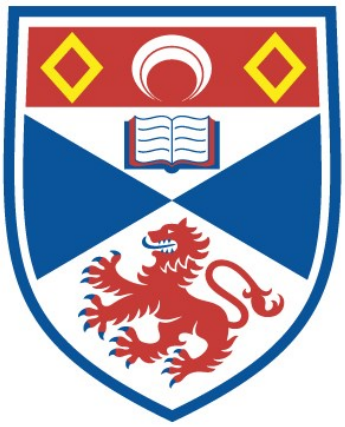
NEW NITRIC OXIDE DONOR DRUGS

Iain Robert Greig

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1997

New Nitric Oxide Donor Drugs

a thesis presented by

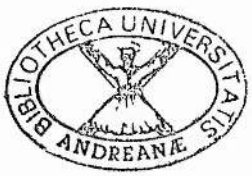
Iain Robert Greig

to the

University of St. Andrews

in application for

The Degree of Doctor of Philosophy



St. Andrews

August 1997

Declaration

I, Iain Robert Greig, hereby certify that this thesis, which is approximately 56,000 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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I was admitted as a research student in October 1994 and as a candidate for the degree of Ph.D. in September 1995; the higher study for which this is a record was carried out in the University of St. Andrews between 1994 and 1997.

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dedicated to my Grandparents,
where it all began

I spoke about wings,
you just flew

Acknowledgements

I would like to thank my supervisor, Dr Tony Butler for a unique project which has given me a chance to work in a very wide scientific area and to be involved in some fascinating studies. I would particularly like to thank him for his exhausting efforts to set up these collaborations. Finally I would like to thank him for his scientific contacts which have allowed me to continue research in another exciting field.

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Finally thanks to Wendy, you didn't know it but it all happened because of you.

Abstract

Nitric oxide is a recognised dilator of vascular smooth muscle and therefore is central in the control of blood flow. A lack of blood flow in humans can have very important implications in a number of disorders of both cutaneous tissue and internal circulation.

In this thesis we look at the synthesis of new nitric oxide donors, their stabilities and their possible medicinal usage. These donors have been based on the *S*-nitrosothiol group, connected to sugar moieties, simple amino acids or linked glycoaminoacids.

The donors prepared have been used to investigate the skin blood flow and localised responses to nitric oxide, proving that NO has an important role in the maintenance of healthy skin. These will be further investigated as possible treatments for disorders involving a lack of cutaneous blood flow, such as connective tissue disorders and the repeated ulceration often seen in diabetic patients.

A set of clinical trials have been carried out comparing the responses of healthy patients and sufferers of Raynaud's Phenomenon to exogenous nitric oxide. In this we have highlighted a number of differences and have helped to determine a possible cause of the disorder.

We have prepared a number of slow release NO donors which have been shown to produce a sustained vasodilatory response in blood vessels with removed or damaged endothelial cells. These show promise for use in the treatment of patients with circulatory disorders, especially for subjects following treatment for atherosclerosis.

Basic studies investigating the stabilities of these compounds have been carried out, in order to aid our understanding of their mode of breakdown.

Abbreviations

cGMP = Cyclic guanosine monophosphate.

cGRP = Calcitonin gene related peptide.

DTT = Dithiothreitol.

EDTA = Ethylenediaminetetraacetate

GSNO = *S*-Nitrosoglutathione.

L-NAME = *N*-Nitro-L-arginine methyl ester.

L-NMMA = L-*N*-monomethylarginine.

PCTA = Percutaneous transluminal angioplasty.

RBS = Roussin's Black Salt

RP = Raynaud's Phenomenon.

RIG 200 = *N*-(*S*-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose.

RIG 300 = *N*-(*S*-nitroso-*N*-propionylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose.

RIG 500 = N-(*S*-nitroso-*N*-valerylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose.

RIG 700 = N-(*S*-nitroso-*N*-heptanoylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose

SNAG = *S*-nitroso-1-thio-β-D-glucose tetraacetate.

SNAP = *S*-Nitroso-*N*-acetylpenicillamine.

SNOIL = *S*-Nitrosothioglycerol.

SNOIL-Acetate = 2,3-*O*-Acetyl-1-*S*-nitrosothioglycerol.

SNOPHE = *S*-Nitroso-*N*-heptanoylpenicillamine.

SNOPP = *S*-Nitroso-*N*-propionylpenicillamine.

SNP = Sodium nitroprusside.

SNVP = *S*-Nitroso-*N*-valerylpenicillamine.

TG = Thioglycerol.

TGA = Thioglycerol acetate.

Publications

A.R.Butler, I.R.Greig and F.Khan. The transdermal delivery of an NO donor drug: a new approach to Raynaud's Syndrome, *Proceedings of the 4th International Biology of Nitric Oxide Conference*, (1995).

I.L.Megson, I.R.Greig, A.R.Butler, G.A.Gray and D.J.Webb. Vasodilatory properties of a novel nitrosated glyco-amino acid in isolated rat-femoral arteries: potential as a slow release nitric oxide donor drug, *British Journal of Pharmacology*, (1997), **120**, 52.

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A.R.Butler, R.A,Field, I.R.Greig, F.W.Flitney, S.K.Bisland, F.Khan and J.J.F.Belch. An examination of some derivatives of *S*-nitroso-1-thiosugars as vasodilators, *Nitric Oxide: Chemistry and Biology*, (1997), **1**, 211 - 217.

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Chapter 2: Experimental

Foreword

Nitric oxide can be regarded as a bit of an underachiever. The career of NO may be compared with that of dioxygen, somewhat unfavourably. Both were discovered by Joseph Priestley in the late eighteenth century, nitric oxide in 1772 and oxygen in 1774. Oxygen supports vitually all known life forms, supplies us with energy through the combustion of fossil fuels and provides innumerable useful organic and inorganic compounds, notably that most vital of compounds, water. Nitric oxide, on the other hand, is a radical which does not even react with itself. It will react with oxygen to form NO_2, admittedly one of the few coloured gases but hardly an achievement worthy of great acclaim. It is a major contributant to the photochemical smogs which afflict many large cities and is also one of the chemicals responsible for ozone depletion. Oxygen has continued its career steadily acquiring a reputation as a molecule you can trust in times of need and has managed to retain the respect and admiration of the scientific community. Nitric oxide made a dramatic bid for stardom during the late 1980s. Suddenly every chemist, biologist and physiologist wanted a seat on the bandwagon. Nitric oxide leapt into every facet of human life and received multidisciplinary plaudits, claiming an involvement in everything from muscle relaxation and the immune system to long term memory. Nitric oxide was even given the prestigious Molecule of the Year award in 1992[1] and even more significantly gained a mention in Cosmopolitan magazine. Unfortunately, success built overnight can disappear just as rapidly. Tales of its prowess became muddled, contradictory and in some places downright inaccurate. Disputes broke out amongst the fans, some even began to query whether nitric oxide had a significant role to play in the ever changing modern lifestyle. Those who had once unquestioningly thrown money at nitric oxide began to demand a return for their investment or they would have to look elsewhere for a molecule capable of satisfying their needs.

It is at this pivotal point that we join the continuing saga of nitric oxide to find out whether this historic underachiever can fulfil the promise it has recently shown or will sink back into the oblivion from which it came.

Many papers and reviews have been written on both the chemistry of nitric oxide and its physiological role. This is an indication of the interest in nitric oxide and its potential importance. However, that so much has been said on the matter is also an indication of how little understanding we have of its action and how little agreement there is between the various scientists.

There are several major problems which afflict the nitric oxide community. The first is the difficulty in detecting nitric oxide. Various probes have been developed but these are difficult to use due to their extreme sensitivity and are highly inaccurate: results were often more affected by atmospheric conditions than NO concentrations. The probes can be used successfully to show the presence of NO but not the amounts produced. A second problem is to mimic accurately physiological conditions in the laboratory. Experiments may be carried out to explain all of the problems but few of these reflect what may happen in the body where any number of additional factors may be involved.

An additional problem impeding progress in the field of nitric oxide is its interdisciplinary nature. NO is studied by chemists, biologists, biochemists and physiologists. Each of these groups is undoubtedly very competent in a chosen field and yet is capable of causing chaos and confusion when they step outside the area of expertise. Statements which are often little short of ridiculous to even a minimally experienced member of one discipline are routinely published and taken as gospel by members of another discipline. It is perhaps for these reasons that few major tangible benefits have been gained, considering the vast amounts of research performed.

Chapter 1:

A general overview of the chemistry and biology of nitric oxide[2,3,4,5]

Many excellent reviews have been written concerning nitric oxide and there is little value in repeating what has been said in any great detail. Some of what has been said is open to debate and may prove to be inaccurate upon further study. The nitric oxide field moves at such a rapid rate that much of what is currently thought concerning NO may change within a very brief period and may change our understanding of the entire area. This report will make no assumptions about nitric oxide except that it is a vasodilator. We will discuss the favoured explanation of this along with a short review of the other areas of possible NO involvement.

1.1. Chemical Reactions of nitric oxide

1.1.1. The preparation and occurrence of nitric oxide

NO was first prepared by Priestley in 1772 by the action of HNO_3 on various metals. The most common reaction is that with copper (1) a common school experiment.

$$(1) \quad 8HNO_3 + 3Cu \longrightarrow 3Cu(NO_3)_2 + 4H_2O + 2NO$$

NO is an intermediate in the production of nitric acid in both the inefficient and obsolete Birkland - Eyde process (2) from 1903 and the Nobel prize winning Ostwald process (3) from 1908.

$$(2) \quad N_2 + O_2 \xrightarrow{\text{spark}} 2NO$$

$$(3) \quad 4NH_3 + 5O_2 \xrightarrow[900°C]{\text{Pt/Rh cat}} 4NO + 6H_2O$$

It is perhaps unusual that intentional NO production requires large amounts of energy and yet it is a pollutant which forms all too readily when not wanted. The above Birkland - Eyde reaction occurs in automobile internal combustion engines and is a major contributant to the photochemical smogs which afflict many large cities, particularly Los Angeles. It is hoped that catalytic converters, fitted to all new cars, will help alleviate some of this problem. However these converters are easily poisoned and are notoriously

fragile. The reactions (4) and (5) are catalysed by noble metals contained within the converter.

(4) $2NO \longrightarrow N_2 + O_2$

(5) $CO + NO \longrightarrow CO_2 + {}^1/_2N_2$

1.1.2. Ozone Depletion

The widening holes in the ozone layer have become a serious concern in recent years, especially in the southern hemisphere where skin cancers are taking an increasing toll of human life. Much has been said about the influence of chlorofluorocarbons (CFCs) on ozone depletion. Production and usage of these has now been limited. NO, being a radical, also presents a danger to ozone levels. There are particular fears about the NO produced at high altitudes by supersonic aircraft in their exhaust fumes and its reaction with ozone (6). NO is also produced naturally in sunlight from NO_2 (7).

(6) $O_3 + NO \longrightarrow NO_2 + O_2$
$NO_2 + O \longrightarrow NO + O_2$

(7) $NO_2 \longrightarrow NO + O$

It can be seen from (6) that NO not only reacts with O_3 but also produces NO_2 which reacts with oxygen radicals. These in reaction with O_2, would normally produce ozone

(8) $O + O_2 + M \xrightarrow{h\upsilon\ 393nm} O_3 + M$

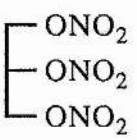
1.2. Nitric oxide donors

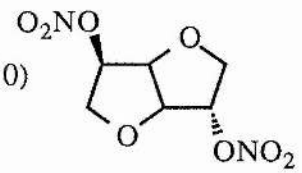
There are now a large number of NO donors available. These fall into two main categories: those which require metabolism in order to produce NO and those which will spontaneously release NO by thermal, chemical or photolytic decomposition. They may also transfer NO to another thiol to create a less stable nitrosothiol, e.g. cysteine. There

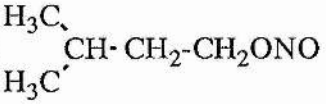
is also a third class of donor: those where the cause of decomposition is either unknown or in debate.

1.2.1. Organic nitrates

Organic nitrates have been known for many years and have proved to be of great therapeutic use. The best known of these is probably glyceryl trinitrate (9), better known as the explosive nitroglycerine. Its incorporation into porous silica to create dynamite was first done by Alfred Nobel. The hypotensive action of NO was made famous by the discovery of abnormally low blood pressure amongst the girls who packed explosives during the First World War.[6] Recently this has been the subject of a documentary on BBC television.[7] In this it was noted that the workers involved in explosives often had heart attacks whilst taking holidays. The explanation for this involved their continual exposure to nitroglycerine during the working week, reducing their blood pressure. Eventually they developed tolerance to this and it had less of an effect on their blood pressure, allowing it to return to, or close to, normal values. However, at weekends they did not have this exposure and the blood pressure would rise to well above the normal levels. During a normal weekend there wasn't ususally time for any great problems to occur, once back at work on the Monday their blood pressure would be reduced again. When there was a long weekend or a holiday the danger was much greater as the blood pressure would rise steadily throughout and a number of workers were unknowingly at risk of heart attacks whenever they took time off work. The study had not run for long enough to determine whether these workers would develope tolerance to such a degree that they would run the risk of a heart attack every weekend.

(9) 

(10) 

(11) 

Both glyceryl trinitrate and isosorbide dinitrate (10) are common ingredients in the puffers used by angina sufferers and are used in medicines to treat other heart conditions. Amyl nitrite (11) has been used as a vasodilator since 1867, when it was used as an inhalant to treat asthma.[8] Nowadays it is most commonly used in the so called Gay Bars in pill form known as poppers. There its muscle dilating capacity and aphrodisiac effects are said to prove useful amongst homosexuals. In a fascinating and controversial article from the Sunday Times[9] (1994) it was suggested that amyl nitrite might be a major contributant to AIDS. This was based on the connection between homosexuals and many of the early cases of AIDS. The study showed amyl nitrite to do more damage to the immune system than HIV. This article provoked a very strong backlash from other members of the scientific community who felt it hindered their efforts to control the disease. Nothing more has been said on this line of investigation and it may be considered to be discredited. The actual mechanism of metabolism of the organic nitrates has not been satisfactorily resolved.[10] It has been proposed that thiols are necessary for the enzymatic transformation into NO, particularly cysteine. However it may be that nitrosothiols are not formed.

1.2.2. Spontaneous NO donors

1.2.2.1. Inorganic Compounds

The best known inorganic donors are sodium nitroprusside (SNP) (12) and Roussin's Black Salt (RBS) (13)

(12) $Na_2[Fe(CN)_5NO]$

(13) $Na[Fe_4S_3(NO)_7]$

Both are highly effective vasodilators, SNP is used in heart surgery to reduce blood pressure. RBS is particularly potent as it not only has 7 NO ligands in each anion but it is more soluble in organic solvents than water. RBS is taken into the endothelial cells because of its lipid solubility and can remain there slowly releasing NO for several

hours.[11] RBS decomposes to produce NO by chemical and photolytic means. The means of NO release from SNP are much studied and little understood.[12] Spontaneous decomposition is unlikely due to the very high formation constants for the hexacyanoferrates and similar compounds. Photolytic decomposition (14) is fairly well documented but fails to explain the vasodilatory activity shown in the absence of light.

(14) $[Fe(CN)_5NO]^{2-} \xrightarrow{h\nu} [Fe(CN)_5]^{2-} + NO^{\cdot}$

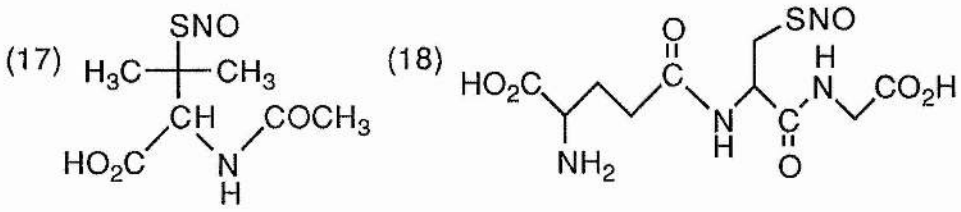
(15) $[Fe(CN)_5NO]^{2-} + RS^{-} \longrightarrow [Fe(CN)_5N(O)(SR)]^{3-} \dashrightarrow RSNO + \text{Fe species}$

A reaction between SNP and thiol has been studied (15). In this nitrosothiols are produced and these decompose to produce disulphide and NO (16). Laboratory tests have supported this theory but suggest it may be too slow to account for the immediate vasodilatory action of SNP.[13] The currently favoured explanation amongst physiologists is that of enzymic involvement.[14]

(16) $RSNO \longrightarrow RS^{\cdot} + NO^{\cdot}$, $RS^{\cdot} \rightarrow RSSR$

1.2.2.2. Nitrosothiols

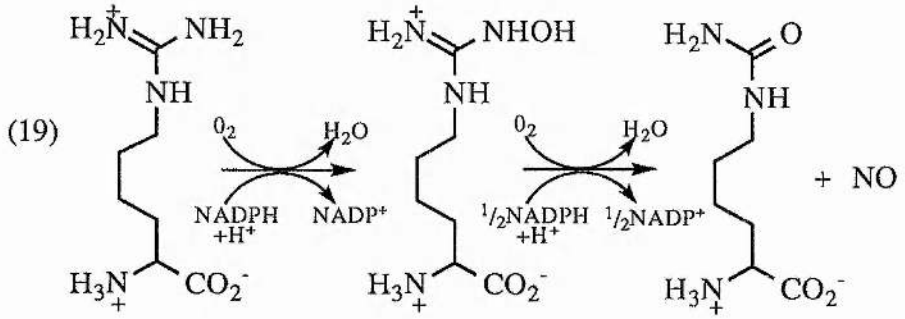
Potentially the most useful NO donors are *S*-nitrosothiols. The nitroso derivative of cysteine is thought to play an important role in the body, however it is too unstable to isolate. Two nitrosothiols with a high degree of stability are *S*-nitroso-*N*-acetylpenicillamine (SNAP) (17) and *S*-nitrosoglutathione (GSNO) (18). These are highly coloured due to the SNO group, making them easy to study.



These are potent vasodilators in whole animals as well as in *ex vivo* experiments, decomposing readily to produce NO (16). The route and speed of decomposition is very much dependent on the nature of the thiol. SNAP is very stable as a solid at room temperature but rapidly decomposes in solution in the presence of copper ions. GSNO may require transnitrosation or enzymatic cleavage.[15] Other nitrosothiols are often thermally decomposed and most are photolytically unstable.

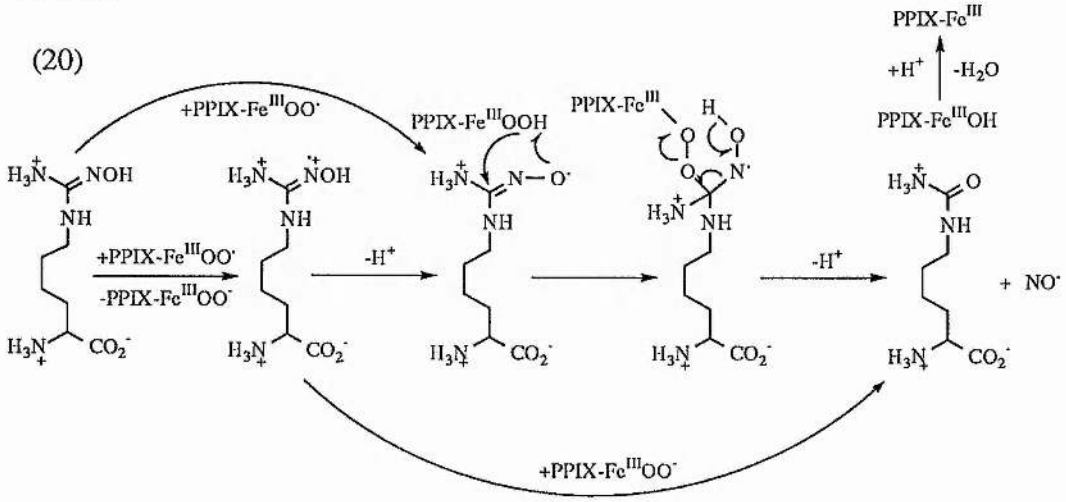
1.3. *In-vivo* production of NO

Nitric oxide is thought to be produced in the body by the conversion of L-arginine to citrulline by enzymes known as NO synthases (19). There are two main NO synthases: constitutive and inducible. Both are NADPH dependent dioxygenases. Constitutive NO synthase (cNOS) is produced by smooth muscle where NO is always being produced to some degree. There is a basal NO production from the endothelial cells which acts against vascular smooth muscle contraction. Activation gives a rapid but short lived response. As NO is toxic only small amounts are produced. Inducible NO synthase (iNOS), involved in the immune system, is only present when the macrophages have been activated. In order to rid the body of infection it is longer lasting than cNOS. A final, less well understood, NOS type is brain or neural (nNOS).



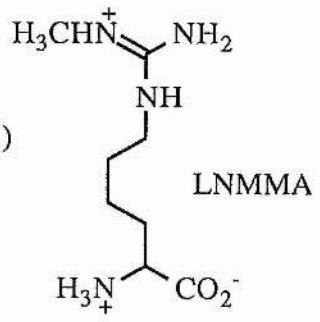
NOS is a haem protein and it is thought that the process is regulated by NO itself. This is presumably by an interaction between NO and the haem iron centre. This could constitute a feedback mechanism preventing overproduction of NO. The accepted mechanism for the conversion of L-arginine to *N*-hydroxyarginine is thought to involve initial one electron oxidation of the guanidine nitrogen and hydroxylation of the amidine nitrogen utilising 1 eq. NADPH and O_2, as shown in the first stage of (19). The second stage is somewhat more complex and involves the iron-protoporphyrin IX component of NOS (20).

(20)

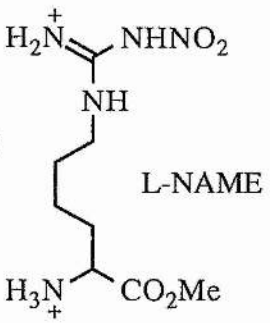


This process can be inhibited by arginine analogues acting as competitive inhibitors, such as L-*N*-monomethylarginine (LNMMA) (21) and the methyl ester of *N*-nitro-L-arginine (L-NAME) (22).

(21) LNMMA



(22) L-NAME



Processes caused by NO can be halted or reduced by the use of NO scavengers such as haemoglobin. In this way it can be shown whether an *in vitro* reaction is being caused by NO or not.

1.4. Physiological role of nitric oxide

1.4.1. NO and the immune system

The first indication of a role for nitric oxide in the immune response came when high levels of nitrate were found in patients suffering from gastroenteritis.[16] Cultured macrophages, one of the main cell groups involved in the non-specific immune response, were found to produce nitrite and nitrate. This was dependent on the presence of L-arginine. In the body NO is produced by the enzymatic action of NO synthase on arginine (section **1.3**). The ability of macrophages to kill abnormal cells such as tumours, was shown to be dependent on L-arginine.[17] This demonstrated that NO must be part of the natural defence of the body against invaders and abnormal cells within the body. It was initially suggested that NO itself might be sufficiently potent to be the cytotoxic agent.[18] Tests have shown that in general it is not capable of killing cells on its own.[19] There are two main routes by which mammalian DNA can be damaged by NO.[20] One involves the reaction of NO with molecular oxygen and the other the reaction of NO with superoxide. The reaction with O_2 produces N_2O_3, a nitrosating agent. N_2O_3 can react with secondary amines to form *N*-nitrosamines (**1.5.1**) which can be carcinogenic or mutagenic. N_2O_3 can also nitrosate primary amines on DNA bases causing deamination and base mutation. The reaction with superoxide produces peroxynitrite ($ONOO^-$). This may either rearrange to give nitrate or decompose to produce reactive oxygen intermediates (ROIs) (23).[21]

(23)
$$NO + O_2^{\cdot} \longrightarrow ONOO^-$$
$$ONOO^- + H^+ \rightleftharpoons ONOOH$$
$$ONOOH \longrightarrow NO_2^{\cdot} + HO^{\cdot}$$

The production of the highly reactive hydroxyl radicals is an appealing solution. However peroxynitrite was not found to be particularly toxic against *Leishmania major*.[22] It is possible that the toxicity of peroxynitrite involves interaction with cellular targets rather than the formation of hydroxyl radicals.[23] This would make it very much tissue specific rather than universally toxic. A final suggestion is that NO, having a high affinity for iron, can react with iron centres to give iron nitrosyls. If this occurs it can destroy the action of the iron sulphur clusters which are part of the bacterial respiratory system.[24] Some of these clusters are involved in DNA synthesis and this will stop the bacterium growing. Yet again, examples where this is found not to occur are available in the literature. NO does not inhibit the enzyme aconitase which contains an important iron-sulphur cluster.[25] The most probable role for NO in the immune system is a combination of the above suggestions, with NO being toxic enough to weaken the cell for other defence mechanisms to be efficient.

1.4.2. Platelet aggregation

When a blood vessel is damaged, tiny fragments contained in the blood adhere to the surface and form a plug. These are called platelets. Further aggregation of these platelets causes a clot to form. This prevents excessive blood loss. Platelets adhere to damaged, altered or abnormal surfaces. Spontaneous platelet aggregation in the blood vessel may cause a thrombus, which can have serious consequences. This can occur in the heart where platelets adhere to non-contractile scar tissue or vessels coated in atherosclerotic plaque. If aggregation takes place then myocardial infarction may occur. When the platelets are activated NO is produced in a feedback mechanism to counteract the substances promoting adhesion and aggregation. NO is produced both by the endothelial cells which the platelets come into contact with and the platelets themselves. It is thought that NO and prostacyclin act synergistically to inhibit platelet aggregation and to break up the aggregates already formed.[26] Both SNAP and GSNO are effective in the prevention of platelet aggregation. This may well provide an important role for such compounds as there are few effective drugs currently available.

1.4.3. Brain

Signals move along nerve cells as electrical impulses but chemical messengers are used to convey the signal across the gaps between the nerve cells (synapses). Two of the major neurotransmitters are the amino acids aspartate and glutamate. These pass from the pre-synaptic cells to the post-synaptic cells. The presence of enhanced levels of cGMP, paralleling that which occurs in smooth muscle relaxation, suggested a role for NO in the nervous system. It is currently thought that NO is acting as a retrograde messenger between the synapses. NO produced by the post-synaptic cell diffuses to the surrounding pre-synaptic cells, strengthening the link between them. In this way the system becomes sensitised to a particular stimulus and will be able to respond more efficiently when next exposed to that stimulus. This may be responsible for functions such as long term memory and complex automated actions - such as the playing of musical instruments. It is also thought that NO in the central nervous system may be responsible for the modulation of pain perception and the mediation of short term electrocortical activation. The best studied role of NO in the nervous system is that which concerns the NANC (non-adrenergic non cholinergic) nerves where NO is released in response to an electrical signal.[27] This then acts upon smooth muscle as covered in **1.4.4.**

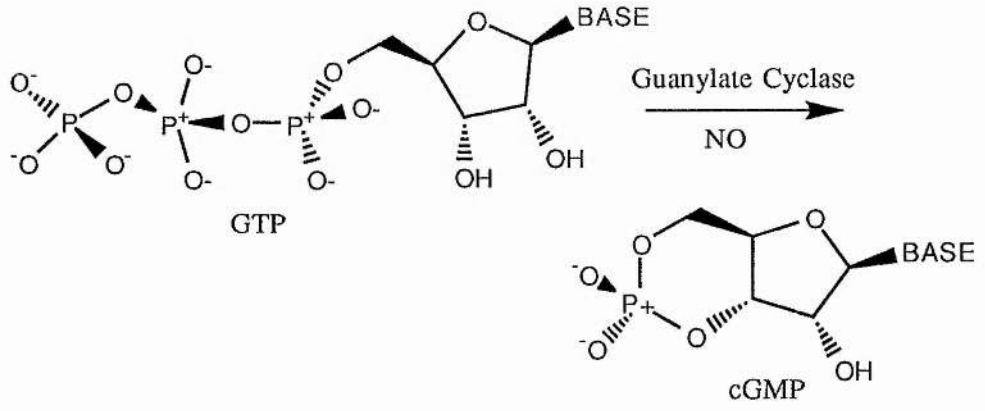
1.4.4. Smooth muscle relaxation

In human physiology there are three types of muscle: skeletal, responsible for the movement of joints; cardiac, responsible for keeping the heart beating and smooth muscle, which surrounds hollow organs and tubes. Smooth muscle controls such actions as peristalsis, by intestinal muscle relaxation, urine continence by the muscle at the bladder neck and for our purposes most importantly, vasodilation and the reduction of blood pressure. It was noted by Furchgott and Zawadski[28] that a precontracted blood vessel with a damaged endothelium was not responsive to the vasodilator acetylcholine. This demonstrated that acetylcholine acted upon the endothelial cells which then

produced a vasodilatory agent. This substance could then act upon the vascular smooth muscle and cause relaxation. The molecule was named the endothelium derived relaxation factor, or EDRF. It is thought that NO is this agent.[29,30] This is still very much a disputed point and there have been suggestions that the EDRF may be a nitrosothiol or related compound.[31]

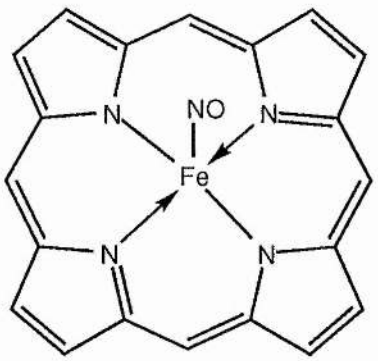
The role that NO is thought to play in smooth muscle relaxation is the activation of the enzyme guanylate cyclase. This causes the conversion of guanosine triphosphate to cyclic guanosine monophosphate (24).

(24)



NO has been shown to interact with the haem component of soluble guanylate cyclase (25). Coordination of the NO ligand to the iron causes a change in spin state of the iron. This forces the iron to move out of the porphyrin ring.

(25)



The process of smooth muscle relaxation is initiated by the arrival of an endothelium dependent vasodilator such as acetylcholine. This triggers the opening of the calcium channels in the endothelial wall. The increase in Ca^{2+} in the cell activates NO synthase. The NO formed passes into the smooth muscle where it acts upon guanylate cyclase. The increase in cGMP results in the activation of a protein kinase. This enyzme facilitates the phosphorylation of proteins eventually leading to muscle relaxation.

1.5. Nitric oxide as a harmful agent

It has been obvious that NO was a very dangerous gas ever since the serious disasters[3] which occurred from anaesthetists using cylinders of laughing gas (N_2O) contaminated with NO.

1.5.1. As a carcinogen[32,33]

Just as NO can kill cancerous cells it would also appear to be quite capable of causing them. Where the dividing line between being a cytotoxic agent and a carcinogen lies is unknown. It thought that the carcinogenic activity lies in the formation of nitrosamines. NO itself will not react with amines, it requires to be oxidised to NO_2. This occurs readily in the presence of oxygen. NO^+ will act as an electrophile and is attacked by secondary amines to give *N*-nitroso compounds. Many of these are known to be potent carcinogens. There is particular concern over the addition of sodium nitrite to meat as a preservative. The nitrite serves two purposes. Firstly it stops the meat from turning brown, by oxygenation of iron in myoglobin, and taking on an unattractive appearance. Much more importantly it prevents the growth of the bacterium which produces the botulinus toxin by interaction with electron transfer ferredoxins.[34] When the meat is cooked the sodium nitrite and amines will react. Cooked bacon is known to contain *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine. Under acidic stomach conditions nitrite and amines may also react. With this knowledge there was a strong campaign to ban nitrite from food. The fears were eased slightly when it was shown that our own intestinal bacteria produce nitrite in quantities which must be just as damaging as

anything we obtain from food. It was also shown that nitrite occurs naturally in many other foods. As a compromise nitrite levels are now kept to a minimum. Cigarettes smoke also contains *N*-nitrosodimethylamine which may contribute to the cancer hazards of both smoking and passive smoking. In a more direct cancer inducing route, NO can cause mutagenesis by nitrosative deamination of DNA base pairs. This has been demonstrated in *Salmonella typhinum*.[35]

1.5.2. NO overproduction

As a toxic agent it is logical to assume that too much NO, even when produced by the body, will be harmful

.

1.5.2.1. Inflammation

Whilst the NO produced by the macrophages is a vital part of the immune response, it can also cause great damage when released where it isn't needed. NO has recently been implicated in arthritis and rheumatism causing symptoms of inflammation as the joints are attacked by their own immune system. Inflammation is closely associated with the activation of iNOS and many anti-inflammatory drugs will inhibit iNOS e.g. dexamethasone and other glucocorticoids.[36]

1.5.2.2. Neurodegeneracy

Probably the most dangerous place for excessive NO production to occur is in the brain. Nitric oxide has been implicated in cerebral damage caused by stroke, Parkinson's disease and AIDS dementia.[37] The overproduction of NO causing cell death in the central nervous system is thought to exist.

1.5.2.3. Septic shock

Following illness or an accident it is possible for a condition known as septic shock to take hold. A progressive reduction in blood pressure occurs which is resistant to vasoconstrictors. It is now believed that excessive NO production is occurring and

contributes to the fall in blood pressure. If unchecked, circulation will cease altogether. The usage of iNOS inhibitors to treat the condition has been demonstrated in a small number of cases.[38]

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36. Biology of Nitric Oxide 4th International Conference Proceeding Abstracts

a) A.Amin, P.Vyas, M.Attur, J.Leszczynka-Piziak, I.Patel, G.Weissmann and S.Abramson.

b) L.Ignarro, E.Aeberhard and S.Henderson.

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Chapter 2: Synthesis of Nitrosothiols.

2.1. Introduction.

The aim of all syntheses discussed in this report was the preparation of *S*-nitrosothiols. These were made for a number of reasons, including simply for the sake of seeing whether such a molecule could exist. However, the most important reason was to prepare compounds which could be used in animal studies. This aim had a strong effect on how such syntheses could be performed and which reagents could be used. In order to prepare compounds on the scale required, the synthetic route had to be relatively inexpensive. It was for this reason that much effort was put into the usage of inexpensive leaving groups such as mesylate and tosylate, when a triflate group could have been used under much simpler conditions and avoided much wasted time. Most of the syntheses encountered here are of a relatively simple nature, the type that could have been performed many years ago had there been reason to do so. This has been dictated by the interdisciplinary nature of the project, where the aim was to make as many useful compounds as possible. Had innovative synthetic routes been used yields might have been enhanced but the number of compounds prepared would have been reduced. Likewise reaction conditions have not been modified to improve yields, as only the production of the final *S*-nitrosothiol in large enough quantities to characterise and test was of importance. The usage of these compounds in human testing has also determined that certain reagents could not be used. Salts of silver, mercury and barium which might have been carried through into the final product and had a dramatic effect on the biological action. Also a product from a reaction involving such reagents would, in all probability, not have gained ethical permission for its usage. These factors have had considerable impact on the synthetic routes chosen. As with many reactions involving a thiol group, a number of the steps described create a very unpleasant odour and are particularly antisocial to perform. Even with great care and attention, especially in the reactions involving a lengthy workup, it is very difficult to contain the odour. It has been found that merely inhaling such odours is sufficient to require a change of clothing and a shower, probably through the thiol finding its way into perspiration. It is for this reason

that a number of reactions may appear to have been terminated without a full investigation of the possibilities having been performed, simply because the end product was not regarded as being sufficiently important to warrant the trouble of synthesising it.

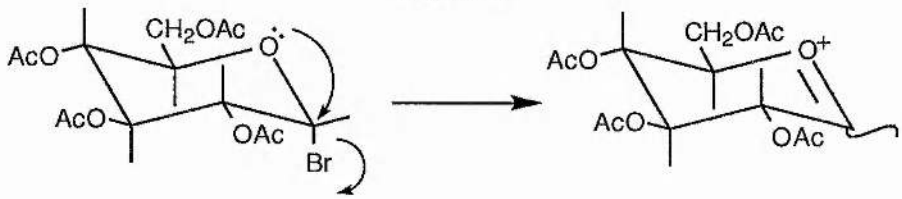
2.2. Discussion

2.3.1. 1-*S*-Nitrosothiosugars

As a starting point for the preparations of new NO-donors based on carbohydrate residues it was decided that simple sugars were an ideal topic of investigation. There are well proven methods for the introduction of a thiol group at the anomeric position of a mono- or disaccharide. These methods can be used with a wide number of derivatives to produce a large range of characteristics. It was hoped that we would be able to change the substituents on the hydroxyl groups of the sugar to affect both the solubility of the molecule and the stability of the -SNO group. Thiosugars have the advantage of small size, allowing their usage in a number of different physiological conditions. Non-acylated thiosugars have been prepared in situ by Matthews and Kerr.[1] In physiological tests these were found to be very potent vasodilators. However, in solution these were found to be very unstable and a product was never isolated.

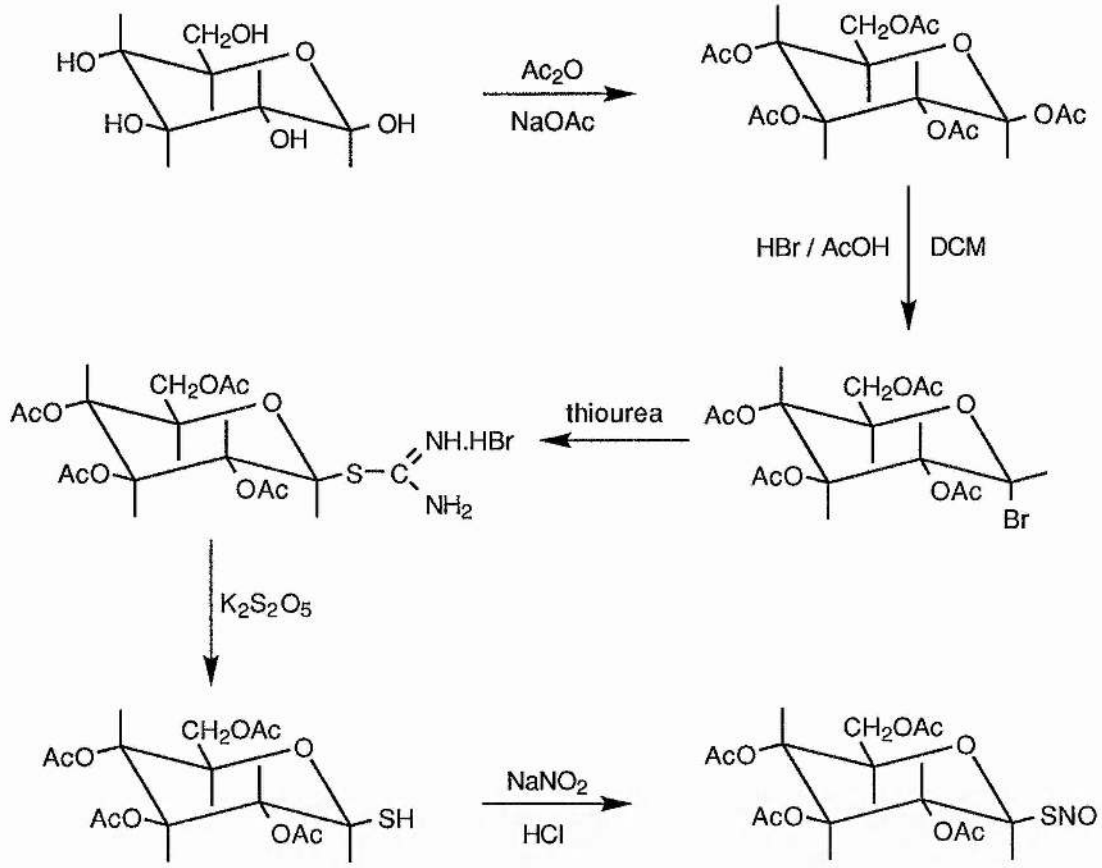
The anomeric position of a sugar is usually the easiest on which to carry out substitution reactions due to the formation of a carbocation as shown in figure 1. This gives the reaction a degree of S_N1 character. It is because of this that relatively mild conditions can be used in this series of reactions.

Figure 1

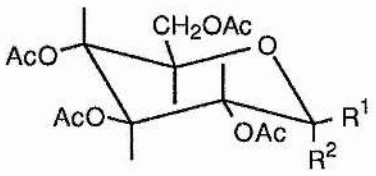


As preparations for a number of peracetylated thiosugars are available in the literature,[2,3,4] these were viewed as being a suitable point to begin our investigations. The route to the preparation of 1-*S*-nitrosothioglucose tetraacetate (SNAG) is shown in figure 2

Figure 2



Acetylations were carried out using acetic anhydride, either with sodium acetate at the temperature of a boiling water bath or with pyridine at room temperature. The base acts as an acid acceptor and moves the equilibrium in favour of acetylated product. In the case of pyridine / acetic anhydride an adduct is formed by the pyridine attacking the anhydride and it is this adduct which acts as the acetylating agent. Acetylation has the benefit of giving the product a low solubility in water, making isolation and purification easier. The reaction mixture is poured into ice water and the peracetylated product precipitated as a white powder in a high yield. The acetylated products of glucose [1], galactose [6], xylose [11], maltose [16] and lactose [22] were made in this manner. The β-form is predominant in the first four sugars listed. In the case of lactose a mixture of α and β product is obtained.



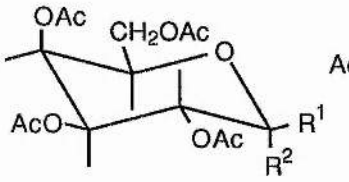
[1] R^1=OAc, R^2=H

[2] R^1=H, R^2=Br

[3] R^1=$SC(NH_2)NH.HBr$ R^2=H

[4] R^1=SH, R^2=H

[5] R^1=SNO, R^2=H



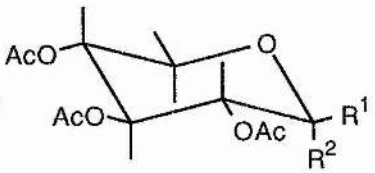
[6] R^1=OAc, R^2=H

[7] R^1=H, R^2=Br

[8] R^1=$SC(NH_2)NH.HBr$ R^2=H

[9] R^1=SH, R^2=H

[10] R^1=SNO, R^2=H



[11] R^1=OAc, R^2=H

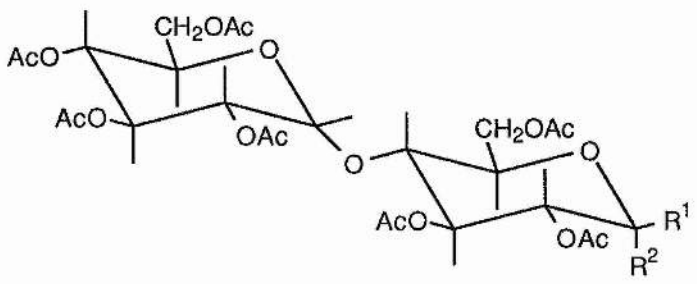
[12] R^1=H, R^2=Br

[13] R^1=$SC(NH_2)NH.HBr$ R^2=H

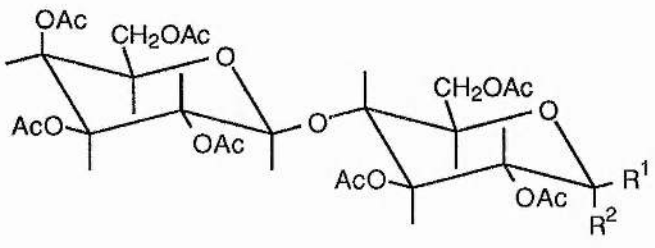
[14] R^1=SH, R^2=H

[15] R^1=SNO, R^2=H

The bromination reaction was carried out using a 30 % solution of HBr in acetic acid. This can be used either as the solvent, in the case of lactose, or in smaller quantities with the peracetylated sugar dissolved in DCM. The workup to obtain the product, evaporation of the solvents which involves utilising toluene as an azeotrope and washing with large quantities of $NaHCO_3$ solution, was done with great care as the bromide is easily lost. It is preferable to convert the product to a more stable compound as quickly as possible. Evaporation of the solvent gives the bromides as fluffy white, or off-white, powders [2], [7], [12], [17], [23]. The instability of the bromide is responsible for the melting points and elemental analyses being inaccurate. The melting point is very much dependent on how rapidly the sample is heated and the elemental analyses dependent on how long the samples have been stored. It was found to be more expensive to buy acetobromoglucose than to make it by this method but as the crystallisation is often difficult, a lot less effort. The α-product is found to be the most common in all but the maverick lactose. This is due to the interaction of the dipoles involved. In the α position the dipoles of the bromide and ring oxygen are in opposite directions making this form more favourable than the usually sterically favoured β form.



[16] R^1=OAc, R^2=H
[17] R^1=H, R^2=Br
[18] R^1=SC(NH_2)NH.HBr
R^2=H
[19] R^1=SH, R^2=H
[20] R^1=SAc, R^2=H
[21] R^1=SNO, R^2=H



[22] R^1=OAc, R^2=H
[23] R^1=H, R^2=Br
[24] R^1=SC(NH_2)NH.HBr
R^2=H
[25] R^1=SH, R^2=H
[26] R^1=SNO, R^2=H

Two convenient methods for thiolation were available. The one used depended upon the stability of the resultant thiol and whether it is acceptable to use heat during the hydrolysis step. Initially for all of the five sugars thiourea was used as the nucleophile. The bromosugar and thiourea are refluxed in dry acetone for fifteen minutes, after which the product should have precipitated. Difficulties were encountered in this reaction. In some cases reflux for an hour was required. Precipitation occurred only after the removal of solvent by evaporation or the addition of cold acetone to the reaction mix. Thiourea has a low solubility in acetone and does not go into solution until reaction has occurred. The product is formed as the isothiouronium hydrobromide salt which also has a low solubility in acetone and precipitates in the reaction mixture. Recrystallisation from acetone requires a large amount of solvent and a lot of patience. For glucose [3], galactose [8] and xylose [13] a white product was precipitated during the reaction and found to be the desired hydrobromide salt. No precipitation was seen in the reactions with the maltose and lactose bromides. In these cases the acetone was removed by evaporation yielding a white oil which slowly solidified. ^{13}C nmr spectroscopy suggested that these were impure samples of the hydrobromide salt in view of the upfield movement of the anomeric carbon. The products were also soluble in water as expected. Attempted recrystallisation from acetone gave a white product found to be unreacted

thiourea or its hydrolysis product urea. It is presumed that it is a higher degree of solubility in acetone rather than formation of a different product, which caused failure to obtain a product. Evaporation of the filtrate gave an oil. As the thiol could be obtained from this, the oil probably contained the thiouronium salt [18], [24].

Hydrolysis was carried out by adding the thiouronium salt to a hot solution of potassium metabisulphite. Addition of DCM to this, followed by stirring at reflux, allowed the thiol to move to the organic layer. Evaporation of the solvent gave a pungent oil which slowly solidified. Thioglucose [5], thioxylose [14] and thiomaltose [19] derivatives were successfully recrystallised from methanol to give large white crystals. Thiogalactose [9] and thiolactose [25] could not be recrystallised and remained as oils which slowly hardened. NMR studies revealed these to be fairly pure. Of these thiols it is noticeable that the most pungent, thioxylose, is also the least stable, whereas the least pungent, thioglucose is the most stable. Thioglucose and thiogalactose are stable in the long term under refrigeration but the other three thiols are prone to dimerisation even under such conditions.

The second method involves the preparation of the anomeric thioacetate from the bromide. Reflux of the bromide of maltose with potassium thioacetate in DCM / ethanol gave a yellow oil which was recrystallised from ethanol to give a white solid [20] . The ^{1}H nmr spectrum of a sample was extremely complex and it is really only necessary to identify the anomeric proton, to gain an idea of the substituent at that position and to determine the orientation of that substituent. However, as it is a good exercise in sugar chemistry, more complete interpretations have been made. The thioacetate was hydrolysed using 1.5 equivalents of benzylamine in THF. Evaporation of the solvent left a yellow oil which solidified on cooling. The solid was stirred with ethanol and filtered and the product obtained by removal of the solvent. The thiol was found to be surprisingly stable and relatively odourless. The ^{13}C nmr spectrum of a two year old sample, presumed to have formed a large quantity of disulphide, was compared to that of

new product and no differences were seen. The ^1H nmr spectrum showed that the -SH peak at δ 2.25 was present.

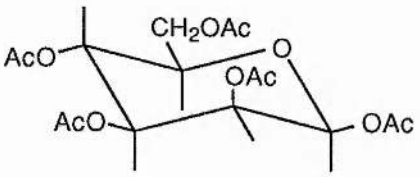
Nitrosation is both the shortest and the most difficult of the processes involved in the synthesis. A thiol can be nitrosated either by N_2O_3 gas or acidified nitrite. However it is isolation of the product which was found to be difficult. The method used for the preparation of the glucose derivative, SNAG [5], involved dissolving the thiol in methanol with a small quantity of water and chilling in ice. A small quantity of EDTA was added. This is to compensate for poor quality distilled water or sodium nitrite which may contain some copper ions. Excess sodium nitrite was added followed by the dropwise addition of conc. HCl to the mixture to give a red solution. The addition of more water to this caused the precipitation of the nitrosothiol, usually as dark red oil. The addition of a small quantity of HCl was then used to facilitate solid formation. A deep pink powder was filtered off and dried under reduced pressure. Despite investigation of a number of different reaction conditions, this method is the only one which yielded a solid precipitate. Unfortunately it is not a particularly reliable method and frequently there is formation of a large amount of disulphide which contaminates the nitrosothiol. The presence of EDTA should reduce the effects of any metal ions in the reagents or solvents used. It has been noted that the use of freshly made thioglucose is necessary for successful nitrosation. Older samples, even when kept in the freezer, usually give large quantities of disulphide upon nitrosation.

When following this method glucose is the only sugar whose nitrosothiol can be precipitated as a solid. The preparation of the nitrosothiol for the other sugars involves extraction into DCM of the red oil produced. Evaporation of this gives a pale pink solid. None of these products are stable however. Somewhat confusingly, when SNAG is prepared by this method it is also found to be unstable. When nitrosothiols are prepared by the other method, i.e. bubbling N_2O_3 through a solution of the thiol in DCM, evaporation also gives an unstable pale pink powder as above. The anomaly is further

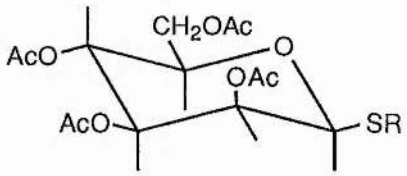
compounded by the fact that the maltose [21] and lactose [26] derivatives are more stable in solution than SNAG, yet much less so in solid form. The only conclusion which can be drawn from this is that the method of isolation would appear to be vital in preparation of the nitrosothiol, with only immediate precipitation being successful. A third method of nitrosation, involving stirring the thiol in DCM with tertiary butyl nitrite, showed no nitrosation.

Totally satisfactory characterisation of all of these products has not been achieved. Only SNAG has been found to be stable enough to allow both ^{1}H and ^{13}C nmr spectra to be run. The ^{1}H spectrum shows the disappearance of the -SH doublet at δ 2.3, whilst the ^{13}C spectrum shows the downfield movement of the anomeric carbon as expected. ^{13}C nmr spectra have been obtained for the galactose [10] and maltose derivatives and these show the same trend. The ^{1}H spectra for the sugars apart from glucose show the disappearance of the -SH doublet but are not very well resolved. This is due to the formation of disulphide. The protons of the disulphide have a very similar chemical shift to those of the nitrosothiol, causing a broadening of the peaks and giving unsatisfactory spectra. It was not possible to obtain a spectrum of the very unstable xylose derivative [15]. Infra red data show the disappearance of the -SH stretch at 2560 cm-1 and in, the case of SNAG, the appearance of an NO stretch at 1550 cm-1. Examination of the uv / visible spectra shows a strong absorbance in the 340 - 350 nm range. This is characteristic of other known nitrosothiols. Other commonly used identification techniques have not been possible. None of the compounds were stable enough to allow elemental analysis. FAB mass spectroscopy or other MS techniques cause sugar fragmentation giving no useful information. Attempts to examine purity using HPLC proved unsuccessful due to the highly non-polar nature of the compounds. The columns at our disposal were not polar enough to allow passage of the nitrosothiol. In view of the data we have and the purity of the precursor thiol we feel confident that the nitrosothiol has indeed been prepared in each case but, apart from SNAG, they are unstable and impossible to obtain in pure form.

