Exploring Novel Catalytic Chalcogenide Antioxidants

HENRIK JOHANSSON
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Abstract

This thesis is concerned with the synthesis and evaluation of regenerable chalcogen containing antioxidants. Various substituted 2,3-dihydrobenzo[b]selenophene-5-ol antioxidants were evaluated in order to gain information about structure/reactivity-relationships. Within the series explored, the most regenerable unsubstituted compound inhibited lipid peroxidation for more than 320 minutes when assayed in a two-phase lipid peroxidation model in the presence of N-acetylcysteine (NAC). α-Tocopherol which could inhibit lipid peroxidation for 90 minutes under similar conditions was therefore easily outperformed. The antioxidant activity of the parent was also documented in an aqueous environment. The best catalyst quenched/inhibited ROS production by neutrophils and PMA-stimulated macrophages more efficiently than Trolox. In addition, over a period of seven days, no disruption in proliferation for the cell lines used was observed when exposed to our synthetic compound or Trolox at a concentration of 60 μM.

3-Pyridinols substituted with alkyltelluro groups in the ortho-position were more regenerable in the two-phase model than their corresponding para-substituted analogues in the presence of NAC and also inhibited autoxidation of styrene in a catalytic fashion in homogenous phase in the presence of N-tert-butoxycarbonyl cysteine methyl ester (LipCys), a lipid-soluble analogue of NAC. The best inhibitors quenched peroxy radicals more efficiently than α-tocopherol. They could also catalyze reduction of organic hydroperoxides in the presence of thiols and therefore mimic the action of the glutathione peroxidase enzymes. Mechanisms for the catalysis are proposed.

Octylthio, octylseleno and octyltelluro analogues of butylated hydroxyanisole (BHA) were synthesized and evaluated. Among these, the tellurium compound was superior to α-tocopherol in the presence of NAC both when it comes to quenching capacity and regenerability.

Organochalcogen substituent effects in phenolic compounds were studied by using EPR, IR and computational methods.

Keywords: antioxidant, tellurium, selenium, catalytic, toxicity, ROS, neutrophil, glutathione peroxidase mimic, macrophage.

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Till minne av Nikolai Kvist
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


VII Appendix: Supplementary Material

Reprints were made with kind permission from the publishers.
The author wishes to clarify his contribution to the papers I–VII presented in this thesis:

Paper I:

Contributed significantly to the synthetic work and performed DFT-calculations. Participated in evaluation experiments using the two-phase lipid peroxidation system described. Contributed to writing of the paper.

Paper II:

Contributed to formulation of the research idea and participated in the planning of most experiments. Contributed to writing of the paper.

Papers III, IV and VII:

Contributed significantly to the synthetic work and to evaluation experiments using the two-phase lipid peroxidation system described. Contributed to writing of the papers.

Paper V:

Contributed to the original research idea; performed all synthetic work and evaluation experiments. Contributed to writing of the paper.

Paper VI:

Contributed significantly to the synthetic work.
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<tr>
<td>ABTS</td>
<td>2,2’–Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>AIBN</td>
<td>2,2’-Azobis(isobutyronitrile)</td>
</tr>
<tr>
<td>AMVN</td>
<td>2,2’–Azobis(2,4-dimethylvaleronitrile)</td>
</tr>
<tr>
<td>BDE</td>
<td>Bond dissociation enthalpy</td>
</tr>
<tr>
<td>BHA</td>
<td>Butylated hydroxyanisole</td>
</tr>
<tr>
<td>BHT</td>
<td>3,5-Di-tert-butyl-4-hydroxytoluene</td>
</tr>
<tr>
<td>CL</td>
<td>Chemiluminescence</td>
</tr>
<tr>
<td>CPS</td>
<td>Counts per second</td>
</tr>
<tr>
<td>DFT</td>
<td>Density functional theory</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ED</td>
<td>Electron donating</td>
</tr>
<tr>
<td>EW</td>
<td>Electron withdrawing</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>ET</td>
<td>Electron transfer</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (γ-glutamyl cysteiny1 glycine)</td>
</tr>
<tr>
<td>HAT</td>
<td>Hydrogen atom transfer</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IP</td>
<td>Ionisation potential</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetylcysteine</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PCET</td>
<td>Proton coupled electron transfer</td>
</tr>
<tr>
<td>PD</td>
<td>Peroxide decomposer</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leucocytes</td>
</tr>
<tr>
<td>PT</td>
<td>Proton transfer</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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1. Introduction

This thesis describes the development of a new generation of catalytic and multifunctional chalcogen containing antioxidants and comprises a continuation of Nature’s work that started millions of years ago. It details the journey from the ‘old fashioned drawing table’, via synthesis of new substances in the laboratory to mechanistic investigations of their mode of action. To some extent it also explores their use as pharmaceutical agents.

Nature’s development of antioxidants has been based on a trial-and-error work from elements provided – a strategy that would be likewise suitable in pursuit of new ones. Still, from the point of view of evolution, selection of some molecular structures over others hands us templates and useful knowledge for further improvements. Before presenting the journey of the goals sought for, a brief background to radical chemistry and the need of antioxidants is provided for appreciation of given discussions.

1.1 Radicals and oxidation

1.1.1 Chemistry of free radicals – history and development

Radicals, or free radicals, impinge our lives whether we want it or not. They are involved in combustion processes, in stratospherical degradation of the ozone layer and in biological systems. Over the years, we have learned to “tame” many highly reactive radical species resulting in applications in the synthesis of small molecules and in polymerization processes for the production of rubbers, plastics and other commercial products. Still, due to their reactivity, they can cause much damage in biological systems and have been implicated in many diseases.

By definition, ‘a free radical is any species capable of independent existence that contains one or more unpaired electrons’. The formation of a radical can be achieved from a non-radical species by loss (oxidation), or gain (reduction) of a single electron or by homolytic cleavage of covalent bonds.

The birth of radical chemistry is often associated with the identification of the first relatively stable organic carbon-centered free radical, the triphenylmethyl radical (1), by Moses Gomberg in 1900.
However, it took until 1937 for radicals to be postulated as intermediates in chemical reactions.\textsuperscript{3} As the World War II demanded development of synthetic rubber industries, polymer chemistry became vitally important and with this, radical chemistry too.\textsuperscript{4} Around this time, useful structure/reactivity relationships in co-polymerizations were introduced by Mayo\textsuperscript{5} and at the end of the 1940s the nature of free radicals as well as chain reactions had become familiar to many scientists.

From 1950, development of electron paramagnetic resonance (EPR) spectroscopy also made it possible for chemists to study the structure and formation of short-lived radicals. Absolute rate constants could also be determined and radicals were suspected to be involved in the ageing process. During this period radical processes involved in the deterioration of oils, fats and food products where also examined closely and autoxidation, studied first by de Saussure in 1820, was investigated in more detail. The discovery of superoxide dismutase in the late 1960’s\textsuperscript{6} led to much understanding of our antioxidant defence systems and the important role of radicals as metabolic products.\textsuperscript{7} Since 1970, the information on absolute rate constants has extended the use of radicals in organic synthesis and the work of Barton,\textsuperscript{8} Hart\textsuperscript{9} and Giese\textsuperscript{10} in the mid 1980’s made the field of radical synthesis flourish. Most polymers manufactured today are produced by radical reactions and we know that not all radical reactions are destructive to man. However, they have been suggested to play key roles in the development of several diseases such as heart disease and cancers.

\subsection*{1.1.2 Oxidation}
\subsubsection*{1.1.2.1 Oxygen – both essential and toxic}
Surrounding us during everyday life, molecular oxygen is essential to maintain many living systems on Earth. Despite this, it is also involved in processes that slowly degrade most organic materials – including our bodies – with time. By closer inspection, the diatomic oxygen molecule, O\textsubscript{2}, has two unpaired electrons, with parallel spins located in different \( \pi^* \) antibonding orbitals. Hence, and in accord with the definition of a free radical, it qualifies as a diradical in its ground state.

If O\textsubscript{2} would accept an electron pair from an atom or molecule in an oxidation process, these electrons must have the same spin. This is unlikely since, according to the Pauli principle, the electrons would have opposite spins.
Due to this, $O_2$ preferably accepts electrons one at a time. The spin-restriction is very fortunate, as oxidation of many non-radical species or biomolecules otherwise would be rapid. However, the possibility of single electron transfer explains why oxygen often reacts fast with radicals, and is involved in many radical reactions as one of our most common toxins.

1.1.2.2 Autoxidation

Autoxidation is a free radical process consisting of three steps: Initiation, propagation and termination. Autoxidation is a problem as soon as any kind of organic material is contacted with oxygen. A proposed mechanism\(^\text{11}\) is shown below (Scheme 1).

![Scheme 1. General mechanisms for autoxidation and the action of antioxidants.](image)

During initiation various environmental stresses (UV-light, transition metals etc.) cause formation of an alkyl radical ($R^\cdot$). A rapid reaction with molecular oxygen then results in formation of a peroxyl radical ($ROO^\cdot$).\(^\text{12}\) Peroxyl radicals quickly abstract hydrogens both from secondary and tertiary positions in hydrocarbon substrates ($RH$) with hydroperoxide ($ROOH$) formation. The new carbon-centered radical thus formed is then part of the propagation sequence. Cleavage of the weak O-O bond of the hydroperoxide can in turn generate reactive oxygen centered radicals such as the hydroxyl radical ($HO^\cdot$) and the alkoxyl radical ($RO^\cdot$). Both these species are capable of starting new chain reactions. In the presence of oxygen, autoxidation proceeds until all oxidizable substrate is consumed, or until the cycle is interrupted. The breaking of the chain could occur via radical-radical recombination (termination) or by the action of antioxidants in different manners\(^\text{11}\) (Scheme 1).
1.2 Antioxidants in prevention and protection

Antioxidant is a term presumably known by everyone even though the meaning of the word may vary. Before further reading it should therefore be defined. As formulated by Halliwell and Gutteridge, an antioxidant is “any substance that delays, prevents or removes oxidative damage to a target molecule”.\(^{13}\)

Antioxidants can be divided into two classes – preventive and chain breaking. Preventive antioxidants, such as peroxide decomposers (PD), destroy the radical precursor before it falls apart. Metal chelators and UV absorbers can also be classified as preventive antioxidants. Chain-breaking antioxidants either donate a hydrogen atom to peroxyl radicals and take them out of the propagation cycle (donating) or compete with dioxygen for the carbon centered radicals (accepting) (Scheme 1).

1.2.1 Non-enzymatic antioxidants

Some of the most prominent non-enzymatic antioxidants are ascorbic acid (Vitamin C, 2), glutathione (3) and Vitamin E. At physiological pH, ascorbic acid predominantly exists in the deprotonated form (HAsc\(^{-}\)) and is therefore often referred to as ‘asorbate’. Whereas plants and many animals can synthesize ascorbate from glucose, man cannot. Therefore it is referred to as an exogenous antioxidant which must be supplied from the dietary intake, for example in the form of fruits and vegetables. Ascorbic acid is regarded both as essential and as a biological antioxidant needed for the action of several hydroxylase enzymes (i.e. lysyl, prolyl and \(\beta\)-hydroxylases), where it acts as a co-factor and reducing agent for active site metals. The concentration of ascorbate in human plasma varies from 30-90 \(\mu\)M and the levels correlate well with the intake of ascorbate-rich food.\(^{14}\) However, even if ascorbate is a powerful scavenger of many radical species \textit{in vitro} at these concentrations, its direct antioxidant function \textit{in vivo} still needs more evidencing support.\(^{15}\)

In contrast to ascorbate, the thiol containing tripeptide glutathione (GSH) is synthesized intracellularly in the aqueous cytoplasm and in mammalian cells it could be present in millimolar concentration.\(^{16}\) Glutathione reacts with several radical species \textit{in vitro} and its high concentration makes it a good radical scavenger also \textit{in vivo}.\(^{17}\) It acts by hydrogen donation to form thyl radicals (GS\(^{\pm}\)) which could recombine to form glutathione disulfide (GSSG). The reduction of GSSG to GSH is brought about enzymatically by glu-
Glutathione reductase. Glutathione is a co-factor for the glutathione peroxidases (GPx). It is also involved in ascorbate\textsuperscript{18} and xenobiotic\textsuperscript{19} metabolism.

