DEBUNKING ENDOGENOUS OZONE
&
TOWARDS TERT-BUTYLATED 3-PYRIDINOLS AND 5-PYRIMIDINOLS

by

Johan Brinkhorst

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Queen's University

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Abstract

Hydrocarbon autoxidation, a free radical chain reaction, is one of the most important chemical processes, and is ubiquitous in biological systems and industry. While it is vital to maintaining cellular homeostasis and plays central roles in the immune and inflammatory responses, it is also believed to play a role in the onset and development of diseases and degenerative disorders when not kept in check. In vivo, this process is generally initiated by the reduction of O_2 to superoxide ($O_2^{\bullet -}$), which can then afford various reactive oxygen species (ROS), such as HOO•, H_2O_2, HO•, and $^1O_2$. Recently, it was suggested that antibodies, as part of the immune system, produce another ROS: ozone. The evidence for endogenous ozone formation was based largely on the isolation of the known cholesterol ozonolysis products in extracts of arterial plaque and brain tissue. Identification was accomplished by derivatization and subsequent HPLC-MS analysis. Herein, an alternative, more likely explanation for the appearance of these two compounds and their derivatized forms is given, via acid-catalyzed Hock cleavage of cholesterol 5α-hydroperoxide.

Radical-trapping chain-breaking antioxidants inhibit hydrocarbon autoxidation; in Nature and as additives in industrial materials, formulations, etc. Nature typically employs phenols in this context, and it is well documented that their potency is based largely on the lability of their phenolic O-H bond. While their reactivity can be improved by making the phenol more electron-rich by introducing electron-donating groups on the aromatic ring, this increases their air (oxygen) sensitivity, leading them to decompose in air and generate ROS themselves! To prevent this, nitrogen(s) can be introduced in the aromatic ring to make 3-pyridinols and 5-pyrimidinols; the most effective air-stable radical-trapping antioxidants reported to date. Unfortunately, introduction of nitrogen in the phenolic ring leads to a concomitant increase in the acidity of the O-H bond, leading to stronger interactions with H-bond accepting solvents. This interaction reduces the efficacy of these compounds as antioxidants in polar and heterogeneous media. Herein we describe our efforts to

minimize the effect of this interaction, thereby maintaining the strong antioxidant activities of 3-pyridinols and 5-pyrimidinols, by introducing two *tert*-butyl moieties flanking the reactive hydroxyl group.

Acknowledgements

Graduate study in a foreign country is a challenging undertaking that can not be completed without the support of many people. I would like to take this opportunity to express my gratitude to those that helped me along the way.

First and most I would like to thank Dr. Derek Pratt, both as advisor and friend, who prompted me to join him in Canada, and helped make my stay a success. For being able to share in his enthusiasm for chemistry and knowledge I am very indebted. I also would like to thank him for being there in good times, but especially for giving a hand in difficult times and being patient and encouraging during the preparation of this document.

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Blessed with the best family one can wish for, I would like to thank them all for their love, support and visits. Finally, I would like to thank my parents; for without their help this would not be possible.

Allemaal ontzettend bedankt!

Statement of Originality

I hereby certify that all of the work described within this thesis is the original work of the author. Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with the standard referencing practices.

Johan Brinkhorst

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Chapter 1

Introduction

1.1 Autoxidation: A Historical Perspective

Although the oxygen-dependent deterioration of lipids, known commonly as rancidity, has been noticed since antiquity, the first scientific citation in this context is usually that of work done by Nicolas Théodore de Saussure, published in Recherches Chimiques sur la Végétation in 1804.[1] Therein, de Saussure described the results of his experiments on the oxidation of walnut oil; specifically, that it became viscous and developed a bad smell following the uptake of oxygen. Antoine-Augustin Parmentier, a pharmacist that introduced potato culture in France, also hypothesized that oxygen, to which fats were exposed in the atmosphere, was the agent of rancidity.[2] Research in the field toward the end of the 19th century and early into the 20th century was slow, but some important discoveries were made. In particular, Moritz Traube proposed a scheme of metal oxidation yielding hydrogen peroxide (Eq. 1.1).[3, 4]

$$Zn + O_2 + 2H_2O \longrightarrow Zn(OH)_2 + 2H_2O_2 \qquad (1.1)$$

Haber proposed that hydrogen peroxide (Eq. 1.2) was formed as an intermediate in the metal-mediated oxidation of various substrates, including SO_2 (Eq. 1.3).[5]

$$As_2O_3 + 2O_2 + 2H_2 \longrightarrow As_2O_5 + H_2O_2 \qquad (1.2)$$

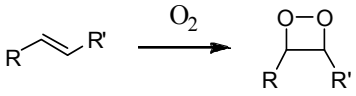
$$SO_2 + H_2O_2 \longrightarrow SO_3 + H_2O \qquad (1.3)$$

Subsequent work - especially that of Carl Engler and co-workers at the turn of the 20th century - demonstrated the formation of substrate-derived peroxides as the primary products in the oxidation of various hydrocarbon substrates in the absence of water.[6] This led to the proposal of the following general scheme for the oxidation of substrate A by O_2 (Eq. 1.4, 1.5):

$$A + O_2 \longrightarrow AO_2 \quad (1.4)$$

$$AO_2 + A \longrightarrow 2AO \quad (1.5)$$

Engler also proposed that the peroxide of olefin oxidation had the structure of a dioxethane (Eq. 1.6).[7-9]



(1.6)

Extensive study on autoxidations, oxidations by oxygen gas at normal temperature and pressure, were carried out on simple olefins, and in 1928, Stephens reported the isolation of a cyclohexene-derived peroxide which was later shown to be hydroperoxide.[10, 11] Around the 1940s, systematic studies of lipid autoxidation begun when Criegee *et al.* established that hydroperoxides are the primary products of hydrocarbon oxidation.[12] The major credit for developing the hydroperoxide hypothesis of lipid autoxidation is due to Farmer and co-workers, who greatly extended the number of hydroperoxides known to be products of olefin oxidation.[13-15] Subsequent work by

especially Bolland, Bateman and colleagues at the British Rubber Producers Research Association clearly defined the role of free radicals in the autoxidation process, which led to a general accepted mechanism for hydrocarbon autoxidation that is still used and considered adequate for explaining most experimental findings (Scheme 1.1).[16-19]

Initiation

$$In^{\bullet} + RH \xrightarrow{k_i} InH + R^{\bullet} \quad (1.7)$$

Propagation

$$R^{\bullet} + O_2 \xrightarrow{k_{O2}} ROO^{\bullet} \quad (1.8)$$

$$RH + ROO^{\bullet} \xrightarrow{k_p} ROOH + R^{\bullet} \quad (1.9)$$

Termination

$$ROO^{\bullet} + ROO^{\bullet} \xrightarrow{k_t} ROH + O_2 + R{=}O \quad (1.10)$$

Scheme 1.1. The radical chain mechanism of hydrocarbon autoxidation.

As do all radical chain reactions, hydrocarbon autoxidation consists of three fundamental types of reactions, initiation, propagation and termination. The first step, initiation, can be essentially any chemical reaction which yields a substrate derived alkyl radical (R•). Initiator radicals (In•) can be generated thermally or photochemically from various sources, including diazo compounds and peroxides. The substrate-derived carbon-centered radical (R•) then reacts with O_2 to form a peroxyl radical (ROO•) at or near a diffusion controlled rate in the first propagation step. In the second propagation step, the peroxyl radical abstracts an H-atom from another molecule of substrate forming a hydroperoxide (ROOH) and a new carbon-centered radical (R•). The chain reaction is

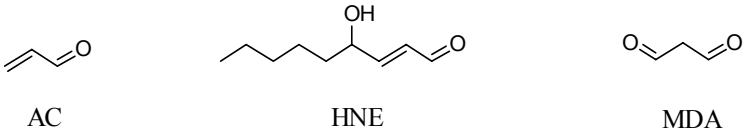
terminated when two substrate-derived peroxyl radicals react to give non-radical products via the so-called Russell termination reaction (Eq. 1.10).[20, 21] This autoxidation radical chain mechanism is believed to operate in the oxidative deterioration of essentially all hydrocarbon materials; such as lubricating oils, fuels, rubber, polymers and other organic materials and therefore of key importance to global society and commerce.

1.2 Lipid Peroxidation

The autoxidation of biological lipids, such as fatty acids (e.g. linoleic and arachadonic acids) and steroids (e.g. cholesterol) is commonly referred to as lipid peroxidation. The primary peroxidation products, lipid hydroperoxides, are themselves not very toxic due to their lack of uptake. However, lipid hydroperoxides formed *in vivo* are of far greater pathological significance. The generation of reactive oxygen species (ROS), which can initiate lipid peroxidation endogenously, is a normal process in human physiology. Essential processes of life, such as mitochondrial respiration, cyclooxygenase activation and phagocytic activity generate these species, such as $O_2\bullet^-$, HOO•, HO•, H_2O_2 and $^1O_2$.

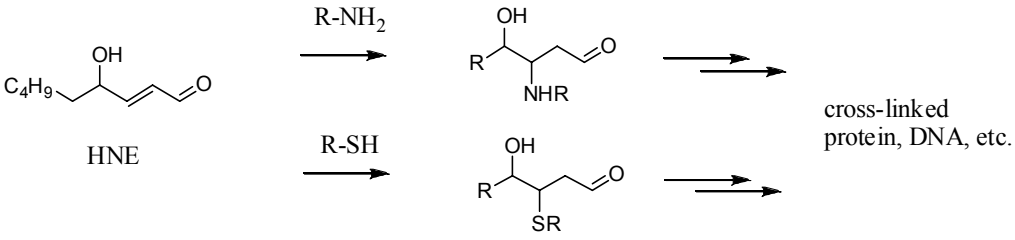
While lipid peroxidation can be carried out in a well-controlled manner by the cyclooxygenases and lipoxygenases, as part of the synthesis of important signaling molecules, e.g., prostaglandins and leukotrienes, ROS-initiated lipid peroxidation results in the formation of a series of cytotoxic compounds.[22] The most prolific are α,β-unsaturated aldehydes, which arise from oxidative cleavage of unsaturated lipid hydroperoxides. Among the most well-studied examples are acrolein (AC), malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). These compounds are good

Michael acceptors and will readily perform unwanted reactions with biomolecules that contain nucleophilic moieties such as amines and thiols.[23]



AC HNE MDA

A particular nasty feature of these α,β-unsaturated aldehydes is that they can serve to cross-link DNA and/or protein together, which can induce mutations that may lead to cancer, and protein misfolding or aggregation that may lead to other degenerative deseases (e.g. Scheme 1.2).[24,25]

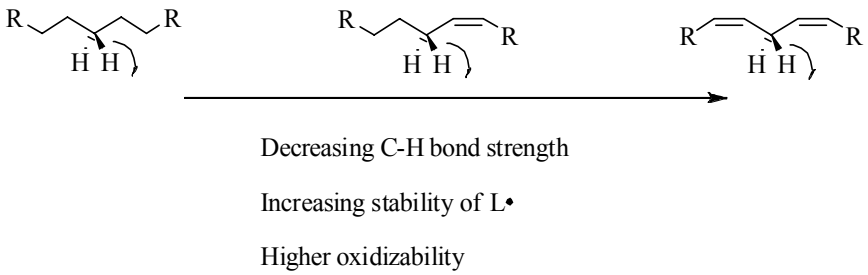


Scheme 1.2. Example of undesired reactions of biomacromolecular amines and thiols with 4-hydroxy-2-nonenal.[26]

A healthy human body will detoxify the reactive intermediates or repair the resulting damage, but when there is an imbalance between the forming of uncontrolled reactive oxygen species and the antioxidant defenses (*vide infra*), in the way that there is antioxidant deficiency, it is referred to as oxidative stress. Chronic oxidative stress leads to higher concentrations of cytotoxic compounds which are associated with numerous

human conditions and diseases such as aging, [27] atherosclerosis, cancer, Parkinson's and Alzheimer's disease.[28] Because the close relation of lipid peroxidation and disease development, many studies have been carried out in order to better understand this process.

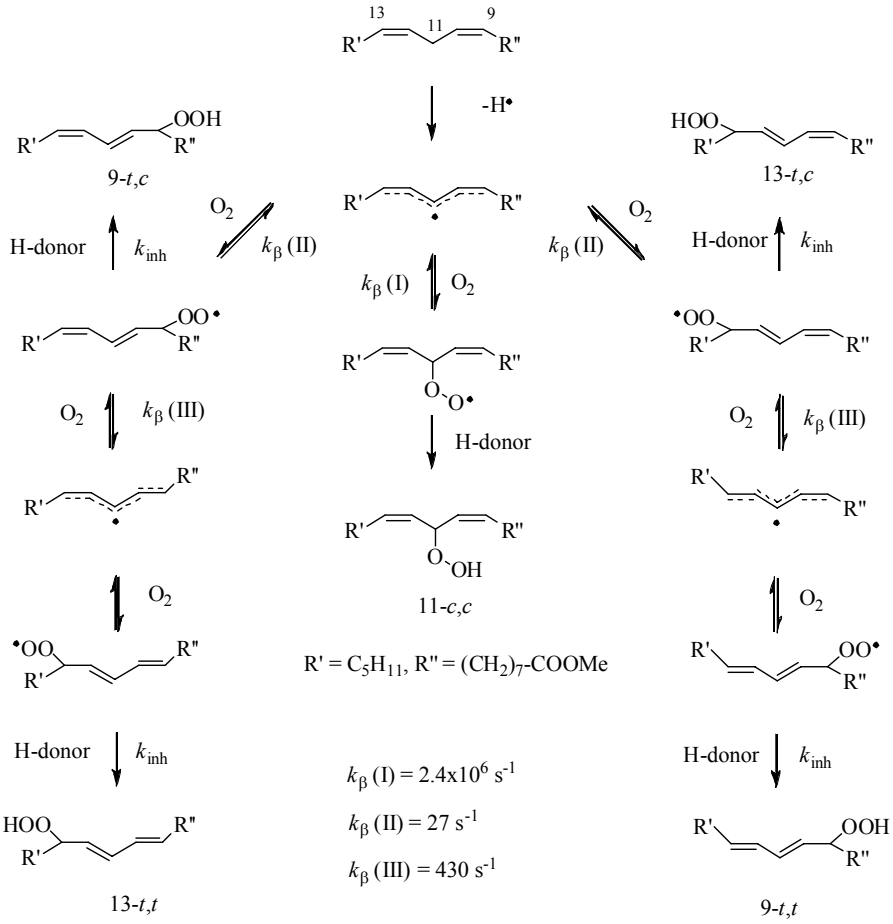
The major factor that makes lipids particularly reactive towards autoxidation is a weak C-H bond. The bond dissociation enthalpy (BDE) of C-H bonds in lipids is especially low at allylic positions of monounsaturated lipids, e.g. oleic acid, and even more so at the bis-allylic position(s) of polyunsaturated lipids, e.g. linoleic and arachidonic acid, where the derived radical is better stabilized by resonance (Scheme 1.3).



Scheme 1.3. Differing reactivities of saturated, monounsaturated and polyunsaturated fatty acids and their esters. BDE_{C-H} of: saturated ~98 kcal/mol, monounsaturated ~82 kcal/mol and polyunsaturated ~73 kcal/mol.[29, 30] k_p of: saturated ~0.01 $M^{-1}s^{-1}$, monunsaturated ~1 $M^{-1}s^{-1}$ and polyunsaturated ~60 $M^{-1}s^{-1}$.

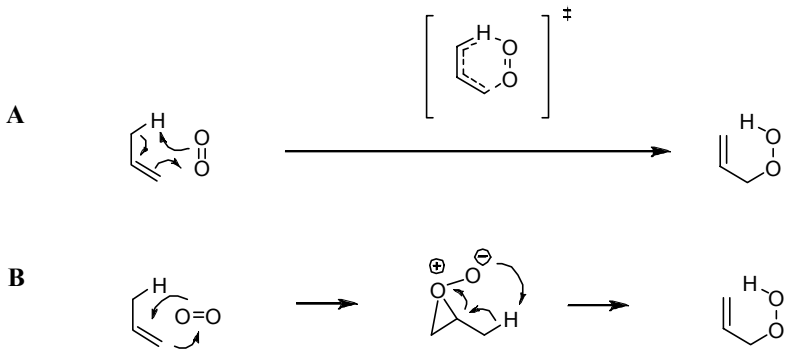
The oxidation of unsaturated lipids is a complex process but the mechanism is well understood thanks to the research of especially Porter *et al*.[31, 32] Illustrated in Scheme 1.4 is the mechanism which accounts for the formation of the five primary lipid hydroperoxides derived from the polyunsaturated fatty acid ester methyl linoleate. Upon abstraction of a hydrogen atom from the bis-allylic position, reversible oxygen addition can occur at 3 different positions, C11, C13 and C15; the first, third and fifth carbon of the pentadienyl radical intermediate. The product distribution is dependent on the values of k_β of each of the intermediate peroxyl radicals, which reflects their relative propensities to fragment back to the alkyl radical and oxygen and either the k_p of the substrate or the k_{inh} of an antioxidant when it is present in the medium – either of which can donate a hydrogen atom to the peroxyl radical, trapping it as the hydroperoxide.

In the absence of antioxidants, the *trans*,*trans* diene hydroperoxides are the major products; which arise from the isomerization and β-fragmentation of the initially formed *trans,cis* diene peroxyl radicals. These are the thermodynamic products. In the presence of good H-donors, these intermediate peroxyls are quenched before they may isomerize, giving way to the *trans*,*cis* diene hydroperoxides as the major products. Recently, it was shown that when high concentrations of very good H-donors (e.g. α-tocopherol, $k_{inh} = 3 \times 10^6$ $M^{-1}s^{-1}$) are present in solution, the non-conjugated diene peroxyl radical can be trapped in competition with its very rapid β-fragmentation ($k_\beta \sim 2 \times 10^6$ s^{-1}) to yield the bis-allylic hydroperoxide product.[33]



Scheme 1.4. Mechanism of peroxidation of linoleic acid and its esters.

Lipid hydroperoxides are also formed by a non-radical mechanism involving singlet oxygen. This reaction, shown here for the oxidation of cyclohexene, was long believed to occur via a concerted pericyclic mechanism (a so-called ene reaction). Migration of the double bond was a hallmark of this type of reaction (Scheme 1.5).



Scheme 1.5. A; Classical mechanism of the reaction of singlet oxygen with an allylic substrate through a pericyclic ene reaction mechanism. **B**; Modern step-wise (no intermediate) mechanism based on KIE mechanistic studies. Both pathways show the allylic hydroperoxide product with migration of the double bond.[34]

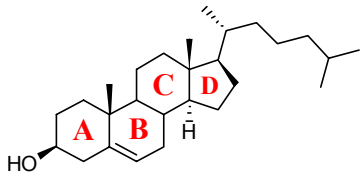
Singlet oxygen ($^1O_2$) is the lowest energy electronically excited spin paired state of molecular oxygen, and lies 22.5 kcal/mol above the ground state, triplet form of molecular oxygen ($^3O_2$). This affords double bond character, which led researchers to believe that it reacted with olefins through a concerted ene reaction, a formally allowed 6-electron pericyclic reaction mechanism. More recently, the mechanism has been described as a step-wise mechanism process, based on the absence of a significant intermolecular H/D kinetic isotope effect, suggesting the transition state for oxygenation occurs before the product determination step pointing towards a stepwise mechanism.[35] The initial attack of $^1O_2$ on an alkene occurs at the center of the π-bond with an early

transition state. After the rate limiting transition state, the mechanism involves a species with the effective symmetry of a per-epoxide (Scheme 1.5).

Unsaturated lipids in cell membranes, including phospholipids and cholesterol, are well known targets for oxidative modification. The focus of this thesis will be on oxidative modifications of cholesterol.

1.3 Cholesterol

Classified as a steroid, cholesterol (**1.1**) consists of the typical 4 ring structure. The A, B and C ring build up with six carbons and the D ring consists of five.



Cholesterol **1.1**

Its name originates from the Greek 'chole' for bile and 'stereos' for solid since it was first identified in solid form in gallstones by François Poulletier de la Salle in 1769. For this reason it was originally named 'cholesterine', but when Berthelot in 1859 reported that 'cholesterine' had an alcohol moiety the chemical suffix –ol was added resulting in cholesterol.[36] It was as early as 1871 that the Russian chemistry professor Alexander Borodin reported that cholesteryl esters were isolated from fatty degenerated heart muscle by one of his students.[37]

The importance of the relationship between cholesterol and heart disease was not realized until the early 1900s. During the 19th century, atherosclerosis, a term introduced

by Marchand in 1904, was well recognized, but it wasn't until 1908 that another Russian scientist, Alexander Ignatowski, suggested a possible relationship between cholesterol rich foods and atherosclerosis.[38] More evidence for the connection between cholesterol and atherosclerosis was obtained by Adolf Windaus, who showed that atheromatous lesions contained 6 times more free cholesterol and 20 times more esterified cholesterol than a normal artery wall. The classic work of Nikolai Anitschkow in 1913 showed the correlation between cholesterol and atherosclerosis by feeding cholesterol to rabbits after which they developed a directly proportional degree of atherosclerotic changes in their intimae.[39] Since his work was not reproducible in rats and dogs this very important work was not followed up until around the 1950's when John Gofman and his associates were the first ones to characterize the full spectrum of lipoproteins in the blood using ultracentrifugation techniques.[40]

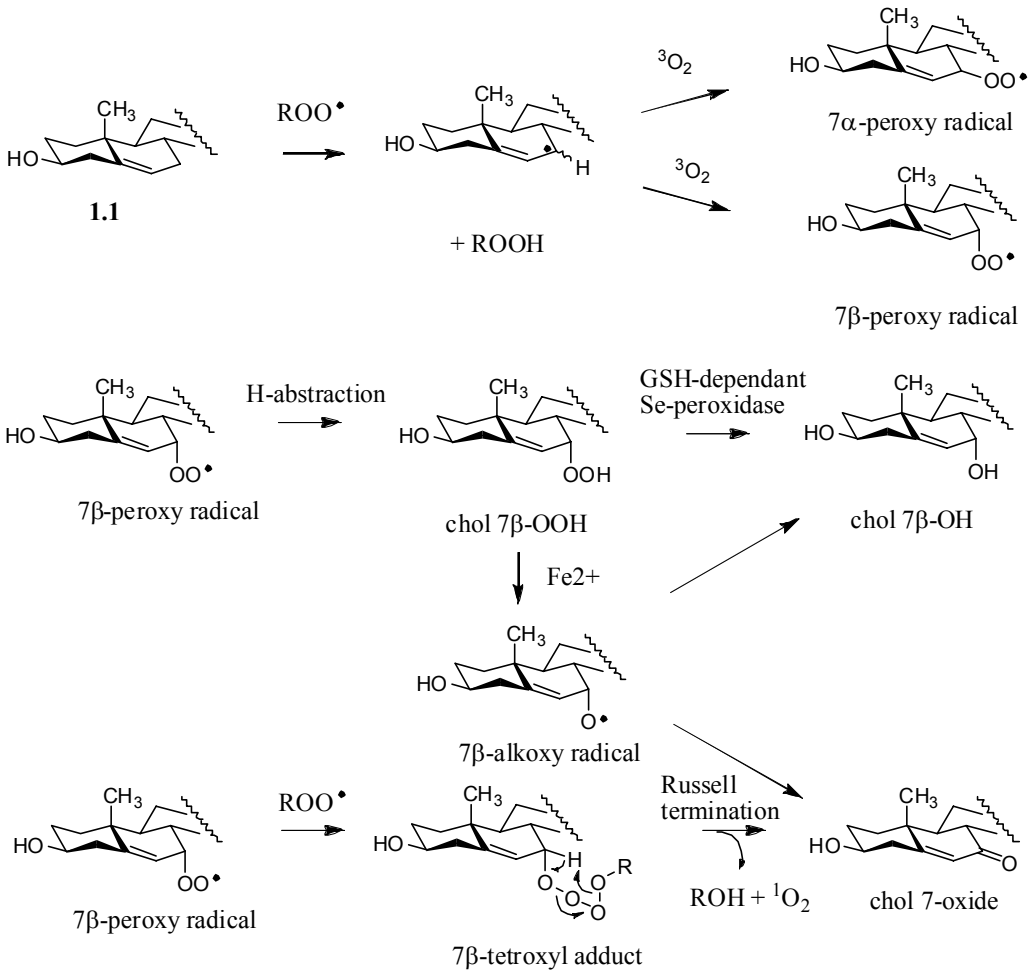
Since lipids are not soluble in blood they are associated with proteins to make them water-soluble. These so called lipoproteins have different densities based on their function. Characterization resulted in the nomenclature of lipoproteins used today dividing them based on their density.[41] The smallest particles have the highest ratio of protein to lipids and are called high density lipoproteins (HDL) and are considered to be 'good cholesterol' since these particles transport endogenous cholesterol from the tissues to the liver. Then there are three related particles with lipid densities in between; very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and low density lipoprotein (LDL).[42] Of these species, LDL is the major carrier of cholesterol in blood and is considered to be 'bad cholesterol'. This is especially true in its oxidized or partly

oxidized form.[43] How the oxidation of LDL exactly takes place, the role of low level inflammation of the artery walls and the macrophages associated with it is not completely clear yet but certain aspects have been elucidated, *vide infra*.[44]

1.4 Cholesterol Peroxidation

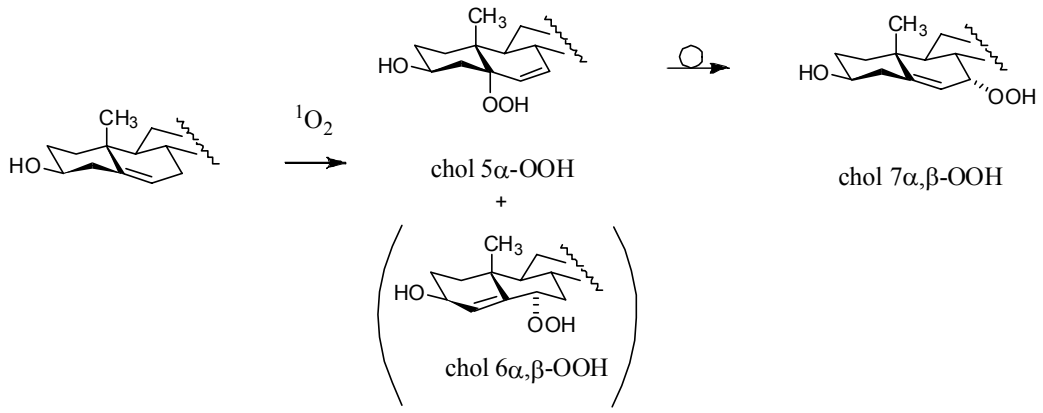
The blood levels of cholesterol both in LDL and HDL for a healthy person are around 1.5-2 mg/ml. The total body count for a person weighing 70 kilogram is 35 grams, and with an internal daily production of 1 gram, cholesterol is one of the most abundant lipids in the human body. Its typical function as a fluidity regulator in the plasma membrane puts it in a position to regularly encounter reactive oxygen species (ROS). Its position, with the hydroxyl group pointing outwards in the lipid bilayer, makes its alkene function more susceptible towards reactions with various radical and nonradical reactive oxygen species.

Oxidation products of cholesterol are generally known as oxysterols. Some of these oxysterols found in tissue are clearly formed in cells, but there is also considerable evidence that there are oxysterols that are of dietary origin. Oxysterols with modifications on the C-17 iso-octyl side chain are mostly beneficial products of enzymatic oxidation (i.e. cytochrome P450 mediated) and will not be discussed here. The reaction of cholesterol with peroxyl radicals is situated around the 5,6 double bond. There are three major observed products of this reaction, cholesterol 7α/β-OOH, cholesterol 7α/β-OH and cholesterol 7-oxide (Scheme 1.6).



Scheme 1.6. Primary cholesterol autoxidation products formed *in vivo*. The C and D ring are not shown for clarity.

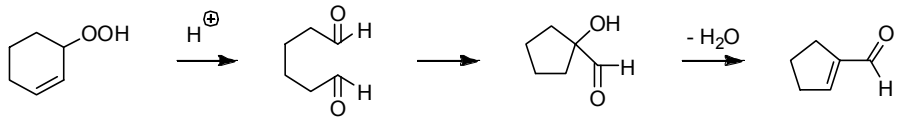
In contrast, the primary cholesterol hydroperoxides formed from reaction with singlet oxygen are cholesterol 5α-OOH (major) and cholesterol 6α/6β-OOH (minor).[45] Normally this is, however, not the isolated product of cholesterol $^1O_2$ oxidation, since it readily rearranges to cholesterol 7α,β-OOH (Scheme 1.7).[46]



Scheme 1.7. Products of cholesterol singlet oxygen oxidation. The main product is cholesterol 5α-OOH that is initially formed and used as singlet oxygen probe. Over time it rearranges to both cholesterol 7α- and 7β-OOH.

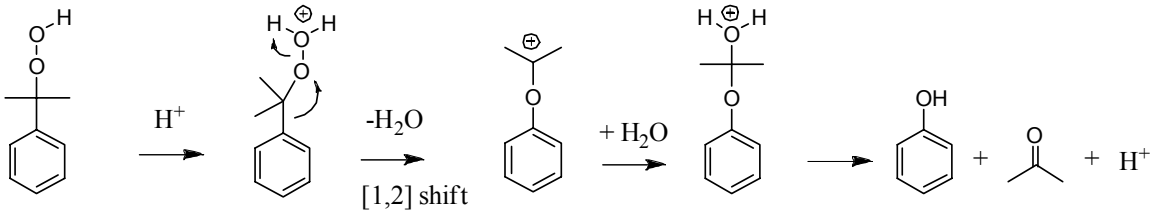
1.5 Hock Cleavage of Alkyl Hydroperoxides

Decomposition of peroxides by acid catalysis is an important synthetic process. This is commonly known as Hock fragmentation, named after its discoverer who reported in 1936 that cyclohexene hydroperoxide gave cyclopentene carboxaldehyde upon standing with sulfuric acid (Scheme 1.8).[47, 48]



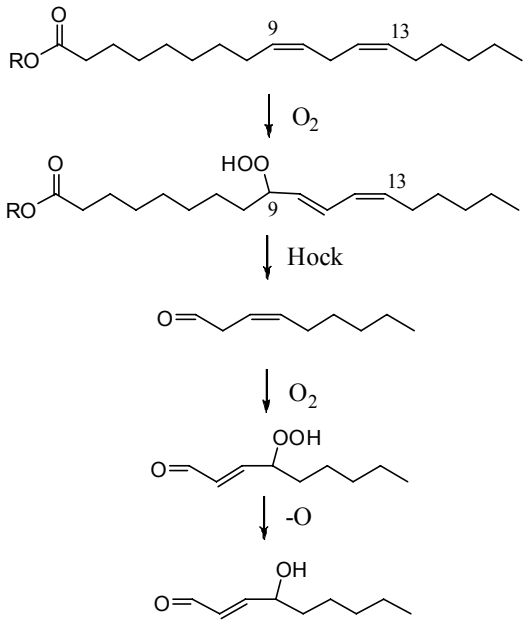
Scheme 1.8. Acid-catalyzed fragmentation of cyclohexene hydroperoxide as observed by Hock in 1936.

The acid catalyzed heterolysis of the O–O bond produces an oxycarbenium ion intermediate through migration of a neighboring substituent (Scheme 1.9). This reaction is used on an industrial scale for the synthesis of phenol (Scheme 1.9). [49, 50] It is known to work with both protic as well as with Lewis acid catalysis.[51]



Scheme 1.9. Industrial synthesis of phenol and acetone from cumene hydroperoxide by acid-catalyzed Hock fragmentation.

The Hock fragmentation of lipid hydroperoxides is widely believed to be at least partly responsible for the formation of reactive carbonyl compounds, such as acrolein, MDA and 4-hydroxynonenal (*vide supra*). A suggested mechanism for formation of HNE from linoleate is shown in Scheme 1.10.



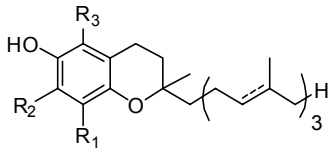
Scheme 1.10. Hock fragmentation of 9-hydroperoxide octadecanoic acid, on the primary linoleate autoxidation products, to give 4-hydro(peroxy)nonenal.

1.6 Antioxidants

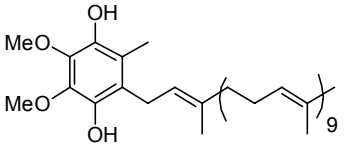
Antioxidants slow the rate of autoxidation. They may do so by either capturing species which initiate radical chain reactions (ROS) or by trapping the chain carrying radicals which propagate the chain (R• or ROO•). In Nature, the elimination of ROS than can initiate autoxidations is mainly the responsibility of enzymes. For example, superoxide dismutases catalyze the conversion of superoxide to oxygen and hydrogen peroxide, catalase converts hydrogen peroxide to oxygen and water, and peroxidases reduce alkyl hydroperoxides to alcohols.[52] In contrast, the trapping of chain-carrying radicals that

propagate lipid peroxidation in membranes and lipoproteins is mainly the responsibility of small molecules, generally referred to as chain-breaking antioxidants.

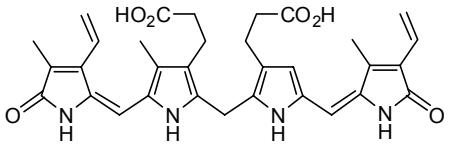
Since lipid peroxidation of membrane lipids and LDL in the bloodstream is implicated in degenerative diseases, the intervention of chain-breaking antioxidants is key in maintaining good health and cell homeostasis. There are several lipophilic antioxidants present in membrane lipids and the LDL particle. They vary from type and concentration based upon diet, but ubiquinol (coenzyme Q_{10}), bilirubin, and Vitamin E are generally found (Scheme 1.11). Vitamin E is the general name of the 4 congeners of each of the tocopherols and tocotrienols. The most abundant and most reactive of which is α-tocopherol, the protoptyical example of a phenolic radical-trapping chain-breaking antioxidant.



α-tocopherol, $R_1=R_2=R_3=CH_3$ γ-tocopherol, $R_1=R_2=CH_3=R_3=H$
α-tocotrienol, $R_1=R_2=R_3=CH_3$ γ-tocotrienol, $R_1=R_2=CH_3=R_3=H$
β-tocopherol, $R_1=R_3=CH_3=R_2=H$ δ-tocopherol, $R_1=R_2=R_3=H$
β-tocotrienol, $R_1=R_2=R_3=CH_3$ δ-tocotrienol, $R_1=R_2=R_3=H$



ubiquinol



bilirubin

Scheme 1.11. Several different lipophilic antioxidants present in low density lipoprotein particles in human serum.

1.7 Phenolic Antioxidants

Phenolic antioxidants work to inhibit lipid peroxidation by trapping chain-carrying peroxyl radicals by transfer of their phenolic H-atom to form a lipid hydroperoxide (Eq. 1.11).

