Development of Novel Nutrapharmaceuticals Combining the Anti-Oxidant and Anti-Inflammatory Properties of n-3 Fatty Acids with a Nitric Oxide Donor – a Potential New Preventative Strategy for Heart Disease

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Abstract

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MRes

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Atherosclerosis is a cardiovascular disease which forms plaques which narrow the arteries, restricting blood flow and thus increasing blood pressure. As this can lead to more serious conditions, such as myocardial infarction, it is important that atherosclerosis is treated to prevent dangerous levels of atherosclerotic plaques forming.

The aim of this project was to create a library of novel PUFA-NO conjugates which would contain the combined beneficial cardioprotective and anti-inflammatory qualities of both groups.

Synthesis of aliphatic-aminothiol conjugates has been achieved using both acid chloride (*N*-decanoyl cysteamine (Compound 1), *N*-decanoyl *L*-cysteine ethyl ester (compound 3)) and peptide coupling based reactions, and full characterisation has been achieved.

The nitration stage of synthesis was achieved using tert-butyl nitrite and 'fuming' NO as the nitrating agents. Successful nitration was determined by MS analysis and by use of the Griess reaction as a colourimetric test to determine the ability of the synthesised compounds (*S*-nitroso *N*-decanoyl cysteamine (compound 7), *S*-nitroso *N*-oleoyl cysteamine (compound 8)) to release nitric oxide.

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Abbreviations

ACE	Angiotensin converting enzyme
CAD	Coronary Artery Disease
CVD	Cardiovascular Disease
DHA	Docosa Hexaenoic Acid
DiPEA	*N*,*N*-Diisopropylethylamine
EDRF	Endothelium Derived Relaxing Factor
EPA	Eicosapentaenoic Acid
HDL	High Density Lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
Hobt	Hydroxybenzotriazole
LDL	Low Density Lipoprotein
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSAID	Non-Steroidal Anti-Inflammatory Drug
PUFA	Polyunsaturated Fatty Acid

SFA	Saturated Fatty Acid
SNO	*S*-Nitrosothiol
TBTU	(2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate)

1.1. Cardiovascular Disease

Cardiovascular disease is the leading cause of death in adults in the United Kingdom. There were almost two hundred thousand related deaths in 2006 attributed to cardiovascular disease, which accounted for almost one third of all deaths in the U.K. that year (British Heart Foundation, 2008). The term refers to a range of conditions that affect arteries and the heart such as myocardial infarction, angina, coronary artery disease, chronic heart failure, pulmonary hypertension and atherosclerosis.

Causes of cardiovascular disease vary, but can include genetic factors, obesity, sedentary lifestyle, diabetes and smoking (World Health Organisation, 2009).

Treatment for cardiovascular disease involves many stages, requiring management such as undertaking lifestyle changes, stress reduction, dietary changes, surgical intervention and drugs (World Health Organisation, 2009).

Secondary treatments for cardiovascular disease, which have been shown to reduce the risk of cardiovascular events, include increasing fruit and vegetable intake and partaking in moderate levels of physical activity on a regular basis (Ignarro *at al.*, 2007). Whilst regular excessive consumption of alcohol is linked to an increase in a range of heart conditions and disease (Klatsky, 2007), some research (Wollin & Jones, 2001) has indicated that moderate intake of alcohol (red wine) can actually confer beneficial cardioprotective effects.

Dietary interventions can also take place in the form of reducing saturated fatty acids in the diet and giving an omega rich polyunsaturated fatty acid diet. Such a diet is a low risk alternative to drug therapy which can prevent the need for surgery and can reduce the effects of cardiovascular disease (CVD) (Patil and Gisleroed, 2006).

Current NHS treatment guidelines for cardiovascular disease include the following management: antiplatelet treatment can be administered as primary prevention, usually in patients with a 10 year risk of 20% or greater for cardiovascular disease, in the form of daily 75 mg doses of aspirin, or 75 mg daily doses of Clopidogrel in cases of aspirin intolerance. The same treatment can be administered as a secondary preventative of CVD where the patient has a recognized cardiovascular disease, such as myocardial infarction, angina, stroke or peripheral arterial disease (NHS, 2009(a)).

Someone defined as 'at high risk' of cardiovascular disease under NHS guidelines is someone who falls under the following categories: being aged 75 years or older, suffers from established CVD, has family history of hypercholesterolaemia, has type 2 diabetes and aged 40 or more. Drug intervention is recommended for high risk CVD patients and treatment can include statin treatment as a primary treatment, antiplatelet prescription as previously mentioned and a lifestyle management plan to minimise further risk factors (NHS, 2009(b)).

1.2. Atherosclerosis

Known as the hardening of the arteries, atherosclerosis is the "leading cause of mortality and morbidity in the developed world" and it is believed it could also become the leading worldwide killer through its "manifestations such as cardiovascular disease and stroke" (Young and Libby, 2007).

Atherosclerosis was initially believed to stem from high cholesterol levels caused by fat intake. In the late 1970s, developments (Hansson, 2009) were made in the field of atherosclerosis pathogenesis leading to a re-evaluation of atherosclerosis as a chronic inflammatory condition (Ross, 1999).

Atherosclerosis is a chronic inflammatory condition caused by a build-up of lipids, such as cholesterol, and macrophages on the endothelial tissue of arteries. These accumulations begin as 'fatty streaks' but can harden and form plaques, effectively narrowing the artery, reducing blood flow and causing high blood pressure. Complications can arise from the atherosclerotic plaques, such as a rupture or calcification of the plaque, which could severely restrict blood flow (Young and Libby, 2007).

Causes of atherosclerosis are similar to those of cardiovascular disease in general, in that poor diet, lack of exercise and smoking can all be contributing factors.

Treatments for atherosclerosis are varied but can include statin treatment, lifestyle changes, diet and dietary supplements, and surgery, such as angioplasty or coronary artery bypass (Young and Libby, 2007).

Statins are a series of drugs which inhibit cholesterol synthesis in the liver (Schoen *at al.*, 2007) and are shown to reduce low density lipoprotein cholesterol levels and thus lower the incidence of ischaemic heart disease greatly (Law *at al.*, 2003). Trials have shown in some cases that high intensity statin treatments can also lead to atherosclerosis regression (Nissen *at al.*, 2006).

The chemical structures of some example statins are shown in figure 1:

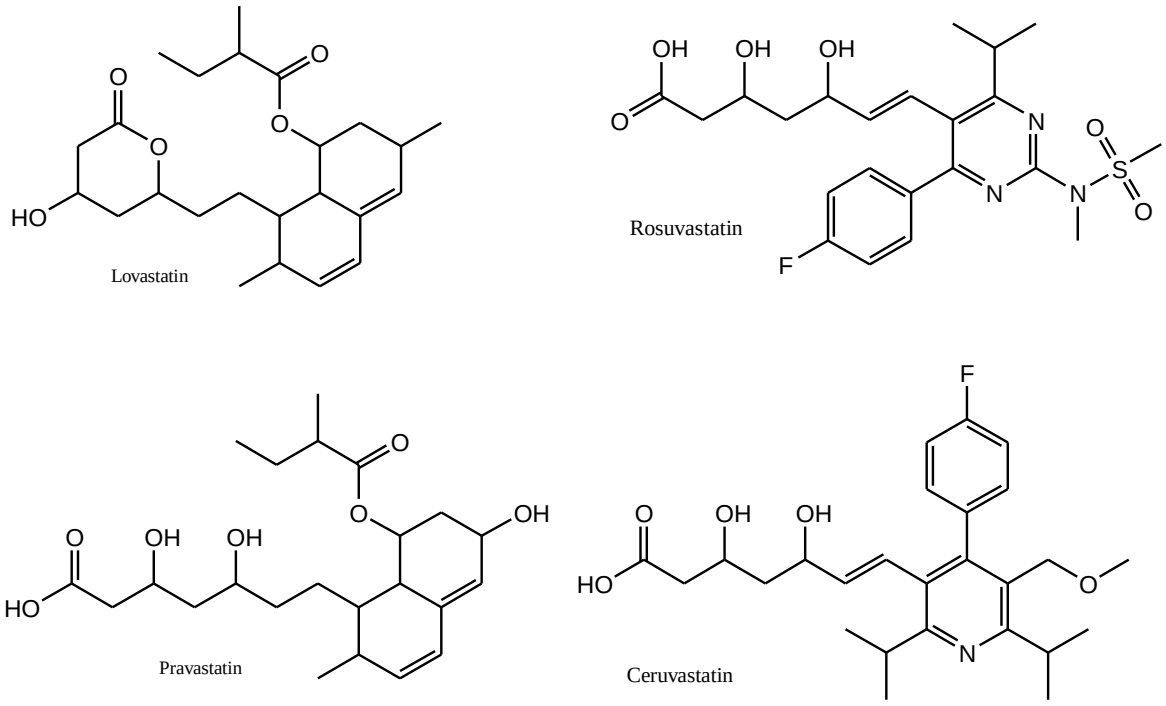


Figure 1: Chemical structures of selected statins.

Lovastatin has been used as a treatment to lower serum cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate limiting enzyme for cholesterol biosynthesis (Zhu at al., 1992). All statins work in this manner, by inhibiting HMG-CoA reductase, and are selected for use based on ability to normalise serum cholesterol levels and cost (Chong *at al.*, 2001).

The development of atherosclerosis occurs in stages, initially changing from a normal artery to an early atheroma. The early atheroma can generate one of two outcomes, a 'stable' plaque and a 'vulnerable' plaque. These 'vulnerable' plaques contain a larger number of inflammatory cells and lipids than the 'stable' plaque and also have a thin fibrous cap (Young and Libby, 2007). The thin fibrous cap is prone to rupture, which leads to thrombus formation which can result in a healed, but much narrowed, artery or have the devastating consequence of acute myocardial infarction.

Atherosclerotic plaque formations can result in varying complications, such as plaque calcification which confers pipe-like rigidity to the vessel wall, leading to fragility. A rupture or ulceration of a vulnerable plaque can lead to vessel blockage, causing an infarction, or alternatively the thrombosis can be incorporated into the plaque, further narrowing an artery. The vessel wall can be weakened by the fibrous plaque exerting constant pressure on the nearby medial tissue, which could cause dilation of the artery and ultimately form an aneurysm (Young and Libby, 2007).

As high LDL cholesterol and abnormal lipid levels lead to a greater chance of atherosclerosis developing, finding effective means of lowering levels is becoming increasingly important. Changes in lifestyle factors can reduce risk, such as replacing a sedentary lifestyle with moderate exercise on a regular basis, stopping smoking and reducing alcohol intake, as well as other dietary factors, such as reduced saturated fat intake.

Studies have shown that statin treatment for at-risk individuals can placate and even, in some cases, regress atherosclerotic plaques. The ASTEROID trial (Nissen *at al.*, 2006) demonstrated that, with a high intensity therapy using the statin rosuvastatin, a significant regression of atherosclerosis can result.

Studies have been undertaken in recent years to determine to what extent increased polyunsaturated fatty acid consumption affects the progression of atherosclerosis. The JELIS trial (Yokoyama *at al.*, 2007) found against a control group taking statins, a trial group taking both statins and PUFA showed a significant decrease of 19% fewer major coronary events.

Other studies, such as the GISSI trial (Marchioli *at al.*, 1999) have also demonstrated control groups taking PUFA supplements are less likely to suffer fatal coronary events from complications, such as atherosclerosis and cardiovascular disease.

1.3. Nitric Oxide

Nitric oxide is one of the simplest and smallest molecules, yet a very important one in the human body, as it is implicated in key biological functions. Chemically, it is a diatomic molecule with an unpaired electron on the nitrogen, causing it to be a radical, and has a bond order of 2.5. It is a relatively stable molecule despite this and resists dimerisation to dinitrogen dioxide, N_2O_2. A key factor accounting for this is delocalisation of the unpaired electron between the nitrogen and the oxygen atoms, rather than being localised on the nitrogen (Butler and Nicholson, 2003).

In human cells, the nitric oxide synthase enzymes, commonly known as NOS, catalyse the oxidation of *L*-arginine causing release of nitric oxide (Al-Sa'Doni and Ferro, 2000). The nitric oxide produced is then used to activate guanylyl cyclase which leads to vasorelaxant effects. Figure 2 shows this in more detail below.