The preparation of a mannose derivative by the above reaction scheme was attempted. The pentaacetate [27] was found to be an oil. ^{13}C nmr spectroscopy showed only one major product but ^{1}H spectra were indistinct and no interpretations could be made from them. The bromide [28] was prepared as before, giving a brown oil. The C-1 shift on the ^{13}C nmr was as expected but no more information could be obtained. Reaction of the bromide with thiourea gave a white solid by precipitation. This solid was filtered and recrystallised from acetone. Somewhat surprisingly it was found to contain little or no organic material. It was clearly not [29]. The solvent was removed from the filtrate to give a white gum with a ^{13}C nmr as expected for [29].



[27]



[29] R=$C(NH_2)NH.HBr$

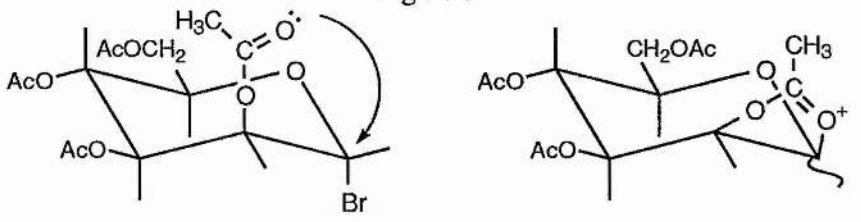
[30] R=H

[31] R=NO

Hydrolysis of this with $K_2S_2O_5$ gave an oil which was assumed to be [30]. The movement of the C-1 in the ^{13}C nmr spectrum was as expected and the ^{1}H spectrum showed a doublet at δ 2.3 with a J value consistent with that of the expected thiol. The product was nitrosated in methanol / water with HCl / $NaNO_2$ giving a red oil [31]. The oil had a strong absorbance at 351 nm and showed no appreciable decomposition. However, ^{13}C nmr showed chemical shifts almost identical to those of the pentaacetate. As an alternative the bromide was reacted with potassium thioacetate to give a yellow oil but the ^{13}C nmr spectrum showed this to be the pentaacetate also. Other studies[5] suggest that mannose is very difficult to deal with and methods of a complexity outwith the time available would have been required to prepare the products in pure form. The major reason for the difficulties with mannose involve the orientation of the acetate at position 2

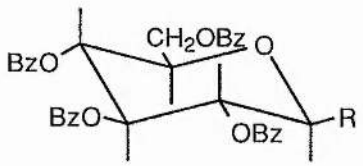
[28]. This is set up perfectly for an elimination of the anomeric bromide as shown in figure 3.

Figure 3



[28]

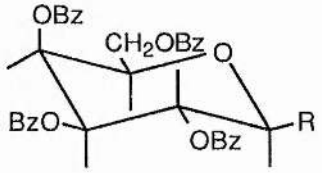
2.3.2. 1-Thiobenzoates. It is possible to displace a benzoate group with a bromide in the same manner as an acetate, thus allowing use of a similar procedure to prepare 1-*S*-nitrosothiosugar perbenzoates. The glucose and galactose products were investigated. Glucose was benzoylated in chilled DCM and pyridine by the slow addition of benzoyl chloride.



[32] R=OBz

[34] R=SAc

[35] R=SH

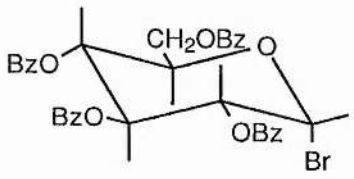


[36] R=OBz

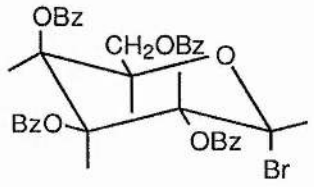
[38] R=SAc

[39] R=SH

Removal of the solvents gave an oil [32] which slowly hardened and could be recrystallised from acetone / water. Bromination, as for the acetates, gave a white powder [32] which was identified from its ^{13}C and ^{1}H nmr spectra but had a poor elemental analysis and a low melting point.



[33]

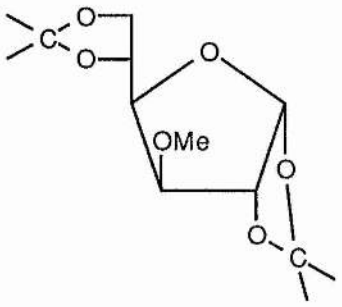


[37]

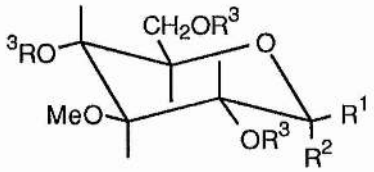
[33] was reacted with potassium thioacetate in DCM / ethanol to give a yellow oil, [34], which solidified on heating in methanol. The 1-thioacetylgalactose tetrabenzoate [38]

was prepared in the same manner. Subsequent reaction gave the pentabenzoate [36] and bromo compounds [37] as gums. The thioacetate was also a white powder. Attempts at hydrolysis of both thioacetates using benzylamine in THF under N_2 were successful in producing the thiols, [35] and [39], but these rapidly dimerised and disulphides were formed. TLC showed the disappearance of the starting material and a strong thiol smell was noted during reaction. Once exposed to the air, TLC showed the formation of another compound which was accompanied by a disappearance of the thiol smell. Reduction of the disulphide with DTT was attempted. Again a thiol smell was noted during reaction but this faded as soon as the mixture was exposed to air. Only disulphide could be isolated despite a very careful workup. The lack of mention of the thioacetates in the literature is perhaps a demonstration of the instability of the thiol and their lack of use as synthetic precursors.

2.3.3. 3-*O*-Methylglucose. To investigate the effect of a partial change of protecting group an attempt was made to introduce a methyl group at position 3, amongst the acetate groups at 2, 4 and 6 and to compare this compound with SNAG. This procedure involved the use of diisopropylidene glucose [40] as described later. The hydroxyl group at position 3 was methylated [41] with dimethyl sulphate. The diisopropylidene groups were removed by overnight reflux in acid to produce 3-*O*-methyl glucose [42] and the product acetylated using acetic anhydride and pyridine to give the tetraacetate [43] as a viscous oil. The oil was found to be a mixture of the α and β products. Bromination was performed as before to give a brown oil [44]. Addition of ether caused precipitation of a white solid. When filtration was attempted the solid become a brown intractable gum, either on contact with air or with moisture. Attempts to purify the gum proved unsuccessful. We felt that this study was a fairly good indication of the unpredictability of sugars and that a change from an acetate to a methyl at a distant site could make an easy reaction nigh on impossible.



[41]

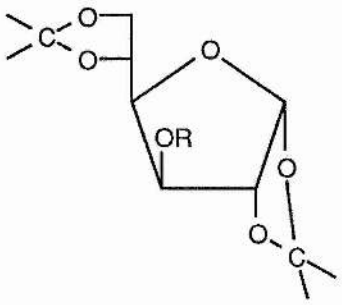


[42] R^1=OH, R^2=H, R^3=H

[43] R^1=OAc, R^2=H, R^3=Ac

[44] R^1=H, R^2=Br, R^3=Ac

2.4. 3-*S*-Nitrosothiosugars. Of the three accessible positions on a sugar ring, the 3 position is the most shielded. The anomeric position has the influence of the ring oxygen whereas the 6 position is at a primary site outwith the ring. The 3 position is readily accessible in both glucose and fructose with the use of diisopropylidene groups to block the other positions and leave the 3- position available for reaction. 1,2:5,6-Diisopropylidene glucofuranose [40] was prepared by stirring glucose in acetone with a $ZnCl_2$ dehydrating agent and a catalytic amount of H_3PO_4. Yields are quite low (~50 %) with much of the glucose remaining unreacted. In view of the low cost of the materials involved, this is not a great problem.. The unreacted glucose was filtered off and the zinc removed as $Zn(OH)_2$ by addition of NaOH. Upon evaporation the product formed as a hard lining inside the flask which had to be chiselled out with care, giving the product in the form of hard sheets. In order to permit further substitution introduction of a number of leaving groups was investigated.

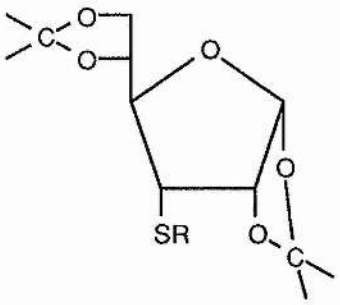


[40] R=H

[45] R=Ms

[46] R=Ts

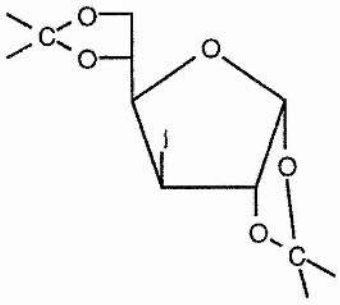
[47] R=Tf



[48] R=Ac

[49] R=H

[50] R=NO



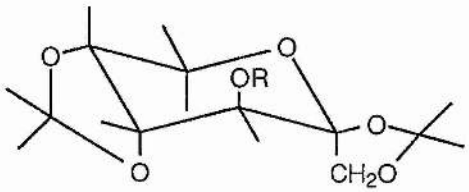
[51]

The mesyl [45] and tosyl [46] derivatives were prepared using mesyl or tosyl chloride in pyridine to give white solids in high yields. These groups proved very difficult to displace. Initially mild conditions were attempted in order to introduce a thiolate nucleophile. Predictably only starting material could be isolated from the reaction between the mesylate and thiourea in acetone or with potassium thioacetate in DCM / ethanol. Attempts to displace the mesyl and tosyl groups with iodine by way of reflux with sodium iodide in DMF also proved unsuccessful giving an impure product. ^{13}C nmr spectroscopy demonstrated that the leaving group remained but that an isopropylidene group had been lost. The method of Nayak and Whistler[6] suggested that the tosyl group could be displaced by heating with potassium thioacetate in DMF, at 115°C for 3 days. Several attempts were made to copy this reaction, including heating to 120°C for 6 days under N_2 with a five-fold excess of thioacetate. At best a 25 % conversion was obtained. Generally only unreacted starting material could be isolated. This method, apart from the inconvenience of the harsh conditions involved, produces a foul smelling dark brown sludge. Workup involved repeated precipitation of unwanted salts in xylene and cannot be recommended for maintaining a pleasant lab atmosphere. A Mitsonobu type reaction using thioacetic acid with triphenylphosphine and diethyl azodicarboxylate was attempted. A grey solid was isolated but found to be neither

starting material nor the desired thioacetate. Other workers have verified that this reaction is not viable on a starting material such as this. The leaving group is simply not labile enough to compensate for the steric crowding around the 3-position. Despite the expense it was concluded that the use of a triflate leaving group was necessary. 1,2:5,6-Diisopropylidene 3-*O*-triflylglucofuranose [47] was produced using triflic anhydride and pyridine well diluted with dry DCM. Despite the fact that this molecule is fairly unstable at room temperature, turning black within a day, the triflate displacement was not found to be easy. Reflux with potassium thioacetate in DCM / ethanol yielded only starting material. Attempts at displacement of the triflate with a more labile iodide [51], using tetrabutylammonium iodide in refluxing toluene, were successful but yields were very low (<15 %). Attempts to displace the triflate using potassium thioacetate in DMF at 110°C gave only a dark brown tar. This was extracted with hot hexane but no product was observed upon evaporation. Successful displacement of the triflate was achieved using tetrabutylammonium thioacetate (from tetrabutylammonium hydroxide and thioacetic acid) in refluxing toluene. Evaporation of this gives a thick dark brown sludge and a more pale brown liquid. This liquid was decanted and large crystals of 3-*S*-acetyl-1,2:5,6-diisopropylidene glucofuranose [48] formed in the liquid. These were repeatedly recrystallised from hexane to remove the thioacetate starting material. The dark brown sludge was also repeatedly extracted with hexane to give an impure product which required many subsequent recrystallisations. Despite all this effort, the yields remained very low (<30 %). Attempts at deacetylation using 1.5 equivalents of benzylamine in THF, under N_2, initially produced a mixture of thiol and disulphide. The disulphide was successfully reduced using excess DTT in DCM. The reacted and unreacted DTT were washed out with water to leave the pure thiol and evaporation gave an oil. The hydrolysis method was later perfected; the procedure was addition of three portions of benzylamine, each of 1.5 equivalents, over a week and progress was monitored by TLC. Flash column chromatography gave the 3-thio product [49] as a pale yellow oil. Attempts to isolate the thiol as its sodium salt using 1 equivalent NaOMe were only partially successful. An orange oil was isolated and this could be nitrosated to give a red

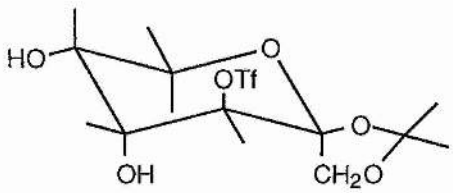
oil. However, the orange oil was not soluble in water and ^{13}C nmr spectroscopy revealed the presence of an extra peak casting doubt upon the identity of the compound. Hydrolysis using a catalytic amount of sodium metal in methanol was also attempted but after stirring for three days, under N_2, only disulphide was detected. 3-Thioglucose was nitrosated in methanol / water with HCl / $NaNO_2$ to give a red solution. Extraction into DCM and evaporation gave a red oil. The oil maintained its colour during long term storage but large amounts of disulphide were detected by ^{13}C nmr spectroscopy. A clean ^{13}C nmr spectrum could be obtained only from a preparation of the nitrosothiol obtained by bubbling N_2O_3 through a solution of the thiol in DCM. This process is difficult to control and even slight over-nitrosation gave a brown solution which rapidly decomposed. *S*-Nitroso-3-thio-1,2:5,6-diisopropylidene glucofuranose [50] can be prepared as a seemingly stable molecule but cannot be isolated in its pure form.

1,2:5,6 diisopropylidenefructose [52] was prepared by stirring fructose in acetone with a catalytic amount of H_2SO_4. As with glucose, the product was isolated as hard white sheets covering the inside of the Buchi flask. Some confusion was caused upon examination of the ^{13}C nmr spectrum as the C-2 is quaternary and therefore gives a much smaller peak than the other sugar carbons and it was seen only with difficulty. The 3-*O*-tosylate [53] was prepared by the standard method using tosyl chloride and pyridine to give a white powder. As with glucose, attempts at the displacement of this tosyl group with a thiolate nucleophile proved unsuccessful and only starting material could be isolated.



[52] R=H

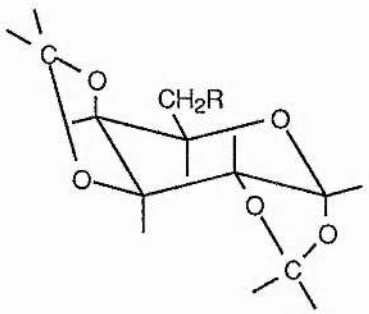
[53] R=Ts



[54]

Following the same recipe as for glucose, the preparation of the 3-*O*-triflyl derivative was attempted. A white powder was isolated with some difficulty as it had a higher than expected solubility in water. This powder showed the same blackening at room temperature as the glucose derivative. ^{13}C nmr spectroscopy showed that only one isopropylidene group remained, explaining the relatively high solubility in water. A possible product is [54]. Study of the reaction was not continued.

2.5. 6-*S*-Nitrosothiosugars. It has been noted that the nitrosoderivative of a tertiary thiol, such as penicillamine, is much more stable than that of a primary thiol such as cysteine. This would suggest that the derivative of a secondary thiosugar, e.g. at positions 1 or 3, would be more stable than that of a primary derivative, at position 6. In order to study this, various routes toward the preparation of 6-*S*-nitrosothiosugars were investigated. The 6-position in most sugars is the second most reactive, after the anomeric position and is therefore readily accessible once the anomeric position is blocked. In a similar manner to the preparation of 3- substituted glucose, a 6- substituted galactose can be prepared using isopropylidene protecting groups to block the 1,2,3 and 4 positions.



[55] R=OH
[56] R=OTs
[57] R=SAc
[58] R=SH
[59] R=SNO

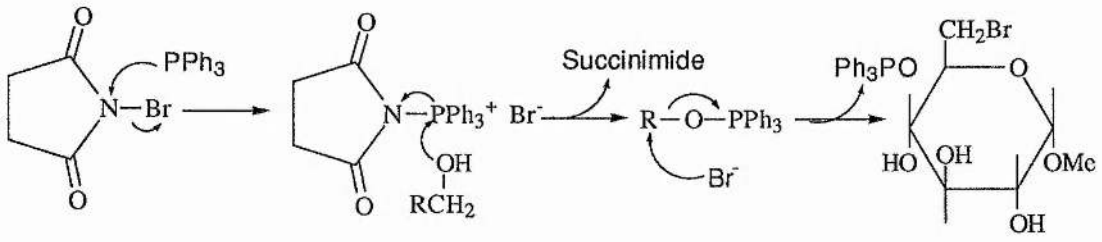
The reaction of galactose with acetone using $ZnCl_2$ and H_2SO_4, gave the 1,2:3,4-*O*-diisopropylidenegalactopyranose [55] as a sticky brown syrup. Tosylation of the oil gives the 6-tosyl [56] product as a white crystalline solid. Attempts at displacement of the tosyl group using tetrabutylammonium thioacetate in refluxing toluene were unsuccessful. However the tosyl group was displaced by potassium thioacetate in DMF.

Distillation of the dark brown gum gave a pungent orange oil shown to be 6-thioacetyl 1,2:3,4-*O*-diisopropylidenegalactopyranose [57]. This method of isolation is particularly effective for this compound . The isopropylidene groups give the molecule a rigidity and stability which allows it to be heated to such a high temperature (up to 175°C) without suffering decomposition. The use of ^{13}C DEPT nmr spectroscopy was applied to identify the chemical shift of the C-6. Conversion to the thioacetate caused it to move upfield by 38 ppm. The thioacetate was hydrolysed to the thiol [58] using a catalytic amount of sodium in methanol. Evaporation and extraction into ether gave the 6-thio product as a pale yellow oil. This very simple method of hydrolysis can be used as the isopropylidene groups, unlike acetates, are resistant to base hydrolysis. The oil was nitrosated to give a red semi-solid [59]. Although this appeared to be stable it was found to be mainly composed of disulphide. This was confirmed by direct oxidation of the thiol to give an identical product. Repeats of the reaction gave increased degrees of dimerisation during the hydrolysis stage. It was found to be impossible to obtain the nitrosothiol in any greater than ~25 % purity.

Tosylation of commercially available 1,2,3,4-*O*-tetraacetylglucose gave the white crystalline 6 tosyl product [60] in good yields. This was stirred in DMF at 120°C with potassium thioacetate to give a thick brown slurry. The solvents were removed by evaporation and the residue stirred with ice cold xylene. The residual salts were filtered off and the solvent evaporated from the filtrate. The process repeated to give a pale brown semi-solid. ^{13}C nmr spectroscopy of this revealed only the presence of thioacetate. No sugar ring carbons or *O*-acetates were observed. It is very unlikely that the desired 6-*S*-acetylglucose tetra-*O*-acetate would have been insoluble in xylene, suggesting that reactions other than its formation have occurred. A previous study[7] involving the preparation and usage of a number of monosaccharide triflates suggested that the 6-triflylgalactoses were stable but that their glucose counterparts were not. The difference is probably that the galactose involved diisopropylidene protection whereas the glucose used acetate protecting groups. It may be that instability which caused loss of the

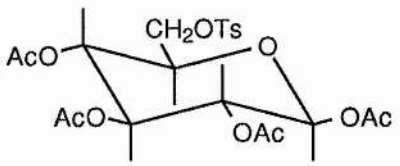
tosyl group was accompanied by acetate rearrangement. This reaction could have been attempted using less harsh conditions, such as potassium thioacetate in ethanol, but in view of the unpleasant smell, the difficulty in selective hydrolysis of the thioacetate and the difficulty of purification without causing *O*-acetate hydrolysis, it was decided not to pursue this reaction further.

2.4.3. A more elaborate procedure for the preparation of a 6-thio substituted glucose starts with methylglucopyranoside and involves the use of a halide leaving group. Bromination at C-6 was accomplished using NBS and Ph_3P in DMF, probably by the mechanism shown.

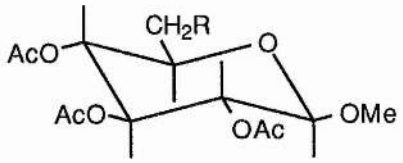


[61]

During evaporation of the solvent it is important not to overheat the solution or the bromide can be easily lost. The product was dissolved in DCM and extracted into water. This is a very easy way of removing the phosphine oxide which can often be a difficult process. This remains in the organic phase. The sugar [61] was then acetylated in acetic anhydride and pyridine and the succinimide washed away with water. Evaporation of the solvents gave a thick brown oil. Recrystallisation from ethanol / water gave a well crystalline white product [62].



[60]



[62] R=Br

[63] R=SAc

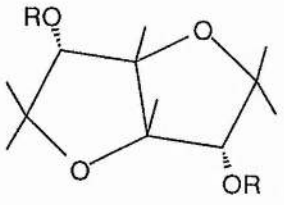
[64] R=SH

[65] R=SC(NH_2)NH.HBr

Substitution of the bromide with thioacetate is an easy process. Potassium thioacetate in DMF (or acetone) is effective as is tetrabutylammonium thioacetate in toluene. The 6 thioacetyl sugar [63] can be isolated either by distillation, to which the sugars are surprisingly resilient, or by precipitation of unwanted salts using xylene. Both methods gave a pure product but in a very low yield (20 - 30 %). Hydrolysis of the thioacetate proved very difficult. Matchell and Roberts[8] reported the preparation of the thioacetate as a pale yellow glass, from which a thiol containing compound was obtained by hydrolysis with NaOMe. For this they reported an IR stretch consistent with a thiol but the elemental analysis was not as expected, suggesting large amounts of disulphide may have been present. The use of benzylamine in THF successfully gave only one product by TLC and no starting material. Flash column chromatography, under the same solvent conditions, gave no product. The disulphide has a much lower rf value so it is thought that dimerisation had occurred on the column. The usage of thiourea as a nucleophile has also been found to be successful. The thiouronium bromide salt [65] is precipitated upon reflux of the bromide with thiourea in acetone. Hydrolysis with $K_2S_2O_5$ gave a pungent smelling white powder [64]. ^1H nmr spectroscopy showed a doublet characteristic of thiol. However, the spectrum revealed the product not to be pure and disulphide was observed. Following two days storage, under refrigeration, the thiol smell had disappeared, suggesting that most of the thiol had oxidised. In view of the difficulties experienced in isolating pure thiol [64] in these reactions it was felt that a C-6 *S*-nitrosothiosugar would not be a stable or viable prospect. Finally, as the C-6 is reactive,

it is possible to mesylate methylglucopyranoside selectively using 1 equivalent of mesyl chloride. This was done, followed by acetylation with acetic anhydride / pyridine to allow isolation of the product. The product was found to contain no mesylate and to be fully acetylated. This suggested that either mesylation was unsuccessful, or that the mesylate was unstable and decomposed allowing acetylation of the C-6.

2.6. 2,5-Dianhydroalditols. The shape of dianhydroalditols make them interesting to study. A substituent may be either exo or endo due to the bent and rigid shape of the molecule. Upon substitution the positioning is reversed. In the case of 1,3,4,6 dianhydromannitol (isomannide) [68] the two hydroxyl groups are endo. An attacking nucleophile approaches from the "outside" of the structure which is relatively easy. In the case of 1,3,4,6-dianhydroglucitol (isosorbide) [75], one hydroxyl group is exo and the other is endo. The endo group is easily displaced but the exo group can only be displaced under forcing conditions as an attacking nucleophile has to approach from "inside" the structure. There is a third derivative, 1,3,4,6-dianhydroiditol [80], in which both of the hydroxyl groups are exo and substitution requires very harsh conditions.

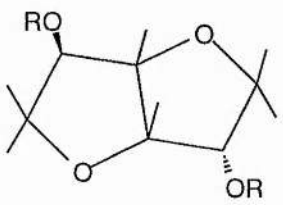


[68] R=H

[69] R=Ms

[70] R=Ts

[71] R=Tf

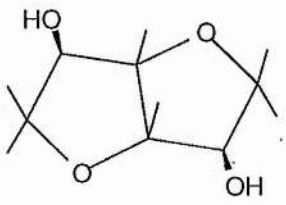


[75] R=H

[76] R=Ms

[77] R=Ts

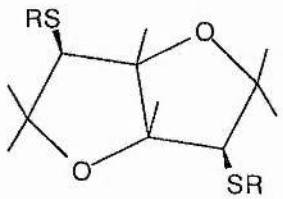
[78] R=Tf



[80]

It had been hoped that the *S*-nitrosothio derivatives for all three could have been made, this would have allowed investigation into the differences in stability between a shielded SNO group and an exposed one. It was also hoped that these molecules would facilitate the preparation of a di-*S*-nitrosothiol and studies into whether this would be twice as

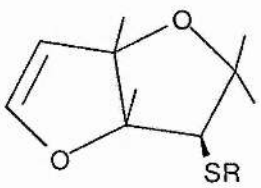
active as a mono derivative. The dimesyl [69], [76] and ditosyl [70], [77] derivatives of isomannide and isosorbide were prepared. These are easy reactions and gave white crystalline products. A variety of thiolation methods were attempted in order to displace the mesyl and tosyl groups viz. thiourea in acetone, potassium thioacetate in DCM / ethanol, thioacetic acid with $ZrCl_4$ in DCM, tetrabutylammonium thioacetate in toluene and phase transfer using tetrabutylammonium hydrogensulphate and finally thioacetic acid and sodium hydroxide in ethyl acetate/water. Unfortunately none of these conditions was strong enough to cause reaction and starting materials were isolated each time. Two harsher sets of reaction conditions involve the use of potassium thioacetate in acetonitrile or in DMF. In both cases a foul smelling brown sludge was produced from which no identifiable product could be obtained by either precipitation or by column chromatography. Distillation of the product from the DMF reaction gave an orange oil. This was found to be the 2,5 dithioacetate [72].



[72] R=Ac

[73] R=H

[74] R=NO

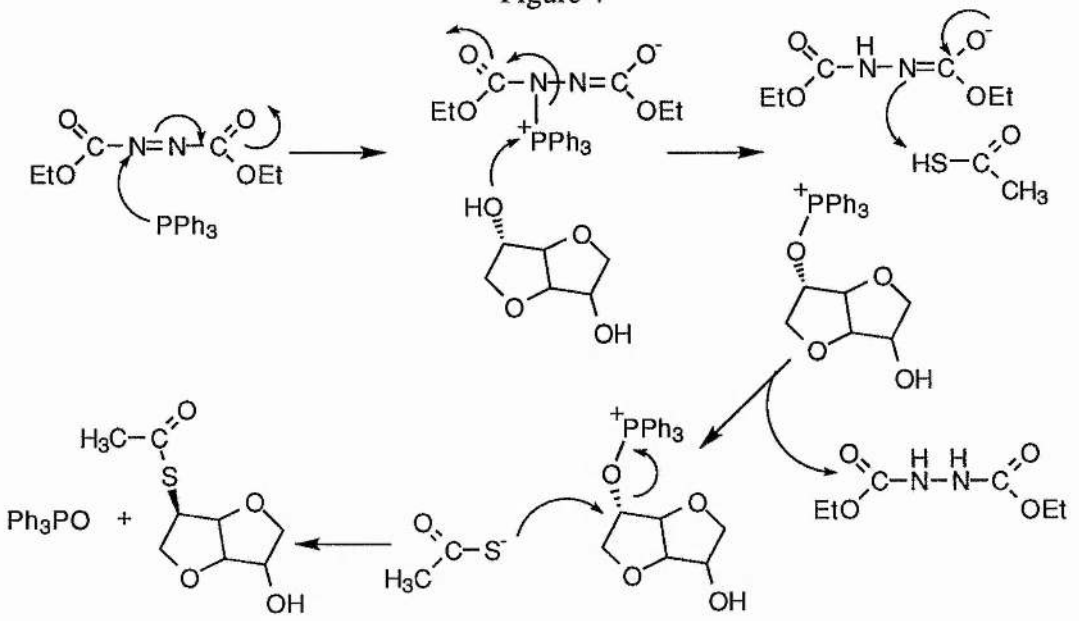


[79] R=Ac

Following the methods of Bladon and Owen,[9] the thioacetate was refluxed in a 2.5% HCl solution in methanol. Evaporation gave a pungent brown oil. The authors claimed that the product is stable enough to allow removal of the HCl by evaporation followed by distillation of the product to give a clear low melting solid (35 - 37°C). Storage of the oil under vacuum with NaOH removed most of the remaining HCl and left a brown semi-solid. This was distilled under vacuum to give a pale orange oil with a very strong odour. The oil solidified under refrigeration. Nmr studies showed it to be a pure sample of [73]. The thiol stretch at 2560 cm^{-1} was seen by i.r. spectroscopy, showing that the

pure thiol has been formed. The thiol would appear to be perfectly stable under acid hydrolysis conditions and remarkably stable towards heat. Considering our apparent inability to prepare the thiol under base hydrolysis condition we had initially supposed this to be a very unstable thiol, prone to oxidisation or polymerisation. These results indicate that the instability is more towards basic conditions. The dithiol was nitrosated to give an impure but seemingly stable red oil. In view of our earlier failures to displace mesyl and tosyl groups, the usage of a triflate leaving group was investigated. Isomannide ditriflate [71] can be prepared from isomannide using triflic anhydride and pyridine in dry DCM. Evaporation gave a yellow oil. Solidification followed by recrystallisation from ethanol produced long needle crystals. The triflate is much more reactive than the mesylate or tosylate and reaction with potassium thioacetate in DCM / ethanol to gave the 2,5-dithioacetylisomannide [72] as an oil. The deacetylation proved difficult. The use of two equivalents of NaOMe lead to the formation of a white solid found to be insoluble in all common laboratory solvents. This was assumed to be the polysulphide polymer. Acetate hydrolysis with benzylamine in THF under N_2 successfully removed all starting material and during the reaction a strong thiol smell was noted. TLC showed the formation of a large number of different products as the reaction continued. Attempts to separate these by flash column chromatography proved ineffective. The thioacetate was also directly produced from isomannide using a Mitsonobu type reaction as shown in figure 4 This produced the desired thioacetate but left a vast amount of Ph_3PO. For 3 g of the product over 20 g of phosphine oxide is formed. Isolation of the pure product from this requires an excessive amount of time and effort involving repeated crystallisations of phosphine oxide from ethyl acetate.

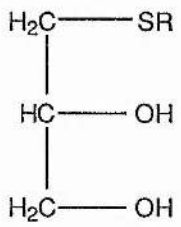
Figure 4



The ditriflate of isosorbide [78] was also produced by the same method as for isomannide and gave a low melting solid. Displacement with potassium thioacetate in DCM / ethanol gave an oil. ^{13}C nmr spectroscopy gave a clean-looking spectrum but showed 13 major peaks for the ring carbons instead of the expected 6. Two thioacetate peaks were seen. A number of spots were shown by TLC. It is assumed that the endo triflate will substitute readily but it is thought possible that, with the exotriflate, elimination may occur more readily than substitution. A possible product from this reaction is [79]. A similar ^{13}C nmr spectrum was obtained for the reaction of the ditosylate with potassium thioacetate in DMF. The hydrolysis of this impure thioacetate was attempted using benzylamine in THF. A strong thiol smell was noted during the reaction but a number of side reactions were indicated by TLC and isolation of the thiol proved impossible. Our results show that an exo thiol group is reactive and prone to dimerisation. If a thiol group could be introduced at an endo position, without causing elimination, then a compound of greater utility for our purposes might be prepared.

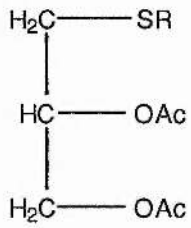
2.7. Thioglycerols. Nitroglycerine is not only a very effective nitric oxide donor but it is also a very effective transdermally delivered molecule. It was hoped that *S*-

nitrosothiol derivatives of glycerol might exhibit the same qualities. Thioglycerol (3-mercapto-1,2-propanediol) [81] is readily available and provides a very useful and inexpensive starting material. The nitrosation of thioglycerol was previously performed by Matthews and Kerr[1] without isolating the product. The nitrosothiol was discarded as too stable and therefore inactive. It has now been shown to be of great interest and other groups are now investigating the route of decomposition. The *S*-nitrosated product (SNOIL) [82] was prepared by nitrosation of with HCl / $NaNO_2$ in methanol / water.



[81] R=H

[82] R=NO



[84] R=Ac

[85] R=H

[86] R=NO

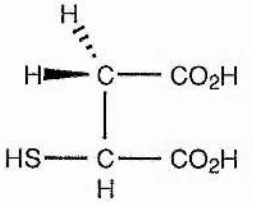
This was then repeatedly extracted with DCM to give a deep red oil in low yields, most of the product remaining in the aqueous phase. A better nitrosation procedure involves dissolving the thiol in DCM and passing N_2O_3 (from HCl dripped onto $NaNO_2$) through the solution. This reaction is difficult to control but it should be possible to follow the loss of thiol using basified nitroprusside as a marker. A purple adduct forms in the presence of thiol. Overnitrosation leads to the formation of a brown solution which decomposes giving none of the desired product. Various reasons for this have been considered including the formation of ClNO, a powerful oxidising agent, from impurities in the solvent. Under-nitrosation obviously leads to unreacted thiol being present. A complex twist to our study of this compound is that in solution SNOIL is a very stable compound. On the other hand, as a pure oil it can be very unstable. When stored in a sealed container in a freezer it can be explosive. Our studies suggest that the isolated pure nitrosothiol is unstable, but material still containing small quantities of unreacted thiol

impurity is indefinitely stable. As far as our transdermal studies were concerned, the water solubility of the non-protected glycerol meant that it was not of use to us. It was hoped that acetylation would allow its use. Three different methods were investigated for the preparation of the diacetyl product. The first involved the initial preparation of the disulphide. Dioxygen was bubbled through a solution of the thiol basified with triethylamine. The disulphide was then acetylated with acetic anhydride / sodium acetate to give the tetraacetate [83] as an oil. This was then stirred in methanol with 1 equivalent of the reducing agent dithiothreitol under N_2 in an effort to regenerate the thiol. Only starting material could be isolated from the reaction. The second method involved direct acetylation of the thioglycerol to give the triacetate [84]. An *S*-acetate is less stable than an *O*-acetate and can be deprotected by selective hydrolysis. This involved stirring the thioacetate in dry THF, DMF or diethyl ether with an amine (1.5 eq). After 3 days stirring under N_2, benzylamine in THF showed 100 % *S*-deacetylation, hydrazine acetate in DMF showed 50 % reaction and diethylamine in ether showed no reaction. Due to the difficulties in removing DMF without causing disulphide formation the hydrazine acetate reaction was not further investigated. The diethylamine reaction would have been an obvious choice had it been successful as both the ether and amine are easily removed by evaporation. The by-product, diethylamine acetate, also has a low boiling point and is easily removed. The benzylamine in THF method was the preferred choice. Flash column chromatography is required to remove the benzylamine acetate and unreacted benzylamine. This produced the pure thiol [85] as a pale brown oil. The thiol was indefinitely stable in a refrigerator. The thioglycerol diacetate was nitrosated by both the N_2O_3 and the HCl / $NaNO_2$ methods. Evaporation gave the nitrosothiol as a deep red oil [86]. The oil required to be kept in the freezer, decomposing overnight under refrigeration. The final method involved the direct acetylation of SNOIL. It was hoped that the high stability of the nitrosothiol might allow acetylation and purification without showing any significant decomposition. The red oil was acetylated in acetic anhydride / pyridine. The product was dissolved in DCM and the unwanted acid and base washed

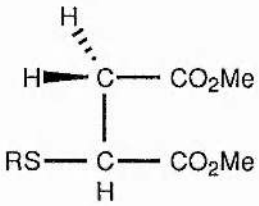
out.. Evaporation produced a thick red oil. NMR revealed that a substantial degree of decomposition had occurred (~30 %). The oil decomposed within 24 h of refrigeration.

Attempts were made to synthesise the dibenzoyl derivative of thioglycerol using benzoyl chloride. This gave a range of products with could not be adequately separated by column chromatography.

2.8. Mercaptosuccinates. Mercaptosuccinic acid is one of the more stable thiols and is found to be quite resistant to disulphide formation. *S*-Nitrosothio derivatives of this were investigated in the hope that the acid group, next to the SNO, might shield the SNO from decomposition. Mercaptosuccinic acid [87] is a crystalline water soluble compound. In order to lower the solubility the methyl and ethyl esters were prepared. Several methods for this were investigated including acetylation followed by methylation. These would have required hydrolysis of the thioacetate produced. These studies were stopped when it was discovered quite how easy it was to make the esters by standard methods. Mercaptosuccinic acid was heated at reflux in the relevant alcohol with H_2SO_4 and a few molecular sieves. The methyl [88] and ethyl [90] esters were successfully prepared as unpleasant smelling oils.

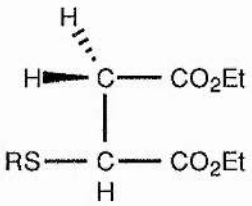


[87]



[88] R=H

[89] R=NO



[90] R=H

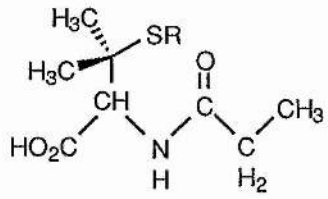
[91] R=NO

No reaction at the thiol centre is seen. The nitrosation of these using either N_2O_3 or HCl / $NaNO_2$ in methanol / water gave a deep red oil. These oils [89], [91] appeared to be almost indefinitely stable, one having been left on a bright fume cupboard window showed very little decomposition. Unusually for a thiol, if anything the odour becomes

more unpleasant upon nitrosation. The smell, quite the worst encountered in these studies, curtailed any further studies on these compounds, whether kinetic or physiological. If not for the smell then these compounds would have been of great physiological interest to us due to their stability.

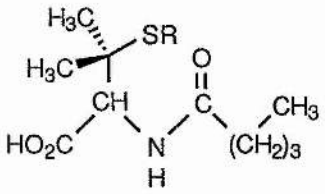
2.9. Penicillamine derivatives.

2.9.1. *N*-Acylated penicillamine derivatives. *S*-Nitroso-*N*-acetyl penicillamine (SNAP) is a well known nitrosothiol. It is very stable as a solid but in solution it usually decomposes fairly rapidly. This has been shown to be due to the formation of a complex with Cu(I), a species which undergoes rapid decomposition. The preparation of similar compounds containing longer acyl chains is of interest for several reasons. It is hoped that the long chain may "shield" the -SNO group and prevent the complexation of copper, giving a much enhanced lifespan to the molecule. It is also a possibility that a long acyl chain will be soluble in the lipid membrane. The rest of the molecule would not be and so the acyl chain would act as an anchor holding the molecule in place and distributing NO in a much less random fashion than a "free" NO donor. This may have the effect of greater activity, with less NO being lost. It may also give a longer lasting effect with the donor being absorbed onto the membrane rather than passing rapidly through. Three different chain lengths have been prepared alongside the two membered acetyl group seen in SNAP: the 3, 5 and 7 membered propionate [99], valerate [103] and heptanoate [107] derivatives. These are prepared by a similar procedure to that of Martin *et al*[10] for the acetylation of cysteine. The thiol precursors to all three could be isolated but the *S*-nitroso products for the propionate and heptanoate were more easily prepared without isolation of the thiol.



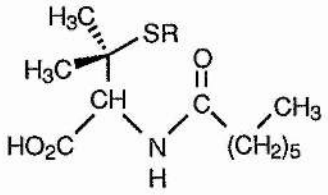
[99] R=H

[100] R=NO



[103] R=H

[104] R=NO



[107] R=H

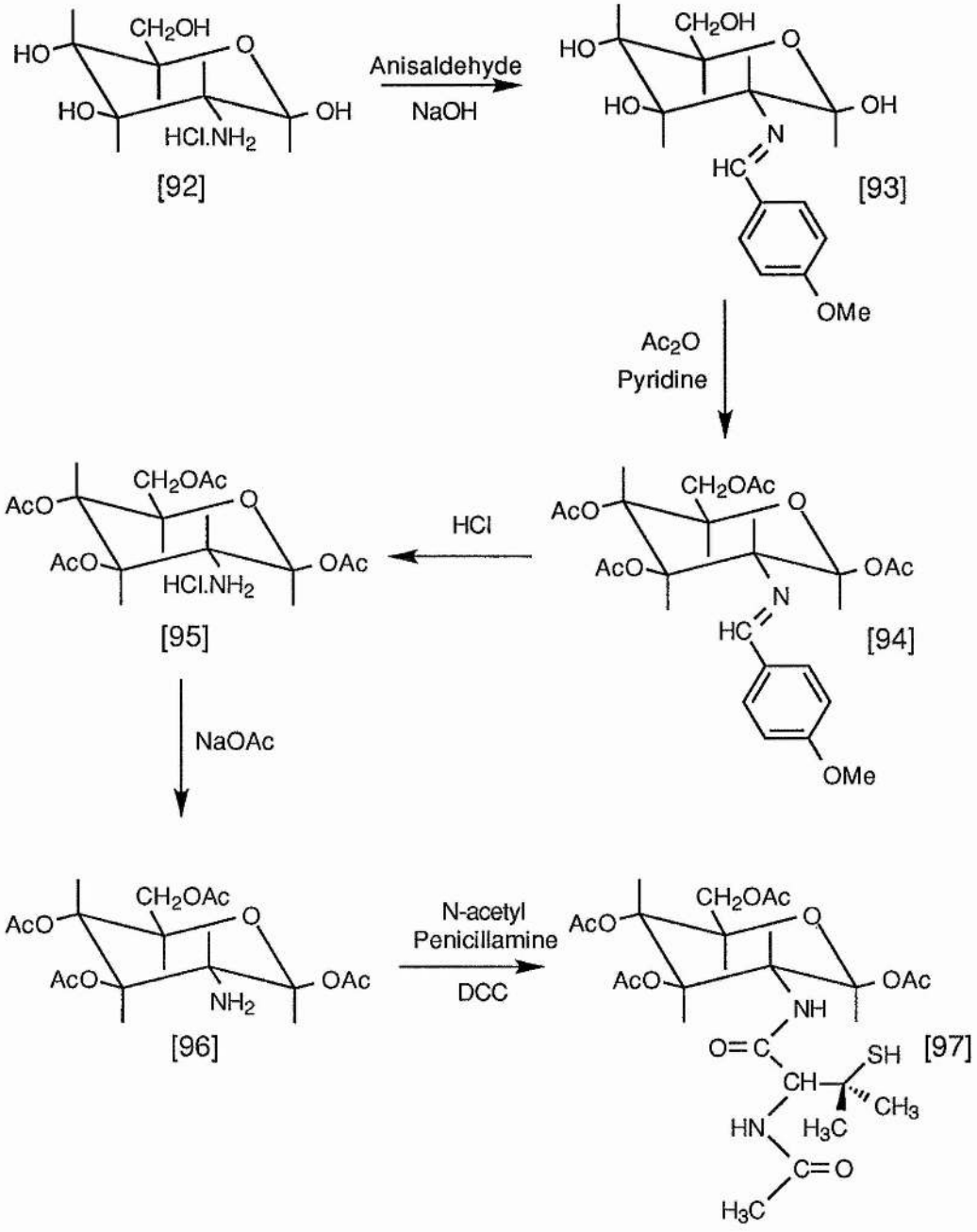
[108] R=NO

N-Propionylpenicillamine was prepared from stirring penicillamine in aqueous THF with 1.2 equivalents of propionic anhydride and 2 equivalents of sodium propionate under N_2. After standing overnight the THF was evaporated to leave a thin oil. The oil was dissolved in water and conc. HCl was added until precipitation began. The mixture was chilled and a large amount of white solid was obtained. The solid could be recrystallised from hot water. However, an easier purification route was to stir it with hexane, where impurities taken out to leave the *N*-propionylpenicillamine in pure form. The *N*-valeryl and *N*-heptanoyl derivatives can be made in the same manner using the appropriate anhydride and sodium salt. The valeryl derivative has a particularly nasty smell. In the direct preparation of *S*-nitroso-*N*-propionylpenicillamine [100] the thin oil from the acylation was dissolved in water and nitrosated with an excess of HCl / $NaNO_2$. A deep red liquid was produced. This was chilled in ice and a green solid appeared. An HPLC study showed only one product present. The ^1H nmr spectrum showed a particularly clear NH - CH coupling. The valeryl derivative [104] was prepared by dissolving the thiol in water and nitrosating as above. The slow addition of excess acid with chilling caused a dark green solid to precipitate. The nitrosothiol was only slightly less pungent than the thiol. The heptanoyl derivative [108] was, like the propionate, prepared via the thin oil from the acylation reaction. The oil was nitrosated in water as above and a red oil was precipitated. The oil was dissolved in DCM, washed and dried. The major impurity was found to be heptanoic acid. The nitrosothiol was very soluble in base, ruling out the use of $NaHCO_3$ as a means to remove the acid impurity. The solvent was evaporated to give a red / green dichroic oil. Refrigeration for several days caused a green solid to form

in the oil. The semi-solid mixture was stirred with hexane and a dark green solid was precipitated. ^{13}C nmr spectroscopy showed the powder to be a mixture of nitrosothiol and unreacted thiol. This was dissolved in DCM and re-nitrosated with N_2O_3. Evaporation and trituration with hexane gave the pure nitrosothiol. Further studies confirmed that this is a relatively slow nitrosation. Whilst all of these products were green and showed the same red/green dichroism as SNAP, their colours were significantly different to that of SNAP, all being of a lighter shade. There also appears to be an increase in the λ_{max} value with increasing chain length. This is also shown in the results from the penicillamine derivatives coupled to glucosamine. All derivatives showed a similar stability to SNAP and a similar response to copper. The chain length appears not to give protection against copper catalysed decomposition and so probably does not prevent copper complexation. The propionyl and heptanoyl derivatives have a very similar physiological activity to that of SNAP, whereas the action of the valerate is startlingly different as described in chapter 3.

2.9.2. Penicillamine-sugar coupling reactions. Results in chapter 4 demonstrate the possibilities for the transdermal delivery of an acetylated sugar moiety and that a compound as such is a much more effective vasodilator than SNAP when delivered transdermally. Since SNAP is a much more stable nitrosothiol (in the solid phase) than SNAG, it was thought that it might be possible to combine the two structures to create a molecule containing the good features of both. Various methods have been looked at for the prevention of copper complexation, and the decomposition this causes, in penicillamine based NO donor compounds (see above). The free acid group plays an important role in this complexation . Were the acid to be linked to a bulky group there was the possibility of inhibiting this copper promoted decomposition. From this came the idea of linking an amino sugar to a penicillamine residue via an amide bond. The most readily available aminosugar is glucosamine (2-amino-2-deoxy glucose) bought as the hydrochloride salt. This otherwise synthetically difficult molecule is extracted from such sources as crab shells. The free base may be prepared by reaction with sodium

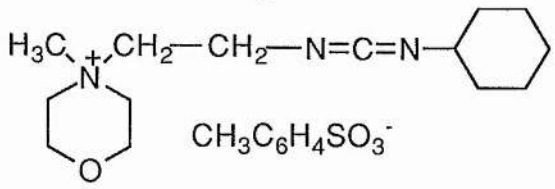
dissolved in methanol, the hydrochloride salt going into solution as the free base whilst sodium chloride is precipitated and can be filtered off. Attempts to use this in coupling reactions with *N*-acetylpenicillamine, either by the mixed anhydride or DCC methods, were thwarted by the lack of a suitable solvent in which to carry out the reaction. This was not of great importance as the coupled product would have had too great a solubility in water to have been of any interest to us. In order to prepare a suitable compound it was necessary to acetylate the product and then selectively remove the *S*-acetate. Whilst this is not overly difficult it did suggest that it would be simpler to introduce the acetate groups onto the sugar prior to coupling with penicillamine. The method of Bergmann and Zervas[11] has been widely used for this, involving the use of an anisaldehyde protecting group for the amine.



The glucosamine hydrochloride [92] was dissolved in sodium hydroxide solution and shaken with anisaldehyde. Initially the anisaldehyde remained as an oily suspension but eventually reacted. Chilling at -20°C gave large quantities of product [93]. It was necessary to dry the product, under vacuum over P_2O_5 at 45°C, for 3 days, before acetylation can be carried out. In the presence of water acetic anhydride will react to form acetic acid. The acid will hydrolyse off the anisaldehyde group allowing unwanted *N*-

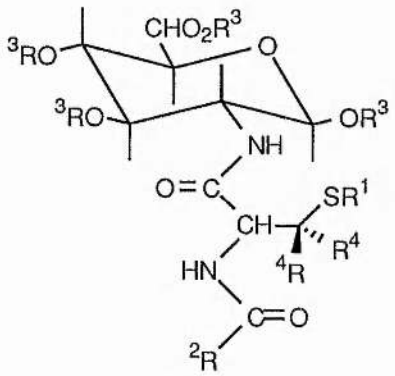
acylation to occur. An *N*-acetate is particularly difficult to remove, being more stable than an *O*-acetate. The dry anisol-glucosamine was acetylated in acetic anhydride / pyridine. When poured into ice water a high yield of acetylated product [94] was obtained. The method of Bergmann and Zervas for the removal of the anisaldehyde group involves dissolving the acetylated product in boiling acetone and the addition of 1 mole of 5 M HCl. This should lead to the precipitation of large crystals. Repeated attempts at this method gave no precipitate. However, the deprotected amine was prepared by a simpler method. The acetate was dissolved in acetone with heating. A small quantity of water was added, followed by dropwise addition of conc. HCl until precipitation of [95] occurred. Chilling caused the precipitation of large amounts of solid until the mixture could no longer be stirred efficiently. Repeated washing and stirring with chilled ether removed the oily yellow anisaldehyde precipitate. The solid material was dried and dissolved in water. The free base [96] was prepared by the addition of 2 equivalents NaOAc. Extraction with DCM gives a white solid in moderate yields. Coupling with *N*-acetylpenicillamine was carried out using the DCC derivative shown in figure 5.

Figure 5



This method has the advantage that the urea by-product is insoluble in DCM and can be filtered off after reaction is complete. Glucosamine acetate, *N*-acetylpenicillamine (1.5 eq) and the DCC agent (1.5 eq) are stirred under N_2 overnight. The reaction follows a particular profile when successful. The reaction mixture is initially cloudy due to the low solubility of *N*-acetylpenicillamine in DCM. After about 15-30 minutes it begins to react, goes into solution and the reaction mixture becomes clear. This lasts for about 5 minutes before the urea begins to precipitate and the solution again becomes cloudy. Washing with acid, base and water removes any remaining starting materials and by-products to

give a white powder. The powder is washed with dry ether to give a very clean product [97]. ^{13}C nmr spectroscopic studies were complicated by the use of D, L-penicillamine as a starting material. This produces two distinctive enantiomers in the product, shown by each peak being doubled or being twice the expected height. The use of a 300 MHz nmr spectrometer and DEPT was successful in assigning each peak. The ^{1}H nmr spectrum proved difficult to interpret. For nitrosation the thiol was dissolved in methanol / water, nitrosated with excess $NaNO_2$ / HCl and extracted with DCM. Evaporation gave a dark green oil which was triturated with dry ether to give a dark green powder [98]. The yield is low (~30 %) but it is interesting that this should be so similar to the yields obtained by Wang[12] for the preparation of the non-acetylated derivatives by a completely different method. Frequently it has been found that the product is not fully nitrosated and still contains large quantities of the thiol precursor. Nitrosation can be completed by passing N_2O_3 through a solution of this in DCM. The nitrosothiol was found to be stable, more resistant towards copper catalysed decomposition and of great physiological interest to us (chapter 3). This compound and its derivatives have been patented for use as a vasodilator, involving a range of R^1 - R^4 substituents.



Where R^3=Ac and R^4=CH_3.

