SYNTHESIS AND ANTIOXIDANT PROPERTIES OF VITAMIN B_6 DERIVATIVES; AND ω-ALKYNYLATED FATTY ACIDS AS SUBSTRATES FOR PREPARATION OF MODIFIED PHOSPHOLIPIDS, NOVEL PROBES FOR EVALUATING LIPID-PROTEIN INTERACTIONS

By

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This dissertation is dedicated to everyone who finds it interesting

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Abbreviation	
AA	arachidonic acid
aAA	ω-alkynylated arachidonic acid
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
aHETE	ω-alkynylated hydroxyeicosatetraenoic acid
aHNE	ω-alkynylated 4-hydroxynonenal
aHODE	ω-alkynylated hydroxyoctadecadienoic acid
aHPETE	ω-alkynylated hydroperoxyeicosatetraenoic acid
aHPODE	ω-alkynylated hydroperoxyoctadecadienoic acid
AIPH	2,2'-azobis(2-(2-imidazolin-2-yl)propane) dihydrochloride
aIsoP	ω-alkynylated isoprostane
aLA	ω-alkynylated linoleic acid
AMP	antioxidant mediated peroxidation
AMVN	2,2'-azobis(2,4-dimethylvaleronitrile)
APCI	atmospheric pressure chemical ionization
Apo B-100	apolipoprotein B-100
aPUFA	ω-alkynylated polyunsaturated fatty acid
AscH	ascorbic acid
$Asc^{\bullet}$	ascorbyl radical
BDE	bond dissociation energy

BHT	2,6-di-*t*-butyl-4-methylphenol
CoQ_{10}	coenzyme Q_{10} (oxidized form)
$CoQ_{10}H_2$	coenzyme Q_{10} (reduced form)
$CoQ_{10}H^{\bullet}$	coenzyme Q_{10} derived phenoxyl radical
COX	cyclooxygenase
DCM	dichloromethane
ECCD	exciton-coupled circular dichroism
ECD	electrochemical detection
ELISA	enzyme-linked immunosorbent assay
ERG	electron-withdrawing group
ESI	electrospray ionization
ET	electron transfer
GC	gas chromatography
GSH	glutathione (reduced form
GSSG	glutathione (oxidized form)
GST	glutathione transferase
HDL	high density lipoprotein
HETE	hydroxyeicosatetraenoic acid
HNE	4-hydroxynonenal
HODE	hydroxyoctadecadienoic acid
HOOH	hydrogen peroxide
$HOO^{\bullet}$	hydroperoxyl radical
$HO^{\bullet}$	hydroxyl radical

HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high pressure liquid chromatography
HPODE	hydroperoxyoctadecadienoic acid
HSA	human serum albumin
IP	ionization potential
IsoP	isoprostane
KODA	9,12-dioxododec-10-enoic acid
LA	linoleic acid
LC	liquid chromatography
LDL	low density lipoprotein
LH	lipid
LOH	lipid hydroxide
LOOH	lipid hydroperoxide
LOX	lipoxygenase
NMBHA	*N*-methyl benzohydroxamic acid
MALDI	matrix-assisted lased desorption ionization
MDA	molonyldialdehyde
MeOAMVN	2,2'-azobis(2,4-dimethyl-4-methoxy-valeronitrile)
MLV	multi-lamellar vesicles
MS	mass spectrometry
$O_2^{\bullet -}$	superoxide radical anion
PBS	phosphate buffered saline
PC	phosphatidylcholine

PLPC	1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine
PLPC-OH	PLPC derived hydroxides
PLPC-OOH	PLPC derived hydroperoxides
PMC	2, 2', 5, 7, 8-pentamethylchromanol
PUFA	polyunsaturated fatty acid
PyrOH	pyridinol
$PyrO^{\bullet}$	pyridoxyl radical
ROS	reactive oxygen species
RP-HPLC	reversed phase high pressure liquid chromatography
SRM	selected reaction monitoring
TMP	tocopherol mediated peroxidation
α-TOH	α-tocopherol
α-$TO^{\bullet}$	α-tocopheryl radical
TpepK	Ac-Ala-Val-Ala-Gly-Lys-Ala-Gln-Ala-Arg
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TsOH	*p*-toluenesulfonic acid

Part One

SYNTHESIS AND ANTIOXIDANT PROPERTIES OF VITAMIN B6 DERIVATIVES

CHAPTER I

INTRODUCTION

Lipid Peroxidation

It is common knowledge that vegetable oils and animal fats turn rancid upon prolonged storage. This phenomenon, which we now associate with lipid peroxidation processes, could not have been explained until the first half of the 20th century, when pioneering studies on isolation of fatty acid components from oils and fats were conducted.[1, 2] About 70 years ago, it was established that addition of molecular oxygen to polyunsaturated fatty acids (PUFAs) yields conjugated hydroperoxides as the primary oxidation products.[3, 4] More and more detailed studies on mechanisms ruling PUFA autoxidation were conducted since the 1960's, when new analytical instrumentation techniques, such as GC-MS and LC-MS, became available. In the early 1980's, the formation of complex hydroperoxide products from PUFAs was explained on the basis of thermodynamic equilibration of participating radical species.[5-7]

Due to the progress in medical and biochemical sciences over last few decades, the importance of lipid peroxidation in human health has also been revealed. The process has been associated with a number of diseases, among which the most extensively studied were: atherosclerosis, diabetes, cancer and neurodegenerative disorders, such as Alzheimer's and Parkinson's disease.[8-12] As a consequence of the discovery of the bio-relevance of lipid oxidation,

the field became interdisciplinary and our knowledge of the topic has quickly expanded since. Figure 1.1 shows the increase in research attention to lipid peroxidation over last 5 decades, based on the number of relevant references input into a scientific database (SciFinder).[13] Recently, a tremendous amount of publications have been focused on oxidation of PUFAs and their derivatives in various *in vivo* settings.[14-16] The establishment of mechanistic foundations on the formation of biologically active lipid derived oxidation products is a desirable goal in current area of lipidomics. Another important part of research is directed towards development of an effective method for inhibition of lipid peroxidation *in vivo* (*vide infra*).

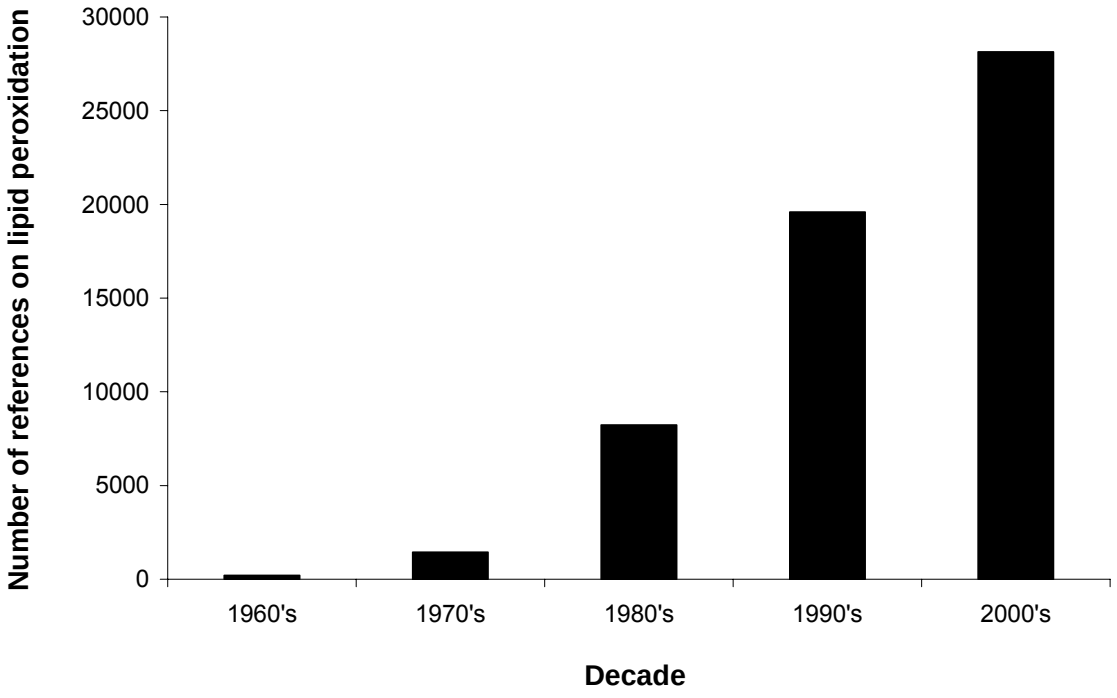


Figure 1.1. Increase in research attention on lipid peroxidation over the last 50 years.[13]

Atherosclerosis, defined as progressive narrowing and hardening of arteries leading to increase risk of heart attack and stroke, is the single most important cause of death in the United States.[17] The condition has been correlated with abnormal transport of cholesterol in the body. Cholesterol, an important cell membrane component and precursor of many hormones, is transported from the liver to cells at various locations with a help of lipoprotein particles, serving as carriers for this water insoluble molecule.[18] Human blood plasma lipoproteins are classified with respect to their increasing density: chylomicrons (CM), very-low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL). All of these different lipoprotein classes play various roles in cholesterol transport and metabolism. However two of them, commonly referred to as good (HDL) and bad (LDL) cholesterol, have attracted most of the attention of human health specialists, due to their involvement in development of cardiovascular diseases.[19] HDL is responsible for cleaning up arteries from cholesterol molecules that have been released from decomposed cells and membranes. LDL, on the other hand, is a major cholesterol transporter, which under some conditions was found to fail serving its function. Elevated plasma level of LDL (>1.3 g/L) and decreased concentration of HDL (<0.35 g/L) indicate increased tendency for development of atherosclerosis and other cardiovascular diseases by an individual.[20]

An average LDL particle, as shown in Figure 1.2, has molecular weight of 2500 kDa, a diameter of 22 nm, and a volume of 4×10^{-21} L.[21, 22] The neutral core

of this dynamic micellar system consists of triacylglicerols and cholesteryl esters (*ca.* 900), whereas the polar coat layer is built out of phospholipids and unesterified cholesterol molecules (*ca.* 600). Sample structures of the fatty ester constituents of LDL are presented in Figure 1.3. The cholesteryl esters found in LDL predominantly contain PUFA residues (arachidonate or linoleate) in their structures, whereas outer coat phospholipids may additionally include more saturated fatty acid residues.[23] The exact composition of LDL is dependent on the fatty acid composition of the diet.[24] The most common fatty acid precursors of the ester components found in LDL are presented in Figure 1.4.

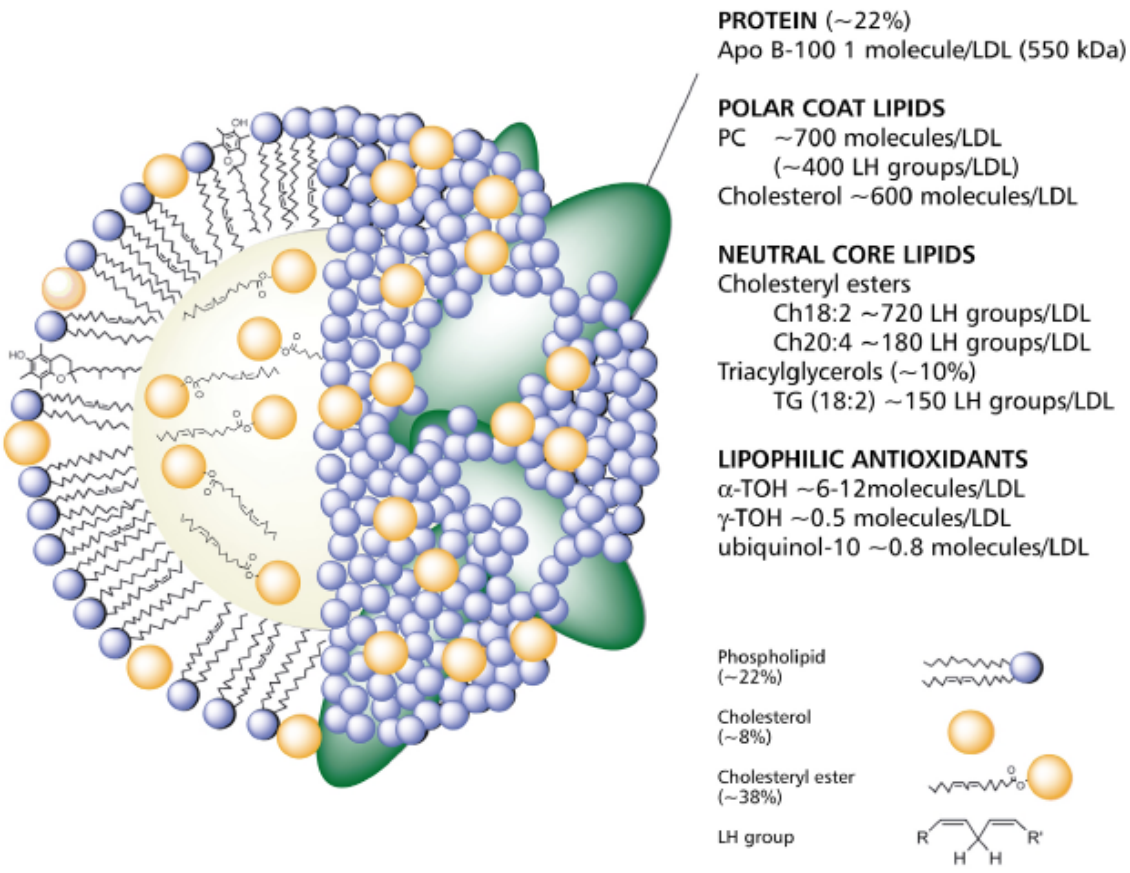


Figure 1.2. Schematic representation of an LDL particle.[22]

Inside the core as well as in the outer layer of the LDL, there are also antioxidant molecules present that help to spare LDL from oxidative modifications.[21] This issue is however addressed separately in the next section of this chapter.

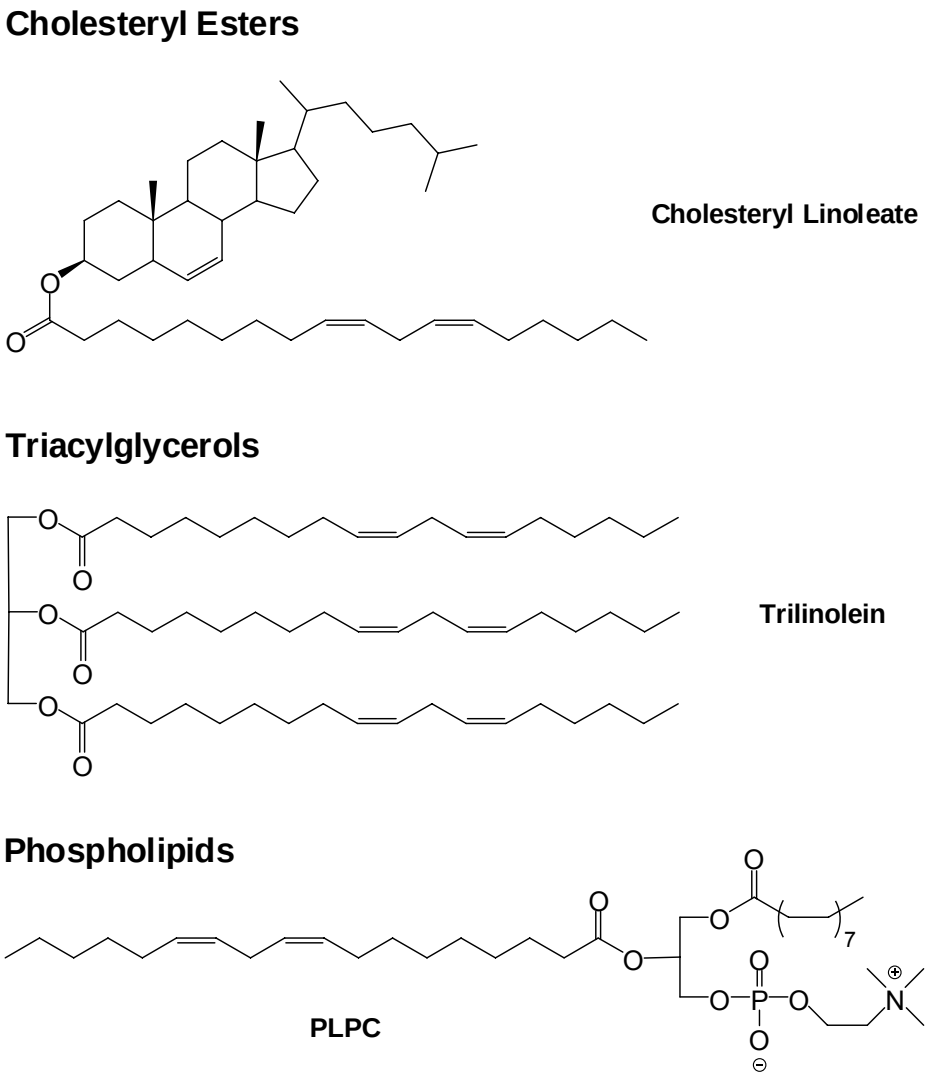


Figure 1.3. Classes of esterified fatty acids found within an LDL particle.

An important ingredient of the LDL structure is the Apo B-100 protein. This biopolymer containing 4536 amino acid residue is anchored to the outer layer of the particle and serves as a recognition site for LDL receptor proteins located on various target cells. Upon recognition, a fusion of the LDL and the target cell is observed, followed by a degradation of the protein and release of cholesterol into the cell.[23]

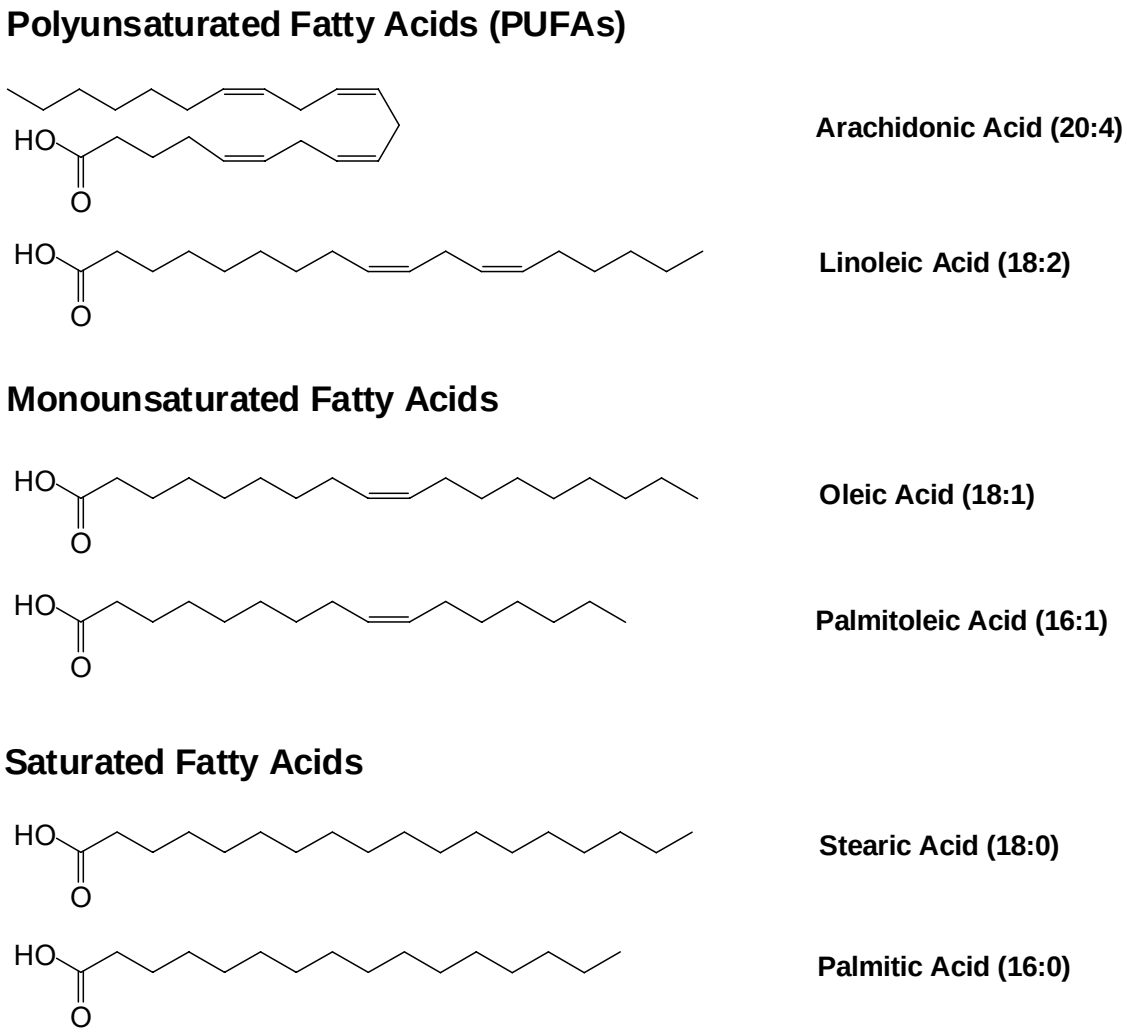


Figure 1.4. Structures of fatty acid residues found in an LDL.

It has been found that upon modifications to Apo B-100 (either due to a free radical attack or as a result of adduction to lipid peroxidation derived electrophiles), the oxidized lipoprotein is no longer recognized by its typical acceptors, but instead it is recognized by scavenger receptors on arterial macrophages (Figure 1.5).[25] Initially serving a protective role by taking up oxidized LDL, macrophages become foam cells upon accumulation of large quantities of modified LDL.[26] These foam cells are responsible for formation of fatty streaks on the internal walls of arteries. This process is recognized as an early hallmark of atherosclerosis.

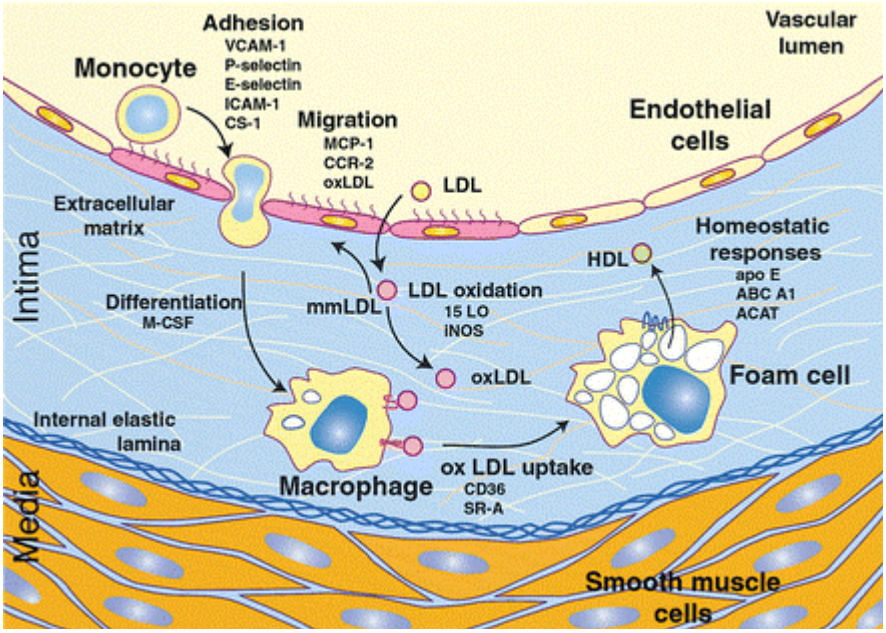


Figure 1.5. Schematic representation of the fate of oxidized LDL in artery.[25]

Under free radical attack, the main LDL associated targets for oxidative damage are the unsaturated fatty acid hydrocarbon chains, such as those

presented in Figure 1.4. Mechanistically, oxidation of these fatty acids is a free radical chain process consisting of initiation, propagation and termination steps (Scheme 1.1).[27]

Initiation

$In^{\bullet} + RH \longrightarrow InH + R^{\bullet}$ **(1)**

Propagation

$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$ **(2)**

$ROO^{\bullet} + RH \longrightarrow R^{\bullet} + ROOH$ **(3)**

Termination

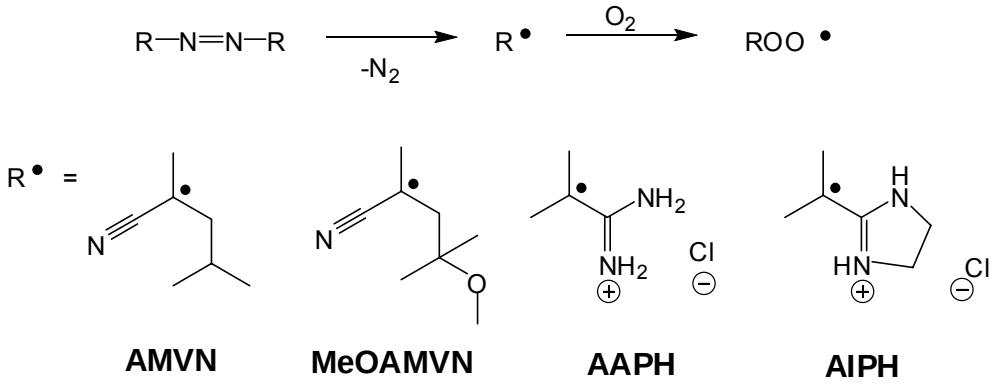
$2ROO^{\bullet} \longrightarrow$ non-radical products **(4)**

Scheme 1.1. Free radical chain in the autoxidation of lipids.

In vivo, the radical chains are initiated by reactive radical species that either escape from their normal sites of operation or are produced as a result of some pathogenic mechanisms.[28] In the laboratory set up, however, thermally activated azo-initiators are used as they provide a constant flux of radicals. Readily available radical initiators, commonly employed for kinetic studies conducted on lipid peroxidation are: 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN), 2,2'-azobis(2,4-dimethyl-4-methoxy-valeronitrile) (MeOAMVN), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2-(2-imidazolin-2-yl)propane) dihydrochloride (AIPH).[29] Structures of these compounds are given in Scheme 1.2. AMVN and MeOAMVN are soluble in lipid

layer and non-polar organic solvents whereas AAPH and AIPH are considered water soluble, and are used typically for oxidations in aqueous buffers.

All azo-initiators undergo thermally induced breakage of C-N bonds yielding gaseous nitrogen and carbon centered radicals that react with oxygen, with a diffusion controlled rate, to form peroxyl radicals (Scheme 1.2). Thus formed initiator derived peroxyl radicals initiate lipid oxidation by hydrogen atom abstraction from the weakest C-H bonds of the lipid hydrocarbon chains (Eq. 1).[30]



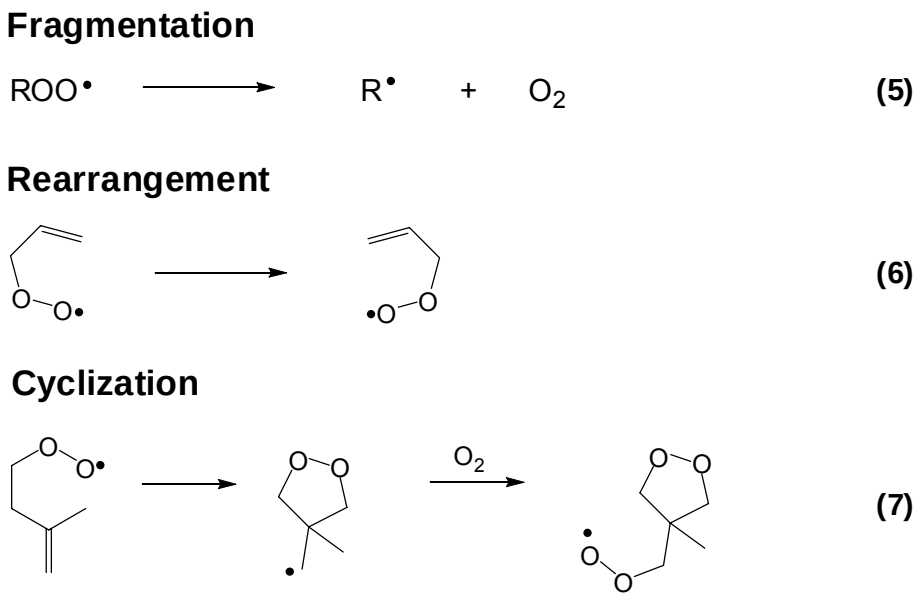
Scheme 1.2. Commonly used free radical azo-initiators.

The propagation element of the free radical process involves two steps. First is a radical coupling between lipid derived carbon centered radical and molecular oxygen, which has a partially free radical character, yielding lipid peroxyl radical (Eq. 2). This step typically occurs at the diffusion controlled rate of *ca.* 10^9 $M^{-1}s^{-1}$.[31] A second propagation step, consisting of a hydrogen atom transfer from the lipid hydrocarbon skeleton to the peroxyl radical (Eq. 3), is much slower. Its rate depends predominantly on the strength of the bond being

broken, which itself is a function of the stability of the carbon centered radical formed in this process. Therefore, abstraction of a bisallylic hydrogen atom (*ca.* 60 $M^{-1}s^{-1}$) is much faster than abstraction of an allylic analogue (*ca.* 1 $M^{-1}s^{-1}$), which is itself much faster than formation of a localized carbon centered radical (*ca.* 10^{-3} $M^{-1}s^{-1}$). As a result only compounds containing bisallylic hydrogens, *e.g.* linoleic and arachidonic acids, in contrast to more saturated lipids, are readily oxidizable at room temperature.[30]

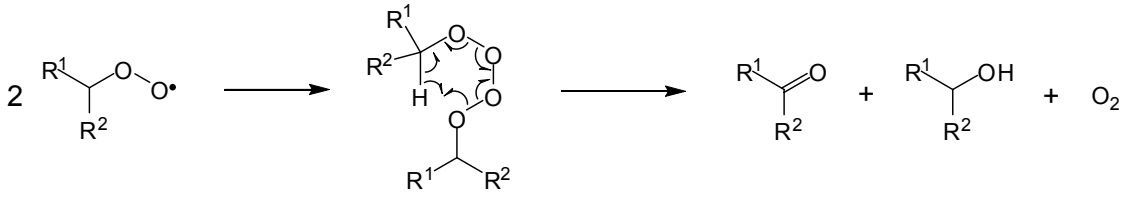
Besides the two aforementioned radical chain propagation types, a radical coupling (Eq. 2) and an atom transfer (Eq. 3), other reactions, such as fragmentations, rearrangements and cyclizations have also been observed during oxidations of lipids (Scheme 1.3). Since the hydrogen atom transfer from lipid hydrocarbon chains to a lipid peroxyl radical is a relatively slow process, the later compound may decompose to reform carbon centered radical and O_2 (Eq. 5). This so called β-fragmentation occurs readily if the radical product is stabilized. The process has been studied extensively using methyl linoleate as a substrate for autoxidation, however rates of β-fragmentation of peroxyl radicals derived from other lipids are also available.[7, 32, 33] Moreover, if β-fragmentation occurs, the solvent caged pair of oxygen and delocalized carbon centered radical may undergo a rearrangement to form a peroxyl radical that is different from the substrate (Eq. 6). Lastly, peroxyl radicals may undergo cyclization reactions forming 5- and 6-membered endoperoxide rings (Eq. 7). The process involving arachidonic acid derived peroxyl radicals has been studied extensively, especially since the cyclic products obtained were found to have very high

biological activity.[34-36] Peroxyl radicals produced from other PUFAs and the cyclic products derived from such species are currently being investigated.[37-39]



Scheme 1.3. Additional propagation reaction types in autoxidation of lipids.

Termination of the radical chain occurs generally via coupling of two peroxyl radicals to form non-radical products (Eq. 4). Typically the two radicals combine to form a tetroxide intermediate, which further decomposes through a 6-membered ring transition state to give a molecule of oxygen, an alcohol and a ketone or aldehyde (Scheme 1.4).[40] This reaction, called Russell termination, occurs readily unless the two interacting species are both tertiary peroxyl radicals. Alternatively, the radical chain process can be terminated in the reaction of the chain carrying radical with a scavenging antioxidant.



Scheme 1.4. Russel termination mechanism for chain carrying peroxyl radicals.

Natural and Synthetic Antioxidants

For aerobic organisms, molecular oxygen is a vital substance but also a lethal toxin. The main drawback of efficient aerobic catabolism is oxidative damage to important endogenous molecules, *i.e.* DNA, proteins, carbohydrates and lipids. Oxidative modification of these important molecules can lead to a series of mutations, cell damage or apoptosis. In order to minimize the damage, biological systems have developed antioxidant defense mechanisms which neutralize and/or prevent the formation of reactive oxygen species (ROS), produced from molecular oxygen.[41]

One electron reduction of molecular oxygen yields superoxide radical anion ($O_2^{\bullet-}$), which can be further converted into uncharged hydrogen peroxide (HOOH). These two ROS together are able to form a potent reducing agent, hydroxyl radical ($HO^{\bullet}$). The reaction between them is very slow in an aqueous environment unless it is catalyzed by free transition metal ions. It has been commonly accepted that Cu^{+} or Fe^{2+} ions acts as catalysts in such transformation. Rapid formation of hydroxyl radical catalyzed by Fe^{2+} is historically known as a metal catalyzed Haber-Weiss reaction (Scheme 1.5),

whereas the second part of the process alone (Eq. 9) is ascribed to as Fenton chemistry.[42, 43] More recent studies indicate that other species such as Ni^{2+}, Co^{2+} and Cr^{5+} also possess the ability to catalyze hydroxyl radical formation.[44, 45] Another form of a reactive oxygen species is singlet oxygen, formed by energy transfer to ground state oxygen from photosensitizers, such as flavin-containing compounds. All of these ROS are potentially dangerous to cellular components, however special precautions apply to $HO^{\bullet}$ since it has the highest reactivity of all reactive oxygen species. No specific antioxidant mechanism against hydroxyl radical is known, therefore prevention of the production of this ROS is favored.[46]

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2 \qquad \textbf{(8)}$$

$$Fe^{2+} + HOOH \longrightarrow Fe^{3+} + HO^{-} + HO^{\bullet} \qquad \textbf{(9)}$$

Scheme 1.5. Formation of hydroxyl radicals via the metal-mediated Haber-Weiss reaction.[43]

The specialized natural antioxidant mechanisms of action can be separated into four categories: prevention, diversion and neutralization, scavenging, and quenching (Table 1.1).[46] Structures of selected naturally occurring non-enzymatic antioxidants are shown in Figure 1.6.

Prevention can be attributed to proteins that are able to bind transition metal ions preventing Fenton chemistry. Transferrin and ferritin bind Fe^{2+} while Cu^{2+} is bound by caeruloplasmin. The dismutation process relies on enzymatic

conversion of superoxide radical anion into HOOH and molecular oxygen. This reaction is catalyzed by superoxide dismutase enzyme (Eq. 10). Diversion of HOOH into H_2O in the presence of enzymes, catalase (Eq. 11) and glutathione peroxidase (Eq. 12), is another safe and efficient antioxidant mechanism. The oxidized form of glutathione (GSSG) is then recycled by another enzyme, glutathione reductase (Eq. 13). This efficient co-operative system effectively diverts the potentially harmful reactive oxygen species into water (Scheme 1.6).[46]

Table 1.1. Types of naturally occurring antioxidants.[46]

Type	Action	Example
Prevention	Protein binding/ inactivation of metal ions	Transferrin, ferritin, caeruloplasmin, albumin
Enzymatic diversion/ neutralization	Transformation of ROS into harmless products	Superoxide dismutase, catalase, glutathione peroxidase
Scavenging	Sacrificial interaction with ROS by expendable substrates	Ascorbic acid, uric acid, bilirubin, glutathione, α-tocopherol, ubiquinol-10
Quenching	Absorption of electrons and/or energy	α-Tocopherol, β-carotene, lycopene

Scavenging usually involves small molecules which react with primary ROS or secondary reactive molecules such as lipid peroxyl radicals. In humans, the most common endogeneous radical scavengers are water soluble uric acid, bilirubin, and glutathione as well as hydrophobic α-tocopherol (α-TOH) and ubiquinol-10 ($CoQ_{10}H_2$). Some of these species can be recycled, whereas others have to be replaced *de novo*. Another important water soluble radical scavenger

is ascorbic acid (AscH). Together with uric acid, this dietary supplement is among the most highly abundant antioxidants in human plasma and tissues. [47, 48]

Quenching is an ability to prevent singlet oxygen formation by competitive energy adsorption from photosensitizers. Plant derived carotenoids, lipophilic compounds possessing a large number of conjugated double bonds in their structures, were found to be highly effective quenching antioxidants.[46]

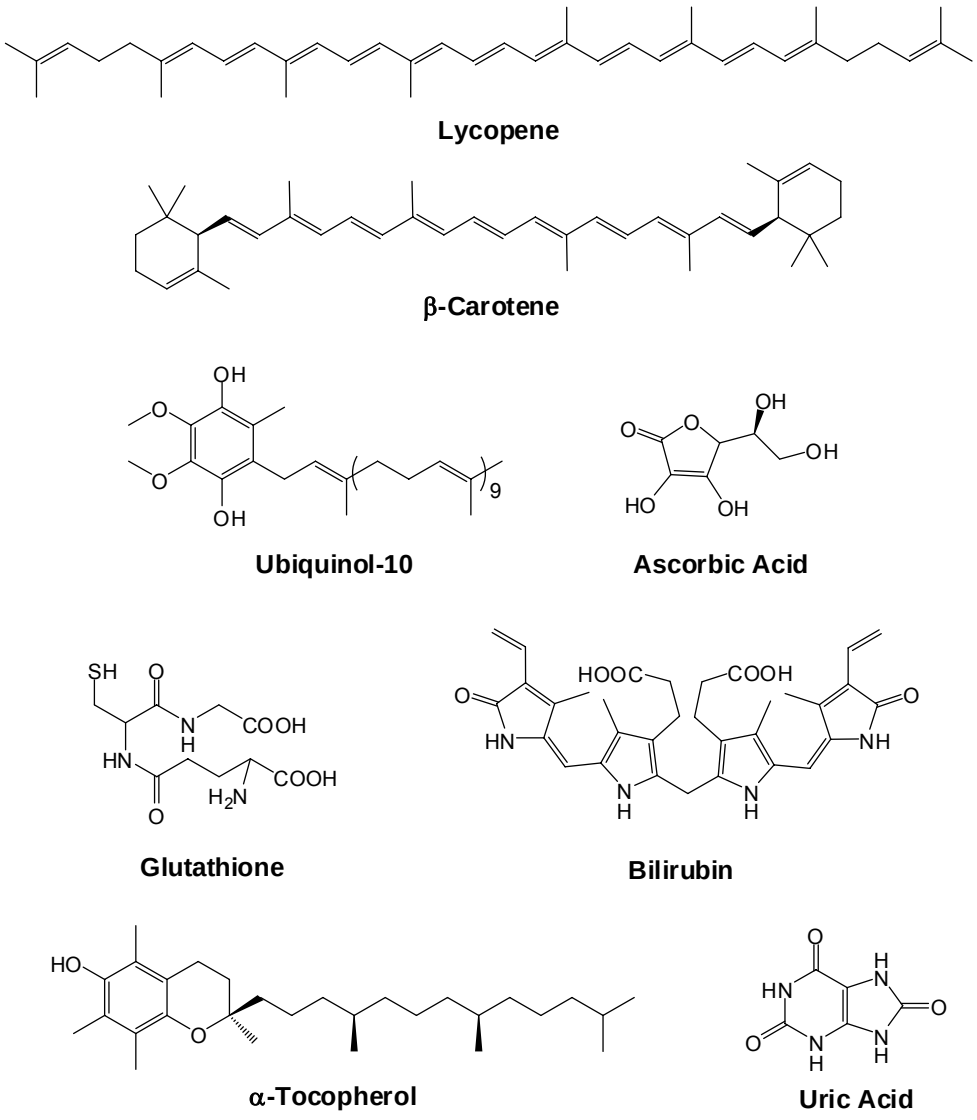


Figure 1.6. Structures of selected naturally occurring antioxidants.

These diverse and coordinated antioxidant mechanisms are effective but not infallible. The radical theory of aging states that organisms age as a result of ROS induced damage to cellular components.[49] In order to minimize the damage, nutritional supplementation of a diet with natural antioxidants has been suggested as a reasonable strategy.[50-52] Additionally, extended research has been conducted to produce synthetic antioxidants able to reduce the rate of the oxidative damage even more profoundly.[53-56] Such compounds could be especially useful for treatment of some serious medical conditions, with which development and/or progression of lipid peroxidation has been associated (*vide supra*).

Superoxide dismutase:	$2\ O_2^{\bullet -} + 2H^+ \longrightarrow HOOH + O_2$		(**10**)
Catalase:	$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$		(**11**)
Glutathione peroxidase:	$2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O$		(**12**)
Glutathione reductase:	$GSSG \longrightarrow 2\ GSH$		(**13**)

Scheme 1.6. Enzymatic diversion of reactive oxygen species into water.

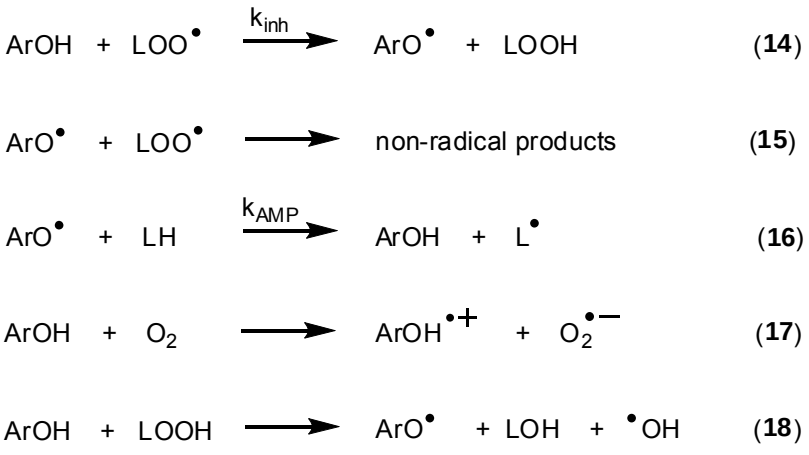
Although there is some interest in mimicking ROS neutralizing and metal chelating properties of enzymes by small molecules, [57-59] synthetic compounds able to scavenge free radical chain oxidation reactions have attracted most of the attention in the scientific community. This has been partially due to the fact that some of the chain breaking antioxidants may serve as ROS neutralizing

agents, and sometimes also as metal ion chelators.[60, 61] On the contrary, only a group of radical scavengers is able to terminate chain oxidation processes that have been already initiated.

An introduction of a scavenging antioxidant into the environment of an ongoing lipid peroxidation effectively quenches the propagating step via hydrogen atom transfer from the antioxidant to a chain-carrying peroxyl radical (Eq. 14). Thus formed antioxidant derived phenoxyl radical usually reacts with a second lipid peroxyl radical to form non-radical products (Eq. 15). Rate constants of the chain propagation (Eq. 3) for polyunsaturated lipid substrates are rather slow (*vide supra*), while the chain breaking rate constant (k_{inh}) for α-TOH, the best natural scavenging antioxidant, is undeniably greater (*ca.* 3.5×10^6 $M^{-1}s^{-1}$).[32] Judging by the difference between rate constants of these two reactions, and the [LH]/[α-TOH] ratio in LDL of *ca.* 160 to 1, one could suspect that this micellar complex is greatly overprotected against peroxidation. This was however found not exactly to be the case.[62]

Experiments in which LDL particles, isolated from human blood plasma, were oxidatively modified (either by Cu^{2+} or a water soluble azo-initiator) showed that relatively little LOOH was formed until α-TOH had been consumed.[63] On the other hand, very poor correlation between [α-TOH] in LDL and the duration of the induction period preceding lipid peroxidation was found.[64] Furthermore, under mild radical flux conditions α-TOH depleted LDL was practically resistant to peroxidation comparing to native LDL particles.[65] That observation led to the conclusion that α-TOH, which serves a crucial antioxidant function during severe

oxidative stress, is also capable of promoting oxidation under milder conditions. The pro-oxidant behavior of α-TOH, associated with its ability to catalyze phase as well as radical chain transfer processes, is referred to as tocopherol mediated peroxidation (TMP) since α-TOH derived phenoxyl radical (α-TO$^{\bullet}$) initiates radical chains by abstraction of a hydrogen atom from lipid residues.[21] An analogous process, but generalized to all phenolic radical scavengers (Eq. 16) is commonly ascribed as an antioxidant mediated peroxidation (AMP). Although the rate of the TMP (k_{TMP}) is not greater than 0.1 $M^{-1}s^{-1}$, the reaction can be efficient since the competitive chain termination reactions occur with extremely low frequency due to the restricted size of the LDL particle.[62]



Scheme 1.7. Ambivalent, pro- and antioxidant behavior of phenolic antioxidants.

It has been proposed that phenolic compounds having lower O-H bond dissociation energies (O-H BDEs) than α-TOH should induce less AMP and react faster with lipid derived peroxyl radicals, and hence be better antioxidants than

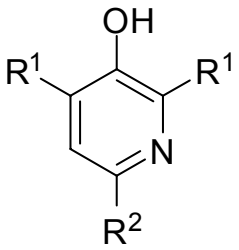
α-TOH.[23] Since the O-H BDE is a measure of stability of phenoxyl radicals formed upon hydrogen atom donation, compounds characterized by lower BDEs are desired because they give up hydrogen atoms quicker and produce more stable radicals that are less likely to react via AMP. Experimental data confirmed the prediction that an increase in the ring electron density caused by electron-donating *ortho*- and *para*- substituents of phenols increased their O-H BDEs and enhanced antioxidant properties of these molecules.[66, 67] Unfortunately, some of them were found to be very unstable upon exposure to the atmosphere.[68, 69] Air-stability of a phenolic antioxidant is related to its ionization potential (IP), defined as the ease in giving up a single electron to another molecule, oxygen in this case (Eq. 17). The lower the IP value, the easier an antioxidant becomes ionized, and hence the more likely it loses its valuable properties with a simultaneous production of reactive superoxide as a byproduct. Moreover, a low IP value for an antioxidant may result in its reactivity with LOOH via Fenton-like mechanism, leading to formation of extremely toxic hydroxyl radical (Eq. 18).

Since O-H BDE depends mostly on resonance π-interactions with *ortho*- and *para*-substituents, while IP is a result of σ-induced ring electron density, it was proposed that incorporation of nitrogen atom into an aromatic ring of phenol should increase the IP value significantly and at the same time only slightly increase the O-H BDE of the pyridinol molecule. According to physico-chemical calculations, for any given electron donating group (ERG) substitution pattern, a pyridinol (PyrOH) possesses significantly higher IP and only slightly increased O-H BDE in comparison with an analogous phenol.[70] For example,

2,4,6-trimethylphenol has an IP = 178.3 and BDE = 80.4, while 2,4,6-trimethylpyridin-3-ol has IP = 186.3 and BDE = 81.1 kcal/mol. Indeed, it has been found that for a series of substituted 3-PyrOHs, the relevant properties measured empirically matched closely with the calculated predictions.[71]

Analyzing the ERG substitution patterns for 3-PyrOHs, it was shown that 2,4-dimethyl-4-dimethylamino substitution was the most efficient one (Table 1.2).[70] It should be emphasized that introduction of 4-amino substituent offers additional adventage. It enables control of lipophilicity for antioxidant molecules by adjusting the lengths of *N*-alkyl chain(s). Partition between aqueous and lipid layers is an important factor in designing biologically active molecules and it will be addressed later in the text. It is also worth mentioning that a vacant 5-position of the aromatic ring raises a possibility of constructing a second ring adjacent to main aromatic ring. Given the opportunity, few potentially highly active bicyclic structures were synthesized and their properties have been measured.[23]

Table 1.2. The computational BDE and IP values for selected pyridinols.[70]

Generic Structure	R^1	R^2	BDE	IP
	H	H	88.2	206.4
	H	Me	85.4	196.6
	Me	Me	81.1	186.3
	H	OMe	82.0	186.1
	Me	OMe	78.1	177.4
	H	NMe_2	77.0	164.6
	Me	NMe_2	73.5	157.7

Based on the calculations and preliminary experimental results presented above, a number of water soluble (**1**-**3**) as well as lipophilic (**4**-**7**) compounds containing 2-amino-5-pyridinol moiety have been synthesized (Figure 1.7).