Vitamin E is a nutritional term that refers to a series of chromanols named tocopherols (\textbf{4a-d}), differing only in the aromatic methyl substitution pattern. Among these, the fully methylated $\alpha$-tocopherol ($\textbf{4a}$) is the most potent antioxidant. With the phytol chain sticking into the cell membrane, Vitamin E is the most important lipophilic chain-breaking antioxidant \textit{in vivo}.\textsuperscript{20} As an exogenous antioxidant it must be supplied by the diet.

![Phytol group](image)

\begin{align*}
\textbf{4a} & \quad R^1 = R^2 = \text{Me} \quad (\alpha\text{-tocopherol}) \\
\textbf{b} & \quad R^1 = \text{Me}, R^2 = \text{H} \quad (\beta\text{-tocopherol}) \\
\textbf{c} & \quad R^1 = \text{H}, R^2 = \text{Me} \quad (\gamma\text{-tocopherol}) \\
\textbf{d} & \quad R^1 = R^2 = \text{H} \quad (\delta\text{-tocopherol})
\end{align*}

$\alpha$-Tocopherol is known to trap two peroxyl radicals before it is converted into non-radical products. In the reduction of peroxyl radicals it would therefore seem wise for Nature to regenerate this valuable molecule. The regeneration of $\alpha$-tocopherol (Figure 1) is brought about by hydrogen atom transfer (HAT) from ubiquinol in the cell membrane.\textsuperscript{21} It is also generally accepted that ascorbate could donate a hydrogen atom at the lipid-aqueous interphase (Figure 1).\textsuperscript{22}

![Figure 1. Regeneration of $\alpha$-tocopherol by ascorbate.](image)
1.2.2 Enzymatic antioxidants

There are three major classes of preventive antioxidant enzymes: Superoxide dismutases (SOD), catalases (CATs) and glutathione peroxidases (GPx). SODs can be divided into four groups depending on the metal involved (Cu/Zn, Mn or Fe and Ni) and catalyse the dismutation of superoxide \( \text{O}_2^- \) into oxygen and hydrogen peroxide, \( \text{H}_2\text{O}_2 \). Hydrogen peroxide formed in the process can be catalytically dismutated into water and ground-state \( \text{O}_2 \) by catalases.

The glutathione peroxidases (GPx) catalyse reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively, with reduced glutathione (GSH) serving as the stoichiometric reductant (GSSG formation). At the active site of glutathione peroxidase, the reduction of hydrogen peroxide is facilitated by selenocysteine (Sec) – the selenium analogue of cysteine. In the catalytic reaction, the selenol moiety (GPx-SeH) is oxidized to a selenenic acid (GPx-SeOH). Further reaction with one equivalent of glutathione generates a selenosulfide (GPx-Se-S-G). The selenol is regenerated by reaction with another equivalent of glutathione with formation of disulfide (GSSG) (Scheme 2).

![Scheme 2. Catalytic cycle for the action of glutathione peroxidase.](image-url)
The family of glutathione peroxidases includes in all seven isoenzymes (GPx1-7) but only five of them are selenoenzymes. Among these, glutathione peroxidase 1 (GPx-1) is the most abundant in humans.

1.2.3 Antioxidants for stabilization of man-made materials

Since all organic matter suffers from degradation or deterioration with time when exposed to molecular oxygen, the need for antioxidant additives is obvious. Stabilizers are used by food technologists to prevent rancidity of food materials and by the oil industry in lubricants. Polymer chemists use antioxidants extensively not only to avoid discoloration but also to improve mechanical properties such as tensile strength of the products.

A large variety of antioxidants are used as food preservatives. Butylated hydroxytoluene (BHT, 5) and butylated hydroxyanisole (BHA, 6) are both common additives. Except for being manufactured as a dietary supplement, ascorbic acid serves as an oxidation protecting agent together with its sodium and calcium salts. Other commercial forms of ascorbic acid such as ascorbyl palmitate and ascorbyl stearate (7) are also used as flavour and color stabilizers, often in combination with vitamin E. They also appear as additives to various cosmetic formulations.

Most antioxidants used as stabilizers in synthetic oils, polymers and plastics are sterically hindered chain-breaking phenols such as BHT or the Irganox family of additives (i.e. Irganox® 1076, 8). Sterically hindered aromatic amines are also frequently used. Ethoxyquin (9) is a chain-breaking antioxidant that acts as a hydrogen donor. It has been used in the rubber industry and as an antioxidant for fishmeal and various foods.

Peroxide decomposers such as organophosphites, i.e. Irgafos® 38 (10), are common stabilizers during polymer melt processing and sulfur derivatives such as thioesters (i.e. dilauryl and distearyl thiodipropionate) serve as preventive antioxidants in the production of polymers. As the volume of...
plastic additives has increased with the growth of the plastic industry,\textsuperscript{31} there is a need for high performance, non-toxic and cheap antioxidants.

1.3 ROS and RNS

ROS is a collective name for Reactive Oxygen Species. It includes oxygen radicals as well as derivatives thereof, but also non-radicals such as hydrogen peroxide, peroxynitrite (ONOO\textsuperscript{−}) and hypochlorous acid (HOCl). RNS is a collective name for Reactive Nitrogen Species such as nitric oxide (NO\textsuperscript{•}) and nitrogen dioxide (NO\textsubscript{2}\textsuperscript{•}). Peroxynitrite, more known as part of the ROS can also be classified as RNS.

1.3.1 ROS and RNS in the immune system

The immune system involves organs, cells and proteins and constitutes in its complexity the defence of the host against viruses or bacteria with the potential to cause disease. Its primary function is to defend the host from infectious organisms, or pathogens, but it also plays an important role during disease evolvement. Pathogens may vary in size and also differ in the way they cause diseases and the immune system must therefore have a broad repertoire in the actions and elimination of threats. One of the elimination mechanisms is phagocytosis – the engulfment and internalization of the pathogens. This process is carried out by phagocytes (i.e. macrophages, neutrophils and dendritic cells).\textsuperscript{32} Macrophages are derived from the bone marrow as monocytes. Monocytes are found in the bloodstream but can differentiate into macrophages after entering damaged tissue or an inflamed area. One essential difference between macrophages and neutrophils is that macrophages are able to phagocytose larger particles whilst neutrophils, also referred to as polymorphonuclear leucocytes (PMN), only can engulf smaller organisms. The killing mechanism of the phagocytes involves ROS and RNS production. As phagocytosis begins phagocytes show an increase in the uptake of oxygen, a phenomenon known as respiratory burst. This is due to the activation of NADPH oxidase that is assembled in the plasma membrane and leads to the production of superoxide (O\textsubscript{2}\textsuperscript{•−}). The fast dismutation of superoxide into H\textsubscript{2}O\textsubscript{2} then gives a useful weapon in bacteria elimination along with the more reactive hydroxyl radical (OH\textsuperscript{•}).

Another pathway involves generation of nitric oxide (NO\textsuperscript{•}) that is toxic to bacteria and also can inhibit viral replication.\textsuperscript{33} Nitric oxide may react with superoxide to generate peroxynitrite (ONOO\textsuperscript{−}). However, there is some controversy as to the role of RNS in the killing of pathogens by neutrophils and macrophages in humans.\textsuperscript{34}
1.3.2 Oxidative stress

“Oxidative stress” is a term that has been frequently used in the literature. It refers to an imbalance between production of ROS/RNS and antioxidant defence systems in favour of the former. Overproduction of ROS and RNS, i.e. superoxide, hydrogen peroxide and peroxynitrite, might lead to damage not only of vital molecules such as DNA and proteins but also lipids - the major constituents of eukaryotic cell membranes. Indeed, ROS/RNS are involved in numerous pathologies\(^35\) such as cancer, allergic and inflammatory conditions\(^36,37\) atherosclerosis\(^38\) and stroke.\(^39\) Also, oxidative stress has been implicated as a factor in ageing.\(^40\) With many oxidative stress related diseases, the interest in pharmacological antioxidants and antioxidant phar-macotherapy has increased considerably.\(^41\)

1.4 Group 16 – the chalcogens

The chalcogens – also known as the oxygen family - are elements of group 16 of the periodic table. They include oxygen, sulfur, selenium, tellurium and polonium. With oxygen already introduced and polonium being radioactive and rarely used in chemistry, a brief introduction to sulfur, selenium and tellurium follows.

1.4.1 Sulfur

Sulfur is a non-metallic pale yellow element that has been known for thousands of years. It exists in many allotropic forms and serves as an important starting material in industrial production of vulcanized rubber. It is also a common element in many pharmaceuticals. As an essential element for human life, sulfur is found in the amino acids methionine and cysteine which are incorporated into many of our proteins.

1.4.2 Selenium

Selenium (from the Latin “selene” meaning “moon”) was discovered by Berzelius in 1817 and was originally identified as a toxin. However, selenium was recognized as an essential trace element in 1957 by Klaus Schwarz.\(^42\) Today selenium deficiency has been suggested as a factor in the pathogenesis of several diseases such as white muscle disease in livestock animals,\(^43\) Keshan disease and Kashin-Beck disease.\(^44,45\)

The UGA codon, which is usually a stop codon, also dictates incorporation of selenocysteine (Sec) into proteins. The human proteome consists of 25 selenoproteins\(^46\) – several with antioxidant functions to the organism. The aforementioned glutathione peroxidase was the first selenoprotein to be discovered.\(^47\)

Organoselenium reagents have been used extensively in organic synthesis since the 1970s,\(^48\) although organoselenium compounds have been known
since 1847. The interest in selenium over the last twenty to thirty years, both among biochemists and practitioners of organic chemistry, has been constantly high.

1.4.3 Tellurium

Tellurium (from the Latin “tellus”, meaning “earth”) was first discovered and extracted from an ore of gold in 1782 by Baron Franz–Josef Müller von Reichenstein. However, it was not until 16 years later that Martin Heinrich Klaproth named the element. The first organotellurium compound, diethyl telluride, was prepared by Friedrich Wöhler in 1840. Although the field of organotellurium chemistry has seen a slow development, thousands of papers have been published to date.

Introduction of tellurium into organic compounds can be achieved in several ways using both electrophilic (e.g. TeCl₄) and nucleophilic forms of the element. Nucleophilic tellurium reagents can be obtained by insertion of the element into Grignard and organolithium reagents or via reduction of diorganyl ditellurides.

No nutritional role has been reported for tellurium. Although organotellurium compounds have been reported as toxic, literature data are still scarce. Nevertheless, many tellurium containing compounds have shown interesting antioxidant properties, and various types of therapy using tellurium compounds has been tried, for instance with the immunostimulant AS-101 (11).
2. Antioxidant design

2.1 General principles for antioxidant design

Already existing antioxidants in Nature would seem as good templates in the design of new ones. Many antioxidants used today, such as BHA and BHT are phenols structurally related to α-tocopherol, which is one of the best chain-breaking antioxidants known. It is generally recognized to act by hydrogen atom transfer (HAT) in the reduction of peroxyl radicals. However, electron transfer (ET) coupled with proton transfer (PT) or proton tunneling may have also serve as a mechanism for its action (Scheme 3). The major factor that influences the reaction rate by HAT is the homolytic bond dissociation enthalpy (BDE) of the O-H group. The OH bond strength in α-tocopherol and in phenols depends on the nature and number of aromatic substituents, where their effect is mainly electronic. Electron donating groups substituted ortho and para decrease the BDEOH and thereby increase reactivity while the opposite is true for electron withdrawing groups. The contribution of substituents is approximately additive.

