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Synthesis of natural products, their synthetic analogues and evaluation of various associated biological activities

Khairan

Synthesis of natural products, their synthetic analogues and evaluation of various associated biological activities

Dissertation

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Diese Dissertation entstand unter der Anleitung von **Prof. Dr. Claus Jacob** in der Arbeitsgruppe für Bioorganische Chemie, Fachrichtung 8.2 Pharmazie der Naturwissenschaftlich-Technischen Fakultät III der Universität des Saarlandes im Zeitraum von Oktober 2009 bis August 2013.

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Saarbrücken, Datum
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Dedicated to the memory of my loving Mother

I would like to dedicate my thesis to my father, brothers and sister.

I also want to dedicate my thesis to my loving wife and daughter.

Abstract

In history, garlic (*Allium sativum*), onions (*Allium cepa*) and other *Allium* plants were used due to their antimicrobial-, antifungal- and desinfection abilities.[1-3] These plants contain a wide and rich range of sulfur-containing molecules, such as alliin, allicin and various polysulfanes (formerly named polysulfides) which do show high reactivity in several medicinal (*e.g.* cancer chemoprevention) and agricultural system (*e.g.* as “green pesticides”).[1-16]

The main objective of this PhD study is to further investigate the chemistry, reactivity, biological activity of natural disulfanes and chemically related species.

Sulfur and selenium and containing compounds were used to examine the different activity and toxicity of the agents based on the two chalcogen elements. Various biological assays were used to examine the activity of the chalcogen compounds against mammalian cells, such as Neuro A2 cell line (from murine neuroblastoma) and agricultural of relevant organism (*e.g. Botrytis cinerea, Steinernema feltiae*) to find new lead structures for a potential pro-drug or possible new phytoprotectants and ‘green’ pesticides. Cyclic voltammetry was used to analyze the redox behavior of the different chalcogen compounds and their potential reactivity against glutathione (GSH) and other thiol-containing groups in cells.

SEM-EDX microanalysis was employed to investigate the distribution of chalcogen atoms inside the Neuro 2A cells. Sulfur atoms from the polysulfanes and the sulfur nanoparticles as well as selenium nanoparticles could be detected as part of a very preliminary study employing the EDX methodology to chalcogen-containing material. Cell fractionation was subsequently used to investigate further where the model tetrasulfane DPhTTS is located inside Neuro 2A cells.

Kurzfassung

Bereits in Zeiten des Altertums wurden Knoblauch (*Allium sativum*), Zwiebeln (*Allium cepa*) und andere Pflanzen der *Allium*-Spezies wegen ihrer antimikrobiellen und antimykotischen Eigenschaften, sowie ihrer desinfizierenden Wirkung in vielen Kulturen weltweit eingesetzt. Die Pflanzen dieser Gattung enthalten einen hohen Anteil an hoch reaktiven, schwefelhaltigen Substanzen, die in wissenschaftlichen Versuchen bereits ein signifikantes Potential im medizinischen (z.B. in der Krebstherapie) als auch im landwirtschaftlichen (z.B. als „grüne“ Pflanzenschutzmittel) Bereich aufgezeigt haben.

Das Hauptaugenmerk der hier vorliegenden Doktorarbeit lag daher auf der Vertiefung der bereits früher durchgeführten Analysen, um nähre Erkenntnisse zu den unterschiedlichen Reaktionsmechanismen und Reaktivitäten der chalkogenhaltigen Naturstoffe und deren synthetischen Derivate, zu erhalten.

Schwefel- und Selen- haltige Verbindungen wurden synthetisiert und in den biologischen Experimenten mit den Schwefelverbindungen verglichen, um mehr über die Reaktivität und die Verteilung der einzelnen Chalkogene in der Zelle oder weiteren biologischen Materialien (z.B. Pilzkulturen, Nematoden) zu erfahren. Innerhalb der hier vorliegenden Dissertation wurden Experimente an Nervenzellen von Nagetieren (Neuro 2A Zelllinie) und Experimente an landwirtschaftlich bedeutsamen Systemen (*Botrytis cinerea, Steinernema felitae*) durchgeführt, sowie grundlegende Methoden zur Kontrolle der Redox-Aktivität (Zyklische Voltammetrie) der einzelnen Verbindungen beschrieben.

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I am deeply thankful to my loving wife, Nadia Zainal, and my loving daughter, Shazia Yossa Zamara Khairan for supporting and helping me throughout my PhD studies, in good and bad days and for providing me the moral, patient and emotional support that I needed to complete my dissertation.

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Abbreviation list

°C	degree Celsius
µl	micro liter
µM	micro molar
1,2-VDT	1,2-vinyldithiin or 3-vinyl-4H-1,2-dithiin
1,3-VDT	1,3-vinyldithiin or 2-vinyl-4H-1,3-dithiin
1,5-DTCO	1,5-dithiacyclooctane
^{13}C-NMR	carbon nuclear magnetic resonance
^{1}H-NMR	proton nuclear magnetic resonance
ABC	ATP binding casettes transporters
ACN	acetonitrile
ADT	anetholedithiolethione or 5-(4-Methoxyphenyl)-3H-1,2-dithiole-3-thione
APT	α-pinene trithione or bicycloalkyldithiolethione
AU	absorption unit
BnTTSPs	3-(benzyltetrasulfanyl)propanoic acid or benzyltetrasulfanepropylacid
Calcein-AM	calcein acetoxymethyl ester
$CDCl_3$	deuterated chloroform
CV	cyclic voltammetry
Cys	cysteine
d	duplet

DADS	1,2-diallyldisulfane or diallyldisulfide
DAS	diallylmonosulfide
DATS	1,3-diallyltrisulfane or diallyltrisulfane
DATTS	1,4-diallyltetrasulfane or diallyltetrasulfane
DBnTTS	1,4-dibenzyltetrasulfane or dibenzyltetrasulfane
DEETTS	1,4-bis(2-ethoxyethyl)tetrasulfane or diethylethertetrasulfane
DMSO	dimethyl sulfoxide
DPhTTS	1,4-diphenyltetrasulfane or diphenyltetrasulfane
DPSEETTS	diethyl 3,3'-tetrasulfanediyldipropanoate
DPSTTS	3,3'-tetrasulfanediyldipropanoic acid dipropylacidtetrasulfane
DPTS	1,3-dipropyltrisulfane or dipropyltrisulfane
DPTTS	1,4-dipropyltetrasulfane or dipropyltetrasulfane
DT	dithiolethione or 3*H*-1,2-dithiole-3-thione
dt	duplet of triplets
EDX	energy-dispersive *X*-ray spectrometry
Ep_a	peak anodic potential
Ep_c	peak catodic potential
ESI	energy spray ionisation
EtOAc	ethanolic acid
EtOAc	ethyl acetate
EtOH	ethanol

FCS	fetal calf serum
GSH	glutathione
GSSG	glutathione disulfide
GST	gluthathione-*S*-transferase
H_2O_2	hydrogen peroxide
HBr	hydrogen bromide
HO	haarlem oil
HPLC-UV-MS	high performance liquid chromatography-UV-mass spectroscopy
keV	kilo electron volt
LA	α-lipoic acid or 5-(1,2-dithiolan-3-yl)pentanoic acid
LAm	lipoamide or 5-(1,2-dithiolan-3-yl)pentanamide
LC-MS	liquid chromatography-mass spectroscopy
LD_{50}	lethal doses at 50 percent
m	multiplet
M	Molar
MCA	multi channel analyzer
MDR	multidrug resistance
MeOH	methanol
MFS	major facilitator superfamily transporters
MFS	major facilitator superfamily transporters
MfsM2	major facilitator superfamily transporter involved in MDR2

MHz	mega Hertz
MIC_{50}	minimum inhibitory concentration at 50 percent
Mp	melting point
Mrr1	multidrug resistance regulator 1
n_{D}	refractive index
Neuro 2A	murine neublastoma cell line
NPS	sulfur nanoparticles
NPSe	selenium nanoparticles
NQO1	NAD(P)H/ quinine oxidoreductase 1
Oltipraz	5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione
OS	oxidative stress
OSCs	organosulfur compounds
PDI	polydispersity index
PE	petroleum ether
PhTTSBn	1-benzyl-4-phenyltetrasulfane or phenyltetrasulfanebenzyl
PhTTSPs	3-(phenyltetrasulfanyl)propanoic acid or propylacidterasulfanedephenyl
PI	propidium iodide
ppm	parts per million
PrTTSPs	3-(propyltetrasulfanyl)propanoic acid or propyltetrasulfanepropylacid
R_f	retention factor
RNOS	reactive nitrogen oxygen species

RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	rounds per minute
RSS	reactive sulfur species
s	singlet
SEM	scanning electron microscopy
t	triplet
TLC	thin layer chromatography
V	volt
δ	chemical shift

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1. Introduction

1.1 Oxidative stress

In biochemistry, Oxidative Stress (OS) is related to a increase of the intracellular concentration of oxidizing species.[1] In biology, OS describes the imbalance between the “normal” conditions of a healthy cell and the increased amounts of oxidising processes with does often take place when the cell degenerates or turns into an abnormal cell, *e.g.* cancer or inflammatory cell.[2-4] OS in cells is characterized by a disturbed (anti)oxidant defense system, the precence of high (nearly toxic) concentrations of redox active species such as free radicals and non-radical reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive nitrogen oxygen species (RNOS) and reactive sulfur species (RSS). Several diseases, including cancer, hypoxia, inflammation, but also aging, are related to OS and the antioxidant levels in biological materials.[5-7] Figure 1.1 indicates the damages to biological material that are related to ROS and the presence of free radicals.

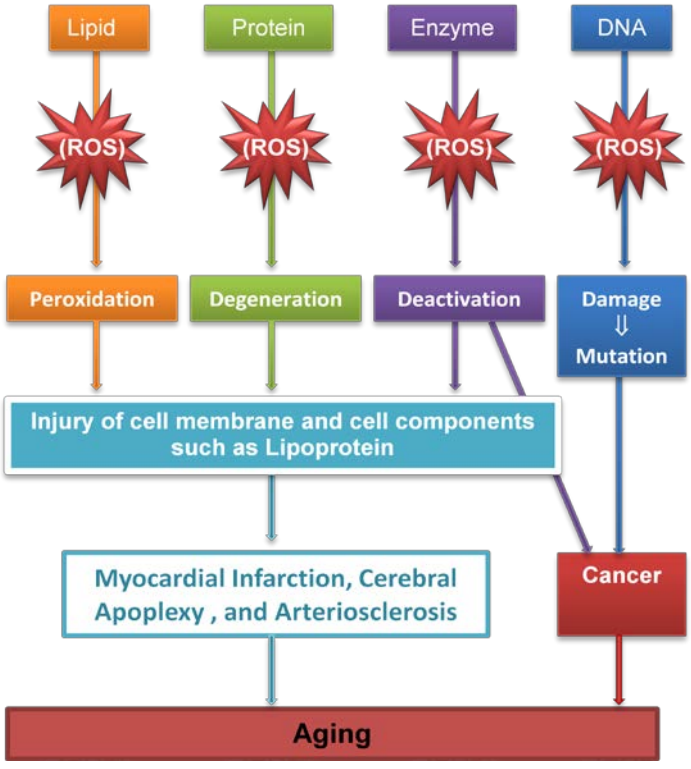
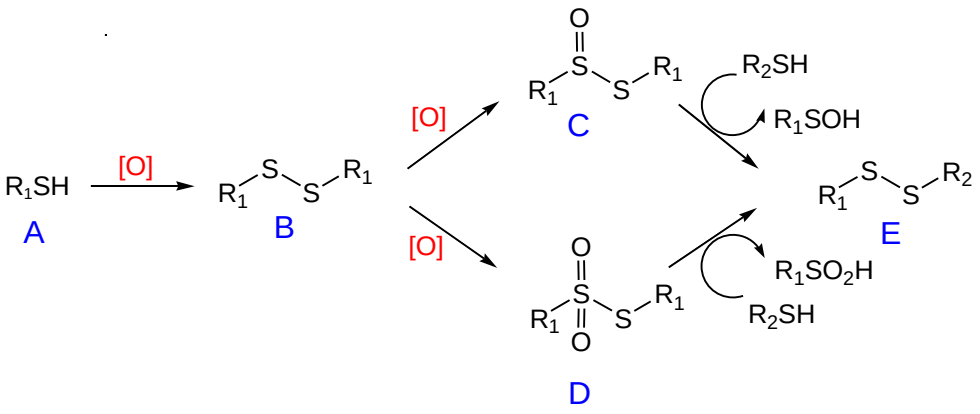


Figure 1.1: Cells and tissues damaged by reactive oxygen species (ROS) (adopted from Ref. [8])

While oxygen containing molecules normally act as pro-oxidants, other chalcogen (sulfur, selenium, and tellurium) containing compounds could be used as pro-oxidants as well as anti-oxidants, regarding their molecular structures and the redox-environment inside specific cells.[4,6,9-17] Sulfur- and selenium-containing molecules can in particular react with the sulfur-containing available in the cells, like glutathione (GSH), cysteine (Cys) and other thiol (RSH) groups. The 'cellular thiolstat', an expression invented by Prof. Dr. Claus Jacob in Saarbruecken in 2011, gives an overview about the redox active sulfur groups and their possible reactions inside the cells.[18-21]

Allicin (diallyldisulfide-*S*-monoxide) from garlic, as one of the best described sulfur-containing natural compound, reacts very well with the thiols inside biological material due to disulfide/thiol-interaction and sulfur-exchange reactions (Scheme 1.1). It is the high reactivity of the poly-sulfur groups (further named as 'polysulfanes') which gives molecules like allicin and alliin the antimicrobial, antifungal and antiviral activities for which they were used since ancient times by different cultures worldwide.[22-24]

Disulfanes, such as allicin, and their oxidized forms, like disulfide-*S*-dioxide, are known to easily undergo sulfur-sulfur-exchange reactions to produce new mixed disulfanes.



Scheme 1.1: Establishment of disulfide-*S*-oxides and the mixed disulfide through oxidative reaction. The disulfide compound (B) is constructed from thiol through oxidation. The disulfides then undergo further oxidation to form disulfide-*S*-monoxide (C) and disulfide-*S*-dioxide (D). The S-S bonds are activated through sulfur oxidation that encourages the reaction to produce mixed disulfide (E) as well as sulfenic and sulfinic acid.[3]

The different oxidation states of the sulfur compounds are summarized in Table 1.1. The table shows various ROS and RSS, the oxidation state of the chalcogen and the number of electrons that is formally required to complete the reductions of a thiol. As also shown in the table, disulfide-*S*-oxides have a higher oxidation state compared to other RSS.

Table 1.1 Summary of the oxidation states of various sulfur compounds. (Table adopted from Ref.[3])

Reactive species	Structure	Oxidation state of chalcogen	Electrons required for complete reduction
Thiyl radical	**$RS^{\bullet}$**	**–1**	**1**
Disulfide	**RSSR**	**–1**	**2**
Disulfide-*S*-monoxide	**RS(O)SR**	**+1, –1**	**4**
Disulfide-*S*-dioxide	**$RS(O)_2SR$**	**+3, –1**	**6**
Sulfenic acid	**RSOH**	**0**	**2**
Hydroxyl radical	**$HO^{\bullet}$**	**–1**	**1**
Peroxide	**ROOR**	**–1**	**2**
Superoxide	**$O^{\bullet}{}_2^-$**	**–0.5**	**3**

1.2 *Allium* plants as source of natural compounds

Garlic (*Allium sativum*), onion (*Allium cepa*) and other members of the *Allium* family have been used as part of nutrition since ancient times. Since thousands of years, these plants are also used as medicinal plants to heal all kind of diseases and for their antimicrobial activity.[24-26]

Nowadays, the allium plants are back in the focus of science due to their rich amount of sulfur containing molecules with high reactivity against microbes, fungi and bacteria and their possible application in cancer therapy and cardiovascular diseases.[13,27-34]

The garlic (and also the other *Allium* species) defense system against animals and other potential enemies uses an effective and potent chemical reaction which will take place as soon as the garlic plant is hurt or damaged. Alliin (a nonprotein sulfur containing amino acid and a secondary metabolite)[35,36] is enzymatically esseantially found in garlic via enzymatic degradation, which is catalysed by alliinase to form ammonium piruvate and sulferic acid (Figure 1.2). Alliin is transformed by alliinase to allicin within seconds, a reaction which produces the typical smell and taste of garlic. This intense smell and the reactive allicin prevent animals to eat the garlic plant. Allicin also attacks bacteria and fungi which otherwise may affect the damaged part of the garlic plant.[24,37,38]

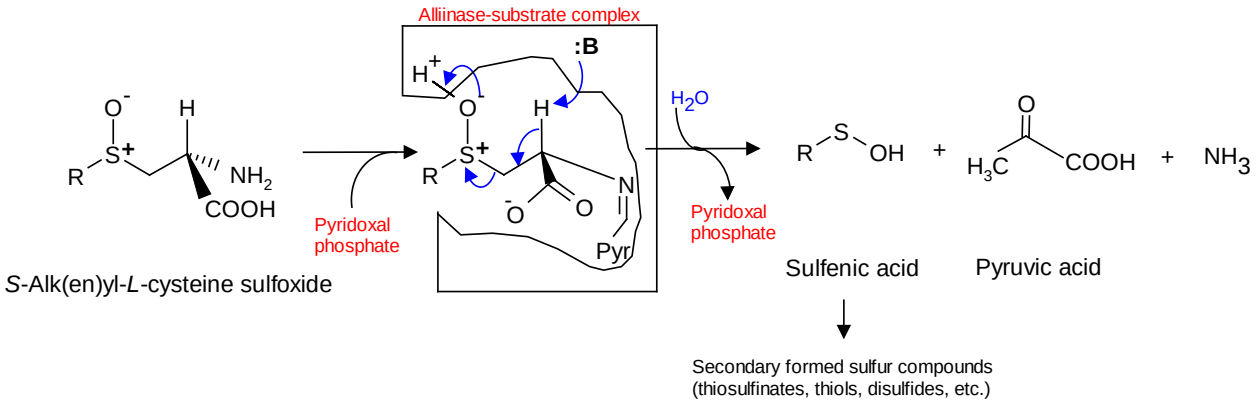
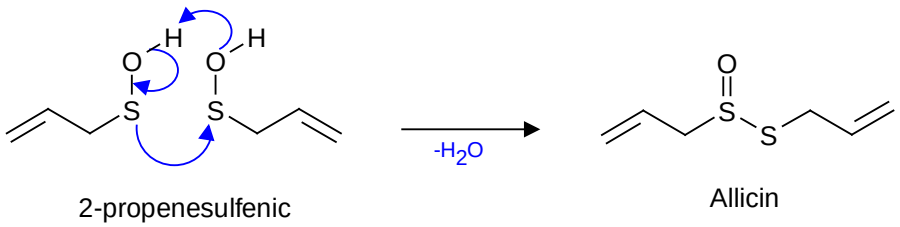


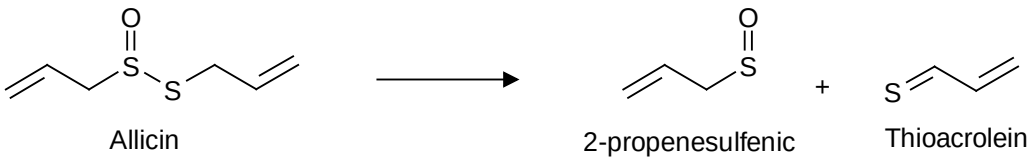
Figure 1.2: Enzymatic synthesis of allicin from alliin by alliinase.[39]

2-Propensulfenic acid is an unstable molecule and very reactive at room temperature. It easily undergoes self-condensation (elimination of water) to form allicin (Scheme 1.2).



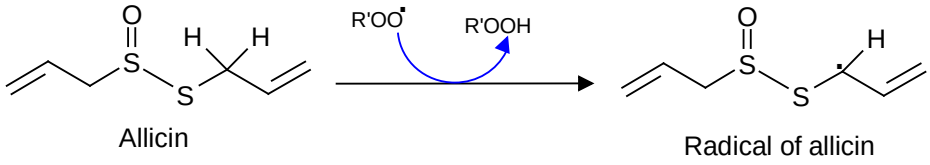
Scheme 1.2: Condensation of 2-propenesulfenic to allicin.[40]

It should be mentioned that allicin is also easily prone to Cope reaction, an elimination reaction to form alkenes. At room temperature it can easily form 2-propenesulfenic acid and thioacrolein (Scheme 1.3).[40,41]

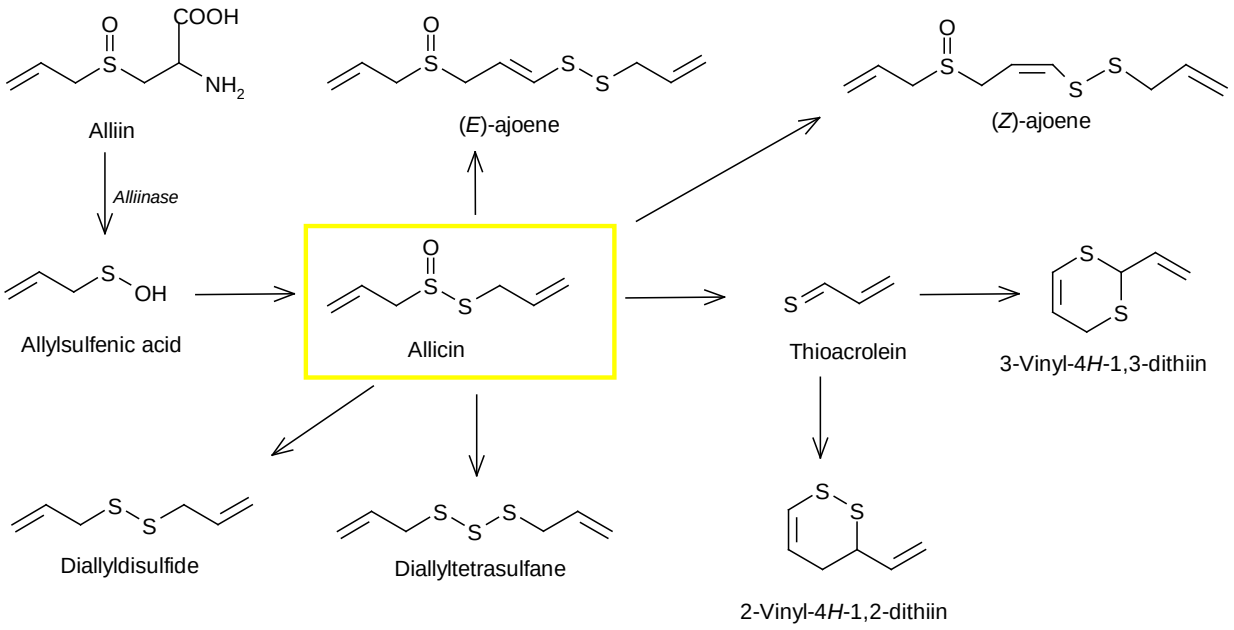


Scheme 1.3: Cope elimination of allicin to form 2-propenesulfenic and thioacrolein.[41]

Okada *et al*. have found that the -S(O)SCH$_2$CH=CH$_2$ moiety of allicin could contribute to the antioxidant properties, due to the fact that it involves a mechanism of abstraction H-atoms from the ally-group next to the *S*-*S*-bond.[16] H-atom transfer, which is shown in Scheme 1.4 might play an important role for radical-trapping activities of allicin.



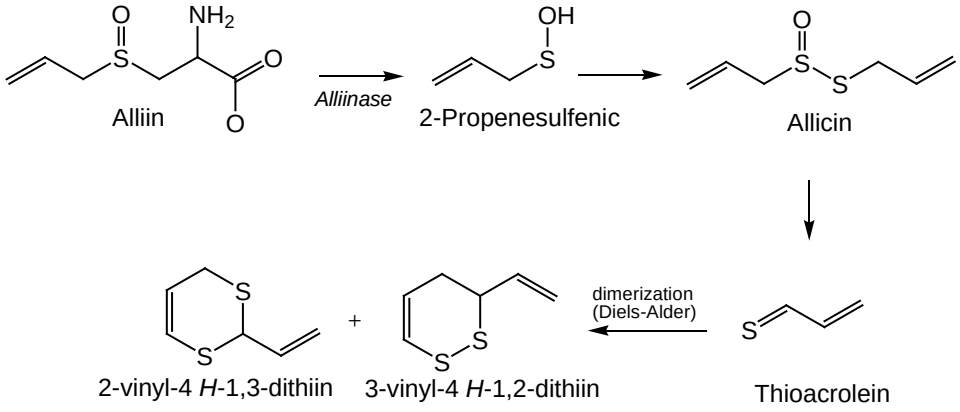
Scheme 1.4: Mechanism of H-atom abstraction from allicin.[16]



Scheme 1.5: Reactivity and biotransformation of allicin. (Figure adapted from Ref.[42])

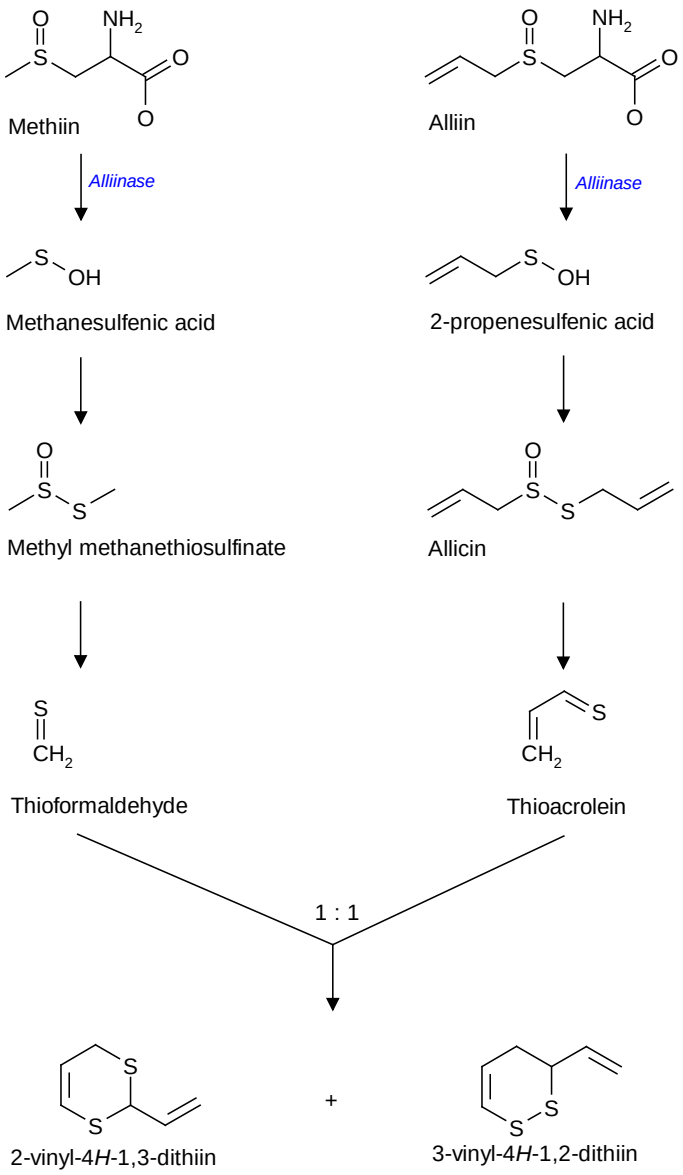
Allicin is also able to form vinyldithiins in non-polar solvents such as hexane or various oils like pine oil. The reactivity and the biotransformation of allicin is shown on Scheme 1.5. The reaction follows a Diels-Alder (D-A) dimerization mechanism.[43] According to Beslin *et al.*, the transformation of allicin leads to 2-vinyl-4*H*-1,3-dithiin as main product in non-polar medium. A

further important product, 3-vinyl-4*H*-1,2-dithiin is formed following the reaction processes shown in Scheme 1.6.[44]



Scheme 1.6: Transformation of allicin to form vinyldithiins.[44]

It was proposed that the dithiins were formed by the enzymatic condensation reaction between thioacrolein and thioformaldehyde by alliinase. It is known that thioacrolein is formed by the alliinase-mediated breakdown of alliin. Thioformaldehyde is also formed by the alliinase-mediated breakdown of methiin.[42,45] The formation mechanisms of 1,2- and 1,3-dithiins are shown in Scheme 1.7.



Scheme 1.7: Formation of 1,2- and 1,3-vinyldithiins from methiin and alliin, respectively.[45]

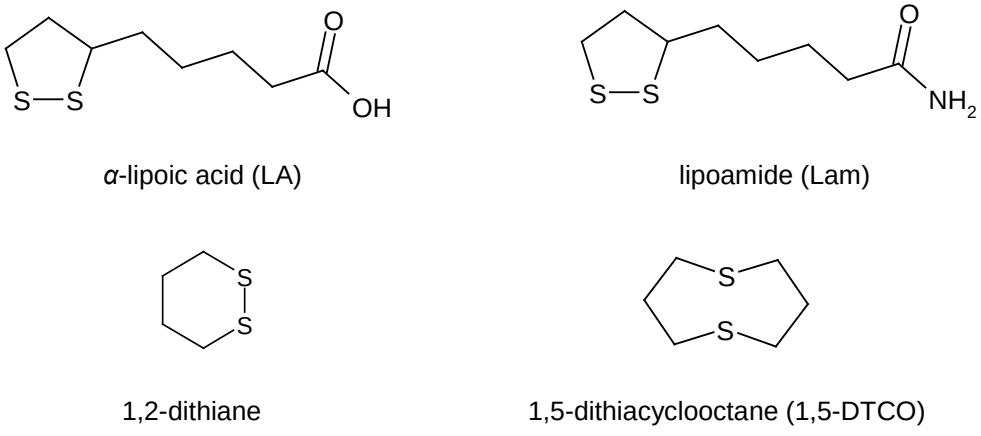
Vinyldithiins are regioisomeric, heterocyclic compounds with two sulfur atoms and a better stability compared to allicin.[39] The pharmacological studies indicate that 3-vinyl-4*H*-1,2-dithiin is more lipophilic than 2-vinyl-4*H*-1,3-dithiin. Most recent studies show that 3-vinyl-4*H*-1,2-dithiin has a tendency to accumulate inside fat tissues. Besides that, this molecule also easily undergoes biotransformation by microsomes from murine liver tissue.[46] Assays using blood showed that this substance can decrease the amounts of cholesterol in the blood and could be useful as nutritional supplement.[47]

Polysulfanes are also found naturally in garlic. Bioactive decomposition products from garlic include diallyltrisulfide (DATS) and diallyltetrasulfide (DATTS). Various diallypolysulfanes, including DATS and DATTS, can easily interact with intracellular thiols via thiol/polysulfane exchange reactions. Therefore these compounds do have an huge influence on the cellular thiolstat, cell signaling and different apoptosis pathways which do involve cysteine containing enzymes and proteins.[18,21]

One of the main reaction partners in cells for the polysulfanes could be glutathione (GSH) which will then be transferred into glutathione disulfide (GSSG).[35,48] DATS and DATTS have a very broad impact on biological processes and can cause cell cycle arrest (in the G2/M phase) and apoptosis especially when they are used against oxidative stressed cell lines (*e.g.* certain cancer cell lines).[35]

Other disulfide containing compounds such as *α*-lipoic acid (LA) and lipoamide (LAm) are known to have antioxidant properties.[12]

Disulfide compounds which were not so much in the focus in the past, such as 1,2-dithiane and 1,5-dithiacyclooctane, are known to play important roles in biological and chemical as well processes.[49] The structure of these compounds are shown in Figure 1.3.



α-lipoic acid (LA) lipoamide (Lam)

1,2-dithiane 1,5-dithiacyclooctane (1,5-DTCO)

Figure 1.3: The chemical structures of several active sulfur containing molecules

Several other (synthetic) compounds containing polysulfane groups, such as 3*H*-1,2-dithiole-3-thione (dithiolethione, DT), 5-(4-Methoxyphenyl)-3*H*-1,2-dithiole-3-thione (anetholedithiolethione,

ADT) 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (Oltipraz), and bicycloalkyldithiolethione (*α*-pinene trithione, APT), are known for their chemo protective and antitumor activity.[50] The structures of some of these compounds are shown in Figure 1.4.

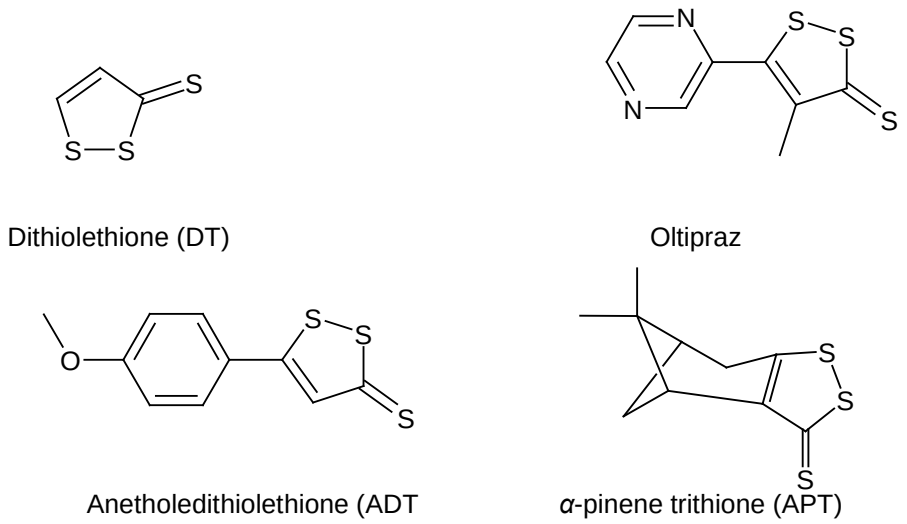


Figure 1.4: Chemical structures of selected dithiolethiones.

Recent studies have demonstrated, that several dithiolethione compounds, particularly anetholedithiolethione (ADT), exhibit cardioprotective activities due to H_2S-release from the parent dithiole-thione moiety.[51] Munday *et al.* reported that DT has a higher activity to induce Phase 2 enzymes in rodents compared to Oltipraz due to its ability to stimulate the transcription factors of enzymes such as gluthathione-*S*-transferase (GST) and NAD(P)H/quinine oxidoreductase 1 (NQO1) via activation of Nrf2.[52]

In case of *α*-pinene trithione (APT), this compound was synthesized from *α*-pinene oil and elemental sulfur by a base-catalyzed reaction. The so called Haarlem Oil (HO) is a semisynthetic (natural) product made from terpene oil and elemental sulfur by reaction them together under the high temperatur. Haarlem Oil was first introduced in the Netherlands in the 16th-Century. Thomas Monsieur was the first using HO in scientific studies and marketed the oil in France in 1924. In the 1980s and 1990s, HO has been further studied due to the presence of various sulfur compounds and the potentially use in medicinal treatment.[53,54]

The cardioprotective activities of these compounds are well known but not fully understood. The knowledge about the biological activity, especially with regard to the redox-modulatory behaviour and the antinematicidal and antifungal activity, is still very limited.