[97] R^1=H, R^2=CH_3

[98] R^1=NO, R^2=CH_3

[101] R^1=H, R^2=CH_2CH_3

[102] R^1=NO, R^2=CH_2CH_3

[105] R^1=H, R^2=$(CH_2)_3CH_3$

[106] R^1=NO, R^2=$(CH_2)_3CH_3$

[109] R^1=H, R^2=$(CH_2)_5CH_3$

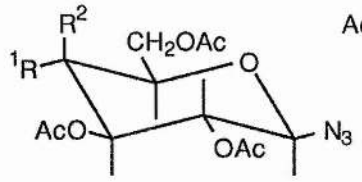
[110] R^1=NO, R^2=$(CH_2)_5CH_3$

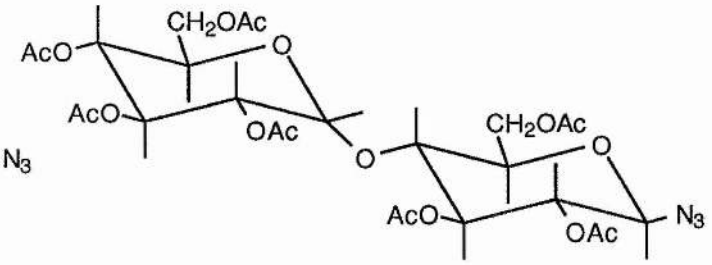
Compounds containing other alkyl groups were synthesised. The same methods as above were used to link *N*-propionyl, *N*-valeryl and *N*-heptanoylpenicillamine to glucosamine [101], [105], [109]. The reaction with the propionoate is similar to that of the acetate. The heptanoate was prepared as a white oil which required trituration and

chilling with dry ether in order to obtain a solid product. The valerate showed the same profile as the heptanoate but appeared to be more soluble in ether. Trituration gave a product but only in very small amounts. These three were nitrosated as above [102], [106], [110], the heptanoate again proving very resistant to nitrosation. The three have similar stabilities to RIG 200 but the longer acyl chain leads to solubility problems which has made physiological testing difficult. The nomenclature of these, RIG 200 etc, was created due to a seminar being presented on the physiological results before the patent had been finalised. The original name "GLUPEN" was felt to give an idea on the structure and, as prior disclosure can invalidate a patent, it was decided to use the original experiment number, IRG 200, as the name. A mistake lead to this being changed to the more pleasing RIG 200. This somewhat conveniently refers to a compound with a 2 carbon chain. The other compounds, RIG 300, 500 and 700 have been named accordingly as have their thiol precursors.

2.9.3. Synthesis of 1-aminosugars for glycopeptide preparation. Having successfully prepared glyco-aminoacids based on 2-aminoglucose we were interested in preparing other similar derivatives. The possibility of other acyl groups on the penicillamine residue has been discussed above. Another possibility was to move the penicillamine-sugar linkage to a different part of the sugar ring. This has been done in the study performed by Wang *et al* on related compounds. It should be noted here that our study was begun 6 months before the publication of the above work and the patent for RIG 200 also submitted before that work. As in Wang's study we felt that the 1 and 6 positions were the most worthy of investigation. Both of these positions are very different from positions 2, 3 and 4. They are also the most easily accessible. In order to couple the sugar to the penicillamine moiety it is necessary to introduce an amine group into the structure. One of the easiest methods is via the azide. The bromides of per-acetylated glucose, galactose and maltose were stirred in DMF at 100°C with excess sodium azide for several hours. The 1-azido products [111], [115], [117] were

precipitated when the mixture was poured into ice water. Recrystallisations from methanol gave attractive pale yellow crystals.



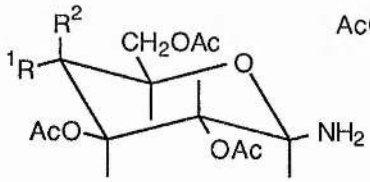


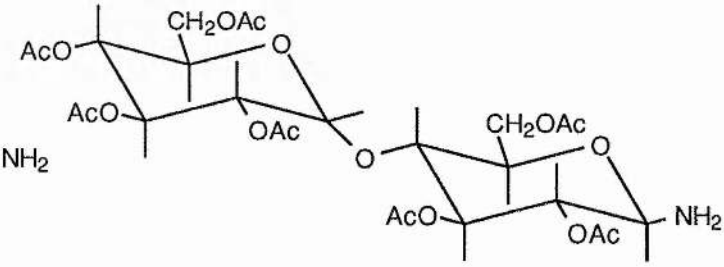
[111] R^1=OAc, R^2=H

[115] R^1=H, R^2=OAc

[117]

Hydrogenation of the azide was attempted using Raney Nickel in ethyl acetate but only starting material was isolated. The three 1-azido products were successfully hydrogenated using 10 % Pd on carbon in a large volume of methanol, under an atmosphere of hydrogen. In the cases of glucose [112] and galactose [116], filtration and evaporation gave a clear oil which slowly solidified. Recrystallisations from ethanol gave the 1-amino sugars as white solids. In the hydrogenation of 1-azido maltose the amino sugar was precipitated during the reaction. DCM was added and the mixture heated until solution occurred. The catalyst was removed by filtration through hot celite. As the solution cooled the amine was re-precipitated as a white powder [118].



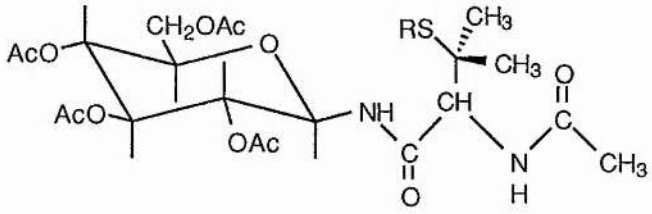


[112] R^1=OAc, R^2=H

[116] R^1=H, R^2=OAc

[118]

The coupling reactions were carried out in the same manner as for 2-aminoglucose. The reactions usually followed the same pattern as before, whereby the penicillamine goes into solution and the urea by-product is precipitated. Both these factors, along with the strong thiol smell, suggested a successful reaction [113]. In the cases of glucose and galactose a thick oil was produced. Unlike the 2-aminosugar coupling, this was found to be soluble in ether. ^{13}C nmr spectroscopy revealed most of the desired peaks to be present along with a number of major peaks which could not be assigned. No suitable solvents could be found for a successful trituration.

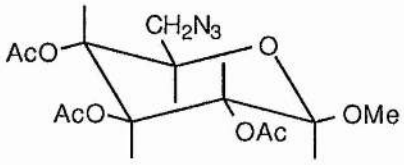


[113] R=H

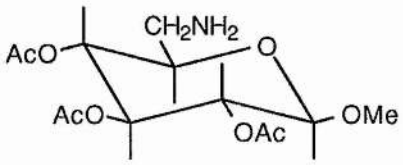
[114] R=NO

The oil was nitrosated in methanol / water with HCl / $NaNO_2$ and a green / brown oil produced. Again the oil was soluble in ether and trituration was not possible. ^{13}C nmr spectroscopy of the nitrosothiol [114] revealed it to be impure and gave few indications as to the structure of the molecule. In the case of maltose the reaction followed the same pattern but a white solid was isolated in large quantities (>70 % yield). Despite the strong thiol smell, this was found to be starting material. It is not clear why products with analyses resembling those expected, with the expected odour, from reactions following very distinctive profiles should prove to be non-isolable. The products from the 2-aminoglucose reactions are insoluble in ether, which provides a very convenient method for the removal of impurities. Why a similar compound based on 1-aminoglucose should have a completely different solubility is not readily apparent. It is possible that the desired product has not been prepared due to a molecular rearrangement of an undefined nature.

2.9.4. Synthesis of 6-aminosugars for glycopeptide preparation. 6-Aminogalactose was prepared from 6-bromomethyl glucopyranoside triacetate via the azide by the same route as the 1-amino sugars. The azido product [66] was a crystalline white solid and the amino product [67] a clear oil which eventually hardened. The coupling followed the same pattern as those above, giving a pungent oil which could not be purified. Similarly nitrosation gave a green oil which could not be characterised



[66]



[67]

2.10. Summary.

We have made a variety of interesting products and investigated a number of different routes toward the synthesis of such compounds. The compounds prepared have fallen into a number of different categories:

a) Stable compounds which may be of use for physiological studies and which may have potential therapeutic usage. These include SNAG, SNOPP, SNVP, SNOPHE, RIG 200, RIG 300, RIG 500 and RIG 700.

b) Stable compounds which cannot be used due to their unpleasant odour. These are the mercaptosuccinates.

c) Semi-stable compounds of which some usage may be made but their full potential is difficult to assess without further study concerning their mode of decomposition. In particular these are the thioglycerol derivatives.

d) Semi-stable compounds which cannot be prepared to the degree of purity necessary for physiological usage, such as the 3-*S*-nitrosothiol derivatives of glucose, the 6-*S*-nitrosothiol derivatives of galactose and the 2,5-*S*-nitrosodithioisomannide.

e) Unstable compounds of no possible physiological usage due to this instability, such as the *S*-nitrosothiol derivatives of galactose, xylose, maltose and lactose.

f) Compounds for which the S-nitrosothiol derivative could not be made either because of thiol instability or because pure thiol could not be obtained. These include the derivatives of benzoylated glucose and galactose, the derivatives of isosorbide and isomannide, the coupled products of the 1-penicillaminyl aminosugars and the 6-penicillaminyl aminosugars.

g) Synthesis which failed after a few steps. These include reactions involving fructoses and 3-*O*-methyl glucose.

Many different nitrosothiols have been prepared but it is convenient that the most interesting products developed all came from relatively easy, user-friendly reactions. These gave products in a solid form which were easy to handle. The products which could be obtained from the more difficult and less user friendly reactions appeared to be

of little interest as either the reagents were too expensive, the yields were too low, the products too unstable or simply the products were unsuitable for testing.

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Chapter 3: Vasodilatory Properties of Nitric Oxide Donors.

3.1. Introduction.

Amongst the many roles nitric oxide is known to play, or thought to play, the maintenance of vascular tone is possibly the most important. As discussed in chapter 1, nitric oxide is responsible for relaxation of the smooth muscle which surrounds blood vessels. By this method blood pressure can be controlled. Nitric oxide is produced by the thin layer of cells lining the vessels, the endothelium. Endothelial dysfunction, resulting in a reduced level of nitric oxide synthesis, is thought to play a major part in a number of disorders such as atherosclerosis. Nitric oxide acts as an inhibitor of platelet aggregation and of muscle cell proliferation. When it is not present in sufficient quantities the walls of the blood vessel are susceptible to a build up of platelets which form clots and allow the deposition of fatty substances which narrow the blood vessel. Similarly, an increase in muscle cells in the vessel also acts to narrow the artery. The reduction in vessel diameter restricts the amount of blood which can pass through and causes an increase in blood pressure. This places additional strain on the heart and increases the risks of a clot forming and blocking the artery. This may then cause a heart attack. A common treatment for this condition is percutaneous transluminal angioplasty. This involves the insertion of an inflatable tube into the artery via the blood vessels at the top of the leg. The tube is then inflated to fill the artery and forcibly removed. This act of removal scrapes away the deposits lining the artery, but also destroys the very delicate endothelium. The lack of endothelial production of nitric oxide to inhibit platelet aggregation, allows further deposits to gather in the artery and frequently leads to a reoccurrence of the problem.[1] Following angioplasty it is common for the endothelium to take as much as two weeks to fully recover. During this period a hardening and scarring of the vessel, known as restenosis, may occur. Without the continual changes in dimension encountered by a normal blood vessel, in response to nitric oxide, the vessel may lose its ability to dilate. Consequently it would be desirable to supply nitric oxide to the afflicted areas during this healing period to prevent damage to the arteries. GSNO has been used for this purpose, inhibiting platelet aggregation as well as acting as a

vasodilator.[1] This usage, whilst patented, is not terribly effective as the GSNO is not very stable and gives only very short term relief. The other drawback encountered is the difficulty in containing the response to only involve the desired blood vessels. If a non-specific NO donor, such as GSNO, is used then it will cause a general increase in blood flow throughout the body. As there is no overall increase in blood volume, there is no benefit to the damaged areas. In practice a systemic response may be positively harmful due to vascular steal. When a damaged area is in competition with a healthy area it will receive a smaller blood flow, the blood taking the route of least resistance. When a non-specific vasodilator is given, the healthy area will be able to respond more efficiently, dilating more rapidly and to a greater degree than the damaged one. Due to this the healthy area will steal an even greater proportion of the available blood and, instead of helping, the situation will be exacerbated. This highlights the necessity of developing a specific NO donor. GSNO is a stronger inhibitor of platelet aggregation than SNAP, however it is a poorer vasodilator,[2] showing a useful degree of specificity. If this could be exploited and a more stable derivative prepared, then it could be of therapeutic benefit. Another useful avenue of investigation is the preparation of NO donors which exhibit tissue specificity, showing a greater degree of activity towards damaged tissue. It is these compounds we have concentrated upon. The addition of a long aliphatic chain to a stable NO donor was thought to have the possibility of being absorbed into the tissue lining the blood vessel. A possibility for this involves the preparation of bipolar molecules with a strongly hydrophobic portion and a strongly hydrophilic portion. It was envisaged that the hydrophobic area would be taken into the tissue whilst the hydrophilic part would remain on the surface in the lumen. The NO donor part could be on either the hydrophobic or hydrophilic portion of the molecule giving two completely different types of donors. Nitric oxide, delivered intra-arterially from a compound remaining in the blood flow, can only have a transient effect upon the blood vessels as it will move through them at such great speed. Were the molecule to be held in place, delivering the nitric oxide to one particular area, then it could have a much higher potency. This effect would be even greater were the NO to be released inside the tissue

rather than in the blood and so another line of investigation is to create a molecule with the correct solubility as to pass into the surrounding tissue in its entirety. In a similar manner to RBS (chapter 1) this could act as a long term local supply of nitric oxide.

Another important vascular use for nitric oxide donors is the treatment of angina pectoris, the intense pain caused by the spasmic contraction of the cardial muscles. The organic nitrates nitroglycerine, isosorbide mononitrate and isosorbide dinitrate as well as amyl nitrite have been used for this for many years.[3] Their usage is limited by the development of tolerance (chapter 4). This is probably due to depletion of the body supply of thiols, which are thought to be used in the metabolic processes involved in the production of nitric oxide from organic nitrates. A nitrosothiol, not requiring metabolic processing, is a viable alternative. This could provide a continuous supply of nitric oxide without the need for rest periods as for organic nitrates. It may also be possible to alternate between the two types of donor to provide full and effective treatment for more serious sufferers. In order to do this a nitrosothiol with a greater degree of stability than those previously known would be required. It is probable that this would involve resistance to copper ion catalysed decomposition whilst maintaining the thermal stability of SNAP. A final usage for these donors is in the field of heart by-pass surgery. This involves an artery being transferred from the leg to act as a new vessel in the area of the heart. A problem frequently encountered in this area is that the vessel may suffer an irreversible contraction during this transferral period. A donor which could be absorbed into the vessel and keep it dilated during the process, would obviously be of great benefit in these operations.

With very able support from a number of physiologists we have been able to investigate the possibilities for fulfilling these requirements from the nitric oxide donors that have been prepared. These have been tested upon isolated rat arteries. Early studies were performed on the tail artery. This is a relatively large and easily accessible artery. The drawback of this, as a large artery, is that it has little relevance to the control of blood

pressure. It is changes in tone of the smallest blood vessels, the resistance vessels, which are responsible for major alterations in blood pressure. Therefore it is preferable to use the smallest possible vessels which can be handled. Perhaps a more valid complaint is that humans don't have tails and therefore no valid comparisons can be made. Later studies were performed using the smaller rat femoral arteries. Further work may involve use of the even smaller mesenteric arteries. Several delivery methods for the donor to the artery were investigated. The donor may be delivered as a bolus injection, whereby increasing doses are passed through the artery where they remain for only a very brief period of time. The artery is allowed time to recover between doses. From this a dose response curve for each compound can be constructed. The donor may also be delivered by superfusion, where the donor is added to the Krebs buffer and the artery is continually bathed in it. Increasing doses are then added to the perfusate without the artery recovering to its original level of contraction. The artery can then be washed out with fresh buffer to determine the time taken for a full recovery to be made. It is also of importance to add a nitric oxide scavenger, such as haemoglobin or an L-arginine analogue to the perfusate to determine that the dilation is NO mediated. The co-superfusion of the donor with a scavenger is a valuable method of separating a sustained response from a transient one. With this method the transient response may be seen but a sustained one is suppressed by the scavenger.

The most important aspect of these studies was found to be the difference in vasodilatory activity between arteries with an intact endothelium and those with the endothelium removed. In order to validate the relevance of these studies limited testing upon humans has been done. Ethical permission for the use of the more promising compounds has been obtained and so full *in vivo* studies on humans will be performed in time. A lack of time, and other commitments for those involved, has determined that only a select number of our compounds have been tested. It is felt that these are probably the most likely to show results of interest to us.

From these studies we have shown our compounds to be active as NO mediated vasodilators. We have gained an idea of their relative potencies and the length of time the response may be sustained.

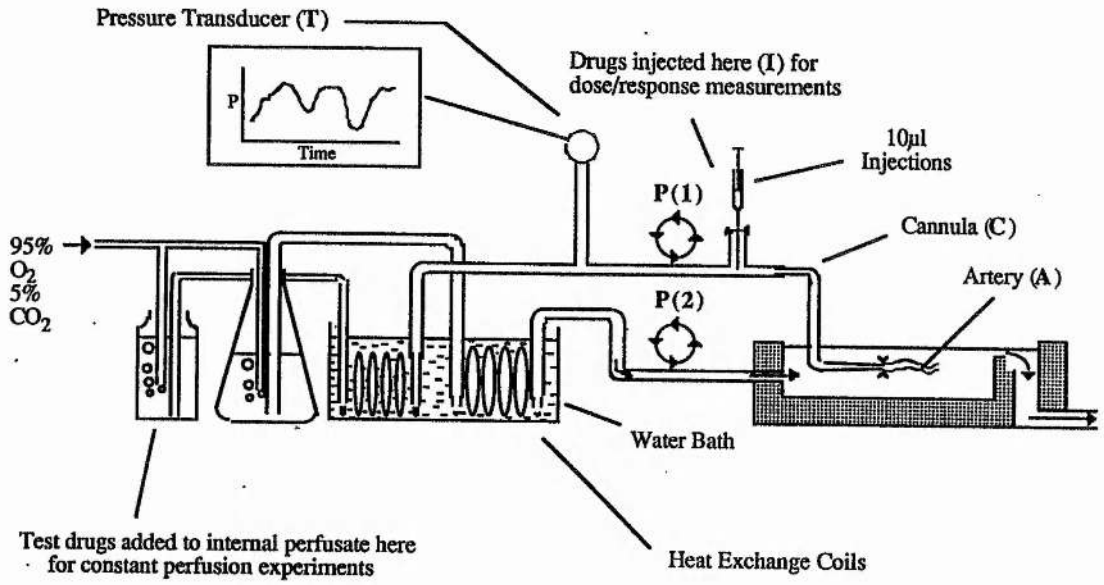
3.2. Isolated rat tail artery studies.

In order to carry out any kind of a study on a nitric oxide donor, it is necessary to first ascertain that it does indeed act as a vasodilator. Our initial studies were carried out using SNAG as the vasodilator and the animal model studies solely to support the results obtained from human testing. Later studies, performed by a more experienced physiologist using blood vessels more closely related to those found in humans represented full studies in their own right.

Experimental set-up

A set-up depicted below (figure 1) was used. In this case it was the tail artery rather than the femoral that was used in this experiment. The perfusion system used is similar to that previously described.[4]

Figure 1



Segments of tail artery (1 - 2 cm long) were dissected from normotensive male wistar rats (300 - 400 g) following cervical dislocation. These were cannulated and perfused internally with pre-warmed Krebs solution pH 7.3 - 7.4 (composition in mmols dm^{-3}: NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; NaH_2PO_4, 115; $CaCl_2$, 2.5; $MgCl_2$, 1.1; glucose, 5.6 and purged with 95 % O_2 / 5 % CO_2) at a constant flow rate of 2 ml / min. A Gilson Minipuls peristaltic pump was used to draw the internal perfusate (Krebs) from a reservoir. The temperature was maintained at 32 ± 2°C by superfusing the preparations with pre-warmed Krebs solution, drawn from a second reservoir (flow rate 8 ml / min) by means of a peristaltic pump.

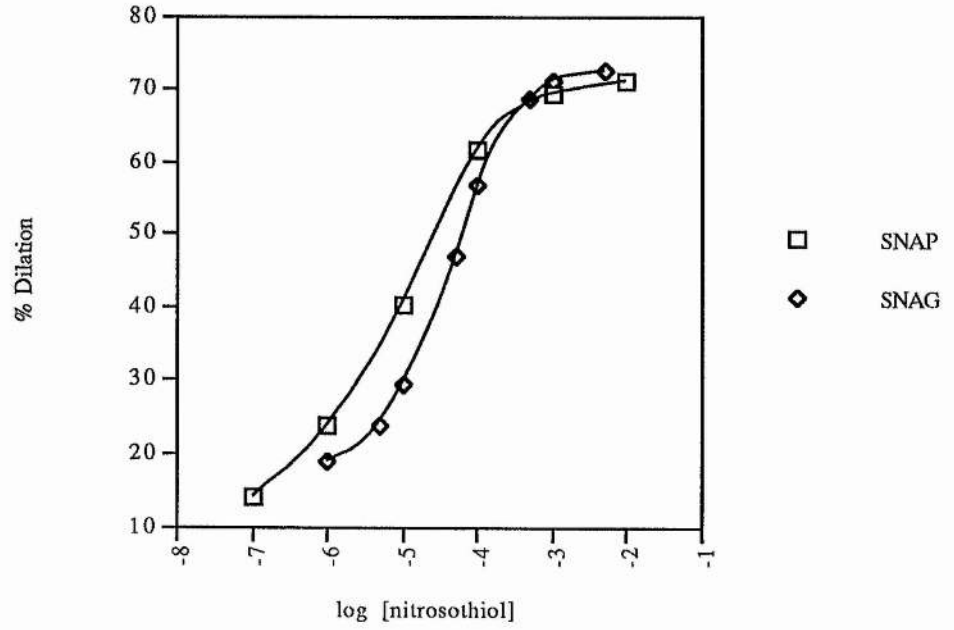
The perfusion pressure was monitored using a differential pressure transducer (sensym type SCX 150 NC, Farnell Electronic components, Leeds) located upstream from the vessel. The electrical output from the vessel was fed to a MacLab type 4e A/D converter and printed out. The printer traces were analysed by hand as described later. A short

side-tube, which terminated in a resealable rubber spectrum, allowed bolus injections of SNAP and SNAG to be delivered into the internal perfusate. The transit time from the injection site to the lumen was three to four seconds. Each bolus injection remaining inside the artery for approximately 0.3 seconds.

Experimental protocol

The experiments were performed in a darkened laboratory using low intensity lighting (red 15 W lamps) as the nitrosothiols are light sensitive. Phenylephrine (1 - 7 μm) was added to the Krebs internal perfusate to precontract the artery, generating perfusion pressures of 100 - 120 mmHg. Phenylephrine is an analogue of noradrenalin and stimulates Ca^{2+} influx allowing contraction to occur. Stock solutions of SNAP and SNAG (1×10^{-2}M) were made up in 50 % aqueous ethanol and diluted serially to give a range (10^{-7} - 10^{-3}M) of concentrations in aqueous ethanol. The ethanol was necessary due to the low solubility of SNAG. Injections using only the solvent were given and found to have little effect upon the level of arterial tone. Bolus injections (10μl) of the nitrosothiols were then delivered. These produced transient responses which were allowed to recover fully between successive injections. The vasodilator responses were calculated by expressing the drop in internal pressure as a percentage of the pressure immediately prior to injection (active pressure). The passive pressure (pressure before the vessel was precontracted with phenylephrine) is used as the baseline rather than zero pressure. Representative traces are shown in figure 3

Figure 2



Both nitrosothiols were tested upon the same piece of artery to allow direct comparison. The combined dose response results (where n = 12 for each) are shown in figure 2. The two response curves are very similar and demonstrate that the two drugs are fairly closely matched in vasodilatory activity. The addition of haemoglobin to the perfusate completely inhibits the vasodilatory action of SNAG on the arteries at all concentrations below 5 x 10^{-4}M and greatly reduced the activity at the highest concentrations. This indicates that the vasodilatory action is due to the release of NO.[5] These results are conclusive in showing that SNAG is an effective vasodilator.

3.3. Rat femoral artery studies.

Selection of compounds.

Problems with solubility determined that only a small selection of the compounds prepared were suitable for testing. The femoral artery, when used with this system, was found to be sensitive to both DMSO and ethanol. Both of these caused substantial dilation of the artery and so, with the time available, we were only able to use such compounds as could be dissolved in an aqueous system at concentrations of 10^{-3} and

below. In this series of experiments we used the *N*-acyl penicillamines, SNOPP, SNVP and SNOPHE, the derivatives bearing 3, 5 and 7 membered chains respectively. We were also able to use RIG 200, which was at its solubility limits at ~2.5 x 10^{-3}M. Sonication was necessary to dissolve these compounds. Absorbance studies verified that this did not cause any loss of the NO group. In each of these studies the artery response to the *S*-nitrosothiol was compared to that of SNAP.

Methods.

A protocol similar to that of 3.2 was used. Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (400 - 550 g; n=36). The perfusion system was as described for rat tail artery perfusion. The animals were killed by cervical dislocation and the femoral arteries exposed and canulated immediately distal to the epigastric arterial branch. Cannulated arterial segments (7 - 8 mm long) were dissected free and transferred to a perspex organ bath chamber (1 ml volume) at 37°C. The rest of the procedure was as 3.2, except that the signal from the converter was fed to a MacIntosh LCIII computer as to make data storage and analysis easier.

Endothelial denudation.

The endothelium is an essential part of the arterial machinery for the production of NO. It is of interest to note any differences in response to NO donors in the presence and absence of the endothelium. The endothelium is easily damaged during exposure and cannulation of the artery but can be surprisingly difficult to remove intentionally. In human studies it is enough to pass water through the blood vessel, whereby the osmotic pressure kills the endothelium. In animal model studies it is easier to pass air through the artery which also kills the endothelium. In order to determine the endothelial viability, the pressure after denudation is compared to that of the healthy artery in the presence of an NO scavenger such as haemoglobin. In both cases there should be no nitric oxide and so the pressure should be identical.

The apparatus allows the use of three different modes of *S*-nitrosothiol delivery to the vessel:

a) Bolus injection (10 μl) through a resealable rubber septum into the perfusate immediately upstream of the vessel. The artery is exposed to the drug for ~300ms.

b) Addition of the drug to the perfusate, allowing the inside of the artery to be continuously exposed to the drug.

c) Addition of the drug to the superfusate, continuously exposing the outside of the artery to the drug.

Bolus injection studies.

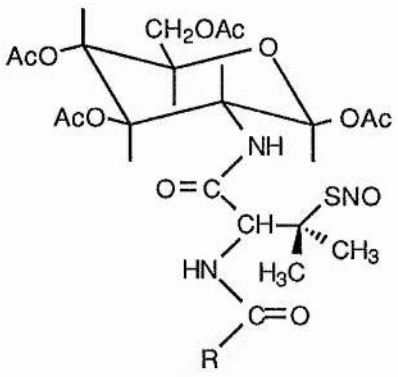
Bolus injections of *S*-nitrosothiol (10μl; 10^{-8} - 10^{-3}M) were made sequentially into the perfusate of the precontracted arteries. The was performed on endothelium intact and endothelium denuded arteries. The responses were deemed to have recovered once pressure was maintained for more than $2^1/_2$ min. Following the highest dose (10^{-3}M) the arteries were allowed to recover for periods of between 15 min and 5 h before being perfused with haemoglobin (10 μM).

Results.

Experiments using endothelium intact artery showed the nitrosothiol to have only a transient effect. Injections of all of the nitrosothiols showed dose dependent vasodilation which rapidly recovered to their original pre-injection pressures, as expected. The results for endothelium denuded vessels varied substantially between compounds and will be dealt with separately, in comparison with SNAP.

3.3.1. RIG 200

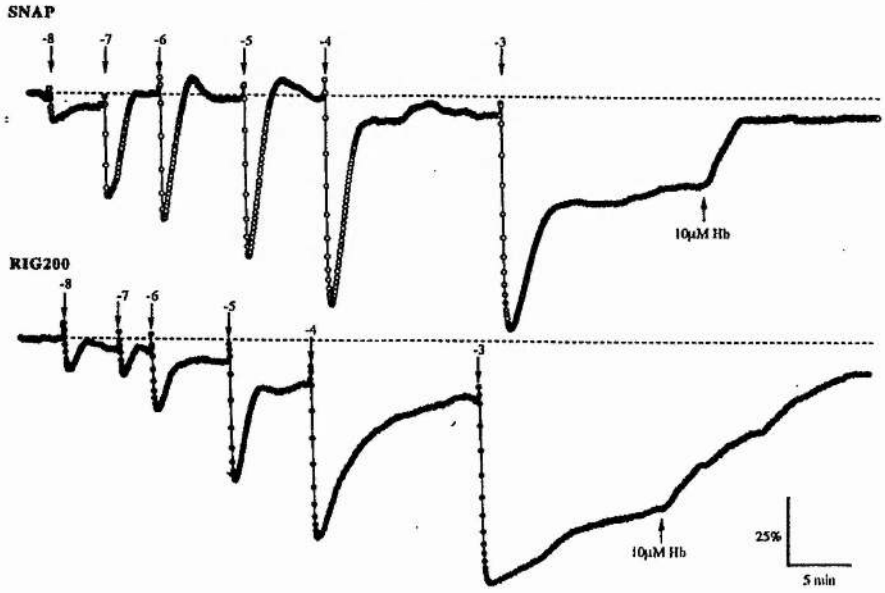
Injections of RIG 200 into endothelium intact arteries gave a dose dependent increase in dilatory response. The arteries recovered fully to their pre-injection pressures. The studies showed it to have a lesser effect than that caused by SNAP. Bolus injections of



RIG 200: R = CH_3
RIG 300: R= CH_2CH_3
RIG 500: R = $(CH_2)_3CH_3$
RIG 700: R = $(CH_2)_5CH_3$

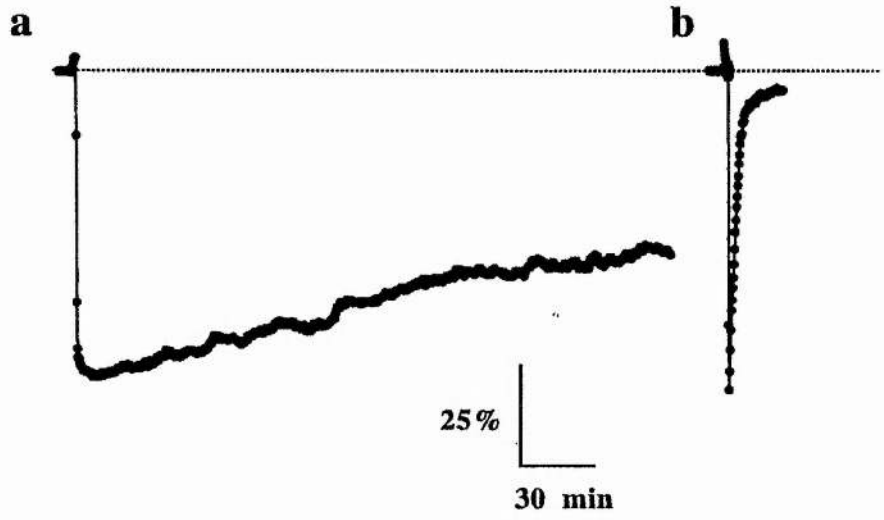
RIG 200 into endothelium denuded arteries showed a full recovery following 10^{-8} and 10^{-7}M injections but thereafter failed to recover to pre-injection pressure. There appears to be no difference in the sensitivity to the NO donors between the endothelium denuded arteries and those with intact endothelia. The results are shown in figure 3. The upper trace shows the bolus injection of increasing doses of SNAP. At all but 10^{-3}M the pressure recovers fully. The trace shows the recoveries to be rapid. At the 10^{-3}M dose there is a rapid initial recovery which then tails off. The addition of haemoglobin, via perfusion, gives a rapid return to original pre-injection pressure.

Figure 3



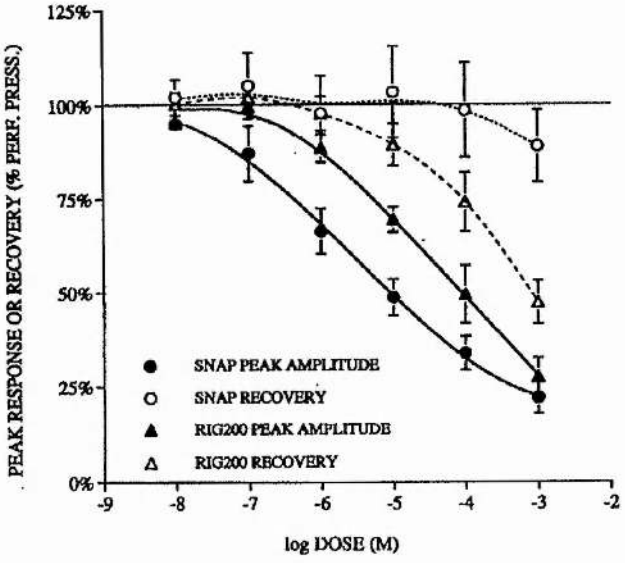
The lower trace shows the bolus injection of RIG 200 in increasing doses. The recovery between injections is much less rapid and to a lesser degree, leading to a sustained depression of tone Typically recovering to only about 50 % of pre-injection pressure following a 10^{-3}M bolus injection. The perfusion of haemoglobin gives a very slow rise in pressure. The difference between the recovery periods for SNAP and RIG 200 is demonstrated by the traces in figure 4. Trace a) relates to RIG 200, a slow and incomplete recovery, trace b) relates to SNAP, a full and rapid recovery of tone.

Figure 4



The comparisons between SNAP and RIG 200 are shown in figure 5 below. The graph gives the maximum dilation (as a percentage of perfusion pressure) caused by increasing doses of each drug. The graph also shows the degree of recovery shown by the artery at each dose. From the graph we can see that there is a strong dose dependency for both drugs, with an increase in peak amplitude with increasingly concentrated bolus injections. SNAP (filled circles) is shown to be more potent at all but the highest dose than RIG 200 (filled triangles). Following bolus injections of SNAP the artery is shown to recover fully (100 %) at all but the highest doses (empty circles). The artery shows a dose dependent decrease in recovery following doses of RIG 200 (empty triangles) and only recovers fully at the lowest doses.

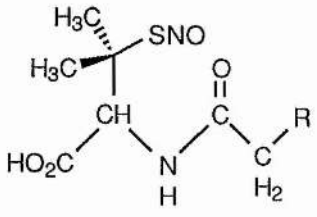
Figure 5



3.3.2. Penicillamine derivatives.

As the penicillamines prepared are close analogues of SNAP it is reasonable to expect that their activities and stabilities should be of similar magnitudes to that of SNAP. The acyl chain, increasing the lipophilicity of the molecule, enhances prospects of entering the tissue lining the lumen. However, unlike RIG 200, they still retains some degree of hydrophilicity through the free acid group. This makes the handling of the compound somewhat more simple. It is easier to get these derivatives into solution than RIG 200 and consequently it is possible to use them in higher concentrations.

Penicillamine derivatives



SNOPP: R = CH_3

SNVP: R = $(CH_2)_2CH_3$

SNOPHE: R = $(CH_2)_4CH_3$

Figure 7

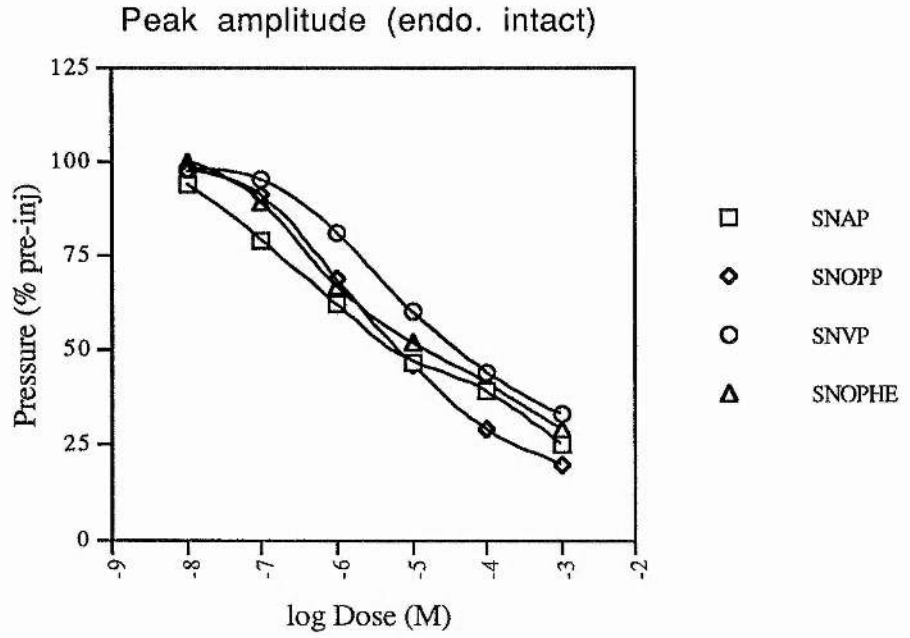
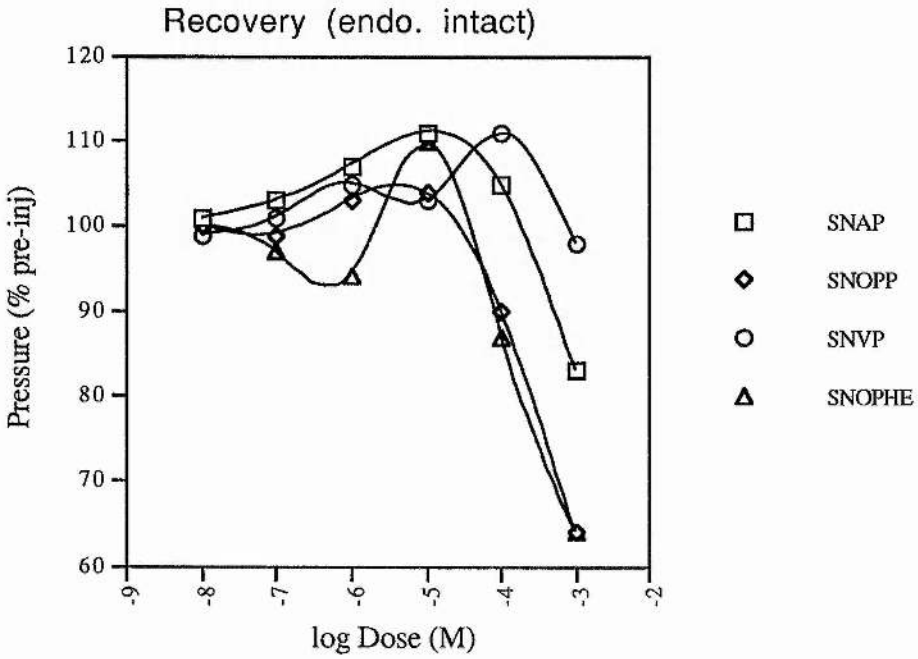


Figure 8



Bolus injections of the nitrosothiols gave transient dilations as expected. When vessels with intact endothelia were used, very similar patterns for the penicillamines and SNAP were seen. Figure 7 demonstrates the increase in arterial dilation with increasing doses of nitrosothiol. The graph shows very little difference between any of the derivatives..

Figure 8 shows the recovery of the arteries with increasing dose. The arteries recover to their initial pressures at all but the highest doses. Statistical analyses have shown the differences to be non-significant.

A similar pattern to that shown for the endothelium intact vessels (figure 7) is shown for the dose dependent dilations of endothelium denuded vessels (figure 9). There are no significant differences in the activities of any of the four nitrosothiols. In view of the variance in the stabilities of the four compounds, especially with respect to copper catalysed decomposition (chapter 6), it is perhaps surprising that there is not more of a difference in the activities of these compounds. This suggests that there are possibly more factors involved for *in vivo* decomposition than those used in our model decomposition studies and that these are of a significant importance. The amplitude of the response to each nitrosothiol was very similar, however, when looking at the individual response traces (figure 10) it is apparent that there is a very significant trend in the response of the endothelium denuded artery to each drug. As was hoped, the longer chain did appear to allow the nitrosothiols to be taken into the lining of the blood vessel in a similar manner to that of RIG 200.

Figure 9

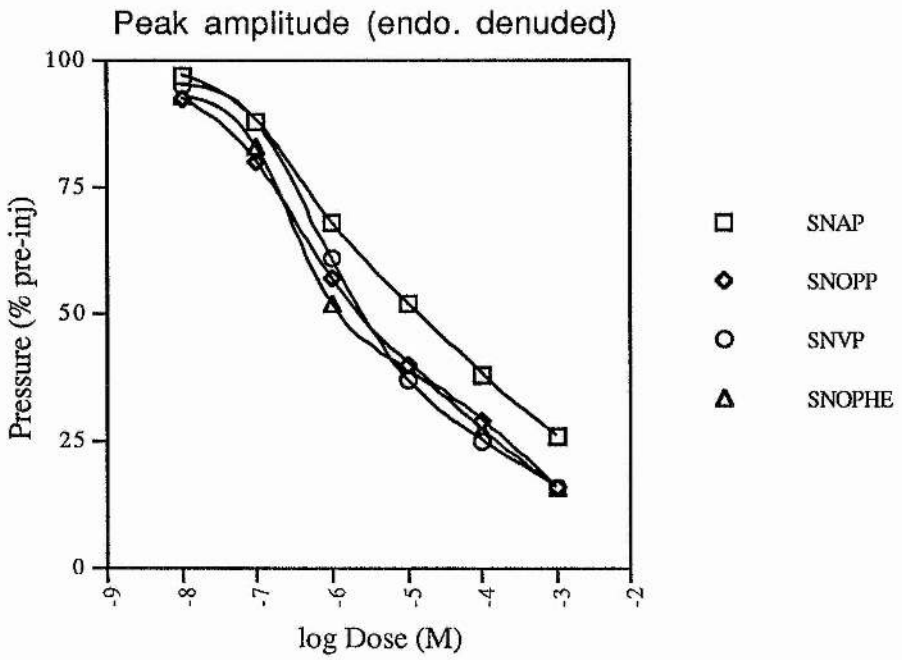


Figure 10

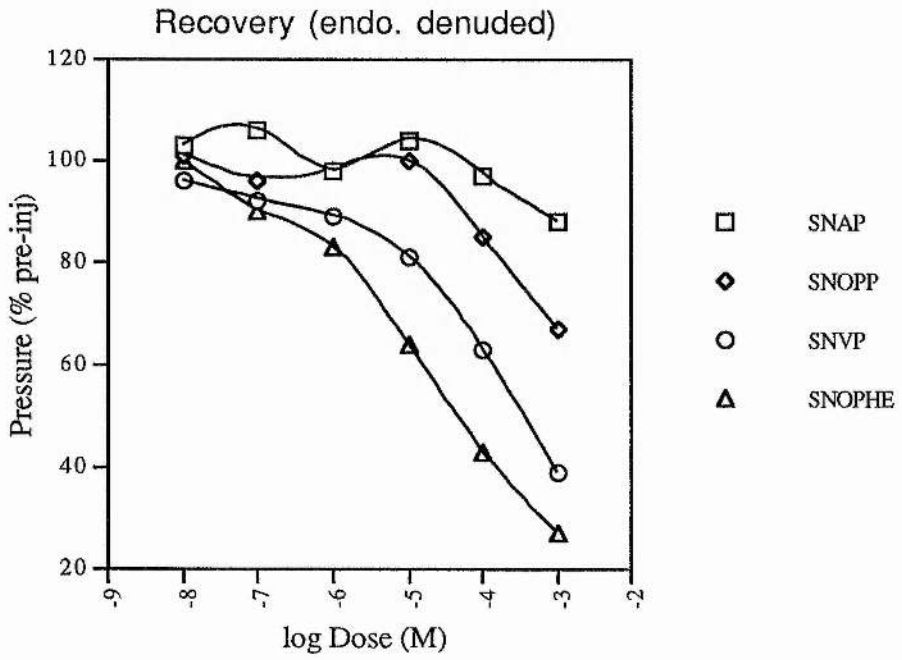


Figure 10 demonstrates that the response is more sustained with increasing chain length. The heptanoyl derivative showing the lowest degreee of recovery, the valeryl derivative giving a slightly lesser response (greater recovery) and the propionyl and acetyl derivatives giving the most transient responses. The results are discussed individually.

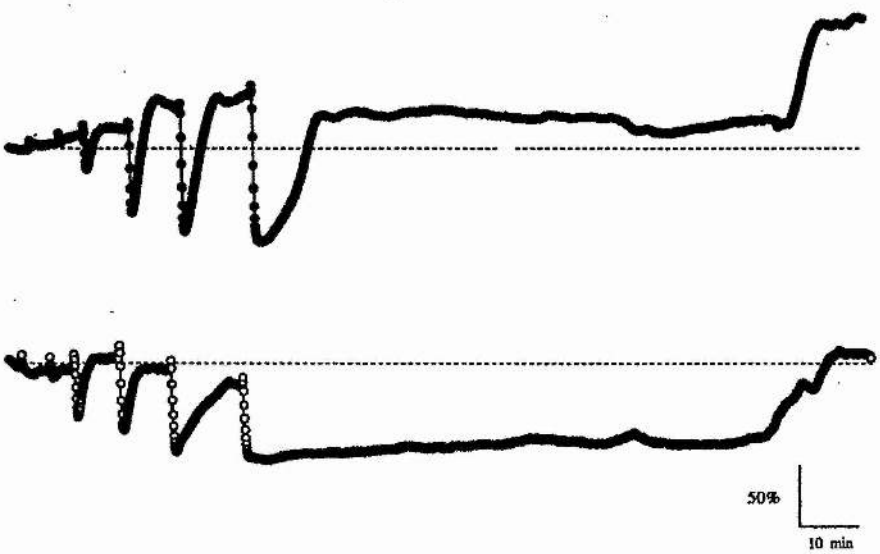
3.3.2.1. SNOPP.

The propionyl derivative, with only one carbon more in the acyl chain than SNAP, shows very similar results to SNAP. In endothelium denuded tissue there is a less of a recovery at the highest two doses. It is a stable compound which is easy to use and prepare, but, as a vasodilator, shows only slight benefits over SNAP.

3.3.2.2. SNVP.

Figures 9 and 10 demonstrate that SNVP is a very good vasodilator and also acts as to give a sustained response. Figure 11 shows an individual trace comparing the effects of increasing bolus injection doses of SNVP on endothelium intact and endothelium denuded vessels. The full recovery of the artery to pre-injection pressure, and above, after microinjections of SNVP to an artery with its endothelium intact is shown in the upper trace. The substantial increase in pressure at the end of the run marks the addition of 1μM haemoglobin. The lower trace shows the lack of recovery of an endothelium denuded vessel to the highest dose for SNVP. The artery only recovers to its initial pressure with the addition of haemoglobin. This recovery is noticeably slower than that shown in the upper trace.

Figure 11



3.3.2.3. SNOPHE

The heptanoyl derivative is shown by figure 10 to be the best of the four nitrosothiols as regards degree of recovery of arterial tone. The long chain makes it less soluble and therefore less easy to use than the other three. It was also noted that the shelf-life of SNOPHE was not as great as those for the other derivatives. This is possibly due to the more hygroscopic nature of the compound.

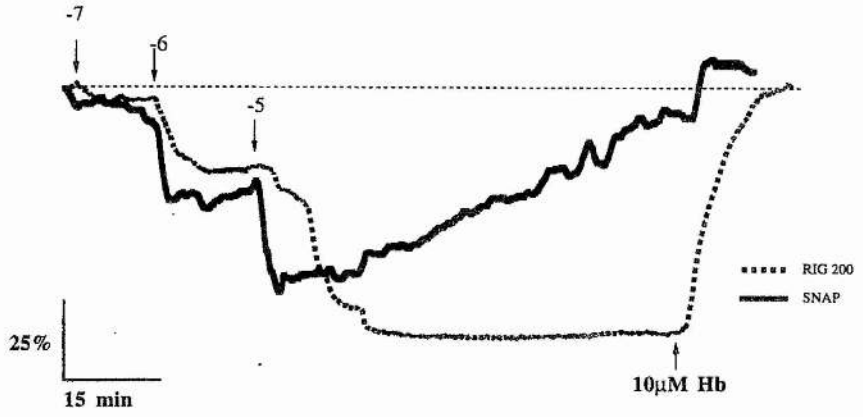
3.4. Current and future studies

This work represents an on-going project and the bolus injections studies, regarded as the most valuable, are the only ones fully investigated thus far. The end to these studies will involve testing upon human subjects. Preliminary trials have already been carried out. These have shown RIG 200 to be an active dilator of human blood vessels. The studies remained incomplete due to difficulties in fully removing the endothelium from the vessel and verifying its complete removal.

Trials investigating the transdermal capabilities of these compounds will also be performed, probably in a similar manner to those described in chapter 4. Preliminary studies have been carried out involving the addition of RIG 200 and SNAP to the

superfusate reservoir. Whilst this does not show the ability of the drug to be delivered transdermally, it does demonstrate the ability for it to pass through the outside covering of the blood vessel to the muscle cells. The results shown below (figure 12) demonstrate the effects of superfusing 10^{-7} - 10^{-5}M SNAP and RIG 200 into the artery. Superfusion of SNAP gives a dose dependent decrease in pressure, which is rapidly reversed upon washing out with fresh buffer or on addition of haemoglobin to the perfusate. Superfusion of RIG 200 gives a dilation of lesser magnitude to that of SNAP. Following superfusion with 10^{-5}M RIG 200, the vessel remained dilated for over 4 h, showing no recovery of pressure. These results suggest that RIG 200 has the ability to pass all the way through the blood vessel in order to reach the elastic lamina where it is absorbed **(3.5)**. Superfusion with haemoglobin or L-NAME was required to obtain a recovery to the pre-injection pressure.

Figure 12



3.5. Summary

These results suggest that the endothelium acts as a physical barrier preventing the nitrosothiol from entering the tissue. The layer under this, known as the internal elastic lamina, is composed of cells embedded in a glycoprotein matrix. It is possible that the nitrosothiols, particularly RIG 200 with its sugar component, have an affinity to this extracellular fluid. Our results strongly indicate that the more hydrophobic compounds have a higher affinity for this layer and are therefore more soluble in it. The more

hydrophilic donors with the shorter chains, SNAP and SNOPP, appear to be unable to penetrate this layer. RIG 200 and SNOPHE are being used at the limits of their solubilities, i.e. they are about as hydrophobic as we can utilise with this delivery system. From this it is fair to say that these compounds will remain in the elastic lamina for as long as any compounds we could use and it is only the potency that we could improve on. From these compounds we have tested, the best sustained response is seen with SNOPHE. However this compound is relatively difficult to handle due to its hygroscopic nature. The next best donor compound is SNVP, which can be made in large quantities and is very stable. The one drawback with this compound is the unpleasant smell from the valerate portion of the molecule, which may be a factor against its use in human testing. Despite being less potent and having a slightly less well sustained response, RIG 200 is the most useful of these compounds, especially for human testing. Further work may also involve the other similar derivatives, RIG 300, 500 and 700. These have been shown to be more stable than RIG 200 (chapter 6) and may prove useful for inducing long term vasodilation. This will require a slightly different delivery system to overcome the solubility problems.

3.6. Conclusions

A number of new NO donor drugs have been prepared. These have been found to be active vasodilators of about the same potency as SNAP. Three of these have been found to be taken into the tissue lining the blood vessel when the endothelium has been removed. This has very important medical implications especially in the treatment of conditions where the endothelium has been damaged. The usage of these drugs for the treatment of patients following PCTA, for removal of atherosclerotic plaque, has been envisaged. As the endothelium is removed in this treatment, the donors prepared here would be of particular benefit. As these compounds are only taken into the tissue when the endothelium is removed, it is feasible that following delivery they would pass harmlessly through the healthy tissue, where the endothelium is intact. These could then gather in the damaged areas, where the endothelium is damaged or removed, and act as

the source of nitric oxide no longer available to the blood vessel. In this they would act as tissue specific donors, specific to damaged tissue and hopefully allow recovery of the endothelium without the blood vessels sustaining any more damage. As effective long term NO donors these compounds also have the potential to be used in a number of other roles such as the treatment of cardiovascular disorders. The work on these compounds will continue, both to determine the potential of those already prepared and to synthesise similar compounds which may prove to be more effective.