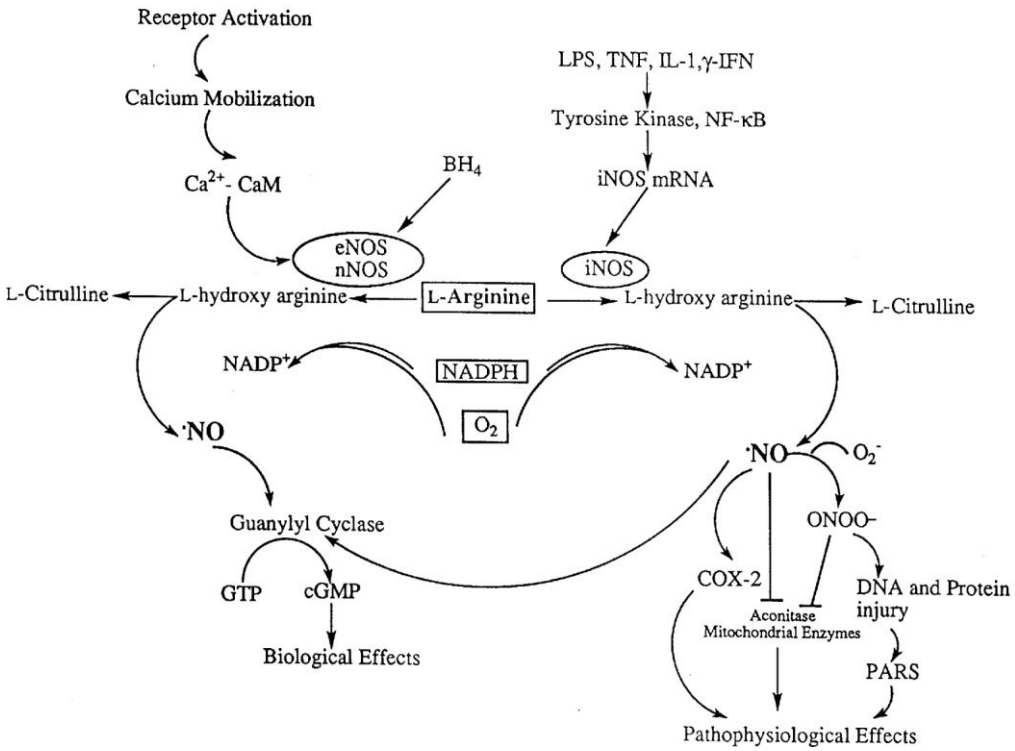


Figure 2: NO release via NOS enzymes activating guanylyl cyclase to give vasorelaxant effects, Al-Sa'Doni and Ferro, 2000

There are three types of NOS enzymes, two of which are dependent on elevated levels of Ca^{2+} and the third of which acts independent of Ca^{2+} levels. Neuronal NOS, known as nNOS, and endothelial NOS (eNOS) are dependent on raised Ca^{2+} levels (Fleming and Busse, 1999) and are constitutive enzymes in their respective areas (Pagliaro, 2003) whilst it is inducible NOS (iNOS) that is independent from Ca^{2+} and induced by "inflammatory cytokines or ischemic stimulus" (Pagliaro, 2003).

Endothelial NOS activity has been implicated in vasodilation and maintenance of vascular tone, indeed a deficiency of the eNOS enzyme has been related to cases of hypertension (Awolesi *at al.*, 1995). Links have also been made between eNOS and the inhibition of platelet aggregation and adhesion and inhibition of smooth muscle cell proliferation (Marletta, 1993)

Nitric oxide (NO) has been recognised as an endothelial derived relaxing factor (EDRF) (Furchgott and Vanhoutte, 1989) and was shown to relax the smooth muscle in the arterial vessel in patients afflicted by atherosclerotic plaques, resulting in increased blood flow. A simplified view of the biological mechanism of action is illustrated in figure 3.

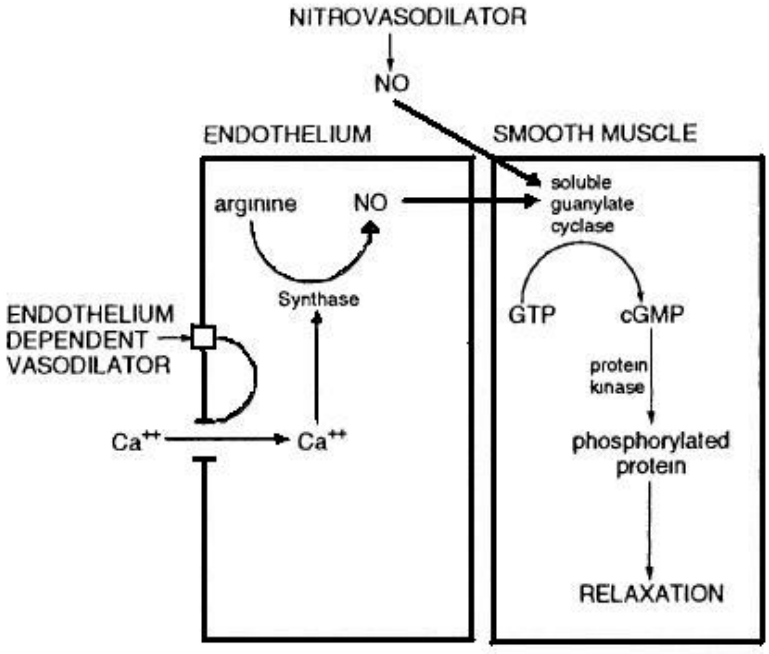


Figure 3: EDRF action of NO (Butler and Williams, 1993)

In the body, nitric oxide is generated in the endothelium and can diffuse into the vascular smooth muscle, whereby it acts as a vasorelaxant. One of the reasons it can diffuse like this is due to its small molecular size (Butler and Nicholson, 2003). Nitric oxide generated in the endothelium is constitutively expressed by eNOS, although certain factors can still affect the amount of NO produced. It has been reported that in cases of high levels of oxidised low density lipoprotein there is a reduced expression of eNOS enzyme whilst in cases of exercised subjects, the eNOS enzyme is expressed in greater amount, leading to more NO available for production (Harrison, 1997).

Inhaled nitric oxide has been shown to have vasodilating effects in cases of pulmonary hypertension (Kovalchin *at al.*, 1997). In addition, it has long been known that *S*-nitrosothiols (Ignarro *at al.*, 1981) can give the same vasodilation effects by means of nitric oxide release. It has been reported that nitric oxide is present almost exclusively as *S*-nitrosoglutathione in the human airway, which acts as an intermediary in the release of NO giving a bronchodilator effect (Nevin and Broadley, 2002), which implicates nitric oxide or NO donors as potentially useful in the field of asthma treatment.

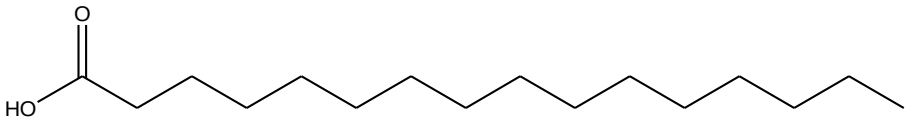
Nitric oxide also has a role to play in cancer. It has been shown that cancer cell lines of many different types of cancer, including lung, prostate and lung cancer, have their growth hindered by application of a NO donating drug (more specifically a NO donating non-steroidal anti-inflammatory drug) (Bonavida *at al.*, 2006). Developments along these lines show that nitric oxide and nitric oxide donors could have an important role to play in developing new chemotherapeutic drugs. In the body, macrophages produce nitrates, and the production of these nitrates is dependent on the availability of arginine. The arginine-NO pathway is present in macrophages, thus Stuehr (e*t al.* 1989) implied that nitric oxide is a natural cytotoxic agent and is synthesised by the body as a defence mechanism against foreign or abnormal cells.

When an atherosclerotic plaque ruptures, the result is the endothelium is damaged and sub-endothelial tissue is then exposed. This exposed layer consists mainly of collagen and platelets interact strongly with this collagen, inducing shape change causing them to adhere and activate. This leads to fibrin aggregating over platelets; this process leads to closure of the rupture in the vessel, which, in atherosclerotic plaques, can cause dangerous vessel constriction (Rang and Dale, 1991). Nitric oxide has been shown to inhibit this adhesion of fibrils and endothelial cells (Radomski *at al.*, 1990) and thus can be of vital help in cases of atherosclerotic plaque rupture by avoiding dangerous constriction of the artery.

1.4. Fatty Acids

Much of the study within the area of polyunsaturated fatty acids (PUFAs) has centred on the beneficial effects they can confer as an anti-inflammatory agent with cardioprotective qualities.

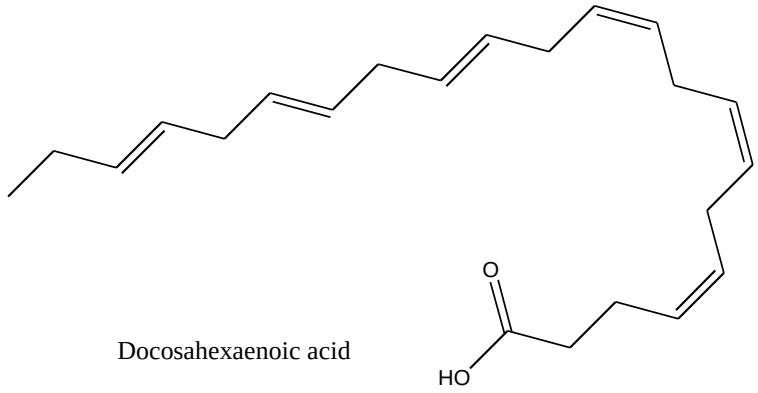
A saturated fatty acid (SFA) is a molecule with a polar carboxylic acid head with a long chain aliphatic hydrocarbon tail, denoted as saturated because each carbon is saturated with sp^3 hybridised C-H bonds. As the chain is straight the molecule can be densely packed. An example of a SFA is given in figure 4.



palmitic acid

Figure 4: Example of a saturated fatty acid

Polyunsaturated fatty acids are somewhat similar but differ to SFAs in key areas. The carboxyl acid head of the molecule is the same, but the hydrocarbon chain differs in that it has multiple double bonds between adjacent carbons, C=C, such as the example in figure 5. Monounsaturated fatty acids also exist, the key difference between a monounsaturated fatty acid and a polyunsaturated fatty acid is that the monounsaturated fatty acid contains only one C=C bond.



Docosahexaenoic acid

Figure 5: Cis conformation docosahexaenoic acid.

The conformation means the cis-PUFA will not accumulate in the same manner as the straight trans molecules. The puckering effect is caused by rigidity introduced by the C=C bond. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are denoted as n-3 PUFAs as the first double bond in the chain starts on the third carbon from the methyl end.

Large scale controlled trials have given evidence that the omega-3 polyunsaturated fatty acids EPA and DHA both confer cardioprotective effects (Lee *at al.*, 2008). These PUFAs can be taken as a measure to prevent the onset of arterial plaques and heart disease. Indeed, it is reported that atherosclerotic plaques are stabilised by the n-3 PUFAs EPA and DHA, and believed that this stability effect leads to reduced cardiovascular incidents (Thies *at al.*, 2003).

Omega-3 PUFAs, such as EPA and DHA are essential fatty acids (Grundt and Nilsen, 2008) which the body is unable to generate itself, so must be obtained either in the diet (such as eating omega oil rich fish) or through supplements.

It is believed that the ratio of n-6 to n-3 PUFAs is very important as there is competition between the enzymatic pathways of both: an overabundance of either n-3 or n-6 will reduce the metabolic process of the other, which leads to a lack of its beneficial results. Western diet is heavily weighted in favour of n-6 PUFAs, but many diets, such as oily fish, contain n-3 fatty acids in sufficient levels that increasing consumption of oily fish in the diet will go some way to rebalancing the n-3: n-6 ratio (Thies *at al.*, 2003).

Many human dietary intervention trials have been carried out with an aim to better understand the beneficial properties which n-3 polyunsaturated fatty acids confer. As far back as 1989, the DART trial (Burr *at al.*, 1989) showed that fish based fatty acids could provide a secondary means to preventing coronary heart disease. The MRFIT trial (Dolecek *at al.*, 1991) displayed a control group taking fish derived n-3 PUFAs to suffer a reduced rate of mortality from coronary artery disease (CAD) and CVD.

In the late 90s, the "Indian Experiment of Infarct Survival" (Singh *at al.*, 1997) published results indicating that a control group taking PUFA supplements had reduced incidence of cardiac events, such as non-fatal myocardial infarction and the SCIMO trial (von Schacky *at al.*, 2001) produced encouraging results that patients taking fish oil based n-3 PUFA had less progression, and even a small amount of regression, of atherosclerotic plaques. The GISSI trial (Marchioli *at al.*, 1999) again demonstrated that patients taking PUFA supplements in a controlled intervention experienced decreased rate of non-fatal myocardial infarction and death. As previously mentioned, the JELIS trial (Yokoyama *at al.*, 2007) showed that PUFA in conjunction with statin treatment results in a significantly reduced incidence of major coronary events.

Whilst these numerous intervention trials leave little doubt about the effectiveness of n-3 polyunsaturated fatty acids as cardioprotective agents, the manner in which they act as such is still the subject of much research.

It is proposed that PUFAs can protect against cardiovascular disease via multiple methods, such as acting as an antiatherogenic agent, slightly lowering blood pressure, decreasing inflammatory response, inhibition of thrombosis, by decreasing susceptibility to arrhythmias and by improving endothelial function (Kris-Etherton *at al.*, 2003).

Omura (Omura *at al.* 2001) proposed that eicosapentaenoic acid caused enzymatic inducement of nitric oxide, via the eNOS cycle and activating endothelium dependent vasorelaxation.

1.5. Nitric Oxide Donors

As nitric oxide confers beneficial effects, partly due to the EDRF, "direct delivery of NO is expected to be effective in the prevention and/or treatment" of various cardiovascular disorders (Herman and Moncada, 2005). The effect of nitric oxide applied to smooth muscle cells in this manner is to activate the guanylyl cyclase enzyme, which then catalyses the production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate, leading to smooth muscle relaxation (Levick, 2003). The ability of NO donors to bypass the nitric oxide generating mechanism of the endothelium and deliver NO directly to the arterial walls (Butler and Williams, 1993) in need of vasorelaxation can make them potentially very useful.

Glyceryl trinitrate, figure 6, is used in the treatment of angina (National Health Service, 2006) and has been known as a vasodilator for many years and acts as such by breaking down to release nitric oxide *in vivo* (Salvemini *at al.*, 1993). Two further nitric oxide donors are shown in figure 6, sodium nitroprusside and isosorbide mononitrate.