1.3 Chalcogen nanoparticles

As shown before, polysulfanes do have a high biological activity. Interestingly, many of the chemical properties related to the polysulfanes are also found in the S_8 ring system of elemental sulfur. For instance, both, polysulfanes and S_8 contain sulfur-sulfur bonds which may react with biological materials such as cysteine containing proteins and enzymes. Both, polysulfanes and S_8 are able to form superoxide radical anion ($O_2^{\cdot-}$), under physiological conditions. They can bind to diverse metal ions (*e.g.* iron, copper, zinc) and react with proteins and cellular membranes.

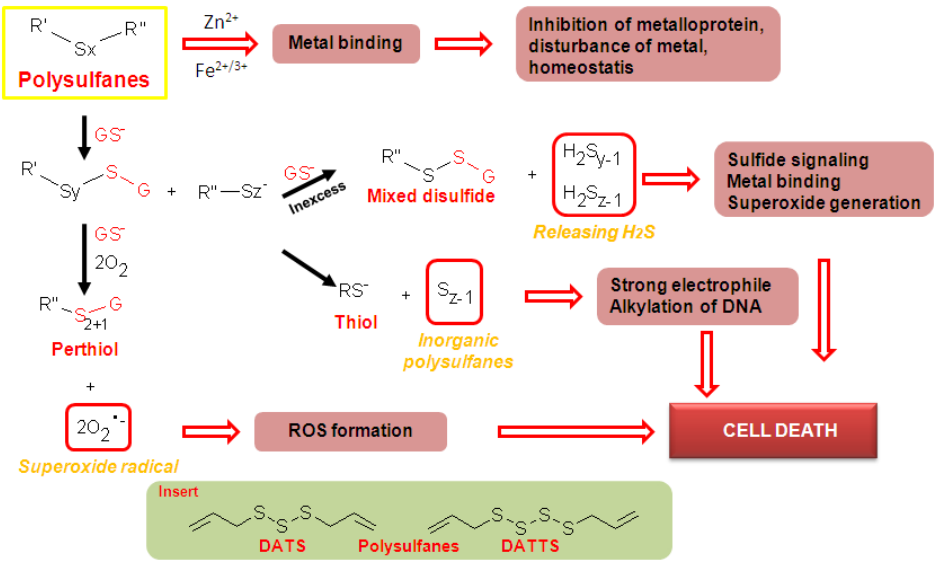


Figure 1.5: Reactivity of polysulfanes and their interactions with biomolecules. (Figure adopted from Ref. [35,55]).

Chemically, elemental sulfur is more stable and, as a solid, also easier to use. S_8 is odorless compared to polysulfanes, which increases the chance for a possible industrial application.

Recent studies have shown that elemental sulfur shows activity against bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus areus*, as well as some antimicrobial activity.[56]

1.4 Biological activity

1.4.1 Antimicrobial activity

Allicin posseses high activity in inhibiting the growth of several bacteria such as *Streptococci, Staphylococci, Eberthella typhosa, Bacillus dysenteriae, Bacillus enteritidis* and *Vibrio chlorae*.[57]

Polysulfanes, in particular diallyldisulfanes, diallyltrisulfanes and diallyltetrasulfanes do show high antiviral activity when exposed to *Cryptococcal meningitis*.[58] Yoshida *et al.* reported that DATS, which is isolated from oil-macerated garlic extract, has high antimicrobial activity against several different types of bacteria.[59] Several studies showed that the antimicrobial activities were affected by alk-(en)-yl groups, and therefore, Yoshida *et al.* suggested that the order for antimicrobial activity should be allyl ≥ methyl > propenyl.[59]

1.4.2 Antifungal activity

In history, extracts from garlic, onion and other *Allium* plants were used to test their antifungal activity. These extracts are potent against several fungi families, for example *Candida*, *Cryptococcus*, *Trichophyton*, *Epidermophyton*, and *Microsporum*. The *Allium* extracts were however less active or even inactive against the *Aspergillus* family.*[26]* Shams-Ghahfarokhi *et al.* reported that 2 mg/ml of aqueous onion extract and 65 to 125 µg/ml of aqueous garlic extract do have activity against *Malassezia furfur*, *Candida albicans* and other *Candida spp*.[26] In contrast, López-Díaz *et al.* have found that garlic extracts have no or very low activity against the growth of *Penicillium olsonii* and *Penicillium nalgiovense*.[60]

Botrytis cinerea (‘grey mold’) acts in infected crops as a necrotrophic fungus, an organism that kills a part or the entire plant before deriving nutrients from it. *B. cinerea* is induced to the damaged parts of the plant and spreads to healthy tissue thus causing necrotic lesion.[61]

1.4.3 Nematicidal activity

To investigate the activity of the sulfur compounds from *Allium* plants and their synthesized mimics, on the organisms, the compounds were tested on different nematodes (small round worms), like *Caenorhabditis elegans* and *Steinernema feltiae*.[25]

Diallylpolysulfanes such as DATS have been examined and found to be more active than diallydisulfide (DADS) against *Bursaphelencus xylophilus*, a pine wilt nematode (PWN) that infects pine trees and cause pine wilt.[25]

Steinernema feltiae is a transparent nematode, maximum 5 mm long, used as a 'phytoprotectant' by eating fly eggs and larvae. Due to the fact that *S. feltiae* is not a pest but a helpful organism for gardening, these nematodes can be used in normal laboratories without any special security protocols. This is also commercially available via the internet by the company Sautter and Stepper, Ammerbuch, Stuttgart, Germany. This nematode is easy to culture and simple toxicity screens using a normal light microscope are possible.[62] Even though these nematodes are not pests by themselves, they represent a standard model for related environmental and agricultural vermins and pests.

This nematode is an entomophatogenic nematode, which means that it is itself a parasite for other parasites, especially insects and their eggs. *S. feltiae* and other nematodes are normally used by gardeners as a biological defence against garden pests like flies, bugs, snails and various other organisms.[63] In the past few years, nematodes are also attracting more and more attention as part of laboratory experiments. These small organisms are easy to handle, cheap and they do not require any specific handling due to the fact that they are useful animals in the environment with a very short lifespan.

1.5 Cell assay

Cell assays are a major system for biological research. Such *in vitro* assays are becoming more and more important due to the high costs of animal tests and the ethical conflicts such experiments are creating. The simplest experiments using different cells and cell lines are toxicity tests. Here, cells show different colors regarding if they are alive or dead. Such viability assays are standardly done using two-color fluorescence dyes (green and red), like calcein acetoxymethyl ester (calcein-AM) and propidium iodide (PI). The cells are counted simultaneously by a fluorescence reader.

Calcein-AM is highly lipophilic compared to PI. Therefore, once this dye enters the cell and is retained in the cytoplasm due to hydrolyis by esterases in viable cells. It produces a strong green fluorescence. In contrast, PI cannot reach beyond the viable cell membrane, it only reaches the nucleus by passing through membranes of dead cells and then intercalates with the DNA double strand to produce a red fluorescence (Figure 1.6). Calcein-AM and PI can be used separately or together to identify viable and death cells.[64]

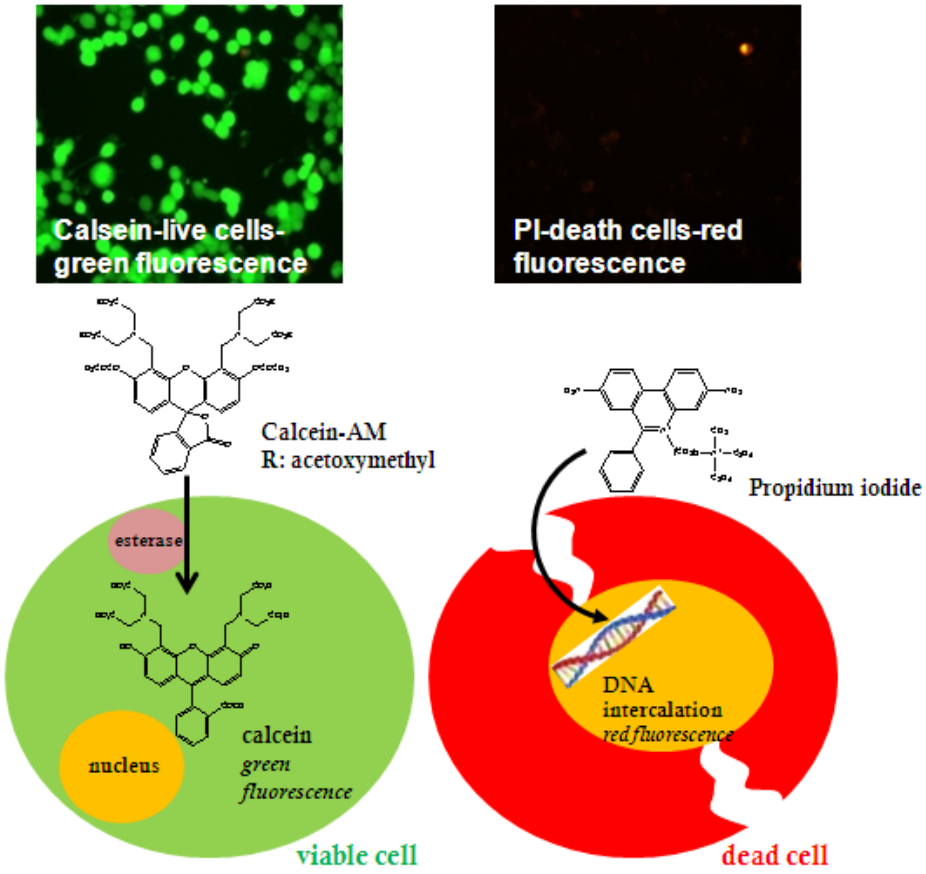


Figure 1.6: Calcein-AM and PI staining to determine viable and dead cells.(Adopted from Ref.[64])

In this study, calcein-AM and propidium iodide were used to determine cell viability of Neuro 2A cells, a murine neuroblastoma cell line. There are three reasons why we were used a Neuro 2A cell in associated to know the activity of the sulfur compound in this cell. Firstly, due to the high content of GSH in Neuro 2A cells. It is known, that the Neuro 2A cell line does contain up to four to five times more glutathione than other cell lines, such as the PC12 cell line (derived from a

transplantable rat pheochromocytoma).[65] Second, this cell is widely used as model for the explanation of the basic functions of neuronal system and the third, Neuro 2A cells can easily be differentiated with retinoic acid so they are much nearer to “real neuron”.

1.6 Analytical methods

1.6.1 Cyclic voltammetry

Cyclic voltammetry (CV) is an electroanalytical technique used to study the redox state of different (natural) compounds. CV is often used to study possible biological activity of freshly synthesized, novel compounds. The comparison of the redox values of the compound with the redox state of the cells at particular pH-levels provides a hint about the reactivity of such a compound.[66-68]

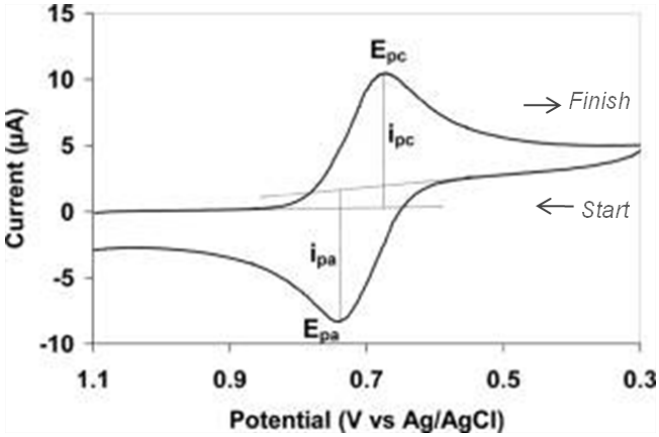


Figure 1.7: The graph of a simple cyclic voltammogram.[66]

CV is a potent and powerful method to examine preliminary biological effects of redox active compounds. The potential is applied between the reference electrode and the working electrode and the current is measured between the working electrode and the counter electrode (3-electrode setup).[67,68]

More details about the experimental setup and the theory of the electrochemical method are given in the experimental part of this thesis.

1.6.2 Energy-dispersive *X*-ray spectrometry

Energy-dispersive *X*-ray spectroscopy (EDX, EDS or XEDS) is a technique or method to analyze or to characterize elements in a specific sample such as a geological specimen or a biological material.[69,70] Powerful *X*-rays allow to scan the entire area of a biological material and to determine the different (heavy) elemental atoms inside this material. With this method, the mapping of different elements inside the sample is possible.

The EDX spectrometry is consists of three main parts: an *X*-ray detector, used to detect and transform emitted *X*-rays into electronic signals; a Pulse Processor, to measure the electronic signals to specify the energy that produces each *X*-ray detected; and a multi-channel Analyzer (MCA) with data management system (computer, software) which displays and calculates the interpretation of the *X*-ray data as a histogram of intensity versus the energy voltage (keV) (Figure 1.8).

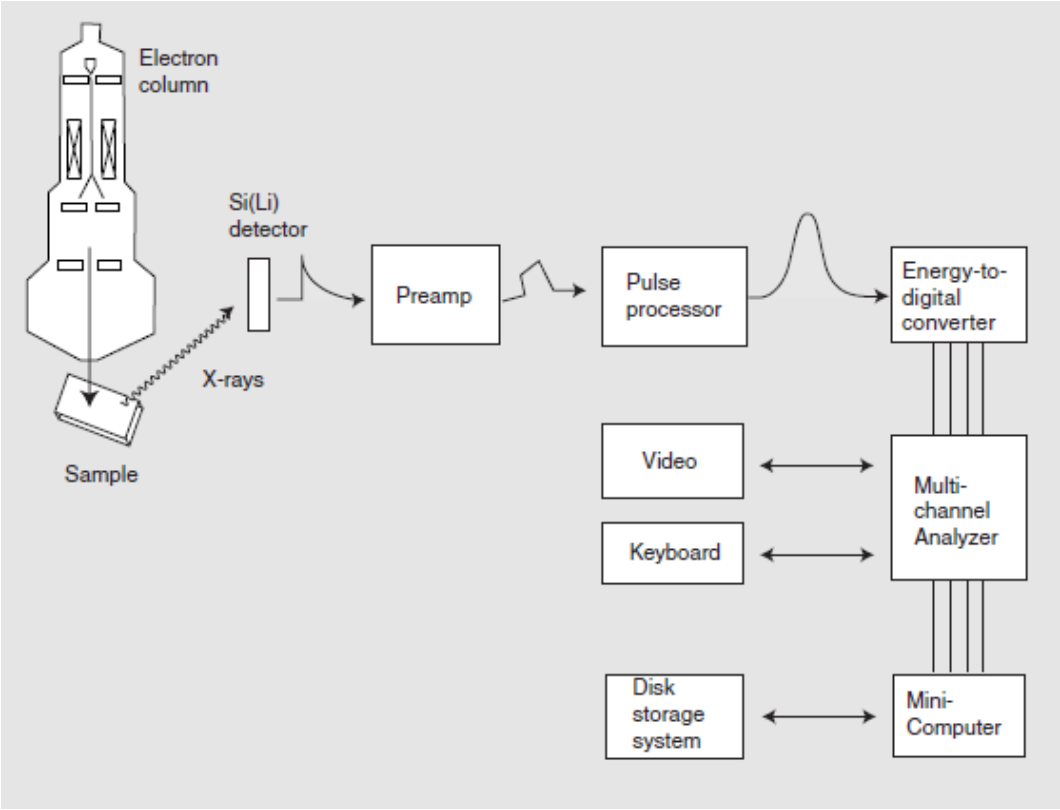


Figure 1.8: The EDX spectrometry (Figure adopted from Ref. [69])

1.7 Objectives of the present work

The first objective of this project was to investigate the biological and redox-modulatory behaviour of the polysulfanes and nanoparticles using cyclic voltammetry. Another aim of this study was to investigate the biological activities of these compounds against selected modul organisms, such as *Botrytis cinerea*, *Steinernema feltiae*, cancer cells and Neuro 2A cells lines. The possibility of creating for the first time a “cellular map” of various chalcogen atoms within the cells using EDX was also of high interest.

Due to the highly interdisciplinary character of this project, it was divided into eight main research areas:

1. Synthesis of the polysulfanes and dithiole thione compounds.
2. Electrochemical studies to investigate the redox behavior of the polysulfanes.
3. Evaluation of the activity of the polysulfanes on several cell lines.
4. Evaluation of the nematicidal activity of polysulfanes against *Steinernema feltiae*.
5. Evaluation of the antifungal activity of polysulfanes against *Botrytis cinerea*.
6. Basic screening of Neuro 2A cell lines to highlight the activity of polysulfanes.
7. “Cellular mapping” using EDX-microanalysis.
8. Detection of the main location for (re-)activity of such compounds in neuroblastoma cells using advanced HPLC/UV-MS analysis.

2. Results and Discussion

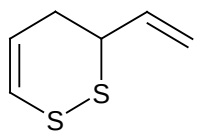
2.1 Synthesis of organosulfur compounds

2.1.1 Synthesis of vinyldithiins

Vinyldithiins were synthesized from acrolein and hydrogen sulfide according to the standard procedure developed by Beslin.[44] The characterization of 1,2-vinyldithiin and 1,3-vinyldithiin was performed using ^{1}H-NMR. The molar mass of 1,2-VDT and 1,3-VDT was confirmed using LC-MS. Both compounds exhibited the expected m/z ratio 144.04 [M^{+}] and 144.03 [M^{+}], respectively. The peak area percentage of both compounds was 100%, indicating a high purity.

All the results obtained were in very good agreement with those reported in the literature.

1,2-Vinyldithin (1,2-VDT)



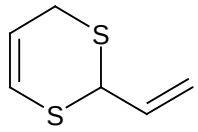
1,2-VDT

1,2-VDT was purified by column chromatography on silica gel with petrol ether:ethyl acetate = 99:1. It was obtained as a yellow oil. The total yield was only 1.2 % compared to 24 % reported by Beslin. [44] *TLC* (petrol ether: ethyl acetate = 99.5:0.5): R_f = 0.4.

^{1}H NMR ($CDCl_3$, 500 MHz): 2.13-2.38 (m, 2H), 3.25 (m, 1H), 5.08-5.16 (m, 1H), 5.57 (m, 1H) 5.96 (m, 1H), 6.08 (d t, 1H) ppm.

Literature: ^{1}H NMR ($CDCl_3$, 500 MHz): 2.3-2.7 (m, 2H), 3.62 (m, 1H), 5.1-6.5 (complex m, 5H) ppm.[44].

1,3-Vinyldithin (1,3-VDT)



1,3-VDT

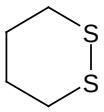
1,3-VDT was purified by column chromatography on silica gel with petrol ether:ethyl acetate = 99:1. It was obtained as a yellow oil. The yield was slightly better than the one from 1,2-VDT, but still just 2.4 % compared to 24 % % reported by Beslin. [44] *TLC* (petrol ether: ethyl acetate = 99.5:0.5): R_f = 0.6.

^{1}H NMR ($CDCl_3$, 500 MHz): 3.06-3.16 (m, 2H), 4.26 (d, 1H), 5.08-5.16 (m, 1H), 5.79 (m, 1H) 5.96 (m, 1H), 6.13 (d t, 1H) ppm.

Literature: ^{1}H NMR ($CDCl_3$, 500 MHz): 3.17-3.36 (m, 2H), 4.68 (d, 1H), 5.17-6.4 (complex m, 5H) ppm.[44].

2.1.2 Synthesis 1,2-dithiane

Cyclic disulfanes are difficult to synthesize due to the competing intermolecular reaction which ultimately leads to the formation of oligomers. Using a dispersion of dithiols on a large surface area provided by silica gel avoids intermolecular reactions. The 1,2-dithiane was synthesized according to literature method of Ali and McDermott, using molecular bromine on hydrated silica gel in non-aqueous media as oxidizing agent.[71] Silica gel also removes the hydrogen bromide formed intermediately in the reaction and turns it unavailable for side reactions. The silica gel acts both as a heat sink and as hydrogen bromide (HBr) scavenger. This procedure leads to the desired compounds.



1,2-dithiane

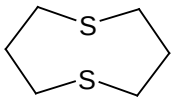
The product was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 95:5. It was obtained as a white crystallized solid (white crystals) with an R_f-value of 0.64. The yield was 15.0 %.

^{1}H NMR ($CDCl_3$,): δ 1.73-2.06 (s, 4 H); 2.8 (s, 4 H).
The melting point of the final product was 28-30 °C.

Literature: ^{1}H NMR ($CDC1_3$): δ 1.95 (s, 4 H); 2.8 (s, 4 H).[72]
Literature: 32-33 °C.[72]

2.1.3 Synthesis of 1,5-dithiacyclooctane

1,5-dithiacyclooctane (1,5-DTCO) was synthesized from 1,3-dibromopropane and 1,3-propanedithiol according to the literature method of Clennan and Liao. [62]



1,5-DTCO

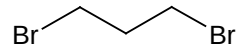
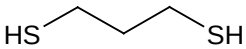
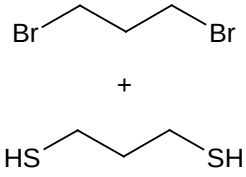
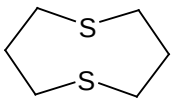
The crude product was purified by column chromatography on silica gel with hexane: ethyl acetate = 9:1. It was obtained as colorless liquid with a R_f-value of 0.56. Yield = 197 mg (1.3 mmol; 3.2 %).

^{1}H NMR ($CDCl_3$) δ 2.1 (m, 4H), 2.8 (m, 8H).

Literature: ^{1}H NMR ($CDCl_3$) δ 2.1 (m, 4H), 2.8 (m, 8H).[62]

Additionally, the refractive index of the starting material and of 1,5-DTCO was measured at 20 °C. The values of the refractive indices are presented in Table 2.1.

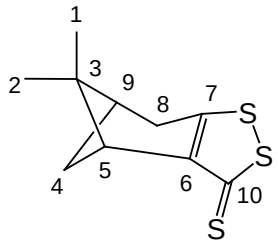
Table 2.1: The refractive indices of starting materials, their mixtures and the product (data are presented as mean ± SD)

	Structure of the molecule	**Exp. values of refractive index, n_D**
Starting material		1.5230 ± 0.0
		1.5388 ± 0.0
Mix		1.5270 ± 0.0
Product		1.5743 ± 0.0

2.1.4 Synthesis of *α*-pinene trithione

α-Pinene trithione (APT) was synthesized from *α*-pinene and elemental sulfur following the procedure of Djerassi and Luttringhaus.[8]

The product was purified by column chromatography on silica gel with petrol ether. It was obtained as a red-brown solid with a R_f*-value of* 0.58 and in an yield of 5.2%.



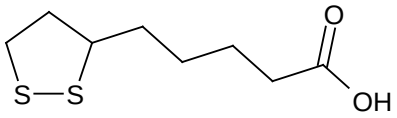
APT

^{13}C NMR ($CDCl_3$) δ 211.53 (C_{10}), 167.75 (C_6), 153.76 (C_7), 43.72 (C_1), 40.29 (C_2), 38.88 (C_3), 33.90 (C_4), 31.71 (C_5), 24.44 (C_8), 21.03 (C_9).

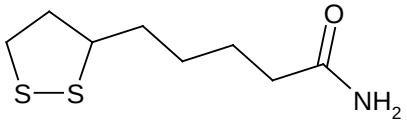
Literature: ^{13}C NMR ($CDCl_3$) δ 211.29 (C_{10}), 167.76 (C_6), 153.80 (C_7), 43.75 (C_1), 40.32 (C_2), 38.91 (C_3), 33.98 (C_4), 31.75 (C_5), 25.50 (C_8), 21.10 (C_9).[73]

2.2 Selected organosulfur compounds

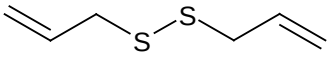
Other disulfane compounds were selected and used as a part of this research project for comparison. Such compounds include as *α*-lipoic acid (LA), lipoamide (LAm), diallyldisulfide (DADS). All of these compounds were purchased from Sigma Aldrich. The other disulfide compounds such as 3*H*-1,2-dithiole-3-thione, dithiolethione (DT), 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione(Oltipraz), 5-(4-methoxyphenyl)-3*H*-1,2-dithiole-3-thione, anetholedithiolethiones (ADT) were purchased from LKT Laboratories Inc. Haarlem Oil (HO) was a gift from Laboratoire du Dr. J. Lefevre. Chemical structures of the relevant disulfane molecules are shown in Figure 2.1.



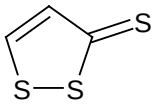
5-(1,2-dithiolan-3-yl)pentanoic acid
or α-lipoic acid (LA)



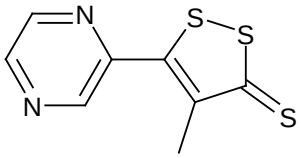
5-(1,2-dithiolan-3-yl)pentanamide
or lipoamide (LAm)



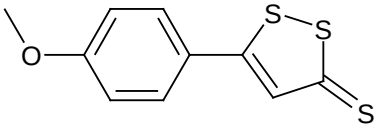
1,2-diallyldisulfane or
diallyldisulfide (DADS)



3*H*-1,2-dithiole-3-thione or dithiolethione (DT)



4-methyl-5-(pyrazin-2-yl)-3H-1,2-dithiole-3-
thione or Oltipraz

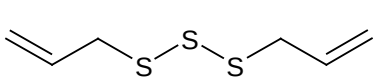


5-(4-methoxyphenyl)-3H-1,2-dithiole-3-thione or
anetholedithiolethione (ADT)

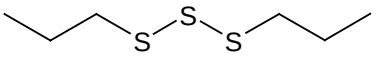
Figure 2.1: The chemical structures of cyclic disulfides compounds used.

Some of the polysulfane compounds synthesized in the laboratory of Prof. Dr. Claus Jacob during the last few years were also used as a part of this project (diallyltrisulfide (DATS), dipropyltrisulfide (DPTS), diallyltetrasulfide (DATTS), dipropyltetrasulfide (DPTTS), dibenzyltetrasulfide (DBnTTS), dipropylacidtetrasulfide (DPSTTS), diethylethertetrasulfide (DEETTS) and dipropylethanoicacidtetrasulfide (DPSEETTS). These polysulfane compounds were synthesized and purified by Brigitte Czepukojc.

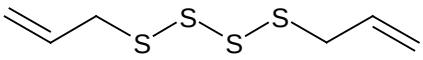
Other polysulfanes, such as diphenyltetrasulfide (DPhTTS), benzyltetrasulfidepropylacid (BnTTSPs), propyltetrasulfidepropylacid (PrTTSPs), phenyltetrasulfidebenzyl (PhTTSBn) and propylacidterasulfidephenyl (PhTTSPs) were synthesized and purified by Uma M. Viswanathan. The chemical structures of these polysulfanes are shown in Figures 2.2. and 2.3.



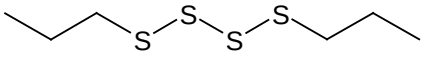
1,3-diallyltrisulfane or
diallyltrisulfide (DATS)



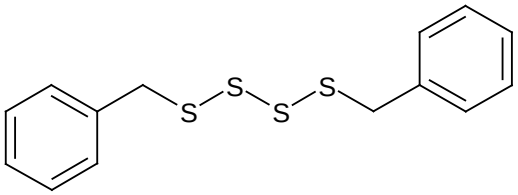
1,3-dipropyltrisulfane or
dipropyltrisulfide (DPTS)



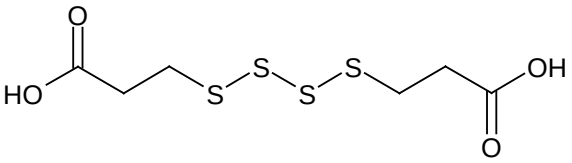
1,4-diallyltetrasulfane or
diallyltetrasulfide (DATTS)



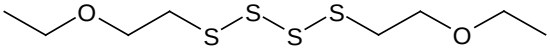
1,4-dipropyltetrasulfane or
dipropyltetrasulfide (DPTTS)



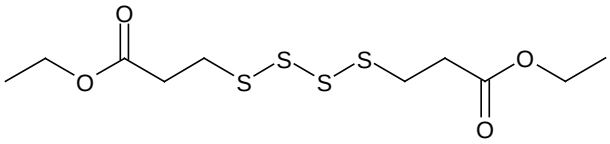
1,4-dibenzyltetrasulfane or
dibenzyltetrasulfide (DBnTTS)



3,3'-tetrasulfanediyldipropanoic acid
dipropylacidtetrasulfide (DPSTTS)

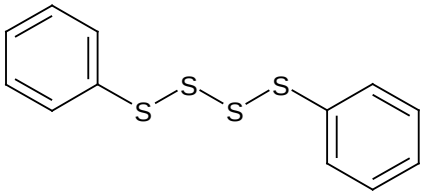


1,4-bis(2-ethoxyethyl)tetrasulfane or
diethylethertetrasulfide (DEETTS)

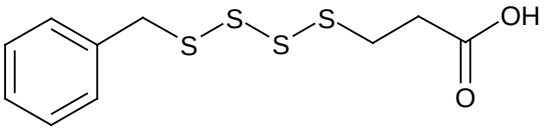


diethyl 3,3'-tetrasulfanediyldipropanoate or
dipropylethanoicacidtetrasulfide (DPSEETTS)

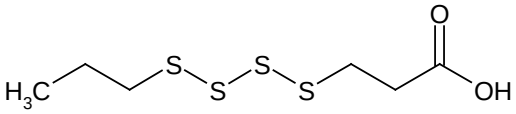
Figure 2.2: Chemical structures of polysulfane compounds prepared by Brigitte Czepukojc.



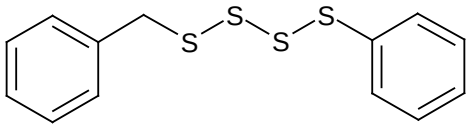
1,4-diphenyltetrasulfane or
diphenyltetrasulfide (DPhTTS)



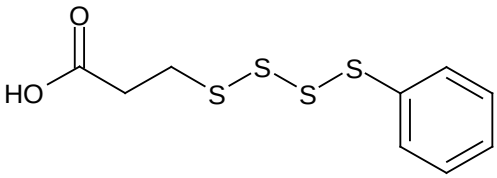
3-(benzyltetrasulfanyl)propanoic acid or
benzyltetrasulfidepropylacid (BnTTSPs)



3-(propyltetrasulfanyl)propanoic acid or
propyltetrasulfidepropylacid (PrTTSPs)



1-benzyl-4-phenyltetrasulfane or
phenyltetrasulfidebenzyl (PhTTSBn)



3-(phenyltetrasulfanyl)propanoic acid or
propylacidterasulfidephenyl (PhTTSPs)

Figure 2.3: Chemical structures of polysulfane compounds prepared by Uma M. Viswanathan.

2.3 Synthesis of the chalcogen nanoparticles

Sulfur nanoparticles (NPS) were synthesized and purified based on the procedure reported by Bomhard and Lange.[74] Selenium nanoparticles (NPSe) were synthesized and purified according to the literature by Chen *et al*. [75]

All nanoparticles were characterized by the Zetasizer Nano from Malvern Instruments, Ltd. Germany. The Zeta potentials of the nanoparticles were performed at 25 °C, pH 7 following standard procedures. The Zeta potential is the potential difference across phase boundaries between solids and liquids. The polydispersity index (PDI) describes the ratio of weight average molecular mass to the number average molecular mass. These data represents the results of three independent experiments. The respective sizes of the nanoparticles are presented in Figure 2.4.

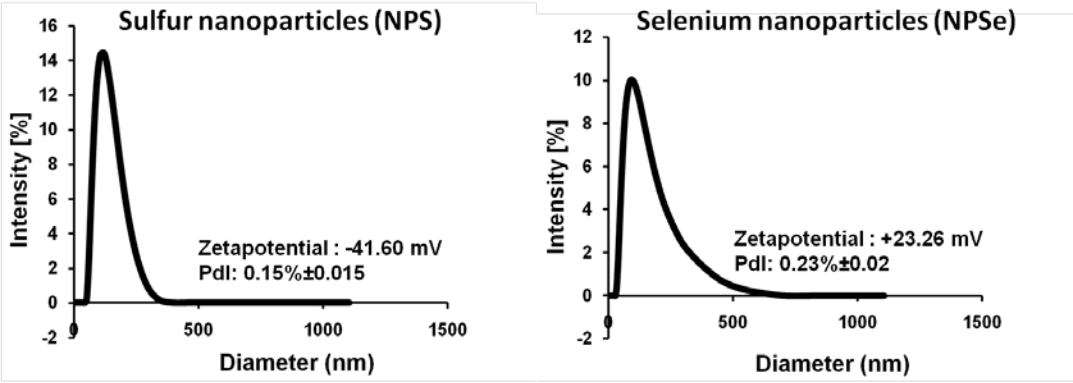


Figure 2.4: Zeta potentials of nanosulfur (NPS) and nanoselenium (NPSe) particles.

The NPS possesses an average diameter of 117.0 nm with a zeta potential of approximately -41.6 mV and a PDI of 0.15 % ± 0.015, while the NPSe possesses an average diameter of 99.0 nm with a PDI of 0.23 % ± 0.02. The selenium nanoparticles are smaller than the sulfur particles and have a zeta potential of approximately +23.26 mV.

2.4 Nematode toxicity studies

The purpose of this assay is to investigate the effect of organosulfur compounds on higher organisms, here on the nematode *Steinernema feltiae*.

In the nematode assay, the different sulfur compounds under investigation were used at several concentrations to check their reactivity and toxicity and their potential application in a agricultural context. The results of these nematode assay are provided in the following sections.

2.4.1 Activity of the cyclic disulfide compounds in *Steinernema feltiae*

The compounds 1,2-VDT and 1,3-VDT were tested to evaluate their potential effect against the nematode *Steinernema feltiae* (*S. feltiae*) and to investigate if the S-S-bond plays an important role for the activity of these compounds. Comparing the results in Figure 2.5., it is obvious that the reactivity of 1,3-VDT is considerably lower than the one of 1,2-VDT. This finding clearly indicates that the S-S-bond plays a major role as far as the activity of such cyclic compounds concerned.