Inhibition

$$ArOH + ROO^{\bullet} \underset{(\leftarrow)}{\xrightarrow{k_{inh}}} ArO^{\bullet} + ROOH \quad (1.11)$$

Termination of inhibited autoxidation

$$ArO^{\bullet} + O_2 \xrightarrow{very\ slow} Ar(=O)OO^{\bullet} \quad (1.12)$$

$$ArO^{\bullet} + RH \xrightarrow{very\ slow} ArOH + R^{\bullet} \quad (1.13)$$

$$ArO^{\bullet} + ArO^{\bullet} \xrightarrow{fast} Products \quad (1.14)$$

$$ArO^{\bullet} + ROO^{\bullet} \xrightarrow{fast} Non\text{-}radical\ products \quad (1.15)$$

This mechanism is historically believed to be a H-atom transfer (HAT) mechanism but is recently being revised and there may be a more complex mechanism at work namely proton-coupled electron transfer (PCET).[53] In this mechanism, complex formation between the phenol and peroxyl radical precedes the reaction, wherein the phenolic hydrogen is H-bonded to the peroxyl radical. This hydrogen is then moved as a proton from the phenol to the lone pair on the peroxyl radical along with the coupled movement of an electron from the 2p lone pair of the phenolic oxygen to the orbital containing the unpaired electron on the peroxyl radical, such that the electron travels between two formally non-bonding orbitals (Figure 1.1).

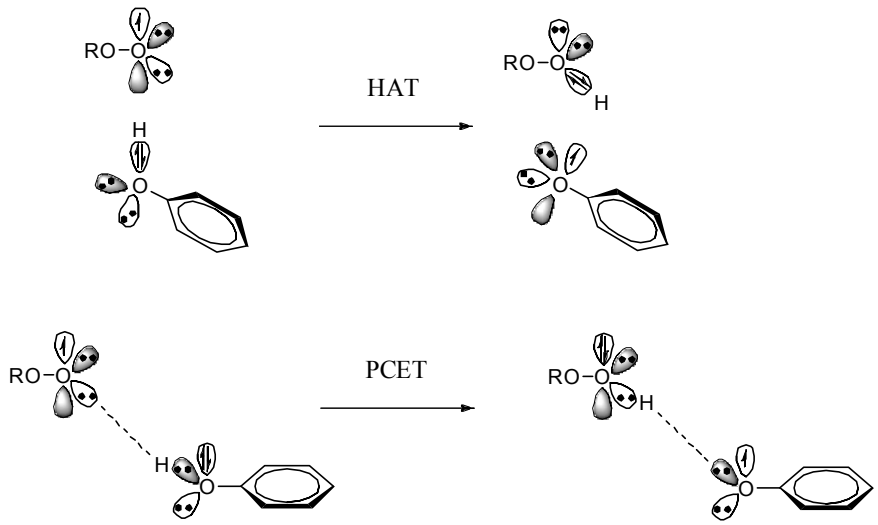
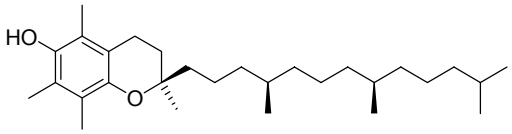


Figure 1.1. Hydrogen atom transfer mechanism versus proton coupled electron transfer mechanism.

So why are phenols such good chain breaking antioxidants? For breaking the radical chain reaction, the antioxidant has to intercept the radical in the propagation step and form a phenoxyl radical that is unreactive to either oxygen (Eq. 1.12) or the substrate (Eq. 1.13). Hence, the phenoxyl radical decays either through reacting with itself (Eq. 1.14) or more likely reacting with another chain-carrying radical (Eq. 1.15). Assuming that chain termination occurs only via the two consecutive reactions 11 and 15 and taking quasi-stationary conditions, the rate of inhibition under a steady rate of initiation (R_i) and low [ROOH] is given by Eq. 1.16.

$$R_{O_2}^{inh} = \frac{k_p[RH]R_i}{nk_{inh}[ArOH]_0} \tag{1.16}$$

In this equation, k_p and k_{inh} represent the rate constants for the propagation (Eq. 1.9) and inhibition (Eq. 1.11) steps, respectively, and n represents the stoichiometric factor. The stoichiometric factor is the number of chain reactions halted by a single molecule of phenol (i.e., number of peroxyl radicals trapped by each phenol), and is generally close to two, due to Eq. 1.11 and Eq. 1.15). It is shown that in many cases the rate follows this law at least in the early stage (induction period) of inhibition of oxidation.[54] It also shows that that for ArOH to be efficient k_{inh} must be several orders of magnitude greater then k_p. So antioxidant ability is not an absolute property of ArOH, but also depends on the substrate. Substrates that are difficult to oxidize can be protected by relatively slow antioxidants. The substrates in the human body are, however, rather easily oxidized and need fast phenolic antioxidants for protection, e.g. α-tocopherol **1.2** (α-TOH).



α-Tocopherol **1.2**

So what factors play a role in the antioxidant ability of a phenol? The most important is the ability to give up a hydrogen atom as seen in Eq. 1.11. This ability is reflected in the bond dissociation enthalpy (BDE) of the phenolic O–H bond. An increase of the electron density of the aromatic ring leads to a lower BDE by stabilizing the electron deficient phenoxyl radical. An electron donating group (ED) in the *para* position

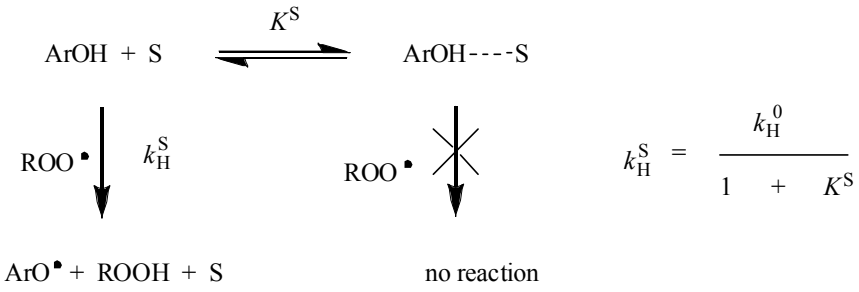
as shown by Pedulli *et al.* lowered the O–H BDE by several kcal/mol, *p*-MeOH phenol has an O–H BDE of 82.8 ± 0.8 versus 88.3 ± 0.2 for plain phenol.[55 56]

The classic form of expressing substituent (Y) effects on rates and chemical equilibria is the Hammett equation. Applied to ArO–H BDEs for *para* substituted phenols, both theoretical as well as experimental values show an excellent linear correlation with σ^+ (Y).[57] It was also established that the effect of a substituent on the H atom abstraction from ArOH by radicals was due to substituent induced change in ArO–H BDE and not to polar effects of the substituent on the transition state.[58] So, phenols with electron-donating substituents in the *ortho* and *para* positions would make faster antioxidants and, as exemplified the case of α-TOH, which possesses a *para* alkoxy moiety and *ortho* alkyl groups.

1.8 Kinetic Solvent Effects

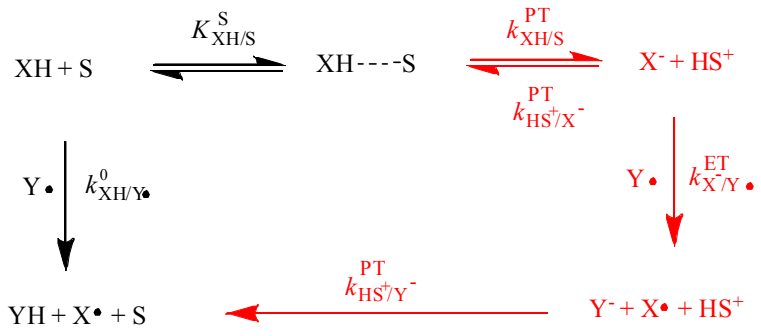
Another important mechanistic consideration does not depend on the intrinsic ability of the antioxidant to donate a hydrogen atom, but on the environment in which it operates. Since antioxidants are normally used in solutions, the solvent in which this operate also has an influence on the rate. This kinetic solvent effect (KSE) is mainly based on the hydrogen-bonding ability of the solvent itself or other additives in the medium. It can be easily envisioned that when the rate of an antioxidant depends on how fast it donates a hydrogen, if something (solvent or additive) interacts with this hydrogen it will influence its rate (Scheme 1.12).

Solvents that are strong hydrogen bond acceptors (HBA), such as those which contain carbonyls, amines or nitrile functionalities, tend to slow the rate of hydrogen donation. Switching, for example, from carbon tetrachloride to ethanol, results in a 100-fold reduction of the antioxidant capability of phenol at room temperature.[59] The solvent has no effect on the abstraction of a hydrogen atom from a hydrocarbon by a peroxyl or alkoxyl radical, making this effect obviously relevant to the abilities of antioxidants to fulfill their role.



Scheme 1.12. Kinetic solvent effect on the reaction of phenols with peroxyl radicals.[60]

Another mechanistic consideration also depends heavy on solvent characteristics, both polarity as well as hydrogen bonding ability and is called sequential proton loss electron transfer (SPLET, shown in Scheme 1.13). In this mechanism, the proton migrates to the solvent first, followed by an electron transfer from the anion to the radical, sequentially the proton transfers from the solvent to the newly formed radical. This mechanism prevails in highly ionizing solvents, such as alcohols and water.



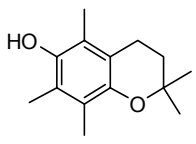
Scheme 1.13. SPLET mechanism in red versus HAT mechanism in black.

1.9 Improving Antioxidant Activity

There is a great interest in the development of antioxidants with increased reactivities to alkyl and peroxyl radicals. With the previously mentioned substituent effect in mind, this would appear to be rather easy. However, there is a limitation to this approach: while the introduction of increasingly electron-donating groups on the phenolic ring leads to a decrease in the O-H BDE, it also leads to a decrease in the ionization potential (IP) - which represents the ease of loss of an electron from the phenol (Eq. 1.18). If this is sufficiently low, the phenol will react readily with O_2 in the atmosphere yielding a ROS that can initiate lipid peroxidation.

$$ArOH + O_2 \longrightarrow ArOH^{\blacksquare +} + O_2^{\bullet -} \qquad (1.18)$$

Unfortunately, the IP of substituted phenols has also a linear correlation with σ^+ (Y). So, by introducing ED substituents in the *ortho* and *para* positions of a phenol, the O–H BDE decreases, but so does the IP, making the compound air-sensitive. This has been found to be a general problem in the improvement of reactivity of phenolic antioxidants. In fact, until recently, only a single useful improvement to the antioxidant activity of the pentamethylated chromanol (**1.3**) group found in α-tocopherol has been made. This was done by contraction of the heterocyclic ring from the 6-membered chroman nucleus to a 5-membered furan (**1.4**), which leads to a 2-fold higher k_{inh} as shown by Ingold *et al.*[61] This is attributed to a better overlap of the heteroatom lone pair(s) with the aromatic ring system. The introduction of a more electron-donating heteroatom in the ring (**1.5**, **1.6**), or extending the conjugation in the aromatic system (**1.7**) , leads to very fast antioxidants, but unfortunately also results in low IP's making them air-sensitive.



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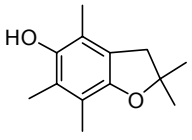

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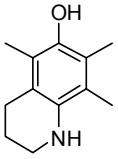

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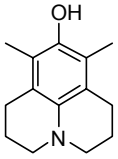

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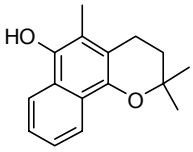
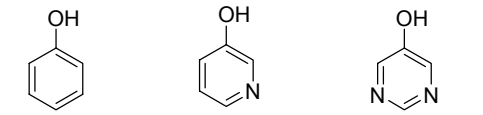


1.7

A solution to this problem was presented by Pratt *et al.* in 2001.[62] The ArO–H BDE and its IP are partly influenced by different electronic effects. The BDE is highly governed by resonance π-interaction with *o*- and *p*-substituents while the IP is more a

result of σ-induced electron density in the ring. It was shown that including nitrogen(s) into the aromatic ring making a pyridinol or pyrimidinol ring system affected both the BDE and the IP but the latter far more because destabilization of the more electron deficient radical cation produced by one-electron oxidation upon incorporating of N (Scheme 1.14). Hence, the BDEs changed to only a small extent, 1.1 kcal/mol on going from phenol to 3-pyridinol, and 1.4 kcal/mol from 3-pyridinol to 5-pyrimidinol, respectively. The IPs, however, increased dramatically, by 11 kcal/mol on going from phenol to 3-pyridinol, and then another 13.3 kcal/mol from 3-pyridinol to 5-pyrimidinol.



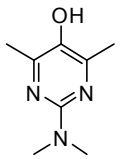
BDE = 87.1
IP = 195.4

BDE = 88.2
IP = 206.4

BDE = 89.6
IP = 219.7

Scheme 1.14. Similar bond dissociation enthalpies and higher ionization potential for pyridinol and pyrimidinol compared to phenol.

By introducing ED substituents in the *ortho*/*para* position(s) of the 3-pyridinol and 5-pyrimidinol it is possible to create compounds with a (very) low BDE but which are still air stable. For example, the pyrimidinol **1.8** was 2-fold faster then α-TOH and the 3-pyridinols **1.9**, **1.10** and **1.11** were 5-, 28- and 88-fold more effective in intercepting peroxyl radicals.[63]



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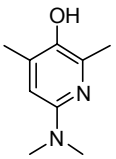

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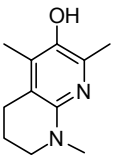

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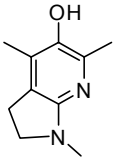
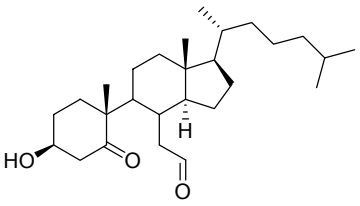


1.11

1.10 Research Objectives

Lipid peroxidation has long been an area of high scientific interest. In order to understand the mechanisms at work, and assess their possible roles in the onset and development in pathological conditions, it is necessary to carry out careful studies of the fates of the primary oxidation products formed in these processes. In a series of high profile papers, Wentworth *et al.* presented an unorthodox view of a possible oxidation mechanism of cholesterol that was purported to be of relevance in atherosclerosis.[64, 65] This body of work, starting from 2001, was based on several observations.[66] Since then a number of people have expressed their reservations, and even shown alternative explanations, for some of these observations.[67, 68] However, for one observation concerning the oxidation of cholesterol, to form the so-called Atheronals A (**1.12**) and B (**1.13**), there was no alternative explanation. One research project described in this thesis will be to provide a more likely explanation for the observation of these cholesterol oxidation products.



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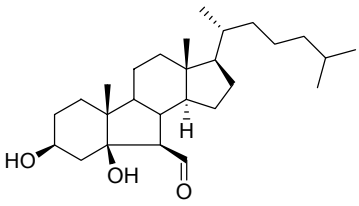
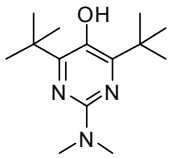


1.13

Radical-trapping chain-breaking antioxidants serve to protect cholesterol and other lipids from peroxidation. It has long been of interest to develop synthetic compounds with increased efficacy for trapping peroxyl radicals as compared to the prototypical phenolic antioxidant, α-tocopherol. Both substituted 3-pyridinols and 5-pyrimidinols have been shown to be very effective antioxidants.[69, 70] Unfortunately, the study of these compounds has been limited to very few substitution patterns on the aromatic ring. It is believed that the ideal substitution pattern would be one incorporating *tert*-butyl groups next to the hydroxyl moiety and a strongly electron-donating dialkylamino substituent in the 2-position (**1.14** and **1.15**). Unfortunately, synthetic methods available to access these compounds have been low-yielding (**1.14**) or simply don't exist (**1.15**).[71] The other research object described is this thesis is a synthetic journey setting out to find conditions to easily access different *tert*-butylated 3-pyridinols and 5-pyrimidinols.



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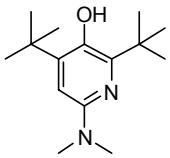

1.15

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Chapter 2

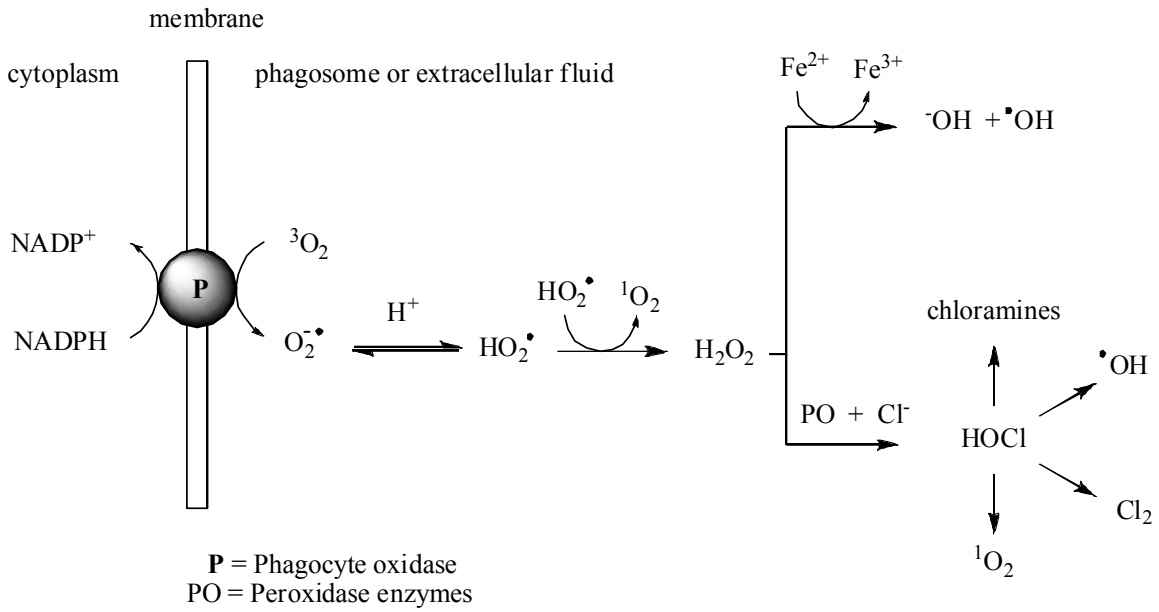
Debunking Endogenous Ozone

2.1 Introduction

In the early 2000s, a series of three very interesting papers appeared in Science.[1-3] Described therein are the findings of Wentworth and co-workers regarding the capacity of antibodies to not only recognize antigens, but also to destroy them through the generation of reactive oxygen species (ROS). On the basis of their experimental results, Wentworth and co-workers proposed that antibodies catalyze the oxidation of water by singlet oxygen to yield a trioxidic species with the chemical signature of ozone (O_3). This stemmed from a series of four observations: 1) irradiated antibodies generated hydrogen peroxide (H_2O_2) at an unusually high rate when exposed to UV light; 2) isotope-labeling experiments suggested that the mechanism of this hydrogen peroxide formation involves a trioxidic intermediate; 3) hydrogen peroxide production alone was insufficient to explain the efficacy of the antibacterial response of phagocytes; 4) an oxidant with the chemical signature of ozone appeared to be formed when antibodies are irradiated directly, or when phagocytes were stimulated in vivo.

It has long been thought that reactive oxygen species are generated by phagocytes as part of the immune response, summarized in Scheme 2.1.[4,5] This stems from the single electron reduction of ground state molecular oxygen ($^3O_2$) by the NADPH-dependent transmembrane phagocyte oxidase system, which generates the superoxide radical anion ($O_2^{\bullet -}$).[6] Although the superoxide anion itself is not considered to be cytotoxic, it occupies a critical position in the cycling of reactive oxygen species *in vivo*. It is a direct precursor to hydrogen peroxide (H_2O_2), which in turn can lead directly to highly toxic derivatives such as hydroxyl radical ($HO^{\bullet}$) and hypochlorous acid (HOCl). Another key ROS, generated from HOCl and also from the self reaction of hydrogen peroxide, is

singlet molecular oxygen ($^1O_2$). While singlet oxygen is not a radical which can initiate radical chain reactions (see Chapter 1), it does react quickly with unsaturated lipids (e.g. cholesterol) to afford similar oxidation products (e.g. hydroperoxides).[7]



Scheme 2.1. The generation of ROS by phagocytes as part of the immune response.

In 2000, Wentworth and co-workers discovered that the irradiation of antibodies led to unusually high rates of hydrogen peroxide formation. They found that the near UV-irradiation (312 nm) of a variety of human, horse, murine and sheep polyclonal immunoglobulins (Ig's) all resulted in the same linear formation of roughly 20 mole equivalents of H_2O_2 over the course of 3 hours. The same experiment employing non-immunoglobulin proteins (e.g. chick ovalbumin, β-galactosidase or α-lactalbumin) resulted in the production of only ~3 mole equivalents of H_2O_2. Intrigued by these results

they undertook further experiments in an attempt to understand the mechanism of hydrogen peroxide formation when antibodies are irradiated.

The most interesting of the limited mechanistic studies conducted by Wentworth and co-workers involved determining the isotopic incorporation of ^{18}O derived from either ^{18}O-enriched water or molecular oxygen into the H_2O_2 produced upon irradiation of the antibodies. The isotopic content of the hydrogen peroxide was assayed indirectly following its reduction with tris(carboxyethyl)phosphine (TCEP) and followed by mass spectral analysis of the phosphine oxides formed. The results were striking. When horse poly IgG was irradiated under air in ^{18}O enriched water ($H_2{}^{18}O$), the ratio of ^{18}O-labeled TCEP oxide to ^{16}O containing TCEP oxide was 1:2. When the same experiment was carried out, but using $^{18}O_2$ enriched air in unlabelled water, a ratio of 2:1 of ^{18}O-labeled to unlabeled TCEP oxide was obtained. These results were rationalized by a mechanism wherein the reaction of $^1O_2$ with water yields a trioxidic species, believed to be H_2O_3. The intermediate H_2O_3 was then suggested to undergo a S_N2-type disproportionation reaction to form H_2O_2 and oxygen ($^3O_2$), *vide infra*. While the reaction of $^1O_2$ with water is calculated to be highly unlikely in the gas phase (activation energy of 64.7 kcal/mol),[1] when additional water molecules are included in the transition state for the reaction, theoretical calculations suggest that the activation energy drops significantly (i.e. E_a = 31.2 and 12.0 kcal/mol upon incorporation of 2 and 3 molecules of H_2O, respectively, Eq. 2.1).[8]

$$^1O_2{}^* + yH_2O \rightleftharpoons [H_2O_3(y\text{-}1)H_2O] \rightarrow H_2O_2 + (x\text{-}1)\,{}^3O_2 \qquad (2.1)$$

Subsequently, Wentworth and co-workers demonstrated that irradiated antibodies are effective in killing *Escherichia coli* regardless of the antigen specificity of the

antibody. Given the foregoing results, it seemed possible that with the formation of hydrogen peroxide or singlet oxygen, antibodies are not only capable of recognizing antigens, but are also involved in their destruction. However, when the same strain of *E. coli* was subjected to either $^1O_2$ or hydrogen peroxide at similar concentrations to those produced when antibodies are irradiated, they survived. The obvious ensuing question was thus: what bactericidal agent was produced upon antibody irradiation? Could it be hydrogen trioxide, the believed precursor to the hydrogen peroxide produced in the antibody-catalyzed oxidation of water?

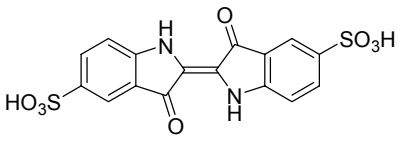
Due to the difficulty associated with experimental investigations of the mechanism of formation of trioxidic species from the antibody-catalyzed oxidation of water pathway, quantum chemical calculations[1, 5] were employed to probe the likelihood of various mechanisms to explain the foregoing observations. Two mechanistic possibilities with reasonable thermodynamics (activation energies) were presented (Eq. 2.2, 2.3). This provided an explanation for the intervention of another oxidant in the bactericidal action of antibodies, but ozone had yet to be directly detected in these systems.[9]

$$HOOOH + HOOOH \xrightarrow{E_a = 24.2 \text{ kcal/mol}} H_2O + O_3 + H_2O_2 \quad (2.2)$$

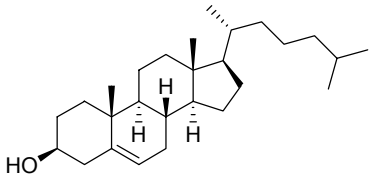
$$HOOOH + {}^1O_2 \xrightarrow{E_a = 15.2 \text{ kcal/mol}} O_3 + H_2O_2 \quad (2.3)$$

Unfortunately, obtaining direct evidence in support of the role of ozone in the immune response was complicated by the difficulty associated with its direct detection due to its short life time, and the fact that its spectroscopic properties overlap with those of biological molecules. Therefore, Wentworth and co-workers sought out indirect

evidence based on its "chemical signature", i.e. reaction products believed to derive uniquely from reaction with ozone. Two substrates were considered, indigo carmine (**2.1)** and cholesterol (**2.2**), both of which were believed to undergo reactions which yielded well-defined products unique to ozonolysis.

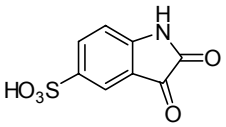


2.1

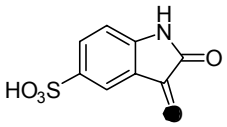


2.2

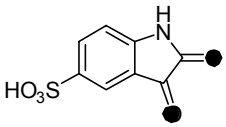
When sheep polyclonal IgG was irradiated with UV light in phosphate buffered saline (PBS) in the presence of a photosensitizer [hematoporphyrin IX (HPIX)] and **2.1**, the formation of two molecules of isatin sulfonic acid (**2.3**) was observed with incorporation of a water molecule in an unsymmetrical fashion (Scheme 2.2).[2] Although the authors acknowledge that $^1O_2$ could also be responsible for cleavage of the central bond of **2.1** to yield **2.3**, the unsymmetrical incorporation of water into the amide carbonyl of **2.3** was considered to be specific to ozone. This was revealed by experiments performed in $H_2{}^{18}O$, where the reaction products were analyzed via negative ion mass spectroscopy (MS), and a peak was found for $[M\text{-}H]^-$ = 230. This peak corresponds to double incorporation of ^{18}O in **2.3**; one ^{18}O from $H_2{}^{18}O$ via the cleavage mechanism and the other from exchange of the carbonyl oxygen. This fragment was apparently not found when **2.1** was oxidized using $^1O_2$.



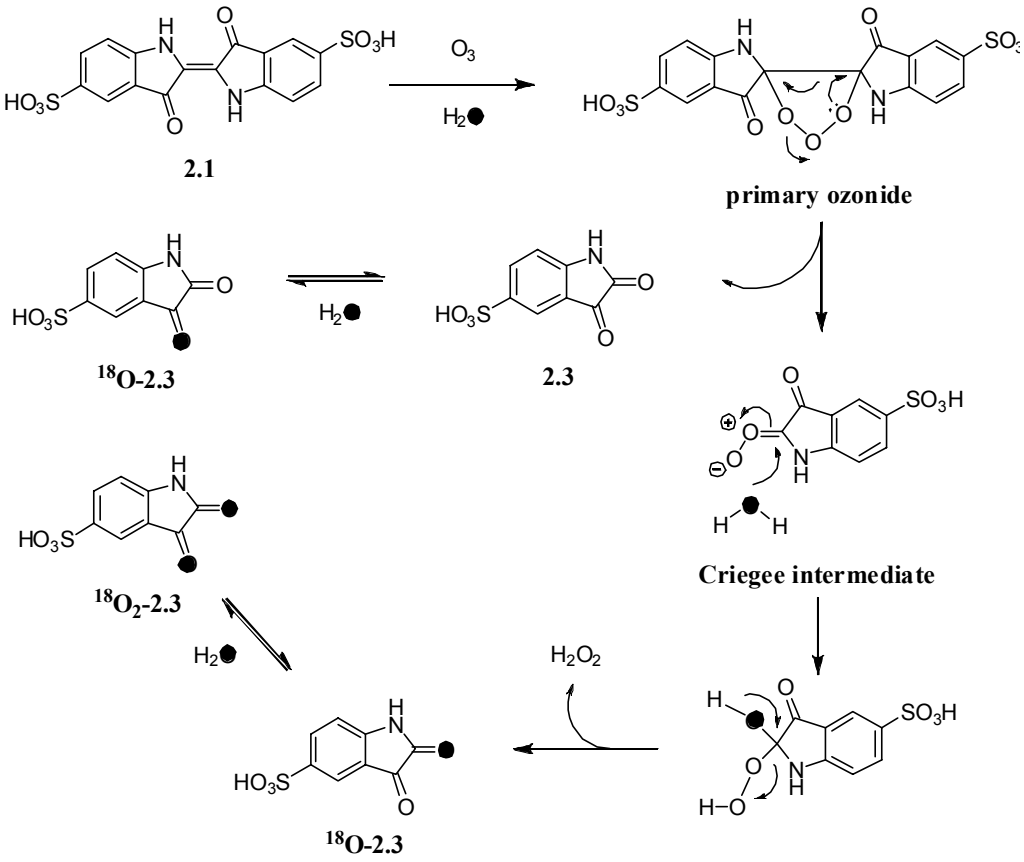
2.3
$[M\text{-}H]^-$ = 226



^{18}O-2.3
$[M\text{-}H]^-$ = 228



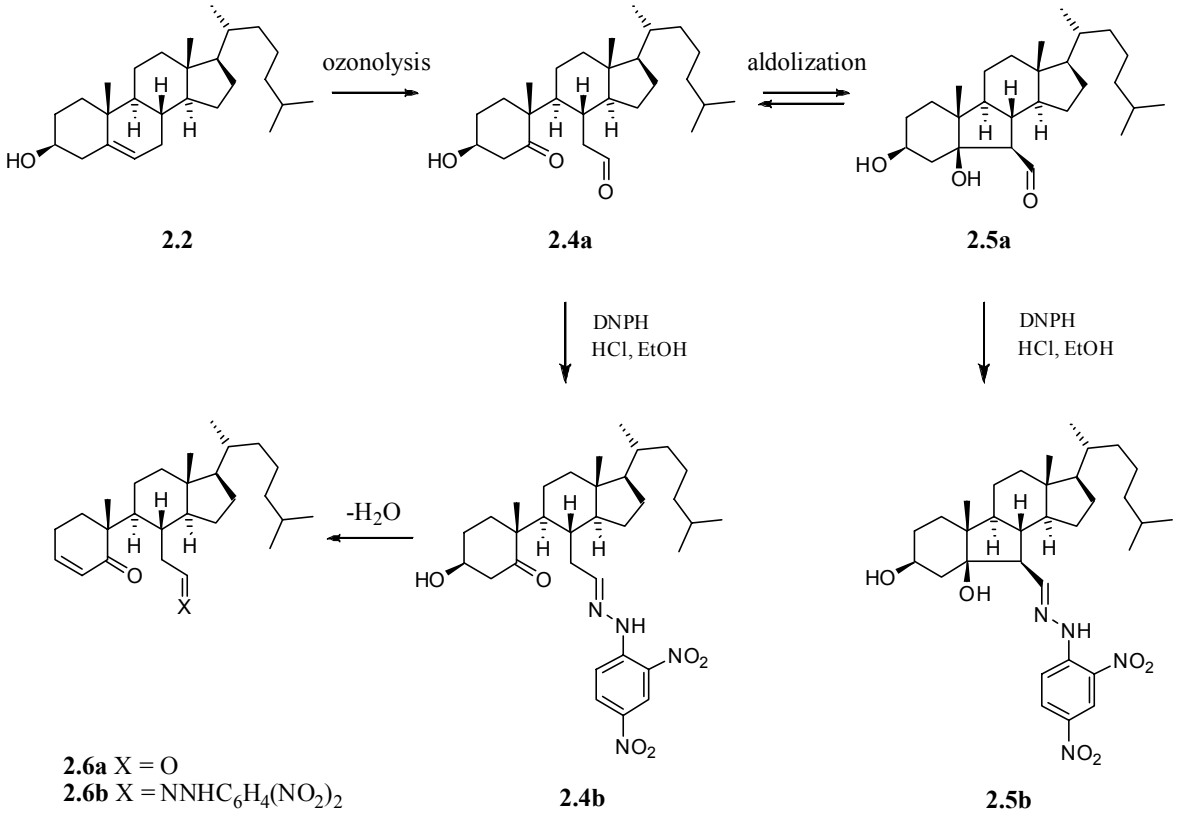
$^{18}O_2$-2.3
$[M\text{-}H]^-$ = 230



Scheme 2.2. Proposed mechanism of cleavage of indigo carmine (**2.1**) to two molecules of isatin sulfonic acid (**2.3**) with ozone in $H_2{}^{18}O$.

More interesting was when Wentworth and co-workers provided evidence for ozone formation *in vivo*; the key to which was the identification of two cholesterol oxidation products featuring a cleaved $\Delta^{5,6}$ double bond in both arterial plaque extracts and diseased brain tissues.[10] The only previously documented reaction yielding these type of products – the 5,6-secosterol **2.4a** and its aldolized form **2.5a** – was ozonolysis, studied some time ago by Pryor *et al.*[11] It should be pointed out that the identification of these cholesterol oxidation products was indirect, i.e., the arterial plaque and brain tissue

extracts were analyzed by HPLC/MS following derivatization with 2,4-dinitrophenylhydrazine (DNPH), such that the 2,4-dinitrophenylhydrazones **2.4b** and **2.5b** (as well as **2.6b**, the dehydration product of **2.4b**) were actually identified. This is summarized in Scheme 2.3



Scheme 2.3. Cholesterol ozonolysis products and their DNPH derivatives.

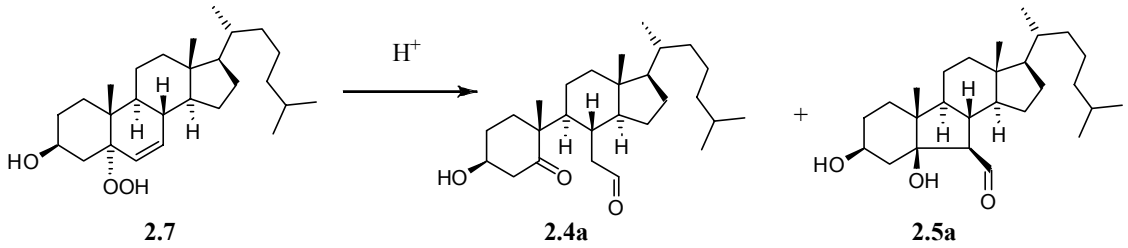
The foregoing evidence for endogenous ozone formation from the antibody-catalyzed oxidation of water has been met with skepticism by some of the most well-respected investigators in oxidation chemistry; for example the late Leland Smith,[12] Helmut Sies,[13] Chris Foote and William Pryor[14]. Since the products were not directly isolated and analyzed it was suggested that the mass spectral *m/z* 597 ion found for the

DNPH derivatization products **2.4b** and **2.5b** could also arise from a different oxysterol with the formula $C_{27}H_{46}O_3$.[15] Alternatively, it was suggested that the reaction of $^1O_2$ with cholesterol may yield a 1,2-dioxetane at carbons 5 and 6, which could subsequently decompose to yield the 5,6-secosterol **2.4a**.[16]

The relevance of proving an alternative pathway for the formation of **2.4a** and **2.5a** *in vivo* is not purely academic. The suggestions of both the antibody-catalyzed oxidation of water and endogenous ozone formation, which have appeared in the most prestigious of scientific publications, have received much attention and resulted in a significant amount of follow-up research. The apparent identification of **2.4a** and **2.5a** in human atherosclerotic plaques prompted Wentworth *et al.* to name them atheronal A and atheronal B, respectively, and much subsequent attention has been directed at examining their proatherogenic potential.[10] Likewise, the identification of **2.4a** and **2.5a** in diseased brain tissue has prompted extensive studies of their ability to induce protein misfolding, such as that which is believed to be a key factor in plaque development in Alzheimer's patients. By August 2008, Wentworth *et al.*'s 2003 paper describing the identification of **2.4a** and **2.5a** in atherosclerotic plaque extract has been cited 64 times, in research papers published in some of the most prestigious journals in science. This clearly reflects the interest in the medicinal and biochemical science community on this subject.

