Errata to Ph.D thesis titled "Blind Linear Multiuser Detection for DS-CDMA Wireless Networks"

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Typing Errors

- The first part of Eq. (7.14) on p.158 should be written as follows

$$\mathbf{P}_{1,n}^{\perp} \triangleq \mathbf{I}_N - \mathbf{s}_{1,n}\mathbf{s}_{1,n}^{\dagger},$$

- The equation given in Step 3 on top of p.164 should be written as follows

$$\hat{\eta}_1 = \arg\max_n \sum_{i=i_s}^{Q_{CMA}+i_s-1} Z_{1,n}^2[i]$$

- The sample cross-correlation matrix of the received signal in Eq. (7.34) on p.165 should be written as follows

$$\widehat{\mathbf{R}}_{\mathbf{yy}} = \frac{1}{Q_R}\sum_{i=1}^{Q_R}\mathbf{Y}[i]\mathbf{Y}^T[i]$$

Amendments to content

- In the light of new third generation (3G) RTT proposals, some information pertaining to 3G standards in Section 1.2.3 on p.15 have became obsolete or incomplete. The hybrid combination of wideband CDMA and TDMA is known as TD-CDMA which is one option within the European UMTS standard. In Jan. 1998, wideband CDMA (WCDMA) has been selected as the UMTS terrestrial air interference scheme for FDD frequency bands in ETSI. However, there are still currently much difference between WCDMA and USA's 3G standard (known as cdma2000) in terms of their chip rates, bandwidth and spreading codes [1, 2, 3].

- In section 2.2, it should be noted that all emerging 3G standards (e.g., cdma2000 [3]), except the TD-CDMA option in UMTS, all use long codes, as in the IS-95. Furthermore, centralised multiuser detection has now been specified in those 3G standards in BS for the reverse link. The first sentence on p.22 "As reflected in the apparent divorce between the IS-95 CDMA cellular standard and most CDMA literature....", has became obsolete. The argument that long codes is used to model MAI as AWGN, was the assumption made by the matched filter detection in the IS-95. However, the usage of long codes in 3G is to reduce the cross-correlation between different sequences. The first sentence of the last paragraph on p.24 "The short code design approach facilitates the use of multiuser detection..." is incorrect. There are multiuser detection schemes proposed for long codes that have been reported in the literature, such as Kalman filtering [4] and linear array MF-MMSE [5]. However, in comparison with short codes, long codes can place more computational burden on the blind adaptation (see reference [23] in the thesis).

- In Section 2.3.3, if significant fading rate or Doppler spread is present (see ch.2 in reference [28] in the thesis), the channel response in Eq. (2.10) can be described by a Gauss-Markov model [6]. This model assumes that channel variations are modelled as symbol-to-symbol changes of channel tap gains. A second-order auto-regressive fading process is commonly used, since it represents a good match to mobile radio channel. However, multipath effect considered in ch. 4 and 5 in this thesis models all multipath channels as LTI filters, as in Eq. (2.27) on p.37, under the assumption in Section 2.3.5.

- In Section 2.5.7, a useful reference which examines the transient behavior of adaptive MMSE-based multiuser detection is [7].

- In ch. 3, it is clarified that the modified MOE detector using Frost's constraint projection in Eq. (3.17) is more robust to finite precision errors in comparison to the original MOE detector (see reference [3] in the thesis). Also, it avoids the unreliable bit decision if $Z_{MF,1}[i] \simeq Z_{X,1}[i]$, as discussed in Section 3.3.4. Futhermore, the constraint constant a in Eq. (3.19) can be adjusted for tradeoff between recovery of desired signal and noise enhancement.

- In ch. 4, it is noted that the tracking properties of CMA in the RAKE-type receiver in the presence of fast fading, remains an open problem. Depending on the fading rate, inputs to the CMA-based adaptation should be taken over a time interval when the path gains are relatively constant. However, as long as channel undermodelling is not excessive (as discussed in Section 4.7.4) and not all L paths undergo deep fades simultaneously, the RAKE-type receiver should still give reliable bit decision. This is due to the fact that space-time diversity is induced to improve the detection performance over that using any single copy of the received signal (as discussed in Section 4.3.1).

References

[1] T.Ojanpera and R.Prasad, *Wideband CDMA for Third Generation Mobile Communications,* Artech House, 1998.

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[6] M.Stojanovic and Z.Zvonar, "Performance of Multiuser Detection with Adaptive Channel Estimation," *Proc. ICC,* 1997.

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CONTROL OF POLYUNSATURATED FATTY ACID OXIDATION

A thesis submitted for the degree of

Doctor of Philosophy of

The Australian National University

Research School of Chemistry

by

Ling Xia
BSc, MSc, China
MSc, UK

April 1999

To Mum And Dad, My Husband And Son

Declaration

I declare that the work described in this thesis is the original work of the candidate. It contains no material published or written by another person or submitted for a degree or diploma in any other university or college, except where due reference has been made in the text. This research work was carried out at the Research School of Chemistry, the Australian National University, during the period from February 1996 to April 1999, under the supervision of Dr. Chris J. Easton.

Ling Xia

April 1999

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Abstract

In pursuit of compounds that have potential pharmaceutical value for the control of physiological problems associated with the oxidation of polyunsaturated fatty acids (PUFAs), nine nitro analogues of PUFAs were designed, synthesised and purified. They include saturated and polyunsaturated nitroalkanes, a saturated and an unsaturated 4-nitro fatty acid, and a saturated and an unsaturated 4-(2-carboxyethyl)-4-nitro fatty acid. α,β-Unsaturated and β,γ-unsaturated nitroalkanes were also synthesised, but pure samples were not obtained due to decomposition. Unsucceseful attempts were also made to prepare 1-nitro fatty acids and 2-nitro fatty acids.

Having obtained nitro analogues of PUFAs, lipoxygenase activity of these compounds was investigated through *in vitro* enzyme assays using a soybean 15-lipoxygenase (15-LO), a potato 5-LO and a porcine leukocyte 12-LO. This showed that (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoic acid is a substrate for the 15-LO and the 12-LO and undergoes oxidation to generate hydroperoxides. The K_m and V_{max} values are 8 μM and 24 μM min^{-1} μg^{-1} for the 15-LO, and 15 μM and 1.52 min^{-1} for the 12-LO. The values for this substrate with the 15-LO are comparable to those of arachidonic acid, which is a primary substrate of the enzyme.

The main interest in the nitro analogues of PUFAs was as potential inhibitors of LOs. This was investigated by examining their effects on the oxidation of arachidonic acid catalysed by the 5-LO, 12-LO and 15-LO. 4-Nitrohenicosanoic acid, 4-(*all-Z*)-nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dicarboxylic acid and 4-heptadecyl-4-nitroheptane-1,7-dicarboxylic acid were found to selectively inhibit 5-LO, 12-LO and 15-

LO, respectively. This is the first report of inhibitors that are selective for 15-LO *vs.* 5-LO.

A kinetic study was conducted on 15-LO catalysed oxidation of arachidonic acid in the presence of 4-heptadecyl-4-nitroheptane-1,7-dicarboxylic acid. The inhibitor displayed a mixed inhibition pattern, *i.e.*, it binds to both the free enzyme and the enzyme/substrate complex. The inhibition constants were determined to be 24 μM (K_i) for formation of the enzyme/inhibitor complex and 64 μM (K_I) for formation of the enzyme/substrate/inhibitor complex.

Despite showing potent inhibtion of 12-LO catalysed oxidation of arachidonic acid, 4-(*all-Z*)-nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dicarboxylic acid appeared to be unstable in the presence of the 12-LO and its inhibitory effect was not long-lasting. Preliminary experiments showed that it is metabolised by the 12-LO.

(8*Z*,11*Z*,14*Z*,16*E*)-18(*S*)-hydroperoxy-4-nitrotricosa-8,11,14,16-tetraenoic acid and (8*Z*,11*Z*,14*Z*,16*E*)-18(*S*)-hydroxy-4-nitrotricosa-8,11,14,16-tetraenoic acid were prepared *via* 15-LO catalysed oxidation of (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoic acid. Enzyme assays showed that the hydroperoxide inhibits 15-LO catalysed oxidation of arachidonic acid with an IC_{50} value of 65 μM.

4-Nitrohenicosanoic acid and 4-(*all-Z*)-nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dicarboxylic acid were shown to activate arachidonic acid oxidation catalysed by the 15-LO and the 5-LO, respectively. 4-Nitrohenicosanoic acid and 4-heptadecyl-4-nitroheptane-1,7-dicarboxylic acid also activated 12-LO catalysed oxidation of arachidonic acid.

In order to examine the structural basis for the antioxidant behaviour of 3-[(*all-Z*)-(eicosa-5,8,11,14-tetraenylthio]propionic acid, a series of analogues was designed and

synthesised. These included an unsaturated γ-thia fatty acid, saturated γ- and β-thia fatty acids, and saturated and unsaturated sulfides. The effect of these compounds, along with others already available in the laboratory, on arachidonic acid autoxidation, was examined using a Petri-dish thin film oxidation assay with HPLC analysis. All compounds except the unsaturated and saturated β-thia fatty acids exhibited significant antioxidant behaviour. This shows that the antioxidant activity is associated with the sulfur and adversely affected by a nearby carboxyl group.

Analysis of arachidonic acid oxidation in the presence of 3-tetradecylthiopropionic acid showed that the thia fatty acid reacted to form the corresponding sulfoxide probably through interaction with arachidonic acid hydroperoxides. This suggests that the antioxidant behaviour of the γ-thia fatty acids and sulfides is due to their reaction with, and thus destruction of, the hydroperoxide products of PUFA autoxidation, which are initiators of free radical oxidation chain processes. This conclusion is supported by the observation that a γ-thia fatty acid reacted more rapidly with *tert*-butyl hydroperoxide than a corresponding β-thia fatty acid coinciding with high antioxidant activity for the former and the lack of antioxidant behaviour of the latter.

Abbreviations

The following abbreviation have been used through this thesis:

AIBN	azobisisobutyronitrile
Bu_4NCl	tetra-n-butylammonium chloride
Bu_4NI	tetra-n-butylammonium iodide
n-BuLi	butyl lithium
CoA	coenzyme A
CBr_4	carbon tetrabromide
CH_2Cl_2	dichloromethane
DBU	1,8-diazabicyclo[5,4,0]undec-7-ene
DME	1,2-dimethoxyethane
DMF	*N*,*N*-dimethylformamide
E	enzyme
EI	enzyme/inhibitor complex
EPR	electron paramagnetic resonance
EFA	essential fatty acid
ES	enzyme/substrate complex
ESI	enzyme/substrate/inhibitor complex
ε	molar extinction coefficient
FLAP	five-lipoxygenase activating protein
GC/MS	gas chromatography-mass spectrometry
HETE	hydroxyeicosatetraenoic acid
HMPA	hexamethylphosphoramide
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
I	inhibitor
IC_{50}	inhibitor concentration required for 50% inhibition of substrate metabolism
IR	infrared
J	coupling constant
K_i, K_I	inhibition constant
K_m	Michaelis constant
K'_m	Michaelis constant at [I]
LO	lipoxygenase
LT	leukotriene

LX	lipoxin
MMC	magnesium methyl carbonate
M.P.	melting point
m/e	mass to charge ratio
NADH	nicotinamide adenine dinucleotide (reduced form)
NEt_3	triethylamine
NMR	nuclear magnetic resonance
ODS	octadecylsilane
PPh_3	triphenylphosphine
PUFA	polyunsaturated fatty acid
RBL	rat basophilic leukemia cells
RH	lipid
RI	refractive index
pK_a	dissociation constant
ROOH	lipid hydroperoxide
RT	room temperature
S	substrate
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
TocH	tocopherol
V_{max}	maximum rate
V'_{max}	maximum rate at [I]

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Results and Discussion

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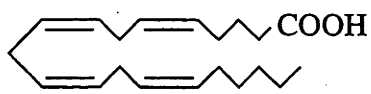
Chapter 1

Introduction

1.1. Polyunsaturated fatty acids and their roles

Polyunsaturated fatty acids (PUFAs) are one of the most intensely studied classes of compounds due to their important role in biological systems. Naturally occurring PUFAs contain 16 or more carbons with two or more methylene-interrupted *cis* double bonds.[1] For instance, arachidonic acid **1** is a typical PUFA, which consists of 20 carbons and four methylene-interrupted *cis* double bonds commencing six carbons from the methyl group terminus. The nomenclature of PUFAs includes trivial names, systematic names and a numeric system.[2] The structure and nomenclature of arachidonic acid **1** as an example are shown in Fig. 1.1.



1

Trivial name: arachidonic acid

Systematic name: (5Z,8Z,11Z,14Z)-eicosatetraenoic acid

Numeric system: 20:4(n-6)

Fig. 1.1: Structure and nomenclature of arachidonic acid **1**

PUFAs can be divided into four families,[3] based on the parent fatty acids from which they are derived *in vivo*: linoleic acid (18:2(n-6)) **2**, α-linolenic acid (18:3(n-3)) **3**, oleic acid (18:1(n-9)) **4** and palmitoleic acid (16:1(n-7)) **5** as shown in Fig. 1.2.

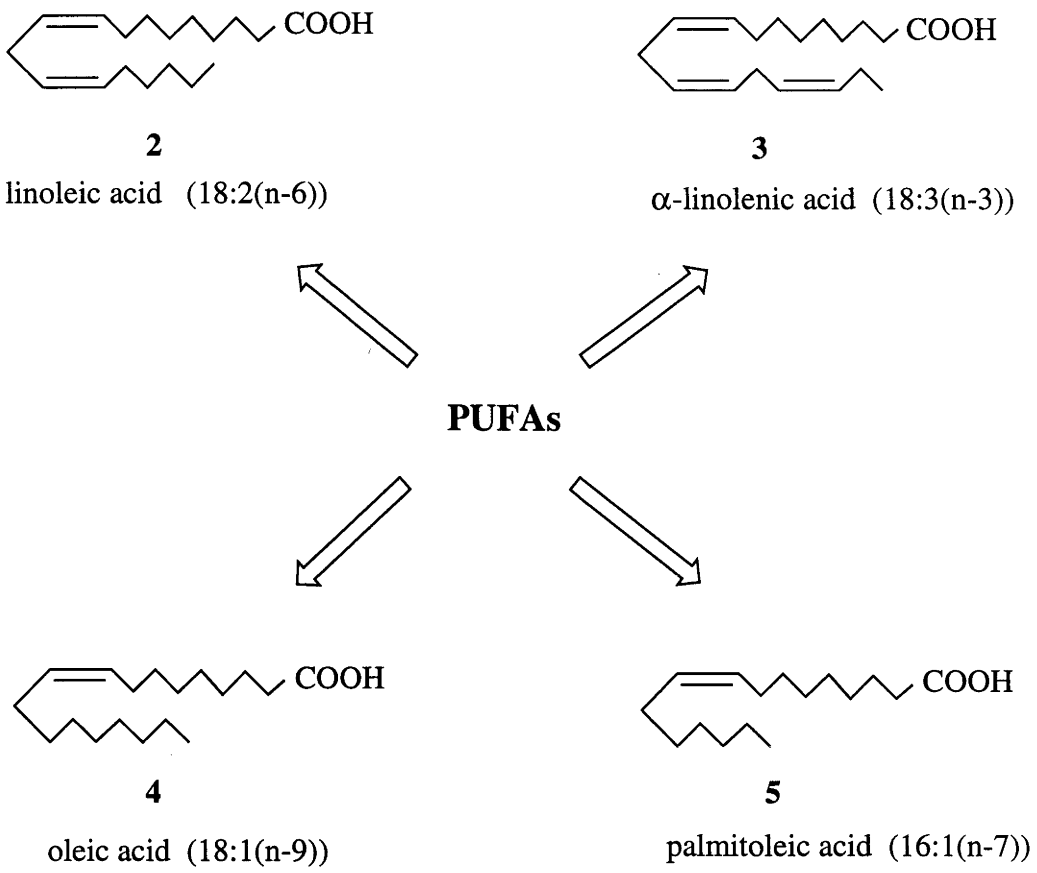


Fig. 1.2: Four parent fatty acids for the four families of PUFAs

The two families derived from the n-6 and n-3 PUFAs, linoleic acid **2** and α-linolenic acid **3**, are known as the essential fatty acids (EFAs).[4] They cannot be synthesised by the human body and are acquired indirectly through desaturation or elongation of linoleic acid **2** and α-linolenic acid **3** (Fig. 1.3), which must be supplied through the diet.[3] The two families of EFAs have distinct nutritional and metabolic effects and should be differentiated. Arachidonic acid **1** can be obtained from the diet or from linoleic acid **2** indirectly through γ-linolenic acid **6**. The other two families from oleic acid **4** and palmitoleic acid **5** are considered to be non-essential fatty acids, because they can be synthesised by humans from saturated fatty acid precursors[3]. Interconversion among these four families of unsaturated fatty acids does not occur in humans.

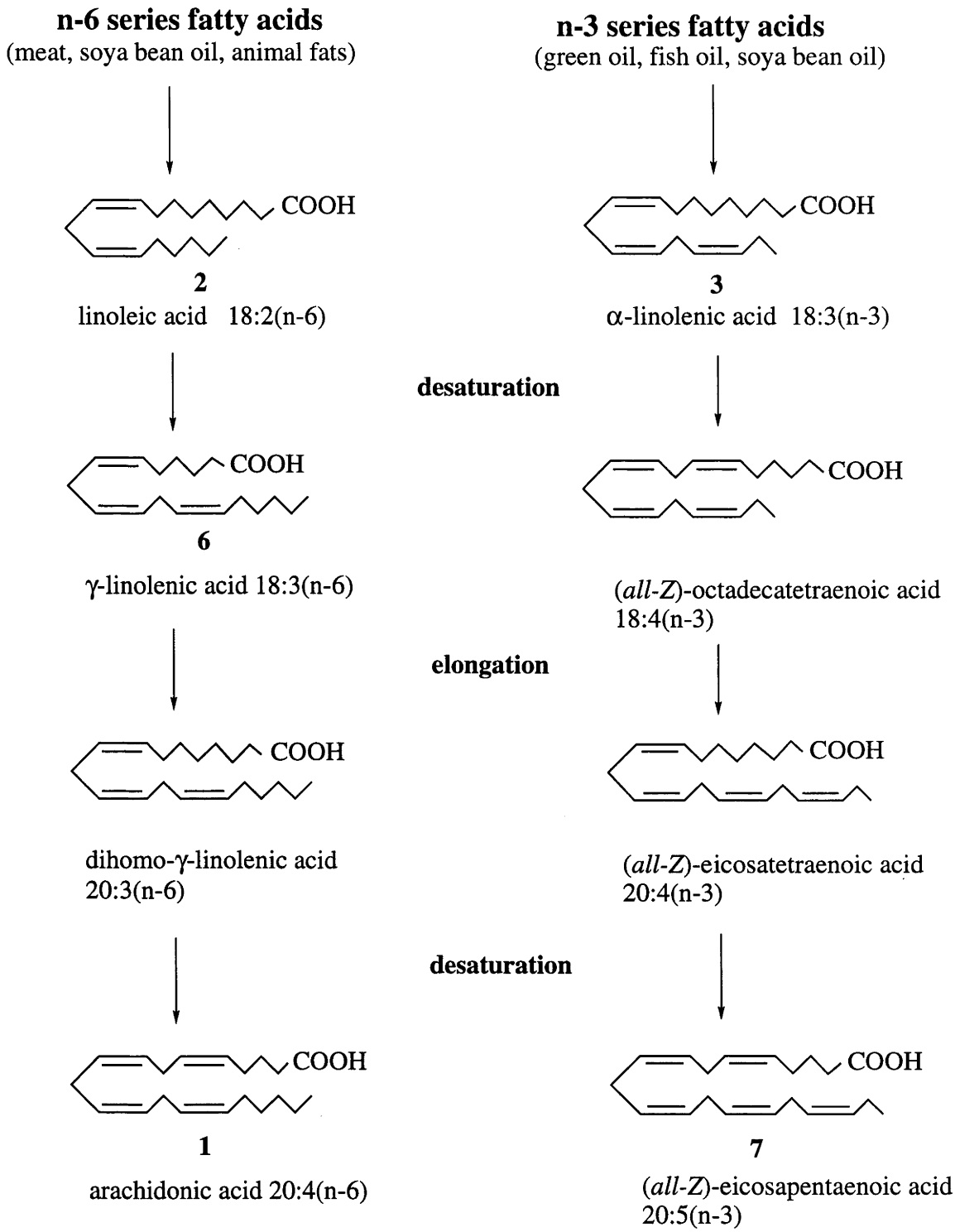


Fig. 1.3: The main pathways of desaturation and elongation of the essential fatty acids

PUFAs and monounsaturated fatty acids are a main source of energy consumed by humans in their daily diet. PUFAs contribute about 5-6% of dietary energy.[3] Besides being used for energy production, the dietary fatty acids can also be maintained as two

types of fat in the body:[3] storage fat, located mostly in adipose tissue rich in triglycerides, and structural fat, consisting of phosphoglycerides and cholesterol esters. The structural fatty acids are integral parts of biomembranes and are often polyunsaturated.

The beneficial effects of PUFAs, particularly EFAs, such as arachidonic acid **1** on the cardiovascular system are well known. In human studies, unsaturated fatty acids cause a reduction in blood viscosity and blood pressure, and a decrease in platelet aggregation associated with prolonged bleeding times.[5] Epidemiological studies in Greenland Eskimos have correlated a low mortality due to coronary heart disease with a high intake of marine n-3 PUFAs.[6] Clinical trials have demonstrated that an intake of marine fatty acids has a hypotriglyceridaemic and possibly hypocholesterolemic effect.[7] It has also been suggested that EFAs are important modulators of neoplastic development because they are capable of decreasing the size and number of tumours as well as increasing the lagtime of tumour appearance.[8]

Some investigators have found that n-3 fatty acids influence the fatty acid profiles of various tissues both in animals and humans,[9] which in turn affects the immune system and various enzyme activities such as the Ca^{2+}-transport system desaturase[10] as well as protein kinase.[11]

Recently, Wang *et al.*[12] reported that five compounds including α-linolenic acid **3** and linoleic acid **2**, which inhibited the amidolytic activity of soluble tissue factor/activated factorVII complex (sTF/VIIa), were isolated from two traditional Chinese medicinal plants commonly used in the treatment of cardiovascular and cerebrovascular diseases. Exploration of the structure-activity relationship of these active molecules showed that at least one *cis* double bond was essential for the inhibitory activity, and that fatty acids containing two or three *cis* double bonds were optimal.

Arachidonic acid **1** plays a central role in biological control systems. It acts both as a

modulator and messenger, particularly of signals triggered at the level of cell membranes.[13] The fatty acids in the cell membranes modulate the configuration of all the membrane-associated proteins, and are able to influence directly or indirectly almost every second messenger system which controls cell function.[14]

1.2. Oxidation of polyunsaturated fatty acids

PUFAs react by three major oxidation pathways: β-oxidation, nonenzymatic oxygenation (*i.e.*, autoxidation) and enzymatic oxidation (*e.g.*, lipoxygenase catalysed oxidation). These pathways lead to degradation of PUFAs and are also involved in important biological signalling processes. Therefore, control of PUFA oxidation has long been an important target of research in pharmacology and organic chemistry.

1.2.1. β-Oxidation pathway of fatty acid metabolism

1.2.1.1. β-Oxidation

The β-oxidation pathway as one of the major oxidative pathways in fatty acid metabolism leads to energy production in mammals as do glycolysis and the oxidation of carbohydrates. There are two different β-oxidation systems in animal cells: one in mitochondria and the other in peroxisomes. Although intermediates formed in the two systems are identical,[15] the first reaction of the β-oxidation in peroxisomes is catalysed by an acyl-CoA oxidase in contrast with the mitochondrial acyl-CoA dehydrogenase. β-Oxidation involves removing sequentially a two-carbon fragment from a fatty acid. Free fatty acid **8** is first activated by condensation with coenzyme A before it is oxidised. In mitochondrial β-oxidation as an example[1] (shown in Fig. 1.4), acyl-CoA synthetase converts the fatty acids **8** to energy-rich fatty acyl-CoA thioesters **9**, which are further converted to *trans*-Δ^2-enoyl-CoA thioesters **10** by fatty acyl-CoA dehydrogenase.

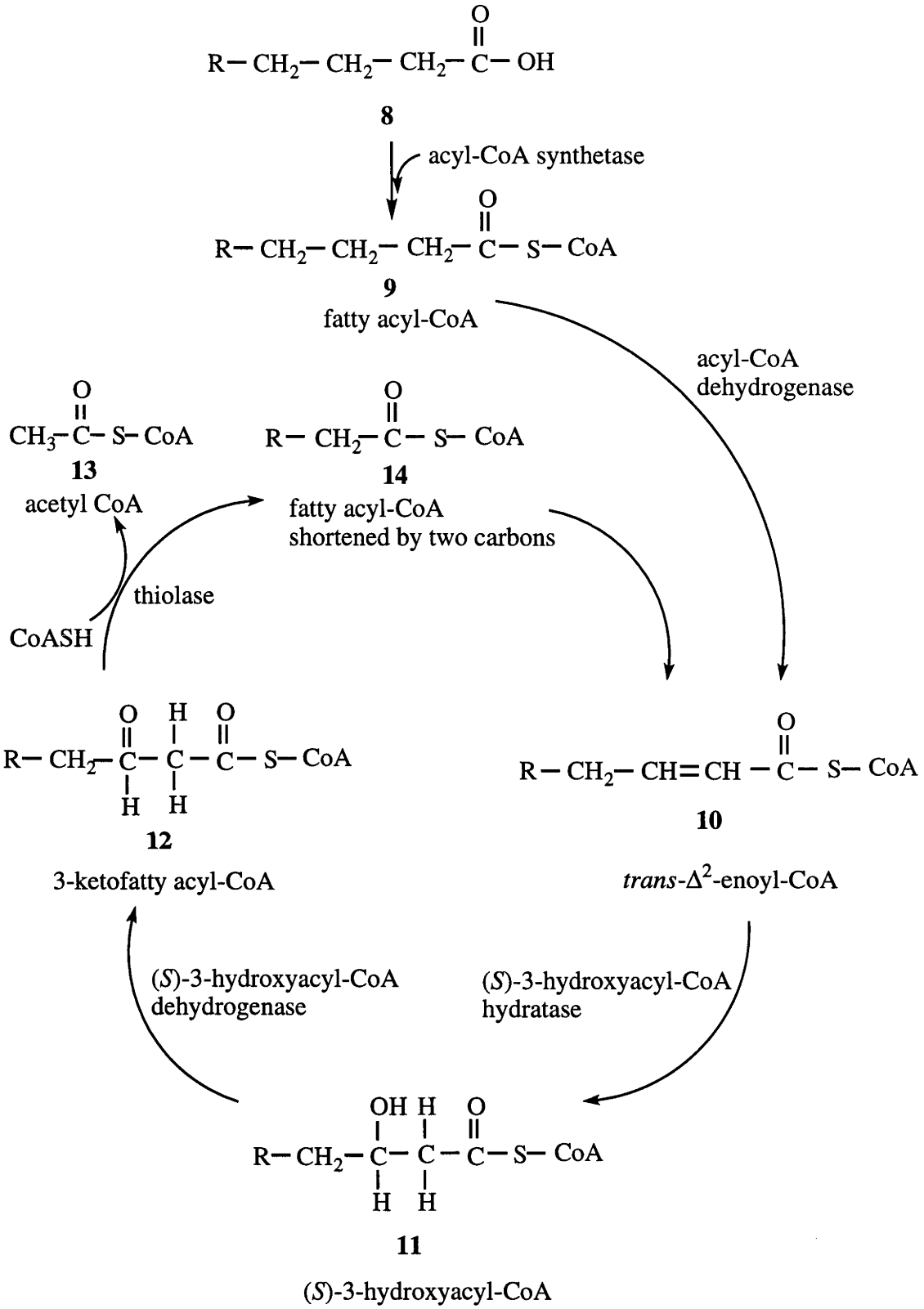


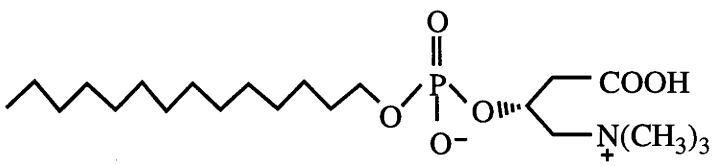
Fig. 1.4: Mitochondrial β-oxidation of fatty acids

Hydration of *trans*-Δ^2-enoyl-CoA **10** is catalysed by (*S*)-3-hydroxyacyl-CoA hydratase

to give (*S*)-3-hydroxyacyl-CoA **11**. The final oxidative step of the β-oxidation cycle is catalysed by NAD^+-dependent (*S*)-3-hydroxyacyl-CoA dehydrogenase, which produces 3-ketoacyl-CoA **12** and NADH. The 3-ketoacyl-CoA **12** is cleaved by thiolase to yield acetyl CoA **13** and a fatty acid acyl-CoA molecule **14** that is two carbons shorter than the fatty acyl-CoA molecule **8** in Step 1.

1.2.1.2. Control of β-oxidation

In mammals, excessive fatty acid oxidation leads to decreased fatty acid levels and is therefore implicated in the pathology of a variety of diseases, such as inherited fatty acid oxidation disorder.[16] Fatty acid oxidation may be inhibited indirectly by reducing the availability of fatty acids or directly by decreasing fatty acid oxidation. The first inhibitor of fatty acid oxidation to be studied was hypoglycin, which is believed to cause hypoglycemia as a consequence of inhibiting β-oxidation *via* an inhibition of butyryl-CoA dehydrogenase.[17] Other known inhibitors of fatty acid oxidation are 2-bromooctanoic acid[18] and 4-bromocrotonic acid.[19] In patients with non-insulin-dependent diabetes mellitus, free fatty acid levels are elevated and contribute to excessive fatty acid oxidation-induced glucose production in the liver. SDZ-CPI-975 **15** potently inhibits fatty acid oxidation by restricting the transport of long-chain fatty acids into the mitochondria, which leads to lower blood glucose and free fatty acid levels in several animal models, accompanied by lowering of β-hydroxybutyrate levels, the end product of β-oxidation.[20]



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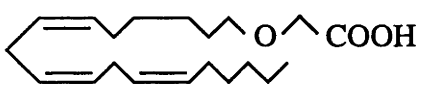
As mentioned earlier, PUFAs play an important role in mammalian systems and the chronic absence of PUFAs such as arachidonic acid **1** and linoleic acid **2** will lead to a

disease known as essential fatty acid deficiency.[2] However, PUFAs are not widely used as therapeutics, because the degradation by β-oxidation leads to their limited availability *in vivo*. To overcome the problem of β-oxidation, some work has been done to design and synthesise modified PUFAs, which are resistant to β-oxidation while retaining the biological activity of naturally occurring PUFAs. A series of polyunsaturated β-oxa, β-thia and γ-thia fatty acids has been prepared,[21,22] of which the structures of five are shown. *In vitro* β-oxidation studies using acyl-CoA oxidase from *arthrobacter* species indicate that (*Z*,*Z*,*Z*)-(octadeca-6,9,12-trienyloxy)acetic acid **16**, (*Z*,*Z*,*Z*)-(octadeca-9,12,15-trienyloxy)acetic acid **17**, (*all-Z*)-(eicosa-5,8,11,14-tetraenylthio)acetic acid **18** and (*all-Z*)-eicosa-5,8,11,14-tetraenylthio)succinic acid **20** do not undergo oxidation, whereas 3-[(*all-Z*)-(eicosa-5,8,11,14-tetraenylthio)]propionic acid **19** is oxidised approximately 40% as efficiently as arachidonic acid **1**.[22,23]



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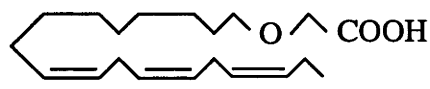

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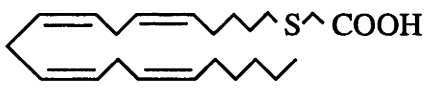

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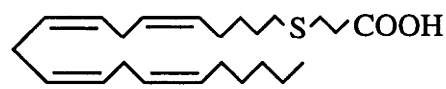

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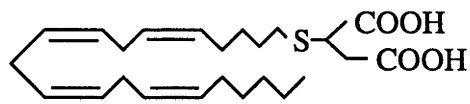

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Other studies showed that 3-tetradecylthiopropionic acid and 3-octylthiopropionic acid can cause powerful inhibition of mitochondrial and peroxisomal β-oxidation although both compounds themselves undergo one cycle of β-oxidation.[24] Thus oxa- and thia-substituted PUFAs may have potential as pharmaceuticals, since they are resistant to degradation through β-oxidation.

1.2.2. Autoxidation and enzyme catalysed oxidation of polyunsaturated fatty acids

Lipid autoxidation is a nonenzymatic oxygenation of both free and esterified PUFAs. The process is a complex one in which molecular oxygen and the lipid react in a free-radical chain sequence to form hydroperoxides.

In mammalian cells, enzyme-catalysed oxidation of PUFAs is one of the major oxidation processes. Arachidonic acid **1**, which is a principal precursor for many important biological mediators, is released from phospholipids[25] and oxidised by a variety of oxygenases. There are three major classes of oxygenases:[26] cyclooxygenases, lipoxygenases, and cytochromes. A cyclooxygenase generates a labile endoperoxide from which prostaglandins and thromboxanes are derived. The various lipoxygenases lead initially to hydroperoxyeicosatetraenoic acids, which in turn can be converted to leukotrienes, lipoxins and hepoxilins. Cytochrome P_{450} can serve as the catalyst for the biotransformation of arachidonic acid **1** to a variety of oxygenated metabolites, including epoxides and a series of fatty acid alcohols.

The physiologically important metabolites from oxidation of PUFAs are collectively known as eicosanoids. These compounds are only produced in nanomolar quantities and are low molecular weight substances, but they possess potent biological properties. Eicosanoids have mild antiatherosclerotic, antithrombotic and antiinflammatory properties.[2] Other functions attributed to eicosanoids include control of transport across membranes, modulation of synaptic transmission and neutrophil chemotaxis.[2]

However, excessive eicosanoid generation through both nonenzymatic[27] and enzymatic[28] lipid oxidation has been implicated in the pathology of a variety of diseases such as bronchial asthma, cardiovascular disease, inflammatory disorders, immunological allergy, aging and cancer. For instance, several indications imply that changes in the course of hypertensive and inflammatory disorders may be achieved by altering the

availability of eicosanoid precursors (*i.e.*, PUFAs).

As well as by altering the precursor availability, the eicosanoid level can be affected by antioxidants, or by inhibitors of the various oxygenases and peroxide-metabolising enzymes.[29,30] Therefore, a subject of continuing interest to researchers is the search for selective inhibitors of the various enzymes in the arachidonic acid cascade and antioxidants to prevent the oxidation of PUFAs.

1.2.2.1. Autoxidation of polyunsaturated fatty acids

i) Mechanism of autoxidation

Lipid peroxidation (autoxidation) leads to the degradation of natural compounds such as rubber and waxes. The process has been of interest to the chemical and biological communities since lipids were first purified and were shown to be reactive with oxygen. The first systematic study of lipid peroxidation was begun in the 1940's by Bateman and Bolland.[31,32] Random autoxidation of PUFAs appears to be an important process *in vivo* as is evidenced by the expiration of pentane and ethane, known fatty acid oxidation products, by organisms under free-radical stress.[33]

The kinetics of autoxidation processes have been studied extensively by Howard and Ingold.[34,35] Autoxidation is a free radical chain process consisting of chain initiation, propagation and termination steps, which can be represented as shown in Scheme 1.1.

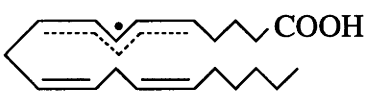
The key event in the initiation of lipid (RH) autoxidation is the formation of a lipid radical R·. This can occur by hydrogen atom abstraction from RH by an initiator free radical. Peroxyl radicals and transition metals such as Fe^{2+}/Fe^{3+} are reactive species which initiate lipid peroxidation.[36] The propagation steps comprise the addition of molecular oxygen to R·, then the rate-limiting step, which is abstraction of hydrogen atom from RH by peroxyl radical ROO· to generate ROOH and another radical R·.[37]

initiation	$In\cdot + RH \rightarrow InH + R\cdot$
propagation:	$R\cdot + O_2 \rightarrow ROO\cdot$
	$ROO\cdot + RH \rightarrow R\cdot + ROOH$
termination	$2ROO\cdot \rightarrow [ROOOOR] \rightarrow$ nonradical products, O_2
	$ROO\cdot + R\cdot \rightarrow ROOR$

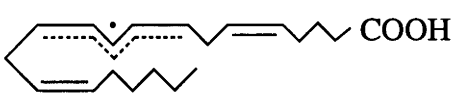
Scheme 1.1

In the past three decades, with wide application of powerful analytical methods such as GC/MS and NMR spectroscopy,[38,39] the products from lipid peroxidation have been well characterised. This has resulted in good understanding of the chemical mechanism of free radical oxidation of unsaturated lipids including monoene (methyl oleate)[39,40], diene (methyl linoleate)[41,42] and polyene (α-linolenic acid **3**) lipids.[36]

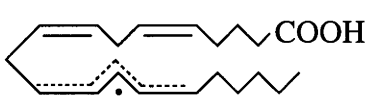
Autoxidation of polyene lipids leads to a much more complex mixture of products than does that of diene lipids. Arachidonic acid **1**, as an example, has three centres, at C-7, C-10, and C-13, each flanked by two double bonds. These are readily attacked by chain-carrying peroxyl radicals. Thus the three pentadienyl radicals **21-23** are formed as intermediates in arachidonic acid **1** autoxidation.[37] These all have *cis,trans,trans,cis* geometry as does the pentadienyl radical formed from autoxidation of linoleic acid **2**.



21



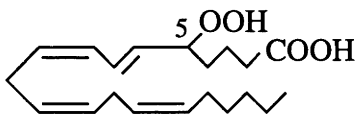
22



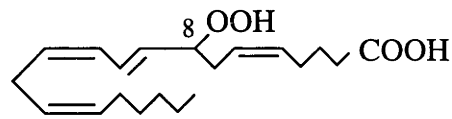
23

Oxygen addition occurs at either end of the three pentadienyl radicals **21-23** giving six

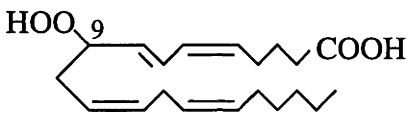
peroxyl free radicals, each having *trans,cis*-diene geometry. Hydrogen atom abstraction by these peroxyl radicals gives the six primary conjugated diene hydroperoxides, hydroperoxyeicosatetraenoic acids (HPETEs). These major HPETE regioisomers **24-29** as shown all have *trans,cis*-diene stereochemistry with hydroperoxide substitution being at carbons 5, 8, 9, 11, 12, or 15.[36] However, the yields of the six products are quite different. For instance, autoxidation of arachidonic acid **1** in benzene or chlorobenzene solution (0.24 M) gives rise to 15-HPETE **29** as the major hydroperoxide (40%). Other hydroperoxides are formed as follows: 5-HPETE **24**, 27%; 8-HPETE **25**, 7%; 9-HPETE **26**, 9%; 11-HPETE **27**, 11% and 12-HPETE **28**, 6%.[43]



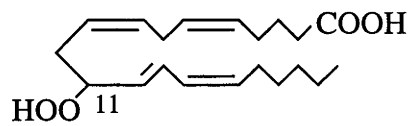
24
5-HPETE



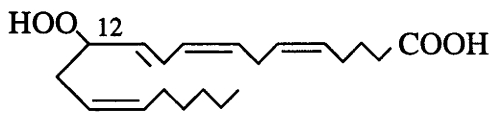
25
8-HPETE



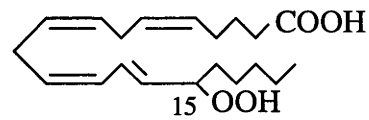
26
9-HPETE



27
11-HPETE



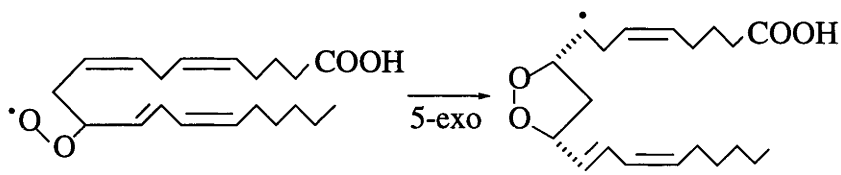
28
12-HPETE



29
15-HPETE

Two factors, selective hydrogen abstraction and peroxyl radical cyclisation, are responsible for this unequal distribution of products.[37,43] In solution, the C-13 hydrogen atom is abstracted more readily than the hydrogens at C-10 or C-7, to give 15-HPETE **29** and 11-HPETE **27**, although the reason is unclear. The peroxyl radicals derived from arachidonic acid **1** that are homoallylic undergo cyclisation reactions which lead to cyclic peroxides.[37] Four of the six peroxyl radicals which have oxygen substitution at C-8, C-9, C-11 or C-12 are homoallylic such that 5-*exo* cyclisations can

occur to give 1,2-dioxolane products. Cyclisation of the 11-peroxyl radical **30** to form compound **31** is shown in Fig. 1.5. The peroxyl radicals substituted at positions C-5 and C-15 are not homoallylic and no 1,5-cyclisation products are observed from these radicals. This may account for the relatively high yields of the 5-HPETE **24** and 15-HPETE **29** in comparison with those of the 8-, 9-, 11- and 12-HPETE (**25-28**). In addition, Porter *et al.*[44] noted that only *exo* cyclisation occurred and *endo* cyclisation is highly disfavoured.



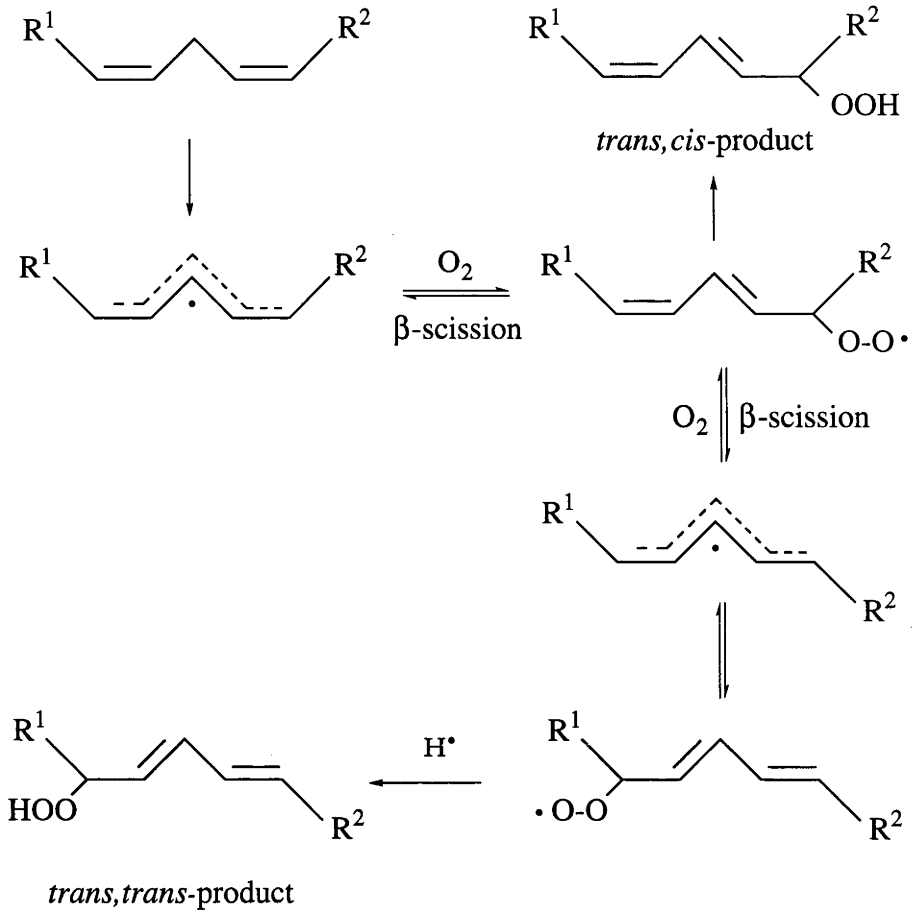
30 **31**

Fig. 1.5: An example of 5-*exo* cyclisation, of the 11-peroxyl radical **30** of arachidonic acid **1**

Apart from the dominant *trans,cis*-hydroperoxide products, relatively small amounts of *trans,trans*-hydroperoxides are also formed in arachidonic acid **1** autoxidation.[45] This is through reversible oxygen addition to the intermediate pentadienyl radicals. The β-scission reaction (Fig. 1.6) is a slow reaction[36] compared with 1,5-cyclisation of the homoallylic peroxyl radicals and the formation of *trans,cis*-hydroperoxides.

ii) Antioxidants and their mechanism of action

Antioxidants can delay or inhibit lipid peroxidation. Modes by which they act include: 1) decreasing localised O_2 concentration (*e.g.*, by sealing foodstuffs under nitrogen); 2) preventing free radical chain initiation by scavenging initiating radicals such as HO·; 3) binding metal ions in forms that will not generate such initiating species as HO· or $Fe^{2+}/Fe^{3+}/O_2$ and/or will not decompose lipid peroxides to peroxyl or alkoxyl radicals;



$R^1 = (CH_2CH{=}CH)_2(CH_2)_3COOH$

$R^2 = C_5H_{11}$

Fig. 1.6: Formation of *trans,cis-* and *trans,trans*-products in arachidonic acid **1** autoxidation

4) decomposing peroxides by converting them to non-radical products, such as alcohols; and

5) free radical chain breaking, *i.e.*, scavenging intermediate radicals such as peroxyl and alkoxyl radicals to prevent continued hydrogen abstraction. Chain-breaking antioxidants are often phenols or aromatic amines.

It is well known that vitamin E (α-, β-, γ- and δ-tocopherol) inhibits lipid peroxidation in

food and biological systems. Since tocopherols are only synthesised by plants, they are very important dietary nutrients for humans and animals. The mechanism involved has been studied extensively by several investigators.[46-48]

Tocopherols act as effective antioxidants and can terminate a free radical chain reaction by donating a hydrogen atom to chain-propagating peroxyl radicals (ROO·) (Scheme 1.2).[49]

$$ROO\cdot + TocH \rightarrow ROOH + Toc\cdot$$

$$ROO\cdot + Toc\cdot \rightarrow \text{inactive products}$$

Scheme 1.2

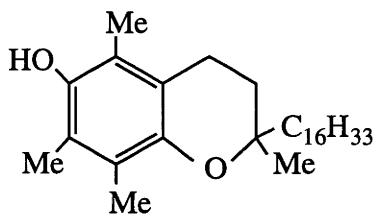
This results in the formation of hydroperoxides and relatively stable tocopheroxyl radicals (Toc·). Tocopheroxyl radicals are consumed by reaction with peroxyl radicals or with each other to give dimers. Intervention of tocopherols not only slows the autoxidation of PUFAs, but also alters the yields of hydroperoxide regioisomers and geometric isomers. When Porter *et al.*[45] proposed an autoxidation mechanism for linoleic acid **2** involving β-scission of intermediate peroxyl radicals, they demonstrated that the concentration of added α-tocopherol, as an effective antioxidant, affects the distribution of *trans,cis*- and *trans,trans*-hydroperoxide product isomers. Addition of α-tocopherol **32** (below 0.4 M) leads to an increased *trans,cis-/trans,trans*-peroxide product ratio, for example, from 0.23 in the absence of α-tocopherol **32** to 25 with as little as 0.25 M α-tocopherol **32**.[45] The reason for this is that the competition between hydrogen-atom abstraction by and β-scission of peroxyl radicals determines the *trans,cis-/trans,trans*-product ratio. α-Tocopherol **32** transfers hydrogen atom to intermediate peroxyl radicals with rates of the order of 10^5-10^6 M^{-1} s^{-1}, which is about four orders of magnitude faster than the normal propagation rates for atom transfer from hydrocarbons to peroxyl radicals. Thus, the initial peroxyl radicals are more efficiently converted to *trans,cis*-products in the presence of α-tocopherol **32** and the slow β-scission reaction (140 s^{-1}) is even more disfavoured

than in the absence of the antioxidant.[36]

However, it has been shown that α-tocopherol **32** at high concentrations acts as a prooxidant during lipid peroxidation.[46-48] The prooxidant effect of α-tocopherol **32** leads to an increase of hydroperoxides with a conjugated diene structure. Loury *et al.*,[50] and Terao and Matsushita[51] proposed that the α-tocopheroxyl radical (α-Toc·) participates in this prooxidant effect through the following mechanism (Scheme 1.3).

$$\alpha\text{–Toc}\cdot + RH \rightarrow \alpha\text{–TocH} + R\cdot$$

$$\alpha\text{–Toc}\cdot + ROOH \rightarrow \alpha\text{–TocH} + ROO\cdot$$

α-TocH = 

32

Scheme 1.3

α-Toc· reacts reversibly with unperoxised lipids (RH) and with lipid hydroperoxides (ROOH) by radical chain-transfer generating alkyl and peroxyl radicals,[49] respectively. The production of peroxyl radicals results in α-tocopherol **32**-induced hydroperoxidation and the regeneration of α-tocopherol **32**.

