to confirm that the process under investigation is NO-dependent.

There are two main reactions between NO and Hb, one with oxyhaemoglobin (HbO_2) and one with deoxyhaemoglobin (Hb).[65,66]

$$HbO_2 + NO \longrightarrow NO_3^- + metHb \qquad \text{(Eqn. 12)}$$

$$Hb + NO \longrightarrow Hb\text{-}NO \qquad \text{(Eqn. 13)}$$

NO can react with HbO_2 to give nitrate and methaemoglobin (metHb) (Eqn. 12), or it can react with Hb and form a stable iron-NO complex (Eqn.13). The picture is further complicated by the fact that Hb is a tetramer and can be partially, as well as fully, oxygenated.

It was pointed out[67] that the concentration of Hb in blood is such that, given certain simplifying conditions, all the NO produced in the endothelium would be scavenged and therefore none would be able to diffuse into the smooth muscle and cause relaxation (Figure 1.6). It was therefore concluded, arguably somewhat rashly, that an insufficient amount of NO to activate sGC would enter the smooth muscle and that it could not be the molecule that brought about vasodilation. There are, however, a number of simplifications to the model described by Lancaster and three groups[68-70] have refined his model to arrive at very different conclusions from the original paper.

The principal effect of blood flow is to create a red blood cell-free zone adjacent to the endothelium. However, Liao and coworkers[71] have shown experimentally that, in the absence of flow, NO consumption of red blood cells is one thousand times slower than that by free Hb. This cannot be due to the lipid membrane containing the Hb as NO is more soluble in lipid than water. An explanation proposed by Liu et al[69] is that there is a layer of water surrounding each red blood cell and diffusion across this reduces the rate of reaction between NO and Hb by a factor of 10^3. The presence of a red blood cell-free zone and the layer of water surrounding red blood cells could account for the 10^6 reduction in the rate of reaction between NO and Hb *in vivo* when compared to a homogeneous medium *in vitro*. It is therefore still reasonable to conclude that NO is the EDRF.

When – following Lancaster's paper - there appeared to be considerable doubt that locally produced NO was responsible for vessel dilatation, Stamler's group[72] suggested that the NO required for vasodilation was transported around the body on Hb, probably as a *S*-nitrosothiol. What is most significant in this paper is the report that, in the reaction between NO and Hb to give Hb-Fe(II)-NO, much of the NO is unaccounted for and it is proposed to nitrosate the cysteine on the β-chain of Hb to give Hb-SNO. Exactly how this happens is not clear, as NO itself will not nitrosate thiols, although NO could be oxidised to N_2O_3 by dioxygen present in the plasma and then nitrosate the thiol (Eqn. 14):

$$\text{Hb-SH} + N_2O_3 \longrightarrow \text{Hb-SNO} + HNO_2 \qquad \text{(Eqn. 14)}$$

This is an unsatisfactory aspect of Stamler's hypothesis as the rate of reaction between NO and dioxygen at physiological concentrations is slow,[73] a much slower reaction than that between NO and the iron from Hb. However, there is no doubt that

formation of Hb-SNO occurs and it is this species that was proposed by Stamler[72] to be responsible for the transport of NO around the body. The nitrosation reaction is proposed to occur in the lungs, where there is an abundance of NOS.[72]

Stamler's ideas also seem to overlook the presence of NOS in the endothelium. In fact, there would be no need for eNOS to produce NO if it was carried to oxygen-depleted tissues by Hb.

Moncada and coworkers[74] examined the nitrosation of Hb by low molecular weight *S*-nitrosothiols and it is clear, by mass spectroscopy, that more than one thiol on Hb can be nitrosated. The vasodilatory action of Hb-SNO is completely annulled by the presence of HbO_2 and this makes it difficult to see how, in the presence of oxygenated blood, it could function as an endogenous vasodilator.

In summary, although various groups have attempted to determine the role of NO transportation by Hb, it is still not fully understood how this comes about. Hb can bind NO at the iron or at a cysteinyl thiol, but the physiological significance of either reaction is still unknown.

1.5. Current treatments for NO-related diseases

As shown in previous sections, NO is involved in a number of physiological pathways and in each of these a slight imbalance in the amount or the location of NO production could lead to serious pathological implications. Here we examine a number of common diseases caused by a lack of NO and discuss the possible therapeutic benefit of NO-donor compounds.

1.5.1. Coronary bypass surgery

NO regulates the vascular tone of blood vessels.[61] For a number of different reasons, including continuous smoking or a high cholesterol diet, fatty deposits can accumulate in various areas of the circulatory system.[75] These fatty deposits damage the underlying endothelium and therefore the production of NO is impaired. This leads to an even greater accumulation of fatty substances in the area and an influx of platelets.

The platelets, whose role is to repair any damage, actually have a negative effect in this situation, eventually leading to the formation of a blockage in the blood vessel. This can then lead to myocardial infarction (heart attack) and cerebral ischaemia (stroke), depending on where the blockage is formed.[76]

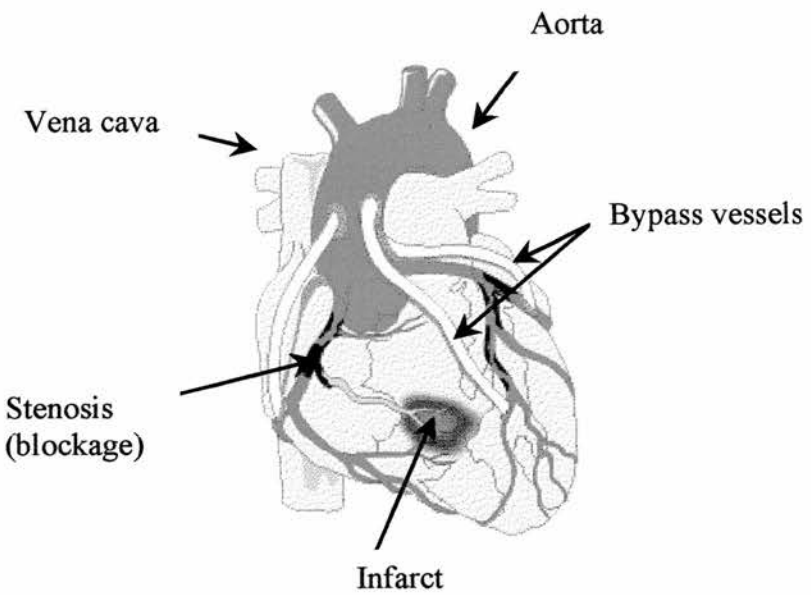


Figure 1.7: Schematic of a by-passed coronary artery (Diagram reproduced from Bodyworks 6.1 CD Rom with permission by the publishers The Learning Company)

Figure 1.7 shows one of the current treatments for blocked coronary arteries, namely by-pass surgery. The areas of the artery that are blocked are represented as black and the veins used to bypass the blockage are represented in white. Although a commonly used and very successful procedure, by-pass surgery is not 100% safe. The pieces of vein or artery used to by-pass the blockages are taken from the patient's leg or chest and, in the effort to check for any leakages, saline solution is pumped through them for a number of minutes. This, unfortunately, removes the layer of endotheial cells, hence removing the only source of NO within the vein. It is no surprise, therefore, that about 5% of patients experience a blockage in the by-pass grafts shortly after surgery; this is due to accelerated artherosclerosis, favoured by the absence of NO. This effect is named restenosis.[76] The endothelial layer within the vein grafts regrows within a couple of weeks, after which the tone of the vessels will be restored and the possibility of restenosis is much diminished.

Currently, during surgery, patients are given vasodilators to help keep the blood vessels relaxed and anticoagulants to stop thrombosis. Another possible solution is to

bathe the vein in a vasodilator (more specifically a vasodilator that targets endothelium-denuded vessels and has a sustained effect of a number of hours) before the veins are grafted onto the patient's heart. By doing so, the vasodilator will be administered in the most direct and specific way possible, therefore not affecting the vascular tone of the rest of the circulatory system.

1.5.2. Percutaneous transluminal balloon angioplasty

For patients who do not require by-pass surgery but that have partially blocked arteries, a commonly used solution is percutaneous transluminal balloon angioplasty (PTBA) (Figure 1.8).

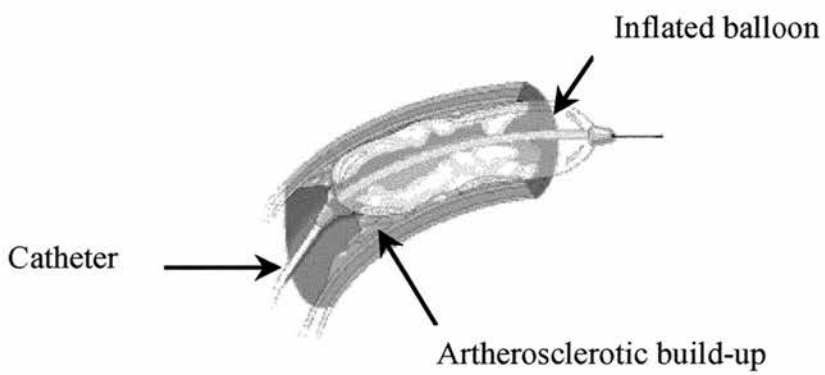


Figure 1.8: Schematic representation of balloon angioplasty (Diagram reproduced from Bodyworks 6.1 CD Rom with permission by the publishers The Learning Company)

This technique involves entering the patient's femoral artery at the groin level with a special catheter. This is manipulated along the patient's coronary artery to the site of blockage where a special ballon is inflated. This pushes the walls of the blood vessel and the blockage outward, hence creating a free passage for the blood to flow. The balloon is then deflated and the cannula taken out. This procedure is a highly invasive one and it has a 25% failure rate, i.e. the patient develops restenosis and the blockage reappears in a matter of days.[77] Endothelial damage caused by passing the cannula and balloon into the artery, is a key event in restenosis. Here too the patients are given vasodilators and antithrombotic drugs to help maintain the artery relaxed and avoid restenosis. A variation of balloon angioplasty places a metal stent over the area affected by the blockage, hence keeping it open for a longer period of time.[78]

Unfortunately restenosis also tends to occur with stents, but to a lesser degree than with angioplasty. Research on the possible coating of the metal stent with an NO donor is currently ongoing.[79]

1.5.3. NO as the illness and as the cure

NO itself can be successfully used in the treatment of certain diseases; such is the case for respiratory diseases. Inhaled NO can reverse pulmonary hypertension and low concentrations of NO can protect against adult respiratory distress syndrome.[80,81]

Even though a release of NO can be beneficial, an excess of NO in the vascular system can lead to septic shock and eventually to death.[1] A treatment for this consists of the inhibition of NOS with specific inhibitors, but the dosage of these must be very carefully regulated so that there is no hypertension and platelet aggregation due to the sudden lack of NO in the system.[82] It is therefore vital that the concentration of NO in the vascular system is maintained within very strict margins.

As far as the nervous system is concerned, overproduction of NO is associated with cerebral ischaemia and epilepsy as well as with Alzheimer's, Parkinson's and AIDS-related dementia.[83] Within the peripheral nervous system, NO is known to be crucial in maintaining the tone of a number of organs. A reduction in the levels of NO can lead to conditions such as impotence and incontinence.[35,36] Arthritis and inflammation can be directly linked with overproduction of NO and therefore treated with NOS inhibitors.[84,85]

It is clear that NO has a dual nature. On one side it is protective and on the other it is highly destructive. Any imbalance of the concentration of NO can lead to the development of a number of life-threatening diseases.

1.6. NO Donors

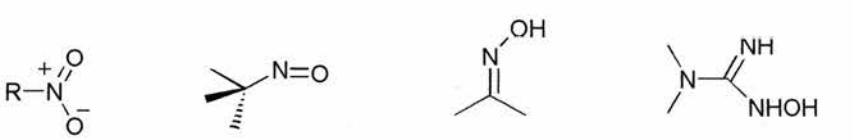
Our group has mainly focused on conditions brought about by a lack of NO and more specifically on conditions within the circulatory system. A number of NO-donor compounds are currently used as treatment for conditions such as angina and as a postoperative prophylaxis in bypass surgery and balloon angioplasty.

Wang and co-workers[86] have recently published a comprehensive review describing current trends in the development of NO-donors. It is therefore redundant to attempt to do the same here. Instead, a brief overview of the various classes of NO-donors will be given, with a specific emphasis on *S*-nitrosothiols, the class of molecules we chose to work with.

The majority of NO-donors are organic compounds, with only a small number of transition metal-NO complexes. All NO-donors can be classified into 6 different categories, depending on what atom the NO is attached to. The are *C*-NO donors, *N*-NO donors, *O*-NO donors, *S*-NO donors, heterocyclic-NO donors and transition metal-NO donors.

1.6.1. *C*-NO donors

C-NO donors consist of nitro and nitroso compounds and guanidine or oxime related compounds. The *C*-nitro compounds are relatively unstable and release NO photochemically or thermally. The guanidine and oxime compounds release NO upon oxidation by an enzyme.



Nitro Nitroso Oxime *N*-Hydroxyguanidino

Figure 1.9: Examples of *C*-NO donors

1.6.2. *N*-NO donors

There are four classes of *N*-NO donors: *N*-nitrosamines, *N*-hydroxy-*N*-nitrosamines, *N*-nitrosoamides and diazeniumdiolates.

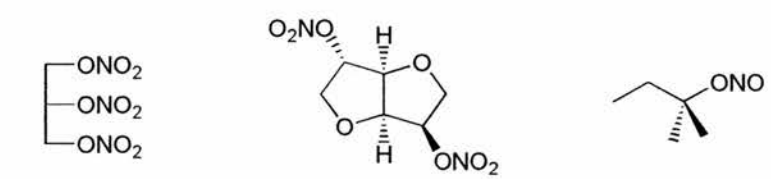
N-Nitrosamines | *N*-Hydroxy-*N*-nitrosamines | *N*-Nitrosoamides | Diazeniumdiolates

Figure 1.10: Examples of *N*-NO donors

The *N*-nitrosamines and *N*-nitrosoamides are known carcinogens and therefore are not used clinically as NO-donors. *N*-Hydroxy-*N*-nitrosamines are widely employed as metal chelators and they are believed to generate NO *via* biotransformations. The diazeniumdiolates are widely used as *in vitro* NO-donor compounds. They are stable as solids and spontaneously decompose in solution to give two molecules of NO. Their half-lives range from 2 seconds to 2 hours at physiological pH and 37°C.

1.6.3. *O*-NO donors

O-NO donors consist of organic nitrates and nitrites. The most widely used for the treatment of angina is glyceryl trinitrite, invented by Alfred Nobel over a century ago and still prescribed today. The exact pathway by which organic nitrates and nitrites are activated *in vivo* is still not known.



Glyceryl trinitrate | Isosorbide dinitrate | Amyl nitrite

Figure 1.11: Examples of *O*-NO donors

1.6.4. Heterocyclic NO-donors

Heterocyclic NO-donors are mainly sydonimines or furoxans.

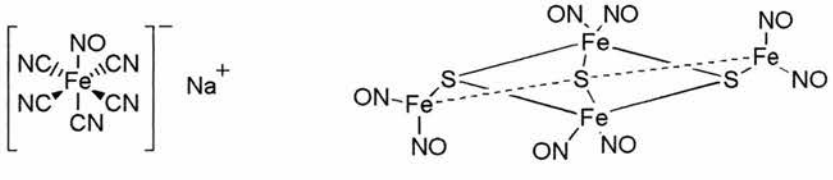
Sydonimines Furoxans

Figure 1.12: Examples of heterocyclic-NO donors

The sydonimines are initially thought to be metabolized by liver esterase and finally oxidised to release NO and the superoxide anion. This then produces peroxynitrite and therefore leads to a series of side reactions. The furoxans need thiols to aid the production of NO. Unfortunately a number of these compounds have been found to have mutagenic properties.

1.6.5. Transition metal-NO donors

Sodium nitroprusside is currently one of the most administered NO-donors in the clinical situation.[87] It has been used for over a century, despite a number of cases of cyanide poisoning arising from its use.[88]



Sodium nitroprusside Roussin's black salt

Figure 1.13: Examples of transition metal-NO donors

The exact mechanism of NO release is still unclear, however it is known that NO is released from the metal as NO^+ and therefore there must be a subsequent single-electron reduction step. Other transition metal NO donors are generally iron-sulfur clusters. The mechanism by which they release NO is still unclear.

1.6.6. *S*-Nitrosothiols

S-Nitrosothiols are formed by the electrophilic nitrosation of a thiol group.[89]

$$RSH + HNO_2 + H^+(cat.) \longrightarrow RSNO + H_2O$$

This conversion can be achieved by using other nitrosating agents such as alkyl nitrites,[90] nitrosyl chloride,[91] N_2O_3[92] or N_2O_4.[93] Effectively, any carrier of NO^+ will convert a thiol to an *S*-nitrosothiol.

S-Nitrosothiols are red or green and have absorptions at 330-350 nm ($\varepsilon \sim 1000$ mol^{-1} dm^3 cm^{-1}) and a smaller absorption at 550-600 nm ($\varepsilon \sim 50$ mol^{-1} dm^3 cm^{-1}). They decompose thermally or photochemically to give the disulfide and NO:

$$2RSNO \rightarrow RSSR + 2NO \qquad \text{(Eqn. 15)}$$

In 1993 the Williams group, in conjunction with the Butler group,[94,95] identified copper ions as an other agent that can bring about the decomposition of *S*-nitrosothiols. It was found that Cu^+ was the active catalyst for decomposition but Cu^{2+} could readily be converted to Cu^+ by small amounts of thiolate anion, usually present as impurities is *S*-nitrosothiol samples (Eqn. 16 and 17).

$$Cu^{2+} + RS^- \rightarrow RS^\bullet + Cu^+ \qquad \text{(Eqn. 16)}$$

$$RSNO + Cu^+ \rightarrow RS^- + Cu^{2+} + NO^\bullet \qquad \text{(Eqn. 17)}$$

S-Nitrosothiols can also be converted to disulfides by reducing agents such as $NaBH_4$[96] and they can be oxidised to *S*-nitrosothiols by oxidising agents such as HNO_3 and N_2O_4.[96,97]

Biologically, one of the most important reactions undergone by *S*-nitrosothiols is *trans*-nitrosation (Eqn. 18)[93,98]

$$RSNO + R'SH \rightarrow RSH + R'SNO \qquad \text{(Eqn. 18)}$$

The facility with which *S*-nitrosothiols transfer NO to other thiols has been exploited by various groups in the attempt to inhibit enzymes which possess an active-site cysteine. Nitrosation at the active-site thiol can reversibly inhibit an enzyme. Targets for this type of modification include HIV protease[99] and transcriptional activators.[100]

1.7. Penicillamine

The amino acid penicillamine was first described in 1943 by Abraham, Chain and Barker[101] who identified it as a product of the decomposition of penicillin. It was first used therapeutically by Walshe[102] in 1956 as a treatment for Wilson's disease, an inborn defect in copper metabolism. In the 1960s and 70s, D-penicillamine was established as an effective treatment of rheumatoid arthritis. However, a high number of undesirable side effects are associated with the use of penicillamine and therefore there is considerable scepticism about its use.

Penicillamine is a trifunctional amino acid and differs from cysteine in that it has two methyl groups replacing the hydrogen atoms at the β-carbon. The thiol group is therefore much more hindered than in cysteine (Figure 1.14).

Figure 1.14: cysteine and penicillamine

As a result, the thiol group is much less reactive than that of cysteine.[103] This has an effect on the number of reactions that penicillamine can undergo *in vivo*. The most common reactions are metal chelation and thiazolidine formation.[103-105] The ability of penicillamine to bind copper(II) ions had already been recorded by Walshe,[102] but it was Birker and Freeman[106] who first showed by X-ray diffraction studies that penicillamine forms a Cu^{I}, Cu^{II} cluster complex. Schonback *et al.*[107] found that the thiazolidine rings formed by D-penicillamine are approximately 12 times more stable than the corresponding cysteine thiazolidines. This could imply that D-penicillamine could, *in vivo,* form stable thiazolidine derivatives with the pyridoxal moiety of PLP-dependent

enzymes (Figure 1.15), thus interfering with their normal catalytic activities. This, in fact, could be one of the main causes of the adverse side effects associated with D-penicillamine treatment.

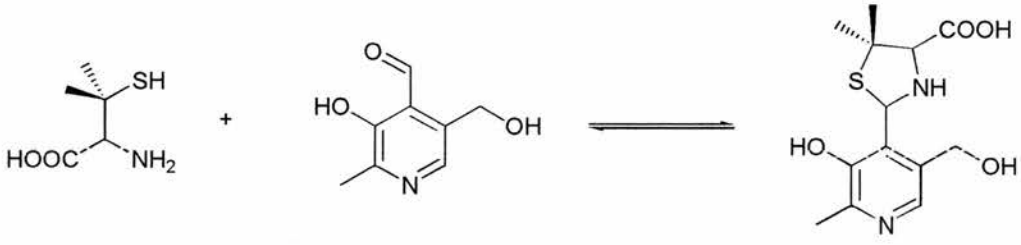


Figure 1.15: Reaction of penicillamine with pyridoxal

1.8. *S*-Nitroso-*N*-acetylpenicillamine (SNAP)

Aside from its role in copper complexation, penicillamine is extremely important within the field of NO research as, in its nitrosated form, it provides one of the most commonly used *S*-nitrosothiols, SNAP (Figure 1.16).

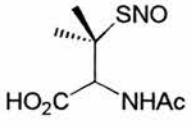
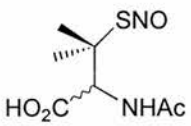


Figure 1.16: *S*-Nitroso-*N*-acetylpenicillamine (SNAP)

In 1981, Ignarro and co-workers[108] used *S*-nitrosocysteine to activate soluble guanylate cyclase. Having observed that this *S*-nitrosothiol markedly increased activation of sGC, they examined other *S*-nitrosothiols, including SNAP. A year later, the same group reported the capacity of *S*-nitrosothiols to inhibit platelet aggregation.[109] Among those tested, SNAP appeared to be the most stable. Thereafter, possibly due to the ease of synthesis, SNAP has been used routinely as a standard for vasodilation.[110-112]

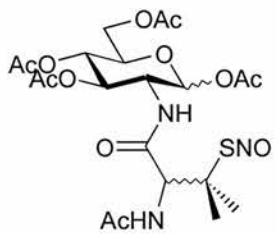
Chapter 2: Results and Discussion - Chemistry

The work carried out during this project is the continuation of a study of *S*-nitrosothiols. The Butler group has already identified two main classes of *S*-nitrosothiol (Figure 2.1) which show good vasodilatory properties.[113-115]



(1)

S-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP)

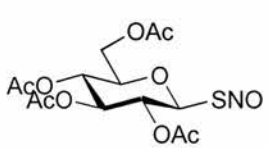


(2)

N-(*S*-Nitroso-*N*-acetyl-D,L-penicillaminyl -1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy -β-D-glucopyranose (GLACSNAP)

Figure 2.1: Two examples of compounds prepared by the Butler group

As discussed earlier, SNAP **(1)** is routinely used as an NO-donor. The synthesis of GLACSNAP **(2)** was achieved by the Butler group in an attempt to increase the uptake of **1** by attaching it to a sugar. It was thought that the presence of the acetylated sugar moiety would aid the transdermal absorption of the compound, based on the observation that SNAG **(3)** (Figure 2.2) acted as a good transdermally delivered vasodilator.[116,117]



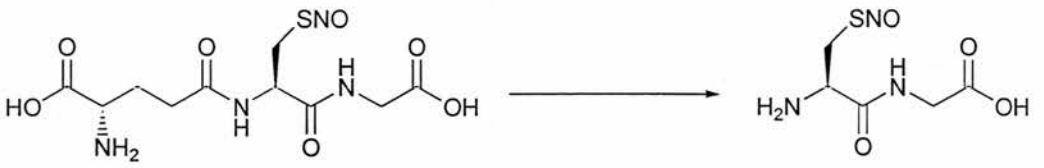
(3)

2,3,4,6-Tetra-*O*-acetyl-1-deoxy-*S*-nitroso-1-thio-β-D-glucopyranose (SNAG)

Figure 2.2: SNAG **(3)**

The aim of this project was to explore structure-activity relationships for **1** and **2** in order to improve their efficacy as vasodilators. Furthermore, the examination of the structure-activity relationships for these classes of molecule would help clarify one of the areas of debate within the NO world. It has been suggested that an enzyme might be involved in the decomposition of *S*-nitrosothiols *in vivo*.[118,119] It is known, for example, that *S*-nitrosoglutathione (GSNO) **(4)**, an endogenous *S*-nitrosothiol, is initially

activated by the removal of the *C*-terminal glutamic acid by the enzyme γ-glutamyltranspeptidase to give *S*-nitroso-cysteinylglycine **(5)** (Scheme 2.1), a much more unstable species than GSNO **(4)** itself, which decomposes readily to give NO and the disulfide.[120]



(4) **(5)**

S-Nitroso-glutathione (GSNO) *S*-Nitroso-cysteinylglycine

Scheme 2.1: Action of γ-glutamyltranspeptidase upon GSNO **(4)**

This enzyme, however, is specific to GSNO **(4)** and does not act upon other *S*-nitrosothiols, such as **1** or **2**.[120] The idea that an enzyme might be involved in *S*-nitrosothiol decomposition has been asserted many times,[118,119] but no hard evidence has been put forward. It was our belief, before this work was undertaken, that such an enzyme was unlikely to exist. *S*-Nitrosothiols decompose so readily, photochemically, thermally or by copper ion catalysis, that the need for enzyme-catalysed decomposition seemed redundant.

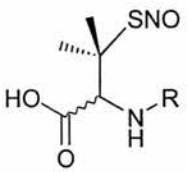
The synthesis of a number of *S*-nitrosothiols, different in physical and electronic properties, could serve as a vehicle for demonstrating the presence, or otherwise, of enzyme-catalysed NO liberation from *S*-nitrosothiols. If all compounds synthesised decomposed *in vitro* and *in vivo* with similar profiles, the involvement of an enzyme would seem unlikely. A number of modifications of both SNAP **(1)** and GLACSNAP **(2)** were therefore explored. These will be described in the following sections.

2.1. SNAP series

2.1.1. Simple SNAP analogues

As part of a study into the activity of SNAP **(1)** as a vasodilator, the Butler group have investigated the effect of *N*-substitution on the biological activity of this

compound.[121] The compounds synthesised and tested were the *N*-propionyl, *N*-valeryl and *N*-heptanoyl derivatives of *S*-nitroso-D,L-penicillamine (Figure 2.3).

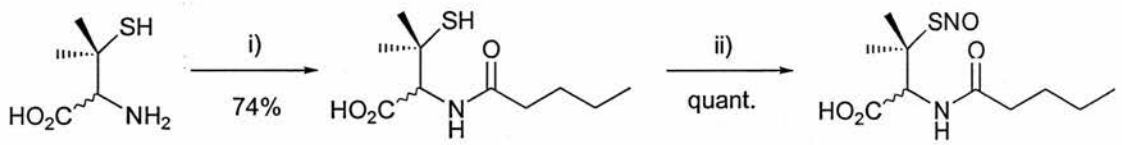


R =	$COCH_3$	*S*-Nitroso-*N*-acetyl-D,L-penicillamine	(SNAP)	**(1)**
	$COCH_2CH_3$	*S*-Nitroso-*N*-propionyl-D,L-penicillamine	(SNPP)	**(6)**
	$CO(CH_2)_3CH_3$	*S*-Nitroso-*N*-valeryl-D,L-penicillamine	(SNVP)	**(7)**
	$CO(CH_2)_5CH_3$	*S*-Nitroso-*N*-heptanoyl-D,L-penicillamine	(SNHP)	**(8)**

Figure 2.3: *N*-substituted analogues of **1**

This study was aimed at the determination of the importance of chain length of the acyl substituent. The vasodilatory properties of each *S*-nitrosothiol were investigated to see if a correlation between chain length and vasodilatory activity could be made. It was found that compound **7** was decidedly better than any of the others in that it had a longer half life (3.5 hours, compared to 1 hours for SNAP) and exerted a more pronounced and longer lasting vasodilation.[121]

The propionyl and heptanoyl *N*-acetyl-D,L-penicillamine derivatives, **6** and **8**, were synthesised by other members of the Butler group. Compound **7** and its enantiomerically pure forms **15** and **16** were prepared in the study reported herein, by the method outlined in Scheme 2.2.



D,L **(9)**
D **(10)**
L **(11)**

D,L **(12)**
D **(13)**
L **(14)**

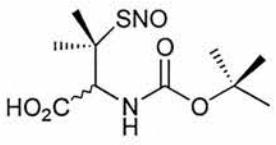
D,L **(7)**
D **(15)**
L **(16)**

Scheme 2.2: Preparation of *S*-nitroso-*N*-valeryl-penicillamines
Reagents: i) sodium valerate, valeric anhydride, THF/water; ii) sodium nitrite, HCl

2.1.2. Carbamate analogues of SNAP

The introduction of longer chains in place of the acetyl group in SNAP **(1)** seemed the natural step to take in determining structure-activity relationships. After having determined that the valeryl chain constituted the optimum length for activity, it was decided to explore different options from straight chain acyl groups.

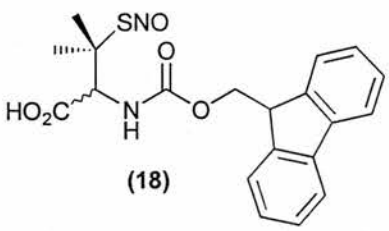
The groups that were linked to the penicillaminyl nitrogen were the butoxycarbonyl (Boc) group, the 9-fluorenylmethyloxycarbonyl (Fmoc) group and the phenylacetyl group (Figure 2.4).



(17)

N-Butoxycarbonyl-*S*-nitroso-D,L-penicillamine

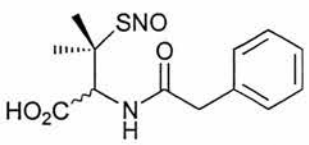
SNBocP



(18)

N-9-Fluorenylmethyloxycarbonyl-*S*-nitroso-D,L-penicillamine

SNFmocP



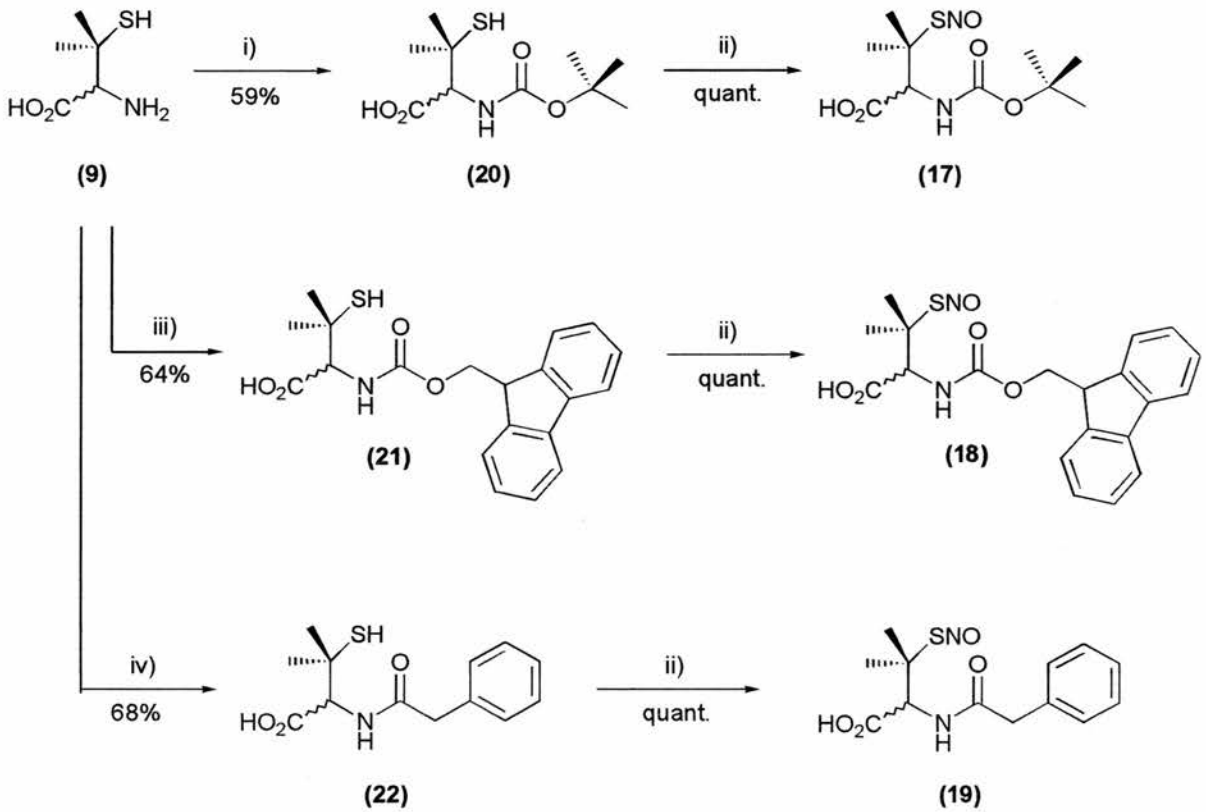
(19)

N-Phenylacetyl-*S*-nitroso-D,L-penicillamine

SNPAP

Figure 2.4: Boc, Fmoc and phenylacetyl substituted penicillamine

All compounds were prepared from D,L-penicillamine **(9)** following known literature procedures. The thiols were then nitrosated with sodium nitrite and HCl (Scheme 2.3).



Scheme 2.3: Preparation of SNBocP **(17)**, SNFmocP **(18)** and SNPAP **(19)**
Reagents: i) Di-tert-butyldicarbonate, NaOH(aq), dioxane/water; ii) sodium nitrite, HCl; iii) 9-fluorenylmethyl chlorocarbonate, sodium bicarbonate (aq), dioxane; iv) phenylacetyl chloride, sodium bicarbonate (aq).

The *S*-nitrosothiols were obtained in good yield and isolated as green oils or solids. Their decomposition and vasodilatory activity are discussed in chapter 3.

2.2. GLACSNAP series

2.2.1. Aims

As mentioned earlier, GLACSNAP **(2)** has been found by the Butler group to be a good vasodilator, with activity comparable to that of SNAP **(1)** and with a longer retention time within the artery.[113] It was decided to examine the structure-activity relationships for this molecule and to prepare a series of analogues that would help clarify the debate on whether an enzyme might be involved in the decomposition of *S*-nitrosothiols *in vivo*.

Modifications of GLACSNAP **(2)** can be made at a variety of positions in the molecule (Figure 2.5).

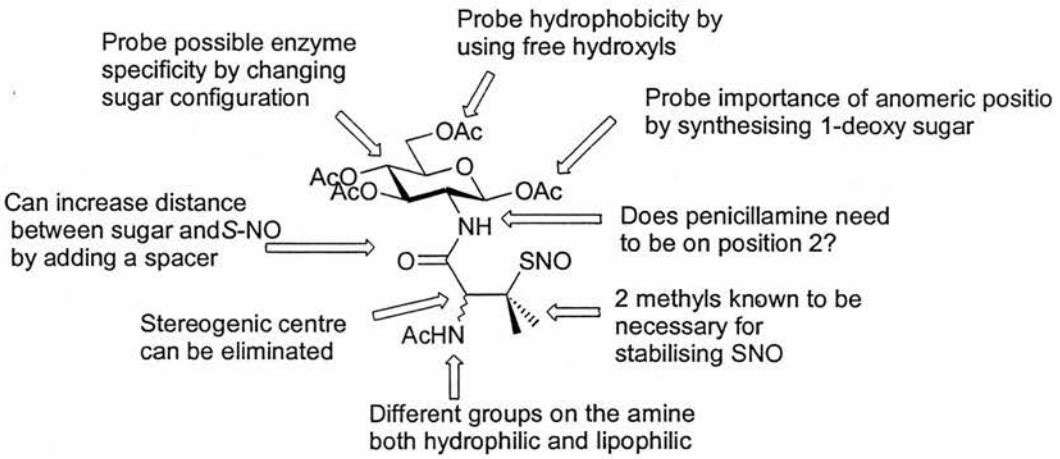


Figure 2.5: Sites of possible modifications of GLACSNAP **(2)**

It is not known why GLACSNAP **(2)** has a sustained vasodilatory effect. Biological testing has shown that vasodilation is sustained even after washout of the relaxed artery with saline. Compound **2** is probably retained within the walls of the artery where it then decomposes to release NO. It is not known what confers this property to **2**, but it is known that SNAP **(1)** does not possess sustained vasodilatory activity and therefore the effect must be associated with the sugar portion of the molecule. The preparation of a number of analogues of **2** could help in investigating the reason for this retention in the artery and at the same time could lead to the preparation of compounds with long half lives and sustained vasodilatory activity.

There are a number of properties of **2** that could be exploited for the introduction of modifications.

- Hydrophobicity

  By changing the degree of lipophilicity and monitoring the vasodilatory activity, it would be possible to determine which tissue the molecule bound to. LogP studies could give an indication of what tissue types the molecule could penetrate. Hydrophobicity would be greatly reduced by deprotecting the sugar hydroxyls and further tuning could be achieved by modifying the protecting group on the amino acid amine, as had been done for SNAP **(1)** (Section 2.1).

- Stereochemistry

a) If an enzyme were responsible for the decomposition of *S*-nitrosothiols *in vivo*, it would likely possess a certain substrate specificity. This could be probed by changing the absolute configuration (using D or L penicillamine) as well as by changing the relative configuration of the sugar moiety, for example by using galactose or glucose.

b) Another possibility would be to synthesise the 1-deoxy sugar, so as to ensure the synthesis of a single diastereoisomer. Compound **2** is, in fact, a mixture of 4 diastereoisomers (D,L amino acid plus α,β sugar) and this can lead to complications with purification and characterisation. This problem can be overcome by preparing a single isomer at the amino acid centre and eliminating the diastereomeric element at the carbohydrate anomeric centre; this can be done by preparing the 1-deoxy sugar.

c) Further simplification of the diastereomeric mixture could be achieved by eliminating the amino group from the amino acid moiety, for example by using β-mercaptoisovaleric acid **(23)** instead of *N*-acetyl-D,L-penicillamine **(24)** (Figure 2.6).

(23) **(24)**

β-mercapto isovaleric acid *N*-Acetyl-D,L-penicillamine

Figure 2.6: Similarity of β-mercaptoisovaleric acid **(23)** to *N*-acetyl-D,L-penicillamine **(24)**

- Regiochemistry

a) A further way of probing the substrate specificity of the postulated enzyme would be to increase the distance between the sugar and the SNO moiety, for example by adding a spacer between SNAP **(1)** and the sugar (Figure 2.7).

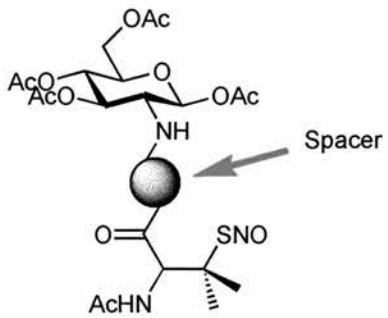


Figure 2.7: Addition of spacer between sugar and SNO

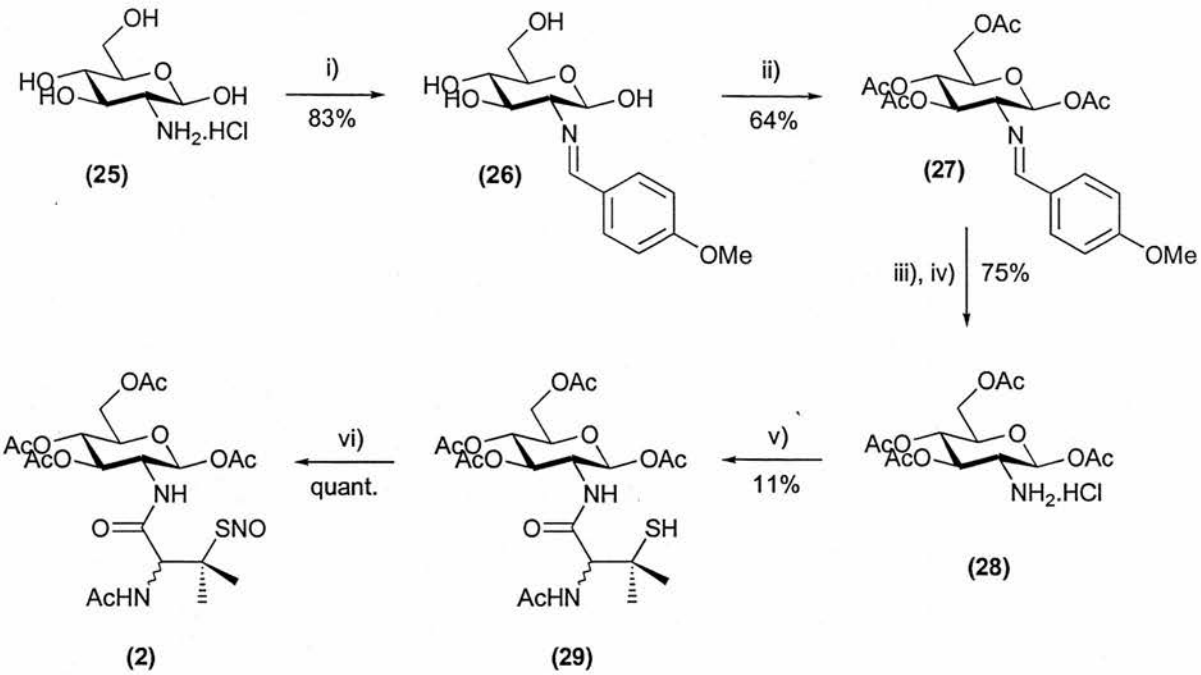
This would also allow the measuring of the impact of the sugar on the uptake and retention of the compound by the arteries.

b) Further regiospecificity could be probed by changing the position of the SNAP moiety on the sugar, for example by preparing the compound with SNAP liked to 6-amino-6-deoxy sugar. This type of compound would possess all the building blocks that make up GLACSNAP **(2)**, but assembled in a different orientation.