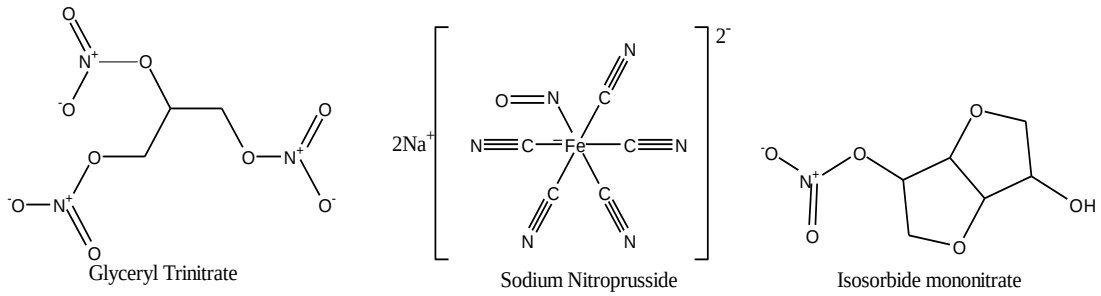


Figure 6: Currently used NO donors.

Isosorbide mononitrate is also used in the treatment of angina, preferred as a long term treatment to glyceryl trinitrate which is administered in cases of acute angina related pain. Sodium nitroprusside has been used in hospitals in cases where there is an urgent need for vasodilation, but the drug is limited due to the difficulties in administering it, as well as the cyanide groups contained in the structure (McDowall *at al.*, 1974).

As well as conventional nitric oxide donor drugs, there have been many studies (Keeble and Moore, 2002; Ongini *at al.*, 2004; Martelli *at al.*, 2006) on developing nitric oxide hybrid drugs, where a NO donating moiety is stably attached to a clinically active drug. The perceived advantages to this strategy are that the beneficial effects of NO are then incorporated into the current drug and in some cases can even reduce some of the negative side effects of the parent molecule.

One such example would be of the NO-NSAID (non steroidal anti inflammatory drug) "NicOx" compounds, which were nitric oxide releasing aspirin derivative drugs. As extended treatments of aspirin in cardiovascular cases can lead to unwanted side effects, such as gastrointestinal bleeding (Keeble and Moore, 2002), and NO has beneficial properties in this area which can counter the negative effect, the final drug was shown to retain the aspirin drug effect whilst minimising the negative side-effects. In addition to conferring anti-inflammatory properties, NO-NSAIDs have also shown promise as a potential treatment for neurological conditions, such as Alzheimer's disease (Keeble and Moore, 2002).

Nitric oxide releasing derivatives of statins are another example of the use of a hybrid NO donor compound. As previously discussed in section 1.2, statins are beneficial and indeed a frontline treatment in high cholesterol related cardiovascular disease, and a nitric oxide releasing hybrid statin could offer further vasorelaxing properties to the drug in treatment of cardiovascular diseases such as atherosclerosis. One such study of NO-statin hybrids (Ongini *at al.*, 2004) concluded that NO donor hybrid statins offered a multiple mechanism approach to treating atherosclerosis which could potentially give better results than normal statins.

Nitric oxide donating derivatives of salbutamol, which is used to treat chronic asthma, have been created and have been shown in studies to be more potent as a bronchodilator than the salbutamol parent molecule. NO-paracetamol has been shown to reduce the toxic effects of paracetamol in the liver, (Keeble and Moore, 2002) giving two further instances whereby a NO donating moiety attached to a parent molecule with an established pharmacological effect can prove beneficial.

Two types of cardiovascular drugs, ACE-inhibitors and sartans, treat hypertension by acting upon the renin angiotensin system in differing ways to prevent a hypertensive effect. NO donating derivatives of both types of drug have been synthesised and tested; *S*-nitrosocaptopril (being the *S*-nitrosylated form of the ACE-inhibitor captopril) enhances the pharmacological effect of the parent drug by means of the NO donor (Martelli *at al.*, 2006). Nitric oxide donor hybrids of sartans have also enhanced their effect as cardiovascular drugs and gave the hybrid drug greater vasorelaxing properties (Martelli *at al.*, 2006).

There are a wide range of molecules which act as NO donors (Chakrapani at al., 2008), such as the nitro, nitroso and oxime C-NO donors illustrated below in figure 7:

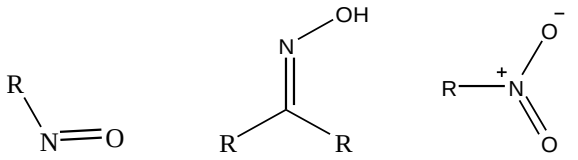


Figure 7: Example of nitroso, oxime and nitro C-NO donors.

In addition to this, there are also a range of O-NO donors, such as the aforementioned examples of glyceryl trinitrate and isosorbide mononitrate. Figure 8 displays the structure of glyceryl mononitrate which also acts as an O-NO nitric oxide donor (Martelli *at al.*, 2006).

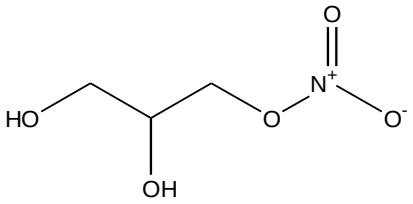


Figure 8: Glyceryl mononitrate.

Transition metal complexes such as sodium nitroprusside shown in figure 9 can also act as NO donors (Martelli *at al.*, 2006).

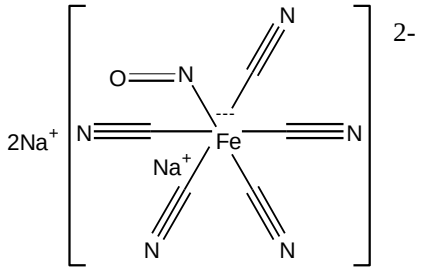


Figure 9: sodium nitroprusside

There are many *S*-NO nitric oxide donors, which shall be discussed in more detail in section 1.6, and an example of one such *S*-NO compound, *S*-nitroso *N*-Acetyl penicillamine (Soulere at al., 2001), is illustrated in figure 10.

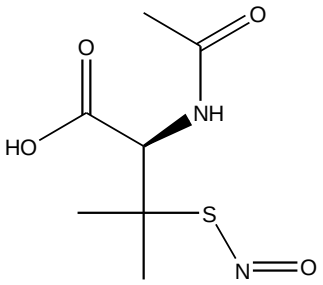


Figure 10: *S*-nitroso-*N*-acetylpenicillamine

1.6. *S*-Nitrosothiols

S-nitrosothiols (RSNOs) have garnered significant interest since the discovery of their physiological role in the early 1990's (Giustarini at al., 2003). It is believed that RSNOs act as a transporter/donor of NO *in vivo* and that they have a potential medical application as NO donors (Williams, 1999). The NOS enzymes cause nitrosative chemistry to occur, leading in turn to *S*-nitrosylation, which accounts for the presence of RSNOs *in vivo* (Foster *at al.*, 2003)

S-nitrosothiols break down to form a disulphide, releasing NO, shown in figure 11; *L*-(-)-glutathione breaking down to form its disulphide, releasing NO.

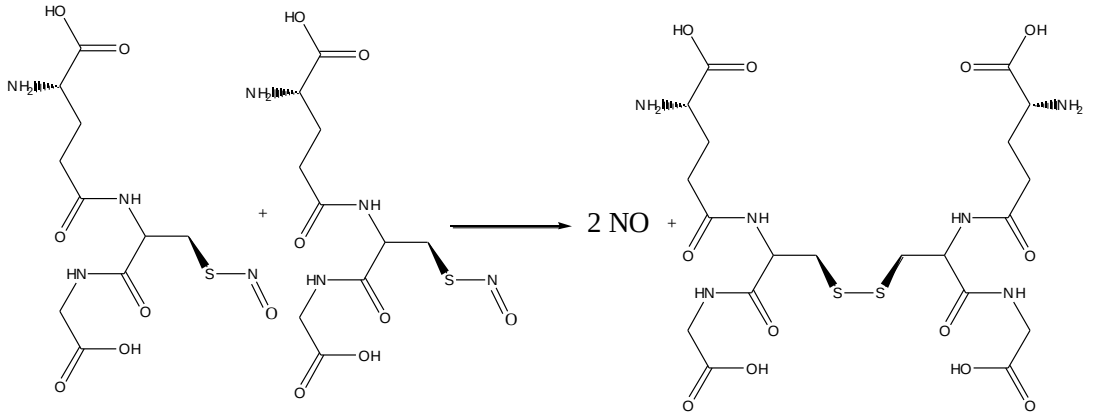


Figure 11: NO generated from disulphide formation.

S-nitrosothiols are generally red or green in colour (Wang *at al.* 2002), with the red colour being attributed to a primary or secondary *S*-nitrosothiol such as *S*-nitrosoglutathione, and the green colour presented by a tertiary *S*-nitrosothiol, such as *S*-nitroso-*N*-acetyl penicillamine (Hogg, 2000).

Some RSNOs are relatively stable solids at room temperature (Williams, 1998), but the nature of the S-N bond means there a number of factors which can cleave the bond and cause release of the NO group and the majority of RSNOs can thus be unstable at room temperature (Wang *at al.* 2002).

Three main causes of *S*-nitrosothiol instability are thermal decomposition, photochemical decomposition and decomposition in solution due to trace metal ions, such as Cu^{2+}.

In the case of photodecomposition, *S*-nitrosothiols are sensitive to UV light and in the case of *S*-nitrosoglutathione irradiation by UV light at the absorption maximum at 340nm or 545nm leads to the release of NO and thiyl radicals (Sexton *at al.*, 1994). The thiyl radicals can then react with GSNO to form a disulphide and release more NO.

With thermal decomposition, the thermally labile S-NO bond is cleaved homolytically giving an NO radical and a thiyl radical (Wang *at al.*, 2002) following which the thiyl radical dimerises to form a disulphide.

Trace copper ions in solution leads to accelerated decomposition of *S*-nitrosothiols and hence NO release is enhanced (Singh *at al.*, 1996). In studies, copper ions bind to GSNO leading to NO release in a method which differs from both thermal and photo decomposition in that it appears to not decompose via thiyl radical intermediates.

Amongst different *S*-nitrosothiols, decomposition can also be affected by the NO donor molecule. Friedman (1977) reports that in thiol groups with the thiol attached to a primary carbon, such as in cysteine or glutathione, the SH group was far more reactive than the reactivity of the thiol group present in penicillamine. As illustrated below in figure 12, cysteine and penicillamine are similar molecules, but the greater steric effect present in penicillamine lowers the reactivity of it's SH group relative to that of cysteine. Thus it can be expected that *S*-nitroso penicillamine would be less reactive and less likely to decompose than *S*-nitroso cysteine.

S-nitrosothiols such as *S*-nitrosoglutathione are powerful inhibitors of human platelet aggregation (Sogo *at al.*, 2000; Gordge *at al.*, 1998) and as such have provided an interesting avenue of research for medicinal chemists. *S*-nitrosothiols are also potent bronchodilators, and indeed deficiencies of RSNOs are seen in near fatal cases of asthma (Gaston *at al.*, 1998).

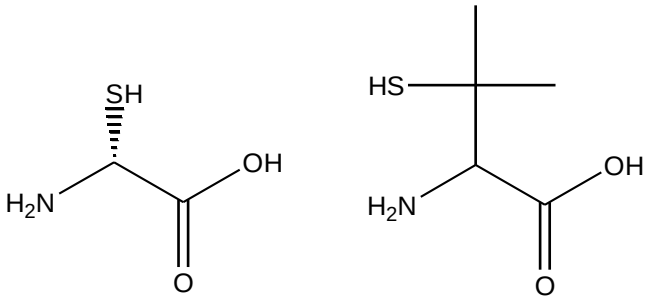


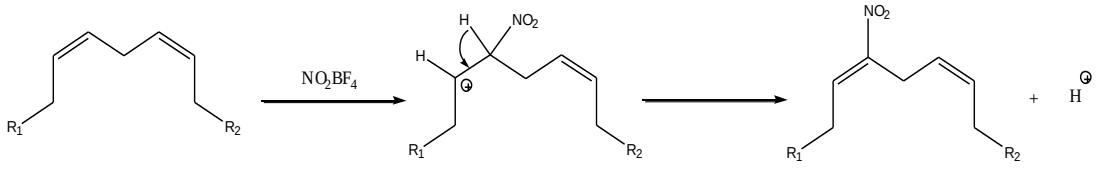
Figure 12: Cysteine (left) and penicillamine.

As both NO and PUFA demonstrate anti-inflammatory properties in the area of heart disease prevention, a conjugate combining the benefits of PUFA in diet, the EDRF of NO and the ability to release NO would be provide a greater benefit than a normal PUFA treatment. *S*-nitrosothiols are a somewhat unique donor of nitric oxide in that they display the same properties of NO.

1.7. Nitrated Fatty Acids

A nitrated fatty acid, or nitro lipid, can be described as a polyunsaturated fatty acid containing a nitrite group. The ability of this compound to then release nitric oxide *in vivo* can lead to vasorelaxation (Lima *at al.*, 2005). Some nitrolipids have been detected in small quantities in human blood (Baker *at al.*, 2009) and there is ongoing research into the field of chemical synthesis of novel nitrated fatty acids.