Herein we describe an alternative explanation for the apparent identification of **2.4a** and **2.5a** from arterial and brain tissue; one which does not invoke ozone as the reactive oxygen species responsible for their formation, but instead a well-known and accepted biological oxidant, and the suggested precursor to endogenous ozone: singlet oxygen. In particular, we will show that cholesterol 5α-hydroperoxide (**2.7**), the major

product of the reaction of singlet oxygen with cholesterol, when subjected to acidic conditions, readily undergoes cleavage of the C5-C6 bond resulting in the 5,6-secosterol **2.4a** and its aldolized partner **2.5a** (Scheme 2.4).



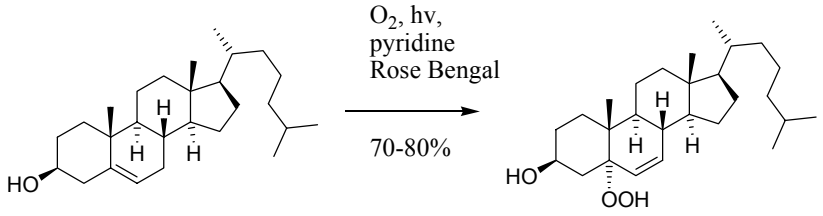
2.7 **2.4a** **2.5a**

Scheme 2.4. Cholesterol 5α-hydroperoxide upon reaction with acid forms aldehydes **2.4a** and **2.5a**

2.2 Results

2.2.1 Hock Fragmentation of Cholesterol 5α-Hydroperoxide

Cholesterol 5α-hydroperoxide (hereafter chol 5α-OOH or **2.7**) was prepared by photosensitized oxidation of cholesterol in pyridine as described by Beckwith *et al.* (Eq. 2.4).[19] Although yields were high (70-80%), successive recrystallization required to produce high purity chol 5α-OOH for our experiments led to lower overall yields.



2.2 **2.7** (2.4)

Following the isolation of pure **2.7**, preliminary ^{1}H-NMR experiments where carried out to provide an idea of the stability of this hydroperoxide and its degradation

products under acidic conditions. Although cholesterol and its derivatives have a relatively complex structure – and consequently a relatively complex ^{1}H-NMR spectrum – the peaks corresponding to the protons of interest show up distinctly in the downfield region, i.e. > 2.2 ppm (Figure 2.1).

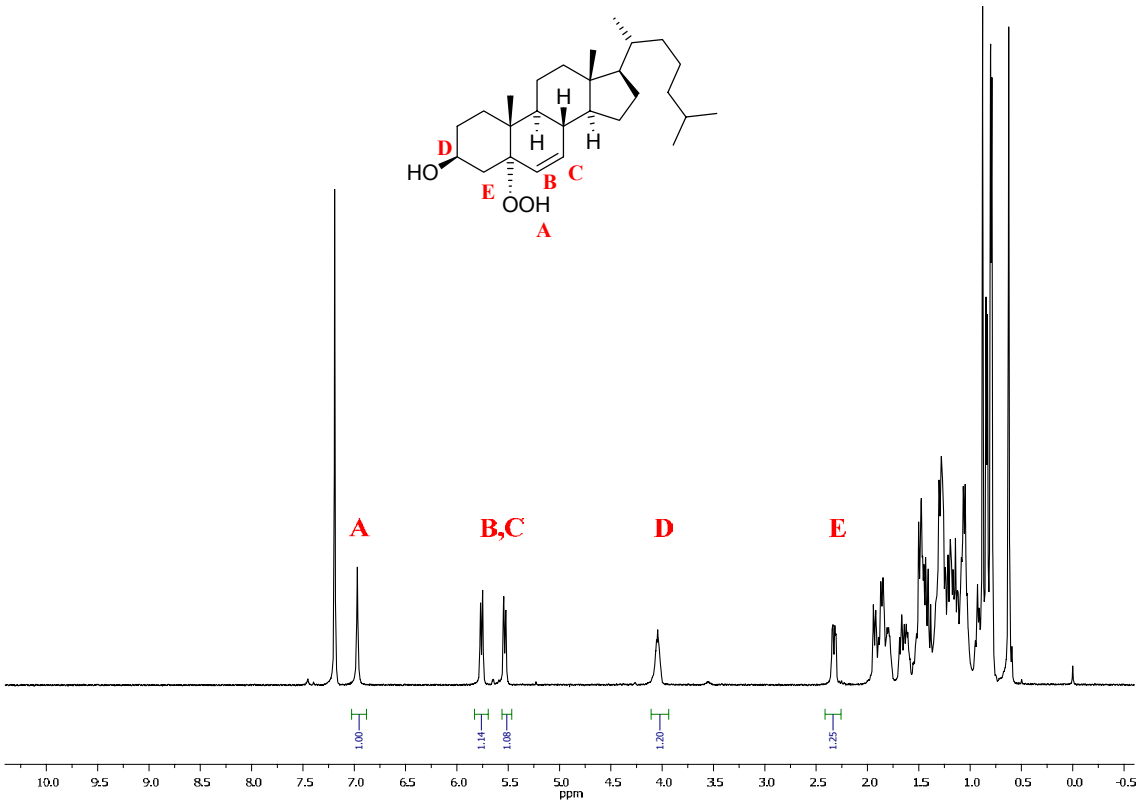


Figure 2.1. ^{1}H-NMR spectrum of cholesterol 5α-hydroperoxide in $CDCl_3$. Obtained at 500 MHz with chemical shifts reported in ppm relative to tetramethylsilane (TMS).

In our initial experiment, when a NMR sample of **2.7** (~5 mg in 0.5 ml $CDCl_3$) was subjected to one drop of TFA (ca. 50 µl), the hydroperoxide cleanly decomposed within 2 minutes. The resulting ^{1}H-NMR spectrum showed, very much to our interest, a peak between δ 9.5 ppm and δ 10 ppm in the aldehyde region (labeled **A**, Figure 2.2). To

unambiguously confirm the identity of this product, authentic **2.4a** and **2.5a** were made as suggested by Wentworth and co-workers. The ozonolysis of cholesterol in a mixture of chloroform/methanol gave the corresponding ozonide which was directly reduced with zinc dust in glacial acetic acid to yield authentic **2.4a**. Authentic **2.5a** was subsequently prepared via intramolecular aldolization of **2.4a** catalyzed by L-proline in a 9:1 mixture of acetonitrile (ACN) and water (Scheme 2.5).[3, 11]

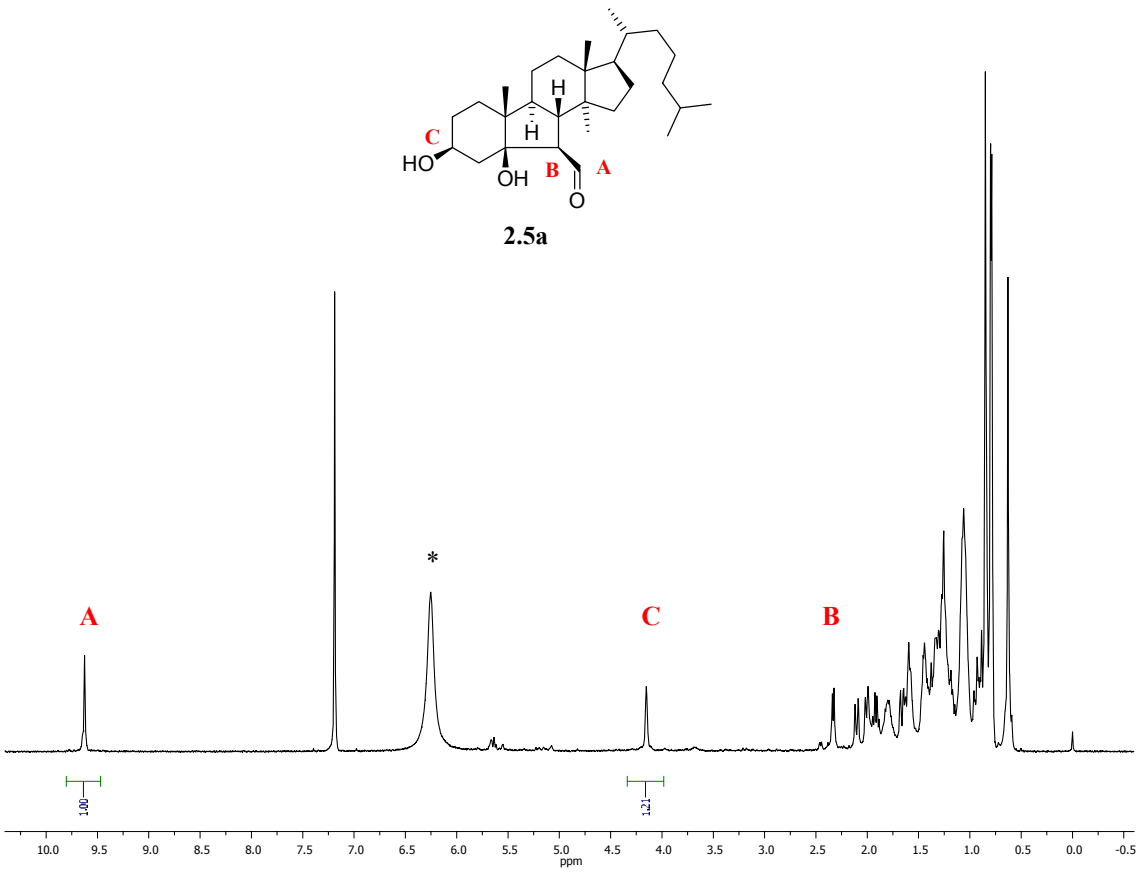
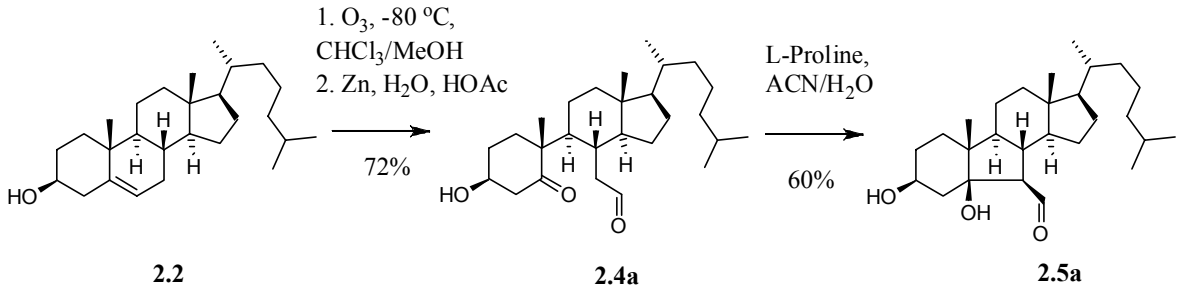


Figure 2.2. ^{1}H-NMR spectrum of **2.7** in $CDCl_3$ following treatment with a drop of TFA. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS. (* = H_2O).



2.2 **2.4a** **2.5a**

Scheme 2.5. Preparation of authentic **2.4a** via ozonolysis of cholesterol and L-proline catalyzed intramolecular aldolization to yield **2.5a**.

In comparing authentic **2.4a** to authentic **2.5a** by ^{1}H-NMR it was clear that the chemical shift of the aldehyde proton of **2.4a** in $CDCl_3$ is clearly distinguishable from that of the aldehyde proton of **2.5a**; where that of **2.4a** displays a resonance at δ 9.533 ppm, compared to δ 9.689 ppm for **2.5a**, leaving no doubt that under the conditions of our initial experiments which employed several different concentrations of chol 5α-OOH and TFA, no **2.4a** was formed. It is interesting to note that **2.5a** could even be detected in NMR samples to which no acid was added, presumably due to adventitious DCl in the $CDCl_3$ (Figure 2.3).

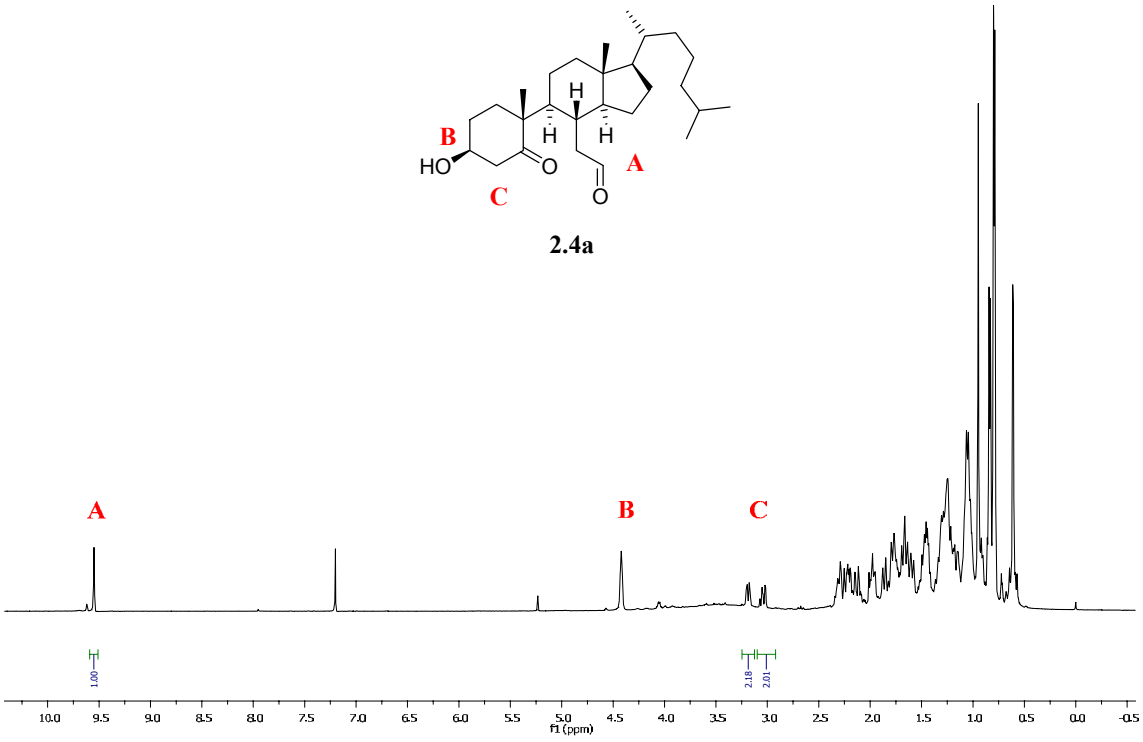


Figure 2.3. ^{1}H-NMR spectrum of the cholesterol 5,6-secosterol **2.4a** in $CDCl_3$. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS. Note that some **2.5a** is formed due to aldolization of **2.4a** in the NMR sample.

2.2.2 Solvent Effects on Product Distribution of Hock Fragmentation of Cholesterol 5α-Hydroperoxide

Proof of principle and the synthesis of the authentic atheronals **2.4a** and **2.5a** opened the way to research the Hock fragmentation of cholesterol 5α-OOH further. We moved on to carry out a set of experiments in different solvents, intent on uncovering what effect, if any, the medium has on the conversion of chol 5α-OOH to **2.5a**. This was prompted by a preliminary experiment in which we saw a minor peak for **2.4a** in reactions carried out in EtOH (Figure 2.4).

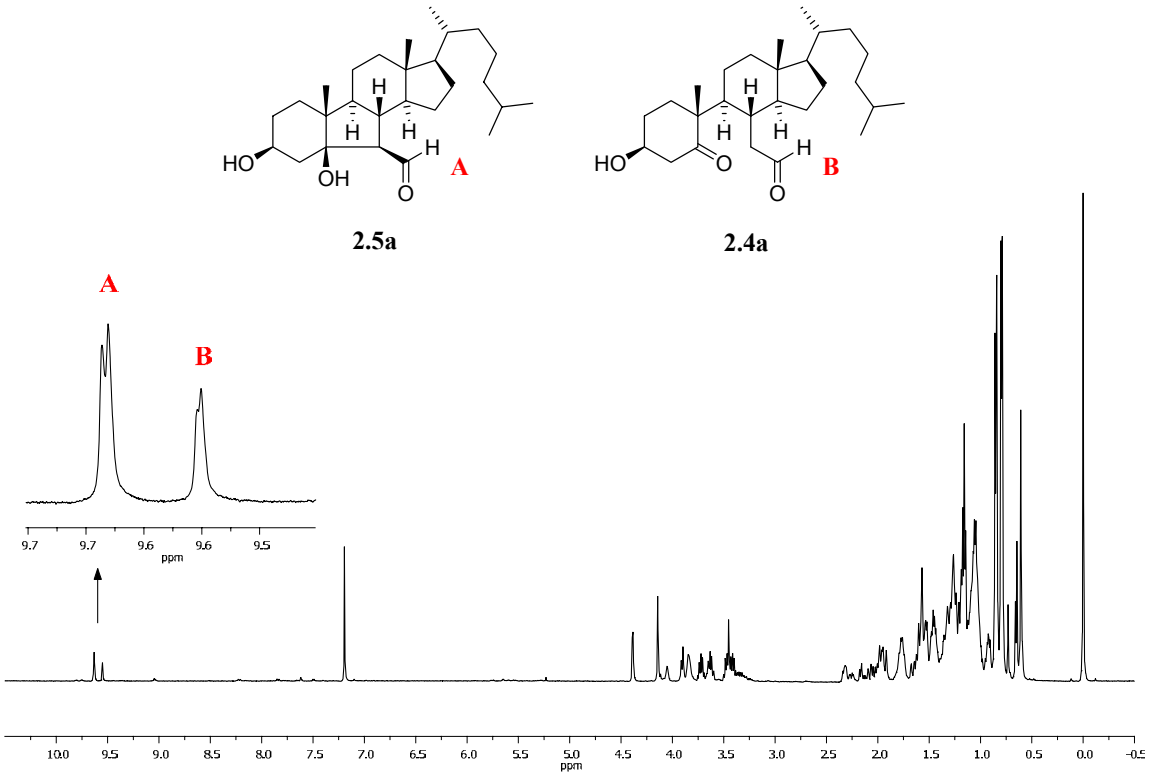


Figure 2.4. ^{1}H-NMR spectrum of the reaction products obtained from treatment of **2.7** with HCl (conc.) in EtOH, analyzed after workup. Obtained at 500 MHz in $CDCl_3$ with chemical shifts reported in ppm relative to TMS.

Experiments in the various solvents listed in Table 2.1 were performed at a concentration of 2 mg/ml chol 5α-OOH. Conversion was determined relative to an internal standard, 2,6-di-*tert*-butyl-4-methylphenol (BHT), chosen for two reasons: First, its two aromatic protons show up at δ 6.908 ppm where they do not interfere with any starting material or product signals. This also holds true for the methyl and *tert*-butyl protons, which show up in the 1.5 to 0.5 ppm region. This region shows the bulk of the cholesterol protons and is of no particular relevance to our analytic purposes. Second, BHT inhibits the rearrangement of cholesterol 5α-OOH to the more stable cholesterol

7α-OOH.[19] To these solutions, 10 equivalents of the various acids listed in Table 2.1 were added. After stirring the solutions for 1 hour, the reactions were quenched by the addition of CH_2Cl_2 and water. The relative amounts of **2.4a** and **2.5a** formed under these conditions are given as a function of solvent in Table 2.1.

Table 2.1. Relative amounts of **2.4a** and **2.5a** formed upon treatment of chol 5α-OOH with 10 equivalents of acid in various solvents.[a]

Solvent	Acid	**2.4a** (%)	**2.5a** (%)
Dichloromethane	TFA	0	100
Acetonitrile	TFA	0	100
Methanol	HCl	17	83
Ethanol	HCl	22	78
Chloroform	PTSA	0	100
THF	PTSA	0	100

[a] Reactions were performed by the same technique. To a Chol 5α-OOH solution (10 mg/5 mL), 10 eq. acid were added and the reactions were stirred for 1 h at rt. Ratios of **2.4a** to **2.5a** were determined by integration of the respective aldehyde peaks in ^{1}H-NMR after workup except MeOH and EtOH, *vide infra*.

When methanol and ethanol were used as the solvent the spectra of the samples were more complicated displaying a series of peaks between 3 and 4 ppm, due to the formation of dimethyl and diethyl acetals respectively. For example, when reactions were performed in ethanol, acetals **2.8** (Figure 2.5) and **2.9** (Figure 2.6) derived from the aldehydes **2.4a** and **2.5a**, respectively, were present in significant amounts.

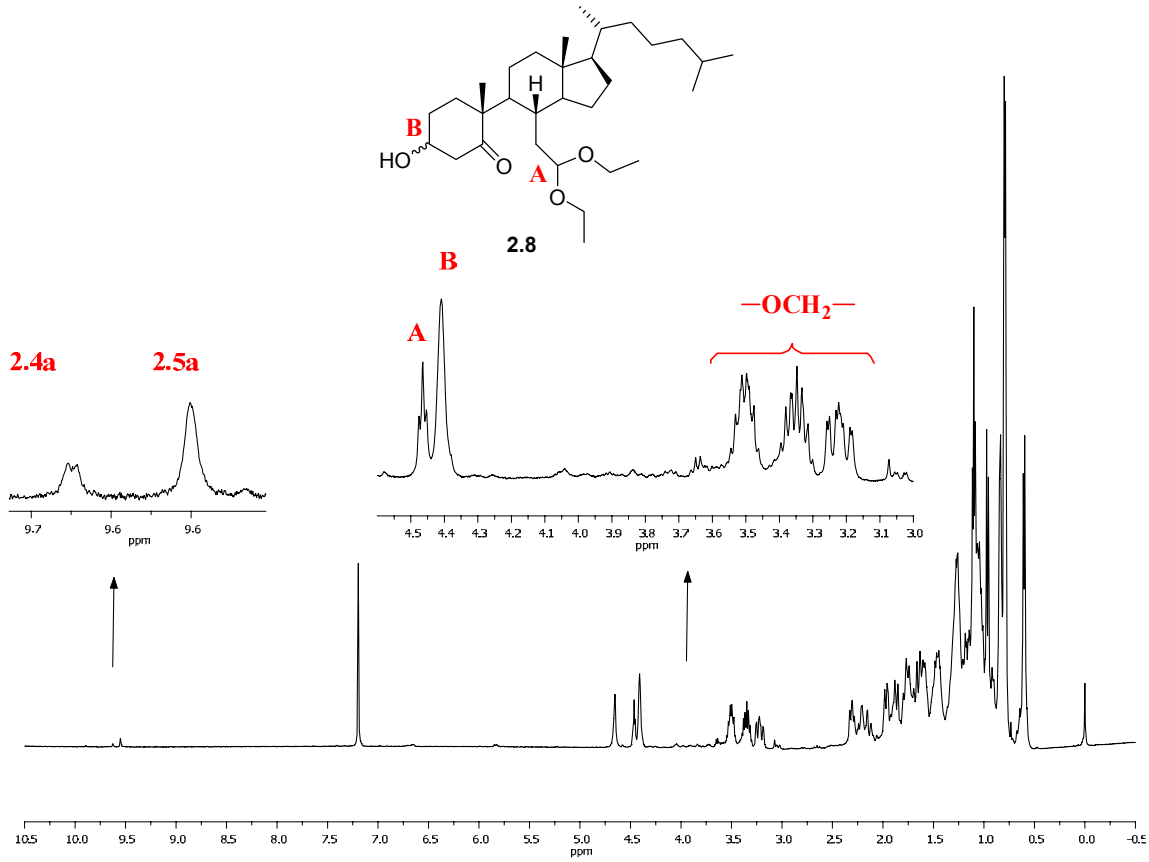


Figure 2.5. ^{1}H-NMR spectrum of **2.8**, the acetal derived from **2.4a**, in $CDCl_3$. Note that some **2.5a** is formed due to aldolization of **2.4a** upon acetalization. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS.

Despite the aqueous work-up, the acetals predominate in the ^{1}H-NMR spectra of the reactions carried out in ethanol; especially for **2.5a**, for which hydrolysis would undoubtedly be slower. Nevertheless, since resolution of the acetal protons of **2.8** and **2.9,** showing up as a triplet at δ 4.477 ppm and a doublet at δ 4.391 ppm, respectively, was possible, it wasn't necessary to hydrolyze the acetals completely in order to determine the ratio of **2.4a** and **2.5a** formed in reactions carried out in ethanol.

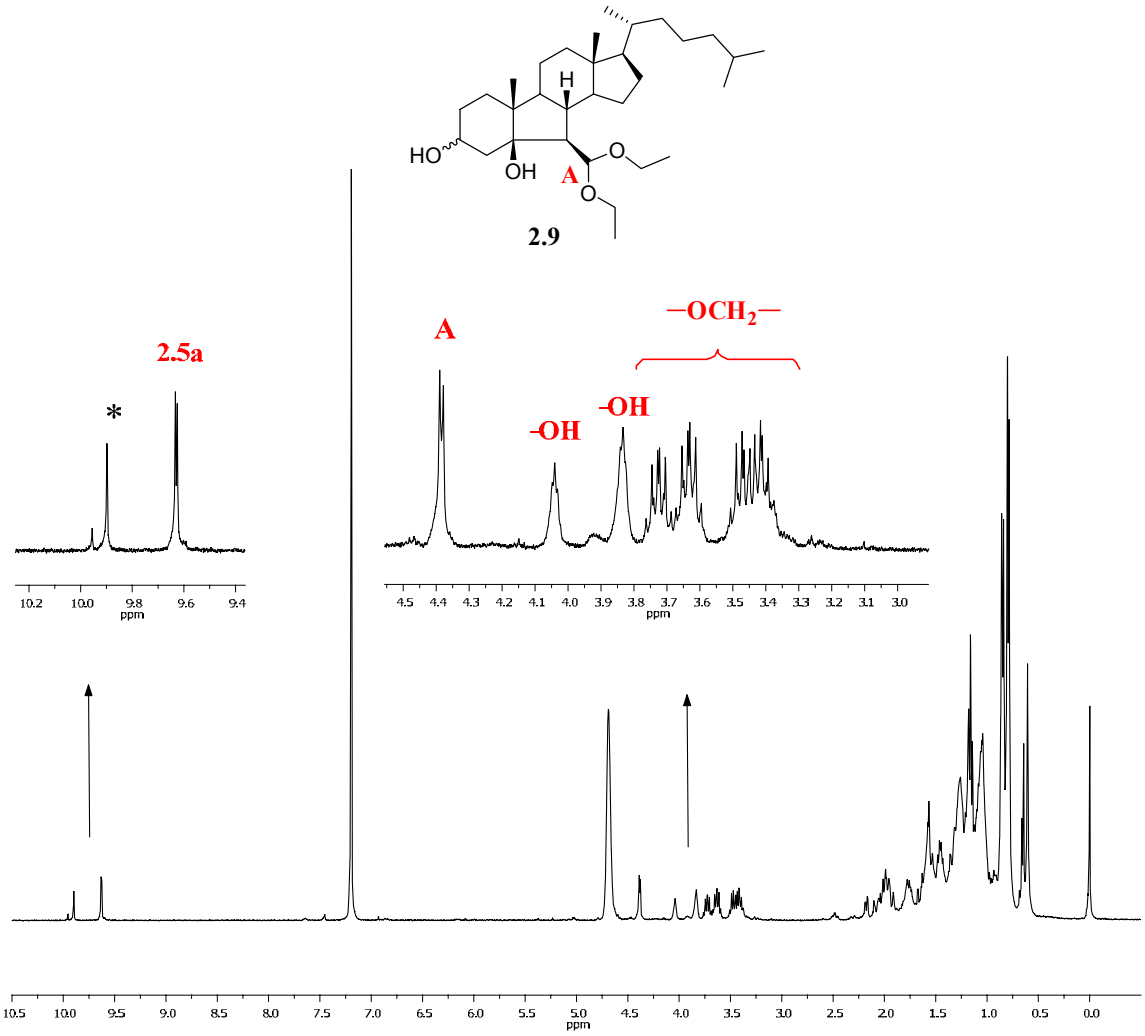


Figure 2.6. ^{1}H-NMR spectrum of **2.9**, the acetal derived from **2.5a** in $CDCl_3$. Note that some dehydration product (*) is formed on treatment of **2.5a** with acid for acetalization (confirmed by LC/MS *vide infra*). Spectrum obtained at 500 MHz with chemical shifts reported in ppm relative to TMS.

The ratios of **2.4a** to **2.5a** formed in these reactions could be determined by integration of the aldehyde proton of **2.5a** and that of its corresponding acetal proton in **2.9** combined relative to the aldehyde proton of **2.4a** and that of its corresponding acetal proton in **2.8**. This yielded a ratio of **2.4a** to **2.5**a of ~1:5.

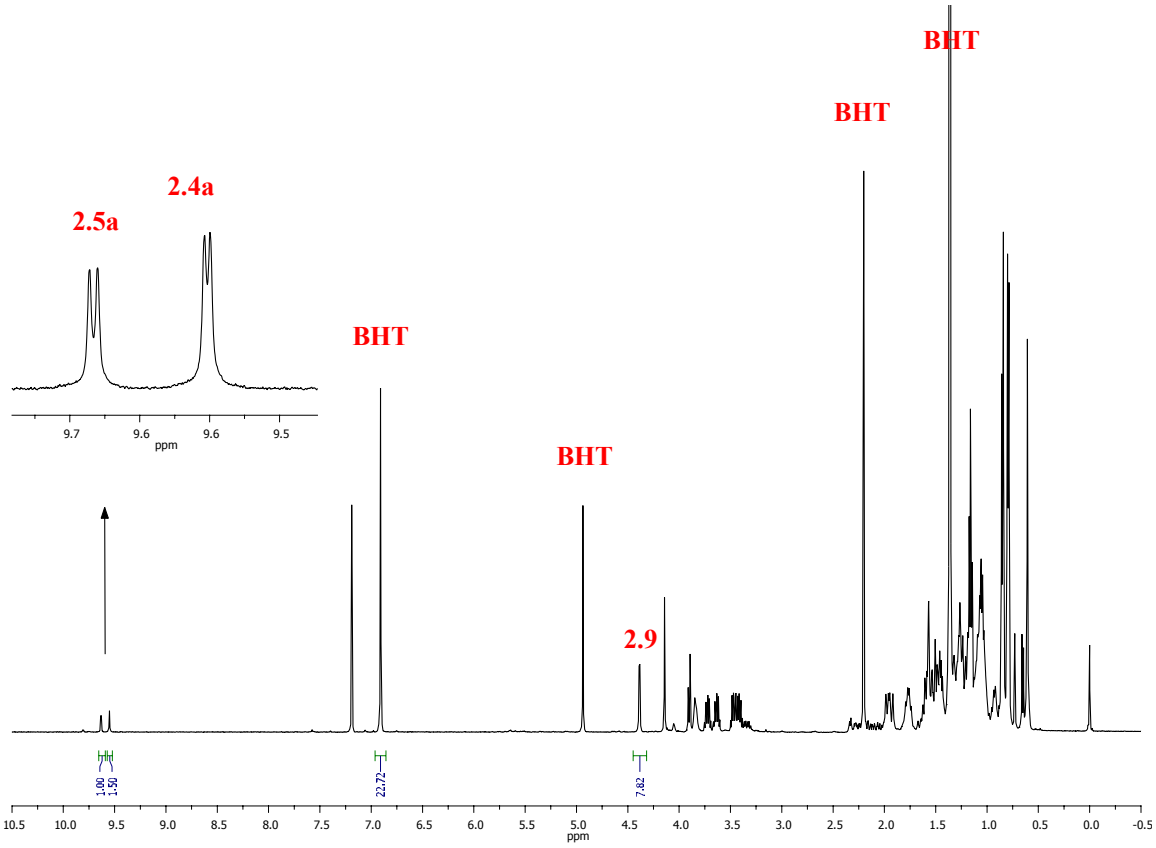


Figure 2.7. ^{1}H-NMR spectrum of the mixture of **2.4a**, **2.5a** and **2.9a** formed upon treatment of **2.7** with HCl in EtOH. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS.

In addition to examining the effect of solvent on the reaction, we also examined the effect of acid concentration using the HCl/EtOH system. We varied the acid concentration over four orders of magnitude (0.1 M – 0.0001 M) and monitored the conversion of chol 5α-OOH to product (relative to the internal standard) with time by ^{1}H-NMR spectroscopy as described above. The results are shown in Figure 2.9 and clearly establish that the rate of conversion depends on acid concentration and also that complete conversion could be observed at sub-stoichiometric amounts of acid.