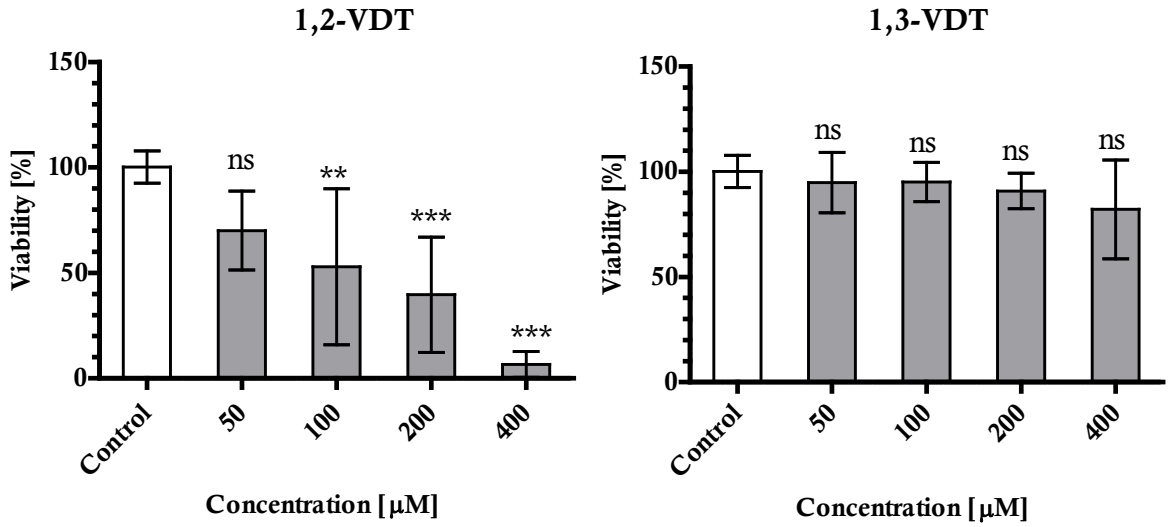


Figure 2.5: *S. feltiae* treated with 1,2-VDT and 1,3-VDT for 24 h, the control containing 1 % of DMSO, The control was normalized to 100 % viability. Significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Other cyclic disulfide compounds such as 1,2-dithiane and 1,5-dithiacyclooctane (1,5-DTCO) were also tested to comparable data in the nematode screen. The choice of 1,5-DTCO because the existence of two S*p*-type lone pairs of this compound which approach each other due to repulsive interaction between the sulfur atoms.[76] The results show that 1,2-dithiane has a moderate activity against the nematodes (67 %) at 400 µM. Nearly the same result is given for 1,5-DTCO, which has a less effect against *S. feltiae* (62 %) at 400 µM as well. The results are given in Figure 2.6.

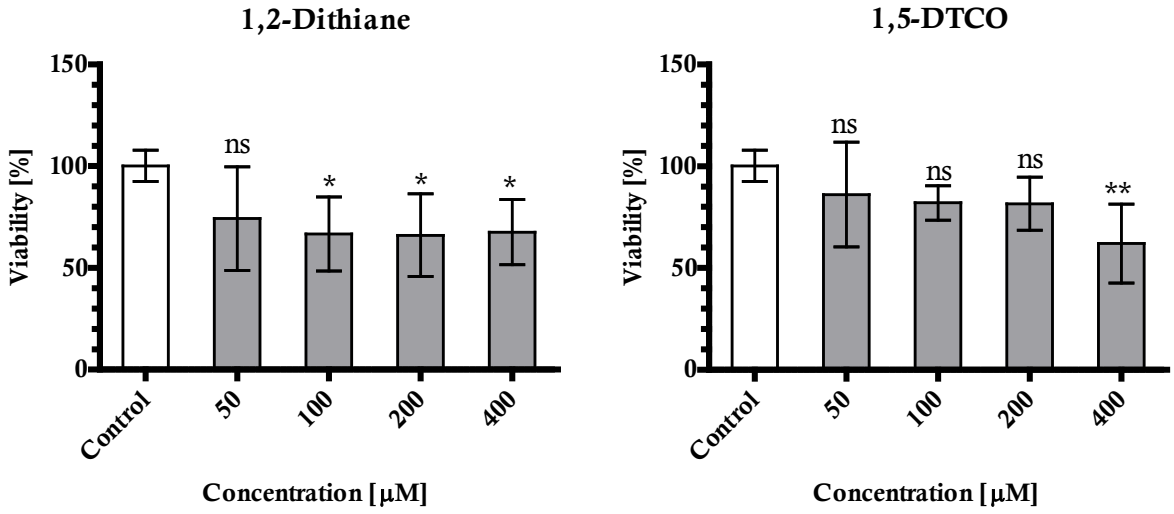


Figure 2.6: *S. feltiae* treated with 1,2-dithiane and 1,5-DTCO for 24 h, the control containing 1 % of DMSO, The control was normalized to 100 % viability. Significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Due to its cyclic structure, *α*-lipoic Acid (LA) and lipoamide (LAm) were also tested for comparison compounds. The data shows that LA and LAm have only low activity against *S. feltiae* (Figure 2.7.).

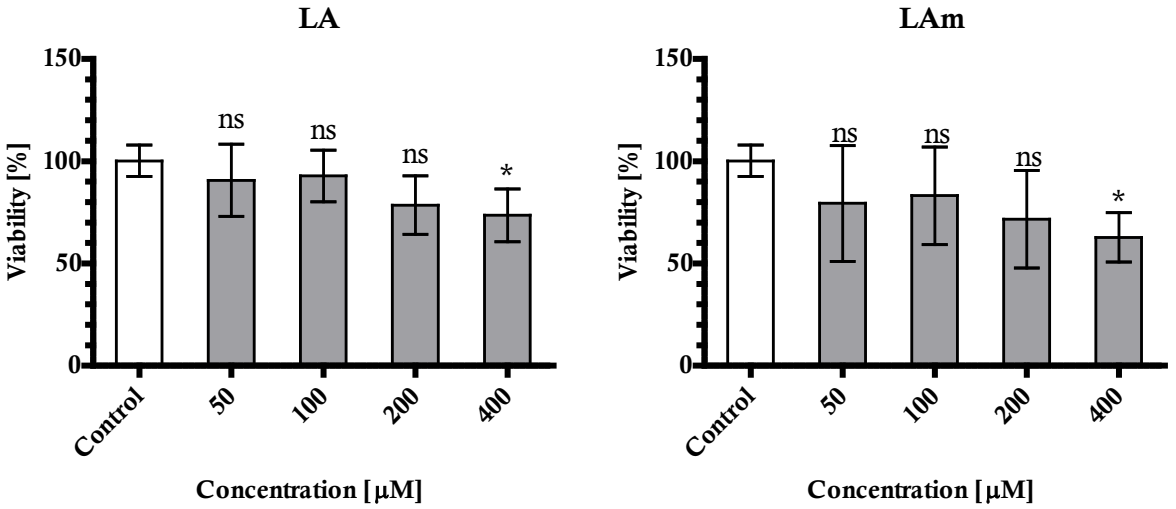


Figure 2.7: *S. feltiae* treated with LA and LAm for 24 h, the control containing 1 % of DMSO, The control was normalized to 100 % viability. Significances are expressed to the control. Data presented as viability % ± SD. Significances: Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The other cyclic disulfane compounds were also considerably tested. These compounds with dithiolethione ring system, such as DT, ADT, APT and Oltipraz were chosen due to modified at sulfur-sulfur ring with unsaturated sulfur-sulfur, *S*=*S* bond. Structurally, compared to the other cyclic disulfide these compounds are more stable. The results showed that these compounds do exhibit a rather pronounced activity against *S. feltiae*. As shown in Figure 2.8., dithiolethione (DT) possesses considerable toxicity against *S. feltiae* at concentrations above 100 µM. Compared to the other compounds and this study, DT shows the highest activity at concentrations of 200 and 400 µM. The activity of DT is even higher than the one of Oltipraz which is used as a commercially available schistosomicide reference compound Both molecules contain the *S*-*S*-bond in a five-ring membered system. DT is smaller and the molecular weight is lower, which may help the molecule to penetrate the nematode membrane easier and faster than Oltipraz. Also the double bond sulfur atom in DT may interact with sulfur proteins inside the biological membrane as well.

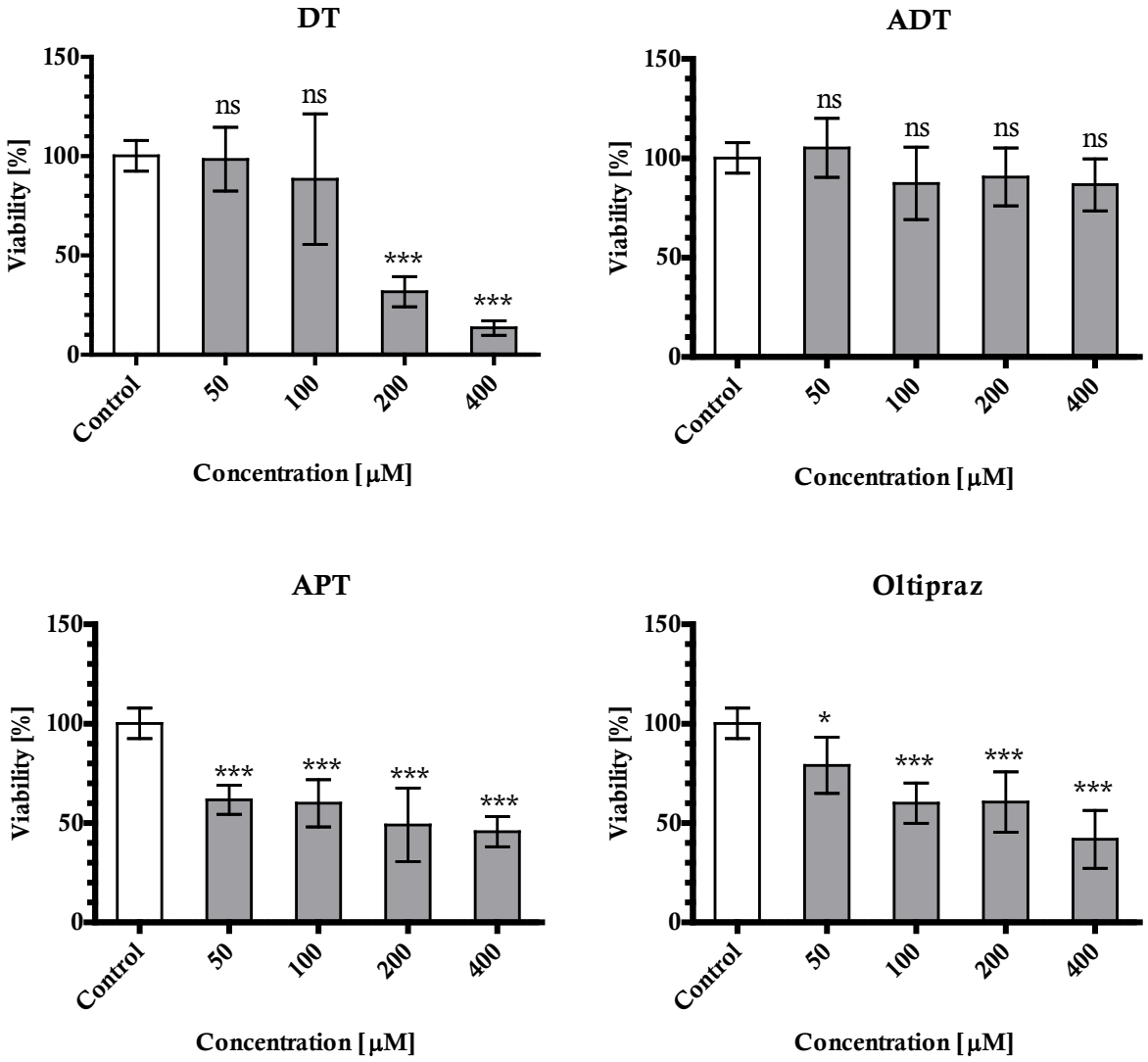


Figure 2.8: *S. feltiae* treated with DT, ADT, APT and Oltipraz for 24 h, the control containing 1 % of DMSO, The control was normalized to 100 % viability. Significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Overall, the cyclic disulfane compounds tested show some interesting, but not the highest activity in the *S. feltiae* assay. 1,2-VDT, the cyclic disulfide from garlic, and DT do have higher activities then other tested cyclic disulfane compounds. The linear DADS shows some activity against *S. feltiae* (30%) which proves that the reactivity is more likely due to the general

presence of the *S*-*S*-bond and not just to the same number of sulfur atoms or the ring system like into angular consistence (as in 1,3-VDT).

Additionally to the synthetic compounds tested, Harleem Oil (HO) was also used in the nematode assay. Due to the low solubility, the HO solution was sonicated or, alternatively centrifuged before it was applied to the nematodes. The two different preparations did not show any toxic effect against *S. feltiae* (Figure 2.9.).

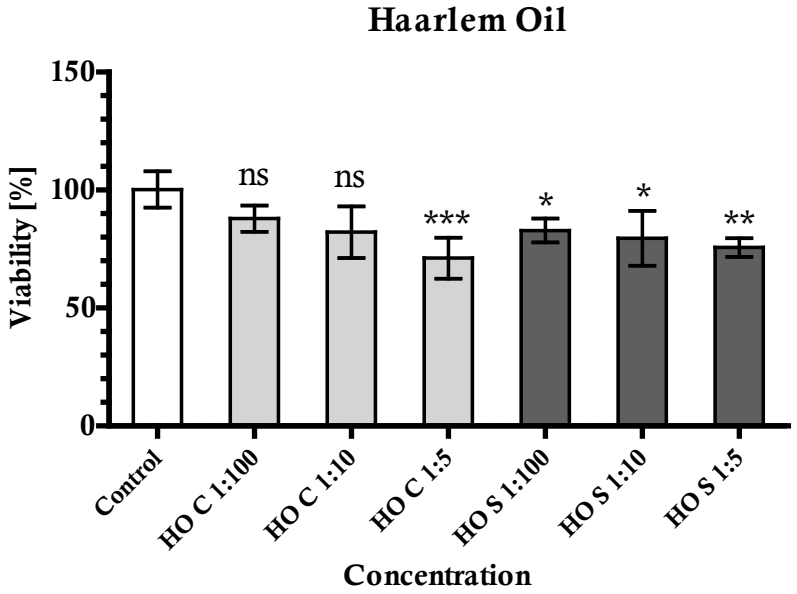


Figure 2.9: *S. feltiae* treated with HO for 24 h, the control containing only water was normalized to 100 % viability. Significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

To compare with cyclic disulfide, a linier disulfide compound such as diallydisulfide (DADS) was also tested. Here, DADS is used as the lead structure and all other cyclic disulfide compounds are used to see if the various structure can actually improve the activity of the disulfide bond.

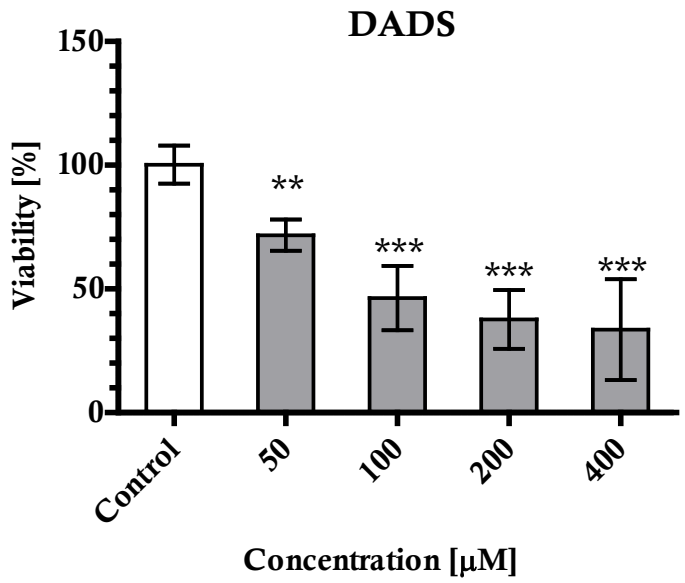


Figure 2.10: *S. feltiae* treated with DADS for 24 h, the control containing 1 % of DMSO, The control was normalized to 100 % viability. Significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.4.2 Activity of the nanoparticles in *Steinernema feltiae*

The activity of the polysulfanes such as diallyltrisulfide (DATS) and diallyltetrasulfide (DATTS) has been known against nematodes for several years. Intersetingly, as the activity of these compounds is a direct result of a longer sulfur-sulfur chain, the chemistry of the S_8 ring in elemental sulfur should be comparable to the one of polysulfanes due to the presence of similar sulfur-sulfur chains.[56] Following the procedure from the nematode assay used before to test the cyclic disulfanes, the nanoparticles were used in the same assay in amounts between 1 and 500 µg/ml. Figure 2.11. shows some nematodes before and after 24 h treatment with NPS at a sulfur concentration of 250 µg/ml.

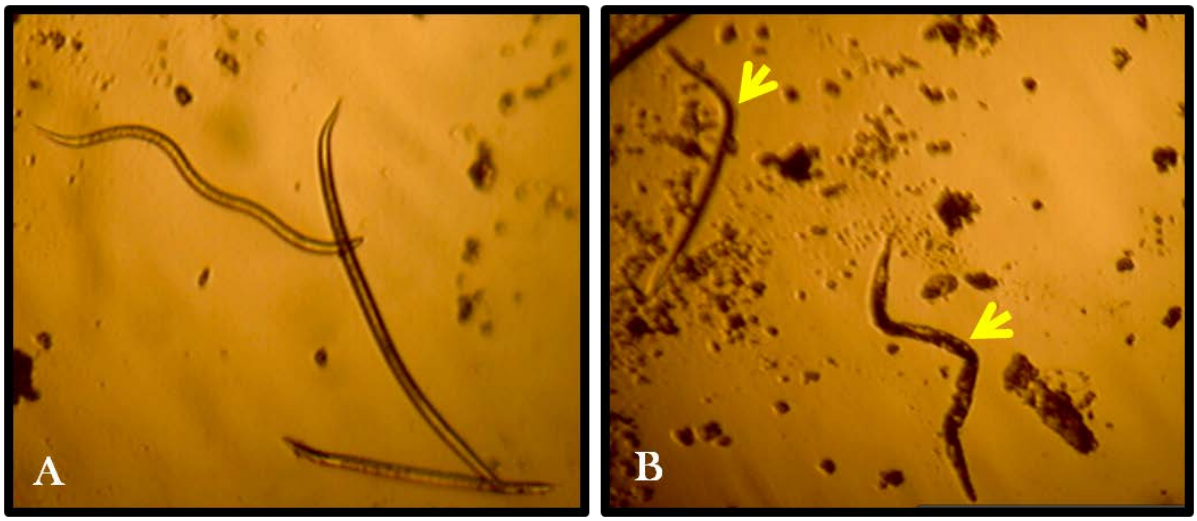


Figure 2.11: Effect of sulfur nanoparticles (NPS) on *Steinernema feltiae*. (A) in the control (the nematodes were very active); (B) after 24 h treatment, the nematodes are dead (yellow arrow). The viability was measured at four-fold magnification.

The activity of nanoparticles on *S. feltiae* after 24 h exposure is presented in Figure 2.12., the results show that sulfur-based nanoparticles exhibit considerable toxicity against *S. feltiae* with a LD_{50} value at around 6.99 µg/ml after 24 h incubation. This LD_{50} value is considerably lower than the ones from the polysulfanes such as DATS and DATTS .[56]. The NPS here killing *S. feltiae* (100 % death at 250 µg/ml). The selenium nanoparticles have also some activity, yet the latter is significantly lower than the one of NPS, the LD_{50} of NPSe is around 43.50 µg/ml.

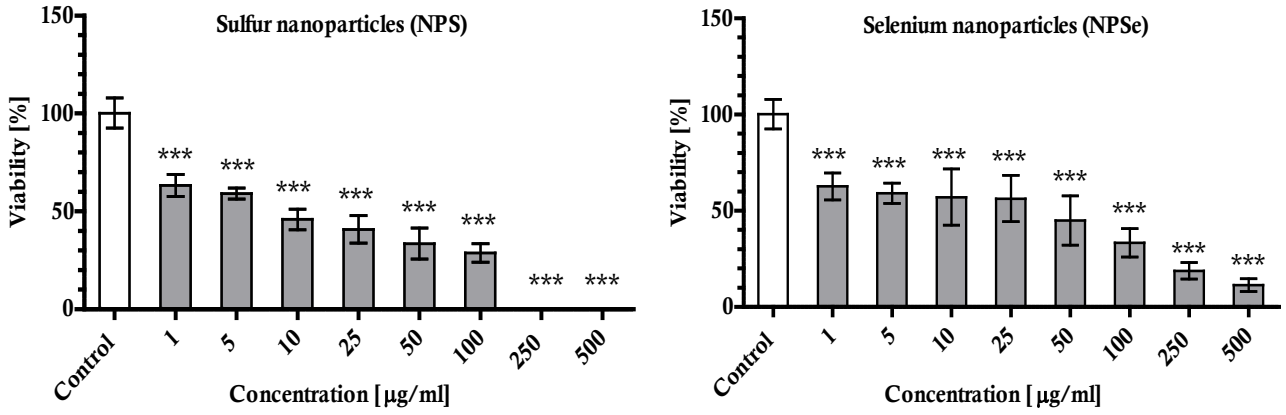


Figure 2.12: Nematicidal assay on *S. feltiae* treated with the nanoparticles for 24 h, the control containing water was set at 100 % viability. Significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.5 *Botrytis assay*

In this assay four different strains of *Botrytis cinerea* were used. These strains are shown in Table 2.2. The multidrug resistant (MDR) phenotypes are usually correlated with increased drug efflux through the overexpressed transporters.[61]

Some of the compounds tested do show toxic effects only in the case of the wild-type strain, but not against the mutants. This is most likely due to the fact that some compounds are selectivity transported out of the fungus through specific transport systems.

Table 2.2: The strains of *Botrytis cinerea* are used in *Botrytis* assay.

Strain	Description
BO5.10 (WT)	a sensitive lab strain, wild type
MDR 1	multi drug resistant strain due to the over expression on ABC and atrB transporter.
MDR 1*	multi drug resistant strain due to the higher over expression on ABC and atrB transporters
MDR 2	multi drug resistant strain due to the over expression on MFS transporter MfsM2.

In this assay, ethanol and DMSO were used as solvents to dissolve the compounds (between 0.05 and 1.36 % (v/v) for EtOH, between 0.01 to 1.5 % (v/v) for DMSO). The concentrations of the compounds tested were between 0.05 mM and 3.2 mM.

Overall, 3*H*-1,2-dithiol-3-tihone or dithiolethione (DT) and *α*-lipoic acid (LA) show lower MIC_{50}-values compared to the other compounds. In contrast, Oltipraz exhibited a higher MIC_{50}-values in all strains tested. Table 2.3. shows that DT and LA were more active than the other compounds tested in all the strains.

Table 2.3: The MIC_{50} values of cyclic disulfide compounds obtained against *Botrytis cinerea* strains after 48 h exposure. Data presented as means .±.SD.

Cyclic disulfides compounds	**MIC_{50} values [mM]**			
	Strains of *Botrytis cinerea*			
	WT	**MDR 1**	**MDR 1***	**MDR 2**
1,2-VDT	1.27 ± 0.0	0.20 ± 0.0	0.37 ± 0.1	1.01 ± 0.3
1,3-VDT	1.27 ± 0.0	1.17 ± 0.1	1.0 ± 0.3	1.22 ± 0.0
LA	0.33 ± 0.0	0.36 ± 0.0	0.32 ± 0.0	0.32 ± 0.1
DT	0.08 ± 0.0	0.23 ± 0.0	0.21 ± 0.0	0.51 ± 0.0
Oltipraz	1.38 ± 0.0	1.38 ± 0.2	1.47 ± 0.1	1.52 ± 0.0

Figure 2.13. shows that DT is very active against the spores of the MDR 2 strain at 0.4 mM. At concentrations of 0.4 and 0.6 mM DT also shows some good activity against the MDR 1, MDR 1* strains and WT strain. *α*-lipoic acid (LA) behaved similar to DT in its inhibition of the fungi or the fungal growth.

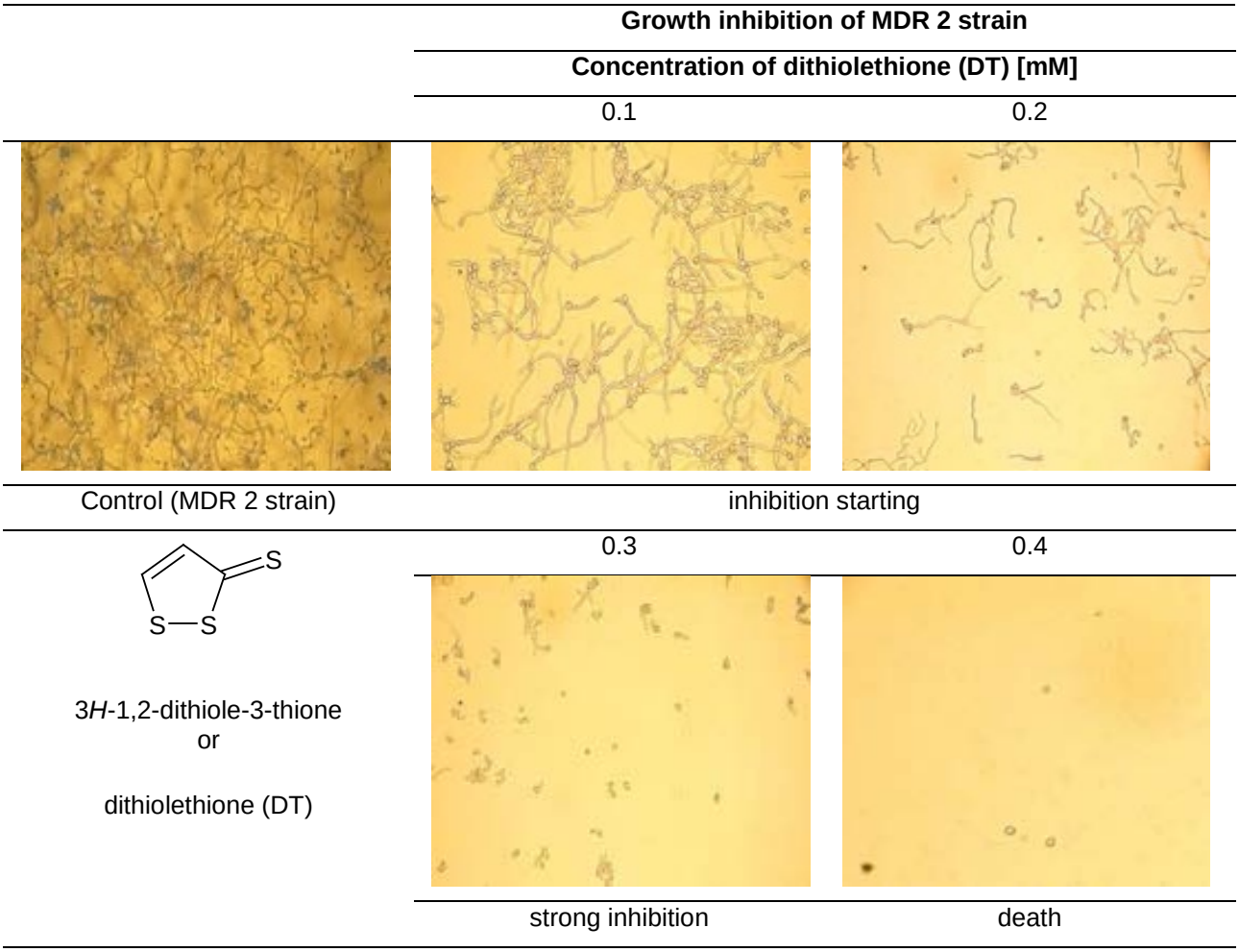


Figure 2.13: The MDR 2 strains treated with dithiolethione (DT) for 48 h, incubation at room temperature. The strains were observed under Nicon Eclipse TS 100 microscope. Magnification 100x.

Interestingly, 1,2-VDT which contains the cyclic S-S structure is once more active than 1,3-VDT (the isomer without a S-S-bond) against of the MDR1 and MDR1* strains at 0.20 and 0.37 mM

respectively. In contrast, Oltipraz shows little activity against the fungal growth in all of the strains tested.

Overall, cyclic disulfide compounds with cyclic S-S structure show some moderate inhibition against the fungal growth.

2.6. Impact of the organo sulfur compounds on the survival of the Neuro 2A cells

In this project, the Neuro 2A cell line was used to investigate the effect of OSCs on such cells in the presence and absence of H_2O_2. In the past, some specially designed chalcogen molecules showed interesting antioxidant effects in cells when incubated together with H_2O_2. Selenium containing compounds and in particular selenium nanoparticles are here for their high antioxidant activity, while sulfur compounds generally do show a higher prooxidant activity. Nonetheless it often depends on the cellular environment if the chalcogen compounds do act as pro- or antioxidants.[21,65,77-80]

Oxidative stress (OS) induces cell injury, or moreover, can cause cell damage or direct cell death. In neuronal cells, a variety of OS mechanisms have been proposed. H_2O_2 causes apoptosis in cortical neurons by attacking DNA immediately or through activation of endonucleases which could harm DNA and ultimately cause cell death.[77,78] Another effect of H_2O_2 is interference of the mitochondrial function by preventing the accumulation of mitochondrial Ca^{2+}. This will consequently reduce ATP production inside the mitochondria which will ultimately lead to cell death.[77,81]

2.6.1 Activity of H_2O_2 on Neuro 2A cell line

Figure 2.14 shows the effects of H_2O_2 on the Neuro 2A cell line at different concentrations of this oxidative stress (100–1000 µM). At 100 µM, only 75 % of the cells are still viable which is already a significant decrease compared to the control. At 1000 µM, H_2O_2 is absolutely toxic against the Neuro 2A cells with a survival rate of just 5 %. The difference between 100 and 250 µM is not significant. A concentration of 250 µM of H_2O_2 was therefore used in the subsequent experiments to investigate the effects of the OSCs on the oxidative stressed cells in the absence and in the presence of H_2O_2. Due to the high GSH level, the Neuro 2A cells should be more

resistant against OS than other cells. Figure 2.14 shows that the resistance is so significant against OS.

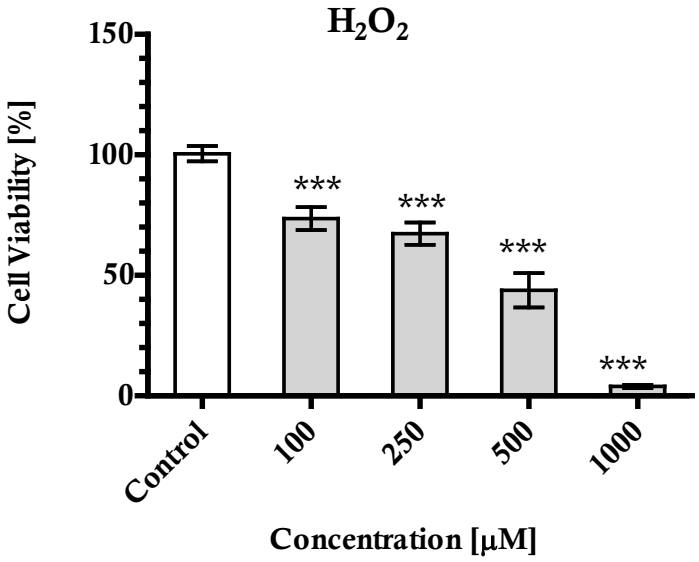


Figure 2.14: Survival assay of H_2O_2 on Neuro 2A cells for 24 h on 96-well plate. The density of the cells was 10,000 cells per well. The control containing water was normalized to 100 % viability, significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.6.2 Activity of cyclic disulfide compounds on Neuro 2A cell line

Neuro 2A cells were incubated with various concentrations of the cyclic disulfide compounds in the absence and in the presence of 250 µM H_2O_2. The final concentration of the compounds tested in this assay was between 1 and 100 µM. The results are shown Figure 2.15. Surprisingly, none of the sulfur containing compounds used did show any significant toxicity against the Neuro 2 A cells in absence of H_2O_2 (white bars) my be due to high levels of GSH in the cells. In the presence of H_2O_2 (gray bars) all the substances tested showed a comparably weak, but not statistically significant cytotoxicity.