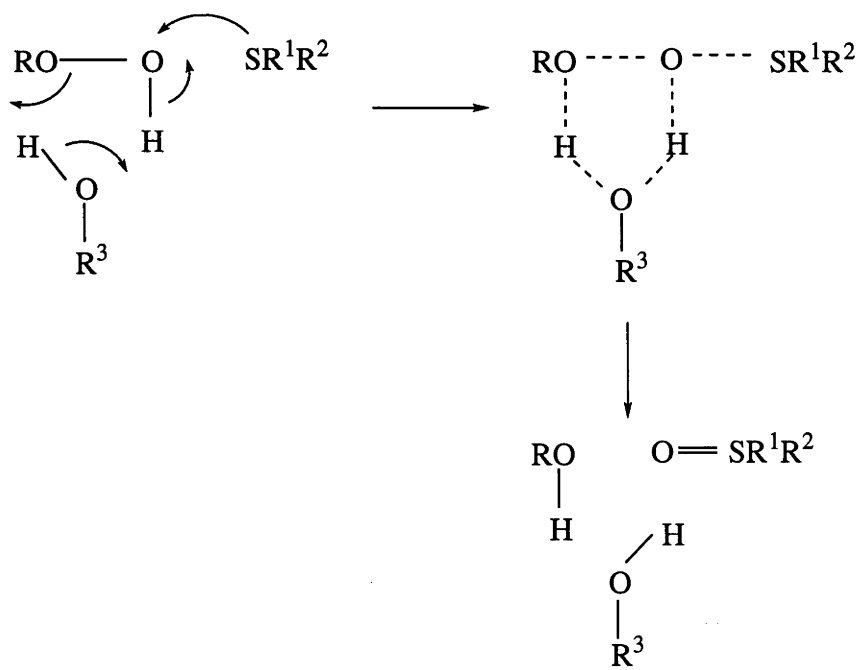
It has been reported that some monosulfides are effective inhibitors of autoxidation of hydrocarbons through destruction of hydroperoxides, which normally function as initiators of the free radical oxidation chain process. The interaction of hydroperoxides with monosulfides is represented in Scheme 1.4.

$$R_2S + ROOH \rightarrow R_2SO + ROH$$

Scheme 1.4

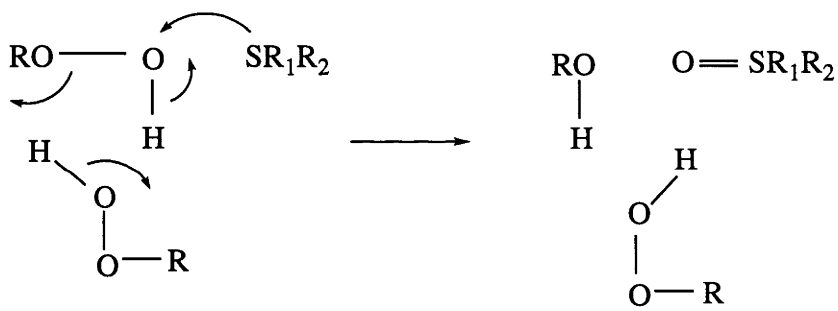
However, the reaction of sulfides with hydroperoxides is more complex. Bateman and Hargrave[52] proposed two mechanisms based on the reaction of saturated sulfides and *t*-butyl hydroperoxide in hydrocarbon solvents and alcoholic solvents, respectively.

In the reaction of cyclohexyl methyl sulfide with *tert*-butyl hydroperoxide in alcoholic solutions, the major features of reaction are: (i) it is first order with respect to both the sulfide and hydroperoxide; (ii) a specific oxygen is transferred; (iii) it is sensitive to the nature of the alcohol solvent; (iv) there is no detectable free radical character; and (v) there is a large negative entropy of activation ($\Delta S^{\ddagger}$) term. Thus, to fulfil these conditions satisfactorily, a mechanism for the sulfide to sulfoxide conversion has been proposed as shown in Scheme 1.5. Here R^3OH represents the solvent molecule. Formation of a cyclic complex between the hydroperoxide and a hydrogen-donating molecule (solvent molecule) precedes reaction with the sulfide. Oxygen transfer to the sulfide is promoted by a synchronous hydrogen exchange process within the complex.



Scheme 1.5

However, the reaction of cyclohexyl methyl sulfide with *tert*-butyl hydroperoxide in hydrocarbons is quite different from that in alcoholic solvents based on the kinetic characteristics and the complexities of the hydrocarbon case, although the sulfoxide yield is quantitative in both cases. For instance, Bateman and Hargrave[53] found that the reaction with hydroperoxide in benzene or cyclohexane is insensitive to the presence of oxygen, there is no evidence of free radical character, and the reaction has orders of one with respect to sulfide and two with respect to hydroperoxide. They proposed a mechanism as shown in Scheme 1.6. The reaction may also be considered as a bimolecular reaction between the hydroperoxide dimer and the sulfide. The actual oxidant is a bimolecular hydroperoxide complex. The experimental data also indicate that acetic acid catalyses the reaction and changes the hydroperoxide order from 2 to 1, suggesting that acetic acid may act in the same way as alcohols.



Scheme 1.6

The reactions of allylic sulfides with several organic hydroperoxides have been investigated.[54] The results show that the yields of sulfoxides are always less than theoretical. The non-sulfoxide products from the reactions of allylic sulfides such as 1-methylbut-2-enyl butyl sulfide with *tert*-butyl hydroperoxide in benzene appear to arise from C-S bond cleavage and consist of dibutyl disulfide and a complex mixture of oxygenated, sulfur-free substances, including alcohols, ethers, peroxides, water, and some polymeric materials.

Some investigators[55] have reported that sulfides show antioxidant effect only after a small amount of oxygen has been absorbed, implying that it is one or more of the oxygenated derivatives instead of the sulfides which functions to inhibt oxidation. These derivatives could be sulfoxides, sulfones, sulfinates and sulfonates.[56] Some evidence has been presented that sulfonates and sulfoxides are more active as inhibitors than sulfides. However, their action is more short-lived and, therefore, sulfides may still provide a reservoir from which more active inhibitors can be generated *in situ.*

1.2.2.2. Lipoxygenase catalysed oxidation of polyunsaturated fatty acids

i) Lipoxygenases and lipoxygenase pathways

Lipoxygenases are widely distributed throughout the plant and animal kingdoms and play a central role in PUFA metabolism.

In the mammalian system, the predominant substrate for these enzymes is arachidonic acid **1**, and lipoxygenases are classified according to their positional specificity with respect to reaction of arachidonic acid **1**. The main enzymes are 5-lipoxygenase (5-LO), 12-lipoxygenase (12-LO) and 15-lipoxygenase (15-LO).[2] The three lipoxygenase pathways are outlined in Fig. 1.7. These lead first to the generation of hydroperoxides and then to the biosynthesis of eicosanoids, such as leukotrienes (*e.g.*, LTA_4, LTB_4 and LTC_4) in the 5-LO pathway and lipoxins (LXA_4 and LXB_4) in the 15-LO pathway.[2]

In plants, the primary substrates are linoleic acid **2** and linolenic acids **3**, and the biosynthetic products have both growth promotion and pest resistance properties.[57] In addition, the initial hydroperoxide products undergo a variety of lyase-promoted cleavage and rearrangement reactions to form short-chain alcohols, vinyl ethers, aldehydes, and oxo acids.[58] The most studied of these is jasmonic acid, a cyclopentanoic acid derived from the 13-hydroperoxide of linoleic acid **2**. Jasmonic acid has been shown to induce growth inhibition, senescence and gene expression leading to production of defensive

proteins such as proteinase-inhibitors.[59]

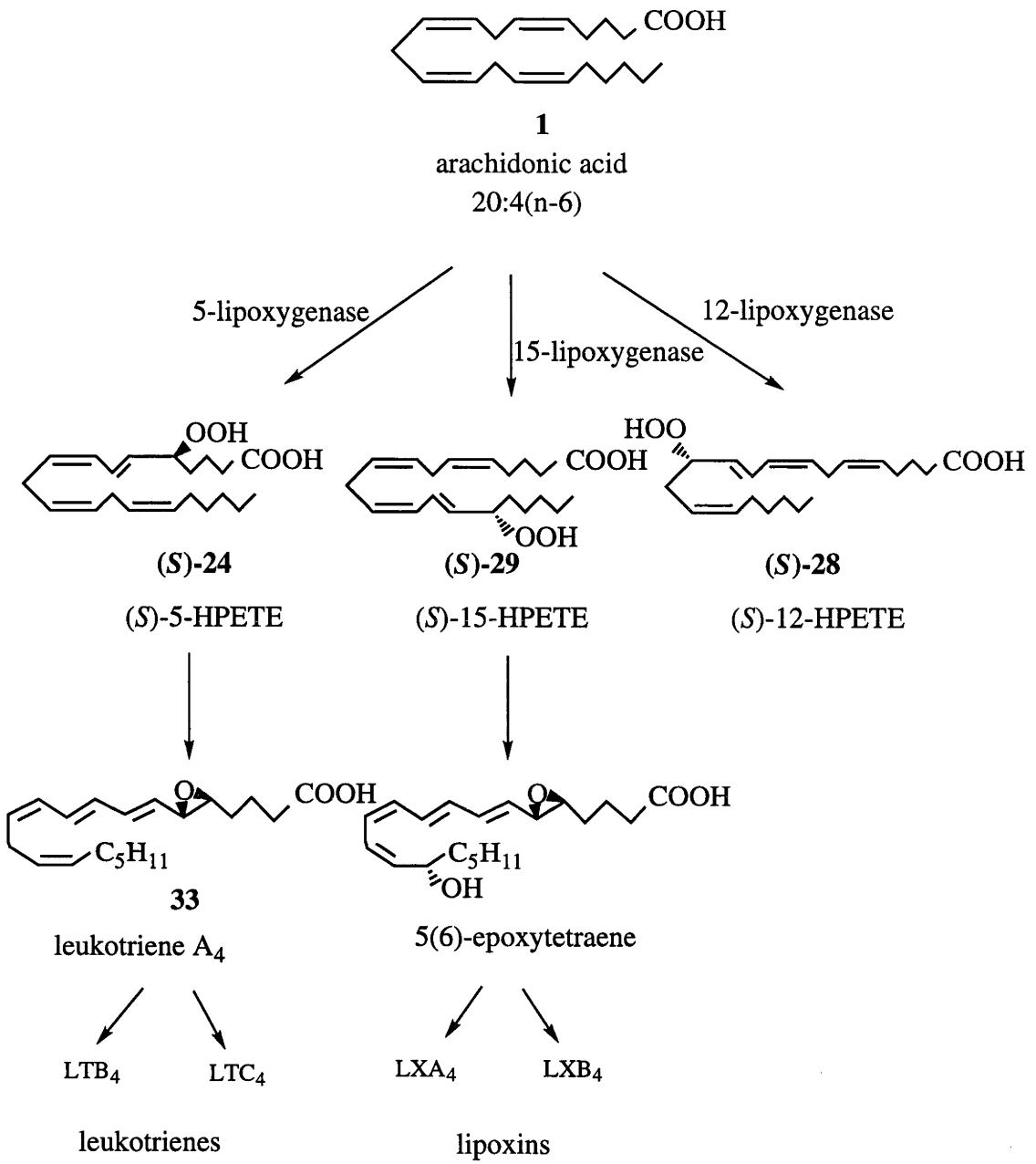


Fig. 1.7: Lipoxygenase pathways for oxidation of arachidonic acid **1**

Plant lipoxygenases have been known for over 50 years and several have been purified. For instance, 15-LO from soybean has been available in a highly purified state since 1947.[60] However, the structure and mechanism of action of lipoxygenases has only been elucidated more recently. The first complete cDNA sequence, pure crystalline enzyme and three-dimensional structure, and mechanistic studies involved the soybean

15-LO enzyme.[61-63]

The first mammalian enzyme, the 12-LO from human platelets, was described in 1974.[64] With the discovery of leukotrienes in 1979,[65,66] emphasis shifted toward the study of mammalian lipoxygenases. The 5-LO pathway has been the major focus of study due to the pronounced pro-inflammatory role of the leukotrienes. Because leukotrienes have been implicated in a variety of diseases including asthma, arthritis and allergy, inhibition of 5-LO is a promising therapeutic target for the development of new and more effective treatments for these conditions.

ii) Catalytic mechanism of lipoxygenases

Lipoxygenases are dioxygenase enzymes. The enzymes, whether of plant or animal origin, catalyse the stereospecific insertion of molecular oxygen into PUFAs containing an unconjugated (*Z*,*Z*)-1,4-pentadiene moiety. All mammalian lipoxygenases isolated so far have proven to be (*S*)-lipoxygenases.[2] They selectively add a single oxygen molecule to the pro-*S* face of the diallyl radical formed by hydrogen atom abstraction from the unsaturated fatty acid substrate, and the products formed are optically active (*S*)-hydroperoxides. Lipoxygenases with (*R*)-specificity are known from lower animal phyla including the 12(*R*)-LO from echinoderms.[67] The first purification, partial sequencing and cloning of an (*R*)-lipoxygenase from the gorgonian *p. homomalla* was completed in 1996.[68] Although (*R*)-lipoxygenases have not yet been isolated from higher animals, (*R*)-hydroxyeicosatetraenoic acid (HETE) metabolites have been widely observed in tissues like cornea and skin and these could arise from the action of this type of enzyme.[69,70]

The catalytic activity of lipoxygenases of both plant and animal origin depends on the presence of a unique non-heme, non-sulfur iron cofactor.[71] In a typical lipoxygenase catalysed reaction, the time course with the native enzyme is characterised by a rate increase immediately after the start, generally referred to as the induction or lag period. It

has been noted that soybean seed 15-LO L-1 (one of the isozymes of 15-LO in soybean seed) is activated by its product, *i.e.*, (*S*)-13-hydroperoxy-9,11-*cis*,*trans*-octadienoic acid, when its substrate is linoleic acid **2**.[72] Later it was shown that the active enzyme exhibits a strong electron paramagnetic resonance (EPR) signal, in contrast to the resting enzyme which is EPR-silent under the same conditions.[73,74] This change is attributed to a conversion of Fe^{2+} to Fe^{3+} in the active enzyme.

Kinetic mechanisms for lipoxygenase activity fall into two classes, which are the multiple-site model and the two-step model. In the multiple-site model, Fe^{2+} and Fe^{3+} lipoxygenases are both catalytically active, although not necessarily to the same extent.[75-77] The fatty acid substrate and product bind not only to a catalytic site but also to one or more regulatory binding sites. All multiple-site models have been proposed specifically to explain the occurrence of the initial rate increase.

In the two-step model,[78-80] the resting enzyme is activated when a trace of lipid hydroperoxide oxidises the Fe^{2+} to Fe^{3+}. The Fe^{3+} lipoxygenase can then catalyse the first step of the reaction, oxidising the central methylene group in the pentadiene region of the fatty acid, causing the release of a proton and leaving an intermediate with an unpaired electron. In the second step, the resulting free radical undergoes addition of dioxygen to form a peroxyl radical. Electron pairing is then accomplished by reduction of the radical intermediate to the peroxide anion by the Fe^{2+} which is thus restored to the Fe^{3+} form, completing the iron cycle as shown in Fig. 1.8. In this model, the substrate and product compete for a single binding site on the lipoxygenases.

Schilstra *et al.*[81] reported a detailed investigation into the relationship between the dioxygenation rate and the ratio of Fe^{2+} to Fe^{3+} lipoxygenase and provided strong evidence to support the two-step model, particularly the hypothesis that only Fe^{3+} lipoxygenase can catalyse the hydrogen abstraction step and thus be regarded as the active enzyme species. Therefore, the two-step model for lipoxygenase catalysis appears to be the more reasonable mechanism.

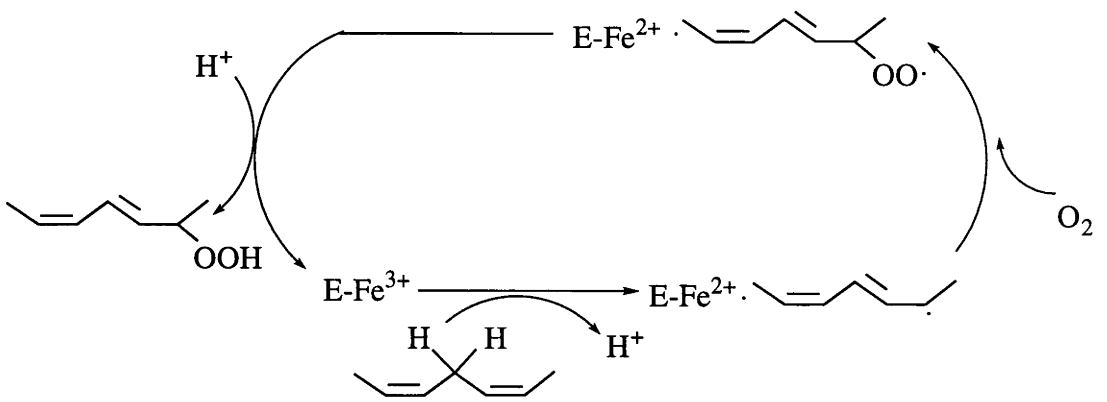


Fig. 1.8: Cycling between Fe^{2+} and Fe^{3+} in the two-step model of lipoxygenase activity

Because of the important role of iron in the mechanism of lipoxygenase catalysis, the characterisation of the non-heme iron binding site has become a high priority. When the three major isozymes of soybean seed 15-LO, L-1, L-2 and L-3, were first sequenced in 1987-1988,[82-84] it was found that the lipoxygenases share a highly conserved region of 38 amino acids, with 5 being histidine residues, suggesting this region as a putative iron binding site.[63] A sixth conserved histidine occurs about 190 residues closer to the carboxyl terminus. During the following 4-5 years, sequences of numerous lipoxygenases from both higher plants and mammalian species became available,[85-87] which all contain a well-conserved version of the 38-residue region and have retained all of the conserved histidines. The first three-dimensional structure of a lipoxygenase, soybean 15-LO L-1, was reported in 1993,[88] and the iron was found to be five-coordinate in the shape of an octahedron with one coordination site vacant. The ligands were identified as His^{499}, His^{504}, His^{690}, Asn^{694}, and the C-terminal Ile^{839}.[89] The role of Asn^{713} in iron atom incorporation and catalysis in soybean lipoxygenase L-3 was also investigated using site-directed mutagenesis and the result showed that residue Asn^{713} in lipoxygenase L-3 corresponds to Asn^{694} in the sequence of lipoxygenase L-1.[90]

Lipoxygenases in general require in substrates a methylene-interrupted diene system of

cis double bonds, and the products are conjugated diene hydroperoxides. Soybean 15-LO has a broad substrate specificity.[50] The enzyme is active with numerous unsaturated C18, C20 and C22 fatty acids. Oxygenation sites for the soybean 15-LO have been shown to be at C-13 of α-linolenic acid **3** and at C-15 of arachidonic acid **1**.[92] Mammalian 15-LOs closely resemble the soybean 15-LO. The rabbit reticulocyte 15-LO shows a broad substrate specificity in terms of the carbon chain length.[93] In contrast, the 5-LO from rat and guinea pig reacts predominantly with C20 fatty acids.[94]

Hamberg and Samuelsson[92] found that the most extensively studied form of the enzyme, 15-LO L-1 from soybean, shows a strong preference for oxygenation of the substrate (*e.g.*, linoleic acid **2** and arachidonic acid **1**) at the n-6 position when the reaction is carried out at pH 9. Increasing the number of carbons in the hydrophobic substituent of the fatty acid drastically reduces the rate of oxygenation. Studies on series of synthetic diene and triene substrates have confirmed these earlier findings.[95] Thus the terminal structure of the substrates is critical to the specificity of the lipoxygenases.

Arachidonate 12-LO is found in platelets, leukocytes and other tissues of various animal species. The enzyme has two isoforms, platelet- and leukocyte-types,[96] which can be distinguished by substrate specificities and primary structures. The human platelet 12-LO is an arachidonic acid **1**-specific lipoxygenase with positional selectivity for C-12. The only other substrate fatty acid for this enzyme is (*all-Z*)-eicosapentaenoic acid **7**. The enzyme is essentially inactive toward linoleic acid **2**.[97] In contrast, the porcine leukocyte 12-LO has a broader substrate specificity reacting with C18 and C22 unsaturated fatty acids as fast as with arachidonic acid **1**.[98]

Amino acids of the lipoxygenases which are responsible for the positional specificity have been investigated. Sloane *et al.*,[99] using amino acid sequence alignment and site-directed mutagenesis, identified amino acid residues at positions 417 and 418 in the human platelet 12-LO and human reticulocyte 15-LO as important features of the binding site for the methyl end of the fatty acid substrate. The atomic resolution structure of soybean 15-LO

L-1[88] reveals a solvent-accessible cavity with the catalytic domain in the protein lined with residues Thr^{556} and Phe^{557}.

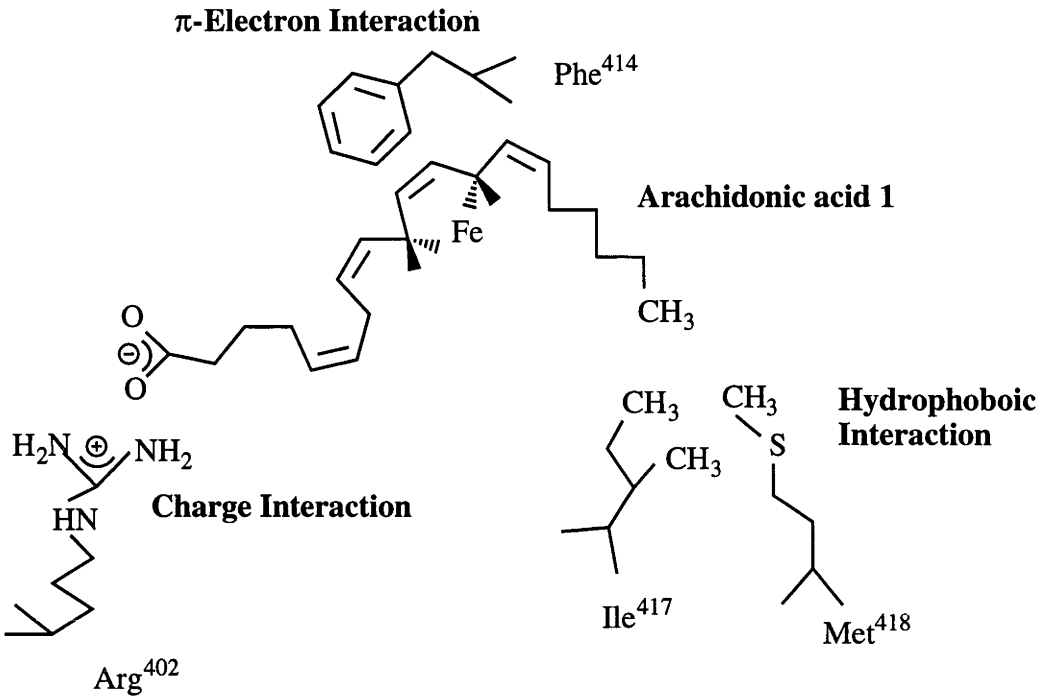


Fig. 1.9: Diagram of the proposed substrate binding determinants of human 15-lipoxygenase[101]

Since the structure of soybean 15-LO L-1 was solved in the absence of any bound substrate or inhibitor,[61] it is difficult to assign functional relevance to particular structural features. In order to define the arachidonic acid **1** binding site of human 15-LO, Gan *et al.*[101] developed a possible docking mode for arachidonic acid **1**. They postulated that there may be an interaction of a positively charged residue in the binding site with the carboxyl group of arachidonic acid **1** and a π-electron interaction between an aromatic residue and the *cis* double bonds of the substrate. In their working model, the methyl end of arachidonic acid **1** was docked near the narrow neck of the inner cavity defined by Thr^{556} and Phe^{557} in soybean 15-LO L-1. The carboxyl group of arachidonic acid **1** was docked near Lys^{260}, as this orientation of the substrate allows the reactive 1,4-*cis*,*cis*-pentadiene moiety to be very close to the catalytic iron. Validation of this model is provided by site-directed mutagenesis of human 15-LO, despite the low amino

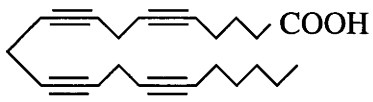
acid sequence identity between the soybean and mammalian enzymes. Both a positively charged amino acid residue (Arg^{402}) and an aromatic amino acid residue (Phe^{414}) in human 15-LO, corresponding to Lys^{260} and Trp^{500} in soybean 15-LO L-1, respectively, were identified as critical for the binding of fatty acid substrates in human 15-LO as shown in Fig. 1.9.

iii) Inhibitors of lipoxygenases

As mentioned earlier, mammalian lipoxygenases have attracted considerable attention as targets of drug design because of their role in inflammatory response. The mammalian 5-LO pathway has been the major focus of study due to the pronounced pro-inflammatory role of the leukotrienes (*i.e.*, LTB_4, LTC_4, LTD_4, LTE_4).[101] Efficient inhibitors of 5-LO have therapeutic potential in the treatment of inflammatory and allergic diseases.[102]

Inhibitors of lipoxygenases exert their effects by preventing or reducing the formation of lipoxygenase products. Synthetic lipoxygenase inhibitors include analogues of the natural substrates (*e.g.*, arachidonic acid **1**) and products, and transition-state analogues.

Substrate analogues enzyme inhibitors have been designed based on knowledge of the enzyme's mechanism of action and substrate specificity. The simplest modification of arachidonic acid **1** involved changing its level of unsaturation (*e.g.*, by substitution of one or more of the double bonds for triple bonds). Lipoxygenases and cyclooxygenases share arachidonic acid **1** as a substrate and involve similar mechanisms. Not surprisingly, therefore, some inhibitors show poor selectivity for lipoxygenases over cyclooxygenases, as is the case with compound **34**, which is an inhibitor of cyclooxygenases and mammalian 12-LO and 5-LO.[103-105] However, 4,5-dehydroarachidonic acid **35** is a selective potent inhibitor of only 5-LO. In this case, inhibition is irreversible and time dependent.[105,106]



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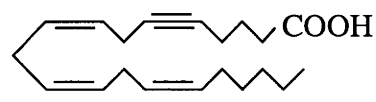


35

A number of analogues of compound **35** and arachidonic acid **1** in which the carboxyl group is replaced by various nonanionic groups, *e.g.*, carboxamide, were also found to be potent inactivators of 5-LO from rat basophilic leukemia (RBL-1) cells.[107] Based on these results and the key role of iron in catalysis by lipoxygenases, several amide analogues of arachidonic acid **1**, such as *N*-hydroxyarachidonamides,[107,108] were prepared and found to be powerful inhibitors of lipoxygenases. The *N*-hydroxyamides are known to be excellent ligands for Fe^{3+} as will be described later.

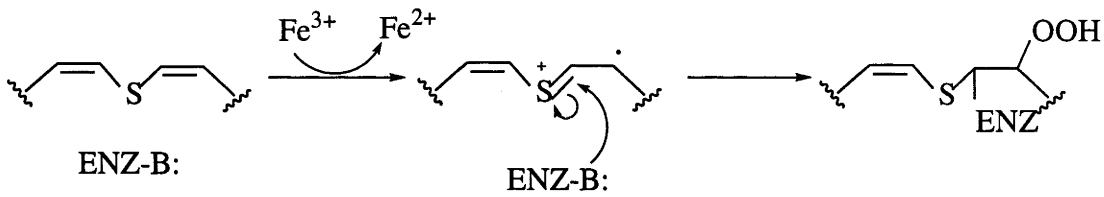
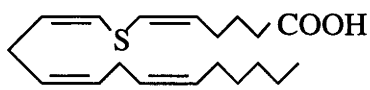


Fig. 1.10: Lipoxygenase inhibition by thiaarachidonic acids



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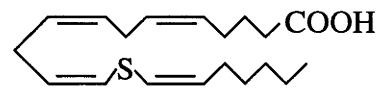

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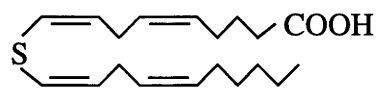

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Fig. 1.11: Examples of thiaarachidonic acids

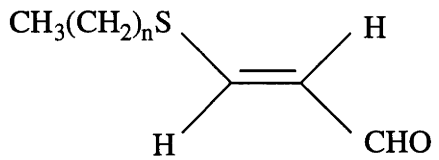
Another type of substrate analogue is represented by the thiaarachidonic acids[108,109] in which the methylene which loses hydrogen upon lipoxygenation is replaced by a sulfur. These compounds are mechanism-based enzyme inactivators (Fig. 1.10). Examples of such compounds are shown in Fig. 1.11. 7-Thiaarachidonic acid **36** inhibits 5-LO from RBL-1 cells but not cyclooxygenases, while 13-thiaarachidonic acid **37** inhibits soybean

15-LO and 10-thiaarachidonic acid **38** behaves as a good substrate for this lipoxygenase. The thiohydroxamate of arachidonic acid **1** was also prepared and found to inhibit 5-LO.[102] Interestingly, incorporation of a hydroxamate moiety into compound **36** converts it from a non-competitive irreversible inhibitor into a competitive inhibitor.

Product analogue enzyme inhibitors have been designed based on those products which are reported to inhibit the lipoxygenases. As discussed above, arachidonic acid **1** is oxidised by lipoxygenases to form hydroperoxyeicosatetraenoic acids (HPETEs) in the first step. Lipoxygenases may also catalyse the further metabolism of HPETEs to hydroxy (HETEs) and epoxy (leukotriene A_4, **33**) acids. It has been reported[110] that 15-HETE is an effective inhibitor of 5-LO in rabbit leukocytes and 12-LO in rabbit platelet, whereas similar inhibitory potencies were observed for 5-HETE and 12-HETE acting on the 15-LO in rabbit leukocytes. Leukotriene A_4 **33** and 5-HPETE **24** are reported to be inhibitors of 5-LO. Kishimoto *et al.*[96] found that 15-HPETE **29** leads to suicide inactivation of the leukocyte-type 12-LO because it is transformed to 14,15-leukotriene A4, which then becomes covalently bound to the enzyme. Analogues of 15-HETE, such as the corresponding acetate and ketone and methyl ester derivatives, have been prepared[110] and show comparable activity to 15-HETE. Kerdesky *et al.*[111] also designed and synthesised a series of 5-substituted eicosanoid analogues and evaluated them *in vitro* for inhibitory activity against RBL-1 5-LO. The results showed that compounds containing the hydroxamic acid functionality exhibited potent inhibitory activity (IC_{50} = 0.19-2.8 μM). The most potent inhibitor was 5-[[(hydroxyamino)-carbonyl]methyl]-6,8,11,14-eicosatetraenoic acid, which was 10 times more active than the C-1 hydroxamates of arachidonic acid **1** or 5-HETE. The plausible mechanism of inhibition involves chelation of functional groups to the iron moiety in the 5-LO active site.

Competitive inhibition of soybean 15-LO L-1 has been reported for relatively few compounds. Straight chain alcohols with even numbers of carbons (C4 - C12) have been found to be competitive inhibitors of the enzyme with K_i values in the range of 0.1-50

mM.[112] It was concluded that the alcohols inhibit the enzyme by binding the aliphatic substituent of the inhibitor to the enzyme active site through hydrophobic interactions. Zhu *et al.*[113] also prepared a series of aldehydes **39** containing sulfur and led to the discovery of another class of competitive inhibitors.



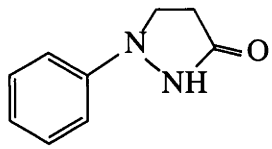
n = 2 - 7

39

Other diverse compounds have been prepared with the goal of developing lipoxygenase inhibitors. The structures of these compounds vary widely, but they may be classified as either redox inhibitors, iron ligands or non-redox inhibitors according to the mechanism of enzyme inhibition.

Redox inhibitors reduce the active site iron of the enzyme to the ferrous form (Fe^{2+}), or reduce one of the radical intermediates, and keep the enzyme in its inactive state. Examples of redox inhibitors are phenidone **40** and BW-755C **41**.[114] However, redox inhibitors generally show poor selectivity for lipoxygenases because they also interact with other biological redox systems. In addition, despite displaying potent activity *in vitro*, redox inhibitors are often weak or inactive enzyme inhibitors *in vivo* when dosed orally.

Iron ligands have been prepared as a class of drugs that inhibit leukotriene synthesis by chelating the iron in the catalytic centre of lipoxygenases. Most of the compounds of this class are hydroxamic acid or *N*-hydroxyurea derivatives.[115] For example, Corey *et al.*[107] described the *in vitro* 5-LO inhibitory properties of arachidonyl hydroxamic acid and its *N*-alkyl derivatives. As with the redox inhibitors, achieving effective enzyme inhibition *in vivo* has been a major challenge with this class of compounds.



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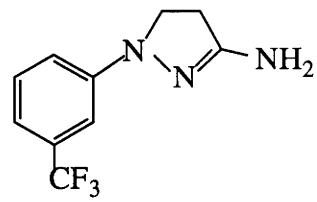

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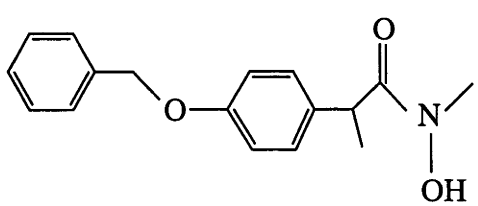
Two distinct types of hydroxamate inhibitors have been investigated, represented by compounds **42** and **43**.[114] Although both types show potent *in vitro* activity, and are well absorbed, those typified by compound **42** undergo rapid metabolism to the corresponding inactive carboxylic acids, and therefore show short half-lives and low *in vivo* potency. In contrast, those typified by compound **43** do not undergo such metabolism, show longer half-lives and are potent inhibitors *in vivo*. Further development of compounds of these classes led to the discovery of the *N*-hydroxyurea zileuton **44**,[116] which is approximately 15 times more potent against 5-LO than against cyclooxygenases and does not inhibit either 12- or 15-LO. Zileuton **44** is an orally active inhibitor of 5-LO in rats and dogs and is orally effective at inhibiting leukotriene-dependent inflammation in mice and inflammatory cell influx in rats.

In parallel, Jackson *et al.*[117] identified a series of aryl hydroxamic acids, including BWA4C **45** as lipoxygenase inhibitors. Compound **45** exhibits approximately 20-fold selectivity for 5-LO compared to cyclooxygenases and is an orally active inhibitor. As with redox inhibitors, structure-activity relationships of compounds of this type are dominated by lipophilicity and there is no direct evidence that these compounds interact with the enzyme in a specific way.



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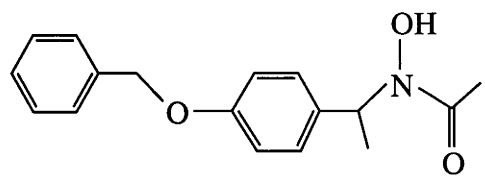

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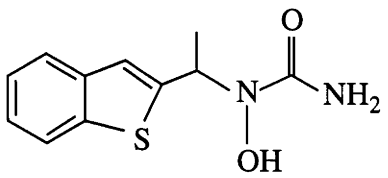

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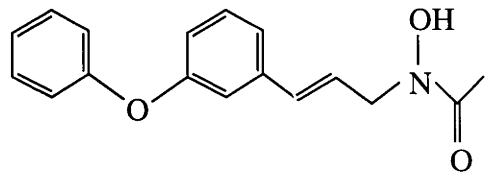

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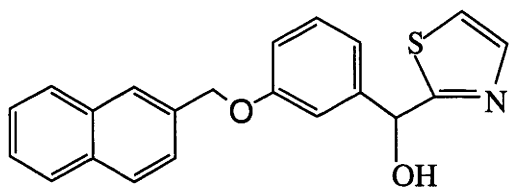
Owing to the fact that many redox inhibitors and iron ligands lack high levels of potency and selectivity, specific and highly potent non-redox 5-LO inhibitors have been developed based on a simple hypothetical active-site model. These compounds, such as the hydroxyalkylthiazole **46** and its derivatives, inhibit 5-LO effectively *in vitro*.[118,119] For example, ICI211965 **47** is a potent inhibitor of 5-LO *in vitro* (IC_{50} = 8 nM in isolated macrophages) and does not inhibit cyclooxygenases.[118,120]



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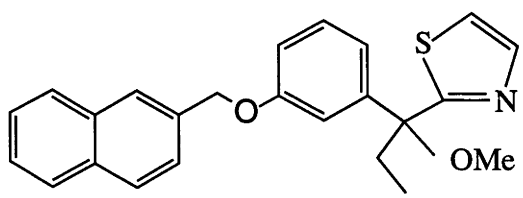

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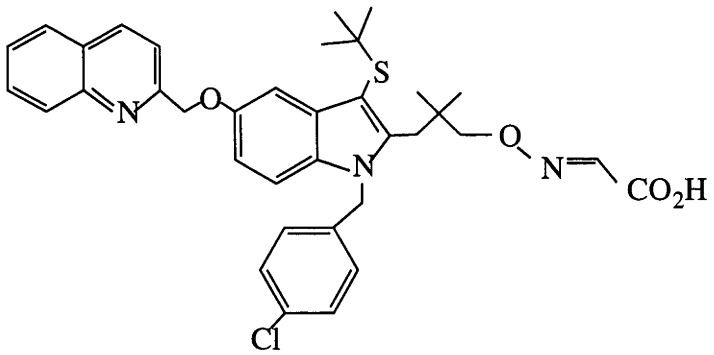
Since it was found that 5-LO undergoes activation through an interaction with a 5-LO activating protein (FLAP) in a nuclear membrane site,[121] a novel class of leukotriene biosynthesis inhibitors, exemplified by MK0886,[122] which can bind to FLAP with high affinity, have been developed.[123,124] Representative examples are A-86885 **48** and A-86886 **49** with *in vitro* IC_{50}s of 21 and 9 nM and *in vivo* rat oral ED_{50}s of 0.9 and 1.7 mg/kg, respectively, for leukotriene biosynthesis inhibition.[125]



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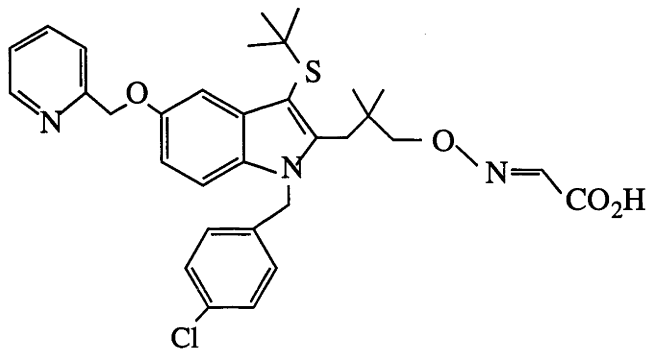

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1.3. Aims of this project

The main aim of this project was to design and synthesise analogues of PUFAs, and to assess their biological activity in order to identify compounds which have potential pharmaceutical value.

The main target compounds were nitro analogues of PUFAs, which include polyunsaturated nitroalkanes, and nitro-substituted PUFAs. Nitroalkanes were selected as targets for several reasons. The nitro group resembles the carboxyl group with regard to size, shape and polarity (Fig. 1.12). The similarity extends to carboxylates, particularly if the nitroalkanes are deprotonated. However, nitroalkanes are weak acids that are predominantly not ionised at physiological pH.[126] Nitroalkanes are relatively stable compounds and nitro analogues of PUFAs would be expected to be resistant to β-oxidation because they would not be susceptible to CoA thioester production. As

discussed above, this is the first step in the β-oxidation of fatty acids. In addition, nitroalkanes would not be likely to form phosphoglycerides or cholesterol esters. Nevertheless it was anticipated that nitro analogues of PUFAs might act as substrates and inhibitors of lipoxygenase enzymes. As outlined above, selective inhibition of the lipoxygenase pathways is the principal basis on which therapeutic compounds are designed for control of pathological processes mediated by arachidonic acid **1** metabolites.

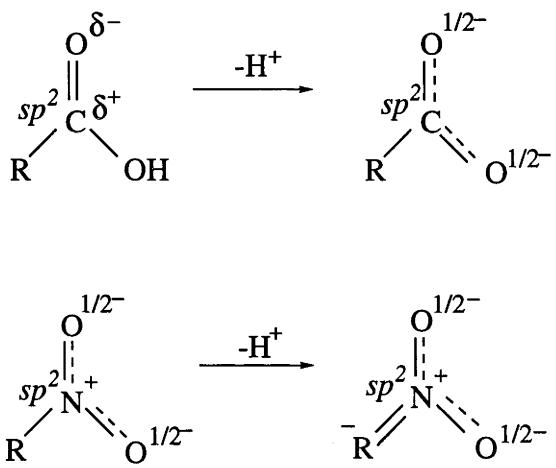


Fig. 1.12: Similarities between the carboxyl and nitro groups

No drugs yet exploit the similarity of the nitro group to the carboxyl group. However, nitroalkanes which serve as inhibitors or substrates of enzymes have been reported. For instance, nitroethane inactivates D-amino acid oxidase in the presence of cyanide.[127] 3-Nitropropanoate **50** inactivites the flavoenzyme succinate dehydrogenase.[128] It is probably oxidised by the enzyme to 3-nitroacrylate **51**, which then alkylates a nucleophilic group of the apoenzyme without diffusing from the active site (Fig. 1.13).

3-Nitrolactate not only inhibits fumarase but is also a substrate for malate dehydrogenase.[129] Nitroalkanes and nitrosaminoalkanes bearing acidic α-carbons are generally good substrates for heme-dependent oxidases and peroxidases.[127] Oxidation of nitroalkanes by horse radish peroxidase was reported by Little in 1957.[130]

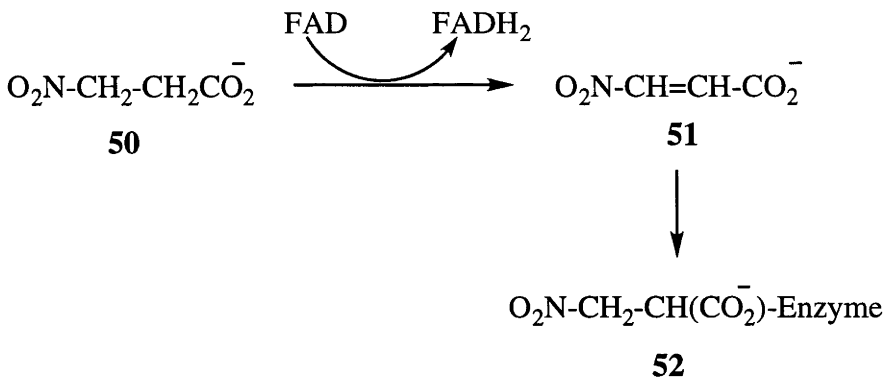


Fig. 1.13: Inhibition of succinate dehydrogenase by 3-nitropropanoate

The polyunsaturated nitroalkanes in this work were to be synthesised from commercially available polyunsaturated alcohols, through the corresponding alkyl halides,[131] and subsequently to be used as starting materials for the synthesis of other nitro-substituted fatty acids. Once compounds had been synthesised, their interaction with lipoxygenases was to be assessed based on their effect on lipoxygenase-catalysed oxidation of arachidonic acid **1**. Lipoxygenases to be used in this work were 15-LO from soybean, 5-LO from potato and 12-LO from porcine leukocyte. The soybean 15-LO shares many important characteristics with those from other sources, and it is commercially available in pure form and good quantity that allows detailed mechanistic and physical studies.[132] The potato tuber possesses a 5-LO which can be readily isolated.[133] Thus, crude potato 5-LO was to be isolated and used for preliminary examination of activity of the nitro compounds. 12-LO is not easily obtained from plant sources. In animal tissues, the cytosol of porcine leukocytes is one of the most abundant sources of 12-LO.[134] Therefore the commercially available 12-LO from porcine leukocyte was to be used for this work.

Lipoxygenase-catalysed oxidation of arachidonic acid **1** was to be determined by measuring the ultraviolet absorbance at 234-237 nm of the hydroperoxide products, which absorb at this wavelength because they contain a conjugated *E*,*Z*-diene chromophore.[135] The reactions were to be conducted at pH values corresponding to

maximal lipoxygenase activity, which according to the literature are 9 for the soybean 15-LO,[136] 6-7 for the potato 5-LO[137] and 7.4 for porcine leukocyte 12-LO.[138]

The other analogues of PUFAs of interest in this project were thia fatty acids, due to their potential as antioxidants. As discussed above compounds of types **16-19** were made as PUFAs analogues, which would be resistant to β-oxidation. Subsequently, the autoxidation of these compounds and their effect on the autoxidation of arachidonic acid **1** were investigated.[139] In these experiments, a thin film assay method was employed. For each reaction, arachidonic acid **1** was mixed with one of the synthetic compounds **16, 17, 18** or **19** at a 1:1 ratio with or without the radical initiator, azobisisobutyronitrile (AIBN). A reverse phase HPLC method was used to simultaneously measure the relative amounts of arachidonic acid **1** and the synthetic compounds **16, 17, 18** or **19** recovered following 60 or 70 h or 7 days of thin film autoxidation. Part of the results are summarised in Table 1.1

As shown in Table 1.1, arachidonic acid **1** underwent rapid autoxidation in the presence of compound **16,** as reflected by reduction in the percentage of recovered arachidonic acid **1** (23% or 17%) after 7 days of autoxidation without the additive AIBN or after 60 h with 10% AIBN. The data showed that arachidonic acid **1** also underwent substantial degrees of autoxidation in the presence of compounds **17** and **18** during the same periods. In contrast, autoxidation of arachidonic acid **1** was completely inhibited during the testing periods when the thin film reaction was carried out in the presence of the γ-thia fatty acid, 3-[(*all-Z*)-(eicosa-5,8,11,14-tetraenylthio)]propionic **19**, even when the reaction contained the radical initiator AIBN. The result indicates that compound **19** is an antioxidant.

The specific objective of this project in regard to the thia fatty acids was to examine the basis of the selective activity of the γ-thia fatty acid **19**. This was to be done by synthesis of a series of analogues of compound **19** and subsequent investigation of their effect on arachidonic acid **1** autoxidation. The analogues include an unsaturated γ-thia fatty acid

with two methylene-interrupted *cis* double bonds, which brings unsaturation closer to the sulfur than is the case in compound **19**, saturated γ-thia and β-thia fatty acids, and unsaturated and saturated sulfides. A thin film method on Petri-dishes was to be employed for assessing autoxidation of arachidonic acid **1** in the presence of the thia fatty acids and sulfides, in conjunction with a reversed phase HPLC technique for analysis of recovered arachidonic acid **1** and thia fatty acids and sulfides. The aim was to examine if the degree of unsaturation, the carboxyl group and the location of sulfur in the thia fatty acids affects their antioxidative activity.

Table 1.1 Percentages of arachidonic acid **1** and compounds **16-19** recovered following thin film autoxidation (the initial ratio of arachidonic acid **1** to each other PUFA compound is 1:1)

Compound	Percentage of compounds recovered		
	Reaction conditions		
	no additive 70 h	no additive 7 day	10% AIBN 60 h
arachidonic acid **1**	97%	23%	17%
16	88%	27%	11%
arachidonic acid **1**	92%	30%	44%
17	102%	41%	49%
arachidonic acid **1**	98%	68%	87%
18	99%	28%	57%
arachidonic acid **1**	101%	102%	100%
19	98%	96%	96%

Chapter 2

Results and Discussion

Synthesis of Nitro Analogues of Polyunsaturated Fatty Acids

2.1. Synthesis of nitroalkanes

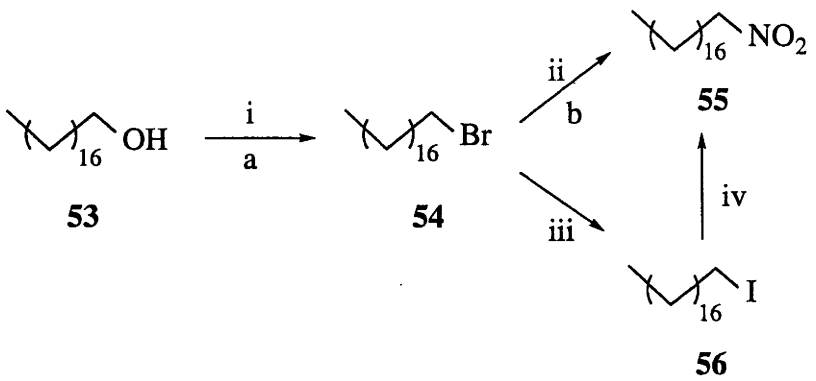
The first series of compounds targeted for synthesis were nitroalkanes with chain lengths of 18 to 22 carbons, and 3 to 5 methylene-interrupted double bonds.

In general, nitroalkanes can be prepared from alkanes by nitration,[140] haloalkanes by reaction with sodium or silver nitrite in a dipolar solvent,[141] aliphatic amines by oxidation,[142] and from azides by ozonolysis.[143] Because of the commercial availability of polyunsaturated fatty alcohols, the basic route used in this work was to convert alcohols to nitroalkanes through alkyl halides employing the methods described by Hayashi *et al.*[144] Since the unsaturated alcohols are relatively expensive to obtain and unstable, a saturated long chain alcohol, octadecan-1-ol **53**, was used as the starting material for establishing the synthetic methods under mild conditions.

Steps a and b in Scheme 2.1 represent the procedure employed for the first attempt at the synthesis of 1-nitrooctadecane **55**.