The synthesis of the analogues referred to above will be discussed later in this chapter.

2.2.2. Original synthesis of GLACSNAP

It was decided that a good synthetic route to GLACSNAP **(2)** was necessary. The synthetic route previously used by the Butler group,[122] and initially adopted here, consisted of the protection of glucosamine hydrochloride **(25)** to give the *N*-anisylidene protected glucosamine **26**.[123] This was then acetylated with pyridine and acetic anhydride[124] to give 1,3,4,6-tetra-*O*-acetyl-*N*-anisylidene-*β*-D-glucosamine **(27)**. Removal of the anisylidene group was achieved by treatment with HCl to afford the acetylated glucosamine hydrochloride **28**.[123] The free base form of **28** was prepared *in situ* by reaction with sodium acetate and then coupled directly onto *N*-acetyl-D,L-penicillamine **(24)** using 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimidemetho-*p*-toluenesulfonate (CMEC),[125] a water-soluble derivative of DCC. This afforded the glycosylated amino acid **29** in poor yield. Thiol **29** was then nitrosated to give GLACSNAP **(2)** (Scheme 2.4).

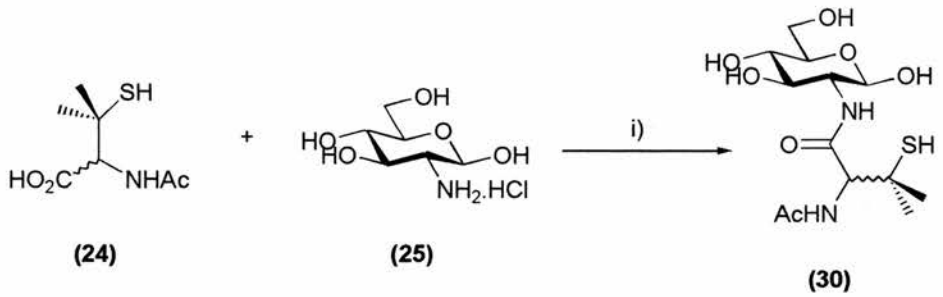


Scheme 2.4: Original route to GLACSNAP **(2)**
Reagents: i) anisaldehyde, NaOH(aq); ii) acetic anhydride, pyridine; iii) HCl, acetone / water; iv) sodium acetate (aq); v) *N*-acetyl-D,L-penicillamine **(24)**, CMEC, DCM; vi) sodium nitrite, HCl

In the current study, the preparation of **28** was achieved in high yield and purity by following the standard 1931 literature preparation described by Bergman and Zervas.[123] Unfortunately, the yield for the coupling to *N*-acetyl-D,L-penicillamine **(24)** was very poor. It was therefore decided, especially in view of the fact that GLACSNAP **(2)** would have to be prepared in multi-gram quantities to allow thorough testing, to explore different synthetic routes for its synthesis.

2.2.3. Synthesis of 2 via the mixed anhydride method

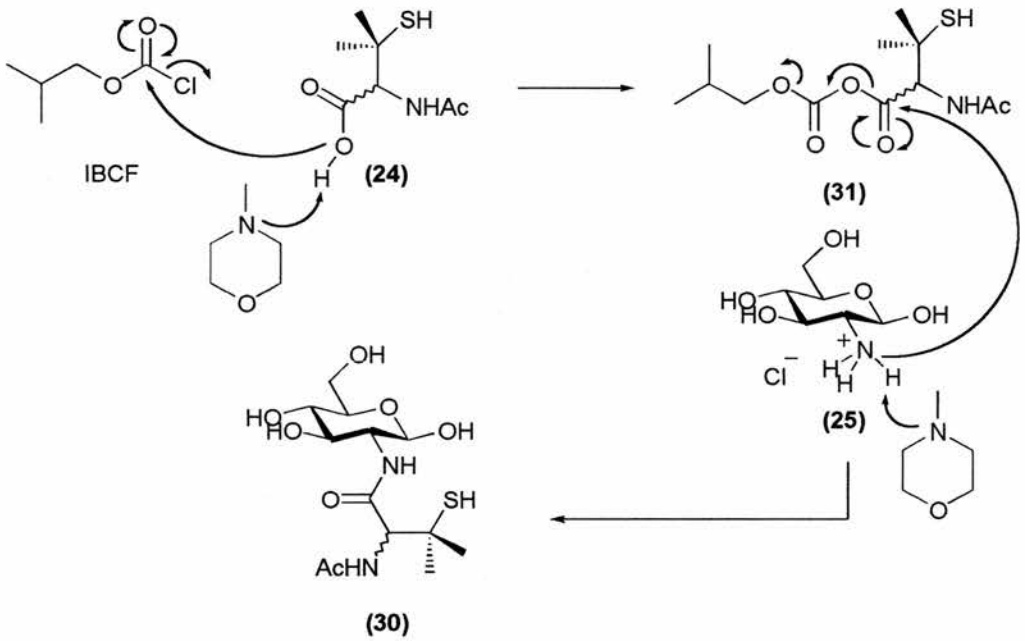
It was thought that a mixed anhydride approach would allow the coupling of glucosamine hydrochloride **(25)** and *N*-acetyl-D,L-penicillamine **(24)** to yield precursor **30** (Scheme 2.5).



(24) (25) (30)

Scheme 2.5: Planned synthesis *via* the mixed anhydride method
Reagents: i) NMM, IBCF, THF / DMF

The mixed anhydride approach used was that described by Vaughan.[126] We expected the mechanism of reaction to be the following (Scheme 2.6).

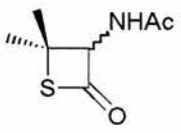


Scheme 2.6: Expected mechanism of the mixed anhydride reaction

As for any mixed anhydride peptide coupling, it was expected that initially the mixed anhydride of *N*-acetyl-D,L-penicillamine **(24)** and isobutylchloroformate (IBCF), intermediate **31**, would be formed. The penicillamine carbonyl group would then be rendered more prone to nucleophilic attack by glucosamine **(25)**, therefore yielding the coupled product **30**. The outcome of the reaction was, in fact, quite different.

2.2.3.1. Outcome of mixed anhydride reaction

^{13}C and ^{1}H NMR spectroscopy showed that the mixed anhydride coupling of *N*-acetyl-D,L-penicillamine **(24)** and glucosamine hydrochloride **(25)** had given a cyclised penicillamine, in 70% isolated yield. This compound, **32**, had been previously used by Wang and coworkers in their synthesis of *S*-nitrosothiols[127] (Figure 2.8) and it was clearly a potentially useful synthetic intermediate for our purposes.

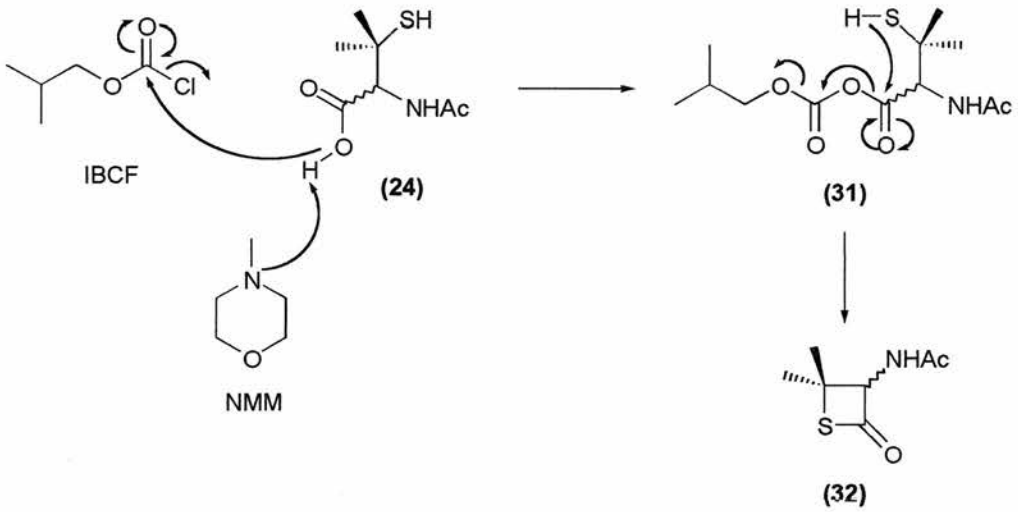


(32)

(3R,S)-3-Acetamido-4,4-dimethylthietan-2-one

Figure 2.8: The isolated product of mixed anhydride reaction

The likely mechanism for formation of thietanone **32** is outlined in Scheme 2.7.



Scheme 2.7: Mechanism of formation of intermediate **32**

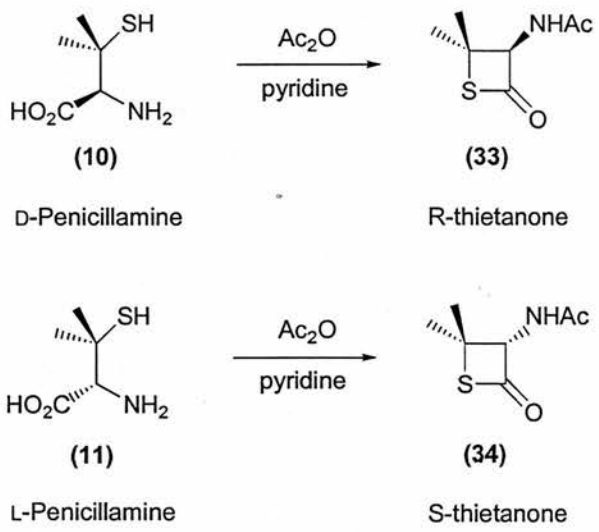
The penicillamine thiol was clearly sufficiently nucleophilic to permit intramolecular attack on its own carbonyl group before the sugar amine was even introduced into the reaction mixture.

2.2.3.2. The intermediate thietanone

Thietanone **32** was initially characterised in 1955 by Knuyants *et al.*[128] who were analysing the synthesis of polypeptides containing mercapto amino acids. They noticed the formation of cyclic derivatives of penicillamine when the sulfur moiety was unprotected and coupling *via* a mixed anhydride method was attempted. They therefore proceded to synthesise and characterise these compounds.

In 1973, Field *et al.*[129] were investigating D-penicillamine **(10)** as a possible anti-arthritic compound. Whilst examining its chemical reactivity, they came across (3*R*)-acetamido-4,4-dimethylthietan-2-one **(33)**. They found this molecule to be inherently stable to protic solvents, so much so, that no change in the NMR spectrum was seen even after 1-3 days in *t*-BuOH, MeOH or THF/H_2O at reflux. Although R-thietanone **33** did not show the same anti-arthritic and metal-chelating properties as **10**, they acknowledged its use as a protected version of **10** in the synthesis of dipeptides.

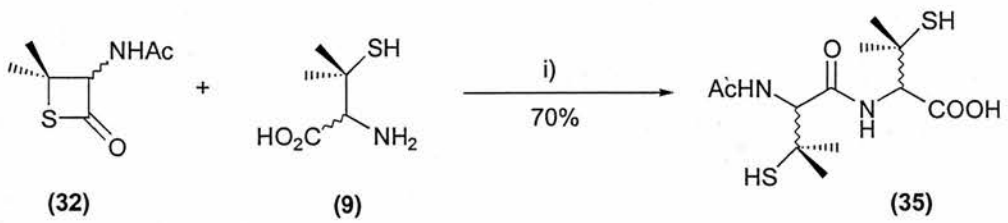
In 1983, Stoodley's group[130] investigated thietanone chemistry and prepared the two enantiomerically pure forms of **32** (Scheme 2.8).



Scheme 2.8: Preparation of enantiomerically pure thietanones

Since then, a number of groups have used racemic thietanone **32** and chiral thietanones **33** and **34** as alternatives to the sulfur protection of penicillamine in the synthesis of peptides and glycopeptides. Two main examples are those of Roberts[131] and Wang.[127] Roberts prepared racemic thietanone **32** both from D,L-penicillamine **(9)** and from *N*-acetyl-D,L-penicillamine **(24)**. The first method involves the use of pyridine and acetic

anhydride. These reagents initially acetylate the free amine and then perform the cyclisation of the thiol onto the carboxylic acid. By using *N*-acetyl-D,L-penicillamine **(24)** directly with isobutylchloroformate and triethylamine in chloroform, the yield was raised. Reaction of racemic thietanone **32** with D,L-penicillamine **(9)** in a chloroform / NaOH(aq) mixture then yielded the dipeptide *N*-(*N*-acetyl-D,L-penicillaminyl)-D,L-penicillamine **(35)** in 70% yield as a mixture of diastereoisomers (Scheme 2.9).



Scheme 2.9: Roberts' preparation of penicillamine dipeptide *via* intermediate **32**
Reagents: i) $CHCl_3$ / NaOH(aq)

Whilst the study reported in this thesis was ongoing, Wang adopted the same idea as Roberts and reacted thietanone **32** with sugar amines to produce a number of glycoamino acids, which were subsequently nitrosated. (Figure 2.9).[132]

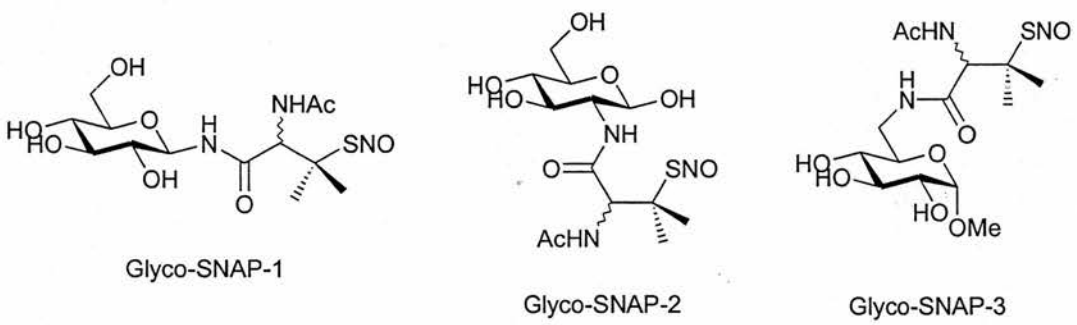


Figure 2.9: Glycoamino acids prepared by Wang's group *via* intermediate **32**

2.2.3.3. Crystal structure of intermediate thietanone

In order to investigate the structure and reactivity of **32**, we undertook the elucidation of its crystal structure (Figure 2.10).

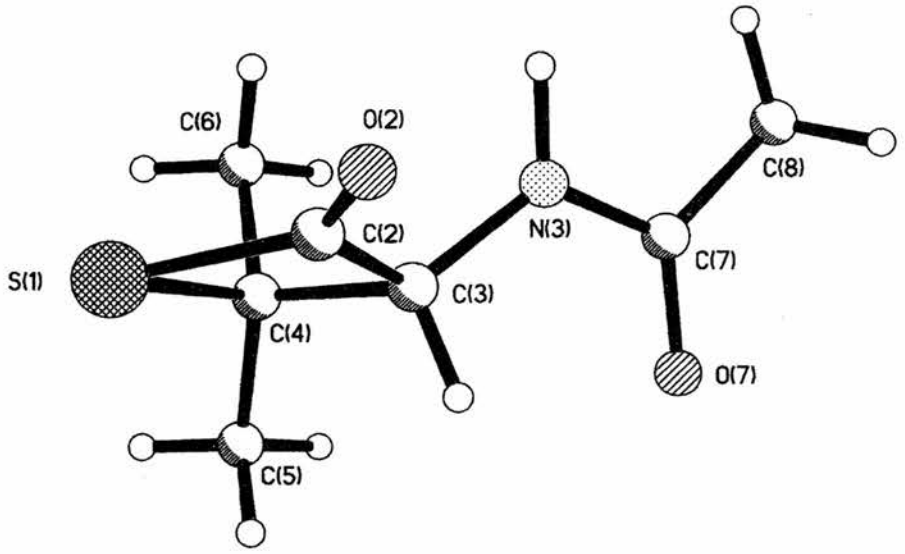
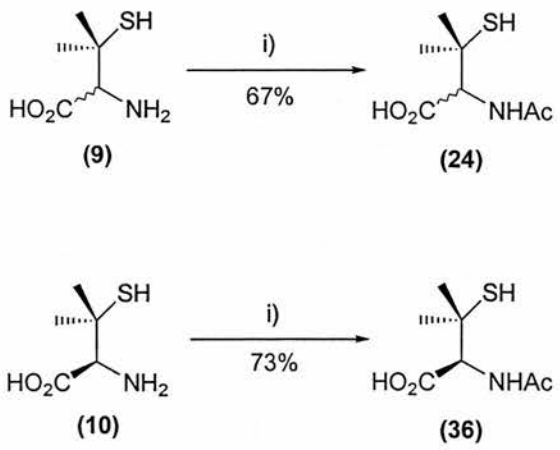


Figure 2.10: Crystal structure of thietanone **32** (full coordinates in appendix)

The two methyl groups on the β-carbon are positioned in such a way as to impede the access of an incoming nucleophile to the carbonyl carbon. It is known, in fact, that nucleophiles attack carbonyl groups at a defined angle, the Burgi-Dunitz angle[133] and the two methyls in this case are on the path defined by such an angle. This might explain the relative unreactivity of this molecule with respect to other thioesters.[129]

It was while looking at the crystal structure of what we presumed to be racemic **32** that we realized that what we had prepared was in fact the R isomer, **33**. The optical rotation for the compound was, in fact, -97.6 (*c* 0.1 in $CHCl_3$), which was comparable to the literature value for **33**: –113 (*c* 0.16 in $CHCl_3$)[131] We then examined the optical rotation for *N*-acetyl-D,L-penicillamine **(24)**, bought from Aldrich, and found it to have a value of +45.4 (*c* 1 in 1M NaOH), which clearly indicated that it was not a racemic mixture, as stated on the bottle. In an attempt to clarify this matter, we prepared *N*-acetyl-D,L-penicillamine **(24)** and *N*-acetyl-D-penicillamine **(36)** from D,L-penicillamine **(9)** and D-penicillamine **(10)**, respectively (Scheme 2.10).



Scheme 2.10: Preparation of **24** and **36**
Reagents: i) MeOH, Acetic anhydride

Compounds **24** and **36** had different optical rotations; 0 and +52.7, respectively. We also prepared the two thietanones, **32** and **33**, from **24** and **36**, respectively, and found their rotations to be 0 and -102 respectively (*c* 1 in $CHCl_3$). The NMR spectra recorded for **32** and **33** were, of course, found to be identical.

The two thietanones, **32** and **33**, were then coupled to glucosamine hydrochloride **(25)** to give **30** and their respective ^1H NMR spectra were recorded (Figure 2.11).

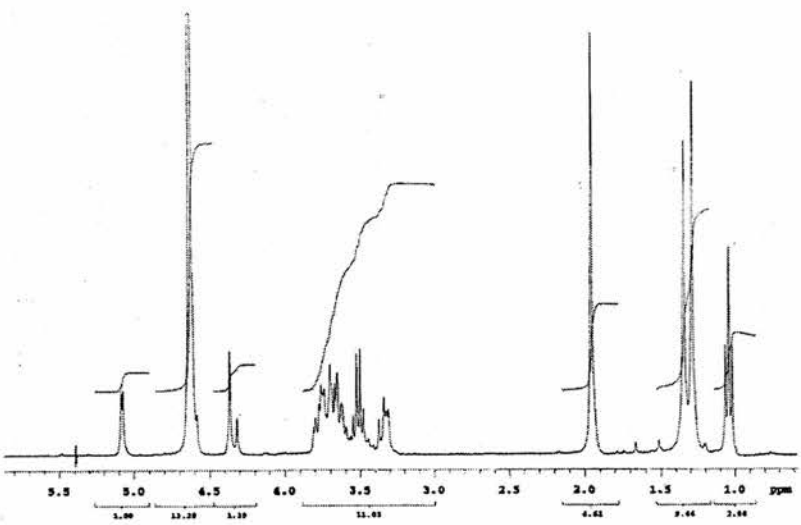


Figure 2.11: NMR spectra for thietanone **32** coupled to glucosamine hydrochloride

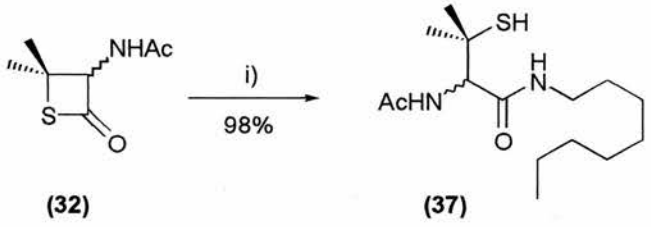
The two spectra proved to be too complex, with a large multiplet between 3.3 and 3.8 ppm comprising most of the molecule's proton signals. Discrimination of the two diastereomeric compounds was shown to be unachievable solely by ^{1}H NMR spectroscopy.

To date Aldrich have not responded to several requests for clarification of this unfortunate and misleading situation.

2.2.3.4. Susceptibility of racemic thietanone 32 to nucleophilic attack

Racemic thietanone **32** was prepared from a batch of *N*-acetyl-D,L-penicillamine which did have an optical rotation of 0°.

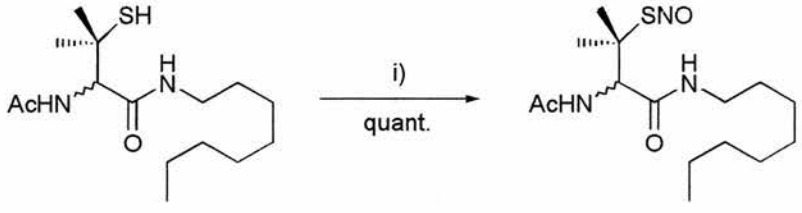
The susceptibility of compound **32** to nucleophilic attack was probed by reaction with *n*-octylamine (Scheme 2.11).



(32) **(37)**

Scheme 2.11: Reaction of **32** with *n*-octylamine
Reagents: i) *n*-octylamine, THF

The reaction yielded *N*-acetyl-D,L-penicillamine octyl amide **(37)** in high purity and good yield. The reaction with glucosamine was therefore attemped. Before doing so, however, compound **37** was nitrosated to give **38** (Scheme 2.12).



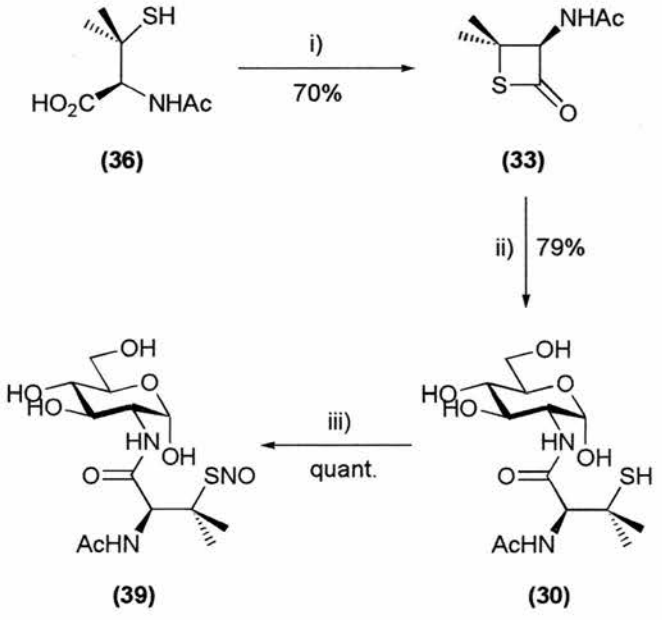
(37) **(38)**

Scheme 2.12: Preparation of SNAPOA **(38)**
Reagents: i) sodium nitrite, HCl

Compound **38** was tested along with the other SNAP derivatives prepared in section 2.1; see chapter 3.

2.2.3.5. Coupling of R-thietanone 33 to glucosamine

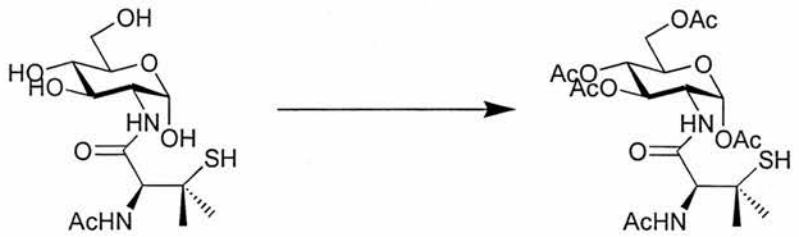
It was decided, for the coupling to glucosamine **(25)**, to use enatiomerically pure **33** instead of racemic **32**, as this would simplify characterisation. Coupling was achieved following the method described by Wang and coworkers.[127] (Scheme 2.13).



Scheme 2.13: Revised route towards **39**, using intermediate **33**
Reagents: i) NMM, IBCF, THF / DMF; ii) glucosamine, $CHCl_3$, H_2O; iii) sodium nitrite, HCl

The single diastereoisomer of **30** was prepared from R-thietanone **33** in good yield. Characterisation by ^{13}C and ^{1}H NMR spectroscopy proved that the compound obtained was the α anomer ($J_{1,2}$ 3.3 Hz). Nitrosation to give GLUSNAP **(39)** was also successful.

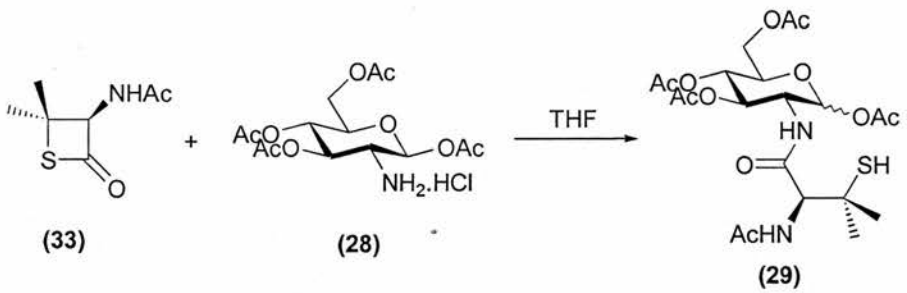
2.2.3.6. Attempts to acetylate compound 30a



(30) (29)

Scheme 2.14: Acetylation of **30**

A variety of reagents were assessed for performing this reaction, including acetic anhydride with pyridine,[124] iodine[134] or sodium acetate,[124] but in all cases acetylation occurred both on the sugar hydroxyls and on the thiol. Selective deprotection of the thioacetate was attempted with benzylamine in THF at 0°C,[135] but this yielded the entirely deprotected molecule, *i.e.* **30** itself. Reaction of R-thietanone **33** and acetylated glucosamine **28** was then attempted, thinking it would yield the acetyl-protected coupled product **29** directly (Scheme 2.15).



(33) (28) (29)

Scheme 2.15: Attempted reaction between **33** and **28**

Unfortunately the reaction was unsuccessful. R-thietanone **33** was recovered unreacted, but the sugar had decomposed. The reasons for the reaction not taking place could be two-fold. Firstly, the amine of acetylated sugar **28** is less reactive than that of the unprotected **25**,[136,137] *i.e.* it is "disarmed", to use Fraser-Reid terminology. Secondly, the acetyl on the anomeric oxygen could migrate onto the free amine and hence prevent it from reacting (Scheme 2.16).[138] It was in fact found, by ^{1}H and ^{13}C NMR spectroscopy, that 70% of **28** had undergone acetyl migration.

(28) (40)

Scheme 2.16: Acetyl migration

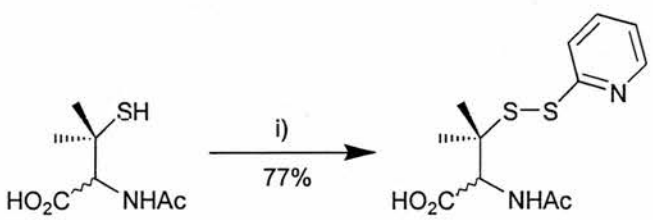
It became apparent, therefore, that the best solution to the synthesis of GLACSNAP would be to use *N*-acetylpenicillamine and protect the thiol prior to coupling.

2.2.4. Sulfur protection on penicillamine

At this point it was decided to investigate *N*- and *S*-protected penicillamine and the use of standard coupling conditions with glucosamine.

2.2.4.1. *S*-Pyridyl disulfide protection

It was initially decided to keep the *N*-acetyl protection and later, once the chemistry had been proven, this would be replaced by *N*-Boc protection. The first thiol protecting group tried was pyridyl disulfide[139] (Scheme 2.17).



(24) (41)

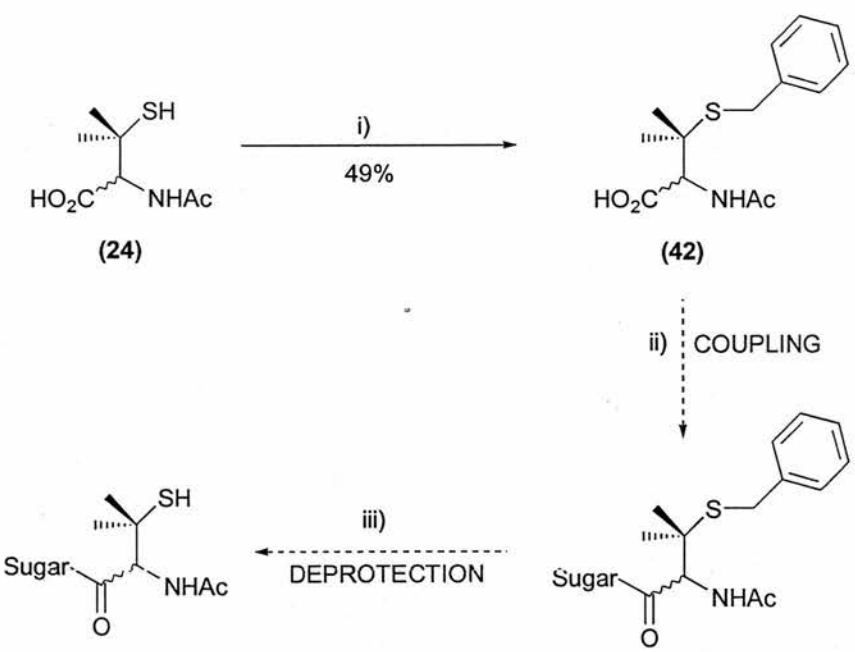
Scheme 2.17: Synthesis of pyridyl disulfide-protected *N*-acetyl-D,L-penicillamine **(41)**
Reagents: i) dipyridyl disulfide, MeOH

N-Acetyl-D,L-penicillamine-*S*-pyridyl disulfide **(41)** was successfully synthesised and purified following the derivatisation procedure described by Kempin *et al.*[140] Coupling of **41** was attempted with unprotected glucosamine **25** and acetylated glucosamine **28**.

None of the coupling reactions, using the CMEC, DCC or the mixed anhydride methods were successful. It became apparent that the pyridyl disulfide protecting group was being removed under the basic coupling conditions. However, the reaction was unsuccessful even when no base (other than the sugar free base) was introduced in the reaction mixture.

2.2.4.2. *S*-Benzyl protection

In view of the pyridyl disulfide protection being removed under the basic coupling conditions, it was decided that a base-stable protection should be used and the preference fell on a benzyl protection since the removal procedure, hydrogenation,[141] was a clean method that would not interfere with any other part of the molecule (Scheme 2.18).



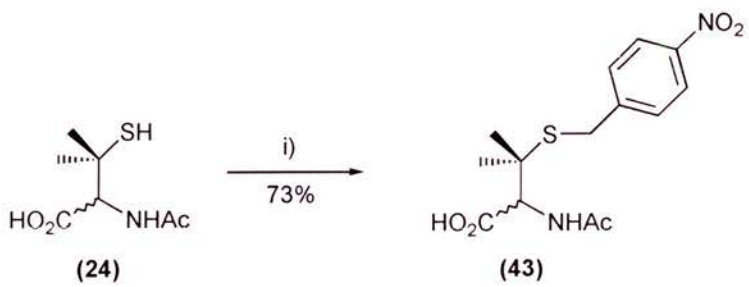
Scheme 2.18: Planned benzyl protection strategy
Reagents: i) benzyl chloride, EtOH, NaOH(aq); ii) standard coupling conditions; iii) hydrogen (gas), Pd/C, MeOH

S-Benzylpenicillamine derivative **42** was prepared in moderate yield but good purity by reaction of *N*-acetyl-D,L-penicillamine **(24)** with benzyl chloride in aqueous base, as described by Bodanszky and Bodanszky.[142] Before coupling, it was thought wise to attempt *S*-deprotection on **42** itself. This reaction was attempted a number of

times, but with no success. Hydrogenation was attempted with various catalytic loadings of palladium on charchol and even at 4 Bar of hydrogen pressure. The other methods of deprotection described in the literature[143] involved the use of mercury salts. It was thought inappropriate in this case, seeing that the compounds might eventually be tested *in vivo*.

2.2.4.3. *S-p*-Nitrobenzyl protection

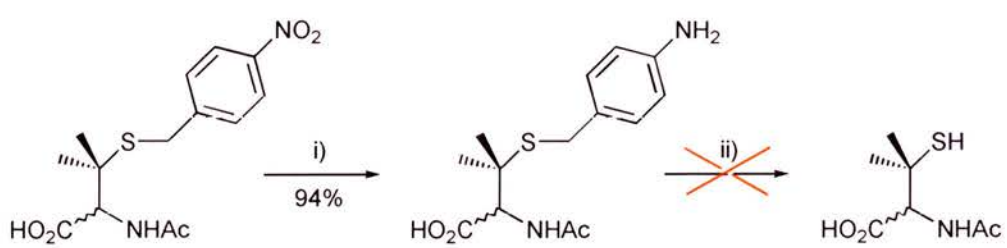
It was then thought that a *p*-nitrobenzyl protecting group might be more susceptible to hydrogenation, as shown by Bachi and Ross-Peterson.[144,145] and therefore *N*-acetyl-*S*-*p*-nitrobenzyl-D,L- penicillamine **(43)** was prepared (Scheme 2.19).



(24) (43)

Scheme 2.19: *S-p*-Nitrobenzyl protection
Reagents: i) *p*-nitrobenzyl chloride, EtOH, NaOH(aq)

Again, hydrogenation was carried out as a test, before coupling, but with no success. The nitro group was reduced to an amine, as judged by NMR, IR and MS, but the benzyl group could not be removed from the thiol (Scheme 2.20).



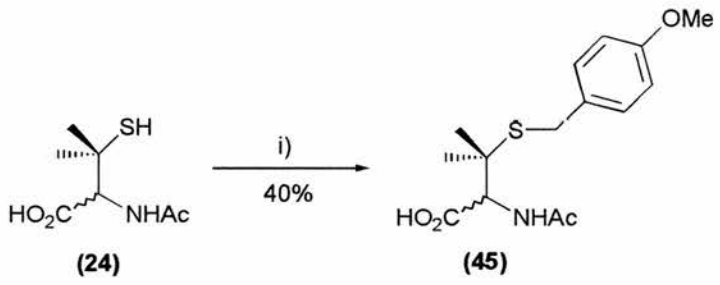
(43) (44) (24)

Scheme 2.20: Outcome of hydrogenation reaction
Reagents: i) and ii) hydrogen (gas), Pd/C, MeOH

It seems likely that the sulfur in the molecule acts as a poison to the catalyst.

2.2.4.4. *S-p*-Methoxybenzyl protection

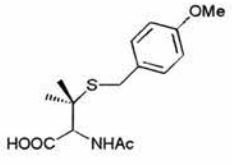
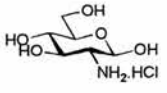
A different type of protection was then tried, the *p*-methoxybenzyl protecting group (Scheme 2.21).



Scheme 2.21: *S-p*-Methoxybenzyl protection
Reagents: i) *p*-methoxybenzyl chloride, EtOH, NaOH(aq)

This group could be removed by using TFA[146] or an oxidant such as DDQ or CAS[143] and would withstand the basic coupling conditions. Furthermore, the *p*-methoxybenzyl group could be used in combination with *N*-Boc protection so that both groups could be removed in one single step,[143] yielding the free thiol and the free amine. The amine could then be acylated with a number of different groups and the thiol could be nitrosated.

N-Acetyl-*S-p*-methoxybenzyl-D,L-penicillamine **(45)** was obtained in moderate yield and good purity and was used for a series of test couplings under a variety of conditions (Table 2.1).

Aminoacid	Sugar	Coupling conditions	Outcome
 (45)	 (25)	CMEC, DCM	Recovered SM
		CMEC, THF	Recovered SM
		CMEC, DMF	Recovered SM
		CMEC, MeOH	Recovered SM
		DCC, DMF	Recovered SM
		NMM, IBCF, THF/DMF	Recovered SM
		NMM, IBCF, THF/H2O	Recovered SM
		PyBOP, DIPEA, MeCN	Recovered SM
		HATU, DIPEA, DMF	Recovered SM

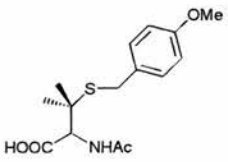
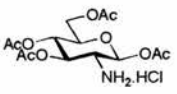
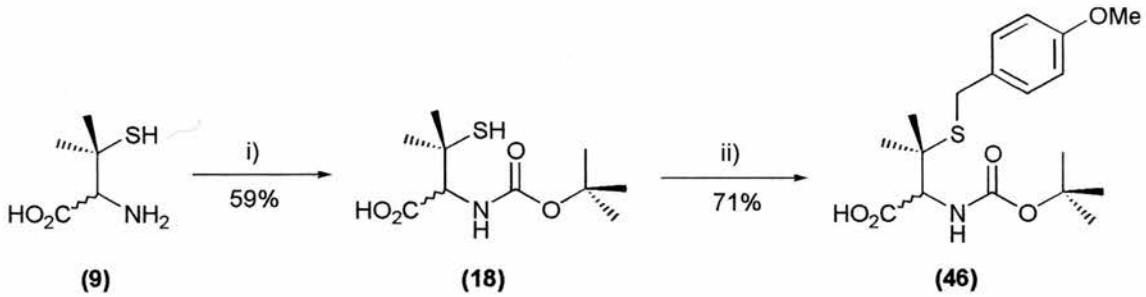
 (45)	 (28)	CMEC, DCM	Recovered SM
		DCC, DCM	Recovered SM
		NMM, IBCF, THF	Recovered SM
		PyBOP, DIPEA, MeCN	Recovered SM
		HATU, DIPEA, DCM	Recovered SM

Table 2.1: Couplings of **(45)**

As shown in table 2.1, a wide variety of coupling conditions were examined. Couplings using the acetylated glucosamine **28** mainly yielded the product resulting from acetyl migration, 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetylglucose **(40)**, judging by ^{1}H and ^{13}C NMR. The amino acid moiety was generally lost in work-up. Couplings using the non-acetylated glucosamine **25** presented a solubility problem, which was eventually solved by the use of DMF or a THF/water mixture. Isolation of the coupled product often proved difficult, hence acetylation of the reaction mixture was carried out. Unfortunately, in all cases, none of the desired product was isolable.

2.2.4.5. *S-p*-Methoxybenzyl-*N*-butoxycarbonyl protection

At this point it was thought that the *N*-acetyl protection was slowing down the coupling and rendering the amino acid less susceptible to nucleophilic attack. It is, in fact, known that *N*-acetylated amino acids are prone to racemisation during coupling and have low reactivity.[147] It was therefore decided to synthesise the *N*-butoxycarbonyl-*S-p*-methoxybenzyl protected penicillamine **(46)** (Scheme 2.22).