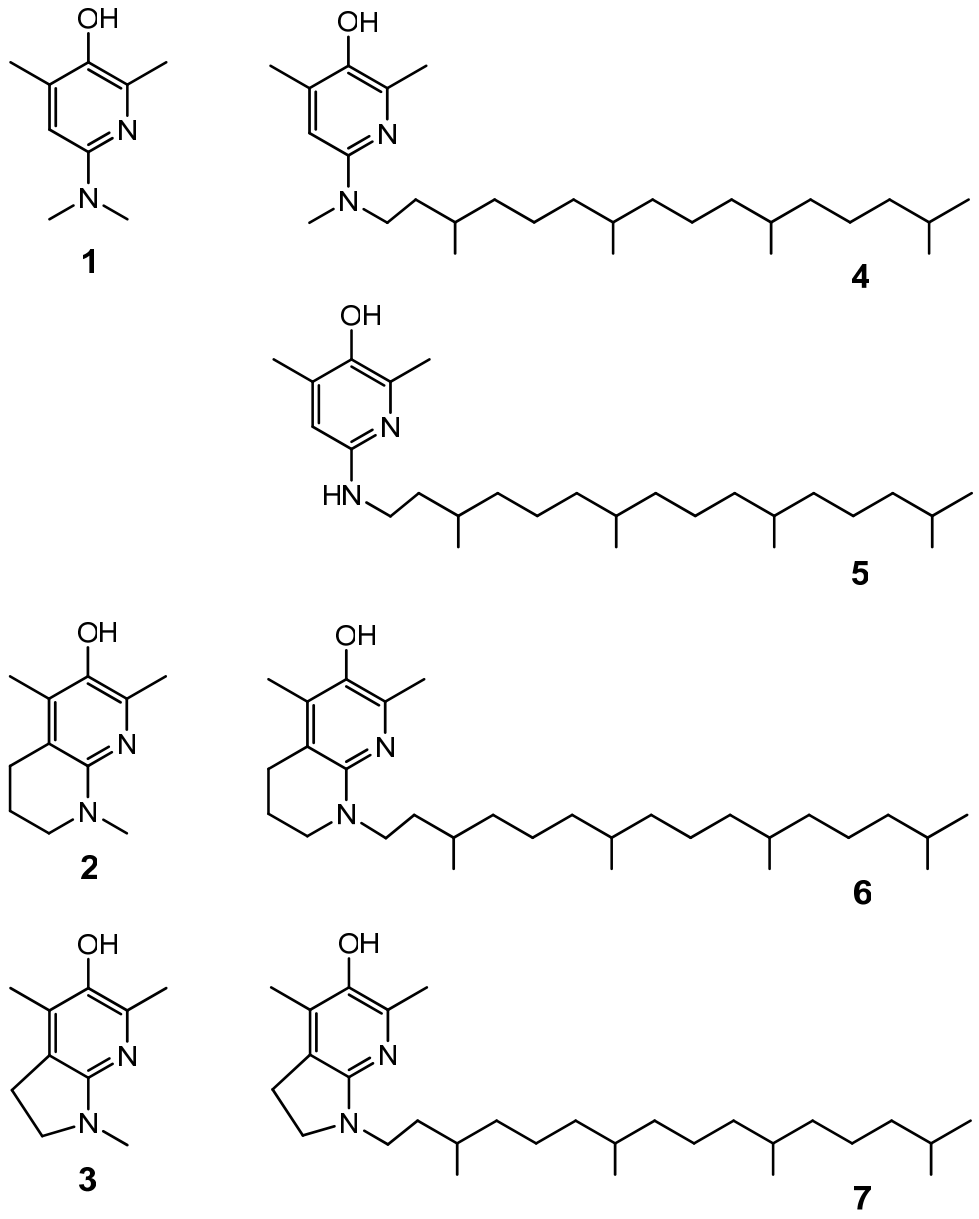


Figure 1.7. Powerful pyridinolic chain breaking antioxidants.

The pyridinols have then been tested in non-polar homogeneous solutions (**1**-**3**) and in aqueous LDL suspensions (**4**-**7**) in order to measure their antioxidant behavior by several available techniques. The experimental results confirmed that these novel compounds were excellent hydrogen atom donors toward peroxyl radicals and thus very good oxidation inhibitors. The inhibition rate constants (k_{inh}s) for PyrOHs **1**, **2** and **3** were found to be 5, 28 and 88 times greater than the corresponding value ascribed to α–TOH.[71] The studies also revealed that electron transfer from the PyrOHs to molecular oxygen (Eq. 17) occurs as electron density in the ring increased, but the rates for the process in an aprotic organic solvent were not as significant as corresponding rates for highly electron-rich phenolic compounds presented in the literature.[23, 68, 69, 71] The most unstable pyridinol **3** decayed for *ca.* 20% over a period of 3h in *t*-butylbenzene at 37°C.[23]

Human LDL particles supplemented with lipophilic compounds **4**-**7** and subjected to water soluble radical initiator AIPH, have been efficiently protected against oxidative damage until vast majority of PyrOH molecules was consumed. Additionally, antioxidant consumption studies revealed that α-TOH, a native LDL component, was spared until PyrOH was severely depleted from the lipoprotein.[23, 72] These observations may indicate that indeed, PyrOHs scavenge peroxyl radicals much faster than α-TOH, and promote much less oxidation via AMP processes. In these studies, the best antioxidant performance was ascribed to pyridinol **5**, as it has protected LDL most efficiently. Compounds **4** and **6** were also found to be relatively good radical scavengers, whereas

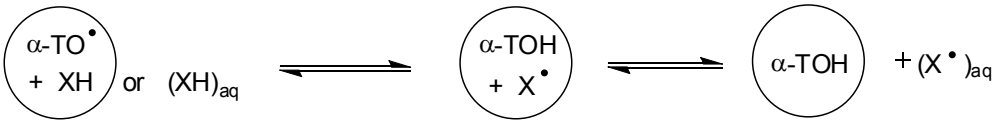
PyrOH **7** did not serve its designated function well, since it was readily depleted (*ca.* 3 times faster than compound **5**).[23] This happened most likely via electron transfer mechanisms (Eqs. 17 and 18), due to a relatively high IP value associated with antioxidant **7**. Overall the novel pyridinols **1**-**6** were shown to possess high antioxidant potential against lipid peroxidation.

It should be also mentioned, that during the time the research presented in this thesis was conducted, some of the 2-amino-5-pyridinols presented herein were tested as potential inhibitors of prostaglandin H_2 synthase enzymes.[73] Indeed, they were found to be reversible inhibitors of peroxidase sites of the enzymes, and therefore could be potentially used as anti-inflammatory agents. This, being in agreement with previous observations that 2-substituted methylamino-5-(hydroxy or alkoxy)pyridines inhibited leukotriene synthesis and therefore could be potentially utilized in treatment of pulmonary, anti-inflammatory, dermatological, allergic and cardiovascular diseases.[74] Aside from ability to inhibit lipid peroxidation, the above examples of biological activity of pyridinols prove that it is an interesting group of compounds which should be studied in more details.

Another important property of some formal hydrogen atom donors, *i.e.* ability to serve as co-antioxidants in the α-TOH containing systems, has been observed during the initial studies that lead to discovery of tocopherol-mediated peroxidation. It was found that during oxidative damage to an isolated LDL, induced by AAPH or AMVN, the minor lipoprotein associated antioxidant - ubiquinol-10 ($CoQ_{10}H_2$) was always consumed prior to α-TOH, and

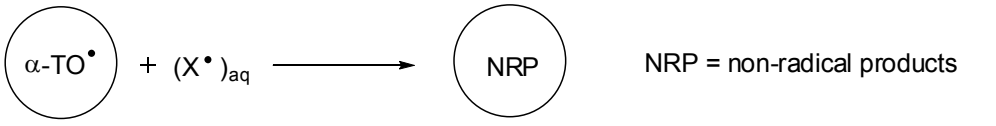
the disappearance of $CoQ_{10}H_2$ corresponded closely with the onset of rapid oxidation phase. Furthermore, in the presence of ascorbic acid (AscH), the $CoQ_{10}H_2$ was still consumed first but the oxidation was inhibited until AscH was also depleted.[75, 76] Since $CoQ_{10}H_2$ is 10 times less reactive toward peroxyl radicals than α-TOH, it was expected that it had little or no effect on the lipid peroxidation rate.[77] Also, water soluble AscH was expected to have little effect on the peroxidation rate in AMVN-initiated LDL due to its limited ability to cross phospholipid membranes.[78] In order to rationalize these observations, three mechanisms of action were proposed: radical export, interparticle radical transport (Eq. 19) and radical import (Eq. 20). All of these mechanisms retard TMP in LDL by transforming α-TO$^{\bullet}$ back into α-TOH or into non-radical products. They can act independently and in co-operative mode, and they are referred to as anti-TMP mechanisms (Scheme 1.8).[62]

Radical Export / Transport



(19)

Radical Import



(**20**)

Scheme 1.8. Radical anti-TMP mechanisms.[62]

Radical export is defined as an irreversible transfer of a radical center from an LDL lipid to an aqueous phase. Rapid reduction of α-TO$^{\bullet}$ to α-TOH on the surface of LDL by aqueous ascorbate $(Asc^{-})_{aq}$ could be an example of that mechanism (Eq. 21). Transformation of α-TO$^{\bullet}$ into α-TOH in the presence of $CoQ_{10}H_2$ is also thought to proceed via this mechanism, the only difference is that the radical export occurs indirectly (Scheme 1.9). Since long alkyl chain (*ca.* 50 carbon atoms) prevents ubisemiquinone radical ($CoQ_{10}H^{\bullet}$) from diffusing out of the LDL into the aqueous environment, the radical export was proposed to be mediated by reduction of molecular oxygen (Eq. 22). Thus obtained superoxide radical ($HOO^{\bullet}$) is highly hydrophilic and therefore it is readily expelled out of the LDL particle (Eq. 23).[62]

$$\alpha\text{-TO}^{\bullet} + (\text{Asc}^{-} + \text{H}^{+})_{aq} \longrightarrow \alpha\text{-TOH} + (\text{Asc}^{\bullet})_{aq} \qquad (21)$$

$$\alpha\text{-TO}^{\bullet} + \text{CoQ}_{10}\text{H}_2 \longrightarrow \alpha\text{-TOH} + \text{CoQ}_{10}\text{H}^{\bullet} \qquad (22)$$

$$\text{CoQ}_{10}\text{H}^{\bullet} + \text{O}_2 \longrightarrow \text{CoQ}_{10} + \text{HO}_2^{\bullet} \longrightarrow (\text{H}^{+} + \text{O}_2^{\bullet -})_{aq} \qquad (23)$$

Scheme 1.9. Direct and indirect radical export mechanisms.

The radical import mechanism enhances chain termination via formation of non-radical products from LDL-trapped tocopheroxyl radical $(\alpha\text{-TO}^{\bullet})_{LDL}$. This is achieved by an amphiphilic radical $(X^{\bullet})_{aq}$ crossing from the aqueous environment into the LDL, whereas interparticle radical transport inhibits TMP by combined radical export and radical import mechanisms. In order to effectively act via

the latter mechanism, an antioxidant (XH) needs to react readily with $(\alpha\text{-TO}^{\bullet})_{LDL}$ yielding antioxidant radical ($X^{\bullet}$) that diffuses rapidly to another particle and terminates rapidly with another $(\alpha\text{-TO}^{\bullet})_{LDL}$ forming non-radical products. The effectiveness of several potential natural and synthetic co-antioxidants during LDL oxidation, induced by azo-initiators, has been studied extensively.[21, 62] It was found that molecules containing a long alkyl chains in their structures act much worse via this mechanism, compared to their short alkyl chains analogues. This can be explained by the fact that long alkyl chain of XH retards diffusion of $X^{\bullet}$ from the LDL to the aqueous environment disabling formation of non-radical products with a second $(\alpha\text{-TO}^{\bullet})_{LDL}$. Another important observation that quinols, catechols and aminophenols used in these experiments were superior co-antioxidants with respect to the similar phenolic structures, was explained by the fact that, unlike phenoxyl, semiquinone radicals are strongly reducing agents that react very rapidly with $\alpha\text{-TO}^{\bullet}$.[62]

It can be expected that small, amphiphilic 2-amino-5-pyridinols will act well via these anti-TMP mechanisms, especially if the amino-substituent is monoalkylated. Of course, *N*-monoalkylated aminopyridinols are also thought to be an efficiently lipid peroxidation inhibitors in the absence of α-TOH, since they possess two hydrogen atoms that can be transferred to chain-carrying oxygen centered radicals. In fact, belonging to that category PyrOH **5** was found to protect LDL from an oxidative damage the most efficiently among the compounds tested (*vide supra*). Large scale syntheses of *N*-monoalkylated 2-aminopyridin-5-

ols and evaluation of important properties of these compounds became therefore the major target of the project described in the next two chapters of these thesis.

Research Aims

The Aims of the research presented in Chapter II are:

1. To establish a general low-cost synthetic method that enables the large scale, efficient preparation of *N*-monoalkylated 2-aminopyridin-5-ols.
2. To establish a general low-cost synthetic method that enables the large scale, efficient preparation of *N*,*N*-dialkylated 2-aminopyridin-5-ols.

The Aims of the research presented in Chapter III are:

3. To evaluate important antioxidant parameters of the various pyridinol antioxidants in highly polar environments, such as aqueous buffers.
4. An evaluation of important antioxidant parameters of the above compounds in non-polar environments, such as aprotic organic solvents.
5. An evaluation of important antioxidant parameters of the above compounds in heterogeneous systems, such as liposomal suspensions in aqueous buffers.

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CHAPTER II

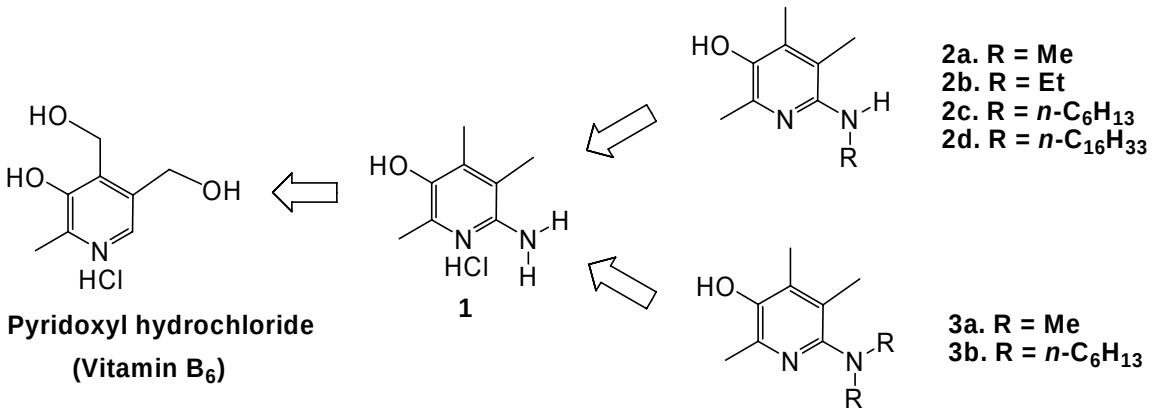
SYNTHESES OF NOVEL 2-AMINO-PYRIDIN-5-OL BASED ANTIOXIDANTS

Introduction

Even though theoretical calculations and primary experimental results confirmed that pyridinols presented in the previous chapter possessed excellent antioxidant properties, the studies performed on these compounds were rather limited. The number of experiments was constrained mostly due to the lack of an efficient method of preparation of these radical scavengers. The key hydroxylation step utilized typically proceeded in low yield and required a tricky chromatographic purification of the final product. A number of other hydroxylation protocols were investigated without success in syntheses of the desired compounds.[1-5] The method found to be the most efficient relied on the conversion of an aryl bromide into an aryl lithium followed by quenching with a nitroarene.[6-8] Among the aryl lithium quenching reagents that have been utilized, 1,3-dimethyl-2-nitrobenzene gave the most reproducible results, therefore it was applied in the preparation of all the compounds presented in the previous chapter.[1, 9]

The inspiration for an alternative method of preparation of the pyridinolic antioxidants came from an article describing the synthesis of a pyridoxamine derivative that contained the 2-amino-pyridin-5-ol moiety in its structure.[10] Undoubtedly, the existence of the hydroxyl moiety in the starting substrate is

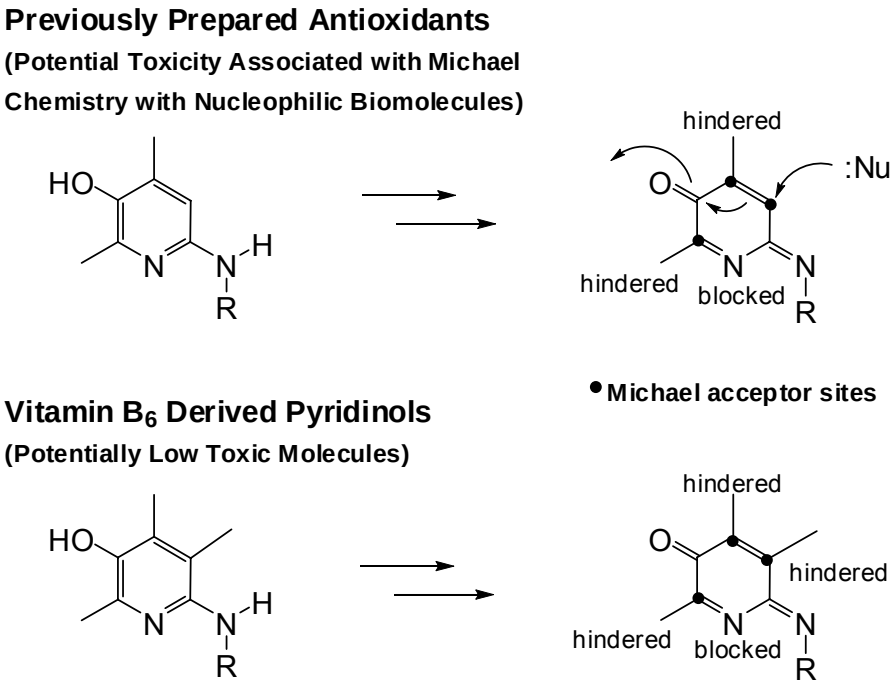
a potentially great advantage of the proposed methodology. The wide availability and extremely low price of pyridoxyl hydrochloride (vitamin B_6) made it additionally attractive as a starting material for organic preparations.[11] It was therefore decided to establish a large scale procedure for syntheses of selected monocyclic (**1**, **2a**-**d** and **3a**-**b**) and bicyclic (**8** and **9**) PyrOHs utilizing vitamin B_6 as a starting material (Scheme 2.1). The methodology for syntheses of bicyclic antioxidants from vitamin B_6 is, however, only briefly mentioned in this chapter since it was developed by another group member.[12]



Scheme 2.1. Retro-synthetic scheme for preparation of pyridinolic antioxidants.

It should be noted that the compounds presented above differ from the monocyclic pyridinols for which the antioxidant properties have been established (*vide supra*) by a presence of a methyl group in the 3-position of the aromatic ring. This substitution was predicted not to have a significant effect on the O-H BDEs of the PyrOHs, therefore not to alter the key properties of these radical scavengers. It is also of interest that the imino-pyridinone products of

oxidation from these compounds would have blocked or hindered Michael acceptor sites (Scheme 2.2).



Scheme 2.2. Potentially decreased toxicity of the vitamin B_6 derivatives.

In case of *N*,*N*-dialkylated pyridinols (**3**), additional mono-dealkylation by cytochrome P-450 or peroxidases is required whereas pyridinols **1** and **2** are expected to form imino-pyridinone products even in the absence of these enzymes.[13, 14] Formation of the products that have inactivated Michael acceptor sites may be an important from the toxicological point of view. It is known that compounds such as catechols or aminophenols are good radical scavengers, [15, 16] but their utility *in vivo* is limited since they are converted into

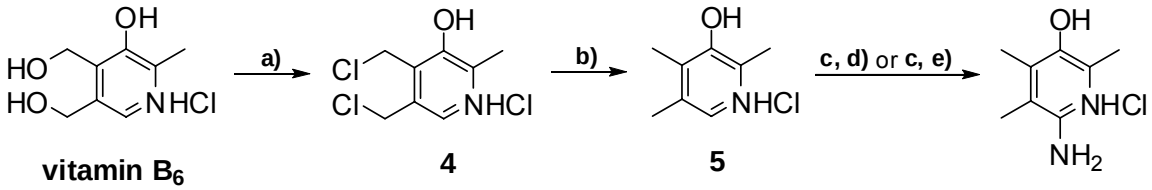
toxic quinones or quinone-imines. They apparently react with a variety of protein or nucleic acid nucleophiles that results in cyto-toxicity.[17-20]

Vitamin B_6 Derived Precursor for Synthesis of Radical Scavengers

The first step towards preparation of pyridinols **2** and **3** was the development of the core compound **1**, which could be easily converted into either *N*-mono or *N*,*N*-dialkylamino products, such as those shown in Scheme 2.1. So far, compound **1** has been found to be very stable upon prolonged storage, therefore it proved to be a convenient key precursor for synthesis of theoretically more potent pyridinols **2** and **3**.[21]

Pyridoxyl hydrochloride was converted into compound **1** in a sequence of four basic transformations (Scheme 2.3). Benzylic hydroxyl groups were successfully reduced in two steps by conversion to the dichloride (99% yield) followed by reduction of the dichloride by zinc and acetic acid (90% yield).[22, 23] Several direct dehydroxylation pathways were also tested, but they did not lead to the desired product **5**. Instead, high-temperature Wolf-Kishner reduction yielded 5-(hydroxymethyl)-2,4-dimethylpyridin-3-ol in approximately 80% yield. The same product was quantitatively obtained via direct reaction of pyridoxyl hydrochloride with zinc in acetic acid. Although potentially useful in preparation of some bicyclic pyridinols or pyridinolic dimers, this partially reduced product did not present substantial value in preparation of the key precursor **1**.[24, 25] Introduction of the amino group into the 6-position of the aromatic ring was also accomplished by a two-step strategy. An azo-coupling followed by

heterogeneous catalytic hydrogenation gave satisfactory results leading to formation of 6-amino-2,4,5-trimethylpyridin-3-ol hydrochloride (**1**) in *ca.* 70% yield over four steps from vitamin B_6.[10] Alternatively, if working with more than 40 grams of the azo-intermediate, reduction by zinc in acetic acid was preferred as a final step yielding key compound **1** in up to 66% overall yield.[26] It should be noted that large-scale preparation of the base compound **1** was achieved without necessity of chromatographic purification. Compounds **4**, **5** as well as the product **1** were all characterized as crystalline materials (especially as corresponding hydrochloric salts), therefore they could be easily purifiried via recrystalization or precipitation. This advantage has been possible most likely due to an existence of hydroxyl moiety incorporated into aromatic cores of all of these compounds, since structurally similar 3-bromopyridines were characterized as oils.[1,9] Therefore, the utilization of vitamin B_6 as a starting material shows additional, advantages compared to the previously established routes.



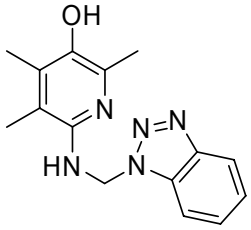
vitamin B_6 **4** **5** **1**

Reagents and Conditions: (a) $SOCl_2$, DMF (cat.), reflux, 30min, 99%; (b) Zn/AcOH, reflux, 2h, 90%; (c) $PhNH_2$, $NaNO_2$, H_2O, pH 8, 0°C, 1h, 87%; (d) H_2, Pd/C, MeOH, rt, 24-48h, 90%; (e) Zn/HCOOH, MeOH, reflux, 3h, 85%.

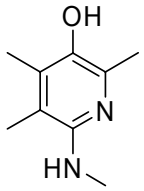
Scheme 2.3. Preparation of the key compound **1**.

Preparation of *N*-Alkylated 2-Amino-5-pyridinols

The general three-step strategy leading to preparation of *N*-monoalkylated pyridinols from the precursor **1** has been developed and successfully applied to produce compounds **2b**-**d** (Scheme 2.4) in multi-gram scale. Application of *N*,*O*-diacylation reaction followed by selective *O*-acyl hydrolysis yielded *N*-(5-hydroxy-3,4,6-trimethylpyridin-2-yl) amides. These compounds were reduced by borane-tetrahydrofuran complex (BH_3•THF) to give the desired *N*-monoalkylated products.[27, 28] An alternative pathway was considered and examined via a sample preparation of methylamino-pyridinol **2a**. In the two-step strategy, preparation of 6-((*1H*-benzo[1,2,3]triazol-1-yl)methylamino)-2,4,5-trimethylpyridin -3-ol (from *1H*-hydroxyalkylbenzotriazole and compound **1**) was followed by reduction of this compound in the presence of sodium borohydride.[1, 29, 30] Even though both of the reactions in the sequence were almost quantitative, the test trials revealed severe problems with separation of the product **2a** from the reaction mixture. And since this problem could not be solved utilizing standard purification techniques, the pathway was abandoned.[31]



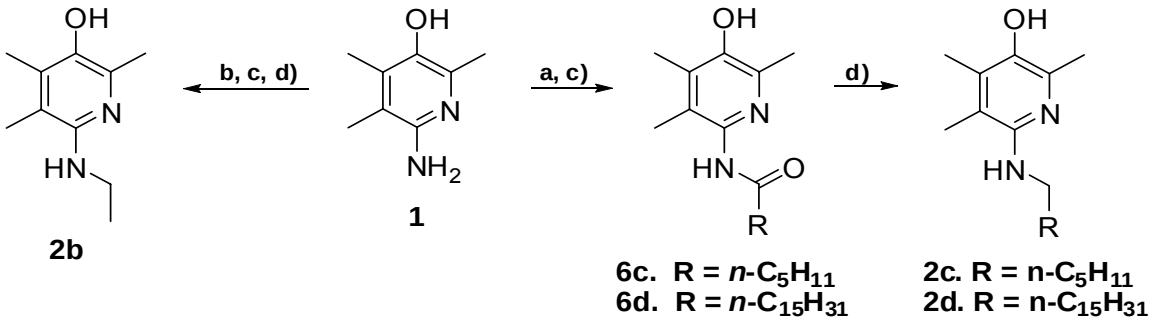
6-((1*H*-benzo[1,2,3]triazol-1-yl)methylamino)-2,4,5-trimethylpyridin-3-ol



2a

Figure 2.1. A synthetic intermediate for preparation of compound **2a**.

Pyridinol **2a** could be alternatively prepared based on the reaction sequence presented in Scheme 2.4, applying a mixed formic-acetic anhydride in the first step.[27] This sequence was not pursued, however, due to the availability of **2a** (*ca.* 0.5g formed by the alternative method) and the very close structural similarity of the compound to **2b**.[32]



Reagents and Conditions: (a) RCOCl, 1,2-dichloroethane, reflux, 4h; (b) $(AcO)_2$, THF, rt,12h; (c) NaOMe, MeOH, rt, 30-90min; (d) BH_3•THF, THF, reflux, 2-3h, 57-64% (for a-c-d) or 74% (for b-c-d).

Scheme 2.4. Preparation of *N*-monoalkylated pyridinols **2b**-**d**.

The syntheses presented in Scheme 2.4 were repeated at least 3 times starting from different amounts of key compound **1** (1, 3 and 5g) in order to investigate reproducibility and test if scaling up has any effect on the overall yield of products **2b**-**d**. It was found that total yields for compound **6b** and **6c** were consistent within trials (74±1% and 64±1% respectively) regardless of the scale of the reaction (within the range given). In case of compound **2b**, purification was only performed after the last step, whereas during synthesis of pyridinol **2c**,

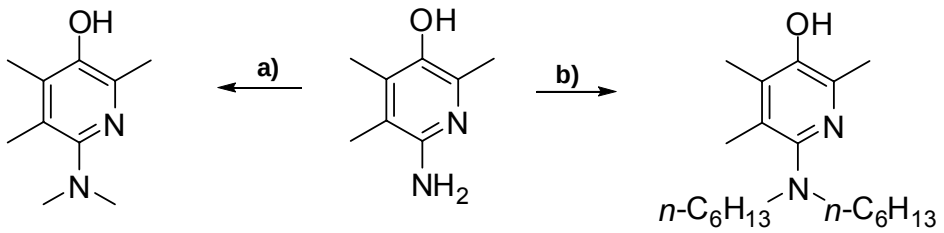
purification procedures were applied before and after BH_3•THF reduction. This strategy was found to give the best yield of PyrOH **2c**. Production of compound **2d** was more difficult, and in order to obtain satisfactory purity, a silica gel adsorption–desorption technique was applied before and after the final step. In that case, the isolated product yield for the three-step process was found to be 64, 62 and 57% for reactions starting from 1, 3 and 5g of precursor **1**, respectively.

It is worth mentioning that all synthetic pathways leading from vitamin B_6 to compounds **2b** and **2c** did not require use of any separation/purification technique other than filtration, extraction, precipitation or crystallization. Exclusive use of these simple techniques potentially enables the synthesis to be applied in large scale. Although the silica gel adsorption–desorption purification method could be potentially scaled up, limited oversized equipment availability prohibits increasing the scale of the reaction in a typical academic organic laboratory. Furthermore, the time required for purification using silica gel was not optimized according to the scale of the reaction. This might be a reason why relatively higher standard deviation for total yield of PyrOH **2d** was obtained (61±4%).

Preparation of *N*,*N*-Dialkylated 2-Amino-5-pyridinols

A one-pot reductive alkylation methodology was successfully applied to produce *N*,*N*-dialkylated derivatives of compound **1** in good yields (Scheme 2.5). The Eishweiler-Clark reaction was utilized to synthesize dimethylated pyridinol **3a** (85% yield), while the *N*,*N*-dihexyl derivative **3b** was obtained in the reaction of

precursor **1** and *n*-hexan-1-al in the presence of sodium triacetoxyborohydride (80% yield).[33, 34]



3a **1** **3b**

Reagents and Conditions: (a) HCOOH, HCHO, H_2O, reflux, 42h, 85%; (b) hexanal, $Na(AcO)_3BH$, AcOH, THF, rt, 6h, 80%.

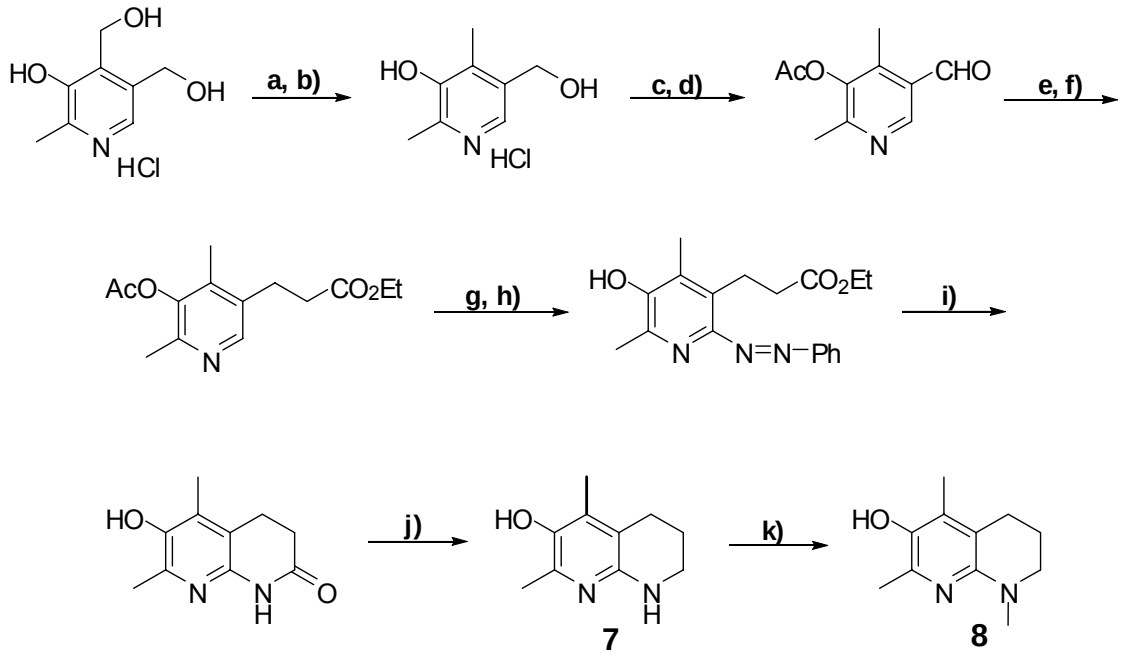
Scheme 2.5. Preparation of *N*,*N*-dialkylated 2-amino-5-pyridinols **3a** and **3b**.

Antioxidant **3a** was conveniently purified via recrystallization, whereas oily compound **3b** failed to yield crystals (even as a hydrochloric salt) and had to be purified chromatographically. The silica gel adsorption–desorption technique, used to purify pyridinol **2d**, could potentially be applied to efficiently prepare larger quantities of compound **3b**. However, no such attempt has yet been pursued.

Preparation of Naphthyridinolic Antioxidants

As already mentioned, bicyclic pyridinols can be prepared from pyridoxyl hydrochloride. The applied methodology for synthesis of 2,4-dimethyl-5,6,7,8-tetrahydro-1,8-naphthyridin-3-ol (**7**) involved ten steps and gave over 60% overall

yield, Scheme 2.6.[12] The *N*-methylated analogue of this antioxidant (**8**) was obtained utilizing a reductive alkylation procedure. The experimental details for these preparations will be presented elsewhere.[35]

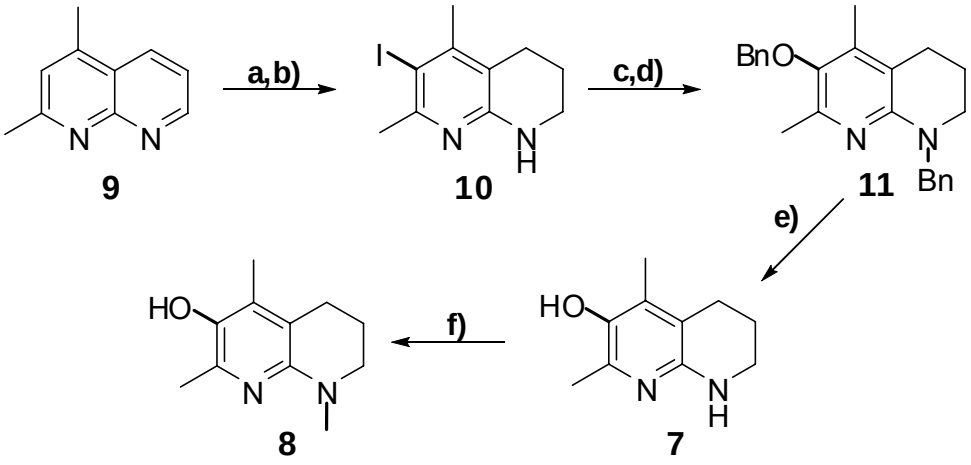


Reagents and Conditions: (a) Zn, AcOH, reflux, 3 h; (b) 2M HCl in ether, MeOH, reflux, 1 h, 93% (for a-b); (c) Ac_2O, $NaHCO_3$, H_2O, rt, 10 min; (d) NaOCl, TEMPO, $NaHCO_3$, CH_2Cl_2, rt, 1 h, 93% (for c-d); (e) NaH, $(EtO)_2P(O)CH_2CO_2Et$, CH_2Cl_2, rt, 1 h; (f) H_2, 10% Pd/C, EtOH, rt, 3 h, 91% (for e-f); (g) K_2CO_3, EtOH, rt, 2 h; (h) $PhNH_2$, 6M-HCl, $NaNO_2$, 2.5M-NaOH, H_2O-THF, 0 °C to rt, 1 h, 93% (for g-h); (i) Zn, AcOH, reflux, 1 h, 92%; (j) BH_3•THF, THF, reflux, 6 h, 93%; (k) HCHO, AcOH, $NaCNBH_3$, MeOH, H_2O, rt, 2h, 80%.

Scheme 2.6. Synthesis of bicyclic pyridinols **7** and **8** from vitamin B_6.[12]

For the purpose of the investigation presented in the next chapter, however, compounds **7** and **8** were prepared from 2,4-dimethyl-1,8-naphthyridine

(**9**), Scheme 2.7. This was due to availability of multiple grams of this bicyclic precursor. The completed synthesis involved an improved method for hydroxylation of pyridines and naphthyridines utilizing copper-catalyzed coupling of iodo- pyridines/naphthyridines with benzylic alcohol as a key step.[25, 36] Iodide **10** was prepared from the substrate **9** in two steps, which involved selective reduction of non-alkylated aromatic ring and subsequent electrophilic substitution in the remaining aromatic ring. It was found that the key coupling step did not work unless the *p*-amino group was di-substituted. Therefore a protective group was typically put on the sp^2 nitrogen atom.



Reagents and Conditions: (a) H_2, Pd/C, MeOH, rt, 48h; (b) NIS, TFA, MeCN, rt, 1h, 76% (for a-b); (c) NaH, DMF, rt, 30min; then $PhCH_2Br$, NBu_4I, DMF, rt, 12h; (d) $PhCH_2OH$, $CsCO_3$, CuI, 90°C, 48h, 35% (for c-d); (e) H_2, Pd/C, MeOH, rt, 48h, 96%; (f) HCHO, AcOH, $NaCNBH_3$, MeOH, H_2O, rt, 2h, 78%.

Scheme 2.7. Preparation of naphthyridinols, an alternative route.

Introduction of benzyl protecting group was advantageous since it could be removed in the hydrogenation step following the copper-catalyzed coupling. Compound **7** was prepared from **9** with *ca.* 26% efficiency over five transformations, which is not comparable to the overall yield for the synthesis starting from vitamin B_6. However, the route presented above was much quicker, and its efficiency would most likely be greater if optimization studies were conducted. It should be noted that bicyclic PyrOH **7** may be *N*-alkylated with hydrocarbon chains of different length to form compounds of varying lipophilicity. However, for the purpose of the investigation presented herein only methylated analogue was prepared, Scheme 2.7.

Experimental Section

General. Unless indicated otherwise, all reactions were carried out under an air atmosphere. Zinc dust applied in reduction reactions was activated prior to use according to the standard procedure.[37] 2,4-dimethyl-1,8-naphthyridine was prepared from 4,6-dimethylpyridin-2-amine via Skraup reaction.[1, 25] Purifications by adsorption-desorption and column chromatography were carried out with silica gel 60 (230-400 mesh) from EMD Chemicals Inc. Progress of reactions was monitored using F_{254} silica coated TLC plates purchased from EMD Chemicals Inc. Spots were visualized with UV, phosphomolybdic acid or potassium permanganate. Unless indicated otherwise, all chemicals were purchased either from Acros Organics or Sigma-Aldrich and used without further purification. NMR spectra were taken on 300 MHz Bruker instrument. Peaks were calibrated on

$CDCl_3$ or DMSO-d_6 and reported with respect to TMS. Melting points were taken on a Thomas Hoover melting point apparatus and were uncorrected. ESI-HRMS measurements (positive ion mode) were performed at Ohio State University or at Texas A&M University. Elemental analysis was conducted in Atlantic Microlab, Inc. (Norcross, Ga).

4,5-Bis(chloromethyl)-2-methylpyridin-3-ol hydrochloride (4). This compound was prepared according to the literature with similar yield and purity.[22] The journal this synthesis has been published in is however rarely available, therefore the following detailed procedure was presented. Pyridoxyl hydrochloride (100g, 0.486mol) was refluxed in a mixture of DMF (3.7mL) and thionyl chloride (370mL, 4.86mol). After 30min the product precipitated out of the reaction mixture. Then, it was subsequently filtered and washed with ether (2×200mL). Drying under high vacuum yielded a white powder (117g, 99%). Mp 203-204ºC; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.43 (s, 1H), 5.00 (s, 2H), 4.97 (s, 2H), 2.65 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 152.6, 144.7, 138.7, 133.8, 132.8, 40.3, 35.3, 15.9; HRMS for $C_8H_{10}NOCl_2^+$ $[M\text{-}Cl]^+$ 205.0056, found 205.0039.

2,4,5-Trimethylpyridin-3-ol hydrochloride (5). Compound **4** (117g, 0.482mol) was dissolved in glacial acetic acid (420mL, 4.44mol) and zinc dust (98g, 1.5mol) was added in 3 portions. The resulting suspension was well stirred under reflux for 2h. The reaction mixture was cooled to the rt, unreacted zinc along with insoluble byproducts were filtered off and washed with acetic acid

(2×90mL). The filtrate was neutralized with 6M NaOH yielding an orange precipitate, which was filtered and washed with a small amount of brine (30mL). The orange precipitate was dissolved in 10M HCl solution and an obtained HCl adduct was salted out with solid NaCl. The crude product obtained after filtration was recrystallized from EtOH – ether system yielding white crystals (76g, 90%). Mp 217-218ºC (216ºC lit)[38]; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.5 (s, 1H), 8.10 (s, 1H), 2.57 (s, 3H), 2.30 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.4, 145.4, 139.6, 136.0, 131.4, 16.4, 14.7, 13.1; HRMS for $C_8H_{11}NO^+$ $[M\text{-}Cl]^+$ 137.0835, found 137.0846.

6-Amino-2,4,5-trimethylpyridin-3-ol (1). In a beaker equipped with a mechanical stirrer and pH electrode and immersed in an ice bath, substrate **5** (76g, 0.44mol) was dissolved in water (850 mL) and the pH of the solution was adjusted to 8 with 2.5M NaOH. Diazonium salt, freshly prepared by slow mixing of aniline in 6M HCl (40mL, 0.44mol; 450mL) with aqueous $NaNO_2$ (30g, 0.44mol; 150mL) in ice bath, was added to the substrate solution portionwise along with 2.5M NaOH to maintain pH 8. After 1h, a red precipitate that formed in the reaction was filtered off and partially dissolved in chloroform. The organic solvent was dried over $MgSO_4$, and condensed under vacuum forming dark red powder. The powder was partially dissolved in EtOH (400mL), insoluble material was removed by filtration and washed with the same solvent (2×75mL). 1M HCl solution in ether (500mL) was then added to the combined EtOH solutions in order to precipitate orange crystals, which were filtered off and washed with ether (2×75mL). Drying under high vacuum yielded a light orange product (104g, 87%).

The orange product (40g, 0.14mol) was dissolved in MeOH (600mL) and palladium on carbon (10 wt. %, wet, Degussa type E101 NE/W) was added carefully to the solution. The resulting suspension was stirred under hydrogen atmosphere (hydrogenation balloon) at rt. After 48h catalyst was filtered off through celite and the filtercake was washed with MeOH (2×100mL). Filtrate was condensed, the residue was suspended in ether (600mL) and stirred for 1h at rt. The product was filtered off, washed with ether (2×50mL) and dried under high vacuum yielding **1** as pale-pink powder (24g, 78% over 2 steps). Mp 242-243ºC dec; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.93 (s, 1H), 7.03 (s, 2H), 2.33 (s, 3H), 2.20 (s, 3H), 2.04 (s, 3H); Anal. calcd. for $C_8H_{13}ClN_2O$: C, 50.93; H, 6.95; Cl, 18.79, found: C, 50.81; H, 7.06; Cl, 18.84.

Alternatively, the light orange azo-compound (100g, 0.36mol) was dissolved in a mixture of MeOH (650mL) and formic acid (82mL, 2.2mol) and zinc dust (141g, 2.2mol) was added portionwise. After 2h of reflux, the reaction mixture changed color from red to dark green and the unreacted zinc along with insoluble byproducts were removed by hot filtration and washed with hot MeOH (2×125mL). The majority of MeOH was evaporated and the resulting white precipitate was filtered off, washed with ether, dried, dissolved in hot water and brought to pH 8 with 6M NaOH. Upon cooling the solution, a white precipitate formed. The precipitate was filtered off, dissolved in EtOH and filtered through silica pad. After solvent removal and drying under high vacuum a pale yellow powder (free base form of **1**) was obtained (36.3g, 74% over 2 steps). Mp 170-172ºC; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.41 (s, 1H), 4.84 (s, 2H), 2.15 (s, 3H),

2.03 (s, 3H), 1.90 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.2, 141.2, 140.2, 134.7, 112.5, 19.3, 13.3, 12.7;. HRMS for $C_8H_{13}N_2O^+$ $[M+H]^+$ 153.1022 found 153.1024.

6-(Dimethylamino)-2,4,5-trimethylpyridin-3-ol (3a). Free base form of pyridinol **1** (5.00g, 32.8mmol) was refluxed for 48 h in a mixture of formic acid (12mL) and 36% formalin (12.0mL, 318mmol). Due to the volatility of formaldehyde, additional formalin (12.0mL, 318mmol) was added each 12h. When all starting material was consumed, the reaction mixture was cooled to rt and neutralized with 2.5M NaOH (to pH 8). The product was then extracted with EtOAc, organic extracts were combined, dried over $MgSO_4$ and concentrated under vacuum. In order to obtain highly pure material, the crude product was recrystallized from benzene yielding white (85%, 5.03g). Mp 138-140ºC; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.00 (s, 1H), 2.55 (s, 6H), 2.25 (s, 3H), 2.09 (s, 3H), 2.06 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 154.4, 145.0, 139.6, 134.5, 121.4, 42.8, 19.6, 14.2, 12.6; HRMS for $C_{10}H_{16}N_2ONa^+$ $[M+Na]^+$ 203.1155 found 203.1147.

6-(Dihexylamino)-2,4,5-trimethylpyridin-3-ol (3b). To a suspension of free base form of compound **1** (500mg, 3.28mmol) in THF (10mL) were added acetic acid (0.76mL, 13.1mmol) and *n*-hexan-1-al (0.89mL, 7.23mmol). Then sodium triacetoxyborohydride (2.09g, 9.85mmol) was added to the mixture in small portions. The resulting suspension was stirred for 6h at rt. After removal of THF by evaporation, the residue was diluted with water and then neutralized with sat. $NaHCO_3$ solution. Extraction with EtOAc was performed and the combined

organic layers were washed with brine, dried over $MgSO_4$ and concentrated. The residue was purified by flash silica gel chromatography (10:1 hexanes/EtOAc) to give **3b** (842mg, 80%) as a light yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.52 (br s, 1H), 2.70 (t, 4H, *J*=7.2 Hz), 2.14 (s, 3H), 1.95 (s, 3H), 1.92 (s, 3H), 1.11-1.21 (m, 4H), 0.95-1.10 (m, 12H), 0.63 (t, 6H, *J*=6.8 Hz); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 155.1, 145.2, 139.7, 134.0, 125.9, 53.4, 32.1, 28.3, 27.5, 23.0, 19.1, 14.7, 14.4, 13.0; HRMS for $C_{20}H_{37}N_2O^+$ $[M+H]^+$ 321.2906 found 321.2903.

2,4,5-Trimethyl-6-(methylamino)pyridin-3-ol (2a). Free base form of **1** (1.85g, 12.1mmol) and *1H*-hydroxymethylbenzotriazole (1.81g, 12.1mmol) were independently dissolved in refluxing absolute EtOH (35mL, 25mL). The saturated solutions were mixed and refluxed for 30min. The mixture was then cooled slowly and stirred for 2h at rt. Reaction mixture was then cooled to -10ºC and stirred for 15min. The solid product was filtered off and washed with cold EtOH (5ml) and ether (2×25mL). Solvent removal yielded a yellow powder (3.0g, 89%).

The powder (3.0g, 11mmol) was dissolved in refluxing 1,4-dioxane (180mL) and powderized $NaBH_4$ (1.4g, 33mmol) was added slowly to the refluxing solution, which was kept under a stream of argon. After 1h, reaction was quenched with water, solvents were evaporated, a small amount of water was added and the pH of the resulting solution was brought to 8.5. The product was then extracted with chloroform; extracts were combined, dried over $MgSO_4$, and concentrated under vacuum. The crude material was purified multiple times by flash column chromatography (5-10% MeOH in CH_2Cl_2) which finally yielded pure pale-yellow product (0.50g, 31%). Mp 160-162 ºC; ^{1}H NMR (300 MHz,

DMSO-d_6) δ 7.39 (s, 1H), 5.21 (s, 1H), 2.73 (d, 3H, J=2.1Hz), 2.20 (s, 3H), 2.04 (s, 3H), 1.90 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.3, 140.5, 139.3, 134.6, 113.1, 29.3, 19.8, 12.8; HRMS for $C_9H_{15}N_2O^+$ $[M+H]^+$ 166.1101 found 166.1091.

6-(ethylamino)-2,4,5-trimethylpyridin-3-ol hydrochloride (2b HCl). To a solution of **1** (5.00g, 26.5mmol) in warm MeOH (50mL) triethylamine (15mL, 0.11mol) was added and the solution was stirred for 15min at 50ºC. Solvents were evaporated and residual mixture was suspended in THF (30mL), triethylamine (7.5mL, 53mmol) was added and acetic anhydride (20mL, 0.21mol) was dropped slowly at 0ºC. The mixture was brought to rt and stirred for 12h. Solvents were evaporated and extraction between chloroform and saturated aqueous $NaHCO_3$ solution was performed. The organic layers were combined and dried over $MgSO_4$. Evaporation of solvent followed by high vacuum drying yielded 6.66g of white powder (crude 6-acetamido-2,4,5-trimethylpyridin-3-yl acetate).

The crude product was dissolved in MeOH (90mL) and reacted with 30% sodium methoxide solution in MeOH (5.5mL, 29mmol) added dropwise at rt. The mixture was stirred for 30min, and then neutralized with 2M HCl in ether to pH 7-8. Solvents were evaporated and the residue was dried under high vacuum yielding 6.94g of white powder (crude N-(5-hydroxy-3,4,6-trimethylpyridin-2-yl)acetamide).

To the crude acetamide suspension in dry THF (140mL) under argon atmosphere, 1M borane-tetrahydrofuran complex in THF (90mL, 90mmol) was added dropwise at 0ºC. The mixture was brought to reflux temperature, stirred for

2h and cooled back down to 0ºC. MeOH (25mL) was added very slowly at 0ºC to quench excess of the reagent. The mixture was further refluxed for 1h and cooled to rt at which 2N HCl in ether (35mL) was added. Mixture was stirred for 20min at that temperature and then condensed under reduced pressure. An extraction between chloroform and saturated aqueous $NaHCO_3$ solution was performed, organic layers were combined and dried over $MgSO_4$. Solvent was evaporated yielding crude 6-(ethylamino)-2,4,5-trimethylpyridin-3-ol, which was subsequently recrystallized from benzene. Thus obtained crystals were dissolved in MeOH (20mL) and acidified with 2M HCl in ether (30mL, 60mmol). Additional 50mL of ether was added and the mixture was stirred for 15min, during which final product precipitated out. The white precipitate was filtered off, washed with 0.2M HCl in ether and dried under high vacuum yielding 6-(ethylamino)-2,4,5-trimethylpyridin-3-ol hydrochloride (4.31g, 75%). Mp 234-235ºC dec; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.95 (s, 1H), 7.34 (s, 1H), 3.53-3.58 (m 2H), 2.49 (s, 3H), 2.21 (s, 3H), 2.08 (s, 3H), 1.16 (t, 3H, J=7.0 Hz); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 147.1, 145.5, 140.5, 129.7, 117.7, 37.2, 14.2, 14.1, 14.0, 13.1; Anal. calcd. for $C_{10}H_{17}ClN_2O$: C, 55.42; H, 7.91; Cl, 16.36, found: C, 55.17; H, 8.02; Cl, 16.61.