In this procedure, octadecan-1-ol **53** was treated with triphenylphosphine (PPh_3) and carbon tetrabromide (CBr_4) in dichloromethane overnight at room temperature.[144] After

purification by flash column chromatography, 1-bromooctadecane **54** was obtained in good yield (96%).



i: PPh_3/CBr_4, CH_2Cl_2, RT

ii, iv: $AgNO_2$, diethyl ether, RT

iii: NaI, dry acetone, RT

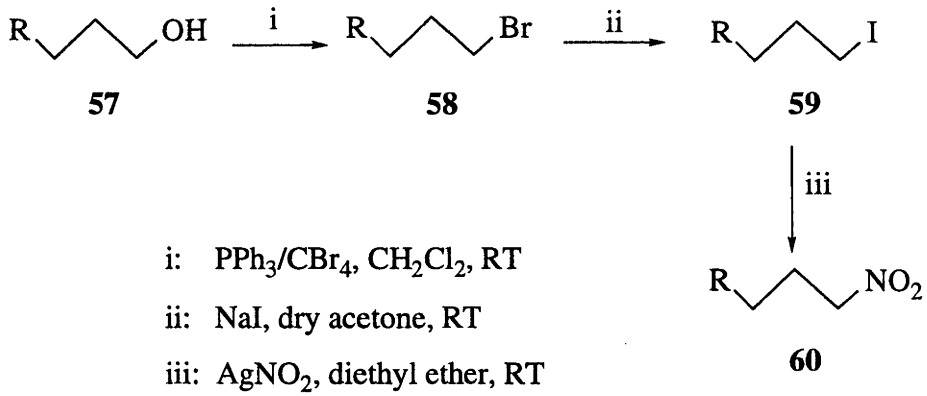
Scheme 2.1

A reported method, which gave good yields of C_4-C_{10} nitroalkanes,[145] was first tried for the conversion of 1-bromooctadecane **54** to 1-nitrooctadecane **55**. Silver nitrite (1.1 eq.) was added into 1-bromooctadecane **54** in anhydrous ether and the reaction was carried out for 36 h at room temperature. However, the reaction gave only a poor yield of 1-nitrooctadecane **55** (<10%), even when the reaction time was changed from 36 h to 1 week and the amount of silver nitrite was increased from 1.1 eq. to 3 eq. The slow reaction may be attributed to the lengthy chain of the 1-bromooctadecane **54**. In order to improve the yield of 1-nitrooctadecane **55**, the bromooctadecane **54** was first converted to the iodooctadecane **56**.[146] Alkyl iodides are more reactive than alkyl bromides as iodide is a better leaving group than bromide.[147] Therefore, compound **56** was expected to be more readily converted to compound **55**. Sodium iodide (2 eq.) was added to a solution of 1-bromooctadecane **54** in dry acetone (Scheme 2.1). After standard workup, the residue was analysed by ^1H NMR. The spectrum showed that 1-bromooctadecane **54** had been completely converted to 1-iodooctadecane **56**. The signal at δ3.40 which

corresponds to the methylene protons at C1 of 1-bromooctadecane **54** had disappeared, to be replaced by a new signal at δ3.20, characteristic of the methylene protons at C1 of iodoalkanes.[148] The crude iodide **56** was then converted to the nitroalkane **55** by treatment with silver nitrite in dry ether for 3 days at room temperature. The yield of purified 1-nitrooctadecane **55** reached 51%, accompanied by a recovery of 9% of the crude iodide **56** that could be reused. The ^{1}H NMR spectrum of 1-nitrooctadecane **55** showed a triplet peak at δ4.37, which is characteristic of the methylene protons at the carbon (C1) attached to the nitro group.[148a] The identity of the compound was further supported by ^{13}C NMR spectral analysis, which gave a peak at δ76.32 that corresponds to the methylene carbon C1. In addition, the electron ionisation mass spectrum displayed a molecular ion at *m/e* 299, in agreement with the formula of the compound, $C_{18}H_{37}NO_2$.

The polyunsaturated nitroalkanes **60a-60d** were then synthesised (Scheme 2.2) based on the procedure developed for the synthesis of 1-nitrooctadecane **55**. The polyunsaturated alcohols **57a-57d** were converted to the corresponding bromides **58a-58d**. These compounds were purified by flash column chromatography and analysed by ^{1}H NMR, which showed characteristic peaks at δ3.40-3.42 and δ1.80-2.00 corresponding to the methylene protons at C1 and C2, respectively. Multiplet peaks at δ5.31-5.47 and δ2.78-2.86 remained the same as those of the starting materials **57a-57d**, and are due to the olefinic protons and the methylene protons at the carbons flanked by double bonds, respectively. These data confirmed the formation of the expected bromoalkanes **58a-58d**.

The bromoalkanes **58a-58d** were subsequently converted to the iodoalkanes **59a-59d** using the conditions developed for the synthesis of 1-iodooctadecane **56**. The crude iodides **59a-59d** were then used to synthesise the nitroalkanes **60a-60d**. Owing to their relatively high polarity in comparison to 1-nitrooctadecane **55**, the polyunsaturated nitroalkanes **60a-60d** were purified by flash column chromatography using 5% diethyl ether in hexane as eluant instead of hexane that had been used in the purification of 1-nitrooctadecane **55**.



(a): R = (Z,Z,Z)-$(CH_2)_4(CH_2CH{=}CH)_3CH_2CH_3$

(b): R = (Z,Z,Z)-$CH_2(CH_2CH{=}CH)_3(CH_2)_4CH_3$

(c): R = (*all*-Z)-$(CH_2CH{=}CH)_4(CH_2)_4CH_3$

(d): R = (*all*-Z)-$CH{=}CH(CH_2CH{=}CH)_5CH_2CH_3$

Scheme 2.2

The ^1H NMR spectra of compounds **60a-60d** contained characteristic peaks at δ4.35-4.41 corresponding to the methylene protons at C1, and multiplet peaks at δ5.31-5.47 due to the olefinic protons as well as peaks at δ2.78-2.86 corresponding to the methylene protons at the doubly allylic carbons. The nitroalkanes **60a-60d** showed characteristic integration ratios for the peaks at δ5.31-5.47 and δ2.78-2.86 (Table 2.1) and characteristic patterns around δ127.58-132.61 in the olefinic region of their ^{13}C NMR spectra.

The reactions of silver nitrite with alkyl halides **58a-58d** only gave rise to nitroalkanes **60a-60d**. By-product alkyl nitrites were not formed under the experimental conditions. This was shown by the characteristic peaks of nitroalkanes **60a-60d** at δ4.35-4.41 in their ^1H NMR spectra corresponding to the methylene protons at C1 and at δ75.42-76.32 in their ^{13}C NMR spectra which correspond to C1 carbons. The characteristic peaks of alkyl nitrites at δ4.8[148a] in their ^1H NMR spectra and at δ66-68[148b] in their ^{13}C NMR spectra were not observed.

When exposed to air at room temperature during the synthesis and purification processes, compounds **58a-58d** and **60a-60d** appeared to undergo rapid decomposition. For instance, TLC analysis detected multiple spots from compound **58d**, which had been exposed to air. The ultraviolet spectrum of this material showed absorbance at 234 nm and its ^{1}H NMR spectrum also exhibited complex multiplet peaks at δ5.5-6.8, which are characteristic of a conjugated *cis,trans*-diene moiety with an allylic hydroperoxy substituent. This probably reflects that these compounds are highly susceptible to autoxidation due to the presence of the reactive methylenes flanked by double bonds.

Table 2.1 Yields of the nitroalkanes **60a-60d** and the ratios of integration of their ^{1}H NMR signals at δ5.31-5.47 and δ2.78-2.86

Compound	Yield (%)	Ratio of integration
60a	53	6 : 4
60b	51	6 : 4
60c	56	8 : 6
60d	53	12 : 10

In order to avoid autoxidation of these compounds, all the synthesis processes were conducted under nitrogen in the dark at room temperature. After purification, the polyunsaturated bromoalkanes **58a-58d** and nitroalkanes **60a-60d** were stored at -30 °C in tightly-sealed containers that had been flushed with nitrogen.

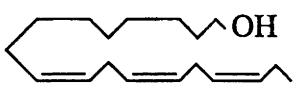
Table 2.2 Resonances between δ127.57-132.62 in the ^{13}C NMR spectra of compounds **57a-57d, 58a-58d** and **60a-60d.**

57a	**58a**	**60a**
127.67	127.67	127.66
128.23	128.29	128.35
128.82 (2C)	128.81(2C)	128.78
130.89	130.83	128.86
132.52	132.51	130.74
		132.54
57b	**58b**	**60b**
128.16	128.14	128.09
128.51	128.65	128.51
128.72	128.71	129.03(2C)
128.90	128.96	129.89
130.57	130.33	131.03
130.99	131.03	
57c	**58c**	**60c**
128.11	128.09	128.06
128.46	128.42	128.35
128.64(2C)	128.74 (2C)	128.54
128.88	129.01	128.90
129.10	129.13	129.19(2C)
130.44	129.93	129.62
131.04	131.05	131.09
57d	**58d**	**60d**
127.57	126.99	127.58
128.44	127.85	128.27
128.66(2C)	127.94	128.42
128.72	128.06	128.55
128.77	128.11(2C)	128.60
128.83	128.18(2C)	128.87(3C)
128.86	128.24	129.05
129.04	128.55	129.15
129.15	129.46	130.94
130.01	132.01	132.61
132.62		

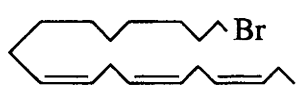
Compounds **58a-58d** and **60a-60d** obtained under these carefully-controlled reaction conditions showed no ultraviolet absorbance at 234 nm and a lack of signals around δ5.5-6.8 in their ^{1}H NMR spectra. This indicates that autoxidation of the compounds has not

occurred. A key feature of the polyunsaturated compounds **58a-58d** and **60a-60d** is their *cis*-double bonds and it was important to know if these remained intact. Normally, disubstituted *cis*- and *trans*-double bonds can be differentiated based on the coupling constants of the olefinic protons in ^{1}H NMR spectra, which are 6-12 Hz for *cis*-protons and 12-18 Hz for *trans*-protons.[148a] However, the olefinic protons of compounds **58a-58d** and **60a-60d** are in similar chemical environments, so their resonances are indistinguishable and coupling constants could not be calculated. In ^{13}C NMR spectra, it has been reported[149] that the chemical shifts of allylic carbons are within the range of δ27-28 when adjacent to a *cis*-double and δ32-33 when adjacent to a *trans*-double bond. However, compounds **58a-58d** and **60a-60d**, and the starting materials **57a-57d** have other methylene carbons which give signals in these ranges. Therefore, the configuration of the double bonds for the bromides **58a-58d** and the nitroalkanes **60a-60d** could not be determined using this approach. Compounds **58a-58d** and **60a-60d** share high similarity to their respective starting materials **57a-57d** with regard to the patterns of peaks at δ5.31-5.47 and δ2.78-2.86 in their ^{1}H NMR spectra, and at δ127.00-132.62 in their ^{13}C NMR spectra. The latter data of compounds **57a-57d**, **58a-58d** and **60a-60d** are shown in Table 2.2. This correlation is good evidence that isomerisation of the *cis*-double bonds does not occur during formation of compounds **58a-58d** and **60a-60d**.

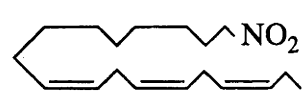
The yields of the bromoalkanes **58a-58d** range from 92-96% and those of the nitroalkanes **60a-60d** from 51-56%. Therefore, the method illustrated in Schemes 2.1 and 2.2 is suitable for the synthesis of both saturated and unsaturated long-chain nitroalkanes.



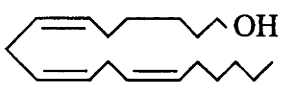
57a



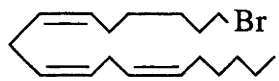
58a



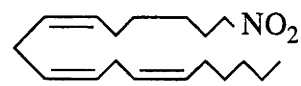
60a



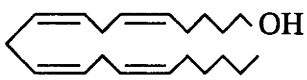
57b



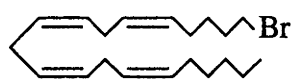
58b



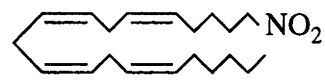
60b



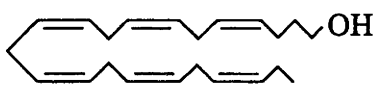
57c



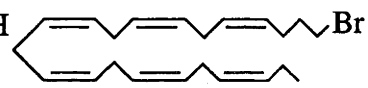
58c



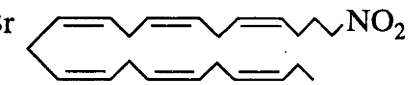
60c



57d



58d



60d

2.2. Synthesis of 2-, 3- and 4-nitro-substituted fatty acids

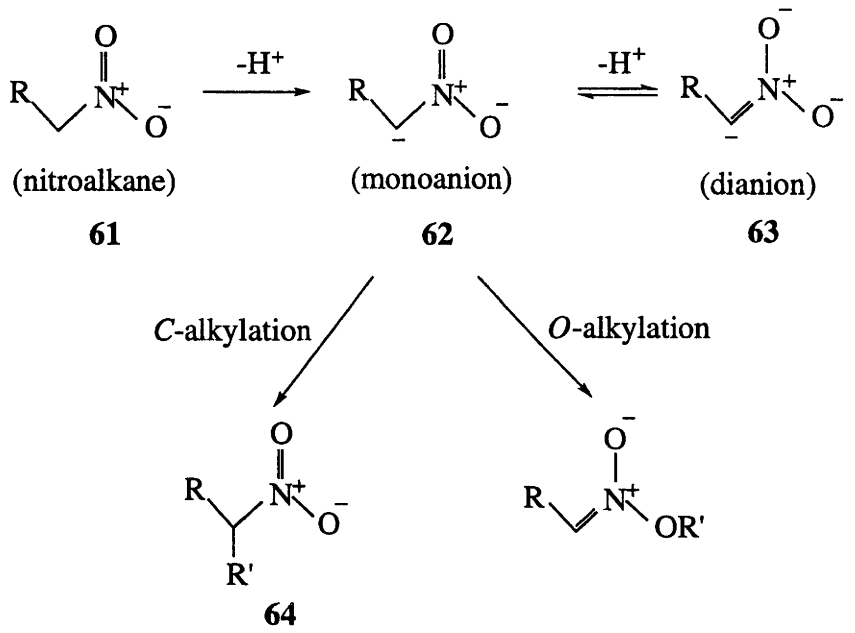
Having obtained the nitroalkanes **55** and **60a-60d**, the next compounds targeted for synthesis were fatty acids with a nitro group at either the C2, C3 or C4 position. The nitroalkanes **55** and **60a-60d** described in Section 2.1 were used as starting materials.

2.2.1. General reactivity of nitroalkanes

Nitroalkanes can be considered as versatile building blocks and intermediates in organic synthesis. They are readily available, can be used in a variety of C-C bond-forming processes which the nitro group facilitates, and can be readily transformed into other classes of compounds using a wide range of efficient methods.[150]

A nitro group, acting as a strong electron-withdrawer, can activate a neighbouring carbon-hydrogen bond. Therefore, nitroalkanes can be regarded as weak acids (*e.g.*, the pK_as of CH_3NO_2 and $CH_3CH_2NO_2$ are 10.2 and 8.5, respectively).[151] Deprotonation of a nitroalkane **61** under basic conditions generates the corresponding alkyl nitronate (monoanion) **62** and dianion **63**, and the anions are capable of covalent bond formation at

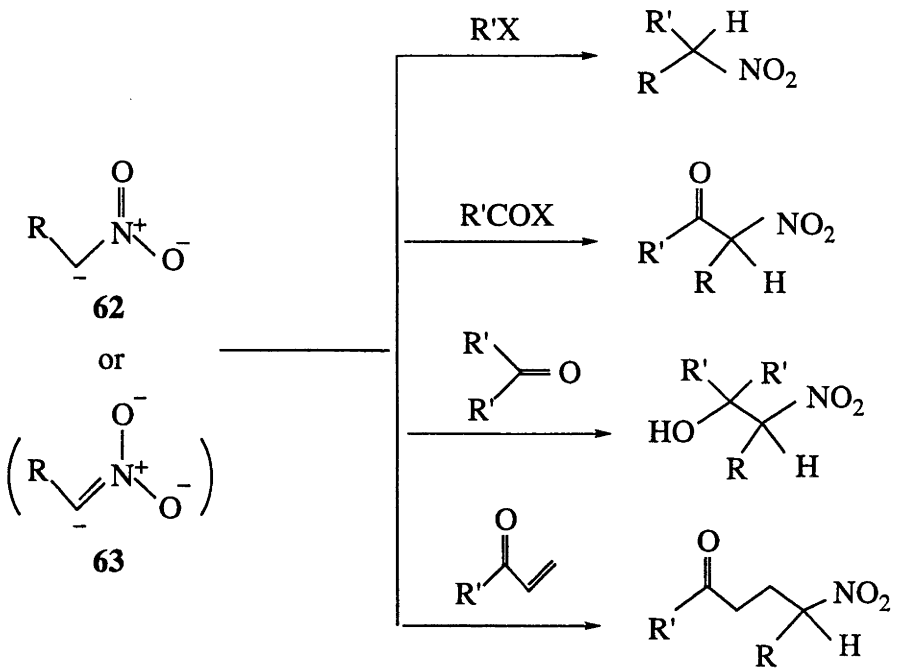
either carbon or oxygen (Scheme 2.3) through reaction with a range of electrophiles.[152,153]



Scheme 2.3

For nitroalkane monoanions **62**, the regioselectivity of alkylation at carbon or oxygen depends on the kind of electrophile used. The cases of *C*-alkylation of monoanions **62** have been attributed to an electron transfer mechanism.[154-156] However, doubly deprotonated nitroalkanes **63** react as typical nucleophiles to give reasonable yields of *C*-alkylated products **64**.[157-159] Some important C-C bond-forming reactions of nitroalkanes are shown in Scheme 2.4.

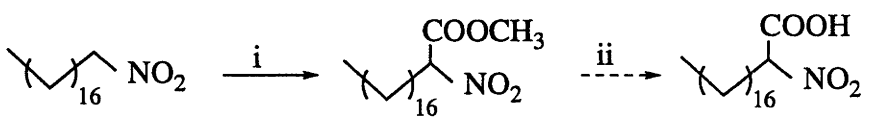
A nitroalkane monoanion **62** or dianion **63** can undergo *C*-acylation through reaction with activated carboxylic acids.[160] For saturated and conjugated carbonyl compounds, Henry and Michael additions, respectively, lead to 1,2- and 1,4-difunctionalised derivatives (Scheme 2.4), in which both the nitro and hydroxy or nitro and carbonyl groups are amenable to conversion into other groups by oxidation, reduction, hydrolysis and dehydration.[150]



Scheme 2.4

2.2.2. Attempted synthesis of a 2-nitro fatty acid

It has been reported that doubly deprotonated nitroalkanes (C_1-C_4) can be *C*-alkylated with methyl chloroformate to give 2-nitro esters.[160] Accordingly, preparation of the long chain 2-nitro fatty acid, 2-nitrononadecanoic acid **66**, was attempted through synthesis of the corresponding 2-nitro ester, methyl 2-nitrononadecanoate **65**, followed by hydrolysis of the ester **65** in alkaline solution (Scheme 2.5).



55 **65** **66**

i: $ClCOOCH_3$, n-BuLi, THF/HMPA, -78 °C

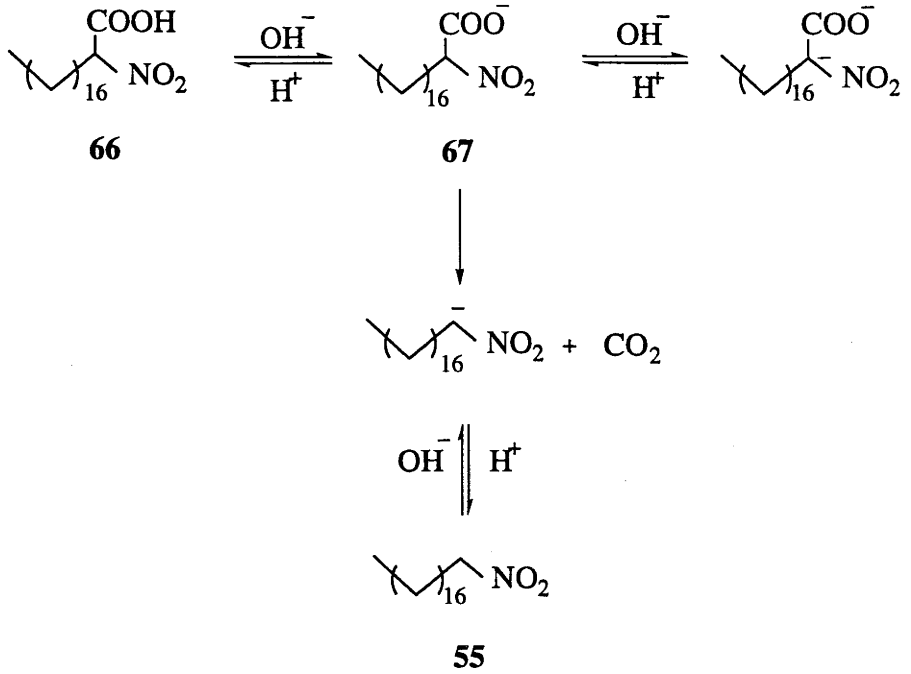
ii: LiOH, DME, RT

Scheme 2.5

The saturated nitroalkane, 1-nitrooctadecane **55**, was used as the starting material. The synthesis of the 2-nitro ester **65** was carried out at -78 °C in THF/HMPA (5:1) containing the strong base, n-butyl lithium (2.5 eq.), and the electrophile, methyl chloroformate (2.5 eq.). ^{1}H NMR spectral analysis of the crude product mixture after workup showed the disappearance of the peak at δ4.38 corresponding to the methylene protons at C1 of the starting material **55** and the appearance of a multiplet peak at δ5.10 which represents the methine proton at the carbon attached to the nitro group and the carboxylate. In addition, the singlet peak at δ3.83, corresponding to the ester methyl protons, had a similar integration value to the triplet peak at δ0.88 for the ω-methyl protons, which indicates the formation of the 2-nitro ester **65**. After purification by flash column chromatography, the product **65** was obtained in a yield of 30%.

Hydrolysis of the 2-nitro ester **65** was attempted by treatment with lithium hydroxide in 1,2-dimethoxyethane (DME) for 1 h. This method has been used in the literature for converting sensitive esters to the corresponding acids.[161] TLC analysis of the reaction mixture, after it had been acidified with dilute hydrochloric acid, showed a single product that was less polar than the starting material **65**. The mixture was extracted with ethyl acetate and the resulting extract was concentrated, and the residue was analysed by ^{1}H NMR. The spectrum showed no signal at δ5.10 for the starting material **65**, but a resonance at δ4.38 had appeared, characteristic of the methylene protons at C1 of the nitroalkane **55**. The mass spectrum of this material confirmed that the product was 1-nitrooctadecane **55**.

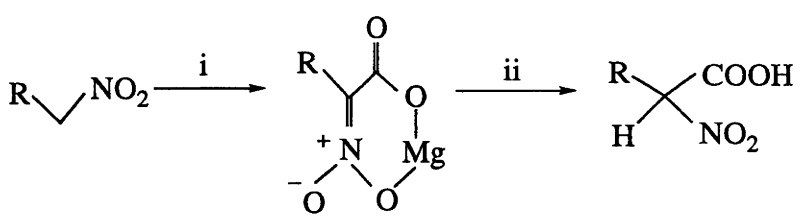
Scheme 2.6 is proposed to explain the above result. It has been reported[162-163] that 2-nitroacetic acid and its dianion are quite stable at room temperature, but that the monoanion decarboxylates rapidly. The production of the nitroalkane **55** is thus likely to be due to decarboxylation of the monoanion **67** in the basic reaction medium.



66 **67** **55**

Scheme 2.6

Whereas 2-nitro acids decarboxylate with ease, the reverse carboxylation reaction can also be readily accomplished under certain conditions. Finkbeiner *et al.*[164,165] succeeded in preparing magnesium and aluminium salts of nitroacetic acid and have utilised the stability of magnesium salts to provide a simple synthesis of 2-nitro acids from primary nitroalkanes and carbon dioxide (Scheme 2.7).



68 **69** **70**

i: magnesium methyl carbonate, 60 °C; ii: HCl

(a): R = CH_3 (b): R = C_2H_5

(c): R = $(CH_2)_2CH_3$ (d): R = $(CH_2)_3CH_3$

Scheme 2.7

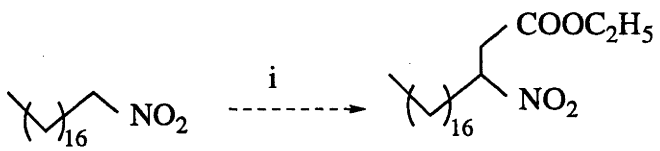
In their work, magnesium methyl carbonate (MMC), that forms in solution from the saturation with carbon dioxide of a magnesium methoxide suspension in *N,N*-dimethylformamide (DMF) was used as the reagent for the introduction of the carboxyl group onto primary nitroalkanes **68** at the C1 position. Acidification of the magnesium salts **69** gave the 2-nitro acids **70** by avoiding formation of the unstable monoanions. They prepared 2-nitro esters, *via* the corresponding 2-nitro acids **70**, from C_2-C_5 nitroalkanes **68** in yields of 40-60%.

In the present work, the same method was employed in an attempt to synthesise the long chain 2-nitro acid **66**, using 1-nitrooctadecane **55** as the starting material. 1-Nitrooctadecane **55** was treated with MMC at 60 °C under nitrogen, and after approximately 16 h the mixture was cooled, acidified using ice-cooled hydrochloric acid and extracted with ether. The resulting extracts were concentrated. However, analysis of the residue using ^{1}H NMR spectroscopy showed the presence of only the starting material **55**, indicating that no reaction had occurred. A parallel experiment was carried out using the short-chain nitroalkane, 1-nitropropane **68b**, as the staring material. In this case, the expected 2-nitro acid **70b** was successfully obtained, as indicated by the appearance of a multiplet at δ5.10 in the ^{1}H NMR spectrum, which represents the methine proton at C2 attached to the nitro and carboxyl groups. It is unclear why the long chain nitro compound **55** failed to react under the same conditions, but the chain length of the nitro compound **55** appears to be a major factor that prevents reaction.

Based on the observation that 2-nitro acids readily decarboxylated, it was expected that they would probably be unstable under physiological conditions. It was therefore decided that the synthesis of the long chain 2-nitro acids would not be pursued further.

2.2.3. Attempted synthesis of a 3-nitro fatty acid

Nitroalkanes can be *C*-alkylated at the C1 position through treatment with alkyl halides in the presence of n-butyl lithium (2 eq.) at temperatures below -78 °C.[166] An attempt was made to prepare the 3-nitro ester **71** by using ethyl bromoacetate as the alkylating reagent (Scheme 2.8). The procedure and reaction conditions employed were the same as those used for the synthesis of the 2-nitro ester **65** described in section 2.2.2. However, both TLC and ^{1}H NMR spectral analyses indicated that no reaction occurred. When ethyl bromoacetate was replaced by ethyl iodoacetate, again no reaction occurred. The failure of the method is probably due to the strongly basic conditions, under which the ethyl haloacetates are likely deprotonated at C2 and their anions will not be susceptible to attack by nitroalkane anions as nucleophiles.

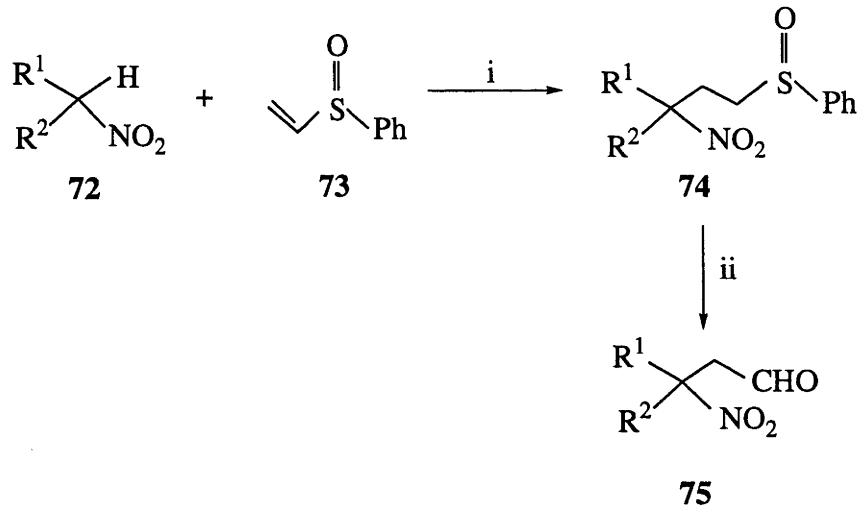


55 **71**

i: $BrCH_2COOC_2H_5$, n-BuLi, -78 °C

Scheme 2.8

An alternative approach to synthesising the 3-nitro fatty acids, based on work done by Ono *et al.*,[167] was tested. These authors reported the synthesis of α,β-unsaturated aldehydes from nitroalkanes **72** through the intermediate 3-nitro aldehydes **75**. Preparation of the 3-nitro aldehydes **75** was accomplished by Michael addition of nitroalkanes **72** to phenyl vinyl sulfoxide **73** in the presence of a base, 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), followed by oxidation (Scheme 2.9). Analogous reactions of phenyl 1-propenyl sulfoxide were also described.



i: DBU/CH_3CN, RT, 24-28 h

ii: $(CF_3CO)_2O$, aqueous $NaHCO_3$

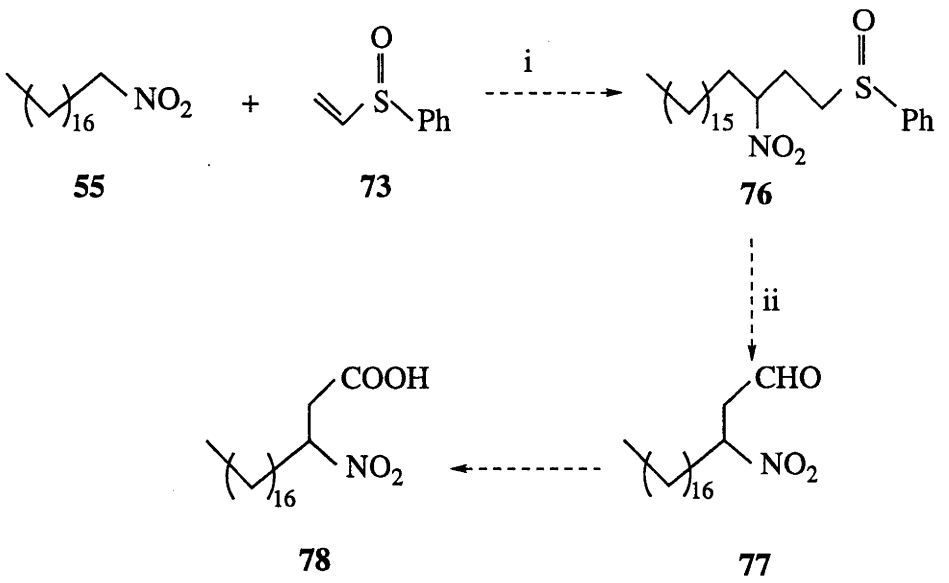
(a): $R^1 = CH_3$, $R^2 = CH_3$

(b): $R^1 = CH_3$, $R^2 = C_2H_5$

(c): $R^1 = CH_3$, $R^2 = CH_2CH(CH_3)_3$

Scheme 2.9

In the present work, the same method was used in an attempt to synthesise the long chain 3-nitro aldehyde **77**, as a precursor of the 3-nitro acid **78** (Scheme 2.10). 1-Nitrooctadecane **55** was treated with phenyl vinyl sulfoxide **73** and DBU. However, ^1H NMR spectral analysis showed none of the expected product. Parallel experiments were carried out using 2-nitropropane **72a** in the place of 1-nitrooctadecane **55**. The expected 3-methyl-3-nitro-1-(phenylsulfinyl)-butane **74a**, was successfully synthesised based on ^1H and ^{13}C NMR spectral analyses of the crude product. In the ^1H NMR spectrum of the crude product, two singlet peaks at δ1.52 and 1.58 correspond to protons of the two methyl groups. The multiplet peak at δ7.50-7.59 is characteristic of aromatic protons. In the ^{13}C NMR spectrum, peaks were observed at δ26.36 and 26.61 for the carbons of two methyl groups, and at δ124.47, 129.96 and 131.85 for aromatic carbons. In addition, a peak at δ87.64 corresponds to the carbon adjacent to the nitro group. However, when 2-nitropropane **72a** was replaced with 1-nitropropane **68b**, no reaction occurred. This result suggests that the method may not be suitable for primary nitroalkanes.



i: DBU/CH_3CN, RT, 24 h

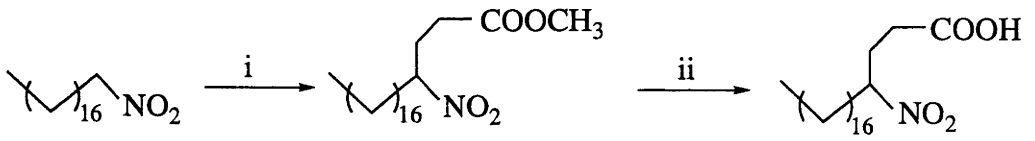
ii: $(CF_3CO)_2O$, aqueous $NaHCO_3$

Scheme 2.10

2.2.4. Synthesis of 4-nitroalkanoic acids

Michael addition of nitroalkanes to acrylate esters has been reported in the literature for the synthesis of short chain 4-nitroalkanoate esters,[168,169] as precursors of 4-nitroalkanoic acids. The reactions are typically run as homogeneous solutions of the reagents in an organic solvent and using a soluble organic base. However, Chasar[170] reported an alternative method for the preparation of 4-nitroalkanoate esters using a two-phase system of water and dichloromethane and an inorganic base (*e.g.*, sodium hydroxide). The advantage of the two-phase method is that the formation of by-products is minimised.

An attempt was made to prepare methyl 4-nitrohenicosanoate **79** following the two-phase method (Scheme 2.11).[170] Under these conditions, 1-nitrooctadecane **55** was treated for 24 h at room temperature with methyl acrylate (1 eq.) in water and CH_2Cl_2 in the presence of sodium hydroxide (0.12 eq.). However, no reaction occurred.



55 **79** **80**

i: methyl acrylate, NaOH, CH_2Cl_2, n-Bu_4NI, reflux

ii: LiOH, DME, RT

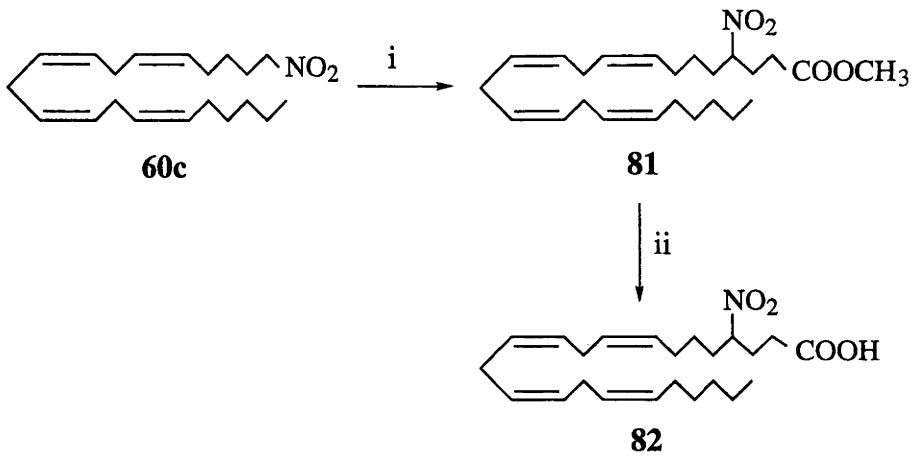
Scheme 2.11

Tetra-n-butylammonium iodide (n-Bu_4NI, 0.2 eq.), a phase transfer catalyst,[171] was subsequently introduced into the system to improve the effective base concentration in the organic solvent. After 18 h with continuous stirring, a new compound was detected, which had a lower mobility than the starting material **55** on TLC. The residue obtained by concentrating the organic phase was analysed by ^1H NMR and the spectrum contained a multiplet at δ4.54, which is consistent with a methine proton adjacent to a nitro group. In addition, there was a singlet peak at δ3.69 corresponding to methyl ester protons, whose integration value was the same as that for the signal due to the protons of the ω-methyl group. This result indicated that the 4-nitroalkanoate ester **79** had formed. However, the yield of the product after purification was only 10%. In order to improve the yield, the temperature of the reaction was increased from room temperature to reflux. In addition, the ratio of methyl acrylate, base and catalyst was optimised. The best result was obtained from a 20 h reaction performed at reflux, with a ratio of nitroalkane **55** : NaOH : n-Bu_4NI : methyl acrylate of 1 : 2 : 0.25 : 3. Methyl 4-nitrohenicosanoate **79** prepared in this manner was purified by flash column chromatography to give a 76% yield.

Based on the procedure and the optimised reaction conditions described above, the unsaturated 4-nitroalkanoate ester, methyl (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoate **81**, was also synthesised (Scheme 2.12) and isolated in a yield of 72%. The ^1H NMR spectrum of compound **81** contains not only similar characteristic peaks to those of

compound **79**, at δ4.54 and 3.68, but also peaks typical of the polyene system. The multiplet peaks at δ5.36 and δ2.81 represent the olefinic protons and the methylene protons at doubly allylic carbons, respectively, and the 8:6 ratio of the integrations of these two peaks is as expected and in good agreement with that seen for the starting material **60c**. A multiplet peak at δ2.11 corresponds to the methylene protons at the two allylic carbons C7 and C19. The ^{13}C NMR spectrum of compound **81** contains resonances at δ88.22, 52.48 and 172.92, which correspond to the carbon adjacent to a nitro group, the ester methyl carbon and the carboxyl carbon, respectively. Signals for the eight olefinic carbons were observed at δ128.07, 128.35, 128.55, 128.87, 129.17(2C), 129.60 and 131.07. These values are very similar to those of the olefinic carbons of the starting material, (*all-Z*)-1-nitro-5,8,11,14-eicosatetraene **60c**, which are δ128.06, 128.35, 128.54, 128.90, 129.19(2C), 129.62 and 131.09. This shows that the double bonds of compound **81** remain in the *cis*-configuration. The mass spectrum of compound **81** exhibits a molecular ion at *m/e* 405, which corresponds to the formula $C_{24}H_{39}NO_4$.

4-Nitrohenicosanoic acid **80** was prepared by hydrolysis of the corresponding ester **79** with a saturated lithium hydroxide solution in 1,2-dimethoxyethane (DME)[162] (Scheme 2.11). After 24 h, the reaction mixture was acidified with dilute HCl and extracted with ethyl acetate. The extract was concentrated and the residue was column chromatographed to give compound **80** in a yield of 85%. The ^{1}H NMR spectrum of compound **80** is basically the same as that of compound **79** except for the disappearance of the singlet peak at δ3.69 corresponding to the methyl ester protons. The ^{13}C NMR spectrum of compound **80** displays a peak at δ177.51, which is consistent with the presence of the carboxylic acid group. The structure of compound **80** was further verified by its infrared spectrum, which showed a broad absorption at 3500-2600 cm^{-1} due to the carboxylic acid O-H stretch and a strong absorption at 1698 cm^{-1} due to the C=O double bond stretch.



60c **81** **82**

i: methyl acrylate, NaOH, CH_2Cl_2, n-Bu_4NI, reflux

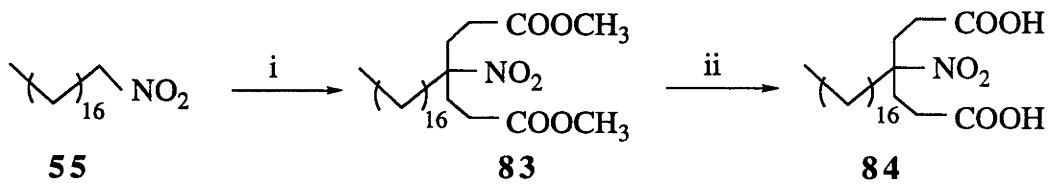
ii: LiOH, DME, RT

Scheme 2.12

(*all-Z*)-4-Nitrotricosa-8,11,14,17-tetraenoic acid **82** was synthesised from compound **81** using similar conditions to those employed to prepare compound **80** (Scheme 2.12), and the yield after purification by flash column chromatography was 93%. The ^1H NMR spectrum of compound **82** is very similar to that of compound **81** except for the lack of the singlet peak of the methyl ester protons at δ3.68. In both ^1H NMR spectra, the absence of any signal at δ5.5-6.8 suggests that no conjugated *cis,trans*-diene has formed. In the ^{13}C NMR spectrum of compound **82**, a peak at δ176.77 is characteristic of the carbon of the carboxylic acid group. Eight peaks at δ128.07, 128.36, 128.53, 128.89, 129.13, 129.20, 129.65 and 131.10 represent the olefinic carbons, and are consistent with those seen for compound **81**. Once again this shows that isomerisation of the *cis*-double bonds has not occurred. In the infrared spectrum of compound **82,** a broad absorption at 3611-3317 cm^{-1} and a strong absorption at 1714 cm^{-1} were observed, which are consistent with the carboxylic acid O-H stretch and the C=O double bond stretch, respectively.

2.2.5. Synthesis of 4-(2-carboxyethyl)-4-nitro fatty acids

Michael addition of primary nitroalkanes using organic bases (*e.g.*, tetraalkylammonium hydroxides) is often accompanied by multiple Michael addition leading to by-products.[169] However, such multiple Michael addition can be used to advantage in synthesis, if a relatively strong base is used.



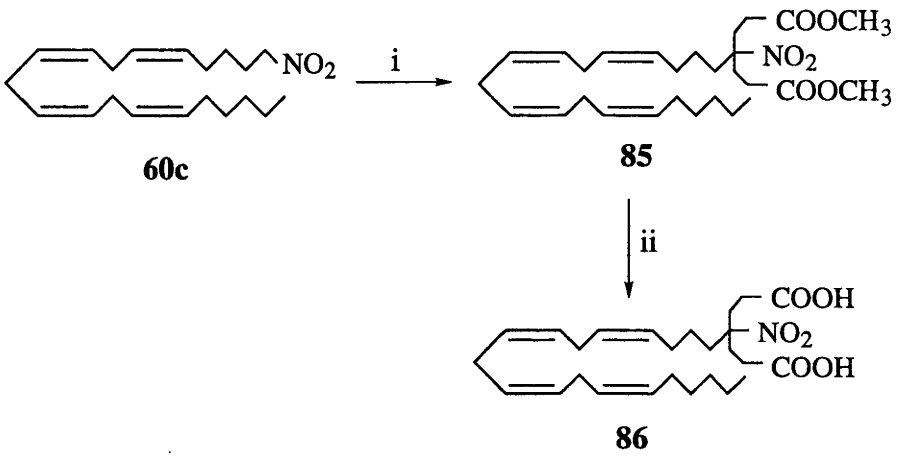
55 **83** **84**

i: methyl acrylate, DBU, CH_2Cl_2, RT, 24 h
ii: LiOH, DME, RT

Scheme 2.13

In the present work, DBU, a strong base, was introduced to the Michael addition of the long chain nitroalkane, 1-nitrooctadecane **55**, to make dimethyl 4-heptadecyl-4-nitroheptane-1,7-dioate **83** (Scheme 2.13). After 24 h the starting material **55** had disappeared, based on TLC analysis, and a single product was detected. After isolation by extraction, concentration of the extracts and flash column chromatography of the residue, the product was obtained in a yield of 95%. In the ^1H NMR spectrum, the peak at δ4.38, corresponding to the methylene protons at C1 of the starting material **55**, had disappeared, and a singlet peak at δ3.69 which corresponds to ester methyl protons was observed. The ratio of the integrations of the peaks at δ3.69 and δ0.88, the latter corresponding to the ω-methyl protons, was 2 : 1. In addition, a multiplet peak at δ2.27 is consistent with the four methylene groups at C2, C3, C5 and C6. The ^{13}C NMR spectrum of compound **83** shows peaks at δ52.53, 93.30 and 172.96 which are consistent with the ester methyl carbon, the carbon adjacent to the nitro group and the carboxyl carbon, respectively.

Dimethyl 4-[(*all-Z*)-nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dioate **85** was synthesised using the same procedure and conditions employed to prepare compound **83** (Scheme 2.14). After purification by flash column chromatography, the product was obtained in a yield of 86%.



60c **85** **86**

i: methyl acrylate, DBU, CH_2Cl_2, RT, 24 h
ii: LiOH, DME, RT

Scheme 2.14

The ^1H NMR spectrum of compound **85** contains characteristic peaks at δ3.69 and 2.27, which are identical to those of compound **83**, and peaks corresponding to a polyene system. The latter include a multiplet peak at δ5.38 corresponding to the olefinic protons and a multiplet peak at δ2.81 due to the methylene protons at doubly allylic carbons, and the ratio of integration of these peaks is 8:6. No peaks at δ5.5-6.8 were observed, indicating that *cis,trans*-conjugated dienes had not formed. The ^{13}C NMR spectrum of compound **85** contains several peaks which resemble those of compound **83**, at δ52.57, 93.21 and 172.92. In addition, resonances at δ128.07, 128.34, 128.48, 128.93, 129.05, 129.21, 129.89 and 131.09, which correspond to the eight olefinic carbons show not only the existence of the polyene system, but also that the *cis*-configuration of the double bonds has been retained since these resonances are similar to those of the nitroalkane **60c**. Mass spectral analysis of compound **85** showed the expected molecular ion at *m/e* 491, confirming the formula of $C_{28}H_{45}NO_6$.

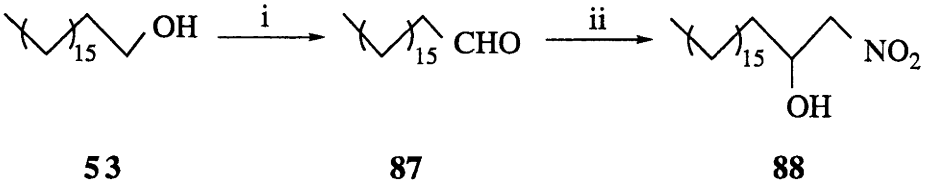
As shown in Scheme 2.13, 4-heptadecyl-4-nitroheptane-1,7-dicarboxylic acid **84** was obtained by hydrolysis of compound **83** with a saturated lithium hydroxide solution in 1,2-dimethoxyethane. The yield of purified compound **84** was 90%. The ^{1}H NMR spectrum of compound **84** lacks the peak at δ3.69 which corresponds to the ester methyl protons of the starting material **83**. The ^{13}C NMR spectrum of compound **84** contains a peak at δ93.83, characteristic of the carbon attached to the nitro group. It also contains a peak at δ179.23, which is consistent with the presence of the carboxylic acid groups. The conversion of the diester **83** to the dicarboxylic acid **84** was further confirmed by the infrared spectrum which shows a broad absorption at 3600-2700 cm^{-1} for the carboxylic acid O-H stretch and a strong absorption at 1740 cm^{-1} for the C=O double bond stretch.

4-[(*all-Z*)-Nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dicarboxylic acid **86** was prepared from compound **85** in a yield of 88% (Scheme 2.14), using the same hydrolysis procedure as that employed for the conversion of compound **83** to compound **84**. The structure of compound **86** was verified by ^{13}C NMR and infrared spectral analysis. In the ^{13}C NMR spectrum of compound **86**, signals at δ93.37 and 178.84 were detected as expected for the carbon adjacent to the nitro group and the carboxyl carbons, respectively. Peaks at δ128.05, 128.31, 128.43, 128.94(2C), 129.21, 129.95 and 131.09 represent the carbons of the *cis*-disubstituted olefins. The infrared spectrum of compound **86** shows a broad absorption at 3400-2300 cm^{-1} due to the carboxylic acid O-H stretch and strong absorptions at 1742 and 1714 cm^{-1} from the C=O double bond stretches. The mass spectrum of compound **86** exhibits a molecular ion at *m/e* 463, which corresponds to the formula $C_{26}H_{41}NO_6$.

2.3. Attempted synthesis of α,β-unsaturated nitroalkanes

α,β-Unsaturated nitroalkanes are another group of PUFA analogues that were pursued in this work. It has been reported[172] that this type of compound can be synthesised by dehydration of 2-hydroxy nitroalkanes. 2-Hydroxy nitroalkanes are readily available by aldol-type addition of nitroalkanes to aldehydes and ketones (the Henry reaction).

The procedure outlined in Scheme 2.15 was followed in this work, for the synthesis of the long chain 2-hydroxy nitroalkane, 1-nitrononadecan-2-ol **88**. Octadecan-1-ol **53** was used as the starting material.



53 **87** **88**

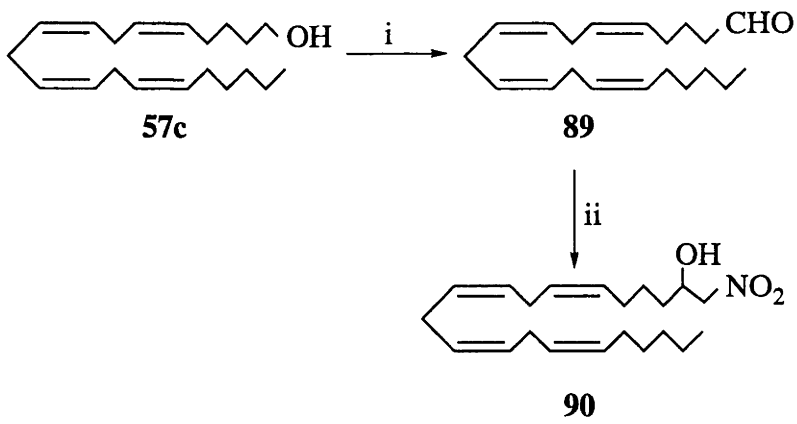
i: pyridinium chlorochromate, CH_2Cl_2, RT, 24 h

ii: CH_3NO_2, Amberlyst A-21, CH_2Cl_2, reflux, 2 days

Scheme 2.15

Initially, octadecan-1-ol **53** was converted to the aldehyde **87** by oxidation at room temperature with pyridinium chlorochromate in dichloromethane.[173] The yield of the product **87** was 81% after purification by flash column chromatography. The ^1H NMR spectrum of compound **87** is consistent with the predicted structure; a peak at δ3.65 in the spectrum of compound **53** due to the methylene protons at C1 has disappeared, to be replaced by a singlet peak at δ9.76 and a triplet peak at δ2.42, which correspond to the aldehyde proton and the methylene protons at C2, respectively. The ^{13}C NMR spectrum of compound **87** contains a characteristic peak at δ203.56 corresponding to the aldehyde carbon. The existence of the aldehyde group is also confirmed by the infrared spectrum, which shows a strong absorption at 1730 cm^{-1} from the C=O stretch.