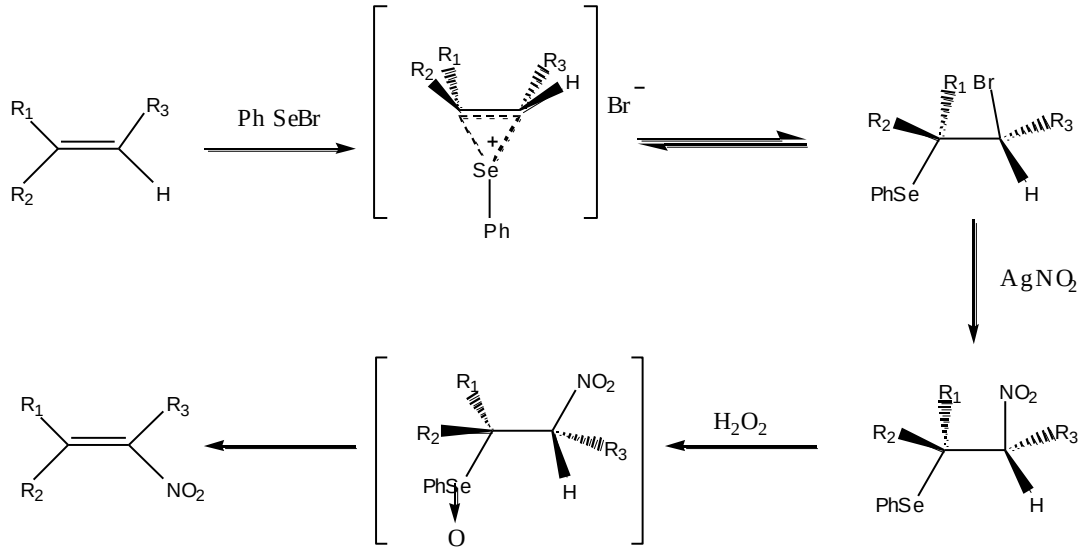
One proposed mechanism for chemical synthesis of a nitrolipid is electrophilic substitution upon reaction of an unsaturated fatty acid and a suitable nitrating agent such as nitronium tetrafluoroborate or nitrogen dioxide (O'Donnell *at al.,* 1998) whereby the NO_2 group would attack a double bond on the fatty acid chain leading to loss of hydrogen and attachment of the nitrite group (Manini *at al.*, 2008). An example of this type of reaction is demonstrated below in scheme 1.



Scheme 1: Nitronium tetrafluoroborate nitration

Hayama *at al.* (1982) suggested a method of synthesising conjugated nitroalkenes from simple alkenes in a manner that could also be applied to polyunsaturated fatty acids. The method relies upon "electrophilic addition of organoselenium reagents across the carbon-carbon double bond" to provide nitration of the alkenes, with the nitration occurring in a stereospecific manner.

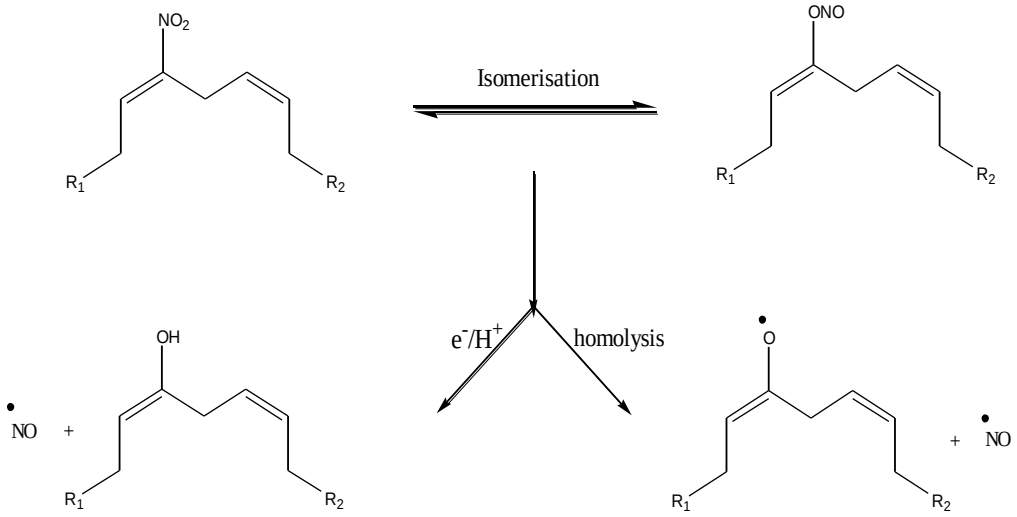
Scheme 2 below demonstrates the reaction of the alkene with benzeneselenyl bromide followed by nitration via silver nitrate and subsequent removal of benzeneselenyl group with hydrogen peroxide and reformation of the carbon-carbon double bond.



Scheme 2: Organoselenium reaction route.

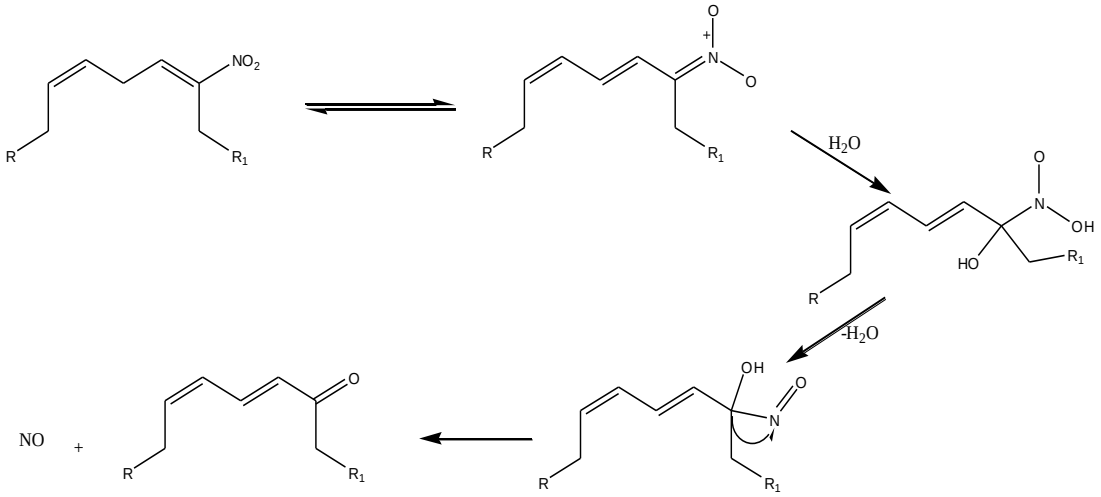
Gorczynski (*at al.*, 2006) demonstrated that these two syntheses are viable yet not specific as they give regioisomeric mixtures of nitroalkene products. Gorczynski proposed a 9 step synthesis for a regiospecific and stereospecific synthesis of (E)-9- and (E)-10-nitrooctadec-9-enoic acid. The advantages in a specific compound may be outweighed by the difficulties presented by introducing many steps in to the synthetic route. One consideration might be that over a nine step reaction the overall yield would be low as a result of multiple product purifications and multiple analyses at each stage of synthesis.

In scheme 3 (adapted from Lima *at al.*, 2005) a possible mechanism for nitric oxide release from a nitrolipid decomposing is shown in which the nitrolipid isomerises to the nitrite derivative and releases nitric oxide via either electron reduction or homolysis.



Scheme 3: Possible mechanism of NO release.

Manini (*at al.* 2008) proposed another mechanism for NO release from a nitrated lipid, which was based on the nef reaction as shown in scheme 4. The nef reaction involves NO release and formation of a ketone from a nitroalkane by acid hydrolysis of a nitroalkane salt. In the modified nef reaction of Manini's experiment, the nitroalkene is converted to a nitronate anion, before transformation into a hydroxynitroso intermediate which is then severed to give nitric oxide and an alkene.



Scheme 4: Example of NO release from nitrolipid

The anti-inflammatory properties of nitric oxide and polyunsaturated fatty acids are discussed elsewhere in sections 1.4 and 1.5, but there have been a range of papers published recently (see below) discussing the anti-inflammatory characteristics of nitrated polyunsaturated fatty acids.

Trostchansky *at al.* (2007) demonstrated the successful nitration of the n-6 PUFA arachidonic acid. They then determined the NO release capabilities by both electrochemical detection using an NO selective electrode and oxyhemoglobin oxidation recording a spectrum in the presence of the nitrated lipid. The results demonstrated that NO was released in aqueous media and that the NO released from their nitrolipid "induced vasorelaxation in an endothelium independent manner" by activation of the soluble guanylate cyclase cycle.

Nitrated derivatives of oleic and linoleic acid (Cui *at al.*, 2006) have been studied and shown to be endogenous anti-inflammatory signalling mediators which generate anti-inflammatory effects independently of NO formation.

Coles (*at al.*, 2002) suggested an example of similar benefits from a nitrated lipid; nitrolinoleate is given which also referred to the vascular protective properties via inhibition of platelet activation and by stimulating vasorelaxation. This same paper also concludes that the nitrated fatty acid "potently blocks multiple proinflammatory responses" and can assuage "leukocyte migration and subsequent activation in atherosclerotic lesions" and hence display additional vascular protective effects beyond its antiplatelet and vasorelaxing effects.

2.1 Aims and Objectives

The aim of this project was the synthesis and characterisation of a library of novel nitric oxide releasing molecules which could potentially be used as nutrapharmaceuticals.

Nutrapharmaceuticals, or nutraceuticals are known as food products or supplements which can provide beneficial medicinal or health related effects and can be used as a preventative measure for disease.

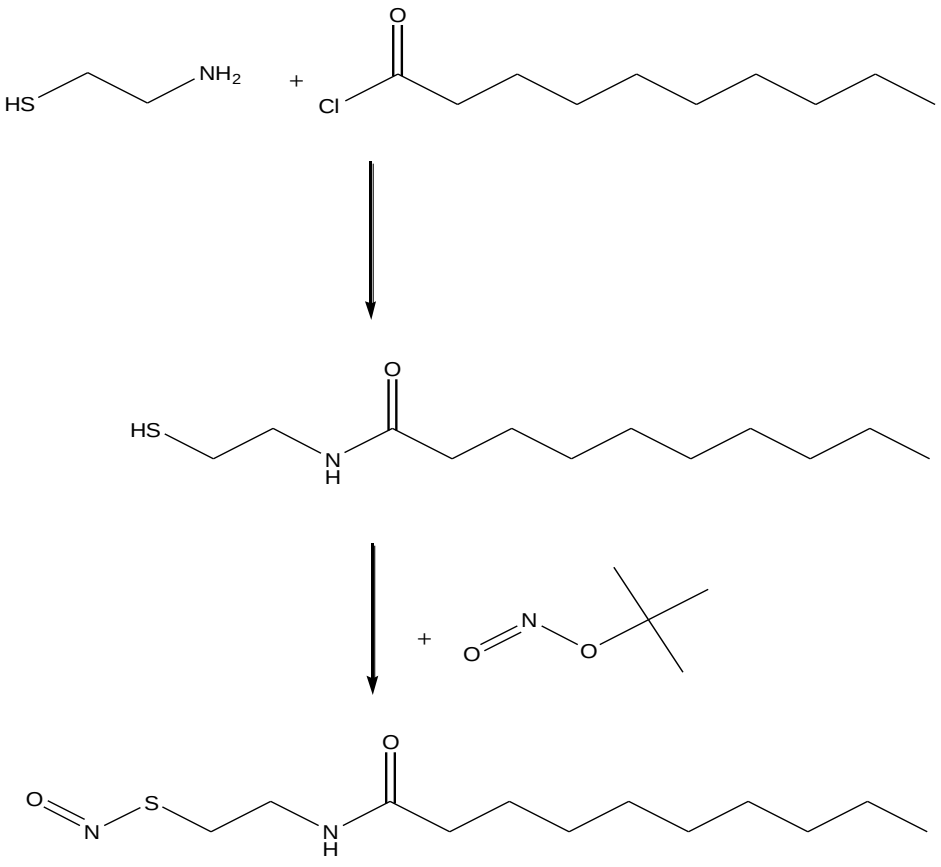
Synthesis of these molecules involved stably attaching a series of low molecular weight aminothiols to a polyunsaturated fatty acid before *S*-nitrosylation to give a final product.

Whilst the idea of nitrosation of aminothiols dates back some time (Hart, 1985), there are no recorded cases of a PUFA-NO releasing *S*-nitrosothiol conjugates being synthesised especially with regard to their use as a nutrapharmaceutical.

Once the library was synthesised and characterised it was intended to then examine the nitric oxide releasing properties of the compound via a colourimetric test.

2.2 Fatty Acids

As stated, the aim was the development of a library of compounds to be synthesised whereby a saturated and unsaturated fatty acid molecule was stably attached to a NO donating linker group. Initial work involved attaching aminothiols to decanoic acid ($C_{10}H_{20}O_2$) followed by nitrosation of the appropriate group on the attached linker molecule to give a final product. Once this was achieved, and the synthesised compounds purified and characterised, the decanoic acid in the reactions was replaced with an unsaturated fatty acid such as oleic acid. An example of the general reaction schemes involved can be seen in scheme 5 and a list of prepared compounds is shown in table 1.



Scheme 5: Proposed general reaction scheme

Table 1: Prepared intermediate compounds

Acid	**Linker Group**	**Product**
Decanoic Acid	Cysteamine HCl	*N*-decanoyl cysteamine
Decanoic Acid	*L*-Cysteine Ethyl Ester HCl	*N*-decanoyl *L*-cysteine ethyl ester
Decanoic Acid	*N*-Alloc - 1,4 Butanediamine HCL	*N*-Alloc - 1,4 Butanediamine Decanoate
Dodecanoic Acid	Cysteamine HCl	*N*-dodecanoyl cysteamine
Dodecanoic Acid	*L*-Cysteine Ethyl Ester HCl	*N*-dodecanoyl *L*-cysteine ethyl ester
Oleic Acid	Cysteamine HCl	*N*-oleoyl cysteamine

The first stage of the synthesis involved reaction between a relevant fatty acid chloride and the specific aminothiol/linker molecule in a basic solution. The purpose of the base was to capture hydrogen chloride released in situ from the reaction and to aid dissolution of the linker molecules. Completion of this stage yielded a library of compounds consisting of long aliphatic chains attached to a linker molecule via an amide bond. These products were then purified by re-precipitation and column chromatography. Full characterisation was then undertaken using analytical mass spectrometry and NMR spectroscopy.