***N*-(5-hydroxy-3,4,6-trimethylpyridin-2-yl)hexanamide (6c)**. To a solution of **1** (5.00g, 26.5mmol) in warm MeOH (50mL) triethylamine (15mL, 0.11mol) was added and the solution was stirred for 15 minutes at 50ºC. Solvents were evaporated and residual mixture of neutralized compound **1** and triethylammonium chloride was further dried under high vacuum for 30min to ensure the complete removal of MeOH. To thus obtained dry solid mixture

suspended in 1,2-dichloroethane (200mL), triethylamine (15mL, 0.11mol) was added and hexanoyl chloride (15.5mL, 106mmol) was dropped slowly at 0ºC. The mixture was brought to rt and then refluxed for 4h. Solvents were evaporated and extraction between chloroform and saturated aqueous $NaHCO_3$ solution was performed. The organic layers were combined and dried over $MgSO_4$. Evaporation of solvent followed by high vacuum drying yielded 18g of sticky pale yellow solid (crude 6-hexanamido-2,4,5-trimethylpyridin-3-yl hexanoate).

The crude product was dissolved in MeOH (120mL) and reacted with 30% sodium methoxide solution in MeOH (14mL, 75mmol) added dropwise at rt. The mixture was stirred for 90min, and then neutralized with 2M HCl in ether to pH 7-8. Solvents were evaporated to give a solid mixture from which organic compounds were extracted with chloroform. The solvent was condensed to about 20% of the original volume and mixed with equal volume of toluene. The mixture was further condensed until the chloroform and half of the volume of toluene was evaporated. Thus obtained toluene solution was placed at -10ºC for 1h. A precipitate formed upon cooling was filtered off, washed with cold toluene then cold pentane and dried under high vacuum yielding white crystalline material (5.24g, 69%). Mp 187-188ºC; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.58 (br s, 1H), 8.46 (s, 1H), 2.27 (s, 2H), 2.22 (t, 2H, *J*= 7.5Hz), 2.10 (s, 3H), 1.93 (s, 3H), 1.55 (m, 2H), 1.28 (m, 4H), 0.86 (t, 3H, *J*=6.9 Hz); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 173.2, 148.0, 142.1, 139.1, 134.6, 126.8, 36.4, 31.2, 25.1, 22.1, 18.4, 14.3, 13.7, 12.0; HRMS for $C_{14}H_{23}N_2O_2^+$ $[M\text{-}Cl]^+$ 251.1760 found 251.1755.

6-(hexylamino)-2,4,5-trimethylpyridin-3-ol hydrochloride (2c HCl). To the solution of **6c** (5.24g, 18.3mmol) in dry THF (70mL) under argon atmosphere, 1M borane-tetrahydrofuran complex in THF (75mL, 75mmol) was added dropwise at 0ºC. The mixture was brought to reflux temperature, stirred for 3h and cooled back down to 0ºC. MeOH (25mL) was added very slowly at 0ºC to quench excess of the reagent. The mixture was further refluxed for 1h and cooled to the rt at which 2M HCl in ether (35mL) was added. Mixture was stirred for additional 20min and then condensed under reduced pressure. An extraction between chloroform and saturated aqueous $NaHCO_3$ solution was performed; organic layers were combined and dried over $MgSO_4$. Solvent was evaporated yielding crude 6-(hexylamino)-2,4,5-trimethylpyridin-3-ol, which was subsequently recrystallized from heptane. Thus obtained white crystals were dissolved in MeOH (20mL) and acidified with 2M HCl in ether (40mL, 80mmol). The mixture was stirred for 20min; solvents were evaporated yielding thick oil which solidified under high vacuum. Thus obtained solid was washed with small amount of 0.2M HCl in ether (10mL) and dried again under high vacuum yielding 6-(hexylamino)-2,4,5-trimethylpyridin-3-ol hydrochloride (4.77g, 95%). Mp 151-152ºC; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.00 (br s, 1H), 7.29 (s, 1H), 3.47-3.51 (m, 2H), 2.47 (s, 3H), 2.22 (s, 3H), 2.00 (s, 3H), 1.52-1.61 (m, 2H), 1.20-1.39 (m, 6H), 0.84-0.88 (m, 3H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 147.4, 145.7, 140.7, 129.7, 118.8, 42.5, 31.3, 28.5, 26.2, 22.4, 14.3, 14.2, 14.1, 13.3; Anal. calcd. for $C_{14}H_{25}ClN_2O$: C, 61.64; H, 9.24; Cl, 13.00, found: C, 61.11; H, 9.28; Cl, 12.83.

***N*-(5-hydroxy-3,4,6-trimethylpyridin-2-yl)palmitamide (6d)**. To a solution of **1** (5.00g, 26.5mmol) in warm MeOH (50mL) triethylamine (15mL, 0.11mol) was added and the solution was stirred for 15 minutes at 50°C. Solvents were evaporated and residual mixture of neutralized compound **1** and triethylammonium chloride was further dried under high vacuum to ensure the complete removal of MeOH. To thus obtained dry solid mixture suspended in 1,2-dichloroethane (200mL), triethylamine (15mL, 0.11mol) was added and palmitoyl chloride (33mL, 0.11mol) was dropped slowly at 0°C. The mixture was brought to rt and then refluxed for 4h. Solvents were evaporated and extraction between chloroform and saturated aqueous $NaHCO_3$ solution was performed. The organic layers were combined and dried over $MgSO_4$. Evaporation of solvent followed by high vacuum drying yielded 35g of sticky white solid (crude 2,4,5-trimethyl-6-palmitamidopyridin-3-yl palmitate).

The crude product was dissolved in 1:10 MeOH in CH_2Cl_2 solution (250mL) and reacted with 30% sodium methoxide solution in MeOH (15mL, 80mmol) added dropwise at rt. The mixture was stirred for 90 minutes, and then neutralized with 2M HCl in ether to pH 7-8. Solvents were evaporated to give a solid residue which was suspended in chloroform (1.5L). Silica gel (100g) was then added and the suspension was stirred at rt. After 1h solid particles were filtered off (filtrate was discarded), washed with chloroform (2×150mL) then re-suspended in 1:3 MeOH in chloroform solution (1.5L) and stirred for 1h at rt. Adsorbent particles were filtered off again, washed with 1:3 MeOH in chloroform solution (2x150mL). Filtrates were combined and condensed under reduced

pressure yielding white solid (7.89g, 70%). Mp 136-137ºC; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.56 (br s, 1H), 8.46 (s, 1H), 3.32 (s, 2H), 2.27 (s, 3H), 2.22 (t, 2H, *J*=7.3 Hz), 2.10 (s, 3H), 1.93 (s, 3H), 1.52 (m, 2H), 1.23 (m, 24H), 0.84 (t, 3H, *J*=6.6 Hz); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.3, 147.5, 141.3, 140.8, 133.6, 126.2, 35.2, 31.3, 29.0, 28.9, 28.8, 28.7, 25.2, 22.1, 19.2, 14.5, 14.0, 12.5; HRMS for $C_{24}H_{43}N_2O_2^+$ $[M\text{-}Cl]^+$ 391.3325 found 391.3319.

6-(hexadecylamino)-2,4,5-trimethylpyridin-3-ol hydrochloride (2d HCl). To the solution of **6d** (7.89g, 18.5mmol) in dry THF (120mL) under argon atmosphere, 1M borane-tetrahydrofuran complex in THF (70mL, 70mmol) was added dropwise at 0ºC. The mixture was brought to reflux temperature, stirred for 3h and cooled back down to 0ºC. MeOH (25mL) was added very slowly at 0°C to quench excess of the reagent. The mixture was further refluxed for 1h and cooled to rt at which 2M HCl in ether (35mL) was added. Mixture was stirred for 20 minutes at that temperature and then condensed under reduced pressure. An extraction between chloroform and saturated aqueous $NaHCO_3$ solution was performed; organic layers were combined and dried over $MgSO_4$. The solution was acidified using 2M HCl in ether (30mL), solvents were evaporated to give a solid residue which was suspended in chloroform (600mL). Silica gel (80g) was then added and the suspension was stirred at rt. After 1h adsorbent particles were filtered off (filtrates were discarded), washed with chloroform (2×100mL) then re-suspended in 1:3 MeOH in chloroform solution (600L) and stirred for 1h at rt. Solids were filtered off again, washed with 1:3 MeOH in chloroform solution (2×100mL) and filtrate was condensed under reduced pressure. The residual

pale yellow oil was treated with 2N HCl in ether (10mL) to give pale-yellow solid which was then recrystallized from toluene to give bright white crystalline product (6.77g, 89%) upon drying. Mp 86-87ºC; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.98 (s, 1H), 7.29 (s, 1H), 3.36-3.45 (m 2H), 2.48 (s, 3H), 2.22 (s, 3H), 2.09 (s, 3H), 1.52-1.65 (m, 2H), 1.15-1.37 (m, 26H), 0.79-0.86 (m, 3H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 147.4, 145.6, 140.7, 129.7, 117.9, 42.5, 31.6, 29.4, 29.3, 29.1, 29.0, 28.5, 26.5, 22.4, 14.2, 14.1, 13.3; Anal. calcd. for $C_{24}H_{45}ClN_2O$: C, 69.78; H, 10.98; Cl, 8.58, found: C, 68.96; H, 10.97; Cl, 8.16.

2,4-dimethyl-5,6,7,8-tetrahydro-1,8-naphthyridin-3-ol hydrochloride (7 HCl). This compound was prepared in five steps. The synthetic intermediates have been purified only briefly and have not been fully characterized.

First step, the transformation of naphthyridine **9** into 5,7-dimethyl-1,2,3,4-tetrahydro-1,8-naphthyridine was performed according to the previously published procedure, with a comparable yield.[1]

To a solution of this partially hydrogenated compound **9** (3.3g, 21mmol) in MeCN (70mL) was added NIS (4.7g, 64.7mmol) and TFA (0.47g, 4.1mmol) at rt. After 1h of stirring, solvents were evaporated, and the residue was partitioned between sat. $NaHCO_3$ solution and AcOEt. The combined organic layers were dried over $MgSO_4$ and condensed to yield 5.8g of the solid product **10**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.08 (s, 1H), 3.31 (m, 2H), 2.65 (t, 2H, 6.6Hz), 2.54 (s, 1H), 2.28 (s, 1H), 1.89 (q, 2H, J = 5.7Hz).

To a solution of compound **10** (5.8g, 20mmol) and NaH (0.83g, 40mmol) in DMF (80mL) was added NBu_4I (2.0g, 15mmol) and benzyl bromide (3.3mL,

67mmol). After the solution was stirred overnight at rt, sat. NH_4Cl solution was added and the multiphase solution was stirred for 15min. Solid $NaHCO_3$ was added till pH 7 and the product was extracted with DCM. The organic layers were combined, washed well with water, dried over $MgSO_4$ and condensed. The residue was purified chromatographically (0-5% EtOAc/hexanes) to give 5.0g of 1-benzyl-6-iodo-5,7-dimethyl-1,2,3,4-tetrahydro-1,8-naphthyridine as a pale violet solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.25 (m, 5H), 4.87 (s, 2H), 3.22 (m 2H), 2.69 (t, 2H, *J* = 5.7Hz), 2.57 (s, 3H), 2.30 (s, 3H), 1.52 (m, 2H).

To a solution of this pale violet product (5.0g, 13mmol) in benzyl alcohol (200mL) were added portionwise (at 90ºC) Cs_2CO_3 (167g, 0.5mol) and CuI (1.4g, 7mmol). After 48h of stirring at 90ºC, most of the solvent was distilled off. The residue was partitioned between water and DCM. The organic layers were combined, washed with brine, dried over $MgSO_4$ and condensed. The residue was purified chromatographically (0-5% EtOAc/hexanes) to give 2.4g of oily compound **11**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.48-7.20 (m, 10H), 4.77 (s, 2H), 4.66 (s, 2H), 3.19 (m 2H), 2.58 (t, 2H, *J* = 6.0Hz), 2.23 (s, 3H), 2.06 (s, 3H), 1.86 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 151.1, 145.1, 143.1, 139.7, 138.2, 137.7, 128.5, 128.4, 128.2, 128.1, 127.7, 126.7, 113.4, 74.6, 50.9, 46.6, 24.3, 21.4, 19.4, 11.9.

To a solution of compound **11** (2.4g, 6.7mmol) in EtOH (55mL) and THF (55mL) was added 10% Pd/C (0.55g). After 48h of stirring under an atmosphere of hydrogen at rt, insoluble materials were filtered off and washed with EtOH. The filtrate was condensed to yield crude product, which was washed with 10:1

Et_2O/EtOH mixture (2 x 10mL), and then with plain Et_2O (3 x 15mL) to yield of pure compound **7** as a white solid (1.2g, 95%). To this solid dissolved in MeOH (3mL) was added 2M HCl/Et_2O (5mL). After 15min of stirring, the mixture was condensed, and washed with Et_2O to yield **7 HCl** as a white powder. Mp 219-220ºC dec; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.87 (br s, 1H), 7.35 (s, 1H), 3.30 (t, 2H, *J*=5.4 Hz), 2.63 (t, 2H, *J*=6.2 Hz), 2.31 (s, 3H), 2.15 (s, 3H), 1.80 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 146.1, 145.2, 140.1, 129.8, 116.4, 22.6, 19.6, 13.9, 12.9; HRMS for $C_{10}H_{15}N_2O^+$ [M-Cl] 179.1184 found 179.1186.

2,4,8-trimethyl-5,6,7,8-tetrahydro[1,8]naphthyridin-3-ol hydrochloride (8 HCl). To a solution of compound **7** (400mg, 2.25mmol) in MeOH (20mL) were added acetic acid (6.75mL, 0.12mol), 36% formalin solution (8.0mL, 0.21mol) and $NaCNBH_3$ (0.74mg, 12.4mmol). The resulting mixture was stirred for 2h at rt. After removal of MeOH by evaporation, the residue was diluted with water (25mL) and then neutralized with sat. $NaHCO_3$ solution. Extraction with EtOAc was performed and the combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated. The residue was purified by silica gel flash chromatography (2:1 hexanes/EtOAc) to give a pale-yellow solid, which was subsequently dissolved in MeOH (0.6mL) and treated with 5mL of 1M HCl/Et_2O. The mixture was stirred for 15min, condensed and washed with Et_2O to yield pale-yellow solid (82mg, 80%). Mp 164-166ºC dec; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.94 (br s, 1H), 3.40 (t, 2H, *J*=5.7 Hz), 3.18 (s, 3H), 2.65 (t, 2H, *J*=6.3 Hz), 2.43 (s, 3H), 2.17 (s, 3H), 1.87 (m, 2H) ; ^{13}C-NMR (75 MHz, DMSO-d_6) δ 146.1,

144.3, 140.2, 130.7, 118.2, 50.2, 39.1, 23.3, 19.9, 14.0, 13.3; HRMS for $C_{11}H_{17}N_2O^+$ [M-Cl] 193.1341 found 193.1334.

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CHAPTER III

ANTIOXIDANT PROPERTIES OF THE VITAMIN B_6 DERIVED PYRIDINOLS

Introduction

As described in Chapter I, a combination of different properties makes a molecule a good antioxidant. A key factor is an ability to donate hydrogen atom(s) to chain carrying peroxyl radicals in reasonably fast manner. Nevertheless, interactions of an antioxidant or the radical derived from it with oxygen (autoxidizability), natural antioxidants (co-antioxidant behavior) or other *in vivo* abundant molecules are also important. In order to establish which processes are likely to occur in lipid like surroundings, some experiments have been conducted in non-polar, aprotic solvent. On the other hand, to investigate the behavior of pyridinols in a polar environment, a series of experiments in an aqueous buffer of physiologically relevant pH have also been performed. Finally to better mimic behavior of PyrOH under conditions more likely to occur *in vivo*, the molecules have been investigated in heterogeneous systems. A model that has been commonly applied to mimic oxidation occurring in bio-membranes, a system consisting of oxidizable multi lamellar liposomes dispersed in an aqueous buffer, has been employed in these latter studies.[1-6] All of the experiments were of a comparative nature. Two compounds structurally related to α-TOH, for which reasonably good antioxidant properties have been established, were chosen as reference compounds. 2, 2', 5, 7, 8-Pentamethylchromanol (PMC) was utilized in

non-polar systems, whereas its water soluble analogue, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was chosen for studies in aqueous buffers.[7-11] Additionally, α-TOH was used as a reference compound in liposomal suspensions. Structures of the PyrOHs that were investigated are presented together with the structures of the reference α-TOH analogues in Figure 3.1.

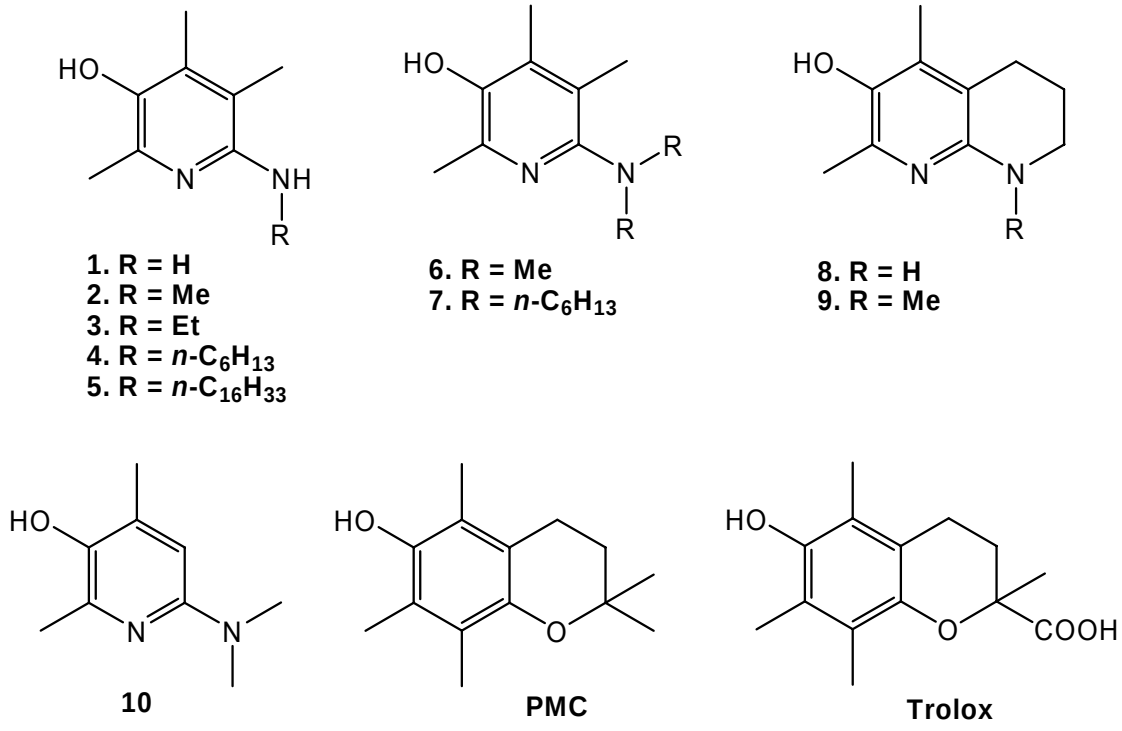


Figure 3.1. Pyridinols under investigation and relevant reference compounds.

Although the pyridinols **1**-**10** have been stored as their hydrochloric salts, due to solubility issues for the experiments in organic solvents, they were converted into the free base forms before use. For all other experiments the salts

of compounds **1**-**10** were used.[12] To avoid additional numbering, throughout this chapter, PyrOHs **1**-**10** will represent either the salts or their free bases.

Properties in non-polar environment

Air stability as well as tests of reactivity of selected pyridinols with peroxyl radicals were investigated. For air stability measurements, a representative group of pyridinols was incubated in benzene at 37°C in a closed vial that was opened from time to time to withdraw aliquots. After a quick sample preparation that included the addition of an internal standard (a molecule of similar electro activity and slightly different polarity), mixtures were analyzed by HPLC-ECD.[13, 14] From the results collected in Table 3.1, it can be seen that these compounds may be divided into two categories: stable for at least two days (all monocyclic antioxidants) and stable for a few hours (bicyclic pyridinols). The bicyclic compounds are considerably less stable due to the increased electron density of their aromatic rings compared to the monocyclic compounds. A fused ring on the pyridinol causes a decrease in dihedral angle between the exo-ring nitrogen's p-type lone pair and the p-obrital of an adjacent aromatic carbon.[15, 16] This increase in electron density makes IP values for compounds **8** and **9** smaller than the corresponding values for monocyclic pyridinols (**1**, **3**, **6** and **10**), thus increasing the reactivity of the former compounds with electron acceptors such as molecular oxygen (*vide supra*).

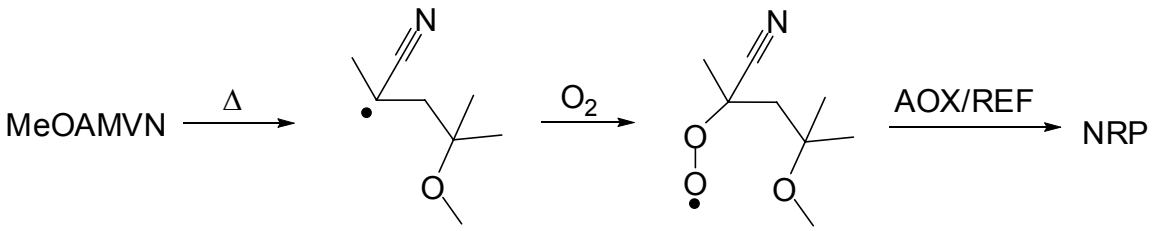
Table 3.1. Some properties of the representative PyrOHs measured in benzene.

Compound	Air stability [h][a]	n[b]	λ_{max} [nm]
1	≥ 48	2.1	307
3	≥ 48	2.0	314
6	≥ 48	1.9	298
8	≥ 3	1.9	325
9	≥ 2	1.7	326
10	≥ 48	2.0	321

[a] Time period during which no consumption of the compound occurred, based on HPLC-ECD measurements. [b] Stoichiometric factors calculated with respect to PMC (n = 2.0), based on HPLC-ECD measurements.

Due to structural similarity of compounds **2**-**5** as well as **6** and **7**, only one member of each group was investigated. It is believed that no stereo-electronic differences exist within these groups of antioxidants therefore the measured properties can be associated with every group member.

To investigate the reactivity of pyridinols with peroxyl radicals, benzene solutions of these pyridinols were incubated with an excess of radical initiator, MeOAMVN.[17] The radicals formed from the initiator were scavenged by an antioxidant resulting in its consumption. Rates of disappearance of antioxidants in the presence of MeOAMVN were compared with the rate of consumption of the reference compound, PMC, measured under identical conditions (Scheme 3.1).[18, 19]



NRP = non-radical products; AOX = antioxidant; REF = reference antioxidant

$$n_{AOX} = \frac{n_{REF} \times t_{AOX}}{t_{REF}} \qquad (\mathbf{24})$$

Scheme 3.1. Scavenging of MeOAMVN derived radicals by antioxidants.

The stoichiometric factor (*n*) is defined as a number of radicals that can be scavenged by one molecule of antioxidant, and it is an important property of radical scavengers. For PMC, it was found equal to two in several previous studies.[7, 11] The *n* values associated with pyridinols were calculated according to the generalized Eq. 24, where t_{AOX} is the time needed for complete consumption of the investigated antioxidant in the presence of radical initiator and t_{REF} is the time required to deplete a reference antioxidant, PMC in this case. The stoichiometric factors measured for the representative pyridinols **1**, **3**, **6** and **8**-**10** are included in Table 3.1. The applied initiator to antioxidants ratio of 20:1 allows zero order kinetics over the course of the experiment. Regressions performed to obtain *t* values showed high linearity (R>0.99).[20] A typical plot of relative disappearance of antioxidants during an initial period of incubation with MeOAMVN is presented in Figure 3.2.

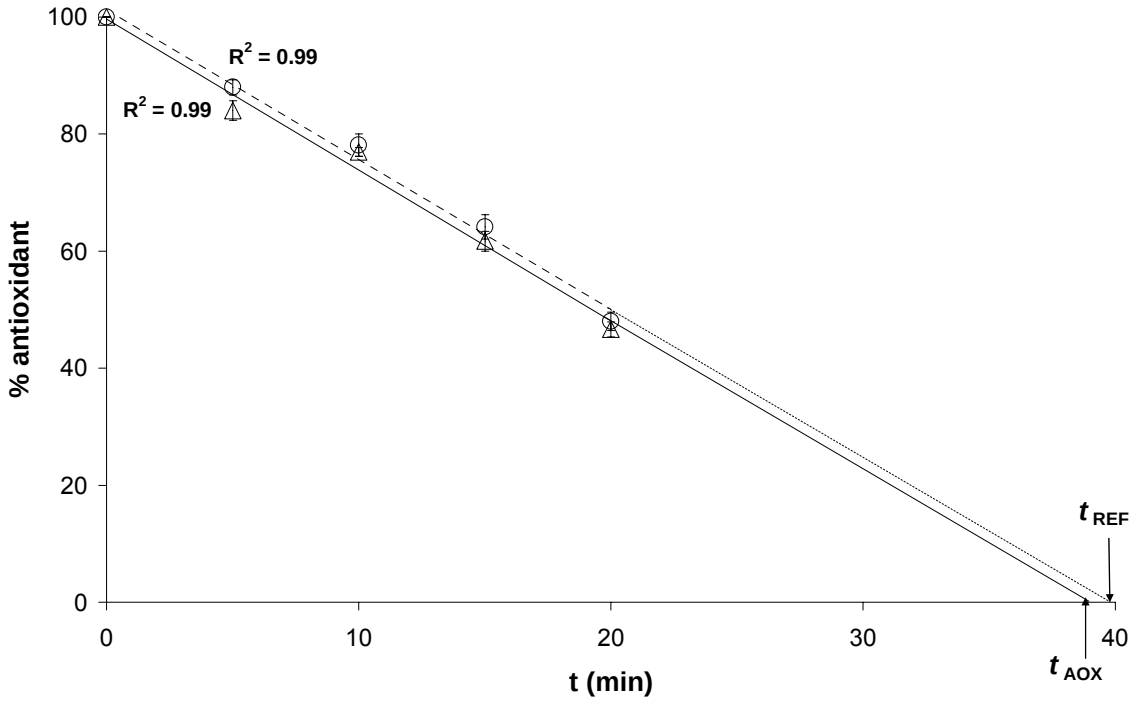
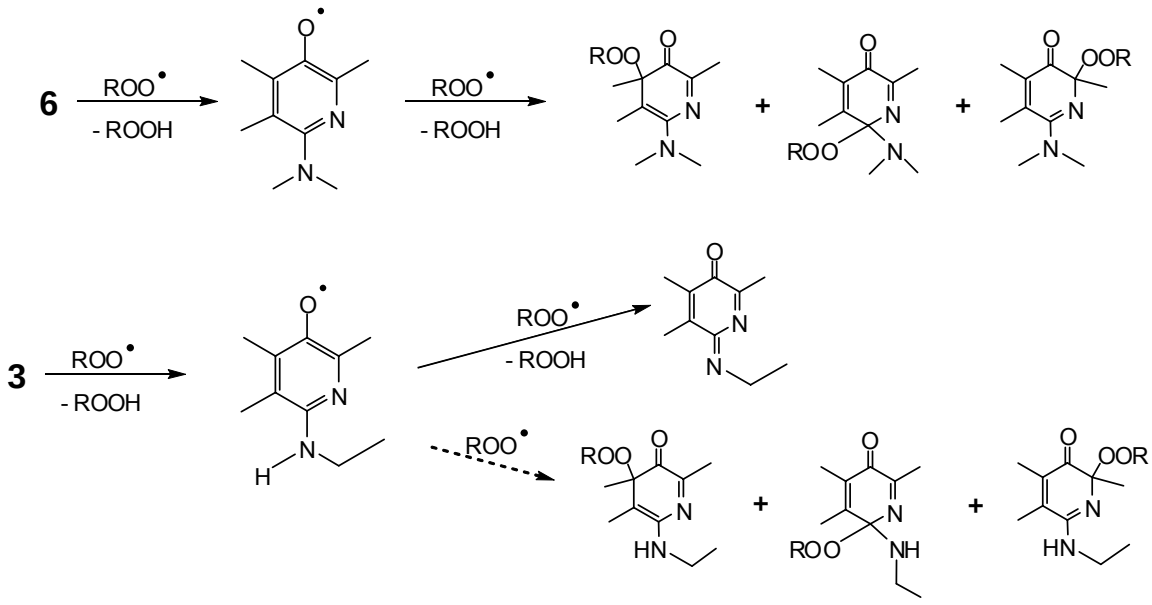


Figure 3.2. The disappearance of 0.1mM PyrOH **3** (triangles) and 0.1mM PMC (circles) in the presence of 2.0mM MeOAMVN in benzene at 37ºC. Linear trend lines for depletation of PyrOH **3** (solid) and PMC (dashed) are also presented.

As can be seen in Table 3.1, stoichiometric factors for all pyridinols tested are close to two and the conventional mechanism may be proposed to explain these results. When a pyridinol donates hydrogen atom to peroxyl radical it is converted to phenoxyl radical that could further react with another peroxyl radical by means of radical-radical coupling reaction to the *ortho*- or *para*- position of the aryloxyl radical.[18] Alternatively, antioxidants that have the ability to donate two hydrogen atoms (**1**-**5**, **8**) could scavenge two peroxyl radicals via two hydrogen atom transfers to yield imino-pyridinone products (Scheme 3.2).[7, 21] The fate of the pyridinols however, remains unknown at this time, since multiple

attempts performed to isolate or even characterize the products generated from these compounds in the mixture have failed.



Scheme 3.2. Proposed radical scavenging mechanisms for PyrOHs **3** and **6**.

Routine UV measurements performed on compounds under investigation revealed an interesting observation. Analysis of π-π* transition energies (via comparison of λ_{max} values found for compounds presented in Table 3.1) indicates that the transition energy for *N*,*N*-dialkylated 2-aminopyridin-5-ol **6** is higher than the corresponding energy for 2-alkylaminopyridin-5-ol **3**, and even higher than the energy for 2-aminopyridin-5-ol **1**. This is unexpected since dialkylamino substitution gives better electron donation than the monoalkylamino or amino analogues. It is therefore proposed that steric interactions exist between the 3-methyl substituent and 2-dimethylamino group of **6**, causing

a decrease in the dihedral angle between the 2-nitrogen's p-type lone pair and the p-orbital of the adjacent aromatic carbon. This can be simply visualized as a twist of the nitrogen's lone pair out of the plane of best interaction between p-orbitals (Figure 3.3).

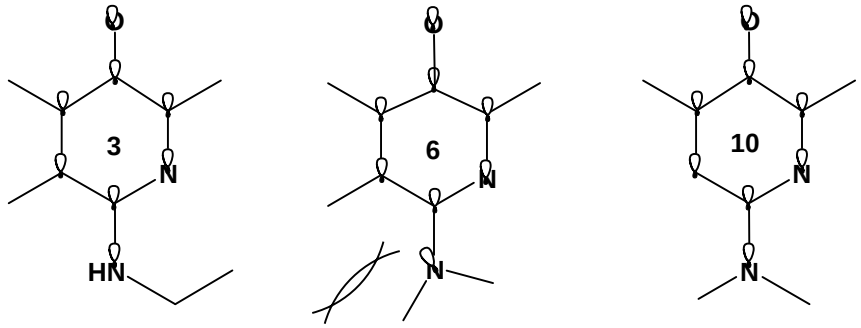


Figure 3.3. An overlap of orbitals in compounds **3**, **6** and **10**.

Such steric interactions do not exist in the case of compounds that have a hydrogen atom substituted in the 3-position of the aromatic ring (**10**) or have at least one hydrogen atom bonded to the nitrogen atom of the 2-amino substituent (**1** and **3**). This twisting of **6** is further supported by the fact that the ring nitrogens of **3**, **1** or **10** are more basic than the corresponding nitrogen of **6** (*vide infra*).

Since the stronger the interactions between the 2-nitrogen's lone pair and an aromatic ring, the lower the OH-BDE of an 2-amino-pyridin-5-ol (*vide supra*), it was predicted that compounds **3** and **10** should donate hydrogen atoms much more readily than PyrOH **6**. Measurements recently carried out in our laboratory gave k_{inh} values for **3** ($1.4\pm0.2 \times 10^7$ $M^{-1}s^{-1}$), **6** ($1.6\pm0.3 \times 10^6$ $M^{-1}s^{-1}$) and **10** ($1.7\pm0.2 \times 10^7$ $M^{-1}s^{-1}$) that were consistent with that prediction.[22] Overall,

the experiments described in this section suggest that *N*-monoalkylated 2-amino-3,4,6-pyridin-5-ols are better hydrogen atom donors than their *N*,*N*-dialkylated analogues, hence they are better antioxidants (at least in a non-polar environment).

Properties in aqueous media

The stability of selected water soluble pyridinols in the presence and absence of a radical azo-initiator was investigated in phosphate buffered saline (PBS) at physiologically relevant pH = 7.4. Experiments were conducted using the same general protocol that was applied in non-polar solvent. The key changes include the usage of a different initiator, AIPH instead of MeOAMVN,[23, 24] and a different reference hydrogen atom donor Trolox in place of PMC. The results of air stability tests together with *n* values found for the representative pyridinols **1**, **3**, **6** and **8**-**10** are presented in Table 3.2. Again, compounds may be divided into two categories, stable for over 8h (all monocyclic antioxidants) and unstable (bicyclic pyridinols). The bicyclic compounds decayed linearly throughout the incubation and disappeared within a few hours, whereas monocyclic pyridinols retained their initial concentrations for at least 8h of incubation. Water, a solvent that is known to favor ionic reactions by stabilization of charged transition states is presumably an excellent medium for a rapid electron transfer (ET) from an antioxidant or phenoxyl radical to molecular oxygen, whereas aprotic benzene is not likely to facilitate such reactions. That is likely why compounds **8** and **9** are far less stable in aqueous media than in non-

polar organic solvent. Monocyclic pyridinols, on the other hand, are much less electron rich, compared to bicyclic compounds (*vide supra*) and therefore they do not readily undergo autoxidation even in an aerated aqueous environment.

Table 3.2. Some properties of the representative pyridinols in aqueous media.

Compound	Air stability [h][a]	n[b]	pK_a[c]	$E_{p,ox}$ [V][d]
1	≥ 8	1.4	6.9	0.16 (R)[g]
3	≥ 8	0.92	7.3	0.12 (R)[g]
6	≥ 8	1.4	6.2	0.18
8	0 (4.0×10^{-9} M/s)[e]	0.48 (0.58)[f]	7.5	0.14 (R)[g]
9	0 (9.2×10^{-9} M/s)[e]	0.23 (0.28)[f]	7.5	0.11
10	≥ 8	0.90	7.2	0.16

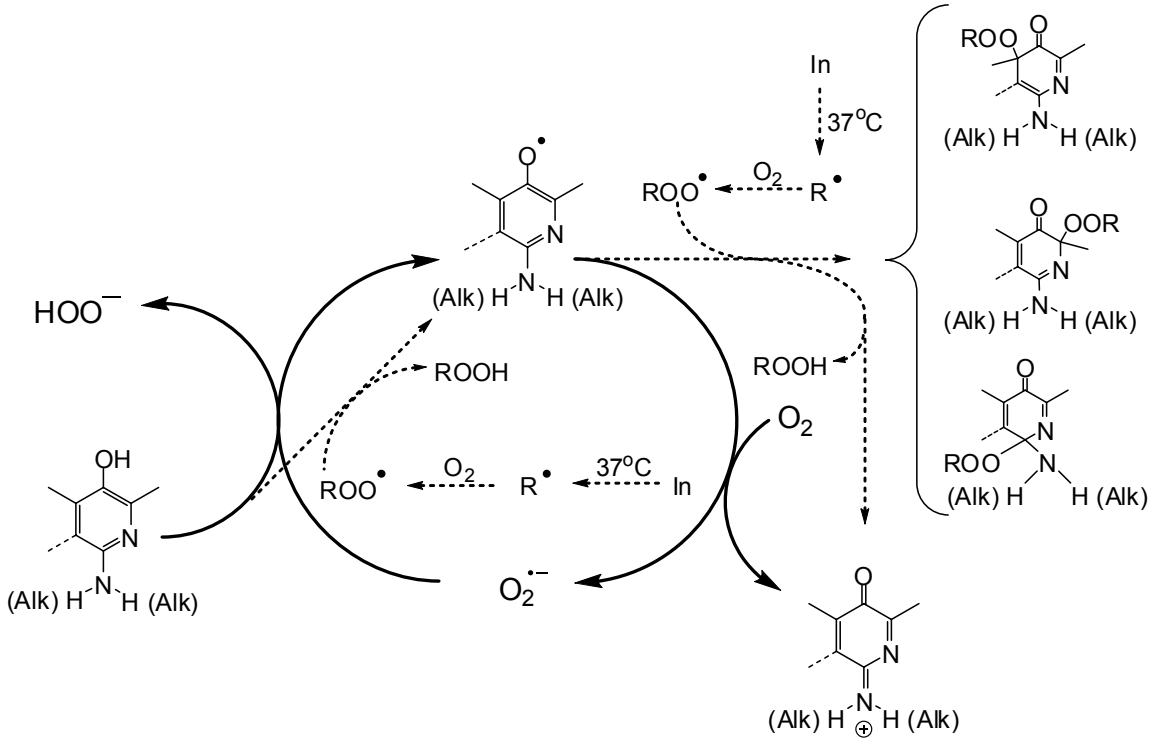
[a] Time period during which no consumption of the compound occurred in PBS pH 7.4 at 37ºC, based on HPLC-ECD measurements. [b] Stoichiometric factors in PBS pH 7.4 at 37ºC, calculated with respect to Trolox (n = 2.0), based on HPLC-ECD measurements. [c] The pK_a value for the protonated form of the pyridinol measured in 25% aqueous solution of methanol at 25ºC. [d] The 2e oxidation peak's maximum reported with respect to Ag/AgCl electrode, measured in degassed phosphate buffer at 25ºC. [e] The rate of autoxidation of the compound is presented in parenthesis. [f] Values in parentheses are corrected for autoxidation [g] The R indicates reversible process, two 1e reduction peaks were observed in each case (vide infra).

Stoichiometric factors for pyridinols dissolved in aqueous buffers, calculated with respect to Trolox (n = 2.0), were smaller than expected.[8] As can be seen in Table 3.2, all n values were less than 2 suggesting that initiator-

derived peroxyl radicals were not the exclusive hydrogen atom acceptors in these systems. It was proposed previously that electron rich phenols undergo autoxidation in the presence of molecular oxygen dissolved in the reaction solvent (*vide supra*). This may be the case for unstable compounds **8** and **9**, but unlikely for much less electron-rich monocyclic antioxidants shown in Table 3.2. The proposed alternative mechanism for autoxidation of pyridinols in the presence of radical generator in aqueous media is illustrated in Scheme 3.3.

In the presence of radical initiator peroxyl radicals are formed. These species are scavenged by a pyridinol to form hydroperoxide and phenoxyl radicals. It is proposed that the phenoxyl radical could either scavenge another peroxyl radical or transport an electron to molecular oxygen to form superoxide and thermodynamically stable pyridinoneiminium salt. The concentration of oxygen is relatively high in the system, whereas peroxyl radicals are generated rather slowly.[23, 25] Therefore, the reduction of oxygen by pyridinol derived radical should be favored. Superoxide radical anion could then dismute (*vide supra*) or it could be reduced to hydroperoxide anion in reaction with the pyridinol. The concentration of superoxide in the system is much smaller than the concentration of the pyridinol. Therefore, it is more probable that the latter reaction occurs, especially since it was shown that the rate constants for both types of transformations are similar.[26, 27] The stronger π-electron-donating interactions of *p*-amino group with the remaining molecular π-system, the better the charge stabilization in the pyridinoneiminium cation, hence the more likely it forms. Furthermore, the more electron rich the PyrOH, the easier the reaction with

superoxide should occur. That is the reason why the most active pyridinols are most readily consumed in aqueous media. Extremely electron rich compounds **8** and **9** were depleted even in the absence of radical initiator (most likely via their reaction with O_2). However, in the presence of AIPH autoxidation of bicyclic pyridinols was about four times faster than their disappearance without added initiator, suggesting that AIPH induced autoxidation was a predominant process. The solvent effect on autoxidizability of active pyridinols must be emphasized, by comparing *n* values measured in benzene and PBS it is clear that a polar environment is necessary for the autoxidation to occur.



Scheme 3.3. The proposed mechanism for the disappearance of pyridinols in aqueous media.

It should be noted that the mechanism presented in Scheme 3.3 has not yet been directly validated. Attempts to detect superoxide or hydrogen peroxide in the reaction mixture failed due to poor specificity of analytical assays currently available. Cytochrome *c* assay, epinephrine assay, Cypridina luciferin analog chemiluminescence assay, and number of colorimetric assays for HOOH detection were found to be incompatible with a system containing so many different redox active species.[28-33] In addition, products of oxidation of the investigated pyridinols in aqueous environment could not be identified. Currently, some toxicological experiments in cell cultures are being conducted on these and related molecules, presenting an opportunity to resolve this issue.

The autoxidation mechanism presented here is similar to one suggested for hydroquinols.[26, 27, 34, 35] It was proposed that a *p*- or *o*-hydroxyphenoxyl radical, formed upon formal hydrogen atom transfer from a hydroquinol, reacts with oxygen to form a *p*- or *o*-quinone and superoxide. However, no direct evidence for the presence of superoxide in such system was presented. This lack of confirmatory experiments is most likely due to the already mentioned unavailability of a suitable analytical method.

Another important factor in the determination of antioxidant properties for pyridinolic compounds is the basicity of their aromatic ring nitrogen atoms. It is crucial for pyridinols to have their ring nitrogen mostly deprotonated in the environment of action in order to act as powerful hydrogen atom donors. If pyridinols were to be used as antioxidants *in vivo*, pK_a values for their ring nitrogens should not be greater than 7.4 allowing a majority of antioxidant

molecules to be active. The experiment revealed the pK_a values for monocyclic pyridinols were smaller than 7.4, whereas bicyclic compounds gave the dissociation constant above the physiological pH value (Table 3.2). Therefore, it can be concluded that monocyclic PyrOHs will be predominantly deprotonated, whereas bicyclic pyridinols will be predominantly protonated under the relevant conditions. One might consider increased protonation of PyrOHs **8** and **9** at pH 7.4 as a mechanism for sparing these compounds from autoxidation. However, the observed fast disappearance of these pyridinols from aqueous media indicates that such sparing is rather ineffective. The phenolic pK_a values could not be obtained from the titrations performed, possibly due to the rapid autoxidation of phenoxyl anions. The phenolic pK_a reported for 3-pyridinol was equal to 8.7,[36] and since the pyridinols under investigation are more electron rich than the latter compound, it is believed that at physiological pH their hydroxyl groups are predominantly protonated facilitating H-atom transfer from these antioxidants.[36, 37] A sample titration curve is shown for compound **3** in Figure 3.4.

It was previously suggested that pyridinols or their mono-radical forms are able to transfer electrons to molecular oxygen or superoxide (*vide infra*). It should also be mentioned that pyridinols are able to reduce metal ions at higher oxidation states. It was shown that compounds **1** and **3** (reductive properties of other pyridinols presented in this chapter have not been tested) effectively reduce ferryl myoglobin to its ferric form in a dose dependent manner. Because of their reducing properties, and also their ability to scavenge peroxyl radicals, pyridinols might be considered as important inhibitors of some serious

pathologies, such as myoglobinuric lipid peroxidation associated with rhabdomyolysis.[38] It was also found that ferric cytochrome *c* is reduced to its ferrous form in the presence of PyrOHs **1** and **3** in a dose-dependent manner. It is however unclear at this point what *in vivo* relevant consequences that reduction of cytochrom *c* may yield. It was also suggested recently that 2-amino-pyridin-5-ols interact with heme containing enzymes, such as cyclooxygenases (*vide supra*).

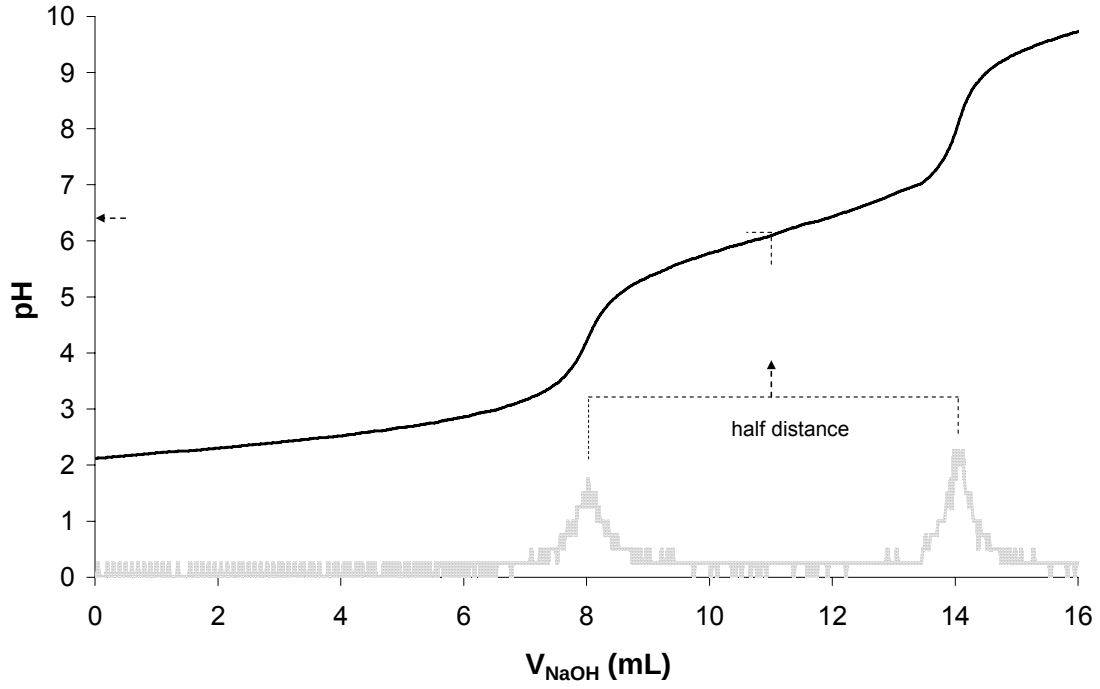


Figure 3.4. pH Titration curve (solid line) and its first derivative (dotted line) for 0.5mmol of PyrOH **3** dissolved in 10mL of aq. HCl (0.1 mM) containing 25% MeOH and titrated with 25 μL portions of aq. NaOH (0.1 mM) containing 25% MeOH. pKa Value was found from the half distance between two maximums of the first derivative reflected on the pH axis via titration curves (dashed lines).

On the other hand, in the environments of increased levels of free transition metal ions, the excellent reducing properties of these compounds may promote formation of ROS, analogously to the process observed in the presence of Fe^{3+} and ascorbate.[39] This issue is recommended for further exploration during the next phase of evaluation of the pyridinols.

Redox properties of pyridinols are also of interest from the perspective of interactions between these compounds with other natural antioxidants. As it was already mentioned, aqueous Asc^- as well as hydrophobic $CoQ_{10}H_2$ were found to be able to transform α-tocopheryl radical into α-TOH.[40, 41] The oxidation potential measured for α-TOH (0.48V ref. Ag/AgCl) was much higher than corresponding values found for Asc^- (0.28V) and CoQ_{10} (0.20V).[42] It was that difference in the oxidation potential values that made regeneration of α-TOH thermodynamically possible. Oxidation potentials were therefore measured for pyridinols **1**, **3**, **6** and **8**-**10** (Table 3.2) to predict if any redox reaction with these natural antioxidants is suitable. Sample cyclic voltammetry curves obtained for PyrOH **1** and **10** are shown in Figure 3.5. Theoretically, a pyridoxyl radical could be reduced in the presence of Asc^- or CoQ_{10}, and/or parent pyridinol could serve to regenerate α-TOH from its radical. Judging by the oxidation potential values obtained, only the latter interaction would be thermodynamically possible for all of the pyridinols under investigation.