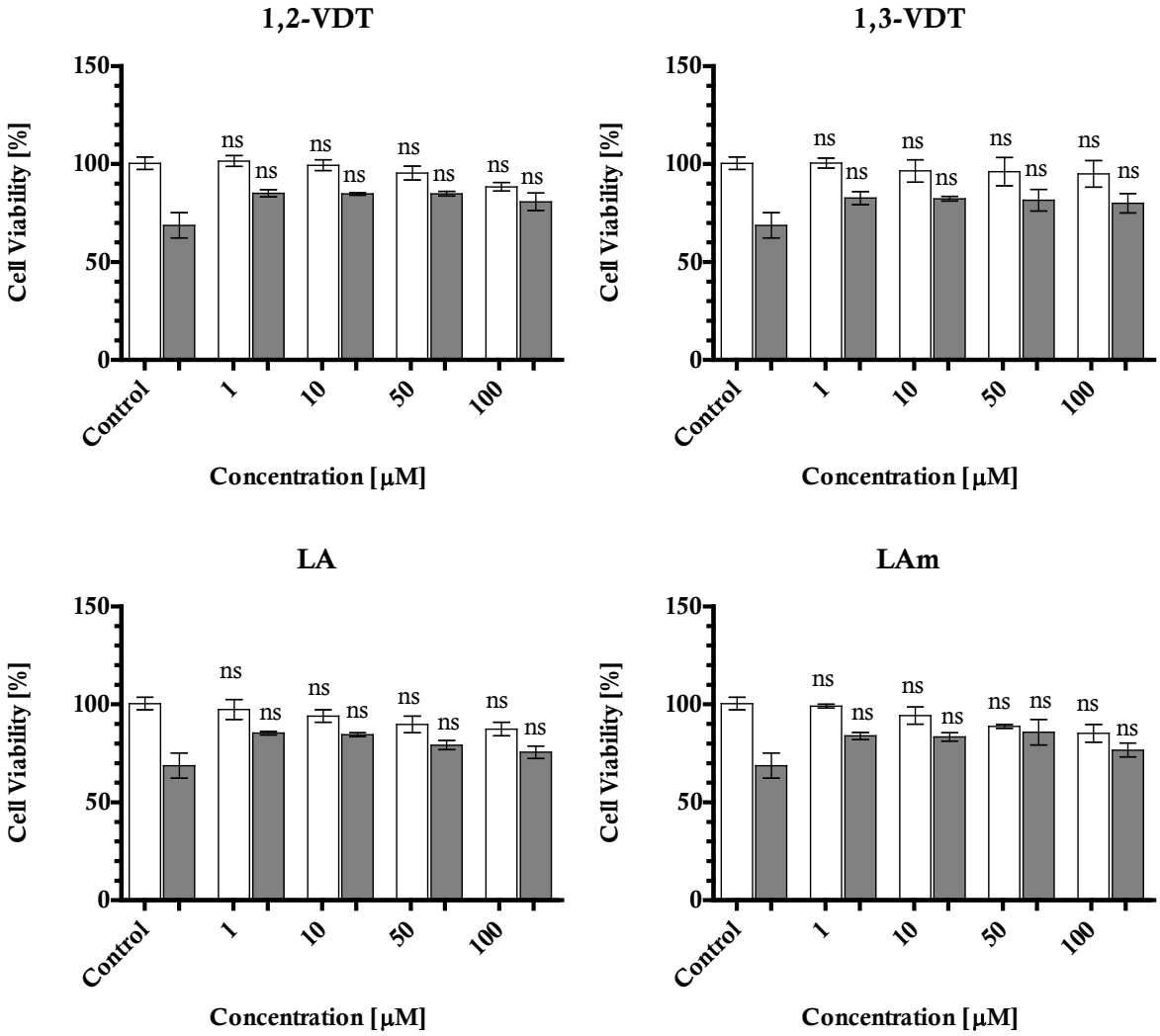


Figure 2.15: Survival assay of 1,2-VDT, 1,3-VDT, LA and LAm on Neuro 2A cells for 24 h on 96-well plate. The density of the cells was 10,000 cells per well. White bars, the compounds were tested in the absence H_2O_2 and gray bars the compounds were tested in the presence H_2O_2. The control containing 1 % of DMSO was normalized to 100 % viability, significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In contrast, 1,2-dithiane and 1,5-DTCO exhibited a stronger toxican effect in the neuronal cells at 100 µM in absence of H_2O_2 (Figure 2.16). When these compounds were tested in the presence of H_2O_2 (gray bars) however they showed less toxicity. This could be a hint for the prooxidant character of these two compounds.

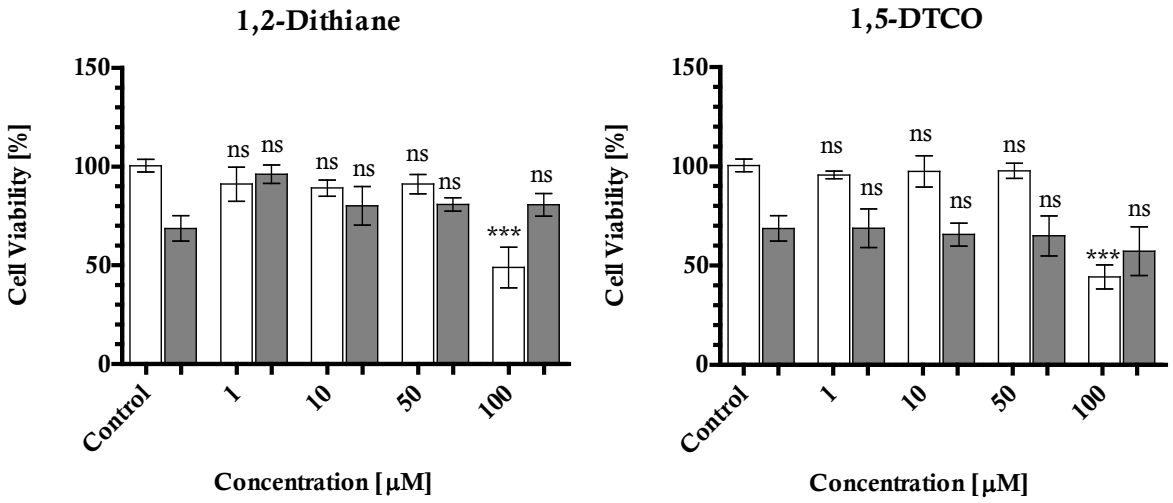


Figure 2.16: Survival assay of 1,2-dithiane and 1,5-DTCO on Neuro 2A cells for 24 h on 96-well plate. The density of the cells was 10,000 cells per well. White bars, the compounds were tested in the absence H_2O_2 and gray bars the compounds were tested in the presence H_2O_2. The control containing 1 % of DMSO was normalized to 100 % viability, significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The morphological structures of the Neuro 2A cells treated with 1,2-dithiane and 1,5-DTCO are presented in Figure 2.17. The pictures show that morphological changes were apparent in bubbling cell bodies when examined 24 h after treatment. The cells were also stained with calcein-AM and propodium iodide (PI) to see the difference between living and dead cells. Living cells emit strong green fluorescence due to the calcein-AM enters to the cell and is retained in the cytoplasm due to hydrolyis by esterases in viable cells, while the dead cells emit red fluorescence due to PI reaches the nucleus by passing through membranes of dead cells and then intercalates with the DNA double strand to produce a red fluorescence.

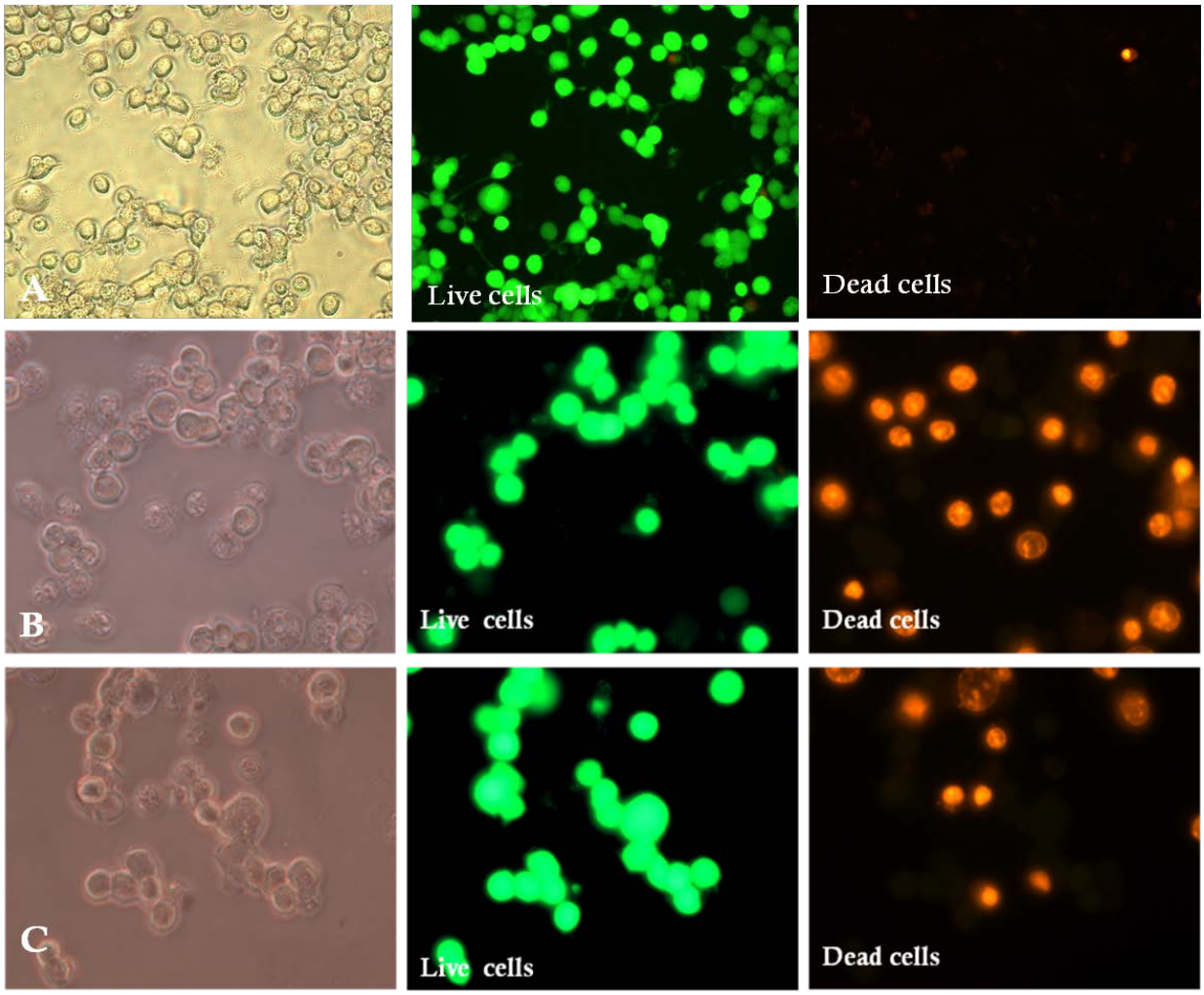


Figure 2.17: Survival assay of Neuro 2A cells upon treatment with 1,2-dithiane and 1,5-DTCO. Neuronal cells were plated at a density of 10,000 cells/well in 96-well tissue culture plate. After 24 h, the culture medium containing the organosulfur compounds was replaced with 0.2 ml of dye solution (calcein-AM and PI) and cells were observed under the microscope (Magnification 40 x). A. Control (untreated cells); B. Cells treated with 100 µM of 1,2-dithiane; and C. Cells treated with 100 µM of 1,5-DTCO.

The other cyclic disulfide compounds such as, DT, ADT, APT and Oltipraz, were also tested on Neuro 2A cells. The results presented in Figure 2.18 show that these compounds do not have any toxic effects on Neuro 2 A cells in the absence (white bars) or in the presence (gray bars) of H_2O_2.

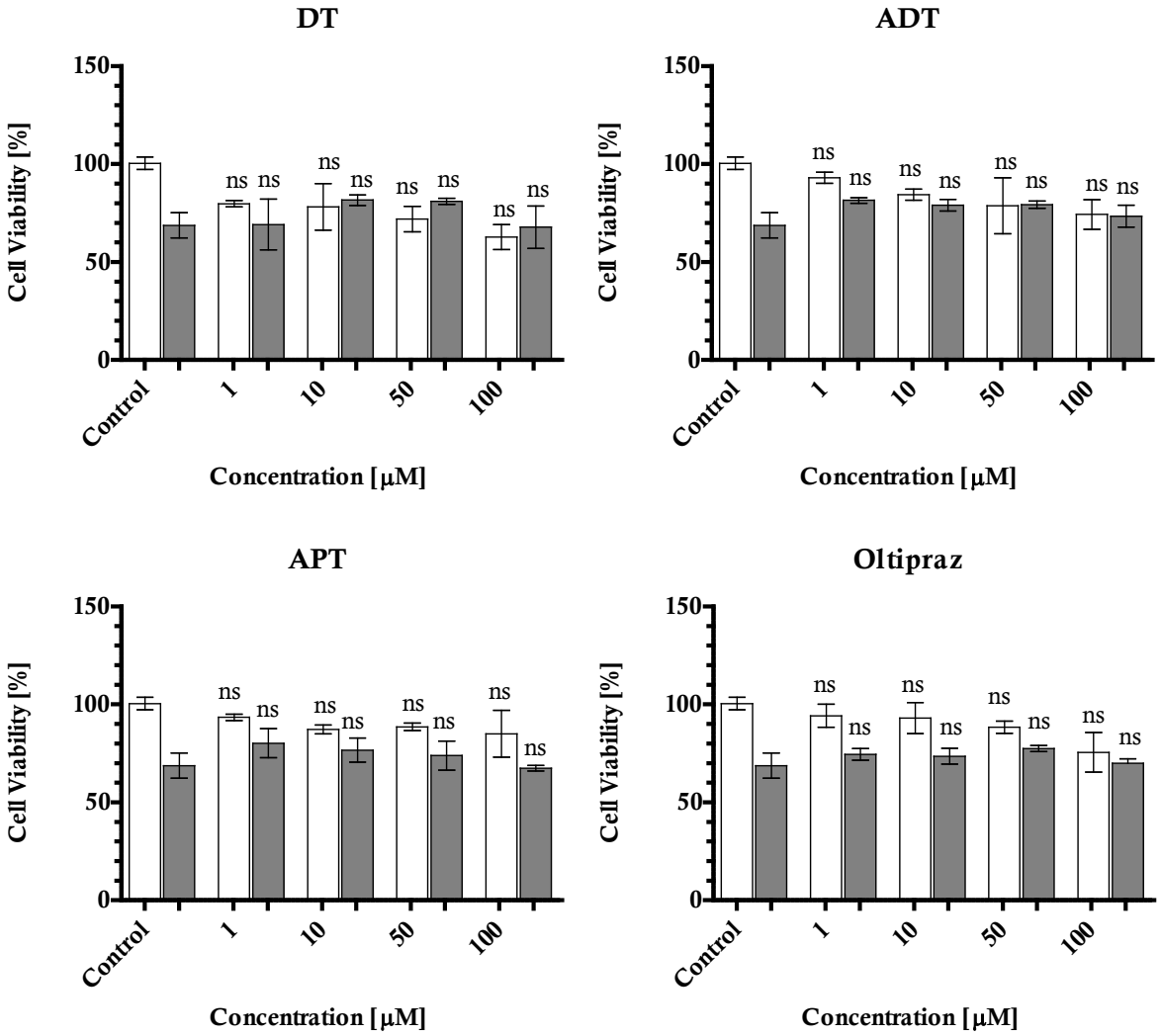


Figure 2.18: Survival assay of DT, ADT, APT and Oltipraz on Neuro 2A cells for 24 h on 96-well plate. The density of the cells was 10,000 cells per well. White bars, the compounds were tested in the absence H_2O_2 and gray bars the compounds were tested in the presence H_2O_2. The control containing 1 % of DMSO was normalized to 100 % viability, significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In the case of Haarlem Oil (HO), the experiment shows moderate activity of HO against the Neuro 2A cells at a dilution of 1:1 (viability of 6% in the absence of H_2O_2). In presence of H_2O_2 (gray bars), HO shows moderate toxicity, which is somewhat surprising as HO is sold as an

antioxidant. Nonetheless, the concentrations of HO applied in this experiment are unlikely to be found in the human body after oral consumption of HO.

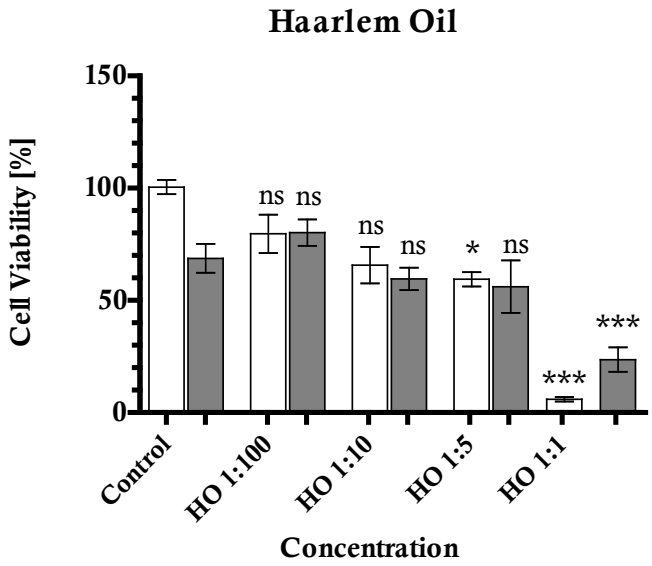


Figure 2.19: Survival assay of HO on Neuro 2A cells for 24 h on 96-well plate. The density of the cells were 10.000 cells per well. White bars, the compounds were tested in the absence H_2O_2 and gray bars the compounds were tested in the presence H_2O_2. The control containing water was normalized to 100 % viability, significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The morphological structures of the Neuro 2A cells after treatment with Haarlem Oil are shown in Figure 2.20. Changes in morphology lead to bubbling cells when the cells were exposed to HO at dilution 1:5 for 24 h. As in the previous experiments cells were stained with calcein-AM and PI to analyse the ratio of living against dead cells.

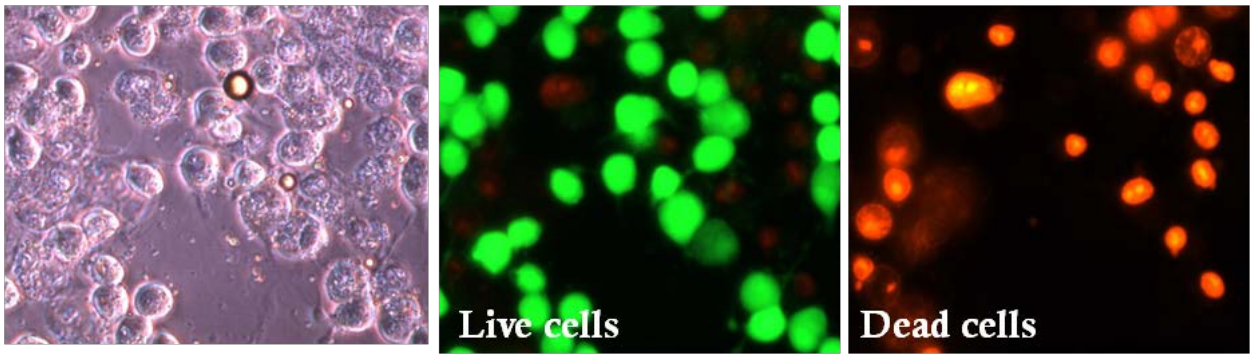


Figure 2.20: Survival assay of Neuro 2A cells upon treatment with HO. Neuronal cells were plated at a density of 10,000 cells/well in 96-well tissue culture plate. After 24 h, the culture medium containing the HO was replaced with 0.2 ml of dye solution (calcein-AM and PI) and cells were observed under the microscope (Magnification 40 x). Cells treated with HO 1:5.

2.6.3 Activity of polysulfane compounds on Neuro 2A cell line

Linear disulfide compound such as DADS and polysulfane compounds, such as DATS, DPTS, DATTS and DPTTS showed different activities in the neuronal cell line. DADS, DATS and DPTS showed no activity on Neuro 2A cells, while DATTS and DPTTS do slighty a significant toxicity. One reason for this result may be the different sulfur-sulfur chain length found in these molecules. The longer the *S-S* chain length is seem to be the activity. Similar results were already found in other experiments with other biological material in the past.[56] Interestingly the compounds with an allyl-group showed more activity compared to the compounds with a propyl group. The results are shown in Figure 2.21.

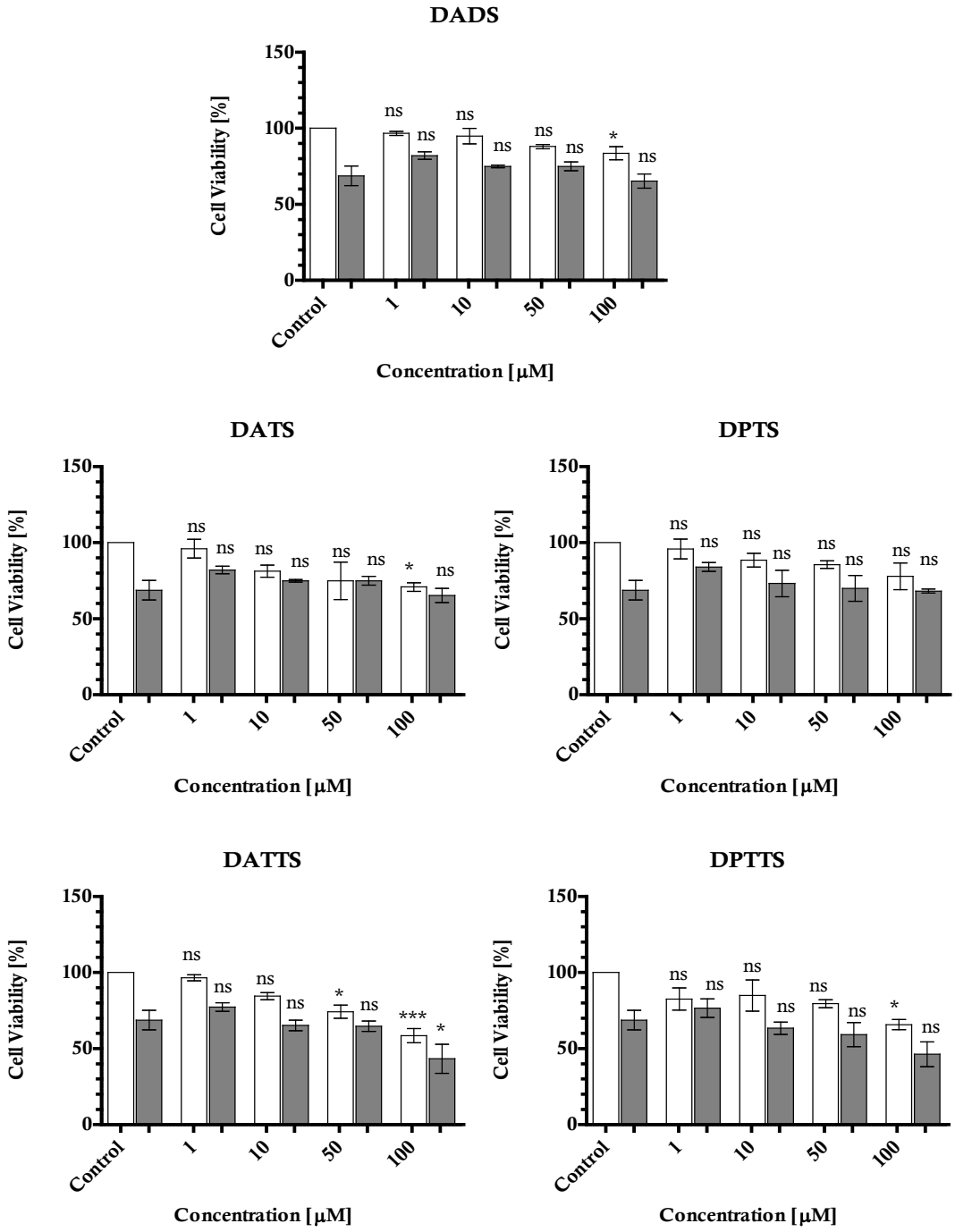


Figure 2.21: Survival assay of DADS, DATS, DPTS, DATTS and DPTTS on Neuro 2A cells for 24 h on 96-well plate. The density of the cells was 10,000 cells per well. White bars, the compounds were tested in the absence H_2O_2 and gray bars the compounds were tested in the presence H_2O_2. The control containing 1 % of DMSO was normalized to 100 % viability, significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

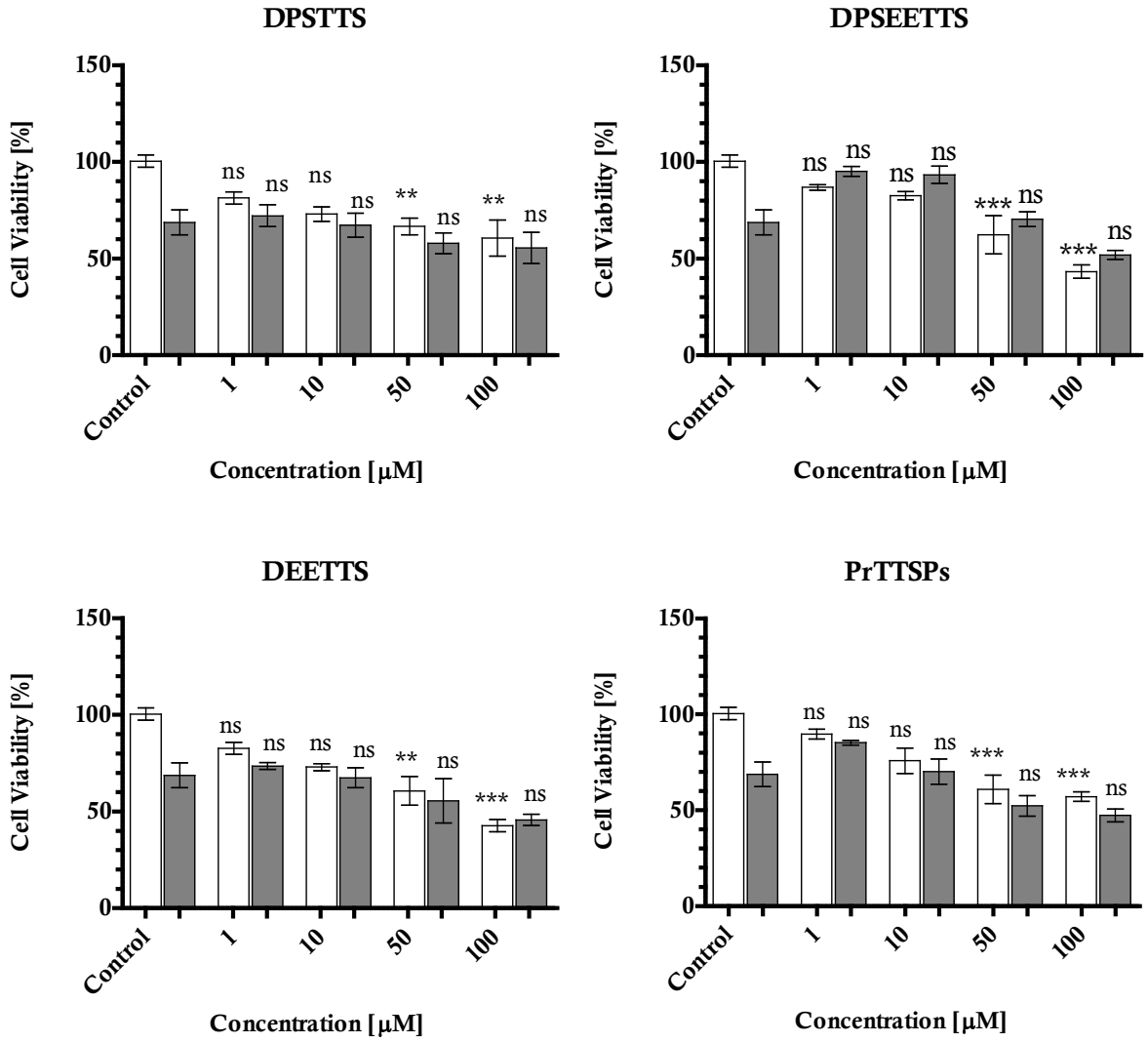


Figure 2.22: Survival assay of DPSTTS, DPSEETTS, DEETTS and PrTTSPs on Neuro 2A cells for 24 h on 96-well plate. The density of the cells was 10,000 cells per well. White bars, the compounds were tested in the absence H_2O_2 and gray bars the compounds were tested in the presence H_2O_2. The control containing 1 % of DMSO was normalized to 100 % viability, significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Another derivatives of polysulfanes, including DPSTTS, DPSEETTS, DEETTS, and PrTTSPs were also tested under the same conditions on the Neuro 2A cells. All these compounds contain a tetrasulfur chain and show a weak cytotoxic effect on neuronal cells at concentrations of around 50 to 100 µM (Figure 2.22).

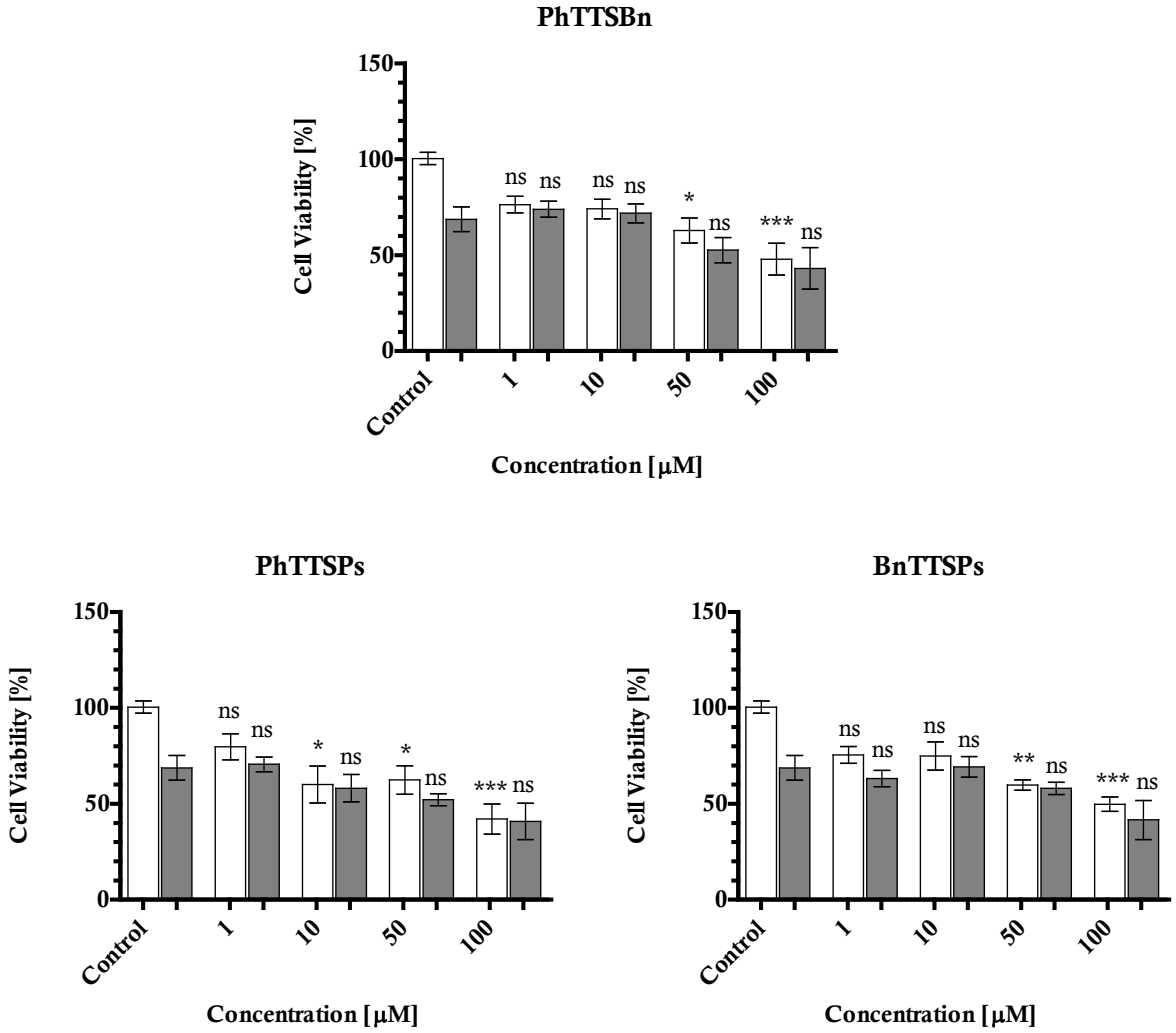


Figure 2.23: Survival assay of PhTTSBn, BnTTSPs and PhTTSPs on Neuro 2A cells for 24 h on 96-well plate. The density of the cells was 10,000 cells per well. White bars, the compounds were tested in the absence H_2O_2 and gray bars the compounds were tested in the presence H_2O_2. The control containing 1 % of DMSO was normalized to 100 % viability, significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The asymmetric aromatic tetrasulfanes such as PhTTSBn, BnTTSPs, PhTTSPs and do both show a weak activity against Neuro 2A cells in the absence and in the presence of H_2O_2 which is shown in Figure 2.23.

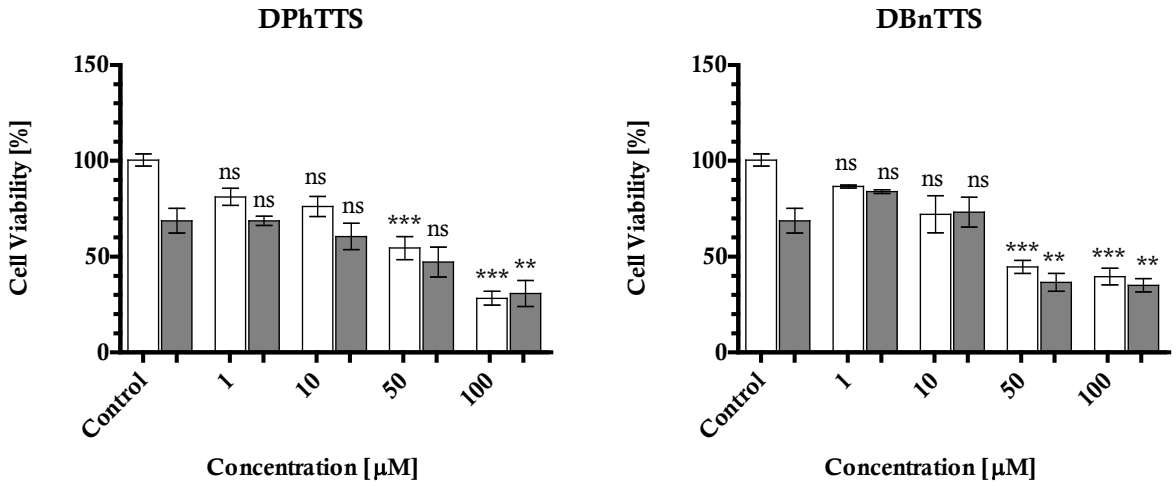


Figure 2.24: Survival assay of DPhTTS and DBnTTS on Neuro 2A cells for 24 h on 96-well plate. The density of the cells was 10,000 cells per well. White bars, the compounds were tested in the absence H_2O_2 and gray bars the compounds were tested in the presence H_2O_2. The control containing 1 % of DMSO was normalized to 100 % viability, significances are expressed to the control. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Comparing the data, DPhTTS was the most active compound and showed a greater cytotoxicity than other compounds, with only around 28 % of cell viability left when used in a concentration of 100 µM (at the same concentration, DBTTS resulted in around 38 % of remaining viability). These results also confirm a different activity as far as benzyl- and phenyl-side chains. Phenyl containing compound slightly more active than benzyl one. (Figure 2.24).

2.6.4 The summary of IC_{50} values of organo sulfur compounds in the Neuro 2A cell line

The IC_{50} values of the various OSCs are presented in Table 2.4.