Many thanks to Dr Ian Megson of Western General Hospital, Edinburgh, for carrying out the bulk of this work

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Chapter 4: Transdermal Delivery Studies[1, 2]

4.1. Introduction

There are many ways of introducing a therapeutic agent into the body. Commonly drugs are delivered orally or intravenously. A method which has shown great promise in recent years is that of transdermal drug delivery (TDD). In this the drug is delivered directly through the skin and into the bloodstream. The main advantages that accompany the use of TDD are:

1. Avoidance of breakdown of the drug in the stomach and hepatic first pass metabolism. e.g. the bioavailability of nitroglycerine is reduced to 1% by its short half- life and first pass metabolism.[3]

2. A constant rate of drug delivery and consequently a sustained plasma drug concentration can be attained.

3. Ease of delivery and reduction of dosing frequency.

4. Avoids the pain and possibility of infection associated with injections.[4]

5. The drug can be delivered in a lower dose or less often. However this may be offset by the initial cost of the TDD system compared with the costs of oral delivery.[5]

There are many considerations necessary in TDD, particularly as it involves transporting a substance across a barrier specifically designed to resist such a process:

1. The skin is a very effective barrier and so the drug must be very potent.

2. The drug must have the correct solubility to pass through the skin. It must be a small molecule, generally under 1000 Da. The molecule must have a low solubility in water, though some degree of water solubility appears to be necessary.

3. The drug must not irritate the skin.

4. The drug must have the correct diffusion and partitioning characteristics as it is necessary for the drug to: i) Diffuse through the delivery system, ii) Partition from the system to the stratum corneum, iii) Diffuse through the stratum corneum, iv) Partition from the stratum corneum into the viable epidermis, v) Diffuse through the viable tissue

and finally, vi) Enter the cutaneous microcirculation. These are complex demands to place on the shoulders of any prospective candidate for TDD.

Despite the fact that topical drug application has been used since Egyptian times, most topical drugs are intended for local action and not for the general circulation. TDD has been mostly ignored as demonstrated by there having been only two drugs licensed in the USA for transdermal delivery in 1985. By 1994 there were seven marketed transdermal systems. These were for: nitroglycerine, oestradiol, clonidine, fentanyl, nicotine, scopolamine and noresthisterone acetate.[6] The usage of these, in particular nitroglycerine, will be discussed later.

Whilst there are a sizeable number of delivery systems, most are similar and follow the same basic design:

a) Drug reservoir - the required amount of drug for the prescribed program is stored in a stable form.

b) Rate controlling element - maintains the prescribed rate of drug administration throughout the operating life of the system.

c) Energy source - effects the transfer of the drug molecule from the reservoir to the selected point of entry in the body.

d) Delivery portal - provides an exit for the drug from the delivery module so that it can reach the target organ.

e) Platform - houses the drug and delivery modules. This must be compatible with the local tissues, i.e. it must not irritate the skin.

The system is attached to the skin by an adhesive which contains a priming dose designed to saturate skin sites before establishment of a steady state input of drug from the reservoir. The rate controlling microporous membrane is chosen to ensure that the delivery rate of the drug reservoir is much less than the rate at which the impermeable skin can absorb the drug. This ensures that the system, and not the skin, determines the

rate of delivery. Were this not so then patients with high skin permeabilities or damaged skin might receive a harmfully high dose.

A good example of a delivery system is Ciba-Geigy's "Transderm-Nitro" for the delivery of nitroglycerine. The reservoir is composed of nitroglycerine absorbed onto lactose and dispersed in a colloidal suspension of silicon dioxide and silicone medical fluid. The amount of nitroglycerine contained in the medium is five times that delivered to the body and thus serves as the energy source for drug diffusion. The microporous membrane is composed of ethylene vinyl acetate co-polymer. The membrane pores are of such a size as to limit *in vivo* delivery from the reservoir to $0.5mg/cm^2/24hrs$. The surface area of skin covered by the membrane determines the total amount of drug diffused. The system is attached by a hypoallergenic adhesive which is protected during storage by a non-permeable peelstrip. Approximately 8% of the nitroglycerine diffuses into the adhesive layer during storage . Upon application, this amount of drug in the adhesive may serve as a priming dose. Finally the reservoir is protected by an outer layer of aluminised plastic which is impermeable to the nitroglycerine housed in the adjacent reservoir. The system which we have used in testing involves a less complex setup where the skin controls the rate of adsorption. This will be discussed later.

As nitric oxide donors are already used for transdermal delivery, nitroglycerine and the other organic nitrates are obviously of great interest to us.

Organic nitrates are safe, effective and relatively inexpensive for the management of ischaemia related to coronary heart disease.[7] The benefits of these have been known for over 100 years, with the usage of nitroglycerine in the treatment of acute attacks of cardiac angina.[8] There is considerable evidence to suggest that the site of the metabolic conversion to produce NO occurs in the vascular smooth muscle.[9] However the actual mechanism by which this occurs remains undefined.[10] Organic nitrates are known to cause vasodilation and vasorelaxation of coronary arteries. A platelet aggregation

inhibitor role has also been suggested. There are three nitrate compounds currently available in the USA: nitroglycerine, isosorbide dinitrate and isosorbide mononitrate. These are available as rapid acting but short-lived sublingual and transmucal doses for the relief from angina pectoris attacks, or as longer lasting oral and transdermal formulations for the relief of chronic ischaemia and myocardial infarction. Transdermal nitroglycerine is more effective than oral nitroglycerine for cardiovascular therapy, though it does lead to a higher incidence of adverse effects such as headaches, dizzinesss and skin irritation.[5] Tolerance to the organic nitrates, rendering them useless, can be a major problem: a once daily application of isosorbide dinitrate cream produces tolerance and a complete lack of efficacy within a week. Nitroglycerine patches are claimed by the manufacturers to provide 24 hr anti-ischaemic and anti-anginal efficacy, provided the use and dosage is strictly controlled.[11] Despite this it is more commonly thought that a drug free resting period is required to avoid tolerance. Removal of the patch during the night appears to be a favoured option.[2] Transdermal delivery of nitroglycerine has been used in the treatment of Raynaud's Phenomenon. It was found to be effective in reducing the number and severity of the attacks, but was found to induce frequent headaches making it an unsuitable treatment.[12]

In recent years nicotine patches such as nicorette and nicotinell have become popular cigarette substitutes for those "requiring" nicotine. These deliver nicotine into the body in a manner currently thought to be less harmful than smoking. The usage of transdermal nicotine has been shown to double the success rate of smoking cessation. However the patches are costly and should only be recommended for smokers unable to quit by simpler means and those likely to suffer severe nicotine withdrawal symptoms.[13]

Two of the other TDD systems involve the use of clonidine, a mild hypertensive agent, and oestradiol, used in female hormone treatment.

One of the first TDD systems was for scopolamine. This is a powerful narcotic and sedative commonly used for the prevention of motion sickness and also as a wartime truth drug. In a slightly unusual study, transdermal scopolamine has been shown to reduce drooling in developmentally delayed children. In one third of the subjects a complete cessation of drooling was observed whilst the patch was being worn.[13] Whilst few scientists would regard this as a major development, it does show the range of possiblities for the usage of TDD.

The range of drugs which can be delivered transdermally is limited to smaller molecules. In general this means that larger molecules cannot be passed through the skin. However, laboratory techniques have been developed to either push drugs through the skin (such as iontophoresis) or to make the skin more permeable, (such as sonophoresis). In iontophoresis, a potential difference is set up through the skin so that a charged molecule can be pushed through. This can be used for the delivery of sodium nitroprusside or acetylcholine as well as many other drugs. Sonophoresis[4] involves the usage of low frequency ultrasound (1 MHz) to increase the permeability of the skin. This has been used for the delivery of insulin (~6000 Da) and erythropoeitin (~48000 Da). The ultrasound increases the size of air pockets in the stratum corneum. This allows these drugs, to which the skin would normally be an impermeable barrier, to pass through. Upon termination of treatment the skin quickly regains its normal properties. At present these methods of delivery are possible only under laboratory or medical supervision but in future years it may be possible to make use of these techniques for drug delivery.

4.2. Selection of a Transdermal Agent

In order to initiate a series of studies involving transdermal delivery on humans, a number of criteria had to be met. The compound being used had to be relatively easy to synthesise on a moderately large scale. It had to be easy to handle, preferably a solid and stable enough to be stored for prolonged periods of time. Most importantly it had to be shown to be completely safe in order to obtain permission for its usage. *S*-nitrosothioglucose tetraacetate (SNAG) was felt to satisfy these criteria. It is a pink powder (stable under refrigeration) and can be synthesised from commercially available acetobromoglucose in three short steps. The first step involves the usage of thiourea, a known carcinogen. There is no chance of any of this being present in the final product. Any thiourea not removed by recrystallisation of the first product is likely to be hydrolysed to urea or washed away during the second and third steps. In a sample of SNAG the only likely impurities are unreacted thiol (TGA) and the disulphide formed by nitrosothiol decompostion. *In vivo* the thiol may also be produced by enzymatic reduction of the disulphide.[15] It is also probable that in the body the acetyl groups are lost by enymatic cleavage[16] to give the free thiosugar (TG).

Consultation of a number of toxicological databases has not suggested any notable adverse effects of any of the above compounds. Further to this TG and TGA have been put to a number of physiological uses. TGA is used in a the treatment of rheumatoid arthrits as a ligand in the gold complex aurafin.[17] TG and TGA complexed to copper have been tested sucessfully as antiinflammatory agents.[18] The use of TG and TGA as detoxification agents have been suggested and patented.[19,20] Thioglycosides are found in a number of plants used in human foodstuffs, such as sprouts, horseradish and mustard. In the cooking of these it is likely that some TG is formed. No harmful effects have been noted, in fact sprouts are regarded as being a particularly healthy food.

With this information began the lengthy process of obtaining ethical permission for an initial pilot testing on humans. After a long delay this was finally granted. The delay

was necessary in order to determine legal responsibilities for any side effects, to make sure all involved parties had the necessary insurance and also to satisfy the commitee that the identity of the compound was as stated. It is because of this timespan that we have not been able to investigate other NO donors for their transdermal delivery capabilities.

It is not certain whether it is SNAG passing through the skin and decomposing internally to deliver NO, or SNAG decomposing externally and NO passing through the skin. Negative results obtained using a very hydrophilic NO donor suggest that it may be the former explanation which is correct.

4.2. Preliminary studies.

Studies were carried out in collaboration with members of the Department of Medicine at Ninewells Hospital, Dundee. Large amounts of time were spent working out a suitable protocol for a full scale trandermal study on SNAG. The situation was somewhat complicated by the change from a "single point" to a "scanning" laser Doppler flowmeter which changed the methods used. Laser Doppler flowmetry is a useful non-invasive method for studying the skin.[21] It is commonly used for studying a number of conditions:

a) Rheumatology. Many painful conditions involve changes in skin blood flow. The microvascular changes disorders such as Raynaud's phenomenon are easily observed by laser Doppler imaging.

b) Burns. Laser Doppler Images help the plastic surgeon to assess burn depth, the regions most affected and the regions where blood flow has been sufficiently maintained as for recovery to occur without surgery. Following treatment, graft progress can be monitored.

c) Dermatology. The degree and involvement of irritant and allergic responses can be monitored over a number of sites to determine the extent of the reaction and to determine the nature of the irritant.

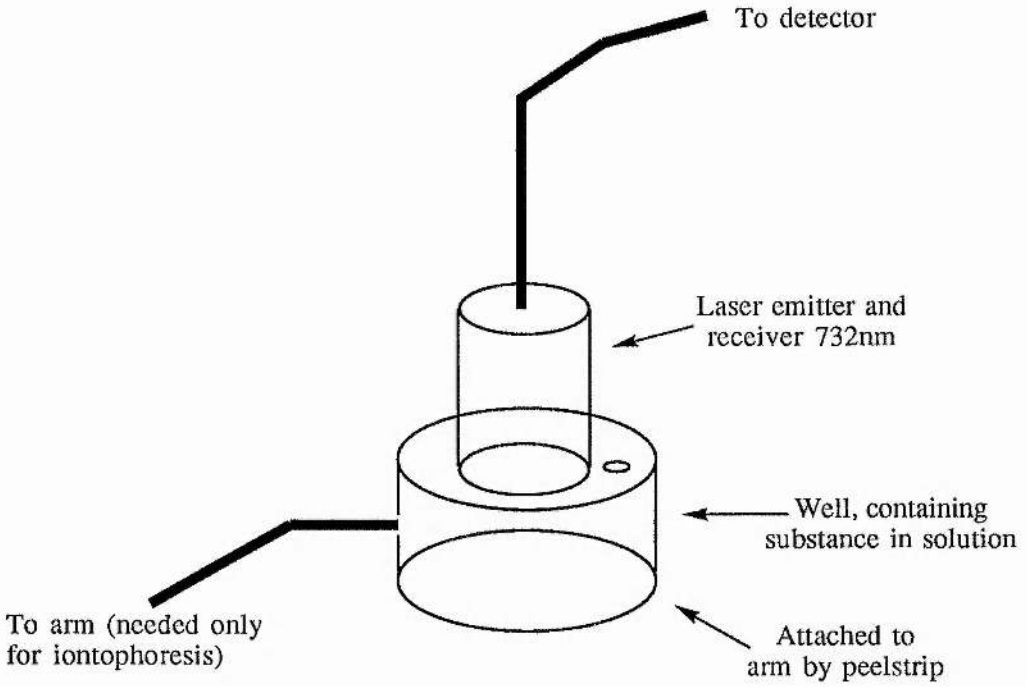
d) Other studies include monitoring post angioplasty subjects and wound healing.

Both single point and scanning LDF work on a simple principle involving the differences between transmitted and reflected light. A beam is emitted from the probe and is reflected back off the skin to a detector in the probe. The beam is powerful enough to penetrate 1 mm into the skin. It is the microcirculation in this area that is of interest to us. If the laser beam comes into contact with a moving object i.e. a blood cell, then the frequency will be changed. The probe measures the frequency differences between the transmitted and reflected beams and from this calculates a figure termed the "flux". Flux is made up from the product of two values: the number of cells passing a point per unit time and the average velocity of these blood cells. It is possible to break the arbitrary figure into its individual components. For our purposes this is unnecessarily complex and an increase

in flux can be regarded as an increase in blood flow. As suggested by their names, the single point Doppler flowmeter measures over a very small area and gives the flux as a continuous readout. The scanning Doppler imager has a beam which passes rapidly back and forth in lines across the desired area until it has taken a reading over every part. This gives a colour coded picture made up of individual pixels. Each pixel represents the blood flow in a volume of skin approximately equal to 1 mm^3. The scanner can be set to take readings at any time interval to build up a series of pictures of blood flow changes with time. It is also possible to obtain and follow the exact readings for any individual pixels, but it is more informative to look at the whole picture.

4.2.1. Single Point Studies.

Measurements were made using a Moor MBF3/D laser Doppler Flowmeter (MOOR Instruments, Axminster, UK). The perpex well had an outer diameter of 30 mm, a central chamber, responsible for holding the probe, of 3.5 mm diameter and an inner chamber, in which the solution is held, of 8 mm diameter. The laser records the blood flow for a 1 mm^3 volume.



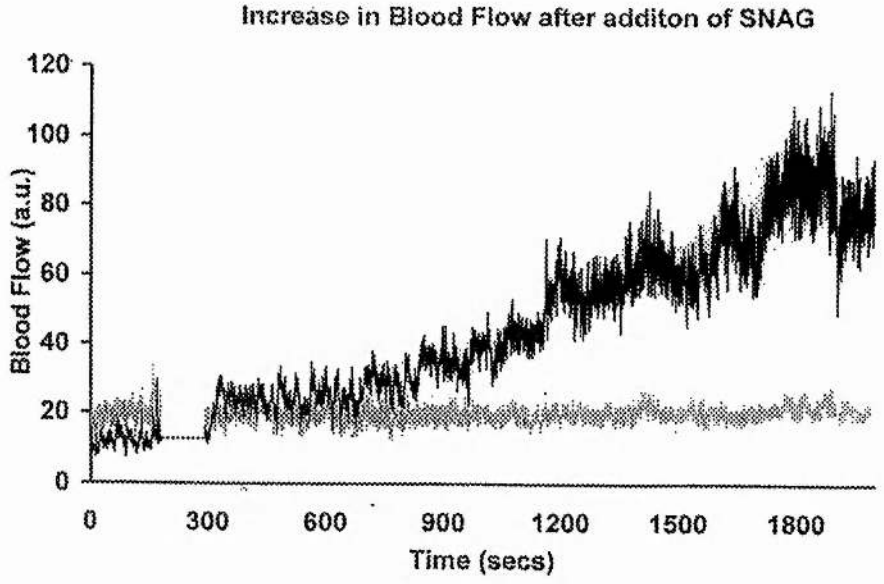
Our original, technique involving the use of the single point apparatus, had a number of flaws. We now know that some human subjects will always show a very low response

to vasodilatory drugs, age often playing an important role in this. There are many other factors which may influence the result such as the emotional state of the volunteer and the weather outside. It was also found that some ususally responsive subjects could show little or no response at times simply because of items they may have eaten or drunk. The picture generated by the scanner often contained "rogue" pixels. These are single pixels in which the blood flow, often quite different to that of surounding areas, does not change in spite of any drastic overall changes in the skin blood flow. Whilst this is not a problem when using the scanner, it is quite possible that some of our early negative results, using the single point apparatus, were caused by attempts to study such an area. Initial studies were carried out using ethanol as the solvent. SNAG (1 % in ethanol) was placed in the small plastic well into which the probe is inserted. The well is attached to the inside of the forearm with double sided adhesive rings prior to this. Areas on the forearm with no large underlying veins were chosen and cleaned with ethanol wipes. A second probe, in a holder containing only ethanol, was used as a baseline control. A suitable area of skin is one with a stable blood flow which falls within a range of flux values, typically 20 to 40 arbitrary flux units. A maximum possible blood flow, seen by heating the skin, is usually between 200 and 250 units. Once the solutions have been added the blood flow is displayed as a continuous reading on a monitor screen as shown. When the trace has filled the monitor screen it will begin to overwrite the previous trace. This makes it easy to see any changes in the blood flow. The major problem with this system is its sensitivity. The patient cannot talk, laugh, change their position or even take a deep breath without having a disruptive effect on the blood flow trace. The position in which the arms must be held, outstretched with forearms at heart level facing upwards, is incomfortable and difficult to maintain for long periods of time.

Figure 1 shows the increase in blood flow for a 1% solution of SNAG compared with a control. Very little increase in flux was observed over the first six minutes. The flux then gradually climbed to give a maximum eight - fold increase after 20 minutes. At this

stage a plateau was usually reached and the flux value levelled off, sometimes with a slight decrease until the end of the experiment at 30 minutes.

Figure 1



SNAG was compared with solutions of SNAP, isosorbide dinitrate and isosorbide mononitrate (all 1 % in ethanol). Neither of the nitrates gave any response. An increase of up to three-fold was observed with SNAP. This lower response to SNAP may be due to it being a poorer transdermal agent - less able to pass through the skin, or if it is NO passing throught he skin (see 4.1) it may be because SNAP has a higher stability and is simply giving off NO at a reduced rate.

A dose dependent study was set up, involving concentrations of 0.25, 0.5, 1.0, 1.5 and 2 % SNAG in ethanol. However it was now found that none of these gave any response. In several successive tests no positive results were seen. An explanation for this could not be given until it was noted that there was a discolouration of the skin near to the site of observation. A red ring at distance of up to 2 cm from the site under study was being produced despite the negative result of the test. A reddening of the skin is usually indicative of an increase in blood flow, suggesting that vasodilation is occurring

but not in the area under scrutiny. Simple experiments involving holding a vial containing a solution of SNAG in ethanol upturned against the skin also demonstrated this phenomenon. Little or no reddening was seen for the areas in contact with the solution but a very distinctive and persistent red ring around the area was noted. These very basic tests were very important in showing that the vasodilator was indeed active but that our delivery system was at fault. The ethanol, by some hitherto unknown route, was transporting the vasodilator away from the site, either across the surface of the skin or through the microvessels. It is not understood why a ring is observed rather than a general reddening of the skin around the site.

For human usage there are generally thought to be three possible solvents, water, ethanol and DMSO. We discounted DMSO due to the problems with absorption through the skin and the subsequent unpleasant taste the patient experiences. SNAG is not soluble enough in water and ethanol had proved ineffective for our purposes. Despite the simplicity of the idea, changing from neat ethanol to an ethanol / water mix was a major step. Using this system the results were not only reproducible but faster to obtain. With the ethanol system an initial lag phase was seen. This was impossible to distinguish from a study in which no increase in blood flow was going to occur. A lot of time was often spent waiting to determine whether it was a negative result or merely a prolonged lag phase. With ethanol / water an immediate result increase in blood flow was seen. The addition of a drop of his solution to the skin led to a reddened patch, at the site of the drop, within 30 s. This made for shorter testing times and therefore more acceptable to the subject. Upon removal of the probe and holder a reddening at the site was observed. A ring, distant to the site, was no longer seen. Despite the succes of these results it was decided to move our studies onto the newly aquired scanning apparatus.

4.2.2. Scanning Studies

The scanning laser Doppler Flowmeter is pictured in use on page 17. It gives a blood flow over a much larger area than the single point apparatus and so probably provides us

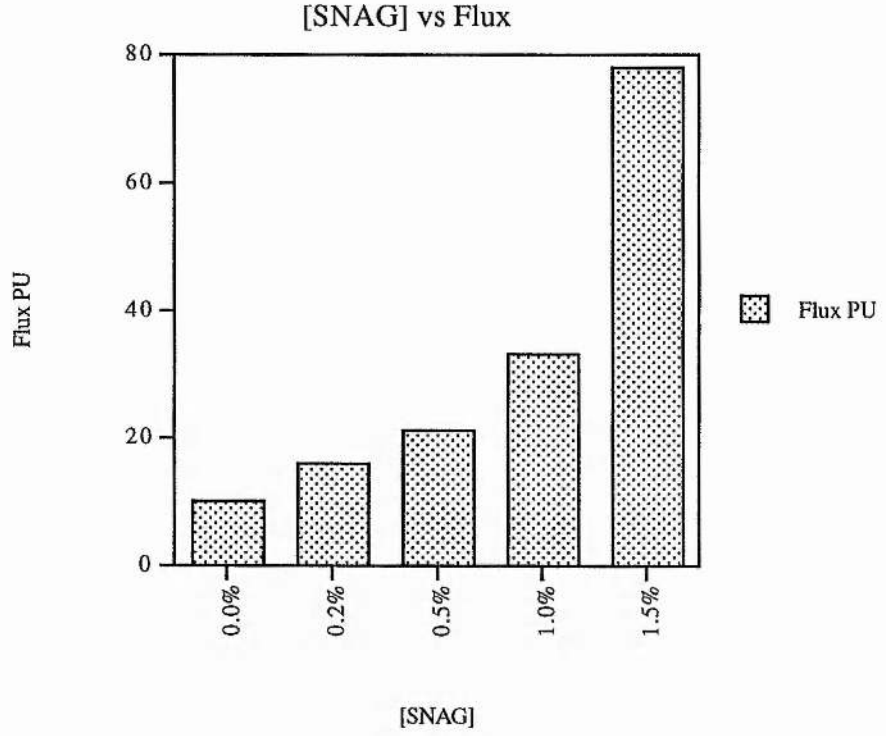
with a more accurate indication of the blood flow variation. As readings are only taken at various time intervals it is only necessary for the subject to remain completely quiet and still when the actual scan is being done. This method also lends itself to other possible delivery methods such as a KY jelly base.

In order to fit both a baseline and an "active" area under the scanner at once, only one arm was used in this study. In this we make the assumption that any changes in blood flow will be local and will not influence one another.

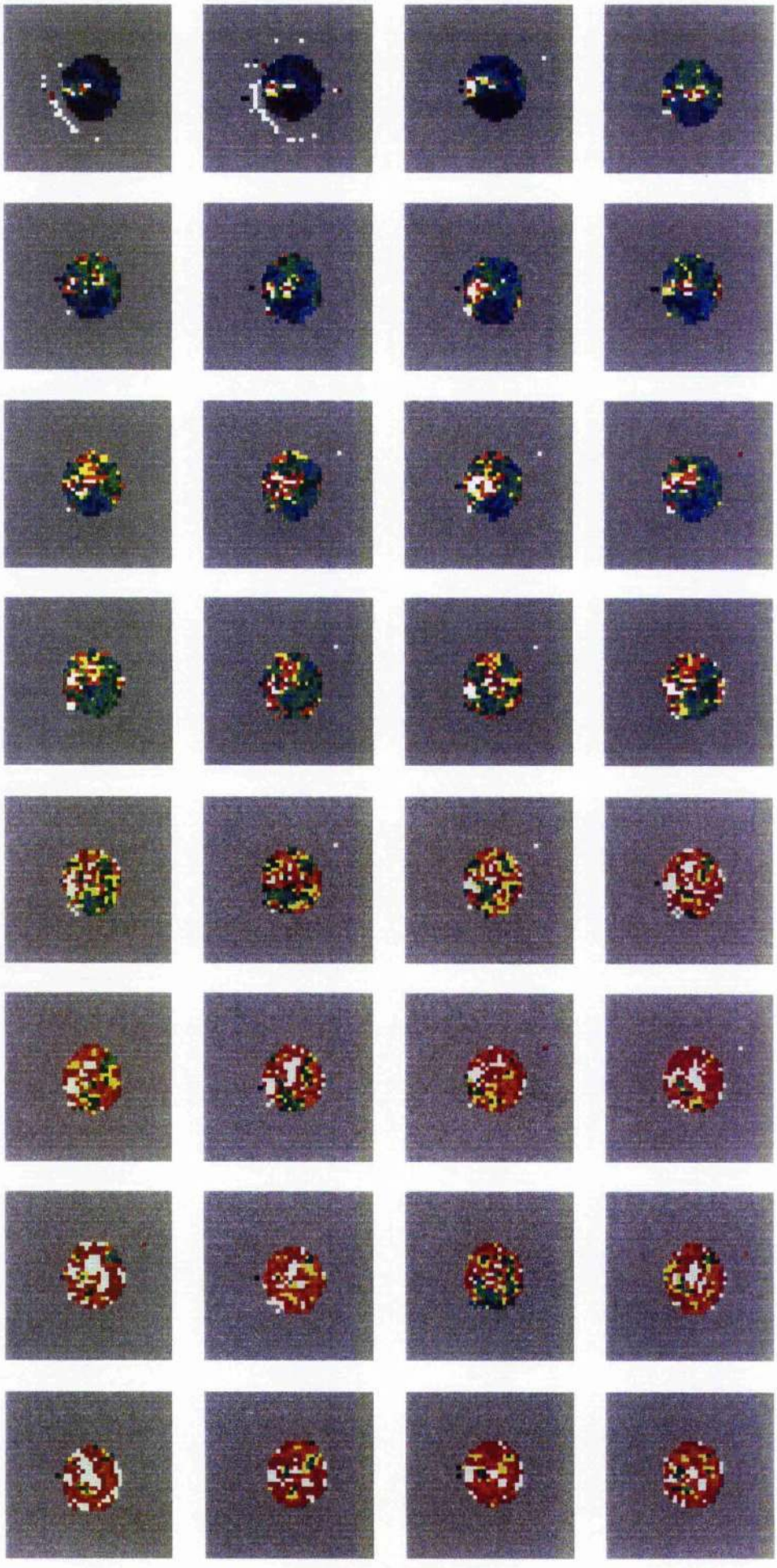
The plastic wells, larger than for the single point study, are attached to the forearm and filled with a solution of SNAG in a 1 : 1 ethanol : water mix. All other areas of skin and exposed plastic are covered with black material to avoid confusion and to visually improve the results. On the monitor screen is displayed two circles , each filled with coloured pixels. Each colour represents a particular flux level, according to a scale on the screen. Increases are easily observed as the basal flow is usually shown as a dark blue colour. An increase in the blood flow leads to a lightening of these colours, becoming more vibrant as the flow further increases. The computer with dedicated software can then be used to calculate the average blood flow for the designated area. This is actually taken as the median rather than the mean value. The reason for this concerns problems encountered with reflections leading to enormously high flux values. It was possible to obtain a photographic image from the DC signal of the area being studied. This shows clearly where any reflecting areas may be. These can then be isolated and such areas removed from the calculation. Even with this there are still anomalous regions which will give an incorrect estimate of the blood flow were the mean value to be taken. Usage of the median value is an approximation based on the assumption that there will be as many abnormally high values as abnormally low values and that these will cancel each other out. The laser scans over the desired areas, through the solvent, giving a series of images on the monitor screen. Figure 2 (page 98) shows the gradual increase in blood flow on an area of forearm skin. The dark blue of the early pictures developes into the

lighter and brighter colours of the later pictures as the vasodilatory effects occur. A dose dependent study was carried out using 0.25, 0.5, 1.0 and 1.5 % solutions of SNAG in 1: 1 ethanol : water. The results, shown in figure 3 demonstrate a sizeable increase in skin perfusion with increasing dosage.

Figure 3



An eight-fold increase with the 1.5 % solution is the maximum obtained. A more concentrated solution cannot be prepared due to solubility problems. The results from a study of blood flow against time for a 1 % solution are shown in figure 4. This demonstrates an immediate effect, which builds up to its full intensity over a period of 15 to 20 mins. This maximum effect is then sustained for over 10 minutes. These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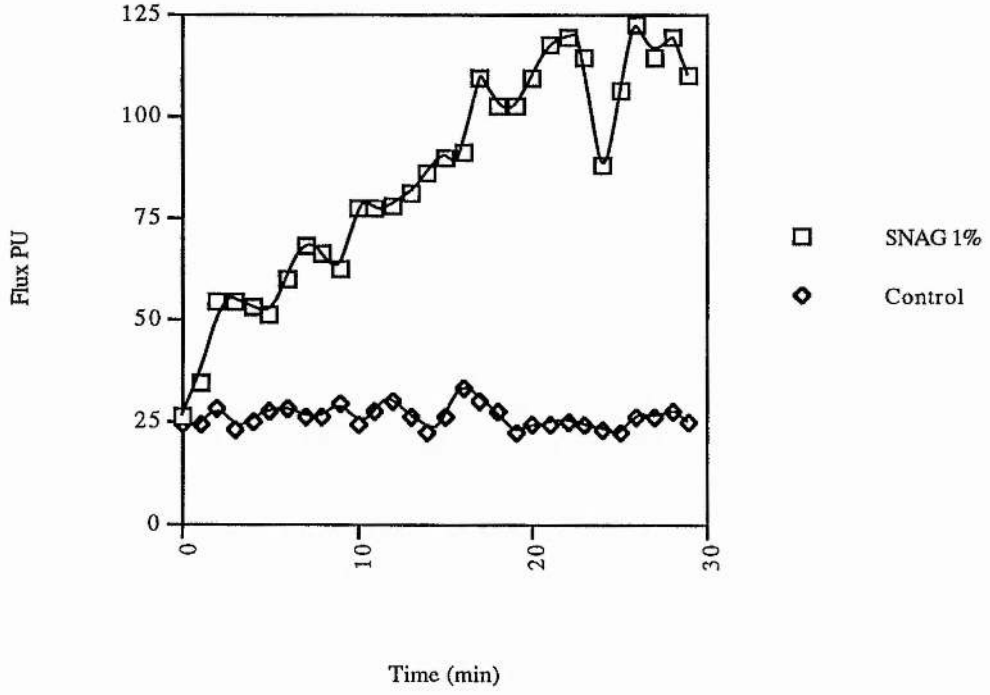
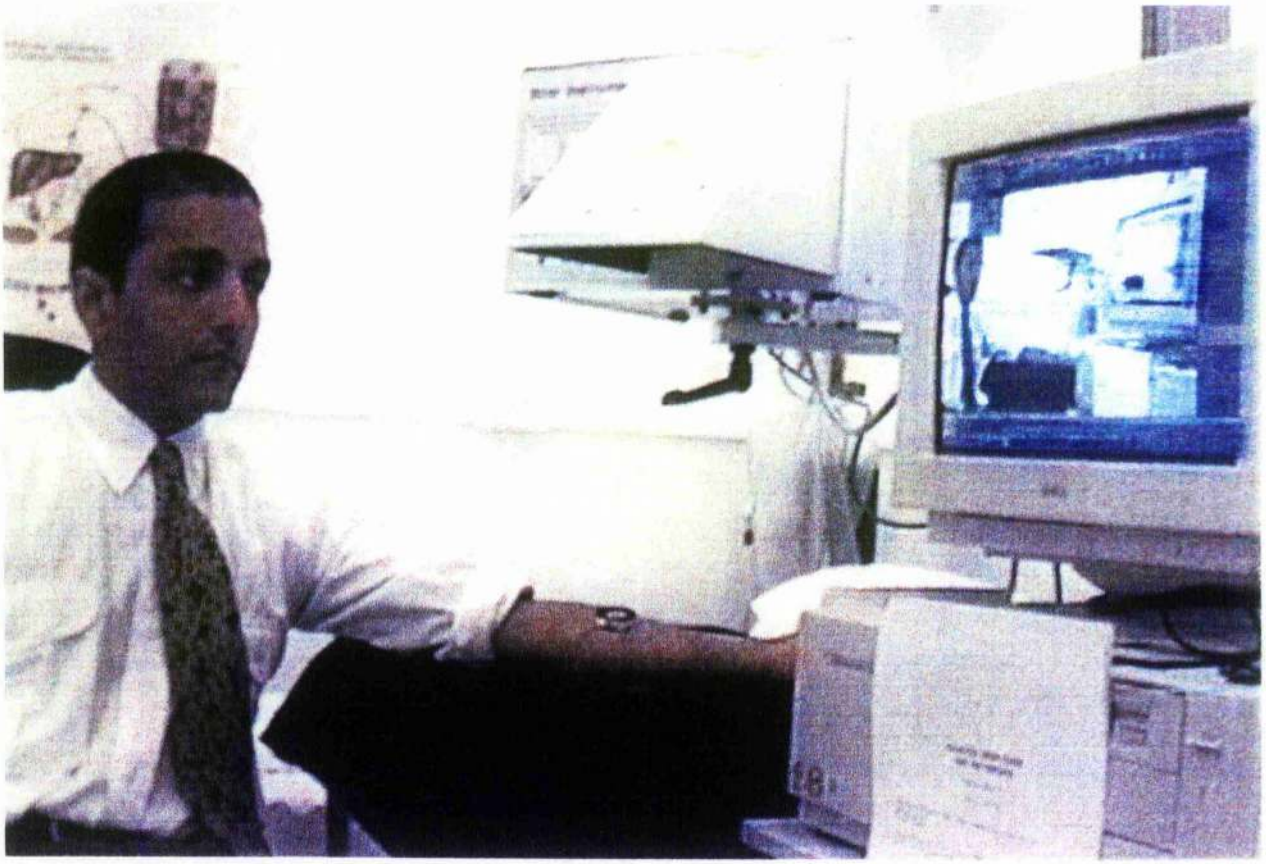
results are also shown pictorially in figure 2 (page 98). The scale at the bottom of the page explains that the blue dots represent the areas of lowest bloodflow, seen at the beginning of the experiment. The pink and white dots, seen at the end of the experiment, represent areas of higher blood flow. This picture clearly shows the development of an increase in bloodflow following the application of SNAG.

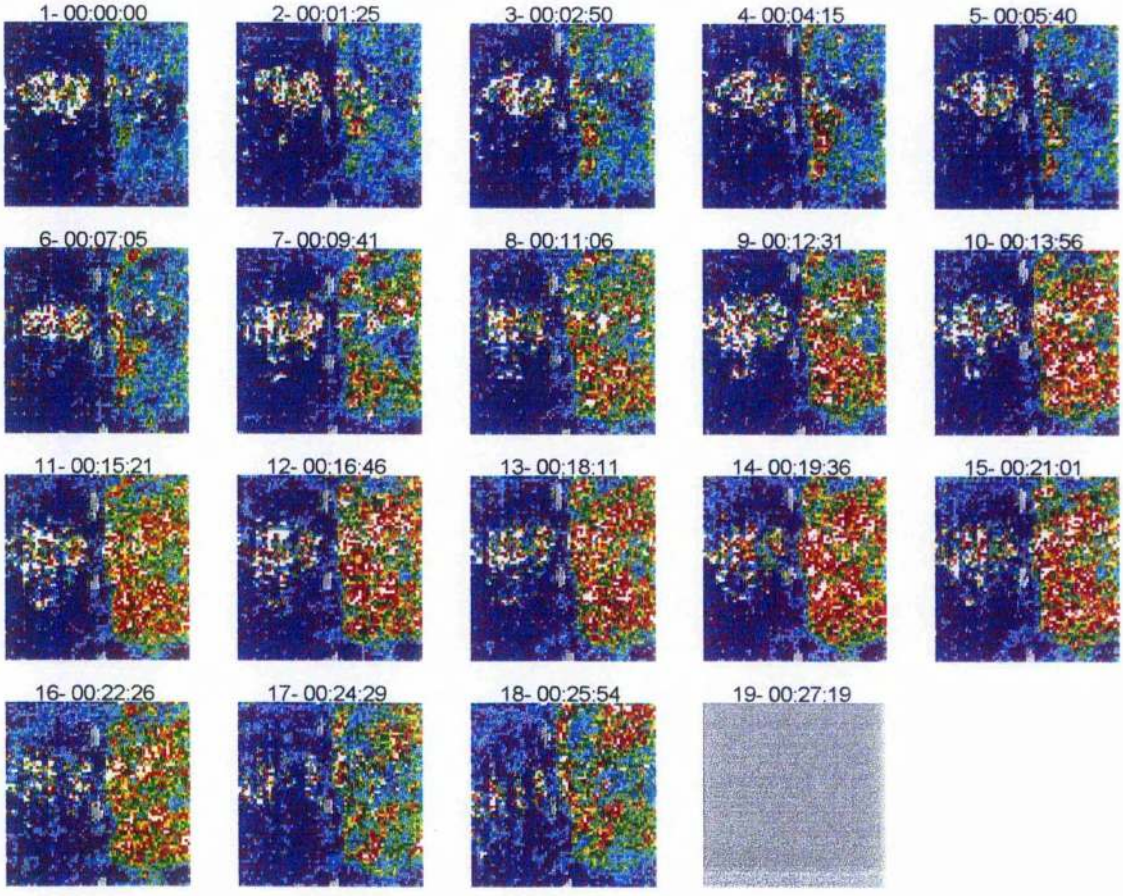
Figure 4

SNAG 1% in EtOH/ H_2O



In a study more relevant for therapeutic purposes, we also tested SNAG delivered in KY jelly. In order to do this SNAG was dissolved in a small quantity of ethanol, 10 % of final volume. This was then mixed with KY jelly to make up the final desired concentration, giving a pink gel. The gel is rubbed gently onto the skin and can then either be scanned through or wiped off prior to scanning. In the picture shown (figure 5, page 100) a sizeable area of the forearm has been studied (5 cm x 4 cm). Half of this was smeared with KY jelly : ethanol 9 : 1 and the other half with a 1 % SNAG solution in the same solvent system. These were scanned through over a period of 20 minutes. The initial flux levels were the same on both halves. Figure 5 shows a very distinctive

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8- 00:11:06
9- 00:12:31
10- 00:13:56
11- 00:15:21
12- 00:16:46
13- 00:18:11
14- 00:19:36
15- 00:21:01
16- 00:22:26
17- 00:24:29
18- 00:25:54
19- 00:27:19

increase in flux on the side bearing the SNAG solution. When these were wiped clean it was noted that the skin on the SNAG covered area was bright red whereas the control side showed no discolouration. This is a good example of how localised the vasodilatory effects of this system are.

Figure 6

SNAG 1 % in KYJelly

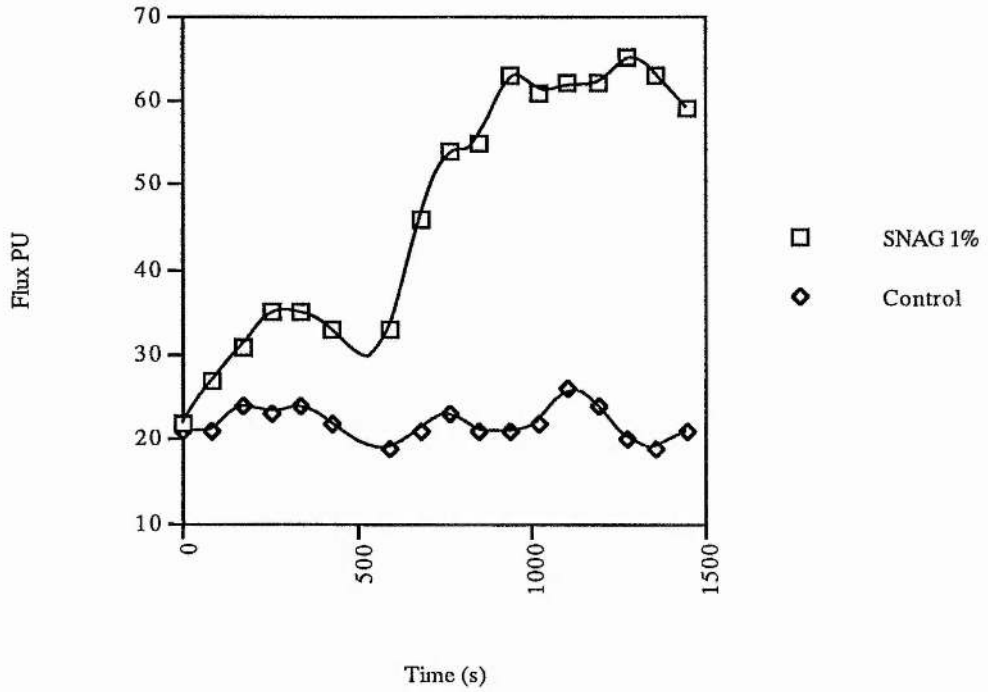


Figure 6 shows the variation of flux with time in graph format from the above study. When spread over a large area as above the increase in flux is much smaller (3-fold) then when the same quantities are used over a smaller area (8-fold). It should be noted that an 8-fold increase has been seen with all three delivery systems, suggesting that this may be the maximum obtainable with this compound. It is a surprise to find such closeness between the differing systems as there are many factors which should inhibit comparisons between the methods. When KY jelly is used it is difficult to quantify the amount or degree of "rubbing" given, though this will be the same for the control site. The rubbing is necessary for the jelly to pass through the skin. The uneven thickness of the jelly may also be of importance. This may affect the laser transmission and also the

amount of SNAG locally available to the skin at the particular site. We must also take into account the cooling effects of ethanol used in the solvent and the probable variation in stability of SNAG in these different solutions. In an ethanol solution there will probably be fewer of the copper ions present than in a water / ethanol mix. The stability of SNAG in KY jelly has not been studied but all in all three systems SNAG visually decomposes, i.e. loses its colour, at an equivalent rate.

4.3. Studies on blood flow variance in human cutaneous tissue following transdermal delivery of an NO donor.

As part of a trial in collaboration with Ninewells Hospital, funded by the Arthritis and Rheumatism Council, we are required to perform a study investigating the localised effects of nitric oxide, delivered transdermally from SNAG, on human skin blood flow. The full tests will involve fifteen healthy volunteers and thirty primary and secondary Raynaud's patients. The tests will not be completed in the lifetime of this study and therefore only preliminary results, from a smaller sample, can be reported here. However, with six healthy patients and nine Raynaud's patients, a sufficient number of tests have been performed for the results to be viable. The results of the tests on Raynaud's patients and their comparison with the data from healthy patients will be discussed in chapter 5. The results and implications of the trials on healthy volunteers are reported in this chapter. The study on the six healthy volunteers was reported as a full paper in its own right. Whilst this has been rewritten, it will be reported in a similar form.

4.3.1. Introduction

Skin ischaemia, with possible tissue necrosis, ultimately results from a blood flow problem in the nutritive microvessels. 80 - 90 % of the blood flow in the skin serves a thermoregulatory purpose, with only the small percentage remaining being involved with nutrition. However it is this remaining component which is important clinically and a failure at this level which may lead to a number of vascular problems as encountered in

disorders such as diabetes mellitus[22] and Raynaud's phenomenon.[23] Since the endothelium and NO are important regulators of vascular tone, it is therefore essential to determine the responsiveness of cutaneous microvessels to nitric oxide. In order to investigate this, different areas of cutaneous tissue with different thermoregulatory to nutritive vessel ratios have been studied. NO is known to be an important regulator of vascular tone at a number of different levels of circulation. However it is questionable whether cutaneous microvessels are responsive to it. This uncertainty arises from the variable effects of L-NMMA, an inhibitor of NO production, in cutaneous blood vessels. Coffman[24] showed that the brachial artery infusions of L-NMMA significantly reduced basal skin blood flow at the dorsum of the finger and the forearm. In contrast, Noon *et al* [25] demonstrated that L-NMMA did not reduce basal skin blood flow at the dorsal surface of the finger but did at the pulp where thermoregulatory arteriovenous shunts are abundant. However, a role for NO in the thermoregulatory control of skin blood flow in humans has been refuted. Thus while NO may be involved in the basal regulation of skin blood flow, the stimulated release of NO from cNOS appears to be small. Indeed Khan *et al* [23] have shown that L-NMMA does not inhibit cutaneous vascular responses to acetylcholine or methacholine. Studies have demonstrated that providing more L-arginine for NO production does not improve cutaneous vascular reactivity. Known vasodilators such as acetylcholine and sodium nitroprusside have been shown to have a relatively small effect on cutaneous blood flow. Even when sodium nitroprusside is infused intra-arterially, the cutaneous vascular response tends to be small[26] or even completely absent.[27] Of the previous studies concerning the effects of transdermally delivered NO upon cutaneous tissue, the donors have usually required metabolism, such as nitroglycerine, rendering them ineffective in the vascular beds being studied. These do not contain the necessary apparatus to effect this transformation (chapter 4.1). Others, such as sodium nitroprusside, require active transport across the skin. One method for this is via iontophoresis, whereby the current used may also be having an effect on the skin. Nitroprusside may also require extensive metabolism (chapter 1) and so results obtained may not be relevant to our studies.

Therefore, in this study, we are reporting for the first time the effect of an NO donor drug requiring no metabolic conversion to produce NO, on the cutaneous microvasculature in human subjects. From this we aim to highlight any variance in the response to NO between thermoregulatory and nutritive blood vessels.

4.3.2. Methods

Subjects.

Six healthy adult males were recruited as volunteers for the study. Ethical approval was granted from the Tayside Committee on Medical Research Ethics and informed consent was obtained . The mean age of the sample was 29 years (range 24 - 41). Blood pressure was 123/77 ± 14/12 mm/Hg and heart rate was 69 ± 13 beats per minute.

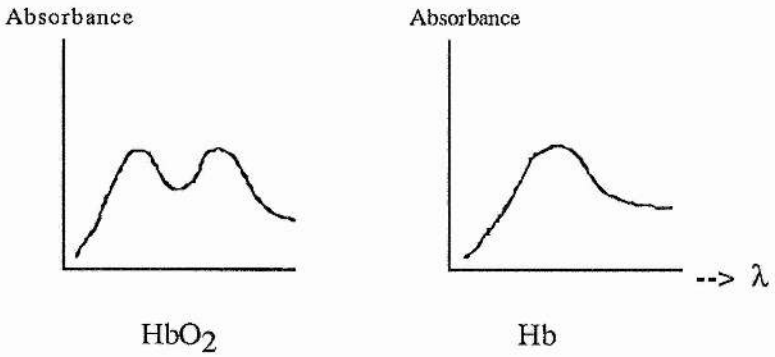
Measurement of skin erythrocyte flow.

The levels of blood flow in cutaneous tissue shows a considerable level of variance over any given area. To overcome this potential problem we used scanning laser Doppler imaging. Skin perfusion was measured in ambient light conditions using the MoorLDI scanning laser Doppler imager (Moor Instruments, Axminster, UK) which scans a low power laser beam (1.5mW helium neon , wavelength 632.8 nm) in a raster pattern over the skin surface. Moving blood in the microvasculature causes a Doppler shift which is processed to build a colour coded image of blood flow (erythrocyte flow). The image is stored on a computer which uses dedicated Windows software for image processing and analysis. The colour image is processed to give a blood flow in arbitrary perfusion units (PU). The scanner was placed 50 cm above the measuring area for all scans. The spatial resolution for the scan area was set at 100 x 100 pixels and the scan speed was 4ms/pixel. The median value was determined for each scan area.

Measurement of Skin Oxygenation

Oxygen saturation in the skin was measured with micro-lightguide spectrophotometry (EMPHO II, BGT, Gmbh, Germany). In this technique the absorption spectrum of the light reflected from the skin, between 500 and 620 nm, is analysed to provide an estimate of the concentration of the oxygenated haemoglobin, expected as a percentage of total haemoglobin. One optical fibre, of 250 μm internal diameter, transmits light to the skin and six similar fibres detect the reflected light. The catchment volume of the instrument is estimated to encompass predominantly capillary blood. For each measurement, the probe was scanned evenly over the skin and a set of 20 spectra were recorded continuously at a rate of one per second. The absorption spectra for haemoglobin and oxyhaemoglobin are visually quite different (figure 7) allowing interpretation of the spectra obtained. These spectra were analysed by fitting with set spectra of known oxygen saturation, to produce 20 values of blood oxygenation, from which the mean was calculated.

Figure 7



Statistical analysis

Changes in skin perfusion and oxygenation were determined using one-way analysis of variance (ANOVA) for repeated measures, followed by post-hoc paired t-tests for individual concentration when a significance was found. The null hypothesis was rejected at $p<0.05$.

4.3.3. Experimental Protocol.

The subjects were seated comfortably in a slightly cooled room (19°C) and allowed to equilibrate over a 20 minute period. The right arm was supported on a table at about heart level. Measurements were made over three different areas, the volar forearm, the dorsum of the finger and the finger pulp. Each site was cleaned gently with alcohol wipes. The first site to be studied was the forearm. The subject's arm was set up as to have the forearm facing upwards. Two circular perspex wells (diameter 2 cm, height 0.7 cm) were attached to the forearm with double sided adhesive tape. The edges of the wells were and all external areas of skin were covered with black material. The scanner was set to cover an area of skin containing both wells and for the scanning time to be approximately 45 seconds. The scans were taken every one and a half minutes, allowing time for the skin oxygenation readings to be taken in between. A scan was over each area to obtain a "dry" baseline for skin perfusion and oxygenation. The wells were then filled with 50 % aqueous ethanol and "wet" baselines for perfusion and oxygenation recorded. This was to assess the influence of the solvent on readings, as regards cooling etc. One well was then emptied and refilled with a solution of 0.5 % SNAG in aqueous ethanol. Scans were then recorded over a period of 15 - 20 minutes, until no more increases in readings were observed. The wells were then emptied, moved to a different site, and the process repeated using 1 % and then 1.5 % solutions of SNAG. After the tests were complete, the subject was allowed 5 minutes to stretch the arm and normalise the blood flow. The arm was turned over as to have the back of the hand facing upwards and the fingers flat on the table. Three wells were constructed for the fingers out of caulking compound. Blu-tac was found to be the most effective substance for this purpose. These wells, of 1 cm diameter and 0.8 cm height, were firmly attached to the dorsum of the middle three fingers using double sided adhesive tape. As before, both dry and wet baselines for perfusion and oxygenation were recorded. The aqueous ethanol was dried from the wells and these were then filled with 0.5, 1.0 and 1.5 % solutions of SNAG. These were then scanned as for the forearm. Care needed to be taken to avoid a leakage of the contents from the well. In order to avoid the choice of finger having any bearing

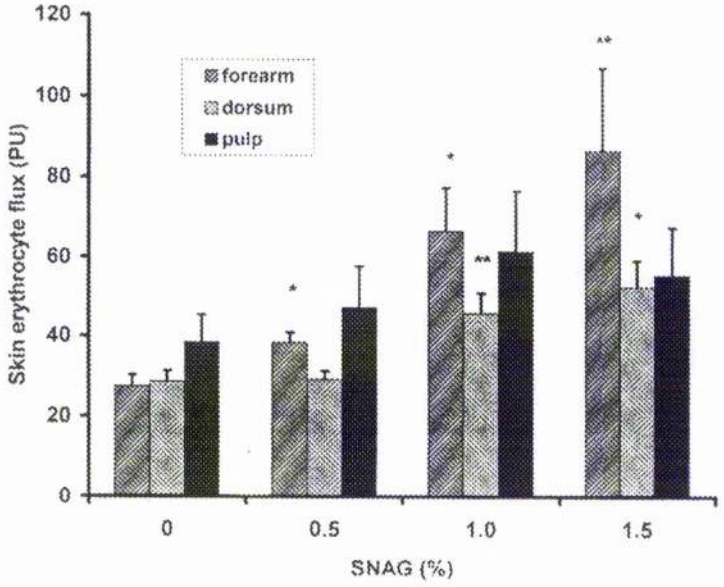
on the results, different concentrations of SNAG were used on different fingers with each volunteer. Finally the same procedure was repeated on the pulp of the middle three fingers. This is a relatively uncomfortable position for the patient to maintain and the hand requires to be supported for the fingers to be at the correct angle.