Another interesting observation was that all of the compounds possessing at least one hydrogen on the 2-nitrogen atom (for example pyridinol **3**) underwent semi-reversible electrochemical processes, whereas the *N*,*N*-dialkylamino

PyrOHs (for example compound **10**) oxidized irreversibly. This result may be explained by formation of imino-pyridinones from the earlier group of compounds in two subsequent one electron processes with formation of semi-iminopyridinones, easily oxidizable intermediates. In the past similar reversible electrochemical processes have been observed for quinols.[43-45]

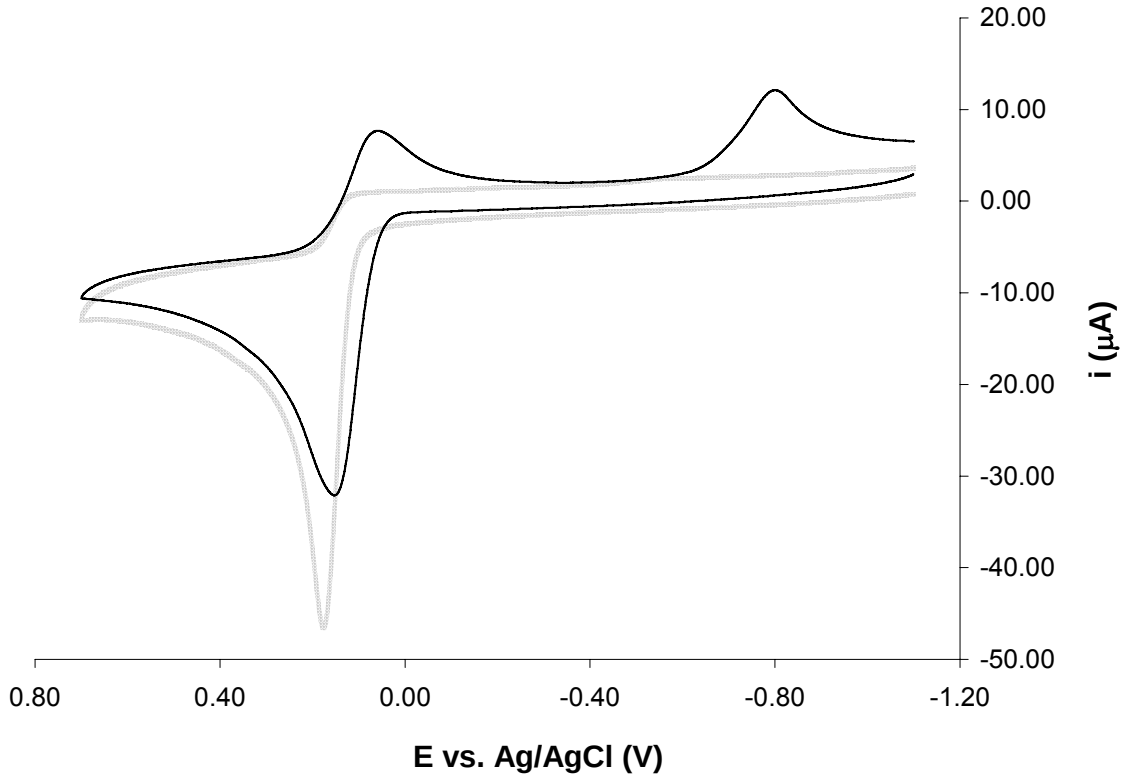


Figure 3.5. Cyclic voltammetry curves for 1mM PyrOHs **3** (solid line) and **10** (dotted line) dissolved in degassed 100mM phosphate buffer. Glassy carbon was used as a working, platinum as a counter, and Ag/AgCl as a reference electrode.

Unfortunately, even though multi-milligram scale electrochemical oxidations of PyrOHs **1** and **3** appeared to be nearly completed (bulk electrolysis on glassy

carbon electrode, 12-24h, 1.2V, with stirring), spectroscopic analysis (^{1}H NMR, ESI-MS) of the produced mixtures did not unambiguously support formation of the predicted products.

Properties in heterogeneous systems

Due to the fact that pyridinols behaved differently in a polar, aqueous environment than in non-polar, aprotic media, it was important to investigate their properties in a heterogeneous system. The heterogeneous system of choice consisted of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) multi-lamellar liposomes (MLV) suspended in PBS (pH 7.4) at 37ºC.[4, 8, 17] Water soluble AIPH acted as a radical initiator in the system, whereas antioxidants were applied to inhibit lipid peroxidation. For these experiments, both classes of pyridinols, highly water soluble (**1**, **3**, **6**, **10**), as well as water insoluble (**4**, **5** and **7**) were used. Also reference compounds belonging to each of these categories were utilized: Trolox and uric acid, and PMC and α-TOH, specifically. Bicyclic PyrOHs **8** and **9** were not studied due to their high instability in aqueous media.

Stoichiometric factors (*n*) and rates of oxidation during the inhibited phase ($R_{ox,\ inh}$) were extracted from plots of [PLPC-OOH] *versus* time. An example of such a plot is shown in Figure 3.6. The formation of PLPC-OOHs was monitored via HPLC-UV analysis of methyl linoleate alcohols (MeLin-OHs) obtained upon reduction of PLPC-OOHs with PPh_3 and trans-esterification with alkali methoxide in MeOH.[46] Information about the quantity (*n*) and quality ($R_{ox,\ inh}$) of the inhibition by pyridinols and reference antioxidants is collected in Table 3.3.

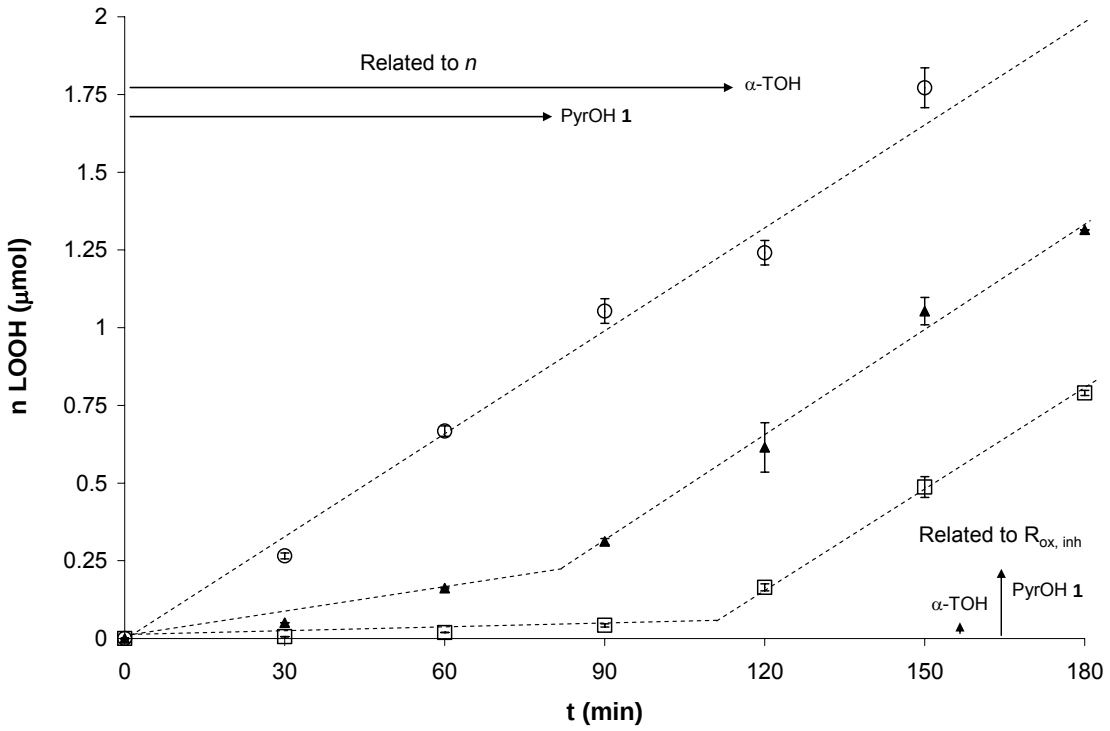


Figure 3.6. Formation of PLPC-OOH during oxidation of 26.6μmol of PLPC (MLV) suspended in PBS pH 7.4 (2mL) at 37°C initiated by 1.06μmol of AIPH: uninhibited reaction (circles), inhibited by 26.6nmol of PyrOH 1 (triangles) or α-TOH (squares). Dashed lines and solid arrows demonstrate how the relevant information can be extracted from the plots.

Hydrogen atom donors in these systems were consumed in reactions with initiator derived and lipid chain-carrying peroxyl radicals, and potentially in autoxidation reactions. In an early stage of oxidation, when antioxidants were present in the system, relatively small rates of PLPC-OOHs formation were observed, followed by a rapid PLPC oxidation taking place after consumption of antioxidants. This phenomenon is illustrated in Figure 3.6, where oxidation profiles of systems containing α-TOH and PyrOH **1** are compared.

Table 3.3. Antioxidant properties of pyridinols in PLPC liposomes (MLV).

Compound	$R_{ox,\ inh}$ [nmol/min][a]	n[b]
1	1.9	1.3
3	0.24	1.4
4	0.10	2.2
5	0.12	2.1
6	0.23	1.6
7	0.24	2.0
10	0.18	1.4
Uric acid	1.9	2.0
Trolox	0.23	2.1
PMC	0.22	2.0
α-TOH	0.24	2.0
n/a	17	n/a

[a] The rate of oxidation during the inhibited phase based on the formation of PLPC-OOHs analyzed as MeLin-OHs by UV-HPLC. Composition of the mixture: PLPC (26.6μmol), AIPH (1.06μmol), antioxidant (26.6nmol), PBS pH 7.4 (2mL).
[b] Stoichiometric factors in PLPC (MLV) / PBS pH 7.4 suspension at 37ºC calculated with respect to α-TOH (n = 2.0) based on the formation of PLPC-OOHs analyzed as MeLin-OHs by UV-HPLC.

Stoichiometric factors for antioxidants could be easily found from lengths of lag-period preceding the rapid phase of the peroxidation (Figure 3.6). All of the n factors obtained for antioxidants in the heterogeneous systems were calculated according to Eq. 24, where t_{AOX} was the lag-period found in the presence of

investigated antioxidant and t_{REF} was the lag-period found in the presence of α-TOH (n = 2.0).[11, 21]

It is notable (Table 3.3) that, except for compound **1**, peroxidation rates in the presence of pyridinols are similar or smaller than the corresponding rates obtained in the presence of reference phenolic antioxidants (Trolox, PMC, α-TOH). It is proposed that pyridinol **1** and urate have a limited access to chain-carrying lipid peroxyl radicals as well as to a part of initiator derived peroxyl radicals that diffused into lipid phase,[13, 47] and as a result relatively higher peroxidation rates were observed in the presence of these compounds. It is believed that nearly all of the radical scavenging processes performed by these antioxidants took place in an aqueous environment. It was previously shown that urate is extremely hydrophilic and its presence had a rather small impact on processes localized in phospholipid bilayers.[13, 47] On the contrary, more lipophilic phenols are able to penetrate the lipid more efficiently, therefore protecting oxidizable substrates better.[8, 48]

A distribution of compound **1** between aqueous phase and the bilayer was studied and revealed that under experimental conditions over 92% of the antioxidant molecules were present in the aqueous phase, suggesting high hydrophilicity of this pyridinol. Distribution between the phases was also measured for Trolox and PyrOH **3**, for which a relatively high solubility in aqueous media have been observed on various occasions.[8, 49] It was found that 25% of Trolox and 52% of compound **3** partitioned into the lipid phase. These measured partition properties indicate better accessibility of Trolox and

pyridinol **3** to radicals localized inside the bilayer and therefore better protection against oxidation for PLPC.

Stoichiometric factors found for pyridinols in the heterogeneous systems were generally greater than values measured in an aqueous buffer. A close look at these values revealed that the distribution of antioxidant between the two phases had an important effect on inhibition of lipid peroxidation not only in a qualitative but also a quantitative sense. Although the distribution among compounds having very short *N*-alkyl chains was investigated only for compound **3**, it is reasonable to predict that due to close proximity of the calculated LogP values (2.5-3.0),[50] PyrOHs **10** and **6** will partition between liposomes and aqueous buffer similarly to compound **3**. The increase in *n* factors observed in heterogeneous system, compared to the values measured in PBS, can be explained simply by the partition of antioxidant between layers. If pyridinols are partially present in lipid phase, their abilities to autoxidize decreases, therefore their stoichiometric factors increase. Among compounds that share stereo-electronic properties (for instance **3**-**5** or **6** and **7**) antioxidants that partitioned into the lipid phase significantly (**4**, **5** and **7**) had greater *n* values than compounds that partitioned into both layers (**3** and **6**). Furthermore, pyridinol **1** in liposomal suspension gave an *n* factor close to the value measured in PBS, most likely because it partitioned almost exclusively into the aqueous phase. All of the observations described above show that partition between phases is one of the most important parameters to consider in investigating properties of pyridinols in heterogeneous systems.

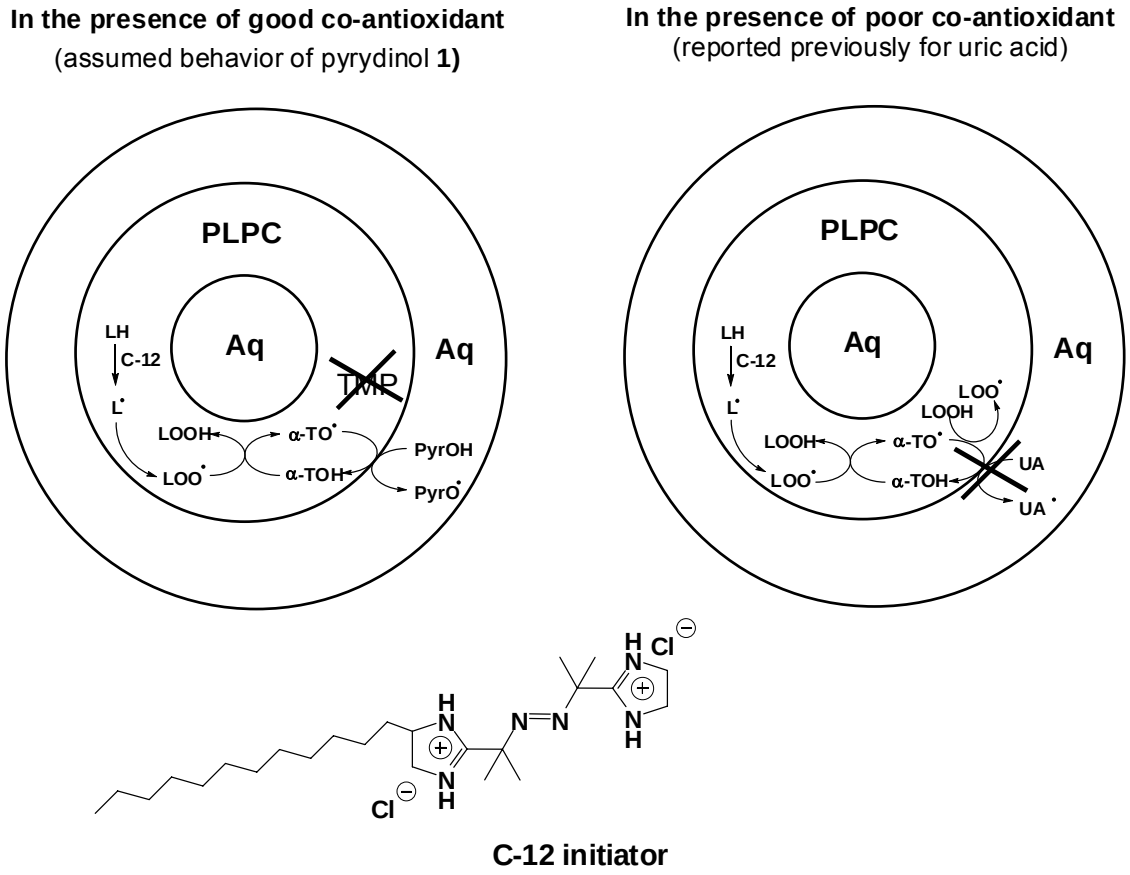
Additional series of experiments were conducted in order to evaluate if pyridinols act as co-antioxidants and convert tocopheryl radical into its precursor. It can be seen in Table 3.3 that peroxidation of PLPC was slower in the presence of α-TOH, than it was in the presence of pyridinol **1** or urate. This is most likely due to the relatively high lipophobicity of these compounds that decreases their accessibility to lipid peroxyl radicals (*vide supra*). An application of a lipid associated radical azo-initiator, instead of water soluble AIPH, should therefore additionally diminish antioxidant properties of these water soluble compounds compared to lipophilic α-TOH, hence their co-antioxidant properties will be easier to investigate. It can be predicted that when mixture of both types of antioxidants is introduced into PLPC (MLV) suspension containing such initiator, the lipophilic hydrogen atom donor (α-TOH) should be consumed first if an aqueous antioxidant does not react with tocopheryl radicals, or the water soluble scavenger should be consumed initially if it is able to act as co-antioxidant. Additionally TMP should occur in the absence, but be diminished in the presence of a good co-antioxidant (Scheme 3.4). An asymmetric azo-initiator, 2,2'-azo [2- (2-imidiazolin-2-yl) propane] {2- [2- (4-n-dodecyl) imidazolin-2yl]propane} dihydrochloride (C-12), presented in scheme 3.4, was chosen for use in these experiments since it was shown to partition almost completely into the lipid phase and react with α-TOH, but poorly with aqueous urate in soy lecithin (MLV) suspension.[47] The disappearance of equimolar mixtures of antioxidants from PLPC (MLV) suspended in PBS in the presence of C-12 azo-initiator was monitored by HPLC-ECD, whereas formation of PLPC-OOH

was monitored via HPLC-UV analysis of MeLin-OHs (*vide supra*). The results are presented in Figures 3.7 and 3.8.

It can be seen in Figure 3.7 that α-TOH is consumed first in the co-oxidation with uric acid. Therefore it is concluded that this aqueous scavenger is not a good co-antioxidant. The observed small protective effect of urate on α-TOH was associated with its ability to scavenge a portion of the radicals generated by the unsymmetrical azo-initiator that diffused out of the lipid bilayer. The possibility of scavenging the radicals derived from that initiator by uric acid is not in agreement with a previous report,[13] but it can be rationally explained. The C-12 initiator is asymmetric hence it generates both lipophilic and hydrophilic radicals. It is possible that a fraction of the hydrophilic species manage to escape into the aqueous phase where they may be scavenged by aqueous antioxidants. In the absence of a water soluble scavenger however, these aqueous radicals may diffuse back into the bilayer to initiate peroxidation or be scavenged by lipophilic antioxidant.

In the mixture containing pyridinol **1** and α-TOH the order of consumption of antioxidants was quite different (Figure 3.8). The pyridinol was consumed first and the initial concentration of α-TOH was preserved until scavenger **1** was depleted, suggesting strong co-antioxidant activity of the water soluble compound. In both antioxidant mixtures, the length of the lag-phase was closely correlated with the disappearance of α-TOH from the system. The rates of PLPC (MLV) peroxidation ($R_{ox,\ inh}$) initiated by the C-12 azo-initiator in the presence of

radical scavengers and their mixtures are collected together with stoichiometric factors found for these antioxidants (Table 3.4).



Scheme 3.4. TMP *vs.* co-antioxidant properties of aqueous radical scavengers.

The *n* value for α-TOH in the mixture with urate was found slightly higher than the corresponding value measured in the absence of the aqueous antioxidant. This phenomenon was explained by the ability of urate to scavenge radicals that diffused into the aqueous phase. Stoichiometric factors measured for aqueous antioxidants, **1** and uric acid were, interestingly, quite different from

one another. Due to a nearly exclusive (**1**) or complete (urate) partition of these antioxidants into aqueous phase, their scavenging interactions were very likely limited only to a fraction of radicals that diffused out of lipid bilayer.

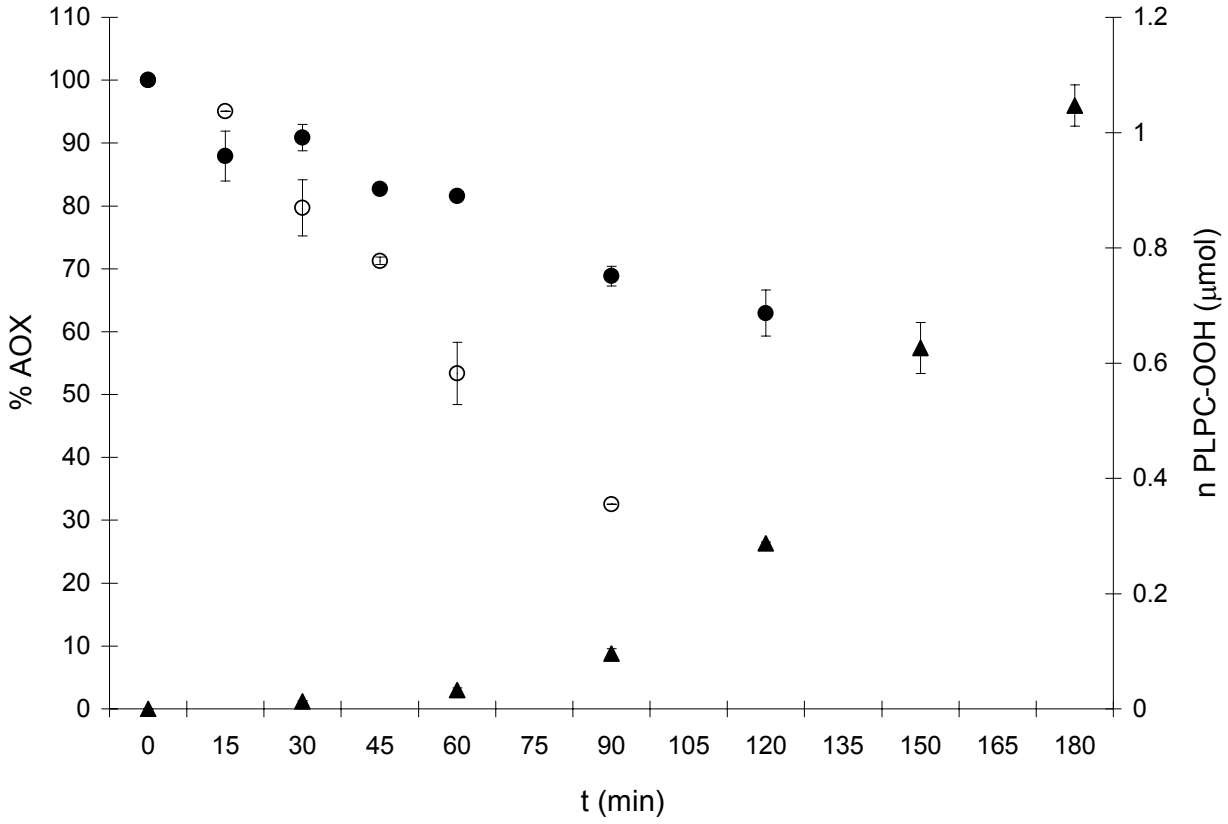


Figure 3.7. The disappearance of the antioxidant mixture: 13.3nmol of α-TOH (open circles) and 13.3nmol of uric acid (closed circles), and formation of PLPC-OOH (closed triangles) during peroxidation of 26.6μmol of PLPC (MLV) suspended in PBS pH 7.4 (2mL) at 37°C initiated by 2.12μmol of lipid associated C-12 azo-initiator.

A relatively large *n* factor calculated for urate is in agreement with its limited scavenging ability in the system, whereas an unusually low *n* value found for

compound **1** was associated with increased autoxidazibility of the pyridinol under given conditions. In the heterogeneous systems, hydrophilic radicals generated from the C-12 initiator had to undergo an interface diffusion process to be found in an aqueous phase.

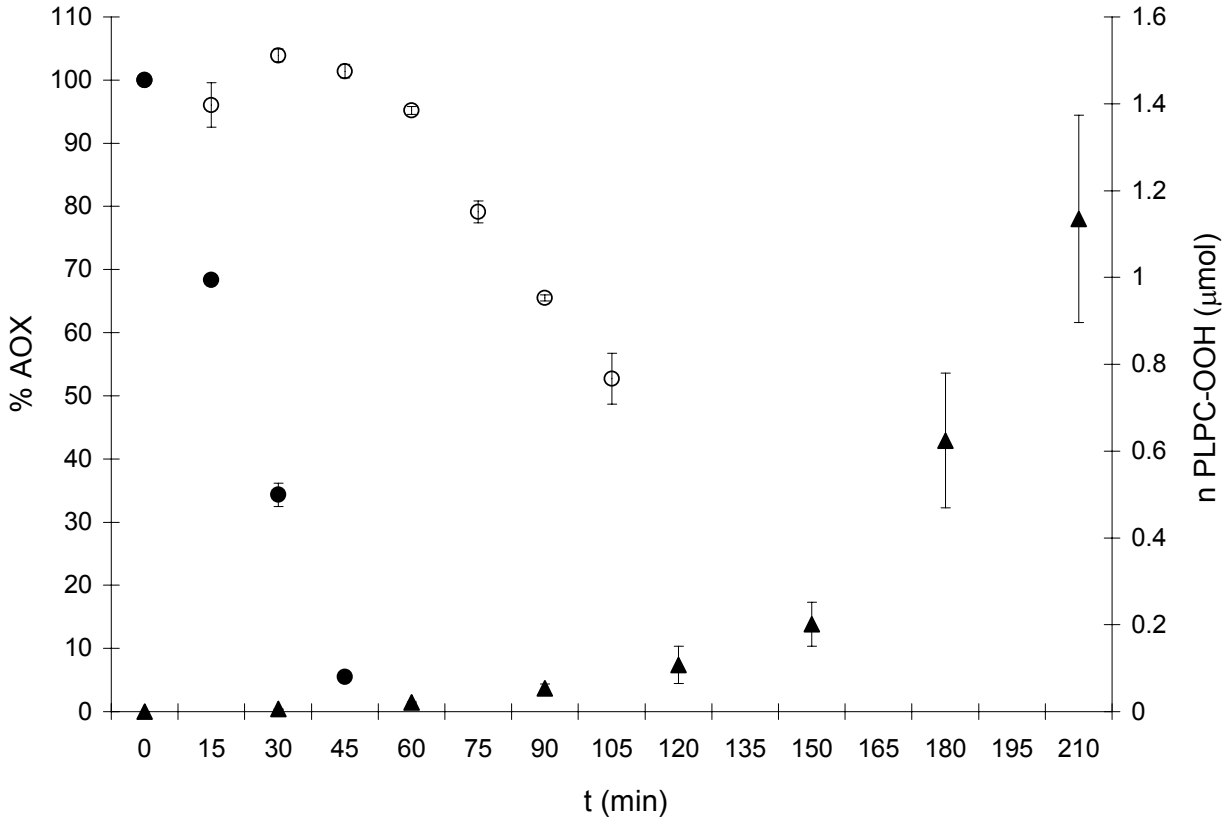


Figure 3.8. The disappearance of the antioxidant mixture: 13.3nmol of α-TOH (open circles) and 13.3nmol of compound **3** (closed circles), and formation of PLPC-OOH (closed triangles) during peroxidation of 26.6μmol of PLPC (MLV) suspended in PBS pH 7.4 (2mL) at 37°C initiated by 2.12μmol of lipid associated C-12 azo-initiator.

The effective concentration of radicals in the buffer was therefore relatively low. Hence, the reactivity of pyridoxyl radicals with initiator derived radicals was decreased and the competitive self-wasting reactivity of pyridoxyl radicals with oxygen was enhanced (Scheme 3.3).

Table 3.4. PLPC (MLV) oxidizibility in the presence of antioxidant mixtures.

Compound	$R_{ox,\ inh}$ [nmol/min][a]	*n* (dLOOH/dt)[b]	*n* (dAOX/dt)[c]
1	1.4	1.0	0.9
UA	17	N/D[d]	4.5
α-TOH	0.28	2.0	2.0
α-TOH + **1**	0.17	3.3	3.3
α-TOH + UA	0.27	2.6	2.5[e]
no antioxidant	22	N/A	N/A

[a] The oxidation rate during the inhibited phase, calculated based on initial time points (at least 4) obtained via measurements of formation of PLPC-OOHs (UV-HPLC analysis of MeLin-OHs). Composition of the mixture: PLPC (26.6μmol), C_{12} azo-initiator (2.12μmol), H-atom donor (13.3nmol of single antioxidant or 13.3nmol of each in the mixture of two), α-TOH (13.3nmol, if used), PBS (2mL). [b] Stoichiometric factors in PLPC (MLV) / PBS pH 7.4 at 37ºC, calculated with respect to α-TOH (*n*=2.0), based on the formation of PLPC-OOHs analyzed as MeLin-OHs by UV-HPLC. [c] Stoichiometric factors based on disappearance of antioxidants from the system, reported with respect to α-TOH (*n*=2.0), based on HPLC-ECD measurements. [d] No distinctive lag period was found, however, a small decrease in lipid peroxidation rate was observed. [e] The *n* value based on disappearance of α-TOH only (consumption of urate was not counted).

The cumulative *n* factor calculated for a mixture of α-TOH with compound **1** was found to be close to the sum of the corresponding values measured for individual antioxidants, which is in agreement with expectations.

When radicals were generated inside the lipid bilayer, lipophilic scavengers had relatively easy access to a majority of these radicals, whereas water soluble antioxidants reacted mainly with radicals that escaped into the aqueous phase. This is the reason why α-TOH protected PLPC (MLV) against peroxidation much more efficiently than aqueous urate or compound **1**. The difference between rates of the peroxidation in the presence of urate and pyridinol **1** could be also explained by a difference in distribution of these compounds between the phases of the heterogeneous system. More lipophilic scavenger **1** would have slightly better access to lipid chain-carrying peroxyl radicals than extremely hydrophilic urate. Therefore, it would better protect the lipid bilayer from peroxidation.

It would be expected that two antioxidants introduced as a mixture into an oxidizable system undergoing peroxidation would compete for radicals to scavenge them. The rates of peroxidation in the presence of α-TOH alone and as a mixture with urate were similar, suggesting that location of α-TOH caused it to unconditionally win the competition with the aqueous scavenger. On the other hand, addition of pyridinol **1** to α-TOH significantly decreased the rate of the formation of PLPC-OOHs. It is proposed that due to its relatively poor ability to scavenge lipid peroxyl radicals (Table 3.3), compound **1** did not decrease the rate of peroxidation acting as antioxidant, but it rather inhibited tocopherol

mediated peroxidation by transferring a formal hydrogen atom to tocopheryl radical. The anti-TMP activity of pyridinol **3,** observed as a result of good co-antioxidant properties of this compound, could be potentially associated with all of the pyridinols presented in this manuscript. All of these compounds have oxidation potential lower than α-TOH, and all of the remaining pyridinols are more electron rich than compound **1** (maybe except for **6** and **7**). Therefore, they should react with tocopheryl radical even more readily. It was, however, convenient to investigate highly hydrophilic compound **1**, instead of other more lipophilic pyridinols . To distinguish if amphiphilic pyridinols acts via radical export mechanism alone or in combination with radical import, additional studies should be conducted. At the current level of our knowledge, it seems unlikely that highly oxidizable pyridoxyl radicals (*vide supra*) survive in aqueous media long enough to be diffused onto the surface of another lipid aggregate.

Experimental

General. Trolox, δ-TOH, α-TOH, PMC, uric acid, *p*-aminophenol, *p*-methoxyphenol and *p*-methylphenol, 2,6-di-*t*-butyl-4-methylphenol (BHT) were purchased from Sigma Aldrich and purified (except for uric acid) by flash column chromatography prior to use. Monocyclic pyridinol **10** was prepared as previously described,[37] whereas bicyclic PyrOHs **8** and **9** were prepared utilizing methodology recently developed in our laboratory to obtain closely related PyrOHs.[36] AIPH and MeOAMVN was purchased from Wako Chemicals and used without further purification. 2,2'-Azo[2-(2-imidiazolin-2-yl)propane]{2-[2-(4-

ndodecyl imidazolin-2-yl]propane} dihydrochloride was synthesized by a former group member from AIPH according to previously published procedure.[47, 51] All four isomers of conjugated MeLin-OHs were purchased from Cayman whereas all inorganic chemicals were purchased from Sigma Aldrich. An ion pairing cocktail containing 0.5M solution of dodecyl triethylammonium phosphate was obtained from Regis Technologies, Inc. (Morton Grove, IL). Phosphate-buffered Saline (PBS, pH 7.4, 50 mM) was prepared from NaCl (8.00 g), Na_2HPO_4 (5.00 g), NaH_2PO_4 (1.96 g) in deionized water (1.0 L) followed by adjustment of the pH to 7.4 with 10M aqueous NaOH. Phosphate buffer (pH 7.4, 100mM) was prepared from Na_2HPO_4 (10.00 g) and NaH_2PO_4 (3.92 g) in deionized water (1.0 L) followed by adjustment of the pH to 7.4 with 10M aqueous NaOH. Both solutions were stored over Chelex 100 resin (*ca*. 3 g / L) for 24 h, then the resin was removed by filtration and the filtrate was stored at 5ºC. Benzene (HPLC grade) was passed through column of neutral alumina and stored over molecular sieves. PLPC was purchased as a solution in chloroform (25mg/mL) from Avanti Polar Lipids. After aliquots (20mg) were withdrawn from the solution, they were kept under argon at -40ºC until used in experiments (no later than 5 days after aliquoted). UV-spectra of antioxidants were recorded on Helwett Packard HP8453A spectrometer. HPLC analysis was performed with Waters 600 Controller, 717 Autosampler, Waters 996 photodiode array detector and electrochemical detector (ECD; LC-4C, from BAS). All HPLC Analyses of phenols and pyridinols were carried out on a single Supelco 5 μm Discovery C-18 column (4.6 mm x 25 cm) using reverse phase conditions (mixture of MeOH

and water, both containing $LiClO_4$ (1.06g/L) and pyridine (2mL/L)). A composition of solvents and speed of an isocratic elution depended on the antioxidant's structure: 1mL/min, 30%MeOH/H_2O (**1**); 1mL/min, 45%MeOH/H_2O (**3**, **6**, **8**-**10** and Trolox); 1.25mL/min, 70%MeOH/H_2O (**4** and PMC); 1.5mL/min, 100%MeOH/H_2O (**5** and α-TOH). HPLC analyses of uric acid were carried out on a single Supelco 5 μm Discovery C-18 column (4.6 mm x 25 cm) using reverse phase conditions (1mL/min, 0.54mM EDTA/5mM dodecyl triethylammonium phosphate/7.5% MeOH/40mM acetate buffer pH 4.75. *p*-Methoxyphenol (**1**), *p*-methylphenol (**3**, **6**, **8**-**10**, Trolox and PMC), *p*-aminophenol (uric acid) and δ-TOH (α-TOH) were used as internal standards in HPLC-ECD analyses of antioxidants. The electrochemical detector was set for all analyses on 500mV (except for uric acid: 650mV). The working electrode was cleaned regularly with nonionic detergent, washed with water and dried under argon stream. All HPLC analyses of MeLin-OHs were carried out on single Beckman 5μm ultrasphere column (4.6mm x 25cm) using normal phase conditions (1.5mL/min, 0.5%IPA/Hex, 30min). Cinnamyl alcohol was applied as an internal standard in these HPLC-UV analyses. pH Titration curves were obtained using Corning pH meter 340 equipped with Beckman calomel electrode.

Stability of antioxidants in aerated benzene. Antioxidants (1.00 μmol) in 10mL of benzene were placed in sand bath at 37^{o}C. Aliquots (400 μL) were withdrawn every 20min for 3h (**8** and **19**) or every 8h for 48h (**1**, **3**, **6** and **10**) and added to an ice cold mixture of BHT and $P(OMe)_3$ (2.0μmol each) in benzene

(100 μL). Internal standards were added in 80μL of benzene and the mixtures were vortexed for 30 seconds and solvent was blown off under a stream of argon. The residue was dissolved in 65% MeOH/H_2O (150μL) and placed in the HPLC autosampler at 5^oC prior to sample injection on HPLC. Information about stability was extracted from charts: [antioxidant] *versus* time.

Stability of antioxidants in aerated PBS. Experiment were conducted as in benzene, except aliquots were withdrawn every 1h for 8h (**1**, **3**, **6** and **10**) or every 15min for 2h (**8** and **9**). Compounds were incubated in PBS pH 7.4 (50mM) and a mixture of BHT and $P(OMe)_3$ (2.0μmol each) was added in MeOH (500μL). Internal standards were also added in MeOH and no solvent evaporation prior to analysis was required.

Oxidation of antioxidants in benzene in the presence of MeOAMVN. Experiments were conducted similarly to stability measurements in benzene, except antioxidants were incubated in the presence of MeOAMVN (20μmol) and aliquots were withdrawn every 5min for 20min. The time periods needed to consume antioxidants were extracted from charts: [antioxidant] *versus* time.

Oxidation of antioxidants in PBS in the presence of AIPH. Experiments were conducted similarly to stability measurements in PBS, except antioxidants were incubated in the presence of AIPH (20μmol) and aliquots were withdrawn every 15min for 90min (**1**, **3**, **6** and **10**) or every 5min for 25min (**8** and

9). The time periods needed to consume antioxidants were extracted from charts: [antioxidant] *versus* time.

Oxidation of PLPC (MLV) in PBS in the presence of AIPH. Antioxidants (26.6nmol) in methanol (100μL) were added to PLPC (20mg) in chloroform (800μL). Solvents were blown off under a stream of argon to form thin films. PBS (1.90mL) was added to those films followed by an addition of AIPH (1.06 μmol) in PBS (100μL). Samples were vortexed for 2min and incubated at 37ºC for 4h. Every 30min, aliquots (200μL) were withdrawn, vortexed (20s) with an ice cold BHT/PPh_3/MeOH (1.0mg/1.2mg/1mL), then vortexed (20s) with 1mL of 0.50M KOH/MeOH and left for 15min at rt. After that time, mixtures were vortexed (20s) with 1mL of aqueous NH_4Cl (5%) and a hexanes extraction (2 × 1mL) was performed. Organic layers were combined and dried over Na_2SO_4, and then solvents were blown off under a stream of argon. The residual material was dissolved in 100μL of 12.5μM cinnamyl alcohol/hexanes and placed in the HPLC autosampler at 5^{o}C prior to sample injection on HPLC. The lag-phase duration periods were extracted from charts: [PLPC-OOHs] *versus* time.

Oxidation of PLPC (MLV) in PBS in the presence of 2,2'-azo[2-(2-imidiazolin -2-yl) propane] {2- [2- (4-n- dodecyl) imidazolin-2-yl] propane} dihydrochloride (C-12). α-Tocopherol (13.3nmol) in methanol (100μL) was added (if applicable) to PLPC (20mg) in chloroform (800μL). Solvents were blown off under a stream of argon to form thin films. PBS (1.85mL) was added to those

films followed by an addition of an aqueous antioxidant (13.3nmol) in PBS (100μL), if applicable, or additional 100μL of PBS in case aqueous antioxidant was not used. Samples were vortexed for 2min, C-12 assymetrical initiator (2.12μmol) in MeOH (50μL) was added, and milky mixtures were vortexed for additional 20s and incubated at 37ºC for 120min (single antioxidant) or 210min (two antioxidants). To monitor disappearance of antioxidants, aliquots were withdrawn every 15min for at least 1h. In case of aqueous antioxidants, 100μL aliquots were vortexed (20s) with BHT/PPh_3/MeOH (0.25mg/0.30mg/400μL) and 50μL of an internal standard in MeOH. Samples were centrifuged (2min at 10kRPM), 300μL of supernatants withdrawn, and solvents were blown off under a stream of argon. Residues were dissolved in 70μL of 70% aqueous MeOH (**1**) or 100μL of 50% aqueous MeOH (uric acid), and mixtures were placed in the HPLC autosampler at 5^{o}C prior to sample injection on HPLC. For α-TOH analysis, 200μL aliquot was vortexed (20s) with BHT/PPh_3/MeOH (0.50mg/0.60mg/700μL) and 100μL of internal standard in MeOH. Highly lipophilic components were extracted with hexanes (2 × 1mL), organic layers were combined and dried over Na_2SO_4. Solvents were blown off under a stream of argon, residues were dissolved in hexanes (60μL) and placed in the HPLC autosampler at 5^{o}C prior to sample injection on HPLC. Analyses of MeLinOHs were performed as for oxidations initiated by AIPH. Antioxidant consumption profiles were shown on charts: [antioxidant] versus time; whereas the lag-phase duration periods were extracted from [PLPC-OOHs] *versus* time.

Partition of antioxidants between PLPC (MLV) and PBS. Antioxidants (52.2nmol) in methanol (100μL) were added to PLPC (20mg) in chloroform (800μL). Solvents were blown off under a stream of argon to form thin films. Degassed PBS (2.00mL) was added to those films, mixtures were vortex (2min) and further degassed for 5min with bubbling argon. Vials containing milky suspensions were then carefully closed and incubated for 2h at 37ºC. An aqueous phase was separated from the mixtures by ultrafiltration (3 × 5min at 3500RPM with 10kD cut off). Filtrates obtained after the initial 5min of centrifugation were discarded, 400μl of the remaining supernatants were vortexed with 400μl of an internal standard in MeOH and mixtures were placed in the HPLC autosampler at 5°C prior to sample injection on HPLC. The extent of partitioning of an antioxidant into liposomes was assign by comparison of $[antioxidant]_{aq}$ found in samples incubated with and without PLPC and otherwise identically following the above procedure.

UV studies. Solutions of antioxidants (20μM) in benzene were transferred into quartz cuvettes and UV absorbance spectra were recorded.

pH Titrations curves. Solutions of pyridinols (0.05 mmol) in 10 mL of aqueous HCl (0.1 mM) containing 25% MeOH were titrated with 25 μL portions of aqueous NaOH (0.1 mM) containing 25% MeOH. After addition of each portion of titrant, pH value was recorded (waiting for 5s for full mixing). The approximate first derivative was obtained by ploting the slope between the two direct neighbors for a certain data point. pKa Values were found from the half distances

between two maximums of the first derivative reflected on the pH axis via titration curves.

Cyclic voltammetry. Solutions of antioxidants (1mM) were prepared in 100mM phosphate buffer and degassed for at least 30min with bubbling nitrogen prior to measurements. Glassy carbon was used as a working electrode, platinum as a counter electrode, and Ag/AgCl as a reference electrode. The platinum electrode was flame burned whereas glassy carbon electrode was polished with diamond paste and sonicated in deionized water for 30s prior to each measurement. To ensure cleanness of electrodes blank measurement of degassed buffer was taken between measurements of solutions of antioxidants.

Reproducibility of reported results. All experiments described herein were repeated at least three times and an average value (stoichiometric factor, rate of lipid peroxidation, rate of hydrogen atom donor autoxidation, UV absorption maximum, dissociation constant, percent distribution in aqueous and lipid phase) was reported for each measurement. In homogeneous systems, the repeated values were within 10% margin from the reported value. In heterogeneous systems, the highest standard deviation was close to 13%. Stability tests in aerated solvents were also repeated at least three times yielding consistent with each other results.

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50. According to Chemdraw Ultra 10.0 (Cambridgesoft), the compounds possess similar calculated partition coefficients: LogP = 2.5 (**3**), 2.5 (**10**) and 3.0 (**6**).

51. The C12 initiator (used in experiments described in this chapter) was synthesized by Dr. Soen Culbertson.

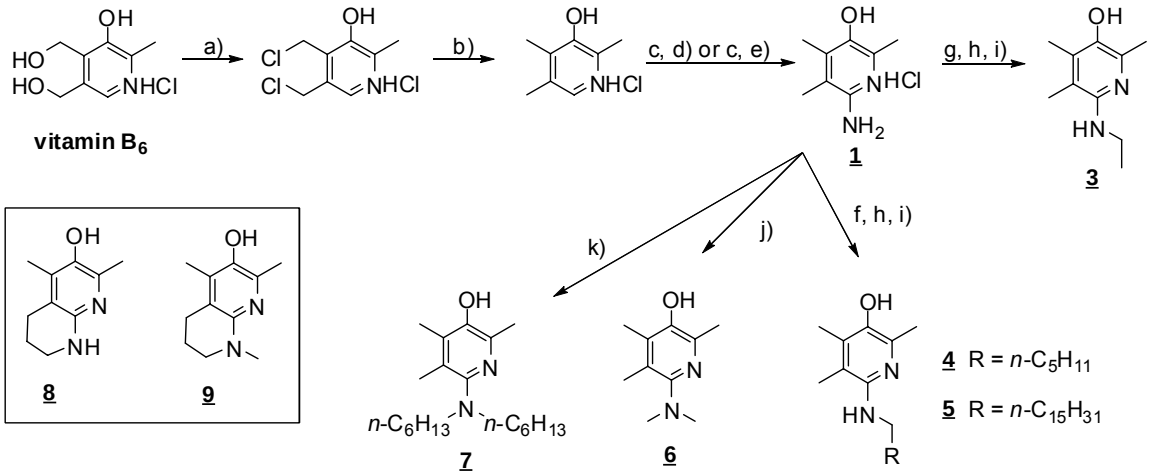
Research Summary (Part One)

Free radical chain oxidation of lipids has been associated with a number of cardiovascular and neurodegenerative diseases. *In vivo*, α-tocopherol (α-TOH) is a major scavenger of lipid derived chain-carrying peroxyl radicals. It has been shown, however, that α-TOH-derived phenoxyl radicals under some conditions act as pro-oxidants, abstracting hydrogen atoms from lipids and initiating their peroxidation. Therefore, an interest exist in developing an antioxidant that is a better hydrogen atom donor but also yields phenoxyl radicals that are more persistent than α-TOH derived species. It was previously demonstrated that some *p*-aminopyridinols possess such properties. However, limited quantities of these compounds could be prepared utilizing previously published synthetic methodology. Therefore, development of a new preparation route was essential to enable more extended studies on this class of compounds.

An efficient multi-gram scale synthesis of 2-amino-5-pyridinols based on transformations of vitamin B_6 was applied to address this issue (shown in a scheme below). An important advantage of this approach is the presence of a hydroxyl moiety in the desired position of the aromatic ring. As a consequence, a low-yielding ring hydroxylation step may be avoided. The new method also utilizes only simple reagents and purification methods, thus opening the possibility for easy reaction scale-up.

Antioxidant efficiency of a compound is a combination of several properties; its stability in the absence of radicals, its stoichiometric factor *n* (number of radicals that are quenched per antioxidant molecule), rate at which

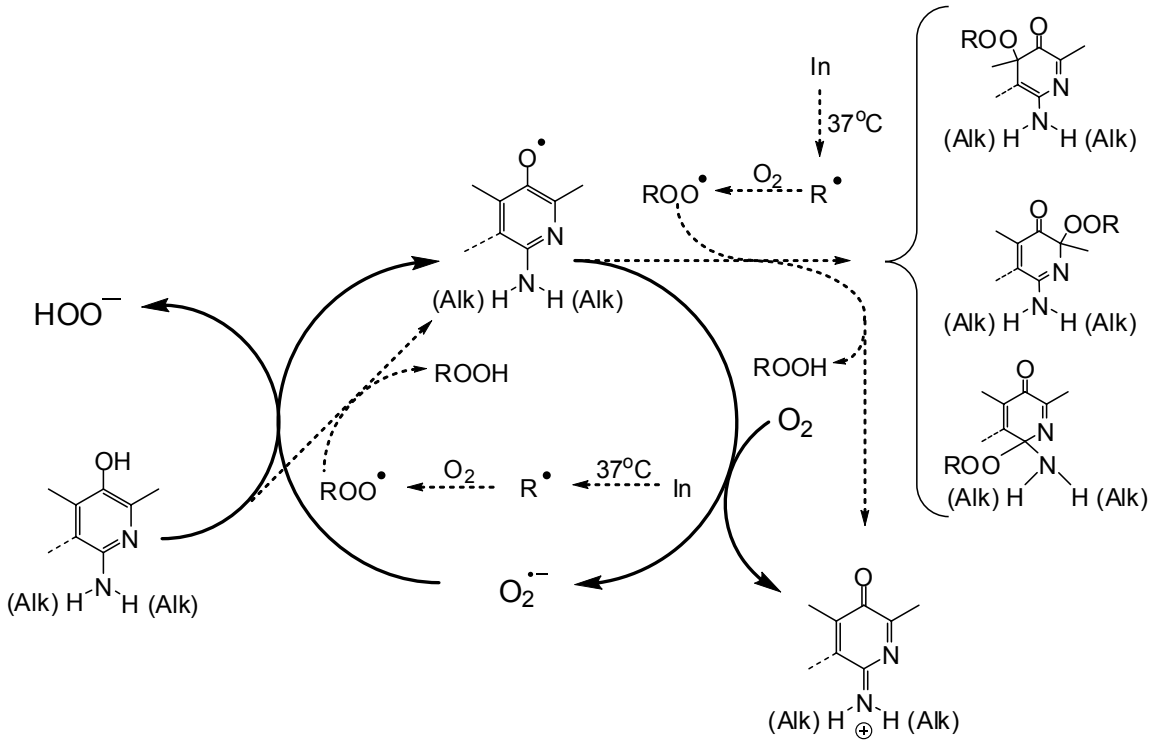
the radicals are quenched by antioxidant, and persistency of the antioxidant-derived radicals. The above properties of the newly synthesized compounds were studied in aprotic, aqueous as well as in heterogeneous environments.



Reagents and Conditions: (a) $SOCl_2$, DMF (cat.), reflux, 30min, 99%; (b) Zn/AcOH, reflux, 2h, 90%; (c) $PhNH_2$, $NaNO_2$, H_2O, pH 8, 0°C, 1h, 87%; (d) H_2, Pd/C, MeOH, rt, 24-48h, 90%; (e) Zn/HCOOH, MeOH, reflux, 3h, 85%. (f) RCOCl, 1,2-dichloroethane, reflux, 4h; (g) $(AcO)_2$, THF, rt, 12h; (h) NaOMe, MeOH, rt, 30-90min; (i) BH_3•THF, THF, reflux, 2-3h, 57-64% (for f-h-i) or 74% (for g-h-i). (j) HCOOH, HCHO, H_2O, reflux, 42h, 85%; (k) hexanal, $Na(AcO)_3BH$, AcOH, THF, rt, 6h, 80%.

It was found that *N*-monoalkylated 2-amino-3,4,6-trimethyl-5-pyridinols (**3**-**5**) are superior to *N*,*N*-dialkylated analogues (**6** and **7**). This was due to steric interactions present in the latter molecules, which causes an increase in dihedral angle between 2-nitrogen's p-type lone pair and the p-orbital of the adjacent aromatic carbon. In aerated aprotic systems, *N*-monoalkylated 2-amino-3,4,6-trimethyl-5-pyridinols do not autoxidize and their stoichiometric factors are close

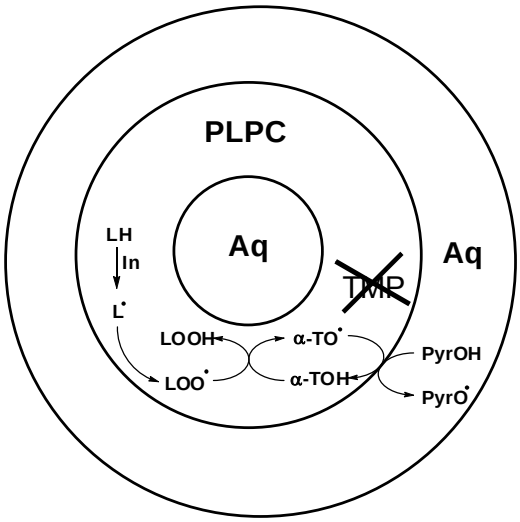
to two, which is consistent with known decomposition mechanism for vast majority of phenolic antioxidants. In aerated aqueous solutions, all the tested 2-amino-5-pyridinols are considered stable, but they autoxidize in the presence of a radical source (*n*= 0.9-1.45). A mechanism for the disappearance of these compounds in such systems was proposed (scheme immediately below).