(9) **(18)** **(46)**

Scheme 2.22: Synthesis of *N*-butoxycarbonyl-*S-p*-methoxybenzyl-D,L-penicillamine **(53)**
Reagents: i) di-tert-butyldicarbonate, sodium bicarbonate (aq);
ii) *p*-methoxylbenzyl chloride, EtOH, NaOH(aq)

Compound **46** was prepared by initially *N*-butoxycarbonyl protecting D,L-penicillamine **(9)** following the procedure described by Bodanszky and Bodanszky[142] and then by adding the *S*-*p*-methoxybenzyl group, again following the method described by Bodanszky and Bodanszky.[142]

Once compound **46** had been synthesised, a number of couplings were tried both with unprotected glucosamine **25** and with acetylated glucosamine **28**. (Table 2.2).

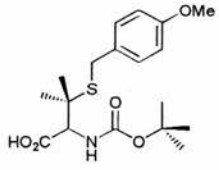
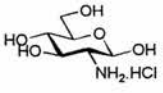
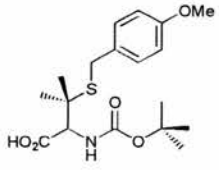
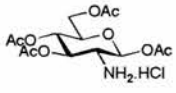
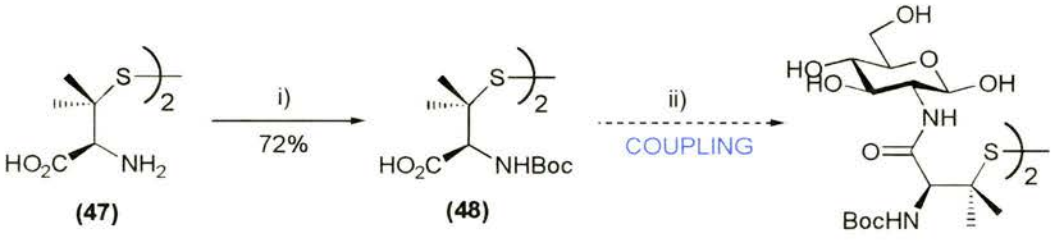
Aminoacid	**Sugar**	**Coupling conditions**	**Outcome**
 **(46)**	 **(25)**	DCC, DMF	Recovered SM
		NMM, IBCF, THF/DMF	Recovered SM
		PyBOP, DIPEA, MeCN	Recovered SM
		HATU, DIPEA, DMF	Recovered SM
 **(46)**	 **(28)**	DCC, DCM	Recovered SM
		CMEC, DCM	Recovered SM
		NMM, IBCF, THF	Recovered SM
		PyBOP, DIPEA, MeCN	Recovered SM
		HATU, DIPEA, DMF	Recovered SM

Table 2.2: Coupling of *N*-Boc-*S*-*p*-methoxybenzyl-D,L-penicillamine **(46)**

Once again, unfortunately, none of the couplings yielded the desired product. The reason for the unreactivity of penicillamine in this case was thought to be two-fold. On one side there is considerable steric bulk. The Boc and *p*-methoxybenzyl groups as well as the two methyl groups on the *β*-carbon have a great steric influence over the approach of any incoming nucleophile. On the other side, these groups also have an electronic influence, which may be one of the reasons for the poor reactivity of the carbonyl group. The combined effect of all the protecting groups and, perhaps most importantly, the *gem*-dimethyl group, is to greatly reduce the reactivity of the amino acid. It is apparent, therefore, that the unreactivity of the amino acid, combined with the problems of solubility or acetyl migration of the sugars, rendered the coupling of the two extremely difficult.

2.2.4.6. *S*-Disulfide protection

As a last attempt, it was decided to protect the thiol as its own disulfide. D-Penicillamine disulfide **(47)** can, in fact, be purchased directly from Aldrich and then reacted with di-tert-butyldicarbonate and sodium bicarbonate to give *N*-butoxycarbonyl-D-penicillamine disulfide **(48)**. A new synthetic plan was thus drawn up (Scheme 2.23).



Scheme 2.23: Using the disulfide as a protecting group for the thiol
Reagents: i) di-tert-butyldicarbonate, sodium bicarbonate (aq); ii) **(25)**, standard coupling agents

After coupling, the disulfide could easily be reduced to the thiol by use of triphenylphosphine.[148,149]

A variety of conditions were attempted for the coupling of disulfide **48** with sugar amines **25** and **28** (Table 2.3), again without success.

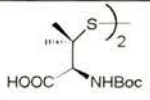
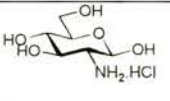
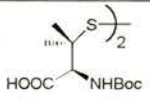
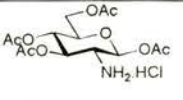
Aminoacid	Sugar	Coupling conditions	Outcome
 **(48)**	 **(25)**	NMM, IBCF, THF/H_2O	Recovered SM
		DCC, DMF	Recovered SM
		PyBOP, DIPEA, MeCN	Recovered SM
 **(48)**	 **(28)**	NMM, IBCF, THF	Recovered SM
		DCC, DCM	Recovered SM
		PyBOP, DIPEA, MeCN	Recovered SM

Table 2.3: Couplings of *N*-butoxycarbonyl-D-penicillamine disulfide **(48)**

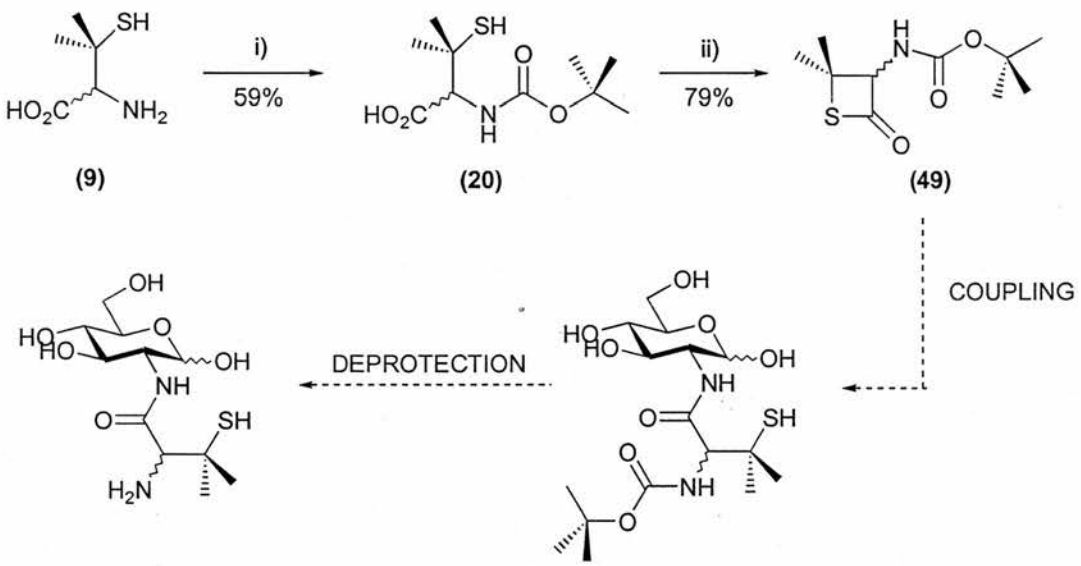
It became apparent, therefore, that GLACSNAP **(2)** would have to continue to be prepared by the route described in scheme 2.6, however wasteful this seemed to be.

2.2.5. Different *N*-protection on thietanone 32

It was thought that it might be useful to synthesise a series of analogues of GLUSNAP **(39)** with different groups on the amine. The strategy applied in this case was to *N*-protect D,L-penicillamine **(9)**, giving **20**, cyclise this to obtain the intermediate thietanone **49** and then open this up with glucosamine **25** to give an *N*-protected analogue of **30**. The protection could then be removed and a number of different groups coupled to the free amine.

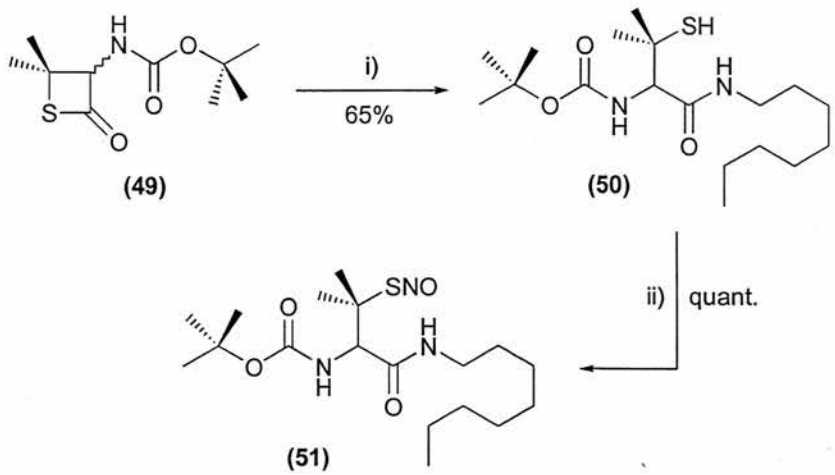
2.2.5.1. *N*-Butoxycarbonyl protection

The first protecting group to be investigated was the butoxycarbonyl (Boc) group. (Scheme 2.24).



Scheme 2.24: Strategy for Boc-protection
Reagents: i) Di-tert-butyldicarbonate, NaOH(aq), dioxane / water; ii) NMM, IBCF, THF

N-Butoxycarbonyl-D,L-penicillamine **(20)** was prepared following the procedure described by Bodanszky and Bodanszky[142] in good yield. The protected penicillamine was then cyclised, as before, to give 3-butoxycarbonylamido-4,4-dimethylthietan-2-one **(49)**. The susceptibility of **49** to nucleophilic attack was tested, as for **32**, by reaction with *n*-octylamine (Scheme 2.25).



(49) (50) (51)

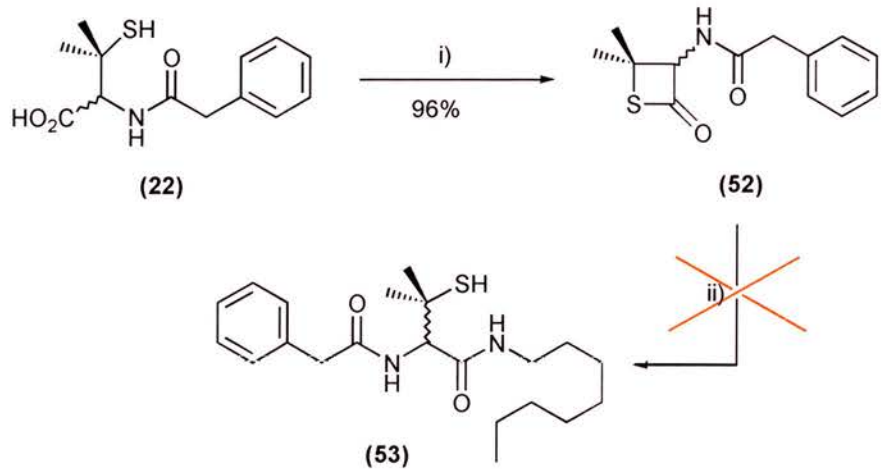
Scheme 2.25: Reaction of **49** with *n*-octylamine and nitrosation to give **51**
Reagents: i) *n*-octylamine, THF; ii) sodium nitrite, HCl

The reaction proceeded in good yield. Thiol **50** was then nitrosated to give **51**, which was tested along with other SNAP derivatives (see chapter 3).

Coupling with glucosamine **25** was then attempted. Unfortunately the coupling of **49** with **25** in H_2O / $CHCl_3$ did not take place at all. This was probably due to the difference in solubility between acetyl-thietanone **32** and Boc-thietanone **49**. It was suspected that the Boc group was preventing the thietanone from entering the aqueous layer where it would then react with the sugar. Both molecules were recovered unreacted after 16 hours vigorous stirring. The reaction was then attempted in methanol and in THF, solvents in which both species were soluble, but it did not take place, indicating that the problem was not solely one of solubility.

2.2.5.2. *N*-Phenylacetyl protection

It was decided that a more water-soluble group should be used on the thietanone, such as the phenylacetyl group. This group also possessed the advantage of being easily removable, under mild conditions, by the action of enzyme penicillin G acylase.[150] *N*-Phenylacetyl-D,L-penicillamine **(22)** was therefore synthesised following the derivatisation procedure described by Bodanszky and Bodanszky.[142] Intermediate thietanone **52** was synthesised in the usual way and reaction with *n*-octylamine was attempted (Scheme 2.26).



Scheme 2.26: Preparation of **52** and reaction with *n*-octylamine
Reagents: i) NMM, IBCF, THF; ii) *n*-octylamine, THF

In this case the reaction did not take place, indicating that the carbonyl group in **52** was somewhat less susceptible to nucleophilic attack than in the case of **32** and **49**. Reactions with glucosamine **25** in H_2O / $CHCl_3$ and in methanol were tried nonetheless but, unsurprisingly, both starting materials were recovered unreacted.

2.3. Modification of sugar moiety in GLACSNAP (2)

It was decided to concentrate on a number of modifications to the sugar moiety of **2**. As mentioned earlier, it was thought that the possibility that an enzyme was responsible for the activity of *S*-nitrosothiols *in vivo* could be probed by changing the regio- and stereo-chemistry of the molecule used. For this reason, the 6-amino-6-deoxygalactose, 3-aminopropyl glucoside and the 2-amino-1,5-anhydro-2-deoxy glucitol series were synthesised.

2.3.1. 6-Amino-6-deoxygalactose series

It was decided to synthesise methyl 6-amino-6-deoxy-β-D-galactopyranoside **(54)** and couple it to *N*-acetyl-D,L-penicillamine **(24)** to give an analogue of **2**, namely compound **55** (Figure 2.12).

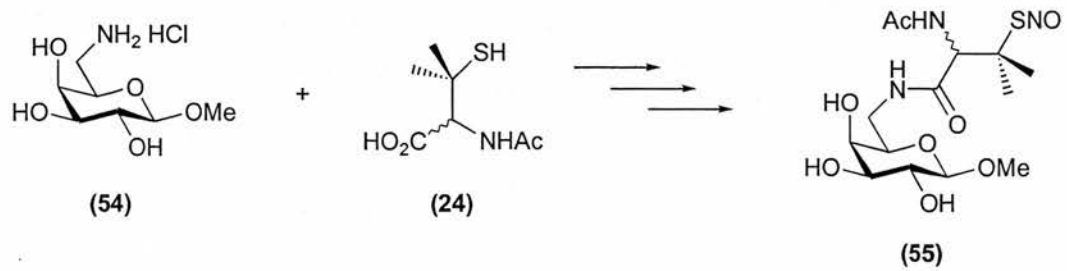
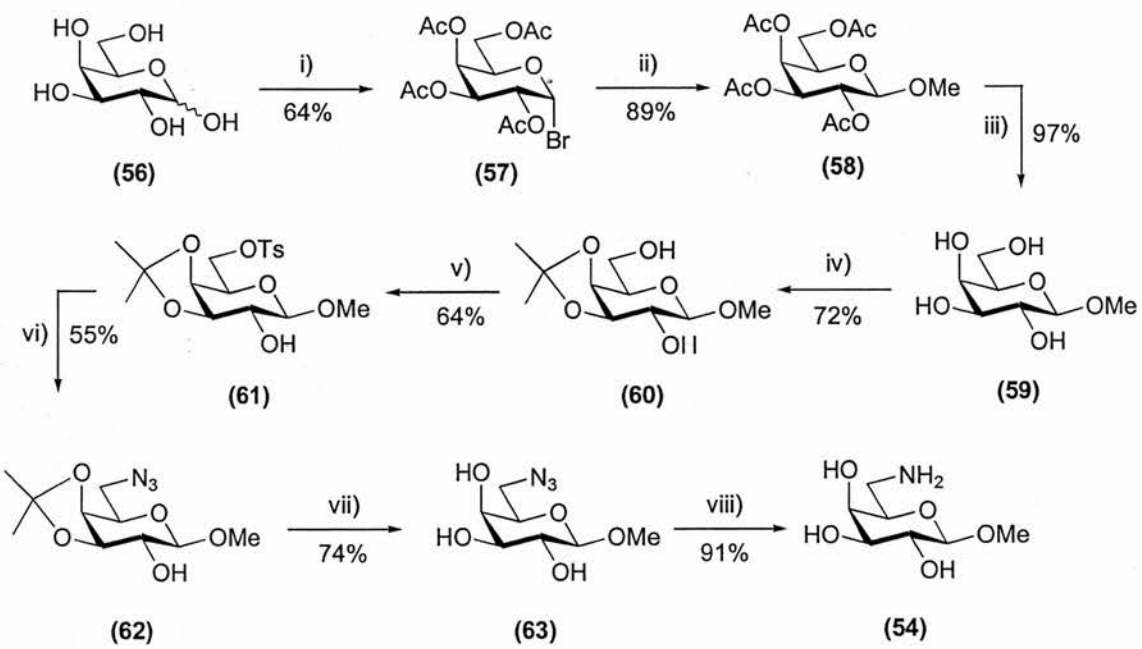


Figure 2.12: Coupling of **54** with **24**

The synthesis of methyl 6-amino-6-deoxy-β-galactoside hydrochloride salt **(54)** was achieved by initially converting D-galactose **(56)** to 2,3,4,6-tetra-*O*-acetyl-α-D-galactosyl bromide **(57)** following the procedure described by Kartha and Jennings.[151] Methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **(58)** was then obtained by addition of methanol and iodine.[134] De-acetylation proceeded rapidly with the use of sodium in methanol[152] to give the unprotected methyl β-galactopyranoside **59**. This was protected at positions 3 and 4 with an isopropylidene group to give acetal **60** and then tosylated at position 6 to give **61**, as described by Brimacombe and Ching.[153] The tosyl group was then displaced with sodium azide to give the 6-azido sugar **62**. The isopropylidene protection was removed with TFA and azide **63** was then converted to 6-amino compound **64** by catalytic hydrogenation. (Scheme 2.27).



Scheme 2.27: Synthesis of methyl 6-amino-6-deoxy-β-galactopyranoside **(54)**
Reagents: i) 45% HBr in AcOH; ii) I_2, MeOH; iii) Na, MeOH;
iv) 2,2-dimethoxypropane, tosic acid (cat.); v) tosyl chloride, acetone, pyridine; vi) sodium azide, DMF;
vii) 80% TFA(aq); viii) H_2, Pd/C, MeOH

2.3.1.1. Couplings of methyl 6-amino-6-deoxygalactopyranoside (54)

Once compound **54** had been obtained, as described above, a series of coupling reactions were attempted with a number of penicillamine derivatives (Table 2.4).

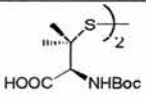
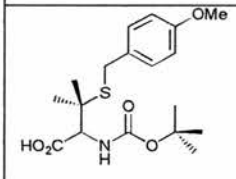
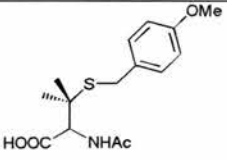
Aminoacid	Coupling conditions	Outcome
 **(48)**	DCC, DMF	Recovered SM
	PyBOP, DIPEA, MeCN	Recovered SM
	NMM, IBCF, THF/H_2O	Recovered SM
 **(46)**	DCC, DMF	Recovered SM
	PyBOP, DIPEA, MeCN	Recovered SM
 **(45)**	DCC, DMF	Recovered SM
	PyBOP, DIPEA, MeCN	Recovered SM

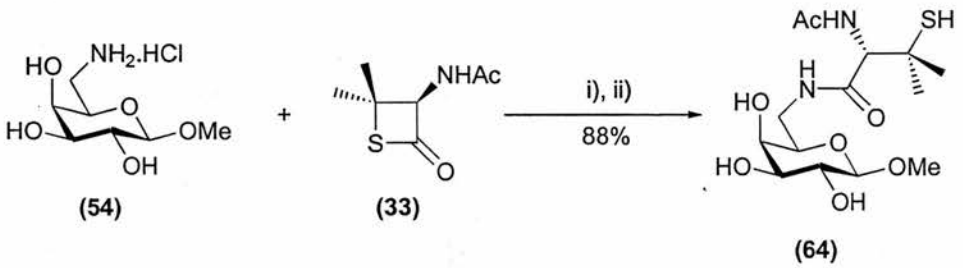
Table 2.4: Couplings of **54**

Due to the water-solubility of the coupled products, which rendered separation from the starting materials and from the coupling agents quite complex, in all cases, the products were acetylated directly after coupling with pyridine and acetic anhydride[124] and purification was attempted on the acetylated compounds rather than the non-acetylated ones. In all cases, none of the expected products were isolated.

It is not clear why none of the coupling procedures yielded the expected product. In most cases the sugar was recovered in its acetylated form and the amino acid was lost during the aqueous work-up following acetylation. The only explanation that can be attempted is that the amino acids are all unreactive towards nucleophilic attack by the sugar amine, a problem already encountered during couplings to glucosamine **(25)**.

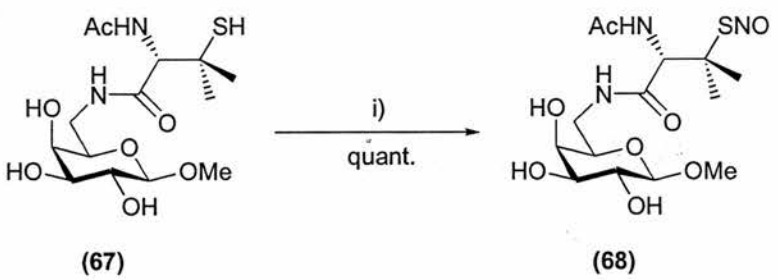
2.3.1.2. Coupling of 54 to R-thietanone 33

At this point it was thought that R-thietanone **33** would be reactive enough to couple to sugar amine **54** (Scheme 2.28).



Scheme 2.28: Reaction of **54** and **33**
Reagents: i) Na, MeOH; ii) water / chloroform

The reaction did, in fact, give **64** in good yield, proving that sugar amine **54** is a good enough nucleophile in this case. Acetylation of the product of the reaction was not attempted due to the problems encountered in *S*-Ac removal (see section 2.2.3.6.). The product itself was nitrosated to give **55** (Scheme 2.29), which was tested along with the other *S*-nitrosothiols (see chapter 3).

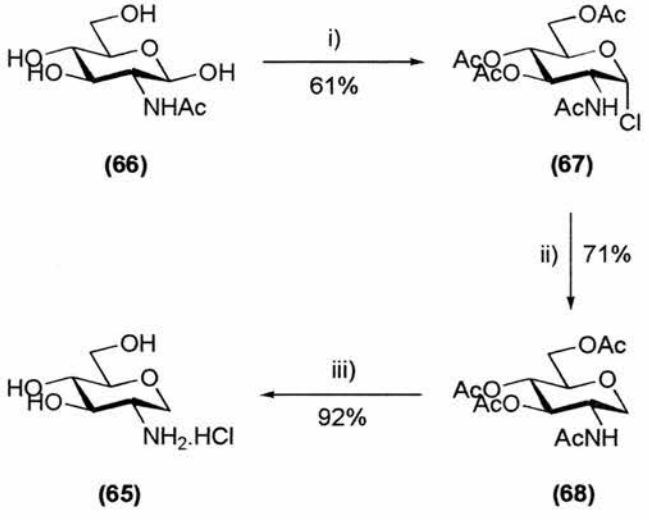


Scheme 2.29: Nitrosation of **64**
Reagents: HCl, sodium sulfite

2.3.2. 2-Amino-1,5-anhydro-2-deoxy glucitol series

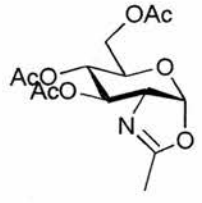
One of the main problems with GLACSNAP **(2)** is that it has routinely been prepared as a mixture of diastereoisomers. It was therefore decided to synthesise an analogue of **2** that lacked any group at the anomeric position and to test its biological activity.

The synthesis of known 2-amino-1,5-anhydro-2-deoxy-D-glucitol hydrochloride **(65)**[154] was undertaken by initially preparing 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride **(67)**, as described by Horton,[155] from 2-acetamido-2-deoxy-β-D-glucopyranoside **(66)** and acetyl chloride. Chloride **67** was then reacted with tributyltin hydride and AIBN in refluxing toluene to give the acetylated 1,5-anhydro glucitol **68**, following the procedure used by Bamford *et al.*[156] Acetylated glucitol **68** was then deprotected using refluxing HCl as described by Schafer *et al.*[154] to give **65** in 75% overall yield from *N*-acetylglucosamine **66** (Scheme 2.30).



Scheme 2.30: synthesis of 2-amino-1,5-anhydro-2-deoxy glucitol hydrochloride **(65)**
Reagents: i) AcCl; ii) tributyltin hydride, AIBN, toluene; iii) HCl

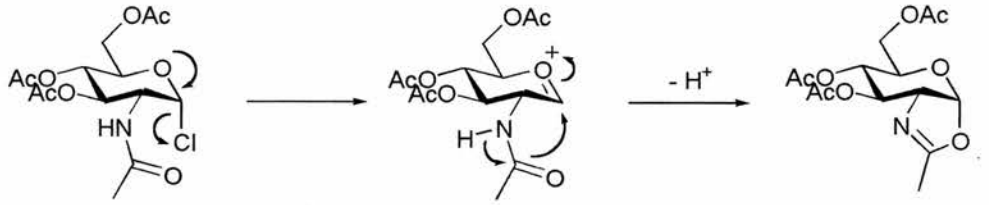
It is worth pointing out that the radical reaction of **67** with tributyltin hydride and AIBN did not, initially, proceed as expected. The main product obtained, as confirmed by ^{1}H and ^{13}C NMR, was in fact oxazoline **69**, which was the result of a non-radical cyclisation reaction rather than a radical reaction (Figure 2.13).



(69)

Figure 2.13: Oxazoline product

The mechanism by which oxazoline **69** is formed is thought to be the following (Scheme 2.31).



Scheme 2.31: Mechanism for formation of oxazolidine **69**

After a number of failed attempts at the synthesis of **68**, a new batch of AIBN was purchased and extra care was taken in degassing the solution prior to reaction. Thereafter the expected product was routinely obtained.

2.3.2.1. Coupling of 2-amino-1,5-anhydro-2-deoxyglucitol (65)

Once **65** had been prepared, coupling was attempted with *N*-butoxycarbonyl-*S*-*p*-methoxybenzyl-D,L-penicillamine **(46)** and with *N*-butoxycarbonyl-D,L-penicillamine disulfide **(48)** (Table 2.5).

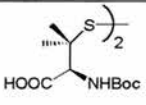
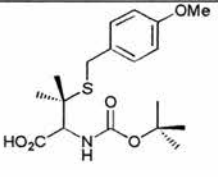
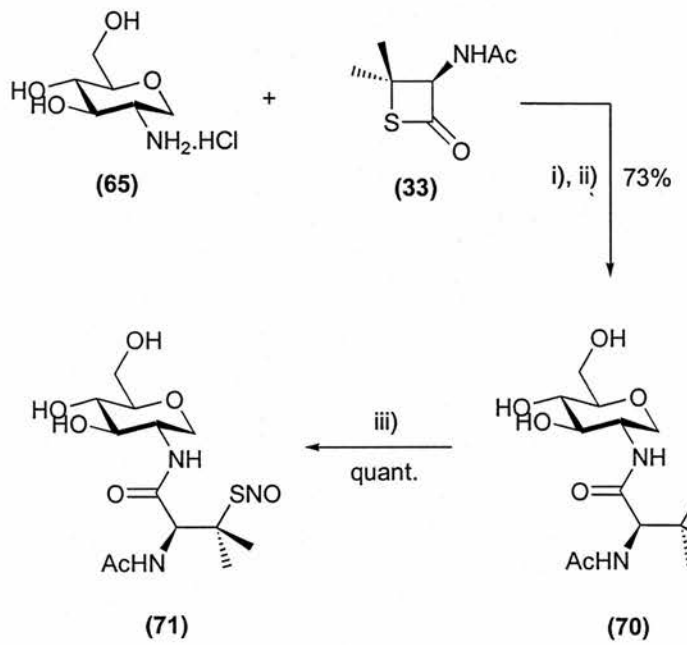
Aminoacid	**Coupling conditions**	**Outcome**
 **(48)**	DCC, DMF	Recovered SM
	PyBOP, DIPEA, MeCN	Recovered SM
	NMM, IBCF, THF/H_2O	Recovered SM
 **(46)**	DCC, DMF	Recovered SM
	PyBOP, DIPEA, MeCN	Recovered SM

Table 2.5: Couplings of 2-amino-1,5-anhydro-2-deoxy-D-glucitol **(65)**

Once again, all couplings were unsuccessful.

2.3.2.2. Coupling of 65 with R-thietanone 33

It was decided to try the coupling of **65** to cyclic intermediate **33**, as for amine **54** (Scheme 2.32).



Scheme 2.32: Coupling of 2-amino-1,5-anhydro-2-deoxy-D-glucitol **(65)** with **33**
Reagents: i) sodium, methanol; ii) water / chloroform; iii) sodium nitrite, HCl

The reaction proceeded in good yield and resulting thiol **70** was nitrosated to give **71** which was tested with all other *S*-nitrosothiols (see chapter 3).

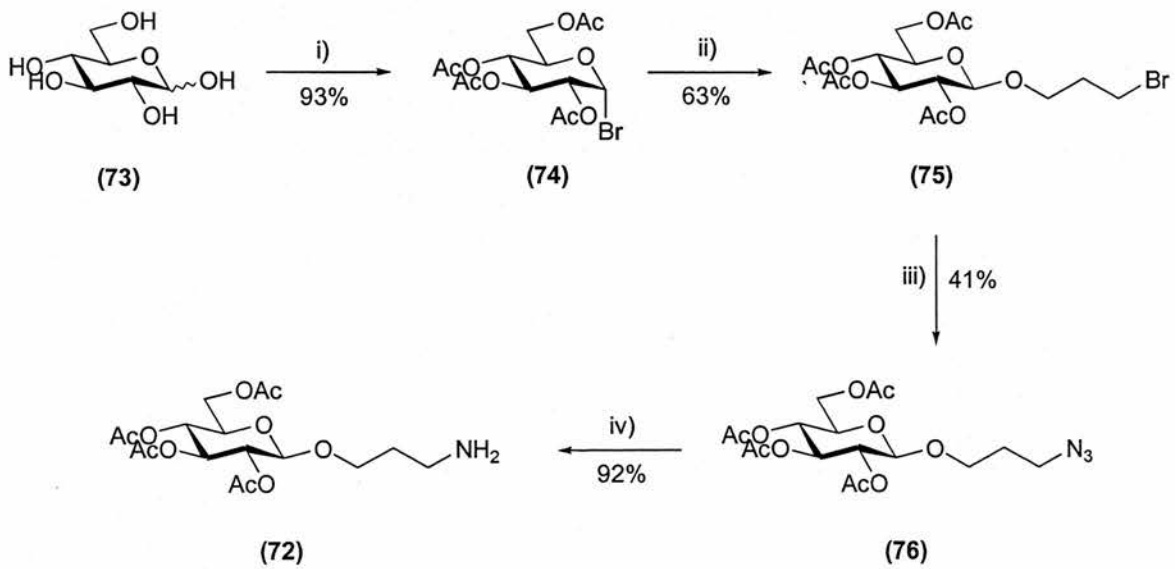
2.3.3. 3-Aminopropyl glucoside series

It was proposed to add a spacer between the sugar and the aminoacid moieties of GLACSNAP **(2)**, hence rendering the molecule different in shape but not in its components.

It was thought that 3-aminopropyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside **(73)** would be suitable candidate to couple to SNAP **(1)**. The distance between the sugar and the aminoacid would be significant enough to change its recognisability by an enzyme.

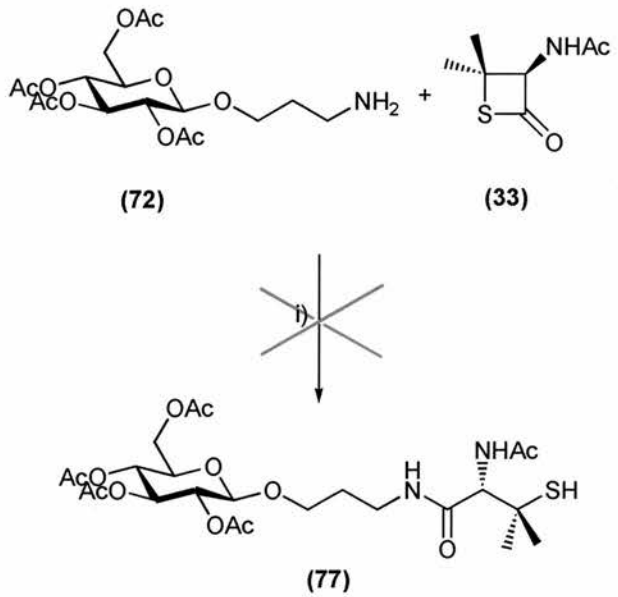
The synthesis began with the conversion of D-glucose **(73)** to 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **(74)** *via* the method published by Kartha and

Jennings.[151] This was then subjected to nucleophilic attack by 3-bromopropanol in dry acetonitrile in the presence of iodine, a method well established within our group.[134] This gave the 3-bromopropyl glucoside **75** in 63% yield. Alkyl bromide **75** was then converted to azide **76** in the presence of sodium azide and tetrabutylammonium triflate[157] and this was then reduced to amine **72** by catalytic hydrogenation (Scheme 2.33).



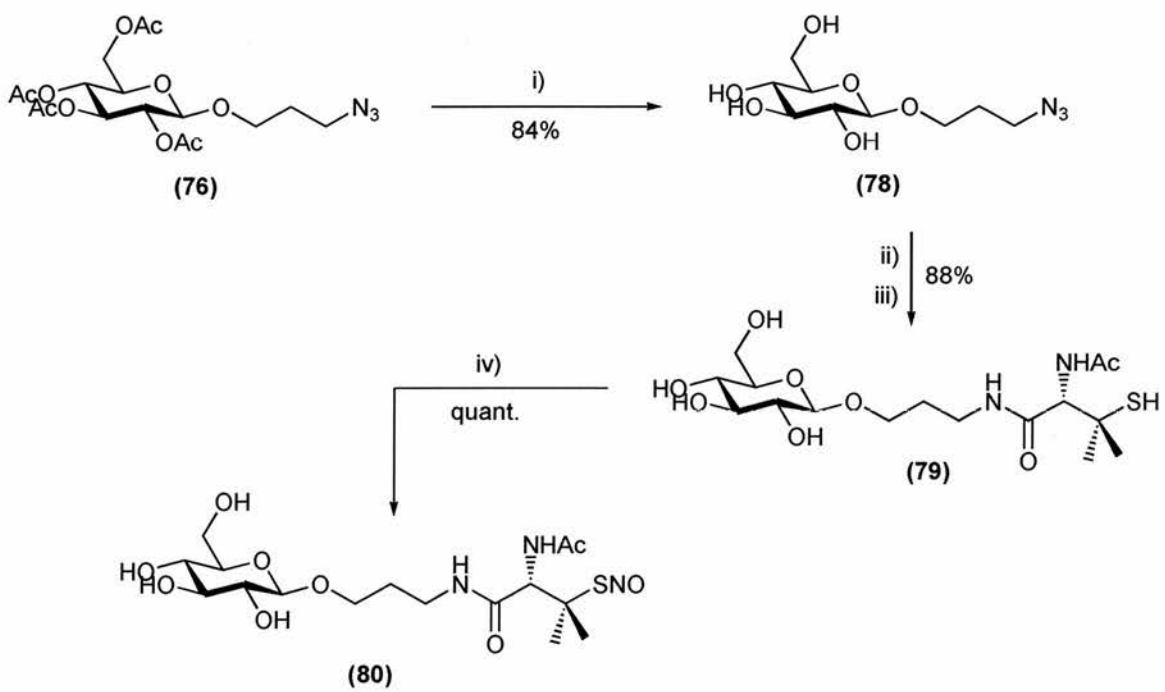
Scheme 2.33: synthesis of aminopropylglucoside **72**
Reagents: i) 45% HBr in AcOH, acetic anhydride; ii) 3-bromopropanol, Iodine, MeCN; iii) sodium azide, tetrabutylammonium triflate; iv) hydrogen (gas), Pd/C, MeOH

In this case, strong in the knowledge that the couplings with the *N*- and *S*-protected penicillamines would most likely not take place, we decided not to attempt them but to opt directly for the coupling with thietanone **33** (Scheme 2.34).



Scheme 2.34: Coupling of **72** and **33**
Reagents: i) THF

Surprisingly, the reaction did not take place. Thietanone **33** was recovered unreacted, whilst the sugar was found to have decomposed yielding a mixture of compounds. As it was possible that acetyl migration might take place before the coupling, we opted for total deprotection of the sugar before reaction with **33** (Scheme 2.35).

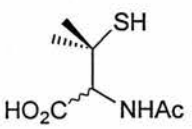


Scheme 2.35: Preparation of **78** and coupling to **33**
Reagents: i) Na, MeOH; ii) hydrogen (gas), Pd/C, MeOH; iii) **33**, water / chloroform; iv) sodium nitrite, HCl

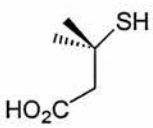
The acetyl groups in **76** were removed by reaction with sodium in methanol[152] to give non-acetylated azide **78**. The free amine obtained by catalytic hydrogenation, was reacted directly with thietanone **33** in a bi-phasic mixture of water and chloroform to yield the coupled product **79**. This was then nitrosated to give **80**, which was tested along with other *S*-nitrosothiols (see chapter 3).

2.3.4. Mercaptoisovaleric acid as a substitute for penicillamine

As an extension of the elimination of chirality from GLACSNAP **(2)**, it was decided to eliminate the chirality at the amino acid α carbon. For this reason, it was decided to use β-mercaptoisovaleric acid **(23)** instead of *N*-acetyl-D,L-penicillamine **(9)** in the couplings to sugars (Figure 2.14).



(9)

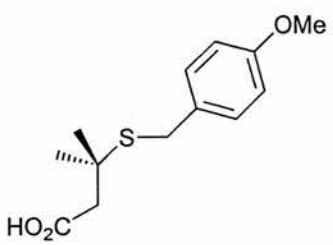


(23)

Figure 2.14: Penicillamine and mercaptoisovaleric acid

In doing this, the chirality of the amino acid part of the molecule would be eliminated, rendering purification easier and biological evaluation less ambiguous. It should be noted that mercaptoisovaleric acid does possess the *gem*-dimethyl groups, so important for *S*-nitrosothiol stability.

For synthetic purposes, mercaptoisovaleric acid was used as its *S*-p-methoxybenzyl protected derivative **81** (Figure 2.15).



(81)

Figure 2.15: *S-p*-Methoxybenzylmercaptoisovaleric acid

S-p-Methoxybenzylmercaptoisovaleric acid was coupled to all of the sugar amines prepared as described in the previous sections using one or other of the coupling methods described earlier, followed by acetylation with acetic anhydride and pyridine[124] (Table 2.6).

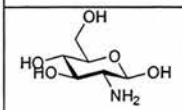
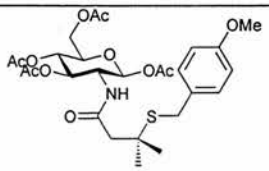
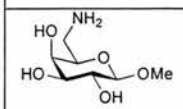
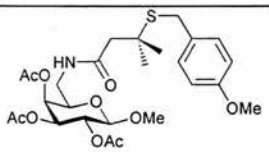
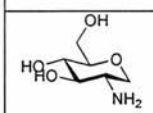
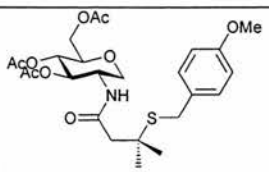
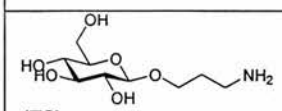
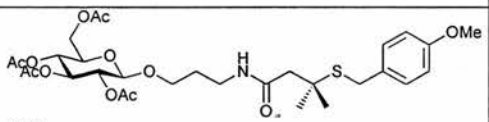
Sugar	Product	Couplings used	Yield
 **(25)**	 **(83)**	NMM, IBCF THF/water	31%
		PyBOP, MeCN DIPEA	28%
 **(54)**	 **(84)**	NMM, IBCF THF/water	31%
		PyBOP, MeCN DIPEA	26%
 **(65)**	 **(85)**	NMM, IBCF THF/water	20%
		PyBOP, MeCN DIPEA	23%
 **(78)**	 **(86)**	NMM, IBCF THF/water	18%
		PyBOP, MeCN DIPEA	14%

Table 2.6: Couplings of *S-p*-methoxybenzylmercaptoisovaleric acid

In this case, as opposed to all couplings attempted before, all couplings did yield the expected products. The yields varied between 14 and 31%, with the mixed anhydride method proving the more efficacious in all cases.

The success achieved in the couplings outlined above, gave further strength to the theory that the carbonyl in the penicillamine adducts is quite severely hindered. The absence of the amine group in mercaptoisovaleric acid, renders the carbonyl group decidedly better prone to nucleophilic attack by the sugar amines. In all cases the products were acetylated directly after coupling and purified by column

chromatography thereafter.