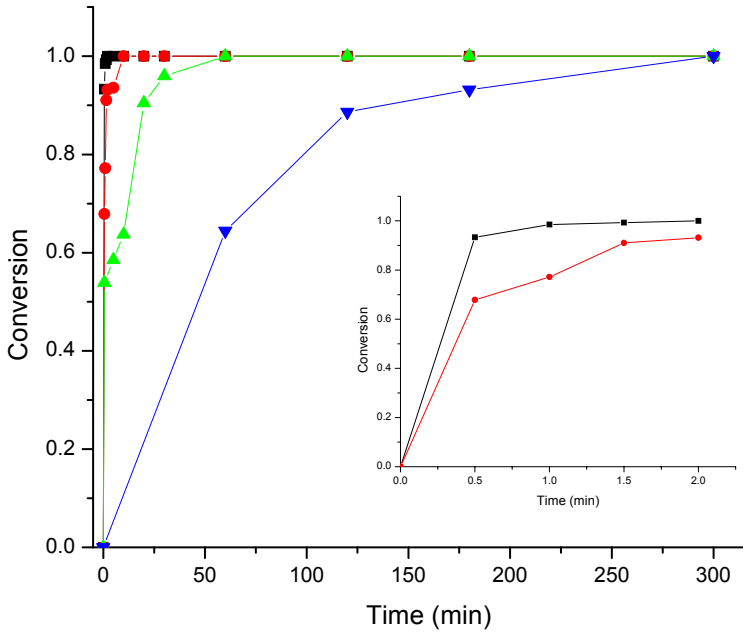
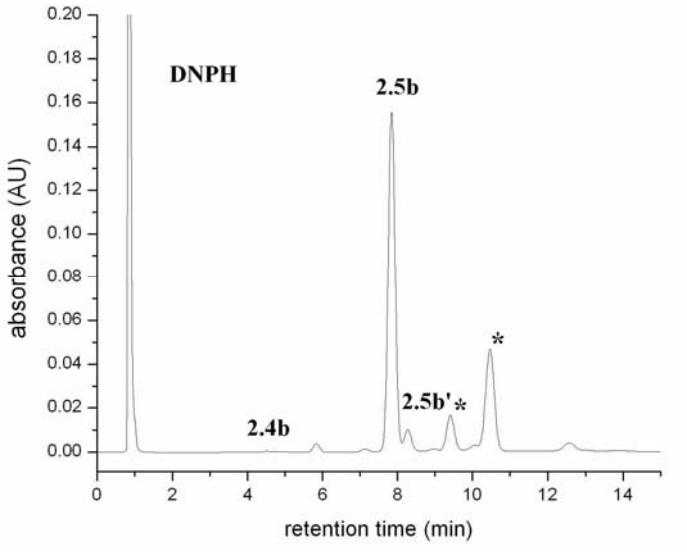


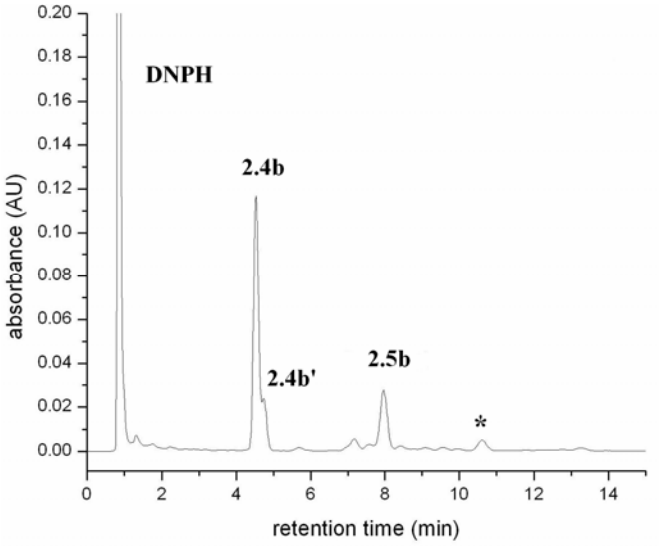
Figure 2.8. Conversion of cholesterol 5α-OOH (4,8 mmol/ml in EtOH) to **2.4a** and **2.5a** as a function of time at different concentrations of HCl (■, 0.1 M; ●, 0.01 M; ▲, 0.001 M; ▼, 0.0001 M).

2.2.3 Hock Fragmentation of Cholesterol 5α-OOH Under Derivatization Conditions

Since the derivatization of both arterial plaque and brain tissue extracts that led to identification of **2.4a** and **2.5a** in vivo was carried out under acidic conditions, we studied whether Hock fragmentation of chol 5α-OOH could occur under these conditions and what ratio of **2.4a** to **2.5a** arises under these conditions. First chol 5α-OOH was subjected to the specific derivatization conditions as reported in literature: 0.1 M HCl and 0.2 mM DNPH in EtOH and the products compared to those formed from derivatization of **2.4a** directly. Analysis of these reaction products was carried out by reverse-phase HPLC with detection of the DNPH chromophore at 360 nm.[11] Chromatograms are shown in Figure 2.9.[3]

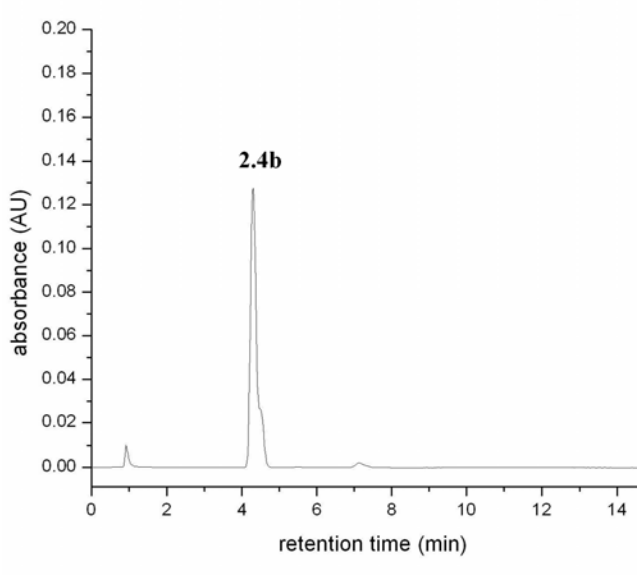


A

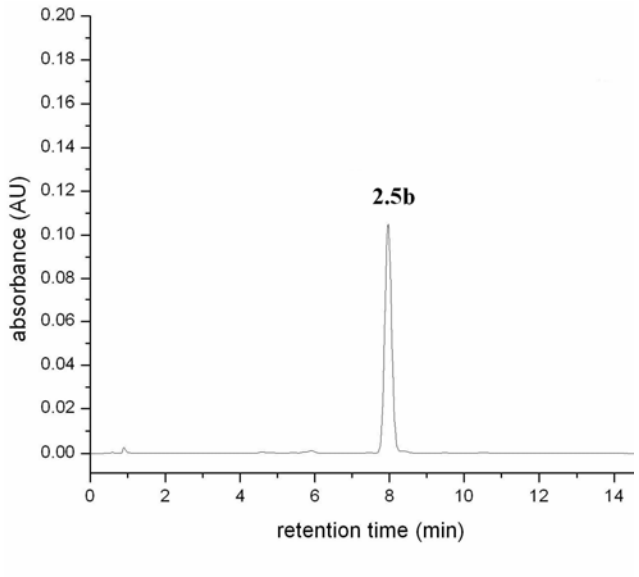


B

Figure 2.9 A: Chromatogram obtained following DNPH derivatization of cholesterol 5α-OOH **2.7** as described above. **B:** Chromatogram obtained following DNPH derivatization of secosterol **2.4a** as described above ({**2.4b'** and **2.5b'** are the cis isomers of **2.4b** and **2,5b** respectively} *dehydration products confirmed by LC/MS analysis $[M-H]^-$ = 579 as observed in ref. 3).



A



B

Figure 2.10 A: Chromatogram of authentic **2.4b**, **B:** Chromatogram of authentic **2.5b**, both prepared as described in ref. 3.

The reaction was also monitored using ^{1}H-NMR spectroscopy. A solution of cholesterol 5α-OOH (2 mg/ml) was subjected to DNPH derivatization conditions (0.1 M HCl, 0.2 mM DNPH in EtOH) and the reaction was quenched and analyzed at different time points. Under these conditions, Hock fragmentation is fast relative to derivatization and after 1 minute no cholesterol 5α-OOH was detectable by ^{1}H-NMR (Figure 2.11). Aldehydes **2.4a** and **2.5a** and the acetal of the latter (**2.9**) are clearly visible at 1 minute, as well as the hydrazone **2.5b**, but no **2.4b** could be detected (Figure 2.13).

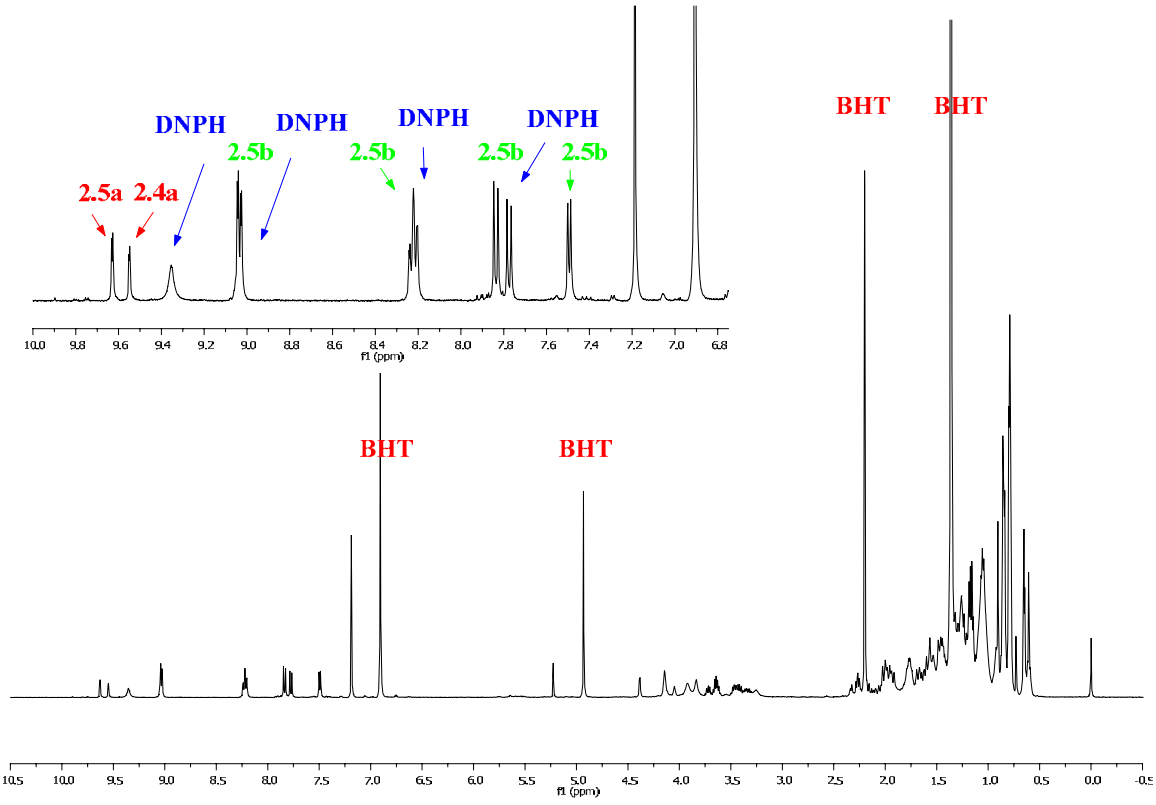


Figure 2.11. ^{1}H-NMR spectrum of the mixture of **2.4a**, **2.5a**, **2.9** and **2.5b** in $CDCl_3$ one minute after subjecting **2.7** to the DNPH derivatization conditions followed by work-up. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS.

At 2 hours the aldehyde **2.5a** (and its acetal **2.9**) was completely converted to **2.5b** with a trace amount of **2.4a** left. No **2.4b** is observed over the time course of 2 hours (Figure 2.12).

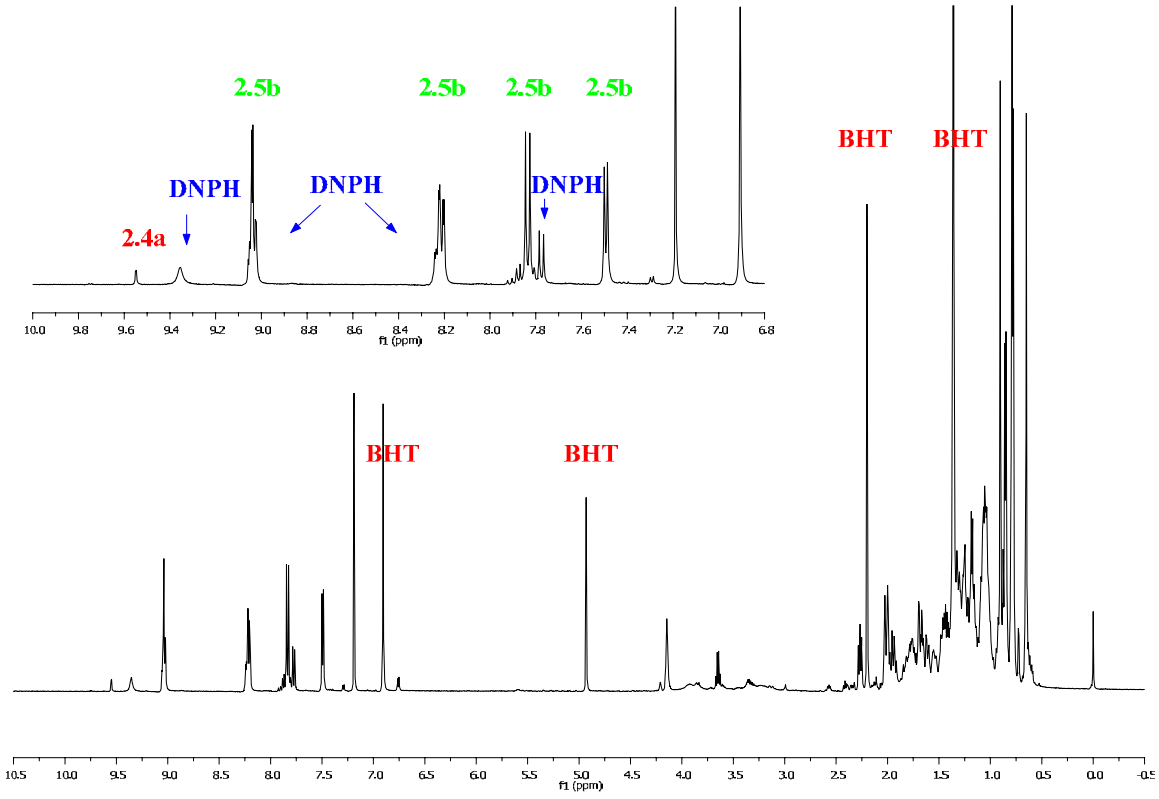


Figure 2.12. ^{1}H-NMR spectrum of the mixture of **2.4a** and **2.5b** in $CDCl_3$ 2 hours after subjecting **2.7** to the DNPH derivatization conditions followed by work-up. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS.

We also monitored the DNPH derivatization of secosterol **2.4a** using ^{1}H-NMR spectroscopy as described above in order to compare to the corresponding chromatogram obtained following derivatization (Figure 2.9B). These experiments reveal that derivatization is indeed complete within 2 hours (Figure 2.13) and that the product ratio (where **2.4b** predominates over **2.5b**) is essentially the same as that seen by HPLC.

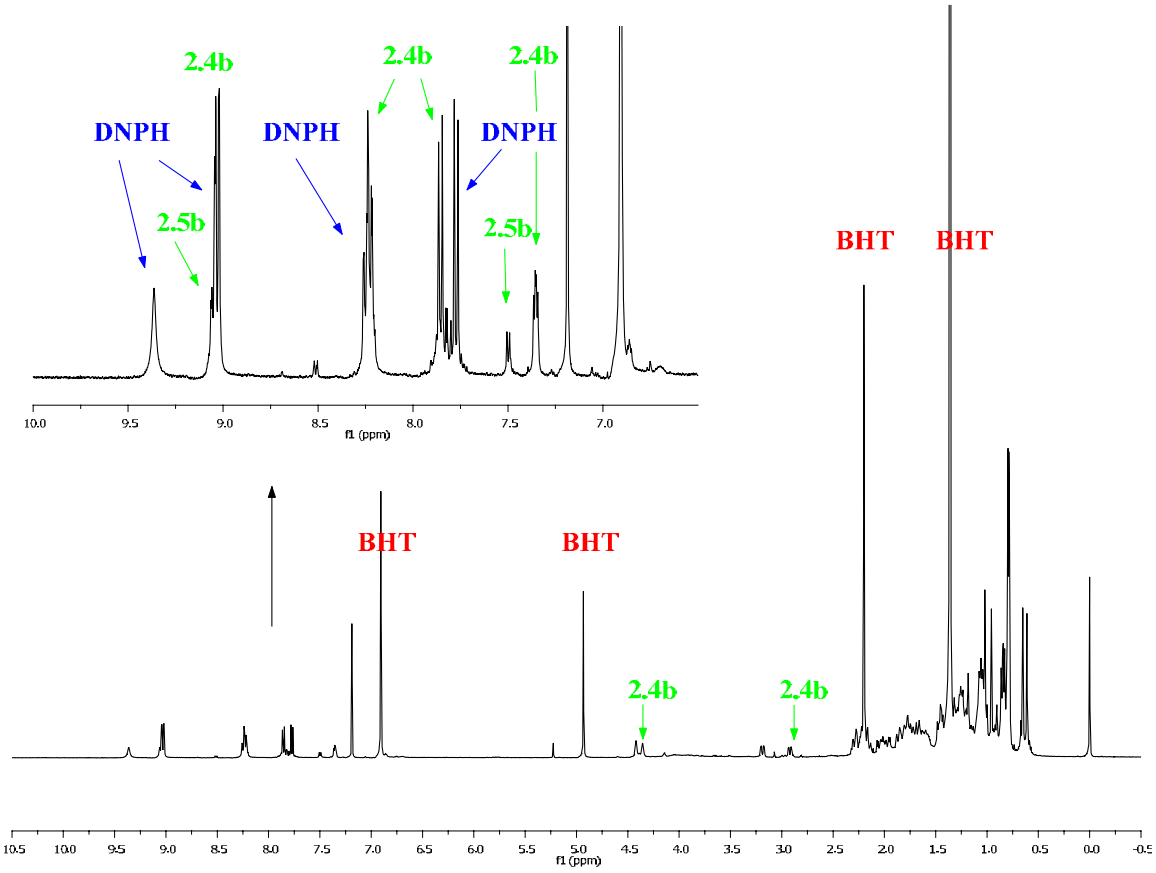


Figure 2.13. ^{1}H-NMR spectrum of the mixture of **2.4b** and **2.5b** in $CDCl_3$ 2 hours after subjecting **2.4a** to the DNPH derivatization conditions followed by work-up. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS.

The ^{1}H-NMR spectra of the authentic samples of **2.4b** and **2.5b** are shown in Figure 2.14 and Figure 2.15, respectively, for comparison with the foregoing spectra.

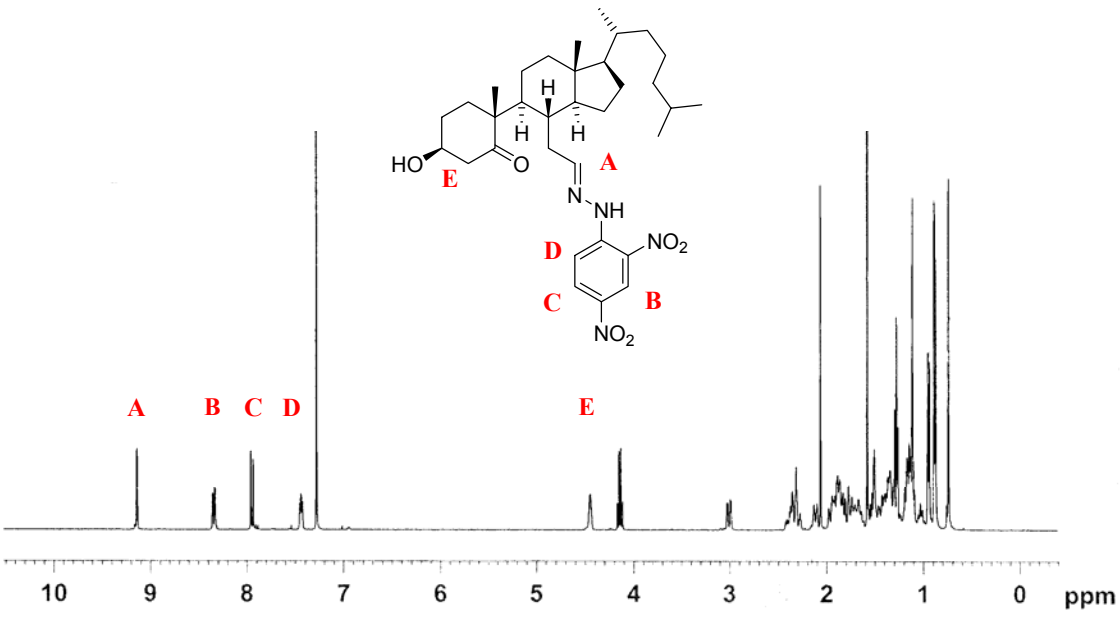


Figure 2.14. [1]H-NMR spectrum of authentic DNPH derivatized secosterol **2.4b**. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS.

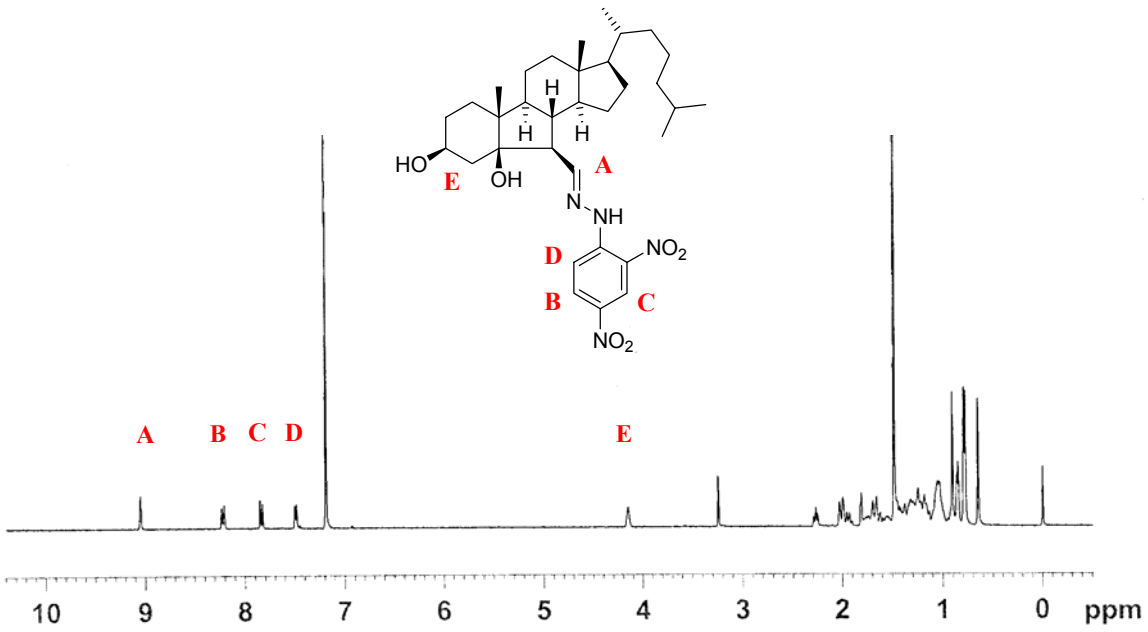


Figure 2.15. [1]H-NMR spectrum of authentic DNPH derivatized secosterol **2.5b**. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS.

2.2.4 Hock Fragmentation of Cholesterol 7α-Hydroperoxide

A few scouting reactions were performed to determine the relative propensity of the isomeric cholesterol 7α-hydroperoxide (hereafter chol 7α-OOH, **2.10)** to undergo Hock fragmentation. Compound **2.10** was easily obtained upon stirring chol 5α-OOH for 24 h in $CDCl_3$. This straightforward synthesis gives as a side reaction the epimerization of chol 7α-OOH to chol 7β-OOH.[19] This isomer was found to be difficult to remove, even following successive recrystallizations, but chol 7β-OOH was readily identified in ^{1}H-NMR spectra of **2.10** (Figure 2.16).

Initial trials using the same TFA concentration (2.7 mM) as that used in the initial experiments with chol 5α-OOH showed a very small peak in the aldehyde region after 2 hours. Using a higher concentration of TFA (13 mM) gave conversion to 2 distinct aldehyde peaks with the corresponding disappearance of starting material over 25 minutes (e.g. Figure 2.17), but the spectra looked far less clean compared to the initial chol 5α-OOH conversion (Figure 2.2), over the time course of the 25 minutes of this experiment. Initially the peak at δ 9.68 ppm was dominant but over the span of 25 minutes the peak at δ 9.75 ppm was clearly overtaking the peak upfield (see inset, Figure 2.17).

Although the reaction products have been not further characterized at this time, it is clear that over time several peaks appeared at the expense of the three downfield peaks of chol 7α-OOH alkene, (δ = 5.65 ppm) and the associated methine protons of the 3β-OH (δ = 3.55 ppm) and α-OOH (δ = 4.08 ppm). In particular, the two peaks at δ 5.341 ppm and at δ 5.09 ppm in the approximately the same rate as cholesterol 7α-OOH disappeared.

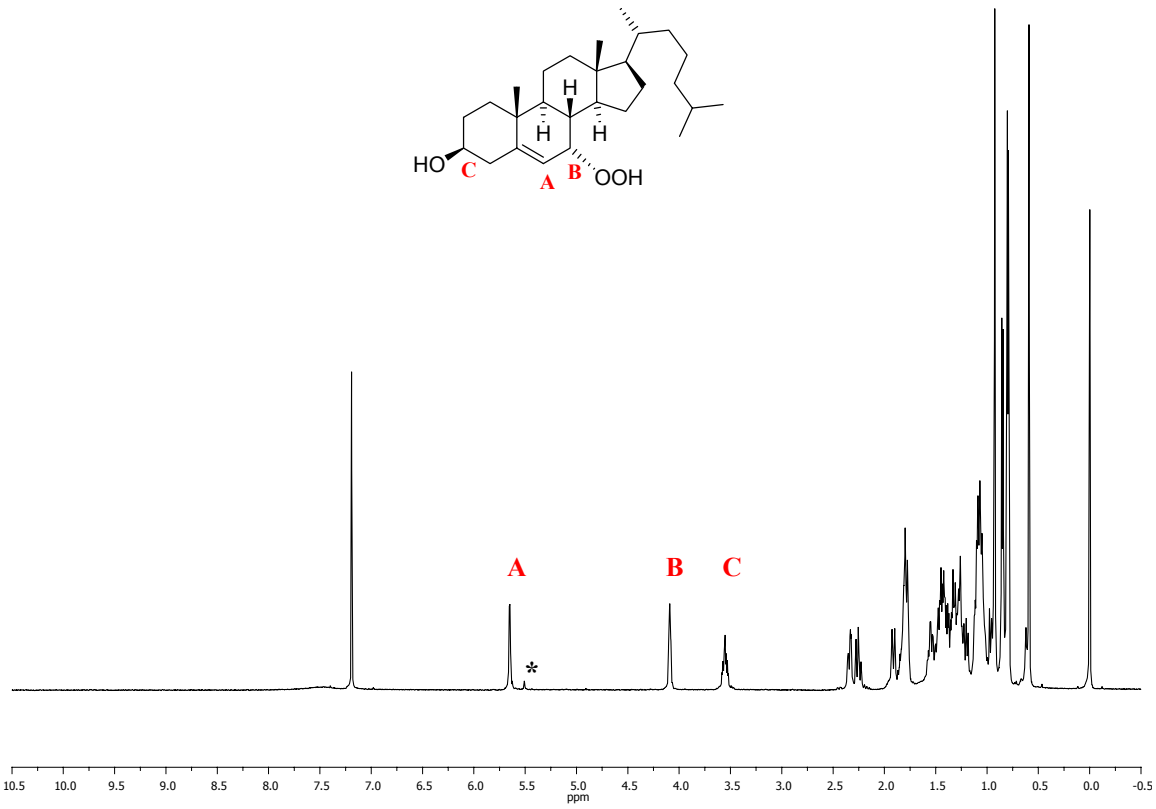


Figure 2.16. ^{1}H-NMR spectrum of cholesterol 7α-OOH in $CDCl_3$. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS. (*Trace amount of chol 7β-OOH).

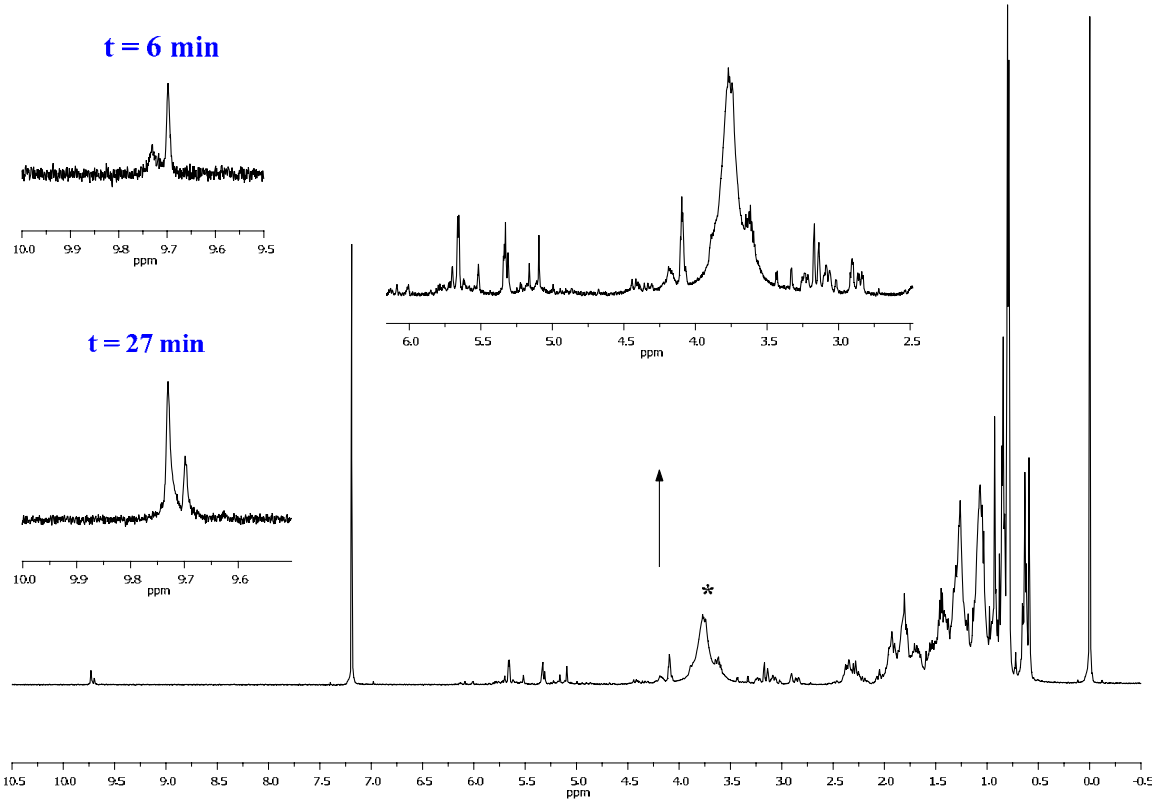


Figure 2.17. ^{1}H-NMR spectrum of chol 7α-OOH in $CDCl_3$ following treatment with TFA (2.7 mM). Insets show aldehydes peaks distribution at t = 6 minutes and t = 27 minutes. Obtained at 500 MHz with chemical shifts reported in ppm relative to TMS. (* = H_2O).

2.3 Discussion

The generation of reactive oxygen species (ROS) under physiological conditions is of great interest to science and human health. Since ROS can be generated *in situ* and are able to damage most biomolecules, notably DNA, lipids and proteins, it is widely believed that their production is a key factor in the pathogenesis of various diseases and degenerative disorders. Well known are, for example, superoxide ($O_2\bullet^-$), hydrogen

peroxide (H_2O_2), organic hydroperoxides (ROOH), singlet oxygen ($^1O_2$) and hydroxyl radical (•OH). Although very reactive, ozone (O_3) is generally thought not to be physiologically relevant due to our low level of exposure from environmental sources. Ozone levels are higher in the stratosphere (>10 km) at 2-8 ppm, but the global background concentration is 0.04 ppm. Due to smog formation this can, however, rise to 0.3 to 0.4 ppm; well above the World Health Organization exposure limit of 0.05-0.010 ppm (1 hour exposure time).[20] In smog, O_3 is produced by photolysis of NO_2 (eq 2.5-2.6).

$$NO_2 \xrightarrow{h\nu\ (\lambda\ 295\text{-}430\ nm)} NO + O \qquad (2.5)$$

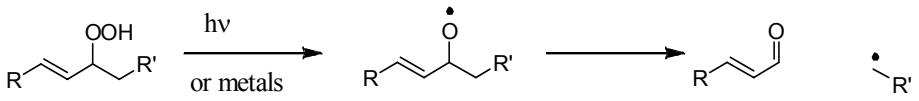
$$O_2 + O \xrightarrow{M} O_3 \qquad (2.6)$$

(M = third-body molecule absorbing excess energy of the reaction)

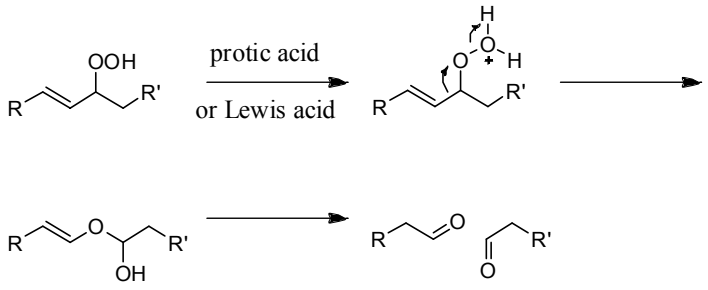
The exposure to ozone as an air pollutant has direct implications on human health mainly, as could be expected, in the respiratory system. The mechanisms of O_3 toxicity in biological systems are complex, but has the typical signature of ROS, that is, formation of free radicals, initiation of lipid peroxidation, oxidative loss of functional groups and activities of biomolecules, alteration of membrane permeability and function, induction of inflammation and initiation of secondary processes.[21] The peroxidation of membrane lipids is thought to be the most important mechanism of O_3 injury. Evidence of *in vivo* oxidation of lipids derived from O_3 exposure has been found in lung tissue and lavage fluid.[20] Animal studies have shown that polyunsaturated fatty acids in lung fibroblasts are the primary targets for ozone oxidation.[22]

When cholesterol, an essential component of cell membranes, is subjected to ozonolysis, the primary product is the cholesterol 5,6-secosterol **2.4a** which can undergo an intramolecular aldolization to give the aldehyde **2.5a**. Recently, these aldehydes were detected by Wentworth and co-workers as their DNPH derivatives **2.4b** and **2.5b** in atherosclerotic plaque tissue and diseased brain tissue extracts, prompting these workers to suggest that ozone is also produced endogenously. The startling proposal could have serious implications given that it was hitherto believed that one's only exposure to this highly toxic compound came only from exogenous environmental sources, and was thus largely localized to the respiratory system.

It is well known that lipid hydroperoxides can decompose to give carbonyl compounds.[23] The two mechanisms that are generally invoked are β-fragmentation of alkoxyls and Hock fragmentation of hydroperoxides (Scheme 2.7).

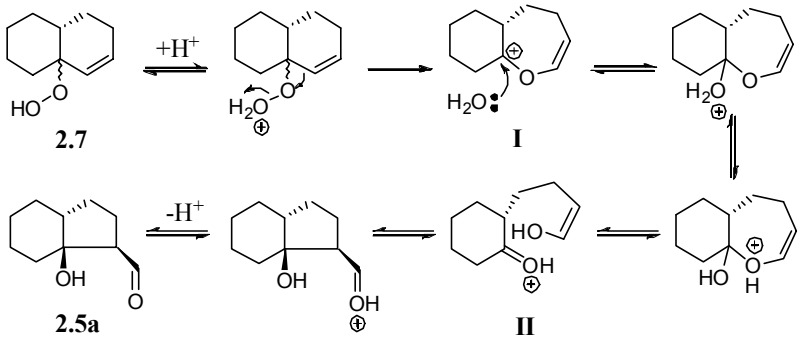


Scheme 2.6. β-Fragmentation of an alkoxyl derived formal allylic hydroperoxide.