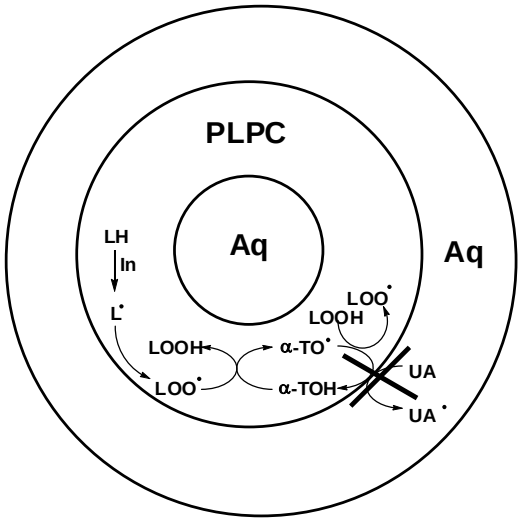
In a heterogeneous environment consisting of oxidizable multi-lamellar vesicles suspended in aqueous buffer, *n* factors for pyridinols were generally greater than corresponding values measured in the buffer alone. It was found that distribution of pyridinolic antioxidants between phases of the heterogeneous system was crucial for their autoxidizability. To the extent that a pyridinol partitioned into lipid bilayers, the better it was protected from self-consuming

reactions. It was also found in the heterogeneous systems that water soluble compound **1** acts as a co-antioxidant and regenerates α-TOH from the corresponding phenoxyl radical, hence protecting lipids from oxidation by inhibiting tocopherol mediated peroxidation, TMP (scheme immediately below).

In the presence of good co-antioxidant
(assumed behavior of pyrydinol **1)**



In the presence of poor co-antioxidant
(reported previously for uric acid)



Such regeneration of α-TOH in heterogeneous systems is typical for water soluble quinols and ascorbic acid, but not for plasma abundant uric acid, which was used as a control aqueous radical scavenger. It is proposed that other structurally similar 2-amino-5-pyridinols possesses similar properties.

Rates of hydrogen atom transfer from the pyridinols to oxidizable fatty esters were measured in our laboratory, where the bimolecular reaction under investigation competed with unimoleular transformations of fatty esters derived radicals. It was found that monoalkylated pyridinols (1.4×10^7 $M^{-1}s^{-1}$) gave up

a hydrogen atom much faster than α-TOH (3.5×10^6 $M^{-1}s^{-1}$). Some bicyclic pyridinols, *e.g.* compounds **8** and **9**, have been shown to be the most reactive in these studies. However, they were also found to undergo very fast autoxidation in aqueous buffers in the presence of a radical source (*n* = 0.23-0.48). Additionally, they decomposed reasonably fast even in the absence of radical initiators ($4.0\text{-}9.2 \times 10^{-9}$ Ms^{-1}). The instability of these compounds is expected to decrease their bio-utility compared to monocylic vitamin B_6 analogues, which are proposed to be potent lipid peroxidation inhibitors *in vivo* (ongoing investigation).

Part Two

ω-ALKYNYLATED FATTY ACIDS AS SUBSTRATES FOR PREPARATION OF MODIFIED PHOSPHOLIPIDS, NOVEL PROBES FOR EVALUATING LIPID-PROTEIN INTERACTIONS.

CHAPTER IV

INTRODUCTION

Phospholipids as Components of Biological Membranes

All living cells are surrounded by a membrane that separates their constituents from an external milieu. In other words: ”a membrane provides identity to a cell”.[1] Sublime pathways of communication of a cell with the external environment are only possible because of the semi-permeability of the membrane that surrounds the cell. This very property allows the membrane to participate in important cellular processes, such as transport, excitability, intercellular interaction, morphological differentiation, and fusion.[1]

Biological membranes can be considered as two-dimensional matrixes consisting of the phospholipid bilayer that is interrupted by the presence of integral proteins (Figure 4.1).[2] This so-called fluid mosaic model of membrane states that the hydrophobic interior of the membrane forms a fluidic matter. The components of the bilayer are held together as a consequence of non-covalent interactions. The lipid chains adhere to one another and to hydrophobic residues of the proteins due to van der Waals forces, whereas interactions between polar groups at the bilayer surface are mostly of coulombic and hydrogen-bonding types.[1]

As a consequence of the nature of these interactions, both the phospholipids and the integral proteins are able to move laterally in the plane

of the bilayer, but movement from either one face of the bilayer to another is restricted. Additionally, specific interactions between the components of a membrane produce their selective ordering and results in their segregation in the plane of the membrane, leading to a formation of a long-range order and co-operativity within the segregated domains.[1]

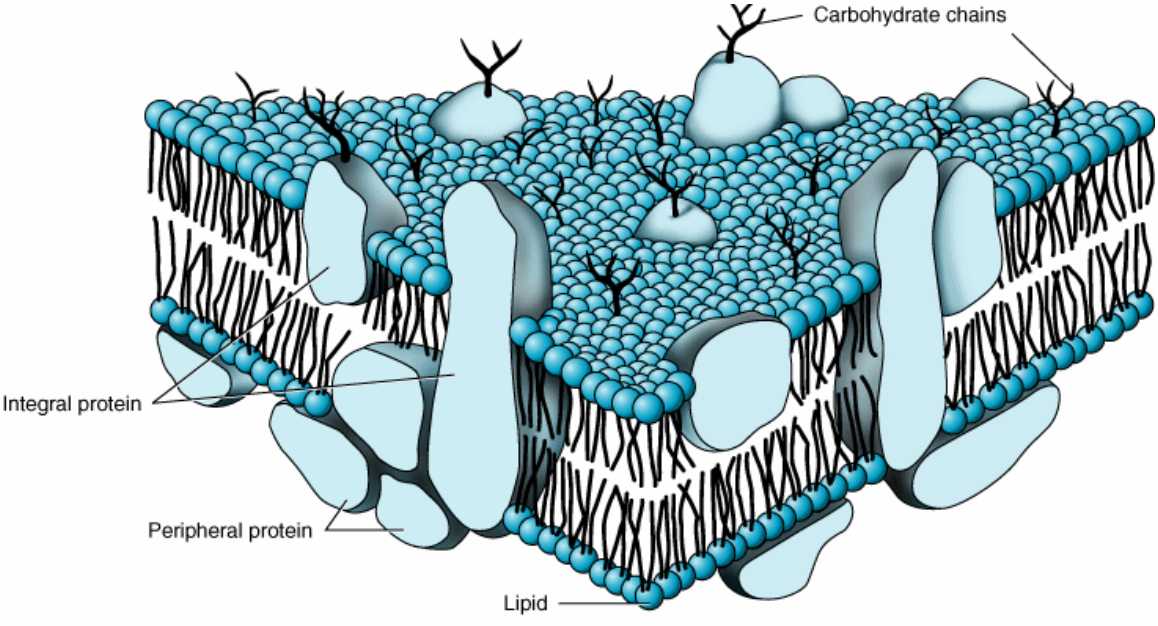


Figure 4.1. The fluid mosaic model of a cell membrane.[2]

Besides lipids (30-80%) and proteins (20-60%), biological membranes contain small amount of carbohydrates (up to 10%). The carbohydrates are exposed at the external surface of the cell and serve as recognition sites for signaling molecules, and other cells. However, the amount of protein embedded in the bilayer is of special importance, since it is responsible for a number of metabolic functions of the membrane.[1]

The most abundant lipid membrane components, phospholipids consist of fatty acids esterified to a phosphate containing head group, derived from a glycerol molecule (Figure 4.2). Within this class of lipids, phosphatidylcholines (PCs) are major components of animal membranes. PCs typically contain both saturated (at *sn*-1 position) and unsaturated (at *sn*-2 position) fatty acid residues (Figure 5.2).[1] In animals, the most abundant acyl chains are: saturated [palmitate (16:0) and stearate (18:0)] and polyunsaturated [linoleate (18:2) and arachidonate (20:4)].[1]

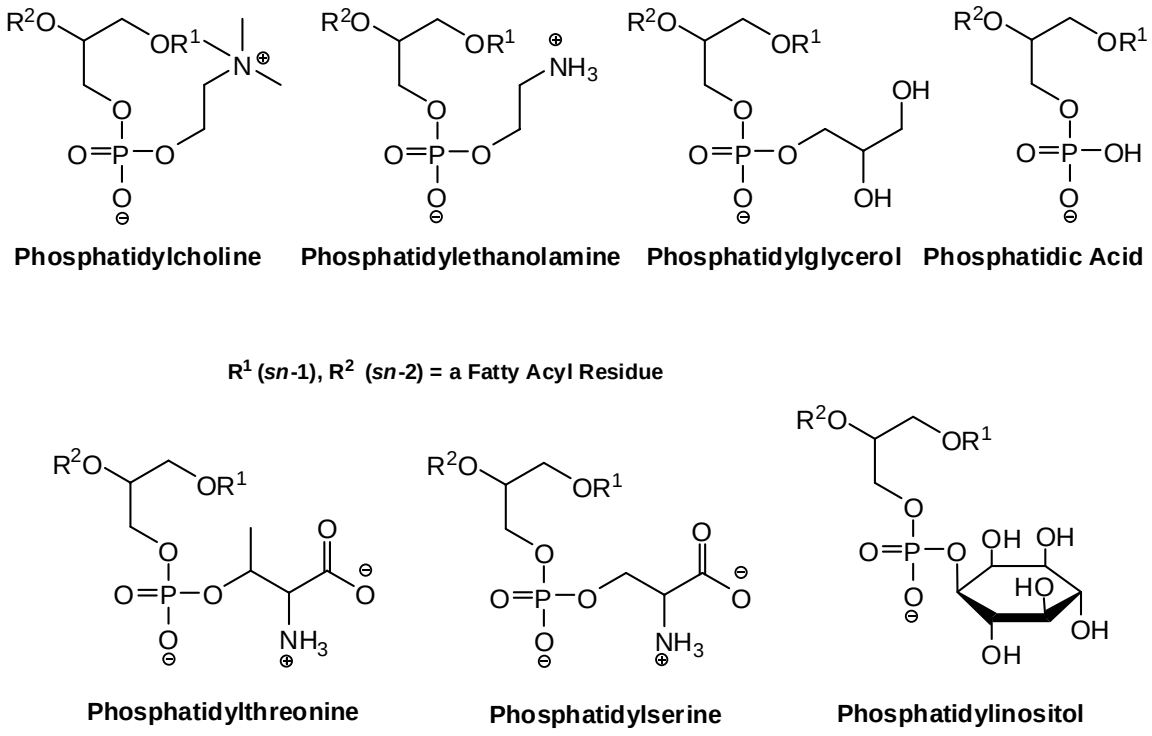


Figure 4.2. Phospholipids classified with respect to their polar head groups.

Linoleic and Arachidonic Acid Derived Reactive Molecules

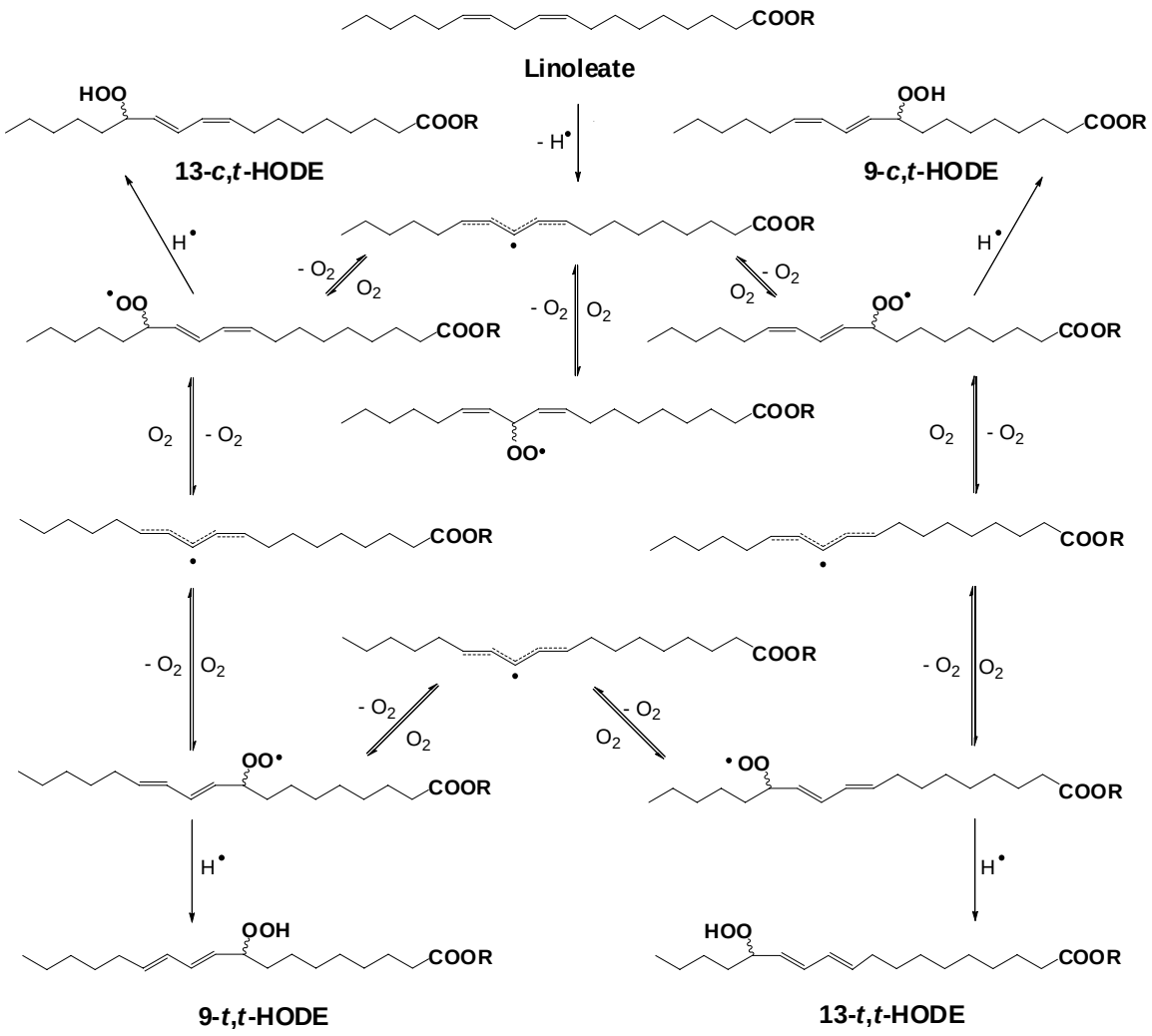
Taking into account their relatively high abundance in biological membranes, it is not surprising that linoleic and arachidonic acid esters have been studied most intensively. It was found that in the presence of a free radical source, membrane constituents containing these acyl groups undergo chain oxidation processes that give rise to a rather complex mixture of products.[3-7]

The pool of primary oxidation products obtained from a linoleic acid ester precursor is relatively small. During uninhibited lipid peroxidation, the predominant products are two regioisomeric *t*,*t*-conjugated diene hydroperoxides (9- and 13-*t*,*t*-HPODE), and the minor products are two regioisomeric hydroperoxides containing two conjugated *c*,*t*-double bonds (9- and 13-*c*,*t*-HPODE) in their structures, Scheme 4.1.[8-10] The enhanced formation of *t*,*t*-dienes is thermodynamically driven. Furthermore, each of the four HPODEs exists as mixture of two enantiomers.[11]

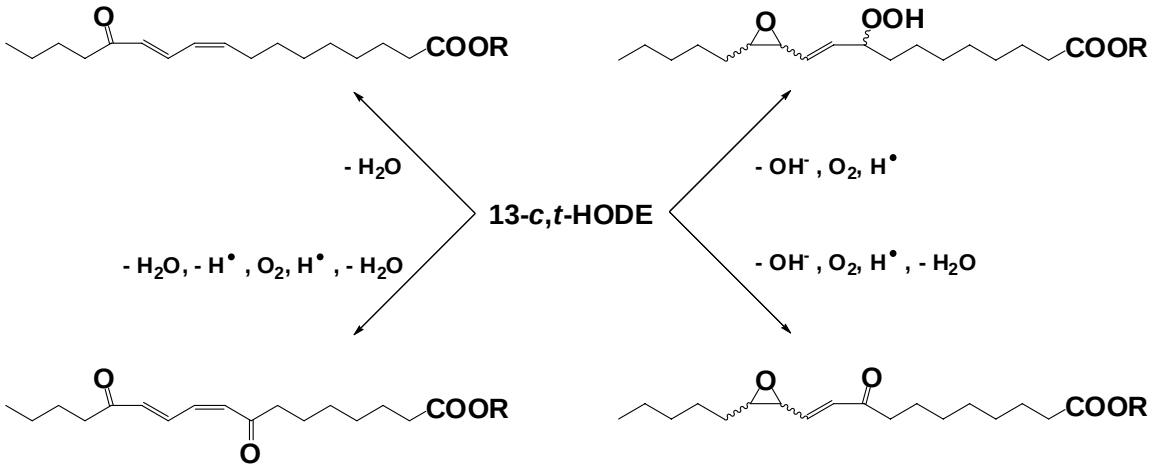
All of these compounds may be further transformed via dehydrations and/or one electron reductions into a large number of secondary products, such as the electrophilic dienones and α,β-unsaturated epoxides shown in Scheme 4.2.[5, 12, 13] In addition, all of the primary and secondary products with preserved α,β-unsaturated hydroperoxide moiety may undergo Lewis acid catalyzed fragmentation, referred to as Hock rearrangement, to form highly electrophilic aldehydes (Scheme 4.3).[14, 15]

The formation of reactive electrophiles is important for a number of patho-physiologically relevant reasons (*vide infra*) and mechanisms have been written

for the formation of some of the compounds observed as peroxidation products. γ-Hydroxy-α,β-unsaturated aldehydes, such as 4-hydroxy-nonenal (HNE) are probably the most widely studied lipid derived electrophiles, since they have been found to be extremely cytotoxic.[13, 15-18] One of the proposed pathways of the formation of HNE from 13-*c*,*t*-HPODE is presented in Scheme 4.3.[15]

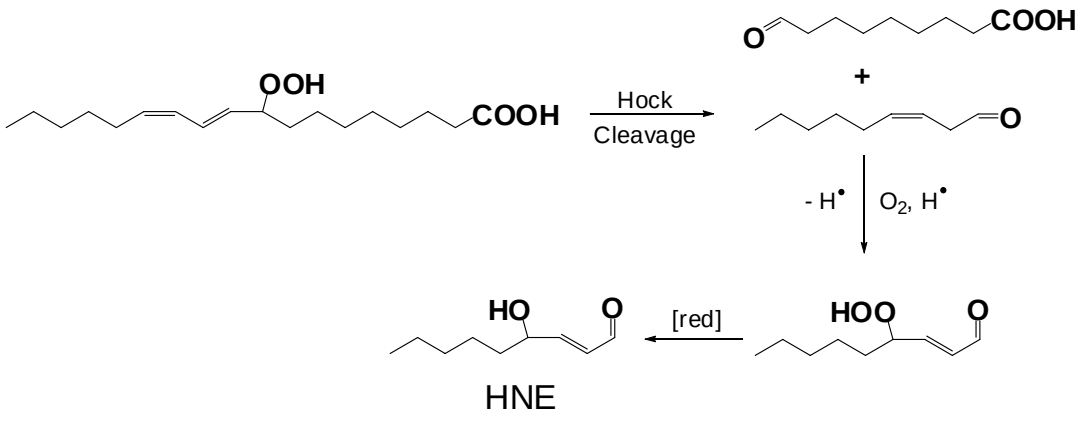


Scheme 4.1. Formation of the primary linoleate oxidation products.



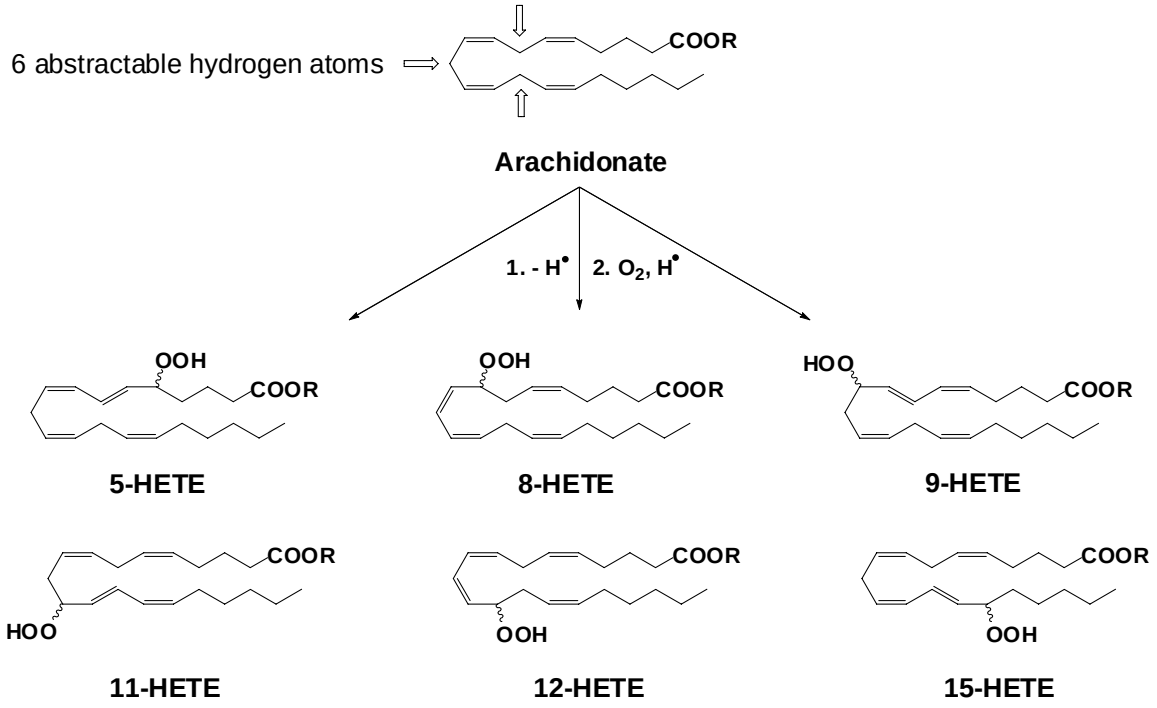
Scheme 4.2. Formation of selected electrophiles from 13-*c*,*t*-HODE.

Arachidonic acid, on the other hand, has six easily abstractable hydrogen atoms and its oxidation yields six regioisomeric *c*,*t*-conjugated diene hydroperoxides (5-, 8-, 9-, 11-, 12- and 15-HPETE) as primary products, each of which is a mixture of two enantiomers (Scheme 4.4).[8, 19, 20]



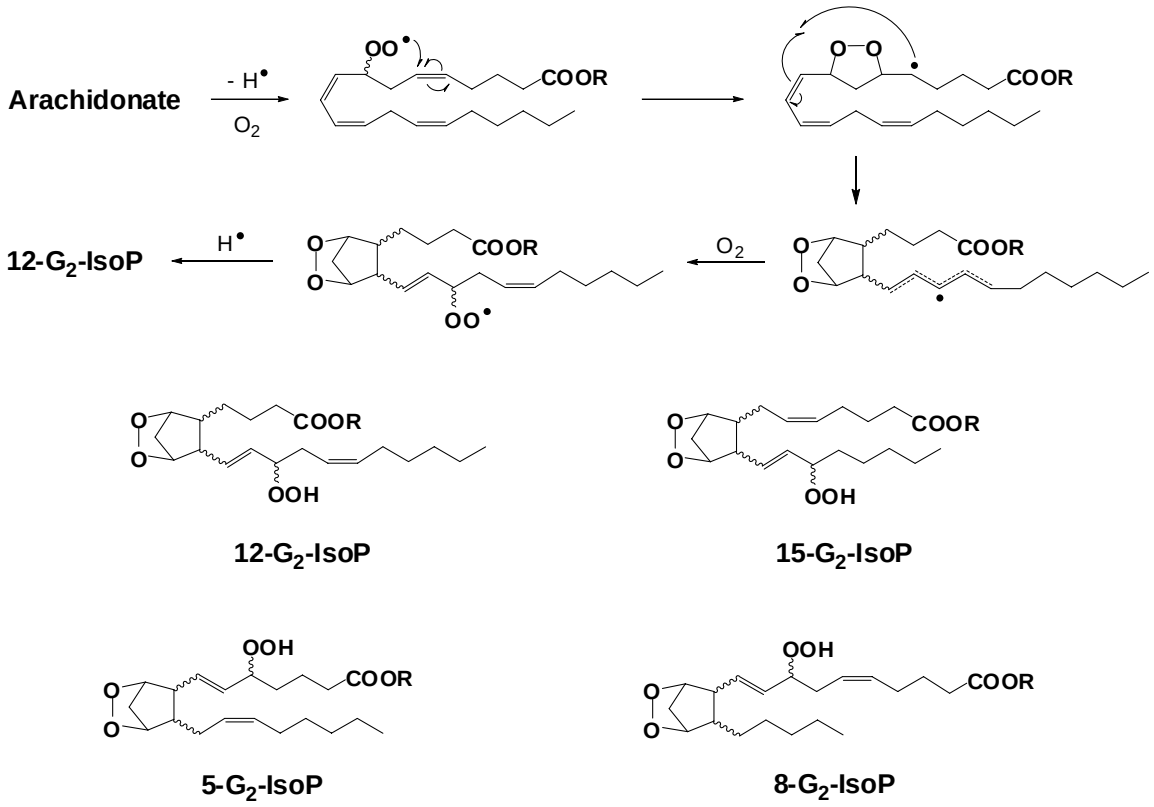
Scheme 4.3. A Mechanism for the Formation of HNE from 9-*c*,*t*-HPODE.[15]

As a result of intramolecular radical cyclization processes, a mixture of four regioisomeric bicyclic endoperoxides referred to as G_2-isoprostanes (5-, 8-, 12- and 15-G_2-IsoPs) is formed as additional primary oxidation products of arachidonate oxidation (Scheme 4.5). Each of the G_2-IsoPs exits as a mixture of eight diastereoisomers.[8]



Scheme 4.4. Formation of acyclic hydroperoxides (HETEs) from arachidonate.

In addition to the aforementioned primary oxidation products, formation of monocyclic and serial cyclic peroxides has been observed during the autoxidation of arachidonate. These compounds are believed to form as a result of a single or multiple 5-*exo* radical cyclization reactions.

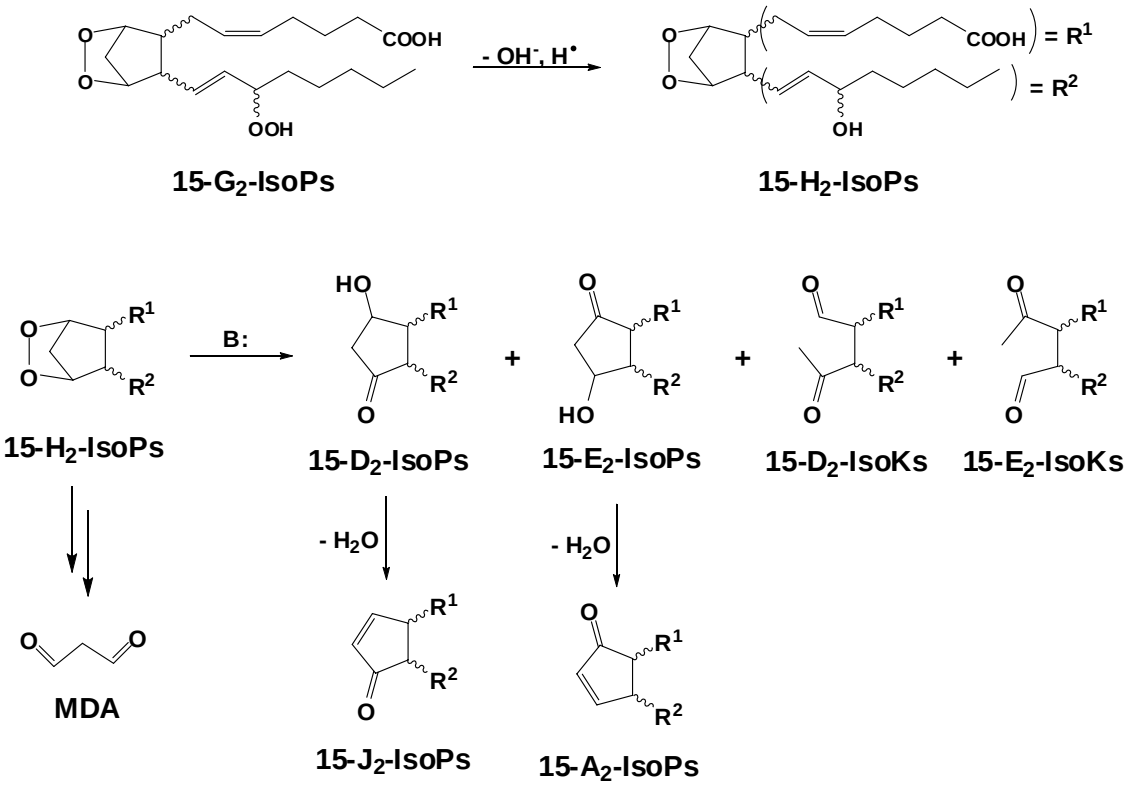


Scheme 4.5. Formation of cyclic endoperoxides from arachidonate.

All of these primary oxidation products can be further converted to α,β-unsaturated ketones and epoxides in ways similar to those presented for linoleate derivatives. Additionally, Hock fragmentation of the arachidonate derived α,β-unsaturated hydroperoxides yields much more complex mixture of highly electrophilic aldehydes than the one obtained from linoleate substrate.

A number of extremely reactive electrophiles, such as isoketals, malondialdehyde (MDA) and cyclopentadienone isoprostanes can be generated from bicyclic G_2-IsoPs (Scheme 4.6).[21-23] Isoprostanes H_2 (H_2-IsoPs) are formed upon one electron reduction of the hydroperoxide moiety of G_2-IsoPs.[24] As a result of a base catalyzed endoperoxide ring opening, H_2-IsoPs can be

further converted into new classes of compounds, D_2-IsoPs and E_2-IsoPs, and corresponding isoketals (D_2-IsoK and E_2-IsoK) if retro aldol condensation occurs. A dehydration of D_2 and E_2 isoprostanes yields cyclopentadienone J_2 and A_2-IsoPs, respectively. On the other hand, a multistep degradation of bicyclic endoperoxide moiety leads to formation of MDA. [18, 21-23, 25-27]



Scheme 4.6. Isoketals, pentadienone isoprostanes and MDA, the reactive electrophiles formed from bicyclic endoperoxides (shown for 15-series of IsoPs).

The PUFAs are also substrates for important enzymatic transformations. A family of oxygenase enzymes is mainly responsible for oxygen addition to fatty acids and their derivatives. Lipoxygenases convert linoleate into HODEs and

arachidonate into HETEs, whereas cyclooxygenases facilitate transformation of arachidonate into bicyclic endoperoxides. Once formed these primary oxidation products may undergo further conversion into reactive electrophiles.[28, 29]

Since the enzymatic catalysis is highly selective, a very stereo-specific set of products is formed. Therefore, the origin of the reactive electrophiles or their primary PUFA oxidation product precursors may be determined by evaluation of the enantiomeric and diastereomeric composition of the isolated products. In fact, an assay has been developed, which is used to evaluate the extent of free radical lipid oxidation *in vivo* that is based upon measurements of individual diastereoisomers of stable isoprostane products - 15-F_2 series (15-F_2-IsoPs), Figure 4.3.[30] Since only $PGF_{2\alpha}$ isomer is derived enzymatically, non-enzymatic peroxidation-derived products can be assigned by determination of the amount of $PGF_{2\alpha}$ in the total of 15-F_2-IsoP product mixture.[30] It is of note that metabolism can complicate the analysis because stereo-isomeric species may be differentially metabolized. For comparison, most assays used in the past to quantitate oxidative damage to lipids did not distinguish between enzymatically formed compounds and non-enzymatic products. The most commonly used method of such, known as a TBAR assay was based on a measurement of a fluorescence response of the products obtained from a reaction between thiobarbituric acid and mixtures of low molecular weight products of lipid oxidation, mainly MDA.[31] This relatively unspecific and misleading assay has been substituted by analysis of F_2-IsoPs, which are generally referred to as gold standard biomarkers of oxidative stress *in vivo*.[30, 32-34] Furthermore, the origin of

secondary oxidation products that retain chiral information can most likely be investigated on similar bases.

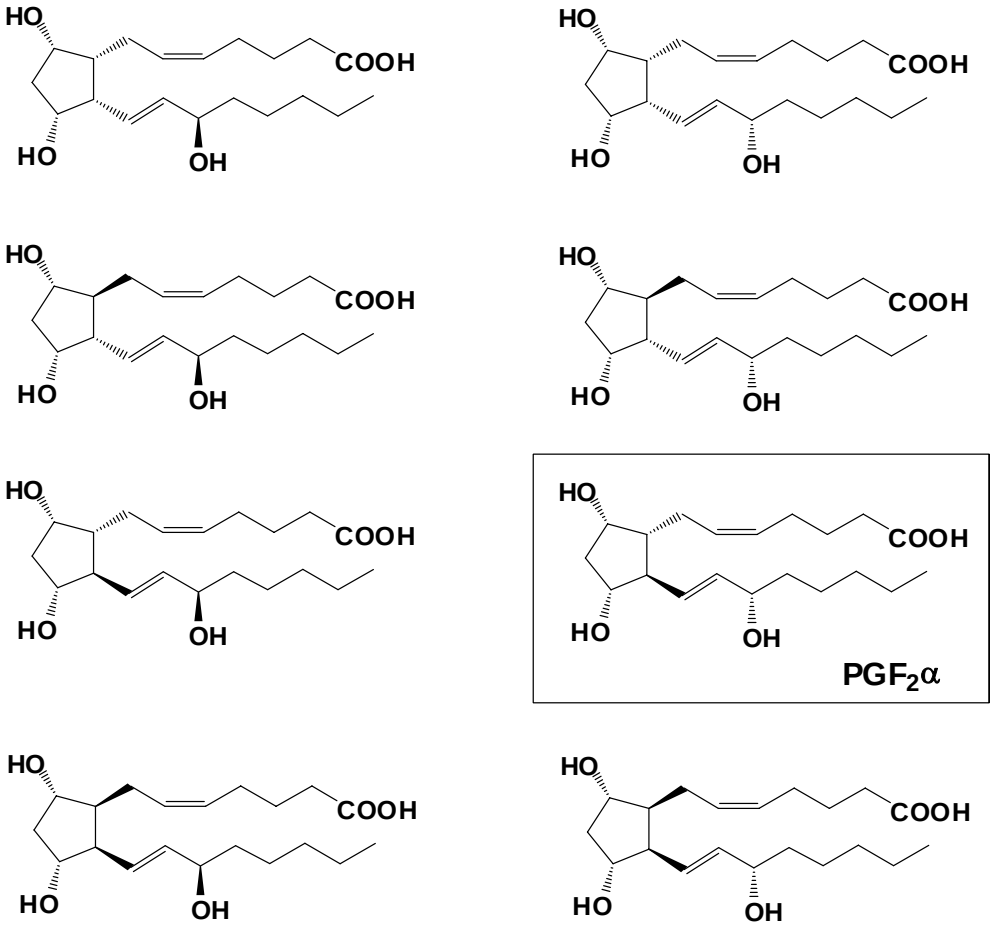


Figure 4.3. The diasereoisomers of the 15-F_2-isoprostane subfamily.

Assaying for isoprostanes requires utilization of either an enzyme-linked immunosorbent assay (ELISA) or mass spectrometry (MS).[30, 32, 33, 35] For instance, PGF_2 diastereoisomers can be separated utilizing high pressure liquid chromatography, followed by detection on MS upon electrospray (ESI) or

atmospheric pressure chemical ionization (APCI).[30] The procedures based on LC-MS typically operate in selected reaction monitoring mode (SRM) that usually has excellent sensitivity, which is important while analyzing complex mixtures of products.[8, 30]

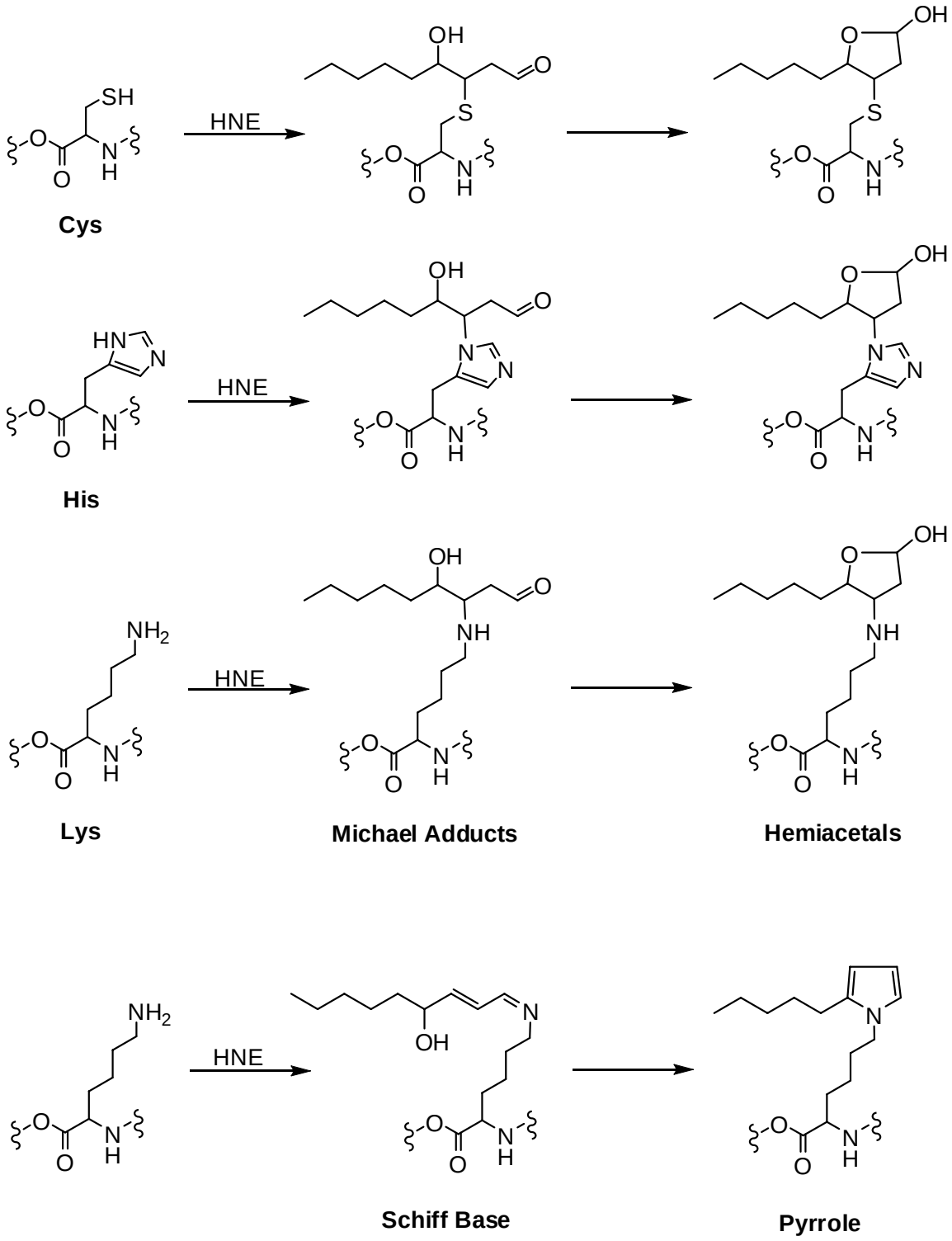
The electrophilic oxidation products derived from linoleate and arachidonate form adducts with nucleophilic residues on nucleic acids, proteins and other biologically relevant molecules.[36-41] The adduction of these electrophiles to nucleic acids, has to date been investigated the most extensively. However, the majority of the published research was focused on the reactivity of only a few lipid derived electrophiles. HNE, a compound that is highly abundant in biological membranes (up to 10μM),[42] is the electrophile most widely investigated. It was shown that interactions of this electrophile with DNA cause modulations in the expression of various genes, which may lead to serious disruption of important cellular functions. Furthermore, HNE affects the cellular redox balance (*i.e.* highly induces intracellular peroxide formation and a stress-signaling pathway), and stimulates unfolded protein aggregation.[43-45]

Covalent adduction of PUFA derived electrophiles to proteins has been studied less extensively. However, evidence accumulated so far suggested strong pathological implications of such processes. Significant amounts of covalently modified proteins have been found in cells of patients suffering from cardiovascular diseases, neurodegenerative disorders and malignancies.[38, 46-48] Again, HNE has been the most widely studied substrate for investigations regarding covalent modifications of proteins or peptides. For instance, it was

proposed that the adduction of HNE to Apo B-100 in LDL played an important role in the mechanism by which conversion of LDL into its atherogenic form occurred.[46] Also, HNE may deplete cellular glutathione (GSH) by adduction, leading to apoptosis.[49]

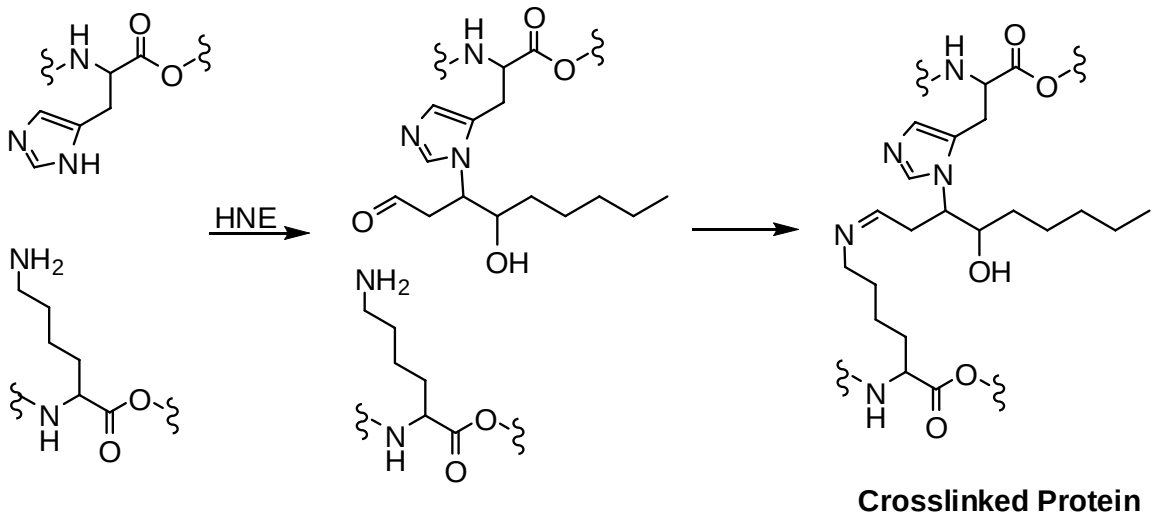
The protein and peptide sites which are reactive towards lipid-derived electrophiles are side chains of cysteine (Cys), lysine (Lys) and histidine (His). All of these nucleophiles may form Michael adducts with lipid peroxidation products that contain α,β-unsaturated carbonyl moieties in their structures. Additionally, ε-amino group of lysine residue may form Schiff base adducts with lipid-derived aldehydes. The possible adduction modes between nucleophilic amino acid residues and the model electrophile, HNE are presented in Scheme 4.7.[50] The hydroxyl moiety of γ-hydroxylated α,β-unsaturated aldehydes serves a dual function. It increases the electrophilic character of the β-carbon facilitating 1,4-addition, and enables formation of a hemiacetal, thus stabilizing the adduct. Additionally, it enables the conversion of a Schiff base to a thermodynamically stable pyrrole derivative, via an enamine intermediate (Scheme 4.7). The ability of a covalent adduct to be converted into a stable molecule may be essential for its stability, since the initial key steps, Michael addition and Schiff base formation are essentially reversible under numerous conditions.[50, 51]

Due to an existence of two electrophilic sites, α,β-unsaturated aldehydes via Michael addition and subsequent Schiff base formation can cause inter- and intramolecular protein crosslinking.



Scheme 4.7. Formation of covalent adducts between HNE and nucleophilic residues (Cys, Lys and His) of proteins or peptides.[50]

This phenomenon is illustrated in Scheme 4.8.[52] Multiple sites of such crosslinking may lead to a cytotoxic protein aggregation.[53, 54]



Scheme 4.8. A possible mechanism of protein crosslinking by HNE.[52]

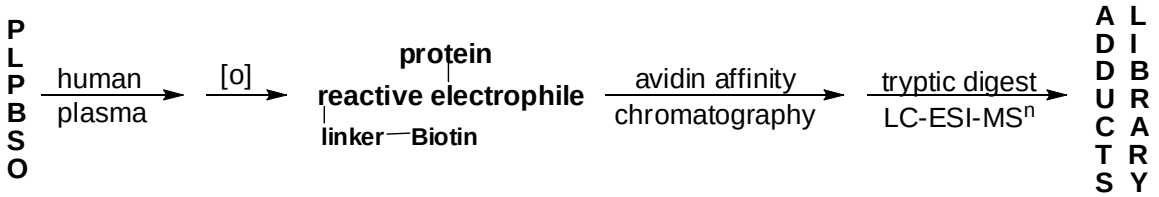
A Novel Tool for Evaluating Phospholipid-Protein Interactions

Besides HNE, MDA and other low molecular weight aldehydes, one can imagine an enormous pool of electrophilic peroxidation products, generated in membranes, which are attached to a phospholipid head group. These products have been studied only briefly to date. However, there exists preliminary evidence suggesting that such compounds actively participate in atherogenesis. Products of this type, formed on the surface of LDL were shown to serve as potent ligands for scavenger receptor CD-36 presented on macrophages, leading to the LDL uptake and formation of foam cells.[55] Furthermore, these electrophiles

may promote transport of monocytes, from which the macrophages are differentiated, into a vessel wall.[56]

Even though the chemical basis for adduct formation, *i.e.* Michael addition and Schiff base formation are unquestionable, mechanisms that governs site selectivity of the adductions remain unknown. Furthermore, an extremely limited number of studies have monitored adduction of more that one electrophile to a protein simultaneously. Therefore, structural features that control reactivity of mixtures of electrophiles in biologically relevant settings are yet to be established.

In a recently published report, a new tool for simultaneous evaluation of multiple adducts of phospholipid derived electrophiles to proteins was presented. The principles of this innovative methodology involved a synthetic preparation of a phospholipid molecule modified with a biotin unit, its insertion into biological membranes and upon adduction, isolation of the biotinylated adducts via avidin affinity chromatography followed by their proteomics MS analysis (Scheme 4.9).[57]



Scheme 4.9. A strategy proposed for simultaneous evaluation of mixtures of phospholipid derived electrophiles adducted to plasma proteins.

The structures of the synthetic phospholipid (PLPBSO) and its native analogue, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) are shown in Figure 4.4. The modified phospholipid retains zwitterionic headgroup of the PCs and was concluded to have similar calorimetric and colloidal properties to those of PLPC.[57]

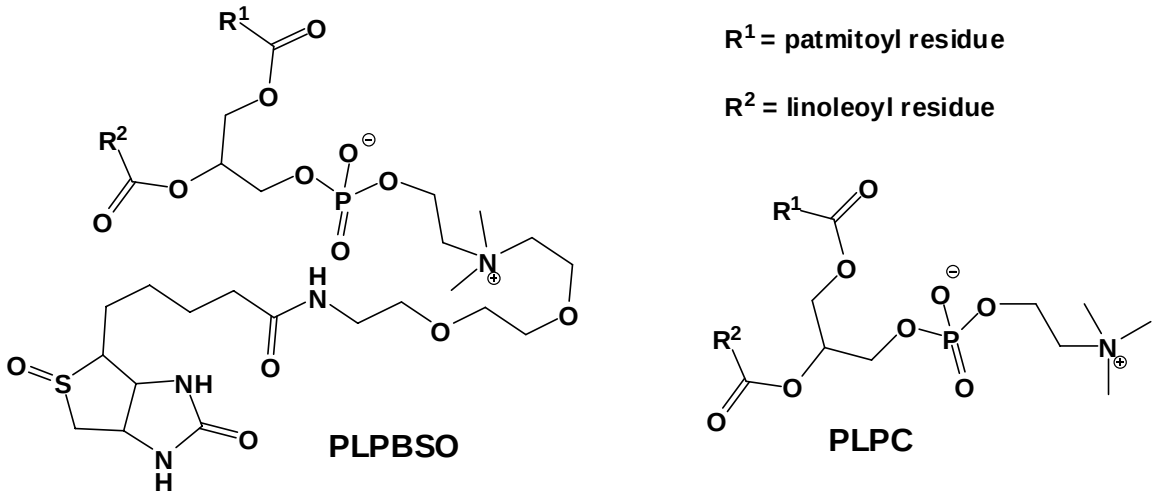


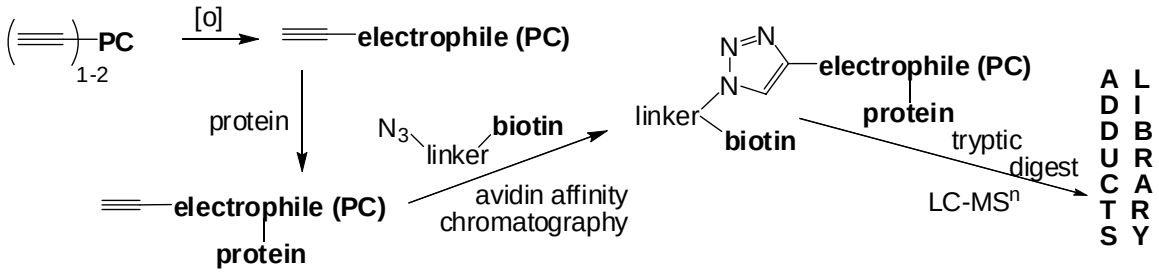
Figure 4.4. PLPBSO, a synthetic analogue of PLPC.