Standard deprotection of the *p*-methoxybenzyl group consists of treatment with TFA.[143] This reaction was attempted on all coupled products but with no success as, in all cases, a complex mixture was obtained. Unfortunately, due to time limitations, it was not possible to explore further the conditions for this deprotection.

Chapter 3: Results and Discussion - Physiology

The *S*-nitrosothiols prepared were tested both *in vitro* and *ex vivo* to determine their chemical stability and efficacy as vasodilators, respectively. The main aim of this study was to draw a comparison between a number of *S*-nitrosothiols so that a structure-activity profile could be determined. The methods employed are outlined in the following sections.

3.1. Chemical decomposition studies

All *S*-nitrosothiols were tested *in vitro* to determine their thermal decomposition patterns. *S*-Nitrosothiols decompose spontaneously, albeit slowly, to give the disulfide and NO; the decomposition can be followed spectrophotometrically at 340 nm since all *S*-nitrosothiols have an absorbtion band at that wavelength. The extinction coefficient (ε) for this absorbance is generally around 10^3 dm^3 cm^{-1} mol^{-1},[158] hence the concentration required for a sufficiently strong signal to be obtained is approximately 2 x 10^{-3} M. This concentration is, unfortunately, much higher than the concentrations used in the *ex vivo* experiments (10^{-8} to 10^{-3} M) and therefore the comparison between the two sets of data is not straightforward.

The decompositions studies were carried out as direct comparisons between compounds using the same freshly prepared Krebs buffer to eliminate the problem of the unknown quantity of copper ions in the buffer itself. Sections 3.3 and following, describe the comparisons of different classes of *S*-nitrosothiols; the specific decompositions of such compounds will be discussed therein.

3.2. Vasodilatory testing methodology

The vasodilatory activity of compounds was assessed at the Department of Clinical Pharmacology, Edinburgh University by Mark Miller and Naoki Sogo under the supervision of Dr. Ian Megson.

Experiments were carried out on isolated segments of femoral arteries from adult male Wistar rats. Cannulated arterial segments were perfused and superfused at constant flow rates by 95% O_2 / 5% CO_2 saturated Krebs solution. The solution was

kept at the constant temperature of 37°C and pumped through the vessel at a constant rate (0.6 ml/min) throughout the experiment. A diagram of the apparatus is shown below (Figure 3.1).

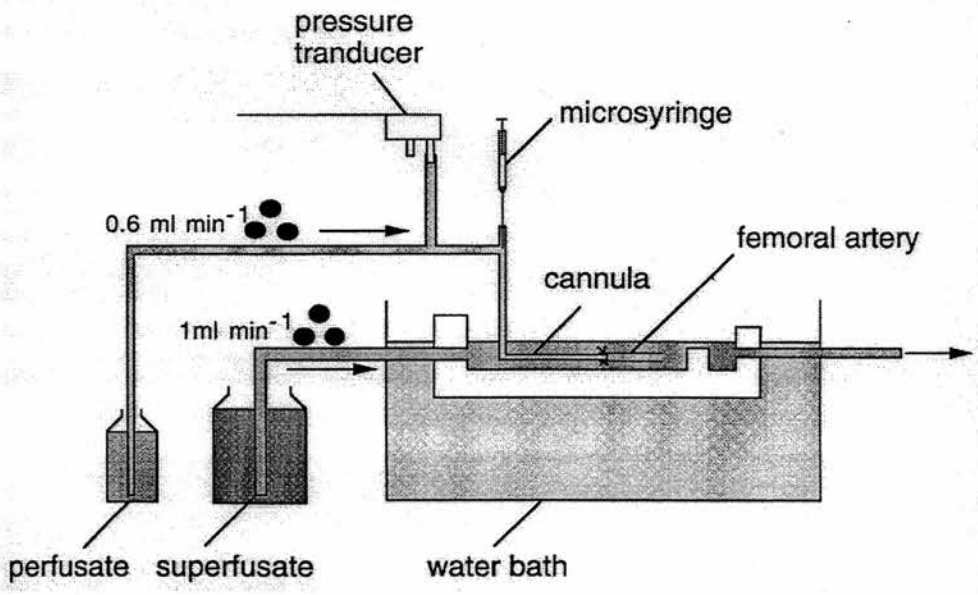
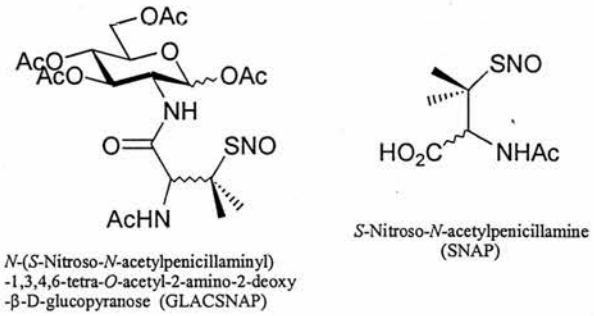


Figure 3.1: Apparatus used for biological testing

The vessels were precontracted with phenylephrine and the perfusion pressure was monitored by a differential pressure transducer. Test drugs were injected through a rubber resealable cap just upstream from the arteries. Injection of the vehicle (Krebs buffer) had no effect on perfusion pressure. Vasodilator responses in control vessels could be compared to those perfused with supramaximal concentrations of either the recognised NO scavenger ferrohemoglobin (10 μM),[159] or the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (200 μM).[160] All experiments were carried out in a darkened laboratory in order to protect the photolabile compounds and to prevent the photorelaxation of the blood vessels.[115] All compounds were dissolved in Krebs solution and kept on ice before use. In experiments requiring endothelium-denuded vessels, the endothelium was removed by passing air through the lumen until the vessel became unresponsive to carbachol (10^{-2} M), an endothelium-dependent vasodilator (5 – 10 min). Removal of the endothelium invariably caused an increase in pressure due to the loss of endothelium-derived NO. Pressure was restored to its original level by appropriate reduction in phenylephrine concentration (~ 0.5 x original concentration).

3.3. Vasodilatory activity of GLACSNAP and SNAP

As mentioned earlier, GLACSNAP had initially been prepared in the Butler group prior to this study commencing. Thorough testing of this compound, however, has been carried out as part of the current project. (Figure 3.2)



N-(*S*-Nitroso-*N*-acetylpenicillaminyl)
-1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy
-β-D-glucopyranose (GLACSNAP)

S-Nitroso-*N*-acetylpenicillamine
(SNAP)

Figure 3.2: GLACSNAP and SNAP

GLACSNAP was compared to SNAP for its vasodilatory properties and its decomposition pattern as described elsewhere.[113] Here we will simply outline the results found at that time, as comparison to these is pivotal for all new compounds evaluated.

3.3.1. Decomposition patterns for SNAP and GLACSNAP

Decomposition of both SNAP and GLACSNAP was assessed by monitoring the absorbance at 340 nm in oxygenated Krebs buffer. SNAP was found to have a half life ($t_{½}$) of approximately 44 minutes, while $t_{½}$ for GLACSNAP was found to be approximately 24 hours. (Figure 3.3)

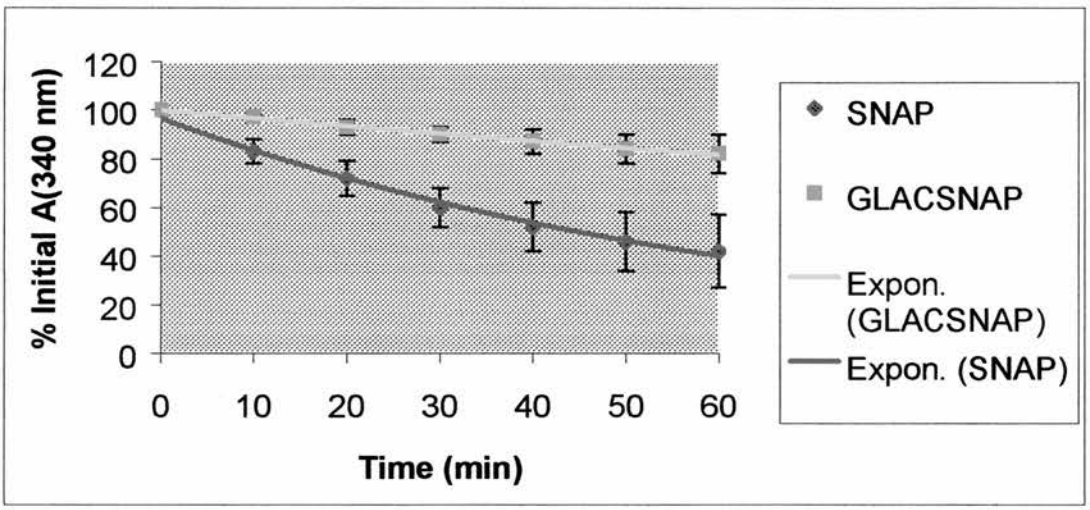


Figure 3.3: Decomposition curves for SNAP and GLACSNAP at 24°C. The trendlines represent the calculated first order decay plots.

Both compounds decomposed following first order kinetics (symbols), as shown by the good fit to first order decay plots (lines).

The effect of copper-aided decomposition was also assessed. The decompositions of both compounds were monitored in Krebs buffer, in Krebs buffer containing 10^{-5} M Cu^{2+} and in Krebs buffer containing neocuproine (NCu), a copper chelating agent. (Figures 3.4 and 3.5)

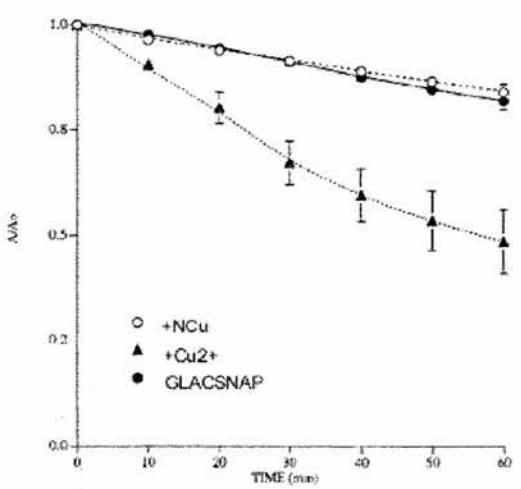


Figure 3.4: Effect of copper on GLACSNAP thermal decomposition

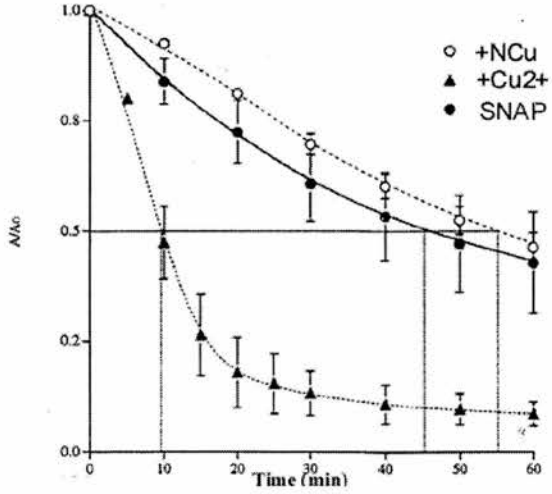


Figure 3.5: Effect of copper on SNAP decomposition

As can be seen in figures 3.4 and 3.5, the addition of copper markedly reduces the half life of both compounds. SNAP was found to have a half life of 10 minutes, while GLACSNAP had a half life of approximately one hour.

3.3.2. Vasodilatory activity of SNAP and GLACSNAP

The vasodilatory responses to both compounds were recorded, as described in section 3.2., on endothelium-denuded vessels. Representative traces shown in figure 3.6.

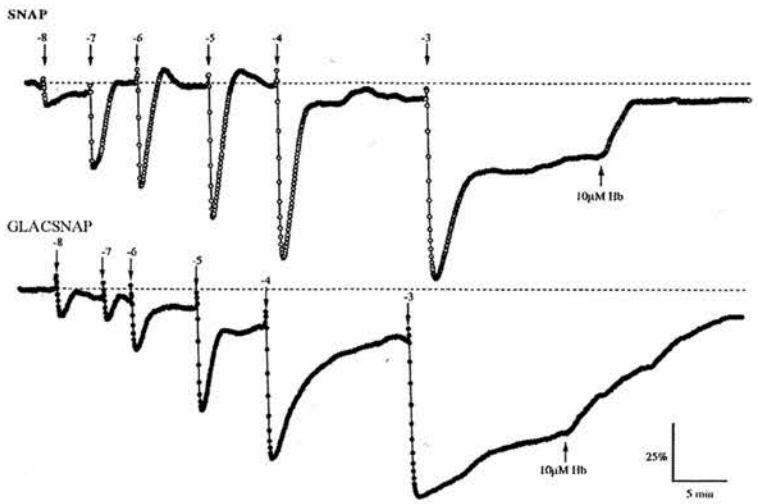


Figure 3.6: Response curves for SNAP and GLACSNAP on endothelium-denude vessels. The arrows indicate bolus injections of *S*-nitrosothiol at concentration of 10^{-8} to 10^{-3} M.

Persistent vasodilation occured with lower doses of GLACSNAP than of SNAP and it was reversed by perfusing with 10 μM Hb. The log-dose response curves show that at a given concentration of SNAP and GLACSNAP, the latter induces a vasodilatory response that is lower in magnitude but less reversible than the former.

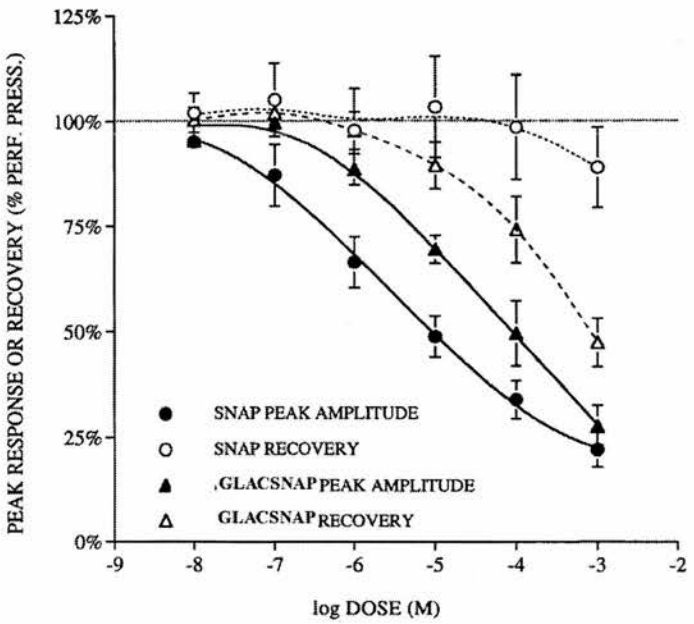


Figure 3.7: Log dose response curves for SNAP and GLACSNAP

This indicates that, although SNAP is a better vasodilator, GLACSNAP has a longer lasting effect. This can be more clearly seen in figure 3.8.

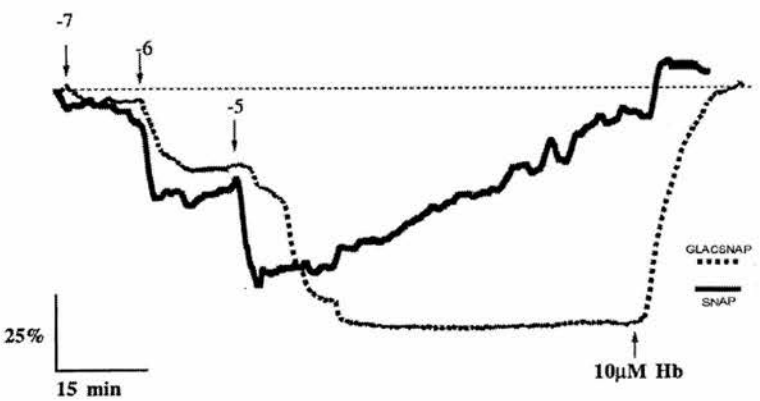


Figure 3.8: Response curves for SNAP and GLACSNAP

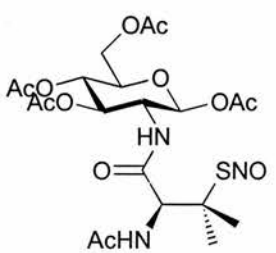
Here SNAP and GLACSNAP have been added to the superfusate and the vasodilatory response to cumulative doses investigated. The initial concentration of the compounds

was 10^{-7} M and gradually increased to 10^{-5} M over a period of 30 minutes. The vessels were then kept at a constant concentration of 10^{-5} M for a further 60 minutes. At t = 90 10 μM ferrohaemoglobin was added to remove any NO present in the system and therefore to restore the vessels to their original pressure. As can be seen, although initially the pressure in GLACSNAP-treated vessels was higher that in SNAP-treated ones, the dilation caused by GLACSNAP remained at its peak value for the duration of the experiment until treatment with Hb. SNAP-treated vessels gradually recovered to their original pressure despite the concentration of SNAP being kept constant.

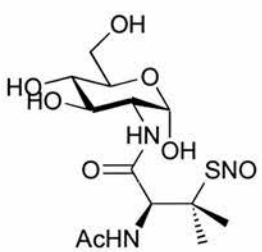
Overall, GLACSNAP has a longer lasting vasodilatory response than SNAP and would therefore be a better choice for a drug candidate. The reason we postulate GLACSNAP to have a sustained effect, most marked in endothelium-denuded vessels, is that the compound may be retained in the sub-endothelial layer from where it gradually releases NO.

3.4. Acetylated *versus* non-acetylated sugars

This part of the study was aimed at the comparison of GLACSNAP and its non-acetylated derivative GLUSNAP, so that the importance, or otherwise, of the acetyl groups could be assessed (i.e. ability, or otherwise, to reside in the sub-endothelial layer).



N-(*S*-Nitroso-*N*-acetyl-D-penicillaminyl) 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose (GLACSNAP)



N-(*S*-Nitroso-*N*-acetyl-D-penicillaminyl) 2-amino-2-deoxy-α-D-glucopyranose (GLUSNAP)

Figure 3.9: GLACSNAP and GLUSNAP

As well as possible recognition by the postulated *S*-nitrosothiol-decomposing enzyme, the acetyl groups will also influence the lipophilicity of the compound, and hence its uptake by the endothelial cells or the collagen layer immediately underlying it.

3.4.1. *In vitro* decomposition studies

Decomposition curves for GLACSNAP and GLUSNAP were recorded. The results are outlined in figure 3.10.

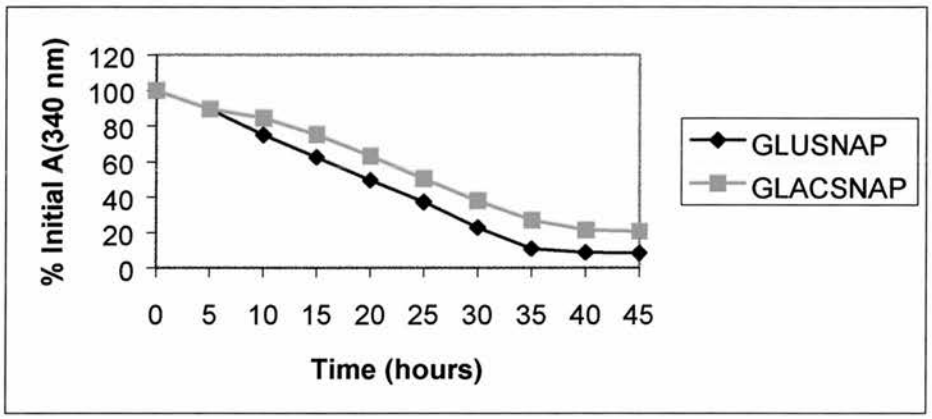


Figure 3.10: Decomposition curves for GLACSNAP and GLUSNAP

The curves obtained are comparable, indicating that the two compounds release NO at similar rates. The half lives of GLACSNAP and GLUSNAP were found to be approximately 20 and 24 hours, respectively.

3.4.2. Vasodilation studies

Bolus injections of 1mM GLACSNAP and GLUSNAP were administered to both endothelium-intact and endothelium-denuded arteries and the following curves were obtained (Figure 3.11).

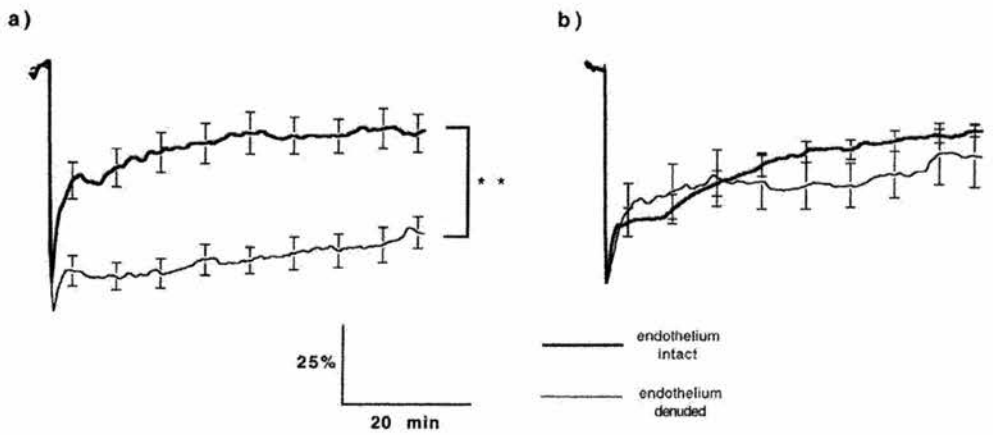


Figure 3.11: Means traces (n = 6) for a) GLACSNAP and b) GLUSNAP after a 1 mM bolus injection. The X-axis defines time, while the Y-axis is the percentage of vasodilation of the vessel.

It is notable that for both compounds, where endothelium-intact vessels were used, the pressure dropped upon injection but the response was transient and pressure slowly recovered back to its initial level. The response that the compounds cause in endothelium-denuded vessels is, however, quite different. With GLACSNAP the pressure drops significantly and the response was sustained over a period of several hours, whilst with GLUSNAP the profile is comparable to that obtained in endothelium-intact vessels.

Not only does GLACSNAP have a more sustained response than GLUSNAP, the response is also a larger one, as shown in Figure 3.12.

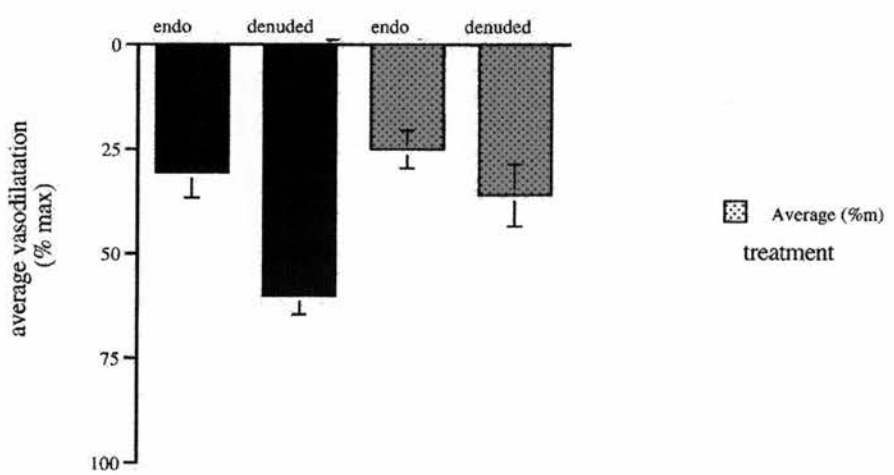


Figure 3.12: Average vasodilation (n = 8) to 1 mM NO-donor over 1 hour, expressed as a percentage of remaining vasodilation. Black: GLACSNAP; Red: GLUSNAP

We have postulated that the reason why GLACSNAP causes such an extensive and sustained vasodilation is because it is being retained in the sub-endothelial layer, where it slowly decomposes to release NO. GLUSNAP is much less lipophilic than GLACSNAP and therefore might not be as efficiently taken up by the vascular tissue.

3.5. Tolerance studies

Organic nitrates are currently one of the most commonly used classes of NO-donor compounds. Glyceryl trinitrate (GTN) is used for treatment of angina, cardiac ischaemia, myocardial infarction and heart failure. Its therapeutic use, however is limited by the development of tolerance, which can occur in a period as short as 24 hours.[161] The current remedy is to limit therapy to only 12 hours at a time, allowing the patient a nitrate-free period, usually at night. This is not satisfactory and the search for a therapy that could be administered without causing tolerance is still ongoing. *S*-Nitrosothiols have been found to be endogenous[162,163] and therefore may not induce self-tolerance. *S*-Nitroso-*N*-acetyl-D,L-penicillamine itself has been shown to develop less tolerance than GTN,[112] therefore we have investigated a selection of our *S*-nitrosothiols to determine whether they to engender tolerance. The compounds tested were *S*-nitrosoglutathione (GSNO), GLACSNAP and D-SNVP.

3.5.1. Materials and methods

All experiments were carried out on isolated segments of femoral artery from adult male Wistar rats in the perfusion system described in section 3.2. Tolerance was initially induced by perfusing the vessels with the NO-donors (10 μM) or Krebs buffer as a control. The vessels were perfused overnight at 25°C to optimise survival. At $t = 20$ h, the original phenylephrine-containing Krebs buffer was re-perfused at 0.6 ml/min at 37°C. In GTN-treated vessels and control vessels, at $t = 20$ h, bolus injections of increasing concentration of NO-donor (10 μl, 10^{-8} – 10^{-3} M) were made sequentially into the perfusate. Responses were deemed to have recovered once pressure was maintained for more than 2.5 min, at which time the next dose was injected. To confirm

viability in those vessels which did not re-develop tone with phenylephrine following 20 h *S*-nitrosothiol perfusion, these were washed out with Krebs buffer until maximum pressure was restored. In *S*-nitrosothiol-treated vessels, at t = 20, the NO scavenger ferrohaemoglobin (10 μM)[159] was added to the internal perfusate to prove the vasodilatory effect was NO-linked.

3.5.2. Results

Perfusion with GTN (10 μM) caused an initial vasodilation of 72 ± 3%. The vessels gradually recovered to 35 ± 10% dilation. After overnight incubation the pressure was not significantly different from the control value (10 ± 10%).

Perfusion with GSNO, GLACSNAP and D-SNVP (10 μM) produced greater vasodilations (91 ± 2%, 93 ± 1% and 84 ± 3% respectively) which were maintained over the 20 h period.

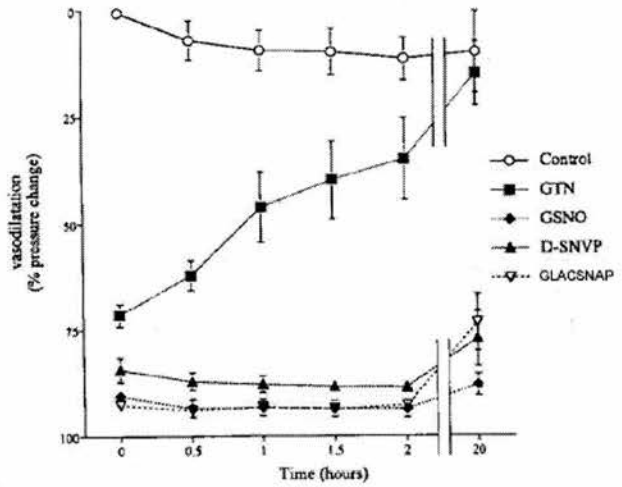


Figure 3.13: Effect of perfusing NO donors on perfusion pressure in preconstricted arteries

Bolus injections of GTN (10 µl, 10^{-8} – 10^{-3} M) produced transient vasodilations. In control vessels 10^{-3} M GTN produced a vasodilation of 65 ± 7%; in vessels perfused with GTN for 20 h (GTN-tolerant vessels) the response was markedly attenuated (19 ± 4%). Equivalent injections of *S*-nitrosothiols also produced transient vasodilations (69 ± 6%, 70 ± 3% and 70 ± 6% for GSNO, GLACSNAP and D-SNVP respectively).

However, the concentration response curves for the *S*-nitrosothiols in GTN-tolerant vessels were not significantly different from those in control vessels

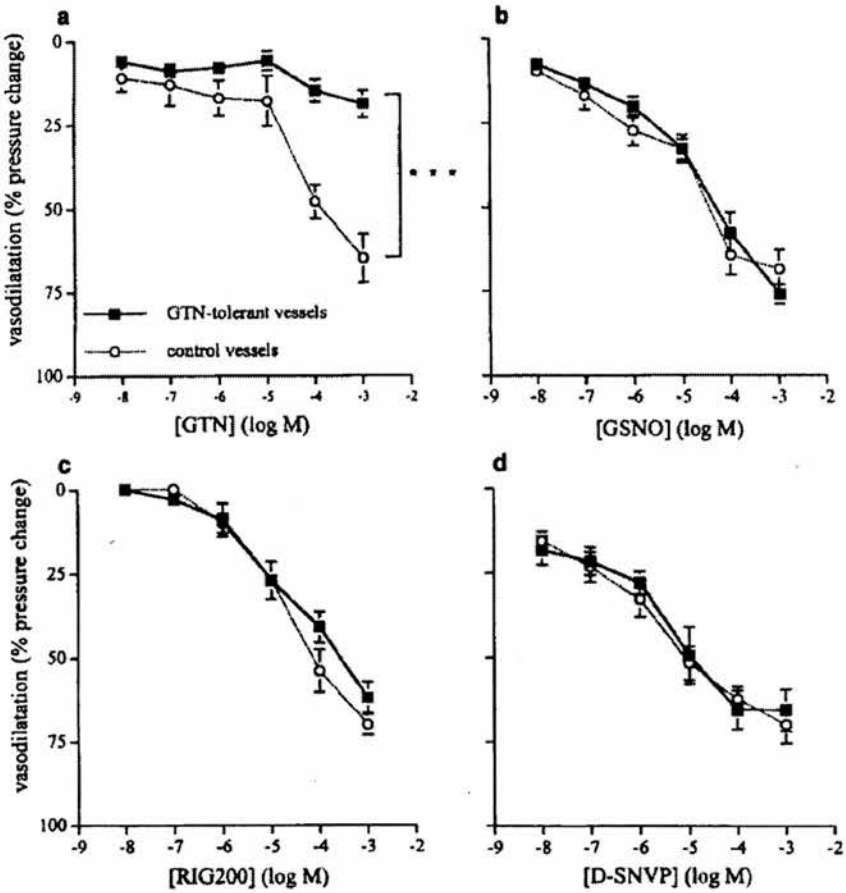


Figure 3.14: Log concentration response curves showing the vasodilatory effect (% pressure change) of bolus injections of a) GTN, b) GSNO, c) GLACSNAP and d) D-SNVP in control (open circles) and GTN-tolerant (filled squares) vessels.

At $t = 20$ h, the *S*-nitrosothiols and GSNO were removed from the internal perfusate. On washout pressure recovered to levels that were not significantly different from the preconstriction pressures before the perfusion. Pressure rapidly recovered in 5.5 ± 0.9, 6.5 ± 0.8 and 11.1 ± 4.5 min for GSNO, GLACSNAP and D-SNVP respectively. The time course for D-SNVP washout was significantly slower than for GSNO and GLACSNAP.

3.5.3. Discussion

Results from the experiments discussed show that tolerance to GTN develops rapidly in rat femoral arteries, within 20 hours of exposure, resulting in a marked decrease in the vessels' response to bolus injections of GTN. The *S*-nitrosothiols tested did not induce tolerance within 20 hours of exposure and did not exhibit cross-tolerance to the GTN-tolerant vessels.

The *S*-nitrosothiols tested relaxed the vessels to a similar extent, producing a 90% dilation throughout the 20 hour period, despite the slow decomposition of the compounds in the perfusate reservoir. This study demonstrates that these particular *S*-nitrosothiols do not engender tolerance. Following the 20 hour period, washout rapidly restored vessel pressure, hence demonstrating the viability of the vessels and confirming the reversibility of the *S*-nitrosothiol-mediated dilation.

Our finding that *S*-nitrosothiols do not engender tolerance and that they remain active in GTN-tolerant vessels, reinforces the conclusion given by Ignarro *et al.*[108] that the underlying cause of nitrate tolerance is upstream of NO release.

3.6. SNAP analogues

As mentioned earlier, SNAP has been used by a number of groups as a standard for *S*-nitrosothiols, but in previous sections it has been demonstrated that not all *S*-nitrosothiols exhibit the same vasodilatory properties. SNVP and GLACSNAP, in fact, have very different profiles to SNAP, although the structural differences may not appear to be extreme.

It was decided to carry out a direct comparison between all SNAP-like *S*-nitrosothiols synthesised, to determine whether there was a pattern to the difference in their vasodilatory activity or whether the differences were entirely random.
The compounds chosen for investigation were the following (Figure 3.15).

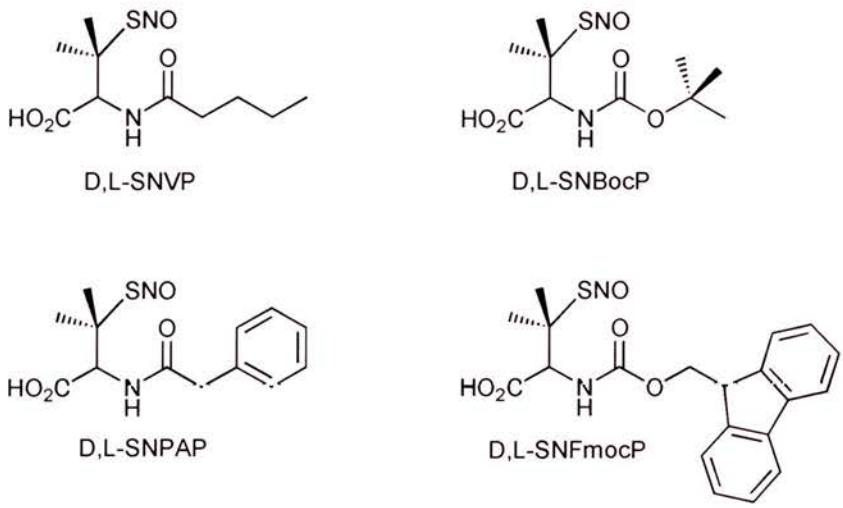


Figure 3.15: SNAP-like *S*-nitrosothiols tested

3.6.1. Decomposition studies

The decompositions of the four compounds were carried out as described in section 3.1. All compounds were dissolved in a 1:1 mixture of Krebs buffer and DMSO due to the poor solubility of SNBocP and SNFmocP in Krebs buffer alone.

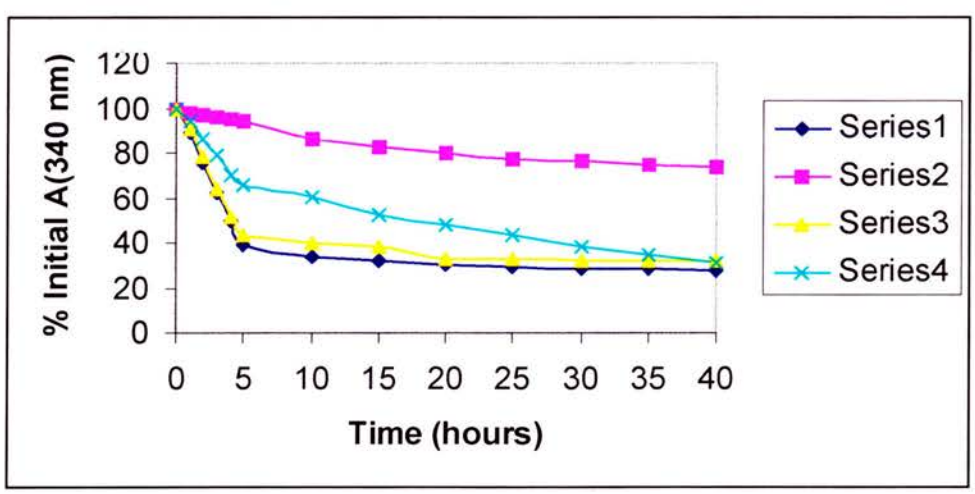


Figure 3.16: Comparison of the decompositions of the four compounds

As can be seen above, DL-SNVP, DL-SNPAP and DL-SNFmocP decompose in a similar fashion. The half lives for the four compounds were found to be 4 hours for SNVP and SNPAP and 18 hours for SNFmocP. SNBocP did not decompose to less than 80% of its initial absorbance in the duration of the experiment (40 hours), indicating the

high stability of this compound.

The results of the *ex vivo* testing do not seem to mirror the *in vitro* decompositions at all.

3.6.2. Vasodilatory activity studies

The vasodilatory activity of the 4 SNAP analogues was determined using the methodology described in section 3.2. The traces obtained for the compounds are shown in figure 3.17.

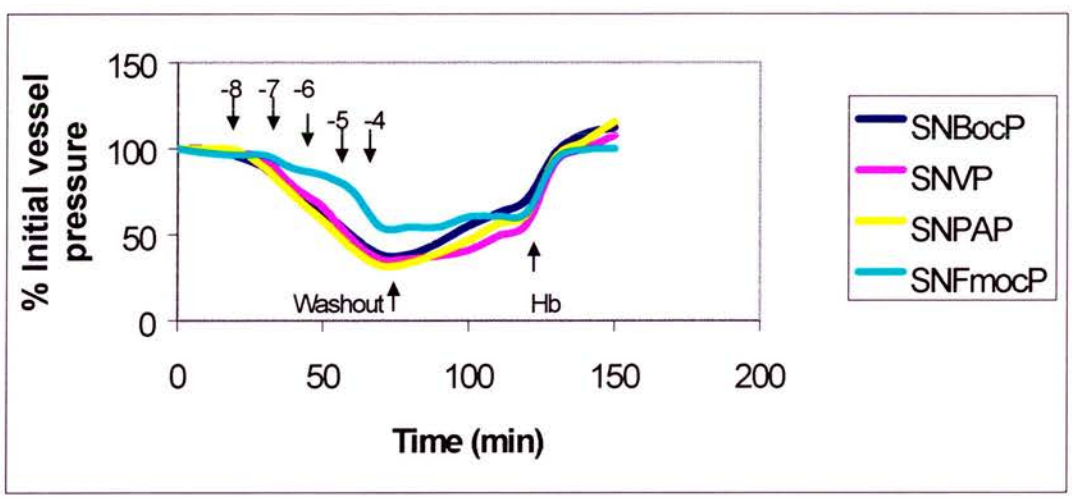


Figure 3.17: Vasodilation traces for the SNAP derivatives

The isolated arteries were injected with bolus injections of *S*-nitrosothiols at increasing concentrations (10^{-8} to 10^{-4} M) in 1:1 Krebs buffer / DMSO. At t = 75 min the arteries were perfused with Krebs buffer so that any *S*-nitrosothiol in the lumen would be washed out. At t = 125 min 10 μM Hb was added to remove any NO present in the system and therefore restore the arteries to their original pressure.

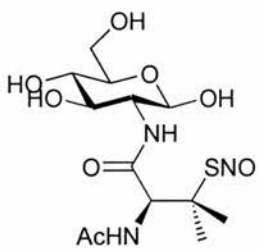
It is immediately noticeable from the traces above that SNVP, SNPAP and SNBocP have a very similar vasodilatory profile, reaching 30% of the initial vessel pressure, whilst SNFmocP is quite different. SNFmocP is a poorer vasodilator, reaching only about 50% of the initial vessel pressure, but its response is a much more sustained one than the other compounds. Even after washout the vessel treated with SNFmocP shows a 10% recovery as opposed to the 25-35% recovery for the other three

vasidilators, indicating that the compound must be somehow retained within the vessel wall. This is consistent with data for GLACSNAP and GLUSNAP, i.e. the more hydrophobic compounds have a longer-lasting effect.

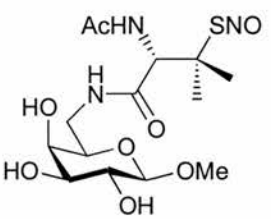
The mechanisms by which S-nitrosothiols bring about vessel dilation is not yet fully understood. It could be argued that the *S*-nitrosothiols initially release NO in the lumen of the arteries and then it is NO itself to be retained within the vessel walls, but the decomposition studies do not support this conclusion. If that were the case, SNBocP should be the least effective vasodilator, since it has a very long half life (greater than 40 hours). The activity in our *ex vivo* model is obviously determined by the capability of the *S*-nitrosothiol to be retained within the walls of the vessel, not by its ability to release NO.

3.7. GLACSNAP analogues

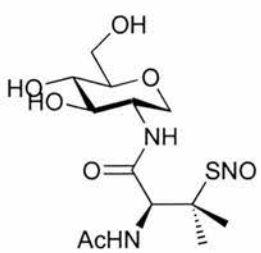
The comparison between GLACSNAP and its non-acetylated analogue GLUSNAP has already been discussed (section 3.4), here we describe the comparison between GLACSNAP and three other glycosylated *S*-nitrosopenicillamines (Figure 3.18).