The ED methyl groups in α-tocopherol stabilize the phenoxyl radical formed and thereby lower the OH BDE. This explains why α-tocopherol is more reactive than β-,γ- and δ-tocopherol which are lacking one or several methyls. However, too bulky alkyl groups in the vicinity of the hydroxyl group might lower the rate of HAT as they hinder the approach of the peroxyl radical. Moreover, the fused heterocyclic ring of the tocopherols also plays an important role. The 2p-type lone pair of the chromane oxygen adopts an orientation perpendicular to the aromatic ring plane and thereby stabilizes the developing phenoxy radical.

Scheme 3. Direct hydrogen atom transfer (HAT) and ET coupled PT.

When it comes to the rate of ET, the adiabatic ionisation potential (IP) of the antioxidant is an important factor. As electron donating substituents lower the IP it favours reaction via ET. However, if the IP is low enough the antioxidant might transfer a single electron to molecular oxygen and thus get
consumed. Both BDE and IP are therefore key parameters in the design of novel antioxidants.

Lipophilicity is also a design parameter. If we are aiming for antioxidant protection in an aqueous environment we should strive for low lipophilicity. On the contrary, if we go for novel stabilizers for polymeric materials, they should comply with the lipophilic environment present in materials such as polyethylene or polypropene.

Environmental aspects also put demands on the antioxidant structure. Substances that are predicted or chemically evaluated to be good candidates must meet the criteria of low toxicity to become ‘a promising lead antioxidant’ for further use in food or pharmaceutical applications. Stabilizers for polymers must also meet certain toxicity requirements if the plastic is to be used for manufacturing of medicinal articles or toys or if going to be contacted with foodstuffs or drinking water.

2.2 Improvements in retrospect

2.2.1 Chain-breaking antioxidants

Several structural modifications of the α-tocopherol skeleton have been done. Water soluble analogues of Vitamin E, such as Trolox (12) were evaluated for their scavenging effect of oxygen-derived free radicals. \[ \text{HO} \]

\[ \text{HO} \]

\[ \text{HO} \]

\[ \text{COOH} \]

\[ \text{12} \]

Trolox is nowadays used as a reference substance when evaluating aqueous phase antioxidants or complex antioxidant mixtures and Trolox Equivalent Antioxidant Capacity (TEAC) is often a method for comparison of activity. It is defined as the concentration of Trolox which has an equivalent antioxidant potential as a 1 mM solution of the compound investigated.

\[ \text{13} \]

\[ \text{HO} \]

\[ \text{HO} \]

\[ \text{COOH} \]

\[ \text{14} \]

Variations in the heterocyclic ring have also been frequently explored for improving antioxidant capacity. Ring-contraction to a five-membered structure 13 increased antioxidant capacity as compared with α-tocopherol. A more lipophilic analogue 14 with \textit{tert}-butyl groups flanking the phenolic moiety has been prepared as a drug for inhibition of lipid peroxidation.

The strategy to introduce electron donating (ED) groups to lower the OH BDE can only be perused until the IP will become so low that a spontaneous ET with molecular oxygen occurs. Substitution in the phenolic ring was a method found to circumvent this problem. Incorporation of nitrogen in the 3- and 5-positions (15) was predicted and shown to remarkably increase the IP.
while the increase in BDE was minimal.\textsuperscript{68,69} Other nitrogen heterocycles such as 3-pyridinol (16) were investigated for the same reason. Substitution with ED groups, for example compound 17, resulted in high antioxidant activity by way of lowering the BDE but with IPs high enough for air stability.\textsuperscript{69}

![Chemical structures](image)

Further introduction of methyls in the aromatic moiety of 3-pyridinol and an electron-donating amino group para to the hydroxyl group in a fused five-memered ring afforded the most powerful chain-breaking antioxidant 18 reported so far.\textsuperscript{70} The more vitamin E-like naphthyridinol (19) has also been prepared and evaluated.\textsuperscript{71}

![Chemical structures](image)

The chromanol ring oxygen of \(\alpha\)-tocopherol has also been a target for substitution. Sulfur is known to stabilize a neighbouring radical better than oxygen.\textsuperscript{72} The racemic sulphur analogue 20a of \(\alpha\)-tocopherol\textsuperscript{73} was prepared and evaluated. However, it turned out to be a slightly poorer hydrogen donor than the parent. Also, the all-racemic selenium analogue 20b was synthesised and found to have a slightly higher OH BDE than \(\alpha\)-tocopherol.\textsuperscript{74}

### 2.2.2 Preventive antioxidants

The interest in simple compounds that could mimic the action of the peroxide decomposing selenoprotein glutathione peroxidase (GPx) has increased over the years. Many low-molecular-weight organoselenums (e.g. benzoselenazolinones,\textsuperscript{75} bensoselenazinones,\textsuperscript{76} camphor-derived selenenamides,\textsuperscript{77} diaryl diselenides,\textsuperscript{78} cyclic seleninate esters and spirodioxselenuranes\textsuperscript{79}) have been synthesised for this purpose. One compound that gained early interest and has served as a benchmark for these studies is ebselen (21). In the presence of glutathione (GSH) the labile Se-N bond is cleaved and the resulting selenosulfide may undergo a similar series of transformations as was proposed for the action of the real enzyme (Scheme 2). Moreover, ebselen has been evaluated in clinical trials as a possible drug in the treatment of stroke.\textsuperscript{80}
Nature probably selected selenium because it is easily redox cycled. Organotellurium compounds are even better in this respect. This makes them interesting scavengers of all kinds of oxidizing species such as hydrogen peroxide, organic hydroperoxides, peroxynitrite, hypochlorite and ozone. The proposed mechanism shown in Scheme 4 involves rate-determining oxidation of the telluride followed by reduction of the resulting tellurium (IV)-species brought about by thiol. The tellurapyrylium dye 22 was the first organotellurium compound to be reported with GPx-like activity. After that, diorganyl ditellurides and diorganyl tellurides, such as cyclodextrin derivative 23, have been described as peroxide decomposing agents.

![Scheme 4. Proposed mechanism for diorganyl telluride catalyzed decomposition of oxidants.](image)

Synthetically prepared tellurocysteine (TeCys), with a lower redox potential than SeCys, also showed GPx-like activity when introduced into a protein.
2.3 Towards regenerable and multifunctional chalcogenide antioxidants

2.3.1 Regeneration of chalcogen containing antioxidants

Despite weak evidence for α-Toc regeneration by ascorbate in vivo, suppression of oxidation in liposomes was prolonged in the presence of ascorbate in the aqueous phase, and a synergistic behaviour was seen in homogenous phase. Other lipid soluble co-antioxidants such as α-tocopheryl hydroquinone, flavonoids, phenothiazines, catechols and phenols have also been reported to recycle α-tocopherol. A kinetic mechanism for such regeneration in homogenous solution has been suggested.

Water soluble agents other than ascorbate, such as the biologically active antioxidant glutathione, have been considered as co-reductant in regeneration of α-tocopherol. However, glutathione seems incapable of regenerating α-tocopherol in a lipid environment.

Some time ago, 2,3-dihydrobenzo[b]furan-5-ol (24a) and its 1-thio (24b), 1-seleno (24c) and 1-telluro (24d) analogues were assayed in a two-phase lipid peroxidation system (described in section 4.1.1). Interestingly, the selenium analogue 24c showed a remarkably long inhibition time in the presence of N-acetylcysteine (NAC, 25), a water soluble thiol. In contrast, α-tocopherol could not be regenerated by NAC in the system used. The all-racemic α-selenotocopherol (20b) could also not inhibit peroxidation more efficiently in the presence of NAC. This seems to indicate that the substitution pattern in the aromatic moiety could be crucial for regenerability.

\[
\begin{align*}
24a & \quad X = O \\
24b & \quad X = S \\
24c & \quad X = Se \\
24d & \quad X = Te \\
25 & \quad OCH_{2}SH
\end{align*}
\]

2.3.2 Present work

The following parts of the thesis are concerned with synthesis and evaluation of 2,3-dihydrobenzo[b]selenophene-5-ols and other regenerable antioxidants that could also cause decomposition of organic hydroperoxides and, thus, act in a multifunctional fashion. With antioxidant pharmacotherapy in mind, cytotoxicity and ability to quench ROS-production was studied in some detail with one of the compounds.
3. Synthesis

3.1 Synthesis of 2,3-dihydrobenzo[b]selenophene-5-ol antioxidants (Paper I)

In order to systematically investigate the catalytic properties of 2,3-dihydrobenzo[b]selenophene-5-ol antioxidants, an easy access to derivatives differently substituted in the aromatic moiety was sought for. Stefani and co-workers reported a seleno-Claisen rearrangement/intramolecular hydroselelation some time ago\(^9\) that turned out to be useful for the synthesis of the target Vitamin E analogues\(^\text{28}\). The allyl aryl selenides\(^\text{26}\) required could be prepared from commercially available methyl substituted 4-bromo-anisoles. In the synthesis of\(^\text{28}\), the appropriate 4-bromoanisole was converted to a Grignard reagent followed by insertion of elemental selenium to the carbon-magnesium bond, air oxidation, borohydride reduction of the diselenide formed and finally allylation of the resulting arneselenolates. After microwave-assisted seleno-Claisen rearrangement/ring-closure, affording 5-methoxy-substituted dihydroselenophenes\(^\text{27}\) final \(O\)-demetylation with\(\text{BBr}_3\) gave the phenolic target compounds\(^\text{28}\) (Scheme 5).

![Scheme 5](image)

Scheme 5. (a) (i) Mg, THF (heated at reflux); (ii) Se; (iii) air oxidation. (b) (i) \(\text{NaBH}_4\), EtOH, 0 °C, r.t. 30 min; (ii) allyl bromide, 0 °C, r.t. 30 min, 32-96 %. (c) Microwaves, quinoline, 220-230 °C, 13-59 %. (d) \(\text{BBr}_3\), CH\(_2\)Cl\(_2\), -78 °C, r.t. 20 h, 47-98 %.
In all, seven structurally different 2-methyl-2,3-dihydro-benzo[b]-
selenophene-5-ol antioxidants (28a-g) were prepared by using the synthetic
route described. With an interest to vary the substitution pattern in the vicin-
ity of selenium, a few analogues with alkyl groups in the 2-position were
prepared. Compounds 29a and 29b were synthesised according to Scheme 5
using 2-methyl-2-propene-1-yl bromide and (E/Z)-3-methyl-2-propen-1-yl
bromide, respectively, in the allylation step instead of allyl bromide.

The synthesis of compound 33, with a longer aliphatic chain next to sele-
nium required a somewhat different approach (Scheme 6). The alcohol 30
was generated from 2-bromo-5-methoxybenzyl bromide, via substitution of
the benzylic bromide using BuTeLi and lithium tellurium exchange to afford
a benzyllithium species that was trapped with 2-hexanone. Conversion of the
aryl bromide into the corresponding benzylseleno species set the scene for an
intramolecular homolytic substitution at chalcogen. The required tertiary
carbon-centered radical was obtained by using the Barton-Crich procedure.97
Final O-demethylation gave the phenolic analogue 33.