Scheme 2.7. Hock fragmentation of an allylic hydroperoxide.

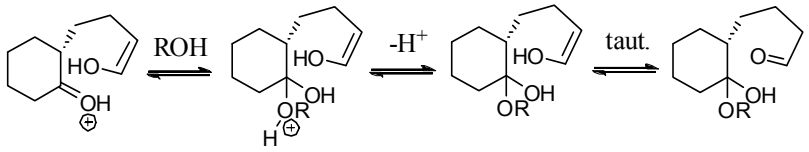
While it is difficult to envision an alternative pathway leading to aldehydes **2.4a** and **2.5a** invoking alkoxyls, one which involves Hock fragmentation is a distinct possibility.[24,25] Indeed we have shown this to be the case. When freshly synthesized and purified cholesterol 5α-hydroperoxide was treated with various acids (e.g. HCl, TFA, PTSA) in various organic solvents (e.g. $CHCl_3$, THF, CH_3CN), the hydroperoxide was rapidly and quantitatively converted to aldehyde **2.5a**. Interestingly, no **2.4a** was observed under these conditions. When the mechanism of the Hock fragmentation is taken into account this can be readily understood (Scheme 2.8).[26]



Scheme 2.8. The proposed mechanism of Hock cleavage of cholesterol 5α-OOH in non-participating solvents. Substituents are omitted on both the A and B ring of cholesterol for clarity.

The first step in the Hock fragmentation is protonation of the hydroperoxide. This leads to migration of the sp^2 carbon to the electron deficient oxygen concerted with departure of water. The resulting oxocarbenium ion (**I**) is then rapidly captured by the recently departed water yielding the protonated hemiacetal. Proton transfer to the ring oxygen followed by ring-opening results in a second oxocarbenium ion (**II**) which can

undergo ring closure to give **2.5a** following return of the proton to solvent. When the Hock fragmentation was performed in alcoholic solvents a different product distribution was observed. Under these conditions, the oxocarbenium ion **II** can instead be captured by nucleophilic attack preventing ring closure (Scheme 2.9). This explains the appearance of aldehyde **2.4a** when cholestrol 5α- hydroperoxide underwent Hock fragmentation in alcoholic solvents.



II

Scheme 2.9. The proposed mechanism of Hock cleavage of cholesterol 5α-OOH in participating (alcoholic) solvents. Substituents are omitted on both the A and B ring of cholesterol for clarity.

The ratio of **2.4a** to **2.5a** formed in these reactions depends on the relative rate of ring closure (an unimolecular process) and capture by the solvent (a pseudo unimolecular process). Determining the exact product distribution of **2.4a** to **2.5a** was complicated by the formation of their acetals under the acidic conditions. Careful deconvolution of the product mixture, aided by the well resolved acetal protons of **2.8** and **2.9** allowed us to determine this ratio as ~1:5. In support of the Hock fragmentation mechanism for this transformation, reactions were performed with sub-stoichiometric amounts of acid, smoothly went to complete conversion. In fact, we surveyed HCl concentrations over 4

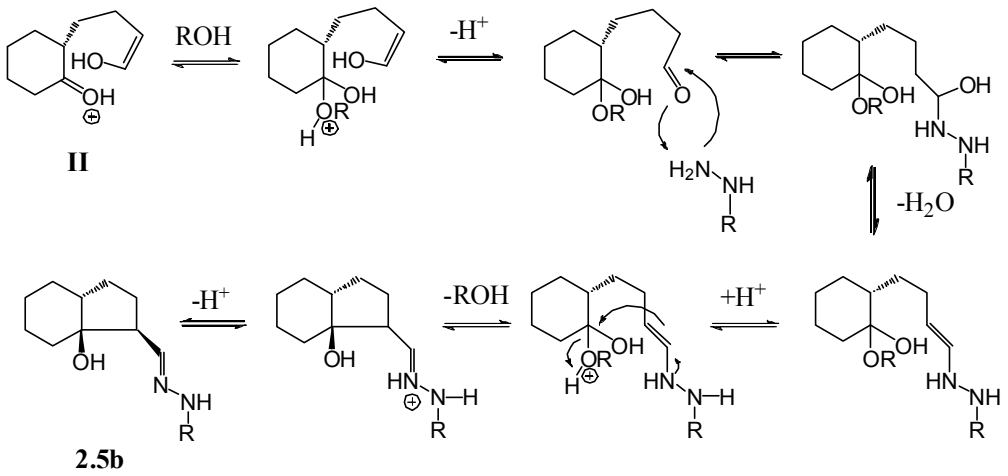
orders of magnitude; the lowest of which (1×10^{-4} M) still resulted in complete conversion, but required 5 hours to do so.

While the foregoing firmly established that the Hock fragmentation of cholesterol 5α-OOH leads to **2.4a** and **2.5a**, whose ratio depends on the solvent in which the reaction is carried out, it was not clear what was responsible for their identification in arterial plaque and brain tissues. Hence, we sought to investigate this further. Our first concern was whether the derivatization process of Wentworth and co-workers (0.1 M HCl, 2 mM DNPH, EtOH) was directly responsible for the products **2.4a** and **2.5a**, i.e., whether they were formed simply as the result of the derivatization process on extracted **2.7** and therefore only artifactually identified *in vivo*.

When cholesterol 5α-OOH **2.7** was exposed to standard DNPH derivatization conditions, the hydrazones **2.5b** and **2.4b** were both formed, but only a minimal amount of **2.4b** was detected by HPLC, with the rest of **2.7** being smoothly converted to **2.5b** as the major product (along with some dehydrated products derived there from). While this was consistent with the trend found by Wentworth *et al.* insofar as that they also found **2.5b** as the major product in arterial and brain tissue extracts, there was much more **2.4b** in their derivatized arterial plaque extracts (**2.4b**:**2.5b** ~ 1:3) than in our reactions starting with **2.7** (**2.4b**:**2.5b** <5:95). It is important to note that when **2.4a** is derivatized directly, the ratio of **2.4b** to **2.5b** formed as a result is roughly 4:1 (after 2 hours under the standard conditions). Although **2.4a** is the primary ozonolysis product, Wentworth *et al.* argued that aldolization *in vivo* catalyzed by amino acids (e.g. proline), was likely to be responsible for the predominance of **2.5b** in the derivatized plaque extracts.

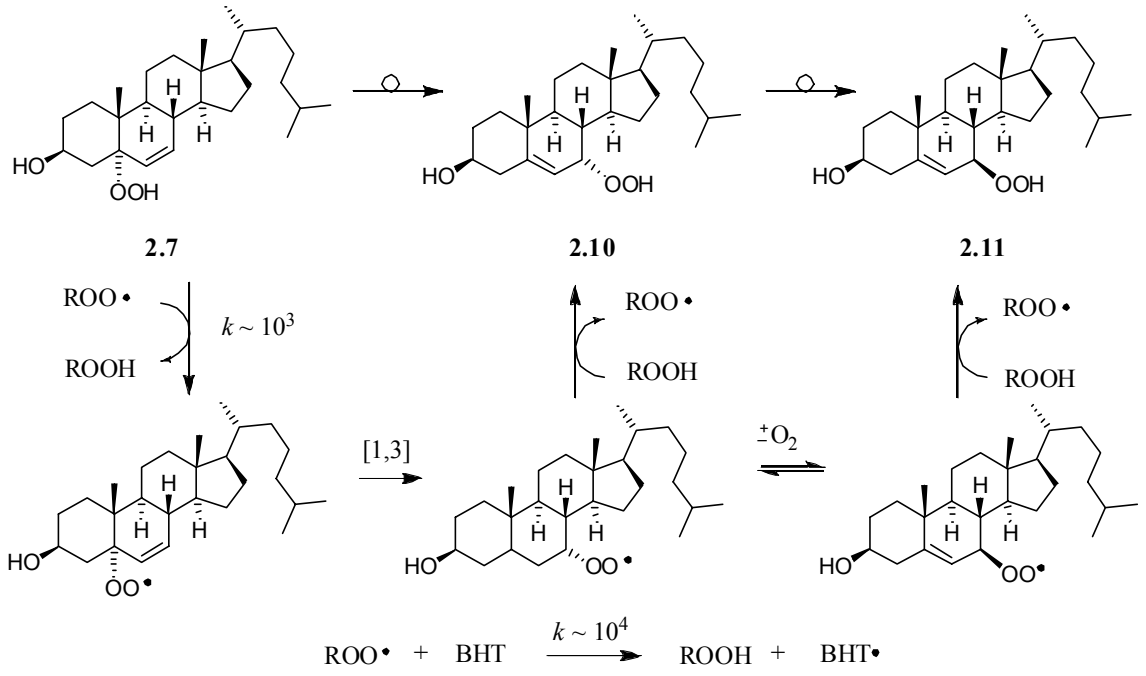
Regardless, the possibility that the detection of **2.4b** and **2.5b** in tissue samples ultimately resulted from Hock fragmentation of **2.7** would be stronger if the product ratios presented by Wentworth *et al.* could be reproduced. Therefore we pursued a series of experiments with varying DNPH derivatization conditions to explain if this changed the ratios of the produced hydrazones formed. Changing the HCl, cholesterol 5α-OOH or DNPH concentration had no significant effect on the ratio of **2.4b** to **2.5b** that was observed by HPLC. Higher acid concentration and/or longer reaction times did, however, give rise to a new peak, which following mass spectral analysis, we attributed to a precedented dehydration product.[3]

When we followed the fate of **2.7** under the derivatization conditions by ^{1}H-NMR, the same trends were observed. However, we could also clearly see that derivatization was very slow compared to the Hock fragmentation of **2.7**. With the acid concentration used under derivatization conditions, 0.1 M, the Hock fragmentation of **2.7** is very fast, almost complete in 1 minute, which we know results in a mixture of roughly 1:5 of **2.4a** to **2.5a** (and also it acetal **2.9**). However, when DNPH is included in the reaction mixture, it becomes enriched in the aldolized derivative **2.5b** relative to **2.4b** to the point where the ratio is >95:5. The reason for the change in product distribution in the presence of DNPH is not clear. One possibility is that following hydrazone formation on the (hemi)ketal form of **2.4a** (generated following capture of the oxocarbenium ion **II** as in Scheme 2.9), the enamine would readily cyclize upon formation of any oxocarbenium intermediate upon interconversion of the (hemi)ketal and ketone (Scheme 2.10).



Scheme 2.10. Hock cleavage of **2.7** under DNPH derivatization conditions. Formation of oxocarbenium **II**, capture by alcoholic solvent, subsequent DNPH derivatization and closing acid catalyzed closing of the enamine resulting in **2.5b**.

Regardless, from the product ratios, it would appear that Hock fragmentation of **2.7** to **2.4b** and **2.5b** upon derivatization of the arterial plaque extracts is unlikely to be the explanation for the apparent identification of **2.4a** and **2.5a** in vivo. If we take the stability of **2.7** into account this could be expected. Although cholesterol 5α-hydroperoxide **2.7** is the main product of singlet oxygen oxidation of cholesterol (>98%), once formed it can isomerize to its thermodynamically more stable isomer, cholesterol 7α-hydroperoxide **2.10** which itself can epimerize to the 7β-hydroperoxide **2.11** (Scheme 2.11). These processes are believed to involve the formation of allylperoxyl radical intermediates, which is one of the reasons we chose to add BHT as both internal standard and inhibitor in experiments with **2.7**.[19,27]



Scheme 2.11. Rearrangement of cholesterol 5α-OOH **2.7** in non-hydrogen bonding solvent to cholesterol 7α-OOH **2.10** which can epimerize to cholesterol 7β-OOH **2.11**. Both processes involve allylperoxy radicals and as such are inhibited by antioxidants such as 2,6-di-tert-butyl-4-methylphenol, BHT.

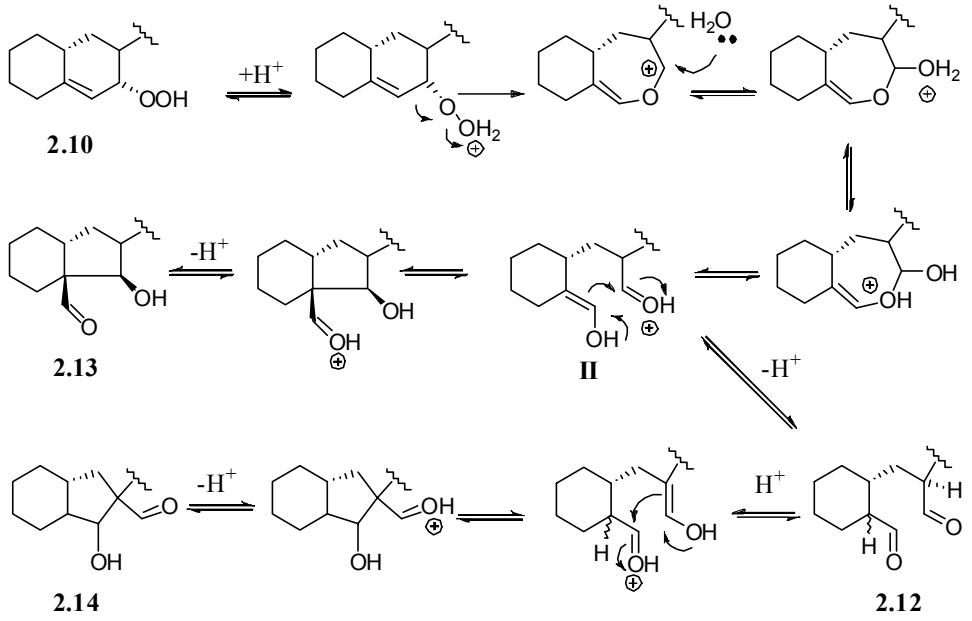
Given the facile rearrangement of **2.7** as well as the speed of the Hock cleavage upon exposure to catalytic amounts of acid it is plausible that if cholesterol 5α-OOH **2.7** is present in the tissue samples it will not survive surgical removal, homogenation, extraction and concentration under aerobic conditions. A more likely path of events is that the Hock cleavage of **2.7** occurs, completely or partly, *in vivo*. The direct results of an in vivo Hock fragmentation are hard to assess but one can envision that endogenously more **2.4a** is formed which subsequently will lead to a higher **2.4b** concentration leading to the ratio **2.4b** to **2.5b** as found by Wentworth *et al*. Taking Schemes 2.18 and 2.19 into

account, if Hock cleavage takes place in the presence of a better nucleophile than an alcohol the oxocarbenium ion **I** will be quenched at a higher rate resulting in more **2.4a** formation. With amines or thiols present in biological systems were cholesterol oxidation takes place, such a pathway could very well take place. Another possibility for the different product distribution observed by Wentworth *et al.* could be that they observed a DNPH derivatization product of a different cholesterol oxidation product. Although their synthesis of authentic **2.4b** and **2.5b** and subsequent analysis gave almost identical retention times by reverse phase HPLC, a similar cholesterol dinitrophenyl hydrazone product with the same mass can not be completely ruled out. Taking the similarity of other cholesterol oxidation products (such as chol 7α-OOH) in account it could be that upon DNPH derivatization conditions a product forms with the same mass and similar retention time on HPLC as **2.4b** or **2.5b**.

The cholesterol 7-OOH is the major product of cholesterol autoxidation and the product of cholesterol 5α-OOH rearrangement. Cholesterol 7α-hyroperoxide **2.10** is commonly found in biological systems and is isolated from atherosclerotic lesions and oxidized LDL.[28] Although surely found as a product of cholesterol autoxidation itself, one could easily speculate its origin arises from the rearrangement of **2.7**.

To investigate this possibility, we carried out analogous experiments on chol 7α-OOH, and upon presenting it to acidic conditions, aldehyde formation was indeed observed. It was found to be slower and the formation of several products resulted in more complicated ^{1}H-NMR spectra. Two aldehydes peaks were clearly distinguishable. It is likely to be **2.13** and **2.14** since the aldehyde **2.12** would be expected to very readily aldolize under acidic conditions, much more rapidly than the ketoaldehyde cholesterol

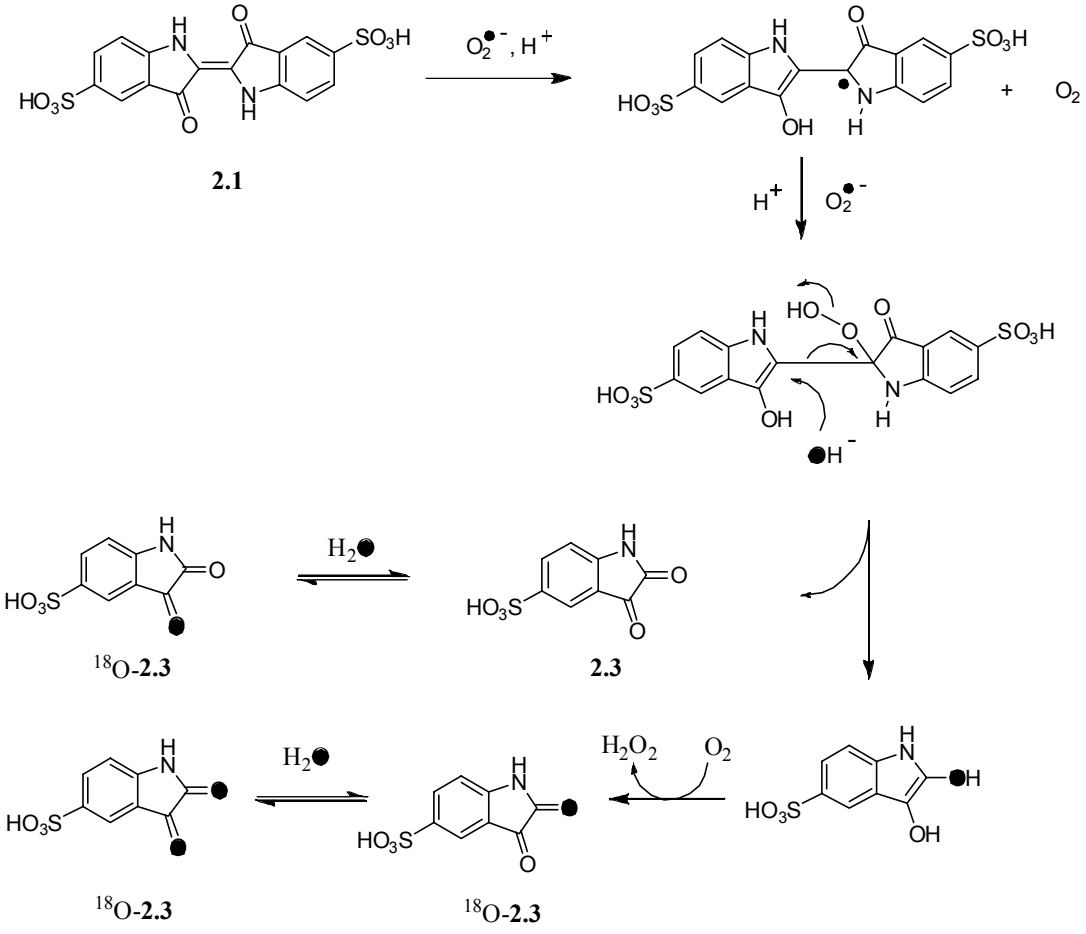
5,6-secosterol. One can envision the sequence in scheme 2.19 operating to afford these aldehydes.



Scheme 2.12. Proposed mechanism for Hock cleavage of cholesterol 7α-OOH in $CDCl_3$ to afford aldehydes **2.13** and **2.14**. Substituents are omitted on both the A and B ring of cholesterol for clarity, except the C ring residue at the 8 position.

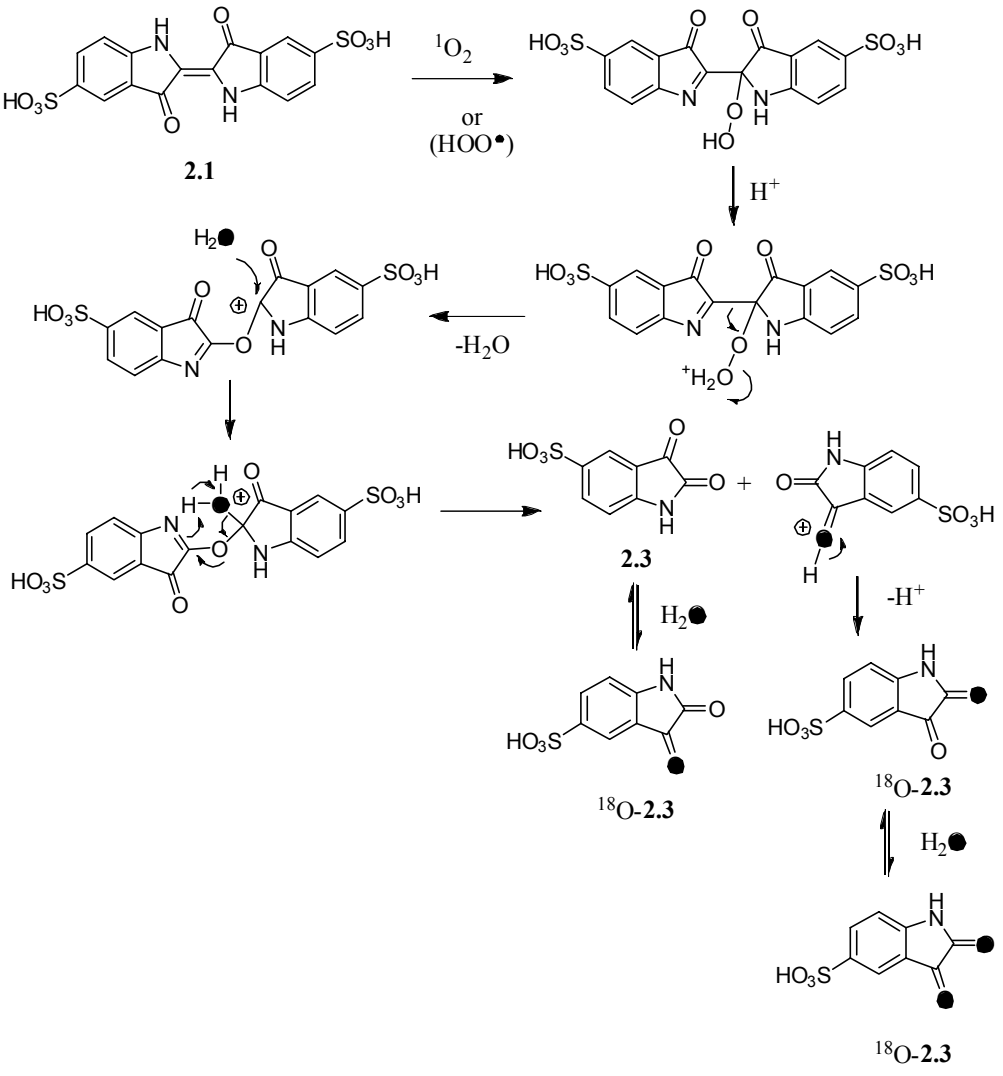
Current efforts are directed at characterizing the aldehydes formed. Once characterized the retention times of the hydrazones formed upon DNPH derivatization will have to be determined under the same conditions as for **2.4b** and **2.5b**. This would provide a possible explanation for Wentworth *et al.*'s different product ratios and it would provide a standard to search for cholesterol 7-OOH derived carbonyls that are likely to be much more prevalent *in vivo*, and they also may have similar or more potent proatherogenic properties then **2.4a** and **2.5a**.

It should be pointed out that the other reaction which was deemed to be unique to ozonolysis, and as such, used to provide evidence for ozone in vivo provided by Wentworth *et al*. has also been shown not to be unique to ozone. Winterbourn and co-workers have shown that indigo carmine subjected to superoxide in $H_2{}^{18}O$ clearly results in the formation of doubly labeled **2.3**. Cleavage of the central bond of indigo carmine with symmetric incorporation of ^{18}O in the lactam carbonyl of the product isatin sulfonic acid was found when reactions were carried out with superoxide ($O_2^{\bullet -}$) generated by xanthine oxidase (XO), an enzyme found in human liver (Scheme 2.13).[29, 30]



Scheme 2.13. The reaction of superoxide with indigo carmine as proposed by Winterbourn and co-workers.[30]

Although the result is clear, the suggested mechanism is somewhat suspicious, given that it involves a reduction of **2.1** by superoxide. We wonder whether a mechanism involving either $^1O2$ or autoxidation to yield a hydroperoxide followed by Hock fragmentation is not more likely. Hock cleavage would be expected to yield the same distribution of isotopically-labelled products (Scheme 2.14). This is currently under investigation.



Scheme 2.14. Proposed cleavage of indigo carmine with singlet oxygen via Hock cleavage.

2.4 Conclusions

Although the idea of endogenous ozone formation as reported by Wentworth *et al.* is very appealing, the scientific evidence to support this claim is not as solid as one may expect from high profile scientific publications. Since the appearance of this claim in 2003, scientists such as Winterbourn and co-workers were able to show that some products described to be unique to ozonolysis can also have a different origin. Interestingly enough, for two related products that were found in tissue samples and were claimed to be unique to ozonolysis, no alternative formation pathway could be found. These two products, atheronal A and B (compounds **2.4a** and **2.5a** in this thesis) are cholesterol derivatives and are believed to have cytotoxic properties since their dominant appearance in atherosclerotic artery tissue and diseased brain tissue. Here we very clearly demonstrated that these products can be readily derived from cholesterol 5α-hydroperoxide (**2.7**) upon acid catalyzed Hock cleavage. The Hock cleavage appeared to be very fast even under slightly acidic conditions and not acid specific. Subsequent DNPH derivatization to make HPLC detectable chromophores as used by Wentworth et al. was slow compared to Hock cleavage. The product distribution of Hock cleavage of **2.7** varied greatly on solvent used as well as on the presence of nucleophiles, namely DNPH. The reaction always produced **2.5a** as the predominant product, in non-nucleophilic solvents (ACN, CH_2Cl_2 etc.) exclusively but when the reaction was performed in alcoholic solvents the presence **2.4a** could be observed typically around 20%. If however DNPH was present in this mixture the DNPH derivative of **2.5a,** namely **2.5b**, proved to be far dominant again, in 95% yield. And while both cholesterol oxidation products, **2.4a** and **2.5a**, are easily obtained via Hock cleavage of **2.7** their

ratios vary greatly with several factors influencing them. The ratios of DNPH derivatives **2.4b** and **2.5b** as observed by Wentworth *et al.* upon analyzing tissue extracts suggest that the Hock cleavage of **2.7** happens *in vivo*.

This finding does not only clearly invalidate the endogenous ozone claim but also raises the question where and under what circumstances are these cholesterol oxidation products formed in the human body. By showing the same reaction takes place on a different more prevalent cholesterol oxidation product, cholesterol 7α-OOH, one wonders what are the health implications of the products formed from this reaction? Thus, answering the endogenous ozone formation claim negatively by showing an alternative reaction pathway that can be responsible for the formation of atheronals A and B, the Hock cleavage of cholesterol hydroperoxides does reveal the formation of a whole series of products that are of critical importance in normal and pathological situations.

2.5 Experimental

Materials and Methods. Cholesterol, Rose Bengal, solvents and acids were purchased from commercial sources and used as received. ^{1}H-NMR spectra were collected on a Bruker AMX-500 spectrometer. Mass spectra were obtained on an Applied Biosystems/MDS Sciex QSTAR XL QqTOF mass spectrometer. Reverse phase HPLC was carried out on a Waters XBridge C18 2.5µm column (4.6 × 75 mm) with a mobile phase consisting of 75% acetonitrile, 20% methanol and 5% water at 1 ml/min. on a Waters Delta 600 binary pump equipped with a Waters 2487 Dual Wavelength UV-Visible Detector (234 and 360 nm). LC/MS data were obtained on a Waters Alliance 2695 equipped with a Waters ZQ Single Quadrupole electrospray ionization mass detector operating in the negative ion mode with the same column and conditions.

5α-Hydroperoxy-3β-hydroxycholest-6-ene (**2.7**). A solution of cholesterol **2.2** (1.0 g) and Rose Bengal (14 mg) in pyridine (15 ml) was vigorously stirred and cooled to 0 ^{o}C. Oxygen was bubbled through at 100 ml/min and the mixture was irradiated with a HPS 400 W sodium lamp at a distance of 5 cm. After 6 hours TLC indicated no more starting material. Pyridine was removed under reduced pressure and the residue was dissolved in dichloromethane and absorbed on silica. The dichloromethane was removed under reduced pressure and the product coated silica was added on top of a preparative silica column and eluted with ethyl acetate-heptane (1:1) to give 0.82 g (76%) of the hydroperoxide. Recrystallization from aqueous methanol gave pure 5α-hydroperoxy-3β-hydroxycholest-6-ene. Analytical and spectral data were in accordance with previously reported.[19]

3β-Hydroxy-5-oxo-5,6-secocholestan-6-al (**2.4a**). A solution of cholesterol **2.2** (3.0 g) in a mixture of chloroform-methanol (9:1, 300 ml) was ozonized at – 80 ^{o}C for 20 min. The reaction mixture was concentrated under vacuum and redissolved in water-acetic acid (1:9, 150 ml). Zinc powder (1.95 g) was added and the mixture was stirred 3 h at room temperature. The reduced mixture was diluted with dichloromethane (300 ml) and washed with water (3 x 150 ml). The organic fraction was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was purified over a preparative silica column [ethyl acetate-heptane (1:2)] to give title compound (**2.4a**) as a white solid (2.11 g, 65%). Analytical and spectral data were in accordance with previously reported.[3]

3β-Hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (**2.5a**). To a solution of ketoaldehyde **2.4a** (150 mg) in acetonitrile-water (20:1, 10 ml) was added L-proline (42 mg). The reaction mixture was stirred for 2 h at room temperature, evaporated to dryness *in vacuo*. The residue was dissolved in ethyl acetate (20 ml) and washed with water (3 x 10 ml) The combined organic fractions were dried over sodium sulfate and evaporated in vacuo. The residue was purified over a preparative silica column [ethyl acetate-heptane (1:4)] to give title compound (**2.5a**) as a white solid (94 mg, 63%). Analytical and spectral data were in accordance with previously reported.[3]

2,4-Dinitrophenylhydrazone of 3β-Hydroxy-5-oxo-5,6-secocholestan-6-al (**2.4b**). To a solution of ketoaldehyde **2.4a** (100 mg, 0.24 mmol) in acetonitrile was added 2,4-dinitrophenylhydrazine (52 mg, 0.26 mmol) and *p*-toluenesulfonic acid (1 mg, 0.0052 mmol). The reaction mixture was stirred at room temperature for 4 h. The mixture was

concentrated under reduced pressure and the residue was redissolved in ethyl acetate (10 ml). After washing with water (3 x 20 ml) the organic layer was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was purified by silica gel chromatography [ethyl acetate-heptane (1:4)] to give the title compound **2.4b** as a yellow solid (90 mg, 58%). Analytical and spectral data were in accordance with previously reported.[3]

2,4-Dinitrophenylhydrazone of 3β-Hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (**2.5b**). To a solution of aldehyde **2.5a** (100 mg, 0.24 mmol) in acetonitrile was added 2,4-dinitrophenylhydrazine (52 mg, 0.26 mmol) and hydrochloric acid (12 M, 60 μl). The reaction mixture was stirred at room temperature for 4 h. The mixture was concentrated under reduced pressure and the residue was dissolved in ethyl acetate (10 ml). After washing with water (3 x 20 ml) the organic layer was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was purified by silica gel chromatography [ethyl acetate-heptane (1:4)] to give the title compound **2.5b** as a yellow solid (83 mg, 53%). Analytical and spectral data were in accordance with previously reported.[3]

7α-Hydroperoxy-3β-hydroxycholest-5-ene (**2.10**). A solution of cholesterol 5α-OOH **2.7** (250 mg, 0.6 mmol) in chloroform (15 ml) was stirred for 12 h at rt. Solvent was removed under vacuum and resulting solid absorbed on silica. The product coated silica was added on top of a preparative silica column and eluted with ethyl acetate-heptane (1:1) to give a mixture of hydroperoxides chol 7α-OOH to its epimer chol 7β-OOH.

Double recrystallization from aqueous methanol gave 95% pure 7α-hydroperoxy-3β-hydroxycholest-5-ene (160 mg, 64%). Analytical and spectral data were in accordance with previously reported.[19]

Hock fragmentation of cholesterol 5α-OOH by p-TsOH in tetrahydrofuran. To a solution of cholesterol 5α-OOH **2.7** (0.024 mmol, 0.01 g) in tetrahydrofuran (5 ml), *p*-toluenesulfonic acid (0.24 mmol, 0.041 g) was added and stirred at rt for 1 h. The reaction was quenched by addition of a 1:1 mixture of water and methylene chloride (50 ml). The product was extracted in methylene chloride and concentrated under reduced pressure. The residue was placed under high vacuum for 1 h and analyzed by ^{1}H-NMR and MS without any further purification. The spectra were consistent with literature data and those obtained from an authentic sample of **2.4a** and **2.5a** prepared as described in ref. 1.

Hock fragmentation of cholesterol 5α-OOH by trifluoroacetic acid in chloroform. To a solution of cholesterol 5α-OOH (0.024 mmol, 0.01 g) in chloroform (5 ml), trifluoroacetic acid (0.24 mmol, 18 μL) was added and stirred at rt for 5 min. The reaction was quenched by addition of a 1:1 mixture of water and methylene chloride (50 ml). The product was extracted in methylene chloride and concentrated under reduced pressure. The residue was placed under high vacuum for 1 h and analyzed by ^{1}H-NMR and MS without any further purification. The spectra were consistent with literature data and those obtained from authentic samples of **2.4a** and **2.5a** prepared as described in ref. 3.

Hock fragmentation of cholesterol 5α-OOH by HCl in ethanol. To a solution of cholesterol 5α-OOH (0.024 mmol, 0.01 g) in ethanol (5 ml), concentrated HCl (12.6 M) was added to a concentration of 0.1 M and stirred at rt for 5 min. The reaction was quenched by addition of a 1:1 mixture of water and methylene chloride (50 ml). The product was extracted in methylene chloride and concentrated under reduced pressure. The residue was placed under high vacuum for 1 h and analyzed by ^{1}H-NMR and MS without any further purification. The spectra showed a mixture of **2.4** and **2.5** and their corresponding diethyl acetals **2.8** and **2.9**. The signals for **2.4** and **2.5** were consistent with literature data and those obtained from authentic samples of **2.4** and **2.5** prepared as described in ref. 1. The chemical shifts corresponding to **8** and **9** were assigned based on their preparation from **2.4** and **2.5** under the exact same experimental conditions. The ratio of **2.5** (+**2.9**) to **2.4** (+**2.8**) was ~5:1.

2.8: ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 4.47 (t, J = 7.4 Hz, acetal C-H), 4.42 (bs, H-3), 3.55-3.45 (m, acetal -OCH_2-), 3.40-3.29 (m, acetal -OCH_2-), 3.23 (dd, J = 13.5, 3.8 Hz, H-4). Resonances at δ < 2.5 ppm for alkane C-H's are omitted due to their complexity.