Using the same procedure, the unsaturated long chain aldehyde, (*all-Z*)-eicosa-5,8,11,14-tetraenal **89**, was synthesised (Scheme 2.16) in a yield of 76% after purification by flash column chromatography.



57c **89** **90**

i: pyridinium chlorochromate, CH_2Cl_2, RT, 24 h

ii: CH_3NO_2, Amberlyst A-21, CH_2Cl_2, reflux, 2 days

Scheme 2.16

The ^1H NMR spectrum of compound **89** shows signals at δ9.78, 5.38 and 2.80, which are consistent for the aldehyde proton, the olefinic protons and the methylene protons at doubly allylic carbons, respectively. In addition, peaks at δ2.45 and 2.12 correspond to the methylene protons at C2, C4 and C16. The ^{13}C NMR spectrum of compound **89** contains a peak at δ202.90, characteristic of the aldehyde carbon. The ^{13}C NMR signals at δ127.92, 128.22, 128.44, 128.68, 129.01, 129.21, 129.48 and 130.91 resemble the corresponding resonances of the starting material **57c**, indicating that no isomerisation of the double bonds has occurred.

Nitroaldol reactions are generally performed under basic conditions.[150] Inorganic bases normally used include alkali metal hydroxides, carbonates and bicarbonates, and magnesium and aluminium ethoxides. Among the organic bases, primary and tertiary amines and ammonium acetate and fluoride have proven useful. Some heterogeneous catalysts have also been reported to be efficient in this reaction. For instance, Ballini *et al.*[174] introduced Amberlyst A-21 for the synthesis of 2-nitroalcohols under mild conditions, and achieved good yields with primary and secondary nitroalkanes.

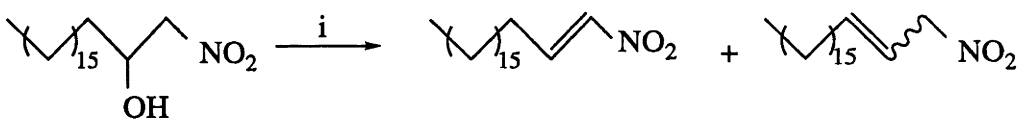
In the present work, the method of Ballini *et al.*[174] was applied to preparing 1-nitrononadecan-2-ol **88** (Scheme 2.15). In the initial experiment, 1-octadecanal **87** was treated for 24 h at room temperature with nitromethane in ether, in the presence of Amberlyst A-21. A product was detected by TLC analysis, but most of the starting material remained unreacted. Even when the reaction time was extended to 7 days, only about 20% of the 1-octadecanal **87** was converted to product based on ^{1}H NMR spectral analysis of the reaction mixture. A high yield of the hydroxy product **88** was achieved in dichloromethane after increasing the reaction temperature from room temperature to reflux. Stirring at reflux for two days resulted in complete conversion of 1-octadecanal **87** to 1-nitrononadecan-2-ol **88**, giving a yield of 89% after purification by flash column chromatography.

1-Nitrononadecan-2-ol **88** was characterised by NMR and infrared spectral analysis. In the ^{1}H NMR spectrum, a multiplet at δ4.28-4.46 is observed. The chemical shifts of methylene protons adjacent to nitro groups fall in this region. However, the 1:1 ratio of the integrations of this multiplet and that of the ω-methyl protons indicates that the δ4.28-4.46 peak comprises overlapping signals. The other is consistent with the methine proton at C2.[174] The broad peak at δ2.22-2.43 in the ^{1}H NMR spectrum and the absorption at 3500-3300 cm^{-1} in the infrared spectrum of compound **88** confirm the presence of the hydroxyl group. ^{13}C NMR spectral analysis shows signals at δ81.20 and 69.24, which are characteristic for carbons C1 and C2, respectively.

Based on the optimised reaction conditions for the synthesis of compound **88**, the unsaturated 2-hydroxy nitroalkane, (*all-Z*)-1-nitrohenicosa-6,9,12,15-tetraen-2-ol **90**, was synthesised from compound **89** (Scheme 2.16) and purified by flash column chromatography to give a 90% yield. The ^{1}H NMR spectrum of compound **90** contains not only peaks at δ4.29-4.45 and at δ2.40-2.60, which are similar to those present in the spectrum of compound **88** due to the protons at C1 and C2, and the hydroxyl proton, respectively, but also peaks at δ5.36 and 2.81 corresponding to the polyene system and resembling those of compound **89**. In the ^{13}C NMR spectrum of compound **90**, peaks at

δ80.98 and 68.91 are observed, corresponding to the carbons C1 and C2, respectively. Resonances at δ127.88, 128.20, 128.46, 128.63, 128.99, 129.11, 129.51 and 130.92 resemble the corresponding olefinic resonances of compound **89**, suggesting that all the double bonds retain their *cis*-configuration and conjugation has not occurred. The electron ionisation mass spectrum of compound **90** shows a molecular ion at *m/e* 349, consistent with the molecular formula of $C_{21}H_{35}NO_3$.

Dehydration of the 2-hydroxy nitroalkane **88** to synthesise the α,β-unsaturated nitroalkane **91** was carried out under mild conditions according to a reported method.[175] As shown in Scheme 2.17, the dehydration was performed by mixing 1-nitrononadecan-2-ol **88** with 1 eq. of methanesulfonyl chloride (CH_3SO_2Cl) and 4 eq. of triethylamine (NEt_3) in dry dichloromethane at 0 °C. The reaction was monitored by TLC from 5 min to 2 h. A fluorescent product could be seen after 5 min, which was significantly less polar than the starting material **88**. After 10 min, another non-fluorescent product was observed along with the first product, and both migrated similarly on the TLC plate. The co-existence of the two products persisted during the 2 h reaction period.



88 **91** **92**

i: CH_3SO_2Cl, NEt_3, CH_2Cl_2, 0 °C

Scheme 2.17

The reaction mixture was washed with dilute HCl and water, and then the solvent was evaporated. The resulting residue was analysed by ^1H NMR. The spectrum indicates the presence of two types of olefin, one of which is represented by a doublet at δ7.00 and a multiplet at δ7.27 and the other by multiplets at δ5.50 and 5.95. This suggests that each of the two products detected by TLC contains a double bond. Based on the ratio of

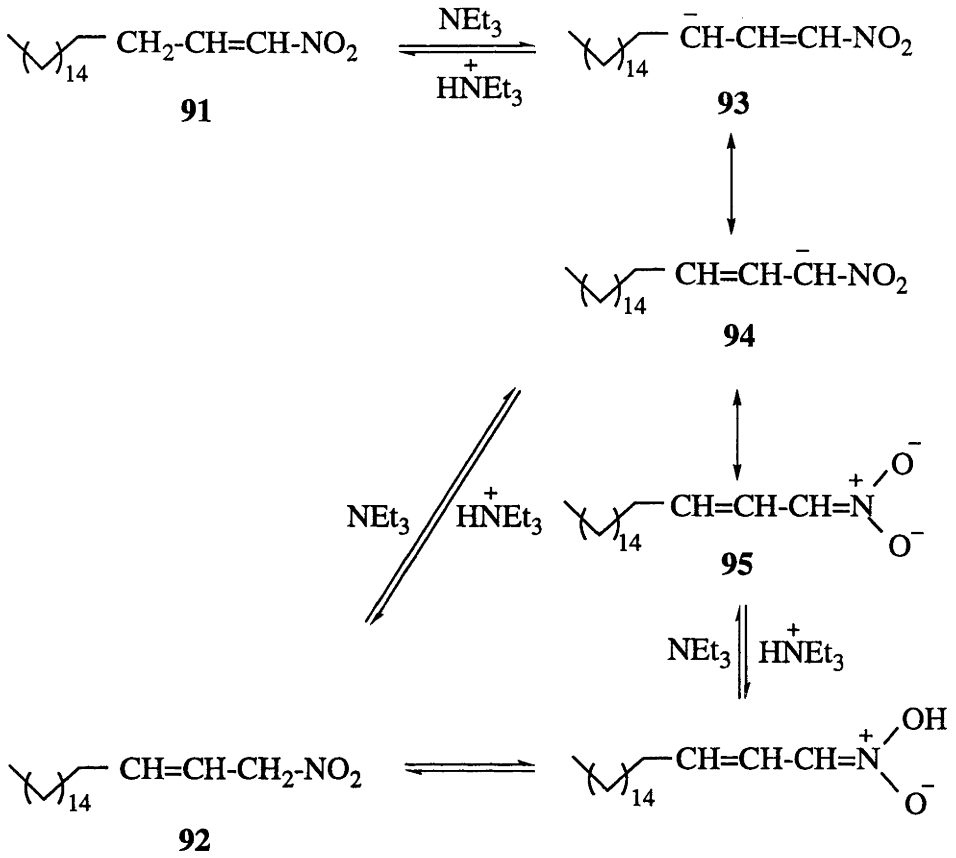
integrations of the peaks corresponding to the olefinic protons, the products are present in similar amounts. The products were partially separated by flash column chromatography, but pure compounds were not obtained because of their rapid decomposition.

The partially separated products were nonetheless analysed by ^{1}H and ^{13}C NMR, which indicates that the fluorescent (*i.e.*, the first appearing) product is the α,β-unsaturated nitroalkane **91** and the non-fluorescent product is the β,γ-unsaturated nitroalkane **92** (Scheme 2.17).

The ^{1}H NMR spectrum of compound **91** contains a doublet at δ7.00 and a multiplet at δ7.27 for the olefinic protons at C1 and C2. These proton signals exhibit a coupling constant of 14 Hz, suggesting that the α,β-double bond has the *trans*-configuration.[148] The ^{13}C NMR spectrum of compound **91** shows resonances at δ140.08 and 143.42 corresponding to the olefinic carbons. In the electron ionisation mass spectrum, a molecular ion at *m/e* 311 is observed, which confirms the formula of $C_{19}H_{37}NO_2$.

The ^{1}H NMR spectrum of compound **92** contains a multiplet at δ4.44 corresponding to the methylene protons at C1 and multiplets at δ5.52 and 5.96 due to the olefinic protons. The chemical shifts of the resonances of the olefinic protons of compound **92** are substantially lower than those of the olefinic protons of compound **91**, consistent with the conjugated structure of compound **91** and the non-conjugated structure of compound **92**. The structure of compound **92** was further verified by ^{13}C NMR spectral analysis, which shows resonances at δ85.94, characteristic of the carbon adjacent to the nitro group, and at δ121.14 and 142.05, for the carbons of the double bond. However, the configuration of the double bond in compound **92** could not be determined by ^{1}H and ^{13}C NMR spectral analysis.

Syntheses of short chain α,β-unsaturated nitroalkanes (C3-C7) have already been reported.[175] It is noteworthy that some of the reported compounds were also obtained as mixtures with the nonconjugated isomers.[175]

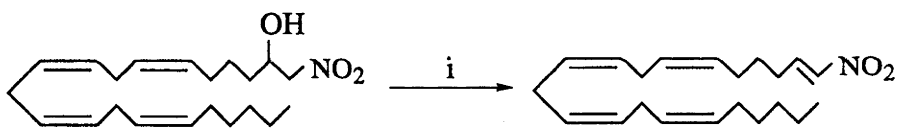


Scheme 2.18

The mechanism illustrated in Scheme 2.18 is proposed to explain this phenomenon. Comparing the acidity of the protons at C1 and C3 of the 2-hydroxy nitroalkane **88** and of the mesylate intermediate in the reaction of the alcohol **88**, it is obvious that those at C1 are the more acidic. Consequently, they are more easily removed under basic conditions. Thus, reaction of the 2-hydroxy nitroalkane **88** only generates the corresponding conjugated compound **91** in the early stages of reaction. The nonconjugated compound **92** is likely to be formed from the conjugated compound **91** through an equilibration process. Deprotonation of compound **91** generates an anion, which can be represented by the series of resonance contributors **93**, **94** and **95**. Protonation then gives a mixture of compounds **91** and **92**.

Compound **96** was synthesised from compound **90** (Scheme 2.19) based on the procedure and reaction conditions used to prepare compound **91** from compound **88**.

TLC analysis of the reaction mixture showed a fluorescent product after 5 min, followed by an additional and non-fluorescent product after 10 min. Based on the reaction of compound **88**, it was expected that the first product would be the α,β-unsaturated nitroalkane **96**. In order to minimise the conversion of the α,β-unsaturated product **96** to the β,γ-unsaturated product, a reaction was quenched after 8 min by addition of dilute HCl. After evaporation of the solvent from the organic phase, the residue was analysed by ^{1}H NMR. The spectrum contains only peaks characteristic of compound **96**. A doublet at δ7.00 and a multiplet at δ7.26 correspond to the new olefinic protons and are very similar to resonances of compound **91**. Multiplets at δ5.37 and 2.81 are similar to those of compound **90**, and represent the protons in the polyene system. The allylic protons at C3, C5 and C17 are represented by multiplets at δ2.25 and 2.08. The coupling constant displayed by the new olefinic protons is 14 Hz, suggesting that this olefin has the *trans*-configuration. Compound **96** was isolated by flash column chromatography, but as was the case with compound **91**, pure product was not obtained due to decomposition.



90 **96**

i: CH_3SO_2Cl, NEt_3, 0 °C, 8 min

Scheme 2.19

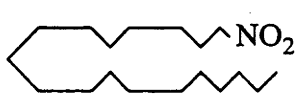
Chapter 3

Results and Discussion

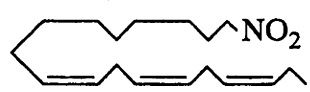
Lipoxygenase Activity of Nitro Polyunsaturated Fatty Acids

As described in Chapter 2, compounds **55**, **60a-60d**, **80**, **82**, **84** and **86** were synthesised, purified and characterised. These were made to investigate their interaction with lipoxygenases.

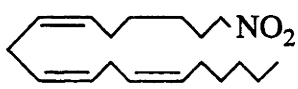
As mentioned in the introduction, lipoxygenases are widely distributed in plants and animals and they are responsible for catalysing the oxidative metabolism of a range of unsaturated fatty acids.[50,92] The products of the lipoxygenase pathways, such as leukotrienes and lipoxins, play an important role in immune response and in inflammatory processes.[115] Therefore, there is considerable pharmaceutical interest in compounds that inhibit lipoxygenase enzymes. In the work described in this chapter, the interactions of the nitro PUFA analogues **55**, **60a-60d**, **80**, **82**, **84** and **86** with 5-, 12- and 15-LO, were investigated.



55



60a



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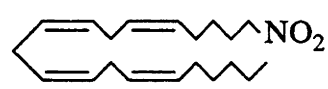
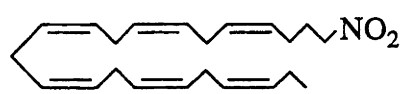
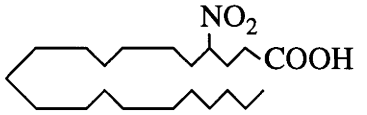


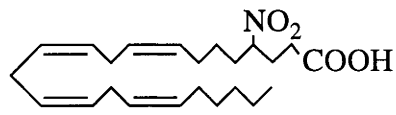
60c



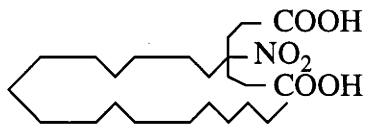
60d



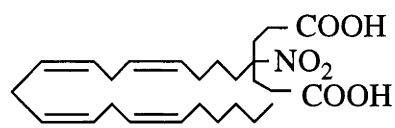
80



82



84



86

3.1. 15-Lipoxygenase assay

3.1.1. Substrate behaviour of nitro compounds with 15-lipoxygenase

As mentioned earlier, one of the problems with the natural PUFAs with regard to pharmaceutical utility is that they are relatively unstable and subject to β-oxidation.[1] The nitro PUFA analogues **60a-60d**, **82** and **86** were expected to be resistant to β-oxidation. The purpose of this work was to examine whether the unsaturated nitro PUFA analogues **60a-60d**, **82** and **86** would be substrates for soybean 15-LO. In this work, arachidonic acid **1** was used as a standard for assessing substrate behaviour.

Determination of substrate activity with lipoxygenases was based on monitoring the formation of hydroperoxide products. These contain a conjugated *E*,*Z*-diene chromophore that generates absorbance at 234-237 nm.[176] The variation of the ultraviolet absorbance over time thus reflects the formation of hydroperoxides and the relative rate of lipoxygenase catalysed oxidation of the substrate.

The conditions for assaying 15-LO catalysed oxidation of arachidonic acid **1**, including the concentrations of arachidonic acid **1** and 15-LO, were first established. Basically, arachidonic acid **1** (40 μM) was incubated with 15-LO (1 μg/ml) at pH 9.0 (0.1 M KH_2PO_4, 25 °C), and the absorbance at 234 nm was recorded for a period of 200 seconds after the addition of 15-LO. The nitro compounds **60a-60d**, **82** and **86** were

assayed using the same procedure and conditions simply by using them instead of arachidonic acid **1**. Fig. 3.1 shows a typical result for arachidonic acid **1**, compound **82** and compound **60c**.

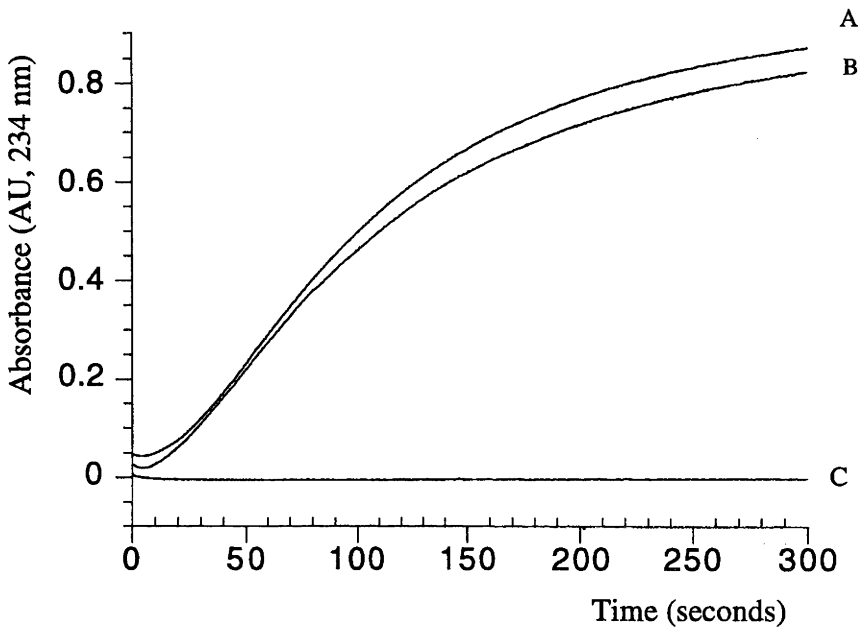


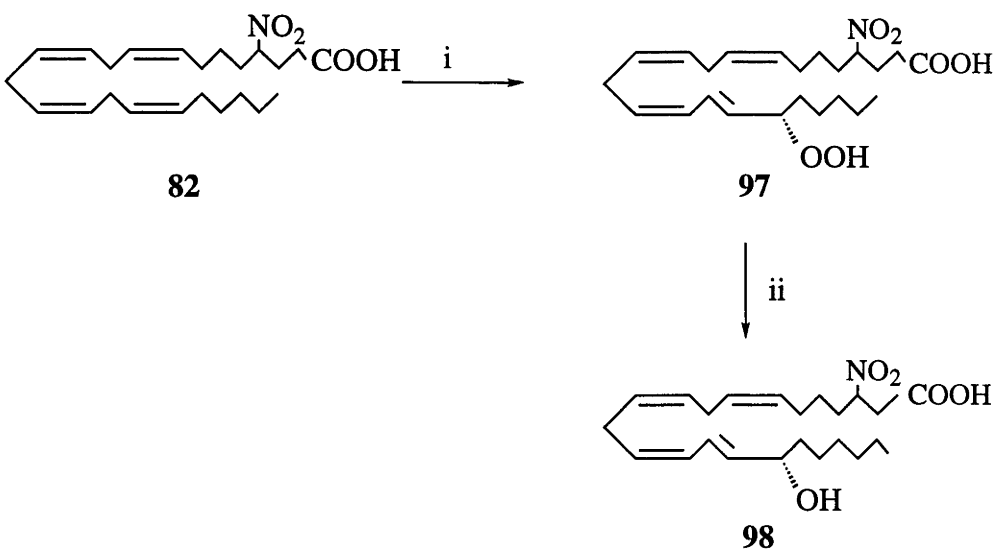
Fig. 3.1: Progress curves for 15-LO catalysed oxidation of arachidonic acid **1** (curve A), compound **82** (curve B) and compound **60c** (curve C) at 40 μM concentration of each compound. (Temperature: 25 °C)

As can be seen from Fig. 3.1, compound **82** gave a very similar progress curve to arachidonic acid **1**, indicating that the oxidation of compound **82** is similar to that of arachidonic acid **1** when tested with 15-LO. Incubation of compound **60c** showed no such oxidation (curve C) and an increase of the concentration of compound **60c** from 40 μM to 100 μM did not affect this result. This indicates that compound **60c** is not a substrate for 15-LO. The same result was obtained for compounds **60a**, **60b**, **60d** and **86**, *i.e.*, no oxidation occurred for any of these four compounds. Thus, compound **82** is the only one among the six tested that is a substrate for 15-LO.

3.1.2. Synthesis and characterisation of the hydroperoxide and alcohol from 15-lipoxygenase catalysed oxidation of (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoic acid

It has been reported that hydroperoxyeicosatetraenoic acids (HPETEs) and hydroxyeicosatetraenoic acids (HETEs) formed by lipoxygenase-catalysed oxidation of arachidonic acid **1** possess a variety of biological activities[177,178] (*e.g.*, 15-HETE inhibits leukotriene biosynthesis).[179] Since compound **82** is a substrate of 15-LO, it was anticipated that its products could have biological activity. Therefore, our subsequent interest was to synthesise and identify the product or products from 15-LO catalysed oxidation of compound **82**.

Procedures for the synthesis, purification and identification of isomeric HETEs and HPETEs have been well established in the literature.[180,181] In this work, the synthesis of hydroperoxide and alcohol derivatives of compound **82** was performed according to the method described by Maguire *et al.*[182]



82 **97** **98**

i: 15-LO, pH 9.0 buffer, O_2, 0 °C

ii: $NaBH_4$, CH_2Cl_2

Scheme 3.1

For the synthesis of the hydroperoxide **97**, compound **82** was incubated with 15-LO at 0 °C in pH 9.0 buffer with oxygen continuously bubbled through the mixture (Scheme 3.1). The reaction was monitored by measuring the ultraviolet absorbance at 234 nm. After 30 min, the absorbance reached a maximum and thereafter began to decrease, indicating that the oxidation had stopped and the product had begun to decompose. In order to minimise decomposition, the subsequent product purification procedure, which consists of acidification, extraction, evaporation of solvent and flash column chromatography, was carried out at 0-4 °C. Owing to the difficulty in separating the product **97** from the starting material **82** by flash column chromatography, the final sample still contained a small proportion of compound **82.**

In comparison with the ^{1}H NMR spectrum of the starting material **82**, the spectrum of compound **97** shows new signals at δ5.60, 6.00 and 6.60 which represent the olefinic protons H_b, H_d and H_c, respectively, and at δ4.40 corresponding to the allylic proton H_a (Fig. 3.2). These resonances are similar to those of 15-HPETE **29**[183] and are characteristic of a dienyl hydroperoxide. However, the position of the hydroperoxy group and the stereochemistry could not be determined from the ^{1}H NMR spectrum. These are considered below.

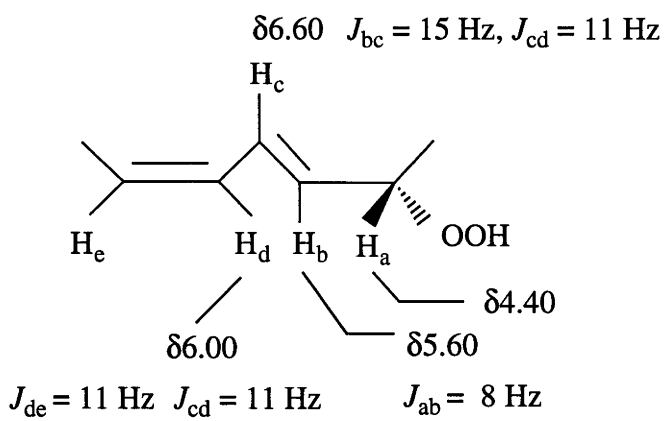


Fig. 3.2

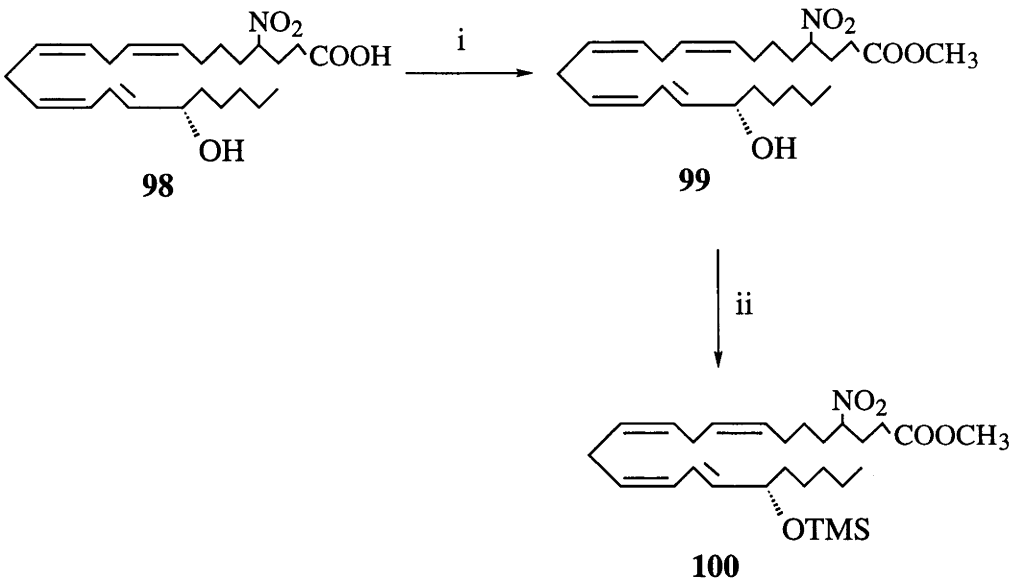
The purity of compound **97** was estimated as 87% based on the ratio (8.1:27.8) of the integrations of the ^{1}H NMR signals at δ4.40 for H_a of compound **97** and δ0.88 for the protons of the ω-methyl groups of both the starting material **82** and compound **97**.

Compound **98** was prepared by $NaBH_4$ reduction of the hydroperoxide **97** (Scheme 3.1). It was obtained pure by flash column chromatography in 43% yield. The ^{1}H NMR spectrum of compound **98** is similar to that of compound **97**. The two products can be distinguished by small differences in the chemical shifts of the signals for H_a and H_b which are δ4.40 and 5.60 for compound **97** and δ4.20 and 5.70 for compound **98**, respectively. Since the position of the hydroxy group could not be determined by NMR spectroscopy, negative ion tandem electrospray mass spectrometry was performed on compound **98**.[180] This showed a deprotonated molecular ion $(M-1)^-$ at *m/e* 406 and an ion $(M-OH)^-$ at *m/e* 390, which are consistent with the predicted formula of $C_{23}H_{37}NO_5$. However, it does not give enough information to assign the position of the hydroxy group.

An alternative approach in the literature[184] to determine the structures of HETEs and other hydroxy acids is to convert them to ester/ether derivatives (*e.g.*, the methyl ester/trimethylsilyl (TMS) ether derivatives) followed by GC/MS analysis using electron ionisation (EI). Therefore, compound **98** was converted to the methyl ester **99** and this was converted to the TMS ether derivative **100**. The procedure and reaction conditions are shown in Scheme 3.2.

The methyl ester **99** was prepared by treating compound **98** with ethereal diazomethane for 15 min at 0 °C. The hydroxy group of compound **99** was then trimethylsilylated at 75°C for 30 min with *N,O*-bis-(trimethylsilyl)trifluoroacetamide containing 10% trimethylchlorosilane. The reaction mixture was then analysed by GC/MS. Compound **100** gave a mass spectrum in which a significant ion at *m/e* 173 was observed. This indicates that the position of the hydroxy group is at C18 (Fig. 3.3). In turn this indicates that the location of the hydroxy and hydroperoxy groups in compounds **98** and **97** is

also at C18. Based on the reports in the literature where the products from soybean 15-LO catalysed oxidation have the *S*-configuration,[2] the stereochemistry of compounds **97-100** is similarly assigned.



i: diazomethane, ether, 0 °C

ii: *N,O*-bis-(trimethylsilyl)trifluoroacetamide containing 10% trimethylchlorosilane, 75 °C

Scheme 3.2.

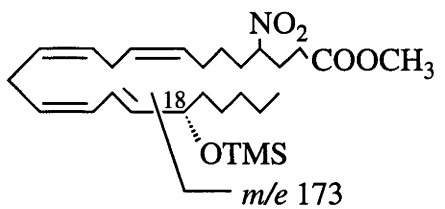


Fig. 3.3.

3.1.3. Determination of the K_m and V_{max} values for 15-lipoxygenase catalysed oxidation of (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoic acid

In order to more fully assess the substrate behaviour of compound **82** with 15-LO, the Michaelis constant K_m and the maximum turnover rate V_{max} were determined.

A single-substrate enzyme catalysed reaction can be represented by the Michaelis-Menten equation, the Lineweaver-Burk equation or the Hanes equation (Scheme 3.3) based on some reasonable assumptions (*e.g.*, the concentration of the enzyme must be very small compared to [S]). In this work, K_m and V_{max} for compound **82** were calculated using the Hanes equation.[185]

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \longrightarrow E + P$$

$$\frac{1}{v} = \frac{V_{max}[S]}{[S] + K_m} \qquad \text{Michaelis-Menten equation}$$

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \qquad \text{Lineweaver-Burk equation}$$

$$\frac{[S]}{v} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}} \qquad \text{Hanes equation}$$

(v - observed rate, V_{max} - maximum rate, K_m - substrate concentration which gives half the maximum rate, E - enzyme, S - substrate, P - product)

Scheme 3.3

To measure the oberved rate (v) for the oxidation of compound **82**, it was necessary to first determine the extinction coefficient (ε) for the conjugated *E,Z*-diene-containing product **97**. A number of molar extinction coefficients have been reported in the literature for arachidonic acid hydroperoxides (HPETEs) at 235 nm and the values range from

23000-30000 M^{-1} cm^{-1}.[176,186] A molar extinction coefficient of 29000 M^{-1} cm^{-1} was also reported[187] for HETEs. Since both HPETEs and HETEs contain conjugated dienes which give rise to the characteristic absorbance at 234-236 nm, the ε values are similar. In the present work, the value to be used for the hydroperoxide **97** was determined using the alcohol **98**, as the latter is more stable. This was done by measuring the absorbance at 234 nm of solutions containing a series of concentrations of compound **98**. The ε value was obtained from the slope of the plot of absorbance *vs* concentration (data shown in Table 3.1), and is 26000 M^{-1} cm^{-1}. This is within the reported range for compounds containing conjugated dienes.

Table 3.1 Data used to calculate the molar extinction coefficient at 234 nm of compound **98**

Concentration of compound **98** (μM)	Absorbance (AU)
5	0.154
10	0.296
15	0.385
20	0.531
25	0.680
30	0.805

For calculation of the K_m and V_{max} values for the 15-LO catalysed oxidation, solutions having different concentrations of compound **82** ranging from 5 to 30 μM were incubated with 15-LO. The reactions were initiated by addition of 25 μl of enzyme solution (0.1 μg/μl) to 2.5 ml of buffer (pH 9.0, 0.1 M KH_2PO_4)[136] containing the required amount of compound **82** and product formation was measured at 25 °C by ultraviolet spectroscopy at 234 nm.[139] Table 3.2 lists the concentrations of compound **82** ([S]) and the corresponding observed rates (v) as well as the [S]/v values for the 15-LO catalysed reactions. The K_m and V_{max} values were then deduced from the slope and the vertical axis intercept of the Hanes plot (Fig. 3.4), and are 8 μM and 24 μM min^{-1} μg^{-1}, respectively.

Table 3.2 Data used to calculate K_m and V_{max} for 15-LO catalysed oxidation of compound **82** (ε = 26000 M^{-1} cm^{-1}, [E] = 1 μg/ml, 25 °C)

[S] (μM)	v (μM/min)	[S]/v (min^{-1})
5	8.77	0.57
10	13.15	0.76
15	16.12	0.93
20	17.24	1.16
25	18.38	1.36
30	18.86	1.59

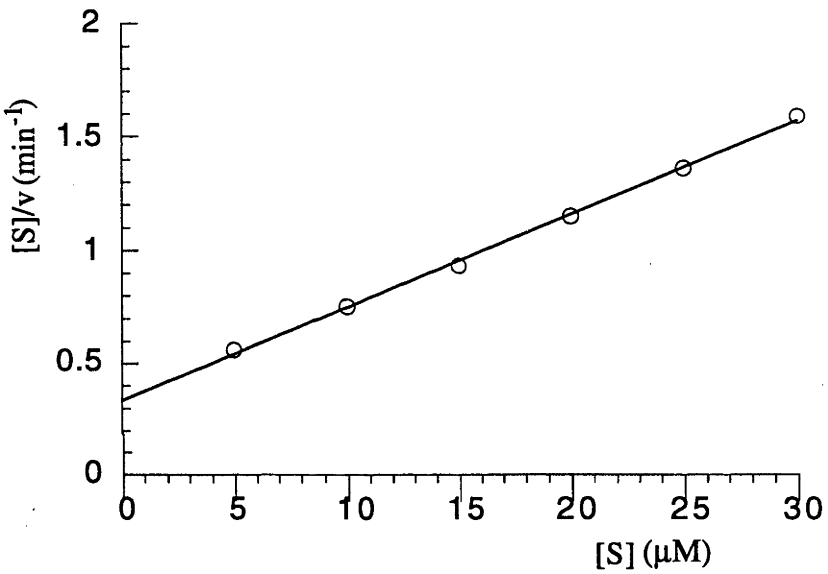


Fig. 3.4: The Hanes plot of 15-LO catalysed oxidation of compound **82**

For comparison of the substrate behaviour of compound **82** and arachidonic acid **1**, the K_m and V_{max} values for arachidonic acid **1** were determined under identical conditions with the assumption that the extinction coefficient of the hydroperoxide product from arachidonic acid **1** is the same as that for compound **98**, *i.e.* 26000 M^{-1} cm^{-1}. The data used for calculation of K_m and V_{max} for arachidonic acid **1** oxidation are given in Table 3.3, and the values are 12 μM and 26 μM min^{-1} μg^{-1}, respectively. This K_m value is consistent with that reported in the literature (13.3 μM).[188]

Table 3.3 Data used to calculate K_m and V_{max} for 15-LO catalysed oxidation of arachidonic acid **1** (ε = 26000 M^{-1} cm^{-1}, [E] = 1 μg/ml, 25 °C)

[S] (μM)	v (μM/min)	[S]/v (min^{-1})
10	11.65	0.858
15	14.43	1.040
20	15.95	1.254
25	17.61	1.420
30	18.38	1.632

The K_m and V_{max} values for compound **82** are similar to those for arachidonic acid **1**. Compound **82** and arachidonic acid **1** both contain four double bonds beginning six carbons from the methyl terminus. They differ at the carboxyl terminus but the similarities in K_m and V_{max} suggest that this does not affect their binding affinity to or oxidation rate by 15-LO.

As mentioned in the introduction, soybean 15-LO shows a preference for oxygenation of substrates at the n-6 position.[92] Datcheva *et al.*[189] have demonstrated the regioselective oxygenation of nonnatural substrates, showing that the lipoxygenase enzyme can tolerate functional group modifications in addition to variations in the chain length between the reactive centre and the carboxyl group. Consistent with these reports, the presence of the nitro group in compound **82** is not critical to the oxygenation by 15-LO nor is the increase in chain length by comparison with arachidonic acid **1**.

3.1.4. Effect of nitro PUFA analogues on 15-lipoxygenase catalysed oxidation of arachidonic acid

The effect of the nitro compounds **60a-60d**, **80**, **84** and **86** on 15-LO catalysed oxidation of arachidonic acid **1** was investigated. This was done through determination of the initial rate of arachidonic acid **1** oxidation in the presence and absence of each nitro compound. For each reaction, 2.5 μg of 15-LO was added to 2.5 ml of 40 μM arachidonic acid **1** in pH 9.0 buffer (25 °C) containing one of the nitro PUFA analogues

60a-60d, **80**, **84** and **86**, whose concentrations ranged from 0 to 100 μM. Ultraviolet absorbance at 234 nm was recorded for a period of 300 seconds after addition of the enzyme.

The results showed that compounds **60a-60d** and **86** had no effect on 15-LO catalysed oxidation of arachidonic acid **1**. Incubation of arachidonic acid **1** with 15-LO in the presence of each of these compounds gave identical progress curves to that of arachidonic acid **1** alone.

Compounds **60a-60d** and **86** are thus neither substrates nor inhibitors of 15-LO. This indicates that they do not bind to the active site of 15-LO. The main difference between compounds **60a-60d** and arachidonic acid **1** is the substitution of a nitro group for a carboxyl group. Apparently this prevents binding to 15-LO.

In a model of arachidonic acid **1** docked into the substrate binding site of soybean 15-LO, it is proposed[101] that the interaction of the positively charged Lys^{260} of the enzyme with the negatively charged carboxylate of the substrate is important in positioning the substrate accurately in the binding site. Presumably the nitro group of compounds **60a-60d** does not form such interactions, particularly at pH 9.0. Although 15-LO has optimal activity at this pH, the pK_as for nitro compounds are about 11,[151] so under these conditions, each of the compounds **60a-60d** will exist predominantly in its neutral form. Compound **86** retains a carboxyl group, but still does not bind to 15-LO.

Compound **80** has a mild activating effect on 15-LO catalysed oxidation of arachidonic acid **1**. Fig. 3.5 shows the progress curves with the concentrations of compound **80** ranging from 0 to 20 μM. The rate of product formation from arachidonic acid **1** oxidation is higher in the presence of compound **80**, as reflected by the higher ultraviolet absorbance at 234 nm. However, further increasing the concentration of compound **80** above 20 μM (*i.e.*, 20-80 μM) did not further increase the rate of arachidonic acid **1** oxidation.

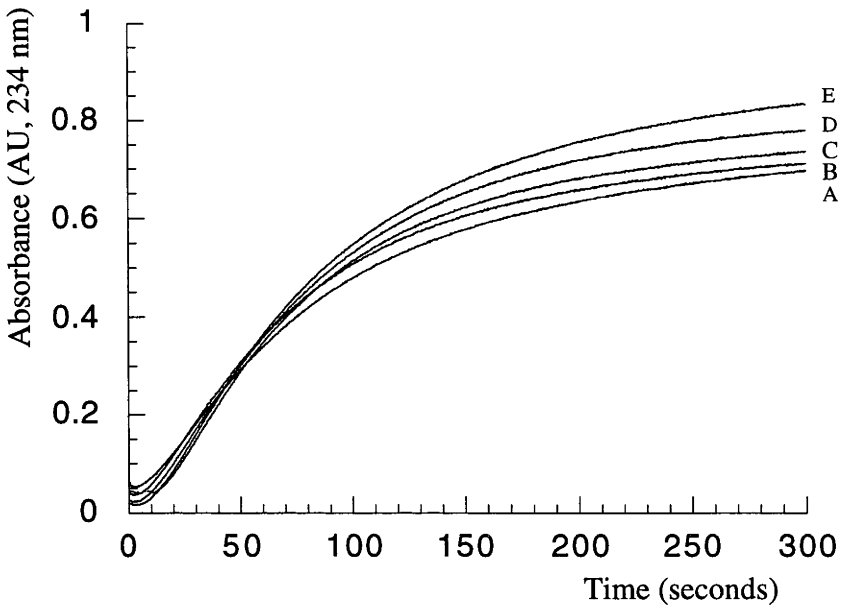


Fig. 3.5: Progress curves for 15-LO catalysed oxidation of arachidonic acid **1** (40 μM) with different concentrations of compound **80**: A, 0 μM; B, 1.25 μM; C, 5 μM; D, 10 μM; E, 20 μM. (Temperature: 25 °C)

The enhancement of the 15-LO activity by compound **80** suggests that the enzyme may be subject to allosteric regulation. Compound **80** may bind to the enzyme at an allosteric site, or separate to the substrate binding site, thus inducing a modified conformation of the enzyme that has higher affinity for the substrate.

Activators of lipoxygenases have not been widely reported. 5-HETE was reported to selectively stimulate the human neutrophil 15-LO to metabolise endogenous substrate.[190] The main efforts of researches have been focused on the search for efficient inhibitors of lipoxygenases which have therapeutic potential in the treatment of a range of allergic and inflammatory conditions such as asthma, allergic rhinitis and rheumatoid arthritis. However, lipoxygenase activators may serve as effective tools for investigation of the structure, catalytic mechanism and regulation of the enzymes. In addition, activators may be useful to increase lipoxygenase activity when it is necessary.

The other nitro PUFA analogue, compound **84**, showed inhibition of 15-LO catalysed oxidation of arachidonic acid **1**. Fig. 3.6 illustrates the oxidation of arachidonic acid **1** (40 μM) in the absence and presence of compound **84**.

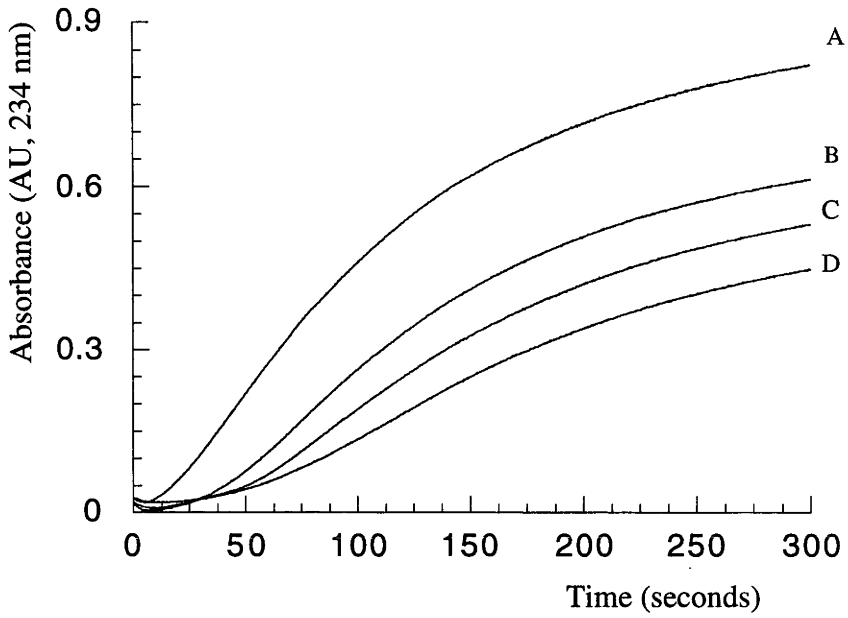


Fig. 3.6: Progress curves for 15-LO catalysed oxidation of arachidonic acid **1** (40 μM) with different concentrations of compound **84**: A, 0 μM; B, 40 μM; C, 60 μM; D, 100 μM. (Temperature: 25 °C)

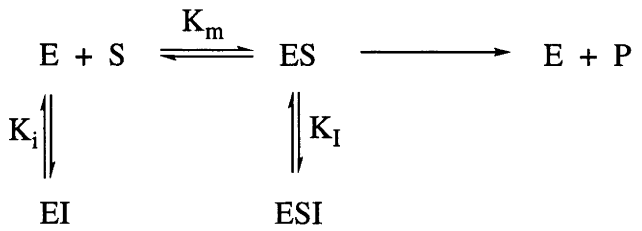
To further understand this inhibition by compound **84**, more detailed experiments were conducted to determine the mode of inhibition.

3.1.5. Kinetics of inhibition by 4-heptadecyl-4-nitroheptane-1,7-dicarboxylic acid of 15-lipoxygenase catalysed oxidation of arachidonic acid

The inhibition of most simple single-substrate enzyme catalysed reactions obeys Michaelis-Menten kinetics.[185] The inhibitor may bind to either the enzyme E or the enzyme/substrate complex ES or both to form enzyme/inhibitor EI or

enzyme/substrate/inhibitor ESI complexes with the respective inhibition constants K_i and K_I. It is reasonable to assume that the formation of EI and ESI is reversible.

In the case where the inhibitor binds to both E and ES, the inhibition is known as mixed inhibition (Scheme 3.4).[185]



(E - enzyme, S - substrate, I - inhibitor, P - product, K_I and K_i - inhibition constants, EI and ESI are assumed to be inactive)

Scheme 3.4

The rate of the enzyme catalysed reaction v as a proportion of the maximum rate V_{max} is then given by:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left[1 + \frac{[I]}{K_i}\right]\frac{1}{[S]} + \frac{1}{V_{max}}\left[1 + \frac{[I]}{K_I}\right]$$

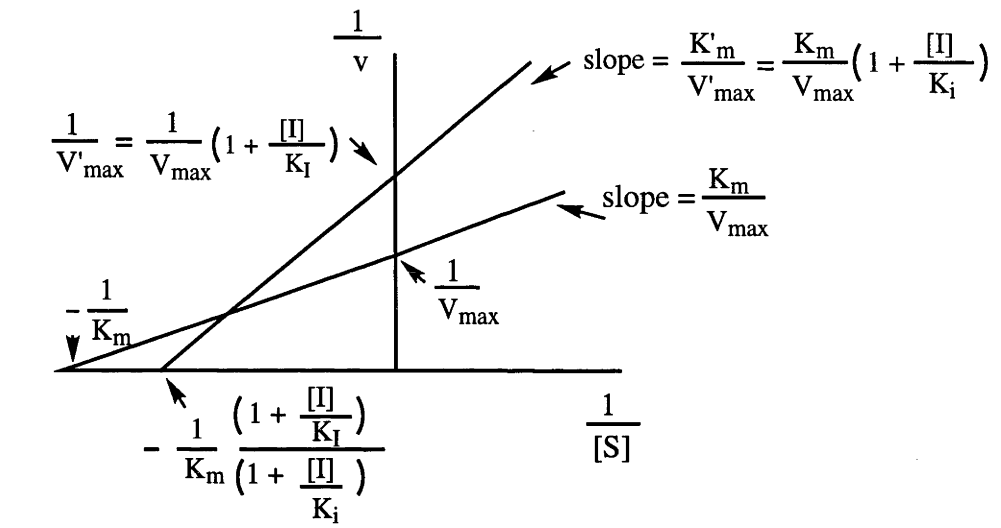
This equation can be written as a Lineweaver-Burk equation:

$$\frac{1}{v} = \frac{K'_m}{V'_{max}} \cdot \frac{1}{[S]} + \frac{1}{V'_{max}}$$

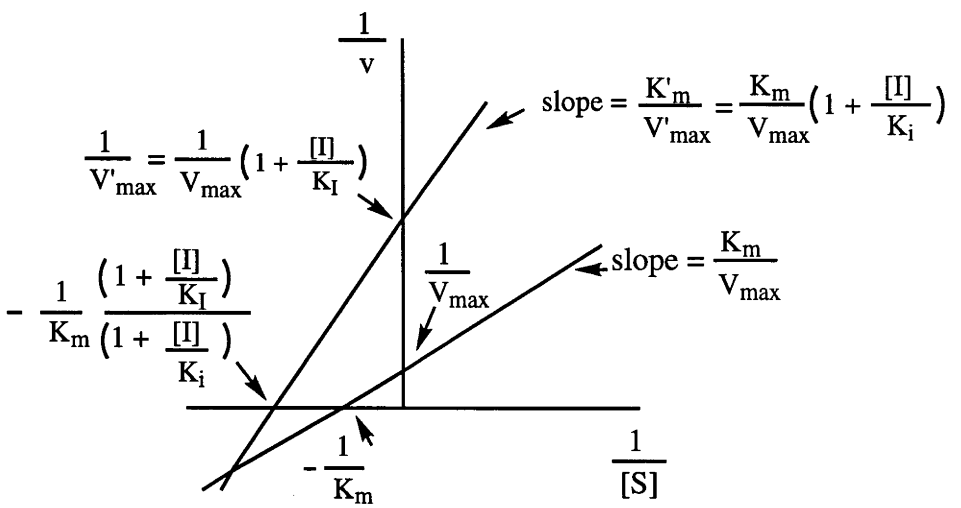
$$\text{where} \quad V'_{max} = \frac{V_{max}}{\left(1 + \frac{[I]}{K_I}\right)} \qquad K'_m = K_m \frac{\left(1 + \frac{[I]}{K_i}\right)}{\left(1 + \frac{[I]}{K_I}\right)}$$

V'_{max} is the V_{max} at [I] and K'_m is the apparent K_m under the same conditions.