Table 2.4: The IC_{50} values of OSCs on Neuro 2A cell line (data are presented as mean ± SD)

Compound	IC_{50} value in the absence of H_2O_2 [μM]	IC_{50} value in the presence of H_2O_2 [μM]
A. Linear disulfide		
diallyldisulfide (DADS)	>100	>100
B. Cyclic disulfides		
1,2-VDT	>100	>100
1,3-VDT	>100	>100
1,2-dithiane	79.5 ± 0.1	>100
1,5-DTCO	80.9 ± 8.0	>100
LA	>100	>100
LAm	>100	>100
DT	>100	>100
ADT	>100	>100
APT	>100	>100
oltipraz	>100	>100
C. Polysulfanes		
diallyltrisulfide (DATS)	>100	>100
dipropyltrisulfide (DPTS)	>100	>100
diallyltetrasulfide (DATTS)	>100	>100
Dipropyltetrasulfide (DPTTS)	>100	>100
dipropylacidtetrasulfide (DPSTTS)	>100	>100
Dipropylethanoicacidtetrasulfide (DPSEETTS)	65.3 ± 13.7	52.5 ± 3.0
diethylethertetrasulfide (DEETTS)	81.6 ± 16.7	40.2 ± 18.2
propyltetrasulfidepropylacid (PrTTSPs)	>100	>100
phenyltetrasulfidepropylacid (PhTTSPs)	76.8 ± 5.5	52.0 ± 22.2
benzyltetrasulfidepropylacid (BnTTSPs)	>100	>100
phenyltetrasulfidebenzyl (PhTTSBn)	>100	51.9 ± 12.1
dibenzyltetrasulfide (DBnTTS)	34.4 ± 18.3	37.6 ± 13.0
diphenyltetrasulfide (DPhTTS)	60.9 ± 25.0	65.5 ± 8.8

2.6.5 Activity of nanoparticles on Neuro 2A cell line

As seen before, sulfur nanoparticles and selenium nanoparticles exhibit a strong toxicity against *S. feltia*. In this experiment, the nanoparticles were therefore tested on the Neuro 2A cells to compare their activity with the one of di- and polysulfanes.

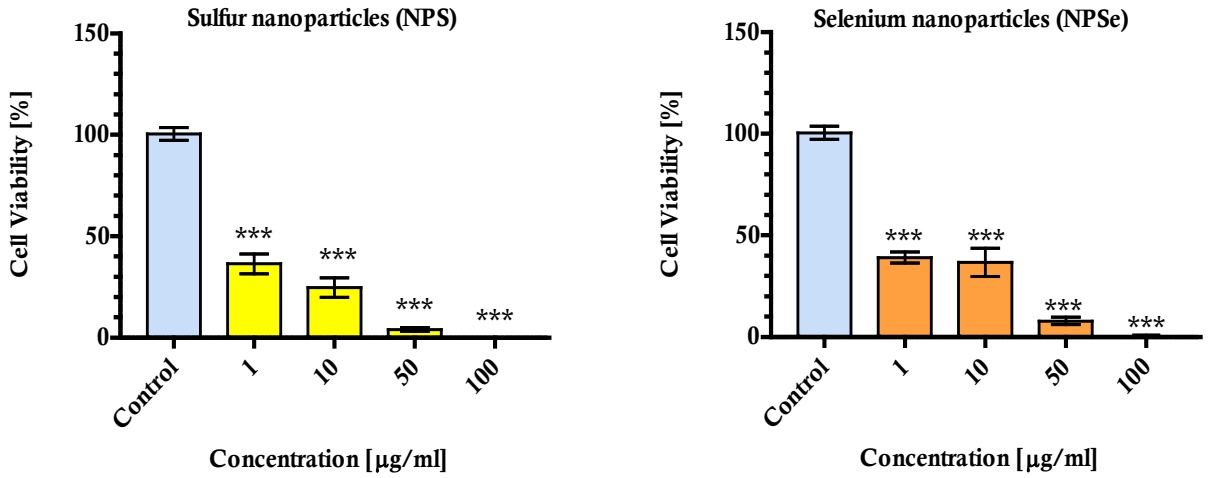


Figure 2.25: Survival assay of Neuro 2A cells exposed to chalcogen nanoparticles for 24 h. The assay was performed on 96-well plate, the density of the cells was 10,000 cells per well. After these treatments, cells were washed with PBS and viability was assessed by dyes solution (calcein-AM and PI). The control containing water was set at 100 % viability. Significances are expressed to the control. Data shows means of three independent experiments. Data presented as viability % ± SD. Significances: *ns* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The results show that both of the chalcogen nanoparticles, *i.e.* NPS and NPSe are active against Neuro 2A cells when employed at a concentration of at around 1µg/ml (Figure 2.25.). The IC_{50}-values determined for them are below 1 µg/ml for NPS and NPSe respectively.

2.7 Electrochemical analysis: Cyclic Voltammetry

Cyclic voltammetry (CV) is an electrochemical technique to analyse the redox properties of organic and inorganic compounds.[67,82] In this particular study, the different redox potentials of OSCs could provide a hint for the chemical reactivity and biological activity of the different molecules. Also statements about the antioxidant or prooxidant behavior of the OSCs could be derived from their redox potentials. To analyze the OSCs under appropiate conditions (*e.g.* in phosphate buffer at pH 7.4), a dropping mercury was used [83,84] All measurements were performed using a platinum wire counter electrode and a silver/silver-chloride electrode as a reference electrode with a standard scan rate of 250 mV/s. The experiment was carried out together with Uma M. Viswanathan, a PhD student in Prof. Jacob's group and under supervise by Dr. Torsten Burkholz.

2.7.1 Electrochemistry of cyclic disulfide compounds

1,2-VDT, 1,2-dithiane and 1,5-DTCO were analyzed in the absence and in the presence of glutathione (GSH) as an internal reference standard. Anodic signals (Ep_a) are obtained due to oxidation of the reduced forms of the compounds, while reduction signals (Ep_c) are obtained due to reduction of the oxidized forms of the compounds.[85] As expected, 1,3-VDT shows no oxidation or reduction signal (no *S*-*S* bond in the molecule). The voltammogram obtained in the presence of this compound is therefore not shown.

Table 2.5: Summary of oxidation potentials (Ep_a) and reduction potentials (Ep_c) of cyclic disulfide compounds in the absence and in the presence of GSH. Data presented as mean ± SD.

Potentials	Compound	GSH	mix (Compound + GSH)
	1,2-VDT)		
Ep_a (mV)	-618 ± 1.0		-615. ± 2.5
		-412 ± 4.3	-409 ± 3.8
Ep_c (mV)	-650 ± 1.6		-656 ± 1.9
		-470 ± 1.0	-426 ± 7.2
	1,2-dithiane		
Ep_a (mV)	-540 ± 1.6		-540 ± 0.0
		-412 ± 4.3	-404 ± 31.0
Ep_c (mV)	-561 ± 1.2		-585 ± 1.2
		-470 ± 1.0	-422 ± 1.9
	1,5-DTCO		
Ep_a (mV)	-535 ± 3.8		-535 ± 4.1
		-412 ± 4.3	-352 ± 3.4
Ep_c (mV)	-556 ± 1.0		-556 ± 4.0
		-470 ± 1.0	-418 ± 3.3

The CVs of 1,2-VDT, 1,2-dithiane and 1,5-DTCO show more negative potentials compared to GSH (here GSH used as an internal reference standard). The reduction (Ep_c) signals for these compounds were not affected when GSH was presence together (the Ep_c value of the compounds only are -650, -561 and -556 mV for 1,2-VDT, 1,2-dithiane and 1,5-DTCO respectively, while the Ep_c value of the compounds in the presence of GSH are -656, -585 and -556 mV for 1,2-VDT, 1,2-dithiane and 1,5-DTCO respectively). The reduction (Ep_c) signals for GSH alone (Ep_c = -470 mV) however were shifted positively towards higher potentials when GSH was present together with the compounds (Ep_c = -426, -422, and -418 for 1,2-VDT, 1,2-dithiane and 1,5-DTCO respectively) (Table 2.5).

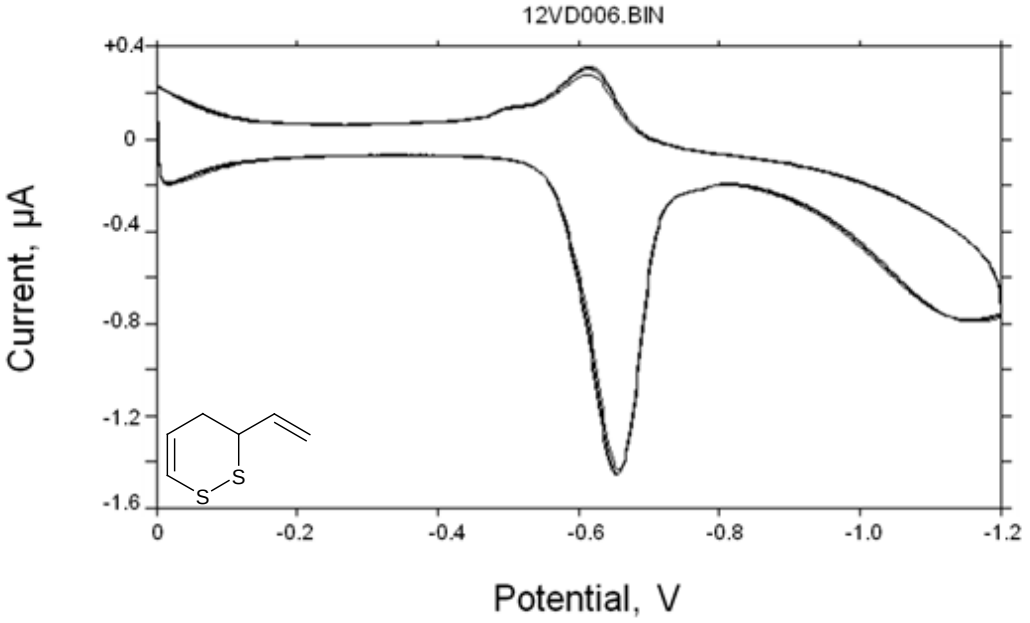


Figure 2.26: The cyclic voltammogram of 1,2-VDT, Ep_a = -618 mV, Ep_c = -650.

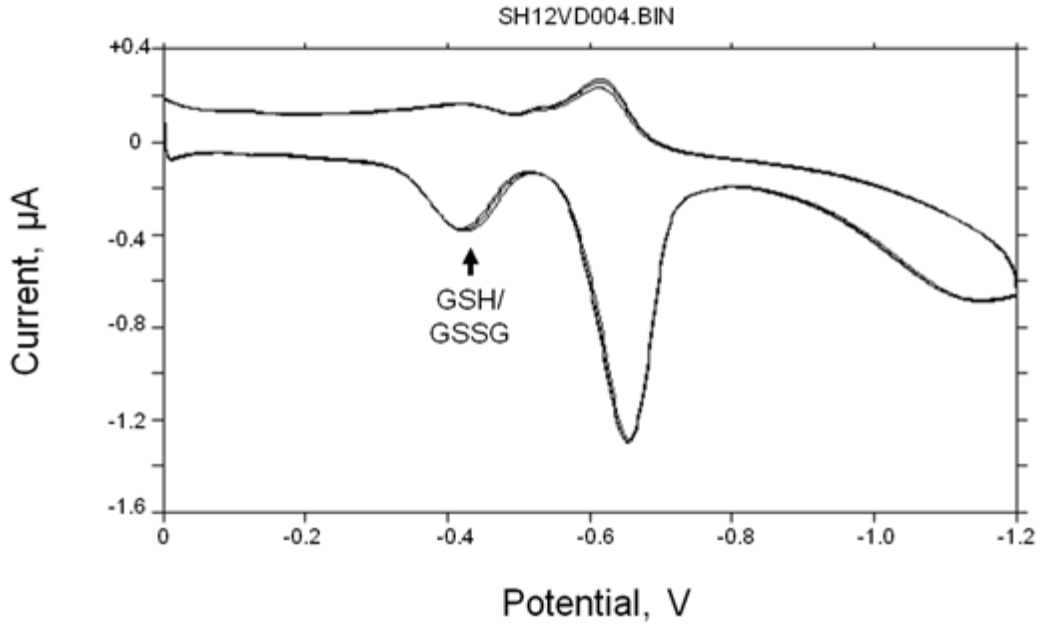


Figure 2.27: The cyclic voltammogram of 1,2-VDT in the presence of GSH, Ep_a = -615 mV, Ep_c = -656.

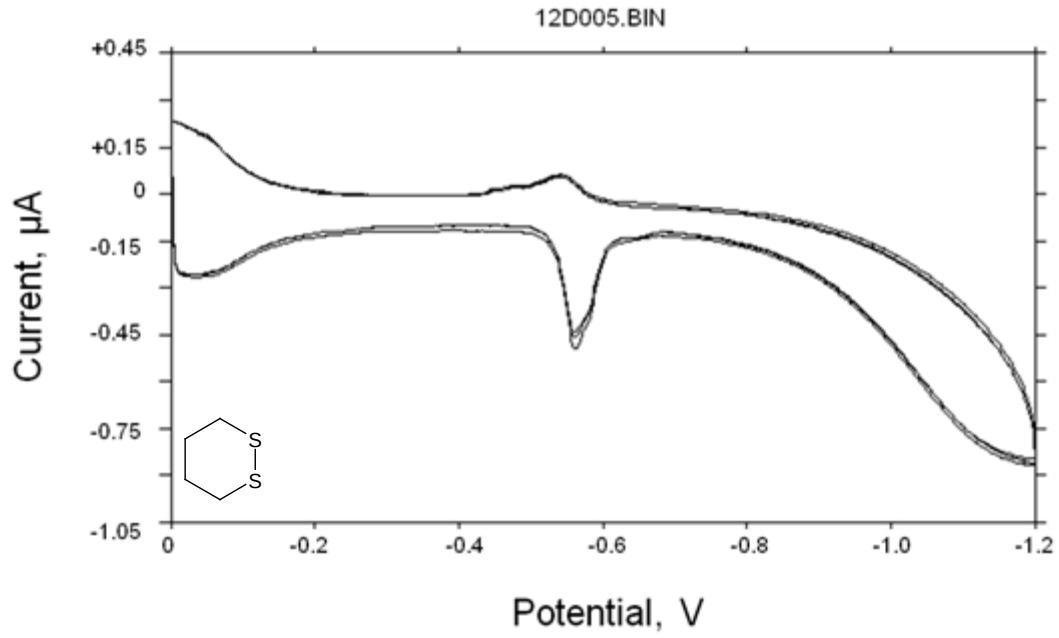


Figure 2.28: The cyclic voltammogram of 1,2-dithiane, Ep_a = -540 mV, Ep_c = -561.

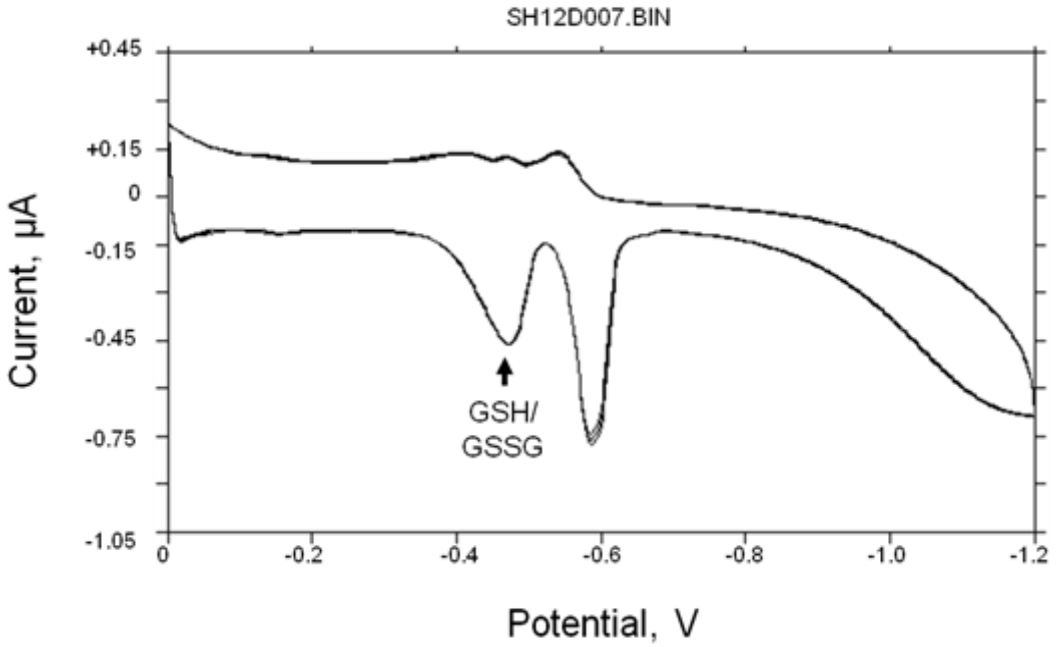


Figure 2.29: The cyclic voltammogram of 1,2-dithiane in the presence of GSH, Ep_a = -540 mV, Ep_c = -585.

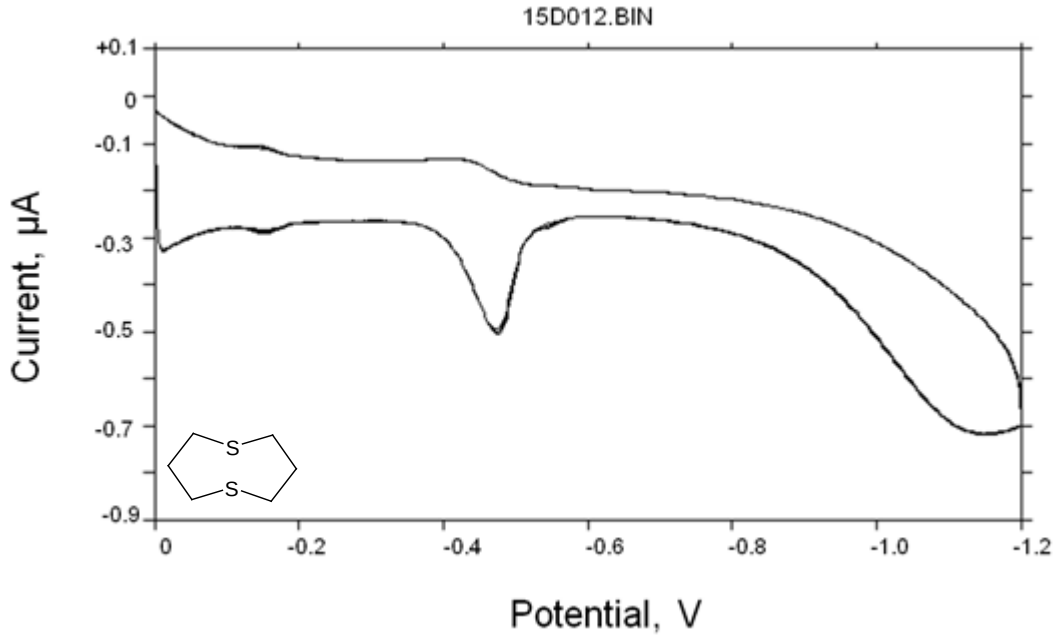


Figure 2.30: The cyclic voltammogram of 1,5-DTCO, Ep_a = -535 mV, Ep_c = -556.

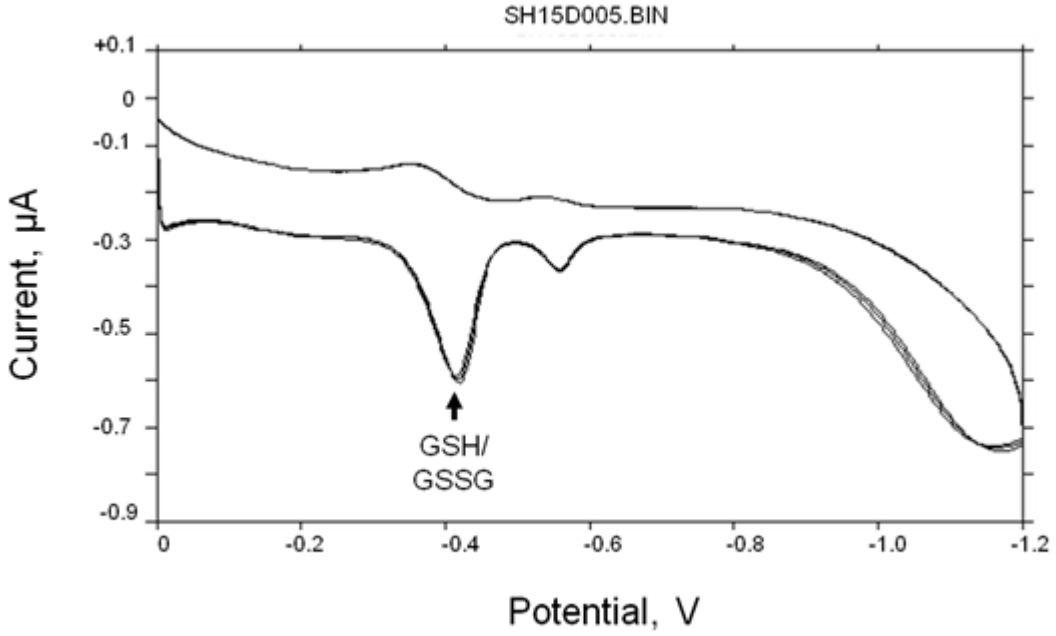


Figure 2.31: The cyclic voltammogram of 1,5-DTCO in the presence of GSH, Ep_a = -535 mV, Ep_c = -556.

DT, ADT, APT and Oltipraz were also measured using similar conditions. Similarly to 1,2-VDT, these cyclic disulfide compounds showed more negative potentials when compared to GSH. Unfortunately, the signals for GSH/GSSG are not clearly visible in the case Oltipraz is recorded in the presence of GSH. The redox potentials obtained are summarized in Table 2.6. The voltammograms of these compounds are presented in Figures 2.32. to 2.38.

Table 2.6: Summary of oxidation potentials (Ep_a) and reduction potential (Ep_c) of cyclic disulfides compounds in the absence and in the presence of GSH. Data presented as mean ± SD.

Potentials	Compound	GSH	mix (Compound + GSH)
	DT		
Ep_a (mV)	-686 ± 1.2		-660 ± 9.6
	-569 ± 7.7		-547 ± 11.8
		-412 ± 4.3	-
Ep_c (mV)	-960 ± 9.6		-986 ± 2.0
	-627 ± 13.3		-705 ± 8.9
		-470 ± 1.0	-445 ± 1.9
	ADT		
Ep_a (mV)	-677 ± 7.0		-687 ± 6.4
			-570 ± 1.9
		-412 ± 4.3	-351 ± 6.0
Ep_c (mV)	-721 ± 3.5		-721 ± 5.3
	-621 ± 12.7		-603 ± 4.1
		-470 ± 1.0	-434 ± 15.3
	APT		
Ep_a (mV)	-752 ± 2.8		-744 ± 0.0
	-570 ± 1.0		-564 ± 0.0
		-412 ± 4.3	-392 ± 11.7
Ep_c (mV)	-788 ± 0.0		-803 ± 1.0
	-602 ± 8.2		-621 ± 11.0
		-470 ± 1.0	-406 ± 0.0

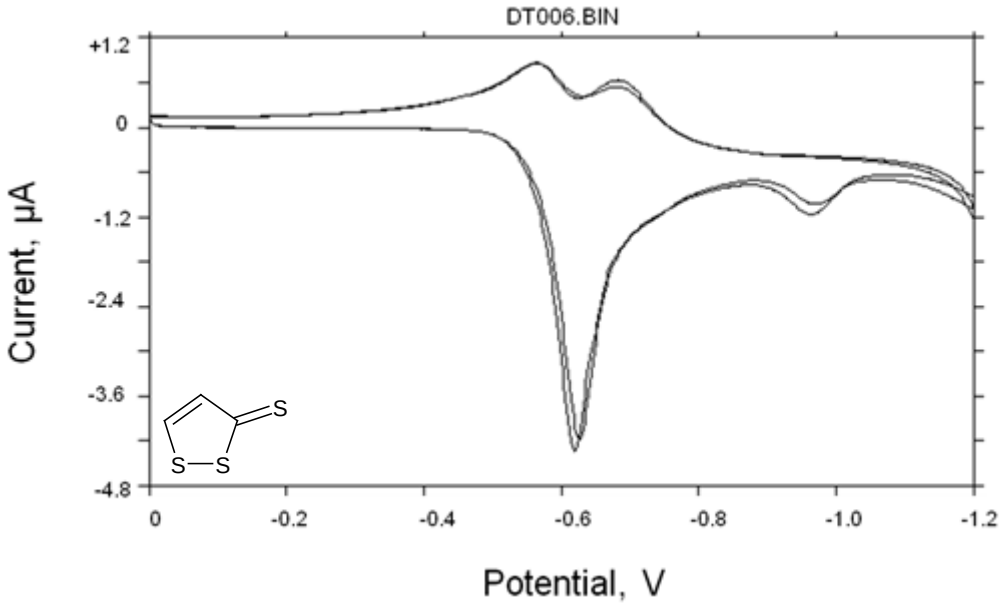


Figure 2.32: The cyclic voltammogram of DT, Ep_a = -686 mV, Ep_c = -960 mV.

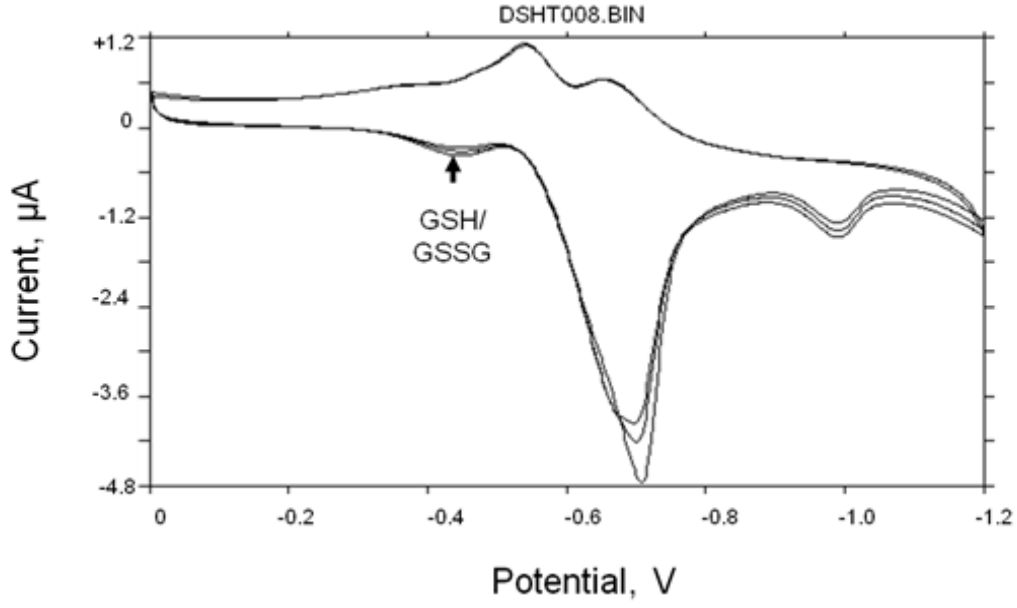


Figure 2.33: The cyclic voltammogram of DT in the presence of GSH, Ep_a = -660 mV, Ep_c = -986 mV.

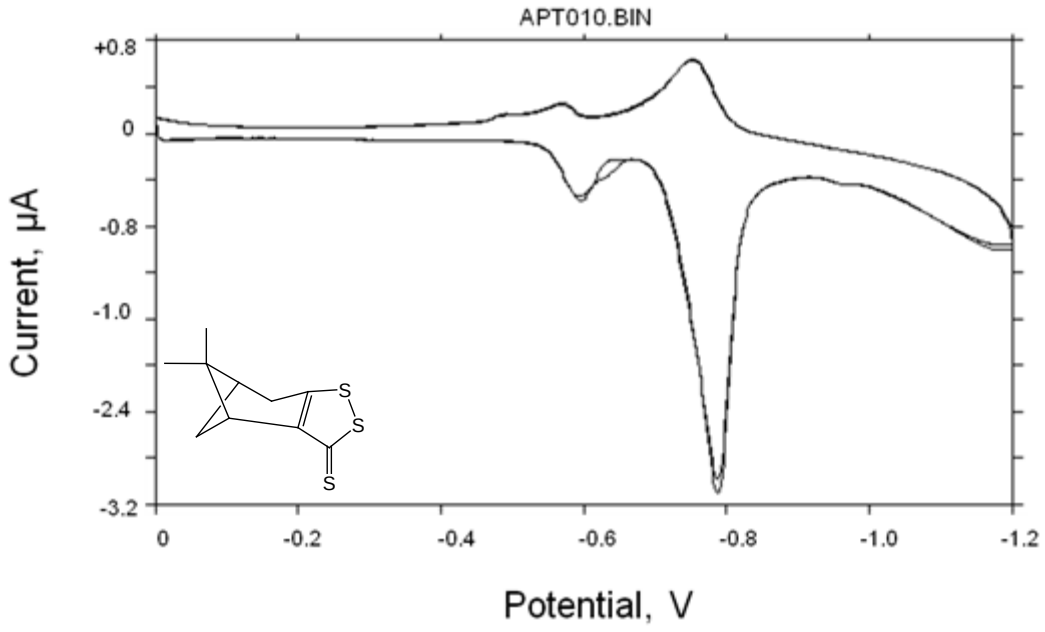


Figure 2.34: The cyclic voltammogram of APT, Ep_a = -752 mV, Ep_c = -788 mV.

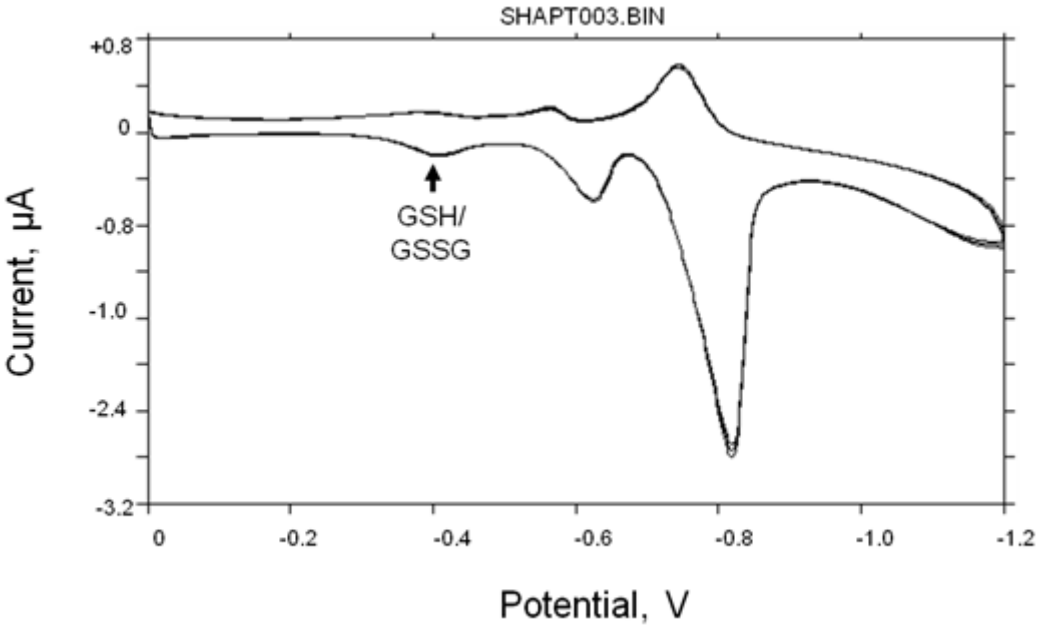


Figure 2.35: The cyclic voltammogram of APT in the presence of GSH, Ep_a = -744 mV, Ep_c = -803 mV.

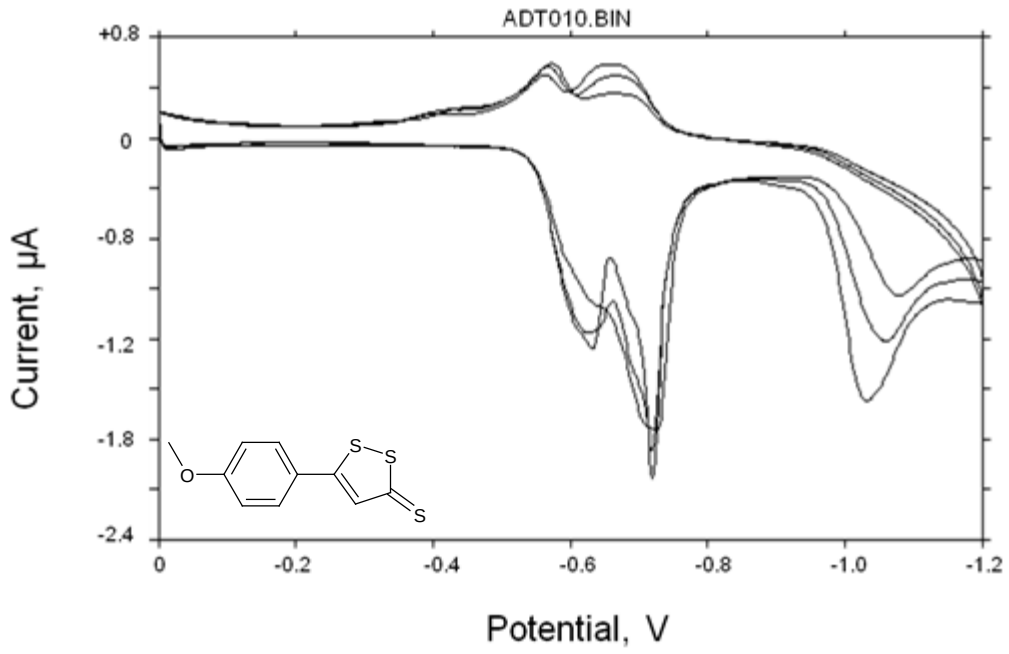


Figure 2.36: The cyclic voltammogram of ADT, Ep_a = -677 mV, Ep_c = -721 mV.