Scheme 6. (a) (i) n-BuTeLi; (ii) n-BuLi, -78 °C; (iii) 2-hexanone, -115 °C, 85 %. (b)
(i) t-BuLi, THF, -100 °C, r.t. 20 min; (ii) Se, -78 °C, r.t. 5 h; (iii) air oxidation; (iv)
NaBH₄, EtOH, 0 °C, 30 min, BnBr, 1 h, 43 %. (c) (i) Oxalyl chloride, benzene, 18 h;
(ii) 2-mercaptopyridine-N-oxide Na salt/DMAP, reflux, 34 %. (d) BBr₃, CH₂Cl₂, -78
°C, r.t., 20 h, 91 %.
In addition to the methyl substituted analogues 28a-g, the fluorine derivative 35 was prepared as an example of a compound carrying an electron withdrawing substituent in the phenolic ring (Scheme 7). Starting from commercially available 3-fluoro-4-hydroxybromobenzene, lithium-halogen exchange followed by selenium insertion, oxidation, borohydride reduction and allylation gave allyl 3-fluoro-4-hydroxyphenyl selenide (34) in a moderate yield. Seleno-Claisen rearrangement/intramolecular hydroselenation was then performed as described above.

Scheme 7. (a) (i) t-BuLi, THF, -78 °C; (ii) Se; (iii) air oxidation; (iv) NaBH₄, EtOH, 0 °C, r.t. 0.5 h; (v) Allyl bromide, 0 °C, r.t. 1h, 57 %. (b) Microwaves, quinoline, 220-230 °C, 18 %.

3.2 Synthesis of chalcogen containing pyridinol antioxidants (Papers III, IV and VII)

Lithium-halogen exchange, followed by chalcogen insertion, is a general method for introduction of chalcogens. As a result, halogenated 3-pyridinols were thought of as good starting materials for the antioxidants we had in mind. In order to facilitate purification (i.e. by extraction and column chromatography), lipophilic n-octyl bromide was initially used as an alkylating agent throughout. In the synthesis of pyridinols 37, 6-bromo-3-pyridinol 36, prepared from 2-bromo-5-amino-pyridine, was treated with 3 eq tert-BuLi to form the corresponding 6,0-dilithiated species. In the preparation of alkyl telluro compound 37a, finely ground elemental tellurium was added to form the corresponding lithium tellurolate, followed by air oxidation, which provided the corresponding ditelluride. Borohydride reduction followed by alkylation afforded 37a in 60 % yield. Readily available di-n-octyl diselenide and di-n-octyl disulfide, respectively, were added to the organolithium to produce selenium and sulphur analogues 37b and 37c (Scheme 8).
Scheme 8. (a) HBF₄ (50 % in water), NaNO₂, 0 °C 1h, reflux 5 h, 60 % (b) 3 eq. t-BuLi, THF, -78 °C. (c) 37a: (i) Te; (ii) air oxidation; (iii) NaBH₄, EtOH; (iv) n-octyl bromide, 66%. (d) 37b: Di-n-octyl diselenide, 65 %. (e) 37c: Di-n-octyl disulfide, 84 %.

By using a similar procedure, pyridinol compounds substituted with alkyl-chalcogeno groups ortho to the pyridinol group (38 a-c) were synthesized using 2-bromo-3-pyridinol as a starting material.

Telluride 39a, carrying an additional methyl group was prepared from lithium-halogen exchange according to Scheme 8 using commercially available 2-iodo-6-methyl-3-pyridinol as a starting material. Selenium and sulfur analogues 39b and 39c were prepared using a microwave-induced and copper-catalyzed coupling reaction (Scheme 9) as previously reported.ARYLchalcogeno derivatives 40a-c were also synthesized in line with this literature procedure.

Scheme 9. (a) 39b: Di-n-octyl diselenide, CuI, bipyridyl, Mg, DMF, microwaves, 200 °C, 6 h, 60 %. (b) 39c: Di-n-octyl disulfide, CuI, bipyridyl, Mg, DMF, microwaves, 200 °C, 6 h, 65 %.

Further introduction of electron donating methyl groups into the 3-pyridinol scaffold was synthetically more demanding. The desirable starting material
44 for preparation of compounds 45a–c were prepared in a short sequence of transformations from commercially available 3-cyano-4,6-dimethyl-2-pyridinol (Scheme 10).

\[
\begin{align*}
\text{CN} & \xrightarrow{a} \text{CN} & \text{CN} & \xrightarrow{b} \text{NH}_2 & \xrightarrow{c} \text{NH}_2 \\
\text{OH} & \text{Br} & \text{OH} & \text{Br} & \text{Br} \\
41 & 42 & 43 & \\
\end{align*}
\]

Scheme 10. (a) Bu₄NBr, P₂O₅, toluene, reflux, 14 h, 91 %. (b) H₂SO₄, 100 °C, 3 h, 68 %. (c) KOH, Br₂, 84 %. (d) HBF₄ (in water), NaNO₂, 0 °C 1 h, reflux 5 h, 40 %.
(e) 45a: (i) t-BuLi, THF, -78 °C; (ii) Te; (iii) air oxidation; (iv) NaBH₄, EtOH, (v) n-Octyl bromide, 44 %. (f) 45b: (i) t-BuLi, THF, -78 °C; (ii) di-n-octyl diselenide, 60 %.
(g) 45c: (i) t-BuLi, THF, -78 °C; (ii) Di-n-octyl disulfide, 35 %.

Reaction with tetrabutylammonium bromide and phosphorous pentoxide in refluxing toluene afforded the 2-brominated cyanopyridine 41 in 91 % yield. Subsequent hydrolysis of nitrile to amide occurred in concentrated sulfuric acid to give 42 in 68 % yield. Following Hoffman rearrangement to give amine 43, diazotization in water furnished 2-bromo-4,6-dimethyl-3-pyridinol (44). Lithium-halogen exchange in line with chemistry described in Scheme 8 afforded the alkyl pyridyl chalcogenides 45a-c (Scheme 10).

To probe the effect of a further increase in the electron density on chalcogen an additional para-methoxy group was desired. Prepared according to literature, 99 3-cyano-5-methoxy-4,6-dimethyl-2-pyridinol (46) served as a starting material that could be transformed in analogy with Scheme 10 into compounds 47a and 47b with approximately the same yields.

\[
\begin{align*}
\text{MeO} & \text{CN} & \text{OH} & \text{X-n-Octyl} \\
\text{46} & 47a & 47b \ 
\end{align*}
\]

The relative positioning of pyridinol and chalcogen groups appeared to be an issue worth investigating. It occurred to us that amide 48, formed en route to compounds 47 could be hydrodeaminated (diaziotization followed by hypo-
phosphorous acid-induced reduction) and O-demethylated (BBr₃) to provide a starting material 49 suitable for conversion to compounds 50 (Scheme 11).

![Scheme 11](image)

Scheme 11. (a) (i) HNO₂/H₃PO₂, 37 %; (ii) BBr₃, -78 °C, 78 %. (b) (i) t-BuLi, THF; -78°C; (ii) Te; (iii) air oxidation; (iv) NaBH₄, EtOH; (v) n-Octyl bromide, 47%. (c) 50b: (i) t-BuLi, THF, -78 °C; (ii) Di-n-octyl diselenide, 54 %. (d) 50c: Di-n-octyl disulfide, 64 %.

To investigate the antioxidant mechanism, the O-methylated analogue 52 of compound 45a, unable to act as hydrogen donor, was synthesized. O-Methylation of 44 occurred on treatment with methyl iodide and base and conversion of bromide 51 to the desired telluride 52 involved lithiation followed by treatment with di-n-octyl ditelluride (Scheme 12).

![Scheme 12](image)

Scheme 12. (a) K₂CO₃, DMF, MeI, 100 %. (b) t-BuLi, THF, -78°C; (ii) Di-n-octyl ditelluride, 57 %.

As divalent organotellurium compounds rapidly get oxidized to the tetravalent state, the antioxidant capacity of some telluroxides was interesting to study. Compounds 38a and 45a were therefore treated with a slight excess of tert-butyl hydroperoxide in methanol to give the corresponding telluroxides 53 and 54.

![Scheme](image)
After evaporation of all volatiles, the final products were difficult to characterize. $^1$H NMR spectra recorded using CDCl$_3$ as solvent showed broad peaks which could indicate exchange or oligomerization processes. Mass spectral analysis also was not satisfactory. However, the polar telluroxide products always returned the corresponding alkyl pyridyl tellurides quantitatively upon reduction with sodium disulfite.

In order to vary the lipophilicity of our antioxidants, compounds $^{55a-c}$ were prepared from $^{44}$ in line with chemistry described in Scheme 10 using different alkylating agents (Scheme 13).

$$
\begin{align*}
44 & \xrightarrow{\text{a}} 55a & R = \text{Ethyl} \\
& & \text{b } R = \text{Pentyl} \\
& & \text{c } R = \text{Cetyl}
\end{align*}
$$

Scheme 13. (a) (i) t-BuLi, THF, -78 °C; (ii) Te; air oxidation; (iii) NaBH$_4$, EtOH; (iv) $^{55a}$: Ethyl bromide, 43 %. $^{55b}$: n-Pentyl bromide, 22%. $^{55c}$: n-Cetyl bromide, 29 %.

3.3 Improving on a commercial antioxidant (Papers V and VI)

In commercially available BHA, 3-tert-butyl-4-hydroxyanisole is the major component. Prepared according to literature, 100 bromophenol $^{56}$ was thought to be a good starting material for preparation of chalcogen analogues $^{57a-c}$ via lithiation and reaction with the corresponding di-n-alkyl dichalcogenides (Scheme 14).
The O-methylated tellurium containing BHA-analogue 59 was prepared by first reacting 56 with methyl iodide in DMF (Scheme 15). Surprisingly, alkylation product 58 could only be obtained if methyl iodide was present when the base was added. The reversed order of addition led to rapid degradation of the halogenated phenol.

Further treatment of 58 with two equivalents of tert-BuLi, addition of finely ground tellurium powder followed by air-oxidation, provided the corresponding diaryl ditelluride. Borohydride reduction and alkylation with n-octyl bromide finally gave the telluride 59 in 33 % yield.
4. Evaluation

4.1 Antioxidant profile of novel antioxidants

Several methods and techniques were used in the evaluation of the synthesized compounds. Two systems were used more frequently and also served for initial screening of catalytic properties of antioxidants. A shorter description of these is given below before further interpretations and discussions.

4.1.1 The two-phase lipid peroxidation model

A two-phase (chlorobenzene/water) lipid peroxidation system earlier developed in our group\(^9\) was used to study regeneration of antioxidants across an aqueous-lipid interphase (Figure 2). The compound to be investigated was added to a stirred solution of linoleic acid in chlorobenzene at 42 °C and peroxidation initiated with 2,2’-azobis(2,4-dimethylvaleronitrile) (AMVN). A stoichiometric amount of a reducing agent (\(N\)-acetylcysteine) was contained in the aqueous phase. Autosampling of the organic phase and analysis for linoleic acid hydroperoxide (conjugated diene absorbing at 234 nm) was done every 10 min. Only a very slight increase in the concentration of conjugated diene is observed with efficient antioxidants, whereas the increase is substantial if the reaction proceeds uninhibited. This is also seen when the antioxidant is all consumed. The time from the start of the experiment until peroxidation proceeds with an uninhibited rate, is defined as \(T_{\text{inh}}\). Plotting concentration of conjugated diene versus time gives the inhibited rate of peroxidation, \(R_{\text{inh}}\), as the slope of the line.

Regenerability and efficiency of antioxidants prepared was studied by recording \(T_{\text{inh}}\) and \(R_{\text{inh}}\) in the absence/presence of the thiol reducing agent in the aqueous phase. A good regenerable antioxidant inhibits peroxidation
with a low $R_{inh}$ and a high $T_{inh}$ in the presence of NAC. A typical peroxidation trace using $\alpha$-tocopherol is presented in Figure 3.

Figure 3. Typical peroxidation trace (linoleic acid hydroperoxide concentration vs time) recorded using $\alpha$-tocopherol.