In the situation where $K_I > K_i$, plots of 1/v *vs* 1/[S] at different inhibitor concentrations (at fixed [E]) cross to the left of the 1/v axis but above the 1/[S] axis (Fig. 3.7a). In the situation where $K_I < K_i$, the same plots cross to the left of the 1/v axis and below the 1/[S] axis (Fig. 3.7b).[185]



(a)



(b)

Fig. 3.7: Lineweaver-Burk plots of mixed inhibition

(a) $K_I > K_i$; (b) $K_I < K_i$

In either case, K_i and K_I can be determined using secondary plots. For mixed inhibition, each Lineweaver-Burk equation at a fixed concentration of inhibitor gives an intercept value $1/V'_{max}$ (on the 1/v axis) and a slope value K'_m/V'_{max}. A secondary plot of $1/V'_{max}$ *vs* [I] or K'_m/V'_{max} *vs* [I] will be linear, based on the equations $1/V'_{max} = 1/V_{max}(1+[I]/K_I)$ and $K'_m/V'_{max} = K_m/V_{max}(1+[I]/K_i)$. The K_i and K_I values can then be calculated from the intercepts on the [I] axes of the plots (Fig. 3.8 and Fig. 3.9).[185]

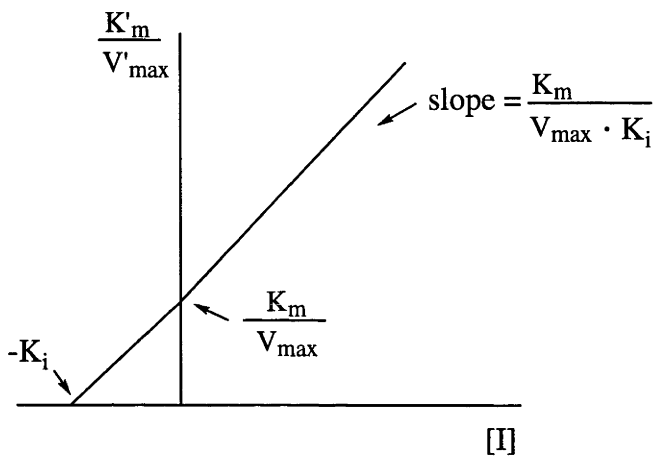


Fig. 3.8

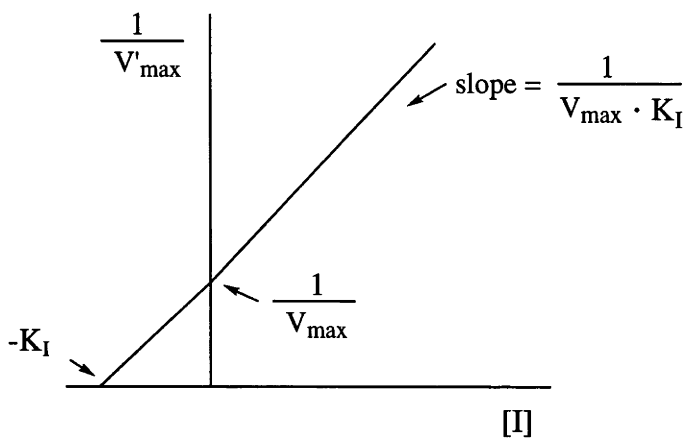


Fig. 3.9

In the case of competitive inhibition, the inhibitor binds only to the free enzyme to form EI with V'_{max} equal to V_{max}, but K'_m equal to $K_m(1+[I]/K_i)$. Since V_{max} is unaltered, the Lineweaver-Burk plots (1/v *vs* 1/[S]) in competitive inhibition have a common intersection point on the 1/v axis (Fig. 3.10).[185]

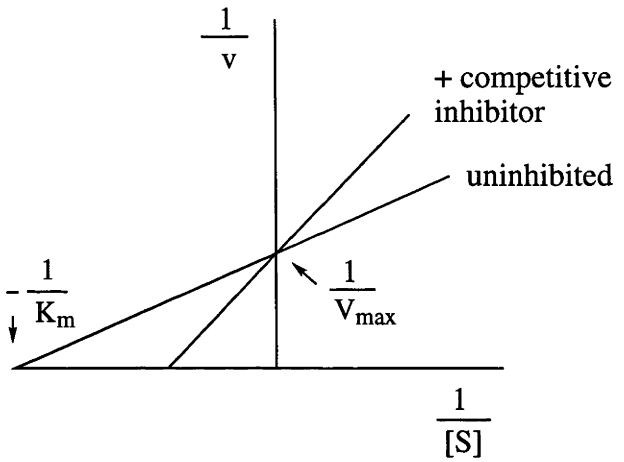


Fig. 3.10

For uncompetitive inhibition, the inhibitor binds only to the enzyme/substrate complex to form ESI with $1/V'_{max}$ equal to $1/V_{max}(1+[I]/K_I)$ and $1/K'_m$ equal to $1/K_m(1+[I]/K_I)$. In this case, the slope of the Lineweaver-Burk plot is not altered by the presence of the inhibitor, but the intercepts on both the 1/v and the 1/[S] axes change (Fig. 3.11).[185]

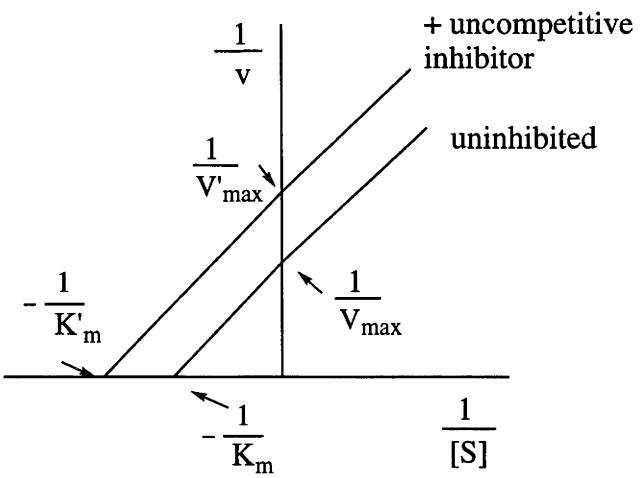


Fig. 3.11

For non-competitive inhibition, the inhibitor binds to the free enzyme and the enzyme/substrate complex to form EI and ESI with the same inhibition constant ($K_i = K_I$). In this case, the K_m value remains constant, but the V'_{max} value changes [$V'_{max} = V_{max}/(1+[I]/K_i)$]. Lineweaver-Burk plots for non-competitive inhibition therefore have a common intersection point on the 1/[S] axis (Fig. 3.12).[185]

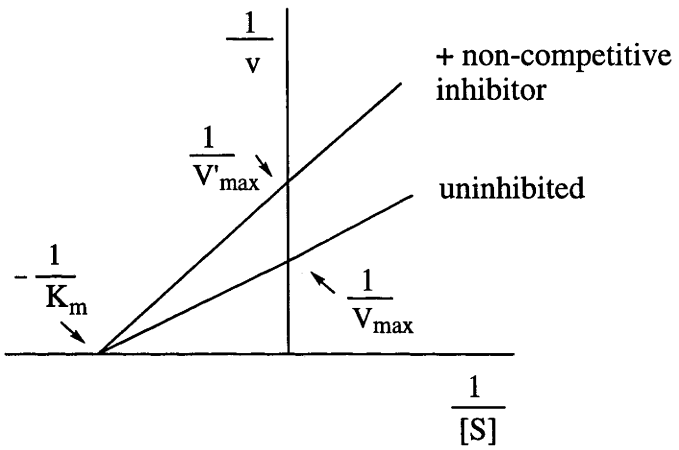


Fig. 3.12

In this work, Lineweaver-Burk plots were produced for several concentrations (0, 20, 50 and 80 μM) of the inhibitor **84**, with a fixed concentration of 15-LO (1 μg/ml). For each plot, the concentration of compound **84** was fixed, and the concentration of arachidonic acid **1** ranged from 10 μM to 40 μM. The values of the initial rate (v) of the oxidation reaction were calculated using kinetics software. The data are summarised in Table 3.4, and the corresponding Lineweaver-Burk plots are shown in Fig. 3.13.

As shown in Fig. 3.13, the four plots have no common intersection point, probably due to experimental error. However, it is clear that the plots do not intersect on the 1/v or the 1/[S] axis, nor are they parallel lines, which would represent competitive, non-competitive or uncompetitive inhibition. The four 1/v *vs* 1/[S] plots cross on the left of the 1/v axis and above the 1/[S] axis, suggesting that compound **84** is a mixed inhibitor with two different inhibition constants K_i and K_I. For calculation of the K_i and K_I values,

the co-ordinates of the common intersection point were needed. It was not feasible to calculate these co-ordinates, so an approximation was made that the intersection of the lines bisecting the tetragon ABCD (Fig. 3.13) represents the common intersection point. Including these values in the plot for each inhibitor concentration gave equations for these plots (Table 3.5). The inhibitor constants K_I and K_i were then calculated based on these equations using the methods described above. The values obtained are 24 μM for K_i and 64 μM for K_I.

Table 3.4 Lineweaver-Burk analysis of inhibition by compound **84** of 15-LO catalysed oxidation of arachidonic acid **1** (25 °C, pH 9.0, [15-LO] = 1 μg/ml)

1/[S] (10^{-2} μM^{-1})	[I] = 0 μM 1/v (10^{-2} μM^{-1} min)	[I] = 20 μM 1/v (10^{-2} μM^{-1} min)	[I] = 50 μM 1/v (10^{-2} μM^{-1} min)	[I] = 80 μM 1/v (10^{-2} μM^{-1} min)
10.00	8.58	13.33	20.31	27.22
6.67	6.93	10.88	15.85	22.61
5.00	6.27	9.81	14.13	19.12
4.00	5.68	8.90	12.09	16.88
3.33	5.44	8.61	11.35	15.95
2.50			9.77	14.44

The mixed inhibition pattern shows that compound **84** is capable of binding to the enzyme both in the presence and absence of bound arachidonic acid **1**. This suggests that compound **84** binds to the enzyme at a different site from the substrate binding site. It is known that an inhibitor may destroy the catalytic activity of an enzyme either by binding directly to and blocking the catalytic site or by binding to an allosteric site which induces a conformational change and affects the enzyme activity. Obviously, compound **84** falls in the latter category, *i.e.*, it is an allosteric inhibitor. The value of the inhibition constant K_i is smaller than that of K_I, suggesting that compound **84** binds to 15-LO alone relatively more tightly than to the 15-LO/arachidonic acid **1** complex. The K_m value of arachidonic acid **1** (12 μM) is smaller than the K_I and K_i values of compound **84**, indicating that

arachidonic acid **1** has a higher affinity for the substrate binding site than compound **84** has for the allosteric site.

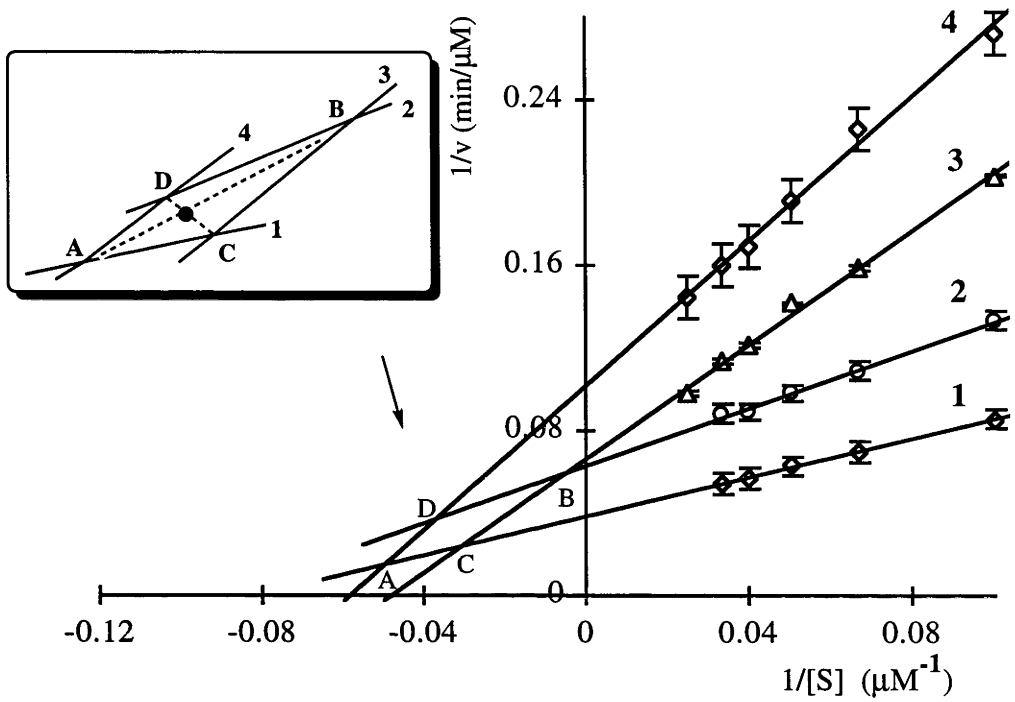


Fig. 3.13: Lineweaver-Burk plots of 15-LO catalysed oxidation of arachidonic acid **1** in the presence of compound **84**
([I] = 0, 20, 50 and 80 μM for lines 1, 2, 3 and 4, respectively)

Table 3.5 Equations for plots 1-4 of Fig. 3.13 after including the values of the common intersection point

Plot	Equation	R-Value
1	y = 1.86x + 0.095	0.9975
2	y = 1.33x + 0.070	0.9982
3	y = 0.79x + 0.057	0.9967
4	y = 0.43x + 0.041	0.9977

3.1.6. Inhibition by (8*Z*,11*Z*,14*Z*,16*E*)-18(*S*)-hydroperoxy-4-nitrotricosa -8,11,14,16-tetraenoic acid and (8*Z*,11*Z*,14*Z*,16*E*)-18(*S*)-hydroxy-4-nitrotricosa-8,11,14,16-tetraenoic acid of 15-lipoxygenase catalysed oxidation of arachidonic acid

The hydroperoxide **97** and the alcohol **98** synthesised as described above were examined as potential inhibitors of 15-LO catalysed oxidation of arachidonic acid **1**.

Compound **97** containing approximately 14% of the parent **82** and compound **98** were each mixed with arachidonic acid **1**, and the mixtures were incubated with 15-LO. The concentration of arachidonic acid **1** was kept constant (40 μM) for each reaction, and the concentrations of compounds **97** and **98** varied from 0-60 μM and 0-120 μM, respectively, with the concentration of the enzyme fixed at 1 μg/ml. The initial rates of arachidonic acid **1** oxidation were determined by measuring changes in the ultraviolet absorption at 234 nm, and the results are summarised in Table 3.6.

Table 3.6 Initial rates of 15-LO catalysed oxidation of arachidonic acid **1** in the presence of various concentrations of compound **97** or compound **98**.

Compound **97** [μM]	v (10^{-3}·AU/sec)	Compound **98** [μM]	v (10^{-3}·AU/sec)
0	5.53	0	6.45
20	4.71	40	6.27
40	3.77	80	6.11
80	3.01	120	4.48

Plotting the initial rates of arachidonic acid **1** oxidation against the concentrations of compounds **97** and **98** yields the curves shown in Fig. 3.14.

Curve A shows that increasing the concentration of compound **97** (0-60 μM) results in a proportional decrease in the rate of arachidonic acid **1** oxidation. This indicates that compound **97** inhibits 15-LO catalysed oxidation of arachidonic acid **1**. The inhibition

mode of compound **97** was not determined due to its instability, but the IC_{50} value[184] of 65 μM indicates its effectiveness.

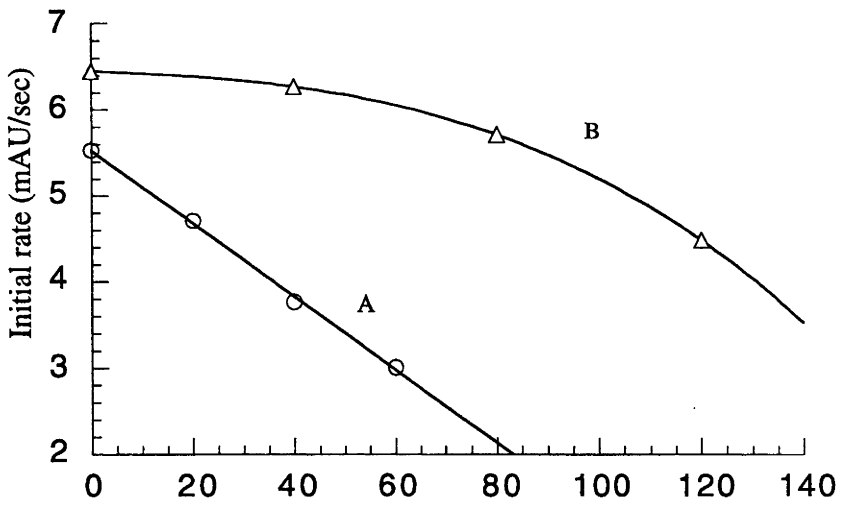


Fig. 3.14: Effect of compounds **97** (curve A) and **98** (curve B) on 15-LO catalysed oxidation of arachidonic acid **1**.

Curve B also shows decreasing oxidation rates of arachidonic acid **1** in the presence of increasing concentrations of compound **98** (0-120 μM). However, the shape of the curve is different to that expected for a standard inhibitor. Normally, the decrease in the rate of an enzyme-catalysed reaction is proportional to the concentration of the inhibitor, [I], at lower [I], with higher [I] having proportionately less effect as the active site of the enzyme becomes saturated by the inhibitor. However curve B shows disproportionately large decreases in the rate of arachidonic acid **1** oxidation with higher concentrations of compound **98**. A possible explanation for this is that the decreasing rate of arachidonic acid **1** oxidation is not due to enzyme inhibition by compound **98**, but rather due to the formation of micelles. At higher concentrations of compound **98**, lipid micelles may form and this will decrease the concentration of arachidonic acid **1** in solution and consequently the oxidation rate. Such an effect would become more pronounced with higher concentrations of compound **98**.

3.2. 5-Lipoxygenase assay

The saturated nitroalkane **55**, the unsaturated analogue **60c** and the nitro fatty acids **80**, **82**, **84** and **86** were evaluated with 5-LO.

3.2.1. Substrate behaviour of nitro compounds with 5-lipoxygenase

The unsaturated nitro compounds **60c**, **82** and **86** were investigated as potential substrates of 5-LO. 5-LO was extracted from potato tubers, and the crude extract in pH 6.3 buffer (0.1 M KH_2PO_4, 0.005% Tween 20) was used for experiments.[137] Conditions for the enzyme assays were established using arachidonic acid **1** as the substrate. Basically, arachidonic acid **1** (60 μM) was incubated with 25 μl of the 5-LO extract in pH 6.3 buffer (25 °C). The reaction was monitored for a 5 min period by ultraviolet spectroscopy at 234 nm. The progress curve showed a high background absorbance due to the turbidity of the 5-LO extract, but it was of sufficient quality for application with the other compounds.

Using the conditions employed with arachidonic acid **1**, compounds **60c**, **82** and **86** were tested as substrates of 5-LO. However, no change in ultraviolet absorbance was detected with these compounds indicating that they are not substrates. Typical results for arachidonic acid **1** and compound **60c** are shown in Fig. 3.15.

3.2.2. Effect of nitro PUFA analogues on 5-lipoxygenase catalysed oxidation of arachidonic acid

The assay used with 5-LO is similar to that developed with 15-LO and described above. The effect of the nitro compounds **55**, **60c**, **80**, **82**, **84** and **86** on 5-LO catalysed oxidation of arachidonic acid **1** was investigated by measuring the rate of reaction in the absence and presence of each of the nitro compounds **55**, **60c**, **80**, **82**, **84** and **86**. For each reaction, 25 μl of the 5-LO extract was added to 2.5 ml of 60 μM arachidonic acid **1**

in pH 6.3 buffer (0.1 KH_2PO_4, 0.005% Tween 20, 25 °C) containing one of the nitro compounds **55**, **60c**, **80**, **82**, **84** and **86**, whose concentration ranged from 40-100 μM. Ultraviolet absorbance at 234 nm was recorded for a period of 300 seconds after addition of the 5-LO.

Compounds **55**, **60c** and **84** had no effect on the change in ultraviolet absorbance at 234 nm, when compared to the reaction of arachidonic acid **1** alone. These compounds are neither inhibitors nor activators of 5-LO.

The effect of compound **82** on 5-LO catalysed oxidation of arachidonic acid **1** is shown in Fig. 3.16. The rate of arachidonic acid **1** oxidation increases, but the effect is small.

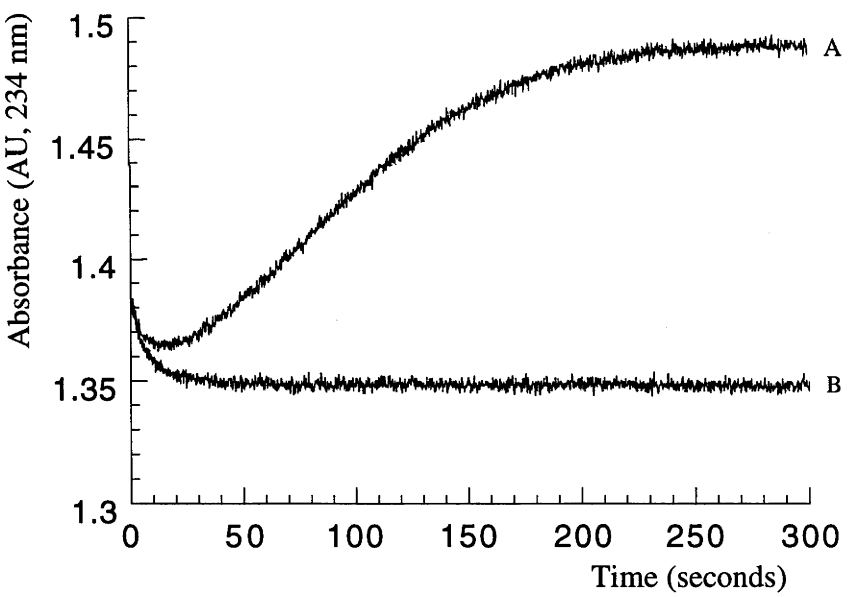


Fig. 3.15: Progress curves for the incubation of arachidonic acid **1** (curve A) and compound **60c** (curve B) with 5-LO at 60 μM concentration of each compound. (Temperature: 25 °C)

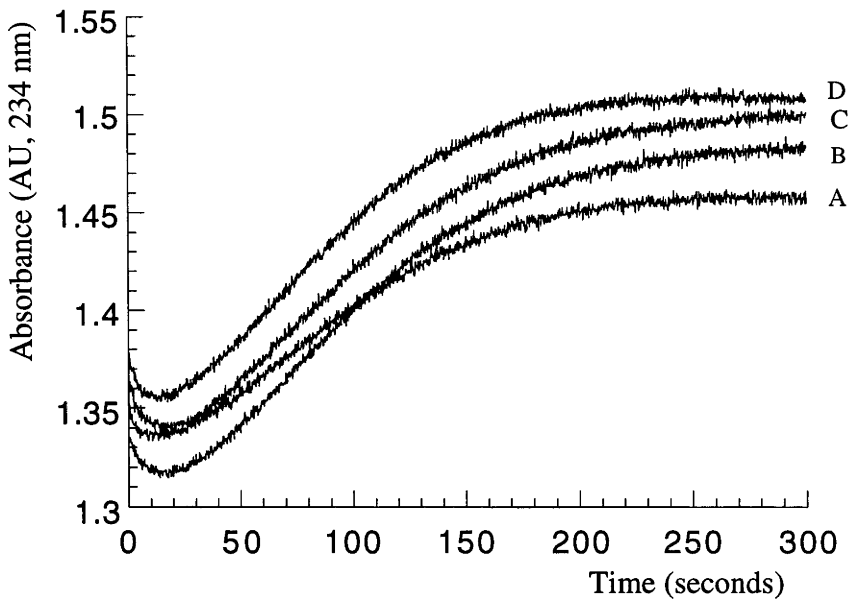


Fig. 3.16: Progress curves for 5-LO catalysed oxidation of arachidonic acid **1** (60 μM) with different concentrations of compound **82**: A, 0 μM; B, 40 μM; C, 60 μM; D, 100 μM. (Temperature: 25 °C)

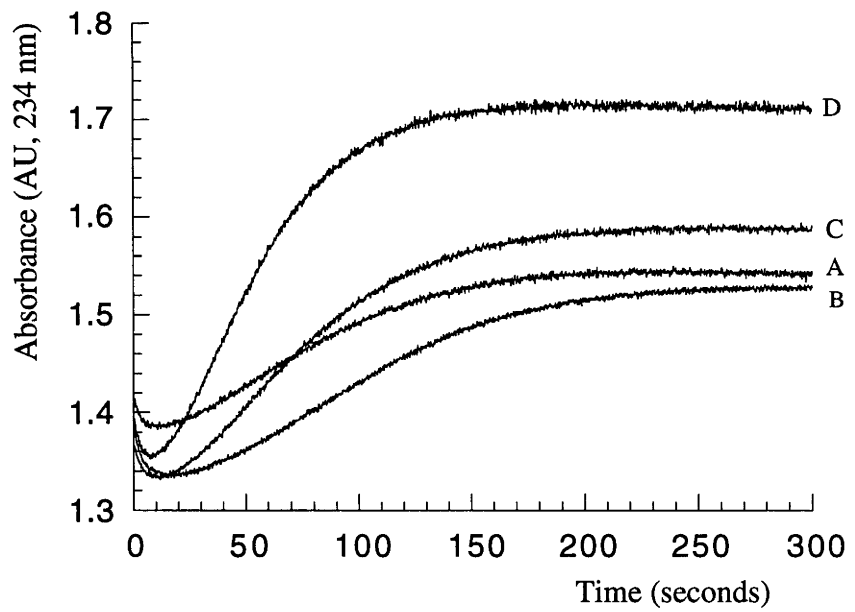


Fig. 3.17: Progress curves for 5-LO catalysed oxidation of arachidonic acid **1** (60 μM) with different concentrations of compound **86**: A, 0 μM; B, 40 μM; C, 60 μM; D, 100 μM. (Temperature: 25 °C)

Compound **86** showed more substantial activation of 5-LO catalysed oxidation of arachidonic acid **1** (Fig. 3.17). The presence of compound **86** at 100 μM almost doubled the total oxidation over the assay period and more than quadrupled the initial oxidation rate.

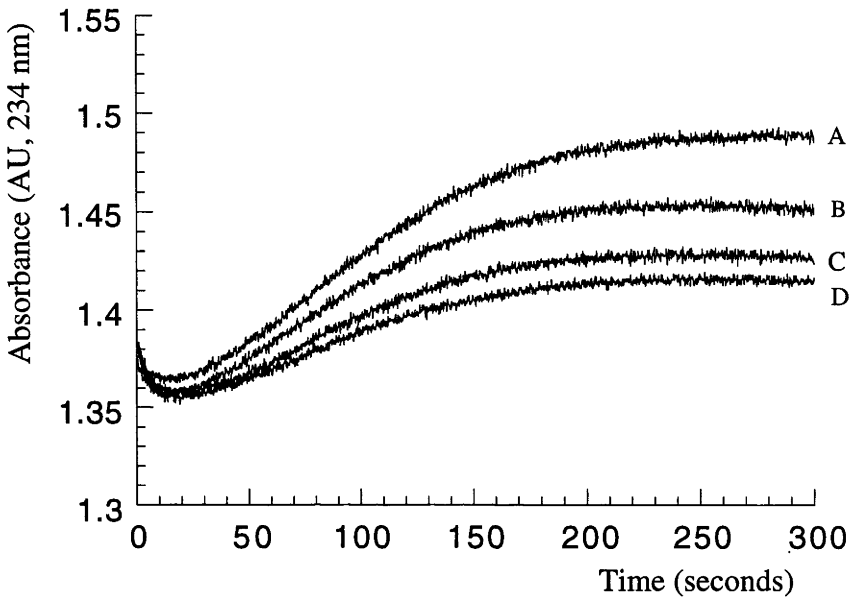


Fig. 3.18: Progress curves for 5-LO catalysed oxidation of arachidonic acid **1** (60 μM) with different concentrations of compound **80**: A, 0 μM; B, 40 μM, C, 80 μM; D, 100 μM. (Temperature: 25 °C)

Among the compounds tested, compound **80** was the only one that showed inhibition of 5-LO catalysed oxidation of arachidonic acid **1**. As shown in Fig. 3.18, the initial rates of arachidonic acid **1** oxidation and the total amount of the oxidation product decreased significantly with increasing concentrations of compound **80** from 40 to 100 μM. The IC_{50} value of compound **80** was calculated to be 90 μM. Further investigation of this inhibition was not carried out due to the instability of the 5-LO extract.

The relationship between the structures of the nitro compounds **55**, **60c**, **80**, **82**, **84** and **86** and their behaviour with 5-LO is not obvious. Nevertheless, the data obtained support

the view that potato 5-LO is an allosteric enzyme.[191] The nitro compounds **80**, **82** and **86** probably bind to the 5-LO enzyme at a different site to the substrate binding site, resulting in changes to the conformation of the enzyme that either enhance or suppress the catalytic activity.

Compound **80** inhibits potato 5-LO but not soybean 15-LO, so it may be useful for selective blocking of a specific lipoxygenase pathway. Compounds such as **82** and **86** which activate potato 5-LO are rare. However, Butovich and coworkers[192] have reported that (*R*,*S*)-2-hydroxy-2-trifluoromethyl-*trans*-octadec-4-enoic acid is a powerful activator of this enzyme.

3.3. 12-Lipoxygenase assay

3.3.1. Substrate behaviour of nitro compounds with 12-lipoxygenase

12-LO from porcine leukocytes is commercially available and its interaction with the nitro compounds **60c**, **82** and **86** was investigated. The conditions for assaying 12-LO activity were established using arachidonic acid **1**. Arachidonic acid **1** at 40 μM concentration was incubated with 50 μl of 12-LO (7.4 μg/μl) in 2.5 ml of pH 7.4 buffer (50 mM Tris-HCl, 0.03% Tween 20, 25 °C),[138] and the ultraviolet absorbance at 234 nm was recorded over 1000 seconds. Using the same conditions, but replacing arachidonic acid **1** with the unsaturated nitro compounds **60c**, **82** and **86**, their behaviour with 12-LO was determined. Typical results for arachidonic acid **1** and compounds **60c**, **82** and **86** are shown in Fig. 3.19.

No change in absorbance at 234 nm was observed with compounds **60c** and **86**, showing that these compounds are not converted to dienyl hydroperoxides by 12-LO. Compound **82** underwent 12-LO catalysed oxidation.

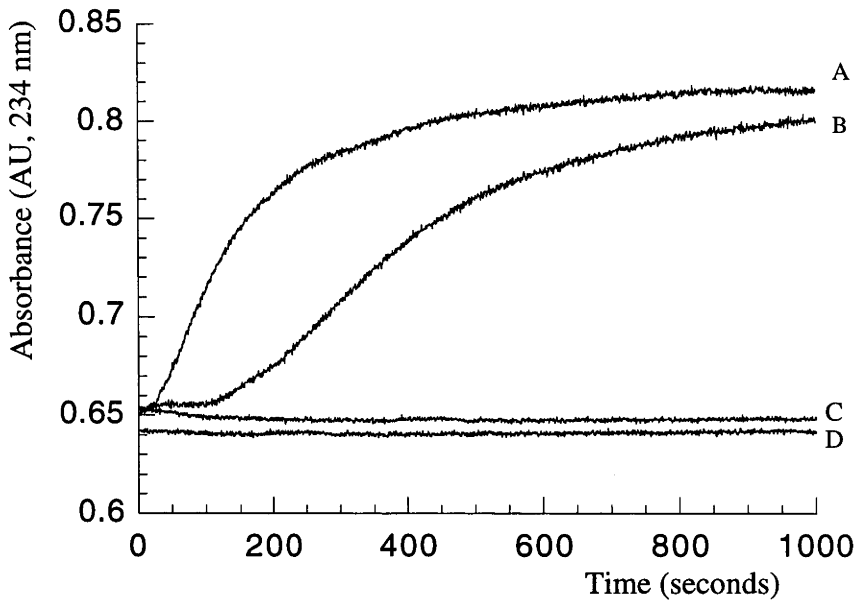


Fig. 3.19: Progress curves for 12-LO catalysed oxidation of arachidonic acid **1** (curve A), compound **82** (curve B), compound **60c** (curve C) and compound **86** (curve D) at 60 μM concentration of each compound. (Temperature: 25 °C)

3.3.2. Determination of the K_m and V_{max} values for 12-lipoxygenase catalysed oxidation of (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoic acid

In order to more fully assess the substrate behaviour of compound **82** with 12-LO, the K_m and V_{max} values were determined, using the method employed to determine the K_m and V_{max} values for 15-LO catalysed oxidation of compound **82**. It was assumed by analogy with other examples discussed above that the extinction coefficient of the hydroperoxide formed from compound **82** is 26000 M^{-1} cm^{-1}. The data used to calculate the K_m and V_{max} values are summarised in Table 3.7.

As described above, the values of K_m and V_{max} were obtained from the slope and the intercept of the Hanes plot shown in Fig. 3.20, and they are 15 μM and 1.52 min^{-1}, respectively. The K_m value has been reported for arachidonic acid **1** with 12-LO from

human and murine glomeruli, as 44 μM.[193] The smaller K_m value of compound **82** with porcine leukocyte 12-LO indicates a higher binding affinity. However, the K_m value for arachidonic acid **1** has not been reported using 12-LO from this source, nor was it determined in the present work due to the limited amount of enzyme available. Therefore, a more direct comparison could not be made.

Table 3.7 Data used to calculate the K_m and V_{max} values for 12-LO catalysed oxidation of compound **82** ($\varepsilon = 26000\ M^{-1}\ cm^{-1}$, [E] = 0.15 μg/μl, 25 °C)

[S] (μM)	v (μM/min)	[S]/v (min^{-1})
5	0.42	11.90
10	0.65	15.38
30	0.93	32.26
40	1.03	38.83
60	1.24	48.39
80	1.30	61.54

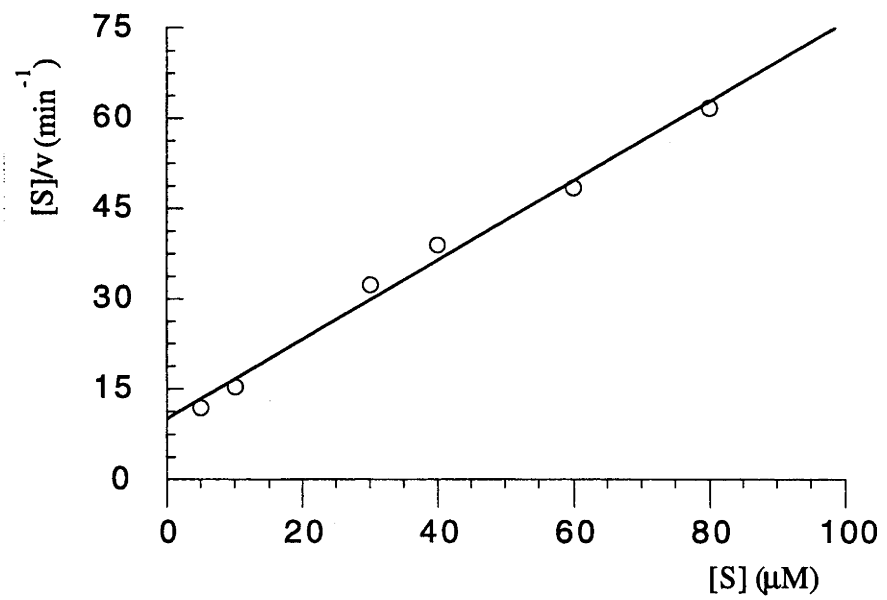


Fig. 3.20: The Hanes plot of 12-LO catalysed oxidation of compound **82**.

3.3.3. Effect of nitro PUFA analogues on 12-lipoxygenase catalysed oxidation of arachidonic acid

Preliminary experiments were carried out to investigate the effects of the nitro compounds **55**, **60c**, **80**, **84** and **86** on 12-LO catalysed oxidation of arachidonic acid **1**. Arachidonic acid **1** (60 μM) was mixed with an equimolar amount of each of the compounds **55**, **60c**, **80**, **84** and **86** in pH 7.4 buffer (50 mM Tris-HCl, 0.03% Tween 20, 25 °C), and the mixtures were incubated with 12-LO (0.15 μg/μl). The reactions were monitored by ultraviolet spectroscopy at 234 nm.

Oxidation of arachidonic acid **1** was not affected by the presence of compounds **55** and **60c**, indicating that these compounds do not interact with 12-LO.

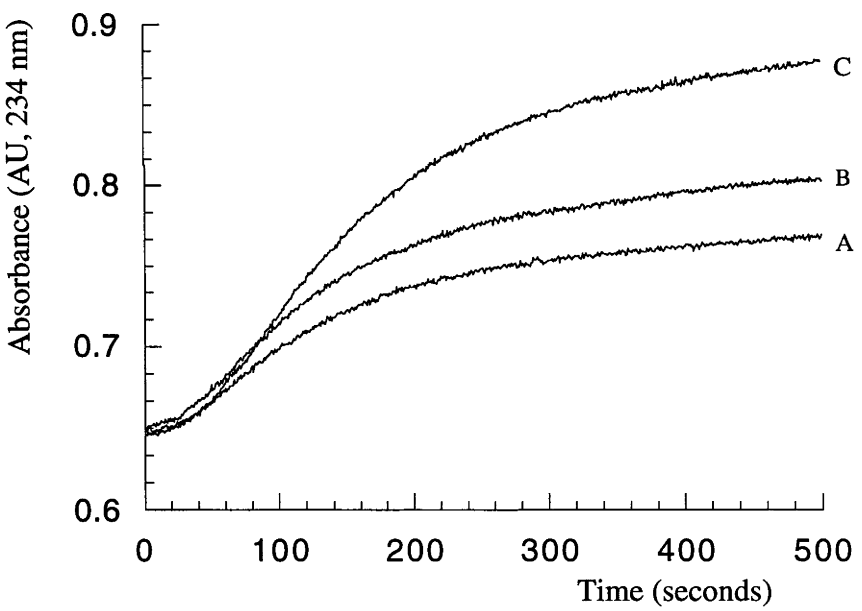


Fig. 3.21: Effect of compounds **80** and **84** on 12-LO catalysed oxidation of arachidonic acid **1**: A, arachidonic acid **1** alone (60 μM);
B, arachidonic acid **1** (60 μM) in the presence of compound **80** (60 μM);
C, arachidonic acid **1** (60 μM) in the presence of compound **84** (60 μM).
(Temperature: 25 °C)

As shown by the progress curves in Fig. 3.21, the addition of compounds **80** and **84** significantly increased the rate of product formation. Since compounds **80** and **84** are not themselves substrates for 12-LO, this suggests that they enhance 12-LO catalysed oxidation of arachidonic acid **1**.

Compound **86** substantially inhibited 12-LO catalysed oxidation of arachidonic acid **1**. The inhibition was complete under the assay conditions when a 10 μM concentration of compound **86** was used but at lower concentrations the inhibition was only temporary and the delay before recovery of 12-LO activity increased with increasing concentrations of compound **86** (Fig. 3.22). A reasonable explanation for this phenomenon is that compound **86** is a substrate as well as an inhibitor of the enzyme and the metabolic product does not inhibit 12-LO.

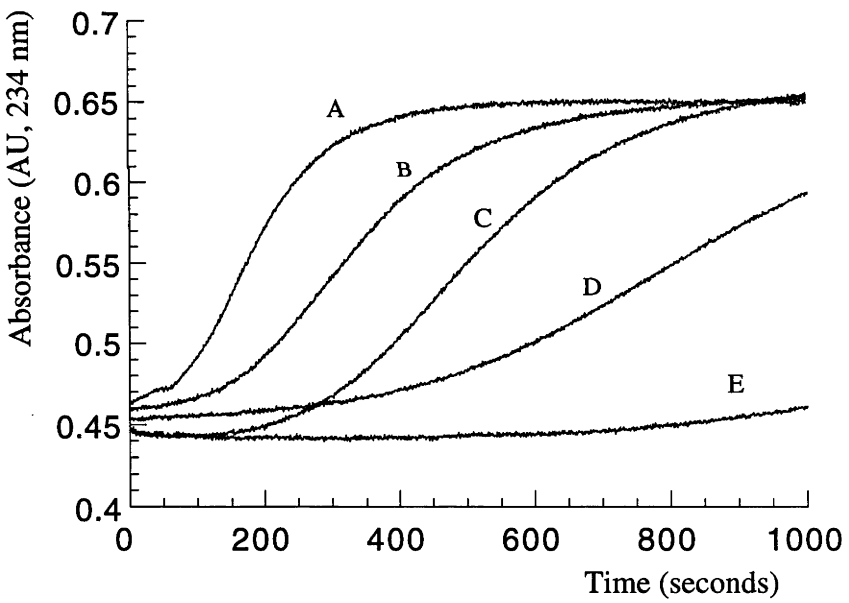


Fig. 3.22: Effect of compound **86** at different concentrations on 12-LO catalysed oxidation of arachidonic acid **1** (60 μM):

A, 0 μM; B, 2.5 μM; C, 5 μM; D, 8 μM; E, 10 μM. (Temperature: 25 °C)

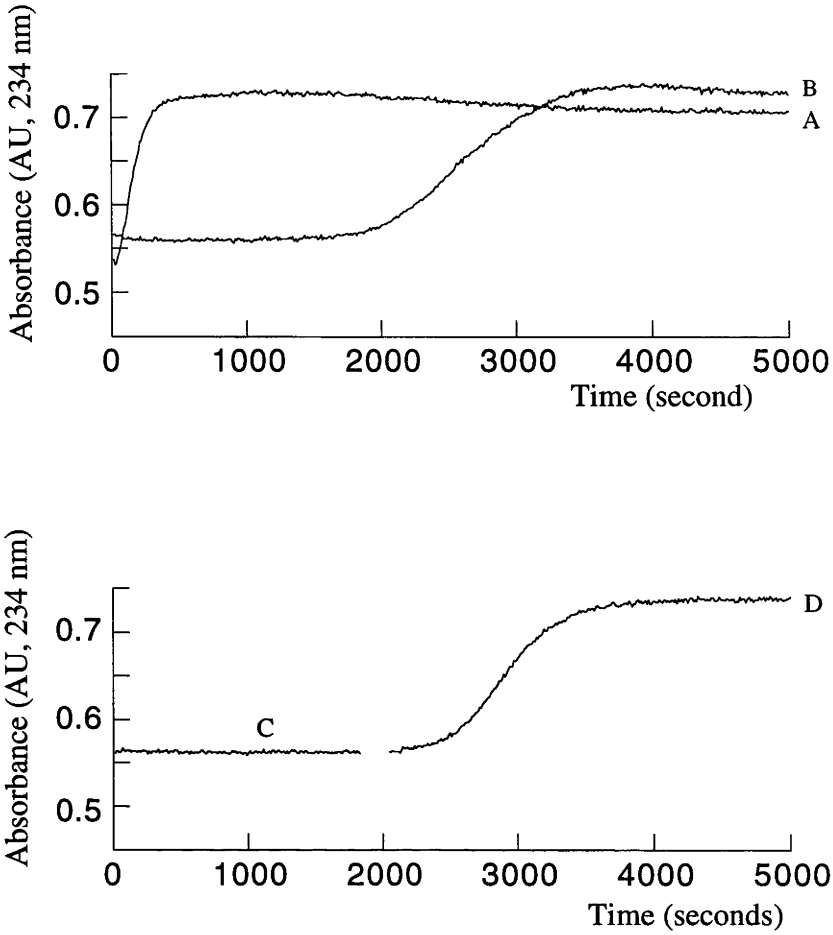


Fig. 3.23: Effect of compound **86** on 12-LO catalysed oxidation of arachidonic acid **1**:

A, oxidation of arachidonic acid **1** alone (60 μM);

B, oxidation of arachidonic acid **1** (60 μM) in the presence of compound **86** (20 μM);

C, incubation of compound **86** (20 μM) with 12-LO;

D, followed by addition of arachidonic acid **1** into the compound **86** / 12-LO reaction mixture that had already proceeded for 1800 seconds.

(Temperature: 25 °C)

In order to substantiate this hypothesis, further experiments were performed. Arachidonic acid **1** (60 μM) and compound **86** (20 μM) were mixed before incubation with 12-LO (0.15 μg/μl). The reaction was monitored by ultraviolet spectroscopy at 234 nm for 5000

seconds. As shown by curve B in Fig. 3.23, the lag time of the reaction under these conditions was approximately 1800 seconds. In another experiment, compound **86** (20 μM) was first incubated alone with the enzyme (0.15 μg/μl) for 1800 seconds, during which the progress curve (C) showed no increase in absorbance. Arachidonic acid **1** (60 μM) was then added to the mixture, and the reaction was continued to be monitored. As shown by curve D, the oxidation of arachidonic acid **1** then occurred almost immediately. In addition, the combined change in absorbance of curves C and D is similar to that of curves A and B. This confirms that compound **86** is indeed metabolised by 12-LO, and the product does not inhibit the enzyme.

3.3.4. HPLC analysis of 12-lipoxygenase mediated metabolism of 4-[(*all-Z*)-nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dicarboxylic acid

To further confirm metabolism of compound **86** by 12-LO, the diacid **86** (0.3 mg) and lauric acid (2 mg) as an internal reference were dissolved in 1 ml of pH 7.4 buffer (50 mM Tris-HCl, 0.03% Tween 20), and 1 ml of 12-LO solution (7.4 μg/μl) was then added. A 0.8 ml aliquot of the mixture was withdrawn after 5 min and then again after 4 h incubation. These were extracted with diethyl ether. After concentration of the extracts, the residues were analysed by HPLC. The results are shown in Fig. 3.24 and 3.25. After 5 min incubation of compound **86** with 12-LO, the ratio of peak areas for lauric acid and compound **86** was 1.05 (Fig. 3.24). After 4 h incubation, however, compound **86** had almost completely gone (Fig. 3.25) and the ratio increased to approximately 25. This indicates that the majority (96%) of compound **86** was metabolised after the 4 h incubation with 12-LO under the experimental conditions. However, the metabolic product was not observed by HPLC, so it could not be identified.

HPLC analysis was also carried out on a mixture of lauric acid and compound **86** in the absence of 12-LO. The ratio of peak areas was then unchanged during 4 h incubation, indicating that compound **86** does not react in the absence of the enzyme.

It would be interesting to identify the metabolic product of compound **86**. However, this was not feasible because of the expense of 12-LO.

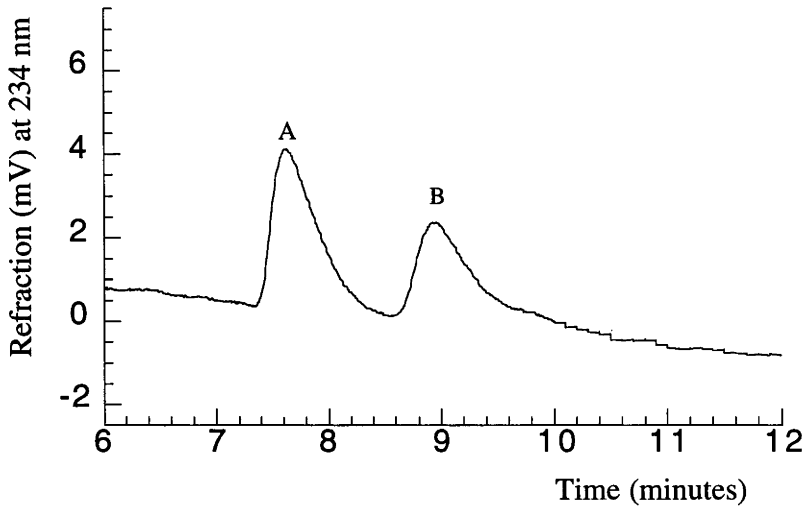


Fig. 3.24: HPLC spectrum of a mixture of lauric acid (A) (80 μg/μl) and compound **86** (B) (12 μg/μl) after 5 min incubation with 12-LO (0.3 μg/ml).

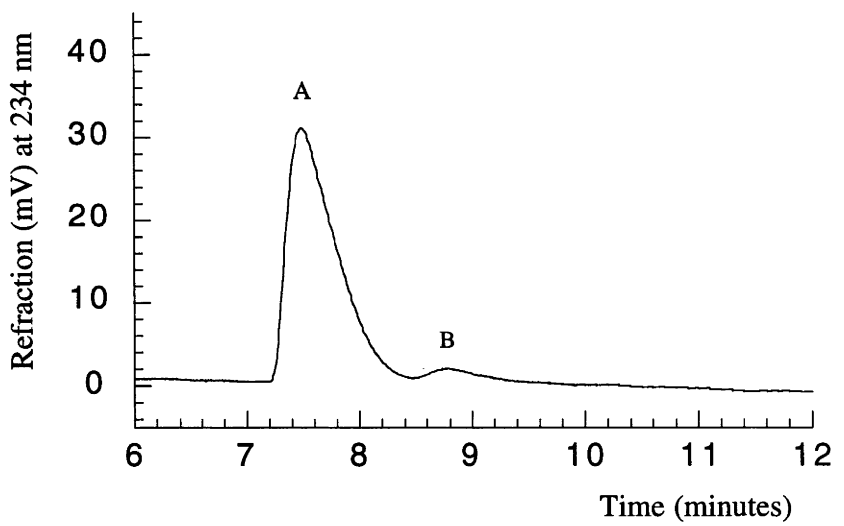


Fig. 3.25: HPLC spectrum of a mixture of lauric acid (A) (100 μg/μl) and compound **86** (B) (15 μg/μl) after 4 h incubation with 12-LO (0.4 μg/ml).

Chapter 4

Results and Discussion

Antioxidant Behaviour of Thia Fatty Acids

As mentioned in the introduction, fatty acid autoxidation is one of the three main pathways for PUFA destruction. Compounds with antioxidant properties have potential pharmaceutical value and therefore have been extensively pursued. As described in the introduction, previous work conducted in this laboratory showed that among synthetic β-oxa fatty acids, and β-thia and γ-thia fatty acids analysed, the thia fatty acid, 3-[(*all-Z*)-(eicosa-5,8,11,14-tetraenylthio)]propionic acid **19**, exhibited strong inhibition of arachidonic acid **1** autoxidation.[139] This suggests that such thia fatty acids may be very useful as antioxidants. The aim of this work was to explore the relationship between the structural components of thia fatty acids and their antioxidant behaviour.