After purification and characterisation of the intermediate molecules nitrosation was attempted using a range of nitrosating agents and methods until a successful nitrosation method was found. Presence of the *S*-Nitroso or C-Nitroso group on the molecule was confirmed both in reaction solution and in isolated solid product by MS and NO release was measured using the Griess test, a colourimetric method of determining the presence of organic nitrites.

Past studies have shown (Williams, 1985) that many *S*-nitrosothiols can be very unstable due to the inherent weakness of the S-N bond. Of the range of aminothiols to be used, some have been shown to form stable *S*-nitrosothiols (Park and Means, 1989) whilst other aminothiols may form less stable *S*-nitrosothiols but it was hoped the long chain acid would act as a stabilising agent, thus preventing the molecule from releasing NO via degradation as opposed to biological mediation.

2.3 Synthesis of Nitric Oxide Compounds

The library of molecules listed in section 2.2 was further developed in the attempted synthesis of a series of nitric oxide donors.

It was necessary in the course of the research to investigate a number of different nitrosation methods of these molecules.

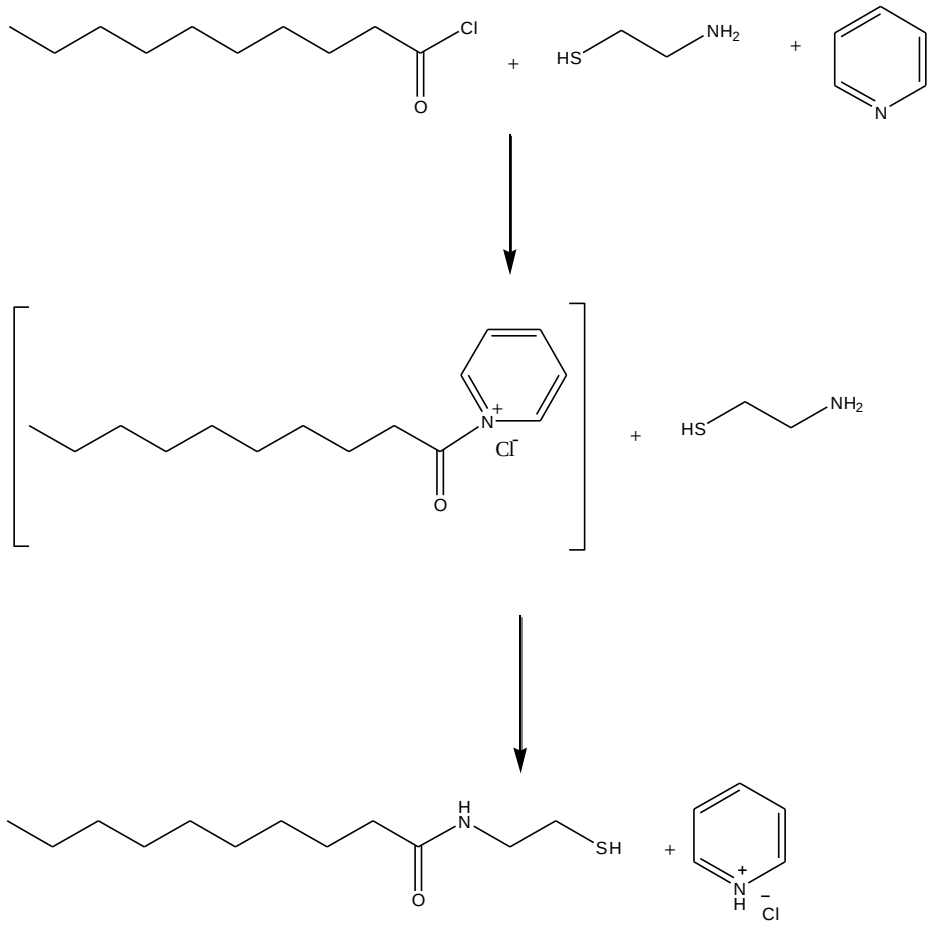
Whilst the attempted nitrosations were met with varying levels of success, *S*-nitroso cysteamine and *S*-nitroso octadec-9-enoate were synthesised and characterised thus giving both a saturated and unsaturated fatty acid linked to a nitric oxide donor. Unfortunately these molecules both displayed instability and decomposed quickly even when stored in the best possible conditions – namely, the sample was stored in a darkened round bottomed flask which had been purged with nitrogen and was kept in a freezer. This was observed as MS analysis a few days after synthesis showed more abundant fragmentation signals for the un-nitrosated starting compound and much less abundant fragmentation peaks for the target product when compared to MS analysis taken directly after the completion of the reactions.

2.4 Saturated Fatty Acids

The main challenge found in coupling a saturated fatty acid to the aminothiols was in choosing a solvent for the reaction. The primary difficulty was the poor solubility of many of the aminothiols, notably *L*-cysteine and to a lesser extent *DL*-homocysteine, in many solvents (such as methanol, DCM, DMF, chloroform) and a second issue was the extraction of the product from the solvent after the completion of the reaction.

Montalbetti *at al.*, 2005, demonstrated that pyridine could be used as a solvent for this type of reaction. Therefore pyridine was adopted into the reaction scheme and during experimentation it was found that the solubility of all aminothiols increased greatly. Though MS analysis indicated expected product fragmentation signals there was the challenge of isolating a solid. Initial attempts to this end involved use of water to precipitate a solid, which proved unfruitful, but the use of toluene enabled the solid product to be extracted from solution.

One disadvantage in using pyridine was that it proved to be a difficult solvent to remove completely from the product. This was in part due to the high boiling point making it difficult to remove completely via rotary evaporation even with toluene acting as an azeotrope. Also the basic nature of pyridine meant it would react with excess HCl in the reaction mixture to form the pyridine hydrochloride salt, as demonstrated in scheme 6. This salt required additional washing with water to remove the salt. Unfortunately, both of these processes had the potential to encourage disulphide formation.

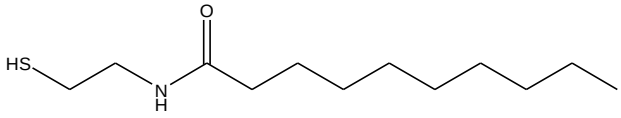


Scheme 6: Reaction scheme involving pyridine solvent.

After re-precipitation of the extracted solid, NMR analysis displayed proton signals not corresponding to the product, implying the presence of impurity. These proton signals were in the aromatic region, implying that pyridine remained in the final solid. Further TLC analysis resulted in two spots on the TLC plate, one representing the product and another on the baseline indicating a non-starting material. Unfortunately the resolution which was present in the TLC was not able to be replicated with column chromatography as the 2 spots co-eluted even using solvents which have low polarity.

Due to synthetic and product isolation difficulties, it was decided to shift focus away from the pyridine solvent based reaction and concentrate on finding potential linker molecules which were more readily soluble in organic solvents to react with the fatty acids. The solubility of a range of molecules was evaluated and it was found that esters and hydrochloride salts of aminothiols were sufficiently soluble in solvent with excess base to make reactions viable.

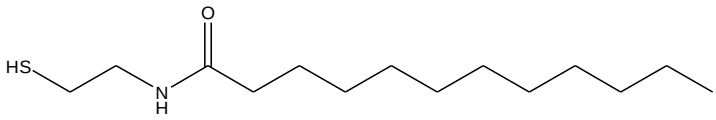
2.4.1 Synthesis of *N*-decanoyl cysteamine



Compound 1: *N*-decanoyl cysteamine.

Synthesis of *N*-decanoyl cysteamine (Compound 1, exp 4.1.1) was successfully undertaken reacting cysteamine HCl dissolved in DMF solvent with triethylamine base with the decanoyl chloride before precipitating a solid by addition of deionised water. On the NMR spectrum, a signal that integrated for 1 proton was detected at 7.93 ppm and assigned to the NH group. The methylene next to the C=O was detected at 2.0 ppm indicating the presence of an amide bond. A large broad multiplet integrating for 12 protons was centred at 1.2 ppm was assigned to the alkyl chain. MS analysis of the isolated solid gave an M-H signal of m/z 230.0 corresponding to the product molecular weight of 231.

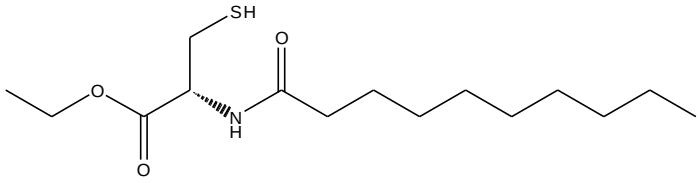
2.4.2 Synthesis of *N*-dodecanoyl cysteamine



Compound 2: *N*-dodecanoyl cysteamine.

N-dodecanoyl cysteamine (compound 2, exp 4.1.3) was synthesised utilising the same method, substituting lauroyl chloride for decanoyl chloride as the acid chloride to be reacted with cysteamine hydrochloride. MS analysis of the purified isolated solid displayed signals determined to be M+H at m/z 260.2 and M+Cl at m/z 294.2 which corresponded to the product molecular weight of 259. On the NMR spectrum, an NH signal integrating for 1 proton was detected at 6.35 ppm and the CH_2 next to the C=O was detected at 2.15 ppm indicating the presence of an amide bond. A 16 proton signal assigned to $(CH_2)_8$ was centred at 1.2 ppm confirming the presence of the long alkyl chain in the molecule.

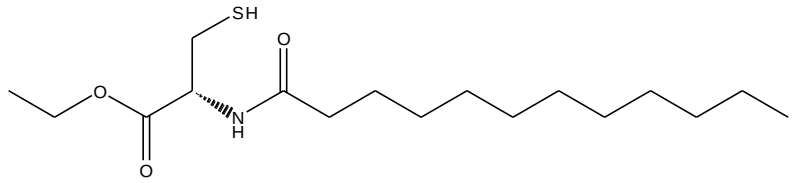
2.4.3 Synthesis of *N*-decanoyl *L*-cysteine ethyl ester



Compound 3: *N*-decanoyl *L*-cysteine ethyl ester.

Whilst *L*-cysteine had proved to be too insoluble for the purpose of previous synthesis attempts, the *L*-cysteine ethyl ester hydrochloride derivative was suitably soluble in a mixture of organic solvents and DiPEA base. As a result, synthesis of *N*-decanoyl *L*-cysteine ethyl ester (compound 3, exp 4.1.2) was completed by reaction of decanoyl chloride with *L*-cysteine ethyl ester HCl. MS analysis of the purified isolated solid displayed signals at m/z 302.2 (M-H) and m/z 338.2 (M+Cl) corresponding to the product molecular weight of 303. NMR analysis detected a signal, assigned to the NH, which integrated for 1 proton at 8.3 ppm, the CH near the peptide bond at 4.5 ppm and the CH_2 next to the CO on the amide bond was detected at 2.2ppm. The CH_3 with a signal integration showing 3 protons of the alkyl chain was seen at 0.85 ppm whilst the CH_3 of the ethyl ester, a triplet signal integrating for 3 protons, was seen at 1.23 ppm.

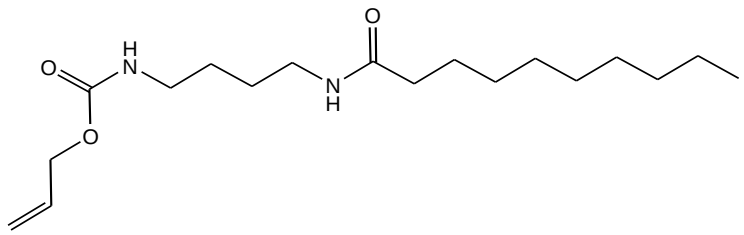
2.4.4 Synthesis of *N*-dodecanoyl *L*-cysteine ethyl ester



Compound 4: *N*-dodecanoyl *L*-cysteine ethyl ester.

N-dodecanoyl *L*-cysteine ethyl ester (compound 4, exp 4.1.4) was synthesised utilising the same technique, replacing decanoyl chloride with lauroyl chloride. NMR analysis detected the NH signal integration for 1 proton at 6.4 ppm, the CH near the amide bond at 4.8 ppm and the CH_2 next to the CO on the amide bond was detected at 2.2 ppm, integrating for 2 protons. The CH_3 , integrating for 3 protons, of the alkyl chain was seen at 0.8 ppm whilst the CH_3 of the ethyl ester was seen at 1.2 ppm, with a signal integration representing 3 protons. MS analysis of the product solid displayed a signal at m/z 367.2 for M+Cl corresponding with the expected product molecular weight of 333.

2.4.5 Synthesis of *N*-Alloc – 1,4 Butanediamine Decanoate



Compound 5: *N*-Alloc 1,4 butanediamine decanoate.

N-Alloc 1,4 butanediamine hydrochloride was reacted with decanoic acid as the acid chloride route was deemed unsuitable in this case. Instead, a peptide coupling cocktail, consisting of TBTU, Hobt and DiPEA, was utilised to create an amide bond in the synthesis of *N*-Alloc 1,4 butanediamine decanoate (compound 5, exp 4.1.5). Following purification and characterisation, the product was ready for nitrosation attempts. The product was confirmed by MS analysis, with MS peaks at m/z 327.2 (M+H) and m/z 361.2 (M+Cl) corresponding to the expected product molecular weight of 326. NMR analysis showed the NH signal integration for 1 proton at 4.7 ppm and the CH_2 next to the CO of the amide bond at 2.2 ppm, demonstrating the formation of a peptide bond between decanoic acid and *N*-Alloc 1, 4 butanediamine HCl. On the *N*-alloc moiety, the NH signal integration for 1 proton was seen at 5.6 ppm and the C=CH was seen at 5.9 ppm, integrating for 1 proton.