In order to evaluate the analytical methodology, a series of oxidations in the presence and absence of nucleophiles have been performed in liposomal suspensions containing the modified phospholipid and non-oxidizable PCs. The biotinylated products were then isolated and analyzed. It was found that during free radical initiated oxidations performed in liposomal emulsions, a number of PLPBSO oxidation products had formed. Overall the set of products obtained from the modified phospholipid was found to be similar to the one obtained upon oxidation of the natural analogue.[57]

It was further observed that during oxidations of PLPBSO initiated in the presence of a lysine containing model peptide, a number of peptide adducts formed. The compounds obtained have been successfully analyzed by tandem mass spectrometry. Following data collection, the proteomic software P-Mod has been used to screen for adducts.[57]

MALDI-MS analysis of the biotinylated compound mixture, obtained upon oxidation of a liposome emulsion in the presence of human serum albumin (HSA) followed by an avidin affinity separation, revealed a significant adduction of lipids to the protein. It was further demonstrated that HSA was a major substrate for modifications in human plasma, but other proteins were also found to be biotinylated.[57]

Even though, the applied methodology yielded promising results for experiments conducted *in vitro*, one can argue that biotinylation of its polar head group may alter reactivity of the phospholipid *in vivo*. If the probe is to be used in complex biological settings, in cells or in living animals, it has been recommended that structural alterations to phospholipids should be minimized to ensure that modified compounds undergo the same metabolic pathway as their native analogues. Incorporation of alkyne unit at the ω-positions of fatty acyl chains only slightly alter their structures, and yet allow for their coupling to azido-reagents via triazole-forming click chemistry. Upon oxidation of bio-membrane incorporated ω-alkynylated phospholipids, the phospholipid-protein adducts are to be coupled with azido-biotin, and they are to be purified via avidin affinity chromatography, prior to a tandem MS analysis (Scheme 4.10).



Scheme 4.10. A revised strategy proposed for simultaneous analyses of mixtures of phospholipid derived electrophiles adducted to protein targets.

ω-Alkynylated fatty acids that have been chosen to be substrates for modified phospholipids [linoleic (aLA), arachidonic (aAA) and palmitic acids (aPA)] are presented in Figure 4.5. Additional advantage of possessing these free terminally modified fatty acids is a possibility of their bio-catalyzed incorporation into a variety of phospholipids produced by living systems.

In the initial studies evaluating the new methodology two model phospholipids will be utilized: PLPC ω-alkynylated at *sn*-1 (aPLPC) or at both *sn*-1 and *sn*-2 positions (aPaLPC), Figure 4.5. Depending on the results obtained from those preliminary experiments, phospholipids containing alkynylated arachidonyl residues may also be prepared and applied in oxidation/adduction studies. Introduction of the alkyne group on the saturated lipid chain will allow for capturing of phospholipid bound adducts only, whereas ω-alkynylation of both acyl residues will increase the pool of the click-captured products by the adducts derived from small tail electrophiles. Depending on a particular problem to be solved, one of these classes of compounds will be utilized.

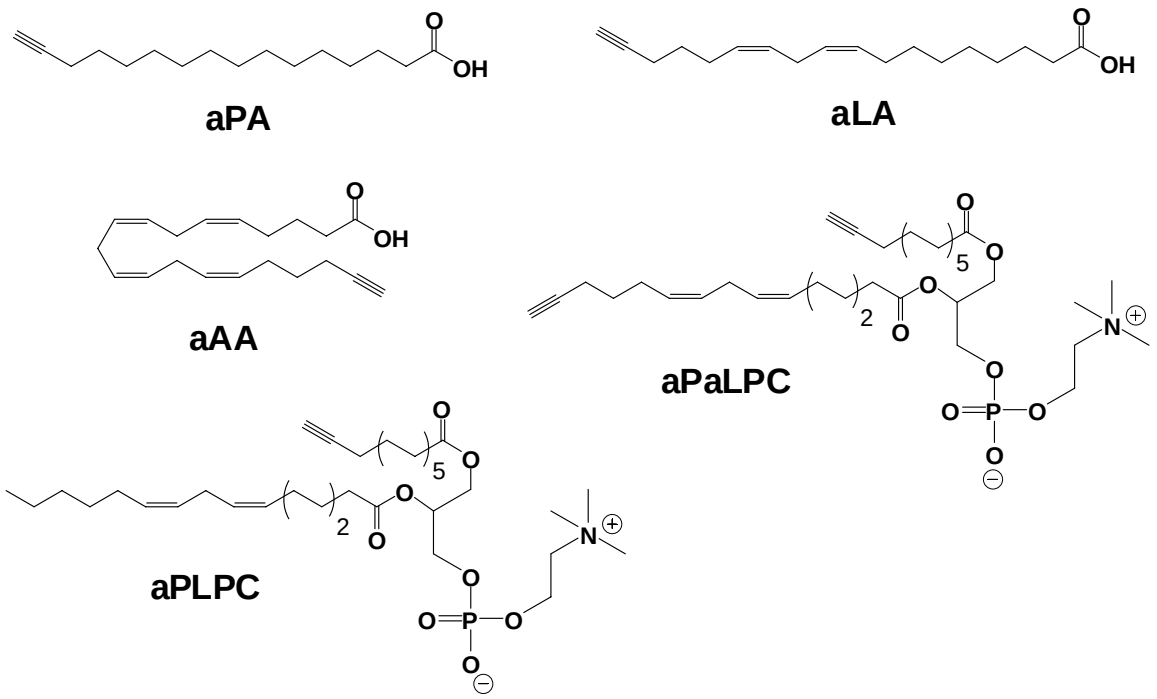


Figure 4.5. ω-Alkynylated fatty acids and sample phospholipids that can be prepared from them.

Research Aims

The aims of the research presented in Chapter V are:

1. To establish a methodology for efficient multi-gram scale preparation of ω-alkynylated linoleic acid, which will be utilized in syntheses of modified phospholipids.
2. To establish a methodology for efficient preparation of ω-alkynylated arachidonic acid, which will be utilized in synthesis of modified phospholipids.

The aims of the research presented in Chapter VI are:

3. An evaluation of oxidizability of ω-alkynylated fatty acids; characterization of the primary oxidation products obtained during their chemical and enzymatic transformations.
4. An evaluation of the formation of secondary electrophilic oxidation products from ω-alkynylated fatty acids.

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CHAPTER V

SYNTHESES OF TERMINALLY-MODIFIED FATTY ACIDS

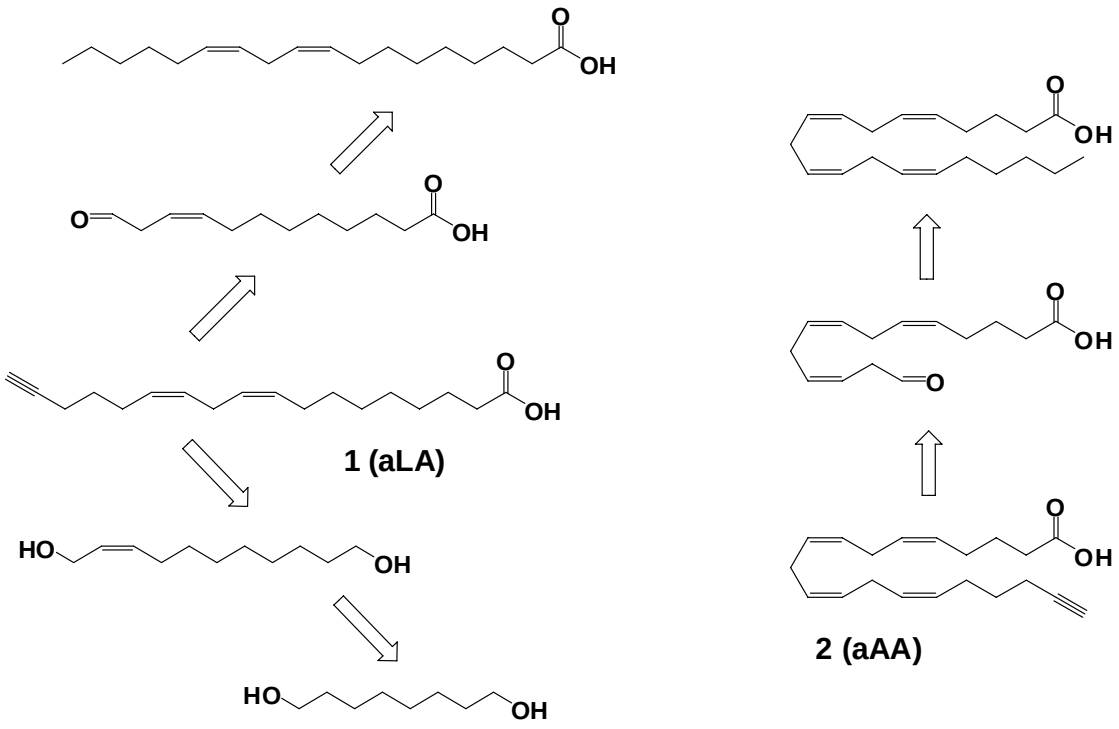
Introduction

ω-Alkynylated fatty acids may prove to be useful for preparation of phospholipid-based probes that can be used to investigate lipid-protein interactions. The least complicated of this class of compounds to synthesize, ω-alkynylated palmitic acid has been made previously in two steps.[1, 2] The key step involved an alkyne migration, commonly referred to as a zip reaction.[1, 3, 4] One can expect that preparations of ω-alkynylated polyunsaturated fatty acids will be more complicated. This is mainly because skipped polyenes are easily oxidizable (*vide supra*). Therefore, special care should be applied during chemical transformations as well as in purification procedures in order to minimize loses of these materials. Another synthetic challenge is a result of necessity of building up molecules that have multiple double bonds of specified (*Z*) geometry in their structures. Even though many C=C formation methods have been discovered to date, their stereo-selectivities are typically highly dependent on the applied substrates and reaction conditions.[5]

Since linoleic acid (LA) contains only two double bonds, synthesis of its ω-alkynylated analogue (aLA) was approached first. Two different approaches were employed. First one involved building the fatty acid from scratch (starting from the unsaturated chain and introducing two alkene units in separate steps).

Syntheses of terminally modified linoleic acids have been successfully applied using this approach in the past.[6] Another strategy involved a cleavage of the 12,13-ene unit in the natural linoleic acid and reconstruction of that double bond with a simultaneous incorporation of a modification at the ω-position.

Arachidonic acid (AA) is more unsaturated than linoleate, so the strategy based on constructing double bonds one after another would not be very efficient, if used. The methodology involving the 14-15-ene unit breakage followed by reconstruction of this bond with an introduction of a modification was therefore chosen to be applied in order to prepare ω-alkynylated arachidonic acid (aAA).



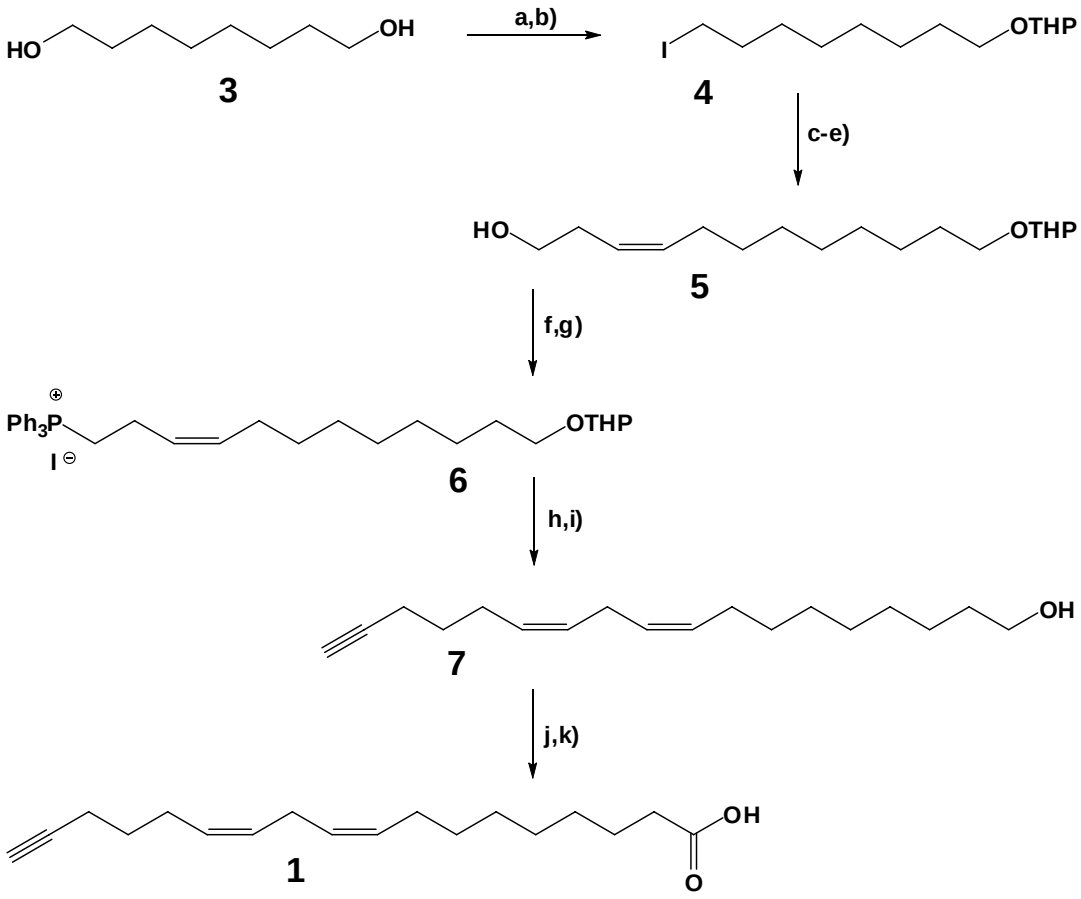
Scheme 5.1. Retro syntheses of ω-alkynylated PUFAs.

The methodology involving the 14-15-ene unit breakage followed by reconstruction of this bond with an introduction of a modification was therefore chosen to be applied in order to prepare ω-alkynylated arachidonic acid (aAA). This approach has also been used in the past to synthesize terminally modified arachidonic acid derivatives.[7-11] The retro syntheses of ω-alkynylated polyunsaturated fatty acids (aPUFAs) are presented in Scheme 5.1.

Synthesis of ω-Alkynylated Linoleic Acid from Scratch

The successful synthetic pathway consisted of eleven steps and was used to prepare multi-gram quantities of aLA, Scheme 5.2. 1,8-Octadiol was chosen as starting material, since it is inexpensive and easily obtainable. One of the hydroxyl groups of the substrate was protected throughout the synthesis and following deprotection was oxidized to the carboxylic acid moiety in the last step. The second hydroxyl moiety was used to build up the lipid chain of the aLA. Coupling of compound **4** and *t*-butyldimethylsilyl (TBDMS) protected 3-butynol in the presence of a strong base yielded internal alkyne, which upon terminal OH deprotection and controlled hydrogenation was converted to mono-ene compound **5**. The hydroxyl group of **5** was then transformed into phosphonium iodide **6** via standard procedures.[5] Compound **6** has already been reported in the literature and a small modification of the published procedure was applied in order to increase the overall efficiency of the synthesis.[6] The coupling between compound **4** and 3-butynol was found low yielding. This was most likely due to interactions between the alkoxyl moiety of the alkyne and the electrophilic

iodinated carbon, since application of TBDMS protected 3-butynol, in place of the unprotected analogue, increased the yield of the coupling significantly.[2]



Reagents and Conditions: (a) 2,3-Dihydro-*2H*-pyran (DHP), tosic acid (TsOH), CH_2Cl_2 (DCM), rt, 1h; (b) I_2, PPh_3, Im, Et_2O, MeCN, 0ºC -> rt, 90min, 50% (for a-b); (c) 3-butynyloxy-TBDMS, *n*-BuLi, THF, DMPU, 0ºC -> rt, overnight; (d) TBAF, THF, rt, 1h; (e) H_2, $Ni(OAc)_4$, $NaBH_4$, $Et(NH_2)_2$, EtOH, rt, 3h, 52% (for c-e); (f) I_2, PPh_3, Im, Et_2O, MeCN 0ºC -> rt, 90min; (g) PPh_3, PhMe, MeCN, reflux, 72h, 94% (for f-g); (h) *n*-BuLi, 5-hexynal, THF, HMPA, 0ºC -> rt, 2h; (i) TsOH, MeOH, rt, 2h, 42% (for h-i); (j) DMP, DCM, 0ºC -> rt, 15min; (k) Jones oxidation, 0ºC, 15min, 80% (for j-k).

Scheme 5.2. Synthesis of aLA from scratch.

Wittig reaction between phosphonium iodide **6** and 5-hexynal yielded THP protected (9,12)-octadeca-9,12-dien-17-yn-1-ol in reasonable yield. The newly formed double bond was found to have the desired *Z* geometry in *ca.* 95%.[12] An alternative route leading to this compound would involve Wittig reaction between the carboxylic acid analogue of compound **6** and 5-hexynylphosphonium halide.[13, 14] The strategy involving such transformation would be theoretically more efficient, since it would require fewer transformations of the longer chain substrate. This route has however not been tested, since the reaction involving carbonylated analogue of compound **6** with similar short chain phosphonium salt was reported to proceed with poor stereo-selectivity.[6]

Upon deprotection of the terminal hydroxyl group, compound **7** was obtained, which was further oxidized in two steps to yield aLA. Direct Jones oxidation of alcohol **7** required a few hours to reach completion, and during that time some conjugated diene hydroperoxides formed. The hydroperoxides were further transformed into corresponding dienones, under these strong dehydrating conditions. Even though the amount of these byproducts did not exceed 10%, they were not readily separable from the desired product. Apparently, conversion of the alcohol into the corresponding aldehyde was problematic in the Jones reagent, since that aldehyde underwent oxidation to form the desired carboxylic acid in a matter of minutes, under the same reaction conditions.

A conversion of the alcohol into the corresponding aldehyde was achieved in the presence of Dess-Martin periodinane. The reaction was very fast and almost quantitative and was followed by Jones oxidation to the target aLA.

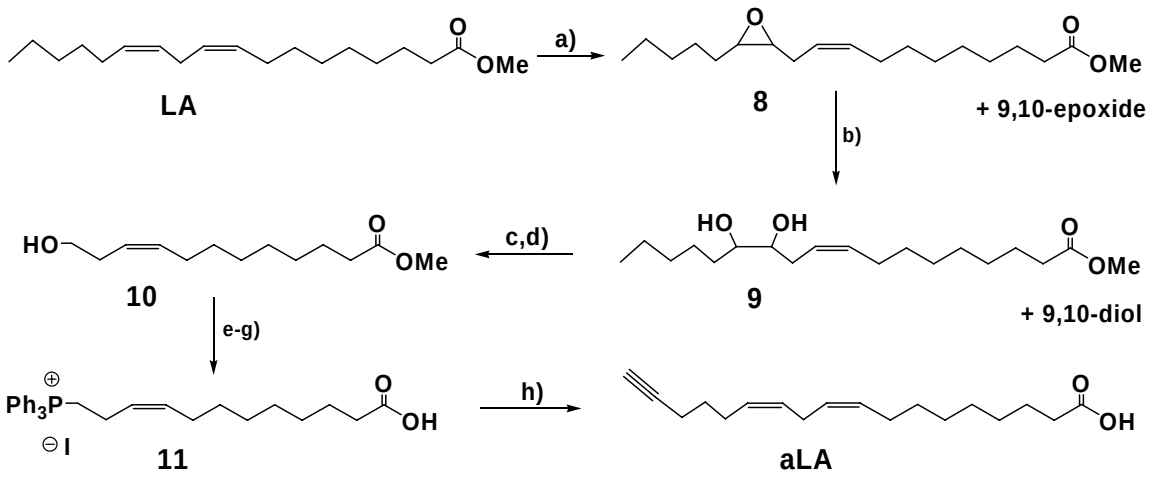
Chromatographic purification of the final product yielded compound **1** in *ca.* 8% yield over eleven steps. The high purity of the product (≥96% *Z*,*Z*) was confirmed by NMR and HPLC-UV.

Preparation of ω-Alkynylated Linoleic Acid from Methyl Linoleate

An alternative methodology has also been applied to prepare aLA (Scheme 5.3). This synthetic route starts from inexpensive methyl linoleate and involves fewer steps than the approach outlined above.[15] On the other hand, the overall yield for this methodology was only *ca.* 4% (a half of efficiency of the "*de novo*" route), therefore relatively large amounts of the starting material had to be utilized in order to obtain multiple grams of the final product. This required the use of oversized laboratory equipment. A big chromatography column, which could separate dozens of grams of oily reaction mixtures at a time proved especially useful.

The first step, oxidation of the double bonds of methyl linoleate in the presence of *m*-CPBA, yielded a regioisomeric mixture of epoxides (9,10- and 12,13-). This step was mainly responsible for decreasing the overall yield of the method. This essentially eqiumolar mixture was further hydrolyzed to yield a corresponding mixture of vicinal diols.[16-18] Oxidative cleavage of the mixture by periodate led to the formation of 4 aldehydes which were subsequently reduced to the corresponding alcohols: hexanol, 3-nonen-1-ol, methyl 9-hydroxynonanoate and (*Z*)-methyl 12-hydroxydodec-9-enoate (**10**). The unsaturated fatty ester alcohol **10** was then converted into phosphonium

iodide **11** utilizing standard protocols. The Wittig reaction between compound **11** and 5-hexynal led to the formation of the product **1**, which was found to be highly pure (≥96% *Z*,*Z*) by NMR and HPLC-UV.



Reagents and Conditions: (a) *m*-CPBA, DCM, 5ºC, 5h; (b) $HClO_4$, H_2O, THF, 5ºC, overnight; (c) $NaIO_4$, H_2O, THF, 0ºC, 60min; (d) $NaBH_4$, MeOH, 0ºC, 45min, 18% (for a-d); (e) I_2, PPh_3, Im, Et_2O, MeCN 0ºC -> rt, 2h; (f) LiOH, H_2O, THF, 5ºC, overnight, 80% (for e-f); (g) PPh_3, PhMe, MeCN, reflux, 48h, 77%; (h) $LiNH(TMS)_2$, 5-hexynal, THF, HMPA, -78ºC -> rt, 2.5h, 37%.

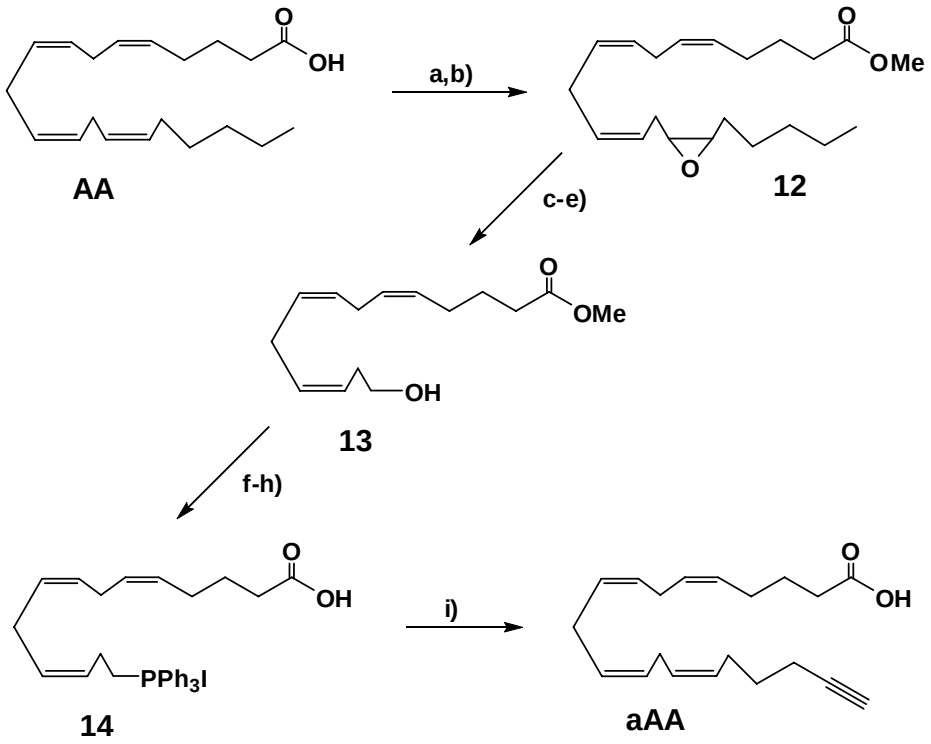
Scheme 5.3. Synthesis of aLA from methyl lineleate.

For syntheses of small amounts (<1g) of aLA, the method based on transformations of methyl linoleate is recommended. The same synthetic pathway may be applied during up-scaled preparations of the fatty acid, but only if bulky laboratory equipment is available. Otherwise, from scratch methodology is recommended to prepare multi-gram quantities of compound **1**.

Preparation of ω-Alkynylated Arachidonic Acid from its natural analogue

As already mentioned, due to the presence of four unsaturated bonds, the synthesis of modified arachidonic acid from small-molecule precursors would be difficult. The alternative approach involves the use of arachidonic acid as the precursor. A number of modifications to the ω-position of AA have been done by breaking the 14,15-double bond followed by its reconstruction by Wittig methodology.[7-11] All of these protocols were based on the strategy developed for selective 14,15-ene cleavage by Corey that involves regiospecific intramolecular epoxidation of AA followed by hydrolysis and oxidative cleavage of the 14,15 vicinal diol.[19] We describe here the use of this methodology to the synthesis of ω-alkynylated AA.

The majority of the double bond reconstructive strategies toward AA reported involve the Wittig reaction between (5*Z*,8*Z*,11*Z*)-methyl 14-oxotetradeca-5,8,11-trienoate (an aldehyde analogue of compound **13**, Scheme 5.4) with a short chain phosphonium salt. Although, this would provide a more direct route to aAA, it was observed that reaction between such an aldehyde and 5-hexynyltriphenyl-phosphonium salt led to formation of the AA isomer possessing a conjugated 11*Z*,13*E*-diene moiety in its structure.[20] The aldehyde was rarely isolated and characterized in the past due to its extreme instability. Since the bisallylic moiety formed in an alternative Wittig protocol (*vide supra*) was found stable, it is suggested that the formation of the conjugated product has occurred due to the double bond migration from the 11,12- to the 12,13-position of the aldehyde prior to its reaction with the ylid.



Reagents and Conditions: (a) Im_2CO, DCM, rt, 40min; then H_2O_2, LiIm (cat.), Et_2O, 0ºC -> rt, 12h; (b) Im_2CO, DCM, rt, 45min; then MeOH, rt, 2h, 55% (for a-b); (c) $HClO_4$, H_2O, THF, 5ºC, overnight; (d) $NaIO_4$, H_2O, THF, 0ºC, 1h; (e) $NaBH_4$, MeOH, 0ºC, 40min, 55% (for c-e); (f) I_2, PPh_3, Im, Et_2O, MeCN, 0ºC -> rt, 2h; (g) LiOH, H_2O, THF, 5ºC, overnight; h) PPh_3, PhMe, MeCN, reflux, 4d, 49% (for f-h); (i) $LiNH(TMS)_2$, 5-hexynal, THF, HMPA, -78ºC -> rt, 2.5h, 54%.

Scheme 5.4. Synthesis of aAA from its natural analogue.

An alternative Wittig transformation, the reaction between compound **14** and 5-hexynal was found to be relatively high yielding, Scheme 5.4. The phosphonium iodide was prepared in good yield by a standard protocol.[7] Utilizing the strategy outlined in the Scheme, aAA has been synthesized with *ca.* 8% overall yield over nine steps, and the high purity of the product

(≥96% *Z*,*Z*,*Z*,*Z*) has been confirmed by NMR and HPLC-UV. Due to relative cost of the starting material (AA) preparations of aAA have been conducted on a much smaller scale compared with syntheses of aLA.[15]

Experimental Section

General. All reagents and solvents were used as supplied commercially, except for HMPA, which was distilled from CaH_2 and stored over molecular sieves. Fatty acids/esters were purchased from Nu-Check Prep Inc. (Elysian, MN), whereas ω-alkynylated aldehydes were obtained from TCI America. All other chemicals were purchased from Sigma-Aldrich Company. Ethereal hydrogen peroxide was obtained by extraction of 30% aqueous H_2O_2 with diethyl ether. The concentration of the reagent in the ethereal layer was found by iodometric titrations. Commercially unavailable 5-Hexyn-1-ol was obtained via PCC oxidation of the corresponding alcohol.[21] TBDMS protected 3-butynol was synthesized in the reaction between the corresponding alcohol and TBDMS chloride.[22] Lithium imidazole was prepared in reaction of imidazole with butyl lithium.[23] All of the reactions described in this section, were performed under an atmosphere of argon. Purifications via flash column chromatography were carried out with silica gel 60 (230-400 mesh) from EMD Chemicals Inc. utilizing automated purification system from Biotage AB. Progress of reactions was monitored using F_{254} silica coated TLC plates purchased from EMD Chemicals Inc. Spots were visualized with UV and phosphomolybdic acid. ^1H and ^{13}C NMR spectra were recorded on 300MHz spectrometer in $CDCl_3$; individual peaks are

reported as (multiplicity, number of hydrogen atoms, coupling constants). HRMS analyses were performed in Laboratory for Biological Mass Spectrometry at Texas A&M University, College Station, TX.

2-(8-iodooctyloxy)tetrahydro-2H-pyran (4). This compound has been prepared from the diol **3** according to a previously published procedure, with a comparable yield.[6]

(*Z*)-12-(tetrahydro-2H-pyran-2-yloxy)dodec-3-en-1-ol (5). This compound has been prepared form the iodide **4** via a modified literature procedure.[6] To a solution of TBDMS protected 3-butynol (22.5g, 0.12mol) in THF (120mL) was added 2.5M *n*-BuLi in THF solution (55mL, 0.14mol) at 0ºC. After 30min of stirring, the mixture was cooled down to -78ºC and a solution of compound **4** in a mixture of HMPA and THF (30.3g, 89mmol; 40mL; 120mL) was added dropwise. The mixture was stirred overnight (during that time it was gradually brought to rt), quenched with water and extracted with EtOAc. The combined organic extracts were washed with brine, dried over $MgSO_4$ and condensed. The residue was chromatographed (1-10% EtOAc/Hexanes) to yield 26g of a colorless oil.

To this oily product (26g) dissolved in THF (270mL) was added 1M solution of TBAF in THF (120mL, 0.12mol). After 90min of stirring at rt, the mixture was poured onto brine and extracted with EtOAc. The combined organic layers were dried over $MgSO_4$ and condensed. The residue was chromatographed (10-30% EtOAc/Hexanes) to yield 13.5g of a colorless oil.

To the mixture of $Ni(OAc)_2$ x $4H_2O$ (11.8g) and $NaBH_4$ (1.78g) in EtOH (550mL) under H_2 atmosphere was added ethylenediamine (12mL) followed by a solution of oily alkyne product (13.5g) in EtOH (75mL). After 3h of stirring at rt, the mixture was filtered through celite, washed with EtOH, and the filtrate was condensed to leave *ca.* 80mL of the solution. Water was then added into it, and the product was extracted with hexanes. Organic layers were combined, dried over Na_2SO_4 and condensed. The residue was chromatographed (10-30% EtOAc/Hexanes) to yield a colorless oil (13.1g, 52% over 3 steps). NMR spectra of thus obtained compound **5** were identical with previously reported ones.[6]

(*Z*)-triphenyl(12-(tetrahydro-2H-pyran-2-yloxy)dodec-3enyl)phosphonium iodide (6). This compound has been prepared from the alcohol **5**, essentially according to a previously published procedure, with a comparable yield.[6] The only change that was made, involved modification of the conditions for the last reaction in the sequence (72h under reflux rather than 48h at 60ºC).

(9*Z*,12*Z*)-octadeca-9,12-dien-17-yn-1-ol (7). To a solution of compound **6** (28.4g, 43mmol) in HMPA (26mL) and THF (380mL) was added 2.5M *n*-BuLi solution in THF (17.2mL, 43mmol) at 0ºC. After 40min of stirring, 5-hexynal (3.9g, 41mmol) in THF (140mL) was added dropwise at 0ºC. The reaction mixture was stirred for additional 90min, and during that time it was gradually brought to rt. The reaction was quenched with water and the product was extracted with hexanes. Organic layers were combined, dried over $MgSO_4$ and condensed. The residue was chromatographed (0-5% EtOAc/Hexanes) to yield 8g of a pale

yellow oil. ^{1}H NMR (300MHz) δ 5.34 (m, 4H), 4.54 (m, 1H), 3.83 (m, 1H), 3.70 (m, 1H), 3.47 (m, 1H), 3.34 (m, 1H), 2.77 (t, 2H, *J* = 6.0Hz), 2.16 (m, 4H), 2.02 (q, 2H, *J* = 6.6Hz), 1.92 (t, 1H, *J* = 2.7Hz), 1.50-1.85 (m, 10H), 1.28 (m, 10H); ^{13}C NMR (300MHz) δ 130.3, 129.2, 128.6, 98.8, 84.5, 68.3, 67.6, 62.3, 30.7, 29.7, 29.6, 29.4, 29.2, 28.3, 27.2, 26.2, 26.1, 25.6, 25.4, 19.6, 17.8; HRMS Calcd for $C_{23}H_{38}O_2Li^+$ $(M+Li)^+$ 353.3032, found 353.3027.

To a solution of this oily product (8g) in MeOH (250mL) was added TsOH x H_2O (1.2g) at rt. After 2h of stirring, reaction was quenched with 5% Na_2CO_3 (30mL), the mixture was concentrated and partitioned between aqueous phase and hexanes. Organic layers were combined, dried over $MgSO_4$ and condensed. The residue was chromatographed (5-20% EtOAc/Hexanes) to yield 4.8g of a pale yellow oil. ^{1}H NMR (300MHz) δ 5.36 (m, 4H), 3.64 (t, 2H, *J* = 6.6Hz), 2.80 (t, 2H, *J* = 6.0Hz), 2.20 (m, 4H), 2.05 (m, 2H), 1.96 (t, 1H, *J* = 2.7), 1.59 (m, 4H), 1.31 (m, 10H); ^{13}C NMR (300MHz) δ 130.2, 129.2, 128.6, 127.7, 84.3, 68.3, 63.0, 32.7, 29.6, 29.4, 29.3, 29.2, 28.3, 27.2, 26.1, 25.7, 25.6, 17.8; HRMS Calcd for $C_{18}H_{30}OLi^+$ $(M+Li)^+$ 269.2457, found 269.2468.

(9*Z*,12*Z*)-octadeca-9,12-dien-17-ynoic acid (1). To a solution of alcohol **7** (4.8g, 18mmol) in DCM (100mL) was added Dess-Martin periodinone (8.2g, mmol) at 0ºC. The reaction mixture was stirred 10min at 0ºC and then for 5min at rt. A precipitate formed during the reaction was filtered off and washed with Et_2O. The filtrate was condensed and chromatographed (0-10% EtOAc/Hexanes) to yield 4.4g of a pale yellow oil.

To a solution of this oily product (4.5g, 17mmol) in acetone (350mL) was added Jones reagent (5.1mL of the mixture prepared from 2g of CrO_3, 2mL of fuming H_2SO_4 and 6mL of H_2O) at 0ºC. After stirring for 15min, a precipitate formed during the reaction was filtered off, washed with acetone, and the filtrate was condensed. The residue was partitioned between an aqueous phase and EtOAc. Organic layers were combined, washed with brine, dried over $MgSO_4$ and condensed. The residue was chromatographed (1-30% EtOAc/hexanes cont. 0.1% AcOH) to yield the product as a pale yellow oil (4.0g, 80% over 2 steps). ^{1}H NMR δ 5.34 (m, 4H), 2.77 (t, 2H, *J* = 6.0Hz), 2.33 (t, 2H, *J* = 7.5Hz), 2.17 (m, 4H), 2.01 (m,2H), 1.94 (s, 1H), 1.60 (m, 4H), 1.30 (m, 8H); ^{13}C NMR (300MHz) δ 179.5, 130.1, 129.1, 128.6, 127.8, 80.5, 68.3, 33.9, 29.5, 29.1, 29.0, 28.9, 28.3, 27.1, 26.1, 25.6, 24.6, 17.8; HRMS Calcd for $C_{18}H_{27}0_2^-$ $(M-H)^-$ 275.2011, found 275.2023.

This compound was also prepared via an **alternative route**. To a solution of phosphonium iodide **11** (10.8g, 18.5mmol) in THF (225mL) and HMPA (55mL), was added over a 10min $LiNH(TMS)_2$ solution (1M in THF, 38.6mL) at -78ºC. After addition of the base, the reaction mixture was stirred at -40ºC for 1h, and then it was brought back to -78ºC and 5-hexynal (1.74g, 18.1mmol) in THF (60mL) was added in few portions. The mixture was then allowed to gradually warm up to rt. over 1.5h. The reaction was quenched with water, and the pH of the mixture was brought to *ca.* 3 with 1M HCl. The mixture was partitioned between water (250mL) and EtOAc (4 x 200mL), and organic layers were combined and condensed. The residual liquid was dissolved in Et_2O (150mL)

and it was washed with brine (5 x 150mL), the ethereal layer was then dried $MgS0_4$ and condensed. The residue was chromatographed (1-30% EtOAc/hexanes) to yield the product as a pale yellow oil (1.89g, 37%). Analytical data for thus obtained compound **1** matched well with the data collected for this products prepared from the alcohol **7** (vide supra).

Methyl (*Z*)-12-hydroxydodec-9-enoate (10). To a solution of methyl linoleate (100g, 0.34mol) in DCM (2.6L), was added *m-CPBA* (75g, 0.43mol) at 5ºC. After 5h, the reaction mixture was poured into sat. $NaHCO_3$ solution (1L). Phases were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were dried with $MgSO_4$, condensed, and the residue was chromatographed (1-6% EtOAc/hexanes) to yield 80g of a colorless oily mixture of epoxide **8** and its regioisomer.[4]

To the mixture of epoxides (80g, 0.26mol) in THF (1.8L) at 5ºC was added dropwise a cold 5% $HClO_4$ solution (650mL). The mixture was stirred overnight at 5ºC, and neutralized with 10% $NaHCO_3$ solution. The majority of the organic solvent was evaporated, and the residual material was extracted with ethyl acetate. The combined organic layers were condensed to *ca.* 1L, washed with brine, and dried over $MgSO_4$. Evaporation of solvents gave yellowish oil, which was chromatographed (5-30% EtOAc/hexanes) to yield 42g of the colorless oily mixture of diol **9** and its regioisomer.

To the mixture of diols (42g, 0.13mol) in THF (420mL) and water (160mL) at 0ºC, solid $NaIO_4$ (42g, 0.20mmol) was added, and the resulting suspension was vigorously stirred for 1h. The mixture was then partitioned between water

and EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4 and condensed. The residual scented oil was dissolved in MeOH (400mL), and it was treated with solid $NaBH_4$ (16g, 0.42mol) at rt. After 45min, water was added (800mL) and the mixture was stirred for additional 5min, followed by an extraction with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$ and condensed. The residue was chromatographed (10-22% EtOAc/hexanes) to yield compound **10** as a colorless oil (13.6g, 18% over 3 steps). ^{1}H NMR (300MHz) δ 5.55 (m, 1H), 5.37 (m, 1H), 3.67 (s, 3H), 3.65 (t, 2H, *J* = 6.6Hz), 2.32 (m, 4H), 2.06 (q, 2H, *J* = 6.6), 1.62 (m, 2H), 1.31 (m, 8H); ^{13}C NMR (300MHz) δ 174.4, 133.3, 125.0, 62.3, 51.4, 34.0, 30.8, 29.5, 29.0, 28.9, 27.2, 24.9; HRMS Calcd for $C_{13}H_{24}LiO_3^+$ $(M+Li)^+$235.1885, found 235.1890.

(*Z*)-11-(carboxyundec-3-enyl)triphenylphosphonium iodide (11).

To a solution of compound **10** (13.6g, 60mmol) in Et_2O (220mL) and MeCN (80mL) were added imidazole (5.73, 85mmol), PPh_3 (23.6g, 90mmol) and iodide (21.5g, 85mmol) at 0ºC. After 30min, mixture was brought to rt. and stirred for additional 1.5h. The resulting suspension was washed with 10% Na_2SO_3 solution (350mL), organic layer was subsequently dried over $MgSO_4$ and condensed. The residual material was chromatographed (1-5% EtOAc/hexanes) to yield 18g of a mixture of methyl (*Z*)-12-iodododec-9-enoate and PPh_3 *(ca. 20:1* mol/mol).

The mixture was dissolved in THF (850mL) and water (160mL), and the solution was degassed by argon bubbling for 30min before and 30min after addition of 1M LiOH solution (120mL) at 5ºC. The Mixture was reacted overnight

at 5ºC, and the pH of the solution was brought to 3 with 1M HCl. THF was evaporated, and the residual material was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$ and condensed. The residue was chromatographed (6-40% EtOAc/hexanes) to yield 15.4g of (*Z*)-12-iodododec-9-enoic acid as a colorless oil. ^{1}H NMR (300MHz) δ 5.52 (m, 1H), 5.32 (m, 1H), 3.14 (t, 2H, *J* = 7.2Hz), 2.63 (q, 2H, *J* = 7.2Hz), 2.35 (t, 2H, *J* = 7.5Hz), 2.02 (m, 2H), 1.62 (m, 2H), 1.32 (m, 8H); ^{13}C NMR (300MHz) δ 180.1, 132.5, 127.8, 34.0, 31.4, 29.3, 29.1, 29.0, 28.9, 27.3, 24.6; HRMS Calcd for $C_{12}H_{21}ICIO_2^-$ $(M+Cl)^-$ 359.0275, found 359.0282.

To a solution of compound the above acid (15.4g, 48mmol) in toluene (105mL) was added PPh_3 (18.1g, 69mmol) in MeCN (37mL). The mixture was refluxed for 96h, solvent was removed and the residual thick yellow oil was chromatographed (1-17% MeOH/AcOEt) yielding a mixture of compound **11** and silica gel, upon condensation. The mixture was partially dissolved in DCM, and the silica particles were filtered off. The filtrate was condensed to give the product as a yellow semi-solid (21.6g, 62% over 3 steps). ^{1}H NMR (300MHz) δ 7.77 (m, 15H), 5.53 (m, 1H), 5.49 (m, 1H), 3.73 (m, 2H), 2.44 (m, 2H), 2.33 (t, 2H, *J* = 7.2Hz), 1.78 (m, 2H), 1.58 (m, 2H), 1.21 (m, 8H); ^{13}C NMR (300MHz) δ 177.7, 135.2, 135.1, 133.8, 133.6, 132.7, 130.6, 130.5, 125.9, 125.7, 118.6, 117.5, 33.9, 29.0, 28.8, 28.7, 27.1, 24.6, 23.7, 23.1, 20.6; HRMS Calcd for $C_{30}H_{36}O_2P^+$ $(M-I)^+$ 459.2453 , found 459.2446.

Methyl (5*Z*,8*Z*,11*Z*)-14,15-epoxyeicosa-5,8,11-trienoate (12). This compound was prepared based on previously published procedures that somewhat differed in experimental detailes.[7, 10, 19] The obtained yield was much lower then expected. Therefore, the detailed procedure that has been applied is reported. To a solution of arachidonic acid (5.0g, 16.4mmol) in DCM (50mL) was added 1,1'-carbonyldiimidazole (2.8g, 17.3mmol). After 40min of stirring, the resulting arachidonyl imidazolide solution was added over a 5min to a cold (*ca.* 5ºC) 3.6M ethereal solution of hydrogen peroxide (120mL) containing lithium imidazole (25mg, 0.34mmol). Followed the addition, mixture was stirred for 5min, diluted with DCM (125mL) and treated with powdered anhydrous $KHSO_4$ (35g). The mixture was stirred for another 5min, solids were then removed by filtration and the resulting filtrate was kept over anhydrous Na_2SO_4 (10g) overnight at rt., under an argon atmosphere. The drying agent was filtered off, and the filtrate was washed with brine (5 x 50mL). The organic layer was then dried over $MgSO_4$, and solvents were removed yielding a colorless oil. The oil was dissolved in DCM (45mL) and it was treated with 1,1'-carbonyldiimidazole (4.2g, 26mmol). After 45min, MeOH (8mL) was added and the mixture was stirred for additional 2h. Solvents were evaporated, and the residue was partitioned between water and ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$ and condensed. The residue was chromatographed (1-7% EtOAc/hexanes) to yield compound **12** as a colorless oil (3.0g, 55%). Spectral characterization data set for thus obtained product was identical with already published data set.[10]

Methyl (5*Z*,8*Z*,11*Z*)-14-hydroxytetradeca-5,8,11-trienoate (13). The hydrolysis part of the following protocol was written based a literature report,[10] whereas the conditions for other reactions have been optimized experimentally. To a solution of compound **12** (3.0g, 9.1mmol) in THF (80mL) at 5ºC was added dropwise a cold 10% $HClO_4$ solution (27mL). The mixture was stirred overnight at 5ºC, and it was then neutralized with solid $NaHCO_3$. The majority of the organic solvent was evaporated, and the residual material was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$ and condensed. The residue was chromatographed (10-30% EtOAc/hexanes) to yield 2.4g of (5*Z*,8*Z*,11*Z*)-methyl 14,15-dihydroxyicosa-5,8,11-trienoate as a colorless oil.

To a mixture of that diol (2.4g, 6.8mmol), THF (30mL) and water (12mL) at 0ºC, solid $NaIO_4$ (2.18g, 10.2mmol) was added, and the resulting suspension was vigorously stirred for 1h. Mixture was then partitioned between water (40mL) and EtOAc (4 x 20mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and condensed. The residual scented oil was dissolved in MeOH (20mL), and it was treated with solid $NaBH_4$ (0.86g, 22.7mmol) at 0ºC. After 35min, water was added (40mL) and the mixture was stirred for additional 5min, followed by an extraction with Et_2O. The combined organic layers were washed with brine, dried over $MgSO_4$ and condensed. The residue was chromatographed (10-30% EtOAc/hexanes) to yield the product as a colorless oil (1.25g, 55% over 3 steps). Analytical data for thus obtained compound **13** matched well with the data presented for this compound in the literature.[7]

(3*Z*,6*Z*,9*Z*)-13-carboxytrideca-3,6,9-trienyl)triphenylphosphonium iodide (14). To a solution of compound **13** (1.25g, 5.0mmol) in Et_2O (18mL) and MeCN (6mL) was added imidazole (0.47g, 7.0mmol), PPh_3 (1.97g, 7.5mmol) and iodide (1.78g, 7.0mmol) at 0ºC. After 30min, mixture was brought to rt. and stirred for additional 1.5h. The resulting suspension was washed with 10% Na_2SO_3 solution (25mL), organic layer was subsequently dried over $MgSO_4$ and condensed. The residual material was chromatographed (1-5% EtOAc/hexanes) to yield 1.6g of a mixture of Methyl (5*Z*,8*Z*,11*Z*)-14-iodotetradeca-5,8,11-trienoate and PPh_3 (*ca.* 20:1 mol/mol).

The mixture was dissolved in THF (60mL) and water (12mL), and the solution was degassed by argon bubbling for 20min before and 20min after addition of a cold 1M LiOH solution (9mL) at 5ºC. Mixture was reacted overnight at 5ºC, and the pH of the solution was brought to 3 with 1M HCl. THF was evaporated, and the residual material was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$ and condensed. The residue was chromatographed (10-40% EtOAc/hexanes) to yield 1.4g of (5*Z*,8*Z*,11*Z*)-14-iodotetradeca-5,8,11-trienoic acid as a colorless oil. ^{1}H NMR (300MHz) δ 5.52 (m, 1H), 5.37 (m, 5H), 3.15 (t, 2H, *J* = 7.2Hz), 2.81 (m, 4H), 2.67 (q, 2H, *J* = 7.2Hz), 2.38 (t, 2H, *J* = 7.5Hz), 2.14 (m, 2H), 1.72 (m, 2H); ^{13}C NMR (300MHz) δ 179.9, 130.3, 128.9, 128.8, 128.4, 128.3, 127.7, 33.4, 31.4, 26.4, 25.8, 25.6, 24.4; HRMS Calcd for $C_{14}H_{21}IO_2Cl^-$ $(M+Cl)^-$ 383.0275, found 383.0270.

To a solution of compound this oily compound (1.4g, 4.0mmol) in toluene

(9.6mL) was added PPh_3 (1.57g, 6.0mmol) in MeCN (3.8mL). After the mixture was refluxed for 96h, solvent was removed and the residual thick yellow oil was chromatographed (1-23% MeOH/AcOEt) yielding upon condensation a mixture of product and silica gel. The mixture was partially dissolved in DCM, and the silica particles were filtered off. The filtrate was condensed to give salt **14** as a yellow semi-solid material (1.5g, 49%). ^{1}H NMR (300MHz) δ 7.77 (m, 15H), 5.53 (m, 1H), 5.30 (m, 5H), 3.75 (m, 2H), 2.63 (m, 4H), 2.49 (m, 2H), 2.34 (t, 2H, *J* = 7.2Hz), 2.05 (m, 2H), 1.64 (m, 2H); ^{13}C NMR (300MHz) δ 177.4, 135.2, 135.1, 133.7, 133.6, 130.7, 130.6, 130.5, 129.0, 128.7, 128.5, 127.0, 126.3, 126.1, 118.5, 117.4, 33.4, 26.4, 25.6, 24.4, 23.5, 22.9, 20.3; HRMS Calcd for $C_{32}H_{36}O_2P^+$ $(M\text{-}I)^+$ 483.2453, found 483.2447.