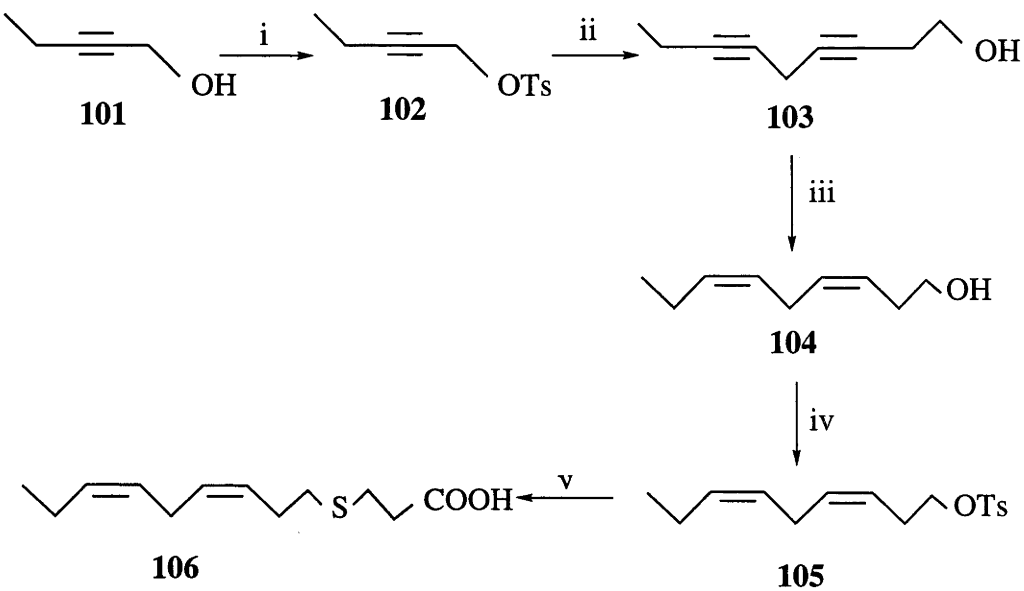
4.1. Synthesis of analogues of 3-[(*all-Z*)-(eicosa-5,8,11,14-tetraenylthio)]propionic acid

The strategy for investigating the roles of the structural components of compound **19** in its antioxidant behaviour was to first synthesise a series of analogues and then to examine their antioxidant activity. The analogues were designed such that they could be used to examine specifically the importance of the carboxyl group, the position and extent of unsaturation, and the distances between the sulfur, the carboxyl group and the unsaturation.

4.1.1. Synthesis of 3-[(3Z,6Z)-nona-3,6-dienylthio]propionic acid

3-[(3Z,6Z)-Nona-3,6-dienylthio]propionic acid **106** contains two methylene-interrupted double bonds with the dienyl moiety closer to the sulfur than is the case in compound **19**. It was designed to probe the effect of the distance between the dienyl moiety and the sulfur on the antioxidant behaviour.

Compound **106** was synthesised following the procedure shown in the Scheme 4.1.



i: *p*-toluenesulfonyl chloride, pyridine, chloroform, 0 °C, 4 h

ii: but-3-yn-1-ol, Na_2CO_3, CuI, *n*-Bu_4NCl, -30 °C, RT, 24 h

iii: Pd / $CaCO_3$ / Pb, quinoline, methanol, hydrogen, 2.5 h

iv: *p*-toluenesulfonyl chloride, pyridine, chloroform, 15 °C, 24 h

v: 3-mercaptopropionic acid, sodium methoxide, 40 °C, 48 h

Scheme 4.1

Tosylation of alcohols is a common transformation which is often used to facilitate subsequent nucleophilic substitution reactions. In the present work, 2-pentyn-1-ol **101** was converted to its tosylate **102** based on a reported method.[194] The alcohol **101** was

treated with *p*-toluenesulfonyl chloride and pyridine in distilled chloroform at 0 °C with the ratio of the alcohol **101** / tosyl chloride / pyridine as 1 : 1.5 : 2. After 4 h, the mixture was washed with 1N HCl, 5% $NaHCO_3$ and water, then it was dried with Na_2SO_4, and the solvent was removed. The residue was purified by flash column chromatography to give pent-2-ynyl *p*-toluenesulfonate **102** in 65% yield.

The structure of compound **102** was confirmed by NMR spectral analysis. The ^1H NMR spectrum contains the characteristic peak at δ4.69 which represents the protons of the methylene flanked by the alkyne and the *p*-toluenesulfonate ester, the peaks at δ7.35 and 7.82 which correspond to aromatic protons, and the singlet at δ2.44 which is consistent with the protons of the methyl group attached to the aromatic ring. The ^{13}C NMR spectrum of compound **102** exhibits peaks at δ128.69, 130.30, 133.90 and 145.47 corresponding to the four types of aromatic carbons. The alkyne carbons are represented by peaks at δ71.72 and 92.33, and the carbon adjacent to oxygen is reflected by the peak at δ59.35.

The 1,4- or skipped-(Z,Z)-diene unit represents a key feature of many biologically active natural products (*e.g.*, arachidonic acid **1** contains consecutive skipped-(Z,Z)-diene units). Different routes for synthesis of these types of compounds have been reported.[195-197] A classical procedure for the synthesis of arachidonic acid **1** and other *cis*-polyenes was introduced by Osbond *et al.*,[198] in which the corresponding polyacetylenic acids were first prepared, followed by selective hydrogenation of the acetylenic bonds to give rise to *cis*-olefins. The polyacetylenic compounds were built up through successive condensations of acetylenic Grignard reagents with propargylic bromides. Alternatively, Jeffery *et al.*[199] reported the treatment of propargylic halides and tosylates with 1-alkynes, in the presence of copper(I) iodide, sodium carbonate and tetra-n-butylammonium chloride (*n*-Bu_4NCl), in *N*,*N*-dimethylformamide or acetonitrile, without formation of acetylenic Grignard intermediates. In this manner, functionalised skipped diynes were prepared without protection and deprotection of functional groups and were formed without by-product isomeric alkenes. As shown in Scheme 4.1, the

same approach and reaction conditions were employed in this work to synthesise nona-3,6-diyn-1-ol **103**. The reagents, including pent-2-ynyl *p*-toluenesulfonate **102** (1.1 eq.), but-3-yn-1-ol (1 eq.), CuI (1 eq.), *n*-Bu_4NCl (1 eq.) and Na_2CO_3 (1.5 eq.), were mixed at -20 °C and the mixture was stirred for 24 h at room temperature. The product **103** was obtained in 62% yield, after being purified by flash column chromatography.

In the ^1H NMR spectrum of compound **103**, the triplet at δ3.69 and the broad peak at δ1.96 are characteristic of an alcohol, corresponding to the protons of the methylene adjacent to the hydroxyl and the hydroxyl proton, respectively. The multiplet at δ3.12 is consistent for the methylene flanked by two alkynes. The ^{13}C NMR spectrum of compound **103**, which shows peaks at δ75.08, 76.83, 78.46, 82.42 and 61.95, further confirms the existence of two alkyne moieties and an alcohol. In the infrared spectrum of compound **103**, a broad absorption at 3650-3100 cm^{-1} is observed, which is consistent with the alcohol O-H stretch.

Lead-poisoned palladium on calcium carbonate (Lindlar catalyst) is frequently used for selective conversion of alkynes to *cis*-alkenes.[200] Thus, as shown in Scheme 4.1, compound **103** was readily converted to (3*Z*,6*Z*)-nona-3,6-dien-1-ol **104** by hydrogenation in the presence of quinoline in methanol over the Pd / $CaCO_3$ / Pb catalyst under atmospheric pressure. Compound **104** was purified by flash column chromatography and obtained in a yield of 92%. In the ^1H NMR spectrum of compound **104**, a multiplet at δ3.12 from the starting material **103** was replaced by a multiplet at δ2.81, which corresponds to the protons of the doubly allylic carbon. There are also multiplets at δ5.27-5.56, which represent the olefinic protons. The ^{13}C NMR spectrum of compound **104** shows characteristic peaks at δ125.90, 127.40, 132.04 and 132.74 corresponding to the four carbons that comprise the two double bonds, and a peak at δ62.77 due to the carbon adjacent to the hydroxyl group. The mass spectrum of compound **104** shows a molecular ion at *m/e* 140, which is consistent with the molecular formula $C_9H_{16}O$.

Based on the procedure and the conditions shown in Scheme 4.1, (3*Z*,6*Z*)-nona-3,6-dienyl *p*-toluenesulfonate **105** was synthesised. The reaction was initially conducted using the conditions employed to prepare compound **102**, but the reaction of the alcohol **104** was found to be substantially slower. After 4 h at 0 °C, TLC analysis showed that only half of the starting material **104** had reacted. The reaction conditions were subsequently modified by increasing the amount of pyridine from 1 eq. to 4 eq., the reaction temperature from 0 to 15 °C, and by extending the reaction time from 4 h to 24 h. TLC analysis showed that under these conditions the yield of the product improved, although the conversion was still incomplete. After purification by flash column chromatography, (3*Z*,6*Z*)-nona-3,6-dienyl *p*-toluenesulfonate **105** was obtained in 57% yield, accompanied by a recovery of 9% of compound **104**. Compared with the ^{1}H NMR spectrum of compound **104**, that of compound **105** shows a characteristic peak at δ4.00 in place of the peak at δ3.64, both of which represent the methylene protons at C1. In addition, the feature of the toluenesulfonate ester is reflected by peaks at δ7.33, 7.80 and 2.45, which correspond to the aromatic and benzylic methyl protons. In the ^{13}C NMR spectrum of compound **105**, peaks at δ123.53, 126.94, 128.47, 130.37, 132.61, 132.92 and 145.28 are consistent with the presence of the olefinic and aromatic carbons. A peak at δ70.20 represents the C1 carbon.

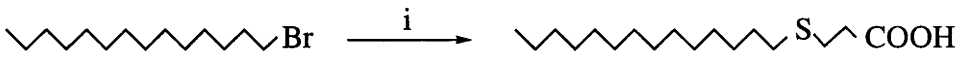
3-[(3*Z*,6*Z*)-Nona-3,6-dienylthio]propionic acid **106** was synthesised following the procedure illustrated in Scheme 4.1, which is a modification of the method described by Pitt *et al.*[21] Compound **105** was treated with 3-mercaptopropionic acid in a solution containing sodium methoxide at 40 °C for 48 h. After purification by flash column chromatography, compound **106** was obtained in a yield of 41%. The ^{1}H NMR spectrum of compound **106** displays peaks at δ2.59 and 2.67, which are characteristic of the protons of the methylenes adjacent to the sulfur, a multiplet at δ2.80 which corresponds to the protons of the methylene adjacent to the carboxyl group and the protons of the methylene flanked by the two double bonds, and peaks at δ2.07 and 2.36 corresponding to the protons at the allylic carbons C2 and C8. The ^{13}C NMR spectrum of compound **106** contains peaks at δ127.37, 127.97, 130.53 and 132.72 corresponding

to the olefinic protons, and a peak at δ178.66 due to the carbon of the carboxyl group. The existence of the carboxyl group is further confirmed by the infrared spectrum, which shows a broad absorption at 3400-2500 cm^{-1} for the O-H stretch.

The configuration of the double bonds in compounds **104-106** can be assigned based on the chemical shifts of the allylic carbon signals in their ^{13}C NMR spectra because it has been reported[201] that the signal for carbon adjacent to two *cis*-double bonds, two *trans*-double bonds and *cis/trans*-double bonds is observed at δ25-26, δ35-36 and δ30-31, respectively. The chemical shifts for the doubly allylic carbons in compounds **104-106** are 26.20, 26.12 and 26.20, respectively, indicating that the two double bonds in each compound have the *cis*-configuration.

4.1.2. Synthesis of 3-tetradecylthiopropionic acid

In order to examine the effect of the unsaturation of compound **19** on its antioxidant behaviour, the saturated γ-thia fatty acid, 3-tetradecylthiopropionic acid **108**, was synthesised.



107 **108**

i: 3-mercaptopropionic acid, sodium methoxide, RT, 16 h

Scheme 4.2

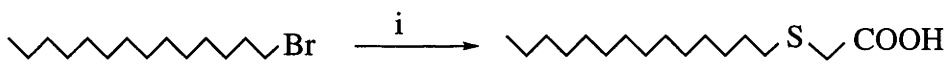
The procedure and reaction conditions employed for the synthesis of 3-tetradecylthiopropionic acid **108** are the same as those described in the literature,[21] which are shown in Scheme 4.2. In this method, 1-bromotetradecane **107** was treated with 3-mercaptopropionic acid in a solution containing sodium methoxide at room temperature. The reaction proceeded for 16 h, and the product **108** was obtained in a 73% yield after purification by flash column chromatography.

The ^{1}H NMR spectrum of compound **108** contains peaks at δ2.54 and 2.67, which correspond to the protons of the methylenes adjacent to the sulfur, and a peak at δ2.79 which is due to the protons of the methylene adjacent to the carboxyl group. The ^{13}C NMR spectrum of compound **108** shows a peak at δ178.50 which is characteristic of the carboxyl carbon. The existence of the carboxyl group is further indicated by the infrared spectrum, which shows a broad absorption at 3200-2600 cm^{-1} for the carboxyl group O-H stretch.

4.1.3. Synthesis of 2-tetradecylthioacetic acid

The unsaturated β-thia fatty acid **18** has no antioxidative activity, unlike the unsaturated γ-thia fatty acid **19**.[139] This suggests that the location of the sulfur with respect to the carboxyl group is important to the antioxidant activity. In order to examine this, the saturated β-thia fatty acid, 2-tetradecylthioacetic acid **109**, was synthesised.

The synthesis of 2-tetradecylthioacetic acid **109** followed a similar procedure to that used to obtain compound **108** except that 3-mercaptopropionic acid was replaced with 2-mercaptoacetic acid. The procedure is shown in Scheme 4.3.



107 **109**

i: 2-mercaptoacetic acid, sodium methoxide, RT, 16 h

Scheme 4.3

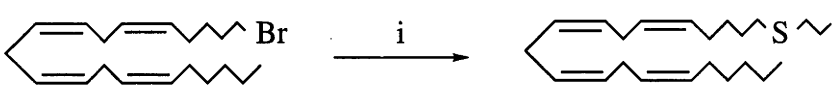
1-Bromotetradecane **107** reacted with 2-mercaptoacetic acid in a solution containing sodium methoxide for 16 h. After being purified by flash column chromatography, compound **109** was obtained in a 77% yield.

The structure of compound **109** was determined by NMR and infrared spectral analyses. The ^{1}H NMR spectrum contains a singlet at δ3.26 corresponding to the protons of the methylene adjacent to the carboxyl group, and a triplet at δ2.66 due to the protons of the other methylene adjacent to sulfur. The ^{13}C NMR spectrum exhibits a peak at δ177.57 which is characteristic of the carboxyl carbon. The existence of the carboxyl group is confirmed by the infrared spectrum, which shows a broad absorption at 3200-2600 cm^{-1} for the O-H stretch.

4.1.4. Synthesis of propyl (*all-Z*)-eicosa-5,8,11,14-tetraenyl sulfide

The role of the carboxyl group of the γ-thia fatty acid **19** in its antioxidant behaviour was investigated by synthesising the analogue **110**, which retains the four *cis*-double bonds and the sulfur, but has no carboxyl group.

Using the procedure described for the preparation of 3-tetradecylthiopropionic acid **108**, compound **110** was synthesised in a 15 h reaction of the (*all-Z*)-1-bromo-5,8,11,14-eicosatetraene **58c** with propanethiol and sodium methoxide (Scheme 4.4). After purification by flash column chromatography, compound **110** was obtained in a 75% yield.



58c **110**

i: propanethiol, sodium methoxide, RT, 15 h

Scheme 4.4

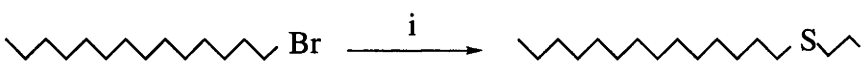
Compared with the ^{1}H NMR spectrum of compound **58c**, that of compound **110** contains similar peaks at δ5.37, 2.82 and 2.07, which correspond to the olefinic protons,

the protons of the doubly allylic carbons and those of the allylic carbons, respectively. Other peaks appear which are characteristic for compound **110**, including one at δ2.51 corresponding to the protons of the two methylenes adjacent to the sulfur and triplets at δ0.89 and 0.99 representing the protons of the terminal methyl groups at C20 and C3', respectively. The presence of these methyl groups is confirmed by the ^{13}C NMR resonances at δ14.13 and 14.67. The mass spectrum of compound **110** shows a molecular ion at *m/e* 348, consistent with the molecular formula $C_{23}H_{40}S$.

4.1.5. Synthesis of propyl tetradecyl sulfide

Compound **111** is similar to compound **110** except that it has no double bonds. It was therefore of interest in examining the role of the sulfur in the absence of both unsaturation and the carboxyl group.

The procedure employed for the synthesis of propyl tetradecyl sulfide **111** is shown in Scheme 4.5, and is the same as that shown in Scheme 4.4 for the synthesis of compound **110**. 1-Bromotetradecane **107** reacted with propanethiol in a solution containing sodium methoxide for 15 h, to give propyl tetradecyl sulfide **111** in 89% yield after purification by flash column chromatography.



107 **111**

i: propanethiol, sodium methoxide, RT, 15 h

Scheme 4.5

The structure of compound **111** was determined by NMR and mass spectral analyses. The ^{1}H NMR spectrum shows a multiplet at δ2.49 which corresponds to the protons of the two methylenes adjacent to the sulfur, and triplets at δ0.87 and 0.99 which represent

the protons of the methyl groups at C14 and C3'. In the ^{13}C NMR spectrum, peaks corresponding to the carbons of the methyl groups are observed at δ14.13 and 14.71. The electron ionisation mass spectrum exhibits a molecular ion at *m/e* 272, consistent with the molecular formula $C_{17}H_{36}S$.

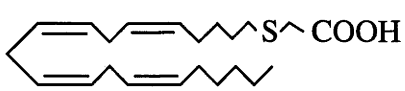
4.2. Effects of thia polyunsaturated fatty acids and sulfides on autoxidation of arachidonic acid

Having prepared analogues of compound **19**, the subsequent aim was to investigate their effects on autoxidation of arachidonic acid **1**. Based on previous work,[139] a thin film method was employed for this purpose, in conjunction with a reverse phase HPLC technique for analysis of the recovered arachidonic acid **1** and thia PUFAs and sulfides, with lauric acid as an internal standard.

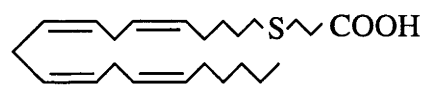
Autoxidation of arachidonic acid **1** was conducted in the presence of compound **19** and lauric acid, using the procedure described by Robertson.[139] Stock solutions of arachidonic acid **1**, compound **19** and lauric acid in dichloromethane with equal concentrations were added to a 25 ml round-bottomed flask, and the solvent was evaporated to leave a thin film on the internal surface of the flask. The flask was then filled with oxygen and kept in the dark. The percentages of arachidonic acid **1** and compound **19** remaining after 7 days were measured by HPLC. The same assay was carried out simultaneously with several flasks but the results were not reproducible. The variation was attributed to differences in oxygen concentration and the surface area of the thin films formed in the flasks. Therefore, in order to establish a reproducible assay for analysis of the autoxidation of arachidonic acid **1**, Petri-dishes with uniform size (80 mm in diameter) were used instead of flasks for thin film formation and the oxidation was carried out by placing the Petri-dishes in a desiccator filled with oxygen. To assess this method, thin films of arachidonic acid **1** with lauric acid as a standard were prepared on six Petri-dishes using identical treatment, and then subjected to oxidation in the same desiccator filled with oxygen. After 24 h, the percentage of arachidonic acid **1** recovered

following autoxidation in each Petri-dish was determined by HPLC. The results showed that the variations in the data obtained for the six samples were smaller than 7%. The advantage of using a Petri-dish over a flask is that the thin films on each Petri-dish are spread over the same area, and each Petri-dish is exposed to oxygen to the same extent.

Using the Petri-dish assay method, the effects of the thia PUFAs and sulfides **106** and **108-111**, along with compounds **18**, **19** and 3-[(*Z*,*Z*,*Z*)-(octadeca-9,12,15-trienylthio)]propionic acid **112** which were available in the laboratory, on the autoxidation of arachidonic acid **1**, were examined. In addition, the stability of these compounds in the presence of arachidonic acid **1** was also investigated. Arachidonic acid **1** and lauric acid as a standard were mixed with each sulfur compound at different ratios and the mixtures were subjected to thin film autoxidation. The mixtures were analysed by HPLC after 1, 2, 3, 5 and 7 days. The results are summarised in Tables 4.1-4.9 below. The yields given in the tables are the mean values of at least duplicate experiments, which showed good reproducibility with standard errors within ±12%.



18



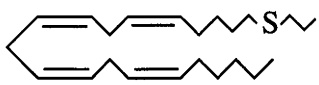
19

106

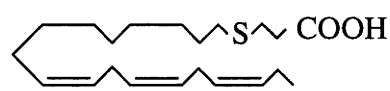
108

109

110



111



112

Table 4.1 Percentage of arachidonic acid **1** recovered following autoxidation

Autoxidation time (days)	Arachidonic acid **1** (%)
1	91
2	20
3	16
5	12
7	N.D.

N.D.- None detectable

Table 4.2 Percentages of arachidonic acid **1** and compound **18** recovered following autoxidation

using a ratio of arachidonic acid **1** and compound **18** of 1:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **18** (%)
1	68	26
2	29	7
3	10	N.D.
5	7	N.D.
7	5	N.D.

Table 4.3 Percentages of arachidonic acid **1** and compound **19** recovered following autoxidation

A: using a ratio of arachidonic acid **1** and compound **19** of 1:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **19** (%)
1	99	99
2	100	100
3	99	99
5	99	98
7	98	99

B: using a ratio of arachidonic acid **1** and compound **19** of 2:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **19** (%)
1	100	98
2	99	96
3	100	96
5	98	94
7	97	90

C: using a ratio of arachidonic acid **1** and compound **19** of 2:1, with AIBN at 10% the amount of arachidonic acid **1**

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **19** (%)
1	100	98
2	98	95
3	94	80
5	49	47
7	35	43

D: using a ratio of arachidonic acid **1** and compound **19** of 10:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **19** (%)
1	99	95
2	99	87
3	87	38
5	42	N.D.
7	17	N.D.

Table 4.4 Percentages of arachidonic acid **1** and compound **106** recovered following autoxidation

A: using a ratio of arachidonic acid **1** and compound **106** of 1:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **106** (%)
1	99	98
2	98	96
3	99	98
5	100	97
7	101	98

B: using a ratio of arachidonic acid **1** and compound **106** of 2:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **106** (%)
1	98	97
2	99	98
3	98	97
5	99	97
7	99	100

C: using a ratio of arachidonic acid **1** and compound **106** of 10:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **106** (%)
1	101	94
2	101	84
3	99	59
5	79	N.D.
7	16	N.D.

Table 4.5 Percentages of arachidonic acid **1** and compound **108** recovered following autoxidation

using a ratio of arachidonic acid **1** and compound **108** of 1:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **108** (%)
1	98	99
2	93	91
3	79	86
5	30	44
7	N.D.	37

Table 4.6 Percentages of arachidonic acid **1** and compound **109** recovered following autoxidation

using a ratio of arachidonic acid **1** and compound **109** is 1:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **109** (%)
1	53	50
2	10	16
3	N.D.	17
5	N.D.	17
7	N.D.	16

Table 4.7 Percentages of arachidonic acid **1** and compound **110** recovered following autoxidation

A: using a ratio of arachidonic acid **1** and compound **110** of 1:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **110** (%)
3	100	99
7	100	100

B: using a ratio of arachidonic acid **1** and compound **110** of 10:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **110** (%)
1	97	85
2	89	22
3	67	N.D.
5	24	N.D.
7	10	N.D.

Table 4.8 Percentages of arachidonic acid **1** and compound **111** recovered following autoxidation

A: using a ratio of arachidonic acid **1** and compound **111** of 1:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **111** (%)
1	100	101
2	98	91
3	98	92
5	98	92
7	97	90

B: using a ratio of arachidonic acid **1** and compound **111** of 10:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **111** (%)
1	99	81
2	99	63
3	97	38
5	68	N.D.
7	17	N.D.

Table 4.9 Percentages of arachidonic acid **1** and compound **112** recovered following autoxidation

A: using a ratio of arachidonic acid **1** and compound **112** of 1:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **112** (%)
1	100	100
2	99	98
3	98	97
5	99	97
7	93	86

B: using a ratio of arachidonic acid **1** and compound **112** of 10:1

Autoxidation time (days)	Arachidonic acid **1** (%)	Compound **112** (%)
1	82	25
2	33	N.D.
3	17	N.D.
5	4	N.D.
7	N.D.	N.D.

The results in Table 4.1 show that arachidonic acid **1** undergoes autoxidation readily. After 2 days, only 20% of the arachidonic acid **1** remained. As shown in Tables 4.3A and 4.4A, the introduction of either compound **19** or **106** at a ratio of 1:1 results in almost complete prevention of autoxidation of arachidonic acid **1**, even over the extended 7 days assay period, indicating that compounds **19** and **106** are both effective antioxidants. When the concentration of compounds **19** and **106** was reduced to one-tenth that of arachidonic acid **1** (Tables 4.3D and 4.4C), autoxidation of arachidonic acid **1** was very slow over the first 3 days, but faster after that period, coinciding with decomposition of compounds **19** and **106**. The antioxidative activity of compounds **19** and **106** is quite similar. Compound **112** was also effective as an antioxidant when used

in a 1:1 ratio with arachidonic acid **1** (Table 4.9A), but it was less effective than either compound **19** or **106** at the lower concentration (Table 4.9B).

The unsaturation of compounds **19**, **106** and **112** is not essential for antioxidant activity. Neither is the carboxyl group. Compound **111** is saturated and neither compound **110** nor **111** possesses a carboxyl group. Yet when present in a 1:1 ratio with arachidonic acid **1**, both of the sulfides **110** and **111** effectively inhibit the oxidation of arachidonic acid **1** (Tables 4.7A and 4.8A). Even when the amount of the sulfides **110** and **111** used was reduced to one-tenth that of arachidonic acid **1**, they were still effective antioxidants (Tables 4.7B and 4.8B). Apparently the sulfur alone is the key to the antioxidant activity of compounds **19**, **106**, **108** and **110-112**.

By contrast the autoxidation of arachidonic acid **1** is not significantly inhibited by either of the β-thia fatty acids **18** or **109** (Tables 4.2 and 4.6). To examine possible reasons for this lack of antioxidative activity, the chemical stability of compounds **18** and **109** in the absence of arachidonic acid **1** was investigated. For comparison, the stability of compounds **19**, **106**, **108** and **110-112** was also examined. Each compound was studied as a thin film under oxygen as described above. Samples were removed and analysed by ^{1}H NMR spectroscopy regularly for up to six weeks. The results showed that compounds **19**, **106** and **108-112** are all stable under these conditions. However, the unsaturated β-thia PUFA **18** decomposed after 7 days. The product mixture was analysed by ^{1}H NMR, which showed a complex mixture of products. The results for compound **109** show that β-thia PUFAs are not inherently unstable, so the decomposition of compound **18** presumably relates to its unsaturation. This is consistent with compound **109** decomposing in the presence of arachidonic acid **1**, but not alone.

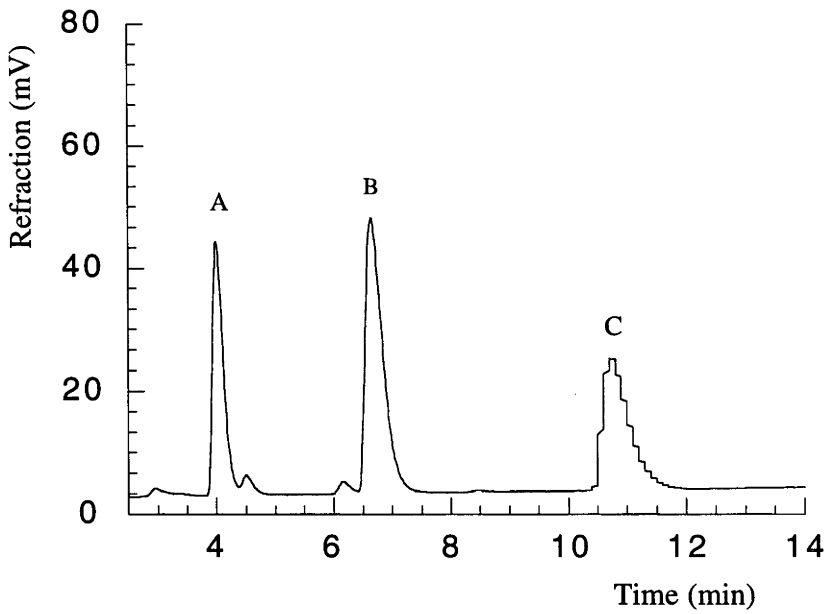


Fig. 4.1a: HPLC analysis of a mixture containing arachidonic acid **1**, compound **108** and lauric acid prior to thin film autoxidation.
(peak A: lauric acid; peak B: arachidonic acid **1**; peak C: compound **108**)

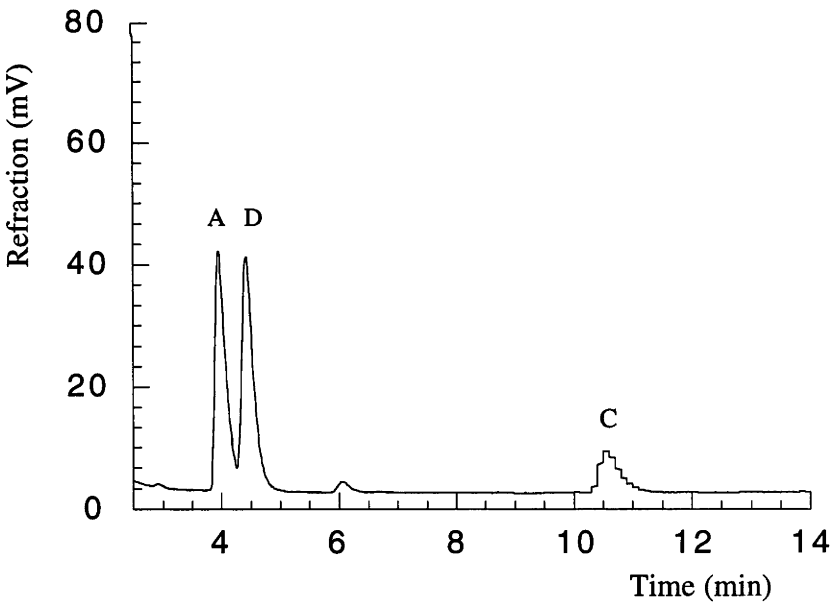
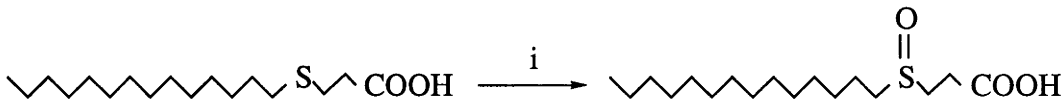


Fig. 4.1b: HPLC analysis of a mixture containing arachidonic acid **1**, compound **108** and lauric acid after 7 days of thin film autoxidation.
(peak A: lauric acid; peak C: compound **108**; peak D: a product)

It is apparent from the results shown in Tables 4.3D, 4.4C, 4.5 and 4.9B that the γ-thia PUFAs **19**, **106**, **108** and **112** react on thin film oxidation in the presence of arachidonic acid **1**. This was studied further in the case of the saturated PUFA **108**, where normal polyene oxidation is not possible. HPLC analyses were conducted of mixtures of compound **108**, arachidonic acid **1** and lauric acid, prior to and after 7 days of autoxidation. As shown in Fig. 4.1a and Fig. 4.1b, after 7 days of treatment, most of the thia PUFA **108** had reacted and a new compound was detected (peak D in Fig. 4.1b). The products of arachidonic acid **1** autoxidation are not observed in the HPLC, probably because they decomposed. In order to characterise the observed product, a sample containing arachidonic acid **1** and compound **108** but without lauric acid was subjected to autoxidation. After 7 days, components of the mixture were separated by HPLC. The fraction corresponding to peak D was collected, and analysed by NMR and mass spectroscopy. This showed that the product is 3-(tetradecylsulfinyl)propionic acid **113**. In the ^{1}H NMR spectrum of compound **113**, peaks at δ2.74 and 2.88 represent the protons of the methylenes adjacent to the sulfur and correspond with peaks at δ2.54 and 2.67 for the corresponding protons of compound **108**. Other peaks at δ3.00 and δ3.07 are due to the protons of the methylene adjacent to the carboxyl group, and these chemical shifts are slightly higher than those for the corresponding protons of compound **108** (δ 2.79). The chemical ionisation mass spectrum of compound **113** shows an ion at *m/e* 319 which is the protonated molecular ion (*i.e.*, MH^+).



108 **113**

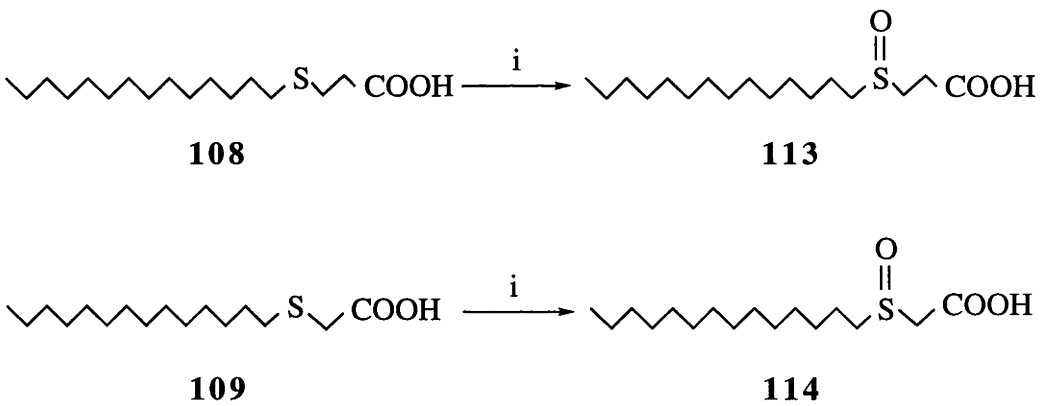
i: arachidonic acid **1**, O_2, 7 days

Scheme 4.6

4.3. Mechanism of antioxidant activity

The saturated γ-thia PUFA **108** is converted to the sulfoxide **113** on autoxidation in the presence of arachidonic acid **1**, but not alone. Therefore it seems likely that hydroperoxides of arachidonic acid **1** are responsible for production of the sulfoxide **113**. Presumably the mechanism of reaction of compound **108** with the hydroperoxides is as described in the introduction.

As mentioned earlier, the γ-thia fatty acids **19**, **106**, **108** and **112** are effective antioxidants but the β-thia fatty acids **18** and **109** are not. To further investigate the possible involvement of hydroperoxide-induced sulfoxide formation in the antioxidant behaviour of the γ-thia and β-thia fatty acids **18**, **19**, **106**, **108**, **109** and **112**, compounds **108** and **109** (in a ratio of 1:1 in CH_2Cl_2) were allowed to react with *tert*-butyl hydroperoxide (Scheme 4.7).



i: *tert*-butyl hydroperoxide, CH_2Cl_2, RT

Scheme 4.7

The reaction was monitored by TLC and ^1H NMR analysis. This showed that 55% of compound **109** was converted to the product **114** after 9 h while compound **108** was completely converted to the corresponding sulfoxide **113** during the same period. NMR spectral analysis of the product from compound **109** isolated after completion of the

reaction (48 h) showed that it is the sulfoxide **114**. The ^{1}H NMR spectrum of compound **114** contains two multiplets at δ2.88-3.07 and two doublets at δ3.63-3.86 corresponding to the methylene protons on the carbons adjacent to sulfur. The ^{13}C NMR spectrum shows characteristic peaks at δ52.27 and 53.47 representing the corresponding carbons.

This shows that both the γ-thia fatty acid **108** and the β-thia fatty acid **109** react with organic hydroperoxides to form sulfoxides, but the reaction rate is much faster for the γ-thia fatty acid **108**. This explains why the γ-thia fatty acid **108** is a much better antioxidant than the β-thia fatty acid **109**. The former reacts fast with and destroys hydroperoxides, which are initiators of free-radical oxidation chain processes. Consequently, it functions as an effective antioxidant. In contrast, the saturated β-thia fatty acid **109** reacts relatively slowly with hydroperoxides and therefore is ineffective as an antioxidant.

Time constraints prevented further studies in this area but to the extent that general conclusions may be drawn from these preliminary experiments, it appears that β-thia fatty acids such as compound **18** and **109** may be ineffective as antioxidants due to the proximity of the sulfur to the carboxyl group. This may affect the nucleophilicity of the sulfur or introduce steric hindrance in the reactions with hydroperoxides. In β-thia fatty acids, the carboxyl group is relatively close to the sulfur and consequently the nucleophilicity of the sulfur may be weakened because of the electron-withdrawing nature of the carboxyl group. The proximity of the carboxyl group to the sulfur in the β-thia fatty acids may also cause steric hindrance to the nucleophilic substitution process. In the γ-thia fatty acids **19**, **106**, **108** and **112**, and the sulfides **110** and **111**, however, the carboxyl group is either absent or more remote.

Earlier studies indicated that some sulfoxides are more effective inhibitors of hydrocarbon autoxidation than the parent sulfides.[55] However, the results of the present work show that sulfides but not sulfoxides have antioxidant activity. For instance, conversion of the

sulfide **108** to the sulfoxide **113** coincides with rapid autoxidation of arachidonic acid **1** (Table 4.5).

α-Tocopherol (vitamin E) is a widely used, naturally occurring, phenolic antioxidant which inhibits free-radical chains in biological systems. The γ-thia fatty acids **19**, **106**, **108** and **112**, and the sulfides **110** and **111**, of the present work should be more readily miscible in lipids than is α-tocopherol. Therefore, they may be more effective antioxidants than α-tocopherol in this environment.

Chapter 5

Experimental

5.1. General

^{1}H NMR and ^{13}C NMR spectra were recorded on a Gemini 300 MHz or a Unity Inova 500 MHz spectrometer with tetramethylsilane (TMS) as the internal standard (δ 0.00 ppm). Samples were run in deuterochloroform (99.8% D) unless indicated otherwise. The following abbreviations are adopted: s (singlet); d (doublet); t (triplet); m (multiplet); dd (doublet of doublets); bs (broad singlet).

Infrared (IR) spectra were recorded on a Perkin-Elmer 683 and 7700 infrared spectrophotometers. The following abbreviations are used: br (broad), w (weak), m (medium), s (strong).

Ultraviolet spectra were recorded on a Shimadzu UV 2101 PC spectrophotometer with a temperature controller and kinetic software.

Low and high resolution electron ionisation (EI) mass spectra and chemical ionisation (CI) mass spectra were run on a Fisons VG Autospec. A Fisons VG Instrument Quatro II mass spectrometer was used for negative ion electrospray mass spectra. Gas chromatography-mass spectrometry (GC-MS) was carried out with a HP 5970 mass selective detector connected to a HP 5890 gas chromatography with a 12.5 m BP-1 column.

Melting points were determined using a Reichert microscope with a Kofler heating stage and are uncorrected.

Buffers were adjusted to the required pH using a model 520A pH meter.

Microanalyses were conducted by the Microanalytical Laboratory, Research School of Chemistry, Australian National University.

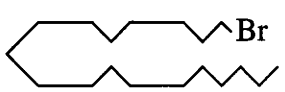
HPLC was performed using a Waters HPLC system with ultraviolet (UV) or refractive index (RI) detection. The column used contained Alltech Spherisorb octadecylsilane (ODS) (4.6 mm x 250 mm, 3 μm). The mobile phase was comprised of acetonitrile (or methanol) and phosphoric acid (30 mM) solution in the ratios indicated in the text, with a flow rate of 1 ml/min.

Column chromatography was carried out using Merck Silicagel 60 as the absorbent. Analytical TLC was performed on Merck Silicagel 60 F_{254} silica on aluminium baked plates.

15-LO was obtained from Sigma Chemical Company, and 12-LO from Cayman Chemical Company. Arachidonic acid **1**, linolenyl alcohol **57a**, gamma linolenyl alcohol **57b**, arachidonyl alcohol **57c** and docosahexaenyl alcohol **57d** were purchased from Nu-Chek Prep. Inc. Elysian, Minnesota, USA. Other chemicals were commercially available from Aldrich Chemical Company.

5.2. Experimental for Chapter 2

1-Bromooctadecane 54



Octadecan-1-ol **53** (520 mg, 1.92 mmol) and triphenylphosphine (550 mg, 2.10 mmol, 1.1 eq.) were dissolved in dichloromethane (25 ml) under nitrogen. The mixture was

cooled in an ice bath and carbon tetrabromide (630 mg, 1.90 mmol) was added with stirring. The mixture was allowed to warm to room temperature and was stirred under nitrogen overnight. The crude reaction mixture was concentrated under a stream of nitrogen and the residue was purified by flash column chromatography, being eluted with hexane to afford 605 mg (96%) of 1-bromooctadecane **54** as a waxy solid. The spectral data of this compound are consistent with those reported previously.[202]

M.P. 26-28 °C.

IR(KBr): ν_{max} 2920 (s), 2848 (s), 1468 (s), 1378 (w), 1254 (w), 1144 (m), 720 (m), 658 (s) cm^{-1};

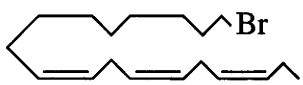
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.87 (3H, t, *J* = 6.7 Hz, C18-H_3), 1.25-1.32 (30H, m, (C3-17)-H_2)), 1.82-1.85 (2H, m, C2-H_2), 3.40 (2H, t, *J* = 6.8 Hz, C1-H_2);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.66, 23.22, 28.71, 29.30, 29.90, 29.97, 30.08, 30.16, 30.23, 32.46, 33.37, 34.59;

MS (EI) *m/e* (relative intensity): 334 (M^+, 8%), 332 (M^+, 10%), 253 (25), 151 (27), 149 (28), 137 (67), 135 (69), 113 (19), 97 (30), 85 (50), 71 (70), 57 (100);

HRMS: Calculated for $C_{18}H_{37}Br$ *m/e*: 334.205816 (M^+) and 332.207863 (M^+). Found: 334.207007 and 332.208636.

(*Z,Z,Z*)-1-Bromo-9,12,15-octadecatriene 58a



From linolenyl alcohol **57a** (102 mg, 0.39 mmol), using the procedure described above for the preparation of 1-bromooctadecane **54**, (*Z,Z,Z*)-1-bromo-9,12,15-octadecatriene **58a** was obtained as a colourless oil (118 mg, 93%). The spectral data of this compound are consistent with those reported previously.[203]

IR (smear): ν_{max} 3001 (s), 2960 (s), 2920 (s), 2850 (s), 1460 (m), 1430 (m), 1395 (w), 1270 (w), 720 (w) cm^{-1};

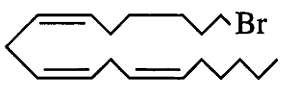
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.98 (3H, t, *J* = 7.5 Hz, C18-H_3), 1.30-1.45 (10H, m, C3-H_2, C4-H_2, C5-H_2, C6-H_2, C7-H_2), 1.81-1.88 (2H, m, C2-H_2), 2.03-2.11 (4H, m, C8-H_2, C17-H_2), 2.80-2.83 (4H, m, C11-H_2, C14-H_2), 3.41 (2H, t, *J* = 6.8 Hz, C1-H_2), 5.30-5.42 (6H, m, C9-H, C10-H, C12-H, C13-H, C15-H, C16-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.88, 21.14, 26.10, 26.19, 27.79, 28.74, 29.32, 29.76, 29.91, 30.17, 33.40, 34.60, 127.67, 128.29, 128.81(2C), 130.83, 132.51;

MS (EI) *m/e* (relative intensity): 328 (M^+, 14%), 326 (M^+, 14%), 272 (42), 270 (41), 149 (13), 135 (28), 121 (33), 108 (92), 95 (53), 79 (100), 67 (72), 55 (59);

Analysis: Calculated for $C_{18}H_{31}Br$: C, 66.05; H, 9.54. Found: C, 65.82; H, 9.32%.

(*Z,Z,Z*)-1-Bromo-6,9,12-octadecatriene **58b**



From gamma linolenyl alcohol **57b** (143 mg, 0.54 mmol), using the procedure described above for the preparation of 1-bromooctadecane **54**, (*Z,Z,Z*)-1-bromo-6,9,12-octadecatriene **58b** was obtained as a colourless oil (170 mg, 96%).

IR (smear): ν_{max} 3002 (s), 2950 (s), 2920 (s), 2850 (s), 1460 (s), 1378 (w), 1260 (w), 715 (m), 648 (m) cm^{-1}.

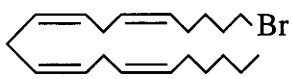
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, *J* = 6.8 Hz, C18-H_3), 1.29-1.45 (10H, m, C3-H_2, C4-H_2, C15-H_2, C16-H_2, C17-H_2), 1.82-1.91 (2H, m, C2-H_2), 2.02-2.17 (4H, m, C5-H_2, C14-H_2), 2.79-2.83 (4H, m, C8-H_2, C11-H_2), 3.40 (2H, t, *J* = 6.7 Hz, C1-H_2), 5.30-5.41 (6H, m, C6-H, C7-H, C9-H, C10-H, C12-H, C13-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.67, 23.16, 26.21, 27.58, 27.80, 28.39, 29.33, 29.91, 32.11, 33.29, 34.46, 128.14, 128.65, 128.71, 128.96, 130.33, 131.03;

MS (EI) *m/e* (relative intensity): 328 (M^+, 10%), 326 (M^+, 8%), 228 (50), 230 (49), 150 (66), 135 (15), 121 (25), 107 (32), 93 (59), 79 (100), 67 (95), 55 (64);

HRMS: Calculated for $C_{18}H_{31}Br$ *m/e*: 326.160912 (M^+) and 328.158866 (M^+). Found: 326.161148 and 328.159185.

(*all-Z*)-1-Bromo-5,8,11,14-eicosatetraene 58c



From arachidonyl alcohol **57c** (740 mg, 2.54 mmol), using the procedure described above for the preparation of 1-bromooctadecane **54**, (*all-Z*)1-bromo-5,8,11,14-eicosatetraene **58c** was obtained as a colourless oil (826 mg, 93%).

IR (smear): ν_{max} 3012 (s), 2958 (s), 2927 (s), 2856 (s), 1653 (m), 1456 (m), 1394 (m), 1251 (m), 1199 (w), 1041 (m), 915 (w), 807 (w), 715 (s) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, *J* = 6.8 Hz, C20-H_3), 1.29-1.38 (6H, m, C17-H_2, C18-H_2,C19-H_2), 1.47-1.56 (2H, m, C3-H_2), 1.83-1.93 (2H, m, C2-H_2), 2.03-2.14 (4H, m, C4-H_2, C16-H_2), 2.80-2.83 (6H, m, C7-H_2, C10-H_2, C13-H_2), 3.42 (2H, t, *J* = 6.8 Hz, C1-H_2), 5.30-5.41 (8H, m, C5-H, C6-H, C8-H, C9-H, C11-H, C12-H, C14-H, C15-H);

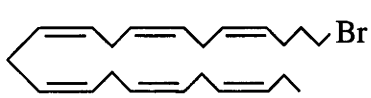
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.68, 23.17, 26.22, 26.89, 27.81, 28.65, 29.91, 32.11, 32.87, 34.31, 128.09, 128.42, 128.74(2C), 129.01, 129.13, 129.93, 131.05;

MS (EI) *m/e* (relative intensity): 354 (M^+, 5%), 352 (M^+, 6%), 283 (8), 281 (8), 256 (15), 254 (15), 216 (25), 214 (25), 150 (34), 119 (29), 105 (36), 93 (53), 91 (56), 79 (100), 67 (75);

HRMS: Calculated for $C_{20}H_{33}Br$: *m/e*: 352.176562 (M^+) and 354.174516 (M^+). Found: 352.177193 and 354.174805;

Analysis: Calculated for $C_{20}H_{33}Br$: C, 67.98; H, 9.41. Found: C, 68.05; H, 9.28%.

(*all-Z*)-1-Bromo-4,7,10,13,16,19-docosahexaene 58d



From docosahexaenyl alcohol **57d** (201 mg, 0.64 mmol), using the procedure described above for the preparation of 1-bromooctadecane **54**, (*all Z*)-1-bromo-4,7,10,13,16,19-docosahexaene **58d** was obtained as a colourless oil (221 mg, 92%).

IR (smear): ν_{max} 3008 (s), 2960 (s), 2928 (s), 2868 (s), 1650 (m), 1434 (s), 1392 (s), 1348 (w), 1322 (w), 1266 (s), 1244 (s), 1068 (m), 1044 (m), 928 (m), 714 (s) cm^{-1}.

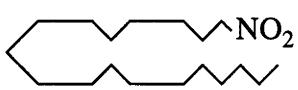
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.98 (3H, t, *J* = 7.5 Hz, C22-H_3), 1.85-2.30 (6H, C2-H_2, C3-H_2, C21-H_2), 2.80-2.90 (10H, m, C6-H_2, C9-H_2, C12-H_2, C15-H_2, C18-H_2), 3.42 (2H, t, *J* = 6.6 Hz, C1-H_2), 5.31-5.45 (12H, m, C4-H, C5-H, C7-H, C8-H, C10-H, C11-H, C13-H, C14-H, C16-H, C17-H, C19-H, C20-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.38, 20.54, 25.52, 25.64, 32.47, 33.24, 126.99, 127.85, 127.94, 128.06, 128.11(2C), 128.18(2C), 128.24, 128.55, 129.46, 132.01;

MS (EI) *m/e* (relative intensity): 378 (M^+, 10%), 376 (M^+, 10%), 349 (20), 347 (20), 309 (46), 307 (53), 244 (75), 242 (74), 227 (49), 202 (30), 200 (30), 173 (12), 133 (34), 119 (45), 108 (50), 91 (65), 79 (100), 67 (66);

HRMS: Calculated for $C_{22}H_{33}Br$ *m/e*: 376.176562 (M^+) and 378.174516 (M^+). Found: 376.175998 and 378.174181.