4.1.2 Inhibition studies in homogeneous phase

In addition to the two-phase lipid peroxidation system, several compounds were also evaluated in homogenous phase. The capacity to inhibit autoxidation of styrene in chlorobenzene (or acetonitrile) at 30°C (303 K), initiated with azobis(isobutyronitrile) (AIBN) was investigated. Oxygen consumption with time was studied and reactions (initiation, propagation and termination steps) and rate constants involved are shown in equations 1-6.56

\[
\begin{align*}
\text{initiator} & \xrightarrow{R_i} R^* & \text{(eq. 1)} \\
R^* + O_2 & \xrightarrow{k_{p}} ROO^* & \text{(eq. 2)} \\
ROO^* + RH & \xrightarrow{2k_i} \text{ROOH} + R^* & \text{(eq. 3)} \\
ROO^* + ROO^* & \xrightarrow{k_{inh}} \text{products} & \text{(eq. 4)} \\
ROO^* + ArOH & \xrightarrow{k_{inh}} \text{ROOH} + \text{ArO}^* & \text{(eq. 5)} \\
ROO^* + ArO^* & \xrightarrow{} \text{products} & \text{(eq. 6)}
\end{align*}
\]

From the experimental data, the absolute rate constant for reaction of an antioxidant with peroxy radicals, $k_{inh}$, can be calculated (eq 5). From the length of the inhibition period, the stochiometric factor $n$ (the number of peroxy radicals trapped by each molecule of antioxidant) can be obtained. Many phenolic compounds (such as $\alpha$-tocopherol), can trap two peroxy
radicals before they are converted to non-radical products \((n = 2)\). A typical oxygen uptake kinetic trace for \(\alpha\)-tocopherol is shown in Figure 4.

![Typical oxygen uptake kinetic trace during homogenous phase autoxidation of styrene in chlorobenzene inhibited by \(\alpha\)-tocopherol.](image)

**Figure 4.** Typical oxygen uptake kinetic trace during homogenous phase autoxidation of styrene in chlorobenzene inhibited by \(\alpha\)-tocopherol.

### 4.2 Exploring the antioxidant capacity of 2,3-dihydrobenzo[\(b\)]selenophene-5-ol antioxidants (Papers I and II)

#### 4.2.1 Inhibition studies in the two-phase model

When assayed in the two-phase lipid peroxidation system the majority of compounds 28 were regenerable by \(N\)-acetylcysteine to some extent as can be seen from the prolonged inhibition times recorded in the presence of the thiol (Table 1).
Table 1. Inhibition times ($T_{inh}$) and inhibited rates of peroxidation ($R_{inh}$) for compounds 28a-g in the absence and presence of NAC in the aqueous phase (uninhibited rate is ca 650 μM/h).

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>$R_{inh}$ (μM/h)</th>
<th>$T_{inh}$ (min)</th>
<th>With NAC in the aqueous phase</th>
<th>$R_{inh}$ (μM/h)</th>
<th>$T_{inh}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image.png" alt="Image of 28a" /></td>
<td>46</td>
<td>50</td>
<td>69</td>
<td>&gt;320</td>
<td></td>
</tr>
<tr>
<td><img src="image.png" alt="Image of 28b" /></td>
<td>31</td>
<td>50</td>
<td>44</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td><img src="image.png" alt="Image of 28c" /></td>
<td>43</td>
<td>60</td>
<td>51</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td><img src="image.png" alt="Image of 28d" /></td>
<td>25</td>
<td>60</td>
<td>30</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td><img src="image.png" alt="Image of 28e" /></td>
<td>29</td>
<td>50</td>
<td>34</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td><img src="image.png" alt="Image of 28f" /></td>
<td>29</td>
<td>50</td>
<td>39</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td><img src="image.png" alt="Image of 28g" /></td>
<td>26</td>
<td>50</td>
<td>30</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>24 ± 2</td>
<td>80</td>
<td>24 ± 2</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

By traversing the series of compounds evaluated, it was clear that the methyl substitution pattern in the aromatic moiety is of vast importance for the catalytic performance. Interestingly, compound 28a lacking methyl groups inhibited peroxidation for more than 320 min in the presence of thiol and therefore outperformed the reference α-tocopherol ($T_{inh} = 80$ min)(Figure 5). The oxidation of NAC to the corresponding disulfide during the inhibited period was confirmed by sampling and analysis of the aqueous phase. Introduction of methyls ortho and meta to the hydroxyl group (28b, 28c) markedly decreased the inhibition time ($T_{inh} = 230$ and 190 min) as compared to the parent. The inhibition times continued to decrease upon further substitu-
tion with methyl groups (28d $T_{inh} = 80$ min, 28e $T_{inh} = 130$ min; 28f $T_{inh} = 130$ min). The fully methylated antioxidant 28g could not be regenerated by N-acetylcysteine under the experimental conditions (as observed previously for its selenium analogue, selenotocopherol).

![Figure 5](image.png)

Figure 5. Peroxidation trace (linoleic acid hydroperoxide concentration vs time) recorded for compound 28a and α-tocopherol in the presence of NAC in the aqueous phase.

Table 2. Inhibition times ($T_{inh}$) and inhibited rates of peroxidation ($R_{inh}$) for compounds 29a, 29b, 33 and 35 in the absence and presence of NAC in the aqueous phase (uninhibited rate is ca 650 μM/h).

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>$R_{inh}$ (μM/h)</th>
<th>$T_{inh}$ (min)</th>
<th>With NAC in the aqueous phase</th>
<th>$R_{inh}$ (μM/h)</th>
<th>$T_{inh}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image.png" alt="29a" /></td>
<td>28</td>
<td>60</td>
<td></td>
<td>29</td>
<td>100</td>
</tr>
<tr>
<td><img src="image.png" alt="29b" /></td>
<td>30</td>
<td>60</td>
<td></td>
<td>35</td>
<td>80</td>
</tr>
<tr>
<td><img src="image.png" alt="33" /></td>
<td>45</td>
<td>70</td>
<td></td>
<td>59</td>
<td>&gt;320</td>
</tr>
<tr>
<td><img src="image.png" alt="35" /></td>
<td>51</td>
<td>70</td>
<td></td>
<td>93</td>
<td>300</td>
</tr>
</tbody>
</table>
Structural variations in position 2 (compounds 29a and 29b) did not affect catalytic performance much (Table 2). Compound 33, substituted with an additional butyl group in the 2-position but otherwise identical to compound 28a was still highly regenerable in presence of NAC ($T_{\text{inh}} > 320$ min). Fluorine derivative 35, due to the electron withdrawing substituent, was slightly less regenerable ($T_{\text{inh}} = 300$ min) than the parent 28a.

The majority of the organoselenium antioxidants were poorer quenchers of peroxy radicals than $\alpha$-tocopherol with $R_{\text{inh}}$-values exceeding $R_{\text{inh}} = 24 \pm 2$ recorded for the natural antioxidant. Furthermore, in the absence of thiol, they inhibited peroxidation for shorter times than $\alpha$-tocopherol. Thus, their stoichiometric factors are less than 2 ($n < 2$).

### 4.2.2 Inhibition studies in homogeneous phase

From the two-phase peroxidation experiments performed, antioxidants 28a and 28g were selected as candidates for further evaluation in homogenous phase. In accord with the two-phase peroxidation experiments, compound 28a ($k_{\text{inh}} = 3.8 \pm 0.4 \times 10^5$) was found less reactive towards peroxy radicals than 28g ($k_{\text{inh}} = 1.5 \pm 0.3 \times 10^6$) (Table 3). The stoichiometric factors where found to be 2 and 1.7 respectively. The lower value for 28g is believed to be due to spontaneous air-oxidation of the compound during handling.

Table 3. Rate constants, $k_{\text{inh}}$, for the reaction of compounds 28a and 28g with peroxy radicals in styrene/chlorobenzene at 30°C (303 K) and the number of radicals trapped, $n$, by each antioxidant molecule. $\alpha$-Tocopherol is included as reference.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>$k_{\text{inh}}$ (M$^{-1}$s$^{-1}$)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>28a</td>
<td>$3.8 \pm 0.4 \times 10^5$</td>
<td>2.0</td>
</tr>
<tr>
<td>28g</td>
<td>$1.5 \pm 0.3 \times 10^6$</td>
<td>1.7</td>
</tr>
<tr>
<td>$\alpha$-tocopherol</td>
<td>$3.2 \times 10^6$</td>
<td>2.0</td>
</tr>
</tbody>
</table>

### 4.2.2.1 Catalysis in homogenous phase

In order to explore regeneration by thiol in homogenous phase, variable amounts of lipophilic 1-octylmercaptan were added up to 160 times the concentration of antioxidant. However, no catalytic activity was found and oxygen consumption traces remained the same as recorded for experiments without thiol.

### 4.2.3 DFT calculations, OH BDEs and lipophilicity

To get a deeper insight into the catalytic process, bond dissociation energies (BDEs) as well as adiabatic ionization potentials (IPs) were calculated. DFT models for such predictions have been reported, but are not directly applicable to antioxidants containing heavy chalcogens such as selenium or tellurium. Therefore, calculations were performed by utilizing a more convenient
(RO)B3LYP/LANL2DZdp//B3LYP/LANL2DZ model previously developed in our group.\textsuperscript{102}

As expected, BDEs decreased within the series of compounds investigated with increasing methyl substitution in the aromatic moiety. Also, IPs were found to decrease in a similar fashion when traversing the series from \textit{28a} to \textit{28g} (Table 4). Both IPs and BDE\textsubscript{OH} values showed a linear correlation when plotted against inhibited rates of peroxidation.

Table 4. Calculated BDE\textsubscript{OH} and IP-values for antioxidant \textit{28a} and \textit{28g}. Experimental BDE\textsubscript{OH} measured by radical equilibration EPR in benzene at 25 ºC (298 K) with \textalpha-tocopherol included as a reference.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>BDE\textsubscript{OH} (kcal/mol)\textsubscript{calc}</th>
<th>IP (kcal/mol)\textsubscript{calc}</th>
<th>BDE\textsubscript{OH} (kcal/mol)\textsubscript{exp}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{28a}</td>
<td>80.8</td>
<td>163.2</td>
<td>81.6\textsuperscript{a}</td>
</tr>
<tr>
<td>\textit{28g}</td>
<td>76.3</td>
<td>156.0</td>
<td>77.6 ± 0.5</td>
</tr>
<tr>
<td>\textalpha-tocopherol</td>
<td>-</td>
<td>-</td>
<td>77.15 ± 0.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Estimated value.

To confirm calculated BDE values for compounds \textit{28a} and \textit{28g} experimentally, a radical EPR equilibration technique was used. The equilibrium constant (K\textsubscript{e}) for the hydrogen atom transfer (HAT) between the compound (ArOH), a reference phenol (Ar´OH, in this case 2,6-di-\textalpha-tert-buty-4-methoxyphenol) and the corresponding phenoxyl radicals was measured. (Scheme 16).

\[
\text{ArOH} + \text{Ar}´\text{O}^\cdot \xrightleftharpoons{}^{K_e} \text{ArO}^\cdot + \text{Ar}´\text{OH}
\]

Scheme 16. HAT equilibrium between two phenols and their corresponding phenoxyl radicals.