(5*Z*,8*Z*,11*Z*,14*Z*)-eicosa-5,8,11,14-tetraen-19-ynoic acid (2). To a solution of phosphonium iodide **14** (1.5g, 2.4mmol) in THF (30mL) and HMPA (7mL) was added dropwise 1M solution of $LiNH(TMS)_2$ in THF (4.8mL, 4.8mmol) at -78ºC. After addition of the base, the mixture was stirred at -40ºC for 40min, and then it was brought back to -78ºC and 5-hexynal (0.23g, 2.4mmol) in THF (9mL) was added dropwise. The mixture was then allowed to gradually warm up to rt. over a period of 100min. The reaction was quenched with water, and the pH of the mixture was brought to 3 with 1M HCl. The mixture was partitioned between water (50mL) and EtOAc (4 x 30mL), organic layers were combined and condensed. The residual liquid was dissolved in Et_2O (30mL) and it was washed with brine (5 x 30mL), the ethereal layer was then dried over $MgSO_4$ and condensed. The residue was chromatographed (1-28% EtOAc/hexanes) to yield

compound **2** as a pale yellow oil (0.39g, 54%). ^{1}H NMR (300MHz) δ 5.35 (m, 8H), 2.82 (m, 6H), 2.35 (t, 2H, *J* = 7.5Hz), 2.18 (m, 6H), 1.94 (s, 1H), 1.70 (m, 2H), 1.60 (m, 2H); ^{13}C NMR (300MHz) δ 179.5, 129.0, 128.9, 128.8, 128.7, 128.3, 128.2, 128.1, 128.0, 127.9, 84.4, 68.4, 33.2, 28.2, 26.4, 26.1, 25.6, 25.5, 24.4, 17.8; HRMS Calcd for $C_{20}H_{27}0_2^-$ $(M-H)^-$ 299.2011, found 299.2009.

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CHAPTER VI

PROPERTIES OF ω-MODIFIED FATTY ACIDS

Introduction

The ultimate use of alkynylated phospholipids is the investigation of protein-lipid interactions in biological fluids, tissues and isolated cells (*vide supra*) that result from lipid peroxidation. We report in this chapter several studies that confirm the utility of the compounds. We first investigated the radical oxidation of ω-alkynylated linoleate and arachidonate to determine if their radical oxidation chemistry was analogous to the authentic lipids. We chose to study the oxidation chemistry of alkynylated fatty acids instead of modified phospholipids. It was anticipated that free PUFAs would undergo essentially the same types of transformations as their esters and the analysis of aLA and aAA oxidation products was expected to be easier than analysis of products derived from oxidation of phospholipids.[1] All of the studies presented in this chapter involved side by side oxidations of aPUFAs with their natural analogues and the products obtained from these transformations were directly compared. The analyses were typically qualitative; however some quantitative data has also been collected. Major primary oxidation products of aLA and aAA have been well characterized, whereas identification of aPUFAs secondary oxidation products, due to their number, have only been investigated in preliminary studies. ω-Alkynylated 4-hydroxynonenal (aHNE) was an exception to this general approach, its

formation from aLA and aAA was confirmed in several studies.[2, 3] This compound was also used as a model electrophile in adduction studies to lysine containing peptides. Formation of other highly reactive electrophiles, alkynylated isoketals and cyclopentenone isoprostanes, has been confirmed in peptide/protein adduction studies and enzymatic transformations of aLA and aAA have been investigated as well, Products of the lipids promoted by lipoxygenase and cyclooxygenase were characterized and relevant kinetic parameters have been measured.

Formation of Primary Oxidation Products from Alkynylated Linoleic Acid

On peroxidation of linoleic acid four conjugated diene hydroperoxides (HPODEs) are produced as primary products. Formation of analogous, ω-alkynylated compounds (aHPODEs) was therefore expected to result from oxidation of aLA. Both LA and aLA were therefore incubated in benzene, in the presence of radical initiator MeOAMVN. After the radical reaction was terminated by addition of an antioxidant, PPh_3 was added to the reaction mixtures in order to reduce (a)HPODEs to the corresponding alcohols (a)HODEs, which are more stable and better suited for analysis. Sample chromatograms profiling conjugated dienols formed during these incubations are shown in Figure 6.1.

Upon incubation of an equimolar mixture of both substrates, similar amounts of HODEs and aHODEs were obtained. The quantification statements presented above were based on the assumption that extinction coefficients (ε_{235nm}) for HODEs and aHODEs were equal.

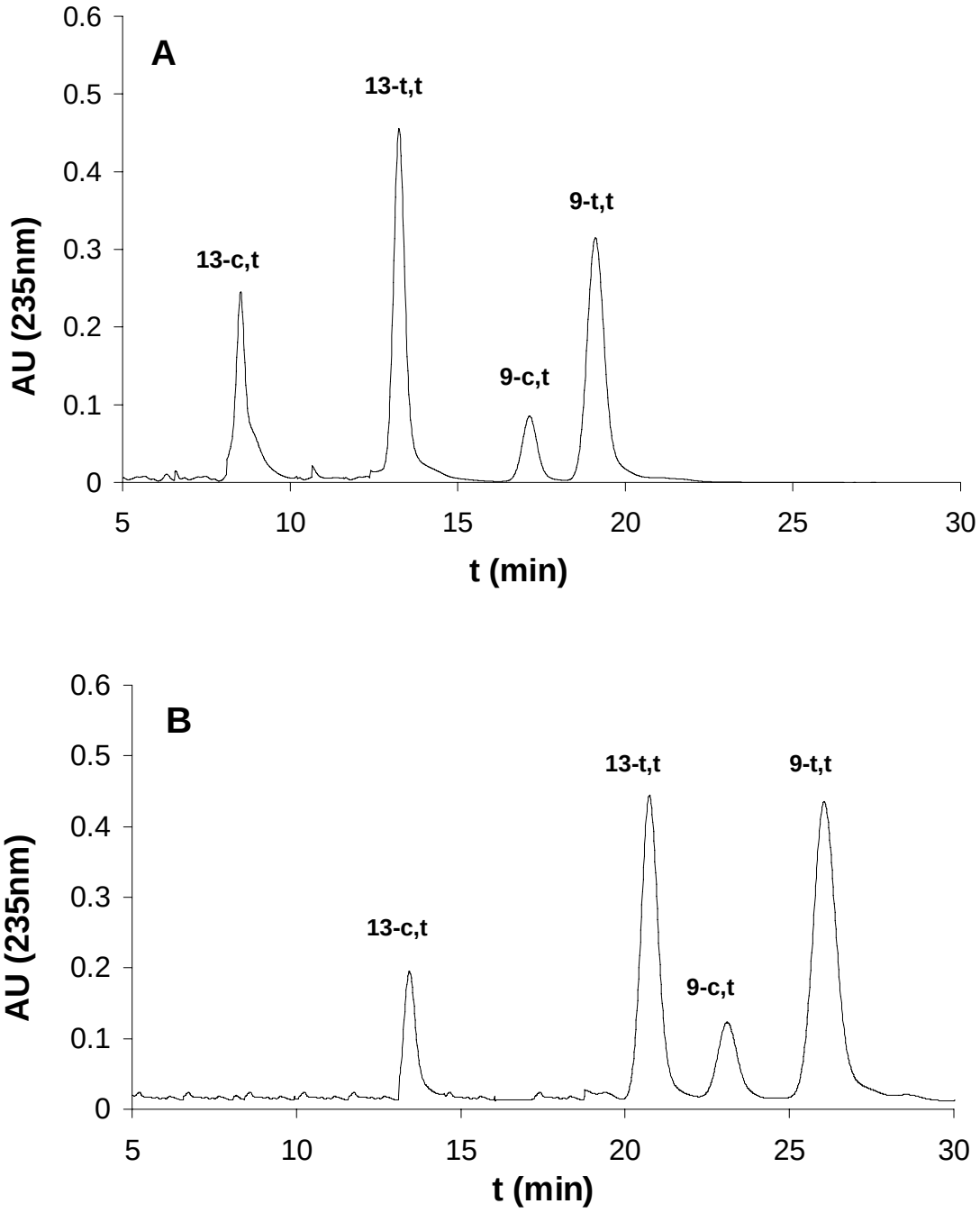


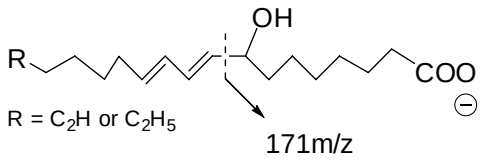
Figure 6.1. Elution profiles for conjugated dienol products obtained upon incubation of 4.5mg of LA (**A**) or aLA (**B**) with MeOAMVN (1.8mg) in benzene (200μL) for 45min at 37ºC, followed by reduction of the corresponding hydroperoxides with PPh_3. Separation conditions are given in the experimental section.

This assumption was justified, since an isolated alkyne was found not to significantly absorb light at 235nm, and the strong chromophores - conjugated dienols, were identical in both classes of compounds. Additional support for equal oxidizability of modified and natural linoleic acid was obtained from to similarities of the observed ratios of *c*,*t*/*t*,*t* oxidation products.[4] From these ratios of kinetic (*c*,*t*) to thermodynamic (*t*,*t*) products, it could be concluded that bisallylic hydrogen atoms abstractabilities in LA and aLA were approximately equal.[5]

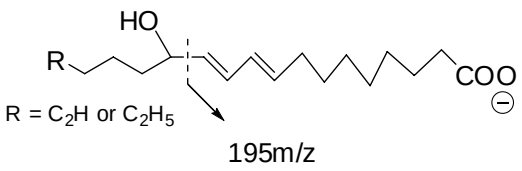
To confirm that aHODEs were the products obtained from modified linoleic acid, the compounds were isolated by HPLC and analyzed by MS and NMR. The MS fragmentation profiles for HODEs have been previously reported.[6] Upon cleavage of the weakest bonds of the deprotonated HODEs (bonds between hydroxylated carbon and the neighboring sp^2 carbon atom), fragments of 171m/z (9-isomers) and 195m/z (13-series) have been observed. Since these fragments do not contain ω-carbons of the precursor in their structures, identical ions are expected to be observed during fragmentation of aLA, Scheme 6.1. MS analyses of the isolated aHODEs were performed via direct injections of solutions of HODEs into the ESI-MS source. Negatively charged parent ions at 291m/z were fragmented in the triple quadrupole setup, and locations of the hydroxyl moieties were deduced from the observed daughter ions (171 or 195m/z).

Even though the above MS^2 experiments suggested that the two initially eluted peaks in Figure 6.1B were 13-aHODEs, whereas the remaining two peaks were 9-aHODEs, the geometry of the double bonds was unknown. In order to solve this issue, NMR experiments were conducted.[7]

9-HODEs (295m/z) **/ 9-aHODEs** (291m/z)



13-HODEs (295m/z) **/ 13-aHODEs** (291m/z)



Scheme 6.1. Regiospecific fragmentation of (a)HODEs.

Determination of coupling constants between vinyl protons permits the geometry of a carbon-carbon double bond to be deduced. Typically, if a coupling constant (*J*) is greater than 14Hz, the bond has trans geometry.[7, 8] Spectra of the second and last eluting compounds in Figure 6.1B showed that all the vinyl protons coupled with 15±0.5Hz frequency, suggesting *t*,*t* geometry of these dienes. Spectra of the first and third eluting compounds showed only two vinyl protons coupled with *J*=15Hz, therefore the geometry of these dienes was assigned to be *c*,*t*. Furthermore, 2-D COSY NMR experiments confirmed that the compounds of interest were indeed conjugated dienols.[9] Structural analysis analogous to the one presented above was performed in the past for linoleate-derived HODEs.[7, 10] Based on the above results, it is concluded that the elution order of the isomeric ω-alkynylated HODEs is the same as the elution order observed for native linoleic acid oxidation products - HODEs, Figure 6.1.[11] Sample proton spectra for 9-HODEs are shown in Figure 6.2.

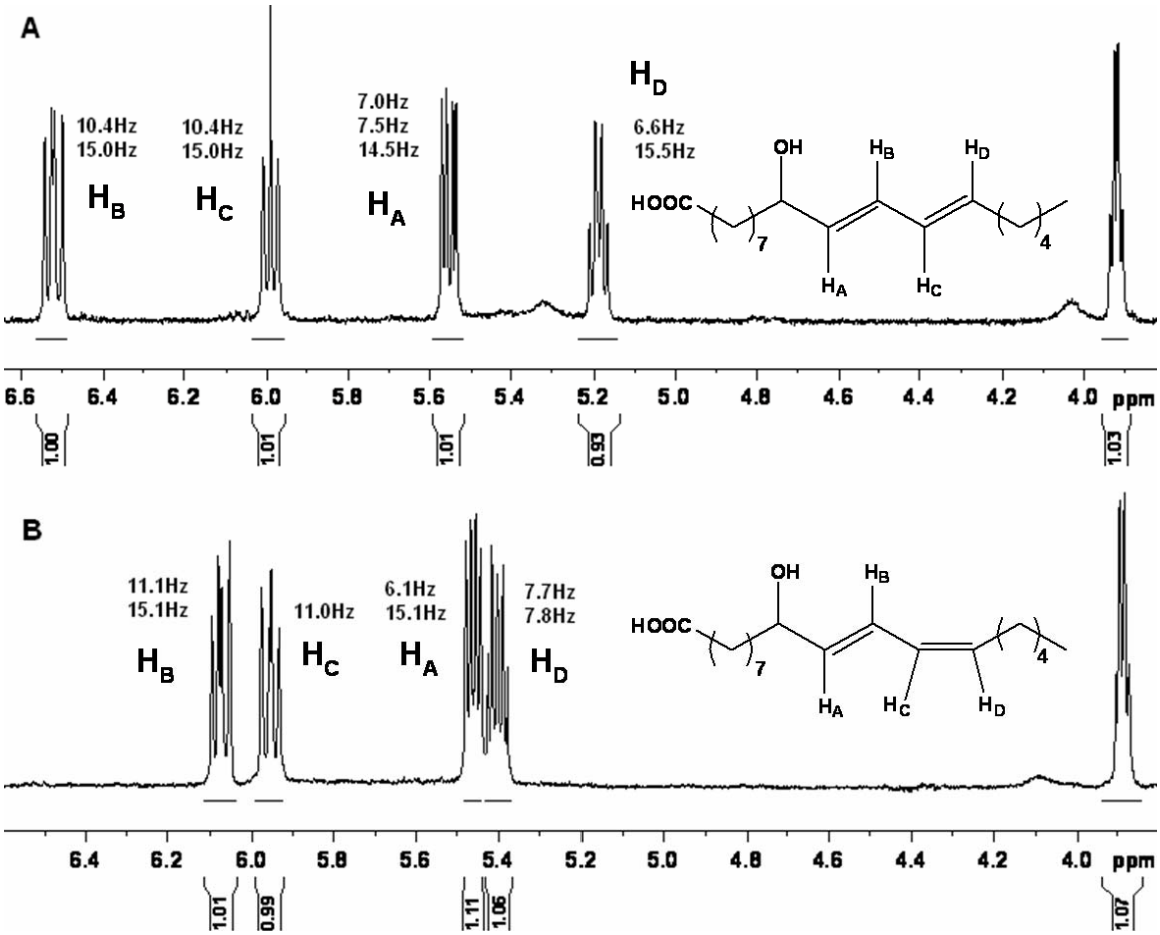


Figure 6.2. Sample ^{1}H NMR spectra of 9-*t,t*-aHODE (A) and 9-*c,t*-aHODE (B).

Formation of Acyclic Primary Oxidation Products from Alkynylated Arachidonic Acid

Peroxidation of arachidonic acid yields a mixture of acyclic (HPETEs) and cyclic hydroperoxides as primary products (vide *supra*). Due to formation of numerous products, the isolation of sufficient amounts of the acyclic compounds that were required for their full characterization was problematic under typical, MeOAMVN initiated, uninhibited oxidation condition. On the other hand, when

α-TOH was present during the oxidation as a hydrogen atom donor, the formation of HPETEs (or their ω-alkynylated analogues, aHPETEs) was nearly exclusive, but the yields obtained from inhibited peroxidation was understandably poor. In order to produce quantities (*ca.* 100μg) of aHPETEs sufficient for analysis, oxidations were conducted in the presence of *N*-methyl benzohydroxamic acid (NMBHA), an efficient catalyst for radical based oxygen insertion reactions.[12] Analysis was performed on (a)HETEs, formed by reduction of the corresponding hydroperoxides. Since six regioisomeric HETEs are formed during oxidation of AA, the same number of products is expected from oxidation of aAA.[13] Sample chromatograms profiling conjugated dienols obtained from the oxidations in the presence of α-TOH are shown in Figure 6.3.

As shown in the below chromatograms, the elution profile for aHETEs was similar to the profile obtained for their natural analogues. In both mixtures, ratios of the products were close to 1:1:1:1:1:1, which was expected from the inhibited peroxidation reaction.[14] Furthermore, total amounts of acyclic hydroperoxides formed from AA and its alkynylated analogue were nearly identical.[15] Additionally, oxidation of equimolar mixtures of AA and aAA yielded nearly equimolar mixtures of HETEs and aHETEs, suggesting similar oxidizabilities for both substrates (data not shown).

To confirm the structures of peroxidation products obtained from modified arachidonic acid, compounds were isolated by HPLC and analyzed by MS and NMR. The MS analysis of aHETEs were similar to those observed for aHODEs.[7]

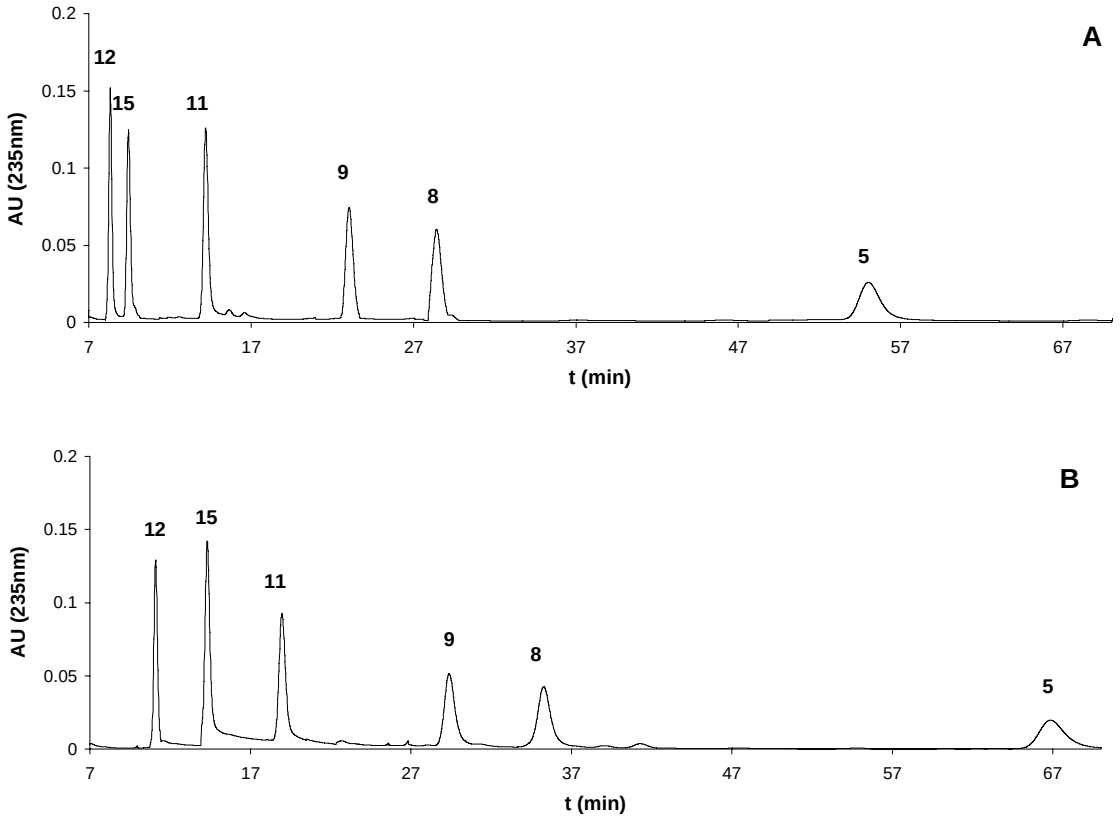
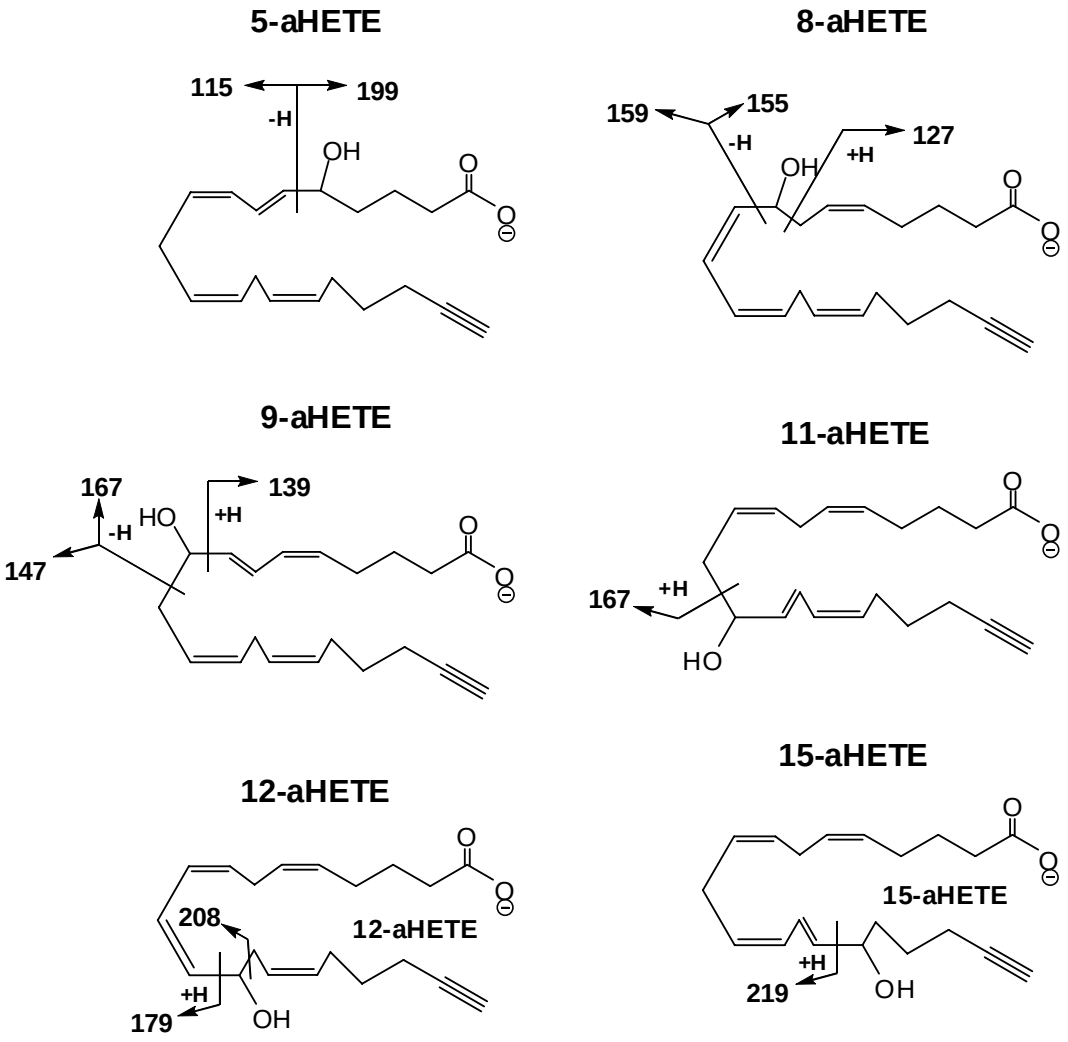


Figure 6.3. Elution profiles for conjugated dienol products obtained upon incubation of 2mg of AA (**A**) or aAA (**B**) with α-TOH (100μg) in thin film for 24h at 37ºC, followed by reduction of the corresponding hydroperoxides with PPh_3. Separation conditions are given in the experimental section.

The fragmentation patterns for HETEs, however, gave typically more than one characteristic ion, and some of the spectra showed ω-terminal fragments of the precursor.[16] Fragmentation of the deprotonated aAA yielded the expected daughter ions (Scheme 6.2) from which locations of the hydroxyl moieties were deduced. A sample tandem mass spectrum for 5-aHETE is presented in Figure 6.4.



Scheme 6.2. Regiospecific fragmentation of aHETEs.

Since all of the conjugated dienes formed during inhibited peroxidation of AA were shown to have *c*,*t*-geometry,[17] NMR structural analysis has been conducted on the isolated aHETEs to support the expected analogy in the double bond geometry. 1-D and 2-D NMR experiments were conducted in the way similar to the aHODEs. Careful analyses of the spectra (data not shown) supported the expected *c*,*t*-configuration of the conjugated dienes in aHETEs.

All the above experiments taken together confirmed that the elution order of the isomeric ω-alkynylated HETEs is the same as the elution order observed for native arachidonic acid oxidation products, HETEs (Figure 6.3).[13]

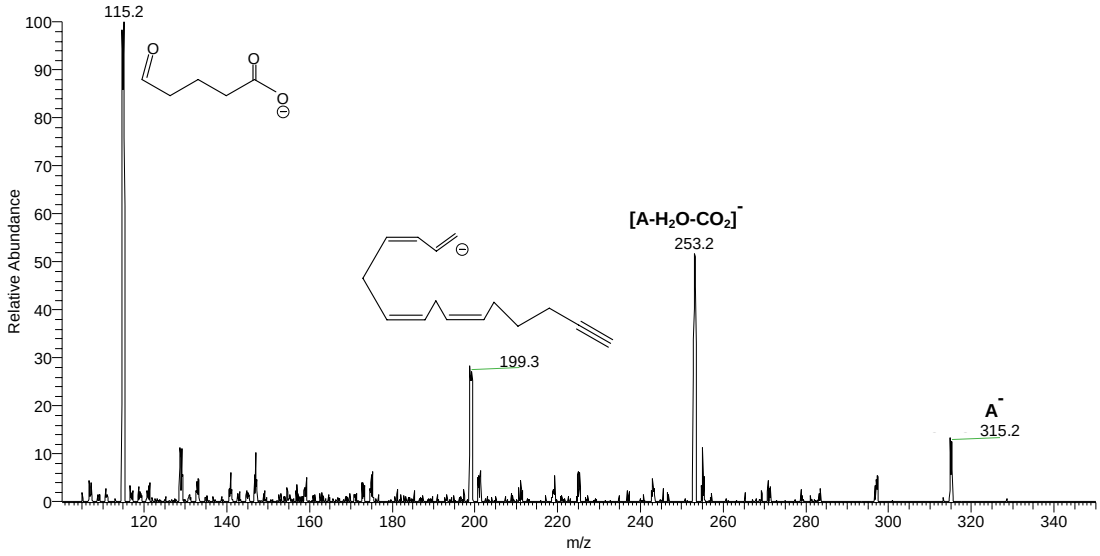


Figure 6.4. Sample ESI-MS^2 spectrum obtained for isolated 5-aHETE.

Formation of Some Cyclic Primary Oxidation Products from Alkynylated Arachidonic Acid

Besides HETEs, a number of cyclic primary oxidation products are obtained from AA during free radical oxidation (*vide supra*). Bicyclic endoperoxides (G_2-IsoPs) are of special interest, since they are known to be precursors for many biologically active compounds.[18-21] In order to confirm the formation of ω-alkynylated analogues of bicyclic endoperoxides (aG_2-IsoPs) from aAA, it was incubated in aprotic solvent in the presence of the free radical

initiator MeOAMVN.[22] The aG_2-IsoPs generated in this way were further converted into the alkynylated F_2 isoprostanes (aF_2-IsoPs) by $P(OMe)_3$ in wet acetonitrile. Arachidonic acid was subjected to identical transformations in experiments carried out side by side with aAA. HPLC elution profiles for major (a)F_2-IsoPs are shown in Figure 6.5.

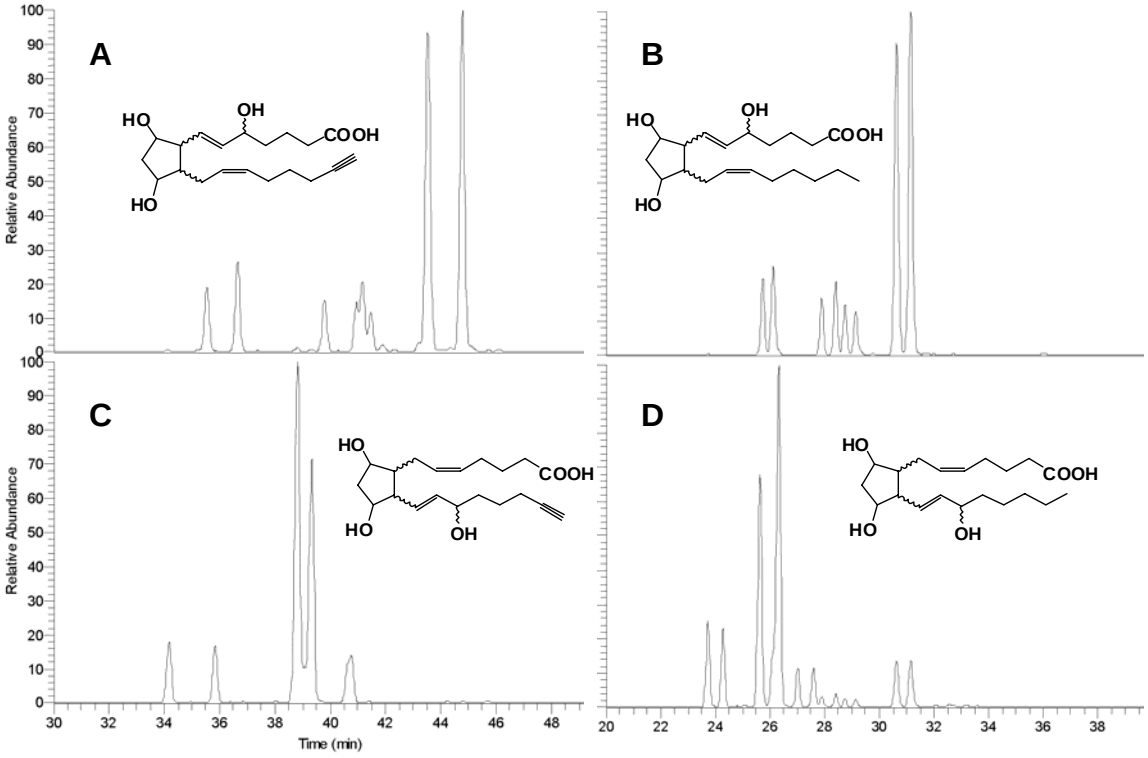


Figure 6.5. Reverse-phase HPLC/MS chromatograms of aAA and AA oxidations detected by Selected Reaction Monitoring (SRM). Isoprostanes [**A** (5-aIsoPF_2), **B** (5-IsoPF_2), **C** (15-aIsoPF_2), **D** (15-IsoPF_2)] formed upon oxidation of 2mg of AA (**B** and **D**) or aAA (**A** and **C**) in the presence of MeOAMVN (0.3mg) in benzene (60μL) for 24h at 37ºC, followed by reduction with $P(OMe)_3$ in MeCN/H_2O. SRM was as follows: 5-aIsoPF_2; 349m/z->115m/z, etc. Separation conditions are given in the experimental section.

Chromatograms of the 5- and 15-series (a)F_2-IsoPs are shown in the Figure since these compounds are formed in a large access compared to the 8- and 12-regioisomes.[22] The preference for formation of the 5- and 15-regioisomers has been observed in the oxidation reactions of arachidonic acid and a mechanism proposed to account for the observation.[22, 23] Similar regio-selectivity is observed for the transformations of aAA.

In order to obtain the chromatograms presented in Figure 6.5, an ESI-MS triple quadrupole instrument was operated in selected reaction monitoring (SRM) mode. More precisely, transitions of negatively charges ions of 349m/z (aF_2-IsoPs) or 353m/z (F_2-IsoPs) into 115m/z (5-series) and 193m/z (15-isomers) have been monitored. During past studies, these fragmentations have been shown to be regio-specific and to occur very readily.[22] Full MS^2 spectra of individual species, represented by peaks at Figures 6.5A and 6.5C, did confirm that the product ions chosen for SRMs gave the strongest signals.

The elution profiles observed for the 5- and 15-F_2-IsoPs (Figure 6.5B and 6.5D, respectively) were consistent with previous reports, and were similar to those obtained for the ω-alkynylated analogues (Figures 6.5A and 6.5C).[22] As can be seen in the chromatograms, not all of the aF_2-IsoP diastereisomers can be separated under the reverse phase HPLC conditions applied. Attempts were made to improve the separation, but they were not successful. Separation of the diastereoisomeric aF_2-IsoPs was observed to be somewhat worse compared to separation of the non-alkynylated compounds, Figure 6.5.

Terminal Alkyne Reactive Electrophiles Derived from aLA and aAA

Due to availability of the substrates as well as the expected relative simplicity of product mixtures (*vide supra*), reactive electrophiles formed during aLA oxidation were analyzed first. Initial studies were planned to provide evidence for the formation of as many electrophilic products as possible. Identification of compounds analogous to those proposed to form during the oxidation of LA is of special interest.[2, 3, 24] Upon side by side incubation of aLA and LA in the presence of the free radical initiator MeOAMVN, mixtures were treated with BHT (to stop radical processes) and PPh_3 (to reduce hydroperoxides into corresponding alcohols). This reaction sequence was followed by analysis by RP-HPLC-UV or RP-HPLC-MS. [25]

A number of peaks were detected in UV chromatograms (data not shown), while monitoring wavelengths in the range of 220-280nm.[3, 10, 26] Unfortunately, the identity of a majority of them could not be confirmed by MS studies. Compounds that were tentatively identified were 9,12-dioxododec-10-enoic acid (KODA) possibly formed upon Hock cleavage of 9-aHPODEs followed by dehydration of thus produced 9-hydroperoxy-12-oxo-10-dodecenoic acids,[3] and conjugated dienones resulted from dehydration of aHPODEs (Figure 6.6).[10] Their UV and MS characteristics (not shown) matched well or were analogous to the previously reported ones.[3, 10]

Upon oxidation of aLA, the product mixtures were incubated in aqueous solutions of a lysine containing model peptide, TpepK (Ac-Ala-Val-Ala-Gly-Lys-Ala-Gln-Ala-Arg). During these incubations, aLA derived electrophiles were

thought to be adducted to TpepK. Similar experiments have been conducted using native LA, side by side with its alkynylated analogue. Since the initial processes, Michael adduction and Schiff base formation are reversible (*vide supra*), adducts have been stabilized by reduction in the presence of $NaCNBH_3$.[27] The mixtures were separated by RP-HPLC and analyzed in an ion trap mass spectrometer.

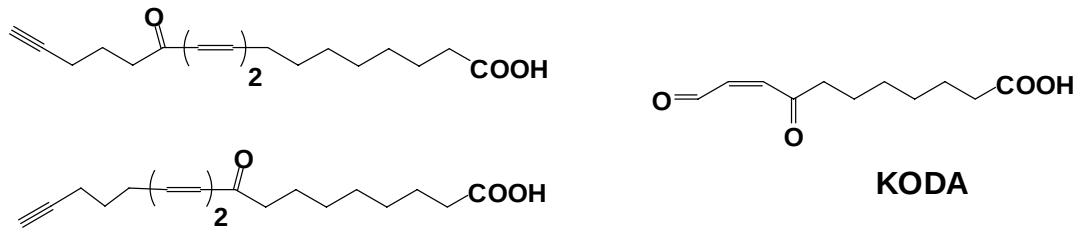


Figure 6.6. Structures of the tentatively identified aLA secondary oxidation products.

Formation of adducts of (a)LA derived electrophiles to TpepK were confirmed by the observed m/z signals in ESI-MS as well as by the fragmentation patterns observed in MS^2. In peptides, amide bonds are the easiest bonds to be broken under typical MS conditions.[28] Such fragmentations yield so-called b (N-terminal fragment) and y (C-terminal fragment) cations. Analysis of b and y ions has been used for a number of purposes, ranging from sequencing of the unknown peptides to localization of modifications to proteins or peptides.[28] For the purpose of the investigation described here, analysis of these ions was performed to confirm that the relative increase of the peptide mass due to the adduction was localized at the lysyl residue of TpepK.[27]

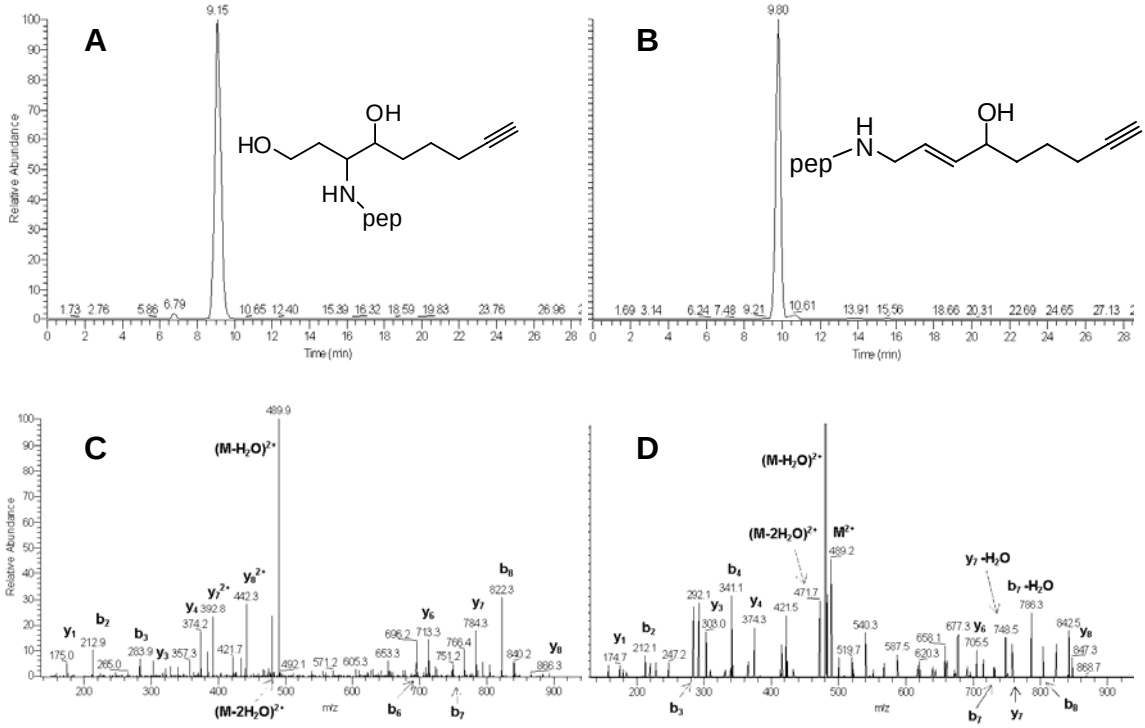


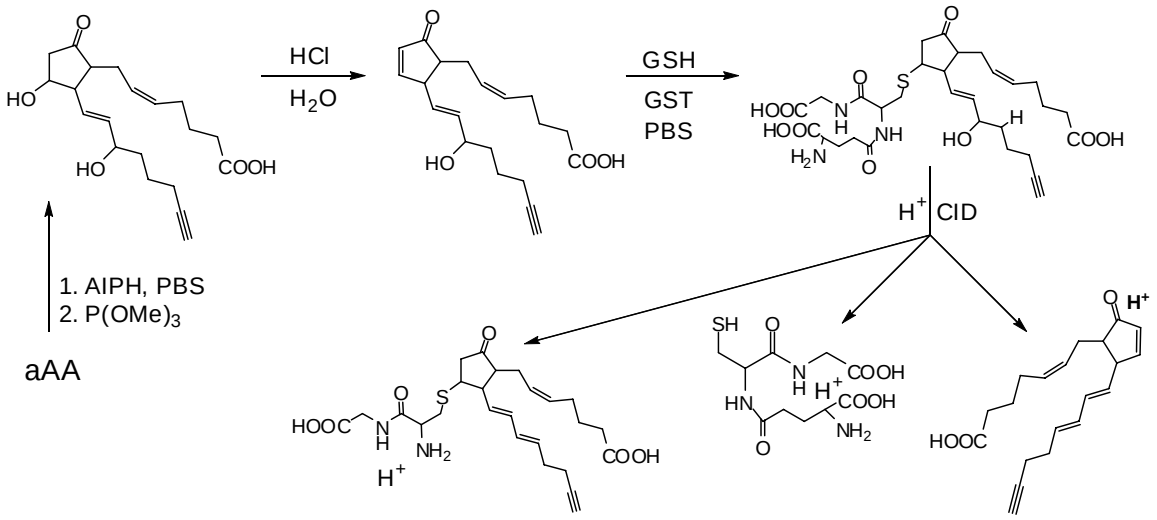
Figure 6.7. Relative RP-HPLC/MS elution observed for the 1,4- (**A**) and 1,2- (**B**) adducts of aHNE (from aLA peroxidation) in the presence of TpepK (Ac-Ala-Val-Ala-Gly-Lys-Ala-Gln-Ala-Arg). Detection was by HPLC/MS monitoring species at 490.8±0.4m/z (**A**) and 481.8±0.4m/z (**B**) respectively. Fragmentation patterns (b and y ions) obtained upon breakage of the peptide bonds of the Michael (**C**) and Schiff base (**D**) parent ions are shown. Separation conditions are given in the experimental section.

Analyses of the mixtures obtained upon incubation of oxidized aLA in the presence of the model peptide confirmed formation of Michael as well as Schiff base adducts of aHNE to TpepK, Figure 6.7. MS^2 spectra of the ions corresponding to the doubly charges aHNE-TpepK adducts contained nearly all of the expected b and y ions. Furthermore, synthetic aHNE prepared in our laboratory,[29] upon incubation with TpepK and analogous LC-MS analysis yielded peaks of identical retention times and fragmentation patterns. On the other hand,

HNE-TpepK adducts obtained by incubation of LA oxidation products with the model peptide, eluted as predicted a bit later then their alkynylated analogues.

For both HNE and aHNE adducts, the Michael-type adduct was eluted earlier than the Schiff base. It was also observed that the peak areas of both adducts of HNE were very close to the areas of aHNE adducts obtained side by side. This suggests that both compounds form and are adducted to TpepK with similar rates. However, in order to support this suggestion, detailed kinetic experiments would need to be performed.

Since AA derived cyclopentenone isoprostanes and isoketals have been shown to possess biologically significant activity,[17-20] it was decided to focus on detection of ω-alkynylated versions of these compounds produced from aAA. In order to confirm the formation of aA_2/J_2-IsoPs, aAA was subjected to a series of transformations. First, it was oxidized in aqueous solution of AIPH. Then the resulting mixture was treated with reducing agent, $P(OMe)_3$, and products of interest were dehydrated on prolonged incubation in the presence of HCl. The mixture was then incubated with glutathione (GSH) in the presence of glutathione transferase (GST) and the resulting GSH-aIsoP Michael adducts were analyzed by ESI-MS^2, using a triple quadrupole instrument. Similar protocols have been applied to prove or quantitate formation of A/J-IsoPs, in the past.[30-32] A schematic representation of the transformations applied to aAA is shown in Scheme 6.3.



Scheme 6.3. Schematic representation of the methodology (involving formation of GSH adducts) that was used to confirm formation of alkynylated cyclopentenone isoprostanes from aAA (shown for 15-aA_2-IsoPs).

The above scheme shows a typical fragmentation of the GSH-IsoP ions.[31] The S-C bond formed in the Michael adduction fragments most readily in the MS.

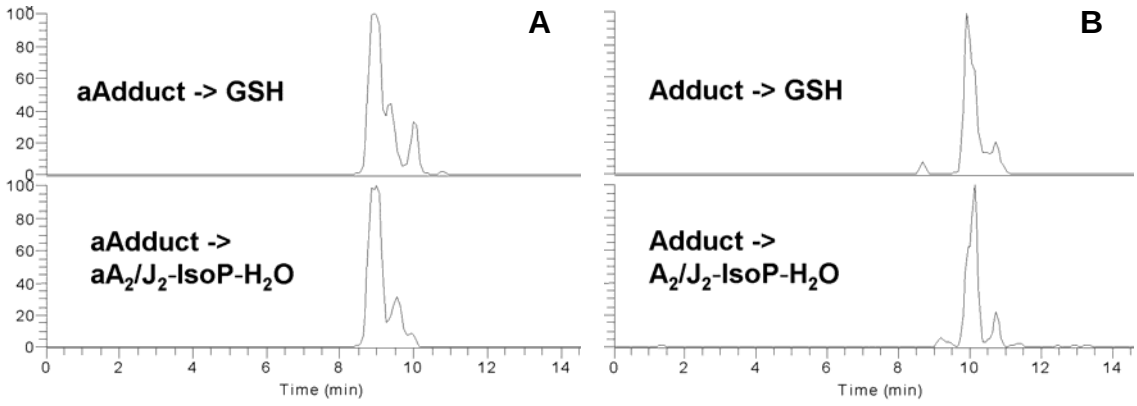


Figure 6.8. RP-HPLC elution profiles observed for aA_2/J_2-IsoPs (**A**) and A_2/J_2-IsoPs (**B**) adducted to GSH, while monitoring conversions of the parent ions into protonated GSH and dehydrated (a)A_2/J_2-IsoPs. Separation conditions are given in the experimental section.

Cleavage of this bond yields GSH and A/J-IsoPs, which are subsequently dehydrated. Alternatively, side chain dehydration can occur prior to S-C bond cleavage. Elution profiles for A_2/J_2-IsoP-GSH adducts and their, side by side produced, aAA-derived analogues are shown in Figure 6.8. Full MS^2 characterization of the parent ions of interest is also presented (Figure 6.9).

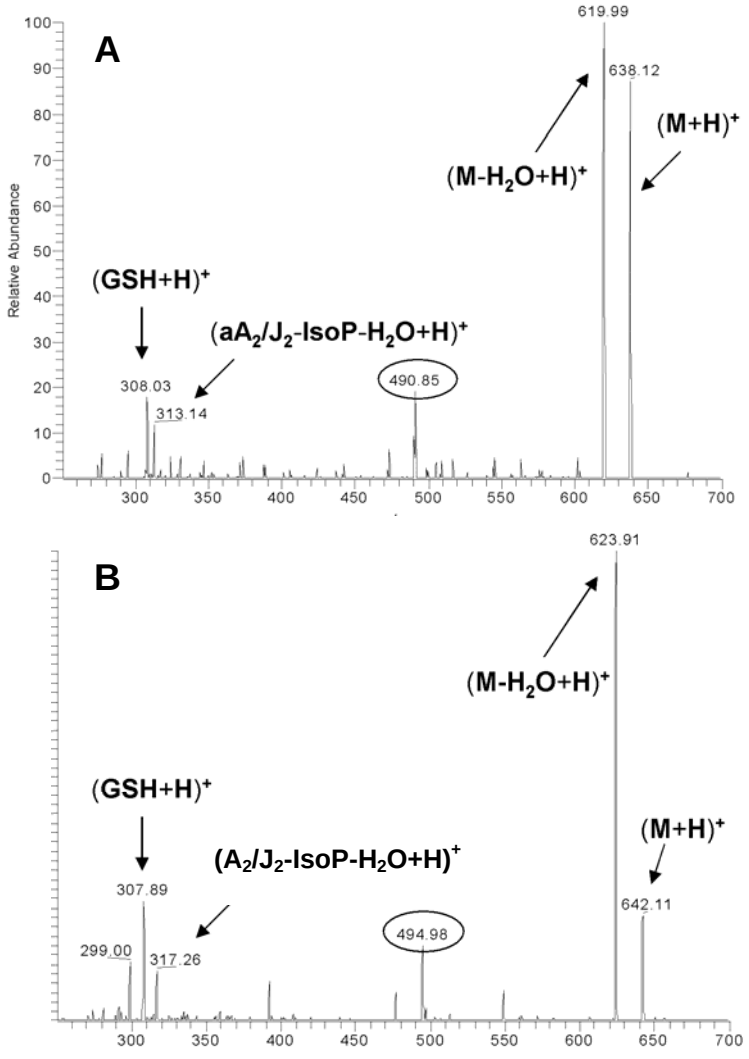
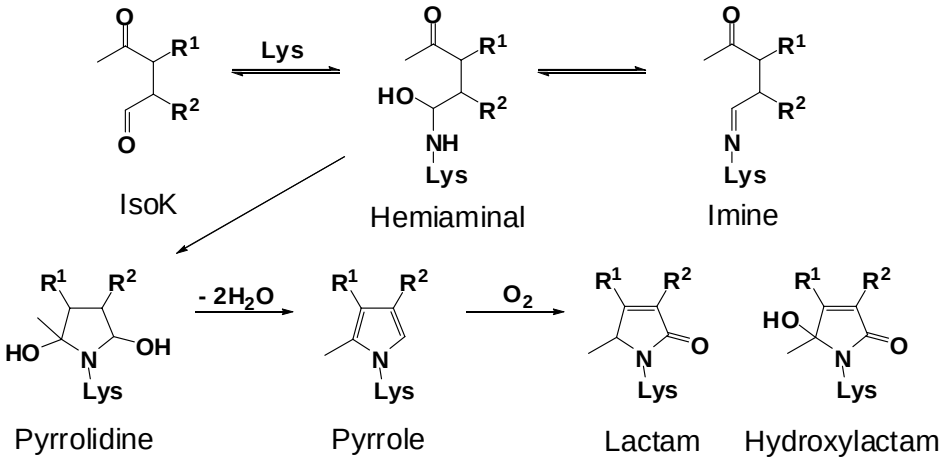


Figure 6.9. Full MS^2 characterization of the parent ions of GSH adducts to A_2/J_2-IsoP (**B**) and their alkynylated analogues (**A**).