4.3.4. Results

Skin erythrocyte flux.

Baseline skin erythrocyte flux was not significantly different at any of the three sites. Figure I shows that transdermal application of SNAG produced significant concentration-dependent increases in skin erythrocyte flux at both the forearm ($p<0.01$, ANOVA) and dorsum of the finger ($p<0.005$, ANOVA). For the forearm, post-hoc t-tests showed significant increases in skin erythrocyte flux from baseline at all three concentrations ($p<0.02$ for all concentrations, figure 1). At the 1.5 % concentration, skin erythrocyte flux increased from 27 ± 3 PU to 86 ± 21 PU. With the 0.5 % solution it was difficult to observe any increase in flux by way of a colour change on the computer screen. The increase was visually noticeable at the higher concentrations. At the dorsum of the finger, post-hoc testing showed a significant increase in skin erythrocyte flux at 1% ($p<0.02$) and 1.5% ($p<0.02$) concentrations with no significant increase at 0.5 %. Skin erythrocyte flux increased from a baseline value of 29 ± 3 PU to 52 ± 7 PU after application of 1.5 % SNAG. The results for erythrocyte flow in the pulp shown in figure 8 suggest that SNAG has caused an increase. However, statistical analysis demonstrates that the increase is not significant. The increase shown was mainly due to an outlier from one subject who showed some increase in blood flow. Visually there was no increase in flux during the experiments on the other subjects.

Figure 8

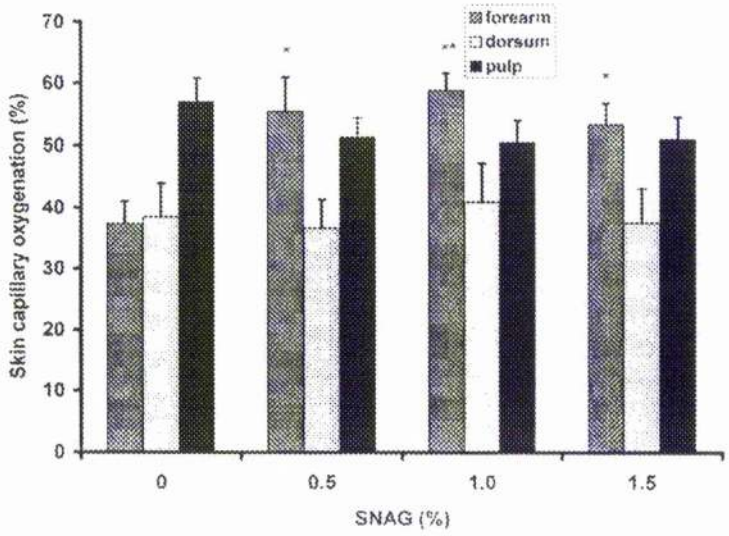


The application of aqueous ethanol was found to produce no changes in skin erythrocyte flux at any of the sites in question.

Skin oxygenation

Figure 9 shows that at the forearm SNAG produced a 40 % increase in oxygen saturation ($p<0.001$, ANOVA) and post-hoc testing showed this to be significant for all three doses ($p<0.05$ for 0.5 % and 1.5 %, $p<0.01$ for 1.0 %, paired t-tests). In practise the value for oxygen saturation probably reached its maximum with the 0.5 % dose and no further increase was possible. At the dorsum of the finger, plots of oxygen saturation over time for individual subjects showing highly fluctuating values but with no significant change overall. At the finger pulp there was no significant increase in oxygen saturation from the baseline at any concentration of SNAG. It is shown (figure 9) that the baseline was higher for the pulp than forearm or dorsum and this may have had some bearing on the results. Were the saturation already at its maximum then we would not have seen an increase even had the vessels been responsive.

Figure 9



4.3.5. Discussion

The three areas of cutaneous tissue were selected as they represent good examples of the two different blood vessel functions. The perfusion in the forearm and dorsum of the finger serves a mainly nutritive role and plays little part in the control of body temperature via dilation or constriction. The fingertips, however, are rich in arteriovenous shunts. These can be viewed as channels which may divert large quantities of blood through them to help in thermoregulation. The increase in perfusion flux in the forearm and dorsum, with no increase in the pulp, shows a very definite difference in the responsiveness of the different types of cutaneous microvessels. Our results demonstrate conclusively that nutritive microvessels are responsive to exogenous NO. The significant increase in oxygen saturation of the forearm is also suggestive of an increase in capillary perfusion. The catchment volume for this technique is thought to encompass mainly capillary flow,[28] where the oxygen is being consumed. The initial levels of oxygen saturation, upon entering the capillary system, should be the same irrespective of the blood flow. However, the tissue may only use a certain amount of this oxygen as the blood passes through, therefore the percentage consumed from a higher blood flow will be less than that consumed from a lower blood flow. It is because of this that we see an increase in the levels of oxygen saturation rather than an increase in the uptake of oxygen by the

blood. From this we can equate a higher blood flow with a higher level of oxygen saturation of haemoglobin. To explain this increase a decrease in oxygen consumption is unlikely. Results from the dorsum of the finger are more difficult to interpret. No overall change in capillary oxygen saturation is seen but individual values fluctuated between high and low values throughout the period of measurement. This gives an average zero response. These results, alongside the increase in perfusion flux, suggest that SNAG is having an effect on the dorsum cutaneous microvessels. From these results we cannot say exactly what the effect is. Despite the confusing nature, the fluctuating values for oxygen saturation did turn out to be of significance when compared with the corresponding results from Raynaud's patients (chapter 5). The results from the tests upon the finger pulp suggest that microvessels with a thermoregulatory function do not respond to exogenously delivered NO. This interpretation was hindered by the suggestion that the skin at the finger pulp site may be of a different nature to the skin on the forearm and the dorsum and that this may be the factor causing this difference. If this skin were tougher or thicker or have a thicker layer of dead cells covering it then it might affect the results. To try and answer this, a number of methods for removing any dead cells were investigated. Sticky tape was applied to the fingertips and sharply removed to pull off any loose tissue. The perfusion and oxygenation results were similar to those above. It is possible to shave off large amounts of dead skin from the fingertip with careful usage of a scalpel blade. This was performed the evening before testing to allow the finger time to recover. When SNAG was applied, the results were identical to those seen before. Finally the injection of a very small quantity of SNAG solution into the finger pulp was tried. This proved very painful and the area surrounding the injection site showed a large degree of trauma, disguising any potential vasodilation. As suggested above, the lack of rise in the oxygenation of the finger pulp blood flow may have been due to the levels already being at their maximum. To investigate this we cooled the subject (room temperature of 14°C) to obtain a lower baseline. Despite the low baseline we did not see any increase upon addition of 1.5 % SNAG solution. From

our results we felt we could confidently say that the microvasculature in the finger pulp was not being affected by the exogenous NO being supplied to it.

4.3.6. Conclusions

The finding that SNAG causes significant concentration dependent vasodilation of nutritive cutaneous microvessels is consistent with these blood vessels being responsive to exogenous NO. SNAG has a considerable advantage over other commonly used donors such as nitroglycerine in its mode of action and its mode of delivery. It is rapidly metabolised such that the vasodilatory effects remain local and there are no systemic side effects such as headache, flushing and nausea. Considering the small scale applications involved it has been no surprise that we have not encountered any problems with tolerance. With the apparent thermal decomposition of SNAG, without the need for transnitrosation, we do not expect tolerance to develop were SNAG to be used for therapeutic purposes. A compound such as this may have important therapeutic implications in several areas of microvascular dysfunction. The ability to increase nutritive capillary flow through NO may be of particular importance in patients with diabetes mellitus who are prone to skin ulceration and infection. It has been suggested that vascular sensitivity to NO is diminished in patients with diabetes mellitus,[22] perhaps due to oxidative stress[29] or quenching of NO by the formation of advanced glycosylation end-products.[30] Providing NO transdermally to compromised areas of tissue may enable NO levels to be elevated sufficiently to overcome its inactivation. Furthermore, local, transdermal application of an NO donor could limit the "steal" phenomenon seen with other non-specific vasodilators. In the "steal" phenomenon ischaemia can be worsened due to the diversion of blood through non-nutritive thermoregulatory vessels but also to existing healthy blood vessels. With direct application of SNAG to an affected area it should be possible to avoid this occurrence.

The rapid action of SNAG makes it an ideal compound upon which to base these studies. Work carried out on isolated rat-tail arteries has shown it to be an effective NO donor.

The vasodilatory action is inhibited by the addition of haemoglobin, demonstrating that it is indeed the NO and not some other facet of the molecule having this effect.

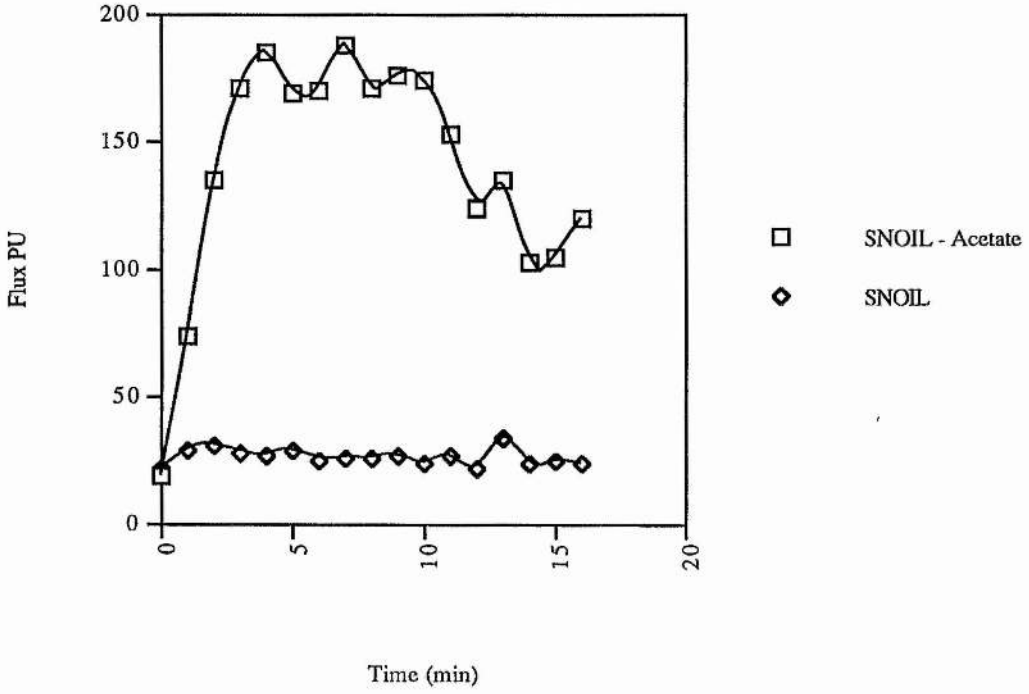
4.4. Other transdermal delivery studies.

As stated in 4.2, a lack of time has prevented us from obtaining permission for full transdermal tests on other compounds. We were able to carry out preliminary tests, using ourselves as volunteers, on other compounds which showed potential.

The two derivatives of thioglycerol, known as SNOIL and SNOIL-acetate were investigated. We were particularly interested in these compounds due to their structural similarity to nitroglycerine, one of the most effective transdermal agents and one of the most efficient NO donors. Despite their apparent similarities, the two thioglycerol derivatives have very different solubilities. SNOIL is very hydrophilic and SNOIL - acetate is fairly hydrophobic. They are both very stable compounds under certain conditions but are sometimes found to be very unstable, explosively so at times. Solutions of the two compounds (1 % in ethanol / water) were placed in the plastic wells and the blood flow investigated using the scanning laser Doppler. The results (figure 10) demonstrate a completely different effect between the two. SNOIL has no effect on the blood flow whereas SNOIL - acetate has given a rapid and vast increase, larger than we had seen for any vasodilatory compound. This is in accordance with suggestions that a molecule must have a low degree of water solubility to pass through the skin. It is hoped that further work, both on the kinetics and the physiological usage of these compounds will be possible and that their potential be fully investigated.

Figure 10

SNOIL and SNOIL Acetate



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Chapter 5: Investigations into Raynaud's Phenomenon.

5.1. Introduction

Also known as symmetric gangrene or local asphyxia, this was first described by Raynaud in 1862. However, despite the disease being known for over 130 years, the etiology remains an enigma. Between 1991 and 1993 at least 38 different therapies were advocated and seventy different theories put forward as to the underlying cause.[1] These will be discussed later. Interest in the disease has increased dramatically over the last few years with over two hundred papers published each year and a well supported charity. As more is learnt about the disease and its importance and relevance to other disorders its significance receives greater appreciation, especially as the disruption to the individual and to society becomes apparent.

It is a disease that afflicts approximately five percent of the population, though the figure varies drastically, depending on the exact definition of the disease.[2] Of these up to 80 % are females.[3] This may be due to the observation that women have a lower hand blood flow than men.[4] The disease tends to be prevalent in those under the age of about forty years. Not surprisingly it is most common in the colder parts of the world.[5]

Raynaud's Phenomenon (RP) is a paroxsymal or intermittent spasm of the digital arteries[6] and possibly involves other factors such as platelet aggregation[3] disrupting the blood flow. The symptoms encountered by a sufferer involve a loss of blood circulation to the affected area, usually the hands. This leads to the hands becoming numb and useless. Intense pain is felt when the circulation is restored. Sufferers tend to have lower blood flow in the digital microcirculation than normal healthy subjects and have a lengthier recovery period. The skin microvessels contract and the blood flow is blocked. This is most usually caused by exposure to the cold and emotional stress. In a susceptible individual temperatures as high as 17°C can cause an onset of the symptoms.[7] In the most severe cases temperatures of 25°C can be sufficient to initiate an attack, i.e. well above the normal maximum temperature both outside in northern Europe and indeed

inside a normal house. In a mild case of the disorder the only symptoms are pale and numb fingers.[8] Sufferers may have dry, cracked hands with ulcers on the fingertips and skin with a prematurely aged appearance.[9] In a more advanced case the extremities become painful and useless, taking on a livid red colour accompanied by a throbbing pain. In the most serious of cases, cyanosis and superficial gangrene can occur. The gangrene can be of a moist or dry nature and can lead to the loss of the digits involved. Damage caused generally takes a long time to heal and can leave the fingers looking shiny, smooth and claw-like.[10] While the fingers are the most frequently affected part of the body, it is also possible for the toes, ears, nose and tongue to be afflicted.

RP is a heterogeneous disorder with many different stimuli producing the same symptoms. The name is generally used to describe the symptoms of loss of circulation irrespective of the cause. RP can be divided into primary and secondary. Secondary is taken to mean RP is associated with an underlying disease, whereas Primary RP is taken to mean that no underlying disease co-exists. This is referred to as Raynaud's Disease (RD) which is often hereditary. RD is usually diagnosed when there is an absence of any symptoms or signs of systemic disease that might account for the occurrence of RP. There are two main theories to explain RP: an increase in sympathetic nervous activity or a local fault such as a drop in CGRP-like nerves or an increase in α-adrenoreceptor activity. The cause of these is as yet unknown.

Of the many prospective causes of RP, one is not in dispute. In early 1996 miners suffering the disorder won the right to compensation in the courts.[10] Workers suffering RP through the long term usage of vibrational tools were recognised as victims of the so called Vibrational Induced White Finger (VWF).[11,12] This is very common amongst workers exposed to excessive vibration which damages blood vessels. This includes miners, chainsaw operators and those involved in polishing metal especially in the aerospace industry. This may frequently demand a career change, particularly difficult for some professions such as secretaries involved in typing. The disorder often affects

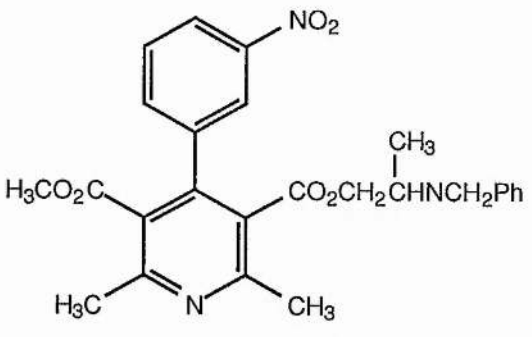
piano players in later life. There are also a number of chemicals implicated in the onset of RP such as PVC. Similarly those exposed to the cold on an intense or long term basis stand a good chance of developing RP. Prolonged changes in environmental temperature may alter thermosensitivity in humans. A good example is shown in that after 10 or more years of daily exposure to iced fish, 90 % of frozen fish filleters developed RP.[13]

Patients with RP have been shown to have abnormally increased platelet activity.[14] Hyperactive platelets may further impede blood flow in the already contracted blood vessels of the sufferers and platelet aggregation may have a pathophysiological role in RP. Exposure to silica[15] and silicone[16] from breast implants has been shown to lead to an incidence of RP much above the average. Patients receiving chemotherapy, particularly antineoplastic agents, cis-platin and bleomycin as used in the treatment of testicular cancer, are very likely to develop RP.[17,18] Other commonly suggested triggers include an enhanced response to catecholamines[19] and female sex hormones.[20]

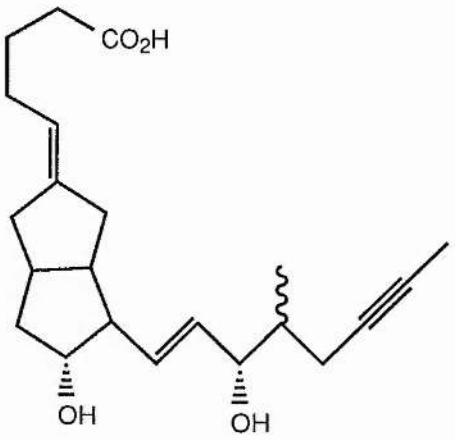
The suggestion that nitric oxide is involved is not a new one. In tests the response to nitroprusside, a well known, if inadequately studied, NO donor, has not been diminished in Raynaud's sufferers, whereas that of bradykinin has. This suggests that endothelium dependent venodilation is impaired in peripheral vessels in RP, possibly due to diminished release of nitric oxide and may contribute to the pathogenesis of the disorder.[19] Levels of cGMP (involved in the pathway leading to smooth muscle relaxation and hence increased bloodflow) increased in a control group after exposure to the cold but levels remained constant in RP sufferers, suggesting they lack the physiological response of cGMP to cold exposure.[21] A deficiency of immunoreactive CGRP nerve fibres has been demonstrated in the digital cutaneous micro-vasculature of patients with RP. The distribution of other nerve fibres seems to be unaltered.[22] Raynaud's suggestion that RP is caused by overactivity of the sympathetic nerves is still regarded as being a possibility.[13]

A final contributing factor toward RP would appear to be lifestyle itself, attacks are more common in smokers and those taking oral contraceptives and are often brought on by stress and other strong emotions.

When stress management and relaxation training fail to control symptoms then various medications may be prescribed. These include calcium channel blockers such as nicardipine.[23] This is generally well tolerated and can give effective improvement in RP patients provided there are no underlying diseases.[24] A well tested treatment is the prostacyclin analogue iloprost.[25] The action of this is not defined but it is known to inhibit platelet aggregation and provide cytoprotection.[26] Giving evidence of the nitric oxide involvement, L-arginine, from which NO is produced in the body, has been shown to be of some use in the treatment of RP[27] although recent studies have conflicted with this conclusion.[28] The most commonly used treatment at the moment involves a sympathectomy, whereby doses of phenol are given to the affected areas. This basically kills the nerve endings, removing their ability to cause vasospasm. This is a fairly effective treatment but usually leads to a reoccurrence of the problem within a short period of time.



Nicardipine



Iloprost

The lack of suitable treatment is highlighted by some of the more comical suggestions seen for the control of RP symptoms. The Sunday Times[29] suggests massaging the feet with a few drops of essential oil of black pepper diluted in a tablespoon of almond oil.

This is probably as effective as any other suggestion yet put forward. Finally the suggestion of Raynaud himself involving, "a galvanic current with the positive pole applied over the fifth cervical vertebra and the negative over the last lumbar vertebra".[8] With a moderately strong current applied for five to ten minutes daily this treatment appeared to be effective in some cases. Whilst this is not a common treatment at present, the usage of spinal cord stimulation has been proposed for the treatment of RP "because of the high probability of failure of other medical or surgical therapy."[30]

Treatment of the disease is limited in that the most effective drugs for vasodilation are ineffective on the skin microcirculation. The most commonly used vasodilators are glyceryl trinitrate and isosorbide dinitrate. However these are compounds that require to be metabolised in order to release nitric oxide. The necessary machinery to metabolise these is only contained in microvessels of diameter larger than about 100 μm. The skin surface microvessels are only about 20 μm in diameter. Therefore a nitric oxide donor which can spontaneously emit NO is preferable. In these compounds the decomposition is caused thermally, photolytically or by transnitrosation or metal ion catalysis. It is on this basis that we feel it worthwhile synthesising a nitric oxide donor which has similar characteristics to glyceryl trinitiate and isosorbide dinitrate in size and solubility but will emit nitric oxide spontaneously. These, it is hoped, will aid our understanding of the disease and assist in the formulation of a successful treatment by showing whether the defect lies in a lack of nitric oxide production, a lack of ability to use it, or neither.

However, the most vital information this may supply will be the connection of RP to other vascular disorders where a deficiency in NO production is involved. RP often heralds connective tissue disorder,[31] in fact 50% of secondary RP sufferers are likely to develop a connective tissue disorder such as CREST (calcinosis, Raynaud's oesophageal dysmotility, sclerodactyly, telangiectasia) and other collagen vascular diseases including scleroderma.[32] RP can also be used as an early warning of an underlying problem, notably testicular cancer.[33] The prevalence of migraine is significantly increased in

subjects with RP,[34] with as much as twice the normal incidence.[35] This suggests that these conditions may share a common pathogenic defect or mechanism.

5.2. Vasodilatory Responses of Raynaud's Patients.

As stated in chapter 4, the aim of the ARC funded trial was to study a number of sufferers of Raynaud's disease and their skin blood flow responses to the vasodilator SNAG. These were then to be contrasted with the results from tests upon healthy volunteers. From this study we hoped to learn a little about the disease and possibly suggest a treatment for it and similar conditions.

5.2.1. Selection of Candidates.

Ten volunteers from the Tayside area, suffering from Raynaud's disease, were accepted as part of the trial. Out of these, four showed primary Raynaud's and six showed secondary Raynaud's phenomenon. The ages ranged from 16 to 65. A number of these candidates had visible symptoms of Raynaud's, such as the pinched appearance of the face, particularly the mouth. Some of the secondary patients also suffered from systemic sclerosis and had slightly deformed hands. It is possible that a thickening of the skin in some of the sufferers may have affected the ability of the drug to pass through to the living tissue. It is also fair to postulate that we are dealing with a different type of skin in the volunteers with such conditions as scleroderma and systemic sclerosis. However, comparisons between these patients and those with primary Raynaud's and no other symptoms, suggest that it has had little influence upon the results. The degree to which each of the patients was affected by the disorder varied dramatically, from those who suffered several attacks per week, to those who only found problems during periods of cold weather. The stimuli causing the attacks also showed considerable variation, some subjects were affected by emotional stress and others found it had little or no bearing on the onset of an attack. For the completed study ($n = 45$) there will be an equal number of primary and secondary sufferers. Each of these will be matched with a healthy patient of the same age as to eliminate the natural changes in circulation which occur with increasing age. The full study will eliminate the errors caused by the heterogeneous nature of the sample. With the smaller numbers discussed in this report we can only look at the general pattern of response.

5.2.2. Methods

The effects of SNAG upon the cutaneous blood flow were as discussed in section 4.3. The blood flow in the forearm, dorsum of the finger and pulp of the finger were investigated at 0.5, 1.0 and 1.5 % solutions of SNAG using scanning laser Doppler Imaging. The % oxygen saturation was also followed using micro-lightguide spectroscopy. The overall time for the tests was between $1^1/_2$ and 2 h per patient.

5.3. Results

The results of the study are shown in comparison to those from the tests carried out upon healthy patients from chapter 4.

5.3.1. Skin erythrocyte flux.

The results shown below compare the blood flow changes in response to SNAG for healthy patients and Raynaud's sufferers. These have all been calculated as % changes relative to the baseline rather than as individual flux values shown in chapter 4. It is because of this that some of the values appear to be contrary to those already shown.

Figure 1 shows that transdermal application of SNAG produces a dose dependent increase in blood flow in the Raynaud's patients. However it is noticeable that this increase is dramatically smaller than that obtained in healthy patients. At the 0.5 % dose the increases for Raynaud's and healthy patients were 29.5 and 44 % respectively. The differences are greater at the 1.0 (40 and 123 %) and 1.5 % concentrations (59 and 144 %). A similar pattern is seen on the dorsum of the finger at the higher concentrations (figure 2). At the 0.5 % concentration, the % increase for blood flow for the Raynaud's and healthy patients were 3.8 and 2.0 % respectively. A large difference in the % increase was seen at 1.0 (10 and 60 %) and 1.5 % (-1.2 and 84)%. The results for the erythrocyte flux in the pulp are more difficult to interpret (figure 3) as they show no pattern and were not found to be of statistical significance ($p>0.05$ for all values).

Figure 1

% Increase in blood flow over baseline on forearm

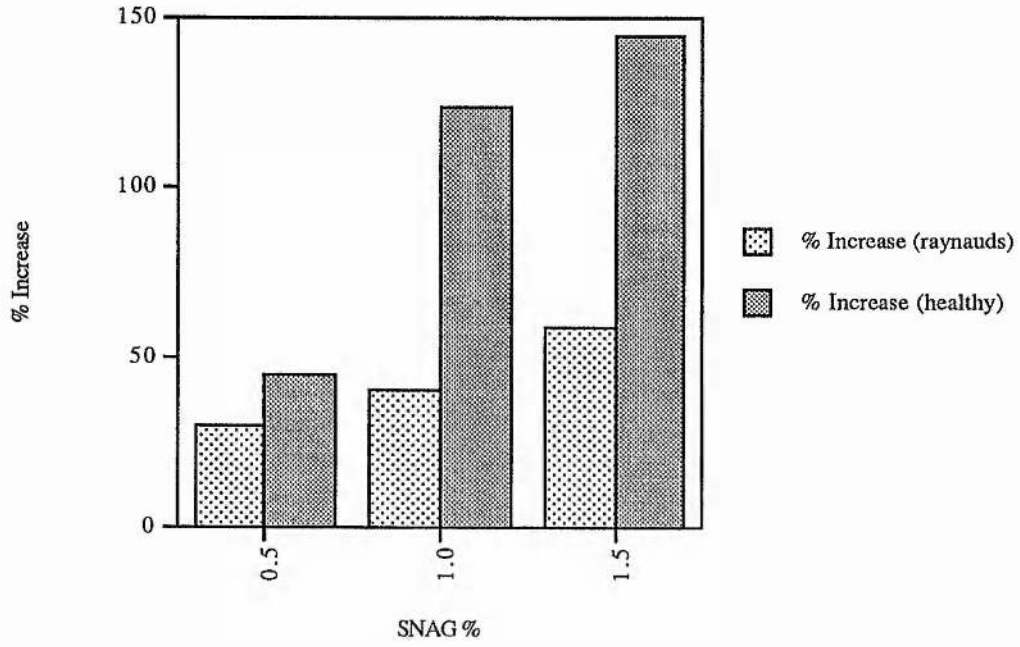


Figure 2

% Increase in bloodflow over baseline on dorsum

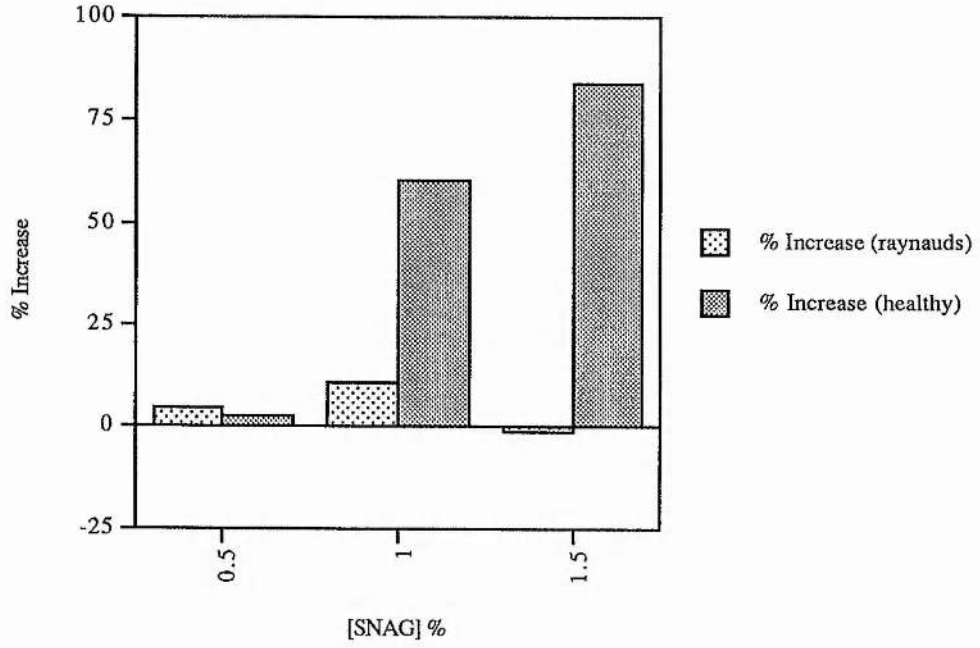
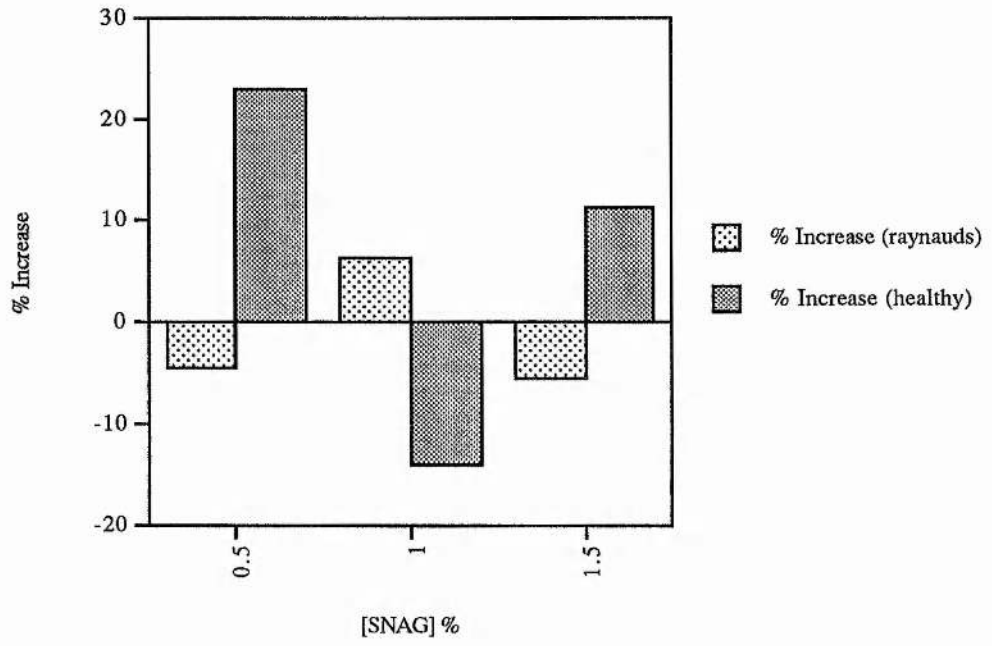


Figure 3

% Increase in blood flow over baseline in pulp



5.3.2. Skin Oxygenation. The results below compare the levels of oxygen saturation in the skin of Raynaud's sufferers and healthy patients. The results are shown as actual levels of skin oxygenation rather than % increases to highlight important differences, especially in figure 6. Statistical analysis was carried out as chapter 4.

The effects of transdermally delivered SNAG on the forearm are shown in figure 4. These demonstrate a similar pattern between both sets of patients, both showing a significant increase in pO_2 upon application of SNAG ($p<0.001$). The 0.5 % dose causes relatively high increases in pO_2 of 57 and 51.5 % for the Raynaud's and healthy patients respectively. In healthy patients this would appear to be about as much of an increase as is possible, the 1.0 and 1.5 % SNAG doses giving pO_2 increases of 54 and 46 % respectively. In the Raynaud's patients a further increase in saturation is seen with pO_2 levels of 74.5 and 76.5 % for the 1.0 and 1.5 % solutions respectively. Whilst there

Figure 4

Forearm

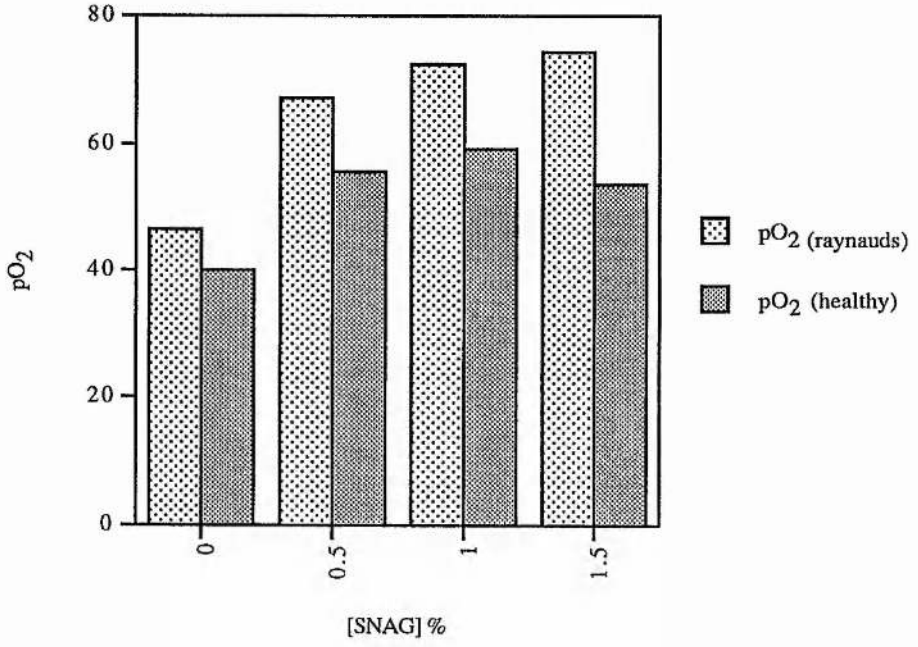
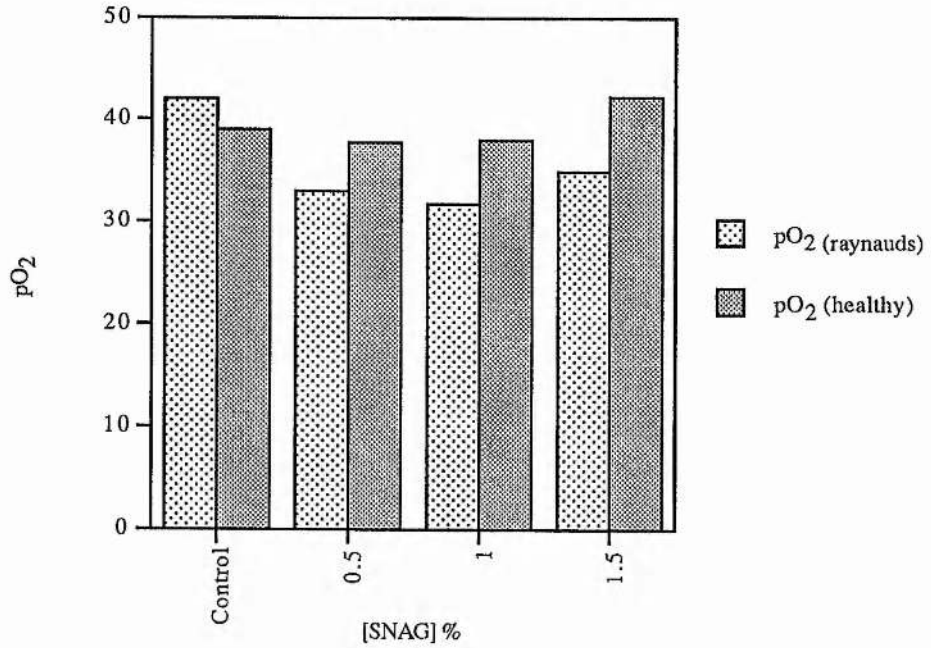


Figure 5

Dorsum

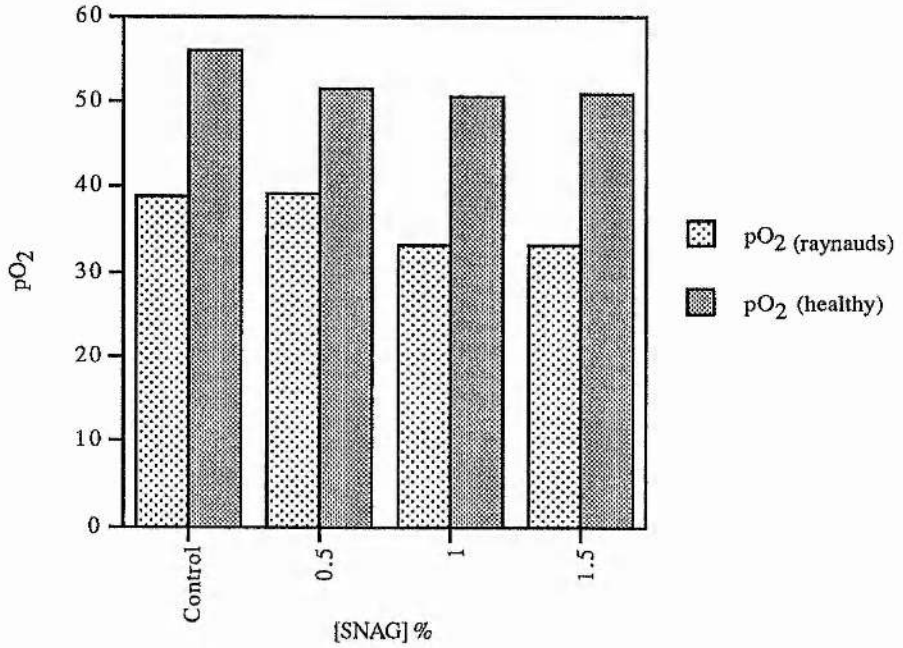


does appear to be a trend showing a higher 0_2 saturation in the Raynaud's patients, this is not borne out be rigorous statistical analysis ($p>0.05$).The results shown in figure 5 compare the change in oxygen saturation in the dorsum of the finger between the two sets of patients and demonstrate a lack of any significant change in pO_2, ($p>0.05$). This is explained in 5.4.

The changes in pO_2 levels in the pulp of the finger for the two sets of patients are compared in figure 6. The results demonstrate that there were no significant changes in the levels of oxygen saturation for either the healthy patients or the Raynaud's sufferers, in fact the graphs show a slight decrease. It is noticeable that there is a markedly higher level of pO_2 in the finger pulp of healthy patients. These patients showed an average initial pO_2 level of 56 % whilst the Raynaud's patients had an average initial reading of 39 %.

Figure 6

Pulp



5.4. Discussion

The results show that the two groups of patients show very different responses to exogenous nitric oxide. This response is quite difficult to quantify and the results have shown that there may be little correlation between the two methods that have been used. This is most obvious in the results for the forearm, whereby the healthy patients show a much larger increase in blood flow, but the Raynaud's patients show a slightly larger increase in pO_2. The blood flow differences are very dramatic and cannot be discounted, demonstrating that Raynaud's patients have a lesser ability to respond to exogenous NO. The micro-lightguide spectroscopy technique studies the blood at a very superficial vascular level and the ability of Raynaud's patients to respond at this level may have little bearing on the disorder. As this technique is not as fully tested as laser doppler imaging, connections between the two sets of results cannot be made with 100 % certainty. The results from the dorsum for both sets of patients showed no significant increase in the levels of saturation as shown in 4.3. However, though not described by the graph, the results were very different. The pO_2 figure is calculated from the mean of a number of values as described previously. With the Raynaud's sufferers there was very little deviation from the mean and it was obvious that SNAG was having little or no effect upon the area studied. With the healthy patients there was a large variation in the results with alternating high and low readings being recorded. This suggested that SNAG was having an effect upon the subject. Therefore calculation of the mean, giving a non-significant change in pO_2 for both sets of patients, does not accurately reflect the effects of SNAG upon the levels of oxygen saturation in the dorsum. From this it is possible to say that there is a difference in the dorsum response between Raynaud's and healthy patients, with Raynaud's patients giving a lesser response. However it is not possible to quantify what this difference may be. In view of the lack of response in the pulp of healthy patients it was not surprising to note the lack of response in Raynaud's patients. Though the pO_2 did not change during the experiments, there was a much lower starting value in the Raynaud patients. This low value in the finger pulp may turn out to be of

major importance in the etiology of Raynaud's Phenomenon but, as the pulp is unresponsive to exogenous NO, this will be difficult to investigate.

These results have highlighted that the ability to respond to exogenous NO is an important factor in the defect involved in RP and that there is a marked difference between sufferers and non-sufferers. From these we can suggest that the blood vessels of Raynaud's patients either have a diminished ability to dilate, in response to nitric oxide, or that there are other factors which over-ride this external stimulus, such as sympathetic nerve activity.

5.5. Conclusions

Despite our hopes that an NO donor could be of therapeutic benefit in the treatment of skin disorders, it became apparent from an early stage that SNAG would not be a suitable candidate for this purpose. SNAG has too low a stability to be used as a drug and the vasodilatory responses it causes are much too transient. However, this rapid action, short term response characteristic makes SNAG an excellent compound for a study such as this. It has no known side effects and the vasodilation caused is very much localised, allowing many sites to be tested without affecting each other another. It also gives a rapid response allowing patients to be tested quickly. This has allowed us to carry out the first study investigating the effects of exogenous nitric oxide on Raynaud's patients. It has also allowed us to make the first study that directly compared the differences in the response between Raynaud's sufferers and healthy patients. From an investigative point of view the nature of the results were unimportant as long as they were reproducible. Two of the possible causes of RP involved the sufferer either not producing sufficient quantities of NO, or the blood vessels not having the ability to respond to it. It is possible that the two are connected, a long term lack of NO leading to damage of the tissue and so reducing its capability to dilate when exposed it NO. From our point of view the first cause would have been of more interest as it would have allowed for the possibility of using an NO donor in the treatment of RP. As it was, our results point

toward the second cause being of more significance. This dictates that NO donors are unlikely to be a particularly effective treatment for RP.

Despite finding our compounds to be of little value in the treatment of RP, the study has been instrumental in demonstrating one of the defects present in sufferers. It has, however not told us how this has come about. Further studies clarifying the effects of L-arginine in both RP and healthy patients, as well as investigations into the levels of L-arginine in RP sufferers, will be necessary to determine how much of the NO pathway is damaged. It may be that it is only the lack of NO induced vasodilation which is different in RP patients or it may be that there are several defects in the pathway. This information will be of use in the continuing search for a treatment or cure for Raynaud's phenomenon.

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Chapter 6: Kinetic Studies

6.1. Introduction

In recent years a large amount of the work performed with nitrosothiols has involved investigations into their mode of decomposition.[1] When studying the decomposition of SNAP, in order to determine the mechanism of NO release, it was often difficult to obtain reproducible values for the half life. In view of the fact that SNAP is very stable as a solid and yet sometimes decomposed very rapidly in solution, it was concluded that something about the solvent must have been responsible for causing the decomposition. Differences in the half life were shown to occur for different batches of distilled water, suggesting that some carried a higher level of impurities. When EDTA, a metal chelating agent, was added to a solution of SNAP, the rate of decomposition was greatly reduced. Studies, using a range of metal salts, revealed copper to be the major influence upon decomposition.[2] A number of later studies showed that it was in fact the Cu(I) ion which was responsible.[3] This was found to be slightly peculiar in that Cu(I) is unstable and is rapidly oxidised to Cu(II) under physiological conditions. The addition of neocuproine, a specific Cu(I) chelator, dramatically reduced or even halted decomposition.[4] A reaction mechanism for this is shown in figure 1.

Figure 1[4]

(1) $RSH + OH^- \rightleftharpoons RS^- + H_2O$

(2) $RSNO + 2OH^- \rightleftharpoons RS^- + NO_2^- + H_2O$

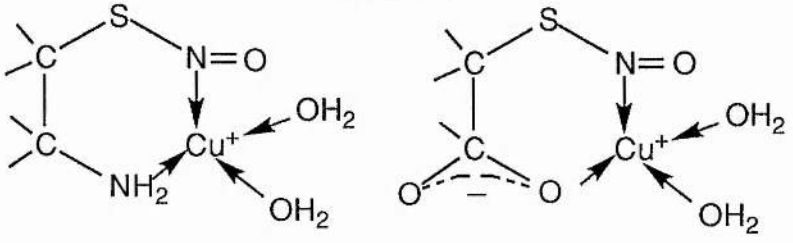
(3) $RS^- + Cu^{2+} \rightleftharpoons [RSCu^+] \xrightarrow{SLOW} RS^{\bullet} + Cu^+$

(4) $RSNO + Cu^+ \rightleftharpoons [RSNOCu^+] \xrightarrow{FAST} RS^- + Cu^{2+} + NO^{\bullet}$

(5) $2RS^{\bullet} \longrightarrow RSSR$

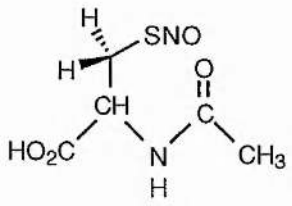
The first steps involve the production of $RS^{\cdot}$. This may be from a thiol impurity (1) or from the reaction of the nitrosothiol with OH^- (2). These steps may be regarded as the initiation stages, whilst the concentration of RS^- is building up. During this, a lag phase in the decomposition is sometimes seen. When thiol is added to the solution, or an impure sample used, a more rapid decomposition, without a lag phase is seen. The reaction is autocatalytic, regenerating RS^- and Cu(II) so that the reaction may continue producing NO. Finally thiyl radicals will combine to produce disulphide. Despite the complexity of the full reaction scheme, the rate of decomposition remains dependent upon the concentrations of RSH and Cu(II). When step (3) is slow and step (4) fast there is zero order dependence on RSNO concentration. In the human body there is about 0.1 g of copper per 75 kg body weight.[5] This is mostly present in a bound form, as serum albumin. Very little copper is present as free ions. Experiments have been carried out using bound copper as a model for serum albumin. These have shown the rate of decomposition of SNAP to be increased by bound copper, though not to the same degree as when using free copper. These results suggest that the copper catalysed decomposition might also be relevant in vivo. Possible Cu-nitrosothiol complexes are shown in figure 2.

Figure 2

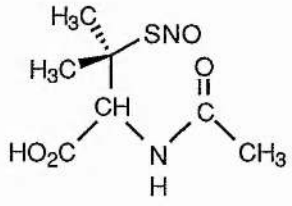


The formation of these are favoured by the attraction of the electron rich NH_2 or CO_2 groups to the copper and the stability of the six membered ring intermediate. The importance of the amine and acid groups is verified by experiments involving the blocking of these groups. The *S*-nitroso derivatives of *N*- or *O*- protected penicillamines are more stable than the unprotected molecule.[2] When these sites are blocked, typically with an *N*-acetyl and an *O*-methyl, it is more difficult for the copper to coordinate and it

therefore has a smaller effect. However, the fully protected derivative has a low solubility in water and is of little use in physiological studies. Another important factor in the stability involves the other groups on the thiol-bearing carbon. The penicillamine derivative is much more stable than the cysteine derivative,[2] for instance. It is fairly easy to envisage the methyl groups of the penicillamine hindering coordination to the copper. The effect of these is more notable in the solid form, where SNAP is stable but S-nitroso acetyl cysteine (SNAC) is not. This suggests an electronic influence of the methyl groups as logic would suggest that more steric crowding would be more likely to lead to displacement of the NO to ease this crowding. In solution it is also possible to halt the decomposition of SNAC using a Cu(I) chelating agent[5] i.e. the lack of dimethyl grouping does not appear to make the nitrosothiol more susceptible to copper catalysed decomposition.



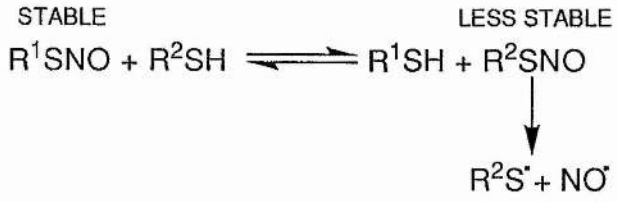
SNAC



SNAP

There are of course other factors involved in S-nitrosothiol decomposition. The most important are thermal and photolytic. Most RSNO compounds are thermally unstable and most will rapidly decompose when exposed to light. Another route for NO production is transnitrosation, whereby the NO is transferred to a thiol which will give a less stable nitrosothiol (figure 3). This may then spontaneously decompose to produce NO.[6,7]

Figure 3

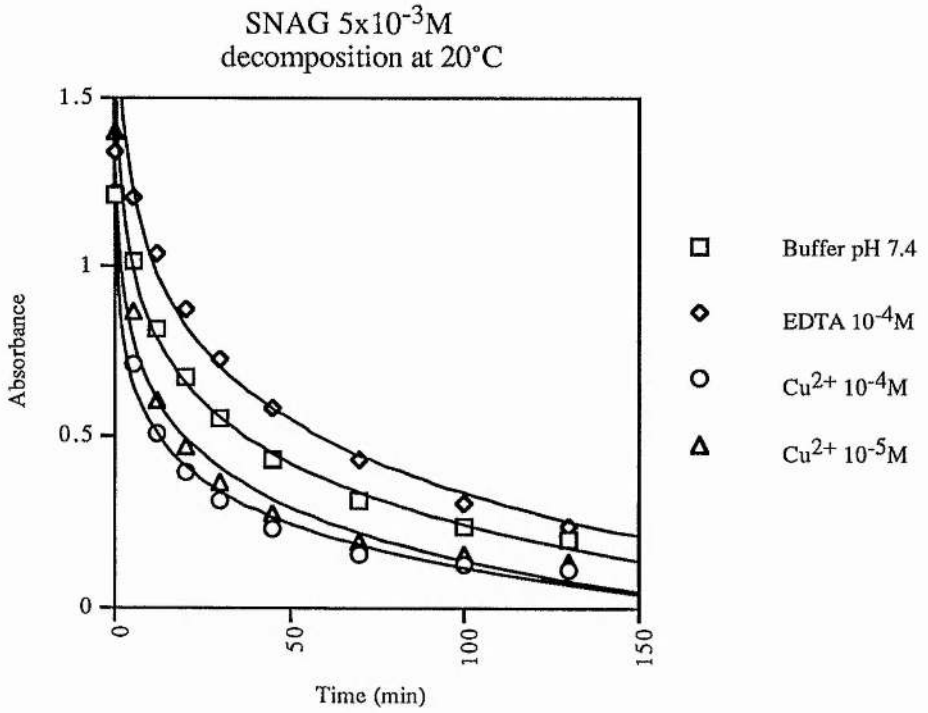


6.2. Decomposition Studies.

The original intention had been to study the effect of copper ions on the rate of nitrosothiol decomposition in aqueous buffer. However, this has been performed only for a small number of the nitrosothiols prepared, due to problems with impurities and instability. The compounds can be divided into two classes: those based on penicillamine and those based on thiosugars. The decomposition of the penicillamine derivatives have been fairly easy to investigate and are covered in 6.2.2. The thiosugar derivatives were more difficult to study as they were often impossible to obtain in a pure form and decomposed so rapidly as to be difficult to follow the decomposition using our apparatus.