1-Nitrooctadecane 55



To a solution of 1-bromooctadecane **54** (480 mg, 1.44 mmol) in dry acetone (25 ml) at room temperature was added sodium iodide (430 mg, 2.87 mmol, 2 eq.). The reaction mixture was stirred at room temperature overnight, then the solvent was removed *in vacuo*. After addition of 25 ml of saturated aqueous sodium bisulphite, the organic material was extracted with diethyl ether (3 x 25ml). The combined extracts were dried (Na_2SO_4) and the solvent was removed *in vacuo*. The residue (502 mg) was then dissolved in anhydrous diethyl ether under nitrogen and silver nitrate (406 mg, 2.64

mmol) was added. After 3 days of stirring, the reaction mixture was filtered through a bed of celite and the solvent was evaporated under a stream of dry nitrogen. Purification of the crude residue by flash column chromatography on silica (5% ether in hexane for elution) provided 220 mg of 1-nitrooctadecane **55** (51%) as a white solid and 97 mg of recovered crude iodide **56**.

M.P. 41-42 °C.

IR (smear): ν_{max} 2954 (s), 2919 (s), 2850 (s), 1563 (s), 1470 (m), 1385 (w), 1147 (w), 742 (w), 720 (m), 650 (w) cm^{-1};

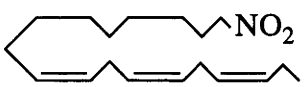
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 6.6 Hz, C18-H_3), 1.25-1.34 (30H, m, (C3-C17)-H_2), 1.96-2.05 (2H, m, C2-H_2), 4.38 (2H, t, *J* = 7.1 Hz, C1-H_2);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.70, 23.26, 26.73, 27.97, 29.41, 29.82, 29.93, 30.02, 30.14, 30.20, 30.26, 32.50, 76.32;

MS (EI) *m/e* (relative intensity): 299 (M^+, <1%), 282 (4), 264 (20), 252 (7), 238 (7), 224 (7), 210 (5), 196 (4), 154 (5), 139 (7), 125 (20), 111 (40), 97 (74), 83 (87), 69 (95), 57 (100), 55 (96);

Analysis: Calculated for $C_{18}H_{37}NO_2$: C, 72.19; H, 12.45; N, 4.68. Found: C, 72.33; H, 12.77; N, 4.57%.

(*Z*,*Z*,*Z*)-1-Nitro-9,12,15-octadecatriene 60a



According to the procedure described for the preparation of 1-nitrooctadecane **55**, sodium iodide (77 mg, 0.51 mmol, 2 eq.) was added to a solution of (Z,Z,Z)-1-bromo-9,12,15-octadecatriene **58a** (79 mg, 0.24 mmol) in dry acetone (10 ml) and the mixture was stirred overnight at room temperature. The reaction flask was covered with aluminium foil during the reaction processes. After workup, 87 mg of residue was obtained. The residue was then treated with silver nitrate (74 mg, 0.48 mmol) in anhydrous diethyl ether for 3 days. Purification by flash column chromatography (5% ether in hexane) produced 37

mg of (*Z,Z,Z*)-1-nitro-9,12,15-octadecatriene **60a** (53%) as a colourless oil and 12 mg of recovered crude iodide **59a**.

IR (smear): ν_{max} 3011 (s), 2962 (s), 2929 (s), 2856 (s), 1652 (w), 1554 (s), 1463 (m), 1435 (m), 1383 (m), 1268 (w), 1148 (w), 1069 (w), 968 (m), 912 (w), 724 (m), 614 (w) cm^{-1};

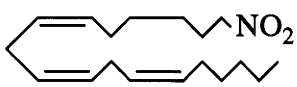
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.98 (3H, t, *J* = 7.5 Hz, C18-H_3), 1.25-1.33 (10H, m, C3-H_2, C4-H_2, C5-H_2, C6-H_2, C7-H_2), 1.97-2.06 (6H, m, C2-H_2, C8-H_2, C17-H_2), 2.79-2.81 (4H, m, C11-H_2, C14-H_2), 4.37 (2H, t, *J* = 7.1 Hz, C1-H_2), 5.36-5.40 (6H, m, C9-H, C10-H, C12-H, C13-H, C15-H, C16-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.85, 21.12, 26.10, 26.18, 26.77, 27.74, 27.96, 29.37, 29.64, 29.71, 30.10, 76.29, 127.66, 128.35, 128.78, 128.86, 130.74, 132.54;

MS (EI) *m/e* (relative intensity): 293 (M^+, 24%), 276 (14), 264 (5), 246 (5), 237 (32), 224 (17), 135 (26), 121 (35), 108 (63), 95 (84), 93 (75), 91 (69), 79 (100), 67 (95);

Analysis: Calculated for $C_{18}H_{31}NO_2$: C, 73.67; H, 10.65; N, 4.77. Found: C, 73.69; H, 10.57; N, 4.85%.

(*Z,Z,Z*)-1-Nitro-6,9,12-octadecatriene 60b



According to the procedure described for the preparation of 1-nitrooctadecane **55**, sodium iodide (115 mg, 0.77 mmol, 2 eq.) was added to a solution of (*Z,Z,Z*)-1-bromo-6,9,12-octadecatriene **58b** (122 mg, 0.37 mmol) in dry acetone (10 ml) and the mixture was stirred overnight at room temperature. After work-up, 129 mg of the residue was obtained. The residue was then treated with silver nitrate (106 mg, 0.69 mmol) in anhydrous diethyl ether for 3 days. Purification by flash column chromatography (5% ether in hexane for elution) gave 56 mg of (*Z,Z,Z*)-1-nitro-6,9,12-octadecatriene **60b** (51%) as a colourless oil and 15 mg of recovered crude iodide **59b**.

IR (smear): ν_{max} 3012 (s), 2956 (s), 2928 (s), 2858 (s), 1652 (m), 1555 (s), 1464 (s), 1435 (s), 1382 (s), 1266 (m), 1159 (w), 1067 (w), 1040 (w), 970 (w), 914 (w), 720 (s), 614 (w) cm^{-1};

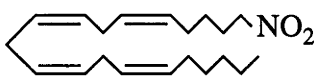
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 7.1 Hz, C18-H_3), 1.29-1.43 (10H, m, C3-H_2, C4-H_2, C15-H_2, C16-H_2, C17-H_2), 2.01-2.08 (6H, m, C2-H_2, C5-H_2, C14-H_2), 2.78-2.82 (4H, m, C8-H_2, C11-H_2), 4.38 (2H, t, *J* = 7.1 Hz, C1-H_2), 5.34-5.40 (6H, m, C6-H, C7-H, C9-H, C10-H, C12-H, C13-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.65, 23.15, 26.20, 26.40, 27.37, 27.78, 27.88, 29.38, 29.90, 32.09, 76.18, 128.09, 128.51, 129.03(2C), 129. 89, 131.03;

MS (EI) *m/e* (relative intensity): 293 (M^+, 31%), 276 (25), 258 (12), 246 (4), 222 (7), 195 (72), 150 (36), 137 (18), 105 (25), 91 (84), 81 (80), 80 (79), 79 (100), 67 (82), 55 (60);

Analysis: Calculated for $C_{18}H_{31}NO_2$: C, 73.67; H, 10.65; N, 4.77. Found: C, 73.56; H, 10.56; N, 4.74%.

(*all-Z*)-1-Nitro-5,8,11,14-eicosatetraene 60c



According to the procedure described for the preparation of 1-nitrooctadecane **55**, sodium iodide (687 mg, 4.58 mmol, 2 eq.) was added to a solution of (*all-Z*)-1-bromo-5,8,11,14-eicosatetraene **58c** (782 mg, 2.21 mmol) in dry acetone (25 ml) and the mixture was stirred overnight at room temperature. After workup, 826 mg of the residue was obtained. The residue was then treated with silver nitrate (682 mg, 4.43 mmol) in anhydrous diethyl ether for 3 days. Purification by flash column chromatography (5% ether in hexane for elution) yielded 397 mg of (*all-Z*)-1-nitro-5,8,11,14-eicosatetraene **60c** (56%) as a colourless oil and 71 mg of recovered crude iodide **59c**.

IR (smear): ν_{max} 3013 (s), 2957 (s), 2928 (s), 2857 (s), 1648 (w), 1555 (s), 1457 (m), 1435 (m), 1381 (s), 1267 (w), 1106 (w), 1047 (w), 969 (w), 914 (w), 716 (m) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, *J* = 6.8 Hz, C20-H_3), 1.20-1.51 (8H, m, C3-C_2, C17-H_2, C18-H_2, C19-H_2), 1.99-2.16 (6H, m, C2-H_2, C4-H_2, C16-H_2), 2.79-2.86 (6H, m, C7-H_2, C10-H_2,C13-H_2), 4.39 (2H, t, *J* = 7.0 Hz, C1-H_2), 5.32-5.43 (8H, m, C5-H, C6-H, C8-H, C9-H, C11-H, C12-H, C14-H, C15-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.64, 23.14, 26.21, 26.69, 26.90, 27.47, 27.79 29.89, 32.09, 76.13, 128.06, 128.35, 128.54, 128.90, 129.19(2C), 129.62, 131.09;

MS (EI) *m/e* (relative intensity): 319 (M^+, 6%), 302 (14), 220 (27), 205 (15), 190 (11), 181 (24), 177 (20), 164 (25), 150 (41), 119 (48), 105 (63), 91 (90), 79 (100), 67 (97), 55 (77);

Analysis: Calculated for $C_{20}H_{33}NO_2$: C, 75.19; H, 10.41; N, 4.38. Found: C, 74.92; H, 10.40; N, 4.43%.

(*all-Z*)-1-Nitro-4,7,10,13,16,19-docosahexaene 60d

According to the procedure described for the preparation of 1-nitrooctadecane (**55**), sodium iodide (131 mg, 0.87 mmol, 2 eq.) was added to a solution of (*all-Z*)-1-bromo-4,7,10,13,16,19-docosahexaene **58d** (165 mg, 0.44 mmol) in dry acetone (15 ml) and the mixture was stirred overnight at room temperature. After workup, 176 mg of the residue was obtained. The residue was then treated with silver nitrate (128 mg, 0.83 mmol) in anhydrous diethyl ether for 3 days. Purification by flash column chromatography (10% ether in hexane for elution) yielded 80 mg of (*all-Z*)-1-nitro-4,7,10,13,16,19-docosahexaene **60d** (53%) as a colourless oil and 27 mg of recovered crude iodide **59d**.

IR (smear): ν_{max} 3014 (s), 2962 (s), 2926 (s), 2873 (s), 2854 (s), 1653 (m), 1554 (s), 1434 (s), 1381 (s), 1352 (m), 1267 (m), 1069 (w), 917 (w), 712 (s), 611 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.98 (3H, t, *J* = 7.6 Hz, C22-H_3), 2.05-2.23 (6H, m, C2-H_2, C3-H_2, C21-H_2), 2.78-2.85 (10H, m, C6-H_2, C9-H_2, C12-H_2, C15-H_2, C18-

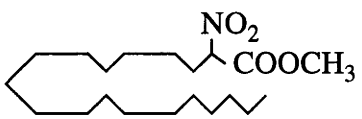
H_2), 4.38 (2H, t, J = 6.7 Hz, C1-H_2), 5.31-5.47 (12H, m, C4-H, C5-H, C7-H, C8-H, C10-H, C11-H, C13-H, C14-H, C16-H, C17-H, C19-H, C20-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.84, 21.13, 24.39, 26.11, 26.20, 27.70, 75.42, 127.58, 128.27, 128.42, 128.55, 128.60, 128.87(3C), 129.05, 129.15, 130.94, 132.61;

MS (EI) *m/e* (relative intensity): 343 (M^+, 10%), 326 (59), 314 (21), 274 (44), 215 (55), 207 (42), 167 (16), 145 (18), 131 (16), 119 (36), 105 (48), 91 (77), 79 (100), 67 (78), 55 (42);

Analysis: Calculated for $C_{22}H_{33}NO_2$: C, 76.92; H, 9.68; N, 4.08. Found: C, 76.52; H, 9.87; N, 4.26%.

Methyl 2-nitrononadecanoate 65



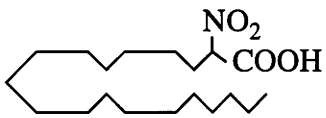
To a solution of tetrahydrofuran (10 ml) and hexamethylphosphoramide (2 ml) maintained at -78 °C, a solution of butyllithium (1.55 M in hexane, 0.60 ml, 0.93 mmol, 2.5 eq.) was added dropwise, followed by 1-nitrooctadecane **55** (110 mg, 0.37 mmol) in THF (2 ml) and methyl chloroformate (87 mg, 0.93 mmol, 2.5 eq.). The reaction mixture was stirred at -78 °C for 24 h. Acetic acid (0.2 ml) was then added to quench the reaction. The mixture was dissolved in ethyl acetate (25 ml), and that mixture was washed with saturated aqueous sodium bicarbonate (3 x 25 ml), dried over Na_2SO_4 and concentrated. The residue was flash column chromatographed on silica to give the title product **65** (40 mg, 30%) as a white solid. This material was not fully characterised as subsequent reactions were not successful.

IR (KBr): ν_{max} 2956 (m), 2920 (s), 2851 (s), 1759 (s), 1560 (s), 1469 (m), 1439 (m), 1372 (m), 1259 (m), 1104 (s), 899 (w), 802 (w), 720 (w), 697 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, J = 6.7 Hz, C19-H_3), 1.18-1.36 (30H, m, (C4-C18)-H_2), 2.07-2.18 (H, m, C3-H), 2.23-2.35 (H, m, C3-H'), 3.83 (3H, s, OCH_3), 5.09-5.14 (H, dd, J = 5.4, 9.5 Hz, C2-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.69, 23.26, 26.15, 29.31, 29.69, 29.93, 30.11, 30.26, 30.83, 32.49, 54.07, 88.60, 177.62.

Attempted preparation of 2-nitrononadecanoic acid 66



Method 1:

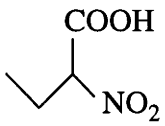
Methyl 2-nitrononadecanoate **65** (20 mg, 0.06 mmol) was dissolved in 1 ml of 1,2-dimethoxyethane and 0.6 ml of aqueous lithium hydroxide (1.5 M) was then added. The reaction was left for 1 h. The mixture was acidified with dilute HCl (10%, 5 ml) and extracted with ethyl acetate (2 x 5 ml). The extracts were concentrated under a stream of dry nitrogen and the residue was flash column chromatographed on silica gel (10% diethyl ether in hexane for elution) to afford 1-nitrooctadecane **55** (5 mg) as a white solid, which was identical in all respects to the sample obtained as described above.

Method 2:

Magnesium methyl carbonate solution (1.65 M in DMF, 500 ml) was first prepared according to the procedure in the literature.[165]

Magnesium methyl carbonate solution (0.065 M in DMF, 10 ml, 0.65 mmol, 5 eq.) was heated, while stirring, to 60 °C under a carbon dioxide stream. When the temperature had stabilised at approximately 60 °C, 1-nitrooctadecane **55** (40 mg, 0.13 mmol) was added, and the carbon dioxide stream was replaced by a slow nitrogen stream. After overnight reaction and workup, the residue was tested by ^{1}H NMR. The spectrum only showed the starting material, 1-nitrooctadecane **55**.

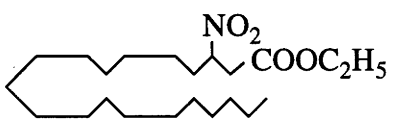
Synthesis of 2-nitrobutanoic acid 70b



Magnesium methyl carbonate solution (0.83 M in DMF, 11 ml, 9 mmol, 5 eq.) was heated, while stirring, to 60 °C under a carbon dioxide stream. When the temperature had stabilised at approximately 60 °C, 1-nitropropane **68b** (160 mg, 1.80 mmol) was added, and the carbon dioxide stream was replaced by a slow nitrogen stream. The reaction was left for 6 h. After stirring overnight at 60 °C, the mixture was cooled to 10 °C with an ice bath, and then the mixture was poured into 10 ml of concentrated HCl and 10 g of ice which had been overlayed with 10 ml of ether. The ether was separated and the aqueous layer was extracted three times with 10 ml portions of ether. The combined extracts were dried over Na_2SO_4. After the extracts were evaporated, the residue was analysed by ^1H NMR. The spectrum showed the formation of 2-nitrobutanoic acid **70b**. This procedure is based on a literature method[145] and compound **70b** was prepared to establish the viability of the protocol. For this reason compound **70b** was analysed crude and not further characterised.

^1H NMR ($CDCl_3$, 300 MHz): δ 1.06 (3H, t, J = 7.4 Hz, C4-H_3), 2.19-2.34 (2H, m, C3-H_2), 5.06-5.11 (1H, dd, J = 5.6, 9.2 Hz, C2-H), 8.0 (1H, bs, COOH).

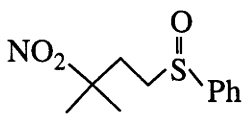
Attempted synthesis of ethyl 3-nitroicosanoate 71



A solution of butyllithium (1.55 M in hexane, 0.39 ml, 0.6 mmol, 3 eq.) was added dropwise to a solution of 1-ntrooctadecane **55** (60 mg, 0.2 mmol) in tetrahydrofuran (2 ml) and hexamethylphosphoramide (0.4 ml) maintained at -78 °C. A solution of ethyl

bromoacetate (67 mg, 0.4 mmol, 2 eq.) in tetrahydrofuran (1 ml) was then added at -78 °C. After 21 h at that temperature, acetic acid (100 µl) was added. The mixture was dissolved in ethyl acetate (10 ml), and that solution was washed with saturated aqueous sodium bicarbonate, dried over Na_2SO_4 and concentrated. The residue was examined by ^1H NMR, which showed only the starting material **55**.

3-Methyl-3-nitro-1-(phenylsulfinyl)butane 74a

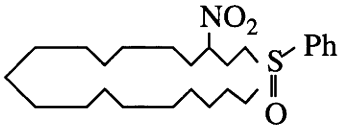


A solution of 2-nitropropane **72a** (100 mg, 1.12 mmol), DBU (170 mg, 1.12 mmol, 1 eq.) and phenyl vinyl sulfoxide **73** (170 mg, 1.12 mmol, 1 eq.) in 4 ml of acetonitrile was kept at room temperature for 12 h. The reaction mixture was then poured into water and extracted with diethyl ether. The ether extract were washed with 1N HCl and dried over Na_2SO_4. The residue after evaporation of the solvent was analysed by ^1H NMR and ^{13}C NMR. The spectra indicated the formation of 3-methyl-3-nitro-1-(phenylsulfinyl)butane **74a**. This procedure is based on a literature method[167] and compound **74a** was prepared to establish the viability of the protocol. For this reason compound **74a** was analysed crude and not further characterised.

^1H NMR ($CDCl_3$, 300 MHz): δ 1.52 (3H, s), 1.58 (3H, s), 2.07-2.11 (1H, t, *J* = 12 Hz, d, *J* = 4.8 Hz), 2.31-2.38 (1H, t, *J* = 12 Hz, d, *J* = 4.4 Hz), 2.62-2.72 (1H, t, J = 12Hz, d, *J* = 4.8 Hz), 2.79-2.88 (1H, t, *J* = 12 Hz, d, *J* = 4.4 Hz), 7.50-7.59 (5H, m, ArH);

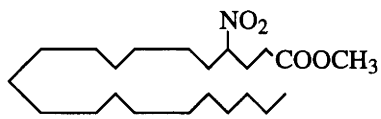
^{13}C NMR ($CDCl_3$, 300 MHz): δ 26.36, 26.61, 32.76, 51.61, 87.64, 124.47, 129.96, 131.85.

Attempted synthesis of 3-nitro-1-(phenylsulfinyl)icosane 76



According to the procedure described for the preparation of 3-methyl-3-nitro-1-(phenylsulfinyl)butane **74a**, a solution of 1-nitrooctadecane **55** (50 mg, 0.17 mmol), DBU (26 mg, 0.17 mmol, 1 eq.) and phenyl vinyl sulfoxide **73** (26 mg, 0.17 mmol, 1 eq.) in 2 ml of acetonitrile was kept at room temperature for 24 h. After workup, the residue was analysed by ^{1}H NMR. However, the expected product, 3-nitro-1-(phenylsulfinyl)icosane **76** was not obtained.

Methyl 4-nitrohenicosanoate 79



Sodium hydroxide (136 mg, 3.4 mmol, 2 eq.) and tetra-n-butylamonium iodide (158 mg, 0.43 mmol, 0.25 eq.) in water (10 ml) were added to a solution of 1-nitrooctadecane **55** (510 mg, 1.70 mmol) and methyl acrylate (442 mg, 5.13 mmol, 3 eq.) in dichloromethane (10 ml) at room temperature. The mixture was stirred at 45-50 °C for 24 h, and the layers were then separated. The organic phase was washed with water (2 x 25 ml) and dried with Na_2SO_4. The solvent was evaporated and the residue was purified by flash column chromatography (ether : hexane = 5 : 95 for elution), giving methyl 4-nitrohenicosanoate **79** (498 mg, 76%) as a waxy solid.

IR (Nujol): ν_{max} 2924 (s), 2853 (s), 1744 (s), 1554 (s), 1466 (m), 1439 (m), 1367 (m), 1201 (m), 1175 (m), 1120 (m), 829 (w), 722 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.87 (3H, t, J = 6.7 Hz, C21-H_3), 1.19-1.25 [30H, m, (C6-C20)-H_2], 1.69-1.78 (H, m), 1.92-2.30 (3H, m), 2.32-2.40 (2H, m, C2-H_2), 3.69 (3H, s, OCH_3), 4.50-4.59 (H, m, C4-H);

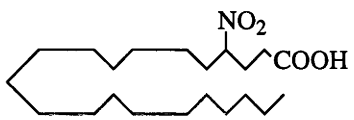
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.69, 23.26, 26.23, 29.15, 29.48, 29.82, 29.94, 30.00, 30.14, 30.23, 30.26, 30.50, 32.49, 34.46, 52.47, 88.38, 172.97;

MS (EI) *m/e* (relative intensity): 386 ($(M+1)^+$, 25%), 368 (12), 354 (18), 339 (20), 305 (24), 287 (28), 263 (18), 221 (15), 193 (10), 179 (15), 165 (21), 151 (26), 137 (31), 123 (36), 111 (52), 97 (76), 83 (86), 69 (88), 55 (100);

HRMS: Calculated for $C_{22}H_{44}NO_4$ *m/e*: 386.327034 $(M+H)^+$. Found 386.327461;

Analysis: Calculated for $C_{22}H_{43}NO_4$: C, 68.53; H, 11.24; N, 3.63. Found: C, 68.39; H, 11.53; N, 3.50%.

4-Nitrohenicosanoic acid 80



Methyl 4-nitrohenicosanoate **79** (147 mg, 0.38 mmol) was dissolved in 2 ml of 1,2-dimethoxyethane and 2 ml of saturated aqueous lithium hydroxide solution was then added. The reaction was left for 24 h. The reaction mixture was then acidified with dilute HCl (10%, 10 ml) and extracted with ethyl acetate (2 x 10 ml). The extracts were concentrated under a stream of dry nitrogen and the residue was flash column chromatographed on silica gel (ether : hexane = 100 : 20 → ether : hexane : acetic acid = 60: 40 : 1 for elution) to afford the title compound **80** (121 mg, 85%) as a white solid.

M.P. 55-56 °C.

IR (KBr): ν_{max} 3500-2600 (br), 2955 (m), 2919 (s), 2849 (s), 1698 (s), 1615(w), 1543 (s), 1467 (m), 1445 (m), 1413 (w), 1360 (w), 1334 (w), 1266 (w), 923 (w), 827 (w), 723 (w), 612 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.87 (3H, t, *J* = 7.1 Hz, C21-H_3), 1.20-1.28 (30H, m, (C6-C20)-H_2), 1.69-1.78 (H, m), 1.98-2.30 (3H, m), 2.39-2.48 (2H, m, C2-H_2), 4.53-4.60 (H, m, C4-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.69, 23.26, 26.22, 28.83, 29.49, 29.80, 29.93, 30.00, 30.12, 30.26, 30.33, 32.49, 34.43, 88.19, 177.51;

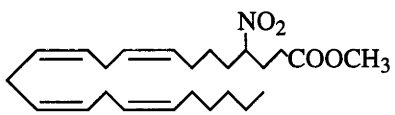
MS (CI) *m/e*: 389.3 $(M+NH_4)^+$;

MS (EI) *m/e* (relative intensity): 354 ($(M-OH)^+$, 2%), 323 (19), 321 (19), 305 (17), 287 (14), 263 (12), 236 (5), 221 (9), 193 (10), 179 (15), 165 (15), 151 (17), 137 (20), 125 (25), 110 (73), 97 (100), 83 (64), 69 (64), 55 (73);

HRMS: Calculated for $C_{21}H_{40}NO_3$ *m/e*: 354.300820 $(M-OH)^+$. Found 354.300575;

Analysis: Calculated for $C_{21}H_{41}NO_4$: C, 67.88; H, 11.12; N, 3.77. Found: C, 67.58; H, 11.08; N, 3.81%.

Methyl (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoate 81



Sodium hydroxide (162 mg, 4.05 mmol, 2 eq.) and tetra-n-butylammonium iodide (187 mg, 0.51 mmol, 0.25 eq.) in water (10 ml) were added to a solution of (*all-Z*)-1-nitro-5,8,11,14-eicosatetraene **60c** (650 mg, 2.03 mmol) and methyl acrylate (524 mg, 6.09 mmol, 3 eq.) in dichloromethane (10 ml) at room temperature. The mixture was stirred at 45-50 °C for 24 h, and the layers were then separated. The organic phase was washed with water (2 x 25 ml) and dried with Na_2SO_4. The solvent was evaporated and the residue was purified by flash column chromatography (ether : hexane = 5 : 95 for elution), giving methyl (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoate **81** (594 mg, 72%) as a colourless oil.

IR (smear): ν_{max} 3065 (w), 3013 (m), 2956 (s), 2930 (s), 2859 (m), 1737(s), 1552 (s), 1439 (m), 1363 (w), 1267 (w), 1263 (w), 1259 (w), 1204 (m), 1178 (m), 981 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 6.8 Hz, C23-H_3), 1.24-1.45 (8H, m, C6-H_2, C20-H_2, C21-H_2, C22-H_2), 1.70-1.81 (H, m), 1.91-2.27 (3H, m), 2.32-2.40 (2H, m, C2-H_2), 2.73-2.83 (6H, m, C10-H_2, C13-H_2, C16-H_2), 3.68 (3H, s, OCH_3),

4.51-4.58 (H, m, C4-H), 5.29-5.44 (8H, m, C8-H, C9-H, C11-H, C12-H, C14-H, C15-H, C17-H, C18-H);

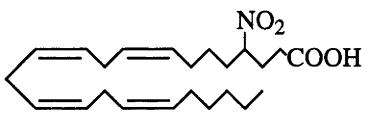
^{13}C NMR ($CDCl_3$, 300 MHz) δ 14.65, 23.14, 26.12, 26.20, 26.92, 27.79, 29.16, 29.89, 30.26, 30.47, 32.08, 33.88, 52.48, 88.22, 128.07, 128.35, 128.55, 128.87, 129.17(2C), 129.60, 131.07, 172.92;

MS (EI) *m/e* (relative intensity): 405 (M^+, 7%), 374 (8), 359 (5), 327 (4), 307 (15), 294 (6), 267 (4), 229 (5), 215 (10), 190 (13), 177 (27), 164 (33), 150 (36), 147 (24), 131 (35), 119 (43), 105 (54), 91 (70), 79 (93), 67 (100), 55 (56);

HRMS: Calculated for $C_{24}H_{39}NO_4$ *m/e*: 405.287909 (M^+). Found 405.287025;

Analysis: Calculated for $C_{24}H_{39}NO_4$: C, 71.08; H, 9.69; N, 3.45. Found: C, 71.50; H, 10.03; N, 3.34%.

(*all-Z*)-4-Nitrotricosa-8,11,14,17-tetraenoic acid 82



Methyl (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoate **81** (230 mg, 0.57 mmol) was dissolved in 2.5 ml of 1,2-dimethoxyethane and 2 ml of saturated aqueous lithium hydroxide solution was then added. The reaction was left for 24 h. The reaction mixture was acidified with dilute acetic acid to pH reached 4-5 and then it was extracted with ethyl acetate (2 x 10 ml). The extracts were concentrated under a stream of dry nitrogen and the residue was flash column chromatographed on silica gel (ether : hexane = 100 : 20 → ether : hexane : acetic acid = 60: 40 : 1 for elution) to afford the title compound **82** (207 mg, 93%) as a colourless oil.

IR (smear): ν_{max} 3611-3317 (br), 3013 (m), 2922 (s), 2852 (m), 2693 (m), 2361 (w), 1714 (s), 1551 (s), 1441 (s), 1379 (m), 1360 (m), 1270 (m), 1071 (m), 969 (w), 916 (m), 844 (m), 824 (w), 720 (m) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, J = 7.1 Hz, C23-H_3), 1.27-1.44 (8H, m, C6-H_2, C20-H_2, C21-H_2, C22-H_2), 1.70-1.82 (H, m), 1.93-2.27 (3H, m), 2.40-2.48 (2H, m, C2-H_2), 2.78-2.86 (6H, m, C10-H_2, C13-H_2, C16-H_2), 4.56-4.59 (H, m, C4-H), 5.30-5.43 (8H, m, C8-H, C9-H, C11-H, C12-H, C14-H, C15-H, C17-H, C18-H);

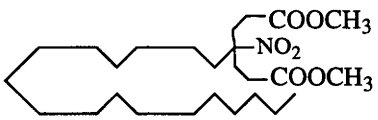
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.65, 23.14, 26.10, 26.20, 26.91, 27.79, 28.86, 29.89, 30.21, 32.09, 33.86, 88.05, 128.07, 128.36, 128.53, 128.89, 129.13, 129.20, 129.65, 131.10, 176.77;

MS (EI) *m/e* (relative intensity): 391 (M^+, 8%), 345 (8), 320 (4), 293 (13), 280 (8), 253 (10), 203 (15), 190 (25), 177 (28), 164 (42), 150 (46), 131 (34), 110 (100), 91 (72), 79 (93), 67 (97);

HRMS: Calculated for $C_{23}H_{37}NO_4$ *m/e*: 391.272259 (M^+). Found 391.272504;

Analysis: Calculated for $C_{23}H_{37}NO_4$: C, 70.55; H, 9.52; N,3.58. Found: C, 70.29; H, 9.86; N, 3.43%.

Dimethyl 4-heptadecyl-4-nitroheptane-1,7-dioate 83



A solution containing 1-nitrooctadecane **55** (50 mg, 0.17 mmol), methyl acrylate (88 mg, 1.02 mmol, 6 eq.) and DBU (13 mg, 0.085 mmol, 0.5 eq.) in 2 ml of dichloromethane was kept at room temperature for 24 h and the mixture was then acidified with 5 ml of HCl (10%). The organic material was extracted twice with 10 ml of CH_2Cl_2. Combined extracts were dried with Na_2SO_4 and concentrated, and the residue was purified by flash column chromatography (EtOAc : petroleum spirit = 15 : 85 for elution), giving dimethyl 4-heptadecyl-4-nitroheptane-1,7-dioate **83** (76 mg, 95%) as a colourless oil.

IR (smear): ν_{max} 2954 (m), 2914 (s), 2849 (s), 1744 (s), 1732 (s), 1537 (s), 1470 (s), 1458 (s) 1439 (s), 1378 (s), 1355 (s), 1319 (s), 1298 (s), 1203 (s), 1180 (s), 1129 (s),

1110 (m), 1071 (m), 1022 (m), 986 (s), 894 (s), 864 (m), 842 (s), 826 (s), 807 (m), 788 (m), 717 (s), 705 (m) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 6.8 Hz, C17'-H_3), 1.16-1.25 [30H, m, (C2'-C16')-H_2], 1.85-1.91 (2H, m, C1'-H_2), 2.23-2.28 (8H, m, C2-H_2, C3-H_2, C5-H_2, C6-H_2), 3.69 (6H, s, OCH_3);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.67, 23.25, 24.14, 29.12, 29.83, 29.92, 30.05, 30.12, 30.25, 30.88, 32.48, 35.99, 52.53, 93.30, 172.96;

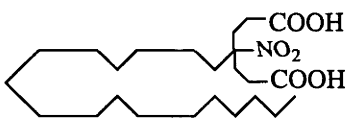
MS (EI) *m/e* (relative intensity): 440 ($(M-OCH_3)^+$, 9%), 425 (28), 393 (100), 392 (73), 392 (83), 364 (19), 333 (18), 305 (14), 194 (11), 168 (42), 138 (82), 109 (35), 81 (53);

MS (CI) m/e: 489 $(MNH_4)^+$;

HRMS: Calculated for $C_{25}H_{46}NO_5$ *m/e*: 440.337599 $(M-OCH_3)^+$. Found 440.337909;

Analysis : Calculated for $C_{26}H_{49}NO_6$: C, 66.21; H, 10.47; N, 2.97. Found: C, 66.63; H, 10.91; N, 2.71%.

4-Heptadecyl-4-nitroheptane-1,7-dicarboxylic acid 84



Dimethyl 4-heptadecyl-4-nitroheptane-1,7-dioate **83** (138 mg, 0.29 mmol) was dissolved in 2 ml of 1,2-dimethoxyethane, and then 2 ml of saturated aqueous lithium hydroxide solution was added. The reaction was left for 22 h and the mixture was then acidified with dilute HCl (10%, 10 ml) and extracted with ethyl acetate (2 x 10 ml). The extracts were concentrated under a stream of dry nitrogen and the residue was purified by flash column chromatography on silica gel (ethyl acetate : petroleum spirit 40-60 °C = 15 : 85 for elution) to afford the title compound **84** (93 mg, 90%) as a white solid.

M.P. 102 °C.

IR (Nujol): ν_{max} 3600-2700 (br), 2919 (s), 2852 (s), 1740 (s), 1700 (w), 1652 (w), 1534 (s), 1467 (m),1454 (m), 1428 (m), 1353 (w), 1323 (m), 1282 (m), 1267 (w), 1354 (w), 1322 (m), 1234 (m), 1224 (s), 894 (w), 834 (w), 814 (w), 721 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 6.8 Hz, C17'-H_3), 1.17-1.30 (30H, m, (C2'-C16')-H_2), 1.85-1.91 (2H, m, C1'-H_2), 2.26-2.40 (8H, m, C1-H_2, C2-H_2, C4-H_2, C5-H_2);

^{13}C NMR ($CDCl_3$,300 MHz): δ 14.69, 23.26, 23.90, 29.08, 29.40, 29.81, 29.90, 29.93, 30.02, 30.11, 30.23, 30.26, 32.49, 37.69, 93.83, 179.23;

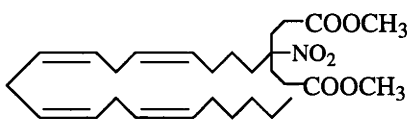
MS (EI) *m/e* (relative intensity): 426 ((M-OH)$^+$, 1%), 397 (3), 379 (68), 377 (70), 359 (56), 350 (28), 332 (42), 323 (56), 305 (30), 168 (77), 157 (100), 138 (56), 129 (56), 111 (58), 97 (58), 81 (58), 71 (64), 57 (68);

MS (CI) *m/e*: 461 (M+NH_4)$^+$;

HRMS: Calculated for $C_{24}H_{44}NO_5$ *m/e*: 426.321949 (M-OH)$^+$. Found 426.322876;

Analysis: Calculated for $C_{24}H_{45}NO_6$: C, 64.98; H, 10.22; N, 3.16. Found: C, 64.55; H, 10.69; N, 2.81%.

Dimethyl 4-[(*all-Z*)-Nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dioate 85



A solution of (*all-Z*)-1-nitro-5,8,11,14-eicosatetraene **60c** (96 mg, 0.30 mmol), methyl acrylate (155 mg, 1.80 mmol, 6 eq.) and DBU (23 mg, 0.15 mmol, 0.5 eq.) in 5 ml of dichloromethane was kept at room temperature for 24 h, and the mixture was then poured into water and then acidified with dilute HCl. The organic material was extracted twice with CH_2Cl_2 and the combined extracts were dried with Na_2SO_4 and concentrated. The residue was purified by flash column chromatography (EtOAc: PS = 20 : 100 for elution), giving dimethyl 3-[(*all-Z*)-nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dioate **85** (127 mg, 86%) as a colourless oil.

IR (smear): ν_{max} 3012 (m), 2955 (m), 2929 (m), 2857 (m), 1742 (s), 1540 (s), 1438 (m), 1379 (w), 1351 (m), 1321 (m), 1260 (m), 1200 (m), 1176 (m), 990 (w), 721 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 6.8 Hz, C19'-H_3), 1.25-1.35 (8H, m, C2'-H_2, C16'-H_2, C17'-H_2, C18'-H_2), 1.86-1.92 (2H, m, C1'-H_2), 2.03-2.10 (4H, m, C3'-H_2, C15'-H_2), 2.25-2.37 (8H, m, C2-H_2, C3-H_2, C5-H_2, C6-H_2), 2.78-2.86 (6H, m, C6'-H_2, C9'-H_2, C12'-H_2), 3.69 (6H, s, OCH_3), 5.31-5.43 (8H, m, C4'-H, C5'-H, C7'-H, C8'-H, C10'-H, C11'-H, C13'-H, C14'-H);

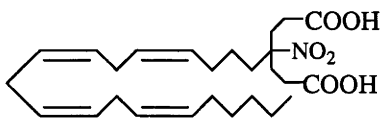
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.64, 23.14, 24.06, 26.22, 27.35, 27.79, 29.12, 29.89, 30.92, 32.08, 35.39, 52.57, 93.21, 128.07, 128.34, 128.48, 128.93, 129.05, 129.21, 129.89, 131.09, 172.92;

MS (EI) *m/e* (relative intensity): 491 (M^+, 16%), 460 (72), 444 (50), 429 (28), 413 (70), 393 (42), 381 (28), 357 (36), 333 (14), 301 (50), 207 (26), 181 (32), 164 (34), 150 (40), 133 (40), 121 (50), 106 (71), 93 (86), 80 (78), 79 (100), 67 (98), 55 (60);

HRMS: Calculated for $C_{28}H_{45}NO_6$ *m/e*: 491.324689 (M^+). Found 491.324673;

Analysis: Calculated for $C_{28}H_{45}NO_6$: C, 68.40; H, 9.22; N, 2.85. Found C, 68.77; H, 9.57; N, 2.85%.

4-[(*all-Z*)-Nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dicarboxylic acid 86



Dimethyl 4-[(*all-Z*)-nonadeca-4,7,10,13-tetraenyl]-4-nitropentane-1,7-dioate **85** (110 mg, 0.22 mmol) was dissolved in 2 ml of 1,2-dimethoxyethane and then 2 ml of saturated lithium hydroxide solution was added. The reaction was left for 24 h. The reaction mixture was acidified with dilute acetic acid to pH 4-5 and extracted with ethyl acetate (2 x 10 ml). The resulting extract was concentrated under a stream of dry nitrogen

and the residue was chromatographed on silica gel (ethyl acetate : petroleum 40-60°C = 15 : 85 for elution) to afford title compound **86** (90 mg, 88%) as a white solid.

M.P.: 50-51 °C

IR (smear): ν_{max} 3400-2300 (br), 3013 (s), 2955 (s), 2927 (s), 2855 (s), 2734 (m), 2630 (m), 1742 (s), 1714 (s), 1538 (s), 1439 (s), 1353 (s), 1321 (s), 1291 (s), 1231 (s), 1068 (m), 989 (m), 918 (s), 833 (s), 807 (m), 803 (m), 732 (m), 678 (m), 622 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, *J* = 6.9 Hz, C19'-H_3), 1.21-1.38 (8H, m, C2'-H_2, C16'-H_2, C17'-H_2, C18'-H_2), 1.85-1.91 (2H, m, C1'-H_2), 2.03-2.09 (4H, m, C3'-H_2, C15'-H_2), 2.26-2.38 (8H, m, C1-H_2, C2-H_2, C4-H_2, C5-H_2), 2.77-2.86 (6H, m, C6'-H_2, C9'-H_2, C12'-H_2), 5.25-5.47 (8H, m, C4'-H, C5'-H, C7'-H, C8'-H, C10'-H, C11'-H, C13'-H, C14'-H);

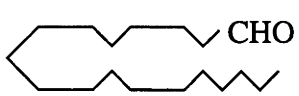
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.66, 23.14, 23.93, 26.21, 27.19, 27.78, 29.23, 29.74, 29.88, 32.07, 36.37, 93.37, 128.05, 128.31, 128.43, 128.94(2C), 129.21, 129.95, 131.09, 178.84;

MS (EI) *m/e* (relative intensity): 463 (M^+, 16%), 446 (4), 416 (24), 397 (6), 365 (4), 343 (8), 305 (6), 278 (10), 245 (12), 231 (12), 217 (14), 203 (22), 192 (20), 177 (56), 164 (42), 157 (38), 145 (30), 138 (50), 119 (54), 106 (72), 93 (82), 91 (76), 80 (72), 79 (100), 69 (46), 67 (98), 55 (64);

HRMS: Calculated for $C_{26}H_{41}NO_6$ *m/e*: 463.293388 (M^+). Found 463.294170;

Analysis: Calculated for $C_{26}H_{41}NO_6$: C, 67.36; H, 8.91; N, 3.02. Found: C, 67.51; H, 9.23; N, 2.92%.

Octadecanal 87



Pyridinium chlorochromate (6 g, 27.83 mmol, 1.5 eq.) was suspended in dichloromethane (30 ml), and octadecan-1-ol **53** (5.02 g, 18.57 mmol) in dichloromethane (15 ml) was then rapidly added at room temperature. The solution

become briefly homogeneous before the deposition of the black insoluble reduced reagent. After 2 h, the black mixture was diluted with five volumes of anhydrous diethyl ether, the solvent was decanted, and the black solid was washed twice with ether. The crude product was isolated by filtration of the organic extracts through Florisil and evaporation of the solvent under reduced pressure. Purification by flash column chromatography (ether : hexane = 4 : 96 for elution) gave the title compound **87** (4.02 g, 81%) as a white solid.

M.P. 43-44 °C.

IR (Nujol): ν_{max} 2960 (s), 2910 (s), 2850 (s), 2705 (w), 1730 (s), 1460 (s), 1375 (s), 720 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 6.4 Hz, C18-H_2), 1.28 (28H, m, C4-C17)-H_2), 1.58-1.65 (2H, m, C3-H_2), 2.42 (2H, t, J = 7.3Hz, C2-H_2), 9.76 (H, s, CHO);

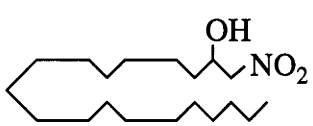
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.69, 22.65, 23.26, 29.73, 29.92, 29.99, 30.14, 30.26, 32.49, 44.49, 203.56;

MS (EI) *m/e* (relative intensity): 268 (M^+, 4%), 250 (34) , 224 (17), 222 (18), 208 (6), 194 (10), 182 (8), 166 (8), 152 (10), 137 (20), 124 (30), 110 (42), 96 (74), 82 (100), 71 (82), 69 (69), 57 (53), 55 (57);

HRMS: Calculated for $C_{18}H_{36}O$ *m/e*: 268.276616 (M^+). Found: 268.276466;

Analysis: Calculated for $C_{18}H_{36}O$: C, 80.53; H, 13.51. Found: 80.46, H, 13.49%.

1-Nitrononadecan-2-ol 88



To a solution of octadecanal **87** (2.22 g, 8.28 mmol) and nitromethane (1.52 g, 24.90 mmol, 3 eq.) in anhydrous ether (10 ml), Amberlyst A-21 (1.2 g) was added at room temperature. The mixture was stirred at reflux for 48 h. After the removal of Amberlyst

A-21 by filtration, the extract was concentrated under reduced pressure. Purification of the residue by flash column chromatography (eluted with 5% ethyl acetate in petroleum spirit 40-60 °C) gave the title compound **88** (2.41 g, 89%) as a white solid.

M.P. 55-56 °C.

IR (Nujol): ν_{max} 3500-3300 (br), 2960 (s), 2910 (s), 2850 (s), 1550 (m), 1460 (s), 1375 (s), 720 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.86-0.90 (3H, m, C19-H_3), 1.26 [30H, m, (C4-C18)-H_2], 1.43-1.55 (2H, m, C3-H_2), 2.22-2.43 (H, bs, OH), 4.28-4.46 (3H, m, C1-H_2, C2-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.66, 23.25, 25.73, 29.88, 29.92, 30.00, 30.08, 30.17, 30.25, 32.49, 34.29, 69.24, 81.20;

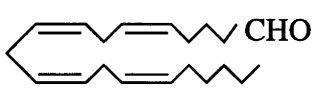
MS (EI) *m/e* (relative intensity): 311 ($(M-H_2O)^+$, 3%), 294 (32), 282 (9), 276 (27), 267 (31), 250 (34), 240 (6), 222 (15), 208 (8), 194 (9), 179 (7), 165 (10), 151 (16), 137 (37), 123 (62), 109 (85), 97 (95), 95 (100), 83 (100), 69 (88), 57 (92), 55 (92);

MS (CI) *m/e*: 347 $(M+NH_4)^+$;

HRMS: Calculated for $C_{19}H_{37}NO_2$ m/e: 311.282430 $(M-H_2O)^+$. Found: 311.283095;

Analysis: Calculated for $C_{19}H_{39}NO_3$: C, 69.25; H, 11.93, N, 4.25. Found: C, 69.54, H, 12.18, N, 4.13%.

(*all-Z*)-Eicosa-5,8,11,14-tetraenal 89



According to the procedure described above for the preparation of octadecanal **87**, to a suspension of pyridinium chlorochromate (458 mg, 2.12 mmol, 1.5 eq.) in dichloromethane (4 ml) was added arachidonyl alcohol **57c** (402 mg, 1.38 mmol). The mixture was stirred for 2 h. After workup, the residue was chromatographed on a silica gel column (eluted with 4% ether in hexane) to afford 303 mg of the title compound **89** (76%) as a colourless oil.

IR (smear): ν_{max} 3005 (s), 2960 (s), 2910 (s), 2850 (s), 1730 (s), 1460 (w), 1390 (w), 1160 (w), 920 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, J = 6.8 Hz, C20-H_3), 1.28-1.34 (6H, m, C17-H_2, C18-H_2, C19-H_2), 1.69-1.74 (2H, m, C3-H_2), 2.04-2.14 (4H, m, C4-H_2, C16-H_2), 2.42-2.45 (2H, m, C2-H_2), 2.79-2.85 (6H, m, C7-H_2, C10-H_2, C13-H_2), 5.34-5.40 (8H, m, C5-H, C6-H, C8-H, C9-H, C11-H, C12-H, C14-H, C15-H); 9.78 (H, s, CHO);

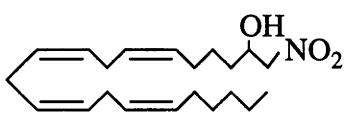
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.51, 22.34, 23.00, 26.05, 26.89, 27.64, 29.74, 31.94, 43.70, 127.92, 128.22, 128.44, 128.68, 129.01, 129.21, 129.48, 130.91, 202.90;

MS (EI) *m/e* (relative intensity): 288 (M^+, <1%), 244 (1), 234 (1), 217 (2), 203 (3), 177 (9), 164 (13), 150 (30), 131 (12), 119 (19), 106 (59), 93 (56), 91 (64), 80 (77), 79 (100), 67 (93), 55 (43);

HRMS: Calculated for $C_{20}H_{32}O$ *m/e*: 288.245316 (M^+). Found: 288.244932;

Analysis: Calculated for $C_{20}H_{32}O$: C, 83.27; H, 11.18. Found: C, 83.28; H, 11.12%.

(*all-Z*)-1-Nitrohenicosa-6,9,12,15-tetraen-2-ol 90



According to the procedure described above for the synthesis of 1-nitrononadecan-2-ol **88**, to a solution of (*all-Z*)-eicosa-5,8,11,14-tetraenal **89** (220 mg, 0.76 mmol) and nitromethane (139 mg, 2.28 mmol, 3 eq.) in anhydrous ether (5 ml), Amberlyst A-21 (200 mg) was added at room temperature. The mixture was stirred at 50 °C for 3.5 days. Purification of the crude product by flash column chromatography (eluted with 5% ethyl acetate in petroleum spirit 40-60 °C) gave the title compound **90** (240 mg, 90%) as a colourless oil.