Formation of ω-alkynylated IsoKs has been supported using a quite different method. The experiments were conducted by Dr. Sean Davies of Vanderbilt University Department of Clinical Pharmacology, an expert in the field of isoketals formation and reactivity.[33] After incubation of ovalbumin in the presence of aAA and a radical initiator, *t*-butyl hydroperoxide (TBHP) to form lipid modified protein, the modified protein was proteolytically digested by pronase and aminopeptidase M to its amino acid constituents. This mixture of amino acids was subjected to MS analysis in order to investigate if any Lys-IsoK adducts were formed. Adduction of lysine to IsoKs initially leads to formation of hemiaminals that may be further converted to Schiff bases or pyrroles. The latter typically undergo facile oxidation to form more stable lactams and hydroxylactams, Scheme 6.4.[18]



Scheme 6.4. Mechanism for formation of lysine-isoketal adducts.[18]

Routine analysis focuses on LC-MS^2 (SRM mode) identification of the lactams, since they are usually the most abundant forms of adducts.[19]

The elution profile for Lys-aIsoK adducts compared to a Lys-IsoK standard mixture is shown in Figure 6.10. Retention times for alkynylated analytes were found to be a bit smaller than corresponding values for terminally unmodified adducts. Under RP-HPLC conditions, this was in agreement with our expectations based on the observed chromatography of other alkynyl adducts.

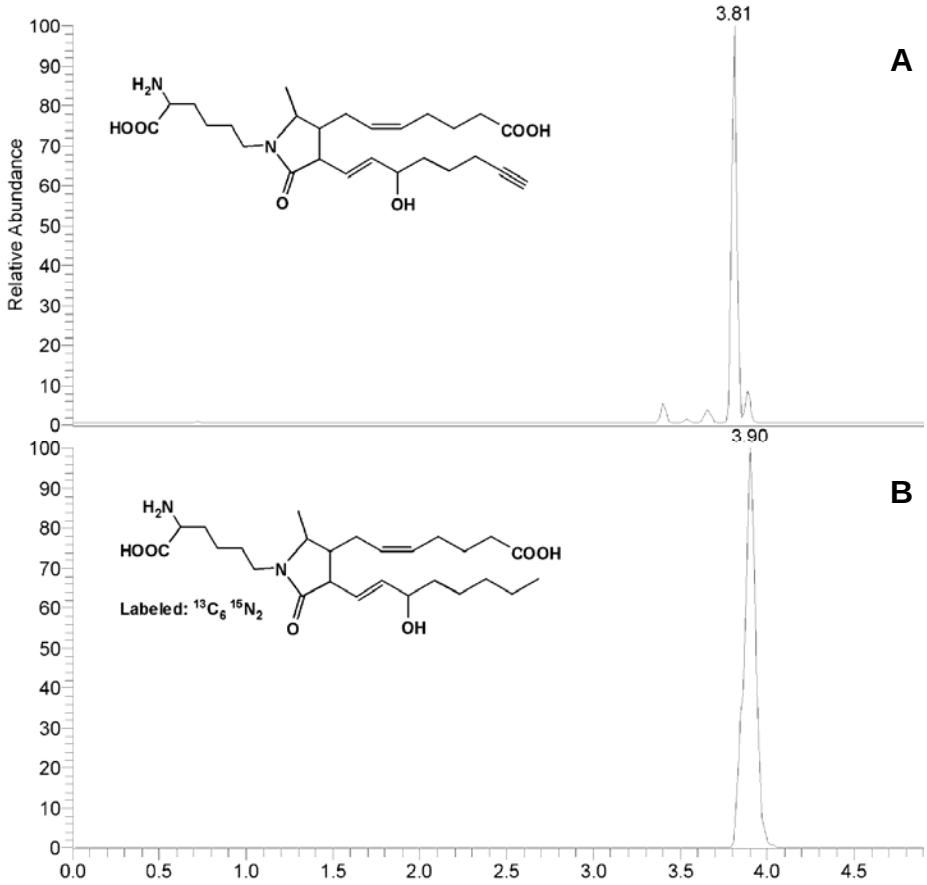


Figure 6.10. RP-HPLC-MS^2 elution profiles for lysine adducts to aIsoKs (**A**), 475 ->84m/z, and IsoKs (**B**), 487->90m/z . aIsoK-Lys adducts were obtained upon oxidation (10μmol TBHP) of aAA (25μg) in the presence of ovalbumin (10mg), followed by degradation of the protein to its constituent amino acids. Radio-labeled IsoK-Lys adducts were formed in reaction of $^{13}C_6{}^{15}N_2$-Lys with a mixture o 15-IsoKs. Sample structures of adducts are shown for lactam adducts of lysine to 15-series (a)IsoKs.[33] LC/MS conditions used as reported previously.[18,19]

The preliminary data presented suggests that aPUFAs undergo similar transformations to ones reported for their natural analogues, yielding electrophilic products. Since oxidizabilities of PUFAs and aPUFAs have been found to be similar, proximal relevant properties of natural and alkynylated phospholipids are also expected.

Transformations of ω-Alkynylated Polyunsaturated Fatty Acids in the Presence of Oxygenase Enzymes

Behavior of the alkynylated compounds in the presence of two widely available classes of bio-catalysts, lipoxygenases (LOXs) and cyclooxygenases (COXs), is of special interest since products of reactions catalyzed by these enzymes may be further transformed into reactive electrophiles (*vide supra*).

In an initial set of experiments, aLA and aAA were incubated in the presence of highly oxygenated solutions of soybean lipoxygenase, and the products of enzymatic conversion were characterized. The natural PUFAs were incubated under identical conditions, to insure that the enzymes and assays were working as expected. LA and AA were exclusively transformed into 13(*S*)-*c*,*t*-HPODE and 15(*S*)-HPETE (data not shown)[34] and similar transformations of aLA and aAA were observed. RP-HPLC elution profiles of product mixtures obtained during chemical and enzymatic aLA oxidation are shown in Figures 6.11A and 6.11C.

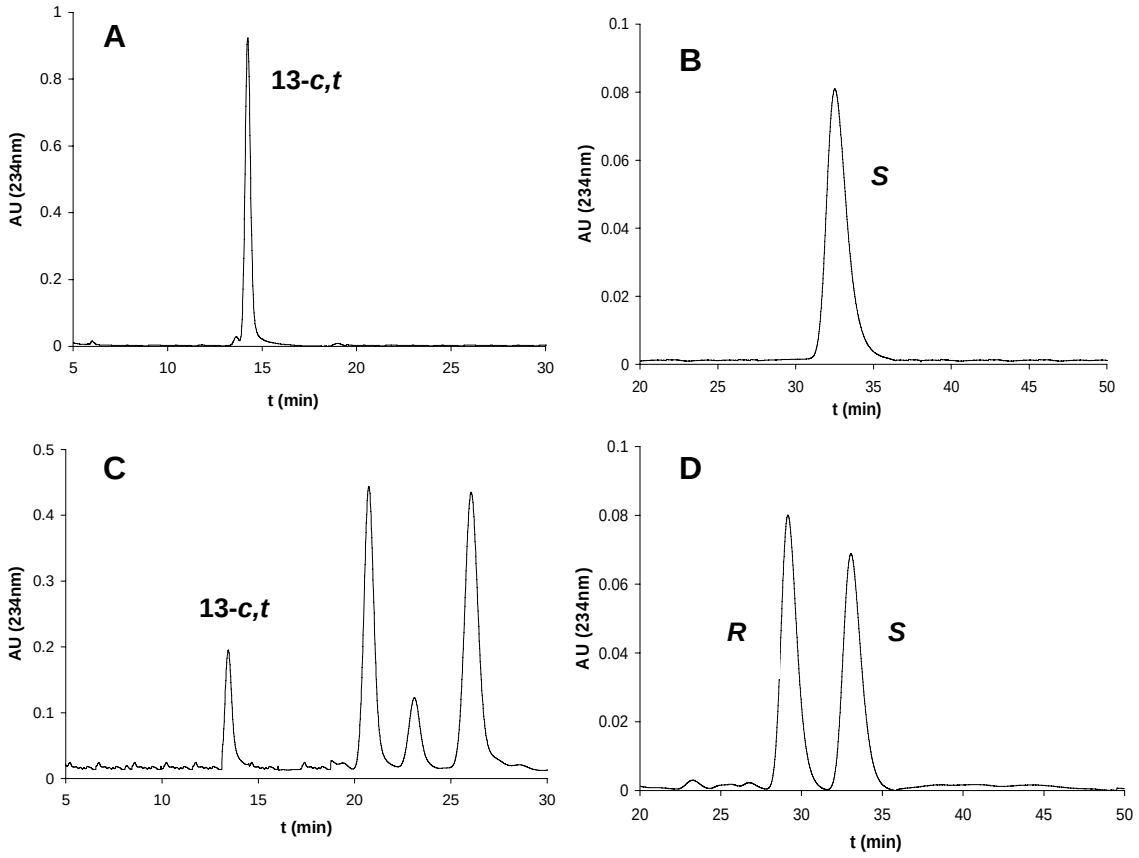


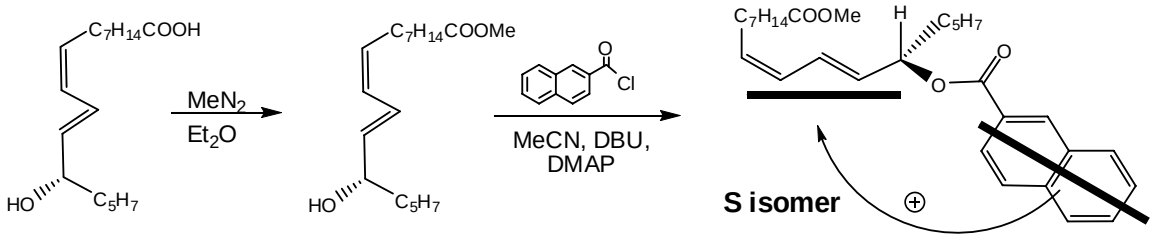
Figure 6.11. Elution profiles for aHODEs obtained from enzymatic (**A**) and chemical (**C**) oxidation. Chiral phase elution profiles for 13-*c*,*t*-HODEs methyl esters isolated from enzymatic (**B**) and chemical (**D**) oxidation mixtures. Separation conditions are given in the experimental section.

As shown in the Figure, the retention time of the single product obtained from aLA in the presence of soybean lipoxygenase corresponded to 13-*c*,*t*-aHODE. It was also established that the amounts of the HODE or the HETE formed from LA or AA in the presence of soybean LOX were similar (<20% difference) to the corresponding amounts of the aHODE or the aHETE produced from aLA and aAA, respectively. Furthermore, equimolar mixtures of aLA and LA as well as aAA and AA subjected to enzymatic transformations

yielded comparable amounts of alkynylated and non-alkynylated products on total consumption of starting materials. On the other hand, when a reaction was quenched before the substrates were completely consumed, it was found that natural substrates were oxidized somewhat faster than the modified fatty acids (data not shown).

The optical purity of the LA lipoxygenase product was assigned based on HPLC chiral analysis. Chiralpak AD columns have been utilized in the past to separate mixtures of fatty ester based enantiomers,[35] and in the present investigation they were found to be useful in separation of aHETE and aHODE methyl ester enantiomers. A sample separation of 13(*R*)- and 13(*S*)-c,t-HODE on one of these columns is presented in Figure 6.11D, whereas Figure 6.11B shows that only one enantiomer (*S*) has been obtained in the soybean LOX catalyzed oxidations of aLA.

The absolute configuration of the product can be assigned based on the observation that *R* enantioners always elute first during separation of hydroxy-fatty esters on Chiralpak AD columns.[35] In order to confirm this assignment, exciton-coupled circular dichroism (ECCD) spectra of naphthoyl derivatives of the two 13-c,t-HODE ester enantiomers were obtained, Figure 6.11.[36] The preparation of the naphthoyl ester is illustrated in Scheme 6.5. It has been empirically established that scanning from longer wavelengths towards shorter ones, if a maximum is followed by a minimum in the CD spectrum curve, *S* configuration is to be assigned to the chiral carbon present in proximity of two interacting chromophores, Figure 6.12.



Scheme 6.5. Preparation of an *S*-naphthoyl ester enantiomer for ECCD studies.

If the minimum is followed by maximum in such curves, the relevant carbon atoms will have *R* configuration.[36] An analogous analysis was carried out for aAA oxidation mixtures to conclude that 15(*S*)-HETE was the only aAA product formed in the presence of soybean LOX.

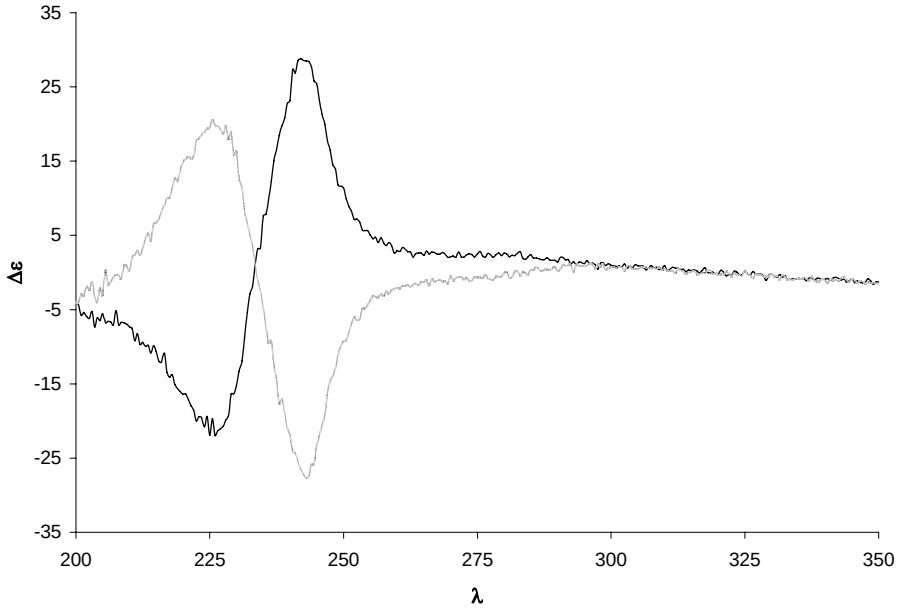


Figure 6.12. Electron coupled circular dichroism spectra of naphthoylated 13(*S*)-HODE (solid line) and 13(*R*)-HODE (dotted line) methyl esters, obtained from chemical oxidation followed by chiral separation of 13-HODE methyl esters and further derivatization.

Once it was established that ω-alkynylated substrates underwent oxidations in the presence of the model lipooxygenase, interactions with other oxygenases became of interest. This research was focused on aAA since, unlike aLA, it was thought to be potentially a good substrate for both LOX and COX enzymes. These experiments involved both enzymatic kinetics and product identification studies.

COX activities were quantified by following oxygen consumption using a polarographic electrode with an oxygen monitor, and maximal reaction velocity data were obtained from the linear portion of the oxygen uptake curves. On the other hand, LOX activities were quantified by spectrophotometric (UV) monitoring of the formation of conjugated diene products and maximal reaction velocity data was obtained from the linear portion of the absorbance curves.[37] These kinetics experiments were conducted by Melissa Turman in the Marnett laboratories at Vanderbilt. The important kinetic parameters measured during bio-transformations of aAA in the presence of available LOX and COX enzymes are presented in Table 6.1.[38]

The observed small differences in k_{cat}/K_M values suggest that aAA is nearly as good a substrate for cyclooxygenases as its native analogue. The catalytic efficiency of human platelet-type 12-LOX in the presence of aAA was also found similar to the efficiency observed for AA as a substrate. On the other hand, porcine leukocyte-type 12-LOX and rabbit reticulocyte 15-LOX-1 did not oxidize aAA nearly as efficiently as AA, as illustrated by relatively large differences in ν_{max}/K_M values. Noticeably lower catalytic

efficiencies for these enzymes in the presence of aAA are due mainly to much lower v_{max} values observed in the presence of the modified substrate. It could be speculated that the ω-alkyne unit of aAA was responsible for partial inhibition of the enzymes, but confirmation of this proposal would required additional studies.

Table 6.1. Enzymatic kinetic parameters obtained for aAA *vs.* AA substrate.[38]

Enzyme	k_{cat} (s^{-1})	v_{max} (μMs^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	v_{max}/K_M (s^{-1})
oCOX-1	57±6 (51±3)	n/a	6.2±0.8 (3.4±0.6)	9±6 (15±3)	n/a
hCOX-2	11±1 (14.7±0.5)[39]	n/a	4.5±0.7 (6.1±0.6)[39]	2±1 (2.4)[39]	n/a
plt12-LOX	n/a	4.53±0.08 (13.3±0.3)[37]	7.0±0.3 (9.5±0.7)[37]	n/a	0.6±0.3 (1.4±0.7)[37]
lk12-LOX	n/a	1.37±0.09 (13.1±0.7)[37]	4±1 (7.8±1.3)[37]	n/a	0.3±1 (2±1)[37]
r15-LOX-1	n/a	0.61±0.05 (8.6±0.4)[37]	7±2 (20±3)[37]	n/a	0.09±2 (0.4±3)[37]

Numbers given in brackets are the relevant values previously reported for AA. Abbreviations: oCOX-1, ovine COX-1; hCOX-2, human COX-2; plt12-LOX, human platelet-type 12-LOX; lk12-LOX, porcine leukocyte-type 12-LOX; r15-LOX-1, rabbit reticulocyte 15-LOX-1. Conditions used to obtain the reported kinetic parameters are outlined in Experimental Section.

Mixtures of products obtained from the enzymatic conversions of aAA were analyzed by RP-HPLC-MS^2 in order to investigate selectivity of the bio-catalyses. Oxidation mixtures obtained in the presence of COXs were analyzed

for aPGD_2, aPGE_2 and aHETEs, whereas the analysis of the mixtures obtained in the presence of LOXs focused on identification of aHETEs only. The COX oxidations of aAA were accompanied by side by side transformations of the native substrate performed under identical conditions. As expected, (a)PGE_2 and (a)PGD_2 were the major products obtained in the presence of both COX-1 and COX-2, and only traces of (a)HETEs were observed.[40] In the absence of enzyme insignificant amounts of the products were formed.[38] As shown in Figure 6.13, the total amount of the (a)PGD_2/E_2 products obtained in the presence of COX-1 was larger then the amount of the products produced in the presence of COX-2. This was in agreement with the kinetic data presented in Table 6.1.

Analysis of mixtures obtained in the presence of lipoxygenases confirmed that, as expected, 12-aHETE was the major product of transformations catalyzed by investigated 12-LOXs, whereas 15-HETE was the major product obtained in the presence of the 15-LOX used.[37] Besides the fact that the observed aHETE products yielded expected daughter ions in MS^2, their identities have been additionally confirmed by spiking the mixtures with previously isolated aHETEs (data not shown).

The presented data suggests that aPUFAs are substrates for the important COX and LOX oxidizing enzymes and this fact should not be forgotten during *in vivo* studies on alkynylated PUFAs or phospholipids. First of all, the enzymatic conversions of alkynylated substrates into precursors for reactive electrophiles may account for hypothetical background formation of lipid-protein adducts, in the absence of radical initiator. Furthermore, chiral

analysis of adducts that retain stereochemical information may lead to biased conclusions if stereospecific enzymatic conversions are not taken into account.

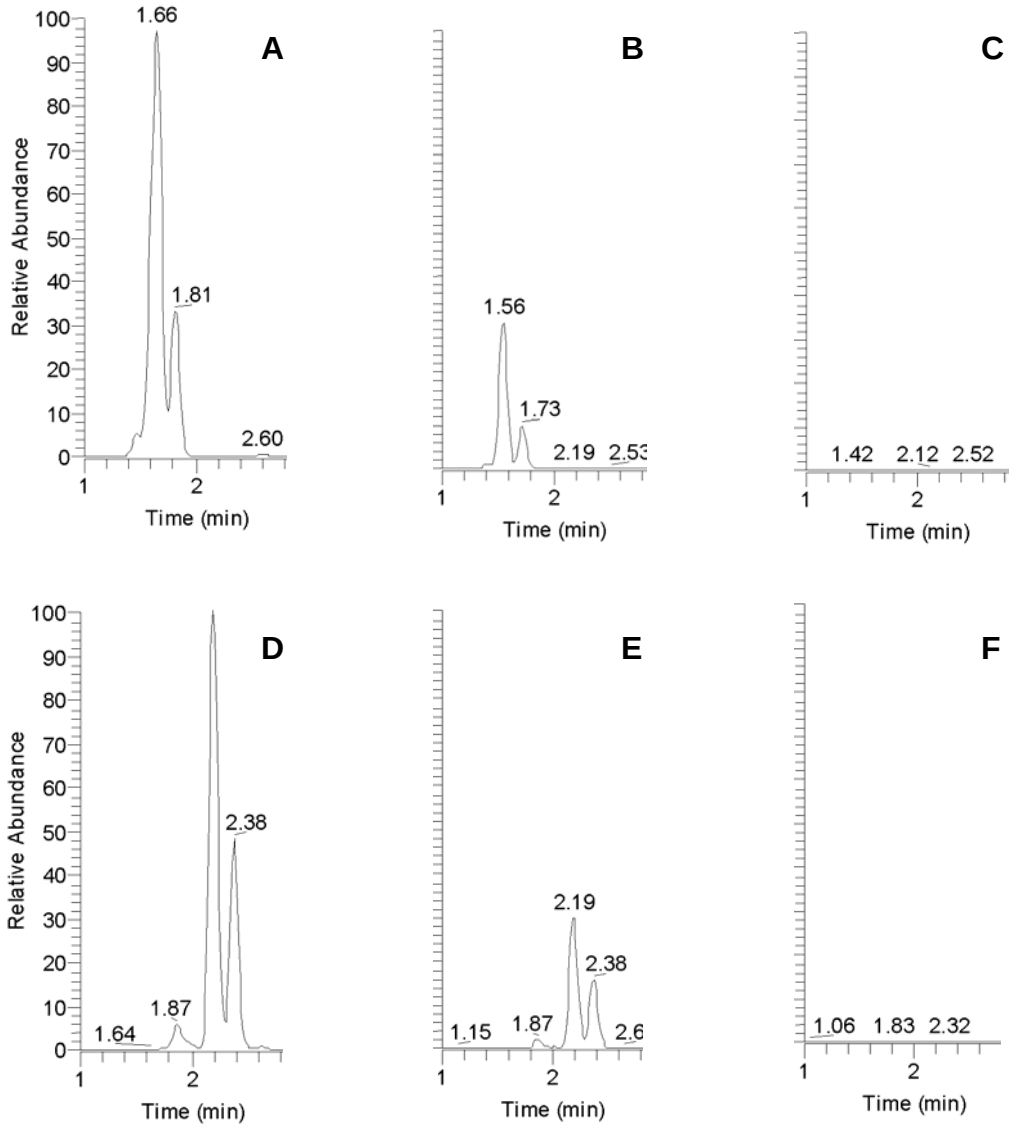


Figure 6.13. RP-HPLC-MS^2 (SRM) monitored formation of prostaglandins D_2/E_2 (**A**-**C**), 351->271m/z, or their alkynylated analogues (**D**-**F**), 347->267m/z, in the presence of ovine COX-1 (**A** and **D**), human COX-2 (**B** and **E**) and in the absence of an enzyme (**C** and **F**). Conditions for enzymatic transformation and LC separations are given in the Experimental Section.

Experimental

General. Unless stated otherwise, all the chemicals were purchased from Sigma Aldrich. NMBHA has been prepared as previously described.[12] AIPH and MeOAMVN were purchased from Wako Chemicals and used without further purification. Benzene (HPLC grade) was passed through column of neutral alumina and stored over molecular sieves. Commercial α-TOH has been chromatographed before use. Diazomethane was prepared by portion wise addition of nitrosomethylurea (500mg) into heterogeneous mixture of 40% aqueous KOH (1.25mL) and Et_2O (5mL) at 0ºC.[41] The deep yellow organic layer was decanted and dried over NaOH. The dried ethereal MeN_2 was used immediately. Phosphate-buffered Saline (PBS, pH 7.4, 50 mM) was prepared from NaCl (8.00 g), Na_2HPO_4 (5.00 g), NaH_2PO_4 (1.96 g) in deionized water (1.0 L) followed by adjustment of the pH to 7.4 with 10M aqueous NaOH. Buffer solutions were stored over Chelex 100 resin (*ca*. 3 g / L) for 24 h, then the resin was removed by filtration and the filtrate was stored at 5ºC (for no longer than a month). Ammonium Bicarbonate Buffer (pH 7.4, 100mM) has been prepared by dissolution of $NaHCO_3$ (791mg) in deionized water (100mL) followed by adjustment of the pH to 7.4 with 5M aqueous HCl. The buffer solution was stored at 5ºC (for no longer than 2 weeks). Borate Buffer (pH 9.5, 100mM) was prepared by dissolving boric acid (1.24 g) in deionized water (200 mL) followed by adjustment of the pH to 9.5 with 5M aqueous NaOH. This buffer was stored at 5ºC (for no longer than a month). HPLC-UV analyses were performed with Waters 600 Controller, 717 Autosampler and Waters 996 photodiode array

detector. All HPLC Analyses of (a)HETEs and (a)HODEs, as well as separations of naphthoyl derivatived of conjugated dienols, were carried out on a single Beckman 5μm ultrasphere column (4.6mm x 25cm) using isocratic normal phase conditions (1.2% IPA in hexanes contining 0.1% AcOH). Chiral HPLC analyses of HETE and HODE methyl esters were performed on a single Chiralpak AD column (4.6mm x 25cm) produced by Chiral Technologies Inc., Exton, PA. aHETE products have been eluted with 2% EtOH in hexanes, whereas aHODEs were eluted with 5% MeOH in hexanes. DLI-MS experiments were performed on ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer, whereas all HPLC-MS analyses, except for TpepK adducts studies, were conducted on the same instrument coupled with a Surveyor MS Pump and Surveyor Autosampler (for RP-HPLC) or with Waters Alliance 2690 Separation Module (NP-HPLC). Analyses of TpepK adducts were conducted on ThermoFinnigam LTQ (3-D ion-trap instrument) coupled with Waters Aquity UPLC system. All RP-LC analyses of aPUFA oxidation products and their adducts were performed on single Phenomenex 5μm Luna column (4.6mm x 25cm), except for identification of PGE_2/D_2 products that was performed using a 3μm Luna column (2.0mm x 5cm). Detailed information about solvent gradients and MS settings applied during these analyses is given in the appropriate protocols presented below. Unless stated otherwise, all the HPLC separations were conducted with rate of 1mL/min. ECCD experiments were conducted on an Aviv 215 Circular Dichroism spectrophotometer equipped with temperature controller. NMR spectra were recorded on Bruker Biospin instruments (500MHz, 600MHz or 800MHz

depending of availability) equipped with CryoProbes. Oxygen uptake measurements have been performed using polarographic electrode with YS5300 oxygen monitor (Yellow Springs Instrument Co. Inc., Yellow Springs, OH). UV assays (used for enzymatic kinetics studies) were monitored using a Hewlett Packard 8453 diode array spectrophotometer equipped with a thermostatted cuvette, with possibility of stirring of the reaction vessel.

MeOAMVN initiated oxidation of aLA. To a solution of aLA (4.5mg) in benzene (180μL) was added 1.8mg of MeOAMVN in benzene (20 μL), and the mixture was incubated at 37ºC. After 45min, solution of BHT and PPh_3 in benzene (3.26μmol/32.6μmol, 20μL) was added, and the mixture was vortexed for 1min. Benzene was evaporated under a stream of argon, and the residual material was reconstituted in 1.0mL of 1.2% IPA in hexanes (+0.1% AcOH), from which 50μL was used for a single UV-HPLC analysis. Side by side oxidations of LA were performed using analogous procedure as well. For DLI-MS studies of aHODEs, eluted peaks from a single 50μL injection were collected, and solvents were evaporated under a stream of argon, then the residues were reconstituted in MeOH. For NMR studies, corresponding peaks collected from three 100μL injections were combined, solvents were evaporated and the residues were dried under highvac for 2h. These dried materials were reconstituted in 150μL of benzene-d_6 and placed into 1.7mm OD sample tubes. Spectra were collected for samples were chilled to 5ºC.

Thin film oxidations of aAA. Solutions of aAA (2.0mg) in benzene (200μL) containing of α-TOH (100μg), have been evaporated while the reaction vials (20mL) were rotated, to for thin lipid films. After these films were incubated at 37ºC for 20h, PPh_3 solution in benzene (10μmol, 200μL) were added, and the mixtures were vortexed for 2min. Benzene was removed under a stream of argon and the remaining material was reconstituted in 200μL of 1.2% IPA in hexanes (+0.1% AcOH), from which 60μL was used for a single UV-HPLC analysis. Side by side oxidations of AA were performed using analogous procedure.

Oxidations of aAA in the presence of NMBHA. To a mixture of aAA (1mg) and NMBHA (1mg) in MeCN (120μL) was added MeOAMVN (100μg.) in MeCN (40μL). After 35h of incubation at 37ºC, BHT/PPh_3 in MeCN (1μmol/10μmol, 40μL) was added, and the mixture was vortexed for 2min. Acetonitrile was removed under a stream of argon and the remaining material was reconstituted in 0.5mL of 1.2% IPA in hexanes (+0.1% AcOH). For DLI-MS studies of aHODEs, eluted peaks from a single 50μL injection were collected, and solvents were evaporated under a stream of argon, then the residues were reconstituted in MeOH. For NMR studies, corresponding peaks collected from three 100μL injections were combined, solvents were evaporated and the residues were dried under highvac for 2h. These dried materials were reconstituted in 150μL of benzene-d_6 and placed into 1.7mm OD sample tubes. Spectra were collected for samples were chilled to 5ºC.

Oxidations of aAA in the aqueous AIPH solution. To a solution of aAA (2mg) in benzene (40μL) was added MeOAMVN in benzene (20μL) and the mixture was incubated at 37ºC for 24h. Afterwards, solvent was evaporated, the residue was treated with mixture of BHT and $P(OMe)_3$ in MeCN/H_2O (1mmol/ 10mmol, 150μL/50μL), and vortexed for 5min and kept at -40ºC prior to analysis. Injections of 10μL were performed, and the following solvent gradient has been applied: 10% MeCN (+5% MeOH) in 2mM aq. NH_4OAc (0-10min); 10-25% MeCN (+5% MeOH) in 2mM aq. NH_4OAc (10-55min). ESI- was used, and the important mass spectrometer parameters were optimized for commercially available $PGF_{2\alpha}$. The following MS fragmentations were monitored during the analyses: 349->115m/z (5-aF_2IsoPs); 349->127m/z (8-aF_2IsoPs); 349->151m/z (12-aF_2IsoPs); 349->193m/z (15-aF_2IsoPs). Side by side oxidations of AA were performed using analogous reaction conditions, however slightly different analytical conditions were applied to analyze the AA oxidation products [Solvent gradient applied: 20% MeCN (+5% MeOH) in 2mM aq. NH_4OAc (0-10min); 20-40% MeCN (+5% MeOH) in 2mM aq. NH_4OAc (10-50min). MS fragmentations monitored: 53->115m/z (5-F_2IsoPs); 353->127m/z (8-F_2IsoPs); 353->151m/z (12-F_2IsoPs); 353->193m/z (15-F_2IsoPs).

Adduction of aPUFAderived electrophiles to TpepK. Solutions of aLA (200μg) or aAA (200μg) in benzene (200μL) were condensed in the vials (20mL), which were rotated, in order to form thin lipid films. After incubation for 16h at 37ºC, to these films was added TpepK (130μg) in ammonium bicarbonate pH 7.4

buffer (100μL). The mixtures were vortexed for 5min, and stirred well for 4h at 37ºC. Then 1M aqueous $NaCNBH_3$ (5μL) was added to the mixtures, followed by addition of 1M aqueous HCl (5μL). Mixtures were then stirred at rt. for additional 2h. Afterwards, they were diluted with 10% MeCN in H_2O (400μl) and stored at -40ºC until analyzed. Injections of 20μL were performed, and the following solvent gradient has been applied: 0-50% MeCN in H_2O (0-25min); 50-100% MeCN in H_2O (25-35min); 100% MeCN in H_2O (35-50min); both solvents contained 0.1% AcOH. ESI+ mode was used, and the important mass spectrometer parameters were optimized for TpepK. Data dependent MS^2 as well as MS^2 for number of selected ions, which were expected to be found in the mixtures, have been performed. The above protocol was also applied to LA and AA, side by side with their alkynylated analogues.

Adduction of aAA derived cyclopentenone alsoPs to GSH. To a solution of aAA (5mg) in EtOH (100μL) was added AIPH (3mg) in PBS pH 7.4 (4.9mL), and the mixture was incubated at 37ºC. After 5h, a solution of $P(OMe)_3$ in water (30μmol, 100μL) was added, and the mixture was vortexed for 2min. The solution was acidified to *ca.* pH 3 with 0.1M aqueous HCl, and the compounds of interest were extracted with AcOEt (2 x 2mL). Organic solvents were combined and evaporated under a stream of argon. The residual material was reconstituted in 0.1M aqueous HCl (5mL) and the mixture was stirred at 37ºC. After 14h, 10x PBS pH 7.4 (2mL) was added to the mixture, and the pH was brought to 6.5 with 1M aqueous NaOH, followed by an addition of GSH

(4mg) and human placenta GST (250μg of 25-125U/mg) in water (300μL). The mixture was stirred for 2h at 37ºC, centrifuged at 3500rpm for 15min using Amicon Ultracell 10kD tube, and the filtrate was condensed to *ca.* 1mL. The condensed mixture was placed on a solid phase extraction cartridge (360mg Sep-Pak Plus C18), washed with 20mL of water (+1% AcOH), and eluted with 6mL of MeCN (+1% AcOH). Acetonitrile was evaporated, and the residue was reconstituted in 100μL of 20% MeCN in water (+0.1% AcOH). Injections of 10μL were performed, and the following solvent gradient has been applied: 0-50% MeCN in H_2O (0-15min); both solvent contained 0.1% AcOH. ESI+ mode was used, and the important mass spectrometer parameters were set as previously reported for analysis of analogous non-alkynylated adducts. Full MS^2 (250-700m/z) as well as SRM experiments (638->308m/z and 638->313m/z) were conducted. Side by side modifications to AA were performed using analogous conditions, however slightly different MS transitions were monitored to analyze the AA derived GSH adducts (642->308m/z and 642->317m/z).

Adduction of aAA derived alsoKs to ovalbumin. A detailed procedure by Dr S. Davies will be published elsewhere.

Oxidation of aPUFAs in the presence of soybean LOX. To a solution aLA (1mg in 100μL EtOH) or AA (1mg in 100μL EtOH) in borate buffer pH 9.5 (4mL) was added soybean LOX-1 (0.25mg, 20000U) in the buffer (1mL) and the mixture was vigorously stirred (while bubbling O_2 into it) at *ca.* 0ºC. After 30min (or 5min, for uncompleted reaction), to the mixture were added 1M HCl (300μL)

and brine (1mL), and the products were extracted with DCM containing 10μmol PPh_3 (2 x 2mL). Organic layers were combined, and solvent was evaporated under a stream of argon. The residue was reconstituted in 0.5mL of 1.2% IPA in hexanes (+0.1% AcOH). For Chiral analysis, peaks eluted from three 100μL injection were collected, solvent was evaporated and the residue was treated with an excess of freshly prepared CH_2N_2 in Et_2O. Solvent was evaporated, and the residue was reconstituted in 120μL of 5% MeOH in hexanes (if started from aLA) or 2% EtOH in hexanes (if started from aAA), from which 15μL was injected for a single HPLC-UV analysis. For further transformations, peaks eluted from 90μL injections were collected, and solvents were evaporated. The residue was reconstituted in dry MeCN (50μL) containing 2% DBU, and few crystals of 2-naphthoyltriazole were added to that mixture followed by vortex mixing (2min). After 16h of incubation at rt, solvent was evaporated, the residue was reconstituted in 2mL of DCM, and the solution was washed with water (3 x 1mL). Organic layer was dried with Na_2CO_3, and condensed under a stream of argon. The residue was dissolved in 100μL of 0.2% IPA in hexanes (+0.1% AcOH). Peaks from 95μL injections were collected, solvents were evaporated and the residues were reconstituted in dry MeCN for ECCD analysis. Chiral analysis of products formed during chemical oxidations of aLA and aAA were performed on similar basis.

Oxidation of aAA in the presence of animal LOX enzymes. The enzyme reactions included reaction buffer [50mM Tris-HCl (pH 7.4) with 0.03%

Tween-20] and substrate, and were initiated by the addition of enzyme (approximately 50U). Compounds were dissolved in acetonitrile (ACN) containing 10 % acetic acid before addition to the reaction buffer; ACN was kept below 1% reaction volume (2ml). For kinetic experiments, substrate concentration was varied (1-50μM), and maximal reaction velocity data were obtained from the linear portion of the absorbance curves. Rates were converted from absorbance units/s to μM HETE/s using a absorption coefficients reported in the literature. Similar studies were conducted in order to obtain kinetic parameters in the presence of AA. For the product analyses, to the most concentrated samples was added $Na_2S_2O_3$ and the products of interest were extracted with Et_2O. Solvent was evaporated and the residue was reconstituted in 200μL of 1.2% IPA in hexanes containing 0.1% AcOH.[38]

To characterize (a)HETEs formed in enzymatic reactions, injections of 20μL of product mixtures were performed, and an isocratic elution with 1.2% IPA in hexanes (+0.1% AcOH) was applied. Analysis was conducted in APCI- mode, and the important mass spectrometer parameters were optimized for commercially available 12-HETE. Full MS^2 (100-400m/z) as well as SRM experiments [315->115m/z (5-aHETE), 315->155m/z (8-aHETE), 315->139m/z (9-aHETE), 315->167m/z (11-aHETE), 315->179m/z (12-aHETE), 315->219m/z (15-aHETE)] were conducted.

Oxidation of aAA in the presence of COX enzymes. Substrates were solubilized in dimethyl sulfoxide (DMSO). Activity assays were performed in 100mM Tris-HCl buffer containing 500 μM phenol, with hematin-reconstituted

protein [oCOX-1 (50nM), hCOX-2 (100nM)]. For kinetic experiments, substrate concentration was varied (1-50μM), and maximal reaction velocity data were obtained from the linear portion of the oxygen uptake curves. For the product analyses, to the most concentrated samples was added $Na_2S_2O_3$ and the products of interest were extracted with Et_2O. Solvent was evaporated and the residue was reconstituted in 200μL of 20%MeCN in water containing 0.1% AcOH (for aPGD_2/E_2 analysis) or in 200μL of 1.2% IPA in hexanes containing 0.1% AcOH (for aHETEs analysis).[38]

For the analysis of prostaglandins, injections of 10μL were performed, and the following solvent gradient has been applied: 20% MeCN in water (0-1min); 20->35% MeCN in water (1-6min); aqueous phase contained NH_4OAc (5mmo/L), and MeCN contained 5% of the aqueous phase; HPLC column was heated to 40ºC during separation and low flow rate of 300μL/min was applied. Analysis was conducted in ESI- mode, and the important mass spectrometer parameters were optimized for commercially available $PGF_{2\alpha}$. Full MS^2 (200-800m/z) as well as SRM experiments (347->267m/z) were conducted. For analysis of products derived from AA, different HPLC solvent gradient was applied: 30% MeCN in water (0-1min); 30->45% MeCN in water (1-3.25min); aqueous phase contained NH_4OAc (5mmol/L), and MeCN contained 5% of the aqueous phase; HPLC column was heated to 40ºC during separation and low flow rate of 300μL/min was applied. Also, different MS fragmentation was monitored, in case of AA derivatives: (351->271m/z). For the analysis of aHETEs, injections of 20μL were performed, and an isocratic elution with 1.2%

IPA in hexanes (+0.1% AcOH) was applied. Analysis was conducted in APCI-mode, and the important mass spectrometer parameters were optimized for commercially available 12-HETE. Full MS^2 (100-400m/z) as well as SRM experiments [315->115m/z (5-aHETE), 315->155m/z (8-aHETE), 315->139m/z (9-aHETE), 315->167m/z (11-aHETE), 315->179m/z (12-aHETE), 315->219m/z (15-aHETE)] were conducted. For analysis of products derived from AA, different MS fragmentations were monitored [319->115m/z (5-aHETE), 319->155m/z (8-aHETE), 319->139m/z (9-aHETE), 319->167m/z (11-aHETE), 319->179m/z (12-aHETE), 319->219m/z (15-aHETE)].

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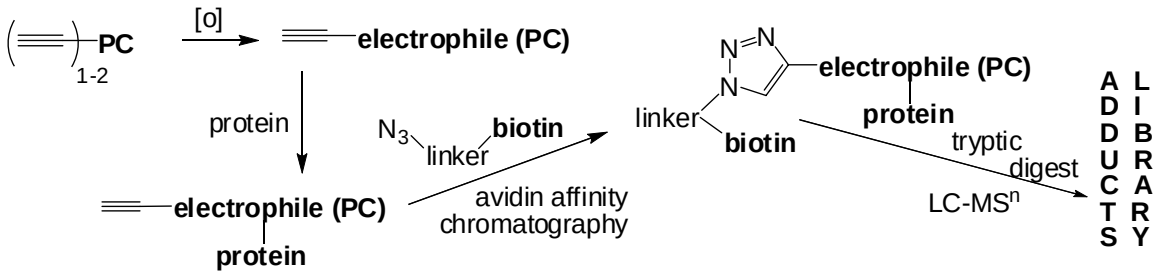
Research Summary (Part Two)

Covalent modifications of proteins may severely alter their critical functions *in vivo*. A number of studies have suggested that formation of covalent adducts between reactive electrophiles formed in the course of free radical oxidation of phospholipids and the nucleophilic sites of peptides and proteins. A novel tool has been developed to provide information about the sites of electrophile adduction as well as the nature of the protein-lipid adducts. An important part of this strategy is the modification of the ω-position of fatty acids and phospholipids with an alkynyl moiety. This structural modification is considered to be a minimal perturbation of the natural substrates but it will potentially allow for an easy separation of the alkynylated peptide adducts from complex protein mixtures.



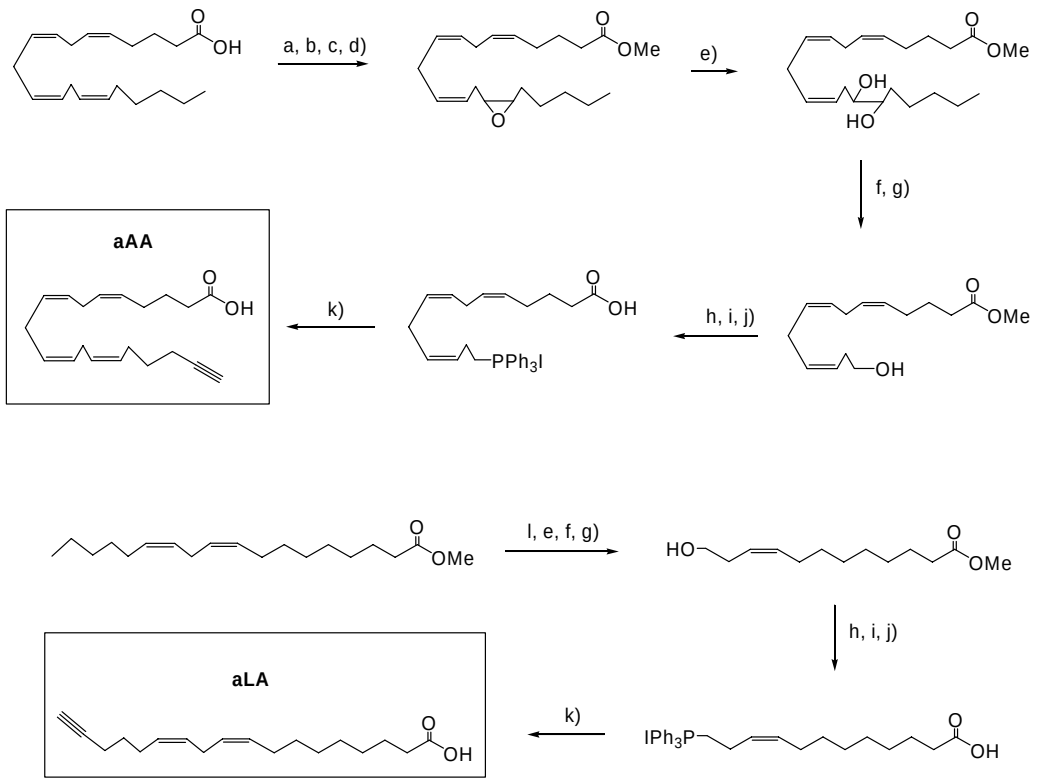
The designed isolation method is based on a coupling of protein adducts derived from alkynylated phospholipids to an azido-biotin compound via triazole forming click chemistry, and subsequent purification of the adducts of interest by avidin affinity chromatography followed by their release. Such isolated species may be analyzed utilizing tandem MS techniques. The strategy is presented in the above scheme.

Chapters IV-VI of this thesis summarized the part of the project that focused on syntheses and properties of ω-modified polyunsaturated fatty acids (aPUFAs), precursors for modified phospholipids that have been designed to act as novel probes for investigation of interactions between membrane phospholipids and systemic proteins.

ω-Alkynylated linoleic (aLA) and arachidonic (aAA) acids have been prepared utilizing simple organic reactions as shown in the scheme below. The synthetic strategy applied is relatively general and could be utilized to prepare other ω-alkynylated PUFAs. Alternative strategies were also tested but were found to be less efficient.

The terminally modified fatty acids (aLA and aAA) were oxidized in thin films and in solutions, in the presence of radical initiators. The analysis of acyclic hydroperoxides formed as primary oxidation products in these reactions, revealed that oxidations of the unnatural fatty acids parallel those of native linoleic (LA) and arachidonic (AA) acids. It is known that these hydroperoxides, HPETEs and HPODEs, formed from AA and LA, respectively, may be decomposed to yield reactive electrophiles, that react with proteins and peptides. We then inquired if the ω-alkynylated versions of these compounds (aHODEs and aHETEs) undergo similar decomposition processes. Some preliminary evidence was found that aHNE, an alkynylated analogue of highly cytotoxic 9-hydroxynonenal (HNE), was produced upon prolonged oxidation of both aLA and aAA. Its formation was confirmed via tandem ESI-MS by the identification of an aHNE adduct to a lysine-containing model peptide (AcAVAGKAGAR).

It was found that aHNE adducted to the peptide in both a 1,2- (imine) as well as a 1,4-(Michael) fashion, and the amount of the adducts formed was comparable to the amount of adducts obtained in the presence of HNE.



Reagents and Conditions: (a) Im_2CO, CH_2Cl_2, rt., 40min,; (b) H_2O_2, Et_2O, 0°C -> rt., 12h; (c) Im_2CO, CH_2Cl_2, 0°C, 3h; (d) MeOH, rt, 1h, 53% (for a-b-c-d); (e) $HClO_4$, THF, H_2O, 5°C, 12h, 75%. (f) $NaIO_4$, H_2O, THF, 0°C, 1h; (g) $NaBH_4$, MeOH, rt, 30min, 73% (for f-g); (h) I_2, PPh_3, Im, Et_2O, MeCN, 0°C -> rt, 2h; (i) LiOH, H_2O, THF, 5°C, 12h; (j) PPh_3, MeCN, PhMe, reflux, 72h, 46% (for h-i-j); (k) i. $LiNH(TMS)_2$, HMPA, THF, -78°C, 40min; ii. 5-hexynal, THF, -78 -> rt., 2h, 54%; (l) *m*-CPBA, CH_2Cl_2; 5°C, 6h.

Additional types of reactive electrophiles are obtained via formation of cyclic PUFA-hydroperoxides and their subsequent decomposition. Upon

extended oxidation of aAA, production of cyclic peroxides was detected. Furthermore, the relative isomeric distribution as well as total amount of cyclic products was similar to the respective to the product set obtained upon oxidation of AA under the same conditions. Formation of cyclopentenone isoprostanes from aAA was confirmed in tandem ESI-MS studies by the identification of a Michael adduct of these compounds to a cysteine-containing peptide. It was reported previously that such electrophiles formed from AA reacted readily with glutathione (GSH) in the presence of glutathione transferase (GST) to form 1,4-type adducts. Formation of alkynylated analogues of these adducts was observed upon extended oxidation of aAA followed by dehydration and incubation with GSH in the presence of GHT. Identical conditions were applied to AA to show that indeed, similar yields of the comparable adducts were obtained from the terminally modified and native fatty acids. Additionally, evidence for oxidative conversion of aAA into extremely reactive alkynylated isoketal species *in vivo* was obtained in collaborative studies with Sean Davies of Vanderbilt University.

Since the general strategy for investigation of protein-lipid or peptide-lipid interactions is going to be eventually applied for cellular studies, transformations of aPUFAs by several oxygenase enzymes, for which native fatty acids are substrates, were also studied. Upon incubation of aLA and aAA with various dioxygenase enzymes, oxidative transformations have been explored. For instance, soybean lipoxygenase-1 transformed aLA into 13(*S*)-c,t-HPODE and aAA into 15(*S*)-HPETE, in agreement to regio- and stereoselectivity

observed during oxidations of native PUFAs. Additionally, a number of mammalian cyclooxygenases were shown to actively convert aAA to cyclic endoperoxide products analogous to those obtained from native AA. Furthermore, rates of the enzymatic transformations of aPUFA were found to be similar to the respective rates for transformation of unmodified fatty acids. The latter results were obtained in collaborative studies with Melissa Turman of Vanderbilt University.