Attempts were made to react penicillamine with a saturated fatty acid chain using both the acid chloride route and the peptide coupling reaction route. Reactions involving penicillamine and decanoyl chloride yielded no positive results, with MS analysis only detecting peaks assignable to the starting materials. Another method was attempted, using a mixed anhydride synthesis which had shown some success as demonstrated by Megson (at al., 1999). Initial attempts in recreating this method showed some success (with MS analysis detecting an expected product fragmentation signal) although a pure solid could not be isolated, due to time constraints and no way of progressing the reaction to an unsaturated fatty acid chain, it was decided to not pursue this reaction route.

2.5 Unsaturated Fatty Acids

As the acid chloride route employed for the generation of compounds 1-5 as described in section 2.4 had yielded some positive results, synthesis of an unsaturated fatty acid-linker conjugate via the acid chloride synthetic route using oleoyl chloride was undertaken. Oleoyl chloride (pictured below in figure 15) is an eighteen carbon chain unsaturated fatty acid with a carbon carbon double bond at the 9 position.

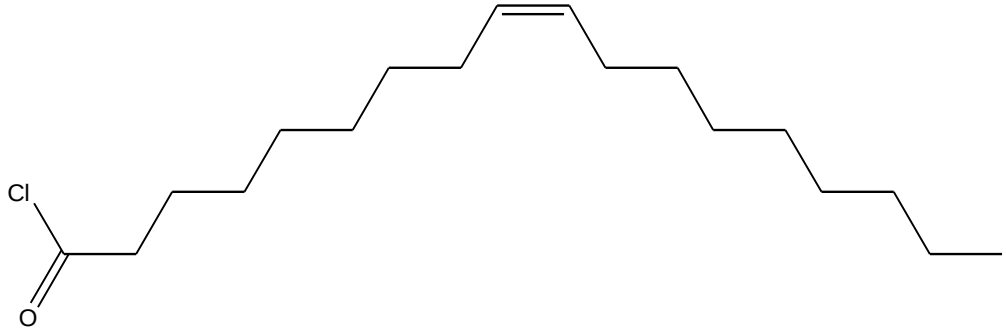
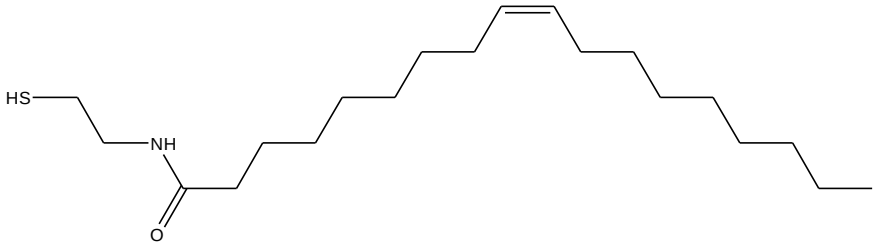


Figure 15: Oleoyl chloride

Synthetic attempts were made to link penicillamine, *L*-cysteine ethyl ester HCl and cysteamine HCl with oleoyl chloride. The most successful of these syntheses was in using cysteamine HCl. Cysteamine HCl was dissolved in a solution of DMF and triethylamine before the drop wise addition of oleoyl chloride. After the reaction was allowed to proceed to completion, deionised water was added to precipitate a solid. This was then purified and characterised to give *N*-oleoyl cysteamine (Compound 6, Exp4.1.6). MS analysis confirmed the expected molecular weight of 341 with signals corresponding to M-H at m/z 340.2 and M+Cl at m/z 376.2. NMR analysis displayed the NH integration signal representing 1 proton at 8.0 ppm, with the CH_2 next to the amide bond seen at 2.7 ppm representing 2 protons. The hydrogens of the C=C of the alkyl chain were seen at 5.35 ppm.



Compound 6: *N*-oleoyl cysteamine

Whilst attempts to create a saturated fatty acid - penicillamine conjugate had been unsuccessful, the reaction between penicillamine and oleoyl chloride proved more successful, yielding a solid which displayed signals of m/z 414.2 (M+H) and m/z 412.6 (M-H) under MS analysis conforming to the expected product molecular weight of 413.

However, investigation using NMR showed a mixture of signals indicating the presence of 2 molecules very similar in structure present in the solid. Signals were interpreted which would account for the long alkyl chain, there were two broad multiplets in close proximity (at δ 1.35 and 1.40) which could have represented the alkyl chain and similarly two triplets (at δ 1.1 and 1.2) which would likely represented the CH_3. There were also signals which indicated the presence of NH (δ 6.7), $COCH_2$ and HC=CH (δ 5.35) which is a strong indicator that the spectrum showed a mixture of two compounds similar in structure

As acid chlorides are very reactive and thus lack specificity, it is believed that the mixture in the solid could be as the acid chloride has attached to both the NH_2 and the OH sites on penicillamine in equal amounts. A solution to this could be to protect the OH group, however due to time constraints; it was not possible to focus on this avenue in the course of the research.

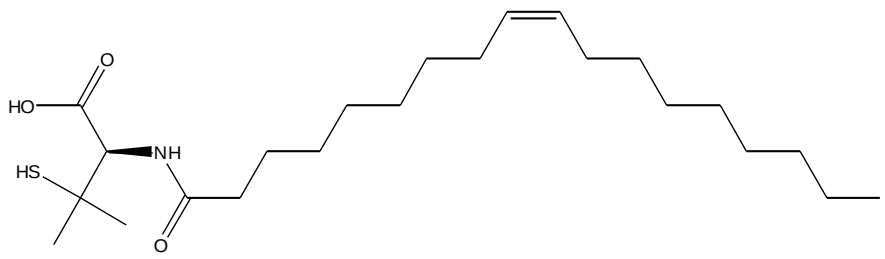


Figure 14: Penicillamine octadec-9-enoate

2.6 Nitric Oxide Compounds

In order to generate a library of nitrosated compounds (from compounds 1-6) a number of different methods of nitrosation were considered and attempted, with varying levels of success.

2.6.1 Sodium Nitrite Technique

Initial attempts were made to nitrosate these compounds by addition of sodium nitrite (Hart, 1985) as described in 4.3.4 as sodium nitrite is a common nitrosating agent. After a series of attempted nitrosations no sign of nitrosated product was detected by MS analysis so it was decided to move on to other nitrating agents. Nitrosation attempts by this method of *N*-decanoyl cysteamine and *N*-decanoyl *L*-cysteine ethyl ester yielded no success, as MS analysis displayed no sign of product, with fragmentation signals only indicating starting materials present.

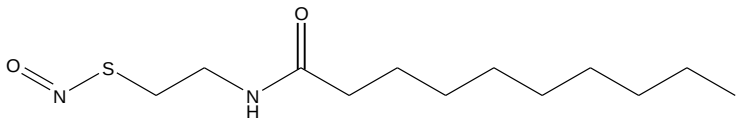
2.6.2 Nitronium Tetrafluoroborate Technique

Nitronium tetrafluoroborate was deemed to be a suitable nitrating agent in the case of *N*-alloc 1, 4 butanediamine decanoate, which has a C=C bond as opposed to an S-H group as the proposed site of nitrosation. It was hoped that nitronium tetrafluoroborate could provide successful nitrosation as it had been used in nitrosation of C=C bonds (O'Donnell *at al.*, 1999) successfully as reported in literature. Nitrosation attempts were carried out, as described in section 4.2.5, by addition of one equivalent of nitronium tetrafluoroborate to the fatty acid-linker conjugate dissolved in organic solvent. Analysis both during and after this reaction showed this method of nitrosation to be unsuccessful as MS analysis failed to show any expected product signal, instead only providing signals which corresponded to the molecular weight of the starting material.

2.6.3 Tert-Butyl Nitrite Technique

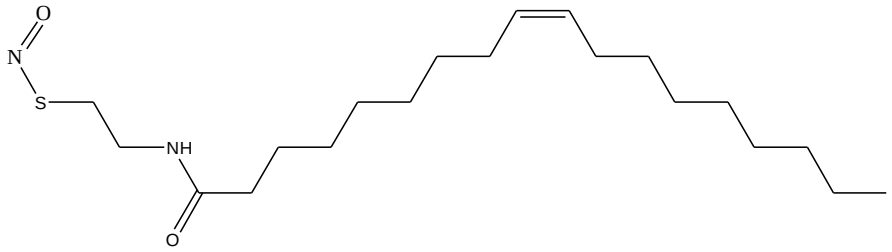
Other methods of nitrosation were explored, one of which was nitrosation by tert-butyl nitrite (Koley at al., 2009). Initial experimentation using one equivalent to nitrate proved unsuccessful so it was decided to add an excess of tert-butyl nitrite.

N-decanoyl cysteamine was dissolved in a small amount of DCM and a drop of HCl in a darkened flask, cooled with ice and purged with nitrogen. To this was added five equivalents of tert-butyl nitrite, following which the reaction was observed by TLC and MS. Colour changes were observed and MS analysis displayed the expected product signals for *S*-nitroso *N*-decanoyl cysteamine (compound 7, exp 4.2.1) with a signal of (M-H) m/z 259.0 corresponding to the molecular weight of 260.



Compound 7: *S*-nitroso *N*-decanoyl cysteamine

This method was also applied to *N*-oleoyl cysteamine (compound 8, below) as described in exp4.2.2 and also yielded success in providing a nitrated product, with MS analysis indicating a signal of M+H at m/z 372.2, corresponding to the product molecular weight of 371.



Compound 8: *S*-nitroso *N*-oleoyl cysteamine

In both cases the DCM solvent was evaporated to give a solid. The evaporation of the solvent was performed at low temperatures due to the inherent lability of the S-N bond.

Unfortunately nitrosation attempts of the other compounds using this method proved to be unsuccessful.

2.6.4 Nitric Oxide 'Fuming' Technique

Another method attempted was by 'fuming' (Mazzei, 2001) nitrous oxide fumes directly into a solution containing *N*-oleoyl cysteamine in organic solvent. This was performed by dropping concentrated HCl directly onto sodium nitrite pellets and directing the flow of NO gas given off to bubbling through the *N*-oleoyl cysteamine solution. MS analysis determined that this was also an effective method of nitrating the sample as indicated by the presence of a signal (M+Cl) at m/z 404.2 corresponding to the expected product mass. Proton NMR analysis of the product showed the absence of the C=C hydrogen peaks, which had been present in the starting material at δ: 5.35, indicating that the fuming method of nitrosation was a stronger nitrosation method than the tert-butyl nitrite method and was likely also nitrating the C=C bond on the fatty acid chain in addition to the S-H group on the cysteamine linker.

2.6.5 Summary of Results

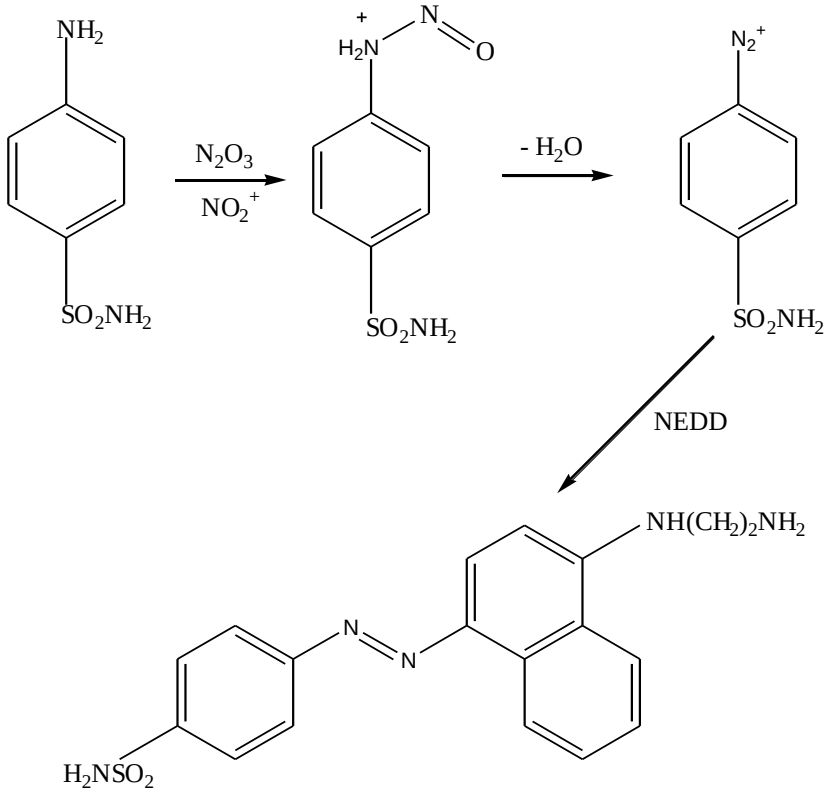
Nitric oxide containing molecules were successfully synthesised using a range of techniques and were characterised by NMR and MS. Some nitrating agents proved more successful than others and the created compounds are displayed below in table 2.

Table 2: Synthesised NO containing compounds.