2.9: ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 4.39 (d, J = 5.9 Hz, acetal C-H), 4.05 (bs, H-3), 3.85 (dq, J = 10 Hz, acetal -OCH_2-), 3.72 (dq, J = 10 Hz, acetal -OCH_2-), 3.64 (dq, J = 10 Hz, acetal -OCH_2-), 3.43 (dq, 10 Hz, acetal -OCH_2-). Resonances at δ < 2.5 ppm for alkane C-H's are omitted due to their complexity.

Hock fragmentation of cholesterol 5α-OOH by HCl in ethanol at different acid concentrations. To a stirred solution of 2mg/mL cholesterol 5α-OOH in dueterated ethanol was added an equivalent of *t*-butylated hydroxytoluene (BHT). BHT was used both as an internal standard and to prevent isomerization of the cholesterol 5α-OOH to the more thermodynamically stable cholesterol 7α-OOH in lengthy experiments.[2] An HCl solution in ethanol was then added to give the desired final acid concentration (0.1 M, 0.01 M, 0.001 M, 0.0001 M). The solution was stirred at room temperature and aliquots of 5 ml were taken at appropriate time intervals. The aliquots were quenched with 30 ml of a 2/1 methylene chloride/water mixture which was subsequently washed twice more with 10 ml water and the organic layer concentrated under vacuum and analyzed by ^{1}H-NMR. Conversion was determined by the relative integration of the downfield alkene C-H doublet of the cholesterol 5α-OOH (δ = 5.76 ppm) to that of the aromatic C-H singlet of BHT (δ = 6.90). The ratio of **2.5** (and corresponding acetal **2.9**) to **2.4** (and corresponding acetal **2.8**) formed was also quantified and determined to be essentially invariant with acid concentration and time, averaging ~5:1, i.e., for 0.1 M HCl, **2.5**: **2.4** = (4.51 ± 0.86); 0.01 M HCl, **2.5**: **2.4** = (3.90 ± 0.98); 0.001 M HCl, **2.5**: **2.4** = (6.03 ± 1.26); 0.0001 M HCl, **2.5**: **2.4** = (5.02 ± 0.39).

Hock fragmentation of cholesterol 5α-OOH under DNPH derivatization conditions. To a solution of cholesterol 5α-OOH (0.005 mmol, 0.002 g) in ethanol (0.9 ml), a solution of 2,4-dinitrophenyl hydrazine (0.1 ml, which was 2 mM with respect to DNPH and 1N with respect to HCl) was added and stirred at rt for 2 h. The reaction mixture was

then analyzed by reverse phase HPLC with detection at 360 nm. The chromatogram is shown in Figure S2A.

DNPH derivatization of 2.4. To a solution of **2.4** (0.005 mmol, 0.002 g) in ethanol (0.9 ml), a solution of 2,4-dinitrophenyl hydrazine (0.1 ml, which was 2 mM with respect to DNPH and 1N with respect to HCl) was added and stirred at rt for 2 h. The reaction mixture was then analyzed by HPLC. The chromatogram is shown in Figure S2B.

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Chapter 3

Towards *tert*-Butylated 3-Pyridinols and 5-Pyrimidinols

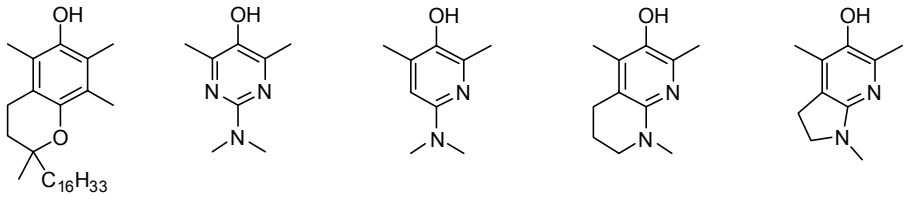
3.1 Introduction

Lipid peroxidation is believed to play a key role in the onset and development of several diseases and degenerative disorders as discussed in Chapter 1.[1, 2] As a result, it has been of longstanding interest to understand the role of antioxidants in inhibiting these unwanted radical chain oxidation processes. This is not only significant due to the application of this knowledge in human health and medicine, but also in food preservation and the protection of organic materials and petroleum products from degradation (e.g. lubricating oils, rubbers and plastics).[3]

The prototypical example of a radical-trapping chain-breaking antioxidant is vitamin E, consisting of several tocopherols and tocotrienols of which α-tocopherol (α-TOH, **3.1**) is the most reactive; possessing a rate constant for reaction with chain-carrying peroxyl radicals of k_{inh} = 3.2 x 10^6 $M^{-1}s^{-1}$ at 25°C. The reactivity of phenolic chain-breaking antioxidants relies on their phenolic O-H moiety, which has a low bond dissociation enthalpy (BDE) of 78-88 kcal/mol, and undergoes very fast proton-coupled electron transfer reactions (formally a H-atom transfer) with peroxyl radicals. As such, attempts to optimize the activity of phenols beyond that of α-TOH have centered on substituting the phenol with increasingly electron-donating groups to minimize the O-H bond strength. This has met with limited success, since as the electron density of the aromatic ring is increased, the compounds become reactive to oxygen in the air.

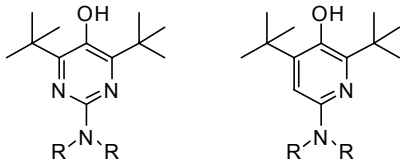
Recently, Pratt *et al.* have demonstrated that by incorporation of nitrogen atoms in the phenolic ring, it is possible to design and synthesize phenols with increasingly electron-donating groups (e.g. amino), thereby possessing lower O-H BDEs and higher k_{inh}, but that remain stable in air.[4] The most effective 5-pyrimidinols (**3.2**) and 3-

pyridinols (**3.3-3.5**) studied to date have an amine moiety *para* to their hydroxyl group and methyl groups in both *ortho* positions, akin to the substitution in α-tocopherol (**3.1**), but with a more electron-donating heteroatom (N vs. O).



3.1 **3.2** **3.3** **3.4** **3.5**

This substitution pattern makes them very efficient antioxidants, with k_{inh} values exceeding that of α-TOH. For example, pyrimidinol **3.2** has an inhibition rate constant 2-fold higher than α-tocopherol in benzene,[5] and 3-pyridinols **3.3**, **3.4** and **3.5** react 5-fold, 28-fold and 88-fold faster under similar conditions.[6] Although the absolute rate constants differ slightly depending on the method by which they were determined, it is clear that these compounds are indeed very good radical-trapping chain-breaking antioxidants. As a further improvement on these classes of antioxidant, which we shall elaborate on below, we aim to prepare analogs of **3.2** and **3.3** with *tert*-butyl substitution in place of methyl substitution *ortho* to the hydroxyl group (**3.6**, **3.7**).



3.6a R = H
3.6b R = Me

3.7a R = H
3.7b R = Me

3.1.1 Rationale

While phenols are extremely effective antioxidants in homogenous non-polar organic solvents, it has been shown that in certain media their efficacy is reduced dramatically and these effects may have serious consequences on *in vivo* activities. Under these conditions, phenols that give rise to highly stabilized phenoxyl radicals upon H-atom abstraction (e.g. α-tocopherol), can actually mediate the lipid peroxidation process instead of inhibiting it. This is sometimes referred to as antioxidant mediated peroxidation (AMP), or more generally as chain-transfer. It occurs at low steady-state radical concentrations where the phenoxyl radical has sufficient lifetime that it can serve as the chain carrying radical (hence the term chain transfer). Thus, following the typical radical-trapping, chain-breaking reaction (Eq. 2.1), the aryloxyl radical (ArO•) can react with a lipid to carry on the chain reaction (Eq. 2.2, 2.3, 2.4). The key chain-transfer reaction (Eq. 2.2) occurs with a second order rate constant k_i of 0.01-0.1 $M^{-1}s^{-1}$ for the aryloxylradical derived from α-tocopherol, depending on reaction conditions.

Inhibition

$$ArOH + ROO\bullet \xrightarrow{k_{inh}} ArO\bullet + ROOH \quad (2.1)$$

Initiation and propagation

$$ArO\bullet + RH \xrightarrow{k_i} ArOH + R\bullet \quad (2.2)$$

$$R\bullet + O_2 \xrightarrow{k_{O2}} ROO\bullet \quad (2.3)$$

$$RH + ROO\bullet \xrightarrow{k_p} ROOH + R\bullet \quad (2.4)$$

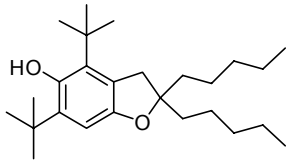
Termination

$$ArO\bullet + ArO\bullet \xrightarrow{k_{t1}} \text{Non radical products} \quad (2.5)$$

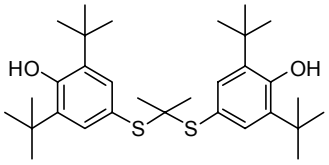
$$ArO\bullet + ROO\bullet \xrightarrow{k_{t2}} \text{Non radical products} \quad (2.6)$$

This is believed to be of particular significance in biological systems where the steady state concentration of peroxyls is low and where the α-tocopherol radical, once generated, is trapped within a lipoprotein particle or region of a lipid bilayer where it is unable to freely diffuse to encounter either another α-tocopheroxyl radical or peroxyl radical, thereby leading to non-radical products (Eq. 2.5, 2.6). These termination events are more easily carried out in solution.

The rate of antioxidant-mediated peroxidation of lipids can be slowed either by making the aryloxyl radical more stable or by making the aryloxyl radical more persistent. As introduced in Chapter 1, the presence of ED groups on the phenolic ring serves to stabilize the aryloxyl radical via both delocalization of the unpaired electron spin and provision of electron density to the electron-poor aryloxyl radical. The persistence of the aryloxyl radical can be dramatically increased by introducing steric bulk around the oxygen atom, for example by the inclusion of *tert*-butyl groups in the *ortho* positions relative to the aryloxyl oxygen. This design strategy has been incorporated into the synthetic radical-trapping chain-breaking antioxidants BO-653 and probucol, the former of which is in phase 2 clinical trials as an anti atherogenic agent and the latter of which is a widely used cholesterol lowering drug.



BO-653



Probucol

Recently, we have studied the effects of 3-pyridinol antioxidants on the oxidation of cholesterol esters in human low density lipoproteins. While they were found to substantially inhibit the rate of peroxidation of lipids in the LDL (expected based on their high values of k_{inh}) they did still undergo some antioxidant-mediated peroxidation – this despite giving way to an aryloxyl radical ca. 2 kcal/mol more stable than that derived from α-tocopherol upon H-atom transfer. Hence, it would be of interest to incorporate *tert*-butyl substitution into our compounds in an effort to suppress antioxidant-mediated peroxidation with our 3-pyridinols even further.

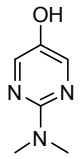
It is also now well demonstrated that solvent effects greatly influence the rate of reaction of phenolic radical-trapping chain-breaking antioxidants. For example, while the rate constant for the reaction of α-tocopherol with peroxyls in hexane is 2.0×10^7 $M^{-1}s^{-1}$ at 25 ^oC, it drops by two orders of magnitude in ethyl acetate. Ingold and co-workers have worked to demonstrate that this is nicely accounted for by a model wherein the H-bond acceptor (HBA) ability of the solvent and the H-bond donor (HBD) ability of the phenol are taken into account.[7] Thus, the rate constant for the reaction of a phenol with a peroxyl radical, (the inhibition rate constant) k_{inh}, can be expressed as in any given solvent S as:

$$\log k_{inh}^{S} = \log k_{inh}^{alkane} - 8.3\, \alpha_2^H \beta_2^H \qquad (2.7)$$

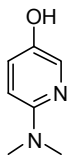
Where k_{inh} determined in a non-H-bond accepting solvent (i.e. alkane) is corrected for the strength of the interaction between the H-bond donating phenol and the H-bond accepting solvent (given by the product of Abraham's H-bond acidity parameter α^H_2 and H-bond basicity parameter β^H_2).[7] Recently, in our studies of pyridinol and pyrimidinol radical-trapping antioxidants, it has become apparent that the greater electron deficiency of the pyridine and pyrimidine rings compared to benzene, which leads to a

greater acidity of the pyridinol and pyrimidinol O-H bond, will lead to stronger H-bonding interactions with solvents, reducing their efficacy. For example, the k_{inh} of pyrimidinol **3.8** (pK_a = 8.15) drops 21-fold on going from benzene to propionitrile. In comparison, the k_{inh} of α-tocopherol drops only 10-fold (pK_a = 11.9). The pyridinol **3.9** (pK_a = 9.22) has an intermediate solvent effect, which drops k_{inh} by roughly 17-fold.

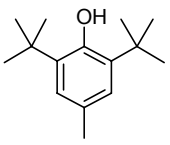
We surmised that incorporation of *tert*-butyl groups in the *ortho* positions relative to the phenolic hydroxyl would serve to disrupt H-bond formation between the pyridinol or pyrimidinol and the H-bond accepting solvents, thereby recovering the improvement we gained relative to phenol in developing the pyridinols and pyrimidinols as radical-trapping antioxidants. Indeed, in our recent studies of solvent effects on the reactions of phenols with peroxyl radicals, we found that while BHT (**3.10**) is roughly 5-fold less reactive to peroxyls than 2,4,6-trimethylphenol (**3.11**) in benzene, it is of the same reactivity in the H-bond accepting solvents acetonitrile or ethyl acetate.



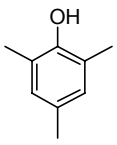
3.8



3.9



3.10

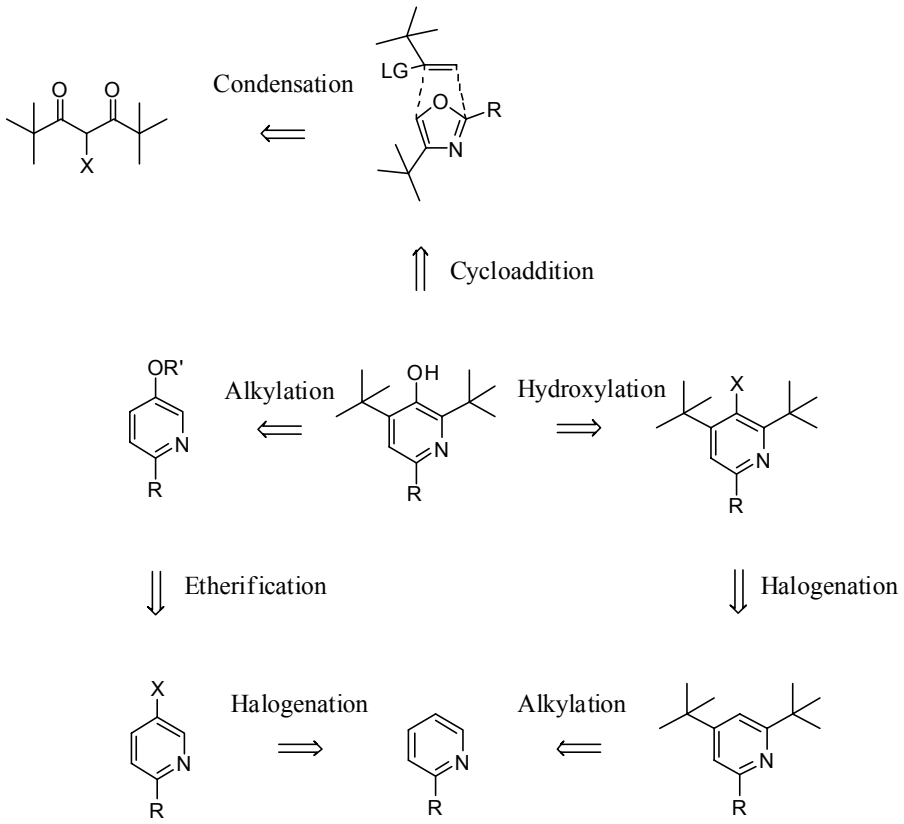


3.11

3.1.2 Approaches to *t*-Butylated 3-Pyridinols

There is a vast literature on the preparation of substituted pyridines, comprising both substitution reactions on the ring and ring construction strategies.[8] For the *de novo* synthesis of substituted pyridines, the starting materials can vary dramatically based on

the approach chosen. Most common is ring construction from two fragments; such that either a [5+1], [4+2] or [3+3] combination of fragments to arrive at the eventual pyridine nucleus can be envisioned. While the literature shows various examples of these approaches, they generally require high temperatures and/or high pressures and/or sophisticated catalysts. The yields are also generally not too impressive (20-60%), even with relatively simple fragments. Our target compound (**3.7**), a sterically-crowded 3-pyridinol, with the generally difficult to access 2,3,4-substitution on the pyridine ring, would have little chance of success using most condensation methods. It was, however, surmised that if a fragment was constructed with most (or all) substituents already in place and one robust reaction could form the pyridine ring this route would be highly economical. A [4+2] approach, employed on an appropriate substituted oxazole and dienophile, was found that had all these factors (Scheme 3.1). Another approach to make pyridinol **3.7** could be to start with a commercially available pyridine ring system and to change the substituent pattern towards our desired compound. With the series of substituents that we desire, the difficulty is the order in which each substituent needs to be introduced. This accounts especially for 2,3,4-substitution required of *t*-butyl group, hydroxyl group and another *t*-butyl group. Ideally, both *t*-butyl groups would be introduced in a single reaction. Leaving the question if the hydroxyl group should be introduced before or after alkylation? If introduced before alkylation it has to be protected since its reactivity is the reason to synthesize these class of compounds. If introduced after alkylation, would this position still be accessible for manipulation? In brief these compounds will give rise to several synthetic challenges. The proposed approaches are summarized in Scheme 3.1.



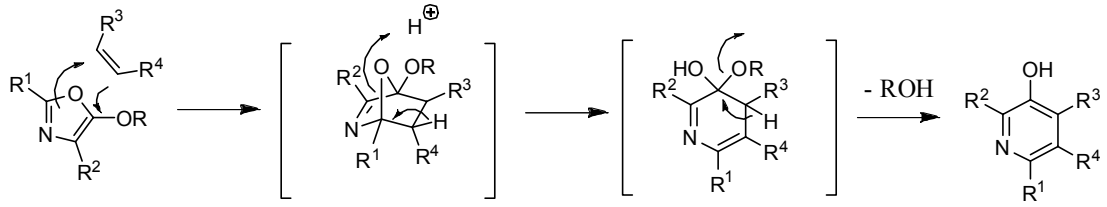
Scheme 3.1. Suggested approaches to 2,4-di-*tert*-butylated-3-pyridinols.

3.1.2.1 3-Pyridinols by Cycloaddition

The cycloaddition of an electron poor dienophile to an electron rich oxazole is a known procedure for the preparation of 3-pyridinols (Scheme 3.2),[9] and the possibility of adapting this approach to make 2,4-di-*tert*-butyl-3-pyridinol antioxidants via an intermediate with the *t*-butyl groups already in place would clearly be of advantage. In this way manipulation at the sterically crowded 2, 3 and 4 positions could be avoided.

The preparation of pyridinols via the [4+2] cycloaddition of dienophiles to oxazoles was first reported in 1962 in an effort to discover a new synthesis of pyridoxines. Thus, oxazole was reacted with either of diethylmaleate (R^3=R^4=CO_2Et),

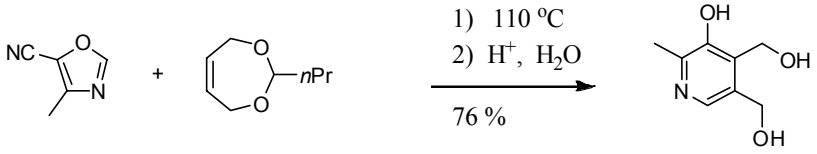
fumaronitrile (R^3=R^4=CN) or 2,5-dihydrofuran followed by treatment with acid to catalyze decomposition of the bicyclic cycloaddition product.



Scheme 3.2. Hetero Diels-Alder reaction of a 2-alkoxy oxazole with dienophile to yield 3-pyridinols.

While the fumaronitrile reacted readily at reflux in MeOH for 5 hours to yield 75% of product, the less reactive dienophile diethylmaleate required 110 ^{o}C for 2 hours and the least reactive dihydrofuran required heat in a Parr bomb at 175 ^{o}C for 3 hours to yield 58%.[10]

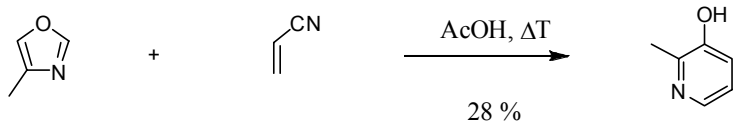
Indeed, over the years, many more examples have appeared employing different combinations of oxazoles and dienophiles – including that which is used for the industrial synthesis of vitamin B_6 (Scheme 3.4).[11]



Scheme 3.3. Synthesis of vitamin B_6 from 4-methyloxazole-5-carbonitrile and (Z)-4,7-dihydro-2-propyl-1,3-dioxepine through a hetero Diels-Alder reaction.

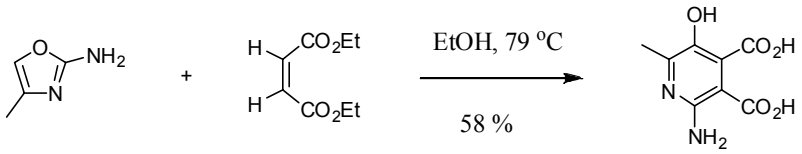
In 1965, Naito *et al.* demonstrated that substituted oxazoles, when treated with acrylonitrile, yielded unexpectedly 3-pyridinols as their main product (Scheme 3.4).[12-14]

In this case, the leaving group (cyanide), which permits aromatization, is on the dienophile as opposed to the oxazole. The reaction with acrylonitrile, although relatively low yielding, led directly to the mono alkylated 3-pyridinol. The reaction also proceeded in toluene without additional acetic acid.



Scheme 3.4. Example of the pyridinol synthesis as performed by Naito and co-workers.

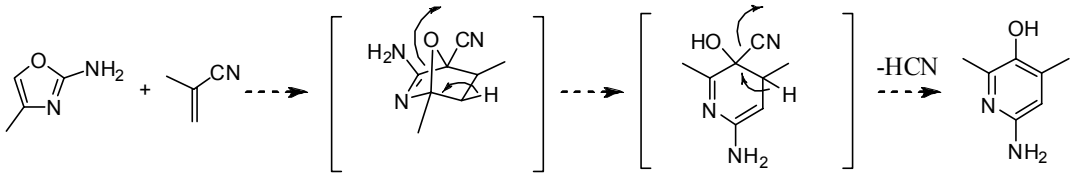
The success of this reaction, carried out with a slightly electron-rich oxazole and slightly electron-poor dienophile was very encouraging. Even more so was an example which employed a 2-amino oxazole as a starting material. By making the oxazole more electron-rich and the dienophile more electron-poor, the reaction now proceeds smoothly in EtOH (Scheme 3.5).[15]



Scheme 3.5. The reaction of 2-amino-5-methyloxazole with diethylmaleate to form 2-amino-5-hydroxy-6-methylpyridine-3,4-dicarboxylic acid.

Given the foregoing, we surmised that 2-amino-5-methyloxazole and methacrylonitrile would provide a route to the N,N-demethylated analog of **3.3** and that

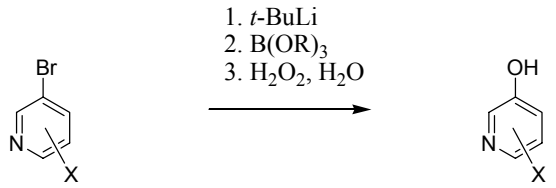
replacing the methyl substitution with *t*-butyl substitution in the oxazole and dienophile may lead to the target compound **3.7** itself.



Scheme 3.6. Proposed Hetero Diels Alder reaction of 2-amino-5-methyloxazole with methacrylonitrile resulting in 6-amino-2,4-dimethyl-3-pyridinol.

3.1.2.2 3-Pyridinols by Hydroxylation

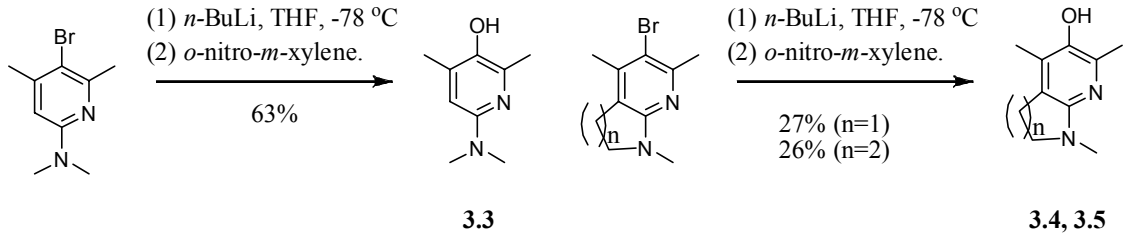
The conversion of easily accessible pyridyl halides to pyridyl alcohols has been the goal of many researchers. Despite this, few useful approaches have been developed.[16, 17] The most common involves generation of a pyridolithium by lithium/halogen exchange, followed by quenching with an oxidant, or first a boronate and then an oxidant (Scheme 3.7).[18]



Scheme 3.7. Typical method of converting a pyridyl bromide to pyridyl alcohol.

More recently, several pyridinols were made by Pratt and co-workers in which the electron-rich character of the aminopyridine substrates prevented the use of peroxides for fear of oxidizing the ring nitrogen. As an alternative, the reaction was executed via a pyridolithium intermediate, but after the lithium/bromide exchange, a nitroarene was used

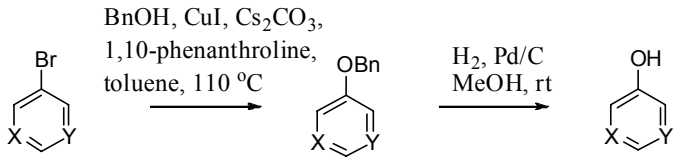
as the oxidizing agent.[19] Although successful, this reaction is very low yielding with two methyl substituents flanking the 3-position (Scheme 3.8).[6]



3.3 **3.4, 3.5**

Scheme 3.8. 3-Pyridinols prepared by lithium/halogen exchange from the corresponding pyridyl bromide followed by oxidation with a nitroarene.

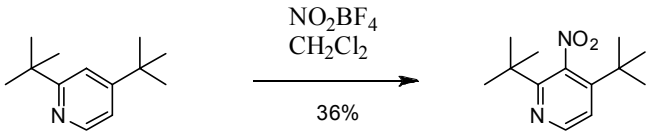
More recently, Pratt and co-workers have developed an alternative which has greater substrate scope and does not require the usual lithium reagents. Specifically, a Cu-catalyzed benzyloxylation appropriate substituted pyridyl halides, followed by catalytic hydrogenation actively leaves a hydroxyl moiety behind.[20-22] The increased electron deficiency of the pyridine and pyrimidine ring systems allows the aryl bromides to convert smoothly to the benzyl ethers, when no reaction occurs with bromobenzene. Interestingly the reaction worked on both pyrimidine as well as on a pyridine ring system but gave no yield when bromo or iodo benzene was used (Scheme 3.9).



X	Y	Yld (%)
CH	CH	0
CH	N	86
N	N	88

Scheme 3.9. 3-Pyridinols prepared by benzyloxylation of the corresponding pyridyl bromide followed by catalytic hydrogenation.

Neither the lithium/halogen exchange-oxidation sequence nor the benzyloxylation-hydrogenation sequence have been attempted on such hindered substrates as required for making target compound **3.7** with *t*-butyl groups flanking the reaction site. Nevertheless, *tert*-butylated pyridines have been shown to be substrates for electrophilic aromatic substitution (Scheme 3.10),[23] suggesting that electrophilic bromination followed by hydroxylation may be possible. Of course, this requires access to *tert*-butylated pyridines.

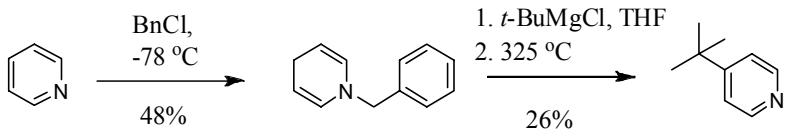


Scheme 3.10. Nitration of the 3 position in 2,4-di-*tert*-butylpyridine.

3.1.2.3 Nucleophilic Alkylation of Pyridines

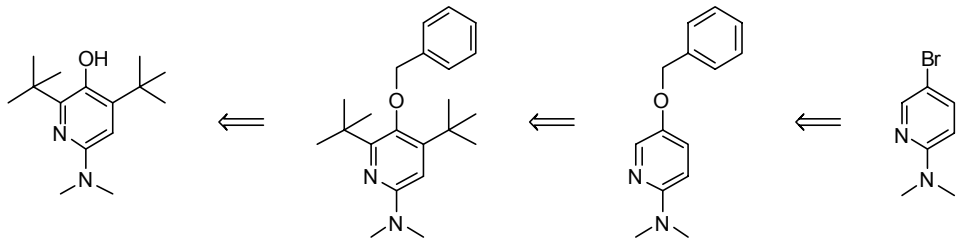
The alkylation of pyridine ring systems can be accomplished in various ways.[24, 25] Substitution, both electrophilic and nucleophilic, is the usual method for introducing alkyl moieties into aromatic ring systems. For a pyridine ring system, where the nitrogen unshared electron pair is not part of the aromatic sextet, electrophilic alkylation will target this electron pair. To prevent this reaction these electrons can be tied up through the use of (temporary) substituents. This will quarternize the ring nitrogen leaving the ring too electron poor for electrophilic substitution. It will, however, activate the ring for nucleophilic alkylation. While nucleophilic substitution of unactivated pyridines is possible, as shown by Brown *et al.*, it is generally proven best to activate the ring first.[26]

For this purpose, several N-substituents can be applied to make the less electron rich dihydropyridine which can be subsequent be alkylated using a Grignard reagent. For example a benzyl group (Scheme 3.11).[27]



Scheme 3.11. Alkylation of pyridine with Grignard reagent via the N-benzyldihydropyridine according to literature procedure.

Given the difficulty we may encounter with conversion of the hindered pyridyl bromide to its benzyl ether, we envisioned a related sequence where we first introduce the benzyl ether followed by alkylation of the pyridine ring (Scheme 3.12). In this case, activation is likely to be absolutely necessary due to the electron richness of the ring.

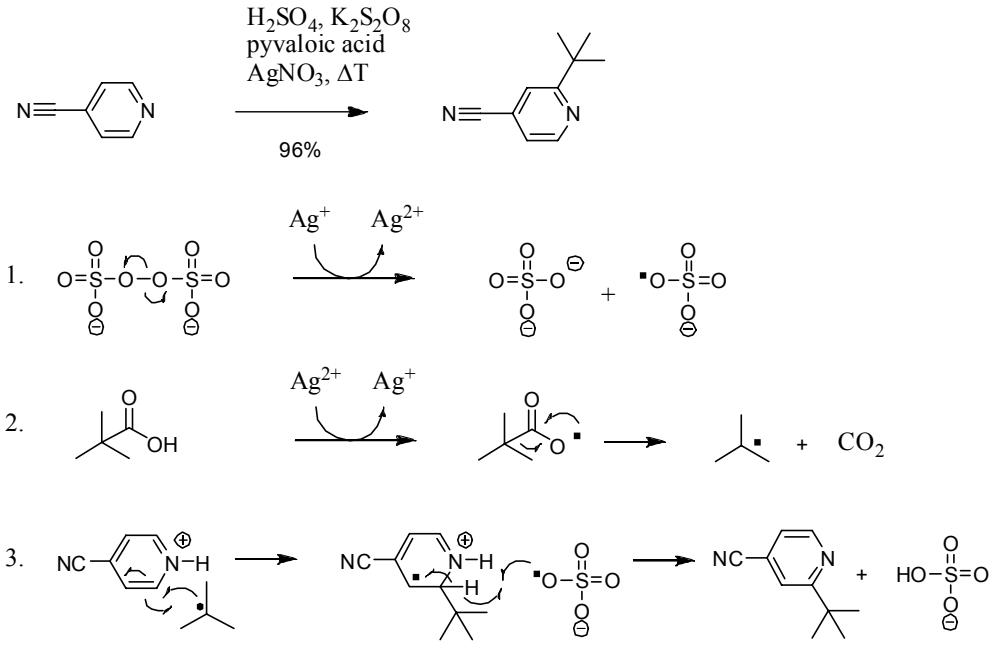


Scheme 3.12. Alkylation sequence envisioned with introduction of the hydroxyl group as protected benzyl ether in the first step.

3.1.2.4 Radical Alkylation of Pyridines

An alternative approach for the alkylation of pyridines utilizes alkyl radicals. These can be generated directly from organomercury compounds or from acids or alcohols using

silver salts.[28, 29] The latter has been widely studied and used, especially by Minisci *et al*., since it avoids organomercury compounds (Scheme 3.13).[25, 30, 31] This reaction sequence uses silver nitrate to initiate a radical reaction that generates pivalic acid radicals. The pivalic acid radical readily loses carbon dioxide to form the more stable isopropyl radical. Tertiary isopropyl radicals are long lived enough to react with activated (electron poor) pyridine ring systems resulting in *tert*-butylation of pyridine. Substrates with electron withdrawing substituent(s) are preferred. The reaction is performed in aqueous solution which makes (additional) activation of the pyridine ring system easy by adding sulfuric acid to protonate the ring nitrogen (step 3, Scheme 3.13). To make this reaction sequence valuable a persulfate salt is used as co-oxidant reducing the amount of silver nitrate necessary to catalytic proportions.

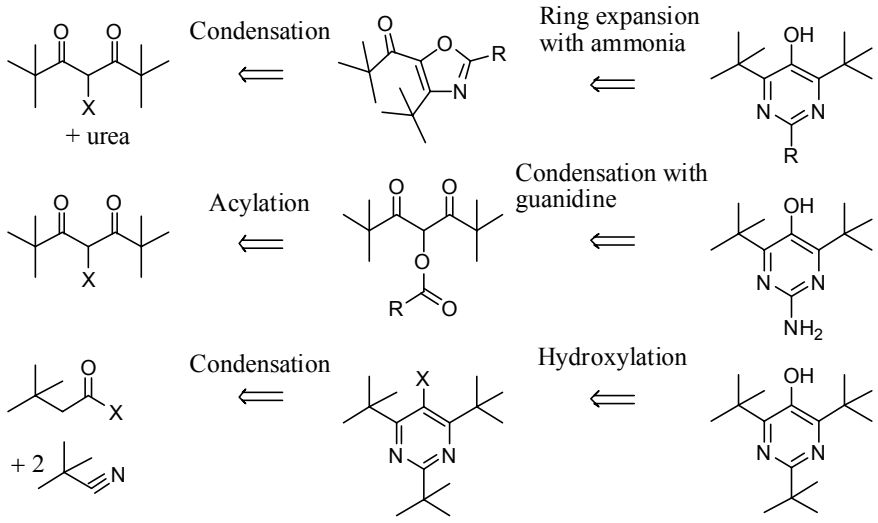


Scheme 3.13. A typical example of the radical alkylation chemistry of pyridines as developed by Minisci and co-workers. Here the *t*-butylation of 4-cyanopyridine is shown.