Compound and reference were dissolved in benzene containing di-\textalpha-tert-butyl peroxide and radicals were generated by photolysis. BDE\textsubscript{OH} values were calculated from eq. 7 with the known BDE\textsubscript{OH} of the reference phenol as 77.2 kcal/mol.

\[
\text{BDE(ArO-H)} \approx \text{BDE(Ar}´\text{O-H)} – RT \ln (K_e) \quad \text{(eq. 7)}
\]

The experimental BDE\textsubscript{OH} of compound \textit{28g} was found to be 77.6 ± 0.5 kcal/mol which was ~ 1.3 kcal higher than the calculated value. The EPR equilibration technique requires a relatively high persistence of radicals to give accurate results. This was not the case with compound \textit{28a}. Estimating from \textit{28g}, two \textit{ortho} methyl groups would add 3.5 kcal/mol to the BDE\textsubscript{OH} and a \textit{meta} methyl another 0.5 kcal/mol.\textsuperscript{60} Therefore, BDE\textsubscript{OH} for \textit{28a} would be 81.6 kcal/mol, a value that correlates reasonably well with the calculated value of 80.8 kcal/mol.
4.2.4 Mechanisms of catalysis

Radical quenching would be facilitated by an increasing number of aromatic methyl groups, as observed, whether the mechanism involves HAT, proton coupled electron transfer (PCET)\textsuperscript{103} or proton tunneling.\textsuperscript{59} In order to find out about this we included the O-methylated compound \textsuperscript{27} (unsubstituted in the aromatic moiety) in the study. No antioxidant activity whatsoever was found in the two-phase system, indicative of initial phenoxyl radical formation. Selenoxides were considered as intermediates in catalysis. They would be readily reduced by NAC. However, if selenoxides form by electron transfer (ET), a low IP would be expected for good antioxidants. By calculation, the inactive precursor \textsuperscript{27} was found to have a 3.8 kcal lower IP than \textsuperscript{28a}. Thus, direct ET does not seem to be involved in the catalytic mechanism.

One could also speculate that selenoxides form via further oxidation of phenoxyl radical and reaction of the resulting selenonium ion with water (Scheme 17). However, this process would also be facilitated by electron donating substituents which is not in line with the results.

![Scheme 17. Formation of a selenoxide as an intermediate in the catalytic process.](image)

For direct HAT to phenoxyl radical to occur, the BDE of the co-reductant should generally be equal to or below that of the catalyst.\textsuperscript{92} Literature data for S-H BDEs (BDE\textsubscript{SH}) are however rare and the reported works describe very system specific results. One could assume that the BDE\textsubscript{SH} of N-acetylcysteine would be in the range 85-90 kcal/mol,\textsuperscript{104} significantly higher than the calculated values for compounds \textsuperscript{28a-g}. This would make hydrogen atom transfer from thiol highly unlikely to be involved in the regeneration process. The α-C-H bond of NAC would in fact be weaker due to captodative stabilization. The corresponding α-C-H bond has been estimated to be 82.7 ± 2.4 kcal/mol for cysteine in proteins,\textsuperscript{105} and is indeed closer to the BDEs of compounds \textsuperscript{24a-d} (BDE\textsubscript{OH} = 81.3, 80.5, 80.3, 80.5 respectively)\textsuperscript{95} and also \textsuperscript{28a}. Nevertheless, it would not explain the prolonged inhibition time found for the selenium analogue \textsuperscript{24c} within the former series of compounds, unless another mechanism than HAT is involved.

In line with the above reasoning it is also not strange that 1-octylmercaptan was not able to regenerate the catalysts in homogenous phase. Assuming a BDE\textsubscript{SH} similar to the 88.7 kcal/mol determined for ethanethiol,\textsuperscript{106} the values are approximately 7 and 10 kcal/mol higher, respectively, than the calculated BDE\textsubscript{OH} for \textsuperscript{28a} and \textsuperscript{28g}. Having evaluated other possibilities, we therefore felt inclined to speculate that electron trans-
fer or proton coupled electron transfer could be involved in the catalytic mechanism as outlined in Scheme 18. Such processes may be favored in polar solvents such as water.

Scheme 18. Proposed mechanism involving ET from thiol to phenoxyl radical followed by proton transfer and dimerization of thiyl radicals.

Considering that we are using a two-phase system for evaluation of our antioxidants, parameters such as hydrophilicity may also be important. In fact, while compounds are becoming less and less regenerable as we traverse Table 1, lipophilicity increases.

4.2.5 Antioxidant capacity in aqueous solution

Among catalysts screened in the two-phase system, compound 28a was also evaluated in an 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS\(^{+}\)) assay to get a rough idea about its radical scavenging ability in an aqueous environment.

Figure 6. Absorbance (734 nm) of the ABTS\(^{+}\)-radical cation with time after addition of compound 28a, sodium ascorbate, Trolox and N-acetylcysteine.
The capacity of compound 28a to quench the relatively stable ABTS⁺-radical cation was compared in this study with sodium ascorbate, Trolox and N-acetylcysteine as three water soluble reference substances (Figure 6). With a Trolox Equivalent Antioxidant Capacity (TEAC)-value of 1.6, 28a outperformed both Trolox and sodium ascorbate and is therefore a potent antioxidant also in aqueous solution. TEAC is defined as the concentration of Trolox which has an equivalent antioxidant potential as a 1 mM solution of the investigated compound.66

4.2.6 Inhibition of ROS-production and toxicity

4.2.6.1 Inhibitory effects on ROS Production

Compound 28a was further investigated for its capacity to quench ROS produced by human neutrophils and Phorbol Myristate Acetate (PMA)-stimulated THP-1 cells. Trolox and ascorbic acid were once again used as references. Modified luminol enhanced chemiluminescence (CL) was used to measure the total ROS production (i.e. both intra- and extra-cellular production).

Figure 7. CL measurements in counts per second (CPS) for freshly isolated human neutrophils exposed to compound 28a at 20, 40 and 60 μM concentrations respectively in comparison to control (Hanks balanced salt solution, HBSS)

Selenide 28a was shown to quench/inhibit ROS production from human neutrophils dose-dependently in a concentration range of 20-60 μM (Figure 7). Thus, it is more efficient than Trolox, the reference compound used (Figure 8).
Figure 8. CL measurements in counts per second (CPS) for freshly isolated human neutrophils exposed to compound 28a and Trolox at 20 μM concentrations.

PMA-stimulated THP-1 cells were used in order to investigate the capacity of 28a to quench ROS production over a longer time. As observed with neutrophils, compound 28a dose-dependently quenched/inhibited ROS production and outperformed Trolox at equal concentrations (Figures 9 and 10).

Figure 9. CL measurements in relative light units (RLU) for PMA-stimulated THP-1 cells exposed to compound 28a at various concentrations (1, 10 and 20 μM). Hanks balanced salt solution (HBSS) was used as control.
4.2.6.2 Cell viability and cytotoxicity

Using Almar Blue as a metabolic indicator for viable cells, relative cell growth patterns for five human cell types (MG-63, HEK-293, SHSY-5y, MRC-5 and CaCo-2) exposed to compound 28a at a 60 μM concentration were evaluated over a period of 7 days. Trolox was used as a reference. No sign of disruption in cellular proliferation could be seen during the period of exposure for any of the antioxidants in comparison to medium control (Figure 11).

As a complement to the cell viability study, release of lactate dehydrogenase (LDH) as a result of cell damage or cell death was monitored during the same period of time. No significant indication of toxicity for any of the cell types could be observed for antioxidant 28a (Figure 12).

Figure 10. CL measurements in relative light units (RLU) for PMA-stimulated THP-1 cells exposed to compound 28a and Trolox at 20 μM concentrations.

Figure 11. Relative viability of 5 human cell types (MG-63, HEK-293, SHSY-5y, MRC-5 and CaCo-2) exposed to 28a (60 μM) and Trolox (60 μM). Almar Blue measurements (570 nm) were made after 1, 3 and 7 days.
We therefore feel antioxidant 28a could be a good candidate for future drug development for prevention of disorders related to or caused by radical-mediated tissue damage.

4.3 Evaluation of chalcogen containing 3-pyridinol antioxidants (Papers III, IV and VII)

4.3.1 Inhibition studies in the two-phase model

A comparison of 3-pyridinols substituted in the 2-position with octylthio, octylseleno and octyltelluro groups clearly showed an increase in regenerability in the two-phase model as one traverses the periodic table. The sulfur and selenium analogues were usually poor quenchers of peroxyl radicals with $R_{\text{inh}}$-values ranging from 45 μM/h for the most efficient, electron-rich quencher 47b, to mediocre 328 μM/h for compound 40c (Table 5).

All alkyltelluro substituted pyridinols failed to inhibit peroxidation of linoleic acid in the absence of thiol ($T_{\text{inh}} = 0$ min) but were active when NAC was added to the water layer. This is because tellurium is oxidized to the tetravalent state by residual linoleic acid hydroperoxides always present in commercial samples of linoleic acid and telluroxides are very poor chain-breaking antioxidants. The $O$-methylated compound 52 inhibited peroxidation for 60 min in the presence of NAC. As HAT is not possible, chain-breaking is more likely to proceed via ET, and the resulting radical cation formed is probably not efficiently reduced by thiol. Consumption of NAC was monitored during the inhibited phase of peroxidation using catalyst 45a by sampling of the aqueous phase using reversed phase HPLC. Oxidation to the corresponding disulfide was the only ongoing process.
Table 5. Inhibition times ($T_{inh}$) and inhibited rates of peroxidation ($R_{inh}$) for compounds 37, 38, 39, 40, 45, 47, 50 and 52 in the absence and presence of NAC in the aqueous phase. α-Tocopherol is included as a reference.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Chalcogen</th>
<th>$R_{inh}$ (μM / h)</th>
<th>$T_{inh}$ (min)</th>
<th>With NAC in the aqueous phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>a X = Te</td>
<td>542</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>b X = Se</td>
<td>119</td>
<td>100</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>c X = S</td>
<td>121</td>
<td>110</td>
<td>115</td>
</tr>
<tr>
<td>38</td>
<td>a X = Te</td>
<td>726</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>b X = Se</td>
<td>152</td>
<td>90</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>c X = S</td>
<td>115</td>
<td>120</td>
<td>203</td>
</tr>
<tr>
<td>39</td>
<td>a X = Te</td>
<td>652</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>b X = Se</td>
<td>57</td>
<td>80</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>c X = S</td>
<td>68</td>
<td>90</td>
<td>45</td>
</tr>
<tr>
<td>40</td>
<td>a X = Te</td>
<td>627</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>b X = Se</td>
<td>85</td>
<td>80</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>c X = S</td>
<td>166</td>
<td>110</td>
<td>328</td>
</tr>
<tr>
<td>45</td>
<td>a X = Te</td>
<td>600</td>
<td>0</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>b X = Se</td>
<td>58</td>
<td>70</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>c X = S</td>
<td>99</td>
<td>70</td>
<td>88</td>
</tr>
<tr>
<td>47</td>
<td>a X = Te</td>
<td>479</td>
<td>0</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>b X = Se</td>
<td>54</td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>50</td>
<td>a X = Te</td>
<td>490</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>b X = Se</td>
<td>78</td>
<td>70</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>c X = S</td>
<td>78</td>
<td>70</td>
<td>82</td>
</tr>
<tr>
<td>52</td>
<td></td>
<td>677</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td></td>
<td>24 ± 2</td>
<td>80</td>
<td>24 ± 2</td>
</tr>
</tbody>
</table>

To our surprise, 3-pyridinols carrying octyltelluro groups ortho instead of para to the hydroxyl group were more regenerable and more potent quench-
ers of peroxyl radicals. Compound 39a could match α-tocopherol when it comes to inhibition but the inhibition time exceeded 360 minutes. Further introduction of electron-donating substituents improved both regenerability and the capacity to inhibit peroxidation of linoleic acid.

As can be seen in Figure 13, α-tocopherol (R_{inh} = 24 ± 2, T_{inh} = 90) is outperformed in all respects by organotelluriums 45a and 47a (R_{inh} = 8 ± 2, T_{inh} > 400 min). Due to the tendency of the para-methoxy-substituted 47a to decompose more rapidly during handling and storage, 45a was used for further comparison, analysis and evaluation in homogenous phase.