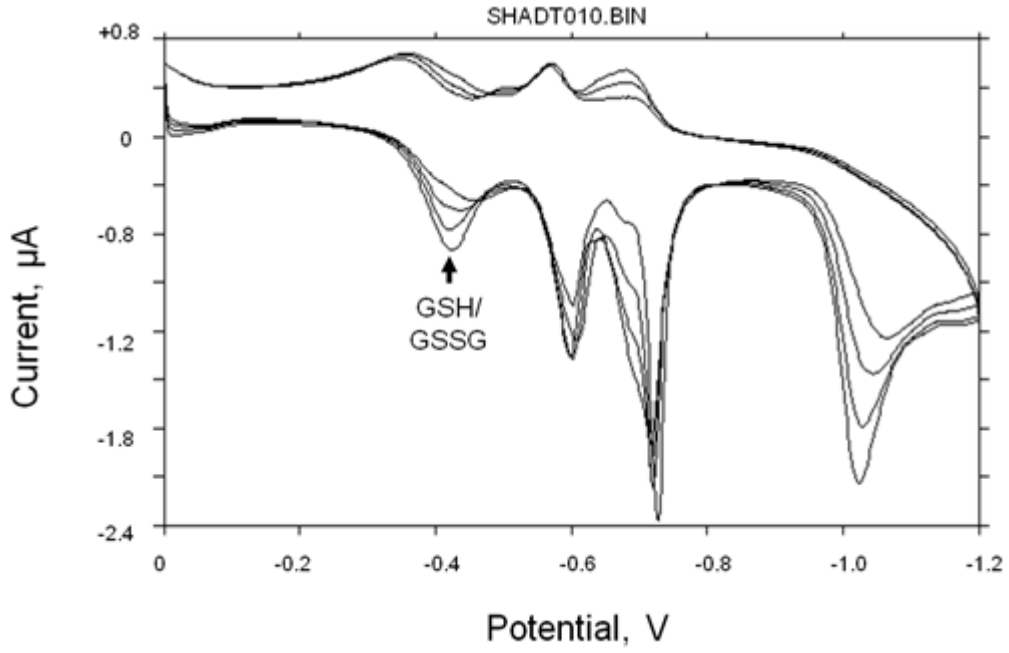


Figure 2.37: The cyclic voltammogram of ADT in the presence of GSH, Ep_a = -687 mV, Ep_c = -721 mV.

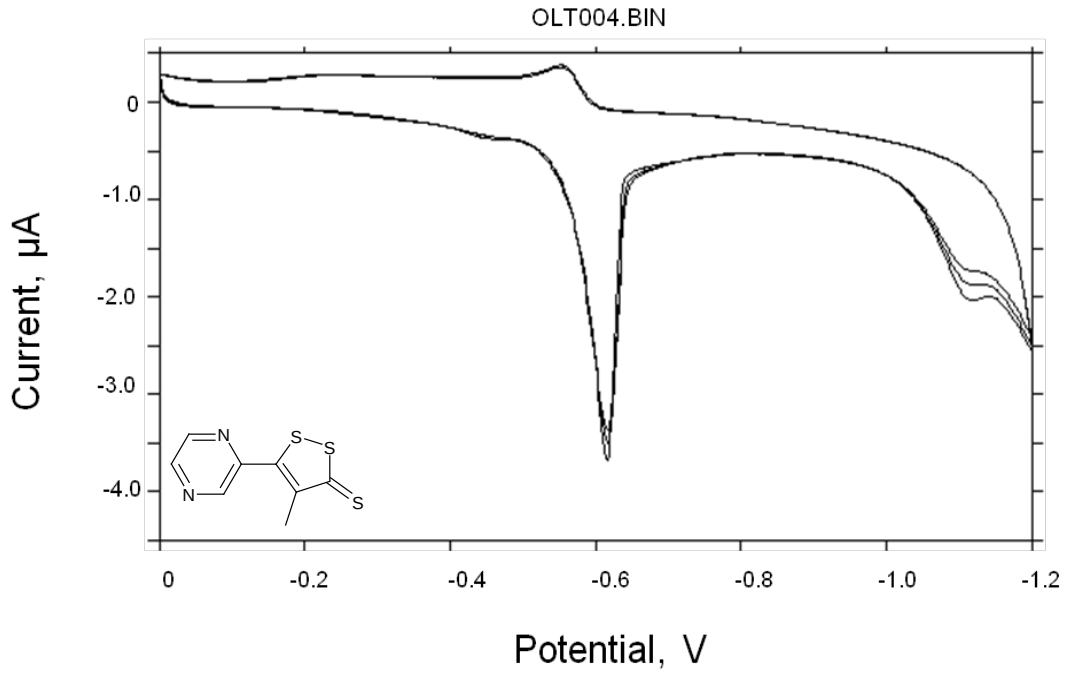


Figure 2.38: The cyclic voltammogram of oltipraz, Ep_a = -555 mV, Ep_c = -617 mV.

Table 2.7: Summary of oxidation potentials (Ep_a) and reduction potential (Ep_c) of cyclic disulfides compounds in the of GSH. Data presented as mean ± SD.

Potentials	Compound
	Oltipraz
Ep_a (mV)	-555 ± 3.5
Ep_c (mV)	-617 ± 2.0

2.7.2 Electrochemistry of diallyldisulfide and polysulfanes

Diallyldisulfide (DADS) and the polysulfanes exhibited at least two reduction and oxidation signals, of which one reduction signal (Ep_c) and one oxidation signal (Ep_a) were dominant. The summary of the redox potentials of the DADS and linear polysulfanes is presented in Table 2.8. The CVs of DADS and linear polysulfanes are shown in Figure 2.39 to 2.42.

Table 2.8: Summary of oxidation potentials (Ep_a) and reduction potential (Ep_c) of polysulfanes in the absence of GSH. Data presented as mean ± SD.

Potentials	Compound
	DADS
Ep_a (mV)	-462´± 1.9
Ep_c (mV)	-603 ± 1.2
	-522 ± 1.0
	DATTS
Ep_a (mV)	-579 ± 1.0
Ep_c (mV)	-651 ± 1.0
	-528 ± 1.6
	DPSTTS
Ep_a (mV)	-574 ± 1.0
	-387 ± 1.0
Ep_c (mV)	-644 ± 0.0
	-411 ± 1.0
	DPSEETTS
Ep_a (mV)	-576 ± 0.0
Ep_c (mV)	-642 ± 0.0
	-532 ± 0.0

In the first instance, the reduction signal (Ep_c) are generally tendency more negative with increased sulfur-sulfur chain, *i.e*. DATTS is more negative than DADS. This tendency is clearly visible at physiological pH 7.4 where DADS and DATTS exhibit a Ep_c value of -603 mV and -651 mV respectively. In case of the novel tetrasulfane derivatives Ep_a and Ep_c values, do not differ fiercely from the values obtained under physiological pH 7.4 for DATTS (a reference compound for tetrasulfanes). The values generally are obtained around -644 mV for DPSTTS and -642 mV for DPSEETTS. Interestingly, these Ep_c values are significantly more negative compared to the value of the reduction signal obtained of GSH/GSSG (-470 mV), which appear that the

tetrasulfanes are, at least, considerably less oxidizing than GSSG. This finding is in good agreement at least in theory. Based on this initial electrochemistry studies showed that polysulfanes behave as strong oxidants.[29]

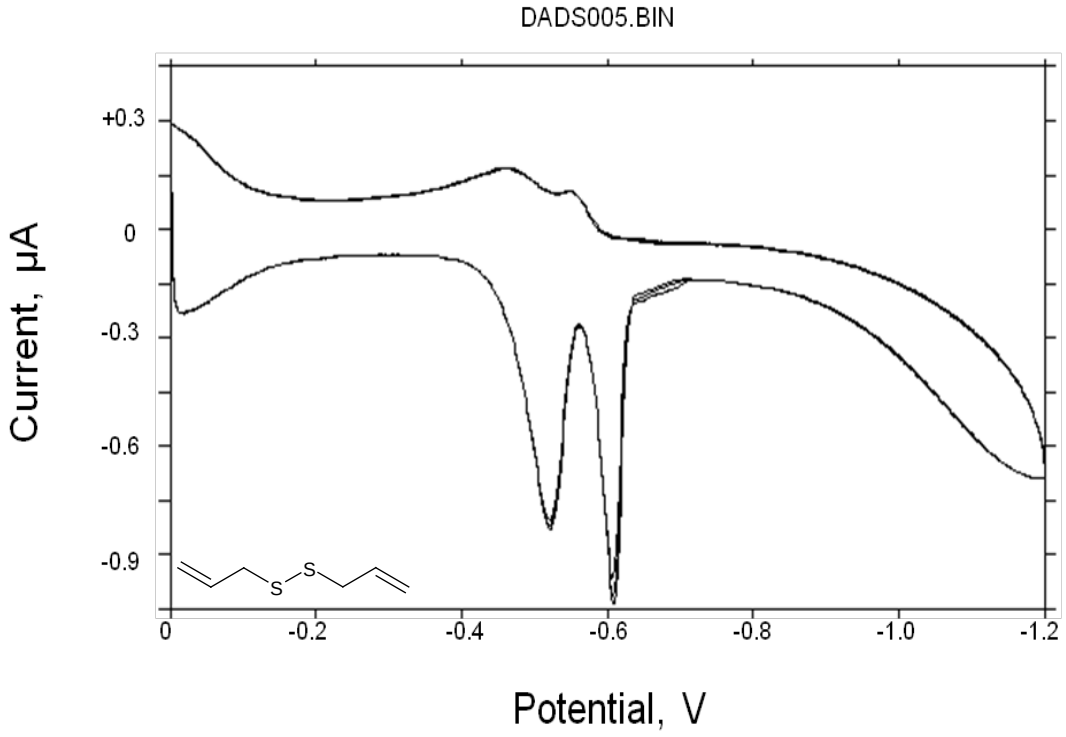


Figure 2.39: The cyclic voltammogram of DADS, Ep_a = -462 mV, Ep_c = -603 mV.

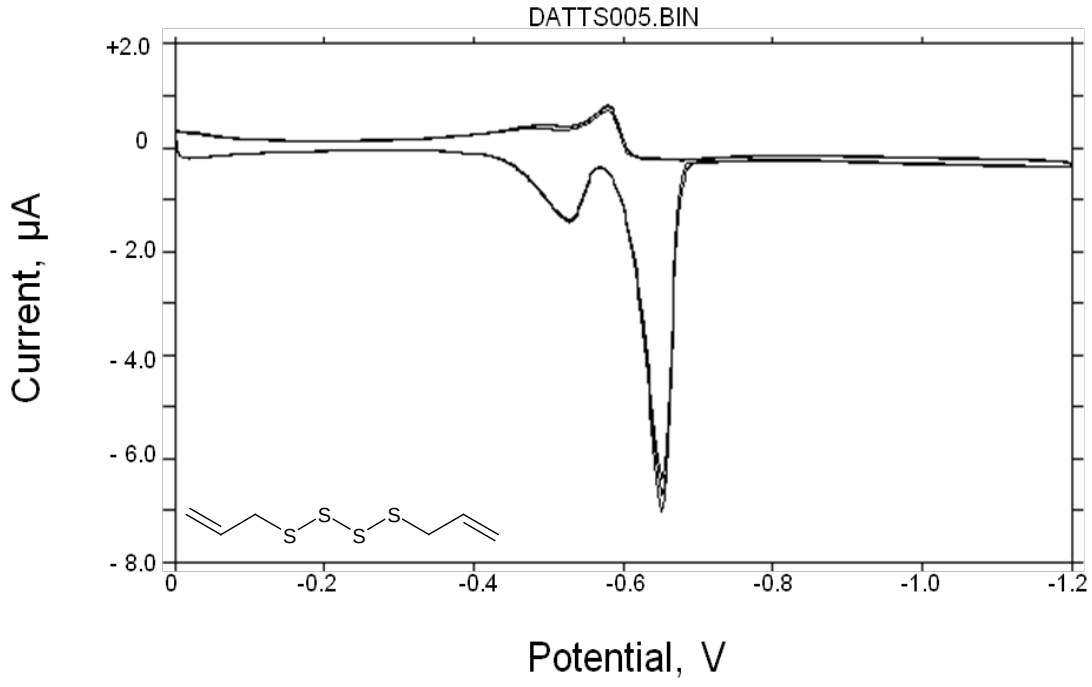


Figure 2.40: The cyclic voltammogram of DATTS, Ep_a = -579 mV, Ep_c = -651 mV.

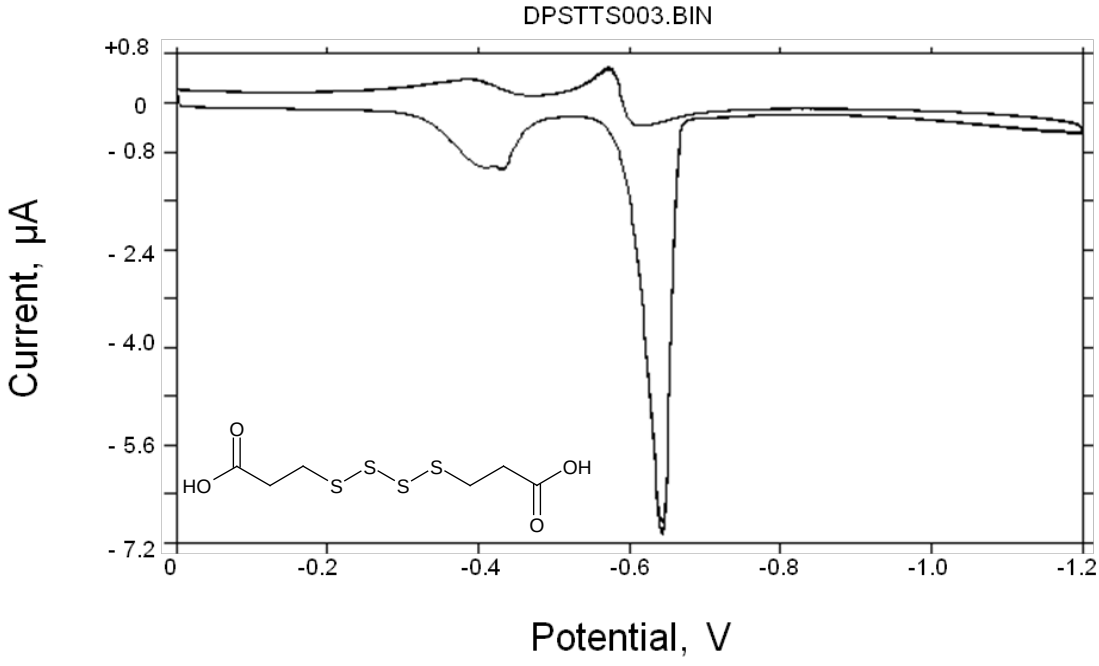


Figure 2.41: The cyclic voltammogram of DPSTTS, Ep_a = -574 mV, Ep_c = -644 mV.

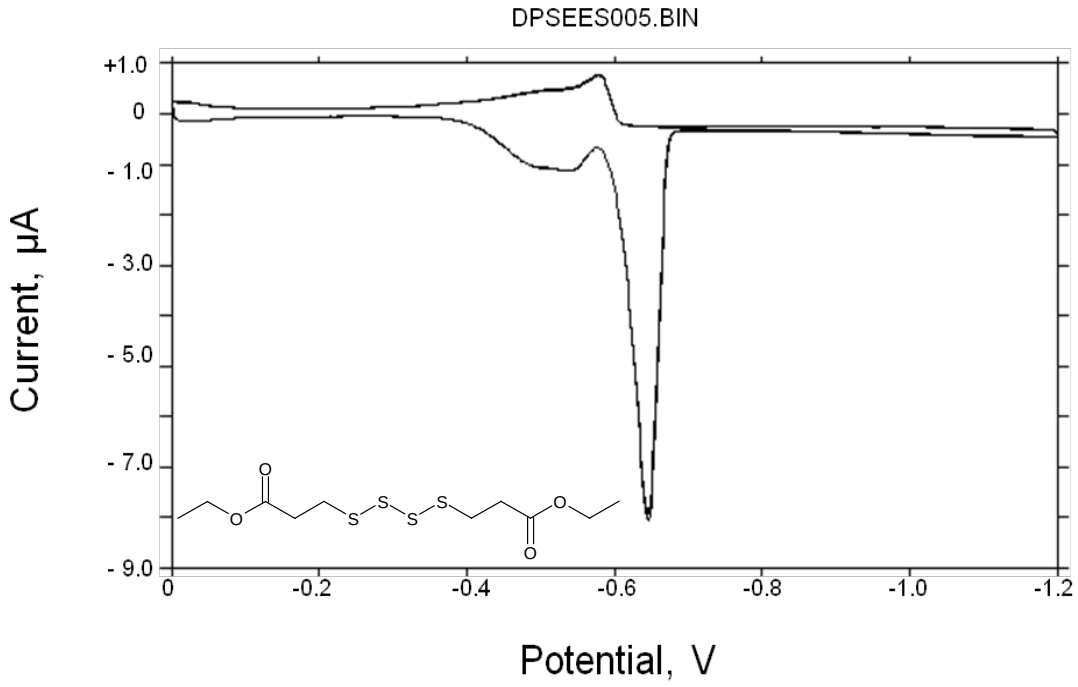


Figure 2.42: The cyclic voltammogram of DPSEETTS, Ep_a = -576 mV, Ep_c = -642 mV.

The summary of the oxidation and reduction potentials obtained under the same condition for the aromatic tetrasulfane derivatives such as DBnTTS in the absence or in the presence of GSH in phosphate buffer of pH 7.4 is provided in Table 2.9. The data showed that Ep_c values obtained for the aromatic compound are significantly more negative than the Ep_c value obtained for GSH/GSSG. The Ep_c value of DBnTTS are found around -616 mV and the Ep_c value obtained for glutathione disulfide (GSSG) under the same experimental conditions are obtained around -470 mV. This implies that aromatic tetrasulfane compound is less oxidizing than GSSG. The CVs of DBnTTS in the absence or in the presence of GSH are shown in Figures 2.43 and 2.44.

Table 2.9: Summary of oxidation potentials (Ep_a) and reduction potential (Ep_c) of polysulfanes in the absence and in the presence of GSH. Data presented as mean ± SD.

Potentials	Compound	GSH	Mix Compound + GSH

	dibenzyltetrasulfide (DBnTTS)		
***E*p$_a$ (mV)**	-565 ± 1.0		-572 ± 0.0
		-412 ± 4.3	-
***E*p$_c$ (mV)**	-616 ± 0.0		-626 ± 0.0
		-470 ± 1.0	-463 ± 3.5

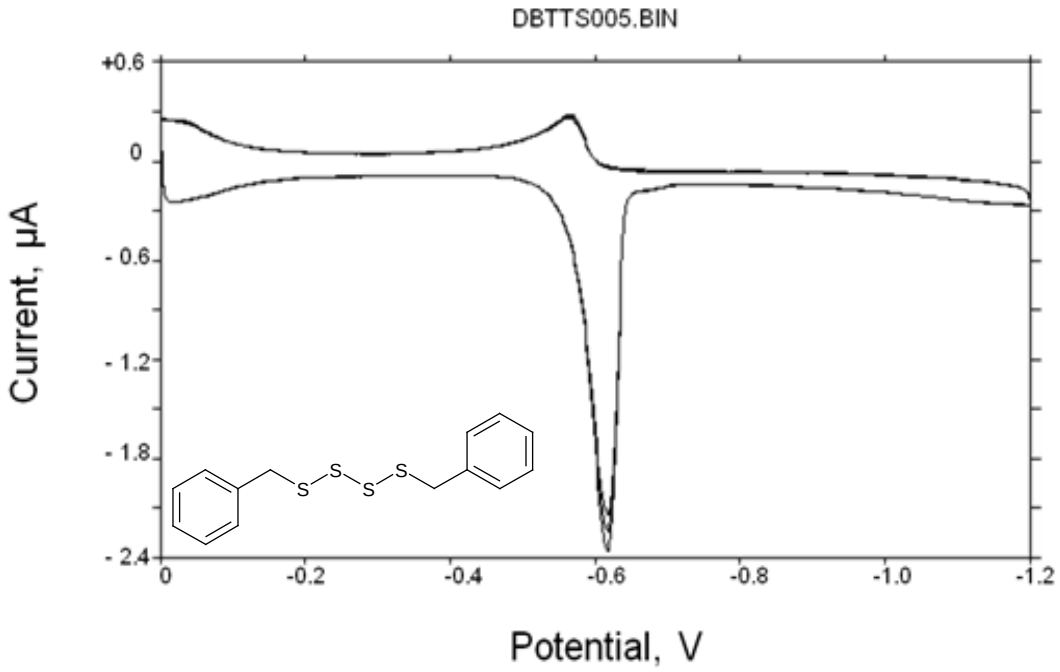


Figure 2.43: The cyclic voltammogram of DBnTTS, *E*p$_a$ = -565 mV, *E*p$_c$ = -616 mV.

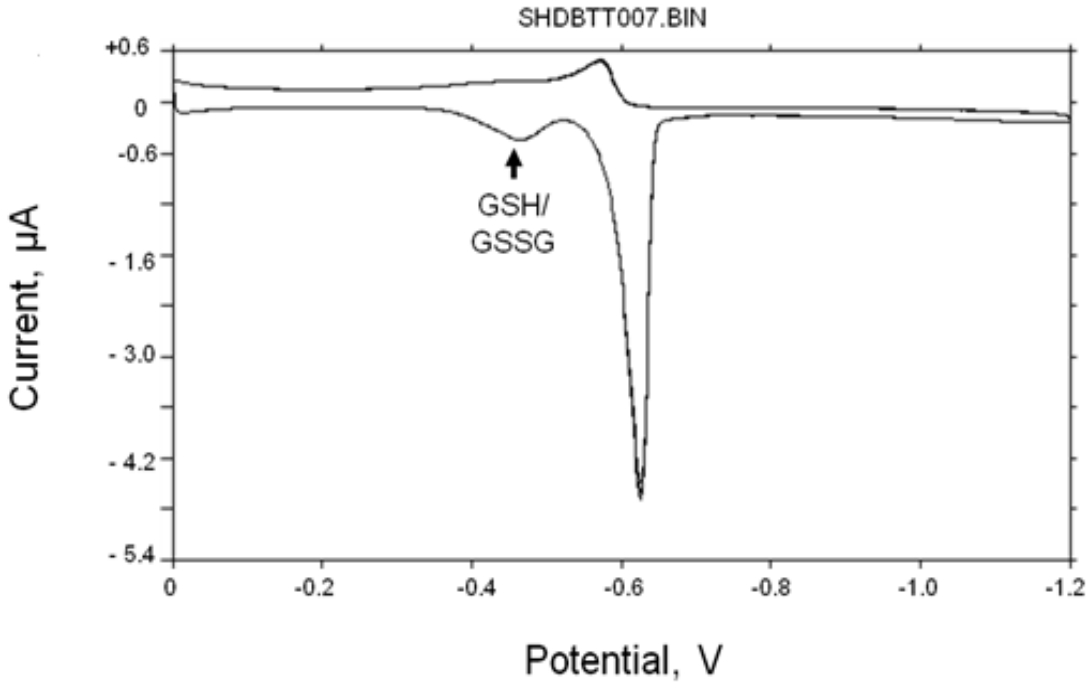


Figure 2.44: The cyclic voltammogram of DBnTTS in the presence of GSH, Ep_a = -572 mV, Ep_c = -626 mV.

2.8 Energy-dispersive *X*-ray spectroscopy microanalysis

Energy-dispersive *X*-ray spectroscopy (EDX) is an analytical technique used for elemental analysis and chemical characterization of diverse samples. The aim of this study is to analyze and to identify the different distribution of sulfur and selenium in Neuro 2A cells. The intensity or relative proportions of the elements are presented in mass percentages.

The different OSCs and chalcogen-based nanoparticles were analyzed due to their different behavior inside the cells. Overall, nine different chemical elements were determined in this study: carbon, nitrogen, oxygen, sulfur, phosphorus, selenium, silicon, sodium, and aluminium. The treatment and preparation of the cells for the EDX analysis was as follows. The cells were fixed with formaldehyde and glutaraldehyde in cacodylate buffer on glass coverslips (the cells were grown adherent on a glass coverslip) and incubated with the compounds for 24 h. Subsequently, analysis of the cells was carried out in order to established the overall distribution of the defined elements.

2.8.1 EDX microanalysis of untreated cells

It was known that most of biological redox system present in the human cell is not based on the metal ion or flavin, but on the redox active sulfur. The latter found, GSH which present in most mammalian cells was around 10 mM (most as GSH, but also in oxidized as GSSG, glutathione disulfide). GSH is a primary part of the “cellular redox buffer” which protect cells effectively against oxidative stress.[86]

Figure 2.45. shows the elemental distribution of the defined elements over the area of Neuro 2A cells without pretreatment with compounds (control). The defined elements were scanned across the cell and across the coverslip, to determined the differentiation of the elements. Silicon and oxygen are obviously detected in a cell-covered glass coverslip. Interestingly, sulfur is found within the scanned area of the cell without pretreatment with compounds (control) with the mass percentage of 0.90 % (± 0.2). Even though this finding not as much as if we compared to the literature, but this result showed that sulfur resulting from the presence of GSH in the cell. The mass percentage of the sulfur found was depend on the spot of the analyzer and homogeneity of the cell.

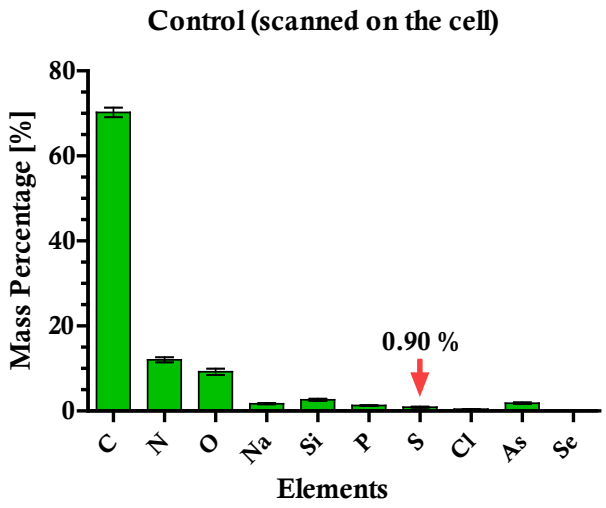


Figure 2.45: Mass percentages of the defined elements scanned by EDX analysis across neuronal cells (untreated).

2.8.2 *X*-ray mapping of Neuro 2A cells treated with diphenyltetrasulfide

The concentration of DPhTTS used in the Neuro 2A cells in this assay was 100 µM. The distribution of elements in Neuro 2A cells incubated with this compound are displayed in Figure 2.46. As shown in Figure 2.46, sulfur atoms were detected in every area (yellow color) of the cell, with the mass percentage of 1.18 %.

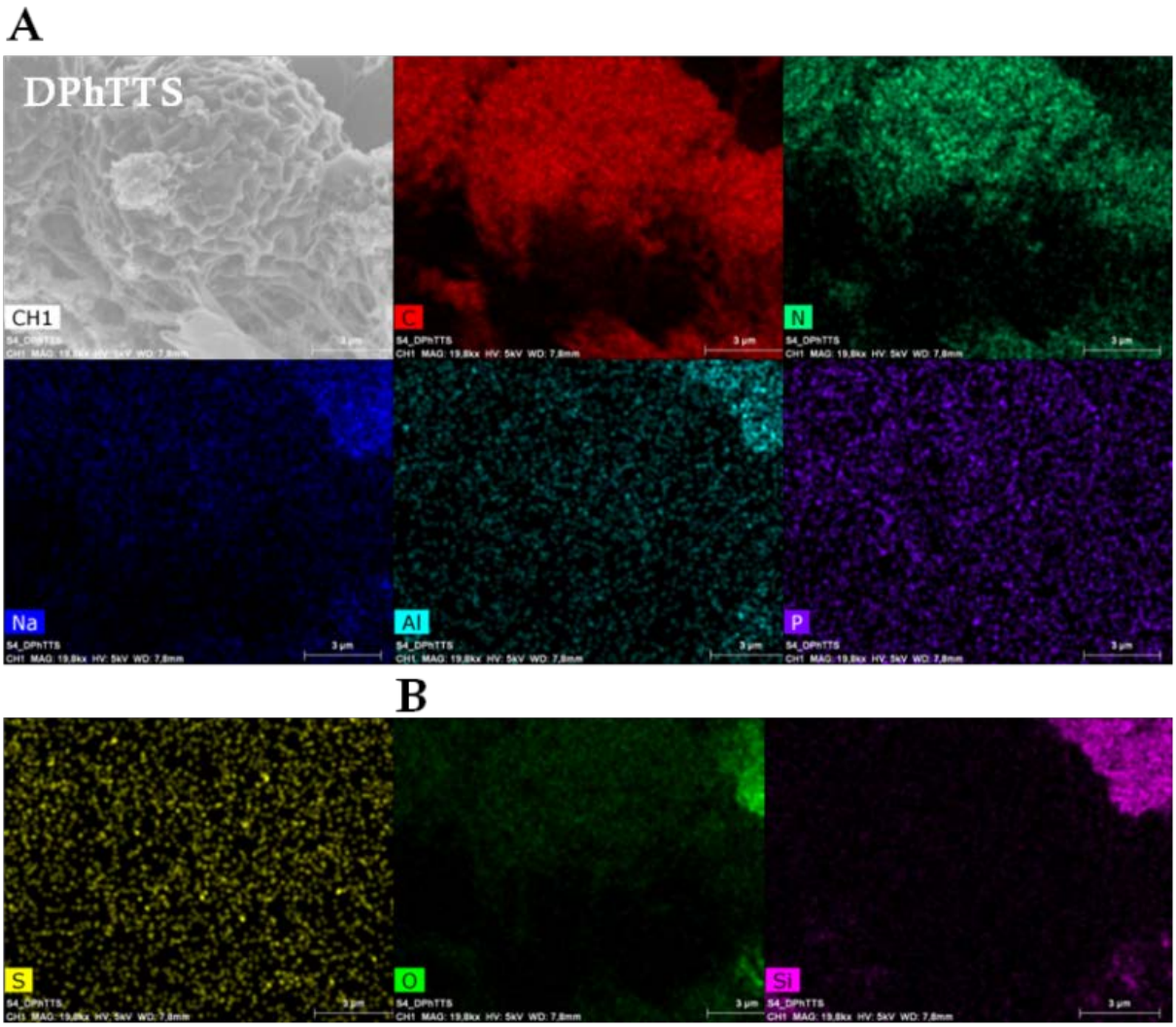


Figure 2.46: *X*-ray mapping of defined elements over the scan area of Neuro 2A cell lines; A. Distribution of defined elements scanned across the cell (cells treated with 100 µM of

DPhTTS); B. Distribution of defined elements scanned across the coverslip. X-ray mapping was performed by a EDX microanalyzer (SUPRA^{TM} 40, Carl Zeiss AG).

2.8.3 *X*-ray mapping of Neuro 2A cells treated with dibenzyltetrasulfide

The concentration of DBnTTS used in the Neuro 2A cells in this assay was 100 µM. The distribution of elements in Neuro 2A cells incubated with this compound are displayed in Figure 2.47. Similarly to DPhTTS, the sulfur atoms were detected with the mass percentage was around 4 %.

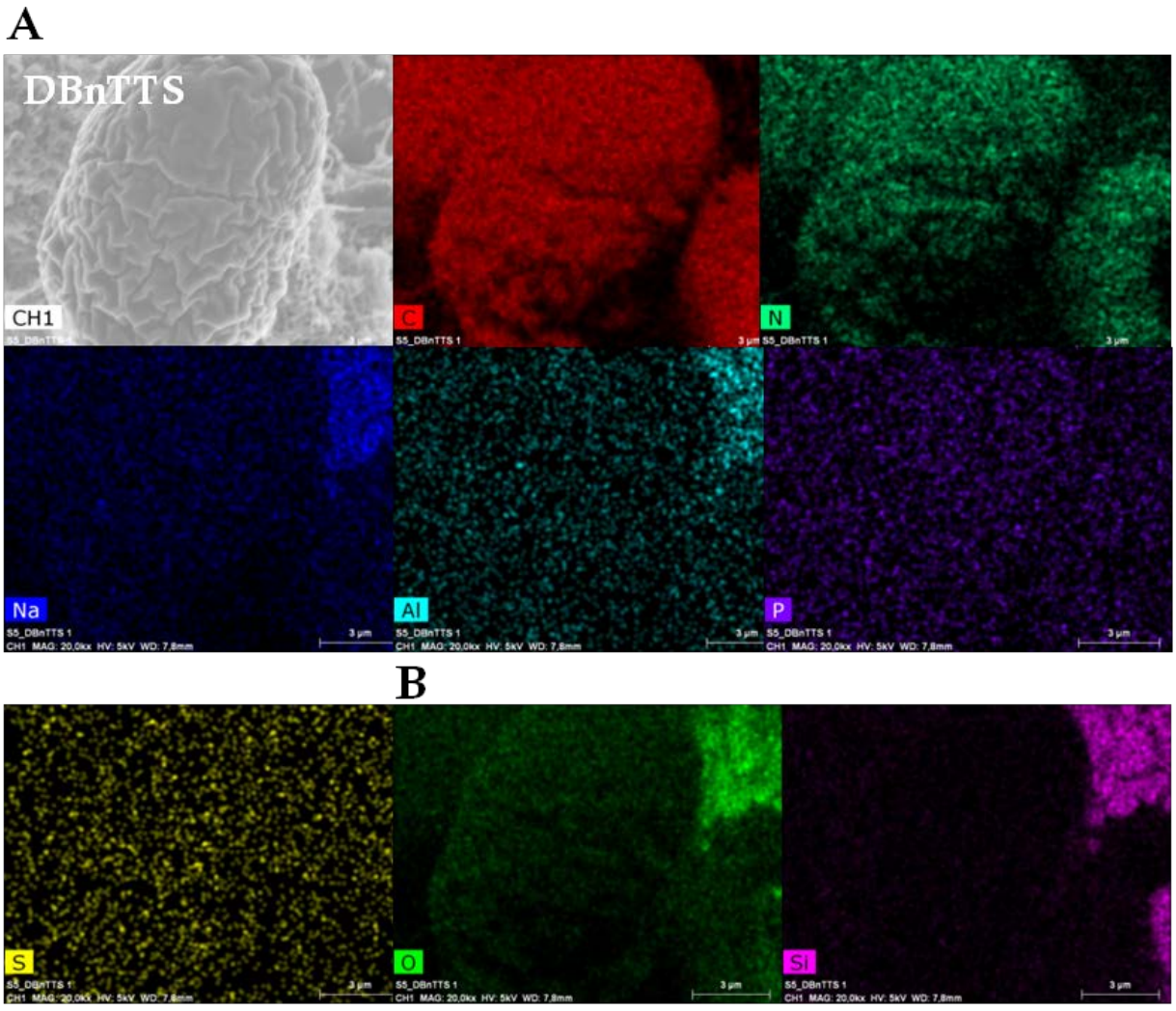


Figure 2.47: *X*-ray mapping of defined elements over the scan area of Neuro 2A cell ines; A. Distribution of defined elements scanned across the cell (cells treated with 100 µM of

DBnTTS); B. Distribution of defined elements scanned across the coverslip. X-ray mapping was performed by a EDX microanalyzer (SUPRA^{TM} 40, Carl Zeiss AG).