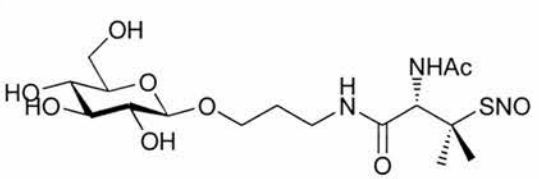
N-(*S*-nitroso-*N*-acetyl-D-penicillaminyl)-2-amino-2-deoxy-*β*-D-glucopyranose **(GLUSNAP)**



Methyl *N*-(*S*-nitroso-*N*-acetyl-D-penicillaminyl)-6-amino-6-deoxy-*β*-D-galactopyranoside **(Sugar 1)**



2-Amino-1,5-anhydro-2-deoxy-*N*-(*S*-nitroso-*N*-acetyl-D-penicillaminyl)-D-glucitol (Sugar 2)



3-Amino-[*N*-(*S*-nitroso-*N*-acetyl-D-penicillaminyl)]-propyl-*β*-D-glucopyranoside (Sugar 3)

Figure 3.18: GLACSNAP analogues

The decomposition of the four compounds were recorded at 340 nm (Figure 3.19).

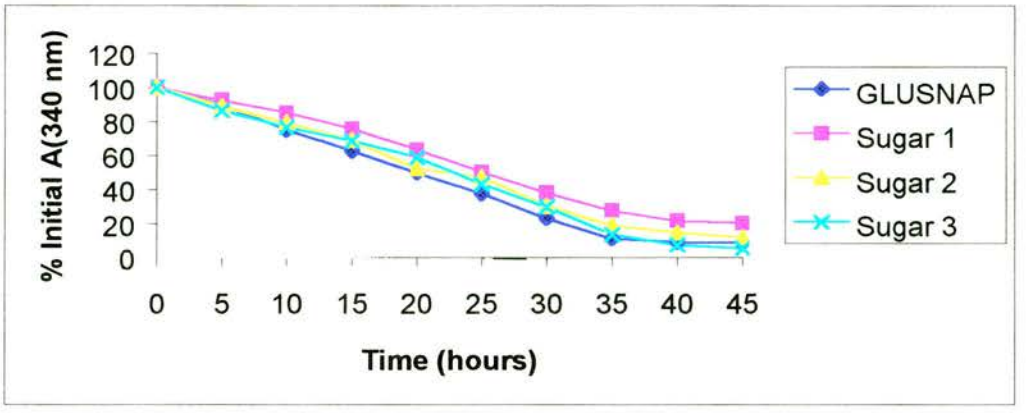


Figure 3.19: Decompositions of GLACSNAP analogues

As can be quite clearly seen, the four compounds decompose with a similar pattern and with a half life of 20 to 25 hours. The decompositions are described by the following equation: $y = ax^2 - bx + cx$. This clearly shows that the decomposition does not follow standard first order kinetics ($[A] = [A_0]e^{-kt}$) and therefore there must be an other component aiding decomposition. The most likely candidate is Cu^+ which is present in Krebs buffer.

As trace copper is present in the water used to prepare Krebs buffer, one of the explanations for these trends could be the following. The copper ions present could be initially catalysing RSNO decomposition (as discussed in section 1.6.6.); as the concentration of disulfide increases, the copper ions become complexed by the disulfide itself and hence are unavailable to induce further decomposition.

At present the vasodilatory ability of sugars 1 to 3 is in the process of being assessed by the *ex vivo* method described in section 3.2.

3.8 Summary and conclusions

In summary, a number of *S*-nitrosothiols were tested *in vitro* and in an *ex vivo* model. Both the thermal stabilities of the compounds tested and their vasodilatory activities are varied, but there seems to be no correlation between *ex vivo* activity and inherent *in vitro* stability of the molecules, as shown in table 3.1.

Compound	Half life (hours)	Maximum vasodilation at 1mM (% of initial pressure)
SNAP	0.75	23 %
GLACSNAP	24	27 %
GLUSNAP	20	25 %
SNVP	4	35 %
SNBocP	> 40	40 %
SNPAP	4	30 %
SNFmocP	20	55 %
Sugar1	25	To be determined
Sugar2	20	To be determined
Sugar3	23	To be determined

Table 3.1: Correlation between *in vitro* and *ex vivo* data

One conclusion that can be drawn is that the more hydrophobic compounds seem to have a more sustained effect in the *ex vivo* testing. This is most likely due to the fact that these compounds are more readily retained in the sub-endothelial layer, from where they can gradually release NO. The extent to which these compounds bring about vasodilation is quite varied (23% to 55%) hence not supporting or confuting that their activity is mediated by an enzyme. However, the structural differences between the compounds seem too marked for an enzyme to be able to recognise and process all of them. Unfortunately the results offer no proof one way or the other.

We have demonstrated that at least two of the compounds prepared had an advantage over existing drugs in that they do not engender tolerance. This might be common to the whole *S*-nitrosothiol family and studies to investigate this point are ongoing.

It appears that *S*-nitrosothiols are good candidates for vasodilator drugs. These compounds are relatively new and extensive work has to be done on their optimisation and testing, however preliminary results have shown that they possess good activity, they give rise to potentially non-toxic metabolites and have been proven not to engender tolerance.

Chapter 4: Experimental

4.1. General methods

All reagents and solvents were dried prior to use according to standard methods.[164] Commercial reagents were otherwise used without further purification. Analytical TLC was performed on silica gel 60-F_{254} (Merck) with detection by fluorescence and/or by charring following immersion in a dilute ethanoic solution of sulfuric acid or phosphomolybdic acid. Column chromatography was performed with silica gel 60 (Fluka).

Optical rotations were measured at the sodium D-line and at ambient temperature, with an Optical Activity AA-1000 polarimeter. $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Melting points were measured using a Gallenkamp melting point apparatus and are uncorrected. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 spectrometer at 300 MHz and 75 MHz, respectively. ^1H NMR spectra were referenced to the following internal standards: $CHCl_3$, δ_H 7.26 ppm in $CDCl_3$; CD_2HOD, δ_H 3.35 ppm in CD_3OD, 4.75 ppm in D_2O. ^{13}C NMR spectra were referenced to the following internal standards: δ_C 77.23 ppm in $CDCl_3$; δ_C 49.15 ppm in CD_3OD. *J*-values are given in Hz. UV-vis spectra were recorded on a Phillips PU8700 UV-visible scanning spectrophotometer.

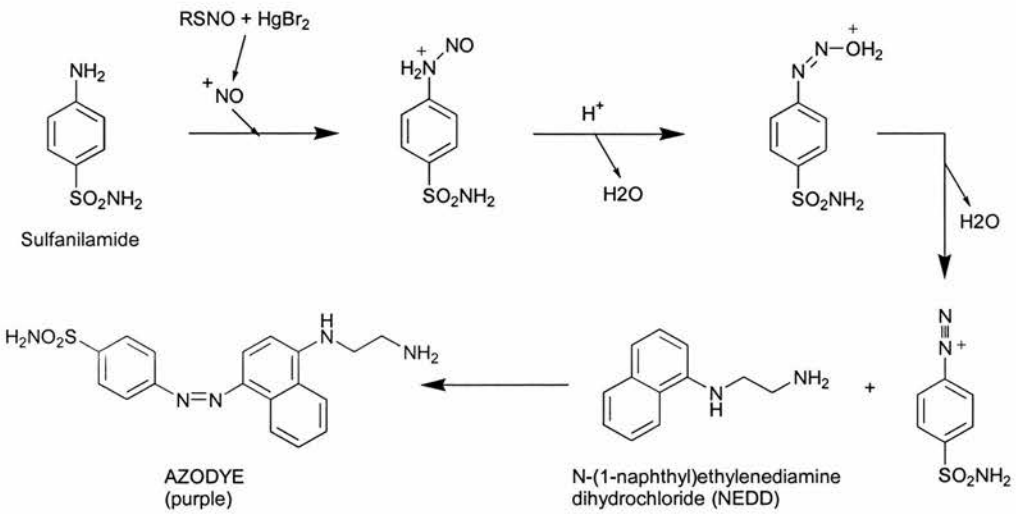
– Mass spec? IR

4.2. Preparation of *S*-nitrosothiols *via* the fuming method

Unless otherwise stated, all *S*-nitrosothiols were prepared *via* the fuming method. This consisted of dropping conc. HCl onto sodium nitrite and bubbling the fumes that were produced (known as 'nitrous fumes') directly into a solution of the thiol in which ever solvent it was soluble in. The solvent was then removed *in vacuo* to yield the *S*-nitrosothiol in near quantitative yield.

4.3. Determination of *S*-nitrosothiol purity *via* the Griess Test

S-Nitrosothiol purity was determined by the following colourimetric reaction:



Scheme 4.1: Mechanism of the Griess test

The Griess test is based on the fact that *S*-nitrosothiols react with mercury(II) salts to release NO^+.[165] After reaction of an *S*-nitrosothiol with mercury bromide, the resulting NO^+ is reacted with sulfanilamide. Subsequent dehydration yields the diazonium salt, which is then allowed to react with NEDD, giving rise to the formation of an azodye, a purple species which absorbs strongly at 550nm. Determination of the UV absorption is a quantitative analysis of the purity of the initial *S*-nitrosothiol. All *S*-nitrosothiols used in biological testing had >97% purity, as judged by this method.

Experimental:

A millimolar solution of *S*-nitrosothiol (2.5 ml) was added to doubly distilled water (20 ml), a millimolar solution of $HgBr_2$ (2.5 ml) and a solution of sulfanilamide (500 mg in 100 ml water, 20% HCl) (0.5 ml). The solution was allowed to stand at room temp. for 5 min, after which a solution of NEDD (300 mg in 100 ml water, 1% HCl) (0.5 ml) was added. The solution rapidly turned purple and the absorbance at 550 nm was recorded. The purity of the *S*-nitrosothiol tested was determined by fitting the absorbance value obtained to the equation of a calibration curve previously obtained for a number of concentrations of NEDD.

4.4. Sugar-amino acid coupling procedures

A. DCC coupling:

The amino sugar (free base) (1 mol. eq.) and the *N*-protected amino acid were dissolved in a suitable dry solvent (1 ml / 100 mg of sugar) and DCC (1.2 mol. eq.) was added. The solution was stirred at room temp and the reaction monitored by TLC. Once the reaction had proved complete, the DCU precipitate was removed by filtration. In the case of the unprotected sugar, the solvent was evaporated *in vacuo* to give an oil which was then purified by column chromatography or, alternatively, directly acetylated with pyridine and acetic anhydride. In the case of the protected sugar, the organic solvent was washed with 1M HCl, sat. $NaHCO_3$ solution and water, then dried ($MgSO_4$) and concentrated *in vacuo*. The resulting oil or solid was purified by column chromatography, eluting with ethyl acetate / hexane mixtures, to yield the coupled compound.

B. CMEC coupling:

The amino sugar (free base) (1 mol. eq.), and the *N*-protected amino acid were dissolved in a suitable dry solvent (1 ml / 100 mg of sugar) and CMEC (1.2 mol. eq.) was added. The solution was stirred at room temp and the reaction monitored by TLC and the Kaiser test. Once the reaction had proved complete, in the case of the unprotected sugar, the solvent was evaporated *in vacuo* to give an oil which was then purified by column chromatography or, alternatively, directly acetylated with pyridine and acetic anhydride. In the case of the protected sugar, the organic solvent was washed with 1M HCl, sat. $NaHCO_3$ and water, then dried ($MgSO_4$) and concentrated *in vacuo*. The resulting oil or solid was purified by column chromatography, eluting with ethyl acetate / hexane mixtures, to yield the coupled compound.

C. Mixed anhydride coupling:

The *N*-protected amino acid (1 mol. eq.) was dissolved in a suitable dry solvent (1 ml / 100 mg aminoacid) and NMM (1.1 mol. eq.) was added. The solution was then cooled to -10°C. IBCF (1 mol. eq.) was then added and the solution was stirred at -10°C for 30 min. In the mean time, the sugar amine HCl salt (1.1 mol. eq.) was dissolved in a suitable solvent (1 ml / 100 mg sugar). In the case of unprotected sugars, water was

added to THF until it was fully saturated. Triethylamine (1.1 mol. eq.) was then added. The sugar solution was then added to the amino acid solution and the mixture was stirred at room temperature. The reaction was followed by TLC and by the Kaiser test. Once the reaction had proved complete, in the case of the unprotected sugar, the solvent was evaporated *in vacuo* to give an oil which was then purified by column chromatography or, alternatively, directly acetylated with pyridine and acetic anhydride. In the case of the protected sugar, the organic solvent was washed with 1M HCl, sat. $NaHCO_3$ and water, then dried ($MgSO_4$) and concentrated *in vacuo*. The resulting oil or solid was purified by column chromatography, eluting with ethyl acetate / hexane mixtures, to yield the coupled compound.

D. PyBOP coupling:

The *N*-protected amino acid (1mol. eq.) and PyBOP (2 mol. eq.) were dissolved in dry MeCN (1 ml / 100 mg aminoacid) and DIPEA (3 mol. eq.) was added. The sugar amine HCl salt (1 mol. eq.) was then added and the solution stirred at room temp. The reaction was followed by TLC and by the Kaiser test. Once the reaction had proved complete, in the case of the unprotected sugar, the solvent was evaporated *in vacuo* to give an oil which was then purified by column chromatography or, alternatively, directly acetylated with pyridine and acetic anhydride. In the case of the protected sugar, the organic solvent was washed with 1M HCl, sat. $NaHCO_3$ and water, then dried ($MgSO_4$) and concentrated *in vacuo*. The resulting oil or solid was purified by column chromatography, eluting with ethyl acetate / hexane mixtures, to yield the coupled compound.

E. HATU coupling:

A solution of the sugar amine HCl salt (1 mol. eq.), the *N*-protected amino acid (1 mol. eq.) and DIPEA (3 mol. eq.) in a suitable dry solvent (1 ml / 100 mg sugar) was stirred at 0°C under nitrogen atmosphere. A solution of HATU (1.5 mol. eq.) in the same solvent (1 ml / 100 mg HATU) was added and the solution was allowed to warm to room temp. The reaction was followed by TLC and by the Kaiser test. Once the reaction had proved complete, in the case of the unprotected sugar, the solvent was evaporated *in vacuo* to give an oil which was then purified by column chromatography or, alternatively, directly acetylated with pyridine and acetic anhydride. In the case of the protected sugar, the organic solvent was washed with 1M HCl, sat. $NaHCO_3$ and water,

then dried ($MgSO_4$) and concentrated *in vacuo*. The resulting oil or solid was purified by column chromatography, eluting with ethyl acetate / hexane mixtures, to yield the coupled compound.

4.3 Determination of completion of coupling *via* the Kaiser test

The determination of consumption of free amine in a coupling reaction was carried out by use of the Kaiser test.[166] The test is a colourimetric test for the presence of primary amines. A positive test would indicate an incomplete reaction, whilst a negative test would confirm that the reaction had taken place. The Kaiser test consists of the addition of one drop of the crude reaction mixture to one drop of each of the following three solutions:

SOLUTION A: 500 mg ninhydrin in 10 ml EtOH;
SOLUTION B: 80 g phenol in 20 ml EtOH;
SOLUTION C: 2 ml of 1 mM aqueous solution of KCN in 100 ml pyridine.

Upon heating to 80 °C for 2 min, the solution turned blue (positive test) in the presence of unreacted amine; the solution remained yellow (negative test) if all amine had reacted and formed an amide bond.

4.6. Preparation of Krebs buffer

The buffer was prepared by adding the following to 1 litre of distilled water:

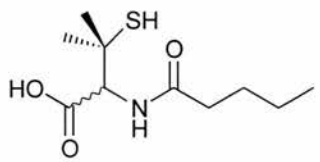
- NaCl (6.9 g)
- D-Glucose (1 g)
- $NaHCO_3$ (2.1 g)
- 10% KCl sol. (3.5 ml)
- 10% $MgSO_4.6H_2O$ sol. (2.9 ml)
- 10% K_2HPO_4 sol. (1.6 ml)

The solution was then bubbled with 95% O_2 / 5% CO_2 for 10 min, after which a 1 M $CaCl_2.6H_2O$ sol. (3.7 ml) was added and the bubbling continued for a further 5 min. The final ion content of the buffer was calculated to be the following:

- $[Na^+] = 144$ mM
- $[K^+] = 4.7$ mM
- $[Cl^-] = 130$ mM
- $[Ca^{2+}] = 2.5$ mM

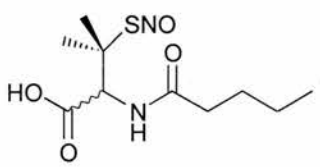
4.7. Synthesised compounds

N-Valeryl-D,L-penicillamine (12)



D,L-Penicillamine **(9)** (3 g, 20 mmol) and sodium valerate (3.9 g, 40 mmol) were added to a chilled solution of THF / water 4:1 (20 ml). Valeric anhydride (4 ml, 20 mmol) was added and the mixture stirred at room temp. overnight. The THF was evaporated *in vacuo* and more water (30 ml) was added. Conc. HCl was added dropwise until a white precipitate formed, which was filtered and crystallised from hexane / DCM 1:1 to yield the *title compound* as white crystals (5.5 g, 99%). m.p. 145-147°C; found: C, 51.18; H, 8.40; N, 5.77%, $C_{10}H_{19}NO_3S$ requires: C, 51.48; H, 8.21; N 6.00%; δ_H (300 MHz, CD_3OD): 0.95 (3H, t, *J* 6.2 Hz, -$(CH_2)_3\underline{C}H_3$), 1.37 (2H, m, $C\underline{H}_2$), 1.40 (3H, s, Me), 1.50 (3H, s, Me), 1.60 (2H, m, $C\underline{H}_2$), 2.30 (2H, m, $CO\underline{C}H_2CH_2$-), 4.55 (1H, s, C<u>H</u>); δ_C (75 MHz, CD_3OD): 14.2 (-$(CH_2)_3\underline{C}H_3$), 23.4 and 29.2 (2 x $\underline{C}H_2$), 30.6 and 30.7 (2 x Me), 36.6 ($CO\underline{C}H_2CH_2$-), 46.2 ($\underline{C}Me_2$) 63.1 (<u>C</u>H), 173.5 and 176.4 (2 x <u>C</u>O).

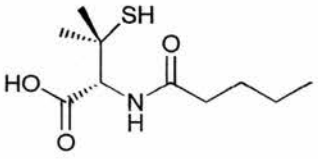
S-Nitroso-*N*-valeryl-D,L-penicillamine (SNVP) (7)



The *title compound* was prepared quantitatively from *N*-valeryl-D,L-penicillamine **(12)** *via* the "fuming method" and was obtained as dark green crystals. m.p. 87-89°C; λ_{max}

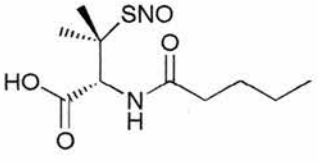
/nm (Krebs / DMSO 1:1) 341.1 (ε/ dm^3 mol^{-1} cm^{-1} 950.5).

_N_-Valeryl-L-penicillamine (14)



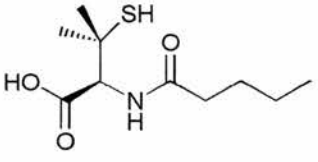
L-Penicillamine **(11)** (500 mg, 3.3 mmol) and sodium valerate (653 mg, 6.7 mmol) were added to a chilled solution of THF / water 4:1 (20 ml). Valeric anhydride (0.67 ml, 3.3 mmol) was added and the mixture stirred at room temp. overnight. The THF was evaporated *in vacuo* and more water (10 ml) was added. Conc. HCl was added dropwise until a white precipitate formed, which was filtered and crystallised from hexane / DCM 1:1 to yield the *title compound* as white crystals (584 mg, 76%). m.p. 124-125°C; found C, 51.48; H, 8.32; N, 5.72%, $C_{10}H_{19}NO_3S$ requires: C, 51.48; H, 8.21; N, 6.00%; $[\alpha]_D$ +11.5 (*c* 0.1 in $CHCl_3$); δ_H (300 MHz, CD_3OD): 0.84 (3H, t, *J* 7.3 Hz, -$(CH_2)_3C\underline{H}_3$), 1.20-1.25 (2H, m, $C\underline{H}_2$), 1.35 (3H, s, Me), 1.37 (3H, s, Me), 1.40-1.45 (2H, m, $C\underline{H}_2$), 2.05-2.1 (2H, m, $CO\underline{CH}_2CH_2$-), 4.40 (1H, s, $C\underline{H}$); 7.96 (1H, d, $J_{CH,NH}$ 9.2 Hz, $N\underline{H}$); δ_C (75 MHz, CD_3OD) 12.7 ($(CH_2)_3\underline{C}H_3$), 21.9 and 27.7 (2 x $\underline{C}H_2$), 29.0 and 29.1 (2 x Me), 35.0 ($CO\underline{C}H_2CH_2$-), 44.7 ($Me_2\underline{C}$), 61.4 ($\underline{C}H$), 171.7 and 175.1 (2 x $\underline{C}O$).

_S_-Nitroso-_N_-valeryl-L-penicillamine (L-SNVP) (16)



The *title compound* was prepared quantitatively from *N*-valeryl-L-penicillamine **(14)** *via* the "fuming method" and was obtained as dark green crystals. m.p. 82-84°C; $[\alpha]_D$ +105 (*c* 0.5 in $CHCl_3$); λ_{max} / nm (Krebs /DMSO 1:1) 341.5 (ε/ dm^3 mol^{-1} cm^{-1} 910.5).

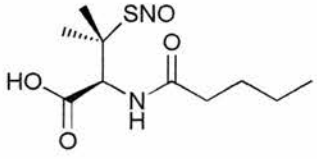
_N_-Valeryl-D-penicillamine (13)



D-Penicillamine **(10)** (500 mg, 3.3 mmol) and sodium valerate (653 mg, 6.7 mmol) were added to a chilled solution of THF / water 4:1 (20 ml). Valeric anhydride (0.67 ml, 3.3

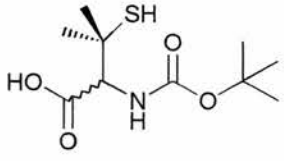
mmol) was added and the mixture stirred at room temp. overnight. The THF was evaporated *in vacuo* and more water (10 ml) was added. Conc. HCl was added dropwise until a white precipitate formed, which was filtered, dissolved in DCM (20 ml), washed with water (2 x 50 ml), dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow oil. This was crystallised from hexane / DCM to yield the *title compound* as white crystals (411 mg, 53%). m.p. 126-128°C; found C, 51.36; H, 8.40; N, 5.75%, $C_{10}H_{19}NO_3S$ requires C, 51.48; H, 8.21; N, 6.00%; $[\alpha]_D$ –12.2 (*c* 0.1 in $CHCl_3$); δ_H (300 MHz, CD_3OD): 0.93 (3H, t, *J* 7.0 Hz, -$(CH_2)_3CH_3$), 1.3-1.4 (2H, m, CH_2), 1.40 (3H, s, Me), 1.47 (3H, s, Me), 1.6-1.7 (2H, m, CH_2), 2.2-2.3 (2H, m, $COCH_2CH_2$-), 4.58 (1H, s, CH), 7.88 (1H, d, $J_{CH,NH}$ 9.2 Hz, NH); δ_C (75 MHz, CD_3OD): 12.7 (-$(CH_2)_3CH_3$), 21.9 and 27.7 (2 x CH_2), 29.0 and 29.1 (2 x Me), 35.0 ($COCH_2CH_2$-), 44.7 (Me_2C), 61.4 (CH), 171.7 and 175.0 (2 x CO).

_S_-Nitroso-_N_-valeryl-D-penicillamine (D-SNVP) (15)



The *title compound* was prepared quantitatively from *N*-valeryl-D-penicillamine **(13)** *via* the "fuming method" and was obtained as dark green crystals. m.p. 78-80°C; $[\alpha]_D$ –113 (*c* 0.5 in $CHCl_3$); λ_{max} / nm (Krebs /DMSO 1:1) 340.8 (ε / dm^3 mol^{-1} cm^{-1} 999.0).

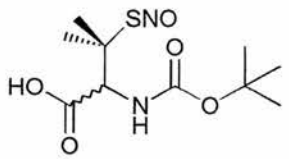
_N_-Butoxycarbonyl-D,L-penicillamine (20)[131]



The *title compound* was prepared by the protection method described by Bodanszky and Bodanszky.[142] D,L-Penicillamine **(9)** (1.49 g, 10 mmol) was dissolved in a mixture of dioxane (20 ml), water (10 ml) and 1M NaOH (10 ml) and stirred while cooling (ice-bath). Di-tert-butyldicarbonate (2.4 g, 11 mmol) was added and the stirring continued overnight. The solution was then concentrated *in vacuo* to about 15 ml and EtOAc (30 ml) was added. The mixture was cooled to 0°C and acidified with dilute $KHSO_4$ to pH 3. The aqueous phase was extracted with ethyl acetate. The organic phase was dried ($MgSO_4$) and concentrated *in vacuo* to give a white solid, this was crystallised from

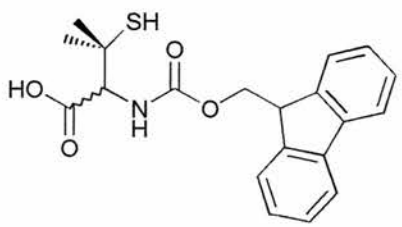
hexane / EtOAc 1:1 to yield the *title compound* as white crystals (1.46 g, 59%). m.p. 154-155°C (lit.,[131] 151-152°C); found: C, 48.04; H, 7.65; N, 5.64%, $C_{10}H_{19}NO_4S$ requires: C, 48.17; H, 7.68; N, 5.62%; δ_H (300 MHz, DMSO): 1.35 (15H, s, 5 x Me), 4.05 (1H, d, $J_{CH,NH}$ 9.3 Hz, C<u>H</u>), 6.90 (1H, d, $J_{CH,NH}$ 9.3 Hz, N<u>H</u>); δ_C (75 MHz, DMSO): 28.6 (3 x Boc-Me), 29.8 and 30.6 (2 x Me), 45.6 ($Me_2\underline{C}$), 63.9 (<u>C</u>H), 79.0 (Boc-$\underline{C}Me_3$), 156.2 (Boc-<u>C</u>O) and 172.2 ($\underline{C}O_2H$).

_N_-Butoxycarbonyl-_S_-nitroso-D,L-penicillamine (17)



The *title compound* was prepared quantitatively from *N*-butoxycarbonyl-D,L-penicillamine **(20)** *via* the "fuming method" and was obtained as a green oil. λ_{max} / nm (Krebs / DMSO 1:1) 341.8 (ε / dm^3 mol^{-1} cm^{-1} 840.5).

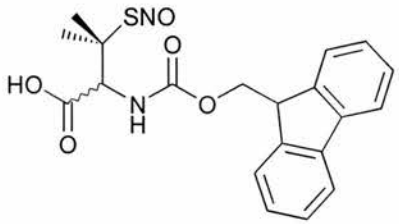
_N_-(9-Fluorenylmethyloxycarbonyl)-D,L-penicillamine (21)



The *title compound* was prepared following the protection procedure described by Bodanszky and Bodanszky.[142] D,L-Penicillamine **(9)** (1 g, 6.7 mmol) was dissolved in 10% $NaHCO_3$ (15 ml) and cooled to 0°C. A solution of 9-fluorenylmethyl chlorocarbonate (1.759 g, 6.8 mmol) in dioxane (2 ml) was added and stirring continued at room temperature overnight. The solution was diluted with water (100 ml) and washed twice with ether (2 x 150 ml). The aqueous layer was then acidified with conc. HCl and precipitation occurred. The precipitate was extracted into DCM (100 ml), dried ($MgSO_4$) and concentrated *in vacuo* to give a clear oil. This was triturated with hexane to give white crystals (1.6 g, 64%). m.p. 67-69 °C; δ_H (300 MHz, $CDCl_3$): 1.41 (3H, s, Me), 1.56 (3H, s, Me), 4.23 (1H, t, *J* 6.7 Hz, 9-H), 4.4-4.5 (2H, m, CH_2), 5.82 (1H, d, $J_{NH,CH}$ 9.3 Hz, CH), 6.31 (1H, broad s, NH), 7.32 (2H, t, *J* 7.1 Hz, 2 and 7-H or 3 and 6-H), 7.39 (2H, t, *J* 7.1 Hz, 2 and 7-H or 3 and 6-H), 7.59 (2H, broad s, 1 and 8-H), 7.76 (2H, d, *J* 7.4 Hz, 4 and 5-H); δ_C (75 MHz, $CDCl_3$): 30.8 and 31.6 (2 x Me), 46.3

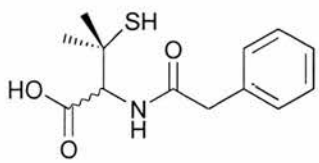
(CMe$_2$), 47.1 (C9), 62.8 (CH), 67.5 (CH_2), 120.1, 125.2, 127.3, 127.9, 141.5 and 143.7 (aromatics), 156.5, 174.6 (2 x CO); m/z (FAB) 371.1296 (M^+ $C_{20}H_{22}NO_4S$ requires 371.1191).

_N_-(9-Fluorenylmethyloxycarbonyl)-_S_-nitroso-D,L-penicillamine (19)



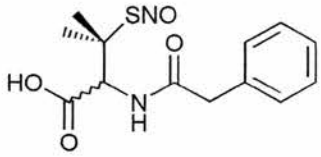
The *title compound* was prepared quantitatively from *N*-(9-Fluorenylmethyloxycarbonyl)-D,L-penicillamine **(21)** *via* the "fuming method" and was obtained as green crystals. m.p. 78-80 °C; λ_{max} / nm (Krebs / DMSO 1:1) 333.6 (ε / dm^3 mol^{-1} cm^{-1} 502.5).

_N_-Phenylacetyl-D,L-penicillamine (22)



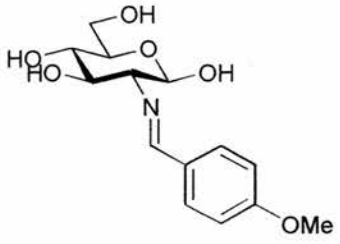
The *title compound* was prepared following the protection procedure described by Bodanszky and Bodanszky.[142] D,L-Penicillamine **(9)** (1 g, 6.7 mmol) was dissolved in a saturated solution of sodium bicarbonate (50 ml) and cooled to 0°C. Phenylacetyl chloride (1 ml, 7.5 mmol) was added dropwise and the mixture was stirred overnight. The solution was then washed with EtOAc (2 x 50 ml), then the aqueous layer was acidified to pH 3 with conc. HCl and extracted into DCM (4 x 50 ml). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo* to yield the *title compound* as a white solid, which was then crystallised from ether / DCM to give white crystals (1.22 g, 68%). m.p. 102-104°C; δ_H (300 MHz, $CDCl_3$): 1.23 (3H, s, Me), 1.45 (3H, s, Me), 3.64 (2H, s, CH_2-Ph), 4.58 (1H, d, $J_{CH,NH}$ 9.3 Hz, CH), 6.51 (1H, d, $J_{CH,NH}$ 9.3 Hz, NH), 7.2-7.4 (6H, m, aromatics); δ_C (75 MHz, $CDCl_3$): 30.2 and 31.3 (2 x Me), 41.6 (CMe$_2$), 61.3 (CH), 128.1, 128.4, 129.4, 129.9, 130.2 and 134.7 (aromatics), 172.9 and 174.1 (2 x CO); m/z (CI) 268.8171 (M^+ $C_{13}H_{18}NO_3S$ requires 268.8199).

_N_-Phenylacetyl-_S_-nitroso-D,L-penicillamine (19)



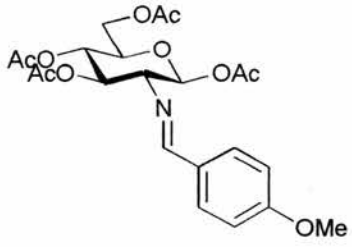
The *title compound* was prepared quantitatively from *N*-Phenylacetyl-D,L-penicillamine **(22)** *via* the "fuming method" and was obtained as a green oil. λ_{max} / nm (Krebs / DMSO 1:1) 348.9 (ε / dm^3 mol^{-1} cm^{-1} 603.5).

2-Amino-_N_-anisylidene-2-deoxy -β-D-glucopyranose (26)[123]



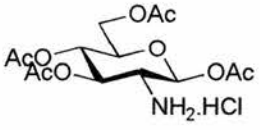
The *title compound* was prepared as described by Bergman and Zervas.[123] Glucosamine hydrochloride **(25)** (10 g, 47 mmol) was dissolved in 1M NaOH (47 ml, 47 mmol). *p*-Anisaldehyde (5.7 ml, 47 mmol) was added and the mixture shaken vigorously for about 10 min until precipitation occurred. The mixture was then cooled (ice-bath) for 30 min. The product was filtered, washed with ice-cold water and ice-cold ether and dried, then recrystallised from EtOH / H_2O 1:1 to yield the *title compound* as white crystals (11.5 g, 83%). m.p. 161-163°C (lit.,[123] 166°C); $[\alpha]_D$ +37.0 (*c* 0.1 in pyridine) (lit.,[123] +33); δ_H (300 MHz, DMSO): 2.75 (1H, t, $J_{1,2 \text{ and } 2,3}$ 8.5 Hz, 2-H), 3.25 (1H, m, 5-H), 3.80 (3H, s, $OC\underline{H}_3$), 4.50 (1H, t, $J_{3,4}$ and $J_{4,5}$ 8.5 Hz, 4-H), 4.65 (1H, d, $J_{3,4}$ and $J_{2,3}$ 8.5 Hz, 3-H), 4.80 (1H, d, $J_{5,6}$ 7.7 Hz, 6 or 6'-H), 4.90 (1H, d, $J_{6,6}$ 7.7 Hz, 6 or 6'-H), 6.50 (1H, d, $J_{1,2}$ 8.5 Hz, 1-H), 6.95 (2H, d, J_{AB} 8.8 Hz, Ar), 7.65 (2H, d, J_{AB} 8.8 Hz, Ar), 8.10 (1H, s, N=C$\underline{H}$); δ_C (75 MHz, DMSO): 55.7 (OMe), 61.5 (C2), 70.9, 75.1, 77.3 and 78.7 (C3-C6), 96.2 (C1), 114.4, 129.7 and 130.2 (aromatics) and 161.8 (N=$\underline{C}$H);

1,3,4,6-Tetra-*O*-acetyl-2-amino-2-deoxy-*N*-anisylidene-β-D-glucopyranose (27)[123]



The *title compound* was prepared as described by Bergman and Zervas.[123] 2-Amino-*N*-anisylidene-2-deoxy-β-D-glucopyranose **(26)** (11.2 g, 37.8 mmol) was added to acetic anhydride (35 ml) and dry pyridine (55 ml) and stirred at room temp. overnight. The mixture was poured onto cracked ice (300 ml) and precipitation occurred. The product was filtered, washed with ice-cold water and crystallised from EtOH / MeOH 2:1 to give the *title compound* as a white crystals (11.23 g, 64%). m.p. 185-187°C (lit.,[123] 188°C); $[\alpha]_D$ +88.2 (*c* 0.1 in $CHCl_3$) (lit.,[123] +98.6); δ_H (300 MHz, DMSO): 1.93 (6H, s, 2 x COC<u>H</u>$_3$), 1.95 (6H, s, 2 x COC<u>H</u>$_3$), 3.42 (1H, t, $J_{4,5}$ and $J_{3,4}$ 9.0 Hz, 4-H), 3.75 (3H, s, OC<u>H</u>$_3$), 4.0 – 4.3 (3H, m, 5, 6 and 6'-H), 4.95 (1H, t, $J_{3,4}$ and $J_{2,3}$ 9.0 Hz, 3-H), 5.40 (1H, dd, $J_{2,3}$ 9.0 and $J_{1,2}$ 8.2 Hz, 2-H), 6.05 (1H, d, $J_{1,2}$ 8.2 Hz, 1-H), 6.95 (2H, d, J_{AB} 8.8 Hz, aromatics), 7.65 (2H, d, J_{AB} 8.8 Hz, aromatics), 8.25 (1H, s, N=C<u>H</u>); δ_C (75 MHz, DMSO): 18.9, 20.6, 20.8 and 20.9 (4 x CO<u>C</u>H$_3$), 56.5 (O<u>C</u>H$_3$), 62.1 (C2), 68.3, 72.0, 72.7 and 72.8 (C3-C6), 93.1 (C1), 114.8 and 130.5 (aromatics), 165.0 (N=<u>C</u>H), 169.2, 169.6, 170.1 and 170.7 (4 x <u>C</u>OCH$_3$).

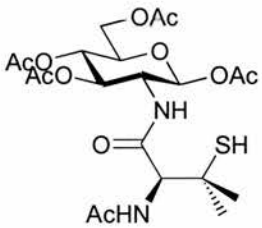
1,3,4,6-Tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride (28)[123]



The *title compound* was prepared as described by Bergman and Zervas.[123] 1,3,4,6-Tetra-*O*-acetyl-2-amino-2-deoxy-*N*-anisylidene-β-D-glucopyranose **(27)** (11.213 g, 24.1 mmol), was dissolved in acetone (30 ml) and warmed to boil. Water (5 ml) was added and the solution cooled. Conc. HCl was added dropwise until precipitation occurred. The precipitate was filtered, washed with cold diethyl ether and dried ($MgSO_4$) to give the *title compound* as white crystals these were recrystallised from EtOH / H_2O 3:1 (7.01 g, 75 %). m.p. 228-230°C dec. (lit.,[123] 230°C dec.); $[\alpha]_D$ +27.2 (*c* 0.1 in H_2O) (lit.,[123] +29.7); δ_H (300 MHz, D_2O): 1.95-2.05 (12H, m, 4 x COC<u>H</u>$_3$), 3.60 (1H, dd, $J_{1,2}$ 8.8 and $J_{2,3}$ 9.6 Hz, 2-H), 4.00-4.05 (2H, m, 5 and 6'-H), 4.25 (1H, dd, $J_{5,6'}$ 4.4 and $J_{6,6'}$

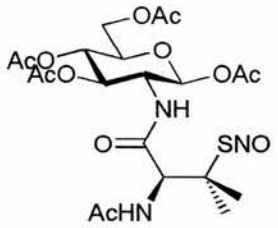
(1H, t, $J_{3,4}$ and $J_{4,5}$ 9.6 Hz, 4-H), 5.35 (1H, t, $J_{3,4}$ and $J_{2,3}$ 9.6 Hz, 3-H), 5.80 (1H, d, $J_{1,2}$ 8.8 Hz, 1-H); δ_C (75 MHz, D_2O): 19.8, 19.9 and 20.1 (4 x $CO\underline{C}H_3$), 52.3 (C2), 61.4 (C6), 70.0, 70.8 and 72.1 (C3-C5), 90.4 (C1), 173.1 and 173.8 (4 x $\underline{C}OCH_3$).

1,3,4,6-Tetra-*O*-acetyl-*N*-(*N*-Acetyl-D-penicillaminyl)-2-amino-2-deoxy-β-D-glucopyranose (29)[122]



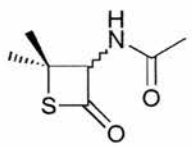
The *title compound* was prepared as described by Greig[122] for the preparation of the corresponding mixed diastereoisomers. 2-Amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose hydrochloride **(28)** (1 g, 2.6 mmol) was dissolved in a minimum amount of water (about 30 ml). Dry sodium acetate (640 mg, 7.8 mmol) was added and the solution stirred at room temp. for 15 min. DCM (50 ml) was then added and washed with water (2 x 75 ml), then dried ($MgSO_4$) and concentrated *in vacuo* to give a white solid. This was added to *N*-acetyl-D-penicillamine **(36)** (574 mg, 3 mmol) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimidemetho-*p*-toluenesulfonate (1.271 g, 3 mmol) in dry DCM (5 ml) and stirred at room temp. overnight. DCM (100 ml) was added and washed with 1 M HCl (150 ml), sat. $NaHCO_3$ (150 ml) and water (150 ml), then dried ($MgSO_4$) and concentrated *in vacuo* to give a clear oil which, on addition of Et_2O turned into white crystals (286 mg, 21%). m.p. 161-163 °C (not reported in lit.); $[\alpha]_D$ +34.5 (*c* 0.55 in $CHCl_3$), (not reported in lit.); δ_H (300 MHz, $CDCl_3$): 1.18 (3H, s, Me), 1.25 (3H, s, Me), 2.0-2.1 (15 H, m, 5 x $COC\underline{H}_3$), 3.86 (1H, m, 5-H), 4.1-4.4 (4H, m, 2, 6 and 6'-H and C$\underline{H}$), 5.10 (1H, t, $J_{3,4}$ and $J_{4,5}$ 9.6 Hz, 4-H), 5.43 (1H, t, $J_{2,3}$ and $J_{3,4}$ 9.6 Hz, 3-H), 5.89 (1H, d, $J_{1,2}$ 9.1 Hz, 1-H), 6.53 (1H, d, $J_{NH,2}$ 9.1 Hz, N$\underline{H}$), 6.88 (1H, d, $J_{NH,CH}$ 9.3 Hz, N$\underline{H}$Ac); δ_C (75 MHz, $CDCl_3$): 20.6, 20.7, 20.8 and 20.9 (4 x $CO\underline{C}H_3$), 23.1 ($NHCO\underline{C}H_3$), 28.7 and 31.0 (2 x Me) 45.3 ($\underline{C}Me_2$), 52.7 (C2), 60.6 (C6), 61.9 ($\underline{C}H$), 68.2, 71.7 and 72.8 (C3-C5), 92.4 (C1), 168.8, 169.9, 171.0, 172.6, 173.4 and 175.4 (6 x $\underline{C}OCH_3$); ν_{max} / cm^{-1} 2362.9 (SH); m/z (CI) 521.1776 (M^+ $C_{21}H_{33}N_2O_{11}S$ requires 521.1706).