6.2.1. Decomposition of 1-*S*-nitrosothiosugars.

The compound was dissolved in 50 % aqueous methanol and the disappearance of the SNO absorbance at 340 nm monitored. Routinely samples were made up in four separate cuvettes. Into each was placed 1 ml of either: buffer pH 7.4, EDTA 2 x 10^{-4} M in pH 7.4 buffer, Cu^{2+} 2 x 10^{-4}M in buffer or Cu^{2+} 2 x 10^{-5}M in buffer. A sample of the nitrosothiol ~1 x 10^{-2}M was made up in methanol. Immediately prior to scanning, 1 ml of the nitrosothiol solution was added to each cuvette, to give a final nitrosothiol concentration of ~5 x 10^{-3}M. The results for all four sugars other than SNAG [5] were found not to be reproducible but suggested little difference in the rate of decomposition between the four samples. The results of the decomposition of SNAG are shown below, indicating a small rate increase for the copper containing samples, the slowest rate being found for the sample containing the least copper (EDTA x 10^{-4}M) and the fastest rate for the sample containing the most copper (Cu^{2+} x 10^{-4}M).



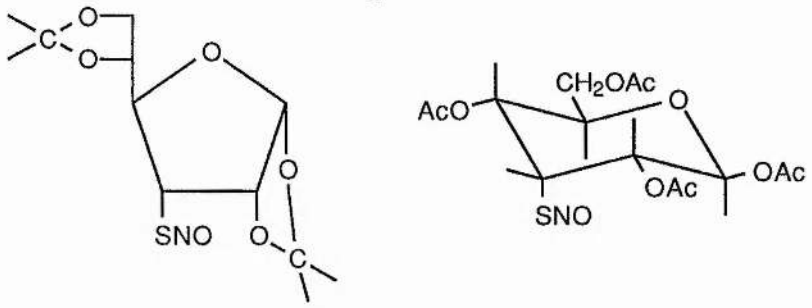
Likewise, the sample containing Cu^{2+} at the lower concentration still shows a faster rate of decomposition than the sample containing only buffer. A large increase in the rate of decomposition was seen when the temperature was increased from 20 to 30°C, the half life falling by 50 %. This, and the observation that the removal of copper does not halt the decomposition as with SNAP, suggests that the decomposition of SNAG is mainly a thermal process, with only a small role for copper.

Other compounds studied include SNOIL-acetate [86], 3-*S*-nitrosothioglucose [50], 6-*S*-nitrosothiogalactose [59] and di-*S*-nitrosothioisomannide [74]. Results for these were difficult to interpret. The variable levels of disulphide already present probably retarded the decomposition rate by complexing Cu^{2+}, making the results meaningless.

Further studies of a range of compounds is necessary to determine the effects of copper on *S*-nitrosothiosugar decomposition. Various studies of interest which should be carried out include comparisons between 3-*S*-nitrosothioglucose [50] with its

diisopropylidene protecting groups which should not show any copper complexation, and the acetylated form which may.

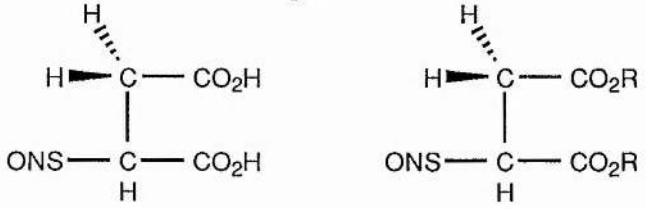
Figure 4



[50]

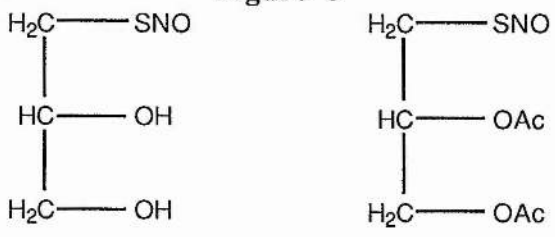
This would possibly highlight the involvement of the thermal reaction in the decomposition. A study involving the mercaptosuccinates (figure 5) would have been very informative, comparing the decomposition of the unprotected derivative, containing two acid groups, and the protected derivatives with their two ester groups.

Figure 5



Studies by associated groups are in progress, studying the decomposition of SNOIL [82] and SNOIL-acetate [85], figure 6.

Figure 6



[82] [85]

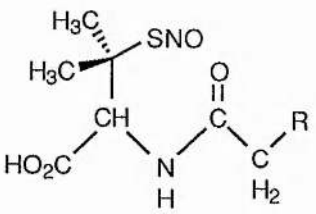
These are showing some interesting results which may further our understanding of the groups necessary for copper chelation to occur.

The study of copper ion catalysis is incomplete because of competing demands as the research developed. However, this work is necessary before a complete understanding of this class of NO-donor compounds can be obtained

6.2.2. Penicillamine Derivatives

The decomposition of SNAP has been studied in great detail and a full understanding of the mode of breakdown elucidated only fairly recently. A number of new derivatives of penicillamine have been prepared. These show interesting variations in physiological activity (chapter 3) and so it was of interest to learn if the added groups had an effect on either the rate of decomposition or the mechanism of decomposition. The new derivatives all had an acyl chain, of various lengths, attached to the amine group of penicillamine. One possible complex between the amino acid and the copper ion involves the amide of SNAP. Therefore it was expected that a long aliphatic chain in this region might inhibit the complexation to a degree. This might occur either by inhibiting an interaction between the nitrogen and the copper, or in the case of longer chains which could coil around the molecule, inhibit complexation to other parts of the molecule. A long chain might also act as a 'cage' around the SNO group and prevent the loss of nitric oxide.

The three derivatives prepared were the propionyl, valeryl and heptanoyl derivatives of penicillamine. These have been named SNOPP, SNVP and SNOPHE respectively.



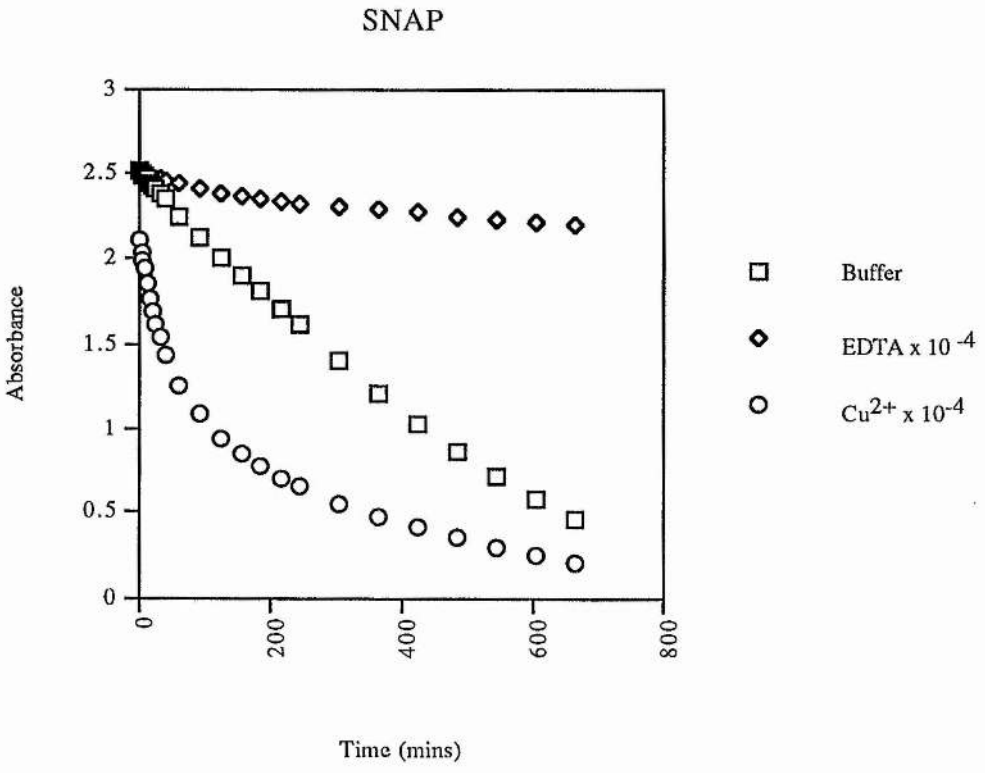
SNOPP: R = CH_3

SNVP: R = $(CH_2)_3CH_3$

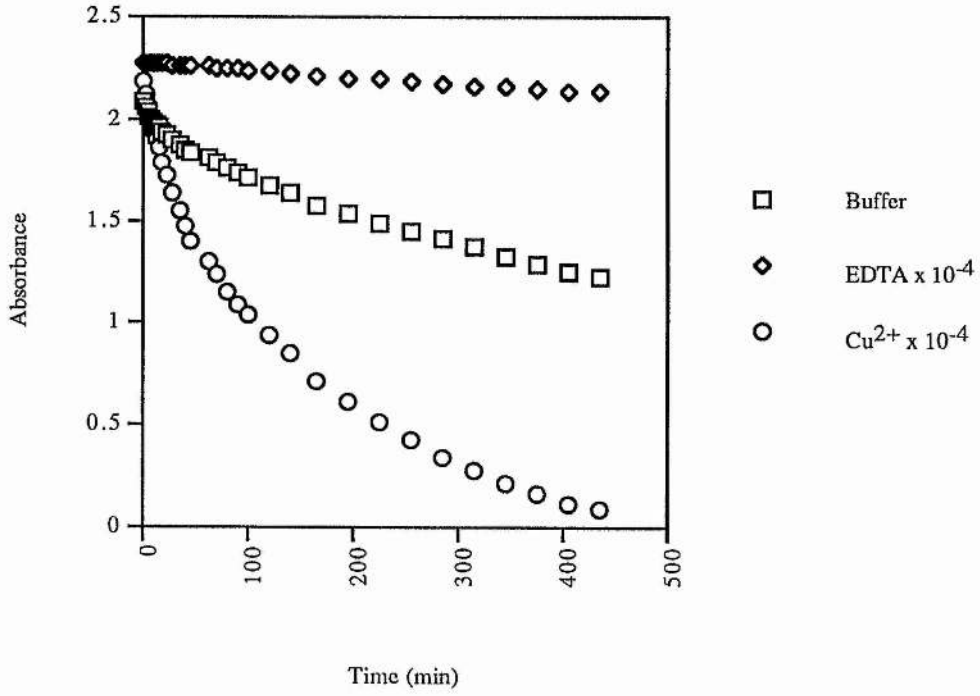
SNOPHE: R = $(CH_2)_5CH_3$

Decomposition studies for these were carried out and comparison of these with SNAP were made. The decomposition curves are shown below. Nitrosothiol (1 ml, 3 mM in methanol) was mixed with either buffer (pH 7.4), EDTA 2 x 10^{-4}M in buffer solution or

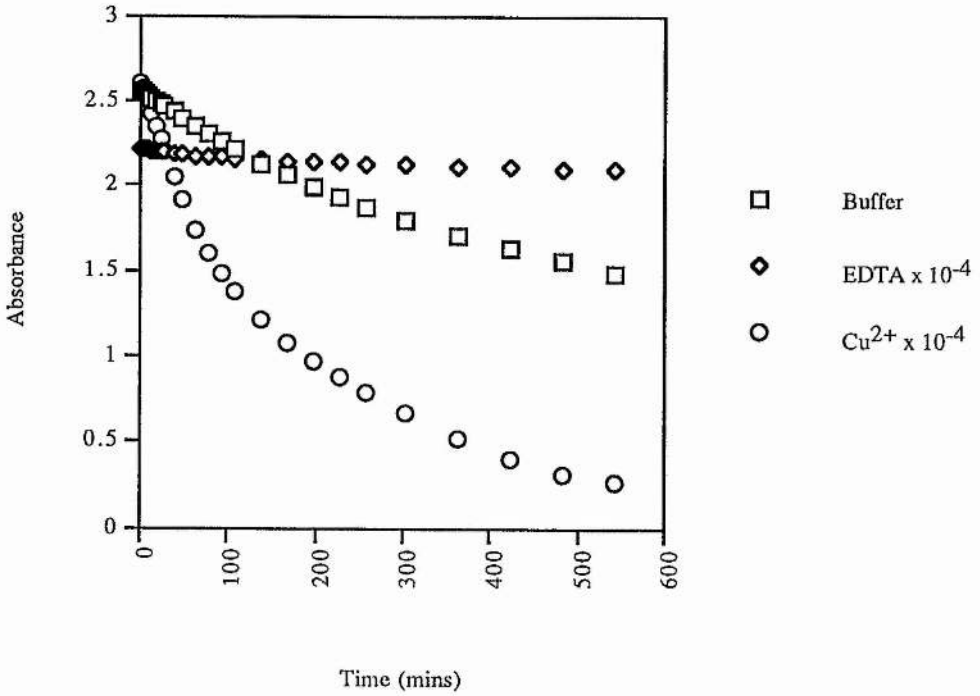
Cu^{2+} 2 x 10^{-4}M in buffer (1 ml). The fall in absorbance at 340 nm was recorded. SNAP was synthesised by the method described by Field *et al.*[8]

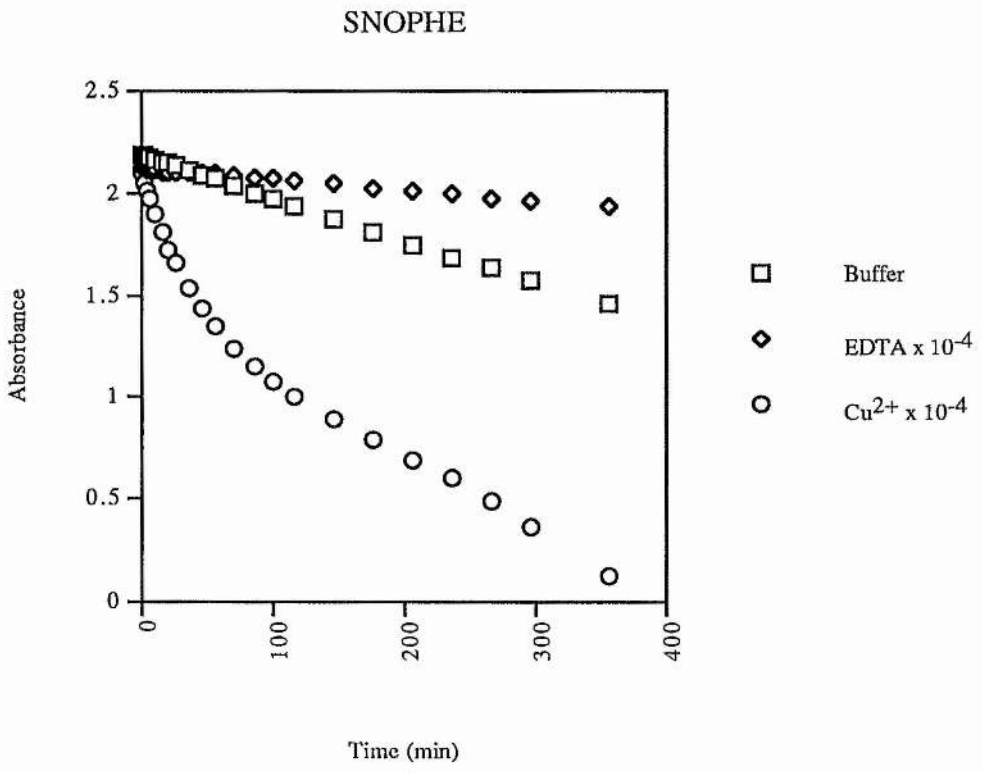


SNOPP


Absorbance
2.5
2
1.5
1
0.5
0
0
100
200
300
400
500
Time (min)
Buffer
EDTA x 10^{-4}
Cu^{2+} x 10^{-4}

SNVP


Absorbance
3
2.5
2
1.5
1
0.5
0
0
100
200
300
400
500
600
Time (mins)
Buffer
EDTA x 10^{-4}
Cu^{2+} x 10^{-4}



The above graphs demonstrate the similarities between the four compounds. All show a high stability in the presence of the metal chelating agent EDTA. All show a marked lack of stability in the presence of Cu^{2+} ions. The results, summarised in table form below, indicate that the longer chain does appear to confer a higher degree of stability toward decomposition catalysed by trace amounts of copper. When only buffer is present the decomposition of SNOPP, SNVP and SNOPHE is each significantly slower than that of SNAP. The decomposition catalysed by larger quantities of copper appears not to be hindered by the longer chain with SNOPP and SNOPHE having shorter half-lives than SNAP in the presence of 10^{-4}M Cu^{2+}. It is not obvious how the longer chain would aid complexation. The fact that the five membered chain, seen in SNVP, appears to give a more stable molecule than the three or five membered chains seen in SNOPP and SNOPHE, suggests that there is not necessarily a logical or simple reason for the way this series of compounds decompose.

	ε ($mol^{-1}dm^3cm^{-1}$)	$t_{1/2}$/mins in buffer	$t_{1/2}$/mins with $[Cu^{2+}] = 10^{-4}M$
SNAP	1360	350	105
SNOPP	1370	620	90
SNVP	1550	720	120
SNOPHE	1360	560	80

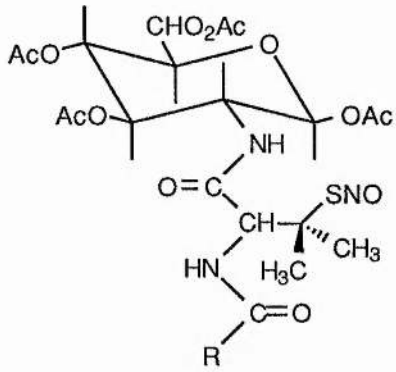
Decomposition after 400 minutes at 20°C

	Buffer	EDTA x $10^{-4}M$	Cu^{2+} x $10^{-4}M$
SNAP	55 %	8.3 %	79 %
SNOPP	40 %	5.9 %	96 %
SNVP	35 %	4.6 %	78 %
SNOPHE	36 %	9.1 %	100 %

The decomposition rate is very much affected by the purity of the sample and the concentration of thiol. The stability will also be affected by the position of the equilibrium between the nitrosothiol and thiol + NO^+ as well as the stability of the pure nitrosothiol. Because of this, these numbers can only give a very inaccurate impression of nitrosothiol stability.

The complexation of copper to the penicillamine moiety of SNAP can occur through the carboxyl or the amine groups. The above results have indicated to us that blocking the amine group with a longer chain is of limited value in reducing the influence of copper on the rate of decomposition.

We had also prepared another series of compounds, containing the same selection of chain lengths on the amine, but also with a sugar ring attached to the carboxyl group. One of these has proven to be of great physiological interest (chapter 3).



RIG 200: R = CH_3

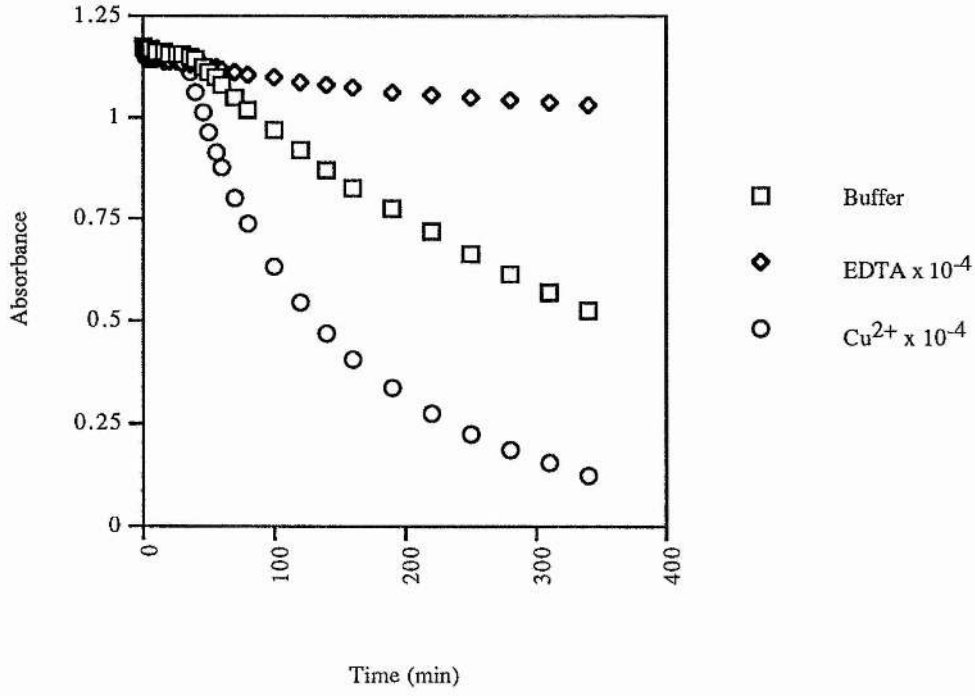
RIG 300: R= CH_2CH_3

RIG 500: R = $(CH_2)_3CH_3$

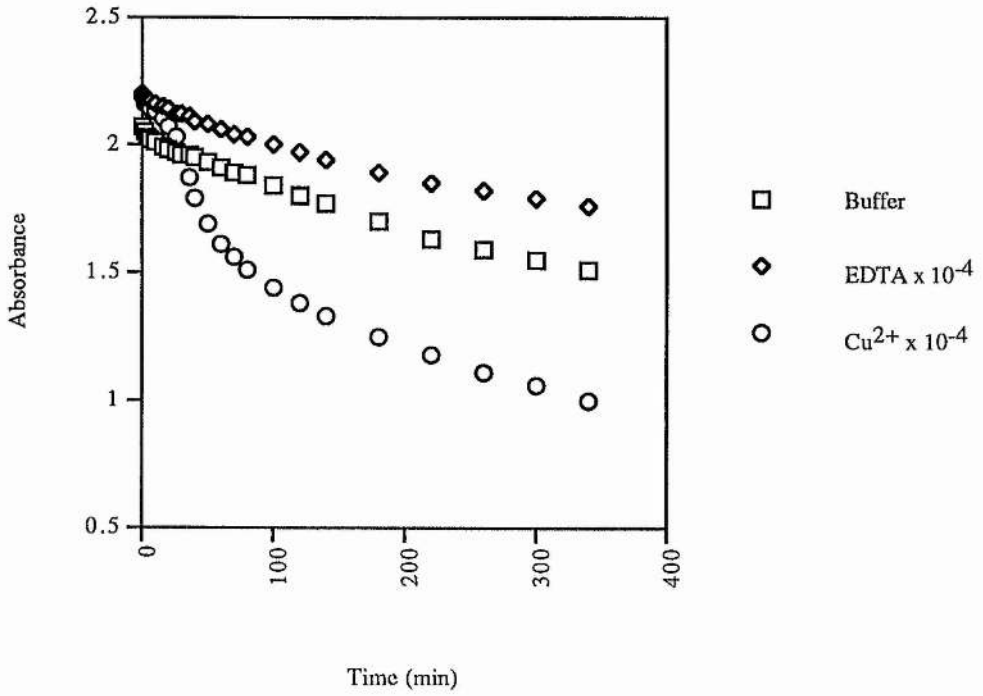
RIG 700: R = $(CH_2)_5CH_3$

The limited solubility of derivatives with an acyl chain other than the acetate, determined that these could not be tested under the same conditions as other NO donors investigated. Due to this we were unable to get any idea of their potential as NO donors. Whilst it is not as satisfactory a study, an investigation into the decomposition of these molecules has been very important in assessing their potential. The results obtained show that the addition of a longer chain gives a quite different type of NO donor. The results are shown in the graphs below.

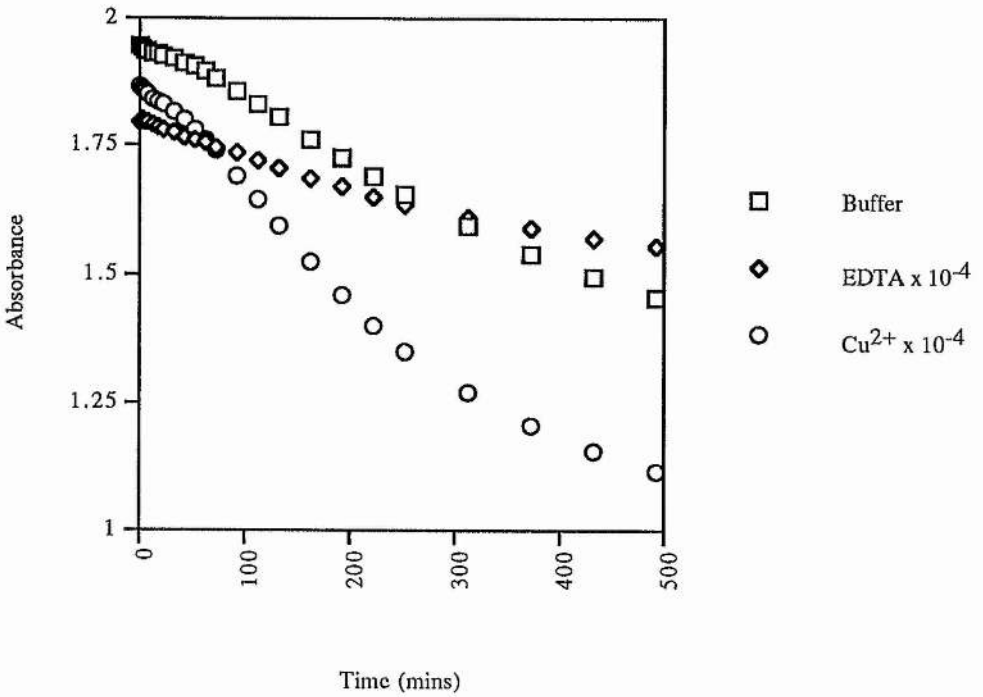
RIG 200



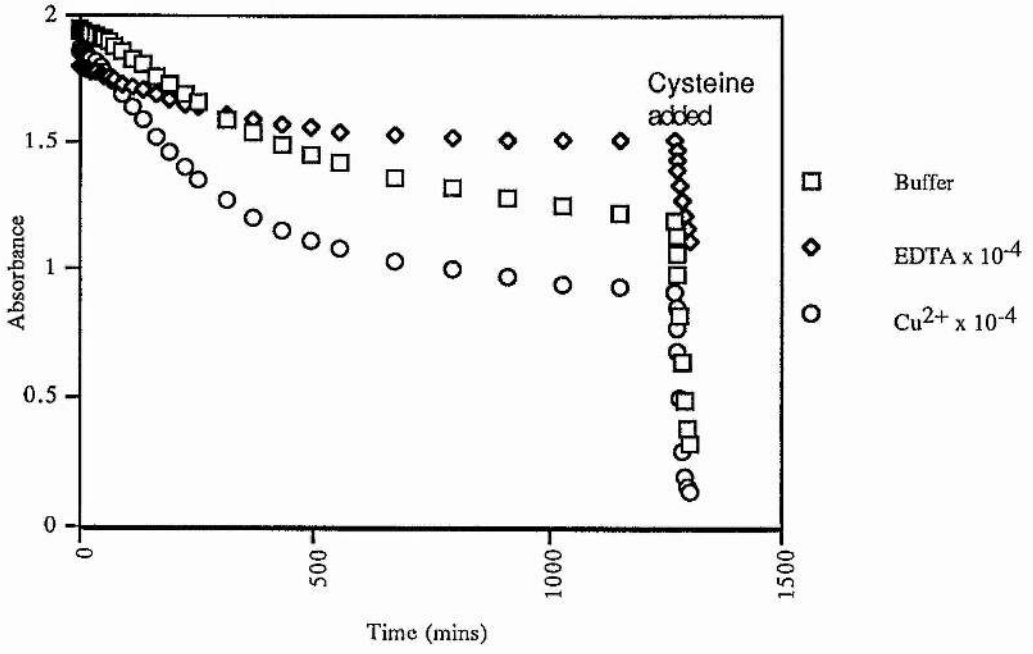
RIG 300



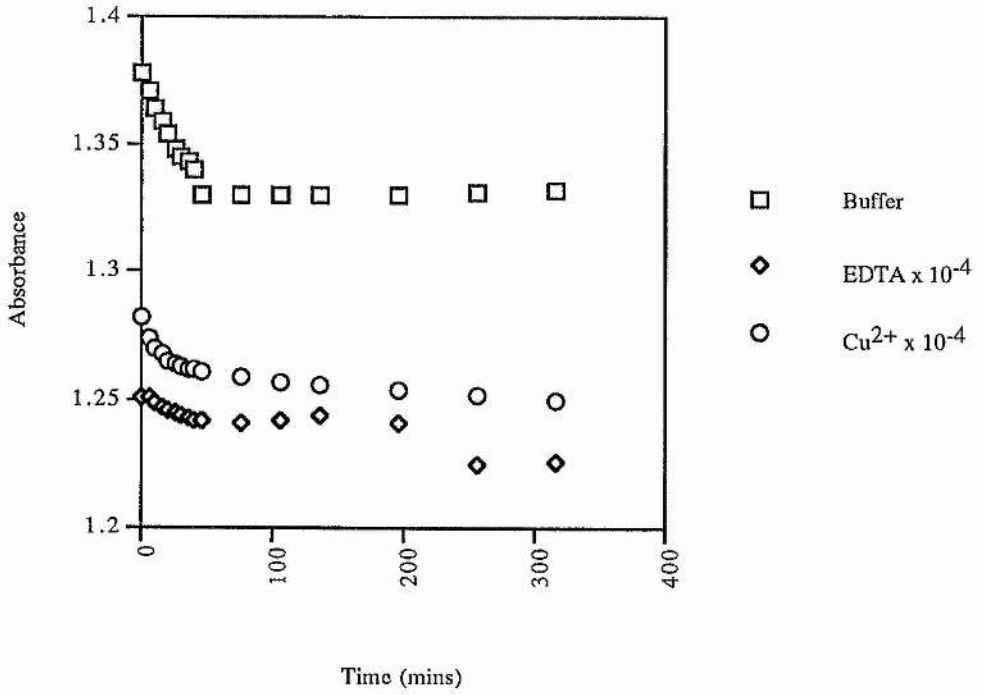
RIG 500

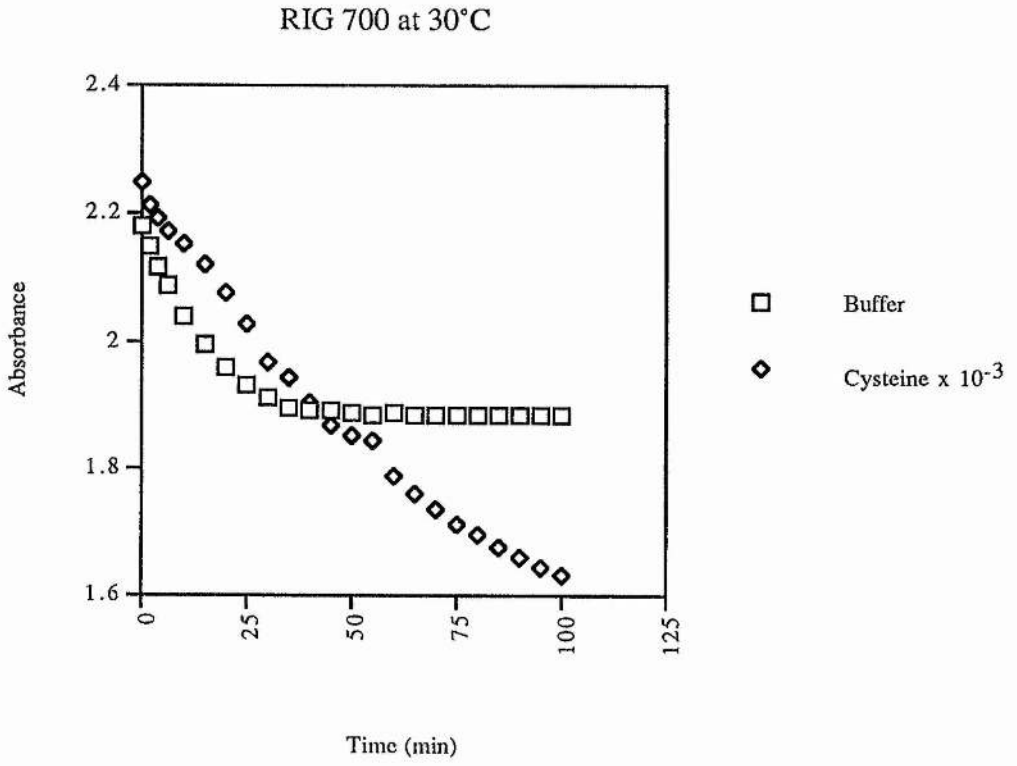


RIG 500 continued



RIG 700 at 20°C





The above graphs appear to split the four compounds into two very distinctive categories. RIG 200 and RIG 300 give very similar profiles whilst RIG 500 and RIG 700 show the same pattern of decomposition. RIG 200 is very similar in stability to SNAP in both half lives and % decomposition for the three solvent conditions examined. RIG 300 shows the same decomposition pattern but is very much more stable toward trace copper catalysed decomposition. The limitations of both half life and % decomposition values are demonstrated here in comparing RIG 200 with RIG 300. They have similar half lives for the decomposition in the presence of 10^{-4}M copper and yet the % decomposition for RIG 300 at 400 minutes is much lower than for RIG 200. This is probably due to the rate slowing down as the decomposition continues, in the same manner as shown for RIG 500 and RIG 700. Both compounds show a significant lag phase at the start of the decomposition curves for the buffer and Cu^{2+} samples. This can be explained as the period whilst the levels of thiol are building up as shown in figure 1. The decomposition of RIG 500 and RIG 700 is much slower than for the first two compounds. In the case

of RIG 500 the decay appears to proceed smoothly, but when the study is continued for a longer period (>100 min) it is noted that absorbance levels out and no further decomposition is seen. This is shown more dramatically in the case of RIG 700 where this levelling off is seen after only a very small degree of initial decay. This is also seen in investigations into the decomposition of GSNO. The decay of GSNO is very complex but involves inhibition of the decomposition by the disulphide which halts the reaction.. The addition of 1 mmol cysteine to the halted RIG 500 decomposition causes an instant response and rapid breakdown of the nitrosothiol. At 30°C the initial decomposition of RIG 700 proceeds to a greater degree before the decay is halted. An increase in temperature appears to cause no further decomposition. The addition of 1 mmol cysteine allows the reaction to continue but does not increase the initial rate.

The data from these experiments are summarised in the tables below.

	ε($mol^{-1}dm^3cm^{-1}$)	$t^1/_2$ / mins in buffer	$t^1/_2$ / mins with $[Cu^{2+}]=10^{-4}M$	$t^1/_2$ / mins with [cysteine]=$10^{-3}M$
RIG 200	1100	300	110	
RIG 300	1150	1000	105	350
RIG 500	1280	>24 h	1140	
RIG 700	1220	N/A	N/A	400

Decomposition after 400 minutes at 20°C

	Buffer	EDTA x $10^{-4}M$	Cu^{2+} x $10^{-4}M$
RIG 200	63.5 %	11.9 %	100 %
RIG 300	29.5 %	21.8 %	57.4 %
RIG 500	21.9 %	12.1 %	36.6 %
RIG 700	3.1 %	2.1 %	3.0 %

6.3. Conclusions

Our results show no obvious pattern between the blocking of the acid and amine groups of penicillamine and the inhibition of copper catalysed decomposition. The addition of various groups to penicillamine does appear to increase the thermal stability i.e. the rate of decomposition in the absence of copper is reduced. It is presumed that the decomposition is a mainly thermal process in these cases. The rate of decomposition in the presence of only trace amounts of copper is also reduced. However, it is only in the cases of RIG 500 and RIG 700 that the rate of decomposition in the presence of larger amounts of copper is reduced. This suggests that we have not hindered the complexation of copper to the nitrosothiol to any major degree.

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Chapter 7:

Experimental

β-D-Glucose pentaacetate [1] was prepared by stirring D(+) glucose (10 g, 55 mmol) with anhydrous sodium acetate (8 g, 97 mmol), dissolved in acetic anhydride (50 ml, 0.52 mol), in a boiling water bath for 1.5 h. The resultant mixture was poured onto 500 ml crushed ice and scratched to induce crystallisation from the oil formed. The white solid produced was washed with copious amounts of water and dried, yielding white crystals (18.2 g, 84%), m.p. 129-130°C, (lit.[1] 132-133°C). δ_H (200 MHz, $CDCl_3$), 2.10 (15 H, s, OAc), 3.85 (1 H, m, 5-H), 4.1 (1 H, dd, J = 13 and 5.2 Hz, 6 or 6'-H), 4.3 (1 H, dd, J = 13 and 5.2 Hz, 6 or 6'-H), 5.2 (3 H, 3 t, 2, 3, 4-H) and 5.7 (1 H, d, J = 10 Hz, 1-H). δ_C (50 MHz, $CDCl_3$), 21.00, 21.14 and 21.25 (OAc), 61.85, 66.13, 70.52, 73.10 and 73.17 (C2 - C6), 92.09 (C1), 169.38, 169.67, 169.81, 170.51 and 171.01 (OAc).

Acetobromoglucose [2] was prepared by stirring, in an ice bath, β-D-glucose pentaacetate (17 g, 44 mmol) dissolved in DCM (50 ml) with 30% HBr in acetic acid (17 ml) and acetic anhydride 12 ml for 2 hours. The resultant mix was evaporated after the addition of toluene several times and the acetic acid removed as an azeotrope. A pale brown oil was produced. The oil was diluted with DCM, washed with saturated $NaHCO_3$ solution and evaporated to a clear oil. Ether was added and a white solid appeared on refrigeration. This was filtered yielding white crystals (11.4 g, 63%), m.p. 88-90°C, (lit.[2] 88-89°C). δ_H (200 MHz, $CDCl_3$), 2.1 (12 H, s, OAc), 4.15 (1 H, m, 5-H), 4.3 (2 H, m, 6 and 6'-H), 4.85 (1 H, dd, J = 9.4 and 4.2 Hz, 2-H), 5.15 (1 H, t, J = 10.2 Hz, 3 or 4-H), 5.55 (1 H, t, J = 10.5 Hz, 3 or 4-H) and 6.65 (1 H, d, J = 4.2 Hz, 1-H). δ_C (50 MHz, $CDCl_3$), 21.04, 21.11 and 21.15 (OAc), 61.41, 67.62, 70.62, 71.06 and 72.60 (C2 - C6), 87.04 (C1), 169.88, 170.45 and 171.32 (OAc).

2,3,4,6 Tetra-*O*-acetyl-β-D-glucopyranosyl isothiouronium bromide [3] was prepared by the addition of acetobromoglucose (6.75 g, 16 mmol) to thiourea (1.25

g, 16 mmol) in dry acetone (30 ml). The mixture was refluxed for 20 minutes until a white solid apppeared. The mixture was cooled to room temperature, filtered and washed with acetone. Recrystallisation from acetone gave a white solid product (4.5 g, 57.7%), m.p.193-194°C, (lit.[3] 193-196°C). δ_H (200 MHz, D_2O), 2.0 (12 H, m, OAc), 4.15 (2 H, m, 6 and 6'-H), 4.3 (1 H, dd, 5-H) and 5.05 - 5.35 (4 H, m, 1, 2, 3 and 4-H). δ_C (50 MHz, D_2O), 22.3 and 23.1 (OAc), 64.8, 70.4, 71.3, 76.2, 78.7 and 83.9.

1-Thio-β-D-glucose tetraacetate [4] was prepared by the addition of the above product **[3]**, with stirring, to a solution of $K_2S_2O_5$ (1.68 g, 7.56 mmol) in water (20 ml) at 85°C. To this was added DCM (25 ml) and the mixture was refluxed for 15 minutes with stirring. Upon cooling the DCM layer was separated and washed with water (3 x 25 ml) then dried ($MgSO_4$) and evaporated to give an oil. A white solid formed from the oil. Recrystallisation from methanol gave white crystals (2.1 g, 62.4%), m.p. 108°C, (lit.[4] 110-112°C). δ_H (200 MHz, $CDCl_3$), 2.05 (12 H, s, OAc), 2.3 (1 H, d, J = 10.6 Hz, HS-), 3.75 (1 H, m, 5-H), 4.2 (2 H, 2 dd, J = 12.7 and 5.3 Hz, 6 and 6'-H), 4.55 (1 H, t, J = 10.6 Hz, 1-H) and 5 - 5.2 (3 H, 3 t, 2, 3 and 4-H). υ_{max}/cm^{-1} 2555 (SH stretch). δ_C (50 MHz, $CDCl_3$), 21.087 and 21.255 (OAc), 62.427, 68.469, 73.913, 73.915, 76.716 and 79.156. (Found: C, 46.14; H, 5.66%. $C_{14}H_{20}O_9S$ requires C, 46.15; H, 5.49%)

***S*-Nitroso-1-thio-β-D-glucose tetraacetate [5]** was prepared by dissolving the thiol **[4]** in the minimum amount of methanol followed by the addition of an equivalent amount of water. Excess $NaNO_2$ and HCl were added. A pink solid was filtered off and dried. λ_{max} 342 nm. $t_{1/2}$ of 30 - 50 mins in aqueous buffer / methanol at 20°C. υ_{max}/cm^{-1} 1550 (NO stretch), no signal at 2555. δ_H (200 MHz, $CDCl_3$), 2.05 (12 H, s, OAc), 3.75 (1 H, m, 5-H), 4.20 (2 H, m, 6 and 6'-H), 4.65 (1 H, d, J = 10.1 Hz, 1-H) and 5.10 - 5.30 (3 H, m, 2-, 3-, 4-H). δ_C (50 MHz, $CDCl_3$), 21.08, 21.17 and 21.31 (OAc), 62.04 , 68.32, 70.17, 74.32, 76.62 (C2 - C6) and 87.69 (C1).

β-D-galactose pentaacetate [6] was prepared by the same method as glucose, using D(+)-galactose. White crystals were produced (12.3 g, 56.8%). m.p. 137°C, (lit.[4] 140-142°C). δ_H (200 MHz, $CDCl_3$), 2.1 (15 H, m, OAc), 4.1 (3 H, m, 5, 6 and 6'-H), 5.05 (1 H, dd, J = 12.5 and 3.75 Hz, 2-H), 5.35 (1 H, t, 4-H), 5.45 (1 H, dd, 3-H) and 5.7 (1 H, d, J = 10 Hz, 1-H). δ_C (50 MHz, $CDCl_3$), 20.98, 21.09 and 21.25 (OAc), 61.52, 67.27, 68.26, 71.24 and 72.09 (C2 - C6), 92.55 (C1), 169.47, 169.87, 170.45, 170.63 and 170.85 (OAc).

Acetobromogalactose [7] was prepared by the same method as acetobromoglucose except the reaction was carried out at room temperature. White crystals were produced (7.2 g, 57%). m.p.75°C, (lit.[5] 84-85°C). δ_H (200 MHz, $CDCl_3$), 2.1 (12 H, m, OAc), 4.15 (2 H, m, 6 and 6'-H), 4.45 (1H, t, J = 7 Hz, 5-H), 5.05 (1 H, dd, J = 11 and 4.4 Hz, 2-H), 5.45 (2 H, dd, 3 and 4-H) and 6.7 (1H, d, J = 4.4 Hz, 1-H). δ_C (50 MHz, $CDCl_3$), 21.05, 21.10, 21.22 (OAc), 61.31, 67.44, 68.22, 68.44 and 71.52 (C2 - C6) and 88.60 (C1).

2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl isothiouronium bromide [8] was prepared by dissolving acetobromogalactose (4.5 g, 11 mmol) in dry acetone (4.5 ml) and refluxing with thiourea (0.85 g, 11.1 mmol) until a white solid appeared (20 mins). The solid was filtered off and washed with acetone then recrystallised from acetone to give white crystals (4.25 g, 79.7%). m.p. 166°C, (lit.[6] 169°C), δ_H (200 MHz, D_2O), 2.0 (12 H, m, OAc), 4.15 (2 H, d, 6 and 6'-H), 4.3 (1 H, t, 5-H) and 5.2 - 5.5 (4 H, m, 1, 2, 3 and 4-H). δ_C (50 MHz, D_2O), 22.89, 22.97 and 23.05 (OAc), 64.95, 69.49, 70.74, 74.38 and 78.30 (C2 - C6) and 84.26 (C1).

1-Thio-β-D-galactose tetraacetate [9] was prepared by the addition of the above salt **[8]** (3.5 g, 7.2 mmol) to a solution of $K_2S_2O_5$ (1.25 g, 5.6 mmol) in water (4 ml) heated to 85°c with stirring. DCM (5.5 ml) was added and the mixture refluxed for 15 minutes. The DCM layer was separated and washed with water (3 x 10 ml). The water

layer was washed with DCM and the organic extracts combined and dried ($MgSO_4$). The mix was filtered and evaporated leaving a thick oil. Upon standing this slowly solidified yielding a white solid (1.65 g, 63.1%) which could not be recrystallised. υ_{max}/cm^{-1} 2560. δ_H (200 MHz, $CDCl_3$), 2.05 (12 H, m, OAc), 2.4 (1 H, d, J = 10 Hz, -SH), 3.95 (1 H, m, 5-H), 4.15 (2 H, m, 6 and 6'-H), 4.55 (1 H, t, J = 10 Hz, 1-H), 5.0 (1 H, dd, J = 10 and 3.6 Hz, 3-H), 5.2 (1 H, m, 2-H) and 5.45 (1 H, d, J = 3.6 Hz, 4-H). δ_C (50 MHz, $CDCl_3$), 21.07, 21.20 and 21.34 (OAc), 61.98, 67.73, 71.31, 72.05 and 75.42 (C2 - C6) and 79.66 (C1). (Found: C, 46.19; H, 5.11%. $C_{14}H_{20}O_9S$ requires C, 46.15; H, 5.49%)

***S*-Nitroso-1-thio-β-D-galactose tetraacetate [10]** was prepared by dissolving the above thiol **[9]** in the minimum amount of methanol and adding water until just before precipitation occurs. Excess $NaNO_2$ and HCl were added with stirring to give a deep red solution. This was extracted into DCM and the solvent removed by evaporation without heating. A pink powder was produced with λ_{max} at ~344 nm. $t_{1/2}$ of 5 - 10 mins in aqueous buffer / methanol at 20°C. The material was unstable as a solid making the ^1H NMR spectrum indistinct. δ_C (50 MHz, $CDCl_3$), 21.16 and 21.23 (OAc), 61.89, 67.79, 66.80, 72.59, 75.87 and 85.75.

Xylose tetraacetate [11] was prepared as for glucose (using D-xylose) giving white crystals (11.8 g, 55.9%). m.p. 118-120°C, (lit.[4] 120°C). δ_H (200 MHz, $CDCl_3$), 2.05 (12 H, m, OAc), 3.5 (1 H, dd, J = 12 and 7.5 Hz, 5a-H), 4.25 (1 H, dd, J = 12.5 and 5 Hz, 5b-H), 5.0 (2 H, m, 2-, 3-H), 5.2 (1 H, t, J = 8.75 Hz, 4-H) and 5.7 (1 H, d, J = 10 Hz, 1-H).

Acetobromoxylose [12] was prepared by the addition of 10 g of the above product **[11]** to 30% HBr in acetic acid solution (13 ml) and acetic anhydride (1 ml) in dry DCM (32 ml). This was covered in foil and stirred for 2 hours. The resultant mix was evaporated using toluene to azeotrope away the acetic acid, yielding a pale brown oil.

The oil was diluted with DCM and washed (saturated $NaHCO_3$ solution). The resultant solution was then dried ($MgSO_4$) and evaporated to an oil. Ether was added and the solution left to crystallise in the fridge. Large white crystals were filtered off (4.8 g, 45%). m.p. 98-99°C, (lit.[4] 98-100°C). δ_H (200 MHz, $CDCl_3$), 2.05 (9 H, OAc) 3.85 (1 H, t, J = 11.5 Hz, 5a-H), 4.05 (1 H, dd, J = 11.5 and 7 Hz, 5b-H), 4.75 (1 H, dd, J = 10.4 and 4.3 Hz, 2-H), 5.05 (1 H, td, J = 10.4 and 6 Hz, 4-H), 5.55 (1 H, t, J = 9.7 Hz, 3-H) and 6.6 (1 H, d, J = 4.3 Hz, 1-H)

2,3,5-Tri-*O*-acetyl-β-D-xylopyranosyl isothiouronium bromide [13] was prepared by the reflux of acetobromoxylose (3.29 g, 9.7 mmol) in dry acetone (3.5 ml) with thiourea (0.74 g, 9.7 mmol) for 15 mins. A white solid was precipitated. This was filtered off, washed with acetone and recrystallised from acetone yielding a white powder (2.69 g, 66.8%). m.p. 159°C, (lit.[7] 176-178°C). δ_H (200 MHz, D_2O), 2.1 (9 H, m, OAc), 3.75 (1 H, dd, J = 12.9 and 5.9 Hz, 5a-H), 4.4 (1 H, dd, J = 12.9 and 4.3 Hz, 5b-H), 4.95 (1 H, m, 4-H), 5.15 (2 H, m, 2-,3-H) and 5.7 (1 H, d, J = 7.5 Hz, 1-H).

1-Thio-β-D-xylose triacetate [14] was prepared by the addition of the above product (2.82 g, 6.8 mmol) to a solution of $K_2S_2O_5$ (1.41 g, 6.3 mmol) in H_2O (5.5 ml) at 85°C. DCM (7 ml) was added and the mixture allowed to reflux with stirring for 15 mins. The lower organic phase was separated and washed with water (3 x 25 ml), dried ($MgSO_4$) and evaporated to an oil. The oil solidified upon refrigeration and was then recrystallised from methanol yielding white crystals (0.6 g, 30%) m.p. 120°C, (lit.[7] 123°C). δ_H (200 MHz, $CDCl_3$), 2.1 (9 H, m, OAc), 2.3 (1 H, d, J = 10 Hz, -SH), 3.4 (1 H, t, J = 11 Hz, 5b-H), 4.2 (1 H, dd, J = 12 and 5 Hz, 5a-H), 4.6 (1 H, t, J = 9.2 Hz, 1-H), 4.95 (1 H, t, J = 9.2 Hz, 2/3-H), 5.0 (1 H, dd, J = 9.2 and 5.4 Hz, 4-H) and 5.2 (1 H, t, J = 9.2 Hz, 2/3-H). (Found: C, 44.94; H, 5.39%. $C_{11}H_{16}O_7S$ requires C, 45.21; H, 5.48%).

***S*-Nitroso-1-thio-β-D-xylose tetraacetate [15]** was prepared by dissolving the above product in the minimum amount of methanol followed by the addition of the maximum amount of water possible without inducing precipitation. Excess $NaNO_2$ was added followed by excess HCl with stirring. The red liquid produced was extracted into DCM and evaporated without heating. A pale pink solid was produced (0.32 g, 48.5 %) with λ_{max} at ~350 nm. $t_{1/2}$ of 3 - 5 mins in aqueous buffer / methanol at 20°C. The product was unstable in the solid form making NMR studies impossible.