Starting compound	**Nitrating agent**	**Product**
N-decanoyl cysteamine	Tert-butyl nitrite	*S*-nitroso *N*-decanoyl cysteamine
N-oleoyl cysteamine	Tert-butyl nitrite	*S*-nitroso *N*-oleoyl cysteamine
N-oleoyl cysteamine	NO fuming method	*S*-nitroso *N*-oleoyl cysteamine

2.7 Griess Reagent

The Griess reaction is a means by which to detect organic nitrites originally developed in 1879 as a colourimetric test (Griess, 1879). During the reaction, the Griess reagent (a solution containing sulfanilic acid and *N*-(1-naphthyl)-ethylenediamine dihydrochloride(NEDD)) undergoes a diazotisation reaction with nitric oxide to form a diazo compound, which is a red azo dye. The reaction is shown in scheme 7 (adapted from Nirode, 2006). The Griess reaction can be viewed as a simple colourimetric test, with positive nitrite presence indicated by formation of a red colour.



Scheme 7: Griess reaction

Demonstrated below in figure 15 are three sample vials of Griess reagent (1ml) to which has been added, from left to right; 1ml methanol (blank control sample), *S*-nitroso *N*-oleoyl cysteamine as nitrosylated via the fuming method and finally *S*-nitroso *N*-oleoyl cysteamine as nitrosylated by reaction with tert-butyl nitrite.

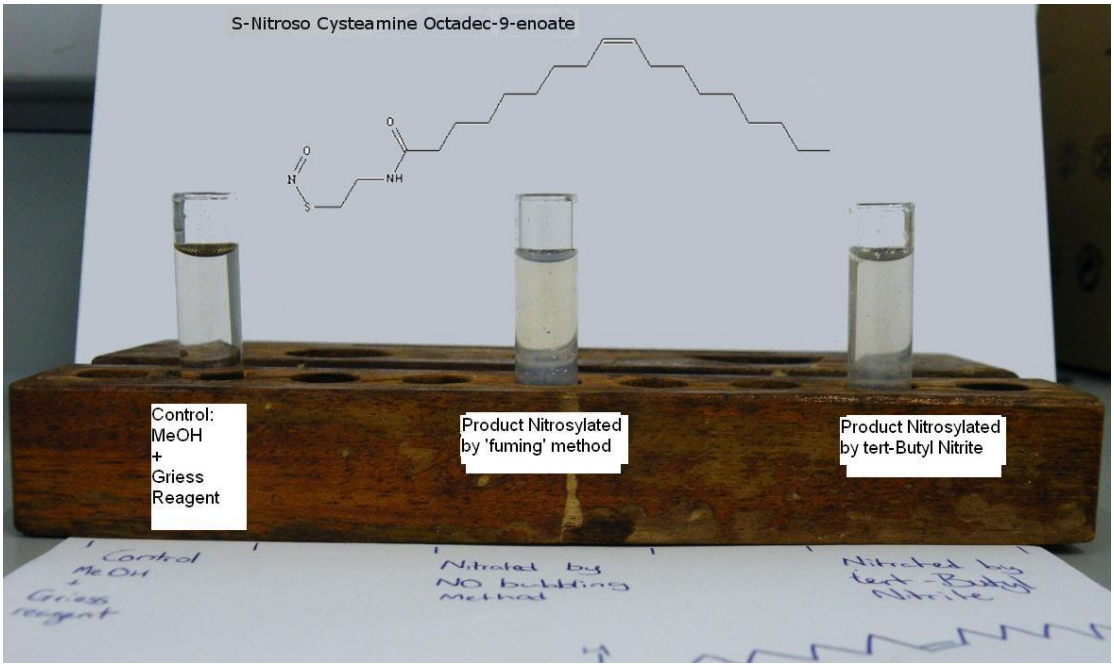


Figure 15: Photograph taken shortly after the respective samples were added to the Griess reagent.

Following this, the sample tubes were incubated at 37°C for 15 minutes.

It was observed, figure 16, that the control sample remained colourless whilst both *S*-Nitroso *N*-oleoyl cysteamine samples had developed a pink/red colour indicating that a red azo dye had been formed as a result of the presence of NO in both solutions. Thus it can be inferred that *S*-Nitroso *N*-oleoyl cysteamine was releasing nitric oxide in the presence of the Griess reagent.

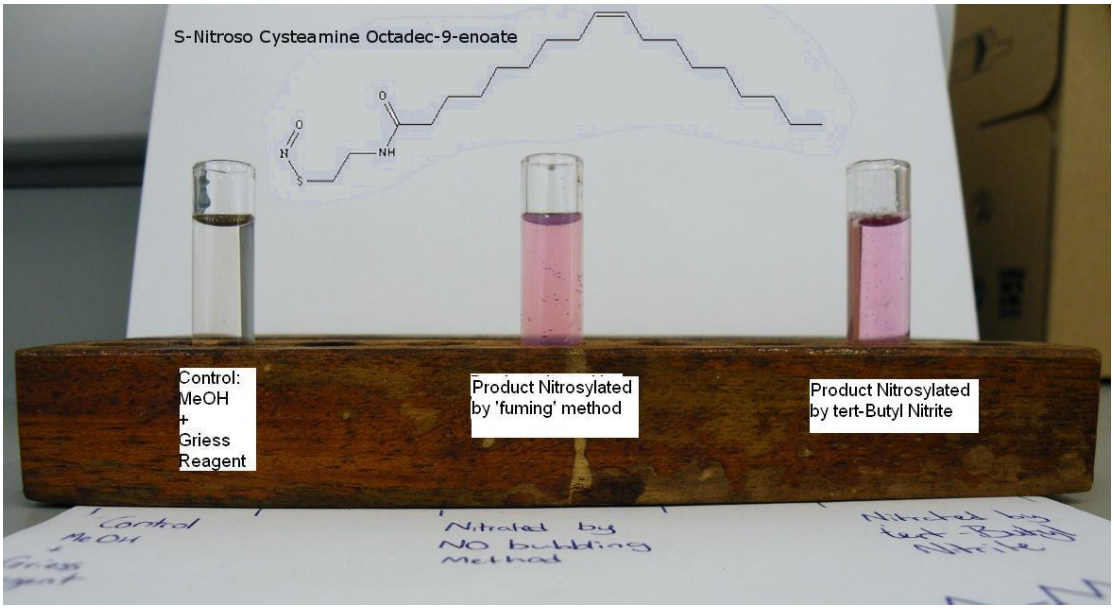


Figure 16: Griess samples after incubation period.

The observations made from all Griess testing of synthesised potential NO donor compounds are displayed in Table 3.

Table 3: Observations of Griess testing

Compound observed	**Colour change noted**
S-nitroso *N*-decanoyl cysteamine	Pinkish red colour change noted
S-nitroso cysteamine octadec-9-enoate	Pinkish red colour change noted
S-nitroso cysteamine octadec-9-enoate	Pinkish red colour change noted
Blank methanol control sample	No change

3.0 Conclusions and Future Work

During this research a number of methods were used to synthesise fatty acid chains attached to linker molecules. From this work a small library of compounds was synthesised and work was undertaken to nitrate the linker molecules via a range of nitrosation methods.

As a result both *S*-nitroso cysteamine and *S*-nitroso octadec-9-enoate were synthesised and characterised. The ability of these molecules to release nitric oxide was then qualitatively studied via the Griess reaction, which demonstrated that both molecules released NO. When compared to a blank, both molecules dissolved in methanol and Griess reagent developed a red colour whilst the blank remained colourless.

Due in part to the inherent instability of *S*-nitrosothiols in general, both of the synthesised nitric oxide donors exhibited poor stability and short shelf life, although a full decomposition study was not undertaken in this research. As a result, it is unlikely that either of the synthesised molecules would have much practical application.

As an avenue for future study, it should be noted that the synthesis of a penicillamine-unsaturated fatty acid conjugate could be completed with more time to dedicate to the separation of the product mixture alluded to in section 3.1. With the steric effect exhibited by penicillamine, it is likely a successfully created and nitrated molecule would be relatively stable.

Further study could also focus on different types of NO donors, such as transition metal NO donors, C-NO donors or O-NO donors (Martelli at al., 2006). Replacing the *S*-NO spacer molecules with C-NO spacer molecules would likely give a more stable final compound than those synthesised in the course of this research. Aiming towards a final product with more likely stability than that typically seen in RSNOs could result in a library of more stable final NO donating compounds.

4.0 Experimental

4.0.1 Methods used to characterise synthesised compounds

NMR

^{1}H NMR spectra obtained using a Bruker WP250 (400MHz) NMR spectrometer.

MS

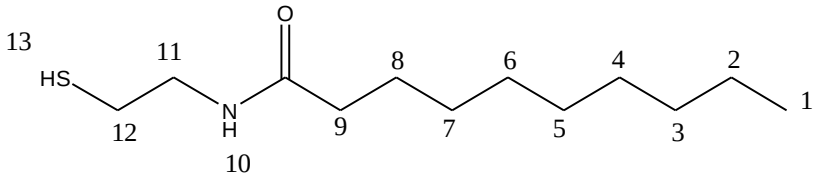
Low resolution electrospray mass spectra recorded using an Agilent Technologies 6130 Quadruple LC/MS instrument (mass range m/z 4000, maximum resolution 3000). Samples were dissolved in methanol prior to analysis.

Chromatography

Thin layer chromatography performed on polygram Sil G UV 0.2mm silica gel plates. TLC plates were stained using an iodine tank to visualise spots. Solvent used was Methanol:water (9:1)

Column chromatography used silica gel and solvent used was hexane:chloroform (9:1 and 99:1)

4.1.1 Synthesis of *N*-decanoyl cysteamine



To a solution of cysteamine hydrochloride (0.5g, 4.4mmol) dissolved in DMF (5ml) was added triethylamine (0.61ml, 4.4mmol). This mixture was allowed to stir for 20 minutes before decanoyl chloride was (0.91ml, 4.4mmol) added dropwise. The reaction was allowed to proceed for 2 hours, after which time 5ml deionised H_2O was added to precipitate a solid. The solid was filtered off and washed with 5ml cold deionised H_2O, followed by 5ml of ether. The solid was then dried in a vacuum oven for 2 hours prior to characterisation by NMR and MS.

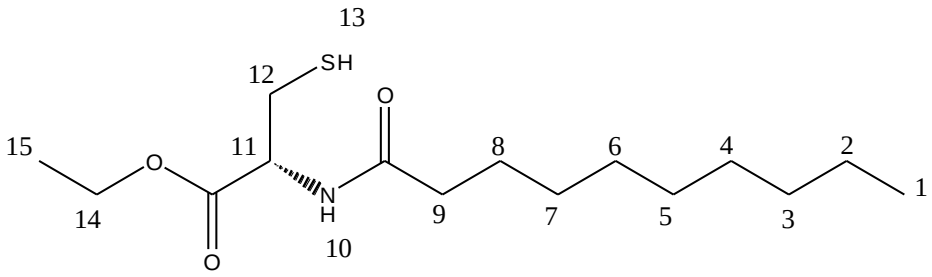
Found:

Yield: 52.6%

MS: m/z at 230.0 (100%) (M-H)

^{1}H NMR: (DMSO) δ: 0.8(t, 3H, CH_3)1; δ: 1.2 (bm, 12H, $(CH_2)_6$)2-7; δ: 1.42 (t, 2H, CH_2)8; δ: 2.0 (t, 2H, CH_2)9; δ: 2.7 (bm, 2H, CH_2)12; δ: 3.23 (m, 2H, CH_2)11; δ: 7.93 (t, 1H, NH)10

4.1.2 Synthesis of *N*-decanoyl *L*-cysteine ethyl ester



L-cysteine ethyl ester hydrochloride(1g, 5.4mmol) was dissolved in a 1:1 mixture of dichloromethane and ethyl acetate (10ml:10ml), to this solution was added DiPEA (diisopropylethlamine) (18.6 ml, 1.025mol). The mixture was allowed to stir for 30 mins, upon which time decanoyl chloride (5.3mmol, 1.34ml) was added. After 3 hours, the reaction solution was rotary evaporated to dryness. The solid was then heated to 60°C then reprecipitated to purify and dried at 50°C in a vacuum oven for 2 hours before being analysed by NMR and MS.

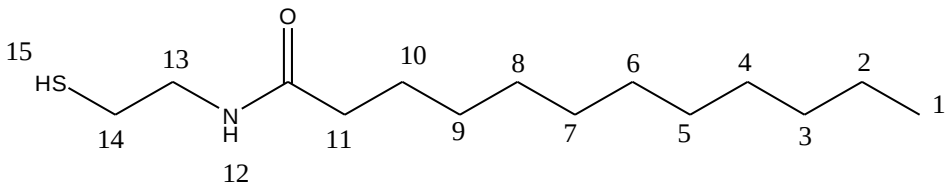
Found:

Yield: 42.3%

MS: (-) m/z 302.2 (100%) (M-H); 338.2 (18%) (M+Cl)

^{1}H NMR: (DMSO) δ: 0.85(t, 3H, CH_3)1; δ: 1.23(t, 3H, CH_3)15; δ: 1.4 (bm, 12H, $(CH_2)_6$)2-7; δ: 1.49 (t, 2H, CH_2)8; δ: 2.2 (t, 2H, CH_2)I ; δ: 2.9 (m, 1H, CH_2)12; δ: 3.1 (m, 1H, CH_2)12; δ: 4.1 (q, 2H, CH_2)14; δ: 4.5 (m, 1H, CH)11; δ: 8.3 (d, 1H, NH)10

4.1.3 Synthesis of *N*-dodecanoyl cysteamine



Cysteamine hydrochloride (1g, 8.8mmol) was dissolved in a solution of DMF (20ml), triethylamine (2.45ml, 17.6mmol) and allowed to stir for 20 mins. After this time lauroyl chloride (2.04ml, 8.8mmol) was added dropwise and the reaction was then allowed to proceed for 2 hours. After this time deionised water (20ml) was added and a solid precipitate, which was then filtered off before being dissolved in ethanol at 60°C and allowed to reprecipitate then vacuum oven dried at 40°C for 2 hours. Following drying, the solid was characterised by MS and NMR.