![Figure 13. Peroxidation trace (linoleic acid hydroperoxide concentration vs time) recorded for compounds 45a and 47a and α-tocopherol in the presence of NAC in the aqueous phase.](image)

4.3.2 Inhibition studies in homogeneous phase

In homogeneous phase, all three organotelluriums 37a, 38a and 45a inhibited autoxidation of styrene in chlorobenzene in the absence of a thiol reducing agent. Pyridinols carrying the octyltelluro group ortho to the pyridinol where found to quench peroxyl radicals approximately three times faster than α-tocopherol. However, the stociometric factor, n, was much lower than the ideal value of 2 recorded for α-tocopherol (Table 6).
Table 6. Rate constants, $k_{inh}$, for the reaction of 37a, 38a, 45a, 53 and 54 with peroxyl radicals in styrene/chlorobenzene or acetonitrile at 30 °C (303 K) with α-tocopherol included as a reference.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>chlorobenzene</th>
<th>acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{inh}$ (M$^{-1}$s$^{-1}$)</td>
<td>$n$</td>
</tr>
<tr>
<td>37a</td>
<td>$(7.5 \pm 0.9) \times 10^5$</td>
<td>$0.3 \pm 0.1$</td>
</tr>
<tr>
<td>38a</td>
<td>$(1.0 \pm 0.3) \times 10^7$</td>
<td>$0.5 \pm 0.1$</td>
</tr>
<tr>
<td>45a</td>
<td>$(9.2 \pm 2.0) \times 10^6$</td>
<td>$0.4 \pm 0.1$</td>
</tr>
<tr>
<td>53</td>
<td>$&lt; 100$</td>
<td>$&lt; 100$</td>
</tr>
<tr>
<td>54</td>
<td>$&lt; 100$</td>
<td>$&lt; 100$</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>$3.2 \times 10^6$</td>
<td>2.0</td>
</tr>
</tbody>
</table>

This observation strongly suggests that the reaction with peroxyl radicals occurs in a different manner than observed for phenols (or pyridinols). Formation of telluroxides, inactive as judged from the two-phase model, could be an explanation. Telluroxides 53 and 54 (corresponding to 38a and 45a) were therefore studied and their poor chain-breaking activity ($k_{inh} < 100$) documented.

The octyltelluro group had a dramatic effect on the antioxidant capacity of pyridinols. Thus, compound 45a was approximately 400 times more reactive than the corresponding methyl substituted analogue. This indicates that the chalcogen analogues react by a different mechanism somehow related to the heteroatom present. Although the alkyltelluro group may be electron donating, thereby lowering the BDEOH, it would not explain the higher reactivity of the ortho substituted 38a and 45a as compared to the para substituted 37a and 47a.

### 4.3.2.1 Catalysis in homogenous phase

When catalytic performance of octyltelluro pyridinol 45a was studied in homogenous phase, simple alkylmercaptans failed to increase inhibition times. However, when N-tert-butoxycarbonyl cystein methyl ester (LipCys), a lipid-soluble analogue of NAC, was used the inhibition period could be extended. In fact, it was found to vary linearly with the amount of added thiol. Less substituted pyridinol 38a was also found to be regenerable under these conditions and the corresponding telluroxide 53 gave almost identical oxygen consumption traces when LipCys was present. In support of a catalytic mechanism involving telluroxides, addition of thiol after all antioxidant was consumed made it active again. In contrast, para analogue 37a was not as efficiently regenerated and higher concentrations of LipCys were required in order to extend the inhibition time.

To get an idea of the catalytic efficiency of these antioxidants with respect to thiol consumption, $n$(SH), the number of radical chains that is interrupted by each molecule of thiol, was determined. Values of compounds 37a, 38a,
45a, 53 and 54 were in the range of ~ 0.1-0.4. This is an indication that thiol is also consumed in processes that do not involve chain-termination.

4.3.3 Mechanisms of catalysis

Ortho-substituted octyltelluro pyridinols were always found to be more reactive than the corresponding para substituted isomers when evaluated in homogenous phase. They were also more regenerable in the presence of thiol both under two-phase and homogenous phase conditions. Changing the solvent from apolar chlorobenzene to polar and hydrogen-bonding acetonitrile lowered the reactivity of the compounds (Table 6), suggesting that the reactions proceed by HAT or PCET. Telluroxides are clearly involved in the catalytic mechanism. They form initially by oxidation of residual hydroperoxide present in the oxidizable substrate but they may also form during the course of peroxidation via oxygen atom transfer from peroxy radical formed. In fact, competing HAT and oxygen transfer processes may explain the different antioxidant characteristics of ortho and para-substituted octyltelluro pyridinols as shown in Schemes 19 and 20. Whether or not thiol is present, pathway A describes the HAT mechanism for quenching of peroxyl radicals by phenolic and pyridinolic compounds. Pathway B describes oxygen atom transfer to tellurium, resulting in the formation of telluroxide and alkoxy radical.

Scheme 19. Proposed antioxidant mechanisms for reactions in the absence of NAC or LipCys.
In the solvent cage, the alkoxyl radical can abstract the pyridinolic hydrogen from the telluroxide. This is favorable only for ortho-substituted compounds. If the reactive alkoxyl radical escaped into the bulk solution (pathway C) it would abstract a hydrogen and start a new chain. In pathways B and C (Scheme 20) several molecules of thiol are used up for reduction of telluroxide to telluride. This explains the low efficiency of the process in terms of \( n(\text{SH}) \), the number of peroxyl radicals destroyed by each molecule of thiol.

![Scheme 20. Proposed antioxidant mechanisms for reactions in the presence of NAC or LipCys.](image)

4.3.4 Lipophilicity as a parameter for regeneration
Replacement of the octyltelluro group of pyridinol 45a with ethyltelluro, pentyltelluro or hexadecyltelluro groups did not effect antioxidant capacity much (\( R_{\text{inh}} \)-values were similar; Table 7). However, inhibition times changed considerably and less lipophilic compounds (55a and 55b) were not as regenerate as 45a whereas an increase in lipophilicity (55c) did not have much of an effect.
Table 7. Inhibition times \( T_{\text{inh}} \) and inhibited rates of peroxidation \( R_{\text{inh}} \) in the absence and presence of NAC in the aqueous phase.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Aliphatic chain</th>
<th>( R_{\text{inh}} ) (μM / h)</th>
<th>( T_{\text{inh}} ) (min)</th>
<th>With NAC in the aqueous phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( R_{\text{inh}} ) (μM / h)</td>
<td>( T_{\text{inh}} ) (min)</td>
<td></td>
</tr>
<tr>
<td>a R = Ethyl</td>
<td>602</td>
<td>0</td>
<td>8</td>
<td>240</td>
</tr>
<tr>
<td>b R = n-Pentyl</td>
<td>600</td>
<td>0</td>
<td>8</td>
<td>280</td>
</tr>
<tr>
<td>c R = n-Cetyl</td>
<td>606</td>
<td>0</td>
<td>8</td>
<td>&gt;400</td>
</tr>
</tbody>
</table>

The poorer performance of compounds 55a and 55b is likely to reflect their distribution between the two phases with a lower concentration in the organic phase where peroxidation occurs.

4.3.5 Catalytic decomposition of hydroperoxides

In addition to their catalytic chain-breaking capacity, octyltelluro pyridinols could also catalyze reduction of hydroperoxides and thus mimic the action of the GPx-enzymes. To compare their catalytic performance as peroxide decomposers, the initial rate of hydrogen peroxide reduction was measured by monitoring formation of diphenyl disulfide (PhSSPh) in the presence of benzenethiol (eq 8). UV spectroscopy at 305 nm was used for detection as described in the literature\textsuperscript{108} and reactions were performed in methanol in the presence of 1 mol-% catalyst. Diphenyl diselenide was used as a reference. The data is presented in Table 8.

\[
\text{H}_2\text{O}_2 + 2 \text{PhSH} \xrightarrow{\text{catalyst}} 2 \text{H}_2\text{O} + \text{PhSSPh} \quad \text{(eq. 8)}
\]

All tellurium compounds studied catalyzed \( \text{H}_2\text{O}_2\)-reduction more efficiently than diphenyl diselenide. Compound 47a was found to be the best catalyst among the investigated antioxidants. The rate limiting step in the catalysis is probably nucleophilic attack by tellurium on oxygen. Therefore, steric and electronic effects would influence catalytic activity, as seen with the electron donating methoxy group of compound 47a.
Table 8. Thiol peroxidase activity for tellurium containing 3-pyridinols.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Thiol Peroxidase Activity (μM / min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37a</td>
<td>4.8 ± 1.1</td>
</tr>
<tr>
<td>38a</td>
<td>7.8 ± 1.0</td>
</tr>
<tr>
<td>39a</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>40a</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>45a</td>
<td>15.4 ± 2.2</td>
</tr>
<tr>
<td>47a</td>
<td>17.8 ± 3.0</td>
</tr>
<tr>
<td>50a</td>
<td>9.8 ± 1.8</td>
</tr>
<tr>
<td>PhSeSePh</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>

* Initial rate of hydrogen peroxide reduction in the presence of thiophenol and antioxidant. Data are mean ± SD for triplicates.

4.4. Improving on a commercial antioxidant (Papers V & VI)

4.4.1 Inhibition studies in the two-phase system

The commercial antioxidant BHA (6, 3-tert-butyl-4-hydroxyanisole isomer) inhibited peroxidation of linoleic acid for 100 min in the two-phase system. The inhibition period could not be extended by addition of NAC to the aqueous phase (Table 9). It inhibited peroxidation with \( R_{inh} = 34 \, \text{μM} \) and is thus a poorer quencher of peroxyl radicals than \( \alpha \)-tocopherol. Introduction of octyltelluro, octylseleno or octylthio groups \( ortho \) to hydroxyl in the BHA-scaffold caused a reduction in inhibition time (\( T_{inh} = 80 \, \text{min} \)) and chain-breaking capacity was poor (\( 252 < R_{inh} < 295 \, \text{μM/h} \)) in the absence of thiol (Table 9). Whereas the performance of organoselenium compound 57b and organosulfur compound 57c remained unchanged with NAC in the aqueous phase, tellurium compound 57a was superior to \( \alpha \)-tocopherol both when it came to inhibition time (\( T_{inh} = 365 ± 12 \)) and rate of inhibition (\( R_{inh} = 14 ± 2 \)) (Figure 14).
Table 9. Inhibition times ($T_{\text{inh}}$) and inhibited rates of peroxidation ($R_{\text{inh}}$) for BHA-analogue in the absence and presence of NAC in the aqueous phase.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Chalcogen</th>
<th>$R_{\text{inh}}$ (μM / h)</th>
<th>$T_{\text{inh}}$ (min)</th>
<th>$R_{\text{inh}}$ (μM / h)</th>
<th>$T_{\text{inh}}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a X = Te</td>
<td>252</td>
<td>80</td>
<td>14 ± 2</td>
<td>365 ± 12</td>
<td></td>
</tr>
<tr>
<td>b X = Se</td>
<td>249</td>
<td>80</td>
<td>252</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>c X = S</td>
<td>295</td>
<td>80</td>
<td>278</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>OH X-n-Ocyl</td>
<td>0</td>
<td>664</td>
<td>19</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>OH Te-n-Ocyl</td>
<td>65</td>
<td>100</td>
<td>34</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>BHA, 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>24 ± 2</td>
<td>80</td>
<td>24 ± 2</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

The $O$-methylated tellurium analogue 59 did not inhibit peroxidation of linoleic acid in the absence of thiol but some antioxidant activity was observed when NAC was present in the water layer ($R_{\text{inh}}$=19 μM/h, $T_{\text{inh}}$=90 min). As HAT is not possible, inhibition is likely to involve ET. NAC is needed to keep the organotellurium in its reduced form as observed for the tellurium containing pyridinols. The retardation of peroxidation observed for compound 57a in the absence of thiol is probably due to the corresponding oxide formed initially in the experiment.