IR (smear): ν_{max} 3600-3300 (br), 3005 (s), 2960 (s), 2910 (s), 2850 (s), 1650 (w), 1550 (s), 1460 (m), 1440 (m), 1380 (s), 1260 (w), 910 (w), 720 (s) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.87-0.91 (3H, m, C21-H_3), 1.27-1.39 (6H, m, C18-H_2, C19-H_2, C20-H_2), 1.50-1.56 (4H, m, C3-H_2, C4-H_2), 2.02-2.16 (4H, m, C5-H_2, C17-H_2), 2.40-2.60 (H, bs, OH), 2.80-2.86 (6H, m, C8-H_2, C11-H_2, C14-H_2), 4.29-4.45 (3H, m, C1-H_2, C2-H), 5.30-5.45 (8H, m, C6-H, C7-H, C9-H, C10-H, C12-H, C13-H, C15-H, C16-H);

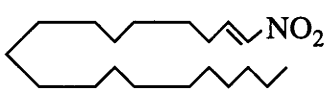
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.49, 22.97, 25.52, 26.03, 27.13, 27.61, 29.71, 31.91, 33.53, 68.91, 80.98, 127.88, 128.20, 128.46, 128.63, 128.99, 129.11, 129.51, 130.92;

MS (EI) *m/e* (relative intensity): 349 (M^+, <1%), 314 (1), 251 (2), 234 (1), 217 (2), 203 (3), 177 (6), 164 (10), 150 (24), 131 (13), 119 (21), 106 (43), 93 (57), 91 (71), 79 (100), 67 (92), 55 (48);

HRMS: Calculated for $C_{21}H_{35}NO_3$ *m/e*: 349.261694 (M^+). Found: 349.261372;

Analysis: Calculated for $C_{21}H_{35}NO_3$: C, 72.17; H, 10.09, N, 4.01. Found: C, 72.25, H, 9.91; N, 3.64%.

(*E*)-1-Nitrononadec-1-ene 91



To a solution of 1-nitrononadecan-2-ol **88** (215 mg, 0.65 mmol, 1 eq.) in CH_2Cl_2 (4 ml), methanesulfonyl chloride (74 mg, 0.65 mmol) was added in one portion and triethylamine (263 mg, 2.60 mmol, 4 eq.) was added dropwise at 0 °C. The mixture was stirred at 0 °C for 30 min and then it was washed with 5% HCl, water and brine, and concentrated. The residue was chromatographed on a silica gel column (eluted with 10% ether in hexane) to yield an impure compound **91** due to decomposition (63 mg, 31%) as a white solid.

IR (Nujol): ν_{max} 2960 (s), 2910 (s), 2850 (s), 1700 (w), 1530 (s), 1460 (s), 1375 (m), 1350 (m), 960 (w), 720 (w) cm^{-1};

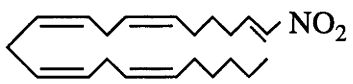
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, *J* = 6.9 Hz, C19-H_3), 1.18-1.28 (28H, m, (C5-C18)-H_2)), 1.46-1.56 (2H, m, C4-H_2), 2.22-2.30 (2H, m, C3-H_2), 6.96-7.00 (H, d, *J* = 14 Hz, C1-H), 7.23-7.33 (H, m, C2-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.69, 23.26, 26.27, 29.02, 29.65, 29.82, 29.93, 30.01, 30.14, 30.26, 32.48, 140.08, 143.42;

MS (EI) *m/e* (relative intencity): 311 (M^+, 1%), 294 (30), 276 (62), 250 (8), 222 (6), 180 (6), 152 (8), 137 (16), 123 (32), 109 (64), 97 (78), 95 (78), 83 (88), 81 (76), 69 (88), 57 (98), 55 (100);

HRMS: Calculated for $C_{19}H_{37}NO_2$ *m/e*: 311.282430 (M^+). Found: 311.282590.

(1*E*,6*Z*,9*Z*,12*Z*,15*Z*,)-1-Nitrohenicosa-1,6,9,12,15-pentaene 96



To a solution of (*all-Z*)-1-nitrohenicosa-6,9,12,15-tetraen-2-ol **90** (60 mg, 0.17 mmol) in CH_2Cl_2 (4 ml), methanesulfonyl chloride (19 mg, 0.17 mmol, 1 eq.) was added in one portion and triethylamine (69 mg, 0.68 mmol, 4 eq.) was added dropwise at 0 °C. The mixture was stirred at 0 °C for 10 min and then it was washed with 5% HCl, water and brine, and concentrated. The residue was chromatographed on a silica gel column (eluted with 10% ether in hexane) to give an impure compound **96** due to decomposition (27 mg, 47%) as a colourless oil.

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, *J* = 6.8 Hz, C21-H_3), 1.26-1.38 (6H, m, C18-H_2, C19-H_2, C20-H_2), 1.57-1.65 (2H, m, C4-H_2), 2.02-2.18 (4H, m, C5-H_2, C17-H_2), 2.24-2.30 (2H, m, C3-H_2), 2.79-2.86 (6H, m, C8-H_2, C11-H_2, C14-H_2), 5.32-5.46 (8H, m, C6-H, C7-H, C9-H, C10-H, C12-H, C13-H, C15-H, C16-H), 6.96-7.01 (H, d, *J* = 14 Hz, C1-H), 7.26-7.30 (H, m, C2-H), ;

MS (EI) *m/e* (relative intensity): 331 (M^+, 4%), 314 (30), 296 (6), 269 (4), 230 (5), 216 (24), 199 (10), 190 (14), 177 (27), 175 (22), 164 (26), 150 (49), 131 (40), 119 (49), 105 (63), 91 (74), 79 (100), 67 (99), 55 (57);

HRMS: Calculated for $C_{21}H_{33}NO_2$ *m/e*: 331.251130 (M^+). Found: 331.251134.

5.3. Experimental for chapter 3

5.3.1. Buffers for lipoxygenase assays

Buffers used for the 5-LO, 12-LO and 15-LO assays are shown in Table 5.1.

Table 5.1 pH values of buffers and contents for lipoxygenase assays

pH	Purpose	Content
6.3	5-LO assay	0.1 M KH_2PO_4, 0.005% Tween 20
7.4	12-LO assay	50 mM Tris-HCl 0.03% Tween 20
9.0	15-LO assay	0.1 M KH_2PO_4
6.3	Extraction of 5-LO from potato tuber	0.1 M KH_2PO_4 2 mM $Na_2S_2O_5$ 2 mM ascorbic acid 1 mM EDTA
6.3	Dialysis of 5-LO extract	40 mM KH_2PO_4

5.3.2. Preparation of 5-lipoxygenase from potato tuber

Based on the procedure described by Reddanna,[133] 160 g of red potato tuber was minced and homogenised in a blender for 30 sec with 500 ml buffer. The homogenate was filtered through two layers of cloth and the filtrate was centrifuged at 10,000 rpm for 20 min. The pellet was discarded and to the supernatant (360 ml) was added 28.8 g of $(NH_4)_2SO_4$ with continuous stirring to give a 15% saturated solution. The suspension was then adjusted to pH 6.3 with 1M NH_4OH and stirred for 1 h. Precipitated protein was pelleted by centrifugation at 15,000 rpm for 15 min and discarded. The resulting supernatant was brought to 45% saturation by further addition of 65.8 g solid $(NH_4)_2SO_4$, then it was stirred for 1 h, and centrifuged at 15,000 rpm for 15 min. The pellet was resuspended in 100 ml of 40 mM KH_2PO_4 (pH 6.3). The suspension was dialysed for 24 h against 2 litres of 40 mM KH_2PO_4 (pH 6.3) with two changes.

5.3.3. Determination of K_m and V_{max} values of 15-lipoxygenase catalysed oxidation of (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoic acid

(*all-Z*)-4-Nitrotricosa-8,11,14,17-tetraenoic acid **82** (4.5 mg) was dissolved in 5 ml of dichloromethane to make a 0.9 mg/ml stock solution. Varying volumes of the solution (43, 87, 130, 175, 220, 260 or 350 µl) were added separately to seven volumetric flasks. Dichloromethane was then evaporated under nitrogen flow and to each flask was added 20 ml of 0.1 M KH_2PO_4 (pH 9.0) to make a series of diluted solutions (5, 10, 15, 20, 25, 30, 40 µM). 2.5 ml of each diluted solution was added to a 1 cm quartz cuvette, followed by addition of 25 µl of 0.1 mg/ml 15-LO. The absorbance of the solution was measured by ultraviolet spectroscopy at 234 nm. Based on these concentrations of samples and the corresponding initial rates of 15-LO catalysed oxidation, a Hanes plot was obtained and the K_m and V_{max} values were then deduced from the slope and the [S]/v axis intercept of the plot.

5.3.4. Determination of K_m and V_{max} values of 12-lipoxygenase catalysed oxidation of (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoic acid

(*all*-Z)-4-Nitrotricosa-8,11,14,17-tetraenoic acid **82** (7 mg) was dissolved in 5 ml of dichloromethane to make a 1.4 mg/ml stock solution. Varying volumes of the solution (28, 56, 168, 224, 336 and 448 μl) were added separately to six volumetric flasks. Dichloromethane was then evaporated under nitrogen flow and to each flask was added 20 ml of 50 mM Tris-HCl, 0.03% Tween 20 (pH 7.4) to make a series of diluted solutions (5, 10, 30, 45, 60 and 80 μM). 2.5 ml of each diluted solution was added to a 1 cm quartz cuvette, followed by addition of 25 μl of 12-LO (7.4 μg/μl). The absorbance of the solution was measured by ultraviolet spectroscopy at 234 nm. Based on these concentrations of samples and the corresponding initial rates of 12-LO catalysed oxidation, a Hanes plot was obtained and the K_m and V_{max} values were then deduced from the slope and the [S]/v axis intercept of the plot.

5.3.5. Determination of the inhibition constants K_I and K_i of 4-heptadecyl-4-nitroheptane-1,7-dicarboxylic acid for 15-lipoxygenase catalysed oxidation of arachidonic acid

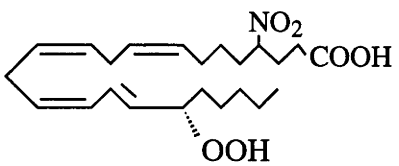
Arachidonic acid **1** (2.38 mg) and 4-heptadecyl-4-nitroheptane-1,7-dicarboxylic **84** (9.15 mg) were separately dissolved in dichloromethane (5 ml) to make two stock solutions. Four series of diluted solutions were prepared. In each series of solutions, six solutions were made with different concentrations of arachidonic acid **1** (10, 15, 20, 25, 30 or 40 μM) at a fixed concentration of compound **84** (0 , 20, 50 or 80 μM), 2.5 ml of each solution was loaded to a 1 cm cuvette, and after addition of 15-LO (25 μl, 0.1 μg/μl), the initial rate of arachidonic acid **1** oxidation was recorded by ultraviolet spectroscopy at 234 nm. The inhibitor constants K_i and K_I were calculated based on the method described in section 3.1.5.

5.3.6. Analysis of metabolic reaction of 4-[(*all-Z*)-nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dicarboxylic acid in the presence of 12-lipoxygenase

4-[(*all-Z*)-Nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dicarboxylic acid **86** (0.2 mg) and lauric acid (2 mg) were dissolved in 1.0 ml of buffer (50 mM Tris-HCl, pH 7.4) and mixed with 1 ml of 12-LO (7.4 μg/μl). After zero h (and 4 h) incubation, 0.8 ml aliquots were removed and extracted with 2 ml of diethyl ether. The extracts were concentrated under a nitrogen flow, and the residues were dissolved in 100 μl of the HPLC mobile phase (acetonitrile : 30 mM H_3PO_4 = 70 : 30), 60 μl of the sample was analysed by HPLC with refractive index detection. The HPLC column used contained Alltech Spherisorb octadecylsilane and the rate of the mobile phase was 3 ml/min.

5.3.7. Synthesis

(8*Z*,11*Z*,14*Z*,16*E*)-18(*S*)-hydroperoxy-4-nitrotricosa-8,11,14,16-tetraenoic acid 97

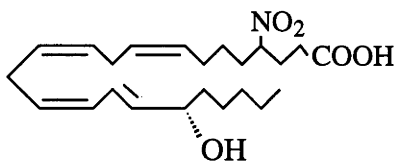


Phosphate buffer (0.1 M, pH = 9.0, 60 ml) in a 250 ml conical flask was cooled to 0 °C in an ice bath. Soybean 15-LO (6 mg) was added, followed by addition of a solution of (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoic acid **82** (45 mg, 0.12 mmol) in ethanol (2 ml). The mixture was stirred and oxygen was bubbled through the mixture. The reaction was monitored by ultraviolet spectroscopy at 234 nm. After 30 min, the mixture was acidified with hydrochloric acid (10%, 10 ml) to pH 3. The product was extracted with cold dichloromethane (2 x 100 ml, 0 °C). The combined extracts were dried over sodium sulphate and concentrated *in vacuo*. Purification by flash column chromatography (eluted with ether : hexane : acetic acid = 60 : 40 : 1) at 0-4 °C gave (8*Z*,11*Z*,14*Z*,16*E*)-18(*S*)-

hydroperoxy-4-nitrotricosa-8,11,14,16-tetraenoic acid **97** 26 mg as a colourless oil with a purity of 87% according to ^{1}H NMR analysis. Further characterisation was not possible due to the instability of this compound.

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 6.8 Hz, C23-H_3), 1.29-1.51 (8H, m, C6-H_2,C20-H_2, C21-H_2, C22-H_2), 1.66-1.78 (2H, m), 1.96-2.24 (6H, m), 2.40-2.47 (2H, m, C2-H_2), 2.74-2.82 (2H, m, C10-H_2), 2.96-3.00 (2H, m, C13-H_2), 4.36-4.43 (H, m, C18-H), 4.53-4.61 (H, m, C4-H), 5.31-5.50 (5H, m, C8-H, C9-H, C11-H, C12-H, C14-H), 5.57-5.65 (H, dd, *J* = 8, 15 Hz, C17-H), 6.00-6.07 (H, t, *J* = 11 Hz, C15-H), 6.56-6.65 (H, dd, *J* = 11, 15 Hz, C16-H);
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.57, 23.06, 25.52, 26.03, 26.22, 26.74, 26.88, 28.82, 30.39, 32.29, 33.08, 33.79, 87.35, 87.96, 128.04, 128.36, 129.14, 129.27, 129.45, 130.14, 131.81, 132.50, 177.61.

(8*Z*,11*Z*,14*Z*,16*E*)-18(*S*)-Hydroxy-4-nitrotricosa-8,11,14,16-tetraenoic acid 98



Phosphate buffer (0.1 M, pH = 9.0, 80 ml) in a conical flask (250 ml) was cooled to 0 °C in an ice bath. Soybean 15-LO (12.5 mg) was added, followed by addition of a solution of (*all-Z*)-4-nitrotricosa-8,11,14,17-tetraenoic acid **82** (100 mg, 0.26 mmol in 2 ml of ethanol). The mixture was stirred and oxygen was bubbled through the mixture at 0 °C. The reaction was monitored by ultraviolet spectroscopy at 234 nm. After 30 min, dichloromethane (50 ml) was poured into the reaction mixture and to the resulting turbid mixture was added sodium borohydride (90 mg). The mixture was stirred at 0 °C for 1 h and was then it was acidified with hydrochloric acid (10%, 10 ml) to pH 3. The product was extracted with dichloromethane (2 x 100 ml). The combined extracts were dried over sodium sulphate and concentrated *in vacuo*. Purification by flash column chromatography

(eluted with ether : hexane : acetic acid = 60 : 40 : 1) gave (8*Z*,11*Z*,14*Z*,16*E*)-18-hydroxy-4-nitrotricosa-8,11,14,16-tetraenoic acid **98** (44 mg, 42%) as a colourless oil.

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.87-0.90 (3H, m, C23-H_3), 1.19-1.47 (8H, m, C6-H_2, C20-H_2, C21-H_2, C22-H_2), 1.52-1.57 (2H, m), 1.72-1.80 (H, m), 1.97-1.23 (5H, m), 2.32-2.47 (2H, m, C2-H_2), 2.80-2.84 (2H, m, C10-H_2), 2.96-3.00 (2H, m, C13-H_2), 4.19-4.25 (H, m, C18-H), 4.56-4.61 (H, m, C4-H), 5.30-5.46 (5H, m, C8-H, C9-H, C11-H, C12-H, C14-H), 5.66-5.74 (H, dd, *J* = 7, 15 Hz, C17-H), 5.98-6.05 (H, t, *J* = 11 Hz, C15-H), 6.51-6.60 (H, dd, *J* = 11, 15 Hz, C16-H);
^{13}C NMR ($CDCl_3$, 300MHz): δ 14.58, 23.07, 25.53, 26.03, 26.22, 26.74, 26.89, 28.83, 30.35, 32.29, 33.08, 87.34, 87.98, 128.04, 128.35, 129.14, 129.27, 129.43, 130.13, 131.81, 132.53, 177.71;
MS(electrospray MS/MS) *m/e*: 406 $(M-1)^+$.

5.4. Experimental for Chapter 4

5.4.1. Determination of stability of thia fatty acids and sulfides

Compounds **110** (4.3 mg) and **106** (6 mg) were each dissolved in 5 ml of dichloromethane and added into 250 ml one-neck flasks. compound **18** (20 mg), and compounds **19, 108, 109** and **111-112** (14-20 mg) were each dissolved in 10 ml of dichloromethane and added into 500 ml flasks. The solvent dichloromethane was then evaporated with continuous rotation of the flasks, allowing the compounds to form thin films. The flasks were flushed with oxygen, sealed, and kept in darkness. The compounds in the flasks were redissolved in chloroform-*d* and analysed by ^{1}H NMR every two weeks for up to six weeks.

5.4.2. Determination of antioxidant behaviour of 3-[(3Z,6Z)-nona-3,6-dienylthio]propionic acid on arachidonic acid autoxidation

This is a typical autoxidation assay designed to investigate the antioxidant properties of thia fatty acids and sulfides in the autoxidation of arachidonic acid **1**.

Table 5.2 HPLC mobile phase and retention time of thia fatty acids and sulfides

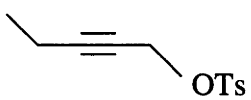
Compound	Mobile phase (buffer = 30 mM H_3PO_4)	Retention time (min) (arachidonic acid **1**)	Retention time (min) (Lauric acid)	Retention time (min) (compound)
18	acetonitrile : buffer (80 : 20)	6.53	4.23	8.75
19	acetonitrile : buffer (80 : 20)	6.80	4.44	10.91
106	acetonitrile : buffer (70 : 30)	14.71	7.13	3.15
108	methanol : buffer (90 : 10)	6.71	4.00	10.74
109	methanol : buffer (90 : 10)	6.82	4.05	9.38
110	acetonitrile : buffer (95 : 5)	3.48	3.09	14.05
111	acetonitrile : buffer (95 : 5)	3.38	3.05	21.57
112	acetonitrile : buffer (90 : 10)	5.24	3.80	6.97

A stock solution in dichloromethane (2 ml) containing 18 mg arachidonic acid **1** and 3-[(3Z,6Z)-nona-3,6-dienylthio]propionic acid **106** (18 mg), as well as lauric acid (18 mg) as an internal standard, was prepared. Samples of the stock solution (100 µl) were added onto glass Petri-dishes followed by addition of 400 µl ethanol. After evaporation of the solvent, a well-distributed thin film was formed on each Petri-dish. The Petri-dishes were then placed in a desiccator, which was evacuated, then filled with oxygen and stored in

the darkness. Dishes were removed from the desisccator after 1, 2, 3, 5 and 7 days, and the mixture on each dish was redissolved in diethyl ether, and transferred to a 2 ml vial. After evaporation of the solvent, the residue was dissolved in 100 μl of the HPLC mobile phase and 10% of the solution was analysed by HPLC using a reverse phase column (octadecylsilane) (4.6 mm x 250 mm, 3 μm) and a refractive index detector. Table 5.2 shows the mobile phases used for different thia fatty acids and sulfides, and their retention times by HPLC.

5.4.3. Synthesis of analogues of 3-[(*all-Z*)-(eicosa-5,8,11,14-tetraenyl-thio)]propionic acid

Pent-2-ynyl *p*-toluenesulfonate 102



2-Pentyn-1-ol **101** (1.03 g, 12 mmol) was dissolved in chloroform (10 ml) and the mixture was cooled in an ice bath. Pyridine (1.90 g, 24 mmol, 2 eq.) was then added, followed by the addition of *p*-toluenesulfonyl chloride (3.43 g, 18 mmol, 1.5 eq.) in small portions with constant stirring. The reaction was complete in 4 h (monitored by TLC). Ether (30 ml) and water (7 ml) were added and the organic layer was washed successively with 1 N HCl (7 ml), 5% $NaHCO_3$, water (7 ml) and brine (20 ml), and then dried with Na_2SO_4. The solvent was removed under reduced pressure and the crude tosylate was flash column chromatographed (ether: hexane = 20 : 80 for elution) on a silica gel column to yield the title compound **102** (1.85 g, 65%) as a colourless oil.

IR (smear): ν_{max} 2980 (m), 2940 (w), 2878 (w), 2240 (m), 1598 (s), 1495 (w), 1450 (m), 1360 (s), 1180 (s), 1175 (s), 1095 (s), 1020 (m), 1000 (m), 960 (s), 940 (s), 840 (s), 815 (s), 735 (s), 662 (s) cm^{-1};

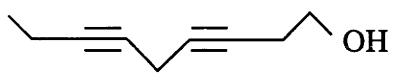
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.98-1.03 (3H, m, C5-H_3); 2.04-2.10 (2H, m, C4-H_2), 2.44 (3H, s, ArCH_3), 4.69 (2H, m, C1-H_2), 7.35 and 7.82 (4H, dd, J = 8.3, 8.7 Hz, ArH);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 12.91, 13.72, 22.22, 59.35, 71.72, 92.33, 128.69, 130.30, 133.90, 145.47;

MS (EI) *m/e* (relative intensity): 238 (M^+, <0.1%), 209 (1), 155 (24), 139 (100), 129 (6), 117 (18), 107 (10), 92 (42), 91 (87), 83 (29), 66 (50), 65 (48);

Analysis: Calculated for $C_{12}H_{14}SO_3$: C, 60.48; H, 5.92; S 13.45. Found C, 60.24; H, 5.93; S, 13.22%.

Nona-3,6-diyn-1-ol 103



Pent-2-ynyl *p*-toluenesolfonate **102** (1.37 g, 5.78 mmol, 1.1 eq.) was added at -30 °C under nitrogen to a well-stirred suspension in DMF (15ml) of but-3-yn-1-ol (368 mg, 5.25 mmol, 1 eq), sodium carbonate (834 mg, 7.87 mmol, 1.5 eq.), tetrabutylammomium chloride (1.46 g, 5.25 mmol) and copper(I) iodide (1.00 g, 5.25 mmol, 1 eq). The mixture was stirred at room temperature for 48 h. Ether (30 ml) and 1N HCl (30 ml) were then added. After filtration through a bed of celite, the organic phase was washed with brine, dried over sodium sulphate and the solvent was evaporated under reduced pressure. Purification of the residue by flash column chromatography on silica gel (40% ether in hexane for elution) gave the product **103** (442 mg, 62%) as a colourless oil.

IR (smear): ν_{max} 3650-3100 (br), 2975 (s), 2938 (s), 2905 (s), 2880 (s), 2500 (m), 1415 (m), 1375 (w), 1320 (s), 1180 (w), 1120 (w), 1040 (s), 900 (m), 735 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.10 (3H, t, J = 7.4 Hz, C9-H_3), 1.96 (H, bs, OH), 2.13-2.20 (2H, m, C8-H_2), 2.41-2.45 (2H, m, C2-H_2), 3.11-3.13 (2H, m, C5-H_2), 3.69 (2H, t, J = 6.1 Hz, C1-H_2);

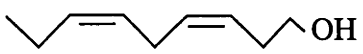
^{13}C NMR (ACETONE, 300 MHz): δ 10.14, 13.07, 14.72, 24.03, 61.95, 75.08, 76.83, 78.46, 82.42;

MS (EI) *m/e* (relative intensity): 135 ($(M-H)^+$, 12%), 121 (44), 107 (30), 105 (51), 103 (29), 93 (44), 91 (100), 79 (58), 77 (80), 65 (41), 63 (29), 57 (14), 53 (27), 51 (37);

HRMS Calculated for $C_9H_{11}O$ *m/e*: 135.080990 $(M-H)^+$. Found 135.081144;

Analysis: Calculated for $C_9H_{12}O$: C, 79.37; H, 8.88. Found C, 79.55; H, 8.82%.

(3*Z*,6*Z*)-Nona-3,6-dien-1-ol 104



Nona-3,6-diyn-1-ol **103** (198 mg, 1.45 mmol) was hydrogenated at atmospheric pressure, in the presence of a mixture of 44 mg of quinoline and 100 mg of palladium (5%) on calcium carbonate, poisoned with lead in 25 ml of methanol. The reaction was stopped after 2.5 h when the uptake of hydrogen was 61 ml. Removal of methanol *in vacuo*, followed by silica gel column chromatography (35% ether in hexane for elution) to remove quinoline, gave 187 mg (92%) of (3*Z*, 6*Z*)-nona-3,6-dien-1-ol **104** as a colourless oil.

IR (smear): ν_{max} 3500-3160 (br), 3011 (s), 2960 (s), 2930 (s), 2870 (s), 1462 (m), 1377 (m), 1050 (m), 722 (m) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.97 (3H, t, *J* = 7.6 Hz, H9-H_3), 2.01-2.12 (2H, m, C8-H), 2.32-2.40 (2H, m, C2-H_2), 2.79-2.84 (2H, t, *J* = 7.1 Hz, C5-H_2), 3.64 (2H, m, C1-H_2), 5.27-5.43 (3H, m), 5.49-5.56 (H, m);

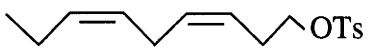
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.82, 21.14, 26.20, 31.33, 62.77, 125.90, 127.40, 132.04, 132.74;

MS (EI) *m/e* (relative intensity): 140 (M^+, 2%); 122 (15), 111 (7), 109 (12), 107 (22), 98 (12), 96 (19), 95 (21), 93 (72), 91 (33), 81 (39), 79 (56), 68 (31), 67 (100), 55 (59), 54 (21), 53 (21);

HRMS Calculated for $C_9H_{16}O$ *m/e*: 140.120115 (M^+). Found 140.120290;

Analysis: Calculated for $C_9H_{16}O$: C, 77.09; H, 11.50. Found C, 77.42; H, 11.75%.

(3Z,6Z)-Nona-3,6-dienyl *p*-toluenesulfonate 105



(3Z,6Z)-Nona-3,6-dien-1-ol **104** (167 mg, 1.19 mmol) was dissolved in chloroform (5 ml) and the solution was cooled in an ice bath. Pyridine (376 mg, 4.76 mmol, 4 eq.) was then added, followed by the addition of *p*-toluenesulfonyl chloride (340 mg, 1.78 mmol, 1.5 eq.) in small portions with constant stirring. The mixture was stirred for 24 h at 15 °C. Ether (15 ml) and water (5 ml) were added and the organic layer was washed successively with 1 N HCl (10 ml), 5% $NaHCO_3$, water (10 ml), and brine (10 ml), and then dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude tosylate was flash column chromatographed (ether: hexane = 20 : 80 for elution) on a silica gel column to yield the title product **105** (201 mg, 57%) as a colourless oil, and the starting material (15 mg, 9%).

IR (smear): ν_{max} 3005 (m), 2960 (s), 2930 (m), 2870 (m), 1599 (m), 1462 (m), 1377 (s), 1310 (w), 1290 (w), 1189 (s), 1178 (s), 1100 (s), 1020 (w), 973 (s), 815 (s), 770 (m), 660 (s) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.95 (3H, t, *J* = 7.6 Hz, C9-H_3), 2.00-2.05 (2H, m, C8-H_2), 2.38-2.44 (2H, m, C2-H_2), 2.45 (3H, s, ArCH_3), 2.69-2.74 (2H, t, *J* = 7.0 Hz, C5-H_2), 3.99-4.04 (2H, m, C1-H_2), 5.20-5.28 (2H, m), 5.34-5.50 (2H, m) 7.33, 7.80 (4H, dd, *J* = 8.2, 8.7 Hz, AA'BB', ArH);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.78, 21.09, 22.20, 26.12, 27.64, 70.20, 123.53, 126.94, 128.47, 130.37, 132.61, 132.92, 145.28;

MS (EI) *m/e* (relative intensity): (277 $(M-OH)^+$, 1%), 155 (25), 139 (2), 122 (67), 107 (47), 93 (100), 91 (77), 79 (66), 67 (47), 55 (32);

MS (CI) *m/e*: 312 $(M+NH_4)^+$;

Analysis: Calculated for $C_{16}H_{22}SO_3$: C, 65.28; H, 7.53; S, 10.89. Found: C, 65.17; H, 7.44; S, 11.27%.

3-[(3*Z*,6*Z*)-Nona-3,6-dienylthio]propionic acid 106

3-Mercaptopropionic acid (150 mg, 1.41 mmol, 1.5 eq.) was added, under an atmosphere of dry nitrogen, to a stirred solution of sodium methoxide, prepared from sodium (64 mg, 2.78 mmol, 3 eq) and methanol (20 ml). After the initial white precipitate had dissolved, a solution of (3*Z*,6*Z*)-nona-3,6-dienyl *p*-toluenesulfonate **105** (276 mg, 0.94 mmol) in diethyl ether was added. After the mixture was stirred at 40 °C for 2 days under nitrogen, 20 ml of hydrochloric acid (10% v/v) and 20 ml of diethyl ether were poured into the crude reaction mixture. The organic phase was separated and washed with water and brine, and dried over sodium sulphate. After removal of the solvent, the residue was purified by flash column chromatography (ether : hexane : acetic acid = 60 : 40 : 2 for elution) to afford 3-[(3*Z*,6*Z*)-noca-3,6-dienylthio]propionic acid **106** (88 mg, 41%) as a colourless oil.

IR (smear): ν_{max} 3400-2500 (br), 3005 (m), 2960 (m), 2910 (m), 2870 (w), 1713 (s), 1459 (m), 1377 (w), 1264 (m), 1195 (w), 1140 (w), 940 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.97 (3H, t, *J* = 7.8 Hz, C9'-H_3), 2.05-2.08 (2H, m, C8'-H_2), 2.34-2.39 (2H, m, C2'-H_2), 2.57-2.60 (2H, t, *J* = 7.4 Hz C1'-H_2), 2.65-2.69 (2H, t, *J* = 7.3 Hz, C3-H_2), 2.78-2.82 (4H, m, C5'-H_2, C2-H_2), 5.27-5.32 (H, m), 5.37-5.47 (3H, m), 5.50-6.10 (H, bs, COOH);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.83, 21.14, 26.20, 27.19, 27.95, 32.62, 35.21, 127.37, 127.97, 130.53, 132.72, 178.66;

MS (EI) *m/e* (relative intensity): 228 (M^+, 34%), 169 (14), 159 (18), 155 (45), 133 (8), 122 (54), 119 (42), 113 (12), 107 (44), 93 (100), 89 (66), 79 (57), 77 (53), 67 (52), 61 (33), 55 (43);

HRMS calculated for $C_{12}H_{20}SO_2$ *m/e*: 228.118402 (M^+). Found 228.118179;

Analysis: Calculated for $C_{12}H_{20}SO_2$: C, 63.12; H, 8.83; S, 14.04. Found: C, 62.90; H, 8.73; S, 14.01%.

3-Tetradecylthiopropionic acid 108

According to the procedure described for the preparation of 3-[3Z,6Z)-nona-3,6-dienylthio]propionic acid **106**, 3-mercaptopropionic acid (261 mg, 2.46 mmol, 1.2 eq.) was added, under an atmosphere of dry nitrogen, to a stirred solution of sodium methoxide, prepared from sodium (142 mg, 6.17 mmol, 3 eq.) and methanol (20 ml). After the initial white precipitate had dissolved, a solution of 1-bromotetradecane **107** (568 mg, 2.05 mmol) in diethyl ether (2 ml) was added The reaction mixture was stirred for 16 h at room temperature. After workup and purification by flash column chromatography (ether : hexane = 20 : 80 → ether : hexane : acetic acid = 60 : 40 : 1 for elution), 450 mg of the title compound **108** (73%) was obtained as a white solid.

M.P. 67 °C.

IR (Nujol): ν_{max} 3100-2600 (br), 2965 (s), 2910 (s), 2840 (s), 1680 (s), 1460 (s), 1405 (w), 1375 (m), 1265 (m), 1255 (w), 1231 (w), 1210 (w), 1200 (m), 1080 (w), 915 (m), 725 (m) cm^{-1};

^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.88 (3H, t, *J* = 6.7 Hz, C14'-H_3), 1.25-1.38 (22H, m, (C3'-C13')-H_2), 1.56-1.61 (2H, m, C2'-H_2), 2.54 (2H, bs, C1'-H_2), 2.65-2.68 (2H, t, J = 6.6 Hz, C3-H_2), 2.79 (2H, bs, C2-H_2);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.69, 23.26, 27.16, 29.44, 29.80, 29.93, 30.02, 30.10, 30.17, 30.23, 32.49, 32.78, 35.25, 178.50;

MS (EI) *m/e* (relative intensity): 302 (M^+, 21%), 230 (24), 229 (100), 185 (2), 161 (4), 119 (8), 106 (24), 97 (15), 89 (21), 83 (22), 69 (25), 55 (32);

HRMS: Calculated for $C_{17}H_{34}SO_2$ *m/e*: 302.227952 (M^+). Found: 302.227166;

Analysis: Calculated for $C_{17}H_{34}SO_2$; C, 67.50; H, 11.33; S, 10.60. Found: C, 67.32; H, 11.32; S, 10.41%.

2-Tetradecylthioacetic acid 109

2-Mercaptoacetic acid (288 mg, 3.13 mmol, 1.2 eq.) was added, under an atmosphere of dry nitrogen, to a stirred solution of sodium methoxide, prepared from sodium (180 mg, 7.83 mmol, 3 eq) and methanol (20 ml). After the initial white precipitate had dissolved, a solution of 1-bromotetradecane **107** (725 mg, 2.61 mmol) in diethyl ether (2 ml) was added and the mixture was stirred for 16 h at room temperature under nitrogen . The crude reaction mixture was poured into an equal volume of hydrochloric acid (10% v/v), and the organic phase was separated and washed with water and brine, and dried over sodium sulphate. After removal of the solvent, the residue was purified by flash column chromatography (diethyl : hexane = 20 : 80 → diethyl ether : hexane : acetic acid = 60 : 40 : 2 for elution) and crystallised to afford 2-tetradecylthioacetic acid **109** (580 mg, 77%) as a white solid.

M.P. 68 °C.

IR (Nujol): ν_{max} 3200-2600 (br), 2950 (s), 2910 (s), 2840 (s), 1680 (s), 1460 (s), 1425 (w), 1375 (s), 1265 (m), 1140 (w), 908 (w), 725 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 6.6 Hz, C14'-H_3), 1.26-1.40 (22H, m, (C3'-C13')-H_2), 1.56-1.64 (2H, m, C2'-H_2), 2.64-2.69 (2H, t, *J* = 7.4 Hz, C1'-H_2), 3.26 (2H, s, C2-H_2);

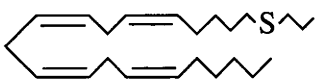
^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.68, 23.26, 29.30, 29.46, 29.75, 29.93, 30.06, 30.15, 30.22, 32.49, 33.36, 34.05, 177.57;

MS (EI) *m/e* (relative intensity): 288 ($M^{+\cdot}$, 12%), 230 (21), 229 (100), 111(6), 97 (17), 83 (27), 69 (30), 55 (34);

HRMS: Calculated for $C_{16}H_{32}SO_2$ *m/e*: 288.212302. Found: 288.212125;

Analysis: Calculated for $C_{16}H_{32}SO_2$; C, 66.61; H, 11.18; S, 11.11. Found: C, 66.46; H, 10.93; S, 10.83%.

Propyl (*all-Z*)-eicosa-5,8,11,14-tetraenyl sulfide 110



Using the procedure described for the preparation of 3-tetradecylthiopropionic acid **108**, propanethiol (26 mg, 0.34 mmol, 1.2 eq.) was added, under an atmosphere of dry nitrogen, to a stirred solution of sodium methoxide, prepared from sodium (20 mg, 0.87 mmol, 3 eq) and methanol (10 ml). After the initial white precipitate had dissolved, a solution of (*all-Z*)-1-bromo-5,8,11,14-eicosatetrane **58c** (101 mg, 0.29 mmol) in diethyl ether (1 ml) was added. The reaction mixture was stirred for 15 h at room temperature. After workup, purification by flash column chromatography (eluted with hexane) gave 75 mg of the title compound **110** (75%) as a colourless oil.

IR (smear): ν_{max} 3005 (s), 2950 (s), 2920 (s), 2850 (s), 1650 (w), 1450 (m), 1390 (w), 1375 (w), 1290 (w), 1260 (w), 1230 (w), 910 (w), 720 (m) cm^{-1};

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, t, *J* = 6.8 Hz, C20-H_3), 0.99 (3H, t, *J* = 7.2 Hz, C3'-H_3), 1.26-1.35 (6H, m, C17-H_2, C18-H_2, C19-H_2), 1.43-1.48 (2H, C3-H_2), 1.57-1.64 (4H, m, C2-H_2, C2'-H_2), 2.05-2.13 (4H, m, C4-H_2, C16-H_2), 2.50-2.51 (4H, m, C1-H_2, C1'-H_2), 2.80-2.86 (6H, m, C7-H_2, C10-H_2, C13-H_2), 5.32-5.43 (8H, m, C5-H, C6-H, C8-H, C9-H, C11-H, C12-H, C14-H, C15-H);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.13, 14.67, 23.17, 23.60, 26.22, 27.41, 27.81, 29.44, 29.91, 32.11, 32.54, 34.79, 128.12, 128.48, 128.64(2C), 128.90, 129.11, 130.40, 131.06;

MS (EI) *m/e* (relative intensity): 348 (M^+, 44%), 305 (38), 273 (4), 251 (6), 237 (14), 205 (17), 177 (19), 161 (36), 150 (27), 131 (29), 119 (40), 105 (48), 93 (77), 91 (76), 81 (79), 79 (95), 67 (100), 55 (69);

HRMS: Calculated for $C_{23}H_{40}S$ *m/e*: 348.285073 (M^+). Found: 348.285378;

Analysis: Calculated for $C_{23}H_{40}S$: C, 79.24; H, 11.56; S, 9.20. Found: C, 78.91; H, 11.38; S, 8.96%.

Propyl tetradecyl sulfide 111

Using the procedure described above for the synthesis of propyl (*all-Z*)-eicosa-5,8,11,14-tetraenyl sulfide **110**, propanethiol (165 mg, 2.16 mmol, 1.2 eq.) was added, under an atmosphere of dry nitrogen, to a stirred solution of sodium methoxide, prepared from sodium (82 mg, 3.56 mmol, 2 eq.) and methanol (10 ml). After the initial white precipitate had dissolved, a solution of 1-bromotetradecane **107** (500 mg, 1.80 mmol) in diethyl ether (2 ml) was added. The reaction mixture was stirred for 15 h at room temperature. After workup, purification by flash column chromatography (eluted with hexane) gave 435 mg of the title compound **111** (89%) as a colourless oil.

IR (smear): ν_{max} 2960 (s), 2910 (s), 2850 (s), 1460 (s), 1375 (w), 1290 (w), 1270 (w), 890 (w), 720 (w) cm^{-1};

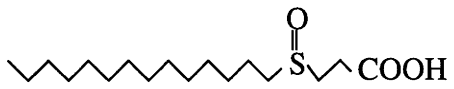
^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.87 (3H, t, J = 6.5 Hz, C14-H_3), 0.99 (3H, t, J = 7.4Hz, C3'-H_3), 1.25 (22H, m, (C3-C13)-H_2), 1.54-1.63 (4H, m, C2-H_2, C2'-H_2), 2.47-2.51 (4H, m, C1-H_2, C1'-H_2);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.13, 14.71, 23.28, 23.59, 29.55, 29.85, 29.94, 30.12, 30.18, 30.23, 30.33, 32.50, 32.69, 34.78;

MS (EI) *m/e* (relative intensity): 272 (M^+, 52%), 243 (18), 229 (100), 196 (8), 187 (2), 168 (5), 145 (6), 131 (15), 111 (14), 97 (22), 89 (34), 83 (27), 76 (33), 69 (32), 57 (30), 55 (44);

Analysis: Calculated for $C_{17}H_{36}S$: C, 74.92; H, 13.31; S, 11.76. Found: C, 75.05; H, 13.27; S, 11.50%.

3-(Tetradecylsulfinyl)propionic acid 113



Arachidonic acid **1** (175 mg) was dissolved in 5 ml of dichloromethane to make a stock solution (35 mg/ml). 3-Tetradecylthiopropanoic acid **108** (10 mg, 0.03 mmol), arachidonic acid **1** (10 mg, 0.03 mmol, 284 µl) and dichloromethane (10 ml) were added into a one-neck flask (500 ml). The solvent was evaporated using a rotary evaporator to allow the reagents to form a thin film on the internal surface of the flask. After vacuuming, the flask was filled with oxygen and placed in darkness for 7 days. Dichloromethane (5 ml) was then added into the flask to dissolve the mixture and the solution was then transferred to a 2 ml vial. After evaporation of the solvent, the residue was dissolved in the mobile phase (methanol : 30 mM phosphoric acid = 90 : 10, 300 µl) and then subject to reverse phase HPLC analysis. The HPLC was performed on an Alltech Spherisorb octadecylsilane (ODS) column with RI detection. The flow rate of the mobile phase was 3 ml/min. Fifty microlitres of the sample was loaded each time. The product with a retention time of 5.49 min was collected and pooled. After evaporation of the solvent at reduced pressure, the product was extracted with diethyl ether (2 ml). The resulting extract was washed with water and dried with Na_2SO_4 and the solvent evaporated, yielding 2 mg of the title compound **113** as a white solid.

M.P. 166-167 °C.

IR (Nujol): ν_{max} 3600-2500 (br), 2965 (s), 2910 (s), 2840 (s), 1695 (m), 1460 (s), 1375 (s), 1330 (w), 1305 (w), 1125 (w), 1040 (w), 1025 (w), 920 (w), 720 (w) cm^{-1};

^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.81 (3H, t, *J* = 7.0 Hz, C14'-H_3), 1.19-1.26 (20H, m, C4'-C13')-H_2), 1.34-1.37 (2H, m, C3'-H_2), 1.68-1.72 (2H, m, C2'-H_2), 2.70-2.76 (H, m), 2.82-2.89 (3H, m), 2.88-3.03 (H, m), 3.05-3.10 (H, m), 7.96 (H, bs, COOH);

^{13}C NMR ($CDCl_3$, 300 MHz): δ 14.67, 23.19, 23.24, 27.78, 29.29, 29.72, 29.91, 30.09, 30.17, 30.20, 32.47, 46.66, 52.53, 174.37;

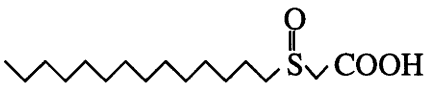
MS (CI) *m/e*: 319 (MH^+);

MS (EI) *m/e* (relative intensity): 301 ((M-OH)$^{+}$, 27%), 246 (21), 245 (16), 229 (100), 196 (5), 121 (15), 94 (22), 97 (22), 83 (29), 71 (32), 70 (34), 57 (51);

HRMS: Calculated for $C_{17}H_{33}SO_2$ *m/e*: 301.220127 (M-OH)$^{+}$. Found: 301.219714;

Analysis: Calculated for $C_{17}H_{34}SO_3$: C, 64.11; H, 10.76. Found: 64.33, H, 10.50%.

2-(Tetradecylsulfinyl)acetic acid 114



2-Tetradecylthioacetic acid **109** (19mg, 0.066 mmol) was dissolved in dichloromethane (2 ml) and *tert*-butylhydroperoxide (11 ml, 0.08 mmol, 1.2 eq.) was added. After 48 h reaction at room temperature, the solvent was removed and the residue was chromatographed (eluted with ether : hexane : acetic acid = 60 : 40 : 2 → methanol) to obtain the white product **114** (17 mg) in a yield of 86% .

^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (3H, t, *J* = 6.4 Hz, C14'-H_3), 1.20-1.29 (20H, m, (C4'-C13)- H_2), 1,44-1.50 (2H, m, C3'-H_2), 1.77-1.82 (2H, m, C2'-H_2), 2.88-2.95 (H, m, C1'-H), 3.02-3.07 (H, m, C1'-H'), 3.63-3.68 (H, d, *J* = 14 Hz, C2-H), 3.81-3.86 (H, d, *J* = 14 Hz, C2-H'), 7.92 (H, bs, COOH);

^{13}C NMR ($CDCl_3$, 300MHz): δ 14.69, 23.20, 23.26, 29.18, 29.70, 29.89, 29.93, 30.09, 30.18, 30.22, 32.49, 52.27, 53.47, 166.93;

MS (EI) *m/e* (relative intensity): 305 ((M+1)$^{+}$, 1%), 287 (50), 243 (60), 229 (94), 196 (12), 168 (6), 149 (6), 125 (10), 111 (21), 97 (45), 83 (63), 69 (74), 57 (100), 55 (91);

HRMS: Calculated for $C_{16}H_{33}SO_3$ *m/e*: 305.215042 (M+1)$^{+}$. Found: 305.215275.

Conclusion

Oxidation of polyunsaturated fatty acids (PUFAs) plays an important role in biological systems and some of the metabolic products from PUFA oxidation are important biological mediators that have been implicated in the pathology of many diseases such as asthma, inflammation and allergy. There are three major oxidative pathways for PUFAs: β-oxidation, autoxidation, and oxidation catalysed by enzymes such as cyclooxygenases and lipoxygenases. The aim of this research was to pursue analogues of PUFAs that are effective in control of both non-enzymatic and lipoxygenase-catalysed PUFA oxidation and would therefore be potentially useful as therapeutic agents for the control of diseases related to the oxidative pathways. Such analogues were required to display certain properties including resistance to β-oxidation, antioxidant activity and selective inhibition of different lipoxygenases.

The main group of compounds targeted in this project were nitro analogues of PUFAs. They were expected to be potentially useful due to their generally high stability and the chemical similarity of the nitro group to the carboxyl group. The other group of compounds investigated were γ-thia fatty acids. The γ-thia fatty acid, 3-[*all-Z*)-(eicosa-5,8,11,14-tetraenylthio)]propionic acid, had been previously shown to inhibit autoxidation of arachidonic acid. Such compounds were expected to be useful lipid antioxidants, due to their miscibility with and structural similarity to natural fatty acids.

From the nine nitro analogues of PUFAs that were synthesised, including long chain nitroalkanes, γ-nitro fatty acids and carboxyethyl nitro fatty acids, (*all-Z*)-4-Nitrotricosa-8,11,14,17-tetraenoic acid has been identified as a good substrate of soybean 15-LO and a 12-LO from porcine leukocyte. The substrate activity of this compound with the soybean 15-LO is comparable to that of arachidonic acid, which is a major substrate of the lipoxygenase.

A more significant outcome of this work was the identification of 4-nitrohenicosanoic acid, 4-(*all-Z*)-nonadeca-4,7,10,13-tetraenyl]-4-nitroheptane-1,7-dicarboxylic acid and 4-heptadecyl-4-nitroheptane-1,7-dicarboxylic acid as selective inhibitors of 5-LO, 12-LO and 15-LO catalysed oxidation of arachidonic acid, respectively. Although a large number of inhibitors have been reported for these three lipoxygenases, so far few inhibitors have entered clinical trials and no agents that are selective for 15-LO *vs* 5-LO (or *vs* 12-LO) are available.[204]

Selective inhibition of a specific lipoxygenase is particularly desirable for treatment of diseases related to these metabolic pathways. Non-selective inhibitors have the disadvantages of causing possible side effects. For instance, asthma has been treated as an inflammatory disease, and corticosteriods are the therapy of choice for the inflammatory component of asthma.[205] Although this class of drugs provides powerful anti-inflammatory effects in most patients, these effects are not specific and in some cases result in serious side effects. Since leukotrienes, a family of inflammatory mediators generated through the 5-LO pathway, have been shown to enhance bronchoconstriction and airway mucus secretion, agents that target the specific inflammatory pathway have been developed to treat asthma by modulating leukotriene activity. So far, specific leukotriene receptor antagonists and synthetic inhibitors have been extensively studied in laboratory-induced asthma and currently show promise in clinical trials; one leukotriene receptor antagonist (zafirlukast) and one 5-LO inhibitor (zileuton) were recently approved for the treatment of asthma.[205]

The identification of the three nitro analogues of PUFAs having selective inhibition activity with the three lipoxygenases may lead toward a new class of drugs with specificity and reduced side effects for treating diseases that are associated with lipoxygenase pathways. It may also provide a structural template for rational design of more potent inhibitors of lipoxygenase. However, further laboratory tests using animal models or cultured cell or clinical trials will be needed to evaluate the pharmaceutical values of the inhibitor compounds. Several *in vivo* properties, such as sufficient aqueous

solubility, long half-lives, good oral activity, low side effects, and high *in vivo* potency and selectivity, will be crucial factors of the clinical usefulness of these compounds. Apart from evaluation of the *in vivo* properties, the animal tests or clinical trials will be important for the nitro compounds under physiological conditions, which may help to optimise the design of nitro analogues of PUFAs with drug activity.

Studies to examine the basis of the antioxidant behaviour of 3-[(*all-Z*)-(eicosa-5,8,11,14-tetraenylthio)]propionic acid suggest that the activity results from interaction with the hydroperoxide products of PUFA autoxidation. Hydroperoxides are initiators of the radical-chain autoxidation process, and decomposition of these compounds through reaction with γ-thia fatty acids and sulfides can therefore reduce the rate of autoxidation. This work showed that the key structural component required for antioxidant activity is a sulfur and neither a carboxyl group nor unsaturation play direct roles. Thus, all the γ-thia fatty acids and sulfides tested showed substantial antioxidant activity on arachidonic acid autoxidation. β-Thia fatty acids were not antioxidants, probably due to their relatively slow reaction with hydroperoxides. The closeness of the carboxyl group to the sulfur in the β-thia fatty acids may cause steric hindrance or reduce the nucleophilicity of the sulfur. These data may provide useful information for the design of antioxidants based on destruction of the hydroperoxide products of PUFA autoxidation.

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