1,3,4,6-Tetra-*O*-acetyl-*N*-(*N*-acetyl-S-nitroso-D-penicillaminyl)-2-amino-2-deoxy-*β*-D-glucopyranose (GLACSNAP) (2)[122]



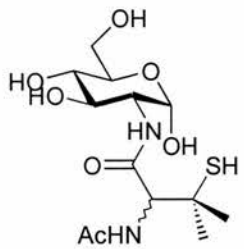
The *title compound* was prepared quantitatively from 1,3,4,6-tetra-*O*-acetyl-*N*-(*N*-acetyl-D-penicillaminyl)-2-amino-2-deoxy-*β*-D-glucopyranose **(29)** *via* the "fuming method" and was obtained as dark green crystals. m.p. 126-128 °C; $[\alpha]_D$ +48 (*c* 0.25 in CHCl3); λ_{max} /nm (Krebs / DMSO 1:1) 340.7 (ε / dm^3 mol^{-1} cm^{-1} 860.0).

(3R,S)-3-Acetamido-4,4-dimethylthietan-2-one (32)[130,131]



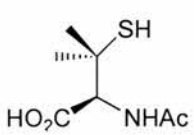
N-Acetyl-D,L-penicillamine **(24)** (1 g, 5.23 mmol) was dissolved in dry THF (30 ml) and dry DMF (5 ml). *N*-methylmorpholine (0.575 ml, 5.23 mmol) was added and the mixture stirred at room temp for 10 min. The mixture was then cooled to -30°C and isobutylchloroformate (684 µl, 5.23 mmol) was added. The mixture was stirred overnight. The precipitates were filtered off and the solvents evaporated *in vacuo* to give a yellow oil. This was purified by column chromatography eluting with EtOAc / hexane 1:1 to give a white powder, this was recrystallised from hexane / EtOAc 5:1 to yield the *title compound* as white needles (645 mg, 71%). m.p. 129-131°C (lit.,[131] 128-130°C); found C, 48.62; H, 6.53; N, 7.90%, $C_7H_{11}NO_2S$ requires C, 48.53; H, 6.40; N, 8.09%; δ_H (300 MHz, $CDCl_3$): 1.63 (3H, s, Me), 1.80 (3H, s, Me), 2.10 (3H, s, Me), 5.60 (1H, d, $J_{CH,NH}$ 8.2 Hz, C<u>H</u>), 6.83 (1H, d, $J_{NH,CH}$ 8.2 Hz, N<u>H</u>Ac); δ_C (75 MHz, $CDCl_3$): 22.5, 26.1 and 30.2 (CO<u>C</u>H_3 and 2 x Me), 51.0 (<u>C</u>Me_2), 77.0 (<u>C</u>H), 169.7 (<u>C</u>OCH_3) and 192.1 (S-<u>C</u>=O).

N-(N-Acetyl-D,L-penicillaminyl)-2-amino-2-deoxy-α-D-glucopyranose (30)[127]



The *title compound* was prepared as described by Wang and coworkers.[127] 2-Amino-2-deoxy-β-D-glucopyranose hydrochloride **(25)** (345 mg, 1.6 mmol) was added to dry methanol (10 ml). Sodium metal (37 mg, 1.6 mmol) was added and the mixture stirred at room temp for about 10 min. The NaCl precipitate was filtered off and the methanol evaporated to give a white solid. This was dissolved in water (15 ml). (3R,S)-3-Acetamido-4,4-dimethylthietan-2-one **(32)** (260 mg, 1.5 mmol) was dissolved in $CHCl_3$ (15 ml). The two-phase system was stirred vigorously overnight. The aqueous phase was separated, washed with $CHCl_3$ (4 x 50 ml) and freeze-dried to give a white solid. This was purified by column chromatography eluting with EtOAc / EtOH 5:1 to yield the *title compound* as a white powder (320 mg, 61%). m.p. 83-85°C, (not reported in lit); δ_H (300 MHz, D_2O): 1.31 (3H, s, Me), 1.35 (3H, s, Me), 1.92 (3H, s, $COC\underline{H}_3$), 3.30-3.80 (6H, m, 2,3,4,5,6 and 6'-H), 4.41 (1H, s, C<u>H</u>), 5.04 (1H, d, $J_{1,2}$ 3.3 Hz, 1-H); δ_C (75 MHz, D_2O): 21.7 ($CO\underline{C}H_3$), 28.9 and 29.4 (2 x Me), 45.1 ($\underline{C}Me_2$), 54.2 (C2), 60.5 (<u>C</u>H), 60.1, 70.1, 70.8 and 71.3 (C3 – C6), 90.7 (C1), 171.5 and 172.3 (2 x <u>C</u>O); m/z (CI) 353.1389 (M^+ $C_{13}H_{25}N_2O_7S$ requires 353.1383).

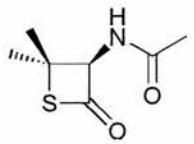
N-Acetyl-D-penicillamine (36)[167]



D-Penicillamine **(10)** (1g, 5.23 mmol) was dissolved in dry MeOH (10 ml) and treated with acetic anhydride (4 ml). The solution was stirred at room temp. and the reaction was monitored by TLC (DCM / MeOH / H_2O 7:3:0.5). Once the reaction had reached completion, the solvent was removed by co-evaporation with toluene. The resulting clear oil was crystallised from MeOH / ether 1:1 to give the *title compound* as white crystals (917 mg, 92%). m.p. 179-181°C (lit.,[167] 178-179°C); found C, 43.99; H, 6.84; N, 7.30%, $C_7H_{13}NO_3S$ requires C, 43.96; H, 6.85; N, 7.32%; $[\alpha]_D$ +57.2 (*c* 1 in MeOH) (lit.,[167] +22); δ_H (300 MHz, MeOD): 1.42 (3H, s, Me), 1.48 (3H, s, Me), 2.03 (3H, s,

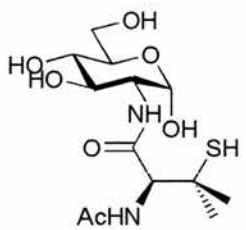
NHCOC$\underline{H}_3$), 4.56 (1H, s, C$\underline{H}$); δ_C (75 MHz, MeOD): 22.2 (NHCO$\underline{C}H_3$), 30.2 and 30.3 (2 x Me), 45.9 ($\underline{C}Me_2$), 62.9 ($\underline{C}H$), 173.6 and 174.0 (2 x CO).

(3R)-3-Acetamido-4,4-dimethylthietan-2-one (33)[130,131]



N-Acetyl-D-penicillamine **(36)** (1 g, 5.23 mmol) was dissolved in dry THF (30 ml) and dry DMF (5 ml). *N*-Methylmorpholine (0.575 ml, 5.23 mmol) was added and the mixture stirred at room temp for 10 min. The mixture was then cooled to -30°C and isobutylchloroformate (684 µl, 5.23 mmol) was added. The mixture was stirred overnight. The precipitates were filtered off and the solvents evaporated *in vacuo* to give a yellow oil. This was purified by column chromatography eluting with EtOAc / hexane 1:1 to give a white powder, this was recrystallised from hexane / EtOAc 5:1 to yield the *title compound* as white needles (500 mg, 55%). m.p. 159-161°C (lit.,[130] 158-160°C); found C, 48.37; H, 6.51; N, 7.82%, $C_7H_{11}NO_2S$ requires C, 48.53; H, 6.40; N, 8.09%; $[\alpha]_D$ –107.2 (*c* 0.1 in $CHCl_3$) (lit.,[131] -113); δ_H (300 MHz, $CDCl_3$): 1.61 (3H, s, Me), 1.82 (3H, s, Me), 2.02 (3H, s, Me), 5.65 (1H, d, $J_{CH,NH}$ 8.2 Hz, C$\underline{H}$), 6.80 (1H, d, $J_{NH,CH}$ 8.2 Hz, N$\underline{H}$Ac); δ_C (75 MHz, $CDCl_3$): 22.5, 26.2 and 30.2 (CO$\underline{C}H_3$ and 2 x Me), 51.1 ($\underline{C}Me_2$), 76.9 ($\underline{C}H$), 169.7 ($\underline{C}OCH_3$) and 192.2 (S-$\underline{C}$=O).

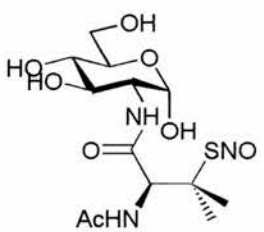
***N*-(*N*-Acetyl-D-penicillaminyl)-2-amino-2-deoxy-α-D-glucopyranose (30)[127]**



The *title compound* was prepared as described by Wang and coworkers[127] for the corresponding mixture of diastereoisomers. 2-Amino-2-deoxy-β-D-glucopyranose hydrochloride **(25)** (345 mg, 1.6 mmol) was added to dry methanol (10 ml). Sodium metal (37 mg, 1.6 mmol) was added and the mixture stirred at room temp for about 10 min. The NaCl precipitate was filtered off and the methanol evaporated to give a white solid. This was dissolved in water (15 ml). (3R)-3-Acetamido-4,4-dimethylthietan-2-one **(33)** (260 mg, 1.5 mmol) was dissolved in $CHCl_3$ (15 ml). The two-phase system

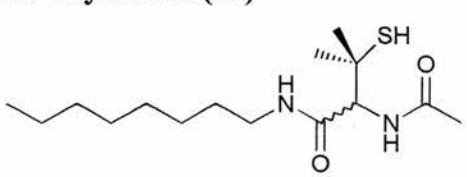
was stirred vigorously overnight. The aqueous phase was separated, washed with $CHCl_3$ (4 x 50 ml) and freeze-dried to give a white solid. This was purified by column chromatography eluting with EtOAc / EtOH 5:1 to yield the *title compound* as a white powder (247 mg, 47%). m.p. 88-90°C, (not reported in lit); $[\alpha]_D$ +38.3 (*c* 0.1 in water), (not reported in lit); δ_H (300 MHz, D_2O): 1.29 (3H, s, Me), 1.35 (3H, s, Me), 1.95 (3H, s, $COC\underline{H}_3$), 3.30-3.80 (6H, m, 2,3,4,5,6 and 6'-H), 4.37 (1H, s, C<u>H</u>), 5.07 (1H, d, $J_{1,2}$ 3.3 Hz, 1-H); δ_C (75 MHz, D_2O): 21.7 ($CO\underline{C}H_3$), 28.8 and 29.4 (2 x Me), 45.2 (<u>C</u>Me_2), 54.1 (C2), 60.5 (<u>C</u>H), 60.1, 70.4, 70.7 and 71.5 (C3 – C6), 90.7 (C1), 171.3 and 172.2 (2 x <u>C</u>O); m/z (CI) 353.1379 (M^+ $C_{13}H_{25}N_2O_7S$ requires 353.1383).

_N_-(_N_-Acetyl-S-nitroso-D-penicillaminyl)-2-amino-2-deoxy-α-D-glucopyranose (GLUSNAP) (39)[127]



The *title compound* was prepared quantitatively from *N*-(*N*-acetyl-D-penicillaminyl)-2-amino-2-deoxy-α-D-glucopyranose **(30a)** *via* the "fuming method" and was obtained as a dark green powder. m.p. 74-76 °C; $[\alpha]_D$ +32.0 (*c* 0.25 in water); λ_{max} / nm (Krebs / DMSO 1:1) 340.8 (ε / dm^3 mol^{-1} cm^{-1} 1191.5).

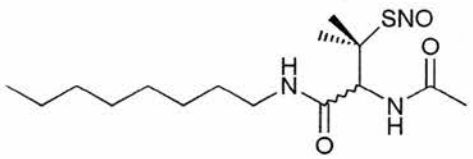
***N*-Acetyl-D,L-penicillamine octylamide (37)**



(3R,S)-3-Acetamido-4,4-dimethylthietan-2-one **(32)** (173 mg, 1 mmol) and *n*-octylamine (129 mg, 1 mmol) were dissolved in dry THF (30 ml). The solution was heated to reflux for 3 hrs. Once the reaction had proven complete by TLC (EtOAc / hexane 1:1) the solvent was evaporated *in vacuo* to yield the *title compound* as a white solid (311 mg, 99%). m.p. 126-128°C; found: C, 59.12; H, 10.26; N, 9.20%, $C_{15}H_{30}N_2O_2S$ requires: C, 59.56; H, 10.0; N, 9.26%; δ_H (300 MHz,$CDCl_3$): 0.84 (3H, t, *J* 6.7 Hz, $-CH_2C\underline{H}_3$), 1.10-1.25 (12H, m, 6 x $C\underline{H}_2$), 1.46 (6H, s, 2 x Me), 2.0 (3H, s, $COC\underline{H}_3$), 2.60 (1H, broad s, S<u>H</u>), 3.20 (2H, m, $NHC\underline{H}_2$-), 4.45 (1H, d, $J_{CH,NH}$ 9 Hz, C<u>H</u>),

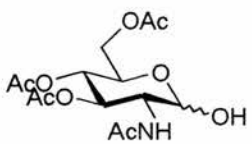
6.73 (2H, m, 2 x NH); δ_C (75 MHz, $CDCl_3$): 14.0 ($CH_2\underline{C}H_3$), 22.5 ($CO\underline{C}H_3$), 23.2 and 23.3 (2 x Me), 26.9, 28.4, 29.1, 29.2, 31.0, 31 7 and 39.5 (7 x $\underline{C}H_2$), 45.9 ($\underline{C}Me_2$), 60.2 ($\underline{C}H$), 169.8 and 170.4 (2 x $\underline{C}O$).

_N_-Acetyl-_S_-nitroso-D,L-penicillamine octylamide (38)



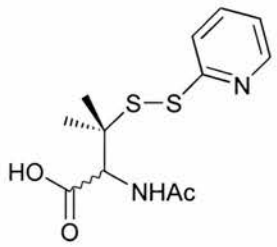
The *title compound* was prepared quantitatively from *N*-Acetyl-D,L-penicillamine octylamide **(37)** *via* the "fuming method" and was obtained as a green oil. λ_{max} / nm (Krebs / DMSO 1:1) 342.2 (ε / dm^3 mol^{-1} cm^{-1} 538).

2-Acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α,β-D-glucopyranose (40)[168]



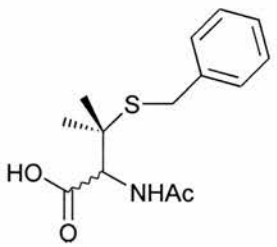
The title compound was obtained as a result of acetyl migration in 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride **(28)**. It was isolated during column chromatography and purified by crystallisation from DCM / ether 1:1 to give white crystals. m.p. 64-66 °C (lit.,[168] 65-75 °C); $[\alpha]_D$ +44.2 (*c* 1 in MeOH), (lit.,[168] +49.4); δ_H (300 MHz, MeOD): 1.93 (3H, s, $COC\underline{H}_3$), 1.96 (3H, s, $COC\underline{H}_3$), 2.00 (3H, s, $COC\underline{H}_3$), 2.03 (3H, s, $COC\underline{H}_3$), 3.88 (1H, dd, $J_{5,6}$ 3.6 and $J_{6,6'}$ 10.7 Hz, 6-H), 4.07 (1H, dd, $J_{5,6'}$ 1.9 and $J_{6,6'}$ 10.7 Hz, 6'-H), 4.15-4.35 (2H, m, 2 and 5-H), 5.09 (0.6H, d, $J_{1\alpha,2}$ 3.3 Hz, 1α-H), 5.11 (0.4H, d, $J_{1\beta,2}$ 8.5 Hz, 1β-H), 5.30 (1H, t, $J_{3,4}$ and $J_{4,5}$ 9.3 Hz, 4-H), 5.44 (1H, t, $J_{3,4}$ and $J_{2,3}$ 9.3 Hz, 3-H), 6.36 (1H, d, *J* 6.9 Hz, NH); δ_C (75 MHz, MeOD): 19.2, 19.3, 19.9 and 20.2 (4 x $COC\underline{H}_3$), 62.3, 66.9, 67.7, 69.2, 69.5 and 71.1 (C2 – C6), 88.6 (C1α), 91.2 (C1β).

N-Acetyl-D,L-penicillamine pyridyl disulfide (41)



N-Acetyl-D,L-penicillamine **(24)** (800 mg, 4.16 mmol) and 2,2'-dipyridyl disulfide (920 mg, 4.16 mmol) were dissolved in dry MeOH (30 ml) and stirred at room temperature. After 30 min, TLC (5% AcOH in EtOAc) proved the reaction complete, so the solvent was removed *in vacuo* to give a yellow oil. This was purified by column chromatography eluting with 5% AcOH in EtOAc to give a yellow solid. This was crystallised from EtOAc / EtOH 5:1 to yield the *title compound* as white crystals (966 mg, 77%). m.p. 160-162°C; δ_H (300 MHz, $CDCl_3$): 1.30 (3H, s, Me), 1.47 (3H, s, Me), 1.96 (3H, s, $COC\underline{H}_3$), 4.74 (1H, d, $J_{CH,NH}$ 8.4 Hz, C<u>H</u>), 6.85 (1H, d, $J_{CH,NH}$ 8.4 Hz, N<u>H</u>Ac), 7.20-7.25 (1H, m, 5-H), 7.42 (1H, d, $J_{3,4}$ 7.4 Hz, 3-H), 7.69 (1H, t, $J_{3,4}$ 7.4 and $J_{4,5}$ 7.9 Hz, 4-H), 8.37 (1H, d, $J_{5,6}$ 4.2 Hz, 6-H); δ_C (75 MHz, $CDCl_3$): 22.4 ($CO\underline{C}H_3$), 23.2 and 26.4 (2 x Me), 52.2 ($\underline{C}Me_2$), 59.0 (<u>C</u>H), 122.2 and 122.4 (C3 and C5), 138.4 (C4), 148.6 (C2), 158.4 (C6), 170.2 and 171.9 (2 x <u>C</u>O); m/z (FAB) 300.9350 (M^+ $C_{12}H_{17}N_2O_3S_2$ requires 300.9365).

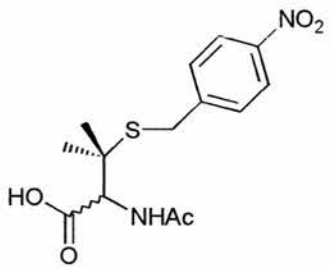
N-Acetyl-S-benzyl-D,L-penicillamine (42)[169]



The *title compound* was prepared following the protection procedure described by Bodanszky and Bodanszky.[142] *N*-Acetyl-D,L-penicillamine **(24)** (9.56 g, 50 mmol), was dissolved in 2M NaOH (50 ml) and EtOH (60 ml). Benzyl chloride (6.36 ml, 55 mmol) was added and the solution was stirred at room temperature. After 1 h the solution was acidified with conc. HCl and precipitation occurred. The white precipitate was filtered and washed with cold ether to give a white solid. This was recrystallised from EtOH to yield the *title compound* as white crystals (6.84 g, 49%). m.p. 172-174°C (lit.,[169] 174°C); δ_H (300 MHz, DMSO): 1.28 (3H, s, Me), 1.37 (3H, s, Me), 1.90 (3H, s,

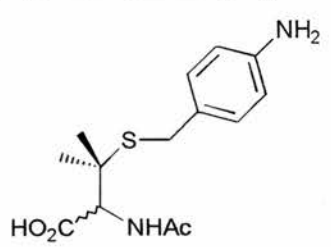
$COC\underline{H}_3$), 3.78 (2H, s, $C\underline{H}_2$), 4.54 (1H, d, $J_{CH,NH}$ 9.0 Hz, C<u>H</u>), 7.1-7.3 (5H, m, aromatics), 8.12 (1H, d, $J_{CH,NH}$ 9.0 Hz, N<u>H</u>); δ_C (75 MHz, DMSO): 22.2 and 22.5 ($CO\underline{C}H_3$), 25.1 and 26.1 (2 x Me), 32.3 ($\underline{C}H_2$), 47.5 ($\underline{C}Me_2$), 58.4 and 58.5 (<u>C</u>H), 126.8, 127.1, 128.3, 128.6, 137.8, 129.1 and 129.4 (aromatics), 169.7 and 171.7 (2 x <u>C</u>O).

_N_-Acetyl-_S_-_p_-nitrobenzyl-D,L-penicillamine (43)



The *title compound* was prepared by the protection method described by Bodanszky and Bodanszky.[142] *N*-Acetyl-D,L-penicillamine **(24)** (5 g, 26.1 mmol) was dissolved in 2N NaOH (25 ml) and EtOH (30 ml). *p*-Nitrobenzyl chloride (4.5 g, 26.2 mmol) was added and stirring continued at room temperature for 2 hours. Once all the material had gone into solution, conc. HCl was added slowly and precipitation occurred. The white solid was filtered and dried in a vacuum dessicator to give a white powder. This was recrytallised from EtOH to yield the *title compound* as white crystals (6.26 g, 73%). m.p. 166-168°C (sublimes); δ_H (300 MHz, DMSO): 1.29 (3H, s, Me), 1.38 (3H, s, Me), 1.91 (3H, s, $COC\underline{H}_3$), 3.93 (2H, s, $C\underline{H}_2$), 4.54 (1H, d, $J_{CH,NH}$ 9.1 Hz, C<u>H</u>), 7.57 (2H, d, J_{AB} 8.8 Hz, aromatics), 8.15 (2H, d, J_{AB} 8.8 Hz, aromatics), 8.17 (1H, d, $J_{CH,NH}$ 9.1 Hz, N<u>H</u>); δ_C (75 MHz, DMSO): 22.8 ($CO\underline{C}H_3$), 25.4 and 26.6 (2 x Me), 32.2 ($\underline{C}H_2$), 48.6 ($\underline{C}Me_2$), 58.8 (<u>C</u>H), 124.1, 130.9 and 147.0 (aromatics), 170.1 and 172.0 (2 x <u>C</u>O); m/z (CI) 327.1026 (M^+ $C_{14}H_{20}N_2O_5S$ requires 327.1015); ν_{max} / cm^{-1} 1486 (NO_2).

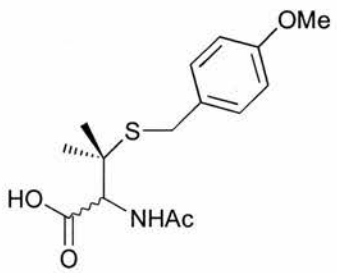
_N_-Acetyl-_S_-_p_-aminobenzyl-D,L-penicillamine (44)



The *title compound* was obtained as the unexpected product of the reaction of *N*-acetyl-*S*-*p*-nitrobenzyl-D,L-penicillamine **(43)** with palladium on charcoal under hydrogen atmosphere. *N*-Acetyl-*S*-*p*-nitrobenzyl-D,L-penicillamine **(43)** (326 mg, 1 mmol) was

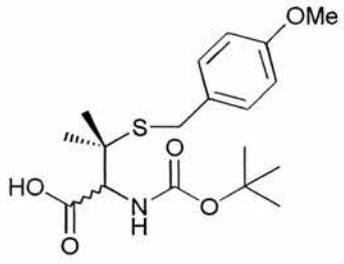
dissolved in dry MeOH (5ml) and treated with 12% Pd/C (20 mg, cat.). The mixture was stirred under hydrogen atmosphere overnight. The catalyst was removed by filtration and the solvent evaporated in vacuo to give a yellow oil. This was crystallised from MeOH / ether 1:1 to give the *title compound* as white crystals (281 mg, 94%). m.p. 175-177 °C; δ_H (300 MHz, MeOD): 1.37 (3H, s, Me), 1.45 (3H, s, Me), 2.02 (3H, s, NHCOC$\underline{H}_3$), 3.73 (2H, s, C$\underline{H}_2$Ar), 4.64 (1H, s, C$\underline{H}$), 6.66 (2H, d, J_{AB} 8.5 Hz, aromatics), 7.07 (2H, d, J_{AB} 8.5 Hz, aromatics); δ_C (75 MHz, MeOD): 25.7 (NHCO$\underline{C}H_3$), 27.3 and 27.5 (2 x Me), 33.8 ($\underline{C}H_2$), 48.4 ($\underline{C}Me_2$), 60.6 ($\underline{C}H$), 117.3, 129.1 and 131.3 (aromatics), 171.8 and 174.0 (2 x CO); m/z (FAB) 297.1265 (M^+ $C_{14}H_{21}N_2O_3S$ requires 297.1273); ν_{max} / cm^{-1} 3346 (NH_2).

_N_-Acetyl-_S_-_p_-methoxybenzyl-D,L-penicillamine (45)



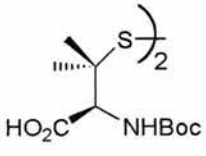
The *title compound* was prepared following the protection procedure described by Bodanszky and Bodanszky.[142] *N*-Acetyl-D,L-penicillamine **(24)** (2 g, 10.5 mmol) was dissolved in 2N NaOH (25 ml) and EtOH (30 ml). 4-Methoxybenzyl chloride (1.44 ml, 10.6 mmol) was added and the mixture stirred at room temperature. The reaction was followed by TLC (EtOAc / EtOH 5:1). Once the reaction had reached completion, conc. HCl was added dropwise until the solution had been neutralised. The EtOH was partially removed by evaporation *in vacuo* until precipitation occurred. The white precipitate was dried overnight in a dessicator to yield the *title compound* as a white powder (1.3 g, 40%). m.p. 168-170°C; found: C, 57.77; H, 7.02; N, 4.50%, $C_{15}H_{21}NO_4S$ requires: C, 57.86; H, 6.98; N, 4.50%; δ_H (300 MHz, DMSO): 1.27 (3H, s, Me), 1.36 (3H, s, Me), 1.91 (3H, s, COC$\underline{H}_3$), 3.72 (2H, s, C$\underline{H}_2$), 4.53 (1H, d, $J_{CH,NH}$ 9.1 Hz, C$\underline{H}$), 6.81 (2H, d, J_{AB} 8.8 Hz, aromatics), 7.18 (2H, d, J_{AB} 8.8 Hz, aromatics), 8.12 (1H, d, $J_{CH,NH}$ 9.1 Hz, N$\underline{H}$); δ_C (75 MHz, DMSO): 16.2 (CO$\underline{C}H_3$), 22.3 (O$\underline{C}H_3$), 25.1 and 26.0 (2 x Me), 31.7 ($\underline{C}H_2$), 47.4 ($\underline{C}Me_2$), 58.5 ($\underline{C}H$), 129.4, 129.8, 130.3 and 130.5 (aromatics), 169.8 and 171.8 (2 x $\underline{C}O$).

N-Butoxycarbonyl-S-p-methoxybenzyl-D,L-penicillamine (46)



The *title compound* was prepared using the protection procedure described by Bodanszky and Bodanszky.[142] *N*-Butoxycarbonyl-D,L-penicillamine **(20)** (6 g, 24.1 mmol) was dissolved in 2M NaOH (20 ml) and EtOH (30 ml). *p*-Methoxybenzyl chloride (4.2 ml, 30 mmol) was added and the solution stirred at room temperature overnight. The solution was acidified with conc. HCl and the product was extracted into EtOAc, dried ($MgSO_4$) and concentrated in vacuo to give a white solid. This was recrystallised from MeOH / DCM to yield the *title compound* as white crystals (6.3 g, 71%). m.p. 125-126°C; δ_H (300 MHz, $CDCl_3$): 1.4-1.5 (15H, m, 3 x Boc-Me and 2 x Me), 3.75 (2H, s, C<u>H</u>$_2$), 3.77 (3H, s, OMe), 4.40 (1H, broad s, C<u>H</u>), 5.45 (1H, broad s, N<u>H</u>), 6.81 (2H, d, J_{AB} 8.4 Hz, aromatics), 7.22 (2H, d, J_{AB} 8.4 Hz, aromatics); δ_C (75 MHz, $CDCl_3$): 25.59 and 26.64 (2 x Me), 28.28 (3 x Boc-Me), 32.59 (MeO), 55.26 (<u>C</u>H), 60.48 (<u>C</u>Me$_2$), 78.93 (Boc-<u>C</u>), 114.14, 129.00 and 130.32 (aromatics), 155.80 and 158.93 (2 x CO); m/z (CI) 370.1636 (M^+ $C_{18}H_{28}NO_5S$ requires 370.1689).

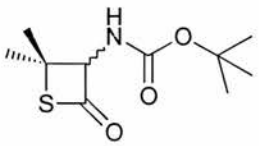
N-Butoxycarbonyl-D-penicillamine disulfide (48)



The *title compound* was prepared following the protection procedure described by Bodanszky and Bodanszky.[142] D-Penicillamine disulfide **(47)** (200 mg, 0.67 mmol) was dissolved in dioxane / 0.5 M NaOH (aq) 1:1 (20 ml) and di-tert-butyldicarbonate (184 µl, 0.8 mmol) was added. The solution was stirred at room temp. overnight. Once the reaction had proved complete, the reaction mixture was washed with ether (100 ml), then cooled to 0 °C and acidified to pH 3 with 2 M citric acid. The product was extracted into EtOAc (5 x 100 ml), then dried ($MgSO_4$) and concentrated *in vacuo* to give the *title compound* as a white powder (118 mg, 72%). m.p. 117-119 °C; $[\alpha]_D$ –23.8 (*c* 0.5 in $CHCl_3$); δ_H (300 MHz, DMSO): 1.26 (3H, s, Me), 1.30 (3H, s, Me), 1.36 (9H,

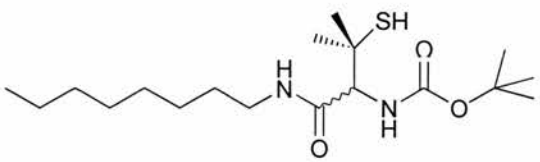
s, 3 x Boc-Me), 4.05 (1H, d, $J_{NH,CH}$ 8.8 Hz, C<u>H</u>), 6.99 (1H, d, $J_{NH,CH}$ 8.8 Hz, N<u>H</u>); δ_C (75 MHz, DMSO): 24.1 and 25.7 (2 x Me), 28.1 (3 x Boc-Me), 50.6, 60.8 and 78.5 (Boc-<u>C</u>, <u>C</u>Me$_2$ and <u>C</u>H), 155.5 and 171.7 (2 x CO); m/z (FAB) 496.2011 (M^+ $C_{20}H_{37}N_2O_8S_2$ requires 496.1992).

3-Butoxycarbonylamido-4,4-dimethylthietan-2-one (49)



N-Butoxycarbonyl-D,L-penicillamine **(20)** (400 mg, 1.6 mmol) was dissolved in dry THF (10 ml). *N*-Methylmorpholine (175 µl, 1.6 mmol) was added and the solution stirred at room temp for 10 min. The solution was then cooled to -30°C and isobutylchloroformate (208 µl, 1.6 mmol) was added. The reaction was followed by TLC (EtOAc / hexane 1:5). Once the reaction had reached completion, the NMM salts were filtered off and the THF concentrated *in vacuo* to give a white powder. This was purified by column chromatography eluting with EtOAc / hexane 1:5 to give a white powder, this was crystallised from hexane / EtOAc 3:1 to yield the *title compound* as white crystals (293 mg, 79%). m.p. 140-142°C; found: C, 51.38; H, 7.61; N, 5.83%, $C_{10}H_{17}NO_3S$ requires: C, 51.05; H, 7.28; N, 5.95%; δ_H (300 MHz, $CDCl_3$): 1.45 (9H, s, 3 x BocMe), 1.63 (3H, s, Me), 1.82 (3H, s, Me), 5.30 (1H, broad s, N<u>H</u>), 5.40 (1H, d, $J_{CH,NH}$ 8.5 Hz, C<u>H</u>); δ_C (75 MHz, $CDCl_3$): 26.2 and 30.0 (2 x Me), 28.1 (3 x BocMe), 51.3 (<u>C</u>Me$_2$), 80.9 (<u>C</u>H), 154.4 (NH-<u>C</u>O) and 191.7 (S-<u>C</u>O).

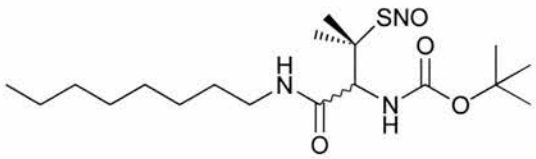
***N*-Butoxycarbonyl-D,L-penicillamine octylamide (50)**



3-Butoxycarbonylamido-4,4-dimethylthietan-2-one **(49)** (800 mg, 3.4 mmol) and *n*-octylamine (439 mg, 3.4 mmol) were dissolved in dry THF (25 ml). The solution was stirred at room temperature and the reaction monitored by TLC (EtOAc / hexane 1:10). After 2 hrs the reaction had reached completion, hence the solvent was evaporated *in vacuo* to give an off-white solid. This was purified by column chromatography eluting with EtOAc / hexane 1:15 to give a white powder. This was recrystallised from hexane

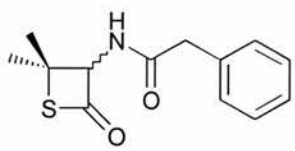
to yield the *title compound* as white crystals (807 mg, 65%). m.p. 95-97°C; found: C, 59.76; H, 10.40; N, 7.59%, $C_{18}H_{36}N_2O_3S$ requires: C, 59.31; H, 9.95; N, 7.68%; δ_H (300 MHz, $CDCl_3$): 0.86 (3H, t, *J* 6.4 Hz, $CH_2C\underline{H}_3$), 1.2-1.5 (28H, m, 6 x CH_2, 2 x Me and Boc), 3.22 (2H, m, $NHC\underline{H}_2$), 4.00 (1H, d, $J_{CH,NH}$ 9.3 Hz, C<u>H</u>), 5.63 (1H, d, $J_{CH,NH}$ 9.3 Hz, N<u>H</u>Boc), 6.32 (1H, broad s, N<u>H</u>CH_2); δ_C (75 MHz, $CDCl_3$): 14.2 ($CH_2\underline{C}H_3$), 22.7, 27.0, 29.6, 31.1, 31.9 and 39.7 (6 x CH_2), 28.4 and 29.3 (2 x Me and Boc), 46.1 ($\underline{C}Me_2$), 62.5 (<u>C</u>H), 82.2 ($\underline{C}Me_3$), 156.7 and 171.9 (2 x <u>C</u>O).

_N_-butoxycarbonyl-_S_-nitroso-D,L-penicillamine octylamide (51)



The *title compound* was prepared quantitatively from *N*-butoxycarbonyl-D,L-penicillamine octylamide **(50)** *via* the "fuming method" and was obtained as a green solid. m.p. 110-112 °C; λ_{max} / nm (Krebs / DMSO 1:1) 342.9 (ε / $dm^3\ mol^{-1}\ cm^{-1}$ 753.5).

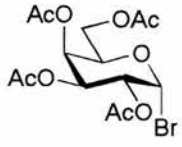
_N_-Phenylacetyl-3-amino-4,4-dimethylthietan-2-one (52)



N-Phenylacetyl-D,L-penicillamine **(22)** (500 mg, 1.87 mmol) was dissolved in dry THF (20 ml) and *N*-methylmorpholine (206 µl, 1.87 mmol) was added. The mixture was stirred at room temp for 10 min. The solution was then cooled to -20°C and isobutylchloroformate (243 µl, 1.87 mmol) was added. The mixture was then stirred at room temp and the reaction followed by TLC (EtOAc / hexane 1:1). After 4 hrs the reaction had reached completion, so the NMM salts were removed by filtration and the solvent evaporated *in vacuo* to give an off-white solid. This was recrystallised from EtOAc / hexane 1:5 to yield the *title compound* as a white crystals (456 mg, 96%). m.p. 116-118°C; found: C, 62.22; H, 6.02; N, 5.60%, $C_{13}H_{15}NO_2S$ requires: C, 62.65; H, 6.06; N, 5.62%; δ_H (300 MHz, $CDCl_3$): 1.44 (3H, s, Me), 1.80 (3H, s, Me), 3.60 (2H, s, $C\underline{H}_2$-Ph), 5.62 (1H, d, $J_{CH,NH}$ 6.9 Hz, C<u>H</u>), 6.31 (1H, d, $J_{CH,NH}$ 6.9 Hz, N<u>H</u>), 7.2-7.4 (5H, m, aromatics); δ_C (75 MHz, $CDCl_3$): 26.0 and 30.3 (2 x Me), 43.2 ($\underline{C}H_2$-Ph), 51.1

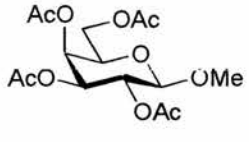
($\underline{C}Me_2$), 76.5 ($\underline{C}H$), 127.9, 129.4, 129.6 and 134.2 (aromatics), 170.8 (NH-$\underline{C}O$), 191.8 (S-$\underline{C}O$).

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide (57)[170]



The *title compound* was prepared as described by Kartha and Jennings.[151] D-Galactose **(56)** (10 g, 55.5 mmol) was added to acetic anhydride (50 ml) and 45% HBr in acetic acid (10 ml) and stirred at room temperature. Once all the sugar had gone into solution, more 45% HBr in acetic acid (50 ml) was added and the solution stirred at room temperature for 18 hours. The acetic acid was partially removed by coevaporation with toluene. The orange residue was then dissolved in DCM (150 ml) and washed extensively with saturated sodium bicarbonate, then dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow oil. Crystallisation from diethyl ether / hexane 1:3 yielded the *title compound* as white crystals (7.37 g, 64%). m.p. 82-84°C (lit.,[170] 84-86°C); $[\alpha]_D$ +193 (*c* 1 in $CHCl_3$) (lit.,[170] +219); δ_H (300 MHz, $CDCl_3$): 2.0-2.2 (12H, 4 x s, 4 x $COC\underline{H}_3$), 4.0-4.2 (2H, m, 6 and 6'-H), 4.47 (1H, t, $J_{5,6}$ and $J_{5,6'}$ 6.7 Hz, 5-H), 5.02 (1H, dd, $J_{1,2}$ 3.8 and $J_{2,3}$ 10.7 Hz, 2-H), 5.45 (1H, dd, $J_{3,4}$ 3.3 and $J_{2,3}$ 10.7 Hz, 3-H), 5.50 (1H, d, $J_{3,4}$ 3.3 Hz, 4-H), 6.68 (1H, d, $J_{1,2}$ 3.8 Hz, 1-H); δ_C (75 MHz, $CDCl_3$): 20.6, 20.7 and 20.8 (4 x $CO\underline{C}H_3$), 61.0, 67.2, 67.9, 68.2 and 71.2 (C2-C6), 88.3 (C1), 170.1, 170.2, 170.4 and 170.6 (4 x CO).