2.8.4 EDX microanalysis of Neuro 2A cells with the nanoparticles

Figure 2.48. shows the elemental distribution of the sulfur and selenium elements over the area of Neuro 2A cells treated with sulfur and selenium nanoparticles. Interestingly, selenium is detected within the scanned area of the cell with the mass percentage of 0.87 % (± 0.44 %) (Figure 2.48.). This implies that Se can be up taken by the neuronal cell either in form of intact NPSe or as metabolic product.

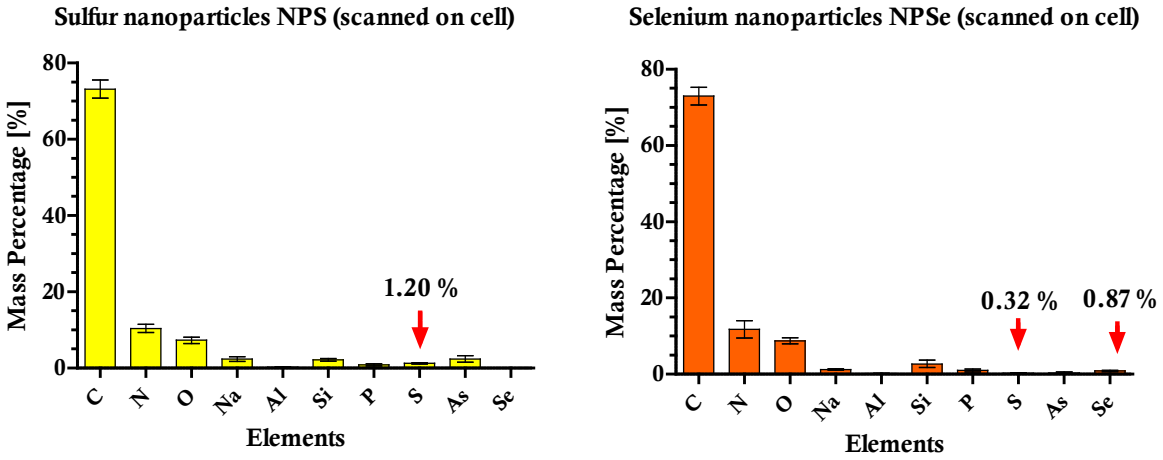


Figure 2.48: Mass percentages of the defined elements scanned by EDX analysis across three neuronal cells treated with NPS and NPSe.

X-ray mapping of Neuro 2A cells treated with the sulfur nanoparticles

The cell culture studies using neuronal cells showed that NPS have a strong toxic effect against the Neuro 2A cell line. The distribution of elements in Neuro 2A cells in the presence of NPS are displayed in Figure 2.49.

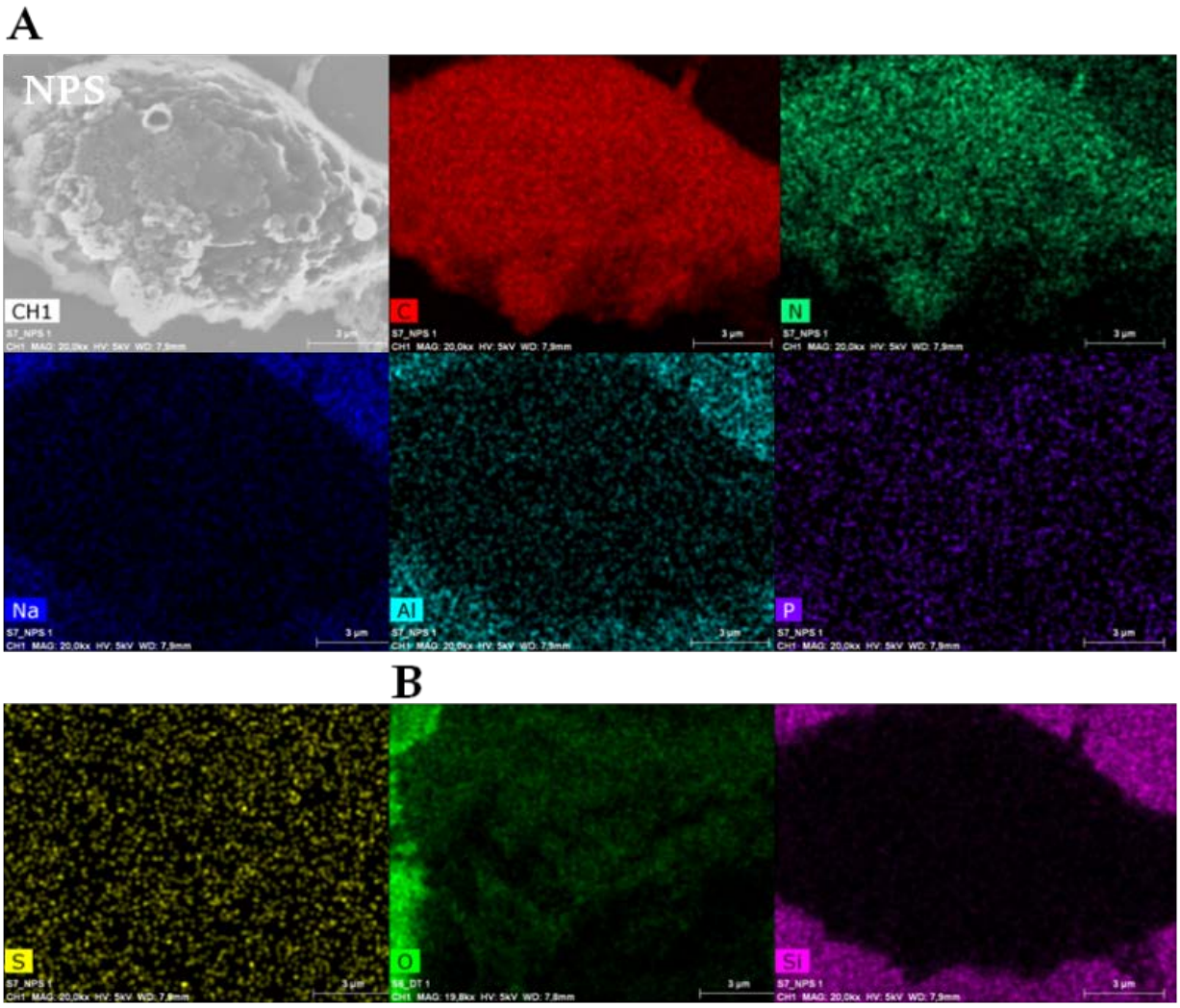


Figure 2.49: *X*-ray mapping of defined elements over the scan area of Neuro 2A cell-lines; A. Distribution of defined elements scanned across the cell (cells treated with 100 µg/ml of NPS); B. Distribution of defined elements scanned across the coverslip. X-ray mapping was performed by a EDX microanalyzer (SUPRA^{TM} 40, Carl Zeiss AG).

X-ray mapping of Neuro 2A cells treated with selenium nanoparticles

The cell cultures studies using NPSe showed a high toxicity on neuronal cells at 1 µg/ml. The distribution of element Se on neuronal cells are presented in Figure 2.50.

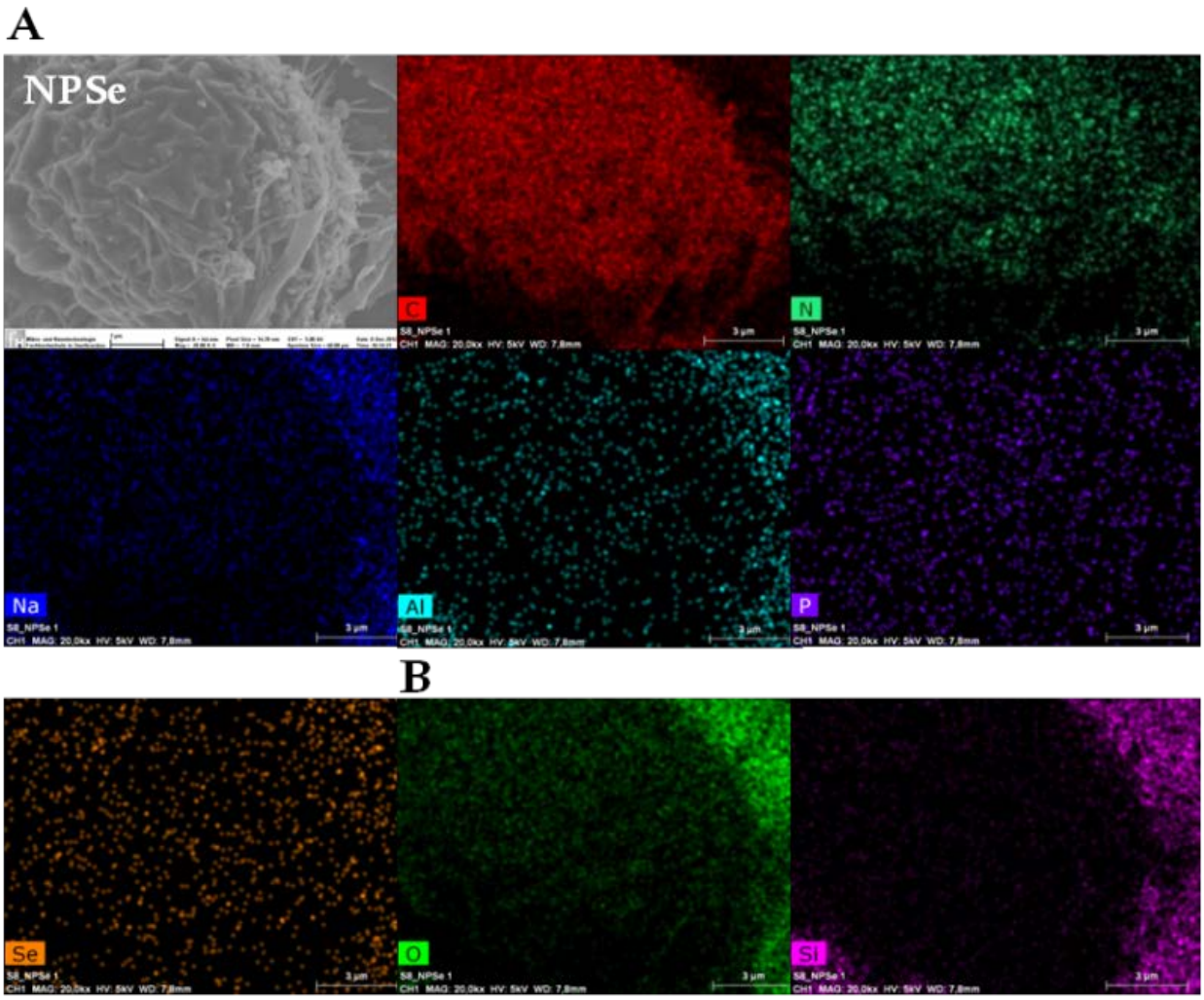


Figure 2.50: **X**-ray mapping of defined elements over the scan area of Neuro 2A cell lines; A. Distribution of defined elements scanned across the cell (cells treated with 100 µg/ml of NPSe); B. Distribution of defined elements scanned across the coverslip. X-ray mapping was performed by a EDX microanalyzer (SUPRA^{TM} 40, Carl Zeiss AG).

2.9 Cell fractionation to investigate the location of the compounds in Neuro 2A cells

In the previous toxicity experiments DPhTTS showed the highest activity in the Neuro 2A cells. Therefore, DPhTTS was chosen as a suitable candidate for the cell fractionation. The aim of this study was to investigate where DPhTTS is located inside the neuronal cells. Cells were therefore incubated with 100 µM of DPhTTS for 24 h, than fractionated into their different constituents: cytosol, cell membrane, nuclear, mitochondria, reticulum endoplasmic-ribosome (RE-Ribosome), and the Golgi apparatus. The cellulars fractions were subsequently analyzed using HPLC/UV-MS to determine the approximate location and distribution of DPhTTS inside the cell.

2.9.1 Intracellular distribution of DPhTTS

To fractionate the cytosol and cell membrane, the neuronal cells were centrifuged at 17.500 rpm using Eppendorf AG Centrifuge 5430 R. The pellet obtained was recognized as cell membrane fraction, while the cytosolic fraction was still in the supernatant.

Table 2.10. The retention times (RT) of DPhTTS and component in the cell membrane as fraction obtained by HPLC/UV-MS analysis.

Retention time (RT) (minute)	
DPhTTS	**cell membrane fraction**
6.26	6.42
6.72	6.73
7.20	7.18
7.69	7.65
DPhTTS	**nuclear fraction**
6.26	6.23
6.72	6.63
7.20	6.97
7.69	7.45

DPhTTS was detected in the cell membrane fraction (Table 2.10.). This implies that this compound enters to the cell membrane. Based on Lipinski's rules this symmetric tetrasulfane

compound poseses clog*P* values of 5.044. DPhTTS was not detected in cytosol, indicating that this compound may become enriched in cell membranes and possibly other lipophilic of the cell.

To separate the nuclear fraction, neuronal cells were dounced three times using dounce homogenizer in hypotonic solution (0.25 M sucrose) to break the cell membranes open. The cells were then centrifuged for 10 min at 3,000 rpm, the resulting pellet contains the nuclear fraction. The top part of the nuclear fraction was centrifuged further at 10,000 g for 20 min to separate the mitochondria fraction. The RE-Ribosome fraction was then separated by the isolation of the surface part of the mitochondria fraction and subsequent centrifugation at 196,000 g for 60 min. The Golgi apparatus fraction was obtained from the top layer of the ER-Ribosome fraction. All the different steps of the subcellular fractionations were carried out in cool condition. The results all of the cellular and subcellular fractions are demonstrated in the Figure 2.51. The results show that DPhTTS might be detected in the nuclear fraction due to the peaks obtained were similar to the peaks obtained from DPhTTS. In addition, some of molecules of DPhTTS were detected in the mitochondria and ER-Ribosome fractions but the intensities are too small to give a clear statement (Table 2.9.).

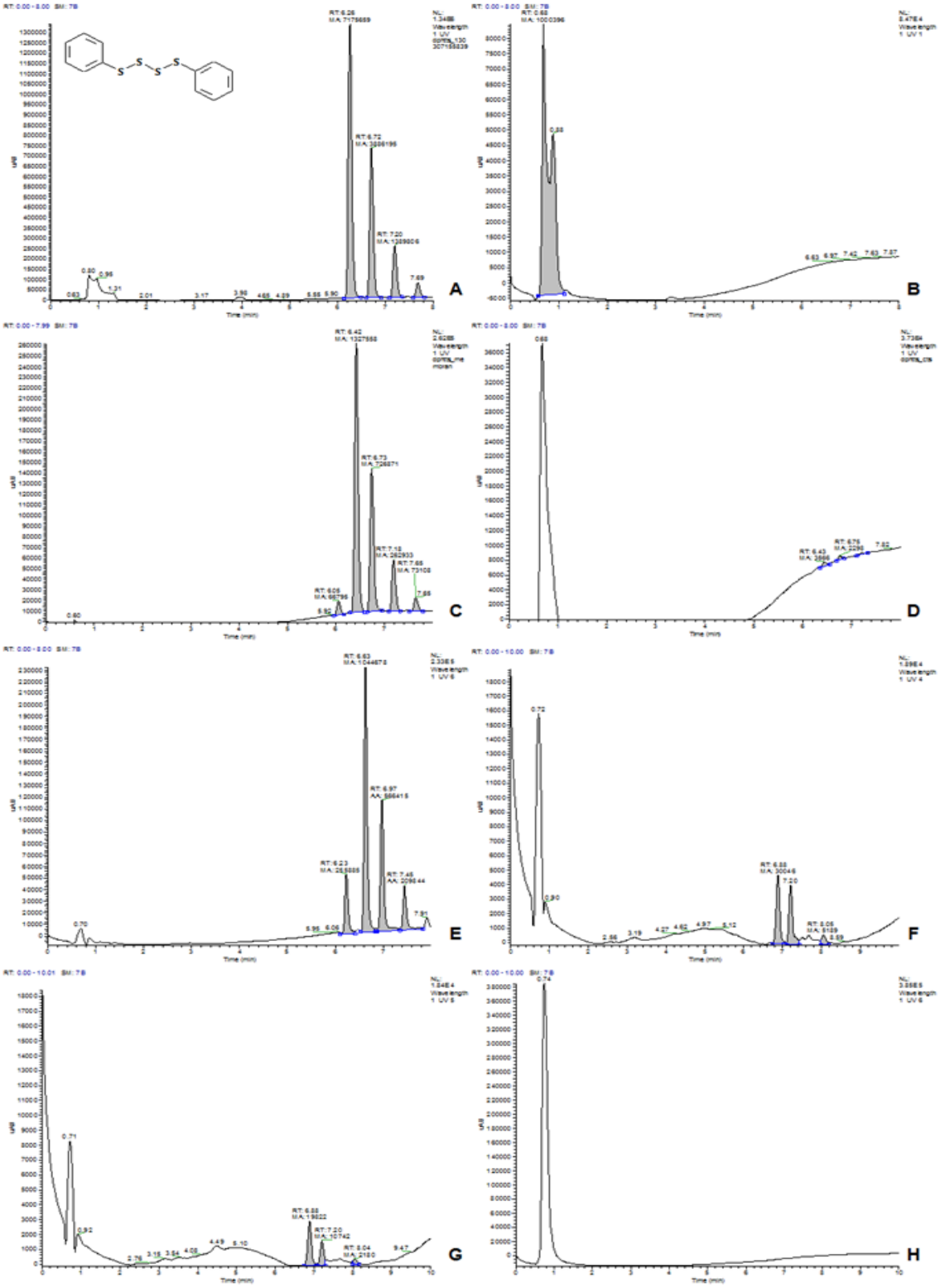


Figure 2.51: HPLC/UV-MS analysis of the cellular and sub cellular fractions. A. HPLC/UV-MS analysis of DPhTTS, B. Buffer, C. Cell membrane, D. Cytosolic, E. Nuclear, F. Mitochondria, G. ER-Ribosome, and H. Golgi fraction.

3. Summary and Outlook

The primary aim of this study was to investigate the redox behaviour, biological activities and a possible use of disulfide-based OSCs and sulfur-based nanoparticles as potential redox-modulating agents in medicine or as phytoprotectans in Agriculture.

1,2-Vinyldithiin (1,2-VDT), 1,3-vinyldithiin (1,3-VDT), 1,2-dithiane, 1,5-dithiacyclooctane (1,5-DTCO), and *α*-pinene trithione (APT) were synthesized successfully in the beginning of this project. The sulfur- and selenium-nanoparticles (NPS and NPSe respectively) were also prepared according to the literature. The structures and the purities of OSCs were confirmed using NMR, HPLC and LC-MS spectroscopy. NPS and NPSe were characterized using a ZetaSizer Nano from Malvern Instruments, Ltd. Germany. The sulfur rich Haarlem Oil was a gift from Laboratoires du Dr. J. Lefevre, France. The other compounds used as part of this project were synthesized and purified by colleagues in the group of Prof. Dr. Claus Jacob at the University of Saarland, especially by Brigitte Czepukojc and Uma M. Viswanathan.

The electrochemical studies were conducted to evaluate aspect of the redox behavior of the various compounds, using cyclic voltammetry (CV) as analytical technique. All experiments were carried out in the presence and absence of glutathione (GSH) to investigate if the different compounds were reacting with GSH or not (also GSH was used as internal reference). In general, all the compounds used were more reducing than GSH and therefore apparently did not react with GSH. As expected, 1,3-VDT did not show any oxidations or reduction signals due to the fact that this compound does not possess a sulfur-sulfur bond which could be reduced.

Furthermore, the activity of the compounds and nanoparticles was measured employing *Steinernema feltiae* nematodes as a model for living (higher) organisms. This studies indicated that 1,2-VDT was more active against the nematodes compared with its isomer (1,3-VDT). The linear disulfide, such as diallyldisulfide, and associated chemical reactivity generally were highly active and toxic against the nematodes, while Harlem Oil did not show such a high toxicity. These results do reflect the already expected high (redox) activity of the sulfur-sulfur bond containing compounds.

Nonetheless the highest activity was found using the nanoparticles. Sulfur nanoparticles, in particular, showed an excellent activity at low concentrations (µg/ml). These results are

interesting and should be followed up by (expensive) agricultural field trials to prepare a highly active and commercial product out of the sulfur and selenium nanoparticles.

In order to investigate the activity of the OSCs against *Botrytis cinerea*, wild type and resistant mutant strain were studied. The results show that dithiolethione (DT) has a strong activity at low concentrations (around 0.3 mM) against all different strains of *B. cinerea*. In the same concentration range, *α*-lipoic acid (LA) has also shown some inhibitory effects against the MDR 2 spores of this fungus.

Calcein-AM-PI dyes were used to performed survival and apoptosis assays on Neuro 2A cells, a standard murine neuroblastoma cell line. In this assay, the compounds were tested in the presence and the absence of hydrogen peroxide to analyze the influence of oxidative stress on the compounds. The toxicity of the molecules was evaluated based on the cell viability of the neuronal cell after 24 h. The results showed that cyclic disulfide compounds, such as 1,2-dithiane, 1,5-DTCO and DT possess some moderate activity. As before, sulfur- and selenium-nanoparticles exhibited the most cytotoxic effects The polysulfane-derivatives (especially the tetrasulfanes) also showed toxic effects at moderate sometimes even at lower concentrations. DPhTTS and DBnTTS, in particular, exhibited high activity at concentrations around 100 µM with cell survival rates below 38%.

SEM-EDX microanalysis was used to study the distribution of chalcogen atoms inside the cells. Sulfur atoms from the polysulfanes and the nanoparticles were expected detect at in every area of the Neuro 2A cells. Interestingly, the selenium was also detected inside the cells with a mass percentage of Se of 0.87 % (± 0.44 %).

DPhTTS showed the highest activity in the neuronal cells, so this compound was used further as part of cell fractionating studies. Cells were fractionated after 24 h incubation to obtain various cellular organelles: cell membrane, nuclear, mitochondria, reticulum endoplasmic-ribosome, and the Golgi apparatus. All of the fractions were analyzed by HPLC/UV-MS detector with ESI (Electron Spray Ionization) at 254 nm. Ultimately, this analysis indicated that the lipophilic tetrasulfane DPhTTS becomes enriched in the cell membrane and also in the nucleus. Some lower concentrations of the compound were also found in the mitochondria and in the RE-ribosome.

In future, several studies to analyze the behaviour of the chalcogen containing compounds in mammalian cells and other biological materials such as fungi and bacteria are required. It may

also be interesting to investigate further the toxicity and the signalling pathway triggered as affected by these compounds. Indeed, it would interesting to see if these compounds do affect primary signaling pathways to induce apoptosis or if the cellular processes are more complicated and complex as differentiated as thought at present.

Finally, it may be worth to subsequently conduct some filed trials with industrial partners to established possible OSCs-brand ‘green’ pesticides or phytoprotectans as commercial products. The preliminary data looks indeed promising and there is a huge market to sell nature-based and cheap phytoprotectans. The same consideration also applies to the sulfur nanoparticles and to the selenium containing compound and to possible application for medicinal and agriculture.

4. Experimental Section

4.1 Materials and methods

4.1.1 Materials

Acrolein, ethyl orthoformate, 1,4-butanedithiol, 1,3-dibromopropane, 1,3-propanedithiol, diallyldisulfide (DADS) and α-pinene were purchased from Sigma Aldrich (Germany) and used without further purification. Anhydrous zinc chloride, sodium sulfate, silica gel, bromine, sodium, anhydrous calcium chloride, sulfur, sodium sulfite, sodium sulfide, sulfuric acid, sodium sulfide nonahydrate, L-cysteine, selenious acid, α-lipoic acid, lipoamide were purchased from Sigma Aldrich (Germany). Meanwhile, Oltipraz, dithiolethione (DT) and anethole dithiole thione (ADT) were purchased from LKT Laboratories Inc. Haarlem Oil was a gift from Dr. J. Lefevre, ZI La Pelouse-55 190, Void Vacon, France.

4.1.2 Nuclear magnetic resonance spectroscopy

NMR spectra (^{1}H NMR and ^{13}C NMR) were recorded on a Bruker Avance 500 at the Department of Pharmaceutical and Medicinal Chemistry, School of Pharmacy, University of Saarland. The chemical shifts are expressed relative to the signal of chloroform ($CDCl_3$), used as a solvent, at 7.26 ppm and 77.16 ppm, for ^{1}H NMR and ^{13}C NMR, respectively. All the spectra recorded and the chemical shifts reported are given in δ (ppm).

4.1.3 Melting points

Melting points were measured using Thermo Scientific 9300, Fisher company, UK and given without correction.

4.1.4 Refractive index

Refractive indices were measured as appropriate using a thermostatically controlled Abbe refractometer (Atago 3T, Japan). For each compound, the refractive index was measured at 25 °C and in triplicate.

4.1.5 Characterization of nanoparticles

Sulfur and selenium nanoparticles of sulfur and selenium were characterized with a Zetasizer Nano (Malvern Instruments Ltd, Germany). The diameter (nm) of particles, polydispersity index (PDI) and Zeta potentials (mV) of the nanoparticles were measured at 25 °C and at pH=7 in triplicate.

4.2 Synthesis of organo sulfur compounds

4.2.1 Synthesis of vinyldithiins

Vinyldithiins were synthesized from acrolein and hydrogen sulfide according to the literature procedure described by Beslin. [44] In brief, into a ice-cooled solution of absolute ethanol (150 ml), acrolein (0.1 mol), ethylorthoformate (0.15 mol) and $ZnCl_2$ (0.002 mol) were added under a continuous stream of H_2S (0.1 ml/min). The solution was stirred for 5 h, afterwards the reaction mixture was poured on ice and extracted with 50 ml of cold pentane (3x). The yellow organic phase was separated, dried over sodium sulfate (Na_2SO_4), filtered and the solvent evaporated using a vacuum rotary evaporator. The vinyldithiins (1,2-VDT and 1,3-VDT) were separated and purified by silica gel column chromatography using petroleum ether and ethyl acetate (PE/EtOAc, 99:1) as solvent. Fractions from the column were collected was done under ice-cooled conditions (*i.e.* the collecting tubes were immerged into an ice bath) because of low thermal stability of the vinyldithiin compounds.

4.2.2 Synthesis of 1,2-dithiane

1,2-Dithiane was synthesized by brominating 1,4-butanedithiol according to the literature.[71] Under ice-cooled and vigorous stirring conditions, flash chromatography grade silica gel (5.0 g) was placed into a 100 ml threenecked round-bottom flask. Distilled water (2.5 ml) was added drop-wise into the flask using a syringe. Stirring was continued until a flowing suspension was obtained. Dichloromethane (DCM) (25 ml) was added to the flask, followed by slow addition of 1,4-butanedithiol (4.02 mmol) in DCM (5 ml). A Solution of elem. bromine (Br_2) in DCM (1.16 M, 3.50 ml, 4.06 mmol) was added drop-wise to the mixture using a syringe. The reaction was terminated once the colour of the solution turned light brown-yellow (fine yellow) and did not change any further for another 10 to 15 minutes (this indicates that the reaction is completed). The reaction mixture was then filtered and washed with 100 ml of distilled water (3x) and dried over anhydrous Na_2SO_4. The organic solvent was removed under reduced pressure to obtain the crude product which was purified further by silica gel column chromatography using petroleum ether and ethyl acetate (PE/EtOAc, 95:5) as solvent.

4.2.3 Synthesis of 1,5-dithiacyclooctane

1,5-Dithiacyclooctane (1,5-DTCO) was synthesized from 1,3-dibromopropane (20 mmol) and 1,3-propanedithiol (20 mmol) under an argon atmosphere, as described by Clennan *et al*. with a few modifications.[62] In short, sodium (1 g, 0.04 mol) was added to anhydrous EtOH (200 ml) and stirred until a fine yellow solution was produced, The solution was then warmed to 50 °C, followed by the simultaneous drop-wise addition of 1,3-dibromopropane (20 mmol) in anhydrous EtOH (50 ml) and 1,3-propanedithiol (20 mmol) in anhydrous EtOH (50 mL) over 1.5 h. The mixture was then heated for another hour upon which it produced a gloomy white solution. The latter was allowed to cool to room temperature. Then the white precipitate was filtered and the ethanol solution obtained was reduced to induce formation of more white precipitate. Water (150 ml) was added to the precipitate and the resulting aqueous solution was extracted four times with 40 ml of hexane. The hexane layers were combined, dried over anhydrous $CaCl_2$, filtered, and the solvent was removed under vacuum. The crude product was purified by silica gel column chromatography using petroleum ether and ethyl acetate (PE/EtOAc, 95:5) as solvent.

4.2.4 Synthesis *α*-pinene trithione

α-Pinene trithione (APT) was synthesized by mixing *α*-pinene (40 mmol) and elemental sulfur (S_8) (280 mmol),and subsequently heating the mixture to 145-150 °C under continuous stirring. Stirring was continued at this temperature for 5 h until the colour of the solution changed from yellow to red-brown. At this point, diethyl ether was added to the reaction mixture and the mixture was stirred overnight to precipitate any unreacted sulfur and then filtered. The organic solvent was removed under vacuum. The crude product obtained was purified by silica gel column chromatography using petroleum ether and ethyl acetate (PE/EtOAc, 95:5) as solvent.[8]

4.2.5 Synthesis of nanoparticles

Synthesis of sulphur nanoparticles

Sulfur nanoparticles (NPS) were synthesized and purified according to the procedure of Bornhard and Lange with small modifications.[74] After synthesis, the nanoparticles then filtered and washed extensively with MilliQ water and centrifuged several times in order to remove all soluble, unreacted impurities. Afterwards the sulfur nanoparticles was reduced to a final volume (20-25 ml) using a vacuum rotary evaporator. The sulfur nanoparticles are then filtered consisting of a acetate membrane (w/0.2 µM). Characterization of NPS according to size, Zeta potential and polydispersity index (PDI) was performed with a Zetasizer Nano (Malvern Instruments Ltd. Germany).

Synthesis of selenium nanoparticles

Selenium nanoparticles (NPSe) were synthesized and purified according to the method described by Chen *et al*.[75] After synthesis, the nanoparticles then filtered and washed several times with HCl (0.01 M, 60 ml), ethanol (120 ml), and washed extensively with MilliQ water and centrifuged several times in order to remove all soluble, unreacted impurities. Afterwards the selenium nanoparticles was reduced using a vacuum rotary evaporator. The selenium nanoparticles then filtered with Whatman Anotop Syringe Filters (0.45 µM). Characterization of NPSe according to size, Zeta potential and polydispersity index (PDI) was performed with a ZetaSizer Nano (Malvern Instruments Ltd. Germany).

4.3 Nematode assays

4.3.1 Nematodes *Steinernema feltiae*

The nematodes *Steinernema feltiae* were purchased from Sautter & Stepper, Ammerbuch, Germany, as a powder cake product and stored at below 12 °C, as *S feltiae* is inactive below this temperature (ideally at 4 °C). A homogeneous mixture of nematodes was prepared by mixing 200 mg of powder cake with 50 ml of distilled water in order to revive of nematodes before use.

4.3.2 Sample preparation

Test preparation samples followed the method described by Sarakbi.[85] In brief, a primary stock solution, 40 mM, of the compound tested was prepared in DMSO. From this main stock solution, a series of dilutions were carried out to prepare 20, 10, and 5 mM stock solution. These secondary stock solution are then used to prepared the working solution. Here, 444.44, 222.22, 111.11, and 55.56 µM solutions of the respective compound was prepared and 88.8 µl of each of these solutions was then placed into a Falcon tube and diluted with distilled water to a final volume of 8 ml. The percentage of DMSO used was 1.1 %. To produce a test medium compound 400, 200, 100, and 50 µM in 1% of DMSO, 900 µl of the serial solutions were transferred into Eppendorf tubes (1 ml) than mixed with 100 µl of a homogeneous mixture of nematodes. The final volume each tube was 1 ml, and concentration of compounds include 50, 100, 200, and 400 µM in 1% DMSO.

To prepare a negative control, 88.8 µl of DMSO was placed into a Falcon tube and diluted with distilled water to yield 1.1 % DMSO solution, 900 µl of this 1.1 % DMSO solution was transfered into an Eppendorf tube (1 ml) and combined with 100 µl of a homogeneous mixture of nematodes (final volume 1 ml). As a blank, 100 µl of a homogeneous mixture of nematodes was added to 900 µl of distilled water.

4.3.3 Assay procedure

The assay was performed according to the procedure of Sarakbi with few modifications.[85] Three replicates at each concentration of a test compound were performed medium (see the Figure 4.1).

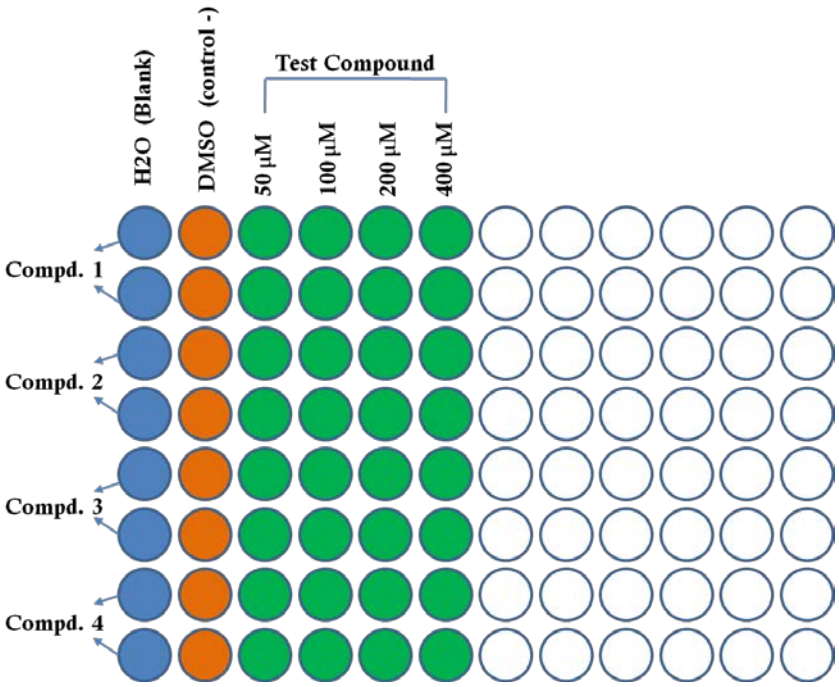


Figure 4.1: Design of the nematode assay using a 96-well culture flask.