Hence we envisioned an analogous sequence as used in the nucleophilic substitution, but utilizing a radical pathway.

3.1.3 Approaches to Improve *t*-Butylated Pyrimidinol Synthesis

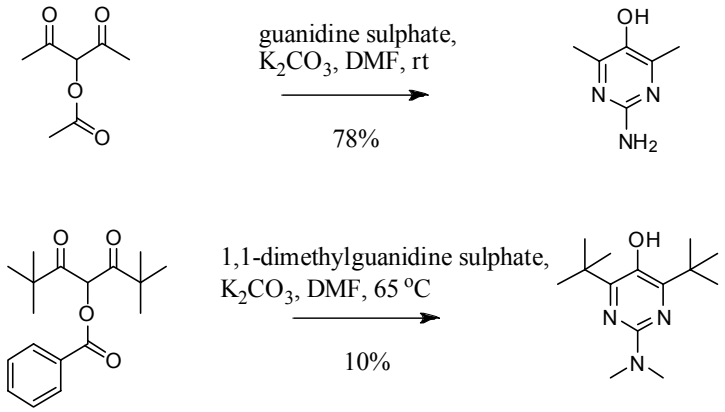
The pyrimidinol of our interest, compound **3.6a**, is a known compound and there are two synthetic pathways as described by Pratt *et al*.[32] The first is a condensation reaction of guanidine with a dione, the second is ring expansion of an oxazole with ammonia. The first route results directly in compound **3.6a**, where the ring opening followed by ring closing leaves room for different substituents at the 2 position (Scheme 3.14). Another possibility would be ring construction in a termolecular approach. This will lead to the pyrimidine with the desired *t*-butyl groups in place but without the hydroxyl group at the 5 position. It does result in a halogen (chloride or bromide) at the 5-position so subsequent hydroxylation would be possible.



Scheme 3.14. Retrosynthetic pathways to access 2,4-di-*tert*-butyl-3-pyrimidinols.

3.1.8.1 Condensation of Guanidines and β-Diketones

An easy method to access substituted pyrimidines is the condensation of a β-diketone with guanidine. When a β-diketone with an acetate or benzoate moiety at the *alpha* position is used basic workup results in a pyrimidine the desired hydroxyl group at the 5-position. This methodology was for example used to make 2-methoxy-4,6-dimethyl-5-pyrimidinol.[4] This chemistry was also used by the same group of Pratt and co-workers to access compound **3.6b** (Scheme 3.15).[33] The only drawback of this method was the low yield, especially when a β-diketone with *t*-butyl groups was used.



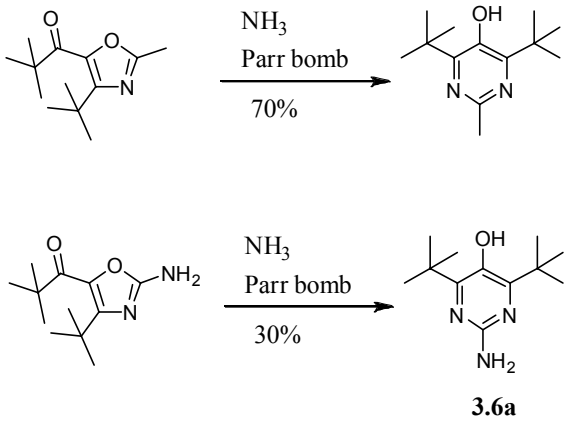
3.6b

Scheme 3.15. An example of the condensation of a β-diketone with an urea to form a substituted pyrimidinol.

3.1.3.2 Ring Expansion of Oxazoles with Ammonia

Another route to make *t*-butylated pyrimidinols is via an oxazole. When an oxazole is subjected to ammonia under the right conditions ammonia will attack the ring, open the oxazole and subsequent closing will result in a pyrimidinol. It is previous shown that this method works well, even with steric hindered oxazoles. For example the synthesis of 2-methyl-4,6-di-*tert*-butyl-5-pyrimidinol, the pyrimidinol analog of the most widely used

industrial phenolic antioxidant BHT, was accomplished this way in good yield (Scheme 3.16). Our target pyrimidinol is also accessible via this route only in lower yield. The fact that oxazoles with less electron donating moieties at the 2 position are much better substrates for this reaction suggests that the difficulty is the initial addition of ammonia to the oxazole. Thus, some optimization in reaction conditions (solvent, temperature) and conversion of the amine group at the 2 position to a less electron donating for the purpose of the ring expansion followed by conversion back to the amine should be attempted. The required oxazole for this reaction can be accessed with relative ease using the same halogenated β-diketone as used in the previous described method.



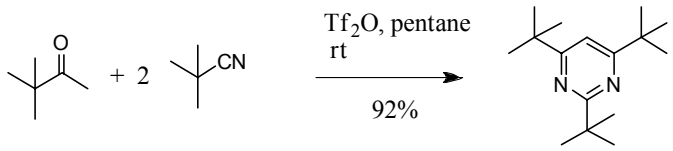
3.6a

Scheme 3.16. Examples of oxazole ring opening reaction with ammonia and subsequent ring closing into target pyrimidinol **3.6a**.

3.1.3.3 Termolecular Approach to Pyrimidines

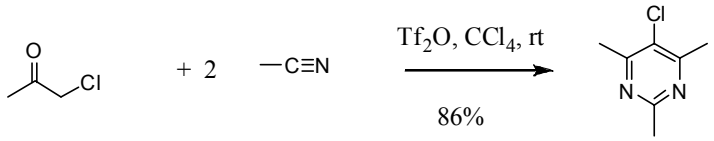
The only other means to prepare *tert*-butylated pyrimidines reported in the literature is a termolecular approach which involves the triflic anhydride mediated condensation of one

molecule of pinacolone and two molecules of pivalonitrile to yield tri-*tert*-butyl pyrimidines (Scheme 3.17).[34]



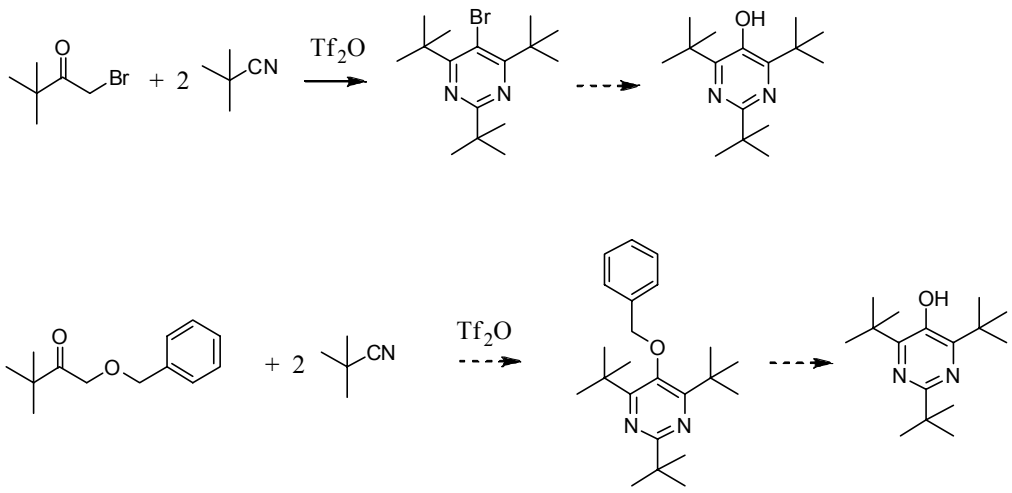
Scheme 3.17. Termolecular synthetic approach to access tri-*tert*-butylated pyrimidine ring system.

It has also been demonstrated that substituents can be introduced in the 5-position by modifying the methyl group of the ketone (Scheme 3.18).[35]



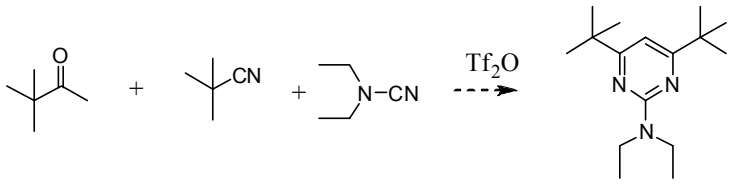
Scheme 3.18. Termolecular synthesis of a substituted pyrimidine ring system with a halogen at the 3 position.

Hence, we surmised that we may be able to use this chemistry to either provide halogenated *tert*-butylated substrates for conversion to the alcohol via a hydroxylation or installing an O-alkyl (e.g. benzyl) ether directly (Scheme 3.19).



Scheme 3.19. Synthetic routes to access tri-*tert*-butylated pyrimidinol starting with termolecular synthesis of the pyrimidine ring system.

If either of these approaches is successful we could then work to modify the approach to incorporate different substituents at the 2 position (Scheme 3.20).



Scheme 3.20. Envisioned reaction using diethylcyanamide to introduce an amine moiety at the 2-position of a *tert*-butylated pyrimidine ring system.

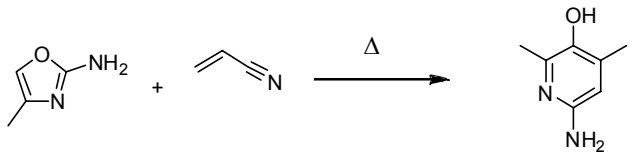
While the optimization of conditions necessary to carry out this transformation is not likely to be trivial, it would be an extremely convenient and inexpensive route to access these compounds.

3.2 Results and Discussion

3.2.1 Cycloaddition Approach to *t*-Butylated 3-Pyridinols

The first reaction pathway explored to access the *t*-butylated target pyridinol **3.7** was via the cycloaddition of an electron poor dienophile to an electron rich oxazole. Since no precedent for this reaction exists in the literature for oxazoles and dienophiles substituted with *tert*-butyl groups, reaction conditions were scouted with a model compound (Scheme 3.21). Oxazole **3.12** was chosen for this purpose due to its ease of accessibility.[36]

When a mixture of hydroxyacetone and cyanamide was heated in water to 42°C, a vigorous reaction took place. Stirring for another hour at 60°C resulted in compound **3.12** in reasonable yields (~75%) that could be used without further purification. Compound **3.12** was then subjected to various reaction conditions with either methacrylonitrile or isopropenyl acetate as dienophile. Methacrylonitrile was chosen because of the precedent in the literature for the reaction of acrylonitrile with an oxazole to yield a 3-pyridinol (Scheme 3.4). Isopropenyl acetate was chosen since the *tert*-butylated analog, necessary for the eventual preparation of our desired product **3.7**, can be easily prepared from *tert*-butyl methyl ketone by deprotonation and acylation.



3.12 **3.6a**

Scheme 3.21. Desired reaction of model oxazole **3.12**.

Initially, we attempted the reaction of oxazole **3.12** with methacrylonitrile. After 24 h at 100^{o}C in toluene, TLC analysis revealed a series of products including a fluorescent spot, which was encouraging since all of the pyridinols we have studied to date are fluorescent. Isolation of this particular product was very difficult and the number of other products formed under these conditions prompted us to look to other conditions. Diels Alder reactions are know to proceed more readily in more polar solvents due to the hydrophobic effect. Unfortunately, upon switching to a MeOH/H_2O mixture both with and without microwave irradiation, no improvement was noted. The solubility, even under elevated temperatures, was too low in this solvent mixture.

Switching dienophiles from methacrylonitrile to isopropenyl acetate did not afford any better results. Long reaction times at elevated temperatures led mostly to polymerization of starting materials. Addition of a Lewis acid, dysprosium triflate, was also investigated since lanthanide triflates are known to very effectively catalyze hetero Diels-Alder reactions and complexation to the oxazole would make it more electrophilic – likely a better match for isopropenyl acetate – a more electron-rich dienophile compared to methacrylonitrile.[37, 38] This yielded an encouraging result, as a new fluorescent spot was observed. Unfortunately, we were unable to isolate this product by column chromatography. Given the lack of success we encountered with the reactions of the unhindered oxazole **3.12** towards cycloadditions with unhindered dienophiles, we surmised that a more sterically-hindered oxazoles bearing a *tert*-butyl moiety with dienophiles having the same bulky group would not result in a feasible synthetic route.

Table 3.1. Overview of reaction conditions surveyed of the hetero Diels Alder cycloaddition of either methacrylonitrile or isopropenylacetate to oxazole **3.12**.

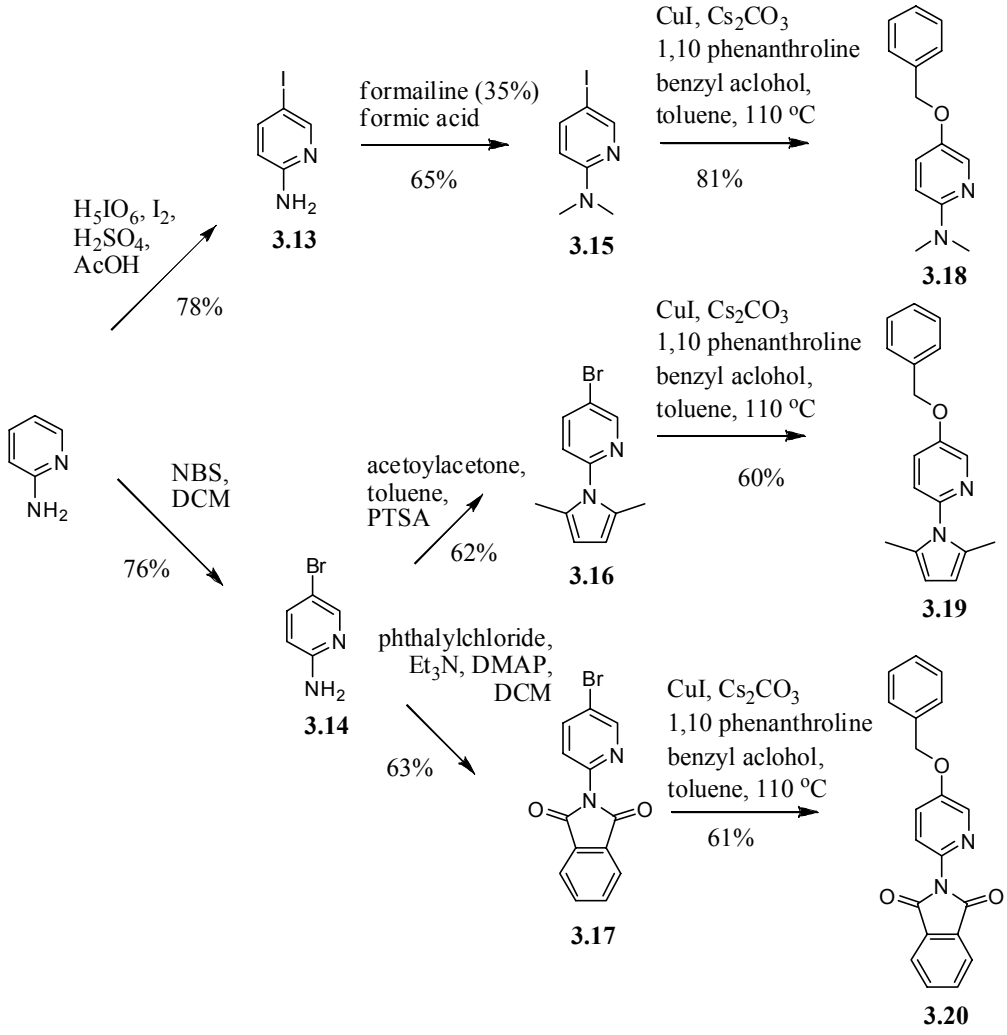
Entry[a]	Solvent	Dienophile	Temp (°C)	Time (h)	Starting material
1[a]	toluene	methylacrylonitrile	110	24	consumed
2[b]	toluene	methylacrylonitrile	110	0.5	polymerizes
3	MeOH/H_2O	methylacrylonitrile	65	24	present
4[b]	MeOH/H_2O	methylacrylonitrile	80	0.5	present
5	ACN	isopropenylacetate	90	24	present
6[c]	ACN	isopropenylacetate	90	72	polymerizes
7	toluene	isopropenylacetate	90	72	polymerizes
8[a,c]	ACN	isopropenylacetate	90	24	consumed

The reactions were performed with a 1:1.5 ratio stoichiometry of oxazole **3.12** to dienophile. [a] TLC gave a new UV active spot indicating product formation. [b] Reaction was performed in a microwave oven. [c] Dysprosiumtriflate was added as Lewis acid catalyst.

3.2.2 Nucleophilic Alkylation of Pyridines

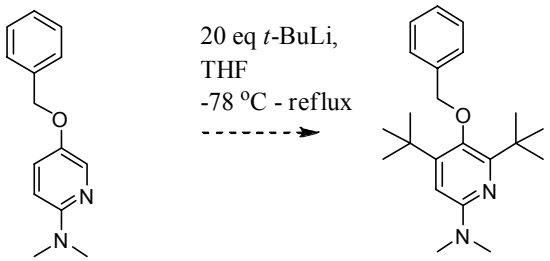
O-Benzyl ethers of 5-amino-3-pyridinols were subjected to a variety of reaction conditions in order to access the desired *tert*-butylated derivatives. The benzyl ethers were prepared via copper-catalyzed Ullmann-type ether synthesis, wherein an appropriately-substituted pyridyl halide was heated with catalytic CuI, 1,10-phenanthroline (ligand), Cs_2CO_3 (base) and benzyl alcohol in toluene (Scheme 3.22).[39, 40] In addition to the N,N-dimethylated derivative **3.18**, both pyrrole (**3.19**) and phthalimide (**3.20**) protected amines were selected as possible substrates for alkylation. While **3.18**,

with its N,N-dimethyl protected amine would, upon alkylation with two *t*-butyl groups and subsequent deprotection of its benzyl ether, result in the target compound, **3.19** and **3.20** were used as starting materials for alkylation since subsequent deprotection of the amines would afford the free amine. This would serve as a versatile handle for further chemistry via halo-dediazotization reactions to yield substrate for further substitution and/or cross-coupling reactions.



Scheme 3.22. O-Benzyl ethers of 2-amino-5-pyridinol derivatives synthesized by Ullmann coupling and used as starting materials for attempted alkylations.

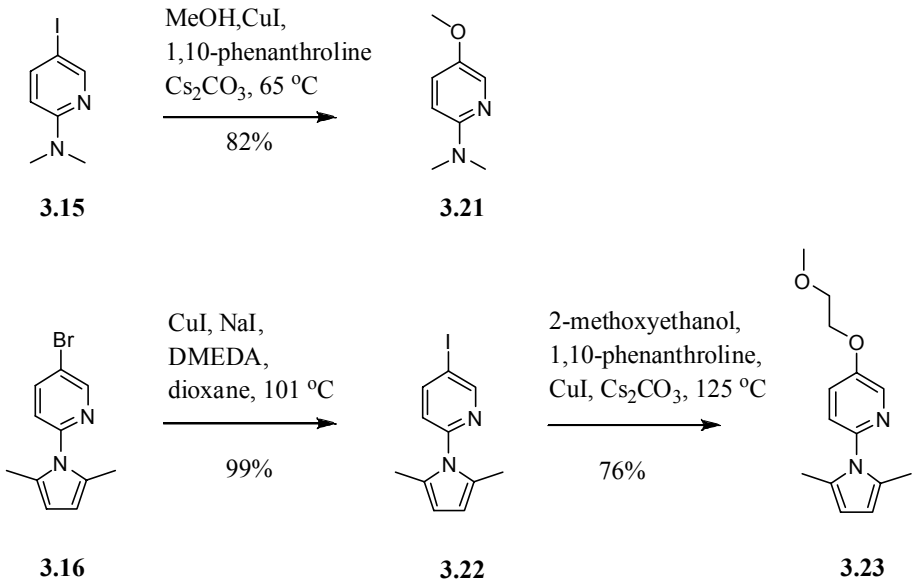
Initial alkylation attempts were carried out on **3.18** at -78°C in THF with 5 equivalents of *tert*-butyllithium (*t*-BuLi) added as a 1.7 M solution in hexanes (Scheme 3.23). This mixture was slowly warmed to room temperature and, although a change in color was observed, and starting material was partly consumed, no product was isolated. Both increasing the amount of *t*-BuLi, from 5 to 20 equivalents, and/or increasing the temperature had no effect on the outcome of the reaction. TLC analysis showed no reaction at low temperature. Upon heating, a series of UV active spots were observed without a dominant product formation. Crude analysis by ^{1}H-NMR gave a series of peaks both in the aliphatic as well as in the aromatic region. The same result was found for reactions carried out on **3.19** and **3.20**.



3.18

Scheme 3.23. Attempt to alkylate 5-(benzyloxy)-N,N-dimethyl-2-aminopyridine **3.18** with excess *t*-butyllithium in tetrahydrofuran/hexane mixture under reflux conditions.

In the event that the presence of an additional aromatic ring in the compound (the benzyl group) is complicating the reaction, leading to multiple products seen by TLC and ^{1}H-NMR, we also carried out the preparation of O-alkyl ethers from two of the pyridyl iodides (Scheme 3.24).

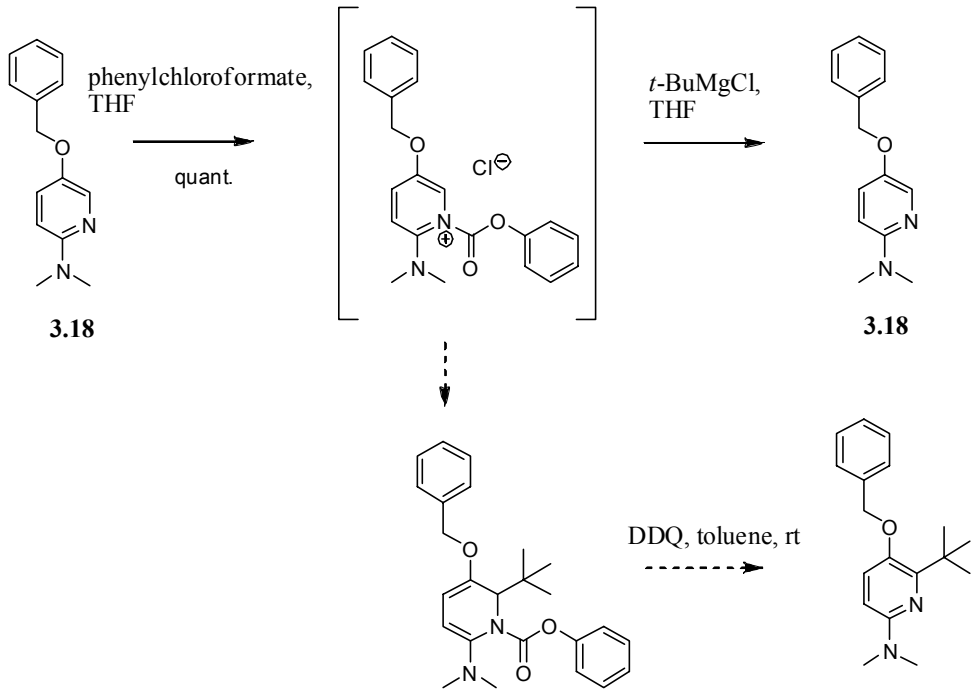


Scheme 3.24. O-alkyl ethers of 6-amino-3-pyridinol derivatives synthesized by Ullmann coupling and used as starting materials for alkylation chemistry.

Unfortunately, attempts to alkylate these substrates under similar conditions also failed. We surmised that this was likely due to the fact that these pyridines were too electron-rich to undergo nucleophilic alkylation, even with *tert*-butyl lithium under reflux in THF. The sluggishness of these reactions then allowed other coupling reactions to result in highly heterogeneous product mixtures. At this point it seemed pertinent to repeat a model nucleophilic alkylation with *t*-BuLi reported in the literature. Thus, following Chen et al., picoline was subjected to *t*-BuLi in THF, but even at elevated temperatures no new spot was observed on TLC and no 2,4-di-*tert*-butylated pyridine was isolated.[41]

Attempts to alkylate **3.18** activated as the N-phenoxyacyl pyridinium salt were carried out next. When **3.18** was treated with phenyl chloroformate in THF, TLC

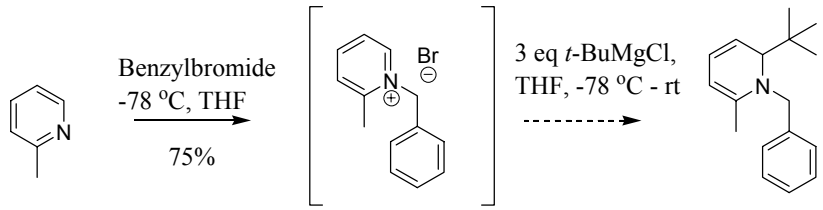
revealed complete consumption of the starting material, the N-(phenoxyacyl)pyridinium chloride. Subsequent cooling to -78 ^{o}C and addition of 1 equivalent of *t*-BuMgCl lead to a new, less polar spot on TLC as well as the deacylated starting material **3.18** (Scheme 3.25). Crude ^{1}H-NMR analysis revealed a mixture of a minimum of two compounds including what appeared to be the mono alkylated 1,2-dihydropyridine product. Subjecting the mixture to oxidation conditions with DDQ in an effort to re-aromatize the monoalkylated 1,2-dihydropyridine yielded a very complex mixture. Since addition of *t*-BuMgCl yielded significant de-acylated starting material, we changed the approach to make use of the more hindered N-*tert*-butoxy acyl pyridinium salt, which would presumably be more stable to deacylation. Unfortunately, similar no-significantly different results were obtained.



Scheme 3.25. Alkylation attempt and subsequent re-aromatization of 4-*tert*-butyl-3-(benzyloxy)pyridine-1(4H)-carboxylate.

Since deacylation, presumably by reaction of the *t*-butyl anion at the acyl group to form the ester (although this was never confirmed) seemed to dominate the reaction pathway under these conditions, we surmised that activation of the pyridine ring by alkylation would be preferable. Rather then subjecting the N-alkyl pyridinium salt of **3.18** to nucleophilic alkylation, we elected to start with a less electron rich, and presumably more reactive, pyridine to explore the chemistry.

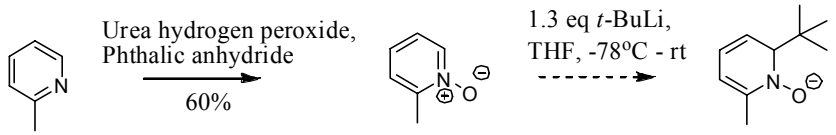
A reaction was attempted with model compound picoline since its quaternary ammonium salt with benzylbromide was easily obtained in 75% yield. The subsequent alkylation attempt with *t*-butylmagnesium chloride did consume starting material but the dark solids formed could not be positive identified by ^{1}H-NMR (Scheme 3.26).



Scheme 3.26. Turning picoline into its benzyl quaternary ammonium salt followed by alkylation attempt with Grignard reagent.

Since it is known that N-oxide formation can also serve to activate pyridine rings to nucleophilic substitution, we prepared picoline N-oxide using urea-hydrogen peroxide complex and phthalic anhydride and attempted to alkylate it using *t*-BuLi. Unfortunately, this also gave a series of product spots according to TLC (Scheme 3.27). Due to the

number of product spots, and picoline being a model compound, the effort was not made to isolate and identify these products.

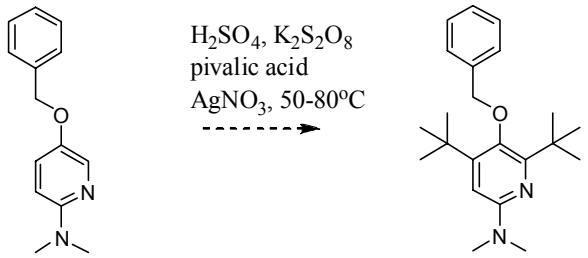


Scheme 3.27. The synthesis of picoline N-oxide followed by alkylation attempt using *t*-butyllithium.

Without being successful in alkylation of our pyridine ring system through nucleophilic approaches this route should not be completely abandoned. The literature shows that, although the reactions are not very clean, pyridine ring systems can be alkylated with *tert*-butyl groups. The reason for the lack of reaction in our cases must lay in the substrates we used. With both an amine in the 2-position and an ether in the 4-position of the pyridine ring, the substrate is clearly too electron-rich to undergo nucleophilic attack. Since we are bound to have this particular substitution pattern options to make changes are limited. A way around this problem is to quarternize the ring nitrogen to create a more electron deficient ring system making it more susceptible towards nucleophilic alkylation. This was done in various ways, but only the N-oxide formation with picoline as model compound gave an indication of reactivity towards alkylation.

3.2.3 Radical Alkylation of Pyridines

Without success using either the cycloaddition approach or the nucleophilic substitution methodology to introduce *t*-butyl moieties into our desired pyridine ring system we next turned to radical alkylation (Scheme 3.2) under various conditions (Table 3.2).



3.18

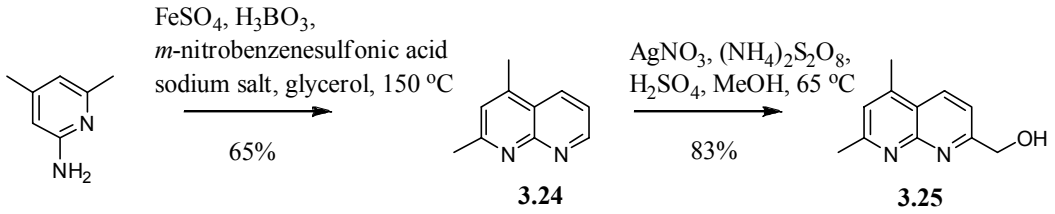
Scheme 3.28. The radical alkylation of **3.18** under the conditions as used by Minisci and co-workers.

Table 3.2. Radical alkylation conditions.

Entry[a]	Startingmaterial	Solvent	Temp (°C)	Time (hour)	SM consumed
1	**3.18**	MeOH	50	2	No
2	3-pyridinol	pivaloic acid/H_2O	80	2	No
3	**3.18**	pivaloic acid/H_2O	80	2	No
4	2-picoline	pivaloic acid/H_2O	80	8	No
5[b]	**3.18**	pivaloic acid/H_2O	80	8	No
6[b,c]	**3.18**	pivaloic acid/H_2O	80	8	No

[a] Reactions were performed with 0.2 eq $AgNO_3$ radical initiator, 2.4 eq $K_2S_2O_4$ and 1 eq H_2SO_4.
[b] Performed with $(NH_4)_2SO_4$. [c] 9 eq H_2SO_4.

To our dismay, the reactions failed in all cases. In fact, no starting material was consumed despite the highly oxidizing conditions. This was surprising given that we had no trouble reproducing the very similar alkylation chemistry performed by a former co-worker, Tae-Gyu Nam; the known hydroxy methylation of a naphthyridine.[42] The substrate for this reaction was acquired via a Skraup reaction of 2,4-dimethyl-6-aminopyridine with crotonaldehyde. This reaction yielded 1,8-naphtyridine **3.24** and subsequent radical hydroxymethylation of this substrate resulted in the desired product **3.25** (Scheme 3.29).[42]



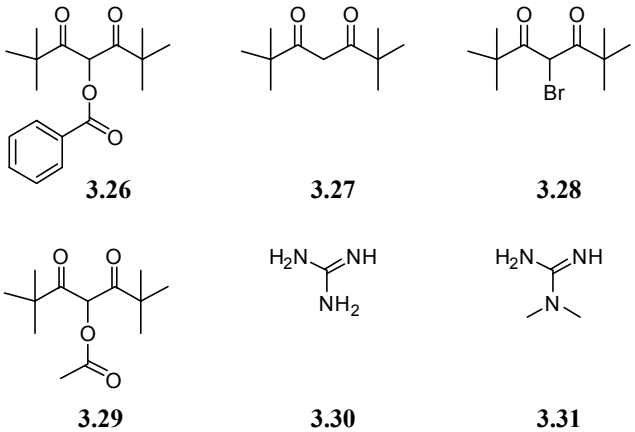
Scheme 3.29. Skraup reaction on 2-amino-4,6-dimethylpyridine resulting in 2,4-dimethyl-1,8-naphthyridine and its hydroxylmethylation confirming radical alkylation methodology.

The same chemistry applied to a pyridine ring system, compound **3.18**, did not yield any product. Even applying the radical alkylation to a more simplified model, 2-picoline, did not result in product formation. A likely reason for this is that the rate of addition is too low, due to electron richness of the ring system, compared to the rate of termination of the alkyl radicals formed. Although the reaction is done with 1.5 equivalents of sulfuric acid, which serves to activate the pyridine ring by protonation, the system is still to electron rich to react fast enough with the generated *t*-butyl radicals.

With a reported relative reactivity of 1:194 methoxypyridine vs. pyridine and our substrate **3.18** being even more electron rich then methoxypiridine, it is assessed that the rate of disproportionation of the *t*-butyl radical is too high compared to the rate of addition to the substrate.

3.2.4 Ring Construction by Condensation

The two component condensation to form the pyrimidine ring system using a dione and guanidine was attempted under different conditions. Several diones were employed as well as both guanidine **3.30** and 1,1-dimethyl guanidine **3.31** (Scheme 3.30). While dione **3.27** is commercially available it can also be made in one step by reacting pinacolone with methylpivolate in THF using sodium hydride as base.[43] Isolation via vacuum distillation gives the dione in good yield. For the condensation chemistry, the same 2,2,6,6-tetramethylheptane-3,5-dione is used in slightly modified form. Given the substantial amount required for both reactions, it was considered more economical to prepare it ourselves (Scheme 3.31).



3.26 **3.27** **3.28**

3.29 **3.30** **3.31**

Scheme 3.30. Compounds used as reagents for the condensation reactions to form pyridiminol **3.6a** or **3.6b**.

It was known that this reaction needed rather forceful conditions to overcome the steric hindrance of the reagents. For this reason the solvent initially used for this reaction, DMF, was switched to more stable higher boiling solvents (e.g. diglyme and dimethylacetamide) since hydrolysis of DMF at high temperatures was thought to interfere in this reaction. This, however, did not have any effect on the outcome. Several combinations of starting materials were tried but without any improvement. The trend shown was slow decomposition of starting material to an unmovable spot on the baseline of a TLC plate, even when highly polar eluent (CH_2Cl_2/MeOH 2:1) was used. Interestingly this baseline spot showed fluorescence in some cases notably when 1,1-dimethyl guanidine was used in a sealed vial (entries 7 and 8, Table 3.3)

Table 3.3. Different reaction conditions and reagents used to access compound **3.6a** or **3.6b**. Reactions resulted in no product isolated.