Maltose octaacetate [16] was prepared by the addition of maltose (20 g) and sodium acetate (20 g) to acetic anhydride (100 ml). The mixture was stirred on a boiling water bath for 3 hrs. The resultant solution was evaporated to an oil and dissolved in DCM. The solution was washed with a saturated solution of $NaHCO_3$ and dried ($MgSO_4$). After filtering, the liquid was boiled with decolourising charcoal and evaporated to an oil. The oil was dissolved in ether and a product crystallised out upon refrigeration, (19.26 g, 51.1%). m.p.151-153°C, (lit.[4] 155-156°C). δ_H (200 MHz, $CDCl_3$), 2.1 (24 H, m, OAc), 3.85 - 4.05, 4.25 and 4.45: (4 H, m) (2 H, m) and (1 H, dd, J = 13.6 and 2.4 Hz), (4a, 5a, 6a, 6a', 5b, 6b and 6b'-H), 4.85 (1 H, dd, J = 10.3 and 4.6 Hz, 2b-H), 5.0 (1 H, t, J = 9.5 Hz), 5.05 (1 H, t, J = 10.9 Hz), 5.3 (2 H, 2 t, J = 9.5 Hz) (2a, 3a, 3b and 4b-H), 5.4 (1 H, d, J = 4.1 Hz, 1b-H) and 5.75 (1 H, d, J = 8.2 Hz, 1a-H). δ_C (50 MHz, $CDCl_3$), 21.02, 21.10, 21.24 and 21.30 (OAc), 61.91, 62.98, 68.42, 69.03, 69.74, 70.46, 71.38, 72.90, 73.43, 75.66, 91.71 and 96.16 (C1a - C6b'), 169.25, 169.89, 170.03, 170.32, 170.50, 170.88, 170.94 and 171.00 (quaternaries). (Found: C, 49.80; H, 5.38%. $C_{28}H_{38}O_{19}$ requires C, 49.51; 5.60%)

Acetobromomaltose [17] was prepared by the addition of 30% HBr in AcOH (8 ml) and acetic anhydride (1.5 ml) to a solution of maltose octaacetate **[16]** (6 g) in dry DCM (25 ml). The mixture was covered and stirred at room temperature for 4 h. Toluene was added and the solvent evaporated to produce a pale brown oil. Repeated solvation in ether followed by evaporation gave a fluffy white powder (4.4 g, 71%). m.p. 69°C,

(lit.[4] 84°C). δ_H (200 MHz, $CDCl_3$), 2.1 (21 H, m, OAc), 3.95 - 4.25 and 4.5 (6 H, m) and (1 H, dd, J = 14.5 and 3.7 Hz) (4a, 5a, 6a, 6a',5b ,6b and 6b'-H), 4.7 (1 H, dd, J = 10.5 and 4.4 Hz, 2b-H), 4.9 (1 H, dd, J = 10.5 and 4.4 Hz, 2a-H), 5.1 (1 H, t, J = 9.6 Hz, 3a or 4a-H), 5.4 (2 H, m, 3b and 4b-H), 5.45 (1 H, d, J = 4.4, 1b-H), 5.6 (1 H, t, J = 9.6 Hz, 3a or 4a-H) and 6.5 (1 H, d, J = 4.4 Hz, 1a-H). δ_C (50 MHz, $CDCl_3$), 21.06, 21.25 and 21.33 (OAc), 61.80, 62.31, 68.36, 69.10, 69.70, 70.47, 71.48, 72.02, 72.80, 73.01, 86.54 and 96.24 (C1a - C6b'), 170.00, 170.34, 170.98 and 171.16 (quaternaries). (Found: C, 45.36; H, 5.01%. $C_{28}H_{35}O_{17}Br$ requires C, 44.64; H, 5.01%).

1-Thio-β-D-maltose heptaacetate [19] was prepared as follows: acetobromomaltose (2.4 g) was dissolved in dry acetone (3 ml) and refluxed with thiourea (0.3 g) for 15 mins. Upon slow evaporation of the acetone, a pale brown oil was produced, which solidified upon standing giving the thiouronium bromide salt **[18]**. This salt was added to a solution of $K_2S_2O_5$ (1.3 g, 5.8 mmol) in water (5 ml) at 80°C. DCM (2 ml) was added and the solution refluxed with stirring for 20 mins. The organic phase was separated and washed with water (3 x 15 ml), dried ($MgSO_4$) and evaporated to an oil. The oil solidified and was recrystallised from methanol to give a white solid (1.15 g, 51%) m.p.156°C (lit.[7] 149 - 152°C). δ_H (200 MHz, $CDCl_3$), 2.1 (21 H, m, OAc), 2.25 (1 H, t, J = 9.4 Hz, SH), 3.7 (1 H, m), 4.0 (3 H, m), 4.2 (2 H, m) and 4.45 (1 H, dd, J = 12.3 and 2.1 Hz) (4a, 5a, 6a, 6a', 5b, 6b and 6b'-H), 4.6 (1 H, t, J = 9.4 Hz, 1a-H), 4.8 (1 H, t, J = 9.3 Hz), 5.05 (1 H, t, J = 9.7 Hz), 5.25 (1 H, t, J = 8.9 Hz) and 5.35 (1 H, t, J = 10.5 Hz) (2a, 2b, 3b and 4b-H), 4.85 (1 H, dd, J = 10.5 and 4 Hz, 3a-H) and 5.4 (1 H, d, J = 3.8 Hz, 1b-H). δ_C (50 MHz, $CDCl_3$), 21.08, 21.18 and 21.37 (OAc), 61.92, 63.45, 68.39, 69.04, 69.73, 70.44, 73.02, 74.78, 76.54, 78.69 (CHs), 96.10 (C1b) (Found: C, 47.32; H, 5.55%. $C_{26}H_{36}O_{17}S$ requires C, 47.81; H, 5.52%).

1-Thioacetyl maltose heptaactetate [20] was prepared as follows: **[17]** (4.5 g) was dissolved in DCM (30 ml). A mixture of KOH (0.62 g) and thioacetic acid (0.9 ml) in ethanol (10 ml) was added and the solution refluxed for 1 h. The mixture was allowed to cool, the precipitated salts filtered and the filtrate evaporated to an oil. The oil was dissolved in DCM, washed with water, dried ($MgSO_4$) and decolourised with charcoal. Evaporation gave a yellow oil which was recrystallised from ethanol to give a white powder, (3.3 g, 73.9 %). m.p. 147 - 149°C (lit.[8] 151-153°C). δ_H (300 MHz, $CDCl_3$), 2.1 (21 H, m, OAc), 2.2 (3 H, s, SAc), 3.80 (1 H, m, 5a-H or 5b-H), 3.95 (1 H, m, 5a-H or 5b-H), 4.0 (1 H, t, J = 9.2 Hz, CH), 4.05 (1 H, dd, J = 12.6, 2.3 Hz), 4.2 (1 H, dd, J = 12.4 and 4.1 Hz), 4.25 (1 H, dd, J = 12.4, 3.8 Hz) and 4.45 (1 H, dd, J = 12.4, 2.5 Hz) (6a, 6a', 6b and 6b'), 4.85 (1 H, dd, J = 10.7, 4.1 Hz, 2b-H), 5.0 (1 H, dd, J = 10.4, 9.1 Hz, CH), 5.05 (1 H, t, J = 9.7 Hz), 5.3 (3 H, m, CH), 5.4 (1 H, d, J = 4.1 Hz, 1b-H). δ_C (75 MHz, $CDCl_3$), 21.08, 21.17, 21.31 and 21.36 (OAc), 31.32 (SAc), 61.91, 63.20, 68.41, 69.02, 69.78, 70.25, 70.49, 72.99, 76.80, 77.03, 80.27 (C1a), 96.15 (C1b), 169.94, 170.09, 170.34, 170.47, 170.94, 171.00 and 171.07 (OAc) and 192.29 (SAc).

1-Thio-β-D-maltose heptaactetate [19] was prepared from the thioacetate as follows: *S*-Acetylmaltose (2 g) was dissolved in dry THF (40 ml) under N_2. Benzylamine (0.47 ml, 1.5 eq) was added and the solution stirred for 4 h when TLC showed that no starting material was present. The solvents were evaporated to a yellow oil. The oil hardened with cooling and was recrystallised for ethanol to give a white solid, (0.74 g, 39.4 %). ^1H and ^{13}C nmr as for the previously described thiol preparation.

***S*-Nitroso-1-thio-β-D-maltose heptacetate [21]** was prepared by passing a stream of N_2O_3 through a solution of **[20]** (1 g) in DCM. The solvent was evaporated to give a pale pink powder, (0.88 g, 84.2 %). $t_{1/2}$ = 50 - 60 mins in aqueous buffer. λ_{max} at ~345 nm (SNO). δ_H (200 MHz $CDCl_3$), no peak at ~2.25. δ_C (50 MHz,

$CDCl_3$), 20.92, 21.08, 21.16 and 21.38 (OAc), 61.94, 63.23, 68.42, 69.12, 69.77, 70.25, 70.52, 73.02, 76.91 and 77.34 (CH), 84.83 (C1a), 96.23 (C1b), 169.95, 170.45 and 171.07.

Lactose octaacetate [22] was prepared by the addition of lactose (20 g) to a mixture of pyridine (60 ml) and acetic anhydride (120 ml). The mixture was heated on a boiling water bath with stirring for 45 minutes by which time all the solid had dissolved. The mixture was poured into ice water (1 l) and stirred. A white solid was filtered off and dried (36.3 g, 96%). m.p. 88-89°C, (lit.[4] 90-91°C). δ_H (200 MHz, $CDCl_3$), 5.7 (0.6 H, d, J = 8.3 Hz, 1b-H) and 6.3 (0.4 H, d, J = 3.5 Hz, 1a-H). (Found: C, 49.82; H, 5.68%. $C_{28}H_{38}O_{19}$ requires C, 49.51; H, 5.60%).

Acetobromolactose [23] was prepared by the addition of 20 g of **[22]** to 30% HBr in acetic acid solution (50 ml). This was stirred at room temperature for 2.5 hrs. The resulting mixture was poured into ice water (500 ml) with stirring. A white precipitate was collected by vacuum filtration. The solid was recrystallised from methanol yielding a white solid (7.12 g, 29%). m.p. 144-145°C, (lit.[9] 145°C). δ_H (200 MHz, $CDCl_3$), 2.1 (21 H, m, OAc), 3.9 (2 H, m) and 4.15 (4 H, m), (5a , 6a, 6a', 5b, 6b, 6b'-H), 4.5 (2 H, m), 4.8 (1 H, dd, J = 10.2 and 4.3 Hz, 2a-H), 4.95 (1 H, dd, J = 10.2 and 3.3 Hz, 3b-H), 5.15 (1 H, m), 5.35 (1 H, d, J = 3.8 Hz, 4b-H), 5.55 (1 H, t, J = 10.2 Hz, 3a-H) and 6.55 (1 H, d, J = 10.2 Hz, 1a-H). (Found: C, 42.93; H, 4.67%. $C_{26}H_{35}O_{17}Br$ requires C, 44.60; H, 5.00%)

Hepta-*O*-acetyl lactose isothiouronium bromide [24] was prepared by the reflux of acetobromolactose (4 g) and thiourea (0.5 g) in dry acetone (5 ml) for 20 mins. The solvent was evaporated slowly to give a white solid (3.5 g, 79%).which could not be satisfactorally recrystallised.

1-Thio-β-D-lactose heptaacetate [25] was prepared by the addtion of **[24]** (3.5 g) to a solution of $K_2S_2O_5$ in water (5 ml) at 75°C. DCM (7 ml) was added and the mixture stirred for 20 mins. The organic phase was separated and washed with water (3 x 20 ml), dried ($MgSO_4$) and evaporated to a white solid which could not be recrystallised, (1.4 g, 45.6 %). δ_H (200 MHz, $CDCl_3$), 2.3 (1 H, d, J = 10.3 Hz, -SH). (Found: C, 47.69; H, 5.25%. $C_{26}H_{20}O_{17}S$ requires C, 47.81; H, 5.52%).

***S*-Nitroso-1-thio-β-D-lactose heptaacetate [26]** was prepared by dissolving **[25]** in the minimum amount of methanol followed by the addition of an equal amount of water. Excess $NaNO_2$ was added followed by excess conc HCl. A dark red solution formed. The product was extracted into DCM and the solvent evaporated under vacuum without heating. A pink powder was produced, (1.13 g, 77.3 %) with λ_{max} at ~345 nm and $t_{1/2}$ of 55 mins in aqueous buffer / methanol. δ_H (200 MHz, $CDCl_3$) as above except no peak at 2.3, indicating loss of -SH.

The attempted preparation of **Mannose pentaacetate [27]** was performed as follows: mannose (20 g) was stirred in pyridine and cooloed in an ice bath. Acetic anhydride (100 ml) was added and the mixture stirred in ice until all of the mannose had dissolved. The solution was stirred at room temperature overnight. The solvents were evaporated, the oil produced was dissolved in DCM and washed with 3 M HCl and saturated $NaHCO_3$ solution. The DCM layer was dried and evaporated to a pale brown oil. δ_C (50 MHz $CDCl_3$), 21.10, 21.19 and 21.29 (OAc), 62.56, 66.01, 68.80, 69.203 and 71.08 (C2 - C6) and 91.065 (C1).

The attempted preparation of **acetobromomannose [28]** was performed as follows: mannose pentaacetate **[27]** (20 g) was dissolved in DCM (55 ml) and acetic anhydride (3 ml). The solution was chilled in ice with stirring. 30% HBr in acetic acid (20 ml) was slowly added, the reaction stirred at 0°C for 2 h and at room temperature for 2 h. The solvents were evaporated with addition of toluene to give a brown oil. δ_C (50 MHz,

$CDCl_3$), 21.02, 21.10 and 21.20 (OAc), 61.89, 65.72, 68.39, 72.65 and 73.28 (C2 - C6), 83.62 (C1), 170.01 and 170.96 (quaternaries).

The attempted preparation of **2,3,4,6-tetra-*O*-acetylmannopyranoysl isothiouronium bromide [29]** was performed as follows: acetobromomannose **[28]** (4 g) was dissolved in acetone (25 ml) and refluxed with thiourea (1.5 g) for 15 min. A white solid was obtained. This was filtered and the liquid evaporated to give a white gum. δ_C (50 MHz, DMSO), 23.06 (OAc), 64.75, 68.32, 72.22, 72.22 and 73.72 (C2 - C6) and 84.81 (C1).

The attempted preparation of **1-thio-2,3,4,6-tetraacetylmannopyranose [30]** was performed as follows: $K_2S_2O_5$ (2 g) was dissolved in water (25 ml) and stirred at 85°C. The thiouronium salt **[29]** (3.5 g) was added slowly, followed by DCM (30 ml). The solution was refluxed for 15 min and allowed to cool. The DCM layer was separated, washed with water, dried ($MgSO_4$) and evaporated to an oil. δ_H (200 MHz, $CDCl_3$), 2.3 (1 H, d, J = 6.8 Hz, -HS). δ_C (50 MHz, $CDCl_3$), 21.16 and 21.32 (OAc), 62.62, 66.54, 68.99, 70.10 and 72.33, (C2 - C6) and 77.41 (C1).

The attempted preparation of **1-*S*-nitrosothio-2,3,4,6-tetraacetylmannopyranose [31]** was performed as follows: the above thiol **[30]** was dissolved in methanol / water. $NaNO_2$ and HCl (2 eq) were added producing a red solution. The product was extracted into DCM and evaporated to give a stable red oil. λ_{max} ~351 nm. δ_C (50 MHz, $CDCl_3$), 21.08, 21.14 and 21.28 (OAc), 63.08, 66.68, 68.78, 69.37, 71.02, and 92.53.

Pentabenzoyl glucose [32] was prepared as follows: dry pyridine (25 ml) and dry DCM (21 ml) were chilled in a 3 necked flask. A chilled solution of benzoyl chloride (21 ml) in DCM (21 ml) was added dropwise. Glucose (10 g) was added slowly with vigorous stirring. The mixture was allowed to stand in an ice bath overnight. The

solution was diluted with DCM, washed with copious quantites of 2 M HCl, saturated $NaHCO_3$, water and dried ($MgSO_4$). Upon evaporation an oil was produced which solidified to give a white powder which was recrystallised from acetone/water, (18.2 g, 46.8%). m.p. 183°C, (lit.[5] 184-186°C). δ_H (200 MHz, $CDCl_3$), 4.6 (3 H, m, 5, 6 and 6'-H), 5.7 (1 H, dd, J = 10.4 and 3.6 Hz, 2-H), 5.9 (1 H, t, J = 9.9 Hz, 4-H), 6.3 (1 H, t, J = 9.9 Hz, 3-H), 6.85 (1 H, d, J = 3.6 Hz, 1-H) and 7.3 - 8.2 (25 H, m, aromatics). δ_C (50 MHz, $CDCl_3$), 62.439, 68.475, 71.113, 71.971 and 73.212 (C2 - C6), 87.382 (C1), 128.9, 130.4, 133.8 and 134.2 (aromatics).

Benzobromoglucose [33] was prepared as follows: **[32]** (5.65 g) was dissolved in DCM (6 ml). 30%HBr in AcOH (5.8 ml) was added and the mixture stirred for 3 h. The mixture was evaporated with toluene to give a brown oil. The oil was dissolved in DCM and washed with large quantities of saturated $NaHCO_3$ and dried ($MgSO_4$). A white powder was produced upon evaporation, (3.46 g, 65.2%). m.p. 58°c, (lit.[5] 88-89°C). δ_H (200 MHz, $CDCl_3$), 4.5 (1 H, dd, J = 13.8 and 4.8 Hz) and 4.7 (2 H, m) (5, 6, and 6'-H), 5.35 (1 H, dd, J=10.3 and 4.2 Hz, 2-H), 5.8 (1 H, t, J = 9.6 Hz, 3 or 4-H), 6.3 (1 H, t, J = 9.6 Hz, 3 or 4-H), 6.9 (1 H, d, J = 4.8 Hz, 1-H), 7.3 - 7.5 (12 H, m, aromatics) and 7.9 - 8.05 (8 H, m, aromatics). δ_C (50 MHz, $CDCl_3$), 62.439, 68.475, 71.113, 71.971 and 73.212 (C2 - C6), 87.382 (C1), 129.0 - 130.5 and 133.8 - 134.2 (aromatics). (Found: C, 64.19, H, 4.37%. $C_{34}H_{27}BrO_9$ requires C, 61.19, H, 4.10%).

1-Thioacetylglucose tetrabenzoate [34] was prepared as follows: benzobromoglucose (8.3 g) was dissolved in a mixture of DCM (30 ml) and ethanol (20 ml). Potassium thioacetate (3.3 g) was added and the mixture refluxed for 3 h. The KBr was filtered off and the solvents evaporated. The yellow oil produced was dissolved in DCM and washed with water, dried ($MgSO_4$) and evaporated to a yellow oil. The oil was heated in methanol and a white solid was precipitated, (4.6 g, 55.8%). m.p. 176 - 178°C. δ_H (200 MHz, $CDCl_3$), 2.35 (3 H, m, SAc), 4.3 (1 H, m, 5-H), 4.5 (1 H, dd,

J = 12.5 and 5.2 Hz, 6 or 6'-H), 4.6 (1 H, dd, J = 12.5 and 3.0 Hz, 6 or 6'-H), 5.7 (3 H, m) and 6.0 (1 H, m) (1, 2, 3, and 4-H), 7.4 (12 H, m) and 8.0 (8 H, m) (aromatics). δ_C (50 MHz, $CDCl_3$), 31.350 (SAc), 63.388, 69.598, 70.333, 74.659, 77.317 and 81.132 (C1 - C6), 128.8 - 130.4 and 133.6 - 134.0 (aromatics). (Found: C, 64.49, H, 4.53%. $C_{36}H_{30}O_{10}S$ requires C, 66.10, H, 4.59%).

The attempted preparation of **1-thio-tetrabenzoylglucose [35]** was carried out as follows: *S*-acetylglucose tetrabenzoate (0.4 g) was dissolved in dry THF (20 ml). Benzylamine (0.1 ml, 1.5 eq) was added and the mixture stirred under N_2 overnight. The reaction was followed by TLC. The formation of thiol could be observed and the solution attained a strong thiol smell, but this could be observed breaking down to the disulphide. The mixture was evaporated to a semi-solid, dissolved in DCM and washed with 3 M HCl (200 ml). The DCM layer was dried, evaporated to a gum and heated in methanol. A white solid was precipitated, presumed to be the disulphide. m.p.106°C. δ_H (200 MHz, $CDCl_3$), 3.9 (1 H, m, 5-H), 4.55 (2 H, m, 6 and 6'-H), 4.95 (1 H, d, J = 10.2, 1-H), 5.55 (1 H, t, J = 9.7 Hz), 5.75 (1 H, t, J = 9.7 Hz) and 6.0 (1 H, t, J = 9.4 Hz) (2, 3 and 4-H), 7.3 - 7.5 (12 H, m, OBz) and 7.8 - 8.1 (8 H, m, OBz). δ_C (50 MHz, $CDCl_3$), 63.31, 69.84, 70.96, 74.23, 77.27 and 90.30 (C1 - C6), 128.91, 130.34, 133.76 and 134.00 (benzoyl).

Pentabenzoylgalactose [36] was prepared as follows: dry pyridine (25 ml) and DCM (21 ml) were chilled in a flask and a chilled solution of benzoyl chloride (21 ml) in DCM (21 ml) was added slowly with vigorous stirring. Galactose (10 g) was added carefully. The mixture was stirred for 30 mins in an ice bath and then left to stand overnight. The mixture was diluted with DCM and washed with large quantities of 2 M H_2SO_4, saturated $NaHCO_3$ and water, dried ($MgSO_4$) and evaporated to a sticky oil which hardened very slowly, (19.2 g, 49.4%). δ_H (200 MHz,$CDCl_3$), 4.5 - 4.9 (3H, m, 5, 6 and 6'-H), 6.1 - 6.25 (3 H, m, 2, 3, 4-H) and 7.0 (1 H, d, J = 3.7 Hz, 1-H). δ_C (50 MHz, $CDCl_3$), 62.369, 68.216, 68.988, 69.055 and 69.965 (C2 - C6), 91.197

(C1), 129.3, 130.3, 133.8 and 134.2 (aromatics). (Found: C, 66.14, H, 4.73%. $C_{41}H_{32}O_{11}$ requires C, 70.29, H, 4.57%).

Benzobromogalactose [37] was prepared as follows: **[36]** (10g) was dissolved in DCM (50 ml). Acetic anhydride (1 ml) and 30% HBr in AcOH (20 ml) were added and the mixture stirred at room temperature for 5 h. The solvents were evaporated with toluene to give a pale brown oil. The oil was diluted with DCM and washed with saturated $NaHCO_3$, dried ($MgSO_4$) and evaporated to a sticky brown oil, (16 g, 71 %).

1-Thioacetylgalactopyranosyl tetrabenzoate [38] was prepared as follows: benzobromogalactose (8 g) was dissoved in DCM (20 ml) and ethanol (20 ml). Potassium thioacetate (2.8 g) was added and the mixture refluxed for 5 hrs. The KBr was filtered off and the solvent evaporated. The oil was dissolved in DCM, washed with water dried ($MgSO_4$) and evaporated to a yellow oil. Successive recrystallisations from methanol gave a white powder, (3.7 g, 46.6%). m.p. 130 - 132°C. δ_H (200 MHz, $CDCl_3$), 2.35 (3 H, s, SAc), 4.35 - 4.65 (3 H, m, 5, 6 and 6'-H), 5.65 (1 H, d, J = 10.3 Hz, 1-H), 5.75 (1 H, dd, J = 10.3 and 3.7 Hz, 3-H), 5.95 (1 H, t, J = 10.3 Hz, 2-H) and 6.10 (1 H, d, J = 3.7, 4-H). δ_C (50 MHz, $CDCl_3$), 31.39 (SAc), 62.52, 67.98, 73.19, 76.22, and 81.42. (Found: C, 65.06, H, 4.39%. $C_{36}H_{30}O_{10}S$ requires C, 66.1, H, 4.59%).

1,2:5,6-Di-*O*-isopropylideneglucofuranose [40] was prepared as follows: glucose (7.5 g) was stirred in acetone (50 ml). Anhydrous $ZnCl_2$ (6 g) was added followed by 85% H_3PO_4 (0.375 ml). The mixture was stirred at room temperature for 2 days. The unreacted glucose was filtered off. A solution of NaOH (4.25 g) in water (42.5 ml) was added and the $Zn(OH)_2$ filtered off and washed with acetone. The organic layers were combined and the solvents evaporated. The oil was diluted with water, extracted with DCM and washed with water. A white powder was produced upon evaporation of the solvent, (6.1 g, 56.5 %). m.p.101°C, (lit.[10] 105-109°C). δ_C (50

MHz, $CDCl_3$), 25.63, 26.66, 27.27 and 27.32 (diisopropylidene), 68.10, 73.84, 75.55, 81.56 and 85.73 (C2 - C6), 105.54 (C1), 110.14 and 112.30 (diisopropylidene).

3-*O*-Methylglucose [42] was prepared as follows: **[40]** (7.8 g) was dissolved in acetone (7.5 ml). Pulverised NaOH (3.2 g) was added and the mixture stirred at 45°C until the NaOH had dissolved. Dimethyl sulphate (4.26 ml) was added over 30 mins. The mixture was warmed to 50°C for 3 h and then kept at 60°C for 3 h. The orange liquid was diluted with water and extracted with DCM. Upon evaporation an oil was obtained **[41]**. The oil was refluxed in water at pH 2.6 (H_2SO_4) overnight. The amber liquid was decolourised with norit charcoal. Ethanol was added and the solvents evaporated to give an oil.

3-*O*-Methylglucose tetraacetate [43] was prepared as follows : **[42]** (5 g) was dissolved in dry pyridine (60 ml). Acetic anhydride (35 ml) and a few crystals of DMAP were added and the mixture stirred for 3 h and then left to stand overnight at room temperature. DCM was added and the mixture washed with 2 M HCl, saturated $NaHCO_3$ solution and dried (Na_2SO_4). The solvents were evaporated to leave a thick oil, (6.45 g, 69.3 %). δ_H (200 MHz, $CDCl_3$), 2.1 (12 H, m, OAc), 3.45 (3 H, s, OMe), 3.7 (1H, t, J = 10.7 Hz, 3-H), 4.0 - 4.2 (3 H, m, 5, 6 and 6'-H), 5.0 - 5.1 (2 H, m, 2 and 4-H), 5.65 (<1 H, d, J = 4.6 Hz, 1-H(α)) and 6.3 (<1 H, d, J = 9.2 Hz, 1-H(β)).

The attempted preparation of **1-bromo-3-*O*-methyl-2,4,6-triacetyl glucose [44]** was carried out as follows: **[43]** (6.75 g) was dissolved in dry DCM (25 ml). Acetic anhydride (1 ml) and 30 % HBr in acetic acid (10 ml) were added. The flask was covered in foil and stirred at room temperature for 1.5 h. Toluene was added and the solvents were evaporated under reduced pressure. The oil produced was dissolved in DCM, washed with saturated $NaHCO_3$ solution , dried ($MgSO_4$) and evaporated to an oil. Upon addition of ether a white solid precipitated. When filtering was attempted the

white solid became a intractable sticky brown gum, either on exposure to moisture or to air. Attempts to purify the gum were unsuccessful.

3-*O*-mesyl-1,2:5,6-diisopropylideneglucofuranose [45] was prepared as follows: **[40]** (5 g) was dissolved in dry pyridine (35 ml) and cooled in an ice bath. Mesyl chloride (3 ml) was added dropwise. The solution was stirred in ice for 15 mins and a white solid precipitated. The mixture was left to stand overnight and then heated on a boiling water bath for 1 h. The brown solution formed was poured onto crushed ice (200 ml) and extracted with DCM (100 ml). The DCM was washed with 2 M HCl (200 ml), dried ($MgSO_4$) and decolourised with charcoal. Evaporation gave a thick oil. The oil was dissolved in ethanol, water was added until precipitation occurred and left under refrigeration to crystallise. A white solid was isolated, (4.0 g, 60 % yield). m.p.60 - 61% (lit.[11] 83 - 84°C). δ_C (50 MHz, $CDCl_3$), 25.71, 26.67, 27.21 and 24.42 (isopropylidene), 38.54 (SO_2Me), 68.49, 73.83, 81.39, 84.01, 85.30 (C2 - C6), 106.88 (C1), 111.07 and 114.00 (isopropylidene). (Found: C, 46.35, H, 6.68%. $C_{13}H_{22}O_8S$ requires: C, 46.15, H, 6.51%).

3-*O*-Tosyl-1,2:5,6-diisopropylideneglucofuranose [46] was prepared as follows: **[40]** (5 g) was dissolved in pyridine (30 ml). Tosyl chloride (7.5 g, 2 eq) was added and the mixture stirred at room temperature for 1 h then allowed to stand overnight. Finally the solution was heated on a boiling water bath for 1 h. The mixture was poured into ice water, extracted into DCM washed with 2 M HCl, dried ($MgSO_4$) and evaporated to a thick oil. The oil was recrystallised from ethanol/water to give a white solid, (6 g, 75.4%). m.p. 103 - 106°C, (lit.[12] 120 - 121°C). δ_C (50 MHz, $CDCl_3$), 22.18 (tosyl), 25.34, 26.67, 27.06 and 27.06 (diisopropylidene), 67.53, 72.21, 80.27, 82.50 and 83.74 (C2 - C6), 105.55 (C1), 109.53, 112.98 (diisopropylidene), 128.93 and 130.16 (tosyl). (Found: C, 55.47, H, 6.43%. $C_{19}H_{26}O_8S$ requires: C, 55.07, H, 6.28%).

3-*O*-Triflyl-1,2:5,6-diisopropylideneglucofuranose [47] was prepared as follows: triflic anhydride (3 ml) in DCM (60 ml) were stirred in an acetone/ice bath. Pyridine (3 ml) in DCM (30 ml) were added slowly. 1,2:5,6-Diisopropylidene glucose **[40]** (3 g) in DCM (30 ml) was added dropwise. The solution was stirred for 30 min and then poured into ice water (200 ml) containing $NaHCO_3$ (0.4 g). The layers were separated, the DCM layer washed with cold 3% HCl, water and saturated $NaHCO_3$ solution. The solution was dried ($MgSO_4$) and evaporated to a solid. Recrystallisation from ethanol / water gave a white powder, (3.9 g, 86.2%). m.p. 69-70°C (lit.[13] 70°C). δ_H (200 MHz, $CDCl_3$), 1.3 - 1.55 (12 H, m, diispropylidene), 4.0 (1 H, m) and 4.2 (3 H, m) (4, 5, 6, 6'-H), 4.75 (1 H, d, J = 3.7 Hz, 2-H), 5.25 (1 H, s, 3-H) and 6.0 (1 H, d, J = 3.7 Hz, 1-H). δ_C (50 MHz, $CDCl_3$), 25.33, 26.71, 27.03 and 27.28 (diisopropylidene), 68.07, 72.16, 80.34 and 83.71 (C2, 4, 5 and 6), 88.64 (C3), 105.47 (C1), 110.34 and 113.58 (diisopropylidene). (Found: C, 40.03, H, 4.89%. $C_{13}H_{19}F_3O_8S$ requires: C, 39.8, H, 4.85%).

3-*S*-Acetyl-1,2:5,6-diisopropylideneglucofuranose [48] was prepared as follows: **[47]** (1.4 g) and tetrabutylammonium thioacetate (4 g) were refluxed in toluene (40 ml) for 2 h. The solution was filtered and evaporated to give a thin brown liquid. The liquid was dissolved in DCM washed with water, dried ($MgSO_4$) and evaporated to give a brown liquid from which a white solid precipitated. The solid was recrystallised from hexane, (0.4 g, 35.2 %). m.p. 89°C (lit.[14] 88-89°C). δ_H (200 MHz, $CDCl_3$), 1.35 - 1.55 (12 H, s, diisopropylidene), 2.4 (3 H, s, SAc), 3.7 (1 H, dd, J = 10.5, 5 Hz, 3-H), 4.05 - 4.2 (4 H, m, 4, 5, 6, 6'-H), 4.7 (1 H, t, J = 3.7 Hz, 2-H), 5.85 (1 H, d, J = 3.7 Hz, 1-H). δ_C (50 MHz, $CDCl_3$), 25.65, 26.82, 26.82 and 27.08 (diisopropylidene), 30.95 (SAc), 46.41 (C3), 65.70, 75.98, 79.84 and 81.38 (C2, C4, C5 and C6), 104.91 (C1), 110.25 and 112.85 (diisopropylidene).

3-Thio-1,2:5,6-diisopropylideneglucofuranose [49] was prepared as follows: **[48]** (1 g) was disolved in dry THF (30 ml) under N_2. Benylamine (0.35 ml, 1.5 eq)

was added and the mixture stirred under N_2 for 3 days. The solvent was evaporated to give a brown liquid. The liquid was purified by flash column chromatography (hexane : EtOAC 3:1) to give a pale yellow oil, (0.42 g, 48.4 %). δ_H (200 MHz, $CDCl_3$), 1.35 - 1.50 (12 H, 4 s, diisopropylidenes), 2.05 (1 H, d, J = 9.7 Hz, -SH), 3.00 (1 H, 2 dd, J = 10.4, 5.1 Hz, 3-H), 3.95 - 4.15 (3-H, m, 4-, 6-and 6'-H), 4.30 (1 H, m, 5-H), 4.60 (1 H, t, J = 4.4 Hz, 2-H) and 5.80 (1 H, d, J = 3.9 Hz, 1-H). δ_C (50 MHz, $CDCl_3$), 25.58, 26.86, 26.94 and 27.07 (diisopropylidenes), 41.65 (C3), 66.10, 76.15, 82.39, 83.46 and 104.48 (C1), 110.33 and 112.48 (quaternaries).

3-*S*-Nitrosothio-1,2:5,6-diisopropylideneglucofuranose [50] was prepared by bubbling N_2O_3 through a sample of [49] in DCM. A red liquid was produced. Evaporation gave a stable red oil. λ_{max} ~341nm. δ_C (50 MHz, $CDCl_3$), 25.47, 26.80 and 27.08 (diisopropylidenes), 50.02 (C3), 66.58, 76.38, 79.83 and 81.92 (C2, and C4 - C6) and 105.04 (C1).

3-Iodo-1,2:5,6-diisopropylideneglucofuranose [51] was prepared as follows: **[47]** (1.2 g) and tetrabutylammonium iodide (2.25 g) were refluxed in toluene (50 ml) for 2 days. After cooling, the solvent was evaporated to give a brown semi-solid slurry. The slurry was extracted with hot hexane (3 x 50 ml) and filtered. The hexane was evaporated to give a brown oil. δ_H (200 MHz, $CDCl_3$), 1.3 (12 H, m, diisopropylidenes), 3.65 (1 H, dd, J = 10, 4.5 Hz) and 3.95 - 4.2 (4 H, m) (3, 4, 5, 6, 6'-H), 4.5 (1 H, t, J = 4.5 Hz, 2-H) and 5.7 (1 H, d, J = 4.1 Hz, 1-H). δ_C (50 MHz, $CDCl_3$), 19.85 (C3), 25.69, 26.91, 27.06 and 27.10 (diisopropylidenes), 66.13, 75.88, 81.86 and 82.13 (C2, C3, C5 and C6), 103.57 (C1), 110.31 and 111.98 (diisopropylidenes).

1,2:4,5-Diisopropylidenefructose [52] was prepared as follows: fructose (7 g) was stirred in dry acetone (140 ml). H_2SO_4 (0.7 ml) was added and the mixture stirred at room temperature for 2 h during which all the sugar had dissolved. NaOH (2.2 g) in

water (20 ml) was added and a yellow oil formed. The oil was extracted into DCM, washed with water, dried ($MgSO_4$) and evaporated to a white powder, (5.4 g, 51.9%). m.p.105°C (lit.[15]104-109°C). δ_C (50 MHz, $CDCl_3$), 26.47, 26.78, 26.88 and 28.47 (diisopropylidene), 61.12, 70.82, 72.69, 73.82 and 77.81 (C2 - C6), 105.01 (C1), 109.88 and 112.36 (diisopropylidene).

3-*O*-Tosyl-1,2:4,5-diisopropylidenefructose [53] was prepared as follows: Diisopropylidene fructose (5 g) was dissolved in pyridine (30 ml) and chilled in an ice bath. Tosyl chloride (7.5 g) was added and the mixture stirred for 30 min. The mixture was left to stand overnight and finally heated on a boiling water bath for 1 h. The solution was poured onto crushed ice (150 ml), extracted with DCM, washed (2 M HCl and water), dried ($MgSO_4$) and evaporated to a white solid (5.9 g, 74.6 %). m.p. 96°C (lit.[16] 97-98°C). δ_C (50 MHz, $CDCl_3$), 22.19 (tosyl), 26.12, 26.69, 27.14 and 28.38 (isopropylidenes), 60.50, 72.08, 74.51, 75.49 and 78.57 (C2 - C6), 103.65 (C1), 110.09 and 113.07 (isopropylidene), 128.52 and 129.98 (tosyl).

The attempted preparation of **3-triflyl-1,2:4,5-diisopropylidenefructose [54]** was as follows: triflic anhydride (7.5 ml) was dissolved in dry DCM (100 ml) in a salt / ice bath. Pyridine (7.5 ml) in dry DCM (50 ml) was added dropwise. Diisopropylidene fructose (7.5 g) dissolved in dry DCM (35 ml) was added over a 10 min period with vigorous stirring. The mixture was stirred for a further 30 min and then poured into ice water (400 ml) containing $NaHCO_3$ (1 g). The layers were separated and the organic phase wahed with 3% HCl (2 x 100 ml), water (100 ml) and saturated $NaHCO_3$ (100 ml). The solution was dried and evaporated to a white oil. The oil was dissolved in ethanol / water and heated until the oil solidified. Filtration and recrystallisation from ethanol gave a white powder, (4.2 g). m.p. 78°C (dec). δ_C (50 MHz, $CDCl_3$), 25.95 and 26.01 (isopropylidene), 65.36, 68.08, 69.80, 71.64, 85.53 (C3), 103.35 (C1), 111.82 (isopropylidene). The results suggest that an isopropylidene group had been lost.

1,2:3,4-diisopropylidenegalactopyranose [55] was prepared as follows: galactose (10 g) was dissolved in acetone (125 ml). $ZnCl_2$ (12 g) and H_2SO_4 (0.4 ml) were added and the mixture stirred at room temperature for 18 h. A solution of Na_2CO_3 (20 g) in water (35 ml) was. The $Zn(OH)_2$ produced was filtered and washed with acetone. The combined filtrates were evaporated to give a yellow oily solid. The product was dissolved in ether and dried by shaking with $MgSO_4$ for 10 min and allowing to stand for 24 h. Filtration and evaporation gave a brown syrup (14.2 g, 98.1 %). δ_C (50 MHz, DMSO), 24.46, 25.12, 26.09 and 26.17 (isopropylidenes), 60.20, 68.46, 70.21, 70.21 and 70.37 (C2 - C6), 95.87 (C1), 107.88 and 108.28 (isopropylidenes).

6-Tosyl-1,2:3,4-diisopropylideneglactose [56] was prepared as follows: 1,2:3,4-diisopropylidenegalactose (16 g) was stirred in pyridine (40 ml) forming a suspension. The suspension was chilled and tosyl chloride (18 g) added slowly. The solution eventually became clear. The solution was stirred at room temperature for 6 h by which time pyridine hydrochloride had formed as a precipitate. The mixture was poured into water and extracted with DCM. The DCM was washed with 2 M HCl and water, dried ($MgSO_4$) and evaporated to an oil. Recrystallisation from methanol/water left an oil which slowly hardened to an amorphous white solid. The solid was recrystallised from ethanol to give white crystals, (21.5 g, 84.5 %). m.p. 91 - 92°C (lit.[17] 91 - 92°C). δ_H (200 MHz, $CDCl_3$), 1.3 - 1.5 (12 H, m, isopropylidenes), 2.45 (3 H, s, tosyl, -CH_3), 4.05 - 4.2 (4 H, m, 2 or 3-H, 5, 6, and 6-H), 4.3 (1 H, dd, J = 4.5, 2.5 Hz, 4-H), 4.6 (1 H, dd, J = 8.2 and 2.5 Hz, 2 or 3-H), 5.45 (1 H, d, J = 4.5 Hz, 1-H), 7.35 (2 H, d, J = 8.2 Hz, tosyl) and 7.8 (2 H, d, J = 8.2 Hz, tosyl). δ_C (50 MHz, $CDCl_3$), 22.09 (tosyl -CH_3), 24.80, 25.37, 26.27 and 26.43 (isopropylidenes), 66.32 (CH), 68.68 (C6), 70.82 (CH), 70.87 (CH), 70.97 (CH), 96.59 (C1), 109.41 and 110.05 (isopropylidenes).

6-Thioacetyl-1,2:3,4-diisopropylidenegalactose [57] was prepared as follows: the above tosylate **[56]**, (3.4 g) was dissolved in DMF (40 ml). Potassium thioacetate (4 g) was addded and the mixture stirred under N_2 at 115°C for 7 h. The slurry was cooled in ice, poured into chilled xylene and stirred for 30 min. A large amount of water soluble precipitate was filtered off. The solvents were evaporated and the process repeated. A dark brown oil was produced. This was dissolved in pyridine (9 ml) and acetic anhydride (6 ml), stirred for 16 h at room temperature and poured into ice water. A brown gum was precipitated. This was collected and the water extracted with toluene. The gum was dissolved in the toluene, dried ($MgSO_4$) and evaporated to an orange oil. The oil was distilled (175°C / 0.05 mmHg) to give a yellow oil, (1.9 g, 72.8 %). δ_H (200 MHz, $CDCl_3$), 1.35 - 1.55 (12 H, m, isopropylidenes), 2.4 (3 H, s, SAc), 3.05 (1 H, dd, J = 13.8, 9.2 Hz, 6 or 6'-H), 3.2 (1 H, dd, J = 13.8, 4.6 Hz, 6 or 6'-H), 3.85 (1 H, m, 5-H), 4.25 (1 H, d, J = 2.8 Hz, 4-H), 4.3 (1 H, dd, J = 5.2, 2.8 Hz, 3-H), 4.6 (1 H, dd, J = 8.1, 2.8 Hz, 2-H), 5.5 (1 H, d, J = 4.6 Hz, 1-H). δ_C (200 MHz, $CDCl_3$), 24.91, 25.47, 26.43 and 26.43 (isopropylidenes), 30.17 (C6), 31.03 (SAc), 67.29, 70.98, 71.40 and 72.51 (C2 - C5), 97.00 (C1), 109.27 and 109.94 (isopropylidenes).

6-Thio-1,2:3,4-diisopropylidene galactose [58] was prepared as follows: **[57]** (1.5 g) was dissolved in methanol (20 ml). Sodium metal (8.5 mg) was added and the solution stirred under N_2 for 3 days. CO_2 was bubbled through the solution to neutralise the NaOMe and the solvent evaporated to give a yellow gum. The gum was extracted into ether and evaporated to a yellow oil with a strong thiol smell, (1.15 g, 88.1 %). δ_H (200 MHz, $CDCl_3$), 1.35 - 1.55 (12 H, m, isopropylidenes), 2.05 (1 H, t, -SH), 2.7 - 2.8 (2 H, m, 6 and 6'-H), 3.8 (1 H, td, J = 7, 1.8 Hz, 5-H), 4.35 (2 H, m, 3 and 4-H), 4.65 (1 H, dd, J = 7.9, 2.4 Hz, 2-H), 5.55 (1 H, d, J = 5.1 Hz, 1-H). δ_C (200 MHz, $CDCl_3$), 24.90 (C6), 24.90, 25.41, 26.44 and 26.53 (isopropylidenes), 70.33, 70.98, 71.31 and 71.73 (C2 - C5), 97.64 (C1), 109.29 and 109.79 (isopropylidenes).

The attempted preparation of **6-nitrosothio-1,2:3,4-diisopropylidene galactose [59]** was performed as follows: the thiol **[58]** (1 g) was dissolved in methanol (8 ml) and water (2 ml). Excess $NaNO_2$ and HCl were added to give a deep red solution. The solution was extracted with DCM and evaporated to give a red sticky solid, λ_{max} 332 nm. This was shown to be mostly disulphide. δ_C (50 MHz, $CDCl_3$), 24.91, 25.49, 26.43 and 26.52 (isopropylidenes), 38.70 (C6) 67.13, 71.02, 71.36 and 71.94 (C2 - C5), 97.10 (C1), 109.24 and 109.74 (isopropylidenes). The nitrosothiol has should have a signal for C6 at 33.76.

6-Tosyl-1,2,3,4-tetraacetylglucose [60] was prepared as follows: 1,2,3,4 tetraacetyl glucose (5 g) was dissolved in pyridine (50 ml) and chilled in an ice bath. Tosyl chloride (10 g) was added slowly with vigorous stirring. The mixture was stirred overnight and poured into ice water (200 ml). The water was extracted with DCM (100 ml) and washed with 2 M HCl (4 x 50 ml). The organic phase was dried ($MgSO_4$) and evaporated to a white solid. The solid was recrystallised from ethanol, (6.16 g, 85.4 %). m.p. 194°C.(lit.[18] 194°C). δ_H (200 MHz, $CDCl_3$), 2.0 (12 H, m, OAc), 2.5 (3 H, s, OTs), 3.85 (1 H, m, 5-H), 4.15 (2 H, m), 5.05 (1 H, dd, J = 9.1, 2.5 Hz) and 5.15 - 5.2 (2 H, m) (2, 3, 4, 6, 6'-H), 5.65 (1 H, d, J = 8.1 Hz, 1-H), 7.35 (2 H, d, J = 8.1 Hz, OTs) and 7.8 (2 H, d, J = 8.1 Hz, OTs). δ_C (50 MHz, $CDCl_3$), 20.98, 21.03 and 21.24 (OAc), 22.18 (OTs), 67.19, 68.33, 70.44, 72.57, 73.02 (C2 - C6), 91.96 (C1), 128.64, 130.34, 133.20 and 145.68 (OTs), 169.26, 169.63, 169.76 and 170.59 (OAc).

6-Bromomethylglucopyranoside [61] was prepared by dissolving methylglucopyranoside (5 g, 26 mmol) and *N*-bromo succinimide (9.15 g, 52 mmol) in DMF (70 ml). This was stirred in an ice bath and 13.5 g Ph_3P was slowly added. The solution was stirred at 50°C for 1.5 hrs and the reaction then quenched with methanol. Butanol was added and the solvents evaporated to give a yellow oil. The oil was dissolved in DCM and washed with water (3 x 50 ml). Ph_3PO remained in the organic phase. The aqueous phases were combined and evaporated to give an orange oil. This

became a milky orange solid upon standing. δ_H (200 MHz, $CDCl_3$), 4.85 (1 H, d, J = 4.5 Hz, 1-H).

Methyl-6-bromoglucopyranoside triacetate [62] was prepared from crude **[61]** by acetylation in acetic anhydride (40 ml) and dry pyridine (60 ml), with stirring at room temperature for 3 h. The solution was then poured into ice water (300 ml) with good stirring and a brown gum was produced. The aqueous solution was extracted with DCM and added to a solution of the brown gum in DCM. The solution was washed with copious quantities of 1 M HCl followed by saturated $NaHCO_3$ solution, dried ($MgSO_4$) and evaporated to a brown oil. Recrystallisation gave from ethanol gave a white well crystallised product, (3.5 g, 35.4 % over 2 steps). m.p. 110°C (lit.[19] 116 - 117°C). δ_H (200 MHz, $CDCl_3$), 2.1 (9 H, m, OAc), 3.4 (1 H, dd, J = 11.3, 6.8 Hz, 6 or 6'-H), 3.45 (3 H, s, OMe), 3.5 (1 H, dd, J = 11.3, 2.6 Hz, 6 or 6'-H), 4.0 (1 H, m, 5-H), 4.9 (1 H, dd, J = 10.0, 3.9 Hz), 5.0 (2 H, m, 1 and 3 or 4-H), 5.5 (1 H, t, J = 9.7 Hz, 3 or 4-H). δ_C (50 MHz, $CDCl_3$), 21.21 (OAc), 31.71 (C6), 56.04 (OMe), 68.95, 70.36, 71.24 and 71.60 (C2 - C5), 97.09 (C1).

6-*S*-Acetyl-2,3,4-triacetylmethylglucopyranoside [63] was prepared by the following methods:

1. **[62]** (1.1 g) and tetrabutylammonium thioacetate (1.8 g) were refluxed in toluene (50 ml) for 2.5 h. Upon cooling the mixture separated into two layers. The pale brown upper layer was decanted into ice cold xylene and stirred for 30 min. A dark brown precipitate was filtered off. The filtrate was evaporated to give a pale brown oil. Distillation under vacuum (175 °C / 0.05 mmHg, lit.[20] 165-170°C / 0.02mmHg) gave an orange oil. (0.53 g, 48.4 %). δ_H (200 MHz, $CDCl_3$), 2.1 (9 H, m, OAc), 2.4 (3 H, s, SAc), 3.1 (1 H, dd, J = 14.3, 6.6 Hz, 6- or 6'-H), 3.2 (1 H, dd, J = 14.3, 3.5 Hz, 6- or 6'-), 3.4 (1 H, s, OMe), 3.95 (1 H, m, 5-H), 4.85 - 4.95 (3 H, m, 1-, 2-, and 3- or 4-H), 5.45 (1 H, t, J = 9.3 Hz, 3- or 4-H). δ_C (50 MHz, $CDCl_3$), 21.19 (OAc), 30.46

(C6), 30.90 (SAc), 55.81 (OMe), 68.65, 70.43, 71.35 and 71.35 (C2 - C6), 97.01 (C1), 170.36 (OAc) and 195.03 (SAc).

2. **[62]** (1 g) and potassium thioacetate (1.2 g, 4 eq) were dissolved in DMF (45 ml) and heated with stirring at 110°C under N_2 for 3 h. The slurry formed was cooled in ice then poured into ice cold xylene and stirred for 30 min. A brown precipitate was filtered. The solvent was evaporated and the process repeated. Evaporation gave a brown oil. The oil was dissolved in acetic anhydride (6 ml) and pyridine (9 ml) and stirred at room temperature for 24 h. The mixture was poured into water with no signs of a precipitate forming. The water was extracted with DCM which was dried and evaporated to give a thick orange oil. The oil was distilled to give the same product as above, (0.28 g, 28.4 %).

3. **[62]** (1 g) and potassium thioacetate (1.1 g) were refluxed in acetone (30 ml) for 5 h. The mixture was allowed to cool, filtered and evaporated to a red sludge. The residue was dissolved in DCM, washed with water and evaporated to a thick red / brown oil. The oil was purified by column chromatography (hexane : EtOAc 3:1) to give the desired product as above, (0.30 g, 30.6 %).

The attempted preparation of **6-thio-2,3,4-triaacetylmethylglucopyranoside [64]** was carried out from the above thioacetates as follows: **[63]** (0.3 g) and benzylamine (0.1 ml, 1.5 eq) were dissolved in dry THF (40 ml) and stirred under N_2 for 3 days. TLC revealed all of the starting material had been hydrolysed. Flash column chromatography using the same solvent system (hexane : EtOAc 3:1) gave no product suggesting that the thiol was breaking down to disulpide on the column.

The attempted preparation of **methyl-2,3,4-triacetyl-6-isothiouronium glucopyranosyl bromide [65]** was carried out by dissolving **[62]** in dry acetone

(10 ml) and refluxing with thiourea (2 g) for 20 mins. Upon slow evaporation, a colourless oil was produced.

The attempted preparation of **methyl-2,3,4-triacetyl-6-thioglucopyranoside [64]** was carried out as follows: **[65]** (4 g) was added to a solution of $K_2S_2O_5$ in water (5 ml) at 75°C. DCM (6 ml) was added and the mixture refluxed with stirring for 15 mins. The DCM layer was separated and washed with water (3 x 15 ml), dried ($MgSO_4$) and evaporated to an amorphous white solid. The solid was recrystallised from methanol to give a white powder (0.75 g). m.p. 56°C (lit.[20] 40°C). δ_H (200 MHz, DMSO), 2.0 (9 H, m, OAc), 2.1 (1 H, d), 3.35 (1 H, m), 3.4 (3 H, s, OMe), 3.55 (1 H, dd, J = 10.8 and 6.5 Hz) and 3.75 (1 H, dd, J = 10.9, and <1Hz) (6 and 6'-H), 3.9 (1 H, t, J = 8.7 Hz), 4.75 (1 H, dd, J = 10 and 3.5 Hz, 2-H), 4.8 (1 H, m), 4.95 (1 H, t, J = 3.9 Hz) and 5.3 (1 H, t, J = 9.8 Hz). It is assumed by the elevated melting point that a high percentage disulphide has been formed. The above product was nitrosated as described previously. A red oil was isolated but could not be solidified before decomposition had occurred.