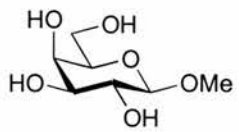
Methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (58)[171]



2,3,4,6-Tetra-*O*-acetyl-α-galactopyranosyl bromide **(57)** (1.028 g, 2.5 mmol) was added to MeOH (10 ml) and cooled to 0°C. Iodine (635 mg, 2.5 mmol) was added and the mixture was allowed to warm up to room temperature. After 24 hours the reaction proved complete. The solvent was removed *in vacuo* to give a yellow solid, which was purified by column chromatography to yield the *title compound* as a white powder (806 mg, 89%). m.p. 90-92°C (lit.,[171] 94°C); $[\alpha]_D$ -17 (*c* 1 in $CHCl_3$) (lit.,[171] -14.0); δ_H (300 MHz, $CDCl_3$): 1.94 (3H, s, OAc), 2.00 (3H, s, OAc), 2.02 (3H, s, OAc), 2.11 (3H, s,

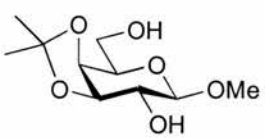
OAc), 3.47 (3H, s, OMe), 3.90 (1H, t, $J_{5,6}$ and $J_{5,6'}$ 6.7 Hz, 5-H), 4.0-4.2 (2H, m, 6 and 6'-H), 4.37 (1H, d, $J_{1,2}$ 7.7 Hz, 1-H), 4.97 (1H, dd, $J_{3,4}$ 3.3 and $J_{2,3}$ 10.4 Hz, 3-H), 5.15 (1H, dd, $J_{1,2}$ 7.7 and $J_{2,3}$ 10.4 Hz, 2-H), 5.35 (1H, d, $J_{3,4}$ 3.3 Hz, 4-H); δ_C (75 MHz, $CDCl_3$): 20.4, 20.5, 20.6, 20.7 (4 x $CO\underline{C}H_3$), 56.9 (OMe), 61.3, 67.1, 68.8, 70.6 and 71.0 (C2-C6), 102.1 (C1), 169.6, 170.3, 170.4 and 170.5 (4 x $\underline{C}OCH_3$).

Methyl β-D-galactopyranoside (59)[172]



Methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **(58)** (362 mg, 1 mmol) was dissolved in dry MeOH (5 ml) and sodium metal (5 mg, cat.) was added. After stirring at room temp. for 10 min, Dowex-50 H^+ resin (250 mg) was added and stirring continued for another 15 min. The resin was then removed by filtration and the methanol concentrated *in vacuo* to give the *title compound* as white crystals (189 mg, 97%). m.p. 175-177°C (lit.,[172] 177-178°C); $[\alpha]_D$ –3.8 (*c* 1 in H_2O) (lit.,[172] 0); δ_H (300 MHz, MeOD): 3.45-3.50 (3H, m, 2,4 and 5-H), 3.52 (3H, s, OMe), 3.74 (2H, m, 6 and 6'-H), 3.82 (1H, m, 3-H), 4.12 (1H, d, $J_{1,2}$ 6.1 Hz, 1-H); δ_C (75 MHz, $CDCl_3$): 58.4 (OMe), 61.1, 69.0, 71.1, 73.6 and 75.3 (C2-C6), 104.7 (C1).

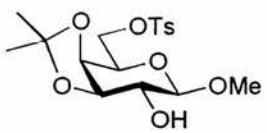
Methyl 3,4-*O*-isopropylidene-β-D-galactopyranoside (60)[153]



The *title compound* was prepared as described by Brimacombe and Ching.[153] Methyl β-galactopyranoside **(59)** (5 g, 26 mmol) was added to 2,2-dimethoxypropane (50 ml) and tosic acid (500 mg, cat.). The mixture was stirred at room temperature for 18 hours. Triethylamine was added dropwise until the pH was neutral and the solvent was then evaporated *in vacuo* to give a clear oil. 50% aqueous TFA (1.5 ml) was added and 5 min later more triethylamine was added until the pH was neutral. The solvent was evaporated *in vacuo* to give a white solid. This was recrystallised from isopropanol to yield the *title compound* as white crystals (4.38 g, 72%). m.p. 135-137°C (lit.,[153] 134-135°C); $[\alpha]_D$ +9.2 (*c* 0.5 in acetone) (lit.,[153] +8); δ_H (300 MHz, $CDCl_3$): 1.33 (3H, s, Me), 1.49 (3H, s, Me), 2.45 (2H, broad s, 2 x OH), 3.50 (1H, m, 2-H), 3.8-3.9 (2H, m, 5

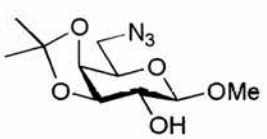
and 6-H), 3.97 (1H, dd, $J_{5,6'}$ 9.0 and $J_{6,6'}$ 13.0 Hz, 6'-H), 4.08 (1H, dd, $J_{3,4}$ 5.6 and $J_{4,5}$ 9.0 Hz, 4-H), 4.10 (1H, d, $J_{1,2}$ 8.1 Hz, 1-H), 4.15 (1H, m, 3-H); δ_C (75 MHz, $CDCl_3$): 26.3 and 28.0 (2 x Me), 57.1 (OMe), 62.4 (C6), 73.5, 73.7, 74.0 and 79.0 (C2-C5), 103.4 (C1), 110.5 ($\underline{C}Me_2$).

Methyl 3,4-*O*-isopropylidene-6-*p*-toluenesulfonyl-*β*-D-galactopyranoside (61)[153]



The *title compound* was prepared as described by Brimacombe and Ching.[153] Methyl 3,4-*O*-isopropylidene-*β*-D-galactopyranoside **(60)** (865 mg, 3.69 mmol) was dissolved in acetone (10.5 ml) and dry pyridine (6.75 ml) and cooled to 0 °C. Tosyl chloride (920 mg, 4.82 mmol) was added and the solution was allowed to warm up to room temperature and stirred for 18 hours. The solution was poured onto cracked ice. The white precipitate was filtered and recrystallised from isopropanol to yield the *title compound* as white crystals (916 mg, 64%). m.p. 150-152°C (lit.,[153] 154-155°C); $[\alpha]_D$ +3 (*c* 0.2 in $CHCl_3$) (lit.,[153] 0); δ_H (300 MHz, $CDCl_3$): 1.28 (3H, s, Me), 1.44 (3H, s, Me), 2.45 (3H, s, Ph-C$\underline{H}_3$), 3.45 (1H, m, 2-H), 3.50 (3H, s, OMe), 4.0-4.1 (4H, m, 1,3,4 and 5-H), 4.2-4.3 (2H, m, 6 and 6'-H), 7.35 (2H, d, J_{AB} 8.2 Hz, aromatics), 7.80 (2H, d, J_{AB} 8.2 Hz, aromatics); δ_C (75 MHz, $CDCl_3$): 21.6 (Ph-$\underline{C}H_3$), 26.2 and 27.9 (2 x isopropylidene Me), 57.1 (OMe) 68.6, 71.1, 72.9, 73.5 and 78.5 (C2-C6), 102.9 (C1), 110.5 (isopropylidene C), 127.9, 129.9, 132.7 and 145.0 (aromatics).

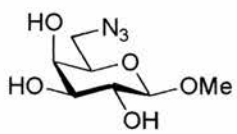
Methyl 6-azido-6-deoxy-3,4-*O*-isopropylidene-*β*-D-galactopyranoside (62)[173]



Methyl 3,4-*O*-isopropylidene-6-tosyl-*β*-D-galactopyranoside **(61)** (300 mg, 0.77 mmol) was dissolved in dry DMF (15 ml) and sodium azide (300 mg, 4.62 mmol) was added. The mixture was heated to reflux for 4 hours. Once all the starting material had been consumed, the mixture was cooled, added to diethyl ether (100 ml) and washed with a dilute solution of sodium chloride (150 ml), then concentrated *in vacuo* to give a white solid. This was purified by column chromatography eluting with EtOAc / hexane 1:3 to yield the *title compound* as white crystals (110 mg, 55%). m.p. 78-80°C (lit.,[173] 80-

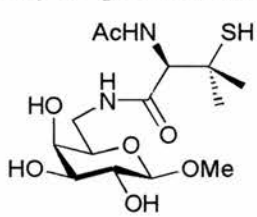
81°C); $[\alpha]_D$ –21.4 (*c* 1 in $CHCl_3$) (lit.,[173] –16.0); δ_H (300 MHz, $CDCl_3$): 1.32 (3H, s, Me), 1.50 (3H, s, Me), 2.60 (1H, broad s, OH), 3.31 (1H, dd, $J_{5,6}$ 4.1 and $J_{6,6'}$ 12.8 Hz, 6-H), 3.51 (1H, m, 2-H), 3.51 (3H, s, OMe), 3.70 (1H, dd, $J_{5,6'}$ 8.2 and $J_{6,6'}$ 12.8 Hz, 6'-H), 3.90-3.95 (1H, m, 5-H), 4.05-4.15 (3H, m, 1,3 and 4-H); δ_C (75 MHz, D_2O): 26.3 and 28.0 (2 x Me), 51.1 (OMe), 57.1 (CH2), 72.6, 73.1, 73.8, 77.5 and 78.8 (C2-C6), 103.3 (C1); ν_{max}/cm^{-1} 2099 (N_3).

Methyl 6-azido-6-deoxy-β-galactopyranoside (63)



Methyl 3,4-*O*-isopropylidene-6-azido-6-deoxy-β-D-galactopyranoside **(62)** (2.42 g, 9.3 mmol) was treated with 80% TFA (4 ml) and stirred at room temperature for 10 min. Once the reaction was proved complete, the solvent was removed by coevaporation with toluene to give a white solid. This was recrystallised from EtOAc / EtOH 3:1 to give white crystals (1.5 g, 74%). m.p. 134-136°C; $[\alpha]_D$ -79.8 (*c* 1 in $CHCl_3$); found: C, 38.56; N, 5.86; H, 19.12%, $C_7H_{13}N_3O_5$ requires: C, 38.36; N, 5.98; H, 19.17%; δ_H (500 MHz, $CDCl_3$): 3.25 (1H, m, 6-H), 3.55-3.60 (2H, m, 2 and 4-H), 3.58 (3H, s, OMe), 3.65 (1H, ddd, $J_{4,5}$ 1.0, $J_{5,6}$ 3.9 and $J_{5,6'}$ 8.3 Hz, 5-H), 5.73 (1H, dd, $J_{5,6'}$ 8.3 and $J_{6,6'}$ 12.7 Hz, 6'-H), 3.80 (1H, m, 3-H), 4.18 (1H, d, $J_{1,2}$ 6.8 Hz, 1-H); δ_C (75 MHz, $CDCl_3$): 52.7 (C6), 57.4 (OMe), 71.0 (C3), 72.5 and 74.8 (C2 and C4), 76.0 (C5), 106.1 (C1); ν_{max}/cm^{-1} 2128 (N_3).

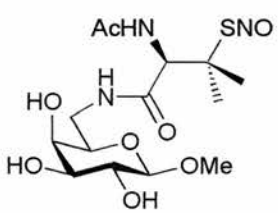
Methyl 6-amino-6-deoxy-6-(*N*-acetyl-D-penicillaminyl)-β-D-galactopyranoside (64)



Methyl 6-azido-6-deoxy-β-D-galactopyranoside **(63)** (100 mg, 0.46 mmol) was dissolved in dry methanol (3 ml). Pd/C (10 mg, cat.) was added and the mixture was stirred at room temperature under hydrogen atmosphere for 4 hours. The catalyst was removed by filtration and the solvent evaporated *in vacuo* to give a white solid. This was dissolved in water (5 ml) and added to a solution of (3R)-3-acetamido-4,4-

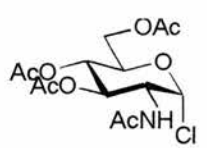
dimethylthietan-2-one **(33)** (80 mg, 0.46 mmol) in chloroform (2 ml). The biphasic mixture was stirred vigorously at room temperature overnight. The aqueous layer was then separated, washed with chloroform (2 x 5 ml) and freeze dried to yield the *title compound* as a white powder (141 mg, 88%). m.p. 210-212 °C; $[\alpha]_D$ –6.4 (*c* 0.25 in H_2O); δ_H (300 MHz, D_2O): 1.30 (3H, s, Me), 1.32 (3H, s, Me), 1.97 (3H, s, $COC\underline{H}_3$), 3.25 (1H, dd, $J_{5,6}$ 9.1 and $J_{6,6'}$ 13.9 Hz, 6-H), 3.38 (1H, dd, $J_{1,2}$ 7.9 and $J_{2,3}$ 9.8 Hz, 2-H), 3.42 (3H, s, OMe), 3.45 (1H, m, 6'-H), 3.52 (1H, dd, $J_{3,4}$ 3.3 and $J_{2,3}$ 9.8 Hz, 3-H), 3.58 (1H, dd, $J_{5,6'}$ 3.6 and $J_{5,6}$ 9.1 Hz, 5-H), 3.79 (1H, d, $J_{3,4}$ 3.3 Hz, 4-H), 4.15 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H), 4.28 (1H, s, C<u>H</u>); δ_C (75 MHz, D_2O): 21.7 ($CO\underline{C}H_3$), 23.3 and 25.8 (2 x Me), 40.1 (C6), 50.9 (CMe2), 57.1 (OMe), 60.6 (CH), 69.2 (C4), 70.7 (C2), 72.7 (C3), 73.0 (C5), 103.8 (C1), 171.1 and 174.1 (2 x CO); m/z (CI) 367.0934, (M^+ $C_{14}H_{26}N_2O_7S$ requires 367.1539).

Methyl 6-amino-6-deoxy-6-(*N*-acetyl-*S*-nitroso-D-penicillaminyl)-*β*-D-Galactopyranoside (55)



The *title compound* was prepared quantitatively from methyl 6-amino-6-deoxy-6-(*N*-acetyl-D-penicillaminyl)-*β*-D-galactopyranoside **(64)** via the "fuming method" and was obtained as a green oil. $[\alpha]_D$ –4.6 (*c* 0.1 in MeOH); λ_{max} / nm (Krebs / DMSO 1:1) 339.4 (ε / dm^3 mol^{-1} cm^{-1} 708.5).

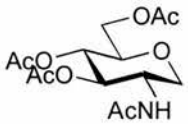
2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*α*-D-glucopyranosyl chloride (67)[155]



The *title compound* was prepared as described by Horton.[155] 2-Acetamido-2-deoxy-D-glucopyranose **(66)** (5 g, 22.6 mmol) was added to acetyl chloride (75 ml) and stirred at room temperature. The reaction underwent spontaneous reflux. After stirring overnight the mixture had become an amber solution. DCM (75 ml) was added and the solution was then poured onto cracked ice (200 ml) and stirred vigorously. The DCM was then

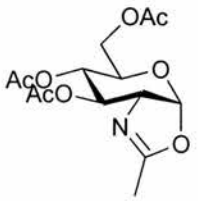
separated and run directly into more cracked ice (250 ml) containing sat. $NaHCO_3$ solution (75 ml). The mixture was stirred vigorously and then the DCM was separated, dried ($MgSO_4$) and concentrated *in vacuo* to a volume of about 15 ml. Diethyl ether (50 ml) was then added and crystallisation occurred. The pink crystals obtained were purified by recrystallisation from EtOAc / hexane 1:1 to give the *title compound* as white crystals (5.49 g, 61%). m.p. 126-128°C (lit.,[155] 127-128°C); $[\alpha]_D$ +115.8 (*c* 1 in $CHCl_3$) (lit.,[155] +110); δ_H (300 MHz, $CDCl_3$): 1.96 (3H, s, $NHCOCH_3$), 2.02 (6H, s, 2 x $OCOCH_3$), 2.08 (3H, s, $OCOCH_3$), 4.0-4.3 (3H, m, 5,6 and 6'-H), 4.5-4.5 (1H, ddd, $J_{1,2}$ 3.8, $J_{NH,2}$ 8.8 and $J_{2,3}$ 10.4 Hz, 2-H), 5.19 (1H, t, $J_{3,4}$ and $J_{4,5}$ 9.6 Hz, 4-H), 5.30 (1H, dd, $J_{3,4}$ 9.6 and $J_{2,3}$ 10.4 Hz, 3-H), 5.88 (1H, d, $J_{NH,2}$ 8.8 Hz, NH), 6.17 (1H, d, $J_{1,2}$ 3.8 Hz, 1-H); δ_C (75 MHz, $CDCl_3$): 20.4, 20.5, 20.6 and 23.0 (4 x $COCH_3$), 53.5 (C2), 61.2 (C6), 67.0, 70.1 and 70.9 (C3-C5), 93.7 (C1), 169.3, 170.3, 170.7 and 171.6 (4 x $COCH_3$).

2-Acetamido-3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-glucitol (68)[156]



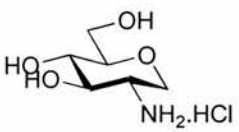
The *title compound* was prepared as described by Bamford *et al.*[156] 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride **(67)** (400 mg, 0.97 mmol) was dissolved in dry toluene (25 ml) and the solution was degassed by bubbling nitrogen through it for 30 min. Tributyltin hydride (325 µl, 1.2 mmol) was added, followed by AIBN (20 mg, cat.). The solution was heated to reflux for 3 hours and TLC (EtOAc) showed the reaction to be complete. The solvent was evaporated *in vacuo* and the resulting solid was purified by column chromatography eluting with EtOAc / hexane 1:1, then EtOAc / hexane 5:1 then EtOAc to yield the *title compound* as white crystals (259 mg, 71%). m.p. 158-160°C (lit.,[156] 158-160°C); $[\alpha]_D$ +6.2 (*c* 1 in $CHCl_3$) (lit.,[156] +4.9); δ_H (300 MHz, $CDCl_3$): 1.93 (3H, s, $NHCOCH_3$), 2.00 (3H, s, $COCH_3$), 2.05 (3H, s, $COCH_3$), 2.10 (3H, s, $COCH_3$), 3.15 (1H, t, $J_{1ax,2}$ and $J_{ax,eq}$ 12.6 Hz, 1_{ax}-H), 3.5-3.6 (1H, m, 5-H), 4.1-4.2 (4H, m, 1_{eq}, 2, 6 and 6'-H), 4.94 (1H, t, $J_{3,4}$ and $J_{4,5}$ 9.6 Hz, 4-H), 5.06 (1H, t, $J_{3,4}$ and $J_{2,3}$ 9.6 Hz, 3-H), 5.70 (1H, d, $J_{NH,2}$ 7.1 Hz, NH); δ_C (75 MHz, $CDCl_3$): 20.7, 20.9, 22.7 and 23.3 (4 x $COCH_3$), 50.7 (C2), 57.6 (C6), 62.5, 68.3 and 68.4 (C3-C5), 74.4 (C1), 169.6, 170.5, 171.1 and 172.5 (4 x $COCH_3$).

2-Methyl-(3,4,6-tri-*O*-acetyl-1,2-deoxyglucopyrano[2,1-*d*]-2-oxazoline (69)[174]



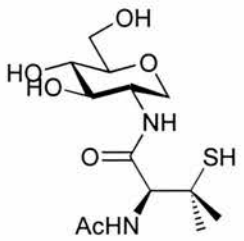
The *title compound* was obtained as the product of the reaction of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride **(68)** with AIBN and tributyltin hydride, before it was noticed that the AIBN used had degraded. The procedure, therefore, is the same as for the preparation of 2-acetamido-3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-glucitol. The *title compound* was obtained as a clear oil (79%). $[\alpha]_D$ +12.1 (*c* 0.5 in $CHCl_3$) (lit.,[174] +16.3); δ_H (300 MHz, $CDCl_3$): 1.93 (3H, s, $COC\underline{H}_3$), 1.97 (3H, s, Me), 1.99 (3H, s, $COC\underline{H}_3$), 2.00 (3H, s, $COC\underline{H}_3$), 3.50 (1H, dt, $J_{4,5}$ 9.3 and $J_{5,6}$ 4.7 Hz, 5-H), 4.0-4.1 (3H, m, 2, 6 and 6'-H), 4.82 (1H, dd, $J_{4,5}$ 9.3 and $J_{3,4}$ 2.4 Hz, 4-H), 5.15 (1H, t, $J_{2,3}$ and $J_{3,4}$ 2.4 Hz, 3-H), 5.87 (1H, d, $J_{1,2}$ 7.42 Hz, 1-H); δ_C (75 MHz, $CDCl_3$): 13.77 (Me), 20.6, 20.7 and 20.8 (3 x OAc), 60.3 (N=$\underline{C}$-Me), 63.3, 64.9, 67.5, 68.4 and 70.3 (C2 – C6), 99.4 (C1), 169.3, 169.6 and 170.6 (3 x CO).

2-Amino-1,5-anhydro-2-deoxy-D-glucitol hydrochloride (65)[154]



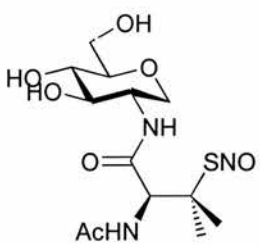
The *title compound* was prepared as described by Schafer *et al.*[154] 2-Acetamido-3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-glucitol **(68)** (200 mg, 0.53 mmol) was added to 2.5M HCl and heated to reflux for 3 hours. The resulting clear solution was evaporated *in vacuo* to give a yellow oil. This was crystallised from EtOH / ether 1:1 to give the *title compound* as white crystals (97 mg, 92%). m.p. 197-199°C, dec. (lit.,[154] 199°C, dec.); $[\alpha]_D$ +24.3 (*c* 1 in H_2O) (lit.,[154] +17.8); δ_H (500 MHz, D_2O): 3.31 (1H, dt, $J_{1eq,2}$ 4.8, $J_{1ax,2}$ 11.2 and $J_{2,3}$ 10.3 Hz, 2-H), 3.4-3.5 (2H, m, 4 and 5-H), 3.57 (1H, t, $J_{1ax,1eq}$ and $J_{1ax,2}$ 11.2 Hz, 1_{ax}-H), 3.67 (1H, dd, $J_{2,3}$ 10.3 and $J_{3,4}$ 8.3 Hz, 3-H), 3.73 (1H, dd, $J_{5,6}$ 5.5 and $J_{6,6'}$ 12.4 Hz, 6-H), 3.90 (1H, dd, $J_{5,6'}$ 2.0 and $J_{6,6'}$ 12.4 Hz, 6'-H), 4.20 (1H, dd, $J_{1ax,1eq}$ 11.2 and $J_{1eq,2}$ 4.8 Hz, 1_{eq}-H); δ_C (75 MHz, D_2O): 51.4 (C2), 60.6 (C6), 65.5 (C1), 69.8 (C4), 73.6 (C3), 80.6 (C5).

2-Amino-1,5-anhydro-2-deoxy-*N*-(*N*-acetyl-D-penicillaminyl)-D-glucitol (70)



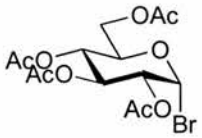
The *title compound* was prepared following the procedure described by Wang and coworkers.[127] 2-Amino-1,5-anhydro-2-deoxy-D-glucitol hydrochloride **(65)** (200 mg, 1 mmol) was added to dry methanol (5 ml). Sodium metal (23 mg, 1 mmol) was added and the mixture stirred at room temp for about 10 min. The NaCl precipitate was filtered off and the methanol evaporated to give a white solid. This was dissolved in water (5 ml). (3R)-3-Acetamido-4,4-dimethylthietan-2-one **(33)** (174 mg, 1 mmol) was dissolved in $CHCl_3$ (15 ml). The two-phase system was stirred vigorously overnight. The aqueous phase was separated, washed with $CHCl_3$ (4 x 15 ml) and freeze-dried to give a white solid. This was purified by column chromatography eluting with EtOAc / EtOH 5:1 to yield the *title compound* as a white powder (175 mg, 52%). m.p. 134-136 °C; $[\alpha]_D$ +11.4 (*c* 0.5 in water); δ_H (300 MHz, D_2O): 1.34 (3H, s, Me), 1.41 (3H, s, Me), 1.99 (3H, s, $COC\underline{H}_3$), 3.2-3.6 (5H, m, 2,3,4,5 and 6-H), 3.75-3.85 (1H, m, 6'-H), 4.09 (1H, dd, $J_{1ax,1eq}$ 4.9 and $J_{1ax,2}$ 11.2 Hz, 1_{ax}-H), 4.34 (1H, s, CH); δ_C (75 MHz, D_2O): 20.5 ($NHCO\underline{C}H_3$), 28.8 and 29.5 (2 x Me), 45.5 ($\underline{C}Me_2$), 51.4 (C5), 60.7 (C6), 66.0 (C1), 69.9 (C4), 72.8 (C3), 78.4 (C2), 171.5 and 173.6 (2 x CO); m/z (FAB) 336.1445 (M^+ $C_{13}H_{25}N_2O_6S$ requires 336.1434).

2-Amino-1,5-anhydro-2-deoxy-*N*-(*N*-acetyl-*S*-nitroso-D-penicillaminyl)-D-glucitol (71)



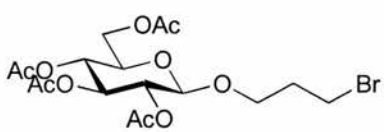
The title compound was prepared quantitatively from 2-Amino-1,5-anhydro-2-deoxy-*N*-(*N*-acetyl-D-penicillaminyl)-D-glucitol **(70)** via the "fuming method" and was obtained as a green solid. m.p. 125-127 °C; $[\alpha]_D$ + 19.2 (*c* 0.5 in H_2O); λ_{max} / nm (Krebs / DMSO 1:1) 341.0 (ε / dm^3 mol^{-1} cm^{-1} 978.1).

2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (74)[151,170]



The *title compound* was prepared as described by Kartha and Jennings.[151] D(+)Glucose **(73)** (5 g, 27.7 mmol) was added to acetic anhydride (25 ml) and 45% HBr in AcOH (5 ml). The mixture was stirred at room temp. After 3 hours the mixture had become a clear solution. More 45% HBr in AcOH (25 ml) was added and stirring continued overnight. TLC (EtOAc / hexane 1:2) proved the reaction complete and the HBr and acetic acid were removed by coevaporation with toluene. The yellow oil obtained was extracted into DCM (150 ml) and washed 10% sodium bicarbonate (100 ml), then dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow solid which was recrystallised from Et_2O / hexane 1:1 to yield the *title compound* as white needles (10.54 g, 93%). m.p. 88-90°C (lit.,[151] 88-89°C); $[\alpha]_D$ +176.6 (*c* 1 in $CHCl_3$) (lit.,[151] +198); δ_H (300 MHz, $CDCl_3$): 2.0-2.1 (12H, m, 4 x $COC\underline{H}_3$), 4.1-4.4 (3H, m, 5,6 and 6'-H), 4.85 (1H, dd, $J_{1,2}$ 4.1 and $J_{2,3}$ 9.9 Hz, 2-H), 5.15 (1H, t, $J_{3,4}$ and $J_{4,5}$ 9.9 Hz, 4-H), 5.55 (1H, t, $J_{3,4}$ and $J_{2,3}$ 9.9 Hz, 3-H) and 6.65 (1H, d, $J_{1,2}$ 4.1 Hz, 1-H); δ_C (75 MHz, $CDCl_3$): 20.5, 20.8, 21.1 and 22.4 (4 x $CO\underline{C}H_3$), 61.1 (C2), 67.4 (C6), 70.4, 70.8 and 72.3 (C3-C5), 86.8 (C1), 169.8, 170.1, 170.8 and 171.0 (4 x $\underline{C}OCH_3$).

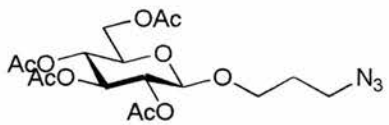
3-Bromopropyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (75)



2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide **(74)** (411 mg, 1 mmol) was added to a solution of 3-bromopropanol (226 µl, 2.5 mmol) in dry MeCN and stirred for 5 min. Iodine (380 mg, 1.5 mmol) was added slowly and the solution stirred at room temperature. The reaction was monitored by TLC (EtOAc / hexane 1:1). Once all the starting material had been consumed, the solution was diluted with DCM (100 ml) and washed with 10% sodium thiosulfate, then dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow oil. This was purified by column chromatography eluting with EtOAc / hexane 1:3 to give the *title compound* as a colourless oil (296 mg, 63%). $[\alpha]_D$ +46.4 (*c* 0.5 in $CHCl_3$); δ_H (300 MHz, $CDCl_3$): 2.0-2.2 (14H, m, 4 x $COC\underline{H}_3$ and $C\underline{H}_2CH_2Br$), 3.45 (1H, m, 5-H), 3.45-3.55 (1H, m, C$\underline{H}$Br), 3.6-3.8 (2H, m, OC$\underline{H}CH_2$- and C$\underline{H}$Br),

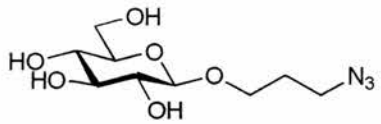
3.9-4.0 (1H, m, OC<u>H</u>CH$_2$-), 4.12 (1H, dd, $J_{5,6}$ 2.5 and $J_{6,6'}$ 12.4 Hz, 6-H), 4.24 (1H, dd, $J_{5,6'}$ 4.9 and $J_{6,6'}$ 12.4 Hz, 6'-H), 4.49 (1H, d, $J_{1,2}$ 8.0 Hz, 1-H), 4.96 (1H, dd, $J_{2,3}$ 9.6 and $J_{1,2}$ 8.0 Hz, 2-H), 5.05 (1H, t, $J_{3,4}$ and $J_{4,5}$ 9.6 Hz, 4-H), 5.18 (1H, t, $J_{2,3}$ and $J_{3,4}$ 9.6 Hz, 3-H); δ_C (75 MHz, $CDCl_3$): 20.8, 20.9, 21.0 and 21.2 (4 x OAc), 30.2, 32.4 and 62.1 (3 x CH_2), 67.6, 68.6, 71.5, 72.1 and 73.0 (C2-C6), 101.3 (C1), 170.3, 172.3, 172.9 and 175.5 (4 x $COCH_3$); m/z (CI) 469.0745 (M^+ $C_{17}H_{26}BrO_{10}$ requires 469.0710).

3-Azidopropyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranoside (76)



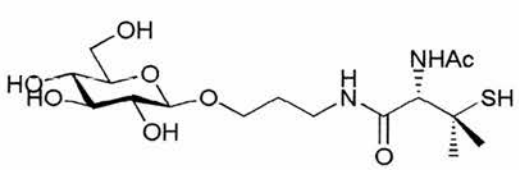
The *title compound* was prepared following the procedure described by Spijeker et al.[157] 3-Bromopropyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranoside **(74)** (1g, 2.13 mmol) was dissolved in dry DMF (20 ml). Sodium azide (554 mg, 8.52 mmol) and tetrabutylammonium triflate (249 mg, 0.64 mmol) were added and the mixture stirred at room temperature under nitrogen atmosphere. Once the reaction had reached completion, the solvent was evaporated *in vacuo*. The white residue was dissolved in diethyl ether (100 ml) and washed with a saturated solution of sodium chloride (100 ml), then dried and concentrated *in vacuo* to give the *title compound* as a colourless oil (377 mg, 41%). $[\alpha]_D$ +49.2 (*c* 1 in $CHCl_3$); δ_H (300 MHz, $CDCl_3$): 1.8-1.9 (2H, m, C<u>H</u>$_2$C<u>H</u>$_2$Br), 2.0-2.1 (12H, m, 4 x OAc), 3.35 (1H, m, 5-H), 3.40-3.45 (1H, m, OC<u>H</u>CH$_2$-), 3.60 (1H, m, C<u>H</u>N$_3$), 3.70 (1H, m, C<u>H</u>N$_3$), 3.93 (1H, dt, *J* 5.5 and 9.9 Hz, OC<u>H</u>CH$_2$-), 4.12 (1H, dd, $J_{5,6}$ 2.7 and $J_{6,6'}$ 12.4 Hz, 6-H), 4.24 (1H, dd, $J_{5,6'}$ 4.6 and $J_{6,6'}$ 12.4 Hz, 6'-H), 4.48 (1H, d, $J_{1,2}$ 8.0 Hz, 1-H), 4.96 (1H, dd, $J_{1,2}$ 8.0 and $J_{2,3}$ 9.6 Hz, 2-H), 5.06 (1H, t, $J_{3,4}$ and $J_{4,5}$ 9.6 Hz, 4-H), 5.19 (1H, t, $J_{2,3}$ and $J_{3,4}$ 9.6 Hz, 3-H); δ_C (75 MHz, $CDCl_3$): 20.6, 21.0, 21.8 and 22.1 (4 x OAc), 28.9, 47.9 and 61.9 (3 x CH_2), 66.5, 68.4, 71.3, 71.9 and 72.8 (C2-C6), 100.9 (C1), 169.6, 169.8, 170.0 and 170.8 (4 x CO); ν_{max} / cm^{-1} 2104.6 (N_3); m/z (CI) 432.1616 (M^+ $C_{17}H_{26}N_3O_{10}$ requires 432.1619).

3-Azidopropyl *β*-D-glucopyranoside (78)



3-Azidopropyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranoside **(76)** (136 mg, 0.3 mmol) was dissolved in dry methanol (2 ml) and sodium metal (10 mg, cat.) was added. After stirring at room temp. for 10 min, Dowex-50 H^+ resin (250 mg) was added and stirring continued for another 15 min. The resin was then removed by filtration and the methanol concentrated *in vacuo* to give the *title compound* as a clear oil (54.4 mg, 84%). $[\alpha]_D$ –6.0 (*c* 0.5 in MeOH); δ_H (300 MHz, MeOD): 1.8-1.9 (2H, m, $C\underline{H}_2CH_2N_3$), 3.17 (1H, dd, $J_{1,2}$ 7.7 and $J_{2,3}$ 9.1 Hz, 2-H), 3.25-3.4 (2H, m, 3 and 4-H), 3.44 (2H, t, *J* 6.9 Hz, $CH_2\underline{N}_3$), 3.6-3.7 (3H, m, 5 and 6-H and $OC\underline{H}CH_2$-), 3.86 (1H, dd, $J_{5,6'}$ 1.9 and $J_{6,6'}$ 11.8 Hz, 6'-H), 3.96 (1H, dt, *J* 6.0 and 10.2 Hz, $OC\underline{H}CH_2$-), 4.25 (1H, d, $J_{1,2}$ 7.7 Hz, 1-H); δ_C (75 MHz, MeOD): 30.3 ($\underline{C}H_2CH_2N_3$), 49.5 ($\underline{C}H_2N_3$), 62.9 (C6), 67.7 ($O\underline{C}H_2$-), 71.7, 75.2, 78.0 and 78.2 (C2 - C5), 104.6 (C1); ν_{max} / cm^{-1} 2104 (N_3); m/z (FAB) 217.0839 (M^+ $C_9H_{18}N_3O_6$ requires 217.0851).

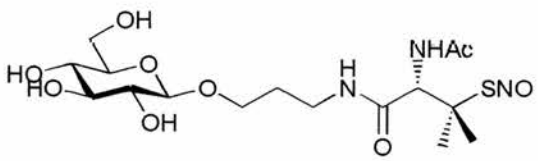
3-Amino-[*N*-(*N*-acetyl-D-penicillaminyl)]-propyl *β*-D-glucopyranoside (79)



3-Azidopropyl *β*-D-glucopyranoside **(78)** (54 mg, 0.25 mmol) was dissolved in dry methanol (3 ml). Pd/C (5 mg, cat.) was added and the mixture was stirred at room temperature under hydrogen atmosphere for 4 hours. The catalyst was removed by filtration and the solvent evaporated *in vacuo* to give a white solid. This was dissolved in water (5 ml) and added to a solution of (3R)-3-acetamido-4,4-dimethylthietan-2-one **(33)** (43 mg, 0.25 mmol) in chloroform (2 ml). The biphasic mixture was stirred vigorously at room temperature overnight. The aqueous layer was then separated, washed with chloroform (2 x 5 ml) and freeze dried to yield the *title compound* as a white powder (82 mg, 88%). m.p. 73-75 °C; $[\alpha]_D$ –8.2 (*c* 0.5 in H_2O); δ_H (300 MHz, D_2O): 1.32 (3H, s, Me), 1.37 (3H, s, Me), 1.7 – 1.8 (2H, m, CH_2), 1.99 (3H, s, $COC\underline{H}_3$), 3.1 – 3.9 (11H, m, 2, 3, 4, 5, 6 and 6'-H, C<u>H</u> and 2x $C\underline{H}_2$), 4.34 (1H, d, $J_{1,2}$ 7.7 Hz, 1-H); δ_C (75 MHz, D_2O): 21.7 ($CO\underline{C}H_3$), 28.3 and 28.9 (2 x Me), 29.6 ($\underline{C}H_2$), 36.2 ($\underline{C}H_2$),

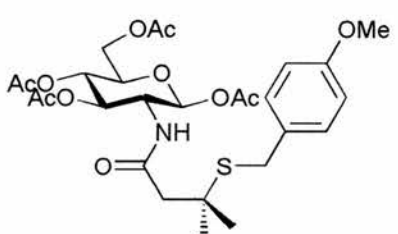
44.9 (<u>C</u>Me$_2$), 60.8 (<u>C</u>H), 62.6 (<u>C</u>H$_2$), 67.8, 69.7, 73.2, 75.8 and 76.0 (C2-C6), 102.3 (C1), 171.2 and 174.3 (2 x CO); m/z (MALDI-TOF) 410.1689 (M^+ $C_{14}H_{29}N_2O_8S$ requires 410.1723).

3-Amino-[*N*-(*N*-acetyl-*S*-nitroso-D-penicillaminyl)]-propyl *β*-D-glucopyranoside (80)



The *title compound* was prepared quantitatively from 3-Amino-[*N*-(*N*-acetyl-D-penicillaminyl)]-propyl *β*-D-glucopyranoside *via* the "fuming method" and was obtained as a green oil. $[\alpha]_D$ –8.9 (*c* 0.5 in H_2O); λ_{max} / nm (Krebs / DMSO 1:1) 340.6 (ε / dm^3 mol^{-1} cm^{-1} 883.9).

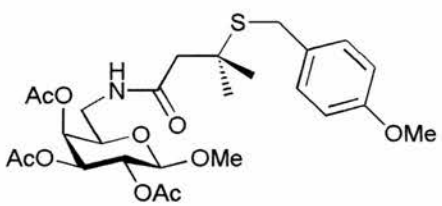
1,3,4,6-Tetra-*O*-acetyl-2-amino-2-deoxy-*N*-(*S*-*p*-methoxybenzylmercaptoisovaleryl) *β*-D-glucopyranose (83)



Glucosamine hydrochloride **(25)** (200 mg, 1 mmol) was added to dry MeOH (2 ml) and treated with sodium metal (23 mg, 1 mmol). After 5 min stirring, the NaCl precipitate was removed by filtration and the solvent concentrated *in vacuo* to give a white solid. This was used in the coupling to *S*-*p*-methoxybenzylmercaptoisovaleric acid **(81)**. The coupling was carried out following one or other of the procedures (coupling A. to E.) described at the beginning of this chapter. The residue obtained after evaporation of the solvent was added to dry pyridine (3 ml) and acetic anhydride (2 ml) and stirred at room temp. overnight. DCM (50 ml) was added and washed with 1 M HCl (100 ml), sat. $NaHCO_3$ (100ml) and water (100 ml), then dried ($MgSO_4$) and concentrated *in vacuo* to give a brown solid. This was purified by column chromatography, eluting with EtOAc / hexane 1:3 to yield the *title compound* as a clear oil (182 mg, 31% - method C). $[\alpha]_D$ +60.8 (*c* 0.25 in $CHCl_3$); δ_H (300 MHz, $CDCl_3$): 1.20 (3H, s, Me), 1.21 (3H, s, Me), 1.79, 1.84, 1.85, 1.87 (4 x 3H, 4 x s, 4 x COC<u>H</u>$_3$), 2.21 (2H, s, COC<u>H</u>$_2$), 3.54 (2H, s,

$C\underline{H}_2Ar$), 3.51 (3H, s, OMe), 3.67 (1H, ddd, $J_{5,6}$ 2.5, $J_{5,6'}$ 4.7 and $J_{4,5}$ 10.2 Hz, 5-H), 3.93 (1H, dd, $J_{5,6}$ 2.5 and $J_{6,6'}$ 11.6 Hz, 6-H), 4.08 (1H, dd, $J_{1,2}$ 8.8 and $J_{2,3}$ 10.4 Hz, 2-H), 4.94 (1H, dd, $J_{5,6'}$ 4.7 and $J_{6,6'}$ 11.6 Hz, 6'-H), 4.87 (1H, dd, $J_{3,4}$ 9.6 and $J_{4,5}$ 10.2 Hz, 4-H), 5.03 (1H, dd, $J_{3,4}$ 9.6 and $J_{2,3}$ 10.4 Hz, 3-H), 5.51 (1H, d, $J_{1,2}$ 8.0 Hz, 1-H), 6.65 (2H, d, J_{AB} 8.8 Hz, aromatics), 7.05 (2H, d, J_{AB} 8.8 Hz, aromatics), 7.10 (1H, d, $J_{NH,2}$ 8.7 Hz, NH); δ_C (75 MHz, $CDCl_3$): 20.1, 20.2, 20.3 and 20.5 (4 x $COC\underline{H}_3$), 28.2 and 28.4 (2 x Me), 32.2 (CH_2), 44.0 (OMe), 52.2, 55.0, 61.6, 68.3, 72.2, 72.3 (C2-C6 and CH_2), 92.0 (C1), 113.9, 129.8 and 130.0 (aromatics), 158.6 (CO), 169.7, 169.9, 170.9 and 171.2 (4 x $COC\underline{H}_3$); m/z (CI) 584.2223 (M^+ $C_{27}H_{38}NO_{11}S$ requires 584.2194).