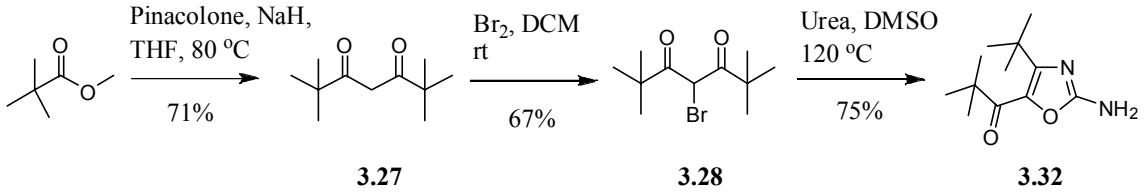
Entry[a]	Reagents[c]	Solvent	Base	Temp (°C)	Time (hour)	SM consumed
1	**3.27** + **3.30**	DMF	NaOAc	150	72	no
2	**3.29** + **3.30**	Diglime	NaOAc	160	148	yes
3[d]	**3.29** + **3.30**	DMAc	NaOAc	160	48	yes
4	**3.26** + **3.30**	DMF	Na_2CO_3	70	24	yes
5	**3.26** + **3.30**	DMSO	NaOAc	150	24	yes
6	**3.26** + **3.30**	DMSO	K_2CO_3	160	24	yes
7[b,d]	**3.29** + **3.31**	DMSO	NaOAc	180	24	yes
8[b]	**3.28** + **3.31**	DMF	Na_2CO_3	60	24	no
9[b,d]	**3.26** + **3.31**	DMF	Na_2CO_3	160	72	yes

[a] Reactions were performed at a 1:1 ratio of startingmaterials with 5 eq of base. [b]Reaction was performed in a sealed vial. [c]Compound **3.30** was used as the HCl salt, compound **3.31** as the H_2SO_4 salt. [d] TLC baseline spot showed fluorescence.

3.2.4.2 Pyrimidinols via Ring Expansion of Oxazoles with Ammonia

The route to make our target *t*-butylated pyrimidinol (**3.6a**) via ring expansion of an oxazole with ammonia is attractive since the required oxazole for this reaction is easily accessible. This approach was deemed more likely to work because adding a small nucleophile to the oxazole is less sterically demanding than adding guanidine to the carbonyl adjacent to the *tert*-butyl group in the foregoing condensations. Oxazole **3.32** is made from the reaction of 4-bromo-2,2,6,6-tetramethylheptane-3,5-dione with urea. The

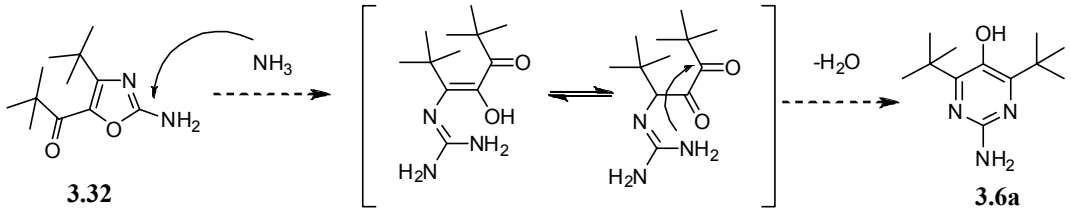
2,2,6,6-tetramethylheptane-3,5-dione in turn is brominated using bromine in dichloromethane (Scheme 3.32).



3.27 **3.28** **3.32**

Scheme 3.31. Synthesis of oxazole **3.12**, starting material for pyrimidine **3.6a** synthesis.

With the oxazole in hand, we first attempted to reproduce the reaction reported in the literature. Oxazole **3.32** was heated in *t*-BuOH with concentrated ammonia in a Parr bomb at 180 °C for 36 hours (Scheme 3.32). [32,44]



3.32 **3.6a**

Scheme 3.32. Oxazole ring opening using ammonia, intermediate and subsequent condensation to yield the pyrimidinol.

Although starting material was consumed according to TLC, these conditions did not result in product formation. For reactions that need a high energy input, performing them in microwave reactors can be of advantage. Several attempts were made varying the power, time, temperature and solvent system, but without success (Table 3.4). It was

noticed that oxazole **3.32** lacked solubility in the *t*-BuOH/ammonia mixture initially used (entries 1 and 2, Table 3.4). Switching to other solvents did indeed improved solubility but resulted in decomposition of starting material. A possible explanation for the lack of reactivity observed is that the harsh conditions used forces ammonia out of solution. Release of excess pressure was however not observed when the microwave oven vials were opened.

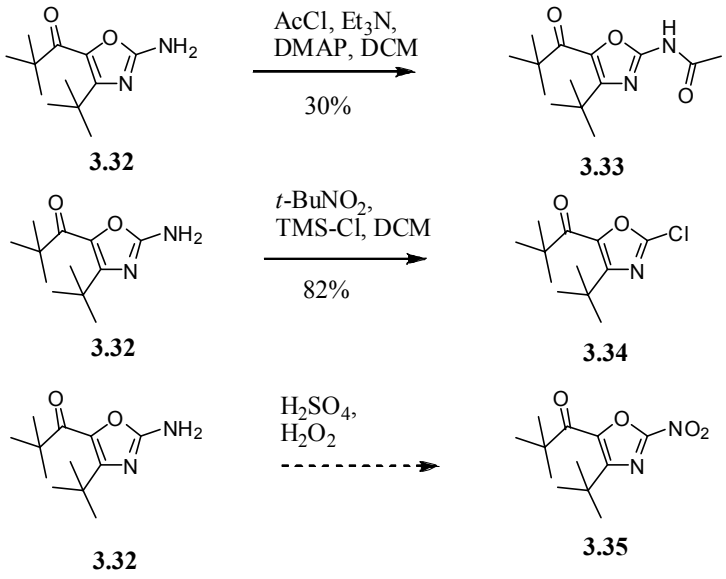
Table 3.4. Reaction conditions of oxazole ring expansion reactions as performed in a microwave oven.

Entry[a]	NH_3 source/Solvent[b]	Temp (^oC)	Time (min)	SM consumed
1	NH_4OH (30%)/ *t*-BuOH	150	15	no
2	NH_4OH (30%)/ *t*-BuOH	200	15	no
3	NH_4OH (30%)/ ACN	150	15	yes
4	NH_4OH (30%)/ DMSO	150	30	yes

[a] Reactions were performed at 0.045 mmole scale in 0.4 ml total volume at 100 Watt. [b] A sovent to ammonia solution of 1:1 was used.

Derivatization of the amine in oxazole **3.32** was tried as well as substitution at the 2-position to change the electronics in an attempt to make the oxazole more electrophilic, and therefore a better substrate for nucleophilic attack by ammonia (Scheme 3.33). Oxazole **3.33** was made by reacting oxazole **3.32** with acylchloride. Upon contact with ammonia it simply deacetylated into oxazole **3.32**. Via diazotization of oxazole **3.32,** the chlorinated oxazole **3.34** was obtained. Under standard reaction conditions (as described

in entry 1, Table 3.4) the ammonia in the reaction mixture substitutes the chloride, resulting in oxazole **3.32**. Oxazole **3.35** with a nitro substituent at the 2-position was not easily accessible, and attention was switched to another project.

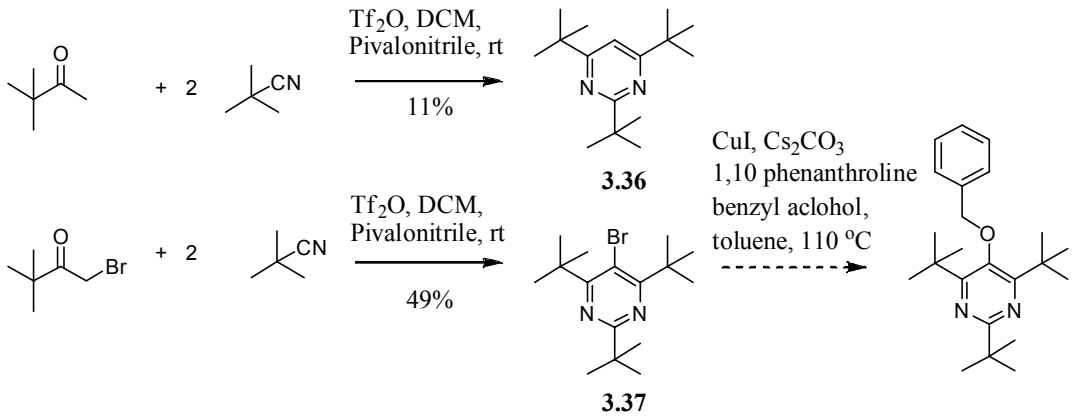


Scheme 3.33. Acetylation, chloro-diazotization and oxidation of oxazole **3.32**.

3.2.4.3 Termolecular Approach to Pyrimidines

Application of the termolecular approach to pyrimidines reported by Martínez and co-workers using triflic anhydride initially gave results. The reaction with pivalonitrile and pinacolone yielded the tri-*tert*-butyl pyrimidine **3.36**, while this compound was of no further synthetic value to us as an intermediate, it did confirm that the termolecular chemistry was a potential candidate to synthesize our desired *tert*-butylated pyrimidines. One step further was the introduction of a synthetic handle at the 5 position to introduce the hydroxyl moiety and access a pyrimidinol. Switching from pinacolone to 1-bromo pinacolone gave the desired pyrimidine **3.37** with a bromide at the 5-position (Scheme

3.34). Unfortunately, subsequent Ullmann chemistry on this substrate to introduce the benzyl ether at this position failed. Switching the bromide for a more reactive iodide using a Cu-catalyzed Finkelstein protocol also failed.[45]



Scheme 3.34. *t*-Butylated pyrimidine synthesis using pyvalonitrile, (substituted) pinacolone and triflic anhydride.

3.3 Prospective

3.3.1 Towards *tert*-Butylated 3-Pyridinols

The target pyridinol **3.7** is of interest since it is very likely that a pyridinol with this substitution pattern, an electron donating group *para* to the hydroxyl group and with two *tert*-butyl groups flanking the hydroxyl group at the 3-position, will be a very effective antioxidant in all media, not only the homogenous hydrogen-bonding organic solvents were it was proved to be one of the best phenolic antioxidants to date. The *tert*-butyl groups will have a positive effect on two parameters that steer antioxidant ability. Their steric bulk will increase the persistency of the pyridinoxyl radical once it has donated its hydrogen atom, making it longer living and less likely to mediate peroxidation. The

same steric bulk will also lower the hydrogen bond ability of the hydroxyl moiety when it is used in hydrogen bond accepting solvent thus minimizing the KSE. Unfortunately, there is no precedent to the 2,4-di-*tert*-butyl-3-pyridinol core structure that we are highly interested in. We investigated three approaches in an attempt to access these structures for subsequent study as radical trapping antioxidants in organic media as well as LDL. The synthetic approaches fall in the following categories: cycloaddition, nucleophilic alkylation and radical alkylation. The cycloaddition approach involved the 4+2 hetero Diels Alder reaction of an appropriate dienophile (methacrylonitrile or isopropenylacetate) to an oxazole (a model oxazole **3.12** was used). This reaction forms an unstable bicyclic intermediate that provided with a good leaving group aromatizes to give a pyridinol with expulsion of said leaving group. Using the appropriate oxazole and dieneophile this reaction could lead to target pyridinol **3.7** in one step.[9] The several trials on model oxazole **3.12** were however not successful. The lack of reactivity likely lies in the improper electronic properties of the diene and dienophile. Although the bulk of the *tert*-butyl groups also have a negative influence on the reactivity, this would be possible to overcome if the HOMO-LUMO gap is small enough. Trials to match these, both normal electron demanding with an electron deficient dieneophile and electron rich diene as well as reverse electron were attempted but not successful. Although one could envision ways to lower the HOMO-LUMO gap by changing substituents on both oxazole and dienophile, a reaction with changed moieties would not lead to target compound **3.5** and significant elaboration to product would likely be required.

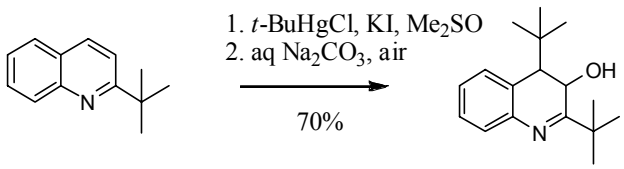
Leading up to the nucleophilic and radical alkylation approaches, it was necessary to develop an alternative approach for introduction of the hydroxyl group. Traditionally

this was done by halogen/lithium exchange followed by oxidation with for example hydrogen peroxide or nitrobenzene.[18, 19] Copper catalyzed Ullmann ether synthesis was found to be highly successful into introducing an oxygen substituent into the ring system. Generally this was done as a benzyl ether but other ethers could be constructed with ease too. The benzyl ether was chosen because it is easily cleaved using hydrogen gas over palladium catalyst resulting in respective pyridinol or pyrimidinol. This chemistry left us with a synthetic tool to make pyridinols but the question of how to introduce flanking *tert*-butyl moieties was not resolved.

The first approach to compound **3.7** was nucleophilic substitution. The literature clearly shows that *tert*-butylated pyridines are accessible via this route. [26, 41] Although these examples use very harsh conditions; up to 20 equivalents *t*-BuLi and refluxing THF, and give moderate yields on simple pyridine compounds such as picoline and pyridine itself. It was thought that if selective and reasonable yielding it was still a useful way to access target pyridinol **3.7**. In our hands isolation proved to be problematic. While literature examples were performed on large scale and the desired product was distilled out, when trials on small scale on several substrates which required multiple steps to access gave no results activation of the substrate was tried. This did not give the wanted result but messy reaction mixtures, some of which may have contained product but this was not established. Disappointed with this result an alternative was found in radical alkylation chemistry. Well documented and starting from commercially available reagents this gave a promising pathway to access compound **3.7**. Initial trails following directly the literature procedure did however not result in di- or even mono alkylated substrate. A switch to a simpler model (picoline) was made also without success. Doubts

about the reagents and/or protocol used, prompted us to follow the literature exactly with a known substrate. This reaction worked as described convincing us that the problem lay in the substrate **3.18** used. Considering the relative ease of which naphthyridine **3.24** reacts under these conditions; changing the substrate **3.18** to a slightly more electron poor pyridine ring system will likely make this synthetic approach successful.

Unbeknownst when we set out to do this chemistry, Russell, in the early 1990's, has reported the radical alkylation of dihydroquinoline **3.38** to give the tetrahydroquinoline **3.39** (Scheme 3.35). When worked up in air with aqueous sodium carbonate this reaction also introduced a hydroxyl moiety as would be desired in target pyridinol **3.7**. Rearomatization of compound **3.39** would lead to a quinoline with the 2,4-di-*tert*-butylated 3-pyridinol substitution pattern sought after. This is a very interesting reaction, since it is sensitive to the amount of KI employed and whether the reaction is worked-up under atmosphere or not. We are currently investigating this chemistry.



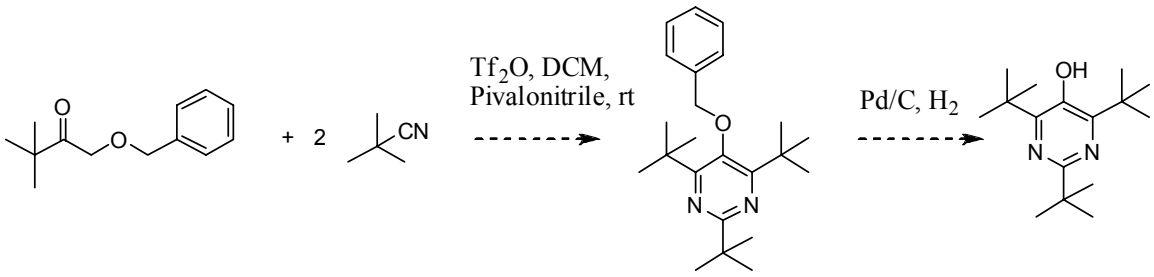
3.38 **3.39**

Scheme 3.35. Radical alkylation of 2-*tert*-butylquinoline to di-*tert*-butyl hydroquinoline **3.39** with the desired substitution pattern of **3.7** at the 2, 3 and 4 positions.

3.3.2 Improving the Routes to *tert*-Butylated 5-Pyrimidinols

Although the *tert*-butylated pyrimidinols **3.6a/3.6b** of our interest are known compounds they proved to be difficult to access. The two reported synthetic pathways to this

molecule, ring expansion of an oxazole ring and condensation, were explored but proved difficult to reproduce let alone improve.[4] The condensation using a β-diketone is straightforward and would be of considerable use if it could be improved. Both the β-diketone as well as the condensation partner, a (alkylated) guanidine, leave after considering the attempts described here little room for further manipulation. The ring expansion pathway, although explored in some depth, leaves room for further investigation. Because utilizing this method would lead in one step to the desired substituent pattern at the 4, 5 and 6 position this is a desirable pathway. A good substrate for this reaction would be compound **3.35** (Scheme 3.33) with a nitro substituent. Although an initial reaction to make compound **3.35** did not resulted in isolation of product, without further attempts made this chemistry can still be developed. The third methodology applied was the termolecular approach using triflic anhydride, pyvalonitrile and pinacolone. The benefits of this reaction are the commercial available reagents and the single construction step to access the *tert*-butylated pyrimidine ring system. It does however not place the hydroxyl functionality at the 5 position. When 1-bromopinacolone is used the pyrimidinol formed has a bromide at the 5 position. Ullmann chemistry at this position was found to be cumbersome due to accessibility that is blocked by the flanking *t*-butyl moieties. The direct introduction of a benzyl ether at this position by using 1-(benzyloxy)-3,3-dimethyl-2-butanone would be a good alternative (Scheme 3.36).



Scheme 3.36. Termolecular synthesis of the *tert*-butyl pyrimidine ring system with the benzyl ether in place.

3.4 Experimental Section

General Methods. All reagents were purchased from commercial sources and used without further purification, unless otherwise indicated. Column chromatography was carried out using Silica-P Flash silica gel (60 Å 40-63 μm, 500 m^2/g) from Silicycle. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 25 °C on a Bruker Avance Spectrometer at 400 MHz and 100 MHz, respectively. Mass spectra were obtained on an Applied Biosystems/MDS Sciex QSTAR XL QqTOF mass spectrometer.

2-Amino-4-methyloxazole (**3.12**). A solution of hydroxyacetone (10 g, 0.14 mol) and cyanamide (6 g, 0.14 mol) in water (20 ml) was slowly heated to 40^{o}C. After a vigourous reaction the mixture was kept at 70^{o}C for 1 h. The pH of the reaction mixture was adjusted to 11 using a sodium hydroxide solution in water (5%). The mixture was extracted with ethyl acetate (3 x 30 ml) and the combined organic layers were dried over sodium sulfate. Concentration in vacuo gave the title compound as a yellow oil (**3.8**) (7.0 g, 53%) Analytical data were in accordance with those reported in the literature.[46]

5-iodo-2-aminopyridine (**3.13**). To a solution of 2-aminopyridine (2.4 g, 25.0 mmol), periodic acid (860 mg, 3.8 mmol) and iodide (2.7 g, 10.7 mmol) in acetic acid (60 ml) was added water (3 ml) and conc. H_2SO_4 (0.5 ml). The mixture was heated for 4 hours at 80 ^oC. The mixture was concentrated on a rotary evaporator and poured into a 10% $Na_2S_2O_4$ solution in water (50 ml). The mixture was extracted with diethylether (3 x 50 ml) and the combined organic layers were dried over $MgSO_4$. Concentration in vacuo yielded a pale yellow solid **3.13** that was not further purified. Yield 5.1 g (91%). Analytical data were in accordance with those reported in the literature.[47, 48]

5-bromo-2-aminopyridine (**3.14**). To a solution of 2-aminopyridine (5.0 g, 53 mmol) in DCM (20 mL) was added N-bromosuccinimide (9.9 g, 56 mmol). The flask was covered with alumina foil and the mixture was stirred overnight. The mixture was concentrated under vacuum and dissolved in ethyl acetate (50 ml). The solution was washed with water (50 ml) and brine (50 ml) and the dried over $MgSO_4$. Concentration and recrystallization from EtOAc gave title compound **3.14** as dark yellow crystals. Yield 7.0 g (76%). Analytical data were in accordance with those reported in the literature.[49]

5-iodo-N,N-dimethyl-2-aminopyridine (**3.15**). The 5-iodo-2-aminopyridine (1.0 g, 4.54 mmol) was heated at reflux overnight in a mixture of formic acid (20 ml) and 37% formalin (20 ml). The reaction mixture was cooled and volatiles removed under reduced pressure. The residue was shaken with a mixture of 1.0 M aq. NaOH and CH_2Cl_2 (twice). The combined organic extracts were washed with brine, dried over Na_2SO_4 and concentrated in *vacuo*. The residue was subjected to flash chromatography (eluent: ethyl

acetate/hexanes). Yield 0.51g (45%). Analytical data were in accordance with those reported in the literature.[50]

5-bromo-2-(2,5-dimethyl-1H-pyrrol-1-yl)pyridine (**3.16**). Substrate 5-bromo-2-aminopyridine **3.14** (2.0 g, 11.6 mmol), 2,5-hexanedione (1.65 mL, 14.0 mmol), and *p*-TsOH (0.023 g, 0.08 mmol) were dissolved in toluene (10 ml) and heated in a Dean-Stark apparatus for 2 h. After cooling, the dark brown reaction mixture was washed with satd. aq. $NaHCO_3$ solution, five times with water, and with brine. After the mixture had been dried with Na_2SO_4, the solvent was removed in *vacuo*. The dark residue was subjected to flash chromatography on silica gel (eluent: ethyl acetate/hexanes 1:1). Yield 1.81g (62%). Analytical data were in accordance with those reported in the literature.[51]

2-(5-bromopyridin-2-yl)isoindoline-1,3-dione (**3.17**). To a solution of 5-bromo-2-aminopyridine **3.14** (0.50 g, 2.89 mmol) in CH_2Cl_2 (20 ml) was added DMAP (70 mg, 0.58 mmol) and Et_3N (0.29 g, 2.89 mmol). The solution was cooled to 0 ^oC and phthaloylchloride (0.59 g, 2.89 mmol) was added. The temperature was allowed to go to rt and the reaction was stirred an additional hour. The mixture was washed with water (3 x 30 ml) and dried over $MgSO_4$. Filtration over a silica plug and concentration under vacuum gave product as dark yellow crystals. Yield 0.67 g (93%). Analytical data were in accordance with those reported in the literature.[52]

5-benzyloxy-2-N,N-dimethylaminopyridine (**3.18**). Dissolve 5-iodo-N,N-dimethyl-2-aminopyridine (0.5 g, 2.0 mmol) in benzylalcohol (2 ml). Add CuI (38 mg, 0.2 mmol(,

DMEDA (35 mg, 0.4 mmol) and Cs2CO3 (1.3 g, 4.0 mmol) and heat to 110 ^{o}C overnight. The mixture was filtered over a silica plug. Concentration *in vacou* gave title compound **3.18** as a light yellow solid. Yield: 364 mg (80%).^{1}H-NMR (300 MHz, $CDCl_3$): δ 3.00 (s, 6H, -N(CH_3)$_2$) 4.28 (s, 2H, -NH-CH_2-Ph), 6.47-6.51 (d, J = 8.9 Hz, 1H, *arom.*), 6.94-6.98 (dd, J = 8.9, 3.0 Hz, 1H, *arom.*) 7.30-7.40 (m, 5H, *arom.*), 7.76-7.77 (d, J = 2.5 Hz, 1H, *arom.*) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 39.6, 50.3, 107.6, 125.5, 127.9, 128.2, 129.3, 134.2, 136.3, 140.2, 154.9 ppm. HRMS (ES^{+}) calculated (M) 227.1422, observed 227.1419.

5-benzyloxy-2-(2,5-dimethyl-1H-pyrrol-1-yl)pyridine (**3.19**). To a solution of 5-bromo-2-(2,5-dimethyl-1H-pyrrol-1-yl)pyridine **3.16** (6.76 g, 22.7 mmol) in benzylalcohol (20 ml) was added CuI (432 mg, 2.27 mmol), DMEDA (597 mg, 4.54 mmol) and Cs_2CO_3. The mixture was stirred at 110 ^{o}C overnight and cooled to room temperature. EtOAc was added (50 ml) and the mixture was filtered over a silica plug. Concentration *in vacou* gave title compound **3.19** as an off white solid. Yield 3.77 g (60%) (^{1}H-NMR (400 MHz, $CDCl_3$*)*: δ 2.18 (s, 6H), 5.21 (s, 2H), 5.90 (s, 2H), 7.04-7.06 (d, J = 8.3 Hz, 1H), 7.34-7.58 (m, 5H, *arom*), 8.11-8.14 (dd, J = 8.2, 2.2 Hz, 1H), 8.83 (d, J = 2.0 Hz, 1H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 13.2, 70.8, 107.5, 114.1, 122.5, 123.5, 127.0, 128.5, 128.8, 136.0, 137.0, 152.0, 154.0 ppm. HRMS (ES^{+}) calculated (M) 278.1419, observed 278.1372. Yield: 60%.

2-(5-benzyloxypyridin-2-yl)isoindoline-1,3-dione (**3.20**). To a solution of 2-(5-bromopyridin-2-yl)isoindoline-1,3-dione **3.17** (100 mg, 0.29 mmol), CuI (6 mg, 0.03

mmol) and Cs_2CO_3 (190 mg, 0.58 mmol) in benzylalcohol (1 ml) was added DMEDA (7.6 mg, 0.06 mmol) and the mixture was stirred overnight at 110 ^oC. The mixture was concentrated on silica gel and was subjected to flash chromatography on silica gel (eluent: ethyl acetate/hexanes 1:9). Yield 58 mg (61%). (^1H-NMR (400 MHz, $CDCl_3$): δ 5.91 (s, 2H), 7.04-7.06 (d, J = 8.3 Hz, 1H) 7.36-7.62 (m, 5H, *arom*), 7.91-7.99 (d J = 1.6 2H) 8.11-8.14 (dd, J = 8.3, 2.0 Hz, 1H), 8.21-8.28 (d J =1.6 2H), 8.83 (d, J = 2.0 Hz, 1H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 92.3, 123.5, 124.1, 131.6, 134.8, 141.0, 127.1, 127.3, 129.6, 145.3, 146.5, 155.7, 166.2 ppm. HRMS (ES^+) calculated (M) 330.1004, observed 330.1124.

5-methoxy-2-N,N-dimethylaminopyridine (**3.21**). To a solution of 5-iodo-N,N-dimethyl-2-aminopyridine (420 mg, 1.7 mmol) in MeOH (3 ml) was added CuI (32 mg, 0.17 mmol), Cs_2CO_3 (827 mg, 2.55 mmol) and DMEDA (44 mg, 0.34 mmol). The mixture was heated in a microwave oven for 90 minutes at 110 ^oC, cooled to rt and concentrated on silica gel. Purification by flash chromatography on silica gel (eluent: ethyl acetate/hexanes 1:9) gave product as a white crystalline solid. Yield 210 mg (82%). Analytical data were in accordance with those reported in the literature.[53]

5-iodo-2-(2,5-dimethyl-1H-pyrrol-1-yl)pyridine (**3.22**). 5-Iodo-2-aminopyridine (5.0 g, 22.7 mmol) was dissolved in toluene (50 ml) and 2,5-hexanedione (3.11 g, 27.2 mmol) was added with a catalytic amount of PTSA (30 mg). The mixture was heated to 120 ^oC under Dean-Stark conditions overnight. After cooling to rt the mixture was washed with aqueous $NaHCO_3$ solution (100 ml) and with water (2 x 100 ml). The organic layer was

dried over $MgSO_4$ and concentration under vacuum gave the title compound 3.25 as light yellow crystals. Yield 6.8 g (99%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.16 (s, 6H), 5.93 (s, 2H), 7.04-7.06 (d, J = 8.4 Hz, 1H), 8.12-8.14 (dd, J = 8.4, 2.4 Hz, 1H), 8.83 (d, J = 2.0 Hz, 1H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 13.3, 91.0, 107.5, 123.6, 128.6, 146.2, 151.2, 155.5 ppm. HRMS (ES^+) calculated (M) 297.9967, observed 297.9972.

5-(2-methoxyethoxy)-2-(2,5-dimethyl-1H-pyrrol-1-yl)pyridine (**3.23**). 5-Iodo-2-(2,5-dimethyl-1H-pyrrol-1-yl)pyridine (500 mg, 1.8 mmol) was dissolved with CuI (34 mg, 0.18 mmol), Cs_2CO_3 (880 mg, 2.7 mmol) and DMEDA (31 mg, 0.36 mmol) in 2-methoxyethanol (3 ml). The mixture was heated overnight at 110 ^{o}C. After concentration under vacuum the mixture was subjected to flash chromatography on silica gel (eluent: ethyl acetate/hexanes 1:9). Yield 337 mg (76%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.31 (s, 6H), 3.77 (d, J = 2 Hz, 2H), 4.13 (d, J = 2 Hz, 2H) 5.80 (s, 2H), 7.10-7.16 (d, J = 8.0 Hz, 1H), 7.31-8.14 (d J = 8.0 Hz, 1H), 8.21 (s, 1H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 13.7, 58.3, 72.3, 74.8, 93.8, 113.6, 128.6, 146.2, 149.5, 151.2, 155.5 ppm. HRMS (ES^+) calculated (M) 246.1368, observed 246.1362.

2,2,6,6-tetramethylheptane-3,5-dione (**3.27**).[43] To a solution of methylpivalate (40 g, 0.35 mol) in dry THF under nitrogen atmosphere sodium hydride (27.7 g, 60% w/w, 0.70 mol) was added. At reflux temperature pinacolone (36 g, 0.36 mol) in THF (50 ml) was added over 1 h. After cooling to room temperature concentrated hydrochloric acid (60 ml) was added and the mixture was poured into 1 L ice water. Extraction with hexanes (2 x 125 ml) and concentration *in vacou* gave yellow oil. Purification by distillation gave title

compound **3.27** (45.8 g, 71%). Analytical data were in accordance with those reported in the literature.[54]

4-bromo-2,2,6,6-tetramethylheptane-3,5-dione (**3.28**). To a solution of 2,2,6,6-tetramethylheptane-3,5-dione (**3.27**) (12.5 g, 68 mmol) in dichloromethane (75 ml) bromine was added (8.2 g, 50 mmol) drop wise. After adding all bromine the mixture was stirred an additional 30 minutes at room temperature. the solvent was removed under reduced pressure and the resulting yellow oil diluted with diethyl ether (200 ml), and washed with water (200 ml), $NaHCO_3$ 0.5 (200 ml), and sodium thiosulphate 0.5 (200 ml). After drying (Na_2SO_4), the solvent was removed and the residue crystallized from hexane at -18 ^oC to yield 11.9 g (67%) of title product (**3.28**) as white crystals. Analytical data were in accordance with those reported in the literature.[4]

4-benzoyl-2,2,6,6-tetramethylheptan-3,5-dione (**3.26**). Sodium benzoate (4.0 g, 28 mmol) and 4-bromo-2,2,6,6-tetramethylheptan-3,5-dione (4.9 g, 18.5 mmol) were mechanically stirred in anhydrous DMSO (50 ml) for 1 hours under nitrogen. After cooling (0 ^oC) and diluting with water the title compound crystallized as a pure white solid. Yield 5.3 g (94%); ^1H NMR (CDCl3): δ 1.25 (s, 18 H), 6.46 (s, 1 H), 7.47 (m, 2H), 7.61 (t, J = 8 Hz, 1H), 8.08 ppm (d, J = 9 Hz, 2 H).[4]

2,2,6,6-tetramethyl-3,5-dioxoheptan-4-yl acetate (**3.29**). 4-Bromo-2,2,6,6-tetramethylheptane-3,5-dione (3.0 g, 11.4 mmol) was dissolved in dry DMSO (10 ml) and sodium acetate (1.9 g, 22.8 mmol) was added. The mixture was stirred for 48 h at 60

^{o}C. The mixture was poured in water (150 ml) and extracted with EtOAc (3 x 100 ml). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. This resulted in title the compound without further purification needed. (2.46 g, 89%). Analytical data were in accordance with those reported in the literature.[55]

2-amino-4-tert-butyl-5-(2,2-dimethylpropanoyl)oxazole (**3.32**). To a solution of 4-benzyloxy-2,2,6,6-tetramethylheptan-3,5-dione (4.5 g, 17.1 mmol) in 25 mL anhydrous DMF was added urea (3.6 g, 60 mmol). The mixture was heated to 120 ^{o}C overnight. After cooling to room temperature the mixture was poured in ice water and the solids were filtered off resulting in pure title compound **3.32**. Yield 2.9 g (75%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.22 (s, 9H) 1.33 (s, 9H), 5.88 (s, 2H –NH_2) ppm. ^{13}C-NMR (400 MHz, $CDCl_3$): δ 26.7, 28.1, 28.8, 33.0, 43.8, 86.8, 158.0, 193.5 ppm. HRMS (ES^{+}) calculated (M) 224.1525, observed 224.1619.

N-(4-tert-butyl-5-(pivaloyl)furan-2-yl) acetamide (**3.33**). To a solution of 2-amino-4-tert-butyl-5-(2,2-dimethylpropanoyl)oxazole (50 mg, 0.22 mmol) in CH_2Cl_2 (1 ml) was added DMAP (5 mg, 0.05 mmol), Et_3N (33 mg, 0.33 mmol) and acetylchloride (21 mg, 0.26 mmol). The mixture stirred for 1 h at rt. Water (10 ml) was added and the mixture was extracted with CH_2Cl_2 (3 x 10 ml). The combined organic layers were dried over $MgSO_4$ and concentrated under vacuum. The compound was used without further purification. Yield 28 mg (30%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.35 (s, 6H), 1.45 (s, 6H), 2.40 (s,

3H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 21.9, 26.8, 28.1, 31.2, 44.8, 112.6, 136.7, 144.2, 177.3, 196.5 ppm.

1-(4-tert-butyl-2-chlorooxazol-5-yl)-2,2-dimethylpropan-1-one (**3.34**). To a solution of 1-(4-tert-butyl-2-aminooxazol-5-yl)-2,2-dimethylpropan-1-one (100 mg, 0.45 mmol) in 1 ml CH_2Cl_2 was added TMS-Cl (97 mg, 0.9 mmol) and *tert*-$BuNO_2$ (206 mg, 1.8 mmol). The mixture stirred for 30 min. at rt. Ice cold aqueous $NaHCO_3$ solution was added (5 ml) and the mixture was extracted with CH_2Cl_2 (3 x 10 ml) The combined organic layers were dried over $MgSO_4$ and concentrated under vacuum. Purification by column chromatography (Heptane/EtOAc 95:5) gave the title compound. Yield 90 mg (82%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.28 (s, 9H) 1.34 (s, 9H), ppm. ^{13}C-NMR (400 MHz, $CDCl_3$): δ 26.5, 28.5, 33.2, 44.4, 145.0, 145.9, 158.9, 195.2 ppm.

2,4,6-tri-tert-butylpyrimidine (**3.36**). To a solution of pyvalonitrile (125 mg, 1.8 mmol) in DCM (3 mL) was added triflic anhydride (131 mg, 1,15 mmol). This mixture was slowly added to a solution of pinacolone (100 mg, 1.0 mmol) in DCM (3 ml). After 5 days additional pivalonitrile was added (50 mg, 0.3 mmol) and stirred for 1 day. Saturated sodium bicarbonate solution in water was added (10 ml) and the mixture was extracted with DCM (3 x 10 ml). The combined organic layers were dried over $MgSO_4$ and concentration *in vacuo* resulted in title compound **3.36** as a yellow semi solid. Yield 28 mg (11%). Analytical data were in accordance with those reported in the literature.[56]

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