6-Azido-2,3,4-triacetylmethylglucopyranoside [66] was prepared as follows: the bromosugar **[62]** (2.4 g) and sodium azide (1.3 g) were stirred in DMF (20 ml) in a boiling water bath for 4 h. The product was poured into ice water (50 ml) and a white precipitate collected, (1.72 g, 79.7 %). m.p.102°C (lit.[21] 103°C) υ_{max} /cm^{-1} 2100. δ_H (200 MHz, $CDCl_3$), 2.05 - 2.15 (9 H, m, OAc), 3.3 (2 H, m, 6 and 6'-H), 3.45 (3 H, s, OMe), 3.95 (1 H, m, 5-H), 4.9 (1 H, dd, J = 9.8, 4.0 Hz, 2-H), 4.95 (1 H, d, J = 4.0 Hz, 1-H), 4.95 (1 H, t, J = 9.8 Hz, 3 or 4-H), 5.45 (1 H, t, J = 9.8 Hz, 3 or 4-H). δ_C (50 MHz, $CDCl_3$), 21.14 (OAc), 51.41 (C6), 56.01 (OMe), 69.00, 70.13, 70.26 and 71.17 (C2 - C5), 97.05 (C1), 170.14, 170.52 and 170.57 (OAc).

6-Amino-2,3,4-tetraacetylmethylglucopyranoside [67] was prepared as follows: 6-azidoglucose (1.7 g) was dissolved in methanol (100 ml). A catalytic amount

of 10 % Pd on C was added and the mixture hydrogenated for 24 h. The suspension was filtered through celite and the filtrate evaporated to give a clear oil which solidified with standing, (0.66 g, 42.1 %). δ_C (50 MHz, $CDCl_3$), 22.67 (OAc), 40.31 (C6), 54.55 (OMe), 70.46, 72.04, 72.13 and 73.14 (C2 - C5), 99.81 (C1) and 170.30 (OAc).

2,5-Dimesyl-1,3,4,6-dianhydromannitol [69] was prepared as follows: isomannide (10 g, 0.068 mol) was dissolved in dry pyridine (60 ml) in an ice bath. Nitrogen was bubbled through the solution for 5 mins. Mesyl chloride (11 ml, 0.142 mol) was added from a dropping funnel with vigorous stirring. Stirring was continued for 10 mins and the solution then left to stand: in the ice bath for 3 h, at room temperature for 1 h and finally on a steam bath for 1 h. Water was added to dissolve the pyridine hydrochloride precipitate and the solution poured into 250 ml cold water. A white precipitate was filtered, washed with water and dried under vacuum to give fine white crystals, (11.6 g, 56%). m.p. 96°C (lit.[22] 101°C). δ_H (200 MHz, $CDCl_3$), 3.05 (6 H, m, $-CH_3$), 3.95 (2 H, dd, J = 9.5 and 6.9 Hz, 1b and 6b-H), 4.15 (2 H, dd, J = 9.2 and 6.6 Hz, 1a and 6a-H), 4.75 (2 H, m, 3 and 4-H) and 5.1 (2 H, m, 2 and 5-H).

2,5-Ditosyl-1,3,4,6-dianhydromannitol [70] as follows: isomannide (9.5 g, 0.065 mol) was dissolved in pyridine (7.5 ml). Tosyl chloride (26 g, 0.136 mol) was slowly added with vigorous stirring. The solution was left to stand at room temperature for 4 hrs and then heated on a steam bath for 1 hour. The solution was allowed to cool and poured onto crushed ice (400 ml). The product was extracted into DCM, washed with 2 M HCl and water, dried ($MgSO_4$) and evaporated to a yellow oil. The oil solidified upon standing and was recrystallised from methanol yielding a white solid (26.1 g, 88.4 %). m.p.91 - 93°C (lit.[23] 89 - 90°C). δ_H (200 MHz, $CDCl_3$), 2.50 (6 H, s, CH_3), 3.70 (2 H, dd, J = 9.5 and 7.7 Hz, 1b and 6b-H), 3.9 (2 H, dd, J = 9.5 and 6.6 Hz, 1a and 6a-H), 4.5 (2 H, m, 3 and 4-H) and 4.85 (2 H, m, 2- and 5-H). δ_C (200 MHz, $CDCl_3$), 22.21 (CH_3), 70.57, 78.37 and 80.43 (ring carbons), 128.46, 130.44, 133.42, 145.85 (aromatics).

2,5-Ditriflyl-1,3,4,6-dianhydromannitol [71] was prepared as follows: isomannide (1.15 g) was dissolved in dry DCM (7.5 ml) and dry pyridine (2.5 ml). Argon was passed over the solution for 5 mins whilst chilling in an ice bath. A solution of triflic anhydride (5 g, 2.8 ml) in DCM (5 ml) was added from a dropping funnel over a period of 15 mins with vigorous stirring. The solution was allowed to warm and was stirred at room temperature overnight then poured into 3 M HCl (50 ml). The product was extraced into DCM and dried (Na_2SO_4). Evaporation yielded a yellow oil which solidified and was recrystallised from ethanol to give fine white needles, (2.4 g, 74.3 %). m.p. 54°C, (lit.[24] 60°C). δ_H (200 MHz, $CDCl_3$), 4.15 (4 H, m, 1a, 1b, 6a and 6b-H), 4.8 (2 H, m, 3 and 4-H) and 5.2 (2 H, m, 2 and 5-H). δ_C (50 MHz, $CDCl_3$), 71.35, 80.81 and 83.89.

2,5-Dithioacetyl-1,3,4,6-dianhydromannitol [72] was prepared by refluxing isomannide ditriflate [71] (1 g) and potassium thioacetate (1 g) in a mixture of dry DCM (10 ml) and dry ethanol (10 ml) for 2 h. The resultant mixture was filtered boiled with decolourising charcoal and evaporated to a brown oil, (0.45 g, 71.1 %). δ_H (200 MHz, $CDCl_3$), 2.4 (6 H, m, SAc), 3.8 (2 H, dd, J = 9.9 and 3 Hz, 1b and 6b-H), 4.0 (2 H, dd, J = 5.3 and 3 Hz, 2 and 5-H), 4.25 (2 H, dd, J = 9.9 and 5.35 Hz, 1a and 6a-H) and 4.55 (2 H, m, 3 and 4-H). δ_C (50 MHz, $CDCl_3$), 31.11, 48.67, 73.37 and 87.93. (Found: C, 43.0, H, 5.23%. $C_{10}H_{14}O_4S_2$ requires: C, 45.8, H, 5.34%).

2,5-Dithioacetyl-1,3,4,6-dianhydromannitol [72] was also prepared as follows: ditosyl isomannide [70] (5 g) and potassium thioacetate (5 g) were stirred in DMF (80 ml) and heated at 110°C in an oil bath for 5 h under N_2. The resulting brown mix was evaporated leaving a dark brown sludge. This was extracted with DCM, washed with water and left to stand over decolourising charcoal and $MgSO_4$. Upon evaporation a pungent brown oil was produced. The oil was distilled under vacuum

(175°C, 0.05 mmHg) to give an orange oil (1.6 g, 55.0 %) which did not solidify upon storage (lit.[25] m.p. 35 - 37°C). Spectral details were as above.

2,5-Dithio-1,3,4,6-dianhydromannitol [73] was prepared as follows: **[72]** (3 g) was refluxed with 2.5 % HCl in methanol (50 ml) for 4 h. The solvent was evaporated to give an orange oil. This was dried under vacuum, over NaOH and chilled to give a brown sludge. The sludge was distilled under vacuum (70°C, 0.05 mmHg) to give a pale orange oil, (1.9 g, 93.2 %). (lit.[25] m.p. 15 - 16°C). δ_H (200 MHz, $CDCl_3$), 1.75 (2 H, d, J = 8.0 Hz, -SH), 3.4 (2 H, m, 2 and 5-H), 3.8 (2 H, dd, J = 9.5 and 2.2 Hz, 1b and 6b-H), 4.15 (2 H, dd, J = 9.5 and 5.0 Hz, 1a and 6a-H), 4.7 (2 H, s, 3 and 4-H). δ_C (50 MHz, $CDCl_3$), 43.82 (C2 and C5), 76.28 and 90.56 (C1, C3, C4 and C6). υ_{max} /cm-1 2565 (SH).

2,5-Di-*S*-nitrosothio-1,3,4,6-dianhydromannitol [74] was prepared from the thiol as follows: **[73]** (1 g) was dissolved in methanol (8 ml) and water (2 ml). Excess $NaNO_2$ and HCl were added to give a deep red liquid. The product was extracted with DCM and evaporated to give a thick red oil. λ_{max} 344 nm. δ_C (50 MHz, $CDCl_3$), 52.31, 73.04 and 88.15.

2,5-Dimesyl-1,3,4,6-dianhydroglucitol [76] was prepared as for isomannide to give a white powder. The powder was recrystallised from ethanol to give fine white needles (17.3 g, 83.6%). m.p. 120°C (lit.[26] 120 - 121°C). δ_H (200 MHz, $CDCl_3$), 3.1 (6 H, m, $-CH_3$), 3.9 (1 H, dd, J = 10.1 and 6.1 Hz, 6b-H), 4.0 (1 H, dd, J = 11.5 and 6.1 Hz, 6a-H), 4.1 (1 H, dd, J = 14 and 3.4 Hz, 1a-H), 4.25 (1 H, d, J = 13.1 Hz, 1b-H), 4.7 (1 H, d, J = 4.7 Hz, 3-H), 4.9 (1 H, t, J= 4.7 Hz, 4-H) and 5.15 (2 H, m, 2 and 5-H). (Found: C, 31.99; H, 4.69%. $C_8H_{14}O_8S_2$ requires C, 31.79; H, 4.64%)

2,5-Ditosyl-1,3,4,6-dianhydroglucitol [77] was prepared as for isomannide to give fine white crystals, (20.75 g, 70.2%). m.p. 95°C, (lit.[22] 100-101°C). δ_H (200

MHz, $CDCl_3$) 2.45 (6 H, s, CH_3), 3.7 (1 H, dd, J = 10.1 and 6.15 Hz, 6b-H), 3.8 (1 H, dd, J = 10.1 and 6.2 Hz, 6a-H), 3.85 (1 H, dd, J = 11.5 and 3.5 Hz, 1a-H), 3.95 (1 H, d, J = 11.6 Hz, 1b-H), 4.45 (1 H, d, J = 4.6 Hz, 3-H), 4.6 (1 H, t, J = 5.1 Hz, 4-H), 4.85 (2 H, m, 2 and 5-H) 7.35 (4 H, m, aromatics) and 7.75 (4 H, m, aromatics). δ_C (50 MHz, $CDCl_3$), 22.18 (OTs), 70.27, 73.71, 78.82, 80.85, 83.76 and 86.07 (ring carbons), 128.32, 128.41, 130.46, 130.63, 133.44, 133.49, 145.84 and 145.99 (OTs). (Found: C, 53.21; H, 4.83%. $C_{20}H_{22}O_8S_2$ requires C, 52.86; H, 4.85%).

2,5-Ditriflyl-1,3,4,6-dianhydroglucitol [78] was prepared as follows: isomannide (1.15 g) was dissloved in dry DCM (7.5 ml) and dry pyridine (2.5 ml). Argon was passed over the solution for 5 mins whilst chilling in an ice bath. A solution of triflic anhydride (5 g, 2.8 ml) in DCM (5 ml) was added from a dropping funnel over a period of 15 mins with vigorous stirring. The solution was then warmed and stirred at room temperature overnight, then poured into 3 M HCl (50 ml). The product was extracted into DCM and dried (Na_2SO_4). Upon evaporation a white powder was produced, (2.4 g, 74.6 %). m.p. 56°C. δ_H (200 MHz, $CDCl_3$), 3.95 (1 H, dd, J = 11.9 and 4.4 Hz, 6b-H), 4.15 (2 H, m, 1a and 6a-H), 4.35 (1 H, d, J = 12.2 Hz, 1b-H), 4.7 (1 H, d, J = 5.5 Hz, 3-H), 5.05 (1 H, t, J = 5.5 Hz, 4-H) and 5.35 (2 H, m, 2 and 5-H). δ_C (50 MHz, $CDCl_3$), 71.86, 73.67, 81.50, 85.41, 86.41 and 88.78.

The attempted preparation of **2,5-dithioacetyl-1,3,4,6-dianhydroglucitol [79]** was as follows: isosorbide ditriflate (1 g) was refluxed with potassium thioactetate (1 g) in a mixture of dry DCM (10 ml) and dry ethanol (15 ml) for 2 hrs. The mixture was filtered, evaporated, dissolved in DCM, washed with water and boiled with decolourising charcoal. Upon evaporation a yellow oil was produced. ^{13}C spectra revealed the oil to be composed either of more than one product, or a product other than that expected. δ_C (50 MHz, $CDCl_3$), 30.96, 31.12, 39.06, 46.45, 48.47, 49.33, 72.24, 73.04, 73.29, 74.10, 82.74, 83.37, 86.07, 87.71 and 88.60.

***S*-Nitrosothioglycerol [82]** was prepared as follows: thioglycerol **[81]** (2 g) was dissolved in DCM (10 ml) and N_2O_3 (from HCl dripped onto $NaNO_2$) was bubbled throught the solution until not more thiol remained (no purple adduct formed with alkaline nitroprusside). The deep red solution was evaporated without heating to form a red oil, (2.5 g, 98.4 %). λ_{max} 332 nm. δ_C (50 MHz, D_2O), 29.43 (C1), 67.08 and 72.90 (C2 and C3).

Dithioglycerol acetate [83] was prepared as follows. thioglycerol (2 g) was dissolved in methanol. A few drops of triethylamine were added and the solution warmed gently with stirring over a 5 day period. Oxygen was bubbled through the solution intermittently. The methanol was evaporated to leave a thin oil. The oil was dissolved in acetic anhydride (25 ml) with NaOAc (4 g) and stirrred in a boiling water bath for 2 h. The product was poured into ice water and an oil formed at the bottom of the beaker. The water was decanted, the oil dissolved in DCM, washed with copious quantities of saturated $NaHCO_3$, water and dried ($MgSO_4$). Evaporation with toluene gave a thin oil, (4.1 g, 57.7 %). δ_H (200 MHz, $CDCl_3$), 2.1 (6 H, s, OAc), 2.95 (2 H, d, J = 7.2 Hz, 1 and 1'-H), 4.15 (2 H, dd, J = 10.8 and 5.2 Hz, 3 or 3'-H), 4.35 (2 H, dd, J = 12.4 and 4.1 Hz, 3 or 3'-H) and 5.25 (2 H, m, 2-H). δ_C (50 MHz, $CDCl_3$), 21.231 and 21.447 (OAc), 39.640 (C1), 64.178 (C2) and 70.270 (C3).

2,3-*O*-Acetyl-1-mercaptopropanediol [85] was not prepared from the above product as follows: the disulphide **[83]** (1 g) was dissolved in DCM (20 ml) and degassed with N_2. Dithiothreitol (0.4 g) was added and the mixture stirred at room temperature under N_2 for 5 days. The solution was washed with water, dried ($MgSO_4$) and evaporated to an oil. ^{13}C NMR revealed the oil to still be the disulphide.

Thioglycerol triacetate [84] was prepared as follows: thioglycerol (4 g) and sodium actetate (8 g) were dissolved in acetic anhydride and stirred in a boiling water bath for 2 hrs. The product was poured onto crushed ice. An oil was observed at the bottom of the

beaker. The water was decanted and the oil dissolved in DCM. The DCM was washed with copious quantities of saturated $NaHCO_3$, water and dried ($MgSO_4$). The solution was evaporated with ethanol to give an oil (4 g, 46.2%) δ_H (200 MHz, $CDCl_3$, 2.1 (6 H, s, OAc), 2.35 (3 H, s, SAc), 3.05 (1 H, dd, J = 14.75 and 6.6 Hz, 1 or 1'-H), 3.25 (1 H, dd, J = 13.9 and 5.7 Hz, 1 or 1'-H), 4.1 (1 H, dd, J = 11.5 and 5.7 Hz, 3 or 3'-H), 4.25 (1 H, dd, J = 12.0 and 4.1 Hz, 3 or 3'-H) and 5.15 (1 H, m, 2-H). δ_C (50 MHz, $CDCl_3$), 21.22 and 21.39 (OAc), 29.78 (C1), 30.96 (SAc), 64.08 (C2) and 70.41 (C3).

2,3-*O*-Acetyl-1-mercaptopropanediol [85] was prepared as follows. thioglycerol triacetate (3.4 g) was dissolved in dry degassed tetrahydrafuran (30 ml). Benzylamine (2.4 ml, 1.5 eq) was added and the solution stirred at room temperature under N_2 for 24 hrs. The reaction was followed by TLC (hexane: EtOAc 2:1) (rf values: disulphide 0.36, S-Acetate 0.53, thiol 0.80). The solvent was evaporated and the oil produced dissolved in DCM. The solution was washed with 3 M HCl, saturated $NaHCO_3$ and water, dried ($MgSO_4$) and evaporated to an oil. The oil was purified by column chromatography (EtOAc: hexane 1:1). (2.1 g, 82.1 %). δ_H (300 MHz, $CDCl_3$), 1.50 (1 H, t, J = 9 Hz, -SH), 2.1 (6 H, m, OAc), 2.7 (2 H, t, J = 6.5 Hz, 1 and 1'-H), 4.25 (1 H, dd, J = 12 and 5.7 Hz, 3 or 3'-H), 4.35 (1 H, dd, J = 11.7 and 3.6 Hz, 3 or 3'-H) and 5.05 (1 H, m, 2-H). δ_C (75 MHz, $CDCl_3$), 21.301 and 21.674 (OAc), 25.317 (C1), 63.918 (C2) and 73.041 (C3).

2,3-*O*-Acetyl-1-*S*-nitrosothioglycerol [86] was prepared by the nitrosation of the above product with N_2O_3 in DCM to give a deep red liquid. The solvent was evaporated to give a red oil with λ_{max} at ~335 nm. $t_{1/2}$ of 50 - 60 mins in aqueous buffer / methanol at 20°C. δ_C (50 MHz, $CDCl_3$), 21.29 and 21.51 (OAc), 33.29 (C1), 64.01 and 69.88 (C2 and C3)

2,3-*O*-Acetylmercaptopropanediol [85] was also prepared as follows: thioglycerol acetate **[84]** (1 g) was dissolved in dry DMF and degassed. Hydrazine acetate (6.8 ml, 1.25 M in DMF) was added and the solution stirred at room temperature under N_2 for 5 hrs. (TLC EtOAc: hexane 2:1). The solvent was evaporated and an oil produced. The oil was dissolved in EtOAc (100 ml), washed (1 M HCl, saturated $NaHCO_3$, H_2O and saturated NaCl), dried ($MgSO_4$) and evaporated to an oil. The pure product was obtained by column chromatography (EtOAc: hexane, 1:1). ^{13}C and ^{1}H spectra as above.

Mercaptosuccinic acid methyl ester [88] was prepared as follows: mercaptosuccinic acid **[87]** (7.5 g) and H_2SO_4 (1.5 ml) were refluxed gently in methanol (40 ml) with a few dried molecular sieves for 7 hrs. The methanol was evaporated and the oil allowed to cool then poured into water (150 ml) in a separating funnel. The product was extracted into ether and washed with saturated $NaHCO_3$ solution. The ether was dried by shaking with $MgSO_4$ for 5 mins and left for 3 hrs to stand. The solvent was evaporated to give a thin oil, (5.7 g, 64.5 %). δ_H (200 MHz, $CDCl_3$), 2.2 (1 H, d, J = 10 Hz, -SH), 2.7 (1 H, dd, J = 17 and 6 Hz, 3 or 3'-H), 3.0 (1 H, dd, J = 17 and 9 Hz, 3 or 3'-H), 3.65 (3 H, s, OMe), and 3.75 (4 H, m, OMe and 2-H). δ_C (50 MHz, $CDCl_3$), 36.403 and 40.069 (C2 and C3), 52.536 and 53.351 (OMe).

***S*-Nitrosomercaptosuccinic acid methyl ester [89]** was prepared as follows: **[88]** (1 g) was nitrosated using N_2O_3 to give an extremely pungent deep red oil, (1.1 g, 94.6 %). λ_{max} at 337nm and $t_{1/2}$ of > 24 hrs. δ_C (50 MHz, $CDCl_3$), 35.80 (C2), 48.06 (C1), 52.71 and 53.42 (OMe).

Mercaptosuccinic acid ethyl ester [90] was prepared as follows: mercaptosuccinic acid (7.5 g) and H_2SO_4 (1.5 ml) were gently refluxed in ethanol (50 ml) with a few molecular sieves for 5 hrs. The ethanol was evaporated to give a thin oil. The oil was

poured into water, extracted into ether, washed with saturated $NaHCO_3$ solution and dried ($MgSO_4$). The solvent was evaporated to give a thin oil, (7.5 g, 72.8 %). δ_H (200 MHz, $CDCl_3$), 1.25 (6 H, m, -$CH_2\underline{CH_3}$), 2.15 (1 H, d, J = 9.4 Hz, -SH), 2.70 (1 H, dd, J = 17 and 6 Hz, 2 or 2'-H), 2.95 (1 H, dd, J = 16.8 and 9 Hz, 2 or 2'-H), 3.70 (1 H, m, 3-H) and 4.15 (4 H, m, -$\underline{CH_2}CH_3$). δ_C (50 MHz, $CDCl_3$), 14.45 and 14.57 (-$CH_2\underline{CH_3}$), 36.66 and 40.32 (C2 and C3), 61.45 and 62.20 (-$\underline{CH_2}CH_3$).

***S*-Nitrosomercaptosuccinic acid ethyl ester [91]** was prepared as follows: **[90]** (1 g) was nitrosated with N_2O_3 to give a pungent deep red oil, (1.1 g, 96.6 %). with λ_{max} at 338nm and $t_{1/2} > 24$ hrs.

Anisylglucosamine [93] was prepared as follows: glucosamine hydrochloride **[92]** (20 g) was dissolved in 1 M NaOH (94 ml). Anisaldehyde was added (11.4 ml) and the mixture shaken until a white precipitate was seen. The flask was cooled in a freezer for 10 mins and filtered. The white solid was washed with ice water and chilled ether. The solid was dried in a vacuum oven to give a white product (26.2 g, 95.1%). m.p. 163°C (lit.[27] 166°C). δ_H (200 MHz, DMSO) 2.85 (1 H, t, J = 8.5 Hz, 2-H), 3.2 (1 H, m, 5-H), 3.85 (3 H, s, OMe), 4.65 (1 H, t, J = 5.6 Hz), 4.75 (1 H, t, J = 7.2 Hz), 4.9 (1 H, d, J = 4.8 Hz) and 5.0 (1 H, d, J = 4.8 Hz) (3,4,6 and 6'-H), 6.6 (1 H, d, J = 6.6 Hz, 1-H), 7.0 (2 H, d, J = 8.6 Hz, aromatics), 7.7 (2 H, d, J = 8.6 Hz, aromatics), 8.15 (1 H, s, CH=N). δ_C (50 MHz, DMSO) 55.54 (OMe), 61.52 (C2), 70.61, 74.80, 77.07 and 78.40 (C3 - C6), 95.85 (C1), 114.18 and 129.94 (aromatics), 161.71 (CHN).

Anisylglucosamine tetra-*O*-acetate [94] was prepared as follows: **[93]** (15 g) was cooled with stirring in a mixture of pyridine (81 ml) and acetic anhydride (45 ml). The mixture was left to stand at room temperature overnight and then poured into ice water with stirring. A white precipitate appeared and was filtered off, dried and recrystallised from ethanol, (20.6 g, 88.0 %). m.p. 187°C (lit.[27] 188°C). δ_H (200 MHz, $CDCl_3$) 1.85 - 2.05 (12 H, m, OAc), 3.4 (1 H, t, J = 8.6 Hz, 2-H), 3.8 (3 H, s,

OMe), 3.95 (1 H, m, 5-H), 4.05 (1 H, dd, J = 12.6, 2.0 Hz, 6 or 6'-H), 4.35 (1 H, dd, J = 12.4, 4.6 Hz, 6 or 6'-H), 5.1 (1 H, t, J = 9.6 Hz, 3 or 4-H), 5.4 (1 H, t, J = 9.4 Hz, 3 or 4-H), 5.95 (1 H, d, J = 8.2 Hz, 1-H), 6.9 (2 H, d, J = 8.6 Hz, aromatics), 7.65 (2 H, d, J = 8.6 Hz, aromatics) and 8.15 (1 H, s, CHN). δ_C (50 MHz, $CDCl_3$) 20.96, 21.1 and 21.24 (OAc), 55.87 (OMe), 62.27 (C2), 68.49, 73.23, 73.37 and 73,70 (C3 - C6), 93.62 (C1), 114.54 and 130.74 (aromatics) and 164.77 (CHN).

Glucosamine tetra-*O*-acetate hydrochloride [95] was prepared as follows: **[94]** (10 g) was was dissolved in the minimum amount of acetone with heating. A small quantity of water was added and the mixture cooled. Concentrated HCl was added dropwise until a precipitate was seen. Chilling was continued whilst stirring with ether, to remove the anisaldehyde. The solid was filtered and washed with chilled ether, (6.1 g, 73.4 %). m.p. dec. (lit.[27] dec). δ_H (300 MHz, D_2O), 2.0 (12 H, m, OAc), 3.6 (1 H, t, J = 10 Hz, 2-H), 4.1 (3 H, m, 5, 6 and 6'-H), 5.0 (1 H, t, J = 10 Hz, 3 or 4-H), 5.4 (1 H, t, J = 10 Hz, 3 or 4-H) and 5.9 (1 H, d, J = 10 Hz, 1-H). δ_C (75 MHz, D_2O) 22.93 and 23.05 (OAc), 55.14, 64.27, 70.83, 73.60, 74.98 (C2 - C6) and 93.16 (C1).

Glucosamine tetra-*O*-acetate [96] was prepared as follows: **[95]** (4.7 g) was dissolved in water. NaOAc (3.4 g, 2 eq) was added and a white suspension formed. The suspension was extracted with DCM (3 x 50 ml), dried ($MgSO_4$) and evaporated. Recrystallisation from ether gave a white solid, (2.9 g, 68.6 %). m.p.138 - 140°C (lit.[27] 143°C). δ_H (200 MHz, $CDCl_3$), 2.05 - 2.20 (12 H, m, OAc), 3.0 (1 H, m, 2-H), 3.80 (1 H, m, 5-H), 4.05 (1 H, dd, J = 12, 3 Hz, 6 or 6'), 4.30 (1 H, dd, J = 12, 4.5 Hz, 6 or 6'), 5.0 (2 H, m, 3 and 4-H), 5.5 (1 H, d, J = 9 Hz, 1-H). δ_C (50 MHz, $CDCl_3$) 21.07, 21.21 and 21.40 (OAc), 55.50, 62.21, 68.68, 73.13 and 75.50 (C2 - C6) and 95.65 (C1).

***N*-(*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (RIG 199) [97]** was prepared as follows: **[96]** (1.3 g) and *N*-

acetylpenicillamine (0.7 g) were stirred in DCM. The penicillamine remained as a suspension. The coupling agent 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-*p*-toluene sulphonate (1.6 g, 1 eq.) was added and the mixture stirred at room temperature. The suspension briefly went into solution before a white precipitate appeared. Stirring was continued for 24 hrs. The solution was washed with 1 M HCl, saturated $KHCO_3$,water, dried ($MgSO_4$) and evaporated. Recrystallisation from ether gave a white solid, (0.61 g, 32.4 %). δ_C (75 MHz, DMSO), 20.13, 20.28, 20.36, 20.45 (OAc), 22.20, 22.22 (NAc), 28.91 / 29.23 and 29.58 / 29.67 (HSC($\underline{C}H_3$), 44.95 / 45.17 (HS$\underline{C}(CH_3)$, 51.56 / 51.61 (C2), 61.53 / 61.53 (C6), 61.90 (NH$\underline{C}$HR), 68.34 / 68.55, 71.38 / 71.60 and 71.60 / 71.91 (C3, 4 and 5), 91.38 / 91.68) (C1) 168.47, 168.55, 169.14, 169.16, 169.31, 169.51 and 169.86 (quaternaries). υ_{max}/cm^{-1} 2560 (SH). (Found: C, 48.46, H, 6.15, N, 5.38%. $C_{21}H_{32}N_2O_{11}S$ requires: C, 48.32, H, 6.10, N, 5.25%).

***N*-(*S*-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (RIG 200) [98]** was prepared by the nitrosation of the above product with HCl / $NaNO_2$ and extraction into DCM. Evaporation and addition of ether gave a stable green solid, (0.96 g, 92.6 %). NMR revealled the presence of two isomers. m.p. 125°C (dec.). λ_{max} at 340 nm. δ_C (75 MHz, DMSO), 20.04, 20.20, 20.30 and 20.37 (OAc), 22.02 / 22.07 (NHAc), 24.73 / 24.78 and 26.19 / 26.29 (ONSC($\underline{C}H_3)_2$, 51.73 / 51.81 (C2), 58.48 / 58.73 (ONS$\underline{C}(CH_3)$, 59.44 / 59.65 (NH$\underline{C}$HR), 61.52 / 61.91 (C6), 68.19 / 68.53, 71.40 / 71.63, and 71.63 / 71.92 (C3, 4 and 5), 91.32 / 91.68 (C1), 168.39, 168.52, 168.80, 168.88, 169.11, 169.20, 169.33 and 169.87 (OAc and NHAc quaternaries). FABMS (*m/e*) 519 (M-NO·)

***N*-Propionylpenicillamine [99]** was prepared as follows: penicillamine (6 g) and sodium propanoate (7.7 g, 2 eq) were stirred in a chilled solution of 90% aqueous THF (20 ml). Propionic anhydride (5.2 ml, 1 eq) was added and the suspension stirred under N_2 at room temperature for 5 h and then left to stand overnight. The THF was

evaporated to give an oily residue. The oil was dissolved in water and conc HCl added until precipitation occurred. After chilling overnight, the solid was filtered, washed and dried under vacuum. The solid was finally purified by washing with hexane, (6.6 g, 79.7 %). δ_H (200 MHz, DMSO), 1.0 (3 H, t, J = 7.6 Hz, $CH_2\underline{CH_3}$), 1.45 (6 H, s, $(CH_3)_2$), 2.25 (2 H, q, J = 7.6 Hz, $\underline{CH_2}CH_3$), 2.95 (1 H, s, SH), 4.45 (1 H, d, J = 9.5 Hz, CH), 8.05 (1 H, d, J = 9.5, NH). δ_C (50 MHz, DMSO), 10.23 ($CH_2\underline{C}H_3$), 28.37 ($\underline{C}H_2CH_3$), 29.79 and 30.19 ($\underline{C}H_3)_2C$), 45.44 ($HS\underline{C}(CH_3)_2$), 61.50 ($R\underline{C}HNH$), 171.64 and 173.67 (NHPr and CO_2H). (Found: C, 46.89, H, 7.27, N, 6.74%. $C_8H_{15}NO_3S$ requires: C, 46.83, H, 7.32, N, 6.83%)

***S*-Nitroso-*N*-propionylpenicillamine [100]** was prepared from the non-isolated product of **[99]**. The oily residue was dissolved in water and treated with $NaNO_2$ / HCl and produced a green / red dichroic solution with cooling. After 10 min a dark green solid was precipitated. The solid was filtered, washed with water and dried under vacuum, (6.1 g, 64.5 % over 2 stages). m.p. dec. at 125°C. λ_{max} 338 nm. HPLC showed the compound to be greater than 99% pure. δ_H (200 MHz, DMSO), 0.95 (3 H, t, J = 7.6 Hz, $CH_2\underline{CH_3}$), 2.00 (6 H, 2s, $C(\underline{CH_3})_2$), 2.20 (2 H, q, J = 7.6, $\underline{CH_2}CH_3$), 5.20 (1 H, d, J = 9.5 Hz, CH), 8.45 (1 H, d, J = 9.5 Hz, NH). δ_C (50 MHz, DMSO), 10.09 ($CH_2\underline{C}H_3$), 25.51 and 26.51 ($C(\underline{C}H_3)_2$), 28.29 ($\underline{C}H_2CH_3$), 58.69 ($\underline{C}(CH_3)_2$), 59.26 (CH), 171.14 (CONH), 173.59 (CO_2H).

***N*-(*N*-propionylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (RIG 299) [101]** was prepared as follows: glucosamine acetate (2 g) and *N*-propionylpenicillamine (1.77 g, 1.5 eq) were stirred in dry DCM (40 ml). 1-Cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluene sulphonate (3.66 g, 1.5 eq) was added. The mixture was stirred under N_2 overnight. The suspension was filtered and the filtrate washed (1 M HCl (25 ml), saturated $KHCO_3$ (50 ml) and water (25 ml), dried ($MgSO_4$) and evaporated to an amorphous white gum. Trituration with ether gave a white solid, (1.1 g, 36.2 %). m.p.175°C. δ_C (50 MHz, DMSO), 10.12

($CH_2\underline{C}H_3$), 20.50, 20.67, 20.76 (OAc), 28.22 ($\underline{C}H_2CH_3$), 29.24 and 29.96 ($C(\underline{C}H_3)_2$), 45.34 / 45.53 ($HS\underline{C}(CH_3)_2$), 51.68 (C2), 61.71 (C6), 62.00 (HN$\underline{C}$HR), 68.38 / 68.64, 71.59 / 72.12 and 71.75 / 71.75 (C2, 3 and 4), 91.57 / 91.89 (C1), 170.32 and 173.31 (R$\underline{C}$ONH and NHPr).

***N*-(*S*-nitroso-*N*-propionylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (RIG 300) [102]** was prepared as follows: **[103]** (1 g) was dissolved in methanol (10 ml). Water (3 ml) was added and the solution chilled in an ice bath. Excess $NaNO_2$ and HCl were added and the green solution extracted with DCM (15 ml). The solvents were evaporated to give a green gum. Trituration with ether gave a dark green solid, (0.8 g, 76.5 %). m.p. 134°C (dec.). λ_{max} 343 nm. δ_C (50 MHz, DMSO), 10.10 ($CH_2\underline{C}H_3$), 20.65 and 20.73 (OAc), 24.98 and 26.58 ($C(\underline{C}H_3)_2$), 28.13 ($\underline{C}H_2CH_3$), 51.88 (C2), 58.88 / 59.11 ($ONS\underline{C}(CH_3)_2$), 59.52 / 59.69 (HN$\underline{C}$HR), 61.70 (C6), 68.22 / 68.61, 71.58 / 71.78 and 71.78 / 72.25 (C3, 4 and 5), 91.48 / 91.86 (C1), 169.42, 169.51, 169.78, 170.27 and 173.31 (quaternaries).

***N*-Valerylpenicillamine [103]** was prepared as follows: penicillamine (3 g) and sodium valerate (3.84 g, 2 eq) were chilled in 80 % aqueous THF (20 ml). Valeric anhydride (3.95 ml, 1 eq) was added slowly and the solution stirred under N_2 overnight. The THF was evaporated leaving an oily residue. The residue was dissolved in water and conc HCl added until a thick white oil was precipitated. The mixture was cooled in an ice bath for 2 h and the oil solidified. The solid was filtered, dried, washed with hexane and dried again to give a white powder, (4.3 g, 91.3 %). m.p. 117°C. υ_{max} /cm^{-1} 2565 (SH). δ_H (200 MHz, DMSO), 0.90 (3 H, t, J = 6.8 Hz, $CH_2\underline{C}H_3$), 1.30 (2 H, m, CH_2), 1.40 (6 H, s, $C(\underline{C}H_3)_2$), 1.50 (2 H, m, CH_2), 2.20 (2 H, m, CH_2), 4.40 (1 H, d, J = 9 Hz, NH$\underline{C}$HR), 8.0 (1 H, d, J = 9 Hz, $\underline{N}$HCHR). δ_C (50 MHz, DMSO), 14.02 (CH_2CH_3), 22.04 and 27.77 (CH_2), 29.81 and 30.20 ($C(CH_3)_2$), 45.38 ($HS\underline{C}(CH_3)_2$), 61.47 (NH$\underline{C}$HR), 171.17 (NHCOVa), 173.54 (CO_2H).

***S*-Nitroso-*N*-valerylpenicillamine [104]** was prepared as follows: **[103]** (1 g) was dissolved in methanol (20 ml). Water (10 ml) was added and the mixture was chilled in an ice bath. Sodium nitrite (2 eq) was added followed by the dropwise addition of HCl until precipitation occurred. The mixture was chilled for a further 30 mins and the dark green solid filtered off. The solid was dried, washed whith hexane and dried again, (1.0 g, 92.4 %). m.p. 103°C (dec.). λ_{max} 338 nm. δ_H (200 MHz, DMSO), 0.9 (3 H, t, J = 6.8 Hz, $CH_2\underline{CH_3}$), 1.35 (2 H, m, CH_2), 1.45 (2 H, m, CH_2), 2.0 (6 H, s, $C(CH_3)_2$), 2.2 (2 H, t, J = 6.8 Hz, $COCH_2$), 5.2 (1 H, d, J = 9 Hz, NH<u>C</u>HR), 8.5 (1 H, d, J = 9 Hz, <u>NH</u>CHR). δ_C (50 MHz, DMSO), 13.94 ($CH_2\underline{C}H_3$), 21.94 (CH_2), 25.45 and 26.58 ($C(\underline{C}H_3)_2$), 27.66 and 34.80 (CH_2), 58.70 (ONS<u>C</u>$(CH_3)_2$), 59.33 (NH<u>C</u>HR), 171.12 and 172.94 (quaternaries).

***N*-(*N*-valerylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose [105]** was prepared as follows: **[103]** (2 g, 1.5 eq) and glucosamine acetate **[96]** (2.3 g, 1 eq) were dissolved in dry DCM (30 ml) and degassed (N_2). 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulphonate (4 g, 1.5 eq) was added and the mixture stirred under N_2 overnight. The by-product urea was filtered and the filtrate washed with 1 M HCl (25 ml), saturated $NaHCO_3$ solution (50 ml) and water (50 ml). The organic layer was dried ($MgSO_4$) and evaporated to a yellow oil. The oil was triturated with dry ether and left in the refrigerator. Small quantities of white powder were produced, (0.42 g, 11.4 %). m.p. 180°C. δ_C (50 MHz, DMSO), 14.00 (valeryl CH_3), 20.49, 20.66, 20.75 (OAc), 22.07 (CH_2), 27.69 (CH_2), 29.20 / 29.40 and 29.95 / 30.08 ($HSC(\underline{C}H_3)_2$), 34.81 (CH_2), 45.44 / 45.64 ($HS\underline{C}CH_3)_2$), 51.68 (C2), 61.53 (C6), 61.53 / 61.76 (CO<u>C</u>HNHR), 68.43 / 68.70, 71.66 / 71.76 and 71.76 / 72.16 (C3 - C5), 91.62 / 91.89 (C1), 168.45, 168.78, 169.02, 169.15 and 169.79 (quaternaries).

***N*-(*S*-nitrosothio-*N*-valerylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose [106]** was prepared as follows: the thiol **[105]** (100 mg) was dissolved in methanol (5 ml) and water (1 ml). Excess $NaNO_2$ and HCl were added, giving a green foam. This was extracted with DCM and evaporated to a green semi-solid. Trituration with ether gave the product as a mid-green powder (42 mg, 40.0 %). m.p. 135 - 137°C. λ_{max} 343 nm. δ_C (50 MHz, DMSO), 13.94 (valeryl-CH_3), 20.41, 20.56, 20.67 and 20.75 (OAc), 21.94 (CH2), 24.91 / 24.94 and 26.62 / 26.68 ($ONSC(\underline{C}H_3)_2$), 27.64 (CH_2), 34.62 (CH_2), 51.89 (C2), 58.98 / 59.21 ($ONS\underline{C}(CH_3)_2$), 59.39 / 59.54 ($CO\underline{C}HNHR$), 61.72 (C6), 68.28 / 68.67, 71.67 / 71.78 and 71.78 / 72.26 (C3 - C5), 91.55 / 91.90 (C1), 168.78, 169.09, 169.16, 169.55, 170.28 and 172.64 (quaternaries).

***N*-Heptanoylpenicillamine [107]** was prepared as follows: penicillamine (6 g) and sodium heptanoate (12.3 g, 2 eq) were stirred in a chilled solution of 90% aqueous THF. Heptanoic anhydride (10.5 ml, 1 eq) was added and the mixture stirred at room temperature overnight. The THF was evaporated and the oily residue dissolved in water. Conc HCl was added until precipitation occurred. The precipitate was filtered, washed with water and dried under vacuum. The white powder was stirred with hexane to remove remaining heptanoic acid, (9.3 g, 88.7 %). m.p. 118-119°C. υ_{max} /cm^{-1} 2560 (SH). δ_H (200 MHz, DMSO), 0.9 (3 H, m, heptanoyl CH_3), 1.25 - 1.5 (15 H, m) and 2.2 (3 H, m) (heptanoyl CH_2s and penicillamine CH_3s), 2.95 (1 H, s, SH), 4.45 (1 H, d, J = 9 Hz, CH), 8.1 (1 H, d, J = 9 Hz). δ_C (50 MHz, DMSO), 14.21, 22.31, 25.59 and 28.54 (NHp), 29.81 and 30.21 ($HSCH_3)_2$), 31.28 and 35.15 (NHp), 45.38 ($HS\underline{C}(CH)_2$), 61.49 ($HN\underline{C}HR$), 171.63 and 172.73 (NHp and CO_2H). .

***S*-Nitroso-*N*-heptanoylpenicillamine [108]** was prepared as for **[107]** to give an oily residue. The oil was dissolved in water and nitrosated with $NaNO_2$/HCl. Extraction into DCM and evaporation gave a red oil. The oil was chilled for several days and a green solid formed. The solid was stirred with hexane and filtered to give a green

powder. NMR revealed the presence of impurities thought to be the thiol. The product was dissolved in DCM and N_2O_3 passed throught he solution. Evaporation and trituration with hexane gave a dark green solid, (6.5 g, 56.1 %) . m.p. 55°C (dec.). λ_{max} 341 nm. δ_H (50 MHz, DMSO), 0.90 (3 H, m, CH_3), 1.30-1.55 (12 H, m, 3 x CH_2 and $(CH_3)_2$), 1.95 (2 H, m, CH_2), 2.15 (2 H, m, CH_2), 5.20 (1 H, d, J = 10 Hz, CH), 8.45 (1 H, d, J = 10 Hz, NH). δ_C (200 MHz, DMSO), 14.12, 22.26, 25.55 and 26.60 ($C(\underline{C}H_3)_2$), 25.25, 28.44, 31.21, 35.08, 58.60 ($\underline{C}(CH_3)_2$), 59.21 (CH), 170.09 and 172.84 (NHHp and CO_2H).

***N*-(*N*-heptanoylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (RIG 699) [109]** was prepared as follows: glucosamine acetate (2 g) and *N*-heptanoylpenicillamine (2.25 g, 1.5 eq) were stirred in dry DCM (30 ml). 1-Cyclohexyl-3-(2-morpholino-ethyl)carbodiimide metho-p-toluene sulphonate (3.66 g, 1.5 eq) was added and the mixture stirred under N_2 for 24 h. The suspension was filtered and the filtrate washed (1 M HCl (25 ml), saturated $NaHCO_3$ (50 ml and water (50 ml), dried ($MgSO_4$) and evaporated to a white gum. Repeated trituration with dry ether gave a white powder (1.3 g, 38.5 %). m.p. 183 - 185°C. δ_C (50 MHz, DMSO), 14.20, ($CH_2\underline{C}H_3$), 20.50, 20.66, 20.76 and 20.86 (OAc), 22.27 (CH_2), 25.44 / 25.49 (CH_2), 28.60 (CH_2), 29.18 / 29.41 ($C(\underline{C}H_3)_2$), 29.96 / 30.03 ($C(\underline{C}H_3)_2$), 31.29 (CH_2), 35.10 (CH_2), 45.43 / 45.64 ($HS\underline{C}(CH_3)_2$), 51.68 (C2), 61.72 (C6), 61.56 / 61.82 (R$\underline{C}$HNH), 68.40 / 68.67, 71.65 / 71.76, and 71.76 / 72.15 (C3, 4 and 5), 91.61 / 91.91 (C1), 168.89, 168.98, 169.56, 169.82, 170.29, 172.52 and 172.62 (quaternaries). (Found, C, 53.26, H, 7.15, N, 4.79 %. $C_{26}H_{42}N_2O_{11}S$ requires: C, 52.88, H, 7.12, N, 4.75 %).

***N*-(*S*-Nitroso-*N*-heptanoylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (RIG 700) [110]** was prepared as follows: **[109]** (1 g) was dissolved in methanol (8 ml). Water (2 ml) was added followed by excess $NaNO_2$ and excess conc HCl with vigorous stirring. The greeen solution was extracted

with DCM (15 ml) and evaporated to give a green semi solid. Trituration with dry ether gave a green powder. NMR revealed the presence of large quantities of thiol. The green solid was dissolved in DCM and N_2O_3 bubbled through the solution for a few seconds. Evaporation and trituration with dry ether gave a dark green solid. (0.64 g, 60.7 %). m.p. 135°C (dec). λ_{max} 345 nm. NMR showed that all of the thiol had been nitrosated. δ_C (50 MHz, DMSO), 14.17 ($CH_2\underline{C}H_3$), 20.41, 20.57, 20.68 and 20.76 (OAc), 22.24 (CH_2), 24.86 ($C(\underline{C}H_3)_2$), 25.45 (CH_2), 26.66 ($C(\underline{C}H_3)_2$), 28.46 (CH_2), 31.22 (CH_2), 34.90 (CH_2), 51.88 (C2), 58.98 / 59.22 ($ONS\underline{C}(CH_3)_2$), 59.36 / 59.54 ($NH\underline{C}HR$), 61.71 (C6), 68.26 / 68.65, 71.66 / 71.79 and 71.79 / 72.27 (C3, 4 and 5), 91.53 / 91.91 (C1), 168.79, 169.08, 169.18, 169.55, 170.29, 172.54 and 172.64 (quaternaries).

1-Azidoglucopyranose tetraactetate [111] was prepared as follows: acetobromoglucose **[2]** (3.3 g) and sodium azide (1.65 g) were stirred in dry DMF (23 ml) over a boiling water bath under N_2 for 2 h. The product was poured into ice water (50 ml) and a brown solid precipitated. The solid was washed with copious amounts of water, dried under vacuum and recrystallised from methanol to give pale yellow crystals (1.75 g, 58.4 %). m.p. 129-130°C (lit.[28] 129°C). υ_{max}/cm^{-1} 2100 (N_3). δ_H (200 MHz, $CDCl_3$), 2.05 (12 H, m, OAc), 3.80 (1 H, m, 5-H), 4.15 (1 H, dd, J = 13.5 and 2.3 Hz), 4.25 (1 H, dd, J = 13.5 and 4.7 Hz), 4.65 (1 H, d, J = 8.8 Hz, 1-H), 4.95 (1 H, t, J = 9.4 Hz, 2/3/4-H), 5.10 (1 H, t, J = 9.4 Hz, 2/3/4-H), 5.20 (1 H, t, J = 9.4 Hz). δ_C (50 MHz, $CDCl_3$), 20.98, 21.12 (OAc), 62.12, 68.36, 71.10, 73.05 and 74.70 (C2 - C6) and 88.34 (C1).

1-Aminoglucopyranose tetraacetate [112] was prepared by the hydrogenation of the above azide **[111]** (2 g) in methanol (150 ml) in the presence of 10% Pd on carbon (300 mg). The solution was stirred for 24 h under H_2. The solution was filtered through celite and evaporated to a clear oil which hardened to an amorphous solid, (1.4 g, 75.2 %). m.p. 121 - 125 (lit.[29] 127°C). δ_H (200 MHz, $CDCl_3$), 2.0 - 2.1 (12 H, m,

OAc), 3.65 (1 H, m, 5-H), 4.1 - 4.25 (3 H, m, 1-, 6- and 6'-H), 4.8 (1 H, t, J = 9.4 Hz, 2/3/4-H), 5.0 (1 H, t, J = 9.4 Hz, 2/3/4-H) and 5.2 (1 H, t, J = 9.4 Hz, 2/3/4-H). δ_C (50 MHz, $CDCl_3$), 21.12 and 21.30 (OAc), 62.74, 69.18, 72.47, 73.15 and 73.60 (C2 - C6), 85.39 (C1), 170.08, 170.74 and 171.24 (OAc).

The preparation of ***N*-penicillaminyl-1-aminoglucose [113]** was attempted as follows: 1-aminoglucose **[112]** (2.5 g) and *N*-acetylpenicillamine (1.4 g) were stirred in dry DCM (40 ml) under N_2. 1-Cyclohexyl - 3(2-morpholinoethyl)carbodiimide-*p*-toluene sulphonate (3.1 g) was added and the mixture stirred under N_2 for 24 h. A suspension formed which briefly became a clear solution before the urea by-product was precipitated. The precipitate was filtered off, the filtrate washed (1 M HCl, saturated $NaHCO_3$, water), dried ($MgSO_4$) and evaporated to an oil. The oil could not be solidified. NMR revealled the presence of a number of impurities, from which the desired product could not be separated. The oil was nitrosated to give a deep green liquid which again could not be adequately purified.

1-Azidogalactose tetraactetate [114] was prepared as follows: acetobromogalactose **[7]** (6 g) and sodium azide (4 g) were stirred in DMF (40 ml) over a boiling water bath for 3 h. The dark browm mixture formed was poured into ice water (200 ml). A brown solid was filtered, washed with copious amounts of water, dried and recrystallised from methanol to give large cubic crystals, 3.8 g, 69.8 %). mp 88°C (lit.[28] 91°C). υ_{max} /cm^{-1} 2100 (N_3). δ_H (200 MHz, $CDCl_3$), 2.0-2.2 (12 H, m, OAc), 4.0 (1 H, m, 5-H), 4.2 (2 H, m, 6, 6'-H), 4.6 (1 H, d, J = 8.5 Hz, 1-H), 5.0 (1 H, dd, J = 10.1 and 3.4 Hz, 3-H), 5.15 (1 H, t, J = 9.0 Hz, 2-H), 5.4 (1 H, dd, J = 3.4 and 1.1 Hz, 4-H). δ_C (50 MHz, $CDCl_3$), 20.99 and 21.14 (OAc), 61.70, 67.31, 68.49, 71.16 and 73.28 (C2-C6), 88.72 (C1), 169.84, 170.45, 170.59, 170.84 (OAc).

1-Aminogalactose tetraacetate [115] was prepared as follows: the above azide **[114]** (2 g) was dissolved in methanol (150 ml) and hydrogenated for 24 h over 10 %

Pd on C. The catalyst was filtered through ceelite and the filtrate evaporated to give an amorphous solid. Recrystallisation from ethanol gave a white powder, (1.6 g, 86.0 %). m.p. 135 - 137°C (lit.[30] 139°C). δ_C (50 MHz, $CDCl_3$), 21.11, 21.18 and 21.42 (OAc), 62.28, 68.03, 70.14, 71.72 and 71.83 (C2 - C6), 85.71 (C1), 171.01 (OAc).

1-Azidomaltose heptaacetate [116] was prepared as follows: acetobromomaltose **[17]** (14 g) and sodium azide (5 g) were stirred in DMF (50 ml) over a boiling water bath for 3 h. The solution was poured into ice water (150 ml). A brown gum formed. The liquid was decanted and the gum repeatedly recrystallised from methanol to give a pale brown solid, (6.3 g, 47.6 %). υ_{max}/cm^{-1} 2105 (N_3). m.p. 92°C (lit.[31] 96°C). δ_C (50 MHz, $CDCl_3$), 21.05, 21.16, 21.26 and 21.32 (OAc), 61.86, 62.94, 68.31, 69.04, 69.65, 70.40, 71.88, 72.69, 74.62, 75.50, 87.87 (C1a), 96.11 (C1b).

1-Aminomaltose heptaacetate [117] was prepared as follows: **[116]** (5 g) was dissolved in methanol (150 ml). 10% Pd on C catalyst (300 mg) was added and the mixture hydrogenation for 24 h. A white precipitate appeared during this time. DCM was added (75 ml) and the mixture heated until the precipitate had dissolved. The mixture was filtered through hot celite. The DCM was evaporated and the product allowed to crystallise in the refrigerator, (2.1 g, 43.7 %). m.p. 189 - 191°C (lit.[31] 191°C). δ_C (50 MHz, $CDCl_3$), 21.05, 21.16, 21.26 and 21.42 (OAc), 61.86, 62.94, 68.31, 69.04, 69.65, 70.40, 71.88, 72.69, 74.62 and 75.50 (CH), 87.87 (C1a), 96.11 (C1b), 169.89, 169.98, 170.42, 170.58, 170.90 and 171.00 (OAc).

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