Figure 14. Peroxidation trace (linoleic acid hydroperoxide concentration vs time) recorded for compound 57a and α-tocopherol in the presence of NAC in the aqueous phase.
4.4.2 Organochalcogen substituent effects

In a search for novel catalytic antioxidants we had the opportunity to compare the effects of organosulfur, organoselenium and organotellurium substituents in 3-pyridinols and phenols. Often, the organotellurium derivative was efficient and highly regenerable whereas the corresponding organosulfur and organoselenium compounds were more mediocre antioxidants. We therefore decided to look closer into substituent effects of organochalcogens.

4.4.2.1 Experimental Phenolic OH BDEs

In order to investigate the electronic effects of ortho- and para alkylchalcogeno groups in phenolic compounds, the radical equilibration technique earlier described (see section 4.2.3) was used to obtain experimental BDE_{OH} values. The compounds investigated are shown below. Reference data for the para alkylseleno substituent could be extracted from previous work (Table 10).74,109

![Chemical Structures](image)

Table 10. Investigated antioxidants and experimental OH BDEs.

<table>
<thead>
<tr>
<th>Antioxidant (ArOH)</th>
<th>BDE_{OH} (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHA, 6</td>
<td>80.3 ± 0.2</td>
</tr>
<tr>
<td>57a</td>
<td>78.9 ± 0.2</td>
</tr>
<tr>
<td>57b</td>
<td>79.8 ± 0.1</td>
</tr>
<tr>
<td>57c</td>
<td>80.6 ± 0.1</td>
</tr>
<tr>
<td>60</td>
<td>78.6 ± 0.3</td>
</tr>
<tr>
<td>61</td>
<td>78.0 ± 0.3</td>
</tr>
<tr>
<td>DBP</td>
<td>81.7</td>
</tr>
</tbody>
</table>

DBP = 2,6-Di-tert-butylphenol

As shown in Table 10, alkylseleno and alkylthio groups (57b and 57c) in the ortho position only has a minor effect on the BDE_{OH} as compared with the unsubstituted compound BHA (6), whereas an alkyltelluro substituent (57a) lowered the BDE_{OH} by approximately 1.4 kcal/mol. Interestingly, alkylchalcogeno groups (60 and 61) positioned para to the OH weakened the O-H bond by more than 3 kcal/mol as compared to unsubstituted analogue DBP. The overall behaviour could be explained by the electron donating character of alkylchalcogeno groups. However, when present in the ortho-position, the weakening effect on the BDE_{OH} is more or less out-balanced by a strengthening effect due to hydrogen bonding to chalcogen.
4.4.2.2 Calculated OH BDEs

DFT calculations performed at the B3LYP/LANL2DZdp level of methyl-chalcogeno compounds lended support to the experimental data as shown in Table 11. When methylchalcogeno groups were placed in the *ortho*-position, the BDE$_{OH}$ decreased as the size of the chalcogen atom increased. However, the opposite trend was seen when the same substituents were placed in the *para* position (Table 11). The differences in BDE$_{OH}$, ΔBDE$_{OH}$, were partitioned into molecule stabilization energies, MSE, and radical stabilization energies, RSE as defined by the isodesmic reactions shown in eq 9 and 10.

\[
\begin{align*}
\text{MeX-ArOH} & \rightarrow \text{C}_6\text{H}_6 \quad \text{MSE} \quad \text{ArOH} & \rightarrow \text{C}_6\text{H}_5\text{-XMe} \\
\text{MeX-ArO}^+ & \rightarrow \text{C}_6\text{H}_6 \quad \text{RSE} \quad \text{ArO}^+ & \rightarrow \text{C}_6\text{H}_5\text{-XMe}
\end{align*}
\]

(eq. 9)

(eq. 10)

Table 11. Calculated substituent contributions as ΔBDE$_{OH}$, Molecule Stabilization Energy (MSE) and Radical Stabilization Energy (RSE), compared to experimental ΔBDE$_{OH}$ (kcal/mol). Value for para-SeR was estimated from previous work.$^{74,109a}$

<table>
<thead>
<tr>
<th>Position</th>
<th>Substituent</th>
<th>ΔBDE$_{exp}$ (kcal/mol)</th>
<th>ΔBDE$_{calc}$ (kcal/mol)</th>
<th>MSE$_{calc}$ (kcal/mol)</th>
<th>RSE$_{calc}$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ortho</em></td>
<td>TeR</td>
<td>-1.4</td>
<td>-2.5</td>
<td>3.3</td>
<td>5.8</td>
</tr>
<tr>
<td><em>ortho</em></td>
<td>SeR</td>
<td>-0.5</td>
<td>-0.8</td>
<td>3.4</td>
<td>4.2</td>
</tr>
<tr>
<td><em>ortho</em></td>
<td>SR</td>
<td>+0.3</td>
<td>-0.3</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td><em>para</em></td>
<td>TeR</td>
<td>-3.1</td>
<td>-2.9</td>
<td>0.5</td>
<td>3.4</td>
</tr>
<tr>
<td><em>para</em></td>
<td>SeR</td>
<td>(-3.4)</td>
<td>-3.5</td>
<td>0.4</td>
<td>3.9</td>
</tr>
<tr>
<td><em>para</em></td>
<td>SR</td>
<td>-3.7</td>
<td>-4.3</td>
<td>-0.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>

While RSEs for the *para*-substituents are approximately the same (3.4 ≤ RSE ≤ 3.9 kcal/mol), the MSEs vary from destabilizing (negative value) for the alkylthio group to stabilizing (positive value) for the alkyltelluro group. This might be due to a region of positive electrostatic potential on the outer side of the chalcogen atom along with the extension of the covalent carbon-chalcogen bond, known as a “sigma-hole”, a term first introduced by Clark and co-workers in 2007.$^{110}$ Such a sigma-hole can interact with the π system if the alkylchalcogeno group is rotated perpendicular to it (Figure 15).

![Electrostatic potential on the molecular surface of 3-methyl-4-hydroxy-5-(methyltelluro)anisole (a) and the corresponding phenoxyl radical (b). Arrows indicate location of sigma-holes.](image-url)
This perpendicular conformation also allows hydrogen bonding of the phenolic O-H to the filled p-orbital of the chalcogen when the groups are oriented ortho. Optimized geometries for investigated phenols showed that the dihedral angle between the aromatic ring and the chalcogen-Me bond was approximately 90° and hydrogen-bonding was indicated by IR and 1H NMR spectroscopies. In the geometry optimized for the corresponding phenoxy radicals this angle was reduced to 0°.

Interaction of the σ-hole with the π system would give rise to an electron withdrawing substituent effect that becomes electron donating as soon as the alkylchalcogeno group rotates in the corresponding radicals. The electron withdrawing effect was supported by a Hammet plot of ν_{OH} vs substituent constants σ which indicated a σ’ value of 0.28 for the –TeR substituent.
5. Concluding remarks

Three new classes of chalcogen containing antioxidants were designed, synthesized and evaluated:

1. Within a series of structurally different 2,3-dihydrobenzo[b]selenophene-5-ol compounds the unsubstituted (in the phenolic moiety) chain-breaking antioxidant 28a was found to inhibit lipid peroxidation for more than 320 min (>320 min) in the presence of NAC, and thereby outperformed \( \alpha \)-tocopherol (\( T_{inh} = 90 \) min), when evaluated in a two-phase lipid peroxidation model. It was also found superior to Trolox – a water soluble derivative of \( \alpha \)-tocopherol – when evaluated for the ability to quench ABTS\(^{+}\) radicals in an aqueous environment. Compound 28a quenched/inhibited ROS production by neutrophils and PMA-stimulated macrophages more efficiently than Trolox. In addition, over a period of seven days, no significant signs of toxicity for either of the cell lines used (MG-63, HEK-293, SHY-5y, MRC-5 and CaCo-2) were observed when exposed to 28a or Trolox at a concentration of 60 \( \mu \)M.

2. Two pyridinols, 45a and 47a, substituted with ortho-octyltelluro groups were found to inhibit lipid peroxidation (\( R_{inh} = 8 \) \( \mu \)M/h) more efficiently than \( \alpha \)-tocopherol (\( R_{inh} = 24 \) \( \mu \)M/h) in the two-phase system with inhibition times exceeding 400 min when NAC was present in the aqueous phase. A catalytic behaviour was also found in homogenous phase using LipCys, a lipid soluble derivative of NAC, as a regenerating agent. Lipophilicity was found to be a critical parameter for regenerability in the two-phase system and a mechanism for regeneration was proposed.

3. Alkylchalcogeno groups were introduced into the commercial antioxidant BHA. The tellurium analogue inhibited peroxidation of linoleic acid efficiently (\( R_{inh} = 14 \) \( \mu \)M/h) and showed good regenerability. The effects of organochalcogen substituents in phenolic antioxidants were studied in a systematic manner by using EPR, IR and computational methods.

Syre i sin naturliga form, O₂, är en livsnödvändig molekyl. Den är även en diradikal i sitt grundtillstånd. Detta gör att syre är involverat i många av de radikalprocesser som bryter ner exempelvis platser eller får mat att oxidera, härskna och bli dålig.

Antioxidanter skyddar mot radikaler och kan fungera på olika sätt. Preventiva antioxidanter förhindrar bildande av radikaler och kedjbrytande antioxidanter bryter de kedjerektioner som radikalerna ger upphov till. Båda dessa typer av antioxidanter finns i kroppen som ett försvar mot fria radikaler. De preventiva antioxidanternas uppgift är att förhindra bildningen av radikaler. Flera av dessa antioxidanter har en central roll i samband med nedbrytningen av potentiellt farliga reaktiva molekyler innan radikalbildning från dessa hinner äga rum. Om mängden radikaler eller reaktiva syreföreningar överskrider vad det kroppsegna antioxidantförsvaret kan hantera så inträder ett tillstånd som brukar betecknas som ”oxidativ stress”.

Bland kedjbrytande antioxidanter förekommer bland andra Vitamin C och Vitamin E. Den förstnämnade, även kallad askorbinsyra, är vattenlös och måste intas med födan i form av frukt eller grönsaker då den inte kan produceras i kroppen. Den tillhör därför de s.k. exogena antioxidanternas. Vid fysiologiskt pH förekommer Vitamin C i sin delvis deprotonerade form och kallas då askorbat (I). Vitamin E är ett samlingsnamn för flera fettlösliga antioxidanter där α-tokoferol (II) är den mest potenta.
Precis som askorbinsyra är den en exogen antioxidant som måste tillföras kroppen genom födan t.ex. i form av vegetabiliska oljor, ägg och mjölk. I reaktionen med radikaler förbrukas E-vitamin såvida den inte kan regenereras. Det är allmänt vedertaget att den fettlösliga α-tokoferolen i våra membraner kan regenereras m.h.a. askorbat i vattenfasen.

Vitamin E är en bra utgångspunkt i sökandet efter nya syntetiska kedjebrytande antioxidanter. Fenolener som BHA (III) och BHT (IV) har länge nyttjats som tillsatser i mat för att skydda mot oxidativ nedbrytning. BHA är även en av vanligt förekommande stabilisatorer för polymera material.

![](image)

Variationer kan exempelvis göras i den aromatiska ringen. Här har kväve-innehållande 3-pyridinoler, så som t.ex. (V) visat mycket goda kedjebrytande egenskaper.


![](image)

Målet är att flertalet av dessa inte bara ska fungera katalytiskt som kedjebrytande antioxidanter utan även preventivt och förebyggande.


Av nedanstående föreningar så har VII och VIII visat sig vara regenererbara och mycket mer potenta kedjebrytande antioxidanter än α-tokoferol i närvaro av tiolen N-acetylcystein.
Med föresatsen att hitta terapeutiska applikationer av våra katalytiska antioxidanter mot oxidativ stress–relaterade sjukdomar har även de toxikologiska egenskaperna för VI undersökts samt dess förmåga att skydda mot radikaler producerade av kroppsegna system dokumenterats.
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References


[64] See ref. 29, Chapt. 1, p. 10.


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