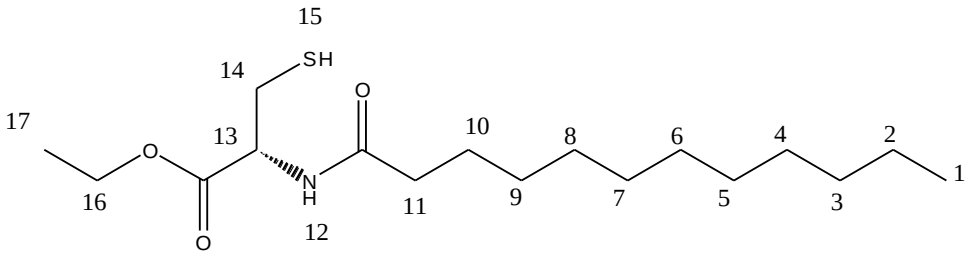
Found:

Yield: 25.7%

MS: (+) m/z 260.2(100%) (M+H); 282.0 (M+Na) (12%), (-) m/z 258.2 (55%) (M-H); 294.2 (35%) (M+Cl)

^{1}H NMR: ($CDCl_3$) δ: 0.8(t, 3H, CH_3)1; δ: 1.2 (bm, 16H, $(CH_2)_8$)2-9; δ: 1.6 (t, 2H, CH_2)10; δ: 2.15 (t, 2H, CH_2)11; δ: 2.75 (t, 2H, CH_2)14; δ: 3.5 (m, 2H, CH_2)13; δ: 6.35 (t, 1H, NH)12

4.1.4 Synthesis of *N*-dodecanoyl *L*-cysteine ethyl ester



L-cysteine ethyl ester hydrochloride(2g, 0.0108mol) was dissolved in a 1:1 mixture of dichloromethane and ethyl acetate (15ml:15ml), to this solution was added DiPEA (30 ml). The mixture was allowed to stir for 30 mins, upon which time lauroyl chloride (1.45ml, 0.0108mol) was added. After 3 hours, the reaction solution was rotary evaporated to dryness. The solid was then heated at 60°C and allowed to reprecipitate to purify and then dried at 50°C in a vacuum oven for 2 hours before being tested by NMR and MS.

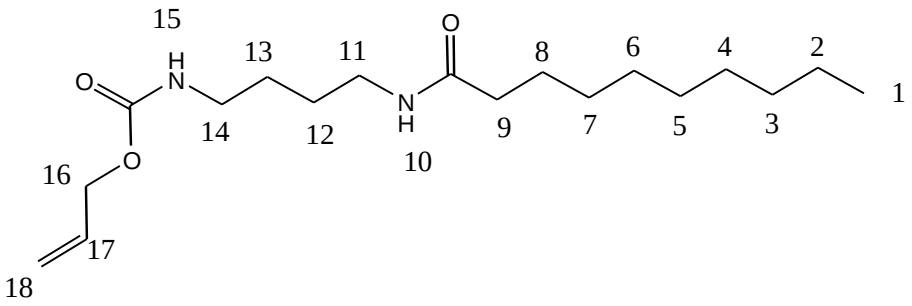
Found:

Yield: 28.6%

MS: (-) m/z 367.2 (100%) (M+Cl)

^{1}H NMR: ($CDCl_3$) δ: 0.8 (t, 3H, CH_3)1; δ: 1.2 (t, 3H, CH_3)17 δ: 1.26 (bm, 16H, $(CH_2)_8$)2-9; δ: 1.6 (t, 2H, CH_2)10; δ: 2.2 (t, 2H, CH_2)11; δ: 3.05 (m, 1H, CH_2)14; δ: 3.15 (m, 1H, CH_2)14; δ: 4.2 (t, 2H, CH_2)16; δ: 4.8 (m, 1H, CH)13; δ: 6.4 (d, 1H, NH)12

4.1.5 Synthesis of *N*-Alloc 1, 4 Butandiamine Decanoate



To a solution of DMF (5ml) and DiPEA (0.17ml, 9.6mmol) was added *N*-Alloc 1,4 butandiamine hydrochloride (0.2g, 9.6mmol), which was allowed to stir for 20 minutes. A second solution comprising of a mixture of decanoic acid (0.1651g, 9.6mmol), TBTU (0.6154g, 19.2mmol), Hobt (0.2589g, 19.2mmol) and DiPEA (0.68ml, 38.3mmol) in DMF (2ml) was prepared and added to the first reaction solution. The reaction was allowed to proceed for 2 hours (with TLC monitoring). Upon completion, the solution was evaporated to dryness and the resultant solid heated in ethanol at 60°C and allowed to reprecipitate then dried in a vacuum oven at 50°C for 2 hours before being tested by NMR and MS.

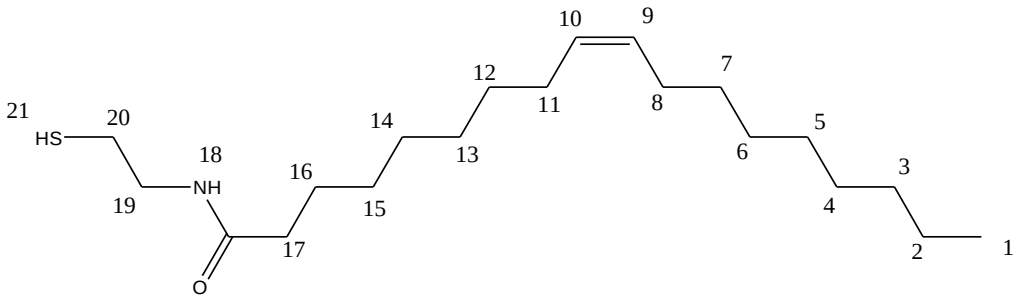
Found:

Yield: 66.1%

MS: (+) m/z 327.2 (100%) (M+H), (-) m/z 361.2 (100%) (M+Cl)

^{1}H NMR: ($CDCl_3$) δ: 0.8(t, 3H, CH_3)1; δ: 1.18 (bm, 12H, $(CH_2)_6$)2-7; δ: 1.49 (t, 4H, CH_2, CH_2)12, 13; δ: 1.51 (t, 2H, CH_2)8; δ: 2.2 (t, 2H, CH_2)9; δ: 3.3 (d, 2H, CH_2)11; δ: 3.4 (d, 2H, CH_2)14; δ: 4.5 (d, 2H, CH_2)16; δ: 4.7 (d, 1H, NH)10; δ: 5.2 (d, 2H, CH_2)18; δ: 5.6 (d, 1H, NH)15; δ: 5.9 (m, H, CH)17

4.1.6 Synthesis of *N*-oleoyl cysteamine



Cysteamine hydrochloride (1g, 8.8mmol) was dissolved in a solution of DMF (20ml), triethylamine (4.91ml, 35.2mmol, 4 eq) and DiPEA (1.53 ml, 8.8mmol) and allowed to stir for 20 mins. After this time of oleoyl chloride (2.91 ml, 8.8mmol) was added dropwise and the reaction was then allowed to proceed for 2 hours. After this time deionised water (20ml) was added to precipitate a solid, which was filtered off before being reprecipitated from ethanol at 60°C and vacuum oven dried at 40°C for 2 hours. Following drying, the solid was tested by MS and NMR.

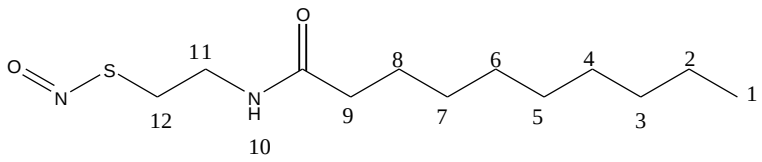
Found:

Yield: 42.8%

MS: (-) m/z 340.2 (15%) (M-H), 376.2 (100%) (M+Cl)

^{1}H NMR: (DMSO) δ: 0.85(t, 3H, CH_3)1; δ: 1.35 (bm, 20H, $(CH_2)_6$ and $(CH_2)_4$)2-7, 12-15; δ: 1.49 (t, 2H, CH_2)16; δ: 1.9 (t, 2H, CH_2)17; δ: 2.1 (4H, 2xCH_2)11,8; δ: 2.7 (t, 2H, CH_2)20; δ: 3.4 (t, 2H, CH_2)19; δ: 5.35 (t, 2H, CH and CH)9,10; δ: 8.0 (t, 1H, NH)1

4.2.1 Nitrosation of *N*-decanoyl cysteamine

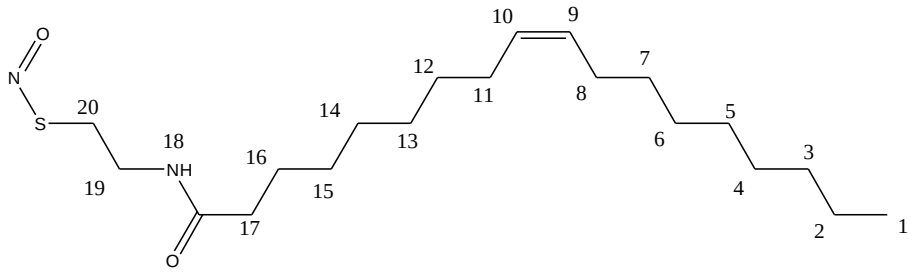


N-decanoyl cysteamine (0.05g, 2.16mmol) was added to a solution of MeOH (5ml) and allowed to stir. In a separate flask, conc. HCl was added dropwise directly onto $NaNO_2$ and the resultant nitrous fumes were introduced into the reaction solution and allowed to bubble through. The reaction was observed by TLC and MS analysis was performed to determine presence of *S*-nitroso-*N*-decanoyl cysteamine. Solvent was removed via rotary evaporation at 30^{o}C to give a white solid powder.

Found:

MS: (-) m/z (100%) (M+Cl)

4.2.2 Nitrosation of *N*-oleoyl cysteamine by tert-Butyl Nitrite

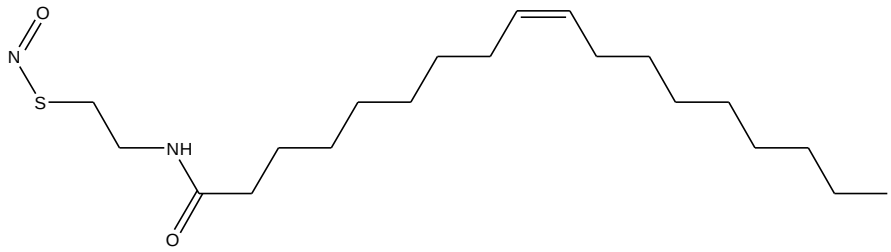


N-decanoyl cysteamine (0.05g, 1.5mmol) was dissolved in anhydrous DCM (3ml) and 2N HCl (2 eq, 0.01ml) and allowed to stir on ice in a darkened 25ml round bottomed flask for 15 minutes. An excess (7.5mmol, 0.087ml) of tert-butyl nitrite was added to this solution, immediately following which Nitrogen gas was purged through the sample flask. A slight colour change from colourless to a mild yellow was observed upon addition of nitrating agent. The solution was dried at 30^{o}C to give a solid powder, which was then analysed by NMR and MS.

Found:

MS: (+) m/z at 372.2 (100%) (M+H), (-) m/z 405.2 (100%) (M+Cl)

4.2.3 Nitrosation of *N*-oleoyl cysteamine by 'fuming' method



N-oleoyl cysteamine (0.05g, 1.5mmol) was added to a solution of MeOH (5ml) and allowed to stir. In a separate flask, conc. HCl was added dropwise directly onto $NaNO_2$ and the resultant nitrous fumes were directed into the reaction solution and allowed to bubble through. The reaction was observed by TLC and MS analysis was performed to determine presence of *S*-nitroso-*N*-oleoyl cysteamine. Solvent was stripped off under mild heat rotary evaporation to give a white solid powder.

Found:

MS: (-) m/z 404.2 (100%) (M+Cl), (+) m/z 408.2 (100%) (M+K)

4.2.4 Nitrosation of *N*-oleoyl cysteamine by Sodium Nitrite

N-oleoyl cysteamine (0.05g, 1.5mmol) was added to a solution of MeOH (5ml) and allowed to stir. One equivalent of sodium nitrite (0.01g) was dissolved in MeOH (3ml) and added dropwise to the reaction solution.

Reaction was observed by MS and TLC but analysis did not determine the presence of *S*-Nitroso_*N*-oleoyl cysteamine.

4.2.5 Nitrosation of *N*-oleoyl cysteamine by Nitronium Tetrafluoroborate

N-oleoyl cysteamine (0.05g, 1.5mmol) was added to a solution of DCM (4ml) and allowed to stir. Nitronium tetrafluoroborate (0.0194g, 1.5mmol) was added in one portion to the reaction solution.

Reaction was observed by MS and TLC but analysis did not determine the presence of *S*-nitroso *N*-oleoyl cysteamine.

The reaction was abandoned when it became apparent the intended product was not being formed.

4.3.1 Griess testing of Nitrosylated compounds

NO containing product (*S*-nitroso *N*-oleoyl cysteamine, 5mg; *S*-nitroso *N*-decanoyl cysteamine, 5mg) was dissolved in 1ml of methanol. To this was added 1ml of the Griess reagent. For comparison purposes, a sample of 1ml blank methanol was also mixed with 1ml Griess reagent. The initial colour was noted and then the sample was incubated at 60^{o}C for 15 minutes. Once removed, any development of colour changes (thus indicating red azo dye formation and thus NO release) was noted.

5.0 References

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