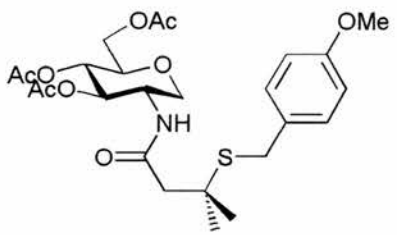
Methyl 6-amino-6-deoxy-*N*-(*S*-*p*-methoxybenzyl-*β*-mercaptoisovaleric acid)-*β*-D-galactopyranoside (84)



Methyl 6-azido-6-deoxy-*β*-D-galactopyranoside **(63)** (220 mg, 1 mmol) was dissolved in dry methanol (5 ml). Pd/C (10 mg, cat.) was added and the mixture was stirred at room temperature under hydrogen atmosphere for 4 hours. The catalyst was removed by filtration and the solvent evaporated *in vacuo* to give a white solid. This was coupled to *S*-*p*-methoxybenzyl-*β*-mercaptoisovaleric acid **(81)** (254 mg, 1 mmol) *via* one or other of the methods described at the beginning of this chapter (Couplings A. to E.) to yield a yellow oil. This was added to dry pyridine (5 ml) and acetic anhydride (3 ml) and stirred at room temp. overnight. DCM (50 ml) was added and washed with 1M HCl (100 ml), sat. $NaHCO_3$ solution (100 ml) and water (100 ml), then dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow oil. This was purified by column chromatography eluting with EtOAc / hexane 1:2 to give the *title compound* as a clear oil (172 mg, 31% - method C). $[\alpha]_D$ 0 (*c* 0.5 in $CHCl_3$); δ_H (300 MHz, $CDCl_3$): 1.37 (3H, s, Me), 1.39 (3H, s, Me), 1.92 (3H, s, $COC\underline{H}_3$), 1.99 (3H, s, $COC\underline{H}_3$), 2.00 (3H, s, $COC\underline{H}_3$), 2.39 (2H, s, $C\underline{H}_2$), 3.3-3.4 (2H, m, 6 and 6'-H), 3.39 (3H, s, sugar-OMe), 3.7-3.8 (1H, m, 5-H), 3.69 (2H, s, $C\underline{H}_2Ar$), 3.73 (3H, s, Ar-OMe), 4.27 (1H, d, $J_{1,2}$ 8.0 Hz, 1-H), 4.93 (1H, dd, $J_{3,4}$ 3.3 and $J_{2,3}$ 10.4 Hz, 3-H), 5.12 (1H, dd, $J_{2,3}$ 10.4 and $J_{1,2}$ 8.0 Hz, 2-H), 5.29 (1H, d, $J_{3,4}$ 3.3 Hz, 4-H), 6.63 (1H, broad s, N$\underline{H}$), 6.78 (2H, d, J_{AB} 7.5

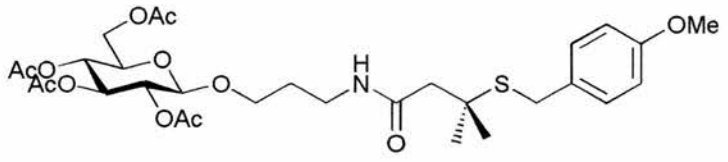
Hz, aromatics), 7.19 (2H, d, J_{AB} 7.5 Hz, aromatics); δ_C (75 MHz, $CDCl_3$): 20.5, 20.6 and 20.7 (3 x $CO\underline{C}H_3$), 28.8 and 29.3 (2 x Me), 32.7 ($\underline{C}H_2Ar$), 39.2 (C6), 48.8 ($\underline{C}H_2$), 55.3 (Ar-OMe), 57.2 (Sugar-OMe), 68.2 (C4), 69.1 (C2), 71.1 and 71.3 (C3 and C5), 102.2 (C1), 114.2, 129.7 and 130.2 (aromatics), 169.8, 170.2, 170.7 and 170.9 (4 x CO); m/z (FAB) 555.2204 (M^+ $C_{26}H_{37}NO_{10}S$ requires 555.2217)

2-Amino-1,5-anhydro-2-deoxy-*N*-(*S*-*p*-methoxybenzylmercaptoisovaleryl)-D-glucitol (85)



2-Amino-1,5-anhydro-2-deoxy-D-glucitol hydrochloride **(65)** (200 mg, 1 mmol) was added to dry MeOH (2 ml) and treated with sodium metal (23 mg, 1 mmol). After 5 min stirring, the NaCl precipitate was removed by filtration. The sugar and *S*-*p*-methoxybenzylmercaptoisovaleric acid **(81)** were coupled via one or other of the methods described at the beginning of this chapter (Couplings A. to E.) to yield a yellow oil. This was added to dry pyridine (3 ml) and acetic anhydride (2 ml) and stirred at room temperature overnight. DCM (50 ml) was added and washed with 1M HCl (100 ml), sat. $NaHCO_3$ (100 ml) and water (100 ml), then dried ($MgSO_4$) and concentrated *in vacuo* to give a brown oil. This was purified by column chromatography eluting with EtOAc / hexane 1:3 to yield the *title compound* as a clear oil (121 mg, 23% - method D). $[\alpha]_D$ –12.5 (*c* 0.75 in $CHCl_3$); δ_H (300 MHz, $CDCl_3$): 1.37 (3H, s, Me), 1.39 (3H, s, Me), 1.98 (3H, s, $COC\underline{H}_3$), 2.02 (3H, s, $COC\underline{H}_3$), 2.08 (3H, s, $COC\underline{H}_3$), 2.34 (2H, d, *J* 3.3 Hz, $C\underline{H}_2$), 3.10 (1H, t, $J_{1ax,1eq}$ and $J_{1ax,2}$ 10.4 Hz, 1_{ax}-H), 3.5-3.6 (1H, m, 5-H), 3.71 (2H, s, $C\underline{H}_2Ar$), 3.79 (3H, s, OMe), 4.1-4.2 (4H, m, 1_{eq}, 2, 6 and 6'-H), 4.95-5.05 (2H, m, 3 and 4-H), 6.17 (1H, d, $J_{NH,2}$ 7.4 Hz, NH), 6.84 (2H, d, J_{AB} 11.5 Hz, aromatics), 7.24 (2H, d, J_{AB} 11.5 Hz, aromatics); δ_C (75 MHz, $CDCl_3$): 20.6, 20.7 and 20.8 (3 x $CO\underline{C}H_3$), 28.8 and 29.0 (2 x Me), 32.6 (CH_2), 44.2 (CMe_2), 48.8 (CH_2), 50.0 (OMe), 55.3, 62.4, 68.1, 68.7 and 73.8 (C2 – C6), 76.5 (C1), 114.2, 129.9 and 130.2 (aromatics), 169.6, 170.4, 170.9 and 171.6 (4 x CO); m/z (MALDI-TOF) 548.1931 (M+Na $C_{25}H_{35}NO_9SNa$ requires 548.1930).

3-Amino-*N*-(*S*-*p*-methoxybenzyl-*β*-mercaptoisovaleryl acid)-propyl β-D-glucopyranoside (86)



3-Azidopropyl *β*-D-glucopyranoside **(78)** (216 mg, 1 mmol) was dissolved in dry methanol (5 ml). Pd/C (10 mg, cat.) was added and the mixture was stirred at room temperature under hydrogen atmosphere for 4 hours. The catalyst was removed by filtration and the solvent evaporated *in vacuo* to give a white solid. This was coupled to *S*-*p*-methoxybenzyl-β-mercaptoisovaleric acid **(81)** (254 mg, 1 mmol) *via* one or other of the methods described at the beginning of this chapter (Couplings A. to E.) to yield a yellow oil. This was added to dry pyridine (5 ml) and acetic anhydride (3 ml) and stirred at room temp. overnight. DCM (50 ml) was added and washed with 1M HCl (100 ml), sat. $NaHCO_3$ solution (100 ml) and water (100 ml), then dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow oil. This was purified by column chromatography eluting with EtOAc / hexane 1:3 to give the *title compound* as a clear oil (115 mg, 18% - method A). $[\alpha]_D$ +7.2 (*c* 0.5 in $CHCl_3$); δ_H (300 MHz, $CDCl_3$): 1.44 (3H, s, Me), 1.47 (3H, s, Me), 2.00 (3H, s, $COC\underline{H}_3$), 2.02 (3H, s, $COC\underline{H}_3$), 2.04 (3H, s, $COC\underline{H}_3$), 2.07 (3H, s, $COC\underline{H}_3$); 2.44 (2H, s, $C\underline{H}_2$), 3.2-3.3 (1H, m, $OC\underline{H}CH_2$-), 3.3-3.4 (1H, m, 5-H), 3.4-3.7 (2H, m, $CH_2C\underline{H}_2NH$), 3.75 (2H, s, $C\underline{H}_2Ar$), 3.79 (3H, s, OMe), 3.9-4.0 (1H, m, $OC\underline{H}CH_2$), 4.11 (1H, dd, $J_{5,6}$ 2.7 and $J_{6,6'}$ 12.1 Hz, 6-H), 4.19 (1H, dd, $J_{5,6'}$ 4.4 and $J_{6,6'}$ 12.1 Hz, 6'-H), 4.44 (1H, d, $J_{1,2}$ 8.0 Hz, 1-H), 4.98 (1H, dd, $J_{1,2}$ 8.0 and $J_{2,3}$ 9.6 Hz, 2-H), 5.06 (1H, t, $J_{3,4}$ and $J_{4,5}$ 9.6 Hz, 4-H), 5.19 (1H, t, $J_{2,3}$ and $J_{3,4}$ 9.6 Hz, 3-H), 6.33 (1H, broad s, NH), 6.84 (2H, d, J_{AB} 7.8 Hz, aromatics), 7.21 (2H, d, J_{AB} 7.8 Hz, aromatics); δ_C (75 MHz, $CDCl_3$): 19.8, 20.6, 20.7 and 21.3 (4 x $COC\underline{H}_3$), 29.0 and 29.2 (2 x Me), 32.6, 36.7 and 37.5 (3 x CH_2), 44.6 (CMe_2), 48.9 and 55.3 (2 x CH_2), 61.7, 68.4, 71.3, 71.9 and 72.7 (C2 – C6), 100.9 (C1), 114.1, 124.6 and 130.2 (aromatics), 156.1, 169.6, 170.0, 170.4 and 170.9 (5 x CO); m/z (MALDI-TOF) 664.2352 (M+Na $C_{30}H_{43}NO_{12}SNa$ requires 664.2404).

REFERENCES

1 P. Vallance and J. Collier, *Br. Med. J.*, 1994, **309**, 453.

2 A. R. Butler and D. L. H. Williams, *Chem. Soc. Rev.*, 1993, **22**, 233

3 R. Furchgott and J. Zawadski, *Nature*, 1980, **288**, 373.

4 M. Fontecave and J.-L. Pierre, *Bull. Soc. Chim. Fr.*, 1994, **131**, 620.

5 S. Pfeiffer, B. Mayer and B. Hemmens, *Angew. Chem. Int. Ed. Engl.*, 1999, **38**, 1714.

6 J. S. Beckman, T. W. Beckman, J. Chen, P. A. Marshall and B. A. Freeman, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 1620.

7 L. J. Ignarro, J. M. Fukuto, J. M. Griscavage, N. E. Rogers and R. E. Byrns, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 8103.

8 A. F. Vanin, I. V. Malenkova and V. A. Serezhenkov, *Nitric Oxide: Biology and Chemistry*, 1997, **1**, 191.

9 A. C. F. Gorren, E. De Boer and R. Wever, *Biochim. Biophys. Acta*, 1987, **916**, 38.

10 Y. Haddad, G. Pataki, P. Hu, C. Galiani, J. S. Beckman and S. Matalon, *J. Clin. Invest.*, 1994, **94**, 2407.

11 J. S. Beckman, H. Ischiropoulos, L. Zhu, M. Van der Woerd, C. Smith, J. Chen, J. Harrison, J. C. Martin and M. Tsia, *Arch. Biochem. Biophys.*, 1992, **298**, 438.

12 M. Miwa, D. J. Stuehr, M. A. Marletta, J. S. Wishnok and S. R. Tannenbaum, *Carcinogenesis*, 1987, **8**, 955.

13 R. S. Lewis, *J. Am. Chem. Soc.*, 1995, **117**, 3933.

14 D. L. H. Williams, *Nitric Oxide: Biology and Chemistry*, 1997, **1**, 522.

15 D. J. Meyer, H. Kramer, N. Ozer, B. Coles and B. Ketterer, *FEBS Lett.*, 1994, **345**, 177.

16 S. C. Askew, A. R. Butler, F. W. Flitney, G. D. Kemp and I. L. Megson, *Bioorg. Med. Chem.*, 1995, **3**, 1.

17 R. Busse and A. Mulsch, *FEBS Lett.*, 1990, **265**, 133.

18 M. A. Marletta, *J. Biol. Chem.*, 1993, **268**, 12231.

19 D. R. Adams, M. Brochwicz-Lewinski and A. R. Butler, *Prog. Chem. Org. Nat. Prod.*, 1999, **76**, 1.

20 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.

21 M. A. Awolesi, W. C. Sessa and B. E. Sumpio, *J. Clin. Invest.*, 1995, **96**, 1449.

22 D. S. Bredt and S. H. Snyder, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 682.

23 A. A. White and G. D. Aurbach, *Biochim. Biophys. Acta*, 1969, **191**, 686.

24 J. G. Hardman and E. W. Sutherland, *J. Biol. Chem.*, 1969, **244**, 6363.

25 C. A. Gruetter, B. K. Barry, D. B. McNamara, D. Y. Gruetter, P. J. Kadowitz and L. J. Ignarro, *J. Cyclic Nucleotide Res.*, 1979, **5**, 211.

26 L. J. Ignarro, B. K. Barry, D. Y. Gruetter, J. C. Edwards, E. H. Ohlstein, C. A. Gruetter and W. H. Baricos, *Biochem. Biophys. Res. Commun.*, 1980, **94**, 93.

27 L. J. Ignarro, J. N. Degnan, W. H. Baricos, P. J. Kadowitz and M. S. Wolin, *Biochim. Biophys. Acta*, 1982, **718**, 49.

28 J. R. Stone and M. A. Marletta, *Biochemistry*, 1996, **35**, 1093.

29 A. J. Hobbs, *TiPS*, 1997, **18**, 484.

30 V. G. Kharitonov, M. Russwurm, D. Magde, V. S. Sharma and D. Koesling, *Biochem. Biophys. Res. Commun.*, 1997, **239**, 284.

31 J. S. Gillespie, X. Liu and W. Martin, *Int. Congr. Ser. - Excerpta Med.*, 1990, **897**, 147.

32 R. A. Lefebvre, E. Baert and A. J. Barbier, *Biol. Nitric Oxide, Proc. Int. Meet., 2nd*, 1992, **1**, 293.

33 S. M. Ward, H. H. Dalzier, K. D. Thornbury, D. P. Westfall and K. M. Sanders, *Biol. Nitric Oxide, Proc. Int. Meet., 2nd*, 1992, **1**, 295.

34 A. L. Burnett, C. J. Lowenstein, D. S. Bredt, T. S. K. Chang and S. H. Snyder, *Science*, 1992, **257**, 401.

35 N. Kim, K. M. Azadzoi, I. Goldstein and I. S. Tejada, *J. Clin. Invest.*, 1991, **88**, 112.

36 A. M. Franchi, M. Chaud, V. Rettori, A. Saburo, S. M. McCann and M. Gimeno, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 539.

37 J. Garthwaite, *Int. Congr. Ser. - Excerpta Med.*, 1990, **897**, 115.

38 N. Miki, Y. Kawabe and K. Kuriyama, *Biochem. Biophys. Res. Commun.*, 1977, **75**, 851.

39 U. Fostermann, L. D. Gorsky, J. S. Pollock, H. H. H. W. Schmidt, M. Heller and F. Murad, *Biochem. Biophys. Res. Commun.*, 1990, **168**, 727.

40 E. W. Ainscough and A. M. Brodie, *J. Chem. Edu.*, 1995, **72**, 686.

41 C. Iadecola, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 686.

42 A. M. Leone, L. E. Gustafsson, P. L. Francis, M. G. Persson, M. P. Wiklund and S. Moncada, *Biochem. Biophys. Res. Commun.*, 1994, **201**, 883.

43 L. E. Gustafsson, D. H. Edwards, M. J. Lewis, A. C. Newby and A. H. Henderson, *Nature*, 1984, **308**, 645.

44 I. R. Hutcheson and T. M. Griffith, *Am. J. Physiol.*, 1991, **261**, H257.

45 G. M. Rubanji, J. C. Romero and P. M. Vanhoutte, *Am. J. Physiol.*, 1986, **250**, H1145.

46 M. A. Marletta, P. S. Yoon, R. Iyengar, C. D. Leaf and J. S. Wishnok, *Biochemistry*, 1988, **27**, 8706.

47 J. B. Hibbs, Z. Vavrin, E. M. Rachlin and R. R. Taintor, *Biochem. Biophys. Res. Commun.*, 1988, **157**, 87.

48 D. Stuehr, S. S. Gross, I. Sakuma, R. Levi and C. F. Nathan, *J. Exp. Med.*, 1989, **169**, 1011.

49 N. V. Blough and O. C. Zafiriou, *Inorg. Chem.*, 1985, **24**, 3502.

50 White, Brock, B. A. Freeman and Tarpey, *Proc. Natl. Acad. Sci. USA*, 1990, **91**, 1044.

51 J.-C. Drapier, C. Pellat and Y. Henry, *J. Biol. Chem.*, 1991, **266**, 10162.

52 M. A. DeGroote, D. Granger, Y. Xu, G. Campbell, R. Prince and F. C. Fang, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 6399.

53 H. P. Rang and M. M. Dale, *'Pharmacology'*, Churchill Livingstone, 1991.

54 M. W. Radomski, R. M. J. Palmer and S. Moncada, *Lancet*, 1987, **2**, 1057.

55 M. W. Radomski, R. M. J. Palmer and S. Moncada, *Br. J. Pharmacol.*, 1987, **92**, 181.

56 B. Furlong, A. H. Henderson, M. J. Lewis and J. A. Smith, *Br. J. Pharmacol.*, 1987, **90**, 687.

57 M. L. Steer and E. W. Salzman, *Adv. Cyclic Nucleotide Res.*, 1980, **12**, 71.

58 M. W. Radomski, R. M. J. Palmer and S. Moncada, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 5193.

59 R. M. J. Palmer, A. Ferrige and S. Moncada, *Nature*, 1987, **327**, 524.

60 L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns and G. Chaudhuri, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 9265.

61 S. Moncada, R. M. J. Palmer and E. A. Higgs, *Pharm. Rev.*, 1991, **43**, 109.

62 J. A. Loskove and W. H. Frishman, *American Heart J.*, 1995, **129**, 604.

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

64 A. R. Butler, F. W. Flitney and P. Rhodes, *Perspectives on Bioinorganic Chemistry*, 1996, **3**, 251.

65 E. Carlosen and J. H. Comroe, *J. Cren. Physiol.*, 1958, **42**, 83.

66 R. E. Eich, T. Li, D. D. Lemon, D. H. Hoherty, S. N. Curry, A. J. Matthews, K. A. Johnson, R. D. Smith, G. N. Phillips and J. S. Osler, *Biochemistry*, 1996, **35**, 6976.

67 J. R. Lancaster, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 8137.

68 M. W. Vaughn, L. Kuo and J. C. Liao, *Am. J. Physiol.*, 1998, **274**, H1705.

69 X. Liu, M. S. Muler, M. S. Joshi, H. Sadowska-Krowicka, D. A. Clark and J. R. Lancaster, *J. Biol. Chem.*, 1998, **273**, 18709.

70 A. R. Butler, I. L. Megson and P. G. Wright, *Biochem. Biophys. Acta*, 1999, **1495**, 168.

71 J. C. Liao, T. W. Hein, M. W. Vaughn, K.-T. Huang and L. Kuo, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 8757.

72 L. Jia, C. Bonaventura, J. Bonaventura and J. S. Stamler, *Nature*, 1996, **380**, 221.

73 D. A. Wink, J. F. Darbyshire, R. W. Nims, J. E. Saavedra and P. C. Ford, *Chem. Res. Toxicol.*, 1993, **6**, 23.

74 M. Woltz, R. J. MacAllister, D. Davis, M. Feelisch, S. Moncada, P. Vallance and A. Hobbs, *J. Biol. Chem.*, 1999, **274**, 28983.

75 D. S. Celermajer, K. E. Sorensen and V. M. Gooch, *Lancet*, 1992, **340**, 1111.

76 G.-W. He, B. Buxton, F. L. Rosenfeldt and J. A. Angus, *Circulation*, 1989, **80(s)**, 1141.

77 E. J. Langford, A. S. Brown, R. J. Wainwright, A. J. De Belder, M. R. Thomas, R. E. A. Smith, M. W. Radomski, J. F. Martin and S. Moncada, *Lancet*, 1994, **344**, 1458.

78 W. W. Parmley and K. Chatterjee, *J. Cardiovasc. Pharmacol.*, 1984, **6**, 888.

79 N. Benjamin and G. Richardson, , 2000.

80 R. Rossaint, K. J. Falke, F. Lopez, K. Slama, U. Pison and W. M. Zapol, *New Engl. J. Med.*, 1993, **328**, 399.

81 J. Pebke-Zaba, T. W. Higenbottam, A. T. Dinh-Xuan, D. Stone and J. Wallwork, *Lancet*, 1991, **338**, 1173.

82 S. Moncada, *Acta Physiol. Scand.*, 1992, **19**, 201.

83 S. Moncada, R. M. J. Palmer and E. A. Higgs, *Biochem. Pharmacol.*, 1989, **38**, 1709.

84 A. Ialenti, S. Moncada and M. DiRosa, *Br. J. Pharmacol*, 1993, **110**, 701.

85 A. Ialenti, A. Ianaro, S. Moncada and M. DiRosa, *Eur. J. Pharmacol*, 1992, **211**, 177.

86 Y.-C. Hou, A. Janczuk and P. G. Wang, *Current Pharmaceutical Design*, 1999, **5**, 417.

87 U. Klinner, D. Ehlers, R. Fermum and P. Meisel, *Acta Biol. Med. Ger.*, 1977, **36**, 1143.

88 A. R. Butler, C. Glidewell, J. McGinnis and W. I. K. Bisset, *Clin. Chem.*, 1987, **33**, 490.

89 D. L. H. Williams, *'Nitrosation'*, Cambridge University Press, Cambridge, 1988.

90 H. Lecher and W. Siefhen, *Chem. Ber.*, 1926, **59**, 1314.

91 H. S. Tasker and H. O. Jones, *J. Chem. Soc.*, 1909, **95**, 1910.

92 R. J. Phillips and R. Moor, *Spectrochim. Acta*, 1961, **17**, 1004.

93 S. Oae, Y. H. Kim, D. Fukushima and K. Shinhama, *J. Chem. Soc., Perkin Trans 1*, 1978, 913.

94 J. McAninly, D. L. H. Williams, S. C. Askew, A. R. Butler and C. Russell, *J. Chem. Soc., Chem. Commum.*, 1993, 1758.

95 A. P. Dicks, H. R. Swift, D. L. H. Williams, A. R. Butler, H. H. Al-Sa'doni and B. G. Cox, *J. Chem. Soc., Perkin Trans. 2*, 1996, **4**, 481.

96 L. Field, R. V. Dilts, R. Ravichandran, P. G. Lenhert and G. E. Carnahan, *J. Chem. Soc., Chem. Commun.*, 1978, 249.

97 Y. H. Kim, K. Shinhama, D. Fukushima and S. Oae, *Tetrahedron Lett.*, 1978, 1211.

98 J. W. Park, *Biochem. Biophys. Res. Commun.*, 1988, **152**, 916.

99 P. K. Sehajpal, A. Basu, J. S. Ogiste and H. M. Lander, *Biochemistry*, 1999, **38**, 13407.

100 S. A. Henderson, P. H. Lee, E. E. Aeberhard, J. W. Adams, L. J. Ignarro, W. J. Murphy and M. P. Sherman, *J. Biol. Chem.*, 1994, **269**, 25239.

101 E. P. Abraham, E. Chain and W. Barker, *Nature*, 1943, **151**, 107.

102 J. M. Walshe, *Br. Med. J.*, 1956, **1**, 588.

103 M. Friedman, *Adv. Exp. Med.*, 1977, **86**, 649.

104 D. Perrett, *Proc. Royal Soc. Med.*, 1977, **70**, 61.

105 R. J. van de Stadt, A. O. Muijsers, A. M. A. Henrichs and J. K. van der Korst, *Scand. J. Rheumatol.*, 1979, **28**, 13.

106 P. J. M. W. L. Birker and H. C. Freeman, *J. Am. Chem. Soc.*, 1977, **99**, 6890.

107 N. D. Schonbeck, M. Skalski and J. A. Shafer, *J. Biol. Chem.*, 1975, **250**, 5343.

108 L. J. Ignarro, H. Lippton, J. C. Edwards, W. H. Baricos, A. L. Hyman, P. J. Kadowitz and C. A. Gruetter, *J. Pharm. Exp. Ther.*, 1981, **218**, 739.

109 B. T. Mellion, L. J. Ignarro, C. B. Myers, E. H. Ohlstein, B. A. Ballot, A. L. Hyman and P. J. Kadowitz, *Mol. Pharmacol.*, 1983, **23**, 653.

110 N. K. Boughton-Smith, I. R. Hutcheson, A. M. Deakin, B. J. R. Whittle and S. Moncada, *Eur. J. Pharmacol.*, 1990, **191**, 485.

111 P. J. Henry, O. H. Drummer and J. D. Horowitz, *Br. J. Pharmacol.*, 1989, **98**, 757.

112 E. A. Kowaluk, R. Poliszczuk and H. L. Fung, *Eur. J. Pharmacol.*, 1987, **144**, 379.

113 I. L. Megson, I. R. Greig, G. A. Gray, D. J. Webb and A. R. Butler, *Br. J. Pharmacol.*, 1997, **122**, 1617.

114 I. L. Megson, I. R. Greig, A. R. Butler, G. A. Gray and D. J. Webb, *Scot. Med. J.*, 1997, **42**, 88.

115 I. L. Megson, F. W. Flitney, J. Bates and R. N. Webster, *Endothelium*, 1995, **3**, 39.

116 A. R. Butler, R. A. Field, I. R. Greig, F. W. Flitney, S. K. Bisland, F. Khan and J. J. F. Belch, *NITRIC OXIDE: Biology and Chemistry*, 1997, **1**, 211.

117 F. Khan, I. R. Greig, D. J. Newton, A. R. Butler and J. J. F. Belch, *Lancet*, 1997, **350**, 410.

118 M. D. Travis, L. L. Stoll, J. N. Bates and S. J. Lewis, *Eur. J. Pharmacol.*, 1996, **318**, 47.

119 P. Gordge, P. Addis, A. A. Noronha-Dutra and J. S. Hothersall, *Biochem. Pharmacol.*, 1998, **55**, 657.

120 T. W. Hart, *Tetrahedron Lett.*, 1985, **26**, 2013.

121 I. L. Megson, S. Morton, I. R. Greig, F. A. Mazzei, R. A. Field, A. R. Butler, G. C. Caron, A. Gasco, R. Fruttero and D. J. Webb, *Br. J. Pharmacol.*, 1999, **126**, 639.

122 I. R. Greig, Ph.D. Thesis , University of St. Andrews, St. Andrews, 1997.

123 M. Bergman and L. Zervas, *Chem. Ber.*, 1931, **64**, 975.

124 M. L. Wolfrom and A. Thompson, , 1963.

125 L. J. Lin and J. F. Foster, *Anal. Biochem.*, 1975, **63**, 485.

126 J. R. J. Vaughan, *J. Am. Chem. Soc.*, 1951, **73**, 3547.

127 J. Ramirez, L. Yu, J. Li, P. G. Braunschweiger and P. G. Wang, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 2575.

128 I. L. Knunyants, O. V. Kildisheva and E. Y. Pervova, *Bull. Acad. Sci. USSR Div. Chem. Sci. Eng. Trans.*, 1955, 613.

129 L. Field, W. S. Hanley, P. L. Kelly, W. J. Sanders and J. E. White, *J. Med. Chem.*, 1973, **16**, 1152.

130 S. M. R. Al-Zaidi, M. M. L. Crilley and R. J. Stoodley, *J. Chem. Soc. Perkin Trans. 1*, 1983, 2259.

131 H. A. Moynihan and S. M. Roberts, *J. Chem. Soc. Perkin Trans*, 1994, **1**, 797.

132 Y. Hou, J. Wang, P. R. Andreana, G. Cantauria, S. Tarasia, L. Sharp, P. G. Braunschweiger and P. G. Wang, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 2255.

133 H. B. Burgi and J. D. Dunitz, *Acc. Chem. Res.*, 1983, **16**, 153.

134 K. P. R. Kartha and R. A. Field, *Tetrahedron*, 1997, **53**, 11753.

135 M. Von Itzstein, *Carboh. Res.*, 1994, **259**, 293.

136 P. Konradsson, D. R. Mootoo, R. E. McDevitt and B. Fraser-Reid, *J. Chem. Soc., Chem. Commun.*, 1990, 270.

137 B. Fraser-Reid, Z. Wu, U. E. Udodong and H. Ottosson, *J. Org. Chem.*, 1990, **55**, 6068.

138 Y. Inouye, K. Onodera, S. Kitaoka and H. Ochiai, *J. Am. Chem. Soc.*, 1957, **79**, 4218.

139 W. H. A. Kuijpers and C. A. A. Van Boeckel, *Tetrahedron*, 1993, **49**, 10931.

140 U. Kempin, L. Henning, D. Knoll, P. Welzel, D. Muller, A. Markus and J. Van Heijenoort, *Tetrahedron*, 1997, **53**, 17669.

141 S. Sugasawa, S. Hatsuno, K. Shigezane and T. Mizoguchi, *Japan. Kokai*, 1974, 5.

142 M. Bodanszky and A. Bodanszky, *'The Practice of Peptide Synthesis'*, Springer-Verlag, 1984.

143 T. W. Greene and P. G. M. Wuts, *'Protective groups in organic synthesis'*, John Wiley & Sons, New York, 1999.

144 M. D. Bachi and K. J. Ross-Petersen, *J. Chem. Soc., Chem. Commun.*, 1974, 12.

145 M. D. Bachi and K. J. Ross-Petersen, *J. Org. Chem.*, 1972, **37**, 3550.

146 S. Akabori, S. Sakakibara, Y. Shimonishi and Y. Nobuhara, *Bull. Chem. Soc. Jpn.*, 1964, **37**, 433.

147 T. Shiraiwa, S. Sakata, H. Natsuyama, K. Fujishima, H. Miyazaki, S. Kubo, T. Nitta and H. Kurokawa, *Bull. Chem. Soc. Jpn.*, 1992, **65**, 965.

148 A. Schonberg and M. Z. Barakat, *J. CHem. Soc.*, 1949, 892.

149 S. Oae and H. Togo, *Bull. Chem. Soc. Jpn.*, 1984, **57**, 232.

150 H. K. Chenault, J. Dahmer and G. M. Whitesides, *J. Am. Chem. Soc.*, 1989, **111**, 6354.

151 R. Kartha and H. J. Jennings, *J. Carbohydr. Chem.*, 1990, **9**, 777.

152 R. L. Whistler and M. L. Wolfrom, *Methods in Carboh. Chem.*, 1963, **2**, 216.

153 J. S. Brimacombe and O. A. Ching, *J. Chem. Soc.*, 1968, 1643.

154 A. Schafer, G. Klich, M. Schreiber, H. Paulsen and J. Thiem, *Carbohydr. Res.*, 1998, **313**, 107.

155 D. Horton, *Methods Carbohydr. Chem.*, 1972, **6**, 282.

156 M. J. Bamford, J. C. Pichel, W. Husman, B. Patel, R. Storer and N. G. Weir, *J. Chem. Soc., Perkin Trans. 1*, 1995, 1181.

157 N. M. Spijker, C. A. Keuning, M. Hooglugt, G. H. Veeneman and C. A. A. van Boecker, *Tetrahedron*, 1996, **52**, 5945.

158 D. L. H. Williams, *Acc. Chem. Res.*, 1999, **32**, 869.

159 W. Martin, G. M. Villani, D. Jothianandan and R. F. Furchgott, *J. Pharmacol. Exp. Ther.*, 1985, **275**, 708.

160 D. D. Rees, R. M. J. Palmer, R. Schultz, H. F. Hodson and S. Moncada, *Br. J. Pharmacol.*, 1990, **101**, 746.

161 J. O. Parker and H.-L. Fung, *Am. J. Cardiol.*, 1984, **54**, 471.

162 J. S. Stamler, O. Jaraki, J. Osborne, D. I. Simon, J. Keaney, J. Vita, D. Singel, C. R. Valeri and J. Loscalzo, *Proc. Natl. Acad. Sci. U.S.A.*, 1992, **89**, 7674.

163 J. S. Stamler, D. I. Simon, J. A. Osborn, M. E. Mullins, O. Jaraki, T. Michel, D. J. Singel and J. Loscalzo, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 444.

164 D. D. Perrin, W. L. Amarego and D. R. Perrin, *'Purification of Laboratory Chemicals'*, Pergamon Press, 1996.

165 H. R. Swift and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1997, **10**, 1933.

166 E. R. Kaiser, L. Colescott, C. D. Bossinger and P. I. Cook, *Anal. Biochem.*, 1970, **34**, 595.

167 T. A. Martin, D. H. Causey and J. R. Corrigan, *J. Med. Chem.*, 1968, **11**, 625.

168 D. H. Leaback and P. G. Walker, *J. Chem. Soc.*, 1957, 4754.

169 R. Marshall, M. Winitz, S. M. Birnbaum and J. P. Greenstein, *J. Am. Chem. Soc.*, 1957, **79**, 4538.

170 D. H. Brauns, *J. Am. Chem. Soc.*, 1925, **47**, 1280.

171 J. K. Dale and C. S. Hudson, *J. Am. Chem. Soc.*, 1930, **52**, 2534.

172 M. L. Wolfrom, J. W. Spoors and R. A. Gibbons, *J. Org. Chem.*, 1957, **22**, 1513.

173 D. Gibson, Ph.D. Thesis, University of St. Andrews, St. Andrews, 2001.

174 R. U. Lemieux and H. Driguez, *J. Am. Chem. Soc.*, 1975, **97**, 4063.

APPENDICES

Table 1: Crystal data and structure refinement for thietanone 33.

Empirical formula	$C_7H_{11}NO_2S$	
Formula weight	173.23	
Temperature	298 K	
Wavelength	0.71073 Å	
Crystal system	Orthorhombic	
Space group	$P2_12_12_1$	
Unit cell dimensions	a = 7.5782 (2) Å	$\alpha = 90°$
	b = 9.5356 (4) Å	$\beta = 90°$
	c = 12.9144 (6) Å	$\gamma = 90°$
Volume, Z	933.23 (6) $Å^3$, 4	
Density (calculated)	1.233 Mg/m^3	
Absorption coefficient	0.302 mm^{-1}	
F (000)	368	
Crystal size	.3 x .1 x .1 mm	
θ range for data collection	2.66 to 23.26°	
Limiting indices	$-8 \leq h \leq 7$, $-10 \leq k \leq 7$, $-11 \leq l \leq 14$	
Reflections collected	4047	
Independent reflections	1340 (R_{int} = 0.0259)	
Absorption correction	Sadabs	
Max. and Min. transmission	1.00000 and .0711470	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	1328 / 1 / 105	
Goodness-of-fit on F^2	0.952	
Final R indices [$I > 2\sigma(I)$]	R1 = 0.0367, wR2 = 0.0926	
R indices (all data)	R1 = 0.0577, wR2 = 0.1075	
Absolute structure parameter	0.1 (2)	
Extinction coefficient	0.015 (4)	
Largest diff. Peak and hole	0.151 and –0.213 e $Å^{-3}$	

Table 2: Atomic coordinates [x 10^4] and equivalent isotropic displacement parameters [Å^2 x 10^3] for thietanone 33. U (eq) is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	*y*	*z*	U(eq)
S(1)	10179 (2)	4182 (1)	9273 (1)	120 (1)
C(2)	10977 (4)	4089 (3)	7997 (3)	68 (1)
O(2)	10578 (3)	3360 (2)	7281 (2)	90 (1)
C(3)	12353 (3)	5219 (3)	8143 (2)	56 (1)
N(3)	14044 (3)	4928 (2)	7690 (2)	60 (1)
C(4)	12190 (4)	5289 (3)	9346 (2)	68 (1)
C(5)	11842 (5)	6741 (3)	9765 (3)	88 (1)
C(6)	13660 (7)	4568 (4)	9905 (3)	119 (2)
C(7)	15107 (4)	5929 (3)	7331 (2)	60 (1)
O(7)	14629 (3)	7165 (2)	7342 (2)	93 (1)
C(8)	16863 (4)	5482 (3)	6940 (3)	83 (1)

Table 3: Bond lengths [Å] and angles [°] for thietanone 33.

S(1)-C(2)	1.758 (3)	S(1)-C(4)	1.856 (3)
C(2)-O(2)	1.195 (3)	C(2)-C(3)	1.511 (4)
C(3)-N(3)	1.436 (3)	C(3)-C(4)	1.560 (4)
N(3)-C(7)	1.332 (3)	C(4)-C(6)	1.495 (5)
C(4)-C(5)	1.509 (4)	C(7)-O(7)	1.233 (3)
C(7)-C(8)	1.486 (4)		
C(2)-S(1)-C(4)	78.12 (14)	O(2)-C(2)-C(3)	133.3 (3)
O(2)-C(2)-S(1)	131.9 (2)	C(3)-C(2)-S(1)	94.8 (2)
N(3)-C(3)-C(2)	115.3 (2)	N(3)-C(3)-C(4)	119.0 (2)
C(2)-C(3)-C(4)	95.8 (2)	C(7)-N(3)-C(3)	122.9 (2)
C(6)-C(4)-C(5)	112.3 (3)	C(6)-C(4)-C(3)	113.7 (3)
C(5)-C(4)-C(3)	114.2 (3)	C(6)-C(4)-S(1)	112.0 (2)
C(5)-C(4)-S(1)	133.3 (3)	C(3)-C(4)-S(1)	89.4 (2)
O(7)-C(7)-N(3)	120.2 (2)	O(7)-C(7)-C(8)	122.8 (3)

Table 4: Anisotropic displacement parameters [Å^2 x 10^3] for thietanone 33. The anisotropic displacement factor exponent takes the form: $-2\pi^2$ [$(ha^*)^2U^{11}$ + ... + $2hka^*b^*U_{12}$]

	U11	U22	U33	U23	U13	U12
S(1)	151 (1)	117 (1)	91 (1)	2 (1)	36 (1)	-54 (1)
C(2)	62 (2)	65 (2)	77 (2)	1 (2)	-7 (2)	-1 (2)
O(2)	84 (2)	89 (2)	98 (2)	-25 (1)	-15 (1)	-14 (1)
C(3)	58 (2)	49 (2)	60 (2)	4 (1)	-2 (1)	2 (1)
N(3)	60 (1)	39 (1)	83 (2)	5 (1)	2 (1)	4 (1)
C(4)	92 (2)	57 (2)	55 (2)	0 (2)	-3 (2)	3 (2)
C(5)	103 (3)	82 (2)	79 (2)	-21 (2)	1 (2)	17 (2)
C(6)	186 (4)	95 (3)	76 (2)	-3 (2)	-38 (3)	51 (3)
C(7)	60 (2)	47 (2)	74 (2)	-5 (1)	-6 (1)	-1 (2)
O(7)	80 (2)	43 (1)	157 (2)	5 (1)	22 (1)	-1 (1)
C(8)	67 (2)	66 (2)	116 (3)	1 (2)	4 (2)	1 (2)

Table 5: Hydrogen coordinates (x 10^4) and isotropic displacement parameters (Å^2 x 10^3) for thietanone 33.

	x	*y*	*z*	U(eq)
H (3A)	11902 (3)	6096 (3)	7850 (2)	67
H (3N)	14473 (37)	3962 (12)	7641 (22)	84 (9)
H (5A)	10890 (5)	7163 (3)	9386 (3)	132
H (5B)	12884 (5)	7303 (3)	9691 (3)	132
H (5C)	11529 (5)	6679 (3)	10484 (3)	132
H (6A)	13833 (7)	3651 (4)	9615 (3)	178
H (6B)	13366 (7)	4484 (4)	10625 (3)	178
H (6C)	14724 (7)	5105 (4)	9833 (3)	178
H (8A)	16968 (4)	4481 (3)	6998 (3)	124
H (8B)	17772 (4)	5922 (3)	7343 (3)	124
H (8C)	16984 (4)	5753 (3)	6228 (3)	124