For the assay, in each replicate, two samples (100 µl of each well, around 50-70 worms are counted per well) were added within 2 wells in 96-well plate flat bottom tissue culture with a lid (Greiner bio-one, Cellstar) and assessed immediately (0 h) under the microscope. Live and dead nematodes in each sample were counted under the microscope at four-fold magnification (VWR International, Belgium); afterwards the plate was incubated at room temperature in the dark. After 24 h, the nematodes were stimulated with 50 µl of warm water (50 °C) to each well and counted as “live” and “dead” once more.

Calculations

The viability of the nematodes obtained at each concentration of compound (three replicates) were expressed as a percentage (%). The viability values were obtained using the number of live nematodes after 24 h, expressed as $W_{24\ h}$ and the number of live nematodes after 0 h, expressed as $W_{0\ h}$. The calculation of the viability of the nematodes after exposing them to the compounds is presented in the equation below:

$$Viability\ (\%) = \frac{W_{24\,h}}{W_{0\,h}}\ x\ 100$$

4.4 Botrytis assay

4.4.1 Composition of the culture medium of *Botrytis cinerea* strains

The strains of *Botrytis cinerea* were kindly provided by the group of Prof. Dr. Matthias Hahn. This assay was performed under supervised by Dr. Michaela Leroch from the Department of Biology, University of Kaiserslautern, Germany. The strains were cultured in media containing Gamborg medium (Gamborg medium mixture (3 g/l), KH_2PO_4 (10 mmol/l), glucose (50 mmol/l) and adjusted to pH = 5.5 by using KOH).

4.4.2 Strains of *Botrytis cinerea*

The strains used in this assay were BO5.10, a sensitive lab strain (wild type strain, WT), MDR1, a multi drug resistance strain that has a high resistance and over expression on ABC transporter. MDR1*, a multi drug resistance strain that has a higher resistance and higher over expression on ABC and *atrB* transporter. MDR2, a multi drug resistance strain that has a higher over expression on MFS-transporter *mfsM2*.

4.4.3 Assay procedure

Preparation of the spore suspensions

The spore suspensions were prepared under aseptic conditions. The spores that were ready to be harvested from the agar plate were suspended by adding sterilized MilliQ water and mixed mechanically using a Drygalski-spatula. The mixture of the mycelium is then strained/washed with the blue tip in to the sterilized 15 ml Falcon tube through another sterilized Falcon tube that is filled with sterilized glass wool. The spore suspensions were washed once more with sterilized MilliQ water and centrifuged at 3,000 rpm for 3 min. Afterwards, the supernatant was removed, and spores were suspended again in sterilized MilliQ water (this step was repeated 3 times).

All the preparations were performed in the fume hood and under aseptic condition. By using a Neubauer improved hemocytometer, spores were counted and multiplied by 5 to obtain the number of spores and then multiplied by 10^4 to obtain the number of spores in 1 ml. The suspension was diluted as required to obtaine a standard concentration of 2×10^5 spores/ml, which was used for the assay. Afterwards, the spore suspension was placed into an Eppendorf tube that was filled with Gamborg medium (final volume is 1 ml). The number of spores suspension obtained was incubated at room temperature in an Eppendorf tube for 2 h.

Setting-up the test

5 µl of the spore-suspensions were added into the centre of each well plate (96-well-plate). Adding into the centre of the well plate was intended to prevent growing on the edge of the well plate and also can be disturbing when using a plate reader to determinate results. Afterwards, 95 µl of the substance (final concentration) was added. The plate was placed in chamber with a wet paper filled (a wet paper used to raise the humidity), then was closed, and incubated for 2 days (48 h) at 20 °C. Furthermore, the optical density (OD) were determined with a plate reader at a wavelength of 595 nm (Dynatech MR5000 plate-reader). The MIC_{50} values were determined using Origin 7.5 software.

4.5 Cell culture studies

4.5.1 Materials and methods

Cell culture assay was performed under sterile conditions at the Department of Microsystems Technique of Prof. Dr. med. Karl-Herbert Schaefer at University of Applied Sciences Kaiserslautern, Campus Zweibruecken under the instructions of Dr. Cornelia Irene Hagl. Neuro 2A cells were kindly provided by the department of Microsystems technique of Prof. Dr. med. Karl-Herbert Schaefer at University of Applied Sciences Kaiserslautern, Campus Zweibruecken.

All plastic- or glass-materials used were sterilised prior to use. All solutions were sterilised by steam autoclaving. Experiments were performed in 96-well from Greiner Bio-one.

Dulbecco's modified Eagle's medium (DMEM) Fetal calf serum (FCS), Penicillin-Streptomycin, Non-Essential Amino Acid (NEAA), Dimethyl sulfoxide (DMSO), Trypsin-EDTA, pure Methanol, calcein-AM and Propidium Iodide were kindly provided by the Department of Microsystems Technique of Prof. Schaefer at University of Applied Sciences Kaiserslautern, Campus Zweibruecken.

4.5.2 Culturing Neuro 2A cell lines

Neuro-2A cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10 % Fetal Bovine Serum (FBS), 1 % penicillin/streptomycin, and non-essential amino acid (NEAA 1 %). Cells were cultured at 37 °C in a 5 % CO_2 atmosphere.

Thawing of cells

Frozen cells in cryo-vials at a temperature of -80°C were allowed to warm in a water bath at a temperature of 37°C. The cells were immediately poured into 10 ml of warmed medium (to minimize the toxic effect of DMSO), the suspension was centrifuged at 800 rpm for 5 min, and the top solution was removed. The pellet was then re-suspended with 10 ml of fresh medium and was placed to cell culture flask and incubated directly at 37 °C. On the next day, the medium was removed and replaced with new fresh medium. When the cells achieved 80 % confluence (2 or 3 days), they were split.

Splitting of cells

At this point, the medium was removed with the 10 ml pipette and poured with 3 ml Trypsin-EDTA. Incubation at 37 °C in 5 % CO_2 atmosphere for 3-5 min in order to detach the cells from the surface of the flask. After incubation, the flask was gently knocked to release the cells on the surface of the flask. To normalize the cells due to a potential enzymatic reaction with Trypsin-EDTA, the cell-trypsin suspension was mixed with fresh medium containing FBS (5 ml) and transferred with the pipette (gentle motion up and down) to the Falcon tube (15 ml, sterilized) and centrifuged at 800-1,000 rpm for 5 min. After centrifugation, the supernatant was removed and the pellet was suspended with fresh medium containing FBS (1 ml). For the next cultivation, 50 µl of cell-suspension was transferred to the cell culture flask containing a fresh medium.

Statistics

Results were expressed as a mean of the number of experiments indicated. The IC_{50}-values for the assays were determined using Origin 7.5 software. The *p*-values were determined using Prism 5 software. Significances: *ns* $p \geq 0.05$, * $p < 0.05$,** $p < 0.01$ and *** $p < 0.001$.

4.5.3 Survival assay (calcein-AM - propidium iodide assay)

Screening the activity of the chemical compounds on 96-well tissue culture plates

Cells were seeded with a density of 10,000 cells per well in 96-well tissue culture plates in appropriate medium, then incubated for 24 h in an atmosphere of 5 % CO_2 in humidified air at 37 °C. After 24 h incubation, the culture medium was replaced with 0.2 ml medium containing H_2O_2, at concentrations of 100, 250, 500 and 1000 µM for 24 h incubation to determine the effect of H_2O_2 on Neuro 2 A cells. For the screening of the chemical compounds, cells were exposed to the compounds at concentrations of 1, 10, 50 and 100 µM in the absence or presence of H_2O_2 then incubated for another 24 h in an atmosphere of 5 % CO_2 in humidified air at 37 °C.

Preparation of staining solution

- *Calcein acetoxymethyl ester* (calcein-AM)*:* 50 µg of calcein was diluted in 50 µl of DMSO.
- *Propiumiodide (PI):* 50 µg of PI was diluted in 50 µl of sterilized distilled water
- *Staining solution:* the staining solution was prepared with diluted 1 µl of calcein-AM and PI into 998 µl of phosphate buffered saline (PBS). This solution was then protected from the light immediately.

Preparation of the culture staining

After the incubation period, the previous medium was removed from each well and washed with 0.2 ml of PBS. Subsequently, the PBS was removed and replaced with 0.2 ml of staining solution and incubated for 15 min in an atmosphere of 5 % CO_2 in humidified air at 37 °C. The plate was immediately protected from light. In this assay, calcein-AM enters the living cells and is cleaved by esterase inside the cells, producing a green fluorescence. In contrast, PI only enters dead cells, interacts and binds to nucleic acid, resulting in a red fluorescence.[87]

Quantification of live and dead cells was performed by fluorescence microscopy. Images were analyzed using the *Image J* software live and dead cells were counted and cell viability was expressed as the percentages of live cells compared to the total number of cells present in the sample analyzed.

4.6 Cyclic Voltammetry studies

Reagent

Phosphate buffer was prepared by mixing potassium dihydrogen phosphate, KH_2PO_4, 174.18 g/mol (0.2 M) and potassium monohydrogen phosphate, K_2HPO_4, 136.09 g/ mol (0.2 M). The phosphate buffer was then adjusted to pH = 7.4 using a pH meter (Hanna Instrument) and stored at 4 °C.

Experimental conditions

The experimental conditions used in cyclic voltammetry were as follows:

- *Working electrodes*: mercury-drop electrode (drop size 16), reference electrode: Ag/AgCl, counter electrode: platinum wire/spiral.
- *Scan rate*: the scan rate used for cyclic voltammetry generally was 250 mV/s.
- *Potential range*: the potential range extended from 0 mV to -1200 mV.
- *Number of cycles*: the number of cycles used in this method was 4 full cycles with 8 segments *i.e.* one full cycle containing two segments. To verify whether the peaks found in the first cycle are also detected in the following cycles.
- *Recording cyclic voltammograms*: Cyclic voltammograms were recorded on a BAS CV-100W workstation (Bioanalytical Sytem Inc., USA).

Preparation of the electrolysis cells

The electrolysis cell was routinely cleaned with acetone, rinsed with distilled water and dried in the oven. A three-electrode system (working electrodes) was used for cyclic voltammetry must be cleaned by rinsing the entirely electrodes with distilled water and methanol after each experiment. The surface of the carbon electrode was also polished on an abrasive surface with circular motion and rinsed with distilled water after used and sonication. Since the mercury-drop electrode was used, a new drop (drop size 16) of the mercury was generated before each point and knocked off after each experiment. When all the measurements were completed, the mercury drops from the electrolysis cell were carefully removed and disposed safely.

Sample preparation

Primary stock solutions of the compounds and glutathione, (10 mM each) were prepared in 1 ml of absolute MeOH. From which, 200 µl of compound stock were transferred to the electrolysis cell containing phosphate buffer (pH = 7.4) and MeOH (30 %) to obtain a final concentration of the compound (100 µM). The total volume in the electrolysis cell was 20 ml. To determine if there are any notable interactions between the compounds tested and GSH they both were mixed together in the same electrolysis cell under the same experimental conditions with the concentration ratio of a GSH : compound of 2 : 1 (100 µM and 50 µM respectively). GSH was used in twofold concentrations to ensure that exchange reaction would go to completion.

4.7 SEM/EDX-microanalysis

Preparation of cells for EDX-microanalaysis

To perform EDX-microanalysis, Neuro 2 A cells were prepared on a coverslip (CS) in a 24-well plate (Greiner Bio-One) at a density of 2 x 10^4 cells per well. This plate was then incubated for 24 h in an atmosphere of 5 % CO_2 in humidified air at 37 °C. After 24 h of incubation, the culture medium was replaced with 0.5 ml medium containing compound and incubated again for another 24 h.

Scanning electron microscopy fixation

Once cells were exposed to the test compounds, were they washed with PBS (1x) and fixed with formaldehyde (1 %) and glutaraldehyde (1 %) in 0.2 M of cacodylate buffer, and subsequently shaken at room temperature for 2 h. They were then washed again with 0.2 M cacodylate buffer (3x) and shaken for another 10 min, followed by washing with 70 % of ethanol (EtOH) (3x) for 10 min with shaking repeated (3x). Afterwards, the same procedure was repeated with a higher concentration of ethanol (80 %, 90 %, 96 % and 100 %) prolonging the time of shaking to 30 min. Cells were washed with absolute ethanol (100 %) and were shaken for 30 min once more and this procedure was repeated.

Then the washing was continued with absolute ethanol (100 %) and hexamethyldisilazane (HMDS) (ratio 1:1) and samples were shaken for 20 min. At the end, a final wash with fresh HMDS was performed twice also following 20 min of shaking. A few drops of a fresh HMDS were then added and the samples were left to evaporate in the fume hood overnight.

Coating the samples

The Neuro 2A cells fixed sample (on a coverslip) was mounted and glued on the sample holders and sputtered with the carbon to perform a carbon coating for SEM. This carbon coating method was performed on a Balzers Sputter Coating Sputtering Device SCD030 with a voltage 240 V. Carbon coating was performed at the Department of Anatomy of Prof. Dr. Frank Schmitz at University of University of Saarland, Homburg, under the instructions of Anna Schuster.

Scanning electron microscopy SEM/EDX analysis

Scanning electron microscopy (SEM) was performed at University of Applied Sciences Kaiserslautern, Campus Zweibruecken by Reiner Lilischki and Olaf Pohl. The SEM/EDX analysis were performed with a 12 - 900,000x magnification, on a Si-Li detector with an acceleration voltage of 0.1 – 30 keV.

4.8 Cell fractionation studies

Cell fractionation studies was performed at the Department of Medical Biochemistry and Molecular Biology of Prof Dr. Richard Zimmerman at University of University of Saarland, Homburg under the supervising of Martin Jung.

Preparation of cells

To fractionate cells into their cell fractions Neuro 2A cells were grown in DMEM medium to a density of 2 x 10^6 cells. The neuronal cells were then incubated with the media containing the compound. To fractionate cells into the subcellular fractions, Neuro 2A cells were grown in DMEM medium to a density of 3 x 10^7 cells then incubated with the media containing the compound.

Cell fractionation

Fractionation of cells

After 24 h exposure of the Neuro 2A cells to the compound tested, cells were spyn down (1,000 rpm, 5 min, RT). The supernatant was removed and the pellet was diluted with 500 µl of distilled water and transfer to a Precellys Ceramic Kit (Peqlab). It was homogenated for 30 s (FastPrep®-24, MP Biomedical, California, USA). The homogenate was then centrifuged for 3 h at 17,500 rpm, 4 °C (Eppendorf AG Centrifuge 5430 R). The pellet obtained at this centrifuged speed is referred to as the cell membrane fraction, while the supernatant is referred as the cytosol fraction.

Subcellular fractionation

After 24 h exposure of Neuro 2A cells to the compound tested, cells were harvested (1,000 rpm, 5 min, RT). The supernatant was removed and the cells were dounced (3x) using a dounce homogenizer (1,300 rpm 10 min for each dounce) in combination with hypotonic buffer (0.25 M sucrose, Table 4.1) used to break the cells. Then, the dounced cell preparation was centrifuged (3,000 rpm, 10 min, 4 °C). The pellet obtained was refered to as nuclear fraction.

Table 4.1: The composition of buffers used as part of subcellular fractionation.

Buffer without DTT/pMSF/CHX/Rnasin	
Composition:	
50 mM HEPES/KOH pH=7.5	10 ml; 1 M
250 mM Sucrose	25 ml; 2 M
50 mM KOAc	2 ml; 5 M
6 mM MgOAc	1,2 ml; 1 M
1 mM EDTA	1 ml; 0.2 M
Add 200 mL H_2O	MilliQ

The top fraction from nuclear fraction was then centrifuged at 10,000 g for 20 min at 2 °C to obtain the mitochondria fraction. The reticulum endoplasmic-ribosome (RE-ribosome) fraction was obtained by isolation of the top mitochondria fraction subsequently centrifugation at 196,000 g for 60 min at 2 °C. The Golgi fraction was obtained from the top fraction of RE-ribosome (Scheme 4.1.). All the steps of the subcellular fractionation steps were performed in the cooled conditions.

Analysis of the subcellular fractions using HPLC/UV-MS

Analysis of all of the fractions was performed using HPLC coupled with a UV-MS detector operated in Electron Spray Ionization (ESI) mode. UV-detection was performed at a wavelength 254 nm with the injection volume of 25 µl and time range 0-8 minutes. The solvents used in part of this analysis were H_2O containing 0.1 % Trifluoroacetic acid (TFA) and acetonitrile consisting 0.1 % TFA at a flow rate of 0.8 ml/min.

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Poster presentation

[1] E. Domínguez Álvarez, C. Jacob, **K. Khairan**, I. Jiménez, A. Calvo, A.L. Holme, L.A. Ba, J.A. Palop, C. Sanmartín, Nematicidal activity of novel selenium derivatives comparation with their cytotoxicity in prostate cancer cells. 1st International HIPS Symposium on Pharmaceutical Sciences devoted to Infection Research, 16.06.2011, Saarbruecken, German.

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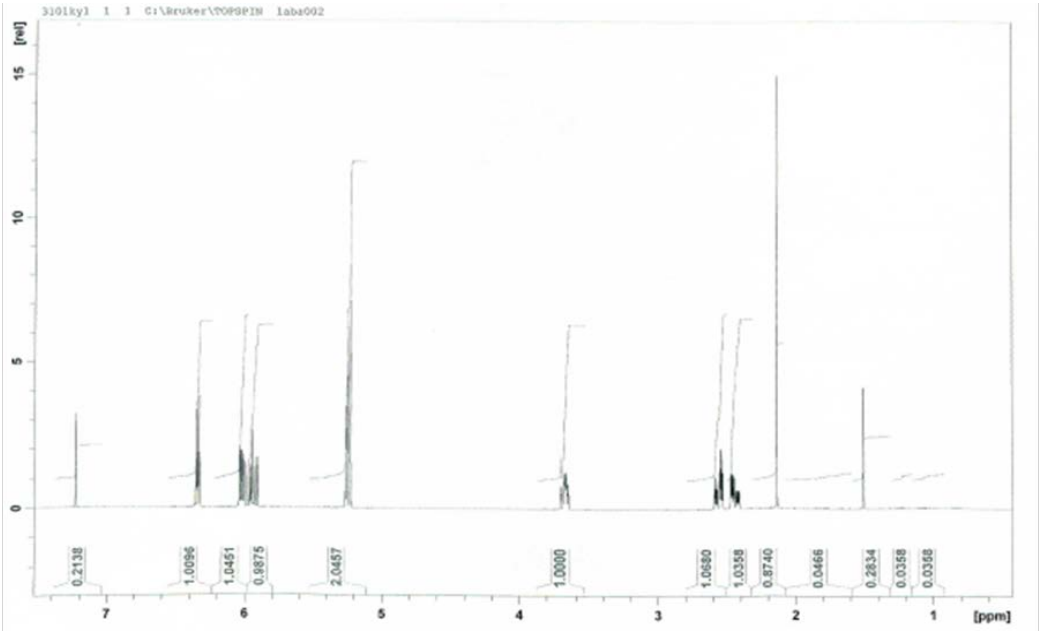
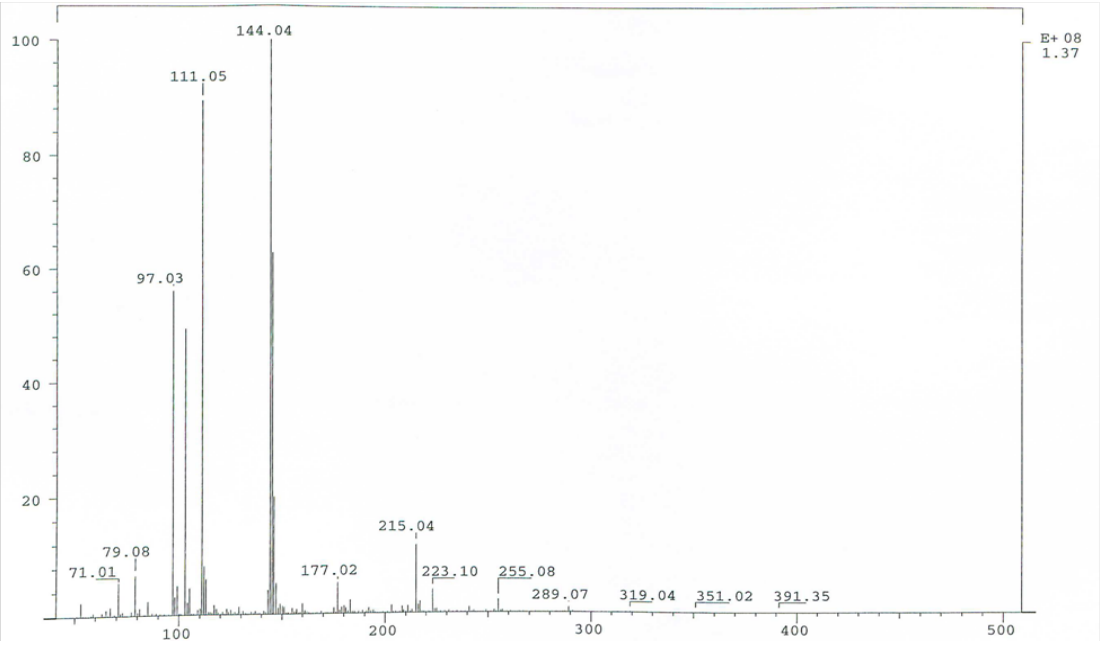
Appendix

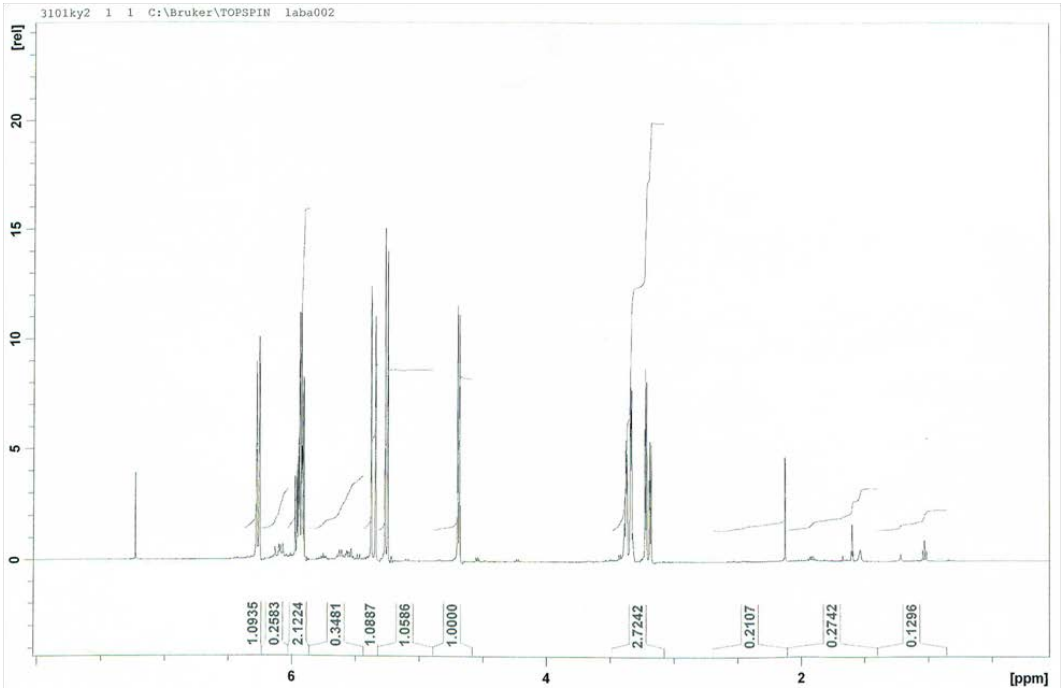
^{1}H NMR, ^{13}C NMR, and LC-MS Results of the synthesized compounds



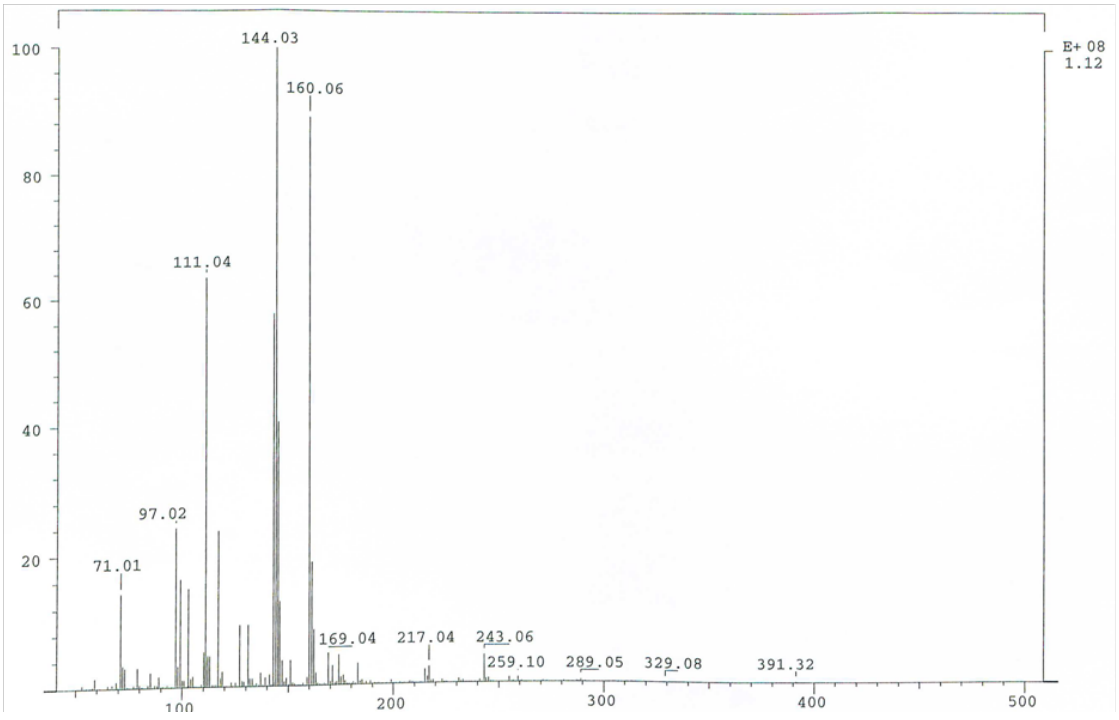
^{1}H NMR spectrum of 1,2-VDT



LC-MS spectrum of 1,2-VDT



^{1}H NMR spectrum of 1,3-VDT



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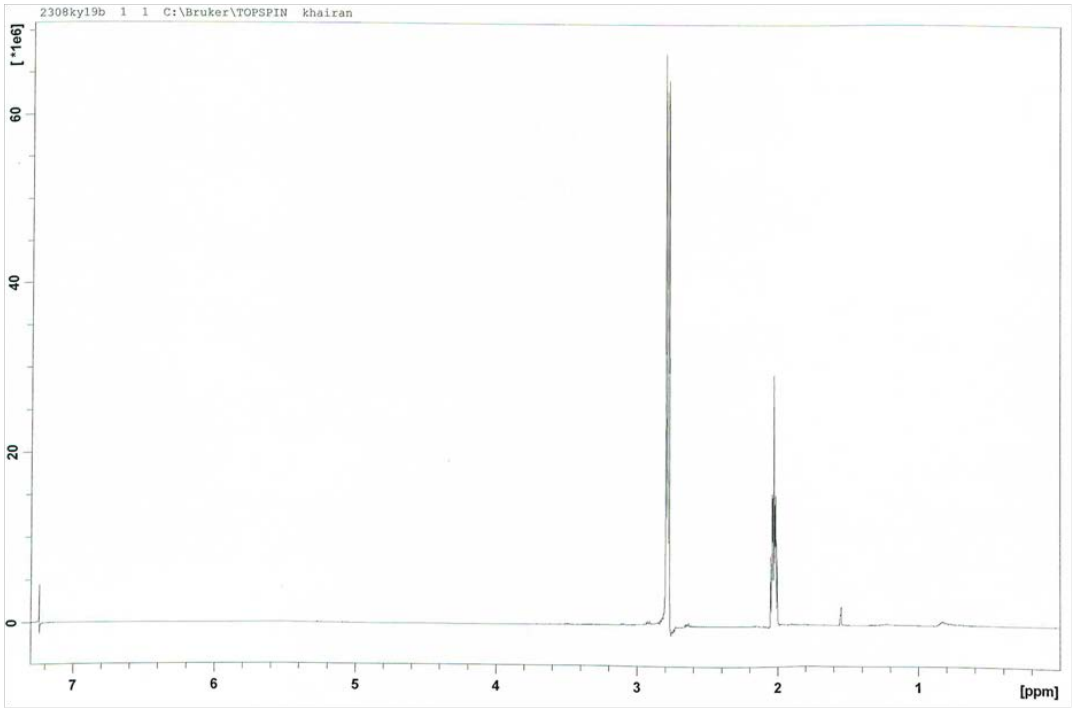
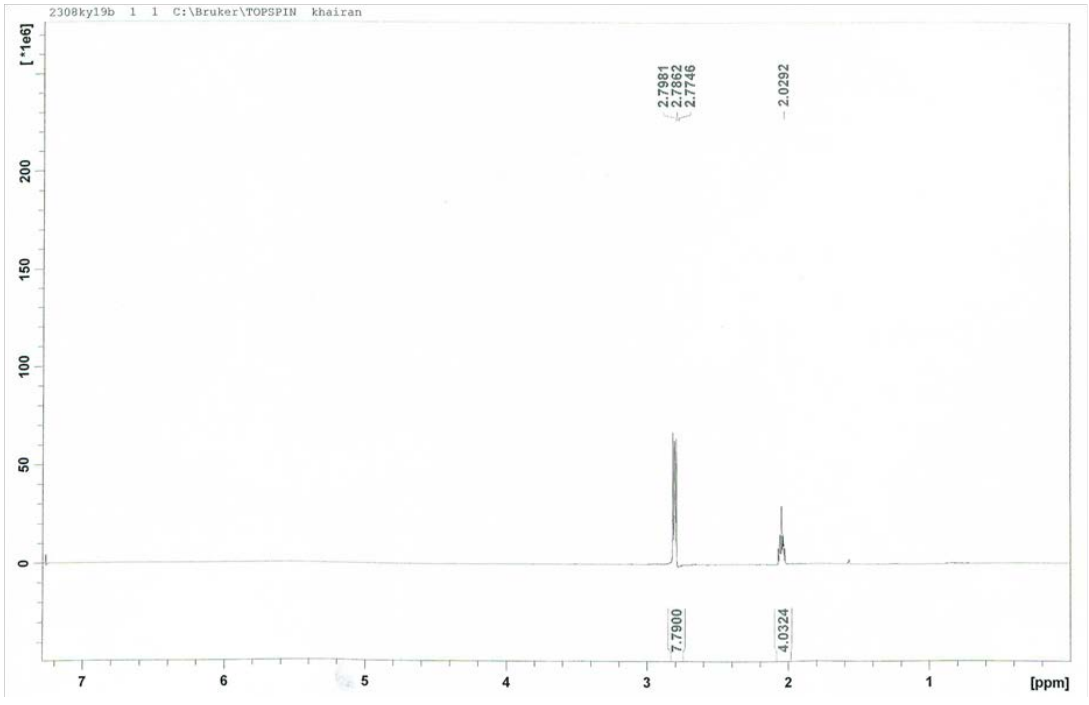
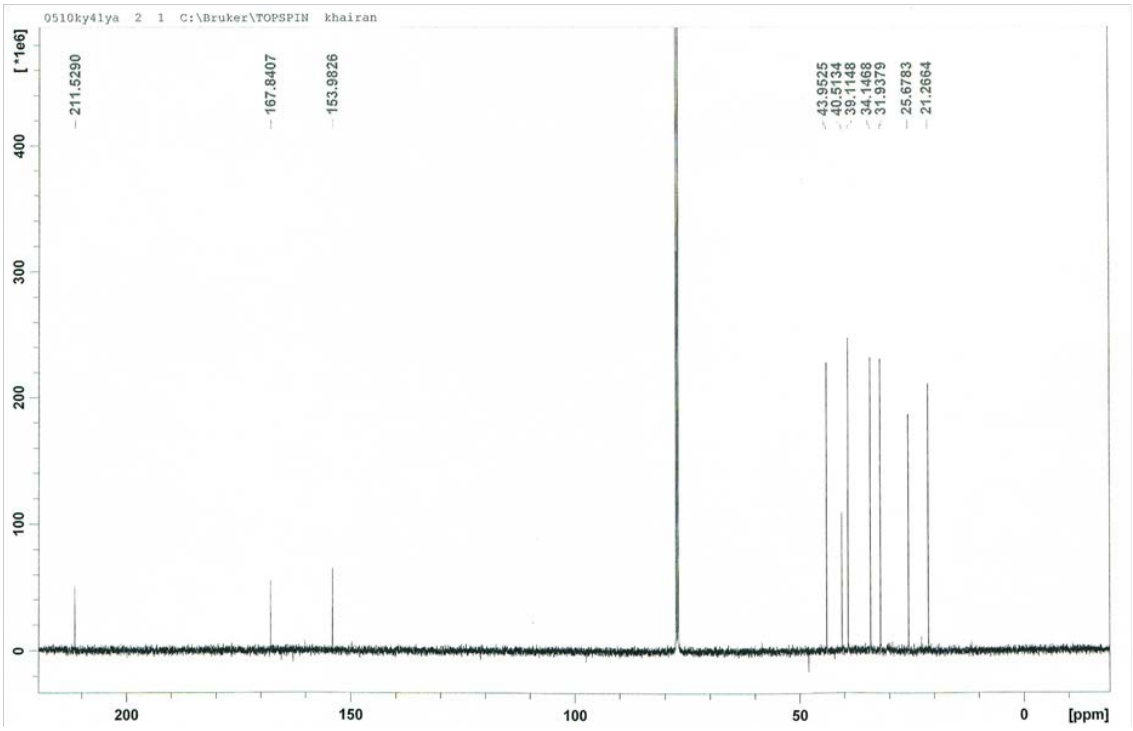


^{1}H NMR spectrum of 1,2-dithiane



^{1}H NMR spectrum of 1,5-DTCO



^{13}C NMR spectrum of α-APT